Modification and Application of Glycosidases to Create Homogeneous Glycoconjugates

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Abstract

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In the post-genomic era, recognition of the importance of sugars is increasing in biological research. For the precise analysis of their functions, homogeneous materials are required. Chemical synthesis is a powerful tool for preparation of homogeneous oligosaccharides and glycoconjugates. Glycosidases are potent catalysts for this purpose because they realize high stereo- and regio- selectivities under conditions benign to biomolecules without repetitive protection/deprotection procedures.

A glycosynthase is an aritificial enzyme which is derived from a glycosidase and is devised for glycosylation reaction. To suppress the mechanistically inherent oligomerization side reaction of this class of biocatalysts, a glycosidase with plastic substrate recognition was engineered to afford the first α-mannosynthase. This novel biocatalyst showed low occurrence of oligomerized products as designed and was applied to prepare a wide range of oligosaccharides.

Glycosidases are also valuable tools for glycan engineering of glycoconjugates, which is a pivotal issue in the development of pharmaceutical agents, including immunoglobulin G (IgG)-based drugs. EndoS, an *endo*-β-*N*-acetylglucosaminidase from *Streptococcus pyogenes*, natively cleaves *N*-glycans on IgG specifically. When the latent glycosylation activity of this enzyme was applied, the *N*-glycan remodelling of full-length IgG was successfully achieved for the first time and a highly pure glycoform was obtained using the chemically synthesized oxazoline tetrasaccharide as glycosyl donor. This biocatalytic reaction allows development of a novel type of antibody-drug conjugates (ADCs) in which drug molecules are linked to *N*-glycans site-specifically. For this purpose, glycans with bioorthogonal reaction handles were synthesized and conjugated to IgG. A model reaction using a dye compound as reaction partner worked successfully and the synthetic method for this newly designed ADC was validated.

Glycan trimming of glycoproteins expressed from *Pichia pastoris* was performed using exoglycosidases to derive homogeneous glycoform. Jack Bean α-mannosidase (JBM) trimmed native *N*-glycans down to the core trisaccharide structure but some of the glycoforms were discovered to be resistant to the JBM activity. Enzymatic analyses using exoglycosidases suggested that the JBM-resistant factor was likely to be β-mannoside.

In summary, this work advanced application of modified glycosidases for preparation of oligosaccharides and also demonstrated biocatalytic utility of glycosidases to produce biologically relevant glycoconjugates with homogeneous glycoforms.

Table of Contents

Chapter 1 1

Introduction

Chapter 2 25

Creation of an α-Mannosynthase from a Broad Glycosidase Scaffold

Chapter 3 127

Development of a Novel Antibody-Drug Conjugate by *N*-glycan Modification

Chapter 4 287

N-glycan trimming by Exoglycosidases to Prepare Homogeneous Glycoproteins

Appendix 349

Chapter 1

Introduction

1.1. Sugars and biology- General considerations 3

1.2. Glycans and glycoconjutates in biology 5

1.2.1. Structural features of oligosaccharides 5

1.2.2. Structures of glycans and glycoconojugates in Nature 6

1.2.3. Metabolism of glycans - Carbohydrate related enzymes 10

1.2.3.1. Construction of glycans- Glycosyltransferases 10

1.2.3.2. Degradation of glycans and recycling of sugar unit-Glycosidases 12

1.3. Glycosidase 15

1.3.1. Structure of glycosidases 15

1.3.2. Reaction mechanisms of glycosidases 18

1.4. Aim of the thesis 21

1.5. Reference 22

1.1. Sugars and biology- General considerations

One of the scientific landmarks in the twentieth century would be the finding that the genetic function of DNA is imprinted in its chemical structure of double helix..[1, 2] This sophistication of Nature fascinated many scientists and since then significant advances have been accomplished in the field of molecular biology. Now, genomes of many species have been decoded, including human genome[3-5] and this achievement has revealed that the genome of *Homo sapiens* is composed of ca. 37,000 genes, whereas other representative species have the numbers of genomes shown in **Table 1.1**. It is surprising that the number of genes falls within one order of magnitude from bacteria (10^{-3} meter size) to human (1 meter size). This result urged scientists to reconsider the notion of complexity of living entities as well as to rectify the simplicity of central dogma.

Table. 1.1. Comparison of the number of genes in various organisms. Data are adopted from the NCBI database[6] (10/May/2013).

organism	type	genome size (Mbp)	genes
Enterobacteria phage Φ-X174sensu lato	virus	0.00539	11
Sulfolobus solfataricus 98/2	archaea	2.67	2,956
Escherichia coli K-12 MG1655	bacterium	4.64	4,497
Saccharomyces cerevisiae S288c	fungi	12.16	6,349
Caenorhabditis elegans	worm	100.28	21,187
Drosophila melanogaster	insect	139.73	15,867
Arabidopsis thaliana	plant	119.67	33,583
Homo sapiens	mammal	3,099.46	37,381

The beauty of the “central dogma” is still at the center of molecular biology, but now scientists are in need for supplemental formulas to describe the complexity of biology. One of such examples is posttranslational modification (PTM) of proteins. Many types of posttranslational modifications have now been reported[7, 8] and glycosylation is one of the major pathways (**Figure 1.1**). Indeed, it is estimated that more than 50% of human proteins are glycosylated.[9]

Sugars have a variety of functions in biological systems. They work as energy sources. They also exist as structural components like extracellular matrix in mammals and cell walls in bacteria. Free sugar molecules play a role as signaling molecules. Therefore, without any doubt, oligosaccharides, i.e. glycans, are considered as the third major component in the living systems along with DNAs and proteins and research into these complex molecules is a key for understanding biology.

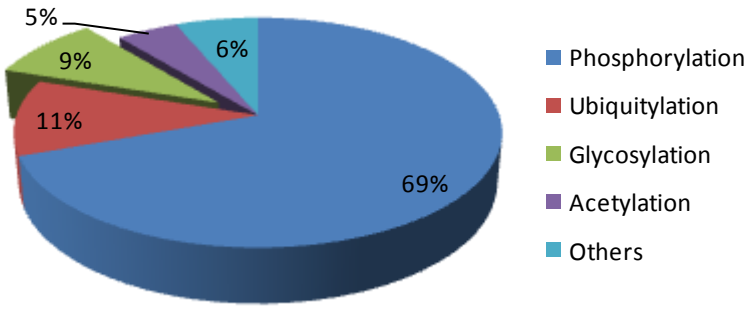


Figure 1.1. The statistics of the experimentally validated posttranslational modification sites on proteins. The data are taken from dbPTM 3.0.[8]

1.2. Glycans and glycoconjutates in biology

1.2.1. Structural features of oligosaccharides

In the field of chemistry, oligosaccharides are still notorious for their complexity and difficulty of their preparation even though carbohydrates have long been synthetic targets of chemistry since the late nineteenth century, as represented by Emil Fischer. The significant progress in the field of molecular biology is partly due to an easy access to synthetic DNA. Therefore, glycochemists are now pursuing efficient synthetic methods to produce oligosaccharides for the biological understanding of their roles.

The difficulty of oligosaccharide synthesis stems from their structural features: multiple reaction sites and two possible stereochemical outcomes (**Figure 1.2**). For example, when two hexoses are combined to produce a disaccharide, the number of possible products is 8 (= 4 reaction sites x 2 stereoconfigurations. For simplicity, the anomeric hydroxyl group is not considered as a reaction site). In comparison, a peptide has only one product when two amino

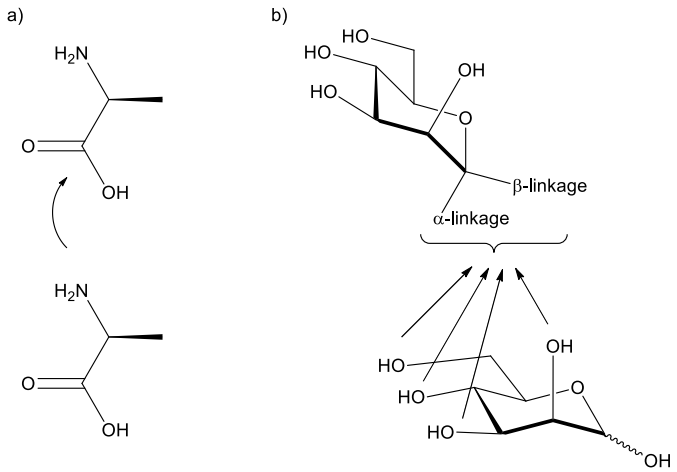


Figure 1.2. Comparison of linkage pattern between (a) two amino acids and (b) two hexoses. When an order of two units are fixed, amino acids have only one possible compound, while hexoses eight.

acids are combined. Obviously, the number of the possible structures increases exponentially as the size of the oligosaccharide becomes larger. Selection of a reaction site from multiple possibilities is crucial for developing *de novo* chemical synthesis to obtain desired products.

1.2.2. Structures of glycans and glycoconojugates in Nature

Even though the number of possible structures that can be constructed from sugars is quite large, the structural variety of glycans in biological systems is limited. Major classes of glycans are summarized in **Figure 1.3**. Though some sugars exist as free forms (unbound to anything), most glycans exist as glycoconjugates and are linked to peptides/proteins or lipids.

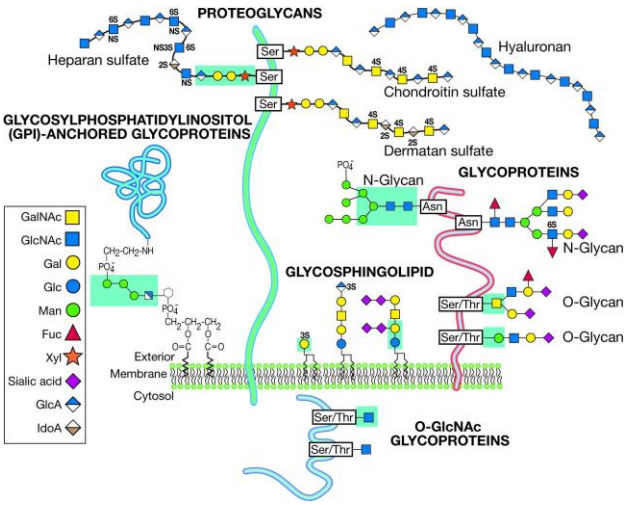


Figure 1.3. Glycans and glycoconjugates found in the biological systems. The figure is adapted from *Essentials of Glycobiology*, 2nd edition.[10]

A glycoprotein is a glycoconjugate which consists of protein backbones and glycans. Depending on which amino acid glycans are attached to, they are subclassed into *N*-glycan and *O*-glycan. The former is bound to the nitrogen atom of an asparagine side chain, while the latter is bound to the oxygen atom of a serine or threonine side chain. Their structures are discrete to

each other (**Figure 1.4**). *N*-Glycans have a core pentasaccharide structure made up from $Man_3GlcNAc_2$ (**Figure 1.4**, left). This core structure is modified at the non-reducing end, constituting three types of *N*-glycans; oligomannose, complex and hybrid (**Figure 1.4**, left). These basic structures can be further modified by, for example, core-fucosylation and/or bisecting GlcNAcation.

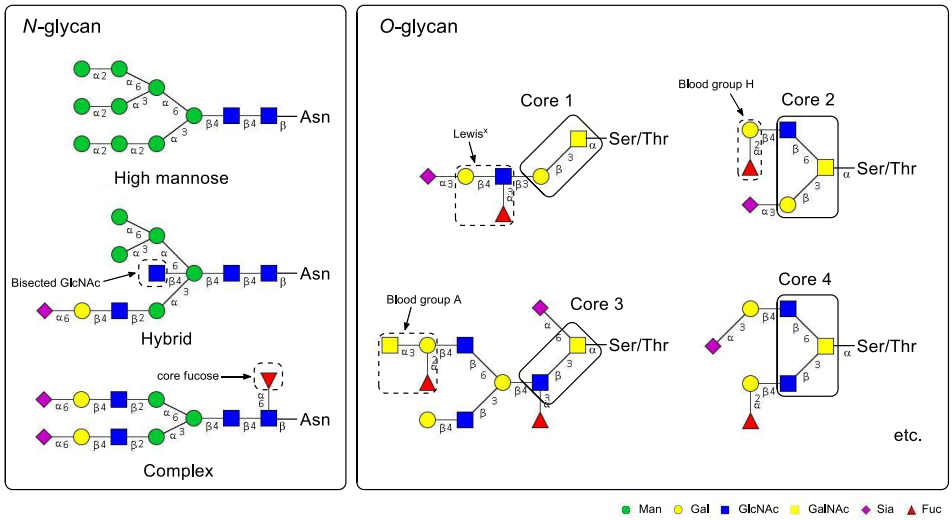


Figure 1.4. Representative structures of *N*-glycans and *O*-glycans. *N*-glycan structures include examples of modification by bisected-GlcNAc and core-fucose. *O*-glycan structures shown here are parts of the core structures (core 1-4) and are modified by fucose, sialic acid and blood group antigenic determinants.

On the other hand, *O*-glycans have more diverse structural varieties. The most common structure contains *O*-GalNAc linked to serine or threonine and is called mucin. This GalNAc at the root of the *O*-glycan is then modified by galactose, GlcNAc and/or GalNAc to form several classes of core structures (**Figure 1.4**, right). These core structures are extended by several epitopes, like the ABO blood group determinants, and may be further decorated with fucose and sialic acid.

Proteoglycan is another class of peptidic conjugate in which a core protein is modified with glycans called glycosaminoglycans. Glycosaminoglycans are linear polysaccharides composed

of repeating disaccharide units. These disaccharide units are made up from a hexosamine (GlcN or GalN) and a galactose or hexuronic acd (GlcA, IdoA). Depending on structural difference of this repeating unit, they are categorized as chondroitin/dermatan sulfate, heparin/heparan sulfate and keratan sulfate (**Figure 1.5**). The former two glycosaminoglycans are attached to the protein core through Gal_2Xyl-Ser linkage, whereas the latter is anchored on non-reducing termini of *N*- or *O*-glycans. Hyaruronan (**Figure 1.5**) is also another type of glycosaminoglycan, though this oligosaccharide normally exists in a free form. These glycosylaminoglycans are frequently decorated by sulfation on amino or hydroxyl groups.

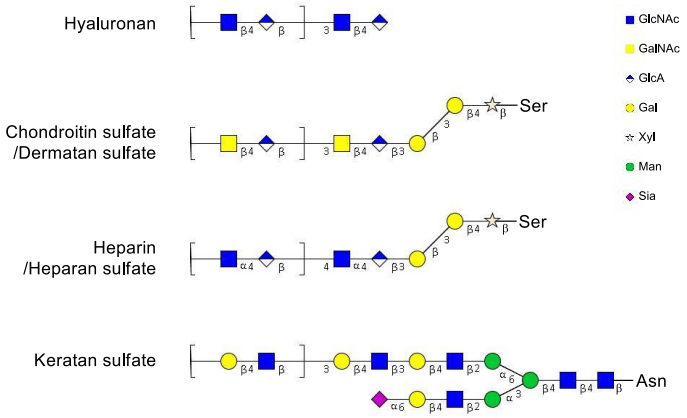


Figure 1.5. General structures of glycosaminoglycans. The repeating disaccharide units are shown in the brackets. Glycosaminoglycans are highly sulfated (except hyaluronan). Epimerization at C5 of GlcA (iduronic acid, IdoA) is also prevalent.

Glycans are also found as conjugates with lipids. Glycosylphosphatidylinositol (GPI) is one of such conjugates and contains the core trimannosyl glucosamine structure (**Figure 1.6**, top). This tetrasaccharide scaffold is linked to inositol of phosphatidylinositol at its reducing terminus, while 6-OH of the non-reducing terminal mannose is linked through phosphate ester with ethanolamine, which forms an amide bond with the C-terminus of the protein. This tetrasaccharide scaffold works as a linker to harbor proteins on the cell surface.

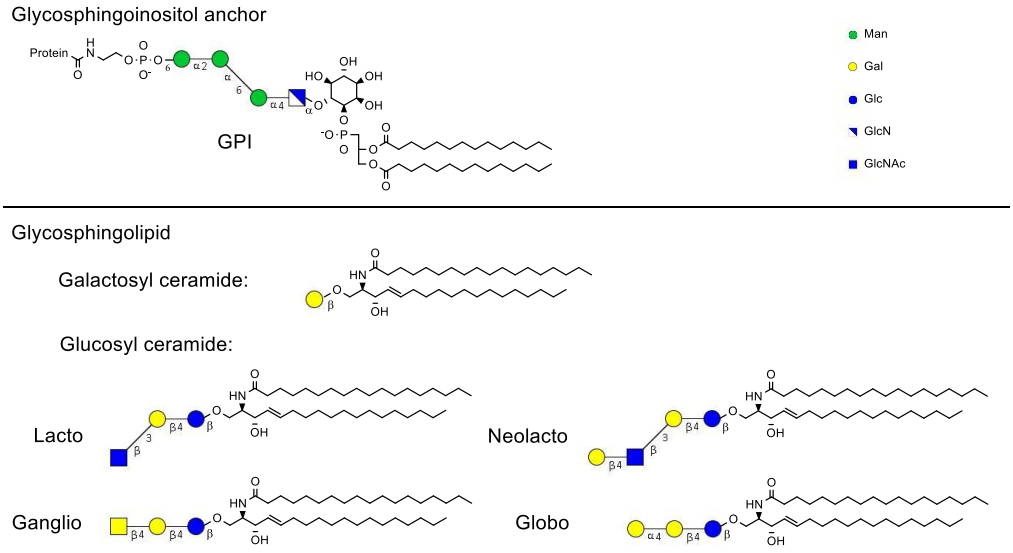


Figure 1.6. Core structures of GPI-anchor and glycosphingolipids.

Glycosphngolipid is another form of glycolipid, in which the core ceramide, which is an amide made from sphingosine and fatty acid, is glycosylated at its terminal primary hydroxyl group. The reducing terminus of the glycan part is glucose or galactose. Galactosyl ceramide is seldom extended, while glucosylceramide is decorated in various manner and forms several classes of glycosphingolipids (**Figure 1.6**, bottom).

One common feature of glycans that should be noted is that their synthesis is not encoded in a specific template, like protein is encoded in DNA. Instead, the synthesis of glycans is performed enzymatically. Therefore, the resultant products dynamically changes depending on the environments like cell types or differentiation stages, which results in the wide heterogeneity of glycan structures.

1.2.3. Metabolism of glycans - Carbohydrate related enzymes

1.2.3.1. Construction of glycans- Glycosyltransferases

Glycosyltransferases (GTs) play a central role in the construction of glycan structures. Therefore, 1-2% of genes in the genome encode this class of enzymes. Basically, these enzymes are known to act in the principle of "one-enzyme to one-linkage" manner[11] and GTs constitute a large family of enzymes. However, they have it in common that they use nucleotide sugars as glycosyl donors along with glycosyl phospholipid donors in some cases.

Sugar units of glycosyl donors are provided externally from food. Uptake of glucose is performed by transporters. Mannose and fucose are also known to be transported through transporters. Another important source is salvage cascade of sugars. Actually, significant parts of sugars are rescued and recycled using this system.

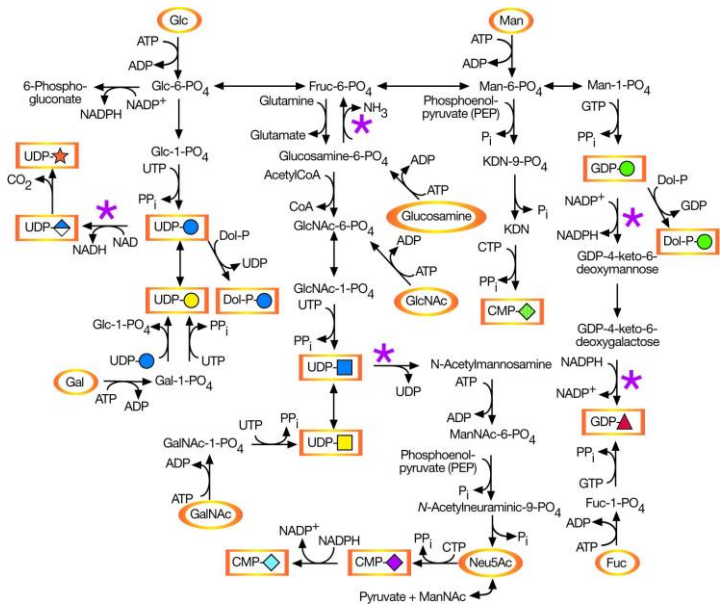


Figure 1.7. Synthesis of the nucleotide sugars and their interconversion pathways. All sugar nucleotides can be synthesized from glucose as the starting material. The figure is adapted from *Essentials of Glycobiology*, 2nd edition.[10]

These sugar components are transformed into nucleotide sugars as summarized in **Figure 1.7**.[10] These reactions take place in the cytoplasm. Using glucose as the starting material, most sugar nucleotides can be synthesized in an interconvertible manner.

As a representative example, the biosynthesis of an *N*-glycan is shown in **Figure 1.8**.[10] The synthetic cascade starts from the formation of GlcNAc-dolichol diphosphate on the surface of the endoplasmic reticulum (ER) in the cytoplasm side. The oligosaccharide scaffold is converted to heptasaccharide sequentially using five different GTs. Then, this glycolipid flips over the membrane and the glycans are exposed to the lumen of the ER. The following glycosylation proceeds in the "one-reaction one-enzyme" manner and the glycolipid ends up in $Glc_3Man_9GlcNAc_2$-dolicol diphosphate, which is the glycosyl donor for OST-catalyzed protein glycosylation reaction. Further maturation of *N*-glycans to the final forms (**Figure 1.4**) is also carried out in the ER as well as in the Golgi by the corresponding GTs.

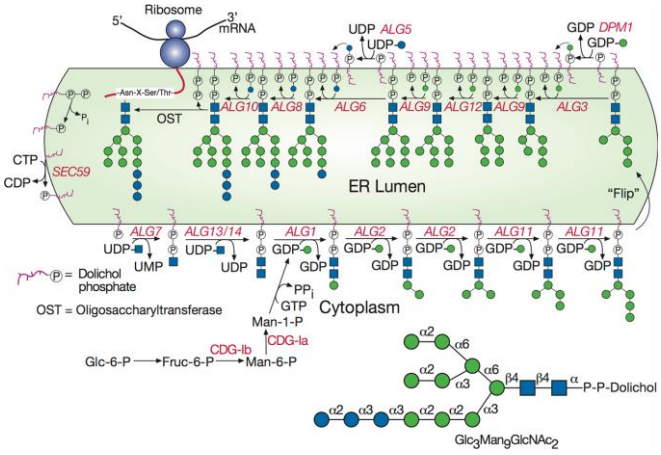


Figure 1.8. Synthesis of the *N*-glycan unit ($Glc_3Man_9GlcNAc_2$) and its transfer to protein backbone. GTs are responsible for each glycosylation step. The figure is adopted from *Essentials of Glycobiology*, 2nd edition.[10]

1.2.3.2. Degradation of glycans and recycling of sugar unit-Glycosidases

As other biological components, glycans are degraded to simpler units, i.e. monosaccharides, and then they are recycled for new syntheses of glycans. Degradation of glycans takes place in lysosomes where a collection of glycosidases is encapsulated. All of these lysosomal enzymes have *N*-glycans containing mannose-6-phosphate, which is used as a signal tag to be delivered to lysosomes. Glycans and glycoconjugates are endocytosed and this endosomes are matured to lysosomes. During this maturation process, pH of these capsules becomes lower to around 4.0-5.5, which is the optimal pH for many of glycosidases.

Degradation of glycans is highly ordered in biological systems. An example is shown by degradation of biantennary *N*-glycans (**Figure 1.9**).[10] Degradation of *N*-glycoprotein starts from hydrolysis of the protein part by proteases, which are estimated to be more abundant than glycosidases in lysosomes. This proteolytic digestion results in an *N*-glycan-Asn structure. Glycan digestion starts from removal of fucose decoration, if any. Then glycosylasparaginase cleaves the bond between GlcNAc and Asn to release a free glycan fragment. This is followed by the cleavage of the chitobiose core by endo-β-*N*-acetylglucosaminidase to afford a truncated *N*-glycan structure. Subsequently, monosaccharide units are sequentially trimmed from non-reducing termini by sialidases, β-galactosidases, β-*N*-acetylglucosaminidases and α-mannosidases. Finally, the remaining disaccharide, Man-β(1-4)-GlcNAc is digested by β-mannosidase.

Degradation of glycoconjugates is sometimes incomplete. In such cases, partially degraded glycoconjugates can be reused for new syntheses, which is especially prominent in glycolipid synthesis. Monosaccharide units return to the cytoplasm through transporters and are recycled in new glycoconjugate synthesis.

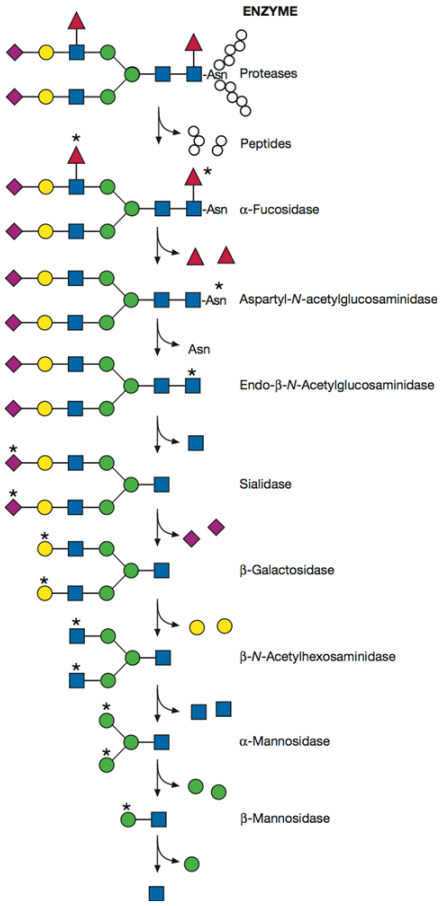


Figure 1.9. Degradation pathway of *N*-glycans. GHs are responsible for each glycosylation step. The figure is adapted from *Essentials of Glycobiology*, 2nd edition.[10]

Today, more than 45 diseases are known to be related to the lack of lysosomal hydrolases, the significant part of which are glycosidases. When a specific glycosidase is lacking, it causes accumulation of the corresponding substrate. Analyses of such accumulated substances in the patients or the corresponding animal models have provided critical information to elucidate the degradation pathway of glycans.[12] For example, it was revealed that degradation pathway of glycans starts from removal of fucoses because intact *N*-glycans bound to asparagines are found from patients lacking fucosidases.[13] Inhibitors for glycosidases also provide informative insights about degradation pathways of glycans or mechanisms of diseases. For example, swainsonine is

a well-known α-mannosidase inhibitor. When this molecule is dosed in sheep, they show temporal neurological retardation symptoms[14] which is observed in α-mannosidosis model.

Diseases caused by lysosomal enzyme deficiency can be treated by supplementing the corresponding enzymes.[15] This therapeutic approach is known as enzyme replacement therapy and has attained many positive results. For example, recombinant α-galactosidase was approved for Fabry disease,[15] which is caused by lack of this enzyme and results in neurological symptoms due to accumulation of undegraded glycolipids.

1.3. Glycosidase

1.3.1. Structure of glycosidases

Glycans can form a very stable structure. It is reported that bacterial cell wall can stand the physical force of up to 7.5 x 10^8 N/m^2, whereas an engine of a space ship can stand up to 2.75 x 10^5 N/m^2. [16] This stability would be attributed to the structural complexity as well as stability of glycosidic bond. The chemical stability of the glycosidic bond is demonstrated by cellulose, which is said to have half-life for spontaneous hydrolysis of 5 x 10^6 years.[17]

However, hydrolysis of glycosidic bonds is carried out in our bodies on a daily basis. This hydrolytic reaction is normally catalyzed by glycosidases. If the reaction catalyzes the glycosidic hydrolysis at the time-scale of seconds, it means the reaction is accelerated by 10^{13}-10^{14} fold. Indeed, there are many reports where they show the acceleration of glycosidic hydrolysis by glycosidase up to 10^{17} fold.[18]

The substrate specificities of glycosidases are defined by the following factors: (i) the anomeric specificities (α/β), (ii) the mode of reaction (retaining/inverting etc.), (iii) the reaction site in glycans (exo/endo) and (iv) the sugar type.

Glycosidases are classified by their substrate specificity by the International Union of Biochimestry and Moleclar Biology[19] (IUB-MB) system as E.C. number. However, glycosidases have relatively promiscuous substrate specificities and sometimes one structural scaffold can accommodate several types of sugars. Conversely, several scaffolds can accept the same substrates. Therefore, it now turns out that this system is not very effective when new enzymes are found and their functions are to be elucidated. Hence, an alternative system was launched, named Carbohydrate-Active enzyme database[20] (CAZy), which provides classification of

glycosidases based on amino acid sequences.

Table 1.3. Clans of glycodidase families and their structural folds and catalytic functions.

Clan	Fold	Catalysis	Families
GH-A	$(\beta/\alpha)_8$ barell	β-retaining	1, 2, 5, 10, 17, 26, 30,35, 39, 42, 50, 51, 53, 59, 72, 79, 86, 113, 128
GH-B	β-jelly roll	β-retaining	7, 16
GH-C	β-jelly roll	β-retaining	11, 12
GH-D	$(\beta/\alpha)_8$ barell	α-retaining	27, 31, 36
GH-E	6-fold β-propeller	α-retaining	33, 34, 83, 93
GH-F	5-fold β-propeller	inverting	43, 62
GH-G	$(\alpha/\alpha)_6$ barell	α-inverting	37, 63
GH-H	$(\beta/\alpha)_8$ barell	α-retaining	13, 70, 77
GH-I	α+β	β-inverting	24, 46, 80
GH-J	5-fold β-propeller	α-retaining	32, 68
GH-K	$(\beta/\alpha)_8$ barell	β-retaining	18, 20, 85
GH-L	$(\alpha/\alpha)_6$ barell	α-inverting	15, 65, 125
GH-M	$(\alpha/\alpha)_6$ barell	β-inverting	8, 48
GH-N	β-helix	α-inverting	28, 49

CAZy database provides useful systematic information to glycoscientists. Now 132 families are catalogued in this database (10/May/2013). These families constitute clans (**Table 1.3**) based on their global folds (**Figure 1.10**). Glycosidases processing a variety of sugars are found in each clan, while the stereoselectivity of substrates (α/β) and the mode of reaction are in common in the same clan. This fact implies these two factors are more related to the global structures, whereas reaction site specificities (endo/exo) and substrate specificities are altered by more local variations.

Other than the global folds of enzymes, an alternative structural features divide glycosidases into three classes by active site topologies (**Figure 1.11**). One class of enzymes has active sites in pockets on the enzyme surfaces (**Figure 1.11a**). This is typical topology for exo-glycosidases, which reasonably corresponds to their substrate specificities (cleaves only non-reducing terminal sugar). The second class forms clefts around active sites (**Figure 1.11b**). They are typically endoglycosidases. Due to their topological feature, they have larger contact surface with substrates and have higher affinity and narrower specificities. In the third class, enzymes evolved a long loop which can cover the cleft found in the second topology and forms a tunnel

(**Figure 1.11c**). This scaffold is also typical to endoglycosidases but seems to acquire the ability of successive processing.

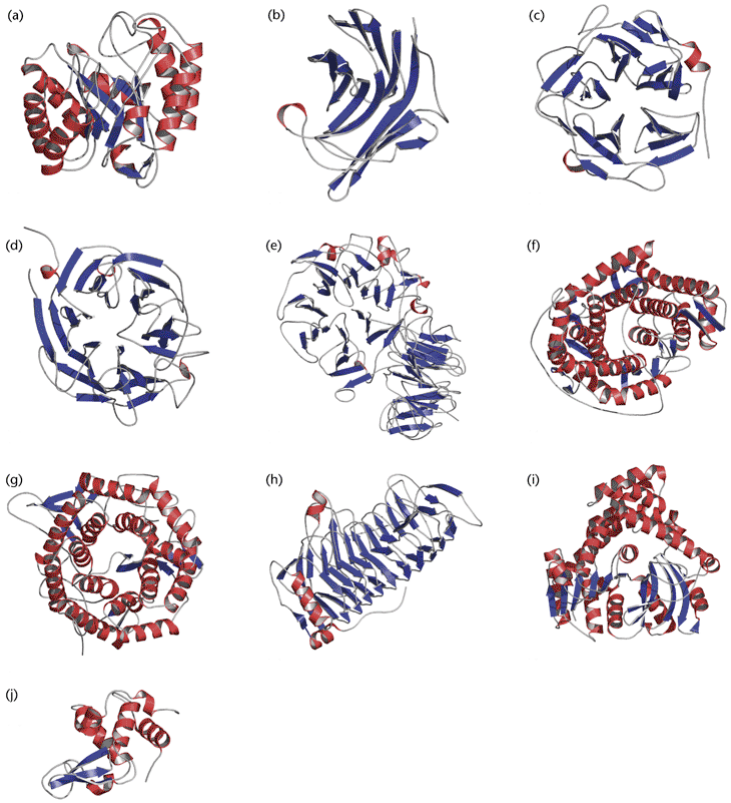


Figure 1.10. The global folds of glycosidases. The figure is adapted from *Encyclopedia of Life Sciences* **2007**.[21] (a) $(\beta/\alpha)_8$ barrel, (b) β-jelly roll, (c) 5-fold β-propeller, (d) 6-fold β-propeller, (e) 7-fold β-propeller, (f) $(\alpha/\alpha)_6$ barrel, (g) $(\alpha/\alpha)_7$ barrel, (h) right-handed β-helix, (i) dehydgronenase-like fold, (j) lysozyme-like fold.

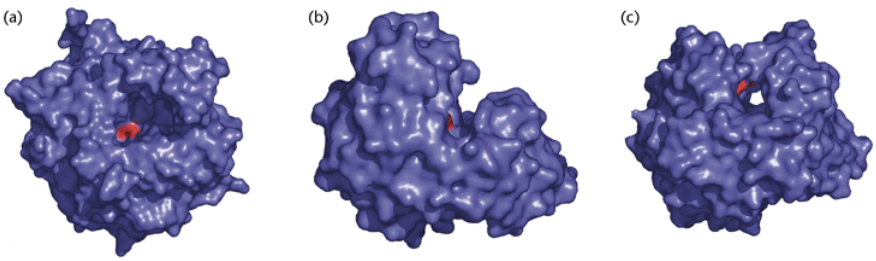


Figure 1.11. Topologies of glycosidases. The figure is adapted from *Encyclopedia of Life Sciences* **2007**.[21] The reaction center shown in red is found in (a) pocket (b) cleft or (c) tunnel.

1.3.2. Reaction mechanisms of glycosidases

The catalysis of glycosidases is exerted in several manners. The reaction mechanisms are summarized in **Figure 1.12**. Glycosidases with retaining double-displacement mechanism (**Figure 1.12.c,d**) are most prevalent and found in the clans GH-A, B, C, D, E, H and J (**Table 1.3**). The mechanism of these enzymes is most investigated. The retaining glycosidases have two conserved amino acids in their catalytic sites and the one works as acid/base catalyst and the other as nucleophile. When the substrate is bound to the active site, the acid coordinates to the oxygen of the glycosidic bond to facilitate the departure of the aglycon part. Meanwhile, the nucleophilic residue attacks the anomeric center from the opposite side of the aglycon to carry out the S_N2-type reaction. This first step of the reaction results in the formation of the covalent intermediate of the enzyme with the substrate. In the second step, the acid/base catalyst works as base to activate the incoming nucleophile and again the S_N2-type reaction proceeds to release the catalytic nucleophile. When the incoming nucleophile is water, the overall reaction gives the hydrolyzed product, while when other nucleophiles are involved in the reaction, the products are transglycosylated substrates. In a few cases, the second step is not the nucleophilic attack but the intramolecular *cis* elimination of the nucleophilic residue (**Figure 1.12g**). This exceptional mechanism is found in GH31 α-glycan lyase.[22]

It should be mentioned that β-*N*-acetylhexosaminidases (**Figure 1.12e**), found in clan GH-K (**Table 1.3**), basically have the same catalytic mechanism as retaining glycosidases and therefore the product has the retained anomeric configuration. However, they do not form covalent intermediates with the substrate, which means they lack the nucleophilic residue. Instead, the acetamide group at the 2-position of the substrate is activated and works as nucleophile to form the oxazoline intermediate.

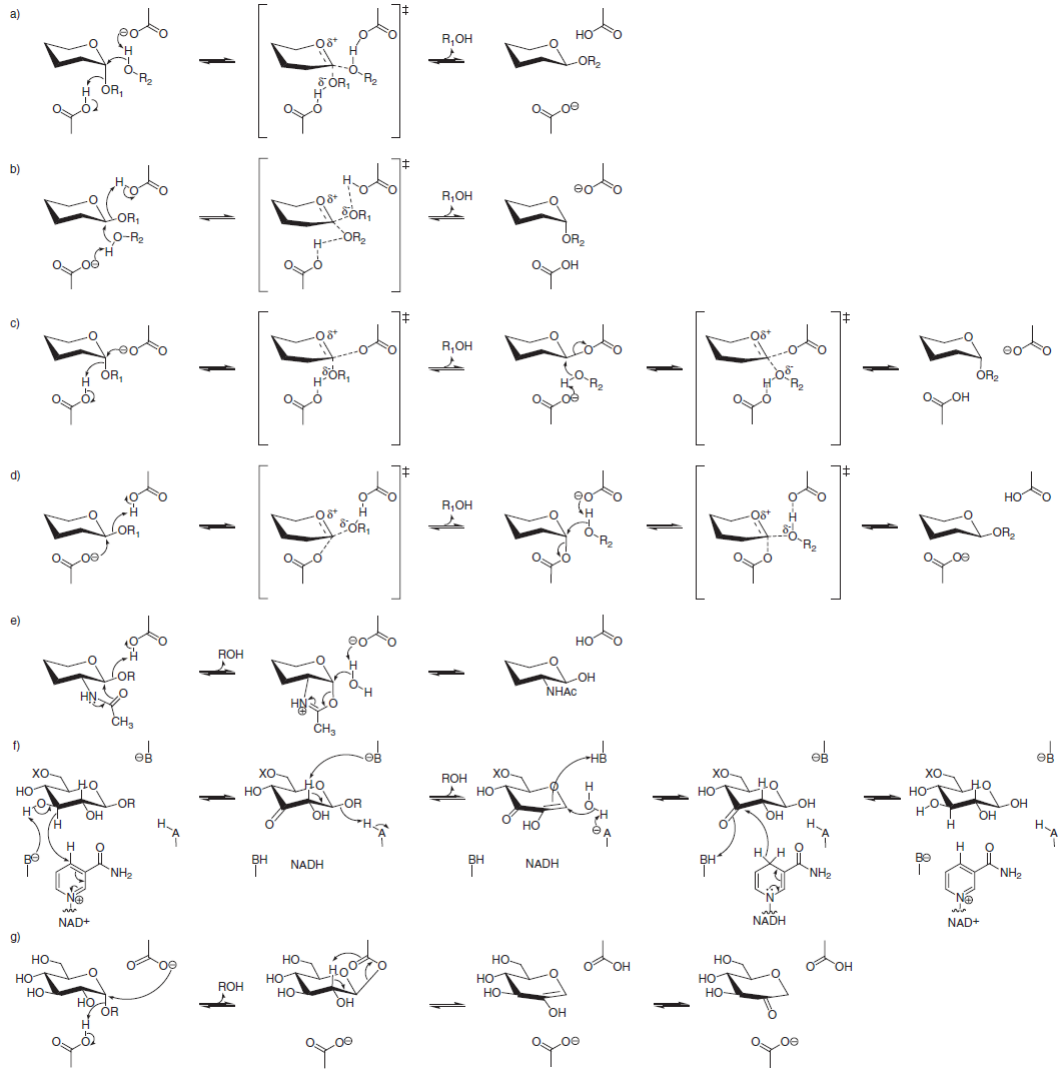


Figure 1.12. Reaction mechanisms of (a) inverting α-glycosidase, (b) inverting β-glycosidase, (c) retaining α-glycosidase, (d) retaining β-glycosidase, (e) *N*-acetyl β-hexosaminidase, (f) NAD^+-dependent glycosidase, (g) α-glycan lyase. The figure is adapted from *Encyclopedia of Life Sciences* **2007**.[21]

Another prevalent catalytic mode is inverting mechanism (**Figure 1.12a,b**). Glycosidases with this reaction mechanism are found in the clans GH-F, G, I, L, M and N (**Table 1.3**). These enzymes also have two conserved amino acids but in these cases, the one is the acid catalyst and the other is the base catalyst. Unlike the retaining glycosidases, inverting glycosidases do not form covalent intermediates and the reaction coordinate is made from only one step. The acid catalyst activates the glycosidic bond through hydrogen bonding and simultaneously the base

catalyst deprotonates the incoming nucleophile. The reaction proceeds in a S_N2-like manner and the hydrolyzed product has the opposite anomeric configuration.

Glycosidases using NAD^+ as a cofactor are found in GH4 and its NAD^+-dependent redox mechanism[23, 24] has been recently postulated (**Figure 1.12f**). In the first step of this catalytic mechanism, C-3 of the substrate is oxidized by hydride transfer to NAD^+. Formation of the carbonyl group at C-3 enhances the acidity of C-2 proton, which is abstracted by a basic residue of the enzyme and facilitates the departure of the aglycon group in the retro-1,4-addition manner. These processes produce the α,β-unsaturated intermediate, which is then subjected to the reverse reaction of the above-mentioned mechanism; i.e. 1,4-addition of water molecule at C-1 position, followed by reduction of C-3 carbonyl group by NADH.

As reviewed here, the reaction mechanisms of glycosidases are well studied to describe how they realize to accelerate hydrolytic reactions of glycosidic bonds by as much as 10^{17} fold. Understanding these mechanisms is essential to engineer glycosidases and modulate their enzymatic activities to develop novel biocatalysts.

1.4. Aim of the thesis

In the post-genomics era, the significance of glycans is ever increasing. Their metabolism is mainly controlled by two classes of enzymes: glycosyltransferases and glycosidases. In particular, the structures of glycosidases are well investigated and their catalytic functions are dissected precisely. Therefore, glycosidases have been applied in various ways in the field of bioengineering as well as chemistry. Such examples have demonstrated their utility when wisely applied.

In this thesis, several glycosidases were investigated as biocatalysts to prepare biologically relevant oligosaccharides or homogeneous glycoconjugates. First, development of a glycosynthase was explored to create a biocatalyst which can produce oligosaccharides without tedious protection/deprotection reactions. In the design of a novel glycosynthase, the issue of undesired oligomerization was addressed. Secondly, preparation of a highly pure glycoform of a therapeutically important glycoprotein, immunoglobulin G (IgG), was investigated using an endoglycosidase with high specificity toward IgG. This enzymatic method of glycan modification on IgG was extended to preparation of a novel type of antibody-drug conjugates. Finally, conversion of heterogeneous glycans on yeast-expressing glycoproteins into homogeneous forms was examined using glycan-trimming activities of exoglycosidases. It is expected that these researches will contribute to expanding the applicability of glycosidases as well as producing homogeneous materials of oligosaccharides and glycoconjugates.

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Chapter 2

Creation of an α-Mannosynthase from a Broad Glycosidase Scaffold

2.1. Introduction 27

2.2. Result and Discussion 32

2.2.1. A novel approach for glycosynthase 32

2.2.2. Expression of MalA 35

2.2.3. Substrate scope and reaction kinetics of MalA WT 40

2.2.4. Mutagenesis- Removal of nuclueophile aspartic acid 44

2.2.5. Preparation of donor substrates 50

2.2.6. Glycosynthase activity of MalA D320G 51

2.2.7. Optimization of mannosynthase reaction 53

2.2.8. Scope of glycosyl acceptor for the α-mannosynthase reaction 62

2.2.9. Kinetics of synthase reactions 75

2.2.10. Broadening donor scope 83

2.2.11. Modification of Glycoprotein 85

2.2.12. Mannosynthase activity of MalA WT 86

2.3. Conclusion 89

2.4. Experimental 91

2.5. Reference 121

2.1. Introduction

Arguably, the most striking landmark of synthetic organic chemistry is synthesis of vitamin B_{12}[1] by Woodward and Eschenmoser. The synthesis of this highly complex molecule has been encouraging to organic chemists by showing that synthesis of any complex molecules can be achievable when much intelligence is gathered and enough time is spent.[2] Now, in modern science, synthetic organic chemistry is a powerful tool for producing research materials, including biomolecules. Syntheses of DNAs and peptides are now routinely performed using solid phase synthesis. *De novo* syntheses of large proteins including glycoproteins[3, 4] also have been achieved since the advent of native chemical ligation.[5] However, there is one missing piece whose routine synthesis has not yet been achieved in spite of its biological importance; that is oligosaccharides.

An analogous strategy to that used for DNA or peptide has been investigated for synthesis of oligosaccharides using solid phase synthesis and some successful results have been reported.[6, 7] However, the current technology is far from delivering unrestricted synthesis of desired oligosaccharide. This significant difficulty for oligosaccharide synthesis comes from their structural features which DNA or peptide does not have (Chapter 1.2.1): several reaction sites with the similar reactivities in one monomer unit, non-linear structure and possible two stereoconfigurations between two monomer units.

Toward these difficulties, synthetic chemists have offered many solutions. Orthogonal protection/deprotection strategies[8] enable site-selective modification in one sugar unit and therefore control of branching structure. The issue of stereochemistry of glycosidic bonds may be resolved by neighboring effect or stereoelectronic control. However, repetitive protection

/deprotection scheme requires up to 7 steps in average for addition of one monosaccharide units.[9] This means that the overall yield of installing one sugar unit falls down to less than 50% even if all the reactions are achieved in 90% yield. A challenge against dependence on protecting groups is an emerging movement in organic chemistry[10] and this wave will reach the field of oligosaccharide synthesis. However, the current mainstream of oligosaccharide synthesis is still based on tedious protection /deprotection reactions.

On the other hand, Nature has developed a rich toolbox of catalysts for oligosaccharide synthesis and is producing and degrading a variety of oligosaccharides every day. Glycosyltransferases play the main role in constructing oligosaccharides (Chapter 1.2.3.1). They have very sophisticated selectivity and normally install one specific glycosyl donor to one specific acceptor.[11] Application of these enzymes to organic synthesis allows us to perform precise as well as straightforward synthesis of oligosaccharides when appropriate enzymes are available for target compounds. However, in chemistry, broad substrate scope is usually anticipated as a synthetic tool.

Glycosidases are responsible mainly for degradation of oligosaccharides in nature (Chapter 1.2.3.2) but these enzymes also have been utilized for chemical synthesis because they have broader substrate scope than glycosyltransferases. Additionally, there are examples where they succeeded in broadening substrate scope.[12] From this viewpoint, glycosidases are more promising candidates for application to organic chemistry. Other advantages to use glycosidases are that they are more stable and more readily available than glycosylransferases and they do not require costly sugar nucleotides as glycosyl donor.

Therefore, many investigations to apply glycosidases to synthetic chemistry have been performed. One of the most clever such examples would be glycosynthase. This artificial enzyme was designed and created by Withers *et al.* in 1998 for the first time.[13] The mechanism

of this smart device is depicted in **Figure 2.1**. In this methodology, one of the carboxylate residues, which acts as nucleophile, in the catalytic site, is deleted and an activated glycosyl donor which has a good leaving group, normally fluoride, with an opposite anomeric configuration to a native substrate is provided as glycosyl donor. When such substrates are bound to the nucleophile-less mutant of glycosidase, the complex (**Figure 2.1**, bottom middle) have equivalent structure to the covalent bound intermediate (**Figure 2.1**, top middle) in the native glycosidase catalytic cycle and incoming nucleophiles capture the glycon moiety. Even in the wild type glycosidase, the reaction gives transglycosylated products when the incoming nucleophile (glycosyl acceptor) is not water but alcohol (**Figure 2.1**, top leftward). However, all the reaction steps are reversible in the wild type enzyme and the formed transglycosylated products are again incorporated in the catalytic cycle. Therefore, in aqueous environments, the thermodynamic equilibration shifts to the hydrolyzed product (**Figure 2.1**, top rightward). On the other hand, when the nucleophile-less mutant is used in combination with the activated glycosyl donor, the glycosylation reaction practically takes place in the kinetically controlled manner. Therefore, when the glycosyl acceptor has good affinity to the glycosidase at the acceptor binding site or is used in excess amount, the hydrolytic side reaction can be suppressed and the glycosylation reaction proceeds dominantly (**Figure 2.1**, bottom leftward) to afford the

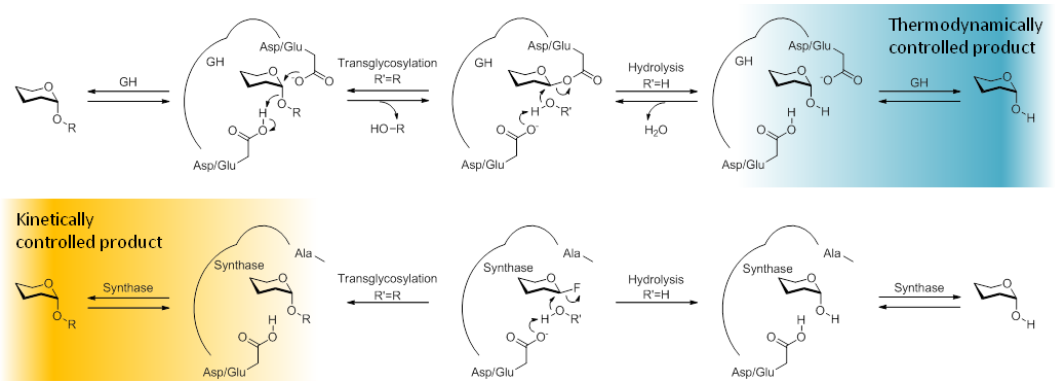


Figure 2.1. Reaction mechanism of glycosidase (top) and glycosynthase (bottom).

desired product in good yields.

Since the first report of the glycosynthase, a number of glycosynthases have been reported from various glycosidases (**Table 2.1**) and now a diverse range of glycosynthases are available. However, when carefully examined, these artificial enzymes are not equally distributed for all glycosidic bonds. Most of the present glycosynthases are for β-glycosides and those for some important glycosidic bonds including α-mannoside, are still missing. Another issue to develop glycosynthases is that they produce undesired oligomeric products[13-18] because most of them have native activity which trims oligosaccharide sequentially from the non-reducing terminus. Aiming at addressing these unmet needs, a novel method to develop glycosynthases will be discussed in this chapter.

Table 2.1. A list of glycosynthases derived from retaining glycosidases. The data are cited from the reference[19] with supplements of recent publications:a),[20] b),[21] c) this work[22] and d).[23] Yellow lines represent α-glycosynthases.

Entry	Donor	Acceptor	Glycosidase (Source)	Mutation	Product linkage
1	α-Glc-F	Ph-β-Glc, *p* NP-β-Glc, *o* NP-β-Glc, DNP-β-Glc(2F), 4MU-β-Glc, Ph-β-Glc(4OMe), *p* NP-β-Xyl, *p* NP-β-Man, *p* NP-β-Cel	Abg (*Agrobacterium* sp.)	E358A	β(1,3)/ β(1,4)
2	α-Glc-F	*p* NP-β-Glc, *p* NP-β-Man, *p* NP-β-Cel	Abg (*Agrobacterium* sp.)	E358S	β(1,3)/ β(1,4)
3	α-Glc-F	*o* NP-β-Glc	Ss (*Sulfolobus solfataricus*)	E387G	β(1,3)/ β(1,4)/ β(1,6)
4	α-Glc-F	Ph-β-Glc, Ph-β-Gal	Tt (*Thermotoga thermophilus*)	E338A	β(1,3)
5	α-Glc-F	Ph-β-Glc, *p* NP-β-Cel, Bn-β-Glc, 4-CN-Ph-β-Glc	Tt (*Thermotoga thermophilus*)	E338G	β(1,3)
6	α-Glc-F	Ph-β-Glc, Ph-β-Gal, *p* NP-b-Fuc, *p* NP-β-Cel, Me-β-Glc	Tt (*Thermotoga thermophilus*)	E338S	β(1,3)
7	α-Glc-F	*p* NP-β-Glc, *p* NP-β-Gal, *p* NP-β-Xyl, *p* NP-β-Man, *p* NP-β-Fuc, *p* NP-β-Lam, *p* NP-β-Cel	*Streptomyces* sp.	E383A	β(1,3)/ β(1,4)
8	α-Glc-F	*p* NP-β-Glc(4S), 4MU-β-Glc(4S)	Abg (*Agrobacterium* sp.)	E358G, E171A	β(1,4)
9	β-Glc-F	*p* NP-α-Glc, *p* NP-α-Xyl, *p* NP-β-Glc, *p* NP-α-Man	Sp (*Saccharomyces pombe*)	D481G	α(1,4)/ α(1,6)
10[a)]	α-Glc-F	*p* NP-β-Fuc, *p* NP-β-Gal, *p* NP-β-Glc, *p* NP-β-Man, *p* NP-β-Xyl, *p* NP-β-Cel	BGlu1 (rice)	E386G	β(1,3)
11	α-Gal-F	*p* NP-β-Glc, *p* NP-β-Xyl, DNP--β-Glc(2-Cl), *p* NP-β-Man, *p* NP-β-Cel, Ph-β-Cel(4OMe)	Abg (*Agrobacterium* sp.)	E358A	β(1,3)/ β(1,4)
12	α-Gal-F	Ph-β-Glc	Tt (*Thermotoga thermophilus*)	E338A	β(1,3)
13	α-Gal-F	Ph-β-Glc, Ph-β-Gal	Tt (*Thermotoga thermophilus*)	E338S	β(1,3)
14	α-Gal-F	Ph-β-Glc, *p* NP-β-Glc, Bn-β-Glc, 4-CN-Ph-β-Glc, Ph-β-Glc(1S)	Tt (Thermotoga thermophilus)	E338G	β(1,3)/ β(1,6)
15	α-Gal-F	Glc (6-Bn), Glc(6-Bz), Glc(6-*p* -nitrobenzyl)	Abg (*Agrobacterium* sp.)	E358S	β(1,2)/ β(1,3)
16	α-Gal-F	*p* NP-β-Glc, *p* NP-Man, *p* NP-β-Cel, *p* NP-GlcNAc	Abg (*Agrobacterium* sp.)	E358S	β(1,3)/ β(1,4)
17	α-Gal-F	Glc-2-AP, Glc-3-AP, Glc-4-AP (Resin bound)	Abg (*Agrobacterium* sp.)	E358G	β(1,3)/ β(1,4)
18	α-Gal-F	*p* NP-β-Glc, *p* NP-β-Gal, *p* NP-β-Xyl, *p* NP-β-Man	*Streptomyces* sp.	E383A	β(1,3)/ β(1,4)
19	α-Gal-F	*p* NP-β-Glc, *p* NP-β-Cel, Ph-β-Glc	LacZ (*Escherichia coli*)	E537S	β(1,6)
20[b)]	β-Gal-N_3	pNP-α-Glc, *p* NP-α-Xyl, *p* NP-β-Xyl, *p* NP-α-Man	TmGalA (*Thermotoga maritima*)	D327G	α(1,2)/ α(1,3)/ α(1,4)/ α(1,6)
21[a)]	α-Gal-F	*p* NP-β-Cel	BGlu1 (rice)	E386G	N.D.
22	α-Man-F	*p* NP-β-Man, DNP-β-Man(2F), *p* NP-β-Glc, *p* NP-β-Xyl, *p* NP-β-Cel, *p* NP-β-Gent	Man2A (*Cellulomonas fimi*)	E519S	β(1,3)/ β(1,4)
23	α-Man-F	*p* NP-β-Glc	Abg (*Agrobacterium* sp.)	2F6	β(1,3)/ β(1,4)
24[a)]	α-Man-F	*p* NP-β-Cel	BGlu1 (rice)	E386G	β(1,4)
25[c)]	β-Man-F	*p* NP-β-Glc, *p* NP-α-Glc, *p* NP-α-Man, *p* NP-α-Xyl, *p* NP-α-D-Rha, *p* NP-α-D-Fuc, *p* NP-α-Mal, *p* NP-β-Cel, *p* NP-α-(1,3)-mannobiose, *myo* -inositol	MalA (*Sulfolobus solfataricus*)	D320G	α(1,2)/ α(1,3)/ α(1,4)
26	α-Xyl-F	*p* NP-β-Glc, *p* NP-β-Xyl, *p* NP-β-$(Xyl)_2$	Abg (*Agrobacterium* sp.)	2F6	β(1,3)/ β(1,4)
27	α-Xyl-F	*p* NP-β-Glc, *p* NP-β-Xyl, *p* NP-β-Man, α-Xyl-F	Gs (*Geobacillus stearothermophilus*)	E335G	β(1,3)/ β(1,4)
28	α-Xyl-2-F	*p* NP-β-Xyl, Xyl(2SBn)	Cf (*Cellulomonas fimi*)	E235G	β(1,4)
29[a)]	α-Xyl-F	*p* NP-β-Cel	BGlu1 (rice)	E386G	N.D.
27	β-Fuc-N_3	*p* NP-β-Xyl, *p* NP-β-GlcNAc, *p* NP-β-Gal	Ss (*Sulfolobus solfataricus*)	D242S	α(1,2)/ α(1,3)/ α(1,4)/α(1,6)
28	β-Fuc-N_3	*p* NP-β-Xyl	Ss (*Sulfolobus solfataricus*)	D242G	α(1,3)/ α(1,4)
29[a)]	α-D-Fuc-F	pNP-β-Cel	BGlu1 (rice)	E386G	β(1,4)
30[d)]	β-Fuc-F	Lac, Lacto-N-biose, LacNAc, Lac(2'Fuc), Lacto-N-tetraose	AfcB (*Bifidobacterium bifidum*)	D703S	α(1,3)/ α(1,4)
31[a)]	α-D-Ara-F	*p* NP-β-Cel	BGlu1 (rice)	E386G	N.D.
32	α-GlcA-F	*p* NP-β-Glc, *p* NP-β-Xyl, pNP-β-Cel, *p* NP-β-GlcA	Tm (*Thermotoga maritima*)	E476A	β(1,3)
33	α-GalA-F	*p* NP-β-Glc, *p* NP-β-Xyl, *p* NP-β-Cel	Tm (*Thermotoga maritima*)	E476A	β(1,3)
34	α-Lac-F	*p* NP-β-Cel	Cel5A (*Clostridium cellulolyticum*)	E307G	β(1,3)
35	α-Lac-F	N^{I}-acetyl-2^{II}-azido-chitobiose	Cel7B (*Humicola insolens*)	E197A, H209A,	β(1,3)
36	α-Lac-F	Bn-Glc, Bn-Xyl, *p* NP-Man(1S), Me-β-Cel, Me-β-Cel($C2^{I}$-Br), GlcN-β(1,4)-GlcNAc, Bn-β-Lam	Cel7B (*Humicola insolens*)	E197A	β(1,4)
37	α-Cel-F	*p* NP-β-Cel	Cel5A (*Clostridium cellulolyticum*)	E307G	β(1,3)/ β(1,4)
38	α-Lam-F	MU-β-Glc, MU-β-Lam, MU-β-Cel	Blg (*Bacillus licheniformis*)	E134A	β(1,4)
39	α-Lam-F	α-Lam-F, *p* NP-β-Xyl, *p* NP-β-Gal, *p* NP-β-Cel, *p* NP-β-Gent	Hv (*Hordeum vulgare*)	E231G	β(1,3)
40	α-Mannobiosyl-F	*p* NP-β-Glc, *p* NP-β-Man, *p* NP-β-Xyl, *p* NP-β-Cel	Man26A (*Culicoides japonicus*)	E320G	β(1,4)

2.2. Result and Discussion

2.2.1. A novel approach for glycosynthase

Conventionally, a glycosynthase to attach a specific monosaccharide unit is developed from the corresponding retaining exoglycosidase[13] which can catalyze hydrolysis of the same glycon unit. However, when exoglycosidases which trim off non-reducing terminal sugars sequentially (**Figure 2.2a**) are converted to glycosynthases, they sometimes face the issue of formation of oligomerized products,[13-18] resulting from the sequential addition of monosaccharide units (**Figure 2.2b**). Therefore, we need additional devices to suppress undesired oligomerization for application of such enzymes to controlled oligosaccharide synthesis.

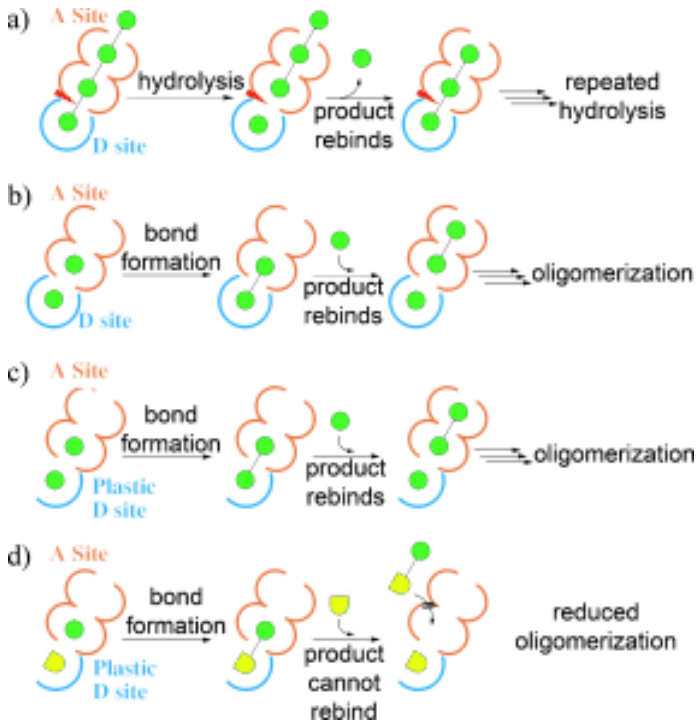


Figure 2.2. Design of a glycosynthase with reduced oligomerization. A glycosidase which trims oligosaccharides from non-reducing termini (a) results in a glycosynthase with activity of oligomerization (b). When the enzyme has plasticity at its D site (c,d), the selection of an appropriate donor can prevent oligomerization (d). The figure is adapted with permission from Yamamoto, K. *et al.*, *Angew. Chem. Int. Ed.* **2012**, *51* (30), 7449-7453. Copyright 2012 John Wiley and Sons.[22]

Theoretically, suppression of oligomerization can be achieved if we can find a set of a glycosyl donor and an enzyme which have high affinity at the donor binding site (D-site in **Figure 2.1**) of the enzyme and poor affinity at the acceptor binding site (A-site in **Figure 2.1**). Validation of this concept is demonstrated by the very first example of glycosynthase.[13] In this study, the β-glycosidase from *Agrobacterium sp.* was used whose native activity is expected to be β-glucosidase,[24] though this enzyme can accept both galactose and glucose at the D-site (**Figure 2.1 c**, **d**). However, the results of the synthase reactions for these two substrates are contrasting; only monoglycosylation was observed when galactose was used as donor (**Figure 2.1 d**) whereas the reaction with glucose gave oligomerized compounds (**Figure 2.1 c**). Our concept is realized by this combination of galactose donor and β-glucosidase from *Agrobacterium sp.*. As exemplified in this example, one of the key points is plasticity which allows an enzyme to accommodate several substrates flexibly in the D-site

A priori prediction of detailed substrate scope of enzymes is difficult. However, glycosidases are enzymes whose enzymatic data are well described. Therefore, application of our concept in a predictive manner would be feasible by examining literature. Among the described glycosidases, our attention was focused on an interesting feature of GH-31 glucosidases.[25, 26] Glycosidases in this family display activities toward 2-deoxyglucosides as well as intact glucosides (**Table 2.2**). This result implies that these enzymes do not use 2-OH of the substrate as recognition handle and the epimer of glucose at 2-OH, i.e. mannose, may be a substrate to this class of enzymes. If so, an enzyme from this family is a candidate to be developed as an α-mannosynthase, which had not yet been prepared.

A campaign was initiated using MalA, an α-glucosidase from *Sulfolobus solfataricus*, because enzymes from such thermophilic organisms are easy to handle due to their stability. Moreover, at this time, MalA was the only α-glucosidase from unicellular organisms, which had a solved

X-ray crystal structure,[27] from which rational design would be possible when needed (now another crystallographic structure[28] is available for GH-31 α-glucosidase from an lower organism).

Table 2.2. Relative activities of GH13- and GH31- α-glycosidases toward the deoxyglucose substrates. The data are from the reference.[25] (ND: not detected)

Source of α-glucosidase	CAZy	Relative rate of hydrolysis (%)		
		Substrate		
		p NP-α-Glc	*p* NP-α-2-deoxy-Glc	*p* NP-α-3-deoxy-Glc
Saccharomyces cerevisiae	GH13	100	ND	ND
Bacillus stearothermophilus	GH13	100	ND	ND
honey bee-I	GH13	100	ND	ND
honey bee-II	GH13	100	ND	ND
rice	GH31	100	175	ND
sugar beet	GH31	100	244	ND
flint corn	GH31	100	231	3.7
Aspergillus niger	GH31	100	259	11.9

2.2.2. Expression of MalA

The *malA* gene encoding MalA was obtained from GenBank under accession No. AAC38215. The DNA sequence was synthesized by GenScript and subcloned into an expression vector pET-29a(+) using *NdeI* and *XhoI* restriction endonucleases. The resultant plasmid vector was

```
 -3 CAT
  1 ATGCAGACAA TAAAAATATA CGAGAACAAA GGCGTTTACA AAGTAGTTAT AGGAGAACCA
 61 TTTCCCCCCA TAGAATTCCC ACTTGAGCAA AAGATATCAT CGAATAAATC TTTATCAGAG
121 TTGGGTTTAA CAATAGTTCA ACAAGGTAAC AAGGTTATTG TCGAGAAATC ATTGGATTTG
181 AAAGAGCACA TTATAGGATT GGGAGAGAAG GCGTTTGAGT TGGATAGAAA GAGGAAAAGG
241 TATGTGATGT ATAACGTTGA CGCTGGGGCT TATAAGAAAT ATCAAGATCC ACTTTACGTT
301 AGTATACCCT TATTTATATC AGTGAAAGAC GGCGTTGCAA CTGGTTACTT CTTCAACTCA
361 GCTTCTAAAG TGATCTTCGA CGTGGGACTT GAGGAATACG ATAAAGTAAT TGTTACAATT
421 CCAGAGGACT CAGTAGAGTT TTACGTGATT GAAGGGCCAA GAATTGAGGA CGTTCTAGAG
481 AAATACACGG AGCTTACCGG AAAACCTTTC CTACCTCCCA TGTGGGCTTT CGGTTACATG
541 ATATCACGCT ACTCTTACTA CCCCCAGGAT AAGGTTGTTG AGTTAGTAGA TATAATGCAA
601 AAGGAGGGTT TTAGAGTAGC TGGAGTATTC TTAGATATAC ACTACATGGA CTCCTATAAG
661 TTATTTACAT GGCATCCTTA TAGGTTCCCA GAACCTAAAA AGCTAATTGA CGAATTACAC
721 AAGAGAAACG TTAAGCTAAT TACAATAGTT GACCACGGAA TAAGGGTTGA TCAGAATTAT
781 TCACCATTTC TTTCCGGAAT GGGAAAATTC TGTGAGATTG AAAGTGGTGA ACTATTCGTA
841 GGTAAAATGT GGCCTGGTAC TACTGTCTAT CCAGACTTCT TCAGGGAGGA TACTAGAGAA
901 TGGTGGGCTG GGTTAATCTC CGAATGGCTT TCACAAGGAG TTGATGGTAT TTGGCTAGAC
961 ATGAATGAAC CAACTGACTT CTCTAGGGCT ATTGAGATCA GAGACGTTTT ATCTTCGTTA
1021 CCCGTACAGT TCAGAGATGA TAGACTTGTT ACCACTTTTC CAGATAACGT AGTTCACTAC
1081 TTGAGGGGAA AGAGGGTTAA ACACGAAAAA GTTAGAAATG CTTATCCTTT ATATGAGGCT
1141 ATGGCAACGT TTAAGGGGTT TAGGACAAGC CATAGGAATG AAATATTTAT CTTGAGTAGA
1201 GCCGGTTATG CCGGAATACA AAGATACGCA TTCATCTGGA CTGGTGATAA TACCCCTTCA
1261 TGGGATGATT TGAAGCTTCA ACTACAATTG GTTCTCGGCT TATCGATTTC TGGTGTACCA
1321 TTTGTAGGTT GTGATATAGG TGGATTTCAA GGCAGGAACT TCGCGGAAAT TGACAACTCT
1381 ATGGATTTAT TAGTCAAATA TTATGCTTTA GCCTTGTTCT TCCCCTTCTA TAGGTCACAC
1441 AAGGCAACTG ATGGTATAGA TACGGAACCA GTTTTCCTGC CAGATTACTA TAAGGAGAAA
1501 GTAAAGGAAA TCGTGGAGTT GAGGTATAAG TTCTTACCCT ATATTTATTC CTTAGCTTTA
1561 GAGGCTAGTG AGAAGGGACA TCCGGTAATT AGACCTCTAT TTTACGAATT CCAGGATGAT
1621 GACGACATGT ATAGAATAGA AGACGAGTAT ATGGTTGGTA AGTATTTGCT TTACGCTCCA
1681 ATTGTAAGTA AAGAGGAGAG TAGGTTAGTA ACATTACCTA GAGGTAAGTG GTACAATTAC
1741 TGGAATGGCG AGATAATAAA CGGTAAGAGT GTTGTTAAGT CTACTCATGA GTTGCCAATT
1801 TACTTGAGAG AAGGATCAAT AATCCCGTTG GAGGGTGACG AGTTAATAGT TTACGGTGAG
1861 ACCTCGTTCA AGCGTTACGA TAATGCTGAA ATTACCTCCT CAAGTAATGA AATTAAGTTT
1921 TCAAGGGAGA TTTATGTATC TAAGCTAACT ATCACATCAG AGAAACCAGT GAGCAAGATA
1981 ATAGTTGACG ATAGTAAGGA AATTCAAGTA GAGAAGACAA TGCAAAACAC TTACGTTGCT
2041 AAGATTAATC AAAAAATTAG GGGAAAGATT AACCTAGAGC TCGAG
```

Figure 2.3. The gene sequence of the modified *malA*. Nucleic acids 1-2079 correspond to the wild type gene sequence. The stop codon (TAG) was removed and the restrictioin sites for *NdeI* and *XhoI* (shown in yellow) were added.

termed as pET-29a(+)-*malA*, which confers kanamycin resistance to the host cell and appends a His_6-tag to the C-terminus of the expresse protein.

Then, BL21(DE3) strain of *E.coli* was transformed by pET-29a(+)-*malA*. The expression of MalA was first performed by IPTG induction. After lysis, a significant amount of the expressed protein was found in the inclusion body, but further purification was performed only for the soluble fraction. Coarse purification[27] was performed by heating up the supernatant of the lysate to 70°C and removing precipitation by centrifugation. To the resultant solution was added ammonium sulphate to precipitate MalA. The precipitate was dissolved in a buffer and the solution was applied to NiNTA affinity column chromatography (**Figure 2.4**). The target protein was successfully purified and the yield was 1.6 mg/ L culture. MalA appears on the SDS-PAGE as aggregated forms when it is not denatured thoroughly.[29]

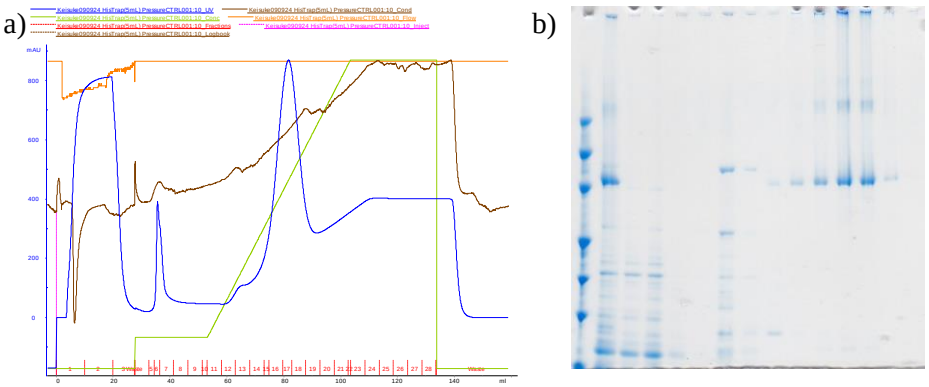


Figure 2.4. Expression of MalA WT using IPTG induction and its purification with NiNTA affinity column chromatography. (a) Chromatogram of the UV trace (blue line) (b) SDS-PAGE analysis of the fractions.

Next, other expression conditions were tested to enhance expression efficiency. Autoinduction[30] as well as IPTG induction was investigated at 28°C and 37°C. OD_{600} and pH of the media were monitored during the course of the culture (**Figure 2.5**). For IPTG induction, IPTG (final concentration: 2 mM) was added when OD_{600} became 0.6-0.9. The resultant density of cells was significantly different between these two induction systems (**Figure 2.5**). OD_{600} of

IPTG induction reached a plateau at less than 3.0 in the cultures at both temperatures, whereas OD_{600} of autoinduction resulted in 7.8 and 5.8 in the 28°C- and 37°C-culture, respectively. The change of pH values in each culture indicates one of the reasons for these results. Both IPTG cultures gradually became basic, ending up in more than 8.0. Conversely, an autoinduction culture at 37°C resulted in acidic pH 5.6. This shift toward acidic pH has been reported previously and is expected to be observed when the acid generated by the metabolism of glucose exceeds the buffering capacity of the medium.[30] On the other hand, an autoinduction culture at 28°C keeps its pH between 6.5 and 7.0 throughout the cell growth, maintaining a comfortable condition for cells. The weight of cell pellets from each culture is consistent to the results of OD_{600}; 4 g from IPTG inductions, while 15 g from autoinduction at 28°C.

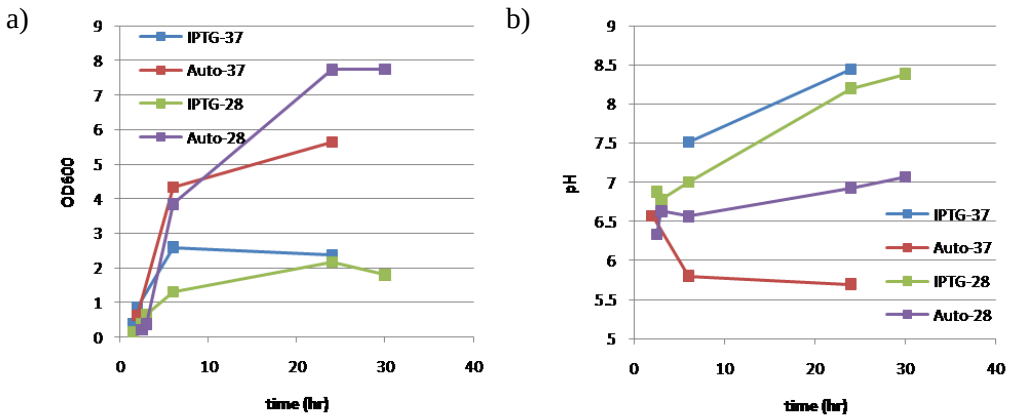


Figure 2.5. Comparison of autoinduction and IPTG-induction. OD_{600} (a) and pH (b) of each culture was monitored.

Purification was performed in the same way as mentioned above and the yields were determined to be 2.4 mg/L culture from IPTG-induction at 37°C and 14.2 mg/L culture from autoinduction at 28°C. This enhancement of expression efficiency by autoinduction is in the range of typical results (7-fold).[30] Later observations confirmed that incubation temperature (28°C or lower) and good aeration (500-mL culture medium in a 2-L baffled flask) were critical

for high efficiency of protein production. Otherwise the culture medium became acidic (pH 4-5) and the cell growth halted in the earlier stage than under the optimal cases, although still the expression efficiency was better than IPTG induction. Therefore, autoinduction at 28°C was used as the first choice of protein expression throughout this thesis.

Purification of MalA expressed in autoinduction is shown in **Figure2.6**. After coarse purification by heating and ammonium sulphate precipitation, NiNTA purification and gel filtration was performed. The elution time of MalA was far quicker than that expected from the molecular weight of the monomer (81 kDa). This result implies it forms aggregates and fits well with the previous observation in the SDS-PAGE[29] and a hexamer form found in X-ray crystallography.[27]

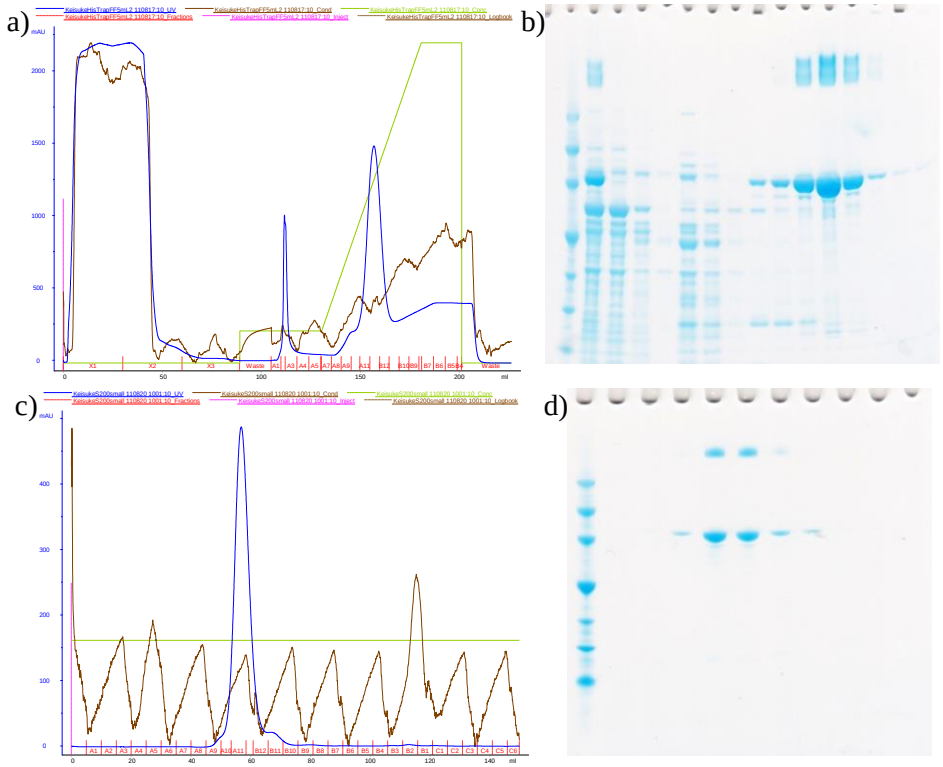
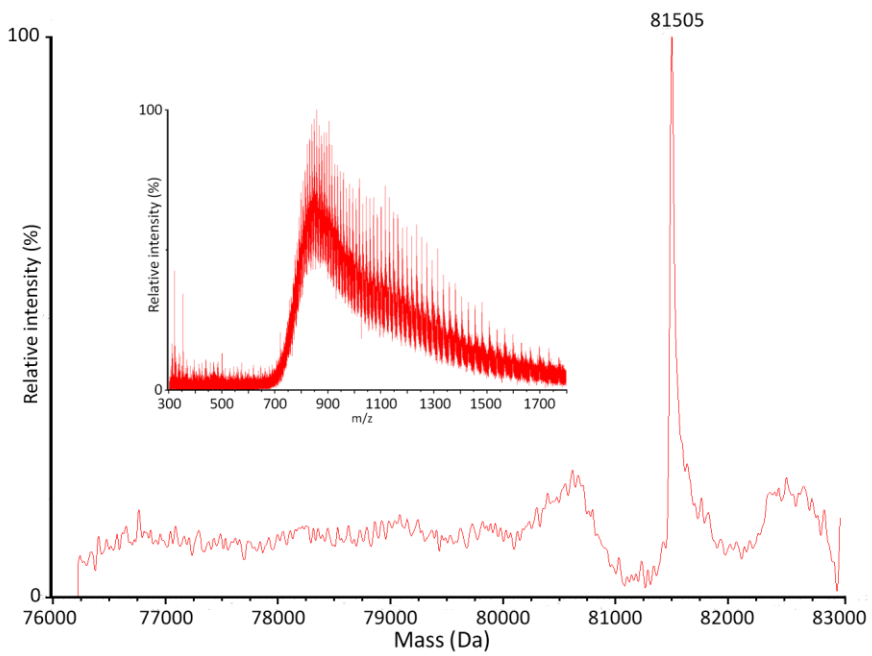


Figure 2.6. Expression of MalA WT using autoinduction and its purification with NiNTA affinity column chromatography (a,b) and gel filtration (c,d). (a,c) Chromatogram of the UV trace (blue line) (b,d) SDS-PAGE analysis of the fractions.

Purified MalA WT was analysed by ESI-MS and the observed molecular weight (81,505 Da) coincided well with the calculated one (81,506 Da).

a)



b)

```
  1 MQTIKIYENK GVYKVVIGEP FPPIEFPLEQ KISSNKSLSE LGLTIVQQGN KVIVEKSLDL
 61 KEHIIGLGEK AFELDRKRKR YVMYNVDAGA YKKYQDPLYV SIPLFISVKD GVATGYFFNS
121 ASKVIFDVGL EEYDKVIVTI PEDSVEFYVI EGPRIEDVLE KYTELTGKPF LPPMWAFGYM
181 ISRYSYYPQD KVVELVDIMQ KEGFRVAGVF LDIHYMDSYK LFTWHPYRFP EPKKLIDELH
241 KRNVKLITIV DHGIRVDQNY SPFLSGMGKF CEIESGELFV GKMWPGTTVY PDFFREDTRE
301 WWAGLISEWL SQGVDGIWLD MNEPTDFSRA IEIRDVLSSL PVQFRDDRLV TTFPDNVVHY
361 LRGKRVKHEK VRNAYPLYEA MATFKGFRTS HRNEIFILSR AGYAGIQRYA FIWTGDNTPS
421 WDDLKLQLQL VLGLSISGVP FVGCDIGGFQ GRNFAEIDNS MDLLVKYYAL ALFFPFYRSH
481 KATDGIDTEP VFLPDYYKEK VKEIVELRYK FLPYIYSLAL EASEKGHPVI RPLFYEFQDD
541 DDMYRIEDEY MVGKYLLYAP IVSKEESRLV TLPRGKWYNY WNGEIINGKS VVKSTHELPI
601 YLREGSIIPL EGDELIVYGE TSFKRYDNAE ITSSSNEIKF SREIYVSKLT ITSEKPVSKI
661 IVDDSKEIQV EKTMQNTYVA KINQKIRGKI NLELEHHHHH H (Cald MW: 81506 Da)
```

Figure 2.7. a) ESI-MS of the purified MalA WT. The deconvoluted peak is shown in the main panel and the ion series is shown in the inset. b) Amino acid sequence of MalA WT. Additional sequence due to the gene modification is colored in yellow.

2.2.3. Substrate scope and reaction kinetics of MalA WT

The enzymatic activity[29] of the expressed MalA was confirmed by hydrolysis of *p*NP-α-D-Glc (**Scheme 2.1**). The reaction was performed under the near-optimal condition in 100 mM sodium acetate (pH 4.5) at 85°C and the concentration of the released *p*-nitrophenol was monitored. The kinetic parameters (**Table 2.4**) were determined by Michaelis-Menten analysis (**Figure 2.9a**) and it turned out these obtained values are comparable to those previously reported.[27]

Scheme 2.1. Kinetic assay of hydrolysis of *p*NP-α-Glc by MalA WT.

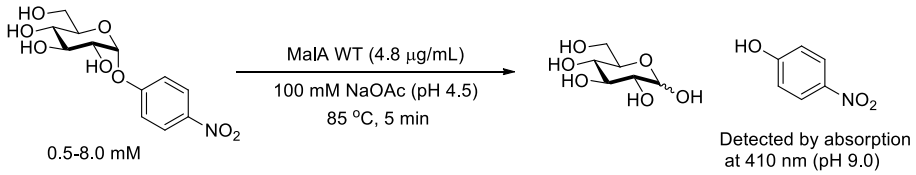


Table 2.4. Kinetic parameters of hydrolytic reaction of *p*NP-sugar substrates with MalA WT. a) Data from the reference.[27]

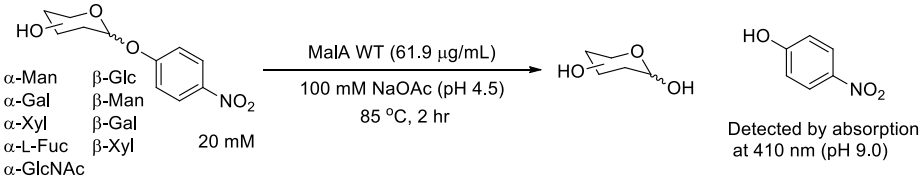
Substrate	K_M (mM)	k_{cat} (s^{-1})	k_{cat} / K_M (s^{-1} mM^{-1})
*p*NP-α-D-Glc	1.74 ± 0.20	38.5 ± 1.6	22.1
	2.0 ± 0.4 [a)]	33 ± 2 [a)]	16.5 [a)]
*p*NP-α-D-Man	3.82 ± 0.30	0.0926 ± 0.0028	0.0242
*p*NP-α-D-Gal	> 80	ND	0.00293 ± 0.00014
*p*NP-α-D-Xyl	> 20	ND	0.00167 ± 0.00006
	> 20 [a)]	ND	0.0016 ± 0.0005 [a)]

Next, the activity toward other *p*NP-sugar substrates, including *p*NP-α-D-Man, was investigated under more intensive conditions (higher concentrations of the substrates and the enzyme, longer reaction time, **Scheme 2.2**). Though it was previously reported that the facile

substrate screening could not detect any evidence of enzymatic activities of GH-31 α-glucosidases toward a mannose substrate,[26] pleasingly MalA showed the hydrolytic activity to *p*NP-α-D-Man (**Figure 2.8**). This finding suggests that hydrolytic activities of GH-31 α-glucosidases towards 2-deoxyglucose can be extended to mannose, a C2-position epimer of glucose. On the other hand, another glucose analogue at 2-OH, GlcNAc, was not hydrolyzed probably due to the steric bulk of an acetamide group.

As reported previously,[27] MalA also showed its hydrolytic acitivty to *p*NP-α-D-Xyl (**Figure 2.8**). Additionally, *p*NP-α-D-Gal showed the similar level of potency as a substrate for MalA (**Figure 2.8**). The other α-configured substrate, *p*NP-α-L-Fuc, was not cleaved probably due to

Scheme 2.2. Hydrolytic activity of MalA WT to various *p*NP-sugars.



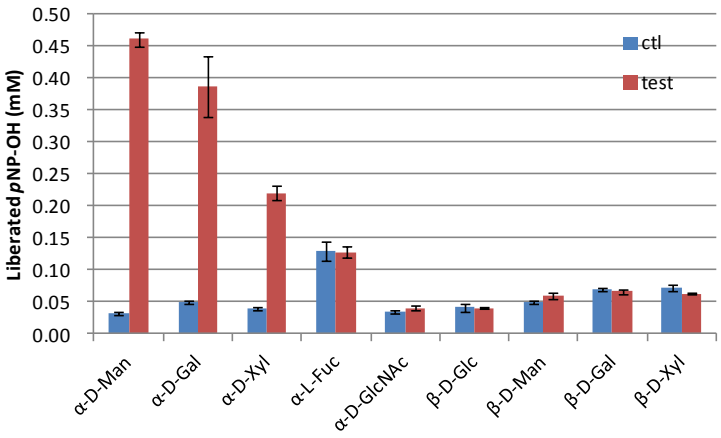
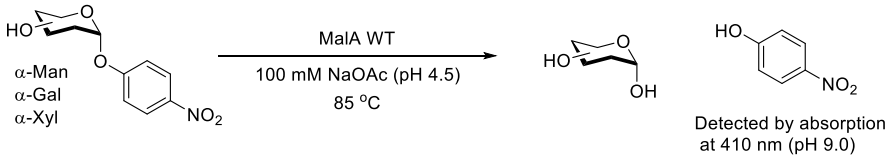


Figure 2.8. Hydrolytic activity of MalA WT to various *p*NP-sugars. The reactions were performed for 20 mM *p*NP-sugars at 85°C using 61.9 μg/mL MalA in 100 mM NaOAc buffer (pH 4.5). In all cases, control reactions were performed in the absence of the enzyme. After incubating for 2 hours, the concentration of the released *p*-nitrophenol was determined by UV absorption at 410 nm in pH 9.

the discrete L-configuration. Reasonably, this enzyme did not hydrolyze any β-sugars.

Michaelis-Menten analysis was then performed for these three substrates (*p*NP-α-D-Man, -Gal, and -Xyl, **Scheme 2.3**, **Figure 2.9b-d**, **Table 2.4**). In case of *p*NP-α-D-Gal and *p*NP-α-D-Xyl, the values of K_M could not be determined because saturation of the reaction rate was not observed even at the maximum concentration of these compounds (80 mM and 20 mM, respectively). On the other hand, K_M of *p*NP-α-D-Man was determined to be 3.82 mM. This value is comparable to that of *p*NP-α-D-Glc (1.74 mM), while the turnover number decreased significantly for *p*NP-α-D-Man (k_{cat}= 0.0926 s^{-1} vs k_{cat} (*p*NP-α-D-Glc)= 38.5 s^{-1}).

Scheme 2.3. Kinetic assay of hydrolysis of *p*NP-α-D-Man. -Gal, and -Xyl by MalA WT.



This low value of K_M for *p*NP-α-Man may be explained by slow reaction rate of deglycosylation step in the reaction mechanism (**Figure 1.12c**) and accumulation of the covalently bound intermediate may result in the "apparent" high affinity of this substrate. However, the catalysis of GH-31 enzymes is known to have its rate-limiting step in glycosylation step rather than deglycosylation step[31, 32] on contrary to β-glycosidase. In such cases, K_M value directly reflects binding affinity of substrates to enzymes. Therefore, it is likely that the low K_M value of *p*NP-α-D-Man suggests its good binding affinity to MalA.

This mismatched combination of the substrate and the enzyme, i.e. mannose and α-glucosidase, is what we desired for developing a glycosynthase with suppressed oligomerization and we were encouraged to develop an α-mannosynthase from this enzyme.

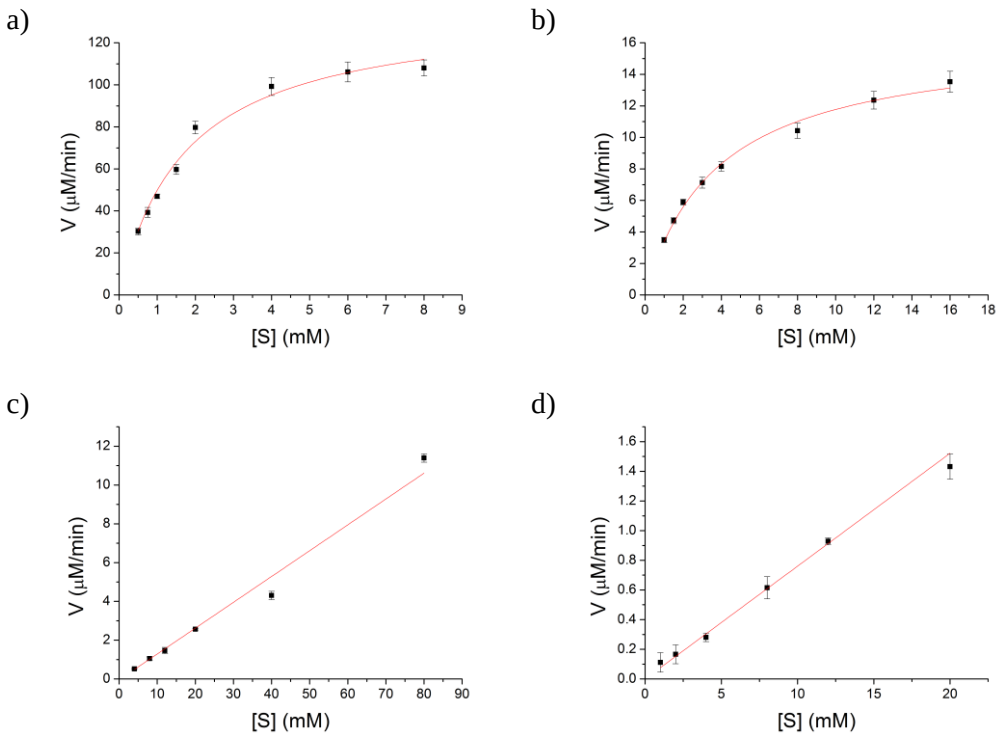


Figure 2.9. Michaelis-Menten analysis of the hydrolytic reactions by MalA. a) *p*NP-α-D-Glc, b) *p*NP-α-D-Man, c) *p*NP-α-D-Gal, d) *p*NP-α-D-Xyl. a) *p*NP-α-D-Glc (0.5-8.0 mM) was hydrolyzed with 4.8 μg/mL MalA at 85°C in 100 mM NaOAc buffer (pH 4.5). The reaction was incubated for 5 minutes and the reaction rate was determined by monitoring the concentration of the released *p*-nitrophenol by UV absorption at 410 nm in pH 9. b) *p*NP-α-D-Man (1.0-16.0 mM) was hydrolyzed with 240 μg/mL MalA at 85°C in 100 mM NaOAc buffer (pH 4.5). The reaction was incubated for 1 hour and the reaction rate was determined in the same way for *p*NP-α-D-Glc. c), d) *p*NP-α-D-Gal (4.0-80.0 mM) or *p*NP-α-D-Xyl (1.0-20.0 mM) was hydrolyzed with 65 μg/mL MalA at 85°C in 100 mM NaOAc buffer (pH 4.5). The reaction was incubated for 1 hour and the reaction rate was determined in the same way for *p*NP-α-D-Glc.

2.2.4. Mutagenesis- Removal of nuclueophile aspartic acid

MalA has the nucleophile and acid/base at D320 and D416, respectively. To develop a glycosynthase, a nucleophile-less mutant D320G was prepared. Mutatgenesis was performed using QuikChange® II XL Site-Directed Mutagenesis Kit. Primers were designed using PrimerX program[33] and its physical properties were checked with Primer Design Tool on the Sigma-Aldrich website.[34] The primers were designed so as to satisfy the following criteria: i) melting point is 70-80 ℃, ii) GC content is >40%, iii) primer dimer formation is not expected, iv) probability of secondary structure formation is low to moderate. The primer sequences for the D320G mutatnt are shown in **Table 2.5** and the mutated plasmid was successfully obtained (**Figure 2.10**).

Table 2.5. Primer sequences and their physical properties for site-directed mutagenesis at D320G.

Mutant		Primer sequence	Tm[a] (°C)	GC[b] (%)	PD[c]	SS[d]
D320G	forward	5' –GATGGTATTTGGCTA G**G**C ATGAATGAACCAAC–3'	74.5	43.7	No	moderate
	reverse	5' –GTTGGTTCATTCAT G**C**C TAGCCAAATACCATC–3'	74.5	43.7	No	moderate
WT	forward	5' –GGAGTTGATGGTATTTGGCTA **GAC** ATGAATGAACCAACTGACTTC–3'				
	reverse	5' –GAAGTCAGTTGGTTCATTCAT **GTC** TAGCCAAATACCATCAACTCC–3'				

a) melting point, b) GC content, c) expectation for primer dimer formation, d) probability of secondary structure formation.

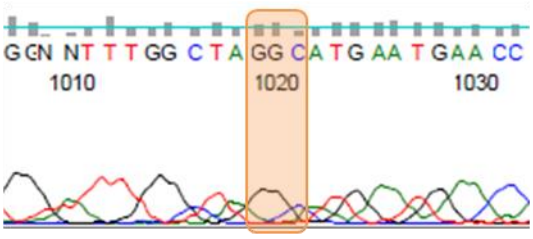


Figure 2.10. DNA sequencing of MalA D320G mutant.

The expression and purification of MalA D320G was performed in the same manner as the wild type. Briefly, MalA D320G was expressed in the BL21(DE3) strain using autoinduction. After lysis and removal of unwanted proteins by heating, the desired MalA D320G was collected by ammonium sulphate precipitation. The protein was purified by NiNTA column and

gel filtration (**Figure 2.11**). The yield of MalA D320G was 14 mg/mL and thus purified MalA D320G was analysed by ESI-MS to find the molecular weight of 81,443 Da, coincided well with the calculated value (81,448 Da).

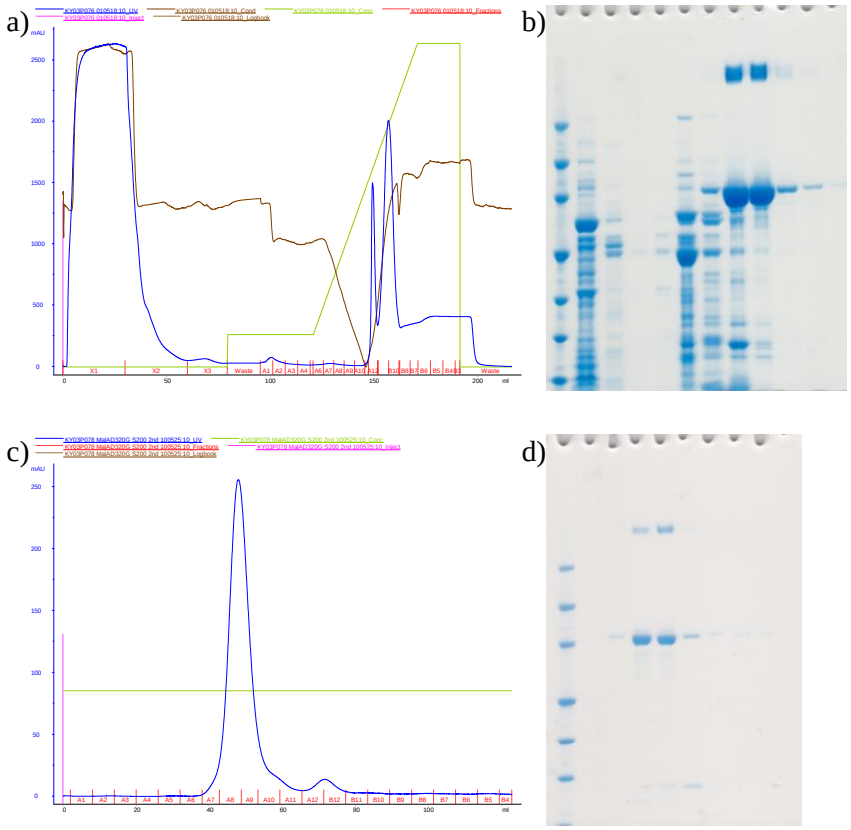
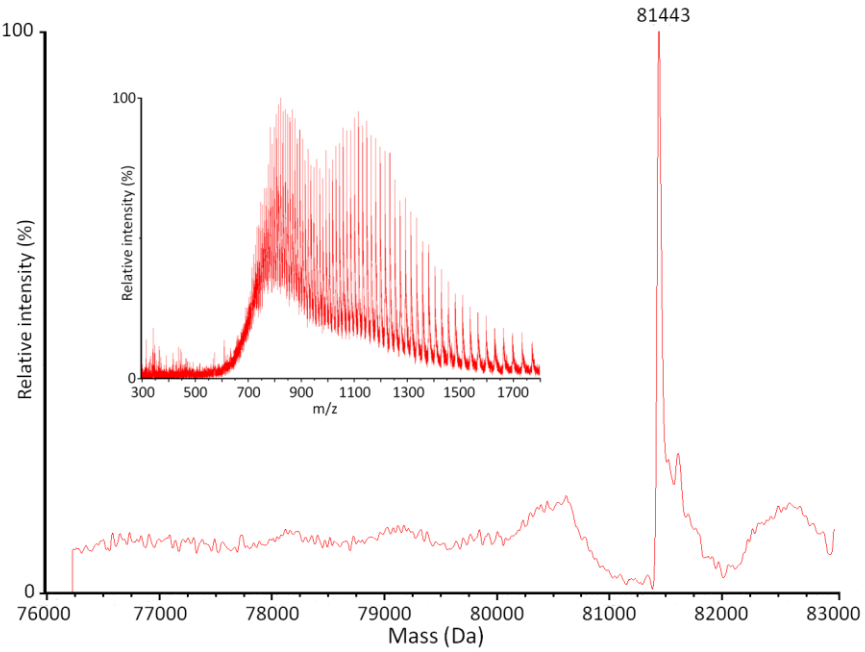


Figure 2.11. Purification of MalA D320G mutant by NiNTA column (a,b) and gel filtration (c,d). (a,c) Chromatogram of the UV trace (blue line) (b,d) SDS-PAGE analysis of the fractions.

With the nucleophile-less mutant in hand, its hydrolytic activity to *p*NP-sugar substrates was tested (**Scheme 2.4**). As expected, the hydrolytic activity decreased significantly and this activity was observed only for *p*NP-α-D-Glc under the test condition, while none of the other substrates were hydrolyzed by this mutant.

a)

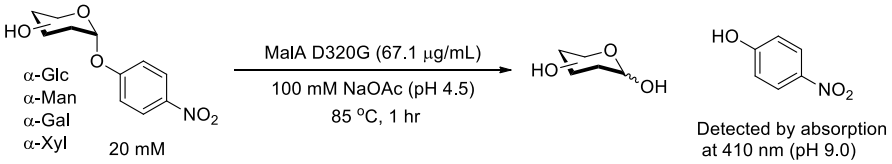


b)

```
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 61 KEHIIGLGEK AFELDRKRKR YVMYNVDAGA YKKYQDPLYV SIPLFISVKD GVATGYFFNS
121 ASKVIFDVGL EEYDKVIVTI PEDSVEFYVI EGPRIEDVLE KYTELTGKPF LPPMWAFGYM
181 ISRYSYYPQD KVVELVDIMQ KEGFRVAGVF LDIHYMDSYK LFTWHPYRFP EPKKLIDELH
241 KRNVKLITIV DHGIRVDQNY SPFLSGMGKF CEIESGELFV GKMWPGTTVY PDFFREDTRE
301 WWAGLISEWL SQGVDGIWLG MNEPTDFSRA IEIRDVLSSL PVQFRDDRLV TTFPDNVVHY
361 LRGKRVKHEK VRNAYPLYEA MATFKGFRTS HRNEIFILSR AGYAGIQRYA FIWTGDNTPS
421 WDDLKLQLQL VLGLSISGVP FVGCDIGGFQ GRNFAEIDNS MDLLVKYYAL ALFFPFYRSH
481 KATDGIDTEP VFLPDYYKEK VKEIVELRYK FLPYIYSLAL EASEKGHPVI RPLFYEFQDD
541 DDMYRIEDEY MVGKYLLYAP IVSKEESRLV TLPRGKWYNY WNGEIINGKS VVKSTHELPI
601 YLREGSIIPL EGDELIVYGE TSFKRYDNAE ITSSSNEIKF SREIYVSKLT ITSEKPVSKI
661 IVDDSKEIQV EKTMQNTYVA KINQKIRGKI NLELEHHHHH H (Cald MW: 81448 Da)
```

Figure 2.12. a) ESI-MS of the purified MalA D320G. The deconvoluted peak is shown in the main panel and the ion series is shown in the inset. b) Amino acid sequence of MalA D320G. Mutated site is colored in green.

Scheme 2.4. Hydrolytic activity of MalA D320G for *p*NP-sugar substrates.



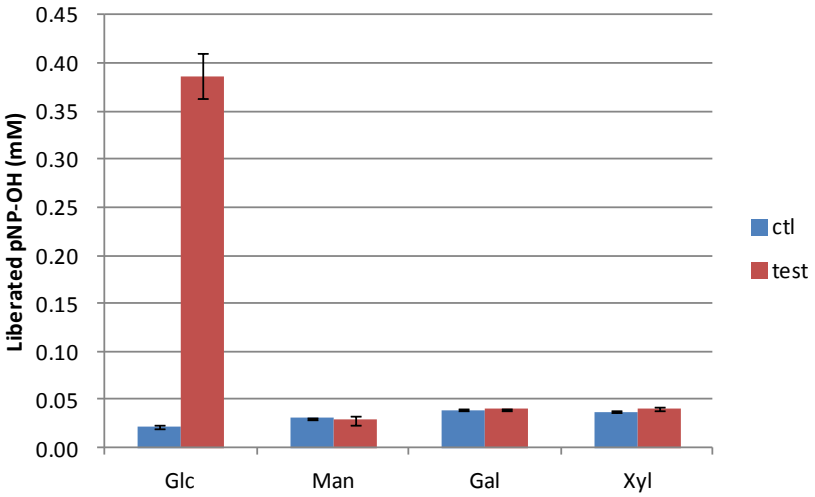


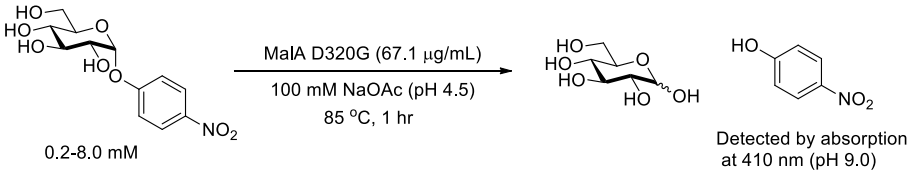
Figure 2.13. Hydrolytic activity of MalA D320G to various *p*NP-sugars. The reactions were performed under the conditions shown in **Scheme 2.4**. The reactions were performed for 20 mM *p*NP-sugars at 85°C using 67.1 μg/mL MalA in 100 mM NaOAc buffer (pH 4.5). In all cases, control reactions were performed in the absence of the enzyme. After incubating for 1 hour, the concentration of the released *p*-nitrophenol was determined by UV absorption at 410 nm in pH 9.

Michaelis-Menten analysis was performed for the hydrolytic reaction of *p*NP-α-D-Glc by MalA D320G (**Scheme 2.5**, **Figure 2.14**). The catalytic constant and the specific constant of D320G mutant were lower than those of the wild type by 360 fold and 170 fold, respectively (**Table 2.6**). On the other hand, the K_M of MalA D320G (0.82 mM) remains in the similar range to that of MalA WT (1.74 mM). Though this decrease of K_M might be explained by the slowness of the hydrolysis by D320G mutant and accumulation of Michaelis complex, it is likely that D320G mutant maintains the affinity to glucose substrate. However, due to the impaired hydrolytic activity towards *p*NP-α-D-Man, the binding affinity of mannose substrate to D320G

could not be estimated from the kinetics of the enzymatic hydrolysis reaction.

It should be noted that this weak hydrolytic activity of MalA D320G toward the glucoside substrate might be due to the undetectable amount of the contaminated wild type derived from, for example, the column used for purification. However, there is a significant difference in K_M values between WT and D320G, which indicates this hypothesis is unlikely. For further confirmation, mechanism-based inhibitor (5-FGlcF) can be used to see if this irreversible inhibitor abrogates the activity completely, but such experiments were not performed because they are out of scope of this project.

Scheme 2.5. Kinetic assay of MalA D320G using *p*NP-α-D-Glc as a substrate.



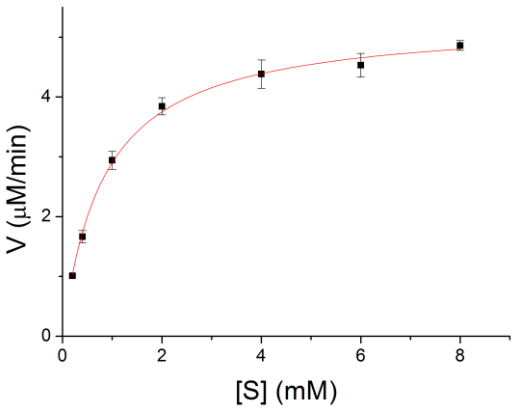


Figure 2.14. Mihaelis-Menten plot for the hydrolytic reaction of *p*NP-α-D-Glc by MalA D320G. *p*NP-α-D-Glc (0.2-8.0 mM) was hydrolyzed with 67.1 μg/mL MalA at 85°C in 100 mM NaOAc buffer (pH 4.5). The reaction was incubated for 1 hour and the reaction rate was determined by monitoring the concentration of the released *p*-nitrophenol by UV absorption at 410 nm in pH 9.

Table 2.6. Comparison of the kinetic parameters of *p*NP-α-D-Glc hydrolysis between MalA wild type and D320G mutant.

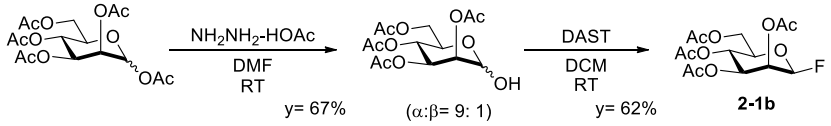
	WT	D320G
K_M (mM)	1.74 ± 0.20	0.821 ± 0.047
k_{cat} (s^{-1})	38.5 ± 1.6	0.107 ± 0.002
k_{cat} / K_M (s^{-1} mM^{-1})	22.1	0.130

2.2.5. Preparation of donor substrates

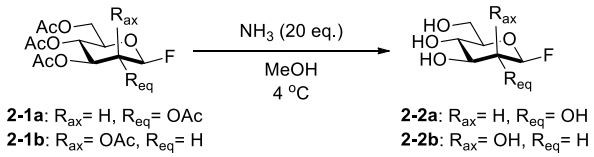
For glycosynthase reactions by MalA D320G, β-fluoride sugars were prepared. Peracetylated β-glucosyl fluoride (**2-1a**) was purchased from Sigma-Aldrich, which required purification due to the poor quality of the product. The donor substrates for the mannosynthase reaction were prepared as shown in **Scheme 2.6**. First, peracetylated mannose was selectively deprotected at 1-OH using hydrazine acetate. The anomeric hydroxyl group was then converted to fluoride using DAST. This reaction afforded the mixture of α- and β-anomers but these epimers could be separated by column chromatography and the β-product (**2-1b**) was isolated in 62% yield, accompanied by the α-isomer in 26% yield. These peracetylated compounds are solid and stable enough for long-term storage at -20°C.

Deprotection of acetyl groups was performed using ammonia-methanol. The reaction completes within 4 hours in the quantitative yield. The free hydroxyl substrates (**2-2a**, **b**) were prepared freshly every time before glycosynthase reactions were performed and were used without further purification after removal of solvents.

Scheme 2.6. Preparation of peracetylated β-mannosylfluoride (**2-1b**).



Scheme 2.7. Deprotection of β-fluoride sugars to afford β-glucosyl fluoride (β-GlcF, **2-2a**) and β-mannosyl fluoride (β-ManF, **2-2b**).



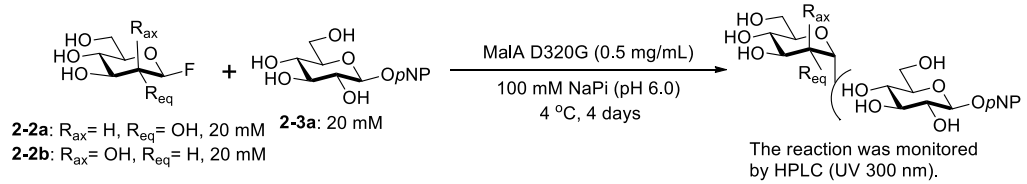
2-1a: R_{ax}= H, R_{eq}= OAc
2-1b: R_{ax}= OAc, R_{eq}= H

2-2a: R_{ax}= H, R_{eq}= OH
2-2b: R_{ax}= OH, R_{eq}= H

2.2.6. Glycosynthase activity of MalA D320G

Glycosynthase activity of MalA D320G was tested for both β-GlcF (**2-2a**) and β-ManF (**2-2b**) as glycosyl donor (**Scheme 2.8**). *p*NP-β-D-Glc (**2-3a**) was selected as acceptor because its UV absorbance makes reaction monitoring easier and it is tolerant to the α-glucosidase activity (**Figure 2.8**).

Scheme 2.8. Glycosynthase reaction using β-GlcF (**2-2a**) and β-ManF (**2-2b**) as glycosyl donor.



Pleasingly, glycosynthase products were observed in both cases (**Figure 2.15**). The conversion of the acceptor was about 30% when the progress of the reactions halted after 4 days. Though the affinity of mannose to this mutant could not be observed in the experiment of *p*NP-α-D-Man hydrolysis (Chapter 2.2.4), this result implied that the D320G mutant maintained the affinity to α-mannosyl substrates. Importantly, only mono-mannosylation was observed in the mannosynthase reaction as anticipated, whereas in glucosynthase reaction, oligomerization reaction proceeded even at this low conversion stage. This result demonstrated the validity of our design concept for development of glycosynthase with reduced oligomerization (**Figure 2.2**).

Isolation of the products revealed that the disaccharide was the mixture of 1,3- and 1,4-products (1,3-product: 1,4-product= 1.5: 1) in the glucosynthase reaction. Similar trend of regioselectivity was observed in the mannoysnthase reaction but selectivity was far better (1,3-product: 1,4-product= 1: 9). This is an additional bonus for applying this enzyme to chemical synthesis.

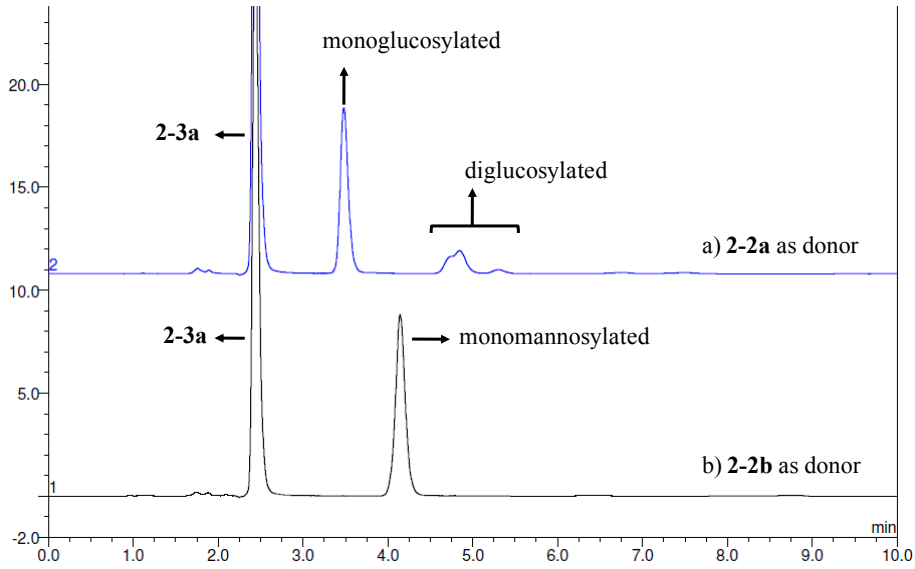


Figure 2.15. Comparison of chromatograms of glucosynthase (a) and mannosynthase (b) reaction after 4 days. 20 mM of *p*NP-β-D-Glc (**2-3a**) was reacted with (a) 20 mM β-GlcF (**2-2a**) or (b) β-ManF (**2-2b**) in 100 mM NaPi (pH 6.0) at 4°C using 0.5 mg/mL MalA D320G.

2.2.7. Optimization of mannosynthase reaction

Now, the glycosynthase based on the novel design was realized. However, in this initial condition, the conversion was not satisfactory (~30%) and the reaction rate was too slow for practical application (4 days). To enhance the efficiency of the mannosynthase reaction, several reaction parameters were optimized.

Temperature

First, the reaction temperature was investigated. Presumably, one of the reasons why the development of α-glycosynthase falls behind that of β-counterpart is because β-glycosyl fluoride is less stable than α-anomer.[36] The quantitative analysis of stability of glycosyl fluorides in several buffers[35] shows that β-anomers tend to have more than ten times shorter half-lives than the corresponding α-isomers (**Table 2.8**). This lability of β-glycosyl fluorides to spontaneous hydrolysis casts concerns about developing α-glycosynthases. To eradicate such issues, α-glycosynthase reactions using β-glycosyl azides in place of β-glycosyl fluorides were recently proposed.[21, 37] Using the thermophilic enzymes, the reactions give moderate to good

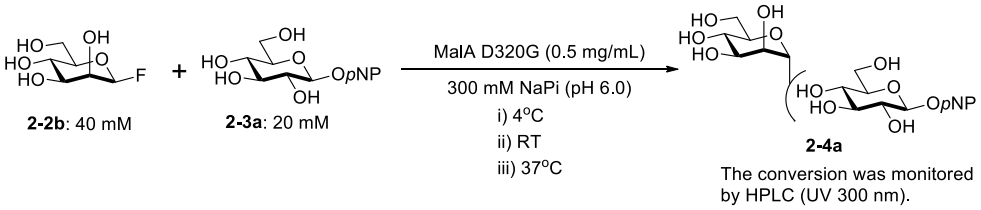
Table 2.8. Half-lives ($t_{½}$ in hour) of glycosyl fluorides in the buffers. The data are from the reference (note: the temperature is not indicated in the literature but presumably room temperature).[35] Buffer1: 90 mM NaOAc (pH 4.5), Buffer2: 90 mM Tris-HCl (pH 7.5), Buffer 3: 90 mM phosphate-citrate (pH 7.0), Buffer 4: 180 mM phosphate-citrate (pH 7.0).

Glycosyl fluoride	Buffer 1	Buffer 2	Buffer 3	Buffer 4
α-Man-F	580	190	290	290
α-Glc-F	290	230	290	290
β-Gal-F	7	7	6	8
β-Cel-F	39	29	39	39
β-Man-F	14	13	14	11
β-Lac-F	14	13	17	11
β-Glc-F	29	39	29	29

yields at 65°C. However, we speculate that the half-life of the donor (β-ManF, **2-2b**) of >11 hour (**Table 2.8**) is sufficient if the rate of the enzymatic reaction is high enough[36] and indeed 30% conversion of the acceptor was observed in the initial reaction condition (**Figure 2.15**).

To determine reaction conditions that balance between the reaction efficiency and spontaneous hydrolysis of donor substrates, three temperatures (4°C, room temperature and 37°C) were tested in the ratio of donor: acceptor= 2: 1 (**Scheme 2.9**).

Scheme 2.9. Investigation on the reaction temperature for the mannosynthase reaction.



The reactions were monitored until they reached plateaus. At 4°C the reaction continued to progress until 4 days. The spontaneous hydrolysis of **2-2b** is expected to be far slower at 4°C than room temperature. In terms of the conversion of the acceptor (**2-3a**), 4°C gave the best result (more than 40%) among these three conditions (**Figure 2.16**). However, the day-scale reaction time is not practical for synthesis. On the other hand, the reaction stopped progressing within one day at higher temperature. This is a reasonable result considering the half-life of the donor substrate. The conversion at 37°C hit a maximum in less than 30%, whereas almost 40% of the acceptor was converted to products at room temperature. Considering the balance between the reaction efficiency and the reaction time, room temperature was selected as an optimal temperature.

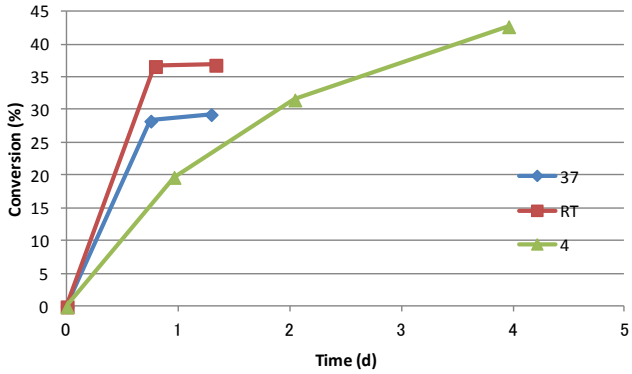
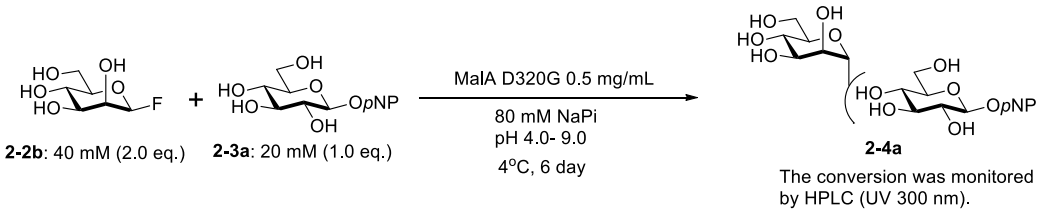


Figure 2.16. The time course of the mannosynthase reactions at different temperatures. 20 mM *p*NP-β-D-Glc (**2-3a**) was reacted with 40 mM β-ManF (**2-2b**) in 100 mM NaPi (pH 6.0) at 4°C, room temperature or 37°C using 0.5 mg/mL MalA D320G.

pH

Then, pH of the reaction buffers was investigated (pH 4.0-9.0, **Scheme 2.10**).

Scheme 2.10. Investigation of pH of the buffer for the mannosynthase reaction.



