

**Synthesis and properties of
oligonucleotides containing triazole
backbone linkages and 2'-modifications
for therapeutic applications**

A thesis submitted to the Board of Mathematical, Physical
and Life Sciences Division, Department of Chemistry in
partial fulfillment for the degree of

Doctor of Philosophy

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*Any knowledge that doesn't lead to new questions
quickly dies out: it fails to maintain the temperature
required for sustaining life.*

Wisława Szymborska

ABSTRACT

Antisense oligonucleotides are short strands of DNA, which bind to their complementary mRNA target to prevent protein translation. Although conceptually appealing, for their practical use as drugs, these oligonucleotides must have better cellular uptake, resistance to enzymatic degradation, and target selectivity. In this work, new synthetic chemistry is established to prepare a novel group of chemically modified oligonucleotides. The anionic phosphodiester backbone is partially replaced with a neutral triazole and, at the same time, the 2'-position of the ribose sugar is functionalised with pyrene, anthraquinone, or guanidine moieties. Being unnatural, the triazole backbone is inherently resistant to enzymatic degradation, while the reduced negative charge potentially improves cell penetration. The limitation of introduction of a triazole backbone into the antisense strand is its destabilising effect on the duplex formation with their complementary target. In this study, the 2'-modifications are used to restore the lost duplex stability and they have been found to be very efficient stabilising moieties. To evaluate the viability of this strategy, reporter gene assays based on splice-switching model are used. Promisingly, these modified oligonucleotides have successfully shown antisense splice-switching activity, suggesting there is further scope for their improvement.

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DECLARATION OF AUTHORSHIP

I, Anna M. J. Dysko declare that this thesis and the work presented in it are my own and have been generated by me as the result of my own original research.

I confirm that:

- This work was done wholly while in candidature for a research degree at this University;
- Where I have consulted the published work of others, this is always clearly attributed;
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;

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ABBREVIATIONS

A	adenine
aq.	aqueous
AS-ON	antisense oligonucleotides
br	broad
bp	base pair
Bz	benzoyl
calc.	calculated
CME	clathrin-mediated endocytosis
conc.	concentrated
CPG	Controlled Pore Glass
CuAAC	Copper-Azide-Alkyne Cycloaddition
C	cytidine
°C	Degree Celsius
d	doublet (NMR)
dd	doublet of doublet (NMR)
dt	double triplet NMR)
DCM	dichloromethane
DCE	1,2-dichloroethane
DIPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	<i>N,N'</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DMT	dimethoxytrityl
DMT-Cl	4,4'-dimethoxytrityl chloride
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eq.	equivalent

ES	electrospray
ESI	electrospray ionization
EtOAc	ethyl acetate
Et ₂ O	diethyl ether
EtOH	ethanol
Fmoc	9-fluorenylmethyl
G	guanine
g	gram
H-bond	hydrogen bond
HPLC	high performance liquid chromatography
HRMS	High Resolution Mass Spectrometry
Hz	hertz
iPrOH	isopropanol
LNA	locked nucleic acids
LRMS	Low Resolution Mass Spectrometry
M	molar concentration
m	multiplet (NMR)
mg	milligram
mL	milliliter
mm	millimetre
MeC	5-methylcytidine
MeCN	acetonitrile
MeOH	methanol
MF	morpholino oligonucleotides
mRNA	messenger RNA
MS	mass spectrometry
Ms	mesyl group
M _w	molecular weight

m/z	mass/charge
NHS	<i>N</i> -hydroxysuccinimide
nm	nanometer
nM	nanomolar concentration
μM	micromolar concentration
NMR	Nuclear Magnetic Resonance
NP	N3'-P5'-phosphoroamidates
ODN	oligodeoxyribonucleotides
ORN	oligoribonucleotides
PCR	polyacrylamide gel electrophoresis
pH	potential of hydrogen
PNA	peptide nucleic acids
ppm	parts per million (NMR)
PPh ₃	triphenylphosphine
PS	phosphorothioate
q	quartet (NMR)
rt	room temperature
RNA	ribonucleic acids
s	singlet (NMR)
T	thymine
t	triplet (NMR)
TBAF	tetrabutylammonium fluoride
TBDMS	<i>tert</i> -butyldimethylsilyl
TCA	trichloroacetic acid
THF	tetrahydrofuran
td	triple doublet (NMR)
TEA	triethylamine
TLC	Thin Layer Chromatography
U	uridine

UV

| ultraviolet

1 INTRODUCTION

1.1 NUCLEIC ACID STRUCTURE

1.1.1 Primary structure

Nucleic acids are the biopolymers responsible for carrying the genetic information of all living organisms. They occur naturally in the form of deoxyribonucleic acids (DNA) and ribonucleic acids (RNA). They are composed of monomers called nucleotides, which in turn contain three components; a heterocyclic base (nucleobase), a pentose sugar and a phosphate linker. The nucleobases can be separated in two families, pyrimidines cytosine (C), thymine (T) and uracil (U), or purines adenine (A) and guanine (G) (Figure 1.1). Cytosine, thymine, adenine and guanine are found in DNA, whereas in RNA thymine is replaced with uracil. In cells, cytosine is occasionally converted into uracil through a deamination process. DNA uses thymine instead of uracil to ensure a more efficient DNA repair process. It allows full discrimination between four different bases C, T, A and G and enables effective mutation repair when C is deaminated to U.

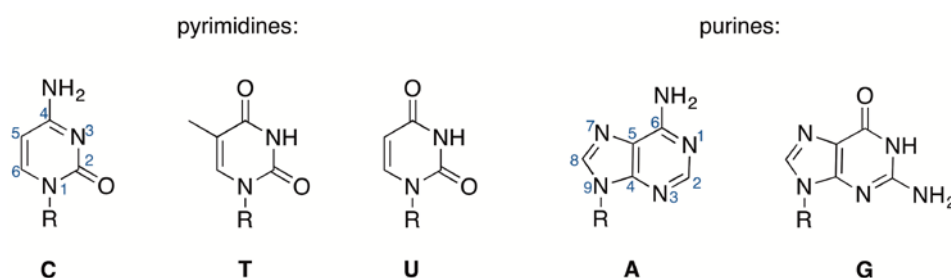


Figure 1.1 Structures of nucleobases.

The sugar in DNA is 2'-deoxy-D-ribose while in RNA it is D-ribose. The pentose sugar is attached to the nucleobase *via* a *N*-glycosidic bond which links the carbon C-1' of the ribose with the N-1 of pyrimidines or N-9 of purines. The nucleobase can be situated on the same face of the sugar ring as the 5'-hydroxyl group (β -anomer) or on the opposite side (α -anomer). In

naturally occurring DNA all nucleosides are in the β -configuration (Figure 1.2). Nucleobases can rotate relative to the pentose sugar, and the two extreme conformations are the *anti*- and *syn*-conformations, where the *anti*-form is exhibited in naturally occurring nucleic acids (Figure 1.2).

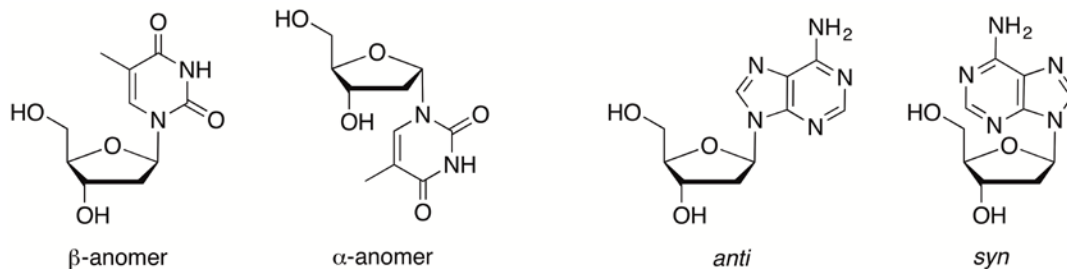


Figure 1.2 Conformations and configurations of nucleosides.

Regarding the sugar ring, to obtain the most stable conformation, the furanose rings twist out of plane. The 'puckering' of the sugar is defined by the displacement of the C-2' and 3' of the ribose from the C1'-O4'-C4' plane. DNA adopts mainly the S-type (Southern) conformation with the C-2' *endo* puckering of the deoxyribose, whereas RNA adopts mostly the N-type (Northern) conformation with C-3' *endo* ribose puckering (Figure 1.3). Sugar conformation determines the secondary structure of nucleic acids and their characteristic, diverse shapes.

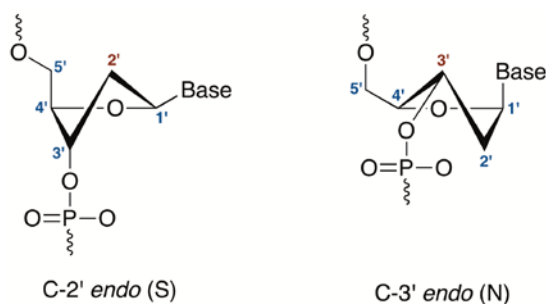


Figure 1.3 2'- *endo* (S) and 3'-*endo* (N) pucker conformations of deoxyribose.

The primary sequence of DNA is the order in which the nucleotides are arranged from the 5'-hydroxyl group at one end to the 3'-hydroxyl group at

the other end of the nucleic acid. Nucleic acids have directionality and the sequence is conventionally written in the 5'- to 3'-direction (Figure 1.4; A).

1.1.2 Secondary and tertiary structure of nucleic acids

In 1949 Chargaff *et al.* found that the ratio of purines to pyrimidines in cells is always the same 1:1.¹ This was followed by the discovery by Maurice Wilkins and Rosalind Franklin, based on X-ray crystallography, who found that the structure of double stranded DNA is helical and varies depending on humidity.²⁻⁴ Finally the tertiary structure of the DNA double helix was established by James Watson and Francis Crick in 1953.⁵ They found that the double stranded helix of DNA is held together by the hydrogen bonds formed between nucleobases in the two opposite strands. They showed that nucleobase A pairs with T and C with G (Watson-Crick base pairs, Figure 1.4; B). It was later discovered that there are other types of less common base pairs such as Hoogsteen base pairs or wobble base pairs (Figure 1.4; C and D).^{6,7}

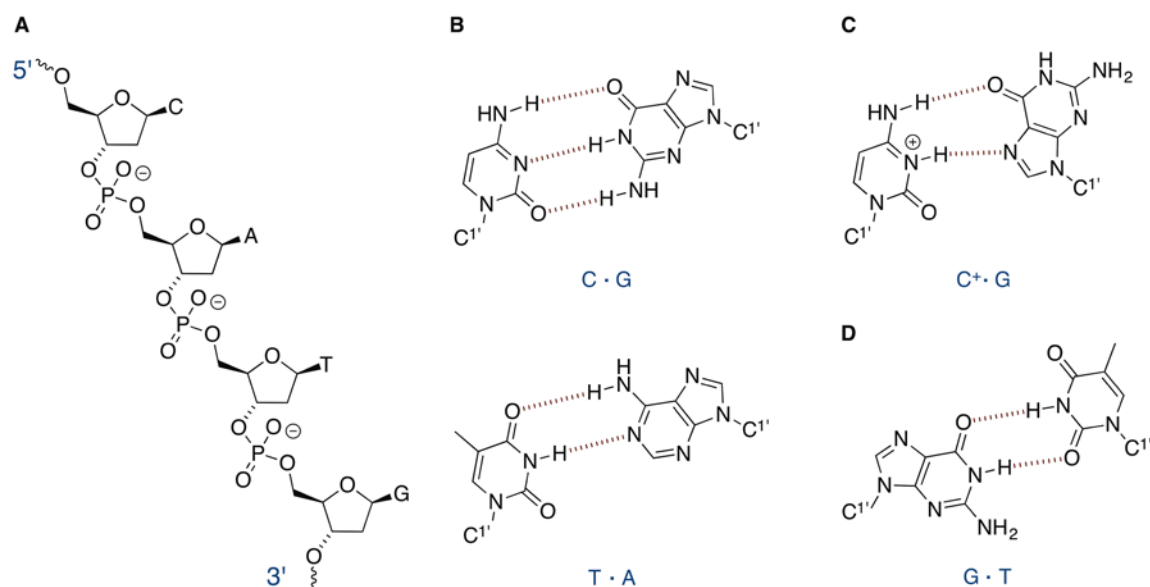


Figure 1.4 DNA primary structure (A); Watson-Crick base pairing; C:G and T:A (B); Hoogsteen C⁺:G base pair (C); wobble base pair G:T (D).

In a duplex, the two complementary strands run antiparallel with the 5'-end of one strand next to 3'-end of second strand. The two strands curl together to form a right-handed double helix, where the negatively charged phosphate backbone forms the outer layer and the base pairs stacking upon each other form a hydrophobic core.

Double stranded DNA generally exists in a 'B-conformation' – a right-handed helical form made of 10-11 base pairs with major and minor grooves of similar depth, but with a wider major groove compared to the minor groove (Figure 1.5). 'A-type' helices are much more common for RNA secondary structures where the sugar takes a *C-3'-endo* pucker and the 2'-hydroxyl group hinders the formation of B-type helix.⁸ The A-form duplex is wider and more compact than the B-form. It has narrow and deep major groove and wide and shallow minor groove (Figure 1.5). Duplexes formed between RNA and DNA strands are also very important in biology and typically adopt the A-form. These RNA:DNA hybrids occur, for example, during transcription when DNA is converted to its complementary messenger RNA in cells.

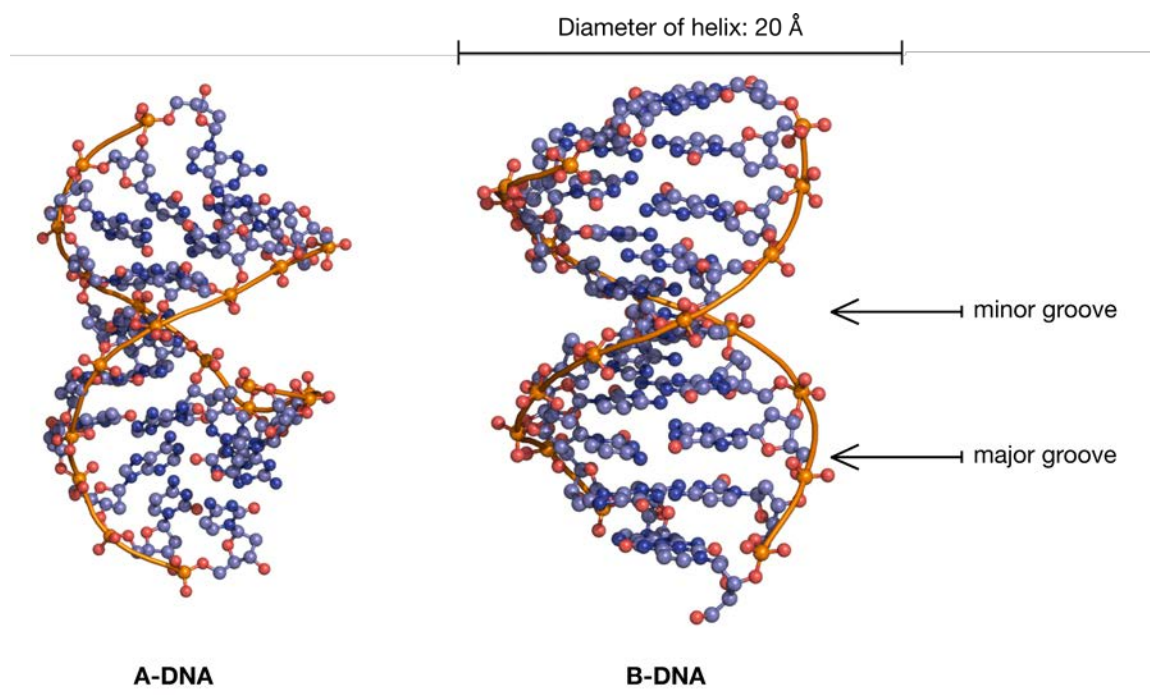


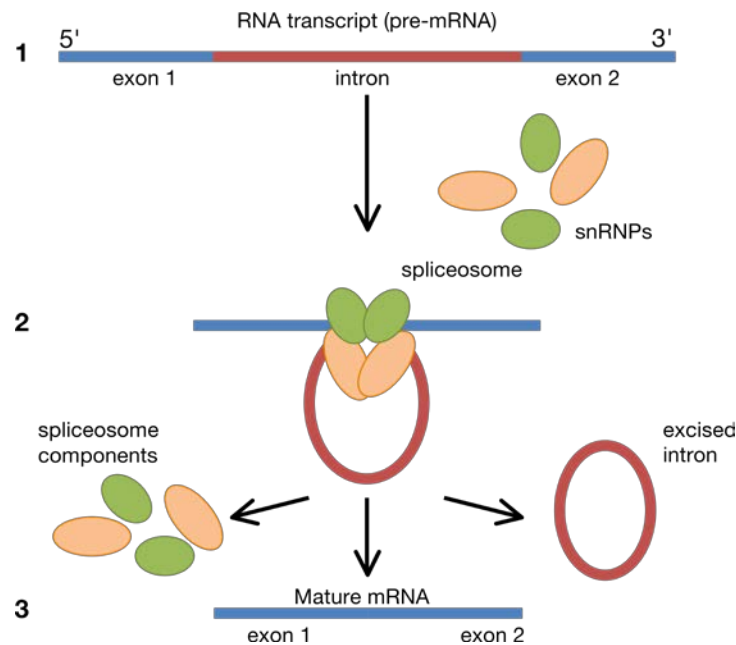
Figure 1.5 A-type and B-type DNA conformations. Figure taken from⁹

1.2 GENETIC CODE

The central dogma of molecular biology is that DNA is transcribed to messenger RNA, which is then translated to protein. The relationship between a sequence of DNA nucleobases and a sequence of the corresponding polypeptide that forms the protein is defined by the genetic code. DNA is a carrier of genetic information and the process of encoding the DNA requires a complex system of proteins and enzymes. The genetic code is read in groups of three nucleotides called codons and each codon represents a single amino acid. As there are four nucleotides in RNA (A, C, G, U) there are 64 possible codons. There are twenty-two natural amino acids that are naturally incorporated into polypeptides, of which twenty are encoded by the universal genetic code. Therefore, many amino acids are encoded by multiple different codons.

There are *ca.* 3×10^9 DNA base pairs in the human genome and they are stored in 23 chromosomes in the nucleus of cells. The human genome encodes about 20,000-25,000 proteins and each protein is encrypted in a DNA code called the gene. The fragments of DNA that code for proteins are called exons. However, 95% of the genome does not code for proteins and these regions are called introns. As DNA is transcribed to RNA it is first transcribed to pre-mRNA, which contains the introns, therefore it needs to be edited to remove the non-coding regions. This process is called RNA splicing and it involves removing the introns, and sticking together the protein coding exon regions (Scheme 1.1). RNA splicing begins with assembly of proteins at the exon-intron junctions, which guide small nuclear riboproteins to form a fully active splicing complex called spliceosome **1**. In the spliceosome two ends of neighbouring exons are brought together. One end of the intron is cut and folds to the other end forming a loop **2**. The spliceosome then cuts the second junction, releasing the intron loop and joining the two exons

together. Each spliceosome complex removes one intron and disassembles after releasing the intron loop, leaving the complete instruction for protein synthesis, the mature mRNA **3**.



Scheme 1.1 Pre-mRNA splicing mechanism.

The biological origin of introns is not very well understood. Some hypotheses state that protein-coding genes were interrupted by introns at the earliest stage of life's evolution, where others suggest they emerged only in eukaryotes.¹⁰ For a long time introns were believed to be unimportant pieces of RNA known as a "junk RNA". It is now understood that they have great functional importance in eukaryotic cells, such as transcription initiation, regulation and termination, alternative splicing or nuclear export.¹¹

1.3 ANTISENSE OLIGONUCLEOTIDES

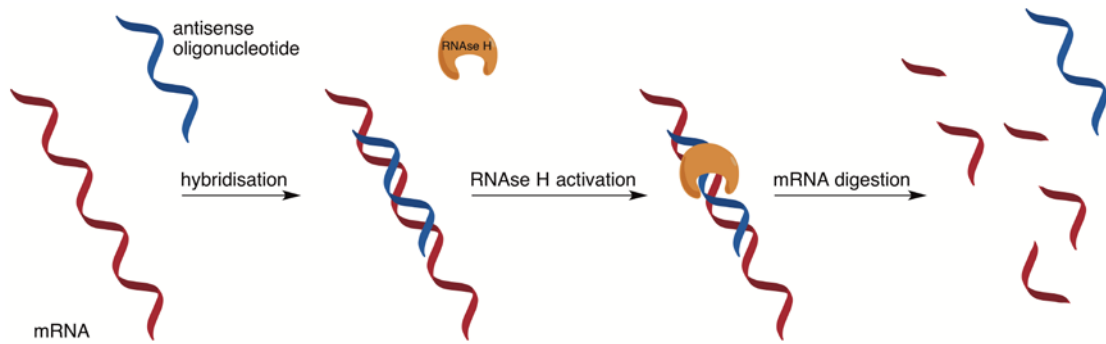
1.3.1 Mechanisms of action of antisense oligonucleotides

Genetic diseases arise from mutations in the genomic DNA. These disorders can be passed down from the parent (hereditary) or occur *de novo*. The

'mutation' can vary from a small change in a single gene, multifactorial changes in many genes (potentially causing the production of an extra chromosome), or the deletion of parts / all of a chromosome. A mutation within a gene causes the production of an aberrant or toxic protein or stops its production completely thereby inhibiting its function. Genetic disorders are fairly rare, although efficient therapies and cures are also rare.

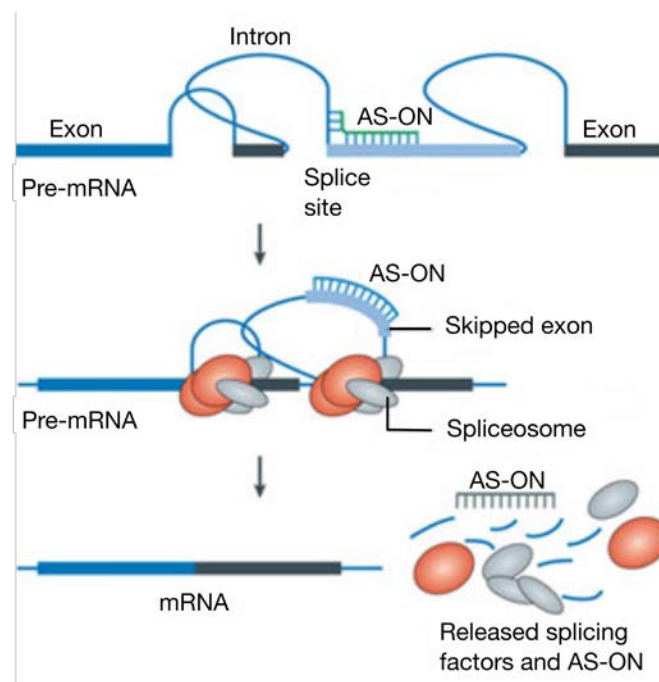
One of the major approaches in the gene therapy area of scientific research is the use of antisense oligonucleotides (AS-ON).¹² The antisense oligonucleotide technique uses pieces of DNA or RNA as pharmaceutical agents to treat genetic disorders by acting on the cell's genetic material.¹³ The potential of oligodeoxynucleotides to be used as antisense agents was first realised by Zamecnik and Stephenson in 1978.¹⁴ It is different from conventional therapeutic approaches, in which a drug binds to an enzyme or receptor causing modulation of its function.

Antisense oligonucleotides are 15 – 30 nucleotides long DNA or RNA strands. Their sequences are complementary to specific regions of messenger RNA (mRNA), to which they bind *via* Watson-Crick base pairing. This hybridisation process disrupts translation of the genetic message and as a result inhibits the synthesis of its intended protein. This happens through two major mechanisms. Firstly, an AS-ON can activate RNase H which cleaves the mRNA in AS-ON:mRNA heteroduplexes (Scheme 1.2). This causes degradation of the mRNA and in doing so stops the further production of the protein. If the AS-ON:mRNA duplex does not activate RNase H, the AS-ON can inhibit the translation process through steric blockage of ribosome. Of the two, activation of RNase H has been found to be the more common mechanism.¹⁵



Scheme 1.2 RNase-H induced antisense oligonucleotide mechanism.

Another very potent antisense oligonucleotides mechanism is exon-skipping or splice-switching. Splice-switching oligonucleotides modulate the pre-mRNA splicing process. By doing so, they repair defective RNA and restore production of the protein, or generate novel protein variants with altered properties. In the mechanism of the exon-skipping and splice-switching, the AS-ON targets exon-intron junction or splice site in pre-mRNA and prevents the correct assembly of the spliceosome on the exon, which redirects the splicing pathway, inducing skipping of the targeted exon (Scheme 1.3).



Scheme 1.3 Exon skipping mechanism. Figure taken from¹⁶

Although antisense approaches are promising in theory, there are several problems that must be resolved in order for AS-ONs to become useful drug candidates. The AS-ON has to bind to its target mRNA; given that RNA is highly structured, finding an accessible site for stable duplex formation is a key challenge. The AS-ON must also be resistant to nuclease attacks, as foreign ONs are rapidly degraded in biological fluids by the wide range of naturally occurring nuclease enzymes. Finally, a sufficient level of cellular uptake needs to be achieved with the desired intracellular localisation.¹⁷

To be able to fulfill these criteria, a variety of different modifications have been made to natural AS-ON molecules. In general, they can be grouped into one of three main areas: nucleobase, sugar or phosphate backbone modifications (Figure 1.6).¹

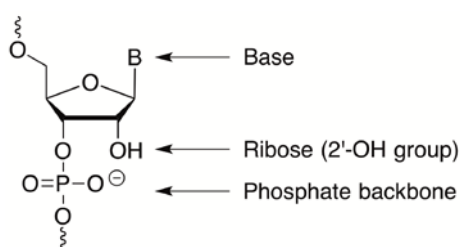


Figure 1.6 Sites for chemical modification of oligonucleotides.

Furthermore, when designing AS-ONs, some sequences should be avoided. Four consecutive guanosine residues are capable of forming intermolecular G-quartets *via* Hoogsteen base pairing, thereby decreasing the 'active' concentration of ONs, and could induce unwanted side-effects.⁵ Usage of CpG dinucleotide steps can be very disadvantageous, as they are more frequently found in viral or bacterial DNA and can bind to the TLR9 protein, stimulating immune responses in mammalian systems.¹⁸

1.3.2 First generation antisense oligonucleotides

'First generation' antisense oligonucleotides focused on backbone modifications where the non-bridging oxygen atoms in the phosphate group

were replaced with sulfur atoms (Figure 1.7). In particular, a single substitution of oxygen by sulfur gave rise to phosphorothioate backbones (PS), first synthesised in the 1960s by Eckstein and colleagues.^{19,20} In DNA the phosphorus atom is a chiral center, therefore a phosphorothioate oligonucleotide is a complex mixture of diastereomers. After chemical synthesis of PS oligonucleotide individual phosphorothioate groups have the Rp or Sp configuration.²¹ Based on X-ray crystallography and NMR studies, negligible structural differences were observed relative to the parent phosphodiester backbone, and they generally bind to enzymes with similar affinities to the phosphodiester oligonucleotides.²²⁻²⁴ PS DNA was first used as an antisense oligonucleotide by Matsukura *et al.* and then Agrawal *et al.* for the inhibition of HIV-1 replication.^{25,26} Whereas unmodified oligonucleotides have a half-life of *ca.* 1 hour in biological media, their PS analogues have half-lives 100 fold higher, thus indicating improved nuclease resistance.^{27,28} Ten years later in 1998 Brautigam and Steitz reported that while the Sp diastereomer was completely resistant to enzymatic hydrolysis the Rp phosphorothioate linkage was only slightly more stable than the unmodified phosphodiester variant.²⁹ X-ray crystallography revealed significant structural similarities between the nuclease enzyme complexed with a phosphodiester and Rp phosphorothioate oligonucleotide. For the Sp PS oligonucleotides however, the sulfur atom displaces the two metal ions in the active site of the nuclease that are essential for enzymatic activity, which naturally impedes RNase H degradation. Unfortunately in comparison to phosphodiesters, phosphorothioate oligonucleotides show stronger non-sequence specific interaction with certain proteins such as heparin that bind to polyanions.³⁰ On the other hand, binding to the plasma proteins can also protect ONs from filtration, improving their pharmacokinetic profile. Finally, PS oligonucleotides have a reduced affinity towards complementary mRNA by

approximately 0.5 °C (melting temperature of heteroduplex) per nucleotide in comparison to the corresponding natural analogues.^{1,28,31}

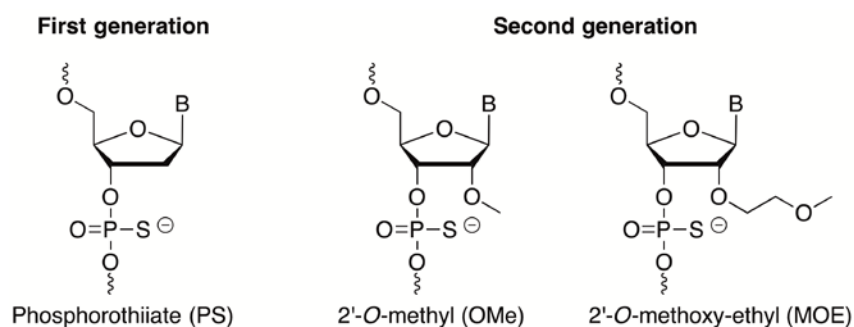


Figure 1.7 First and second generation of antisense oligonucleotides.

1.3.3 Second generation antisense oligonucleotides

'Second generation' antisense oligonucleotides were designed to solve the issues encountered by phosphorothioates by introducing alkyl modifications at the 2' position of the ribose sugar in order to improve the hybridisation properties of the AS-ON to its target. The two most widely used modifications are 2'-O-methyl (OMe) and 2'-O-methoxyethyl (MOE) RNAs (Figure 1.7). These ONs retain nuclease resistance but also show enhanced hybridization affinity and lower toxicity relative to PS oligonucleotides.³² However, the 2'-modification inhibits activation of the RNase H pathway, and acts *via* steric blocking of the ribosome or exon-skipping mode of action.^{1,33} To overcome the RNase H inactivation problem, 'gapmers' were developed. Gapmers contain phosphorothioates in the middle of the oligonucleotide strand and 2'-modified nucleotides at 3'- and 5'-end sides (Figure 1.8).^{32,34} The central part of the oligonucleotide allows for the RNase H binding and the termini improve enzymatic stability and thermal duplex stability. However although this improves the design, this type of modification has an inherent limitation caused by the middle fragment being more susceptible to endonuclease degradation than the termini.

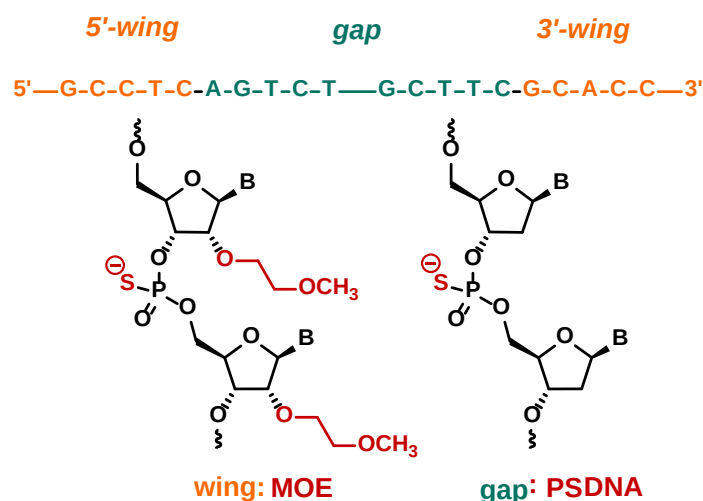


Figure 1.8 Structure of antisense oligonucleotide gapmer.

1.3.4 Third generation antisense oligonucleotides

More recently 'third generation' antisense oligonucleotides and their mimetics aim to further improve target affinity, pharmacokinetics, nuclease resistance and cellular uptake. This generation of ONs includes more extensive modifications on the phosphate backbone as well as on the ribose sugar, of which the most successful are peptide nucleic acids (PNAs), N3'-P5'phosphoramidates (NPs), locked nucleic acids (LNA) and morpholino oligonucleotides (MF) (Figure 1.9).¹

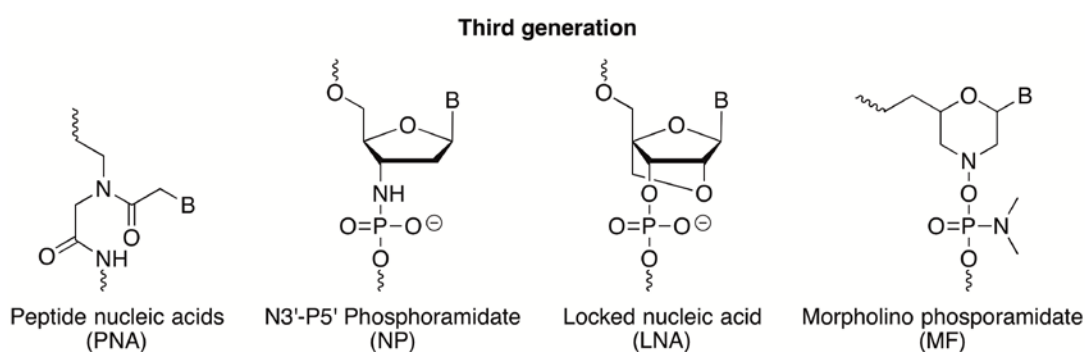


Figure 1.9 Third generation of antisense oligonucleotides.

Introduced by Nielsen and co-workers in 1991, PNAs have polyamide rather than deoxyribose phosphate backbones (Figure 1.9).^{35,36} They are synthesised by standard automated solid-phase peptide synthesis methods using

commercially available monomers protected with ^tBoc or Fmoc.^{37,38} PNA is an excellent structural mimic of DNA, and forms very stable duplexes with complementary DNA and RNA *via* Watson-Crick base pairing.^{6,3739,40} However, as PNA is electrostatically neutral it is not taken up very well by cells, so similarly to DNA it requires the employment of a delivery system such as cell penetrating peptides to improve their cell membrane penetration.^{41,42} There have been a number of examples showing the down regulation of gene expression caused by antisense PNA.⁴³⁻⁴⁵ It is known that PNA-RNA duplexes do not activate RNase H, however they are potentially very useful in processes involved in splicing modulation.^{46,47} PNA exhibits low aqueous solubility, short serum half-life and limited tissue bioavailability due to rapid excretion through the kidneys.⁴⁸ Simple modifications such as cationic peptide conjugates are used to improve these issues and this is now very rapidly developing area of research.⁴⁹

*N*3' - *P*5' phosphoramidites (NPs) are another 'third' generation design where the 3'-hydroxyl is replaced by a 3'-amino group in the phosphodiester backbone (Figure 1.9). NPs are nuclease resistant and have a high affinity towards complementary RNA.⁵⁰ Their antisense activity was shown to down regulate expression of the *c-myc* gene *in vivo*.⁵¹ Since NPs act through steric blockage and not RNase H activation, they physically interfere with the mRNA translation process or induce the exon skipping.

Locked nucleic acids (LNAs) contain a 2'-heteroatom to 4'-C linkage and are one of the most promising modified oligonucleotide analogues (Figure 1.9).⁵² LNAs were first synthesised by Wengel and co-workers at the same time as the Imanishi laboratory in Japan.⁵³⁻⁵⁵ A number of structural analogues of LNA have been synthesised creating a whole LNA oligonucleotide family with the β -D-LNA (parent LNA) being most studied (Figure 1.9). LNAs exhibit great affinity toward complementary RNA and DNA with up to 9 °C increase in melting temperature per modification, retaining their very good sequence

specificity and showing equally good mismatch discrimination compared to native DNA.^{53,56,57} The molecular structure of LNA shows that the sugar adopts a N-type 3'-*endo* conformation.^{58,59} Incorporation of LNA into a DNA strand induces conformational changes in the LNA-DNA:RNA duplex toward the A-type helix. The A-type conformation prevents activation of RNase H and can be used as a steric blocker or splicing inducer.^{60,61} However, studies have shown that LNA can be incorporated in gapmers (LNA modifications at the termini and phosphorothioate in the centre) to re-activate the RNase H mechanism.^{55,62} LNAs shows excellent resistance to nucleases. Fully modified LNA oligonucleotides exhibit complete protection against the 3'-exonuclease snake venom phosphodiesterase.⁶³ The LNA gapmers show a significantly increased half-life in human serum compared to PS-2'-OMe gapmers with a higher resistance to exonucleases and a rather poor resistance to endonucleases, which attack the central phosphodiester or phosphorothioate fragment.^{62,64} LNAs can be efficiently delivered into mammalian cells using standard delivery systems such as lipofectamine but the exact localisation may vary depending on cell type, the carrier or the oligonucleotide sequence.⁵⁵ LNAs are well tolerated in biological systems and due to their low degree of binding to serum proteins in comparison to PS oligonucleotides they exhibit only minor *in vivo* toxicity.^{55,65}

Morpholino oligonucleotides (MF) were first described in 1985 by Summerton.⁶⁶ They are non-ionic DNA analogues where the ribose sugar is replaced by a morpholino moiety and the phosphodiester backbone is changed to a phosphoroamide backbone (Figure 1.9). They exhibit high water solubility due to their very good base stacking, which allows effective shielding of the hydrophobic bases from the polar solvent.^{66,67} Morpholino oligonucleotides are completely stable to nucleases in a serum.⁶⁸ They do not activate RNase H enzymes and can act through the steric blockage or splicing modulation.⁶⁹ Because of the uncharged backbone, MFs are unlikely to bind

the serum proteins ensuring lower toxicity issues. Their hybridisation affinity is similar to that of natural DNA:RNA duplexes but lower than those of PNA, NP or LNA modified nucleic acids.⁶⁶

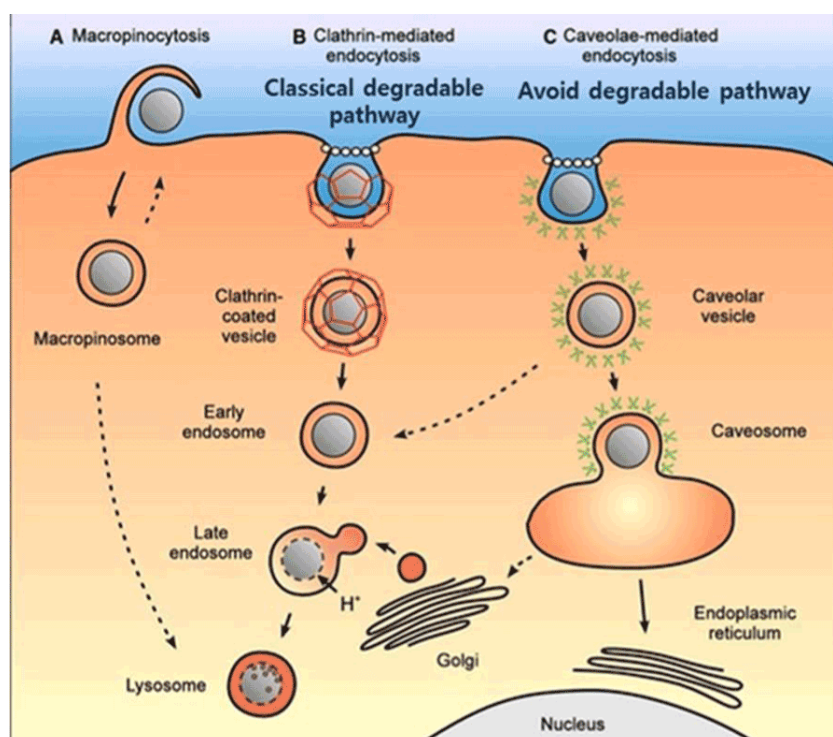
To summarise, most of the recently developed technologies aim to stabilize the AS-ON-mRNA duplex and improve resistant to nuclease attack in biological fluids. Those that do not trigger RNase H activity can perform *via* steric blockage or splicing modulation. However, their poor cellular uptake still remains an issue.

1.3.5 Delivery and distribution of oligonucleotides

Nucleic acids to be used as therapeutic agents have to reach the site of action of their target. Oligonucleotides are typically administered for systemic applications by parenteral injection, either intravenously or subcutaneously. They are then rapidly transferred from the blood into tissue, within minutes to hours.⁷⁰ Negatively charged antisense oligonucleotides bind to plasma proteins, which protects them from renal filtration.⁷¹ Non-charged oligonucleotides do not bind to plasma proteins and therefore exhibit more rapid clearance from blood primarily due to excretion in urine or metabolism in blood.^{48,72} AS-ONs are distributed into most kinds of tissue with more dominant areas being the liver, kidney, bone marrow, adipocytes and lymph nodes. The exception to this is the central nervous system.⁷³⁻⁷⁶

The successful delivery of oligonucleotides to the target cells and their availability at the intracellular site of action are crucial for successful therapy. Macromolecules are internalised into cells by a number of different mechanisms and their fate usually depends on their entry mechanism. The membrane of living cells is a dynamic bilayer structure with the internal layer being very lipophilic. This restricts the cellular entry of large, hydrophilic or charged molecules.⁷⁷ Endocytosis, the vascular uptake of extracellular macromolecules, has been established as the main mechanism for the

internalisation of the oligonucleotides into cells.⁷⁸ A number of mechanisms for endocytosis have been described to date (Scheme 1.4).^{79,80}



Scheme 1.4 Endocytosis pathways. Figure taken from⁸¹

Clathrin-mediated endocytosis (CME) is the foremost and best-characterised endocytotic pathway (Scheme 1.4; B). During endocytosis, oligonucleotides remain trapped in the endosomes, which then fuse with lysosomes where the oligonucleotides are degraded. To enhance cytosolic release, DNA-carrier complexes are formed with the association of targeting ligands such as cationic lipids or cationic polymers such as endosome disrupting peptides, pH sensitive peptides such as GALA which results in an enhanced transfection of oligonucleotides in several cell types.^{82,83} There are other endocytotic pathways that have been shown to be involved in the oligonucleotide delivery such as macropinocytosis or caveolae-mediated endocytosis (Scheme 1.4; A and C).⁸⁴⁻⁸⁷ These pathways have the advantage of avoiding lysosomal degradation and can be accessed by targeting the specific fragment receptors.⁷⁷ The uptake pathways and further intracellular trafficking of the

oligonucleotides are not yet well understood, but it is clear that escape from the endosomes is essential to efficient transfection.

The delivery of “free” oligonucleotides is known to be very inefficient.^{73,88} Oligonucleotides can be modified by appending lipophilic substituents, ligands for cell surface receptors, cell penetrating peptides or nanoparticles to enhance their delivery to the cytoplasm.⁸⁹⁻⁹² Some progress has been made in this area but it remains a very active area of research.

1.3.6 FDA approved antisense drugs and clinical trials

There are two clinically approved antisense drugs on the market.

Fomivirsen sodium salt, marketed as Vitravene, is a “first generation” antisense 21-base long phosphorothioate oligodeoxynucleotide (5'-GCG TTT GCT CTT CTT CTT GCG-3').⁹³ It is complementary to mRNA of the major protein of human cytomegalovirus (CMV) and is a potent and selective antiviral agent for cytomegalovirus retinitis.⁹⁴

Mipomersen, marketed as Kynamro, is a “second generation” antisense 20-mer phosphorothioate and 2'-methoxyethyl gapmer.⁹⁵⁻⁹⁷ The sequence is 5'-GCCUC AGTCTGCTTC GCACC-3', where the underlined residues are 2'-methoxyethyl and middle residues are 2'-deoxynucleosides. It is a cholesterol-reducing agent and it targets the mRNA of apolipoprotein B.⁹⁸ It reduces LDL-C, apolipoprotein B, total cholesterol and non-high-density lipoprotein cholesterol (non-HDL-C) in patients with familial hypercholesterolemia.⁹⁵

Since the FDA approval of the two antisense oligonucleotides: Fomivirsen and Mipomersen, pharmaceutical research has experienced an explosive growth in the number of modified oligonucleotides in clinical trials. The tested agents at different clinical stages listed in a number of recent reviews act *via* different mechanisms including RNase H activation, ribosome steric blockage, siRNA or exon skipping, involve a variety of modifications and target a whole range of diseases.⁹⁹⁻¹⁰³ However, search for potent antisense oligonucleotides is very

challenging, it is yet the only potential alternative in treatment of certain genetic diseases such as various types of muscular dystrophy, hence research in this area is crucial.

1.4 CLICK CHEMISTRY AND THE TRIAZOLE DNA BACKBONE

Since the antisense discovery by Zamecnik and Stephenson, oligonucleotides have been widely modified by changing the phosphodiester group or fully replacing it with a different structure, with the aim, amongst others, to reduce their negative charge and improve their delivery to the cell as well as increase their duplex stability and their resistance to nucleases. Given the great efficiency of the CuAAC reaction and the neutral charge of the triazole ring, triazole is potentially very promising antisense backbone modification.

1.4.1 Click chemistry

The modern concept of click chemistry was first defined by K. B. Sharpless in 2001 as a simple method to link together organic molecules in high yield, under mild conditions and in the presence of various functional groups. In accordance with Sharpless's definition of click chemistry, the reaction should be modular, give a high yield, be stereoselective, should not produce difficulty in purifying the desired product from by-products, should work well on both a small and large-scale and have a wide range of applications.¹⁰⁴ The product should also be stable under physiological conditions.

The best example is the Cu^{I} catalysed [3+2] azide-alkyne cycloaddition reaction (called CuAAC), which was derived from the Huisgen dipolar cycloaddition that has been in use for over 50 years in organic synthesis.¹⁰⁵ The uncatalysed Huisgen reaction is a classic 1,3-dipolar cycloaddition between an azide and a terminal or internal alkyne, which results in a 1,2,3-triazole.¹⁴ The major disadvantages of this cyclisation are its lack of

regioselectivity, giving 1,4- and 1,5-regioisomers (Figure 1.10), the necessity for a high temperature, and the very long time needed for the reaction to come to completion.

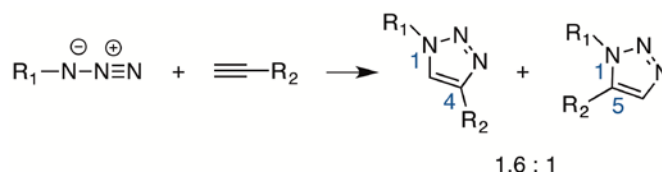


Figure 1.10 Huisgen dipolar azide-alkyne cycloaddition.

To control the regioselectivity of the Huisgen cycloaddition, Rostovtsev *et al.* at the same time as Tornøe *et al.* discovered that the Cu^{I} catalysed reaction gave only a 1,4-disubstituted 1,2,3-triazole (Figure 1.11).^{105,106}

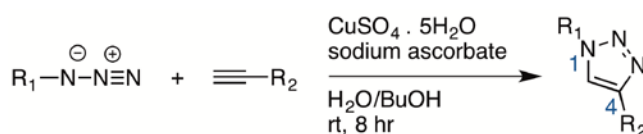
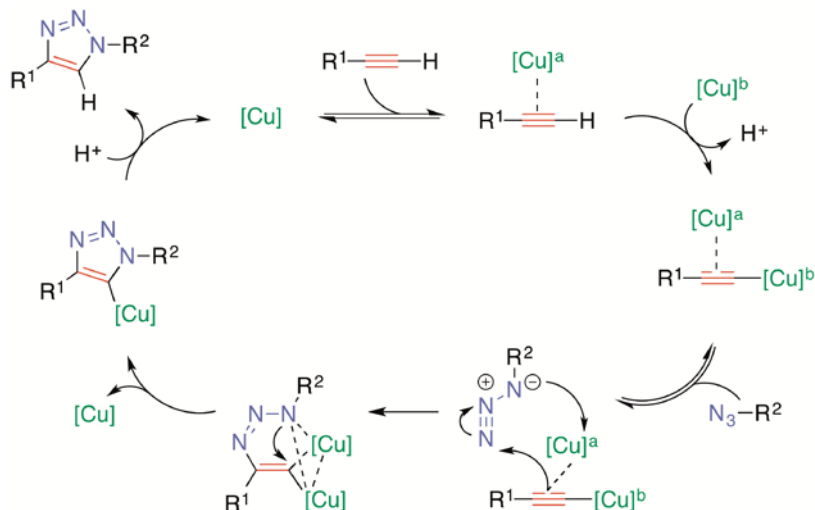


Figure 1.11 Regioselective Cu^{I} catalysed azide alkyne cycloaddition (CuAAC) to give a 1,4-disubstituted-1,2,3-triazole.

There are a number of different sources of Cu^{I} that could be used directly in the reaction, but several studies demonstrated that *in situ* Cu^{I} generation from reduction of Cu^{II} salts with sodium ascorbate was most efficient. The reaction proceeds at room temperature and reaches completion after 6–36 hours and most importantly can be carried out in a variety of solvents including water.¹⁰⁵

The mechanism of the copper-catalysed cyclisation was first proposed in 2002 by Fokin and Sharpless.¹⁰⁵ The catalytic cycle of this ligation starts with the formation of the Cu^{I} acetylide followed by the addition of azide and the formation of six-member copper intermediate. Finally, the rearrangement of the copper complex gives the copper-triazole complex and then the desired final product 1,4-disubstituted 1,2,3-triazole. More recently in 2013, Fokin *et*

al. presented a more detailed CuAAC mechanism demonstrating that two different copper atoms are essential in the cycloaddition intermediate formation (Scheme 1.5).¹⁰⁷



Scheme 1.5 CuAAC reaction mechanism by Fokin *et al.*

Nitrogen-based ligands are used to enhance catalytic activity in this reaction. Their role is to bind to Cu^{I} to stabilise its +1 oxidation state and stop potential Cu^{I} - induced degradation of biological molecules. The two most commonly used ligands are polytriazoles-*tris*-(hydroxypropyltriazolylmethyl)amine (HPTA) and *tris*-(benzyltriazolylmethyl)amine (TBTA) (Figure 1.12).^{108,109}

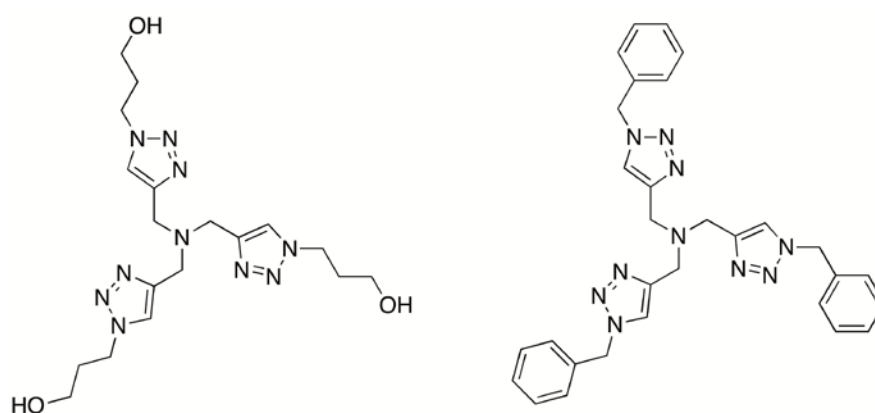


Figure 1.12. Common ligands used to enhance catalytic activity in the CuAAC reaction: HPTA and TBTA.

1.4.2 Triazole backbone in DNA

The success of click chemistry triggered extensive exploration of its use in organic, material and bioorganic synthesis.⁹ The CuAAC ligation has been found to be a very promising reaction in the oligonucleotide field, utilised for linking together two or more strands of DNA, cyclising single and double strands, oligonucleotide labelling, building DNA nanostructures and creating analogues of DNA with chemically modified backbones.⁹

Given the importance of backbone modification on improving AS-ONs, it is only natural to wonder what role the triazole modification could play. Like PS oligonucleotides, it should improve nuclease stability but also has the added benefits of reducing charge in an analogous manner to PNAs. To this end, the literature on the triazole containing ONs is surveyed in the following section.

