

Substrate Analogue Studies on Cephalosporin Biosynthesis Enzymes



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*When I heard the learn'd astronomer,
When the proofs, the figures, were ranged in columns before me,
When I was shown the charts and diagrams, to add, divide, and measure them,
When I sitting heard the astronomer where he lectured with much applause in the lecture-room,
How soon unaccountable I became tired and sick,
Till rising and gliding out I wander'd off by myself,
In the mystical moist night-air, and from time to time,
Look'd up in perfect silence at the stars.*

'When I Heard the Learn'd Astronomer', Walt Whitman

**To my
Mother, Father
and
MLW**

Abstract

The application of β -lactams as chemotherapeutic agents in the 1940s fundamentally changed the course of medicine. Some 80 years later, β -lactam antibiotics still have a central role in modern medicine. The biosynthesis of β -lactams is arguably as remarkable as their applications. The research described in this thesis focussed on the first two steps in the biosynthesis of cephalosporins from penicillin N; the ring-expansion of penicillin N to deacetoxycephalosporin C (DAOC) and the subsequent 3'-hydroxylation of DAOC to deacetylcephalosporin C (DAC). These two steps are catalysed by a single enzyme (DAOCS/DACS) in eukaryotic fungi (*A. chrysogenum*), and by two distinct enzymes (DAOCS and DACS) in prokaryotes (*S. clavuligerus*). These three enzymes are notable both from mechanistic and medicinal perspectives.

Chapter 2 describes updated expression protocols for the production of recombinant DAOCS/DACS; the DAOCS/DACS gene (*cefEF*) has historically proved recalcitrant to soluble expression in *E. coli*. Through the application of modern recombinant expression techniques, such as codon-optimisation and the use of a pCold-derived expression vector, highly active DAOCS/DACS was afforded in pleasing yield and purity. DAOCS/DACS was found to be active under simplified assay conditions compared with previous studies, and was stable after several freeze-thaw cycles. These modified expression conditions for *cefEF* provided a resilient stock of DAOCS/DACS with which mechanistic inquiries could be directed. Procedures for producing recombinant DAOCS and DACS were also developed.

Chapter 3 describes investigations into the hydroxylase activity of DAOCS/DACS using the 3-vinyl cephem substrate analogue. Incubations of the 3-vinyl cephem with DAOCS/DACS produced a diol under standard assay conditions. When the incubations were carried out with addition of 5 mM sodium azide, the product was a cephem hydroxy azide which was isolated by HPLC and robustly characterised by LC-MS and NMR. It is proposed that azide insertion is catalysed by DAOCS/DACS, in which case this would be the first reported enzyme-catalysed incorporation of the azide functional group into the cephalosporin scaffold. DACS was also shown to catalyse the azidation reaction. Derivatisation of the azide functional group may be a route to new cephalosporin antibiotics. More generally, DAOCS/DACS-catalysed azidation suggests that the ability of 2OG oxygenases to catalyse azidation is more widespread than previously thought; the insertion of the azide group into other 2OG oxygenase substrates (*e.g.*, lipids and proteins) should be investigated. In Chapter 4, attempts to crystallise DAOCS/DACS, as well as a mutant of DAOCS (ICM DAOCS) are described. A new co-crystallisation method, coupled with seeding experiments, produced encouraging crystalline precipitates with DAOCS/DACS and ICM DAOCS. Further optimisation is required to yield macrocrystals capable of diffraction. This work provides a foundation for the structural characterisation of DAOCS/DACS, DAOCS, and DACS. Investigations into the 3'-hydroxylation of DAOC to DAC by ^{18}O -labelling experiments are also described in Chapter 4. The results confirm previous observations of incorporation of oxygen from water as well as from O_2 into the hydroxyl group of DAC. By contrast, conversion of the 3-vinyl cephem analogue to give the hydroxy azide products proceeds without incorporation of water derived oxygen; in the case of the diol, one oxygen comes from water and one from O_2 .

Overall, as described in Chapter 5, the work furthers the mechanistic understanding of the hydroxylase activities of DAOCS/DACS and DACS. The results imply they catalyse oxidative transformations by at least two distinct mechanisms, depending on the structure of substrate being converted. It is hypothesised that a HO-Fe(IV)=O species, with the hydroxyl ligand derived from a water molecule, could account for the observed partial incorporation of oxygen from water into some, but not all products. Such an intermediate may be involved in catalysis in other 2OG oxygenases, including those involved in clavam biosynthesis and certain human oxygenases.

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Declaration and contribution list

I declare that the work discussed and the figures presented in this thesis are entirely my own, unless a reference to a person or a published source has been made, with the exception of the following work:

Chapter 2:

The modified pCold I vector used to expression DAOCS/DACS and DACS was a gift from Phillip Zamore (Addgene plasmid #41096) and was kindly donated by Dr Patrick Rabe. The HRV3C protease were kindly gifted by Dr Patrick Rabe.

Chapter 3:

The 3-vinyl cephem G **(31)**, the exomethylene cepham G **(32)**, the epoxide analogue **(34)**, and DAOG were synthesised by Dorian Muntean.

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Abbreviations

2OG	2-Oxoglutarate
6-APA	6-Aminopenicillanic acid
7-ACA	7-Aminocephalosporonic acid
7-ADCA	7-Aminodeacetoxycephalosporin C
7-AVCA	7-Amino-3-vinylcephalosporin C
Amp	Ampicillin
BLI	β -Lactamase inhibitor
Boc	t-Butyloxycarbonyl protecting group
BSA	Bovine serum albumin
<i>C. acremonium</i>	<i>Cephalosporin (C.) acremonium</i> now <i>Acremonium (A.) chrysogenum</i>
COSY	Correlated spectroscopy
CPMG	Carr-Purcell-Meiboom-Gill pulse train
cspA	Cold-shock protein A
DAC	Deacetylcephalosporin C
DACS	Deacetylcephalosporin C synthase
DAG	Deacetylcephalosporin G (DAC but with a benzylic side chain)
DAOC	Deacetoxycephalosporin C
DAOCS	Deacetoxycephalosporin C synthase
DAOCS/DACS	Deacetoxycephalosporin C synthase/Deacetylcephalosporin C synthase
DAOG	Deacetoxycephalosporin G (DAOC but with a benzylic side chain)
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Dpm	Diphenylmethyl protecting group
DSBH	Double-stranded β -helix
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia Coli</i>
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
FIH	Factor inhibiting HIF-1 α (hydroxylase enzyme)
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HMBC	Heteronuclear multiple bond correlations
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
IEX	Ion-exchange chromatography
IMAC	Immobilised metal affinity chromatography
IPNS	Isopenicillin N synthase
IPTG	Isopropyl- β -D-1-thiogalactopyranoside
Kan	Kanamycin
L,L,D-ACV	L-amino adipoyl-D-cysteine-L-valine
LC-MS	Liquid chromatography-mass spectrometry
LIC	Ligation-independent cloning
MEW	Mark Elliott Wood
MPD	2-Methyl-2,4-pentanediol (hexylene glycol)
MQ	Milli-Q [®] pure water
MW	Molecular weight
MWCO	Molecular weight cut-off
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser effect
PCR	Polymerase chain reaction

PMB	<i>p</i> -Methoxybenzyl protecting group
PNB	<i>p</i> -Nitrobenzyl protecting group
RNAP	RNA Polymerase
SDS PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
SEC	Size-exclusion chromatography
TauD	Taurine dioxygenase
<i>t</i> BuOAc	<i>t</i> -Butyl acetate
TEA	Triethylamine
TFA	Trifluoroacetic acid
TfdA	2,4-Dichlorophenoxyacetate dioxygenase
TLC	Thin layer chromatography
t_R	Retention time
Tris	Tris(hydroxymethyl)aminomethane
Tris- d_{11}	Tris(hydroxymethyl- d_3)amino- d_2 -methane
TsOH	<i>p</i> -Toluenesulfonic acid monohydrate
v/v	volume / volume
w/v	weight / volume

Chapter 1

Introduction

1 Introduction

1.1 The discovery of penicillins and cephalosporins

“It [penicillin broth] was used in a few cases as a local antiseptic but although it gave reasonably good results, the trouble making it seemed not worthwhile.”¹

On a late September's day in 1928, Alexander Fleming was inspecting agar plates containing *Staphylococci* colonies which were contaminated with a mould. In the vicinity of the mould, Fleming noticed that the *Staphylococci* colonies were vanishing. He made the prescient observation that the fungus, later identified as *Penicillium notatum*, was secreting a “bacteriolytic substance which spread[s] through the agar and inhibits bacterial growth”.^{2, 3} Fleming cultivated *P. notatum* on surface cultures and demonstrated that the fungus released an antibacterial substance into the liquid broth that was effective against several gram-positive pathogens.³ Despite several attempts by Fleming and others the active material, which Fleming called penicillin, was unstable and degraded readily upon acidified extraction into organic solvent.⁴ Fleming viewed this instability as a major barrier to penicillin's utility, and he envisioned only a limited application for it as a local antiseptic. Seemingly, the potential of penicillin as a chemotherapeutic drug was not readily apparent to him.⁵

The eventual purification of penicillin was achieved by Florey, Chain and co-workers in Oxford.¹ Their demonstration of the therapeutic properties of penicillin, first in mice⁶ and then humans,⁷ stimulated a concerted effort to mass-produce penicillin for use during the Second World War. Work in the United States produced higher-yielding strains of *Penicillium* and lead to the discovery that penicillins with different hydrophobic side chain could be produced by supplementing cultures with the appropriate side-chain precursors.^{8,9} In 1945, crystal structures of the potassium and sodium salts of benzylpenicillin obtained by Dorothy Hodgkin and Barbara Rogers-Low established the 4-membered β -lactam structure.¹⁰ Further developments, such as the production

of 6-aminopenicillanic acid (6-APA) through either the cultivation of *P. chrysogenum* in the absence of side-chain precursors¹¹ or through enzymatic deacylation of benzylpenicillin,¹² paved the way for the development of semi-synthetic penicillins with improved antibacterial properties.

Inspired by work on penicillins, Giuseppe Brotzu began a search for antibiotic producing microorganisms in July 1945. Near a sewage outlet into the sea, Brotzu isolated a fungus resembling *Cephalosporium acremonium* (now *Acremonium chrysogenum*), the secretions of which possessed antimicrobial activity against a range of gram-positive and gram-negative bacteria on agar.^{13, 14} Brotzu cultivated the fungus, but he could not isolate the active substance from culture filtrates. Through a mutual friend, Brotzu contacted Florey at Oxford who agreed to help isolate the active material using techniques refined during his work on penicillins.⁵ Work in Oxford led to the isolation of a hydrophilic antibacterial substance, initially called cephalosporin N, from *C. acremonium* (now *A. chrysogenum*) cultures that accounted for the activity observed by Brotzu. Further investigations of cephalosporin N revealed it to be in fact a penicillin containing a δ -(D- α -aminoadipoyl) side chain. It was subsequently renamed as penicillin N.¹⁵

During the characterisation of penicillin N, a second antibacterial substance, cephalosporin C, was isolated. Cephalosporin C possessed very low antibacterial activity, such that it was not detected during initial antibacterial assays with *Cephalosporium* culture filtrates. It was only because of its concentration during the purification of penicillin N that cephalosporin C was observed at all. Like penicillin N, cephalosporin C contained a δ -(D- α -aminoadipoyl) side chain but, unlike penicillin, it was resistant to hydrolysis by penicillinases. The structural basis of this discrepancy was revealed by an X-ray crystal structure of cephalosporin C, which established the [4,6]-bicyclic ring structure of cephalosporins as distinct from the [4,5]-ring structure of penicillins.¹⁶ The chemical deacylation of cephalosporin C afforded the cephalosporin nucleus (7-aminocephalosporinic acid, 7-ACA),¹⁷ from which multiple semi-synthetic cephalosporins were synthesised, some of which showed improved antibacterial properties compared to cephalosporin C.¹⁸ Derivatisation of the C β '

acetoxymethyl group of cephalosporin C with a range of functional groups further improved antibacterial activity.⁸

1.2 Ferrous iron and 2OG dependent oxygenases: An overview

Whilst oxidative reactions that utilise molecular dioxygen are thermodynamically favoured, there are kinetic barriers that cause them to proceed slowly.¹⁹ Nature has evolved several means of overcoming this kinetic barrier using transition metal centres (iron or copper)²⁰⁻²³ or cofactors (flavins).²⁴ One such superfamily of enzymes are the 2-oxoglutarate-dependent (2OG) oxygenases, which utilise ferrous iron as a cofactor and 2OG (α -ketoglutarate) and O_2 as cosubstrates. There is extensive evidence that, at least many, 2OG oxygenases catalyse oxidations via a reactive ferryl ($Fe(IV)=O$) species that is produced by the oxidative decarboxylation of 2OG in a process that normally also forms succinate and CO_2 .^{25, 26} A diverse range of oxidative transformations are facilitated by this $Fe(IV)=O$ species present within 2OG oxygenase scaffolds, with substrate and intermediate conformations dictating specific reaction outcomes in a manner that is currently only possible within macromolecular protein structures. The most common reaction catalysed by 2OG oxygenases is hydroxylation.^{25, 27} 2OG oxygenases also perform: epimerisation, epoxidation, halogenation, and ring expansion reactions, amongst many others. The substrates for 2OG oxygenases are equally as diverse as their chemistry, with both oligomeric (proteins and lipids) and small molecules accepted as substrates.²⁸ Atypical reactions of 2OG oxygenases are classically performed on small molecules during the biosynthesis of secondary metabolites. One such medically important example of this is the three oxidative enzymes involved in the biosynthesis of cephalosporin C. These enzymes are discussed in detail in **Section 1.3**.

1.2.1 2OG oxygenases: structure

2OG oxygenases possess a highly, but not completely, conserved fold and $Fe(II)$ binding motif.²⁹ The double-stranded β -helix (DSBH) fold forms the central structure in all characterised 2OG oxygenases³⁰ and consists of eight antiparallel β -strands (one of which may be a loop) which are split into two four-stranded β -sheets. These two β -sheets combine to form a squashed β -sandwich

structure.^{31, 32} Hydrophobic interactions between β -sheets within the DSBH fold stabilise the structure.

A minor β -sheet within the core DSBH fold supports the highly conserved 2-His-1-carboxylate Fe(II) binding motif.^{29, 33} The carboxylate group of the triad is typically supplied by an aspartate residue, but a glutamate residue is occasionally employed. The 2-His-1-carboxylate binding motif is not universally conserved. For example, in 2OG dependent halogenases the aspartate is substituted for a non-chelating residue, which opens up a coordination site through which a halide ion can bind to the metal centre.^{34, 35} The human 2OG hydroxylase aspartate/asparagine- β -hydroxylase (AspH) also possesses only two His residues ligated to the Fe-centre. Recent mutational work on AspH has demonstrated that it retains catalytic activity with only a single Fe(II) histidine ligand.³⁶ The use of only two or three Fe-coordinating ligands may enable more catalytic variability in 2OG oxygenases compared to cytochrome P₄₅₀ enzymes, in which the 4-coordinate porphyrin ring may restrict substrate and Fe interactions.³⁷

The binding of Fe(II) and 2OG share common features across all 2OG oxygenases. By contrast, prime substrates have a diverse range of binding modes that reflect the catalytic versatility of 2OG oxygenases.^{29, 38} Prime substrate binding involves the β -sheets from the DSBH fold, as well as ancillary β -strands and C-terminal helices. Interactions between residues in the active site and the substrate serve to orientate the region of the substrate undergoing transformation in relation to the Fe(IV)=O species. As is observed with substrate analogues, suboptimal substrate orientation results in uncoupled 2OG turnover and oxidative damage.^{25, 39} In reactions that are more complicated than hydroxylation, static crystallography likely does not accurately reflect the degree of conformational changes that occur during the reaction cycle.³⁷ Using time-resolved crystallography, extensive rearrangements have been observed in IPNS between substrate binding and product release, including in α -helices that are spatially distant from the active site.⁴⁰

2OG binds to a discrete pocket in the active site that is physically separate from the substrate binding mode;³⁷ however, the two pockets may be connected by dynamic motion during catalysis.⁴¹ Crystal structures of the cephalosporin biosynthesis enzyme deacetoxycephalosporin C synthase (DAOCS) in complex with alternative substrates purport to show overlapping binding modalities of 2OG and substrate.^{42,43} However, solution studies have demonstrated the formation of the DAOCS·Fe(II)·2OG·penicillin N ternary complex.⁴⁴ These results are discussed in more detail in **Chapter 4**.

1.2.2 Hydroxylation: The consensus mechanism

Hydroxylation represents the archetypal reaction catalysed by 2OG oxygenases. An overview of the current mechanism supported by extensive biophysical and kinetic data is given in Figure

1.1.^{27, 29, 31, 32}

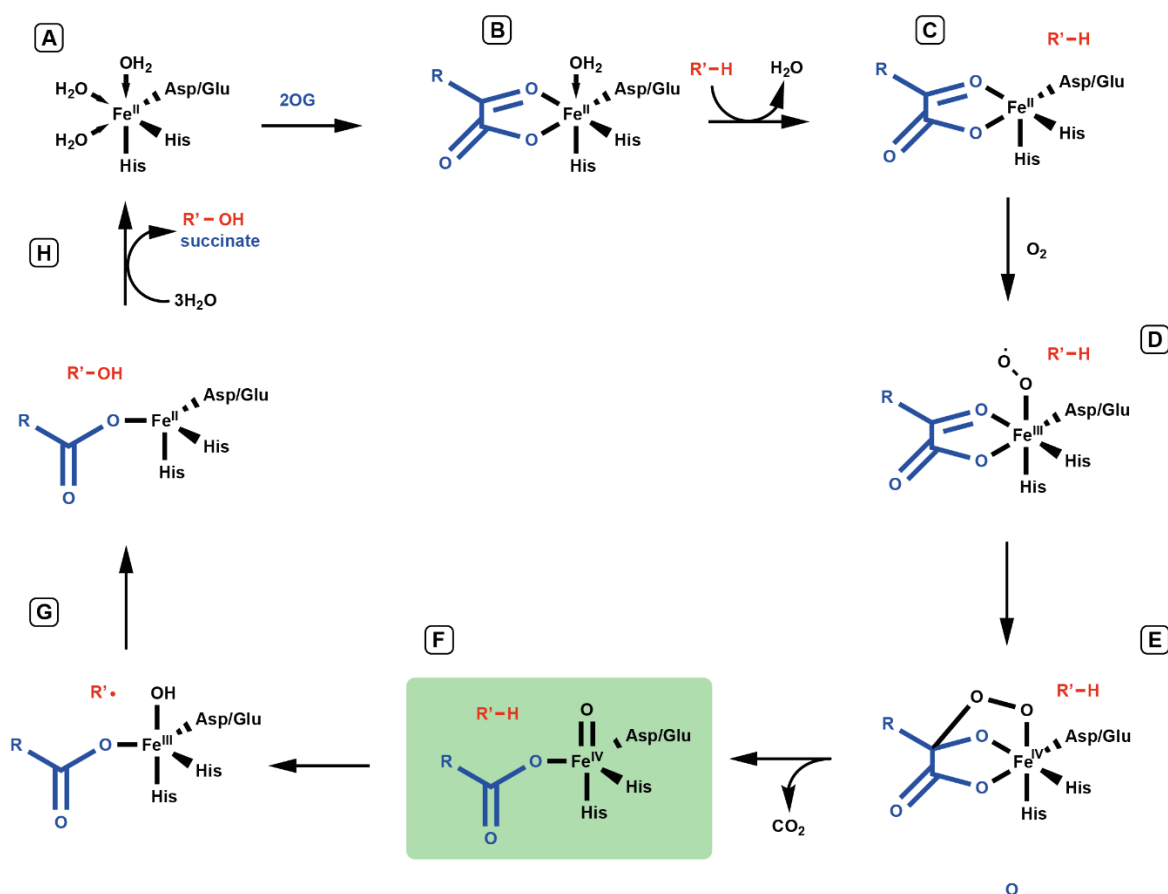


Figure 1.1 | Proposed consensus mechanism for hydroxylation by 2OG oxygenases.

The catalytic cycle begins with Fe(II) coordinated by the 2-His-1-carboxylate facial triad and three water molecules (Figure 1.1, **A**). 2OG binds to the Fe(II) centre in a bidentate manner, displacing two water molecules. The C1 carboxylate group of 2OG binds *trans* to one of the His residues and the keto group of 2OG coordinates opposite the Asp/Glu (Figure 1.1, **B**). The substrate binds to the active site at a position distal to the metal ion, which weakens the Fe(II)–water bond, opening up a coordination site for O₂ (Figure 1.1, **C**).^{23, 45} Upon binding of O₂, an Fe(III)–superoxo species is produced (Figure 1.1, **D**), which reacts with 2OG – possibly via a peroxohemiketal bicyclic intermediate (Figure 1.1, **E**) – to produce CO₂, succinate and the Fe(IV)=O (ferryl) intermediate (Figure 1.1, **F**), which has been characterised.^{27, 31} The ferryl species abstracts a hydrogen from the prime substrate (Figure 1.1, **G**), and the hydroxyl group of the resultant Fe(III)–OH rebounds onto the substrate radical (Figure 1.1, **H**). Current gaps in the mechanistic understanding of 2OG oxygenases include the specific intermediates through which the ferryl species is formed and details on atypical 2OG oxygenase-catalysed transformations, such as the oxidative rearrangement of penicillin N to DAOC.⁴⁶

1.3 2OG oxygenases in cephalosporin biosynthesis

The biosynthesis of β -lactams involves many chemically challenging steps that cannot be efficiently replicated using conventional synthetic methods. As such, the production of penicillin and cephalosporin antibiotics relies on intermediates that are produced via fermentation techniques. The role of 2OG oxygenases in β -lactam biosynthesis was unexpected but investigations have revealed that they perform seminal roles in the biosynthesis of penicillin and cephalosporins.⁴⁷ An overview of the biosynthetic pathway to the penicillins and cephalosporins is given in Figure 1.2. The three most mechanistically interesting steps are arguably isopenicillin N synthesis, ring-expansion of penicillin N to DAOC, and the hydroxylation of the latter to DAC; these steps are all catalysed by enzymes from the 2OG oxygenase structural family.

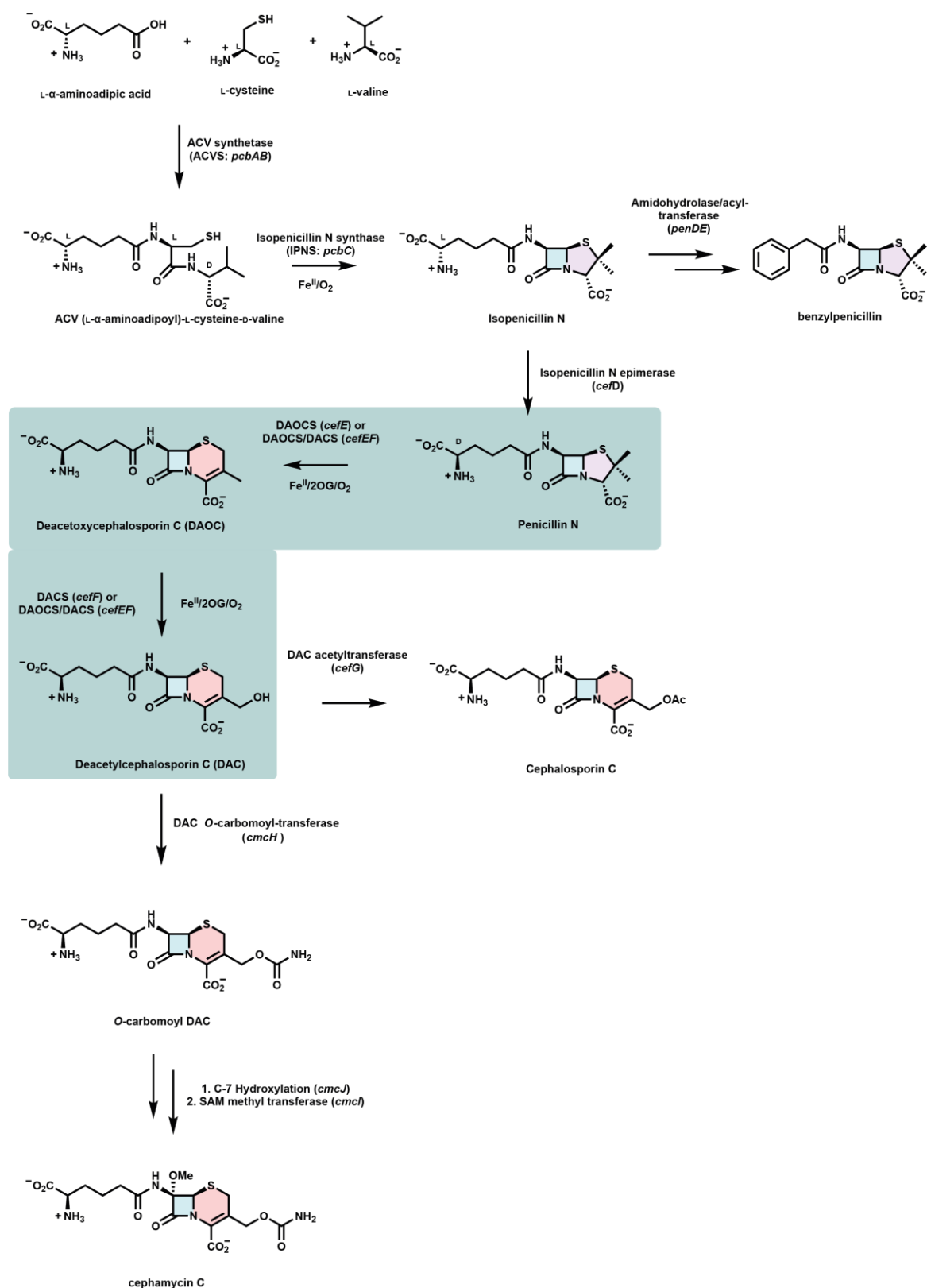


Figure 1.2 | Biosynthesis of penicillin and cephalosporin β -lactam antibiotics. The three most mechanistically interesting steps in the pathway: the formation of isopenicillin N, the ring-expansion of penicillin N to DAOCS and the hydroxylation of the latter to DAC are catalysed by members of the 2OG oxygenases structural superfamily.

1.3.1 Biosynthesis of penicillins

The first enzyme directly involved in the biosynthesis of β -lactams is ACV synthetase, which assembles the tripeptide L,L,D-ACV from L- α -aminoadipic acid, L-cysteine and L-valine.⁴⁸ ACV serves as the precursor for all penicillins and cephalosporins.⁴⁹ In the next step, isopenicillin N synthase (IPNS) forms the [4,5]-fused ring structure of isopenicillin N.⁵⁰ IPNS is structurally homologous to 2OG oxygenases but does not employ 2OG as a cosubstrate. Instead, IPNS exploits the full four electron oxidative power of molecular dioxygen to produce the 4-membered β -lactam and 5-membered thiazolidine rings of isopenicillin N in a single step.⁵¹ After the synthesis of isopenicillin N, the biosynthetic pathway diverges. In *Penicillium* and *Aspergillus*, which do not produce cephalosporins, isopenicillin N acyltransferase exchanges the L- α -aminoadipoyl side chain of isopenicillin N for a hydrophobic aromatic acyl side chain via the penam nucleus 6-APA.^{52, 53} The exact structure of acylated penicillin derivatives depends on the side chain precursors that are added to the fermentation broth. In cephalosporin-producing microorganisms such as *Cephalosporium* and *Streptomyces*, the aminoadipoyl side chain of isopenicillin N is epimerised from L- to D- stereochemistry. In prokaryotes, epimerisation is catalysed by a PLP-dependent enzyme,⁵⁴ whereas a different, three-step epimerisation mechanism is employed in eukaryotes.⁵⁵ The epimerised product, penicillin N, is the precursor to all naturally occurring cephalosporins.

1.3.2 Biosynthesis of cephalosporins

Penicillin N is the substrate for an expandase enzyme, which transforms the 5-membered thiazolidine ring in penicillin N to the 6-membered dihydrothiazine ring of deacetoxycephalosporin C (DAOC). A subsequent step hydroxylates DAOC at the 3'-allylic methyl position to give deacetylcephalosporin C (DAC). In eukaryotic fungi such as *A. chrysogenum*, a single bifunctional enzyme (DAOCS/DACS) is responsible for both expandase and hydroxylase activities.⁵⁶ In prokaryotes such as *S. clavuligerus*, these two steps are catalysed by distinct enzymes (DAOCS and DACS).^{57, 58} This discovery marked the first divergence in the cephalosporin biosynthetic pathway between eukaryotes and prokaryotes. Acetylation of DAC yields

cephalosporin C. Alternatively, further oxidation steps such as 7 α -functionalisation and C3' modifications are involved in the transformation of DAC to give cephamycins.⁴⁶ 2OG oxygenases have also been implicated in the oxidative reactions involved in cephamycin biosynthesis.

1.4 Ring-expansion and hydroxylation reactions

The three enzymes that catalyse expandase and hydroxylase activities in cephalosporin biosynthesis (Figure 1.2, **green box**) were the subject of investigations in this thesis. These two steps are explored in more detail here.

The biosynthetic pathway from penicillins to cephalosporins was established by feeding labelled valine and tripeptide substrates into cell-free extracts of *A. chrysogenum*.⁵⁹ These experiments demonstrated the incorporation of labelled valine into penicillin.⁵¹ The conversion in *A. chrysogenum* extracts of penicillin N to a penicillinase-resistant substrate provided the first evidence that cephalosporins were derived from penicillins.⁶⁰ In extracts of *A. chrysogenum* mutants that did not produce β -lactam antibiotics, the production of a cephalosporin identified as DAOC was shown to be stimulated by the addition of penicillin N.⁶¹ DAOC was confirmed as the first cephalosporin product using feeding experiments with labelled penicillin N.^{62, 63} Further labelling experiments, this time using [3-³H₃C]DAOC established the conversion of DAOC into DAC in cell-free extracts of *A. chrysogenum* and *S. clavuligerus*.⁶⁴ Mutants of *A. chrysogenum* that were defective in cephalosporin C biosynthesis were shown to accumulate DAC to high levels, indicating an intermediary role for DAC in the cephalosporin C pathway.^{65, 66} The conversion of penicillin N to DAOC was shown to require ferrous iron and 2OG, and be stimulated by reducing agents such as ascorbate and DTT.^{67, 68} The same cofactor requirements were established for the conversion of DAOC and DAC.⁶⁹ Partial incorporation of ¹⁸O into cephalosporins was also demonstrated during cultivations of *A. chrysogenum* under an ¹⁸O₂ atmosphere.⁷⁰

That a single, bifunctional enzyme was responsible for both expandase and hydroxylase activities in *A. chrysogenum* was first proposed in 1984, when the two activities could not be separated

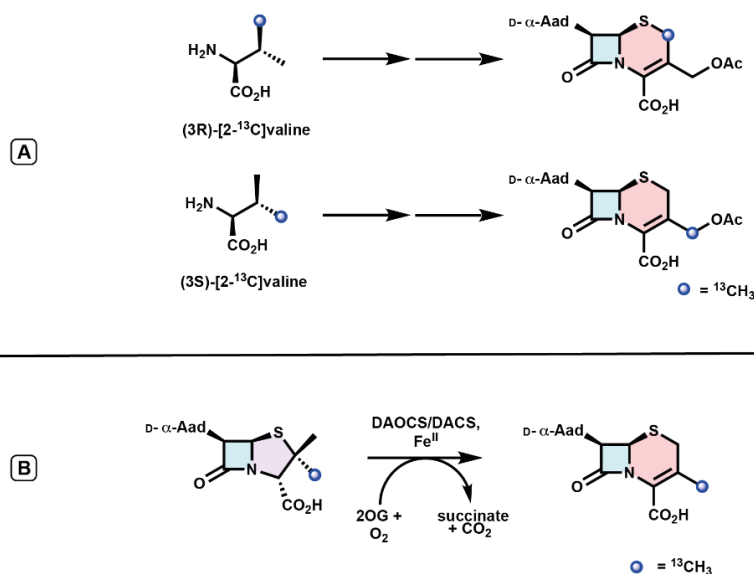
during the partial purification of cell-free extracts by ion-exchange chromatography.⁷¹ At this point it had been established that the expandase and hydroxylase reactions occurred sequentially and had the same cofactor requirements. Further purification of *A. chrysogenum* extracts by gel-filtration, dye ligand, ion-exchange and hydrophobic chromatography could not separate DAOCS and DACS activities.^{72, 73} In contrast, the two activities were easily dissociated in *S. clavuligerus* and *S. lactamdurans*.⁷⁴⁻⁷⁶ Direct evidence for an enzyme of bifunctional nature was obtained by the cloning and recombinant expression of the DAOCS/DACS gene (*cefEF*) in *E. coli*.⁷² Attempts to obtain the amino acid sequence of DAOCS/DACS, purified to near homogeneity from cell-free extracts of *A. chrysogenum*, were unsuccessful likely because of an N-terminal blockage.⁵⁶ To circumvent this issue, the DAOCS/DACS enzyme was digested by trypsin to reveal sequencable peptide fragments. The amino acid sequence of these fragments was used to generate DNA probes and identify the *cefEF* gene within *A. chrysogenum* DNA libraries. Expression of *cefEF* in *E. coli* yielded a protein that possessed both expandase and hydroxylase activities in the presence of the appropriate cofactors.⁵⁶ The DAOCS (*cefE*) and DACS (*cefF*) genes from *S. clavuligerus* were also recombinantly expressed in *E. coli*.^{77, 78} Recombinant expression of the *cefEF*, *cefE*, and *cefF* enabled access to the pure enzymes, which revealed that all three enzymes possessed both expandase and hydroxylase activities, differing only in their respective rates.⁷⁹ The enzymes had similar cofactor requirements and show good sequence homology (approximately 60%),⁸⁰ indicating that all three enzymes contained a single active site. Subtle changes in protein structure and dynamics are therefore likely to account for the observed difference in the relative rates of the two activities between the enzymes.⁷⁹ It has been demonstrated that through a single mutation, DAOCS/DACS could be transformed into an expandase or hydroxylase.⁸⁰

The homology between the amino acid sequences of DAOCS and DACS from *S. clavuligerus* and DAOCS/DACS from *A. chrysogenum* implies a close evolutionary relationship. Horizontal gene-transfer of the β -lactam biosynthetic genes from prokaryote to eukaryote has been proposed to account for this.⁷⁸

1.4.1 Mechanism of the ring-expansion reaction

Mechanistic studies on the conversion of penicillin N to DAOC employed labelled valine and penicillin substrates.⁵⁹ Early mechanistic work was performed on DAOCS/DACS from *A. chrysogenum*. More recently, structural studies have focused on DAOCS from *S. clavuligerus*, with the aim of engineering the enzyme for the conversion of penicillin analogues possessing hydrophobic side chains.⁸¹

A cell-based feeding experiment in *A. chrysogenum* with stereospecifically labelled (3*R*)-[2-¹³C]valine revealed the ¹³C-label was incorporated at the endocyclic C2 position of cephalosporin C. When the feeding experiments were repeated with (3*S*)-[2-¹³C]valine, ¹³C-incorporation was at the exocyclic C3' position of cephalosporin C (Scheme 1.1, **A**).^{82, 83} The (3*R*) methyl group of valine had previously been shown to form the β-methyl group of isopenicillin N.⁸³ That the C2 endocyclic position of cephalosporins was derived from the penicillin β-methyl group was proved definitively through incubations of [2α-¹³CH₃]penicillin N with partially purified DAOCS/DACS from *A. chrysogenum*, which produced DAOC with the ¹³C-label observed exclusively at the C3'-exocyclic position.⁸⁴ (Scheme 1.1, **B**).



Scheme 1.1 | Incorporation of stereospecifically-labelled ^{13}C methyl groups into cephalosporin C. **A** Incubations of ^{13}C -labelled valines with *A. chrysogenum* cells yielded stereospecifically labelled forms of cephalosporin C. **B** Incubations of [2- ^{13}C]penicillin N with DAOCS/DACS yielded DAOC with the ^{13}C label at the 3'-position.

The incorporation of the β -methyl group is analogous to the chemical synthesis of cephalosporins from penicillin β -sulfoxides, known as the Morin rearrangement.⁸⁵ Based on this chemical homologue, α - and β -sulfoxides of penicillin N were proposed as reactions intermediates (Figure 1.3), but neither sulfoxides were accepted as substrates by DAOCS/DACS (Figure 1.3)⁸⁶.

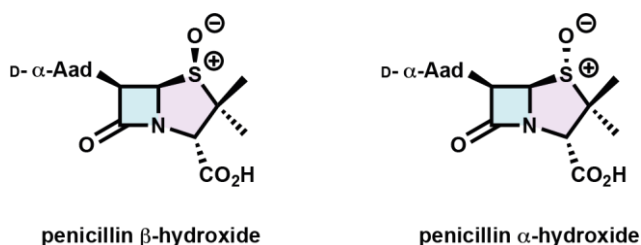
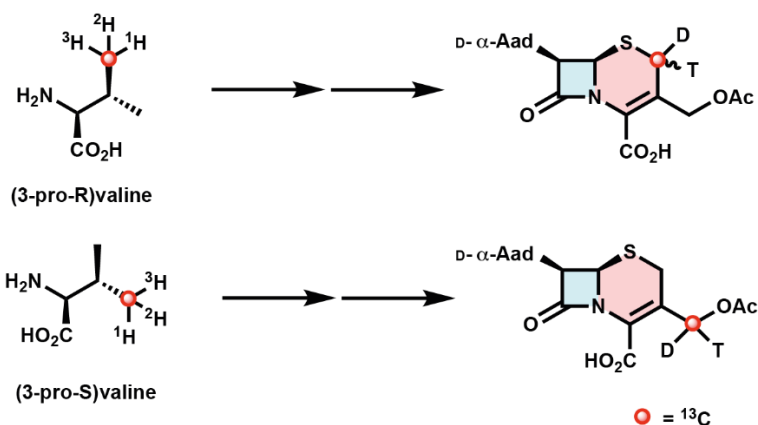


Figure 1.3 | The structure of α - and β -sulfoxides of penicillins

Further cell-based feeding experiments with valine containing a chiral methyl group at the (3-*pro*-R) position showed that the methyl group was incorporated into the cephem C2 position with complete loss of stereochemistry (Scheme 1.2). This result supported the presence of a discrete β -methylene radical (Figure 1.4, **A**), which can undergo rotation before expansion into a cepham.⁸⁷⁻⁸⁹ In contrast, valine with a (3-*pro*-S) stereogenic methyl group was incorporated into the exocyclic C3' methylene group of cephalosporin C with retention of stereochemistry (Scheme 1.2).^{69, 88}



Scheme 1.2 | Incorporation of stereospecifically labelled chiral valines in cephalosporin C. Loss of stereochemistry was observed during incubations of (3-*pro*-R)valine with DAOCS/DACS, supporting the existence of a long-lived β -methylene radical.

The synthetic conversion of a β -bromomethylene penam to a cephalosporin under radical conditions provides a chemical precedence for ring-expansion proceeding via a radical mechanism (Figure 1.4, B).⁹⁰

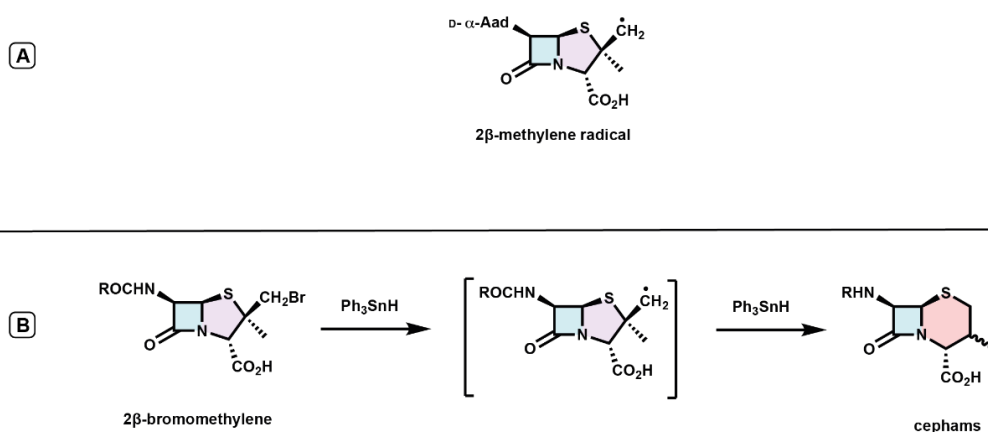


Figure 1.4 | Structure of the proposed 2 β -methylene radical intermediate and a chemical precedent proving the feasibility of ring expansion proceeding via a radical mechanism.