In this initial screening, reactions at pH 6-9 gave better conversion than those at pH 4 or 5 (**Figure 2.17**). When pH of the resultant reaction mixtures was checked, it turned out that it decreased significantly for pH 4 and 5 reactions during the course of the reactions. When the donor substrate reacts with the acceptor or water, it generates HF (pK_a 3.2) as a side product. In the reactions at pH4 and 5, the buffering capacity of the buffer (80 mM NaPi) was probably overwhelmed by the generated HF (40 mM at the maximum), resulting in lower pH than the workable limit of the enzyme.

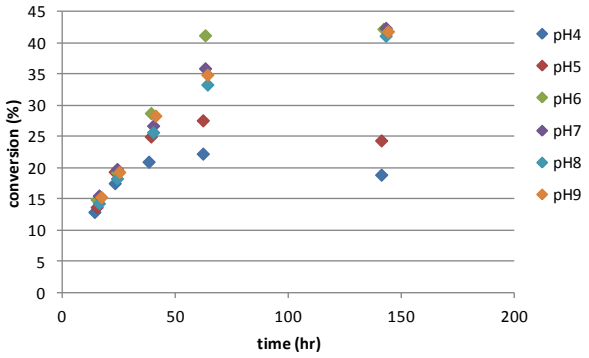
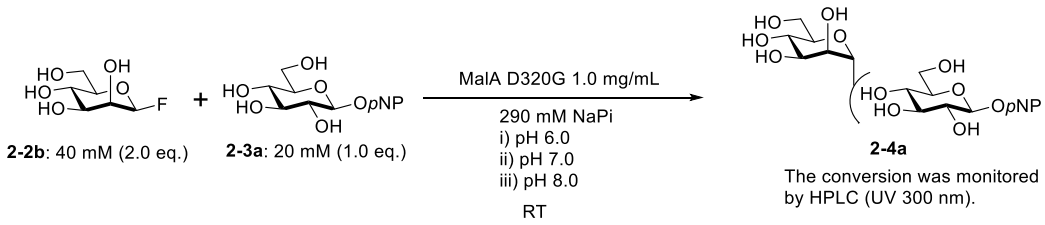


Figure 2.17. pH screening in the broad range (pH 4.0-9.0). 20 mM *p*NP-β-D-Glc (**2-3a**) was reacted with 40 mM β-ManF (**2-2b**) in 80 mM NaPi of various pH values at 4°C using 0.5 mg/mL MalA D320G.

Based on the result above, the next reactions were performed at the higher concentration of the buffer at pH 6-8 using the doubled amount of the enzyme (1.0 mg/mL) (**Scheme 2.11**).

Scheme 2.11. Investigation of pH of the buffer for the mannosynthase reaction.



There was no significant difference in reaction efficiency among these three conditions (**Figure 2.18**) and all reached plateaus around the conversion of 40% in 24 hours. Therefore, the neutral condition (pH 7.0) was chosen for the optimal condition considering the lability of the donor substrate. To push the reactions forward, the donor was sequentially added twice by two equivalents at 24-hour intervals. The conversion was enhanced up to 70% in all cases and no significant decrease of pH was observed.

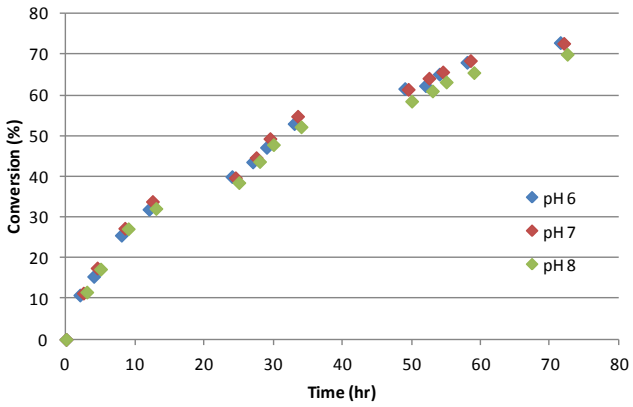
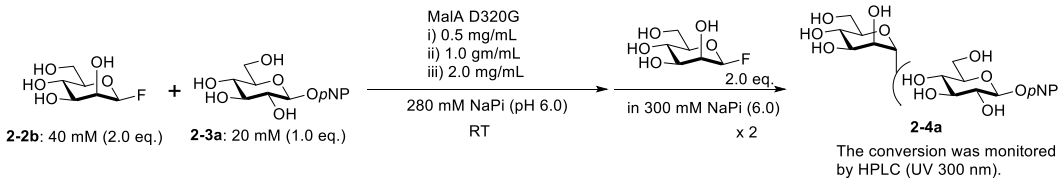


Figure 2.18. The time course of the mannosynthase reactions at three different pH values. The reaction was initiated using 20 mM *p*NP-β-D-Glc (**2-3a**), 40 mM β-ManF (**2-2b**) and 1.0 mg/mL MalA D320G in 290 mM NaPi of pH 6, 7 and 8. The glycosyl donor (**2-2b**) was sequentially added twice at 24-hour intervals.

Enzyme concentration

Next, the effect of enzyme concentration was investigated systematically (**Scheme 2.12**).

Scheme 2.12. Investigation of enzyme concentration for the mannosynthase reaction.



In the same way as the pH investigation, the glycosyl donor (**2-2b**) was sequentially added by 2.0 equivalents at 24-hour intervals twice. In all cases, the reaction proceeded in good conversion (**Figure 2.18**). The higher the concentration of the enzyme was, the higher the conversion of the acceptor (**2-3a**) was. When the concentration of the enzyme was at 2.0 mg/mL, the final conversion exceeded 85% (**Figure 2.18**) and this concentration was chosen for the optimal reaction condition.

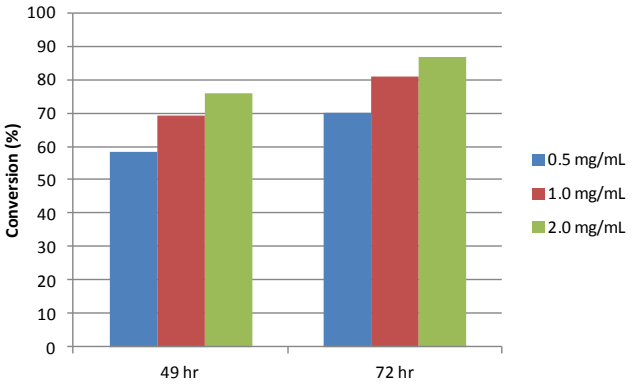
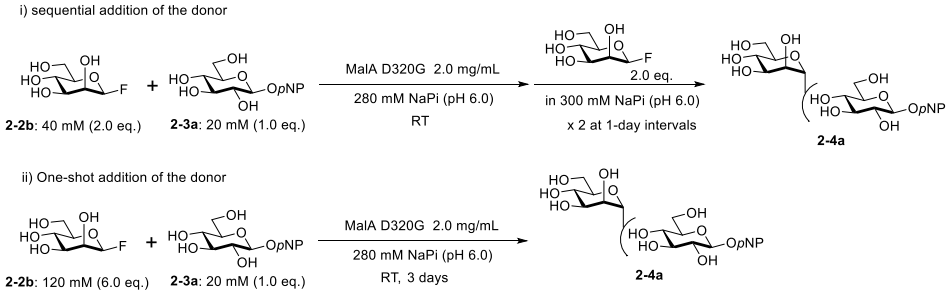


Figure 2.19. The conversioins of the acceptor (**2-3a**) after 49 hours and 72 hours at three different enzyme concentrations. The reaction was initiated using 20 mM *p*NP-β-D-Glc (**2-3a**) and 40 mM β-ManF (**2-2b**) in 290 mM NaPi (pH 6.0) with 0.5, 1.0 or 2.0 mg/mL MalA D320G. The glycosyl donor (**2-2b**) was sequentially added at 24-hour intervals twice.

Sequential addition of the glycosyl donor

From the investigation above, it appeared that the sequential addition of the glycosyl donor was effective to increase the conversion of the acceptor. To confirm the effect of the sequential addition, the same amount of the donor was added in two ways; in one portion and by sequential addition (**Scheme 2.13**).

Scheme 2.13. Investigation of the effect of the sequential addition of the glycosyl donor.



6.0 equivalents of the donor was added in both cases but the difference of the final conversion was significant. When all the 6.0 equivalents were added in one portion at the beginning of the reaction, the progress of the reaction halted in one day and the conversion of the acceptor

remained at 60% whereas it surpassed 85% in the end when the donor was added sequentially by 2.0 equivalents at 24-hour intervals (**Figure 2.20**).

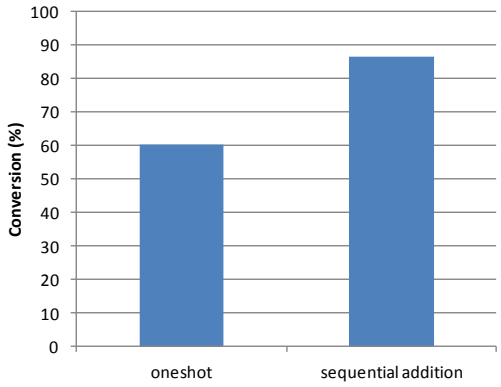


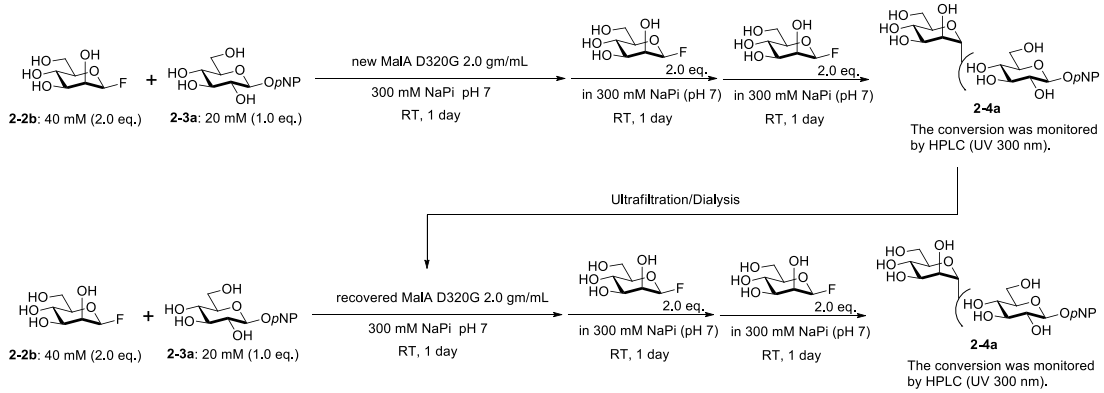
Figure 2.20. The final conversioins of the acceptor (**2-3a**) after the mannosynthase reaction in the different ways of the donor addition. In the in one-shot addition, 20 mM *p*NP-β-D-Glc (**2-3a**) was reacted with 120 mM β-ManF (**2-2b**) in 280 mM NaPi (pH 6.0) at room temperature using 0.5 mg/mL MalA D320G for 3 days. In the sequential addition, the reaction was initiated using 20 mM *p*NP-β-D-Glc (**2-3a**) and 40 mM β-ManF (**2-2b**) in 280 mM NaPi (pH 6.0) with 2.0 mg/mL MalA D320G and the glycosyl donor (**2-2b**) was sequentially added by 2 equivalents at 24-hour intervals twice.

Recovery of the enzyme

High loading of the enzyme (2.0 mg/mL) gave good results and ended up with more than 85% conversion of the mannosynthase reaction. However, it is not economical to use such a high quantity of the enzyme in a consumable manner. Therefore, recycling the enzyme from the finished reaction was investigated (**Scheme 2.14**).

After the reaction with new MalA D320G, the enzyme was separated from small molecules by ultrafiltration (MWCO 10,000). The recovery of the enzyme was usually more than 90% when the ultrafiltration concentrator was pre-treated with 0.1% Triton-X 100. The enzyme thus recovered was used for the same reaction and it showed the equivalent reaction efficiency to find no deterioration of the activity (**Figure 2.21**).

Scheme 2.14. Investigation of recovery of the enzyme.



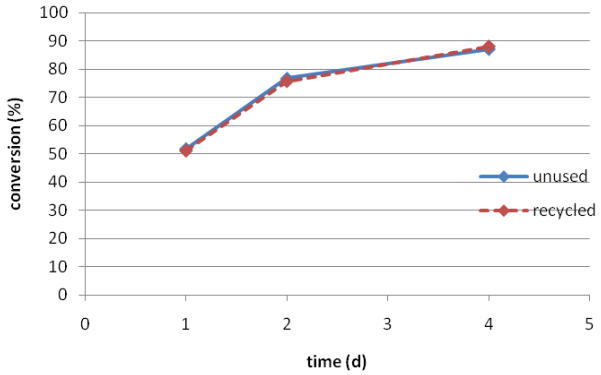


Figure 2.21. Comparison of the activities of the unused enzyme the recycled one. First, 20 mM *p*NP-β-D-Glc (**2-3a**) was reacted with 40 mM β-ManF (**2-2b**) in 300 mM NaPi (pH 7.0) at room temperature using 0.5 mg/mL MalA D320G with addition of 2 equivalents of **2-2b** at 24-hour intervals twice. The enzyme was recovered by ultrafiltration, which was then used for the same reaction.

Organic modifier

As will be mentioned later, not all of the acceptor molecules were soluble in pure water at the required concentration. Addition of an organic solvent is required to dissolve such. The concern is the compatibility of the enzyme to organic solvents.

First, the solubility of *p*NP-α-L-Rha was checked as a representative example of sparingly soluble substrates (**Table 2.9**). It turned out that 15% of DMF, 25% of EtOH or 25% of iPrOH

were required to dissolve *c* at the concentration of 20 mM.

Table 2.9. Solubility of *p*NP-α-L-Rhamnoside.

	5%	10%	15%	25%
MeOH	-	-	×	×
EtOH	-	-	×	✓
iPrOH	-	-	×	✓
DMF	×	×	✓	✓

Then, enzymatic activity in buffers which partially contain an organic solvent was investigated (**Figure 2.22**). Compared to the pure aqueous medium, the conversion decreased to some extent. However, the final conversion in 15% DMF or 25% EtOH was maintained around 90% of that in the pure aqueous system, while it decreased by 20 % in 25% iPrOH. Based on this observation, it was decided that DMF would be added up to 15 % when addition of organic solvent was necessary to dissolve acceptor comounds like *p*NP-α-L-Rha.

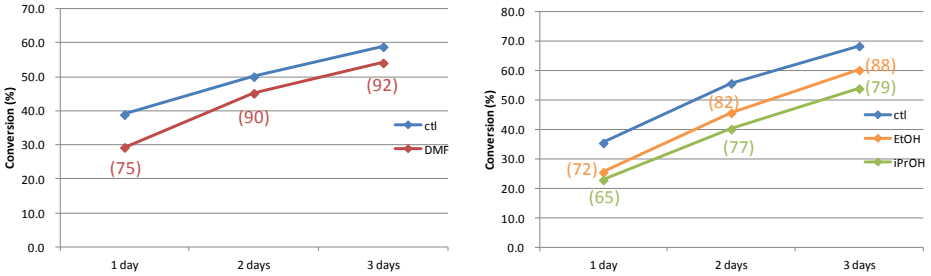
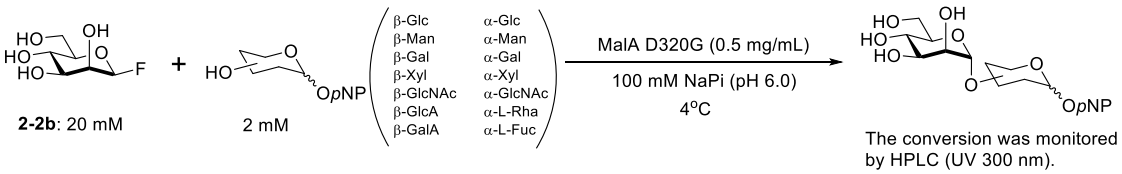


Figure 2.22. Comparison of the time course of the mannosynthase reactions in the pure aqueous buffer and in the buffers with the organic solvents. The reaction was initiated using 30 mM *p*NP-β-D-Glc (**2-3a**) and 60 mM β-ManF (**2-2b**) in 300 mM NaPi (pH 6.0) with or without organic solvents using 0.8 mg/mL MalA D320G. The glycosyl donor (**2-2b**) in 300 mM NaPi (pH 6.0) was sequentially added by 2 equivalents at 24-hour intervals twice. The numbers in the parentheses are the relative conversions in the reactions with organic modifiers to the pure aqueous reaction.

2.2.8. Scope of glycosyl acceptor for the α-mannosynthase reaction

In concurrence with optimizing the conditions of the α-mannosynthase reaction, the scope of glycosyl acceptor was also investigated. A range of *p*NP-sugar substrates were tested (**Scheme 2.15**) in the presence of 10 equivalents of the glycosyl donor (**2-2b**).

Scheme 2.15. Screening of the acceptor scope for the α-mannosynthase reaction.



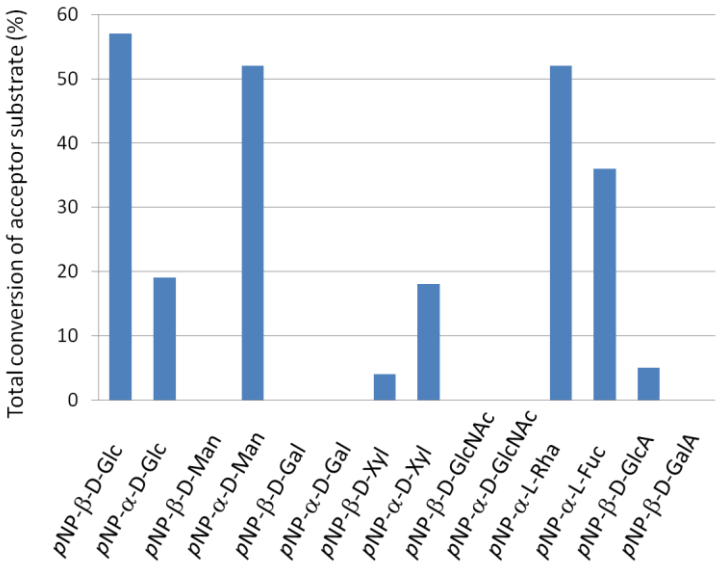


Figure 2.23. Conversion of glycosyl acceptors under the screening condition. 2 mM *p*NP-sugar was reacted with 20 mM β-ManF (**2-2b**) in 100 mM NaPi (pH 6.0) at 4°C using 0.5 mg/mL MalA D320G. After 4 days, the reaction was analyzed by HPLC.

The product formation was analyzed by HPLC (Column: Synergi 4u Fusion-RP 80A, 100x4.60mm, 4 micron, Detection: UV 300 nm, **Figure 2.23**). Surprisingly, a wide range of

sugar substrates were glycosylated by MalA D320G. As well as the known glycosyl acceptor *p*NP-β-D-Glc (**2-3a**), the α-counterpart also gave glycosylated compounds. The lower conversion of *p*NP-α-D-Glc is probably because this compound is a good substrate for the D-site and competes with β-ManF (**2-2b**) to inhibit the desired mannosylation reaction. Nonetheless, about 20% of *p*NP-α-D-Glc was converted to the glycosylated products and no indication of the enzymatic hydrolysis of *p*NP-α-D-Glc was detected in this screening condition.

Mannose substrates gave the contrasting results between two isomers. *p*NP-β-D-Man did not show any indication of glycosylation. The binding mode of the glucoside acceptor with the A-site of MalA is reported (PDB ID: 2G3N).[27] This structure suggests that the hydrogen bonding interaction (distance: 2.8Å) between 2-OH of β-glucoside and carboxylate ($^{\delta}$O) of D87 of MalA at the A-site (**Figure 2.24**). Presumably, the absence of this interaction in the β-mannoside acceptor resulted in the complete loss of the glycosylation to *p*NP-β-D-Man. On

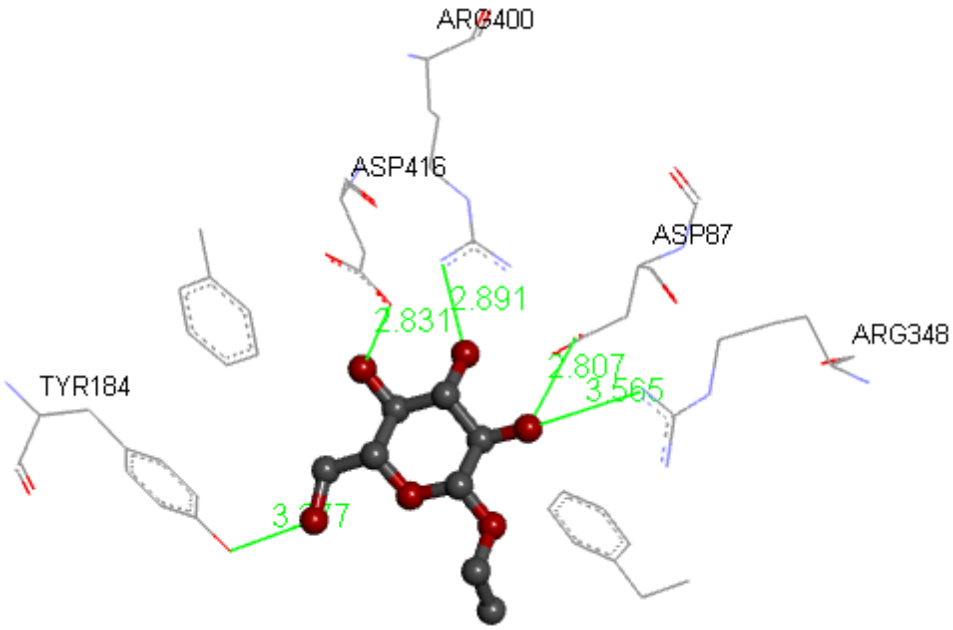


Figure 2.24. Structure of MalA around the A-site (PDB ID: 2G3N).[27] The hydrogen bonds between the ligand (octyl β-glucopyranoside) and MalA was indicated with green lines.

the other hand, *p*NP-α-D-Man was glycosylated in more than 50% conversion. In spite of the same mannose scaffold, the different binding mode of *p*NP-α-D-Man from that of the β-counterpart seemed to allow this compound to be glycosylated efficiently, as will be discussed later in this section. *p*NP-α-D-Man is a potential inhibitor at the D-site. However, presumably because *p*NP-α-D-Man has less binding affinity at the D-site than *p*NP-α-D-Glc, it showed less inhibitory effect to the mannosylation reaction.

Both anomers of xylose gave mannosylated products. The conversion of *p*NP-β-D-Xyl decreased drastically compared with that of *p*NP-β-D-Glc. This setback of the glycosylation efficiency can be explained by the loss of the hydrogen bond (distance: 3.3Å) that the glucose acceptor has at its 6-OH with $^{\eta}$O of MalA Y184 (**Figure 2.24**). On the other hand, *p*NP-α-D-Xyl was glycosylated as efficiently as the corresponding glucosyl acceptor.

Galactoses and *N*-acetylglucosamines (GlcNAcs) did not give any glycosylated products in either anomers. When *p*NP-β-D-Glc was used as glycosyl acceptor, the reaction gave the α(1,4)-linked product as the major product (Chapter 2.2.6). This result implies that the configuration at C-4 of the acceptor is important and hence it is reasonable that galactose, the epimer at this position, is deprived of reactivity in this enzymatic scaffold. GlcNAc has the acetamide group at 2-position, which is likely to have steric repulsion with D87 in MalA at the A-site (**Figure 2.24**). Presumably, this is the reason why GlcNAcs were not good glycosyl acceptor and did not give the mannosylated products.

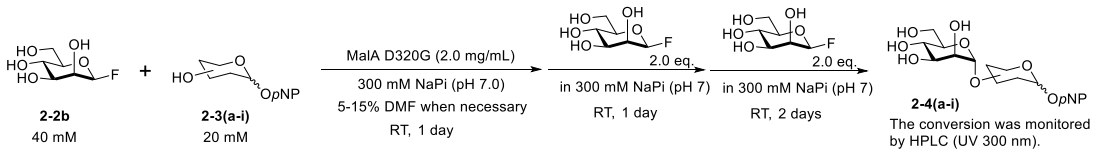
Most surprisingly, both L-sugars, *p*NP-α-L-Rha and *p*NP-α-L-Fuc turned out to be good acceptors for this α-mannosynthase reaction. Simply thinking, three hydroxyl groups of these L-sugars can be in the same position as those of D-sugars when they are flipped over the axis of C1-C4. In such a situation, the aglycon (*p*NP group) sticks to the opposite direction to the case of α-D-sugars, but this area is exposed to the solvent region and so these compounds do not have

steric repulsion with the enzyme. Therefore, this model is fairly reasonable to explain why these L-sugars are glycosylated in such an efficient manner, but these discussions do not go beyond speculation.

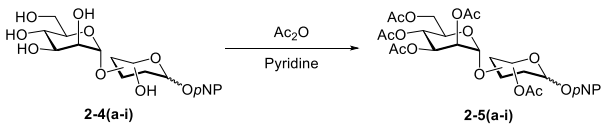
*p*NP-β-D-GlcA also worked as a weak acceptor, though the conversion was lower than 10%. On the other hand, *p*NP-β-D-GalA did not show any indication of product formation. The inefficiency of these compounds as acceptor is probably because the hydrophilic carboxylic acid is not accepted in the relatively hydrophobic environment (**Figure 2.24**). Moreover, the discussion about the configuration of C-4, which is mentioned above, is also applied for *p*NP-β-D-GalA.

To characterize the mannosylated products in these mannosynthase reactions, the acceptors which showed more than 10% conversion in the screening (**2-3(a-f)**) were subjected to the preparative scale synthesis in the optimized condition (**Scheme 2.16**). Three biologically important disaccharides (**2-3(g-i)**) were also tested to see if they work as glycosyl acceptor. The reaction was monitored using NH2 column (Kromasil NH2 100A, 150x4.60mm, 5 micron (Phenomenex)) for good separation by degree of oligomerization. For purification of the products, the reaction solution was first subjected to ultrafiltration to separate and recycle

Scheme 2.16. Mannosynthase reactions under the optimized condition.



Scheme 2.17. Peracetylation of mannosynthase products



the enzyme. The flow-through fraction containing small molecules was desalted by reverse phase HPLC and fractions with absorption at 300 nm were collected. Further separation was performed by preparative scale NH2 column in HPLC. Novel compounds were peracetylated (**Scheme 2.17**) and their structures were determined by NMR.

The results were summarized in **Figure 2.25**. Mannosynthase MalA D320G was able to produce a variety of oligosaccharides in the yield of up to 77%. None of these reactions showed any indication of formation of β-glycosides. In many cases, the regioselectivity was good (>90%, Entry 1, 2, 4, 5, 6, 7) and most of them produced α(1,3)-linkages. It should be noted that this regioselectivity is an important factor in terms of application of this biocatalyst for practical synthesis. Because regioisomeric compounds are more difficult to separate from desired products compared to other side products like over-glycosylated compounds, which are separable when NH2 column is used.

The most noteworthy outcome is that, as designed, the typical ratio of oligomerization was low (OF < 20%, OF: oligomerization factor defined as % of oligomerized compounds out of the total mannosylated products) despite the use of the excess amount of the donor. Particularly, this trend is remarkable when glycosyl acceptor is glucosidic compounds (Entry 1, 2 and 7) probably because they have good affinity at the A-site. It is reasonable that OF increases when acceptors do not have good affinity to the A-site. The less an acceptor has affinity to the A-site of the enzyme, the more it is competed by α-mannosylated products which are formed by the intended reaction. The affinity of α-mannosylated compounds to the A-site is represented by Entry 3. Nevertheless, it is significant that mannosylated compounds were synthesized in fair to good yield (25-77%) in a single step of the enzymatic reaction.



Entry	Acceptor	Product	Yield[a]	Selectivity[b]	OF[c]
1	2-3a	2-4a-I, 2-4a-II	77%[d]	91:9 α1,4: α1,3	12%
2	2-3b	2-4b	26%[d]	>95% α1,3	11%
3	2-3c	2-4c-I, 2-4c-II	55%	60: 40 α1,3: α1,2	32%
4	2-3d	2-4d	35%[d]	93% α1,3	35%
5	2-3e	2-4e	44%[d]	>95% α1,3	16%
6	2-3f	2-4f	35%[d]	92% α1,3	49%
7	2-3g	2-4g	75%[d]	>95% α1,3	11%
8	2-3h	2-4h-I, 2-4h-II	65%[d]	69: 31 α1,4: α1,3	20%
9	2-3i (= 2-4c-I)	2-4i-I, 2-4i-II	25%[d]	76: 24 α1,2: α1,3	27%

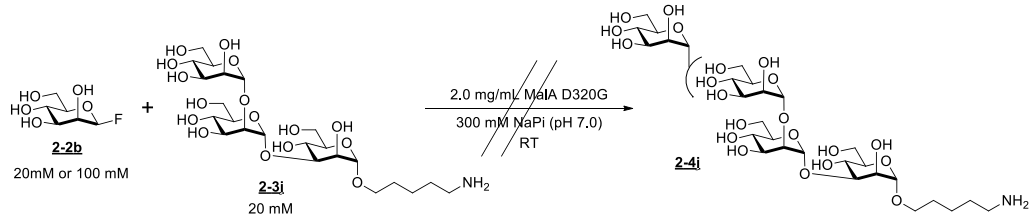
Figure 2.25. α-Mannosynthase-catalyzed reactions.[22] The reaction was initiated at 20 mM *p*NP-β-D-Glc (**2-3a**) and 40 mM β-ManF (**2-2b**) in 300 mM NaPi (pH 7.0) (with DMF if needed) using 2.0 mg/mL MalA D320G. The glycosyl donor (**2-2b**) in 300 mM NaPi (pH 7.0) was sequentially added by 2 equivalents at 24-hour intervals twice. (a) Yields after purification and isolation. (b) Determined by HPLC or Man (1-H) ^{1}H-NMR integratioin. (c) Oligomerization factor (OF) difined as % of oligomannosylated products in total mannosylated products. (d) Isolated as peracetate.

An important exception about the regioselectivity of the products is that *p*NP-β-D-Glc gave an α(1,4)-linked product dominantly (**Figure 2.25**, Entry 1). This outcome seems characteristic to β-glucoside acceptors and *p*NP-β-cellulobiose (**2-3h**) also gave an α(1,4)-linked compound as a major product (**Figure 2.25**, Entry 8).

Another interesting exception is that *p*NP-α-D-Glc (**2-3c**) gave α(1,2)-product as well as α(1,3)-linked compounds. The co-crystalline structure[27] of MalA and octyl β-glucoside at the A-site (**Figure 2.24**) shows that 2-OH of glucose interacts with carboxylate ($^{\delta}$O) of D87 through the hydrogen bond. Details of binding mode of mannosyl compounds are not clear but it is likely that they have a different global orientation in the A-site from glucosyl compounds due to an axial configuration of the hydroxyl group of C-2, resulting in this distinct regioselectivity.

It should be noted that α(1,2)-linkage is characteristically found in high mannose-type *N*-glycan (Chapter 1.2.2) and is abundant in Nature. In particular, D-1 arm scaffold of high mannose glycan is a key motif for neutralizing human antibodies to HIV-1[38] (e.g. 2G12) and therefore its convenient synthesis is required for anti-HIV vaccine design.[39, 40] The unique activity of MalA D320G to construct the Man-α(1,2)-Man motif was further investigated using mannobiose (**2-3i**). This acceptor showed the apparent preference of α(1,2)-linkage though the total conversion was lower than that using monosaccharide acceptor (**2-3c**). A further ambitious reaction using trimannose[41] (**2-3j**) was also performed to construct the complete D-1 arm scaffold (**Scheme 2.17**). Unfortunately, this attempt failed and no indication of D-1 arm formation was observed. This result implies that the non-reducing terminal Man-α(1,2)-Man is not a good glycosyl acceptor for MalA D320G-catalyzed mannosynthase reaction. If this is true, oligomerization takes place dominantly for α(1,3)-linked products in Entry 3 or 9 and the actual regioselectivity of the reaction might be different from what was observed. Unfortunately, the structures of over-mannosylated compounds in these reactions could not be determined due to

Scheme 2.17. Attempt to prepare D1-arm scaffold using α-mannosynthase catalyst.



scarcity of the material and this hypothesis is yet to be conclusive.

Together, these syntheses could provide a number of naturally occurring α-mannoside motifs. Other than oligomannose *N*-glycan scaffold mentioned above, these include several bacterial polysaccharides: Man-α(1,4)-Glc[42] is found as part of lipopolysaccharide in *Bradyrhizobium japonicum*; Man-α(1,3)-Glc[43] is a scaffold of the repeating unit of capsular polysaccharide of *Klebsiella* serotype K64; Man-α(1,3)-L-Rha[44] is the repeating unit of glycans of the S-layer glycoprotein of *Clostridium thermohydrosulfuricum* strain L111-69; Man-α(1,3)-L-Fuc[45] is the repeating unit of an extracellular water-adsorbent polysaccharide of *alcaligenes latus* strain B-16; Man-α(1,3)-Cel[46] is found in the root structure of xanthan from *Xanthomonas campestris*. These biologically relevant oligosaccharide units were prepared in one step in decent yields without conventional multistep protection/deprotection strategy using newly developed α-mannosynthase.

Inositol reaction

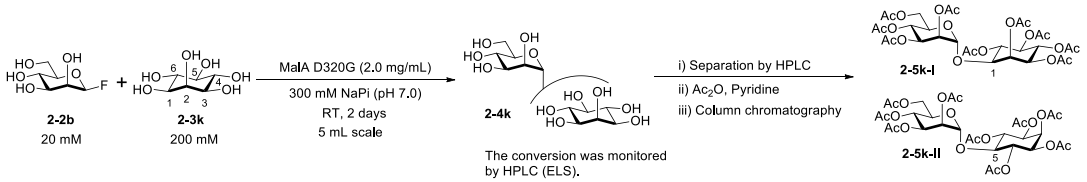
Next, the mannosynthase was applied to a more challenging substrate. Mannosyl inositols are components of the cell wall glycolipids found in *Mycobacterium tuberoculosis* and, as such, are the targets of traditional glycosidase syntheses.[47, 48] Especially, *myo*-inositol (**2-3k**) is a demanding target due to its *meso*-symmetry and controlled desymmetrization is required for derivatization.

An initial attempt under the above-mentioned condition did not give detectable amount of target products and only self-condendation of donor was observed. The sterically congested hydroxyl groups around an inositol ring and thus less affinity to the enzyme may retard the glycosylation reaction. To overcome competition with β-ManF (**2-2b**) at the A-site, the concentration and the stoichiometry of *myo*-inositol (**2-3k**) was increased. Gratifyingly, when **2-3k** was used in 200 mM (10 equivalents to the donor), two newly formed peaks were observed in HPLC (**Scheme 2.18**, **Figure 2.26**). The total conversion of the donor substrate to the mannosylated inositol was 41% (determined by area ratio in HPLC). These peaks were separated by HPLC and further purification was performed by silica gel chromatography after peracetylation (**Scheme 2.18**). It turned out that each peak contained more than one compound but the major component from each peak was isolated. These compounds are characterized as Man-α(1,5)-*myo*-inositol (**2-5k-II**) and Man-α(1,1)-*myo*-inositol (**2-5k-I**).[49] The details of the characterization will be discussed below. It should be mentioned that the formation of Man-α(1,1)-*myo*-inositol by this mannosynthase reaction is a rare example where glycosylation and desymmetrization are simultaneously realized.

Characterization of mannosynthase products from myo-*inositol acceptor*

To determine the structure of the compound isolated from the front peak ^{1}H-NMR, 1D-TOCSY experiments were used. First, the shapes of ^{1}H-NMR peaks can distinguish the protons on *myo*-inositol rings. Triplet-like peaks with large coupling constants (~10Hz; termed as H_T) can be assigned to H_4, H_5 or H_6 and the one with a small coupling constant (~3Hz) to H_2. The other two double doublet peaks (generally termed as H_{dd}) correspond to H_1 or H_3. Among the peaks derived from the inositol ring, H_2 was found at 5.55 ppm and both H_{dd} peaks were found 4.99 and 5.00 ppm. These peaks are in the range of the chemical shift when the corresponding

Scheme 2.18. Mannosynthase reaction using myo-inositol as glycosyl acceptor.



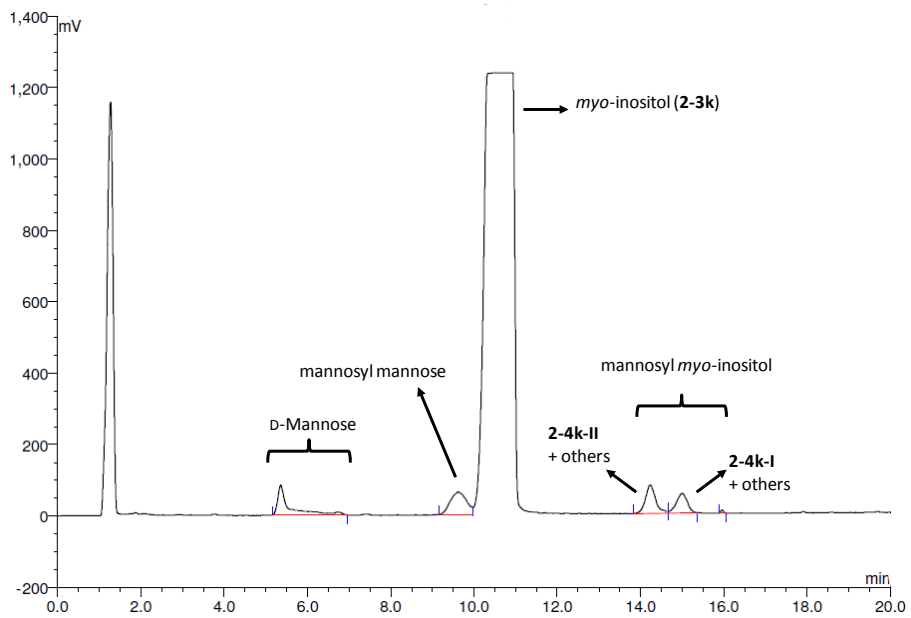


Figure2.23. Chromatogram of mannosynthase reaction with *myo*-inositol as acceptor.

hydroxyl groups are acetylated. Therefore, H_1, H_2 and H_3 can be ruled out from the candidates of the glycosylation site. Among H_T protons (H_4, H_5 or H_6), one is observed in upfield (3.82 ppm) while the other two are found at 5.50 and 5.56 ppm. To investigate which position is glycosylated, a 1D-TOCSY experiment was performed (**Figure2.27**) irradiating the proton signal at 3.82 ppm. When the mixing time was short, the peak of H_2 was not found, while the other two H_T protons were observed in the same signal intensity. In the same manner, both H_{dd} protons showed the same relative signal intensity. When the mixing time was prolonged, the peak of H_2 started to appear, maintaining the symmetry of two H_T protons and two H_{dd} protons. These results indicate that the glycosylation site is the farthest site from C-2 and is on the axis of symmetry of the inositol ring. Hence, the glycosylation site of this compound is

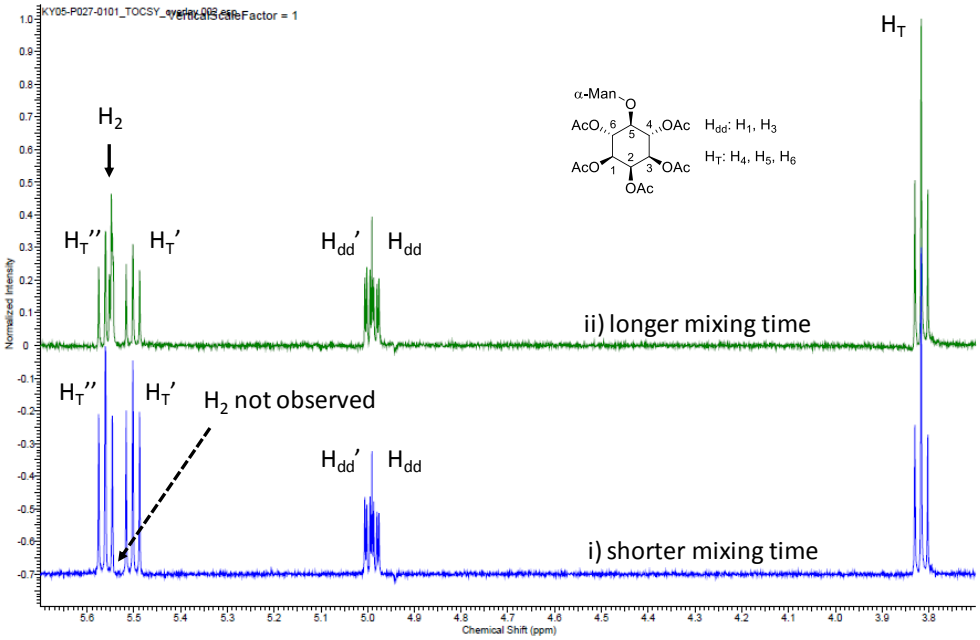


Figure2.27. 1D-TOCSY experiment to determined the structure of **2-5k-II**. When the proton of the glycosylation site is excited, the spectra gave symmetric pattern of the peaks for H_T and H_{dd}, which indicates the glycosylation site is 5-position of *myo*-inositol.

unambiguously determined to be 5-position.

Actually, Man-α(1,5)-*myo*-inositol (**2-4k-II**) and its peracetylated compound (**2-5k-II**) are reported previously[50] but their data are not identical to those shown above. This paper reports isolation of the corresponding compound from the fermentation broth of *Streptomyces coelicolor* A3(2). They also found one of the H_T peaks was upfield (4.12 ppm) after peracetylation. However, grounds to rule out other two possibilities are not mentioned. On the other hand, the data from 1D-TOCSY shown here provides a solid proof to characterize the compound isolated from the mannosynthase reaction as Man-α(1,5)-*myo*-inositol.

To determine the structure of the compound isolated from the rear peak, ^{1}H-NMR, NOESY and HMQC experiments were performed. First, ^{1}H-NMR showed one of the H_{dd} peaks in upfield (3.94 ppm), which means inositol is linked to mannose at either 1- or 3-position. In the NOESY experiment (**Figure2.28**), the strong cross peak between H_1 of mannose ($^MH_1$) and the proton at

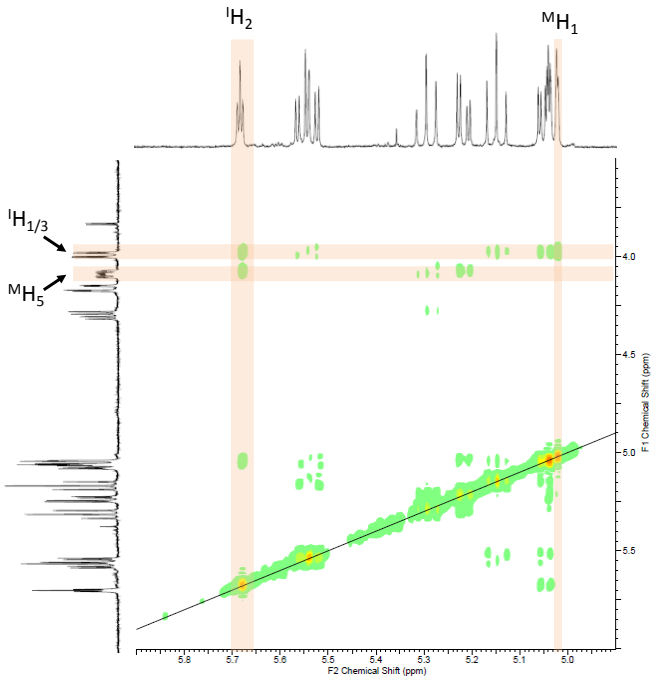


Figure 2.28. NOESY spectrum of **2-5k-I**. The cross-peak of $^{M}H_1$ and $^{I}H_{1/3}$ indicates these protons are in the same face of the ring system. Under this condition, the cross-peak of $^{M}H_5$ and $^{I}H2$ can be possible only for α(1,1)-linked mannosyl *myo*-inositol (see **Figure 2.29a**).

3.94 ppm ($^{I}H_{1/3}$) was observed, which suggests the small dihedral angle of $^{M}H_1$-$^{M}C_1$-O-$^{I}C_{1/3}$. Indeed, the coupling constant between $^{M}H_1$ and $^{I}C_{1/3}$ was determined to be 4.0Hz by the HMQC experiment. This value corresponds to the dihedral angles of 30-40° from the Karplus equation. Though the Karplus equation can also suggest the dihedral angles of 130-140°, such a conformation can be ruled out from the strong cross peak between $^{M}H_1$ and $^{I}C_{1/3}$ in the NOESY spectrum. Therefore, it is concluded that $^{M}H_1$ and $^{I}H_{1/3}$ are sticking out from the same surface of the disaccharide structure.

The most notable point in this NOESY spectrum was the cross peak between H_2 of inositol ($^{I}H_2$) and H_5 of mannose ($^{M}H_5$). If we assume the inositol is linked to mannose at 3-position, $^{I}H_2$ and $^{M}H_5$ have to be placed in the opposite side of the plane formed by $^{M}C_1$-O-$^{I}C_1$ (**Figure 2.29a**, right) and it is unlikely that these two protons show the strong cross peak in the NOESY

spectrum. On the other hand, when we assume the inositol is linked to mannose at 1-position, these protons are located in the same side of the $^{M}C_1$-O-$^{I}C_1$ plane (**Figure 2.26a**, left) and it is plausible that the cross peak corresponding to these protons is observed in the NOESY spectrum. The molecular modelling of Man-α(1,1)-*myo*-inositol gave a reasonable structure which can satisfy the data from the NMR experiments (**Figure 2.29b**), whereas the modelling for Man-α(1,3)-*myo*-inositol did not give any reasonable structures in which the distance between $^{I}H_2$ and $^{M}H_5$ fall in the reasonable nOe range. Hence, it was concluded that the glycosilation site of this compound was 1-position.

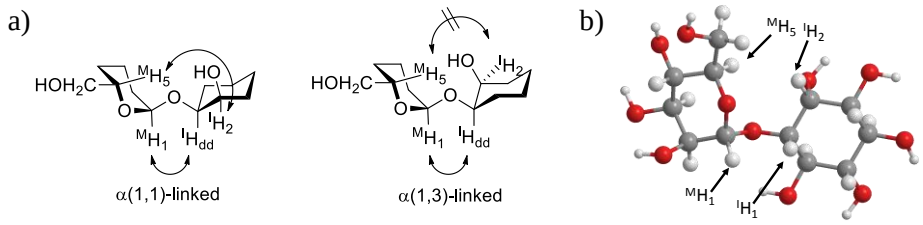


Figure 2.29. (a) Comparison of the α(1,1)- and α(1,3)-linked mannosyl *myo*-inositols. (b)The molecular model of α(1,1)-mannosyl *myo*-inositol. The protons sit in the positions that satisfy the result of the NOESY experiment.

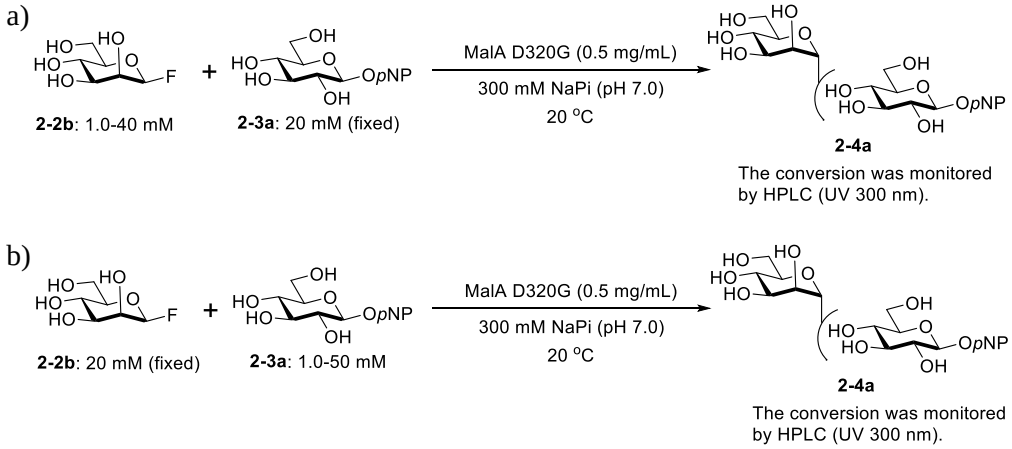
2.2.9. Kinetics of synthase reactions

For further understanding of the glycosynthase reactions catalyzed by MalAD320G, the Michaelis-Menten analysis was performed using *p*NP-β-D-Glc as the acceptor substrate and β-D-ManF or β-D-GlcF as the donor for the manno- or gluco-synthase reaction, respectively (**Scheme 2.19**, **20**). Compared with a number of reported glycosynthases, the corresponding kinetic data are less available (**Table 2.10**). This is partly due to the complex behaviour of the multi-substrate reaction. Especially, the analysis is even more difficult when glycosynthase reaction gives oligo-glycosylated products. All available data of the simple nucleophile-less mutants and representative data of the other evolved mutants are summarized in **Table 2.10**. It should be noted that no kinetic data were reported for α-glycosynthase and the data described

Table 2.10. Kinetic parameters of the glycosynthase reactions. a) α-GlcF: α-D-glucosyl fluoride, α-ManF: α-D-mannosyl fluoride, α-GalF: α-D-galactosyl fluoride, α-XylF: α-D-xylosyl fluoride, α-LamF: α-laminaribiosyl fluoride, α-GlcAF: α-D-glucuronyl fluoride, GM_1OSF: GM_1 oligosaccharyl fluoride. b)*p*NP-β-Cel: *p*NP-β-cellobioside, *p*NP-β-Glc: *p*NP-β-glucoside, *p*NP-β-GlcS: *p*NP-β-4-deoxy-4-thio-glucoside, α-LamF: α-laminaribiosyl fluoride, c), d) and e) the reactions were performed at pH 8.0, 6.9 and 6., respectively.

Enzyme type	Source	Mutation	Donor[a)]	Acceptor[b)]	k_{cat}	K_M	k_{cat}/K_M
					(min^{-1})	(mM)	($min^{-1}\,mM^{-1}$)
exo-β-glucosidase	*Oryza sativa* (rice)	E414G	α-GlcF	pNP-β-Cel	6.08	5.0	1.2
		E414S	α-GlcF	pNP-β-Cel	2.02	4.0	0.50
		E414A	α-GlcF	pNP-β-Cel	0.32	3.7	0.086
exo-β-glucosidase	*Streptomyces* sp.	E383A	α-GlcF	pNP-β-Glc	91.8	1.7	54
exo-β-mannosidase	*Cellulomonas fimi*	E519S	α-ManF	pNP-β-Cel	12	0.70	17
		E519A	α-ManF	pNP-β-Cel	0.33	0.57	0.58
exo-β-glucosidase	*Agrobacterium* sp.	E358S	α-GalF	pNP-β-Glc	177	220	0.800
		E358A	α-GalF	pNP-β-Glc	ND	ND	0.0338
		E358G	α-GalF	pNP-β-Glc	551	316	1.7
		E358G	α-ManF	pNP-β-Glc	18	150	0.12
		E358G, Q248R, M407V	α-GalF	pNP-β-Glc	8282	174	47.3
		E358G, Q248R, M407V	α-ManF	pNP-β-Glc	39	68	0.6
		E358G, Q248R, M407V	α-XylF	pNP-β-Glc	53	1060	0.005
		E171A, E358G	α-GlcF	pNP-β-GlcS	9[c)]	37[c)]	0.24[c)]
		E171A, E358G	α-GlcF	pNP-β-GlcS	1[d)]	6[d)]	0.2[d)]
		E171A, E358G	α-GlcF	pNP-β-GlcS	0.5[e)]	1[e)]	0.5[e)]
endo-β-(1,3)-glucanase	*Hordeum vulgare* (barley)	E231G	α-LamF	α-LamF	0.168	9.7	0.017
		E231S	α-LamF	α-LamF	0.010	19.8	0.00052
		E231A	α-LamF	α-LamF	0.0084	25.9	0.00032
exo-β-glucuronidase	*Escherichia coli*	E504G	α-GlcAF	2-phenylethanol	1.44	0.015	96
endo-glycoceramidase II	*Rhodococcus sp.*	E351S	G_{M1}OSF	sphingosine	13	0.77	16
		E351S, D314Y	G_{M1}OSF	sphingosine	29	0.58	51

Scheme 2.19. The reactions for α-mannosynthase kinetics; a) with the fixed concentration of the acceptor, b) with the fixed concentration of the acceptor.



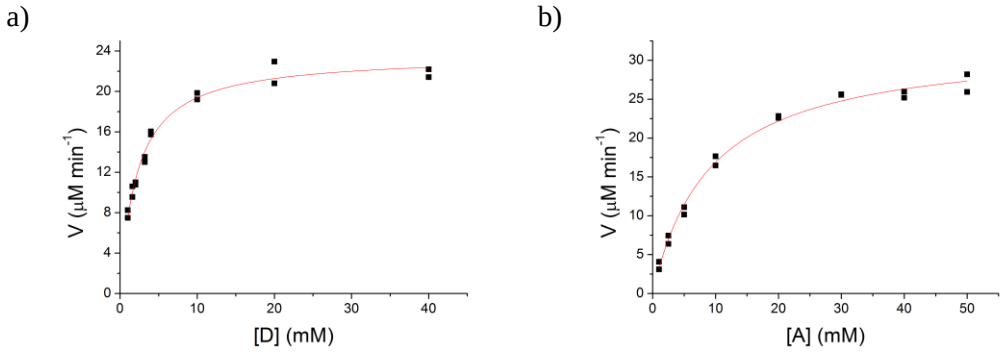
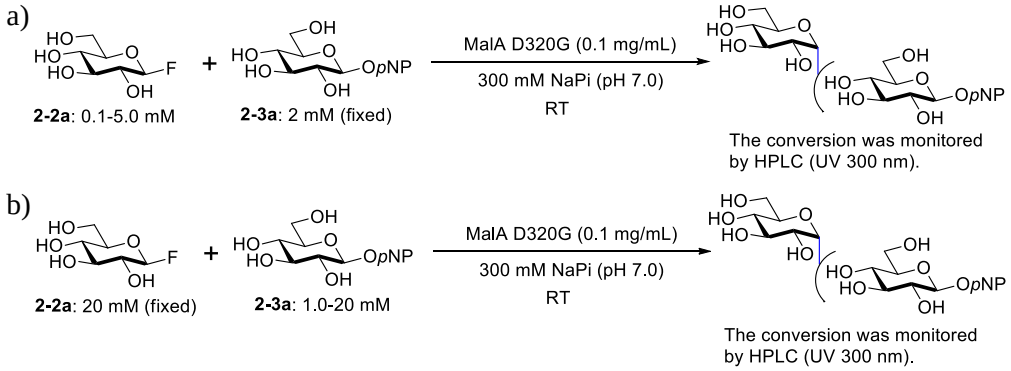


Figure 2.30. Michaelis-Menten analysis on the α-mannosynthase reaction by MalA D320G a) with the fixed concentration of the acceptor ([**2-3a**] = 20 mM), b) with the fixed concentration of the donor ([**2-2b**] = 20 mM).

below are the first such example.

For mannosynthase reaction (**Scheme 2.19**), kinetic parameters could be determined for both the donor and acceptor substrate (**Figure 2.30**, **Table 2.11**). When the acceptor was fixed at 20 mM, the catalytic constant k_{cat} was 3.8 min^{-1} and the Michaelis constant K_M was 2.2 mM, whereas when the donor was fixed at 20 mM, the catalytic constant k_{cat} was 5.3 min^{-1} and the Michaelis constant K_M was 9.1 mM. Though the kinetic parameters differ significantly depending on the used glycosynthases (**Table 2.10**), the typical values of the catalytic constant

Scheme 2.20. The reactions for α-glucosynthase kinetics; a) with the fixed concentration of the acceptor, b) with the fixed concentration of the acceptor.



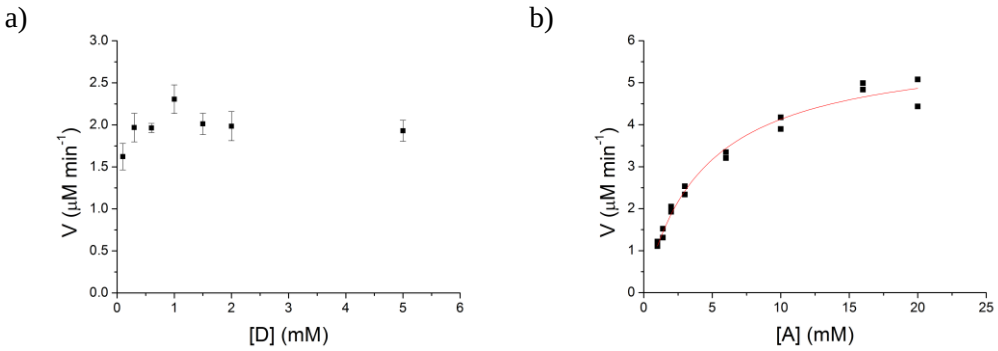


Figure 2.31. Michaelis-Menten analysis on the α-glucosynthase reaction by MalA D320G a) with the fixed concentration of the acceptor ([**2-3a**] = 2 mM), b) with the fixed concentration of the donor ([**2-2a**] = 20 mM).

(k_{cat}) for the simple nucleophile-less mutants are in the range of 10^{-1}-10^{2} min^{-1}, within which the data for the mannosynthase fall. Notably, the turnover number of mannosynthase reaction (k_{cat}= 3.8-5.3 min^{-1}) is far larger than the reaction constant of the spontaneous hydrolysis of the donor substrate[35] (k= (0.8-1.1) × 10^{-3} min^{-1}, **Table 2.8**) and it is a good indicator of the catalytic efficiency of MalA D320G.

Kinetics of the glucosynthase reaction were also measured (**Scheme 2.20**). Though the kinetics for the acceptor substrate could be fitted to the Michaelis-Menten equation (**Figure 2.31b**), that for the donor substrate showed a complex profile (**Figure 2.31a**). To rule out the technical

Table 2.11. Kinetic parameters of synthase reactions by MalA D320G.

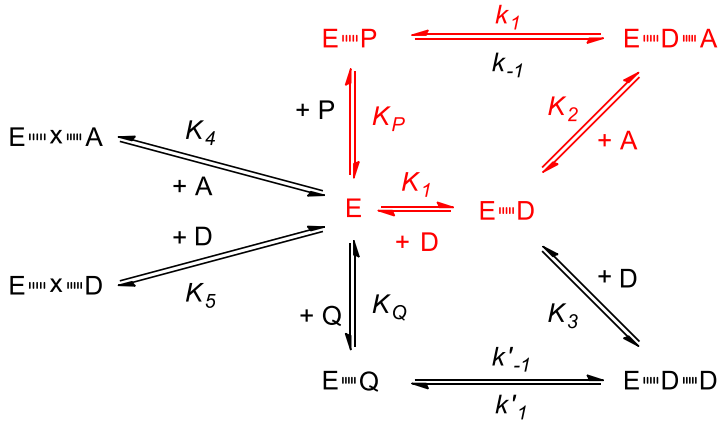
Reaction	Condition	Kinetic parameter		
α-Mannosynthase	[**2-3a**]=20 mM	K_M	2.2 ± 0.1	mM
		k_{cat}	3.8 ± 0.1	min^{-1}
		k_{cat}/K_M	1.7	min^{-1} mM^{-1}
	[**2-2b**]=20 mM	K_M	9.1 ± 0.7	mM
		k_{cat}	5.3 ± 0.1	min^{-1}
		k_{cat}/K_M	0.58	min^{-1} mM^{-1}
α-Glucosynthase	[**2-3a**]=2 mM	K_M	< 0.1	mM
		k_{cat}	1.5-2.0	min^{-1}
		k_{cat}/K_M	N.D.	
	[**2-2a**]=20 mM	K_M	4.3 ± 0.4	mM
		k_{cat}	4.8 ± 0.2	min^{-1}
		k_{cat}/K_M	1.1	min^{-1} mM^{-1}

issues, the experiments were performed at several other concentrations of the acceptor and the enzyme but all the results showed a similar tendency.

For further understanding of these reactions, multisubstrate kinetic analysis was performed by the Cha's modified version[51] of the King-Altman method.[52] We assume bond formation process is far slower than association or dissociation process of substrates on the enzyme.

The following scheme adopts a modified King-Altman analysis that allowed generation of a consistent kinetic model for disaccharide formation from a donor substrate (D) and an acceptor substrate (A). As well as the process of the desired products (P) formation (shown in red), it includes that of the auto-condensation product (Q) formation, which is a potential inhibitory process of the desired product formation and is indeed observed in the absence of the acceptor substrate. Also, it considers binding of substrates at the acceptor site in the absence of donor substrate (these unproductive complexes are expressed as E-x-D and E-x-A); Because we did not observe the hydrolytic activity towards *p*NP-β-D-Glc by wild type MalA we assume its

binding to the donor site is negligible; consequently the enzyme form in which the acceptor binds to the donor site is also explicitly excluded.



The King-Altman method with Cha's rapid equilibrium assumption affords eq. 1 for the rate of the product (P) formation:

$$V = k_1[E\text{-}D\text{-}A] - k_{-1}[E\text{-}P] = \frac{\left(\frac{k_1[D][A]}{K_1K_2} - \frac{k_{-1}[P]}{K_P}\right)[E]_0}{1 + \frac{[D]}{K_1} + \frac{[D][A]}{K_1K_2} + \frac{[P]}{K_P} + \frac{[D]^2}{K_1K_3} + \frac{[Q]}{K_Q} + \frac{[A]}{K_4} + \frac{[D]}{K_5}} \quad \text{eq.1}$$

Because we analyze the initial stage of the reactions as the steady state, we assume concentrations of the disaccharides (P and Q) are negligible (* *vida infra*). For further simplicity, we assume the affinity of substrates at the acceptor site to the enzyme with or without the donor does not change, i.e. $K_4 = K_2$, $K_5 = K_3$. These approximations convert eq.1 into eq.2:

$$V = \frac{\frac{k_1[D][A]}{K_1K_2}[E]_0}{1 + \left(\frac{1}{K_1} + \frac{1}{K_3}\right)[D] + \frac{[A]}{K_2} + \frac{[D]^2}{K_1K_3} + \frac{[D][A]}{K_1K_2}} \quad \text{eq.2}$$

When the concentration of the donor is fixed ($[D] = [D]_0$), the equation is transformed into eq.3 as the function of the concentration of the acceptor:

$$V = \frac{\frac{k_1 [D]_0}{K_2 + [D]_0} [E]_0[A]}{\frac{(K_1 + [D]_0) K_2 (K_3 + [D]_0)}{(K_2 + [D]_0) K_3} + [A]} \qquad \text{eq.3}$$

Indeed, when the concentration of the donor was fixed, experimentally determined kinetics yielded Michelis-Menten-like profiles in both manno- and gluco-synthase reactions (**Figure 2.27**, **2.28**).

When the concentration of the acceptor is fixed ([A] = $[A]_0$), the equation is transformed to eq.4 as the function of the concentration of the donor:

$$V = \frac{\frac{k_1 K_3 [A]_0}{K_1 K_2 + K_2 K_3 + K_3 [A]_0} [E]_0[D]}{\frac{[D]^2}{K_1 + K_3 (1 + \frac{[A]_0}{K_2})} + [D] + \frac{(K_1 + [A]_0) K_2 K_3}{K_1 K_2 + K_2 K_3 + K_3 [A]_0}} \qquad \text{eq.4}$$

This equation contains the second-order term in the denominator.

This equation *only* approximates to a simple M-M-like profile when the following conditions are met:

i) $[D] << K_1$ or ii) $[D] << K_3$ or iii) $[D] << K_3[A]_0/K_2$.

The experimentally determined data for the mannosynthase reaction can be approximated to a simple MM equation (**Figure 2.27**). Since kinetics were determined in the donor concentration range [D]= 0.5- 40 mM and with a fixed concentration of the acceptor, this can be practically interpreted as one of the following conditions having been met:

i) K_1 is very large or ii) K_3 is very large or iii) $K_2 << K_3$.

Since the apparent k_{cat} with the fixed donor concentration (eq.3) was detectable, K_2 can therefore be estimated to be in the similar range as $[D]_0$ (= 20 mM). Thus, condition iii) can be practically regarded as the same situation as condition ii).

If condition i) were true, the apparent K_M of the kinetics determined at fixed concentration of

donor ([D]= $[D]_0$) would be too large to be calculated (see eq. 3) and the apparent k_{cat} of the kinetics with the fixed concentration of the acceptor ([A]= $[A]_0$) would also become too small to be determined (see eq.4). However, this is not the case and so condition i) can be excluded. Hence, we concluded that K_3 is very large in the mannosynthase reaction and eq. 3 and eq. 4 can be approximated to eq. 3' and eq. 4', respectively.

$$V = \frac{\frac{k\,[D]_0}{K_2 + [D]_0}\,[E]_0[A]}{\frac{(K_1 + [D]_0)\,K_2}{(K_2 + [D]_0)} + [A]} \qquad \text{(eq. 3')}$$

$$V = \frac{\frac{k\,[A]_0}{K_2 + [A]_0}\,[E]_0[D]}{\frac{(K_1 + [A]_0)\,K_2}{(K_2 + [A]_0)} + [D]} \qquad \text{(eq. 4')}$$

The implication of the large K_3 value in the mannosynthase reaction is that the mannoside substrate has very weak affinity to the enzyme at the acceptor site. We presume this weak affinity can be extrapolated to the products of the mannosynthase reaction (disaccharides which have D-mannose at non-reducing terminus). These results of the kinetics analyses are strong support for the proposed design strategy.

In the striking contrast, the glucosynthase reaction does not allow fitting of a typical simple MM profile. Previuosly, the failure of the synthase kinetics for the acceptor to fit to the Michaelis-Menten equation was reported.[53] In this case, the acceptor was a substrate for the parent glycosidase and hence it was clear that the acceptor inhibited the binding of the donor to the D-site and the additional term of the inhibition by the acceptor allowed the derivation of a nicely fitting equation (similar to (eq.4); the equation with the second-order term of [A] in the denominator). Conversely, in this glucosynthase reaction by MalA D320G, the failure to fit was observed for the donor substrate. The donor substrate itself is expected to be an inhibitor for the

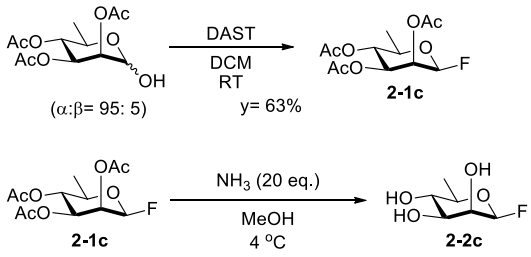
desired reaction due to its affinity to the A-site, resulting in the formation of undesired auto-condensed product. If the inhibitory process is caused only by the donor substrate binding to the A-site, the (eq.4) should fit to the plot. However, even this equation did not give a good fitting to the plot. As discussed above (*), it was assumed that the formed disaccharide (P and Q) were negligible in the initial stage of the reaction. Though this assumption was effective for the mannosynthase reaction, such disaccharides that result from the glucosynthase reaction are likely to have higher affinity for both the D-site and A-site due to their structural similarity to the native substrates (maltoside) and hence might be able to inhibit the desired reaction process even at the very low concentration in the initial stage of the reaction. All in all, there seem to be other factors to be considered for the inhibitory process of the desired reaction than that by the donor substrate itself, resulting in the complex behaviour of the kinetics of the glucosynthase reaction by MalA D320G.

2.2.10. Broadening donor scope

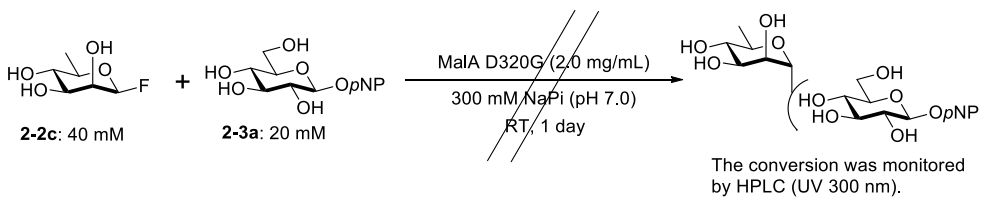
As an attempt to broaden the donor substrate scope, rhamnosynthase reaction was tested. First, β-D-RhaF (**2-2c**) was synthesized (**Scheme 2.21**) as a glycosyl donor, following the preparation scheme for β-ManF (**2-2b**). Triacetyl D-rhamnose[54] with a free hydroxyl group at the anomeric position was reacted with DAST. The reaction gave the mixture of α- and β-anomers as the manno-derivative, but these isomers were separable with the column chromatography. The desired β-anomer was isolated in 63% yield, accompanied by 28% yield of the α-isomer. Deprotected β-D-RhaF was prepared freshly just before the synthase reaction with ammonia methanol. The deprotected product was used without further purification.

The reaction was performed under the optimized condition for the mannosynthase reaction (**Scheme 2.22**). However, the analysis by HPLC did not show any indication of product formation after 1 day (**Figure 2.32**). Rhamnosyl donor has the opposite stereoconfiguration at 2-position to the native glucosyl donor as well as it lacks 6-OH group which is presented in

Scheme 2.21. Preparation of β-D-RhamF.



Scheme 2.22. Attempt of Rhamnosynthase reaction.



the glucosyl and mannosyl donor. Especially, the existence of 4-OH and 6-OH in the sugar substrates are known to be essential for GH-31 α-glucosidases.[25, 26] Therefore, the lack of rhamnosynthase activity would be due to the poor affinity of β-D-RhaF to the A-site in MalA D320G.

When the kinetic data for the previously reported synthases are examined (**Table 2.10**), the K_M values do not have to be low (10^2-10^3 mM range for some of Abg mutants[53, 55]) to create synthases. Therefore, there might be chances to get the galacto- and xylo-synthase reactions using MalA D320G (**Table 2.4**).

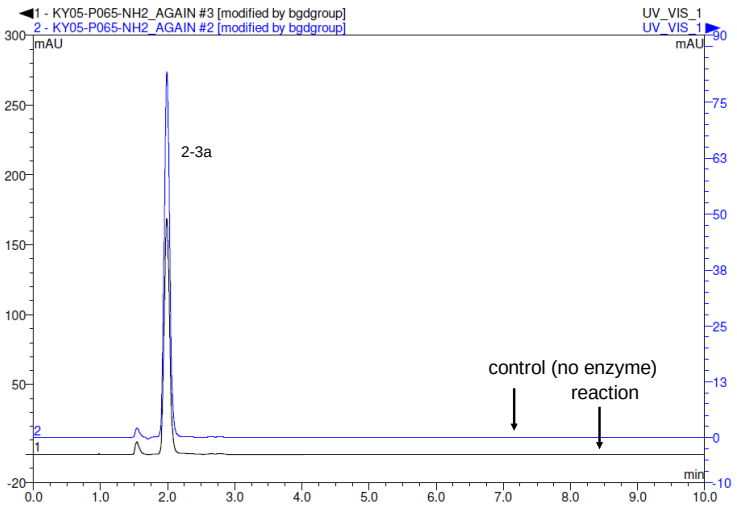
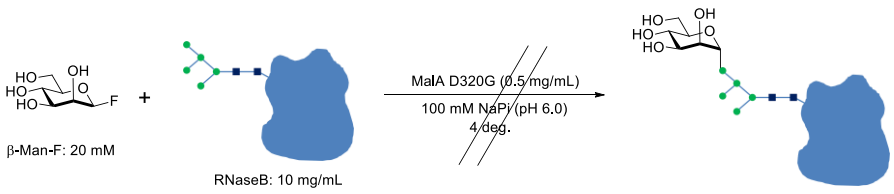


Figure 2.29. Result of rhamnosynthase reaction. No glycosylation of the acceptor was observed under the test condition (**Scheme 2.22**).

2.2.11. Modification of Glycoprotein

Mannosynthase reaction was also applied to glycoprotein modification (**Scheme 2.23**). RNaseB, which has oligomannose *N*-glycan, was used as a model substrate. The structure of oligomannose ranges from $Man_5GlcNAc_2$ to $Man_9GlcNAc_2$. So far, the reaction was tested only under the pre-optimized condition (**Scheme 2.23**). In this condition, no indication of mannosylation was observed by evaluation with ESI-MS (**Figure 2.33**). The reason for the lack of the reactivity is not clear yet but the steric hindrance around the acceptor substrate (the terminal mannoses of *N*-glycan) might impair their easy access to the reaction site. Moreover, the investigation of the substrate scope suggested the terminal Man-α(1,2)-Man is not a good acceptor scaffold (**Scheme 2.17**). Such a motif is presented in more than one of the branches of the oligomannose structures except $Man_5GlcNAc_2$. Therefore, the structure of oligomannoses itself might preclude competence as an acceptor for this reaction.

Scheme 2.23. Attempt of glycoprotein modification by glycosynthase MalA D320G.



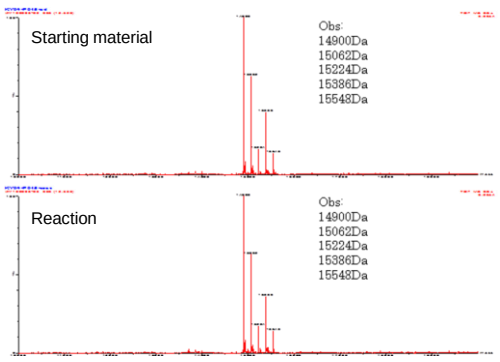


Figure 2.33. ESI-MS of the mannosynthase reaction on RNaseB. Mannosylation of RNaseB was not observed in the test condition (**Scheme 2.23**)

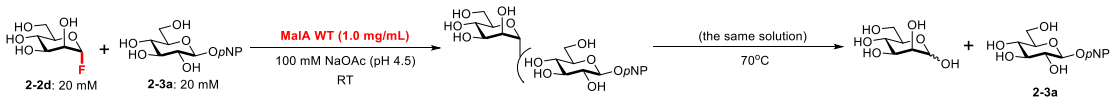
2.2.12. Mannosynthase activity of MalA WT

Transglycosylation activity of MalA WT was also examined. The reaction was performed at room temperature using 1.0 mg/mL MalA WT in 100 mM NaOAc buffer (pH 4.5) (**Scheme 2.24**). Surprisingly, the glycosylation reaction proceeded quite efficiently (**Figure 2.31a**). Even though only one equivalent of the donor substrate was used, the acceptor was converted to the mono-mannosylated substrate in >80% conversion in 24 hours, accompanied by small amount of di-mannosylated compounds. The hydrolytic activity was not apparent at room temperature until 24 hour.

Then, the resultant reaction mixture was heated up to 70°C (**Scheme 2.24**). Now, MalA WT started to exert its hydrolytic activity and the concentration of the acceptor *p*NP-β-D-Glc recovered up to 80% in 4 hours (**Figure 2.31b**). The ttriaccharide byproduct seemed to be tolerant to the hydrolysis even under this heated condition and the concentration of these compounds did not decrease.

All of the glycosidases have latent transglycosylation activity. However, their hydrolytic activities usually surpass the transglycosylation activity under typical conditions. Originally, glycosynthases are developed to suppress such undesired hydrolytic actvities towards the transglycosylated products by deleting their catalytic nucleophiles. However, MalA, an α-glucosidase from the thermophilic archea *sulfolobus solfataricus*, inherently has sparing hydrolytic activity at room temperature. Additionally, the rate-determining step of

Scheme 2.24. Transglycosylation reaction by MalA WT using αManF as donor, followed by heating the same solution.



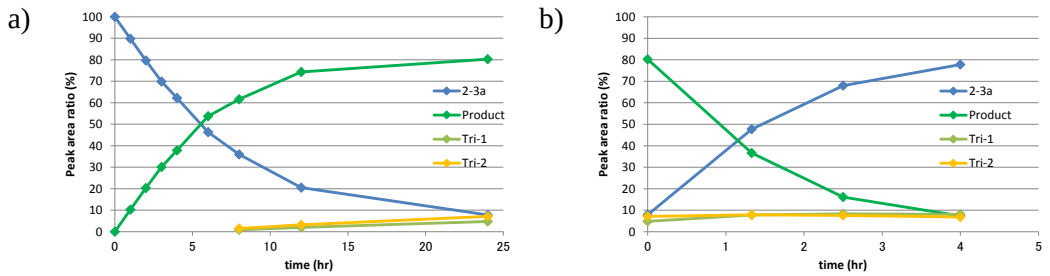


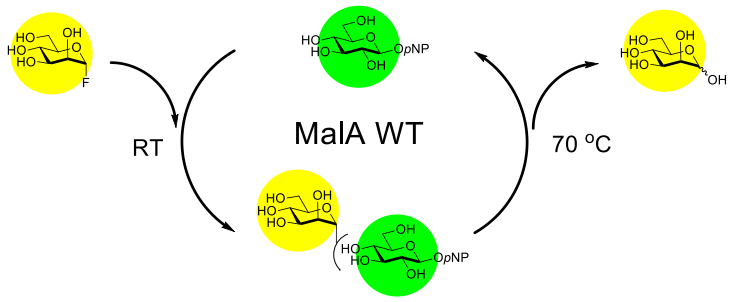
Figure 2.34. Transmannosylation by MalA WT, followed by hydrolysis by the same enzyme under the heated condition. The reaction was initiated using 20 mM *p*NP-β-D-Glc (**2-3a**) and 20 mM α-ManF (**2-2d**) in 100 mM NaOAc (pH 4.5) with 1.0 mg/mL MalA WT at room temperature. The reaction was monitored by UV absorption at 300 nm using HPLC. After 24 hours, the reaction was heated to 70°C.

GH-31 enzymes is reported to be the glycosylation step, not the deglycosylation step. Therefore, only if the first glycosylation takes place, the following hydrolysis or transglycosylation would proceed smoothly. In this experiment, the first glycosylation was facilitated by the fluoride substrate. This activated glycosyl donor enabled the enzyme to overcome the rate-determining glycosylation step, followed by an "easy" transglycosylation. The resultant transglycosylated product has a non-activated glycosidic bond and moreover it is a "mismatched" substrate (mannoside) for MalA. Therefore, it is difficult for MalA to hydrolyze the transglycosylated products, resulting in the excellent conversion of the acceptor substrate. Currently, it is not certain if this highly effective transglycosylation by the wild type glycosidase can be extrapolated to other glycosidases but it seems promising when the following conditions are set together: i) sparing hydrolytic activity at low temperature, ii) a retaining glycosidase which has its rate-determining step in the glycosylation step, iii) its D-site can accommodate mismatched activated donors. The first two conditions are likely to be satisfied by some of α-glycosidases from thermophilic organisms. As to the condition iii), some galactosidases are known to have weak D-fucosidase activity.[26, 56, 57] It is not clearly mentioned which family these enzymes

belong to, galactosidases are mainly found in GH27 and GH36. In GH36, galactosidases from thermophilic organisms (like *Sulfolobus solfataricus* and *Thermotoga maritima*) can be found and these enzymes might be candidates for transfucosidase at room temperature.

Control of the direction of the catalysis by temperature is an intriguing result. Ideally, such a system enables us to switch one compound to another by changing temperature of the reaction system (**Scheme 2.25**). If one or both of these components can trigger some phenotypical effects, this catalyst system might be useful as an oscillo-bio-switch responding to tempetature. The current situation is far from a practical application due to the incompleteness of the conversion and the formation of side procducts. Nevertheless, this experiment provides conceptual inspiration.

Scheme 2.25. A concept of enzymatic oscillation between mono- and di-saccharides using a glycosidase which changes the direction of catalysis oppositely depending on temperature.



2.3. Conclusion

In this chapter, I demonstrated a novel strategy to design glycosynthases. In this method we use a glycosidase which has a plastic structure at the D-site and can accommodate a non-native ligand with good affinity. A glycosynthase devised from such a glycosidase in combination with a non-native glycosyl donor can suppress the formation of undesired oligomerized compounds and catalyze the discrete synthesis of a monoglycosylated product. This new methodology can broaden chances to access more diverse glycosynthase. Now much information is available for glycosidases about their substrate scopes. Examining such data enables us to predict their applicability to the method shown here. Moreover, screening the hydrolytic activity of the wild type glycosidases towards various sugar substrates can also offer implication whether they are convertible to the biocatalyst in this design. Recently, a glycosynthase developed from a rice BGlu1 β-D-glucosidase was reported.[20] This glycosynthase has a very wide donor tolerance and the result of the reactions showed the striking contrast depending on the used donor substrates: when glucosyl fluoride is used, the products of up to tetra-glycosylation was observed as major products, whereas when the other glycosyl fluorides (D-arabinose, D-fucose, Gal, Man, Xyl) were used, monoglycosylated products were obtained almost exclusively. This is another example to validate our strategy to develop glycosynthases for discrete monosaccharide installation.

Our rational design resulted in developing the first example of an α-mannosynthase. This new biocatalyst enabled us to prepare a variety of natural oligosaccharide motifs in one step without repetitive protection/deprotection schemes. Additionally, desymmetrization of *myo*-inositol was realized through glycosylation reaction.

Broadening the scope of donor substrates has not produced an affirmative result yet, but there seem to be much room to be investigated, especially as galacto- and xylo-synthase. Moreover, it is known that directed evolution is an effective way to broaden the scope of the donor and, indeed, the scope of β-gluco-/galacto-synthase was evolved to β-manno-/xylo-synthase.[55] Enhancement of the glycosynthase activity by directed evolution may also be effective for glycoprotein modification, which has not yet been successful.

Unexpectedly, MalA WT showed very high transglycosylation activity at room temperature without any indication of hydrolysis of the products until 24 hours. However, the products were hydrolyzed when the reaction temperature was raised to 70°C. Such exchangable catalysis depending on temperature is expected to render a chance of applications as an oscillatory bio-switch.

2.4. Experimental

Preparation of pET-29a(+)-*malA*

Synthesis of *malA* gene (GenBank accession: AAC38215) and its subcloning to pET-29a(+) vector (Novagen) was performed by GenScript. The original stop codon in *malA* was deleted and the restriction sites for *Nde*I and *Xho*I were introduced at 5'- and 3'-termini, respectively (pET-29a(+)-*malA*), resulting in addition of Leu-Glu and His_6-tag to the native amino acid sequence of MalA in the C-terminus.

Expression of MalA WT by IPTG induction

BL21 (DE3) competent cells (Singles kit, Novagen) were thawed on ice and gently suspended by finger flicks. 1 μL of pET-29a(+)-*malA* (100 ng/mL) was added and mixed by pipetting, followed by incubation on ice for 5 min. Heat-shock was applied to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 250 μL of SOC medium (Novagen) was added and the mixture was incubated at 37°C for 1 hr (250 rpm). On LB-Kan agar was loaded 40 μL of SOC medium with which 5 μL of the transformed cell suspension was mixed, followed by being spread on the plate. The plate was incubated at 37°C for 20 hr and stored at 4°C.

150 mL of LB-Kan medium was inoculated with a single colony and the culture was incubatied at 37°C for 24 hr (250 rpm). 10 mL of this overnight culture was added to 1L of LB-Kan medium. The cultures were grown at 37°C and when the OD_{600} value reached around 0.6, 1 mL of 1M IPTG was added. The culture was further incubated at 37°C for 24 hr. The cells were harvested by centrifugation (8 krpm, 4°C, 15 min) to give a pellet, which was collected and stored at -20°C.

The cell pellet was thawed and suspended in 180 mL of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, which contained 18 mg of lysozyme, 6 mg of DNase I and 1 tablet of Complete (protease inhibitor cocktail, Roche). The cell suspension was stirred for 1 hr at room temperature. The cells were further lysed by sonication on ice (6 x 1.5 min (pulse 1.0 sec; pause 0.5 sec) with 1.0-min intervals). The suspension was heated at 70°C for 20 min to denature the unwanted proteins of *E. coli*. After cooling, the precipitation was removed by centrifugation (20 krpm, 30 min, 4°C).

To the supernatant (180 mL) was added ammonium sulfate (63 g, 50% saturation) at 4°C and the mixture was stirred for 1 hr, followed by centrifugation (20 krpm, 30 min, 4°C). The

supernatant was discarded and the precipitation was redissolved in 16 mL of solvent A (see below). The solution was filtered with 0.45 μm-membrane filter and applied to FPLC for purification. The conditions were as follows:

Column: HisTrap™ HP 5 mL (GE Healthcare)
Solvent A: 20 mM Tris-HCl (pH 7.8), 500 mM NaCl, 5 mM imidazole
Solvent B: 20 mM Tris-HCl (pH 7.8), 500 mM NaCl, 500 mM imidazole
Flow rate: 1.5 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Gradient: 10% Solvent B 5CV
to 100% Solvent B 10CV
100% Solvent B 3CV

After the analysis on SDS-PAGE, the fractions containing the target protein were collected and dialyzed against 100 mM sodium phosphate buffer (pH 6.0), followed by filtration with 0.20 μm-pore membrane. The concentration was determined by BCA assay (General Procedure).

Expression and purification of MalA WT (autoinduction)

BL21(DE3) competent cells in Singles kit (Novagen) were thawed on ice and suspended by finger-flick. 1 μL of pET-29a(+)-*malA* was added and mixed by pipetting, followed by incubation on ice for 5 min. Heat-shock was applied to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 250 μL of SOC medium was added and the mixture was incubated at 37°C for 1 hr (200 rpm). On LB-Kan agar was loaded 40 μL fo SOC medium with which 10 μL of the transformed cell suspension was mixed, followed by being spread on the plate. The plate was incubated at 37°C for 20 hr and stored at 4°C.

A single colony was transferred to 20 mL of LB-Kan medium followed by incubation at 37°C for 20 hr. 4 mL of this preculture solution was added to 500 mL of ZYM-5052-Kan medium in a 2-L baffled flask. The cell culture suspension was incubated for 18 hr at 28°C (200 rpm). When autoinduction works properly, the final OD_{600} is around 8 and the pH is around 7. The cells were harvested by centrifugation (8 krpm, 15 min, 4°C) to give a pellet, which was collected and stored at -20°C.

The cell pellet was thawed and was suspended in 180 mL of 50 mM Tris-HCl (pH 7.5), 100 mM NaCl), which contained 18 mg of Lysozyme, 6 mg of DNase I and 1 tablet of Complete (protease inhibitor cocktail, Roche). The cell suspension was stirred for 1 hr at room temperature. The cells were further lysed by sonication on ice (6 x 1.5 min (pulse 1.0 sec; pause 0.5 sec) with 1.0-min intervals). The suspension was heated at 70°C for 20 min to denature the

unwanted proteins of *E. coli*. After cooling, the precipitation was removed by centrifugation (20 krpm, 30 min, 4°C).

To the supernatant (~180 mL) was added ammonium sulfate (63 g, 50% saturation) at 4°C and the mixture was stirred for 1 hr, followed by centrifugation (20 krpm, 30 min, 4°C). The supernatant was discarded and the precipitation was redissolved in 30 mL of solvent A (see below). The solution was filtered with 0.45 μm-membrane filter and applied to FPLC for purification. The conditions were as follows:

Column: HisTrap™ HP 5 mL (GE Healthcare)
Solvent A: 20 mM Tris-HCl (pH 7.8), 500 mM NaCl, 5 mM imidazole
Solvent B: 20 mM Tris-HCl (pH 7.8), 500 mM NaCl, 500 mM imidazole
Flow rate: 1.5 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Gradient: 10% Solvent B 5CV
to 100% Solvent B 10CV
100% Solvent B 3CV

After the analysis on SDS-PAGE, the fractions containing the target protein were collected, which were concentrated by dialyzed against 100 mM sodium phosphate buffer (pH 6.0), followed by concentration with ultrafiltration. (Note: when the collected fractions were directly concentrated, precipitation was observed.) The resultant solution was filtered with 0.45 μm-membrane filter and applied to FPLC:

Column: HiLoad 26/60 Superdex 200 prep grade (GE Healthcare)
Solvent: 100 mM NaPi (pH 6.0), Isocratic
Flow rate: 2.5 mL/min (Maximum flow rate; Pressure regulation <0.40MPa)
Sample: < 6mL

After the analysis on SDS-PAGE, the fractions containing the target protein were collected and concentrated by ultrafiltration. The concentration was determined by BCA assay (General Procedure). The enzyme solutions were stored as small aliquots at -20°C after snap freezing.

Determination of the molecular weight of MalA WT by ESI-MS

The molecular weight of MalA WT was measured using ESI-MS (General Procedure, Protein MS). Optimal condition to measure molecular weight of MalA was investigated changing a sample cone voltage (25, 35, 45 and 55V) and acid content in eluents (0.1, 0.5 and 1.0%). The

sample volume of MalA WT (1.0 mg/mL) was 20 μL. The best condition was obtained with 25V cone voltage and 0.5% formic acid in the eluent. The molecular weight of MalA WT was found as 81505 Da (Calcd.81506 Da).

Kinetics of hydrolytic reaction by MalA WT to *p*NP-α-D-Glc

100 μL of *p*NP-α-D-Glc (0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0 and 8.0 mM, triplicate) in 100 mM NaOAc (pH 4.5) was preincubated at 85°C for 5 min. 1.0 μL of 0.49 mg/mL MalA solution in 100 mM NaPi (pH 6.0) was added to the test solutions. The solutions were incubated at 85°C and quickly cooled on ice after 0, 5 or 10 min. After centrifugation, the sampled solutions was mixed with 100 mM NaPi (pH 9.0) on a 96-well plate and the concentration of 4-nitrophenol liberated by the enzymatic reaction was determined by UV absorption at 410 nm. The calibration curve was prepared by the dilution series of 4-nitrophenol in 100 mM NaOAc (pH 4.5) and 100 mM NaPi (pH 9.0) on the same 96-well plate. The reaction rates and their errors were determined using Origin 8 (OriginLab). The obtained reaction rates were plotted against the substrate concentrations and the plots were fitted to Michaelis-Menten equation by Origin 8 to determine the kinetic parameters.

Substrate screening for the hydrolytic activity of MalA WT

100 μL of 20 mM *p*NP-glycoside (triplicate) in 100 mM NaOAc buffer (pH 4.5) were preincubated at 85°C for 5 min. 5 μL of MalA solution (1.30 mg/mL in 100 mM NaPi buffer (pH 6.0)) was added to the test solutions and the same amount of 100 mM NaPi buffer (pH 6.0) to the control solutions. The solutions were incubated at 85°C for 2 hr, followed by cooling on ice. After centrifugation, the sampled solutions were mixed with 100 mM NaPi buffer (pH 9.0) on a 96-well plate and the concentration of 4-nitrophenol liberated by the enzymatic reaction was determined by UV absorption at 410 nm. The calibration curve was prepared by the dilution series of 4-nitrophenol in 100 mM NaOAc (pH 4.5) and 100 mM NaPi (pH 9.0) on the same 96-well plate. The solutions of *p*NP-α-D-Xyl formed precipitation when cooled. In such cases, the solutions were warmed for a while until they became clear before mixing with pH 9 NaPi solution.

Kinetics of hydrolytic reaction by MalA WT to *p*NP-α-D-Man, -Gal and -Xyl

The same experiment as *p*NP-α-D-Glc was performed for *p*NP-α-D-Man with the different substrate concentrations (1.0, 1.5, 2.0, 3.0, 4.0, 8.0, 12.0 and 16.0 mM, triplicate), the enzyme concentration (240 μg/mL) and incubation time (0, 30 and 60 min). For *p*NP-α-D-Gal, the

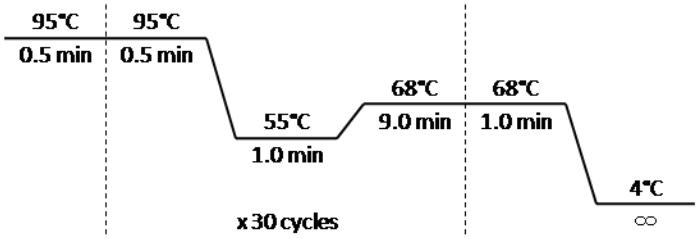
substrate concentrations were 4.0, 8.0, 12.0, 20.0, 40.0 and 80.0 mM (triplicate), the enzyme concentration was 65 μg/mL and the reaction time was 1 hr. For *p*NP-α-D-Xyl, the substrate concentrations were 1.0, 2.0, 4.0, 8.0, 12.0 and 20.0 mM (triplicate) and the enzyme concentration was 65 μg/mL and the reaction time was 1 hr. The reaction rates and their errors were determined using Origin 8 (OriginLab). The obtained reaction rates were plotted against the substrate concentrations and the plots were fitted to Michaelis-Menten equation for the hydrolysis of *p*NP-α-D-Man by Origin 8 to determine the kinetic parameters. The plots for the hydrolysis of *p*NP-α-D-Gal and *p*NP-α-D-Gal were fitted to the linear lines and the slopes were determined as k_{cat}/K_M.

Site-directed mutagenesis of MalA at D320

Mutagensis was performed using QuikChange II Site-Directed Mutagenesis kit (Stratagene). Primers purchased from Sigma-Aldrich were reconstructed to 1 μg/μL solution with water. The reagents were mixed as follows:

5 μL	10x reaction buffer
1 μL	pET-29a(+)-*malA*, 100 ng/μL
1.25 μL	forward primer, 0.1 μg/μL
1.25 μL	reverse primer, 0.1 μg/μL
1 μL	dNTP mix
39.5 μL	H_2O

Then 1 μL of *PfuUltra* HF DNA polymerase in the kit was added and the mixture was cycled using the following cycling parameters.



1 μL of *Dpn* I restriction emzyme (10 U/μL) was added and the solution was mixed by pipetting. The mixture was spun down by centrifugation, followed by incubation at 37°C for 1 hr. The resultant solution was stored at 4°C before use.

XL10-Gold ultracompetent cells in the kit were thawed on ice and 45 μL were dispensed for each transformation in prechilled conical Falcon tubes on ice. 2 μL of the β-ME mix in the kit

was added. The cells were incubated on ice for 10 min with occasional swirling. 2 μL of the *Dpn* I-treated solution was added to the cell suspension, followed by incubation on ice for 30 min. Heat shock was charged to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 0.5 mL of preheated NZY^+ medium was added, followed by incubation at 37°C for 1 hr with shaking at 250 rpm. 250 μL of the transformation reaction was spread on LB-Kanamycin agar. The plate was incubated at 37°C for 16 hr and stored at 4°C before use.

Purification of the the plasmid DNA was performed as in General Procedure and the extract of plasmid DNA (termed as pET-29a(+)-*malA:D320G*) was stored at -20°C. The plasmid DNA solution was diluted to 100 ng/μL with water and submitted to Geneservice (now known as SourceBioScience) for DNA sequencing using T7 promoter primer. The chromatography data were visualized using Finch TV program.

Expression and purification of MalA D320G

Expression and purification of MalA D320G was performed in the same method as described in the autoinduction of MalA WT except using pET-29a(+)-*malA: D320G*. The molecular weight of MalA D320G was found as 81445 Da (Calcd. 81448 Da) in the same condition as MalA WT.

Substrate scope of MalA D320G

100 μL of 20 mM *p*NP-α-D-Glc/Man/Gal/Xyl (triplicate) in 100 mM NaOAc buffer (pH 4.5) were preincubated at 85°C for 5 min. 5 μL of MalA D320G solution (1.41 mg/mL in 100 mM sodium phosphate buffer (pH 6.0)) was added to the test solutions and the same amount of 100 mM sodium phosphate buffer (pH 6.0) to the control solutions. The solutions were incubated at 85°C for 1 hr, followed by cooling on ice. After centrifugation, the sampled solutions were mixed with 100 mM NaPi buffer (pH 9.0) on a 96-well plate and the concentration of 4-nitrophenol liberated by the enzymatic reaction was determined by UV absorption at 410 nm. The calibration curve was prepared by the dilution series of 4-nitrophenol in 100 mM NaOAc (pH 4.5) and 100 mM NaPi (pH 9.0) on the same 96-well plate.

Kinetics of hydrolytic reaction by MalA D320G to *p*NP-α-D-Glc

The same experiment as the hydrolysis of *p*NP-α-D-Glc by MalA WT was performed with the different substrate concentrations (0.2, 0.4, 1.0, 2.0, 4.0, 6.0 and 8.0 mM, triplicate), the enzyme concentration (MalA D320G, 67.1 μg/mL) and incubation time (1 hr). The reaction rates and their errors were determined by Origin 8 (OriginLab). The obtained reaction rates were plotted

against the substrate concentrations and the plots were fitted to Michaelis-Menten equation by Origin 8 to determine the kinetic parameters.

Preparation of 2,3,4,6-tetraacetyl D-(+)-mannopyranosyl fluoride (**2-1b**, **Scheme 2.6**)

To a solution of 1,2,3,4,6-pentaacetyl D-(+)-mannopyranoside (5.00 g, 12.8 mmol, 1.0 eq.) in DMF (12.8 mL) was added hydrazine acetate (1.42 g, 15.4 mmol, 1.2 eq.) and the solution was stirred for 2.5 hr at room temperature. The reaction was diluted with ethyl acetate (50 mL) and washed with 5% NaCl (50 mL x 2). The aqueous phase was extracted with ethyl acetate (50 mL x 2). The combined organic phase was washed with water (50 mL) and brine (50 mL), dried over MgSO4 and concentrated. The residue was purified by Biotage Flash Purification System (cartridge; SNAP KP-Sil 340 g) to afford 2,3,4,6-tetraacetyl D-(+)-mannopyranoside as white foam; 2.97 g (y= 67%). ^{1}H NMR[58] (400MHz, $CDCl_3$, δ (C*H*Cl_3)=7.270): 2.01, 2.06, 2.12, 2.17 (4 x 3H, 4 x s, 3 x OCOC*H*$_3$), 3,23 (1H, br s, OH), 4.15 (1H, dd, $J_{H6\text{-}H6'}$ 11.6Hz, $J_{H6\text{-}H5}$ 2.6Hz, H6), 4.25 (1H, ddd, $J_{H5\text{-}H4}$ 10.1Hz, $J_{H5\text{-}H6}$ 5.1Hz, $J_{H5\text{-}H6'}$ 1.9Hz, H5), 4.27 (1H, dd, $J_{H6'\text{-}H6}$ 11.6Hz, $J_{H6'\text{-}H5}$ 5.1Hz, H6'), 5.26 (1H, s, H1), 5.29 (1H, dd, $J_{H2\text{-}H3}$ 3.3Hz, $J_{H2\text{-}H1}$ 2.0Hz, H2), 5.32 (1H, dd, $J_{H4\text{-}H3}$ 9.9Hz, $J_{H4\text{-}H5}$ 9.9Hz, H4), 5.44 (1H, dd, $J_{H3\text{-}H4}$ 10.1Hz, $J_{H3\text{-}H2}$ 3.4Hz, H3). LRMS (ESI$^+$): m/z 371.1 $[M+Na]^+$. R_f= 0.19 (ethyl acetate: petroleum ether= 1: 1).

To a solution of 2,3,4,6-tetraacetyl D-(+)-mannopyranoside (2.94 g, 8.44 mmol, 1.0eq.) in dichloromethane (16.9 mL) was added DAST (3.32 mL, 25.3 mmol, 3.0 eq.) dropwise at room temperature. The solution was stirred at room temperature for 2 hr. After cooling to 0°C, methanol (3.0 mL) was added slowly to quench DAST. Water (20 mL) was added and the organic phase was separated. The aqueous phase was extracted wth dichloromethane (20 mL). The combined organic phase was washed with sat. $NaHCO_3$ (20 mL) and brine (20 mL), dried over $MgSO_4$ and concentrated. The residue was purified by Biotage Flash Purification System (cartridge; SNAP KP-Sil 340 g, eluent gradient; ethyl acetate/ petroleum ether = 8% -> 66 %, 10CV), to afford the titled compound (**2-1b**) as white solid; 1.73 g (y= 62%). ^{1}H NMR[35] (500MHz, $CDCl_3$, δ (C*H*Cl_3)=7.270): 2.07, 2.09, 2.11, 2.17 (4 x 3H, 4 x s, 3 x OCOC*H*$_3$), 3.92 (1H, ddd, $J_{H5\text{-}H4}$ 11.8Hz, $J_{H5\text{-}H6}$ 5.6Hz, $J_{H5\text{-}H6'}$ 0.6Hz, H5), 4.33- 4.36 (2H, m, H6, H6'), 5.18 (1H, dd, $J_{H3\text{-}H4}$ 7.8Hz, $J_{H3\text{-}H2}$ 3.5Hz, H3), 5.23 (1H, dd, $J_{H4\text{-}H3}$ 8.0Hz, $J_{H4\text{-}H5}$ 6.9Hz, H4), 5.45 (1H, ddd, $J_{H2\text{-}F}$ 10.7Hz, $J_{H2\text{-}H3}$ 3.5Hz, $J_{H2\text{-}H1}$ 1.9Hz, H2), 5.55 (1H, dd, $J_{H1\text{-}F}$ 50.1Hz, $J_{H1\text{-}H2}$ 1.9Hz, H1). LRMS (ESI$^+$): m/z 373.1 $[M+Na]^+$. R_f= 0.16 (ethyl acetate: petroleum ether= 1: 2).

Preparation of 2,3,4-triacetyl D-(+)-rhamnopyranosyl fluoride (**2-1c**, **Scheme 2.21**)

To a solution of 2,3,4-triacetyl D-(+)-rhamnopyranoside[54] (1.00 g, 3.45 mmol, 1.0 eq.) in dichloromethane (6.90 mL) was added DAST (1.36 mL, 10.4 mmol, 3.0 eq.) dropwise at room

temperature. The solution was stirred at room temperature for 1 hr. After cooling to 0°C, methanol (1.0 mL) was added slowly to quench DAST. Water (10 mL) was added and the organic phase was separated. The aqueous phase was extracted wth dichloromethane (10 mL x 2). The combined organic phase was washed with sat. $NaHCO_3$ (10 mL) and brine (10 mL), dried over $MgSO_4$ and concentrated. The residue was purified by Biotage Flash Purification System (cartridge; SNAP KP-Sil 100 g, eluent gradient; ethyl acetate/ petroleum ether = 8% -> 66 %, 10CV), to afford the titled compound (**2-1c**) as white solid; 638 mg (y= 63%): R_f= 0.41 (ethyl acetate: petroleum ether= 1: 2). The α-isomer was also isolated as white solid; 283 mg, (y= 28%): R_f= 0.30 (ethyl acetate: petroleum ether= 1: 2). ^{1}H NMR (lit:[59] the data for the L-form) (400MHz, $CDCl_3$, δ ($CHCl_3$)=7.270): 1.38 (1H, dd, $J_{H6\text{-}H5}$ 6.4Hz, $J_{H6\text{-}F}$ 0.8Hz, H6), 2.03, 2.08, 2.20 (3 x 3H, 3 x s, 3 x OCOC$\underline{H_3}$), 3.65-3.74 (1H, m, H5), 5.05-5.10 (1H, m, H3), 5.09 (1H, dd, $J_{H4\text{-}H3}$ 9.2Hz, $J_{H4\text{-}H5}$ 9.2Hz, $J_{H5\text{-}H6'}$ 6.0Hz, H4), 5.47 (1H, dd, $J_{H1\text{-}F}$ 48.8Hz, $J_{H1\text{-}H2}$ 1.2Hz, H1), 5.51-5.55 (1H, m, H2). R_f= 0.41 (ethyl acetate: petroleum ether= 1: 2).

Deprotection of acetyl groups (**Scheme2.7**, **Scheme 2.21**)

Deprotection of acetyl groups of the donor substrates were performed just before use. β-D-Glucopyranosyl fluoride tetraacetate (**2-1a**) was purchased from Sigma-Aldrich (Part No. 510548). β-D-Mannopyranosyl fluoride tetraacetate (**2-1b**) and β-D-rhamnopyranosyl fluoride triacetate (**2-1c**) were prepared as above. The peracetylated precursor was dissolved in 2M ammonia-methanol at 4°C (0.1 M substrate solution corresponds 20 equivalents of ammonia to the substrate). The reaction solution was stirred at this temperature with monitoring the reaction with TLC (acetonitrile: water= 4: 1). After the complete conversion to the non-protected compound (4 hr), the solvent was removed by N_2 flow and dried *in vacuo* on ice. The residue was dissolved in the buffer used in the following reaction.

Comparison between α-gluco- and α-manno-synthase reactions by MalA D320G (**Scheme 2.8**)

100 μL of 200 mM β-D-Glc-F (**2-2a**) or β-D-Man-F (**2-2b**) in 100 mM NaPi (pH 6.0), 500 μL of 40 mM *p*NP-β-D-Glc (**3a**) in 100 mM NaPi (pH 6.0), 371 μL of 100 mM NaPi (pH 6.0) and 29 μL of 17.4 mg/mL MalA D320G in 100 mM NaPi (pH 6.0) were mixed at 4°C. The reaction mixture was stirred at 4°C for 4 days. The reaction was analyzed by HPLC (Condition A in General Procedure, **Figure 2.15**).

Optimization of the mannosynthase reaction

-Temperature/pH/Enyme concentration/Sequential addition of the donor-

200 mM β-D-Man-F and 40 mM *p*NP-β-D-Glc in the indicated buffer were mixed with the

same buffer and 17.4 mg/mL MalA D320G in 100 mM NaPi (pH 6.0) so the final conditions became as shown in **Scheme 2.9-13** (the final volume was 50 µL). The reactions were incubated at the indicated temperatures with monitoring by HPLC. When needed, the indicated amount of 200 mM β-D-Man-F solutioin in the same buffer was added. The reaction was analyzed by HPLC (Condition B, General Procedure).

Mannosynthase activity of the recovered MalA D320G

10 µL of 200 mM β-D-Man-F (**2-2b**) and 25 µL of 40 mM *p*NP-β-D-Glc (**2-3a**) in 400 mM NaPi (pH 7.0) were mixed. To the mixture were added 400 mM NaPi (pH 7.0), water, new or recycled MalA D320G in 100 mM NaPi (pH 6.0) so that the final concentration of the enzyme and the buffer became 2.0 mg/mL and 300 mM, respectively (the final volume was 50 µL). The reaction solution was incubated at room temperature. After 1 day and 2 days, 10 µL of 200 mM β-D-Man-F (**2-2b**) in 300 mM NaPi (pH 7.0) was added sequentially and the solution was further incubate for another 2 days (total 4 days). The reaction was analyzed by HPLC (Condition B, General Procedure).

Solubility test of *p*NP-α-L-Rha

*p*NP-α-L-Rha (**2-3e**) was dissolved in an organic solvent and the solution was mixed with 100 mM NaPi (pH 6.0) so that the final concentration of *p*NP-α-L-Rha (**2-3e**) became 20 mM with the various concentrations of organic solvents. The solutions were left stand at room temperature and the presence of precipitate was checked by eye.

Effect of organic solvents on the synthase reaction

6.7 µL of 300 mM β-D-Man-F (**2-2b**) and 16.7 µL of 60 mM *p*NP-β-D-Glc (**2-3a**) in 400 mM NaPi (pH 6.0) were mixed at 4°C. The mixture was diluted with 400 mM NaPi (pH 6.0), water and an organic solvent so the final conditions became 40mM (**2-2b**), 20 mM (**2-3a**) and the buffer (300 mM) with 15% DMF, 25% EtOH or 25% iPrOH. For the control reaction, water was added in place of the organic solvent (the final volume was 50 µL). Finally, 1.0 µL of 25.7 mg/mL MalA D320G in 100 mM NaPi (pH 6.0) was added and the reaction mixture was incubated at room temperature. 10 µL of 200 mM β-D-Man-F (**2-2b**) in 400 mM NaPi (pH 6.0) was added sequentially with more than 1-day intervals twice and the solution was further incubate for 2 days. The reaction solution was analyzed by HPLC (Condition B, General Procedure).

Screening of acceptors for mannosynthase reaction

20 μL of 100 mM β-D-Man-F (**2-2b**) and 20 μL of 10 mM *p*NP-glycoside in 100 mM NaPi (pH 6.0) were mixed at 4°C. MalA D320G in 100 mM NaPi (pH 6.0) and 100 mM NaPi (pH 6.0) were added so that the final concentration of the enzyme became 0.5 mg/mL and the final volume was 100 μL. For control reactions, the buffer was added in place of the enzyme solution. The reaction mixture was incubated at 4°C for 5 days and the conversions of the reactions were determined by HPLC (Condition B or B'). For *p*NP-α/β-D-Xyl, *p*NP-α/β-D-GlcNAc, *p*NP-α-L-Fuc and *p*NP-α-L-Rha, Condition B' was used. For the other substrates, Condition B was used.

Preparative-scale mannosynthase reactions using various *p*NP-sugar acceptors

Method 1: without DMF (for **2-3a, b, g, i**)

200 μL of 200 mM β-D-Man-F (**2-2b**) and 500 μL of 40 mM *p*NP-sugar in 400 mM NaPi (pH 7.0) were mixed and MalA D320G in 100 mM NaPi (pH 6.0), 400 mM NaPi (pH 7.0) and water were added so that the final concentration of the enzyme and the buffer became 2.0 mg/mL and 300 mM, respectively (the final volume was 1 mL).

Method 2: with DMF (for **2-3c, d, e, f, h**)

*p*NP-sugar (20 μmol) was dissolved in DMF (**2-3c, d, h**:50 μL, **2-3e**:150 μL, **2-3f**: 100 μL). 200 μL of 200 mM β-D-Man-F (**2-2b**) in 400 mM NaPi (pH 7.0) was mixed and then MalA D320G in 100 mM NaPi (pH 6.0), 400 mM NaPi (pH 7.0) and water were added so that the final concentration of the enzyme and the buffer became 2.0 mg/mL and 300 mM, respectively (the final volume was 1 mL).

The reaction solution was incubated at room temperature. After 1 day and 2 days, 200 μL of 200 mM β-D-Man-F (**2-2b**) in 300 mM NaPi (pH 7.0) was added sequentially and the solution was further incubate for another 2 days (total 4 days). The conversions of the reactions were determined by HPLC (Condition A or A', **Figure 2.35**). For **2-3i**, Condition A' was used. For the other substrates, Condition A was used.

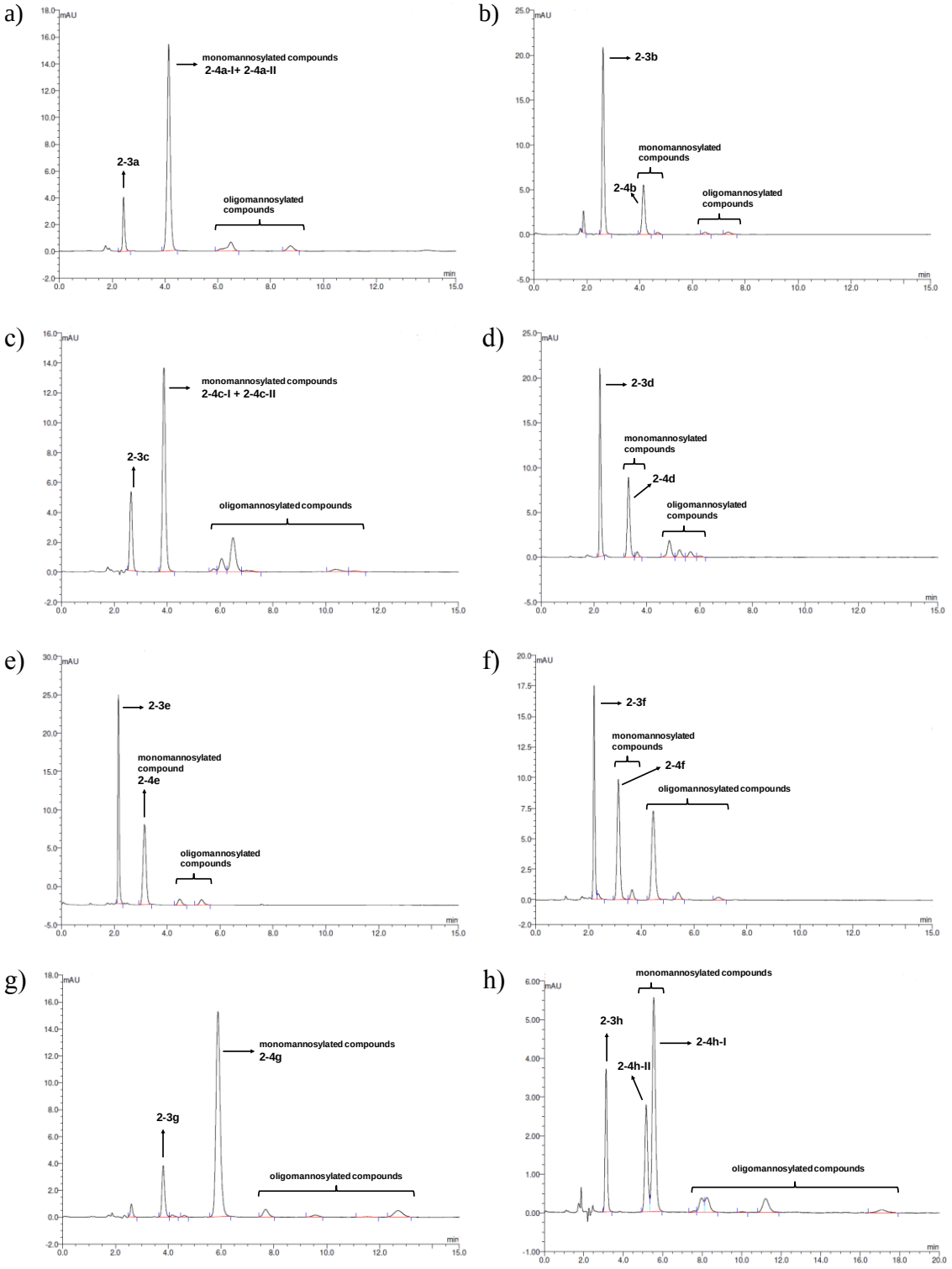


Figure 2.32. Chromatograms of the α-mannosynthase reactions using **2-3(a-i)** as acceptors.

i)

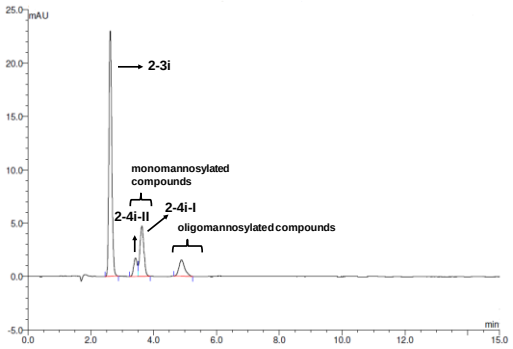


Figure 2.32. (continued).

The reaction solution was applied to vivaspin 6 (MWCO 10,000, Sartorius, pre-treated with 0.1% Triton X-100) to separate the protein fraction and the small molecule fraction. The protein fraction was dialyzed against 100 mM NaPi (pH 6.0) for recycled use. The small molecule fraction was lyophilized and dissolved in 0.8 mL of water. The *p*NP-compounds were separated by the following HPLC condition:

Column: Synergi 4u Fusion-RP 80A, 100x21.20mm, 4 micron (Phenomenex)
Injection volume: 0.8 mL
Flow rate: 7.0 mL/min
Solvent A: Water
Solvent B: Acetonitrile

Gradient: Solvent B	0%	0-4min
	to x%	4- 30 min
	x%	30-34 min
	100%	34-36 min
	100%	36-40 min
	0%	40-44 min

x=20 for the reactions using 2-**3d**, **e**, **f**
x=15 for the other reactions
Detection: UV 300nm

The collected fractions were lyopilized. The residue was dissolved in 0.8 mL of 80% acetonitrile. After filtration with 0.45-μm membrane filter unit, the monomannosylated products were separated by the following HPLC condition:

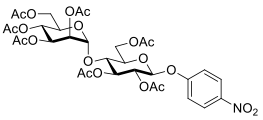
Column: Luna 5u NH2, 250x21.20 mm, 100A, Axia packed (Phenomenex)
Injection volume: 0.8 mL
Flow rate: 15.0 mL/min
Solvent: x% acetonitrile
x=70 for the products of the reactions using **2-3g**, **h**, **i**
x=80 for the products of the other reactions
Detection: UV 300nm

The separated fractions were lyopilized and analyzed by ^{1}H-NMR to determine the purities of the products.

The structures of the products were determined after peracetylation (**Scheme 2.17**) except for the known compounds,[60] 2-**4c-I** and 2-**4c-II**. For peracetylation, the products (**2-4a-i**) were dissolved in 0.5 mL of pyridine and 0.5 mL of acetic anhydride and the solution was stirred for 2 days at room temperature. The solvents were removed by evaporation with toluene. The residues were purified by column chromatography on silica gel and analyzed by NMR to determine the structures of the products.
The spectral data of the products are shown in the next section.

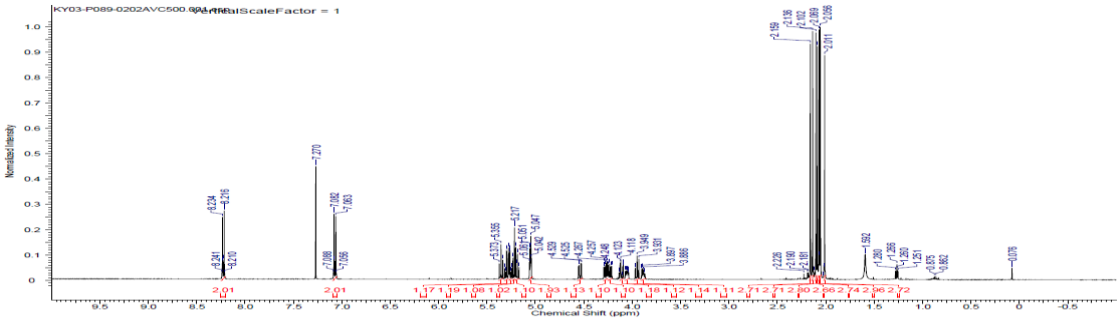
Characterization data of the mannosynthase products

In the NMR spectra, superscripts of a, b and c represent protons/carbons in the first, second and third sugar rings from the reducing termini, respectively. Superscripts of Ar represent protons/carbons in the aromatic rings. The numbering of atoms in the sugar rings follows the conventional nomenclature. The numbering of carbon atoms in the aromatic rings starts from 1, which is the *ipso*-position of the nitro group, ending with 4, which is the *para*-position to the nitro group. The numbering of protons in the aromatic rings follows that of carbon atoms.

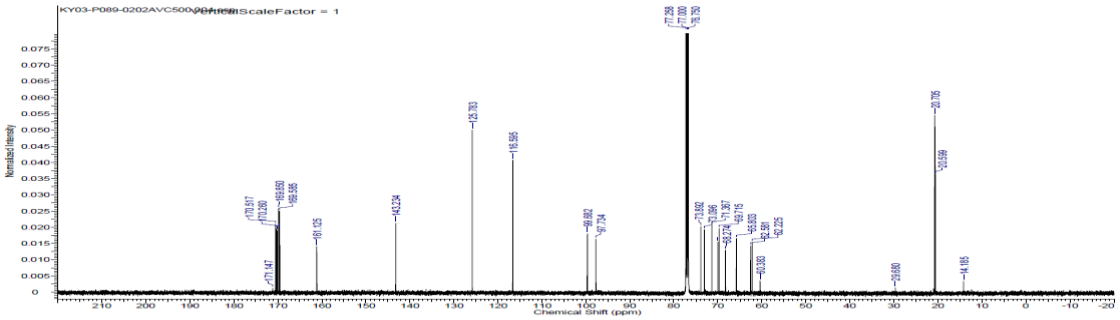
2-5a-I: 

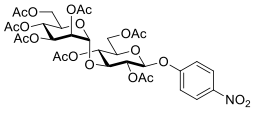
^{1}H-NMR (500MHz, $CDCl_3$, δ(C*H*Cl_3)=7.270): 2.01, 2.06, 2.07, 2.09, 2.10, 2.14, 2.16 (7 x 3H, 7 x s, 7 x OCOC$\underline{H}_3$), 3.88 (1H, ddd, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6}$ 5.5Hz, $J_{H5\text{-}H6'}$ 2.5Hz, ^{a}H5), 3.95 (1H, dd, $J_{H4\text{-}H3}$ 9.0Hz , $J_{H4\text{-}H5}$ 9.0Hz, ^{a}H4), 4.05 (1H, ddd, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6'}$ 5.0Hz, $J_{H5\text{-}H6}$ 2.0Hz, ^{b}H5), 4.11 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{b}H6), 4.23 (1H, dd, $J_{H6\text{-}H6'}$ 12.0Hz, $J_{H6\text{-}H5}$ 5.5Hz, ^{a}H6), 4.27 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 6.0Hz, ^{b}H6'), 4.54 (1H, dd, $J_{H6'\text{-}H6}$ 12.0Hz, $J_{H6'\text{-}H5}$ 2.0Hz, ^{a}H6'), 5.04 (1H, d, $J_{H1\text{-}H2}$ 2.5Hz, ^{b}H1), 5.06 (1H, dd, $J_{H2\text{-}H1}$ 2.5Hz, $J_{H2\text{-}H3}$ 2.5Hz, ^{b}H2), 5.19 (1H, dd, $J_{H2\text{-}H1}$ 7.5Hz, $J_{H2\text{-}H3}$ 9.0Hz, ^{a}H2), 5.22 (1H, d, $J_{H1\text{-}H2}$ 7.5Hz, ^{a}H1), 5.26 (1H, dd, $J_{H3\text{-}H2}$ 2.5Hz , $J_{H3\text{-}H4}$ 9.5Hz, ^{b}H3), 5.30 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{b}H4), 5.36 (1H, dd, $J_{H3\text{-}H2}$ 9.0Hz, $J_{H3\text{-}H4}$ 9.0Hz, ^{a}H3), 7.07 (2H, d, $J_{H3\text{-}H2}$ 9.5Hz, ^{Ar}H3), 8.23 (2H, d, $J_{H2\text{-}H3}$ 9.0Hz, ^{Ar}H2). ^{13}C-NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 20.60, 20.61, 20.63, 20.67, 20.71, 20.71, 20.78 (7 x OCO$\underline{C}H_3$), 62.23 (^{b}C6), 62.58 (^{a}C6), 65.80 (^{b}C4), 68.27 (^{b}C3), 69.72 (^{b}C2), 70.03 (^{b}C5), 71.37 (^{a}C2), 73.10 (^{a}C5), 73.89 (^{a}C3), 76.92 (^{a}C4), 97.73 (^{a}C1), 99.68 (^{b}C1), 116.60 (^{Ar}C3), 125.78 (^{Ar}C2), 143.23 (^{Ar}C1), 161.13 (^{Ar}C4), 169.49, 169.59, 169.69, 169.85, 170.14, 170.26, 170.52 (7 x O$\underline{C}$OCH$_3$). Coupled HSQC: J(^{a}H1-^{a}C1)= 165Hz , J(^{b}H1-^{b}C1)= 174Hz. LRMS (ESI^{+}): m/z 780.3 [M+Na]$^{+}$. HRMS (ESI^{+}): m/z [M+Na]$^{+}$ 780.1952 (found), 780.1958 (calcd for $C_{32}H_{39}NNaO_{20}$). $[\alpha]_D^{20}$= -9.4° (c= 0.50, $CHCl_3$).