Recent studies show very promising features of a new nucleic acid unnatural backbone – the 1,2,3-triazole linkage, including the capability of accurate read through of the modified backbone by both DNA and RNA polymerases.¹¹⁰

One of the advantages of the triazole-modified linkage is that it can be made with a different number of bonds between the 3' and 5' carbons on adjacent nucleosides, which allows examination of a wide range of different types of triazole backbone (Figure 1.13; Figure 1.14).

In 1997, P. von Matt *et al.* published the first 1,2,3-triazole nucleic acid linkage in a thymidine dimer.¹¹¹ In 2007, Andrea Nuzzi *et al.* demonstrated the introduction of the 1,2,3-triazole as an internucleoside linker following the Cu^I-catalysed azide – alkyne ligation strategy synthesising a non-natural codon (Figure 1.13; a).¹¹²

Following Nuzzi's work, the synthesis of this T-T triazole dimer (Figure 1.13; b) as a phosphoramidite and its incorporation into a number of different oligonucleotides was published.^{113,114} The melting studies of oligonucleotides containing single or double addition of this dimer in various positions of the

sequence showed duplex destabilisation on average by 5.3 °C degree per modification.¹¹⁴

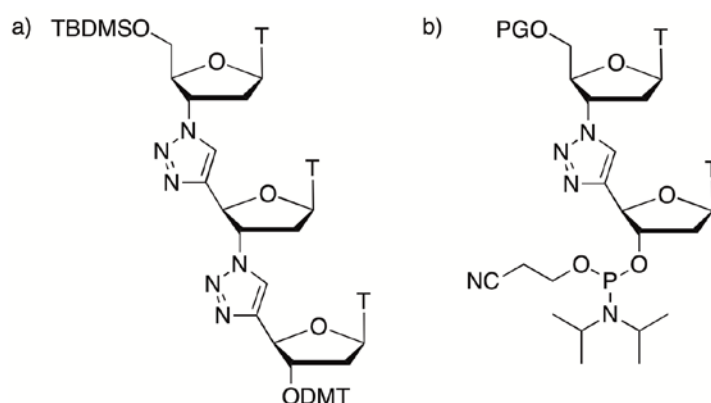
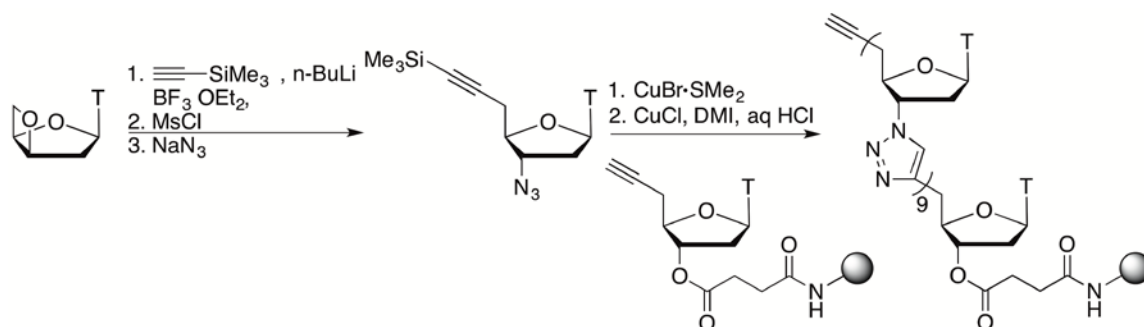


Figure 1.13 Non natural triazole codon by A. Nuzzi (a); T-T triazole dimer phosphoramidite by Chandrasekhar (b).

In 2008, Isobe *et al.* reported the successful synthesis of triazole-linked analogue of DNA, oligothymine consisting of 10 thymidine nucleosides, also using the click chemistry method (Scheme 1.6). Such synthetic oligonucleotide analogues have been shown to form stable duplexes with natural complementary strands of DNA, with a significantly increased melting temperature of the duplex compared to natural DNA duplexes by about 4 °C per modification.¹¹⁵ Further studies describing sequences with all four bases however, were not presented.



Scheme 1.6 Synthesis of triazole 10-mer by Isobe.

In 2011, Madhuri and Kumar published synthesis of two different triazole dimers (Figure 1.14; C and E) as phosphoramidites and incorporated them into 19-mer oligonucleotide in one or two positions.¹¹⁶ These two triazoles were 7 bonds long, therefore longer in comparison to triazole backbones presented before. They found that a single addition of the triazole E decrease thermal stability of the duplex by 11-13.5 °C with complementary DNA or RNA. The double addition of the modification destabilised duplex with complementary DNA or RNA by 17.5 and 23 °C respectively. Incorporation of the triazole backbone C (Figure 1.14; C) had an even bigger destabilising effect, where single addition of C dropped the melting temperature by over 16 °C with DNA and just over 22 °C with RNA. It is important to notice however, that T_m is not the most precise indicator of duplex stability as it strongly depends on the length and the bases content in the sequence.

Efthymiou *et al.* presented the synthesis of the nucleic acid analogues based on a PNA-type scaffold containing single to triple addition of triazole linkage A.^{117,118} The melting studies of these modified oligonucleotides showed destabilising properties decreasing thermal stability with complementary RNA up to 20 °C. They also indicate increase in destabilising effect of this modification the more central the triazole backbone was placed in the PNA oligonucleotide.¹¹⁸

Very recently Palframan and co-workers published the synthesis of morpholino-ribose triazole linked dimer (Figure 1.14; B) and its incorporation to 13-mer oligonucleotide.¹¹⁹ UV melting studies showed that incorporation of the morpholino triazole linked dimer B provided a slight improvement in the melting temperature with complementary RNA when compared to oligonucleotide containing triazole dimer E (Figure 1.14; E) by 1 °C.¹¹⁹

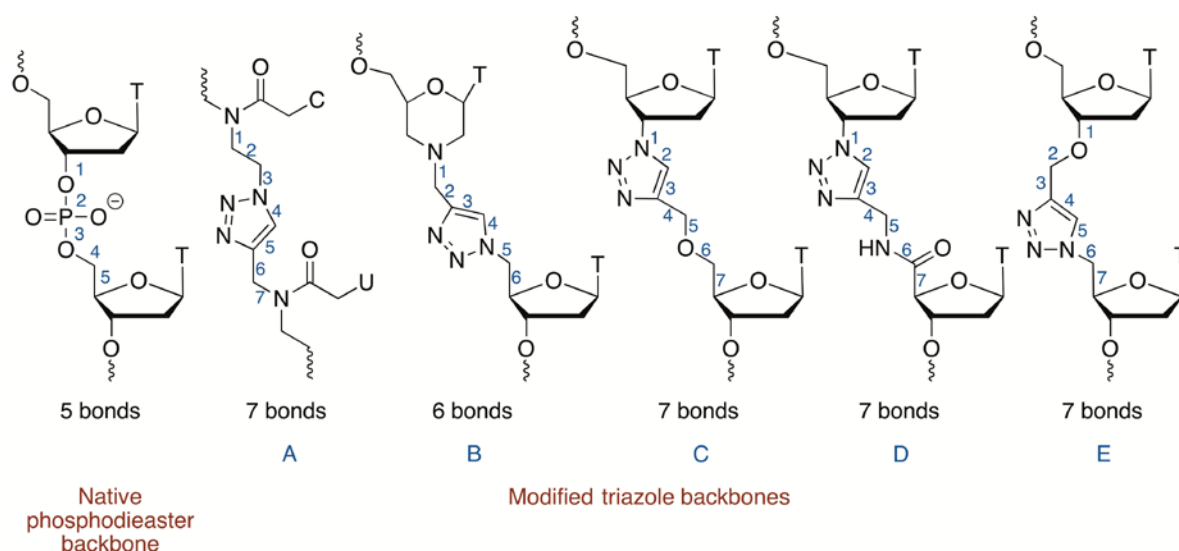


Figure 1.14 Triazole backbone comparison.

In 2008, Brown *et al.* synthesised 7 bonds long unnatural triazole linkage with an amide (Figure 1.14; D).¹²⁰ This click-ligated DNA strand was used as a template in PCR. Amplification of this triazole containing oligonucleotide was successful but only one adenine was found in the complementary strand instead of two at the ligation site.¹²¹ In 2011, Brown *et al.* published synthesis of DNA containing triazole linkage E (Figure 1.14; E), with a more flexible linkage, which was accurately read through by DNA polymerases in *Escherichia coli* and, for the first time, shown to be read in live human cells.^{122,123} Biophysical studies of the triazole backbone E containing oligonucleotide revealed thermal destabilising properties of such modified DNA, showing 8 °C decrease in melting temperature. UV melting studies with triazole and mismatched complementary strands showed a great decrease in melting temperature by 11 °C on average relative to triazole with Watson-Crick base pairs, and substantial loss in base stacking measured by the decrease in hyperchromicity of the UV melting curves compared to native DNA duplex.¹²⁴ These mismatch properties could be very beneficial in potential probe design.

In 2014 A. El-Sagheer and T. Brown published the synthesis of triazole linked oligonucleotide (Figure 1.14; E) with an additional base modification in order to restore the thermal stability that is lowered by the triazole linkage.¹²⁵ The introduction of a G-clamp nucleobase modification on the 3'-side of the triazole dimer (Figure 1.15), was found to be sufficient to increase the duplex stability of the triazole DNA, exceeding that of the natural DNA:DNA duplex by 6.5 °C and by over 9 °C in the case of a DNA:RNA duplex. It has also been revealed that triazole - G-clamp modified ON maintains its high mismatch discrimination properties. Similarly to that of wild type DNA duplex, it showed great destabilisation in the presence of mismatched base pairs close to the modification.¹²⁵

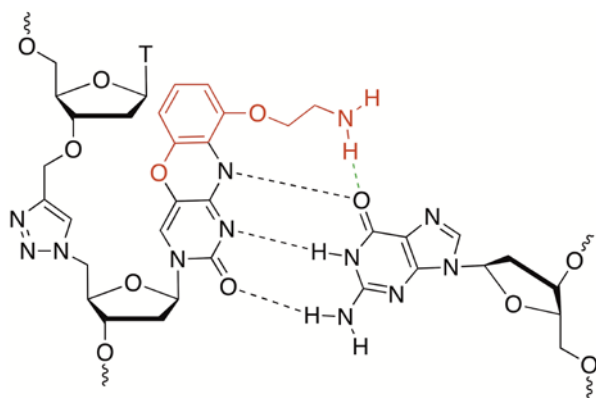
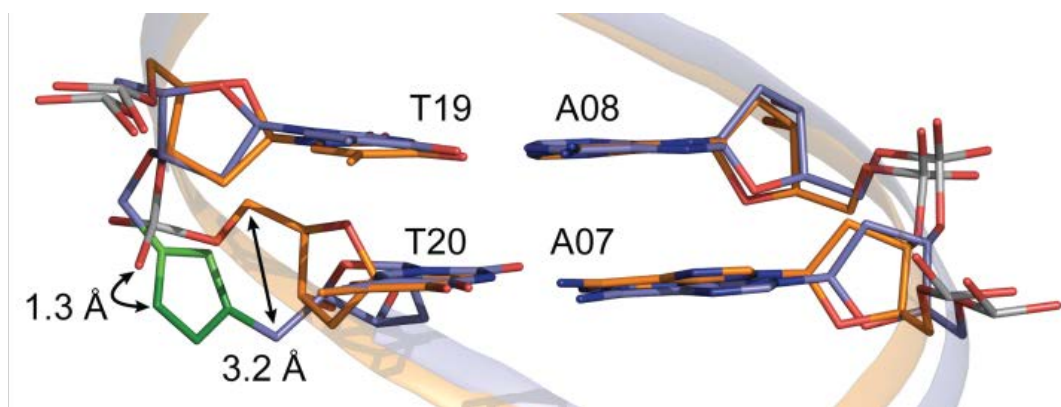


Figure 1.15 Triazole - G-clamp base pair with guanine in complementary DNA. The additional G-clamp nucleobase modification relative to cytosine showed in red; additional hydrogen bonding showed in green.

NMR studies (Scheme 1.7) of a DNA duplex containing a single addition of the 7-bond long triazole backbone E (Figure 1.14; E) showed that the backbone is slightly distorted in order to accommodate the extra length of the modification but that the Watson-Crick base pairing remained intact. This demonstrates that the triazole forms a good mimic of the native DNA phosphate group.¹²⁴



Scheme 1.7 NMR structure of triazole linkage E in DNA duplex. The 5' carbon of the T20 points down and shifts by 3.2 Å relative to its position in the control DNA strand. The triazole ring nitrogen and a phosphate oxygen of DNA control are only 1.3 Å apart. Figure taken from¹²⁴

The chemical ligation of two or more strands of DNA brings potential advantages over enzymatic methods to ligate DNA strands by allowing production of DNA on a larger scale. The properties of triazole DNA open up a wide range of opportunities and possibilities in biochemical and pharmaceutical research.¹¹¹

1.5 AIMS AND THE STRUCTURE OF THE THESES

The aim of this project was to synthesise a novel class of oligonucleotides with reduced backbone charge and increased nuclease stability as antisense oligonucleotides and test them for their antisense splice-switching activity. To this end, the triazole modification was chosen due to its interesting properties. Firstly, incorporation of the neutral triazole in place of the negatively charged phosphodiester should reduce the overall negative charge of the oligonucleotide and potentially improve their cellular uptake. Secondly, being unnatural, the triazole modification should also make the resultant oligonucleotides completely resistant to nuclease degradation.

Based on the work by the Brown group discussed previously, the 3'-methoxytriazolyl thymidine linkage (Figure 1.14; E) is used in this work. Given that this triazole backbone reduces thermal duplex stability, 2'-modifications such as intercalating or groove binding pyrene and anthraquinone derivative or charged guanidine were introduced to potentially counteract this effect. Taking into account these design parameters, phosphoramidite triazole dimer **1** was designed (Figure.1.16).

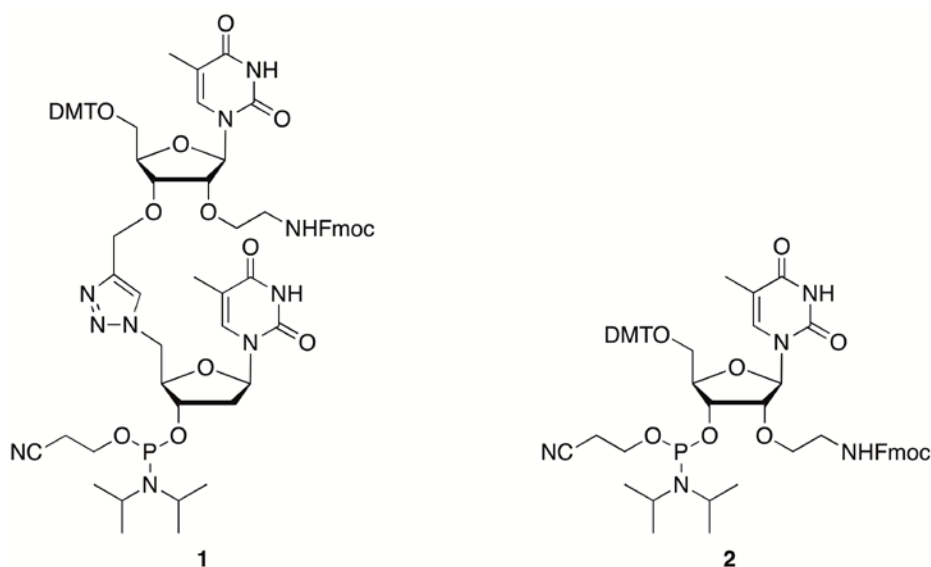


Figure.1.16 Structure of the T-T triazole dimer (1) and 2'-aminoethoxy T (2) phosphoramidite.

Chapter 2 describes synthesis of T-T triazole dimer **1** with the 2'-aminoethoxy group on the 5'-side of the dimer and also describes the 2'-aminoethoxy thymidine phosphoramidite **2** and their single, double and triple incorporation into the oligonucleotide *via* automated solid-phase synthesis. The location of the 2'-aminoethoxy group relative to the triazole backbone was also investigated. The synthesis of the oligonucleotide with a triazole linkage and a 2'-aminoethoxy group at the nucleoside on the 3'-side of the triazole backbone by click ligation of two shorter strands of DNA in solution; one with a 3'-O-propargyl group and the other with 5'-azide and 2'-O-

aminoethoxy groups is discussed. Finally, post-synthetic functionalisation of the amine is described.

Chapter 3 focuses on the biophysical properties of the synthesised modified oligonucleotides. The thermal stability of duplexes containing the modification, the effect on the global structural conformation and effect of mismatch (or off-target) effects are discussed.

Chapter 4 applies the concepts from the previous chapters for more biologically relevant ONs. Synthesis of a new T-C triazole phosphoramidite dimer **38** is described as well as its multiple incorporation into fully phosphorothioate / 2'-OMe modified oligonucleotides. The biophysical properties of the modified oligonucleotides and their duplexes are discussed.

Chapter 5 focuses on the use of the phosphorothioate - 2'-OMe - triazole hybrids from Chapter 4 as antisense oligonucleotides in a splice-switching luciferase assay in cancerous cells.

2 SYNTHESIS

*This chapter describes synthesis of a novel T-T triazole dimer containing 2'-aminoethoxy group **1** and a previously reported 2'-aminoethoxy T **2** as phosphoramidites. Incorporation of these monomers into oligonucleotides via automated solid-phase oligonucleotide synthesis is then discussed followed by post-synthetic labelling of the 2'-aminoethoxy group with previously prepared pyrene NHS ester **30**, anthraquinone derivative active carbonate **35** and guanidine. Synthesis of oligonucleotides functionalised with pyrene via different linker length (one and four-carbon long) between oligonucleotide and pyrene is also presented alongside with oligonucleotides labeled with pyrene via different type of linker (more rigid amide and more flexible amine).*

2.1 INTRODUCTION TO SYNTHESIS OF NUCLEIC ACIDS

2.1.1 Solid-phase oligonucleotides synthesis

The most widely used method for robust and efficient chemical synthesis of short nucleic acids is automated oligonucleotide solid-phase synthesis.¹²⁶ Solid-phase synthesis allows for the usage of large excesses of reagents to quickly drive reactions to completion, with the excess reagents easily washed away. The synthesis proceeds on an insoluble solid support typically made of controlled pore glass (CPG) or polystyrene also referred to as resin.

The solid-phase oligonucleotide synthesis proceeds in the 3'- to 5'- direction and uses phosphoramidite monomers as building blocks.¹²⁷⁻¹²⁹ One nucleotide is added during a single synthetic cycle and each synthetic cycle consists of a number of steps that are presented in Figure 2.1. A standard cycle begins with a protected nucleoside pre-attached to the solid support. To begin synthesis the 5'-*O*-DMTr protecting group (DMTr = 4,4'-dimethoxytrityl) of the support-bound nucleoside is removed, typically using 3% trichloroacetic acid in CH₂Cl₂ (detritylation). The 5'-hydroxyl nucleoside is then reacted with a nucleoside phosphoramidite monomer in presence of an activator (tetrazole or a derivative) in the activation and coupling step (I). Unreacted 5'-hydroxyl nucleoside is removed from the synthetic cycle in a capping step (II), in which the free 5'-hydroxyl group is blocked by acetylation using acetic anhydride, *N*-methylimidazole and pyridine. In the subsequent oxidation step (III) the phosphorus atom of the phosphite-triester (P^{III}) formed in the coupling step is converted to a stable (P^V) form. Due to acid liability of P^{III}, oxidation is performed before the next detritylation step and is achieved using iodine in the presence of water and pyridine. In order to allow addition of the next nucleoside in the cycle, the DMTr protecting group at the 5'-end

of the resin-bound oligonucleotide is removed in the detritylation step (IV). The whole cycle is repeated for each added base until the required oligonucleotide is synthesised. The process is carried out under an atmosphere of argon or nitrogen and with anhydrous solvents, as the phosphoramidite coupling reaction is very sensitive to moisture and oxygen. All solution-phase reagents are used in large excess relative to the support bound oligonucleotide to ensure high efficiency of each step. The efficiency of each cycle can be monitored during the detritylation step, when the 4,4'-dimethoxytrityl cation is liberated, which absorbs light at 495 nm.

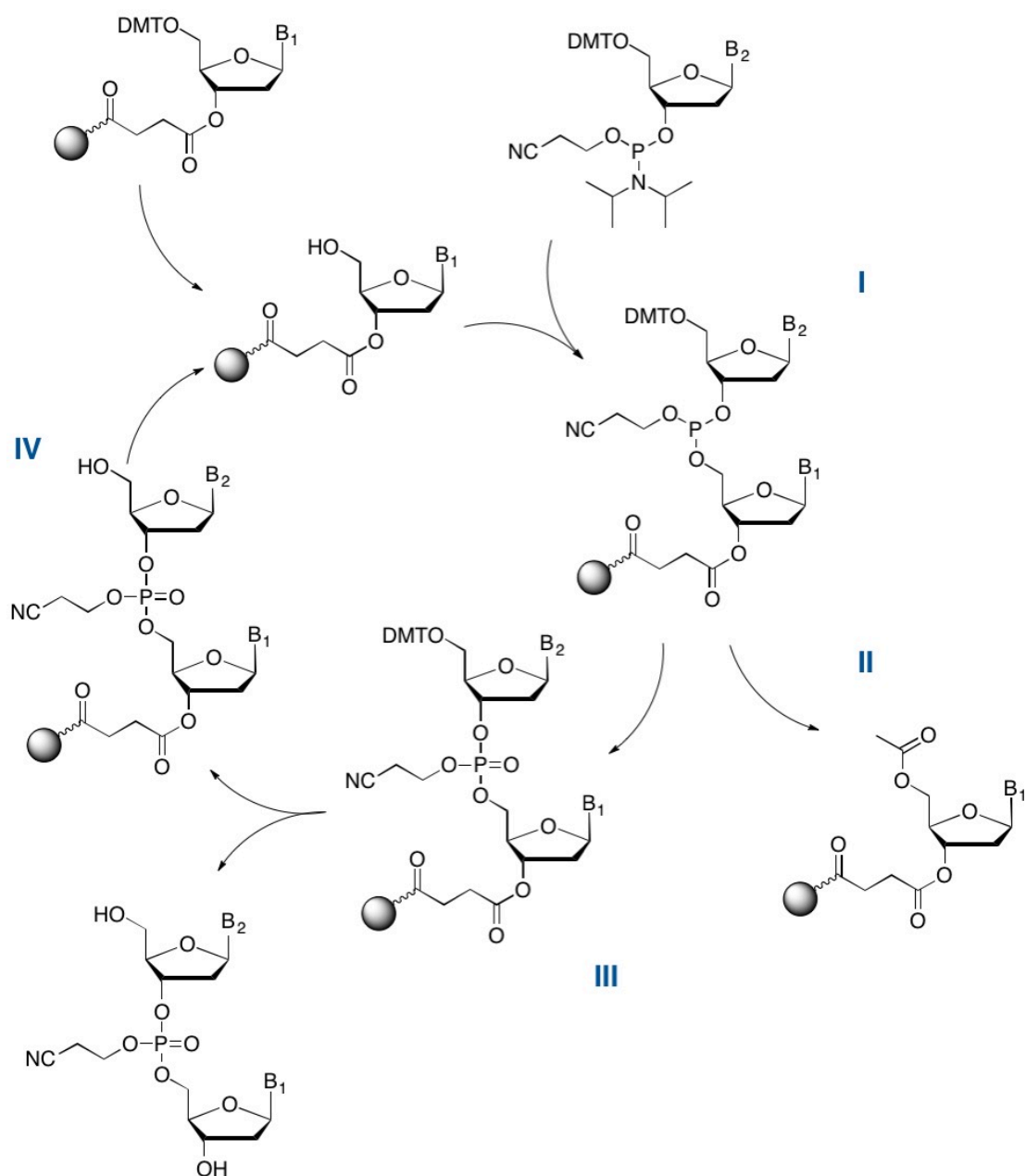


Figure 2.1 Solid-phase oligonucleotide synthesis cycle.

On completion of oligonucleotide assembly the full oligonucleotide can be released from the solid support *via* ester hydrolysis by treatment with 35% ammonium hydroxide. Removal of protecting groups from heterocyclic nucleobases and from phosphates requires further heating in aqueous ammonia.

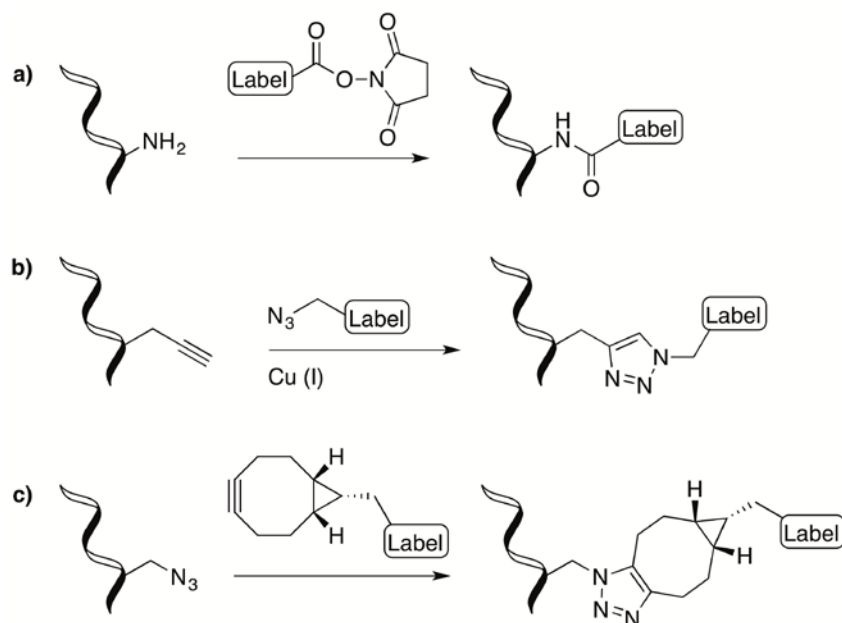
2.1.2 Synthesis of modified nucleosides as phosphoramidite monomers

As described in section 2.1.1 above one of the requirements for automated oligonucleotide synthesis is the availability of monomer building blocks in an appropriate form, where the 5'-hydroxyl is protected with a dimethoxytrityl group (DMTr) and the 3'-hydroxyl is converted to 3'-*O*-(2-cyanoethyl)-*N,N*-diisopropyl phosphoramidite group.^{130,131} When designing phosphoramidite monomers it is also crucial to ensure the compatibility of all the functional groups with the full oligonucleotide solid-phase synthesis cycle. During oligonucleotide synthesis each cycle consists of a capping step that utilises acetic anhydride, which is a very reactive reagent. In some cases when coupled to an unprotected nucleophile in the oligonucleotide it can be very difficult to remove. More importantly, the phosphoramidite species added in each cycle is a very strong electrophile. Nucleotides are rich in various nucleophilic groups and so it is essential to protect these. However, at the end of oligonucleotide synthesis all the protecting groups should be removable with an aqueous ammonia treatment – the standard oligonucleotide deprotection method. Due to these and other requirements, the range of compliant synthetic reactions is limited.

2.1.3 Post-synthetic labelling of modified oligonucleotides

Highly modified synthetic nucleic acids are in high demand in biochemical, molecular biology and biotechnology research. Functionalisation of DNA or RNA strands with various compounds such as intercalators or fluorescent dyes is of vital importance in the preparation of DNA probes for numerous applications such as antisense therapeutics, DNA detection and diagnostics.^{132,133} Various modifications can be incorporated into the DNA strand *via* solid-phase oligonucleotide synthesis in the form of phosphoramidite monomers. However, the synthesis of phosphoramidite monomers is in some cases difficult. An alternative approach is post-synthetic

labelling. There are a number of methods commonly used for post-synthetic oligonucleotide functionalisation, such as: amine-active ester coupling, copper-catalysed azide-alkyne cycloaddition reaction or strain-promoted azide-alkyne cycloaddition reaction (Scheme 2.1).^{134,135}



Scheme 2.1 Post-synthetic labelling strategies; a) amine-active ester coupling; b) copper-catalysed azide-alkyne cycloaddition reaction; c) strain-promoted azide-alkyne cycloaddition reaction.

In the current study amine-active ester coupling was chosen. This reaction requires a nucleic acid bearing a primary amino group and an activated ester such as an N-hydroxysuccinimide (NHS) ester of the label chosen for the functionalisation. The reaction is carried out under basic conditions ranging in pH from 7.2 to 9 and the product is a stable amide. A large excess of the NHS ester is used to drive the reaction to completion because the active ester hydrolyses during the course of the reaction.

Incorporation of an amine-containing monomer into the DNA chain during solid-phase synthesis requires an appropriate protecting group. The most commonly used group is trifluoroacetyl. In the current study it was decided to use the orthogonally removable Fmoc (9-fluorenylmethyloxycarbonyl) group,

thus permitting selective removal of Fmoc while the oligonucleotide remains attached to the solid support. Functionalising an oligonucleotide attached to the solid support with a free amine can be beneficial as the reaction can be then carried out in an organic solvent, which can prove useful when dealing with hydrophobic labels. Reaction in anhydrous organic solvent limits the danger of hydrolysis of the NHS ester of the label, and allows usage of a lower quantity of NHS ester. Furthermore, excess NHS ester can be used to drive the reaction to completion and it can be easily washed away leaving only the labelled oligonucleotide attached to the resin.

2.2 SYNTHESIS OF MODIFIED PHOSPHORAMIDITES

As outlined previously (section 1.5) triazole-containing phosphoramidite dimer **1** is required for the synthesis of triazole-modified oligonucleotides for this project. A small number of publications describe the synthesis of 3'-*O*-methylenetriazole thymidine dimer.

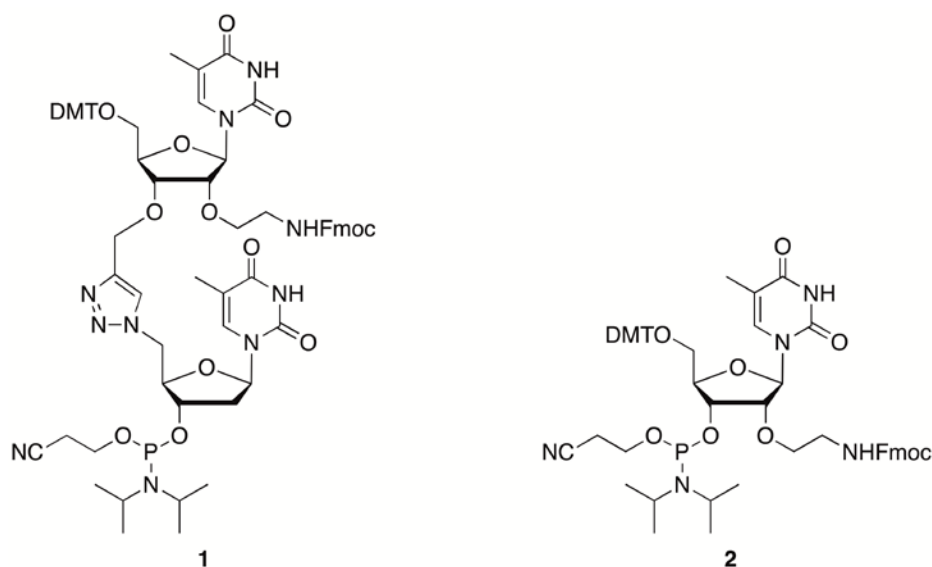
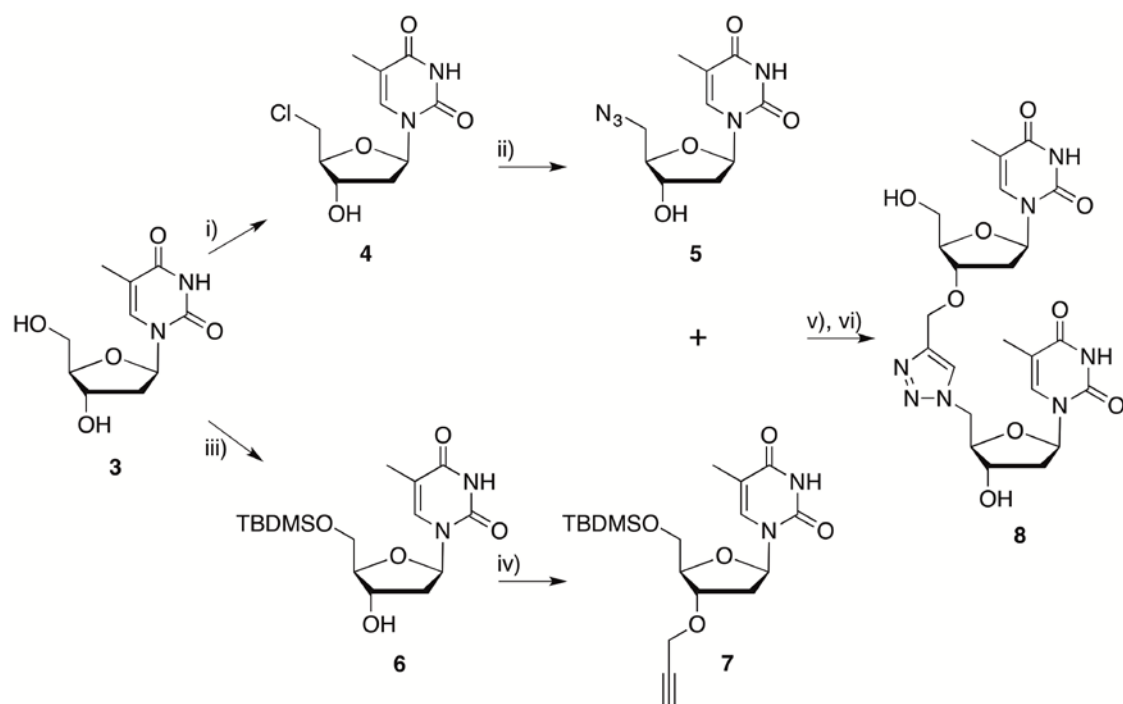


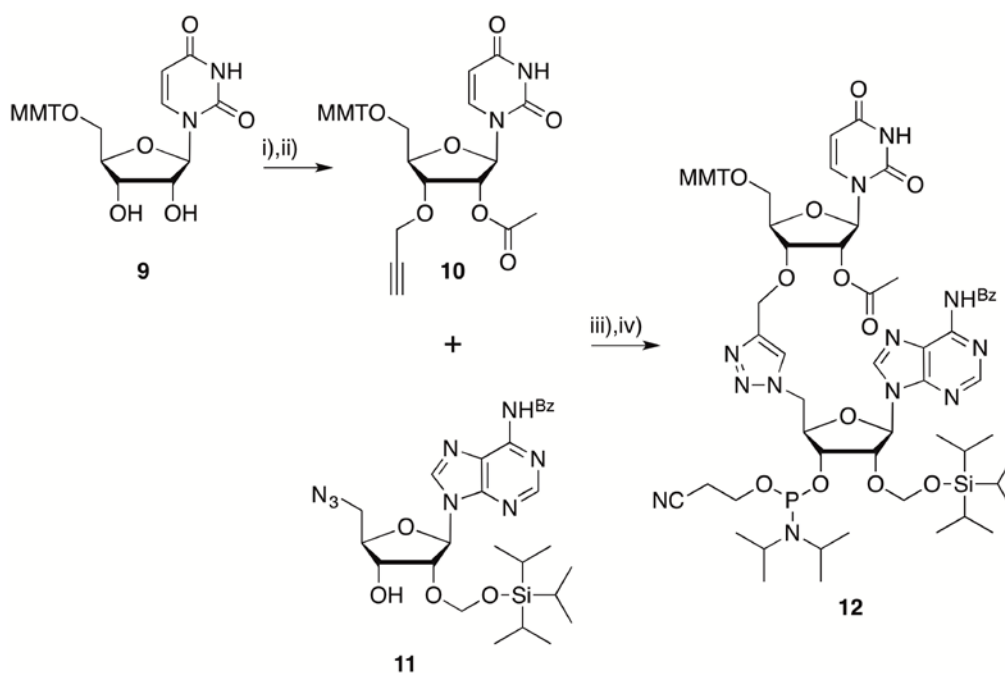
Figure 2.2 Structures of T-T triazole dimer (1) and 2'-aminoethoxy T (2) phosphoramidites.

In 2008 Zerrouki and co-workers published the synthesis of dinucleotide **8** (Scheme 2.2).¹³⁶ Both precursors, the 5'-azido T **5** and 3'-*O*-propargyl T **7** were synthesised from thymidine. First, selective chlorination of the 5'-primary hydroxyl group of the thymidine was performed with tosyl chloride in anhydrous DMF giving the desired 5'-chloro-5'-deoxythymidine **4** in 80% yield. Next, **4** was converted to azide with sodium azide in DMF to deliver 5'-azido-5'-deoxythymidine **5** in 91% yield. Nucleoside **7** was synthesised first by selective protection of the 5'-hydroxyl group using *tert*-butyldimethylsilyl (TBDMS) chloride in presence of DMAP (4-(dimethylamino)pyridine) in anhydrous pyridine, giving 5'-*O*-TBDMS **6** in 98% yield. Next, alkylation of the 3'-hydroxyl was performed using NaH and propargyl bromide in THF, delivering nucleoside **7** in quantitative yields. Finally, click reaction between **5** and **7** was achieved using Cu^I salts with DIPEA in DMF giving the triazole dimer in 85% yield, followed by the deprotection of the 5'-hydroxyl to give dimer **8** in 70% yield.



Scheme 2.2 Synthesis of T-T triazole dimer (8) by Zerrouki and co-workers. (i) TsCl, DMF, MW (80 °C, 300 W, 1 min), 80%; (ii) NaN₃, DMF, MW (80 °C, 300 W, 3 min), 91%; (iii) TBDMS-Cl, DMAP, pyridine, 10 h, 98%; (iv) NaH, propargyl bromide, THF, MW (40 °C, 200 W, 3 min); (v) CuI, DIPEA, DMF, 85%; (vi) TBAF, THF, rt, 7 h, 70%.¹³⁶

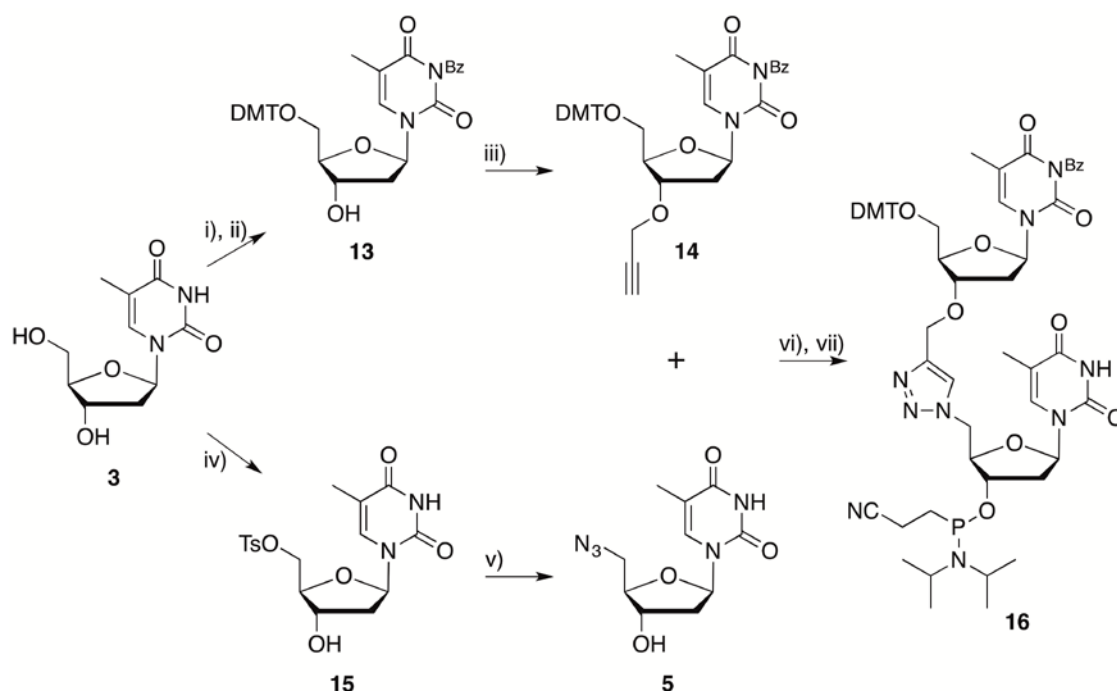
In 2011 Rozners and co-workers published a successful synthesis of the U-A triazole-ribodinucleoside as phosphoramidite **12** (Scheme 2.3).¹³⁷ First, 5'-*O*-4-methoxytrityluridine **9** was treated with dibutyltin oxide and then propargyl bromide to give mixture of 3'- and 2'-*O*-propargyluridine from which the target compound was isolated in 28% yield. Compound **10** was obtained by protection of the 2'-hydroxyl with acetyl. The triazole dimer was formed by reacting compound **10** with 5'-azido-2'-*O*-TOM adenosine **11** in the CuAAC click reaction in 79% yield, followed by standard phosphitylation procedure giving dimer **12** in a respectable yield of 47%.



Scheme 2.3 Synthesis of U-A triazole ribodinucleoside (12) by Rozners and co-workers. (i) Bu_4NBr , Bu_2SnO , DMF, 100 °C, 2 h, then propargyl bromide, 45 °C, 24 h, 28%; (ii) acetic anhydride, pyridine, rt, 5 h, 98%; (iii) CuSO_4 , sodium ascorbate, THF/EtOH/ H_2O , 60 °C, 12 h, 79%; (iv) DIPEA, $\text{CIP}(\text{OCH}_2\text{-CH}_2\text{CN})\text{N}(\text{Pr})_2$, CH_2Cl_2 , rt, 6 h, 47%.¹³⁷

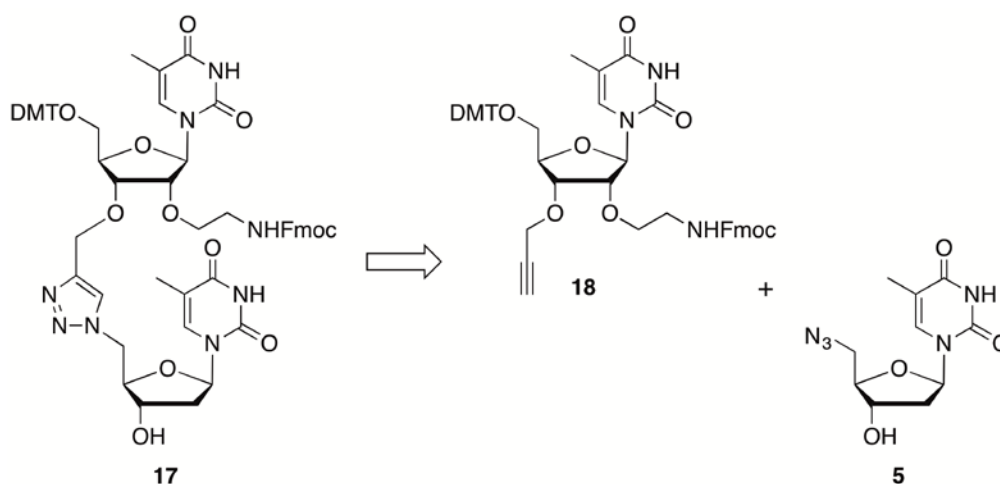
In 2012 Madhuri *et al.* published a very efficient synthetic route for synthesis of the T-T triazole dimer in the phosphoramidite form **16** (Scheme 2.4).¹¹⁶ In this protocol, the 5'-hydroxyl of thymidine **3** was protected as a trimethylsilyl (TMS) ether with TMS-Cl in pyridine, followed by protection of the N3 of the thymidine nucleobase with benzoyl chloride. The TMS group was removed with aqueous ammonia and then derivatised with dimethoxytrityl (DMTr) by treatment with DMTr-Cl in pyridine to give **13** in 85% yield. Compound **13** was then alkylated with propargyl bromide and NaH in DMF to get the 3'-O-alkyne derivative **14** in 55% yield. 5'-Azido-thymidine was synthesised from thymidine **3** through monotosylation at the 5'-position with tosyl chloride in pyridine then treatment with an excess of sodium azide in dry DMF to give compound **5** in 87% yield. Finally, the triazole dinucleoside was synthesised by coupling **5** with **14** in the presence of CuSO_4 and sodium ascorbate in

80% yield, followed by a standard phosphitylation procedure to deliver phosphoramidite **16**.



Scheme 2.4 Synthesis of T-T triazole dinucleoside (16**) by Madhuri and Kumar.** (i) TMS-Cl, pyridine then Bz-Cl then aq NH₃, DMF, 87%; (ii) DMTr-Cl, pyridine, 85%; (iii) propargyl bromide, NaH, DMF, 55%; (iv) Ts-Cl, pyridine, 85%; (v) NaN₃, DMF, 87%; (vi) 0.2% CuSO₄, 0.5% sodium ascorbate, *t*-BuOH/H₂O, 80%; (vii) DIPEA, CIP(OCH₂-CH₂CN)N(*i*Pr)₂, CH₂Cl₂, rt, 87%.¹¹⁶

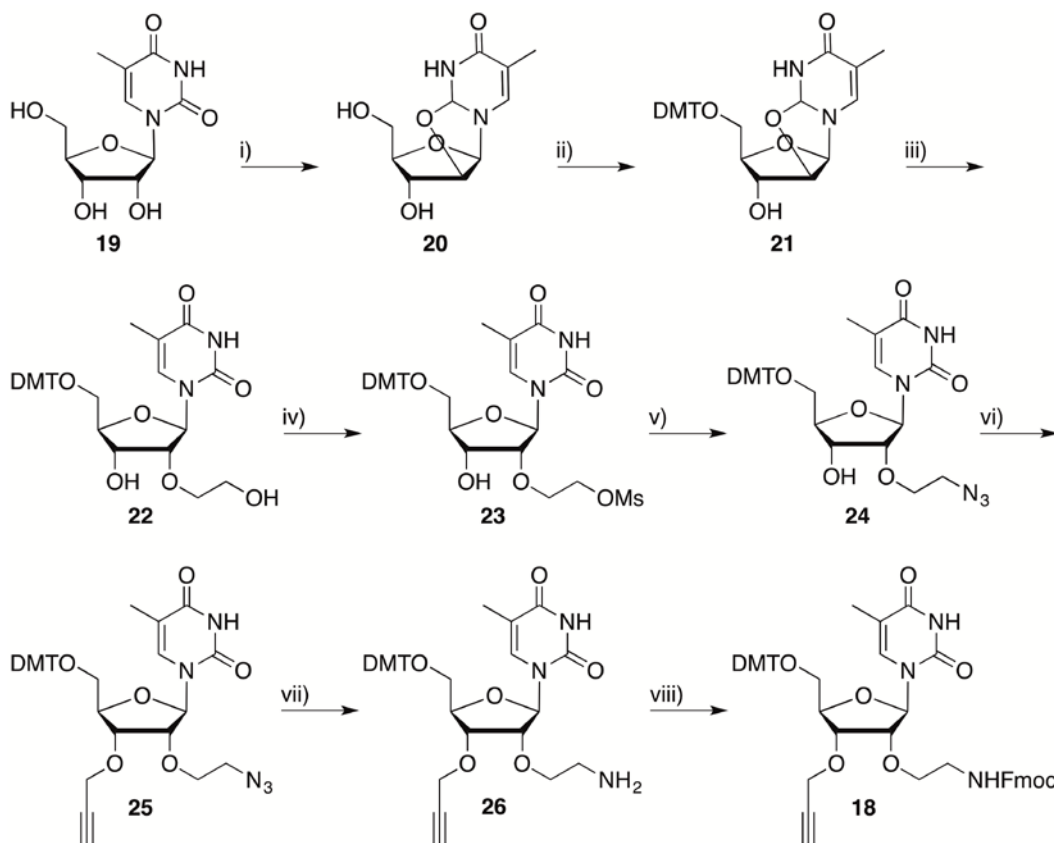
In this project, in order to incorporate the 2'-aminoethoxy group into the triazole dimer, preparation of the T-T triazole dimer **17** required the synthesis of two building blocks: 3'-*O*-propargyl-2'-aminoethoxy T **18** and 5'-azido T **5** (Scheme 2.5).



Scheme 2.5 T-T triazole (17**) retrosynthesis strategy.**

The synthesis of the 3'-*O*-propargyl nucleoside **18** was carried out according to Scheme 2.6. First, five steps were performed in accordance to literature procedures to obtain compound **24**.¹³⁸ The first step gave a very good yield of 78% for 2,2'-anhydro-5-methyluridine **20** formation. Optimisation was necessary for the first step, as the reaction mixture began to gel while heating. The gel was very difficult to dissolve and stopped the reaction from reaching completion. Adding almost double the published amount of solvent and heating the reaction mixture to 120 °C (20 °C higher than reported) allowed the reaction go to completion. Next, 5'-hydroxyl protection with DMT was achieved in 80% yield giving the nucleoside **21**. The third step, synthesis of 2'-*O*-hydroxyethyl derivative **22**, was less efficient giving 56% yield, due to difficulties in purification (related to removing the solid titanium complex from the reaction mixture). The next step, addition of mesyl to the 2'-*O*-hydroxyethyl, gave a moderate yield, due to appearance of a by-product arising from reaction of the mesyl group with the 3'-hydroxyl group. Subsequently, the displacement of the mesyl group with azide in presence of 18-crown-6 ether was successful, giving product **24** in 57% yield. To incorporate the propargyl group, compound **24** was reacted with propargyl bromide in the presence of NaH to give **25** in 72% yield. Next, a Staudinger

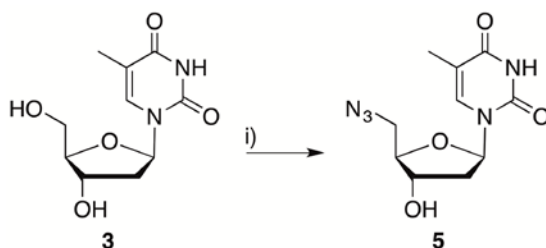
reduction was performed to reduce the azide moiety and give the free amine **26** in 55% yield, which was then protected with Fmoc, giving 2'-*O*-aminoethoxy-3'-*O*-propargyl nucleoside **18** in 87% yield.



Scheme 2.6 Synthesis of 2' -aminoethoxy-3' -*O*-propargyl-5' -*O*-DMT thymidine nucleoside (18**):** (i) diphenyl carbonate, NaHCO₃, DMF, 100 °C, 4 h, 78%; (ii) DMT-Cl, pyridine, rt, 5 h, 80%; (iii) ethylene glycol, Ti(O^{*i*}Pr)₄, NaHCO₃, DMF, 150 °C, 24 h, 56%; (iv) Ms-Cl, Et₃N, DCM, -70 °C, 3 h; (v) NaN₃, DMF, 18-crown-6, 60 °C, 14 h, 57%; (vi) NaH, propargyl bromide, THF, 14 h, 72%; (vii) Ph₃P, H₂O, THF, 45 °C, 14 h, 55%; (viii) Fmoc-OSu, DCM, pyridine, rt, 12 h, 87%.