During the formation of DAOC two hydrogens are lost from penicillin N, one from the 2 β -methyl group and the other from the C3 position. Mixed competitive kinetic isotope experiments with deuterated penicillin analogues (Figure 1.5) revealed that abstraction of the β -methyl hydrogen occurs first.⁹¹

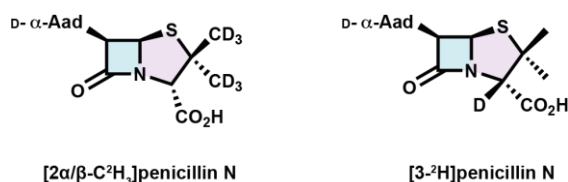
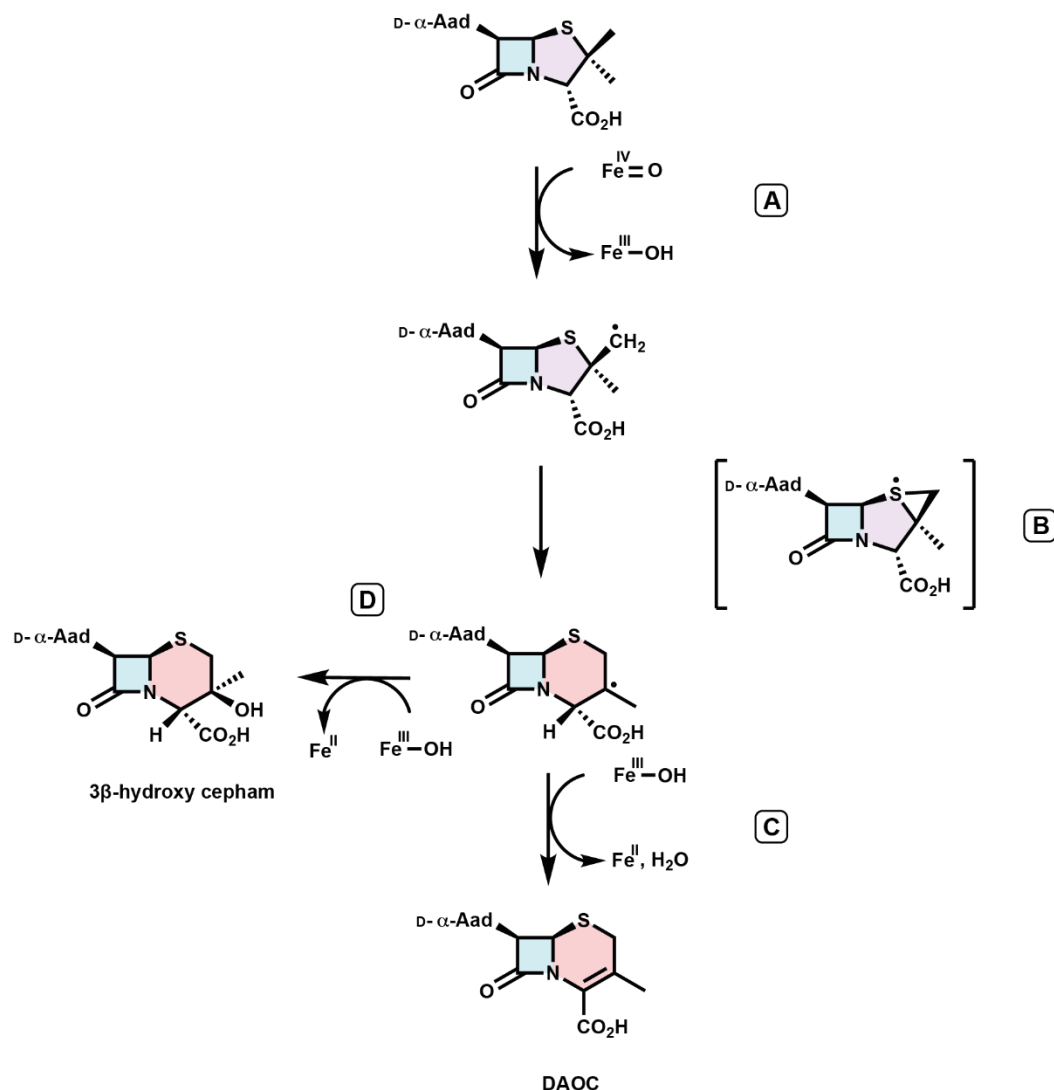


Figure 1.5 | Deuterated penicillin analogous.

During incubations of [3-²H]penicillin N, the 3 β -hydroxy cepham was observed as a major product,^{92, 93} having previously been isolated as a minor component from culture filtrates of *A. chrysogenum* (Scheme 1.3).⁹⁴ The hydroxyl group of the 3 β -hydroxy cepham is derived at least partially from molecular oxygen.⁹³

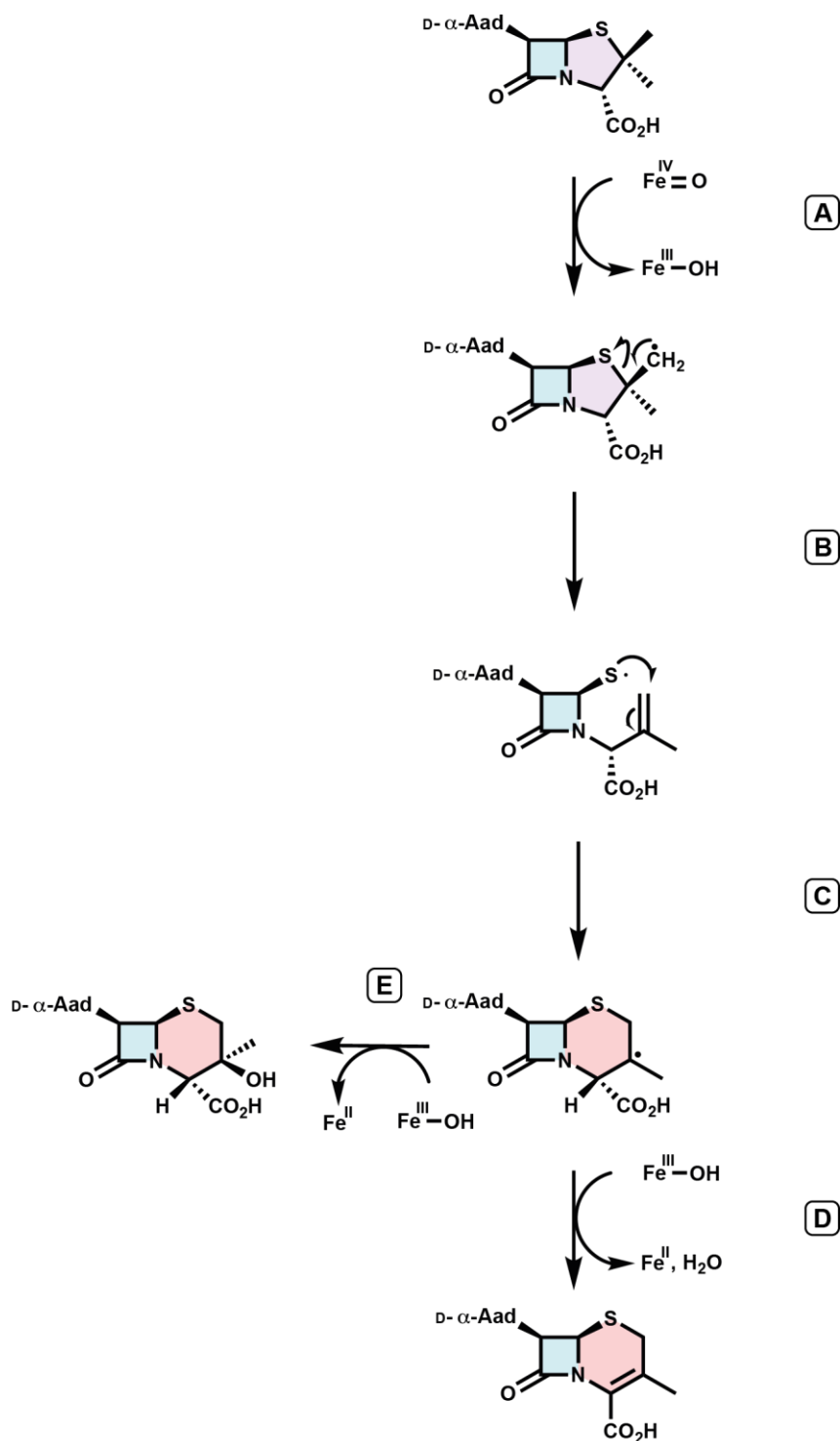
On the basis of the above evidence, a mechanism was proposed for the ring-expansion of penicillin N to DAOC (Scheme 1.3). First, abstraction of a hydrogen atom from the 2 β -methyl group of penicillin N by the ferryl intermediate occurs in an irreversible manner (Scheme 1.3, **A**). The resultant β -methylene radical, which is stabilised by orbital overlap with a sulfur lone pair, undergoes rearrangement to a more stable tertiary cepham radical, likely via an episulfonium intermediate (Scheme 1.3, **B**).

At this point the pathway bifurcates: removal of hydrogen at the C4 position of the cepham radical yields the natural product DAOC (Scheme 1.3, **C**). Alternatively, the hydroxyl group can transfer from the Fe(III)–OH intermediate to the cepham radical to give the 3 β -hydroxy cepham (Scheme 1.3, **D**). This latter pathway is promoted by deuteration at the C3 position of penicillin N, which slows the final elimination step in the production of DAOC.



Scheme 1.3 | Proposed mechanism for the ring-expansion of penicillin N to DAOC and the shunt metabolite 3β-hydroxy cepham. **A** Initial hydrogen abstraction by the Fe(IV)=O intermediate occurs at the 2β-methyl group of penicillin N. **B** The resultant primary radical rearranges to the more stable tertiary cepham radical. **C** Abstraction of a hydrogen atom from the C4 position of the cepham radical gives DAOC. **D** Hydroxyl rebound gives the 3β-hydroxy cepham shunt product.

In an alternative mechanism, the primary β-methylene radical could fragment to give a thiol radical. Readdition of the thiol to the double bond would afford the tertiary cepham radical from which DAOC and 3β-hydroxy cepham could be produced (Scheme 1.4).

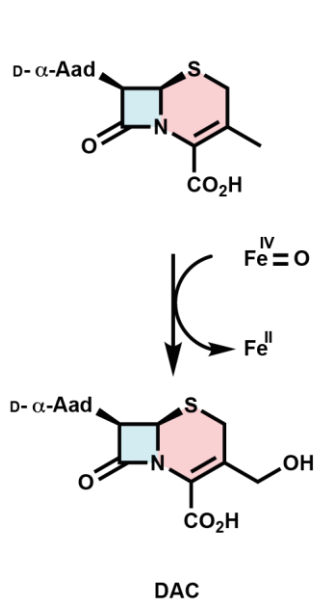


Scheme 1.4 | Mechanism for the conversion of penicillin N to DAOC via a monocyclic intermediate. A Hydrogen abstraction by the $\text{Fe}(\text{IV})=\text{O}$ species. B β -Methylene formation is proceeded by fragmentation. C Addition of the thiol radical to the double bond gives the tertiary cepham radical which, as in Scheme 1.3, can form DAOC (D) or the 3 β -hydroxy cepham (E).

1.4.2 Hydroxylase mechanism

Allylic hydroxylation of DAOC to DAC is purported to proceed via a typical 2OG-dependent oxygenase hydroxylation reaction (Scheme 1.5). 3'-Hydroxylation proceeds with partial retention

of stereochemistry, favouring direct insertion of the hydroxyl group over the formation of a distinct methylene radical.^{57, 95, 96}



Scheme 1.5 | Hydroxylation of DAOC to DAC via a direct insertion mechanism. This mechanism was proposed based on the partial retention of stereochemistry that occurs during DAC formation, which disfavours the formation of a distinct 3'-allylic radical.

1.5 Substrate analogue studies on DAOCS/DACS

Penicillin and cephalosporin substrate analogues have been employed as mechanistic probes to explore both expandase and hydroxylase activities of DAOCS/DACS.⁹⁷⁻¹⁰⁰ In addition to the mechanistic insights, these early studies encouraged protein engineering schemes on DAOCS, with the aim of improving its ability to accept penicillins with hydrophobic side chains that are more amenable to industrial fermentation processes.⁴⁶

1.5.1 Expandase substrate analogues

The expandase activity of DAOCS/DACS has been probed with penicillin analogues.^{98, 101} Penicillins containing adipoyl and *m*-carboxyphenylacetyl side chains were converted to the corresponding cepheams (Figure 1.6, **A** and **B**).¹⁰⁰ A penicillin with a D-(5-carboxymethylcysteine) side chain was also converted by DAOCS/DACS (Figure 1.6, **C**).¹⁰² In contrast, the 5-aminopentanoyl, phenylacetyl and phenoxyacetyl side chains were not accepted by DAOCS/DACS (Figure 1.6, **D**, **E**, and **F**), and neither was isopenicillin N with its L- α -aminoadipoyl side chain (Figure 1.6, **G**).¹⁰⁰ The penam nucleus, 6-APA was also not a substrate for DAOCS/DACS (Figure 1.6, **H**). These studies indicate

that the length of side chain, the presence of the carboxyl group and the stereochemistry of the α -amino group determine substrate selectivity.

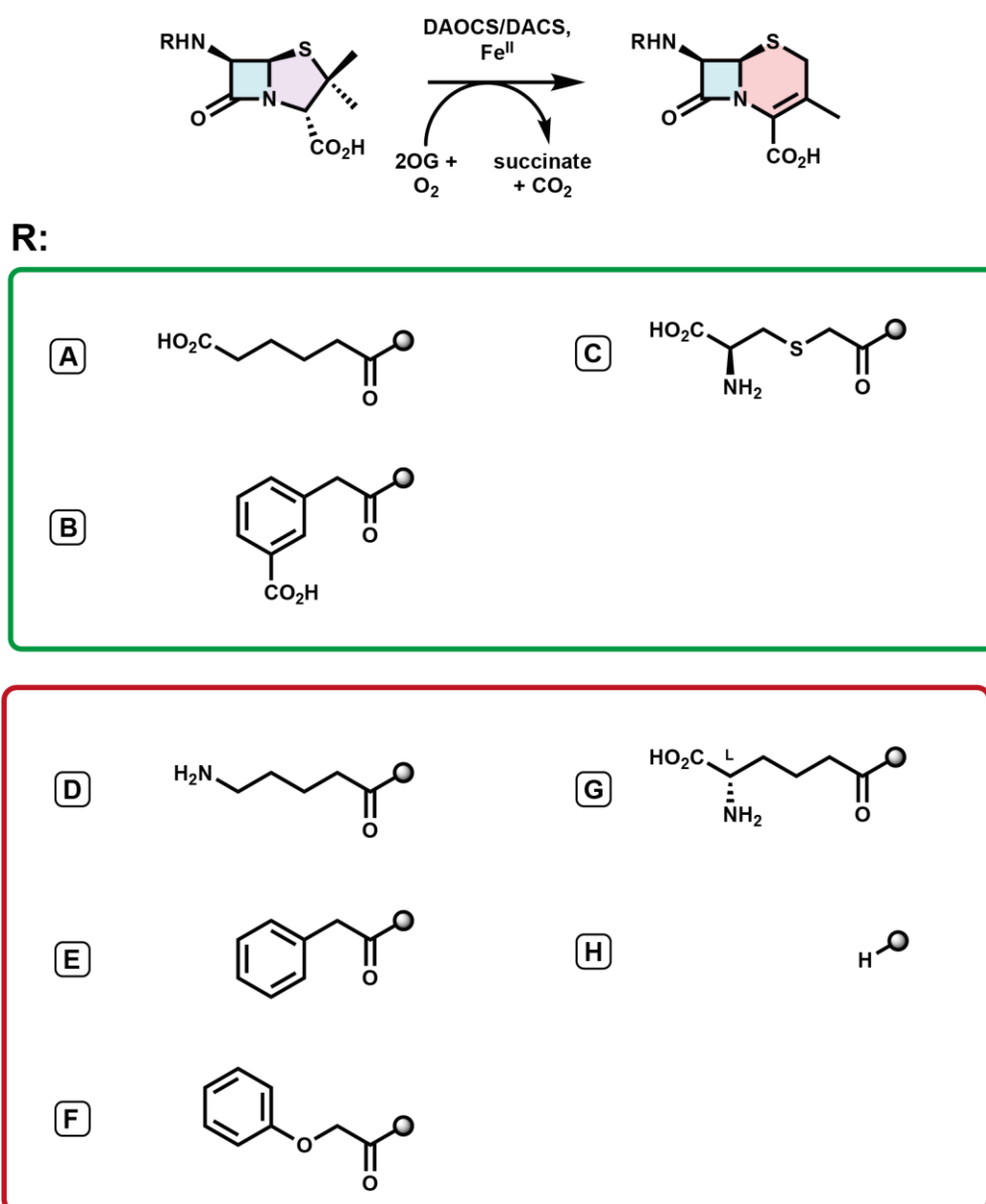


Figure 1.6 | C6-side chain penicillin substrate analogues and their conversion by DAOCS/DACS. Green box These substrates (A, B, and C) are accepted by DAOCS/DACS. Red box Substrate analogues D–H were not turned over by DAOCS/DACS. These results demonstrate that a side chain consisting of a six-carbon chain terminating with a carboxyl group are required for conversion by DAOCS/DACS.

Interestingly, dethio-penicillin N was not converted by DAOCS/DACS, even though it contains the D- α -aminoadipoyl side chain (Figure 1.7, A).⁷⁹ The β -methyl penam was converted to the corresponding cephem (Figure 1.7, B).⁵⁹

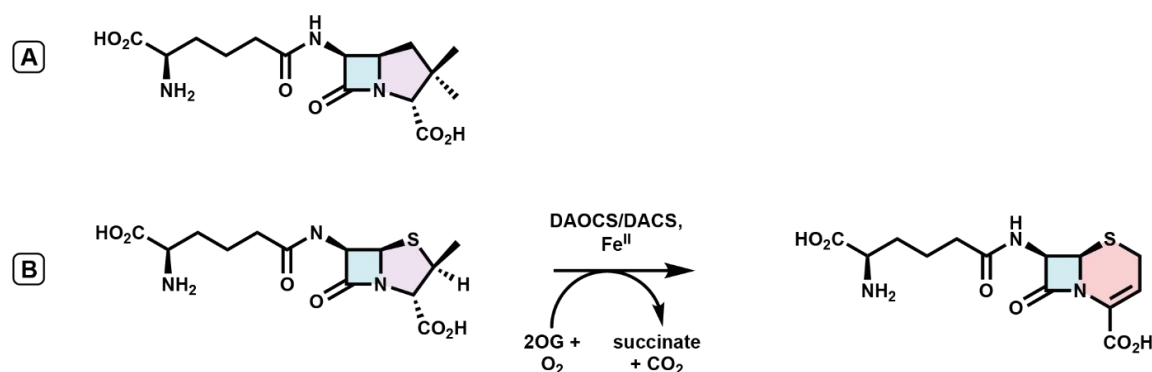


Figure 1.7 | Dethio-penicillin N and β -methyl penicillin N substrate analogues. A Dethio-penicillin N was not accepted as a substrate by DAOCS/DACS. B β -Methyl penicillin was converted to DAOC without the 3'-methyl group.

1.5.2 Hydroxylase substrate analogues

Probing the hydroxylase function of DAOCS/DACS with alternative cepham and cephem substrates has proved much more interesting than the expandase activity. The Δ^2 -isomer of DAOC was converted by DAOCS/DACS to the Δ^2 -DAC isomer (Figure 1.8, **A**).¹⁰³ Overoxidation of DAC to the 3-formyl cephalosporoate was also observed (Figure 1.8, **B**).¹⁰⁴ In a divergence with the expandase activity, carba-DAOC was converted by DAOCS/DACS in good yield (Figure 1.8, **C**).⁷⁹ This demonstrates that whilst sulfur is required for expandase activity, hydroxylation can still proceed in its absence.¹⁰⁵ The C3' ethyl analogue is accepted by DAOCS/DACS (Figure 1.8, **D**),¹⁰⁶ but the sterically similar C3' thiomethyl cephem was not a substrate (Figure 1.8, **E**),¹⁰⁷ suggesting that DAOCS/DACS cannot oxidise sulfur atoms. Conversion of the C3' propyl analogue by DAOCS/DACS was negligible (<5%) and the product has not been identified (Figure 1.8, **F**).¹⁰⁸

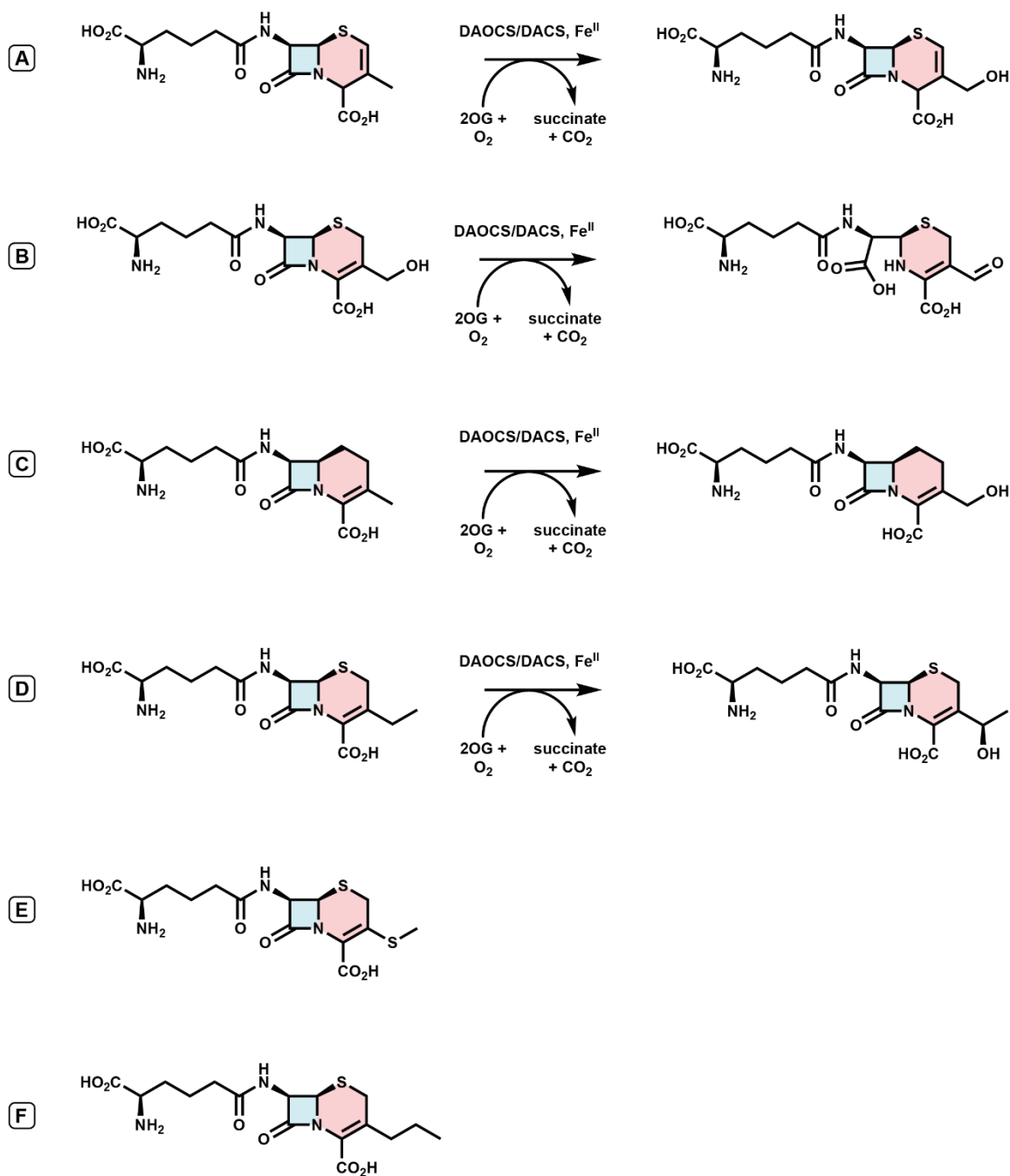


Figure 1.8 | Cephem substrate analogues screened for activity with DAOCS/DACS. **A** The Δ^2 -isomer of DAOC is converted to Δ^2 -isomer of DAC. **B** Oxidation of DAC to cephem aldehyde. **C** carba-DAOC is converted to carba-DAC demonstrating that, unlike the expandase activity, 3'-hydroxylation does not require the ring sulfur. **D** A 3'-ethyl analogy is readily hydroxylated, but the analogous thio-methyl cephem (**E**) is not. **F** 5% conversion of the C3' propyl cephem was observed, but the product was not identified.

Cephem cyclopropyl derivatives have also been screened as substrates for DAOCS/DACS (Figure

1.9). **A** was readily converted to DAC but no turnover was observed with **B** or **C**.

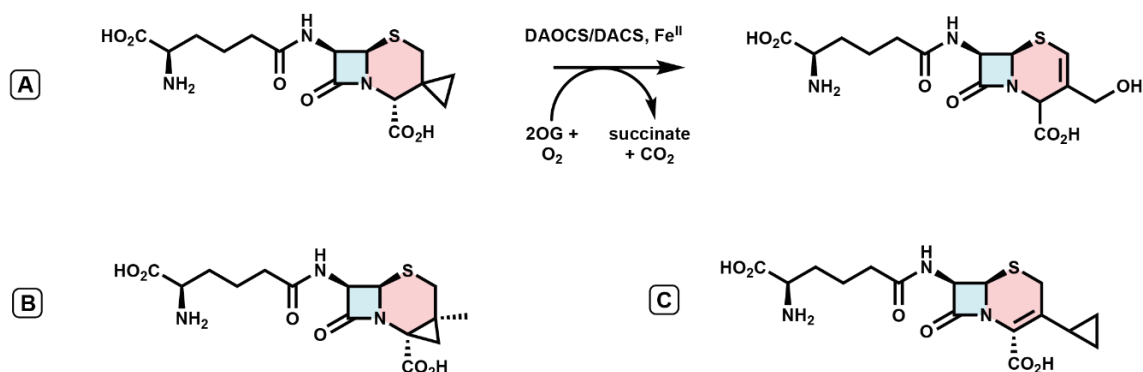


Figure 1.9 | Cyclopropyl cephem/cepham analogues trialled as substrates for DAOCS/DACS. **A** The 3'-cyclopropyl cepham was readily converted to the Δ^2 -isomer of DAC. **B** and **C** Neither cyclopropyl analogues were accepted by DAOCS/DACS

Several analogues possessing unsaturated C3 substituents are accommodated as substrates by DAOCS/DACS. Incubations of the exomethylene cepham with DAOCS/DACS produced DAC (Figure 1.10, **A**).¹⁰⁹⁻¹¹¹ In addition to DAC, conversion of the [4- ^2H]exomethylene cepham by DAOCS/DACS¹¹² and DAOCS⁹⁷ afforded a spiro-epoxide cepham (Figure 1.10, **B**), providing the first evidence of epoxidase activity catalysed by the cephalosporin biosynthesis enzymes. The spiro-epoxide was itself a substrate for DAOCS/DACS and was converted to the ring-opened DAC aldehyde (Figure 1.10, **C**).¹¹² The product of incubations of Z-ethylidene cepham was two diastereomeric alcohols (Figure 1.10, **D**),¹¹³ the minor of which was also formed during incubations of C3' ethyl cepham with DAOCS/DACS (Figure 1.8, **D**). The C3' allyl cepham analogue was not accepted by DAOCS/DACS (Figure 1.10, **E**).¹⁰⁸ The C3' ethynyl cepham analogue could not be evaluated as a substrate for DAOCS/DACS due to a challenging synthesis (Figure 1.10, **F**).¹¹⁴⁻¹¹⁶ Incubations of the 3-vinyl cepham substrate analogue with DAOCS/DACS and DACS are discussed in **Chapter 3**.

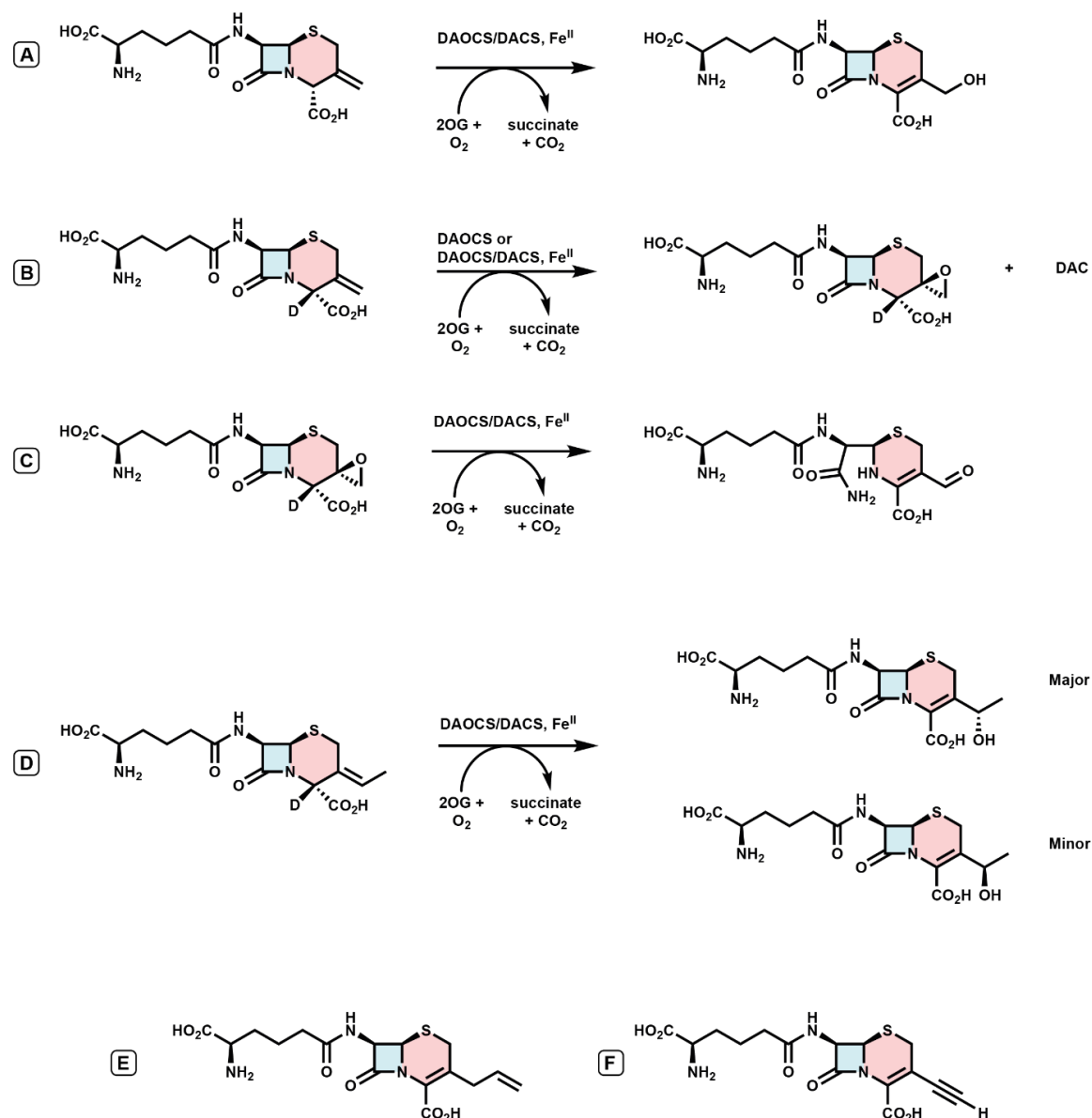


Figure 1.10 | Substrate specificity for 3' hydroxylation of DAOCS/DACS and DAOCS. **A** The exomethylene cepham was readily converted to DAC. **B** The [4-²H]exomethylene cepham was converted to DAC and a spiro-epoxide cepham. **C** Conversion of the spiro-epoxide to a ring-opened aldehyde. **D** The Z-ethylidene cepham was converted to diastereomeric alcohols. **E** The 3'-allyl cephem was not converted by DAOCS/DACS and the 3'-ethynyl cephem (**F**) could not be synthesised.

1.6 Objectives of the work described in this thesis

The aim of the work described in this thesis was to expand the mechanistic understanding of the three enzymes that catalyse the ring expansion of penicillin N to DAOC and the 3' hydroxylation of DAOC to DAC.

Initially, improvements in the expression of the *cefEF* (DAOCS/DACS) gene in *E. coli* were sought, as described in **Chapter 2**. The aim of the work was to develop a platform by which DAOCS/DACS could be produced on a large-scale to facilitate mechanistic probes into this interesting enzyme

by means of substrate analogue and crystallographic studies, both of which require large amounts of enzyme.

In **Chapter 3**, studies on the incubations of DAOCS/DACS with the 3-vinyl cephem substrate analogue are described. These incubations were initially performed 30 years ago, with conversion of the 3-vinyl cephem substrate to a single β -lactam product. However, the structure of the product could not be elucidated. Through the application of modern spectroscopic techniques, it was anticipated that the structure of the product could be determined.

Efforts were also directed towards the structural characterisation of DAOCS/DACS and DAOCS, as described in **Chapter 4**. Crystal structures of DAOCS in complex with the substrate analogue penicillin G have previously been reported, however the structures likely present penicillin G complexed in non-productive binding modes. It was planned that new crystallisation methodologies and screening conditions would be explored in an attempt to produce enzyme-substrate complexes that are more reflective of what occurs during the catalytic cycle of DAOCS. In **Chapter 4**, the mechanism of hydroxylation of DAOC to DAC was also explored using ^{18}O -labelling experiments.

Chapter 2

**Optimising the production of *A. chrysogenum* DAOCS/DACS
in *E. coli***

2 Optimising the production of *A. chrysogenum* DAOCS/DACS in *E. coli*

2.1 Introduction

Since its discovery in the 1960s, significant efforts have been directed towards the elucidation of the cephalosporin biosynthesis pathway. Initial investigations into the pathway involved feeding stereochemically-labelled valines into intact mycelial cells of *A. chrysogenum*.¹¹⁷ However, several issues were associated with the use of intact cells; most critically, their poor uptake of labelled substrates, which hindered efforts to track labelled valines and penicillins through the biosynthetic pathway. Thus, by the early 1970s, intact cell studies had established the precursors of β -lactam biosynthesis, but the exact pathways by which they had been produced were not known.

In 1971, Abraham described the conversion of labelled valine into L,L,D-ACV in a broken cell system of *A. chrysogenum*.¹¹⁸ Broken cells were prepared by the sonication of intact mycelial cells followed by centrifugation. The development of gentler cell disruption methods, such as the preparation of *A. chrysogenum* protoplasts followed by osmotic lysis, yielded cell-free extracts in which the conversion of labelled valine and penicillins into cephalosporins was observed.^{61, 119} Protoplasts of *A. chrysogenum* were prepared using digestive enzymes from *Helix pomatia* or *Cytophaga*. Cell-free extracts were then obtained by either osmotic lysis or gentle sonication without compromising the biochemical activity contained within.¹²⁰ The observation that penicillins were precursors to cephalosporins refuted the established idea at the time that penicillins and cephalosporins were end products of a branched pathway.¹²⁰

Once a reliable cell-free system for *A. chrysogenum* had been established, efforts were directed towards obtaining pure DAOCS/DACS enzyme. Using several methods, native DAOCS/DACS was obtained in ever-increasing purity from cell-free extracts of *A. chrysogenum*. The inability to dissociate expandase and hydroxylase activities during its purification provided the first evidence

for the bifunctional nature of DAOCS/DACS.⁷¹ Incubations of labelled substrates with partially purified DAOCS/DACS achieved higher levels of sensitivity than cell-free extracts, and allowed investigations into the stereochemical incorporation of penicillin N substituents into DAOC (Section 1.4.1).

2.1.1 Recombinant expression

Access to pure DAOCS/DACS permitted its amino acid sequence to be determined. This in turn revealed the *cefEF* gene within *A. chrysogenum*, which was recombinantly expressed in *E. coli*.⁵⁶ *CefEF* was initially expressed using a pUC8-derived vector named pIT-511. Under these expression conditions, DAOCS/DACS accumulated up to 15% of total cell protein. Inclusion body formation was observed, indicating significant insoluble expression of DAOCS/DACS.⁵⁶ DAOCS/DACS was extracted from these bodies using urea and purified by ion-exchange chromatography. After dialysis to remove the urea, the purified recombinant enzyme demonstrated identical substrate selectivity to wild-type DAOCS/DACS.^{56, 103}

In order to improve soluble expression of *cefEF*, the pIT-511 vector was modified to include a *trc* promoter and the spacing between the start codon, ribosome binding site, and promoter were optimised.¹²¹ These changes had previously been shown to increase the soluble expression of IPNS.^{122, 123} Expression of *cefEF* using this new construct, pRH1091, produced DAOCS/DACS at 20% of soluble protein. In 2004, *cefEF* expression was updated to a pET24a(+) vector, which utilised a T7 promoter.⁸⁰ Using this construct, DAOCS/DACS accumulated to 30% total soluble protein in *E. coli* strain BL21(DE3). pET-derived constructs became the preferred recombinant expression system for *cefEF* in the following years¹²⁴⁻¹²⁶

Table 2.1 | Summary of previous DAOCS/DACS production in *E. coli* cell strains. Within each optimisation regime, each subsequent expression protocol built on the previous method. For example, in Route 1 the *cefEF* gene was subcloned from the 1992 vector into pET24a(+) for the expression in 2004.

Optimisation regime	Year ^(ref)	Expression vector	Promoter	% Of total protein	Form
Route 1	1988 ⁵⁶	pUC8	λ P _L	15	Insoluble
	1992 ¹²¹	pUC8	<i>trc</i>	20	Soluble
	2004 ⁸⁰	pET24a(+)	T7	30	Soluble
Route 2	2001 ¹²⁴	pGEM-T	T7	N/A	Soluble
	2005 ¹²⁶	pET30a(+)	T7	N/A	Soluble
	2011 ¹²⁵	pET30a(+)	T7	N/A	Soluble

2.1.2 Project Objectives

A literature search revealed that the recombinant expression of *cefEF* in *E. coli* has been optimised six times (Table 2.1), most recently in 2011¹²⁵ using protocols established in 2004.⁸⁰ The yield and purity in which DAOCS/DACS was obtained compared poorly to that of the related *S. clavuligerus* enzyme DAOCS, restricting mechanistic and particularly structural characterisation of DAOCS/DACS.⁴⁴ An updated expression optimisation regime is therefore required to produce DAOCS/DACS in improved yields and purity in order to facilitate further studies on this crucial enzyme. Importantly, DAOCS/DACS has only been produced in an un-tagged form, precluding the use of affinity chromatography in its purification. Production of DAOCS/DACS as a His₆-tagged fusion protein would enable purification by immobilised metal affinity chromatography (IMAC), yielding higher purity enzyme, more amenable to crystallographic and mechanistic studies. Previous expressions have demonstrated how different promoters can significantly alter the soluble expression levels of recombinant genes. This effect will be explored in the work described in this chapter.

In summary, the objectives of the work described below were as follows:

- Re-optimisation of recombinant DAOCS/DACS production to obtain highly pure and active enzyme in large quantities.

- Production of recombinant DAOCS/DACS in a His₆-tagged form to improve the efficiency of downstream purification processes.
- Extending previous work on the role of expression vector promoters to improve the production of soluble protein.

2.2 Initial expression trials

pNIC28-Bsa4 was the expression vector selected for initial screenings. pNIC28_Bsa4 is a pET-based vector with an N-terminal His₆-[TEV cleavage site] tag. As with all pET vectors, it employs a T7 promoter and its use represented an iterative change on previous recombinant expression methodologies.^{80, 124-126}

A synthetic version of the *cefEF* gene (*cefEF_v1*) was readily available. This gene was subcloned into the pNIC28-Bsa4 vector by Ligation Independent Cloning (LIC) to give a kanamycin-resistant construct (pNIC_DD). pNIC_DD was transformed into *E. coli* BL21(DE3) and expressed under various conditions (Table 2.2). A brief summary of the expression protocols is as follows. BL21(DE3) hosts carrying the pNIC_DD vector were grown to stationary phase by incubation overnight at 37 °C with shaking at 180 rpm. Overnight culture was used to inoculate 100 mL of the same media (1:100 v/v) and grown as before to an OD₆₀₀ = 0.6, then induced with IPTG. At the end of the induction period, cells were harvested by centrifugation and re-suspended in the BugBuster™ Protein Extraction Reagent (Merck). His₆-tagged DAOCS/DACS was purified from clarified lysate on Ni Sepharose High Performance resin using a batch method in microcentrifuge tubes. Elution of the target protein from the Ni-resin was achieved using a high imidazole buffer. For the preparation of insoluble fractions, the cell pellet leftover after clarification was resuspended in low imidazole buffer (1 mL). Ni-resin elution fractions and insoluble fractions were analysed for DAOCS/DACS by SDS-PAGE.

Table 2.2 | Growth conditions investigated during early-phase screenings of *cefEF_v1* expression. Expression from pNIC_DD in BL21(DE3) was explored under these conditions. Cultures were grown to stationary phase overnight at 37 °C and 180 rpm. Overnight culture was expanded into 100 mL of fresh media and grown to OD₆₀₀ = 0.6 before protein expression was induced by IPTG. Cultures were incubated at their post-induction temperature for 15 min before addition of IPTG.

[IPTG] (mM)	Post-induction temperature (°C)	Post-induction time (h)
0	18, 25, 30	16
0.1	18, 25, 30	5, 16
0.2	15, 37	4, 6
0.3	15, 30, 37	3, 4, 16
0.5	30	3
1	25, 30, 37	5, 16

SDS PAGE analysis revealed that DAOCS/DACS was apparently produced exclusively in an insoluble form from pNIC_DD across all expression conditions explored. A representative SDS-PAGE gel is presented in Figure 2.1. No protein was observed in the Ni-resin elution fractions (**Panel A**), whilst there was significant accumulation of protein corresponding to the mass of DAOCS/DACS in the insoluble fractions (**Panel B**). Total expression levels were broadly consistent across all conditions. Overall, soluble expression of *cefEF_v1* from pNIC_DD was not possible across the standard growth conditions that were screened.

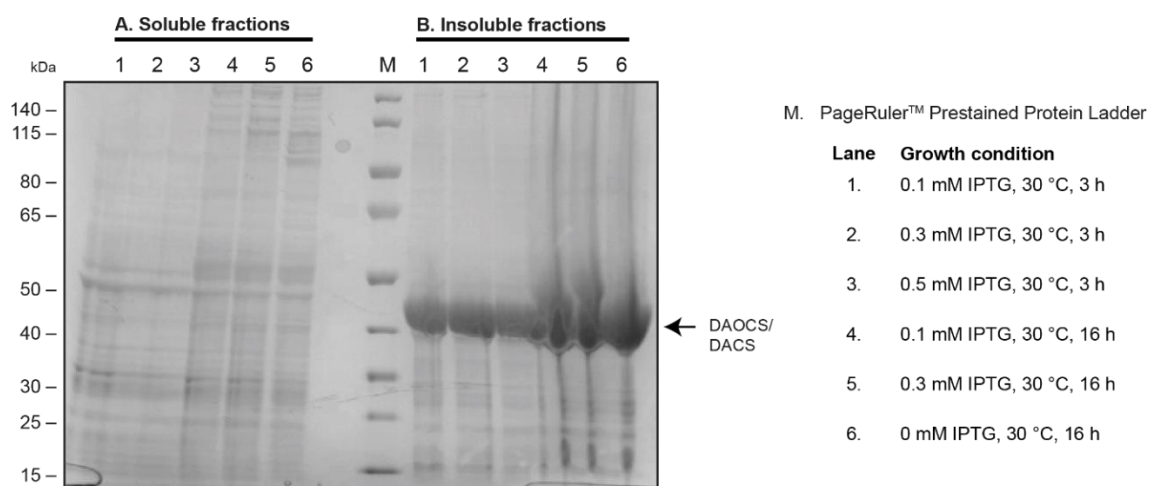


Figure 2.1 | Production of DAOCS/DACS from the pNIC_DD construct expressed in BL21(DE3) under different conditions. Starter cultures of BL21(DE3) containing a pNIC_DD recombinant were grown overnight at 37 °C and 180 rpm before 1 mL was used to inoculate 100 mL of the same media. The cultures were grown as before to an OD₆₀₀ = 0.6 and induced under a range of IPTG concentrations and growth conditions. **Lanes 1–6** represent cultures grown under different conditions, with details supplied in the key (RHS). **Panel A** Elution fractions from Ni Sepharose resin batch purification. **Panel B** Protein levels in the insoluble fraction, obtained by resuspending the cell pellet in low imidazole buffer following clarification and removal of cell lysate.