^{1}H-NMR



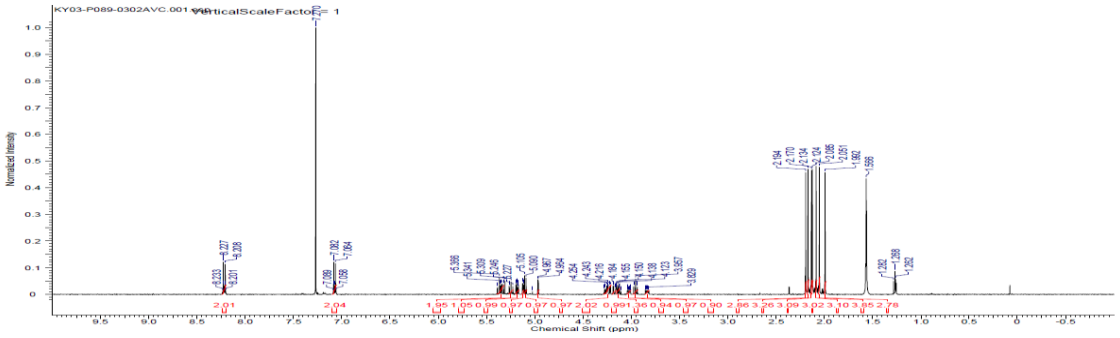
^{13}C NMR



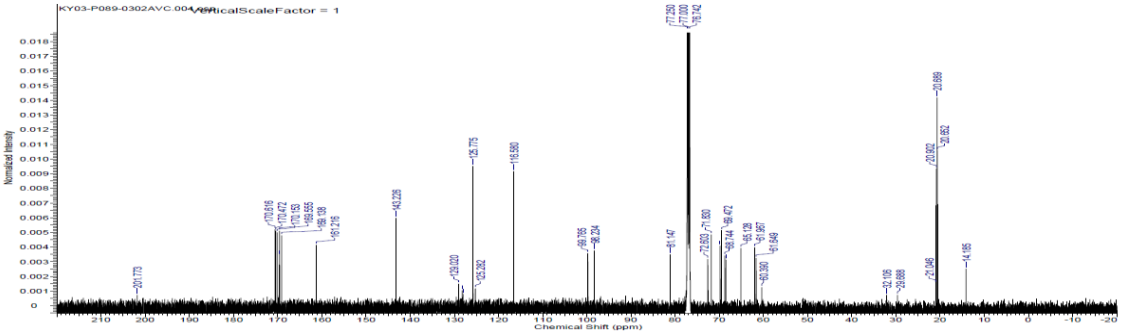
2-5a-II: 

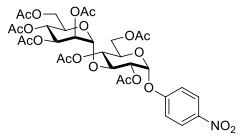
^{1}H NMR (500MHz, $CDCl_3$, δ(C*H*Cl_3)=7.270): 1.99, 2.05, 2.09, 2.12, 2.13, 2.17, 2.19 (7 x 3H, 7 x s, 7 x OCOC$\underline{H}_3$), 3.84 (1H, ddd, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6'}$ 5.5Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{a}H5), 3.96 (1H, dd, $J_{H3\text{-}H2}$ 9.5Hz, $J_{H3\text{-}H4}$ 9.5Hz, ^{a}H3), 4.03 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 3.0Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{b}H5), 4.14 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{b}H6), 4.18 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{a}H6), 4.23 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 3.5Hz, ^{b}H6'), 4.26 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 5.5Hz, ^{a}H6'), 4.97 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{b}H1), 5.10 (1H, d, $J_{H1\text{-}H2}$ 7.5Hz, ^{a}H1), 5.12 (1H, dd, $J_{H2\text{-}H3}$ 3.0Hz , $J_{H2\text{-}H1}$ 2.0Hz, ^{b}H2), 5.18 (1H, dd, $J_{H3\text{-}H2}$ 3.5Hz, $J_{H3\text{-}H4}$ 10.5Hz, ^{b}H3), 5.25 (1H, dd, $J_{H4\text{-}H3}$ 9.5Hz, $J_{H4\text{-}H5}$ 9.5Hz, ^{a}H4), 5.34 (1H, dd, $J_{H2\text{-}H1}$ 7.5Hz, $J_{H2\text{-}H3}$ 9.0Hz, ^{a}H2), 5.37 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{b}H4), 7.07 (2H, d, $J_{H3\text{-}H2}$ 9.0Hz, ^{Ar}H3), 8.22 (2H, d, $J_{H2\text{-}H3}$ 9.5Hz, ^{Ar}H2). ^{13}C NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 20.59, 20.65, 20.69, 20.69, 20.74, 20.90, 20.93 (7 x OCO$\underline{C}H_3$), 61.65 (^{b}C6), 61.97 (^{a}C6), 65.13 (^{b}C4), 68.52 (^{a}C4), 68.74 (^{b}C3), 69.47 (^{b}C2), 69.84 (^{b}C5), 71.83 (^{a}C2), 72.60 (^{a}C5), 81.15 (^{a}C3), 98.23 (^{a}C1), 99.77 (^{b}C1), 116.58 (^{Ar}C3), 125.78 (^{Ar}C2), 143.23 (^{Ar}C1), 161.22 (^{Ar}C4), 169.14, 169.56, 169.59, 169.62, 170.15, 170.47, 170.62 (7 x O$\underline{C}$OCH$_3$). Coupled HSQC: J(^{a}H1-^{a}C1)= 164Hz, J(^{b}H1-^{b}C1)= 173Hz. LRMS (ESI^{+}): m/z 780.2 [M+Na]$^{+}$. HRMS (ESI^{+}): m/z [M+Na]$^{+}$ 780.1965 (found), 780.1958 (calcd for $C_{32}H_{39}NNaO_{20}$). $[\alpha]_D^{20}$= -15.6° (c= 0.33, $CHCl_3$).

^{1}H NMR



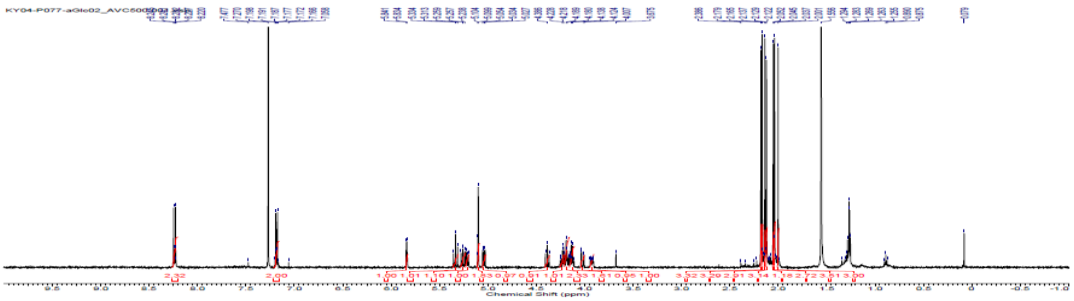
^{13}C NMR



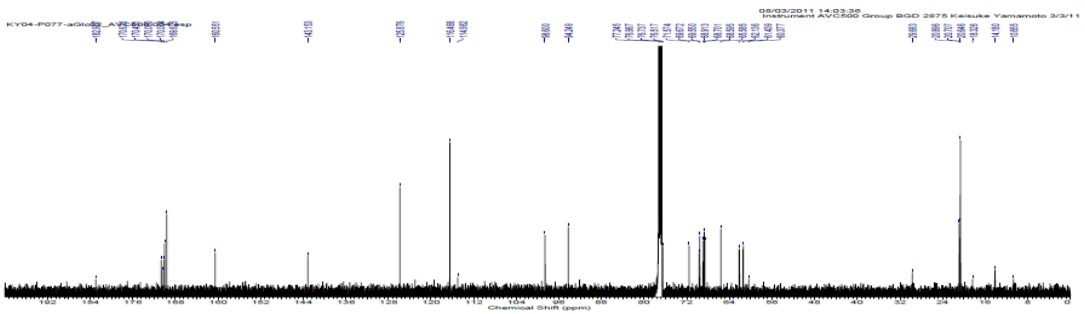
2-5b: 

^{1}H-NMR (500MHz, $CDCl_3$, δ(C*H*Cl_3)=7.270): 2.00, 2.04, 2.05, 2.12, 2.14, 2.17, 2.18 (7 x 3H, 7 x s, 7 x OCOC$\underline{H}_3$), 3.93 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 5.0Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{a}H5), 4.02 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{a}H6), 4.13 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 5.0Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{b}H5), 4.18 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.0Hz, ^{b}H6), 4.20 (1H, dd, $J_{H6'\text{-}H6}$ 13.0Hz, $J_{H6'\text{-}H5}$ 5.0Hz, ^{a}H6'), 4.24 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 5.0Hz, ^{b}H6'), 4.39 (1H, dd, $J_{H3\text{-}H2}$ 10.0Hz, $J_{H3\text{-}H4}$ 10.0Hz, ^{a}H3), 5.04 (1H, dd, $J_{H2\text{-}H3}$ 10.0Hz, $J_{H2\text{-}H1}$ 3.5Hz, ^{a}H2), 5.099 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{b}H1), 5.101 (1H, dd, $J_{H2\text{-}H3}$ 4.0Hz , $J_{H2\text{-}H1}$ 1.5Hz, ^{b}H2), 5.21 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.0Hz, ^{b}H3), 5.26 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{a}H4), 5.33 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{b}H4), 5.84(1H, d, $J_{H1\text{-}H2}$ 3.5Hz, ^{a}H1), 7.18 (2H, d, $J_{H3\text{-}H2}$ 9.5Hz, ^{Ar}H3), 8.24 (2H, d, $J_{H2\text{-}H3}$ 9.0Hz, ^{Ar}H2). ^{13}C-NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 20.61, 20.65, 20.65, 20.68, 20.71, 20.78, 20.90 (7 x OCO$\underline{C}H_3$), 61.44 (^{a}C6), 62.14 (^{b}C6), 65.59 (^{b}C4), 68.60 (^{b}C3), 68.70 (^{a}C4), 68.91 (^{a}C5), 69.55 (^{b}C5), 69.67 (^{b}C2), 71.57 (^{a}C2), 76.52 (^{a}C3), 94.25 (^{a}C1), 98.60 (^{b}C1), 116.47 (^{Ar}C3), 125.88 (^{Ar}C2), 143.15 (^{Ar}C1), 160.55 (^{Ar}C4), 169.63, 169.63, 169.63, 170.00, 170.08, 170.45, 170.63 (7 x O$\underline{C}OCH_3$). Coupled HSQC: J(^{a}H1-^{a}C1)= 175Hz, J(^{b}H1-^{b}C1)= 173Hz. LRMS (ESI^{+}): m/z 780.3 $[M+Na]^{+}$. HRMS (ESI^{+}): m/z $[M+Na]^{+}$ 780.1960 (found), 780.1958 (calcd for $C_{32}H_{39}NNaO_{20}$). $[\alpha]_D^{20}$= +28.0° (c= 0.25, $CHCl_3$).

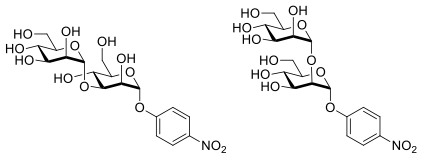
^{1}H NMR



^{13}C NMR

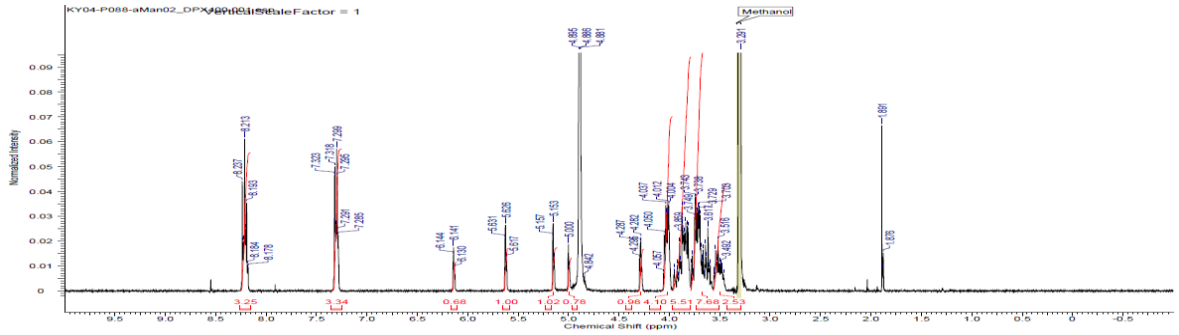


2-4c-I: **2-4c-II**:

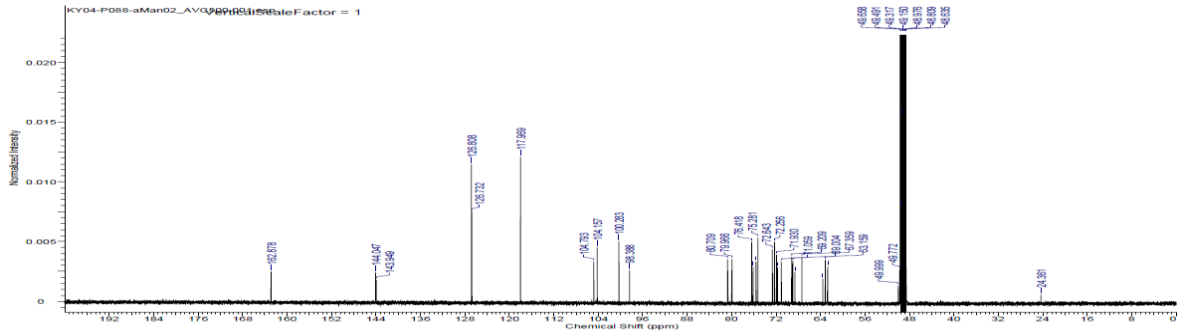


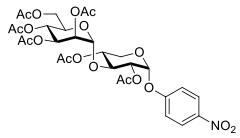
^{1}H NMR[60] (400MHz, CD_3OD, δ(CH_3OH)=3.310), *inter alia*: **4c-I**: 4.29 (1H, dd, $J_{H2\text{-}H3}$ 3.2Hz , $J_{H2\text{-}H1}$ 2.0Hz, ^{a}H2), 5.16 (1H, d, $J_{H1\text{-}H2}$ 1.6Hz, ^{b}H1), 5.63 (1H, d, $J_{H1\text{-}H2}$ 2.0Hz, ^{a}H1), 7.31 (2H, d, $J_{H3\text{-}H2}$ 9.2Hz, ^{Ar}H3), 8.23 (2H, d, $J_{H2\text{-}H3}$ 9.6Hz, ^{Ar}H2). **4c-II**: 5.00 (1H, d, $J_{H1\text{-}H2}$ 1.6Hz, ^{b}H1), 6.14 (1H, d, $J_{H1\text{-}H2}$ 1.2Hz, ^{a}H1), 7.31 (2H, d, $J_{H3\text{-}H2}$ 9.6Hz, ^{Ar}H3), 8.20 (2H, d, $J_{H2\text{-}H3}$ 9.2Hz, ^{Ar}H2). ^{13}C NMR [60] (126MHz, CD_3OD, δ(CH_3OH)=49.15): **4c-I**: 62.70 , 63.16 (^{a}C6, ^{b}C6), 67.36 (^{a}C4), 69.00 (^{b}C4), 71.06 (^{a}C2), 72.26 (^{b}C2), 72.64 (^{b}C3), 75.28 (^{b}C5), 76.42 (^{a}C5), 79.97 (^{a}C3), 100.28 (^{a}C1), 104.16 (^{b}C1), 117.97 (^{Ar}C3), 126.81 (^{Ar}C2), 144.05 (^{Ar}C1), 162.88 (^{Ar}C4). **4c-II**: 62.77, 63.61 (^{a}C6, ^{b}C6), 68.61 (^{a}C4), 69.21 (^{b}C4), 71.76, 71.93 (^{a}C3, ^{b}C2), 72.58 (^{b}C3), 75.59, 76.16 (^{a}C5, ^{b}C5), 80.71 (^{a}C2), 98.39 (^{a}C1), 104.79 (^{b}C1), 118.01 (^{Ar}C3), 126.73 (^{Ar}C2), 143.95 (^{Ar}C1), 162.85 (^{Ar}C4). These data are identical to ones in the literature.[60]

^{1}H NMR



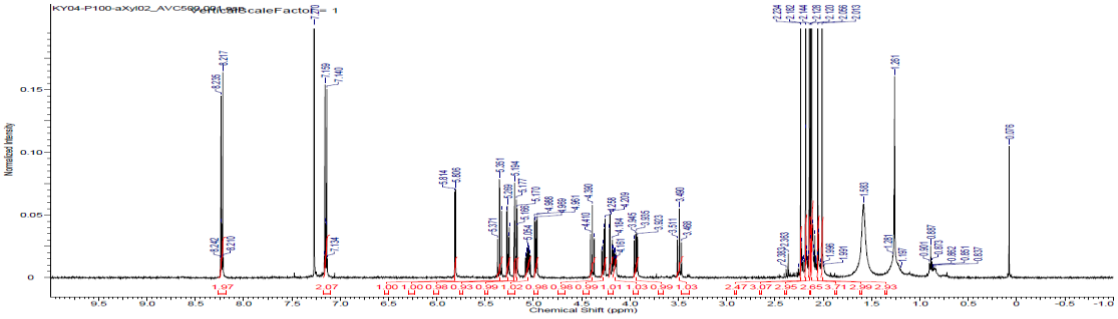
^{13}C NMR



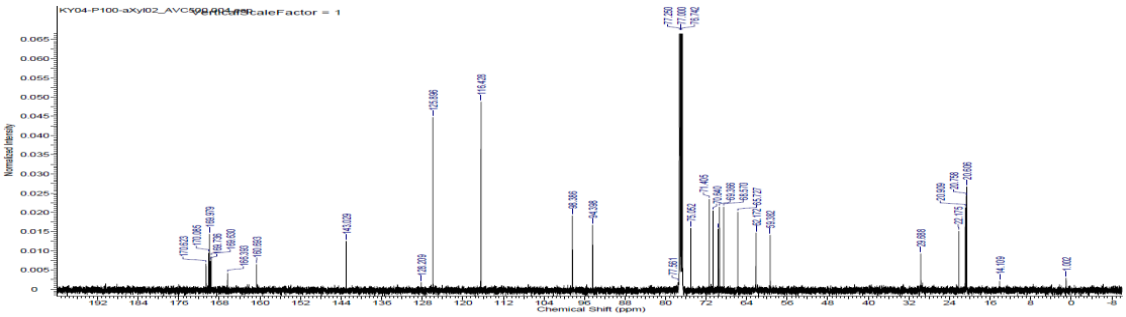
2-5d: 

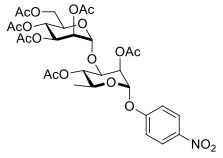
^{1}H-NMR (500MHz, $CDCl_3$, δ($C\underline{H}Cl_3$)=7.270): 2.01, 2.06, 2.12, 2.13, 2.14, 2.18 (6 x 3H, 7 x s, 7 x OCOC$\underline{H}_3$), 3.49 (1H, dd, $J_{H5ax\text{-}H5eq}$ 11.0Hz, $J_{H5ax\text{-}H4}$ 11.0Hz, $^{a}H5_{ax}$), 3.94 (1H, dd, $J_{H5eq\text{-}H5ax}$ 11.0Hz, $J_{H5eq\text{-}H4}$ 6.0Hz, $^{a}H5_{eq}$), 4.16 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 4.5Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{b}H5), 4.20 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{b}H6), 4.28 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 4.5Hz, ^{b}H6'), 4.39 (1H, dd, $J_{H3\text{-}H2}$ 9.5Hz, $J_{H3\text{-}H4}$ 9.5Hz, ^{a}H3), 4.97 (1H, dd, $J_{H2\text{-}H3}$ 9.5Hz, $J_{H2\text{-}H1}$ 4.0Hz, ^{a}H2), 5.06 (1H, ddd, $J_{H4\text{-}H5ax}$ 11.0Hz, $J_{H4\text{-}H3}$ 9.5Hz, $J_{H4\text{-}H5eq}$ 6.0Hz, ^{a}H4), 5.17 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz, $J_{H2\text{-}H1}$ 2.0Hz, ^{b}H2), 5.20 (1H, d, $J_{H1\text{-}H2}$ 2.0Hz, ^{b}H1), 5.26 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{b}H3), 5.35 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{b}H4), 5.81 (1H, d, $J_{H1\text{-}H2}$ 4.0Hz, ^{a}H1), 7.15 (2H, d, $J_{H3\text{-}H2}$ 9.5Hz, ^{Ar}H3), 8.23 (2H, d, $J_{H2\text{-}H3}$ 9.5Hz, ^{Ar}H2). ^{13}C-NMR (126MHz, $CDCl_3$, δ ($\underline{C}HCl_3$)=77.00): 20.61, 20.64, 20.68, 20.74, 20.76, 20.91 (6 x OCO$\underline{C}H_3$), 59.38 (^{a}C5), 62.17 (^{b}C6), 65.73 (^{b}C4), 68.57 (^{b}C3), 69.37 (^{b}C5), 69.65 (^{b}C2), 70.64 (^{a}C4), 71.41 (^{a}C2), 75.05 (^{a}C3), 94.40 (^{a}C1), 98.39 (^{b}C1), 116.43 (^{Ar}C3), 125.90 (^{Ar}C2), 143.03 (^{Ar}C1), 160.69 (^{Ar}C4), 169.63, 169.74, 169.96, 169.98, 170.09, 170.62 (6 x O$\underline{C}$OCH$_3$). Coupled HSQC: J(^{a}H1-^{a}C1)= 175Hz, J(^{b}H1-^{b}C1)= 174Hz. LRMS (ESI^{+}): m/z 708.22 [M+Na]$^{+}$. HRMS (ESI^{+}): m/z [M+Na]$^{+}$ 708.1732 (found), 708.1746 (calcd for $C_{29}H_{35}NNaO_{18}$). $[\alpha]_D^{20}$= +54.4° (c= 0.50, $CHCl_3$).

^{1}H NMR



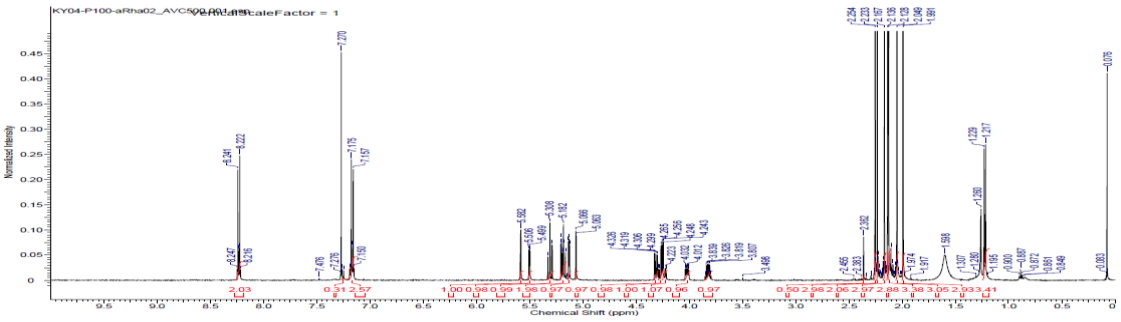
^{13}C NMR



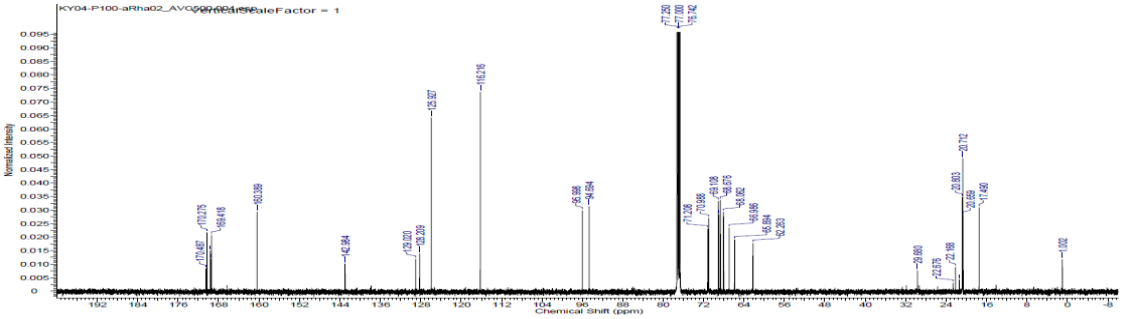
2-5e: 

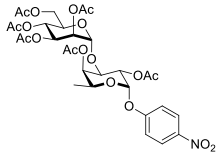
^{1}H-NMR (500MHz, $CDCl_3$, δ(*C*HCl_3)=7.270): 1.23 (3H, d, $J_{H6\text{-}H5}$ 6.0Hz, ^{a}H6), 1.99, 2.05, 2.13, 2.14, 2.17, 2.25 (6 x 3H, 6 x s, 6 x OCOC$\underline{H}_3$), 3.82 (1H, dq, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6}$ 6.0Hz, ^{a}H5), 4.02 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 4.5Hz, $J_{H5\text{-}H6}$ 3.0Hz, ^{b}H5), 4.23 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{b}H6), 4.27 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 4.5Hz, ^{b}H6'), 4.31 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{a}H3), 5.06 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{b}H1), 5.13 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz, $J_{H2\text{-}H1}$ 2.0Hz, ^{b}H2), 5.18 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{a}H4), 5.19 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{b}H3), 5.31 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{b}H4), 5.50 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz, $J_{H2\text{-}H1}$ 2.0Hz, ^{a}H2), 5.58 (1H, d, $J_{H1\text{-}H2}$ 2.0Hz, ^{a}H1), 7.17 (2H, d, $J_{H3\text{-}H2}$ 9.0Hz, ^{Ar}H3), 8.23 (2H, d, $J_{H2\text{-}H3}$ 9.5Hz, ^{Ar}H2). ^{13}C-NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 17.49 (^{a}C6), 20.64, 20.66, 20.71, 20.71, 20.75, 20.80 (6 x OCO$\underline{C}$H$_3$), 62.26 (^{b}C6), 65.89 (^{b}C4), 66.99 (^{a}C2), 68.06 (^{a}C5), 68.68 (^{b}C3), 69.00 (^{b}C2), 69.11 (^{b}C5), 70.99 (^{a}C4), 71.21 (^{a}C3), 94.69 (^{b}C1), 96.00 (^{a}C1), 116.22 (^{Ar}C3), 125.93 (^{Ar}C2), 142.98 (^{Ar}C1), 160.39 (^{Ar}C4), 169.42, 169.56, 169.65, 169.68, 170.28, 170.49 (6 x O$\underline{C}$OCH$_3$). Coupled HSQC: *J*(^{a}H1-^{a}C1)= 174Hz, *J*(^{b}H1-^{b}C1)= 173Hz. LRMS (ESI^{+}): m/z 722.23 [M+Na]$^{+}$. HRMS (ESI^{+}): m/z [M+Na]$^{+}$ 722.1917 (found), 722.1903 (calcd for $C_{30}H_{37}NNaO_{18}$). $[\alpha]_D^{20}$= -7.4° (c= 0.50, $CHCl_3$).

^{1}H NMR



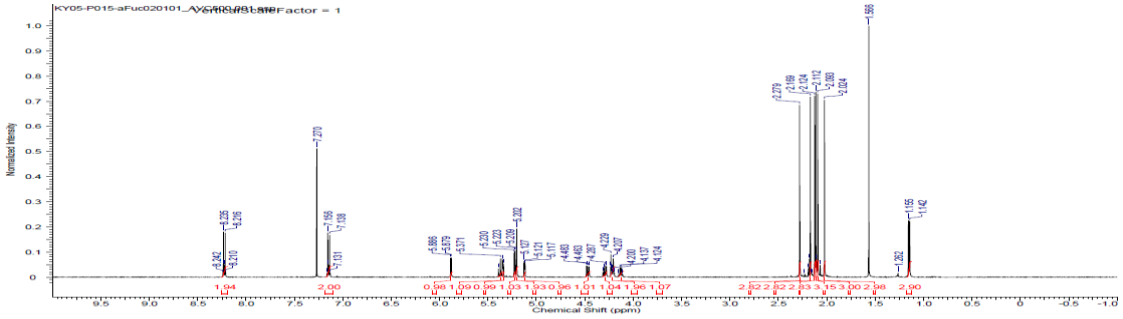
^{13}C NMR



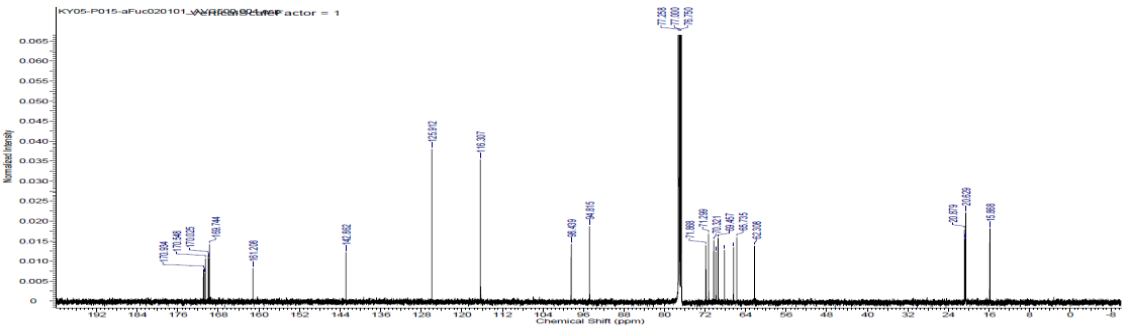
2-5**f**: 

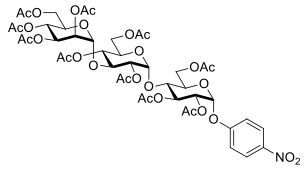
^{1}H-NMR (500MHz, $CDCl_3$, δ(*C*HCl_3)=7.270): 1.15 (3H, d, $J_{H6\text{-}H5}$ 6.5Hz, ^{a}H6), 2.02, 2.09, 2.11, 2.12, 2.17, 2.28 (6 x 3H, 6 x s, 6 x OCOC$\underline{H}_3$), 4.13 (1H, qd, $J_{H5\text{-}H6}$ 6.5Hz, $J_{H5\text{-}H4}$ 1.0Hz, ^{a}H5), 4.18-4.25 (2H, m, ^{b}H5, ^{b}H6), 4.29 (1H, dd, $J_{H6'\text{-}H6}$ 13.0Hz, $J_{H6'\text{-}H5}$ 5.5Hz, ^{b}H6'), 4.47 (1H, dd, $J_{H3\text{-}H2}$ 11.0Hz , $J_{H3\text{-}H4}$ 4.0Hz, ^{a}H3), 5.12 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz , $J_{H2\text{-}H1}$ 1.5Hz, ^{b}H2), 5.20 (1H, d, $J_{H1\text{-}H2}$ 2.0Hz, ^{b}H1), 5.22 (1H, dd, $J_{H2\text{-}H3}$ 10.5Hz , $J_{H2\text{-}H1}$ 3.5Hz, ^{a}H2), 5.22 (1H, dd, $J_{H3\text{-}H4}$ 10.5Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{b}H3), 5.34 (1H, dd, $J_{H4\text{-}H3}$ 3.5Hz , $J_{H4\text{-}H5}$ 1.0Hz, ^{a}H4), 5.37 (1H, dd, $J_{H4\text{-}H3}$ 10.5Hz, $J_{H4\text{-}H5}$ 10.5Hz, ^{b}H4), 5.88 (1H, d, $J_{H1\text{-}H2}$ 3.5Hz, ^{a}H1), 7.15 (2H, d, $J_{H3\text{-}H2}$ 9.0Hz, ^{Ar}H3), 8.23 (2H, d, $J_{H2\text{-}H3}$ 9.5Hz, ^{Ar}H2). ^{13}C-NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 15.87 (^{a}C6), 20.60, 20.63, 20.67, 20.74, 20.79, 20.88 (6 x OCO$\underline{C}H_3$), 62.31 (^{b}C6), 65.74 (^{b}C4), 66.44 (^{a}C5), 68.21 (^{a}C2 or ^{b}C3), 69.46 (^{b}C5), 69.87 (^{b}C2), 70.32 (^{a}C2 or ^{b}C3), 71.30 (^{a}C3), 71.87 (^{a}C4), 94.82 (^{a}C1), 98.44 (^{b}C1), 116.31 (^{Ar}C3), 125.91 (^{Ar}C2), 142.86 (^{Ar}C1), 161.21 (^{Ar}C4), 169.74, 169.90, 170.03, 170.55, 170.62, 170.93 (6 x O$\underline{C}$OCH_3). Coupled HSQC: J(^{a}H1-^{a}C1)= 175Hz, J(^{b}H1-^{b}C1)= 175Hz. LRMS (ESI^{+}): m/z 722.25 $[M+Na]^{+}$. HRMS (ESI^{+}): m/z $[M+Na]^{+}$ 722.1898 (found), 722.1903 (calcd for $C_{30}H_{37}NNaO_{18}$). $[\alpha]_D^{20}$= -65.4° (c= 0.33, $CHCl_3$).

^{1}H NMR



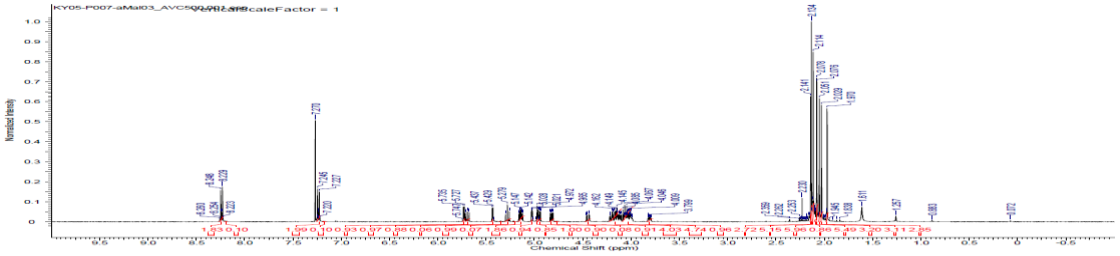
^{13}C NMR



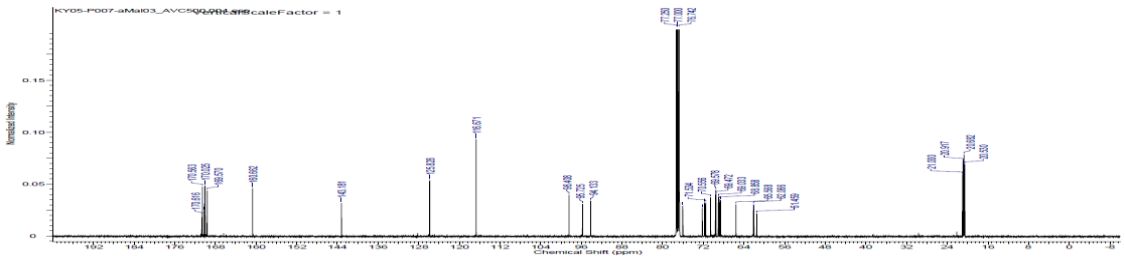
2-5g: 

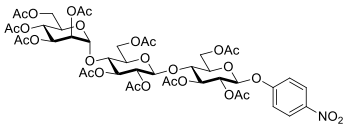
^{1}H NMR (500MHz, $CDCl_3$, δ($CHCl_3$)=7.270): 1.97, 2.03, 2.05, 2.076, 2.078, 2.11, 2.11, 2.13, 2.13, 2.14 (10 x 3H, 10 x s, 10 x OCOC$\underline{H}_3$), 3.81 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6}$ 3.0Hz, $J_{H5\text{-}H6'}$ 3.0Hz, ^{b}H5), 3.97-4.10 (m, 5H, ^{a}H5, ^{b}H6, ^{b}H3, ^{c}H5, ^{a}H4), 4.10-4.23 (m, 4H, ^{c}H6, ^{a}H6, ^{a}H6', ^{b}H6'), 4.45 (1H, dd, $J_{H6'\text{-}H6}$ 12.0Hz, $J_{H6'\text{-}H5}$ 2.5Hz, ^{c}H6'), 4.82 (1H, dd, $J_{H2\text{-}H3}$ 10.5Hz, $J_{H2\text{-}H1}$ 4.5Hz, ^{b}H2), 4.95 (1H, dd, $J_{H2\text{-}H3}$ 10.0Hz, $J_{H2\text{-}H1}$ 3.5Hz, ^{a}H2), 4.97 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{c}H1), 5.03 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz, $J_{H2\text{-}H1}$ 2.0Hz, ^{c}H2), 5.140 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{c}H3), 5.142 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{b}H4), 5.28 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{c}H4), 5.43(1H, d, $J_{H1\text{-}H2}$ 4.0Hz, ^{b}H1), 5.70 (1H, dd, $J_{H3\text{-}H2}$ 10.0Hz, $J_{H3\text{-}H4}$ 9.0Hz, ^{a}H3), 5.73 (1H, d, $J_{H1\text{-}H2}$ 4.0Hz, ^{a}H1), 7.24 (2H, d, $J_{H3\text{-}H2}$ 9.0Hz, ^{Ar}H3), 8.24 (2H, d, $J_{H2\text{-}H3}$ 9.5Hz, ^{Ar}H2). ^{13}C NMR (126MHz, $CDCl_3$, δ($CHCl_3$)=77.00): 20.53, 20.58, 20.67, 20.68, 20.68, 20.68, 20.73, 20.86, 20.92, 21.00 (10 x OCO$\underline{C}H_3$), 61.46 (^{b}C6), 62.07, 62.15 (^{a}C6, ^{c}C6), 65.57 (^{c}C4), 68.59, 68.63 (^{c}C3, ^{b}C4), 68.86 (^{b}C5), 69.03 (^{a}C5), 69.47 (^{c}C5), 69.58 (^{c}C2), 70.56 (^{a}C2), 71.53 (^{b}C2), 71.72 (^{a}C4), 72.19 (^{a}C3), 76.02 (^{b}C3), 94.13 (^{a}C1), 95.73 (^{b}C1), 98.41 (^{c}C1), 116.67 (^{Ar}C3), 125.83 (^{Ar}C2), 143.18 (^{Ar}C1), 160.66 (^{Ar}C4), 169.57, 169.59, 169.61, 170.03, 170.04, 170.11, 170.21, 170.56, 170.62, 170.64 (10 x O$\underline{C}OCH_3$). Coupled HSQC: J(^{a}H1-^{a}C1)= 176Hz, J(^{b}H1-^{b}C1)= 176Hz, J(^{c}H1-^{c}C1)= 172Hz. LRMS (ESI$^+$): m/z 1068.37 $[M+Na]^+$. HRMS (ESI$^+$): m/z $[M+Na]^+$ 1068.2835 (found), 1068.2803 (calcd for $C_{44}H_{55}NNaO_{28}$). $[\alpha]_D^{20}$= +115.9° (c= 1.0, $CHCl_3$).

^{1}H NMR



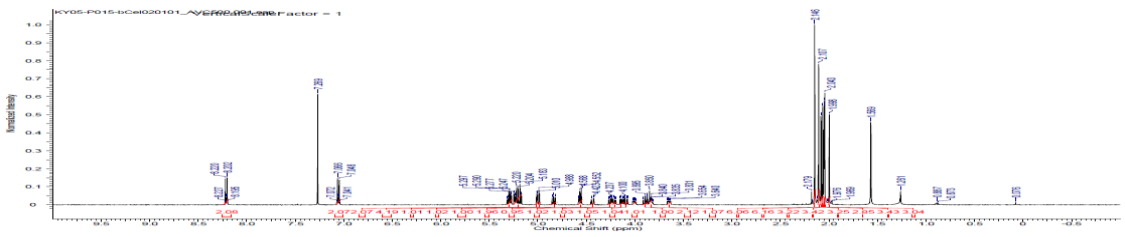
^{13}C NMR



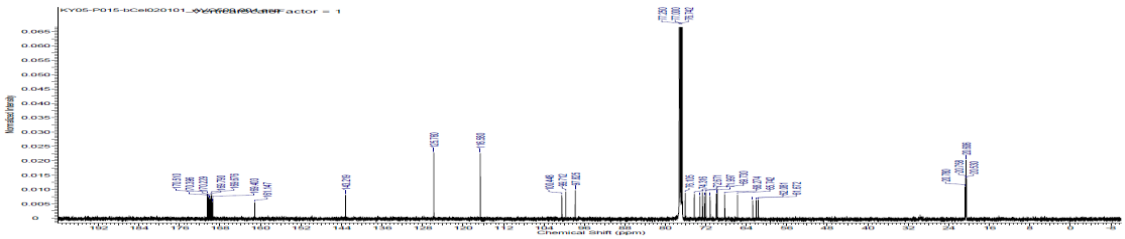
2-5h-I: 

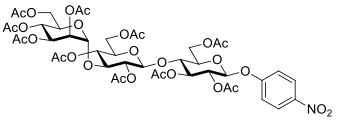
^{1}H-NMR (500MHz, $CDCl_3$, δ(C*H*Cl_3)=7.270): 2.00, 2.04, 2.046, 2.051, 2.06, 2.08, 2.11, 2.11, 2.15, 2.15 (10 x 3H, 10 x s, 10 x OCOC<u>H</u>$_3$), 3.65 (1H, ddd, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6}$ 4.5Hz, $J_{H5\text{-}H6'}$ 2.0Hz, ^{b}H5), 3.80-3.85 (m, 1H, ^{a}H5), 3.85 (1H, dd, $J_{H4\text{-}H3}$ 9.5Hz, $J_{H4\text{-}H5}$ 9.5Hz, ^{b}H4), 3.90 (1H, dd, $J_{H4\text{-}H3}$ 9.5Hz, $J_{H4\text{-}H5}$ 9.5Hz, ^{a}H4), 4.01 (1H, ddd, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6'}$ 4.5Hz, $J_{H5\text{-}H6}$ 2.0Hz, ^{c}H5), 4.09 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{c}H6), 4.13 (1H, dd, $J_{H6\text{-}H6'}$ 12.0Hz, $J_{H6\text{-}H5}$ 6.0Hz, ^{a}H6), 4.21 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 4.5Hz, ^{b}H6), 4.25 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 4.5Hz, ^{c}H6'), 4.44 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 2.0Hz, ^{b}H6'), 4.560 (1H, d, $J_{H1\text{-}H2}$ 8.0Hz, ^{b}H1), 4.565 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 2.0Hz, ^{a}H6'), 4.84 (1H, dd, $J_{H2\text{-}H3}$ 9.5Hz, $J_{H2\text{-}H1}$ 8.0Hz, ^{b}H2), 4.99 (1H, d, $J_{H1\text{-}H2}$ 2.0Hz, ^{c}H1), 5.01 (1H, dd, $J_{H2\text{-}H3}$ 2.5Hz, $J_{H2\text{-}H1}$ 2.5Hz, ^{c}H2), 5.18 (1H, d, $J_{H1\text{-}H2}$ 7.5Hz, ^{a}H1), 5.20 (1H, dd, $J_{H3\text{-}H2}$ 9.5Hz, $J_{H3\text{-}H4}$ 9.5Hz, ^{b}H3), 5.22 (1H, dd, $J_{H2\text{-}H3}$ 9.0Hz, $J_{H2\text{-}H1}$ 7.5Hz, ^{a}H2), 5.24 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{c}H3), 5.29 (1H, dd, $J_{H3\text{-}H2}$ 9.0Hz, $J_{H3\text{-}H4}$ 9.0Hz, ^{a}H3), 5.30 (1H, dd, $J_{H4\text{-}H3}$ 9.5Hz, $J_{H4\text{-}H5}$ 9.5Hz, ^{c}H4), 7.06 (2H, d, $J_{H3\text{-}H2}$ 9.0Hz, ^{Ar}H3), 8.21 (2H, d, $J_{H2\text{-}H3}$ 9.0Hz, ^{Ar}H2). ^{13}C-NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 20.53, 20.57, 20.61, 20.61, 20.64, 20.66, 20.70, 20.74, 20.76, 20.78 (10 x OCO<u>C</u>H$_3$), 61.67 (^{a}C6), 62.08 (^{c}C6), 62.75 (^{b}C6), 65.74 (^{c}C4), 68.27 (^{c}C3), 69.73 (^{c}C2), 69.97 (^{c}C5), 71.16 (^{a}C2), 72.00 (^{b}C2), 72.27 (^{a}C3), 72.67 (^{b}C5), 73.20 (^{a}C5), 74.32 (^{b}C3), 76.11 (^{a}C4), 76.89 (^{b}C4), 97.83 (^{a}C1), 99.71 (^{c}C1), 100.45 (^{b}C1), 116.58 (^{Ar}C3), 125.76 (^{Ar}C2), 143.22 (^{Ar}C1), 161.15 (^{Ar}C4), 169.40, 169.40, 169.57, 169.62, 169.68, 169.79, 170.04, 170.23, 170.40, 170.51 (10 x O<u>C</u>OCH$_3$). Coupled HSQC: J(^{a}H1-^{a}C1)= 165Hz, J(^{b}H1-^{b}C1)= 162Hz, J(^{c}H1-^{c}C1)= 173Hz. LRMS (ESI$^+$): m/z 1068.38 [M+Na]$^+$. HRMS (ESI$^+$): m/z [M+Na]$^+$ 1068.2804 (found), 1068.2803 (calcd for $C_{44}H_{55}NNaO_{28}$). $[\alpha]_D^{20}$= -15.3° (c= 0.33, $CHCl_3$).

^{1}H NMR



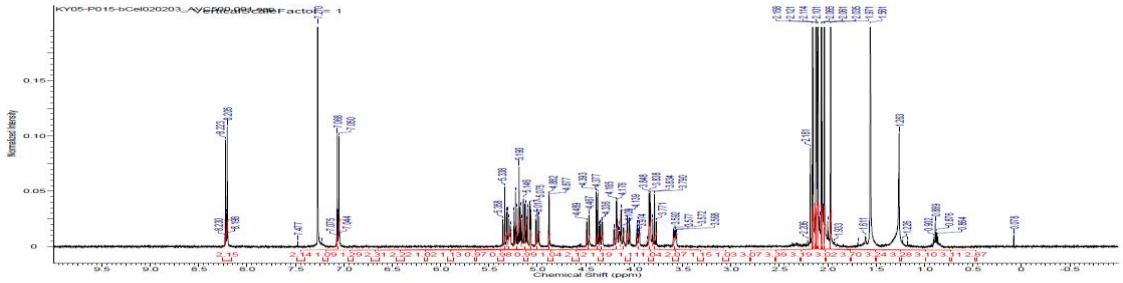
^{13}C NMR



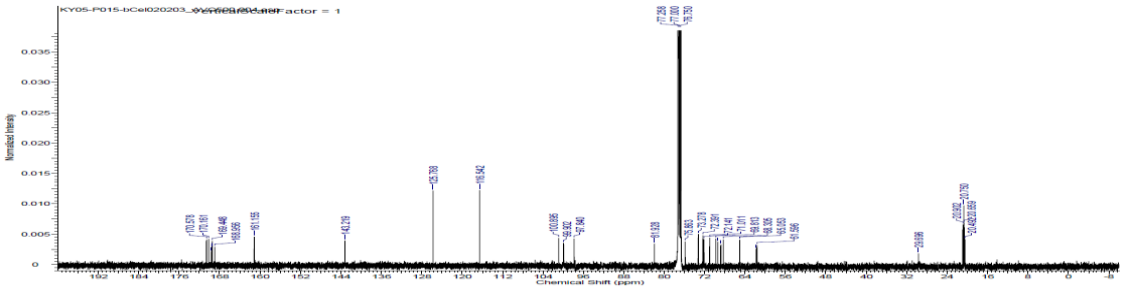
2-5h-II: 

^{1}H NMR (500MHz, $CDCl_3$, δ($CHCl_3$)=7.270): 1.97, 2.04, 2.06, 2.07, 2.10, 2.11, 2.121, 2.124, 2.15, 2.16 (10 x 3H, 10 x s, 10 x OCOC$\underline{H}_3$), 3.58 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 4.5Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{b}H5), 3.79 (1H, dd, $J_{H3\text{-}H2}$ 9.5Hz, $J_{H3\text{-}H4}$ 9.5Hz, ^{b}H3), 3.82-3.88 (m, 2H, ^{a}H4, ^{a}H5), 3.96 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 3.0Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{c}H5), 4.06 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{b}H6), 4.12 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{c}H6), 4.13-4.18 (1H, m, ^{a}H6), 4.19 (1H, dd, $J_{H6'\text{-}H6}$ 13.0Hz, $J_{H6'\text{-}H5}$ 3.5Hz, ^{c}H6'), 4.34 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 4.5Hz, ^{b}H6'), 4.39 (1H, d, $J_{H1\text{-}H2}$ 8.0Hz, ^{b}H1), 4.565 (1H, d, $J_{H6'\text{-}H6}$ 11.0Hz, ^{a}H6'), 4.88 (1H, d, $J_{H1\text{-}H2}$ 2.5Hz, ^{c}H1), 5.00 (1H, dd, $J_{H2\text{-}H3}$ 9.0Hz, $J_{H2\text{-}H1}$ 8.0Hz, ^{b}H2), 5.07 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz, $J_{H2\text{-}H1}$ 2.0Hz, ^{c}H2), 5.12 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{c}H3), 5.15 (1H, dd, $J_{H4\text{-}H3}$ 9.5Hz, $J_{H4\text{-}H5}$ 9.5Hz, ^{b}H4), 5.18 (1H, d, $J_{H1\text{-}H2}$ 7.5Hz, ^{a}H1), 5.23 (1H, dd, $J_{H2\text{-}H3}$ 9.0Hz, $J_{H2\text{-}H1}$ 7.5Hz, ^{a}H2), 5.27-5.32 (1H, m, ^{a}H3), 5.34 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{c}H4), 7.06 (2H, d, $J_{H3\text{-}H2}$ 9.0Hz, ^{Ar}H3), 8.21 (2H, d, $J_{H2\text{-}H3}$ 9.0Hz, ^{Ar}H2). ^{13}C NMR (126MHz, $CDCl_3$, δ($CHCl_3$)=77.00): 20.49, 20.58, 20.62, 20.66, 20.70, 20.72, 20.75, 20.75, 20.90, 20.95 (10 x OCO$\underline{C}H_3$), 61.60, 61.65 (^{b}C6, ^{c}C6), 61.82 (^{a}C6), 65.05 (^{c}C4), 68.31 (^{b}C4), 68.81 (^{c}C3), 69.41 (^{c}C2), 69.81 (^{c}C5), 71.01 (^{a}C2), 72.14 (^{a}C3), 72.23 (^{b}C5), 72.39 (^{b}C2), 73.28 (^{a}C5), 75.86 (^{a}C4), 81.93 (^{b}C3), 97.84 (^{a}C1), 99.90 (^{c}C1), 100.90 (^{b}C1), 116.54 (^{Ar}C3), 125.77 (^{Ar}C2), 143.22 (^{Ar}C1), 161.16 (^{Ar}C4), 168.96, 169.45, 169.53, 169.58, 169.60, 169.67, 170.15, 170.16, 170.58, 170.65 (10 x O$\underline{C}$OCH$_3$). Coupled HSQC: J(^{a}H1-^{a}C1)= 164Hz, J(^{b}H1-^{b}C1)= 161Hz, J(^{c}H1-^{c}C1)= 171Hz. LRMS (ESI$^+$): m/z 1068.38 [M+Na]$^+$. HRMS (ESI$^+$): m/z [M+Na]$^+$ 1068.2816 (found), 1068.2803 (calcd for $C_{44}H_{55}NNaO_{28}$). $[\alpha]_D^{20}$= -16.0° (c= 0.10, $CHCl_3$).

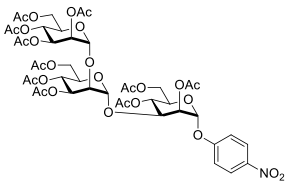
^{1}H NMR



^{13}C NMR

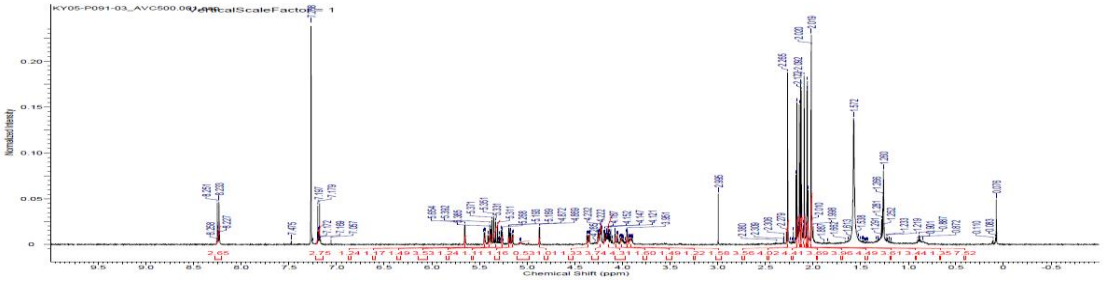


2-5i-I

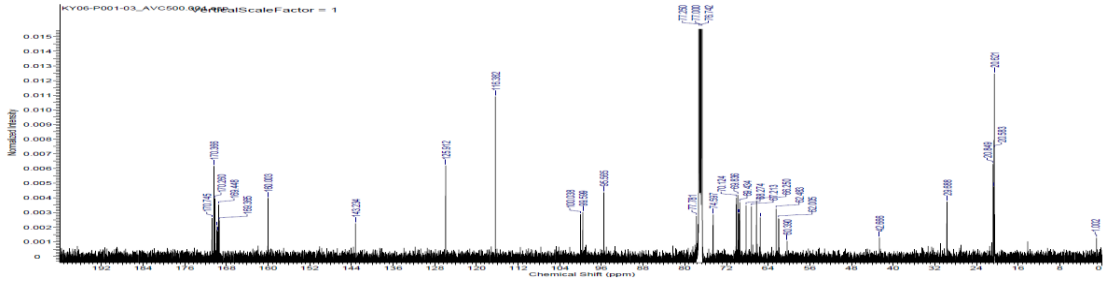


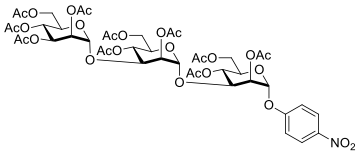
^{1}H NMR (500MHz, $CDCl_3$, δ(C*H*Cl_3)=7.270): 2.019, 2.020, 2.06, 2.07, 2.09, 2.12, 2.13, 2.14, 2.17, 2.27 (10 x 3H, 10 x s, 10 x OCOC$\underline{H}_3$), 3.91 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 5.5Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{a}H5), 3.95 (1H, dd, $J_{H2\text{-}H3}$ 3.0Hz, $J_{H2\text{-}H1}$ 2.5Hz, ^{b}H2), 4.01 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6/6'}$ 4.5Hz, $J_{H5\text{-}H6'/6}$ 2.5Hz, ^{b}H5), 4.06 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{a}H6), 4.10-4.20 (3H, m, ^{c}H5/ ^{b}H6/ ^{c}H6), 4.20-4.27 (3H, ^{a}H6'/^{b}H6'/^{c}H6'), 4.34 (1H, dd, $J_{H3\text{-}H4}$ 9.5Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{a}H3), 4.87 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{c}H1), 5.16 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.0Hz, ^{b}H3), 5.19 (1H, d, $J_{H1\text{-}H2}$ 2.0Hz, ^{b}H1), 5.27 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz, $J_{H2\text{-}H1}$ 1.5Hz, ^{c}H2), 5.31 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz , $J_{H4\text{-}H5}$ 10.0Hz, ^{c}H4), 5.35 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{b}H4), 5.37 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{a}H4), 5.40 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{c}H3), 5.45 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz, $J_{H2\text{-}H1}$ 2.0Hz, ^{a}H2), 5.65 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{a}H1), 7.19 (2H, d, $J_{H3\text{-}H2}$ 9.0Hz, ^{Ar}H3), 8.24 (2H, d, $J_{H2\text{-}H3}$ 9.0Hz, ^{Ar}H2). ^{13}C NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 20.58, 20.58, 20.62, 20.62, 20.67, 20.67, 20.71, 20.74, 20.83, 20.85 (10 x OCO$\underline{C}H_3$), 61.94 (^{a}C6), 62.01, 62.48 (^{b}C6, ^{c}C6), 65.53 (^{b}C4), 66.25 (^{c}C4), 67.21 (^{a}C4), 68.27 (^{c}C3), 69.43 (^{c}C5), 69.53, 69.56 (^{b}C3, ^{c}C2), 69.72 (^{b}C5), 69.84 (^{a}C5), 70.12 (^{a}C2), 74.60 (^{a}C3), 77.78 (^{b}C2), 95.57 (^{a}C1), 99.60 (^{c}C1), 100.04 (^{b}C1), 116.38 (^{Ar}C3), 125.91 (^{Ar}C2), 143.23 (^{Ar}C1), 160.00 (^{Ar}C4), 169.37, 169.45, 169.52, 169.69, 169.83, 170.24, 170.26, 170.37, 170.37, 170.75 (10 x O$\underline{C}$OCH$_3$). Coupled HSQC: J(^{a}H1-^{a}C1)= 176Hz, J(^{b}H1-^{b}C1)= 173Hz, J(^{c}H1-^{c}C1)= 172Hz. LRMS (ESI^{+}): m/z 1068.28 [M+Na]$^{+}$. HRMS (ESI^{+}): m/z [M+Na]$^{+}$ 1068.2815 (found), 1068.2803 (calcd for $C_{44}H_{55}NNaO_{28}$). $[\alpha]_D^{20}$= +74.4° (c= 0.13, $CHCl_3$).

^{1}H NMR



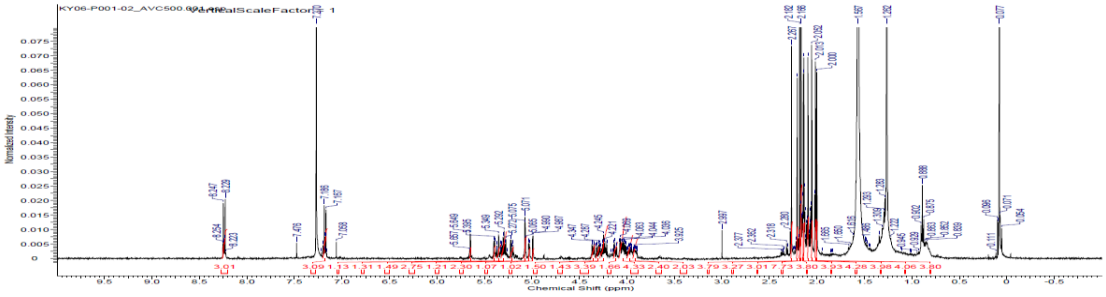
^{13}C NMR



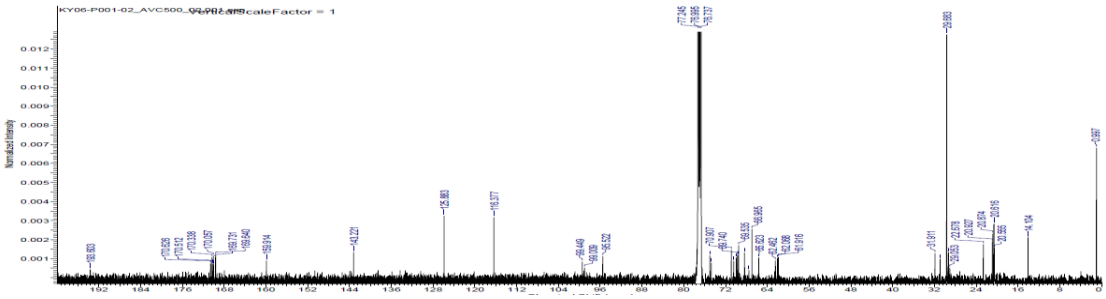
2-5i-II: 

^{1}H NMR (500MHz, $CDCl_3$, δ(C*H*Cl_3)=7.270): 2.00, 2.01, 2.05, 2.09, 2.136, 2.142, 2.17, 2.18, 2.21, 2.27 (10 x 3H, 10 x s, 10 x OCOC$\underline{H}_3$), 3.92 (1H, ddd, $J_{H5\text{-}H4}$ 10.5Hz, $J_{H5\text{-}H6'}$ 5.5Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{a}H5), 3.94-4.00 (1H, m, ^{b}H5), 4.02 (1H, dd, $J_{H3\text{-}H4}$ 9.5Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{b}H3), 4.03-4.09 (1H, m, ^{c}H5), 4.05 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 3.0Hz, ^{a}H6), 4.06 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 3.0Hz, ^{b}H6), 4.12 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{c}H6), 4.23 (1H, dd, $J_{H6'\text{-}H6}$ 13.0Hz, $J_{H6'\text{-}H5}$ 6.0Hz, ^{a}H6'), 4.26 (1H, dd, $J_{H6'\text{-}H6}$ 12.0Hz, $J_{H6'\text{-}H5}$ 6.0Hz, ^{c}H6'), 4.30 (1H, dd, $J_{H6'\text{-}H6}$ 13.0Hz, $J_{H6'\text{-}H5}$ 5.0Hz, ^{b}H6'), 4.35 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 4.0Hz, ^{a}H3), 4.99 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{b}H1), 5.03 (1H, dd, $J_{H2\text{-}H3}$ 4.0Hz, $J_{H2\text{-}H1}$ 1.5Hz, ^{b}H2), 5.05-5.09 (2H, m, ^{c}H1/ ^{c}H2), 5.21 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{c}H3), 5.29 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{c}H4), 5.30 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{b}H4), 5.35 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{a}H4), 5.40 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz, $J_{H2\text{-}H1}$ 1.5Hz, ^{a}H2), 5.65 (1H, d, $J_{H1\text{-}H2}$ 2.0Hz, ^{a}H1), 7.18 (2H, d, $J_{H3\text{-}H2}$ 9.5Hz, ^{Ar}H3), 8.24 (2H, d, $J_{H2\text{-}H3}$ 9.0Hz, ^{Ar}H2). ^{13}C NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 20.56-20.93 (10 x OCO$\underline{C}H_3$), 61.92 (^{a}C6), 62.01 (^{b}C6), 62.46 (^{c}C6), 65.62 (^{c}C4), 66.97 (^{a}C4), 66.97 (^{b}C4), 68.37 (^{c}C3), 69.54 (^{b}C5), 69.66, 69.74 (^{c}C2, ^{c}C5), 69.95 (^{a}C5), 70.46 (^{a}C2), 70.91 (^{b}C2), 74.77 (^{a}C3), 75.00 (^{b}C3,), 95.52 (^{a}C1), 99.01 (^{b}C1), 99.45 (^{c}C1), 116.38 (^{Ar}C3), 125.88 (^{Ar}C2), 143.22 (^{Ar}C1), 159.91 (^{Ar}C4), 169.64, 169.67, 169.73, 169.93, 170.06, 170.16, 170.32, 170.34, 170.51, 170.63 (10 x O$\underline{C}$OCH$_3$). Coupled HSQC: J(^{a}H1-^{a}C1) 176Hz, J(^{b}H1-^{b}C1) 174Hz, J(^{c}H1-^{c}C1) 174Hz. LRMS (ESI^{+}): m/z 1068.21 [M+Na]$^{+}$. HRMS (ESI^{+}): m/z [M+Na]$^{+}$ 1068.2819 (found), 1068.2803 (calcd for $C_{44}H_{55}NNaO_{28}$). $[\alpha]_D^{20}$= +350° (c 0.01, $CHCl_3$).

^{1}H NMR



^{13}C NMR



Attempt for D1-arm synthesis with MalA D320G

5 or 25 μL of 200 mM β-D-Man-F (**2-2b**) and 10 μL of 100 mM trisaccharide[41] (**2-3j**) in 400 mM NaPi (pH 7.0) were mixed and MalA D320G in 100 mM NaPi (pH 6.0), 400 mM NaPi (pH 7.0) and water were added so that the final concentration of the enzyme and the buffer became 2.0 mg/mL and 300 mM, respectively (the final volume was 50 μL). The reaction mixture was incubated at room temperature for 1 day and monitored by HPLC (Condition A' using the ELS detector in place of UV detector).

Mannosynthase reaction using *myo*-inositol as glycosyl acceptor

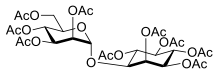
2.5 mL of 400 mM *myo*-inositol (**2-3k**) and 0.5 mL of 200 mM β-D-Man-F (**2-2b**) in 400 mM NaPi (pH 7.0) were mixed and MalA D320G in 100 mM NaPi (pH 6.0), 400 mM NaPi (pH 7.0) and water were added so that the final concentration of MalA D320G and the buffer became 2.0 mg/mL and 300 mM, respectively. The reaction solution was incubated at room temperature for 2 days. The resultant solutions were applied to Vivaspin 6 (MWCO 10,000, Sartorius, pre-treated with 0.1% Triton X-100) and the protein fraction and the small molecule fraction were separated. The protein fraction was dialysed to 100 mM NaPi (pH 6.0) and recycled. The small molecule fraction was lyophilised and dissolved in 4 mL of water. The solution was passed through 10 mL of Amberlite IRA (activated with 2M NaOH) eluting with water. The fractions with the target compounds were collected and the combined solution was passed through 10 mL of Amberlite IRC-505 eluting with water. The fractions with the target compounds were collected and evaporated. The residue was redissolved in 4 mL of water. After filtration with 0.45-μm membrane filter, the solution was analyzed (Condition C) and purified with HPLC. The preparative condition is as follows:

Column: Luna 5u NH2, 250x21.20 mm, 100A, Axia packed (Phenomenex)
Injection volume: <1 mL
Flow rate: 15.0 mL/min
Solvent A: Water
Solvent B: Acetonitrile

Gradient: Solvent B	80%	0-6min
	to 65%	6- 36 min
	65%	36-43 min
	80%	43-48 min

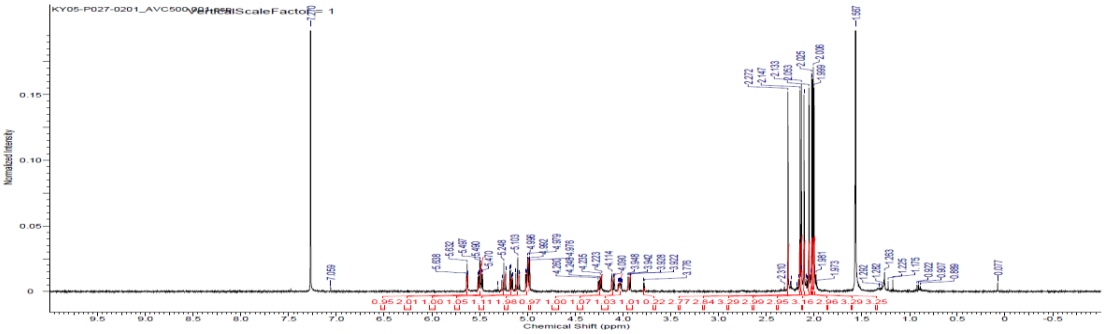
Detection: Evaporative Light Scattering

The separated mannosyl *myo*-inositols were analyzed with MS and NMR. Peracetylation was performed (0.5 mL of pyridine and 0.5 mL of acetic anhydride at RT for 2 days) and **2-5k-II** and **2-5k-I** were isolated as major compounds from the front and rear peaks,respectively.

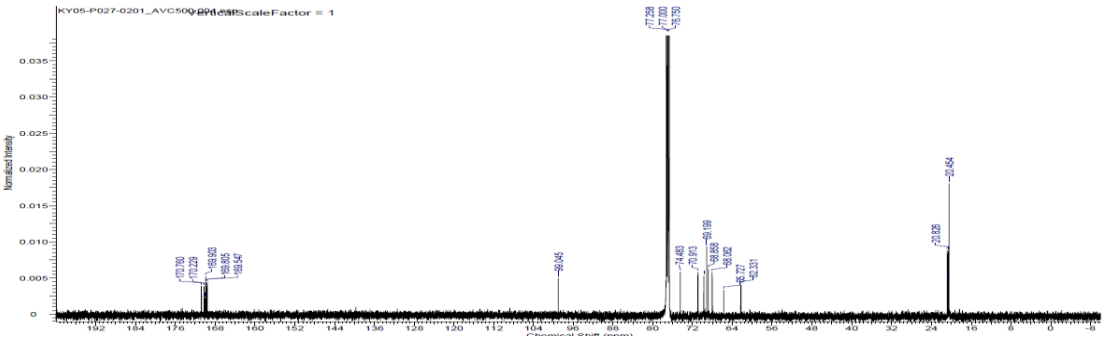
2-5k-I: 

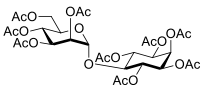
Superscripts of I and M represent atoms in *myo*-inositol and D-mannose, respectively.
^{1}H-NMR (500MHz, $CDCl_3$, δ(C*H*Cl_3)=7.270): 2.00, 2.01, 2.02, 2.03, 2.05, 2.10, 2.13, 2.15, 2.27 (9 x 3H, 9 x s, 9 x OCOC$\underline{H}_3$), 3.94 (1H, dd, $J_{H1\text{-}H6}$ 10.0Hz, $J_{H1\text{-}H2}$ 3.0Hz, ^{I}H1), 4.03 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 6.0Hz, $J_{H5\text{-}H6}$ 2.0Hz, ^{M}H5), 4.10 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.0Hz, ^{M}H6), 4.24 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 6.0Hz, ^{M}H6'), 4.98 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{M}H1), 4.997 (1H, dd, $J_{H2\text{-}H3}$ 3.0Hz, $J_{H2\text{-}H1}$ 2.0Hz, ^{M}H2), 5.005 (1H, dd, $J_{H3\text{-}H4}$ 10.5Hz, $J_{H3\text{-}H2}$ 2.5Hz, ^{I}H3), 5.10 (1H, dd, $J_{H5\text{-}H4/6}$ 10.0Hz, $J_{H5\text{-}H6/4}$ 9.5Hz, ^{I}H5), 5.17 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{M}H3), 5.25 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{M}H4), 5.49 (1H, dd, $J_{H4\text{-}H3'}$ 10.5Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{I}H4), 5.50 (1H, dd, $J_{H6\text{-}H1'}$ 10.5Hz, $J_{H6\text{-}H5}$ 10.0Hz, ^{I}H6), 5.63 (1H, dd, $J_{H2\text{-}H1/3'}$ 3.0Hz, $J_{H2\text{-}H3/1}$ 2.5Hz, ^{I}H2). ^{13}C-NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 20.45, 20.45, 20.49, 20.55, 20.61, 20.63, 20.71, 20.78, 20.83 (9 x OCO$\underline{C}H_3$), 62.33 (^{M}C6), 65.73 (^{M}C4), 68.06 (^{M}C3), 68.86 (^{I}C3), 69.20, 69.20 (^{I}C2, ^{I}C4), 69.73 (^{M}C5), 69.76 (^{M}C2), 70.91, 70.99 (^{I}C6, ^{I}C5), 74.48 (^{I}C1), 99.05 (^{M}C1), 169.55, 169.58, 169.62, 169.77, 169.81, 169.90, 169.99, 170.23, 170.76 (9 x O$\underline{C}OCH_3$). Coupled HSQC: J(^{M}H1-^{M}C1) 174Hz. LRMS (ESI^{+}): m/z 743.25 $[M+Na]^{+}$. HRMS (ESI^{+}): m/z $[M+Na]^{+}$ 743.2009 (found), 743.2005 (calcd for $C_{30}H_{40}NaO_{20}$). $[\alpha]_D^{20}$= +24.0° (c 0.05, $CHCl_3$).

^{1}H NMR



^{13}C NMR

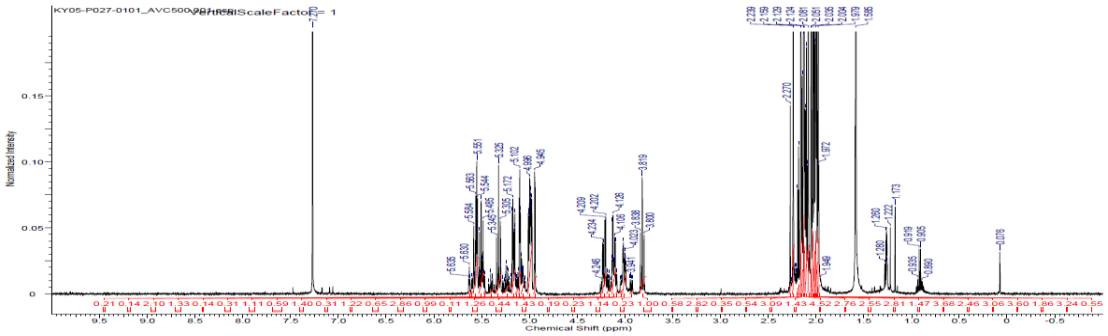


2-5k-II: 

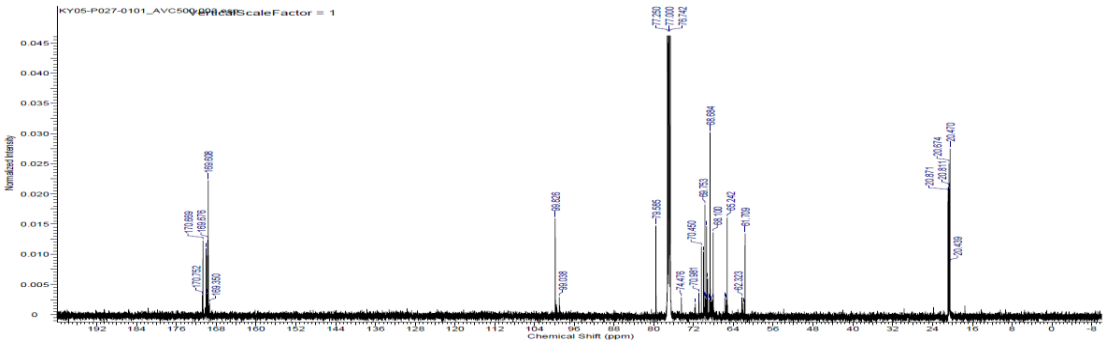
Superscripts of I and M represent atoms in *myo*-inositol and D-mannose, respectively.

^{1}H-NMR (500MHz, $CDCl_3$, δ(C*H*Cl_3)=7.270): 1.98, 2.00, 2.01, 2.04, 2.08, 2.12, 2.13, 2.16, 2.24 (9 x 3H, 9 x s, 9 x OCOC$\underline{H}_3$), 3.82 (1H, dd, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6}$ 9.5Hz, ^{I}H5), 4.01 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 3.5Hz, $J_{H5\text{-}H6}$ 2.5Hz, ^{M}H5), 4.12 (1H, dd, $J_{H6\text{-}H6'}$ 12.5Hz, $J_{H6\text{-}H5}$ 2.5Hz, ^{M}H6), 4.22 (1H, dd, $J_{H6'\text{-}H6}$ 12.5Hz, $J_{H6'\text{-}H5}$ 3.5Hz, ^{M}H6'), 4.94 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{M}H1), 4.99 (1H, dd, $J_{H1/3\text{-}H6/4'}$ 10.5Hz, $J_{H1/3\text{-}H2}$ 2.5Hz, ^{I}H1/3), 5.00 (1H, dd, $J_{H1/3\text{-}H6/4'}$ 10.5Hz, $J_{H1/3\text{-}H2}$ 2.5Hz, ^{I}H1/3), 5.10 (1H, dd, $J_{H2\text{-}H3}$ 3.5Hz , $J_{H2\text{-}H1}$ 2.0Hz, ^{M}H2), 5.17 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{M}H3), 5.33 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, ^{M}H4), 5.50 (1H, dd, $J_{H4/6\text{-}H3/1'}$ 10.5Hz, $J_{H4/6\text{-}H5}$ 9.5Hz, ^{I}H4/6), 5.55 (1H, dd, $J_{H2\text{-}H1'}$ 3.0Hz, $J_{H2\text{-}H3}$ 3.0Hz, ^{I}H2), 5.56 (1H, dd, $J_{H4/6\text{-}H3/1'}$ 10.5Hz, $J_{H4/6\text{-}H5}$ 9.5Hz, ^{I}H4/6). ^{13}C-NMR (126MHz, $CDCl_3$, δ(*C*HCl_3)=77.00): 20.47, 20.47, 20.58, 20.67, 20.67, 20.74, 20.81, 20.87, 20.93 (9 x OCO$\underline{C}H_3$), 61.71 (^{M}C6), 65.24 (^{M}C4), 68.10 (^{I}C2), 68.68 (^{M}C3, ^{I}C1/3, ^{I}C1/3), 69.38 (^{M}C2), 69.75 (^{M}C5), 70.02 (^{I}C4/6), 70.45 (^{I}C4/6), 79.59 (^{I}C5), 99.83 (^{M}C1), 169.57, 169.57, 169.61, 169.61, 169.68, 169.68, 169.93, 170.03, 170.67 (9 x O$\underline{C}$OCH$_3$). Coupled HSQC: J(^{M}H1-^{M}C1) 171Hz. LRMS (ESI^{+}): m/z 743.14 $[M+Na]^{+}$. HRMS (ESI^{+}): m/z $[M+Na]^{+}$ 743.2019 (found), 743.2005 (calcd for $C_{30}H_{40}NaO_{20}$). $[\alpha]_D^{20}$= +15.2° (c 0.25, $CHCl_3$).

^{1}H NMR



^{13}C NMR



Kinetics of the synthase reactions by MalA D320G

α-Mannosynthase kinetics with the fixed concentration of the acceptor. (**Scheme 2.19a**)

Test solutions were prepared with the fixed concentration of the acceptor (*p*NP-β-D-Glc, **2-3a**) at 20 mM and the enzyme at 0.5 mg/mL and with various concentrations of β-D-Man-F **2-2b** (0.5, 1.0, 1.6, 2.0, 3.2, 4.0, 10, 20 and 40 mM, duplicate) in 300 mM NaPi (pH 7.0). The reaction was incubated at 20°C and monitored by HPLC (Condition A'') to determine the initial reaction rate. The obtained reaction rates were plotted against the substrate concentrations and the plots were fitted to Michaelis-Menten equation (**Figure 2.27a**) using Origin 8 to determine kinetic parameters (**Table 2.11**).

α-Mannosynthase kinetics with the fixed concentration of the donor. (**Scheme 2.19b**)

The same experiment was performed with various concentrations of *p*NP-β-D-Glc **2-3a** (1.0, 2.5, 5.0, 10, 20, 30, 40 and 50 mM, duplicate), fixing the concentrations of the donor (β-D-Man-F, **2-2b**) and the enzyme at 20 mM and 0.5 mg/mL, respectively. The Michaelis-Menten plot and the kinetic parameters are shown in **Figure 2.27b** and **Table 2.11**, respectively.

α-Glucosynthase kinetics with the fixed concentration of the acceptor. (**Scheme 2.20a**)

The same experiment was performed with various concentrations of β-D-Glc-F **2-2a** (0.1, 0.3, 0.6, 1.0, 1.5, 2.0, 5.0 mM, quadruplicate), fixing the concentrations of the acceptor (*p*NP-β-D-Glc, **2-3a**) and the enzyme at 2 mM and 0.1 mg/mL, respectively. The Michaelis-Menten plot and the kinetic parameters are shown in **Figure 2.28a** and **Table 2.11**, respectively.

α-Glucosynthase kinetics with the fixed concentration of the donor. (**Scheme 2.20b**)

The same experiment was performed with various concentrations of *p*NP-β-D-Glc **2-3a** (1.0, 1.4, 2.0, 3.0, 6.0, 10, 16, 20 mM, duplicate), fixing the concentrations of the donor (β-D-Glc-F, **2-2a**) and the enzyme at 2 mM and 0.1 mg/mL, respectively. The Michaelis-Menten plot is shown in **Figure 2.28b**.

Attempt for α-rhamnosynthase reaction

10 μL of 200 mM β-D-RhaF (**2-2c**) and 25 μL of 40 mM *p*NP-β-D-Glc (**2-3a**) in 400 mM NaPi (pH 7.0) were mixed. To the mixture were added 400 mM NaPi (pH 7.0), water and MalA D320G in 100 mM NaPi (pH 6.0) so that the final concentration of the enzyme and the buffer became 2.0 mg/mL and 300 mM, respectively (the final volume was 50 μL). The reaction

solution was incubated at room temperature. The reaction was analyzed by HPLC (Condition A', General Procedure).

α-Mannosynthase reaction using RNaseB as glycosyl acceptor

20 μL of 100 mM β-D-Man-F **2-2b** in 100 mM NaPi (pH 6.0) and 50 μL of 20 mg/mL RNase B in 100 mM NaPi (pH 6.0) were mixed at 4°C and MalA D320G in 100 mM NaPi (pH 6.0) and 100 mM NaPi (pH 6.0) were added so that the final concentration of the enzyme became 0.5 mg/mL. For control reactions, 100 mM NaPi (pH 6.0) was added in place of the enzyme solution. The reaction mixture was stirred at 4°C for 4 days. The solution was filtered with 0.45 μm-membrane filter and 20 μL of 1.0 mg/mL protein solution was used for protein MS (General Procedure).

Transglycosylation reaction by MalA WT

12.5 μL of 80 mM α-D-Man-F (**2-2d**) and 12.5 μL of 80 mM *p*NP-β-D-Glc (**2-3a**) in 100 mM NaOAc (pH 4.5) were mixed. To the mixture were added 21.4 μL of 100 mM NaOAc (pH 4.5) and 3.6 μL of 13.9 mg/mL MalA WT in 100 mM NaPi (pH 6.0). The reaction solution was incubated at room temperature for 1 day. After 1day, the reaction solution was heated to 70°C. The reaction was analyzed by HPLC (Condition A'', General Procedure).

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Chapter 3

Development of a Novel Antibody-Drug Conjugate by *N*-glycan Modification

3.1. Introduction 129

3.1.1. Antibody-based drugs and antibody-drug conjugates 129

3.1.2. Endo-β-*N*-acetylglucosaminidase- ENGase 138

3.1.2.1. ENGase as a synthetic tool 141

3.1.2.2 EndoS 151

3.1.3. Aim of the research 153

3.2. Result and Discussion 154

3.2.1. Expression and purification of EndoS 154

3.2.2. Cleavage of glycans from IgG with EndoS 161

3.2.3. Remodelling of glycans on IgG with EndoS 162

3.2.4. Design of glycans for antibody-drug conjugates 166

3.2.5. Preparation of glycans for antibody-drug conjugates 170

3.2.5.1. Tetrasaccharide synthesis 170

3.2.5.2. Decasaccharide synthesis 183

3.2.5.3. Oxazoline formation 189

3.2.6. Introduction of bioorthogonal reaction handles on IgG with EndoS 193

3.2.7. Bioorthogonal reaction on IgG 199

3.3. Conclusion and the scope in the future 202

3.4. Experimental 205

3.5. Reference 274

3.1. Introduction

Penicillin is one of the chemicals that have changed history. It not only cured a lot of infectious diseases but also established the foundation for modern medicines. Penicillin doesn't harm human cells, whereas it "selectively" inhibits cell wall synthesis of bacteria.

Many medical agents have been developed now and even diseases previous know as lethal can be cured. Modern human society has enjoyed significant benefits from pharmaceutical compounds. However, it is also true that the history of drug discovery is always flanked by the risk of unexpected adverse effects.

To minimize such risks, active pharmaceutical ingredients should be homogeneous, i.e. they should contain only the "selected" chemical species without impurities. They should be "selectively" effective on the specific biological target. They should also be delivered to the target organ or cell "selectively." Thus, "selectivity" is the key word even in the modern pharmaceutical industry and the concept proven by the classic drug is still alive in the mind of pharmaceutical scientists as an axiom.

3.1.1. Antibody-based drugs and antibody-drug conjugates

The immune system is, simply speaking, the machinery to detect and eliminate harmful non-self entities. Though it is one of the most sophisticated functions in biology, the central mode of action is based on the complementary binding of a toxin and a "side-chain," as nomenculated by Paul Ehrlich, which are now known as an antigen and an antibody, respectively. Especially, an antibody working in the acquired immune system has such a high specificity toward an antigen that it is natural that pharmaceutical scientists sought to develop

such an antibody as a "magic bullet" to realize the high "selectivity" toward a specific molecular target as Paul Ehrlich himself had wished.

After enourmous technological development in the field of bioengineering, monoclonal antibodies (mAbs) are available including fully humanized ones. Because high selectivity has been the continuous demand, the mAb-based drug was welcomed by the pharmaceutical industry with great enthusiasm. Since first approved by FDA, more than 300 therapeutic antibodies have been reported.[1]

MAb-based drugs are especially effective when the molecular targets are presented on the cell surface or in the extracellular milieu. The binding of mAbs to their molecular targets are the principal mode of action, but there are several ways to exert their efficacies such as:[1]

1. Antagonism through the binding to the molecular target against a native ligand
2. Down regulation of the receptor on the target cell surface
3. Induction of apoptosis of the target cells through the agonistic binding to the apoptosis-inducing receptor
4. Induction of effector functions: antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC)
5. Delivery of effector molecules to the target cells

From the viewpoint of the glycochemist, modulation of effector function of IgG is an interesting topic because the structure of *N*-glycan on IgG-Fc asparagine-297 has significant impact on the effector function. Two *N*-glycans on the heavy chains are sandwiched by the CH2 domains (**Figure 3.1**) and are closely situated with the protein backbone. Therefore, the stuructural variance of *N*-glycans can alter the global conformation of IgG, which results in

changes of its affinity to the FcγRs and the consequent functional outcomes. A simple example is that when IgG_1 loses *N*-glycans, it shows significant decrease in its binding to FcγRIIa and FcγRIIb.[2] A more phamaceutically relevant example is that Herceptin, a mAb-based drug for breast cancer, shows remarkably enhanced ADCC mediated by FcγRIII when *N*-glycan has non-fucosylated glycoform.[3] The terminal sialylation also shows notable effects on the effector function and the antibody with the sialylated *N*-glycan shows enhanced anti-inflamatory properties, whereas the non-sialylated glycoform has higher affinity to FcγR and exert more potent cytotoxicity.[4] Recently, investigation about the precise effect of *N*-glycan structures to the IgG-Fc binding to FcγRs was demonstrated by enzymatic *N*-glycan modification using chemically synthesized oligosaccharides.[5, 6] Chemical and biological modulation of the effector functions by *N*-glycan engineering is intensively investigated for development of more potent mAb-based drugs.

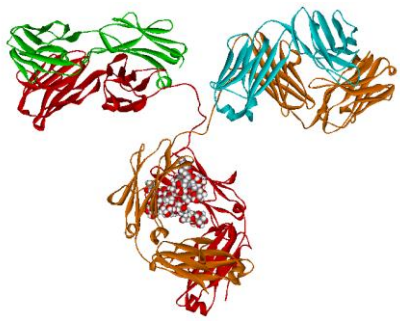


Figure 3.1. The structure model of IgG_1 based on 1IGT (PDB ID). The light chains are shown in green and blue and the heavy chains in red and orange. The *N*-glycans on asparagine-297 depicted in CPK model are sandwiched by the constant region of the two heavy chains.

Another state-of-art technology of mAb drugs is antibody-drug conjugate (ADC)[7] where antibody is used as a carrier of drugs by utilizing its excellent selectivity to the molecular target based on complementary binding. This methodology is especially beneficial for cancer treatment. The drugs for cancer usually have narrow therapeutic windows and therefore the

careful control of administration is required. In spite of such clinical efforts, cancer drugs often lead to severe adverse effects because of the lack of selectivity between normal and malignant cells. Though their differentiation is difficult as long as small molecules are delivered diffusively, antibodies are able to discriminate them if their antigens are expressed in significantly different levels. Indeed, many molecular targets have been reported, which are overexpressed specifically on cancer cells and hence selective delivery of anti-cancer agents using the corresponding antibodies is feasible. Based on this strategy, several ADCs have been developed (**Table 3.1**).

Table 3.1. ADCs under clinical trials (> Phase II study).[8] a) HZ, hydrazone; VC, valine-citrulline; TE, thioether; DS, disulfide. b) CM, calicheamicin MMAE, monomethyl auristatin E; DM1, DM4, maytansinoids; DR, doxorubicin. c) Cleavage mode: A, acid; P, protease; N, non-cleavable; R, reduction. d) Most advanced phases are shown.

ADC	mAb	antibody type	Linker[a)]	Payload[b)]	Cleavage[c)]	Clinical study[d)]
Mylotarg	Gemtuzumab	Humanized IgG4	HZ	CM	A	Approved; withdrawn
Adcetris	Brentuximab	Chimeric IgG1	VC	MMAE	P	Approved
T-DM1	Trastuzumab	Humanized IgG1	TE	DM1	N	PIII
CMC-544	Inotuzumab	Humanized IgG1	HZ	CM	A	PIII
CDX-011	Glembatumab	Human IgG2	VC	MMAE	P	PII
SAR3419	huB4	Humanized IgG1	DS	DM4	R	PII
BT-062	anti-CD138 mAb	Chimeric IgG4	DS	DM4	R	PI/II
IMGN901	Lorvotuzumab	Humanized IgG1	DS	DM1	R	PI/II
MMU-110	Milatuzumab	Humanized IgG1	TE	DR	N	PI/II

The basic structure of ADC is composed of three parts (**Figure 3.2**): i) mAb, ii) linker and iii) payload. To bring out the maximum potency of ADC, these three components should be conjugated to satisfy the following requirements at each stage of the journey to the target:[7]

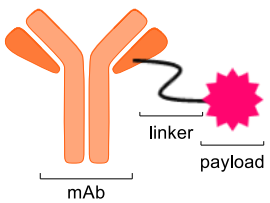


Figure 3.2. Components of ADC. It is composed of i) mAb, ii) linker and iii) payload.

A. Circulation

The linker should be stable during circulation in the blood stream to minimize the exposure of the normal cells to the drug molecules.

B. Antigen binding

The attached linker or the payload should not interfere the binding of mAb to the antigen.

C. Internalization

When the payload brings forth its function inside the cell, the ADC should be internalized. However, this is not necessary when the payload functions on the cell surface, e.g. a radioisotopic compound or an enzyme to activate another prodrug which is administered subsequently.

D. Drug release

Once internalized, the payload should be released or converted to the active form.

E. Drug action

The concentration of the active payload should be sufficient to exert its cytotoxicity. Normally, toxins with 100-1000 times higher cytotoxicity than the traditional anticancer agents are used.

As payload, calicheamicin, maytansine derivatives and auristatine derivatives are preferably used because of their subnanomolar cytotoxicity (Requirement E).[7]

The method to conjugate the payload to the linker is categorized into three groups. The first one is the chemically reversible conjugation (**Figure 3.3**). As mentioned in Requirement A, the payload should be linked to IgG scaffold stably during the circulation in the blood stream. Conversely, it should be released from the protein backbone to bring out its cytotoxicity (Requirement D) once ADC is internalized to the target cell. These obstinate demands can be

resolved by the environmental difference. Once ADC is internalized, it is encapsulated in an endosome, which then matures to a lysosome. It is known that pH of these vesicles (pH 5.0-6.5 in endosome, pH 4.5-5.0 in lysosome) are lower than the usual physiological condition (pH 7.3-7.5). Therefore, acid sensitive functional groups are used to harness the payload to the linker in the blood stream and release it after internalization. Hydrazine was found to have good chemical properties for this stragery and was used in the early examples of ADCs. Recently, 1,2-aminothiol (i.e. N-terminal cystein) was reported as an novel conjugation handle which requires no additional linker between the protein scaffold and the payload, and releases the payload in response to the low pH.[9]

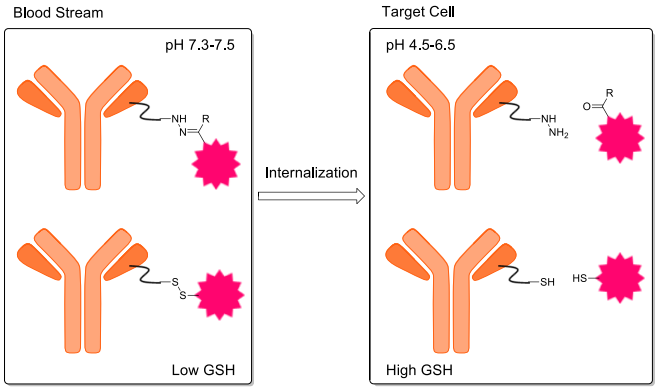


Figure 3.3. The examples of chemically reversible conjugations. Environmental differences between the blood stream and the cell, like pH or the redox condition, allow targeted cleavage of the payloads after internalization.

Alternatively, the diffenrece of the redox condition can also trigger the drug release. The concentration of glutathione is higher in the cytoplasm than in the blood stream by 1000-fold.[7] Especially, tumor cells have the enhanced level of glutathione. Therefore, disulfide bond can be also used as another option for chemically reversible conjugation.

The chemically reversible linkers provided positive results, but it is inevitable that some part of the payload fall off as long as reversible reactions are used. For more precise control of payload

release, enzyme-cleavable linkers were also investigated (**Figure 3.4**).[7] Lysosome is a sack full of digestive enzymes and contains high concentration of proteases, like cathepsin or plasmin. Valine-citruline and phenylalanine-lysine dipeptides are representative examples for protease cleavable linkers.[7] These peptidic linkers are sometimes coupled with 4-aminobenzyl group. This additional linker is deployed between the protease cleavage site and the payload to facilitate the enzymatic hydrolysis. After hydrolysis, the 4-aminobenzyl moiety is eliminated in the 1,6-elimination manner, releasing the free payload.

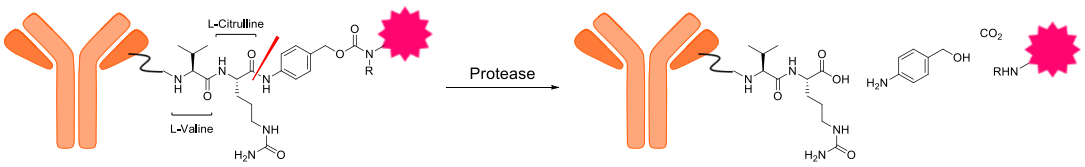


Figure 3.4. An example of lytic peptide linkers to release the payloads in the lysosome.

Finally, the non-cleavable linker is also reported to be effective. Such linkers ensure stability in the blood stream (requirement A). Apparently, the mechanism of the efficacy is due to the degradation of mAb scaffold by protease in lysosome (**Figure 3.5**) and the payload with the linker moiety is released as a working molecule.[7]

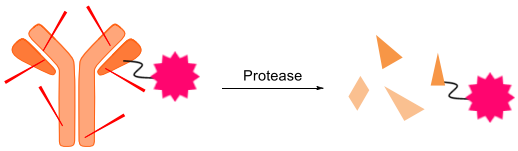


Figure 3.5. Proteolytic degradation of the mAb structure to release the working payload.

Introduction of linkers to IgG scaffold is most commonly implemented on lysine residues (**Figure 3.6**). The amino group of the lysine side chain can be reacted with an activated ester to afford an amide bond under mildly basic condition (pH > 8.0). The abundance of lysines (occurrence in human proteins: 5.9%) allows facile introduction of the linkers on IgG, but at the same time the multiple reaction sites makes it difficult to prepare homogeneous constructs.

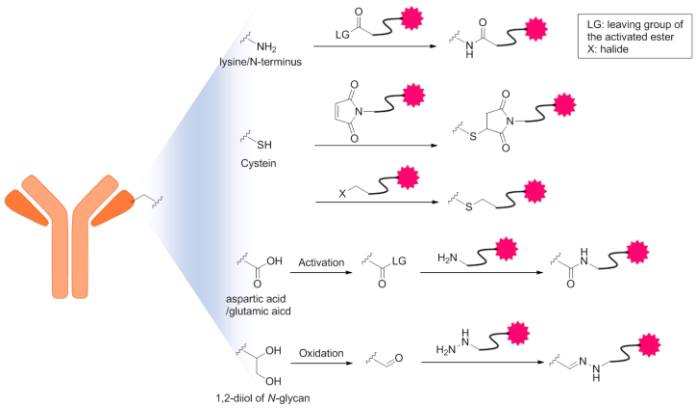


Figure 3.6. (Potential) chemical methods for introduction of linkers on mAbs.

Another common reactive site is the cysteine side chain (**Figure 3.6**). Thiol groups have unique reaction properties and orthogonal reactivities to functioinal groups of other natural amino acides. Maleimide is usually used as a reaction partner to afford a thioether product, though an alkyl halide is also a suitable alternative. The low occurrence of cysteine (1.9%) enables more selective modification. But normally all the cysteines in IgG form disulfide bonds in the intrachains or to cross-link between a light chain and a heavy chain and between two heavy chains. Therefore, reduction of disulfide bonds is required before installing linkers, which can be another cause of heterogeneity of the resultant constructs.

The *N*-terminal α-amino group is another option for the amide formation reaction. Its slightly lower basicity (mostly pK_a 9.0-9.5) than the lysine side chain (pK_a 10.5) might allow for selective modification. Carboxylic acids of aspartic acid and glucamic acid or aldehydes formed by oxidation of *N*-glycans are other potential reaction sites to introduce linkers. However, these reaction handles have not been used in the representative examples of ADCs so far.

As reviewed here briefly, antibody-drug conjugate (ADC) is a smart device integrated by chemistry and biology. Several ADCs have been launched in the market and showed good

clinical results. These achievements prompt further development of ADCs with higher qualities. Heterogeneity of the products, mainly derived from randomly introduced linkers onto lysine or cysteine side chains, is a concern in terms of the quality control of the products. To minimize the risk of adverse events, only “selected” chemical species should be presented as active pharmaceutical ingredients.

Recently biosimilars, which are to come up when the patents of the innovator biopharmaceuticals are expired, are a topic of debate. They have the similar but different structural scaffolds, like glycoform variants, and are produced by different producers, in different plants, using different materials. Therefore, the risk of unexpected outcomes increases unless they are regulated under the product profiles of the precedent innovator drugs. This means at the same time that the quality of the innovator drugs should be definitive and hence its homogeneity is one of the important aspects for the quality control.

This is also true for ADCs. To produce safe drugs, heterogeneity derived from the randomness of the linker positions should be avoided. Therefore, a novel method to introduce payload-linkers in a homogeneous manner is anticipated.

3.1.2. Endo-β-*N*-acetylglucosaminidase- ENGase

Endo-β-*N*-acetylglucosaminidase, ENGase, is an endoglycosidase that hydrolyzes *N*-glycan at the β(1,4)-glycosidic bond between the core chitobiose. These enzymes, found mainly in GH18 and 85 in the CAZy classification,[10, 11] are widely distributed from bacteria to animals and are considered to be involved in various biological functions; e.g. glycan metabolism[12] or bacterial pathogenesis[13-15] in the host. Additionally, some ENGases are often used as biochemical tools[16] to facilitate manupilation of glycoproteins by removing glycan moieties or to elucidate their glycan structures. Representative examples of ENGases are listed in **Table 3.2**.

Hydrolysis by endoglycosidases in both GH18[17-19] and GH85[20-22] is presumed to proceed through the substrate-assisted mechanism. The general reaction mechanism by ENGase is shown in **Figure 3.7**. First, the acid/base catalyst works as acid to activate the leaving aglycon group through hydrogen bonding. Next, the intramolecular S_N2-type reaction by 2-acetamide group cleaves the glycosidic bond. This nucleophilic attack seems to be promoted by another conserved amino acid (aspartic acid for GH18[23-25], asparagine for GH85[20-22]), resulting in the formation of the oxazoline intermediate. Finally, the acid/base catalyst works as base to activate the incoming nucleophile, which attacks the anomeric position to open the oxazoline ring. Since the reaction goes through double inversion, the product has the net retention of the configuration.

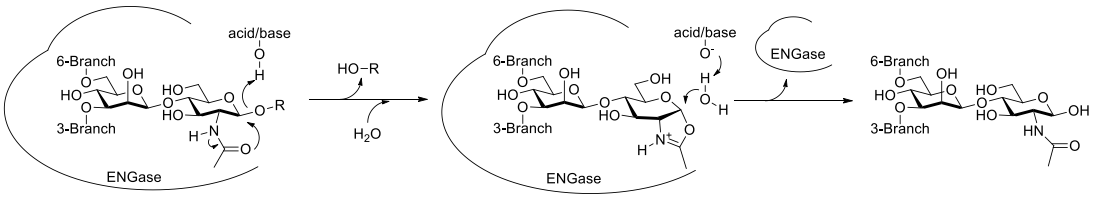


Figure 3.7. General reaction mechanism of glycolysis by ENGase.

The reaction coordinates mentioned above are postulated based on the X-ray structures with oxazoline (analogue) ligands. Though ENGases of both GH18 and GH85 are likely to have this general mechanism in common, there are some structural differences between GH18 and GH85 enzymes: i) GH18 enzymes have aspartic acid[23-25] which interacts with N-H of the nucleophile 2-acetamide group whereas GH85 enzymes have asparagine[20-22] in the equivalent position, ii) the tyrosine of the active site of GH18 has an extra hydrogen bonding interaction with carbonyl oxygen of 2-acetamide group,[17-19, 25] while GH85 enzymes do not have such an interaction, iii) the catalytic diad (the acid/base catalyst-the conserved asparagine) of GH85 is flanked by a tyrosine, which is likely to interact with oxazoline ring through π-stacking to stabilize the transition state.[22] GH18 enzymes do not have a conserved amino acid at the equivalent position.

When the nucleophile is water the reaction gives the hydrolyzed glycon whereas when the nucleophile is other alcohols, it gives the corresponding transglycosylated compounds. Actually, many ENGases are known to show transglycosylation activity (**Table 3.2**). From a synthetic viewpoint, such a reaction is an exquisite tool because it enables us to deliver the gross structure of *N*-glycans in a single reaction onto the peptide/protein backbone. As such, application of transglycosylation reaction by ENGases has been investigated intensively and many magnificent syntheses of biological molecules have been successfully accomplished.

Table 3.2. Representative examples of ENGases.

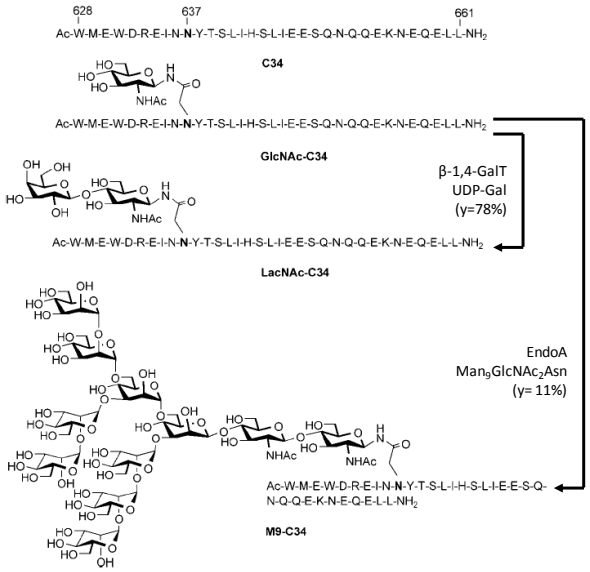
Enzyme Name Source	CAZy[a)]	Substrate [b)]	Fuc [c)]	TG [d)]
EndoA *Arthrobactor protophormiae*	GH85	M [26]	↓ [27]	✓[5, 6, 28-30]
EndoBH *Bacillus halodurans*	GH85	M > H [31]	ND	✓ [31]
EndoD *Streprococcus pneumonia* [e)]	GH85	X[i)] [32, 33]	±[34]	✓ [27]
EndoE *Enterococcus faecalis*	GH18	M, C [15]	±[15]	ND
EndoF1 *Elizabethkingia meningosepticum*[f)]	GH18	M, H [35, 36]	↓ [35, 36]	✓ [37]
EndoF2 *Elizabethkingia meningosepticum* [f)]	GH18	C (b) > M [36]	± [16]	✓ [37]
EndoF3 *Elizabethkingia meningosepticum* [f)]	GH18	C (b, t) [36]	↑[16, 36]	✓ [37]
EndoH *Streptomyces plicatus*[g)]	GH18	M, H [16, 35]	± [16, 35, 36]	✗ [38-41]
EndoS *Streptococcus pyrogenes*	GH18	C [42]	± [42]	✓ [42]
EndoFV *Flammulina velutipes*	GH18	M > H [43]	NA	✓ [43]
EndoM *Mucor hiemalis*	GH85	M > C [44]	↓ [44]	✓[45-49]
EndoT *Hypocrea jecorina*[h)]	GH18	M [50]	ND	ND
EndoCE *Caenorhabditis elegans*	GH85	M > C [51]	↓ [51]	✓ [51]
EndoHO *Gallus gallus domesticus*	NA	M > H, C [52]	ND	ND
ENGase *Homo sapiens*	GH85	M > C [12]	↓ [12]	ND

a) CAZy classification, b) substrates hydrolyzed by the enzymes: M, oligomannose *N*-glycan; H, hybrid *N*-glycan; C, complex *N*-glycan; C(b), biantennary complex *N*-glycan; C(t), triantennary complex *N*-glycan; X, complex glycan treated with neuraminidase, galactosidase and glucosaminidase. c) effects of core-fucose in the substrates to the hydrolytic activity: ↓, ↑ decrease and increase of the activity, respectively; ±, no significant effect; ND, no data available. d) Reported transglycosylation activity: ✓, transglycosylation activity is observed; ✗ transglycosylation activity is NOT observed; ND, No data available. e) Formerly known as *Diplococcus pneumoniae*. f) Formerly known as *Flavobacterium meningosepticum*. g) Formerly known as *Streptomyces griseus*. h) Formerly known as *Trichoderma reesei*. i) Unsubstituted 3-branch mannose is required.

3.1.2.1. ENGase as a synthetic tool

The transglycosylation activity of ENGase has been reported since the early stage of the studies about this class of enzymes. Initially, transglycosylation was accidentally found when $Man_5GlcNAc_2Asn$ was hydrolyzed by EndoF in the presence of glycerol.[38] The analysis of the unexpected compound turned out to be the transglycosylated product onto glycerol in place of the GlcNAcAsn moiety of the starting material. For comparison, EndoH was subjected to the same condition[38] but it did not show such transglycosylation properties. While many other ENGases show transglycosylation activity (**Table 3.2**), extensive studies revealed that EndoH is an exceptional ENGase whose transglycosylation activity has not yet been observed.[39-41]

Among the known ENGases, EndoA has been the most systematically investigated as a synthetic biocatalyst. Its transglycosylation activity was first reported using $Man_6GlcNAc_2Asn$ as glycosyl donor and mono- or di-saccharides as glycosyl acceptor.[53, 54] This transglycosylation reaction with an oligomannosyl *N*-glycoasparagine was applied to preparation of glycopeptides/ glycoproteins[41] and its mimetics[55] as well as a polymer conjugated with oligomannose glycans.[56] Biologically relevant molecules prepared by EndoA include the preparation of C34 glycopeptide, which is the C-terminal ectodomain of the HIV-1 transmembrane envelope glycoprotein, gp41.[57] In this example, the non-glycosylated C34 peptide and the GlcNAc-C34 glycopeptide were prepared by solid phase peptide synthesis (SPPS). The enzymatic glycosylation reactions (**Scheme3.1**), one of which was catalyzed by EndoA, provided several glycoforms of C34 and allowed us to investigate the effect of *N*-glycans on the peptide stucture as well as on the biological activity.



Scheme 3.1. An example of transglycosylation by EndoA. The figure is The figure is adapted with permission from Wang, L. X. *et al.*, *ChemBioChem* **2005**, *6* (6), 1068-1074.[57] Copyright 2005 John Wiley and Sons.

These examples demonstrated the utility of EndoA-catalyzed reaction for preparation of biomolecules. However, transglycosylation using naturally occurring glycans usually gives only poor yields even under optimized conditions. This is mainly because the formed products are good substrates for hydrolysis by EndoA. Addition of organic solvents to the reaction enhanced transglycosylation efficiency and was applicable to preparation of *N*-glycan analogues[58] and small glycopeptides.[55] However, purely aqueous medium is desirable for preparation of glycoproteins.

Alternatively, significant enhancement of yields was observed when synthetic glycan oxazolines with non-native scaffolds were used as glycosyl donor.[59] Such oxazoline glycosyl donors (**Figure 3.8**) mimic the intermediate in ENGase catalysis (**Figure 3.7**). The activated form of these donors facilitates transglycosylation reactions. Moreover, their non-native structures prevent hydrolysis of the formed transglycosylated products by EndoA and hence yields are improved. This approach was applied to preparation of C34 glycopeptide analogues

and afforded good yields (73-75%).[28] Its effectiveness was further demonstrated as replacement of native *N*-glycans ($Man_{5-9}GlcNAc_2$) presented on RNaseB to the artificially prepared non-native analogue in excellent yield (82-96%).[29] Notably, this conversion resulted in preparation of homogeneous glycoproteins.

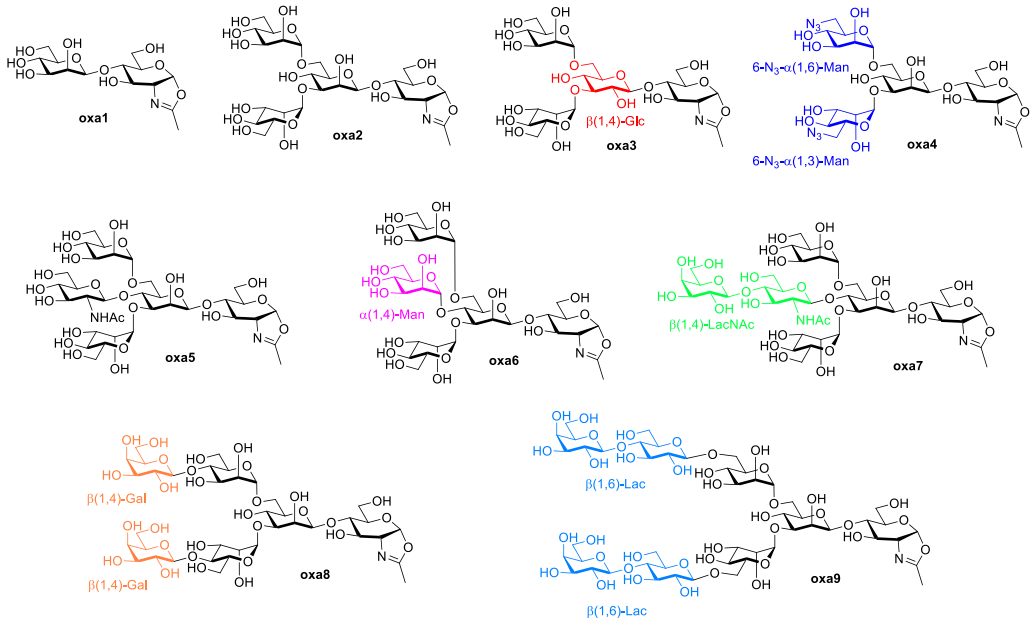
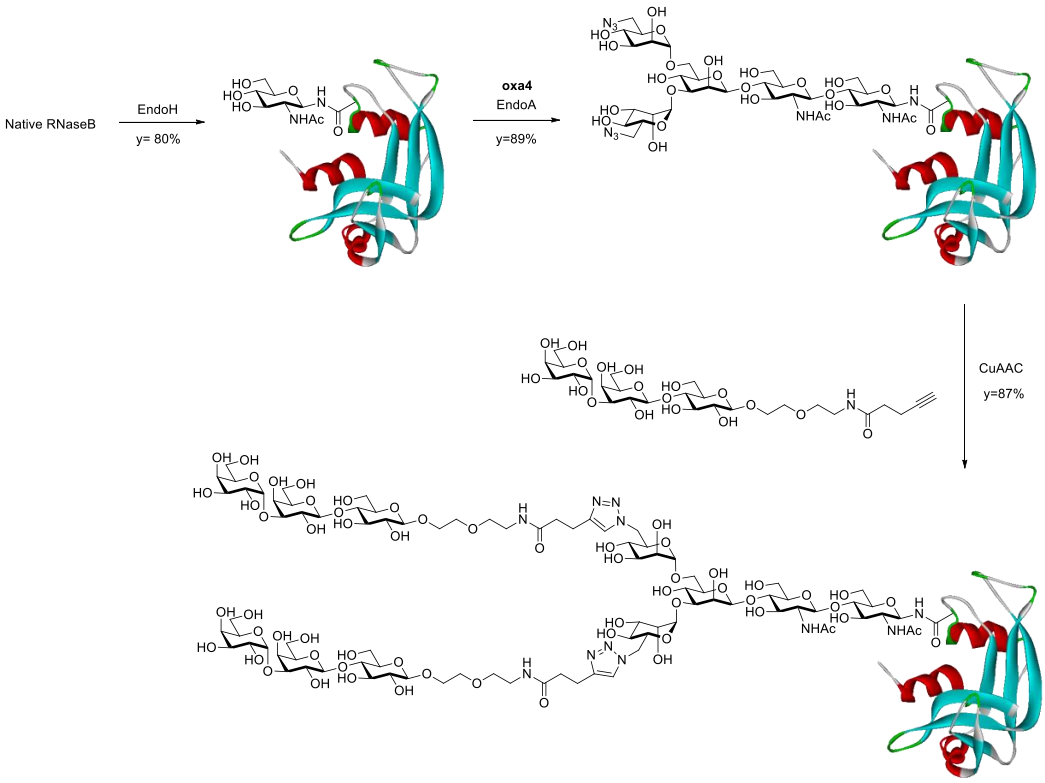


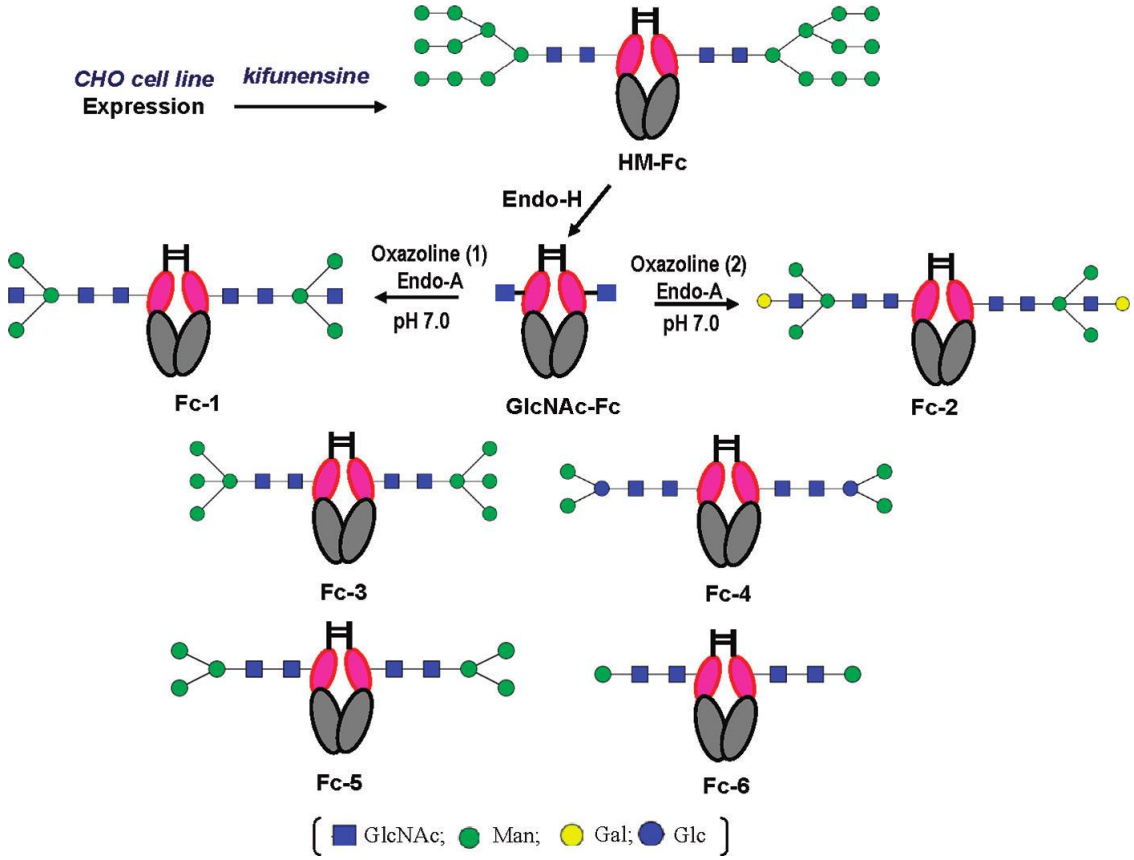
Figure 3.8. Examples of non-native glycan oxazolines used for glycoprotein modificatioin: **oxa1**,[6, 28, 59] **oxa2**,[5, 6, 28-30] **oxa3**,[6] **oxa4**,[30] **oxa5**,[6] **oxa6**,[6, 30] **oxa7**,[6] **oxa8**,[5, 29, 30] **oxa9**.[30]

The structural tolerance of EndoA for oxazoline glycosyl donors turned out to be broad (**Figure 3.8**) and the accepted scaffolds range from disaccharide (**oxa1**) [6, 28, 59] to octasaccharide (**oxa9**)[30] including glycans with bisecting sugars (**oxa5**, **6**, **7**).[6, 30] Surprisingly, the complex-type *N*-glycans were also introduced to the acceptor glycoprotein[60] although it is not the substrate for hydrolysis. Introduction of the glycan with azide groups (**oxa4**) [30] is a useful modulation of glycoproteins because this functional group allows bioorthogonal modification. Indeed, αGal epitopes were introduced onto RNaseB using copper catalyzed alkyne-azide coupling (**Scheme 3.2**). Application of EndoA-catalyzed *N*-glycan modification includes glycan engineering of IgG-Fc (**Scheme 3.3**).[5, 6] Fc expressed in *Pichia pastoris*[5] or CHO cells in the presence of

kifunesine[6] were first treated with EndoH to remove high mannose *N*-glycan, followed by introducing various types of glycans using EndoA. The resultant constructs were tested for binding with Fcγ receptors, FcγIIIa[5, 6] and/or FcγIIb,[6] and dissected to determine the effect of glycan structures on the affinity of Fc to these receptors.



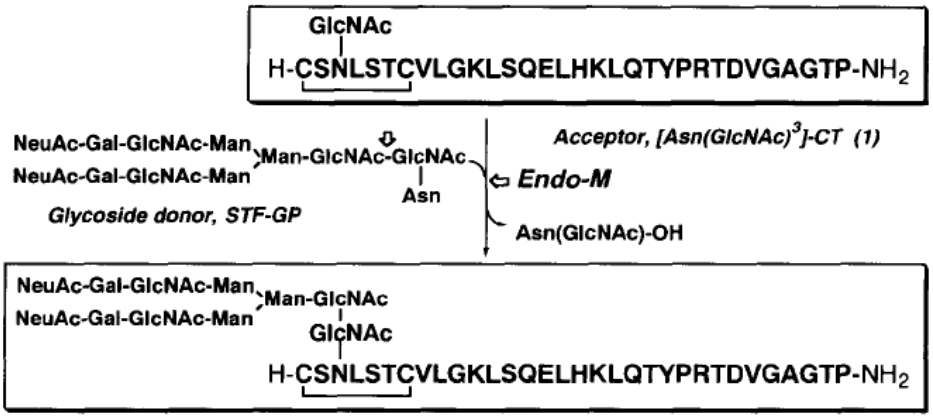
Scheme 3.2. Glycan modification on RNaseB.[30] Native glycans were removed by EndoH, followed by attaching the artificial *N*-glycans with azide. CuAAC was performed to introduce αGal epitopes.