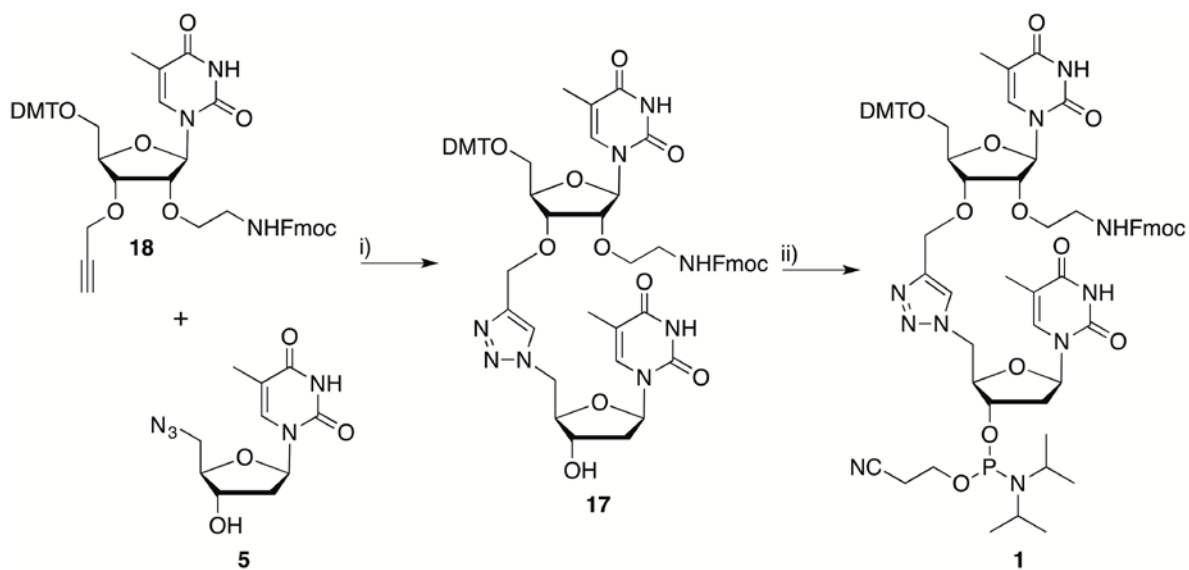
5'-Azido nucleoside **5** was prepared following the procedure reported by Said *et al.*, which was optimised to improve the yield.¹³⁹ The highest efficiency was obtained when reacting thymidine with carbon tetrabromide in the presence of triphenylphosphine in anhydrous DMF at room temperature, until over 50% of the starting material was consumed (monitored by TLC), followed by the

addition of sodium azide and stirring for a further 4 hours at 60 °C (Scheme 2.7).



Scheme 2.7 Synthesis of 5'-azido thymidine (5). (i) CBr_4 , PPh_3 , DMF, NaN_3 , rt to 60 °C, 18 h, 53%.¹³⁹

The two nucleosides **5** and **18** were then coupled using a CuAAC reaction to give triazole dimer **17**, which was finally phosphitylated using 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite and *N,N*-diisopropylamine in CH_2Cl_2 , to give the desired monomer **1** (Scheme 2.8).¹³⁰



Scheme 2.8. Formation of the T-T triazole dimer phosphoramidite (1). (i) CuSO_4 , sodium ascorbate, TBTA ligand, DMF, H_2O , 4 h, rt, 86%; (ii) DIPEA, $\text{CIP}(\text{OCH}_2\text{-CH}_2\text{CN})\text{N}(\text{Pr})_2$, CH_2Cl_2 , rt, 31%.

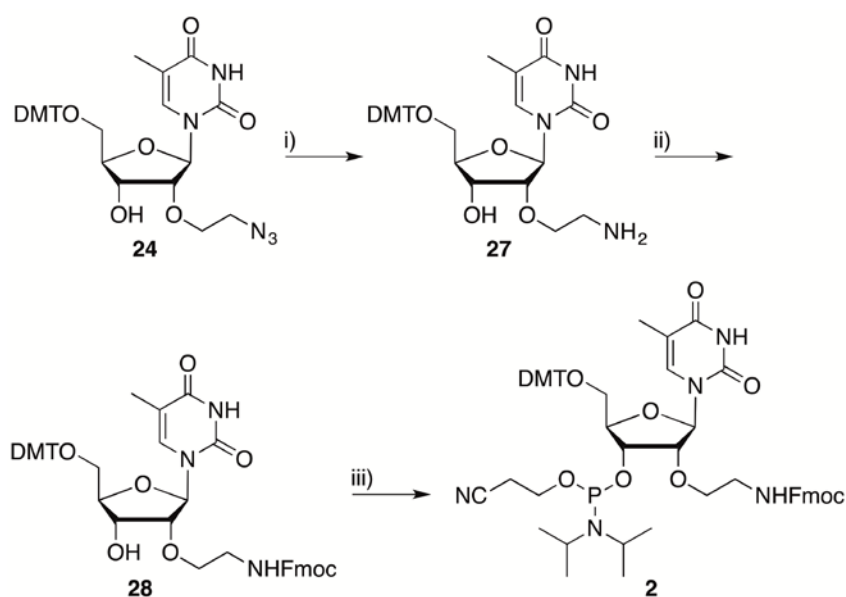
There is very little published work on the synthesis of pyrimidine nucleosides with primary amino groups tethered on the 2'-position of the ribose sugar. For linker longer than two carbon atoms between the 2'-hydroxyl and the

amino group, to this author's knowledge, there are only two synthetic routes reported. In 1995 Manoharan and co-workers described addition of the aminohexyl moiety to the 2'-hydroxyl position of the nucleoside. This reaction gave a mixture of 2'- and 3'- alkylated products in a 55:45 ratio respectively in moderate yield.¹⁴⁰ A year later Griffey *et al.* incorporated 2'-aminopropyl and 2'-aminopentyl units using the phthalimide or trifluoroacetyl – protected derivative of the amine.¹⁴¹

Several methods for the synthesis of the aminoethoxy derivatives have been reported previously. In 1998 Cuenoud *et al.* published a synthesis which started by protecting the nucleoside simultaneously at both 3'- and 5'-hydroxyl positions, using 1,3-dichloro-1,1,3,3-tetra isopropylidisiloxane (Markiewicz reagent).¹⁴² This was followed by the alkylation of the 2'-hydroxyl group with methyl bromoacetate, and finally reduction of the ester group and tosylation. The tosyl group was then displaced with azide (using NaN_3), which could then be reduced to form the desired protected 2'-aminoethoxy nucleoside. In 2002 Darby and co-workers reported the synthesis of the same monomer, following the procedure described above but introducing nitrogen using phthalimide through a Mitsunobu reaction.¹⁴³

Other research groups have also reported alternative approaches to the synthesis of this monomer. Manoharan *et.al.* used the borate ester of *N*-(2-hydroxyethyl)phthalimide to ring open 2,2'-anhydro-5-methyluridine by nucleophilic attack. Subsequently, the nucleoside was protected and finally treated with hydrazine to insert the desired amine.¹⁴⁴ The procedure required a stainless steel bomb heated to 150 °C and gave only around 20% yield after purification. Blommer *et.al.* followed the same approach as described above, opening the 2,2'-anhydro-5-methyluridine by use of the same boron derivative of 2-hydroxy-ethylphthalimide, but in the presence of $\text{Ti}(\text{O}^i\text{Pr})_4$, which increased the yield to 32%.¹⁴⁵

Finally in 2010 Brown and co-workers reported a fully optimised method for synthesis of 5'-*O*-DMT-2'-aminoethoxythymidine-3'-*O*-phosphoramidite **2**, which was used in this project (Scheme 2.9).¹³⁸ Precursor **24** was used from a synthesis of the previously described nucleoside **18** (Scheme 2.6). A Staudinger reduction was performed on **24** to reduce the azide moiety and give free amine **27** in 72% yield, which was then protected with Fmoc giving **28** in 52% yield. Finally phosphitylation was accomplished affording the phosphoramidite monomers **2** in 48% yield.



Scheme 2.9 Synthesis of 2'-aminoethoxy thymidine (2). (i) Ph_3P , H_2O , THF, 45 °C, 14 h, 72%; (ii) Fmoc-OSu, DCM, pyridine, rt, 12 h, 52%; (iii) DIPEA, $\text{CIP}(\text{OCH}_2\text{-CH}_2\text{CN})\text{N}(\text{iPr})_2$, CH_2Cl_2 , rt, 48%.

2.3 SYNTHESIS OF MODIFIED DNA OLIGODEOXYRIBONUCLEOTIDES

Upon successful synthesis of the modified phosphoramidite monomers **1** and **2**, the next step was to synthesise short oligonucleotides containing these modified monomers.

2.3.1 Synthesis of modified oligonucleotides using the backbone and sugar modified phosphoramidite monomers **1** and **2**

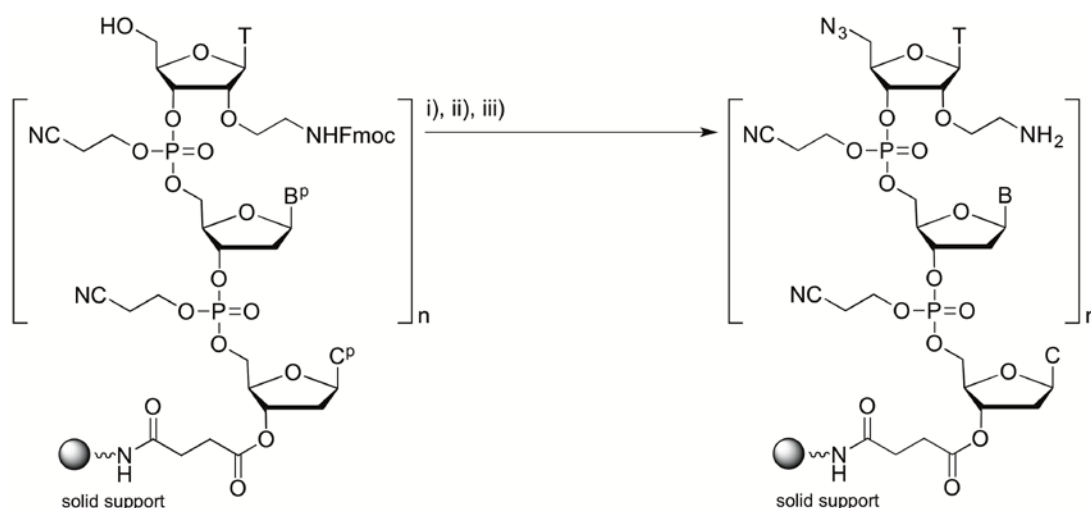
To test our hypothesis, whether an oligonucleotide containing the triazole backbone could potentially make a successful antisense probe, a set of modified oligodeoxyribonucleotides was synthesised: ODN-A-**t1** - with the single addition of triazole dimer **1** in the middle of the sequence, ODN-A-**a1** - with the single addition of 2'-aminoethoxy T **2** and the unmodified DNA oligonucleotide ODN-A-1 with their complementary RNA (ORN-A-2) and DNA (ODN-A-2) strands (Table 2.1). Sequence 'A' – CGACGCTTGCAGC was chosen as it has no secondary structure and is an appropriate length for the duplex thermal stability experiments.

Table 2.1. List of oligonucleotides used in the first study. ^aT = 2'-aminoethoxy T, **t** = triazole linkage, ^{Me}C = 5-methyl C, ^{Me}C^Y = 3'-O-propargyl-5-methyl C, ^aT^Z = 5'-azido-2'-aminoethoxy T.

code	sequence 5' to 3'	calc. mass	found mass
ODN-A-1	CGACGCTTGCAGC	3936	3936
ORN-A-2	GCUGCAAGCGUCG	4155	4156
ODN-A-2	GCTGCAAGCGTCG	3976	3976
ODN-A- t1	CGACGC ^a T ^t TGCAGC	3996	3996
ODN-A- a1	CGACGC ^a TTGCAGC	3995	3997
ODN-A- t2	CGACG ^{Me} C ^t TTGCAGC	4010	4009
ODN-A-3	CGACG ^{Me} C ^Y	1830	1830
ODN-A- a3	^a T ^Z TGCAGC	2156	2156

The position of the 2'-aminoethoxy group relative to the triazole backbone was also investigated. The design of the triazole dimer **1** places the 2'-aminoethoxy group on the nucleoside to the 5'-side of the triazole moiety. We were interested to see whether positioning the 2'-aminoethoxy group on the nucleoside to the 3'-side of the triazole backbone would change the biophysical properties of the oligonucleotide duplex. To achieve this, a new

triazole dimer was required with the 2'-aminoethoxy group on the 3'-side of triazole dimer. This was accomplished by click ligation of two shorter strands of DNA in solution; one with a 3'-*O*-propargyl group - ODN-A-3 and the other with 5'-azido and 2'-aminoethoxy groups - ODN-A-*a*3 (Table 2.1). The 5'-azido oligonucleotide was prepared using methodology originally reported by Kool *et. al*, which requires conversion of the 5'-hydroxyl to azide (Scheme 2.10).^{146,147} Such a functional group conversion has not previously been reported on 2'-aminoethoxy oligonucleotides and it was found that the reaction reached completion very efficiently.



Scheme 2.10 Conversion of the 5'-OH group of the 2'-aminoethoxy oligonucleotide ODN-A-*a*2 to azide ODN-A-*a*3 on solid support. (i) [CH₃P(OPh)₃]⁺I⁻, (ii) NaN₃, 55 °C; (iii) conc. aq. NH₃, 5 h, 55 °C.

Next, short alkyne and azide oligonucleotides (ODN-A-3 7-mer and ODN-A-*a*3 6-mer respectively) were released from the solid support, deprotected and then ligated *via* the CuAAC reaction in the presence of water soluble HPTA ligand, forming a single DNA strand ODN-A-*t*2 (Table 2.1) with the 2'-aminoethoxy group on the 3'-side of the triazole linker.¹⁴⁶ The reaction proceeded efficiently without the need for a complementary splint strand. Encouraged by the preliminary biophysical studies (discussed in detail in Chapter 3) it was decided to expand the range and multiplicity of

modifications in the oligonucleotides. Therefore, synthesis of oligonucleotides with multiple additions of monomers **1** and **2** was the next step of this project. Sequence 'A' needed to be changed to accommodate double and triple additions of the modified monomers. Therefore sequences 'B' - CGTTCGCTTGCAGC and 'C' – CGTTCGCTTGCATGC were designed, allowing double addition of the triazole dimer **1** and triple addition of 2'-aminoethoxy T **2**, with minimal sequence changes. Three new oligonucleotides were successfully synthesised with single and double additions of triazole dimer **1** (ODN-B-**f1**, ODN-B-**f2**, ODN-B-**f3**) and three new oligonucleotides with single, double and triple addition of 2'-aminoethoxy T **2** (ODN-B-**a1**, ODN-B-**a2**, ODN-C-**a1**); (Table 2.2).

Table 2.2 List of oligonucleotides with single, double or triple addition T-T triazole dimer (1) or 2'-aminoethoxy T (2) based on sequence B and C and their complementary RNA and DNA strand.

code	sequence 5' to 3'	calc. mass	found mass
ODN-B-1	CGTTCGCTTGCAGC	4231	4231
ORN-B-2	GCUGCAAGCGAACG	4508	4508
ODN-B-2	GCTGCAAGCGAACG	4298	4299
ODN-B- f1	CG ^a T f TCGCTTGCAGC	4291	4292
ODN-B- f2	CGTTCGC ^a T f TGCAGC	4291	4292
ODN-B- f3	CG ^a T f TCGC ^a T f TGCAGC	4350	4352
ODN-B- a1	CGTTCGC ^a TTGCAGC	4290	4291
ODN-B- a2	CG ^a TTTCGC ^a TTGCAGC	4349	4350
ODN-C-1	CGTTCGCTTGCATGC	4535	4536
ORN-C-2	GCAUGCAAGCGAACG	4597	4597
ODN-C-2	GCATGCAAGCGAACG	4611	4612
ODN-C- a1	CG ^a TTTCGC ^a TTGCA ^a TGC	4712	4713

After the synthesis and cleavage/deprotection the oligonucleotides were purified by RP-HPLC ready for further functionalisation.

2.4 POST-SYNTHETIC FUNCTIONALISATION OF AMINE-MODIFIED

OLIGONUCLEOTIDES

In this study, three labels were chosen for the post-synthetic labelling (Figure 2.3): pyrene, anthraquinone and guanidine (for reasons explained in details in Chapter 3). The length and flexibility of the linker between 2'-aminoethoxy group and the label was also investigated.

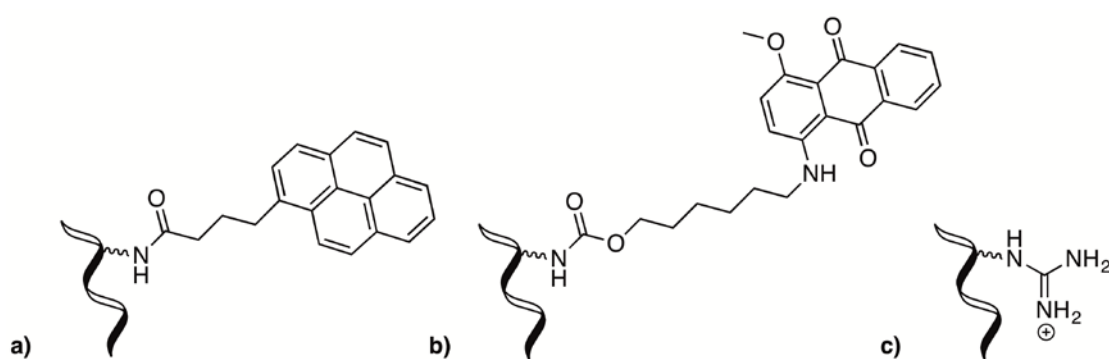
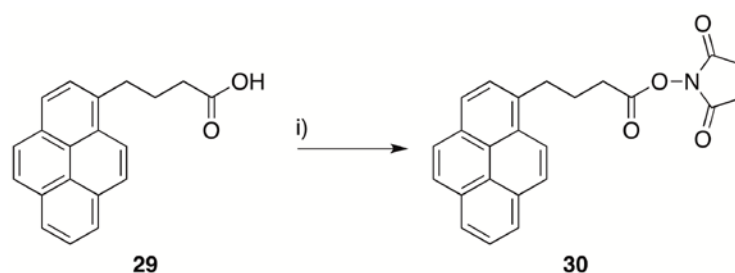


Figure 2.3 Oligonucleotide labelled with: (a) pyrene, (b) anthraquinone derivative, (c) guanidine.

2.4.1 Synthesis of active esters

First, the active esters of the desired label shown in Figure 2.3 were synthesised.

1-Pyrenebutyric acid *N*-hydroxysuccinimide ester **30** was prepared by treatment of 1-pyrenebutyric acid **29** with *N*-hydroxysuccinimide and coupling reagent DCC (*N,N*-dicyclohexylcarbodiimide).¹⁴⁸ This reaction did not give the desired ester. For the second attempt, DIPEA (*N,N*-diisopropylethylamine) was used as a base and 1-pyrenebutyric acid was reacted with *N,N'*-disuccinimidyl carbonate (Scheme 2.11).¹⁴⁹ This method gave the expected compound **30** in 77% yield.



Scheme 2.11 Synthesis of 1-pyrenebutyric acid NHS ester (30). (i) DIPEA, *N*-disuccinimidyl carbonate, 3 h, rt, 77%.

The length of the linker between the 2'-position of the sugar and the label was also investigated. To test this, pyrene NHS ester with the carbonyl group attached directly to pyrene **31** (Figure 2.4) was synthesised according to the method described for compound **30**.

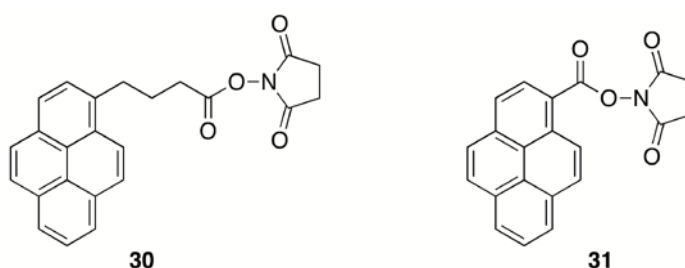
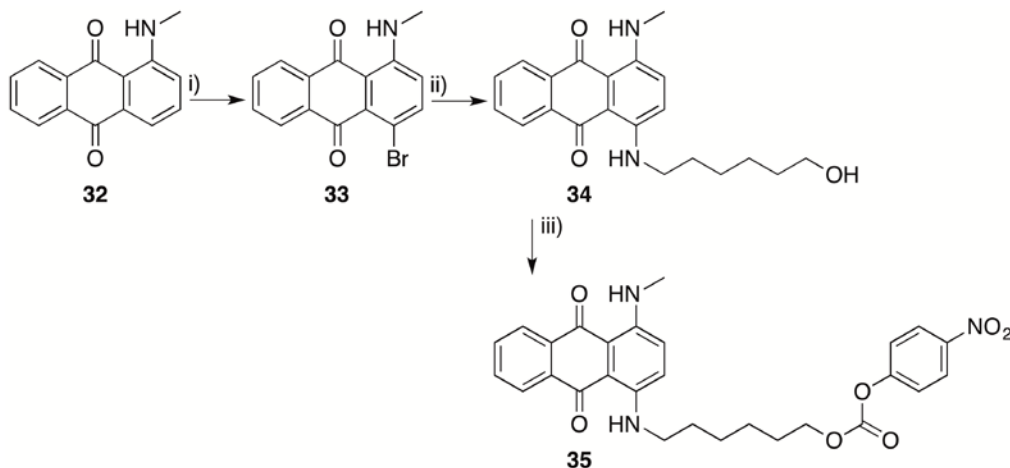


Figure 2.4 Pyrene NHS esters with long (30) and short (31) linkers.

Synthesis of anthraquinone derivative **35** was accomplished in a three-step procedure (Scheme 2.12). The first two steps were performed according to the literature.^{150,151} During the first step of the synthesis bromination (Scheme 2.12), 1-(methylamino)anthraquinone **32** was reacted with bromine in presence of acetic acid, propionic acid and hydrobromic acid. The reaction mixture became a thick suspension.¹⁵⁰ Addition of double amount of all three acids used in the reaction allowed the starting material to go fully into solution. This resulted in a very efficient conversion, giving a dark purple bromo-derivative **33** in high yield (92%). The amino-linked alcohol **34** was obtained from this in a low yield (29%) and was easily purified, allowing recovery of the starting material that could then be reused.¹⁵¹ Formation of

the active carbonate **35** of the anthraquinone analogue was carried out in moderate yield (37%) by reacting alcohol **34** with 4-nitrophenyl chloroformate.



Scheme 2.12 Synthesis of anthraquinone derivative (35). (i) acetic acid, propionic acid, HBr, Br₂, 50 °C and 0 °C, 3 h, 92% (ii) 6-amino-1-hexanol, TEA, toluene, reflux, 14 h, 29% (iii) 4-nitrophenyl chloroformate, pyridine, CH₂Cl₂, rt, 2 h, 37%.

2.4.2 Post-synthetic functionalisation of modified oligonucleotides

First it was investigated whether the labelling proceeds more efficiently in aqueous solution or on the solid support. A portion of the ODN-A-**12** still attached to the resin was treated with aqueous ammonia in order to remove the protecting groups and cleave the oligonucleotide from the resin. A second portion of the ODN-A-**12** on the resin was treated with a solution of 20% piperidine in DMF in order to selectively remove the Fmoc protecting group, leaving the rest of the oligo intact on the resin. The labelling reaction in solution was carried out with ODN-A-**12** dissolved in the buffer at pH 7.2 with excess of pyrene NHS ester **30** dissolved in DMF in 1:1 volume ratio. The reaction mixture was heated for 5 hours. The labelling reaction on solid support was performed entirely in organic solvent (DMF) and the ODN-A-**12** attached to the resin with excess of labelling reagent were added to the solvent with the addition of triethylamine, which served as a base. The

reaction was also heated for 5 hours. Based on the LC-MS, both methods were equally efficient, giving fully labelled product with pyrene attached to the amine *via* an amide bond. It was decided to carry on with the labelling in solution for all future experiments (unless stated differently).

In all cases functionalisation of the 2'-aminoethoxy group with pyrene **30**, in every oligonucleotide containing single or multiple additions of monomer **1** or **2** proceeded efficiently, providing the desired fully labeled products. A full list of labeled oligonucleotides is presented in Table 2.3 below.

Table 2.3 List of oligonucleotides labelled with pyrene (P), anthraquinone derivative (A) and guanidine (G).

ODN	label	code	calc. mass	found mass
ODN-A- <i>t</i> 1	pyrene	ODN-A- <i>t</i> 1-P	4266	4267
	anthraquinone	ODN-A- <i>t</i> 1-A	4374	4375
	guanidine	ODN-A- <i>t</i> 1-G	4039	4040
ODN-A- <i>t</i> 2	pyrene	ODN-A- <i>t</i> 2-P	4279	4280
	short pyrene	ODN-A- <i>t</i> 2-SP	4239	4239
	anthraquinone	ODN-A- <i>t</i> 2-A	4314	4315
	guanidine	ODN-A- <i>t</i> 2-G	3979	3980
ODN-A- <i>a</i> 1	pyrene	ODN-A- <i>a</i> 1-P	4265	4266
	anthraquinone	ODN-A- <i>a</i> 1-A	4373	4374
	guanidine	ODN-A- <i>a</i> 1-G	4038	4040
ODN-B- <i>t</i> 1	pyrene	ODN-B- <i>t</i> 1-P	4561	4562
	anthraquinone	ODN-B- <i>t</i> 1-A	4669	4671
	guanidine	ODN-B- <i>t</i> 1-G	4334	4335
ODN-B- <i>t</i> 2	pyrene	ODN-B- <i>t</i> 2-P	4561	4562
	anthraquinone	ODN-B- <i>t</i> 2-A	4669	4671
	guanidine	ODN-B- <i>t</i> 2-G	4334	4335
ODN-B- <i>t</i> 3	pyrene	ODN-B- <i>t</i> 3-P	4620	4621
	anthraquinone	ODN-B- <i>t</i> 3-A	4728	4729
	guanidine	ODN-B- <i>t</i> 3-G	4354	4355
ODN-B- <i>a</i> 1	pyrene	ODN-B- <i>a</i> 1-P	4560	4561
	anthraquinone	ODN-B- <i>a</i> 1-A	4668	4670
ODN-B- <i>a</i> 2	pyrene	ODN-B- <i>a</i> 2-P	4619	4620
	anthraquinone	ODN-B- <i>a</i> 2-A	4727	4729

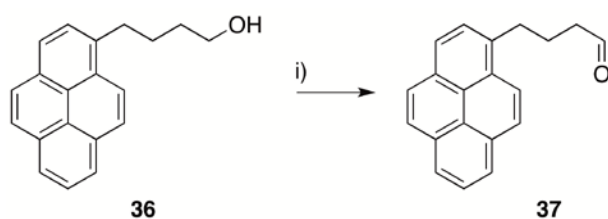
ODN-C- a1	pyrene	ODN-C- a1 -P	4982	4982
	anthraquinone	ODN-C- a1 -A	5090	5091

When using anthraquinone derivative **35**, labelling of oligonucleotides containing multiple additions of free amine presented difficulties. Based on the LC-MS of the crude material it appeared that oligonucleotide was only partially labelled.

An extended reaction time and/or increased label concentration did not improve the reaction efficiency. In order to drive these reactions to completion it was necessary to perform a second labelling reaction on the partially reacted products (Table 2.3).

The functionalisation of the ODNs with the guanidine group was successfully achieved using guanidylating reagent 1*H*-pyrazole-1-carboxamide hydrochloride and imidazole dissolved in water upon heating for 5 hours in an average yield of 50% (Table 2.3).

Different types of linker between the intercalator and the DNA strand were investigated. In previous examples the pyrene was attached to the 2'-aminoethoxy group *via* an amide bond, which is rigid. Exploration of more flexible linkers and investigation whether this additional flexibility would contribute even further to the stabilising properties of pyrene was also undertaken. It was decided to replace the amide bond with a secondary amine. Labelling of ODN-A-**a1** with pyrene through an amine linker instead of amide was achieved through reductive amination of 1-pyrene butyraldehyde **37** with ODN-A-**a1**.¹⁵²⁻¹⁵⁴ 1-Pyrene butyraldehyde **37** was synthesised in a one-step procedure using 1-pyrene butanol **36** and Dess-Martin periodinane in the presence of H₂O in CH₂Cl₂ (Scheme 2.13).¹⁵⁵



Scheme 2.13 Synthesis of 1-pyrene butyraldehyde (37). (i) Dess-Martin periodinane, H₂O, CH₂Cl₂, rt, 81%.

The reductive amination was carried out in a PBS/DMF mixture in the presence of sodium cyanoborohydride at 55 °C. This reaction resulted in two products: a single and a double addition of the pyrene to the amine (Figure 2.5, (A) ODN-A-*a*1-NH-pyr and (B) ODN-A-*a*1-NH-2pyr respectively). Both products were easily separated by RP-HPLC.

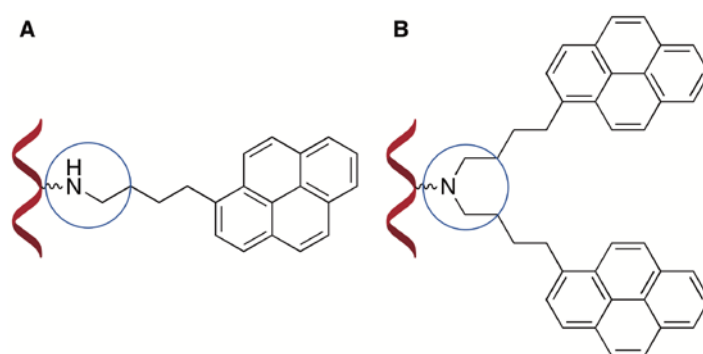


Figure 2.5 Mono (A) and double (B) addition of pyrene through the amine linker.

2.5 SUMMARY AND OUTLOOK

In summary, novel T-T triazole dimer phosphoramidite **1** and previously reported 2'-aminoethoxy T phosphoramidite **2** were successfully synthesised. These monomers were then used in the automated solid-phase oligonucleotides synthesis to form a novel group of oligonucleotides containing neutral triazole backbone and 2'-aminoethoxy ribose modification. These were then successfully functionalised using pyrene NHS ester **30**, anthraquinone derivative active carbonate **35** and guanidine. Functionalisation of oligonucleotides with pyrene *via* one or four-carbon long linker and *via*

more rigid amide or more flexible amine linker was also achieved, providing a wide spectrum of modified oligonucleotides for further biophysical studies.

3

BIOPHYSICAL STUDIES

This chapter describes the biophysical properties of triazole-modified oligonucleotides. The focus is primarily on the thermal stability of the modified nucleic acids hybridised to their complementary RNA and DNA strands. In addition, the effect of single base pair mismatch located at or adjacent to the modification is evaluated, and the conformations of the duplexes are measured by the circular dichroism spectroscopy.

3.1 INTRODUCTION: THERMAL STABILITY AND CIRCULAR DICHROISM

STRUCTURAL STUDIES OF RNA AND DNA DUPLEXES

Understanding the biophysical properties of modified oligonucleotides is of great importance when assessing them for biological applications. Evaluation of the duplex thermal stability with complementary RNA or DNA, and determining their conformational properties and nuclease resistance are the fundamental standard studies to be performed when designing and evaluating modified nucleic acids probes for therapeutic applications. UV-melting is a technique that can be used to measure the thermal stability of structured nucleic acids. Oligonucleotides absorb light in the ultraviolet region, with a maximum around 260 nm depending on the nucleobase composition and sequence. The absorption depends on π -stacking interactions between bases and base pairs. Upon an increase in temperature, the hydrogen bonds between the nucleobases in duplex DNA are broken, disrupting the π -stacking. As a result of base stacking double stranded DNA absorbs less UV light compared to single stranded DNA (hypochromicity). The mid-point of the transition for reversible melting is referred to as the melting temperature (T_m). These characteristics make UV melting a simple and convenient method for measuring and comparing nucleic acid modifications (Figure 3.1).

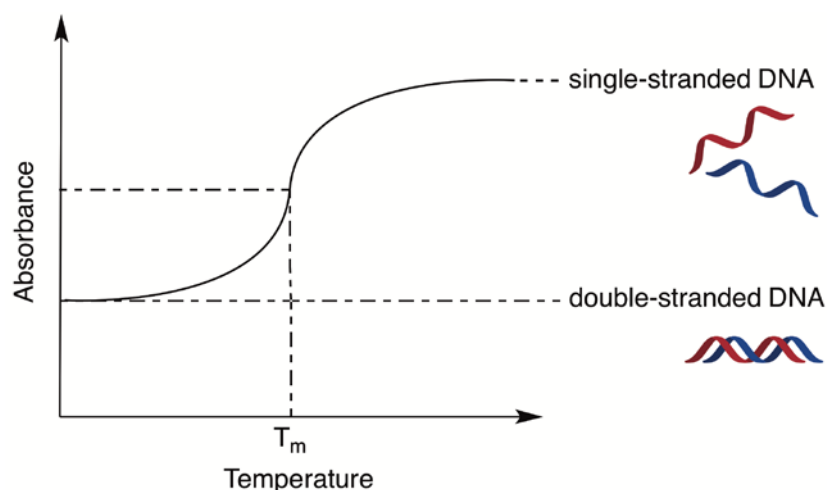


Figure 3.1 UV melting curve of DNA duplex showing the transition from the double-stranded DNA to single-stranded DNA upon increasing temperature. The mid-point of the transition is the melting temperature (T_m).

Circular dichroism (CD) spectroscopy can be used to determine the ensemble conformation of oligonucleotides in solution. CD is based on the differential absorption of circularly polarised light by chiral molecules, which depends on the secondary structure for biopolymers.¹⁵⁶ The quantity directly measured is ellipticity and is expressed in degrees. However, the difference in the molar extinction coefficients is more convenient for characterisation (Equation 3.1).

Equation 3.1 Molar extinction coefficients.

$$\Delta\varepsilon = \varepsilon_{LCP} - \varepsilon_{RCP} = \frac{[\theta]}{3298.2}$$

For nucleic acids, differences in the CD spectra originate from base-base stacking, and a correlation between the pucker of the ribose or deoxyribose sugar ring and the phosphodiester backbone conformation, which lead to a specific type of helix. It is important to determine the secondary structure of the modified nucleic acids for therapeutic applications, as some can only function effectively when they are structured in a conformation that allows them to assemble functional species with specific proteins. For example the

enzyme RNase H can only recognise DNA:RNA duplex to perform its degradation activity on RNA.^{157,158}

The CD spectrum of the B-form DNA duplex is characterised by a strong positive peak at around 280 nm and a smaller negative peak at around 250 nm (Figure 3.2). A small positive peak at 210-220 nm is also visible. The spectrum below 210 nm gives unreliable data due to the instrument limitations. On the other hand, the A-form spectrum of RNA is represented by a dominant positive peak in the 260-280 nm region and a negative peak at around 230 nm.

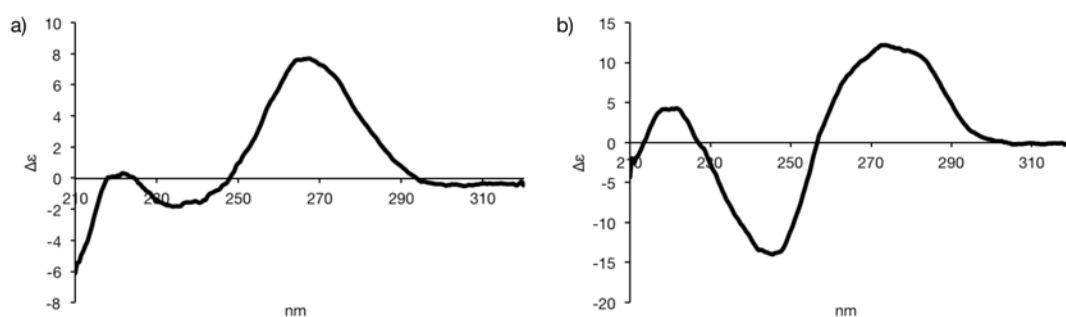


Figure 3.2 Circular dichroism spectra of a) tRNA from baker's yeast (*S. cerevisiae*) and b) DNA sodium salt from calf thymus. The spectra were recorded in 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 200 nM NaCl buffer, pH 7.2, rt.

It is known that triazole backbone modifications destabilise duplexes, therefore addition of the 2'-aminoethoxy group could serve as a handle for post-synthetic labelling with groups that could potentially restore duplex thermal stability. These two modifications, triazole backbone and 2'-modifications, facilitate formation of less negatively charged oligonucleotides. This hypothetically would improve cell permeability and give higher antisense activity of oligonucleotides while also ensuring high nuclease resistance.

In a search for duplex stabilising molecules that could be easily attached to the 2'-aminoethoxy group *via* post-synthetic labelling it was first decided to utilise pyrene modification (Figure 3.3; a). Attachment of a pyrene moiety to

the furanose ring has been widely explored and has been successful in stabilising DNA:DNA duplexes improving the T_m by up to 13 °C per modification due to its aromatic and planar structure, which leads to enhanced stacking interactions.¹⁵⁹⁻¹⁶¹ Yamana *et al.* published NMR studies of DNA:DNA duplex containing a single addition of pyrene on the 2'-position, showing its intercalating properties.¹⁶² In the DNA:RNA duplex however, based on a molecular dynamics simulation, the same group showed pyrene being pushed outside the duplex toward the minor groove, because A-type duplex is more compressed and has less efficient base overlap.^{161,162} It was also decided to functionalise the 2'-aminoethoxy group with the anthraquinone derivative Disperse Blue (Figure 3.3; b). This molecule is mainly used as a fluorescence quencher, but anthraquinone derivatives like this are also known to have DNA duplex and triplex stabilising capability due to their intercalation into DNA.¹⁶³⁻¹⁶⁶ The guanidinium group (Figure 3.3; c), which is highly basic and positively charged at physiological pH, has also been found to be a very successful oligonucleotide stabilising modification and was chosen in this project. It has been reported that guanidine group increases the affinity of oligonucleotides toward RNA or DNA target strands and improves their cellular uptake.¹⁶⁷⁻¹⁶⁹

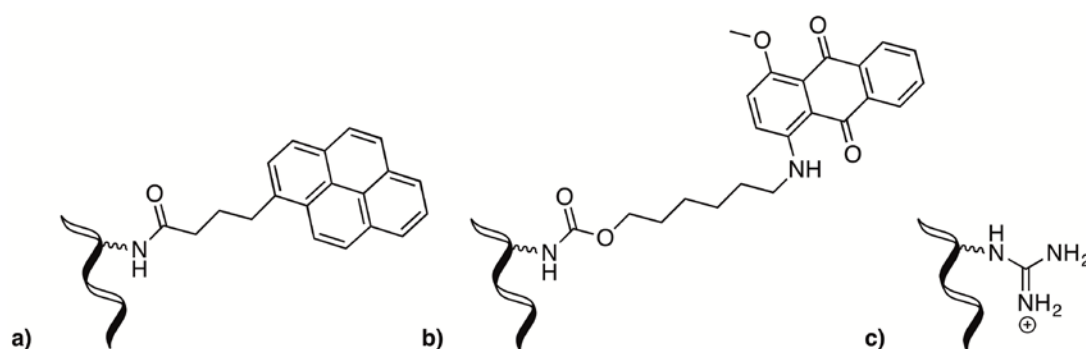


Figure 3.3 Oligonucleotides labels: a) pyrene, b) anthraquinone derivative and c) guanidine.

3.2 BIOPHYSICAL PROPERTIES OF MODIFIED OLIGONUCLEOTIDES WITH THEIR RNA AND DNA COMPLEMENTARY STRANDS

3.2.1 UV melting studies

The thermal stability of oligonucleotides with a single addition of triazole backbone was investigated. ODN-A-*1* contains a 2'-aminoethoxy group on the 5'-side of the triazole, whereas ODN-A-*2* has one on the 3'-side of the triazole. As expected, UV melting temperatures of ODN-A-*1* ($T_m = 54.6\text{ }^{\circ}\text{C}$) and ODN-A-*2* ($T_m = 57.5\text{ }^{\circ}\text{C}$) with the complementary RNA strand were lower than the unmodified DNA:RNA duplex ($T_m = 62.0\text{ }^{\circ}\text{C}$) by 7.4 and 4.5 $^{\circ}\text{C}$ respectively (Figure 3.4). This could be due to different base pairing within the two different triazole linkages and complementary RNA duplexes (T:T, T:T in ODN-A-*1* and C:G, T:T in ODN-A-*2*). It also suggests the 2'-modification placed on the ribose next to the triazole linkage (ODN-A-*1*) additionally distorts the duplex conformation compared to the 2'-modification attached on the ribose below the triazole linkage (ODN-A-*2*).

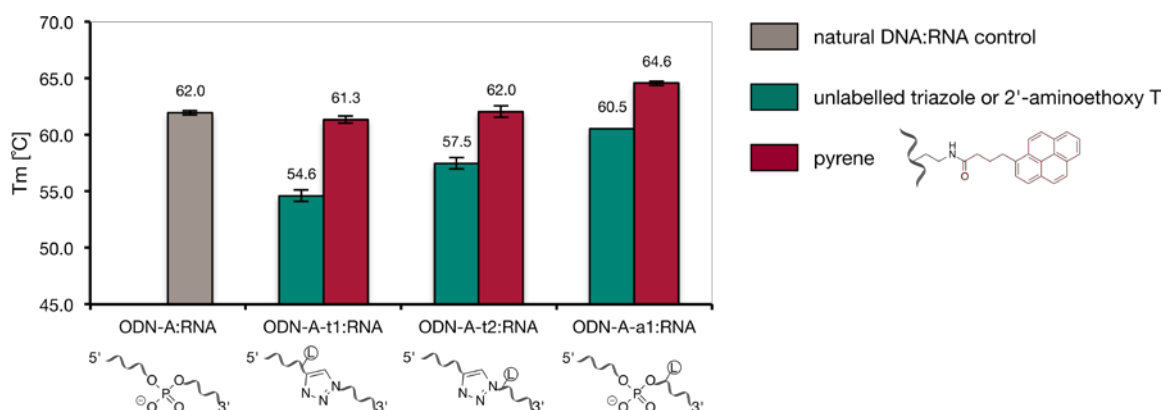


Figure 3.4 Modified oligonucleotides containing triazole linkage and 2'-aminoethoxy on the 5'-side of the triazole (ODN-A-*t*1), 2'-aminoethoxy on the 3'-side of the triazole (ODN-A-*t*2) or only the 2'-aminoethoxy (ODN-A-*a*1) and their functionalisation with pyrene hybridised to the complementary RNA strand. Control unmodified duplex (grey), unfunctionalised 2'-aminoethoxy group (green), functionalised 2'-aminoethoxy group with pyrene (red). Sequences: ODN-A-CGACGCTTGCAGC. The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 mM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 4 μ M.

Next, triazole modified oligonucleotides functionalised with pyrene **30** (ODN-A-*t*1-P and ODN-A-*t*2-P) were compared with the unlabelled oligonucleotides. With the complementary RNA strand, pyrene with a 4-carbon long linker increased the T_m of the ODN-A-*t*1-P (T_m = 61.3 $^{\circ}$ C) and ODN-A-*t*2-P (T_m = 62.0 $^{\circ}$ C) by a significant 6.7 and 4.6 $^{\circ}$ C respectively, restoring the values to those of the unmodified DNA:RNA duplex (Figure 3.4). This suggests that attachment of pyrene on the 5'-end of the triazole linkage (ODN-A-*t*1-P), which is the major force for destabilisation of the formed duplex, allows a more favourable conformation for pyrene to bind, giving higher increase in T_m compared to the pyrene placed on the 3'-end of the triazole (ODN-A-*t*2-P).

In the case of duplexes formed with a DNA complementary strand, the destabilising effect of the single addition of the triazole dimer was greater than with RNA. ODN-A-*t*1 (T_m = 53.0 $^{\circ}$ C) and ODN-A-*t*2 (T_m = 52.2 $^{\circ}$ C) formed

duplexes which were *ca.* 10.0 °C less stable than unmodified DNA:DNA (T_m = 61.9 °C, Figure 3.5). Again, pyrene in the ODN-A-*t*1-P (T_m = 66.0 °C) and ODN-A-*t*2-P (T_m = 66.4 °C) was very efficient in restoring duplex stability by 13.0 °C and 14.2 °C, where the T_m even exceeded that of unmodified DNA duplex by 4.1 °C and 4.5 °C respectively (Figure 3.5). These results show that the pyrene modification on the 3'-side of the triazole with DNA has a higher stabilising effect, which is contrary to the results for the duplex formed with complementary RNA.

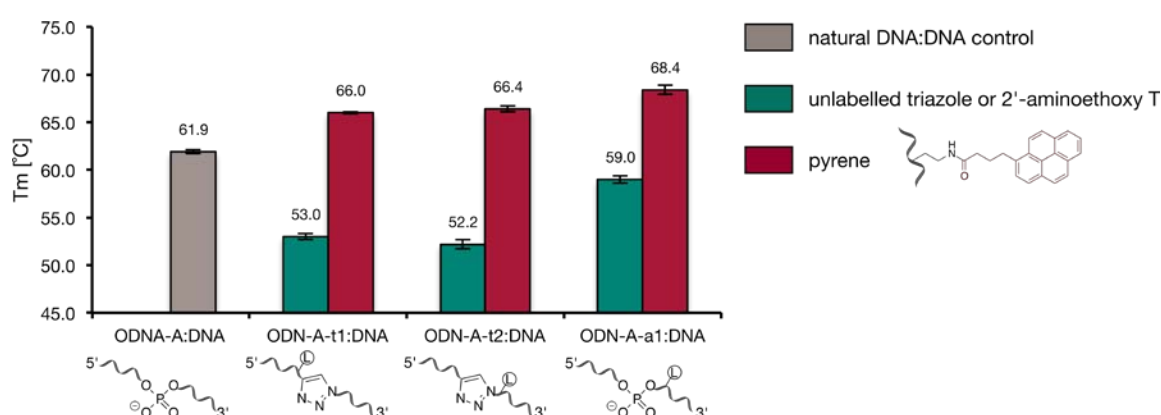


Figure 3.5 Modified oligonucleotides containing a triazole linkage and 2'-aminoethoxy on the 5'-side of the triazole (ODN-A-*t*1), 2'-aminoethoxy on the 3'-side of the triazole (ODN-A-*t*2) or only 2'-aminoethoxy (ODN-A-*a*1) and their functionalisation with pyrene hybridised to the complementary DNA strand. Control unmodified duplex (grey), unfunctionalised 2'-aminoethoxy group (green), functionalised 2'-aminoethoxy group with pyrene (red). Sequences: ODN-A-CGACGCTTGCAGC. The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 mM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 4 μM.

A-type (3'-*endo*) or B-type (2'-*endo*) conformation of the duplex changes the 2'-position of the ribose in the duplex as indicated on the model in Figure 3.6, resulting in different orientations of the modification, which potentially intercalates or binds in the minor groove changing its effect on the duplex thermal stability.

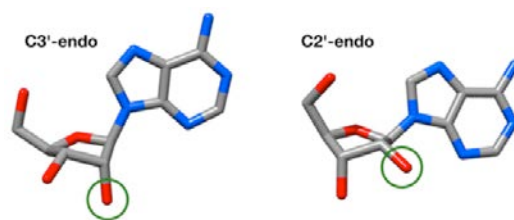


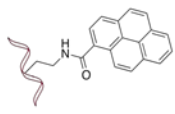
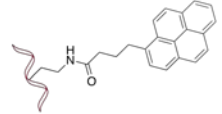
Figure 3.6 3'-endo and 2'-endo conformations of ribose and different position of 2'-modification circled in green.

In order to gain a better understanding of the effect of pyrene itself on the duplex stability, UV melting studies were carried out on oligonucleotides containing only a single 2'-aminoethoxy modification and no triazole. ODN-A-**a1** (unlabeled) and ODN-A-**a1**-P (labeled with pyrene) were tested with their complementary RNA and DNA. The free 2'-aminoethoxy group (ODN-A-**a1** T_m = 60.5 °C) had a very small effect on the duplex stability with RNA, decreasing the T_m by just 1.0 °C. Addition of the pyrene increased the T_m by 3.6 °C and exceeded the T_m of the unmodified DNA:RNA by 2.6 °C (Figure 3.4). When hybridised with DNA, ODN-A-**a1** caused a drop in T_m of 2.9 °C, but addition of pyrene in ODN-A-**a1**-P restored the stability, improving the T_m by 9.4 °C and again forming a more stable duplex than the unmodified DNA:DNA by 6.5 °C (Figure 3.5). It is clear that pyrene has a stabilising effect in all the cases investigated but this is significantly higher in the oligonucleotides containing the triazole modification. This is significant when designing modified oligonucleotides with the neutral but destabilising triazole backbone.

The type of interaction pyrene has with the duplex has been described in literature to be intercalation or groove binding and it is partially determined by the length of the linker used to attach it to the oligonucleotide.¹⁵⁹ Therefore, in order to evaluate the extent of the interaction between pyrene and oligonucleotide containing the triazole linkage, a shorter and more rigid linker between DNA and the label was examined with only one carbon atom

between the amide and the pyrene **31** (in comparison to the 4-carbon long linker used in the previous examples). Hybridisation of ODN-A-**t2**-SP with DNA ($T_m = 62.3\text{ }^{\circ}\text{C}$) revealed that the pyrene with a short linker (Table 3.1) does stabilise duplex formation by $0.4\text{ }^{\circ}\text{C}$, however not as well as the pyrene attached through the longer linker with T_m increase of $4.5\text{ }^{\circ}\text{C}$. It appears that the shorter linker between pyrene and oligonucleotide does not allow efficient enough interaction of pyrene with the duplex and has a significantly lower stabilising effect of the duplex thermal stability than anticipated. Therefore the longer linker was used in further experiments.

Table 3.1 Influence of the length of the linker between DNA and pyrene on the thermal stability of duplex formed with complementary DNA. Sequences: ODN-A-CGACGCTTGCAGC. Experiments carried out in 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 200 mM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = $4\text{ }\mu\text{M}$.

			
	Natural DNA:DNA	ODN-A- t2 -SP:DNA	ODN-A- t2 -P:DNA
$T_m\text{ [}^{\circ}\text{C]}$	61.9	62.3	66.4

Based on these encouraging results, other labels were also investigated. Triazole containing oligonucleotides functionalised with anthraquinone derivative **35** (ODN-A-**t1**-A) when hybridised to the complementary RNA strand increased the T_m by $10.0\text{ }^{\circ}\text{C}$ ($T_m = 64.6\text{ }^{\circ}\text{C}$), forming a duplex almost $3.0\text{ }^{\circ}\text{C}$ more stable than the unmodified DNA:RNA (Figure 3.7; a). The stabilising effect of anthraquinone was higher than the corresponding oligonucleotide labelled with pyrene - ODN-A-**t1**-P ($T_m = 61.3\text{ }^{\circ}\text{C}$). The stabilising effect of the ODN-A-**t1**-A with a complementary DNA was as efficient as ODN-A-**t1**-P with an increase of T_m by $13.1\text{ }^{\circ}\text{C}$. In the non-triazole oligonucleotide ODN-A-**a1**-A the effect of the anthraquinone was also greater than that of pyrene in duplex with RNA, with an increased stability of $8.5\text{ }^{\circ}\text{C}$ and with DNA the

increase was 10.5 °C (Figure 3.7; a and b). Labelling oligonucleotides with the anthraquinone derivative, similarly to pyrene, exhibited a higher stabilising effect when attached to oligonucleotides containing triazole compared to the oligonucleotide with a completely standard backbone and only 2'-modifications.

The impact of the positively charged guanidine group on duplex stability was unexpectedly small in the duplex of ODN-A-**1**-G and ODN-A-**a**1-G with RNA, actually decreasing the T_m by 0.4 °C in a triazole containing oligonucleotide and increasing T_m by only 2.3 °C in the non-triazole one (Figure 3.7; a). With a DNA complementary strand ODN-A-**1**-G and ODN-A-**a**1-G guanidine exhibited also very minor T_m improvement of 0.4 °C and 2.5 °C respectively. This poor stabilising effect might be due to the interaction of guanidine with phosphate group. It perhaps causes narrowing of the minor groove size, which might have an effect on the overall structure of the oligonucleotide, particularly enhanced in the triazole containing oligonucleotides.