2.3 Regulating uninduced expression of *cefEF_v1*

Early screening revealed significant expression of *cefEF_v1* in the absence of IPTG (Figure 2.1, lane B.6). This so-called uninduced or “leaky” expression is common in pET-based systems because T7 is a strong promoter and basal expression of T7 RNA Polymerase (RNAP) in the absence of IPTG, even at low levels, is sufficient to induce expression of the target gene.^{127, 128} The accumulation of recombinant protein to high levels can saturate the protein biogenesis capacity of *E. coli* cells and lead to inclusion body formation.¹²⁸ It was therefore proposed that the observed aggregation of DAOCS/DACS was the result of the expression of *cefEF_v1* to excessive levels, caused primarily by “leaky” expression. Efforts were thus directed toward reducing uninduced expression of *cefEF_v1* from pNIC_DD.

It has been observed that uninduced expression of pET-derived vectors is greatest when expression hosts are grown overnight to stationary phase.¹²⁹⁻¹³¹ This was the protocol used for starter cultures in the pNIC_DD expression trials, during which time high levels of *cefEF_v1* expression could have occurred. As such, DAOCS/DACS protein may have aggregated before the main culture growths. This would explain why production levels of DAOCS/DACS were consistent across all growth conditions (Figure 2.1). To minimise “leaky” expression of *cefEF_v1*, starter cultures were limited to log phase growth ($OD_{600} = 0.6$ to 1.0 , 3 h) prior to their inoculation into main cultures. As before, main cultures were then grown to an $OD_{600} = 0.6$ and induced with IPTG (0.5 mM) for either 3 hours at 27 °C or 37 °C, or 16 hours at 18 °C. Once the culture period was complete, cells were harvested by centrifugation and re-suspended in BugBuster™ Protein Extraction Reagent. His₆-tagged target protein was purified from the clarified lysate using Ni Sepharose resin using a batch method in microcentrifuge tubes. Ni-resin elution and insoluble fractions were analysed by SDS-PAGE (Figure 2.2)

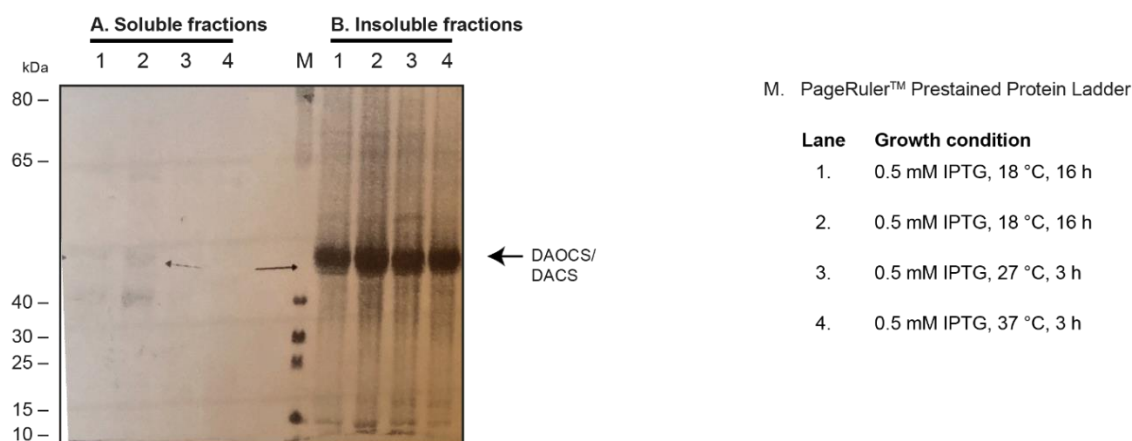


Figure 2.2 | Expression of pNIC_DD in log phase BL21(DE3) cultures. Starter culture growth was halted during the log phase, rather than stationary phase, in an attempt to prevent uninduced expression of *cefEF_v1*. **Panel A** Elution fractions following Ni Sepharose resin purification. **Panel B** Insoluble fractions, prepared by resuspending cell pellets following cell lysis and clarification by centrifugation. Conditions for **Lanes 1–4** are given in the key (RHS).

Limiting starter culture growth did not improve the production of soluble DAOCS/DACS, which was produced in an insoluble form across all growth conditions (Figure 2.2, **Panel B**). Total expression levels of *cefEF_v1* were reduced compared to the previous expression protocol (**Section 2.2**). Nevertheless, any reduction in expression levels was not sufficient to produce soluble protein.

In order to further reduce “leaky” production of DAOCS/DACS, expression of pNIC_DD was moved from BL21(DE3) to the Lemo21(DE3) *E. coli* expression strain. In theory, expression of the bacteriophage T7 RNAP gene in DE3 strains of *E. coli* is regulated by the *lacUV5* promoter and so should be selectively induced by IPTG. In reality, uninduced (basal) expression of T7 RNAP occurs at low levels. T7 RNAP is 5-fold more efficient at transcription compared to *E. coli* RNAP,¹³² so even small amounts of T7 RNAP can result in substantial expression of target genes from pET vectors. Basal T7 RNAP expression can be countered by the concurrent expression of a T7 lysozyme, the natural inhibitor of T7 RNAP.¹³³ Expression of T7 lysozyme suppresses basal T7 RNAP activity, thereby limiting “leaky” expression of the target gene.

Lemo21(DE3) was selected as the preferred T7 lysozyme-producing strain. This cell strain produces a form of the enzyme, lysY, which lacks amidase activity, reducing the risk of non-specific

protein degradation.¹³⁴ Expression of the *lysY* gene is under the control of the well-titratable L-rhamnose promoter on the pLysY plasmid, which is maintained by chloramphenicol. In this system, expression from pET vectors can be screened across a range of T7 RNAP activities by varying the concentration of L-rhamnose.^{135, 136}

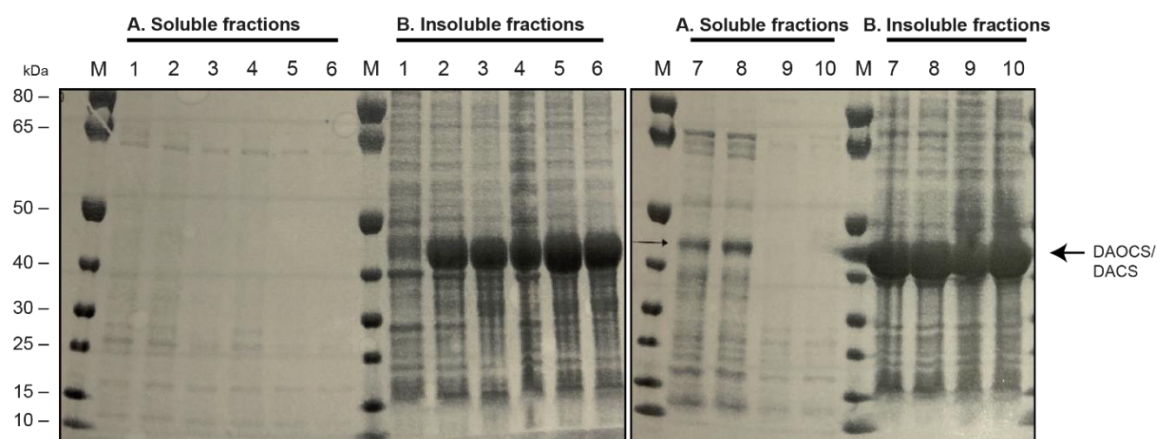
Initially, expression of *cefEF_v1* in Lemo21(DE3) was screened in the absence of L-rhamnose to explore the native T7 lysozyme activity of the strain. In brief, Lemo21(DE3) hosts carrying a pNIC_DD recombinant were grown at 37 °C for 5 h, before 1 mL was used to inoculate 100 mL of the same media. The main cultures were grown as before to an OD₆₀₀ = 0.6 before being induced with IPTG. Cells were collected by centrifugation and pellets resuspended in BugBuster™ Protein Extraction Reagent. As before, batch method Ni Sepharose resin purification was performed. Elution and insoluble fractions were analysed by SDS-PAGE. Conditions screened are reported in Table 2.3.

Table 2.3 | Growth and induction conditions screened for the expression of pNIC_DD in Lemo21(DE3).

[IPTG] (mM)	Post-induction temperature (°C)	Post-induction time (h)
0	25, 30, 37	16
0.1	18, 25, 30, 37	3, 16
0.5	25, 30, 37	3, 16

A representative SDS-PAGE gel of DAOCS/DACS production is given below (Figure 2.3). At 25 °C and above, DAOCS/DACS aggregated as before (lanes **B.1–B.6** and **B.9–B.10**). At 18 °C and 0.1 mM IPTG, bands roughly corresponding to the mass of DAOCS/DACS were observed in the elution fractions (lanes **A.7** and **A.8**). However, the expected mass (43,329 Da) could not be observed when these samples were analysed by LC-MS.

Starter culture growth limited to log phase. Induction for only 3 h



M. PageRuler™ Prestained Protein Ladder

Lane	Growth condition	Lane	Growth conditions
1.	0 mM IPTG, 30 °C, 3 h	7.	0.1 mM IPTG, 18 °C, 16 h
2.	0.1 mM IPTG, 30 °C, 3 h	8.	0.1 mM IPTG, 18 °C, 16 h
3.	0.5 mM IPTG, 30 °C, 3 h	9.	0 mM IPTG, 25 °C, 16 h
4.	0 mM IPTG, 37 °C, 3 h	10.	0.1 mM IPTG, 25 °C, 16 h
5.	0.1 mM IPTG, 37 °C, 3 h		
6.	0.5 mM IPTG, 30 °C, 3 h		

Figure 2.3 | DAOCS/DACS production from Lemo21(DE3) hosts carrying a pNIC_DD recombinant expressed without L-rhamnose. Panel A Elution fractions following Ni Sepharose resin purification. **Panel B** Insoluble fractions, prepared by resuspending cell pellets following cell lysis and clarification by centrifugation. Conditions for lanes 1–10 are given in the key (below).

Additional screening rounds in Lemo21(DE3) sought to minimise the expression of *cefEF_v1*. If soluble protein could not be obtained under these minimised growth conditions it was to be considered unachievable. To this end, the following modifications were introduced:

- Addition of L-rhamnose (0–2 mM) to main cultures to modulate T7 RNAP activity.
- Supplementing starter cultures with glucose (1% w/v), which has been shown to reduce uninduced T7 RNAP expression via catabolite repression.¹²⁹
- Early induction at OD₆₀₀ = 0.4, rather than 0.6.

A brief summary of the minimised expression protocol is given below:

Lemo21(DE3) hosts carrying a pNIC_DD recombinant were grown to log phase (2 h) at 37 °C and 200 rpm. Culture was supplemented with glucose (1% w/v). 200 µL of the culture was inoculated into 10 mL of the same media containing L-rhamnose (0, 0.5, 1 or 2 mM). Cultures were grown as before to an OD₆₀₀ = 0.4, then cooled at 18 °C for 30 min and induced with IPTG (0.4 mM) for 3 h at 18 °C and 250 rpm. Finally, the cells were harvested by centrifugation, resuspended in BugBuster™ Protein Extraction Reagent, and purified using the same Ni Sepharose resin batch method as before. Elution and insoluble fractions were analysed by SDS-PAGE.

SDS-PAGE analysis revealed a single protein band visible across all elution fractions (Figure 2.4). The band was observed in cultures independent of L-rhamnose concentrations or glucose supplementation (Figure 2.4, Lanes **A.1–4** and **B.1–4**). No insoluble expression was observed at L-rhamnose concentrations greater than or equal to 0.5 mM (Figure 2.5, Lanes **A.3–A.4** and **B.2–B.4**).

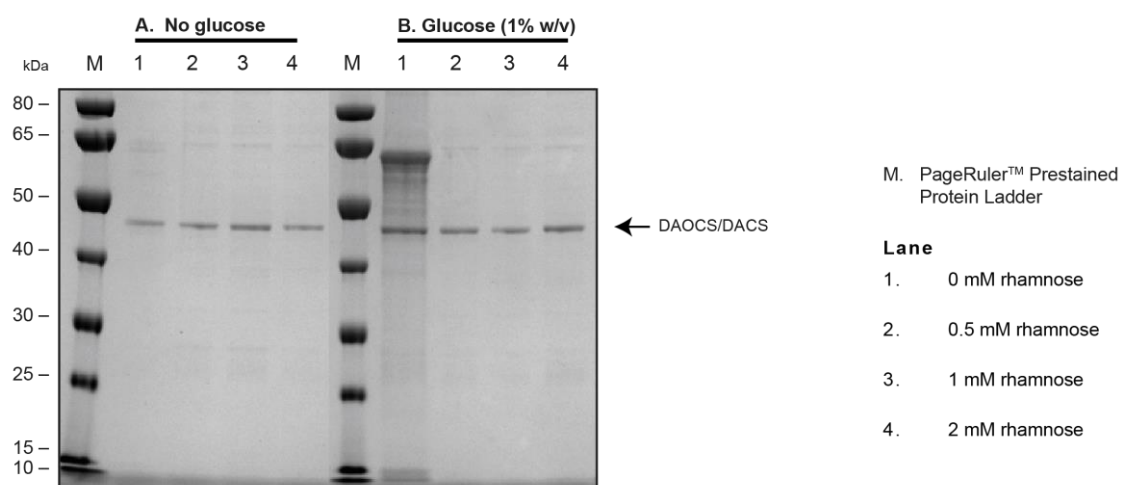


Figure 2.4 | Elution fractions following Ni Sepharose resin purification of clarified cell lysates by batch method. In all cases, cultures were induced with IPTG (0.4 mM) for 3 h at 18 °C, with the amount of L-rhamnose varied. **Lanes 1–4** represent cultures grown at different L-rhamnose concentrations as indicated in the key (RHS). In **Panel A**, starter cultures were not supplemented with glucose. **Panel B** cultures were supplemented with glucose (1% w/v).

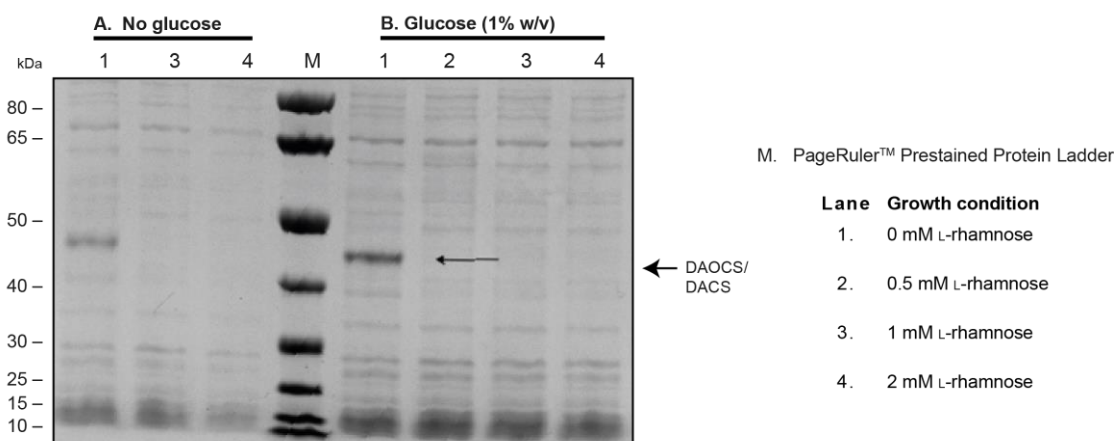


Figure 2.5 | SDS-PAGE analysis of insoluble fractions from minimised growth cultures. Sample A.2 (0.5 mM rhamnose) was lost during preparation and was therefore not included in the gel analysis.

An elution fraction (lane A.1) was analysed by LC-MS (Figure 2.6). A minor peak at 43,330 Da was thought to correspond to DAOCS/DACS (predicted mass: 43,329 Da), but the major peak at 43,194 Da could not be readily identified, even after considering potential glucosylation or fragmentation of C- or N-terminal residues.

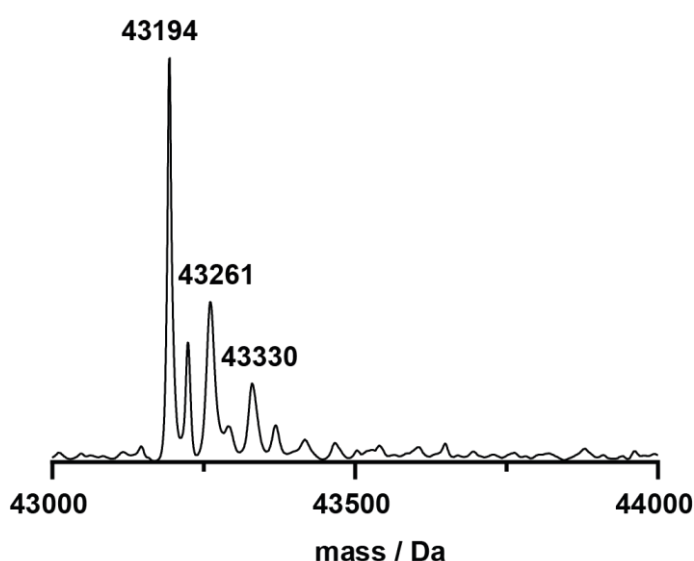


Figure 2.6 | Mass of protein isolated from the batch method Ni Sepharose resin purification of pNIC_DD expression trials in Lemo21(DE3). Sample was analysed using ESI-MS. Mass deconvolution was achieved using MassLynx MS software. Calculated mass for DAOCS/DACS produced from pNIC_DD: 43,329 Da.

Expression of pNIC_DD in Lemo21(DE3) was scaled up to a total culture volume of 7.5 L and a two-column FPLC protocol involving IMAC and size-exclusion chromatography (SEC) was employed. DAOCS/DACS was afforded in very low yield (0.06 mg/L) and moderate purity as judged by SDS PAGE analysis. Incomplete cleavage of the His₆-tag by TEV protease contributed to the poor yield,

as did protein aggregation that was observed throughout the purification, especially following protein concentration. No turnover was observed by LC-MS under reported assay conditions when the isolated DAOCS/DACS was incubated with its natural substrate penicillin N. By contrast, the bacterial enzyme DAOCS, expressed recombinantly using a pET vector in BL21(DE3),⁴⁴ did convert penicillin N. Thus, whilst DAOCS/DACS was produced in a soluble form, the enzyme was inactive and was obtained in yields lower than previously reported literature strategies.^{80, 124-126}

2.4 Producing soluble DAOCS/DACS from codon-optimised *cefEF*

Expression of *cefEF_v1* produced DAOCS/DACS that was prone to aggregation even with minimal expression levels. This observation refuted the existing hypothesis that aggregation was caused by the accumulation of DAOCS/DACS to excessive levels during expression screens. Instead, these findings suggest that DAOCS/DACS produced from *cefEF_v1* has an intrinsic tendency to misfold. Analysis of the *cefEF_v1* sequence revealed it exhibited a codon preference that was poorly suited for its expression in an *E. coli* host (Figure 2.7). Different species display different codon preferences,¹³⁷ and overexpression of a heterologous gene in *E. coli* that contains codons that are rarely used by the latter can cause protein misfolding, loss of activity, aggregation, and inclusion body formation.¹³⁸⁻¹⁴¹ The primary cause of these aberrant effects is translational stalling, resulting from poor availability of tRNAs that recognise these rare codons.¹⁴² Codon optimisation, in which codons in the target gene are replaced with those most abundant in the expression host, is an effective means of overcoming biased codon usage and preventing translational stalling.¹⁴³ This method has been applied successfully,¹⁴⁴ but can also alter translational kinetics unfavourably, leading to misfolding of the target protein in *E. coli*.^{145, 146} The *cefEF* gene originates from the eukaryotic fungus *A. chrysogenum*. Given the large phylogenetic distance between *A. chrysogenum*, and the prokaryotic *E. coli* expression host, it was likely that their codon preferences deviate significantly. Thus, codon optimisation of *cefEF* was explored as a strategy for overcoming insoluble expression.

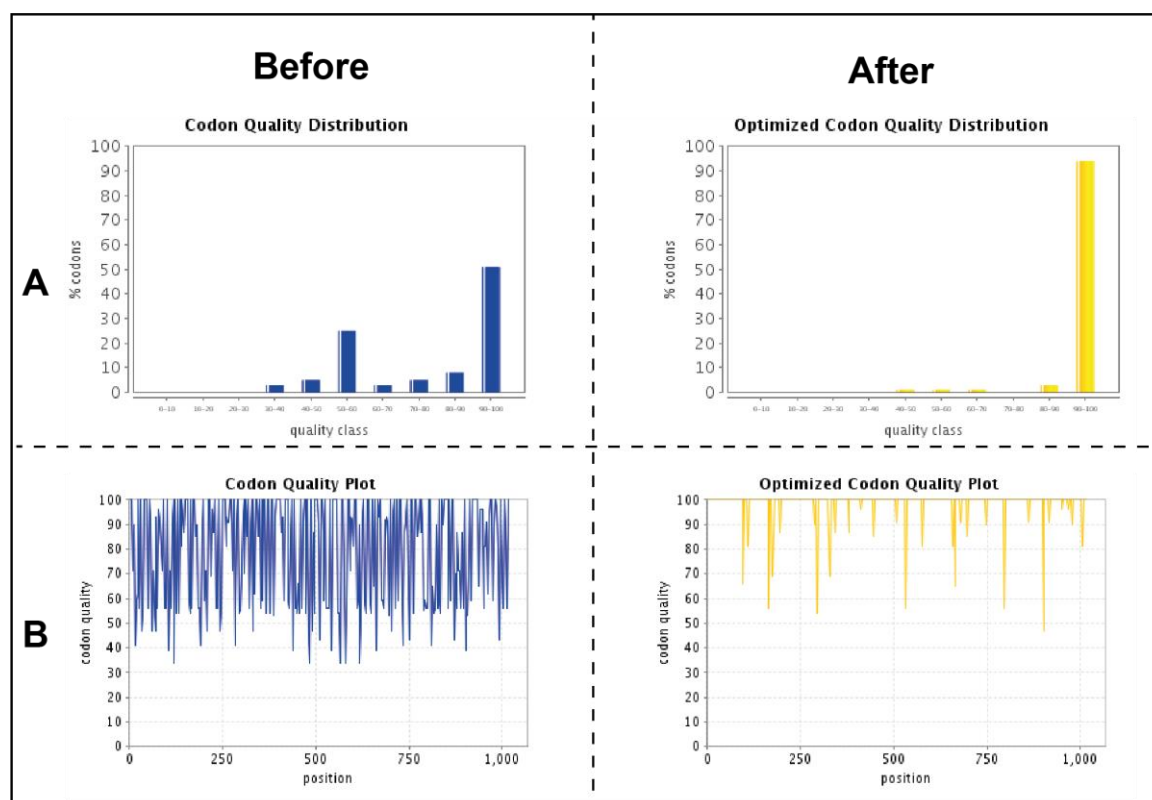


Figure 2.7 | Graphs showing the nature of *cefEF_v1* sequence codons before (blue) and after (yellow) codon optimisation for *E. coli* expression. A Histogram representation. Each codon is assigned to a quality class given a rating based on its frequency of usage by *E. coli*. The most frequently used codon for an amino acid is set to 100, other codons are scaled accordingly. **B** The plots show the quality of the codon used at the given position in the gene sequence. Original *cefEF_v1* (blue) contains a wide range of codon qualities across all positions. After optimisation (yellow), codons are almost exclusively of the highest quality. Sequence optimisation was carried out using software supplied by ThermoFisher during gene synthesis. The figure was prepared using ThermoFisher GeneArt software.

In addition to possessing low codon quality for *E. coli* expression, *cefEF_v1* does not contain a stop codon. As such, transcription of the open reading frame extends to the stop codon supplied by the pNIC_DD expression vector. Consequently, DAOCS/DACS was produced with additional C-terminus residues which may have promoted misfolding. Interactions of the C-terminal region with the active site have been proposed as a mechanism by which the related bacterial enzyme DAOCS aggregated during heterologous expression in *E. coli*.¹⁴⁷

A new version of the *cefEF* gene (*cefEF_v2*) was produced based on the wild-type *cefEF* sequence.⁵⁶ Sall and NdeI restriction sites were added 5' to the ORF with the latter incorporated into the translation initiation site. A NotI site was also introduced at the 3' end, immediately after the stop codon. This sequence was then codon optimised for expression in *E. coli* using

ThermoFisher's GeneArt software. The translated protein sequence was identical to the DAOCS/DACS sequence deposited in UniProt (P11935).

The *cefEF_v2* sequence was subcloned into the pET24a(+) vector, which had been used previously to produce DAOCS/DACS.⁸⁰ Subcloning was achieved using a standard restriction-ligation protocol via NdeI and NotI sites to give pET_AC*. pET24a(+) contains an N-terminal T7 tag, which is lost when subcloning via the NdeI site. A C-terminal His₆-tag was also present, but was not expressed as the *cefEF_v2* insert contains a stop codon. DAOCS/DACS was therefore produced in an untagged form. In addition to pET24a(+), *cefEF_v2* was also subcloned into a pCold vector. Expression of the target gene in pCold vectors is regulated by the cold-shock Protein A (*cspA*) promoter.¹⁴⁸ At low temperatures (10–25 °C), *E. coli* halts “normal” protein production and exclusively upregulates the expression of 13 cold-shock proteins, all of which are regulated by the *cspA* promoter.^{149, 150} Expression of target genes from pCold vectors is induced at lower temperatures. This procedure had two main benefits: the target protein is typically obtained in higher purity, as expression of native *E. coli* proteins is suppressed; and the lower induction temperature also promotes correct protein folding and therefore protein solubility.¹⁵¹ So although total expression levels from the *cspA* promoter are lower than those from the T7 promoter, soluble expression can be higher. As such, pCold vectors are well suited to producing aggregate-prone proteins not amenable to more conventional methods,¹⁵² a desirable feature given the historical production of DAOCS/DACS in an insoluble form.⁵⁶

cefEF_v2 was also subcloned using Sall-NotI site into a modified version of pCold I (Addgene #41096), which contained an N-terminal His₆-[3C human rhinovirus (HRV3C) protease cleavage site]-tag.¹⁵³ This vector was preferred over the pCold I vector since highly efficient cleavage of the His₆-tag with HRV3C protease had previously been achieved using this construct to express IPNS.^{154, 155} Incomplete His₆-tag cleavage from DAOCS/DACS expressed from pNIC_DD had resulted

* AC stands for *Acremonium chrysogenum*, the species from which DAOCS/DACS is obtained.

in reduced yields. It was envisioned this could be avoided by using the pCold system. The resulting construct was called pCold_AC.

pET_AC and pCold_AC constructs were expressed in *E. coli* BL21(DE3) hosts grown under a range of conditions. Cultures expressing pET_AC, which produced un-tagged DAOCS/DACS, were analysed by their total cell extracts (unclarified lysate). For pCold_AC cultures, His₆-tagged DAOCS/DACS was purified using the Ni Sepharose resin batch method established previously. Soluble and insoluble expression was analysed by SDS PAGE (Figure 2.8). In pCold_AC cultures, high-levels of soluble DAOCS/DACS were produced across all growth conditions (lanes **A.1–A.5**). Insoluble expression of DAOCS/DACS from pCold_AC was also observed (lanes **B.1–B.5**). High levels of expression from *cefEF_v2* were also observed in pET_AC cultures.

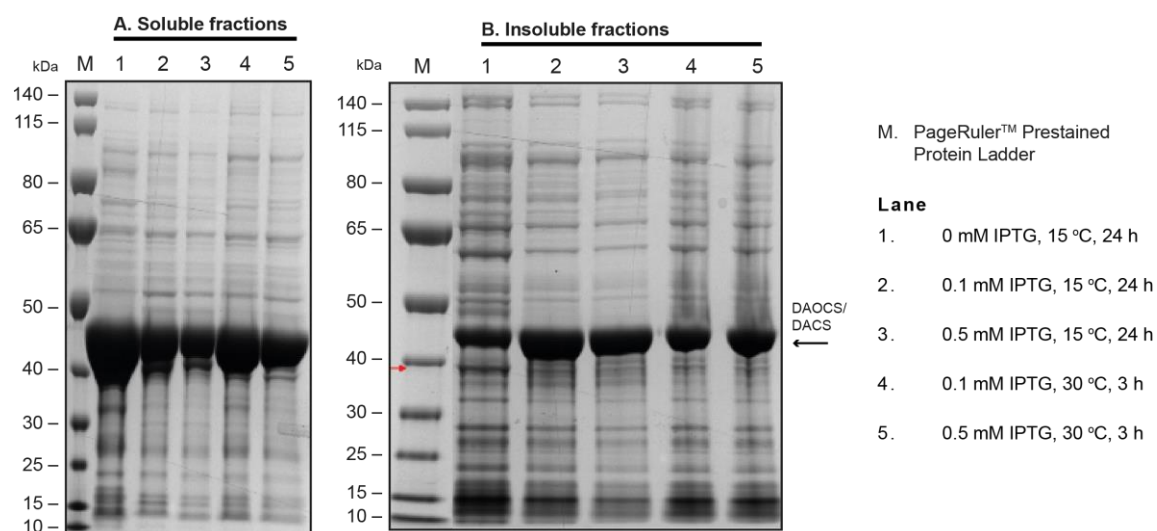


Figure 2.8 | SDS-PAGE analysis of DAOCS/DACS produced in BL21(DE3) from the pCold_AC construct. The molecular ladder, column M, is PageRuler™ Prestained Protein Ladder, 10 to 180 kDa. Key of conditions is on the RHS. **A** Elution fractions from the Ni-bead purification protocol that show high-levels of soluble expression of *cefEF_v2* from the pCold_AC construct. **B** Insoluble expression of *cefEF_v2* was also observed.

2.5 Large-scale production and purification of DAOCS/DACS

The pCold_AC construct was over-expressed in BL21(DE3) using the optimised conditions (Figure 2.8, lane **A.4**) in 7.2 L of culture. Purification of DAOCS/DACS was achieved by IMAC and SEC. In brief, clarified cell lysate was loaded onto a HisTrap HP column (Cytiva, 5 mL) and the target protein was eluted using an imidazole step gradient. His₆-tag cleavage was achieved by overnight incubation of pooled fractions containing purified enzyme with the HRV3C protease (1:100 w/w).

The cleavage mixture was loaded onto a second HisTrap column and untagged DAOC/DACS was isolated in the flowthrough fractions. Further purification on a Superdex S75 column (GE Healthcare, 300 mL) afforded DAOCS/DACS in high purity (>95% by SDS-PAGE) and good yield (6.3 mg/L). LC-MS analysis confirmed the presence of DAOCS/DACS (predicted mass: 36,984.6 Da; observed mass: 36.984.6 Da). A summary of the FPLC purification is given in Figure 2.9.

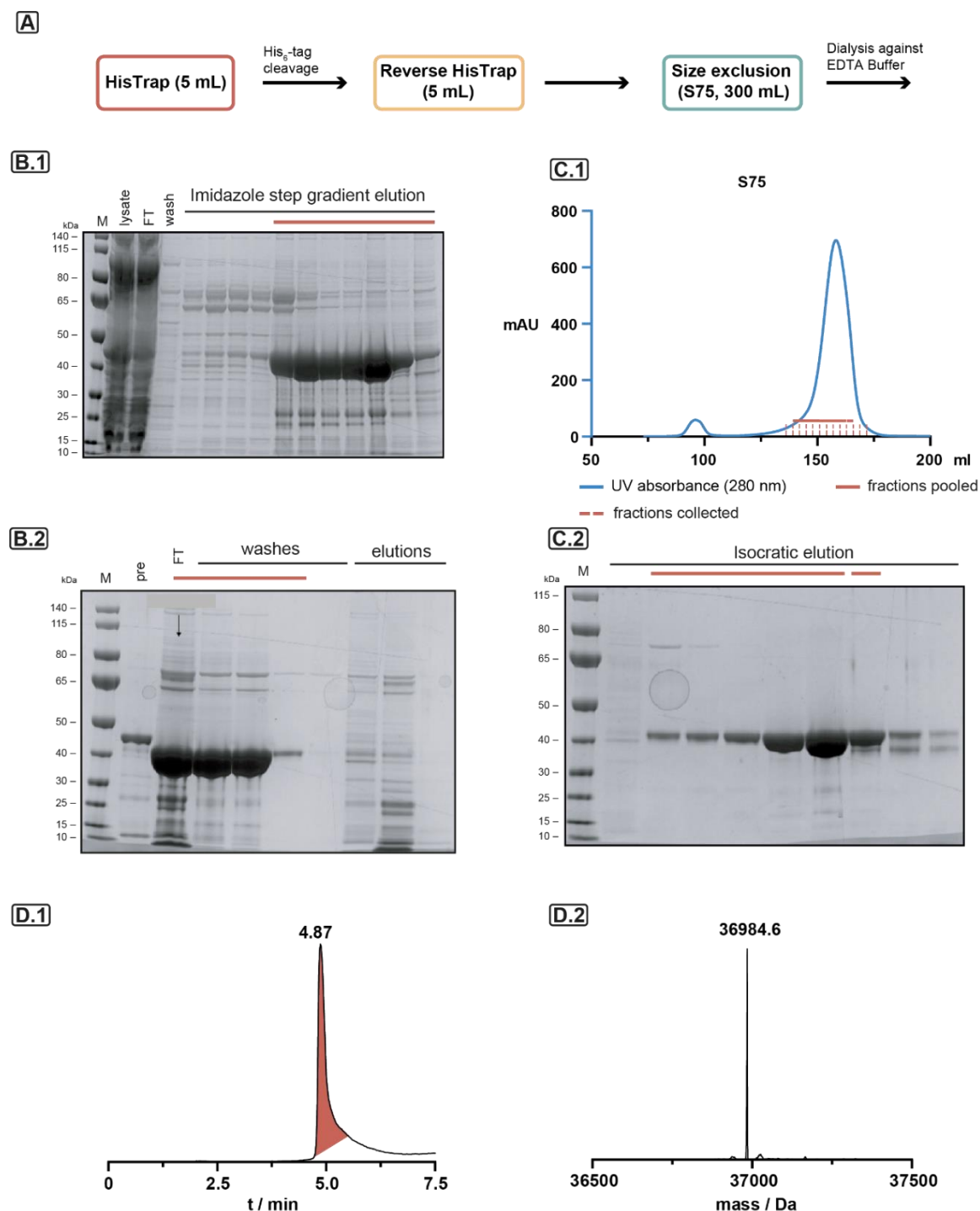


Figure 2.9 | FPLC purification of DAOCS/DACS produced using the pCold_AC construct in BL21(DE3) cells. Panel A summary of the purification workflow. **Panel B** SDS-PAGE gels of HisTrap purifications, normal (**B.1**) and reverse (**B.2**). The molecular ladder (column **M**) is PageRuler™ Prestained Protein Ladder, 10 to 180 kDa. Pooled fractions are highlighted under the red bar. **Panel C** Size-exclusion elution profile (**C.1**) and corresponding SDS-PAGE gel (**C.2**). **Panel D** LC-MS analysis of the isolated protein. A single peak at 4.87 min was observed in the chromatogram (**D.1**) with a mass corresponding to DAOCS/DACS (after mass deconvolution using the MassLynx 4.2 MS software) given in **D.2** (predicted mass: 36,984.6 Da).

DAOCS/DACS was also produced using the pET_AC construct and was purified by anion-exchange and size-exclusion chromatography. A summary of the purification is given below (Figure 2.10).

LC-MS analysis (**Panel D**) showed a minor peak corresponding to DAOCS/DACS (expected mass:

36,479 Da) and a major peak at 36,349 Da, which likely corresponds to the loss of an N-terminal methionine (–130 Da) via N-terminal methionine excision (NME).¹⁵⁶

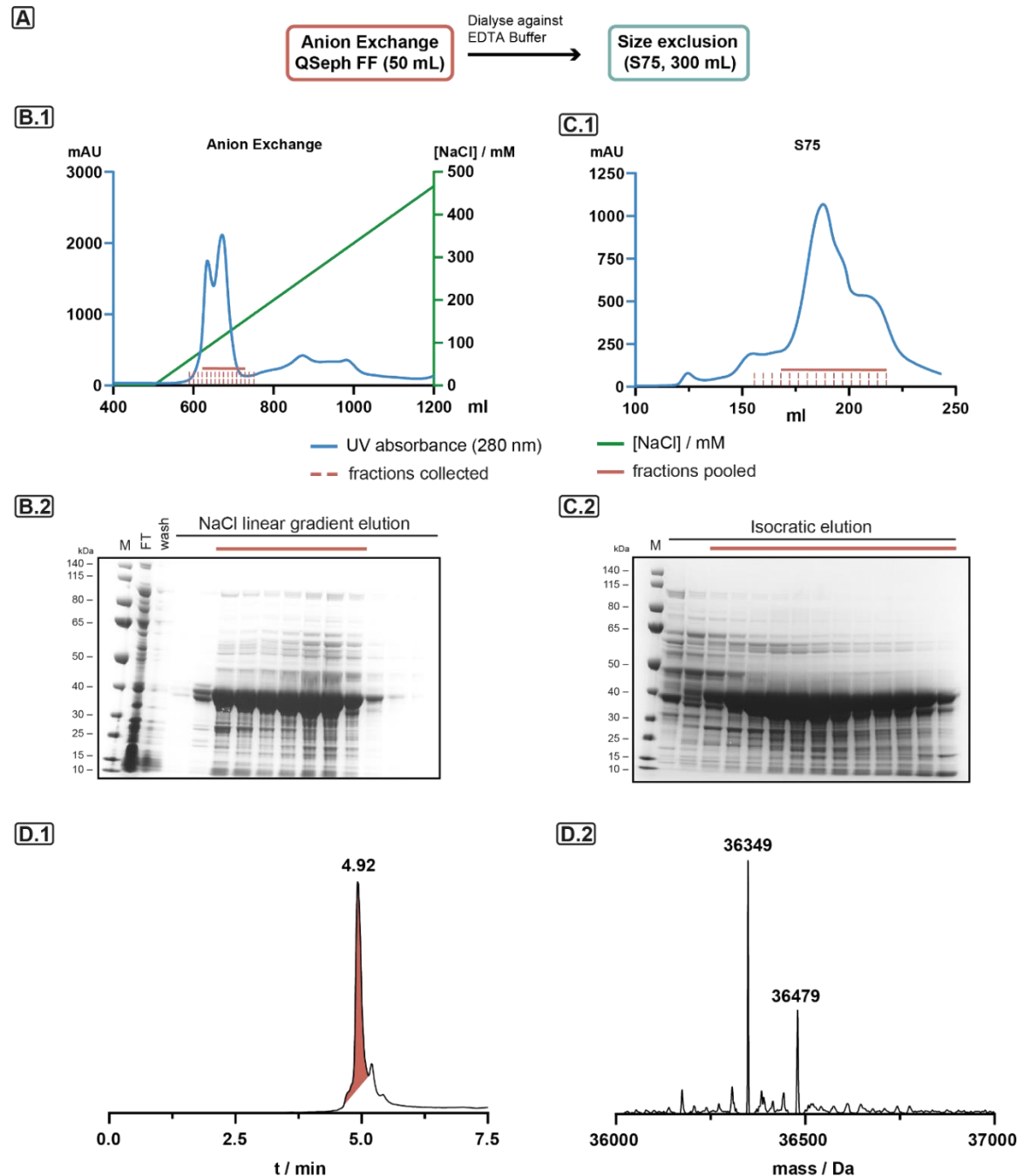


Figure 2.10 | FPLC purification of DAOCS/DACS produced in BL21(DE3) cells using the pET_AC construct. Panel A Purification workflow summary. The recombinant protein did not contain an affinity tag, so affinity chromatography could not be utilised. **Panel B** Elution profiles for anion-exchange (**B.1**) and the corresponding SDS-PAGE gel (**B.2**). The molecular ladder (column M) is PageRuler™ Prestained Protein Ladder, 10 to 180 kDa. **Panel C** Size-exclusion chromatography elution profile (**C.1**) and SDS-PAGE analysis (**C.2**). **Panel D** LC-MS analysis of isolated protein. The peak at 4.92 min (**D.1**) gave the deconvoluted mass spectrum (using MassLynx 4.2 MS software) in **D.2**. The minor peak at 36,479 Da corresponds with the expected mass of DAOCS/DACS. The major 36,349 Da peak (–130 Da) likely corresponds to the loss of an N-terminal methionine (predicted mass: 36,348 Da).

DAOCS/DACS was isolated in higher purity using the pCold_AC vector compared to the pET_AC vectors (Figure 2.9, **C2** cf. Figure 2.10, **C2**), highlighting the benefits of IMAC purification. Conversely, pET_AC (18.4 mg/L) afforded DAOCS/DACS in higher yield than pCold_AC (6.3 mg/L). DAOCS/DACS produced from both pCold_AC and pET_AC was shown to convert its natural substrate, penicillin N, to DAOC and DAC under assays conditions established for DAOCS.⁴⁴

2.6 Changing the selection marker of pCold_AC

Whilst DAOCS/DACS was purified from pCold_AC in high yield and purity, its further use was potentially undermined by the selection marker it employed. All pCold constructs utilise ampicillin resistance for selection during growth. This resistance is conferred by the production of a serine β -lactamase, namely TEM-1, from the *ampR* gene.¹⁵³ Contamination of DAOCS/DACS with TEM-1 would undermine biochemical characterisation of the enzyme, as both penicillin substrates and cephalosporin products would be hydrolysed.

The presence of TEM-1 contaminants in DAOCS/DACS obtained from pCold_AC was investigated in two ways. Firstly, DAOCS/DACS was incubated with penicillin N with and without a β -lactamase inhibitor (BLI). Two BLIs were tested, clavulanic acid and avibactam^{157, 158} (Figure 2.11, **Panel A**). Substrate conversion appeared to be independent of the addition of a BLI to the assay, suggesting no β -lactamase contamination (See Chapter 4, **Section 4.2**). Secondly, DAOCS/DACS was incubated with nitrocefin, a chromogenic cephalosporin that changes colour when hydrolysed (Figure 2.11, **Panel B**).¹⁵⁹ Following incubation with the protein sample for 2 h at 25 °C, a yellow to red colour change was observed, indicating nitrocefin hydrolysis and therefore the presence of a β -lactamase (Figure 2.11, **Panel C**).