Scheme 3.3. Glycan engineering on IgG-Fc using EndoH and EndoA with various types of non-native glycan oxazolines. The figure is adapted with permission from Zou, G. *et al.*, *J. Am. Chem. Soc.* **2011**, *133* (46), 18975-18991.[6] Copyright 2011 American Chemical Society.

EndoM is also applied as a synthetic tool of glycoconjugates. Transglycosylation by EndoM was first reported[45] as the reaction to transfer the complex *N*-glycan of transferrin to monosaccharide GlcNAc. Complex *N*-glycan is not the substrate for EndoA hydrolysis and EndoM has been mainly investigated to introduce such glycans as a reciprocal biocatalyst to EndoA. However, the scope of glycosyl donors for EndoM is not limited to the complex *N*-glycans and EndoM accepts the bisected hybrid *N*-glycan[46] as well as oligomannose *N*-glycan.[47] The synthesis of biologically relevant glycopeptides has been performed using EndoM. Such examples include synthesis of glycosylated peptide T[48] and calcitonin derivatives (**Scheme 3.4**).[47, 49] EndoM was also applied to preparation of several artificial materials like glycosylated cyclodextrin[61] and *N*-glycan-branched chitosan polymer.[62] In analogy to EndoA,

oxazoline donors with non-native scaffolds were applied to EndoM-catalyzed reactions[63] and excellent yields were obtained when di- or tri-saccharides were used (72-98%). It should be mentioned that a comparative study about the transglycosylation reactions by EndoA and EndoM has been performed.[39] Generally, EndoA gives better maximum conversions than EndoM in the course of the reactions though hydrolysis by EndoA becomes dominant earlier than in the case of EndoM.



Scheme 3.4. Preparation of Calcitonin derivative containing complex-type *N*-glycan using EndoM as catalyst. The yield of the reaction was 8.5%. The scheme is reprinted from Haneda, K. *et al.*, Chemo-enzymatic synthesis of calcitonin derivatives containing *N*-linked oligosaccharides, *Bioorg. Med. Chem. Lett.*, **1998**, *8* (11), 1303-1306.[47] Copyright 1998 with permission from Elsevier.

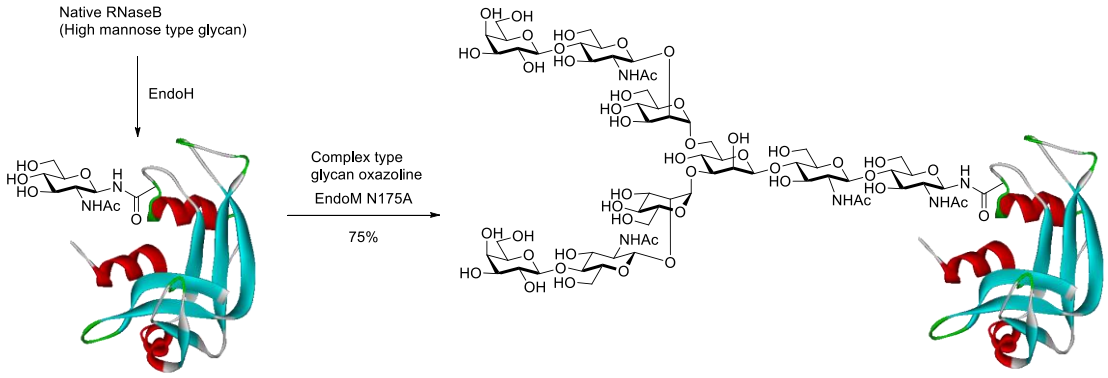
The wild type ENGases afford reasonable yields when oxazoline glycans with non-natural scaffolds are used as glycosyl donor. However, efficient synthesis of glycoproteins with natural glycan structures was still hampered. Application of exoglycosidases for glycoconjugate synthesis had faced the same problems until the breakthrough invention of glycosynthase.[64] Under similar context to that of exoglycosidases, investigation for ENGase mutants with higher transglycosylation efficiency was performed. At that time, X-ray structures of GH85 enzymes had not been resolved. Site-directed mutagenesis of EndoM was performed[65] based on precedent biochemical data.[66, 67] Through combination of bioinformatics as well as the preliminary X-ray data of EndoA, two mutants with the desired properties were identified among those

investigated.

EndoM Y217F was found as a mutant which has the enhanced transglycosylation activity, maintaining the moderate hydrolytic activity. The detailed kinetic data of transglycosylation reaction using the biantennary complex glycopeptide as glycosyl donor show this mutant has more than 10 times lower K_M value for the glycosyl acceptor substrate (4MU-GlcNAc) than the wild type, whereas the kinetic parameters of these two biocatalysts for the hydrolytic activity are in the similar range. These results imply the Y217F mutant has higher affinity to the acceptor molecule than the wild type. This tyrosine is the characteristic residue found in GH85 and is likely to interact with the oxazoline intermediate through π-stacking. The hydroxyl group of the tyrosine might have the role to recruit the incoming water molecule for hydrolysis[65] and hence, the loss of this functional group might reduce the hydrolytic activity as well as provide easier access of the acceptor GlcNAc to the acceptor site.

On the other hand, EndoM N175A mutant shows sparing hydrolytic acitivity and did not catalyze transglycosylation reactions when biantennary complex glycopeptides were used as glycosyl donors. However, the oxazoline derivative of the native high mannose *N*-glycan ($Man_9GlcNAc_2$-oxazoline) served as an excellent glycosyl donor and indeed high mannose type C34 glycopeptide was prepared in 72% yield by the EndoM N175A-catalyzed glycosylation reaction. N175 is the conserved amino acid in GH85 and is likely to play an important role for the formation of the oxazoline intermediate. Therefore, it is reasonable that the N175A mutant loses the hydrolytic acitivity significantly. This mutant corresponds to glycosynthase, the nucleophile-less mutant of the exoglycosidase, and the oxazoline donor corresponds to the glycosyl fluoride used in the glycosynthase reaction. This mutant was further applied to preparation of CD52 glycopeptide[68] as well as replacement of native heterogeneous high mannose type *N*-glycans on RNaseB to homogeneous biantennary complex type *N*-glycan

(**Scheme3.5**).[60, 69] Later, saturation mutagenesis was performed[70] at this position and the N175Q mutant was found to have higher transglycosylation efficiency. It is speculated that the amide group of the N175Q mutant liaises with the aicd/base catalyst E177 to facilitate the nucleophilic attack of the incoming accetptor to the oxazoline intermediate, while the N175A mutant lacks such an interaction.

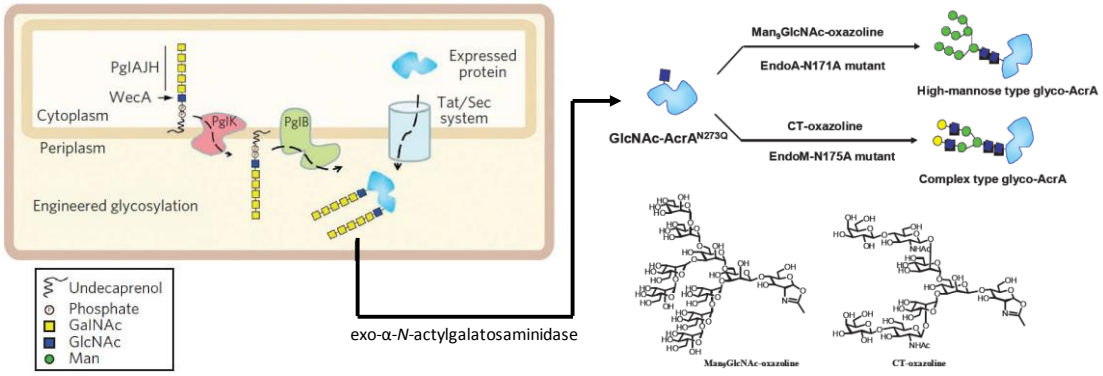


Scheme 3.5. Replacement of *N*-glycan on RNaseB by EndoM N175A from native heterogeneous high mannose type to the homogeneous biantennary complex type.[69]

The same strategy was applied to EndoA and EndoA N171A was developed as endoglycosynthase.[69] This biocatalyst has equivalent kinetic profiles as EndoM N175A in the glycosylation reaction using $Man_9GlcNAc_2$ oxazoline as glycosyl donor, though EndoA N171A has higher thermal stability than EndoM N175A.

Utility of these biocatalysts (EndoA N171A and EndoM N175A) was spectacularly demonstrated by preparation of the homogeneous glycoproteins from *Escherichia coli* as protein expression host (**Scheme 3.7**).[71] In this example, the unusual bacterial glycosylation machinery of *Campylobacter jejuni* was transplanted to *E. coli*, which innately does not have any protein glycosylation machinery as most of other bacteria. This genetically engineered *E. coli* was able to produce the *C. jejuni*-type glycoproteins, which were then treaed with exo-α-*N*-actyl-galatosaminidase to give GlcNAc-glycoproteins. GlcNAc-glycoproteins were then reacted with

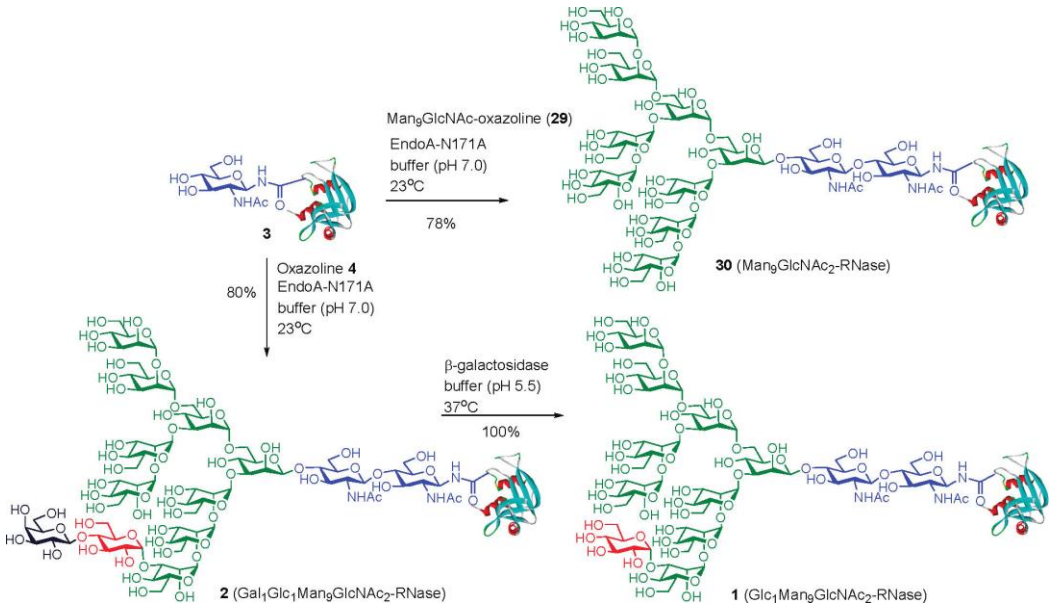
oxazoline glycosyl donors in the presence of the biocatalyst EndoA N171A or EndoM N175A to afford glycoproteins with the high mannose glycan ($Man_9GlcNAc_2$) or asialo-biantennary complex glycan, respectively. Apparently, the efficiency of glycosylation differs from one protein to another, but this example demonstrated the proof-of-concept that eukaryotic glycoprotein can be produced in the most representative bacterial host for protein expression.



Scheme 3.7. Applicatioin of EndoA N171A and EndoM N175A for preparation of the homogeneous glycoprotein from *E. coli* protein expression. The figure is adapted with permission from Schwarz, F. *et al.*, *Nat. Chem. Biol.*, **2010**, *6* (4), 264-266.[71] Copyright 2010 Nature Publishing Group.

EndoA N171A was also applied to preparation of the glycoprotein with monoglucosylated high mannose type *N*-glycan (**Scheme3.8**).[72] It is known that glucose at the non-reducing terminus of D1-arm in the high mannose glycan is essential for recognition by calnexin (CNX)/calreticulin (CRT) system for proper folding of glycoproteins. Preparation of homogeneous glycoproteins with such a glycoform provides more insight about the the system of the quality control for glycoprotein production. Indeed, it was discovered that the binding of the glucosylated high mannose to CRT is tolerant to modification of this glucose moiety with β(1,4)-galatose.

Mutants of the acid/base catalyst are also developed for EndoA (E173Q, E173H)[73] and they show enhanced glycosylation efficiency when tri- or tetrasaccharide oxazolines were used as glycosyl donor.



Scheme 3.8. Application of EndoA N171A to prepare the glycoprotein with monoglucosylated high mannose type N-glycan. The figure was adapted with permission from Amin, G. N. *et al.*, *J. Am. Chem. Soc.* **2011**, *133* (46), 14404-14417.[72] Copyright 2011 American Chemical Society.

As biological research progresses and as society demands safe and effective pharmaceuticals, development of synthetic methods for homogeneous biomolecules with precise quality control is more and more required. Since their discovery, ENGases have been welcome to the field of synthesis of glycoproteins and their unique catalytic features have been investigated. This decade especially saw a huge leap in application of ENGases; preparation of large glycoproteins by ENGase catalyzed reactions, invention of a method to use oxazoline donors, protein engineering by mutagenesis. However, in spite of such huge advances, there are still glycoproteins which are not accessible by the currently used ENGase-catalyzed reactions and hence further investigation of this class of enzymes is required.

3.1.2.2 EndoS

EndoS, an ENGase natively secreted from human pathogen *Streptococcus pyogenes*, was first discovered[13, 14] in the course of investigation to dissect its pathogenicity, accompanied by several other virulence factors like protein H[74] and SpeB.[13, 14] The former has high affinity to human IgG-Fc and the latter is a cysteine protease which shows a hydrotytic activity, for example, toward IgG hinge region. EndoS is also likely to be involved in the bacterial pathogenicity by removing *N*-glycans on IgG to abrogate its effector functions.[13, 14]

EndoS specifically cleaves *N*-glycans on Fc of native IgG[13, 14] including all four human IgG subclasses,[2] whereas it does not show any hydrolytic activities to IgA[14], IgM[14] or even denatured IgG.[13] Moreover, no other glycoprotein has been reported as a substrate of EndoS.[75] Therefore, it is likely that EndoS recognizes not only *N*-glycan structure, but also the protein scaffold of IgG.[13]

The analysis of amino acid sequence and homology modeling of EndoS have revealed that EndoS is composed of three fragments;[76] the signal sequence (amino acids 1-37), the N-terminal domain containing GH18 glycosidase motif (amino acid 37-446) and the C-terminal domain containing leucine-rich repeats (amino acid 446-995). Between the N-terminal domain and the C-terminal domain is observed the amino acid sequence recognized by SpeB, which is a cysteine protease expressed coordinately with EndoS from *S. pyogenes*[76] and is likely to degrade EndoS to modulate the bacterial virulence. Though the catalytic site of EndoS resides in the N-terminal domain, the C-terminal domain is requisite for the hydrolytic acitivty of EndoS.[76] It loses the hydrolytic activity towards IgG *N*-glycan once it is treated with SpeB. The detailed function of the C-terminal domain is unknown, but it is likely that this part is important for binding to IgG.[76]

EndoS is a useful tool to study the effect of *N*-glycan on IgG towards its binding to the Fc-receptors.[2] For example, IgG_1, IgG_3 and IgG_4 lose affinity to FcγRIIa when treated with EndoS, while IgG_2 do not alter its binding ability. Similarly, loss of *N*-glycan on Fc attenuates the binding of IgG_1, IgG_3 and IgG_4 to FcγRIIb whereas IgG_2 binds more strongly when its *N*-glycan was trimmed with EndoS.

Such drastic effects of EndoS on the binding of IgG to FcγRs prompted application of this unique enzyme to the treatment of autoimmune diseases.[77] For example, EndoS was administered to BXSB mice,[78] which develop autoantibodies and show severe glomerulonephritis similar to human systemic lupus erythematosus. In spite of the continuous development of autoantibodies, the treated mice enjoyed reduced severity of symptoms and prolonged life.[77] This behavior is similar to that of BXSB-$\gamma^{-/-}$ mice,[79] which do not have any functional FcγRs. This result implies that EndoS diminished the interaction between autoantibody IgG and FcγR by removing *N*-glycan on IgG and alleviated the fatal pathological conditions. Notably, though injection of EndoS induced the low concentration of IgM, anti-EndoS IgG was not observed, which implies the low risk of severe immunogenic response.

3.1.3. Aim of the research

As has been demonstrated in several examples, ENGases are a powerful tool to prepare homogeneous biomolecules. Among them, EndoA and EndoM are most intensively investigated and have provided magnificent achievements. However, these representative ENGases do not provide unlimited usage to construct all of the natural or artificially designed glycoproteins. For example, these ENGases are not compatible with core fucose[37] (**Table 3.2**) and their application to such a glycoform has not yet been reported. Therefore, other ENGases should be investigated and developed as novel biocatalysts to complement synthetic methodology for glycoproteins or their analogues.

Immunoglobulin G is one of the most important targets among glycoproteins due to its biochemical and phamaceutical application. Though *N*-glycan modification of its truncated part, Fc, had been reported,[5] the unique and valuable character of IgG, that is the specific targeting, comes from Fab region and hence the *N*-glycan engineering of the full-length IgG was required. From this viewpoint, EndoS, which shows the hydrolytic activity specifically to IgG *N*-glycan, was expected to have a potential to be developed as a novel biocatalyst to modify the *N*-glycan of the full-length IgG. Additionally, the artificially prepared IgG using this biocatalytic method is expected to have homogeneous glycoform, which is in increasing demand for biomolecular drugs in terms of the quality control. To investigate the feasibility of this hypothesis, the campaign to apply EndoS for *N*-glycan engineering on IgG was set off.

3.2. Result and Discussion

3.2.1. Expression and purification of EndoS

The *ndoS* gene encoding EndoS was obtained from GenBank under accession No. AF296340. The DNA sequence was optimized for expression in *Escherichia coli* (termed as *ndoS(opt)*, **Table 3.3**) and synthesized by GenScript.[80] The gene was subcloned into an expression vector pGEX-4T-1 using *BamHI* and *NotI* restriction endonucleases. The resultant plasmid vector was termed as pGEX-4T-1-*ndoS(opt)*, which confers ampicillin resistance to the host cell and tags to the expresse protein glutathione-S-transferase (GST) at the N-terminus with a thrombin cleavage site inbetween. The translated protein sequence is shown in **Table 3.4**.

Table 3.4. The amino acid sequence of the protein expressed from pGEX-4T-1-*ndoS(opt)*. The sequence in bold letters is the thrombin cleavage site and the one highlighted in yellow (GST) is cleaved:

```
-226  MSPILGYWKI KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL EFPNLPYYID
-166  GDVKLTQSMA IIRYIADKHN MLGGCPKERA EISMLEGAVL DIRYGVSRIA YSKDFETLKV
-106  DFLSKLPEML KMFEDRLCHK TYLNGDHVTH PDFMLYDALD VVLYMDPMCL DAFPKLVCFK
 -46  KRIEAIPQID KYLKSSKYIA WPLQGWQATF GGGDHPPKSD LVPRGS
   1  MDKHLLVKRT LGCVCAATLM GAALATHHDS LNTVKAEEKT VQVQKGLPSI DSLHYLSENS
  61  KKEFKEELSK AGQESQKVKE ILAKAQQADK QAQELAKMKI PEKIPMKPLH GPLYGGYFRT
 121  WHDKTSDPTE KDKVNSMGEL PKEVDLAFIF HDWTKDYSLF WKELATKHVP KLNKQGTRVI
 181  RTIPWRFLAG GDNSGIAEDT SKYPNTPEGN KALAKAIVDE YVYKYNLDGL DVDVEHDSIP
 241  KVDKKEDTAG VERSIQVFEE IGKLIGPKGV DKSRLFIMDS TYMADKNPLI ERGAPYINLL
 301  LVQVYGSQGE KGGWEPVSNR PEKTMEERWQ GYSKYIRPEQ YMIGFSFYEE NAQEGNLWYD
 361  INSRKDEDKA NGINTDITGT RAERYARWQP KTGGVKGGIF SYAIDRDGVA HQPKKYAKQK
 421  EFKDATDNIF HSDYSVSKAL KTVMLKDKSY DLIDEKDFPD KALREAVMAQ VGTRKGDLER
 481  FNGTLRLDNP AIQSLEGLNK FKKLAQLDLI GLSRITKLDR SVLPANMKPG KDTLETVLET
 541  YKKDNKEEPA TIPPVSLKVS GLTGLKELDL SGFDRETLAG LDAATLTSLE KVDISGNKLD
 601  LAPGTENRQI FDTMLSTISN HVGSNEQTVK FDKQKPTGHY PDTYGKTSLR LPVANEKVDL
 661  QSQLLFGTVT NQGTLINSEA DYKAYQNHKI AGRSFVDSNY HYNNFKVSYE NYTVKVTDST
 721  LGTTTDKTLA TDKEETYKVD FFSPADKTKA VHTAKVIVGD EKTMMVNLAE GATVIGGSAD
 781  PVNARKVFDG QLGSETDNIS LGWDSKQSII FKLKEDGLIK HWRFFNDSAR NPETTNKPIQ
 841  EASLQIFNIK DYNLDNLLEN PNKFDDEKYW ITVDTYSAQG ERATAFSNTL NNITSKYWRV
 901  VFDTKGDRYS SPVVPELQIL GYPLPNADTI MKTVTTAKEL SQQKDKFSQK MLDELKIKEM
 961  ALETSLNSKI FDVTAINANA GVLKDCIEKR QLLKK
```

Table 3.3. The DNA sequence (*ndoS(opt)*) of EndoS optimized for expression in *Escherichia coli* (3002 nucleotides). The sequences highlighted in yellow are restriction sites (*BamHI* and *NotI*):

```
  -6 GGATCC
   1 ATGGACAAAC ATCTGCTGGT TAAACGCACG CTGGGCTGTG TGTGTGCTGC TACGCTGATG
  61 GGCGCTGCTC TGGCGACGCA CCACGATAGC CTGAACACCG TGAAAGCCGA AGAAAAAACG
 121 GTTCAGGTCC AAAAAGGTCT GCCGTCAATC GATTCGCTGC ATTATCTGAG CGAAAACTCT
 181 AAAAAAGAAT TCAAAGAAGA ACTGAGCAAA GCGGGCCAGG AATCTCAAAA AGTTAAAGAA
 241 ATCCTGGCAA AAGCTCAGCA AGCCGATAAA CAGGCACAAG AACTGGCTAA AATGAAAATT
 301 CCGGAAAAAA TCCCGATGAA ACCGCTGCAT GGTCCGCTGT ACGGCGGTTA TTTCCGTACC
 361 TGGCACGATA AAACGTCAGA CCCGACCGAA AAAGACAAAG TCAACTCGAT GGGCGAACTG
 421 CCGAAAGAAG TGGATCTGGC TTTTATTTTC CATGATTGGA CCAAAGACTA CTCTCTGTTT
 481 TGGAAAGAAC TGGCAACGAA ACACGTTCCG AAACTGAACA AACAGGGTAC GCGTGTCATT
 541 CGTACCATTC CGTGGCGCTT CCTGGCTGGC GGTGATAATT CAGGCATCGC GGAAGACACC
 601 TCGAAATATC CGAACACGCC GGAAGGTAAT AAAGCGCTGG CCAAAGCAAT CGTCGATGAA
 661 TACGTGTACA AATACAATCT GGACGGCCTG GATGTGGACG TTGAACATGA TTCAATTCCG
 721 AAAGTGGATA AAAAAGAAGA CACCGCCGGC GTGGAACGTT CGATCCAGGT TTTTGAAGAA
 781 ATTGGTAAAC TGATCGGCCC GAAAGGTGTT GATAAAAGCC GTCTGTTCAT CATGGATTCT
 841 ACCTATATGG CCGACAAAAA TCCGCTGATT GAACGCGGTG CACCGTACAT CAACCTGCTG
 901 CTGGTCCAGG TGTATGGCAG CCAAGGTGAA AAAGGCGGTT GGGAACCGGT GTCTAACCGT
 961 CCGGAAAAAA CCATGGAAGA ACGCTGGCAG GGCTACTCAA AATATATTCG TCCGGAACAA
1021 TACATGATCG GCTTTTCGTT CTATGAAGAA AACGCGCAGG AAGGTAATCT GTGGTACGAT
1081 ATTAATAGTC GCAAAGATGA AGACAAAGCC AACGGCATTA ATACCGATAT CACGGGTACC
1141 CGTGCGGAAC GCTATGCCCG TTGGCAGCCG AAAACCGGCG GTGTTAAAGG CGGTATTTTT
1201 AGCTACGCGA TCGATCGTGA CGGTGTCGCC CATCAGCCGA AAAAATACGC AAAACAAAAA
1261 GAATTCAAAG ATGCTACCGA CAACATCTTC CACAGCGATT ACAGTGTCTC CAAAGCGCTG
1321 AAAACCGTGA TGCTGAAAGA TAAATCTTAC GATCTGATCG ACGAAAAAGA TTTTCCGGAC
1381 AAAGCGCTGC GCGAAGCCGT TATGGCACAG GTCGGCACCC GCAAAGGTGA CCTGGAACGT
1441 TTTAATGGCA CGCTGCGCCT GGATAACCCG GCCATTCAGA GCCTGGAAGG TCTGAATAAA
1501 TTCAAAAAAC TGGCACAACT GGACCTGATT GGCCTGAGCC GTATCACCAA ACTGGATCGC
1561 TCTGTGCTGC CGGCCAACAT GAAACCGGGT AAAGACACGC TGGAAACCGT TCTGGAAACC
1621 TACAAAAAAG ATAACAAAGA AGAACCGGCA ACGATCCCGC CGGTGTCTCT GAAAGTTTCC
1681 GGCCTGACCG GTCTGAAAGA ACTGGATCTG AGCGGCTTTG ACCGTGAAAC GCTGGCAGGT
1741 CTGGATGCGG CCACGCTGAC CAGTCTGGAA AAAGTTGATA TTTCCGGCAA TAAACTGGAC
1801 CTGGCGCCGG GTACCGAAAA CCGCCAGATT TTTGATACGA TGCTGAGTAC CATCTCCAAC
1861 CATGTTGGCA GCAATGAACA GACCGTCAAA TTCGACAAAC AAAAACCGAC GGGCCACTAC
1921 CCGGATACGT ATGGTAAAAC CAGCCTGCGT CTGCCGGTCG CCAACGAAAA AGTGGATCTG
1981 CAGTCTCAAC TGCTGTTTGG CACGGTTACC AATCAGGGTA CCCTGATTAA CAGCGAAGCA
2041 GATTACAAAG CTTACCAAAA CCATAAAATC GCGGGTCGCT CATTTGTGGA TTCGAACTAC
2101 CACTACAACA ACTTCAAAGT TAGTTACGAA AACTACACCG TTAAAGTCAC GGATTCCACC
2161 CTGGGCACCA CGACCGATAA AACGCTGGCC ACCGACAAAG AAGAAACCTA CAAAGTCGAT
2221 TTCTTTAGCC CGGCAGACAA AACGAAAGCG GTGCATACCG CCAAAGTGAT TGTTGGCGAT
2281 GAAAAAACCA TGATGGTGAA CCTGGCTGAA GGTGCGACGG TTATCGGCGG TTCCGCAGAC
2341 CCGGTTAACG CTCGCAAAGT CTTTGATGGC CAGCTGGGTA GTGAAACCGA TAATATTTCC
2401 CTGGGTTGGG ACTCAAAACA GTCGATTATC TTCAAACTGA AAGAAGACGG CCTGATCAAA
2461 CACTGGCGTT TCTTTAACGA TAGTGCCCGC AATCCGGAAA CGACCAACAA ACCGATTCAG
2521 GAAGCATCCC TGCAAATCTT CAACATCAAA GATTACAACC TGGACAATCT GCTGGAAAAC
2581 CCGAATAAAT TCGATGACGA AAAATACTGG ATCACGGTGG ATACCTATAG CGCGCAGGGC
2641 GAACGTGCTA CGGCGTTTAG TAACACCCTG AACAATATTA CGTCCAAATA CTGGCGTGTG
2701 GTTTTCGATA CCAAAGGTGA CCGCTATAGC TCTCCGGTCG TGCCGGAACT GCAGATTCTG
2761 GGCTATCCGC TGCCGAATGC TGATACGATC ATGAAAACCG TGACGACCGC GAAAGAACTG
2821 TCACAGCAAA AAGATAAATT CTCGCAGAAA ATGCTGGACG AACTGAAAAT TAAAGAAATG
2881 GCTCTGGAAA CCAGCCTGAA TTCTAAAATT TTCGATGTTA CGGCGATCAA TGCTAACGCT
2941 GGTGTGCTGA AAGACTGTAT TGAAAAACGC CAACTGCTGA AAAAATGAGC GGCCGC
```

BL21(DE3) strain of *E.coli* was transformed with pGEX-4T-1-*ndoS(opt)*. The expression of GST-EndoS was performed by autoinduction. After 22 hr of incubation at 28°C, the cells were harvested by centrifugation and the pellet was lysed with lysozyme, followed by sonication. The

target protein (calculated molecular weight of GST-EndoS: 138 kDa) was obtained in the supernatant fraction (**Figure 3.9**). Another strong signal was observed just below the target protein, which is likely to be EndoS whose GST-tag was cleaved. Presumably, the cleavage of the GST-tag took place during the culture by the inherent proteases in the host cell. This surmise might be supported by the expression of D233A mutant (data not shown) where the shorter incubation time (16 hours) resulted in reduction of the intensity of this band.

The supernatant was purified with GST affinity column. The capacity of two 5 mL GSTrap columns (GE healthcare) was overflowed by the GST-tagged proteins from the 1.5-L culture due to high efficiency of expression and the large amount of GST-EndoS was eluted out in the flowthrough fractions (**Figure 3.10**, lane X1, X2). Repetitive injections of these fractions improved recovery of the target protein. Purified GST-EndoS was accompanied by the cleaved GST tag which formed during the culture and complete purification was difficult. However, the purity will be improved if the culture condition is optimized and the cleavage of GST tag

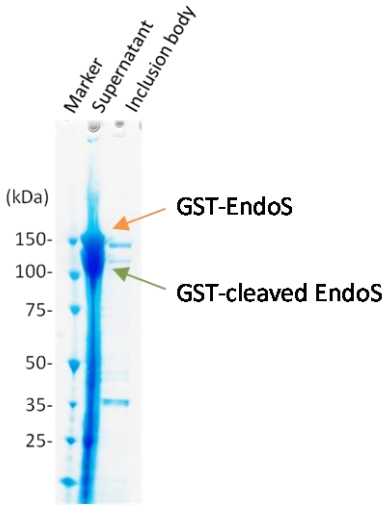


Figure 3.9. Expression of GST-EndoS. GST-EndoS was obtained in the supernatant (orange arrow). Partial cleavage of GST-tag (green arrow) was observed.

is suppressed. The total proteins purified at this stage were 50.0 mg/ L culture and its hydrolytic activity to IgG was equivalent to that of the intensively purified EndoS. Because the cost of thrombin is the bottle neck for the further purification, it is recommended to use this construct for large scale applications. It should be also noted that GST-EndoS thus obtained can be stored at -20°C without significant loss of the activity for months.

To obtain the tag-less construct, GST-EndoS was treated with thrombin (**Figure 3.11**) to afford the band in the expected molecular weight region in SDS-PAGE (calculated molecular weight of the tag-free EndoS: 112 kDa). It should be noted that the cleavage of GST-tag took place even without addition of thrombin (see the central lane, **Figure 3.11**). This result indicates small amount of proteases were still contaminated in the protein solution even after purification by the GST affinity column. The lability of the GST tag corresponds to the observation that its cleavage occurred during the culture. Indeed, when this protein solution was left at 4°C for days,

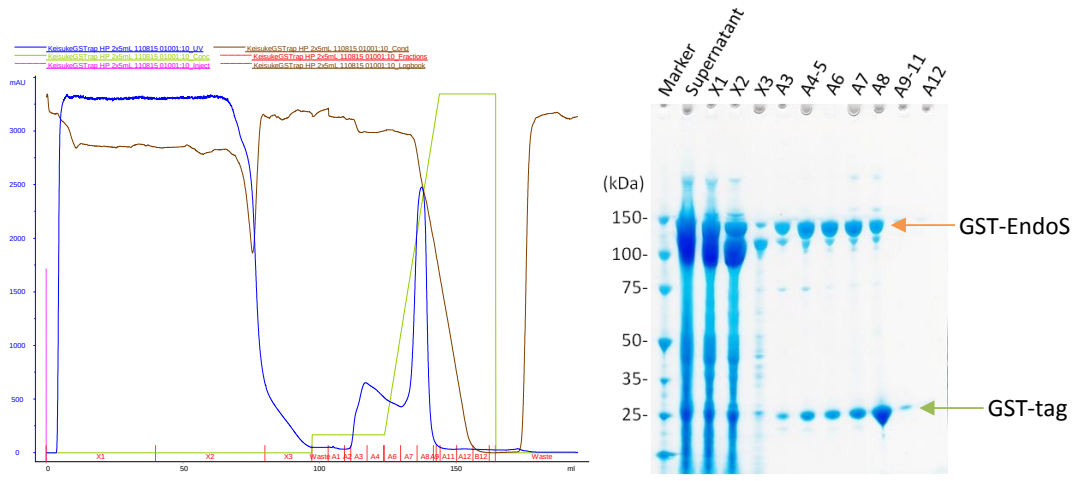


Figure 3.10. Purification of GST-EndoS by GSTrap. GST-EndoS (fraction A3-A8) was successfully purified though GST-tag was partially contaminated. Unbound GST-EndoS (fraction X1-X2) was subjected to purification again to improve recovery.

GST was completely cleaved. Moreover, degradation of EndoS was also observed after prolonged storage at this temperature. This outcome confirmed the lability of EndoS itself to proteases[76] and suggested the necessity to move on to the further purification quickly.

The solution of the thrombin reaction was applied to GSTrap to separate the GST tag from the tag-free EndoS, which was collected in the flowthrough fractions (**Figure 3.12**, A1-A4).

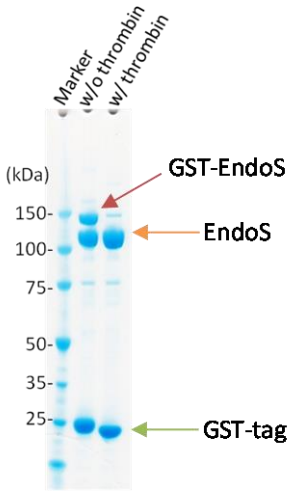


Figure 3.11. Removal of GST with thrombin. The reaction completed in 16 hr at room temperature though cleavage of GST was also found in the control reaction without thrombin.

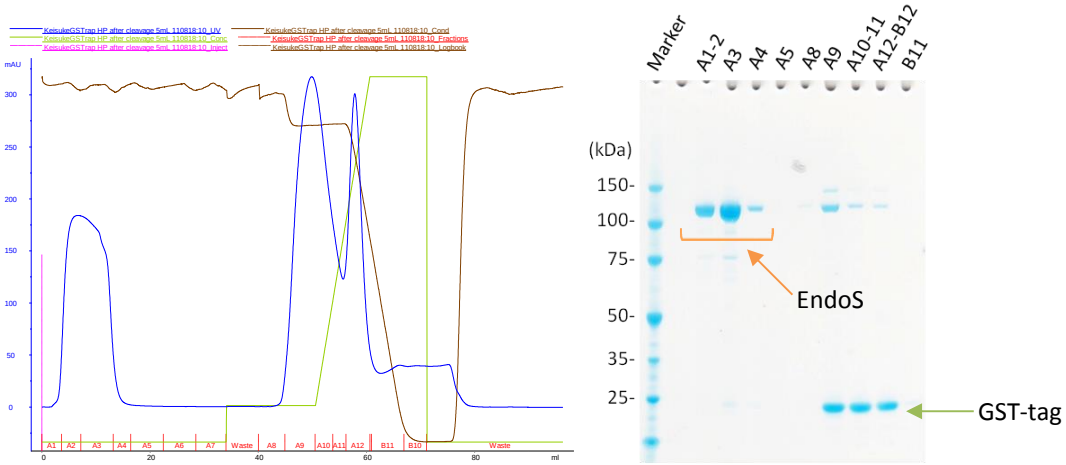


Figure 3.12. Separation of EndoS from cleaved GST tag using GSTrap. Fractions A1-A4 were collected.

After dialysis, the solution was applied to the benzamidine affinity column to remove thrombin (**Figure 3.13**). Further polishment was performed by gel filteration (**Figure 3.14**) to afford highly pure EndoS. After these processes, the yield of EndoS was 9.1 mg/ L culture.

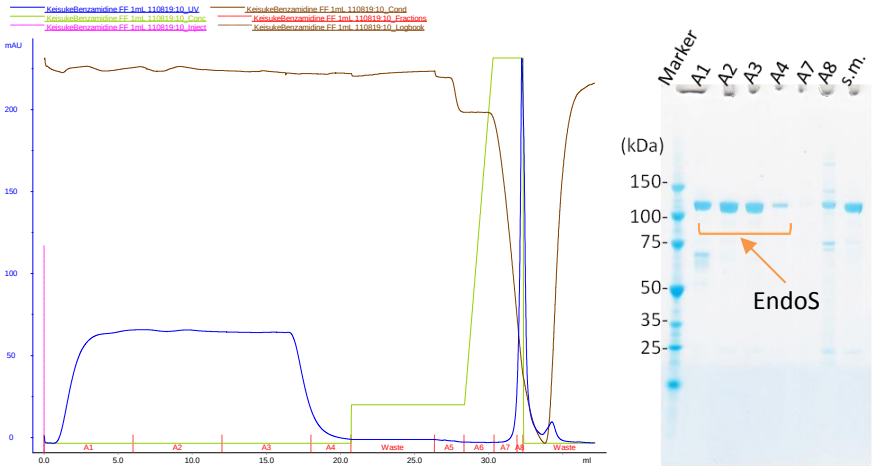


Figure 3.13. Removal of thrombin from the EndoS solution. Fractions A1-A3 were collected.

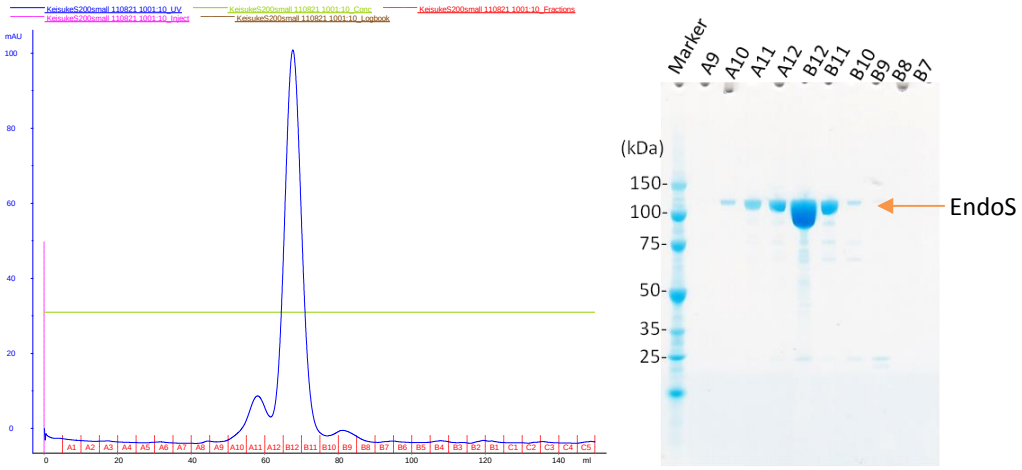


Figure 3.14. Purification of EndoS by gel filtration. Fractions A12-B11 were collected.

The stability test of EndoS after intensive purification was performed (**Figure 3.15**). The solution was left at 4°C and 20°C. It turned out that EndoS is stable at 4°C for 2 weeks, whereas gradual degradation was observed at 20°C even after this intensive purification. This result is useful for selection of conditions for crystallization of this enzyme. It should be also noted that EndoS thus obtained can be stored at -20°C without significant loss of the activity for months.

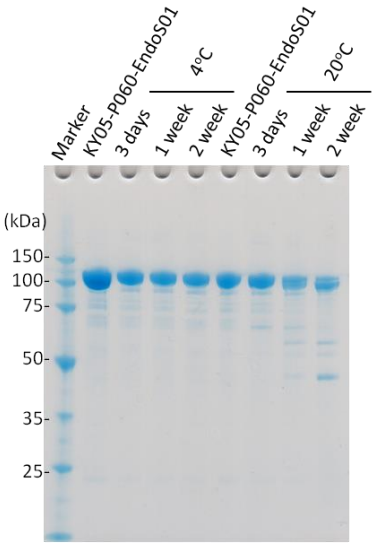


Figure 3.15. Stability test of EndoS. EndoS was stable at 4°C for 2 weeks but degradation was observed at 20°C in 2 weeks.

3.2.2. Cleavage of glycans from IgG with EndoS

To check the activity of EndoS to cleave glycans, 1.0 mg/mL human IgG (Sigma-Aldrich, Part Number: I4506) was treated with a range of concentrations (0.1-10 μg/mL) of EndoS at 20°C and 37°C for 4 hours (**Figure 3.16**). Temperature gave no significant difference, but the reaction proceeded in a dose-dependent manner. When the concentration of EndoS was 10 μg/mL (a mass ratio of IgG: EndoS= 100:1), the band shift corresponding to the molecular weight of *N*-glycan (1.5-2.0 kDa) was observed. At this enzyme concentration, the reaction seemed to complete within one hour and there was no further shift by the longer incubation.

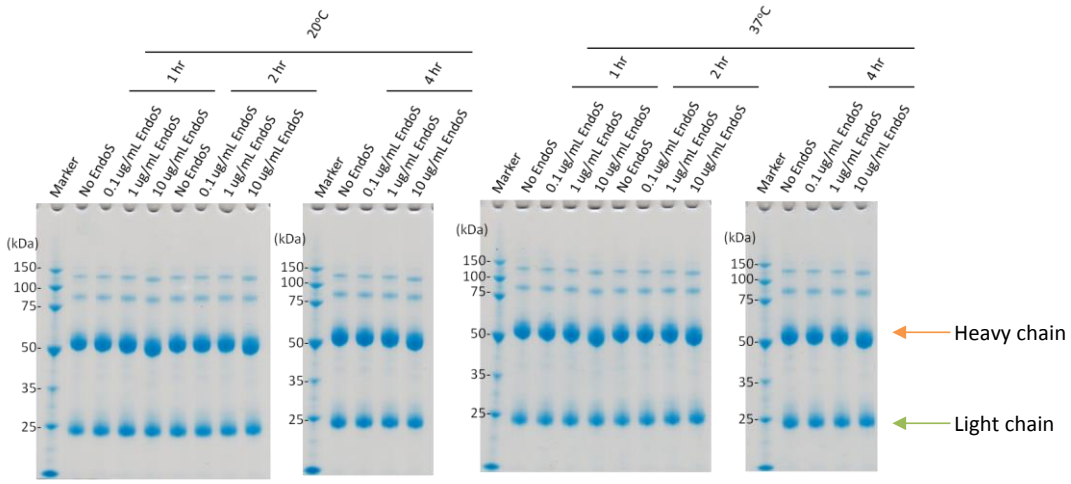


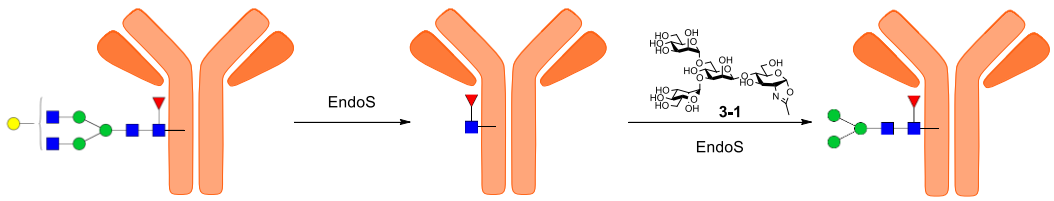
Figure 3.16. Glycan cleavage activity of EndoS. 1.0 mg/mL hIgG was incubated at 20°C or 37°C using various concentrations (0.1-10 μg/mL) of EndoS in PBS. The band shift of the heavy chain of IgG (~50kDa) was observed in a dose-dependent manner. The reaction completed in 1 hourr when EndoS was used in 1:100 mass ratio to IgG at both 20°C and 37°C.

3.2.3. Remodelling of glycans on IgG with EndoS

Next, the remodeling of *N*-glycan was performed using EndoS and tetrasaccharide oxazoline (**3-1**) [42] (**Scheme 3.9**). IgG was first treated in the near identical conditioin to the one set up above (IgG: EndoS= 1.0 mg/mL: 0.01 mg/mL in PBS, room temperature) to remove *N*-glycans. To make sure the cleaved *N*-glycans do not interfere the following glycosylation step, this hydrolytic reaction was performed on the dialysis membrane (MWCO 7,000) floating on the reservoir of the same buffer. After the hydrolytic reaction, the solution was taken up to a reaction tube. For glycosylation reaction, another aliquot of EndoS and tetrasaccharide oxazoline (500 eq.) were added. This transglycosylation reaction was performed at 4°C and the part of the reaction was taken up at 1, 2 and 4 hours for the analysis.

Gratifyingly, this remodeling reaction worked efficiently and the band shift of the heavy chain was observed on the SDS-PAGE (**Figure 3.17**). The reaction was further analyzed by MALDI-TOF-MS after the glycan cleavage by PNGaseF. The major population of the *N*-glycans on native IgG was $(Gal\text{-}GlcNAc)_2Man_3$- GlcNAc(Fuc)GlcNAc with 0-2 galactoses at the non-reducing termini (**Figure 3.18**), accompanied by the small population of these with bisected GlcNAc. Gratifyingly, the peak corresponding to the glycans attached by the tetrasaccharide (m/z= 1079) appeared in the MS spectrum after the remodeling reaction (**Figure 3.18**). For the semi-quantification purpose, a series of the GlcNAc-bisected glycans can be used

Scheme 3.9. Diagram of glycan remodeling on hIgG with EndoS



as internal standards, because it is reported that these glycans are not hydrolyzed by EndoS.[42] The newly formed peak grew up to 4 hours compared to the internal standard (bisected glycans). It should be noted that other peaks (m/z= 1136, 1298 and 1460) were also observed, which correspond to the glycans released from the native IgG. Apparently, these glycans remained in the MS samples even though the reaction solutions were dialyzed during the process for PNGaseF digestion.

Together, the *N*-glycan remodeling on native IgG was demonstrated for the first time.[42] It should be also mentioned that this is the first example of a GH18 enzyme which is able to modify glycoproteins. Previously, this class of enzymes were believed not to be able to process remodeling reactions[65] but the example shown here proved that it was not the case. Only recently other GH18 enzymes ($EndoF_2$ and $EndoF_3$) were reported for their remodelling activities,[37] but their application is yet limited to short peptides. Additionally, EndoS shows discrete activity from EndoA and EndoM in that it can process core fucosylated *N*-glycans.

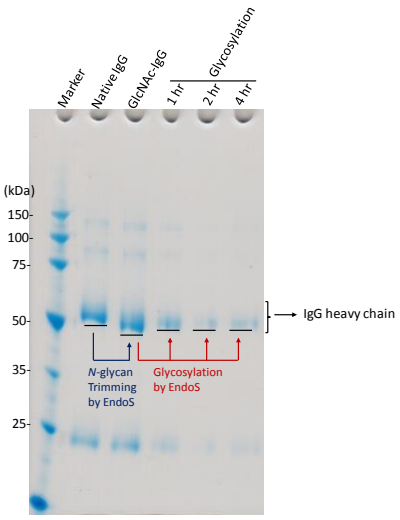


Figure 3.17. SDS-PAGE analsysis of the *N*-glycan remodeling reactions. After cleavage of *N*-glcayn, the glycan-trimmed IgG was reacted with oxazoline tetrasaccharide using EndoS. The band shift of the IgG-heavy chain was observed after the remodeling reaction.

There has been some examples of the remodeling of *N*-glycan on Fc (truncated part of IgG) by EndoA[5, 6] and they provided fundamental information about the influence of the *N*-glycan structures on the binindg of IgG-Fc to the FcγRs. Recently, EndoD was found to be able to remodel Fc with core fucosylated glycoforms[34] to supplement the activity of EndoA or EndoM.

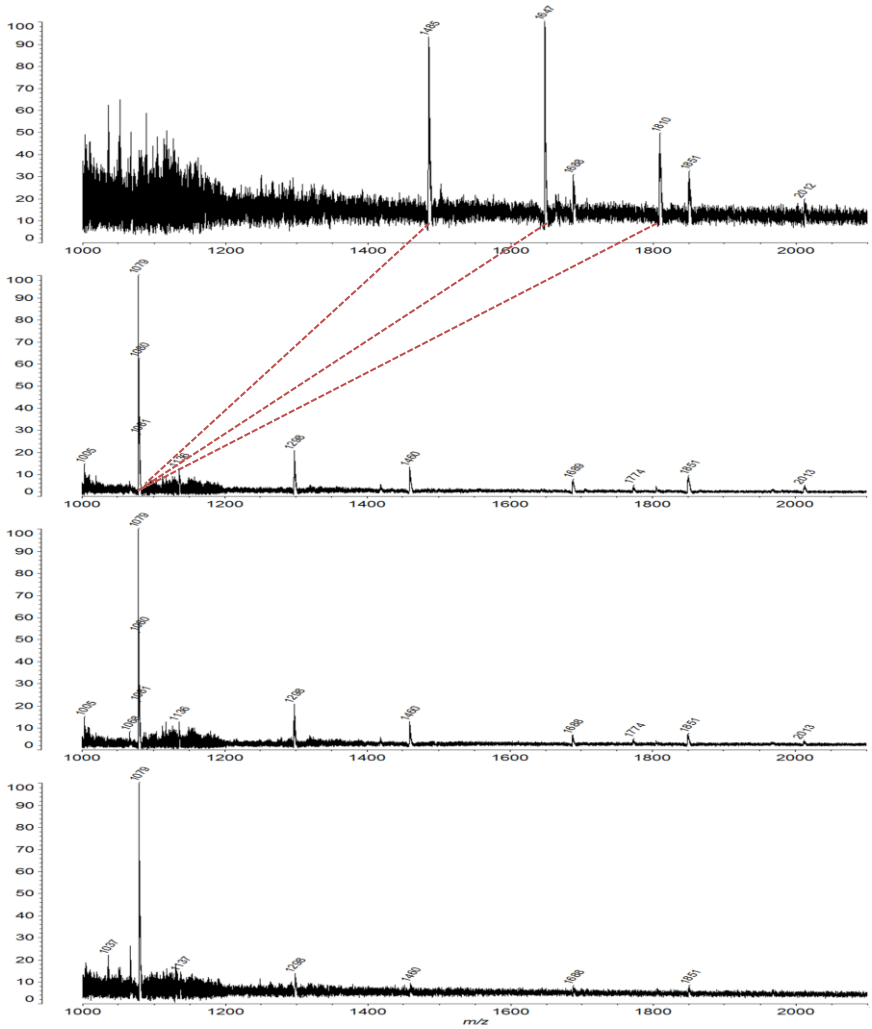


Figure 3.18. MALDI-TOF-MS analysis of the *N*-glycan remodelling reaction. The glycans on the reacted IgG were analyzed after PNGaseF digestion. The top panel shows the glycan profile of native IgG and the following panels are those of IgG after 1, 2 and 4 hourrs of the remodelling reaction.

In terms of clinical application, however, Fab is essential for mAb to exert its targeting property, which is the key for the selectivity and the reason why this glycoprotein is being featured for developing new therapeutics. Therefore, the enzymatic modulation of the full-length IgG by EndoS is the exquisite result. So far, the production of mAbs with the designed glycoforms is dependent on the bioengineering methods,[3, 4] but the reaction demonstrated by EndoS here provides a substantial opportunity to produce clinically applicable mAb-based drugs with the desired glycoforms in the precisely controlled manner.

3.2.4. Design of glycans for antibody-drug conjugates

Now with the IgG modification method in hand, the application of this *N*-glycan remodeling reaction as a novel method to prepare antibody-drug conjugates was investigated. Conventionally, preparation of ADC is performed using the reactivity of lysine or cysteine side chains as described earlier (Chapter 3.1.1). Lysines are normally presented at multiple sites on the protein surface and it is difficult to control regioselectivity of the conjugation reactions precisely (**Figure 3.19**). Cysteines are less abundant and, hence, it should give higher selectivity. However, cysteine is not normally presented on the surface of IgG, and therefore cysteine conjugation should be performed after reduction of disulfile bonds which cross-link between the light chain and the heavy chain or between the two heavy chains. This procedure results in the generation of multiple thiol groups.[81] Moreover, reduction of disulfide bonds attenuates thermal stability of IgG molecules and it might result in shorter lifetime of IgG during the circulation in the body.[82]

Our method aims at installing payloads on *N*-glycans. IgG has only two *N*-glycosylation sites in its whole structure and the efficiency of the enzymatic reaction is high. Therefore the

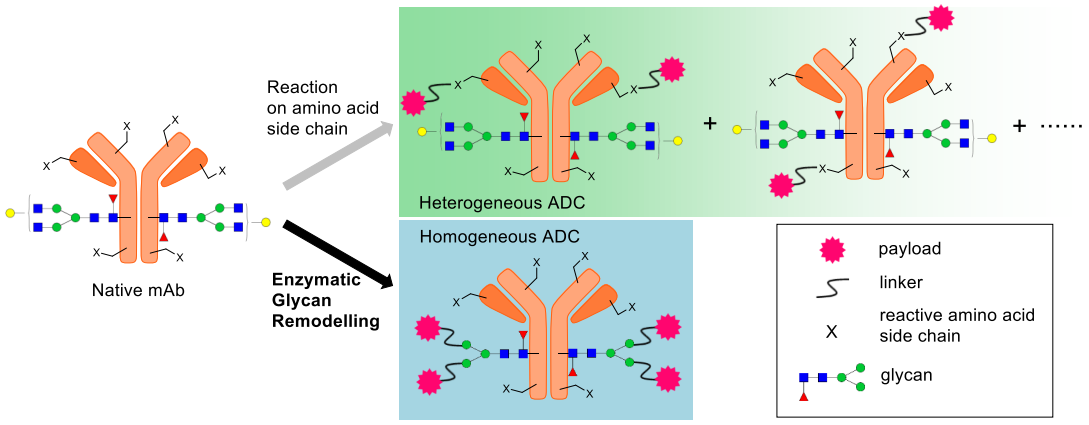


Figure 3.19. Conjugation on IgG with a) reactive amino acid side chaines and b) glycan remodeling.

modification of *N*-glycans by EndoS offers significant chances to realize homogeneity of products. The less number of the modification sites might result in the less number of the payloads on IgG. However, even in the lysine conjugates, the modification sites are controlled around 3-4[83] for the best drug profile under the balance between the efficacy and the stability. Therefore, two payloads on each *N*-glycan site are expected to be enough to satisfy sufficient efficacy.

The payload can be first incorporated in the glycosyl donor, which is then linked with the IgG backbone (**Figure 3.20A**). Alternatively, the glycosyl donor with bioorthogonal reaction handles can be first installed to IgG, followed by conjugating with various drug molecules (**Figure 3.20B**). The latter method requires less steric bulk in the glycosyl donor, which might affect the substrate scope of EndoS. Moreover, it offers chances to test various payloads. Therefore, the method B was taken as the first choice of the trial.

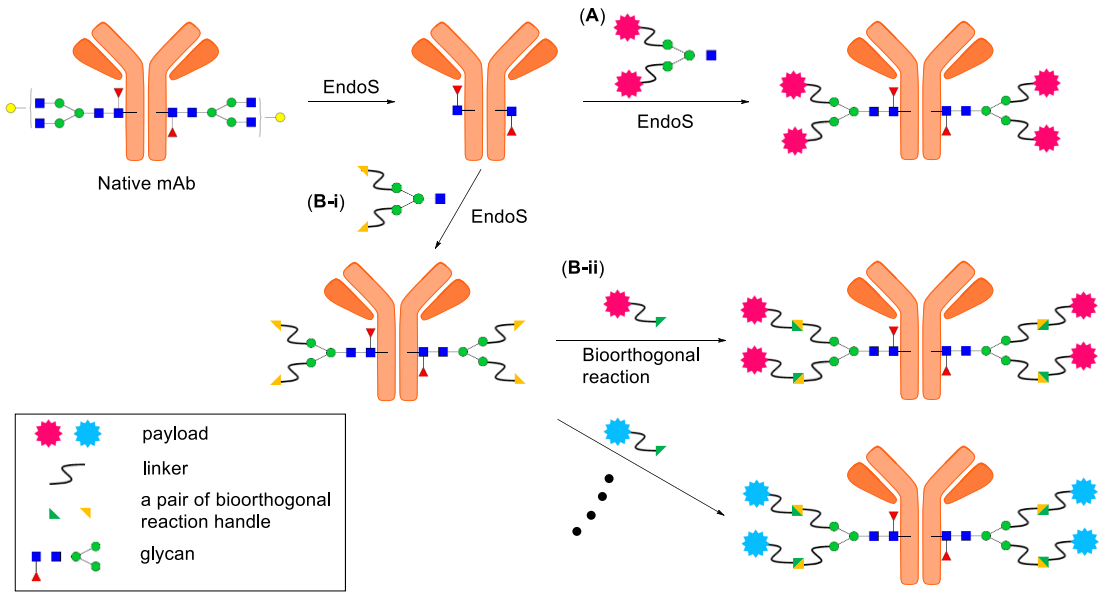


Figure 3.20. Payload installation prior to *N*-glycan conjugation (A) or post to *N*-glycan conjugation (B).

Because of increasing interest in modification of biological molecules, a range of methods are developed to install small molecule payloads on them (**Figure 3.21**). These methods are categorized in the reversible and the irreversible reactions. Previously, the detachment of the payload was considered essential for the efficacy of ADC[7] but recent investigation demonstrates that the release of the payload is not necessarily required to bring out its efficacy.[7] Therefore, irreversible reaction handles are preferable due to its stability at the stage of proof-of-concept. Considering preparation of payloads or glycosyl donors with such reaction handles, the inertness even in organic reactions is also favorable property. In this term, azide and alkye were chosen as the reaction handles and copper catalyzed alkyne-azide coupling (CuAAC) was planned for conjugation chemistry.

As to the basic scaffold of the glycan part, obviously the tetrasaccharide used in the *N*-glycan remodeling reacton is the starting point. From the synthetic viewpoint, the smaller the glycan scaffold is the easier to prepare and di- or trisaccharides are such candidates. However, concerns about these compounds include their affinity to EndoS. Unfortunately, structural information is

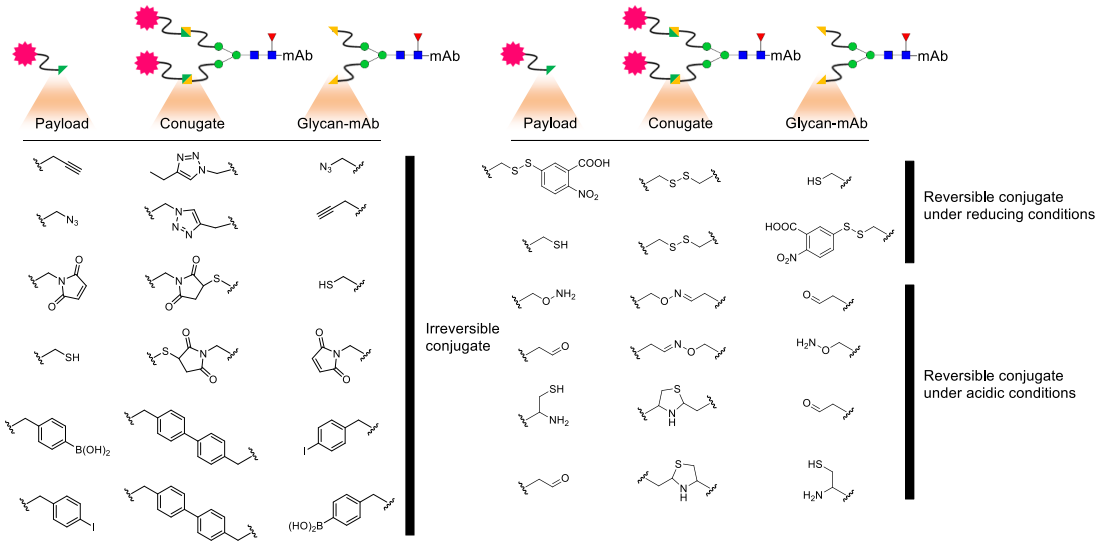


Figure 3.21. Examples of pairs of bioorthogonal reaction handles.

lacking for EndoS and it is difficult to speculate as to its substrate scope rationally. Conversely, the structure of IgG is well studied.[84] The *N*-glycans are deeply buried between two CH2 domains. Their structures are not perturbed in X-ray crystallography and the structures up to GlcNAc in the branch of the biantennary glycans are normally observable. Therefore, these structural components are expected to contribute to stabilization of the whole scaffold of IgG. Recently, the hydrophobic binding of GlcNAc in the branch of the biantennary *N*-glycan to F243 in CH2 domain was found to enhance the thermal stability of IgG significantly.[85] Based on these considerations, I speculated that branching part is necessary for enough structural stability of the final constructs and aimed at preparing tetrasaccharide scaffolds with extension of the branches from 2-OH of Man (**Figure 3.22, left**).

Alternatively, semi-synthesis using natural sources is an option for the desired *N*-glycan scaffolds. Fortunately, glycopeptides with complex type *N*-glycans can be extracted from egg yolk.[86] Additionally, *N*-glycans from egg yolk have sialic acids at the non-reducing termini of both branches. The carboxylic acid of sialic acid can be used as the discrete reaction site to install reactive handles. Therefore, extraction of the glycopeptides with the complex type *N*-glycan and its derivatization was also investigated (**Figure 3.22, right**).

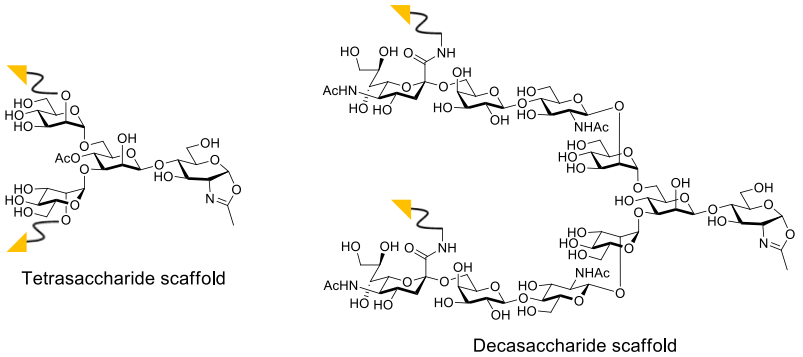


Figure 3.22. Glycans designed for ADC using EndoS-catalyzed *N*-glycan remodeling reaction. Yellow triangles indicate bioorthogonal reaction handles. (See **Figure 3.21**)

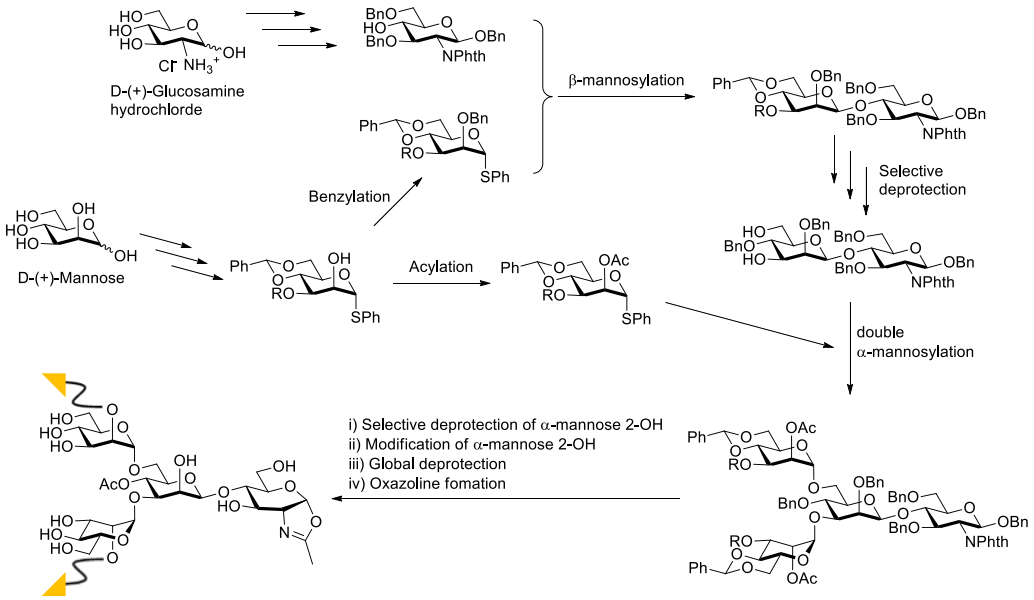
3.2.5. Preparation of glycans for antibody-drug conjugates

3.2.5.1. Tetrasaccharide synthesis

Synthetic plans

In the synthetis of tetrasaccharide scaffold of *N*-glycan, β-mannosylation is a key step (**Scheme 3.7**). Now several indirect approaches[87-91] are available for construction of this difficult linkage but Crich's β-mannosylation[92, 93] is most straightforward. Based on Crich's explanation, the requirement for β-mannosylation is 4,6-benzylidine structure with 2-OH protected by an ether group which is NOT involved in neighboring effect during the glycosylation reaction. The tether between 4- and 6-OH restricts the conformational freedom of the sugar ring system and destabilizes the carbocation (or oxonium cation), which is quenched by counter anion.[94, 95] The formed activated glycosyl donor has α-configuration, which is subjected to S_N2 reaction by the acceptor nucleophile, giving the β-glycoside as major product.

Scheme 3.7. Synthetic plan for the tetrasaccharide oxazoline with bioorthogonal reaction handles.



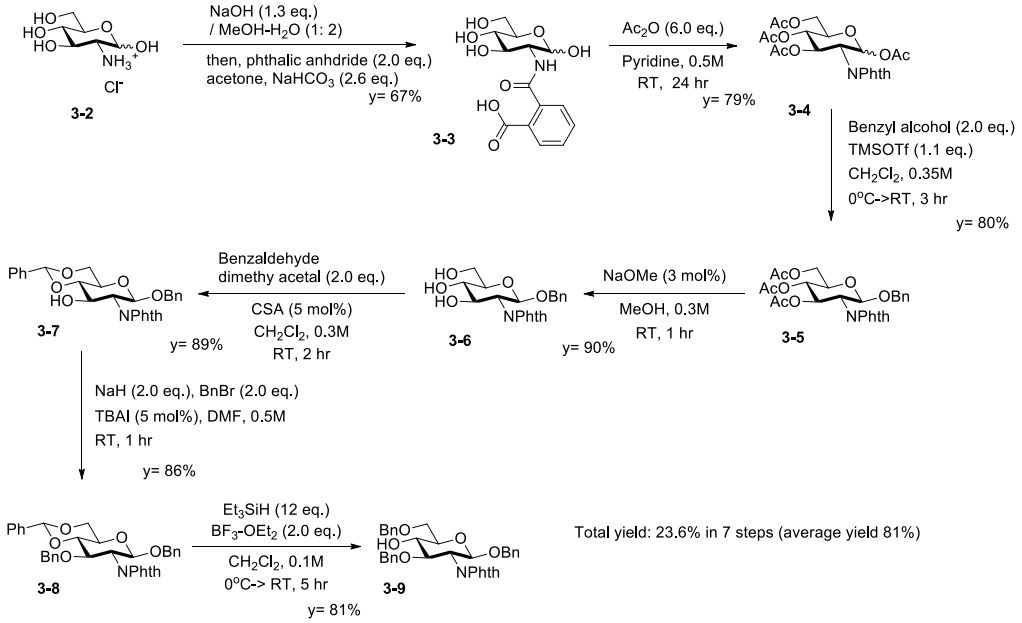
After formation of disaccharide, selective deprotection at 3-OH and 6-OH of β-mannose affords diol which is the glycosyl acceptor for double mannosylation (**Scheme 3.7**). The α-mannosylation donor may be prepared from the common intermediate with β-mannosylation donor by acylation of 2-OH instead of alkylation. These acylated positions allow selective deprotection and to tether the bioorthogonal reaction handles.

Preparation of GlcNAc unit

Preparation of GlcNAc unit is summarized in **Scheme 3.8**. Glucosamine hydrochloride (**3-2**) was first activated by treatment with sodium hydroxide to generate the free amine, which was then reacted with phthalic anhydride to afford the amide **3-3** in the moderate yield (67%).[96] Treatment of this compound with acetic anhydride in pyridine resulted in the ring cylization along with the acetyl protection of hydroxyl groups to give the phthalimide (**3-4**, y= 79%). Anomeric position is then replaced with benzyl group under the glycosylation condition using TMSOTf as activator. Because the β-isomer is more reactive to the glycosylation, the ratio of α- and β-conformer of the starting material affects the reaction time.[97] The reaction of **3-4** in hand (α:β= 1: 0.4) completed in 3 hours to afford the benzyl β-glucosamine **3-5** in 80% yield.

Deprotection of acetyl groups was performed under the Zemplén's condition. While sodium methoxide catalyzes the hydrolysis of the phthalimide, ring opening was detected when 20 mol% of the catalyst was used. Therefore, its amount was reduced as low as 3 mol% maintaining the good yield (90%) of **3-6** in the short reaction time (1 hour). Benzylidene formation between 4- and 6-OH was performed using benzaldehyde dimethyl acetal with camphorsulfonic acid as catalyst to afford **3-7** in 89% yield. The remaining 3-OH was protected with benzyl group by treatment with benzyl bromde in the presence of the catalytic amount of tetrabutylammonium bromide (5 mol%) after activation with sodium hydride to afford the target

Scheme3.8. Synthesis of GlcNAc unit.

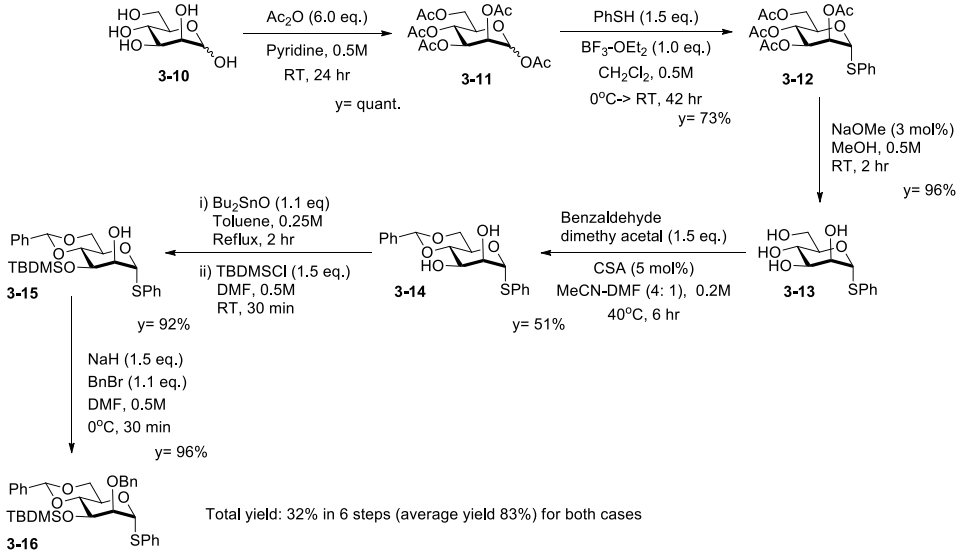


compound **3-8** (y= 86%). Regioselective ring opening of benzylidene was performed by reduction with triethylsilane in the presence of boron trifluoride-diethyl ether complex as lewis acid.[98] No detectable 6-OH compound formed in this condition and the target GlcNAc unit (**3-9**) was obtained in 81% yield. The total yield from glucosamine hydrochloride was 24% in 7 steps.

Preparation of mannose unit

In the first plan, 3-OH of the mannose unit was protected with TBDMS group. Preparation of this compound is shown in **Scheme 3-9**. Mannose (**3-10**) was first peracetylated to afford **3-11** and its anomeric acetate was replaced with thiophenol under glycosylation condition using boron trifluoride-diethyl ether complex as Lewis acid.[99] The reaction gave the α/β mixture (1: 0.16) but these isomers were separable by column chromatography and the desired α-anomer (**3-12**) was isolated in 73% yield. Zemplén deacetylation afforded phenyl α-1-thiomanno-pyranoside (**3-13**), which was subjected to the benzylidene formation reaction. Because the starting material **3-13** was sparingly soluble in pure acetonitrile, DMF was added as co-solvent

Scheme 3.9. Synthesis of Man unit-01.

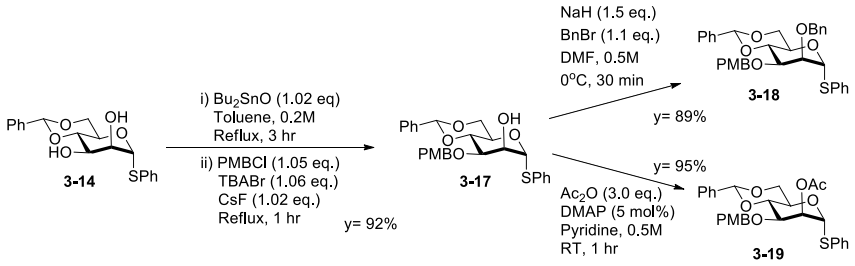


and the mixture was warmed to 40 °C to dissolve it before addition of benzaldehyde dimethyl acetal and camphorsulfonic acid. The product (**3-14**) has sparing solubility in the reaction solvent and was collected by filtration after the reaction as pure material, which needs no further purification (y= 51%).

Silylation of **3-14** was performed by activation of 3-OH by tributyltin oxide followed by addition of TBDMS-Cl.[100] After purification by siliga gel column chromatography, the product was still contaminated by materials derived from tin oxide. However, these materials could be removed by precipitation from diethyl ether and the desired alcohol **3-15** was obtained in 92% yield. Benzylation was performed using benzyl bromide and sodium hydride in DMF to afford the β-mannosylation donor (**3-16**) in 96% yield. In total, the β-mannosylation donor was obtained in 32% yield in 6 steps.

As will be discussed later, the 3-*O*-TBDMS derivative (**3-16**) turned out to be an unfavorable substrate for β-mannosylation. Alternatively, 3-*O*-PMB derivative was prepared starting from the diol **3-14** (**Scheme 3.10**). 3-OH was first activated by tributyltin oxide, followed by

Scheme 3-10. Synthesis of Man unit-02.



treatment with PMB-Cl and cesium fluoride in the presence of tetrabutylammonium bromide to afford the alcohol **3-17**[101] in 92% yield. The same condition was applied for this 3-*O*-PMB derivative as for the 3-*O*-TBDMS derivative to introduce benzyl group at 2-OH to afford the β-mannosylation donor **3-18** in 89% yield. Concurrently, **3-17** was derivatized to the α-mannosylation donor **3-19** in 95% yield by treating it with acetic anhydride in pyridine in the presence of 5 mol% DMAP as catalyst. The total yield of these compounds from mannose were 29% and 31%, respectively, in 6 steps.

β-Mannosylation

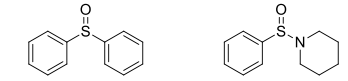
Now with the coupling partners in hand, the reaction condition for β-mannosylation was investigatd (**Scheme 3.11**). As long as 4,6-benzylidene α-1-thiomannoside with 2-ether protected group is used, several methods are now established for β-mannosylation.[94, 95, 102, 103]Among them, due to the accessibility to the reagents, DPSO/Tf_2O activation system[102] was first tested using the 3-*O*-TBDMS derivative (**3-16**) as glycosyl donor. However, it turned out that this condition gave the α-product as the major product (**Table 3.4**, entry 1).

Scheme 3.11. Investigation on the β-mannosylation condition.



Table 3.4. The conditions and the results of the investigation for β-mannosylation.

Entry	Donor	Acceptor	Activator	Tf_2O	DTBMP	Preactivation	Glycosylation	Yield	α: β ratio
1	**3-16** (R= TBDMS)	1.5 eq.	DPSO 2.8 eq.	1.5 eq.	3.0 eq.	-78°C 10 min	-78°C, 30 min -> RT	42%	2: 1
2	**3-18** (R= PMB)	1.5 eq.	DPSO 2.8 eq.	1.5 eq.	3.0 eq.	-78°C 10 min	-78°C, 1.5 hr -> RT	decomp.	
3	**3-18** (R= PMB)	1.0 eq.	BSP 1.1 eq.	1.05 eq.	2.5 eq.	-78°C 10 min	-78°C, 1.0 hr ->RT	73%	1: 5



DPSO: Diphenyl sulfoxide BSP: 1-Benzenesulfinyl piperidine

As this glycosyl donor apparently satisfies the requirements for the β-glycosylation, this poor selectivity was an unexpected outcome. The protecting group at 3-position was the only difference from the previous examples, where benzyl derivative groups are used instead. Therefore, the glycosylation of 3-*O*-TBDMS protected mannose was searched in the reaction database and it turned out that the glycosylation using such a donor had been performed and its poor β-selectivity had been reported.[104, 105] They also observed other cases where β-mannosylation gave poor selectivity when the protecting groups at 3-position were sterically bulky.[104] They reasoned that the steric interactions among protecting groups around the mannose ring are passed on to the reaction site to interfere the nucleophilic attack from the β-face. In detail, the bulky protecting group, like TBDMS, at 3-positioin stays away from the conformationally fixed benzylidene ring (**Figure 3.23**, B or C) in the reaction intermediate glycosyl triflate (or transient ion pair equivalent). Then this 3-position protecting group kicks

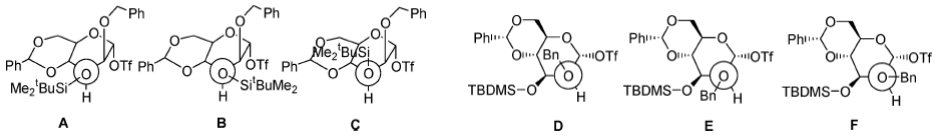


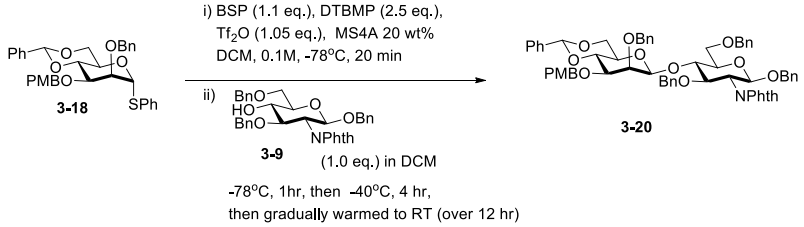
Figure 3.23. Staggered conformations around i) C3-O3 and ii) C2-O2. The figure is adapted with permission from Crich, D. *et al.*, *Org. Lett.* **2005**, *7* (11), 2277-2280.[104] Copyright 2005 American Chemical Society.

away one at the 2-position, increasing the population of the conformer whose β-face of the reaction site is covered. (**Figure 3.23**, F). This steric congestion over the β-face of the anomeric position results in the loss of β-selectivity.

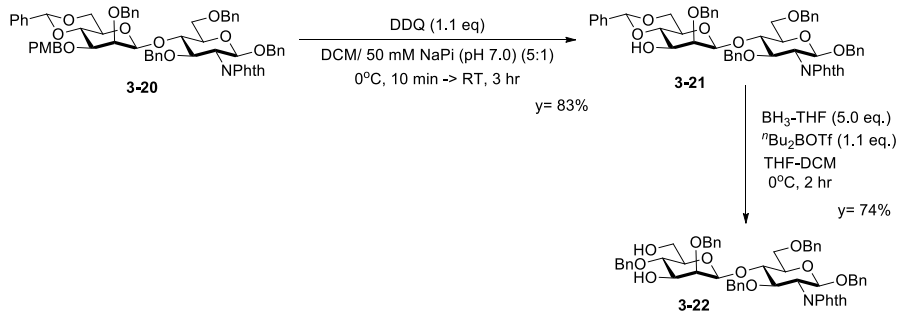
It is difficult to overcome the issue of steric congestion. Therefore, the 3-*O*-PMB derivative (**3-18**) was prepared as mentioned above. However, when the same condition was applied, the reaction gave the complex mixture (**Table 3.4**, entry 2). Apparently, the decomposition of the substrates took place and the TLC analysis did not give any clear spots and isolation or characterization of the formed products were difficult. Because decomposition of the materials occurred after replacement of the protecting group at 3-position, it is reasonable to presume this decomposition is due to the newly installed PMB group. Glycosylation using thioglycosides is an oxidative condition and the different outcome between 3-*O*-TBDMS and the 3-*O*-PMB derivative can be explained by their lability towards oxidation. Indeed, there is no examples where DPSO-Tf_2O activation system is used for PMB-protected sugars as far as I searched in the reaction database. Actually, a combination of DPSO-Tf_2O is a strong activation system and can be used even for disarmed glycosyl donors.[102] This fact implies that protecting groups susceptible to oxidation are not compatible with this condition.

Based on this observation, BSP[95] was then used as a milder activator. Gratifyingly, the reaction worked well (**Table 3.4**, entry 3) and the yield of the disaccharides was more than 70%. The stereoselectivity was also satisfactory (α:β= 1: 5).

Scheme 3.12. The scale-up reaction of β-mannosylation



Scheme 3.13. Preparation of the diol for the double α-mannosylation.



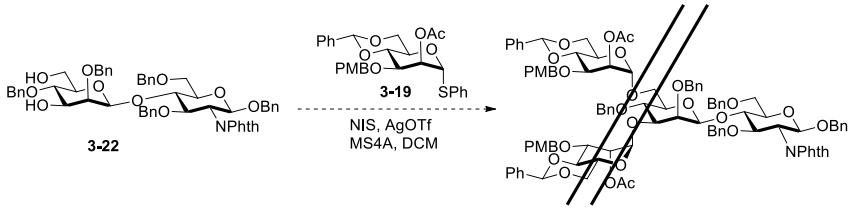
After finely tuning the reaction, for example, as to how to raise the temperature after addition of the acceptor, the reaction was performed using 4.0 g of the donor substrate (**Scheme 3.12**). The stereoselectivity was β: α= 6: 1 (determined by ^{1}H-NMR of the crude reaction mixture) and the isolated yield of the desired product (**3-20**) was 64% after silica gel column chromatography.

Now with the key intermediate (**3-20**) in hand, 3-*O*-PMB was removed by oxidative condition using DDQ to afford the alcohol **3-21** in 83% yield (**Scheme 3.13**). Regioselective ring opening[28] of benzylidene was performed using borane-tetrahydrofuran as reductant in the presence of dibutylboron triflate as Lewis acid to afford the diol (**3-22**) in 74% yield.

Double α-mannosylation

In the first attempt for the double glycosylation on the acceptor (**3-22**) was used the thiomannoside **3-19** as α-mannosylation donor, which was prepared from the same intermediate **3-17** as for the β-mannosylation donor **3-18**. The donor was activated by NIS/AgOTf[106]

Scheme3.14. α-Mannosylation using **3-19** resulted in the complex reaction mixture.

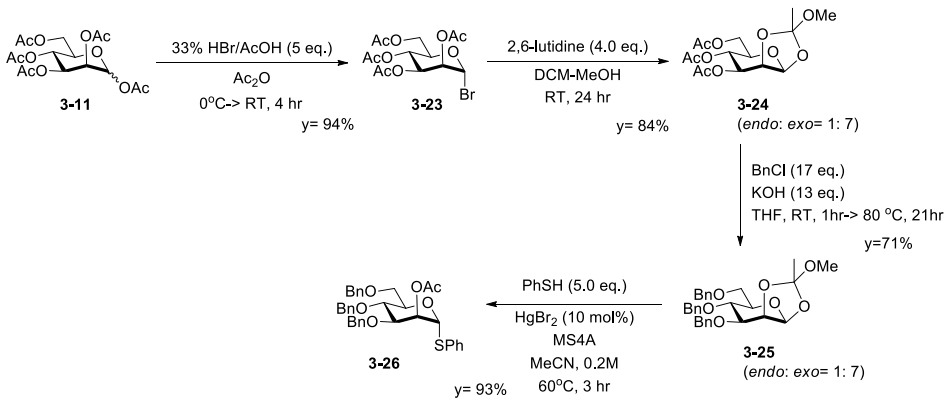


(**Scheme 3.14**). However, the reaction gave the complicated mixture in this condition. Though isolation of the formed compounds was difficult due to the complexity, the peak corresponding to the benzylidene proton was not observed in the ^{1}H-NMR spectrum of the crude reaction mixture. Instead, the peak of benzaldehyde was found. This result implies the incompatibility of benzylidene group for this reaction condition. Probably, addition of a weak base, like DTBMP, can suppress benzylidene cleavage but quenching acid during the course of the reaction is known to cease the isomerization of the initially formed orthoester intermediate to the desired glycosylated product.[107]

The lability of benzylidene under acidic glycosylation conditions required to change the strategy to utilize **3-17** as the common intermediate for the β-mannosyl donor and α-mannosyl donor (**Scheme 3.7**). Therefore, an acid stable mannosyl donor was designed and its preparation was carried out (**Scheme 3.15**).

Peracetylated mannose (**3-11**) was first converted to the bromide **3-23** (y= 94%), which was then treated with lutidine in the presence of methanol to afford orthoester **3-24** in 84% yield. The product was the mixture of *endo*- and *exo*-form of the orthoester in the ratio of 1: 7. Acetyl groups were converted to benzyl groups in the one-step procedure using excess amount of

Scheme 3-15. Preparation of the acid-stable α-mannosyl donor.

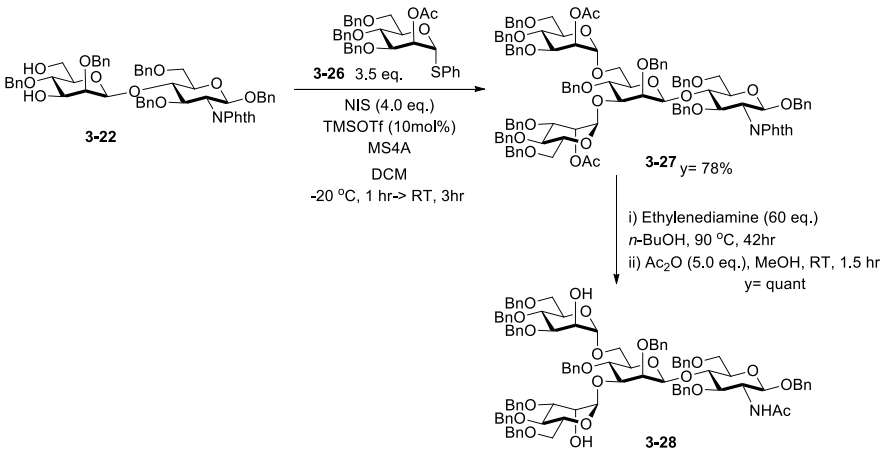


potassium hydroxide and benzyl chloride in tetrahydrofuran to afford **3-25** in 71% yield. Treatment of this orthoester with thiophenol in the presence of mercury bromide as Lewis acid afforded phenyl α-1-thiomannopyranoside (**3-26**) in 93% yield as the α-mannosyl donor. The total yield from pentaacetyl mannose (**3-11**) was 52% in 4 steps.

With the acid-stable α-mannosylation donor **3-26** in hand, the double mannosylation of the diol was tested (**Scheme 3.16**). Pleasingly, when 3.5 equivalent of the donor was used in the presence of TMSOTf as acid catalyst, the target tetrasaccharide was obtained in 78% yield. It should be mentioned that when less amount of the donor was used, more monomannoyslation product was observed and this side product was difficult to separate. Therefore, excess amount of the donor substrate is necessary to obtain the desired product in good yield.

Removal of *N*-phthalimide was performed with ethylenediamine.[108] Before completion of the *N*-phthalimide deprotection, partial deacetylation was observed. Therefore, the reaction time was prolongated to complete deprotection of the 2-*O*-acetyl as well. The resultant amine was treated with acetic anhydride in the absence of base, resulting in *N*-selective acetylation to afford **3-28** in the quantitative yield in these two steps.

Scheme 3.16. Construction of the tetrasaccharide scaffold.

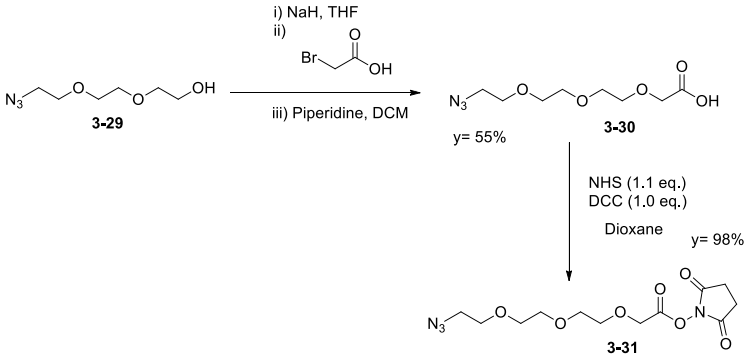


Introduction of the bioorthogonal reaction handle

Azide or alkyne was planned as a bioorthogonal reaction handle to be introduced in the glycan structure. However, these functional groups are not compatible with hydrogenation conditions to remove the benzyl groups presented on the prepared tetrasaccharide scaffold. Therefore, a three-step sequence was required to realize introduction of these groups: i) introduction of a linker with a functional group which will be converted to a selective reactive handle in the presence of free hydroxyl groups, ii) deprotection fo the benzyl groups along with liberation of the reactive handle, iii) reaction of the liberated reactive handle with its reaction partner which bears azide or alkyne. Mono-Cbz-protected ethylenedimine was chosen as such a linker to safisfy these demands: namely, i) carbamate formation with 2-OH of α-mannose, ii) hydrogenation to deprotect benzyl groups along with liberating amino group in the linker, iii) amidation with an activated ester bearing azide or alkyne.

The structure and the preparation scheme of the designed activated ester is shown in **Scheme 3.17**. To ensure the bioorthogonal reaction handle is exposed to the solvent region after conjugation with IgG, an azide compound (**3-29**) with the long PEG-type structure was used as the starting material. Carboxylic acid was introduced by Williamson ether formation using bromoacetic acid.[109] Treatment of the crude reaction mixture with piperidine[109] made the

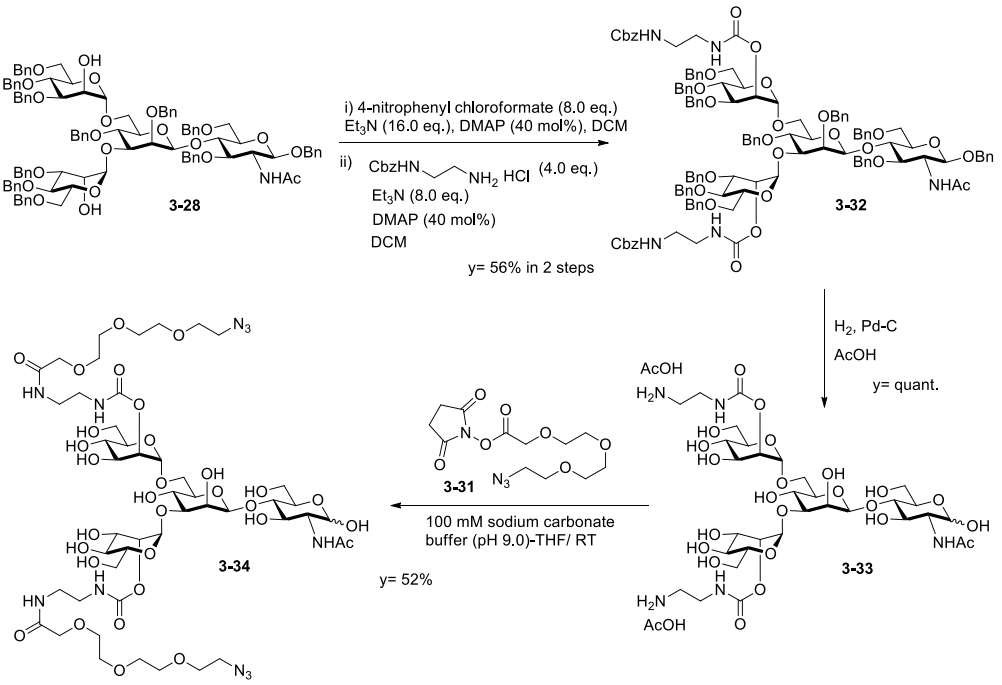
Scheme 3.17. Preparation of the *N*-succinimidyl ester bearing azide.



purification step easier because the unreacted bromoacetic acid could be removed by acid extraction. After removal of solvents, the desired compound (**3-30**) was obtained in 55% yield. This material was reacted with *N*-hydroxysuccinimide mediated by DCC to afford the desired the *N*-succinimidyl ester (**3-31**)[110] in 98% yield.

For the modification on the tetrasaccharide scaffold (**Scheme 3.18**), the two hydroxyl groups on α-mannosyl branches were first activated as *p*-nitrophenyl carbonate. Although this *p*NP-carbonate intermediate was applied to silica gel column chromatography, *p*-nitrophenol was not removed completely from the target compound. Without further purification, this mixture was subjected to the following reaction with the ethylenediamine derivative. These two-step reactions afforded the carbamate compound (**3-32**) in 56% yield. The deprotection of benzyl group as well as the liberation of the terminal amine was carried out by hydrogenolysis. When methanol was used as solvent, the deprotection of the Cbz groups completed quickly but

Scheme 3.18. Modification of 2-OH on the α-mannosyl branches.



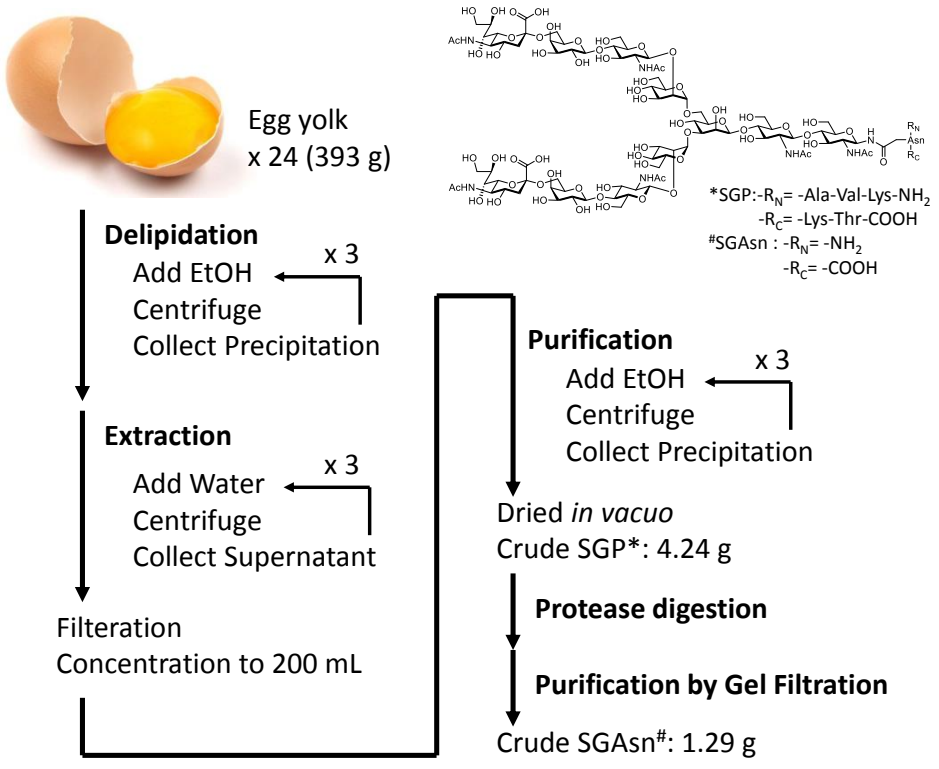
the debenzylation was quite sluggish (not shown in the scheme). It was likely that this slowness of the reaction came from catalyst poisoning by released amines. Therefore, the solvent was changed to acetic acid to suppress catalyst poisoning by the amines and to accelerate the rate of debenzylation. Under this condition, the reaction completed in 24 hours to give the desired diamine as acetic acid salt (**3-33**) in quantitative yield. Finally, the conjugation of the amine (**3-33**) with the activated ester (**3-31**) was performed. The reaction proceeded decently and the starting material was consumed in 2 hours. After coarse separation with gel filteration, the residue was purified by HPLC to afford the target compound (**3-34**) in 52% yield.

3.2.5.2. Decasaccharide synthesis

Extraction and purification of Fmoc-SGAsn

Preparation of the designed decasaccharide started from extraction of sialylglycopeptide[86] (SGP), namely, the hexapeptide with the sialyl biantennary *N*-glycan. The previously reported method[86, 111, 112] was followed for the extraction of SPG from egg. The procedure is summarized in **Scheme 3.19**. Briefly, delipidation was first performed using organic solvents and the precipitate was collected. Then the water soluble fraction from this precipitation was collected. After concentration, SGP was precipitated by addition of ethanol. The target SGP was found in the ^{1}H-NMR spectrum of this material, but it contained unnecessary peptides. To remove these contaminations, the crude SGP was treated with Actinase E (protease mixture obtained from

Scheme 3.19. Summary of the procedure ot extract sialylglycopeptide (SGP) from egg yolk and the structures of SGP and sialylglycoasparagine (SGAsn).

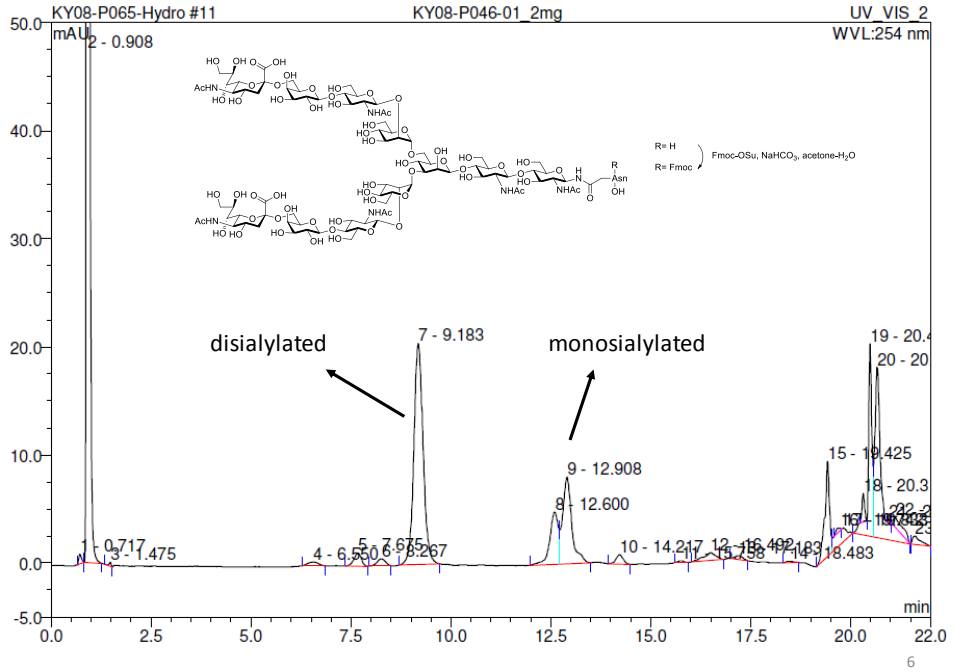


Streptomyces griseus),[112] and the resultant solution was purified by size exclusion column. In this process, the peptide fragment of SGP was also digested to afford sialylglycoasparagine (SGAsn). This crude SGAsn was analyzed by HPLC (**Figure 3.24A**) after derivatization with Fmoc at the amino group of the asparagine. Chromatogram showed a signal (Peak1) at t_R= 9.2 min as well as a twin-peak signal (Peak2) at t_R= 12.6 and 12.9 min. Both fractions were collected and ^{1}H-NMR spectra were measured (**Figure 3.24B**). From the area ratio of H-1 of mannose (5.1 ppm, singlet) and H-3 of sialic acid (1.7 ppm, triplet-like double doublet), it was determined that Peak1 was the the desired disialoglycoasparagine and Peak2 was the mixture of mono-sialylated analogues. All the crude SGAsn was then converted to the corresponding Fmoc derivative and its purification was performed in preparative HPLC to afford pure Fmoc-SGAsn (**3-35**) as triammonium salt (170 mg from 24 eggs).

Introduction of the bioorthogonal reaction handle

Propargyl amine is a readily available reagent and was chosen as the reaction partner with Fmoc-SGAsn to introduce alkyne functional group as bioorthogonal. A wide range of amide coupling reagents are now known,[113] but 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methyl-morpholinium chloride (DMTMM)[114] is outstanding for the reaction in the aqueous phase. In preliminary experiments using DMTMM as a coupling reagent, it turned out that ammonium salt in the reaction mixture deteriorated the desired reaction due to the side reaction to give the primary amide (-$CONH_2$) side products. Therefore, the counter cation of Fmoc-SGAsn (**3-35**) was exchanged before the reaction from ammonium to sodium using cation exchange column, Dowex 50WX8, activated with sodium hydroxide (**Scheme 3.20**). All the characterization of Fmoc-SGAsn was performed using this sodium salt. It should be noted that complete assignment of ^{13}C-NMR peaks of this undecasaccharide compound was performed for the first

A)



B)

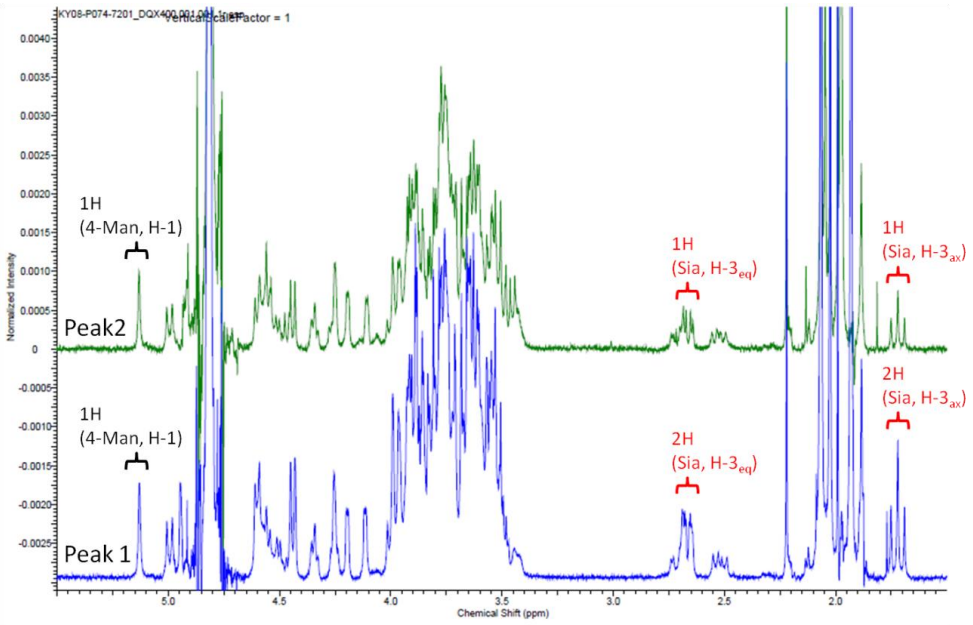


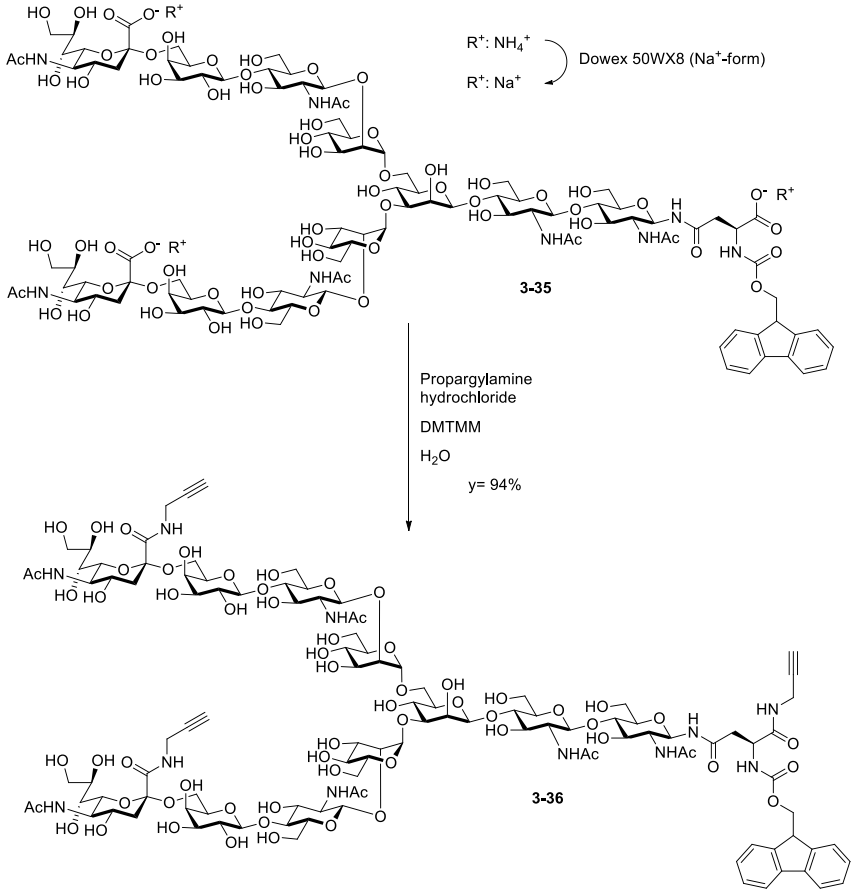
Figure 3.24. UV trace (A) of HPLC for crude SGAsn after Fmoc derivatization. In this experiment, ammonium acetate was used as buffering salt instead of ammonium bicarbonate which was used for the standard HPLC protocol shown in the experimental section. ^{1}H-NMR analysis (B) showed that Peak1 (t_R=9.2 min) was the desired disialoglycoasparagine and Peak2 (t_R=12.6, 12.9 min) was the mixture of monosialo-analogues.

time along with the more detailed assignment of ^{1}H-NMR than the previous report.[112]

This triacid (**3-35**) trisodium salt was subjected to amidation using DMTMM (**Scheme 3.20**). First, the reaction was performed in the buffered 400 mM propargylamine-HCl (pH 7.0 or 8.0) using 40 mM of **3-35** and 400 mM DMTMM. However, the reacion did not complete and halted in 12 hours. Then, the reaction was performed in the non-buffered 400 mM propargylamine-HCl, which afforded the tri-propargylamide (**3-36**) in 12 hours.

The amidation reaction by DMTMM involves three steps: i) anion exchange of DMTMM from chloride to the reactant carboxylate, ii) formation of activated ester from the carboxylate and the triazine, iii) nucleophilic attack of the amine to the activated ester to afford the amide product.

Scheme 3.20. Introduction of propargyl group to the biantennary *N*-glycan scaffold.

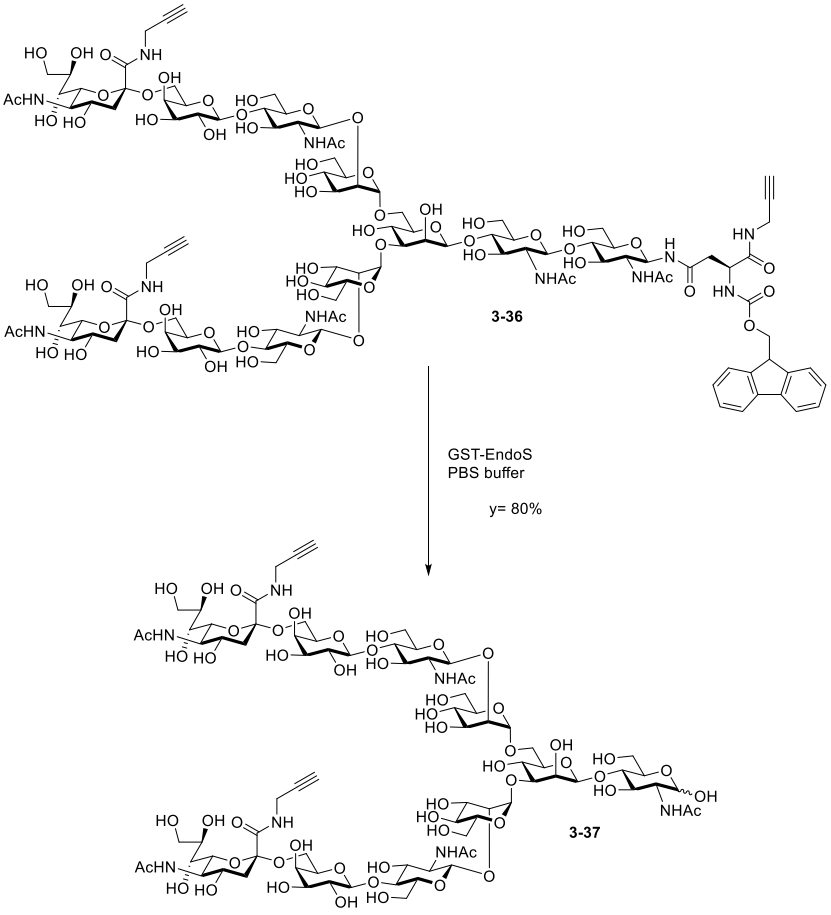


When the reaction is performed in the propargylamine-HCl buffer, there exists free propargyl amine (pK_a of the conjugated acid: 8.2) which is more nucleophilic than the carboxylate and reacts more readily with DMTMM than the carboxylate. Probably, this competing reaction inhibited formation of the activated ester (i, ii) and deteriorated the reaction. On the other hand, when the amine salt is used, the nucleophilicity of the amine is sterilized and the processes i), ii) are not interfered. The process ii) generates a weak base, *N*-methylmorpholine (pK_a of the conjugated acid: 7.4), which mildly activates the reactant amine salt. *N*-methylmorpholine is a tertiary amine and it cannot react with the activated ester. Therefore, only the reactant amine was able to attack the activated ester to afford he desired amide cleanly.

This amidation reaction was performed in the preparative scale (20.0 mg of sodium salt of **3-35**, **Scheme 3.20**) and the desired tripropargyl amide (**3-36**) was obtained in 94% yield after HPLC purification.

Now the glycan with bioorthogonal reaction handles is in hand, this compound can be directly used for transglycosylation onto IgG. Neverthelss, the preparation of GlcNAc-oxazoline compound was investigated to obtain more efficent glycosyl donor. For this purpose, the tripropargyl amide (**3-36**) was treated with EndoS to cleave the chitobiose core of the glycan (**Scheme 3.21**). When 4.4 mg of GST-EndoS was used for 15.0 mg of tripropargylamide (**3-36**), the reaction completed in 4 hr at 37°C. After size exclusion separation and HPLC purification, the desired decasaccharide (**3-37**) was obtained in 80% yield. No optimization of the reaction condition was performed and there is plenty of scope for reduction of the biocatalyst loading.

Scheme 3.21. Cleavage of the chitobiose core of the SGAsn scaffold by EndoS.

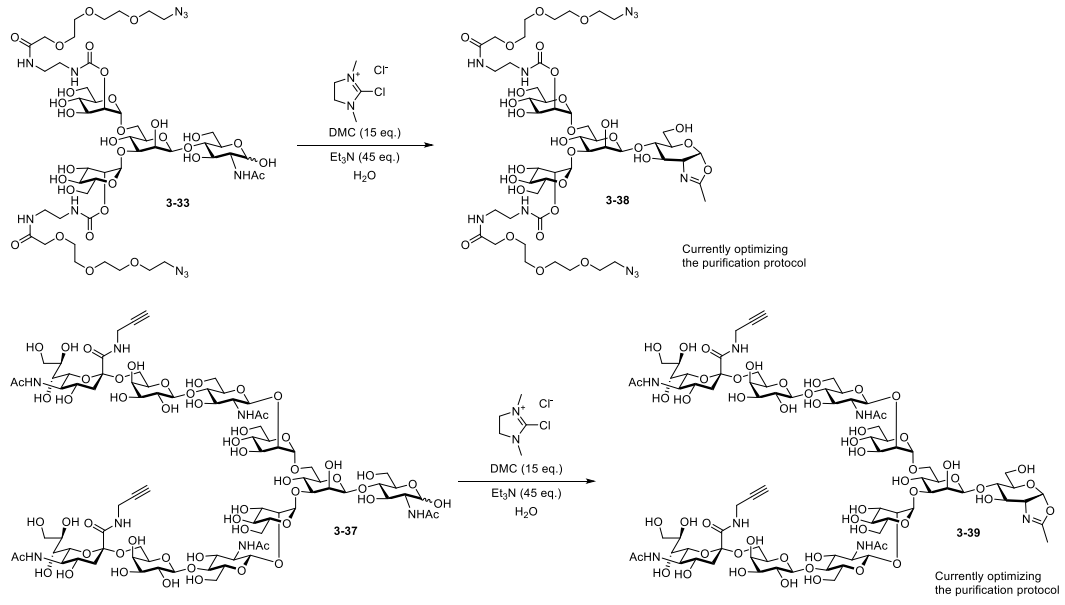


3.2.5.3. Oxazoline formation

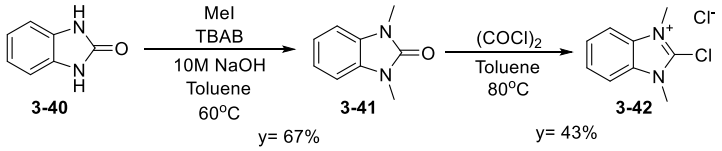
The reaction for oxazoline formation was first carried out using 2-chloro-1,3-dimethyl-imidazolinium chloride[115] (DMC) as dehydrating reagent (**Scheme 3.22**) for both the tetrasaccharide **3-33** and the decasaccharide **3-37**. ^{1}H-NMR spectra of the crude mixtures of the reactions showed that the reaction proceeded in the quantitative conversion in both cases. Several methods were tested to remove the triethylammonium salt and 1,3-dimethyl-2-imidazolidinone (DMI, the side product from DMC). Purification by HPLC using NH2 column (Kromasil or Luna from Phenomenex) resulted in poor recovery of the target material. Alternatively, the triethylammonium salt was removed by treatment with the anion exchange resin (amberlite IRA-400) followed by lyophilization. Significant amount of DMI remained even after 3 days of lyophilization but this material may be used for further reactions.

To obtain highly pure oxazoline compounds, more investigations were performed. Recently, CDMBI (2-chloro-1,3-dimethyl-1*H*-benzimidazol-3-ium chloride) was reported[116] as an

Scheme 3-22. Oxazoline formation using DMC as dehydrating reagent.



Scheme 3.23. Preparation of CDMBI (**3-42**).

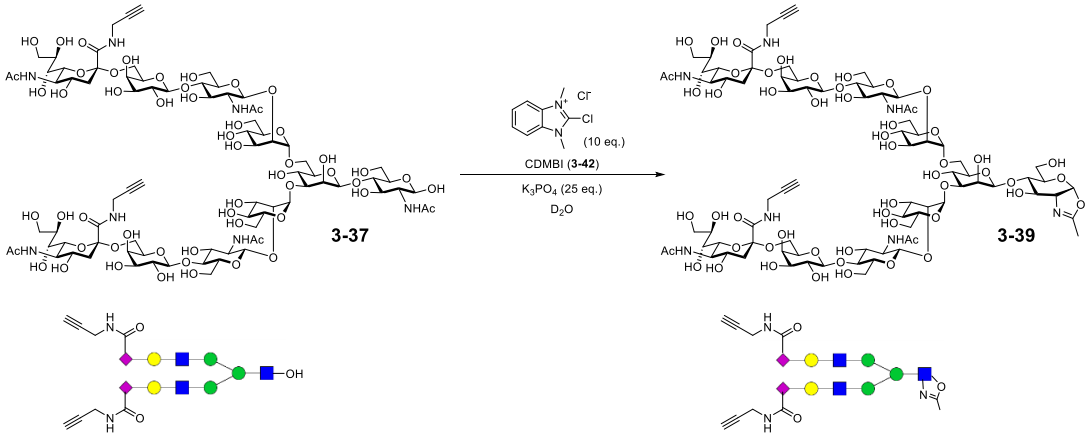


alternative reagent with several advantages over DMC; i) less hygroscopic and easier to handle, ii) the side product (DMBI, 1,3-dihydro-1,3-dimethyl-2*H*-benzimidazol-2-one) is readily removed as precipitation from the dehydration reaction due to its hydrophobicity, iii) inorganic salts like K_2CO_3 and K_3PO_4 can also work efficiently as base for dehydration reaction. In particular, ii) is an attractive property in terms of purification of oxazoline compounds. Therefore, CDMBI was prepared following the reported procedure (**Scheme 3.23**).[116]

2-Hydroxybenzimidazole (**3-40**) was first reacted with methyl iodide in the presence of sodium hydroxide to afford the dimethylated compound **3-41** in 67% yield after recrystallization from acetone-hexane. Then, **3-41** was treated with oxaryl chloride in toluene. The reaction gave the target compound CDMBI (**3-42**) as white precipitation after the reaction in 43% yield. It should be mentioned that CDMBI was gradually hydrolyzed when it was dissolved in purchased DMSO-d_6 for ^{1}H-NMR analysis. Therefore, it was required that DMSO-d_6 was dehydrated with MS4A before use for accurate ^{1}H-NMR analysis of this compound and the spectrum should be measured soon after the sample solution was prepared. However, as long as it is stored as powder at room temperature, it is stable for months.

The oxazoline decasaccharide (**3-39**) was prepared using CDMBI (**Scheme 3.24**). The reaction was performed in D_2O and monitored by ^{1}H-NMR. At first, the amount of the base (K_3PO_4) was mistakenly added less than planned (more than two equivalents to CDBMI are required) but the reaction completed in 1 hour after addition of the required amount of the base. The comparison of ^{1}H-NMR spectra between **3-37** and **3-39** is shown in **Figure 3.25**. The characteristic peak of

Scheme 3.24. Preparation of the oxazoline decasaccharide (**3-39**) using CDMBI.



the oxazoline was found at 6.09 ppm. When the area of the peaks was compared between this oxazoline proton and H-1 of 4-Man (5.12 ppm), the conversion was estimated to be 70 %. After filtration, the crude mixture was desalted with a PD-10 column (GE healthcare) and the fractions containing the target compound (monitored by TLC, 1M NH_4OAc: IPA= 2: 3) were collected and lyophilized. The ^{1}H-NMR spectrum of this material showed that DMBI (the side

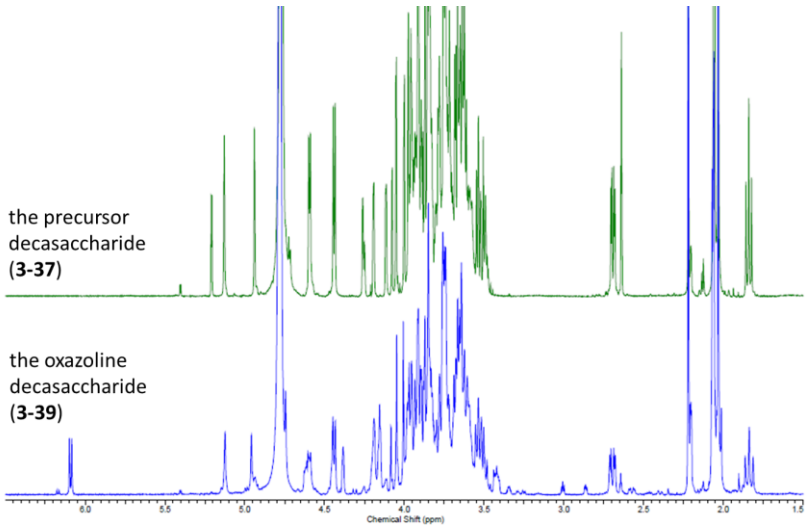


Figure 3.25. Comparison of ^{1}H-NMR spectra between the precursor decasaccharide (**3-37**) and the oxazoline derivative (**3-39**). After oxazoline formation, the characteristic peak corresponding to H-1 of the oxazoline-GlcNAc was found at 6.09 ppm.

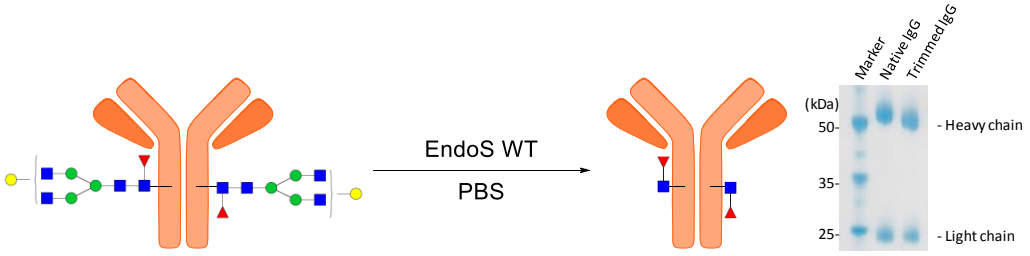
product of CDBMI) was completely removed as expected. However, the apparent yield based on the weight of the material was more than 100%, which unfortunately indicated inclusion of inorganic salts.