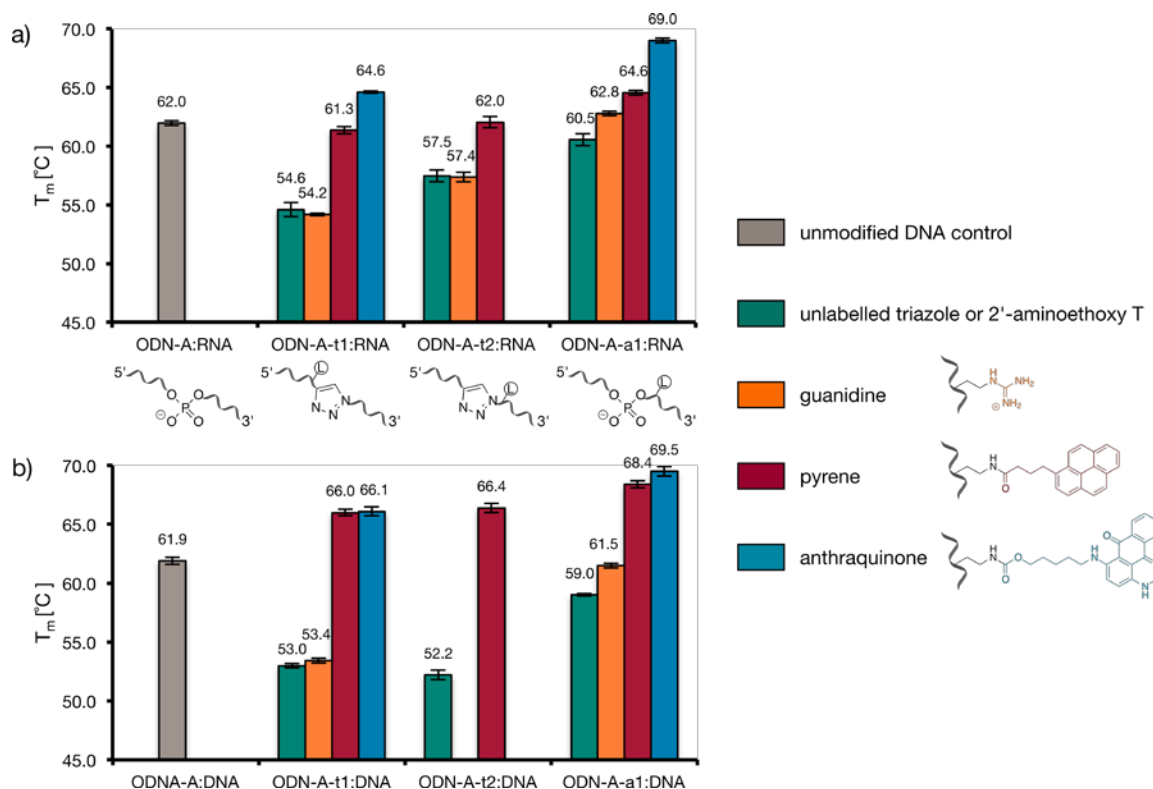


Figure 3.7 Influence of different 2'-labels: guanidine (orange), pyrene (red), anthraquinone derivative (blue) on the thermal stability of oligonucleotides containing triazole linkage (ODN-A-t1 and ODN-A-t2) and 2'-aminoethoxy only (ODN-A-a1) with complementary: a) RNA; b) DNA. Control unmodified duplex (grey), unfunctionalised 2'-aminoethoxy group (green), functionalised 2'-aminoethoxy group with guanidine (orange), functionalised 2'-aminoethoxy group with pyrene (red) functionalised 2'-aminoethoxy group with anthraquinone (blue). Sequences: ODN-A-CGACGCTTGACAGC. The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 4 μ M.

The UV melting studies show that triazole modified oligonucleotides functionalised with pyrene or anthraquinone form stable duplexes with complementary RNA or DNA strands. Both pyrene and anthraquinone are known to be excellent intercalators due to their aromatic and planar structure. Therefore, it was anticipated that this was the driving force behind duplex stabilisation. However, groove binding cannot be excluded without additional structural studies.

In the examples described above, pyrene was attached to the 2'-aminoethoxy group *via* an amide bond and the anthraquinone derivative via a carbamate bond, both being very rigid. Therefore, it was questioned, whether more flexible linkers between the pyrene moiety and the DNA would facilitate stronger intercalation of pyrene. With this in mind the amide bond was replaced with a more flexible secondary amine. Formation of the amine linker was achieved *via* reductive amination between the 2'-aminoethoxy group of the non-triazole ODN-A-**a1** and 1-pyrene butyraldehyde (section 2.4.2). Given the nature of the reductive amination reaction and production of single and double addition of pyrene (Figure 2.6), UV melting studies were carried out with both oligonucleotides ODN-A-**a1**-NH-P and ODN-A-**a1**-NH-2P. With complementary RNA both oligonucleotides were destabilising compared to the ODN-A-**a1**-P (pyrene attached *via* amide bond), lowering the T_m by 2.4 °C and 3.4 °C respectively. In a duplex formed with DNA, ODN-A-**a1**-NH-P improved the T_m by 4.9 °C and ODN-A-**a1**-NH-2P by 6.1 °C, however the increase was not as high as for ODN-A-**a1**-P, suggesting that the amide bond gives the appropriate rigidity to obtain the most stable duplex (Figure 3.8).

The design of potential antisense probes with reduced negative charge obtained by introduction of a neutral triazole backbone instead of an anionic phosphodiester requires multiple additions of the backbone modification. Multiple additions of the triazole backbone however, can potentially have a large negative influence on duplex stability. To test whether 2'-modifications retain their positive effects in restoring the duplex stability of oligonucleotides containing multiple triazoles, ODN-B-**t3** with two additions of T-T triazole dimer **1** and ODN-B-**t3**-P and ODN-B-**t3**-A were examined.

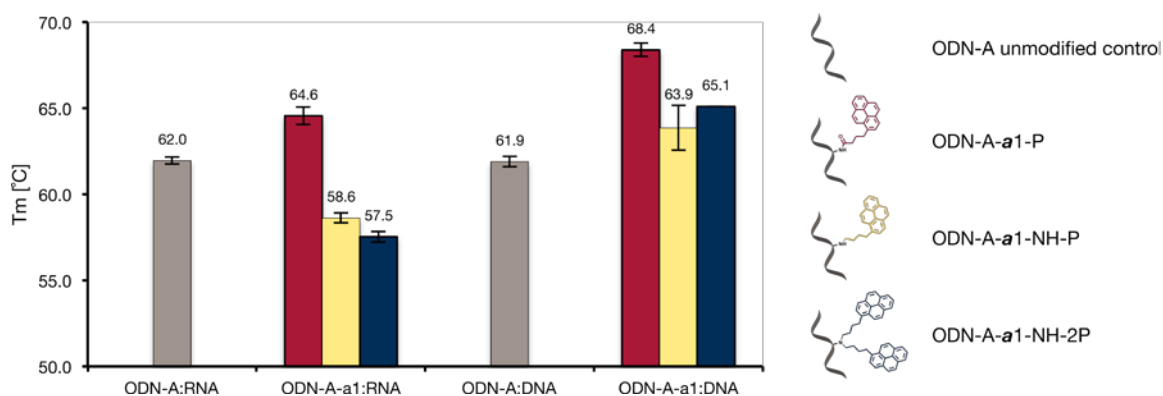


Figure 3.8 Influence of less rigid amine linker between DNA and pyrene on the thermal stability of the duplex with complementary RNA and DNA compared to the more rigid amide linker. Control unmodified duplex (grey), functionalised 2'-aminoethoxy group with pyrene *via* amide bond (red), functionalised 2'-aminoethoxy group with single pyrene *via* amine bond (yellow), functionalised 2'-aminoethoxy group with double pyrene *via* amine bond (dark blue). Sequences: ODN-A-CGACGCTTGACGC. The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 4 μ M.

Hybridisation of the unlabeled ODN-B-**3** (T_m = 37.5 °C) with RNA caused an expected dramatic drop in T_m of over 24 °C but remarkably, addition of pyrene or anthraquinone at both positions of the triazole (ODN-B-**3**-P T_m = 66.0 °C and ODN-B-**3**-A T_m = 63.4 °C) returned the improved duplex stability increasing T_m by 23 and 25 °C respectively (Figure 3.9; a). The ODN-B-**3** forms an even less stable duplex with complementary DNA with a T_m 27 °C lower than unmodified DNA duplex. In this example functionalisation of the 2'-position with pyrene and anthraquinone derivative (ODN-B-**3**-P T_m = 62.2 °C and ODN-B-**3**-A T_m = 64.3 °C) also restored the duplex stability increasing the T_m by over 28 and 25 °C respectively (Figure 3.9; b). The oligonucleotide of the same sequence B but with a single addition of the triazole dimer **1** at each of the positions was also tested to ensure that there were no unusual effects of each of these modifications separately in this particular sequence. Similarly to the ODN-A-**1** (single addition of triazole dimer **1**) described previously, the destabilising effect of triazole in duplexes formed between

ODN-B-*t*1 and ODN-B-*t*2 with RNA or DNA, was on average 9.0 °C. The increase in T_m upon addition of pyrene or anthraquinone derivatives was on average 10.5 °C, and the duplexes were up to 2.5 °C more stable than their unmodified controls (Figure 3.9). Functionalisation of ODN-B-*t*1 and ODN-B-*t*2 with guanidinium group caused further destabilisation of the duplex.

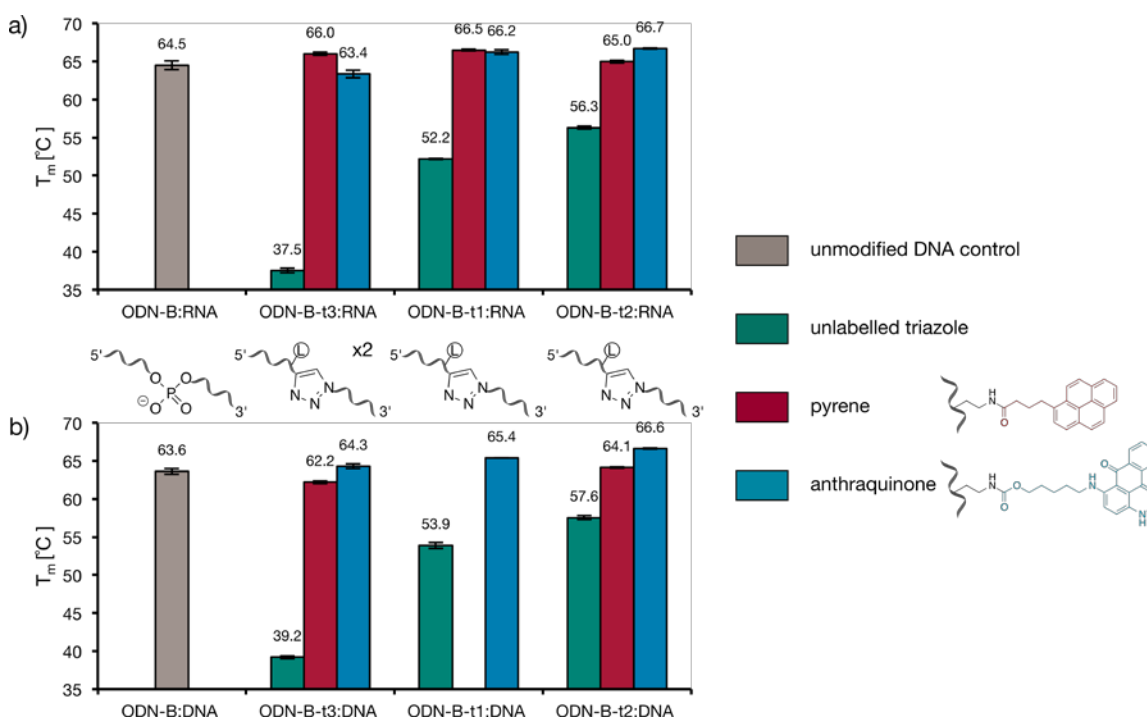


Figure 3.9 Double and single addition of triazole linkage with 2'-aminoethoxy (ODN-B-*t*3 and ODN-B-*t*1, ODN-B-*t*2 in green) and their functionalised with pyrene and anthraquinone derivative oligonucleotides and their effect on the duplex thermal stability compared to unmodified duplex with complementary: a) RNA and b) DNA. Control unmodified duplex (grey), unfunctionalised 2'-aminoethoxy group (green), functionalised 2'-aminoethoxy group with pyrene (red) functionalised 2'-aminoethoxy group with anthraquinone (blue). Sequences: ODN-A-CGACGCTTGCAGC; ODN-B-CGTTCGCTTGCAGC. The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 4 μ M.

Having established the excellent stabilising properties of pyrene and anthraquinone labelled oligonucleotides containing the triazole backbone, the next step was to evaluate the limits of the stabilising capability of these 2'-

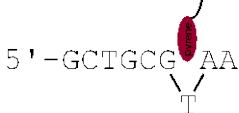
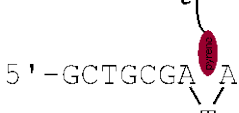
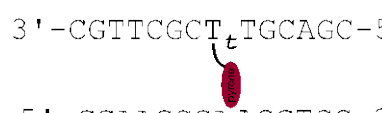
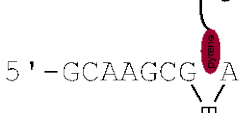
modifications. To achieve this, oligonucleotides with single, double and triple additions of 2'-aminoethoxy T **2**: ODN-B-**a1**, ODN-B-**a2** and ODN-C-**a1** were tested with their complementary RNA and DNA followed by measurements of these functionalised oligonucleotides labelled with pyrene and anthraquinone derivative: ODN-B-**a1**-P, ODN-B-**a1**-A, ODN-B-**a2**-P, ODN-B-**a2**-A and ODN-C-**a1**-P, ODN-C-**a1**-A. A single addition of 2'-aminoethoxy T **2** (ODN-B-**a1**) with RNA lowered the T_m by just 1.0 °C and with DNA by 3.3 °C whereas the ODN-B-**a2** with double addition of 2'-aminoethoxy T **2** caused predictably slightly bigger duplex destabilisation dropping the T_m by 2.5 °C with RNA and 8.5 °C DNA (Table 3.2). Triple addition of 2'-aminoethoxy T **2** (ODN-C-**a1**) however, unexpectedly showed lower destabilisation effect with DNA compared to the double addition, only decreasing the T_m by just 6.6 °C relative to the control. As mentioned in section 2.3.1, sequence 'C' differs from sequence 'B' by one addition of thymidine in order to be able to accommodate three additions of the 2'-aminoethoxy T **2** modification. The extra base pair causes increase of melting temperature by 2.5 °C, which was showed experimentally, where T_m of ODN-C-1 was 58.5 °C and ODN-B-1 56.0 °C. Formation of the more stable duplex by the sequence C could be partially a reason for a 1.9 °C smaller destabilising effect of the triple addition of the modified T comparing to double addition presented in the sequence 'B'. Functionalisation of the ODN-C-**a1** with pyrene or anthraquinone stabilised duplexes with RNA and DNA on average by 3.2 °C per modification. In all cases they formed more stable duplexes than the unmodified controls. A particularly high stabilising effect was exhibited by ODN-C-**a1**-P with a triple addition of pyrenes, increasing the T_m by 15.7 °C and therefore forming an extremely stable duplex, over 9 °C more stable than the unmodified DNA control (Table 3.2).

Table 3.2 Single, double and triple addition of 2'-aminoethoxy T (2) and their functionalised with pyrene and anthraquinone derivative hybrids and their effect on the duplex thermal stability with complementary RNA and DNA. Sequences: ODN-A-CGACGCTTGCAGC; ODN-B-CGTTTCGCTTGCAGC; ODN-C-CGTTTCGCTTGCATGC. The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 4 μ M.

sequence	ODN code	T _m [°C] with RNA	Δ T _m [°C]	T _m [°C] with DNA	Δ T _m [°C]
CGACGC ^a TTGCAGC 	ODN-B-1	64.5		63.6	
	ODN-B- a 1	62.6	-1.9	61.2	-2.4
	ODN-B- a 1-P	65.5	1.0	68.3	4.7
	ODN-B- a 1-A	67.1	2.6	69.5	5.9
CG ^a TTCGC ^a TTGCAGC 	ODN-B-1	64.5		63.6	
	ODN-B- a 2	61.1	-3.4	56.0	-7.6
	ODN-B- a 2-P	66.0	1.5	70.4	6.8
	ODN-B- a 2-A	65.7	1.2	67.4	3.8
CG ^a TTCGC ^a TTGCA ^a TGC 	ODN-C-1	65.1		data not acquired	
	ODN-C- a 1	58.5	-6.9		
	ODN-C- a 1-P	74.2	9.1		
	ODN-C- a 1-A	68.1	3.0		

In 2014, Brown *et al.* published the synthesis of the oligonucleotide with pyrene attached to the G-clamp modified base.¹⁷⁰ They reported enhanced duplex stability when hybridising pyrene-modified oligonucleotide to a bulged DNA strand with an extra base opposite to the location of the intercalated pyrene. To investigate whether a bulged duplex would improve the stabilising properties of the triazole-modified oligonucleotides functionalised with pyrene on the 2'-position of ribose, oligonucleotides with an additional base at the positions where pyrene would potentially be located were synthesised (Table 3.3). UV melting results however, did not show improved T_m, forming duplex on average just over 4 °C less stable comparing to the one without the bulge. These results might suggest that pyrene attached to the 2'-position of ribose does not intercalate and instead it improves the thermal duplex stability *via* groove binding.

Table 3.3 The influence of bulge in the opposite strand on the thermal stability of the duplex formed with oligonucleotide containing triazole and pyrene ODN-A-*t*1-P, compared to duplex with normal complementary DNA. ΔT_m vs unmodified complementary DNA ODN-A-2 or ODN-B-2 where $\Delta T_m = T_m$ (labelled with pyrene) - T_m (ODN-A-2 or ODN-B-2). Sequences: ODN-A-CGACGCTTGCAGC; ODN-B-CGTTTCGCTTGCAGC. The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 4 μ M.

normal duplex	T_m [°C] (ΔT_m)	bulged duplex	T_m [°C] (ΔT_m)
<u>ODN-A-<i>t</i>1-P</u>		3'-CGACGC _t TTGCAGC-5' 5'-GCTGCG _T AACGTCG-3' 	61.3 (-0.6)
3'-CGACGC _t TTGCAGC-5' 5'-GCTGCG _t AACGTCG-3' 	66.4 (4.5)	3'-CGACGC _t TTGCAGC-5' 5'-GCTGCG _t AACGTCG-3' 	59.7 (-2.2)
<u>ODN-B-<i>t</i>1-P</u>		3'-CGTTTCGCT _t TGCAGC-5' 5'-GCAAGCGA _T ACGTCG-3' 	62.0 (0.1)
3'-CGTTTCGCT _t TGCAGC-5' 5'-GCAAGCG _t AACGTCG-3' 	65.0 (3.1)	3'-CGTTTCGCT _t TGCAGC-5' 5'-GCAAGCG _t AACGTCG-3' 	63.1 (1.2)

In summary, the triazole backbone, in accordance with literature, causes significant destabilisation of duplexes with RNA and DNA. Attachment of the molecules such as pyrene or anthraquinone derivative to the 2'-position of ribose restores duplex stability to that of the natural control, and in some cases exceeds it. Although both 2'-modifications in triazole- and non-triazole modified oligonucleotide improve binding to the complementary strand, stronger stabilising effects are observed for triazole-modified oligonucleotides. The UV melting results also show triazole-modified oligonucleotides in the DNA:RNA duplexes, rather than DNA:DNA duplexes, are more stable, which may be related to the A-form duplex structure accommodating the linkage more efficiently. Moreover, this is beneficial for antisense applications where the target is mainly mRNA, meaning it potentially needs less additional duplex stabilising forces.

3.2.2 CD studies

To evaluate whether the triazole backbone and 2'-modifications have a significant influence on the duplex secondary structure circular dichroism studies were performed. CD spectra of oligonucleotide ODN-B-**1** containing single addition of triazole linkage with 2'-aminoethoxy group and its pyrene / anthraquinone functionalised forms (ODN-B-**1**-P and ODN-B-**1**-A) with complementary RNA (Figure 3.10) and DNA (Figure 3.11) showed no significant changes compared to the unmodified DNA:RNA and DNA:DNA control duplexes.

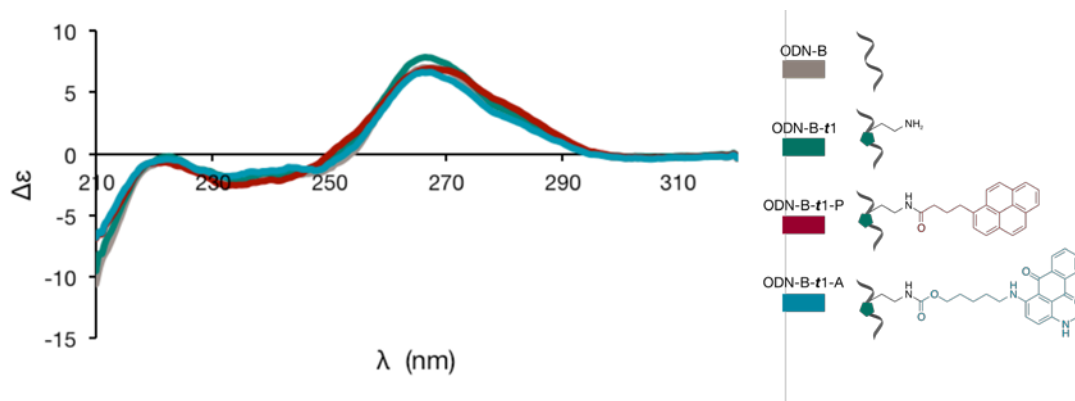


Figure 3.10 The influence of oligonucleotide modifications on the DNA:RNA duplex conformation for: the unmodified DNA:RNA control (ODN-B, grey), triazole linkage with a 2'-aminoethoxy group (ODN-B-*t*1, green), triazole linkage with pyrene (ODN-B-*t*1-P, red) and triazole linkage with anthraquinone derivative (ODN-B-*t*1-A, blue). Sequences: ODN-B-CGTTTCGCTTGACG. The spectra were measured in 10 mM NaH₂PO₄/Na₂HPO₄, 200 mM NaCl buffer, pH 7.2, rt.

When hybridised to the complementary RNA, signals characteristic of an A-type duplex (strong maximum at 270 nm, small minimum at 240 nm) were observed. The green, red and blue spectra, which represent the modified oligonucleotides ODN-B-*t*1, ODN-B-*t*1-P and ODN-B-*t*1-A respectively, overlay the grey spectra, which represents the unmodified DNA:RNA control duplex, almost perfectly (Figure 3.10). CD spectra of duplexes formed with complementary DNA retained the characteristic B-type features of a maximum at *ca.* 280 nm and a minimum at *ca.* 250 nm. The green, red and blue spectra did show however small fluctuations in intensity and relative shifts of the peaks when compared to the unmodified DNA:DNA control.

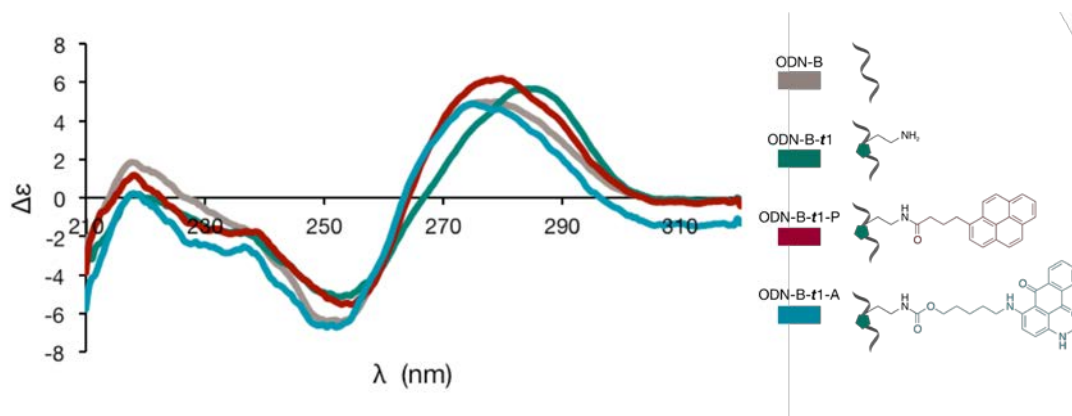


Figure 3.11 The influence of oligonucleotide modifications on the DNA:DNA duplex conformation for: the unmodified DNA:DNA control (ODN-B, grey), triazole linkage with a 2'-aminoethoxy group (ODN-B-*t*1, green), triazole linkage with pyrene (ODN-B-*t*1-P, red) and triazole linkage with anthraquinone derivative (ODN-B-*t*1-A, blue). Sequences: ODN-B-CGTTTCGCTTGCAGC. The spectra were measured in 10 mM NaH₂PO₄/Na₂HPO₄, 200 nM NaCl buffer, pH 7.2, rt.

3.3 MISMATCH PROPERTIES OF MODIFIED DNA OLIGONUCLEOTIDES IN

RNA AND DNA DUPLEXES

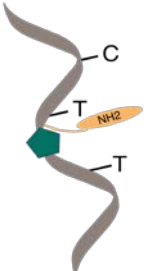
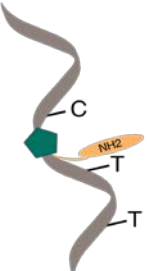
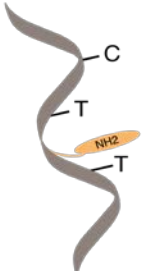
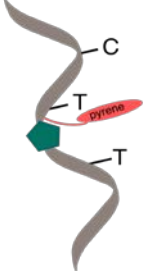
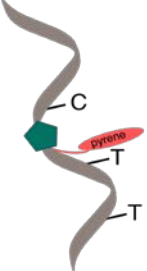
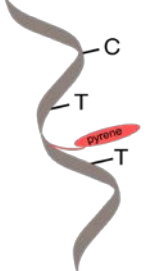
To be able to use modified nucleic acids for therapeutic applications it is critical that they are selective for their designed target. Therefore they must be sensitive to base pair mismatches in order to avoid toxicity generated from off-target binding. To evaluate this, UV melting studies were carried out with modified oligonucleotides hybridised to complementary DNA and RNA strands with a single T:T / T:U mismatch on either side of the triazole or one base away from triazole. Thymidine mismatch at the positions presented in the Table 3.4 generates C:T and T:T mismatches which are two of the most unstable base pairs. Both triazole oligonucleotides, with 2'-aminoethoxy group on the 5'-side and on the 3'-side of the triazole linkage (ODN-A-*t*1 and ODN-A-*t*2) were examined. The T_m values from these experiments were then compared with the pyrene functionalised oligonucleotides: ODN-A-*t*1-P and

ODN-A-*t*2-P hybridised to the same mismatched strands. Finally, the same UV melting experiments were performed on the non-triazole oligonucleotides containing only the 2'-modification ODN-A-*a*1, to study mismatch sensing properties of oligonucleotides containing only pyrene without triazole. The complete set of data is presented in the Table 3.4.

In the DNA:RNA series, unmodified control DNA hybridised with RNA containing a single T:U mismatch exhibited destabilisation of the duplex in the range 12.0 to 21.0 °C. Single T:U mismatch with the oligonucleotides containing triazole with 2'-aminoethoxy and the non-triazole oligonucleotides with only 2'-aminoethoxy (ODN-A-*t*1, ODN-A-*t*2 and ODN-A-*a*1) also showed duplex destabilisation in the range from 13.1 °C to 26.1 °C, varying depending on the position of the mismatch relative to the modification. The most successful mismatch discrimination was exhibited by the ODN-A-*t*1 with a greater average mismatch destabilisation in comparison to the unmodified mismatch control duplex of *ca.* 7.0 °C. In the case of oligonucleotides labelled with pyrene (ODN-A-*t*1-P, ODN-A-*t*2-P and ODN-A-*a*1-P) an average destabilising effect caused by a single T:U mismatch was of 6.8 °C smaller compared to that of not labelled ODN-A-*t*1, ODN-A-*t*2 and ODN-A-*a*1. Overall, for oligonucleotides containing pyrene, the least sensitive mismatch position was the site directly opposite to the 2'-aminoethoxy modification. When single T:U mismatch was incorporated to the duplex, the ODN-A-*t*1-P and ODN-A-*t*2-P directly opposite to the position of pyrene, only small decrease in T_m was showed of 6.5 and 5.0 °C respectively. The moderate mismatch discrimination properties at these positions were to be expected considering the non-sequence specific binding properties of pyrene. Location of a single mismatches one base away from the triazole and pyrene showed significantly better mismatch sensing properties decreasing the T_m by 16.5 °C up to 18.3 °C.

In the context of DNA:DNA hybrid duplexes, the same patterns were observed in the T_m when placing the mismatched base T in the opposite DNA strand. The destabilising effect caused by a single mismatch however, in the DNA:RNA was much greater than in the DNA:DNA by an average of 7.4 °C. This is particularly useful information when considering antisense oligonucleotides applications. Overall these melting studies show that oligonucleotides containing triazole and pyrene are moderately sensitive to the mismatched base pairing and the level of discrimination depends on the position of the mismatch relative to the triazole.

Table 3.4 Mismatch sensing with ODN-A-*t*1-P, ODN-A-*t*2-P and ODN-A-*a*1-P; comparison with unmodified control and unfunctionalised ODN-A-*t*1, ODN-A-*t*2 and ODN-A-*a*1. A single T or U mismatch was introduced at positions underlined in the sequence CGACGCTTGCAGC. Green 5-member ring = triazole, NH₂ = unfunctionalised 2'-aminoethoxy, pyrene = functionalised 2'-aminoethoxy with pyrene. The table shows ΔT_m vs mismatch position where $\Delta T_m = T_m(\text{mismatch}) - T_m(\text{match})$.

ODN-A	RNA	DNA
	-12.1	-21.0
	-7.5	-13.3
	-7.7	-14.5
ODN-A- <i>t</i> 1	RNA	DNA
	-15.9	-26.1
	-1.4	-21.9
	-15.9	-26.1
ODN-A- <i>t</i> 2	RNA	DNA
	-9.0	-16.7
	-2.8	-13.1
	-5.4	-14.4
ODN-A- <i>a</i> 1	RNA	DNA
	-15.5	-19.9
	-4.9	-13.1
	-8.7	-17.1
ODN-A- <i>t</i> 1-P	RNA	DNA
	-12.9	-18.3
	+3.0	-6.5
	-2.2	-6.3
ODN-A- <i>t</i> 2-P	RNA	DNA
	-15.0	-18.2
	-2.1	-5.0
	-5.1	-11.7
ODN-A- <i>a</i> 1-P	RNA	DNA
	-11.5	-16.5
	-3.2	-7.5
	-2.8	-7.4

3.4 SUMMARY AND OUTLOOK

In this chapter the biophysical properties of oligonucleotides containing triazole linkages with additional 2'-modification were examined. In agreement with the literature, oligonucleotides containing triazole linkages and the non-functionalised 2'-aminoethoxy groups presented here decreased the stability of the duplex compared to the unmodified DNA:RNA and DNA:DNA control. It has been shown however, that addition of pyrene or anthraquinone moieties to the 2'-position of ribose next to the triazole is a very efficient method for restoring lost duplex stability caused by the artificial triazole backbone.

Different lengths and flexibility of the linker between the pyrene and oligonucleotide were also examined to attempt to improve the stabilising properties of this moiety. Oligonucleotides functionalised with pyrene *via* short one-carbon linker were examined (compared to the four-carbon linker) but did not show improved duplex binding properties. Oligonucleotides functionalised with pyrene *via* a more flexible amine linker (compared to the more rigid amide) were also tested and also did not show improved hybridisation to complementary RNA and DNA. These results suggest that the four-carbon amide linker allows for most efficient interaction with the complementary strand.

Circular dichroism studies showed that oligonucleotides containing a triazole linkage with an additional 2'-modification have a minimal effect on the secondary structure of duplexes with complementary RNA and DNA.

The mismatch studies carried out with RNA and DNA strands containing single T:U or T:T mismatch on either side of the triazole or one base away from triazole showed moderate mismatch sensing properties. This could be

due to the non-sequence specific and very strong interaction between pyrene and the opposite strand.

As a concluding point, oligonucleotides containing moieties such as pyrene or anthraquinone attached to the 2'-position of ribose next to the unnatural, neutral triazole linkage form very stable duplexes with both RNA and DNA complementary strands. Such modifications do not appear to greatly alter the conformation / secondary structure of duplex. These properties make such modified oligonucleotides an interesting target for further examination for their exon-skipping therapeutic applications.

4

TRIAZOLE MODIFIED PHOSPHOROTHIOATE AND 2'-METHOXY OLIGONUCLEOTIDES FOR ANTISENSE APPLICATIONS

In Chapters 2 and 3 it was shown that it is possible to synthesise oligonucleotides containing a neutral triazole backbone at specific loci. These form very stable duplexes with complementary RNA and DNA upon the addition of 2'-ribose modifications such as pyrene or anthraquinone derivatives. In order for the triazole-modified oligonucleotide to have a chance of exhibiting more significant changes in biophysical and biological properties, it is clear that multiple additions of this backbone in the sequence might be required. This chapter describes the synthesis of new T-C triazole dimer 38. Synthesis of oligonucleotides compatible with a luciferase assay containing single to quadruple additions of the triazole linkage is also discussed. Then, post-synthetic functionalisation is performed according to

the method described in chapter 2 and finally the biophysical properties of the modified oligonucleotides are presented.

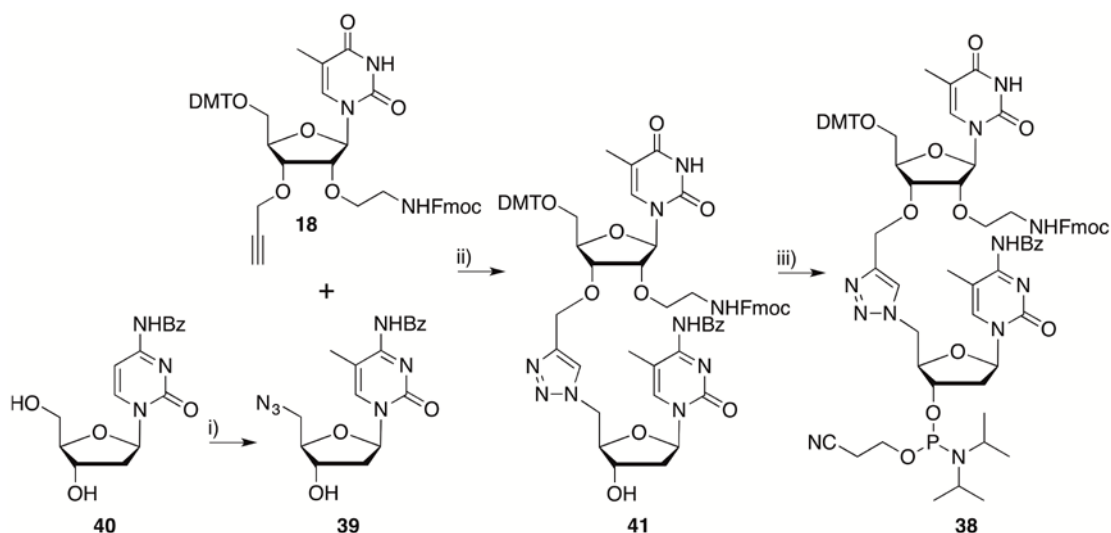
4.1 INTRODUCTION: APPLICATIONS OF PHOSPHOROTHIOATE AND 2'-

METHOXY OLIGONUCLEOTIDES

Nucleases that occur widely in eukaryotic cells and serum readily digest foreign strands of nucleic acids which have unmodified (natural) phosphodiester backbones.¹⁷¹⁻¹⁷⁴ Therefore, unmodified antisense oligonucleotides are cleaved in the cell before they reach their target. The phosphorothioate (PS) linkage is a very well established backbone modification used to enhance the nuclease stability of oligonucleotides in cells and serum.^{27,31} The major disadvantages of this backbone modification are (i) its negative effect on duplex stability and (ii) the fact that PS oligonucleotides are diastereomeric mixtures at phosphorus.²⁰ To improve the hybridisation properties of PS-modified oligonucleotides, 2'-ribose modifications are introduced such as 2'-methoxy (2'-OMe) or 2'-methoxyethyl (2'-MOE). These ribose adducts restore the thermal stability of the duplex such that it is similar to that of the unmodified duplex.^{34,175,176} In the previous chapters triazole containing oligonucleotides functionalised with pyrene and anthraquinone were shown to form stable duplexes with complementary RNA. The triazole linkage, which is a neutral and unnatural modification, should be stable to nucleases and might also improve the cellular uptake of these oligonucleotides. In order to test the antisense exon-skipping activity and cellular uptake properties of such modified oligonucleotides in the luciferase assay, synthesis of appropriate oligonucleotides was necessary. In order to ensure sufficient nuclease stability of the modified oligonucleotide in the cell, it was decided to also incorporate the PS backbone and 2'-OMe sugar modification in the new modified oligonucleotides containing the triazole linkage and 2'-aminoethoxy modifications.

4.2 SYNTHESIS OF T-C TRIAZOLE DIMER PHOSPHORAMIDITE

The sequence required for the exon-skipping luciferase assay used in this study (discussed in more detail in chapter 5) accommodates only two additions of previously synthesised T-T triazole dimer **1**. The T-T dimers are placed instead of the U-U dimers in the desired sequence and are underlined in the following sequence: 5'-CCUCUUACCUCAGUUACA-3'. In order to incorporate multiple additions of the triazole linkage into the desired sequence, synthesis of a new triazole dimer was required. For synthetic tractability it was decided to synthesise T-C triazole dimer **38** which allowed incorporation of another two triazole linkages into the designed sequence. The T-C dimers are placed in position of U-C dimers and these are indicated in bold as follows: 5'-CC**UC**UUACC**UC**AGUUACA-3'. To synthesise this new T-C triazole dimer **38**, the previously prepared alkyne precursor 2'-aminoethoxy-5'-*O*-DMT-3'-*O*-propargyl-thymidine **18** was used, therefore only the synthesis of 5'-azido cytidine **39** was required (Scheme 4.1).



Scheme 4.1 Formation of the T-C triazole dimer phosphoramidite (**38**). (i) CBr_4 , PPh_3 , DMF, LiN_3 , rt – 60 °C, 18 h, 47% (ii) CuSO_4 , sodium ascorbate, TBTA ligand, DMF, H_2O , 4 h, rt, 92%; (iii) DIPEA, $\text{CIP}(\text{OCH}_2\text{CH}_2\text{CN})\text{N}(\text{iPr})_2$, CH_2Cl_2 , rt, 56%.

The 5'-azido-*N*⁴-benzoyl-2'-deoxycytidine **39** was synthesised in one step according to the literature.¹⁷⁷ Triphenylphosphine and carbon tetrabromide were reacted with *N*⁴-benzoyl-2'-deoxycytidine **40**, followed by the addition of LiN₃ (20% solution in water), giving the desired azide **39** in 47% yield. Compound **39** was then reacted with the alkyne monomer **18** *via* the CuAAC click reaction to give the T-C triazole dimer **41** in a very good yield of 92%. Dimer **41** was finally phosphitylated using the standard procedure to deliver the desired phosphoramidite **38** in 56% yield ready for solid-phase oligonucleotides synthesis (Scheme 4.1).

4.3 SOLID-PHASE SYNTHESIS AND POST-SYNTHETIC

FUNCTIONALISATION OF PHOSPHOROTHIOATE 2'-METHOXY

MODIFIED OLIGODEOXYRIBONUCLEOTIDES CONTAINING TRIAZOLE

LINKAGES AND 2'-AMINOETHOXY MODIFICATIONS

Using the two synthesised phosphoramidite triazole dimers: T-T dimer **1** and T-C dimer **38**, three PS/2'-OMe modified oligonucleotides were synthesised with single, triple and quadruple additions of the triazole linkage, in addition to fully PS/2'-OMe control oligonucleotides (Table 4.1). To investigate whether the 2'-ribose modification without triazole could also induce exon-skipping activity, PS/2'-OMe non-triazole oligonucleotides with single to triple additions of monomer 2'-aminoethoxy T **2** were also synthesised (Table 4.1). Synthesis of all ODN-D oligonucleotides was done in collaboration with ATDBioio company.

Table 4.1 List of phosphorothioate and 2'-OMe oligonucleotides with single, triple or quadruple additions of the triazole linkage and single, double or triple addition of 2'-aminoethoxy T, in sequence D; ^aT = 2'-aminoethoxy T, *t* = triazole.

code	sequence 5'-3'	calc. mass	found mass
ODN-D-1	CCUCUUACCUCAGUUACA	6096	6098
ODN-D- <i>t</i> 1	CC ^a T <i>t</i> CUUACCUCAGUUACA	6109	6111
ODN-D- <i>t</i> 2	CC ^a T <i>t</i> CUUACC ^a T <i>t</i> CAG ^a T <i>t</i> TACA	6106	6107
ODN-D- <i>t</i> 3	CC ^a T <i>t</i> C ^a T <i>t</i> TACC ^a T <i>t</i> CAG ^a T <i>t</i> TACA	6118	6119
ODN-D- <i>a</i> 1	CCUCUUACC ^a TCAGUUACA	6140	6141
ODN-D- <i>a</i> 2	CCUC ^a TUACCUCAG ^a TUACA	6183	6184
ODN-D- <i>a</i> 3	CCUC ^a TUACC ^a TCAG ^a TUACA	6226	6227

Synthesis of oligonucleotides with the PS backbone requires changes to the solid-phase oligonucleotide synthesis cycle at the oxidation step in order to replace one non-bridging oxygen atom with a sulfur atom. A large number of PS oligonucleotide drugs are being evaluated in preclinical and clinical studies.¹⁷⁸⁻¹⁸¹ Therefore there was a need to develop a high yielding, robust and economical method for commercial scale production of high quality PS oligonucleotides. A variety of very efficient sulfurizing reagents have been developed to date. Reagents such as 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent) **42**, 3-amino-1,2,4-dithiazole-5-thione (ADTT) **43**, phenylacetyl disulphide (PADS) **44** or 3-ethoxy-1,2,4-dithiazolidin-5-one (EDITH) **45** are most commonly used at present (Figure 4.1).¹⁸²⁻¹⁸⁵

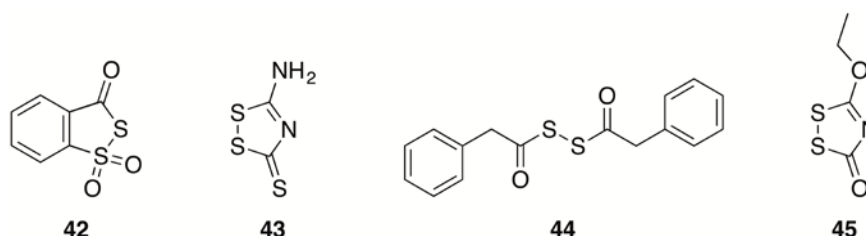


Figure 4.1 Sulfurizing reagents: Beaucage reagent (**42**), ADTT (**43**), PADS (**44**) and EDITH (**45**).

In the synthesis of PS oligonucleotides ODN-D at atdbio, reagent EDITH **45** was utilised. In the synthesis of ODN-E oligonucleotides at GlaxoSmithKline, reagent PADS **44** was used. The choice of the reagents was dictated by the previous sulfurization experience in both companies, with both being considered excellent sulfurizing reagents.

After successful synthesis, deprotection and RP-HPLC purification of all the ODN-D oligonucleotides, post-synthetic functionalisation was carried out with 1-pyrenebutyric acid *N*-hydroxysuccinimide ester **30** and anthraquinone active carbonate **35** as described previously in section 2.4, followed by a final RP-HPLC purification (Table 4.2).

Table 4.2 List of phosphorothioate and 2'-OMe oligonucleotides with single, triple or quadruple additions of the triazole linkage and single, double or triple additions of 2'-aminoethoxy T, in sequence D, functionalised with pyrene (P) and anthraquinone (A); ^aT = 2'-aminoethoxy T, *t* = triazole.

ODN	label	code	calc. mass	found mass
ODN-D- <i>t</i> 1	free amine	ODN-D- <i>t</i> 1	6109	6111
	pyrene	ODN-D- <i>t</i> 1-P	6379	6381
	anthraquinone	ODN-D- <i>t</i> 1-A	6487	6489
ODN-D- <i>t</i> 2	free amine	ODN-D- <i>t</i> 2	6106	6107
	pyrene	ODN-D- <i>t</i> 2-P	6916	6917
	anthraquinone	ODN-D- <i>t</i> 2-A	7240	7242
ODN-D- <i>t</i> 3	free amine	ODN-D- <i>t</i> 3	6118	6119
	pyrene	ODN-D- <i>t</i> 3-P	7198	7200
	anthraquinone	ODN-D- <i>t</i> 3-A	7630	7632
ODN-D- <i>a</i> 1	free amine	ODN-D- <i>a</i> 1	6140	6141
	pyrene	ODN-D- <i>a</i> 1-P	6410	6411
	anthraquinone	ODN-D- <i>a</i> 1-A	6519	6520
ODN-D- <i>a</i> 2	free amine	ODN-D- <i>a</i> 2	6183	6184
	pyrene	ODN-D- <i>a</i> 2-P	6723	6725
	anthraquinone	ODN-D- <i>a</i> 2-A	6941	6941
ODN-D- <i>a</i> 3	free amine	ODN-D- <i>a</i> 3	6226	6227
	pyrene	ODN-D- <i>a</i> 3-P	7037	7039
	anthraquinone	ODN-D- <i>a</i> 3-A	7363	7363

Labelling with pyrene proceeded very smoothly, even with quadruple additions; a fully labeled product was achieved after a 5 hour reaction. Functionalisation with anthraquinone however, presented some difficulties and re-labelling was required to drive the reaction to completion.

4.4 BIOPHYSICAL PROPERTIES OF PHOSPHOROTHIOATE 2'-METHOXY

MODIFIED OLIGODEOXYRIBONUCLEOTIDES CONTAINING TRIAZOLE

LINKAGES AND 2'-AMINOETHOXY MODIFICATIONS

RNA:RNA duplexes are thermally more stable than DNA:DNA duplexes.¹⁸⁶ This indicates that mimicking RNA-like structures could be beneficial in the development of antisense oligonucleotides with favourable binding to RNA targets.

The chemical differences between natural phosphodiester DNA and PS/2'-OMe modified nucleic acids are the presence of the sulfur atom instead of one of the non-bridging oxygen atoms in the phosphate backbone and the 2'-substituent on the furanose ring. Sulfur, which is used to improve the stability of the modified oligonucleotide in serum, does not significantly alter the conformation of DNA.²⁰ Substitution of the 2'-position with an electronegative group such as a methoxy group however, shifts the sugar conformational equilibrium from *C*_{2'}-*endo* toward the *C*_{3'}-*endo* pucker leading to A-form duplexes, characteristic of RNA. The effect of the 2'-OMe modification did indeed improve the thermal stability of this oligonucleotide when hybridized to complementary RNA.¹⁸⁷

Modified oligonucleotides described in chapters 2 and 3 were deoxyribonucleotides; where the 2'-deoxyribose forms a *C*_{2'}-*endo* pucker conformation which is characteristic of the B-form secondary structure. PS/2'-

OMe oligonucleotides with the triazole dimers and 2'-aminoethoxy T shift the conformation of the molecule to the A-form due to the C_3' -*endo* pucker of the ribose with 2'-OMe group, which potentially could be beneficial for their hybridization properties.

The thermal stability of oligonucleotides with single, triple and quadruple additions of triazole linkages and unlabelled 2'-aminoethoxy groups with a complementary RNA strand was first examined. The UV melting measurements (Figure 4.2) showed that the incorporation of the triazole linkage decreased the thermal stability of the duplex on average 4.8 °C per single addition. ODN-D-**1** ($T_m = 65.9$ °C) with a single addition of triazole decreased the T_m by 4.3 °C. Triple additions of triazole (ODN-D-**2** $T_m = 55.5$ °C) decreased the T_m by 14.7 °C and quadruple additions of triazole (ODN-D-**3** $T_m = 49.4$ °C) dropped the T_m by 20.8 °C (Figure 4.2). Chapter 3 describes oligonucleotides with the triazole linkage incorporated to DNA strands with a normal phosphodiester backbone (ODN-A and ODN-B). In these sequences the modified backbone caused a decrease in T_m in a range of *ca.* 8.0 to 11.0 °C in the duplex with complementary RNA, depending on the position of the triazole linkage in the sequence. The triazole incorporated into PS/2'-*O*-Me sequence ODN-D caused a smaller destabilisation of the duplex, in average of only 4.8 °C per addition. This suggests that a triazole backbone is better accommodated in the A-form conformation characteristic for PS/2'-*O*-Me oligonucleotides, potentially causing less structural perturbation.

Based on the results presented in the chapter 3, functionalisation of the 2'-aminoethoxy group with pyrene was expected to compensate for lost duplex stability caused by the addition of triazole. UV melting studies (Figure 4.2) of ODN-D-**1**-P, with a single addition of triazole linkage and pyrene showed an increase of T_m of 5.2 °C compared to the unfunctionalised ODN-D-**1**-P and exceeded the T_m of the PS/2'-*O*-Me control by 0.9 °C.

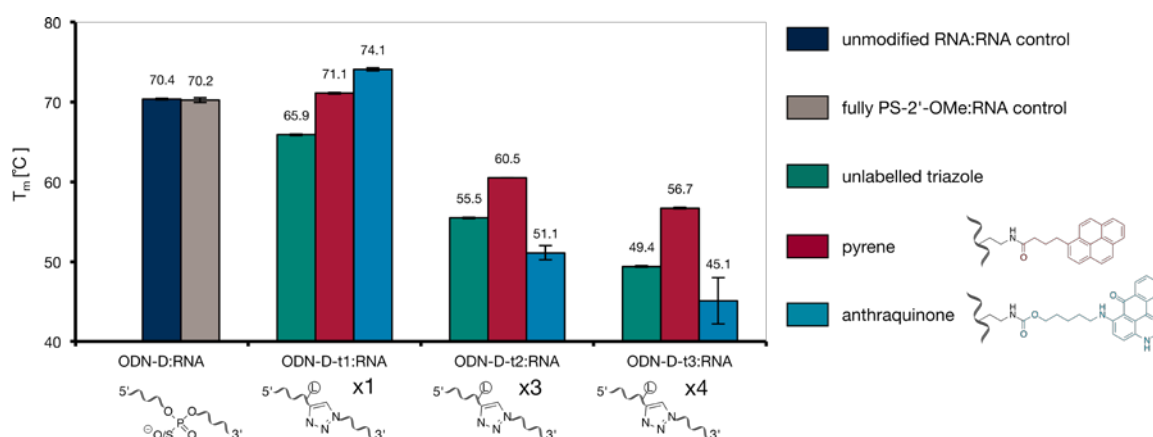


Figure 4.2 Single, triple and quadruple additions of triazole linkage oligonucleotides (ODN-D-*t*1, ODN-D-*t*2, ODN-D-*t*3) and their derivatives functionalised with pyrene and anthraquinone and their effect on duplex thermal stability compared to the unmodified duplex with complementary RNA. Control unmodified duplex (dark blue), control fully PS/2'-OMe (grey), unfunctionalised 2'-aminoethoxy group (green), 2'-aminoethoxy group functionalised with pyrene (red) 2'-aminoethoxy group functionalised with anthraquinone (blue). The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 2 μ M.