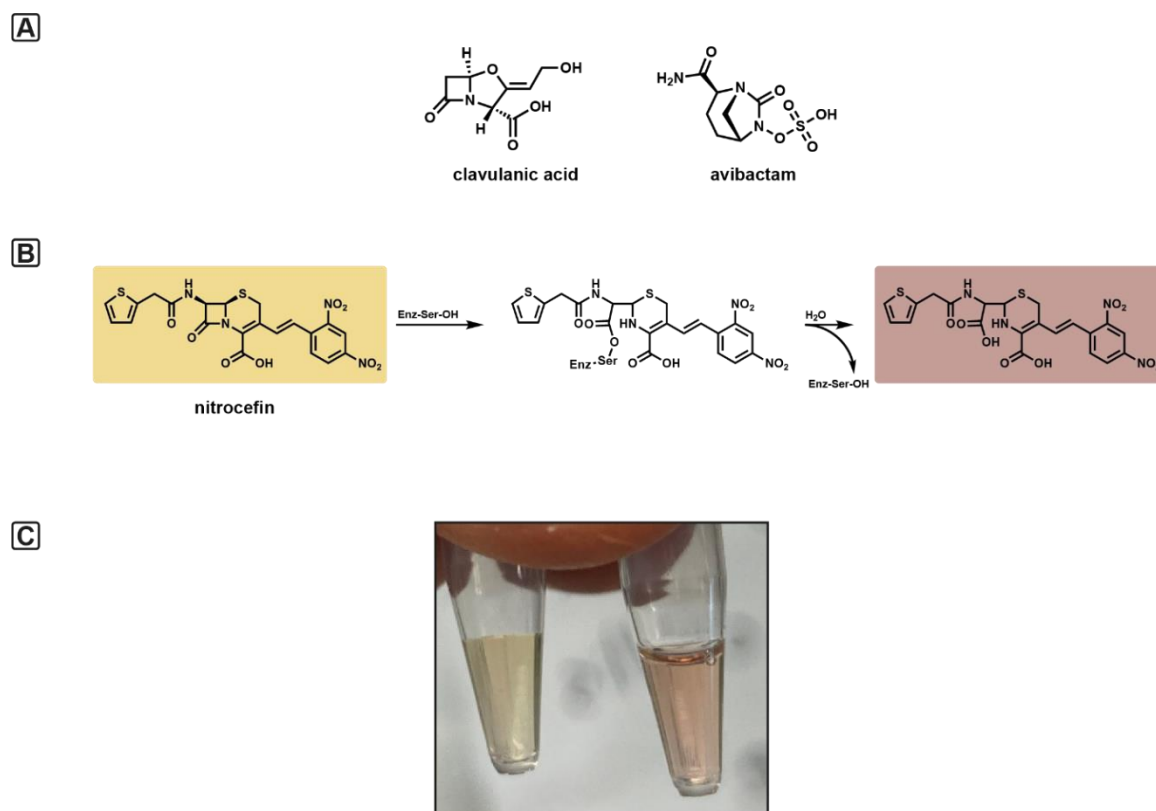


Figure 2.11 | Investigation of β -lactamase contamination in DAOCS/DACS obtained from pCold_AC. Panel A The structure of the two β -lactamase inhibitors used to test for β -lactamase contamination. Panel B Scheme for the degradation of the chromogenic cephalosporin, nitrocefin, and the observed colour change (yellow to red). Panel C The appearance of a red colour when DAOCS/DACS was incubated with nitrocefin (**right**). This colour change was not observed when nitrocefin was incubated in buffer in the absence of DAOCS/DACS (**left**).

In addition to TEM-1 expression from pCold_AC, other origins of the potential β -lactamase contamination were considered. The size-exclusion column used during purification of DAOCS/DACS is also used to purify recombinantly produced β -lactamases. However, the column was washed with 1 M NaOH prior to use to remove any residual protein from previous purifications, making this source of contamination unlikely. In the absence of alternatives, it appeared that TEM-1 was most likely to be responsible for the observed contamination.

Whilst β -lactamase contamination did not appear to affect the specific penicillin N assays tested, its impact on other biochemical or crystallographic experiments was unknown. Accordingly, it was decided to eliminate β -lactamase contamination before moving forward. To achieve this, the ampicillin resistance gene was replaced with a kanamycin selection marker. Kanamycin resistance is conferred through the expression of an aminoglycoside phosphotransferase that does not interact with β -lactam species.¹⁴⁷ Gibson Assembly was the preferred molecular cloning method

to achieve resistance marker substitution. Using this method, DNA fragments can be cloned into a vector rapidly and without the use of restriction enzymes.¹⁶⁰ Linearized vector and DNA fragments are designed with homologous overlapping ends. These fragments are combined with the Gibson Assembly Master Mix, which performs 5' end processing to permit the annealing of homologous ends. Extension and ligation to afford the assembled product then follows. Primers for the production of linearised pCold_AC and a kanR fragment from pET24a(+) were designed using the NEBuilder® Assembly Tool and are given in Table 2.4. pCold_AC primers were designed to exclude the *ampR* gene in the linearized product.

Table 2.4 | Sequences for primer sets used for Gibson Assembly of *kanR* gene into pCold_AC. Sequences are colour coded to show homologous regions across primer sets.

Primer Name	Sequence
pCold_AC_fwd	GTTTTTCTAACTGTCAGACCAAGTTTAC
pCold_AC_rev	TATGGCTCATACTCTTCCTTTTCAATATTATTG
kanR_fwd	AAGGAAGAGTATGAGCCATATTCAACGG
kanR_rev	GGTCTGACAGTTAGAAAACTCATCGAGC

Gibson Assembly was performed using standard protocols. Successful substitution of *ampR* with *kanR* in the construct was confirmed by colony PCR and Sanger sequencing. The new kanamycin-resistant pCold construct was named pCold_kanR_AC. To ensure the *ampR* gene had been removed, the newly isolated pCold_kanR_AC constructs were freshly transformed into XL10 Gold ultracompetent *E. coli* cells and grown on ampicillin agar plates. No colonies were observed after overnight incubation on ampicillin plates, but colonies did grow when the same transformation mixture was incubated overnight on kanamycin agar plates.

As with the pCold_AC construct, expression of pCold_kanR_AC in BL21(DE3) produced active DAOCS/DACS across a range of growth conditions. Protein production was increased in scale to 7.2 L of culture media under optimal expression conditions (0 mM IPTG, 15 °C, 24 h). DAOCS/DACS was purified in the same manner as pCold_AC except that cell lysis was achieved using high

pressure homogenisation, rather than by sonication. In addition, apo-DAOCS/DACS was prepared by dialysing the sample against an EDTA/1,10-phenanthroline buffer prior to SEC. A summary of the purification is given in Figure 2.12.

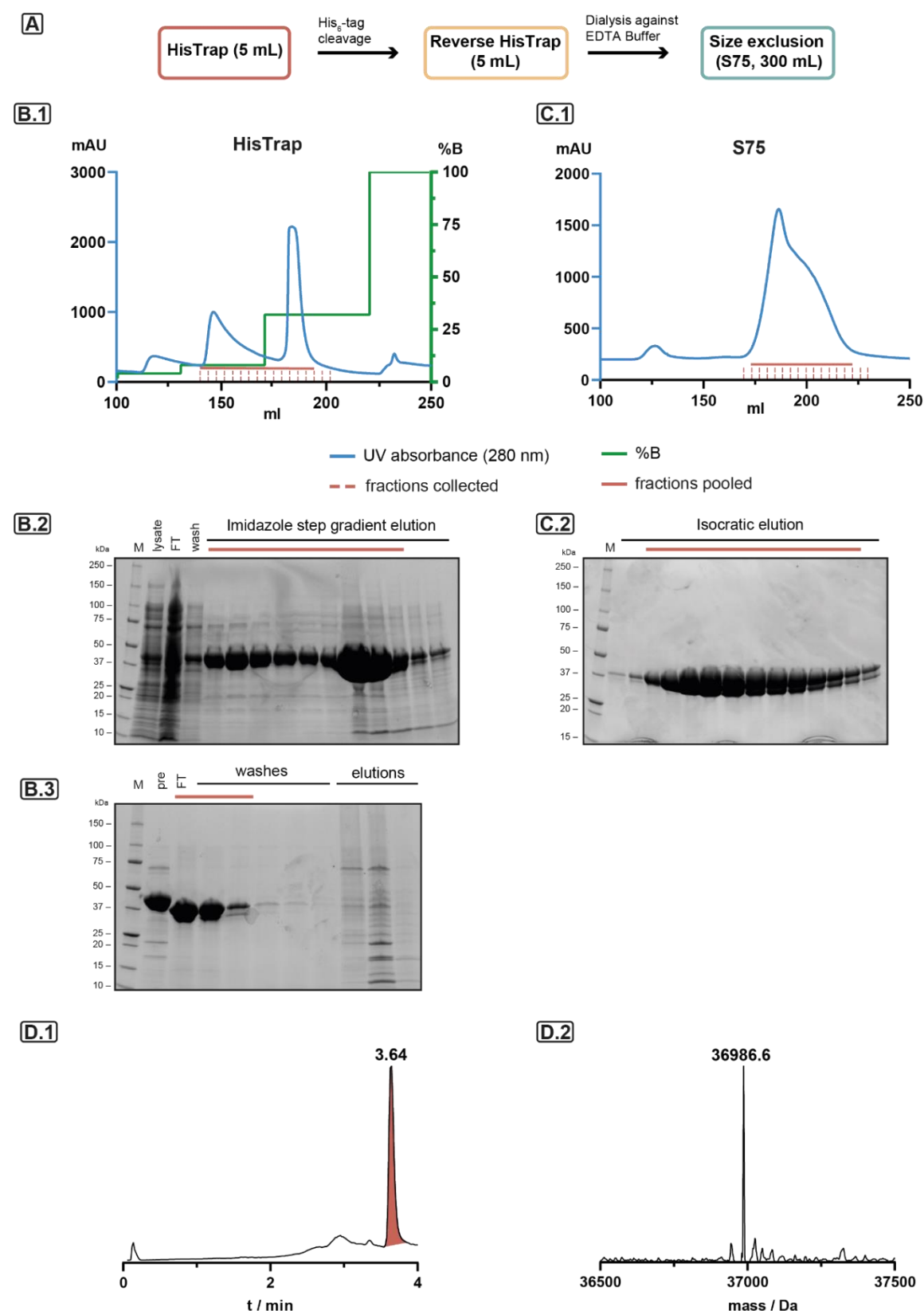


Figure 2.12 | FPLC purification of DAOCS/DACS produced using the pCold_kanR_AC construct in BL21(DE3) cells. Panel A Purification workflow. This represents the optimised protocol in which the apoenzyme is prepared after His₆-tag cleavage and before size-exclusion chromatography. 1,10-phenanthroline and EDTA are removed on the S75 column. **Panel B** HisTrap purifications including HisTrap elution profiles (**B.1**) and SDS-PAGE analysis of fractions from normal (**B.2**) and reverse HisTrap (**B.3**). In **B.1**, 0 and 100% B corresponds to 20 mM 500 mM imidazole respectively. **Panel C** Gel-filtration chromatography, with elution profile (**C.1**) and SDS-PAGE analysis of fractions (**C.2**) HisTrap (**C.1**). LC-MS analysis is shown in **Panel D** Expected mass: 36,984.6 Da, observed mass: 36,986.6 Da (**D.2**).

2.7 Discussion

Crystallographic and substrate analogue studies on DAOCS/DACS from *A. chrysogenum* were planned, with both techniques requiring large amounts of high-purity DAOCS/DACS. Whilst the recombinant production of DAOCS/DACS had been reported several times in recent years, all production regimes utilised expression and purification methodologies originally developed in 2004. As such, there was scope to optimise the production of DAOCS/DACS in *E. coli*.

A version of the *cefEF* gene (*cefEF_v1*) had been produced by a previous Schofield group member and was readily available. However, expression of *cefEF_v1* using a pET-derived vector (pNIC_DD) produced insoluble DAOCS/DACS. A thorough optimisation of expression conditions failed to yield soluble protein. Whilst initially considered to be a by-product of excessively high levels of DAOCS/DACS, issues of misfolding and aggregation persisted under minimised expression conditions in Lemo21(DE3), implying the cause to be the intrinsic instability of the target protein. Given that pET vectors and the BL21(DE3) expression host system had been used previously to yield soluble DAOCS/DACS, the *cefEF_v1* gene was highlighted as the major variable. Sequence analysis of *cefEF_v1* revealed that it shared no homology with the wild-type *cefEF* sequence and was not codon-optimised for *E. coli* expression. A new, codon-optimised version of the *cefEF* gene (*cefEF_v2*) was produced. Expression of *cefEF_v2* gave soluble DAOCS/DACS in high-yield and purity using a range of constructs, summarised in Table 2.5. A flowchart of the optimisation of DAOCS/DACS expression is given in Figure 2.13.

Table 2.5 | Summary of constructs from which DAOCS/DACS production was attempted.

Gene version	Construct name	Description
<i>cefEF_v1</i>	pNIC_DD	pNIC28-Bsa4 vector containing <i>cefEF_v1</i>
	pCold_DD	pCold I containing <i>cefEF_v1</i>
<i>cefEF_v2</i>	pET_AC	pET24a(+) containing <i>cefEF_v2</i>
	pCold_AC	Modified pCold I, in which the His ₆ -tag and HRV3C cleavage site was expressed with the target gene, containing <i>cefEF_v2</i>
	pCold_kanR_AC	pCold_AC in which the ampicillin resistance gene is replaced with a kanamycin selection marker

These results clearly indicate that the successful expression of the *cefEF* gene in *E. coli* to give soluble DAOCS/DACS protein is highly sensitive to codon selection.

The benefits of codon optimisation can be most clearly seen when comparing protein produced from pET_AC and by Lloyd *et al.*⁸⁰ Both strategies used the same pET vector and expression host BL21(DE3) and both employed anion-exchange and size-exclusion chromatography. However, yields from pET_AC were 6-fold higher than reported by Lloyd *et al.*

Table 2.6 | Yields of DAOCS/DACS, defined as mg of protein per L of culture, from various expression strategies. Of the six literature reports in which DAOCS/DACS has been recombinantly produced, only strategies 1 and 2 reported yields. The first recombinant expression⁵⁶ produced insoluble protein. The remaining three strategies¹²⁴⁻¹²⁶ utilised an unoptimized *cefEF* gene in pET expression vectors and followed the same purification protocol as strategy 2.

#	Source	Expression	Yield (mg/L)
1	Literature	Baldwin (1992) ¹²¹	1.4
2		Lloyd (2004) ⁸⁰	3.1
3	This work	pCold_AC	6.3
4		pCold_kanR_AC	11.6
5		pET_AC	18.4

Supplementation of the media with glucose appeared to only make a marginal difference in both soluble and insoluble expression of *cefEF_v1* using pNIC_DD. This is likely because cultures containing glucose were only grown to log phase. Uninduced expression is most prominent during stationary phase growth,¹²⁹ therefore the potential glucose effect was limited by the specific growth conditions.

DAOCS/DACS produced using the pCold-derived constructs (pCold_AC and pCold_kanR_AC) contained 5 additional N-terminal residues (5'-GPVDH-3') compared to the wild-type DAOCS/DACS sequence. These residues are a “scar” resulting from cleavage of the His₆-affinity tag by the HRV3C protease. It has been demonstrated that changes to the N-terminal sequence can influence enzyme properties. For example, an N-terminal His₆-tag allowed DAOCS to be crystallised in a monomeric, rather than trimeric, form.⁴² Thus, DAOCS/DACS containing the N-terminal “scar” might display different, perhaps favourable, crystallographic properties compared with DAOCS/DACS from pET_AC, which is identical to the wild-type sequence.

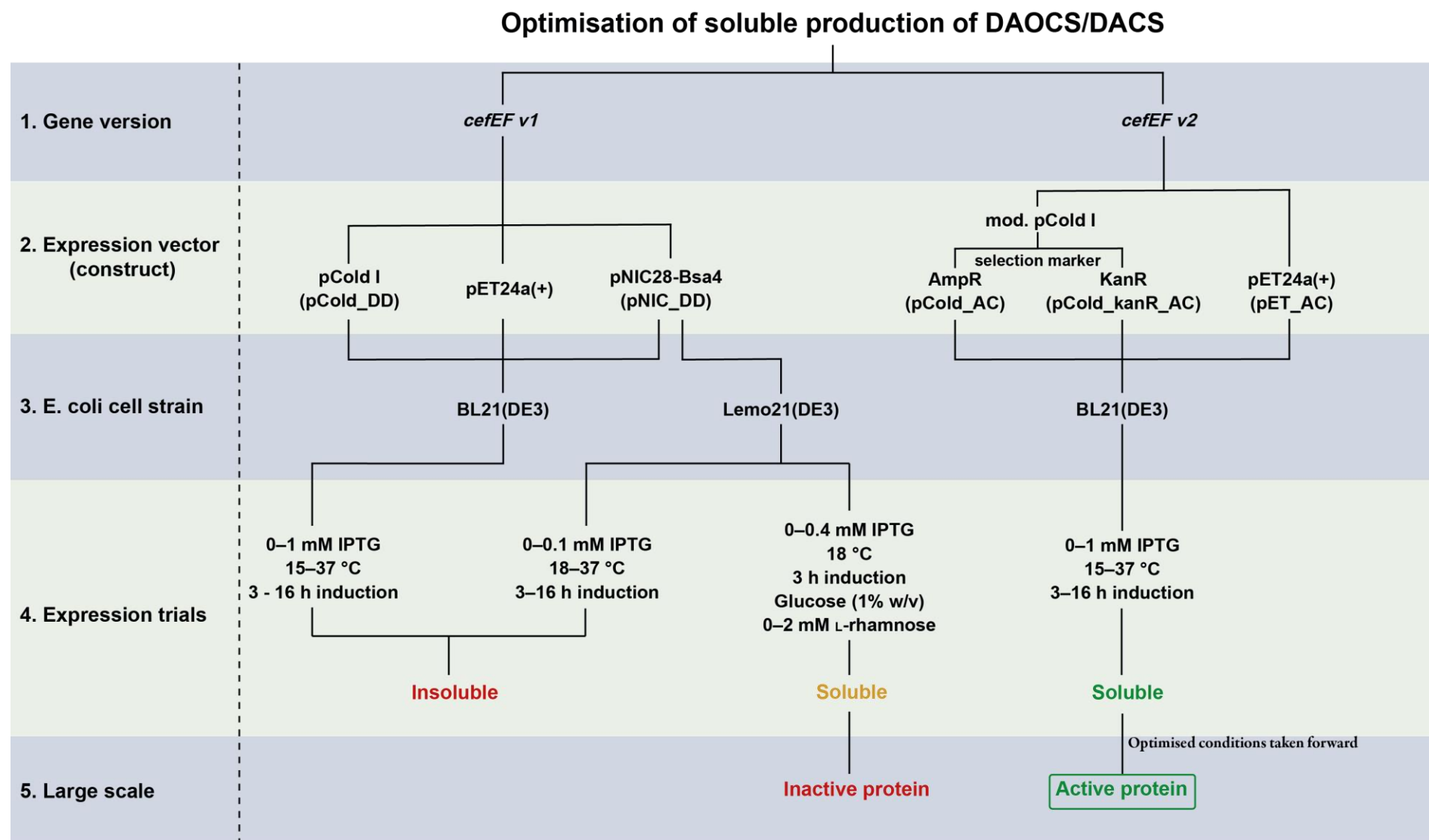


Figure 2.13 | Flowchart for the optimisation of DAOCS/DACS production in *E. coli*.

2.8 Conclusions and future work

After initial setbacks, soluble recombinant DAOCS/DACS was successfully produced in *E. coli* cells in improved yields compared with previous expression strategies (Table 2.6).

Codon optimisation appears to significantly influence the solubility of DAOCS/DACS. Initial expression of an unoptimized *cefEF* gene produced DAOCS/DACS exclusively in an insoluble form. After codon optimising *cefEF* for expression in *E. coli*, soluble forms of DAOCS/DACS were obtained from a range of constructs in yields that compared favourably to previously reported strategies; up to a 13-fold improvement was observed. Isolated DAOCS/DACS displayed high activity towards its natural substrate penicillin N. Whilst soluble expression was achieved, protein aggregation was still observed from pCold_AC and pCold_kanR_AC constructs, suggesting further yield improvements are possible.

The *cspA* promoter has been established as an effective system from which the *cefEF* gene can be expressed to produce soluble protein. This extends previous work on the effect of different promoter regions on the soluble production of DAOCS/DACS.¹²¹ Altering the selection marker of pCold_AC did not appear to influence the yield, purity, or activity of the isolated DAOCS/DACS. This method could be applied to any expression vector containing an ampicillin selection marker conferred by production of TEM-1, increasing the number of vectors compatible with producing β -lactam biosynthesis enzymes.

The isolation of soluble, high purity DAOCS/DACS in a manner amenable to large-scale production expedited biophysical and mechanistic studies on this enzyme. In particular, substrate analogue and crystallographic studies, which require large amounts of highly pure enzyme, became more facile with the improved protein production methods described herein.

Chapter 3

**Incubations of the 3-vinyl cephem substrate analogue with
DAOCS/DACS**

3 Incubations of the 3-vinyl cephem substrate analogue with DAOCS/DACS

3.1 Introduction

3.1.1 3-Vinyl cephem incubations with DAOCS/DACS

As described in the Introduction (**Section 1.5.2**), the 3'-hydroxylation activity of DAOCS/DACS displays great promiscuity and accepts cephem and cepham substrate analogues containing unsaturated groups at the C3 position.

Work carried out in 1990 by Mark E. Wood (MEW) on the 3-vinyl cephem substrate analogue (Figure 3.1, **1**) was of specific interest to this project. When **1** was incubated with DAOCS/DACS, $\approx 46\%$ conversion to a single β -lactam product ("the Wood product") was observed by ^1H NMR.¹⁰⁶ The Wood product was isolated by HPLC and possessed a similar retention time to the starting material (**1**) (Figure 3.1, **A**). Based on the known reactivity of the related iron-dependent cytochrome P450 enzyme superfamily, the cephem epoxide (**2**) was proposed as the product structure (Figure 3.1, **B**), with precedents for DAOCS/DACS epoxidase activity subsequently established for the $[4\text{-}^2\text{H}]$ exomethylene cepham and Z-ethylidene cepham substrates.^{112, 113} However, structure **2** was not supported by the ^1H NMR data of the Wood product, which showed an ABX-type pattern, consistent with a $\text{CH}-\text{CH}_2$ arrangement at the C3' position with electronegative substituents on both carbon atoms (Figure 3.2, **Y** and **Z**). The predicted $[\text{M}+\text{H}]^+$ ion for **2** (381.1 m/z) was also not observed during ESI-MS analysis of the product. Instead, an $[\text{M}+\text{H}]^+$ peak at 429.1 m/z was observed (Figure 3.1, **C**), representing a 59 Da increase over the 3-vinyl cephem (**1**) substrate. This gave the Wood product an even mass (428 Da) which indicated the product contains an even number of nitrogens.¹⁶¹ Since **1** possesses an odd number of nitrogens (15), formation of the Wood product must involve the addition of an odd number of nitrogen atoms. Based on the ESI-MS data, the urethane structure **3** was proposed (Figure 3.1, **B**). Potential sources for the carbonyl and amide functionalities were identified as NH_4HCO_3 present in the

eluant used during HPLC purification, or residual urea used to re-fold DAOCS/DACS from inclusion bodies.

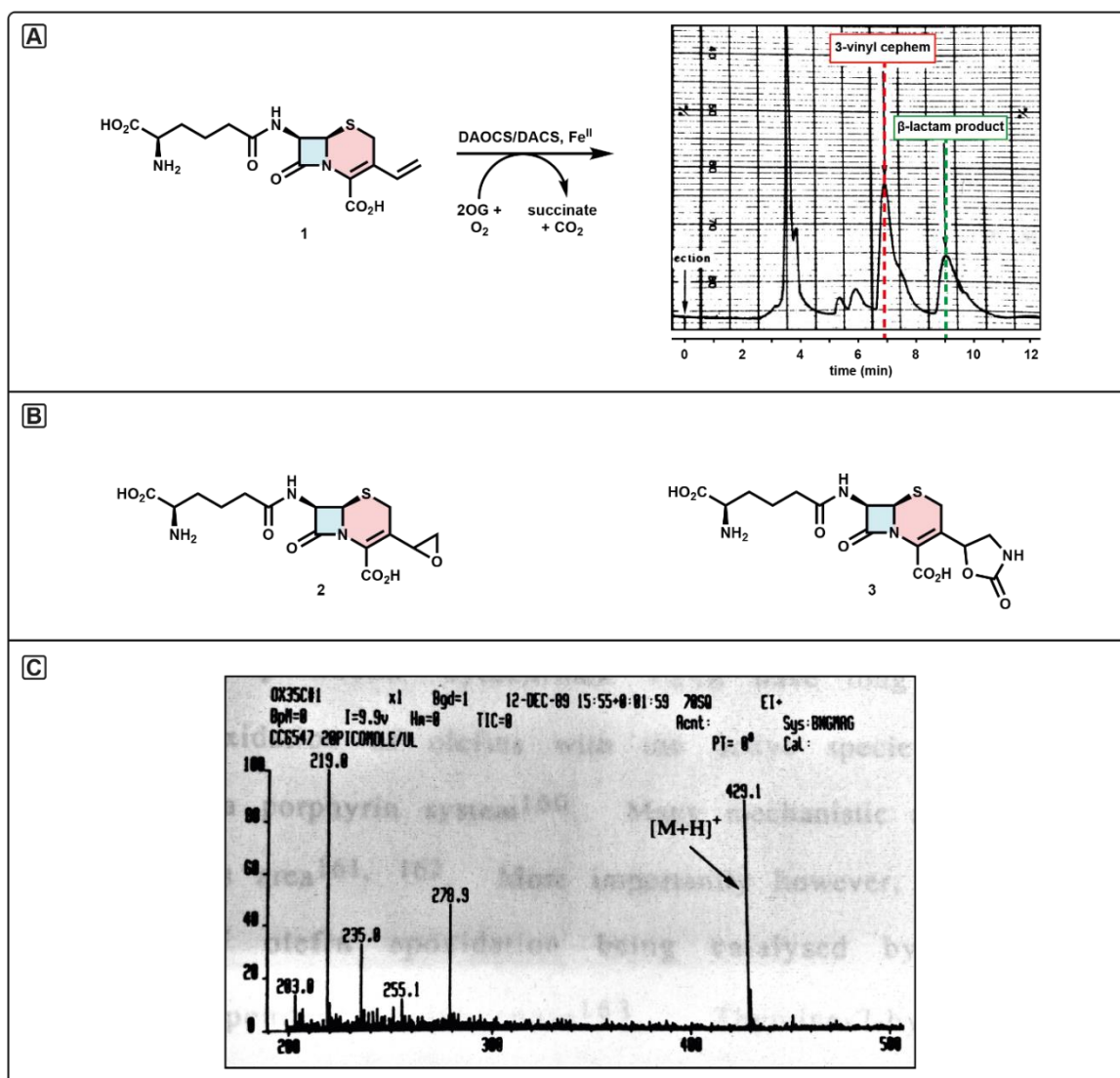


Figure 3.1 | Summary of the 3-vinyl cephem incubations with DAOCS/DACS performed by MEW in 1990. Panel A gives the reaction scheme. The original HPLC chromatogram is presented on the RHS, with monitoring by absorbance at 220 nm. Residual starting material (red), eluted at approx. 7 min, and the single detected β -lactam (Wood) product (green) eluted at 9 min. **Panel B:** the epoxide (2) was proposed based on known activity of cytochrome P450 enzymes. The urethane product structure (3) prediction was made on the basis of the ESI-MS data presented in **Panel C**, which shows an $[\text{M}+\text{H}]^+$ at 429.1 m/z .

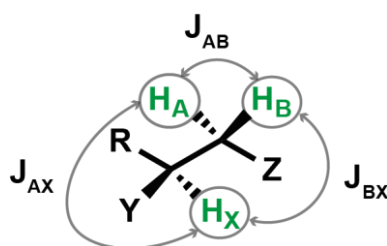


Figure 3.2 | An ABX system, consisting of a CH–CH₂ arrangement with two electronegative substituents (Y and Z). This pattern produces 3 × doublet of doublets in ¹H NMR spectra.

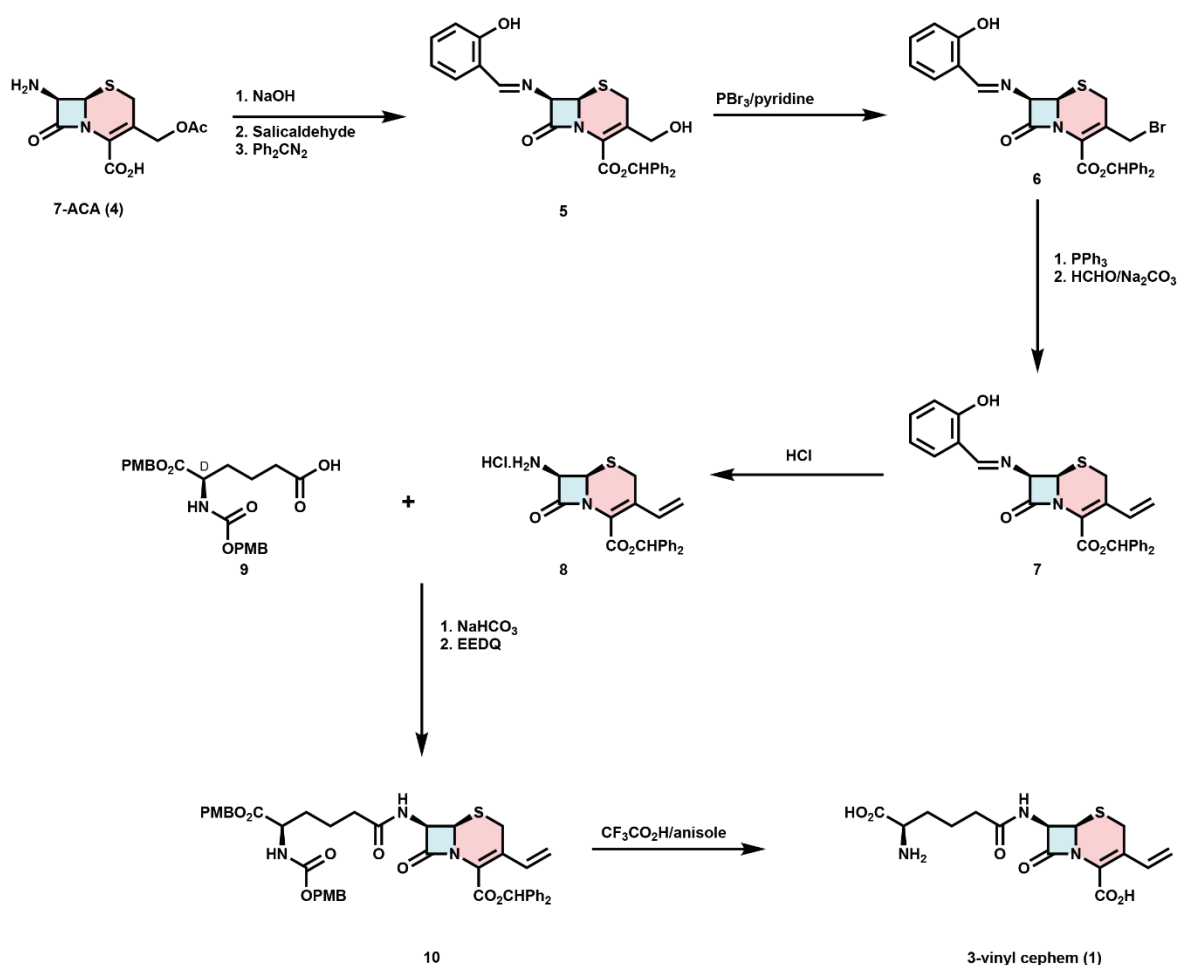
Conversion of **1** to the Wood product by DAOCS/DACS was observed twice, with the latter providing the material for ¹H NMR and LC-MS analysis. After **3** was proposed as the product structure, DAOCS/DACS was de-salted using a PD-10 column to remove trace urea that was hypothesised to contribute to urethane formation. Incubations using de-salted DAOCS/DACS yielded no turnover of **1**. It was proposed that DAOCS/DACS lost activity during de-salting, as has been observed elsewhere.¹²¹ Ultimately, limitations in the analytical techniques of the time prevented the elucidation of the product structure, which has remained unsolved for over 30 years.

3.1.2 Project objectives

The aims of the work described herein were to repeat the incubations of DAOCS/DACS with the 3-vinyl cephem substrate (**1**) performed in 1990 and to characterise the Wood product using modern spectroscopic techniques, leading to the assignment of its structure.

3.2 Synthesis of the 3-vinyl cephem

A synthetic route to the **1** had been previously reported (Scheme 3.1).^{106, 162} Starting from the commercially available 7-ACA (**4**), ester hydrolysis of the 3'-acetoxy group and protection of the C7 amine and C4 acid as an imine and benzhydryl ester respectively afforded **5**. The 3'-OH group of **5** was converted to a bromine in **6**, from which the 3'-vinyl group was installed by Wittig olefination to give **7**. Acid hydrolysis of the C7 imine afforded **8** which was coupled to an appropriately protected D-α-aminoadipic acid (**9**) to give a protected form of the 3-vinyl cephem (**10**). Finally, global deprotection with TFA/anisole yielded the 3-vinyl cephem (**1**), which was isolated by HPLC.



Scheme 3.1 | Previously reported synthetic route to the 3-vinyl cephem, starting from 7-ACA.^{106, 162}

Conversion of the 3'-hydroxyl group to the vinyl group (5 to 7) was a particularly challenging part of this synthetic route. Initially, installation of the vinyl group proceeded via chlorination of the C3'-OH of 5, however, the 3'-chlorinated product was isolated in only a 5% yield.¹⁶² The 3'-halogenation yield was improved to 9% by switching to bromination to give 6. Nevertheless, the vinyl group installation represented a bottleneck in the synthesis of 1.

Since the previously reported synthesis, the de-acylated 3'-vinyl precursor (7-AVCA, 11) has become commercially available. This development means the synthetically challenging installation of the vinyl group can be avoided. 11 could be coupled to D-α-aminoadipic acid (12) to give the 3-vinyl cephem (1). A retrosynthetic analysis involving 7-AVCA is presented in Figure 3.3.

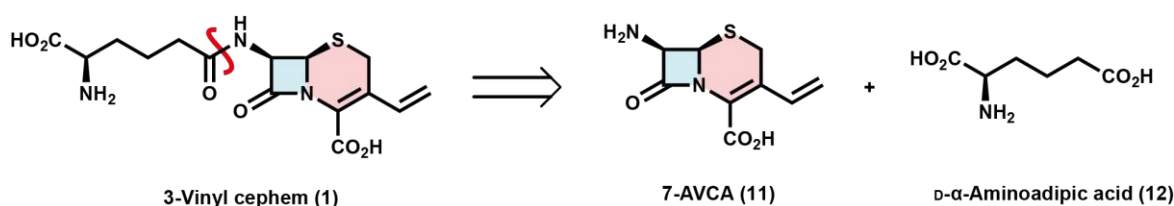
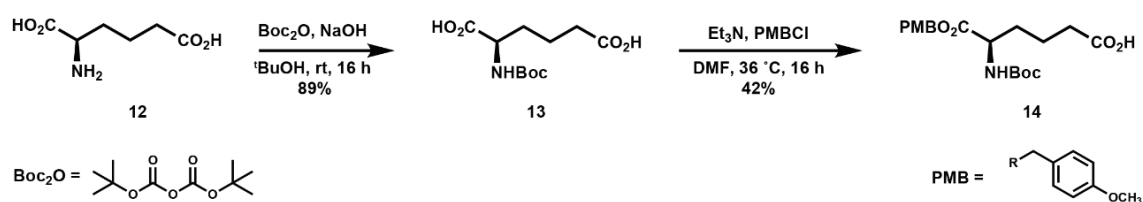


Figure 3.3 | Retrosynthetic analysis of the 3-vinyl cephem substrate analogue. Both 7-AVCA and D-α-amino adipic acid are commercially available.

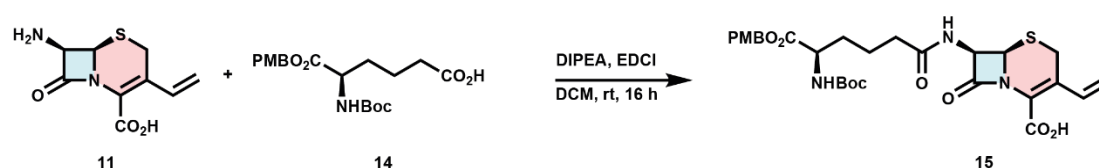
3.2.1 Protection of D-α-amino adipic acid

The D-α-amino adipoyl protection scheme is given in Scheme 3.2. D-α-amino adipic acid (**12**) was reacted with di-*tert*-butyl bicarbonate (Boc anhydride) in the presence of sodium hydroxide to give the α-amino Boc-protected product (**13**).¹⁶³ Protection of the carboxylic acid was achieved with *p*-methoxybenzyl chloride (PMBCl), giving a mixture of α- and δ-protected regioisomers.¹⁶⁴ The desired δ-free acid (**14**) was separated from its regioisomer by column chromatography.



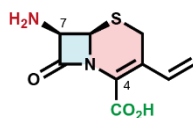
Scheme 3.2 | The protection of D-α-amino adipic acid.

Direct EDCI-mediated coupling of **14** to 7-AVCA (**11**) was attempted (Scheme 3.3). However, the desired product (**15**) could not be isolated by column chromatography.



Scheme 3.3 | The attempted coupling of 14 with 11. The product (**15**) could not be isolated.

¹H NMR analysis of the crude reaction mixture indicated the presence of multiple β-lactam products, suggesting poor levels of regioselectivity during the coupling reaction. This was attributed to the presence of two nucleophilic centres in 7-AVCA: the C7 amine and the C4 carboxylic acid (Figure 3.4).



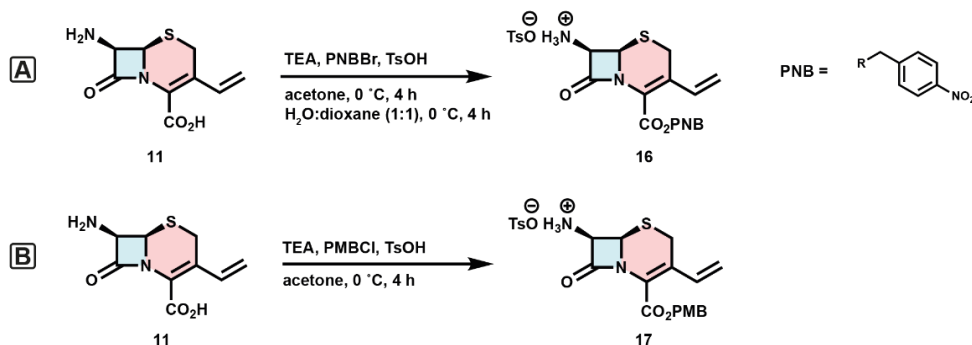
7-AVCA (11)

Figure 3.4 | 7-AVCA with its two nucleophilic groups highlighted: C4 acid (green) and C7 amine (red).

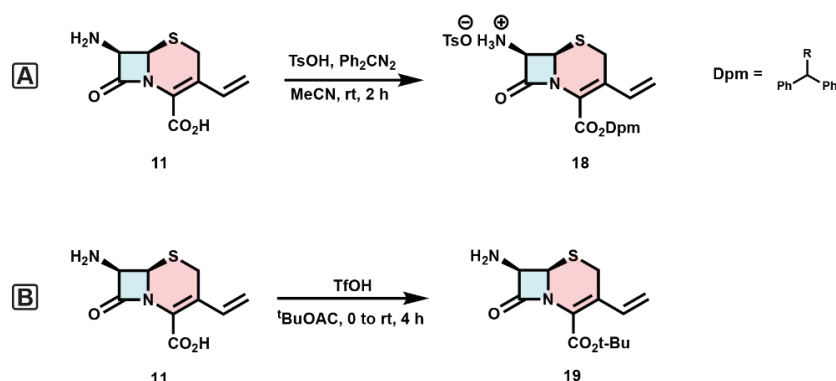
To facilitate regioselective coupling of **14** to the C7 amine of **11**, it was decided to protect the C4 acid of 7-AVCA.

3.2.2 Protection of the C4 acid of 7-AVCA

Protection of the C4 acid of **11** can be achieved either directly or via a three-step orthogonal protecting group strategy in which both the amine and acid are protected before the C7 amine is selectively deprotected. Direct protection was preferred since it required fewer steps, potentially optimising overall yield. Using a protocol established for 6-APA,⁴⁴ formation of the *p*-nitrobenzyl ester of **11** was attempted under basic conditions (Scheme 3.4, **A**). However, the product (**16**) could not be isolated by precipitation as its tosylammonium salt. This protocol was also attempted with *p*-methoxybenzyl chloride to give **17**, similarly without success (Scheme 3.4, **B**). Failure was attributed to the isomerisation of the Δ^3 -double bond to the Δ^2 -position, which is known to occur when esterifying cepheams with alkyl halides under basic conditions.¹⁶⁵⁻¹⁶⁷



Scheme 3.4 | One-step protection of the 7-AVCA C4 acid under basic conditions



Scheme 3.5 | Acid-catalysed esterification strategies for the one-step protection of the 4'-acid of **11**.

Acid-catalysed esterification of **11** was then explored. **11** was treated with TsOH in MeCN, followed by the addition of diphenyldiazomethane (Scheme 3.5, **A**). The product (**18**) however, could not be isolated by chromatography. *t*-Butyl esterification of **11** using triflic acid (TfOH) was then attempted (Scheme 3.5, **B**). This method had been used previously with closely related 7-ADCA (**20**) (Figure 3.5).¹⁶⁸ As with **18**, the desired *t*-butyl ester (**19**) could not be isolated, with ¹H NMR analysis revealing evidence for the loss of the vinyl protons of 7-AVCA, likely due to protonation of the alkene by TfOH.

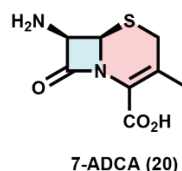
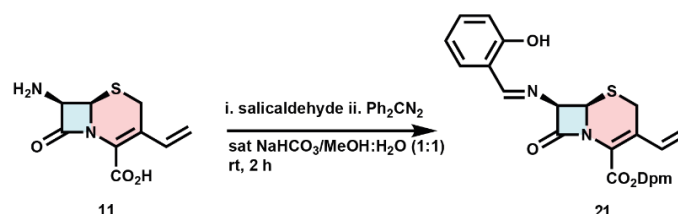


Figure 3.5 | The structure of 7-ADCA (**20**).

After direct esterification of **11** had failed, an orthogonal protecting group strategy was pursued. This involved protection of both the C7 amine and the C4 carboxy groups of **11**, followed by selective deprotection of the amine group. In the previous synthesis of the 3-vinyl cephem (**1**), the C7 amine of 7-ACA (**4**) was protected as its salicaldehyde imine and the C4 acid was protected as a diphenylmethyl ester to give **5** (Scheme 3.1). This strategy was applied to **11** using the reported procedures (Scheme 3.6).^{106, 162} The desired product (**21**) was isolated in much lower yield than was reported for **5** (10% vs 78%).¹⁰⁶ This poor yield was attributed to low solubility of **11** in the reaction solvent (1:1 MeOH/H₂O). The reaction was attempted in saturated NaHCO₃ in order to

improve solubility. Imine formation was also encouraged through the addition of formic acid and heating. Neither changes to the solvent system nor the addition of formic acid substantially improved the yield of **21**.