Further investigations are ongoing to prepare pure oxazoline compounds. The current ideas include i) the use of organic bases for the reaction, which are removable by evaporation after anion exchange, and ii) purification by dialysis with the low MWCO (100~500 Da) membrane. Oxazoline formation for the tetrasaccharide **3-33** using CDBMI has not yet been tested due to the scarcity of the material. Once a purification method is established, it will be applied to the tetrasaccharide **3-33**.

3.2.6. Introduction of bioorthogonal reaction handles on IgG with EndoS

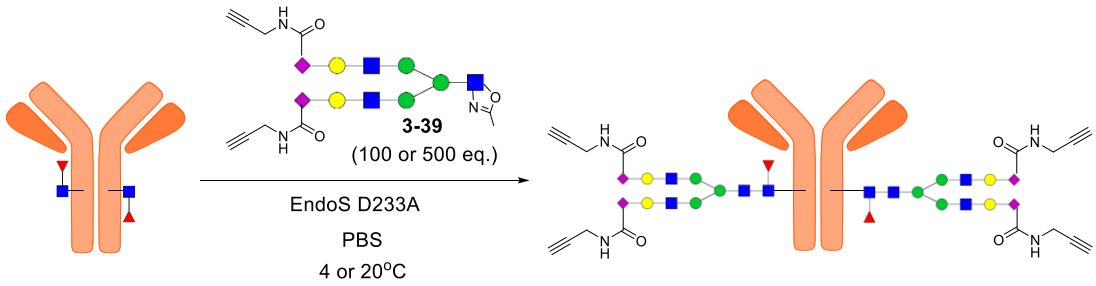
First, the native IgG was treated with EndoS WT (**Scheme 3.25**) to obtain the IgG with the truncated glycans used as the platform for the following glycan remodelling reaction. The reaction proceeded cleanly (**Scheme 3.25,** the picture of the gel) and the target material was purified with a Protein A column.

Scheme 3.25. Preparation of IgG with the truncated glycans.



EndoS D233A mutant was used as biocatalyst for the glycan remodelling reaction because this mutant was supposed not to have the hydrolytic activity due to the lack of the aspartic acid to activate the NHAc group as the intramolecular nucleophile (Chapter 3.1.2). In particular, this acid-less mutant is effective for glycosylation reactions using glycosyl donors with native scaffolds like the oxazoline decasacchride **3-39**. The details of preparation of the corresponding plasmid will be described elsewhere. This enzyme mutant was expressed in a shorter incubation time (16 hours at 28°C) than the wild type to prevent cleavage of GST-tag during the culture (see Chapter 3.2.1). Purification was performed in the same manner as the wild type. After purification by a GST affinity column, GST-EndoS D233A was obtained in the yield of 101 mg protein/ L culture. When further purification was performed after GST-tag cleavage, EndoS D233A was obtained in the yield of 46.3 mg protein/L culture.

Scheme 3.26. IgG remodelling using the oxazoline decasaccharide **3-39** catalized by EndoS D233A.



The glycan remodelling reaction was tested as shown in **Scheme 3.26**. To investigate the optimal condition, the reaction was performed using the different equivalents of the donor (100 or 500 equivalents) at two different temperatures (4°C or 20°C). In all cases, the remodelling reaction proceeded successfully and the band corresponding to the heavy chain of IgG shifted upward by 2 kDa in SDS-PAGE (**Figure 3.26**). However, when the reaction was monitored in a longer time, gradual hydrolysis of glycans of the formed product was observed. When the donor was used in 100 equivalents, the apparent conversions were less than 50% at most at both temperatures. On the other hand, when 500 equivalents of the donor was used, the maximum conversions was around 80% at 20°C in 2 hours and 70% at 4°C in 6 hours. Therefore, the semi-preparative scale reaction using 0.3 mg of IgG was performed at 20°C for 2 hours. The product was purified using a Protein A column and stored at -20°C.

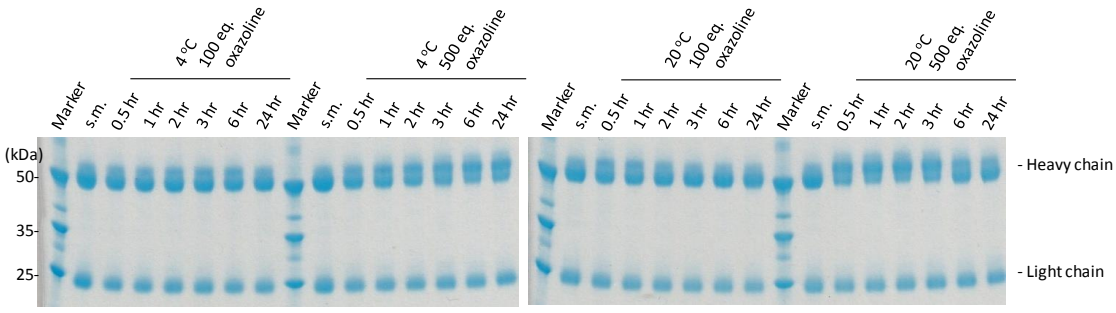


Figure 3.26. The analysis of the glycosylation reaction (Scheme 5) on IgG with EndoS D233A.

The structure of the glycan was confirmed by MALDI-TOF-MS after cleavage by PNGaseF (**Figure 3.27**). The peak of the dodecasaccharide (m/z= 2468) was found as sodium adduct. This result corroborates that the glycosylation took place on the disaccharide platform prepared by the EndoS WT reaction (**Scheme 3.25**).

Now investigations to improve this glycan-remodelling process are ongoing. One possibility for the unexpected glycan hydrolysis is that EndoS WT is contaminated in the remodelling reaction. It may come from the columns used for purification of EndoS D233A. Therefore, EndoS D233A will be purified again using intensively washed columns.

In case the hydrolytic activity is inherent in EndoS D233A, the current ideas to prevent the hydrolysis of the products include A) to change pH of the reaction and B) to develop other mutants. The X-ray structure of $Endo F_3$ with the ligand (**Figure 3.28**)[25] indicates that

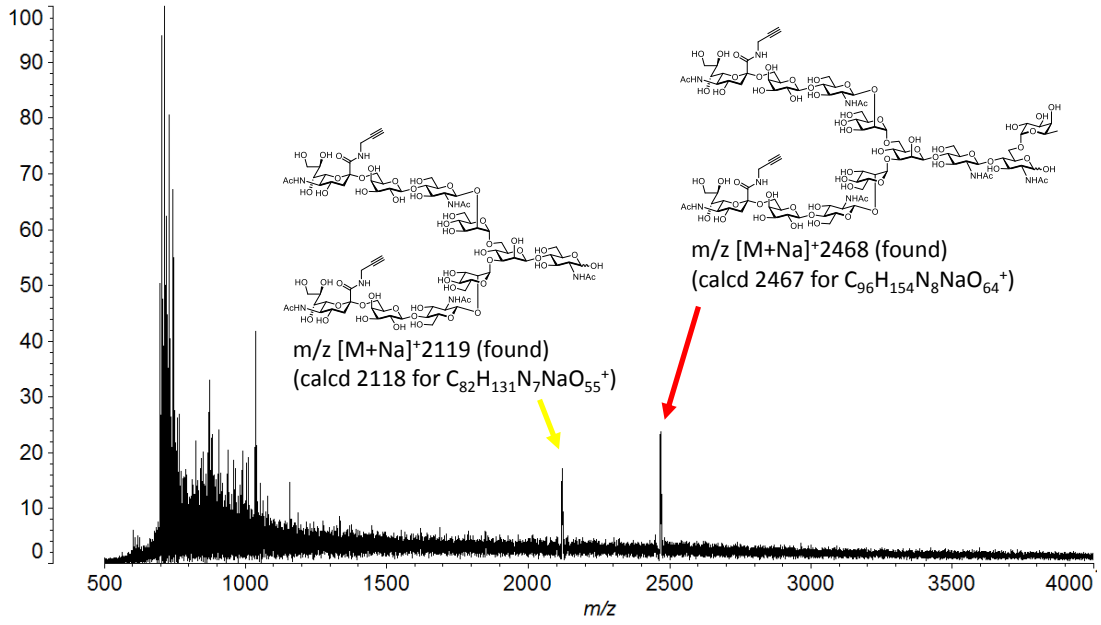


Figure 3.27. The glycan analysis of IgG after the glycan remodelling reaction using **3-39** as glycosyl donor. The found peak corresponding to the dodecasaccharide (m/z= 2468) corroborated that the glycan remodelling reaction proceeded on the disaccharide platform prepared by treatment of IgG with EndoS WT.

GH18 enzymes have the catalytic diad composed of two carboxylic acids, both of which act as acid/base. It is likely that these two functional groups work co-operatively (**Figure 3.29**, top). When one acts as acid to activate the leaving aglycon, the other acts as base to abstract the proton on the nitrogen atom of the acetamide group. This first step converts the substrate to the oxazoline intermediate. In the following step, the roles of these two carboxylic acids are reversed and the former works as base to activate an incoming alcohol (or water), while the latter works as acid to donate proton to the the nitrogen atom of the acetamide group.

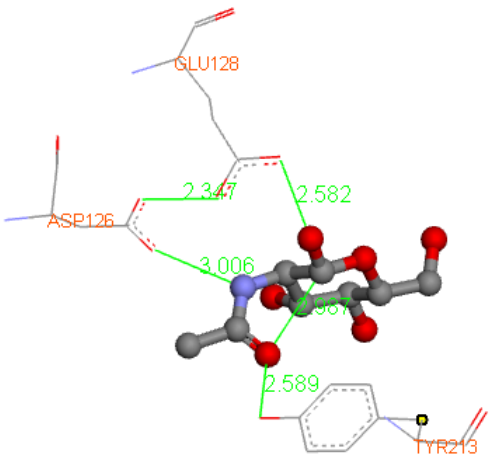


Figure 3.28. The structure of the active site of EndoF_3 with the substrate (PDB ID: 1eom).[25] Asp126 and Glu128 form the catalytic diad to promote the intramolecular nucleophilic attack by the acetamide group of GlcNAc of the substrate to the anomeric position. Tyr213 has the hydrogen bond with the carbonyl group of the acetamide so that it is guided to the appropriate position for the nucleophilic attack. Asp126, Glu128 and Tyr213 in EndoF_3 correspond to Asp233, Glu235 and Tyr305 in EndoS, respectively.

The rationale of the D233A mutant for the enhanced remodelling activity is that its hydrolytic activity is suppressed because the co-operative function of the catalytic diad to form the oxazoline is disrupted by loss of one of these two acids. However, if water molecules can bridge this co-operativity through hydrogen bonding network, the D233A mutant can have weak

hydrolytic acitivy. To suppress the hydrolytic activity further, inceasing pH in the reaction may be effective (**Figure 3.29**, Scheme A). Because the acid residue of the diad is completely deprotonated in sucha a condition and the catalyst has to generate an alkoxide intermediate during the oxazoline formation, which is energetically unfavourable.

GH18 enzymes have a tyrosine which interacts with the carbonyl group of the acetamide through the hydrogen bond. This interaction is likely to guide the nucleophilic carbonyl group to the anomeric position and facilitates the nucleophilic attack to form the oxazoline intermediate. Therefore, if the hydroxyl group of this tyrosine is removed, it makes the productive conformer less favorable (**Figure 3.29**, Scheme B) and suppresses the hydrolytic activity.

Based on these hypotheses, investigations to improve the conversion of the remodelling

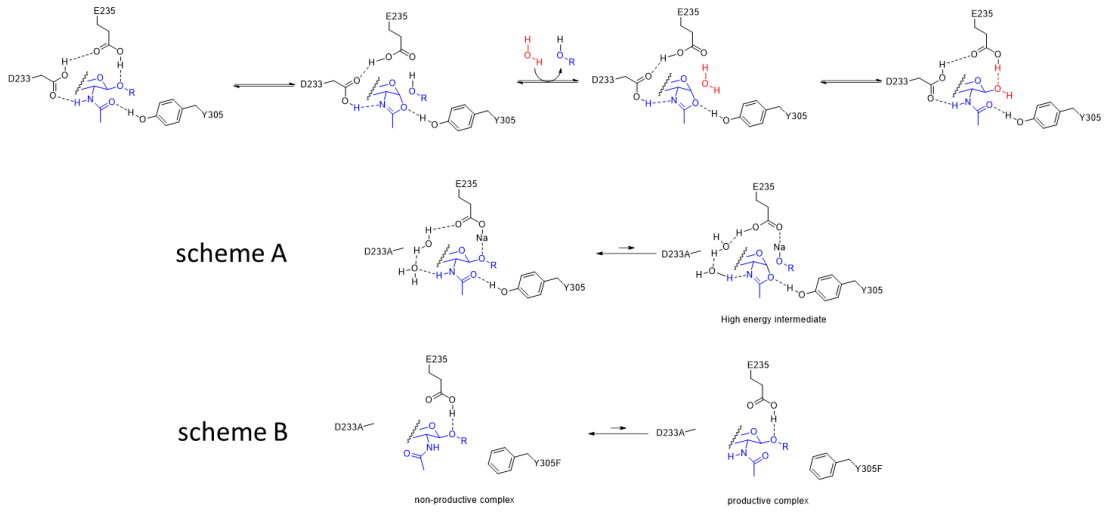


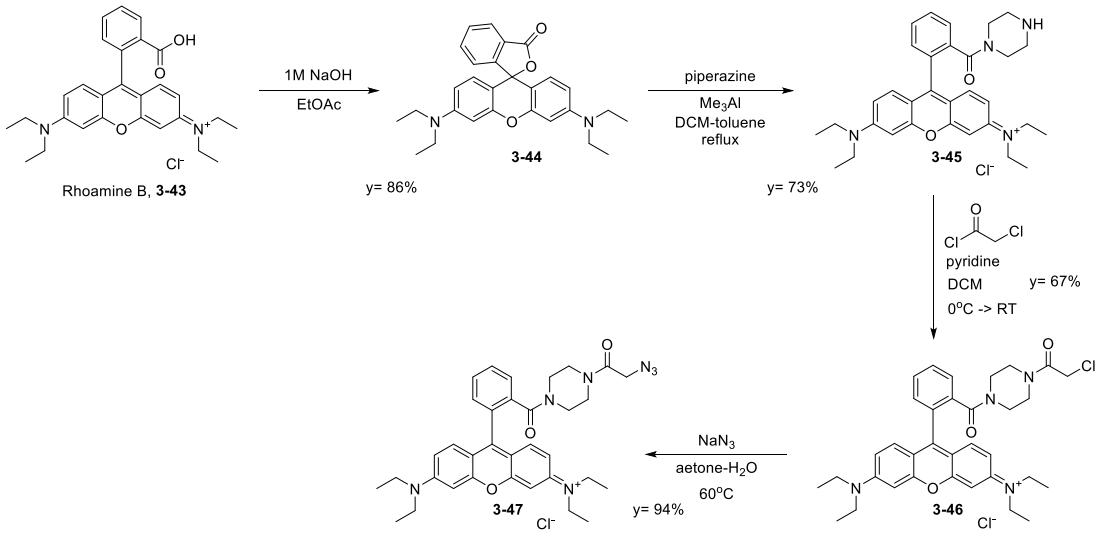
Figure 3.29. The plausible catalytic mechanism of EndoS based on the X-ray structure of EndoF3 (PDB ID: 1eom). Scheme A and B show the ideas to suppress the hydrolytic activity of EndoS D233A if it was inherent to this mutant. Scheme A shows that the basic environment in the reaction may suppress the hydrolytic reaction due to formation of energetically unfavourable alkoxide. Scheme B shows that EndoS D233A Y305F may disrupt the productive conformer for the oxazoline formation and may suppress the hydrolytic reaction.

reaction is ongoing. It is reported that EndoS D233Q also showed the remodelling activity.[117] However, this design is based on the GH85 enzyme, EndoM,[70] which has a different scaffold in the active site and therefore the rationale for the remodelling activity of EndoS D233Q is not clear. It has not escaped from our attentions that improvement of this glycan remodelling reaction is essential for our newly designed antibody-drug conjugates because our aim is to prepare homogeneous constructs.

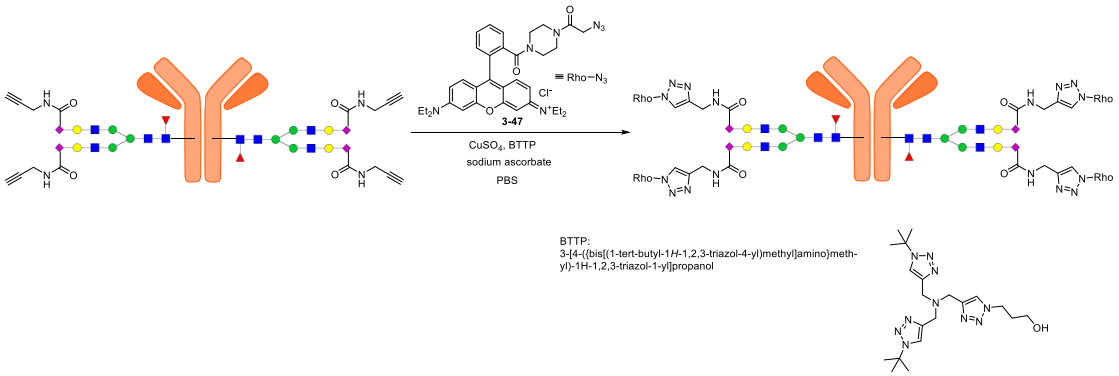
3.2.7. Bioorthogonal reaction on IgG

It was proved that a bioorthogonal reaction handle was introduced to the glycan moiety on IgG utilizing the EndoS-catalyzed reaction. To test the reactivity of the introduced alkyne for copper catalysed alkyne-azide coupling (CuAAC), a model reaction was planned using a dye compound. For this purpose, the rhodaminde derivative with an azide functional group was synthesized (**Scheme 3.27**). Commercially available rhodamine B (**3-43**) was first converted to the cyclic form by treatment with sodium hydroxide to afford the lactone **3-44** in 86% yield. This lactone was reacted with the piperazine-trimethylaluminium complex. The reaction gave the tertiary amide **3-45** in 73% yield. The amino group of **3-45** was then reacted with chloroacetyl chloride to afford **3-46** in 67% yield. Finally, the chloride **3-46** was converted to the azide **3-47** using sodium azide to afford the desired compound in 94% yield.

Scheme 3.27. Preparation of the azide-functionalized rhodamine.



Scheme 3.28. CuAAC between the alkyne-functionalized IgG and the azide-functionalized rhodamine B (**3-47**).



The CuAAC reaction was performed on IgG using the azide **3-47** (**Scheme 3.28**). Copper (II) sulphate was used as pre-catalyst and was activated by sodium ascorbate *in situ*. To accelerate the reaction BTTP[118] was used as the ligand for Cu(I) species. The reaction solution was incubated at 20°C for 2 hours and analyzed by SDS-PAGE (**Figure 3.29**). Gratifyingly, the fluorescence was observed in the region of the IgG heavy chain when the gel was illuminated with UV light. This result indicates that the alkyne attached to the glycans on IgG reacted with

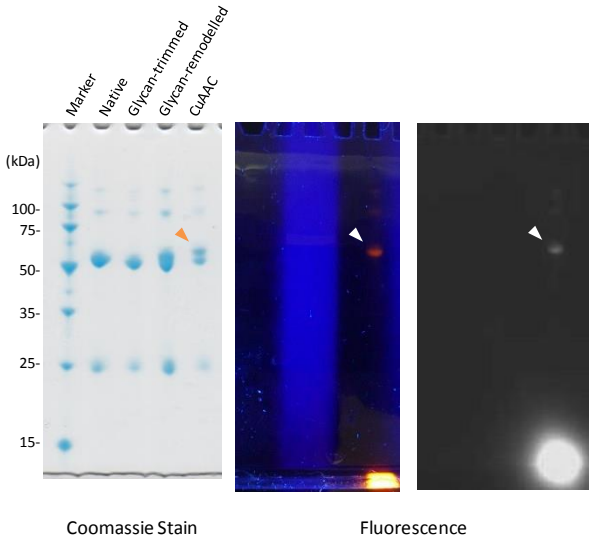


Figure 3.30. The SDS-PAGE analysis of the CuAAC reaction. Fluorescence was observed on the gel when illuminated with UV light (white arrow).

the azide of the rhodamine compound **3-47**. MALDI-TOF-MS was also performed for the glycan analysis after PNGaseF digestion to confirm the structure of the glycans. Unfortunately, the target peak was not observed when the standard protocol (General Procedure) was used for the analysis. Probably, the dicationic glycan was not properly recovered in this protocol and now other methods are now being investigated to confirm the structure.

The model reaction of CuAAC using the rhodamine derivative **3-47** demonstrated a promising result for development of a novel type of ADCs. The analysis of SDS-PAGE showed that the introduced alkynes on IgG have sufficient reactivity with its reaction partner. Now the detailed characterization of the glycans after the reaction is ongoing. Once the validity of this reaction is confirmed, it will be applied to prepare the designed antibody-drug conjugates using drug molecules.

3.3. Conclusion and the scope in the future

In this chapter, a biocatalytic modification of *N*-glycan on full-length IgG was described. EndoS, an ENGase which works specifically on IgG *N*-glycan, was applied for this purpose and indeed the heterogeneous native *N*-glycans were successfully replaced by the synthetic homogeneous analogue.

The structure of *N*-glycan is known to affect significantly to IgG's effector functions.[2-4] The effects of *N*-glycan structures to the Fc-FcγR binding are well investigated using IgG-Fc with various artificial glycoforms introduced by EndoA catalyzed reactions.[5, 6] It is true that these data will provide important insights for development of mAb drugs but EndoA as well as other well-investigated ENGases cannot modulate glycans on full-length IgG. However, the whole structure of IgG is required to exert its full potency as pharmaceutical agent such as targeting property. From this viewpoint, it is remarkable that EndoS is able to remodel glycans on full-length IgG. Notably, EndoS is the only biocatalyst applicable for such purposes so far. Indeed, following our result, the *N*-glycan modification by EndoS was applied[117] to investigate the effect of *N*-glycan structures on the affinities of rituximab with a vatiety of FcγRs. This example demonstrates the usefulness of our method.

Production of mAb with homogeneous glycoform has not been realized but it is important to establish a method to prepare such biopharmaceuticals in terms of quality control of medical products. It is expected that the EndoS-catalyzed reaction offers an excellent opportunity to develop finely-tuned mAb-based drugs by remodelling glycan structures on full-length IgG.

Antibody-drug conjugates are the emerging class of therapeutics which are developed in the interface of chemistry and biology. To extend the applicability of EndoS, a novel method to

prepare antibody-drug conjugates was investigated. In our strategy, bioorthogonal reaction handles are first introduced by the EndoS-catalyzed glycan remodelling reaction, to which payloads are then conjugated. This method is distinguished from the conventional protein modification, which utilizes lysine or cysteine side chains as reaction handle and results in heterogeneous mixtures. For this purpose, two types of glycans with bioorthogonal reaction handles were designed and prepared. One has the tetrasaccharide scaffold with azide groups tethered by PEG-like linkers. The other is the disialyl biantennary-type decasaccharide and has propargyl amide groups in the terminal sialic acid moieties. The synthetic methods for these glycans were established. These procedures are applicable to introduce other reaction handles. In particular, the DMTMM-mediated amidation allows us to prepare various kinds of decasaccharide derivatives using different types of simple amine compounds as reaction partner. Representative examples are shown in **Figure 3.31**. This method facilitates to prepare a library of linkers to conncet payload and mAb. As discussed in Chapter 3.1.1, chemical property of linkers has significant impact on pharmaceutical behavior of ADCs. Therefore, construction of a

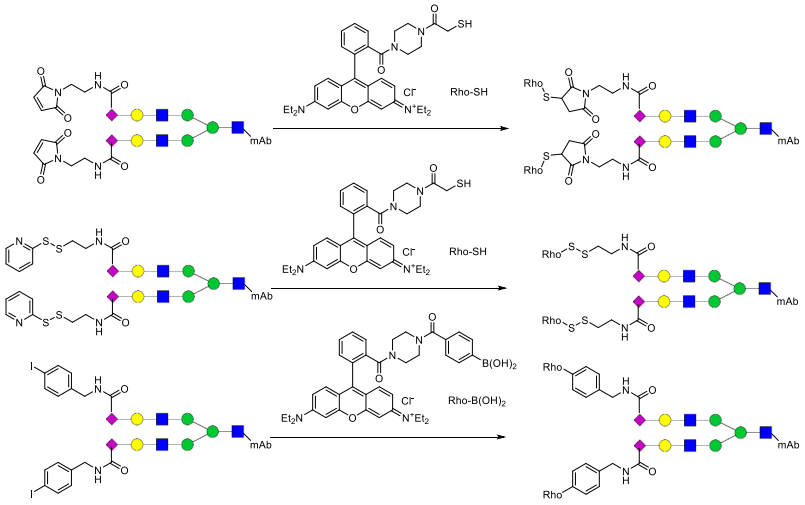


Figure 3.31. Representative examples of a linker library. Various types of reaction handles can be introduced by the DMTMM-mediated amidation (left). They are converted to different types of linkers after bioorthogonal reactions (right). Rhodamine B derivatives are shown as reaction partner.

library of linkers with various properties is of interest for development of novel ADCs.

The oxazoline glycosyl donors were prepared by treatment of the precursor with DMC or CDMBI. Purification protocol is still under investigation but the obtained oxazoline derivatives have enough quality to be used for the EndoS-catalyzed glycan remodelling reactions.

EndoS D233A was used for the glycan remodelling reaction and the glycan with the bioorthogonal reaction handle was successfully introduced on full-length IgG. However, unexpectedly the reaction showed slow glycan hydrolysis of the formed product. Approaches to prevent this undesired reaction were proposed and they are currently under investigation. Development of the analytical method to monitor this reaction is also important to screen the reaction conditions efficiently. We are aware that the complete conversion of this step is quite important to achieve our goal to realize a novel type of homogeneous antibody-drug conjugates.

As the initial example of bioorthogonal reactions, the copper-catalyzed alkyne-azide coupling was performed between the IgG with the alkyne functional groups and the rhodamine derivative with the azide functional group. The reaction successfully proceeded and the fluorescence was observed in the gel of SDS-PAGE when illuminated with UV light. This result demonstrated the validity of our strategy for preparation of antibody-drug conjugates.

Together, it was proved that EndoS is a powerful biocatalytic tool to prepare full-lengh IgG with a homogeneous glycofom. The EndoS-catalyzed glycan remodelling reaction shows a promise for development of a novel type of antibody-drug conjugates which has drug molecules on the glycan scaffold. Our interests about EndoS include its distinct substrate specificity. This enzyme specifically cleaves *N*-glycans on the native IgG and, for example, when IgG is denatured the *N*-glycan is not cleaved any more.[13] This interesting selectivity is scientifically intriguing and the structural analysis using X-ray crystallography and NMR is now ongoing for broader application of this unique enzyme.

3.4. Experimental

Preparation of pGEX-4T-1-*ndoS(opt)*

Synthesis of *ndoS(opt)* gene (an analogue of *ndoS* (GenBank accession: AF296340) optimized for expression in *E. coli*) flanked by *BamHI* and *NotI* restriction sites at 5'- and 3'- termini, respectively, and its subcloning to pGEX-4T-1 vector (GE healthcare) was performed by GenScript. The plasmid vector termed as pGEX-4T-1-*ndoS(opt)* confers ampicillin resistance to the host cell and appends the glutathione-S-transferase (GST)-tag with a thrombin cleavage site to the N-terminus of the the expresse protein.

Expression and purification of EndoS

BL21(DE3) competent cells in Singles kit (Novagen) were thawed on ice and suspended by finger-flick. 1 μL of pGEX-4T-1-*ndoS(opt)* was added and mixed by pipetting, followed by incubation on ice for 5 min. Heat-shock was applied to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 250 μL of SOC medium was added and the mixture was incubated at 37°C for 1 hr (200 rpm). On a LB-Amp agar was loaded 40 μL of SOC medium with which 10 μL of the transformed cell suspension was mixed. The cells were spread on the plate and it was incubated at 37°C for 20 hr and then stored at 4°C.

200 mL of LB-Amp medium was inoculated by a single colony and the culture was incubated at 37°C for 20 hr. 5 mL of this preculture solution was added to 500 mL of ZYM-5052-Amp medium in a 2-L baffled flask (x 6), which was incubated at 28°C for 22 hr with shaking at 200 rpm. The culture was centrifuged (8,000 rpm, 4°C, 15 minutes) and the cell pellet was collected and stored at -20°C.

The half of the cell pellet was resuspended in 80 mL of PBS (pH 7.4) containing 8 mg of lysozyme and 2 mg of DNase I, and the suspension was stirred at 4°C for 1 hr. The cells were further lysed by sonication (4 x 60 seconds, 1.0-sec pulse and 0.5-sec pause, one-minute intervals in between), and the cell debris was removed by centrifugation (20,000 rpm, 4°C, 20 minutes). The supernatant was filtered through 0.2-μm membrane. The resultant solution was applied to FPLC purification with the condition as follows:

Column: GSTrap™ HP 2x5 mL (serial connection) (GE Healthcare)
Eluent A: PBS (pH 7.4)
Eluent B: 50 mM Tris-HCl, 10 mM reduced L-glutathione (pH 8.0)
Flow rate: 2.0 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Sample volume: 66 mL (flow rate: 1.0 mL/min)

Gradient: 0% Eluent B 3CV
5% Eluent B 2CV
to 100% Eluent B 2CV
100% Eluent B 2CV

The flow-through fractions were recovered and applied to the same purification procedure repeatedly to collect as much protein as possible. The combined fractions containing the target protein were concentrated with amicon ultrafiltraton (MWCO 30,000) down to about 15 mL and dialyzed against PBS (pH 7.4). Protein concentration of the recovered solution (15 mL) was determined to be 4.98±0.15 mg/mL by BCA assay.

1.8 mL of 4.98 mg/mL GST-EndoS was diluted to 3.0 mg/mL by adding 1.2 mL of PBS (pH7.4) and 90 µL of 1 unit/µL thrombin (GE healthcare) was added. The solution was incubated at room temperature for 16 hr. The reaction solution was diluted with 6 mL of PBS (pH 7.4), filtered through 0.2-µm membrane and applied to FPLC purification with the condition as follows:

Column: GSTrap™ HP 5 mL (GE Healthcare)
Eluent A: PBS (pH 7.4)
Eluent B: 50 mM Tris-HCl, 10 mM reduced L-glutathione (pH 8.0)
Flow rate: 2.0 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Sample volume: 9.0 mL (flow rate: 0.5 mL/min)
Gradient: 0% Eluent B 5CV
5% Eluent B 2CV
to 100% Eluent B 2CV
100% Eluent B 2CV

The collected fractions were dialysed against 50 mM Tris-HCl, 0.5M NaCl (pH 7.4). The resultant solution was filtered through 0.2-µm membrane and applied to FPLC purification with the condition as follows:

Column: HiTrap Benzamidine™ FF (High Sub) 1 mL (GE Healthcare)
Eluent A: 50 mM Tris-HCl, 0.5M NaCl (pH 7.4)
Eluent B: 50 mM Glycine-HCl (pH 3.0)
Flow rate: 0.50 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Sample volume: 9.0 mL (flow rate: 0.25 mL/min)

Gradient: 10% Eluent B 7CV
to 100% Eluent B 2CV
100% Eluent B 2CV

The collected fraction was concentrated with vivaspin 6 (MWCO 30,000, pretreated with 0.1% Triton X-100) down to about 4 mL. The resultant solution was filtered through 0.2-μm membrane and applied to FPLC purification with the condition as follows:

Column: HiLoad 16/60 superdex 200 prep grade (GE Healthcare)
Eluent: PBS (pH 7.4)
Flow rate: 1.0 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Sample volume: <2.0 mL x2 (flow rate: 1.0 mL/min)

The collected fractions were concentrated with vivaspin 15 (MWCO 30,000, pretreated with 0.1% Triton X-100). Protein concentration of the solution (1.2 mL) was determined to be 1.39±0.15 mg/mL by BCA assay. This corresponds to the yield of 9.1 mg EndoS/ L culture.

Stability test for EndoS

50 μL of 1.0 mg/mL EndoS in PBS (pH 7.4) was mixed with 50 μL PBS (pH 7.4) and the resultant solution was separated into half. One half was incubated at 4°C and the other at 20°C. 10 μL of the solutions were taken up in 3 days, 1 week and 2 weeks. These samples were stored at -20°C until the analysis. The protein profiles of these solutions were analyzed by SDS-PAGE.

Cleavage of glycans on IgG with EndoS

25 μL of 2.0 mg/mL human IgG in PBS (pH 7.4) was diluted with 20 μL of PBS (pH 7.4) and mixed with 5μL of EndoS (1, 10 and 100 μg/mL) or PBS (pH 7.4) for a control reaction. The reaction was incubated at 20°C or 37°C. 10 μL of the solution was sampled at 1, 2 and 4 hr. These samples were stored at -20°C until the analysis. The protein profiles of these solutions were analyzed by SDS-PAGE.

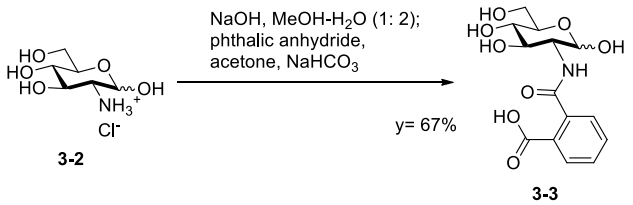
Remodelling of glycans on IgG with EndoS

50 μL of 2.0 mg/mL human IgG in PBS (pH 7.4) was mixed with 40 μL of PBS (pH 7.4) and 10 μL of 0.1 mg/mL EndoS in PBS (pH 7.4) on Slide-A-Lyzer MINI (MWCO 7,000, Thermo Scientific) floated on 500 mL of PBS (pH 7.4). The reaction solution was incubated at room temperature for 3 hr with dialyzing solution stirred. The resultant solution was taken up to a

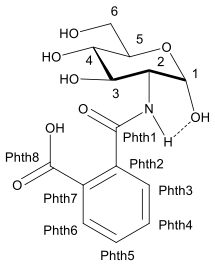
0.5-mL reaction tube and 5 μL of 66.7 mM oxazoline in water and another 10 μL of 0.1 mg/mL EndoS in PBS (pH 7.4) were added. The solution was incubated at 4°C and 30 μL of the reaction solution was sampled at 1, 2 and 4 hours. 5 μL was used for SDS-PAGE analysis. The rest of the samples were digested with PNGaseF (see General Procedure). In this case, the glycan samples released by PNGaseF digestion was cleaned up by SPE column instead of the separation by ultrafiltration. The digested solution was lyophilized and redisslved in 50μL of 5% AcOH. This solution was applied to HyperSep C18 SPE column (200 mg, 3 mL, Thermo Scientific) after the column was conditioned by 5 mL of methanol and 10 mL of 5% AcOH. The glycans were eluted out with 3 mL of 5% AcOH and the collected fraction was lyophilized. The residue was dissolved in 20 μL of water and the solution was used for MALDI-TOF-MS (see General Procedure).

Synthesis of GlcNAc unit

Preparation of 2-deoxy-2-(2-carboxybenzoyl)amino-D-(+)-glucopyranoside[96] (**3-3**)



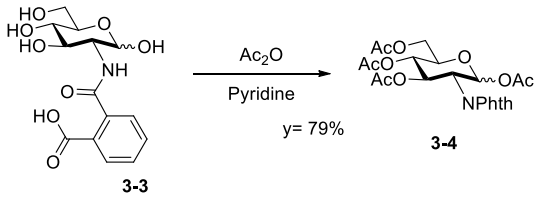
D-(+)-Glucosamine hydrochloride (**3-2**, 10.0 g, 46.4 mmol) was suspended in MeOH (20 mL)-H_2O (40 mL). The suspension was cooled to 0°C and finely ground NaOH (2.41 g, 60.3 mmol, 1.3 eq.) was added. The mixture was stirred for 15 min, followed by addition of a solution of phthalic anhydride (9.95 g, 67.2 mmol, 1.4 eq.) in acetone (80 mL), maintaining the temperature below 10°C. More phthalic anhydride (4.12 g, 27.8 mmol, 0.6 eq.) and $NaHCO_3$ (9.97 g, 119 mmol, 2.6 eq.) was added and the reaction mixture was stirred at room temperature for 4 hr. After the completion of the reaction was confirmed on TLC (EtOAc: AcOH: MeOH: H_2O= 12: 3: 3: 2), the reaction mixture was filtered to remove insoluble materials. The filtrate was adjusted to pH 1 by adding concentrated hydrochloric acid and concentrated using rotary evaporator. When precipitation started to form, the flask was removed from rotary evaporator and left in the cold room (4°C) overnight. The precipitation was collected by filtration, followed by washing with ice-cold water. The white solid was dried in vacuo to give the titled compound (**3-3**) as white solid; 10.2 g (y= 67.1%).



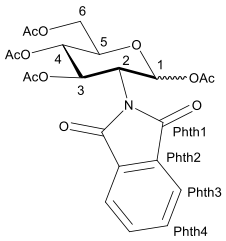
^{1}H NMR (400MHz, DMSO-d_6, δ ((CH_3)$_2$SO)=2.50)):3.19 (1H, dd, $J_{H4\text{-}H3}$ 8.8Hz , $J_{H4\text{-}H5}$ 8.8Hz, H_4), 3.46-3.55 (1H, m, H_6), 3.59-3.70 (3H, m, H_5, H_6', H_3), 3.76 (1H, ddd, $J_{H2\text{-}H3}$ 10.4Hz, $J_{H2\text{-}NH}$ 8.4Hz, $J_{H2\text{-}H1}$ 3.2Hz, H_2), 4.46 (1H, brs, 6-O*H*), 4.54 (1H, brs, 3-O*H*), 4.95 (1H, brs, 4-O*H*), 5.07 (1H, dd, $J_{H1\text{-}OH}$ 3.6Hz, $J_{H1\text{-}H2}$ 3.2Hz, H_1), 6.38 (1H, d, $J_{OH\text{-}H1}$ 4.0Hz, 1-O*H*), 7.46-7.59 (3H, m, $H_{Phth3\text{-}5}$), 7.72 (1H, d, $J_{Hphth6\text{-}Hphth5}$ 7.6Hz, H_{Phth6}), 8.03 (1H, d, $J_{NH\text{-}H2}$ 8.0Hz, N*H*), 12.96 (1H, brs, COO*H*). ^{13}C NMR (101MHz, DMSO-d_6, δ ((*C*H$_3$)$_2$SO)=39.51)): 55.05 (C_2), 61.16 (C_6), 70.50 (C_3), 70.87 (C_4), 72.16 (C_5), 90.61 (C_1), 128.08, 129.00, 129.26, 130.94, 131.04, 138.10 ($C_{Phth2\text{-}7}$), 168.37, 168.54 ($C_{Phth1,\ 8}$). LRMS (ESI$^+$): m/z [M+Na]$^+$ 350.09. $[\alpha]_D^{20}$= +47.6° (c 1.05, DMSO) (lit.[119] $[\alpha]_D^{20}$= +36.5°, c 2.0, H_2O). m.p.= 193-4°C (decomp., acetone-water) (lit.[119]

200°C water-ethanol-diethyl ether). IR (ν_{max}, cm^{-1}, neat): 3311 (broad, O-H stretch), 1711 (carboxylic acid C=O stretch), 1618 (amide-I), 1561 (amide-II), 1266 (carboxylic acid C-O stretch).

Preparation of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-D-(+)-glucopyranoside[96] (**3-4**)



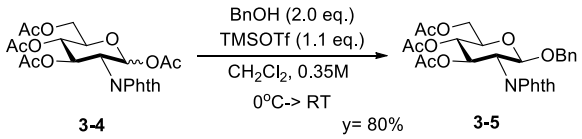
To a solution of 2-deoxy-2-(2-carboxybenzoyl)amino-D-(+)-glucopyranoside (**3-3**, 9.00g, 27.5 mmol) in pyridine (55 mL) was added acetic anhydride (15.6 mL, 165 mmol, 6.0 eq.). The reaction mixture was stirred at room temperature for 24 hr. After concentration by rotary evaporator, the residual reagents were further removed by azeotropic evaporation with toluene (50 mL x2). The residue was dissolved in chloroform (200 mL) and the solution was washed with 10% $KHSO_4$ (200 mL). The aqueous phase was extracted with chloroform (200 mL) and the combined organic phase was washed with water (200 mL x 3) and brine (100 mL), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel: 1,000 mL, eluent: ethyl acetate: petroleum ether= 1: 1) to afford the titled compound (**3-4**) as white foam; 10.3 g (y= 78.6%, α: β= 1: 0.4).



^{1}H NMR[120] (400MHz, $CDCl_3$, δ ($CHCl_3$)=7.27): <u>α-form</u> 1.883, 2.07, 2.10, 2.132 (4 x 3H, 4 x s, 4 x OCOC<u>*H*</u>$_3$), 4.14 (1H, dd, $J_{H6\text{-}H6'}$ 12.0Hz, $J_{H6\text{-}H5}$ 2.4Hz, H_6), 4.32 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 4.40Hz, $J_{H5\text{-}H6}$ 2.0Hz, H_5), 4.38 (1H, dd, $J_{H6'\text{-}H6}$ 12.8Hz, $J_{H6\text{-}H5}$ 4.4Hz, H_6'), 4.73 (1H, dd, $J_{H2\text{-}H3}$ 11.2Hz, $J_{H2\text{-}H1}$ 3.2Hz, H_2), 5.17 (1H, dd, $J_{H4\text{-}H5}$ 10.0Hz, $J_{H4\text{-}H3}$ 9.2Hz, H_4), 6.29 (1H, d, $J_{H1\text{-}H2}$ 3.2Hz, H_1), 6.57 (1H, dd, $J_{H3\text{-}H2}$ 11.2Hz, $J_{H3\text{-}H4}$ 8.8Hz, H_3), 7.74-7.79 (2H, m, H_{Phth4}), 7.84-7.90 (2H, m, H_{Phth3}). <u>β-form</u> 1.876, 2.01, 2.05, 2.126 (4 x 3H, 4 x s, 4 x OCOC<u>*H*</u>$_3$), 4.03 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 4.4Hz, $J_{H5\text{-}H6}$ 2.0Hz, H_5), 4.15 (1H, dd, $J_{H6\text{-}H6'}$ 12.4Hz, $J_{H6\text{-}H5}$ 2.0Hz, H_6), 4.38 (1H, dd, $J_{H6'\text{-}H6}$ 12.8Hz, $J_{H6\text{-}H5}$ 4.4Hz, H_6'), 4.48 (1H, dd, $J_{H2\text{-}H3}$ 10.4Hz, $J_{H2\text{-}H1}$ 8.8Hz, H_2), 5.23 (1H, dd, $J_{H4\text{-}H5}$ 10.0Hz, $J_{H4\text{-}H3}$ 8.8Hz, H_4), 5.89 (1H, dd, $J_{H3\text{-}H2}$ 10.4Hz, $J_{H3\text{-}H4}$

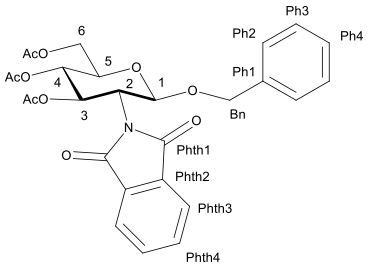
9.2Hz, H_3), 6.52 (1H, d, $J_{H1\text{-}H2}$ 8.8Hz, H_1), 7.76 (2H, dd, *J* 7.2Hz, 3.2 Hz, H_{Phth4}), 7.87 (2H, dd, *J* 7.2Hz, 3.2 Hz, H_{Phth3}). ^{13}C NMR[120] (101MHz, $CDCl_3$, δ (*C*HCl_3)=77.00): α-form 20.62, 20.62, 20.71, 20.95 (4 x OCO*C*H_3), 52.73 (C_2), 61.43 (C_6), 66.92 (C_3), 69.29 (C_4), 70.11 (C_5), 90.46 (C_1), 123.70 (C_{Phth3}), 131.07 (C_{Phth2}), 134.43 (C_{Phth4}), 167.38 (C_{Phth1}), 169.29, 169.50, 169.74, 170.64 (4 x O*C*OCH$_3$). β-form 20.37, 20.59, 20.72, 20.75 (4 x OCO*C*H_3), 53.43 (C_2), 61.47 (C_6), 68.20 (C_4), 70.44 (C_3), 72.57 (C_5), 89.70 (C_1), 123.77 (C_{Phth3}), 131.14 (C_{Phth2}), 134.46 (C_{Phth4}), 167.33 (C_{Phth1}), 168.61, 169.44, 170.00, 170.65 (4 x O*C*OCH$_3$). LRMS (ESI$^+$): m/z [M+Na]$^+$ 500.09. $[\alpha]_D^{20}$= +101.0° (c 1.17, $CHCl_3$, α: β= 1: 0.4) (lit.[121] α-form: +114°, c 1.7, β-form: +59°, c 0.6, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 1752 (ester C=O stretch), 1720 (imide C=O stretch), 1382, 1219, 1150, 1077, 1034, 1014, 757, 723. R_f= 0.29 (Ethyl acetate: Petroleum ether= 3: 2).

Preparation of benzyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-5**)



3-4 y= 80% **3-5**

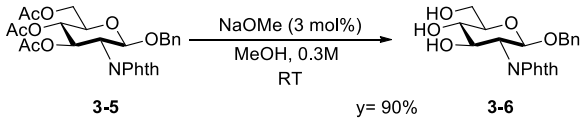
To a solution of 1,3,4,6-tetra-*O*-acetyl-2-deoxy-2-phthalimido-D-(+)-glucopyranoside (**3-4**, 17.3 g, 36.2 mmol, α: β= 0.17-0.30: 1) in dichloromethane (97 mL) was added benzyl alcohol (7.46 mL, 72.4 mmol, 2.0 eq.). The solution was cooled to 0°C and TMSOTf (7.20 mL, 39.8 mmol, 1.1 eq.) was added. The reaction mixture was stirred at this temperature for 30 min and then warmed to room temperature, stirring for additional 3 hr. The reaction mixture was cooled to 0°C and Et_3N (11.1 mL, 79.6 mmol, 2.2 eq.) was added. The resultant solution was washed with sat. $NaHCO_3$ (100 mL). The aqueous phase wash extracted with dichloromethane (100 mL) and the combined organic phase was washed with brine (100 mL), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel: 900 mL, eluent: ethyl acetate: petroleum ether= 2: 3) to afford the titled compound (**3-5**) as white solid; 15.2 g (y= 80.0%).



^{1}H NMR[122, 123] (400MHz, $CDCl_3$, δ (C*H*Cl_3)=7.270)): 1.87, 2.04, 2.14 (3 x 3H, 3 x s, 3 x OCOC*H*$_3$), 3.88 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 4.4Hz, $J_{H5\text{-}H6}$ 2.4Hz, H_5), 4.21 (1H, dd, $J_{H6\text{-}H6'}$ 12.4Hz, $J_{H6\text{-}H5}$ 2.4Hz, H_6), 4.36 (1H, dd, $J_{H6'\text{-}H6}$ 12.4Hz, $J_{H6\text{-}H5}$ 4.8Hz, $H_{6'}$), 4.38 (1H, dd, $J_{H2\text{-}H3}$

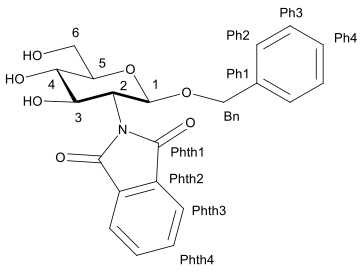
10.4Hz, $J_{H2\text{-}H1}$ 8.4Hz, H_2), 4.54 (1H, d, $J_{HBn\text{-}HBn'}$ 12.0Hz, H_{Bn}), 4.86 (1H, d, $J_{HBn'\text{-}HBn}$ 12.0Hz, H_{Bn}'), 5.20 (1H, dd, $J_{H4\text{-}H5}$ 10.0Hz, $J_{H4\text{-}H3}$ 9.2Hz, H_4), 5.38 (1H, d, $J_{H1\text{-}H2}$ 8.4Hz, H_1), 5.80 (1H, dd, $J_{H3\text{-}H2}$ 10.4Hz , $J_{H3\text{-}H4}$ 9.2Hz, H_3), 7.05-7.16 (5H, m, H_{Ph2}, H_{Ph3}, H_{Ph4}), 7.70-7.76 (2H, m, H_{Phth4}), 7.76-7.87 (2H, br, H_{Phth3}). ^{13}C NMR[122, 123] (101MHz, $CDCl_3$, δ (*C*HCl_3)=77.00): 20.43, 20.61, 20.77 (3 x OCO*C*H_3), 54.59 (C_2), 61.99 (C_6), 68.94 (C_4), 70.62 (C_3), 71.28 (C_{Bn}), 71.79 (C_5), 97.16 (C_1), 123.51 (C_{Phth3}), 127.71, 127.80, 128.21 (C_{Ph2}, C_{Ph3}, C_{Ph4}), 131.35 (C_{Phth2}), 134.13 (C_{Phth4}), 136.59 (C_{Ph1}), 167.45 (C_{Phth1}), 169.46, 170.09, 170.73 (3 x O*C*OCH$_3$). LRMS (ESI^+): m/z $[M+Na]^+$ 548.13. $[\alpha]_D^{20}$= -8.5° (c 1.14, $CHCl_3$) (lit[123]. $[\alpha]_D^{21}$= -11.2°, c= 1.32, $CHCl_3$). m.p.= 105-107°C (ethyl acetate-petroleum ether) (lit.[124] 106-7°C, isopropanol). IR (ν_{max}, cm^{-1}, neat): 1748 (ester C=O stretch), 1717 (imide C=O stretch), 1386, 1367, 1226, 1150, 1077, 1039, 1014, 743, 722, 701 (lit[122]. IR (ν_{max}, cm^{-1}, KBr): 1750 (ester), 1716 (imide), 721 (aromatic)). R_f= 0.25 (Ethyl acetate: Petroleum ether= 2: 3) (lit[122]. R_f= 0.42 (Ethyl acetate: Petroleum ether= 1: 1)).

Preparation of benzyl 2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-6**)



3-5 y= 90% **3-6**

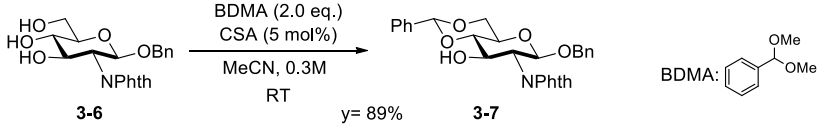
To a solution of benzyl 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-5**, 15.0 g, 28.5 mmol) in methanol (95 mL) was added 25 wt% sodium methoxide in methanol (196 μL, 855 μmol, 3.0 mol%). The solution was stirred at room temperature for 45 min. The reaction was quenched by adding Dowex 50WX2-400 (356 mg, 1.71 meq, 6.0 mol%), followed by concentration. The residue was purified by column chromatography (silica gel: 1,000 mL, eluent: dichloromethane: methanol= 10: 1) to afford the titled compound (**3-6**) as white solid; 10.3 g (y= 90.4%).



^{1}H NMR (500MHz, $CDCl_3$, δ (C*H*Cl_3)=7.270): 2.81 (1H, dd, $J_{6OH\text{-}H6}$ 6.5 Hz, $J_{6OH\text{-}H6'}$=6.5Hz, 6-O*H*), 3.45 (1H, d, $J_{3OH\text{-}H3}$ 6.0 Hz, 3-O*H*), 3.49 (1H, ddd, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6}$ 4.0Hz, $J_{H5\text{-}H6'}$ 3.5Hz, H_5), 3.71 (1H, ddd, $J_{H4\text{-}H3}$ 9.0Hz , $J_{H4\text{-}H5}$ 9.0Hz, $J_{H4\text{-}4OH}$ 4.0Hz, H_4), 3.79 (1H, d, $J_{4OH\text{-}H4}$ 4.0 Hz, 4-O*H*), 3.89 (1H, ddd, $J_{H6\text{-}H6'}$ 12.0Hz, $J_{H6\text{-}6OH}$ 7.5Hz, $J_{H6\text{-}H5}$ 3.5Hz, H_6), 3.93 (1H, ddd,

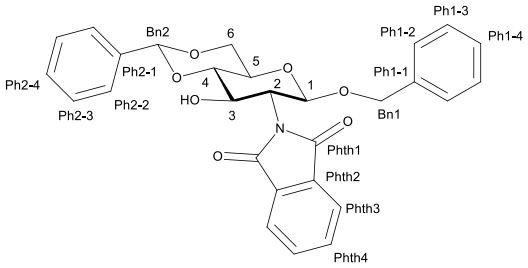
$J_{H6'\text{-}H6}$ 12.0Hz, $J_{H6'\text{-}6OH}$ 5.0Hz, $J_{H6'\text{-}H5}$ 3.0Hz, $H_{6'}$), 4.16 (1H, dd, $J_{H2\text{-}H3}$ 11.0Hz , $J_{H2\text{-}H1}$ 8.5Hz, H_2), 4.33 (1H, ddd, $J_{H3\text{-}H2}$ 11.0Hz , $J_{H3\text{-}H4}$ 9.0Hz, $J_{H3\text{-}3OH}$ 6.0Hz H_3), 4.52 (1H, d, $J_{HBn\text{-}HBn'}$ 12.0Hz, H_{Bn}), 4.80 (1H, d, $J_{HBn'\text{-}HBn}$ 12.5Hz, $H_{Bn'}$), 5.24 (1H, d, $J_{H1\text{-}H2}$ 8.5Hz, H_1), 7.03-7.14 (5H, m, $H_{Ph2\text{-}4}$), 7.66-7.72 (2H, m, H_{Phth4}), 7.73-7.80 (2H, m, H_{Phth3}). ^{13}C NMR (126MHz, $CDCl_3$, δ ($CHCl_3$)=77.00): 56.62 (C_2), 61.94 (C_6), 71.33 (C_{Bn}), 71.53 (C_3), 71.61 (C_4), 75.39 (C_5), 97.67 (C_1), 123.40 (C_{Phth3}), 127.69, 127.69, 128.19 (C_{Ph2}, C_{Ph3}, C_{Ph4}), 131.62 (C_{Phth2}), 133.99 (C_{Phth4}), 137.03 (C_{Ph1}), 168.24 (C_{Phth1}). LRMS (ESI$^+$): m/z $[M+Na]^+$ 422.12. $[\alpha]_D^{20}$= -50.7° (c= 0.41, $CHCl_3$) (lit[124]. $[\alpha]_D^{25}$=-50.7°, c= 0.38, $CHCl_3$). m.p.= 172-3°C (CH_2Cl_2-MeOH) (lit[124]. 169-170°C, ^{i}PrOH). IR (ν_{max}, cm^{-1}, neat): 3446 (broad, O-H stretch), 1775, 1710 (imide C=O stretch), 1389, 1048, 1022, 968, 746, 721, 699, 668, 658. R_f= 0.25 (methanol: dichloromethane= 1: 10) (lit[124]. R_f= 0.44 (methanol: chloroform= 1: 10))

Preparation of benzyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-7**)



3-6 y= 89% **3-7**

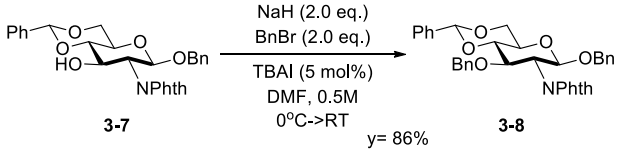
To a solution of benzyl 2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-6**, 10.0 g, 25.0 mmol) in acetonitrile (83 mL) were added benzaldehyde dimethyl acetal (BDMA, 7.53 mL, 50.0 mmol, 2.0 eq.) and (±)-camphor-10-sulfonic acid (CSA, 290 mg, 1.25 mmol, 5 mol%). The solution was stirred at room temperature for 2 hr, followed by neutralization with Et_3N (348 μL, 25.0 mmol, 10 mol%). After concentration, the residue was purified by column chromatography (silica gel: 1,000 mL, eluent: ethyl acetate: petroleum ether= 2: 3 -> ethyl acetate only) to afford the titled compound (**3-7**) as white solid; 10.9 g (y= 89.3%).



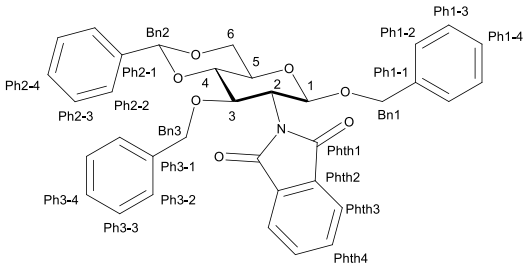
^{1}H NMR[123, 124] (500MHz, $CDCl_3$, δ ($CHCl_3$)=7.270): 2.40 (1H, d, $J_{3OH\text{-}H3}$ 3.5 Hz, 3-O*H*), 3.65 (1H, dd, $J_{H4\text{-}H5}$ 9.5Hz , $J_{H4\text{-}H3}$ 8.0Hz, H_4), 3.68 (1H, ddd, $J_{H5\text{-}H6ax}$ 10.5Hz, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6eq}$ 4.5Hz, H_5), 3.88 (1H, dd, $J_{H6ax\text{-}H6eq}$ 10.5Hz, $J_{H6ax\text{-}H5}$ 10.5Hz, H_{6ax}), 4.32 (1H, dd, $J_{H2\text{-}H3}$ 10.0Hz , $J_{H2\text{-}H1}$ 8.5Hz, H_2), 4.44 (1H, dd, $J_{H6eq\text{-}H6ax}$ 10.5Hz, $J_{H6eq\text{-}H5}$ 4.5Hz, H_{6eq}), 4.54 (1H, d, $J_{HBn1\text{-}HBn1'}$ 12.0Hz, H_{Bn1}), 4.66 (1H, ddd, $J_{H3\text{-}H2}$ 10.5Hz, $J_{H3\text{-}H4}$ 8.5Hz, $J_{H3\text{-}3OH}$ 3.0Hz, H_3), 4.86 (1H, d, $J_{HBn1'\text{-}HBn1}$ 12.5Hz, $H_{Bn1'}$), 5.29 (1H, d, $J_{H1\text{-}H2}$ 8.5Hz, H_1), 5.59 (1H, s, H_{Bn2}), 7.04-7.13 (5H, m,

H_{Ph1-2} H_{Ph1-3}, H_{Ph1-4}), 7.36-7.42 (3H, m, H_{Ph2-3}, H_{Ph2-4}), 7.48-7.53 (2H, m, H_{Ph2-2}), 7.70-7.75 (2H, m, H_{Phth4}), 7.75-7.87 (2H, br, H_{Phth3}). ^{13}C NMR[123, 124] (126MHz, $CDCl_3$, δ ($CHCl_3$)=77.00): 56.52 (C_2), 66.08 (C_5), 68.51 (C_3), 68.71 (C_6), 71.25 (C_{Bn1}), 82.19 (C_4), 97.89 (C_1), 101.99 (C_{Bn2}), 123.40 (C_{Phth3}), 126.29 (C_{Ph2-2}), 127.66, 127.74, 128.23 (C_{Ph2}, C_{Ph3}, C_{Ph4}), 128.41, 129.38 (C_{Ph2-3}, C_{Ph2-4}), 131.64 (C_{Phth2}), 133.99 (C_{Phth4}), 136.81, 136.94 (C_{Ph1-1}, C_{Ph2-1}), 167.90 (C_{Phth1}). LRMS (ESI^+): m/z $[M+Na]^+$ 510.14. $[\alpha]_D^{20}$= -79.7° (c 1.09, $CHCl_3$) (lit[123]. $[\alpha]_D^{21}$=-81.8° (c= 1.67, $CHCl_3$), lit[124]. $[\alpha]_D^{25}$=-81.6° (c= 0.25, $CHCl_3$)). m.p. 181-2°C (EtOAc-petroleum ether) (lit[123]. mp 189°C, lit[124]. mp 187-188°C). IR (ν_{max}, cm^{-1}, neat): 3481 (broad, O-H stretch), 3063, 3032 (C-H stretch), 1775, 1710 (imide C=O stretch), 1388, 1096, 1043, 996, 753, 721, 698, 669. R_f= 0.27 (Ethyl acetate: Petroleum ether= 2: 3) (lit[123]. R_f= 0.44 (Ethyl acetate: Petroleum ether= 3: 7), lit[124]. R_f= 0.57 (Ethyl acetate: toluene= 1: 2)).

Preparation of benzyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-8**)

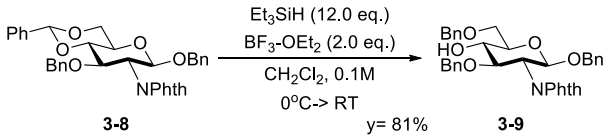


Sodium hydride, 60% in mineral oil (1.64 g, 41.0 mmol, 2.0 eq.) was washed with hexane (10 mL x2), dried in vacuo and suspended in DMF (41 mL). Benzyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-7**, 10.0 g, 20.5 mmol) was added portionwise and the mixture was stirred at room temperature for 10 min, followed by addition of tetrabutylammonium iodide (379 mg, 1.03 mmol, 5 mol%) and benzyl bromide (4.87 mL, 41.0 mmol, 2.0 eq.). The resultant solution was stirred at room temperature for 1 hr. The reaction mixture was poured into a stirred biphasic solution of 5% NH_4Cl (400 mL)-diethyl ether (400 mL). The mixture was filtered through celite (10 g), followed by washing with 5% NH_4Cl (100 mL) and diethyl ether (100 mL). After separation, the aqueous phase was extracted with diethyl ether (400 mL) and the combined organic phase was washed with brine (400 mL), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel: 1,400 mL, eluent: ethyl acetate: petroleum ether= 1: 3 -> 1: 2) to afford the titled compound (**3-8**) as colorless syrup; 10.1 g (y= 85.6%).



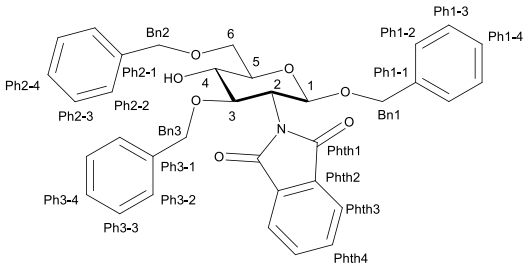
^{1}H NMR[123] (400MHz, $CDCl_3$, δ ($CHCl_3$)=7.270): 3.66 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6ax}$ 10.0Hz, $J_{H5\text{-}H6eq}$ 5.2Hz, H_5), 3.85 (1H, dd, $J_{H4\text{-}H3}$ 9.2Hz, $J_{H4\text{-}H5}$ 9.2Hz, H_4), 3.90 (1H, dd, $J_{H6ax\text{-}H5}$ 10.0Hz, $J_{H6ax\text{-}H6eq}$ 10.0Hz, H_{6ax}), 4.28 (1H, dd, $J_{H2\text{-}H3}$ 10.4Hz, $J_{H2\text{-}H1}$ 8.4Hz, H_2), 4.43 (1H, dd, $J_{H3\text{-}H2}$ 10.4Hz , $J_{H3\text{-}H4}$ 9.2Hz, H_3), 4.45 (1H, dd, $J_{H6eq\text{-}H6ax}$ 10.0Hz, $J_{H6eq\text{-}H5}$ 5.2Hz, H_{6eq}), 4.496 (1H, d, $J_{Bn1/3\text{-}Bn1/3'}$ 12.4Hz, $H_{Bn1/3}$), 4.500 (1H, d, $J_{Bn3/1\text{-}Bn3/1'}$ 12.4Hz, $H_{Bn3/1}$), 4.79 (1H, d, $J_{Bn3/1'\text{-}Bn3/1}$ 12.0Hz, $H_{Bn3/1}$'), 4.82 (1H, d, $J_{Bn1/3'\text{-}Bn1/3}$ 12.0Hz, $H_{Bn1/3}$'), 5.22 (1H, d, $J_{H1\text{-}H2}$ 8.4Hz, H_1), 5.65 (1H, s, H_{Bn2}), 6.82-6.95 (3H, m, $H_{Ph1,3}$), 6.96-7.12 (7H, m, $H_{Ph1,3}$), 7.31-7.46 (3H, m, $H_{Ph2\text{-}3}$, $H_{Ph2\text{-}4}$), 7.48-7.53 (3H, m, $H_{Ph2\text{-}2}$, H_{Phth3}), 7.65-7.76 (2H, br, H_{Phth4}), 7.77-7.95 (1H, br, $H_{Phth3'}$). ^{13}C NMR[123] (101MHz, $CDCl_3$, δ ($CHCl_3$)=77.00): 55.76 (C_2), 65.97 (C_5), 68.75 (C_6), 71.12 (C_{Bn1}), 73.99 (C_{Bn3}), 74.33 (C_3), 82.96 (C_4), 97.82 (C_1), 101.28 (C_{Bn2}), 123.20 (C_{Phth3}), 126.01 ($C_{Ph2\text{-}2}$), 127.31, 127.59, 127.63, 127.96, 128.02, 128.13, 128.26, 128.99($C_{Ph1,3\text{-}2,3,4}$), 131.51 (C_{Phth2}), 133.63 (C_{Phth4}), 136.78, 137.29, 137.84 ($C_{Ph1\text{-}1}$, $C_{Ph2\text{-}1}$, $C_{Ph3\text{-}1}$), 167.50 (C_{Phth1}). LRMS (ESI^+): m/z $[M+Na]^+$ 600.18. $[\alpha]_D^{20}$= +0.1° (c 1.13, $CHCl_3$) (lit[124]. $[\alpha]_D^{25}$= -2.5°, c=0.6, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 3063, 3032, 2944, 2880(C-H stretch), 1775, 1715 (imide C=O stretch), 1387, 1104, 1077, 1028, 999, 751, 722, 698. R_f= 0.28 (Ethyl acetate: Petroleum ether= 1: 3) (lit[123]. R_f= 0.76 (Ethyl acetate: Petroleum ether= 3: 7), lit[124]. R_f= 0.43 (methanol: chloroform= 1: 10)).

Preparation of benzyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-9**)



To a solution of benzyl 3-*O*-benzyl-4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-8**, 9.50 g, 16.4 mmol) in dichloromethane (164 mL) were added triethylsilane (31.4 mL, 197 mmol, 12.0 eq.) and boron trifluoride diethyl etherate (4.05 mL, 32.9 mmol, 2.0 eq.) at 0°C. The reaction mixture was stirred at this temperature for 2 hr and then warmed to room temperature, stirring for additional 5 hr. The resultant solution was poured into sat. $NaHCO_3$ (150 mL). After separation, the aqueous phase was extracted with dichlromethane (150 mL) and the combined organic phase was washed with brine (100 mL),

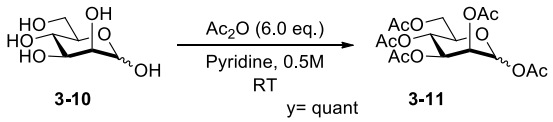
dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel: 1,500 mL, eluent: ethyl acetate: petroleum ether= 1: 2) to afford the titled compound (**3-9**) as colorless syrup; 7.72 g (y= 81.0%).



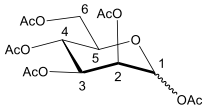
^{1}H NMR[124, 125] (400MHz, $CDCl_3$, δ ($CHCl_3$)=7.270): 2.93 (1H, s, 4-O*H*), 3.66 (1H, dt, $J_{H5\text{-}H4}$ 9.6Hz, $J_{H5\text{-}H6}$ 4.8Hz, H_5), 3.79-3.90 (3H, m, H_4, H_6 x2), 4.19-4.30 (2H, m, H_2, H_3), 4.49 (1H, d, $J_{Bn\text{-}Bn'}$ 12.4Hz, H_{Bn}), 4.53 (1H, d, $J_{Bn\text{-}Bn'}$ 12.4Hz, H_{Bn}), 4.61 (1H, d, $J_{Bn\text{-}Bn'}$ 11.6Hz, H_{Bn}), 4.68 (1H, d, $J_{Bn'\text{-}Bn}$ 12.0Hz, $H_{Bn'}$), 4.74 (1H, d, $J_{Bn'\text{-}Bn}$ 12.4Hz, $H_{Bn'}$), 4.80 (1H, d, $J_{Bn'\text{-}Bn}$ 12.4Hz, $H_{Bn'}$), 5.17 (1H, d, $J_{H1\text{-}H2}$ 6.8Hz, H_1), 6.91-7.00 (3H, m , H_{Ph}), 7.01-7.13 (7H, m, H_{Ph}), 7.30-7.46 (5H, m, H_{Ph}), 7.51-7.64 (1H, br, H_{Phth3}), 7.64-7.75 (2H, br, H_{Phth4}), 7.75-7.87 (1H, br, $H_{Phth3'}$). ^{13}C NMR[124, 125] (101MHz, $CDCl_3$, δ ($CHCl_3$)=77.00): 55.34 (C_2), 70.64 (C_6), 70.78 (C_{Bn}), 73.47 (C_5), 73.75 (C_{Bn}), 74.22 (C_{Bn}), 74.31 (C_4), 78.47 (C_3), 97.33 (C_1), 123.17 (C_{Phth3}), 127.37, 127.52, 127.56, 127.79, 127.84, 127.88, 128.09, 128.09, 128.49 (C_{Ph} x 9), 131.58 (C_{Phth2}), 133.65 (C_{Phth4}), 137.07, 137.59, 138.13 (C_{Ph} x 3), 167.59 (C_{Phth1}). LRMS (ESI$^+$): m/z $[M+Na]^+$ 602.19. $[\alpha]_D^{20}$= -10.3° (c 1.18, $CHCl_3$) (lit[124]. $[\alpha]_D^{25}$=-9.4°, c= 0.8, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 3477 (broad, O-H stretch), 3063, 3030, 2877(C-H stretch), 1775, 1712 (imide C=O stretch), 1388, 1108, 1071, 1028, 738, 722, 698. R_f= 0.31 (Ethyl acetate: Petroleum ether= 2: 3) (lit[124]. R_f= 0.55 (Ethyl acetate: toluene= 1: 2)).

Synthesis of β-Man unit

Preparation of 1,2,3,4,6-penta-*O*-acetyl-D-(+)-mannopyranoside (**3-11**)

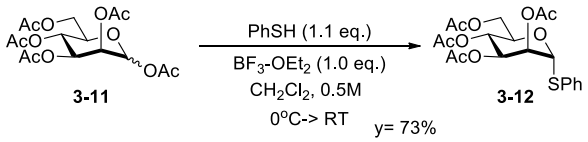


To a solution of D-(+)-mannopyranoside (**3-10**, 20.0 g, 111 mmol) in pyridine (222 mL) was added acetic anhydride (63 mL, 666 mmol, 6.0 eq.). The reaction mixture was stirred at room temperature for 24 hr. After concentration by rotary evaporator, the residual reagents were further removed by azeotropic evaporation with toluene. The residue was purified by column chromatography (silica gel: 1,000 mL, eluent: ethyl acetate: petroleum ether= 1: 1) to afford the titled compound (**3-11**) as colorless syrup; 46.1 g (y= quant, α: β= 0.22- 0.49: 1).

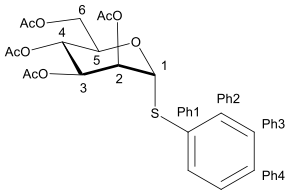


^{1}H NMR[126] (400MHz, $CDCl_3$, δ ($CHCl_3$)=7.270): α-form 2.01, 2.06, 2.10, 2.11, 2.22 (5 x 3H, 5 x s, 5 x OCOCH_3), 3.81 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6'}$ 5.2Hz, $J_{H5\text{-}H6}$ 2.4Hz, H_5), 4.14 (1H, dd, $J_{H6\text{-}H6'}$ 12.8Hz, $J_{H6\text{-}H5}$ 2.4Hz, H_6), 4.31 (1H, dd, $J_{H6'\text{-}H6}$ 12.4Hz, $J_{H6'\text{-}H5}$ 5.2Hz, H_6'), 5.13 (1H, dd, $J_{H3\text{-}H4}$ 10.4Hz, $J_{H3\text{-}H2}$ 3.2Hz, H_3), 5.30 (1H, dd, $J_{H4\text{-}H3}$ 9.6Hz, $J_{H4\text{-}H5}$ 9.6Hz, H_4), 5.49 (1H, d, $J_{H2\text{-}H3}$ 2.8Hz, H_2), 5.86 (1H, s, H_1). β-form 2.01, 2.06, 2.10, 2.17, 2.18 (5 x 3H, 5 x s, 5 x OCOCH_3), 4.01-4.4.10 (1H, m, H_5), 4.10 (1H, dd, $J_{H6\text{-}H6'}$ 12.4Hz, $J_{H6\text{-}H5}$ 2.4Hz, H_6), 4.28 (1H, dd, $J_{H6'\text{-}H6}$ 12.4Hz, $J_{H6'\text{-}H5}$ 4.8Hz, H_6'), 5.26 (1H, dd, $J_{H2\text{-}H3}$ 2.4Hz, $J_{H2\text{-}H1}$ 2.0Hz, H_2), 5.31-5.39 (2H, m, H_3, H_4), 6.09 (1H, d, $J_{H1\text{-}H2}$ 1.6Hz, H_1). ^{13}C NMR[126] (101MHz, $CDCl_3$, δ ($CHCl_3$)=77.00): α-form 20.51, 20.64, 20.69, 20.72, 20.74 (5 x OCOCH_3), 62.00 (C_6), 65.31 (C_4), 68.12 (C_2), 70.59 (C_3), 73.24 (C_5), 90.35 (C_1), 168.35, 169.54, 169.77, 170.19, 170.64 (4 x OCOCH_3). β-form 20.61, 20.63, 20.68, 20.74, 20.83 (5 x OCOCH_3), 62.04 (C_6), 65.45 (C_4), 68.27 (C_2), 68.68 (C_3), 70.55 (C_5), 90.54 (C_1), 168.04, 169.50, 169.72, 169.97, 170.62 (5 x OCOCH_3). LRMS (ESI^+): m/z $[M+Na]^+$ 413.06. R_f= 0.35 (ethyl acetate: petroleum ether= 1: 1).

Preparation of phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-(+)-mannopyranoside (**3-12**)

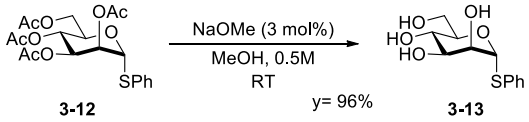


To a solution of 1,2,3,4,6-penta-*O*-acetyl-D-(+)-mannopyranoside (**3-11**, 20.0 g, 51.2 mmol, α: β= 0.22: 1) and thiophenol (7.91 mL, 76.8 mmol, 1.5 eq.) in dichloromethane (102 mL) was added boron trifluoride diethyl etherate (6.32 mL, 51.2 mmol, 1.0 eq.) at 0°C. The reaction mixture was stirred at this temperature for 1 hr and wamed to room temperature, stirring for additional 42 hr. The resultant solution was poured into sat. $NaHCO_3$ (200 mL). After separation, the aqueous phase was extracted with dichloromethane (100 mL x2) and the combined organic phase was washed with brine (100 mL), dried over $MgSO_4$ and concentrated. The residue (α: β= 1: 0.16) was purified by column chromatography (silica gel: 1,000 mL, eluent: ethyl acetate: petroleum ether= 1: 2) to afford the titled compound (**3-12**) as white solid; 16.4 g (y= 72.6%).



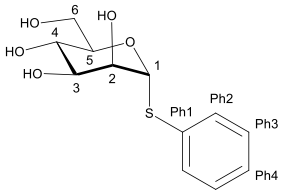
^{1}H NMR[99, 127] (400MHz, $CDCl_3$, δ ($C\underline{H}Cl_3$)=7.270): 2.02, 2.06, 2.08, 2.16 (4 x 3H, 4 x s, 4 x OCOC$\underline{H}_3$), 4.11 (1H, dd, $J_{H6\text{-}H6'}$ 12.4Hz, $J_{H6\text{-}H5}$ 2.0Hz, H_6), 4.31 (1H, dd, $J_{H6'\text{-}H6}$ 12.4Hz, $J_{H6'\text{-}H5}$ 5.6Hz, $H_{6'}$), 4.55 (1H, ddd, $J_{H5\text{-}H4}$ 9.6Hz, $J_{H5\text{-}H6'}$ 5.6Hz, $J_{H5\text{-}H6}$ 2.0Hz, H_5), 5.32 (1H, dd, $J_{H3\text{-}H4}$ 9.6Hz, $J_{H3\text{-}H2}$ 2.8Hz, H_3), 5.34 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, H_4), 5.50 (1H, d, $J_{H1\text{-}H2}$ 1.6Hz, H_1), 5.51 (1H, dd, $J_{H2\text{-}H3}$ 2.8Hz, $J_{H2\text{-}H1}$ 1.2Hz, H_2), 7.27-7.36 (3H, m, $H_{Ph2/Ph3}$, H_{Ph4}), 7.46-7.52 (2H, m, $H_{Ph2/Ph3}$). ^{13}C NMR[99, 127] (101MHz, $CDCl_3$, δ ($\underline{C}HCl_3$)=77.00): 20.60, 20.65, 20.67, 20.83 (4 x OCO$\underline{C}H_3$), 62.39 (C_6), 66.31 (C_4), 69.32 (C_3), 69.45 (C_5), 70.84 (C_2), 85.63 (C_1), 128.07 (C_{Ph4}), 129.15, 132.00 (C_{Ph2}, C_{Ph3}), 132.56 (C_{Ph1}), 169.70, 169.77, 169.87, 170.50 (4 x O$\underline{C}$OCH$_3$). LRMS (ESI$^+$): m/z $[M+Na]^+$ 463.08. $[\alpha]_D^{20}$= +116° (c 1.05, $CHCl_3$) (lit[99]. $[\alpha]_D^{23}$=+107°, c=1.07, $CHCl_3$). m.p. 86-87°C (EtOAc-petroleum ether) (lit.[128] 87°C, diethyl ether-light petroleum). IR (ν_{max}, cm^{-1}, neat): 1746 (ester C=O stretch), 1440, 1369, 1223, 1105, 1051, 749, 692. R_f= 0.24 (ethyl acetate: petroleum ether= 1: 1) (lit[127]. R_f= 0.49 (ethyl acetate: hexane= 1: 1)).

Preparation of phenyl 1-thio-α-D-(+)-mannopyranoside (**3-13**)



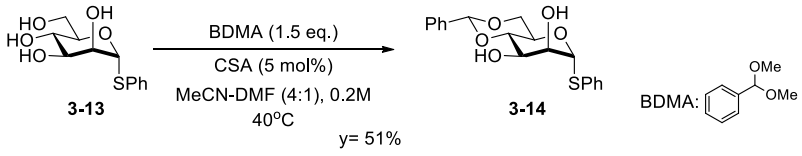
3-12 **3-13**

To a suspension of phenyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-(+)-mannopyranoside (**3-12**, 25.0 g, 56.8 mmol) in methanol (114 mL) was added 25 wt% sodium methoxide in methanol (390 μL, 1.70 mmol, 3.0 mol%). The solution was stirred at room temperature for 1 hr. The reaction was quenched by adding Dowex 50WX2-400 (708 mg, 3.40 meq, 6.0 mol%), followed by concentration. The residue was purified by column chromatography (silica gel: 1,000 mL, eluent: dichloromethane: methanol= 6: 1) to afford the titled compound (**3-13**) as white solid; 14.9 g (y= 96.1%).

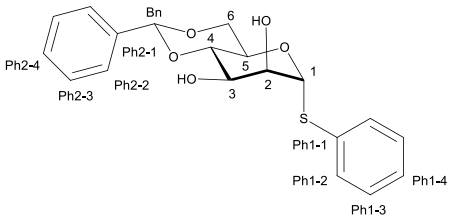


^{1}H NMR[127] (400Hz, CD_3OD,δ (CH_3OH)= 3.310): 3.69 (1H, dd, $J_{H3\text{-}H4}$ 9.2Hz, $J_{H3\text{-}H2}$ 3.2Hz, H_3), 3.73 (1H, dd, $J_{H4\text{-}H3}$ 9.2Hz, $J_{H4\text{-}H5}$ 9.2Hz, H_4), 3.77 (1H, dd, $J_{H6\text{-}H6'}$ 12.0Hz, $J_{H6\text{-}H5}$ 5.2Hz, H_6), 3.82 (1H, dd, $J_{H6'\text{-}H6}$ 12.0Hz, $J_{H6'\text{-}H5}$ 2.8Hz, $H_{6'}$), 4.04 (1H, ddd, $J_{H5\text{-}H4}$ 8.8Hz, $J_{H5\text{-}H6}$ 5.2Hz, $J_{H5\text{-}H6'}$ 2.8Hz, H_5), 4.09 (1H, dd, $J_{H2\text{-}H3}$ 3.2Hz, $J_{H2\text{-}H1}$ 1.6Hz, H_2), 5.43 (1H, d, $J_{H1\text{-}H2}$ 1.6Hz, H_1), 7.24-7.35 (3H, m, $H_{Ph2/Ph3}$, H_{Ph4}), 7.49-7.56 (2H, m, $H_{Ph2/Ph3}$). ^{13}C NMR[127] (101MHz, CD_3OD, δ (CH_3OH)= 49.15): 62.77 (C_6), 68.84 (C_4), 73.31 (C_3), 73.92 (C_2), 75.80 (C_5), 90.60 (C_1), 128.65 (C_{Ph4}), 130.21, 133.08(C_{Ph2}, C_{Ph3}), 136.06 (C_{Ph1}). LRMS (ESI^+): m/z $[M+Na]^+$ 295.06. $[\alpha]_D^{20}$=+252.7° (c 1.04, DMSO) (lit.[128] $[\alpha]_D^{27}$=+253.2°, c 3.0, ethanol)). m.p. 127-9°C (DCM-MeOH) (lit.[128] 128-9°C, Et_2O-EtOH). IR (ν_{max}, cm^{-1}, neat): 3387, 3344, 3205 (broad, O-H stretch), 2969, 2939, 2918, 2873 (C-H sretch), 1581, 1480, 1438, 1404, 1388, 1360, 1326, 1293, 1272, 1254, 1211, 1185, 1159, 1121, 1066, 1040, 1022, 963, 919, 887, 854, 796, 744, 690, 663, 649. R_f= 0.29 (methanol: dichloromethane= 1: 6) (lit[127]. R_f= 0.29 (methanol: chloroform= 1: 5)).

Preparation of phenyl 4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-14**)

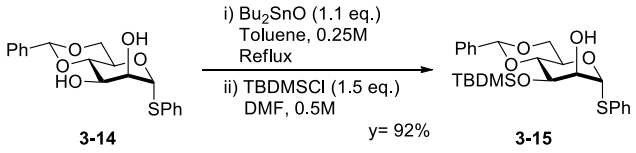


Phenyl 1-thio-α-D-(+)-mannopyranoside (**3-13**, 15.0 g, 55.1 mmol) was suspended in DMF (55 mL)-MeCN (221 mL) and the suspension was warmed up to 40°C to afford a homogeneous clear solution. Benzaldehyde dimethyl acetal (BDMA, 12.5 mL, 82.7 mmol, 1.5 eq.) and (±)-camphor-10-sulfonic acid (CSA, 640 mg, 2.76 mmol, 5 mol%) were added and the solution was stirred at the same temperature for 6 hr, during which precipitation formed. The resultant mixture was cooled to room temperature and Et_3N (461 μL, 3.31 mmol, 6 mol%) was added. The suspension was cooled to 0°C and stirred for 1 hr. The precipitation was collected by filtration, followed by washing with ice-cold MeCN (100 mL). The white solid was dried in vacuo to give the titled compound (**3-14**); 10.2 g (y= 51.3%).



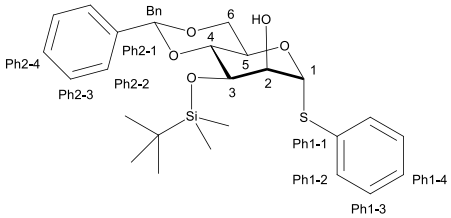
^{1}H NMR (400MHz, DMSO-d_6,δ ((CH_3)$_2$SO)= 2.50): 3.72-3.83 (2H, m, H_3, H_{6ax}), 3.97 (1H, dd, $J_{H4\text{-}H3}$ 9.2Hz, $J_{H4\text{-}H5}$ 9.2Hz, H_4), 4.02 (1H, dd, $J_{H2\text{-}2OH}$ 3.2Hz, $J_{H2\text{-}H3}$ 3.2Hz, H_2), 4.04-4.13 (2H, m, H_5, H_{6eq}), 5.25 (1H, d, $J_{3OH\text{-}H3}$ 6.0Hz ,3-O*H*), 5.48 (1H, s, H_1), 5.57 (1H, d, $J_{2OH\text{-}H2}$ 4.0Hz ,2-O*H*), 5.64 (1H, s, H_{Bn}), 7.28-7.43 (6H, m, H_{Ph}), 7.44-7.51 (4H, m, H_{Ph}). ^{13}C NMR (101MHz, DMSO-d_6,δ ((*C*H$_3$)$_2$SO)= 39.51): 65.31 (C_5), 67.61 (C_6), 68.09 (C_3), 72.42 (C_2), 78.48 (C_4), 89.28 (C_1), 101.21 (C_{Bn}), 126.43, 127.47, 128.04, 128.89, 129.29, 131.32, 133.68, 137.85 (C_{Ph}). LRMS (ESI$^+$): m/z [M+Na]$^+$ 383.08. $[\alpha]_D^{20}$= +280.5° (c 0.41, acetone) (lit[129]. $[\alpha]_D^{27}$=+294.6°, c 0.5, acetone). m.p. 195-8°C (MeCN-DMF) (lit.[129] 182-184°C (methanol), lit.[130] 213-214°C (ethyl acetate), lit.[101]. 203°C (ethyl acetate)). IR (ν_{max}, cm^{-1}, neat): 3334, 3196 (broad, O-H stretch), 2977, 2948, 2894 (C-H sretch), 1474, 1451, 1440, 1401, 1385, 1371, 1353, 1338, 1322, 1288, 1266, 1253, 1217, 1162, 1123, 1080, 1033, 1008, 974, 928, 909, 861, 790, 782, 752, 744, 700, 689, 671, 664, 635. (lit.[130] IR (ν_{max}, cm^{-1}, KBr) 3346, 1452). R_f= 0.29 (ethyl acetate: petroleum ether= 1: 1).

Preparation of phenyl 3-*O*-*tert*-butyldimethylsilyl-4,6-*O*-benzylidene-1-thio-α-D-(+)-manno-pyranoside (**3-15**)



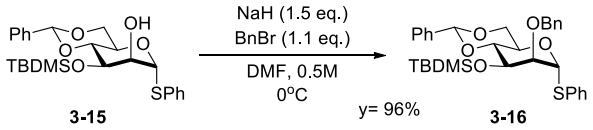
Phenyl 4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-14**, 15.0 g, 41.6 mmol) and dibutyltin oxide (11.4 g, 45.8 mmol, 1.1 eq.) was suspended in toluene (166 mL) and the mixture was refluxed for 2 hr. The resultant clear solution was cooled to room temperature and the solvent was removed by evaporation. The residue was dissolved in DMF (83 mL) and *tert*-butyldimethylsilyl chloride (9.40 g, 62.4 mmol, 1.5 eq.) was added. The solution was stirred at room temperature. The resultant solution was poured into a stirred biphasic solution of 5% NH_4Cl (400 mL)-diethyl ether (400 mL). After separation, the aqueous phase was extracted with diethyl ether (400 mL) and the combined organic phase was washed with brine (200 mL), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel: 1,500 mL, eluent: ethyl acetate: petroleum ether= 1: 6). When contaminated by dibutyltin oxide,

washing with diethyl ether can remove it as precipitation. The titled compound (**3-15**) was obtained as white solid; 18.4 g (y= 92%).



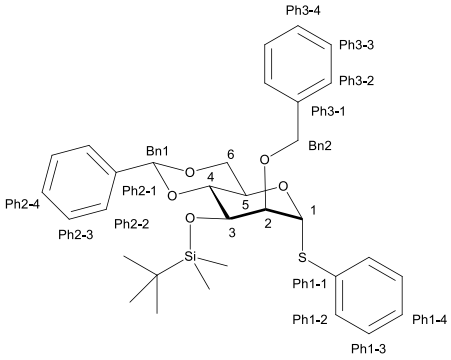
^{1}H NMR (400MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 0.11, 0.16 (3H x 2, s x 2, Si(CH_3)$_2$^{t}Bu), 0.92 (9H, s, $SiMe_2C(CH_3)_3$), 3.04 (1H, s, 2-O*H*), 3.84 (1H, dd, $J_{H6ax\text{-}H5}$ 10.4Hz, $J_{H6ax\text{-}H6eq}$ 10.4Hz, H_{6ax}), 3.95 (1H, dd, $J_{H4\text{-}H3}$ 9.2Hz, $J_{H4\text{-}H5}$ 9.2Hz, H_4), 4.14 (1H, dd, $J_{H3\text{-}H4}$ 8.8Hz, $J_{H3\text{-}H2}$ 3.6Hz, H_3), 4.16 (1H, d, $J_{H2\text{-}H3}$ 4.0Hz, H_2), 4.21 (1H, dd, $J_{H6eq\text{-}H6ax}$ 10.4Hz, $J_{H6eq\text{-}H5}$ 4.8Hz, H_{6eq}), 4.33 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6ax}$ 10.0Hz, $J_{H5\text{-}H6eq}$ 4.8Hz, H_5), 5.57 (1H, s, H_{Bn}), 5.67 (1H, s, H_1), 7.27-7.44 (6H, m, H_{Ph}), 7.46-7.57 (4H, m, H_{Ph}). ^{13}C NMR (101MHz, $CDCl_3$, δ ($CHCl_3$)= 77.00): -5.04, -4.38 (Si(CH)$_2$^{t}Bu), 18.11 ($SiMe_2C(CH_3)_3$), 25.70 ($SiMe_2C(CH_3)_3$), 64.40 (C_5), 68.47 (C_6), 70.09 (C_3), 73.36 (C_2), 79.13 (C_4), 87.46 (C_1), 101.86 (C_{Bn}), 126.09, 127.63, 128.11, 128.90, 129.12, 131.65, 133.36, 137.35 (C_{Ph}). LRMS (ESI$^+$): m/z [M+Na]$^+$ 497.16. $[\alpha]_D^{20}$= +212.2° (c 1.05, $CHCl_3$). m.p. 81-83°C (EtOAc- petroleum ether). IR (ν_{max}, cm^{-1}, neat): 3237 (broad, O-H stretch), 2952, 2929, 2896, 2857 (C-H sretch), 1584, 1473, 1440, 1381, 1311, 1256, 1217, 1153, 1098, 1026, 914, 879, 856, 839, 778, 744, 696, 669, 612. R_f= 0.28 (ethyl acetate: petroleum ether= 1: 5).

Preparation of phenyl 2-*O*-benzyl-3-*O*-*tert*-butyldimethylsilyl-4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-16**)



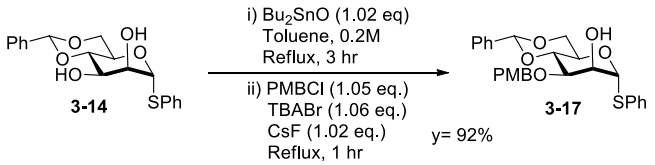
Sodium hydride, 60% in mineral oil (1.14 g, 28.4 mmol, 1.5 eq.) was washed with hexane (10 mL x2), dried in vacuo and suspended in DMF (38 mL). Benzyl bromide (2.48 mL, 20.9 mmol, 1.1 eq.) was added, the suspension was cooled to 0°C and finally (otherwise significant amount of side products was observed) phenyl 3-*O*-*tert*-butyldimethylsilyl-4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-15**, 9.00 g, 19.0 mmol) was added portionwise. The reaction mixture was stirred at the same temperature for 30 min and poured into a stirred biphasic solution of 5% NH_4Cl (200 mL)-diethyl ether (200 mL). After separation, the aqueous phase was extracted with diethyl ether (200 mL). The combined organic phase was washed with brine (100 mL), dried over $MgSO_4$ and concentrated. The residue was purified by column

chromatography (silica gel: 1,000 mL, eluent: dichloromethane: petroleum ether= 3: 2) to afford the titled compound (**3-16**) as colorless syrup; 10.2 g (y= 95.6%).



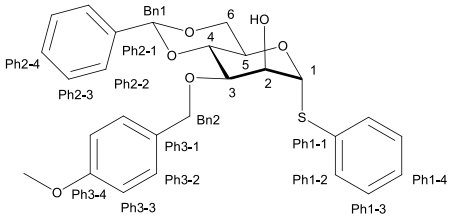
^{1}H NMR[131] (400MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 0.10, 0.14 (3H x 2, s x 2, Si(CH_3)$_2$^{t}Bu), 0.94 (9H, s, $SiMe_2C(CH_3)_3$), 3.88 (1H, dd, $J_{H6ax\text{-}H5}$ 10.0Hz, $J_{H6ax\text{-}H6eq}$ 10.0Hz, H_{6ax}), 3.98 (1H, s, H_2), 4.14 (1H, dd, $J_{H4\text{-}H3}$ 9.6Hz, $J_{H4\text{-}H5}$ 9.6Hz, H_4), 4.18-4.26 (2H, m, H_3, H_{6eq}), 4.30 (1H, ddd, $J_{H5\text{-}H4}$ 9.6Hz, $J_{H5\text{-}H6ax}$ 9.6Hz, $J_{H5\text{-}H6eq}$ 4.8Hz, H_5), 4.73 (1H, d, $J_{HBn2\text{-}HBn2'}$ 12.0Hz, H_{Bn2}), 4.88 (1H, d, $J_{HBn2'\text{-}HBn2}$ 12.0Hz, H_{Bn2}), 5.53 (1H, s, H_1), 5.62 (1H, s, H_{Bn1}), 7.27-7.44 (13H, m, H_{Ph}), 7.46-7.60 (2H, m, H_{Ph}). ^{13}C NMR[131] (101MHz, $CDCl_3$, δ ($CHCl_3$)= 77.00): -4.82, -4.46 (Si(CH)$_2$^{t}Bu), 18.35 ($SiMe_2C(CH_3)_3$), 25.86 ($SiMe_2C(CH_3)_3$), 65.54 (C_5), 68.47 (C_6), 70.77 (C_3), 73.85 (C_{Bn2}), 79.13 (C_4), 80.95 (C_2), 87.62 (C_1), 101.86 (C_{Bn1}), 126.20, 127.53, 127.80, 127.94, 128.06, 128.41, 128.82, 129.10, 131.47, 133.99, 137.54, 137.96 (C_{Ph}). LRMS (ESI$^+$): m/z [M+Na]$^+$ 587.19. $[\alpha]_D^{20}$= +116.8° (c 1.05, $CHCl_3$) (lit.[131] $[\alpha]_D^{26}$=+104.1°, c 1.0, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 3064, 3034, 2953, 2928, 2896, 2857 (C-H sretch), 1584, 1497, 1472, 1456, 1440, 1384, 1360, 1312, 1253, 1214, 1167, 1155, 1134, 1099, 1067, 1027, 1014, 966, 939, 881, 860, 837, 779, 740, 697, 676, 656, 618. R_f= 0.30 (dichloromethane: petroleum ether= 3: 2).

Preparation of phenyl 3-*O*-*p*-methoxybenzyl-4,6-*O*-benzylidene-1-thio-α-D-(+)-manno-pyranoside (**3-17**)



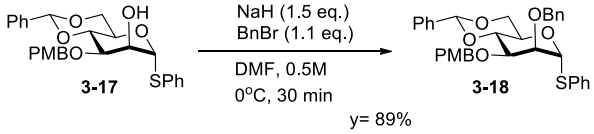
Phenyl 4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-14**, 2.0 g, 5.55 mmol) and dibutyltin oxide (1.41 g, 5.66 mmol, 1.02 eq.) was suspended in toluene (28 mL) and the mixture was refluxed for 3 hr. The resultant clear solution was cooled to room temperature. To the solution were added tetra-*n*-butylammonium bromide (TBAB, 1.90 g, 5.88 mmol, 1.06 eq.), cesium fluoride (CsF, 860 mg, 5.66 mmol, 1.02 eq.) and *p*-methoxybenzyl chloride (790 μL,

5.83 mmol, 1.05 eq.) and the mixture was refluxed for 1 hr. After being cooled, the resultant solution was poured into 5% NH_4Cl (15 mL). After separation, the aqueous phase was extracted with diethyl ether (15 mL) and the combined organic phase was washed with brine (10 mL), dried over $MgSO_4$ and concentrated. The residue was purified by Biotage Flash Purification System (cartridge; SNAP KP-Sil 100 g, eluent gradient; ethyl acetate/ petroleum ether = 8% -> 66 %, 10CV) to afford the titled compound (**3-17**) as white foam; 2.43 g (y= 91%).



^{1}H NMR[101, 130] (400MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 2.09 (1H, d, $J_{2OH\text{-}H2}$ 1.6Hz, 2-O*H*), 3.83 (3H, s, OCH_3), 3.86 (1H, dd, $J_{H6ax\text{-}H5}$ 10.4Hz, $J_{H6ax\text{-}H6eq}$ 10.4Hz, H_{6ax}), 3.96 (1H, dd, $J_{H3\text{-}H4}$ 9.6Hz, $J_{H3\text{-}H2}$ 3.2Hz, H_3), 4.17 (1H, dd, $J_{H4\text{-}H3}$ 9.2Hz , $J_{H4\text{-}H5}$ 9.2Hz, H_4), 4.22 (1H, dd, $J_{H6eq\text{-}H6ax}$ 10.4Hz, $J_{H6eq\text{-}H5}$ 4.8Hz, H_{6eq}), 4.24-4.27 (1H, m, H_2), 4.35 (1H, ddd, $J_{H5\text{-}H4}$ 9.6Hz, $J_{H5\text{-}H6ax}$ 9.6Hz, $J_{H5\text{-}H6eq}$ 4.8Hz, H_5), 4.69 (1H, d, $J_{Bn2\text{-}Bn2'}$ 11.2Hz, H_{Bn2}), 4.84 (1H, d, $J_{Bn2'\text{-}Bn2}$ 11.6Hz, $H_{Bn2'}$), 5.60 (1H, s, H_1), 5.63 (1H, s, H_{Bn1}), 6.90 (2H, d, $J_{HPh3\text{-}3\text{-}HPh3\text{-}2}$ 8.8Hz, $H_{Ph3\text{-}3}$), 7.27-7.35 (5H, m, H_{Ph}), 7.37-7.44 (3H, m, H_{Ph}), 7.44-7.48 (2H, m, H_{Ph}), 7.50-7.55 (2H, m, H_{Ph}). ^{13}C NMR (101MHz, $CDCl_3$, δ ($CHCl_3$)= 77.00): 55.26 (OCH_3), 64.58 (C_5), 68.51 (C_6), 71.36 (C_2), 72.87 (C_{Bn2}), 75.34 (C_3), 78.94 (C_4), 87.76 (C_1), 101.60 (C_{Bn1}), 113.92 ($C_{Ph3\text{-}3}$), 126.06, 127.66, 128.21, 128.95, 129.11, 129.61, 129.77, 131.69, 133.27, 137.43 (C_{Ph}), 159.49 ($C_{Ph3\text{-}4}$). LRMS (ESI^+): m/z $[M+Na]^+$ 503.2. $[\alpha]_D^{20}$= +219.0° (c 1.08, $CHCl_3$) [lit[130]. $[\alpha]_D^{20}$= +209° (c= 1.28, $CHCl_3$)]. m.p. 94-95°C (EtOAc-petroleum ether) [lit.[130] 100-101°C (diethyl ether- hexane)]. IR (ν_{max}, cm^{-1}, neat): 3446 (broad, O-H stretch), 2951, 2932, 2899 (C-H sretch), 1612, 1513, 1456, 1374, 1249, 1174, 1097, 1027, 781, 746, 699. (lit[130]. IR (ν_{max}, cm^{-1}, KBr) 3524, 2953, 2934, 2901, 2856, 1614, 1516, 1452, 1439). R_f= 0.29 (ethyl acetate: petroleum ether= 1: 2).

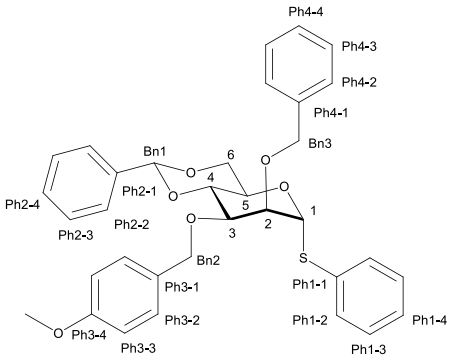
Preparation of phenyl 2-*O*-benzyl-3-*O*-*p*-methoxybenzyl-4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-18**)



y= 89%

Sodium hydride, 60% in mineral oil (125 mg, 3.12 mmol, 1.5 eq.) was washed with hexane (1 mL x2), dried in vacuo and suspended in DMF (4.2 mL). Benzyl bromide (BnBr, 272μL L, 2.29 mmol, 1.1 eq.) was added and the suspension was cooled to 0°C. Finally, phenyl

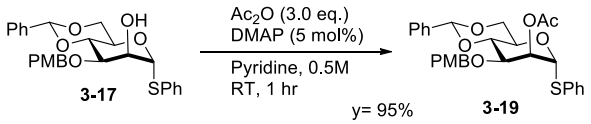
3-*O*-*p*-methoxybenzyl- 4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-17**, 1.00 g, 2.08 mmol, 1.0 eq.) was added portionwise. The reaction mixture was stirred at the same temperature for 30 min and poured into a stirred biphasic solution of 5% NH_4Cl (20 mL)-diethyl ether (20 mL). After separation, the aqueous phase was extracted with diethyl ether (20 mL). The combined organic phase was washed with brine (10 mL), dried over $MgSO_4$ and concentrated. The residue was purified by Biotage Flash Purification System (cartridge; SNAP KP-Sil 100 g, eluent gradient; ethyl acetate/ petroleum ether = 4% -> 34 %, 10CV) to afford the titled compound (**3-18**) as colorless syrup; 1.06 g (y= 89%).



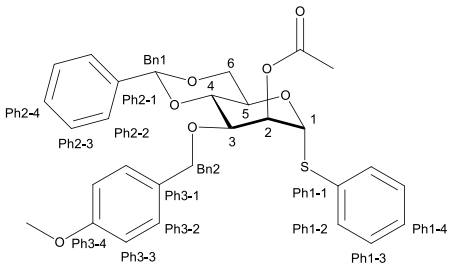
^{1}H NMR[101] (400MHz, $CDCl_3$, δ (C*H*Cl_3)= 7.270): 3.83 (3H, s, OCH_3), 3.90 (1H, dd, $J_{H6ax\text{-}H5}$ 10.0Hz, $J_{H6ax\text{-}H6eq}$ 10.0Hz, H_{6ax}), 3.97 (1H, dd, $J_{H3\text{-}H4}$ 9.6Hz, $J_{H3\text{-}H2}$ 3.2Hz, H_3), 4.02 (1H, dd, $J_{H2\text{-}H3}$ 2.8Hz, $J_{H26\text{-}H1}$ 1.2Hz, H_2), 4.23 (1H, dd, $J_{H6eq\text{-}H6ax}$ 10.0Hz, $J_{H6eq\text{-}H5}$ 4.0Hz, H_{6eq}), 4.27-4.35 (2H, m, H_4, H_5), 4.61 (1H, d, $J_{Bn\text{-}Bn'}$ 11.6Hz, $H_{Bn2/3}$), 4.73 (2H, s, $H_{Bn3/2}$), 4.76 (1H, d, $J_{Bn'\text{-}Bn}$ 12.0Hz, $H_{Bn2/3}$'), 5.51 (1H, d, $J_{H1\text{-}H2}$ 1.2Hz,H_1), 5.66 (1H, s, H_{Bn1}), 6.88 (2H, d, $J_{HPh3\text{-}3\text{-}HPh3\text{-}2}$ 8.4Hz, $H_{Ph3\text{-}3}$), 7.26-7.44 (15H, m, H_{Ph}), 7.52-7.56 (2H, m, H_{Ph}). ^{13}C NMR[101] (101MHz, $CDCl_3$, δ (*C*HCl_3)= 77.00):55.26 (O*C*H_3), 65.46 (C_5), 68.49 (C_6), 68.49 (C_6), 72.73, 73.00 (C_{Bn2}, C_{Bn3}), 75.79 (C_3), 78.02 (C_2), 78.98 (C_4), 87.10 (C_1), 101.47 (C_{Bn1}), 113.73 ($C_{Ph3\text{-}3}$), 126.08, 127.59, 127.81, 128.07, 128.16, 128.40, 128.83, 129.10, 129.32, 130.40, 131.60, 133.76, 137.58, 137.73 (C_{Ph}), 159.18 ($C_{Ph3\text{-}4}$). LRMS (ESI$^+$): m/z [M+Na]$^+$ 593.2. $[\alpha]_D^{20}$= +116.4° (c= 1.16, $CHCl_3$) [lit[101]. $[\alpha]_D$= +106.3° (c= 3.4, $CHCl_3$)]. IR (ν_{max}, cm^{-1}, neat): 3063, 3034, 2932, 2901, 2865 (C-H sretch), 1611, 1585, 1513, 1497, 1455, 1440, 1373, 1303, 1248, 1211, 1173, 1149, 1098, 1068, 1028, 970, 906, 848, 820, 774, 743, 698, 667, 641. R_f= 0.24 (ethyl acetate: petroleum ether= 1: 5).

Synthesis of α-Man unit

Preparation of phenyl 2-*O*-acetyl-3-*O*-*p*-methoxybenzyl-4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-19**)



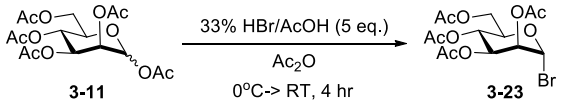
To a solution of phenyl 3-*O*-*p*-methoxybenzyl-4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-17**, 1.00 g, 2.08 mmol) in pyridine (4.2 mL) was added acetic anhydride (590 μL, 6.24 mmol, 3.0 eq.) and DMAP (12.7 mg, 0.104 mmol, 5 mol%). After stirring the reaction mixture for 1 hr, *t*he resultant solution was concentrated and the residue was dissolved in dichloromethane (20 mL) and washed with 10% $KHSO_4$ (20 mL). The aqueous phase was extracted with dichloromethane (20 mL x2) and the combined organic phase was washed with brine (10 mL), dried over $MgSO_4$ and concentrated. The residue was purified by Biotage Isolera Flash Purification System (cartridge; SNAP KP-Sil 50 g, eluent gradient: ethyl acetate/petroleum ether= 6% -> 50%, 10CV) to afford the titled compound (**3-19**) as white foam; 1.04 g (y= 95.4%).



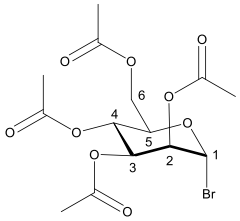
^{1}H NMR (400MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 2.17 (3H, s, $COCH_3$), 3.82 (3H, s, OCH_3), 3.87 (1H, dd, $J_{H6ax\text{-}H5}$ 10.4Hz, $J_{H6ax\text{-}H6eq}$ 10.4Hz, H_{6ax}), 4.01 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz , $J_{H3\text{-}H2}$ 3.2Hz, H_3), 4.14 (1H, dd, $J_{H4\text{-}H3}$ 10.0Hz, $J_{H4\text{-}H5}$ 10.0Hz, H_4), 4.24 (1H, dd, $J_{H6eq\text{-}H6ax}$ 10.4Hz, $J_{H6eq\text{-}H5}$ 4.80Hz, H_{6eq}), 4.37 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6ax}$ 10.0Hz, $J_{H5\text{-}H6eq}$ 4.8Hz, H_5), 4.62 (1H, d, $J_{Bn\text{-}Bn'}$ 11.6Hz, H_{Bn2}), 4.68 (1H, d, $J_{Bn'\text{-}Bn}$ 11.6Hz, $H_{Bn2'}$), 5.47 (1H, d, $J_{H1\text{-}H2}$ 1.2Hz, H_1), 5.62 (1H, dd, $J_{H2\text{-}H3}$ 3.2Hz, $J_{H2\text{-}H1}$ 1.2Hz, H_2), 5.65 (1H, s, H_{Bn1}), 6.88 (2H, d, $J_{HPh3\text{-}3\text{-}HPh3\text{-}2}$ 8.8Hz, $H_{Ph3\text{-}3}$), 7.27-7.38 (5H, m, H_{Ph}), 7.38-7.44 (3H, m, H_{Ph}), 7.44-7.48 (2H, m, H_{Ph}), 7.51-7.55 (2H, m, H_{Ph}). ^{13}C NMR (101MHz, $CDCl_3$, δ ($CHCl_3$)= 77.00): 20.99, ($COCH_3$), 55.24 (OCH_3), 65.20 (C_5), 68.38 (C_6), 71.35 (C_2), 72.01 (C_{Bn2}), 73.61 (C_3), 78.40 (C_4), 87.18 (C_1), 101.58 (C_{Bn1}), 113.81 ($C_{Ph3\text{-}3}$), 126.10, 127.98, 128.17, 128.94, 129.17, 129.51, 129.66, 132.08, 133.03, 137.35 (C_{Ph}), 159.33 ($C_{Ph3\text{-}4}$), 170.06 ($COCH_3$). LRMS (ESI$^+$): m/z $[M+Na]^+$ 545.2. HRMS (ESI$^+$): m/z $[M+Na]^+$ 545.1590 (found), (calcd 545.1604 for $C_{29}H_{30}NaO_7S$). $[\alpha]_D^{20}$= +109.3° (c 1.03, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 3062, 3036, 2956, 2935, 2900, 2873, 2837 (C-H sretch), 1745 (ester C=O

stretch), 1613, 1585, 1559, 1514, 1477, 1456, 1440, 1421, 1372, 1303, 1277, 1231, 1175, 1150, 1101, 1060, 1029, 967, 935, 905, 883, 849, 822, 782, 745, 699, 669, 641, 628. R_f= 0.24 (ethyl acetate: petroleum ether= 1: 5).

Preparation of 2,3,4,6-tetra-*O*-acetyl-α-D-(+)-mannopyranosyl bromide (**3-23**)

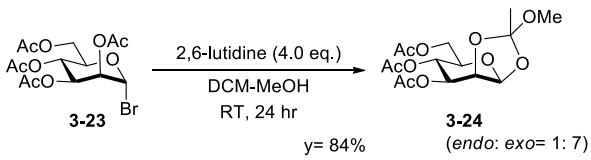


To a solution of 1,2,3,4,6-penta-*O*-acetyl-D-(+)-mannopyranoside (**3-11**, 5.00 g, 12.8 mmol, α: β= 0.22: 1) in acetic anhydride (3.6 mL) was added 33% hydrogen bromide in acetic acid (11.6 mL, 64.1 mmol, 5.0 eq.) dropwise at 0°C. The reaction mixture was warmed to room temperature and stirred for 4 hr. The resultant solution was poured into ice-water (100 mL). After separation, the aqueous phase was extracted with dichloromethane (100 mL x2) and was extracted with dichloromethane (50 mL x2). The combined organic phase was washed with sat. $NaHCO_3$ (50 mL x 2), 10% $Na_2S_2O_3$ (20 mL) and brine (50 mL), dried over $MgSO_4$ and concentrated to afford the titled compound (**3-23**) as pale brown syrup; 4.95 g (y= 94%).



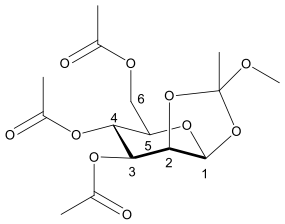
^{1}H NMR[132] (400MHz, $CDCl_3$, δ (C*H*Cl_3)= 7.270): 2.02, 2.09, 2.12, 2.19 (3H x 4, s x4, COC*H*$_3$ x4), 4.15 (1H, dd, $J_{H6\text{-}H6'}$ 12.8Hz, $J_{H6\text{-}H5}$ 2.4Hz, H_6), 4.23 (1H, ddd, $J_{H5\text{-}H4'}$ 10.0Hz, $J_{H5\text{-}H6'}$ 4.8Hz, $J_{H5\text{-}H6}$ 2.0Hz,H_5), 4.34 (1H, dd, $J_{H6'\text{-}H6}$ 12.8Hz, $J_{H6'\text{-}H5}$ 4.8Hz, H_6'), 5.38 (1H, dd, $J_{H4'\text{-}H3}$ 10.0Hz, $J_{H4'\text{-}H5}$ 10.0Hz, H_4), 5.46 (1H, dd, $J_{H2'\text{-}H3}$ 3.6Hz, $J_{H2'\text{-}H1}$ 1.6Hz, H_2), 5.73 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3'\text{-}H2}$ 3.6Hz, H_3), 6.30 (1H, d, $J_{H1\text{-}H2}$ 0.8Hz, H_1). R_f= 0.62 (ethyl acetate: petroleum ether= 1: 1) [lit[132]. R_f = 0.50 (50% EtOAc in hexane)].

Preparation of 3,4,6-tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-β-D-(+)-mannopyranoside (**3-24**)



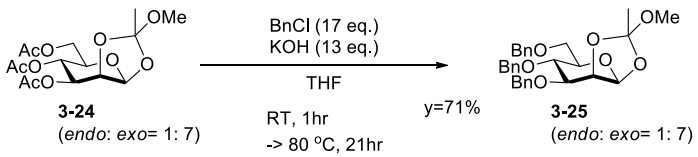
To a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-(+)-mannopyranosyl bromide (**3-23**, 4.95 g, 12.0 mmol, 1.0 eq.) in dichloromethane (20 mL)- methanol (20 mL) was 2,6-lutidine (5.97 mL, 51.2

mmol, 4.0 eq.) dropwise at room temperature. The reaction mixture was stirred for 24 hr. The resultant solution was diluted with dichloromethane (80 mL) and washed with sat. $NaHCO_3$ (50 mL), 10% $CuSO_4$ (50 mL x 4), water (50 mL) and brine (50 mL), dried over $MgSO_4$ and concentrated to afford the titled compound (**3-24**) as pale yellow solid; 3.90 g (*endo*: *exo*= 1: 7, y= 84%).



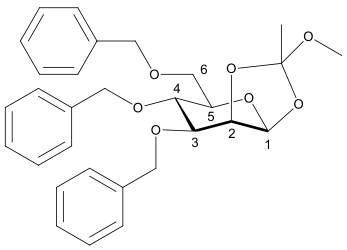
^{1}H NMR[132] (400MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): <u>*Endo*-form</u> 1.53 (3H, s, CH_3 of ethylidene), 2.04, 2.06, 2.13 (3H x 3, s x3, COCH_3 x3), 3.51 (3H, s, OCH_3), 3.68-3.74 (1H, m, H_5), 4.13 (1H, dd, $J_{H6\text{-}H6'}$ 12.0Hz, $J_{H6\text{-}H5}$ 2.4Hz, H_6), 4.30 (1H, dd, $J_{H6'\text{-}H6}$ 12.0Hz, $J_{H6'\text{-}H5}$ 4.4Hz, H_6'), 4.39 (1H, dd, $J_{H2'\text{-}H3}$ 4.0Hz, $J_{H2'\text{-}H1}$ 2.4Hz, H_2), 5.21 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3'\text{-}H2}$ 4.0Hz, H_3), 5.27 (1H, d, $J_{H1\text{-}H2}$ 2.4Hz, H_1), 5.41 (1H, dd, $J_{H4'\text{-}H3}$ 10.0Hz, $J_{H4'\text{-}H5}$ 10.0Hz, H_4). <u>*Exo*-form</u> 1.75 (3H, s, CH_3 of ethylidene), 2.06, 2.08, 2.13 (3H x 3, s x3, COCH_3 x3), 3.29 (3H, s, OCH_3), 3.69 (1H, ddd, $J_{H5\text{-}H4'}$ 9.6Hz, $J_{H5\text{-}H6'}$ 4.8Hz, $J_{H5\text{-}H6}$ 2.8Hz,H_5), 4.15 (1H, dd, $J_{H6\text{-}H6'}$ 12.0Hz, $J_{H6\text{-}H5}$ 2.4Hz, H_6), 4.25 (1H, dd, $J_{H6'\text{-}H6}$ 12.0Hz, $J_{H6'\text{-}H5}$ 5.2Hz, H_6'), 4.63 (1H, dd, $J_{H2'\text{-}H3}$ 4.0Hz, $J_{H2'\text{-}H1}$ 2.4Hz, H_2), 5.15 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3'\text{-}H2}$ 4.0Hz, H_3), 5.31 (1H, dd, $J_{H4'\text{-}H3}$ 10.0Hz, $J_{H4'\text{-}H5}$ 10.0Hz, H_4), 5.50 (1H, d, $J_{H1\text{-}H2}$ 2.8Hz, H_1). LRMS (ESI$^+$): m/z $[M+Na]^+$ 385.1. $[\alpha]_D^{20}$= -19.5° (*endo*: *exo*= 1: 7, c= 1.07, $CHCl_3$) [lit.[133] $[\alpha]_D^{25}$ = -22.3° (c= 1.04, $CHCl_3$)]. IR (ν_{max}, cm^{-1}, neat): 1745 (ester C=O stretch), 1435, 1372, 1227, 1164, 1117, 1048, 895 [lit[132]. IR (ν_{max}, cm^{-1}, neat): 1740]. R_f= 0.32 (ethyl acetate: petroleum ether= 1: 1) [lit.[132] R_f= 0.43 (50% EtOAc in hexane)].

Preparation of 3,4,6-tri-*O*-benzyl-1,2-*O*-(1-methoxyethylidene)-β-D-(+)-mannopyranoside (**3-25**)



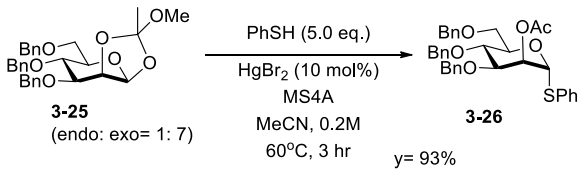
To a solution of 3,4,6-tri-*O*-acetyl-1,2-*O*-(1-methoxyethylidene)-β-D-(+)-mannopyranoside (**3-24**, 3.80 g, 10.5 mmol, 1.0 eq.) in tetrahydrofuran (35 mL) were added benzyl chloride (BnCl, 20.5 mL, 179 mmol, 17 eq.) and powdered potassium hydroxide (KOH, 85% purity, 9.01 g, 137 mmol, 13 eq.) at room temperature. The reaction mixture was stirred for 1 hr and warmed up to 80°C. After 21 hr, the resultant solution was diluted with diethyl ether (100 mL) and washed

with 10% NH_4Cl (100 mL). The aqueous phase was extracted with diethyl ether (100 mL) and the combined organic phase was washed with brine (100 mL), dried over $MgSO_4$ and concentrated. To the residue was added petroleum ether (40 mL) and the precipitation was collected to afford the titled compound (**3-25**) as white solid; 3.75 g (*endo*: *exo*= 1: 7, y= 71%).