The stabilising effect of pyrene attached to the oligonucleotides with triple and quadruple additions of the triazole linkage (ODN-D-*t*2-P and ODN-D-*t*3-P) was not as high as anticipated. In ODN-D-*t*2-P (T_m = 60.5 $^{\circ}$ C) with three triazole linkages, the three pyrenes increased the T_m by only 5.0 $^{\circ}$ C, which makes this duplex almost 10.0 $^{\circ}$ C less stable than the control. ODN-D-*t*3-P (T_m = 56.7 $^{\circ}$ C) with four triazole linkages and four pyrenes increased the T_m by only 7.3 $^{\circ}$ C, which makes this duplex 13.5 $^{\circ}$ C less stable than the control. A single addition of anthraquinone (ODN-D-*t*1-A T_m = 74.1 $^{\circ}$ C) improved the T_m by 8.2 $^{\circ}$ C compared to unfunctionalised ODN-D-*t*1 with a single triazole, forming a duplex almost 4 $^{\circ}$ C more stable than the unmodified control. Addition of three and four anthraquinone moieties next to triazole linkages (ODN-D-*t*2-A T_m = 51.1 $^{\circ}$ C and ODN-D-*t*3-A T_m = 45.1 $^{\circ}$ C) caused further duplex destabilisation compared to the unlabeled ODN-D-*t*2 and ODN-D-*t*3. These results suggest that the addition of more than two triazole linkages to

the short sequence causes significant changes that cannot be efficiently restored by the addition of groove binding or intercalating moieties such as pyrene or anthraquinone. The triazole causes too much conformational flexibility in the oligonucleotide backbone leading to a region in which duplex formation is entropically unfavorable.

UV melting studies of oligonucleotides with single to triple additions of 2'-aminoethoxy groups without triazole linkages and fully PS/2'-OMe (ODN-D-*a*1, ODN-D-*a*2 and ODN-D-*a*3) were also performed (Figure 4.3).

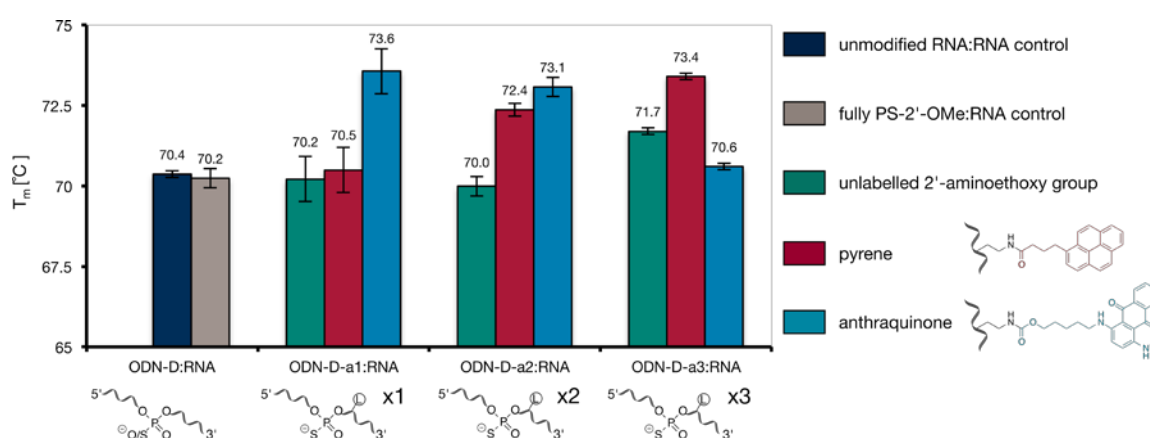


Figure 4.3 Single, double and triple additions of 2'-aminoethoxy T oligonucleotides (ODN-D-*a*1, ODN-D-*a*2, ODN-D-*a*3) and their derivatives functionalised with pyrene and anthraquinone and their effect on duplex thermal stability compared to unmodified duplex with complementary RNA. Control unmodified duplex (dark blue), control fully PS/2'-OMe (grey), unfunctionalised 2'-aminoethoxy group (green), 2'-aminoethoxy group functionalised with pyrene (red) 2'-aminoethoxy group functionalised with anthraquinone (blue). The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 2 μ M.

Single and double additions of the unfunctionalised 2'-aminoethoxy group (ODN-D-*a*1 T_m = 70.2 $^{\circ}$ C and ODN-D-*a*2 T_m = 70.0 $^{\circ}$ C) did not influence the hybridisation of the modified oligonucleotides to complementary RNA compared to the unmodified control. Triple additions of the 2'-aminoethoxy group (ODN-D-*a*3 T_m = 71.7 $^{\circ}$ C) gave a slight stabilising effect, increasing the T_m by 1.5 $^{\circ}$ C compared to the control duplex. This could be due to the

positive charge formed by protonation of the free amine in the pH 7.2 buffer causing a reduction of the electrostatic repulsion of the backbone. This effect was not observed for the oligonucleotides containing the triazole perhaps because of the bigger destabilising effect caused by the triazole linker. The single addition of pyrene (ODN-D-**a1**-P $T_m = 70.5\text{ }^{\circ}\text{C}$) increased the T_m by only $0.3\text{ }^{\circ}\text{C}$ compared to the unfunctionalised ODN-D-**a1**, double addition of pyrene (ODN-D-**a2**-P $T_m = 72.4\text{ }^{\circ}\text{C}$) increased the T_m by $2.4\text{ }^{\circ}\text{C}$ and triple addition of pyrene (ODN-D-**a3**-P $T_m = 73.4\text{ }^{\circ}\text{C}$) increased the T_m by $1.7\text{ }^{\circ}\text{C}$ from the unlabeled oligonucleotides. These results show that the T_m for oligonucleotides labeled with pyrene increases with the growing number of pyrene moieties, however the positive effect per single modification decreases with growing number of pyrenes in the sequence. Oligonucleotides functionalised with the anthraquinone derivative showed also a decreasing stabilising effect with their number in the sequence was increased. However, single and double additions cause formation of a more stable duplex than control. A single addition of anthraquinone (ODN-D-**a1**-A $T_m = 73.6\text{ }^{\circ}\text{C}$) increased the T_m by $3.4\text{ }^{\circ}\text{C}$ compared to unlabeled ODN-D-**a1**, the double addition of anthraquinone (ODN-D-**a2**-A $T_m = 73.1\text{ }^{\circ}\text{C}$) increased the T_m by $3.1\text{ }^{\circ}\text{C}$ and the triple addition of anthraquinone (ODN-D-**a3**-A $T_m = 70.6\text{ }^{\circ}\text{C}$) decreased the T_m by $1.1\text{ }^{\circ}\text{C}$. A similar pattern was found for oligonucleotides containing triazole linkages and anthraquinone as described above (ODN-D-**t1**-A, ODN-D-**t2**-A and ODN-D-**t3**-A). There, also an increasing number of anthraquinones led to an increasingly negative effect on the duplex stability. These results suggest that excessive unwinding of the duplex caused by intercalation of the anthraquinone or structural perturbation caused by its groove binding is too severe, therefore inhibiting efficient hybridisation to the complementary strand.

To check that this moderately stabilising effect of pyrene attached next to the triazole linkage is not due to the particular sequence of ODN-D, a new set of

PS/2'-OMe oligonucleotides of the same length and containing single and double additions of T-T triazole dimer **1** was synthesised and post-synthetically labeled with pyrene and guanidine (Table 4.3). The new sequence ODN-E was designed and prepared in collaboration with GlaxoSmithKline. The positions of triazole dimer incorporation are underlined, where the T-T dimer was placed instead of U-U dimer as follows: 5'-GCU AUU ACC UUA ACC CAG-3'). The choice of this particular sequence was directed by the requirements of the biological assays used at GSK.

Table 4.3 List of phosphorothioate and 2'-OMe oligonucleotides with single or double addition of triazole linkage in sequence E, functionalised with pyrene (P) and guanidine (G); ^aT = 2'-aminoethoxy T, *t* = triazole

code	sequence 5'-3' + label	calc. mass	found mass
ODN-E	GCUAUUACCUUAACCCAG	6160	6161
ODN-E- <i>t</i> 1	GCUA ^a T _t TACCUUAACCCAG	6173	6173
ODN-E- <i>t</i> 1-P	+ pyrene	6443	6444
ODN-E- <i>t</i> 1-G	+ guanidine	6216	6216
ODN-E- <i>t</i> 2	GCUA ^a T _t TACC ^a T _t TAACCCAG	6185	6186
ODN-E- <i>t</i> 2-P	+ pyrene	6725	6726
ODN-E- <i>t</i> 2-G	+ guanidine	6271	6270

The UV melting studies of the ODN-E oligonucleotides showed similar patterns in T_m compared to the ODN-D group of oligonucleotides described above (Figure 4.4). Here, a single addition of triazole (ODN-E-*t*1 T_m = 67.6 °C) destabilised the duplex by 5.2 °C and double additions of triazole (ODN-E-*t*2 T_m = 60.5 °C) destabilised duplex by 12.8 °C compared to the PS/2'-OMe control. This makes an average ΔT_m per triazole modification of -6.0 °C, similar to that seen with the triazole incorporated into the ODN-D (-4.8 °C). Addition of a single pyrene (ODN-E-*t*1-P T_m = 71.5 °C) increased the duplex stability by 3.9 °C and almost fully compensated the lost thermal stability caused by the triazole. Double additions of pyrene next to triazole (ODN-E-

*t*₂-P ($T_m = 68.1\text{ }^{\circ}\text{C}$) also showed a stabilising effect, however this was not sufficient to compensate for the destabilisation caused by the unlabeled two triazole linkers ($\Delta T_m = -4.7\text{ }^{\circ}\text{C}$). These results show that the limited stabilising properties of pyrene attached to the 2'-position of ribose next to the triazole linkage are not likely to be sequence dependent.

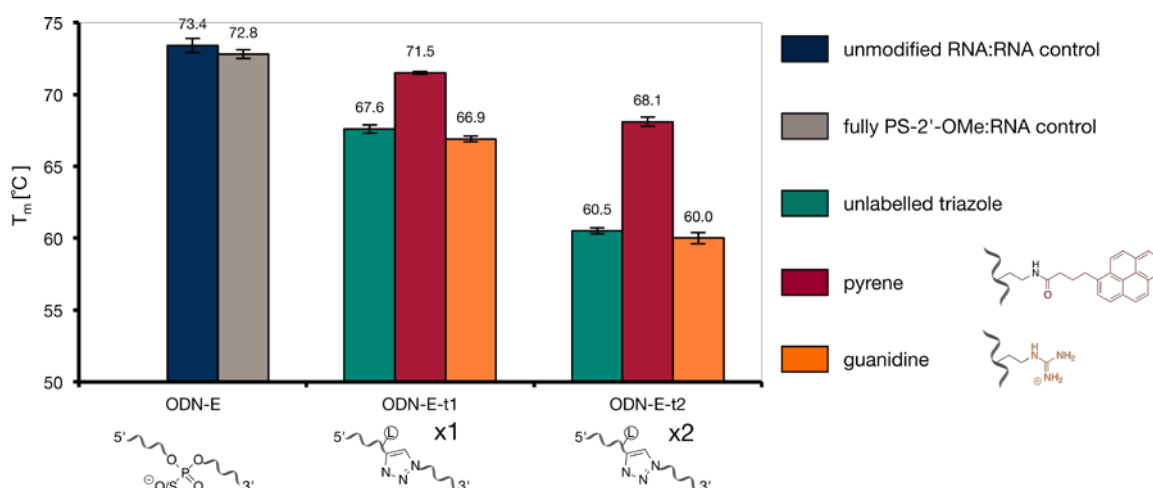


Figure 4.4 Single, and double additions of triazole linkage oligonucleotides (ODN-E-*t*₁, ODN-E-*t*₂) and their derivatives functionalised with pyrene and guanidine and their effect on the duplex thermal stability compared to unmodified duplex with complementary RNA. Control unmodified duplex (dark blue), control fully PS/2'-OMe (grey), unfunctionalised 2'-aminoethoxy group (green), 2'-aminoethoxy group functionalised with pyrene (red) 2'-aminoethoxy group functionalised with guanidine (orange). The experiments were carried out in 10 mM NaH₂PO₄/Na₂HPO₄, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 2 μM .

Chapter 3 described the biophysical properties of oligonucleotides containing the triazole linkage incorporated into the oligodeoxyribonucleotide (B-type) and functionalised with the positively charged guanidine moiety. Those results showed that the guanidine moiety was not able to enhance binding to either complementary RNA or DNA. Guanidine however, has a positive charge, which in combination with neutral triazole could potentially improve the cellular uptake of such modified antisense oligonucleotides. In order to

investigate whether this guanidine moiety could have a higher stabilising effect when incorporated into the oligonucleotide with a RNA-like conformation (A-type), thermal stability studies of ODN-E-**t1**-G and ODN-E-**t2**-G were carried out (Figure 4.4). These UV melting studies demonstrated that a change of conformation of the antisense strand containing triazole and guanidine to the A-type has no significant influence to the binding affinity of this oligonucleotide to the complementary RNA. In both cases, with a single addition of triazole and guanidine (ODN-E-**t1**-G $T_m = 66.9\text{ }^{\circ}\text{C}$) and the double addition (ODN-E-**t2**-G $T_m = 60.0\text{ }^{\circ}\text{C}$), the melting temperatures were almost unchanged from the unfunctionalised ODN-E-**t1** $T_m = 67.6\text{ }^{\circ}\text{C}$ and ODN-E-**t2** $T_m = 60.5\text{ }^{\circ}\text{C}$ counterparts respectively.

4.5 SUMMARY AND OUTLOOK

In summary, the successful synthesis of the novel T-C triazole dimer **38** was accomplished. In addition, the solid-phase synthesis of fully PS/2'-OMe oligonucleotides containing single to quadruple additions of triazole linkages (ODN-D-**t1**, ODN-D-**t2**, ODN-D-**t3** and ODN-E-**t1**, ODN-E-**t2**) or single to triple addition of 2'-aminoethoxy thymidine (ODN-D-**a1**, ODN-D-**a2** and ODN-D-**a3**) was successfully performed. Post-synthetic labelling was also successfully carried out. However, functionalisation with anthraquinone derivatives required a second labelling reaction in order to drive the reaction to completion.

UV melting studies showed that on average, the single addition of the triazole backbone caused a decrease in duplex stability of *ca.* $5.0\text{ }^{\circ}\text{C}$ and *ca.* $6.0\text{ }^{\circ}\text{C}$ per addition in the sequences ODN-D and ODN-E respectively. This was found to be less destabilising compared to the incorporation of the same triazole linkage into the phosphodiester DNA sequence. These results suggest

that such triazole linkages are better accommodated in the RNA-like conformation.

Furthermore, pyrene attached next to the triazole dimer (ODN-D-*t*1-P, ODN-D-*t*2-P, ODN-D-*t*3-P and ODN-E-*t*1-P, ODN-E-*t*2-P) turned out to have a limited stabilising effect, increasing the T_m by only *ca.* 4.0 to 8.0 °C, even with quadruple additions, compared to the unfunctionalised triazole reference oligonucleotides ODN-D-*t*1, ODN-D-*t*2, ODN-D-*t*3 and ODN-E-*t*1, ODN-E-*t*2. The UV melting studies also showed a pattern of decreasing stabilising effects of pyrene per addition as their number increased in the sequence. This decreasing pattern was also exhibited when pyrene was attached to the oligonucleotides without triazole (ODN-D-*a*1-P, ODN-D-*a*2-P and ODN-D-*a*3-P), although the overall T_m values did consistently increase with growing number of pyrenes. These results suggest that there are significant differences in how pyrene interacts with the duplex in oligonucleotides containing triazole linkages and those without triazole. Fluorescence melting studies and structural modeling could be carried out in future to provide insight into the interactions involving pyrene in these different duplexes.

Addition of more than two anthraquinones in the RNA-like PS/2'-OMe oligonucleotides (both triazole and non-triazole oligonucleotides) had a negative effect on the duplex stability, causing a decrease of overall T_m , and leading to formation of duplexes that were less stable than that of the unfunctionalised controls.

Finally, incorporation of guanidine into the RNA-like sequence (ODN-E-*t*1-G, ODN-E-*t*2-G) also did not enhance its duplex stabilising potential. Addition of this positively charged group had no effect on the hybridisation to the complementary RNA, similarly to the DNA oligonucleotides containing guanidine described in chapter 3.

There is no simple rule for how stable a duplex between an antisense oligonucleotide and its RNA target must be to give efficient antisense activity.

It will be interesting to examine whether the moderate T_m values of the described modified oligonucleotides with their complementary RNA targets are high enough to allow the oligonucleotide to perform exon skipping. The oligonucleotides described above do still form a duplex with RNA at 37.0 °C and in combination with the potentially improved cellular uptake given by the neutral triazole linkage, they could form the basis of antisense drugs.

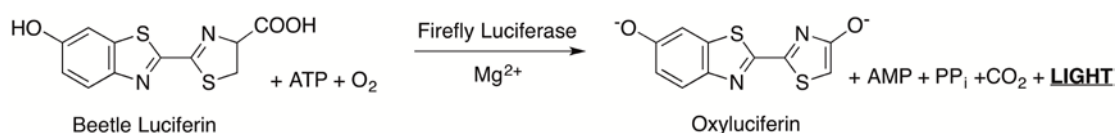
5

SPLICE-SWITCHING ACTIVITY OF PHOSPHOROTHIOATE, 2'-METHOXY, TRIAZOLE AND 2'-AMINOETHOXY MODIFIED OLIGONUCLEOTIDES

This chapter begins with a brief overview of the splice-switching luciferase assay before describing the results obtained using this assay for the modified ODN-D and ODN-E oligonucleotides detailed in chapter 4.

5.1 INTRODUCTION TO THE SPLICE-SWITCHING LUCIFERASE ASSAY

The luciferase assay is a 'reporter' assay commonly used to study gene expression, mRNA processing, and/or protein folding. Luciferase is a class of oxidative enzymes that produce bioluminescence. The signal is produced based on the bioluminescent reaction catalysed by luciferase (Scheme 5.1). Firefly luciferase is a monomeric 61 kDa protein, which catalyses luciferin oxidation using ATP/Mg²⁺. Conversion of the chemical energy of luciferin oxidation through an electron transition to form the product oxyluciferin results in light production.



Scheme 5.1 Bioluminescent reaction catalysed by firefly luciferase.

The firefly luciferase protein is used because:

- The signal of the reporter is available promptly upon translation and no or very little post-translational processing of the protein is required.
- The assay is very sensitive as the bioluminescence quantum yield is very high.
- The luminescence-generating reaction is rapid and requires only a few seconds per sample, ensuring robustness of the assay and also can be automated for high-throughput applications.

The generated luminescent signal is directly related with the amount of expressed protein so it reports the translation process. Due to various cellular translation factors, in some cases, the translation process might not be very efficient although the gene might have been well transcribed to mRNA. In

order to complement the luciferase signal results and measure directly the amount of transcribed mRNA, quantitative RT PCR can be carried out.¹⁸⁸⁻¹⁹¹ For the experiments carried out in this study, HeLa cells were utilised which were stably transfected with a recombinant plasmid pLuc/705 (courtesy of Prof M. Wood, Oxford University). This pLuc/705 plasmid carries the luciferase gene interrupted by the insertion of a single-point mutation from the human β -globin intron 2 (IVS2-705), which causes β -thalassemia in patients.¹⁹² The single T to G mutation at nucleotide 705 in intron 2 creates an additional 5' splice site and also activates a cryptic 3' splice site 126 nucleotides upstream. This mutation in the intron results in aberrant splicing of luciferase pre-mRNA, causing retention of the intron fragment with a stop codon, which prevents translation of the luciferase (Figure 5.1).

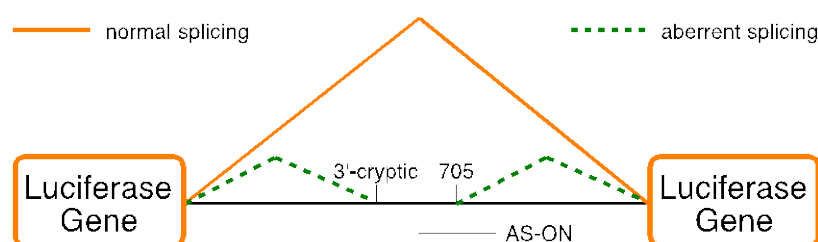


Figure 5.1 Pre-mRNA of modified luciferase gene, pLuc/705. Orange boxes and solid thick black line represent modified luciferase gene sequence with introduced β -globin intron 2 carrying mutation at nucleotide 705. Dashed lines indicate aberrant splicing activated by the mutation. Short line under the position 705 represents antisense oligonucleotide (AS-ON) targeting the aberrant splice site. Orange line indicates correct splicing.

Successful treatment with an antisense oligonucleotide, which targets the aberrant splice sites, prevents recognition of the cryptic splice site so as to induce the correct splicing and restore the production of active luciferase.¹⁹³ Therefore, this is an assay based on the up-regulation of the luciferase gene expression (i.e. a 'turn-on' system), where the effects are sequence specific and concentration dependent. Kang and co-workers, who introduced this system in 1998, showed the sequence specificity of this assay by treatment of

the cells with sequences targeting other regions of the intron 2 and random sequences which did not show any effect on production of luminescent signal.¹⁹³

The aim of using modified oligonucleotides containing unnatural triazole linkages was to improve cellular uptake, a major challenge for the antisense therapy field. Partial replacement of the negatively charged phosphodiester or phosphorothioate backbone with the neutral triazole backbone reduces the overall charge of the oligonucleotide, which in principle should improve uptake of oligonucleotide across the cell membrane. Additionally, the triazole moiety is an unnatural modification, which means it is inherently nuclease resistant. It has already been established that the triazole backbone destabilizes the formation of duplexes with complementary RNA and DNA.^{114,117,124} Therefore, additional 2'-modifications were incorporated on the nucleotides adjacent to the triazole linkage in order to improve binding to the complementary strand. It is now well understood that the strongly anionic and hydrophilic character of oligonucleotides is the limiting factor for their poor cellular delivery.¹⁹⁴ A lot of research has shown that addition of lipophilic and hydrophobic groups to the oligonucleotide, such as cholesterol or peptides, improves the cell uptake of the modified oligonucleotide.¹⁹⁵⁻¹⁹⁷ The drawback of large hydrophobic conjugate groups is also that they may interfere with the intracellular distribution of the oligonucleotide conjugate. That is why linkers susceptible to cleavage by intracellular enzymes, such as disulphide have been designed.¹⁹⁸ There are many reports discussing oligonucleotides with a wide range of modifications and their antisense biological activity in the literature.^{199,200} Pyrene is a very well established and very well studied oligonucleotide modification. It has been extensively used in fluorescent studies and as a duplex and triplex stabilising moiety.²⁰¹⁻²⁰⁵ To my knowledge, there are however no reports on pyrene modified oligonucleotides and their antisense or exon skipping/splice switching activity.

Pyrene and their analogues are known to be toxic molecules.²⁰⁶⁻²⁰⁸ It is important to realise that in presented study pyrene is an example of the duplex stabilising type molecule, and it could be replaced with a wide range of other moieties.

In chapter 3, formation of a very stable duplex between the natural phosphodiester DNA oligonucleotide containing single or double additions of the triazole linkage functionalised with pyrene or anthraquinone with complementary RNA and DNA was presented. In chapter 4, however, when single to quadruple triazole linkages were incorporated into the PS/2'-OMe nucleic acid strand, UV melting studies revealed limited stabilising properties of pyrene and anthraquinone, forming moderately stable duplexes. Nevertheless, the UV spectra recorded during the UV melting experiments showed that at 37 °C duplexes are still formed. Furthermore, in a cell there are far more variables that have an effect on cellular processes, which cannot be controlled and the duplex stability measured *in vitro* is not a direct indication of what happens in the cell environment. Therefore, it was interesting to evaluate whether these oligonucleotides containing neutral triazole backbone can exhibit splice-switching activity.

5.2 EXON-SKIPPING ACTIVITY OF PHOSPHOROTHIOATE 2'-METHOXY

OLIGONUCLEOTIDES CONTAINING TRIAZOLE LINKAGES AND 2'-

MODIFICATIONS

The splice-switching luciferase assay in this study was performed on the HeLa pLuc/705 cell line. Two types of experiments were carried out: *via* transfected delivery and gymnotic delivery. In order to ensure the maximum splice-switching efficacy of the oligonucleotides, they were transfected into the cells

using lipofectamine. This allows for the formation of cationic liposome – anionic nucleic acid complexes, which overcome the electrostatic repulsion of the cell membrane to nucleic acids and facilitate cytoplasm and nuclear localisation. This type of delivery minimises the differences in the efficiency of transfection of different modified oligonucleotides, therefore the obtained luminescent signal is a direct representation of the splice-switching performance of the oligonucleotide. Gymnotic delivery allows to the efficiency of the splice-switching of the oligonucleotides to be determined without additional help to cross the cell membrane.

In the first experiment the cells were treated with oligonucleotides ODN-D, ODN-D-*t*1, ODN-D-*t*1-P, ODN-D-*t*2, ODN-D-*t*2-P, ODN-D-*t*3 and ODN-D-*t*3-P at concentration range 7.8 nM to 180.0 nM in complex with lipofectamine 2000. Higher doses of oligonucleotide-lipofectamine complexes were causing significant cell toxicity.

The data from the luciferase experiments are presented in Figure 5.2. As a control, ODN-D (fully PS/2'-OMe) was used, as it is a well-established anti-sense oligonucleotide modification. This provides reference point for comparing oligonucleotides containing triazole and pyrene modifications.

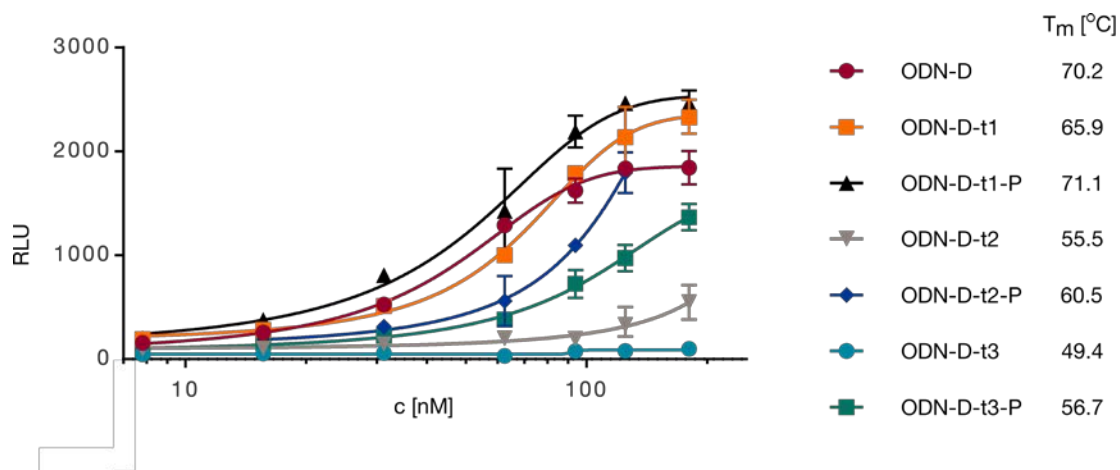


Figure 5.2 Splice-switching activity of transfected antisense oligonucleotides (7.8-180 nM concentration range) in the stably transfected HeLa Luc/705 cell line, treated for 24 h. The RLU (relative light units) are signal normalised to untreated cells. Oligonucleotides used: ODN-D (red), ODN-D-*t1* (orange), ODN-D-*t1*-P (black), ODN-D-*t2* (grey), ODN-D-*t2*-P (blue), ODN-D-*t3* (light blue), ODN-D-*t3*-P (green). Experiments were performed in duplicate in two biological replicates, with data showing standard deviation. T_m values are of modified oligonucleotide with complementary RNA.

The recorded luminescent signals show a clear dose response for all oligonucleotides (except ODN-D-*t3*); with increasing concentration of transfected oligonucleotide, enhanced luminescence was observed. In general the overall dose response curves show that the most to least active oligonucleotides are ODN-D-*t1*-P ($T_m = 71.1$ °C) > ODN-D-*t1* ($T_m = 65.9$ °C) > ODN-D ($T_m = 70.2$ °C) > ODN-D-*t2*-P ($T_m = 60.5$ °C) > ODN-D-*t3*-P ($T_m = 56.7$ °C) > ODN-D-*t2* ($T_m = 55.5$ °C) > ODN-D-*t3* ($T_m = 49.4$ °C). This correlates well with the thermal stability of the duplexes formed by the oligonucleotides *in vitro*, showing the most stable duplexes perform splice switching most efficiently. The luminescence recorded for the least active ODN-D-*t3* (with the triple addition of the triazole linkage) was almost equal to the untreated cells. This could be due to the difficulty to form a duplex between ODN-D-*t3* and the RNA.

The non-triazole oligonucleotides containing the 2'-aminoethoxy T modifications were also tested for their splice-switching activity in the same assay (Figure 5.3).

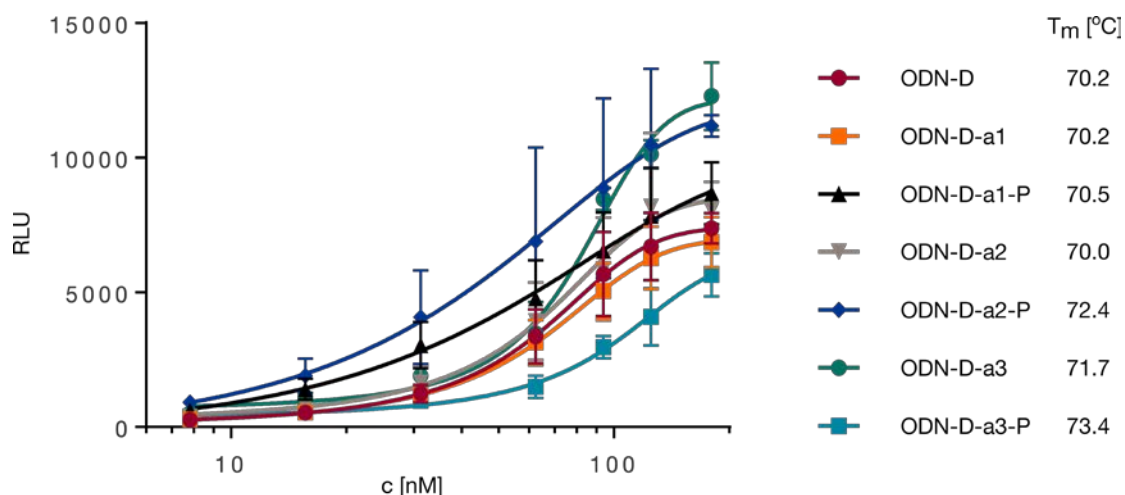


Figure 5.3 Splice-switching activity of transfected antisense oligonucleotides (7.8-180 nM concentration range) in the stably transfected HeLa Luc/705 cell line, treated for 24 h. The RLU (relative light units) are signal normalised to untreated cells. Oligonucleotides used: ODN-D (red), ODN-D-*a1* (orange), ODN-D-*a1*-P (black), ODN-D-*a2* (grey), ODN-D-*a2*-P (blue), ODN-D-*a3* (green), ODN-D-*a3*-P (light blue). Experiments were performed in duplicate in two biological replicates, with data showing standard deviation T_m values are of modified oligonucleotide with complementary RNA.

In this experiment, the recorded luminescent signals also show a clear dose response for all oligonucleotides. In general, the overall dose response curves show that the most to least active oligonucleotides, when considering the luminescent signals at the highest concentration, are: ODN-D-*a3* ($T_m = 71.7$ °C) > ODN-D-*a2*-P ($T_m = 72.4$ °C) > ODN-D-*a1*-P ($T_m = 70.5$ °C) > ODN-D-*a2* ($T_m = 70.0$ °C) > ODN-D ($T_m = 70.2$ °C) > ODN-D-*a1* ($T_m = 70.2$ °C) > ODN-D-*a3*-P ($T_m = 73.4$ °C). However, due to the significance of the errors it is impossible to distinguish the differences more precisely. The ODN-D-*a2*-P shows the highest error bars. The variability of living cells and precision of performed experiments can be an issue and is often a reason for high errors.

The experiments presented in the Figure 5.2 and Figure 5.3 were performed in duplicate in two biological replicates. It would be beneficial to carry out more biological replicates to ensure the accuracy and precision of the experiment. Surprisingly, the oligonucleotide that formed the most stable duplex, ODN-D-**a**3-P (with a triple addition of pyrene), exhibited the lowest splice-switching activity. These results suggest that the addition of pyrene can have a beneficial influence on the splice-switching activity of antisense oligonucleotide compared to their unfunctionalised analogues but only when the number of pyrenes does not exceed two. A higher number of pyrenes decrease the splice-switching activity of this oligonucleotide despite forming a more stable duplex with complementary RNA. This could be related to a variety of factors, such as reduced solubility due to multiple additions of the hydrophobic pyrene, which was observed during *in vitro* experiments by precipitation. Perhaps specific binding with proteins in the cell leading to inhibition of binding with the target RNA. Higher number of pyrenes might also cause difficulties in formation of the lipofectamine complexes or give rise to cell toxicity, which was clearly observed under microscope when cells were treated with oligonucleotides labeled with pyrene in doses higher than 180 nM.

Transfection of oligonucleotides into the cell in the form of a complex with lipofectamine allows for maximum uptake by the cell straight into the cytoplasm and nucleus, therefore, the results of the luciferase assay are a direct measurement of the splice-switching efficiency of the oligonucleotide.

The triazole-modified oligonucleotides were designed to improve the cellular uptake of the modified nucleic acids; an important feature given that transfection reagents are not truly feasible for therapeutic applications. Therefore, the same luciferase assay was performed, but *via* gymnotic ("naked") delivery. In this experiment, the same cells: HeLa pLuc/705 were incubated with oligonucleotides: ODN-D-**a**2-P, ODN-D-**t**3, ODN-D-**t**3-P and

the ODN-D (fully PS/2'-OMe control), for 96 hours at two concentrations: 2 μ M and 10 μ M (Figure 5.4). The recorded luminescent readings showed no significant luminescent signal (typical transfection based values observed in the experiments described above are over 1000 RLU *vs.* ~100 RLU for gymnotic delivery), which meant that no sufficient splice-switching activity was observed. The luminescence for all tested oligonucleotides were comparable to the signal for untreated cells.

The cellular uptake and splice-switching activity of the triazole and pyrene modified oligonucleotides are indeed unknown, however PS/2'-OMe is a well-established modification and has been shown to be taken up in other cells types such as myoblasts and exhibit splice-switching activity.²⁰⁹ Therefore, the stably transfected HeLa cell line may not be a good system for investigating splice-switching activity *via* gymnotic delivery due to the low level of protein expression and/or resistance to cell permeation by oligonucleotides.

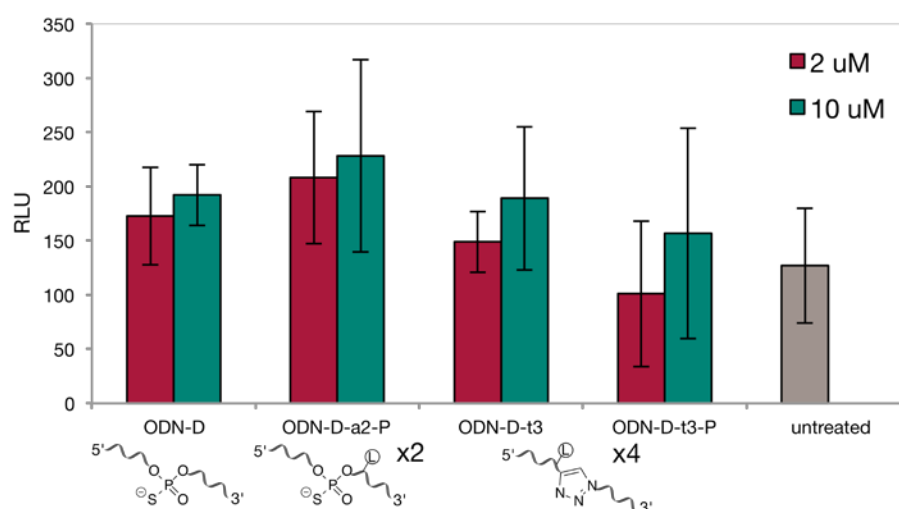


Figure 5.4 Splice-switching activity of antisense oligonucleotides *via* gymnotic delivery in the HeLa Luc/705 cell line, treated for 96 h. The RLU (relative light units) are signal above the background (lysis buffer). Oligonucleotides used: ODN-D, ODN-D-a2-P, ODN-D-t3, ODN-t3-P. Concentration of oligonucleotides: 2 μ M (red) and 10 μ M (green). Experiments were performed in triplicate in one biological replicate.

To examine whether the triazole oligonucleotides could at all show some splice-switching activity *via* gymnotic delivery, it was decided to perform the next set of experiments on a different cell line: MCF7. These experiments were carried out in collaboration with GSK and were performed by Susan Merrihew. The MCF7 cells were transduced with a pHTBV1 plasmid one day prior to the experiment. However the construct of this plasmid differs compared to that of the HeLa pLuc/705, nevertheless the assay principle remains the same. The position of the single mutation in the inserted human β -globin intron 2 was different, where instead of mutation at nucleotide 705, mutation C to T was placed at position 654. These experiments required the DNA sequence of ODN-E, described in chapter 4.

Initially, the splice-switching assay was performed with lipofectamine transfected oligonucleotides ODN-E-*t*1, ODN-E-*t*1-P, ODN-E-*t*2, ODN-E-*t*2-P (with single and double additions of triazole linkage and their functionalised with pyrene hybrids) and the control (fully PS/2'-OMe) ODN-E. The cells were treated with oligonucleotides at 3 different concentrations 2.4 nM, 7.4 nM and 22.2 nM (Figure 5.5).

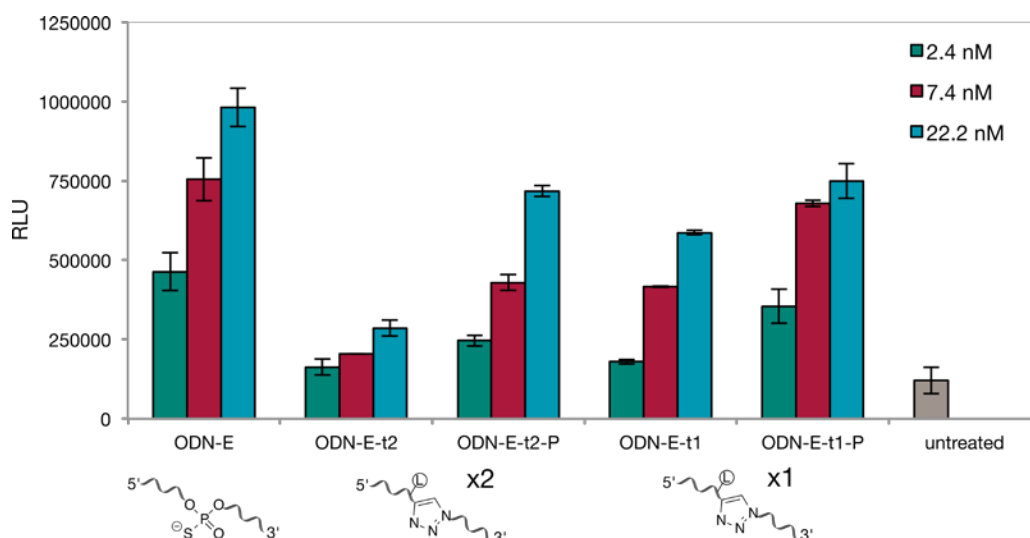


Figure 5.5 Splice-switching activity of transfected antisense oligonucleotides: ODN-E, ODN-E-*t*2, ODN-E-*t*2-P, ODN-D-*t*1, ODN-*t*1-P in the transduced MCF7 cells, treated for 24 h. The RLU (relative light units) are signal above the background (lysis buffer). Concentration of oligonucleotides: 2.4 nM (green), 7.4 nM (red) and 22.2 nM (blue). Experiments were performed in triplicate in one biological replicate.

All tested oligonucleotides showed dose response in their activity, analogous to the results in the HeLa cell line. The two modified oligonucleotides that exhibited the highest exon-skipping activity at the highest concentration (22 nM) were ODN-E-*t*1-P (with a single addition of triazole and functionalised with pyrene; $T_m = 71.5\text{ }^{\circ}\text{C}$) and ODN-E-*t*2-P (with a double addition of triazole and functionalised with pyrene; $T_m = 68.1\text{ }^{\circ}\text{C}$). Both oligonucleotides were, however, slightly less active than the control ODN-E. Of these two, the ODN-E-*t*1-P showed significantly higher activity at the lower doses, which was similar to that of the control ODN-E. Higher activity at the lower doses of the potential drug is very desirable, therefore there may be scope for further studies of ODN-E-*t*1-P. The remaining unfunctionalised oligonucleotides: ODN-E-*t*1 ($T_m = 67.6\text{ }^{\circ}\text{C}$) and ODN-E-*t*2 ($T_m = 60.5\text{ }^{\circ}\text{C}$), characterised by lower duplex stability, performed less efficiently. Overall, these results show a correlation between the thermal stability of the duplex formed between the modified oligonucleotide and the target RNA, and their splice-switching

activity; this supports the data obtained for ODN-D oligonucleotides in HeLa cells.

The luciferase assay using gymnotic delivery of the modified oligonucleotides was also performed. The MCF7 cells were incubated for 24 hours with oligonucleotides ODN-E-**1**, ODN-E-**1**-P, ODN-E-**2**, ODN-E-**2**-P and ODN-E at three different concentrations: 0.2 μ M, 5 μ M and 45 μ M (Figure 5.6). The gymnotic delivery carried out under these conditions was found to be successful. The oligonucleotide that exhibited the highest exon-skipping activity was ODN-E-**1**-P. At a dose of 5 and 45 μ M it was found to be more active than the control ODN-E. It should be noted that only three technical replicates were performed in this experiment and more biological replicates are necessary to ensure the significance of these results. However, the preliminary higher activity of ODN-E-**1**-P than the control ODN-E for gymnotic delivery, compared to the inverse pattern observed in the transfection experiments, suggests that this modification may enhance oligonucleotide uptake.

ODN-E-**1** and ODN-E-**2**-P, with similar thermal duplex stability of *ca.* 68.0 $^{\circ}$ C, exhibited slightly lower activity than control ODN-E. The least stable ODN-E-**2** (with a double addition of the triazole linkage) predictably showed the lowest activity. These results show that cells indeed take up oligonucleotides containing triazole linkages, but formation of a stable duplex with their complementary RNA is an important factor in their activity. The oligonucleotides discussed in this chapter contain a number of different modifications in various combinations. For the gymnotic delivery it is important to understand that each of the used modification can have an individually different influence at the cellular uptake and its distribution.

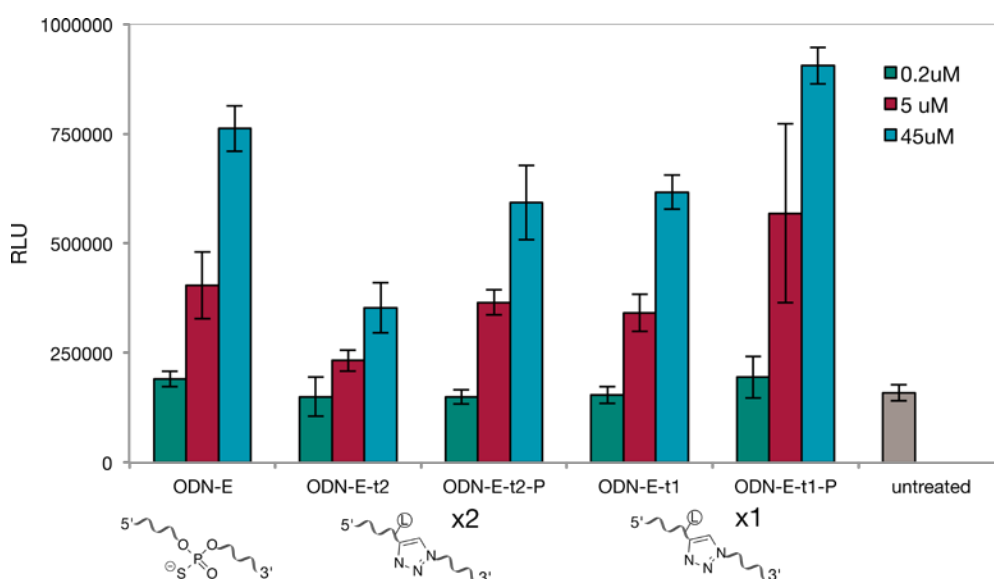


Figure 5.6 Splice-switching activity of antisense oligonucleotides *via* gymnotic delivery in the transduced MCF7 cells, treated for 24 h. The RLU (relative light units) are signal above the background (lysis buffer). Oligonucleotides used: ODN-E, ODN-E-*t*2, ODN-E-*t*2-P, ODN-D-*t*1, ODN-*t*1-P. Concentration of oligonucleotides: 0.2 μM (green), 5.0 μM (red) and 45.0 μM (blue). Experiments were performed in triplicate in one biological replicate.

5.3 SUMMARY AND OUTLOOK

Overall, the results of the splice-switching luciferase assay with a transfection agent showed that the triazole modified oligonucleotides successfully perform splice-switching. In general, there was a correlation between duplex thermal stability of the modified oligonucleotide with its complementary RNA, and the level of splice-switching activity; the more stable the duplex formed, the higher the luciferase activity that was observed. The experiments carried out *via* gymnotic delivery also showed that the triazole-modified oligonucleotides are taken up by the cells and also execute splice-switching in a similar manner.

One of the major limitations of antisense therapy is the limited capability of oligonucleotides to cross the cell membrane. When looking for new therapeutic agents, finding the balance between the therapeutic window and

the therapeutic activity is an important factor. Therefore, if these modified oligonucleotides can enter the cell more efficiently and exhibit similar activity to the ones that are currently used it could make for a significant improvement; as ultimately less compound would become necessary for treatment.

When studying biological activity of modified oligonucleotides *via* gymnotic delivery each of the incorporated modification might have a significant and individual influence at their cell uptake and distribution. In order to study the effects of any one of these modifications in more detail, in future it would be possible to evaluate different oligonucleotides with these modifications in different positions, e.g. 5'-end, 3'-end or internally attached in various ways.

The luciferase signal gives direct information on how much of the luciferase protein was produced. In order to complement these results and quantitatively evaluate the amount of transcribed luciferase mRNA, it would be interesting to perform RT PCR. To gain a better understanding and a more unambiguous insight into the behaviour of triazole-modified oligonucleotides in the cell, it would be also interesting to perform fluorescence microscopy with fluorescently labelled oligonucleotides to visualise the uptake and distribution of these oligonucleotides within the cell, in comparison to the PS/2'-OMe control. This factor is often overlooked but could be highly altered based on the nature of the modifications used.

6 CONCLUSIONS

The question raised in this work was whether oligonucleotides containing triazole linkages and 2'-modifications could serve as potential antisense drug candidates.

In chapter 2, the synthesis of novel phosphoramidite dimer containing a neutral triazole linkage and a 2' sugar modification for solid-phase oligonucleotide synthesis was described. The modified oligonucleotides were successfully functionalised with pyrene, anthraquinone or guanidine moieties after solid-phase synthesis. (Figure.6.1).

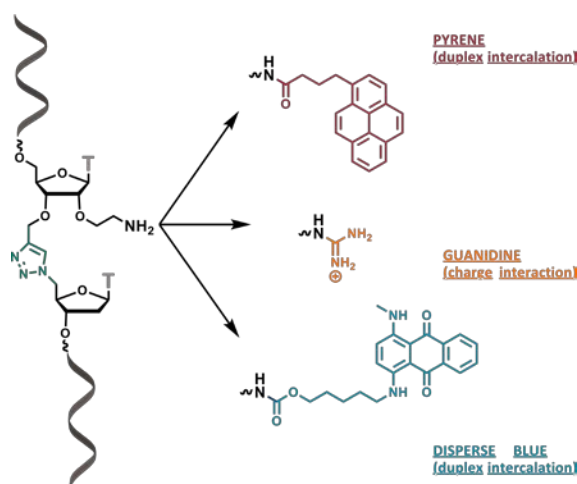


Figure.6.1 Overview of the modifications studied in this work.

In chapter 3, the biophysical properties of oligonucleotides prepared in chapter 2 were evaluated. After confirmation that triazole linkages decrease the stability of DNA:DNA and RNA:DNA duplexes, it was demonstrated that triazole linkages coupled with adjacent 2'-sugar functionalisation can restore and even improve duplex stability. UV melting studies showed that pyrene or anthraquinone moieties are most efficient in this context, restoring lost duplex stability, sometimes by more than 20 °C, and in some cases, improving duplex melting temperature compared to unmodified oligonucleotide by over 4 °C.

In chapter 4, to transfer the new combined triazole / 2'-sugar modifications to the antisense field, between one and four of these modifications were added

to fully PS/2'-OMe-modified antisense oligonucleotides. Compared to the DNA oligonucleotides evaluated in chapter 3, the triazole linkage in the PS/2'-OMe-modified oligonucleotides was less destabilising. This suggests that triazole linkages are better accommodated in an RNA-like conformation. The UV melting studies of these modified PS/2'-OMe oligonucleotides with pyrene attached next to the triazole showed a limited stabilising effect due to the pyrene moiety. Oligonucleotides containing triazole and pyrene were found to be more stable compared to those with triazole but without pyrene. However the magnitude of the stabilising effect of pyrene decreased with their increasing number of pyrene units in the sequence. These results suggest that there are limits to the number of pyrenes that can be usefully incorporated into the sequence in order to improve duplex stability.

In chapter 5, the oligonucleotides prepared in chapter 4 were evaluated for anti-sense activity using a splice-switching luciferase assay. Active delivery of triazole-modified oligonucleotides *via* transfection demonstrated these modifications can indeed initiate splice-switching in pre-mRNA. In general, there was a positive correlation between *in vitro* duplex thermal stability of the modified oligonucleotide with its complementary RNA, and the level of cellular splice-switching activity. Gymnotic delivery showed that the triazole-modified oligonucleotides can be taken up by cells and execute splice-switching. The preliminary experiments showed that in some examples triazole modified oligonucleotides labelled with pyrene exhibited higher splice-switching activity compared to control PS/2'-OMe oligonucleotides but more experiments need to be carried out to confirm these observations. Overall, oligonucleotides containing triazole backbones are taken up by the cells and exhibit splice-switching activity.

This study clearly demonstrates a strategy to create a novel group of antisense oligonucleotides with partially neutral backbone using a triazole linkage and 2'-modifications. The combination of these modifications ensures

that the oligonucleotide has decreased overall negative charge, good duplex thermal stability and stability to nuclease enzymes. However, whether they are better antisense drug remains open to question.

It would be interesting to perform fluorescence microscopy with fluorescently labelled oligonucleotides to visualise the uptake and distribution of these oligonucleotides within the cell. The effect of oligonucleotide structure on subcellular localisation is often overlooked, but could be far more rationally controlled. For example, the number and position of triazole linkages in the sequence might significantly influence cellular uptake. Similarly, exploring other less hydrophobic but equally stabilising 2'-sugar modifications could be also beneficial. The scope of possible modifications for further investigation is very wide. It would be important in the future to supplement these finding with additional NMR and modelling structural studies for better understanding of the type of interactions formed between the antisense strands and target RNA. It is however possible, that the best way to improve efficiency of antisense drugs is by using external delivery systems, which is the subject of very intense studies in the field. Therefore, attachment of modified oligonucleotides to different carriers such as nanoparticles, liposomes or peptides and studies of their activity, cellular uptake and distribution would be an interesting factor to look at. Nevertheless, antisense therapeutic field is of a great importance and further studies will bring the answers required for the development of more efficient drug candidates.