Scheme 3.6 | Benzhydryl esterification of the 7-AVCA C4 carboxy group, via imine formation of the C7' amino group.

Boc protection of **11** was then explored. Using a previously reported procedure which used saturated NaHCO_3 as solvent,¹⁶⁹ **22** was obtained in a 33% yield. This yield was improved to 80% by performing the Boc protection under non-aqueous conditions (MeOH/TEA) (Scheme 3.7).¹⁶⁸ The greater yield of **22** in MeOH/TEA compared to saturated NaHCO_3 was attributed to improved solubility and reduced β -lactam ring hydrolysis.

Benzhydryl esterification of **22** afforded **23** in acceptable yield.¹⁷⁰ Diphenyldiazomethane was initially prepared using HgO ,¹¹⁵ but a MnO_2 based method¹⁷¹ produced Ph_2CN_2 in higher yield without the use of toxic mercury. Selective removal of the Boc group of **23** to give the tosylammonium cephem salt (**24**) was carried out using TsOH in anhydrous THF.¹⁷⁰ Several cycles of concentrating the reaction mixture *in vacuo* before redissolving it in anhydrous THF were required to push the reaction to completion.

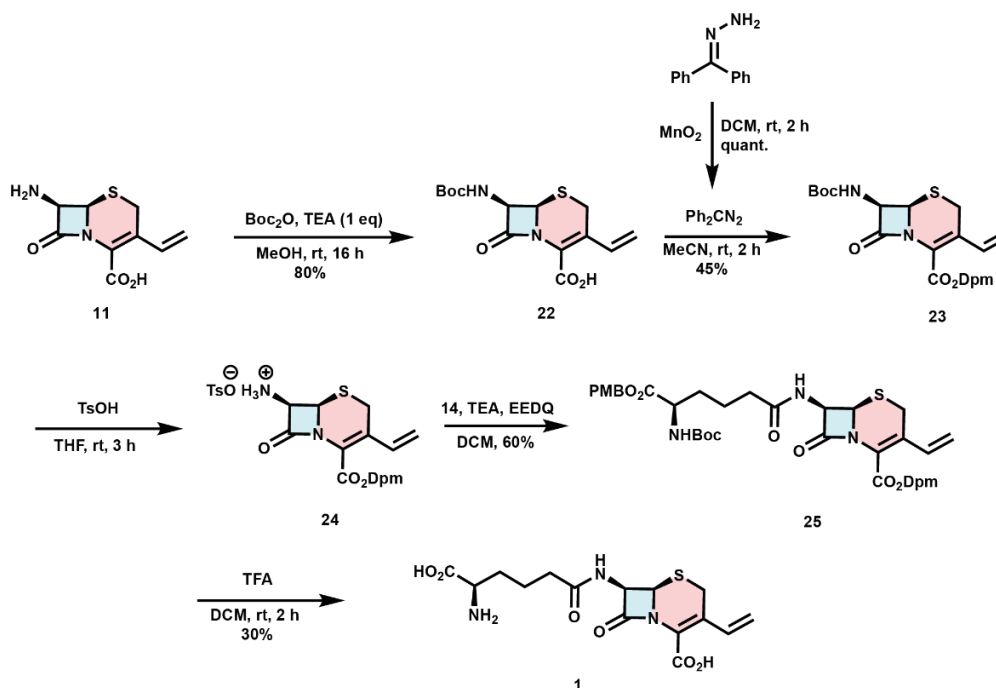
3.2.3 Amide coupling

The suitably protected D- α -amino acid side chain (**14**) was installed on the C7 amine of **24** via amide coupling. EEDQ-mediated coupling,¹⁷² utilised during the previous synthesis of **1**¹⁰⁶ as well as penicillin N,⁴⁴ afforded **25** in reasonable yield (48%). HATU-mediated coupling in DCM gave **25** in lower yields (10%) than via the EEDQ-route.

3.2.4 Global deprotection

Global deprotection of **25** to give the 3-vinyl cephem (**1**) was achieved using TFA in anhydrous DCM.^{173, 174} **1** was isolated by HPLC with an isocratic eluting of 10 mM NH_4HCO_3 and 2% MeCN.

A summary of the partially optimised synthetic route from 7-AVCA (**11**) to give the 3-vinyl cephem (**1**) is given below (Scheme 3.7).



Scheme 3.7 | Overview of the synthetic route to 3-vinyl cephem (**1**) starting from 7-AVCA (**11**).

3.3 Initial incubations of 3-vinyl cephem with DAOCS/DACS

With a synthetic route to the 3-vinyl cephem (**1**) established, incubations with DAOCS/DACS could be undertaken. Recombinant DAOCS/DACS was produced in *E. coli* (Chapter 2) and shown to be highly active towards its natural substrates: penicillin N and DAOC (Chapter 4). Incubations of **1** with DAOCS/DACS were initially performed on an analytical scale (0.1 μmol). Substrate conversion was monitored by ^1H NMR employing a pre-saturation water suppression method and analysing peak formation in the β -lactam region (δ 4–6 ppm), as well as by LC-MS.

Incubations were initially performed under the same conditions in which the Wood product had been observed,¹⁰⁶ however no turnover of the **1** was observed by ^1H NMR (Figure 3.6).

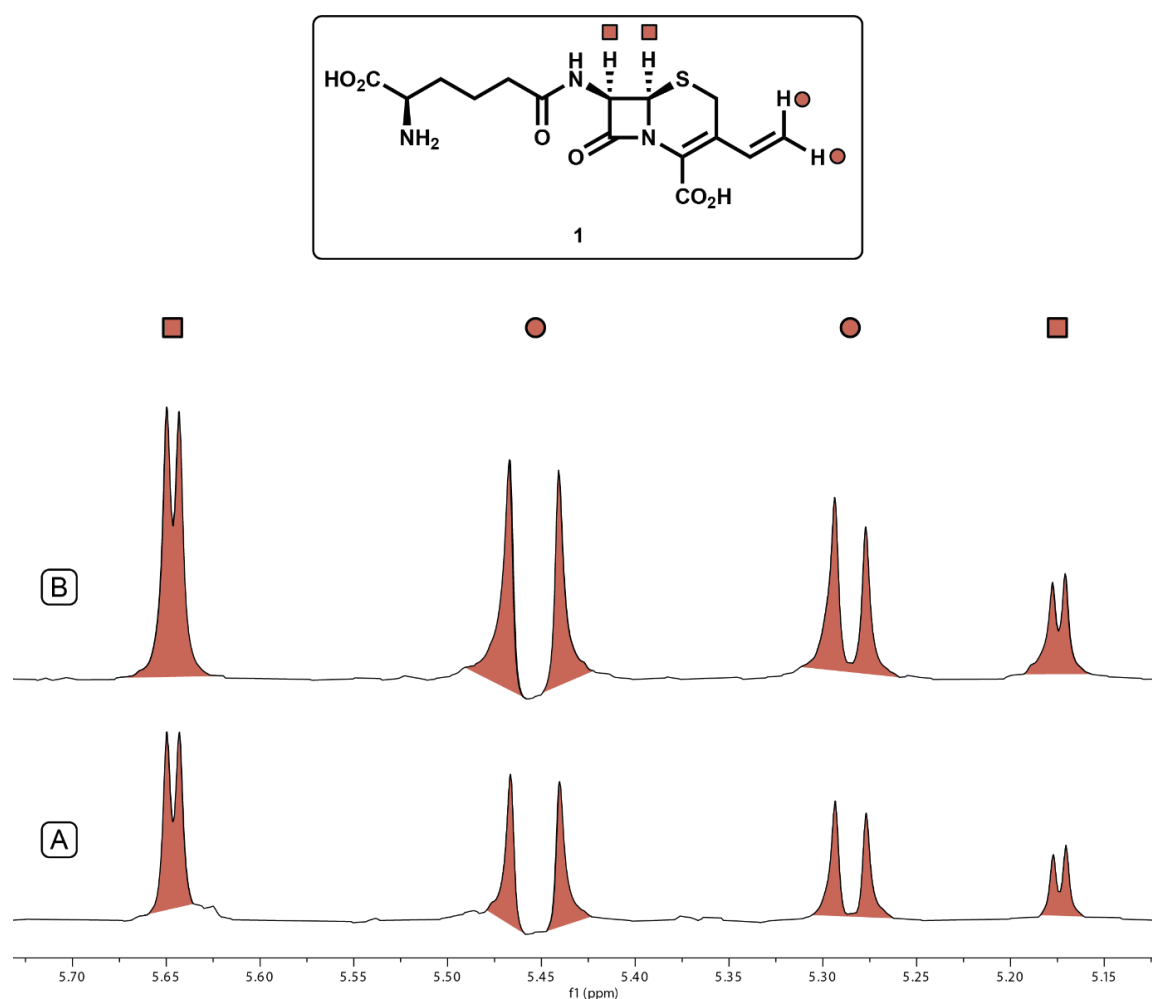


Figure 3.6 | Stacked ^1H NMR (700 MHz) spectra of 3-vinyl cephem incubations under the MEW conditions. A 3-vinyl cephem incubations without enzyme. **B** 3-vinyl cephem incubations with DAOCS/DACS. Assay conditions: DAOCS/DACS (0.1 mM), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.045 mM), L-ascorbic acid (0.83 mM), α -ketoglutaric acid (0.85 mM), DTT (1.68 mM), ammonium sulfate (42 mM), 3-vinyl cephem (1 mM).

Further analysis of the ^1H NMR spectra revealed complete consumption of 2OG during the incubations. In addition to being coupled to prime substrate oxidation, 2OG to succinate conversion is proposed to occur via two non-productive pathways: uncoupled enzymatic prime substrate 2OG conversion and a non-enzymatic reaction with hydrogen peroxide.¹⁷⁵ H_2O_2 is produced by the oxidation of L-ascorbate, a process which is inhibited by Fe(II) .¹⁷⁵ It was hypothesised that increasing the amount of Fe(II) and 2OG in the assay mixture would stimulate the turnover of the 3-vinyl cephem by DAOCS/DACS.

Assay conditions in which full conversion of penicillin N to DACS by DAOCS/DACS was observed were applied to incubations of **1**. Cofactor and cosubstrate concentrations were scaled to account

for the increased ratio of DAOCS/DACS to substrate. Under these conditions, $\approx 40\%$ conversion of **1** to an apparent single β -lactam product was observed by ^1H NMR (Figure 3.7, **B**).

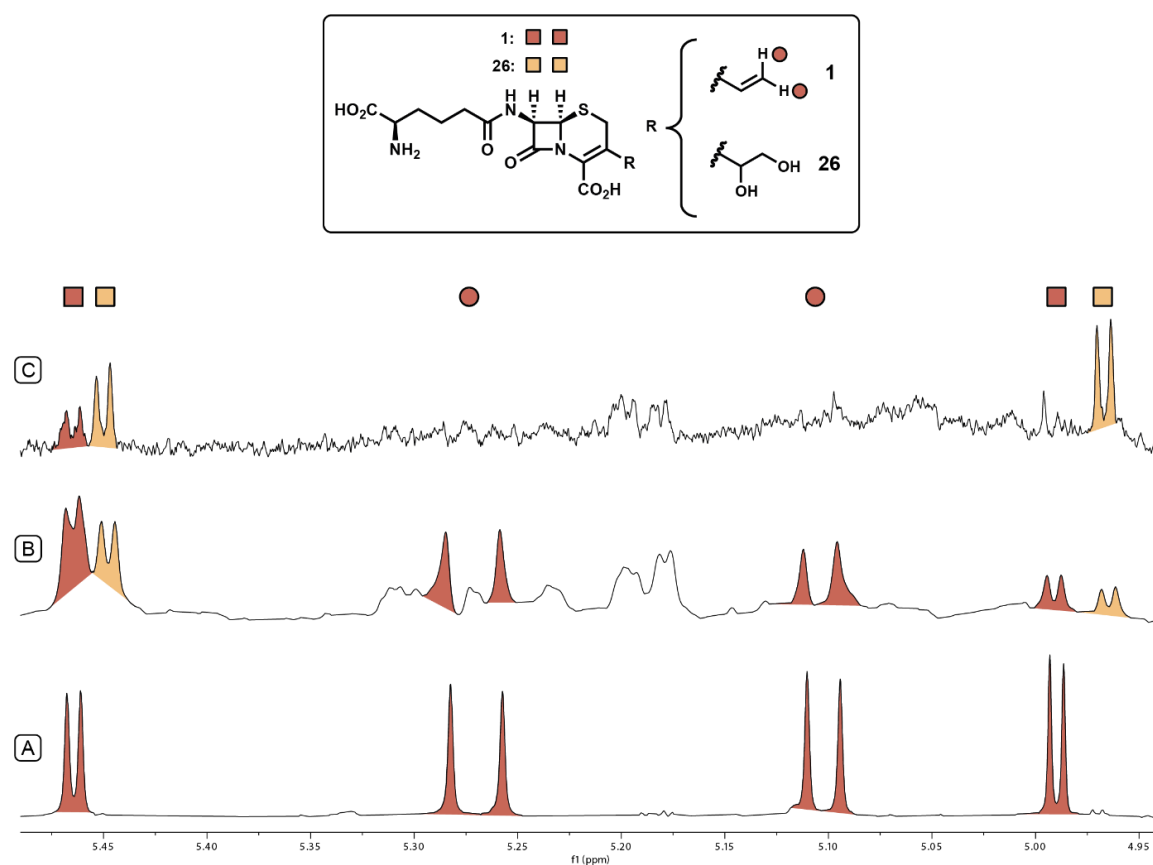


Figure 3.7 | Stacked ^1H NMR (700 MHz) spectra monitoring evidence for the conversion of **1 to a single β -lactam product, proposed to be the diol **26**.** **A** Incubation of **1** without enzyme. **B** 40% conversion of **1** by DAOCS/DACS to a single β -lactam product. **C** 60% conversion of **1** to the same β -lactam product. No additional β -lactam products were observed during assay optimisation. Assay conditions: DAOCS/DACS (0.2 mM), $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.4/0.6 mM), sodium-L-ascorbate (2.4 mM), α -ketoglutarate disodium salt (4 mM), 3-vinyl cephem (1 mM). Assays were performed in 50 mM Tris- d_{11} (pH 7.4) in 100 μL final assay volume.

Based on the ^1H NMR spectra, the observed product was initially thought to be the same as the Wood product, however, LC-MS analysis revealed differences between the two products. The Wood product had a similar retention time to **1** on a reverse-phase C_{18} column and an $[\text{M}+\text{H}]^+$ peak of 429.1 m/z. By contrast, LC-MS analysis of the new product showed no peak with comparable retention to **1** (Figure 3.8, **A** and **B.1**). A mass extracted-ion chromatogram for 429.1 m/z (the mass of the Wood product) also did not identify any clear peaks (Figure 3.8, **B.2**). Increasing the concentration of ferrous ammonium sulfate from 0.4 mM to 0.6 mM increased turnover of **1** to 60% (Figure 3.7, **C**). However, systematic variation of assay components did not result in the formation of any additional β -lactam products.

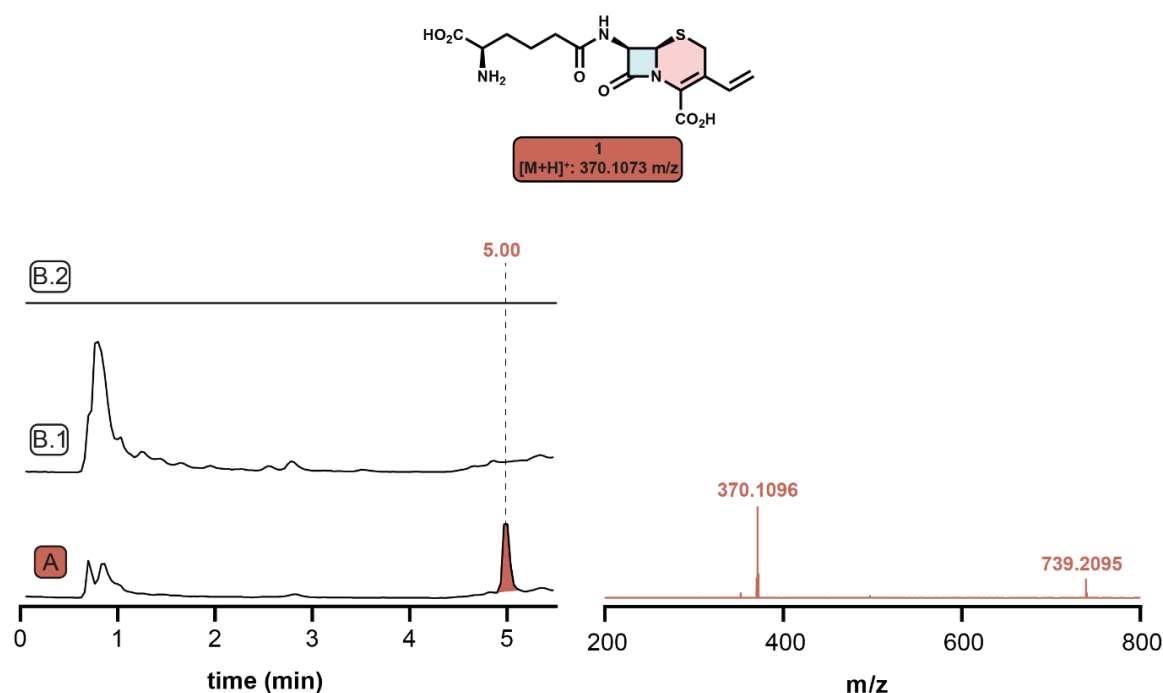


Figure 3.8 | LC-MS analysis of assays in which 1 was converted to a single β -lactam product. LHS: **A** Total ion chromatogram (TIC) of **1** incubated without DAOCS/DACS. **B.1** TIC of **1** incubated with DAOCS/DACS. **B.2** Mass-extracted chromatogram at 429.1 ± 0.2 m/z of the assay that produced the new β -lactam product. No new peaks were observed. RHS Mass spectrum of chromatogram **A** at 5.00 min showing starting material **1**.

Efforts were directed towards characterising the apparently new β -lactam product. Epoxidase activity of DAOCS/DACS towards the $[4\text{-}^2\text{H}]$ exomethylene cepham substrate analogue (**27**) had previously been observed¹¹² (Figure 3.9).

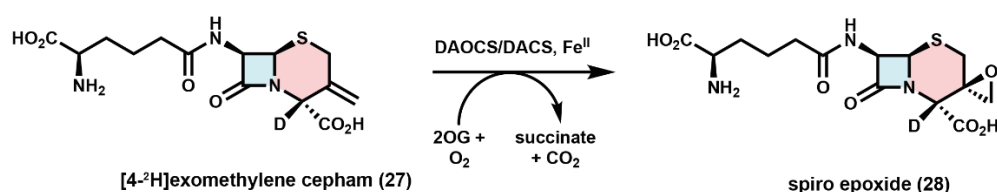


Figure 3.9 | Conversion of $[4\text{-}^2\text{H}]$ exomethylene cepham to an epoxide. The substrate is structurally similar to **1**, and therefore provides a precedent for the potential conversion of **1** to **2**.

Based on the structural similarities between the epoxidized analogue (**27**) and **1**, it was proposed that DAOCS/DACS could convert **1** to the epoxide intermediate **2**, initially proposed as the structure of the Wood product. Ring-opening of **2** by water would then give the diol **26** (Figure 3.10).

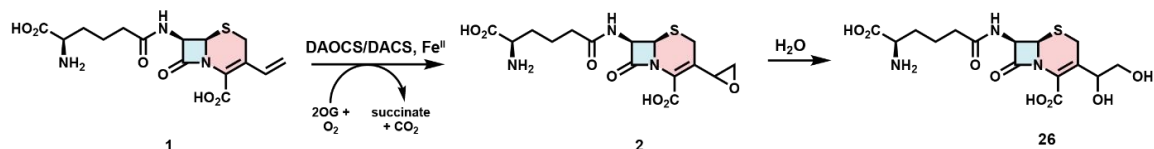


Figure 3.10 | Proposed transformation of 3-vinyl cephem (1) by DAOCS/DACS into the diol (26) via an epoxide intermediate (2).

A mass search of the LC-MS data (Figure 3.8) for **26** (predicted: 404.1127 m/z) revealed a peak in the mass-extracted chromatogram at 1.17 min (Figure 3.11, **B.1** and **B.2**) that was not present when **1** was incubated without DAOCS/DACS (Figure 3.11, **A.1** and **A.2**). The retention time (1.17 min) was comparable to that of the structurally-related cephalosporin DAC. Therefore, the β -lactam product was putatively assigned as the diol **26**.

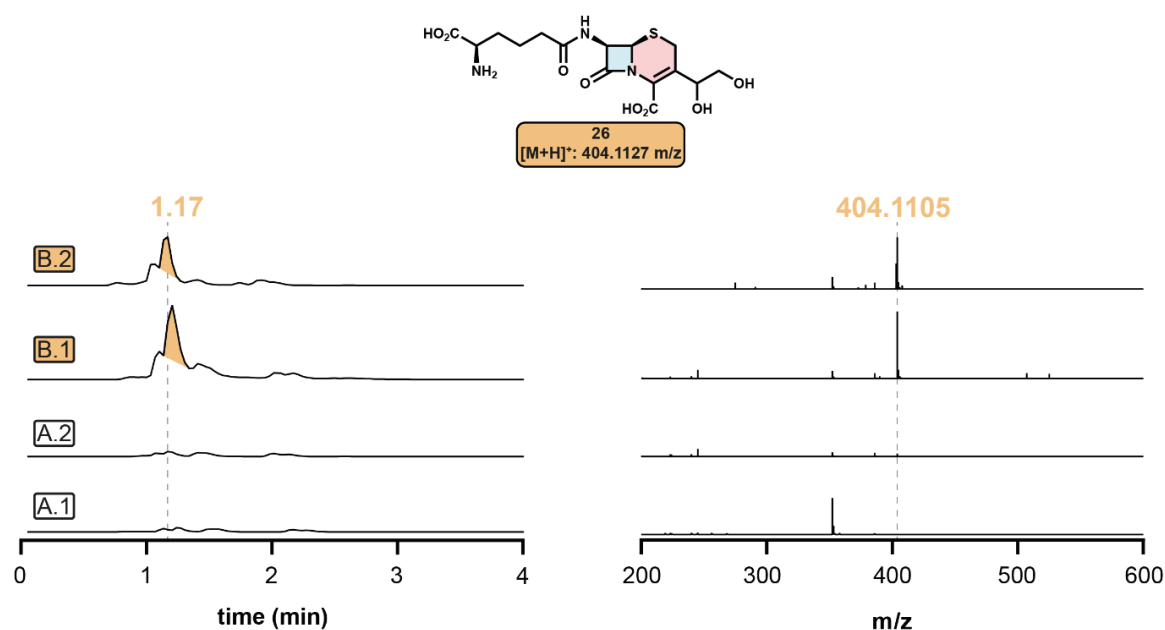


Figure 3.11 | Stacked LC-MS spectra of DAOCS/DACS incubations with 1. The extracted-ion chromatograms for 404.1 ± 0.2 Da are given on the LHS and the mass spectra at 1.17 min are shown on the RHS. **Panel A** are repeats of 3-vinyl cephem incubations without DAOCS/DACS. **Panel B** are repeats of 3-vinyl cephem incubations with DAOCS/DACS. A clear 404.1105 m/z peak is visible in the DAOCS/DACS incubations that is not visible when the incubations were performed without DAOCS/DACS.

The formation of **26** was supported by ^{18}O experiments involving incubations of **1** with DAOCS/DACS under $^{18}\text{O}_2$ or in H_2^{18}O . The results revealed ^{18}O -incorporation into **26** from both labelled sources. Initially, **1** was incubated with DAOCS/DACS under an $^{16}\text{O}_2$ atmosphere. Incubations were run for 2 h, after which time the assay were quenched and analysed by LC-MS. As expected, the formation of **26** was observed (Figure 3.12, **A**). When the incubations were repeated under an $^{18}\text{O}_2$ atmosphere, there was a +2 increase in the mass of **26** (Figure 3.12, **B**)

consistent with the incorporation of a single ^{18}O atom. Singly ^{18}O -labelled **26** was also produced when the assays were performed in H_2^{18}O under a $^{16}\text{O}_2$ atmosphere (Figure 3.12, C). These experiments strongly support the formation of **26** as the product of incubations of **1** with DAOCS/DACS. Moreover, they demonstrate the incorporation of both molecular oxygen and water in **26**, consistent with formation proceeding via the epoxide intermediate **2**. Thus, the initial epoxidation of **1** incorporates an ^{18}O solely from $^{18}\text{O}_2$ and subsequent hydrolysis gives the diol product **26**.

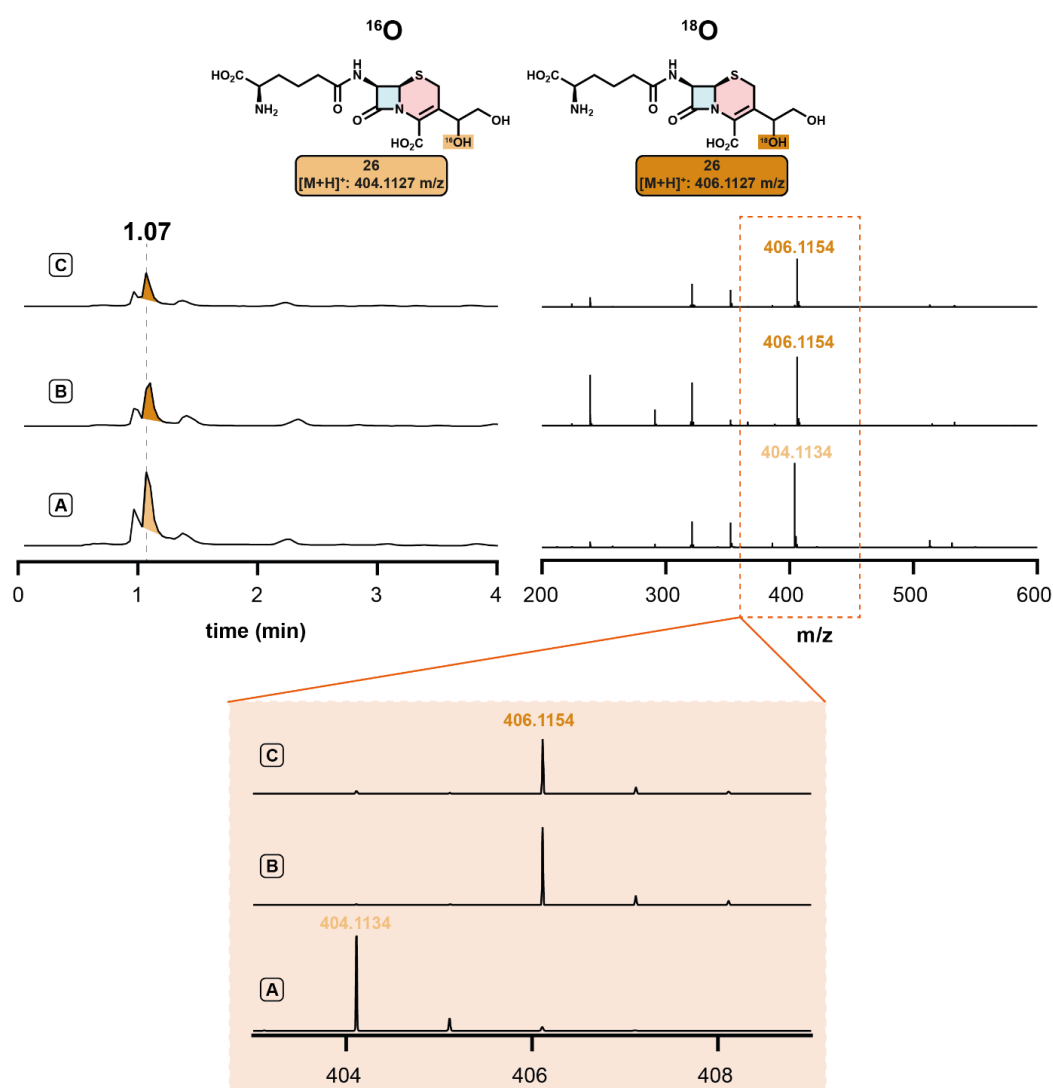


Figure 3.12 | ^{18}O labelling of the diol (26**) occurs when assays are performed in either $^{18}\text{O}_2$ (B) or H_2^{18}O (C).** LC-MS results from three different assays are shown: an $^{16}\text{O}_2$ control in H_2^{16}O buffer (A), $^{18}\text{O}_2$ incubations in H_2^{16}O buffer (B) and a $^{16}\text{O}_2$ incubation in H_2^{18}O buffer (C). Mass-extracted chromatograms are given on the LHS, mass spectra at 1.07 min are given on the RHS. It is not clear which oxygen of **26** is derived from dioxygen and which originates from water. For simplicity, only of the hydroxyl groups of **26** was labelling in the diagram. If diol formation proceeds via the epoxide (**2**) in solution, ring-opening by water would likely occur at the less substituted carbon centre. Therefore, the terminal hydroxyl group would originate from water. Please note, the small peak at 404.1 m/z observed in assay C most likely derives from residual crystal water of solids and pH adjustment made while preparing the assay.

3.4 Formation of a cephem hydroxy azide product

After systematic variation of assay components had failed to reproduce the β -lactam product observed in 1990, alternative sources for the formation of the Wood product were explored.

Whilst searching for the assay conditions used in the 1990 incubations of **1** with DAOCS/DACS, archived laboratory notebooks describing the preparation of DAOCS/DACS were examined. In 1990, DAOCS/DACS was produced in Oxford from the pIT-511 construct gifted by collaborators at Eli Lilly.⁵⁶ pIT-511 was expressed at high levels in *E. coli* NM554 cells, however the resultant DAOCS/DACS was produced in an insoluble form. Active DAOCS/DACS was afforded by extracting and re-folding the protein with urea, followed by ion-exchange chromatography.[†] After purification, the protocol involved buffer exchanging DAOCS/DACS into a storage buffer (10 mM Tris, 1 mM DTT, 0.05% w/v sodium azide, pH 7.4) prior to its long-term storage at -80°C . The addition of sodium azide as an antimicrobial agent was potentially interesting due to the strong nucleophilicity of the azide anion. The molar concentration of sodium azide during the 3-vinyl cephem incubations (7 mM) represented a 10-fold excess with respect to **1**.

It was hypothesised that the azide anion may have been involved in the formation of the Wood product. To explore this, incubations of **1** with DAOCS/DACS which gave **26** were repeated in the presence of 0.5 mM sodium azide. Under these conditions, full conversion of **1** to two β -lactam products was observed by ^1H NMR in an $\approx 2:1$ ratio (Figure 3.13, **C**). The minor β -lactam product (**amber**) was apparently the diol **26**, which was observed as the sole product in the absence of sodium azide (Figure 3.13, **B**). The major β -lactam product (**green**) was a new product that had not been observed in incubations without sodium azide. When the concentration of sodium azide was increased from 0.5 mM to 5 mM, this new β -lactam product (**green**) became the sole product, with no formation of **26** (Figure 3.13, **D**).

[†] Preparations of DAOCS/DACS were performed by Wendy Sobey. Details described in the text were discussed in private communications with Ms. Sobey.

Analytical data for this new β -lactam product corresponded well with the Wood product; the shift differences between β -lactam peaks in ^1H NMR were very similar and, crucially, a 429.1 m/z peak was visible by LC-MS (Figure 3.14). This evidence strongly supported the proposal that this new β -lactam product was the same as the Wood product.

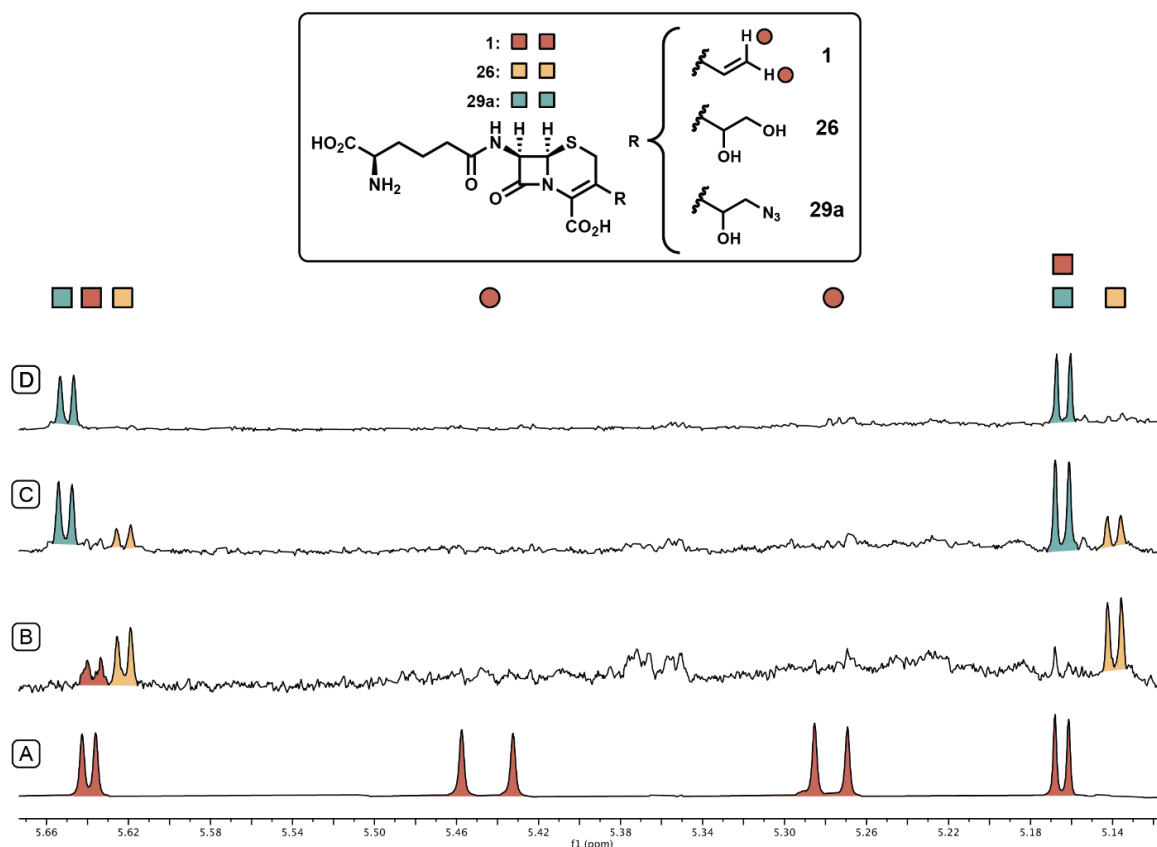


Figure 3.13 | Stacked ^1H NMR spectra of 3-vinyl cephem incubations. **A** 3-vinyl cephem (**1**) incubation with no enzyme. **B** Incubation of **1** with DAOCS/DACS without sodium azide to give the diol (**26**). **C** Incubation of **1** with DAOCS/DACS and sodium azide (0.5 mM) gave **26** as a minor product and a new major product (**green**). **D** Incubation of **1** with DAOCS/DACS and sodium azide (5 mM), in which only the new product (**green**) was produced. Assay conditions: DAOCS/DACS (0.2 mM), $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.6 mM), sodium-L-ascorbate (2.4 mM), α -ketoglutarate disodium salt (4 mM), 3-vinyl cephem (1 mM), $\pm$ sodium azide (0.5/5 mM). Assays were performed in 50 mM Tris- d_{11} (pH 7.4) in 100 μL total assay volume.

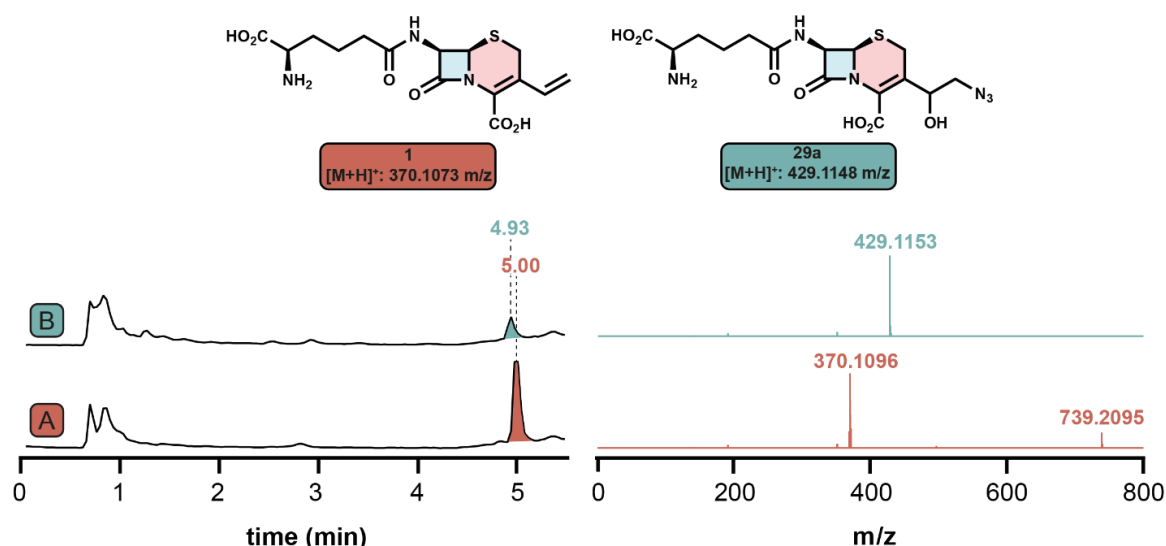


Figure 3.14 | LC-MS analysis of 3-vinyl cephem incubations demonstrating the appearance of a new peak in the presence of sodium azide. Total ion chromatograms (TIC) are given on the left, mass spectra are presented on the right. **A** 3-vinyl cephem incubated with no enzyme. A clear peak is visible at 5.00 min with a mass corresponding to the expected mass of the 3-vinyl cephem substrate (370.1073 m/z). **B** Incubation of **1** with DAOCS/DACS and sodium azide (0.5 mM) yielded a new peak of comparable retention time to **1** and a clear m/z of 429.1, the same as the Wood product.

As with DAOCS/DACS, incubations of **1** with the bacterial hydroxylase DACS in the presence of azide (0.5 mM) produced the diol **26** and the new β -lactam species. Conversion of **1** by DACS was much lower than with DAOCS/DACS; under conditions in which full conversion of **1** by DAOCS/DACS was observed, only $\approx 30\%$ conversion was achieved with DACS (Figure 3.15, **A**). Turnover was increased to $\approx 70\%$ by doubling the concentration of DACS from 0.2 mM to 0.4 mM (Figure 3.15, **B**). At this high enzyme concentration, some enzyme remained after quenching. The presence of residual DACS in the ^1H NMR samples is the cause of the additional peaks observed in **B**.

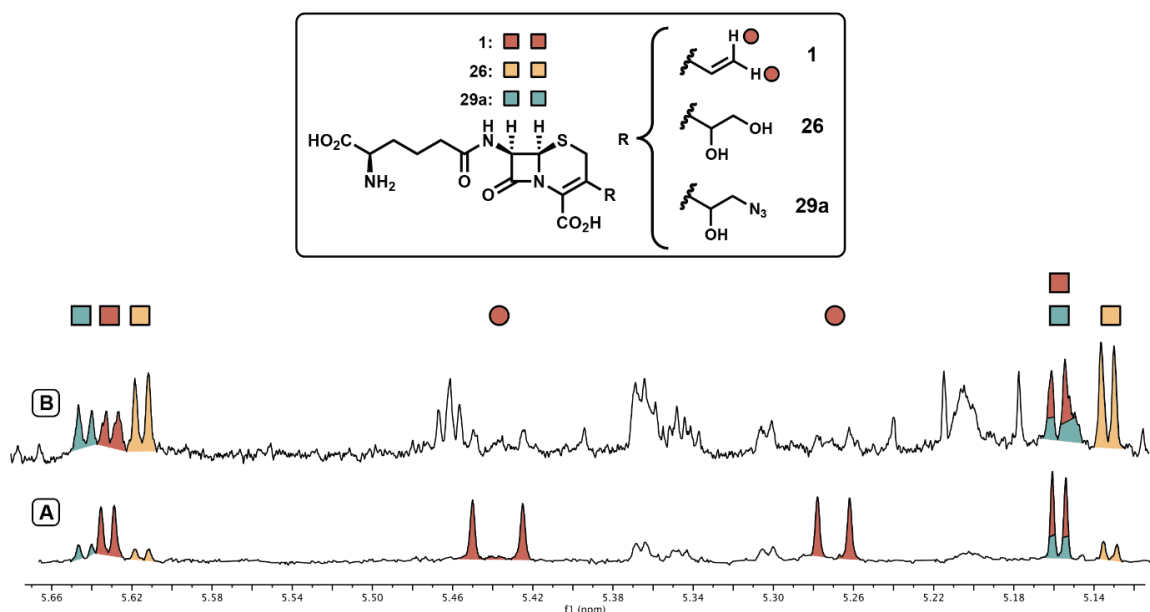


Figure 3.15 | Stacked ^1H NMR spectra of DACS (0.2 mM, A or 0.4 mM, B) incubations with **1 in the presence of sodium azide.** Assay conditions: DACS (0.2/0.4 mM), $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.6 mM), sodium-L-ascorbate (2.4 mM), α -ketoglutarate disodium salt (4 mM), 3-vinyl cephem (1 mM), $\pm$ sodium azide (0.5 mM). Assays were performed in 50 mM Tris- d_{11} (pH 7.4) in 100 μL total assay volume.

The major β -lactam product was putatively assigned as either, or both of the cephem hydroxy azides **29a** or **29b** (Figure 3.16), formed by the ring opening of epoxide **2** by the azide nucleophile. Efforts were thus directed to product isolation on a semi-preparative scale to facilitate full structural characterisation.