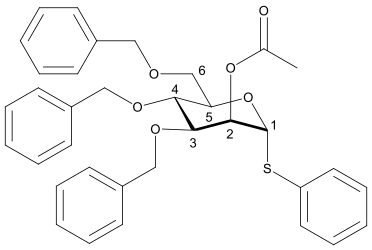
^{1}H NMR[134] (400MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): <u>*Endo*-form</u> 1.51 (3H, s, CH_3 of ethylidene), 3.40-3.47 (1H, m,H_5), 3.45 (3H, s, OCH_3), 3.68-3.80 (3H, m, H_3, H_6 x2), 4.03 (1H, dd, $J_{H4'\text{-}H3}$ 9.6Hz, $J_{H4'\text{-}H5}$ 9.6Hz, H_4), 4.11 (1H, dd, $J_{H2'\text{-}H3}$ 4.0Hz, $J_{H2'\text{-}H1}$ 2.4Hz, H_2), 4.51-4.57 (2H, m, H_{Bn} x 2), 4.64 (1H, $J_{Bn\text{-}Bn'}$ 10.8H, H_{Bn}), 4.75-4.83 (2H, m, H_{Bn} x 2), 4.92 (1H, $J_{Bn\text{-}Bn'}$ 10.8H, H_{Bn}), 5.11 (1H, d, $J_{H1\text{-}H2}$ 2.4Hz, H_1), 7.20-7.45 (15H, m, H_{Ph}). <u>*Exo*-form</u> 1.75 (3H, s, CH_3 of ethylidene), 3.29 (3H, s, OCH_3), 3.43 (1H, ddd, $J_{H5\text{-}H4'}$ 9.6Hz, $J_{H5\text{-}H6'}$ 4.4Hz, $J_{H5\text{-}H6}$ 2.4Hz,H_5), 3.68-3.80 (3H, m, H_3, H_6 x2), 3.93 (1H, dd, $J_{H4\text{-}H3}$ 9.6Hz, $J_{H4\text{-}H5}$ 9.6Hz, H_4), 4.40 (1H, dd, $J_{H2'\text{-}H3}$ 3.6Hz, $J_{H2'\text{-}H1}$ 2.4Hz, H_2), 4.56 (1H, $J_{Bn\text{-}Bn'}$ 12.4H, H_{Bn}), 4.61 (1H, $J_{Bn\text{-}Bn'}$ 10.4H, H_{Bn}), 4.62 (1H, $J_{Bn\text{-}Bn'}$ 12.0H, H_{Bn}), 4.78 (1H, $J_{Bn\text{-}Bn'}$ 12.4H, H_{Bn}), 4.81 (1H, $J_{Bn\text{-}Bn'}$ 12.0H, H_{Bn}), 4.91 (1H, $J_{Bn\text{-}Bn'}$ 10.8H, H_{Bn}), 5.36 (1H, d, $J_{H1\text{-}H2}$ 2.4Hz, H_1), 7.20-7.45 (15H, m, H_{Ph}). LRMS (ESI^+): m/z $[M+Na]^+$ 529.2. $[\alpha]_D^{20}$= +34.5° (*endo*: *exo*= 1: 7, c= 1.12, $CHCl_3$) [lit.[133] $[\alpha]_D^{25}$ = +29.8° (c= 1.04, $CHCl_3$)]. IR (ν_{max}, cm^{-1}, neat): 3088, 3063, 3030, 3002, 2944, 2912, 2866 (C-H stretch), 1497, 1454, 1384, 1314, 1239, 1206, 1134, 1100, 1047, 927, 894, 735, 698. R_f= 0.43 (ethyl acetate: petroleum ether= 1: 2)

Preparation of phenyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio-α-D-(+)-mannopyranoside (**3-26**)



3,4,6-Tri-*O*-benzyl-1,2-*O*-(1-methoxyethylidene)-β-D-(+)-mannopyranoside (**3-25**, 1.50 g, 2.96 mmol, 1.0 eq.) and thiophenol (PhSH, 1.52 mL, 14.8 mmol, 5.0 eq.) were dissolved in acetonitrile (15 mL) and MS4A (1.00 g) was added. After the mixture was stirred for 1 hr, mercury (II) bromide ($HgBr_2$, 107 mg, 0.296 mmol, 10 mol%) was added. The mixture was heated to 60°C and stirred for 3 hr. After being cooled, the mixture was filtered through celite

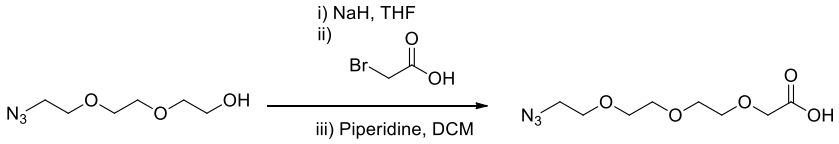
and concentrated. To the residue was added diethyl ether (50 mL) and the precipitation was removed by filtration. The filtrate was concentrated and the residue was purified by column chromatography (first, silica gel: 150 mL, eluent: ethyl acetate/ petroleum ether = 1: 5, then silica gel: 50 mL, eluent: petroleum ether/ dichloromethane = 1: 1 -> dichloromethane/ ethyl acetate= 50: 1) to afford the titled compound (**3-26**) as white solid; 1.61 g (y= 93%).



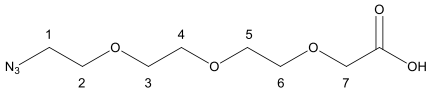
^{1}H NMR[134] (400MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 2.17 (3H, s, $COCH_3$), 3.74 (1H, dd, $J_{H5\text{-}H4'}$ 11.2Hz, $J_{H5\text{-}H6'}$ 1.6Hz, H_6), 3.88 (1H, dd, $J_{H5\text{-}H4'}$ 11.2Hz, $J_{H5\text{-}H6'}$ 4.8Hz, H_6'), 3.96 (1H, dd, $J_{H3\text{-}H4'}$ 9.2Hz, $J_{H3\text{-}H2}$ 3.2Hz, H_3), 4.00 (1H, dd, $J_{H4\text{-}H3}$ 9.2Hz, $J_{H4\text{-}H5}$ 9.2Hz, H_4), 4.31-4.40 (1H, m,H_5), 4.49 (1H, $J_{Bn\text{-}Bn'}$ 12.H, H_{Bn}), 4.54 (1H, $J_{Bn\text{-}Bn'}$ 10.4H, H_{Bn}), 4.60 (1H, $J_{Bn\text{-}Bn'}$ 11.2H, H_{Bn}), 4.69 (1H, $J_{Bn'\text{-}Bn}$ 11.6H, H_{Bn}'), 4.75 (1H, $J_{Bn'\text{-}Bn}$ 11.2H, H_{Bn}'), 4.91 (1H, $J_{Bn'\text{-}Bn}$ 10.8H, H_{Bn}'), 5.56 (1H, s, H_1), 5.63 (1H, s, H_2), 7.19-7.24 (2H, m, H_{Ph}), 7.24-7.40 (16H, m, H_{Ph}), 7.47-7.53 (2H, m, H_{Ph}). LRMS (ESI^+): m/z $[M+Na]^+$ 607.2. $[\alpha]_D^{20}$= +111.2° (c 1.04, $CHCl_3$) [lit.[134] $[\alpha]_D^{20}$= +106° (c 1, $CHCl_3$)]. m.p. 62-65°C (DCM-petroleum ether) [lit[134]. 62-63°C (ethyl acetate-hexane)]. IR (ν_{max}, cm^{-1}, neat): 3088, 3062, 3031, 2868 (C-H stretch), 1744 (ester C=O stretch), 1605, 1584, 1497, 1479, 1454, 1440, 1370, 1318, 1279, 1231, 1156, 1101, 1086, 1045, 1027, 976, 930, 912, 852, 741, 697. R_f= 0.56 (ethyl acetate: petroleum ether= 1: 3).

Synthesis of the linker with bioorthogonal reaction handle

Preparation of 2-(2-[2-(2-azidoethoxy)ethoxy]ethoxy)acetic acid (**3-30**)

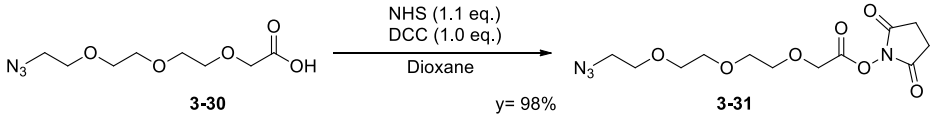


The solvent of a commercially available 2-[2-(2-azidoethoxy)ethoxy]ethanol (**3-29**) solution (10 mL, 0.5M in *tert*-butylmethyl ether) was removed by evaporation. The residue was dissolved in tetrahydrofuran (5.0 mL). Sodium hydride (60% in oil, 801 mg, 20.0 mmol, 4.0 eq.) was washed with hexane (3 mL x2), dried *in vacuo* and suspended in tetrahydrofuran (10 mL). A solution of **3-29** in tetrahydrofuran prepared as above was added dropwise and the mixture was stirred at room temperature for 30 min. After being cooled to 0°C, a solution of bromoacetic acid (1.04 g, 7.50 mmol, 1.5 eq.) in tetrahydrofuran (7.5 mL) was added dropwise. The resultant mixture was warmed to room temperature and stirred for 3 hr. After the solvent was removed by evaporation, dichloromethane (20 mL) was added to the residue and water (10 mL) was added slowly. The aqueous phase was separated and the organic phase was extracted with 0.5M NaOH (10 mL x2). The combined aqueous phase was acidified with 5M HCl to pH <2, extracted with dichloromethane (10 mL x3), dried over $MgSO_4$ and concentrated. The residue was dissolved in dichloromethane (10 mL) and piperidine (1.23 mL, 12.5 mmol, 2.5 mmol) was added. The solution was stirred at room temperature for 1 hr. The resultant solution was diluted with dichloromethane (10 mL) and washed with 4M HCl (10 mL x2). The combined aqueous phase was extracted with dichloromethane (10 mL x2) and the combined organic phase was dried over $MgSO_4$ and concentrated to afford the titled compound (**3-30**) as light brown oil; 646 mg (y= 55%).



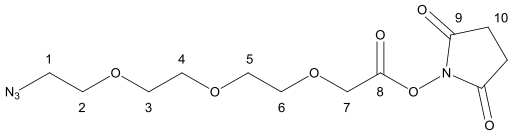
^{1}H NMR[135] (400MHz, $CDCl_3$, δ (C*H*Cl_3)= 7.270): 3.41 (2H, t, $J_{H1\text{-}H2}$ 5.2Hz, H_1), 3.66-3.75 (8H, m, CH_2), 3.76-3.80 (2H, m, CH_2), 4.18 (2H, s, H_7). LRMS (ESI^+): m/z $[M+Na]^+$ 256.2, (ESI^-): m/z $[M-H]^-$ 232.1. IR (ν_{max}, cm^{-1}, neat): 3485 (broad, O-H stretch), 2921, 2876 (C-H stretch), 2108 (N_3 stretch), 1737 (carboxylic acid C=O stretch), 1439, 1348, 1286, 1248, 1121, 936, 853, 669, 654. R_f= 0.35 (acetic acid: methanol: dichloromethane= 0.1: 1: 8).

Preparation of *N*-succinimidyl 2-(2-[2-(2-azidoethoxy)ethoxy]ethoxy)acetate[110] (**3-31**)



3-30 y= 98% **3-31**

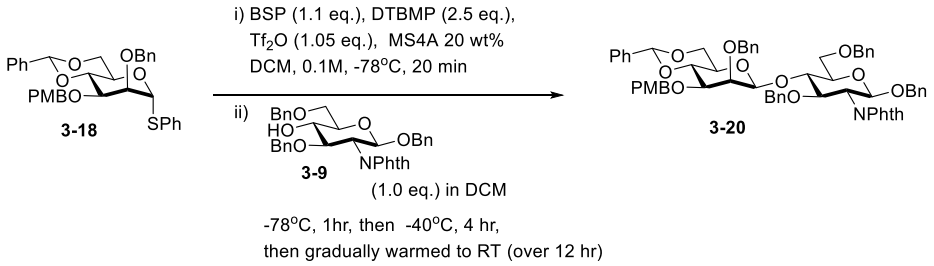
To a solution of 2-(2-[2-(2-azidoethoxy)ethoxy]ethoxy)acetic acid (**3-30**, 100 mg, 0.429 mmol, 1.0 eq.) in dioxane (860 μL) were added *N*-hydroxysuccinimide (54.3 mg, 0.472 mmol, 1.1 eq.) and *N,N'*-dicyclohexylcarbodiimide (88.5 mg, 0.429 mmol, 1.0 eq.). The mixture was stirred at room temperature for 2 hr. Precipitation was removed by filtration through celite and the filtrate was concentrated. The residue was dissolved in tetrahydrofuran (2 mL) and the mixture was cooled to 0°C. Precipitation was removed by filtration through cotton plug. The filtrate was concentrated and the residue was dissolved in toluene (2 mL) and the mixture was cooled to 0°C. Precipitation was removed by filtration through cotton plug. The same procedure was repeated with 0.5 mL of toluene to afford the titled compound (**3-31**) as light yellow oil; 139 mg (y= 98%).



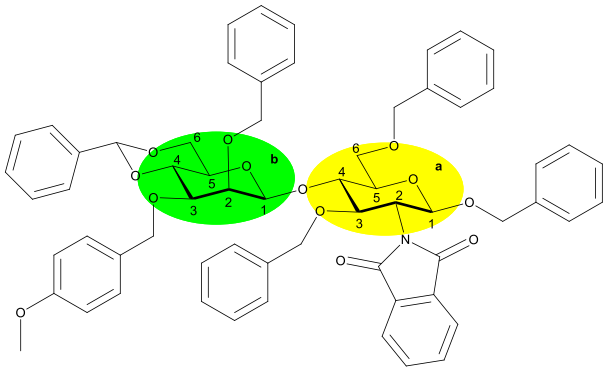
^{1}H NMR (400MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 2.86 (4H, brs, H_{10}) 3.40 (2H, t, J_{H1-H2} 5.2Hz, H_1), 3.66-3.71 (6H, m, CH_2), 3.71-3.75 (2H, m, CH_2), 3.79-3.83 (2H, m, CH_2), 4.54 (2H, s, H_7). ^{13}C NMR (101MHz, $CDCl_3$, δ ($CHCl_3$)= 77.00): 25.54 (C_{10}), 50.64 (C_1), 66.51 (C_7), 70.01, 70.62, 70.65, 70.65, 71.30 (C_2, C_3, C_4, C_5, C_6), 165.99 (C_8), 168.75 (C_9). LRMS (ESI^+): m/z $[M+Na]^+$ 353.1. HRMS (ESI^+): m/z $[M+Na]^+$ 353.1059 (found), (calcd 353.1068 for $C_{12}H_{18}N_4NaO_7$). IR (ν_{max}, cm^{-1}, neat): 2919, 2873 (C-H stretch), 2106 (N_3 stretch), 1819, 1785, 1736 (imide, ester C=O stretch), 1430, 1355, 1299, 1252, 1204, 1076, 1048, 995, 935, 886, 853, 815, 749, 648. R_f= 0.35 (methanol: dichloromethane= 1: 20).

Synthesis of the tetrasaccharide scaffold

Preparation of benzyl 4-*O*-(2-*O*-benzyl-3-*O*-*p*-methoxybenzyl-4,6-*O*-benzylidene-β-D-(+)-mannopyranosyl)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-20**)



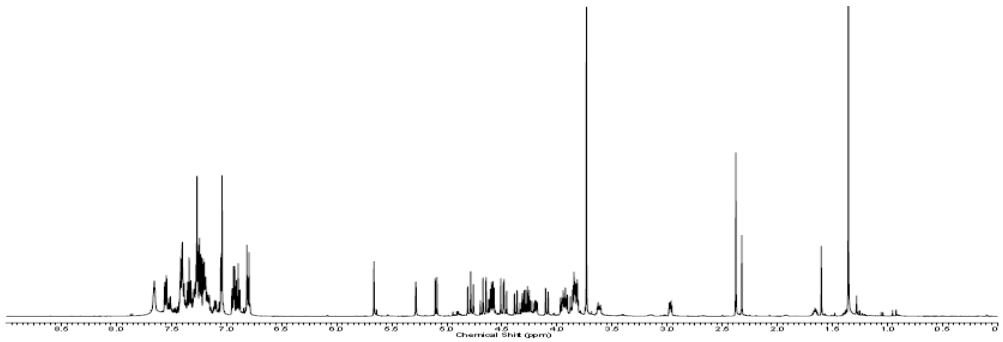
Phenyl 2-*O*-benzyl-3-*O*-*p*-methoxybenzyl-4,6-*O*-benzylidene-1-thio-α-D-(+)-mannopyranoside (**3-18**, 4.00 g, 7.01 mmol), 1-benzenesulfinyl piperidine (BSP, 1.61 g, 7.71 mmol, 1.1 eq.) and 2,6-di-*tert*-butyl-4-methylpyridine (DTBMP, 3.60 g, 17.5 mmol, 2.5 eq.) were dissolved in toluene and solvents were removed by evaporation to dryness. To the residue were added activated MS4Å (800 mg) and dichloromethane (70.1 mL) under N_2 atmosphere. After being stirred for 30 min, the solution was cooled to -78°C. Trifluoromethanesulfonic anhydride (Tf_2O, 1.25 mL, 7.36 mmol, 1.05 eq.) was added and the solution was stirred for 20 min at this temperature. A solution of benzyl 3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-9**, 4.06 g, 7.01 mmol, 1.0 eq.) in dichloromethane (23 mL) was added dropwise and the solution was stirred for 1 hr at -78°C. The mixture was warmed up to -40°C and stirred for 4 hr. The solution was gradually warmed to room temperature over 12 hr. The reaction was quenched by addition of triethylamine (4.88 mL, 35 mmol, 5.0 eq.) and filtered through celite. The filtrate was washed with sat. $NaHCO_3$ (80 mL) and brine (80 mL), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel: 1,200 mL, eluent: ethyl acetate: toluene= 1: 8) to afford the titled compound (**3-20**) as colorless foam; 4.70 g (y= 64%).



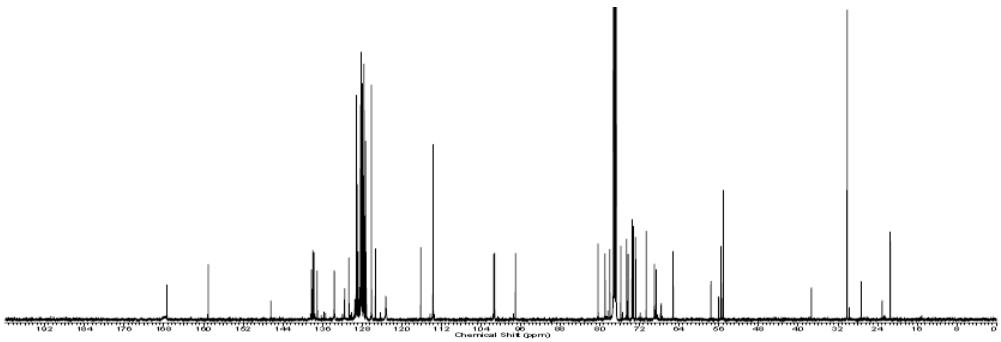
^{1}H NMR[6] (500MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 3.17 (1H, ddd, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6ax}$ 9.5Hz, $J_{H5\text{-}H6eq}$ 4.5Hz, $^bH_5$), 3.44 (1H, dd, $J_{H3\text{-}H4}$ 10.0Hz, $J_{H3\text{-}H2}$ 3.0Hz, $^bH_3$), 3.50 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6}$ 3.0Hz, $J_{H5\text{-}H6'}$ 2.0Hz, $^aH_5$), 3.59 (1H, dd, $J_{H6ax\text{-}H5}$ 10.0Hz, $J_{H6ax\text{-}H6eq}$ 10.0Hz, $^bH_{6ax}$), 3.60 (1H,

dd, $J_{H6\text{-}H6'}$ 11.0Hz, $J_{H6\text{-}H5}$ 3.5Hz, $^{a}H_6$), 3.70 (1H, dd, $J_{H6'\text{-}H6}$ 11.0Hz, $J_{H6'\text{-}H5}$ 2.0Hz, $^{a}H_6$'), 3.75 (1H, d, $J_{H2\text{-}H3}$ 3.5Hz, $^{b}H_2$), 3.81 (3H, s, OCH_3), 4.05 (1H, dd, $J_{H4\text{-}H5}$ 10.0Hz, $J_{H4\text{-}H3}$ 8.0Hz, $^{a}H_4$), 4.09 (1H, dd, $J_{H4\text{-}H3}$ 9.5Hz, $J_{H4\text{-}H5}$ 9.5Hz, $^{b}H_4$), 4.20 (1H, dd, $J_{H6eq\text{-}H6ax}$ 10.5Hz, $J_{H6eq\text{-}H5}$ 4.5Hz, $^{b}H_{6eq}$), 4.22-4.30 (2H, m, $^{a}H_2$, $^{a}H_3$), 4.44 (1H, d, $J_{Bn1\text{-}Bn1'}$ 12.5Hz, H_{Bn1}), 4.44 (1H, d, $J_{Bn2\text{-}Bn2'}$ 12.5Hz, H_{Bn2}), 4.52 (1H, d, $J_{Bn3\text{-}Bn3'}$ 12.5Hz, H_{Bn3}), 4.55 (1H, d, $J_{Bn4\text{-}Bn4'}$ 12.0Hz, H_{Bn4}), 4.58 (1H, s, $^{b}H_1$), 4.687 (1H, d, $J_{Bn1'\text{-}Bn1}$ 12.5Hz, H_{Bn1}'), 4.691 (1H, d, $J_{Bn4'\text{-}Bn4}$ 11.5Hz, H_{Bn4}'), 4.82 (1H, d, $J_{Bn3'\text{-}Bn3}$ 12.0Hz, H_{Bn3}'), 4.87 (1H, d, $J_{Bn2'\text{-}Bn2}$ 12.0Hz, H_{Bn2}'), 4.87 (1H, d, $J_{Bn5\text{-}Bn5'}$ 12.0Hz, H_{Bn5}), 4.90 (1H, d, $J_{Bn5'\text{-}Bn5}$ 12.0Hz, H_{Bn5}'), 5.14 (1H, d, $J_{H1\text{-}H2}$ 8.0Hz, $^{a}H_1$), 5.53 (1H, s, $H_{Benzylidene}$), 6.82-6.90 (4H, m, H_{Ph}), 6.92-6.97 (2H, m, H_{Ph}), 7.02-7.12 (5H, m, H_{Ph}), 7.23-7.41 (15H, m, H_{Ph}), 7.24-7.32 (6H, m, H_{Ph}), 7.32-7.42 (9H, m, H_{Ph}), 7.45-7.59 (4H, m, H_{Ph}), 7.64-7.72 (2H, br, H_{Phth}), 7.74-7.85 (1H, br, H_{Phth}). ^{13}C NMR[6] (126MHz, $CDCl_3$, δ (*C*HCl_3)= 77.00): 55.23 (O*C*H_3), 55.68 ($^{a}C_2$), 67.30 ($^{b}C_5$), 68.49, 68.53 ($^{a}C_6$, $^{b}C_6$), 70.68 (C_{Bn3}), 72.25 (C_{Bn4}), 73.54 (C_{Bn1}), 74.57 (C_{Bn2}), 74.68 ($^{a}C_5$), 74.93 (C_{Bn5}), 76.93 ($^{a}C_3$), 77.01 ($^{b}C_2$), 77.97 ($^{b}C_3$), 78.64 ($^{b}C_4$), 79.46 ($^{a}C_4$), 97.36 ($^{a}C_1$), 101.28 ($C_{Benzylidene}$), 101.95 ($^{b}C_1$), 113.69 (C_{Ph}), 123.14 (C_{Phth3}), 126.06, 126.84, 127.48, 127.53, 127.57, 127.73, 127.78, 127.92, 128.13, 128.19, 128.25, 128.50, 128.77, 129.03, 130.57 (C_{Ph}), 131.61 (C_{Phth2}), 133.56 (C_{Phth4}), 137.17, 137.65, 137.78, , 138.63, 138.73, 159.12 (C_{Ph}), 167.80 (C_{Phth1}). Coupled HSQC: J(^{a}H1-^{a}C1) 165Hz, J(^{b}H1-^{b}C1) 158Hz. LRMS (ESI^+): m/z $[M+Na]^+$ 1062.38. HRMS (ESI^+): m/z $[M+Na]^+$ 1062.4042 (found), (calcd 1062.4035 for $C_{63}H_{61}NNaO_{13}$). $[\alpha]_D^{20}$= -24.2° (c= 0.50, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 3063, 3031, 2867 (C-H stretch), 1775, 1713 (imide C=O stretch), 1612, 1586, 1512, 1496, 1454, 1387, 1364, 1303, 1247, 1210, 1173, 1084, 1048, 1027, 969, 914, 875, 848, 820, 793, 736, 721, 697, 661, 642. R_f= 0.23 (ethyl acetate: toluene= 1: 8).

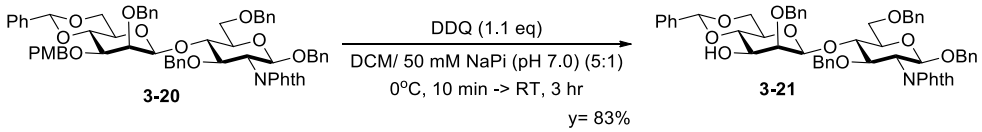
^{1}H NMR



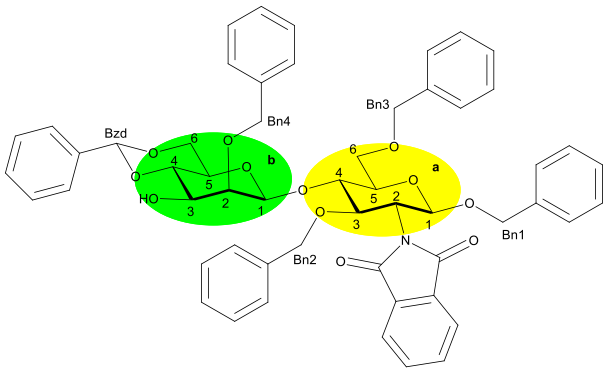
^{13}C NMR



Preparation of benzyl 4-*O*-(2-*O*-benzyl-4,6-*O*-benzylidene-β-D-(+)-mannopyranosyl)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-21**)



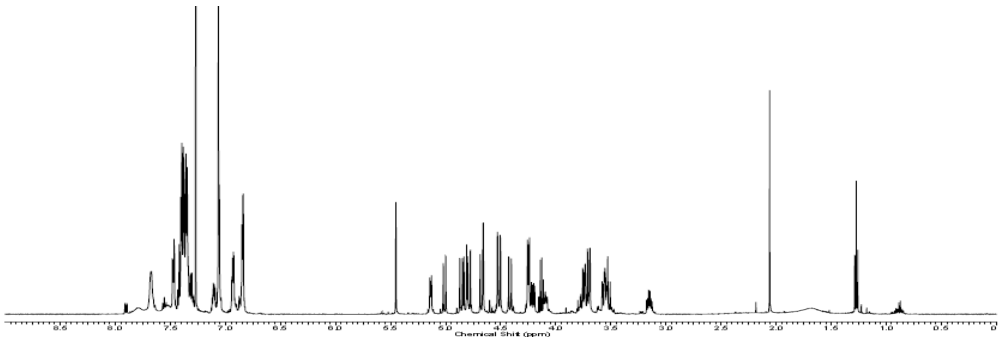
To a solution of benzyl 4-*O*-(2-*O*-benzyl-3-*O*-*p*-methoxybenzyl-4,6-*O*-benzylidene-β-D-(+)-mannopyranosyl)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-20**, 3.50 g, 3.36 mmol, 1.0 eq.) in dichloromethane (34 mL) was added 50 mM sodium phosphate buffer (pH 7.0, 6.7 mL) and cooled to 0°C. 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (DDQ, 840 mg, 3.70 mmol, 1.1 eq.) was added and the solution was stirred for 10 min at this temperature. The solution was warmed to room temperature and further stirred for 3 hr. To the resultant solution was added sat. $NaHCO_3$ (30 mL) and the organic phase was separated. The aqueous phase was extracted with dichloromethane (30 mL) and the combined organic phase was washed with brine (20 mL), dried over MgSO4 and concentrated. The residue was purified by column chromatography (silica gel: 600 mL, eluent: ethyl acetate: petroleum ether= 1: 2 -> 1; 1) to afford the titled compound (**3-21**) as colorless foam; 2.57 g (y= 83%).



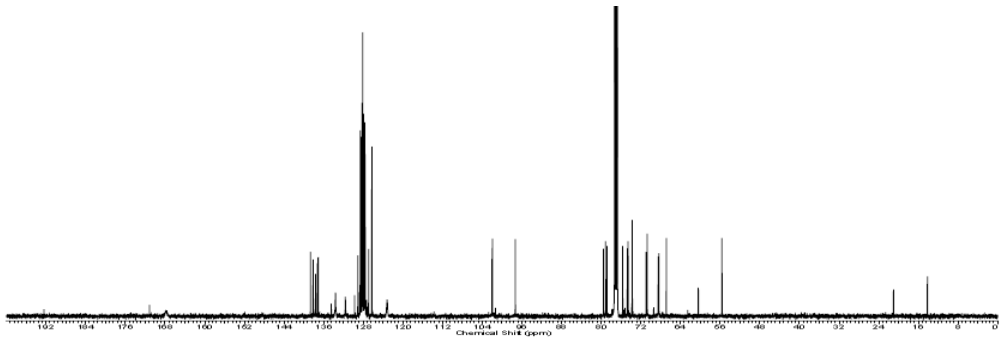
^{1}H NMR (500MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 3.15 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6ax}$ 10.0Hz, $J_{H5\text{-}H6eq}$ 5.0Hz, $^bH_5$), 3.53 (1H, dd, $J_{H6ax\text{-}H5}$ 10.5Hz, $J_{H6ax\text{-}H6eq}$ 10.5Hz, $^bH_{6ax}$), 3.52-3.56 (1H, m, $^aH_5$), 3.57 (1H, dd, $J_{H3\text{-}H4}$ 9.5Hz, $J_{H3\text{-}H2}$ 3.5Hz, $^bH_3$), 3.69 (1H, d, $J_{H2\text{-}H3}$ 3.0Hz, $^bH_2$), 3.71 (1H, dd, $J_{H4\text{-}H3}$ 10.5Hz, $J_{H4\text{-}H5}$ 10.5Hz, $^bH_4$), 3.72 (1H, dd, $J_{H6\text{-}H6'}$ 11.0Hz, $J_{H6\text{-}H5}$ 4.0Hz, $^aH_6$), 3.76 (1H, dd, $J_{H6'\text{-}H6}$ 11.0Hz, $J_{H6'\text{-}H5}$ 2.5Hz, $^aH_6$'), 4.09 (1H, ddd, $J_{H4\text{-}H5}$ 10.0Hz, $J_{H4\text{-}H3}$ 6.0Hz, $J_{H4\text{-}H1}$ 2.5Hz, $^aH_4$), 4.21 (1H, dd, $J_{H6eq\text{-}H6ax}$ 11.0Hz, $J_{H6eq\text{-}H5}$ 5.0Hz, $^bH_{6eq}$), 4.23-4.28 (2H, m, $^aH_2$, $^aH_3$), 4.42 (1H, d, $J_{Bn2\text{-}Bn2'}$ 13.0Hz, H_{Bn2}), 4.51 (1H, d, $J_{Bn1\text{-}Bn1'}$ 12.0Hz, H_{Bn1}), 4.52 (1H, d, $J_{Bn3\text{-}Bn3'}$ 12.5Hz, H_{Bn3}), 4.66 (1H, s, $^bH_1$), 4.68 (1H, d, $J_{Bn4\text{-}Bn4'}$ 11.5Hz, H_{Bn4}), 4.79 (1H, d, $J_{Bn3'\text{-}Bn3}$ 12.0Hz, H_{Bn3}'), 4.82 (1H, d, $J_{Bn1'\text{-}Bn1}$ 12.0Hz, H_{Bn1}'), 4.86 (1H, d, $J_{Bn2'\text{-}Bn2}$ 12.5Hz, H_{Bn2}'), 5.01 (1H, d, $J_{Bn4'\text{-}Bn4}$ 11.5Hz, H_{Bn4}'), 5.14 (1H, dd, $J_{H1\text{-}H2}$ 6.0Hz, $J_{H1\text{-}H4}$ 3.0Hz, $^aH_1$), 5.45 (1H, s, H_{Bzd}), 6.82-6.88 (3H, m, H_{Ph}), 6.91-6.95 (2H, m, H_{Ph}), 7.03-7.14 (5H, m, H_{Ph}), 7.28-7.44 (13H, m, H_{Ph}), 7.45-7.49 (2H, m, H_{Ph}),

7.49-7.59 (1H, br, H_{Phth}), 7.63-7.72 (2H, br, H_{Phth}), 7.73-7.86 (1H, br, H_{Phth}). ^{13}C NMR (126MHz, $CDCl_3$, δ (*C*HCl_3)= 77.00): 55.65 ($^aC_2$), 66.85 ($^bC_5$), 68.36, 68.47 ($^aC_6$, $^bC_6$), 70.72 (C_{Bn1}), 70.92 ($^bC_3$), 73.74 (C_{Bn3}), 74.54 (C_{Bn2}), 74.71 ($^aC_5$), 75.67 (C_{Bn4}), 76.95 ($^aC_3$), 78.82 ($^bC_2$), 79.12 ($^bC_4$), 79.54 ($^aC_4$), 97.32 ($^aC_1$), 101.94, 102.02 (C_{Bzd}, $^bC_1$), 123.18 (C_{Phth3}), 126.28, 126.94, 127.56, 127.63, 127.69, 127.78, 127.90, 127.94, 128.06, 128.13, 128.22, 128.48, 128.62, 129.07 (C_{Ph}), 131.59 (C_{Phth2}), 133.58 (C_{Phth4}), 137.12, 137.25, 137.62, 138.11, 138.66 (C_{Ph}), 167.86 (C_{Phth1}). Coupled HSQC: *J*(^{a}H1-^{a}C1) 163Hz, *J*(^{b}H1-^{b}C1) 158Hz. LRMS (ESI$^+$): m/z $[M+Na]^+$ 942.3. HRMS (ESI$^+$): m/z $[M+Na]^+$ 942.3465 (found), (calcd 942.3460 for $C_{55}H_{53}NNaO_{12}$). $[\alpha]_D^{20}$= -15.2° (c= 0.50, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 3475 (broad, O-H stretch), 3064, 3030, 2870 (C-H stretch), 1775, 1712 (imide C=O stretch), 1611, 1497, 1468, 1454, 1387, 1362, 1313, 1252, 1209, 1180, 1135, 1074, 1051, 1027, 968, 915, 875, 819, 794, 748,721, 696, 666, 641. R_f= 0.21 (ethyl acetate: petroleum ether= 1: 2).

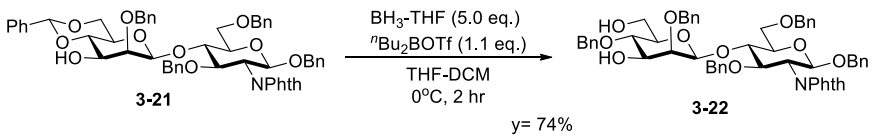
^1H NMR



^{13}C NMR

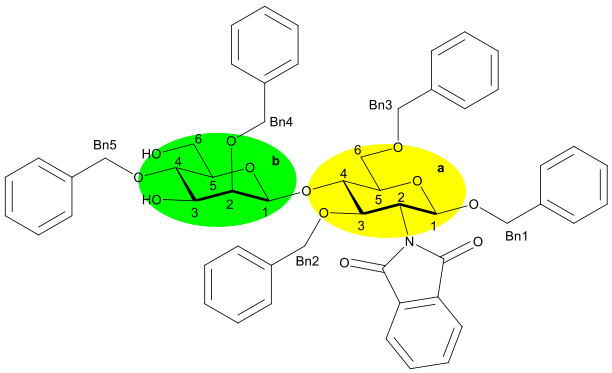


Preparation of benzyl 4-*O*-(2,4-di-*O*-benzyl-β-D-(+)-mannopyranosyl)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-22**)



To a solution of benzyl 4-*O*-(2-*O*-benzyl-4,6-*O*-benzylidene-β-D-(+)-mannopyranosyl)-

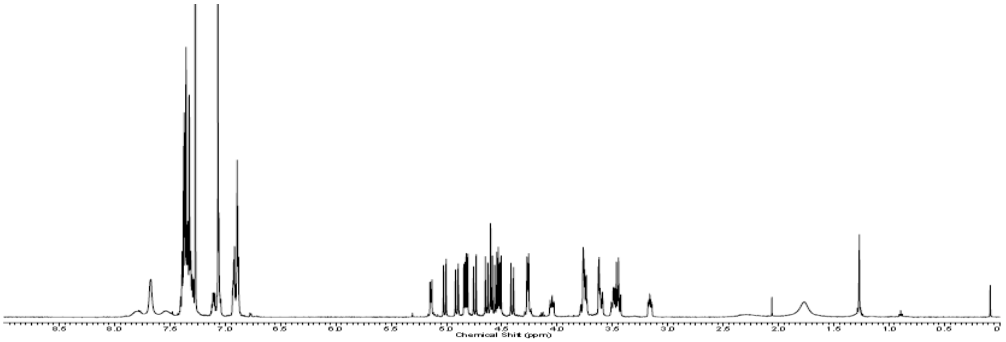
3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-21**, 2.20 g, 2.39 mmol, 1.0 eq.) in tetrahydrofuran (24 mL) was added 1M solution of borane-tetraheydrofuran complex in tetrahydrofuran (12 mL, 12.0 mmol, 5.0 eq.) and 1M solution of dibutylboryl trifluoromethanesulfonate in dichloromethane (2.63 mL, 2.63 mmol, 1.1 eq.). The reaction was stirred at 0°C for 2 hr. The reaction was quenched by adding triethylamine (1.00 mL, 7.17 mmol, 3.0 eq.) and methanol (4.84 mL, 120 mmol, 50 eq.) and stirring at room temperature for 2 hr. After concentration, the residue was purified by column chromatography (silica gel: 600 mL, eluent: ethyl acetate: petroleum ether= 2: 3 -> 1; 1) to afford the titled compound (**3-22**) as colorless foam; 1.62 g (y= 74%).



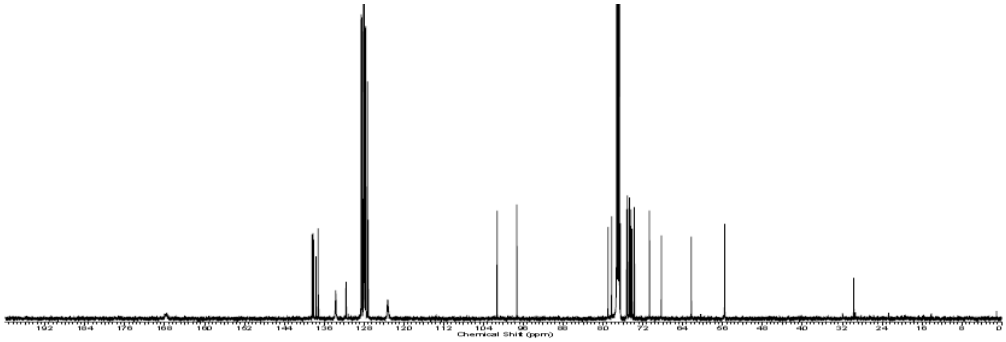
^{1}H NMR (500MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 3.16 (1H, ddd, $J_{H5\text{-}H4}$ 9.0Hz, $J_{H5\text{-}H6}$ 6.0Hz, $J_{H5\text{-}H6'}$ 3.0Hz, $^{b}H_5$), 3.45 (1H, dd, $J_{H4\text{-}H3}$ 9.5Hz, $J_{H4\text{-}H5}$ 9.5Hz, $^{b}H_4$), 3.46 (1H, dd, $J_{H6\text{-}H6'}$ 11.5Hz, $J_{H6\text{-}H5}$ 6.0Hz, $^{b}H_6$), 3.50 (1H, dd, $J_{H3\text{-}H4}$ 9.0Hz, $J_{H3\text{-}H2}$ 3.0Hz, $^{b}H_3$), 3.60 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6}$ 2.5Hz, $J_{H5\text{-}H6'}$ 2.5Hz, $^{a}H_5$), 3.62 (1H, d, $J_{H2\text{-}H3}$ 3.5Hz, $^{b}H_2$), 3.72-3.80 (3H, m, $^{a}H_6$,$^{a}H_6$' ,$^{b}H_6$'), 4.02-4.08 (1H, m, $^{a}H_4$), 4.23-4.30 (2H, m, $^{a}H_2$,$^{a}H_3$), 4.41 (1H, d, $J_{Bn2\text{-}Bn2'}$ 12.0Hz, H_{Bn2}), 4.52 (1H, d, $J_{Bn1\text{-}Bn1'}$ 12.0Hz, H_{Bn1}), 4.53 (1H, d, $J_{Bn3\text{-}Bn3'}$ 12.5Hz, H_{Bn3}), 4.58 (1H, d, $J_{Bn5\text{-}Bn5'}$ 11.0Hz, H_{Bn5}), 4.60 (1H, s, $^{b}H_1$), 4.64 (1H, d, $J_{Bn4\text{-}Bn4'}$ 12.0Hz, H_{Bn4}), 4.74 (1H, d, $J_{Bn3'\text{-}Bn3}$ 12.5Hz, H_{Bn3}'), 4.82 (1H, d, $J_{Bn1'\text{-}Bn1}$ 12.0Hz, H_{Bn1}'), 4.83 (1H, d, $J_{Bn5'\text{-}Bn5}$ 11.0Hz, H_{Bn5}'), 4.91 (1H, d, $J_{Bn2'\text{-}Bn2}$ 12.0Hz, H_{Bn2}'), 5.02 (1H, d, $J_{Bn4'\text{-}Bn4}$ 12.0Hz, H_{Bn4}'), 5.11-5.17 (1H, m, $^{a}H_1$), 6.87-6.94 (5H, m, H_{Ph}), 7.03-7.14 (5H, m, H_{Ph}), 7.27-7.42 (15H, m, H_{Ph}), 7.45-7.60 (1H, br, H_{Phth}), 7.63-7.72 (2H, br, H_{Phth}), 7.73-7.87 (1H, br, H_{Phth}). ^{13}C NMR (126MHz, $CDCl_3$, δ ($CHCl_3$)= 77.00): 55.61 ($^{a}C_2$), 62.27 ($^{b}C_6$), 68.29 ($^{a}C_6$), 70.69 (C_{Bn1}), 73.73 (C_{Bn3}), 74.13 ($^{b}C_3$), 74.39 (C_{Bn2}), 74.66 (C_{Bn5}), 74.77 ($^{a}C_5$), 75.14 (C_{Bn4}), 75.27 ($^{b}C_5$), 76.51 ($^{b}C_4$), 76.92 ($^{a}C_3$), 78.25 ($^{b}C_2$), 79.00 ($^{a}C_4$), 97.30 ($^{a}C_1$), 101.27 ($^{b}C_1$), 123.24 (C_{Phth3}), 127.11, 127.23, 127.56, 127.63, 127.77, 127.81, 127.92, 127.94, 127.98, 128.03, 128.13, 128.40, 128.54, 128.59 (C_{Ph}), 131.57 (C_{Phth2}), 133.65 (C_{Phth4}), 137.14, 137.57, 138.12, 138.24, 138.41 (C_{Ph}), 167.60 (C_{Phth1}). Coupled HSQC: J(^{a}H1-^{a}C1) 163Hz, J(^{b}H1-^{b}C1) 157Hz. LRMS (ESI^{+}): m/z [M+Na]$^{+}$ 944.3. HRMS (ESI^{+}): m/z [M+Na]$^{+}$ 944.3620 (found), (calcd 944.3616 for $C_{55}H_{55}NNaO_{12}$). $[\alpha]_D^{20}$=-1.6° (c= 0.50, $CHCl_3$). IR (ν_{max},

cm^{-1}, neat): 3475 (broad, O-H stretch), 3063, 3030, 2872 (C-H stretch), 1775, 1712 (imide C=O stretch), 1610, 1496, 1454, 1387, 1362, 1315, 1265, 1208, 1159, 1070, 1050, 1027, 933, 912, 875, 819, 793, 735,721, 696, 660, 644. R_f= 0.18 (ethyl acetate: petroleum ether= 2: 3).

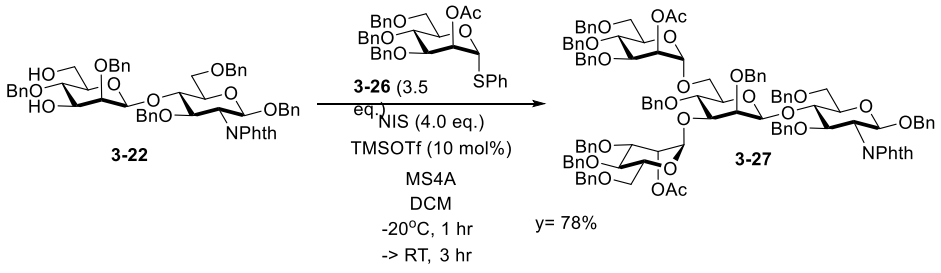
^{1}H NMR



^{13}C NMR

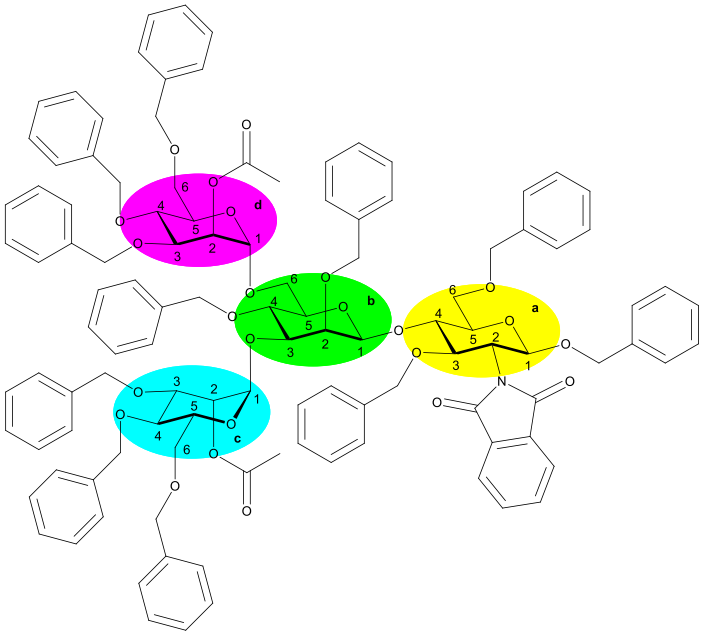


Preparation of benzyl 4-*O*-[3,6-*O*-di-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-(+)-mannopyranosyl)-2,4-di-*O*-benzyl-β-D-(+)-mannopyranosyl]-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-27**)



4-*O*-(2,4-di-*O*-benzyl-β-D-(+)-mannopyranosyl)-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-22**, 300 mg, 0.325 mmol, 1.0 eq.) and phenyl 2-*O*-acetyl-3,4,6-tri-*O*-benzyl-1-thio-α-D-(+)-mannopyranoside (**3-26**, 666 mg, 1.14 mmol, 3.5 eq.) were dissolved in toluene and solvents were removed by evaporation to dryness. To the residue were added activated MS4Å (300 mg), dichloromethane (6.5 mL) and *N*-iodosuccinimide (NIS, 293 mg,

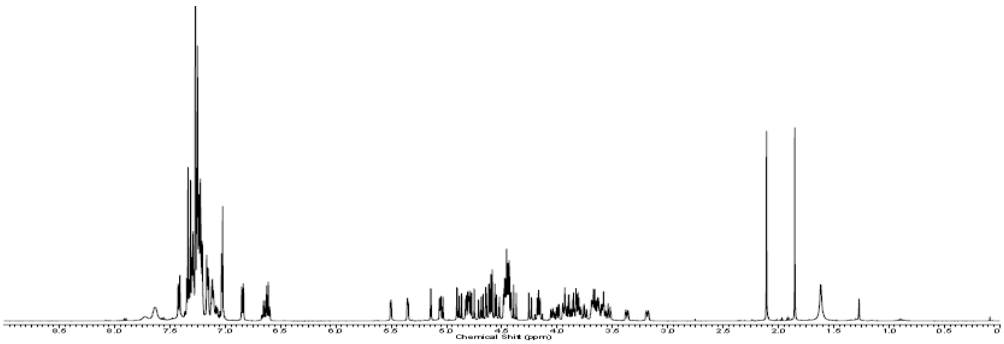
1.30 mmol, 4.0 eq.). After being stirred for 30 min, the solution was cooled to -20°C. Trimethylsilyl trifluoromethanesulfonate (TMSOTf, 5.9 μL, 0.0325 mmol, 10 mol%) was added and the solution was stirred for 1 hr at this temperature. The mixture was warmed up to room temperature and stirred for 3 hr. The reaction was quenched by addition of triethylamine (227 μL, 1.63 mmol, 5.0 eq.) and filtered through celite. The filtrate was washed with 10 wt% $Na_2S_2O_3$ (30 mL) and brine (30 mL), dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel: 200 mL, eluent: ethyl acetate: toluene= 1: 5) to afford the titled compound (**3-27**) as colorless foam; 477 mg (y= 78%).



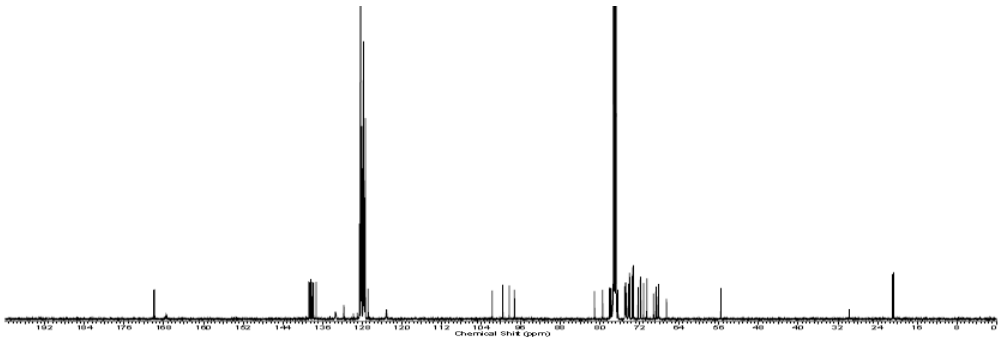
^{1}H NMR (500MHz, $CDCl_3$, δ (C*H*Cl_3)= 7.270): 1.85 (3H, s, COCH_3), 2.11 (3H, s, COCH_3), 3.18 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6}$ 3.0Hz, $J_{H5\text{-}H6}$ 2.0Hz, ^{b}H$_5$), 3.37 (1H, ddd, $J_{H5\text{-}H4}$ 9.5Hz, $J_{H5\text{-}H6}$ 3.0Hz, $J_{H5\text{-}H6}$ 2.0Hz, ^{a}H$_5$), 3.51-3.55 (1H, m, ^{d}H$_6$), 3.55-3.60 (2H, m, ^{a}H$_6$, ^{b}H$_3$), 3.60-3.71 (5H, m, ^{a}H$_6$', ^{c}H$_6$x2, ^{d}H$_5$, ^{d}H$_6$'), 3.75 (1H, dd, $J_{H6\text{-}H6'}$ 11.5Hz, $J_{H6\text{-}H5}$ 1.5Hz, ^{b}H$_6$), 3.77-3.96 (7H, m, ^{b}H$_2$, ^{b}H$_4$, ^{b}H$_6$', ^{c}H$_4$, ^{c}H$_5$, ^{d}H$_3$, ^{d}H$_4$), 3.99 (1H, dd, $J_{H3\text{-}H4}$ 9.5Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{c}H$_3$), 4.04 (1H, dd, $J_{H4\text{-}H5}$ 9.5Hz, $J_{H4\text{-}H3}$ 8.0Hz, ^{a}H$_4$), 4.13-4.21 (2H, m, ^{a}H$_2$,^{a}H$_3$), 4.24 (1H, d, $J_{Bn\text{-}Bn'}$ 11.0Hz, H$_{Bn}$), 4.38 (1H, d, $J_{Bn\text{-}Bn'}$ 11.5Hz, H$_{Bn}$), 4.40-4.50 (8H, m, H$_{Bn}$ x 8), 4.54 (1H, d, $J_{Bn\text{-}Bn'}$ 13.0Hz, H$_{Bn}$), 4.57 (1H, d, $J_{Bn\text{-}Bn'}$ 12.5Hz, H$_{Bn}$), 4.599 (1H, s, ^{b}H$_1$), 4.600 (1H, d, $J_{Bn\text{-}Bn'}$ 13.0Hz, H$_{Bn}$), 4.63 (1H, d, $J_{Bn\text{-}Bn'}$ 11.0Hz, H$_{Bn}$), 4.66 (1H, d, $J_{Bn\text{-}Bn'}$ 12.0Hz, H$_{Bn}$), 4.70 (1H, d, $J_{Bn\text{-}Bn'}$ 11.5Hz, H$_{Bn}$), 4.76 (1H, d, $J_{Bn\text{-}Bn'}$ 12.0Hz, H$_{Bn}$), 4.80 (1H, d, $J_{Bn\text{-}Bn'}$ 10.5Hz, H$_{Bn}$), 4.807 (1H, d, $J_{Bn\text{-}Bn'}$ 12.0Hz, H$_{Bn}$), 4.814 (1H, d, $J_{Bn\text{-}Bn'}$ 13.0Hz, H$_{Bn}$), 4.87 (1H, d, $J_{Bn\text{-}Bn'}$ 11.0Hz, H$_{Bn}$), 4.90 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{d}H$_1$), 5.04 (1H, d, $J_{Bn\text{-}Bn'}$ 12.0Hz, H$_{Bn}$), 5.06 (1H, d, $J_{H1\text{-}H2}$ 8.0Hz, ^{a}H$_1$), 5.14 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, ^{c}H$_1$), 5.35 (1H, dd, $J_{H2\text{-}H3}$ 3.0Hz, $J_{H2\text{-}H1}$ 1.5Hz, ^{d}H$_2$), 5.50 (1H, dd, $J_{H2\text{-}H3}$ 3.0Hz, $J_{H2\text{-}H1}$ 2.0Hz, ^{c}H$_2$),

6.59-6.68 (3H, m, H_{Ph}), 6.84 (2H, d, *J* 7.0Hz, H_{Ph}), 7.00-7.44 (47H, m, H_{Ph}), 7.59-7.68 (2H, br, H_{Phth}), 7.68-7.78 (1H, br, H_{Phth}). ^{13}C NMR (126MHz, $CDCl_3$, δ (*C*HCl_3)= 77.00): 20.75, 21.00 (CO*C*H_3 x2), 55.62 ($^aC_2$), 66.55 ($^bC_6$), 68.15 ($^dC_2$), 68.30 ($^aC_6$), 68.66, 68.71 ($^cC_2$, $^dC_6$), 69.20 ($^cC_6$), 70.53 (C_{Bn}), 71.20 (C_{Bn}), 71.79, 71.84 (C_{Bn}, $^dC_5$), 72.29 ($^cC_5$), 73.28, 73.39, 73.47 (C_{Bn} x3), 74.02, 74.04, 74.17, 74.21 (C_{Bn} x2, $^cC_4$, $^dC_4$), 74.60 ($^bC_4$), 74.76, 74.77, 74.86, 74.89, 74.98 (C_{Bn} x3, $^aC_5$, $^bC_5$), 76.36 ($^aC_3$), 77.71, 77.82 ($^bC_2$, $^dC_3$), 78.08 ($^cC_3$), 79.48 ($^aC_4$), 81.10 ($^bC_3$), 97.23 ($^aC_1$), 98.33 ($^dC_1$), 99.61 ($^cC_1$), 101.74 ($^bC_1$), 123.05 (C_{Phth3}), 126.80, 127.28, 127.32, 127.36, 127.47, 127.54, 127.63, 127.69, 127.76, 127.79, 128.07, 128.13, 128.17, 128.20, 128.29, 128.33, 128.37, 128.54, 129.02 (C_{Ph} x 33), 131.67 (C_{Phth2}), 133.32 (C_{Phth4}), 137.23, 137.78, 137.89, 137.97, 138.01, 138.06, 138.35, 138.51, 138.56, 138.62, 138.76 (C_{Ph}x11), 167.55 (C_{Phth1}), 169.81, 170.02 (*C*O*C*H_3 x2). Coupled HSQC: *J*($^aH1$-$^aC1$) 164Hz, *J*($^bH1$-$^bC1$) 157Hz, *J*($^cH1$-$^cC1$) 173Hz, *J*($^dH1$-$^dC1$) 172Hz. LRMS (ESI^+): m/z $[M+Na]^+$ 1892.8. HRMS (ESI^+): m/z $[M+Na]^+$ 1892.7706 (found), (calcd 1892.7701 for $C_{113}H_{115}NNaO_{24}$). $[\alpha]_D^{20}$= +15.6° (c= 0.50, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 3088, 3064, 3030, 2868 (C-H stretch), 1775, 1746, 1716 (ester C=O stretch, imide C=O stretch), 1497, 1454, 1387, 1368, 1234, 1140, 1077, 1052, 1028, 984, 737, 722, 698, 659. R_f= 0.39 (ethyl acetate: toluene= 1: 4).

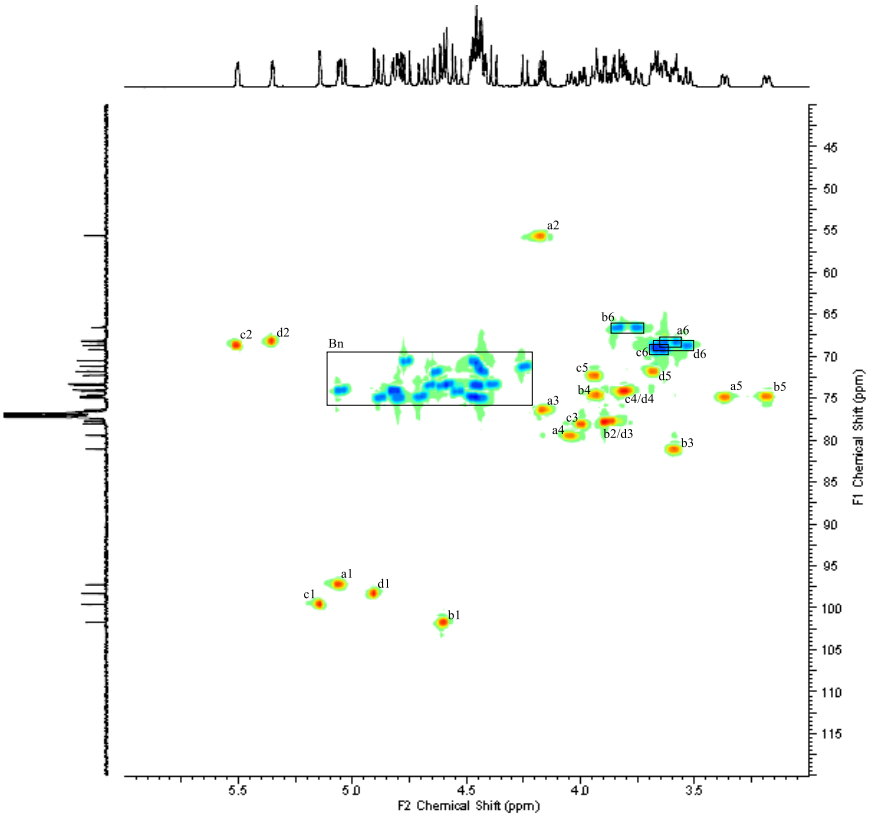
^{1}H NMR



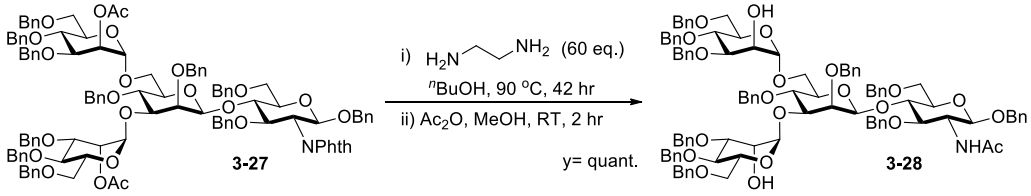
^{13}C NMR



HSQC

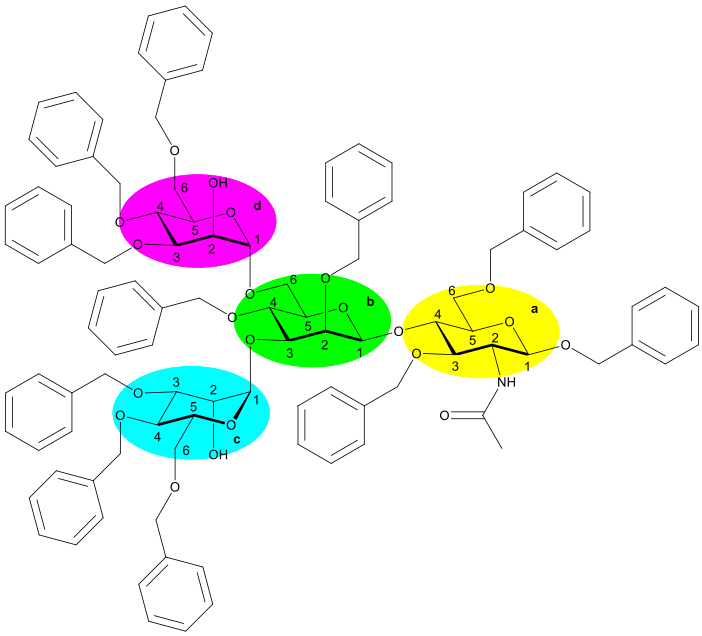


Preparation of benzyl 4-*O*-[3,6-*O*-di-(3,4,6-tri-*O*-benzyl-α-D-(+)-mannopyranosyl)-2,4-di-*O*-benzyl-β-D-(+)-mannopyranosyl]-3,6-di-*O*-benzyl-2-deoxy-2-acetamido-β-D-(+)-glucopyranoside (**3-28**)



To a solution of benzyl 4-*O*-[3,6-*O*-di-(2-*O*-acetyl-3,4,6-tri-*O*-benzyl-α-D-(+)-mannopyranosyl)-2,4-di-*O*-benzyl-β-D-(+)-mannopyranosyl]-3,6-di-*O*-benzyl-2-deoxy-2-phthalimido-β-D-(+)-glucopyranoside (**3-27**, 150 mg, 0.0802 mmol, 1.0 eq.) in *n*-butanol (3.2 mL) was added ehylenediamine (107 μL, 1.60 mmol, 20 eq.). The solution was heated to 90°C and stirred for 5 hr. Additional amount of ethylenediamine (214 μL, 3.20 mmol, 40 eq.) was added and stirred for 37 hr. Solvents were removed by co-evaporation with toluene. The residue was dissolved in methanol (3.2 mL). Acetic anhydride (37.9 μL, 0.401 mmol, 5 eq.) was added and the mixture

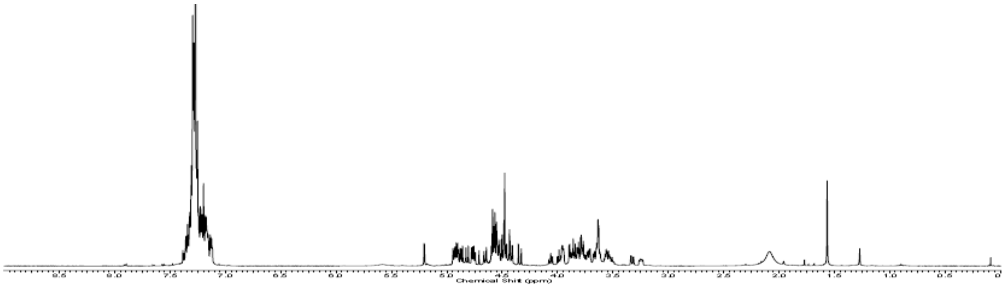
was stirred at room temperature for 2 hr. After removal of solvents and reagents by evaporation, the residue was purified by Biotage Flash Purification System (cartridge; Biotage SNAP 10 g, eluent gradient; ethyl acetate/ petroleue ether = 9% -> 72 %, 10CV) to afford the titled compound (**3-28**) as colorless syrup; 136 mg (y= quant.).



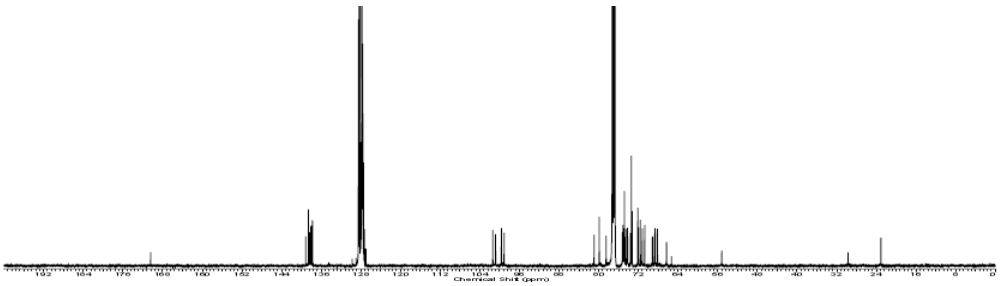
^{1}H NMR (500MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 1.56 (3H, s, $COCH_3$), 3.25 (1H, ddd, $J_{H5\text{-}H4}$ 10.0Hz, $J_{H5\text{-}H6}$ 4.5Hz, $J_{H5\text{-}H6}$ 2.0Hz, $^{b}H_5$), 3.33 (1H, dd, $J_{H6\text{-}H6'}$ 11.0Hz, $J_{H6\text{-}H5}$ 1.5Hz, $^{d}H_6$), 3.47-3.52 (1H, m, $^{a}H_2$), 3.52-3.59 (2H, m, $^{a}H_5$, $^{d}H_6$'), 3.60- 3.69 (5H, m, $^{a}H_6$ x2, $^{b}H_3$, $^{b}H_6$, $^{c}H_6$), 3.69-3.3.74 (2H, m, $^{c}H_6$', $^{d}H_5$), 3.74-3.90 (8H, m, $^{b}H_2$, $^{b}H_4$, $^{b}H_6$', $^{c}H_3$, $^{c}H_4$, $^{c}H_5$, $^{d}H_3$, $^{d}H_4$), 3.93-3.3.97 (2H, m, $^{c}H_2$, $^{d}H_2$), 3.98 (1H, dd, $J_{H4\text{-}H3}$ 7.5Hz, $J_{H4\text{-}H5}$ 7.5Hz, $^{a}H_4$), 4.06 (1H, dd, $J_{H3\text{-}H2}$ 8.0Hz, $J_{H3\text{-}H4}$ 8.0Hz, $^{a}H_3$), 4.34 (1H, d, $J_{Bn\text{-}Bn'}$ 12.0Hz, H_{Bn}), 4.42 (1H, d, $J_{Bn\text{-}Bn'}$ 12.0Hz, H_{Bn}), 4.43 (1H, d, $J_{Bn\text{-}Bn'}$ 11.0Hz, H_{Bn}), 4.45-4.51 (5H, m, H_{Bn} x5), 4.51-4.4.61 (8H, m, H_{Bn} x7, $^{b}H_1$), 4.66 (1H, d, $J_{Bn\text{-}Bn'}$ 11.0Hz, H_{Bn}), 4.75 (1H, d, $J_{Bn\text{-}Bn'}$ 11.5Hz, H_{Bn}), 4.77 (1H, d, $J_{Bn\text{-}Bn'}$ 10.5Hz, H_{Bn}), 4.82 (1H, d, $J_{Bn\text{-}Bn'}$ 11.0Hz, H_{Bn}), 4.87 (1H, d, $J_{Bn\text{-}Bn'}$ 12.0Hz, H_{Bn}), 4.89 (1H, d, $J_{Bn\text{-}Bn'}$ 11.5Hz, H_{Bn}), 4.92 (1H, d, $J_{Bn\text{-}Bn'}$ 11.5Hz, H_{Bn}), 4.92 (1H, d, $J_{H1\text{-}H2}$ 1.5Hz, $^{d}H_1$), 4.94 (1H, d, $J_{H1\text{-}H2}$ 6.5Hz, $^{a}H_1$), 5.20 (1H, d, $J_{H1\text{-}H2}$ 1.0Hz, $^{c}H_1$), 5.46 (1H, d, $J_{NH\text{-}H2}$ 7.5Hz, N*H*), 7.11-7.39 (55H, m, H_{Ph}). ^{13}C NMR (126MHz, $CDCl_3$, δ ($CHCl_3$)= 77.00): 23.20 ($COCH_3$), 55.18 ($^{a}C_2$), 66.30 ($^{b}C_6$), 68.13($^{d}C_2$), 68.62 ($^{c}C_2$), 68.71 ($^{c}C_6$), 69.11, 69.17 ($^{a}C_6$, $^{d}C_6$), 70.71 (C_{Bn}), 71.22 ($^{d}C_5$), 71.62 (C_{Bn}), 71.99 ($^{c}C_5$), 72.10 (C_{Bn}), 73.27, 73.43, 73.43, 73.60 (C_{Bn} x 4), 74.18, 74.38 ($^{c}C_4$, $^{d}C_4$), 74.71 (C_{Bn}), 74.80, 74.80 (C_{Bn}, $^{b}C_5$), 74.98, 75.02 (C_{Bn}, $^{a}C_5$), 75.11 (C_{Bn}), 75.24 ($^{b}C_4$,), 76.90 ($^{a}C_4$), 77.36 ($^{a}C_3$), 78.48 ($^{b}C_2$), 79.93, 79.93 ($^{c}C_3$, $^{d}C_3$), 80.94 ($^{b}C_3$), 99.11 ($^{a}C_1$), 99.64 ($^{d}C_1$), 100.81 ($^{b}C_1$), 101.37 ($^{c}C_1$), 127.25, 127.43, 127.54, 127.62, 127.66, 127.69, 127.75, 127.78, 127.81, 127.86, 127.94, 127.97, 128.07, 128.10 128.23, 128.25, 128.28, 128.35, 128.49 (C_{Ph} x 33), 137.75, 137.75, 137.82, 138.01, 138.03, 138.08, 138.31, 138.37, 138.59, 138.59, 139.16 (C_{Ph}x11), 170.24 ($COCH_3$). Coupled HSQC: J(^{a}H1-^{a}C1) 165Hz, J(^{b}H1-^{b}C1) 156Hz, J(^{c}H1-^{c}C1) 172Hz, J(^{d}H1-^{d}C1) 170Hz. LRMS (ESI^{+}): m/z $[M+Na]^{+}$ 1720.8. HRMS (ESI^{+}): m/z

$[M+Na]^+$ 1720.7544 (found), (calcd 1720.7541 for $C_{103}H_{111}NNaO_{21}$). $[\alpha]_D^{20}$= +16.6° (c= 0.38, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 3425 (broad, O-H stretch, N-H stretch), 3087, 3063, 3030, 2869 (C-H stretch), 1655 (amide-I), 1541 (amide-II), 1497, 1454 (ring C=C stretch), 1364, 1314, 1209, 1072, 1052, 1029, 911, 804, 736, 698. R_f= 0.22 (methanol: dichloromethane= 1: 20).

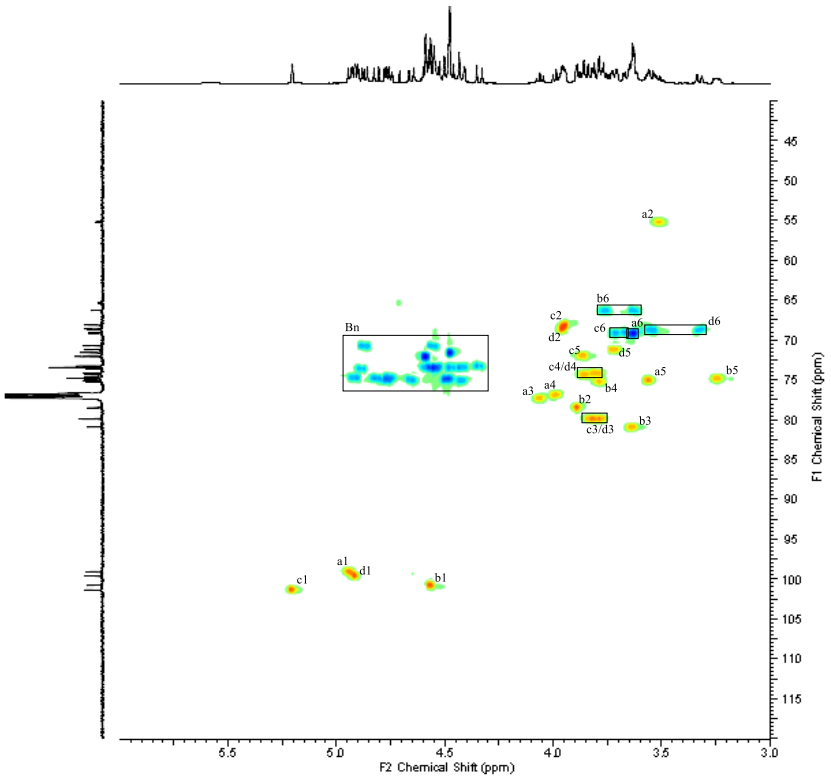
^{1}H NMR



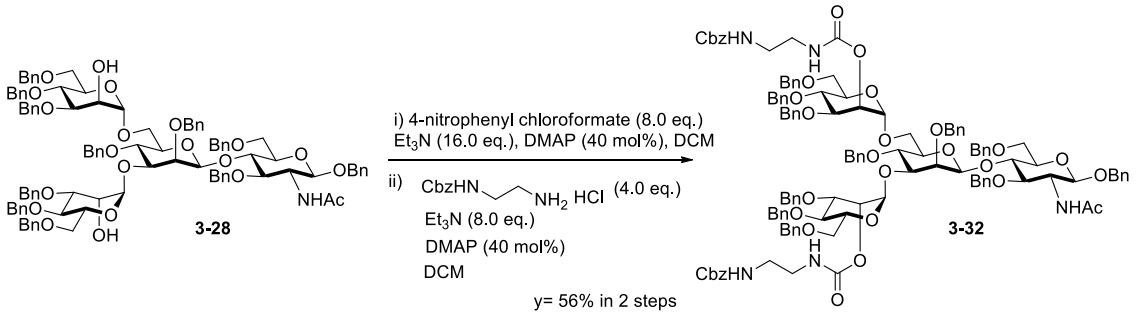
^{13}C NMR



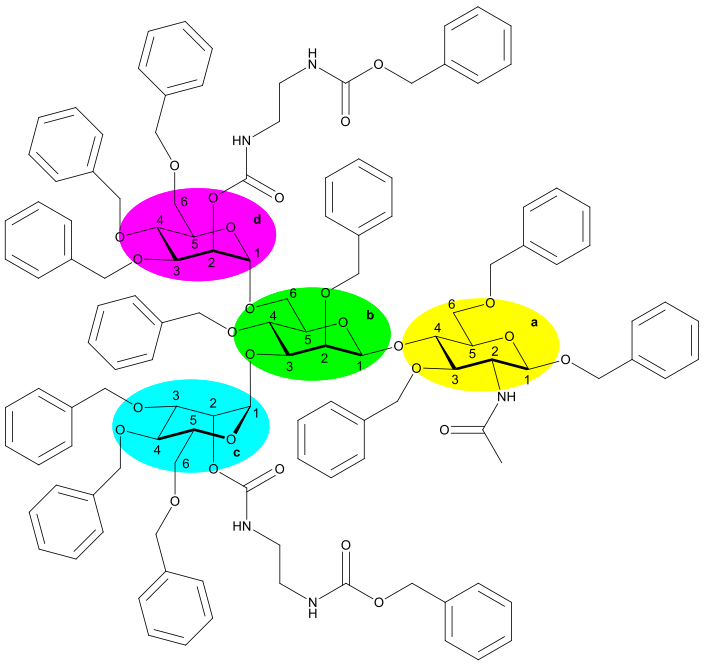
HSQC



Preparation of benzyl 4-*O*-(3,6-*O*-di-[2-*O*-([2-(benzyloxycarbonylamino)ethylamino]carbonyl)-3,4,6-tri-*O*-benzyl-α-D-(+)-mannopyranosyl]-2,4-di-*O*-benzyl-β-D-(+)-mannopyranosyl)-3,6-di-*O*-benzyl-2-deoxy-2-acetamido-β-D-(+)-glucopyranoside (**3-32**)



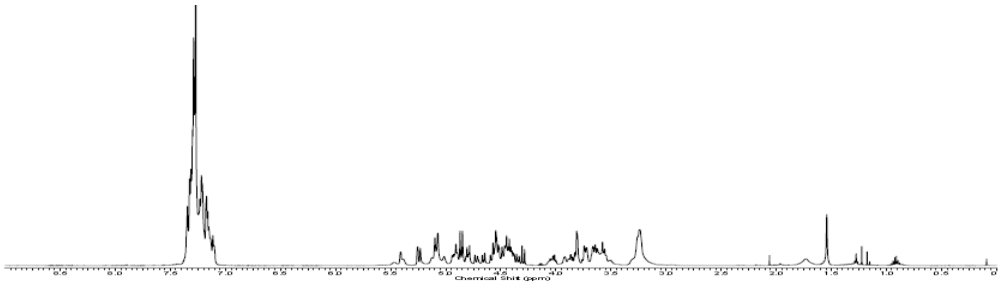
To a solution of benzyl 4-*O*-[3,6-*O*-di-(3,4,6-tri-*O*-benzyl-α-D-(+)-mannopyranosyl)-2,4-di-*O*-benzyl-β-D-(+)-mannopyranosyl]-3,6-di-*O*-benzyl-2-deoxy-2-acetamido-β-D-(+)-glucopyranosi de (**3-28**, 62.3 mg, 0.0367 mmol, 1.0 eq.) in dichloromethane (0.73 mL) were added 4-nitrophenyl chloroformate (29.6 mg, 0.147 mmol, 4.0 eq.), triethylamine (40.9 μL, 0.293 mmol, 8.0 eq.) and *N*,*N*-dimethylaminopyridine (1.8 mg, 0.0147 mmol, 40 mol%). The mixture was stirred at room temperature for 2 hr. Additional portion of 4-nitrophenyl chloroformate (29.6 mg, 0.147 mmol, 4.0 eq.) and triethylamine (40.9 μL, 0.293 mmol, 8.0 eq.) were added and the mixture was stirred at room temperature for 3 hr. The reaction mixture was concentrated and the residue was applied to column chromatography (silica gel: 40 mL, eluent: ethyl acetate: petroleum ether = 1: 2-> 3: 2) to afford the 4-nitrophenyl carbonate intermediate (>90% purity by ^{1}H-NMR containing 4-nitrophenol as contaminant) as light yellow syrup; 52.0 mg. The residue was dissoveld in dichloromethane (0.466 mL) and *N*-Cbz-ethylenediamine hydrochloride (21.5 mg, 0.0932 mmol, 4.0 eq.), triethylamine (26.0 μL, 0.186 mmol, 8.0 eq.) and *N*,*N*-dimethylaminopyridine (1.1 mg, 0.00932 mmol, 40 mol%) were added. The mixture was stirred at room temperature for 2 hr. The mixture was diluted with dichloromethane (5 mL) and washed with 1M HCl (5 mL x2). The aqeous phase was extracted with dichloromethane (5 mL). The combined organic phase was washed with sat. $NaHCO_3$ (5 mL x2) and brine (5 mL), drived over $MgSO_4$ and concentrated. The residue was purified by column chromatography (silica gel: 20 mL, eluent: ethyl acetate: petroleum ether = 5: 4-> 3: 2-> 2: 1) to afford the titled compound (**3-32**) as colorless film; 43.9 mg (y= 56.0 % in 2 steps).



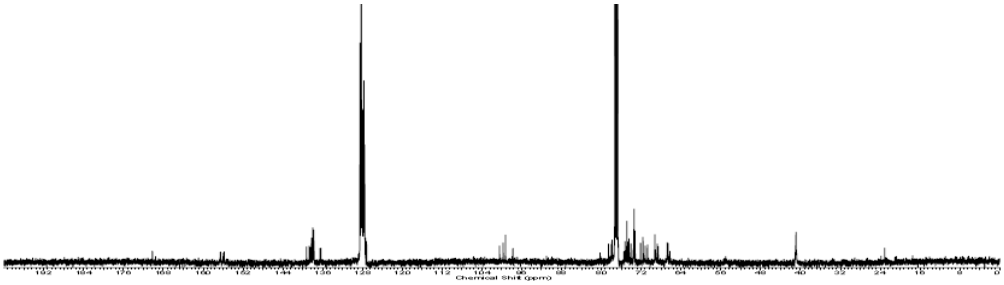
^{1}H NMR (500MHz, $CDCl_3$, δ ($CHCl_3$)= 7.270): 1.54 (3H, s, COCH_3), 3.07-3.33 (8H, m, NHCH_2), 3.19-3.27 (1H, m, $^{d}H_6$), 3.27-3.35 (1H, m, $^{b}H_5$), 3.47-3.54 (1H, m, $^{a}H_2$), 3.53-3.70 (8H, $^{a}H_5$, $^{a}H_6$, $^{b}H_3$, $^{b}H_6$ x2, $^{c}H_6$ x2, $^{d}H_6$'), 3.70-3.77 (3H, m, $^{a}H_6$', $^{b}H_4$, $^{d}H_5$), 3.74-3.85 (4H, m, $^{b}H_2$, $^{c}H_4$, $^{c}H_5$, $^{d}H_4$), 3.88 (1H, dd, $J_{H3\text{-}H2}$ 3.0Hz, $J_{H3\text{-}H4}$ 9.5Hz,,$^{d}H_3$), 3.90-3.95 (1H, m, $^{c}H_3$), 4.01 (1H, dd, $J_{H4\text{-}H3}$ 7.5Hz, $J_{H4\text{-}H5}$ 7.5Hz, $^{a}H_4$), 4.05 (1H, dd, $J_{H3\text{-}H2}$ 7.5Hz, $J_{H3\text{-}H4}$ 7.5Hz, $^{a}H_3$), 4.30 (1H, d, $J_{Bn\text{-}Bn'}$ 12.0Hz, H_{Bn}), 4.35 (1H, d, $J_{Bn\text{-}Bn'}$ 10.5Hz, H_{Bn}), 4.37-4.50 (7H, m, H_{Bn} x7), 4.50-4.60 (5H, m, H_{Bn} x4, $^{b}H_1$), 4.66 (1H, d, $J_{Bn\text{-}Bn'}$ 11.0Hz, H_{Bn}), 4.72 (1H, d, $J_{Bn\text{-}Bn'}$ 12.0Hz, H_{Bn}), 4.80 (1H, d, $J_{Bn\text{-}Bn'}$ 10.5Hz, H_{Bn}), 4.81 (1H, d, $J_{Bn\text{-}Bn'}$ 11.0Hz, H_{Bn}), 4.83-4.88 (3H, m, H_{Bn} x3), 4.88-4.92 (2H, m, H_{Bn}, $^{d}H_1$), 4.94 (1H, d, $J_{H1\text{-}H2}$ 6.0Hz, $^{a}H_1$), 5.01 (1H, brs, OCON*H*), 5.04-5.12 (5H, m, OCOCNHCH_2Ph x2, OCON*H*), 5.13 (1H, brs,OCON*H*), 5.23 (1H, s, $^{c}H_1$), 5.26 (1H, dd, $J_{H2\text{-}H1}$ 2.0Hz, $J_{H2\text{-}H3}$ 2.0Hz, $^{d}H_2$), 5.39 (1H, brs, OCON*H*), 5.41 (1H, brs, $^{c}H_2$), 5.47 (1H, brs, N*H*COCH$_3$), 7.08-7.40 (65H, m, H_{Ph}). ^{13}C NMR (126MHz, $CDCl_3$, δ (*C*HCl_3)= 77.00): 23.12 (CO*C*H$_3$), 40.87, 40.92, 40.92, 41.03 (NH*C*H$_2$ x4), 55.05 ($^{a}C_2$), 66.22 ($^{b}C_6$), 66.60, 66.77 (NHCOO*C*H$_2$Ph x2), 68.59, 68.77 ($^{c}C_6$, $^{d}C_6$), 69.16 ($^{a}C_6$), 69.20, 69.20 ($^{c}C_2$, $^{d}C_2$), 70.72 (C_{Bn}), 71.21 ($^{d}C_5$), 71.62, 71.67 (C_{Bn} x2), 72.10 ($^{c}C_5$), 73.24, 73.41, 73.48, 73.59 (C_{Bn} x4), 74.07 ($^{c}C_4$), 74.42, 74.42 ($^{b}C_5$, $^{d}C_4$), 74.60 (C_{Bn}), 74.88, 74.88, 74.95 (C_{Bn} x 2, $^{a}C_5$), 75.25 (C_{Bn}), 75.46 ($^{b}C_4$), 76.60 ($^{a}C_4$), 77.29 ($^{a}C_3$), 77.89 ($^{b}C_2$), 78.04 ($^{c}C_3$), 78.54 ($^{d}C_3$), 80.22 ($^{b}C_3$), 97.78 ($^{d}C_1$), 99.27 ($^{a}C_1$), 99.74 ($^{c}C_1$), 100.47 ($^{b}C_1$), 127.17, 127.40, 127.49, 127.54, 127.60, 127.69, 127.71, 127.73, 127.78, 127.87, 127.99, 128.04, 128.10, 128.12, 128.19, 128.22, 128.24, 128.28, 128.32, 128.35, 128.44, 128.50 (C_{Ph} x 39), 136.37, 136.52, 137.71, 137.77, 137.83, 137.94, 137.97, 138.01, 138.25, 138.30, 138.48, 138.71, 139.21 (C_{Ph}x13), 155.74 (O*C*ONHCH$_2$, c-ring), 155.95 (O*C*ONHCH$_2$, d-ring), 156.50, 156.55 (NH*C*OOCH$_2$Ph x2), 170.18 (*C*OCH$_3$). Coupled HSQC: J(^{a}H1-^{a}C1) 164Hz, J(^{b}H1-^{b}C1) 157Hz, J(^{c}H1-^{c}C1) 173Hz, J(^{d}H1-^{d}C1) 172Hz. LRMS (ESI^{+}): m/z $[M+H]^{+}$ 2138.61, $[M+Na]^{+}$ 2160.55. HRMS (ESI^{+}): m/z $[M+2Na]^{2+}$ 1091.9572 (found), (calcd 1091.9564 for $C_{125}H_{135}N_5Na_2O_{27}$). $[\alpha]_D^{20}$=-2.94° (c= 0.99, $CHCl_3$). IR (ν_{max}, cm^{-1}, neat): 3355 (broad, O-H stretch, N-H stretch), 3089, 3064, 3031, 2929, 2874 (C-H stretch), 1720

(carbamate C=O stretch, amide-I), 1522 (carbamate N-H bend, amide-II), 1498, 1454 (ring C=C stretch), 1364, 1257, 1137, 1095, 1055, 1028, 911, 849, 737, 698, 669, 652, 612. R_f= 0.28 (ethyl acetate: petroleum ether= 3: 2).

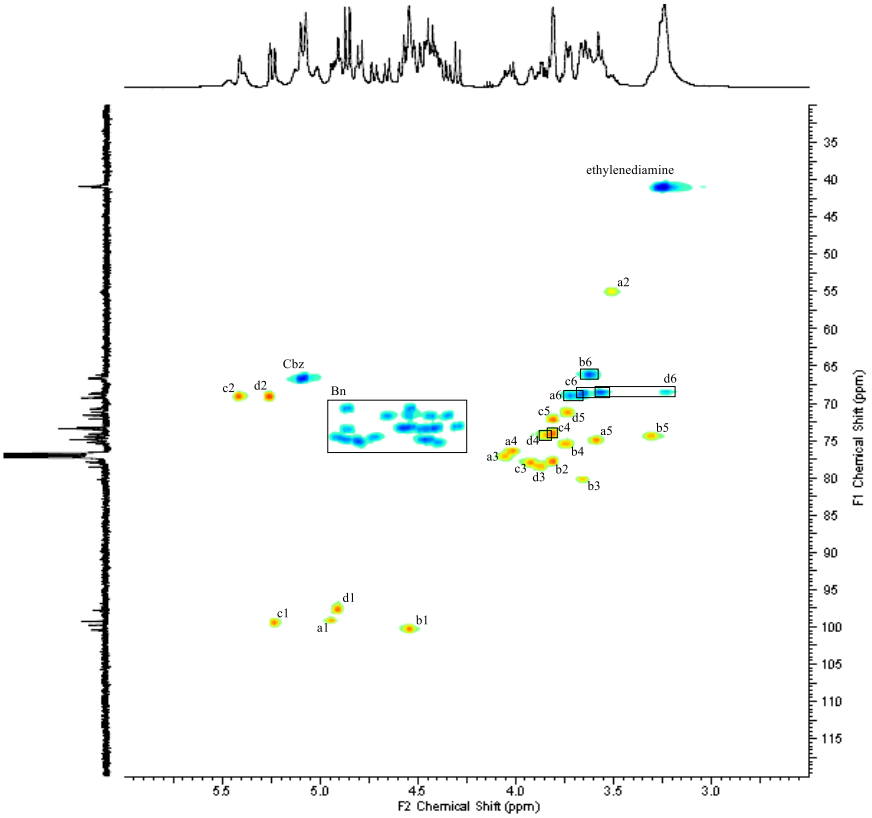
^{1}H NMR



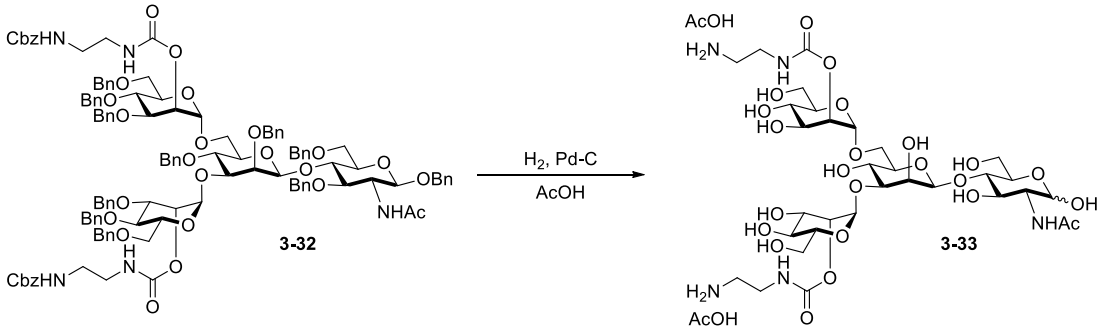
^{13}C NMR



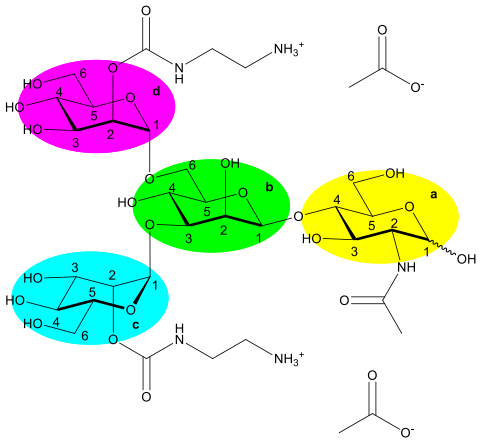
HSQC



Preparation of 4-*O*-(3,6-*O*-di-[2-*O*-(2-aminoethylaminocarbonyl)-α-D-(+)-mannopyranosyl]-β-D-(+)-mannopyranosyl)-2-deoxy-2-acetamido-β-D-(+)-glucopyranoside diacetic acid salt (**3-33**)



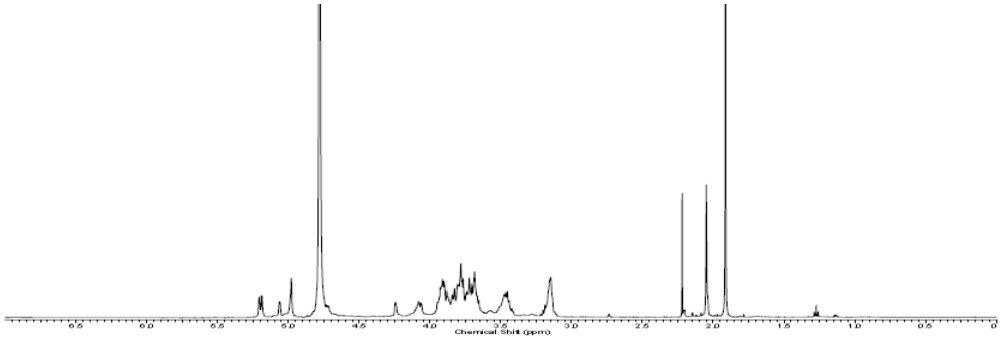
To a solution of 4-*O*-(3,6-*O*-di-[2-*O*-([2-(benzyloxycarbonylamino)ethylamino]carbonyl)-3,4,6-tri-*O*-benzyl-α-D-(+)-mannopyranosyl]-2,4-di-*O*-benzyl-β-D-(+)-mannopyranosyl)-3,6-di-*O*-benzyl-2-deoxy-2-acetamido-β-D-(+)-glucopyranoside (**3-32**, 40.0 mg, 0.0187 mmol, 1.0 eq.) in acetic acid (1.9 mL) were added 10% palladium on carbon (40.0 mg). After the reaction mixture was degassed under reduced pressure, hydrogen gas was charged. This procedure was repeated three times. The reaction mixture was stirred vigorously at room temperature for 24 hr. The reaction mixture was diluted with water (20 mL) and filtered with 0.8-μm syringe filter (cellulose acetate). The filtrate was concentrated under reduced pressure and lyophilized to afford the titled compound (**3-33**) as light brown amorphous; 18.7 mg (y= quant).



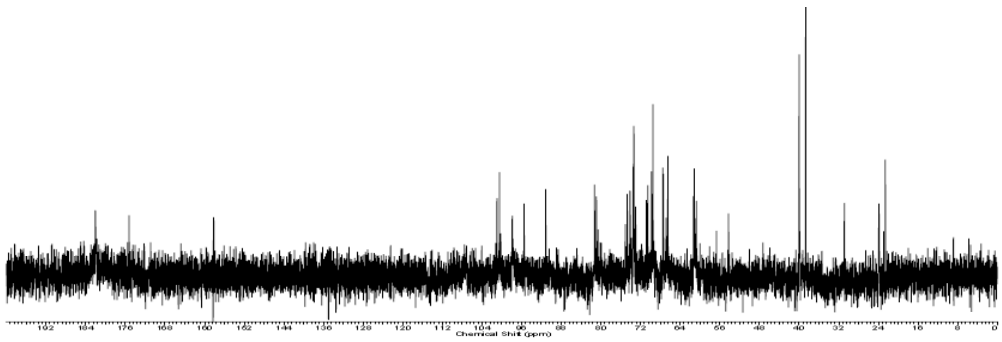
^{1}H NMR (500MHz, D_2O, δ (acetone)= 2.220): 1.92 (6H, s, CH_3COO$^-$ x2), 2.05 (3H, s, COCH_3), 3.10-3.20 (4H, m, CH_2NH$_3^+$ x2), 3.40-3.54 (4H, m, OCONHCH_2 x2), 3.55-3.60 (0.2H, m, $^{β\text{-}a}$H$_5$), 3.63-3.75 (6.4H, m, $^{α\text{-}a}$H$_4$, $^{β\text{-}a}$H$_2$, $^{β\text{-}a}$H$_3$, $^{β\text{-}a}$H$_4$, ^{b}H$_5$, ^{c}H$_4$, ^{d}H$_4$, ^{d}H$_5$, H$_6$), 3.75-3.86 (7.0H, ^{b}H$_3$, ^{b}H$_4$, ^{b}H$_6$, ^{c}H$_5$, H$_6$ x3), 3.86-3.96 (5.4H, $^{α\text{-}a}$H$_2$, $^{α\text{-}a}$H$_3$, $^{α\text{-}a}$H$_5$, ^{b}H$_6$', H$_6$ x2), 4.07 (1H, dd, $J_{H3\text{-}H4}$ 9.5Hz, $J_{H3\text{-}H2}$ 3.5Hz, ^{c}H$_3$), 4.10 (1H, dd, $J_{H3\text{-}H4}$ 9.5Hz, $J_{H3\text{-}H2}$ 3.0Hz, ^{d}H$_3$), 4.24 (1H, dd, $J_{H2\text{-}H1}$ 2.0Hz, $J_{H2\text{-}H3}$ 2.0Hz, ^{b}H$_2$), 4.72 (1H, d, $J_{H1\text{-}H2}$ 9.0Hz, $^{β\text{-}a}$H$_1$), 4.78 (1H, m, ^{b}H$_1$), 4.96-5.01 (2H, m, ^{d}H$_1$, ^{d}H$_2$),

5.05-5.07 (1H, m, $^{c}H_2$), 5.19 (1H, brs, $^{c}H_1$), 5.21 (0.8H, d, $J_{H1\text{-}H2}$ 3.0Hz, $^{\alpha\text{-}a}H_1$). ^{13}C NMR (126MHz, D_2O, δ (acetone)= 30.89): 22.60 (CO*C*H_3), 23.88 (*C*H_3COO^- x2), 38.68 (OCONH*C*H_2 x2), 39.96 (*C*$H_2NH_3^+$ x2), 54.22 ($^{\alpha\text{-}a}C_2$), 56.66 ($^{\beta\text{-}a}C_2$), 60.72, 61.16, 61.34 ($^{a}C_6$, $^{c}C_6$, $^{d}C_6$), 66.48 ($^{b}C_4$), 66.85 ($^{b}C_6$), 67.40, 67.51 ($^{c}C_4$, $^{d}C_4$), 69.51, 69.51 ($^{c}C_3$, $^{d}C_3$), 69.80 ($^{\alpha\text{-}a}C_3$), 70.54 ($^{\alpha\text{-}a}C_5$), 70.81 ($^{b}C_2$), 72.99 ($^{\beta\text{-}a}C_3$), 73.35 ($^{d}C_5$), 73.41 ($^{d}C_2$), 73.49 ($^{c}C_2$), 74.10 ($^{c}C_5$), 74.69 ($^{b}C_5$), 75.08 ($^{\beta\text{-}a}C_5$), 80.89 ($^{a}C_4$), 81.29 ($^{b}C_3$), 91.14 ($^{\alpha\text{-}a}C_1$), 95.50 ($^{\beta\text{-}a}C_1$), 97.89 ($^{d}C_1$), 100.51 ($^{c}C_1$), 100.96 ($^{b}C_1$), 158.14, 158.20 (O*C*ONH x2), 175.25 (*C*OCH$_3$), 182.02 (CH$_3$*C*OO$^-$ x2). Coupled HSQC: J($^{\alpha\text{-}a}$H1-$^{\alpha\text{-}a}$C1) 172Hz, J($^{\beta\text{-}a}$H1-$^{\beta\text{-}a}$C1) 162Hz, J(^{b}H1-^{b}C1) 162Hz, J(^{c}H1-^{c}C1) 174Hz, J(^{d}H1-^{d}C1) 173Hz. LRMS (ESI$^+$): m/z [M+2H]$^{2+}$ 440.68, [M+H]$^+$ 880.39. HRMS (ESI$^+$): m/z [M+Na]$^+$ 902.3309 (found), (calcd 902.3337 for $C_{32}H_{57}N_5NaO_{23}$). $[\alpha]_D^{20}$=+16.1° (c= 0.38, D_2O). IR (ν_{max}, cm^{-1}, neat): 3272 (broad, N$^+$-H, N-H, O-H stretch), 3027, 3016, 2970, 2942 (C-H stretch), 1738, 1727 (carbamate C=O stretch), 1636 (amide-I), 1541 (carbamate N-H bend, amide-II), 1407, 1367, 1259, 1229, 1217, 1207, 1131, 1034, 974, 796, 772, 651. R_f= 0.10 (1M ammonium acetate: isopropanol= 2: 3).

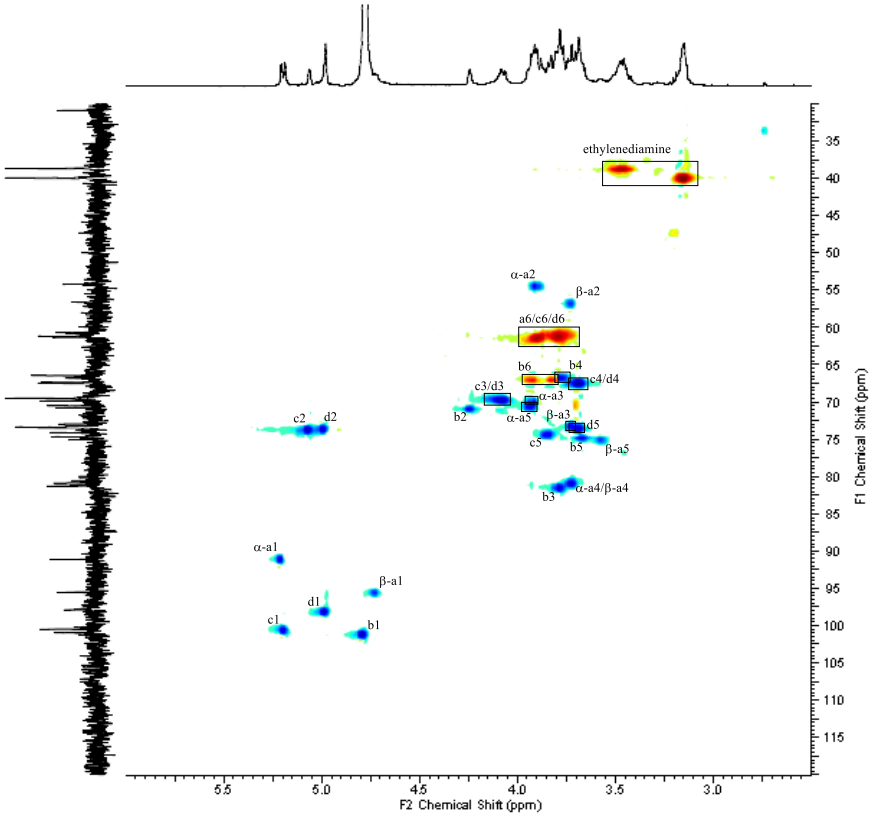
^{1}H NMR



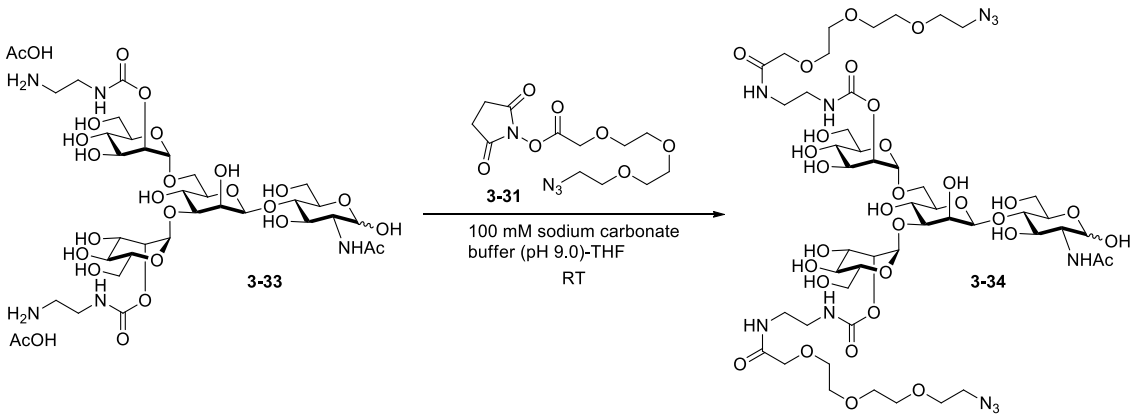
^{13}C NMR



HSQC



Preparation of 4-*O*-[3,6-*O*-di-(2-*O*-[(2-[(2-[2-(2-azidoethoxy)ethoxy]ethoxy)acetyl]aminoethyl-amino)carbonyl]-α-D-(+)-mannopyranosyl)-β-D-(+)-mannopyranosyl]-2-deoxy-2-acetamido-D-(+)-glucopyranose (**3-34**)

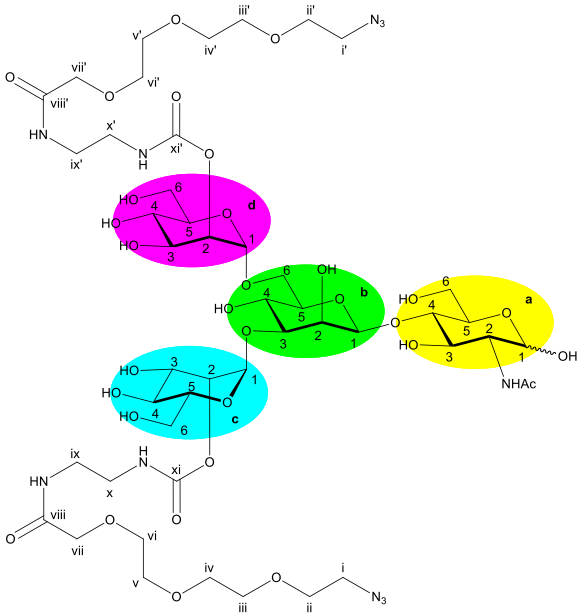


To a solution of 4-*O*-(3,6-*O*-di-[2-*O*-(2-aminoethylaminocarbonyl)-α-D-(+)-mannopyranosyl]-β-D-(+)-mannopyranosyl)-2-deoxy-2-acetamido-β-D-(+)-glucopyranoside diacetic acid salt (**3-33**, 15.0 mg, 0.0150 mmol, 1.0 eq.) in 100 mM sodium carbonate buffer (pH 9.0, 0.75 mL)

was added *N*-succinimidyl 2-(2-[2-(2-azidoethoxy)ethoxy]ethoxy)acetate (**3-31**, 19.8 mg, 0.0600 mmol, 4.0 eq.) in tetrahydrofuran (0.30 mL) and the solution was stirred at room temperature for 2 hr. The resultant mixture was acidified with 5M HCl to pH <2 and washed with diethyl ether (0.30 mL x 2). Residual organic solvents were removed by N_2 flow and the aqueous solution was applied to PD MidiTrap G-10 column for desalting. The target compound was eluted with water (0.25-mL fractions) and the fractions were analyzed with TLC (R_f= 0.42, water: acetonitrile= 1: 3). The collected fractions were lyophilized and the residue was redissoleved in 0.5 mL water. The target material was purified with HPLC under the following condition:

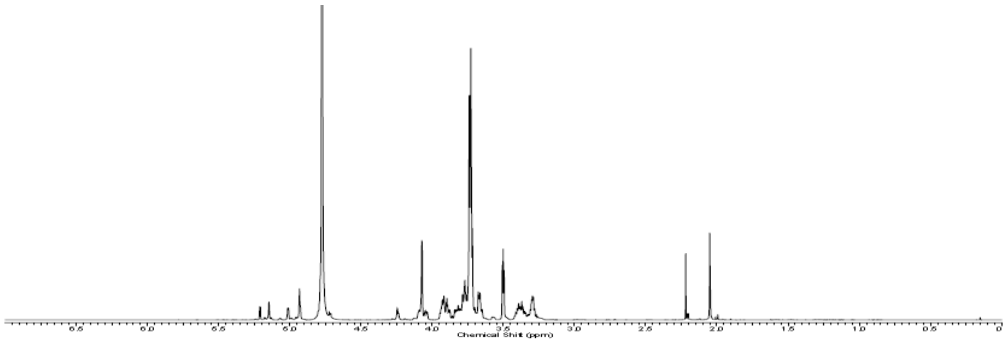
Column: Synergi 4u Hydro-RP 80A, 100 x 21.20 mm, 4 micron
Solvent A: 5 mM ammonium bicarbonate
Solvent B: 5 mM ammonium bicarbonate in 80% acetonitrile
Gradient: 0->2 min 15%B
2->15 min to 30%B
15->17 min 30%B
17->18 min to 100%B
18->20 min 100%B
20->22 min 15%B
Flowrate: 15 mL/min
Detection: 214 nm
t_R= 10.08, 10.34 min (Two peaks corresponding to the anomeric isomers were observed. Even if each peak was separately collected, these two peaks appeared again in the both solutions after incubation at 37°C for 4 hr.)

Fractions containing the target material were collected and organic solvents were removed by rotary evaporator. The resultant aqueous solution was lyophilized to afford the titled compound (**3-34**) as a white lyophilized powder; 10.0 mg (y= 51.8%).

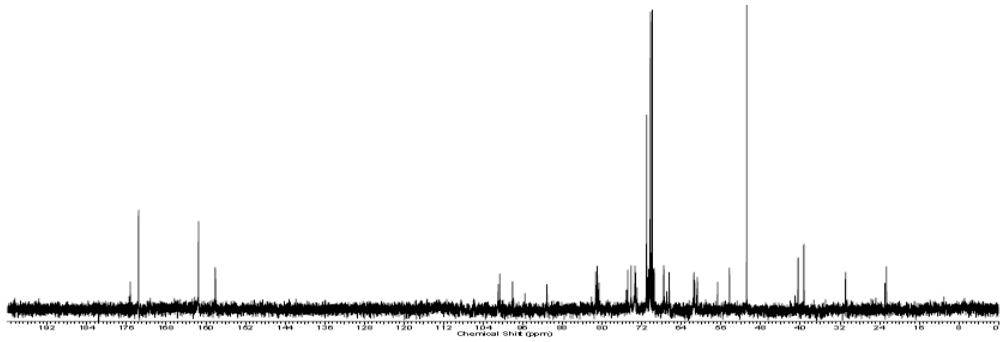


^{1}H NMR (700MHz, D_2O, δ (acetone)= 2.220): 2.05 (3H, s, COCH_3), 3.25-3.33 (4H, m, H_x x2, H_x x2), 3.33-3.43 (4H, m, H_{ix} x2, H_{ix} x2), 3.50 (4H, t, $J_{Hi\text{-}Hii}$ 4.9Hz, H_i x2, $H_{i'}$ x2), 3.55-3.59 (0.3H, m, $^{\beta\text{-}a}H_5$), 3.64-3.69 (4H, m, $^{b}H_5$,$^{c}H_4$, $^{d}H_4$, $^{d}H_5$), 3.69-3.85 (29.6H, $^{\alpha\text{-}a}H_4$, $^{\beta\text{-}a}H_2$, $^{\beta\text{-}a}H_3$, $^{\beta\text{-}a}H_4$, $^{b}H_3$, $^{b}H_4$, $^{b}H_6$, $^{c}H_5$, H_6 x4, $H_{ii\text{-}vi}$ x 10, $H_{ii'\text{-}vi'}$ x 10), 3.85-3.96 (5.1H, m, $^{\alpha\text{-}a}H_2$, $^{\alpha\text{-}a}H_3$, $^{\alpha\text{-}a}H_5$, $^{b}H_6$', H_6 x2), 4.04 (1H, dd, $J_{H3\text{-}H4}$ 9.8Hz, $J_{H3\text{-}H2}$ 2.8Hz, $^{c}H_3$),4.07 (4H, s, H_{vii} x2, H_{vii} x2), 4.06-4.11 (1H, m, $^{d}H_3$), 4.24 (1H, dd, $J_{H2\text{-}H1}$ 2.0Hz, $J_{H2\text{-}H3}$ 2.0Hz, $^{b}H_2$), 4.72 (1H, d, $J_{H1\text{-}H2}$ 7.7Hz, $^{\beta\text{-}a}H_1$), 4.78 (1H, m, $^{b}H_1$), 4.92-4.95 (2H, m, $^{d}H_1$, $^{d}H_2$), 5.00-5.03 (1H, m, $^{c}H_2$), 5.15 (1H, brs, $^{c}H_1$), 5.21 (0.7H, d, $J_{H1\text{-}H2}$ 3.5Hz, $^{\alpha\text{-}a}H_1$). ^{13}C NMR (176MHz, D_2O, δ (acetone)= 30.89): 22.61 (CO*C*H_3), 39.27, 39.31 (C_{ix}, $C_{ix'}$), 40.43, 40.47 (C_x, $C_{x'}$), 50.82 (C_i, $C_{i'}$), 54.27 ($^{\alpha\text{-}a}C_2$), 56.70 ($^{\beta\text{-}a}C_2$), 60.76, 61.32, 61.49 ($^{a}C_6$, $^{c}C_6$, $^{d}C_6$), 66.47 ($^{b}C_4$), 66.96 ($^{b}C_6$), 67.51, 67.61 ($^{c}C_4$, $^{d}C_4$), 69.48, 69.52 ($^{c}C_3$, $^{d}C_3$), 69.73 ($^{\alpha\text{-}a}C_3$), 69.88, 70.22, 70.27, 71.02 ($C_{ii\text{-}vi}$, $C_{ii'\text{-}vi'}$), 70.17 (C_{vii}, $C_{vii'}$), 70.56 ($^{\alpha\text{-}a}C_5$), 70.84 ($^{b}C_2$), 72.95 ($^{\beta\text{-}a}C_3$), 73.26 ($^{d}C_2$), 73.33 ($^{c}C_2$), 73.39 ($^{d}C_5$), 74.14 ($^{c}C_5$), 74.83 ($^{b}C_5$), 75.12 ($^{\beta\text{-}a}C_5$), 80.64 ($^{\beta\text{-}a}C_4$), 80.95 ($^{\alpha\text{-}a}C_4$), 81.28 ($^{b}C_3$), 91.13 ($^{\alpha\text{-}a}C_1$), 95.57 ($^{\beta\text{-}a}C_1$), 98.09 ($^{d}C_1$), 100.61 ($^{c}C_1$), 100.95 ($^{b}C_1$), 158.03, 158.10 (O*C*ONH x2), 173.58 (C_{viii}, $C_{viii'}$), 175.23 (*C*OCH$_3$). Coupled HSQC: J($^{\alpha\text{-}a}$H1-$^{\alpha\text{-}a}$C1) 172Hz, J($^{\beta\text{-}a}$H1-$^{\beta\text{-}a}$C1) 162Hz, J(^{b}H1-^{b}C1) 162Hz, J(^{c}H1-^{c}C1) 174Hz, J(^{d}H1-^{d}C1) 173Hz. LRMS (ESI$^+$): m/z $[M+H]^+$ 1310.6, $[M+Na]^+$ 1332.6. HRMS (ESI$^+$): m/z $[M+2Na]^{2+}$ 677.7497 (found), (calcd 677.7520 for $C_{48}H_{83}N_{11}Na_2O_{31}$). $[\alpha]_D^{20}$=+16.2° (c= 0.47, H_2O). IR (ν_{max}, cm^{-1}, neat): 3316 (broad, N-H, O-H stretch), 2919 (C-H stretch), 2106 (N_3 stretch), 1701, 1649 (amide-I), 1542 (amide-II), 1443, 1349, 1304, 1258, 1116, 1047, 833, 801, 778, 699, 679. R_f= 0.42 (H_2O: acetonitrile= 1: 3).

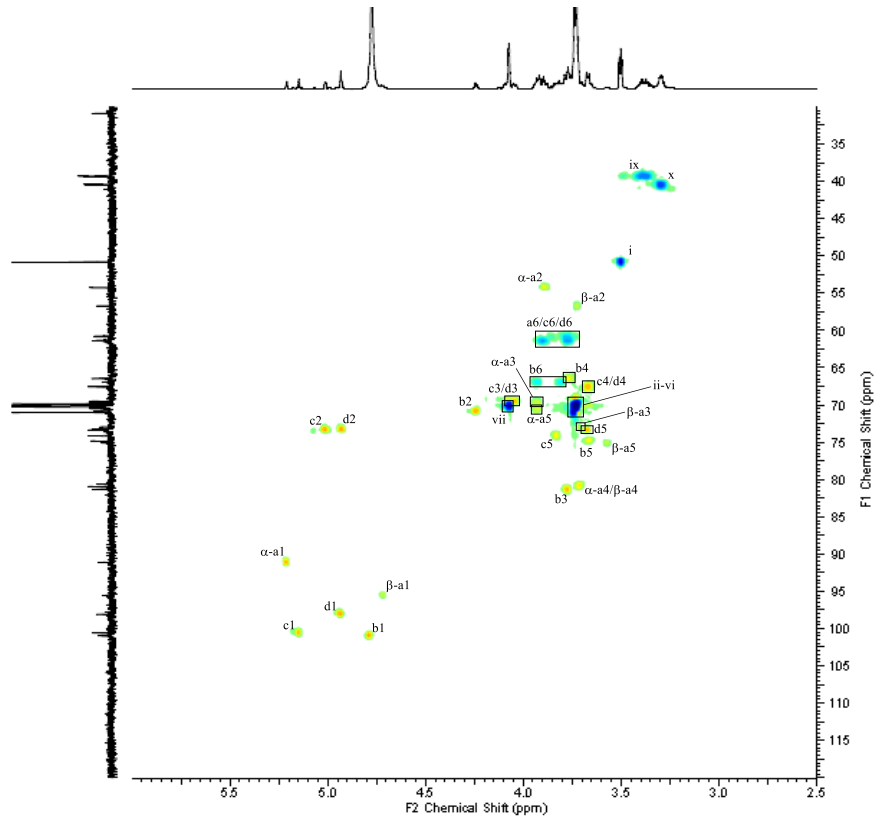
^{1}H NMR



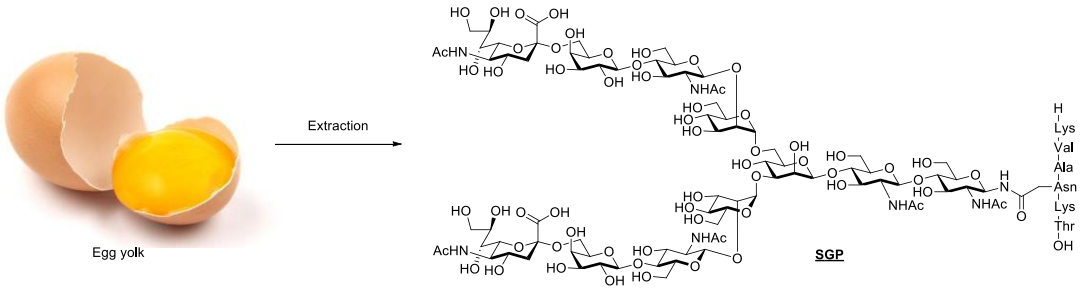
^{13}C NMR



HSQC

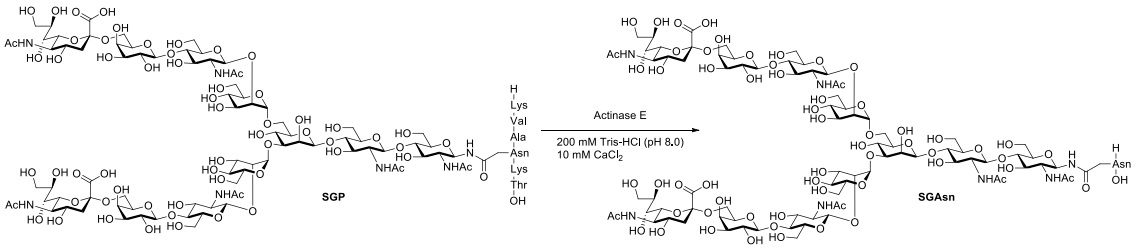


Extraction of SGP



To egg yolk (401 g) from 24 eggs was added ethanol (800 mL) and the suspension was mixed well and centrifuged (8000 rpm, 10 min). After the supernatant was discarded, the precipitation was mixed with ethanol (800 mL), centrifuged (8000 rpm, 10 min) and the precipitation was collected. This procedure was repeated two more times. The collected precipitation was suspended in water (400 mL) and centrifuged (8000 rpm, 10 min). The supernatant was collected and to the precipitation was added water (400 mL), mixed and centrifuged (8000 rpm, 10 min) to collect the supernatant. This procedure was repeated two more times and all the aqueous solutions were combined. When insoluble oil was observed, it was removed at this stage. After filtration to remove insoluble solid material, the aqueous solution was concentrated to 200 mL. The newly formed precipitation was removed by filtration. To the filtrate was added ethanol (1,400 mL) and the precipitation was collected by centrifugation (8000 rpm, 10 min). To the precipitate was added water (200 mL) and the suspension was vigorously stirred. The insoluble material was removed by centrifugation (8000 rpm, 10 min). To the supernatant was added ethanol (1,400 mL) and the precipitation was collected by centrifugation (8000 rpm, 10 min). This precipitation process was repeated one more time. The collected precipitate was dried in vacuo to afford white powder containing SGP; 4.24 g.

Preparation of $^{\gamma}$*N*-sialylglycoasparagine (SGAsn)



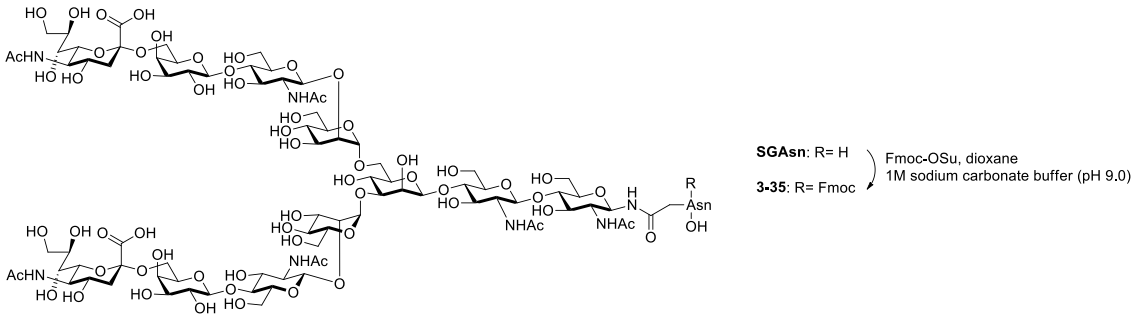
A powder (4.24 g) containing SGP was dissolved in 200 mM Tris-HCl, 10 mM $CaCl_2$, 0.01% NaN_3 (pH 8.0, 150 mL) and actinase E (Sigma-Aldrich, P5147, 424 mg) was added. The reaction solution was incubated at 37°C for 2days. Additional portion of actinase E (212 mg)

was added and the reaction solution was incubated at 37°C for 2 day. Precipitation was removed by centrifugation (8000 rpm, 10 min) and filtration with 0.8-μm membrane. The filtrate was concentrated down to 50 mL. The newly formed precipitation was separated from the supernatant by centrifugation (20,000 rpm, 5 min). The precipitate was washed in 10 mL water and the supernatant was separated from the insoluble material. This process was repeated until the sugar material was extracted completely. The combined supernatant was filtered with 0.22-μm membrane and applied to FPLC:

Column: HiPrep 26/10 desalting column
Eluent: 5 mM ammonium bicarbonate
Injection volume: <8 mL

Fractions were analyzed by TLC (1M ammonium acetate: isopropanol= 2: 3, H_2SO_4 staining, R_f= 0.25) and ones with the target material was collected and lyophilized to afford light brown powder containing SGAsn; 1.29 g.