7 EXPERIMENTAL

7.1 SYNTHESIS

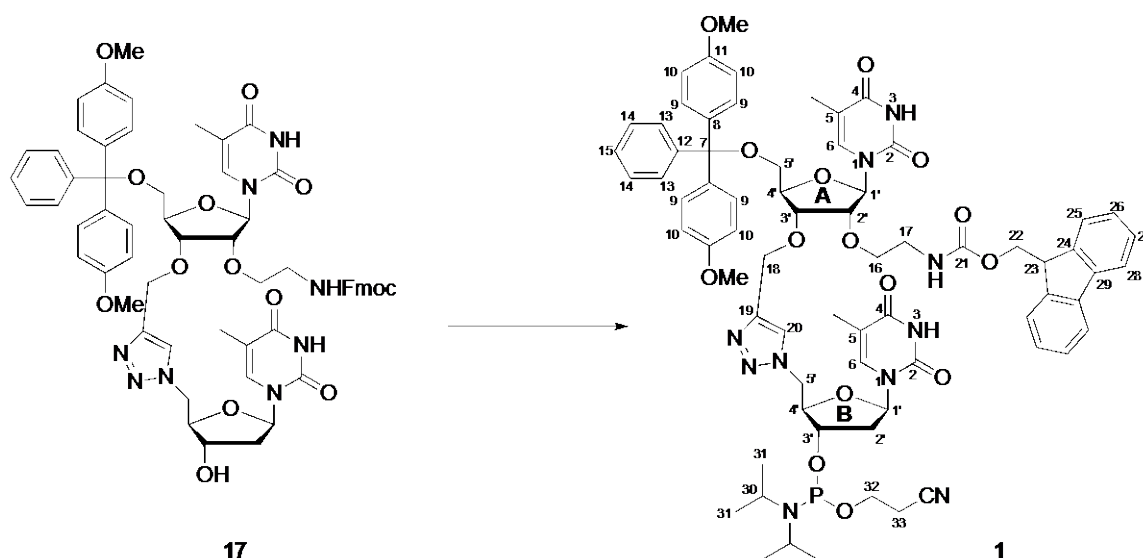
7.1.1 General conditions

All chemicals were purchased from Sigma-Aldrich, Alfa Aesar, Acros Organics or Fisher Scientific and used without further purification. When mentioned, deoxygenated solvents were prepared by bubbling argon through the solvent for 15 min. All air/moisture sensitive reactions were carried out under inert atmosphere (Ar) in oven-dried glassware. Reactions were monitored by thin layer chromatography (TLC) using Merck Kieselgel 60 F24 silica gel plates (0.22 mm thickness, aluminium backed). The compounds were visualised by UV irradiation at 254/365 nm and by staining in *p*-anisaldehyde solution or KMnO₄ (10% aq.). Column chromatography was carried out using Merck Geduran 60 Å (particle size 40-63 micron) silica.

¹H, ¹³C, ³¹P NMR spectra were recorded using Bruker AV300 (300, 75 and 121 MHz respectively), AVIII 400 (400 and 101 MHz respectively) and AVII 500 (500, 126 and 202 MHz respectively) spectrometers. Chemical shifts (δ) are given in ppm and were internally referenced to the appropriate residual solvent signal, all coupling constants (*J*) are quoted in Hertz (Hz). Assignment of compounds was aided by COSY, HSQC, DEPT-135 and HMBC experiments. Low-resolution mass spectra were measured on a Waters LCT premier mass spectrometer and Waters ZMD quadrupole mass spectrometer for +/- electrospray ionisation (ESI). High-resolution mass spectra were recorded on a Bruker μ TOF mass spectrometer. Samples were dissolved in HPLC grade MeOH or MeCN.

7.1.2 Synthesis

7.1.2.1 Synthesis of 2'-aminoethoxy-*N*-Fmoc-5'-*O*-dimethoxytrityl-3'-thymidynyl triazolyl-thymidine phosphoramidite (**1**)

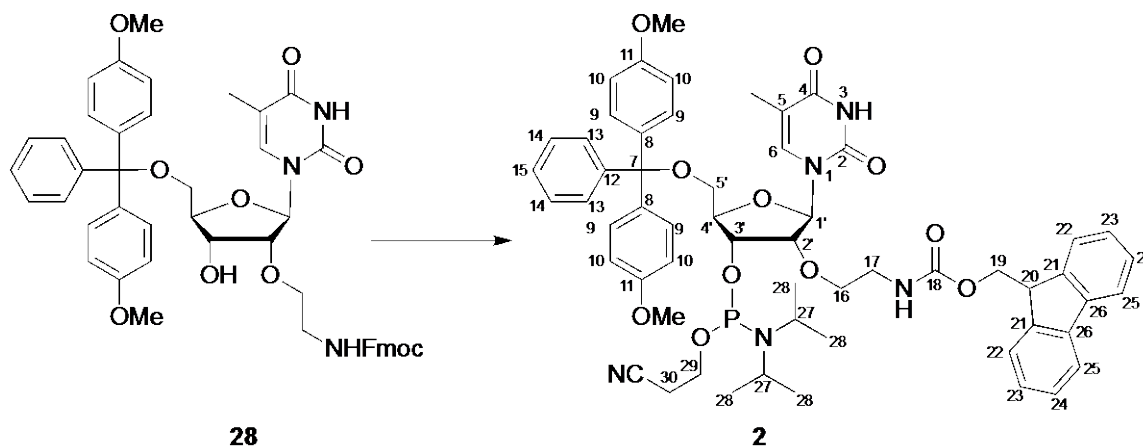


The dimer **17** (0.60 g, 0.53 mmol, 1.0 eq) was co-evaporated with dry pyridine, then dissolved in anhydrous CH₂Cl₂ (8 mL) and kept under argon. DIPEA (0.19 mL, 1.1 mmol, 2.1 eq) was added to the solution, followed by slow addition of 2-cyanoethyl-*N,N*-diisopropylchlorophosphoramidite (0.21 mL, 0.90 mmol, 1.7 eq) at 0 °C. The reaction was allowed to stir under argon at room temperature for 4 hours and then transferred to a separating funnel, under an atmosphere of argon. The reaction mixture was diluted with degassed CH₂Cl₂ and washed with degassed sat. aq. KCl solution, then dried over sodium sulfate and concentrated *in vacuo*. The crude material was purified by column chromatography on silica gel under argon (50-100% ethyl acetate in CH₂Cl₂) affording compound **1** as a white solid (0.22 g, 0.17 mmol, 31%). *M*_W = 1330.50 g/mol (C₇₀H₇₉N₁₀O₁₅P).

^1H NMR (400 MHz, $\text{MeCN-}d_3$) δ 9.05 (br. s, 1H, N-H), 8.99 (br. s, 1H, N-H), 8.57 (s, 1H, H-20), 7.81 – 7.76 (m, 2H, H-Ar), 7.61 (t, $J = 6.7$ Hz, 2H, H-Ar), 7.48 (s, 1H, H-6^A), 7.46 – 7.20 (m, 14H, H-Ar), 6.88 (d, $J = 8.4$ Hz, 4H, H-10), 6.16 (d, $J = 7.0$ Hz, 1H, H-1'^A), 6.06 (td, $J = 6.7, 4.5$ Hz, 1H, H-1'^B), 5.83 (d, $J = 3.2$ Hz, 1H, H-3'^A), 4.71 – 4.48 (m, 5H, H-17, H-18, H-4'^A), 4.41 – 4.22 (m, 3H, H-4'^B, H-23, H-3'^B), 4.22 – 4.14 (m, 2H, H-22), 4.15 – 4.06 (m, 1H, H-2'^A), 3.85 – 3.65 [(m, 9H, H-5'^A or H-5'^B, O-CH₃) containing 3.76 (s, 6H, O-CH₃)], 3.68 – 3.53 (m, 3H, H-30, H-5'^A or H-5'^B), 3.34 – 3.24 (m, 4H, H-16, H-32), 2.64 (t, $J = 5.9$ Hz, 2H, H-33), 2.38 – 2.22 (m, 2H, H-2'^B), 1.77 (s, 3H, 5-CH₃^B), 1.41 (s, 3H, 5-CH₃^A), 1.20 – 1.12 (m, 12H, H-31).

^{31}P NMR (162 MHz, $\text{MeCN-}d_3$) δ 148.86 (s), 148.63 (s)

7.1.2.2 Synthesis of 2'-aminoethoxy-*N*-Fmoc-3'-phosphoramidite thymidine (**2**)



Nucleoside **28** (1.52 g, 1.84 mmol, 1.0 eq) was co-evaporated with dry pyridine, then dissolved in anhydrous CH₂Cl₂ (10 mL) under argon. DIPEA (0.802 mL, 4.61 mmol, 2.5 eq) was added to the solution, followed by slow addition of 2-cyanoethyl-*N,N*-diisopropylchloro phosphoramidite (0.702 mL, 3.13 mmol, 1.7 eq) at 0 °C. The reaction was stirred under argon at room temperature for 4 hours and then transferred to a separating funnel, under argon. The reaction mixture was diluted with deoxygenated CH₂Cl₂ and washed with deoxygenated sat. aq. KCl solution, then dried over sodium sulfate and concentrated *in vacuo*. The crude material was purified by column chromatography on silica gel under argon (30% hexane in ethyl acetate) affording compound **2** as a white solid (1.12 g, 1.09 mmol, 59%). *M*_W = 1026.14 g/mol (C₅₇H₆₄N₅O₁₁P).

¹H NMR (400 MHz, MeCN-*d*₃) δ 8.98 (s, 1H, N-H), 7.82 (d, *J* = 7.6 Hz, 2H, H-Ar), 7.64 (d, *J* = 7.5 Hz, 2H, H-Ar), 7.55 – 7.28 (m, 14H, H-6, H-Ar), 6.88 (dd,

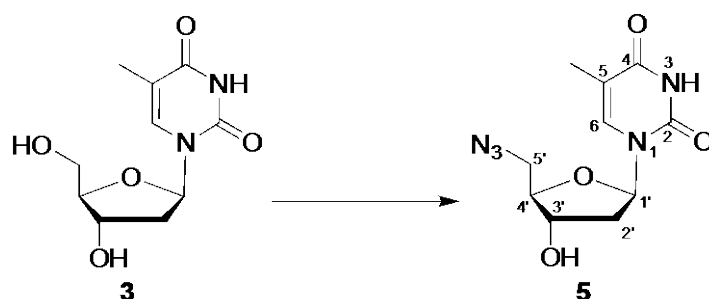
$J = 8.1, 5.4$ Hz, 4H, H-10), 5.90 (dd, $J = 9.3, 3.8$ Hz, 1H, H-1'), 4.55 – 4.40 (m, 1H, H-3'), 4.34 (dd, $J = 13.5, 6.9$ Hz, 2H, H-19), 4.26 – 4.11 (m, 3H, H-2', H-4', H-20), 3.85 – 3.64 [(m, 9H, H-29, H-16 or 17, O-CH₃) containing 3.76 (s O-CH₃)], 3.63 – 3.48 (m, 3H, H-27, H-16 or 17), 3.50 – 3.38 (m, 1H, H-16 or 17), 3.38 – 3.22 (m, 3H, H-5', H-16 or 17), 2.62 (t, $J = 6.3$ Hz, 1H, H-30), 2.48 (t, $J = 6.0$ Hz, 1H, H-30), 1.39 (d, $J = 8.4$ Hz, 3H, 5-CH₃), 1.18 – 0.98 (m, 12H, H-28).

³¹P NMR (162 MHz, MeCN-*d*₃) δ 152.85 (s)

R_f: 0.55 [40% hexane in ethyl acetate + 1% pyridine]

Data consistent with literature.²¹⁰

7.1.2.3 Synthesis of 5'-azido thymidine (**5**)



2'-deoxythymidine **3** (3.00 g, 12.4 mmol, 1.0 eq) was dissolved in dry DMF (30 mL) under argon followed by addition of triphenylphosphine (3.92 g, 14.9 mmol, 1.2 eq) and tetrabromomethane (20.5 g, 61.9 mmol, 5.0 eq). The reaction was stirred at room temperature for 15 hours. Sodium azide (2.41 g, 37.1 mmol, 3.0 eq) was then added and reaction was stirred for further 4 hours at 60 °C. Reaction was next quenched with MeOH and the solvent was removed *in vacuo*. The crude material was purified by column chromatography on silica gel (3-7% MeOH in CH₂Cl₂) affording compound **5** as a white solid (1.76 g, 6.59 mmol, 53%). M_W = 267.10 g/mol (C₁₀H₁₃N₅O₄).

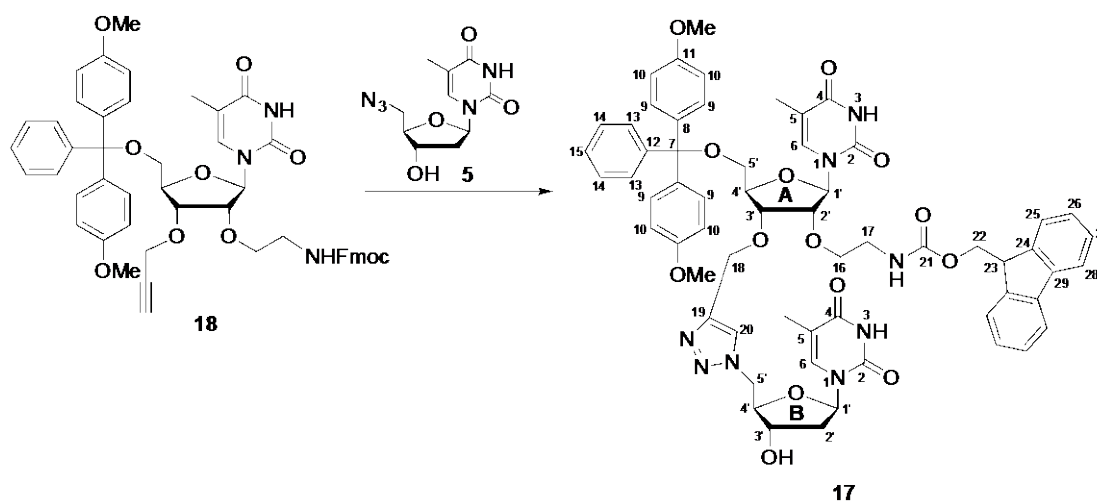
¹H NMR (400 MHz, DMSO-*d*₆) δ 11.32 (s, 1H, N-H), 7.50 (d, *J* = 1.4 Hz, 1H, H-6), 6.20 (t, *J* = 7.0 Hz, 1H, H-1'), 5.42 (d, *J* = 4.4 Hz, 1H, 3'-OH), 4.19 (dq, *J* = 7.5, 3.9 Hz, 1H, H-3'), 3.84 (td, *J* = 5.2, 3.5 Hz, 1H, H-4'), 3.56 (d, *J* = 5.3 Hz, 2H, H-5'), 2.25 (dt, *J* = 13.7, 7.1 Hz, 1H, H-2'), 2.08 (ddd, *J* = 13.5, 6.7, 3.6 Hz, 1H, H-2'), 1.79 (d, *J* = 1.3 Hz, 3H, 5-CH₃).

¹³C NMR (126 MHz, DMSO-*d*₆) δ 163.5 (C-4), 150.3 (C-2), 135.9 (C-6), 109.7 (C-5), 84.4 (C-1'), 83.7 (C-4'), 70.6 (C-3'), 51.5 (C-5'), 37.9 (C-2'), 12.0 (5-CH₃).

LRMS [ESI⁺, MeOH] *m/z*. 268.0 [M+H]⁺, 290.0 (100%) [M+Na]⁺.

Data consistent with literature ³⁴

7.1.2.4 Synthesis of 2'-aminoethoxy-N-Fmoc-5'-O-dimethoxytrityl 3'-thymidinyl triazolyl tymidine (17)



The alkyne compound **18** (0.6 g, 0.7 mmol, 1.0 eq) and azide compound **5** (0.19 g, 0.71 mmol, 1.0 eq) were dissolved in DMF (2 mL). Next TBTA ligand (0.15 g, 0.35 mmol, 0.5 eq) was added followed by addition of CuSO₄ (0.086 g, 0.35 mmol, 0.5 eq) and sodium ascorbate (0.28, 1.4 mmol, 2 eq) solutions in water. Reaction was stirred under argon at room temperature for 4 hours. Upon the reaction reaching completion the solvent was removed *in vacuo*. The residue was redissolved in CH₂Cl₂ (with 5% MeOH) and washed with ethylene diaminetetraacetic acid disodium salt dehydrate (NaEDTA 5% solution) in order to remove Cu salts and then washed with water and brine. The organic layer was dried over sodium sulfate, filtered and the solvent was removed *in vacuo*. The crude material was purified by column chromatography on silica gel (5-10% *i*PrOH in CH₂Cl₂) obtaining compound **17** as a white solid (0.68 g, 0.60 mmol, 86%). M_w = 1130.44 g/mol (C₆₁H₆₂N₈O₁₄).

^1H NMR (400 MHz, DMSO- d_6) δ 11.39 (br. s, 1H, N-H), 11.29 (br. s, 1H, N-H), 8.07 (s, 1H, H-20), 7.86 (d, J = 7.6 Hz, 2H, H-25 or 28), 7.65 (dd, J = 7.8, 2.7 Hz, 2H, H-26 or 27), 7.50 (s, 1H, H-6^A), 7.42 – 7.20 (m, 14H, H-Ar, H-6^B), 6.90 (d, J = 8.5 Hz, 4H, H-10), 6.14 (t, J = 6.9 Hz, 1H, H-1'^B), 5.84 (d, J = 3.9 Hz, 1H, H-1'^A), 5.49 (d, J = 4.2 Hz, 1H, 3'^B-OH), 4.72 – 4.51 (m, 4H, H-17, H-18), 4.38 (t, J = 5.4 Hz, 1H, H-3'^A), 4.34 – 4.23 (m, 4H, H-4'^A, H-3'^B, H-22), 4.18 (t, J = 7.0 Hz, 1H, H-23), 4.13 – 4.03 (m, 2H, H-2'^A, H-4'^B), 3.73 (s, 6H, O-CH₃), 3.67 (dt, J = 10.9, 6.0 Hz, 1H, H-16), 3.57 (dt, J = 10.0, 5.9 Hz, 1H, H-16), 3.26 (dd, J = 10.8, 3.5 Hz, 1H, H-5'^A or H-5'^B), 3.23 – 3.12 (m, 3H, H-5'^A or H-5'^B), 2.18 (dt, J = 13.7, 6.8 Hz, 1H, H-2'^B), 2.09 (ddd, J = 13.5, 6.5, 3.8 Hz, 1H, H-2'^B), 1.77 (s, 3H, 5-CH₃^B), 1.38 (s, 3H, 5-CH₃^A).

^{13}C NMR (101 MHz, DMSO- d_6) δ 164.2 (C-4^A or 4^B), 164.1 (C-4^B or 4^A), 158.7 (C-11), 156.7 (C-21), 150.8 (C-2^B), 150.1 (C-2^A), 145.0 (C-Ar), 144.3 (C-19), 144.2 (C-Ar), 141.2 (C-Ar), 136.5 (C-6^B), 135.8 (C-6^A), 135.5 (C-Ar), 130.2 (C-Ar), 128.4 (C-Ar), 128.1 (C-Ar), 128.0 (C-Ar), 127.5 (C-Ar), 127.3 (C-Ar), 125.6 (C-Ar), 125.2 (C-20), 120.5 (C-Ar), 113.8 (C-10), 110.3 (C-5^A), 109.9 (C-5^B), 87.4 (C-1'^A), 86.5 (C-7), 84.6 (C-1'^B), 84.4 (C-4'^B), 81.2 (C-2'^A), 80.0 (C-4'^A), 75.9 (C-3'^A), 71.2 (C-3'^B), 69.1 (C-16), 65.9 (C-22), 63.1 (C-18), 62.8 (C-5'^A), 55.5 (O-CH₃), 51.7 (C-17), 47.2 (C-23), 40.64 (C-5'^B), 38.4 (C-2'^B), 12.5 (5-CH₃^B), 12.2 (5-CH₃^A)

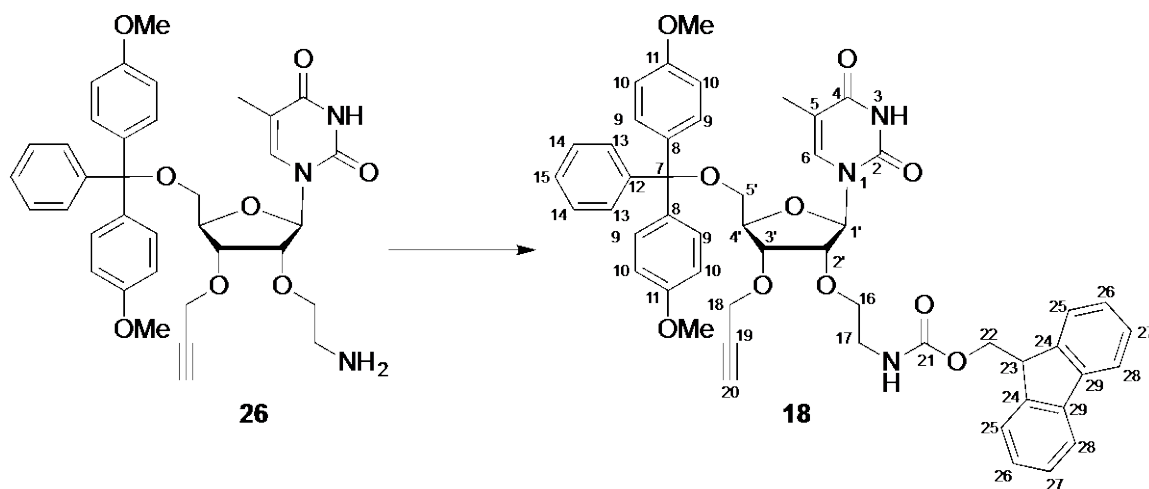
LRMS [ESI⁺, MeOH] m/z 1153.0 (100%) [M+H]⁺.

HRMS [EI, MeOH] calculated mass m/z 1153.42777 for C₆₁H₆₂N₈O₁₄Na [M+Na]⁺; found m/z 1153.42505

HRMS [ESI⁺, MeOH] calculated mass m/z 1153.42777 for C₆₁H₆₂N₈O₁₄Na [M+Na]⁺; found m/z 1153.42480

IR: 2962 (w, C-H alkane), 1687 (s, C=O), 1251 (s, C-O alkyl aryl ether), 1078 (s, C-O secondary alcohol), 791, 742 (s, C-H) $\nu_{\max}/\text{cm}^{-1}$.

7.1.2.5 Synthesis of 5'-O-(4,4'-Dimethoxytrityl)-3'-O-propargyl-2'-O-(2-(fluorenylmethoxyamido)ethyl)-thymidine (18)



The compound **26** (1.0 g, 1.6 mmol, 1.0 eq) was dissolved in anhydrous CH_2Cl_2 (12 mL), followed by addition of pyridine (0.25 mL, 3.1 mmol, 2.0 eq) and then Fmoc-*N*-succinimidyl carbonate (1.8 g, 5.5 mmol, 3.5 eq). The reaction mixture was stirred at room temperature for 2 hours and then was diluted in CH_2Cl_2 and partitioned between sat.aq. NH_4Cl and sat. aq. NaHCO_3 . Next, it was dried over sodium sulfate, filtered and concentrated *in vacuo*. The crude material was purified by flash column chromatography on silica gel (1-3% MeOH in CH_2Cl_2 with constant 1% pyridine) affording compound **18** as a white foam (1.12 g, 1.30 mmol, 83%). $M_W = 863.34$ g/mol ($\text{C}_{51}\text{H}_{49}\text{N}_3\text{O}_{10}$).

^1H NMR (400 MHz, CDCl_3) δ 7.70 (t, $J = 7.7$ Hz, 3H, H-Ar), 7.55 (d, $J = 7.5$ Hz, 2H, H-Ar), 7.40– 7.18 (m, 12H), 6.84 – 6.77 [(m, 4H, H-10) containing 6.80 (d, $J = 8.9$ Hz) and 6.80 (d, $J = 8.9$ Hz)], 5.89 (d, $J = 2.5$ Hz, 1H, H-1'), 4.44 (dd,

$J = 7.1, 5.0$ Hz, 1H, H-3'), 4.35 (d, $J = 6.9$ Hz, 2H, H-22), 4.21 – 4.15 (m, 2H, H-2', H-23), 4.12 – 4.06 (m, 3H, H-4' and H-18), 3.91 – 3.83 (m, 1H, H-16 or 17), 3.79 – 3.68 [(m, 7H, H-16 or 17 and O-CH₃) containing of 3.74 (s, O-CH₃)], 3.55 (dd, $J = 11.0, 2.1$ Hz, 1H, H-5'), 3.49 – 3.30 (m, 3H, H-16 or 17 and H-5') containing 3.34 (dd, $J = 11.1, 2.6$ Hz, 1H, H-5'), 2.34 (t, $J = 2.4$ Hz, 1H, H-20), 1.34 (s, 3H, 5-CH₃).

¹³C NMR (101 MHz, CDCl₃) δ 163.7 (C-4), 158.7 (C-11), 156.5 (C-21), 150.3 (C-2), 144.2 (C-Ar), 144.0 (C-Ar), 141.3 (C-Ar), 136.7 (C-6), 135.3 (C-Ar), 130.1 (C-Ar), 128.1 (C-Ar), 128.0 (C-Ar), 127.6 (C-Ar), 127.0 (C-Ar), 125.0 (C-Ar), 119.9 (C-Ar), 113.3 (C-10), 111.1 (C-5), 88.4 (C-1'), 86.9 (C-7), 81.1 (C-2' and 4'), 78.9 (C-19), 75.8 (C-20), 74.4 (C-3'), 70.0 (C-16 or 17), 66.6 (C-22), 61.4 (C-5'), 57.6 (C-18), 55.2 (O-CH₃), 47.2 (C-23), 40.9 (C-16 or 17), 11.8 (5-CH₃), (32 of 34 resonances observed).

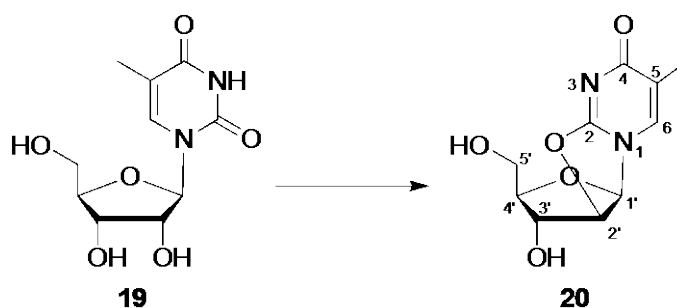
LRMS [ESI⁻, MeOH] m/z 862.2 (100 %) [M-H]⁻.

HRMS [EI, MeOH] calculated mass m/z 886.3310 for C₅₁H₄₉N₃O₁₀Na [M+Na]⁺; found m/z 886.3310

HRMS [ESI⁺, MeOH] calculated mass m/z 886.3310 for C₅₁H₄₉N₃O₁₀Na [M+Na]⁺; found m/z 886.3308

IR: 2161 (w, C≡C), 1687 (s, C=O), 1250 (s, C-O alkyl aryl ether), 758, 741 (s, C-H) $\nu_{\max}/\text{cm}^{-1}$.

7.1.2.6 Synthesis of 2,2'-anhydro thymidine (20)



5-Methyluridine **19** (20.0 g, 77.4 mmol, 1.0 eq), diphenyl carbonate (24.9 g, 116 mmol, 1.5 eq) and sodium hydrogen carbonate (0.650 g, 7.74 mmol, 0.1 eq) were dissolved in anhydrous DMF (25 mL). The reaction mixture was stirred for 3 hours at 100 °C. The reaction turned into a gel after 2 hours. Another portion of DMF (20 mL) was then added and the temperature was increased to 120 °C to dissolve the gel. After stirring the reaction mixture for a further 2 hours the solution was cooled to room temperature. The solvent was removed *in vacuo*. The remaining oil was diluted in a minimum amount of MeOH and added drop-wise to a large amount of Et₂O, forming first a thick brown gum and then upon longer and very vigorous stirring a white solid. The Et₂O was decanted off and the residue was recrystallised from EtOH to give compound **20** as a white powder (16.7 g, 69.7 mmol, 90%). $M_w = 240.07$ g/mol (C₁₀H₁₂N₂O₅).

¹H NMR (300 MHz, DMSO-*d*₆) δ 7.74 (d, $J = 1.3$ Hz, 1H, H-6), 6.29 (d, $J = 5.7$ Hz, 1H, H-1'), 5.86 (br. s, 1H, 3'-OH), 5.17 (d, $J = 5.7$ Hz, 1H, H-2'), 4.95 (br. s, 1H, 5'-OH), 4.37 (s, 1H, H-3'), 4.06 (td, $J = 5.6, 1.7$ Hz, 1H, H-4'), 3.30 – 3.21 (m, 1H, H-5'), 3.20 – 3.10 (m, 1H, H-5'), 1.79 (d, $J = 1.3$ Hz, 3H, 5-CH₃).

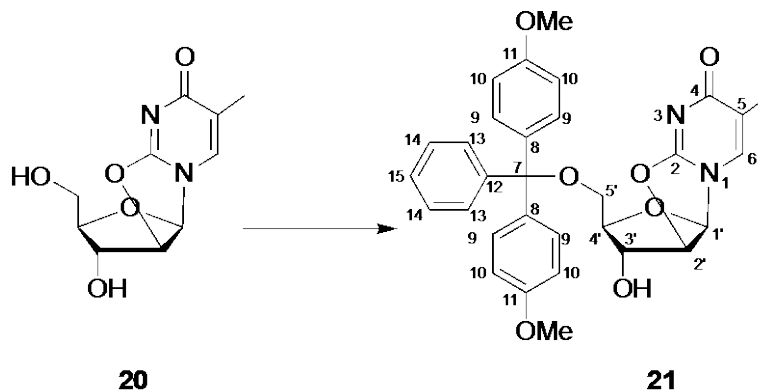
^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 171.6 (C-4), 159.3 (C-2), 132.2 (C-6), 116.6 (C-5), 90.2 (C-1'), 89.1 (C-4') 88.5 (C-2') 74.7 (C-3') 60.8 (C-5') 13.5 (5- CH_3).

LRMS [ESI⁺, MeOH] m/z . 263.0 (100%) [M+Na]⁺, 503.1 (47%) [2M+Na]⁺.

R_f: 0.33 [20% MeOH in CH_2Cl_2]

Data consistent with literature.¹³⁸

7.1.2.7 Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2,2'-anhydro thymidine (21)



2,2'-Anhydro thymidine **20** (4.00 g, 16.7 mmol, 1.0 eq) was suspended in anhydrous pyridine (28 mL). While the suspension was stirred, a solution of 4,4'-dimethoxytrityl chloride (6.78 g, 20.0 mmol, 1.2 eq) dissolved in anhydrous pyridine (20 mL) was added dropwise and then stirred at room temperature under argon for 2 hours. A solution of MeOH (30 mL) and TEA (2 mL) was then added and stirred for a further 30 minutes to quench the reaction before the solvent was removed *in vacuo*. Flash column chromatography on silica gel (1-15% MeOH in CH₂Cl₂ with constant 1% pyridine) afforded compound **21** as a white foam (7.23 g, 13.3 mmol, 80%). $M_W = 542.21$ g/mol (C₃₁H₃₀N₂O₇).

¹H NMR (400 MHz, DMSO-*d*₆) δ 7.84 (d, $J = 1.3$ Hz, 1 H, H-6), 7.30 – 7.22 (m, 4H, H-Ar), 7.23 – 7.14 (m, 1H, H-Ar), 7.16 – 7.10 [(m, 4H, H-9) containing 7.14 (d, $J = 9.0$ Hz) and 7.13 (d, $J = 8.9$ Hz)], 6.86 – 6.78 [(m, 4H, H-10) containing 6.83 (d, $J = 9.0$ Hz) and 6.81 (d, $J = 9.0$ Hz)], 6.31 (d, $J = 5.7$ Hz, 1 H, H-1'), 5.94 (d, $J = 4.3$ Hz, 1 H, 3'-OH), 5.18 (dd, $J = 5.7, 0.9$ Hz, 1 H, H-2'), 4.32 – 4.27 (m, 1H, H-3'), 4.23 (ddd, $J = 7.2, 4.4, 2.7$ Hz, 1H, H-4'), 3.73 (s, 3H,

O-CH₃), 3.73 (s, 3H, O-CH₃), 2.92 (dd, *J* = 10.3, 4.4 Hz, 1 H, H-5'), 2.78 (dd, *J* = 10.3, 7.5 Hz, 1 H, H-5'), 1.79 (s, 3 H, 5-CH₃).

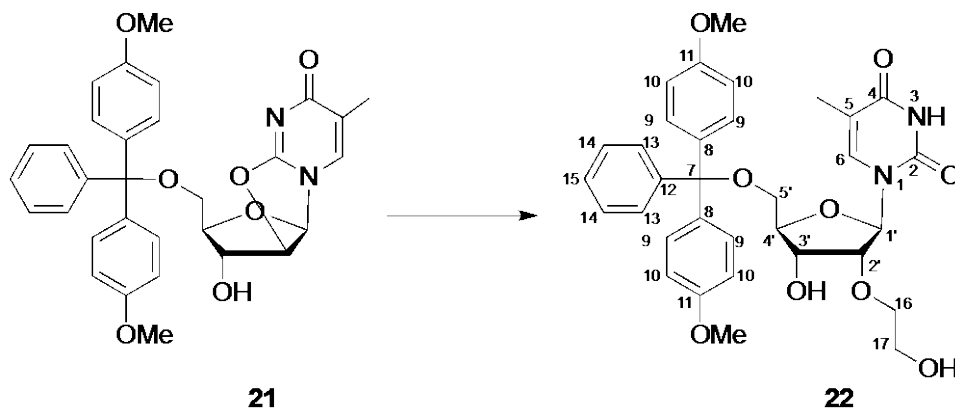
¹³C NMR (101 MHz, DMSO-*d*₆) δ 171.4 (C-4), 158.9 (C-2), 158.0 (C-11), 144.6 (C-Ar), 135.1 (C-8), 132.1 (C-6), 129.5 (C-Ar), 127.8 (C-Ar), 127.4 (C-Ar), 126.6 (C-Ar), 116.9 (C-5), 113.1 (C-10), 90.0 (C-1'), 88.1 (C-2'), 86.9 (C-3'), 85.3 (C-7), 74.8 (C-4'), 63.0 (C-5'), 55.0 (O-CH₃), 13.5 (5-CH₃).

LRMS [ESI⁻, MeOH] *m/z* 587.0 (100%) [M+HCO₂H-H]⁻.

R_f: 0.45 [10% MeOH in CH₂Cl₂ + 1% pyridine]

Data consistent with literature.¹³⁸

7.1.2.8 Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-hydroxyethyl)thymidine (22)



Sodium hydrogen carbonate (0.076 g, 0.90 mmol, 0.5 eq) and ethylene glycol (0.51 mL, 9.2 mmol, 5.0 eq) were dissolved in anhydrous DMF (2 mL) to which titanium(IV) isopropoxide (1.1 mL, 3.7 mmol, 2.0 eq) was added and the reaction mixture was stirred at 160 °C for 3 hours. Compound **21** (1.0 g, 1.8 mmol, 1.0 eq) was dissolved in anhydrous DMF (4 mL) and added to the reaction mixture dropwise over 20 minutes. The reaction was stirred at reflux overnight. After reaching completion the reaction was cooled to room temperature and the solvent was removed *in vacuo*. The remaining residue was dissolved in a large amount of CH₂Cl₂, centrifuged 1000 rpm at 4 °C and supernatant was decanted to remove the solid titanium complex and the solvent was removed *in vacuo*. The crude oil was purified by flash column chromatography on silica gel (1-8% MeOH in CH₂Cl₂ with constant 1% pyridine) affording compound **22** as a white foam (0.31 g, 0.51 mmol, 56%). $M_w = 604.24$ g/mol (C₃₃H₃₆N₂O₉).

^1H NMR (300 MHz, $\text{DMSO}-d_6$) δ 11.41 (s, 1H, N-H), 7.51 (d, J = 1.5 Hz, 1H, H-6), 7.43 – 7.16 (m, 9H, H-Ar), 6.95 – 6.84 [(m, 4H, H-10), containing 6.91 (d, J = 8.9 Hz) and 6.91 (d, J = 8.8 Hz)], 5.86 (d, J = 4.6 Hz, 1H, H-1'), 5.14 (d, J = 6.2 Hz, 1H, 3'-OH), 4.26 (q, J = 5.5 Hz, 1H, H-3'), 4.10 (t, J = 4.9 Hz, 1H, H-2'), 4.03 – 3.94 (m, 1H, H-4'), 3.73 (s, 6H, O-CH₃), 3.70 – 3.50 (m, 4H, H-16, H-17), 3.27 (dd, J = 10.8, 4.1 Hz, 1H, H-5'), 3.19 (dd, J = 10.8, 2.7 Hz, 1H, H-5') 1.39 (d, J = 1.1 Hz, 3H, 5-CH₃).

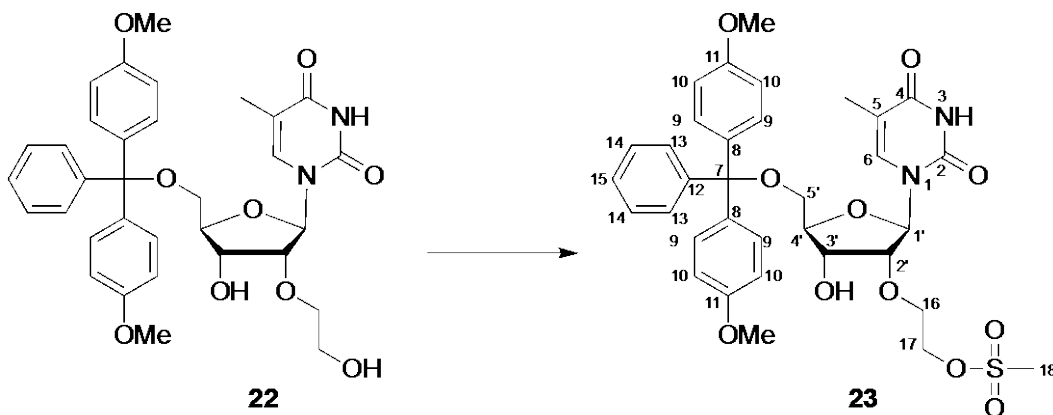
^{13}C NMR (75 MHz, $\text{DMSO}-d_6$) δ 163.7 (C-4), 158.2 (C-11), 150.4 (C-2), 144.6 (C-Ar), 135.6 (C-Ar), 135.1 (C-6), 129.7 (C-Ar), 127.9 (C-Ar), 127.7 (C-Ar), 126.8 (C-Ar), 113.3 (C-10), 109.5 (C-5), 86.6 (C-1'), 85.9 (C-7), 82.9 (C-4'), 81.0 (C-2'), 71.7 (C-16), 68.9 (C-3'), 62.8 (C-5'), 60.3 (C-17), 55.0 (O-CH₃), 11.7 (5-CH₃).

LRMS [ESI⁺, MeOH] m/z 627.1 (100%) [M+Na]⁺, 668.5 (11%) [M+MeCN+Na]⁺.

R_f: 0.67 [10% *i*-PrOH in CH₂Cl₂ + 1% pyridine]

Data consistent with literature.¹³⁸

7.1.2.9 Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-methanesulfonate ethyl) thymidine (23**)**



Nucleoside **22** (2.0 g, 3.3 mmol, 1.0 eq) was dissolved in anhydrous CH_2Cl_2 (35 mL). The reaction was cooled to $-78\text{ }^\circ\text{C}$ (in an acetone/dry ice bath) and TEA (2.3 mL, 1.7 mmol, 2.0 eq) was added, followed by slow addition (over 1.5 hour) of methanesulfonyl chloride (0.31 mL, 4.0 mmol, 1.2 eq) dissolved in of anhydrous CH_2Cl_2 (5 mL). The reaction was stirred for further 16 hours. The solvent was removed *in vacuo*. The reaction mixture was re-dissolved in CH_2Cl_2 (100 mL) and washed with aq. sat. KCl solution. The organic layer was dried over sodium sulfate, filtered and the solvent was removed *in vacuo*. The crude material was purified by flash column chromatography on silica gel (1-3% i PrOH in CH_2Cl_2 with constant 1% pyridine) affording compound **23** as a white foam (1.21 g, 1.77 mmol, 53%). M_w : 682.22 g/mol ($\text{C}_{34}\text{H}_{38}\text{N}_2\text{O}_{11}\text{S}$).

^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ 11.41 (s, 1H, N-H), 7.49 (d, $J = 1.4$ Hz, 1H, H-6), 7.42 – 7.21 (m, 9H, H-Ar), 6.93 – 6.87 [(m, 4H, H-10) containing 6.91 (d, $J = 9.0$ Hz) and 6.90 (d, $J = 9.0$ Hz)], 5.85 (d, $J = 4.0$ Hz, 1H, H-1'), 5.29 (d, $J = 6.6$ Hz, 1H, 3'-OH), 4.36 (t, $J = 4.4$ Hz, 2H, H-17), 4.25 (q, $J = 6.0$ Hz, 1H, H-

3'), 4.07 (t, $J = 4.7$ Hz, 1H, H-2'), 3.98 (m, 1H, H-4'), 3.90 (dt, $J = 12.3, 4.3$ Hz, 1H, H-16), 3.84 (dt, $J = 12.0, 4.5$ Hz, 1H, H-16), 3.74 (s, 6H, O-CH₃), 3.26 (dd, $J = 10.7, 4.4$ Hz, 1H, H-5'), 3.21 (dd, $J = 10.6, 2.6$ Hz, 1H, H-5'), 3.17 (s, 3H, H-18), 1.41 (d, $J = 1.1$ Hz, 3H, 5-CH₃).

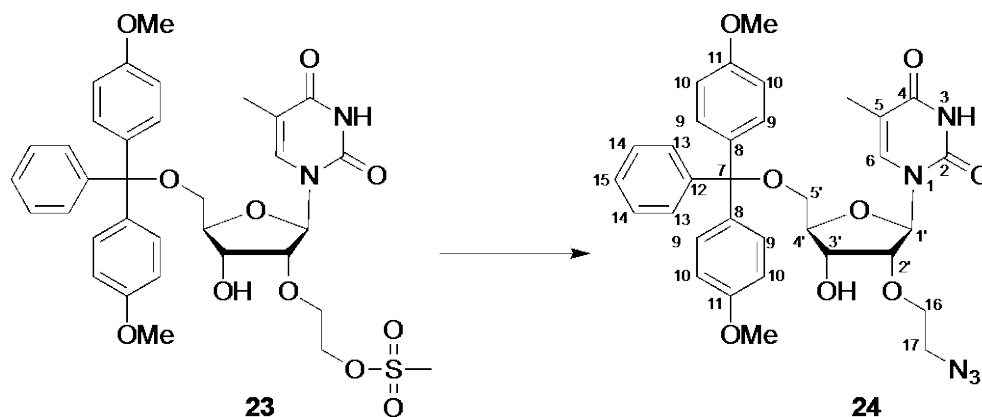
¹³C NMR (126 MHz, DMSO-*d*₆) δ 163.9 (C-4), 158.4 (C-11), 150.6 (C-2), 144.8 (C-Ar), 136.4 (C-8), 135.6 (C-6), 129.9 (C-Ar), 128.1 (C-Ar), 127.0 (C-Ar), 124.1 (C-Ar), 113.5 (C-10), 109.7 (C-5), 87.1 (C-1'), 86.0 (C-7), 82.9 (C-2' or C-4'), 81.3 (C-2' or C-4'), 69.8 (C-3' or C-16 or C-17), 68.8 (C-3' or C-16 or C-17), 68.1 (C-3' or C-16 or C-17), 63.1 (C-5'), 55.2 (O-CH₃), 37.0 (C-18), 12.0 (5-CH₃).

LRMS [ESI⁻, MeOH] m/z 681.0 (42%) [M-H]⁻.

R_f: 0.35 [5% *i*PrOH in CH₂Cl₂ + 1% pyridine]

Data consistent with literature¹³⁸

7.1.2.10 *Synthesis of 5'-O-(4,4'-dimethoxytrityl)-2'-O-(2-azidoethyl)-thymidine (24)*



Compound **23** (1.0 g, 1.5 mmol, 1.0 eq) was dissolved in anhydrous DMF (7.5 mL) and then sodium azide (0.48 g, 7.4 mmol, 5.0 eq) was added. The solution was stirred for 15 hours at 60 °C. DMF was removed *in vacuo*. The colourless residue was dissolved in CH₂Cl₂ and the product was extracted with brine and dried over sodium sulfate. After removing CH₂Cl₂ *in vacuo*, the crude material was purified by flash column chromatography on silica gel (1-25% iPrOH in CH₂Cl₂ with constant 1% pyridine) affording compound **24** as a white foam (0.76 g, 1.2 mmol, 82%). M_W = 629.25 g/mol (C₃₃H₃₅N₅O₈).

¹H NMR (300 MHz, DMSO-*d*₆) δ 11.38 (s, 1H, N-H), 7.50 (d, *J* = 1.4 Hz, 1H, H-6), 7.42 – 7.22 (m, 9H, H-Ar), 6.94 – 6.86 [(m, 4H, H-10) containing 6.90 (d, *J* = 8.8 Hz) and 6.90 (d, *J* = 8.9 Hz)], 5.87 (d, *J* = 4.6 Hz, 1H, H-1'), 5.27 (d, *J* = 6.2 Hz, 1H, 3'-OH), 4.25 (q, *J* = 5.6 Hz, 1H, H-3'), 4.11 (t, *J* = 4.9 Hz, 1H, H-2'), 3.99 (m, 1H, H-4'), 3.86 (ddd, *J* = 11.0, 5.7, 3.4 Hz, 1H, H-16), 3.77 – 3.70 [(m, 7H, H-16 and O-CH₃) containing 3.74 (s, O-CH₃)], 3.47 (ddd, *J* = 13.3, 7.0, 3.4 Hz, H-17), 3.40 (ddd, *J* = 13.3, 5.7, 3.4 Hz, 1H, H-17), 3.27 (dd, *J* = 10.7, 4.4

Hz, 1H, H-5'), 3.20 (dd, $J = 10.7, 2.6$ Hz, 1H, H-5'), 1.40 (d, $J = 1.1$ Hz, 3H, 5-CH₃).

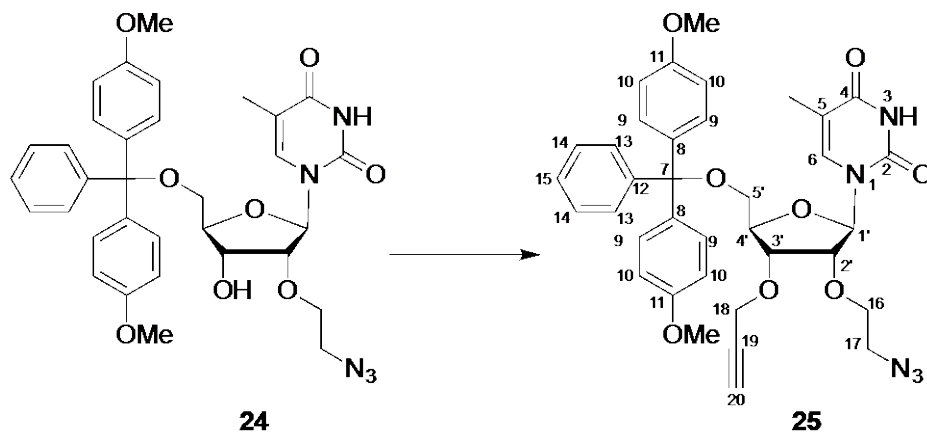
¹³C NMR (126 MHz, DMSO-*d*₆) δ 163.4 (C-4), 157.9 (C-11), 150.2 (C-2), 149.3 (C-Ar), 135.2 (C-6 or C-8), 135.1 (C-6 or C-8), 129.5 (C-Ar), 127.6 (C-Ar), 127.4 (C-Ar), 126.5 (C-Ar), 113.0 (C-10), 109.3 (C-5), 86.2 (C-1'), 85.6 (C-7), 82.6 (C-4'), 80.6 (C-2'), 68.6 (C-16 or 17), 68.4 (C-3'), 62.8 (C-5'), 54.7 (O-CH₃), 49.7 (C-16 or 17), 11.4 (5-CH₃).

LRMS [ESI⁻, MeOH] m/z 628.2 (100 %) [M-H]⁻.

R_f: 0.35 [30% toluene in ethyl acetate + 1% pyridine]

Data consistent with literature.¹³⁸

7.1.2.11 *Synthesis of 5'-O-(4,4'-dimethoxytrityl)-3'-O-propargyl-2'-O-(2-azidoethyl) thymidine (25)*



Compound **24** (1.0 g, 1.6 mmol, 1.0 eq) was dissolved in anhydrous THF (7 mL) and cooled to 0 °C. Sodium hydride (60% dispersion in mineral oil) (0.16 g, 4.0 mmol, 2.5 eq) was added in four portions. After stirring for an hour propargyl bromide (80% in toluene, 0.39 mL, 3.4 mmol, 2.2 eq) was added at 0 °C and stirred at reflux for 14 hours. H₂O was added to quench the reaction and solvent was removed *in vacuo*. The residue was dissolved in CH₂Cl₂ (50 mL) and washed with water and brine. The organic layer was separated, dried over sodium sulfate, filtered and the solvent removed *in vacuo*. The crude material was then purified by flash column chromatography on silica gel (1-3% MeOH in CH₂Cl₂ with constant 1% pyridine) giving compound **25** as a white foam (0.765 g, 1.15 mmol, 72%). M_W = 667.26 g/mol (C₃₆H₃₇N₅O₈).

¹H NMR (400 MHz, CDCl₃) δ 7.76 (d, *J* = 1.4 Hz, 1H, H-6), 7.44 – 7.39 (m, 2H, H-Ar), 7.33 – 7.18 (m, 7H, H-Ar), 6.82 (d, *J* = 8.6 Hz, 4H, H-10), 5.93 (d, *J* = 2.1 Hz, 1H, H-1'), 4.56 (dd, *J* = 7.6, 4.9 Hz, 1H, H-3'), 4.30 (dd, *J* = 16.1, 2.4 Hz, 1H, H-18), 4.27 – 4.19 [(m, 2H, H-4' and H-18) containing 4.22 (dd, *J* = 16.2,

2.5 Hz, H-18)], 4.17 (dd, $J = 5.0, 2.2$ Hz, 1H, H-2'), 4.07 (dt, $J = 10.0, 4.5$ Hz, 1H, H-16), 3.89 – 3.79 (m, 1H, H-16), 3.77 (s, 6H, O-CH₃), 3.55 (dd, $J = 11.2, 2.0$ Hz, 1H, H-5'), 3.43 (t, $J = 5.0$ Hz, 2H, H-17), 3.40 (dd, $J = 11.3, 2.4$ Hz, 1H, H-5'), 2.42 (t, $J = 2.4$ Hz, 1H, H-20), 1.33 (d, $J = 1.1$ Hz, 3H, 5-CH₃).