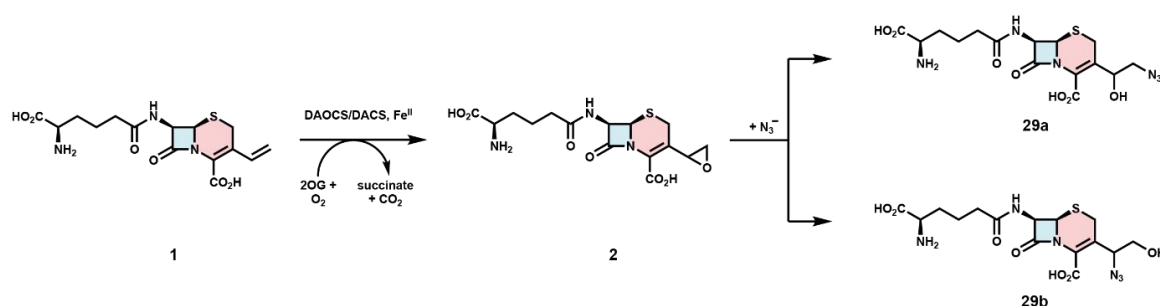


Figure 3.16 | Possible structures proposed for the major β -lactam product observed when the 3-vinyl cephem substrate was incubated with DAOCS/DACS in the presence of sodium azide.

3.5 Cephem hydroxy azide: Isolation and structure elucidation

Incubations of **1** with DAOCS/DACS in the presence of azide (0.5 mM) were scaled up to convert 2.5 mg of substrate. During the optimisation of assay scale-up, oxygen availability was highlighted as a potentially limiting factor; when the analytical scale assays were doubled from 0.1 μmol to 0.2 μmol but performed in the same 1.5 mL microcentrifuge tube, turnover of **1** decreased from

100% to 52% (Figure 3.17, **B**). Limited oxygen availability was likely to be the primary cause of this reduced conversion.

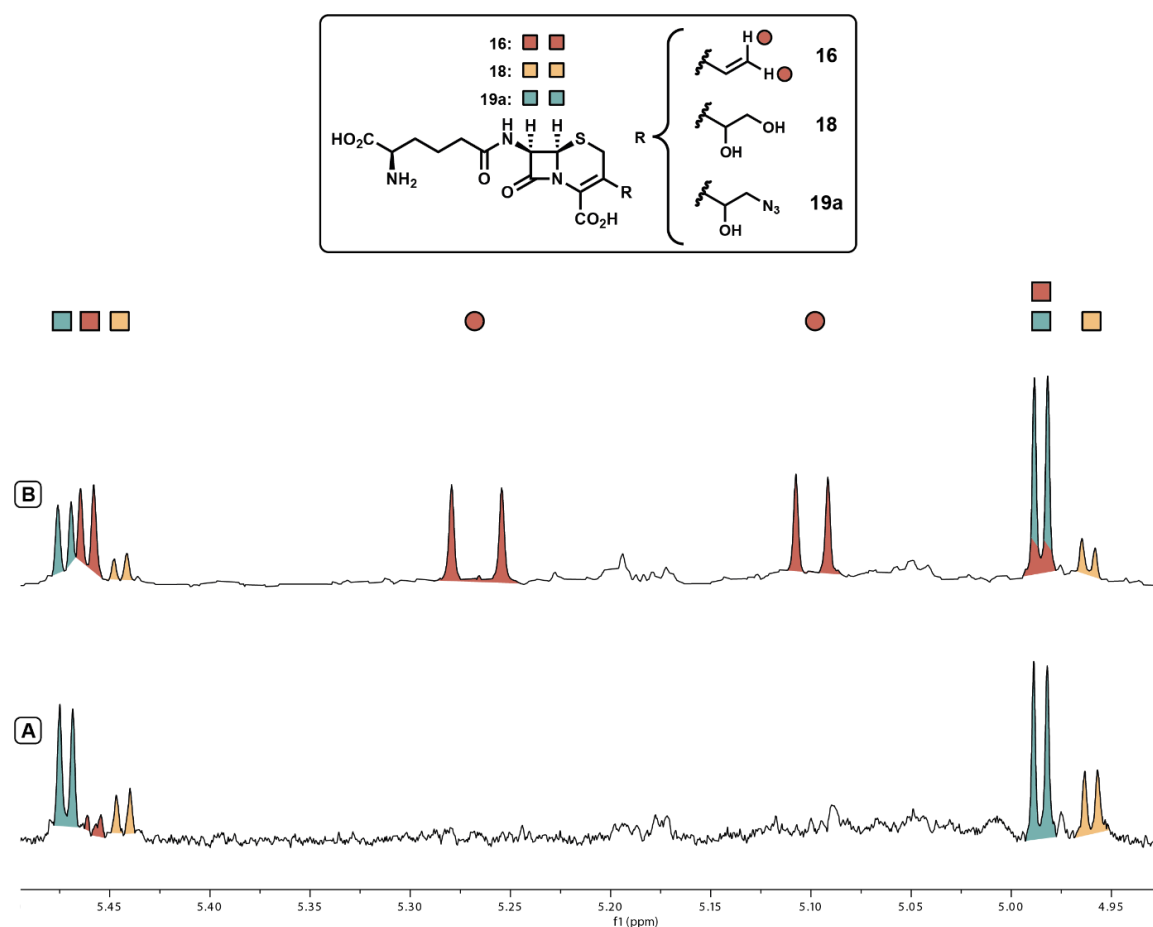


Figure 3.17 | Stacked ^1H NMR spectra showing analytical incubations of **1 with DAOCS/DACS and azide (0.5 mM). A** 0.1 μmol scale (100 μL). **B** 0.2 μmol scale (200 μL). Both assays were performed in a 1.5 mL microcentrifuge tube. Turnover in **B** is $\approx 52\%$, down from $\approx 100\%$ in **A**. Assay conditions: DAOCS/DACS (0.2 mM), $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.6 mM), sodium-L-ascorbate (2.4 mM), α -ketoglutarate disodium salt (4 mM), 3-vinyl cephem (1 mM), $\pm$ sodium azide (0.5 mM). Assays were performed in 50 mM Tris- d_{11} (pH 7.4) in 100 μL total assay volume.

In order to ensure oxygen availability was comparable to the 0.1 μmol scale assays, large-scale incubations of **1** with DAOCS/DACS were split into 2×1 mg and 1×0.5 mg assays performed in 50 mL Falcon tubes. Purification by HPLC was performed on a freshly regenerated C_{18} column using 2% (v/v) MeCN in 10 mM NH_4HCO_3 as eluant. The HPLC UV trace from 5 min to 20 min is given in Figure 3.18.

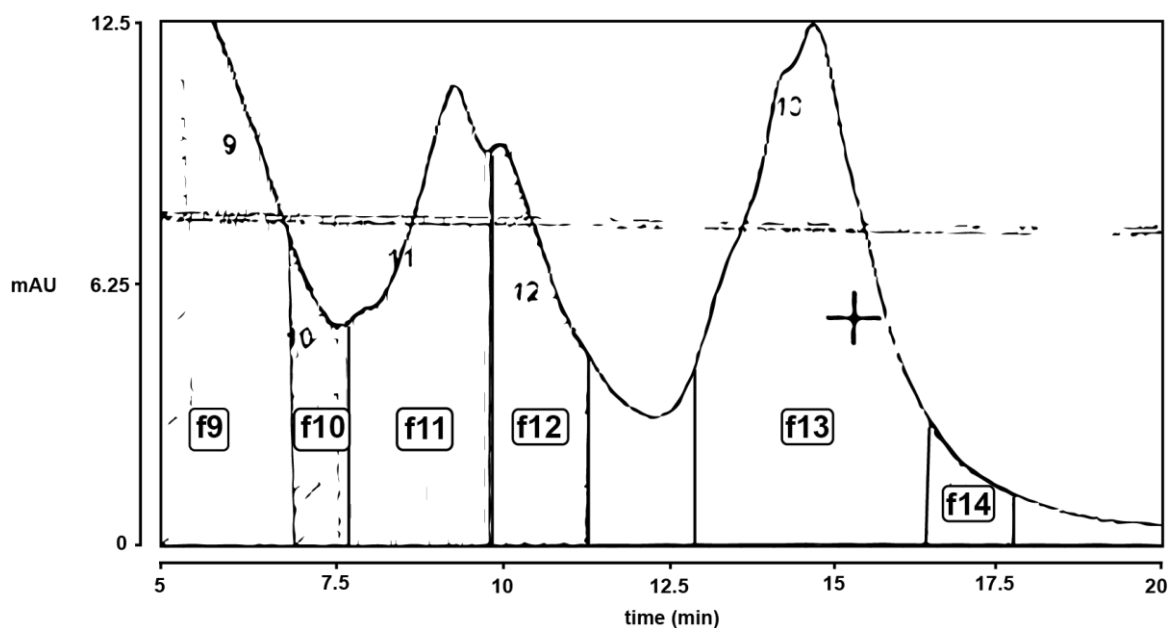


Figure 3.18 | HPLC-UV chromatogram (200 nm) between 5–20 min for the semi-preparative scale purification of 3-vinyl cephem and DAOCS/DACS incubations with sodium azide. Fractions of interest (F9–F14) are highlighted.

No β -lactam protons were visible by ^1H NMR in fractions 1–10 or fraction 15 onwards. Fraction 11 (F11) was identified as starting material **1** by LC-MS (Figure 3.19, **Panel A**). Fraction 13 contained the major β -lactam product (Figure 3.19, **Panel B**) which was observed in the analytical scale assays and putatively assigned as the cephem hydroxy azide **29a/b**.

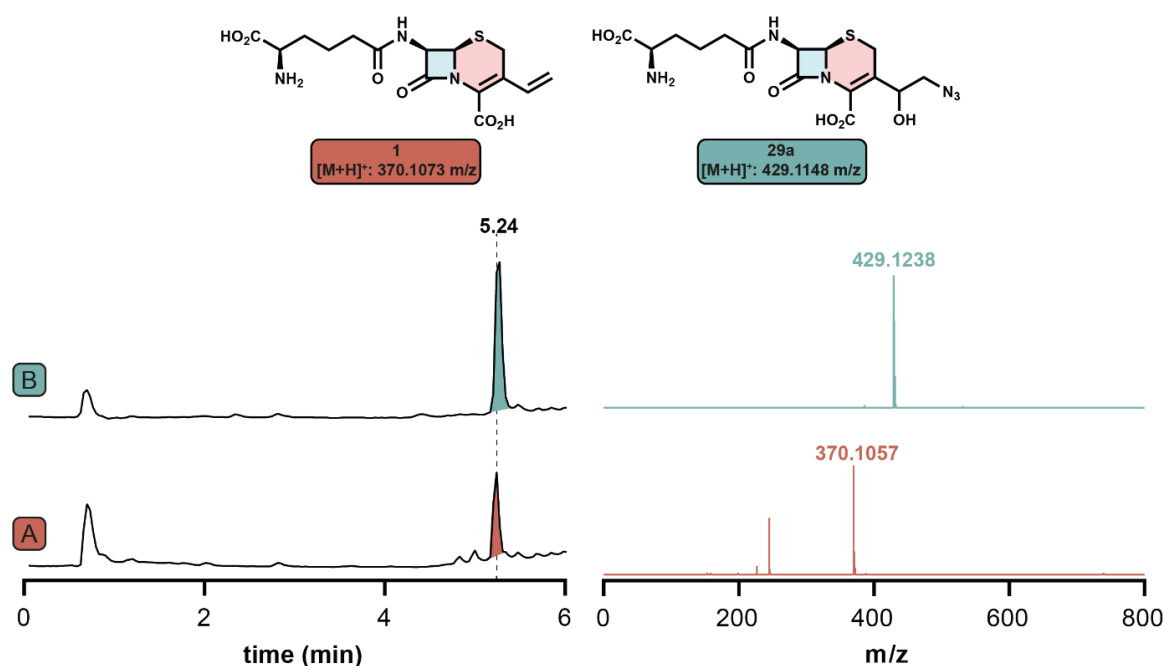


Figure 3.19 | LC-MS analysis of HPLC fractions. Panel A LC-MS analysis of fraction 11, which contained the 3-vinyl cephem starting material. Panel B LC-MS analysis of fraction 13 which contained the major β -lactam product observed on an analytical scale and which was putatively assigned as the cephem hydroxy azide **29a/b**. Total ion chromatograms (TICs) are given on the LHS, with the corresponding mass spectra at 5.24 min presented on the RHS.

Comparing the ^1H NMR spectrum of F13 to that of **1**, the β -lactam product in F13 contained two additional multiplet peaks at 4.75 and 3.68 ppm (Figure 3.20). These two peaks were coupled to each other as evidenced by the ^1H - ^1H COSY spectrum, consistent with an ABX-type system at the 3'-position. From the phase-sensitive HSQC spectrum (Figure 3.20), the peak at 4.75 ppm was assigned to H_X (Figure 3.20, **atom 9**) and the 3.68 ppm peak was assigned to H_{AB} (Figure 3.20, **atom 10**).

The ^{13}C shifts of C9 and C10 were used to assign the product as the regioisomer **29a**, rather than **29b**. In the analogous compounds **30a**¹⁷⁶ and **30b**¹⁷⁷ (Figure 3.21), the C(OH) appears downfield of the C(N_3). From the HSQC spectrum of F13, it can be seen that the C9 peak of the β -lactam product is downfield to the C10 peak in the ^{13}C spectrum (Figure 3.20, **red box**). Thus, C9 was assigned as C(OH) and C10 as C(N_3) and the product was identified as **29a**.

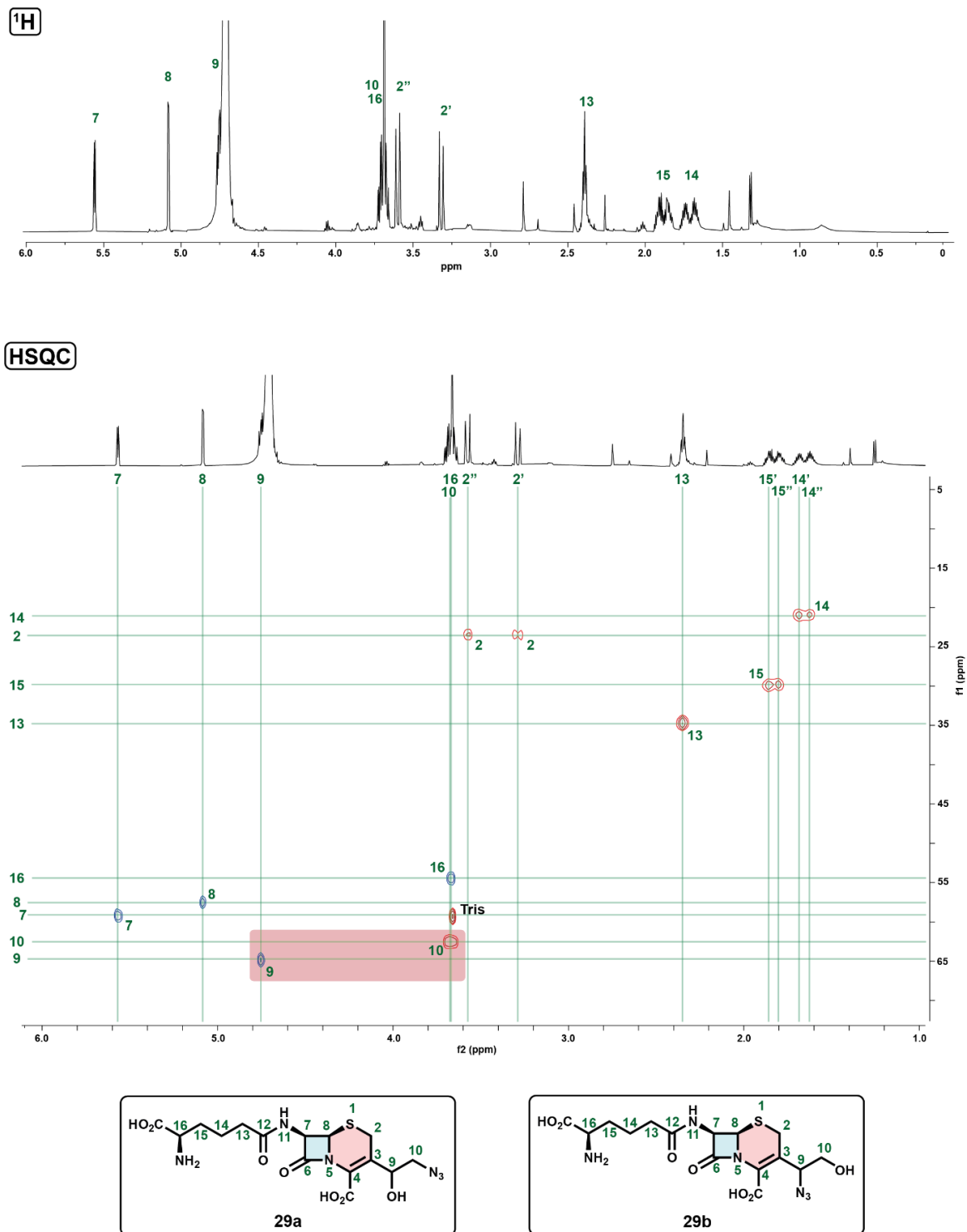


Figure 3.20 | ¹H NMR and 2D HSQC spectra (700 MHz) of F13, containing 29a. The red box in the HSQC spectrum highlights that C9 is downfield to C10 in the ¹³C spectra. Based on analogous compounds 30a/b, the OH group of the hydroxy azide was assigned to C9 and the azide group on C10. Full 2-D spectra (HSQC, HMBC, COSY) are given in Appendix 2: 3-vinyl cephem incubations.



Figure 3.21 | Hydroxy azide regioisomers related to 3-vinyl cephem incubation products. The ^{13}C shifts of azide and alcohol carbons are given. In both cases, the C(OH) is downfield to the C(N_3). This information was used to characterise the new β -lactam product as **29a**.

LC-MS analysis of F12 revealed it to be a mixture of starting material (**1**) and a previously unobserved β -lactam product with an identical mass to **29a** (Figure 3.22).

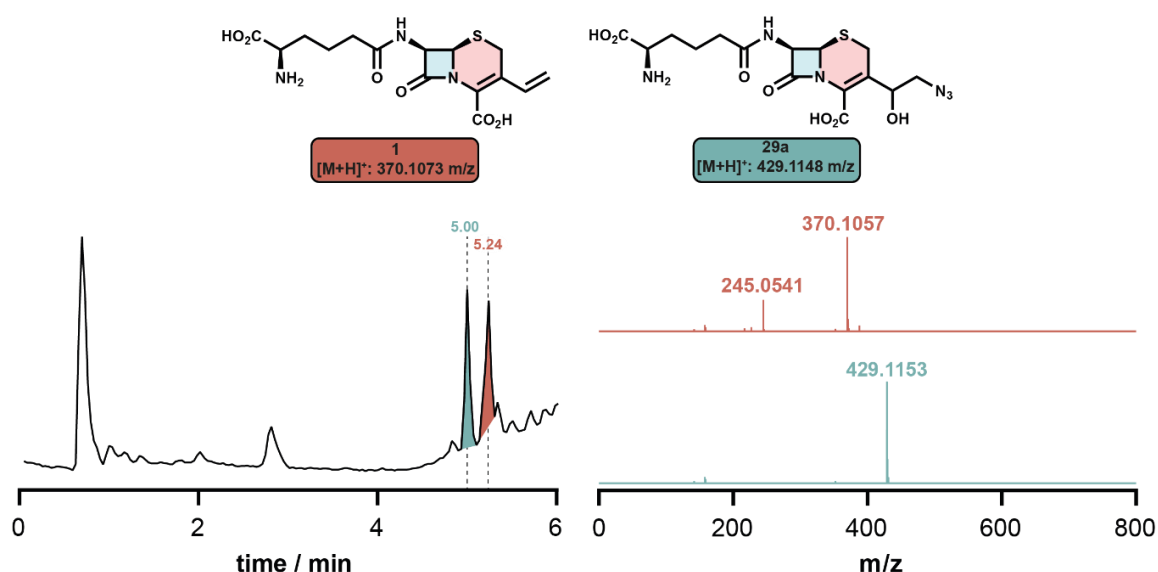


Figure 3.22 | LC-MS analysis of F12. LHS Total ion chromatogram (TIC). RHS Mass spectra for the peaks at 5.00 min (green) and 5.24 min (red). The peak at 5.24 min corresponds to the 3-vinyl cephem. The peak at 5.00 min has an identical mass to **29a** isolated in fraction 13, but possesses a different retention time.

Based on its mass and retention time, it was proposed that this previously unobserved product was either a diastereomer of the major β -lactam product **29a** or its regioisomer **29b**. Whilst insufficient material was isolated for full characterisation of the new product, a low-quality HSQC spectrum was obtained which showed, as with **29a**, the hydroxyl group to be on C9 and the azide on C10, as determined by their respective ^{13}C shifts. Thus, this minor product was assigned as a diastereomer of **29a**.

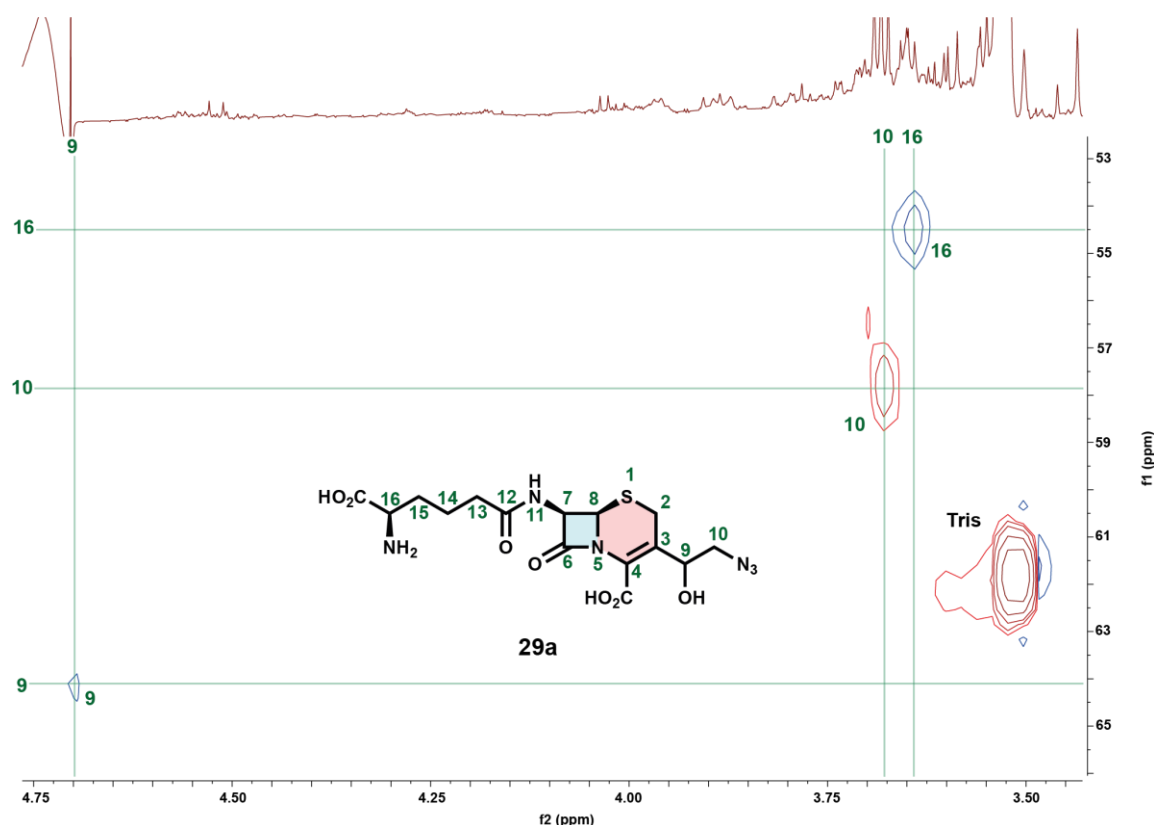
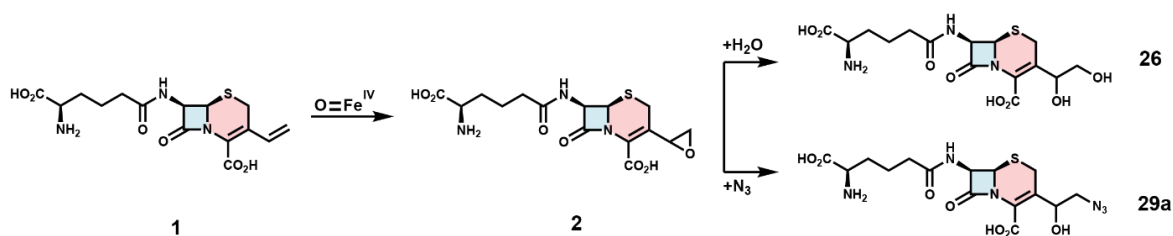


Figure 3.23 | HSQC spectrum of the compound isolated in F12. The C_α carbon shift (atom 9) is downfield to C_{AB} (atom 10) suggesting the OH group is on the C_α , as with the major hydroxy azide product (**29a**) isolated in F13. Full NMR spectra for the minor hydroxy azide product are given in Appendix 2: 3-vinyl cephem incubations.

3.6 Cephem hydroxy azide: Mechanism of formation

In a similar manner to the diol **26**, the formation of **29a** likely proceeds via ring-opening of epoxide **2** by an azide anion (Scheme 3.8). **2** was proposed as the intermediate based on literature precedent for DAOCS/DACS enzyme-catalysed epoxidation of the [4- ^{2} H]exomethylene cepham (**27**) substrate analogue (Figure 3.9).¹¹²

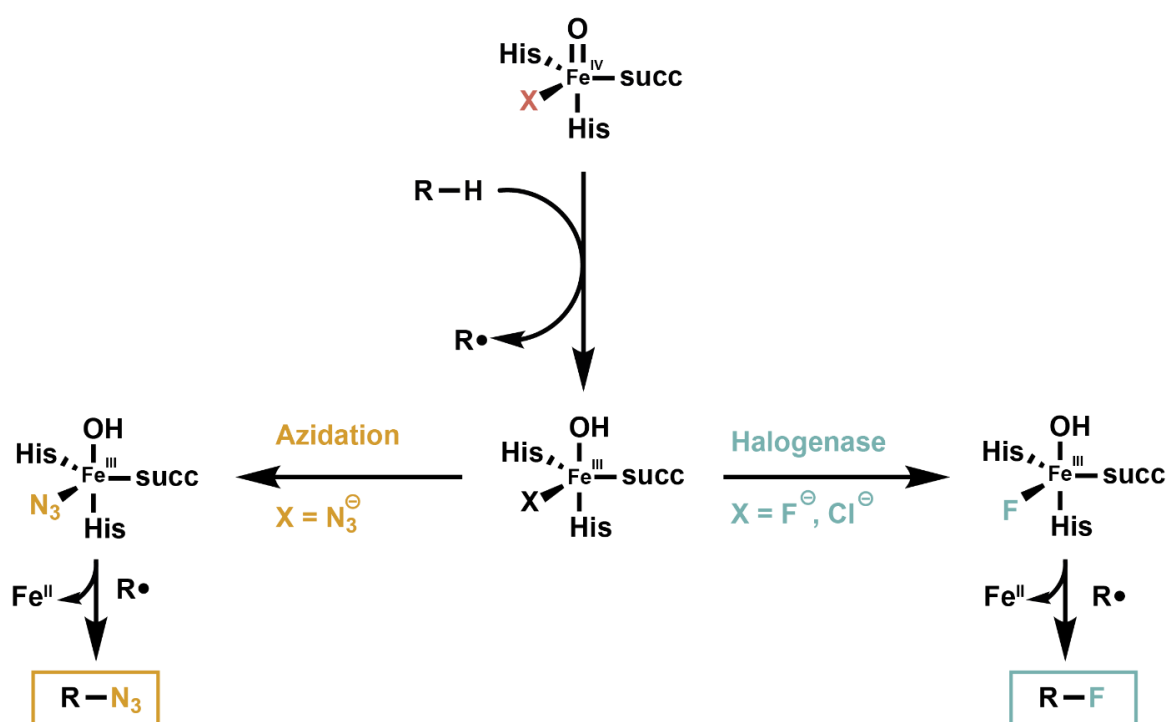


Scheme 3.8 | The formation of 26 and 29a from the common intermediate 2.

Several mechanistic points remained to be clarified, the most pertinent being whether the azidation step that produced **29a** from **2** was enzyme-catalysed or occurred in solution. Based on the $^{18}\text{O}_2$ experiments, it was proposed that ring-opening of **2** with water to produce **26** may occur

in solution. But formation of **29a** might proceed in a different manner; the addition of sodium azide to incubations of **1** with DAOCS/DACS after the enzyme had been quenched yielded only **26**, with formation of **29a** not observed.

Azidation by 2OG oxygenases has been demonstrated for the halogenase SyrB2,¹⁷⁸ In SyrB2, azidation can proceed by a mechanism that is analogous to halogenation, in which a carbon-centred radical is produced by hydrogen abstraction. This radical then couples to an anion, either azide or halide, that is bound to the Fe-centre at a *cis*-coordination site (Scheme 3.9). Close proximity of the anion and prime substrate is required to disfavour rebound of the hydroxyl group which would produce the alcohol product.



Scheme 3.9 | Comparison of azidation and halogenation reactions catalysed by SyrB2. The reaction outcome is determined by which anion is coordinated to the Fe-centre.

Whilst SyrB2 provides a precedent for 2OG oxygenase-catalysed azidation in general, enzyme-catalysed insertion of azides specifically into cephem scaffolds is unprecedented. Efforts were thus directed towards probing the mechanism of the azidation step in the formation of **29a**, to ascertain if azide addition was enzyme catalysed.

3.6.1 ¹⁸O experiments

The origin of the hydroxyl group oxygen atom in the product **29a** was investigated using ¹⁸O-labelling experiments. Incubations of **1** with DAOCS/DACS in the presence of azide (5 mM) under an ¹⁶O₂ atmosphere gave **29a** as the major product (Figure 3.24, **A**). When these incubations were repeated using ¹⁸O₂, a +2-mass increase was observed in **29a** (Figure 3.24, **B**), as had been the case with the diol (**26**). However, when the experiments were performed in H₂¹⁸O, no ¹⁸O-incorporation into **29a** was observed (Figure 3.24, **C**).

The results obtained clearly indicate that the hydroxyl oxygen atom in **29a** was derived solely from molecular oxygen, with no incorporation from water. Analysis of the mass spectra of **26** and **29a** indicated the levels of incorporation of ¹⁸O₂ to be >95%. By contrast, the hydroxyl group oxygen atom of DAC, produced from incubations of penicillin N with DAOCS/DACS, is derived from both O₂ and H₂O, with 50% incorporation from either labelled source (**Section 4.3**). This observation is the same as that previously reported.¹⁷⁹ These results imply that formation of **26** and **29a** from **1** proceeds via a mechanism that is distinct from the mechanism employed by DAOCS/DACS for wild-type hydroxylation.

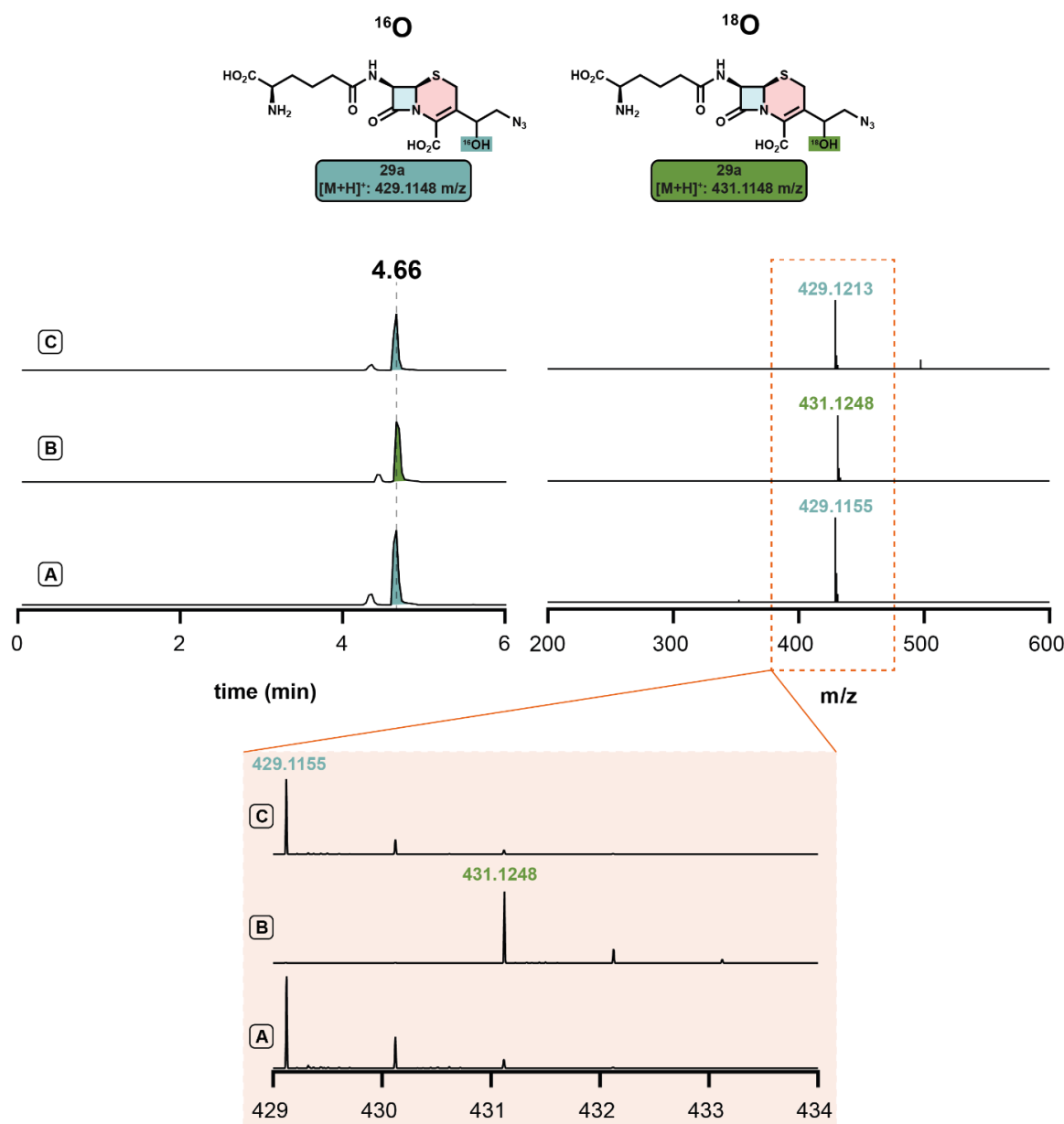


Figure 3.24 | ^{18}O -incorporation into the hydroxy azide 29a product, was observed from incubations of 1 with DAOCS/DACS under $^{18}\text{O}_2$. Mass-extracted chromatograms are presented on the LHS. Mass spectra are given on the RHS. Three different assays are shown: a control under $^{16}\text{O}_2$ in H_2^{16}O buffer (A), incubations under $^{18}\text{O}_2$ in H_2^{16}O buffer (B), or incubations under $^{16}\text{O}_2$ in H_2^{18}O buffer (C).

3.6.2 Inserting different anions into the cephem scaffold

In addition to the azide, the capacity for other functional groups to be incorporated into the cephem scaffold was investigated. **1** was incubated with DAOCS/DACS in the presence of four different anions (Cl^- , F^- , SCN^- , and OCN^-). In all cases, **26** was observed as the sole identified product by ^1H NMR analysis, indicating that none of the functional groups were incorporated (Figure 3.25).

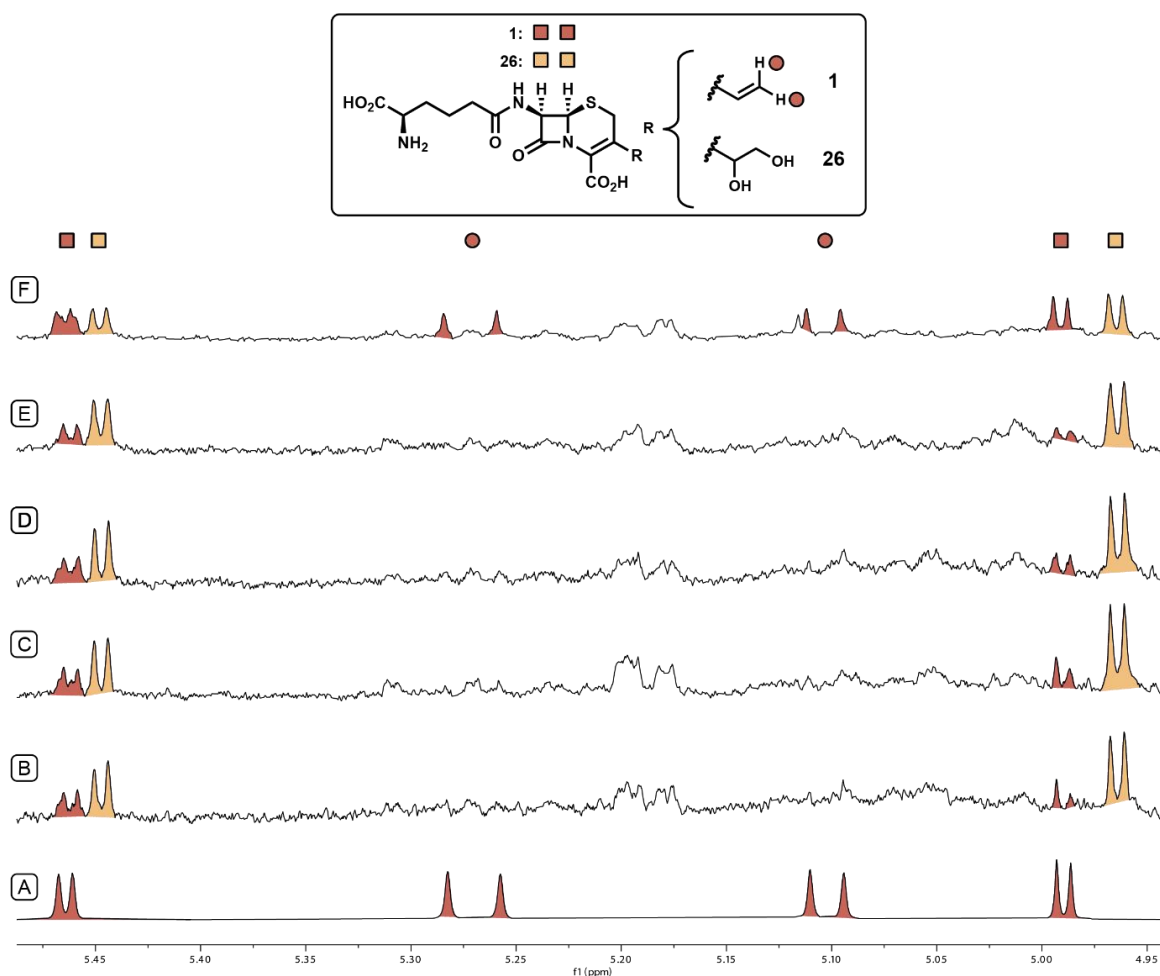


Figure 3.25 | DAOCS/DACS incubations with 1 in the presence of different nucleophiles. A Incubations of 1 without enzyme. B–F Incubations of 1 with DAOCS/DACS with: no nucleophile (B), sodium fluoride (C), sodium chloride (D), sodium thiocyanate (E), and potassium cyanate (F). In assays B–F, the diol 26 was formed exclusively and none of the nucleophiles were incorporated. Assay conditions: DAOCS/DACS (0.2 mM), $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.6 mM), sodium-L-ascorbate (2.4 mM), α -ketoglutarate disodium salt (4 mM), 3-vinyl cephem (1 mM), $\pm$ anion (5 mM). Assays were performed in 50 mM Tris- d_{11} (pH 7.4) in 100 μL total assay volume.

3.6.3 Incubations with other 3'-unsaturated analogues

A limiting factor in the production of **29a** was the availability of the starting material (**1**), the production of which required a multistep synthesis. By contrast, a homologous 3-vinyl cephem substrate with a benzylic side chain (**31**) could be accessed in one step through the coupling of benzoyl chloride and 7-AVCA, facilitating its large-scale production. If DAOCS/DACS accepted **31** as a substrate, the corresponding diol and hydroxy azide products could be produced in larger quantities than **26** and **29a**. Moreover, the unnatural benzylic side chain of **31** might result in slower conversion by DAOCS/DACS, which could permit the epoxide intermediate **2** to accumulate and be isolated. However, no conversion of **31** was observed during incubations with DAOCS/DACS.

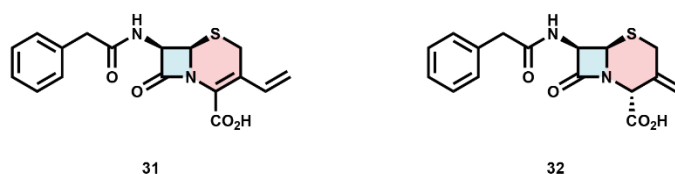


Figure 3.26 | Structures of 3-vinyl cephem G (**31**) and exomethylene cephem G (**32**).

Azidation of the exomethylene cepham G (**32**) was also explored. As mentioned previously, epoxidation of the exomethylene cepham possessing a D- α -amino adipoyl side chain (**27**) has been demonstrated (Figure 3.9).¹¹² The product (**28**) formed from incubations of **27** with DAOCS/DACS is structurally similar to **2**, the proposed intermediate in the formation of **26** and **29a** (Figure 3.27). It was hypothesised that it may be possible to insert an azide nucleophile in the analogues spiro-epoxide that would be the expected product from incubations of **32** with DAOCS/DACS.

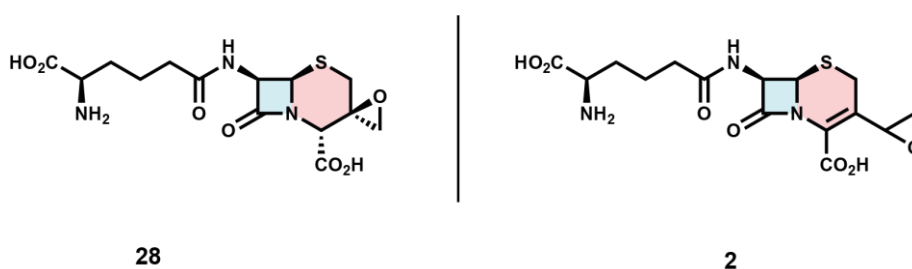


Figure 3.27 | Structural comparison of the spiro-epoxide and **17**.

Whilst **32** was fully converted by DAOCS/DACS to a single β -lactam product, the same product was observed in the presence and absence of azide, suggesting that the azide does not contribute to product formation. Based on previously reported assays,¹¹⁰ the product was assigned as DAG (**33**) (Figure 3.28).