Preparation of $^{\alpha}N$-Fmoc-$^{\gamma}N$-sialylglycoasparagine (**3-35**, Fmoc-SGAsn)



A powder (1.29 g) containing SGAsn was dissolved in 0.5M sodium carbonate buffer (pH 9.0, 13 mL) and Fmoc-OSu (3.87 g) in dioxane (13 mL) was added. The reaction mixture was stirred at room temperature for 2 hr. Fmoc-OSu (0.97 g) was added and was stirred at room temperature for 1.5 hr. The completion of the reaction was monitored by TLC. The resultant mixture was filtered to remove the precipitation. The precipitation was washed with water (10 mL x2) and the combined filtrate was washed with ethyl acetate (13 mL x3). The combined organic phase was extracted with water (5 mL) and the combined aqueous phase was filtered with 0.45-μm membrane and applied to FPLC:

Column: HiPrep 26/10 desalting column
Eluent: 5 mM ammonium bicarbonate

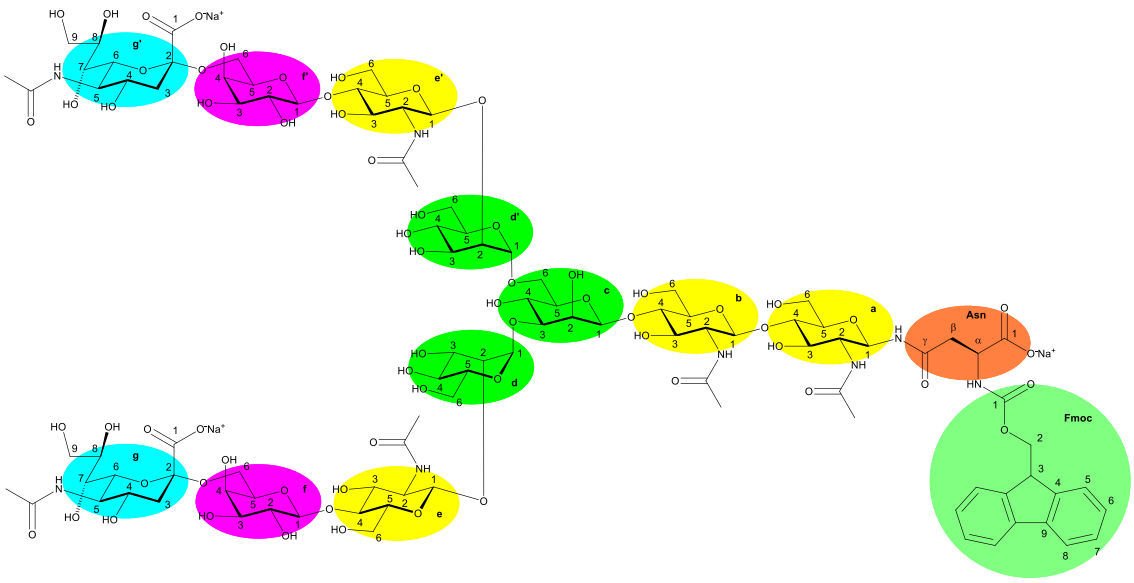
Injection volume: <6 mL

Fractions were analyzed by TLC (1M ammonium acetate: isopropanol= 2: 3) and collected fractions were lyophilized. The residue was dissolved in water (6 mL), filtered with 0.8-μm membrane and applied to HPLC:

Column: Synergi 4u Hydro-RP 80A, 100 x 21.20 mm, 4 micron
Solvent A: 5 mM ammonium bicarbonate
Solvent B: 5 mM ammonium bicarbonate in 80% acetonitrile
Gradient: 0->2 min 3%B
2->15 min to 10%B
15->17 min 10%B
17->18 min to 100%B
18->20 min 100%B
20->22 min 3%B
Flowrate: 15 mL/min
Detection: 254 nm

Collected peaks were lyophilized to afford ammonium salt of Fmoc-SGAsn as white amorphous; 170 mg. (TLC: 1M ammonium acetate: isopropanol= 2: 3, R_f= 0.51)

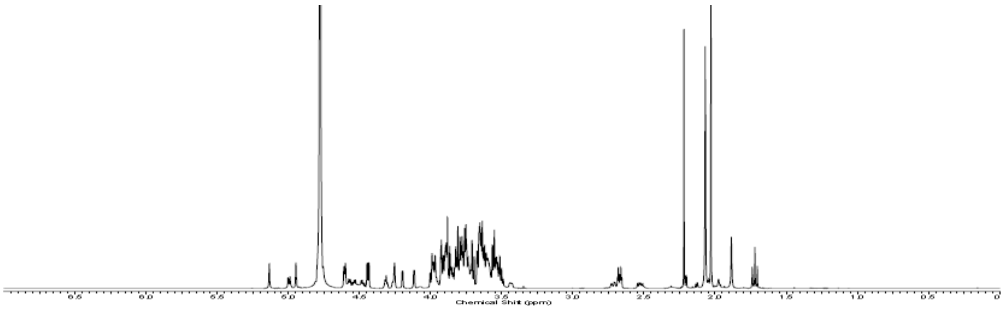
Cation was exchanged from ammonium to sodium by treatment with cation exchange resin. 170 mg of ammonium salt form was dissolved in 3.0 mL of water. 17 mL of Dowex 50WX8 100-200 mesh (1.7 meq/mL) was activated with 1M sodium hydroxide (17 mL x 10) and washed with water (17 mL x 4), with methanol (17 mL x 4) and water (17 mL x 8). The sample solution was applied to the column and eluted with water. Fractions were analyzed with TLC (1M ammonium acetate: isopropanol= 2: 3) and the collected fractions were lyophilized to afford sodium salt of Fmoc-SGAsn (**3-35**) as white lyophilized powder; 157 mg. Characterization of **3-35** was carried out using this trisodium salt.



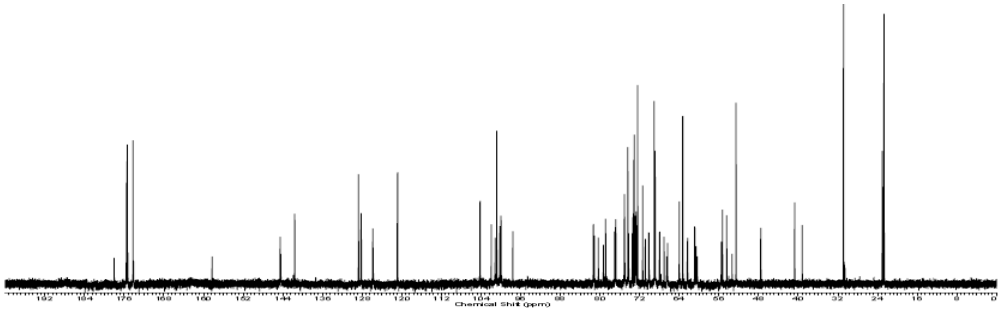
MALDI-TOF-MS (matrix: DHB): [trisodium form + Na]$^+$ 2647.73. $[\alpha]_D^{20}$= -10.2° (c= 0.50, H_2O). IR (ν_{max}, cm^{-1}, neat): 3294 (broad, N-H, O-H stretch), 2930 (C-H stretch), 1611 (amide-I), 1556 (carboxylate antisymmetric stretch, amide-II), 1377 (carboxylate symmetric stretch), 1315, 1222, 1060, 1033, 899, 760. 740, 607. ^{1}H NMR[112] (700MHz, D_2O, δ (acetone)= 2.220): 1.72 (2H, dd, $J_{H3ax\text{-}H3eq}$ 12.6Hz, $J_{H3\text{-}H4}$ 12.6Hz, $^{g}H_{3ax}$, $^{g'}H_{3ax}$), 1.89 (2.25H, s, $^{a}COCH_3$ (major conformer)), 1.98 (0.75H, s, $^{a}COCH_3$ (minor conformer)), 2.03 (6H, s, $COCH_3$ x 2), 2.067, 2.071 (9H, s x 2, $COCH_3$ x 3), 2.27-2.34 (0.25H, m, $^{Asn}H_\beta$ (minor conformer)), 2.48-2.54 (0.25H, m, $^{Asn}H_{\beta'}$ (minor conformer)), 2.53 (0.75H, dd, $J_{H\beta\text{-}H\beta'}$ 15.4Hz, $J_{H\beta\text{-}H\alpha}$ 9.1Hz, $^{Asn}H_\beta$ (major conformer)), 2.669 (1H, dd, $J_{H3eq\text{-}H3ax}$ 12.6Hz, $J_{H3eq\text{-}H4}$ 4.2Hz, $^{g}H_{3eq}$ or $^{g'}H_{3eq}$), 2.675 (1H, dd, $J_{H3eq\text{-}H3ax}$ 12.6Hz, $J_{H3eq\text{-}H4}$ 4.2Hz, $^{g'}H_{3eq}$ or $^{g}H_{3eq}$), 2.72 (0.75H, dd, $J_{H\beta'\text{-}H\beta'}$ 15.4Hz, $J_{H\beta'\text{-}H\alpha}$ 4.2Hz, $^{Asn}H_{\beta'}$ (major conformer)), 3.41-3.46 (0.75H, m, $^{a}H_1$ (major conformer)), 3.46-3.58 (9.25H, m, $^{a}H_5$ (minor conformer), $^{a}H_6$, $^{d}H_4$, $^{d'}H_4$, $^{f}H_2$, $^{f'}H_2$, $^{f}H_6$, $^{f'}H_6$, $^{g}H_7$, $^{g'}H_7$), 3.58-3.68 (17H, m, $^{a}H_4$, $^{a}H_6$', $^{b}H_5$, $^{c}H_5$, $^{d'}H_5$, $^{d}H_6$, $^{d'}H_6$, $^{e}H_4$, $^{e'}H_4$, $^{e}H_5$, $^{e'}H_5$, $^{f}H_3$, $^{f'}H_3$, $^{g}H_4$, $^{g'}H_4$, $^{g}H_9$, $^{g'}H_9$), 3.68-3.84 (20H, m, $^{a}H_2$, $^{a}H_3$, $^{b}H_2$, $^{b}H_3$, $^{b}H_4$, $^{b}H_6$, $^{c}H_3$, $^{c}H_4$, $^{c}H_6$, $^{d}H_5$, $^{e}H_2$, $^{e'}H_2$, $^{e}H_3$, $^{e'}H_3$, $^{f}H_5$, $^{f'}H_5$, $^{g}H_5$, $^{g'}H_5$, $^{g}H_6$, $^{g'}H_6$), 3.84-3.94 (13H, m, $^{b}H_6$', $^{d}H_3$, $^{d'}H_3$, $^{d}H_6$', $^{d'}H_6$', $^{e}H_6$, $^{e'}H_6$, $^{f}H_4$, $^{f'}H_4$, $^{g}H_8$, $^{g'}H_8$, $^{g}H_9$', $^{g'}H_9$'), 3.94-4.02 (5H, m, $^{c}H_6$', $^{e}H_6$', $^{e'}H_6$', $^{f}H_6$', $^{f'}H_6$'), 4.05-4.10 (0.25H, m, $^{Asn}H_\alpha$ (minor conformer)), 4.12 (1H, d, $J_{H2\text{-}H3}$ 3.5Hz, $^{d'}H_2$), 4.20 (1H, d, $J_{H2\text{-}H3}$ 3.5Hz, $^{d}H_2$), 4.25 (1H, s, $^{d}H_2$), 4.25-4.28 (0.75H, m, $^{Asn}H_\alpha$ (major conformer)), 4.31 (1H, t, $J_{H3\text{-}H2}$ 6.3Hz, $^{Fmoc}H_3$), 4.439 (1H, d, $J_{H1\text{-}H2}$ 8.4Hz, $^{f}H_1$ or $^{f'}H_1$), 4.425 (1H, d, $J_{H1\text{-}H2}$ 7.7Hz, $^{f'}H_1$ or $^{f}H_1$), 4.48 (0.75H, dd, $J_{H2\text{-}H2'}$ 11.2Hz, $J_{H2\text{-}H3}$ 6.3Hz, $^{Fmoc}H_2$ (major conformer)), 4.48-4.53 (0.50H, m, $^{Fmoc}H_2$ (minor conformer)), 4.54 (0.75H, dd, $J_{H2'\text{-}H2}$ 11.2Hz, $J_{H2'\text{-}H3}$ 6.3Hz, $^{Fmoc}H_2$' (major conformer)), 4.57 (1H, d, $J_{H1\text{-}H2}$ 7.7Hz, $^{b}H_1$), 4.60 (2H, d, $J_{H1\text{-}H2}$ 6.3Hz, $^{e}H_1$, $^{e'}H_1$), 4.76 (1H, s, $^{c}H_1$), 4.95 (1H, s, $^{d'}H_1$), 5.00 (1H, d, $J_{H1\text{-}H2}$ 9.1Hz, $^{a}H_1$), 5.14 (1H, s, $^{d}H_1$), 7.43 (1H, td, $J_{H7\text{-}H6,8}$ 7.7Hz, $J_{H7\text{-}H5}$ 4.9Hz, $^{Fmoc}H_7$), 7.50 (1H, t,

$J_{H6\text{-}H5,7}$ 7.7Hz, $^{Fmoc}H_6$), 7.70 (1H, dd, $J_{H5\text{-}H6}$ 7.0Hz, $J_{H5\text{-}H7}$ 4.9Hz, $^{Fmoc}H_5$), 7.90 (1H, d, $J_{H8\text{-}H7}$ 7.7Hz, $^{Fmoc}H_8$). ^{13}C NMR (176MHz, D_2O, δ (acetone)= 30.89): 22.62 ($^{a}COCH_3$), 22.71, 22.71, 22.91, 23.07, 23.09 ($COCH_3$ x 5), 39.20 ($^{Asn}C_\beta$), 40.72, 40.73 ($^{g}C_3$, $^{g'}C_3$), 47.53 ($^{Fmoc}C_3$, minor conformer), 47.59 ($^{Fmoc}C_3$, major conformer), 52.54, 52.54 ($^{g}C_5$, $^{g'}C_5$), 53.35 ($^{Asn}C_\alpha$, major conformer), 54.01 ($^{Asn}C_\alpha$, minor conformer), 54.41 ($^{a}C_2$), 55.28, 55.30 ($^{e}C_2$, $^{e'}C_2$), 55.54 ($^{b}C_2$), 60.37 ($^{a}C_6$), 60.60 ($^{b}C_6$), 60.87, 60.90 ($^{e}C_6$, $^{e'}C_6$), 62.28 ($^{d'}C_6$), 62.36 ($^{d}C_6$), 63.31, 63.31 ($^{g}C_9$, $^{g'}C_9$), 63.98, 63.98 ($^{f}C_6$, $^{f'}C_6$), 66.35 ($^{c}C_4$), 66.52 ($^{c}C_6$), 67.07 ($^{Fmoc}C_2$, major conformer), 67.69 ($^{Fmoc}C_2$, minor conformer), 67.94 ($^{d}C_4$), 67.98 ($^{d'}C_4$), 68.864, 68.872 ($^{g}C_4$, $^{g'}C_4$), 69.02, 69.02 ($^{f}C_4$, $^{f'}C_4$), 69.06, 69.06 ($^{g}C_7$, $^{g'}C_7$), 70.08 ($^{d}C_3$), 70.13 ($^{d'}C_3$), 70.85 ($^{c}C_2$), 71.39, 71.40 ($^{f}C_2$, $^{f'}C_2$), 72.38, 72.38 ($^{g}C_8$, $^{g'}C_8$), 72.64 ($^{b}C_3$), 72.71, 72.81 ($^{e}C_3$, $^{e'}C_3$), 73.08, 73.08 ($^{f}C_3$, $^{f'}C_3$), 73.19, 73.20 ($^{g}C_6$, $^{g'}C_6$), 73.37 ($^{a}C_3$), 73.49 ($^{d'}C_5$), 74.20 ($^{d}C_5$), 74.35, 74.35 ($^{f}C_5$, $^{f'}C_5$), 74.99, 75.01 ($^{b}C_5$, $^{c}C_5$), 75.08, 75.09 ($^{e}C_5$, $^{e'}C_5$), 76.78 ($^{a}C_5$), 76.89 ($^{d'}C_2$), 77.05 ($^{d}C_2$), 78.84 ($^{a}C_1$), 79.29 ($^{a}C_4$), 80.29 ($^{b}C_4$), 81.14 ($^{c}C_3$), 81.28, 81.35 ($^{e}C_4$, $^{e'}C_4$), 97.60 ($^{d'}C_1$), 99.93, 99.98 ($^{e}C_1$, $^{e'}C_1$), 100.21 ($^{d}C_1$), 100.80, 100.83 ($^{g}C_2$, $^{g'}C_2$), 101.14 ($^{c}C_1$), 101.95 ($^{b}C_1$), 104.20, 104.22 ($^{f}C_1$, $^{f'}C_1$), 120.85 ($^{Fmoc}C_8$), 125.80 ($^{Fmoc}C_5$, major conformer), 125.87 ($^{Fmoc}C_5$, minor conformer), 128.16, 128.21 ($^{Fmoc}C_6$, two conformers), 128.72, 128.73 ($^{Fmoc}C_7$, two conformers), 141.56 ($^{Fmoc}C_9$), 144.38 ($^{Fmoc}C_4$, minor conformer), 144.53 ($^{Fmoc}C_4$, major conformer), 158.26 ($^{Fmoc}C_1$), 174.10 ($^{Asn}C_\gamma$), 174.20, 174.20 ($^{g}C_1$, $^{g'}C_1$), 175.25, 175.40, 175.40, 175.57, 175.58 ($COCH_3$ x 6), 178.00 ($^{Asn}C_1$). Coupled HSQC: J(^{a}H1-^{a}C1) 156Hz, J(^{b}H1-^{b}C1) 164Hz, J(^{c}H1-^{c}C1) 160Hz, J(^{d}H1-^{d}C1) 171Hz, J($^{d'}$H1-$^{d'}$C1) 170Hz, J(^{e}H1-^{e}C1) 162Hz, J(^{f}H1-^{f}C1) 162Hz.

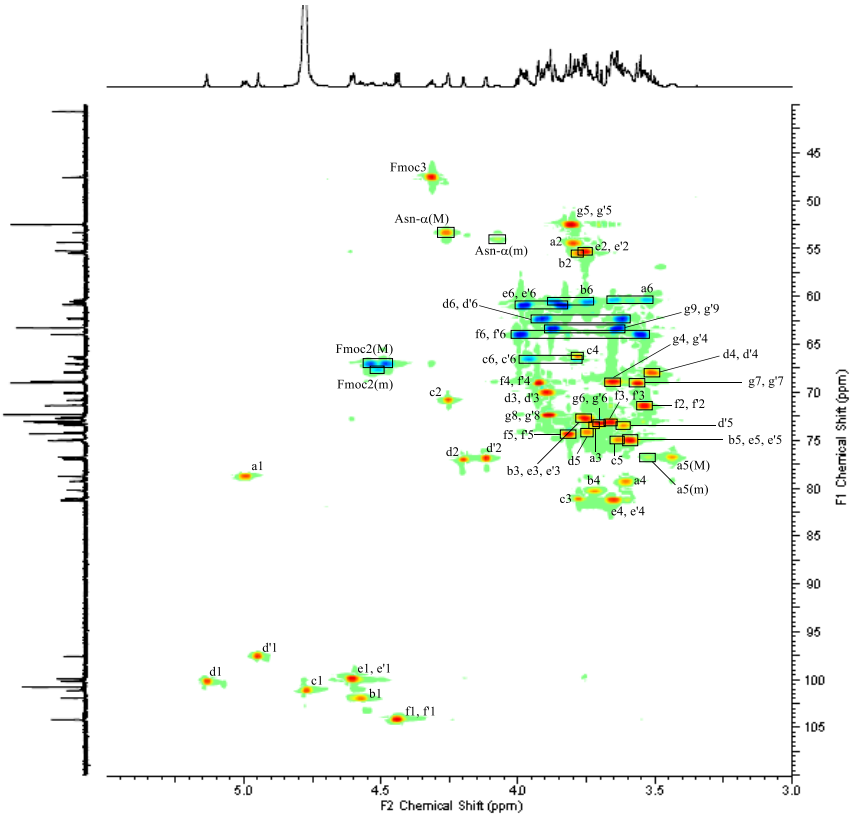
^{1}H NMR



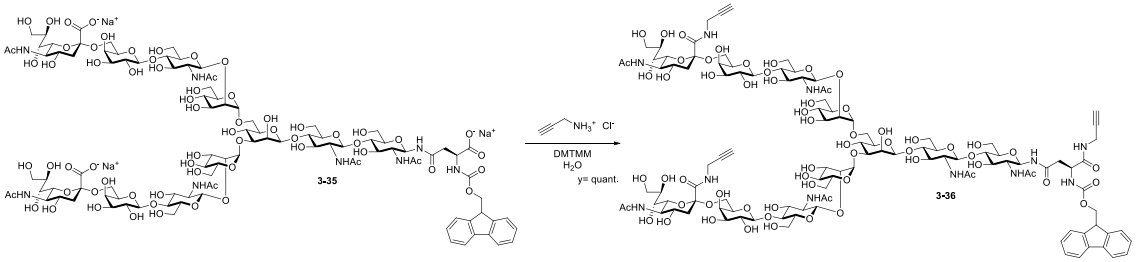
^{13}C NMR



HSQC



Preparation of Fmoc-SGAsn tripropargylamide (**3-36**)



20.0 mg of trisodium salt of **3-35** (7.62 μmol) was dissolved in 381 μL of 400 mM propargyl amine hydrochloride (152 μmol, 20 eq.) in water. Then DMTMM (containing 17% water, 38.1 mg, 114μmol, 15 eq.) was added and the reaction mixture was stirred at 50°C for 12 hr. The completion of the reaction was confirmed by HPLC:

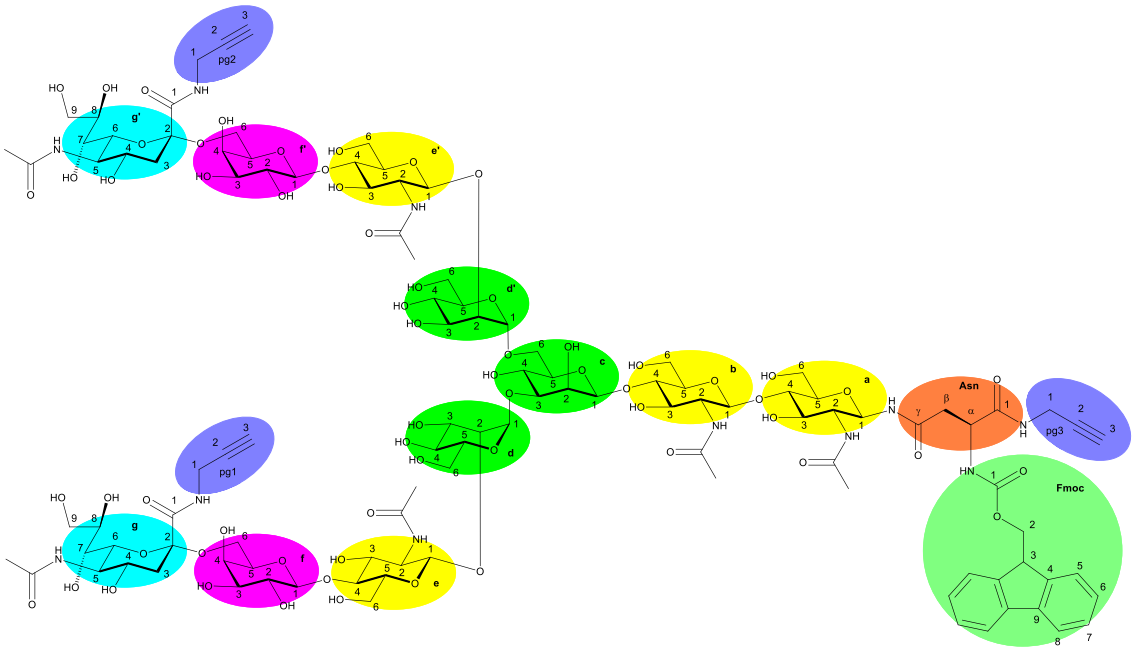
Column: Synergi 4u Hydro-RP 80A, 100 x 4.60 mm, 4 micron
Solvent A: 5 mM ammonium bicarbonate
Solvent B: 5 mM ammonium bicarbonate in 80% acetonitrile

Gradient: 0->2 min 5%B
2->15 min to 100%B
15->20 min 100%B
20->22 min 5%B
Flowrate: 1.0 mL/min
Detection: 254 nm
t_R= 9.99 min

After cooling the reaction to room temperature, the reaction mixture was diluted with 400 μL water. The solution was filtered with 0.20-μm membrane and applied to HPLC purification in the following conditioin:

Column: Synergi 4u Hydro-RP 80A, 100 x 21.20 mm, 4 micron
Solvent A: 5 mM ammonium bicarbonate
Solvent B: 5 mM ammonium bicarbonate in 80% acetonitrile
Gradient: 0->2 min 30%B
2->15 min to 60%B
15->17 min 60%B
17->18 min to 100%B
18->20 min 100%B
20->22 min 30%B
Flowrate: 15 mL/min
Detection: 254 nm
t_R= 7.45 min

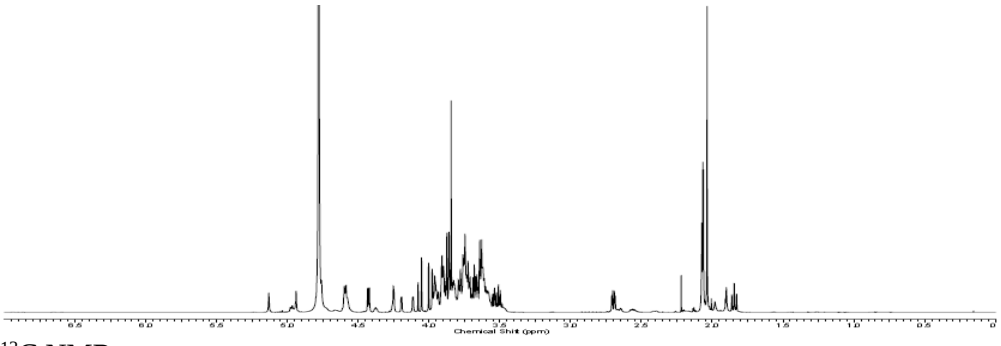
The collected fractions were concentrated by a rotary evaporator to remove organic solvents, followed by lyophilization to afford the desired triamide **3-36** as white lyophilized powder. (19.0 mg, y= 94%).



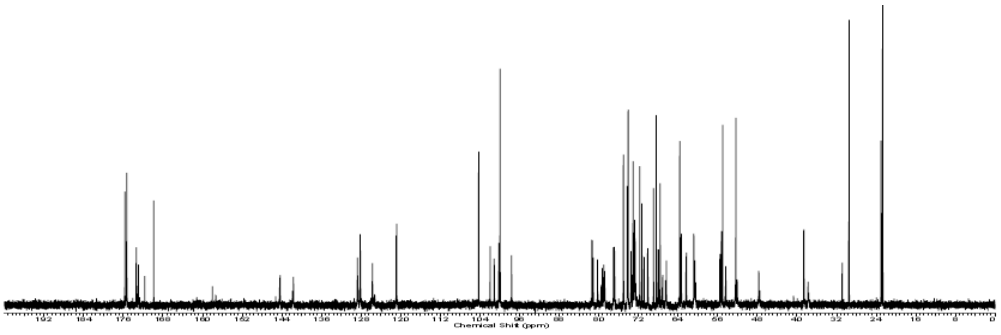
LRMS (ESI$^+$): m/z [M+2Na]$^{2+}$ 1358.3. HRMS (ESI$^+$): m/z [M+2Na]$^{2+}$ 1357.9772 (found), (calcd 1357.9837 for $C_{112}H_{163}N_{11}Na_2O_{63}$). $[\alpha]_D^{20}$= -10.8° (c= 0.95, H_2O). IR (ν_{max}, cm^{-1}, neat): 3285 (broad, N-H, O-H stretch), 2917 (C-H stretch), 2917(alkyl C-H stretch), 1654 (amide-I), 1542 (amide-II) 1373, 1308, 1060, 1034, 869. ^{1}H NMR (700MHz, D_2O, δ (acetone)= 2.220): 1.85 (2H, dd, $J_{H3ax\text{-}H3eq}$ 12.6Hz, $J_{H3\text{-}H4}$ 12.6Hz, $^{g}H_{3ax}$, $^{g'}H_{3ax}$), 1.90 (2.1H, s, $^{a}COCH_3$ (major conformer)), 1.98 (0.9H, s, $^{a}COCH_3$ (minor conformer)), 2.04 (6H, s, $COCH_3$ x 2), 2.063, 2.067, 2.075 (9H, s x 3, $COCH_3$ x 3), 2.15-2.23 (0.3H, m, $^{Asn}H_\beta$ (minor conformer)), 2.37-2.45 (0.3H, m, $^{Asn}H_\beta$' (minor conformer)), 2.51-2.60 (0.7H, m, $^{Asn}H_\beta$ (major conformer)), 2.62-2.71 (0.7H, m, $^{Asn}H_\beta$' (major conformer)), 2.70 (2H, dd, $J_{H3eq\text{-}H3ax}$ 12.6Hz, $J_{H3eq\text{-}H4}$ 4.9Hz, $^{g}H_{3eq}$, $^{g'}H_{3eq}$), 3.45-3.56 (5H, m, $^{a}H_5$, $^{d}H_4$, $^{d'}H_4$, $^{f}H_2$, $^{f'}H_2$), 3.56-3.71 (20H, m, $^{pg3}H_1$, $^{a}H_4$, $^{a}H_6$, $^{b}H_5$, $^{c}H_5$, $^{d'}H_5$, $^{d}H_6$, $^{d'}H_6$, $^{e}H_4$, $^{e'}H_4$, $^{e}H_5$, $^{e'}H_5$, $^{f}H_3$, $^{f'}H_3$, $^{f}H_6$, $^{f'}H_6$, $^{g}H_7$, $^{g'}H_7$, $^{g}H_9$, $^{g'}H_9$), 3.71-3.81 (18H, m, $^{a}H_2$, $^{a}H_3$, $^{b}H_2$, $^{b}H_3$, $^{b}H_4$, $^{b}H_6$, $^{c}H_3$, $^{c}H_4$, $^{c}H_6$, $^{d}H_5$, $^{e}H_2$, $^{e'}H_2$, $^{e}H_3$, $^{e'}H_3$, $^{g}H_4$, $^{g'}H_4$, $^{g}H_6$, $^{g'}H_6$), 3.81-3.93 (19H, m, $^{pg3}H_1$' , $^{a}H_6$' , $^{b}H_6$' , $^{d}H_3$, $^{d'}H_3$, $^{d}H_6$', $^{d'}H_6$', $^{e}H_6$, $^{e'}H_6$, $^{f}H_4$, $^{f'}H_4$, $^{f}H_5$, $^{f'}H_5$, $^{g}H_5$, $^{g'}H_5$, $^{g}H_8$, $^{g'}H_8$, $^{g}H_9$', $^{g'}H_9$'), 3.93-3.98 (5H, m, $^{c}H_6$', $^{e}H_6$', $^{e'}H_6$', $^{f}H_6$', $^{f'}H_6$'), 3.991, 3.992 (1H x2, d x2, $J_{H1\text{-}H1'}$ 17.5Hz, $^{pg1}H_1$, $^{pg2}H_1$), 4.066, 4.067 (1H x2, d x2, $J_{H1'\text{-}H1}$ 17.5Hz, $^{pg1}H_1$', $^{pg2}H_1$'), 4.12 (1H, d, $J_{H2\text{-}H3}$ 2.8Hz, $^{d'}H_2$), 4.18 (1H, d, $J_{H2\text{-}H3}$ 2.8Hz, $^{d}H_2$), 4.25 (1H, s, $^{c}H_2$), 4.24-4.27 (1H, m, $^{Fmoc}H_3$), 4.38 (0.7H, t, $J_{H\alpha\text{-}H\beta}$ 6.3Hz, $^{Asn}H_\alpha$ (major conformer)), 4.428, 4.429 (1H x2, d x2, $J_{H1\text{-}H2}$ 7.7Hz, $^{f}H_1$, $^{f'}H_1$), 4.54-4.60 (1.4H, m, $^{Fmoc}H_2$ (major conformer); 1H, m, $^{b}H_1$), 4.59 (2H, d, $J_{H1\text{-}H2}$ 7.7Hz, $^{e}H_1$, $^{e'}H_1$), 4.64-4.69 (0.3H, m, $^{Fmoc}H_2$ (minor conformer)), 4.71-4.76 (0.3H, m, $^{Fmoc}H_2$' (minor conformer)), 4.76 (1H, s, $^{c}H_1$), 4.94 (1H, s, $^{d'}H_1$), 5.00 (1H, d, $J_{H1\text{-}H2}$ 9.8Hz, $^{a}H_1$), 5.13 (1H, s, $^{d}H_1$), 7.40 (1H, t, $J_{H7\text{-}H6,8}$ 7.0Hz, $^{Fmoc}H_7$), 7.47 (1H, t, $J_{H6\text{-}H5,7}$ 7.0Hz, $^{Fmoc}H_6$), 7.65 (1H, d, $J_{H5\text{-}H6}$ 7.0Hz, $^{Fmoc}H_5$),

7.87 (1H, d, $J_{H8\text{-}H7}$ 7.0Hz, $^{Fmoc}H_8$). $^{Asn}H_\alpha$ (minor conformer), $^{pg1}H_3$, $^{pg2}H_3$, $^{pg3}H_3$ were not found clearly. ^{13}C NMR (176MHz, D_2O, δ (acetone)= 30.89): 22.67, 22.72, 22.92, 23.07 ($COCH_3$ x 6), 29.45 ($^{pg1}C_1$, $^{pg2}C_1$), 29.54 ($^{pg3}C_1$), 37.61 ($^{Asn}C_\beta$, minor conformer), 37.75 ($^{Asn}C_\beta$, major conformer), 38.58, 38.63 ($^{g}C_3$, $^{g'}C_3$), 47.52 ($^{Fmoc}C_3$, minor conformer), 47.69 ($^{Fmoc}C_3$, major conformer), 52.03 ($^{Asn}C_\alpha$, major conformer), 52.32 ($^{g}C_5$, $^{g'}C_5$), 52.42 ($^{Asn}C_\alpha$, minor conformer), 54.37 ($^{a}C_2$), 55.23, 55.27 ($^{e}C_2$, $^{e'}C_2$), 55.57 ($^{b}C_2$), 60.43 ($^{a}C_6$), 60.60 ($^{b}C_6$), 60.81, 60.83 ($^{e}C_6$, $^{e'}C_6$), 62.29 ($^{d'}C_6$), 62.37 ($^{d}C_6$), 63.37, 63.39 ($^{f}C_6$, $^{f'}C_6$), 63.68 ($^{g}C_9$, $^{g'}C_9$), 66.35 ($^{c}C_4$), 66.50 ($^{c}C_6$), 67.11 ($^{Fmoc}C_2$, major conformer), 67.19 ($^{Fmoc}C_2$, minor conformer), 67.63, 67.64 ($^{g}C_4$, $^{g'}C_4$), 67.95 ($^{d}C_4$), 67.99 ($^{d'}C_4$), 68.39 ($^{g}C_7$, $^{g'}C_7$), 68.94 ($^{f}C_4$, $^{f'}C_4$), 70.06 ($^{d}C_3$), 70.12 ($^{d'}C_3$), 70.84 ($^{c}C_2$), 71.30, 71.32 ($^{f}C_2$, $^{f'}C_2$), 71.74, 71.75 ($^{g}C_8$, $^{g'}C_8$), 72.64, 72.73, 72.82 ($^{b}C_3$, $^{e}C_3$, $^{e'}C_3$, $^{pg1}C_3$, $^{pg2}C_3$), 73.08 ($^{f}C_3$, $^{f'}C_3$), 73.34 ($^{a}C_3$), 73.50 ($^{d'}C_5$), 74.09 ($^{g}C_6$, $^{g'}C_6$), 74.21 ($^{d}C_5$), 74.23 ($^{f}C_5$, $^{f'}C_5$), 75.01, 75.03 ($^{b}C_5$, $^{c}C_5$, $^{e}C_5$, $^{e'}C_5$), 76.80 ($^{a}C_5$), 76.88 ($^{d'}C_2$), 77.05 ($^{d}C_2$, $^{pg3}C_3$), 78.83 ($^{a}C_1$), 79.00 ($^{pg1}C_2$, $^{pg2}C_2$), 79.30 ($^{a}C_4$), 79.51 ($^{pg3}C_2$), 80.24 ($^{b}C_4$), 81.11 ($^{c}C_3$), 81.33, 81.41 ($^{e}C_4$, $^{e'}C_4$), 97.62 ($^{d'}C_1$), 99.92, 99.94, 100.00 ($^{e}C_1$, $^{e'}C_1$, $^{g}C_2$, $^{g'}C_2$), 100.20 ($^{d}C_1$), 101.10 ($^{c}C_1$), 101.95 ($^{b}C_1$), 104.26 ($^{f}C_1$, $^{f'}C_1$), 120.83 ($^{Fmoc}C_8$), 125.69 ($^{Fmoc}C_5$), 128.17 ($^{Fmoc}C_6$), 128.73 ($^{Fmoc}C_7$), 141.66 ($^{Fmoc}C_9$), 144.35 ($^{Fmoc}C_4$), 157.99 ($^{Fmoc}C_1$), 169.82, 169.83 ($^{g}C_1$, $^{g'}C_1$), 172.96 ($^{Asn}C_\gamma$), 173.39 ($^{Asn}C_1$), 175.31, 175.37, 175.68, 175.69 ($COCH_3$ x 6). Coupled HSQC: J(^{a}H1-^{a}C1) 157Hz, J(^{b}H1-^{b}C1) 167Hz, J(^{c}H1-^{c}C1) 161Hz, J(^{d}H1-^{d}C1) 170Hz, J($^{d'}$H1-$^{d'}$C1) 171Hz, J(^{e}H1-^{e}C1) 160Hz, J(^{f}H1-^{f}C1) 162Hz.

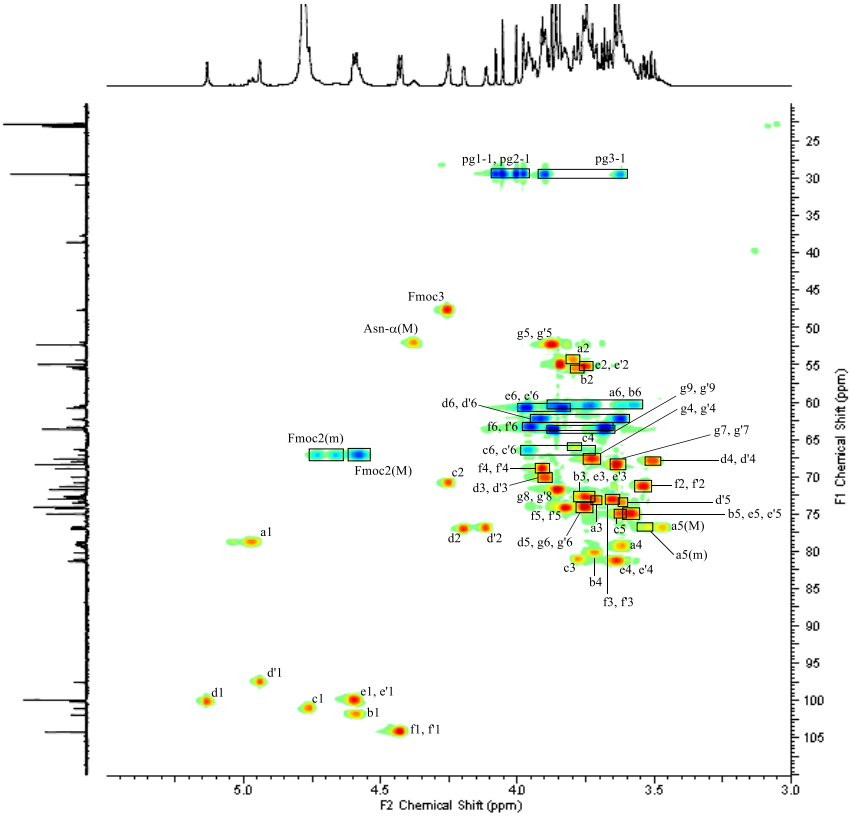
^{1}H NMR



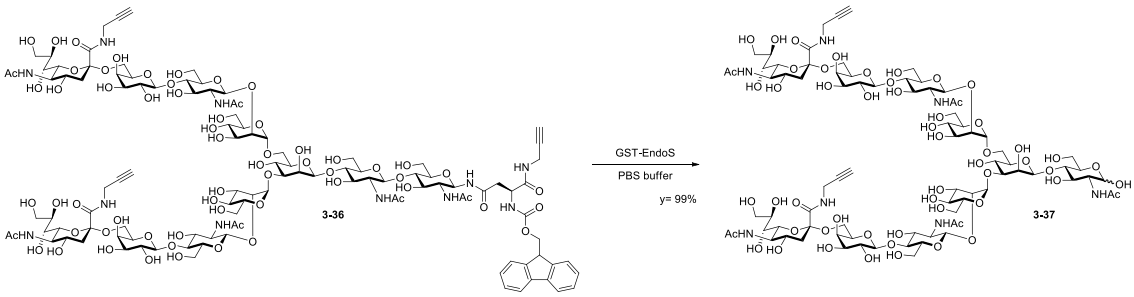
^{13}C NMR



HSQC



Preparation of the decasaccharide dipropargylamine (**3-37**)



15.0 mg of the triamide (**3-36**) was mixed with 300 μL of 14.7 mg/mL GST-EndoS in PBS. The mixture was incubated at 37°C for 4 hr. The completion of the reaction was confirmed by TLC (1M ammonium acetate: isopropanol= 2: 3, R_f(**3-36**)= 0.58, R_f(**3-37**)= 0.41). After filtration of the reaction solution with 0.20-μm membrane, the filtrate was applied to PD-10 column. Fractions were analyzed by TLC and the ones with the target material were collected and lyophilized. The residue was dissolved in 500 μL water and after filtration with 0.20-μm membrane the solution was applied to HPLC purification:

Column: Luna NH2 250 x 21.20 mm, 100A, Axia packed (Phenomenex)

Solvent A: H_2O with 0.1 % formic acid

Solvent B: Acetonitrile with 0.1% formic acid

Gradient: 0->4 min 70%B

4->22 min to 40%B

22->23 min to 10%B

23->28 min 10%B

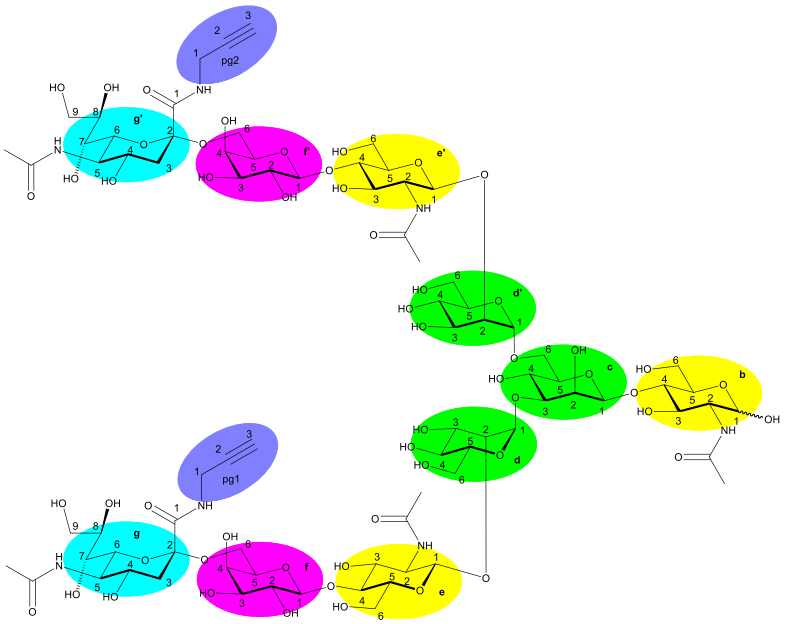
28->33 min 70%B

Flowrate: 22 mL/min

Detection: 214 nm

t_R= 9.64, 9.88 min (Two peaks corresponding to the anomeric isomers were observed. Even if each peak was separately collected, these two peaks appeared again in the both solutions after incubation at 37°C for 4 hr.)

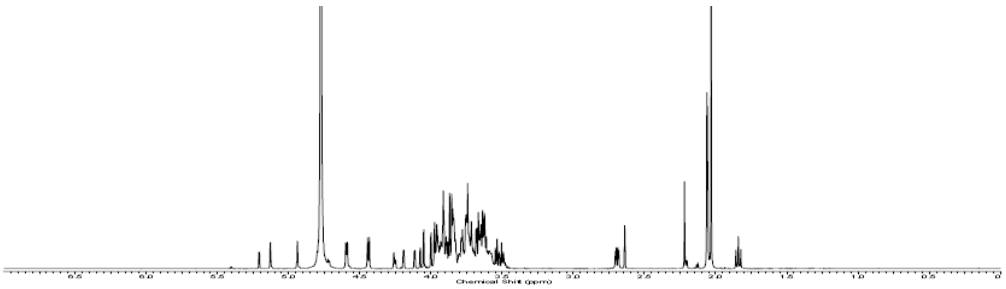
Collected fraction was concentrated by a rotary evaporator to remove organic solvents, followed by lyophilization to afford the target decasaccharide **3-37** as white lyophilized powder: 9.39 mg (y= 80%).



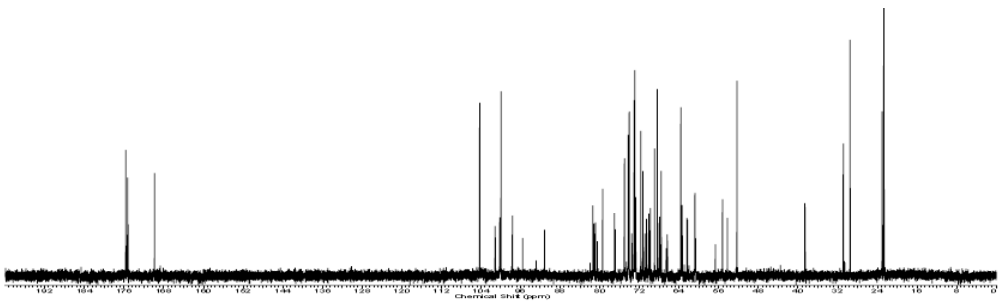
LRMS (ESI^+): m/z $[M+2Na]^{2+}$ 1070.1. HRMS (ESI^+): m/z $[M+2Na]^{2+}$ 1069.8763 (found), (calcd 1069.8728 for $C_{82}H_{131}N_7Na_2O_{55}$). $[\alpha]_D^{20}$= -2.8° (c= 0.47, H_2O). IR (ν_{max}, cm^{-1}, neat): 3292 (broad, alkyne C-H, N-H, O-H stretch), 2921, 2853 (alkyl C-H stretch), 1738, 1643 (amide-I), 1547 (amide-II), 1426, 1377, 1315, 1062, 1034, 963, 901, 781. ^1H NMR (700MHz, D_2O, δ

(acetone)= 2.220): 1.84 (2H, dd, $J_{H3ax\text{-}H3eq}$ 12.6Hz, $J_{H3\text{-}H4}$ 12.6Hz, $^{g}H_{3ax}$, $^{g'}H_{3ax}$), 2.03 (6H, s, COCH_3 x 2), 2.058, 2.061, 2.062 (9H, s x 3, COCH_3 x 3), 2.64 (2H, t, $J_{H3\text{-}H1}$ 2.8Hz, $^{pg1}H_3$, $^{pg2}H_3$), 2.69 (2H, dd, $J_{H3eq\text{-}H3ax}$ 12.6Hz, $J_{H3eq\text{-}H4}$ 4.9Hz, $^{g}H_{3eq}$, $^{g'}H_{3eq}$), 3.47-3.55 (4H, m, $^{d}H_4$, $^{d'}H_4$, $^{f}H_2$, $^{f'}H_2$), 3.55-3.69 (16.4H, m, $^{b\text{-}\beta}H_5$, $^{c}H_5$, $^{d'}H_5$, $^{d}H_6$, $^{d'}H_6$, $^{e}H_4$, $^{e'}H_4$, $^{e}H_5$, $^{e'}H_5$, $^{f}H_3$, $^{f'}H_3$, $^{f}H_6$, $^{f'}H_6$, $^{g}H_7$, $^{g'}H_7$, $^{g}H_9$, $^{g'}H_9$), 3.69-3.82 (15.8H, m, $^{b\text{-}\alpha}H_4$, $^{b\text{-}\alpha}H_6$, $^{b\text{-}\alpha}H_6$', $^{b\text{-}\beta}H_2$, $^{b\text{-}\beta}H_3$, $^{b\text{-}\beta}H_4$, $^{b\text{-}\beta}H_6$, $^{b\text{-}\beta}H_6$', $^{c}H_3$, $^{c}H_4$, $^{c}H_6$, $^{d}H_5$, $^{e}H_2$, $^{e'}H_2$, $^{e}H_3$, $^{e'}H_3$, $^{g}H_4$, $^{g'}H_4$, $^{g}H_6$, $^{g'}H_6$), 3.82-3.99 (22.8H, m, $^{b\text{-}\alpha}H_2$, $^{b\text{-}\alpha}H_3$, $^{b\text{-}\alpha}H_5$, $^{c}H_6$', $^{d}H_3$, $^{d'}H_3$, $^{d}H_6$', $^{d'}H_6$', $^{e}H_6$, $^{e'}H_6$, $^{e}H_6$', $^{e'}H_6$', $^{f}H_4$, $^{f'}H_4$, $^{f}H_5$, $^{f'}H_5$, $^{f}H_6$', $^{f'}H_6$', $^{g}H_5$, $^{g'}H_5$, $^{g}H_8$, $^{g'}H_8$, $^{g}H_9$', $^{g'}H_9$'), 3.99 (2H, dd, $J_{H1\text{-}H1'}$ 18.2Hz, $J_{H1\text{-}H3}$ 2.8Hz, $^{pg1}H_1$, $^{pg2}H_1$), 4.06 (2H, dd, $J_{H1'\text{-}H1}$ 18.2Hz, $J_{H1'\text{-}H3}$ 2.8Hz, $^{pg1}H_1$', $^{pg2}H_1$'), 4.12 (1H, d, $J_{H2\text{-}H3}$ 2.1Hz, $^{d'}H_2$), 4.19 (1H, d, $J_{H2\text{-}H3}$ 2.8Hz, $^{d}H_2$), 4.24-4.27 (1H, m, $^{c}H_2$), 4.44 (2H, d, $J_{H1\text{-}H2}$ 7.7Hz, $^{f}H_1$, $^{f'}H_1$), 4.59 (2H, d, $J_{H1\text{-}H2}$ 7.7Hz, $^{e}H_1$, $^{e'}H_1$), 4.71 (0.4H, d, $J_{H1\text{-}H2}$ 7.7Hz, $^{b\text{-}\beta}H_1$), 4.77 (1H, s, $^{c}H_1$), 4.94 (1H, s, $^{d'}H_1$), 5.13 (1H, s, $^{d}H_1$), 5.21 (0.6H, d, $J_{H1\text{-}H2}$ 2.8Hz, $^{b\text{-}\alpha}H_1$). ^{13}C NMR (176MHz, D_2O, δ (acetone)= 30.89): 22.64, 22.72, 22.97, 23.07 (CO*C*H$_3$ x 5), 29.47 ($^{pg1}C_1$, $^{pg2}C_1$), 38.58, 38.62 ($^{g}C_3$, $^{g'}C_3$), 52.32 ($^{g}C_5$, $^{g'}C_5$), 54.24 ($^{b\text{-}\alpha}C_2$), 55.23, 55.27 ($^{e}C_2$, $^{e'}C_2$), 56.70 ($^{b\text{-}\beta}C_2$), 60.68 ($^{b\text{-}\alpha}C_6$), 60.81, 60.82 ($^{b\text{-}\beta}C_6$, $^{e}C_6$, $^{e'}C_6$), 62.30 ($^{d'}C_6$), 62.38 ($^{d}C_6$), 63.38, 63.40 ($^{f}C_6$, $^{f'}C_6$), 63.68 ($^{g}C_9$, $^{g'}C_9$), 66.38 ($^{c}C_4$), 66.61 ($^{c}C_6$), 67.63, 67.64 ($^{g}C_4$, $^{g'}C_4$), 67.95 ($^{d}C_4$), 68.00 ($^{d'}C_4$), 68.38 ($^{g}C_7$, $^{g'}C_7$), 68.95 ($^{f}C_4$, $^{f'}C_4$), 69.84 ($^{b\text{-}\alpha}C_3$), 70.04, 70.07 ($^{d}C_3$,$^{d'}C_3$), 70.56 ($^{b\text{-}\alpha}C_5$), 70.56 ($^{c}C_2$), 71.31, 71.32 ($^{f}C_2$, $^{f'}C_2$), 71.75 ($^{g}C_8$, $^{g'}C_8$), 72.74, 72.82 ($^{e}C_3$, $^{e'}C_3$), 72.96 ($^{pg1}C_3$, $^{pg2}C_3$), 73.03 ($^{b\text{-}\beta}C_3$), 73.08 ($^{f}C_3$, $^{f'}C_3$), 73.54 ($^{d'}C_5$), 74.09 ($^{g}C_6$, $^{g'}C_6$), 74.21 ($^{d}C_5$), 74.24 ($^{f}C_5$, $^{f'}C_5$), 75.02, 75.03 ($^{c}C_5$, $^{e}C_5$, $^{e'}C_5$), 75.12 ($^{b\text{-}\beta}C_5$), 76.89 ($^{d'}C_2$), 77.05 ($^{d}C_2$), 79.43, 79.45 ($^{pg1}C_2$, $^{pg2}C_2$), 80.48 ($^{b\text{-}\beta}C_4$), 80.86 ($^{b\text{-}\alpha}C_4$), 81.14 ($^{c}C_3$), 81.33, 81.40 ($^{e}C_4$, $^{e'}C_4$), 91.15 ($^{b\text{-}\alpha}C_1$), 95.57 ($^{b\text{-}\beta}C_1$), 97.65 ($^{d'}C_1$), 99.93, 99.95, 100.01 ($^{e}C_1$, $^{e'}C_1$, $^{g}C_2$, $^{g'}C_2$), 100.22 ($^{d}C_1$), 101.11 ($^{c}C_1$), 104.26 ($^{f}C_1$, $^{f'}C_1$), 169.84, 169.85 ($^{g}C_1$, $^{g'}C_1$), 175.19, 175.32, 175.33, 175.44, 175.69 (*C*OCH$_3$ x 5). Coupled HSQC: J($^{b\text{-}\alpha}$H1-$^{b\text{-}\alpha}$C1) 172Hz, J($^{b\text{-}\beta}$H1-$^{b\text{-}\beta}$C1) 162Hz, J(^{c}H1-^{c}C1) 161Hz, J(^{d}H1-^{d}C1) 171Hz, J($^{d'}$H1-$^{d'}$C1) 170Hz, J(^{e}H1-^{e}C1) 161Hz, J(^{f}H1-^{f}C1) 161Hz.

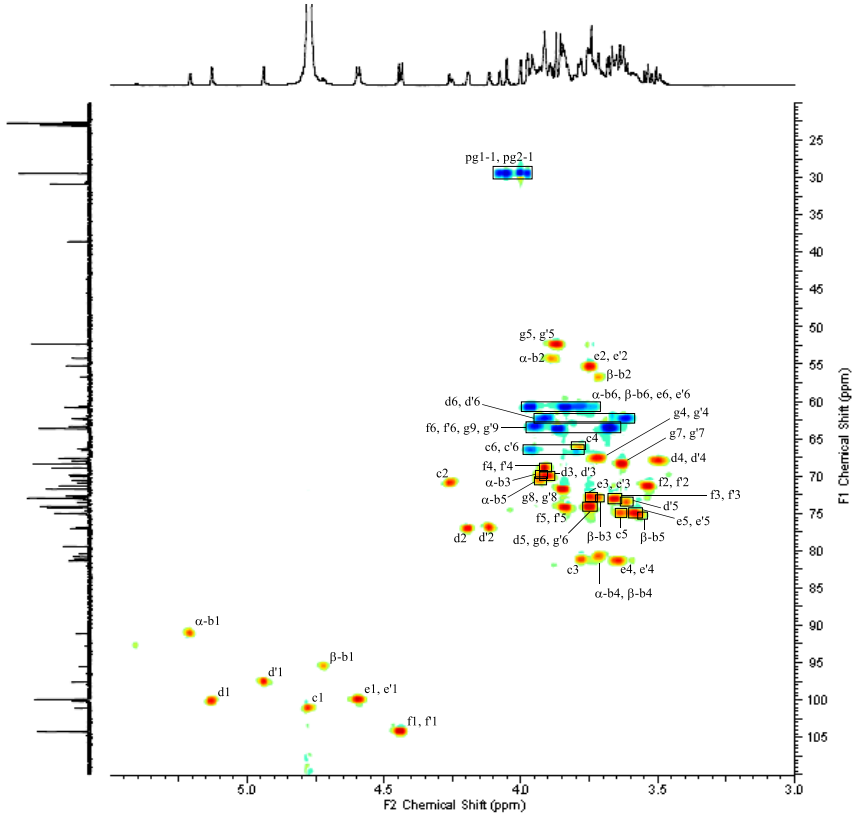
^{1}H NMR



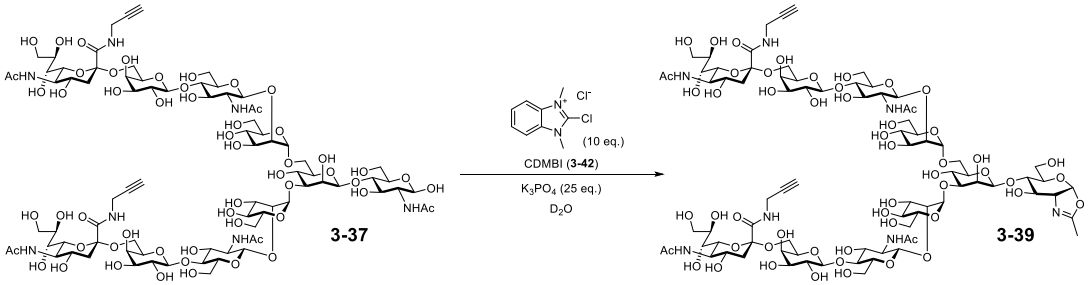
^{13}C NMR



HSQC

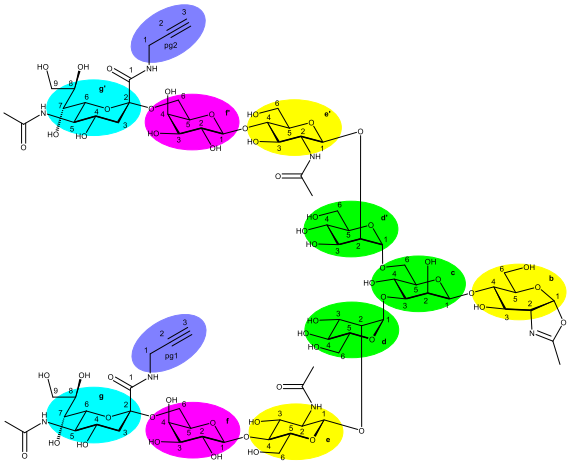


Preparation of the decasaccharide oxazoline (**3-39**)



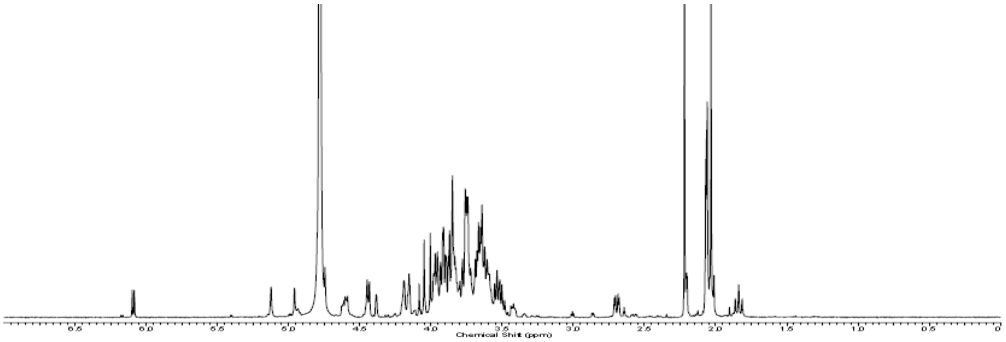
3-37 (4.00 mg, 0.00191 mmol) was dissolved in 38.2 µL of 0.75M K_3PO_4 in D_2O (0.0287 mmol, 15 eq.) and the solution was cooled to 0°C. 4.15 mg of CDMBI (0.0191 mmol, 10 eq.) was added and incubated at 4°C for 2 hr. The reaction mixture was filtered with 0.22-µm membrane and the filtrate was diluted to 550µL with D_2O to check the conversion with ^{1}H-NMR. The spectrum was not clean and it was found the amount of the base was not enough (it should be more than two equivalents to CDMBI). To the resultant solution was added 4.10 mg of K_3PO_4 (10 eq.) and the reaction solution was incubated at room temperature for 1 hr and the complete conversion was confirmed by ^{1}H-NMR. The reaction mixture was applied to PD MidiTrap G-10

(Exclusion limit: 700 Da) and the compound was eluted with water. Fractions were checked by TLC (1M NH_4OAc : IPA= 2: 3) and the collected fractions were lyophilized after 10 μL of 0.2M NaOH was added as stabilizer: 10.34 mg (the apparent yield >100%, the further reactions were performed assuming the yield was quantitative).



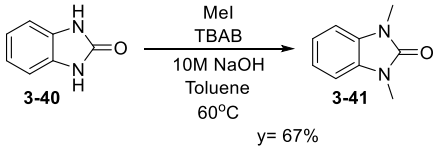
^{1}H NMR (500MHz, D_2O, δ (acetone)= 2.220): (only representative peaks are shown) 1.84 (2H, dd, $J_{H3ax\text{-}H3eq}$ 12.5Hz, $J_{H3\text{-}H4}$ 12.5Hz, $^{g}H_{3ax}$, $^{g'}H_{3ax}$), 2.70 (2H, dd, $J_{H3eq\text{-}H3ax}$ 13.0Hz, $J_{H3eq\text{-}H4}$ 4.5Hz, $^{g}H_{3eq}$, $^{g'}H_{3eq}$), 3.99 (2H, d, $J_{H1\text{-}H1'}$ 17.5Hz, $^{pg1}H_1$, $^{pg2}H_1$), 4.07 (2H, d, $J_{H1'\text{-}H1}$ 17.5Hz, $^{pg1}H_1$', $^{pg2}H_1$'), 4.44 (2H, d, $J_{H1\text{-}H2}$ 8.0Hz, $^{f}H_1$, $^{f'}H_1$), 4.58-4.64 (2H, m, $^{e}H_1$, $^{e'}H_1$), 4.75 (1H, s, $^{c}H_1$), 4.94 (1H, s, $^{d'}H_1$), 5.12 (1H, s, $^{d}H_1$), 6.09 (1H, d, $J_{H1\text{-}H2}$ 7.5Hz, $^{b}H_1$).

^{1}H NMR

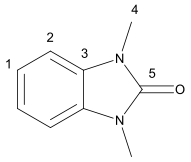


Preparation of CDMBI (**3-42**)

Preparation of 1,3-dimethylbenzimidazoline (DMBI):

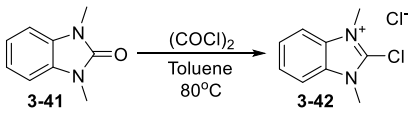


A mixture of 2-hydroxybenzimidazole (20.0 g, 0.149 mol) in toluene (100 mL), 10M sodium hydroxide (59.6 mL, 0.596 mol, 4.0 eq.) and tetrabutylammonium bromide (2.40 g, 7.45 mmol, 5 mol%) was heated to 60°C. Methyl iodide (23.2 mL, 0.373 mol, 2.5 eq.) was added dropwise with occasional manual swirling. The resultant mixture was stirred at the same temperature for 16 hr. After cooling, the organic phase was separated and the aqueous phase was extracted with toluene (50 mL). The combined organic phase was washed with 1M HCl (100 mL), sat. $NaHCO_3$ (100 mL) and brine (100 mL) and dried over $MgSO_4$. The solvents were removed by evaporation to afford crude DMBI (18.3 g). This material was dissolved in warmed acetone (18 mL) and recrystallized by addition of hexane (72 mL) and cooling to 0°C. After filtration, the title compound (**3-41**) was obtained as a pale brown crystal; 16.2 g (y= 67%).



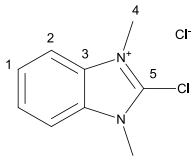
^{1}H NMR[136] (400MHz, $CDCl_3$, δ ($CHCl_3$)=7.270): 3.43 (6H, s, H_4), 6.98 (2H, dd, $J_{H1\text{-}H2}$ 5.6Hz, $J_{H1\text{-}H2'}$ 3.2Hz, H_1), 7.11 (2H, dd, $J_{H2\text{-}H1}$ 5.6Hz, $J_{H2\text{-}H1'}$ 3.2Hz, H_2). IR (ν_{max}, cm^{-1}, neat): 3067, 3054, 2935 (C-H stretch), 1696 (amide-I), 1621, 1609, 1500, 1446, 1423, 1390, 1336, 1267, 1239, 1155, 1132, 1121, 1086, 1032, 1011, 935, 911, 847, 740, 727, 716, 653 [lit.[137] 1703 cm^{-1}]. TLC: R_f= 0.44 (Ethyl acetate: petroleum ether= 6: 1).

Preparation of 2-chloro-1,3-dimethyl-1*H*-benzimidazol-3-ium chloride (CDMBI):



To a solution of 1,3-dimethylbenzimidazoline (10.0 g, 61.7 mmol) in toluene (82 mL) was added oxaryl chloride (13.0 mL, 154 mmol, 2.5 eq.) at room temperature and the resultant solution was heated to 80°C for 42hr. Another portion of oxaryl chloride (13.0 mL, 154 mmol, 2.5 eq.) was added and heated to 80°C for additional 30 hr. (The consumption of the starting

material was monitored by ^{1}H-NMR in the presence of a known amount of 1,2-dichlorobenzene for quantification.) After cooling, precipitation was collected by filtration, washed with toluene and dried in vacuo to afford white powder: 5.72 g (y= 43%).



^{1}H NMR (400MHz, DMSO-d_6, δ ((CH_3)$_2$SO)=2.500): (Note: The material was dissolved in dehydrated DMSO-d_6 by MS4A and NMR spectra were measured soon after the sample preparation) 4.05 (6H, s, H_4), 7.70 (2H, dd, $J_{H1\text{-}H2}$ 6.0Hz, $J_{H1\text{-}H2'}$ 3.2Hz, H_1), 8.13 (2H, dd, $J_{H2\text{-}H1}$ 6.0Hz, $J_{H2\text{-}H1'}$ 3.2Hz, H_2). [Ref:[138] ^{1}H NMR (500 MHz, DMSO-d_6) : δ 8.10(2H, m, Ph), 7.71(2H, m, Ph), 4.05(6H, s, -CH_3)] ^{13}C-NMR (101MHz, DMSO-d_6, δ ((*C*H$_3$)$_2$SO)=39.51): 32.81 (C_4), 113.25 (C_2), 126.70 (C_1), 131.17 (C_3), 141.42 (C_5). [Ref:[138] ^{13}C NMR (125 MHz, DMSO-d_6) : δ 141.4 (C-Cl), 131.2 (Ph), 126.7 (Ph), 113.2 (Ph), 27.0 (-CH_3)] IR (ν_{max}, cm^{-1}, neat): 3048, 3013, 2954 (C-H stretch), 1707, 1638, 1613, 1534, 1508, 1480, 1455, 1414, 1348, 1269, 1255, 1190, 1167, 1130, 1021, 958, 858, 832, 807, 770, 760, 680, 664, 647, 606.

Preparation of IgG with the truncated glycans

To 0.5 mL of 10 mg/mL IgG in PBS was added 50 μL of 1.0 mg/mL EndoS in PBS. The solution was incubated at 20°C for 4 hr. IgG was purified with Protein A affinity column (Nab spin column, Thermo). After the column was equilibrated with Gentle Ab/Ag Binding Buffer (Thermo), the reaction solution was applied. After incubating the column at room temperature with end-over-end mixing for 10 min, the column was centrifuged at 5,000 x g for 1 min. The column was washed with 400 μL of Gentle Ab/Ag Binding Buffer three times. Then IgG was eluted with 400 μL Gentle Ab/Ag Elution Buffer and the fractions were analyzed by Nanodrop. The combined fractions were dialyzed against first 1L TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and then 0.5 L PBS twice. The recovered solution was concentrated with vivaspin 6 (MWCO 30,000) to 3.7 mL of 1.0 mg/mL solution (y= 74%).

Remodelling of glycans on IgG

Optimization

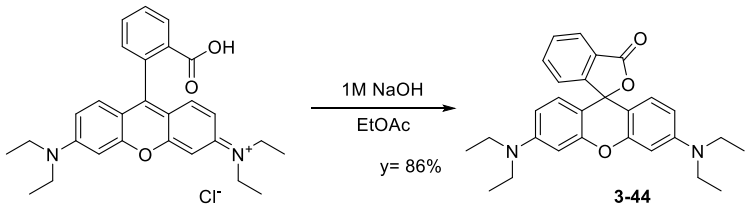
To 50 μL of the glycan-truncated IgG (1.0 mg/mL) was added 0.7 μL or 3.3 μL of 50 mM oxazoline decasacchride (**3-39**). For the former case, 2.6μL of PBS was also added. The reaction was initiated by adding 10μL of 100 μg/mL EndoS D233A and the reaction was incubated at 4°C or 20°C. 7 μL of the reaction solution was taken up at the indicated time and used for SDS-PAGE analsysis.

Preparative scale reaction

To 300 μL of the glycan-truncated IgG (1.0 mg/mL) was added 20 μL of 50 mM oxazoline decasacchride (**3-39**). The reaction was initiated by adding 60μL of 100 μg/mL EndoS D233A and the reaction was incubatedat 20°C for 2 hr. IgG was purified with Protein A affinity column (Nab spin column, Thermo). After the column was equilibrated with Gentle Ab/Ag Binding Buffer (Thermo), the reaction solution was applied. After incubating the column at room temperature with end-over-end mixing for 10 min, the column was centrifuged at 5,000 x g for 1 min. The column was washed with 400 μL of Gentle Ab/Ag Binding Buffer three times. Then IgG was eluted with 400 μL Gentle Ab/Ag Elution Buffer and the fractions were analyzed by Nanodrop. The combined fractions were dialyzed against first 1L TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.4) and then 0.5 L PBS twice. The recovered solution was concentrated with vivaspin 500 (MWCO 10,000) to 220 μL of 0.55 mg/mL IgG solution (protein recovery: 40%). The glycans were released by PNGaseF digestion (see General Procedure) and were analysed by MALDI-TOF-MS (see General Procedure).

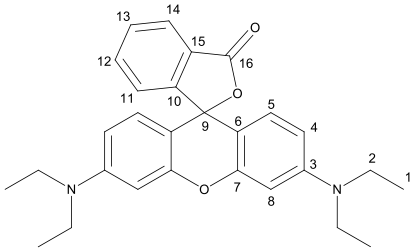
Preparation of the azide-functionalized rhodamine B

Preparation of **3-44**[139]



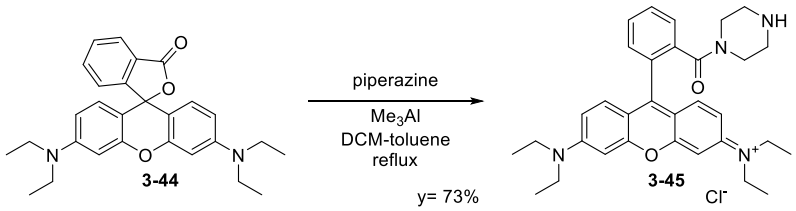
Rhodamine B, **3-43**

15.0 g of rhodamine B was dissolved in 300 mL of 1M NaOH. 300 mL of ethyl acetate was added and two phases were vigorously mixed. After phase separation, the aqueous phase was extracted with 300 mL ethyl acetate twice. The combined organic phase was washed with 100 mL 1M NaOH and 100 mL brine, dried over $MgSO_4$ and concentrated to afford **5** as red-purple foam: 12.0 g (y= 86%).



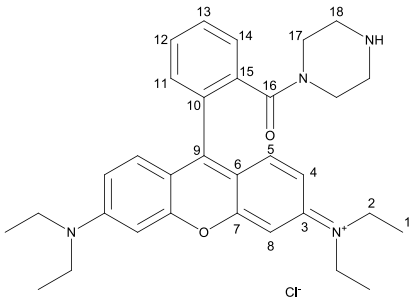
^{1}H NMR[139] (400MHz, CD_3OD, δ (CH_3OH)=3.310): 1.29 (12H, t, $J_{H1\text{-}H2}$ 7.0Hz, H_1), 3.66 (8H, q, $J_{H2\text{-}H1}$ 7.0Hz, H_2), 6.91 (2H, d, $J_{H8\text{-}H4}$ 2.4Hz, H_8), 6.99 (2H, dd, $J_{H4\text{-}H5}$ 9.4Hz, $J_{H4\text{-}H8}$ 2.4Hz, H_4), 7.21-7.27 (1H, m, H_{11}), 7.28 (2H, d, $J_{H5\text{-}H4}$ 9.4Hz, H_5), 7.55-7.70 (2H, m, H_{12}, H_{13}), 8.05-8.13 (1H, m, H_{14}). IR (ν_{max}, cm^{-1}, neat): 2968, 2930, 2896, 2870 (C-H stretch), 1754 (lactone C=O stretch), 1636, 1615, 1584, 1545, 1519, 1465, 1449, 1428, 1405, 1375, 1358, 1331, 1310, 1282, 1270, 1253, 1237, 1220, 1197, 1152, 1122, 1106, 1069, 1014, 954, 939, 922, 867, 852, 829, 807, 792, 777, 761, 728, 700, 666, 650, 623.

Preparation of **3-45**[139]



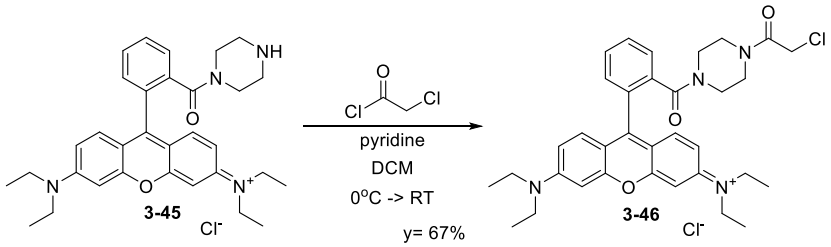
7.79 g of piperazine (90.4 mmol, 4.0 eq.) was dissolved in anhydrous dichloromethane (45 mL) and 22.6 mL of 2.0M trimethylaluminium in toluene (45.2 mmol, 2.0 eq.) was added dropwise

at room temperature and the mixture was stirred for 1 hr during which precipitation formed. To this heterogeneous mixture was added 10.0 g of **3-44** (22.6 mmol, 1.0 eq.) in 20 mL dichloromethane dropwise at room temperature and the resultant mixture was heated to reflux. After 21 hr, the mixture was cooled to room temperature and 0.1M HCl was added dropwise till gas generation ceased (~15 mL). The resultant mixture was filtered and the precipitate was washed with 250 mL dichloromethane and 250 mL dichloromethena-methanol (4:1). The combined filtrate was concentrated to give dark purple metallic foam. This material was dissolved in 200 mL dichloromethane and the insoluble material was removed by filtration. The filtrate was concentrated and the residue was partitioned between 300 mL 1% $NaHCO_3$ and 100 mL ethyl acetate. After phase separation, the aqueous phase was further washed with 100 mL ethyl acetate three times. The aqueous phase was acidified with 5M HCl, saturated with sodium chloride and extracted with isopropanol-dichloromethane (2:1) (300 mL x 4). The combined organic phase was dried over $MgSO_4$ and concentrated. The residue was dissolved in 150 mL methanol and the insoluble material was removed by filtration. The filtrate was concentrated and the residue was again dissolved in 30 mL methanol. To this solution was added diethyl ether (300 mL) and the precipitation was collected by filtration. This dark red powder was dried in vacuo: 9.06 g (y= 73%).

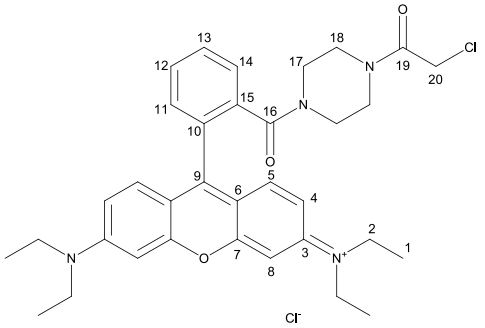


^{1}H NMR[139] (400MHz, CD_3OD, δ (CH_3OH)=3.310): 1.32 (12H, t, $J_{H1\text{-}H2}$ 7.2Hz, H_1), 3.07-3.18 (4H, br, H_{18}), 3.59-3.79 (12H, m, H_2, H_{17}), 6.99 (2H, d, $J_{H8\text{-}H4}$ 2.4Hz, H_8), 7.10 (2H, dd, $J_{H4\text{-}H5}$ 9.6Hz, $J_{H4\text{-}H8}$ 2.4Hz, H_4), 7.27 (2H, d, $J_{H5\text{-}H4}$ 9.6Hz, H_5), 7.51-7.57 (1H, m, H_{11}), 7.75-7.84 (3H, m, H_{12}, H_{13}, H_{14}). LRMS (ESI$^+$): m/z $[M+H]^{2+}$ 256.3, $[M]^+$ 511.3. IR (ν_{max}, cm^{-1}, neat): 3337 (broad, N-H stretch), 2978, 2931 (C-H stretch), 1645, 1621(amide-I), 1590, 1547, 1529, 1510, 1487, 1467, 1444, 1434, 1413, 1392, 1338, 1283, 1271, 1244, 1182, 1160, 1135, 1091, 1072, 1044, 1011, 974, 922, 869, 833, 817, 793, 759, 735, 711, 685, 665, 643, 619 [lit.[139] IR (cm^{-1}): 1630]. mp 234-240°C [lit.[139] mp 218-220°C].

Preparation of **3-46**[139]

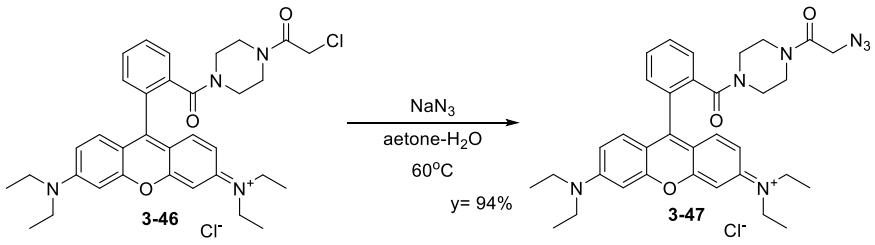


1.00 g of **3-45** (1.83 mmol, 1.0 eq.) was dissolved in anhydrous dichloromethane (18 mL) and the solution was cooled to 0°C. Chloroacetyl chloride (291 μL, 3.66 mmol, 2.0 eq.) and pyridine (355 μL, 4.39 mmol, 2.4 eq.) were added and the resultant solution was stirred at 0°C for 30 min. The reaction was further stirred at room temperature for 3 hr and concentrated. To the residue was added 1.0M K_2CO_3 (150 mL) and this aqueous solution washed with diethyl ether (50 mL x 4). The aqueous phase was saturated with sodium chloride and extracted with isopropanol-dichloromethane (1: 4, 100 mL x 3). The combined organic phase was dried over $MgSO_4$ and concentrated. The residue was purified by column chromatography (siliga gel: 100 mL, eluent: 0.1M HCl: isopropanol: ethyl acetate = 1: 2: 2) to afford **3-46** as dark purple solid: 763 mg (y= 67%).

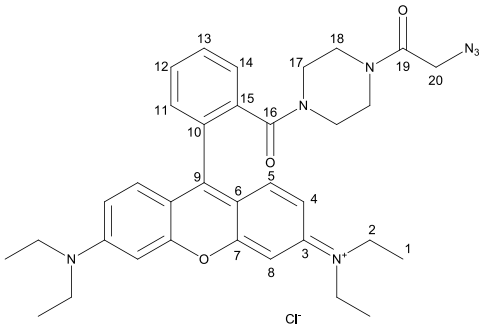


^{1}H NMR[139] (400MHz, CD_3OD, δ (CH_3OH)=3.310): 1.31 (12H, t, $J_{H1\text{-}H2}$ 7.2Hz, H_1), 3.33-3.53 (8H, br, H_{17}, H_{18}), 3.69 (8H, q, $J_{H2\text{-}H1}$ 7.2Hz, H_2), 4.22 (2H, brs, H_{20}), 6.98 (2H, d, $J_{H8\text{-}H4}$ 2.4Hz, H_8), 7.08 (2H, dd, $J_{H4\text{-}H5}$ 9.6Hz, $J_{H4\text{-}H8}$ 2.4Hz, H_4), 7.29 (2H, d, $J_{H5\text{-}H4}$ 9.6Hz, H_5), 7.50-7.57 (1H, m, H_{11}), 7.69-7.75 (1H, m, H_{13}), 7.75-7.82 (2H, m, H_{12}, H_{14}). LRMS (ESI$^+$): m/z $[M]^+$ 587.3. IR (ν_{max}, cm^{-1}, neat): 2974, 2929, 2870 (C-H stretch), 1648, 1629 (amide-I), 1585, 1549, 1528, 1509, 1484, 1466, 1411, 1392, 1334, 1273, 1246, 1199, 1179, 1156, 1131, 1072, 1006, 976, 921, 867, 821, 787, 757, 729, 709, 682 663, 617 [lit. IR (cm^{-1}):[139] 1643.06, 1632.45].

Preparation of **3-47**



100 mg of **3-46** (0.160 mmol, 1.0 eq.) was dissolved in 1.6 mL acetone-water (4: 1) and sodium azide (15.6 mg, 0.241mmol, 1.5 eq.) was added. The reaction solution was warmed up to 60°C and stirred for 3 hr. After cooling, the solution was diluted with 50 mL of water and saturated with sodium chloride. The aqueous phase was extracted with isopropanol-dichloromethane (1: 4, 20 mL x 4) and the combined organic phase was dried over $MgSO_4$ and concentrated to afford **3-47** as dark purple solid: 95.2 mg (y= 94%).



^{1}H NMR (500MHz, CD_3OD, δ (CH_3OH)=3.310): 1.31 (12H, t, $J_{H1\text{-}H2}$ 7.5Hz, H_1), 3.24-3.34 (2H, br, H_{17}/H_{18}), 3.37-3.53 (6H, br, H_{17}/H_{18}), 3.70 (8H, q, $J_{H2\text{-}H1}$ 7.2Hz, H_2), 4.06 (2H, s, H_{20}), 6.97 (2H, d, $J_{H8\text{-}H4}$ 2.5Hz, H_8), 7.08 (2H, dd, $J_{H4\text{-}H5}$ 9.5Hz, $J_{H4\text{-}H8}$ 2.5Hz, H_4), 7.28 (2H, d, $J_{H5\text{-}H4}$ 9.5Hz, H_5), 7.50-7.55 (1H, m, H_{11}), 7.69-7.73 (1H, m, H_{13}), 7.76-7.81 (2H, m, H_{12}, H_{14}). ^{13}C NMR (126MHz, CD_3OD, δ (CH_3OH)=49.15): 12.97 (C_1), 42.53, 42.68, 42.93, 43.23, 45.34, 46.03, 48.32 (C_{17}, C_{18}), 47.04 (C_2), 51.67 (C_{20}), 97.49 (C_8), 115.01 (C_6), 115.57 (C_4), 129.97 (C_{13}), 131.49 (C_{12}, C_{14}), 131.93 (C_{11}), 132.46 (C_9), 133.33 (C_5), 136.58 (C_{15}), 157.12 (C_{10}), 157.37 (C_7), 159.43 (C_3), 168.69 (C_{19}), 169.78 (C_{16}). LRMS (ESI^+): m/z $[M]^+$ 594.3. HRMS (ESI^+): m/z $[M]^+$ 594.3174 (found), (calcd 594.3187 for $C_{34}H_{40}N_7O_3$). IR (ν_{max}, cm^{-1}, neat): 2973, 2930, 2871 (C-H stretch), 2103 (azide stretch), 1647, 1629 (amide-I), 1583, 1549, 1528, 1509, 1484, 1466, 1410, 1392, 1378, 1346, 1333, 1272, 1244, 1199, 1178, 1156, 1131, 1071, 1007, 976, 953, 921, 868, 821, 788, 757, 731, 710, 683, 664, 641, 618.

CuAAC on the glycan of IgG

The stock solution of 10 mM $CuSO_4$ and 20 mM BTTP in water was mixed in the 1: 1 ratio before use. 50 μL of the glycan-remodelled IgG (0.55 mg/mL) in PBS was mixed with 5.0 μL of 40 mM **3-47** in water and 2.5 μL of $CuSO_4$-BTTP (5 mM/10 mM). The reaction was initiated by adding 2.5 μL of 50 mM sodium ascorbate solution. The solution was incubated at 20°C for 2 hr. 5 μL of the reaction solution was taken up and used for SDS-PAGE analysis. The glycans were released by PNGaseF digestion (see General Procedure) from the rest of the solution and were analysed by MALDI-TOF-MS (see General Procedure).

3.5. Reference

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Chapter 4

N-glycan trimming by Exoglycosidases to Prepare Homogeneous Glycoproteins

4.1. Introduction 289

4.2. Result and discussion 294

4.2.1. Engineering MalA to improve α-mannosidase activity 294

4.2.1.1. Design and preparation of D320 mutants 294

4.2.1.2. Development of the screening system and the assay for D320 mutants 297

4.2.1.3. Saturation mutagenesis at W413 302

4.2.1.4. Saturation mutagenesis at H480 305

4.2.1.5. Summary of mutagenesis of MalA to improve α-mannosidase activity 309

4.2.2. Enzymatic trimming of glycans on the yeast-expressed glycoproteins 311

4.2.2.1. Trimming with Jack Bean α-Mannosidase (JBM) 311

4.2.2.2. Trimming with enzyme combination of JBM and MalA 317

4.2.2.3. Trimming with enzyme combination of JBM and β-mannosidase 319

4.2.2.4. Summary of trimming of glycans on the yeast-expressed glycoproteins 323

4.2.3. Chromatographic separation of JBM treated glycoprotein 324

4.3. Conclusion 327

4.4. Experimental 329

4.5. Reference 343

4.1. Introduction

Purification: this technical process has been the first step in many aspects of science since René Descartes proposed the mechanistic study of nature.[1] Any complex systems are to be dissected down to their compositional units and each unit are to be analyzed for its structure and function. Now in the twenty-first century, as science progresses and especially our understanding of biological system advances, the holistic viewpoint becomes more and more required;[2] but still our way to learn science starts from the reductionistic approach.

Pure materials are requisite not only for scientific research but also for industrial products. One of such examples is pharmaceutical medicine, where purity is especially vital because the contamination of impurity can lead to unexpected adverse events. Therefore, purification of therapeutics should be performed with significant care for safety.

As drug discovery of small molecules becomes saturated and experiences more difficulties for spotting safe and potent molecules, scientists started to turn their eyes to biomolecules. They are natively in our bodies and therefore their toxicity is expected to be low. Based on this notion, now many biomolecule-based drugs have been developed.

However, in terms of production of biomolecules, they cast more difficulty in their purification than small molecules due to their structural complexities. There are now many biomolecules launched in the market[3] and therefore they are reasonably “pure” to be clinically used. However, precisely speaking, they are the mixtures of several structural analogues and as the number of biotherapeutics is building up, the risk of the unexpected events may also increase. Additionally, as society requires biomolecular drugs with lower costs, demand for biosimilars is to rise. Therefore, the more precise quality control of biopharmaceuticals is an urgent scientific topic

for social safety.

Currently practical preparation of biologics is performed by expression in host cells, mainly mammalian cells. Production of biologics in mammalian cells is now established and is able to cover medical demands. However, the production of biomolecules in mammalian cells still has several disadvantages compared to lower organism cultures:[4] volumetric productivity, product heterogeneity, media cost, retroviral contamination and time required to generate stable cell lines. Therefore, the search for expression hosts which can overcome these issues has been made.

The most robust system for protein expression would be *Escherichia coli*. However, most of the currently produced biomolecules are glycoproteins although most of prokaryotes including *E.coli* do not have machinery for production of glycoproteins. Recently, it turned out that *Campylobacter jejuni* has an unusual bacterial glycosylation machinery and its incorporation to *E. coli* was achieved.[5] They successfully produced homogeneous human-type glycoproteins using chemoenzymatic modification of *N*-glycans.[5] This result demonstrates the promising future of glycoprotein production in bacterial hosts but the current productivity varies from one protein to another and more investigations are needed for industrial production.

A good candidate to produce glycoproteins in industrial scale is yeast strains, whose culture method is robust and whose expression efficiency is high.[6] However, the issue is that yeast produces highly heterogeneous high mannose-type glycoproteins, whereas most of the clinically used glycoproteins have the complex-type *N*-glycans. It is well known that glycan structures influence biological function or stability of glycoproteins[7-9] and may cause immunogenic response,[10, 11] so modification is required to produce homogeneous glycoproteins for human use in yeast.

Though final structures of yeast glycans and human ones are significantly different, the same glycosylation machinery is shared between these two species in the initial part (**Figure 4.1**, the top compartment). Indeed, genetic engineering of the only downstream part results in producing human-type glycoproteins in yeast.[4, 12-16] Now such a protocol is standardized[17] in *Pichia pastoris* and the vectors needed for these transformations are available for academic uses. In this method, deletion of *och1*, which encodes 1, 6-mannotransferase, is the key step (**Figure 4.1**) to negate hypermannosylation which is characteristic to yeast glycans and also problematic for preparing therapeutic glycoproteins due to its immunogenicity.[18] Other genes for yeast-glycan synthesis are also deleted and at the same time genes encoding enzymes necessary for constructing human-type glycans are introduced. These enzymes include α-mannosidases (Man-I, Man-II), *N*-acetylglucosaminetransferases (GnT-I, Gn-T-II) and galactotransferases (GalT). This established protocol demonstrates the power of bioengineering. However, it is also true that complete replacement of glycosylation machinery significantly impedes growth rate.[17]

An alternative method for homogeneous human-type glycoprotein is a post-expressional approach: to have yeast produce glycoprotein using native glycosylation machinery and modify glycans afterward. The difference of glycan structures between yeast and human resides only in the non-reducing termini, while they share the common core structure, $Man_3GlcNAc_2$. Therefore, if the non-reducing terminal high mannoses of yeast *N*-glycans are removed and then oligosaccharides necessary for complex type-glycan are installed, human type glycoproteins can be prepared using glycoproteins produced in the native yeast strains.

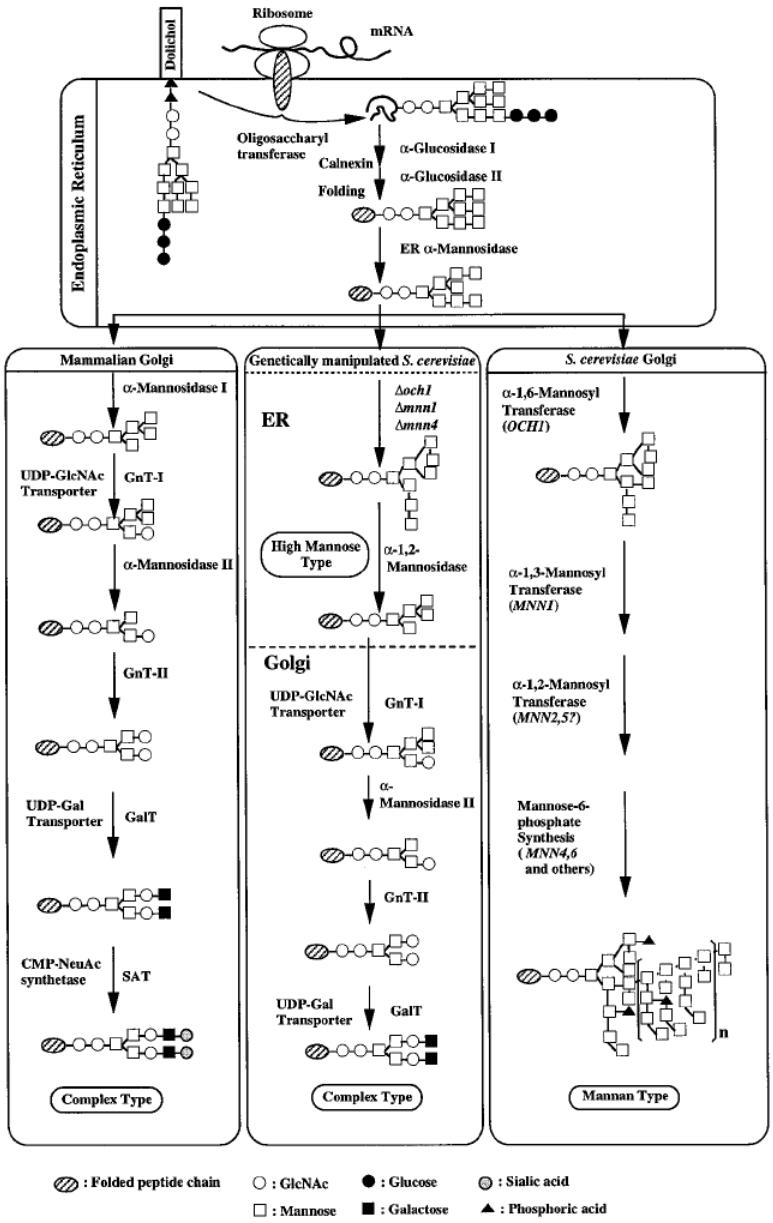


Figure 4.1. Comparison of glycosylation machinery of human (the left column) and *Saccharomyces cerevisiae* (yeast, the right column). The genetic engineering enables production of the human type glycorproteins (the central column). The figure is adapted from Chiba, Y. *et al., J. Biol. Chem.*. **1998**, *273* (41), 26298-26304. Copyright 1998 The American Society for Biochemistry and Molecular Biology.[19]

ENGases, endo-β-*N*-acetylglucosaminidases, are useful biocatalysts for this purpose because a native *N*-glycan can be replaced by a chemically synthesized glycan in an *en bloc* manner. Indeed, some examples perform *N*-glycan remodelling using EndoA on IgG-Fc[20, 21] or EndoS on

full-length IgG.[22] However, these enzymes work at proximity of the protein surface and are susceptible to the backbone structure of the proteins. Therefore, it would be required to investigate which ENGase is workable for each protein. This lack of generality would be the limitation of *endo*-glycosidases. On the other hand, *exo*-glycosidases work at the farther part of glycans from protein scaffold and it is less likely to be influenced by protein structures. This class of enzymes were expected to have a potential of general utility toward any kind of glycoproteins.

In this chapter, development of a new method to use exoglycosidases to modulate glycans on the yeast-expressed glycoproteins will be discussed. Enzymes with α-mannosidase activity were investigated for this purpose. First, mutagenesis of MalA was performed to enhance its α-mannosidase activity. Secondly, the most frequently used α-mannosidase, Jack Bean α-mannosidase (JBM), was applied for trimming of glycans on yeast-expressed glycoproteins. It is expected that these investigations will contribute to application of exoglycosidases to prepare homogeneous glycoproteins from ones expressed in the yeast.

4.2. Result and discussion

4.2.1. Engineering MalA to improve α-mannosidase activity

Glycoproteins expressed in yeast have high mannose-type glycans. This class of glycans is composed of α-mannosides in its exterior part. In the post-expressional approach, trimming these α-mannosides is the initial step to convert heterogeneous glycan structures of the yeast-expressed glycorprteins to a homogeneous glycoform.

In the course of investigation of the enzymatic activity of MalA, an α-glucosidase from *Sulfolobus solfataricus*, it turned out this enzyme has weak α-mannosidase activity.[23] This finding led us to develop the novel type of glycosynthase. To extend applicability of MalA, an investigation to enhance its α-mannosidase activity was performed aiming at applying this activity to modulate the glycan structures on the yeast-expressed glycoproteins.

4.2.1.1. Design and preparation of D320 mutants

It is known that glucoside substrates go through $^{4}H_{3}$ transition state in the hydrolytic reaction coordinate by GH31 enzymes.[24] If the same reaction mechanism works to mannoside analogues, the low hydtolytic activity toward these compounds may come from the diffusion of the negative charge on the catalytic nucleophile by a hydrogen bond with 2-OH of the mannoside substrate.(**Figure 4.2**) Therefore, it was speculated that if the native nucleophile of MalA is replaced by other nucleophiles and if their nucleophilicity is not affected by the hydrogen bond with 2-OH of mannose, the α-mannosidase activity of MalA may be improved.

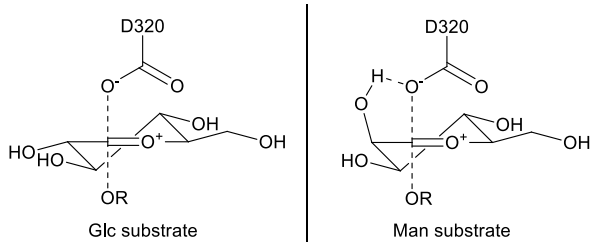


Figure 4.2. The hypothetical sketch about the transition state of the catalysis of MalA toward glucose substrate and mannose substrate.

This hypothesis prompted us to prepare nucleophile mutants, Glu, His, Met and Cys. External nucleophiles[25] can satisfy the hypothetical requirement and the D320G mutant in the presence of external nucleophiles was also investigated.

Mutatgenesis was performed using QuikChange® II XL Site-Directed Mutagenesis Kit. Primers were designed (**Table 4.1**) using PrimerX program[26] and its physical properties were checked with Primer Design Tool in the Sigma-Aldrich website.[27] All the primers were designed so as to satisfy the following criteria: i) melting point is 70-80 ℃, ii) GC content is >40%, iii) primer dimer formation is not expected, iv) probability of secondary structure formation is low to moderate. In all cases mutated plasmids were successfully obtained (**Figure 4.3**).

Table 4.1. Primer sequences and their physical properties for site-directed mutagenesis at D320.

Mutant		Primer sequence	Tm[a] (℃)	GC[b] (%)	PD[c]	SS[d]
D320E	forward	5'-GATGGTATTTGGCTA GA**G** ATGAATGAACCAACTG-3'	73.1	41.1	No	moderate
	reverse	5'-CAGTTGGTTCATTCAT **C**TC TAGCCAAATACCATC-3'	73.1	41.1	No	moderate
D320H	forward	5'-GTTGATGGTATTTGGCTA **C**AC ATGAATGAACCAACTG-3'	76.3	40.5	No	moderate
	reverse	5'-CAGTTGGTTCATTCAT GT**G** TAGCCAAATACCATCAAC-3'	76.3	40.5	No	moderate
D320M	forward	5'-GGAGTTGATGGTATTTGGCTA **ATG** ATGAATGAACCAACTGACTTC-3'	79.3	40.0	No	moderate
	reverse	5'-GAAGTCAGTTGGTTCATTCAT **CAT** TAGCCAAATACCATCAACTCC-3'	79.3	40.0	No	moderate
D320C	forward	5'-GTTGATGGTATTTGGCTA **TG**C ATGAATGAACCAACTG-3'	77.3	40.5	No	moderate
	reverse	5'-CAGTTGGTTCATTCAT G**CA**T AGCCAAATACCATCAAC-3'	77.3	40.5	No	moderate
WT	forward	5'-GGAGTTGATGGTATTTGGCTA **GAC** ATGAATGAACCAACTGACTTC-3'				
	reverse	5'-GAAGTCAGTTGGTTCATTCAT **GTC** TAGCCAAATACCATCAACTCC-3'				

a) melting point, b) GC content, c) expectation for primer dimer formation, d) probability of secondary structure formation.

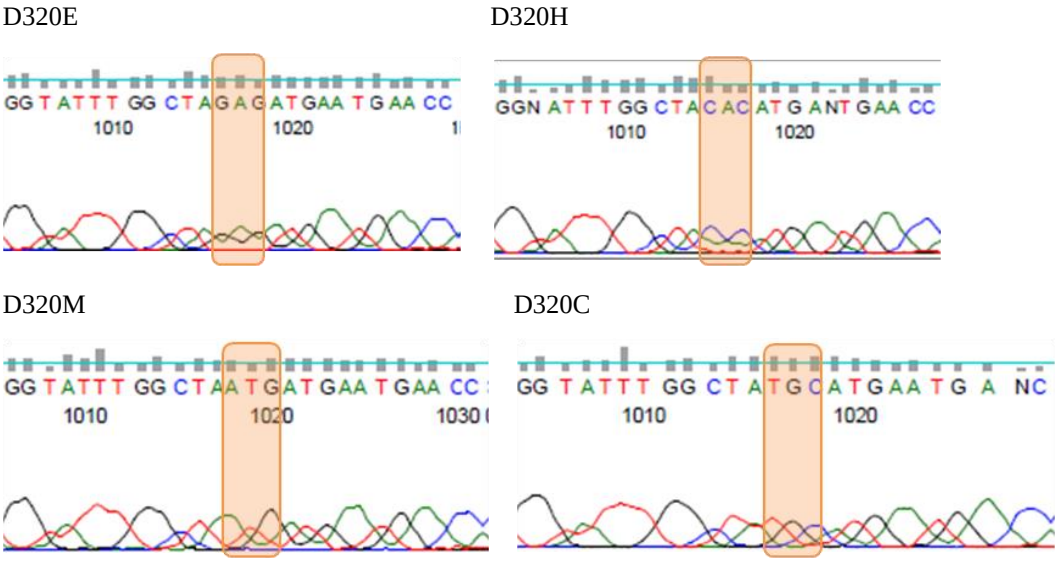


Figure 4.3. The analysis of DNA sequences of mutated plasmid DNA.

4.2.1.2. Development of the screening system and the assay for D320 mutants

As will be discussed below, saturation mutagenesis was also performed for improvement of the α-mannosidase activity of MalA. To handle the large number of small cultures, an efficient screening system was required. Therefore, an assay system which can detect the α-mannosidase activity of the wild type MalA was developed and its validity as a screening system was confirmed using D320 mutants.

First, the size of the multi-well plates for protein expression was investigated. Expression of the protein was performed in a 96-deep well plate or a 24-well plate. After selection by antibiotics was performed in 600 μL of ZYM-505-Kan medium in a 96-deep well plate, 10 μL of these cultures were mixed with 600 μL of ZYM-5052-Kan autoindution medium in either in a 96-deep well plate or a 24-well plate. The cell growth was compared by OD_{600} after incubation at 28°C for 24 hr. The 24-well plate gave much better result than the 96-deep well plate (**Table 4.2**) and the weights of the harvested cell pellets were 34 mg and 18 mg, respectively. Presumably, this difference of the cell growth came from aeration efficiency. Therefore, 24-well plates were chosen for screening.

Cell lysis was performed using BugBuster which was reconstructed from 10xBugBuster reagent and 100 mM NaPi (pH 6). Though pH 4.5 is optimal for the downstream enzymatic assay of MalA, the pH was set at 6.0 because BugBuster contains Benzonase and rLysozyme which are active in the range of pH >6. It is known that MalA shows equivalent activity[28, 29]

Table 4.2. Comparison of expression between 96-deep well plate and 24-well plate.

	OD_{600}	pH	pellet (mg)
96-well	2.5	6.1	18
24-well	12.1	6.8	34

at pH 6.0 compared to pH 4.5 and hence this pH value was selected for BugBuster lysis.

Preliminary experiments showed that endogeneous glycosidases were still active after BugBuster lysis and made the analysis of the screening results difficult. Therefore, to remove such endogeneous enzymes, the lysate with BugBuster was heated at 70°C after dilution with NaOAc buffer (pH 4.5) or NaPi buffer (pH 6.0). The lysate diluted with pH 4.5 buffer formed precipitation after heating and no band was observed by SDS-PAGE in the expected molecular weight region (**Figure 4.4**). To the contrary, the lysate diluted with pH 6.0 buffer did not form precipitation and the target band was observed by SDS-PAGE (**Figure 4.4**). It was confirmed that the glycosidase activity of the endogeneous enzymes became negligible as shown the activity of the lysate solutions from the pET-29a(+) transformant (**Table 4.3**).

To confirm that the glycosidase activity of MalA is maintained after these processes, the MalA solution after the heat treatment was subjected to enzymatic digestion of four *p*NP-sugars (**Table 4.3**). The glycosidase activity was maintained probably due to the thermal stability of MalA and the enhancement of glycosidase activity was observed in the lysate solutions from the

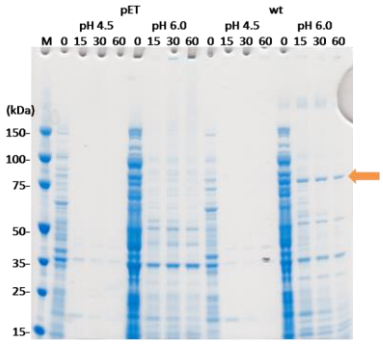


Figure 4.4. Treatment of the cell lysate with heat to remove endogeneous enzymes. Dilution of the lysate was performed with pH 4.5 buffer or pH 6.0 buffer. The diluted solution was incubated at 70°C for the indicated minutes. For compasiron, the lysate from BL21(DE3) transformed with pET-29a(+) without any insert was subjected to the same procedure. The arrow indicates the target protein.

pET-29a(+)-*malA* transformant, compared to that from the pET-29a(+) transformant. As to the heating time of the cell lysate, there is no significant difference when incubated for more than 15 min. Therefore, the heating time for the screening was set at 30 min to ensure complete removal of endogeneous glycosidases.

Table 4.3. OD_{410} after incubating four *p*NP-sugars with the cell lysate treated at 70°C for the indicated time. Enzyme solutions from pET-29a(+)-*malA* transformant showed enhanced hydrolytic activity toward *p*NP-sugars compared to that from pET-29a(+) transformant. a) Time heated at 70°C

	pET-29a(+)-MalA			pET-29a(+)		
	15min[a)]	30 min[a)]	60min[a)]	15min[a)]	30 min[a)]	60min[a)]
p NP-α-D-Glc	2.67	2.91	2.83	0.19	0.27	0.19
p NP-α-D-Man	0.37	0.48	0.35	0.21	0.24	0.21
p NP-α-D-Gal	0.34	0.34	0.29	0.21	0.22	0.27
p NP-α-D-Xyl	0.36	0.36	0.32	0.22	0.23	0.28

Thus establised screening method is summarized as below:

1. Colonies are selected on LB-Kan agar plates.
2. Colonies are added to 600 μL of ZYM-505-Kan medium in 96 deep-well plates and the plates are incubated at 28°C for 18 hr with shaking at 250 rpm.
3. 10 μL of the selection cultures are mixed with 600 μL of ZYM-5052-Kan medium in 24-well plates and the plates are incubated at 28°C for 24 hr with shaking at 250 rpm.
4. The cell suspensions are transferred to microcentrifuge tubes and centrifuged at 13,000 rpm for 20 min at 4°C. The supernatant is discarded. The cell pellets were stored at -20°C if needed.
5. 100 μL of BugBuster (in 100 mM NaPi, pH 6.0) is added to the cell pellets and the mixture is completely homogenized with a vortex mixer. The cell lysates are diluted with 200 μL of 100

mM NaPi (pH 6.0) and heated at 70°C for 30 min. The mixtures are centrifuged at 13,000 rpm for 20 min at 4°C and 250 μL of the supernatants are recovered.

6. 50 μL of the protein solutions are mixed with 50 μL of 10 mM *p*NP-sugar solutions in 100 mM NaPi (pH 6.0) in 96-well plates. To avoid evaporation of the reaction solutions, the reaction solutions are covered with 40 μL of silicone oil and the reaction plates are covered with an adhesive film. The plates are incubated at 85°C for 16 hr in the moisture-saturated container.
7. 40 μL of the reaction solutions are mixed with 200 μL of 100 mM NaPi (pH9.0) and Abs410 is measured.

The D320 mutants were applied to this assay system to validate its screening ability. The obtained results of Abs410 values are summarized in **Figure 4.5**. As expected, the weak α-glucosidase activity of the D320G mutant (Chapter 2.2.4) was detected by this protocol and this outcome demonstrates the feasibility of this screening system. Additionally, D320C mutant was newly found as a weak α-glucosidase. Large-scale preparation of this mutant was performed and the Michaelis-Menten analsysis confirmed the details of its α-glucosidase activity (See experimental). However, unfortutately, the enhancement of mannosidase activity was not observed for any of these designed mutants.

wt				G				C				M				E				H				pET			
1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
2.88	2.89	2.87	2.87	0.41	0.39	0.41	0.42	0.45	0.39	0.42	0.45	0.34	0.32	0.35	0.34	0.38	0.38	0.38	0.39	0.35	0.34	0.31	0.34	0.32	0.30	0.32	0.31
0.77	0.68	0.72	0.75	0.45	0.44	0.47	0.48	0.49	0.41	0.46	0.46	0.47	0.44	0.48	0.49	0.44	0.46	0.48	0.46	0.49	0.43	0.45	0.46	0.43	0.42	0.43	0.43
0.64	0.57	0.60	0.60	0.44	0.45	0.46	0.46	0.50	0.45	0.48	0.45	0.44	0.45	0.48	0.45	0.48	0.44	0.47	0.47	0.48	0.43	0.45	0.46	0.43	0.42	0.42	0.43
0.52	0.44	0.48	0.46	0.35	0.35	0.37	0.38	0.38	0.38	0.38	0.39	0.36	0.39	0.39	0.40	0.37	0.37	0.39	0.39	0.40	0.35	0.36	0.37	0.36	0.34	0.35	0.35

wt				G				C				M				E				H			
1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
2.56	2.58	2.55	2.56	0.09	0.08	0.09	0.11	0.13	0.09	0.10	0.13	0.02	0.02	0.03	0.03	0.06	0.08	0.06	0.07	0.03	0.04	-0.01	0.03
0.34	0.26	0.29	0.31	0.02	0.02	0.04	0.05	0.06	-0.01	0.03	0.03	0.04	0.02	0.05	0.06	0.01	0.04	0.05	0.03	0.06	0.01	0.02	0.02
0.21	0.15	0.19	0.17	0.01	0.03	0.04	0.03	0.06	0.03	0.06	0.02	0.01	0.03	0.07	0.02	0.05	0.03	0.05	0.04	0.04	0.01	0.03	0.03
0.16	0.10	0.13	0.11	-0.01	0.01	0.01	0.03	0.02	0.04	0.03	0.04	0.00	0.05	0.04	0.05	0.01	0.03	0.04	0.04	0.05	0.01	0.01	0.02

Figure 4.5. The assay result of D320 mutants using the screening protocol. The top panel shows the raw data and the bottom shows the subtracted values by the results of the insert-less pET-29a(+) transformants.

Next, addition of external nucleophiles was tested for activty enhancement of the nucleophile-less mutant D320G (**Figure 4.6**). Hydrotylic acitivity for both *p*NP-α-Glc and *p*NP-α-Man was investigated in the presence of external nucleophiles like fluoride, formate[30] and azide.[31] However, enhancement of the activity was not observed in any cases.

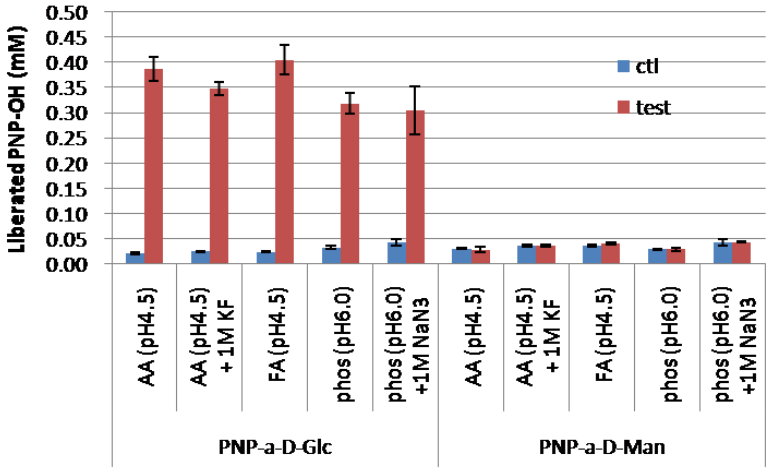


Figure 4.6. The hydrolytic activity of MalA D320G mutant to *p*NP-α-Glc and *p*NP-α-Man with external nucleophiles (fluoride, formate and azide).

4.2.1.3. Saturation mutagenesis at W413

To obtain more insight about the binding mode of substrates to MalA, its X-ray structure (PDB ID: 2g3m)[32] was superimposed with that of GH31 α-xylosidase (YicI) covalently bound with the 5-fluoro-β-xyloside ligand (PDB ID: 1xsk) [24] in D-site (Chapter 2.2.1 for definition, **Figure 2.2**). This analysis (**Figure 4.7**) suggests 2-OH of mannose would not be accepted due to the steric hindrance of W413 residue if mannose takes the same conformation ($^1S_3$) of the intermediate as glucose (or xylose) because the superimposition shows that the distance between C-2 of the substrate and W413 is only 3.4Å. Based on this assumption, alteration of this amino acid was investigated using saturation mutagenesis.

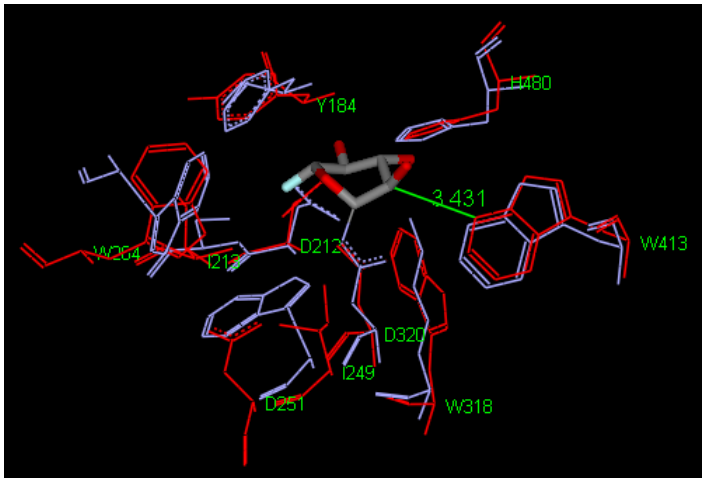


Figure 4.7. Superimposition of MalA (shown in red, PDB ID: 2g3m)[32] and YicI (shown in blue, PDB ID: 1xsk) covalently bound with 5-fluoro-β-xyloside (shown in stick model).[24] The amino acid labelling is based on the sequence of MalA. In this model, the distance between C-2 of the substrate and W413 of MalA is 3.4Å.

The designed primers and their physical properties are shown in **Table 4.4**. Mutagenesis was performed using KOD Hot Start DNA Polymerase kit. Using these degenerated primers, the desired plasmid library was obtained (**Figure 4.7**).

Table 4.4. Primer sequences and their physical properties for saturation mutagenesis at W413.

Mutant		Primer sequence	Tm[a] (°C)	GC[b] (%)	PD[c]	SS[d]
W413X	forward	5' –GGAATACAAAGATACGCATTCATC **NNK** ACTGGTGATAATACCCCTTC–3'	77.1	38.2	No	Weak
	reverse	5' –GAAGGGGTATTATCACCAGT **MNN** GATGAATGCGTATCTTTGTATTCC–3'	76.8	38.2	No	Weak
WT	forward	5' –GGAATACAAAGATACGCATTCATC **TGG** ACTGGTGATAATACCCCTTC–3'				
	reverse	5' –GAAGGGGTATTATCACCAGT **ACC** GATGAATGCGTATCTTTGTATTCC–3'				

N= A, G, T or C, K= G or T, a) melting point, b) GC content, c) expectation for primer dimer formation, d) probability of secondary structure formation.

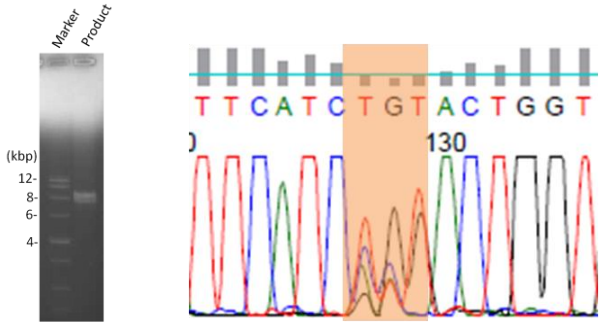
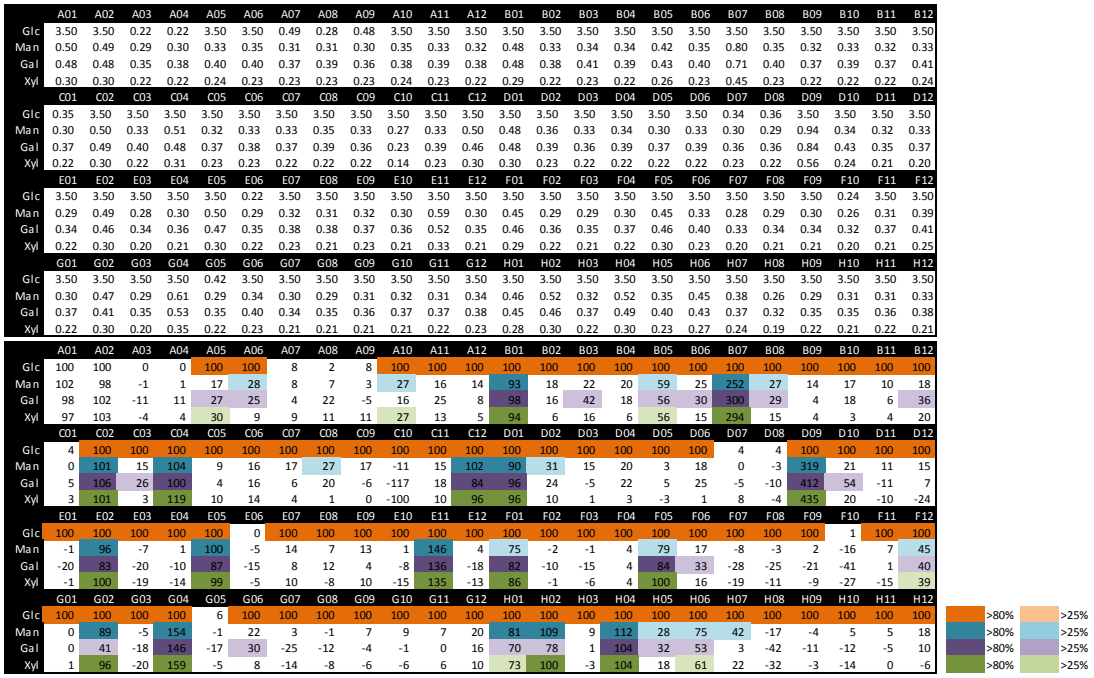


Figure 4.7. The result of PCR to generate W413X plasmid library. Left: agarose gel electrophoresis of PCR. Right: The DNA sequencing of the constructed library. (the target region shown in orange).

BL21(DE3) was transformed with the constructed plasmid library and colonies were grown on LB-Kan agar plates. Following the protocol set up in the previous section, screening for the activities towards *p*NP-α-Glc and *p*NP-α-Man as well as *p*NP-α-Gal and *p*NP-α-Xyl was performed (**Figure 4.8**). In the wells of A1, A2 and A3, A4 were inoculate the transformants of pET-29a(+)-*malA* and pET-29a(+) without insert, respectively, as positive and negative control, respectively. The results in several wells showed the equivalent or enhanced (>80%) hydrolytic acitivities (B01, B07, C02, C04, C12, D01, D09, E02, E05, E11, G02, G04, H01, H02, H04) to the wild type (A1, A2). However, after DNA sequencing of these plasmid DNA, it turned out that all of these had the wild type DNA sequence. The sequencing of the plasmid DNAs from other wells confirmed that the degenerated plasmids were equally introduced to the host *E. coli* and the screening was performed properly. Therefore, the investigation of mutants at this position was discontinued.