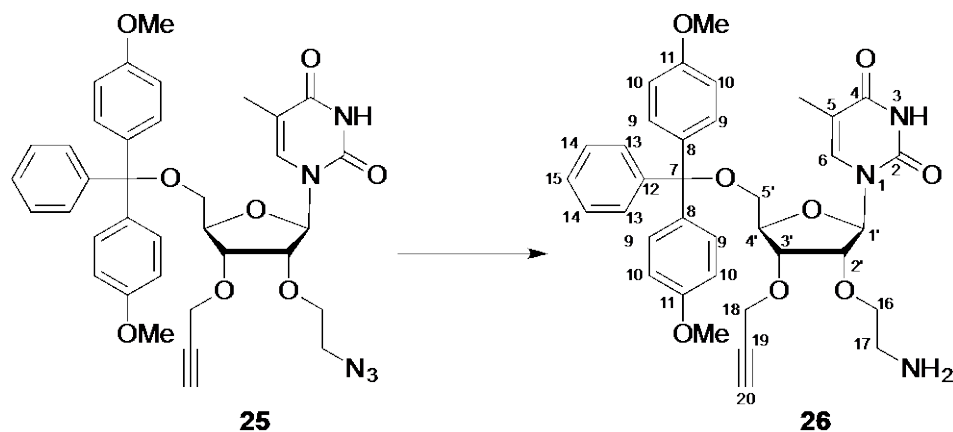
¹³C NMR (101 MHz, CDCl₃) δ 163.8 (C-4), 158.7 (C-11), 150.3 (C-2), 144.3 (C-12), 136.9 (C-6), 135.4 (C-8), 130.1 (C-Ar), 128.1 (C-Ar), 128.0 (C-Ar), 127.1 (C-Ar), 113.3 (C-10), 110.9 (C-5), 88.3 (C-1'), 86.8 (C-7), 81.1 (C-2'), 79.0 (C-19), 75.6 (C-20), 73.8 (C-3'), 69.5 (C-16 or 17), 61.1 (C-5'), 57.5 (C-18), 55.2 (O-CH₃), 50.8 (C-16 or 17), 11.8 (5-CH₃), (24 of 25 resonances observed).

LRMS [ESI⁻, MeOH] m/z 666.0 (100 %) [M-H]⁻.

HRMS [ESI⁺, MeOH] calculated mass m/z 690.2534 for C₃₆H₃₇N₅O₈Na [M+Na]⁺; found m/z 690.2537

IR: 2358 (m, N=N=N), 2108 (m, C≡C), 1687 (s, C=O), 1250 (s, C-O alkyl aryl ether), 828, 702 (s, C-H) $\nu_{\max}/\text{cm}^{-1}$.

7.1.2.12 Synthesis of 5'-O-(4,4'-dimethoxytrityl)-3'-O-propargyl-2'-O-(2-aminoethyl) thymidine (**26**)

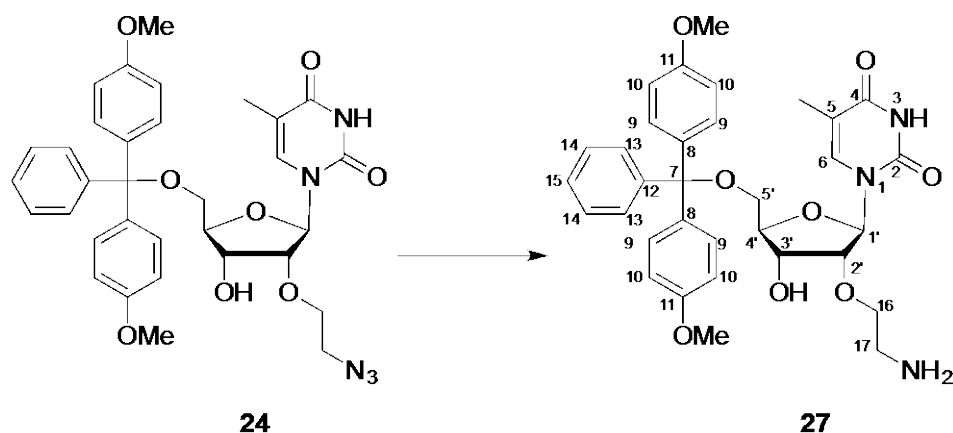


The compound **25** (1.5 g, 2.3 mmol, 1.0 eq) was dissolved in THF (15 mL), then triphenylphosphine (1.2 g, 4.5 mmol, 2.0 eq) was added followed by H₂O (0.19 mL, 11 mmol, 5.0 eq). The reaction mixture was stirred for 2 hours at 45 °C. The solvent was then removed *in vacuo* and the oil was purified by flash column chromatography on silica gel (10-60% MeOH in CH₂Cl₂ with constant 1% pyridine) giving compound **26** as a white foam (1.12 g, 1.75 mmol, 78%). M_W = 641.27 g/mol (C₃₆H₃₉N₃O₈).

¹H NMR (400 MHz, CDCl₃) δ 7.72 (d, *J* = 1.5 Hz, 1H, H-6), 7.50 – 7.38 (m, 1H, H-Ar), 7.36 – 7.18 (m, 7H, H-Ar), 6.84 (d, *J* = 8.6 Hz, 4H, H-10), 5.97 (d, *J* = 2.8 Hz, 1H, H-1'), 4.51 (dd, *J* = 6.9, 5.0 Hz, 1H, H-3'), 4.33 – 4.20 (m, 3H, H-18_{x2} and H-4'), 4.16 (dd, *J* = 5.0, 2.9 Hz, 1H, H-2'), 3.88 (dt, *J* = 10.0, 5.1 Hz, 1H, H-16), 3.79 (s, 6H, O-CH₃), 3.71 (dt, *J* = 9.9, 5.0 Hz, 1H, H-16), 3.57 (dd, *J* = 11.2, 2.1 Hz, 1H, H-5'), 3.40 (dd, *J* = 11.1, 2.6 Hz, 1H, H-5'), 2.93 (t, *J* = 5.2 Hz, 2H, H-17), 2.44 (t, *J* = 2.4 Hz, 1H, H-20), 1.37 (d, *J* = 1.2 Hz, 3H, 5-CH₃).

LRMS [ESI⁺, MeCN] *m/z* 664.1 (18.19%) [M+Na]⁺, 683.4 (34.67%)
[M+MeCN+H]⁺, 705.3 (44.06%) [M+MeCN+Na]⁺.

7.1.2.13 Synthesis of 2'-aminoethoxy thymidine (27)



Compound **24** (2.31 g, 3.67 mmol, 1.0 eq) was dissolved in THF (10 mL), then triphenylphosphine (1.93 g, 4.34 mmol, 2.0 eq) was added followed by H₂O (0.301 mL, 18.4 mmol, 5.0 eq). The reaction mixture was stirred for 2 hours at 45 °C. Solvent was then removed *in vacuo* and the oil was purified by column chromatography on silica gel (10-40% MeOH in CH₂Cl₂ with constant 1% pyridine) giving desired product **27** as a white foam (2.21 g, 3.66 mmol, >99%). $M_W = 603.67$ g/mol (C₃₃H₃₇N₃O₈).

¹H NMR (400 MHz, CDCl₃) δ 7.59 (s, 1H, H-6), 7.41 – 7.33 (m, 2H, H-Ar), 7.30 – 7.15 (m, 7H, H-Ar), 6.79 (d, *J* = 9.0 Hz, 4H, H-10), 5.99 (d, *J* = 4.0 Hz, 1H, H-1'), 4.38 (t, *J* = 5.0 Hz, 1H, H-3'), 4.14 – 4.08 (m, 1H, H-4'), 4.03 (t, *J* = 4.5 Hz, 1H, H-2'), 3.95 – 3.87 (m, 1H, H-16), 3.74 (s, 6H, O-CH₃), 3.63 – 3.55 (m, 1H, H-16), 3.48 (dd, *J* = 11.0, 3.0 Hz, 1H, H-5'), 3.37 (dd, *J* = 11.0, 3.0 Hz, 1H, H-5'), 2.95 – 2.84 (m, 2H, H-17), 1.34 (s, 3H, 5-CH₃).

¹³C NMR (100 MHz, CDCl₃-*d*) δ 163.9 (C-4), 158.7 (C-Ar), 150.5 (C-2), 144.4 (C-Ar), 135.5 (C-Ar), 135.3 (C-6), 130.1 (C-Ar), 128.2 (C-Ar), 127.2 (C-Ar), 127.2

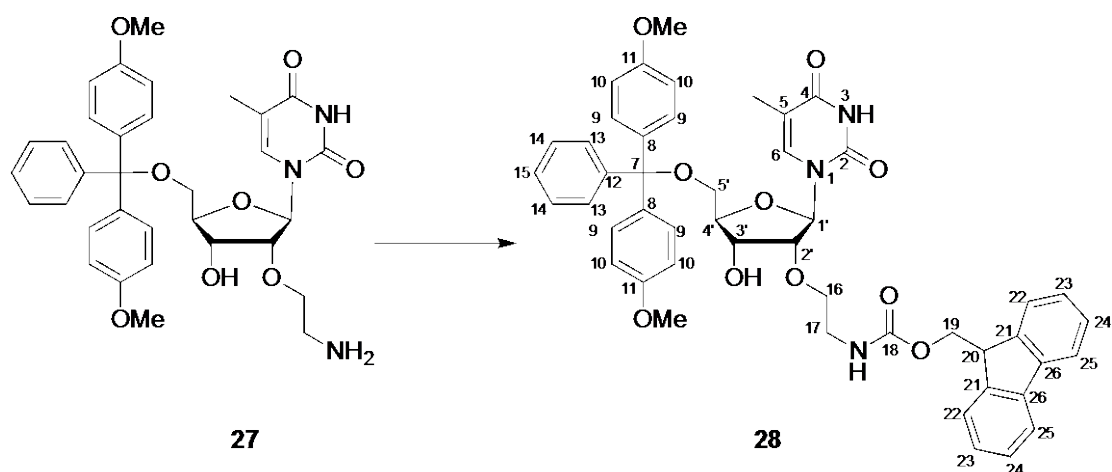
(C-Ar), 113.3 (C-10), 111.2 (C-5), 87.4 (C-1'), 86.9 (C-7), 83.9 (C-4'), 82.9 (C-2'), 72.0 (C-16), 69.6 (C-3'), 62.7 (C-5'), 55.2 (O-CH₃), 41.2 (C-17), 11.7 (5-CH₃).

LRMS [ESI⁺, MeOH] found *m/z*. 626.2 (30%) [M+Na]⁺.

R_f: 0.07 [20% MeOH in CH₂Cl₂ + 1% pyridine]

Data consistent with literature.¹³⁸

7.1.2.14 Synthesis of 2'-aminoethoxy-*N*-Fmoc thymidine (**28**)



Compound **27** (2.2 g, 3.7 mmol, 1.0 eq) was dissolved in anhydrous CH₂Cl₂ (20 mL), followed by addition of pyridine (0.59 mL, 7.3 mmol, 2.0 eq) and then 9-fluorenylmethyl-*N*-succinimidyl carbonate (4.3 g, 13 mmol, 3.5 eq). The reaction mixture was stirred at room temperature for 2 hours and then was diluted in CH₂Cl₂ (100 mL) and partitioned between sat.aq. NH₄Cl and sat. aq. NaHCO₃. The organic layer was then dried over sodium sulfate, filtered and concentrated *in vacuo*. The crude material was purified by flash column chromatography on a silica gel (1-3% MeOH in CH₂Cl₂ with constant 1% pyridine) affording compound **28** as a white foam (2.51 g, 3.04 mmol, 83%). $M_W = 825.92$ g/mol (C₄₈H₄₇N₃O₁₀).

¹H NMR (400 MHz, CDCl₃) δ 7.74 (d, $J = 7.5$ Hz, 2H, H-Ar), 7.69 – 7.66 (m, 1H, H-Ar), 7.58 (d, $J = 7.6$ Hz, 2H, H-Ar), 7.43 – 7.34 (m, 4H, H-Ar), 7.33 – 7.25 (m, 9H, H-Ar), 6.83 (d, $J = 8.1$ Hz, 4H, H-10), 5.93 (d, $J = 2.4$ Hz, 1H, H-1'), 4.48 – 4.35 (m, 3H, H-3', H-19), 4.20 (t, $J = 7.0$ Hz, 1H, H-20), 4.10 – 4.03 (m, 1H, H-4'), 4.02 – 3.97 (m, 1H, H-2'), 3.97 – 3.90 (m, 1H, H-16), 3.85 – 3.73 [(m, 7H, H-16, and O-CH₃) containing 3.77 (s, O-CH₃)], 3.54 (dd, $J = 11.0, 2.0$ Hz, 1H, H-

5'), 3.51 – 3.36 [(m, 3H, H-17, H-5') containing 3.42 (dd, $J = 11.0, 2.9$ Hz, H-5')], 1.36 (d, $J = 1.2$ Hz, 3H, 5-CH₃).

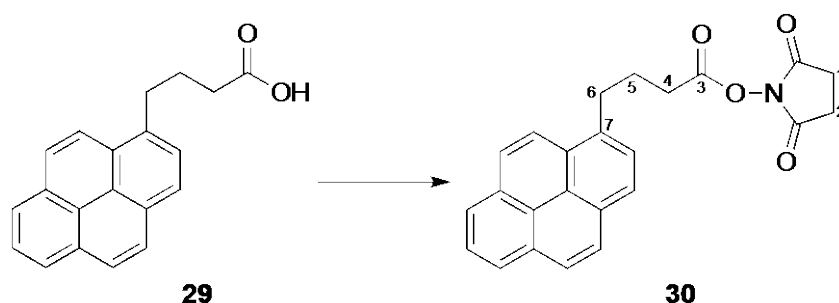
LRMS [ESI⁺, MeOH] found m/z 848.8 (82%) [M+Na]⁺

R_f: 0.8 [10% hexane in ethyl acetate + 1% pyridine]

Data consistent with literature.¹³⁸

7.1.2.15 Synthesis of 1-pyrenebutyric acid *N*-hydroxysuccinimide ester

(30)



1-Pyrenebutyric acid **29** (0.20 g, 0.69 mmol, 1.0 eq) was dissolved in anhydrous CH_2Cl_2 (32 mL) under argon, then activated molecular sieves 3Å were added, followed by anhydrous DIPEA (0.24 mL, 1.4 mmol, 2.0 eq) and *N,N'*-disuccinimidyl carbonate (0.19 g, 0.76 mmol, 1.1 eq). The reaction mixture was stirred for 3 hours at room temperature. After the reaction reached completion, the mixture was diluted with CH_2Cl_2 (40 mL). The organic solution was washed with water, 1 M HCl and brine. The organic layer was then dried over sodium sulfate, filtered and the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography on silica gel (40% hexane in ethyl acetate), affording compound **30** as a white solid (0.20 g, 0.53 mmol, 77%). $M_W = 385.13 \text{ g/mol}$ ($\text{C}_{24}\text{H}_{19}\text{NO}_4$).

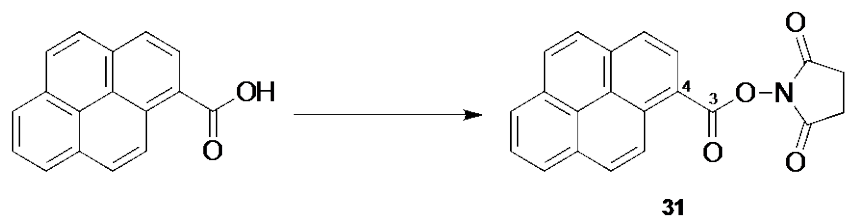
^1H NMR (300 MHz, CDCl_3 -*d*) δ 8.30 (d, $J = 9.3 \text{ Hz}$, 1H, H-7), 8.22 – 8.13 (m, 3H, H-Ar), 8.12 (s, 1H, H-Ar), 8.06 – 7.95 (m, 3H, H-Ar), 7.89 (d, $J = 7.8 \text{ Hz}$, 1H, H-Ar), 3.49 (dd, $J = 8.6, 6.8 \text{ Hz}$, 2H, H-6), 2.86 (br. s, 4H, H-1 and H-2), 2.74 (t, $J = 7.1 \text{ Hz}$, 2H, H-5), 2.32 (p, $J = 7.1 \text{ Hz}$, 2H, H-4).

^{13}C NMR (126 MHz, CDCl_3) δ 169.3 (C-3), 168.7 (NCOCH_2), 135.0 (C-Ar), 131.0 (C-Ar), 130.3 (C-Ar), 128.9 (C-Ar), 127.8 (C-Ar), 127.6 (C-Ar), 127.0 (C-Ar), 126.0 (C-Ar), 125.3 (C-Ar), 125.1 (C-Ar), 125.1 (C-Ar), 125.1 (C-Ar), 125.0 (C-Ar), 123.3 (C-Ar), 32.4 (C-6), 30.7 (C-4), 26.6 (C-5), 25.8 (C-1 and C-2), (20 of 23 resonances observed).

HRMS [EI, MeOH] calculated mass m/z 408.1206 for $\text{C}_{24}\text{H}_{19}\text{NO}_4\text{Na}$ $[\text{M}+\text{Na}]^+$; found m/z 408.1207

Data consistent with literature²¹¹

7.1.2.16 Synthesis of 1-pyrenecarboxylic acid *N*-hydroxysuccinimide ester (31**)**



1-Pyrenecarboxylic acid (0.10 g, 0.41 mmol, 1.0 eq) was dissolved in anhydrous CH_2Cl_2 (15 mL), then activated molecular sieves were added, followed by anhydrous *N,N*-diisopropylethylamine (0.14 mL, 0.82 mmol, 2.0 eq) and *N,N'*-disuccinimidyl carbonate (0.12 g, 0.45 mmol, 1.1 eq). The reaction mixture was stirred for 3 hours under argon at room temperature. Mixture was then diluted with CH_2Cl_2 . The organic solution was washed with water, 1M HCl and brine. The organic layer was then dried over sodium sulphate, filtered and solvent was removed *in vacuo*. The crude product was purified by flash column chromatography on silica gel (30-50% ethyl acetate in hexane) affording desired compound **31** as a yellow solid (0.11 g, 0.32 mmol, 79%). $M_W = 343.08 \text{ g/mol}$ ($\text{C}_{21}\text{H}_{13}\text{NO}_4$).

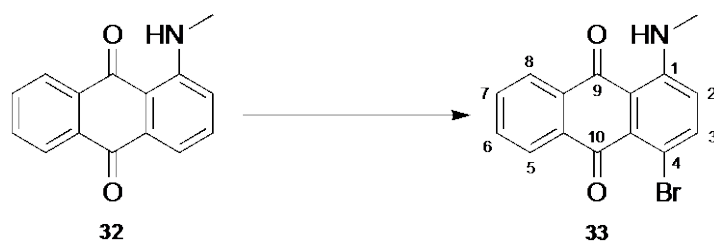
^1H NMR (300 MHz, CDCl_3 -*d*) δ 9.16 (1 H, d, $J=9.5 \text{ Hz}$, H-3), 8.85 (1 H, d, $J=8.4 \text{ Hz}$, H-Ar), 8.04 - 8.37 (7 H, m, H-Ar), 3.01 (4 H, s, H-1 and H-2)

^{13}C NMR (126 MHz, CDCl_3 -*d*) δ 169.8 (C-3), 162.8 (NCOCH_2), 136.0 (C-Ar), 132.5 (C-Ar), 131.0 (C-Ar), 130.8 (C-Ar), 130.4 (C-Ar), 129.1 (C-Ar), 127.3 (C-Ar), 127.2 (C-Ar), 127.1 (C-Ar), 126.8 (C-Ar), 124.9 (C-Ar), 124.4 (C-Ar), 124.3 (C-Ar), 124.0 (C-Ar), 117.5 (C-Ar), 26.0 (C-1 and C-2), (18 of 20 resonances observed).

HRMS [EI, MeOH] calculated mass m/z 366.0737 for $C_{21}H_{13}NO_4Na$
[M+Na]⁺; found m/z 366.0737

Data consistent with literature ²¹²

7.1.2.17 Synthesis of 4-bromo-1-(methylamino)anthraquinone (**33**)



1-(Methylamino)-anthraquinone **32** (0.20 g, 0.84 mmol, 1.0 eq) was suspended in propionic acid (8.4 mL), acetic acid (14.4 mL) and hydrobromic acid (0.96 mL). The reaction mixture (dark red) was stirred at 50 °C for 2 hours. It was then cooled to 0 °C and bromine (1.9 mL, 0.93 mmol, 1.1 eq) was added dropwise. The reaction was stirred for further hour, before sodium metabisulfite in H₂O was added to quench reaction. After stirring for half an hour, the red solid was filtered off and washed with H₂O until the filtrate was of pH=7. The red solid was dried over P₂O₅, obtaining desired compound **33** as a dark red solid (0.24 g, 0.77 mmol, 92%). M_w = 314.99 g/mol (C₁₅H₁₀BrNO₂).

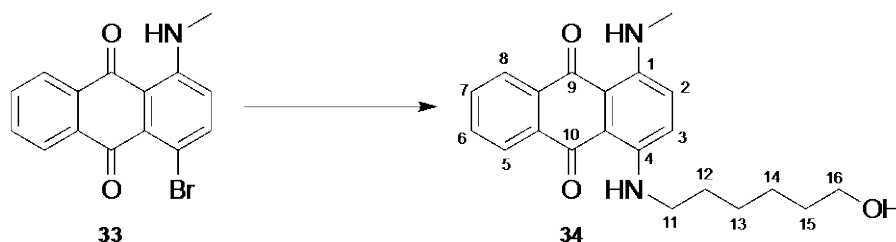
¹H NMR (400 MHz, CDCl₃-*d*) δ 10.01 (s, 1H, N-H), 8.34 – 8.11 (m, 2H, H-5, H-8), 7.89 – 7.60 (m, 3H, H-3, H-6, H-7), 6.89 (d, *J* = 9.3 Hz, 1H, H-2), 3.04 (d, *J* = 4.3 Hz, 3H, H-3).

¹³C NMR (75 MHz, CDCl₃-*d*) δ 184.4 (C-9 or C-10), 183.0 (C-9 or C-10), 152.4 (C-1), 142.4 (C-3), 133.8 (C-6 or C-7), 133.6 (C-5a or C-8a), 132.4 (C-6 or C-7), 131.5 (C-5a or C-8a), 127.0 (C-5 or C-8), 127.2 (C-5 or C-8), 118.1 (C-2), 114.7 (C-4), 108.4 (C-1a, C-4a), 29.8 (NH-CH₃), (14 of 15 resonances observed).

R_f : 0.85 (50% toluene in ethyl acetate)

Data consistent with literature ¹⁵⁰

7.1.2.18 Synthesis of 4-[(6-hydroxyhexyl)amino]-1-(methylamino)anthraquinone (34)



Compound **33** (0.24 g, 0.76 mmol, 1.0 eq) was dissolved in toluene (5 mL) and TEA (1.1 mL, 7.6 mmol, 10.0 eq) was added followed by 6-amino-1-hexanol (0.89 g, 7.6 mmol, 10.0 eq). The reaction mixture was heated at reflux for 24 hours. The reaction solution was diluted with CH₂Cl₂ and washed with H₂O and brine. The organic layer was separated, dried over sodium sulfate, filtered and the solvent was removed *in vacuo*. The crude material was purified by column chromatography on silica gel (50% ethyl acetate in toluene) giving compound **34** as a dark blue solid (0.08 g, 0.2 mmol, 29%). $M_w = 352.43$ g/mol (C₂₁H₂₄N₂O₃).

¹H NMR (400 MHz, DMSO-*d*₆) δ 10.85 (t, $J = 5.5$ Hz, 1H, N-H), 10.67 (q, $J = 5.0$ Hz, 1H, CH₃-N-H), 8.28 – 8.16 (m, 2H, H-5, H-8), 7.82 – 7.75 (m, 2H, H-6, H-7), 7.50 (d, $J = 9.9$ Hz, 1H, H-2 or H-3), 7.45 (d, $J = 9.9$ Hz, 1H, H-2 or H-3), 4.37 (t, $J = 5.2$ Hz, 1H, OH), 3.44 (q, $J = 5.6, 4.5$ Hz, 2H, H-11 or H-16), 3.39 (q, $J = 6.3, 5.1$ Hz, 2H, H-11 or H-16), 3.08 (d, $J = 5.2$ Hz, 3H, NH-CH₃), 1.66 (p, $J = 6.9$ Hz, 2H, H-12), 1.50 – 1.30 (m, 6H, H-13, H-14, H-15).

¹³C NMR (126 MHz, DMSO-*d*₆) δ 180.4 (C-9 or C-10), 180.3 (C-9 or C-10), 146.6 (C-1 or C-4), 145.9 (C-1 or C-4), 133.7 (C-5a, C-8a), 132.1 (C-6, C-7),

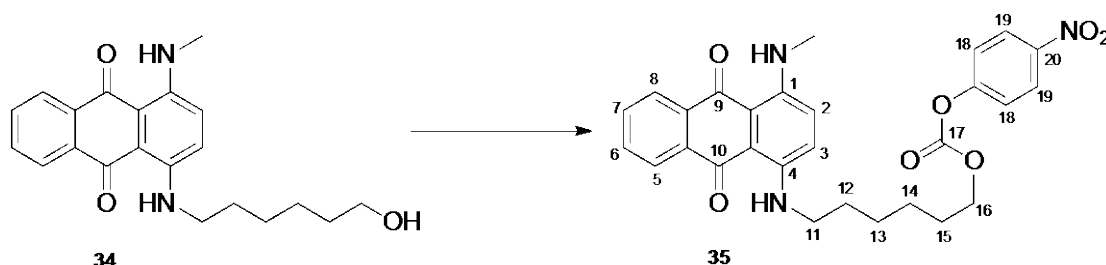
125.5 (C-5 or C-8), 125.5 (C-5 or C-8), 124.4 (C-2 or C-3), 124.2 (C-2 or C-3), 108.2 (C-1a or C-4a), 108.0 (C-1a or C-4a), 60.5 (C-16), 41.9 (C-11), 32.3 (C-15), 29.1 (C-12, NH-CH₃), 26.3 (C-13 or C-14), 25.1 (C-13 or C-14), (18 of 21 resonances observed).

LRMS [ESI⁺, MeOH] *m/z*. 353.2 [M+H]⁺, 375.1 (100%) [M+Na]⁺

R_f : 0.35 (50% toluene in ethyl acetate)

Data consistent with literature ¹⁵¹

7.1.2.19 Synthesis of 1-(methylamino)-4-[(6-O-(4-nitrophenyl)-hexyl)amino]carbonate anthraquinone (35)



Compound **34** (0.05 g, 0.1 mmol, 1.0 eq) was dissolved in anhydrous CH_2Cl_2 (7 mL), followed by addition of 4-nitrophenyl chloroformate (0.057 g, 0.28 mmol, 2.0 eq) and anhydrous pyridine (0.057 mL, 0.71 mmol, 5.0 eq). The reaction was stirred for 6 hours at room temperature. The solvent was removed *in vacuo*. The crude mixture was redissolved in CH_2Cl_2 (20 mL) and washed with H_2O and brine. Organic layer was dried over sodium sulfate and the crude material was purified by column chromatography on silica gel (20-40% ethyl acetate in petroleum ether) delivering compound **35** as dark blue solid (0.045 g, 0.052 mmol, 37%). $M_W = 517.18 \text{ g/mol}$ ($\text{C}_{28}\text{H}_{27}\text{N}_3\text{O}_7$).

^1H NMR (400 MHz, CDCl_3 - d) δ 8.36 – 8.29 (m, 2H, H-5, H-8), 8.26 (d, $J = 9.2 \text{ Hz}$, 2H, H-19), 8.13 (d, $J = 9.1 \text{ Hz}$, 1H, H-3), 7.72 – 7.65 (m, 2H, H-6, H-7), 7.36 (d, $J = 9.2 \text{ Hz}$, 2H, H-18), 6.94 (d, $J = 9.1 \text{ Hz}$, 1H, H-2) 4.30 (t, $J = 6.6 \text{ Hz}$, 3H, NH- CH_3), 1.85 – 1.72 (m, 6H, H-11, H-12, H-16), 1.63 – 1.45 (m, 6H, H-13, H-14, H-15).

^{13}C NMR (126 MHz, CDCl_3 - d) δ 182.9 (C-9 or C-10), 182.8 (C-9 or C-10), 155.9 (C-Ar), 147.3 (C-1 or C-4), 146.5 (C-1 or C-4), 145.7 (C- NO_2), 134.9 (C-5a, C-8a), 132.4 (C-Ar), 126.4 (C-Ar), 125.7 (C-Ar), 123.9 (C-Ar), 123.5 (C-Ar), 122.2

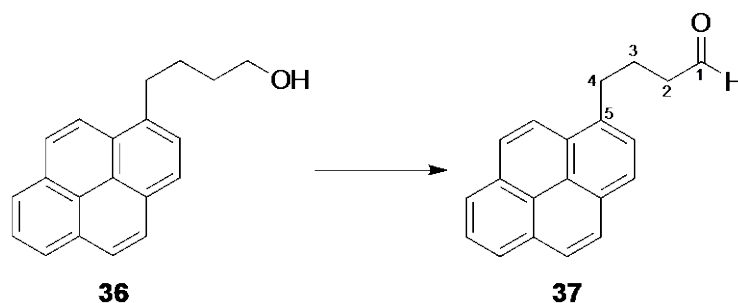
(C-Ar), 110.3 (C-1a or C-4a), 110.2 (C-1a or C-4a), 69.8 (C-16), 43.1 (C-11), 29.9 (C-15), 29.9 (C-12 or NH-CH₃), 28.8 (C-12 or NH-CH₃), 27.2 (C-13 or C-14), 25.9 (C-13 or C-14), (22 of 26 resonances observed).

LRMS [ESI⁻, MeOH] found m/z 517 (100%) [M-H]⁺.

HRMS [ESI⁺, MeOH] calculated mass m/z 518.1922 for C₂₈H₂₇N₃O₇ [M+H]⁺; found m/z 518.1923

HRMS [EI, MeOH] calculated mass m/z 518.1922 for C₂₈H₂₇N₃O₇ [M+H]⁺; found m/z 518.1918

7.1.2.20 Synthesis of 1-pyrenebutyl aldehyde (**37**)



4-Pyrene-10butanol **36** (0.50 g, 1.8 mmol, 1.0 eq) was dissolved in anhydrous CH_2Cl_2 (10 mL), and Dess Martin periodinane (1.2 g, 2.7 mmol, 1.5 eq) was added. A solution of H_2O (36 μL , 2.0 mmol, 1.1 eq) in CH_2Cl_2 (36 mL) was prepared and added dropwise over 30 min to the vigorously stirring reaction mixture. The reaction mixture was stirred for further 30 min at room temperature. The reaction mixture was diluted with CH_2Cl_2 (50 mL) and washed with brine, saturate KCl and sodium bicarbonate. The organic layer was dried over sodium sulfate, filtered and then the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography on silica gel (60% hexane in ethyl acetate) affording desired product **37** as an off white solid (0.398 g, 1.46 mmol, 81%). $M_w = 272.35 \text{ g/mol}$ ($\text{C}_{20}\text{H}_{16}\text{O}$).

^1H NMR (400 MHz, CDCl_3) δ 9.80 (t, $J = 1.5 \text{ Hz}$, 1H, CHO), 8.29 (d, $J = 9.2 \text{ Hz}$, 1H, H-Ar), 8.18 – 8.16 (m, 2H, H-Ar), 8.13 (d, $J = 9.3 \text{ Hz}$, 1H, H-Ar), 8.12 (d, $J = 7.8 \text{ Hz}$, 1H, H-Ar), 8.04 (br. s, 2H, H-Ar), 8.00 (t, $J = 7.6 \text{ Hz}$, 1H), 7.85 (d, $J = 7.8 \text{ Hz}$, 1H, H-Ar), 3.44 – 3.34 (m, 2H, CH_2), 2.58 (td, $J = 7.2, 1.5 \text{ Hz}$, 2H, CH_2), 2.31 – 2.09 (m, 2H, CH_2).

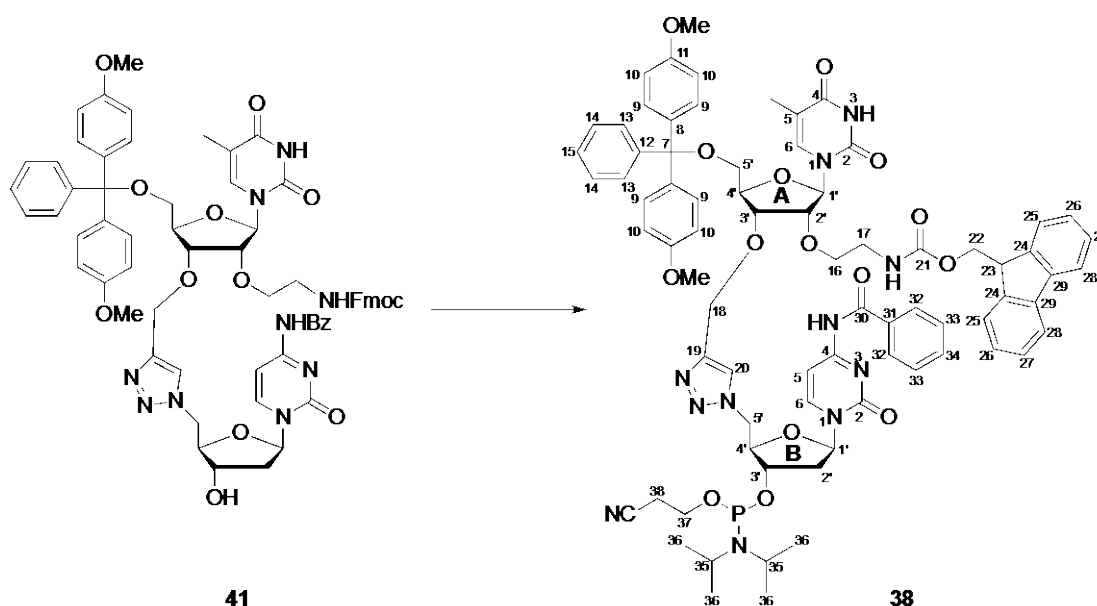
^{13}C NMR (126 MHz, CDCl_3) δ 202.3 (C-1), 135.6 (C-Ar), 131.5, (C-Ar) 131.0 (C-Ar), 130.2 (C-Ar), 128.9 (C-Ar), 127.6 (C-Ar), 127.6 (C-Ar), 127.4 (C-Ar), 126.9 (C-Ar), 126.0 (C-Ar), 125.1 (C-Ar), 125.0 (C-Ar), 124.8 (C-Ar) 123.3 (C-Ar), 43.6 (C-5), 32.7 (C-4), 24.1 (C-3), (18 of 20 resonances observed).

LRMS [ESI⁻, MeOH] found m/z 317 (100%) $[\text{M}+\text{HCO}_2\text{H}-\text{H}]^+$.

R_f : 0.72 (40% hexane in ethyl acetate)

Data consistent with literature ^{213,214}

7.1.2.21 Synthesis of 2'-aminoethoxy-N-Fmoc-5'-dimethoxytrityl-3'-methoxy thymidine- N⁴-benzoyl-2'-deoxy-3'-phosphoramidite-cytidine triazole dimer (**38**)

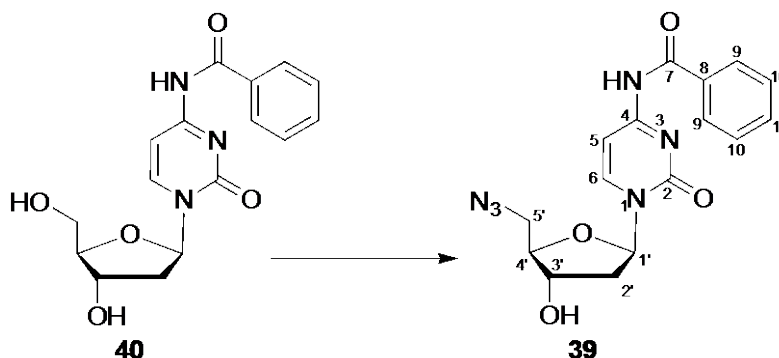


Dimer **17** (0.760 g, 0.623 mmol, 1.0 eq) was co-evaporated with dry pyridine, then dissolved in anhydrous CH₂Cl₂ (6 mL) under argon. DIPEA (0.245 mL, 1.31 mmol, 2.1 eq) was added to the solution, followed by slow addition of 2-cyanoethyl-*N,N*-diisopropylchloro phosphoramidite (0.240 mL, 1.06 mmol, 1.7 eq) at 0 °C. The reaction was stirred under argon at room temperature for 4 hours and then transferred to the separating funnel, under argon. The reaction mixture was diluted with deoxygenated CH₂Cl₂ and deoxygenated sat. aq. KCl solution, and the organic layer was dried over sodium sulfate and concentrated *in vacuo*. The crude material was purified by column chromatography on a silica gel under argon (20% hexane in CH₂Cl₂) affording product **38** as a white foam (0.557 g, 0.392 mmol, 63 %). *M_w* = 1420.53 g/mol (C₇₆H₈₂N₁₁O₁₅P).

^1H NMR (400 MHz, $\text{MeCN-}d_3$) δ 9.12 (br. s, 1H, N-H), 9.03 (br. s, 1H, N-H), 7.92 (br. s, 1H, H-Ar), 7.90 (br. s, 1H, H-Ar), 7.82 (d, $J = 10.9$ Hz, 1H, H-Ar), 7.79 – 7.75 (m, 2H, H-Ar), 7.62 – 7.56 (m, 3H, H-Ar), 7.52 – 7.46 (m, 3H, H-Ar), 7.42 – 7.20 (m, 15H, H-Ar), 6.85 (ddd, $J = 9.0, 1.8, 1.0$ Hz, 4H, H-10), 6.26 (dd, $J = 10.9, 4.3$ Hz, 1H, H-1'B), 5.84 (d, $J = 3.1$ Hz, 1H, H-1'A), 4.75 – 4.50 (m, 5H, H-3'B, H-4'B, C-18, C-22), 4.41 – 4.22 (m, 4H, H-4'A, H-17, H-22), 4.21 – 4.11 (m, 3H, H-3'A, H-16, H-23), 3.83– 3.68 [(m, 9H, H-37, H-16, O-CH₃), containing 3.75 (s, 6H, O-CH₃)], 3.67 – 3.55 (m, 3H, H-30, H-5'A or B), 3.35 – 3.23 (m, 4H, H-2'A, H-5'A or B), 2.68 – 2.60 (m, 2H, H-38), 2.55 – 2.41 (m, 1H, H-2'B), 2.31 – 2.18 (m, 1H, H-2'B), 1.40 (d, $J = 1.2$ Hz, 3H, 5-CH₃), 1.17 (d, $J = 6.7$ Hz, 12H, H-33).

^{31}P NMR (162 MHz, $\text{MeCN-}d_3$) δ 150.39 (s), 150.08 (s)

7.1.2.22 Synthesis of *N*⁴-Benzoyl-5'-azido-2'-deoxycytidine (**39**)



Nucleoside **40** (0.72 g, 2.2 mmol, 1.0 eq) was dissolved in DMF (10 mL) followed by addition of triphenylphosphine (0.68 g, 2.6 mmol, 1.2 eq) and tetrabromomethane (0.86 g, 11 mmol, 5 eq). The reaction was stirred at room temperature for 15 hours under argon. Lithium azide solution 20% in H₂O (0.43 mL, 4.6 mmol, 2.1 eq) was then added and reaction was stirred for further 4 hours at 60 °C. The reaction was next quenched with MeOH and solvent was removed *in vacuo*. The crude material was purified by column chromatography on silica gel (2-10% MeOH in CH₂Cl₂) affording compound **39** as a white solid (0.424 g, 1.19 mmol, 54%). *M*_w = 356.34 g/mol (C₁₆H₁₆N₆O₄).

¹H NMR (400 MHz, DMSO-*d*₆) δ 11.29 (s, 1H, N-H), 8.19 (d, *J* = 7.6 Hz, 1H, H-6), 8.05 – 7.95 (m, 2H, H-Ar), 7.66 – 7.59 (m, 1H, H-Ar), 7.58 – 7.51 (m, 2H, H-Ar), 7.40 (d, *J* = 7.6 Hz, 1H, H-5), 6.21 (t, *J* = 6.5 Hz, 1H, H-1'), 5.48 (d, *J* = 4.4 Hz, 1H, 3'-OH), 4.19 (dq, *J* = 6.3, 4.4 Hz, 1H, H-3'), 3.95 (dt, *J* = 6.4, 4.1 Hz, 1H, H-4'), 3.68 (dd, *J* = 13.2, 6.4 Hz, 1H, H-5'), 3.61 (dd, *J* = 13.2, 4.1 Hz, 1H, H-5'), 2.36 – 2.26 (m, 1H, H-2'), 2.20 (dt, *J* = 13.3, 6.5 Hz, 1H, H-2').

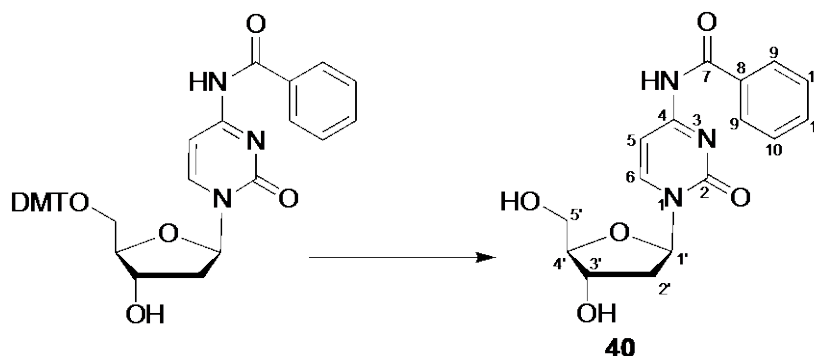
LRMS [ESI⁻, MeOH] *m/z*. 312.0 (100%), 355.2 (67%) [M-H]⁻.

¹³C NMR (126 MHz, DMSO-*d*₆) δ 167.2 (C-7), 163.0 (C-4), 154.2 (C-2), 145.0 (C-6), 132.9 (C-6), 132.6 (C-Ar), 128.3 (C-Ar), 96.3 (C-5), 86.0 (C-4'), 85.0 (C-1'), 70.5 (C-3'), 51.5 (C-5'), 48.5 (C-2'), (13 of 14 resonances observed).

LRMS [ESI⁻, MeOH] *m/z*. 312.1 (100%), 355.1 (75%) [M-H]⁻

Data consistent with literature.¹⁷⁷

7.1.2.23 Synthesis of *N*⁴-Benzoyl-2'-deoxycytidine (**40**)



*N*⁴-Benzoyl-5'-*O*-DMT-2'-deoxycytidine (1.5 g, 2.4 mmol, 1.0 eq) was dissolved in 2% trichloroacetic acid solution in CH₂Cl₂ (100 mL). The reaction was stirred at room temperature for 3 hours and the colour of the reaction mixture turned from clear to orange/pink. The formed suspension was filtered and the filtrate was washed with hexane. The compound **40** was isolated as a white solid (0.50 g, 1.5 mmol, 63%). *M*_W = 331.33 g/mol (C₁₆H₁₇N₃O₅).

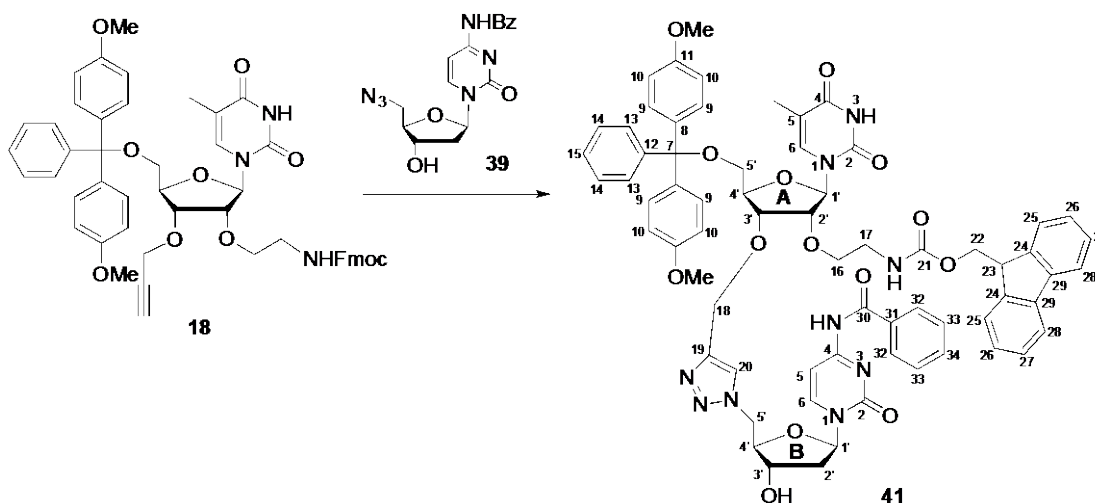
¹H NMR (400 MHz, DMSO-*d*₆) δ 11.17 (s, 1H, N-H), 8.24 (d, *J* = 7.6 Hz, 1H, H-6), 8.04 – 7.97 (m, 2H, H-Ar), 7.61 – 7.53 (m, 1H, H-Ar), 7.52 – 7.40 (m, 2H, H-Ar), 7.18 (d, *J* = 7.4 Hz, 1H, H-5), 6.15 (t, *J* = 6.4 Hz, 1H, H-1'), 5.29 (s, 1H, 3'-OH), 5.09 (s, 1H, 5'-OH), 4.23 (dt, *J* = 6.8, 3.6 Hz, 1H, H-3'), 3.85 (q, *J* = 3.7 Hz, 1H, H-4'), 3.63 (dd, *J* = 11.8, 3.8 Hz, 1H, H-5'), 3.57 (dd, *J* = 11.9, 4.0 Hz, 1H, H-5'), 2.26 (ddd, *J* = 13.2, 6.1, 3.7 Hz, 1H, H-2'), 2.04 (dt, *J* = 13.1, 6.4 Hz, 1H, H-2').

¹³C NMR (126 MHz, DMSO-*d*₆) δ 167.4 (C-7), 163.1 (C-4), 154.7 (C-2), 144.1 (C-6), 133.3 (C-Ar), 132.6 (C-Ar), 128.4 (C-Ar), 96.0 (C-5), 87.6 (C-4'), 86.4 (C-1'), 70.0 (C-3'), 61.0 (C-5'), 41.4 (C-2'), (13 of 14 resonances observed).

LRMS [ESI⁻, MeOH] *m/z* 214.1 (100%), 330.0 (90%) [M-H]⁻.

Data consistent with literature.²¹⁵

7.1.2.24 *Synthesis of 2'-aminoethoxy-N-Fmoc-5'-dimethoxytrityl-3'-methoxythymidine- N⁴-benzoyl-2'-deoxycytidine triazole dimer (41)*



Alkyne **18** (0.785 g, 0.909 mmol, 1.0 eq) and azide **39** (0.324 g, 0.909 mmol, 1.0 eq) monomers were dissolved in DMF (10 mL). TBTA ligand (0.198 g, 0.455 mmol, 0.5 eq) was added followed by addition of CuSO₄ (0.140 g, 0.455 mmol, 0.5 eq) and sodium ascorbate (0.443, 1.82 mmol, 2.0 eq) solutions in H₂O. The reaction was stirred under argon at room temperature for 4 hours. Upon completion the solvent was removed *in vacuo*. The residue was redissolved in CH₂Cl₂ (with 5% MeOH) and washed with NaEDTA (5% solution) in order to remove Cu salts and then washed with H₂O and brine. Organic layer was separated, dried over sodium sulfate, filtered and solvent was removed *in vacuo*. The crude material was purified by column chromatography on silica gel (5-10% *i*PrOH in CH₂Cl₂) obtaining product **41** as a white solid (0.976 g, 0.800 mmol, 88%). M_w = 1220.31 g/mol (C₆₇H₆₅N₉O₁₄).

^1H NMR (500 MHz, DMSO- d_6) δ 11.42 (s, 1H, NH), 11.24 (s, 1H, NH), 8.15 (s, 1H, H-Ar), 8.08 (s, 1H, H-20), 8.06 (d, J = 7.5 Hz, 1H, H-6B), 7.98 (d, J = 7.5 Hz, 2H, H-Ar), 7.85 (d, J = 7.6 Hz, 2H, H-Ar), 7.66 – 7.59 (m, 3H, H-Ar), 7.50 (t, J = 7.7 Hz, 2H, H-Ar), 7.40 – 7.25 (m, 11H, H-Ar), 7.24 – 7.19 (m, 3H, H-Ar), 6.88 (d, J = 8.2 Hz, 4H, H-10), 6.10 (t, J = 6.4 Hz, 1H, H-1'B), 5.84 (d, J = 3.9 Hz, 1H, H-1'A), 5.57 (d, J = 4.3 Hz, 1H, 3'-OH), 4.73 – 4.60 (m, 4H, H-18, H-22), 4.40 (t, J = 5.4 Hz, 1H, H-3'A), 4.30 (t, J = 4.5 Hz, 1H, H-2'A), 4.28 – 4.21 (m, 3H, H-3'B, H-4'B, H-17), 4.19 – 4.14 (m, 2H, H-17, H-23), 4.11 (dt, J = 6.3, 3.0 Hz, 1H, H-4'A), 3.71 (s, 6H, O-CH₃), 3.69 – 3.65 (m, 1H, H-16), 3.60 – 3.55 (m, 1H, H-16), 3.25 (dd, J = 11.1, 3.6 Hz, 1H, H-5' A or B), 3.22 – 3.14 (m, 3H, H-5'A or B), 2.34 – 2.27 (m, 1H, H-2'B), 2.17 (dt, J = 13.2, 6.4 Hz, 1H, H-2'B), 1.35 (s, 3H, 5-CH₃).

^{13}C NMR (126 MHz, DMSO- d_6) δ 167.8 (C-30), 164.2 (C-4A or C-4B), 163.6 (C-Ar), 162.8 (C-4A or C-4B), 158.6 (C-Ar), 158.6 (C-Ar), 156.7 (C-21), 154.7 (C-Ar), 150.8 (C-2A), 145.6 (C-Ar), 145.0 (C-Ar), 144.3 (C-19), 144.2 (C-Ar), 136.7 (C-Ar), 135.8 (C-Ar), 135.5 (C-Ar), 133.2 (C-Ar), 129.2 (C-Ar), 128.9 (C-Ar), 128.5 (C-Ar), 128.2 (C-Ar), 127.5 (C-Ar), 125.6 (C-Ar), 125.3 (C-Ar), 124.7 (C-Ar), 120.5 (C-Ar), 113.8 (C-10), 87.4 (C-1'A), 87.0 (C-1'B), 86.4 (C-7), 81.2 (C-4'A), 80.0 (C-2'A), 75.8 (C-3'A), 71.1 (C-3'B), 69.2 (C-16), 65.9 (C-17), 63.1 (C-18 or 22), 62.7 (C-5'A or 5'B), 60.2 (C-4'B), 55.5 (O-CH₃), 51.8 (C-18 or 22), 47.4 (C-23), 47.1, 40.6 (C-5'A or 5'B), 40.0 (C-2'), 12.2 (5-CH₃), (20 of 23 resonances observed).

HRMS [EI, MeOH] calculated mass m/z 1220.4724 for C₆₇H₆₆N₉O₁₄ [M+H]⁺; found m/z 1220.4707

HRMS [ESI⁺, MeOH] calculated mass m/z 1220.4724 for C₆₇H₆₆N₉O₁₄ [M+H]⁺; found m/z 1220.4705

IR: 2962 (w, C-H alkane), 1688 (s, C=O), 1251 (s, C-N), 1483 (m, C-H alkane), 1257 (s, C-O alkyl aryl ether), 1077 (s, C-O secondary alcohol), 702 (s, C-H) $\nu_{\text{max}}/\text{cm}^{-1}$.

7.2 OLIGONUCLEOTIDES SYNTHESIS

7.2.1 General conditions

DNA reagents including standard DNA and RNA phosphoramidites and solid supports (CPG resin) were purchased from Link Technologies Ltd, Glen Research and GE Healthcare. Oligonucleotides were synthesised on an Applied Biosystems 394 automated DNA/RNA synthesiser or ÄKTA oligopilot plus. Phosphoramidite cycles, including acid-catalysed detritylation, coupling, capping and iodine oxidation or sulfurization steps, were undertaken in 1.0, 15.0 and 30.0 μmole scale. Standard DNA phosphoramidites were used for all the phosphodiester oligonucleotide sequences. 2'-OMe-RNA CE phosphoramidites were purchased from Link Technologies and used for all the phosphorothioate oligonucleotide sequences. PADS or EDITH reagents were used for the sulfurisation step. Coupling efficiencies and overall oligonucleotide yields were determined by the automated trityl cation conductivity monitoring facility of the synthesiser and were $\geq 98.0\%$ for all cases except synthesis of ODN-D-~~1~~**1** and ODN-D-~~3~~**3** where the coupling efficiency of the modified triazole dimers **1** and **38** were $\geq 87.0\%$. Standard and modified phosphoramidite monomers were dissolved in anhydrous acetonitrile to a concentration of 0.1 M immediately prior to use. The coupling time for A, G, C, T and U monomers, was set to 60 s for standard

monomers and up to 20 min for modified ones. The coupling time for 2'-OMe-RNA CE monomers, was set to 360 s for standard monomers and up to 12 min for modified ones. Cleavage of the oligonucleotides from the solid support and deprotection was achieved by exposure to concentrated aqueous ammonia solution for 60 min at room temperature followed by heating in a sealed tube for 5 h at 55 °C.