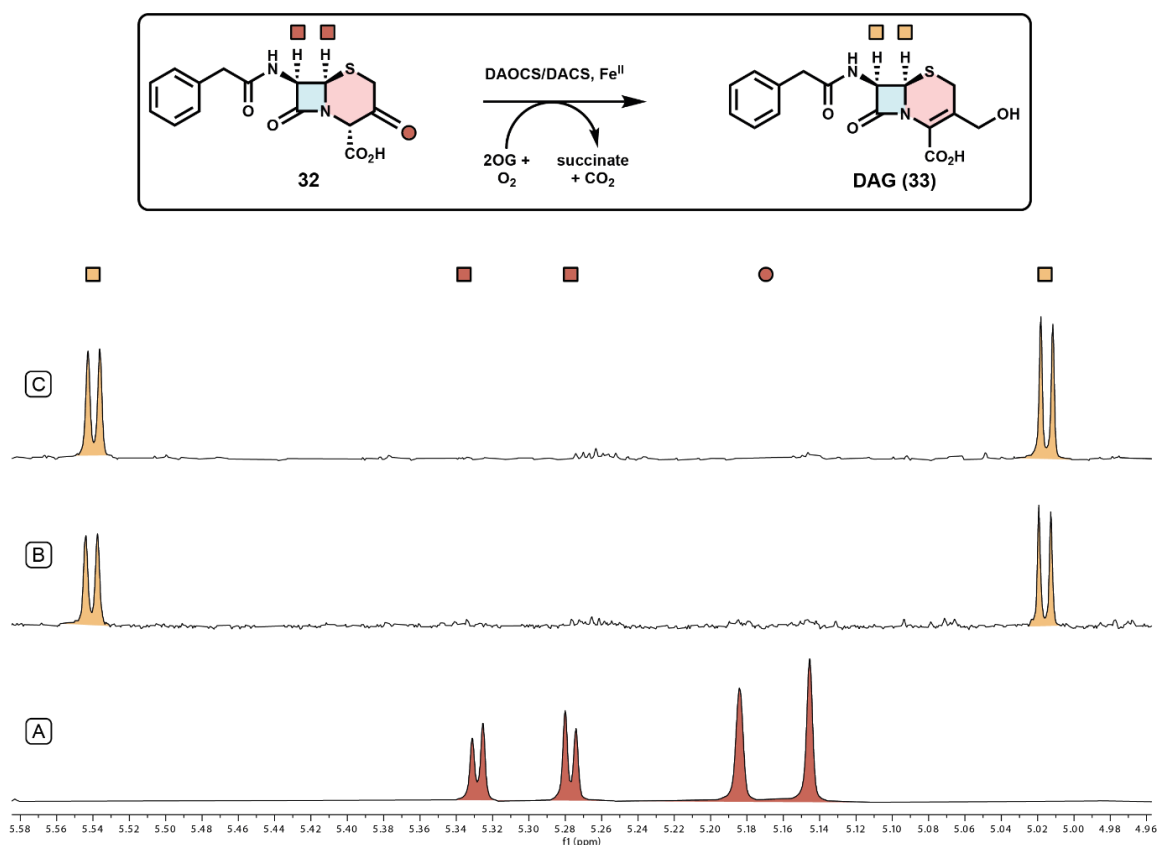
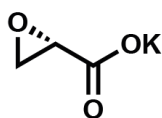


Figure 3.28 | Incubations of 32 with DAOCS/DACS, which produced DAG as a single product in the presence and absence of azide. A Incubations of 32 without enzyme. B Incubations of 32 with DAOCS/DACS with azide (5 mM). C Incubations of 32 with DAOCS/DACS without azide. Assay conditions: DAOCS/DACS (0.2 mM), $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.6 mM), sodium-L-ascorbate (2.4 mM), α -ketoglutarate disodium salt (4 mM), 32 (1 mM), $\pm$ sodium azide (5 mM). Assays were performed in 50 mM Tris- d_{11} (pH 7.4) in 100 μL total assay volume.

3.6.4 Epoxide stability under assay conditions

The stability of **2** towards ring-opening by the azide in solution was a key factor in determining if the azidation step was enzyme-catalysed; if **2** was stable towards azide attack in the absence of DAOCS/DACS it would suggest that DAOCS/DACS was required for azidation. Literature on the stability of epoxides towards azide ring-opening is available as this reaction is used for the synthetic preparation of hydroxy azides. In the absence of a catalyst, epoxides are resistant towards ring-opening by azides, with reflux conditions for 12–48 h typically required to drive the reaction.¹⁸⁰ Under these conditions, ring-opening proceeds with poor selectivity to give a range of hydroxy azide isomers.¹⁸⁰ A number of strategies can improve selectivity, such as: employing a large molar excess of Lewis acids, pH control, the use of metal salts such as $\text{Cu}(\text{NO}_3)_2$ and AlCl_3 , or using organic solvents.^{181–185}

The stability of epoxides towards azide ring opening was explored experimentally using the epoxide **34** (Figure 3.29). Ideally, experiments would be performed with **2**, but it was not readily available. **34** is similar to **2** in that it possesses a carboxylate group adjacent to the epoxide. Moreover, the simple structure of **34** allowed facile reaction monitoring by ^1H NMR.



34

Figure 3.29 | The structure of the epoxide used to explore the capacity for ring-opening by the azide nucleophile in aqueous solvent.

34 was incubated with sodium azide under the assay conditions which produced **29a** (excluding DAOCS/DACS). Incubations were performed at 30 °C and 300 rpm for 2 h and analysed by ^1H NMR (Figure 3.30). No conversion of **34** was observed when the incubations were performed in the absence of sodium azide (**B**). When the incubations were repeated with 5 mM sodium azide (**C**) a similar result was obtained, with no apparent reaction of **34**.

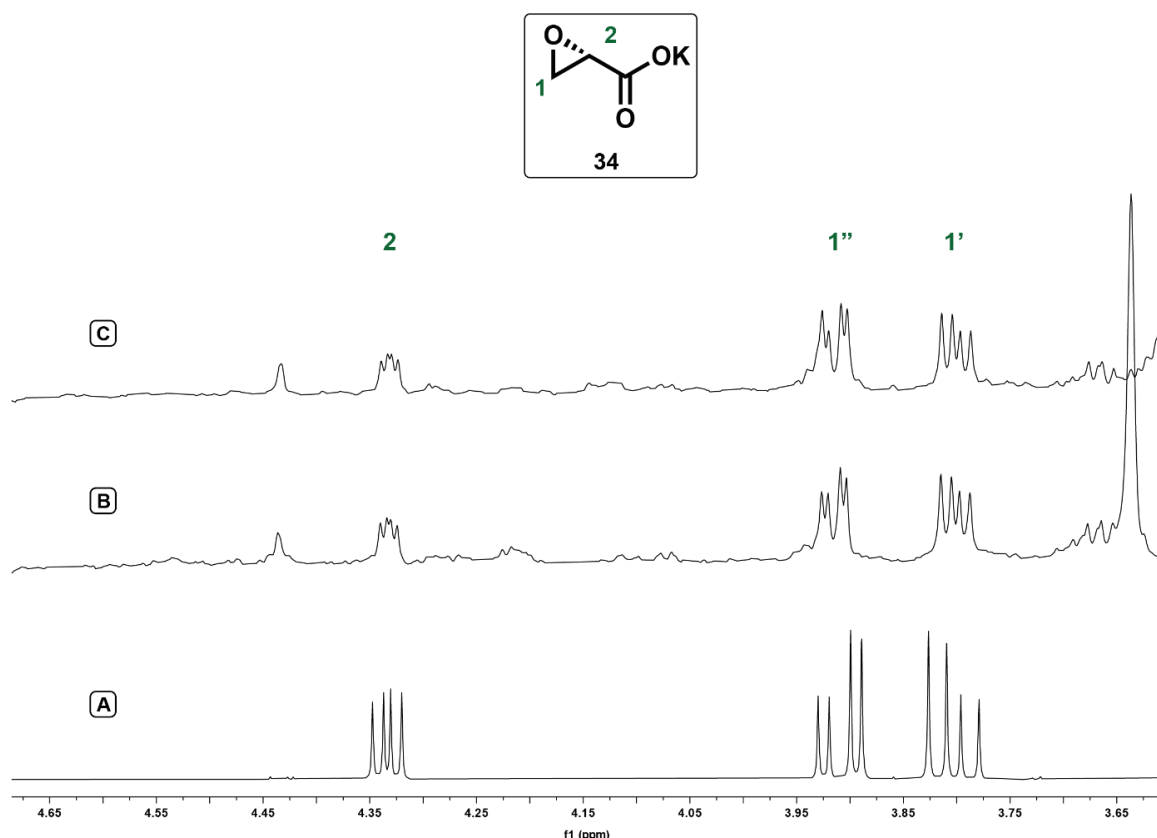


Figure 3.30 | Stacked ¹H NMR spectra following incubations of 34 under conditions which produced 26 and 29a from 1 excluding DAOCS/DACS. A Reference spectrum for 34. B Incubations without sodium azide. C Incubations with sodium azide (5 mM). Assay conditions: (NH₄)₂Fe(SO₄)₂·6H₂O (0.6 mM), sodium-L-ascorbate (2.4 mM), α-ketoglutarate disodium salt (4 mM), 34 (1 mM), ± sodium azide (5 mM). Assays were performed in 50 mM Tris-*d*₁₁ (pH 7.4) in 100 μL total assay volume. Full spectra are given in **Appendix 2: 3-vinyl cephem incubations**. The line-broadening that can be observed in spectra B and C are a result of the paramagnetic effects of Fe(III) being present in solution.

3.6.5 Formation of an Fe–N₃ complex

The azide anion must be capable of binding to DAOCS/DACS in order for the azidation step to be enzyme-catalysed. UV-Vis spectroscopy was used to investigate this binding capacity. UV-Vis has previously been used to probe substrate binding in DAOCS,⁴⁴ and to monitor azide binding to other non-heme iron-dependent enzymes.¹⁸⁶

Two different DAOCS/DACS complexes, DAOCS/DACS·Fe·succinate (**Complex 1**) and DAOCS/DACS·Fe·2OG (**Complex 2**) were prepared anaerobically. After the addition of each ligand, a UV-Vis measurement was taken and a change in absorbance at 330 nm was observed which was taken to represent the ligand binding to DAOCS/DACS (Figure 3.31, **A.1–A.3** and **B.1–B.3**). Upon the addition of 2OG to the DD·Fe complex, a broad peak centred at 510 nm also formed (Figure 3.31, **B.3**). Sodium azide was then added to both complexes and further UV-Vis measurements

were taken. Upon addition of azide to **Complex 1** there was a change in absorbance at 330 nm (Figure 3.31, **A.4**), as had been observed with Fe(II), succinate and 2OG. By contrast, azide addition to **Complex 2** did not induce any spectral changes, either at 330 nm or 510 nm (Figure 3.31, **B.4**). The **B.4** spectrum is identical to the **B.3** spectrum and therefore overlaps with it.

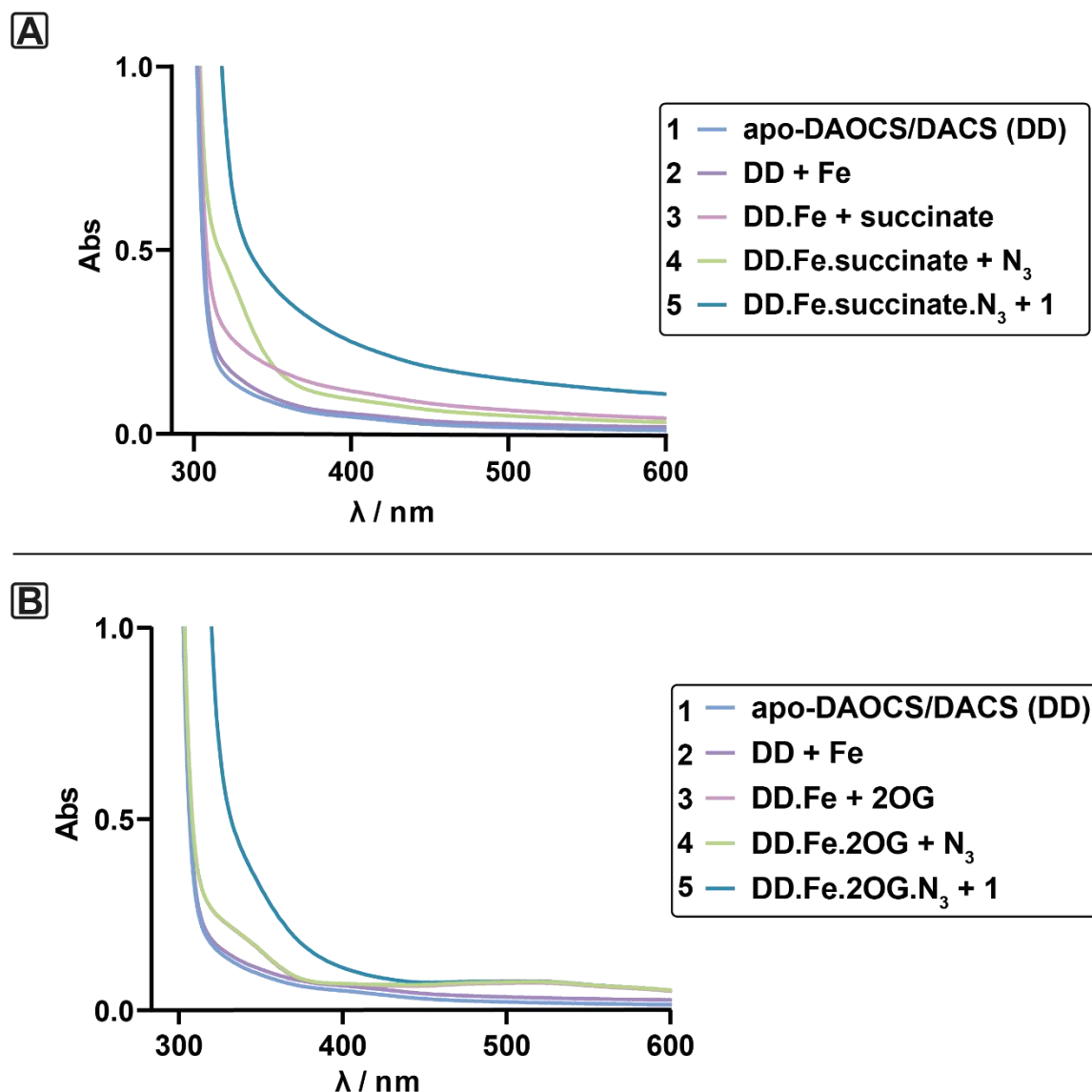


Figure 3.31 | UV-Vis spectra for N₃ addition to DAOCS/DACS complexes. **A** Absorbance spectra for a DAOCS/DACS-Fe(II)-succinate-N₃-**1** complex. **B** Absorbance spectra for a DAOCS/DACS-Fe(II)-2OG-N₃-**1** complex. Because there was no change upon the addition of N₃ (**B.4**), spectrum **B.3** is completely masked by spectrum **B.4**. **1** is the number assigned to the 3-vinyl cephem substrate.

3.6.6 Monitoring substrate conversion in real-time by ¹H NMR

In order to investigate the formation of potential intermediates, such as the epoxide **2**, incubations of **1** with DAOCS/DACS were monitored in real-time by ¹H NMR at 950 MHz, through the use of the PROJECT-CPMG NMR experiments. Proteins, which are much larger than small molecules,

have much shorter T_2 relaxation times. This is exploited in PROJECT-CPMG experiments to filter out protein signals in ^1H NMR spectra, leaving only the signals from small molecules.¹⁸⁷ Incubations of **1** with DAOCS/DACS were prepared in 1.5 mL microcentrifuge tubes and O_2 gas was bubbled into solution to stimulate turnover. The assays were then transferred to a 3 mm NMR tube for analysis (Figure 3.32). Upon O_2 exposure, $\approx 75\%$ conversion of **1** was observed (Figure 3.32, **B**). In addition to the diol (**26**), 9 new peaks (**peaks i–ix**) were present in the β -lactam region (δ 4.5–6 ppm) that were not observed during the analysis of quenched assays. Peaks **i**, **ii**, and **v** disappeared completely after 7 hours (Figure 3.32, **C**). All other peaks reduced in intensity over the 14 hours period in which the sample was monitored (Figure 3.32, **D**).

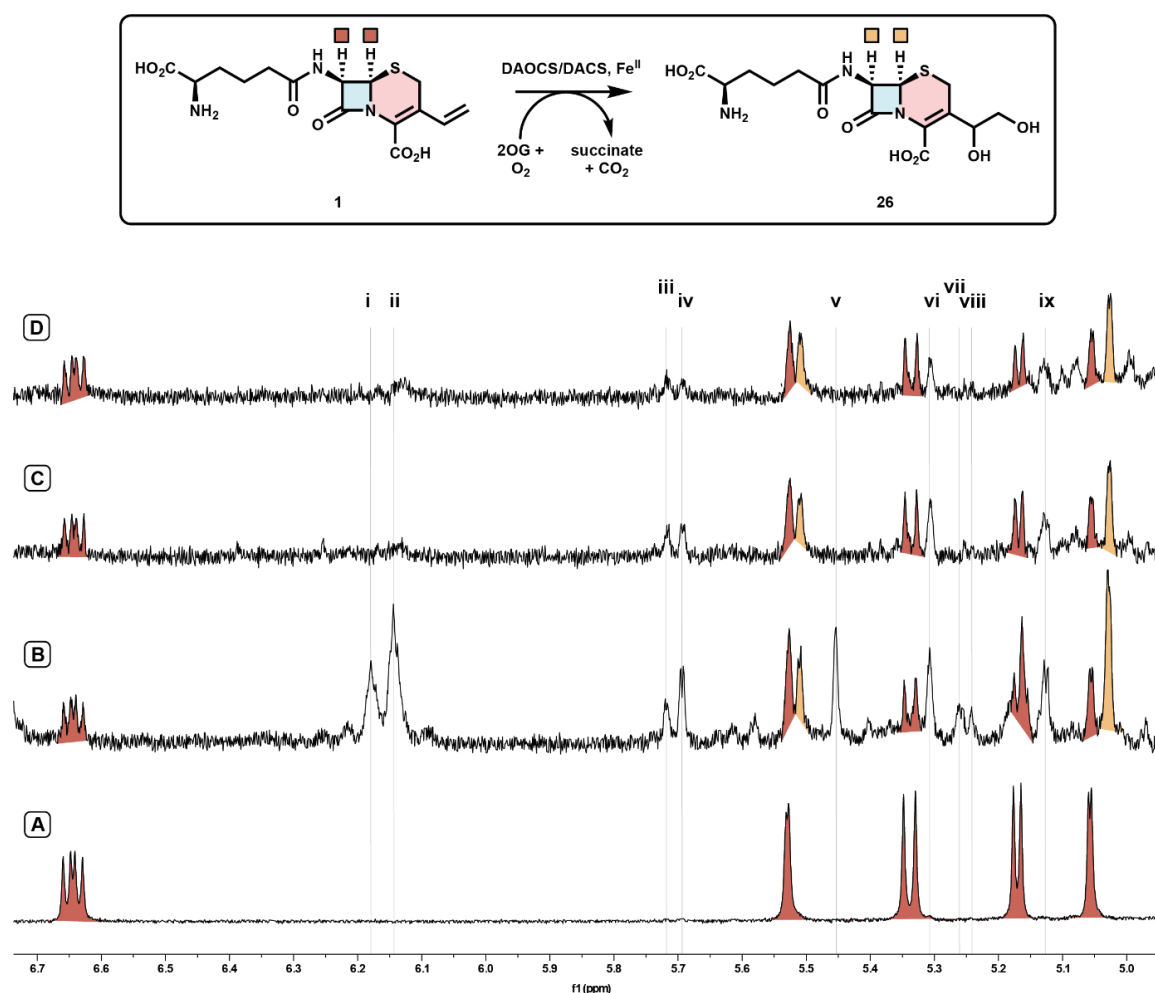


Figure 3.32 | Stacked PROJECT_CPMG spectra monitoring incubations of **1** with DAOCS/DACS in real-time showing the formation of 9 new peaks upon O_2 exposure. **A** Prior to DAOCS/DACS addition and O_2 exposure. **B** Immediately after enzyme addition and O_2 exposure. **C** 7 hours after O_2 exposure. **D** 14 hours after O_2 exposure. Assay conditions: DAOCS/DACS (0.1 mM), $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.3 mM), sodium-L-ascorbate (2.4 mM), α -ketoglutarate disodium salt (4 mM), **1** (1 mM). Assays were performed in 50 mM Tris- d_{11} (pH 7.4) in 180 μL total assay volume.

TOCSY was used to identify if any **peaks i–ix** corresponded to β -lactam species. All β -lactam intermediates will contain a pair of doublets ($J \approx 4\text{--}6\text{ Hz}$) within the β -lactam region ($\delta\ 4.5\text{--}6\text{ ppm}$). Any peaks appearing in this region that are not coupled to another peak within this region were considered unlikely to be β -lactam species.

The TOCSY spectrum is given below (Figure 3.33). Peaks **i** and **ii** had no cross-peaks with other proton signals (data not shown), neither did peaks **v** or **vi**. These peaks likely formed as a result of protein precipitation during O_2 exposure. Their subsequent disappearance at longer time points can be attributed to the aggregated protein settling at the bottom of the NMR tube. Proton-proton correlations in the starting material (**1**, **red**) and diol (**26**, **yellow**) are labelled in Figure 3.33. Of those remaining (peaks **iii**, **iv**, **vii**, **viii**, **ix**), peaks **iv** and **ix** (**blue**) appeared to be the strongest candidates for a β -lactam intermediate, potentially the epoxide **2**, displaying two clear doublets with strong coupling to each other. However, even if peaks **iv** and **ix** did correspond to a β -lactam species, its identity as an intermediate in the formation of **26** is not clear. From the PROJECT-CPMG spectra (Figure 3.32), peaks **iv** and **ix** decay over the course of the experiment, but there is no concomitant increase in the intensity of the two diol (**26**) doublets (**yellow**).

There was an indication of peak **iii** coupling to the shoulder of peak **iv**, as well as peaks **vii/viii** coupling to an unlabelled peak at $\approx 5.40\text{ ppm}$ (**green**). However, these peaks could not clearly be identified as doublets. Efforts to obtain an HSQC spectrum of the sample were unsuccessful, likely due to insufficient material.

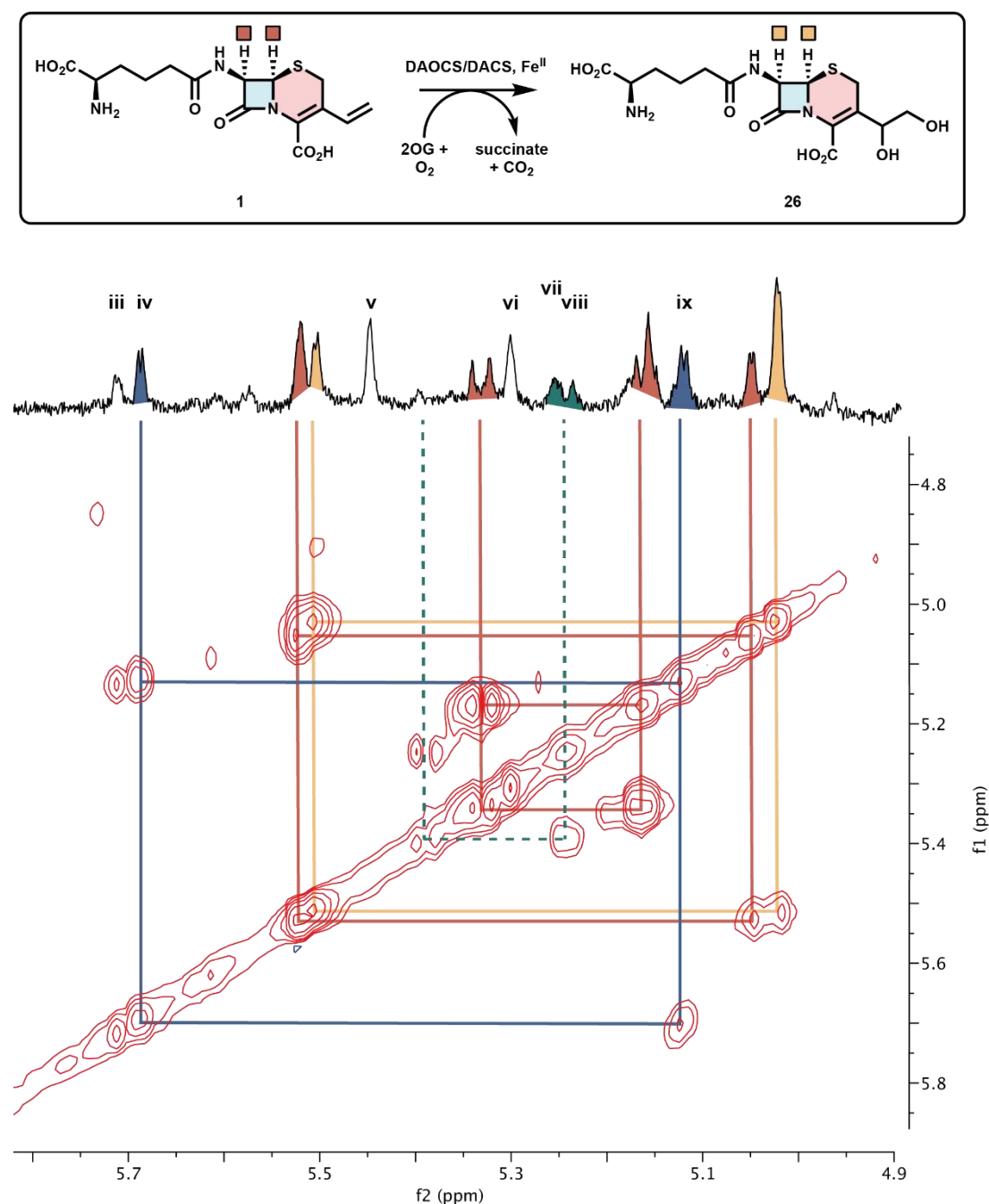


Figure 3.33 | TOCSY showing proton-proton coupling in the β -lactam region during incubations of **1 with DAOCS/DACS.** The 3-vinyl cephem substrate (**1**, red) and diol (**26**, yellow) are clearly visible. Coupling between peaks iv and ix can be observed (blue). There was also potential coupling of peak vii/viii to a region at 5.40 ppm.

3.7 Discussion

30 years ago, MEW demonstrated that DAOCS/DACS was capable of converting the 3-vinyl cephem substrate analogue (**1**) to a single β -lactam (Wood) product, which was isolated by HPLC. Despite ^1H NMR and MS characterisation of the product, its structure was not determined and has remained a mystery ever since. The aim of the work described herein was to repeat MEW's incubations and to characterise and assign the identity of the Wood product.

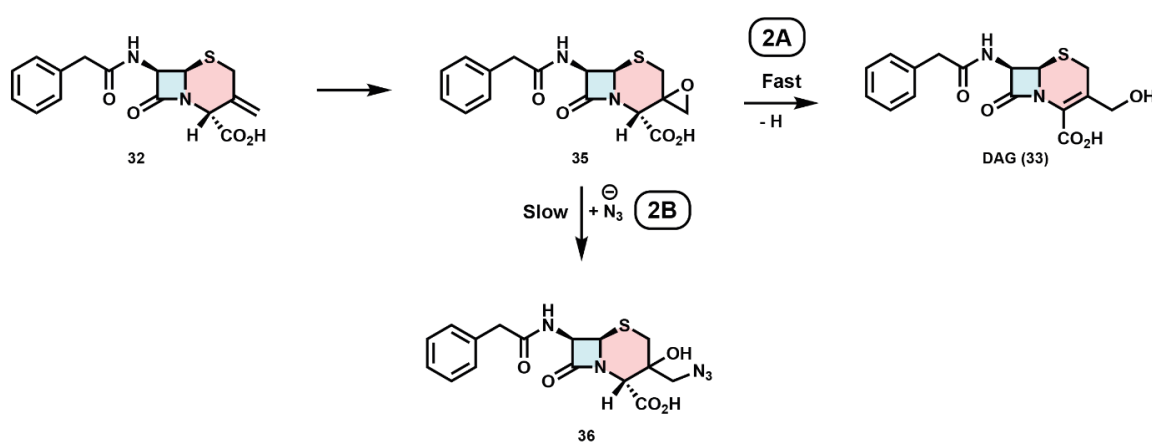
Unbeknownst to MEW, the sample of DAOCS/DACS used for the incubations with **1** contained sodium azide (7 mM), added as an antibacterial agent to the enzyme storage buffer.[‡] Incubations of **1** with DAOCS/DACS in the presence of sodium azide produced a single β -lactam product with identical ¹H NMR and LC-MS characteristics to the Wood product. Using 2-D NMR, the product was formally characterised as the cephem hydroxy azide **29a** with the ¹³C shifts of the C3' carbons used to distinguish between the isomers **29a** and **29b**. In the absence of sodium azide, DAOCS/DACS was shown to convert **1** to the diol **26**. Formal characterisation of **26** is currently ongoing, and will require its isolation by HPLC.

Initial incubations that repeated MEW's assay conditions failed to turn over **1**. This has been attributed to the presence of residual Fe(II) in the DAOCS/DACS sample used by MEW, meaning the actual Fe(II) concentration was higher than stated. By contrast, DAOCS/DACS used for the incubations of **1** described herein was stripped of all divalent metal ions during its purification. Residual Fe(II) has been shown to be sufficient for activity with DAOCS from *S. clavuligerus*, with the addition of exogenous Fe(II) greatly increasing activity of DAOCS towards its natural substrate penicillin N.¹⁸⁸

In this work, **29a** was isolated as a mixture of its diastereomers, the minor of which was not detected in the original 1990 assays, likely because the conversion of **1** was lower; almost complete turnover of **1** was observed by ¹H NMR in this work compared to the $\approx$ 60% conversion reported in 1990. Interestingly, MEW's HPLC trace from 1990 (Figure 3.1, **Panel A**), appears to show a shoulder on the peak of **1** (7 min), analogous to F12 that contained the minor **29a** product (Figure 3.18). This shoulder could correspond to the minor **29a** diastereomer but produced in such small amounts as to be undetectable.

[‡] Personal communication with Ms. Wendy Sobey.

Efforts to incorporate the azide functional group into **32** were not successful. Given that the ^{18}O experiments support the proposal that the conversion of **27** and **1** by DAOCS/DACS proceed by the same mechanism, it is somewhat surprising that the azide could not be inserted into **32**. One theory is that, rather than being a shunt product, the spiro-epoxide (**35**) can be converted into DAG (**33**) via an intramolecular elimination process that outcompetes intermolecular azidation (Scheme 3.10). Alternatively, the formation of the epoxide closer to the ring may sterically hinder azidation.



Scheme 3.10 | Competing formation of the hydroxy azide **36 and DAG (**33**) from the spiro-epoxide intermediate **35**.**
2A Elimination of the C4-hydrogen from the spiro-epoxide to give DAG. **2B** Azidation to produce a **36**. Both routes (**2A** and **2B**) could be enzyme-catalysed or occur in solution.

Once **26** and **29a** had been identified as the product structures, efforts were directed towards establishing a mechanistic understanding of their formation from **1**. DAOCS/DACS demonstrates epoxidase activity with two C3' unsaturated cepham analogues that are comparable in structure to **1** (Figure 3.34).

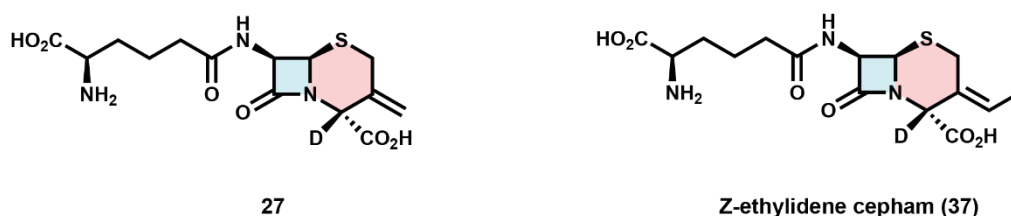
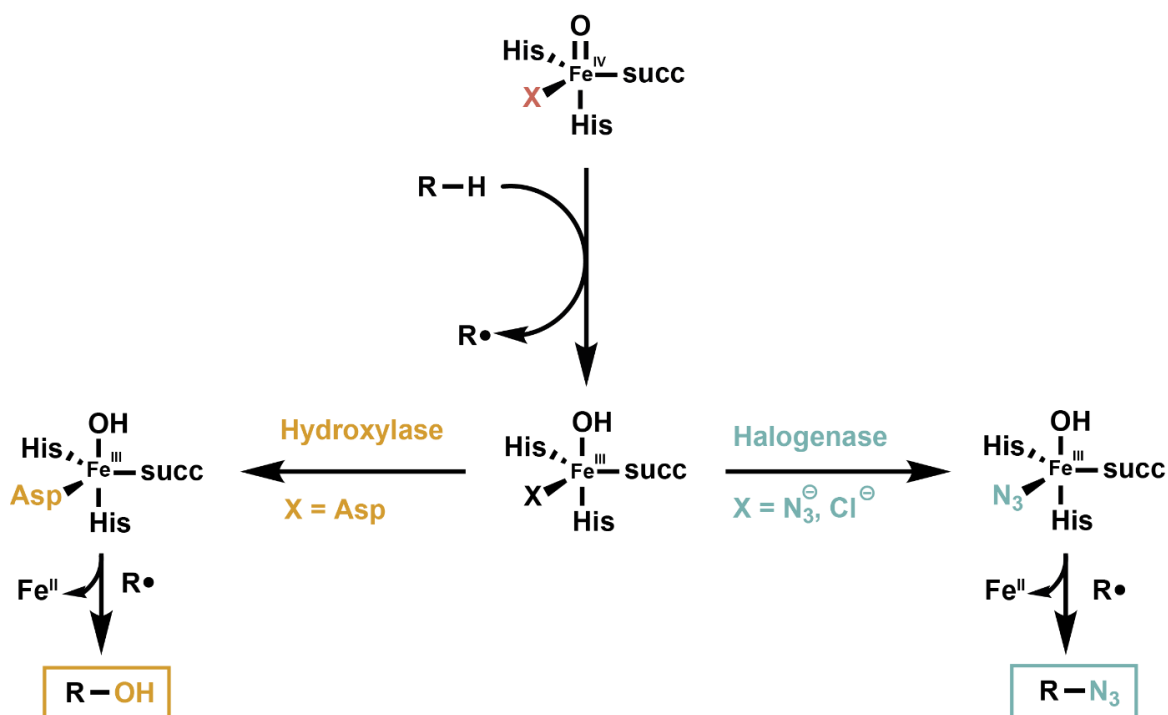


Figure 3.34 | Structure of two 3'-cepham analogues with which DAOCS/DACS displays epoxidase activity. **27 and **37** are structurally homologous to **1**.**

It was therefore proposed that epoxidation of **1** would occur first to produce **2**, from which **26** and **29a** could be formed through nucleophilic attack of the appropriate nucleophile (water or

azide). This proposed mechanism is supported by ^{18}O experiments with molecular oxygen and water. ^{18}O experiments on the wild-type hydroxylation of DAOC to DAC revealed that both O_2 and H_2O are incorporated into DAC during the hydroxylation of the natural substrate DAOC (**Section 4.3**). By contrast, incubations of DAOCS/DACS with $[4\text{-}^2\text{H}]\text{exomethylene cepham (27)}$ produced the spiro-epoxide (**28**) in which the epoxide oxygen atom is derived solely from O_2 .¹⁷⁹ ^{18}O -labelling experiments with **1** similarly demonstrated that the hydroxyl oxygen atom of **29a** is derived exclusively from molecular oxygen. In the diol (**26**), one hydroxyl group each is derived from O_2 and H_2O . This evidence strongly supports the proposal that the formation of **26**, **29a** and **28** proceed (at least initially) via the same mechanism, which is distinct from the wild-type hydroxylase mechanism.

If the addition of the azide nucleophile during the production of **29a** is enzyme-catalysed, the reaction could proceed via several different mechanisms. Azidation by the 2OG halogenase SyrB2 is catalysed via an analogous mechanism to halogenation and requires a free cis-coordination site at the Fe-centre for azide binding.¹⁷⁸ Conventionally, 2OG oxygenases possess a 2-His-1-carboxylate motif that binds to the Fe-centre.²⁹ However, in halogenases such as SyrB2 the carboxylate ligand is replaced by a non-coordinated alanine, permitting coordination of the anion (Scheme 3.11). A 2OG dependent hydroxylase has been converted into a halogenase, but halogenation activity required extensive mutagenesis, including of the coordinating carboxylate group residue.¹⁸⁹



Scheme 3.11 | Simplified mechanisms of hydroxylation and halogenase performed by 2OG dependent oxygenases. In halogenases, the asparagine residue is mutated to a non-coordinating lysine which permit binding of the anion to the Fe-centre from the start of the catalytic cycle.

By contrast to SyrB2, DAOCS/DACS possesses the traditional 2-His-1-carboxylate motif with a saturated coordination sphere at the Fe-centre which prevents binding of the azide from the start of the catalytic cycle. The nonheme iron enzyme *Sav* HppD is also capable of performing azidation and contains a 2-His-1-carboxylate Fe centre, like DAOCS/DACS.¹⁸⁶ However, *Sav* HppD catalyses azidation via a radical-relay mechanism which is initiated by the formation of an amidyl radical. Whilst the Fe-complex of *Sav* HppD contains three coordinating protein residues, it is still unsaturated and the azide nucleophile is bound to the Fe-centre from the start of the catalytic cycle. It would therefore be expected that DAOCS/DACS would utilise a different mechanism for azidation compared to SyrB2 or *Sav* HppD.

In *Sav* HppD, an Fe(III)-N₃ azide intermediate has been observed by Mossbauer and EPR spectroscopy. UV-Vis studies described herein indicate the formation of an analogous DAOCS/DACS-Fe-succinate-N₃ complex. The azide anion was not observed to bind to the DAOCS/DACS-Fe-2OG complex. This result suggests that azide binding to DAOCS/DACS requires a vacant Fe-coordination site; if the azide bound to a hydrophobic pocket in the active site rather

than directly to the Fe-centre, it would be expected to bind to both succinate and 2OG complexes, independent of the Fe-coordination sphere.

In the formation of DAC, oxygen exchange with water is proposed to occur at an Fe(IV)–OH intermediate that is formed by proton abstraction from DAOC by the Fe(IV)=O species.¹⁷⁹ In order to avoid oxygen exchange, the formation of **29a** could proceed via a different mechanism in which **1** reacts directly with the oxygen atom of Fe(IV)=O. Three such mechanisms are proposed below (Figure 3.35). In **Route 1**, formation of the cyclic intermediate could occur initially, with subsequent collapse to the epoxide (**2**) that remains coordinated to the Fe-centre. Insertion of the azide, bound either to an adjacent hydrophobic pocket or at a *cis*-coordination site, would give **29a**. A precedent for the conversion of epoxides by DAOCS/DACS can be found in the spiro-epoxide (**28**), which is converted by DAOCS/DACS to a cephem aldehyde.¹⁷⁹ **2** could also be released from the active site, with ring-opening by N₃ or H₂O occurring in solution (**Route 2**). This is the proposed mechanism by which **26** forms, but is considered unlikely to account for **29a** based on: the stability of the analogous epoxide **34** under assay conditions, the inability to insert other nucleophiles, and the regioselectivity with which **29a** is formed. Alternatively, the conversion of **1** could proceed via an acyclic intermediate, with binding of the azide to the resultant Fe(III) intermediate and insertion to give **29a** (**Route 3**). The mechanism could also proceed via a cationic, rather than radical pathway.

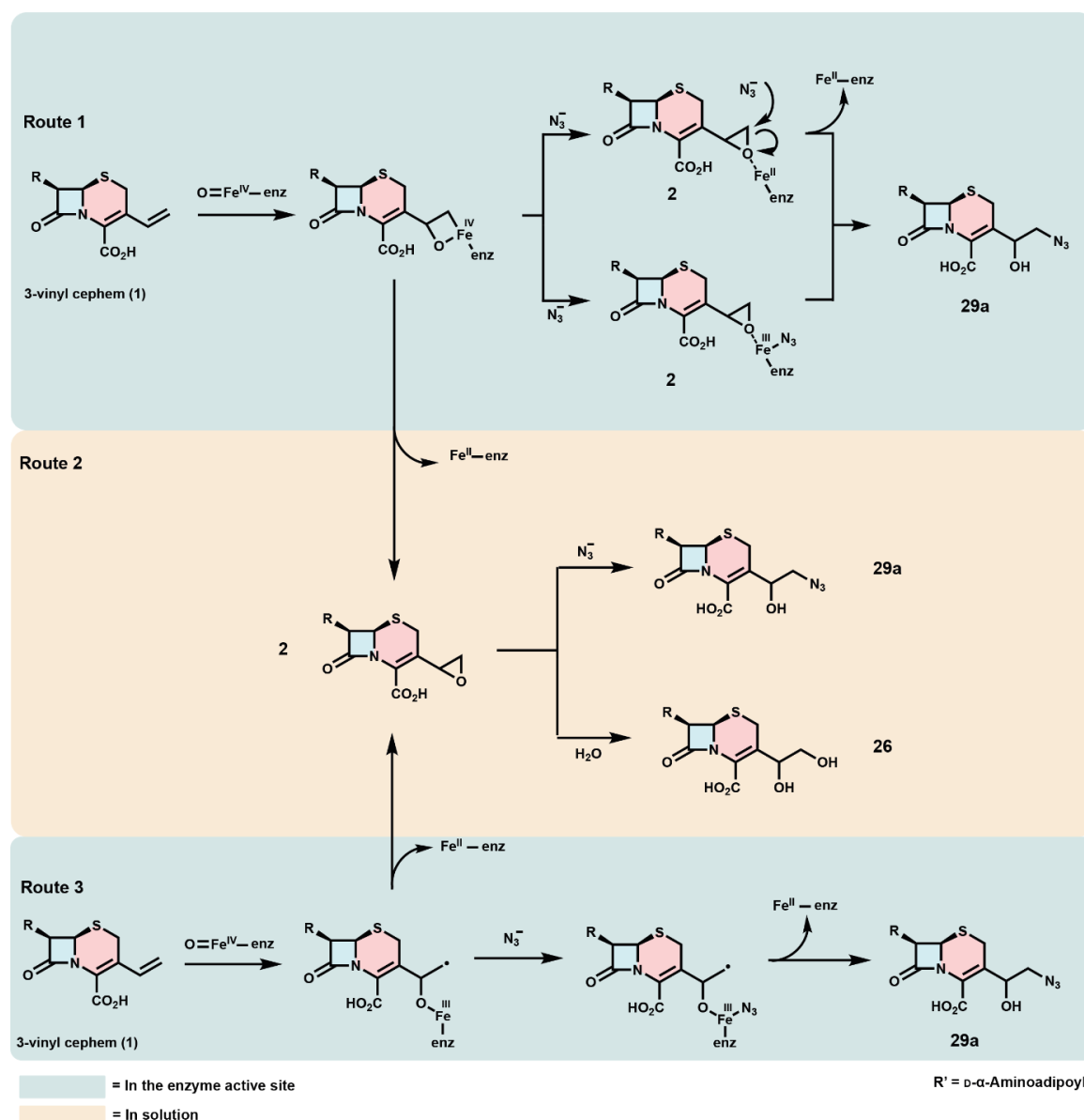


Figure 3.35 | Potential mechanisms for the formation of 26 and 29a from 1. **Route 1** Enzyme-catalysed formation of **29a**, proceeding via a cyclic intermediate which collapses to give the epoxide **2**. The azide anion could be bound in a hydrophobic pocket in the active site (**top**) or, more likely, be bound directly to the Fe-centre (**bottom**). **Route 2** After its formation, the epoxide **2** is released into solution where it forms **26** and **29a**. It is likely that **26** is formed by this route, but is considered less likely to account for the formation of **29a**. **Route 3** DAOCS/DACS-catalysed formation of **29a** proceeding via acyclic, epoxide equivalent intermediates. In both **Route 1** and **3**, the oxygen atom of the $\text{Fe}^{\text{IV}}=\text{O}$ species forms bonds during the reaction mechanism and therefore cannot exchange with water.