	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
Glc	3.50	3.50	0.22	0.22	3.50	3.50	0.49	0.28	0.48	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Man	0.50	0.49	0.29	0.30	0.33	0.35	0.31	0.31	0.30	0.35	0.33	0.32	0.48	0.33	0.34	0.34	0.42	0.35	0.80	0.35	0.32	0.33	0.32	0.33
Gal	0.48	0.48	0.35	0.38	0.40	0.40	0.37	0.39	0.36	0.38	0.39	0.38	0.48	0.38	0.41	0.39	0.43	0.40	0.71	0.40	0.37	0.39	0.37	0.41
Xyl	0.30	0.30	0.22	0.22	0.24	0.23	0.23	0.23	0.23	0.24	0.23	0.22	0.29	0.22	0.23	0.22	0.26	0.23	0.45	0.23	0.22	0.22	0.22	0.24
	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
Glc	0.35	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	0.34	0.36	3.50	3.50	3.50	3.50
Man	0.30	0.50	0.33	0.51	0.32	0.33	0.33	0.35	0.33	0.27	0.33	0.50	0.48	0.36	0.33	0.34	0.30	0.33	0.30	0.29	0.94	0.34	0.32	0.33
Gal	0.37	0.49	0.40	0.48	0.37	0.38	0.37	0.39	0.36	0.23	0.39	0.46	0.48	0.39	0.36	0.39	0.37	0.39	0.36	0.36	0.84	0.43	0.35	0.37
Xyl	0.22	0.30	0.22	0.31	0.23	0.23	0.22	0.22	0.22	0.14	0.23	0.30	0.30	0.23	0.22	0.22	0.22	0.22	0.23	0.22	0.56	0.24	0.21	0.20
	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
Glc	3.50	3.50	3.50	3.50	3.50	0.22	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	0.24	3.50	3.50
Man	0.29	0.49	0.28	0.30	0.50	0.29	0.32	0.31	0.32	0.30	0.59	0.30	0.45	0.29	0.29	0.30	0.45	0.33	0.28	0.29	0.30	0.26	0.31	0.39
Gal	0.34	0.46	0.34	0.36	0.47	0.35	0.38	0.38	0.37	0.36	0.52	0.35	0.46	0.36	0.35	0.37	0.46	0.40	0.33	0.34	0.34	0.32	0.37	0.41
Xyl	0.22	0.30	0.20	0.21	0.30	0.22	0.23	0.21	0.23	0.21	0.33	0.21	0.29	0.22	0.21	0.22	0.30	0.23	0.20	0.21	0.21	0.20	0.21	0.25
	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
Glc	3.50	3.50	3.50	3.50	0.42	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Man	0.30	0.47	0.29	0.61	0.29	0.34	0.30	0.29	0.31	0.32	0.31	0.34	0.46	0.52	0.32	0.52	0.35	0.45	0.38	0.26	0.29	0.31	0.31	0.33
Gal	0.37	0.41	0.35	0.53	0.35	0.40	0.34	0.35	0.36	0.37	0.37	0.38	0.45	0.46	0.37	0.49	0.40	0.43	0.37	0.32	0.35	0.35	0.36	0.38
Xyl	0.22	0.30	0.20	0.35	0.22	0.23	0.21	0.21	0.21	0.21	0.22	0.23	0.28	0.30	0.22	0.30	0.23	0.27	0.24	0.19	0.22	0.21	0.22	0.21

	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
Glc	100	100	0	0	100	100	8	2	8	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Man	102	98	-1	1	17	28	8	7	3	27	16	14	93	18	22	20	59	25	252	27	14	17	10	18
Gal	98	102	-11	11	27	25	4	22	-5	16	25	8	98	16	42	18	56	30	300	29	4	18	6	36
Xyl	97	103	-4	4	30	9	9	11	11	27	13	5	94	6	16	6	56	15	294	15	4	3	4	20
	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
Glc	4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	4	4	100	100	100	100
Man	0	101	15	104	9	16	17	27	17	-11	15	102	90	31	15	20	3	18	0	-3	319	21	11	15
Gal	5	106	26	100	4	16	6	20	-6	-117	18	84	96	24	-5	22	5	25	-5	-10	412	54	-11	7
Xyl	3	101	3	119	10	14	4	1	0	-100	10	96	96	10	1	3	-3	1	8	-4	435	20	-10	-24
	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
Glc	100	100	100	100	100	0	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	1	100	100
Man	-1	96	-7	1	100	-5	14	7	13	1	146	4	75	-2	-1	4	79	17	-8	-3	2	-16	7	45
Gal	-20	83	-20	-10	87	-15	8	12	4	-8	136	-18	82	-10	-15	4	84	33	-28	-25	-21	-41	1	40
Xyl	-1	100	-19	-14	99	-5	10	-8	10	-15	135	-13	86	-1	-6	4	100	16	-19	-11	-9	-27	-15	39
	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
Glc	100	100	100	100	6	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Man	0	89	-5	154	-1	22	3	-1	7	9	7	20	81	109	9	112	28	75	42	-17	-4	5	5	18
Gal	0	41	-18	146	-17	30	-25	-12	-4	-1	0	16	70	78	1	104	32	53	3	-42	-11	-12	-5	10
Xyl	1	96	-20	159	-5	8	-14	-8	-6	-6	6	10	73	100	-3	104	18	61	22	-32	-3	-14	0	-6

Figure 4.8. The screening result of W413 mutant library. A01 and A02 are the results of the wild-type transformants and A03 and A04 are that of the insert-less pET-29a(+) vector transformants. The top panel shows the raw data and the bottom shows the relative values to the wild type (A1, A2), which are colored as indicated.

4.2.1.4. Saturation mutagenesis at H480

In our laboratory, previously we reported broadening of substrate scope of a β-glycosidase, SsβG.[33] In this research, the preference of the wild type SsβG for glucose, galactose and fucose is suppressed by elimination of E432 or W433, which interacts 4-OH or 3-OH of the substrates, respectively. In the latter case, the relative activity of Glc:Man improved from 50: 1 to 4.1: 1 (k_{cat}/K_M). Though mannose is an epimer of glucose at 2-position, the amino acid residue which interacts with 3-OH may have significant influence on the enzymatic activity because glucose and mannose may have different transition-state conformers; glucose goes via $^4H_3$ while mannose $B_{2,5}$.[34] In such a case, the significant positional difference of hydroxyl groups resides not on 2-OH but on 3-OH, which corresponds to the improvement of the relative activity of W433 mutant of SsβG.

The same strategy might be applicable to α-glycosidases. Indeed, enzymes in GH31 family, which MalA belongs to, are reported to take $^4H_3$ iteration for its catalysis.[24] On the other hand,

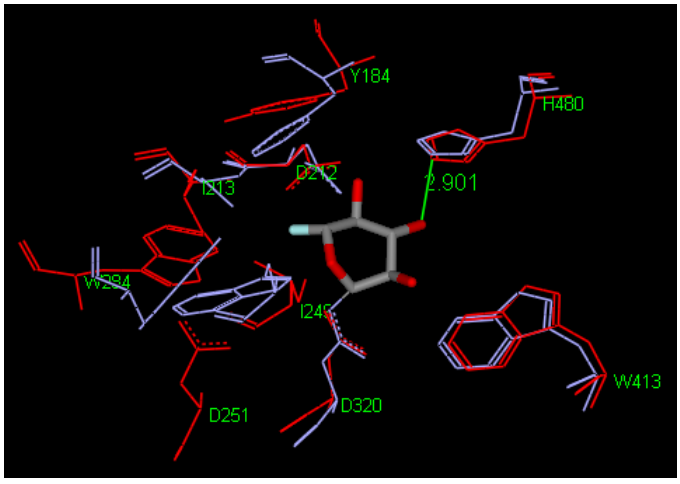


Figure 4.9. Superimposition of MalA (shown in red, PDB ID: 2g3m)[32] and YicI (shown in blue, PDB ID: 1xsk) covalently bound with 5-fluoro-β-xyloside (shown in stick model).[24] The amino acid labelling is based on the sequence of MalA. In this model, the distance between O-3 of the substrate and N^{ε} of H480 is 2.9Å.

Table 4.5. Primer sequences and their physical properties for saturation mutagenesis at H480.

Mutant		Primer sequence	Tm[a] (°C)	GC[b] (%)	PD[c]	SS[d]
H480X	forward	5' –GTTCTTCCCCTTCTATAGGTCA **NNK** AAGGCAACTGATGGTATAGATACG–3'	77.2	40.8	No	Moderate
	reverse	5' –CGTATCTATACCATCAGTTGCCTT **MNN** TGACCTATAGAAGGGGAAGAAC–3'	78.1	40.8	No	Moderate
WT	forward	5' –GTTCTTCCCCTTCTATAGGTCA **CAC** AAGGCAACTGATGGTATAGATACG–3'				
	reverse	5' –CGTATCTATACCATCAGTTGCCTT **GTG** TGACCTATAGAAGGGGAAGAAC–3'				

N= A, G, T or C, K= G or T, a) melting point, b) GC content, c) expectation for primer dimer formation, d) probability of secondary structure formation.

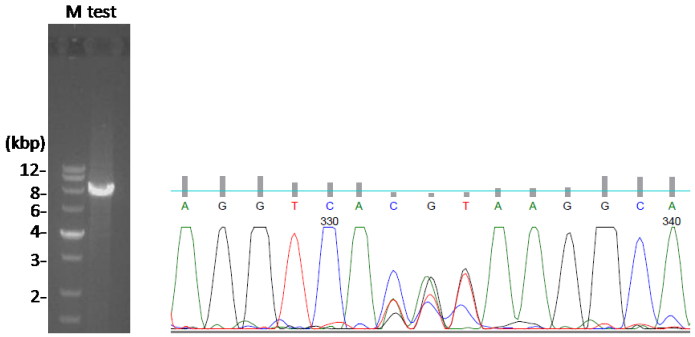


Figure 4.10. The result of PCR to generate H480 plasmid library. Left: agarose gel electrophoresis of PCR. Right: The DNA sequencing of the constructed library.

the catalysis of α-mannosidase ranging in GH38 and GH92 are known to proceed via $B_{2,5}$,[35, 36] which implies that mannose substrates may prefer $B_{2,5}$ conformation as the transition state of hydrolysis. Therefore, it was speculated that mutation at H480, which is likely to interact with 3-OH of glucose (**Figure 4.9**), might improve α-mannosidase activity of MalA and saturation mutagenesis was performed at this position.

The designed primers and their physical properties are shown in **Table 4.5**. Mutagenesis was performed using KOD Hot Start DNA Polymerase kit. Using these degenerated primers, the desired plasmid library was obtained (**Figure 4.10**).

The same screening as the W413 mutants was performed using 96-well plates twice. Several enzyme solutions showed the equivalent or enhanced (>80%) hydrolytic acitivities (**Figure 4.12A,B**: A08, A10, B05, B07, B09, C01, C06, C10, C11, D03, E04, E08, F04, F05, F11, G01, G09; **Figure 4.12C,D**: A05, C01, C02, C03, C09, D02, D07, E06, E11, E12, F01, F10, G11,

H03, H09) than the positive control. However, all of these constructs turned out to be the wild type after DNA sequencing. Other mutants, which have less than 25% glucosidase activity and more than 25% mannosidase activity were also selected (**Figure 4.12A,B**:D06, H01, H07; **Figure 4.12C,D**: B01, B06, D09, D11, D12) and they are assigned to be Val, Ile, Thr, Trp, Pro or Arg. Medium scale culture (200 mL) was performed and mutant proteins were purified to check their hydrolytic activity to *p*NP-α-Man. However, none of them showed significantly enhanced mannosidase activity.

A)

	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
Glc	2.85	2.82	0.28	0.29	1.47	0.31	1.14	2.83	0.35	2.83	2.35	0.31	0.32	0.31	0.69	0.30	2.96	0.97	2.95	1.23	3.01	0.31	0.30	1.56
Man	0.61	0.59	0.38	0.39	0.42	0.42	0.42	0.60	0.40	0.62	0.40	0.40	0.42	0.39	0.39	0.40	0.60	0.44	0.63	0.43	0.62	0.41	0.41	0.40
Gal	0.52	0.52	0.40	0.40	0.43	0.44	0.43	0.54	0.42	0.54	0.41	0.41	0.44	0.42	0.41	0.41	0.52	0.43	0.54	0.44	0.53	0.42	0.42	0.44
Xyl	0.38	0.38	0.28	0.30	0.31	0.32	0.32	0.39	0.31	0.39	0.29	0.30	0.33	0.30	0.30	0.30	0.38	0.33	0.42	0.32	0.40	0.32	0.31	0.31
	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
Glc	2.82	0.30	0.30	0.30	0.75	2.80	0.33	0.33	0.31	2.80	2.79	2.61	0.75	0.76	2.99	0.31	0.33	0.71	1.42	1.05	1.59	0.66	1.09	1.47
Man	0.62	0.40	0.40	0.40	0.42	0.62	0.43	0.43	0.41	0.65	0.63	0.49	0.39	0.40	0.60	0.41	0.43	0.44	0.46	0.43	0.46	0.42	0.42	0.41
Gal	0.54	0.41	0.41	0.42	0.44	0.56	0.45	0.45	0.43	0.58	0.57	0.48	0.42	0.42	0.53	0.42	0.43	0.43	0.43	0.42	0.47	0.41	0.44	0.44
Xyl	0.39	0.30	0.30	0.31	0.31	0.41	0.33	0.34	0.33	0.41	0.40	0.32	0.36	0.29	0.39	0.31	0.31	0.32	0.33	0.32	0.34	0.32	0.33	0.31
	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
Glc	0.29	0.30	0.30	2.77	0.39	0.32	0.32	2.81	0.30	0.77	0.33	0.40	2.79	0.33	0.98	2.98	2.97	0.88	0.75	0.33	2.81	1.25	2.97	0.79
Man	0.40	0.40	0.40	0.58	0.40	0.41	0.41	0.63	0.39	0.39	0.42	0.41	0.46	0.42	0.39	0.61	0.62	0.43	0.43	0.43	0.45	0.42	0.62	0.42
Gal	0.43	0.42	0.42	0.51	0.42	0.43	0.42	0.56	0.41	0.41	0.44	0.41	0.44	0.44	0.39	0.53	0.54	0.42	0.42	0.43	0.44	0.43	0.56	0.47
Xyl	0.31	0.30	0.30	0.37	0.31	0.31	0.32	0.41	0.30	0.29	0.32	0.29	0.33	0.30	0.30	0.39	0.39	0.32	0.33	0.33	0.32	0.32	0.42	0.33
	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
Glc	2.85	0.30	0.30	0.81	0.31	0.49	0.88	0.32	2.81	0.31	0.31	0.31	0.35	0.76	0.31	0.31	0.32	0.88	0.32	0.31	0.39	0.30	1.23	0.90
Man	0.67	0.40	0.40	0.40	0.40	0.40	0.41	0.41	0.62	0.42	0.40	0.40	0.45	0.41	0.39	0.40	0.42	0.43	0.44	0.43	0.42	0.40	0.43	0.40
Gal	0.59	0.42	0.42	0.41	0.43	0.42	0.43	0.43	0.54	0.43	0.43	0.41	0.44	0.43	0.42	0.43	0.43	0.43	0.42	0.41	0.44	0.41	0.42	0.41
Xyl	0.40	0.30	0.30	0.30	0.31	0.30	0.31	0.32	0.39	0.31	0.31	0.31	0.36	0.31	0.31	0.31	0.32	0.33	0.34	0.32	0.33	0.32	0.31	0.30

B)

	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
Glc	101	99	0	0	47	1	33	100	3	100	81	1	1	1	16	1	105	27	104	37	107	1	0	50
Man	103	97	-1	1	18	17	16	102	9	109	9	9	18	4	4	9	102	25	114	22	109	12	13	8
Gal	102	98	-3	3	22	36	27	115	14	116	11	11	30	15	8	10	99	28	113	30	111	21	19	31
Xyl	103	97	-6	6	24	35	30	115	19	110	5	15	40	8	14	12	98	46	146	38	120	37	23	26
	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
Glc	99	0	1	1	18	99	2	2	1	99	98	91	18	18	106	1	2	17	44	30	51	15	31	47
Man	111	7	6	6	16	112	23	24	14	121	116	48	6	7	100	11	21	28	36	23	35	15	16	11
Gal	115	12	7	14	33	134	39	38	27	149	138	65	15	20	107	16	26	28	25	17	59	12	33	34
Xyl	104	12	11	17	25	128	43	53	41	131	124	33	81	1	114	21	24	29	40	31	56	30	41	25
	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
Glc	0	1	1	98	4	1	1	99	0	19	2	4	98	2	27	106	105	23	18	2	99	38	105	20
Man	9	6	6	89	9	10	11	112	3	4	18	12	37	19	5	104	110	20	23	22	32	19	108	17
Gal	29	14	15	93	19	22	21	134	11	6	32	11	37	38	-4	112	117	16	20	27	37	28	135	55
Xyl	19	16	14	84	18	17	27	136	10	5	35	1	43	16	13	105	109	37	45	41	36	33	140	44
	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
Glc	101	1	1	21	1	8	23	1	99	1	1	1	3	19	1	1	1	24	1	1	4	0	37	24
Man	131	7	6	8	9	9	13	13	107	15	6	10	33	12	6	10	20	21	28	22	19	8	21	6
Gal	154	13	15	12	24	17	26	22	115	23	24	11	35	24	21	22	28	24	18	13	31	5	18	12
Xyl	125	14	10	13	18	15	25	28	109	20	21	18	72	22	20	19	35	39	57	33	40	32	26	10

>80% >25%

C)

	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
Glc	2.80	2.79	0.29	0.29	2.81	0.31	0.31	1.47	0.31	0.32	0.73	0.30	0.32	0.29	0.30	0.29	0.33	0.32	1.21	0.66	0.83	1.08	0.31	0.29
Man	0.64	0.61	0.39	0.38	0.67	0.42	0.41	0.45	0.42	0.43	0.41	0.40	0.45	0.39	0.40	0.42	0.40	0.45	0.41	0.39	0.39	0.41	0.42	0.40
Gal	0.56	0.54	0.41	0.40	0.58	0.45	0.44	0.46	0.44	0.44	0.41	0.44	0.45	0.40	0.40	0.43	0.46	0.44	0.41	0.42	0.41	0.41	0.42	0.41
Xyl	0.44	0.40	0.30	0.30	0.45	0.33	0.31	0.32	0.32	0.33	0.31	0.30	0.35	0.32	0.33	0.33	0.33	0.33	0.30	0.30	0.30	0.30	0.34	0.31
	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
Glc	2.86	2.78	2.80	0.30	1.08	0.33	2.65	2.05	2.79	0.75	0.32	0.33	0.31	2.88	0.30	0.30	0.31	0.31	2.92	1.59	0.34	0.33	0.34	0.34
Man	0.59	0.64	0.64	0.41	0.44	0.44	0.48	0.47	0.69	0.43	0.43	0.43	0.42	0.62	0.41	0.39	0.43	0.44	0.59	0.48	0.46	0.44	0.45	0.45
Gal	0.54	0.56	0.56	0.43	0.45	0.45	0.46	0.48	0.60	0.44	0.46	0.47	0.43	0.51	0.43	0.41	0.44	0.43	0.56	0.46	0.45	0.44	0.46	0.46
Xyl	0.40	0.42	0.41	0.31	0.33	0.32	0.33	0.33	0.43	0.33	0.33	0.34	0.32	0.41	0.32	0.30	0.31	0.32	0.41	0.34	0.36	0.32	0.32	0.35
	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
Glc	0.31	0.32	0.76	1.29	1.59	2.76	0.30	1.34	0.85	0.32	2.80	2.76	2.95	0.30	0.29	2.91	0.31	1.55	0.85	0.30	0.30	2.92	0.30	2.77
Man	0.43	0.38	0.41	0.41	0.43	0.55	0.42	0.43	0.43	0.44	0.68	0.66	0.63	0.34	0.39	0.55	0.39	0.43	0.40	0.41	0.41	0.65	0.42	0.55
Gal	0.41	0.40	0.41	0.42	0.42	0.49	0.42	0.44	0.44	0.44	0.57	0.58	0.56	0.38	0.38	0.48	0.41	0.42	0.40	0.41	0.41	0.56	0.42	0.51
Xyl	0.32	0.31	0.31	0.30	0.31	0.36	0.31	0.31	0.31	0.33	0.44	0.41	0.42	0.29	0.28	0.34	0.30	0.32	0.31	0.29	0.30	0.42	0.30	0.38
	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
Glc	0.35	0.28	0.29	1.61	0.30	0.76	1.01	1.00	2.57	0.31	2.77	0.31	0.30	1.54	2.94	0.27	0.28	0.28	2.75	0.36	2.95	1.32	0.31	0.32
Man	0.39	0.38	0.39	0.41	0.40	0.40	0.41	0.41	0.44	0.40	0.62	0.43	0.40	0.41	0.58	0.36	0.37	0.37	0.43	0.41	0.61	0.44	0.41	0.41
Gal	0.41	0.39	0.40	0.41	0.42	0.42	0.42	0.42	0.44	0.43	0.56	0.45	0.42	0.39	0.51	0.37	0.39	0.37	0.43	0.40	0.54	0.45	0.43	0.43
Xyl	0.31	0.29	0.30	0.30	0.31	0.31	0.30	0.32	0.31	0.32	0.42	0.32	0.32	0.30	0.37	0.27	0.29	0.28	0.31	0.31	0.38	0.33	0.32	0.32

D)

	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
Glc	100	100	0	0	101	1	1	47	1	1	18	1	1	0	1	0	2	1	37	15	22	32	1	0
Man	106	94	0	0	121	14	10	28	15	19	12	7	29	2	8	16	6	28	11	3	4	12	14	6
Gal	109	91	3	-3	122	27	20	36	24	21	3	23	32	-7	-8	12	38	19	-2	4	-2	3	7	-2
Xyl	115	85	1	-1	122	20	7	18	11	27	7	-1	36	14	21	21	26	23	-2	-2	-6	-3	31	9
	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
Glc	102	99	100	0	32	2	94	70	100	18	1	2	1	104	1	0	1	1	105	52	2	2	2	2
Man	84	108	108	12	24	23	41	35	128	20	19	20	14	100	9	4	18	22	85	38	31	21	27	28
Gal	94	109	108	18	28	27	35	52	134	25	35	41	17	73	12	0	19	13	112	35	29	22	34	34
Xyl	83	100	95	9	19	18	23	21	110	19	25	27	17	89	11	-7	8	14	89	31	51	13	18	36
	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
Glc	1	1	19	40	52	98	1	42	22	1	100	99	106	1	0	105	1	50	22	0	1	105	1	99
Man	18	-1	11	10	17	69	13	20	19	24	122	114	103	-19	1	69	4	18	5	11	10	110	13	69
Gal	-2	-3	-1	4	10	60	6	20	21	23	116	120	108	-24	-20	48	-2	9	-6	-1	1	109	11	76
Xyl	14	3	3	-6	6	53	4	9	9	25	113	95	96	-8	-17	35	-5	16	3	-8	-7	96	-2	62
	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12
Glc	3	0	0	53	0	19	29	28	91	1	99	1	0	50	106	-1	0	0	98	3	106	41	1	1
Man	4	-2	3	13	6	9	12	12	24	7	98	19	7	11	83	-11	-7	-5	19	13	94	23	9	11
Gal	3	-13	-6	4	5	9	5	10	21	15	105	28	7	-16	76	-28	-15	-26	12	-10	94	32	12	18
Xyl	2	-10	-1	-3	9	4	-1	15	6	14	96	15	14	-3	61	-31	-15	-24	2	2	69	22	14	18

>80% >25%

Figure 4.12. The screening result of H480 mutant library. A01 and A02 are the results of the wild-type transformants and A03 and A04 are that of the insert-less pET-29a(+) vector transformants. The panels of (A) and (C) shows the raw data and the ones of (B) and (D) shows the relative values to the wild type (A1, A2), which are colored as indicated.

4.2.1.5. Summary of mutagenesis of MalA to improve α-mannosidase activity

To enhance the mannosidase acitivity of MalA, three hypotheses were proposed and the experiments were performed to check their validity. First, the nucleophilic residue D320 was replaced by other nucleophilic amino acids (or the nucleophile-less mutant, where external nucelophiles were added in the assays) under the hypothesis that removing an unnecessary hydrogen bond between 2-OH of mannose and the carboxylate of D320 might lead to enhancement of the hydrolytic activity toward mannose substrates. Five mutants were prepared but, unfortunately, none of them showed an enhanced α-mannosidase activity. However, in the course of this investigation, the screening system to detect desired glycosidase activities for this thermophilic protein was developed.

Saturation mutagenesis at W413 was then performed because the side chain of this amino acid was speculated to have steric repulsion against 2-OH of mannose substrates based on the analysis of X-ray crystal structures of MalA (PDB ID: 2g3m) and YicI-5F-Xyl (PDB ID: 1xsk). Saturation mutagenesis at H480 was also tested because the side chain of this residue is likely to interact with 3-OH of mannose substrates, where the significant positional difference from glucose substrates is observed if mannose takes $B_{2,5}$ conformation as its transition state. The plasmid libraries were successfully constructed at both positions but mutants with a desired activity was not found.

To obtain more rational information for enhancement of α-mannosidase activity, crystallization of MalA with manno-configuration ligand (**Figure 4.12**) is ongoing. As an initial trial, crystallization condition without ligand was screened and promising conditions were found. These conditions are similar to the previously reported ones.[32] The crystallines obtained in this screening did not have a sufficient quality for diffraction and further optimization is now

ongoing.

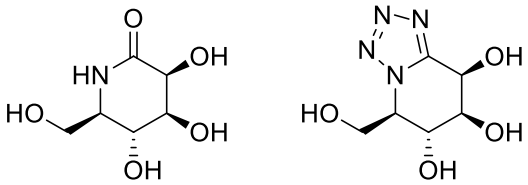


Figure 4.12. Manno-configured ligands. A flat structure around 1- and 2-positioins make these compounds transition state mimetics.

4.2.2. Enzymatic trimming of glycans on the yeast-expressed glycoproteins

4.2.2.1. Trimming with Jack Bean α-Mannosidase (JBM)

To investigate the feasibility about preparation of homogeneous glycofroms from the yeast-expressed glycoproteins, Jack bean α-mannosidase (JBM) was applied. JBM is one of the most frequently used enzymes in glycan analyses. Its scope of the activity is broad and JBM is able to trim all kinds of the α-mannoside linkages with less efficiency toward 1,6-linkage.[37] A commercially available JBM from Sigma-Aldrich (Part Number: M 7257) was used for this purpose.

As a model glycoprotein, a lipase from *Candida antarctica*, CalB, was used. This glycoprotein has one *N*-glycosylation site at N99 (UniProt[38] Entry ID: P41365). CalB was expressed exogenously in *Pichia pastoris* and provided from the collaborator, VTU.[39]

CalB was treated with JBM in the absence (**Table 4.6**: test11, test12) and the presence (**Table 4.6**: test21, test22) of an additional enzyme, α(1,6)-mannosidase (NEBioLabs, Part Number: P0727). The effect of the reaction time (1 day: test11, test21, 2 days; test12, test22) was also investigated with supplemental addition of the enzymes. SDS-PAGE showed shift of bands in all cases (**Figure 4.14**, red arrow). However, the band after the glycosidase reactions was not single but at least two bands were observed.

Table 4.6. Reaction conditions for enzymatic glycan trimming of CalB. The reaction (the final volume: 100-120 μL) was performed for 10 mg/mL CalB in 100 mM NaOAc (pH 4.5) at 37°C for 1 or 2 days. The added enzymes in each condition are shown. a) 136 U/mL JBM solution. b) 2kU/mL α(1,6)-mannosidase solution. The activity units for enzymes are based on the definitions by the suppliers.

	1st day	2nd day
test11	5 μL JBM[a)]	-
test12	5 μL JBM[a)]	5 μL JBM[a)]
test21	5 μL JBM[a)], 5 μL α(1,6)-Man[b)]	-
test22	5 μL JBM[a)], 5 μL α(1,6)-Man[b)]	5 μL JBM[a)], 5 μL α(1,6)-Man[b)]

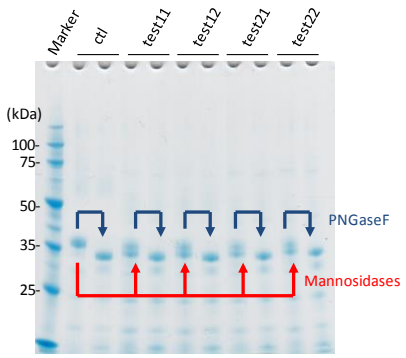


Figure 4.14. SDS-PAGE analysis of CalB after the mannosidase treatment. Treatment with the mannosidases (red arrows) indicated glycan trimming. The "ctl" indicates the reaction without any mannosidases. PNGaseF digestion (blue arrows) was performed to release glycans for MALDI-TOF-MS analysis.

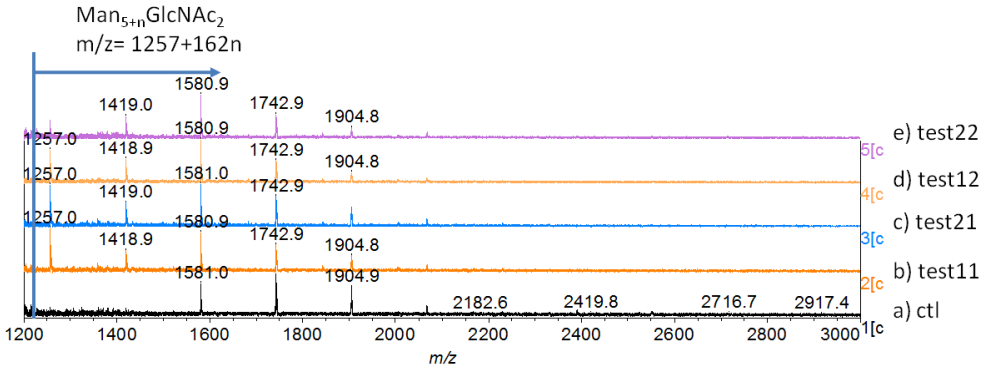


Figure 4.15. MALDI-TOF-MS spectra of the glycans released from CalB treated with the mannosidases.

The resultant solution was then treated with PNGaseF (**Figure 4.14**, blue arrow) to perform glycan analysis in MALDI-TOF-MS (**Figure 4.15**). CalB without any mannosidase treatment showed the peaks of the glycans corresponding to $Hex_7GlcNAc_2$ (m/z= 1581)-$Hex_{10}GlcNAc_2$ (m/z= 2067) as monosodium adduct. They are reasonably assigned as $Man_7GlcNAc_2$-$Man_{10}GlcNAc_2$ based on the basic glycan structures of the *Pichia pasrotis*-expressed glycoproteins. When CalB was treated with the mannosidases, the glycan peaks showed the shift to the lower molecular-weight region. This result indicated the glycans were successfully

trimmed under these conditions. When the effect of α(1,6) -mannosidase was compared (test11 vs 21 or test 12 vs 22), no significant difference was observed in the glycan profiles. On the other hand, when the reaction was incubated for longer time with addition of more enzymes, further processing of glycans took place and the relative intensity of the $Man_5GlcNAc_2$ peak (m/z= 1257) decreased (compare test11 vs test21 or test21 vs test22).

These samples showed complex spectra in the lower molecular-weight region (m/z < 1000) and it was difficult to analyze the existence of smaller glycans. Therefore, they were subjected to permethylation reaction and the analyses for the resultant glycans were also performed in MALDI-TOF-MS (**Figure 4.16**).

Similarly to the unmodified glycans, the permannosylated glycans from the mannosidase reactions showed the peaks corresponding to $Man_5GlcNAc_2$ (m/z= 1987)- $Man_9GlcNAc_2$ (m/z= 2599). Additionally, the lower molecular-weight region was observed after permethylation and

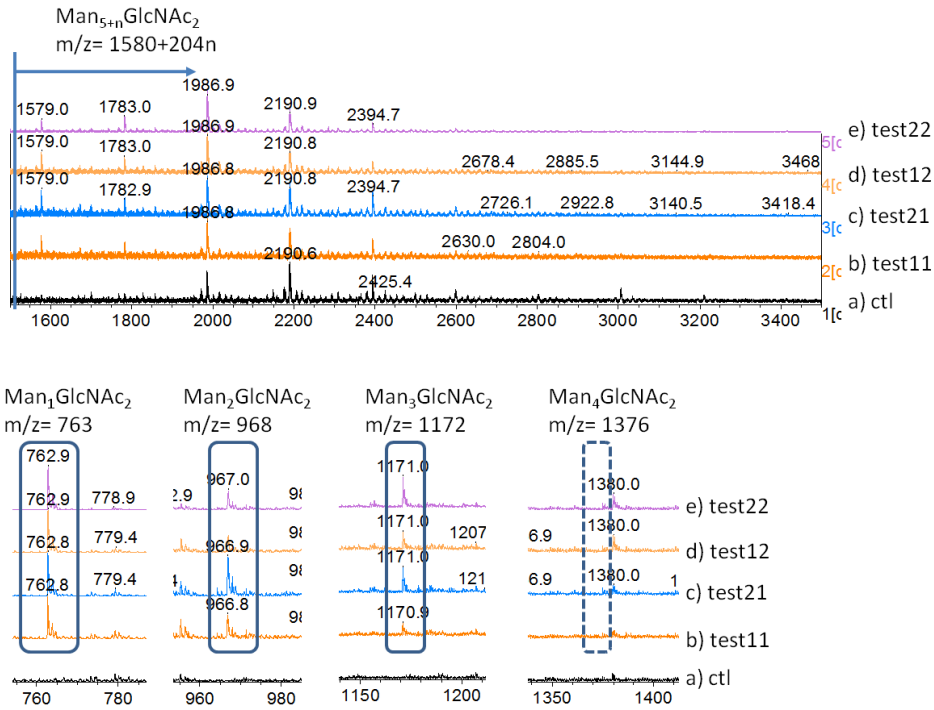


Figure 4.16. MALDI-TOF-MS spectra of the glycans released from CalB treated with the mannosidases after permethylation.

the peaks corresponding to $Man_1GlcNAc_2$ (m/z= 763)- $Man_3GlcNAc_2$ (m/z= 1171) were found in all the reactions. Again, no additional effect by α(1,6)-mannosidase was observed while the longer reaction time resulted in the relative decrease of $Man_2GlcNAc_2$ (m/z= 967) and $Man_3GlcNAc_2$ (m/z= 1171) and the relative increase of $Man_1GlcNAc_2$ (m/z= 763). This result is notable because it demonstrates that JBM has the potency to remove all the α-mannoses on high mannose-type *N*-glycans and to prepare the homogeneous glycoform, $Man_1GlcNAc_2$.

Aiming at the complete trimming by JBM, the effect of the reaction time was investigated up to 3 days. To ensure α-mannosidase activity, additional JBM was supplemented on each day (**Table 4.7**). As the previous experiment, SDS-PAGE showed apparent two bands even after 3 days (**Figure 4.17**). The detailed glycan analysis was performed by MALDI-TOF-MS in the native forms (**Figure 4.18**) as well as in the permethylated form (**Figure 4.19**).

Table 4.7. Reaction conditions for enzymatic glycan trimming of CalB. The reaction (the final volume: 100-110 μL) was performed for 10 mg/mL CalB in 100 mM NaOAc (pH 4.5) at 37°C for 1 or 2 days. The added enzymes in each condition are shown. a) 136 U/mL JBM solution. The activity units for enzymes are based on the definitions by the suppliers.

	1st day	2nd day	3rd day
test1	5 μL JBM[a)]	-	-
test2	5 μL JBM[a)]	5 μL JBM[a)]	-
test3	5 μL JBM[a)]	5 μL JBM[a)]	5 μL JBM[a)]

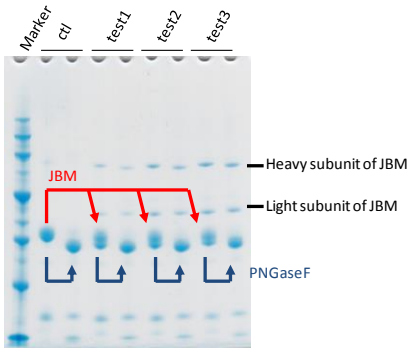


Figure 4.17. SDS-PAGE analysis of CalB after the JBM treatment. Treatment with the mannosidases (red arrows) indicated glycan trimming. The "ctl" indicates the reaction without any mannosidases. PNGaseF digestion (blue arrows) was performed to release glycans for MALDI-TOF-MS analysis.

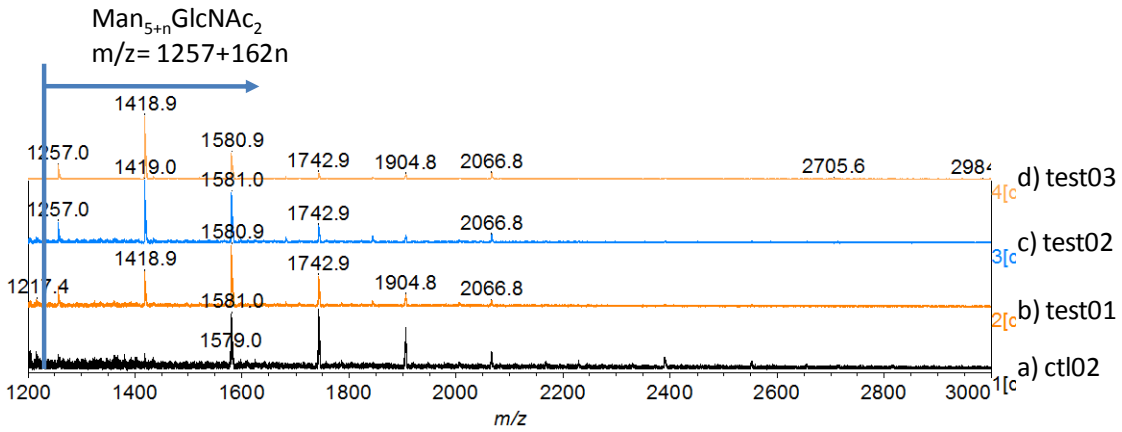


Figure 4.18. MALDI-TOF-MS spectra of the glycans released from CalB treated with the mannosidases.

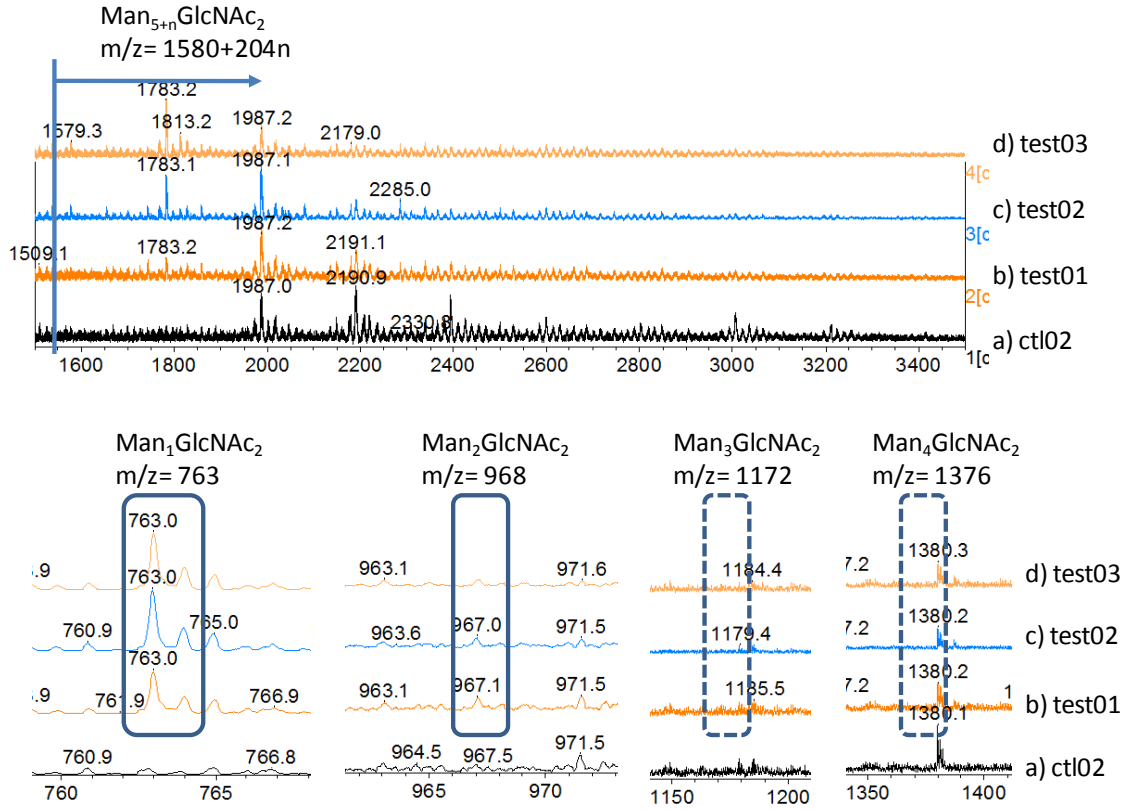


Figure 4.19. MALDI-TOF-MS spectra of the glycans released from CalB treated with the mannosidases after permethylation.

For the lower molecular-glycans, the convergence to $Man_1GlcNAc_2$ was observed in the course of the time as expected (**Figure 4.19**). However, unexpectedly, the shift of the partially trimmed glycans $Man_5GlcNAc_2$-$Man_9GlcNAc_2$ (m/z= 1257-1905 for the unmodified, **Figure 4.18**, 1987-2599 for the permethylated, **Figure 4.19**) stalled after two days.

These data implied that some of *N*-glycans on the *Pichia pastoris*-expressed glycoprotein had $Man_7GlcNAc_2$-$Man_{10}GlcNAc_2$ containing only α-mannosides in their exterior part and hence could be trimmed down to $Man_1GlcNAc_2$ by the intensive treatment with JBM. Therefore, JBM is still a promising biocatalytic tool for preparing the homogeneous glycoform. However, it turned out that there exist other *N*-glycans containing certain factors which were resistant to JBM activity and the glycan trimming by α-mannosidases was hampered. These results indicate that it was necessary to rectify the nomenclature of the glycans on CalB from $Man_7GlcNAc_2$-$Man_{10}GlcNAc_2$ back to the general formula $Hex_7GlcNAc_2$-$Hex_{10}GlcNAc_2$. To realize the complete conversion of the glycans into homogeneity, searching for additional exoglycosidases became needed.

4.2.2.2. Trimming with enzyme combination of JBM and MalA

The result of MALDI-TOF-MS indicated that the glycans resistant to JBM had the general structural formula of $Hex_nGlcNAc_2$ and hence the possible resistant factor is a hexose. Naturally ocurrirng hexoses are limited to glucose, mannose and galactose but it is unlikely that galactose is incorporated in the *N*-glycans based on the glycosylation machinery of *Pichia pastoris*.[6] Glucose is involved in all the eukaryotic glycosylation machinery and it plays a critical role in the quality control of the glycoproteins for their proper folding.[40] Especially, the glycoprotein was produced in the overexpression system and hence the overflow of the quality control system might result in the leakage of unprocessed α-glucosylated constructs.

To test this hypothesis, CalB expressed in *Pichia pastoris* was treated with JBM in combination with MalA as a representative of α-glucosidases. In this experiment, CalB was first treated with JBM for two days as the procedure described above, followed by purification by gel filtration to remove JBM. This JBM-treated CalB was applied to the reaction conditions shown in **Table 4.8** and the glycan profiles of the resultant glycoproteins were analyzed by MALDI-TOF-MS (**Figure 4.20**). Due to the difference of the batch of the used CalB, the JBM treatment gave the different glycan profile, ranging $Hex_6GlcNAc_2$-$Hex_9GlcNAc_2$(**Figure 4.20**,

Table 4.8. Reaction conditions for enzymatic glycan trimming of CalB. The reaction (the final volume: 108 μL) was performed for 6.2 mg/mL the JBM-treated CalB in 100 mM NaOAc (pH 4.5) at 37°C for 24 hr. The added enzymes in each condition are shown. a) 13.9 mg/mL MalA solution. b) 132 U/mL JBM solution. The activity units for enzymes are based on the definitions by the suppliers.

	MalA[a)] (μL)	JBM[b)] (μL)
Rx1	20	-
Rx2	2	-
Rx3	20	8
Rx4	2	8

the top panel). Unfortunately, in spite of the high loading of MalA, no trimming of glycans by MalA was observed (**Figure 4.20**, the second and third panel). When combined with JBM (**Figure 4.20**, the fourth and fifth panel), a slight peak shift was observed but it is likely that this activity was derived not from MalA but from JBM because no dose dependency on MalA was observed (**Figure 4.20**, the fourth panel vs the fifth panel).

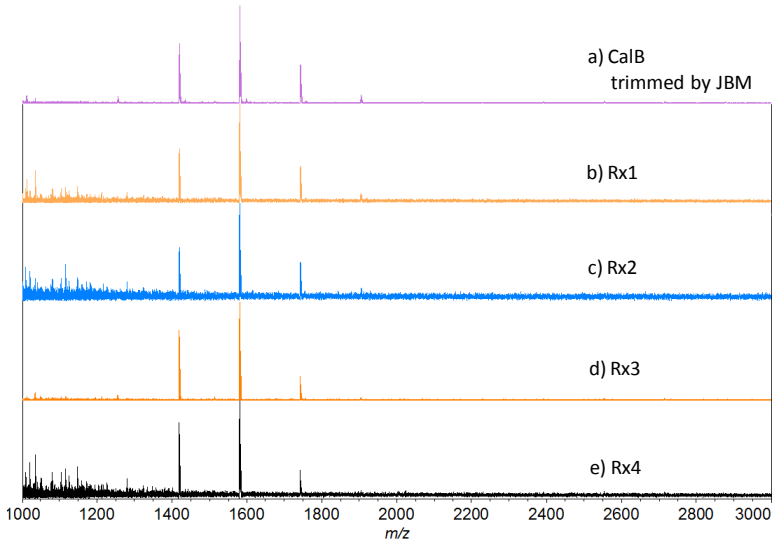


Figure 4-20. MALDI-TOF-MS spectra of the glycans released from CalB treated with MalA in the absence (b,c) or presence (d,e) of JBM.

4.2.2.3. Trimming with enzyme combination of JBM and β-mannosidase

Alternative candidate for the hexose with JBM resistance is β-mannose. Recently, four genes encoding β-mannosyltransfereases were identified[41] and indeed, the existence of JBM-resistant *N*-glycans was reported[42, 43] in the glycoproteins expressed in *Pichia pastoris* which has the humanized glycosylation machinery. Therefore, β-mannosidases were tested to prepare homogeneous glycoproteins enzymatically.

To investigate this possibility, SsβG and *Bt*Man2A were chosen as representative examples of β-mannosidases. SsβG preferably hydrolyzes β-glucosides, β-galactosides and β-D-fucosides but also shows weak β-mannosidase activity.[33] On the other hand, *Bt*Man2A is a native GH2 β-mannosidase found in *Bacteroides thetaiotaomicron*. It is reported that this enzyme has relatively broad substrate scope[44] for the structure of the aglycon moiety.

A plasmid for SsβG was obtained in house.[45, 46] N-His_6-tagged protein (MW: 58 kDa) was expressed efficiently in BL21(DE3) by auto-induction system and the target protein was purified with NiNTA column to afford pure SsβG (**Figure 4.21**).

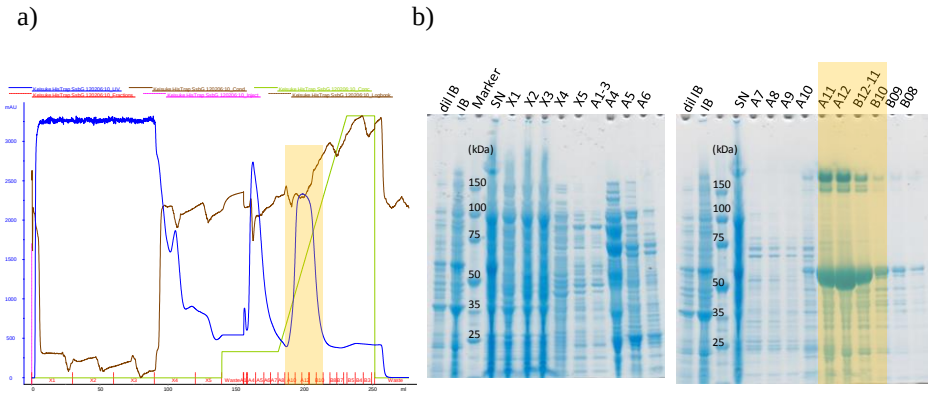


Figure 4.21. Purification of SsβG with NiNTA affinity column chromatography. (a) Chromatogram of UV trace and (b) SDS-PAGE analysis of the fractions. The fractions corresponding to the target protein is colored in yellow.

A plasmid for *Bt*Man2A[44] was a kind gift from Prof. Gideon J. Davies. C-His_6-tagged protein (MW: 98 kDa) was expressed efficiently in BL21(DE3) by auto-induction system and the target protein was purified with NiNTA column, followed by anion exchange column and gel filtration (**Figure 4.22**).

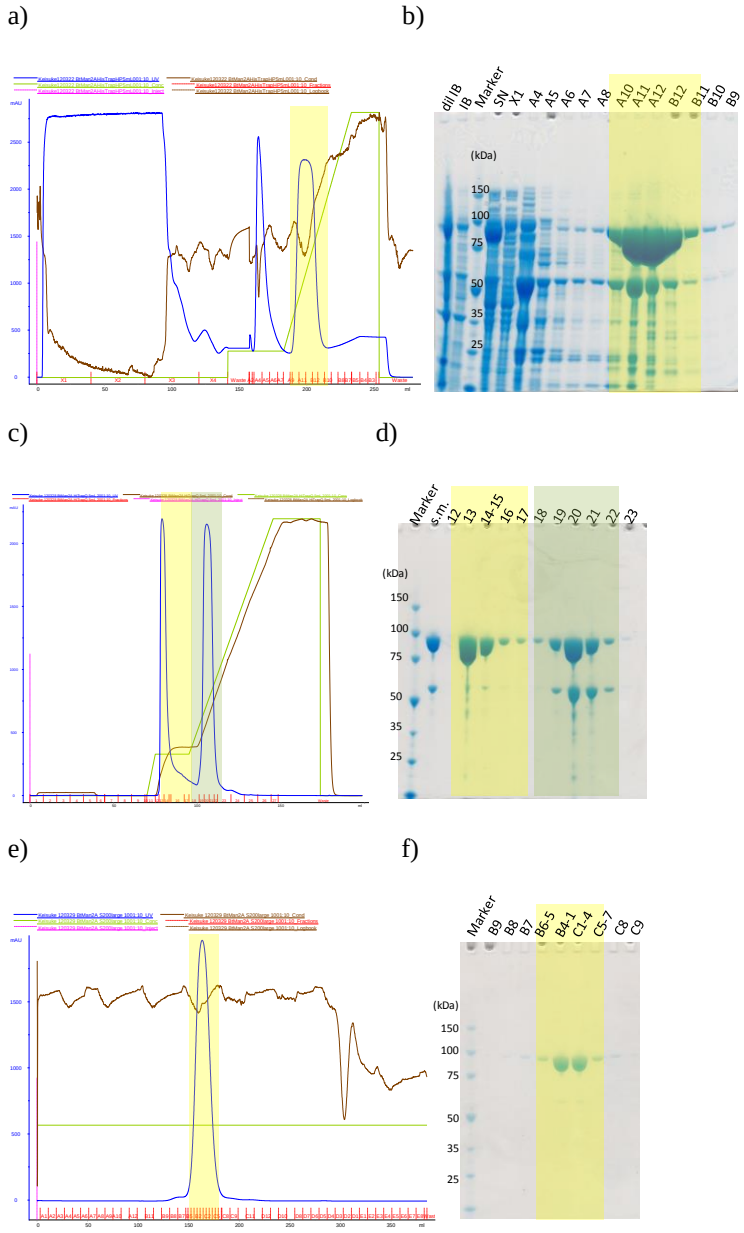


Figure 4.22. Purification of BtMan2A with NiNTA affinity column (a,b), anion exchange column (c,d) and gel filtration (e,f). (a,c,e) Chromatogram of UV trace and (b,d,f) SDS-PAGE analysis of the fractions. The fractions corresponding to the target protein is colored in yellow.

As well as these β-glycosidases, a commercially available β-mannosidase from *Helix pomatia* (MW: 94 kDa, Sigma-Aldrich) was tested to remove JBM-resistant factors. SDS-PAGE analysis of the reactions is shown in **Figure 4.23**. There was observed no significant band shift from JBM-treated CalB. For the detailed analysis of the glycan structures, MALDI-TOF-MS was also performed after releasing the glycans with PNGaseF (**Figure 4.24**).

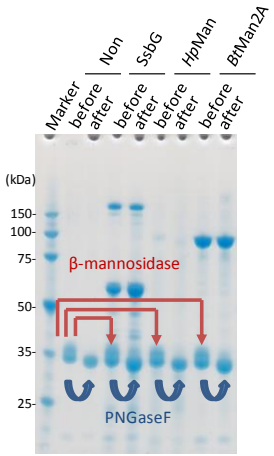


Figure 4.23. SDS-PAGE analysis of β-mannosidase (SsβG, *Hp*Man, *Bt*Man2A) treatment of JBM-treated CalB. Treatment with β-mannosidases is shown in red arrows. Release of glycans with PNGaseF is shown in blue arrows.

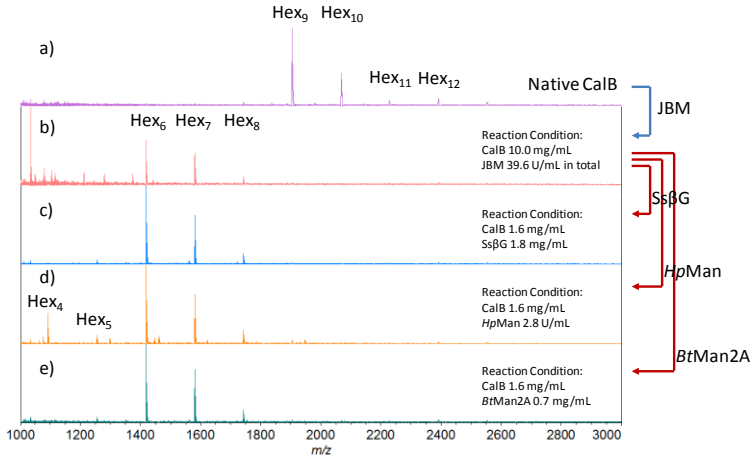


Figure 4.24. MALDI-TOF-MS spectra of the glycans released from native CalB (a) and JBM-treated CalB (b). JBM-treated CalB was further reacted with β-mannosidases, SsβG, *Hp*Man, *Bt*Man2A and the glycan profiles of the resultant glycoproteins are shown in the panel of c), d), e), respectively.

SsβG and *Bt*Man2A did not show sigfinicant change in glycan profiles. However, small amount of Hex_4 and Hex_5 peaks were newly observed when treated with *Hp*Man. The result of *Hp*Man was promising and implied that the JBM-resistant factor might be β-mannoside. However, the trimming of glycans was not complete and hence the enzyme cocktail of JBM and *Hp*Man was tested, expecting the synergistic trimming ability (**Figure 4.25**). However, though the trimming of glycan was observed, no significant improvement was observed.

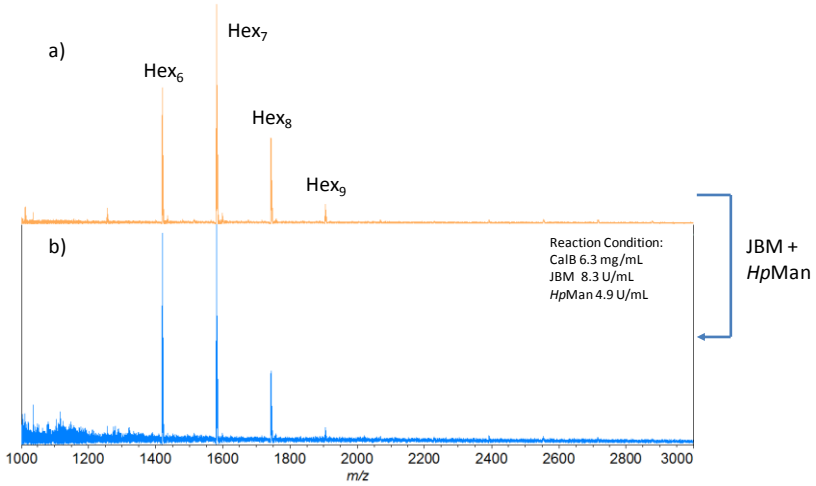


Figure 4.25. MALDI-TOF-MS spectra of the glycans released from JBM-treated CalB (a). JBM-treated CalB was further reacted with the enzyme cocktail of *Hp*Man and JBM and the glycan profile of the resultant CalB is shown in the panel of b).

4.2.2.4. Summary of trimming of glycans on the yeast-expressed glycoproteins

Glycan trimming of glycoprotein expressed in *Pichia pastoris* was performed using several exoglycosidases. Treatment with Jack Bean mannosidase (JBM) trimmed most part of the glycans down to $Man_1GlcNAc_2$ when CalB was used as a model glycoprotein. However, MALDI-TOF-MS analysis revealed the existence of JBM resistant glycan species in glycoproteins expressed in *Pichia pastoris*. To prepare homogeneous glycoprotein, search for enzymes which can remove these JBM-resistant factors was performed.

The first hypothesis was the remaining of glucose cap, which is used for quality control of glycoprotein folding. CalB was treated with MalA, an α-glucosidase to investigate this hypothesis. However, no indication of breakdown of JBM resistance was observed.

Next, the existence of β-mannosides on glycans was hypothesized. Indeed, it was recently reported that β-mannosides are found in the glycoproteins expressed in *Pichia pastoris* strain with the humanized glycosylation machinery.[42, 43] Glycosidases with β-mannosidase activity were expressed (SsβG, *Bt*Man2A) and CalB was treated with these enzymes along with the commercially available β-mannosidase, *Hp*Man. *Hp*Man showed a trend of further trimming, but its efficiency was not enough to get the glycan trimmed completely down to $Man_1GlcNAc_2$. The enzyme cocktail of *Hp*Man and JBM was also tested, but the synergistic effect was not observed.

Along with further search for enzymes to remove JBM resistant factors (now mainly on β-mannosidase), structural analysis of the JBM-resistant glycans is ongoing using MS/MS. Additionally, when enough amount of glycoprotein is available, isolation of glycans will be tried and their structural determination will be performed by ^{1}H NMR.

4.2.3. Chromatographic separation of JBM treated glycoprotein

As discussed in the last section, treatment of JBM results in two distinctive groups in term of their glycan structures; the one has $Man_1GlcNAc_2$, while the other has larger glycans, $Hex_{>5}GlcNAc_2$. If the former glycoform can be separated for the other, preparation of glycoprotein with a single glycoform is achieved. For this purpose, chromatographic separation of these two groups of glycoforms was tested.

In the first attempt, CalB was eluted on a MAPB (*m*-aminophenylboronic acid) column after treatment with JBM (**Figure 4.26**). The glycoprotein was bound to the column, but the separation was not realized and all the glycoforms were eluted out in the single peak.

Next, separation with ConA column was tested (**Figure 4.27**). Some of JBM-treated CalB was eluted out in the flowthrough while the other was bound. SDS-PAGE analysis showed slight difference of the molecular weight and the band width.

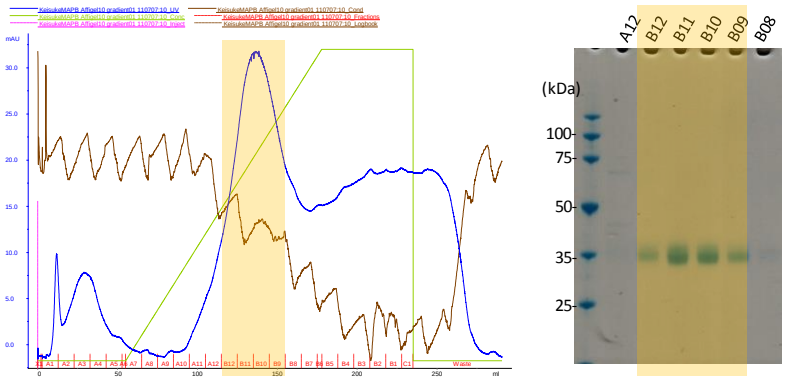


Figure 4.26. Attempt for separation of glycoforms with MAPB column. Left: Chromatogram of UV trace. Right: SDS-PAGE analysis of the fractions.

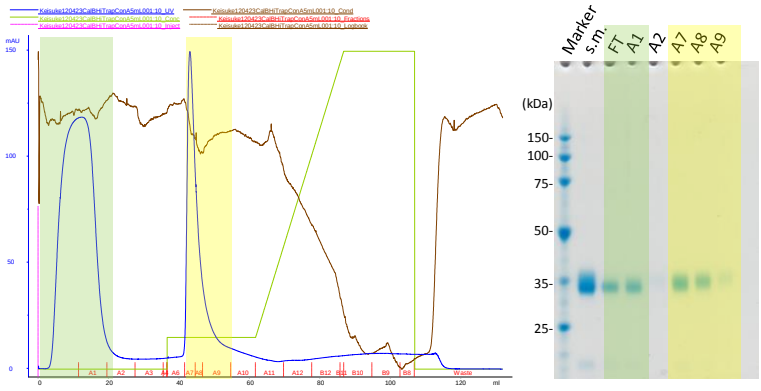


Figure 4.27. Attempt at separation of glycoforms with ConA column. Left: Chromatogram of UV trace. Right: SDS-PAGE analysis of the fractions. Two fractions (colored in green and yellow) were collected separately.

Flowthrough fractions showed smaller molecular weight and the sharper band, while the bound fraction showed the slightly heavier molecular weight and the broader band width. The collected fraction was subjected to MALDI-TOF-MS analysis after PNGaseF digestion (**Figure 4.28**). Gratifyingly, most of $Man_1GlcNAc_2$ glycan was eluted out in the flowthrough fraction along with small amount of $Hex_6GlcNAc_2$. To the contrary, in the bound fractions, only $Hex_{6-8}GlcNAc_2$ glycans were observed and no $Man_1GlcNAc_2$ was observed.

Further purification of the flowthrough fraction is yet to be done, but the present data show a promise for this method. When repeated chromatographic separation with a ConA column is performed glycoproteins with homogeneous glycoproteins might be achievable.

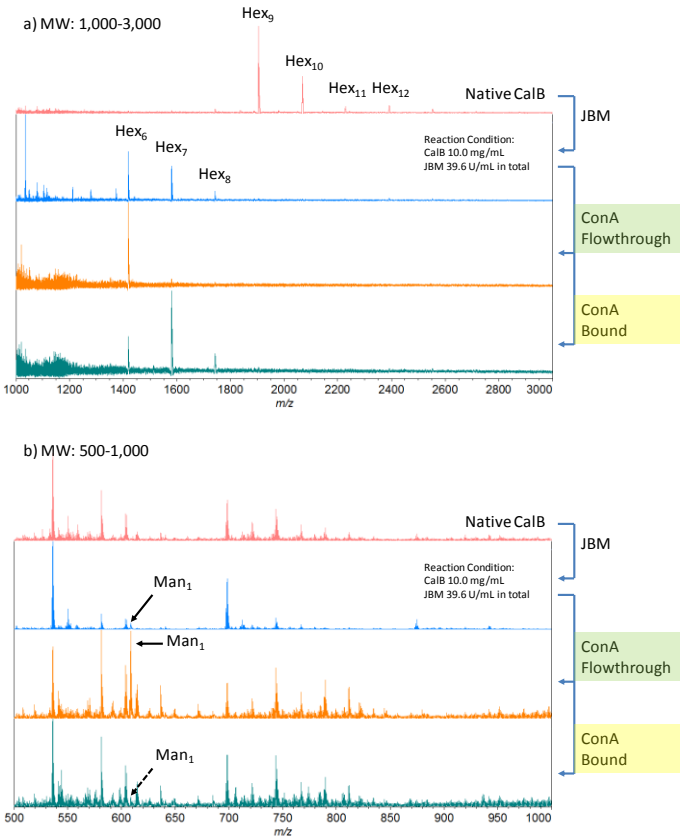


Figure 4.28. MALDI-TOF-MS spectra of the glycans released from the flowthrough fractions and the bound fractions after ConA column separation in the molecular weight range of (a) 1,000-3,000 and (b) 500-1,000.

4.3. Conclusion

In this chapter, several approaches to convert heterogeneous glycans on yeast-expressed glycoproteins into a homogeneous glycoform were investigated. For this purpose, enhancement of α-mannosidase activity of MalA was first attempted. Site-directed mutagenesis and saturation mutagenesis were performed around the catalytic active site. In the course of this investigation, the valid screening system for broadening enzymatic activity was established. Mutagenesis was performed at three sites, but so far a mutant with the desired acitivity has not been obtained. For further rational mutagenesis, X-ray crystallography of MalA with manno-configured ligands is ongoing.

Next, a yeast-expressed glycoprotein was treated with Jack bean mannosidase (JBM). This enzyme has broad substrate scope and is widely used for glycan sequencing. It was revealed that most of the *N*-glycans were trimmed down to $Man_1GlcNAc_2$ but some of the glycans have resistance to the JBM activity. Two hypotheses, the existence of i) glucose cap or ii) β-mannoside, were investigated using corresponding glycosidases. For the latter, a trend of trimming was found when a commercially available β-mannosidase, *Hp*Man was used. However, the trimming efficiency was not enough for complete trimming down to a homogeneous glycoform. Further search for enzymes to remove JBM-resistance is ongoing. At the same time, structural investigations for JBM resistance are planned using MS/MS or ^{1}H NMR.

Finally, separation of homogeneous glycoform, $Man_1GlcNAc_2$, was performed by a MAPB (*m*-aminophenylboronic acid) column and a ConA column. The latter could separate $Man_1GlcNAc_2$-protein from the higher molecular-weight glycoforms. The separation was not complete and in the flowthrough protein with $Hex_6GlcNAc_2$ was observed. Repeated

purification with a ConA column or other lectin columns might give complete separation.

Preparation of a completely pure glycoform has not yet been achieved. However, the results shown here will contribute to development of a general method to prepare homogeneous glycoproteins from yeast-expressed heterogeneous glycoforms.

4.4. Experimental

Site-directed mutagenesis of MalA at D320

Mutagenesis was performed using QuikChange II Site-Directed Mutagenesis kit or QuikChange II XL Site-Directed Mutagenesis kit (Stratagene). Primers purchased from Sigma-Aldrich were reconstructed to 1 μg/μL solution with water. The reagents were mixed as follows:

D320C/M

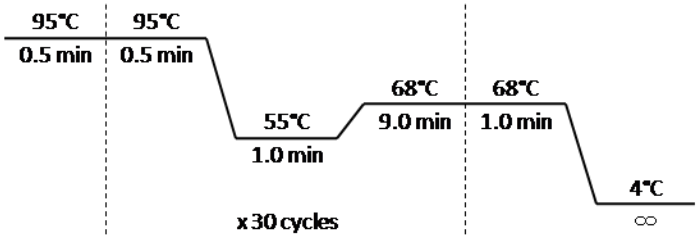
5 μL	10x reaction buffer
1 μL	pET-29a(+)-*malA*, 100 ng/μL
1.25 μL	forward primer, 0.1 μg/μL
1.25 μL	reverse primer, 0.1 μg/μL
1 μL	dNTP mix
39.5 μL	H_2O

D320H/E

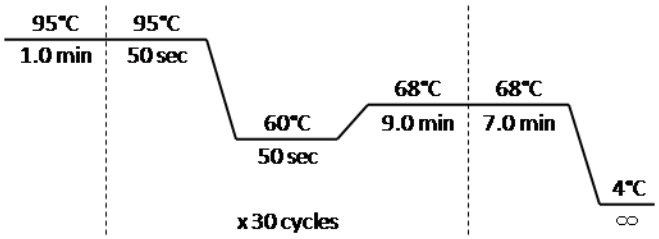
5 μL	10x reaction buffer
1 μL	pET-29a(+)-*malA*, 100 ng/μL
1.25 μL	forward primer, 0.1 μg/μL
1.25 μL	reverse primer, 0.1 μg/μL
1 μL	dNTP mix
3 μL	QuikSolution
37.5 μL	H_2O

Then 1 μL of *PfuUltra* HF DNA polymerase in the kit was added and the mixture was cycled using the following cycling parameters.

D320C/M



D320H/E



1 μL of *Dpn* I restriction emzyme (10 U/μL) was added and the solution was mixed by pipetting. The mixture was spun down by centrifugation, followed by incubation at 37°C for 1 hr. The resultant solution was stored at 4°C before use.

XL10-Gold ultracompetent cells in the kit were thawed on ice and 45 μL were dispensed for each transformation in prechilled conical Falcon tubes on ice. 2 μL of the β-ME mix in the kit was added. The cells were incubated on ice for 10 min with occasional swirling. 2 μL of the *Dpn* I-treated solution was added to the cell suspension, followed by incubation on ice for 30 min. Heat-shock was applied to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 0.5 mL of preheated NZY^+ medium (or SOC medium) was added, followed by incubation at 37°C for 1 hr with shaking at 250 rpm. 250 μL of the transformation reaction was spread on LB-Kanamycin agar. The plate was incubated at 37°C for 16-20 hr and stored at 4°C before use.

Purification of plasmid DNA was performed as General Procedure and the extract of the plasmid DNA (termed as pET-29a(+)-*malA:D320X* where X= C, M, H, E) was stored at -20°C. The plasmid DNA solution was diluted to 100 ng/μL with water and submitted to Geneservice (now known as SourceBioScience) for DNA sequencing using T7 promoter primer. The chromatography data were visualized using Finch TV program.

Validation of the screening system using D320 mutants

BL21(DE3) competent cells in Singles kit (Novagen) were thawed on ice and 1 μL of a mutated plasmid DNA (pET-29a(+)-*malA:D320X*, where X= C, M, H, E), pET-29a(+)-*malA* or pET-29a(+) was added. The suspsension was mixed by pipetting and incubated on ice for 5 min. Heat-shock was applied to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 250 μL of SOC medium was added and the resultant suspension was incubated at 37°C for 1 hr with shaking. On a LB-Kan agar plate was loaded 40 μL of SOC medium, to which 10 μL of the transformed cell suspension was added. After the cells were spread on the agar plate, it

was incubated at 37°C for 20 hr and stored at 4°C before use.

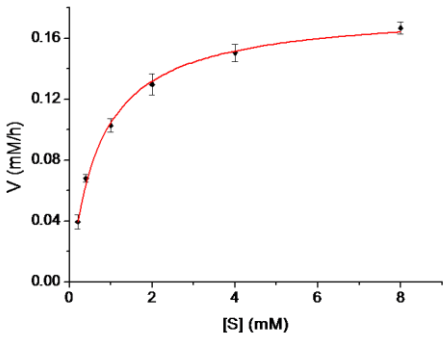
600 μL of ZYM-505-Kan medium was dispensed to each well of a 96 deep-well plate. A single colony (x 4 for each mutant) was inoculated to each well and the plate was covered with a gas permeable film and a lid. The plate was incubated at 28°C for 18 hr with shaking at 300 rpm and 10 μL of this preculture was mixed with 600 μL of ZYM-5052-Kan medium dispensed in 24-well plates, which were covered with gas permeable films and lids. The plates were incubated at 28°C for 21-24 hr with shaking at 250-300 rpm. The cell suspensions were transferred to 1.5-mL microcentrifuge tubes and the cell pellets were collected by centrifugation (13 krpm, 20 min, 4°C). The cell pellets were stored at -20°C if needed. The cell pellets were suspended in 100 μL of BugBuster (reconstructed with 100 mM NaPi, pH 6), and the suspensions were vortexed at RT for 30 min. The resultant mixtures were diluted with 200 μL of 100 mM NaPi (pH 6.0) and heated at 70°C for 30 min. After centrifugation (13 krpm, 20 min, 4°C), 250 μL of the supernatants were recovered. If necessary, another 10 μL of the supernatants were used for SDS-PAGE to confirm the expression of the target proteins.

50 μL of the protein solutions were mixed with 50 μL of 10 mM *p*NP-sugar (*p*NP-α-D-Glc, *p*NP -α-D-Man, *p*NP-α-D-Gal or *p*NP-α-D-Xyl) solutions in 100 mM NaPi (pH 6.0) in 96-well plates. The solution in each well was covered with 40 μL of silicone oil and the plates were covered with an adhesive film. The plates were incubated at 85°C for 16 hr. 40 μL of the reaction solutions were mixed with 200 μL of 100 mM NaPi (pH 9.0) and the absorbance at 410 nm of each well was measured using a platereader.

Michaelis-Menten analysis of MalA D320C

100 μL of *p*NP-α-D-Glc (0.2, 0.4, 1.0, 2.0, 4.0 and 8.0 mM, triplicate) in 100 mM sodium acetate buffer (pH 4.5) was preincubated at 85°C for 5 min. 5 μL of MalA D320C solution (1.39 mg/mL in 100 mM sodium phosphate buffer (pH 6.0)) was added to test solutions and the same amount of 100 mM sodium phosphate buffer (pH 6.0) to a control solution. The solutions were incubated at 85°C for 1 hr, followed by cooling on ice. After centrifugation, 20 μL of the solutions was mixed with 200 μL of 100 mM sodium phosphate buffer (pH 9.0) on a 96-well plate. The concentration of 4-nitrophenol was determined based on the calibration line, which was prepared measuring Abs410 of the mixtures of 20 μL of 4-nitrophenol solution (0.05, 0.10, 0.25, 0.50, 0.75 and 1.00 mM series) in 100 mM sodium acetate buffer (pH 4.5) and 200 μL of 100 mM sodium phosphate buffer (pH 9.0) on the same 96-well plate. These liberated 4-nitrophenol concentrations was divided by the reaction time (1hr) to determine the reaction rate at each substrate concentration. These reaction rates were plotted against the substrate concentration and the plots were fitted to the Michaelis-Menten equation in Origin8 to determine kinetic parameters: k_{cat} 0.061±0.002 s^{-1},

K_M 0.71±0.04 mM, k_{cat}/K_M 0.086 $s^{-1}mM^{-1}$.



Hydrolytic activity of MalA D320G toward *p*NP-α-D-Man in the presence of an external nucleophile

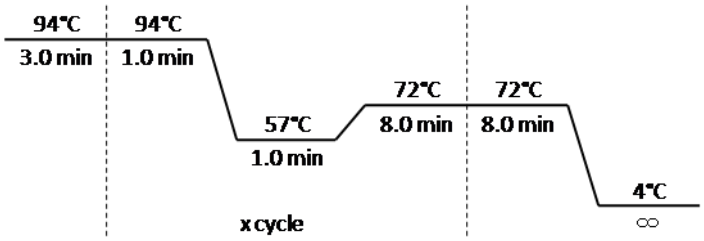
100 μL of 10 mM *p*NP-α-D-Man (triplicate) in the indicated buffer was preincubated at 85°C for 5 min. 5 μL of a MalA solution (1.30 mg/mL in 100 mM sodium phosphate buffer (pH 6.0)) was added to test solutions and the same amount of 100 mM sodium phosphate buffer (pH 6.0) to a control solution. The solutions were incubated at 85°C for 1 hr, followed by cooling on ice. After centrifugation, 20 μL of the solutions was mixed with 200 μL of 100 mM sodium phosphate buffer (pH 9.0) on a 96-well plate. The concentration of 4-nitrophenol was determined based on the calibration curve, which was prepared measuring Abs410 of the mixtures of 20 μL of 4-nitrophenol solution (0.000, 0.025, 0.050, 0.125, 0.250, 0.375 and 0.500 mM) in 100 mM sodium phosphate buffer (pH 6.0) and 200 μL of 100 mM sodium phosphate buffer (pH 9.0) on the same 96-well plate.

Saturation mutagensis

For the construction of the saturation mutagenesis library for W413 and H480, KOD Hot Start DNA polymerase Kit (Novagen) was used. The reagents in KOD Hot Start DNA polymerase Kit (Novagen) were mixed as follows:

Component	volume (μL)	Final concentration
H_2O	25	-
25 mM $MgSO_4$	3	1.5 mM
10 x buffer	5	1 x buffer
dNTPs (2 mM each)	5	0.2 mM each
the forward primer, 2.5 μM	5	0.25 μM
the reverse primer, 2.5 μM	5	0.25 μM
pET-29a(+)-malA, 100 ng/μL	1	2.0 ng/μL
KOD hot start DNA polymerase, 1U/μL	1	20 mM/μL

The mixture was cycled using the following cycling parameters with the cycle number of 15:



After PCR, 2.0 μL of *Dpn* I (20 U/μL) was added and the solution was incubated at 37°C for 90 min. Another 2.0 μL of *Dpn* I (20 U/μL) was added and the solution was incubated at 37°C for 90 min. 10 μL of the solution was used to analyze PCR products in an agarose gel electrophoresis and the rest was used for the further experiment.

XL10-Gold ultracompetent cells (QuikChange II XL Site-Directed Mutagenesis kit (Stratagene)) were thawed on ice and 45 μL was dispensed in a prechilled conical Falcon tube on ice. 2 μL of the β-ME mix in the kit was added. The cells were incubated on ice for 10 min with occasional swirling. 2 μL of the freshly prepared *Dpn* I-treated solution of PCR products was added to the cell suspension, followed by incubation on ice for 30 min. Heat-shock was applied to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 0.3 mL of preheated SOC medium was added, followed by incubation at 37°C for 1 hr with shaking at 250 rpm. 200 μL of the transformation reaction was added to 5 mL of LB-Kan medium and the suspension was incubated at 37°C for 18 hr.

Purification of plasmid DNA was performed as General Procedure and the extract of the plasmid DNA (termed as pET-29a(+)-*malA:W413* or pET-29a(+)-*malA:H480*) was stored at -20°C. The plasmid DNA solution was diluted to 100 ng/μL with water and submitted to Geneservice (now known as SourceBioScience) for DNA sequencing using the following primer, which corresponds to 1082-1099 bases in *malA* gene:

5'-TGAGGGGAAAGAGGGTTA-3'

The chromatography results were visualized using Finch TV program.

Screening Assay for W413 mutants and H480 mutants

BL21(DE3) competent cells in Singles kit (Novagen) was thawed on ice and 1 μL of a saturated mutagenesis library plasmid DNA, pET-29a(+)-*malA* or pET-29a(+) was added. The suspsension was mixed by pipetting and incubated on ice for 5 min. Heat-shock was applied to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 250 μL of SOC medium was added and the resultant suspension was incubated at 37°C for 1 hr with shaking. On a LB-Kan agar plate was loaded 40 μL of SOC medium, to which 10 μL of the transformed cell suspension was added. After the cells were spread on the agar plate, it was incubated at 37°C for 20 hr and

stored at 4°C before use.

600 μL of ZYM-505-Kan medium was dispensed to each well of two 96 deep-well plates. A single colony (x 2 for pET-29a(+)-*malA*- and pET-29a(+)-transformants) was inoculated to each well and the plate was covered with a gas permeable film and a lid. The plate was incubated at 28°C for 18 hr with shaking at 300 rpm and 10 μL of this preculture was mixed with 600 μL of ZYM-5052-Kan medium dispensed in 24-well plates, which were covered with gas permeable films and lids. The plates were incubated at 28°C for 21-24 hr with shaking at 250-300 rpm. The cell suspensions were transferred to 1.5-mL microcentrifuge tubes and the cell pellets were collected by centrifugation (13,000 rpm, 20 min, 4°C). The cell pellets were stored at -20°C if needed. The cell pellets were suspended in 100 μL of BugBuster (reconstructed with 100 mM NaPi, pH 6), and the suspensions were vortexed at RT for 30 min. The resultant mixtures were diluted with 200 μL of 100 mM NaPi (pH 6.0) and heated at 70°C for 30 min. After centrifugation (13 krpm, 20 min, 4°C), 250 μL of the supernatants were recovered. If necessary, another 10 μL of the supernatants were used for SDS-PAGE to confirm the expression of the target proteins.

50 μL of the protein solutions were mixed with 50 μL of 10 mM *p*NP-sugar (*p*NP-α-D-Glc, *p*NP-α-D-Man, *p*NP-α-D-Gal or *p*NP-α-D-Xyl) solutions in 100 mM NaPi (pH 6.0) in 96-well plates. The solution in each well was covered with 40 μL of silicone oil and the plates were covered with an adhesive film. The plates were incubated at 85°C for 16 hr. 40 μL of the reaction solutions were mixed with 200 μL of 100 mM NaPi (pH 9.0) and the absorbance at 410 nm of each well was measured using a platereader.

After the analysis of these data, 10 μL of the selected mutants' precultures in ZYM-505-Kan medium were mixed with 5 mL of LB-Kan medium to grow these transfromants. After incubation for 16 hr at 37°C, purification of plasmid DNA was performed as General Procedure and the extract of the plasmid DNA was stored at -20°C. The plasmid DNA solution was diluted to 100 ng/μL with water and submitted to Geneservice (now known as SourceBioScience) for DNA sequencing using the following primer, which corresponds to 1082-1099 bases in *malA* gene:

5'-TGAGGGGAAAGAGGGTTA-3'

The chromatography results were visualized using Finch TV program.

Trimming of glycans on CalB

Effect of a(1,6)-mannosidase

61 μL of 16.3 mg/mL CalB solution in 100 mM NaOAc (pH 4.5) and 10 μL of 100 mM $ZnCl_2$ in 100 mM NaOAc (pH 4.5) were mixed. For a control reaction, 29 μL of 100 mM NaOAc (pH 4.5) was added for the control reaction. For test 11 and test 12, 24 μL of 100 mM NaOAc (pH 4.5) and 5 μL of JBM (136U/mL, Sigma-Aldrich) were added. For test21 and test22, 19 μL of 100 mM

NaOAc (pH 4.5), 5 μL of JBM (136U/mL, Sigma-Aldrich) and 5 μL of α(1,6)-Man (2kU/mL, NEB Lab) were added.
The reaction solutions were incubated at 37°C for 24 hr. Test 11 and test21 solutions were frozen at this point. Another 5 μL of JBM was added to test 12 and another 5 μL of JBM and 5 μL of α(1,6)-Man were added to test 22. These solutions were further incubated at 37°C for 24 hr.
After the reaction, 10 μL of the resultant solutions were sampled for SDS-PAGE and the rest of the solutions were digested with PNGaseF (see General Procedure) and the liberated glycans were analyzed by MALDI-TOF-MS (see General Procedure).

Longer reaction time for trimming with JBM
60 μL of 16.6 mg/mL CalB solution in 100 mM NaOAc (pH 4.5) and 10 μL of 100 mM $ZnCl_2$ in 100 mM NaOAc (pH 4.5) and 20 μL of 100 mM NaOAc (pH 4.5) were mixed. For test reactions, 10 μL of JBM (136U/mL, a suspension in 3.0M $(NH_4)_2SO_4$, 0.1 mM $Zn(OAc)_2$, pH 7.5 Sigma-Aldrich) was added and for a control reaction 10 μL of 3.0M $(NH_4)_2SO_4$ was added. After incubation at 37°C for 24 hr, 20 μL of 3.0M $(NH_4)_2SO_4$ was added to test01, which was stored at 4°C till analysis. Another 10 μL of 3.0M $(NH_4)_2SO_4$ or JBM was added to the control reaction or the other test reactions, respectively. After incubation at 37°C for 24 hr, 10 μL of 3.0M $(NH_4)_2SO_4$ was added to test02, which was stored at 4°C till analysis. Another 10 μL of 3.0M $(NH_4)_2SO_4$ or JBM was added to the control reaction or the other test reaction, respectively, followed by incubation at 37°C for 24 hr.
After the reaction, 10 μL of the resultant solutions were sampled for SDS-PAGE and the rest of the solutions were digested with PNGaseF (see General Procedure) and the liberated glycans were analyzed by MALDI-TOF-MS (see General Procedure).

Preparation of JBM-trimmed CalB
775 μL of 12.9 mg/mL CalB (in 100 mM NaOAc, pH 4.5) was mixed with 100 μL of 100 mL $ZnCl_2$ (in 100 mM NaOAc, pH 4.5), 100 μL of 132 U/mL JBM and 25 μL of 100 mM NaOAc (pH 4.5). The reaction was incubated at 37 °C for 24 hr. The other portion of 132 U/mL JBM (100 μL) was added and the reaction was incubated at 37 °C for 24 hr. After filtration with 0.2-μm membrane the filtrate was applied to FPLC with the conditions as follows:

Column: HiLoad Superdex 75 pg 16/60 (GE Healthcare)
Buffer: 100 mM sodium acetate, 1 mM $ZnCl_2$, 200 mM NaCl (pH 4.5 at RT)
Flow rate: 1.0 mL/min (Maximum flow rate; Pressure regulation <0.40MPa)
Sample volume: 1 mL

The fractions containing the target protein was collected after SDS-PAGE analysis and

concentrated to 0.4 mL with vivaspin 6 (MWCO 10,000). The concentration was determined to be 8.38 ± 0.67 mg/mL. The solution was stored at -20°C after snap freezing.

Treatment of JBM-trimmed CalB with MalA and JBM

80 µL of JBM-trimmed CalB was mixed with:

Rx1:20µL 13.9 mg/mL MalA in 100 mM NaPi (pH 6.0) and 8µL of 3M $(NH_4)_2SO_4$
Rx2: 2µL 13.9 mg/mL MalA in 100 mM NaPi (pH 6.0), 18µL of 100 mM NaPi (pH 6.0) and 8µL of 3M $(NH_4)_2SO_4$
Rx3: 20µL 13.9 mg/mL MalA in 100 mM NaPi (pH 6.0) and 8µL of 132 U/mL JBM in 3M $(NH_4)_2SO_4$
Rx4: 2µL 13.9 mg/mL MalA in 100 mM NaPi (pH 6.0), 18µL of 100 mM NaPi (pH 6.0) and 8µL of 132 U/mL JBM in 3M $(NH_4)_2SO_4$

The mixture was incubated at 37°C for 24 hr. The reaction mixture was diluted with 400µL of 100 mM NaOAc (pH 4.5), filtered with 0.2-µm membrane and applied to FPLC:

Column: HiLoad Superdex 200 pg 16/60 (GE Healthcare)
Buffer: 100 mM sodium acetate, 1 mM $ZnCl_2$, 200 mM NaCl (pH 4.5 at RT)
Flow rate: 1.0 mL/min (Maximum flow rate; Pressure regulation <0.40MPa)
Sample volume: 0.5 mL

The collected fractions were concentrated to 0.5 mL with vivaspin 20 (MWCO 10,000) and frozen and stored at -20°C till used. The resultant glycoprotein samples were digested with PNGaseF and the liberated glycans were analyzed by MALDI-TOF-MS (see General Procedure).

Expression and purification of SsβG

BL21(DE3) competent cells in Singles kit (Novagen) were thawed on ice and 1 µL of pET-24d(+)-*lacS* was added. The suspsension was mixed by pipetting and incubated on ice for 5 min. Heat-shock was applied to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 250 µL of SOC medium was added and the resultant suspension was incubated at 37°C for 1 hr with shaking. On a LB-Kan agar plate was loaded 40 µL of SOC medium, to which 10 µL of the transformed cell suspension was added. After the cells were spread on the agar plate, it was incubated at 37°C for 20 hr and stored at 4°C before use. A single colony was inoculated to 5 mL LB-Kan and the culture was incubated at 37°C for 13 hr with shaking at 200 rpm and 600 µL of this culture solution and 200 µL of 60% glycerol solution were mixed to

construct a glycerol stock of *E.coli* with pET-24d(+)-*lacS* plasmid.

A pipette scoop of the glycerol stock of BL21(DE3) was inoculated to 200 mL of LB-Kan and the culture was incubatied at 37°C for 17 hr with shaking at 200 rpm. 1 mL of this culture was added to 0.5 L (x2) of ZYM-5052-Kan medium, which was incubated at 28°C for 20 hr with shaking at 200 rpm. The culture was centrifuged (8,000 rpm, 4°C, 15 minutes) and the cell pellet was collected (16.7 g), snap-frozen with liquid nitrogen and stored at -20°C.

The cell pellet was resuspended in 80 mL of Buffer A (see below) containing 8 mg of lysozyme, 2 mg of DNase I and 1 tablet of Complete protease inhibitor cocktail (Roche), and the suspension was stirred at 4°C for 1 hr. The cells were further lysed by sonication (4 x 60 seconds, 1.0-sec pulse and 0.5-sec pause, one-minute intervals in between), and the cell debris was removed by centrifugation (20,000 rpm, 4°C, 20 minutes). The supernatant was filtered through 0.2-μm membrane. The resultant solution supernatant was applied to FPLC with the conditions as follows:

Column: HisTrap™ HP 5 mL (GE Healthcare)
Buffer A: 20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole (pH 7.8 at RT)
Buffer B: 20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole (pH 7.8 at RT)
Flow rate: 1.5 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Sample volume: 89 mL (flow rate: 1.0 mL/min)

Gradient:		
	0% Buffer B	10 CV
	10% Buffer B	5CV
	to 100% Buffer B	10CV
	100% Buffer B	3CV

The fractions containing the target protein was collected after SDS-PAGE analysis and dialyzed against 50 mM NaPi (pH 7.0, 2 L x 2). The recovered solution was filtered through 0.2-μm syringe filter. The concentration was determined to be 3.85 ± 0.10 mg/mL (20 mL, 77 mg protein/ L culture). The solution was stored at -20°C after snap freezing.

Expression and purification of *Bt*Man2A

BL21(DE3) competent cells in Singles kit (Novagen) were thawed on ice and 1 μL of pLT001 (pET-28a(+) with an insert of a gene for mature *Bt*Man2A between NdeI and XhoI restriction sites)[44] was added. The suspsension was mixed by pipetting and incubated on ice for 5 min. Heat-shock was applied to the cells in a 42°C water bath for 30 sec, followed by incubation on ice for 2 min. 250 μL of SOC medium was added and the resultant suspension was incubated at 37°C for 1 hr with shaking. On a LB-Kan agar plate was loaded 40 μL of SOC medium, to which 10 μL

of the transformed cell suspension was added. After the cells were spread on the agar plate, it was incubated at 37°C for 20 hr and stored at 4°C before use.

A single colony was inoculated to 100 mL LB-Kan and the culture was incubated at 37°C for 8 hr with shaking at 200 rpm. 5 mL of the culture was added to 0.5 L (x4) of ZYM-5052-Kan medium, which was incubated at 28°C for 18 hr with shaking at 200 rpm. The culture was centrifuged (8,000 rpm, 4°C, 15 minutes) and the cell pellet was collected (29.6 g), snap-frozen with liquid nitrogen and stored at -20°C.

17.7 g of the cell pellet was resuspended in 180 mL of Buffer A (see below) containing 18 mg of lysozyme, 4.5 mg of DNase I and 1 tablet of Complete protease inhibitor cocktail, and the suspension was stirred at 4°C for 1 hr. The cells were further lysed by sonication (6 x 60 seconds, 1.0-sec pulse and 0.5-sec pause, one-minute intervals in between), and the cell debris was removed by centrifugation (20,000 rpm, 4°C, 30 minutes). The supernatant was filtered through 0.2-μm membrane. The resultant solution supernatant was applied to FPLC with the conditions as follows:

Column: HisTrap™ HP 5 mL (GE Healthcare)
Buffer A: 20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole (pH 7.8 at RT)
Buffer B: 20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole (pH 7.8 at RT)
Flow rate: 1.5 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Sample volume: 80 mL x 2 (flow rate: 1.0 mL/min)

Gradient:		
	0% Buffer B	10 CV
	10% Buffer B	5CV
	to 100% Buffer B	10CV
	100% Eluent B	3CV

The fractions containing the target protein was collected after SDS-PAGE analysis and dialyzed against 20 mM ethanolamine-HCl (pH 7.2 at RT, 2 L x 2). The recovered solution was filtered through 0.2-μm syringe filter and applied to FPLC with the conditions as follows:

Column: HiTrapQ™ HP 5 mL (GE Healthcare)
Buffer A: 20 mM ethanolamine-HCl (pH 7.2 at RT)
Buffer B: 20 mM ethanolamine-HCl, 1M NaCl (pH 7.2 at RT)
Flow rate: 1.5 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Sample volume: 25 mL x 2 (flow rate: 1.0 mL/min)

Gradient:		
	0% Buffer B	10 CV
	15% Buffer B	5CV
	to 100% Buffer B	10CV

100% Eluent B 5CV

The fractions containing the target protein was collected after SDS-PAGE analysis and concentrated to 3 mL with Millipore concentrating filter unit (MWCO 30,000). The solution was filtered through 0.2-μm syringe filter and applied to FPLC with the conditions as follows:

Column: HiLoad Superdex 200 pg 26/60 (GE Healthcare)
Buffer: 50 mM sodium citrate, 200 mM NaCl (pH 5.6 at RT)
Flow rate: 2.5 mL/min (Maximum flow rate; Pressure regulation <0.40MPa)
Sample volume: 3 mL

The fractions containing the target protein was collected after SDS-PAGE analysis and concentrated to 3 mL with Millipore concentrating filter unit (MWCO 30,000). The concentration was determined to be 8.63 ± 0.16 mg/mL (3.8 mL, 32.8 mg protein/ L culture). The solution was stored at -20°C after snap freezing.

Preparation of JBM-trimmed CalB

775 μL of 12.9 mg/mL CalB (in 100 mM NaOAc, pH 4.5) was mixed with 100 μL of 100 mL $ZnCl_2$ (in 100 mM NaOAc, pH 4.5), 100 μL of 132 U/mL JBM and 25 μL of 100 mM NaOAc (pH 4.5). The reaction was incubated at 37 °C for 24 hr and other two portions of 100 μL of 132 U/mL JBM were added with 1-day intervals. The reaction was checked by SDS-PAGE. After filtration with 0.2-μm membrane the filtrate was applied to FPLC with the conditions as follows:

Column: HiLoad Superdex 75 pg 16/60 (GE Healthcare)
Buffer: 100 mM sodium acetate, 1 mM $ZnCl_2$, 200 mM NaCl (pH 4.5 at RT)
Flow rate: 1.0 mL/min (Maximum flow rate; Pressure regulation <0.40MPa)
Sample volume: 1 mL

The fractions containing the target protein was collected after SDS-PAGE analysis and concentrated to 0.5 mL with vivaspin 6 (MWCO 10,000). The concentration was determined to be 5.63 ± 0.35 mg/mL. The solution was stored at -20°C after snap freezing.

Treatment of JBM-trimmed CalB with β-mannosidases

100 μL of JBM-trimmed CalB was dialyzed against reaction buffers; 50 mM sodium phosphate (pH 7.0) for SsβG, 100 mM sodium acetate (pH 4.0) for *Hp*Man, and 50 mM sodium citrate (pH 5.6) for BtMan2A. After dilution of CalB with the reaction buffer to 300μL, each enzyme

solution was added; 50μL of SsβG (12.5 mg/mL) in 50 mM sodium phosphate (pH 7.0), 50μL of HpMan (19.7 U/mL) in 3.0 M ammonium sulfate and 10 mM sodium acetate (pH 4.0) and 50μL of BtMan2A (5.0 mg/mL) in 50 mM sodium citrate and 1M NaCl (pH 5.6). The reaction solutions were incubated for 24 hr at 37°C. The reaction mixture was subjected to PNGaseF digestion (General procedure) and the glycan profile was analyzed by MALDI-TOF-MS (General procedure).

Trimming CalB with the enzyme cocktail of JBM and *Hp*Man

775 μL of 12.9 mg/mL CalB (in 100 mM NaOAc, pH 4.5) was mixed with 100 μL of 100 mL $ZnCl_2$ (in 100 mM NaOAc, pH 4.5), 100 μL of 132 U/mL JBM and 25 μL of 100 mM NaOAc (pH 4.5). The other portions of 100 μL of 132 U/mL JBM were added after 1-day interval and the reaction was incubated for 1 more day at 37°C. 110 μL of the reaction was taken up and 10 μL of JBM and 40 μL of *Hp*Man was added and the mixture was incubated for 24 hr at 37°C. The reaction was diluted with 400 μL of 100 mM sodium acetate (pH 4.5). After filtration with 0.2-μm membrane the filtrate was applied to FPLC with the conditions as follows:

Column: HiLoad Superdex 75 pg 16/60 (GE Healthcare)
Buffer: 100 mM sodium acetate, 1 mM $ZnCl_2$, 200 mM NaCl (pH 4.5 at RT)
Flow rate: 1.0 mL/min (Maximum flow rate; Pressure regulation <0.40MPa)
Sample volume: 0.5 mL

The fractions containing the target protein was collected after SDS-PAGE analysis and concentrated to 0.2 mL with vivaspin 6 (MWCO 10,000). The resultant glycoprotein solution was subjected to PNGaseF digestion (General procedure) and the glycan profile was analyzed by MALDI-TOF-MS (General procedure). When the sample needs to be purified, HyperSep C18 100 mg SPE column (Thermo Scientific) was used. The column was washed with 2.5 mL of methanol and 5 mL of 5% AcOH. After the sample was applied, glycans were eluted with 1.5 mL of 5% AcOH and the collected fractions were lyophilized.

Separation of JBM-trimmed CalB with Boronic acid column

368 μL of 13.6 mg/mL CalB (in 100 mM NaOAc, pH 4.5) was mixed with 100 μL of 100 mL $ZnCl_2$ (in 100 mM NaOAc, pH 4.5), 50 μL of 132 U/mL JBM and 482 μL of 100 mM NaOAc (pH 4.5). The reaction was incubated at 37 °C for 24 hr and other two portions of 50 μL of 132 U/mL JBM were added with 1-day intervals. The reaction was checked by SDS-PAGE. The reaction was dialyzed against Buffer A (see below). After filtration with 0.2-μm membrane the filtrate was applied to FPLC with the conditions as follows:

Column: MAPB (*m*-aminophenylboronic acid) column (CV 25 mL)
Eluent A: 50 mM NaPi, 0.5M NaCl (pH 7.0)
Eluent B: 50 mM NaPi, 0.5M NaCl, 0.2M D-sorbitol (pH 7.0)
Flow rate: 1.0 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Sample volume: 1 mL

Gradient:		
	Eluent A	2CV
	to 100% Eluent B	5CV
	100% Eluent B	2CV
	100% Eluent A	2CV

Fractions were analyzed by SDS-PAGE after concentration with vivaspin (MWCO 10,000).

Separation of JBM-trimmed CalB with HiTrap ConA

775 µL of 12.9 mg/mL CalB (in 100 mM NaOAc, pH 4.5) was mixed with 100 µL of 100 mL $ZnCl_2$ (in 100 mM NaOAc, pH 4.5), 100 µL of 132 U/mL JBM and 25 µL of 100 mM NaOAc (pH 4.5). The reaction was incubated at 37 °C for 24 hr and other two portions of 100 µL of 132 U/mL JBM were added with 1-day intervals. The reaction was checked by SDS-PAGE. After filtration with 0.2-µm membrane the filtrate was applied to FPLC with the condition as follows:

Column: HiLoad Superdex 75 pg 16/60 (GE Healthcare)
Buffer: 20 mM Tris-HCl, 0.5M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ (pH 7.4 at RT)
Flow rate: 1.0 mL/min (Maximum flow rate; Pressure regulation <0.40MPa)
Sample volume: 1 mL

The fractions containing the target protein was collected after SDS-PAGE analysis. After filtration with 0.2-µm membrane the collected fraction was applied to FPLC with the conditions as follows:

Column: HiTrap ConA 5 mL (GE Healthcare)
Buffer A: 20 mM Tris-HCl, 0.5M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$ (pH 7.4 at RT)
Buffer B: 20 mM Tris-HCl, 0.5M NaCl, 0.3M methyl α-D-glucoside (pH 7.4 at RT)
Flow rate: 1.0 mL/min (Maximum flow rate; Pressure regulation <0.30MPa)
Sample volume: 10 mL (flow rate: 0.5 mL/min)

Gradient:		
	0% Buffer B	10 CV
	10% Buffer B	5CV
	to 100% Buffer B	5CV
	100% Eluent B	3CV

Two peaks were observed in the chromatogram and these fractions were collected separately. Both front and rear peaks were concentrated to 0.5 mL with vivaspin 6 (MWCO 10,000), affording the protein solution of 1.38 ± 0.04 mg/mL and 0.41 ± 0.05 mg/mL, respectively. These glycoprotein solutions were subjected to PNGaseF digestion (General procedure) and the glycan profiles were analyzed by MALDI-TOF-MS (General procedure).

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Appendix

General Considerations 351
General Procedure 353
Licenses for adaptation from publications 360
List of Publications 369
Acknowledgment 371

General Considerations

All chemical reagents were purchased from Sigma-Aldrich, Acros, Fluka, Carbosynth, Apollo Scientific or Fisher Scientific. Anhydrous solvents were purchased from Fluka or Acros with the exception of dichloromethane and THF which were dried in an alumina column under nitrogen. All other solvents were used as supplied (Analytical or HPLC grade) without further purification. Petroleum ether refers to the fraction of light petroleum ether boiling in the range 40-60°C. All commercial enzymes were purchased from Sigma-Aldrich, New England Biolab, Novagen, Agilent Technologies or GE healthcare. All competent cells were purchased from Novagen or Agilent Technologies. Deionized water was used for chemical syntheses and preparation of buffers. Milli-Q purified water was used for culture media and preparation of glycan samples for MALDI-TOF-MS. All growth media and applicable equipment was autoclaved at 121 ^{o}C for 20 minutes. For procedures requiring a sterile atmosphere a Heraeus HERAsafe sterile cabinet was used.

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker AV400 (400 MHz), a Bruker AVIII400 (400 MHz), a Bruker DRX500 (500 MHz), a Bruker AVII500 (500 Hz), or a Bruker AVIII700 (700 Hz) spectrometer. Carbon nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker AV400 (101 MHz), a Bruker AVIII400 (101 MHz), a Bruker DRX500 (126 MHz), a Bruker AVII500 (126 Hz), or a Bruker AVIII700 (176 Hz) spectrometer. Peaks in NMR spectra were assigned using COSY, HSQC, HMBC, TOCSY and HSQC-TOCSY. Chemical shifts are quoted on the δ scale in ppm using residual solvent as the internal standard (^{1}H NMR: C*H*Cl$_3$, 7.27; C*H$_3$*OD, 3.31; (C*H$_3$*)$_2$SO, 2.50 and ^{13}C NMR: *C*HCl$_3$, 77.0; *C*H$_3$OD, 49.15; (*C*H$_3$)$_2$SO, 39.51) or acetone (^{1}H NMR: (C*H$_3$*)$_2$CO, 2.22 and ^{13}C NMR: (*C*H$_3$)$_2$CO; 30.89) in D$_2$O. Coupling constants (*J*) are reported in Hz with the following splitting abbreviations: s, singlet; d, doublet; t, triplet, q, quartet; quin, quintet; m, multiplet.

For small molecules, low resolution mass (LRMS) spectra were recorded on a Waters Micromass LCT Premier TOF spectrometer using electrospray ionization. High resolution mass (HRMS) spectra were recorded on a Bruker MicroTOF ESI mass spectrometer. Nominal and exact m/z values are reported in Daltons. For proteins, liquid chromatography-mass spectrometry (LC-MS) was performed on a Micromass LCT (ESI-TOF-MS) coupled with an Agilent 1100 series HPLC.

MALDI-TOF-MS analysis for glycan samples was conducted on a Shimazu AXIMA TOF2 MALDI TOF/TOF mass spectrometer in positive reflection mode.

Infrared (IR) spectra were recorded on a Bruker Tensor 27 Fourier Transform spectrometer using neat materials placed on the lens. Absorption maxima (ν_{max}) are reported in wavenumbers (cm^{-1}).

Melting points were recorded on a Leica Galden III hot stage microscope equipped with a Testo 720 thermocouple probe.

Optical rotations were measured on a Perkin-Elmer 341 polarimeter with a path length of 1.0 dm at 20°C and reported with implied units of 10^{-1} deg cm^2 g^{-1}. Concentrations (c) are given in g/100 mL.

Thin layer chromatography (TLC) was carried out using Merk Kieselgel $60F_{254}$ 0.2 mm precoated aluminium backed plates. Visualization of the silica plates was performed using a UV lamp (λ_{max}= 254 nm) and/or 5% sulfonic acid and/or ammonium molybdate (5% in 2M sulfonic acid). Flash column chromatography was performed using Fluka Kiegselgel 60 220-440 mesh silica gel Merck Geduran Si 60 (40-63 um) silica gel.

High performance liquid chromatography was performed using UltiMate 3000 Basic LC system (Dionex) equipped with Ultimate 3000 Pump and Dionex Ultimate 3000 Variable Wavelength Detector. PL-ELS 2100 ICE (Polymer laboratories) was also used for evaporative light scattering detection. Fast protein liquid chromatography (FPLC) was performed using ÄKTA FPLC system equipped with P-920 (pump), UPC0900 (UV detector) and Frac-950 (fraction collector).

Reading of multi-well plates was performed using SpectraMax Plus (Molecular Devices) or SPECTROstar OMEGA (BMG Labtech).

Gel electrophoresis was carried out using the Invitrogen system (XCell SureLock Mini-Cell, NuPAGE gels, NuPAGE running buffers). Protein molecular weights were approximated by comparison to a protein marker Perfect Protein Markers 15-150 kDa (Novagen). Gels were visualised by Instant Blue (Expedeon).

Concentrations of plasmid DNA samples were determined using NanoDrop ND-1000. Concentrations of protein samples was determined using the BCA (Bicinchoninic Acid) assay or NanoDrop ND-1000.

General Procedure

Purification of plasmid DNA

Purification of plasmid DNA was performed using QIA Spin Miniprep Kit. A transformant of interest was grown in 5 mL of LB medium with proper antibiotics by incubating at 37°C for 6 hr. After centrifugation (3750 rpm, 5 min, 4°C), the supernatant was discarded. The remaining cell pellet was resuspended in 250 μL of Buffer P1 and transferred to a microcentrigure tube, followed by addition of 250 μL of Buffer P2 and 350 μL of Buffer N3, mixed well after each addition. Formed precipitation was removed by centrifugation (13 krpm, 10 min, 4°C) and 700 μL of the supernatant was applied to the QIAprep spin column. Plasmid DNA was adsorbed on the column by centrifugation (13 krpm, 1 min, 4°C) and the flow-through was discarded. 750 μL of Buffer PE was loaded on the column, followed by centrifugation (13 krpm, 1 min, 4°C) to wash the column. After the flow-through was discarded, the column was further centrifuged (13 krpm, 1 min, 4°C) to remove residual washing buffer. The column was dried at room temperature for 5 min and put on a 1.5 mL-microcentrifuge tube. 40 μL of water was loaded and adsorbed on the column standing it for 5 min. The tube was centrifuged (13 krpm, 1 min, 4°C) and the extract of plasmid DNA was stored at -20°C.

BCA assay

The protein samples were diluted in the range of 0.1-1.0 mg/mL and the standard solutions of bovine serum albumin (BSA) were prepared with more than 6 different concentrations in the same buffer. Pierce BCA Potein Assay Reagent A and B were purchased from Thermo Scientific. These reagents were mixed in the ratio of 50:1 (A: B) before use. 200 μL of this BCA reagent was mixed with 10 μL of the protein samples on a 96-well plate and the plate was incubated at 37°C for 30 min. The absorption at 562 nm were measured using a platereader. The concentrations of the protein samples were determined using a calibration line obtained from the BSA standards.

Mass spectrometry of proteins

MS of proteins was measured using a Phenomenex Jupiter 5 μm C4 column (250 x 4.6 mm). The instrumental settings were as follows:

MS setting:

Capillary voltage: 3000V

Sample cone voltage: 25 V

Extraction cone voltage: 3V

RF lens: 250
Desolvation temperature: 250°C
Source temperature: 80°C
Desolvation flow (N_2): 600 L/h
Nebulizer : 100 L/h

LC setting:
Injection volume: 20 μL for MalA WT and MalA D320G, 5 μL for myoglobin (for calibration)
Solvent A: Water: acetonitrile 95: 5 with 0.5% formic acid
Solvent B: Acetonitrile with 0.5% formic acid
Flow rate: 1.0 mL/min

Gradient:	5% Solvent B	0-5 min
	to 95% Solvent B	5-20 min
	95% Solvent B	20-25 min
	to 5% Solvent B	25-30 min
	5% Solvent B	30-35 min

Spectra were calibrated using a calibration curve constructed from a minimum of 17 matched peaks from the multiply charged ion series of equine myoglobin (5 μL, 1.0 mg/mL solution). Total mass spectra were reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.1 from Waters) according to manufacturer's instructions.

Preparation of glycan samples by PNGaseF digestion

Glycoprotein solutions were diluted to less than 2.0 mg/mL with 50 mM Tris-HCl (pH 8.0). To this solution was added the same quantity of the denaturation solution (0.5M Tris-HCl (pH 8.0), 6M GnHCl, 10 mM EDTA, 40 mM DTT) and the mixture was incubated at 50°C for 4 hr. 0.5M iodoacetic acid in 0.5M NaOH (2.0 equivalents to DTT) was added and the solution was incubated at room temperature for 30 min in the dark. The solution was dialysed against 3% AcOH and 50 mM NH_4HCO_3. The recovered solution was filtered with 0.2-μm membrane filter before PNGaseF reaction when precipitation was observed.

The recovered solution was mixed with 500 kU/mL PNGase F (unit definition:NEB Lab, 1kU to 1 mg glycoprotein) and incubated at 37°C for 24 hr. Glycans were separated from protein fragment by vivaspin (MWCO 10,000, Sartorius) and lyophilized.

For permethylation of glycans, the lyophilised glycans was dissolved in 0.5 mL of DMSO in a glass vial. 25 mg of powdered NaOH and 0.3 mL of MeI were added and the suspension was sonicated for 90 min. After cooling the reaction mixture on ice, 1 mL of 5% AcOH was added and the solution was vigorously mixed. The solution was extracted with 0.6 mL of chloroform

(x3). The combined organic phase was washed with 1.8 mL of water (x8). The solvent was removed by N_2 flow and the residue was dissolved in 0.1 mL of MeOH. After HyperSep C18 (200 mg, 3mL) was conditioned with 5 mL of MeOH and 10 mL of water and the crude permethylated glycan solution in MeOH was applied to the column. After washing the column with 15 mL of water and 2 mL of 10% MeCN, the permethylated glycans were eluted with 3 mL of 80% MeCN. After the solvents were removed by N_2 flow and lyophilisation, the residue was dissolved in 20 µL of 50% MeOH solution for MS analysis.

Preparation of glycan samples for MALDI-TOF-MS

For unmodified glycan samples, lyophilized residues were reconstructed with 20 µL of water. They were cleaned up with Nafion membrane. 1 µL of the glycan solution was placed on the membrane floating on MilliQ water reservoir and the solution was contacted with the membrane for 30 min. 0.5 µL of the recovered glycan solution was mixed with 0.5 µL of DHB solution (10 mg/mL in 70% MeCN) and 0.5 µL of NaCl solution (0.1 mg/mL in water) on a MALDI-TOF-MS target plate. After the solvents were dried out, the residue was redissolved with 0.2 µL of EtOH and dried again to obtain the homogenized sample.

For the permethylated glycan solutions, no further purification was performed and 0.5 µL of the permethylated glycan solution was mixed with 0.5 µL of DHB solution (10 mg/mL in 70% MeCN) and 0.5 µL of NaCl solution (0.1 mg/mL in water) on a MALDI-TOF-MS target plate. After the solvents were dried out, the residue was redissolved with 0.2 µL of EtOH and dried again to obtain the homogenized sample.

Recipes of media

LB-Kan agar plate

5 g of LB broth and 4 g of agar were dissolved in 200 mL of water and the solution was autoclaved. After cooling to around 50°C was added 334 µL of 50 mg/mL kanamycin sulfate (filter-sterilized before use) and the solution was dispensed and solidified in petri dishes.

LB-Amp agar plate

5 g of LB broth and 4 g of agar were dissolved in 200 mL of water and the solution was autoclaved. After cooling to around 50°C was added 400 µL of 100 mg/mL ampicillin sodium salt (filter-sterilized before use) and the solution was dispensed and solidified in petri dishes.

LB-Kan medium

2.5 g of LB broth was dissolved in 100 mL of water and the solution was autoclaved. After cooling to around 50°C was added 167 µL of 50 mg/mL kanamycin sulfate (filter-sterilized before use).

LB-Kan medium

2.5 g of LB broth was dissolved in 100 mL of water and the solution was autoclaved. After cooling to around 50°C was added 200 μL of 100 mg/mL ampicillin sodium salt (filter-sterilized before use).

20xM solution

The followings were dissolved in 700 mL of water:

72 g of Na_2HPO_4 (final concentration: 0.5M)

68 g of KH_2PO_4 (final concentration: 0.5M)

53 g of NH_4Cl (final concentration: 1.0M)

14 g of Na_2SO_4 (final concentration: 0.1M)

Then the solution was diluted to 1L and filter-sterilized.

50x505 solution

The followings were mixed:

200 mL of glycerol

600 mL of water

25 g of D-glucose

The solution was diluted to 1L and filter-sterilized.

50x5052 solution

The followings were mixed:

200 mL of glycerol

600 mL of water

25 g of D-glucose

100 g of α-lactose

The mixture was heated to around 50°C to dissolve all the components completely. Then the solution was diluted to 1L and filter-sterilized.

ZYM-505-Kan medium

5 g of trypton and 2.5 g of yeast extract were dissolved in 463 mL of water and the solution was autoclaved. After cooling, the following filter-sterilized solutions were added:

25 mL of 20xM solution

10 mL of 50x505 solution

1 mL of 1M $MgSO_4$

1 mL of 50 mg/mL kanamycin sulfate solution

ZYM-5052-Kan medium

5 g of trypton and 2.5 g of yeast extract were dissolved in 462 mL of water and the followings

were added:

The solution was autoclaved. After cooling, the following filter-sterilized solutions were added:

25 mL of 20xM solution

10 mL of 50x5052 solution

1 mL of 1M $MgSO_4$

1 mL of 50 mM $FeCl_3$

1 mL of 50 mg/mL kanamycin sulfate solution

(Addition of 50x5052 or 20xM before autoclave resulted in poor cell growth.)

ZYM-5052-Amp medium

5 g of trypton and 2.5 g of yeast extract were dissolved in 462 mL of water and the solution was autoclaved. After cooling, the following filter-sterilized solutions were added:

25 mL of 20xM solution

10 mL of 50x5052 solution

1 mL of 1M $MgSO_4$

1 mL of 50 mM $FeCl_3$

1 mL of 100 mg/mL ampicillin sodium salt solution

BugBuster (pH 6.0)

1 mL of BugBuster 10x Protein Extraction Reagent was diluted with 9 mL of 100 mM NaPi (pH 6.0). 1 μL of 250 U/uL Benzonase and 10 uL of 1kU/uL rLysozyme were added.

HPLC conditions

Condition A:

Column: Kromasil NH2 100A, 150x4.60mm, 5 micron (Phenomenex)

Flow rate: 1.0 mL/min

Solvent: Actonitrile: Water= 90: 10 with 0.1% formic acid (Isocratic)

Detection: UV 300nm

Condition A':

Column: Kromasil NH2 100A, 150x4.60mm, 5 micron (Phenomenex)

Flow rate: 1.0 mL/min

Solvent: Actonitrile: Water= 70: 30 with 0.1% formic acid (Isocratic)

Detection: UV 300nm

Condition A'':

Column: Kromasil NH2 100A, 150x4.60mm, 5 micron (Phenomenex)
Flow rate: 1.0 mL/min
Solvent: Actonitrile: Water= 80: 20 with 0.1% formic acid (Isocratic)
Detection: UV 300nm

Condition B:
Column: Synergi 4u Fusion-RP 80A, 100x4.60mm, 4 micron (Phenomenex)
Flow rate: 1.0 mL/min
Solvent A: Water with 0.1% formic acid
Solvent B: Acetonitrile with 0.1% formic acid

Gradient:		
	0% Solvent B	0-2 min
	to 15% Solvent B	2-15 min
	15% Solvent B	15-17 min
	to 100% Solvent B	17-18 min
	100% Solvent B	18-20 min
	0% Solvent B	20-22 min

Detection: 300 nm

Condition B':
Column: Synergi 4u Fusion-RP 80A, 100x4.60mm, 4 micron (Phenomenex)
Flow rate: 1.0 mL/min
Solvent A: Water with 0.1% formic acid
Solvent B: Acetonitrile with 0.1% formic acid

Gradient:		
	0% Solvent B	0-2 min
	to 20% Solvent B	2-15 min
	20% Solvent B	15-17 min
	to 100% Solvent B	17-18 min
	100% Solvent B	18-20 min
	0% Solvent B	20-22 min

Detection: 300 nm

Condition C:
Column: Kromasil NH2 100A, 150x4.60mm, 5 micron (Phenomenex)
Flow rate: 1.0 mL/min
Solvent A: Water
Solvent B: Acetonitrile

Gradient:		
	80% Solvent B	0-3 min
	to 70% Solvent B	3-18 min

70% Solvent B	18-20 min
80% Solvent B	20-22 min

Detection: Evaporative Light Scattering

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Publication:	Nature Chemical Biology
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Author: Mohammed N. Amin, Wei Huang, Rahman M. Mizanur, and Lai-Xi Wang

Publication: Journal of the American Chemical Society

Publisher: American Chemical Society

Date: Sep 1, 2011

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Title: 2-O-Propargyl Ethers: Readily Cleavable, Minimally Intrusive Protecting Groups for β-Mannosyl Donors

Author: David Crich* and and Prasanna Jayalath

Publication: Organic Letters

Publisher: American Chemical Society

Date: May 1, 2005

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Title:	Production of Human Compatible High Mannose-type (Man5GlcNAc2) Sugar Chains inSaccharomyces cerevisiae
Author:	Yasunori Chiba, Misa Suzuki, Satoshi Yoshida, Aruto Yoshida, Hiroshi Ikenaga, Makoto Takeuchi, Yoshifumi Jigami, Eiji Ichishima
Publication:	Journal of Biological Chemistry
Publisher:	The American Society for Biochemistry and Molecular Biology
Date:	Oct 9, 1998

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List of Publications

An endoglycosidase with alternative glycan specificity allows broadened glycoprotein remodeling. Jonathan J. Goodfellow,[§] Kavita Baruah,[§] Keisuke Yamamoto,[§] Camille Bonomelli, Benjamin Krishna, David J. Harvey, Max Crispin, Christopher N. Scanlan, Benjamin G. Davis. ([§]these authors are equally contributed) ***J. Am. Chem. Soc.*, 2012, *134*, 8030-8033.**

Creation of an α-mannosynthase from a broad glycosidase scaffold. Keisuke Yamamoto, Benjamin G. Davis. ***Angew. Chem. Int. Ed.*, 2012, *51*, 7449.**

Acknowledgment

Whether good or bad, I am always trying to be self-contained. This is my nature. However, at the same time, I always find that I cannot be self-contained completely after all. Many people give many things to me. I sometimes realize somebody is supporting me from behind. I sometimes find what somebody made is underneath my feet. Probably, there are many others that I do not even notice. It is a shame that I fail to say my thanks to such people or things. This is why I would like to devote this one paragraph to express my thanks with hearty respect if I miss saying this to anybody. Thank you, to all the people who are involved in my life.

In particular…

This is the thesis to which most of my life in Oxford was dedicated. Why could I be here? How could I write this? I owe these to Professor Ben Davis. He allowed me to join his great team which is full of talent and cutting-edge projects. Being here is stimulating and keeps my eyes wide open. I believe I could give him a small return through the experiments shown in this thesis. But, still there seem much for me to return to him. I will try to pass what I learned here to people around me. I hope he will accept this as a rest part of my return to him. From the island at the eastern edge of Eurasia, to the island at the western edge of Eurasia. It was a big leap in my life. Ben, could I make a big leap in science as well? If so, I have got more to owe to you. And if so, I thank you and your team.

Science is not a hobby of aristocrats any more. In most aspects, this is good because science is now open to anyone who would like to enjoy it. But one difficult thing caused by this change is that we have to raise money to perform science as well as to manage a life of an individual. Therefore, a financial support is critical for the modern science. In my case, I was lucky to have a studentship from VTU during my DPhil course. I really thank all the people there for it. I am not sure if I could contribute to VTU as they had expected through this studentship. If not, I cannot do anything but to say sorry…but please allow me to say that I learned many things during my DPhil supported by VTU and would like to give back as much as I can do to the community of science in the future. I would like to especially thank Dr Thomas Purkarthofer, Dr Roland Weis and Dr Iskandar Dib as industrial advisor for their advice and for being generous about my scientific interests' wobbling around in erratic directions.

My parents. When I think of them, the same words always come up to my mind, “I am sorry.” No, I don’t think I did something bad to them. But why do I feel like that? Maybe partly because I am thinking that this is not the life which they expected me to have. I know that this is my own life and they are happy about my spending my life as I like. But… but still I feel like that. Why? Can I erase this feeling by saying and doing many thanks? I don’t know. But this is an idea I have now. Get an idea and do it. This is the way of science. That’s why I will do it to them.

Myself, thanks for surviving the life in Oxford. Honesty is your belief, faith and philosophy as ever. Enjoy your life with this incomplete verse in mind and think the following lines for yourself;

面白き　こともなき世を　面白く

Keisuke Yamamoto