Oligonucleotides were purified by RP-HPLC using a Gilson HPLC system with ABI Aquapore C8 column (8 mm x 250 mm, pore size 300 Å):

Conditions 1: acetonitrile in triethylammonium bicarbonate (TEAB) (0 to 50% buffer B over 20 min, flow rate 4 mL/min). The following elution buffers were used: buffer A (0.1 M triethylammonium bicarbonate, pH 7.5), buffer B (0.1 M triethylammonium bicarbonate, pH 7.5 with 50% acetonitrile);

Conditions 2: acetonitrile in triethylammonium bicarbonate (TEAB) (0 to 90% buffer B over 30 min, flow rate 4 mL/min). The following elution buffers were used: buffer A (0.1 M triethylammonium bicarbonate, pH 7.5), buffer B (0.1 M triethylammonium bicarbonate, pH 7.5 with 70% acetonitrile);

The fractions from HPLC were evaporated without additional desalting. The phosphorothioate oligonucleotides were purified by RP-HPLC using a Gilson HPLC system with Gemini-NX 10u C18 column (250 mm x 21.2 mm, pore size 110 Å):

Conditions 3: acetonitrile in hexylammonium acetate (HAA) (30 to 80% buffer B over 30 min, flow rate 15 mL/min). The following elution buffers were used: buffer A (0.1 mM hexylammonium acetate, pH 8.0), buffer B (0.1 mM hexylammonium acetate, pH 8.0 with 50% acetonitrile).

The fractions from HPLC were evaporated followed by additional desalting filtration using GE Healthcare columns illustra NAP-25 and NAP-10.

All oligonucleotides were characterized by negative-mode electrospray HPLC-mass spectrometry, using a Bruker micrOTOF mass spectrometer and an Acquity UPLC system with a BEH C18 1.7 μ m column (Waters). Raw data were processed and deconvoluted using the Data Analysis function of the Bruker Daltonics Compass™ 1.3 software package.

7.2.2 Synthesis and purification

7.2.2.1 Synthesis and purification of unmodified DNA and RNA strands

Oligonucleotides were synthesised following the general procedure presented above.

Oligonucleotides were purified by RP-HPLC using a Gilson HPLC system with ABI Aquapore C8 column (8 mm x 250 mm, pore size 300 Å). Oligonucleotides were purified using conditions 1. The fractions from HPLC were evaporated without additional desalting.

Table 7.1 List of unmodified phosphodiester oligodeoxyribonucleotides synthesised and used in this study.

code	sequence	calc mass	found mass
ODN-A-1	CGACGCTTGCAGC	3936	3936
ODN-A-2	GCTGCAAGCGTCG	3976	3976
ODN-A-3	GCTGCAATCGTCG	3951	3951
ODN-A-4	GCTGCATGCGTCG	3967	3966
ODN-A-5	GCTGCAGGCGTCG	3992	3991
ODN-A-6	GCTGCTAGCGTCG	3967	3966
ODN-A-7	GCTGCAAGTGTCG	3991	3990
ODN-A-8	GCTGCAATGCGTCG	4280	4279
ODN-A-9	GCTGCAACGCGTCG	4265	4264
ODN-A-10	GCTGCATAGCGTCG	4280	4280
ODN-A-11	GCTGCACAGCGTCG	4265	4264

ODN-B-1	CGTTCGCTTGCAGC	4231	4231
ODN-B-2	GCTGCAAGCGAACG	4298	4298
ODN-B-3	GCTGCATAGCGAACG	4602	4602
ODN-B-4	GCTGCAAGCGATACG	4602	4602
ODN-B-5	GCTGCAATGCGAACG	4602	4602
ODN-B-6	GCTGCAAGCGAATCG	4602	4601
ODN-C-1	CGTTCGCTTGCATGC	4535	4534
ODN-C-2	GCATGCAAGCGAACG	4611	4610

Table 7.2 List of unmodified phosphodiester oligoribonucleotides synthesised and used in this study.

code	sequence	calc mass	found mass
ORN-A-1	GCUGCAAGCGUCG	4155	4156
ORN-A-2	GCUGCAUGCGUCG	4132	4132
ORN-A-3	GCUGCAAUCGUCG	4116	4116
ORN-A-4	GCUGCUAGCGUCG	4132	4132
ORN-A-5	GCUGCAAGUGUCG	4156	4156
ORN-B-1	GCUGCAAGCGAACG	4508	4508
ORN-C-1	GCAUGCAAGCGAACG	4837	4837
ORN-D-1	CCUCUUACCUCAGUUACA	5572	5572
ORN-D-2	UGUAACUGAGGUAAAGAGG	5859	5859
ORN-E-1	GCUAUUACCUUAACCCAG	5636	5636
ORN-E-2	CUGGGUUAAGGUAAUAGC	5796	5796

7.2.2.2 Synthesis and purification of phosphodiester modified oligonucleotides containing triazole linkages and 2'-aminoethoxy groups

Oligonucleotides were synthesised following the above procedure with some alterations. The coupling time for A, G, C and T monomers was set to 25 s, however, the coupling time for the modified monomer T-T triazole dimer **1**

and 2'-aminoethoxy T **2** were extended to 360 s. After synthesis, oligonucleotides were kept on the solid support.

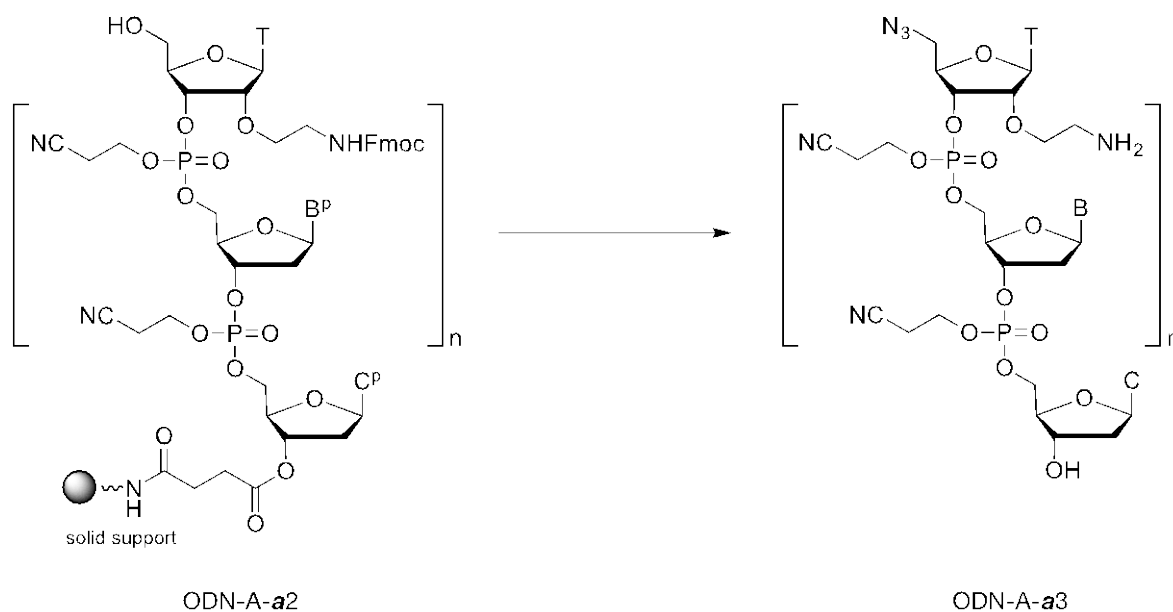
Modified oligonucleotides on resin were treated with diethylamine (20% in DMF) for 20 min in order to selectively remove the cyanoethyl protecting group. Next, oligonucleotides were cleaved from the solid support (CPG resin) and deprotected by suspending the resin in concentrated aqueous ammonia followed by heating in a sealed tube for 5 h at 55 °C. The oligonucleotides were filtered through Chromacol 17mm syringe filter 0.45 µm.

Oligonucleotides were purified by RP-HPLC using a Gilson HPLC system with ABI Aquapore C8 column (8 mm x 250 mm, pore size 300 Å). Oligonucleotides were purified using conditions 1. The fractions from HPLC were evaporated without additional desalting.

Table 7.3 List of phosphodiester oligodeoxyribonucleotides synthesised and used in this study. ^aT = 2'-aminoethoxy T, *t* = triazole linkage, ^{Me}C = 5-methyl C, ^{Me}C^Y = 3'-*O*-propargyl-5-methyl C, ^aT^Z = 5'-azido-2'-aminoethoxy T .

code	sequence	calc. mass	found mass
ODN-A- <i>t</i> 1	CGACGC ^P T ^t TGCAGC	3996	3996
ODN-A- <i>t</i> 2	CGACG ^{Me} C ^t TTGCAGC	4010	4009
ODN-A- <i>a</i> 1	CGACGC ^a TTGCAGC	3995	3994
ODN-A-3	CGACG ^{Me} C ^Y	1830	1830
ODN-A- <i>a</i> 2	^a TTGCAGC	2130	2130
ODN-A- <i>a</i> 3	^a T ^Z TGCAGC	2155	2156
ODN-B- <i>t</i> 1	CG ^a T ^t TCGCTTGCAGC	4291	4290
ODN-B- <i>t</i> 2	CGTTCGC ^a T ^t TGCAGC	4291	4290
ODN-B- <i>t</i> 3	CG ^a T ^t TCGC ^a T ^t TGCAGC	4350	4351
ODN-B- <i>a</i> 1	CGTTCGC ^a TTGCAGC	4290	4290
ODN-B- <i>a</i> 2	CG ^a TTCGC ^a TTGCAGC	4349	4349
ODN-C- <i>a</i> 1	CG ^a TTCGC ^a TTGCA ^a TGC	4712	4711

7.2.2.3 Synthesis of 5'-azido-TTGCAGC (ODN-A-a3)

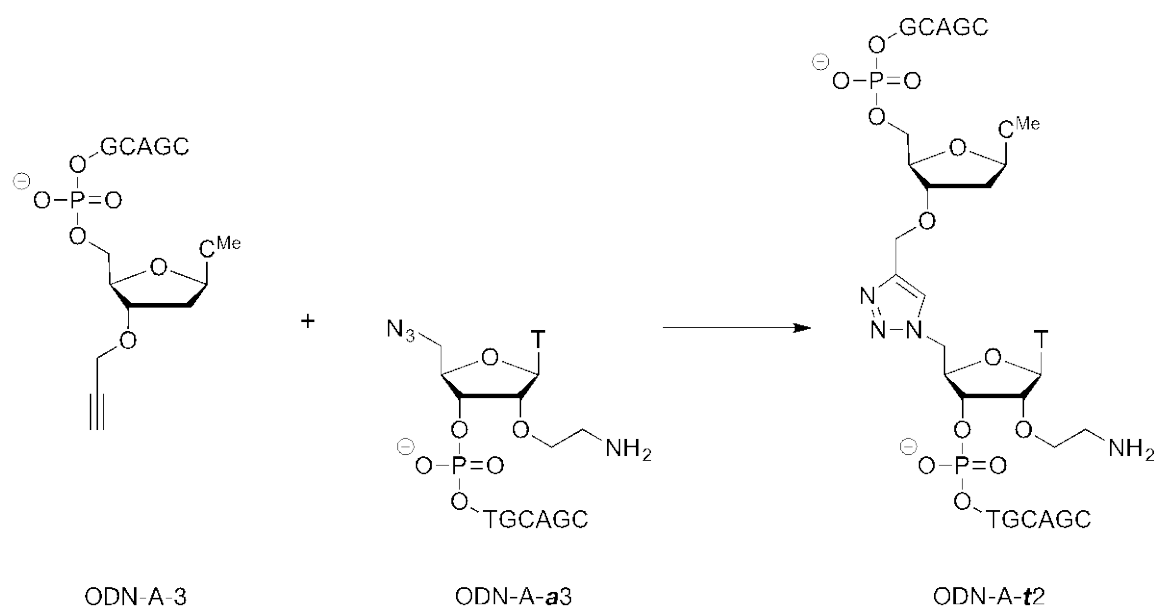


Protected oligomer ODN-A-a2 (1 μmol) attached to the CPG solid support placed in the synthesis column, was treated with 0.5 M solution of methyltriphenoxy phosphonium iodide (0.23 g, 0.5 mmol) in DMF (1 mL) which was periodically passed through the column via two 1 mL syringes over 15 minutes at room temperature. The column was washed with dry DMF four times. Sodium azide (0.03 g, 0.46 mmol) was suspended in dry DMF (1 mL) heated at 70 $^{\circ}\text{C}$ for 10 minutes and then cooled to room temperature. The saturated solution of NaN_3 in DMF was taken into 1 mL syringe and passed back and forth through column and left at room temperature overnight. After 14 hours, the column was washed with DMF and acetonitrile (x2) and dried by passing a stream of argon gas through it. The oligonucleotide was released from the solid support and deprotected by adding conc. aq. NH_3 and heated to 55 $^{\circ}\text{C}$ for 5 hours. The solution was evaporated and purified by RP-HPLC using a Gilson HPLC system with ABI Aquapore C8 column (8 mm x 250 mm, pore size 300 $\text{\AA}$). Oligonucleotides were purified using conditions 1. The ODN-

A-*a*3 product fractions from HPLC were evaporated without additional desalting.

[ES-, H₂O] found: 2181 Da, required: 2181 Da

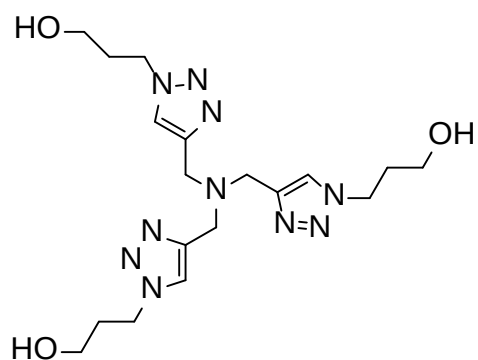
7.2.2.4 Synthesis of 5'-CGACGC-triazole-TTGCAGC-3' (ODN-A-*t*2)



Sodium ascorbate solution in H₂O (80 μ L, 0.5 M) was added to a solution of the HPTA ligand (12 mg) in H₂O (80 μ L) under argon followed by addition of CuSO₄ solution in H₂O (40 μ L, 0.1 M). The ODN-A-*a*3 (200 nmol) and the ODN-A-3 (200 nmol) were dissolved in the above solution. The reaction mixture was kept under the argon for 2 hours. NAP-10 gel filtration column was prepared and the crude material was filtered to remove the reagents. The ligated ODN-A-*t*2 was then purified by RP-HPLC using a Gilson HPLC system with ABI Aquapore C8 column (8 mm x 250 mm, pore size 300 Å) with a gradient of acetonitrile in ammonium acetate (0% to 50% buffer B over 20 min, flow rate 4 mL/min). The following elution buffers were used: buffer A (0.1 M ammonium acetate, pH 7.0), buffer B (0.1 M ammonium acetate, pH 7.0, with 50% acetonitrile). The fractions from HPLC were evaporated with additional desalting filtration using GE Healthcare illustra NAP-10 columns, giving desired oligonucleotide ODN-A-*t*2, with 45% yield.

[ES-, H₂O] found: 4010 Da, required: 4010 Da

*Ligand structure:



7.2.2.5 Synthesis and purification of phosphorothioate modified oligonucleotides containing triazole linkages and 2'-aminoethoxy groups

Oligonucleotides were synthesised following the general procedure with some alterations. The coupling time for 2'-OMe-RNA A, G, C and U monomers was set to 360 s however, the coupling times for the modified monomer T-T triazole dimer **1**, 2'-aminoethoxy T **2** and T-C triazole dimer **38** were extended to 720 s. After synthesis, oligonucleotides were kept on the solid support.

Modified oligonucleotides on resin were treated with a solution of diethylamine (10% in DMF) for 10 min in order to selectively remove the cyanoethyl protecting group. Next, oligonucleotides were cleaved from the solid support (CPG resin) and deprotected by suspending the resin in concentrated aqueous ammonia followed by heating in a sealed tube for 5 h at 55 °C.

Oligonucleotides were purified by RP-HPLC using a Gilson HPLC system with Gemini-NX 10u C18 column (250 mm x 21.2 mm, pore size 110 Å). The phosphorothioate oligonucleotides were purified using conditions 3. The fractions from HPLC were evaporated with additional desalting filtration using illustra NAP-10 and NAP-25 columns.

Table 7.4 List of phosphorothioate oligoribonucleotides synthesised and used in this study. ^aT = 2'-aminoethoxy T, *t* = triazole linkage.

code	sequence	calc. mass	found mass
ODN-D-1	CCTCUUACCUCAGUUACA	6096	6098
ODN-D- <i>t</i> 1	CC ^a T <i>t</i> CUUACCUCAGUUACA	6109	6111
ODN-D- <i>t</i> 2	CC ^a T <i>t</i> CUUACC ^a T <i>t</i> CAG ^a T <i>t</i> UACA	6106	6107
ODN-D- <i>t</i> 3	CC ^a T <i>t</i> C ^a T <i>t</i> UACC ^a T <i>t</i> CAG ^a T <i>t</i> UACA	6118	6118
ODN-D- <i>a</i> 1	CCUCUUACC ^a TCAGUUACA	6140	6141
ODN-D- <i>a</i> 2	CCUC ^a TUACCUCAG ^a TUACA	6183	6184
ODN-D- <i>a</i> 3	CCUC ^a TUACC ^a TCAG ^a TUACA	6226	6227
ODN-E-1	GCUATTACCUUAACCCAG	6160	6161
ODN-E- <i>t</i> 1	GCUA ^a T <i>t</i> TACCUUAACCCAG	6173	6173
ODN-E- <i>t</i> 2	GCUA ^a T <i>t</i> TACC ^a T <i>t</i> TAACCCAG	6185	6186

7.3 POST-SYNTHETIC OLIGONUCLEOTIDES FUNCTIONALISATION

7.3.1 General conditions: post-synthetic modified oligonucleotides functionalisation with pyrene **30** and **31**

Modified oligonucleotides with the 2'-aminoethoxy group were dissolved in 0.5 M NaHCO₃/Na₂CO₃ buffer at pH 8.75 (0.5 mM). 1-Pyrenebutyric acid *N*-hydroxysuccinimide ester **30** or 1-pyrenecarboxylic acid *N*-hydroxysuccinimide ester **31** (~ 100 equivalents) was dissolved in DMF or DMSO in 1:1 ratio to the amount of buffer and added to the oligonucleotide solution. The reaction mixture was then heated at 55 °C for 5 h. The crude mixture was desalted using NAP-25 column and purified by RP-HPLC using a Gilson HPLC system with ABI Aquapore C8 column (8 mm x 250 mm, pore size 300 Å). Oligonucleotides were purified using conditions 2. The fractions from HPLC were evaporated without additional desalting.

Table 7.5 List of phosphodiester and phosphorothioate oligonucleotides functionalised with pyrene (30) and (31), synthesised and used in this study. a^PT = 2'-aminoethoxy T labelled with pyrene (30), $a^{SP}T$ = 2'-aminoethoxy T labelled with short pyrene (31), t = triazole linkage, ^{Me}C = 5-methyl C.

code	sequence	calc. mass	found mass
ODN-A- $t1$ -P	CGACGC a^PT t TGCAGC	4266	4266
ODN-A- $t2$ -P	CGACG ^{Me}C t^{a^PT} TGCAGC	4279	4279
ODN-A- $a1$ -P	CGACGC a^PT TTGCAGC	4265	4266
ODN-B- $t1$ -P	CG a^PT t TCGCTTGCAGC	4561	4561
ODN-B- $t2$ -P	CGTTCGC a^PT t TGCAGC	4561	4561
ODN-B- $t3$ -P	CG a^PT t TCGC a^PT t TGCAGC	4890	4891
ODN-B- $a1$ -P	CGTTCGC a^PT TTGCAGC	4560	4560
ODN-B- $a2$ -P	CG a^PT TTTCGC a^PT TTGCAGC	4890	4889
ODN-C- $a1$ -P	CG a^PT TTTCGC a^PT TTGCA a^PT TGC	5522	5523
ODN-D- $t1$ -P	CC a^PT t CUUACCUCAGUUACA	6379	6381
ODN-D- $t2$ -P	CC a^PT t CUUACC a^PT t CAG a^PT t UACA	6916	6918
ODN-D- $t3$ -P	CC a^PT t C a^PT t UACC a^PT t CAG a^PT t UACA	7198	7200
ODN-D- $a1$ -P	CCUCUUACC a^PT TCAGUUACA	6410	6412
ODN-D- $a2$ -P	CCUC a^PT TUACCUCAG a^PT TUACA	6723	6725
ODN-D- $a3$ -P	CCUC a^PT TUACC a^PT TCAG a^PT TUACA	7037	7039
ODN-E- $t1$ -P	GCUA a^PT t TACCUUAACCCAG	6443	6444
ODN-E- $t2$ -P	GCUA a^PT t TACC a^PT t TAACCCAG	6725	6726
ODN-A- $t2$ -SP	CGACG ^{Me}C $t^{a^{SP}}$ TTGCAGC	4279	4280

7.3.2 General conditions: post-synthetic modified oligonucleotides functionalisation with anthraquinone derivative 35

Modified oligonucleotides with the 2'-aminoethoxy group were dissolved in 0.5 M NaHCO₃/Na₂CO₃ buffer at pH 8.75 (0.5 mM). Anthraquinone carbonate **35** (~ 100 equivalents) was dissolved in DMF or DMSO in 1:1 ratio to the

amount of buffer and added to the oligonucleotide solution. The reaction mixture was then heated at 55 °C for 5 h. The crude mixture was desalted using NAP-25 column and purified by RP-HPLC using a Gilson HPLC system with ABI Aquapore C8 column (8 mm x 250 mm, pore size 300 Å). Oligonucleotides were purified using conditions 2. The fractions from HPLC were evaporated without additional desalting

Table 7.6 List of phosphodiester and phosphorothioate oligonucleotides functionalised with anthraquinone (35), synthesised and used in this study. ^aA^T = 2'-aminoethoxy T labelled with anthraquinone (35), *t* = triazole linkage, ^{Me}C = 5-methyl C.

code	sequence	calc. mass	found mass
ODN-A- <i>t</i> 1-A	CGACGC ^a AT ^t TGCAGC	4374	4375
ODN-A- <i>a</i> 1-A	CGACGC ^a ATTGCAGC	4373	4374
ODN-B- <i>t</i> 1-A	CG ^a AT ^t TCGCTTGCAGC	4669	4668
ODN-B- <i>t</i> 2-A	CGTTCGC ^a AT ^t TGCAGC	4669	4668
ODN-B- <i>t</i> 3-A	CG ^a AT ^t TCGC ^a AT ^t TGCAGC	5106	5106
ODN-C- <i>a</i> 1-A	CG ^a ATTTCGC ^a ATTGCA ^a ATGC	5846	5847
ODN-D- <i>t</i> 1-A	CC ^a AT ^t CUUACCUCAGUUACA	6487	6489
ODN-D- <i>t</i> 2-A	CC ^a AT ^t CUUACC ^a AT ^t CAG ^a AT ^t UACA	7240	7242
ODN-D- <i>t</i> 3-A	CC ^a AT ^t C ^a AT ^t UACC ^a AT ^t CAG ^a AT ^t UACA	7630	7632
ODN-D- <i>a</i> 1-A	CCUCUUACC ^a ATCAGUUACA	6519	6520
ODN-D- <i>a</i> 2-A	CCUC ^a ATUACCUCAG ^a ATUACA	6941	6942
ODN-D- <i>a</i> 3-A	CCUC ^a ATUACC ^a ATCAG ^a ATUACA	7363	7364

7.3.3 General conditions: post-synthetic modified oligonucleotides functionalisation with guanidine

Modified oligonucleotide was dissolved in H₂O (0.5 mM). 1*H*-pyrazole-1-carboxamide hydrochloride (PCH) reagent (~ 300 equivalents) and imidazole (~ 10 equivalents) were added to the oligonucleotide solution. The reaction

mixture was then heated at 55 °C for 5 h. The crude mixture was desalted using NAP-25 column and purified by RP-HPLC using a Gilson HPLC system with ABI Aquapore C8 column (8 mm x 250 mm, pore size 300 Å). Oligonucleotides were purified using conditions 1. The fractions from HPLC were evaporated without additional desalting.

Table 7.7 List of phosphodiester and phosphorothioate oligonucleotides functionalised with guanidine, synthesised and used in this study. ^aG_T = 2'-aminoethoxy T labelled with guanidine, *t* = triazole linkage, ^{Me}C = 5-methyl C.

code	sequence	calc. mass	found mass
ODN-A- <i>t</i> 1-G	CGACGC ^a G _T <i>t</i> TGCAGC	4039	4038
ODN-A- <i>t</i> 2-G	CGACG ^{Me} C ^a G _T <i>t</i> TTGCAGC	4053	4052
ODN-A- <i>a</i> 1-G	CGACGC ^a G _T TTGCAGC	4038	4037
ODN-B- <i>t</i> 1-G	CG ^a G _T <i>t</i> TCGCTTGCAGC	4334	4333
ODN-B- <i>t</i> 2-G	CGTTTCGC ^a G _T <i>t</i> TGCAGC	4334	4333
ODN-B- <i>t</i> 3-G	CG ^a G _T <i>t</i> TCGC ^a G _T <i>t</i> TGCAGC	4436	4435
ODN-E- <i>t</i> 1-G	GCUA ^a G _T <i>t</i> TACCUUAACCCAG	6216	6216
ODN-E- <i>t</i> 2-G	GCUA ^a G _T <i>t</i> TACC ^a G _T <i>t</i> TAACCCAG	6271	6270

7.3.4 General conditions: post-synthetic modified oligonucleotides functionalisation with pyrene aldehyde **30** via reductive amination

Modified oligonucleotide was dissolved in 10 mM phosphate buffer, 200 mM NaCl at pH 7.2 (0.2 mM). 1-Pyrenebutyl aldehyde **37** (~ 50 equivalents) was dissolved in DMF to 0.03 M concentration and was added to the oligonucleotide solution. Sodium cyanoborohydride (~ 400 equivalents) was dissolved in H₂O to 1.0 M concentration and was added to the reaction mixture. The reaction mixture was then heated at 55 °C for 5 h. The crude mixture was desalted using NAP-25 column and purified by RP-HPLC using a Gilson HPLC system with ABI Aquapore C8 column (8 mm x 250 mm, pore

size 300 Å). Oligonucleotides were purified using conditions 2. The fractions from HPLC were evaporated without additional desalting. Reaction resulted in two products with single and double addition of pyrene to the amine.

Table 7.8 List of phosphodiester and phosphorothioate oligonucleotides functionalised with pyrene *via* amine linker, synthesised and used in this study. *aNH-P*T = 2'-aminoethoxy T labelled with single pyrene (37), *aNH-2P*T = 2'-aminoethoxy T labelled with double pyrene (37).

code	sequence	calc. mass	found mass
ODN-A- <i>a</i> 1-NH-P	CGACGC <i>aNH-P</i> TTGCAGC	4251	4267
ODN-A- <i>a</i> 1-NH-2P	CGACGC <i>aNH-2P</i> TTGCAGC	4507	4506

7.4 UV MELTING STUDIES

UV melting analysis was monitored by optical absorption at 260 nm using a Varian Cary 4000 Scan UV-Visible spectrophotometer. The solutions had a total duplex concentration from 2 to 4 µM, with 10 mM phosphate buffer, 200 mM NaCl at pH 7.2. The optical path length was 1 cm. The temperature was in a range between 20 and 85 °C and was changing at rate 1 °C/min. T_m values were calculated using Cary Win UV thermal application software and averaged from six to ten successive T_m values.

7.5 CIRCULAR DICHROISM

Circular dichroism spectra were recorded on a Jasco J-720 spectropolarimeter. Samples were made to 4 µM oligonucleotide concentration in 10 mM phosphate buffer, 200 mM NaCl at pH 7.0 Spectra were recorded between

200-320 nm at a rate of 100 nm/min, with step resolution of 0.2 nm, bandwidth of 1.0 nm and sensitivity of 50 mdeg. Three successive spectra were recorded and an average was taken. A blank (buffer) baseline was subtracted from each spectrum. Spectra were smoothed using Savitzky-Golay filter 7.

7.6 CELL BIOLOGY

7.6.1 General conditions

The HeLa pLuc/705 cells were a courtesy of Prof M. Wood, Oxford University and the luciferase assay experiments were performed with assistance of Dr G. McClorey.

The luciferase assay experiments using MCF7 cells were performed by Susan Merrihew at GlaxoSmithKline.

All reagents were purchased from Invitrogen, ThermoFisher and Promega, unless stated differently.

7.6.2 Compound treatment of HeLa p/Luc 705 cells

HeLa 705 cells were grown in T-75 flask containing 10 mL of DMEM growth media containing 10% FCS, incubated at 37 °C, 5% CO₂. The cells were lysed and then seeded in forty-eight well plates in 250 µL/well, at a density of 30,000 cells/well and incubated for 24 h at 37 °C, 5% CO₂ to allow cells to adhere. After 24 h transfection mixture was prepared by addition of oligonucleotide with Lipofectamine 2000 (ThermoFisher) in a 1:1 wt:wt ratio in OptiMEM, which was incubated for 20 min in room temperature before cell treatment. Cells were treated in duplicates with the transfection mixture at seven oligonucleotides concentrations: 7.81, 15.6, 31.3, 62.5, 93.75, 125 and

180 nM, and each well was filled with OptiMEM to 250 μ L. The cells were incubated for 4 h at 37 °C, 5% CO₂. After 4 h OptiMEM was changed with 250 μ L of DMEM growth media containing 10% FCS and was incubated for additional 18 h at 37 °C, 5% CO₂.

7.6.3 Compound treatment of MCF7 cells

MCF7 cells (BD BioCat™ 117994) were grown in T 225cm² flasks without collagen coating (BD Falcon 353138) with RPMI 1640 media (Invitrogen) containing 10% FBS, 1% GlutaMAX and 1% sodium pyruvate. Cells were split twice in 4 days distance using 0.25% Trypsin.

For the plasmid transduction cells were harvested using 0.25% Trypsin and re-suspended in antibiotic-free media. Cells were counted and viability was determined using ViCell.

MCF7 cells were mixed with 15% BacMam (C654T-B-globin intron 2/luciferase pHTBV1) and diluted to 300,000 cells/mL with growth media. 100 μ L/well of cell transduction slurry was dispensed to ninety-six well collagen-coated white plates (BD BioCoat) to yield a plating density of 30.000 cells/well. Plates were then centrifuged at 1000 rpm for 1 h and then placed in the incubator for 24 h at 37 °C, 5% CO₂ prior to treatment with oligonucleotides.

After 24 h media containing BacMam was aspirated from MCF7 cells and replaced with 100 μ L/well of growth media.

For transfected delivery experiments the transfection mixture was prepared by addition of oligonucleotide with Lipofectamine 2000 in a 1:1 wt:wt ratio in OptiMEM, which was incubated for 20 min in room temperature before the cell treatment. Cells were treated in duplicates with the transfection mixture at three oligonucleotide concentrations: 2.4, 7.4, 22.2 nM, and each well was filled with antibiotic-free cell culture medium to 100 μ L.

For gymnotic delivery experiments cells were treated with oligonucleotides without transfecting agent at three concentrations: 0.2, 5, 45 μ M.

All plates were incubated for 24 h at 37 °C, 5% CO₂.

7.6.4 Luciferase activity

Bright-Glo™ Luciferase Assay System (Promega) was used for the Hela 705 cells experiment. The assay was performed according to the manufacturer's instructions and analysed using a 1420 Victor plate reader. Briefly, the cells were washed with PBS and lysed using Glo Lysis Buffer (Promega) and gently agitated for 5 min. This cell lysate (50 μ L) was then pipetted to a black 96 well plate, followed by addition of the luciferase assay reagent (50 μ L) and mixed gently for 2 min. The luminescence generated was recorded over 1 seconds.

Steady-Glo® Luciferase Assay System (Promega) was used for the MCF7 cells experiment. The assay was performed according to the manufacturer's instructions using a Synergy H4 plate reader. Briefly, 50 μ L of the luciferase reagent was added to each well and plates were incubated for 10 min at room temperature. The luminescence generated was recorded over 1 seconds. Statistical analysis was performed using the nonlinear curve fitting using the equation:

$$y = \text{Bottom} + (\text{X}^{\text{HillSlope}}) * (\text{Top} - \text{Bottom}) / (\text{X}^{\text{HillSlope}} + \text{EC50}^{\text{HillSlope}});$$

(where X: dose, Y: response, increasing as X increases, Top and Bottom: plateaus in same units as Y, ES50: same concentration units as Y, HillSlope: slope factor, unitless) in GraphPad.

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ABBREVIATIONS

A	adenine
aq.	aqueous
AS-ON	antisense oligonucleotides
br	broad
bp	base pair
Bz	benzoyl
calc.	calculated
CME	clathrin-mediated endocytosis
conc.	concentrated
CPG	Controlled Pore Glass
CuAAC	Copper-Azide-Alkyne Cycloaddition
C	cytidine
°C	Degree Celsius
d	doublet (NMR)
dd	doublet of doublet (NMR)
dt	double triplet (NMR)
DCM	dichloromethane
DCE	1,2-dichloroethane
DIPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	<i>N,N'</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DMT	dimethoxytrityl
DMT-Cl	4,4'-dimethoxytrityl chloride
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
eq.	equivalent

ES	electrospray
ESI	electrospray ionization
EtOAc	ethyl acetate
Et ₂ O	diethyl ether
EtOH	ethanol
Fmoc	9-fluorenylmethyl
G	guanine
g	gram
H-bond	hydrogen bond
HPLC	high performance liquid chromatography
HRMS	High Resolution Mass Spectrometry
Hz	hertz
iPrOH	isopropanol
LNA	locked nucleic acids
LRMS	Low Resolution Mass Spectrometry
M	molar concentration
m	multiplet (NMR)
mg	milligram
mL	milliliter
mm	millimetre
MeC	5-methylcytidine
MeCN	acetonitrile
MeOH	methanol
MF	morpholino oligonucleotides
mRNA	messenger RNA
MS	mass spectrometry
Ms	mesyl group
M _w	molecular weight

m/z	mass/charge
NHS	<i>N</i> -hydroxysuccinimide
nm	nanometer
nM	nanomolar concentration
μM	micromolar concentration
NMR	Nuclear Magnetic Resonance
NP	N3'-P5'-phosphoroamidates
ODN	oligodeoxyribonucleotides
ORN	oligoribonucleotides
PCR	polyacrylamide gel electrophoresis
pH	potential of hydrogen
PNA	peptide nucleic acids
ppm	parts per million (NMR)
PPh ₃	triphenylphosphine
PS	phosphorothioate
q	quartet (NMR)
rt	room temperature
RNA	ribonucleic acids
s	singlet (NMR)
T	thymine
t	triplet (NMR)
TBAF	tetrabutylammonium fluoride
TBDMS	<i>tert</i> -butyldimethylsilyl
TCA	trichloroacetic acid
THF	tetrahydrofuran
td	triple doublet (NMR)
TEA	triethylamine
TLC	Thin Layer Chromatography
U	uridine

UV

| ultraviolet

9 APPENDIX

¹H NMR (400 MHz, DMSO-*d*₆)

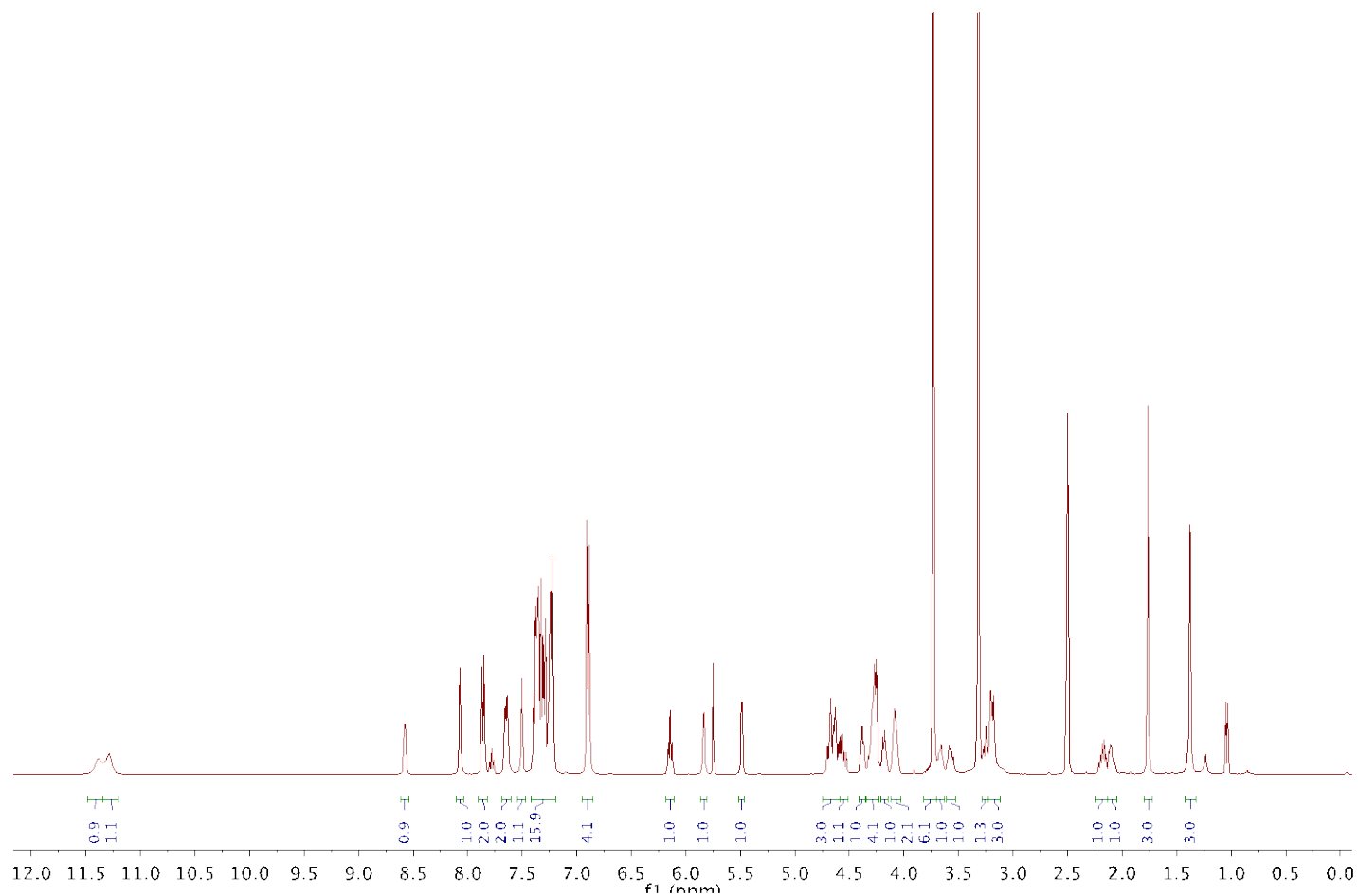


Figure 9.1 ¹H NMR spectrum of T-T triazole dimer (17.)

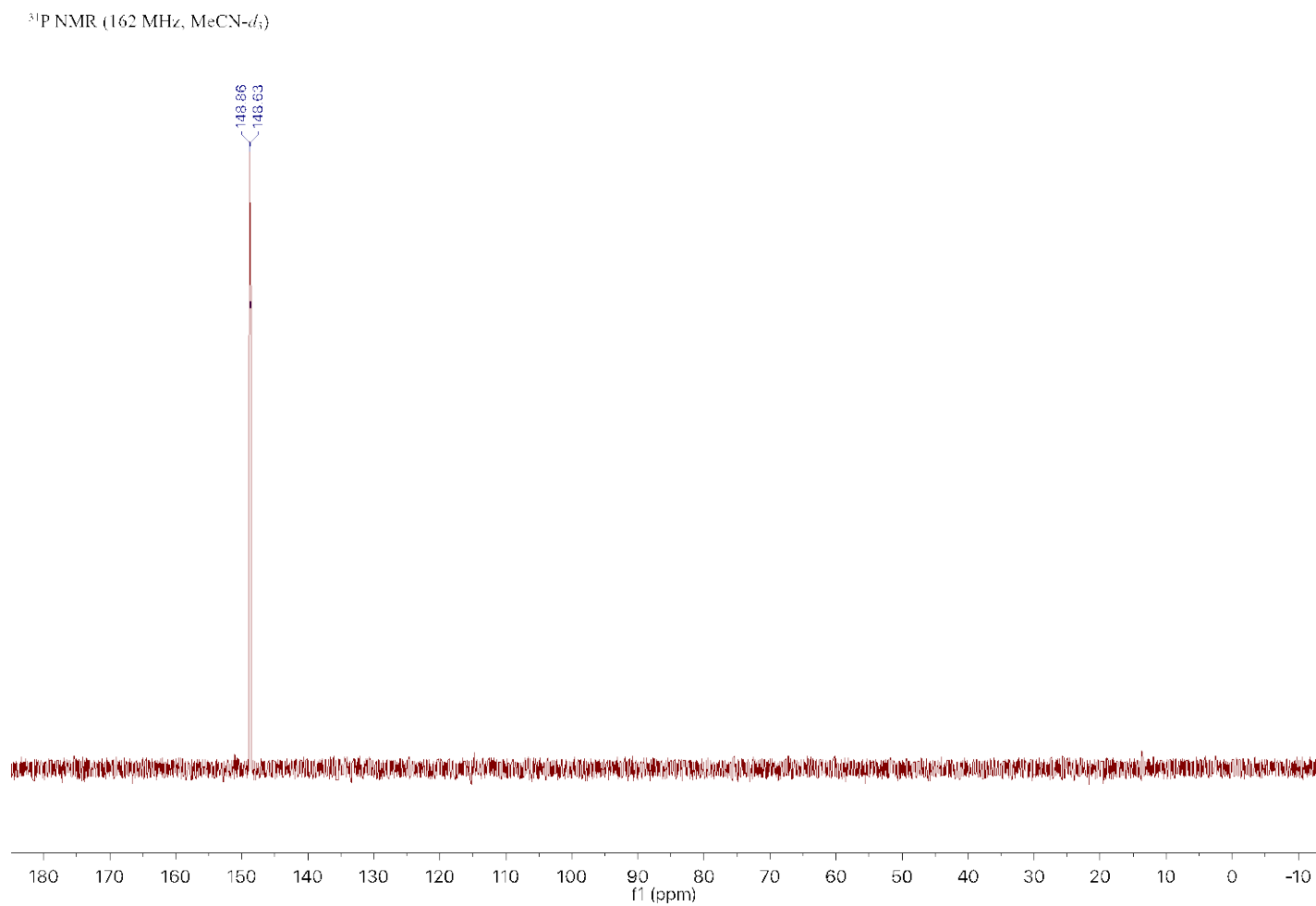


Figure 9.2 ^{31}P NMR spectrum of T-T triazole dimer phosphoramidite (1).

Figure 9.3 ^1H NMR spectrum of T-C triazole dimer (37).

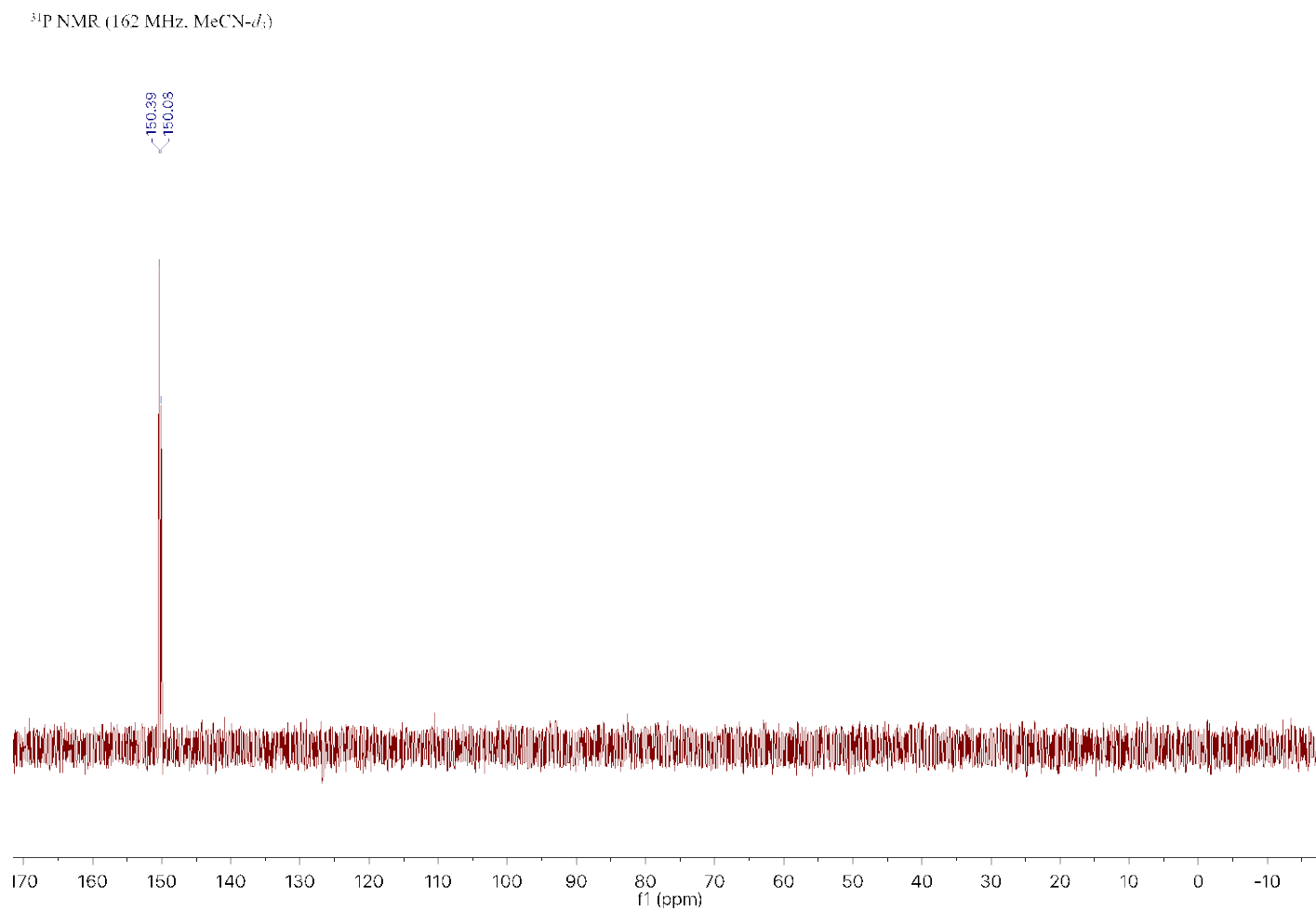


Figure 9.4 ^{31}P NMR spectrum of T-C triazole dimer phosphoramidite (38).

UV MELTING CURVES

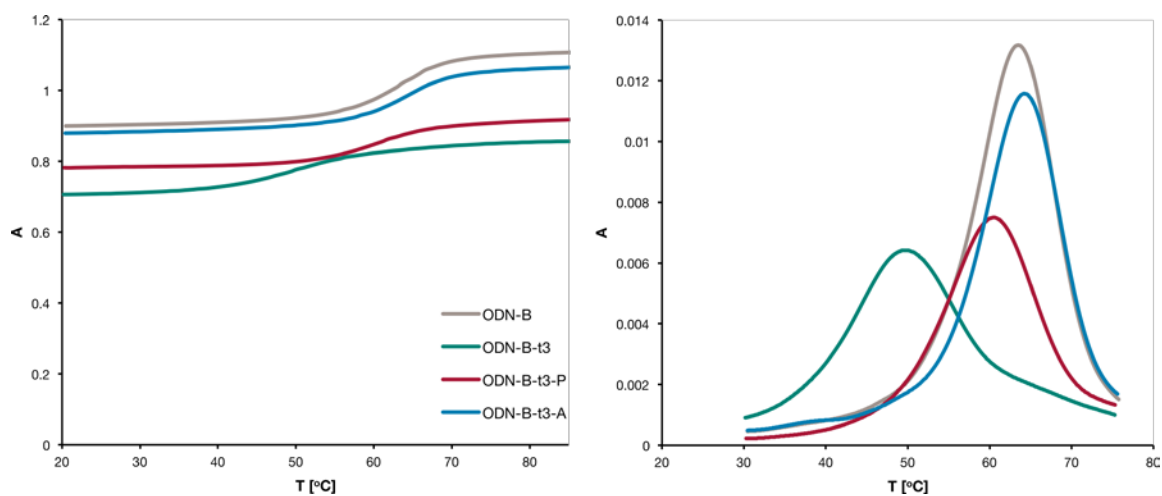


Figure 9.5 UV melting curves and derivative of ODN-B (grey), ODN-B-t3 (green), ODN-B-t3-P (red), ODN-B-t3-A (blue) with complementary RNA. The experiments were carried out in 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 4 μM .

CD SPECTRA

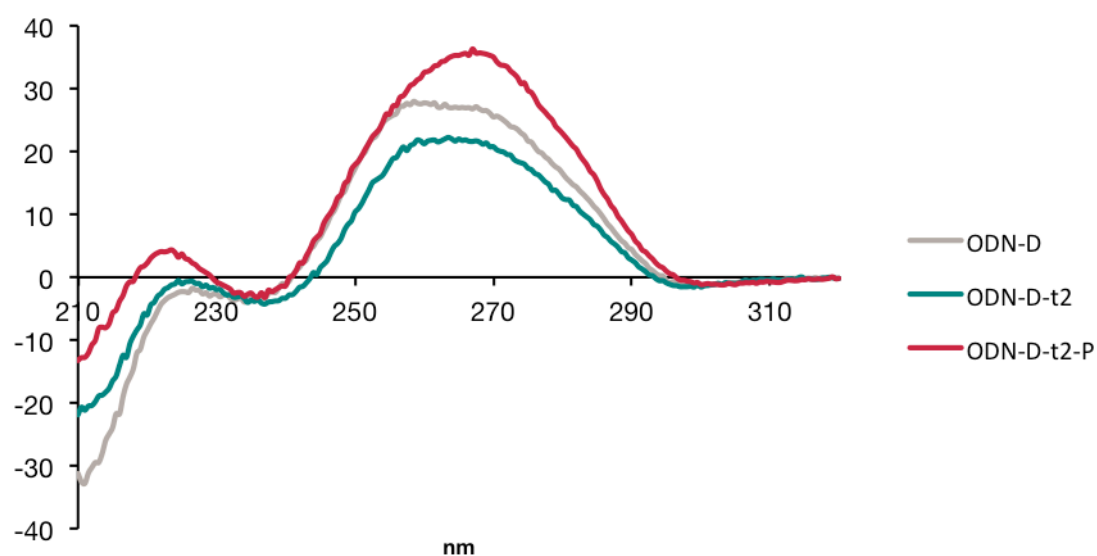


Figure 9.6 CD spectra of ODN-D (grey), ODN-D-t2 (green) and ODN-D-t2-P (red) with complementary RNA. The experiments were carried out in 10 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, 200 nM NaCl buffer, pH 7.2 and oligonucleotide strand concentration = 5 μM .