In *Sav* HppD, efforts to replace the azide with halides or pseudo-halides and install a range of functional groups were unsuccessful, with failure attributed to the weaker binding these anions to the Fe-centre compared to N_3^- .¹⁸⁶ A similar outcome was observed with DAOCS/DACS, with incubations of **1** in the presence of halides and (thio)cyanate yielding only the diol **26**. As with *Sav* HppD, this could be caused by the lower affinity of these anions to the Fe-centre of DAOCS/DACS.

2 was potentially observed during PROJECT-CPMG NMR experiments, however these results need to be validated. The spectra obtained using existing experimental methods suffered from poor resolution, which impaired their analysis. A major limiting factor is the presence of paramagnetic species (Fe(III) and O₂) in the incubations. Paramagnetic effects reduce the difference in T_2 relaxation times between protein and small molecules, which are otherwise governed by molecular size. As a result, the PROJECT-CPMG is less effective at filtering out protein signals from the ¹H spectra, diminishing spectra resolution.

3.8 Future work

In an attempt to reduce the number of steps in the synthesis of **1** and improve yields, further optimisation work could be carried out. A clear area for improvement is in the protection of 7-AVCA (**11**), with direct-protection of the C4 acid reducing this process from three steps to one. Attempts to apply the one-step *t*-Butyl esterification of 7-ADCA to 7-AVCA were unsuccessful, but alternative strategies may be more fruitful.

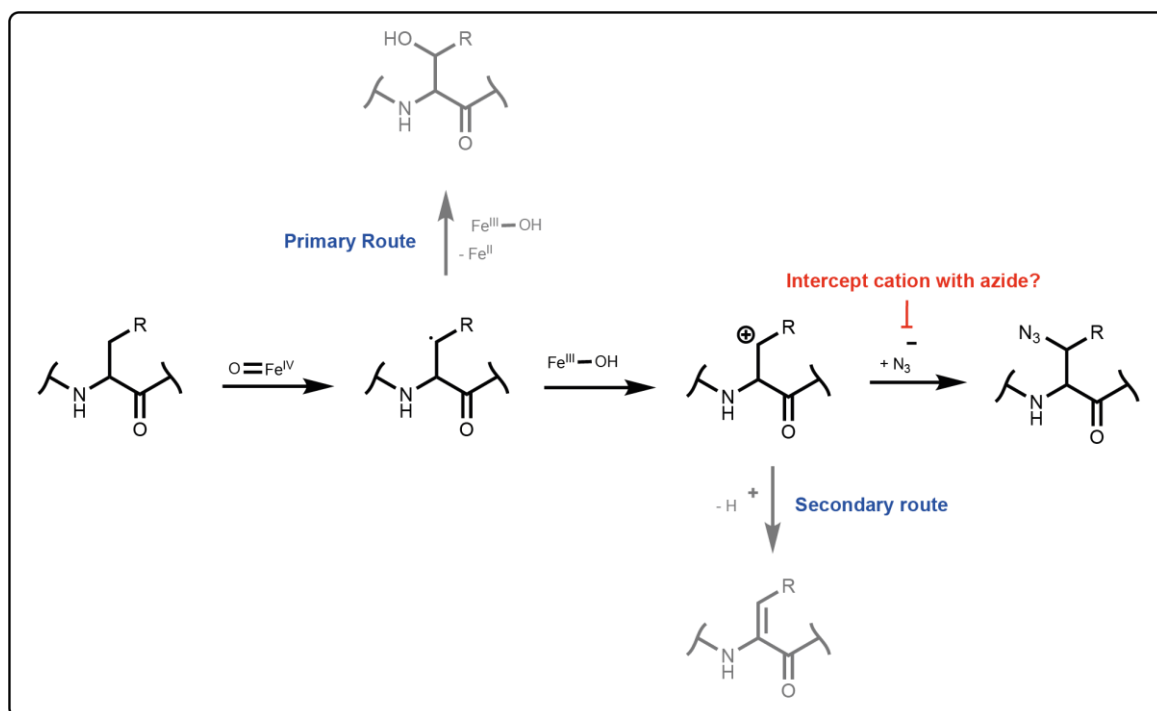
The immediate focus of the CPMG NMR experiments is producing the putative epoxide intermediate (**2**) in sufficient material to permit HSQC spectrum to be obtained. ¹H-¹³C cross-peaks for **2** would allow analysis of the ABX system at the C3' position on the cephem ring. Comparing the ABX shifts of **29a** with the terminal CH₂ group of the spiro-epoxide **27**,¹¹² **2** may be easily identifiable in the assay mixture.

Time-course studies using LC-MS analysis could be used to track changes in the **26:29a** ratio and is complementary to ¹H NMR since LC-MS requires much less material. The capacity of the azide to bind to DAOCS/DACS is being explored further by UV-Vis spectrometry and non-denaturing ESI-MS. In the longer term, it is hoped that Mössbauer spectroscopy can also be established to investigate the capacity of the azide to bind to DAOCS/DACS.

Whilst challenging, the synthesis of **2** would enable the mechanism of azidation to be determined definitively. Previous attempts to convert **1** to **2** using mCPBA resulted in oxidation of the ring sulfur to the corresponding sulfoxide, whilst the C3' vinyl group remained intact. Δ^3 to Δ^2 -double bond isomerisation was also observed (personal correspondence with the author).¹⁰⁶ However, recent advances in epoxidation chemistry might be able to circumvent these issues.

The incorporation of the azide group into a cephem scaffold can enable access to new cephalosporin derivatives via the application of click chemistry.¹⁹⁰ New functionalities can be incorporated via the azide, yielding novel cephalosporin libraries to be screened for antimicrobial potency. Of particular interest is the one-pot synthesis of functionalised cephalosporins by the addition of alkyne reagents to incubations of **1** with DAOCS/DACS. In such an experiment, **29a** would undergo a click reaction with the alkyne as it is produced. Fluorinated cyclooctyne derivatives have been identified as potential alkyne substrate with which this procedure could be explored.¹⁹¹

If DAOCS/DACS is capable of catalysing azidation, it provides another example of azide insertion by a 2OG oxygenase. The capacity of additional 2OG oxygenases to perform azidation could be explored, of particular interest would be the insertion of azides into protein scaffolds. For example, FIH is a 2OG oxygenase that hydroxylates HIF and regulates its transcriptional activity in response to dioxygen availability.^{192, 193} FIH's primary reaction is a typical 'oxygen rebound' mechanism for 2OG oxygenases, but is also been shown to catalyse desaturation.¹⁹⁴ It is hypothesised that desaturation proceeds via a cationic intermediate, which could potentially be intercepted by an azide (Scheme 3.12). This theory should be explored further.



Scheme 3.12 | Potential insertion of the azide nucleophile into proteins by FIH.

Chapter 4

**Characterisation of the cephalosporin biosynthesis
enzymes: crystallisation and incubations with wild-type
substrates**

4 Characterisation of cephalosporin biosynthesis enzymes: crystallisation and incubations with wild-type substrates

4.1 Crystallographic studies on cephalosporin biosynthesis enzymes

4.1.1 Introduction

Of the three cephalosporin biosynthesis enzymes: DAOCS/DACS, DAOCS and DACS, crystal structures have only ever been obtained for DAOCS from *S. clavuligerus*.¹⁹⁵ Attempts to crystallise DAOCS/DACS and DACS were made in the 1980s but they proved recalcitrant to efforts.⁷⁹ In 1998, crystal structures of the DAOCS apoenzyme, as well as the DAOCS·Fe(II) and DAOCS·Fe(II)·2OG complexes became the first to be reported for a 2OG dependent oxygenase.¹⁹⁵ The enzyme-cofactor complexes were obtained by anaerobic soaking of Fe(II) and 2OG solutions into crystals of apo-DAOCS. An identical method was used to obtain further DAOCS·Fe(II) and DAOCS·Fe(II)·2OG complexes,¹⁴⁷ as well as DAOCS structures with 2OG analogues.¹⁹⁶ These crystal structures revealed that DAOCS possesses a distorted double-stranded β -helix (DSBH) core fold flanked by characteristic helices,⁴² structural features that are typical of 2OG oxygenases.²⁹ In the active site, Fe(II) is coordinated by a 2-His-1-Asp facial triad, made up of His183, His243, and Asp185 residues that are contained within the DSBH core (Figure 4.1).¹⁹⁵ Adjacent to the Fe(II) centre of DAOCS is a hydrophobic pocket that is the proposed binding site of 2OG and penicillin substrates.¹⁹⁵

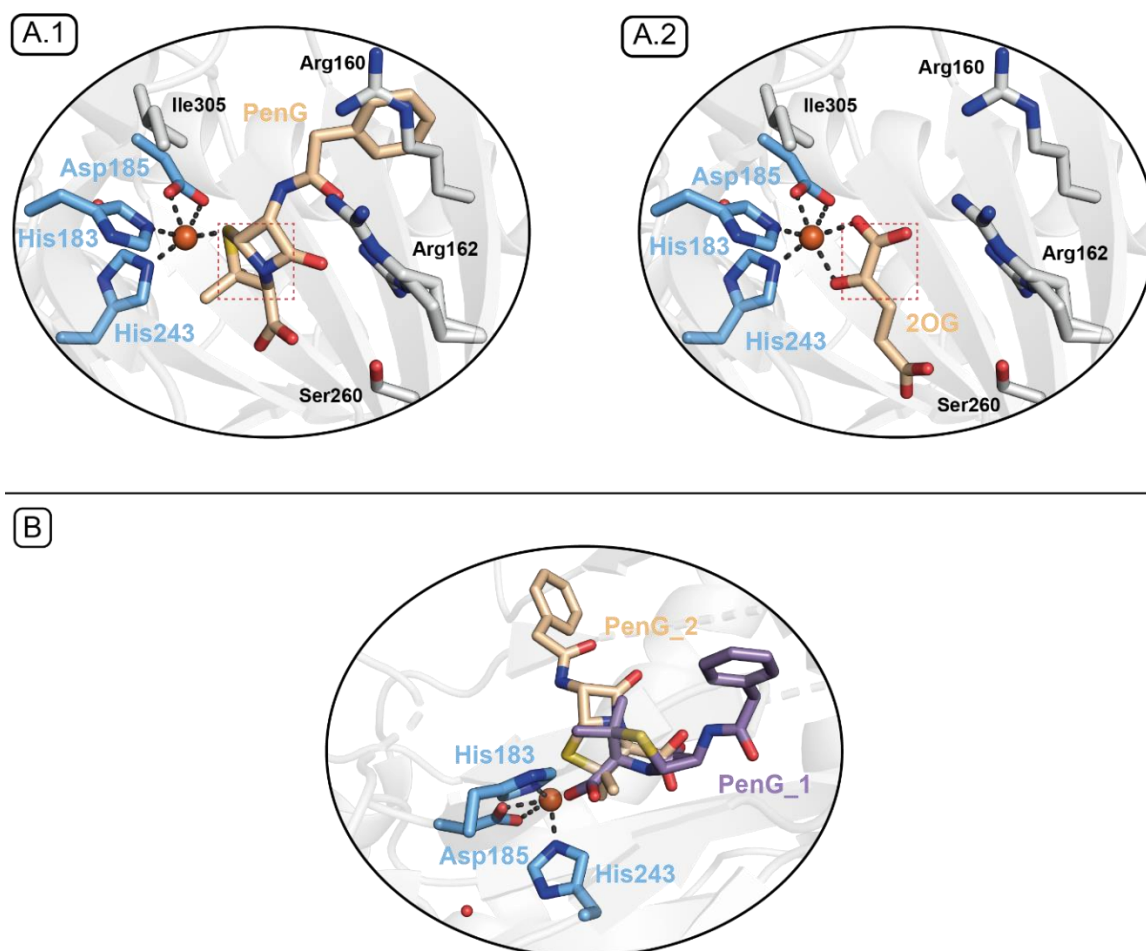


Figure 4.1 | The active site region of DAOCS in complex with (co)substrates. A.1 DAOCS-Fe(II)-penicillin G complex (1.7 Å). **A.2** DAOCS-Fe(II)-2OG complex (1.7 Å). The overlapping binding site of penG and 2-oxoglutarate are highlighted by the red box. **B** Two binding orientations of penicillin G (penG_1 and penG_2) in the active site of DAOCS. Figures were produced from DAOCS structures deposited in the Protein Data Bank (accession codes: 1UOB and 1UOF).⁴³

All crystals of DAOCS produced by anaerobic soaking experiments were merohedrally twinned and of the space group R3. The observed twinning was the result of DAOCS crystallising as a trimer, in which the C-terminal arm (residues 308–311) of one monomer extended into the active site of an adjacent monomer. In these trimeric crystals, access to the active site is partially occluded by this C-terminal arm. Dynamic light-scattering studies performed on DAOCS in solution revealed the apoenzyme to be in equilibrium between monomeric and trimeric forms.¹⁴⁷ In the presence of Fe(II), the monomer is preferred, indicating that this form is catalytically active.¹⁴⁷ Efforts to break trimerization by truncating the C-terminus of DAOCS were unsuccessful, with truncated mutants either being inactive or producing the same crystal form as wild-type DAOCS.¹⁹⁷

Partial occlusion of the active site in DAOCS trimeric crystals prevented access by penicillin substrates or cephalosporin products. As such, enzyme-substrate and enzyme-product complexes were not readily produced. Several years of optimisation were required to find conditions in which penicillin and cephalosporin soaking into DAOCS apoenzyme crystals was successful.⁴³ The resultant enzyme-substrate complexes purported to show overlapping binding sites for 2OG and the penicillin G (penG) substrate (Figure 4.1, A). Based on these structures and limited kinetic data, a novel, “ping-pong” mechanism was proposed, an overview of which is given below (Figure 4.2, 1). In this mechanism, oxidative decarboxylation of 2OG to give succinate, CO₂ and the reactive Fe(IV)=O species occurs before penicillin binds to the active site (**1.b**).⁴³ Release of succinate permits the binding of penicillin (**1.c**), which then reacts with the Fe(IV)=O species and undergoes ring expansion to DAOC.⁴³ New crystal forms of DAOCS, which showed no evidence of trimerization, were subsequently produced by adding an N-terminal His₆-tag.⁴² 3 Å resolution enzyme-product crystal structures of His₆-DAOCS also supported this “ping-pong” mechanism and showed overlapping 2OG and penicillin G binding sites.

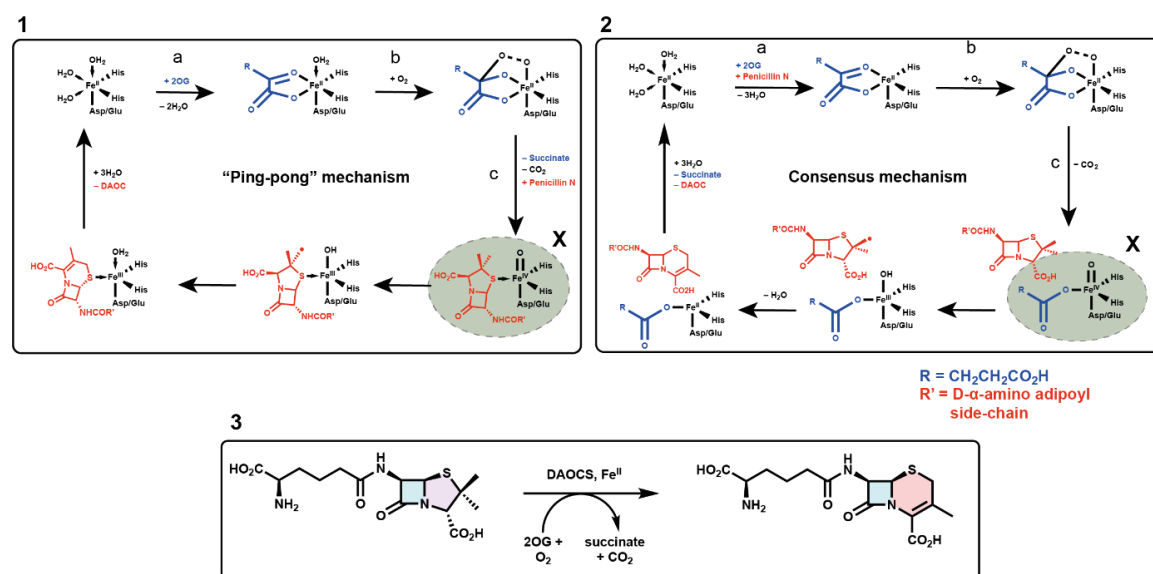


Figure 4.2 | Two mechanisms proposed for the ring-expansion of penicillin N to DAOC by DAOCS. 1 The “ping-pong” mechanism, in which dioxygen binding to DAOCS-Fe(II)·2OG triggers oxidative decarboxylation of 2OG (**step 1.b**). Succinate release is required for penicillin N binding (**step 1.c**). 2 The proposed consensus mechanism, where the ternary complex DAOCS-Fe(II)·2OG·penicillin N forms (**step 2.a**) prior to dioxygen binding (**step 2.b**). In each mechanism, the reactive ferryl species (**1.X** or **2.X**) is highlighted (green). An overview of the ring-expansion of penicillin N to DAOC is also given (**3**). Figure is based on one presented in literature.⁴⁴

The “ping-pong” mechanism marks a significant departure from the well-established 2OG consensus mechanism (Figure 4.2, **2**), in which oxidative decarboxylation of 2OG (**2.c**) does not occur until penicillin N has bound the enzyme’s active site (**2.a**).^{25, 29, 198} Subsequent kinetics and binding studies on DAOCS have cast doubt on the validity of the “ping-pong” mechanism and provide evidence in support of DAOCS-catalysis proceeding via the consensus mechanism.⁴⁴ Crucially, ESI-MS and NMR studies established that 2OG and penicillin N can simultaneously bind to DAOCS in solution. More recent studies have also supported a consensus 2OG mechanism for DAOCS.^{199, 200} Multiple binding modes were observed for penG both in solution and *in crystallo* (Figure 4.1, **B**).⁴³ As such, it is hypothesised that the DAOCS·Fe(II)·penG crystal structures on which the “ping-pong” mechanism was primarily proposed represent penicillin binding modes that are not catalytically productive. Computational studies on the ring-expansion reaction catalysed by DAOCS have demonstrated that the binding orientation of the penicillin substrate in the active site is crucial in determining which proton is abstracted first.²⁰¹ Thus, crystal structures of DAOCS, as well as DAOCS/DACS, in which penicillins are bound productively would be very informative from a mechanistic viewpoint.

4.1.2 Project aims

Currently, a disparity exists between crystallographic^{42, 43} and kinetic data⁴⁴ regarding the mechanism by which DAOCS catalyses the ring expansion of penicillin N to DAOC. Solution-based evidence supports a 2OG consensus mechanism for DAOCS, implying that the existing crystal structures on which the “ping-pong” mechanism is based represent non-productive binding modes for penicillin G. Thus, efforts were to be directed towards the production of new crystal structures of the cephalosporin biosynthesis enzymes. In order to promote productive penicillin binding, a penG optimised DAOCS mutant was identified as a candidate for crystallography. Since this mutant demonstrated more efficient turnover of penG than wild-type DAOCS, it was hypothesised that penG would be more likely to bind the mutant in a catalytically productive orientation compared to wild-type DAOCS. The fungal enzyme DAOCS/DACS was also identified as a target for

crystallisation, facilitated by improvements in its recombinant production (**Chapter 2**). Previous studies have demonstrated the ability to convert the bifunctional enzyme DAOCS/DACS into an expandase or hydroxylase via single mutations.⁸⁰ The mutated residues in DAOCS/DACS were selected based on analysis of DAOCS crystal structures, but structures of DAOCS/DACS would provide more relevant structural insights. To date, crystallisation strategies have soaked cofactor and substrate into apoenzyme crystals under anaerobic conditions.^{42, 43, 147, 195-197} This method could have contributed to the non-productive binding of penG. A new co-crystallisation strategy was developed in which enzyme-metal-substrate complexes were produced in solution prior to crystallisation. It was anticipated that this approach would be more likely to produce structures in which penicillin substrates were bound in a catalytically relevant orientation.

In summary, the aims of the work described were as follows:

- Optimisation of enzyme-penG crystallisation conditions using DAOCS variants that are optimised for penG conversion, to produce crystal structures in which penG is bound in a productive orientation.
- Optimisation of crystallisation conditions of the fungal enzyme DAOCS/DACS.

4.1.3 Selection of ICM DAOCS as a crystallographic target

A DAOCS variant optimised for penG conversion was selected for crystallisation regimes with penG as a substrate. Enzymatic ring-expansion of penG to DAOG is the preferred route to the industrially important precursor 7-ADCA, from which most clinically useful cephalosporin antibiotics are synthesised; penG is commercially available and its hydrophobic side chain permits extraction of the ring-expanded product, DAOG, into organic solvents.⁴⁶ As a result, significant efforts are directed towards improving the activity of DAOCS towards penG.^{202, 203} Optimisation strategies have typically employed directed evolution as means of improving penG conversion. Whilst this approach has identified highly active single mutants,^{43, 125, 203-205} combinations of such mutants

have not been systematically tested.²⁰⁶ This is primarily because large-scale screening of DAOCS is not feasible, given the delicate and labour-intensive screening protocols. In the absence of a high-throughput screening method, a more rational approach to enzyme engineering was required. One option that has been successfully applied to optimise activity in other enzymes is an iterative approach, in which a number of activating mutations are combined and screened across key positions in the enzyme. The mutant demonstrating the most improved specific activity is taken forward to the next round and the cycle is repeated until no activity improvement is observed.²⁰⁷⁻²⁰⁹ Using this technique, combinatorial mutants can be produced from a pool of highly active single mutants with minimal experimental effort.²⁰⁷

Iterative combinatorial mutagenesis (ICM) was applied to DAOCS in 2012, with the aim of generating mutants with improved activity towards penG.²⁰⁶ Combinations of eight single mutations that improved penG conversion in DAOCS were screened across several optimisation rounds. At the end of each round, the most active mutant was taken forward to the next round of mutagenesis. The initial starter enzyme, H4, was a ‘quaternary’ mutant previously reported to have 41-fold increase in k_{cat}/K_m towards penG compared to wild-type DAOCS.²¹⁰ After 3 rounds of mutagenesis, a mutant (H7) demonstrated a 5-fold increase in specific activity towards penG relative to H4 and a 22-fold increase relative to wild-type DAOCS.²⁰⁶ H7, known hereon as ICM DAOCS, represents the DAOCS mutant with the highest specific activity towards penG. As such, ICM DAOCS was selected as a candidate for enzyme-penG crystal optimisation.

4.1.4 ICM DAOCS: recombinant production and penG assay

A gene sequence encoding ICM DAOCS was produced that was codon-optimised for *E. coli* expression. This gene was overexpressed in *E. coli* BL21(DE3) cells, and ICM DAOCS protein was purified using a two-column FPLC protocol: anion-exchange and size-exclusion. ICM DAOCS was afforded in pleasing yield (64 mg/mL) and acceptable purity (Figure 4.3).

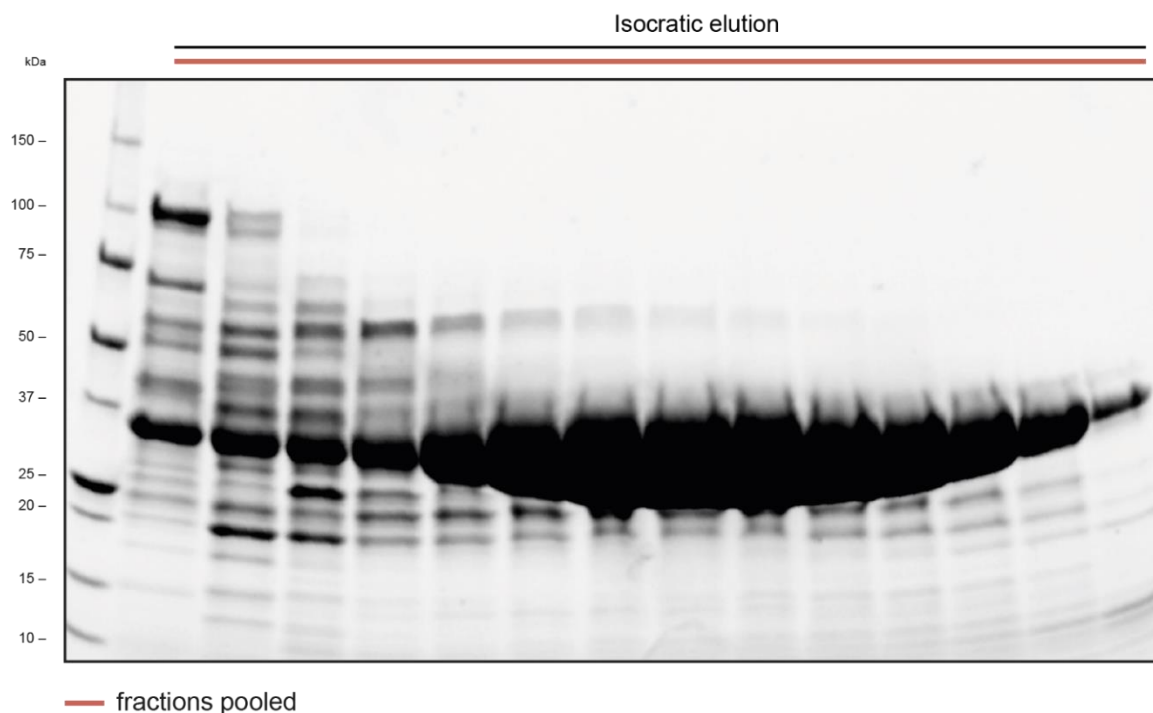


Figure 4.3 | SDS PAGE gel of fractions from the size-exclusion chromatographic step during the purification of ICM DAOCS.

The improved activity of ICM DAOCS towards penG was explored using established assay conditions. ICM DAOCS demonstrated 100% conversion of penG to DAOG (Figure 4.4) under conditions in which wild-type DAOCS/DACS achieved 30% conversion of penG.

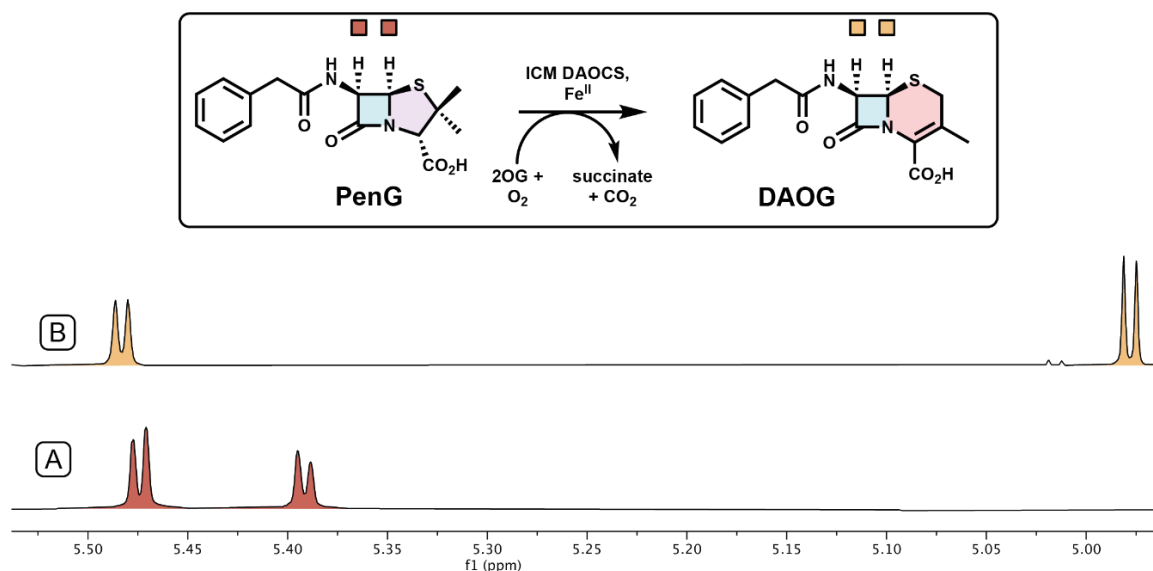


Figure 4.4 | Stacked ^1H NMR spectra showing full conversion of penG by ICM DAOCS. A PenG with no enzyme. B ICM DAOCS and penG under the following conditions: ICM DAOCS (0.2 mM), $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.6 mM), sodium-L-ascorbate (2.4 mM), 2-oxoglutarate disodium salt (8 mM), penG sodium salt (1 mM). DAOG was confirmed as the product by spiking B with an authentic sample of DAOG.

4.1.5 Co-crystallisation of ICM DAOCS

Whilst reported structures of wild-type DAOCS contain Fe(II), these crystals were produced under anaerobic conditions to prevent spontaneous oxidation of Fe(II) to Fe(III) and turnover of the substrate analogue penG. In order to increase the number of conditions explored, an aerobic broad screening approach was adopted for co-crystallisation experiments with ICM DAOCS. As such, an Fe(II) analogue was required to avert substrate turnover.

Mn(II) was chosen as the Fe(II) analogue, and an ICM DAOCS·Mn(II)·2OG·penG complex was selected for initial co-crystallisation experiments. Zn(II) was also trialled as an Fe(II) analogue, but its addition to a solution of ICM DAOCS resulted in significant precipitation. UV-Vis spectroscopic analysis revealed a significant change in the absorbance profile of ICM DAOCS upon the addition of the ligands: Mn(II), 2OG, and penG, suggesting (co)substrate binding to the enzyme. A solution containing ICM DAOCS (0.7 mM), MnCl₂·4H₂O (2 mM), 2OG·2Na (4 mM) and the penG sodium salt (8 mM) was used for crystallisation broad screening attempts. Precipitation and microcrystals were observed across several conditions after three days (Figure 4.5). JCSG+ condition E4 was the most promising condition (Figure 4.5, **A**) with multiple microcrystals observable. Although encouraging, further condition optimisation was required to produce crystals capable of diffracting.

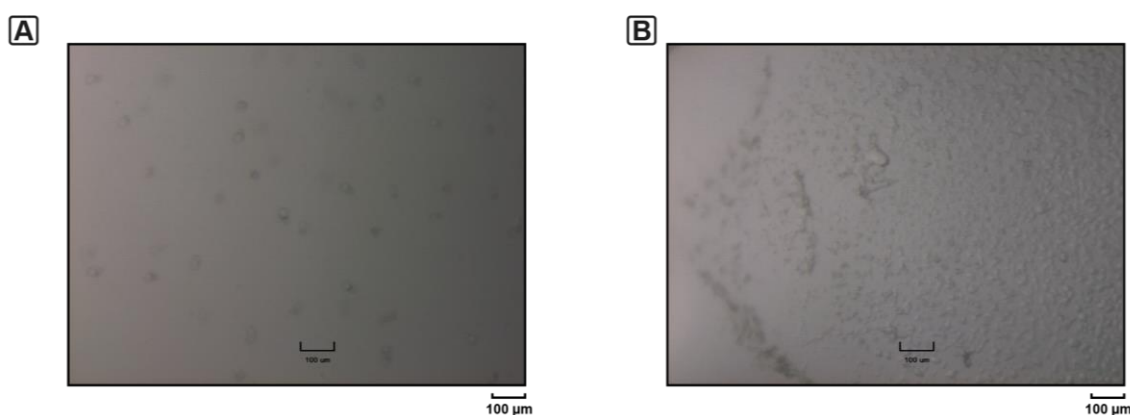


Figure 4.5 | Microcrystals and crystalline precipitate formed during initial co-crystallisation experiments with ICM DAOCS·Mn(II)·2OG·penG. A JCSG+ condition E4 (1.26 M ammonium sulfate, 0.2 M lithium sulfate, 0.1 M Tris, pH 8.5. **B** BCS condition B4 (0.1 M HEPES, pH 7.5, 25% v/v PEG smear medium).

4.1.6 Self-seeding crystallisation experiments with ICM DAOCS

A major challenge in crystallisation is that the conditions that best promote the initial nucleation of crystals (high supersaturation) are not necessarily optimal for their growth into large, single crystals; nucleation is preferred at high supersaturation, whereas crystal growth is favoured at low supersaturation.²¹¹ The challenge of finding crystallisation conditions that satisfy both the need for nucleation and optimum crystal growth is proposed to be the primary basis on which crystallisation attempts fail.²¹² Seeding can provide an effective means of uncoupling nucleation and growth. In this method, previously nucleated microcrystals are supplemented into new crystal growth conditions at lower levels of supersaturation. The seeds serve as nucleation points for new crystals and the low supersaturation levels encourage the growth of large, well-ordered crystals.^{213, 214} Seeding has been applied successfully to produce diffractable crystals for a range of proteins.²¹⁵⁻²¹⁷

Self-seeding experiments, in which the seed and target proteins are the same, were performed with ICM DAOCS. The most promising broad screens from the previous round: JCSG+, Pact Premier, and Index (1+2) were screened again, this time supplemented with microcrystal seeds from JCSG+ condition E4 (Figure 4.5, **A**). Seeding experiments produced macrocrystals of different morphologies and both plate-shaped crystals (Figure 4.6, **A**) and needle clusters (Figure 4.6, **B** and **C**) were observed. These crystals were harvested after addition of cryo-protection, stored under cryogenic conditions and data was collected on the I-24 microfocus beamline (Diamond Light Source, UK); however they did not diffract.

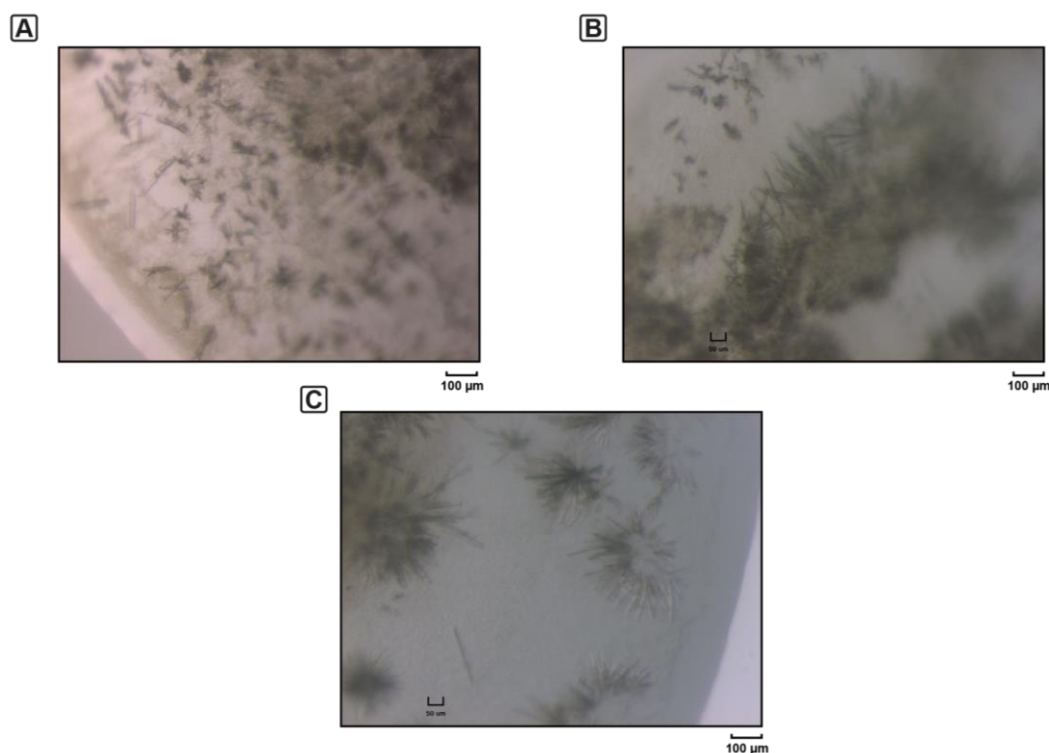


Figure 4.6 | Needle crystals produced from self-seeding experiments using microcrystal seeds. A Index (1+2) condition E1 (0.2 M calcium chloride, 0.1 M bis-Tris pH 6.5, 45% v/v 2-methyl-2,4-pentanediol (MPD)). **B** JCSG+ condition H4 (0.2 M calcium chloride, 0.1 M bis-Tris pH 5.5, 45% v/v MPD). **C** JCSG+ condition H4 (condition as described in **B**).

4.1.7 ICM DAOCS optimisation screens

During harvesting of the crystals from the self-seeding experiment above, the crystals were prone to fragmentation when disrupted. As a result, the crystals on which data collection was performed were much smaller than the ones that were imaged (Figure 4.6). In order to increase the size of the needle-shaped crystals and eventually improve the three-dimensional shape, as well as produce different crystal morphologies, an optimisation screen was performed. The two most promising conditions in the seeding experiment above: JCSG+ H4 and Index (1+2) E1, were very similar (Figure 4.6, **legend**) and were thus used as a starting point for the development of the optimisation screen. Moreover, significant precipitation was observed during the seed screens, an indication that the protein concentration was too high. As such, the concentration of ICM DAOCS was reduced from 0.7 to 0.35 mM. The concentrations of all the other ligands were scaled accordingly.

The buffer chosen for the optimisation screen was bis-Tris pH 6.0, with the screen varying the concentration of hexylene glycol (MPD) (30 to 57.5% v/v in 2.5% increments, horizontal axis) and

the concentration of $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ (0 to 350 mM in 50 mM increments, vertical axis) using 96-well plates (SWISSCI). Crystals obtained from this optimisation screen (Figure 4.7) were larger versions of the same needle morphology as reported previously (Figure 4.6). The crystals were harvested and data was collected on the I-24 microfocus beamline (Diamond Light Source, UK), but as before no diffraction data were collected.

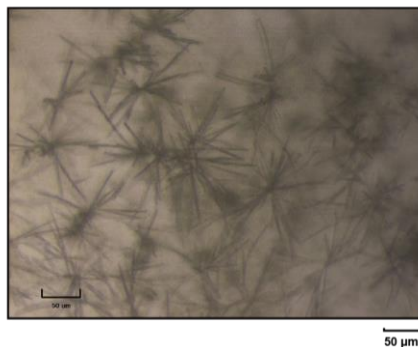


Figure 4.7 | Needle crystals produced from the optimisation screen based on JCSG+ H4 and Index (1+2) E1. Needle crystals pictured were formed under the following conditions: 0.1 M bis-Tris pH 6.0, 200 mM calcium chloride, 32.5% v/v MPD.

Crystal formation occurred most readily at lower calcium chloride and MPD concentrations. To check if crystal growth could be promoted at even lower concentration, a second optimisation screen was performed in which calcium chloride and MPD concentrated were diluted 2-fold. Unexpectedly, no crystals were observed on this plate which had a highest concentration of 175 mM for calcium chloride and 29% v/v for MPD.

4.1.8 Crystallisation of DAOCS/DACS

As a result of the recombinant production of DAOCS/DACS in high-yield and purity (Chapter 2), crystallisation attempts on DAOCS/DACS were now feasible. The co-crystallisation approach used for ICM DAOCS was applied to DAOCS/DACS. A solution containing DAOCS/DACS (20 or 15 mg/mL), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (5 mM), 2OG-2Na (12 mM), and penicillin N (12mM) was used for broad screening approaches at different temperatures including 4 °C and 22 °C. Three salt screens (SaltRx, NeXtal Cation, NeXtal Anion) and three PEG screens (JCSG+, Pact Premier, Crystal Screen) were initially trialled. After seven days, crystals of needle cluster morphology were observed in Crystal Screen 1, condition A3 (0.4 M ammonium phosphate monobasic, pH 4.2) and plate-shaped

crystals were observed in SaltRx, condition E1 (1 M ammonium phosphate monobasic, 0.1 M sodium acetate trihydrate pH 4.6) (Figure 4.8). These crystals were harvested and data was collected on the I-24 microfocus beamline (Diamond Light Source, UK); however, they failed to cause diffraction.

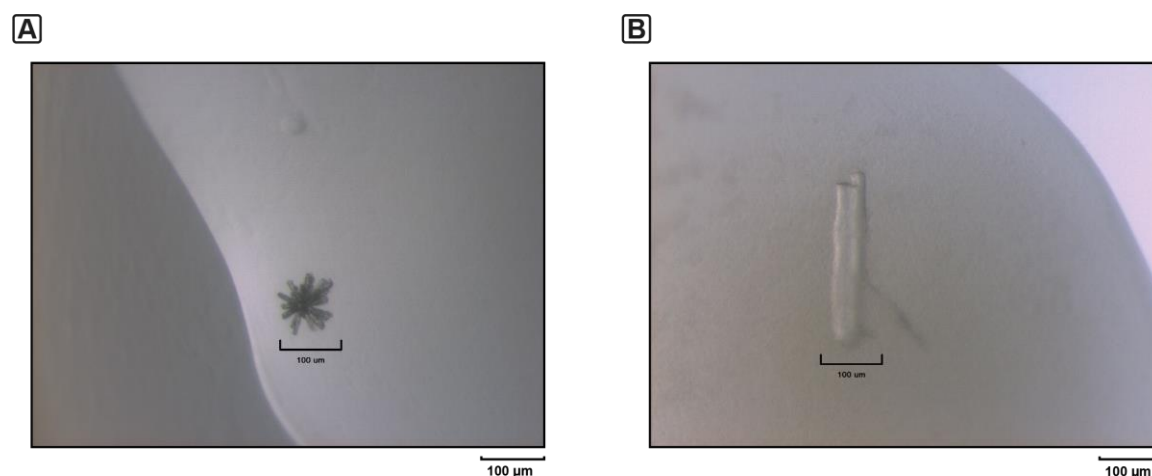
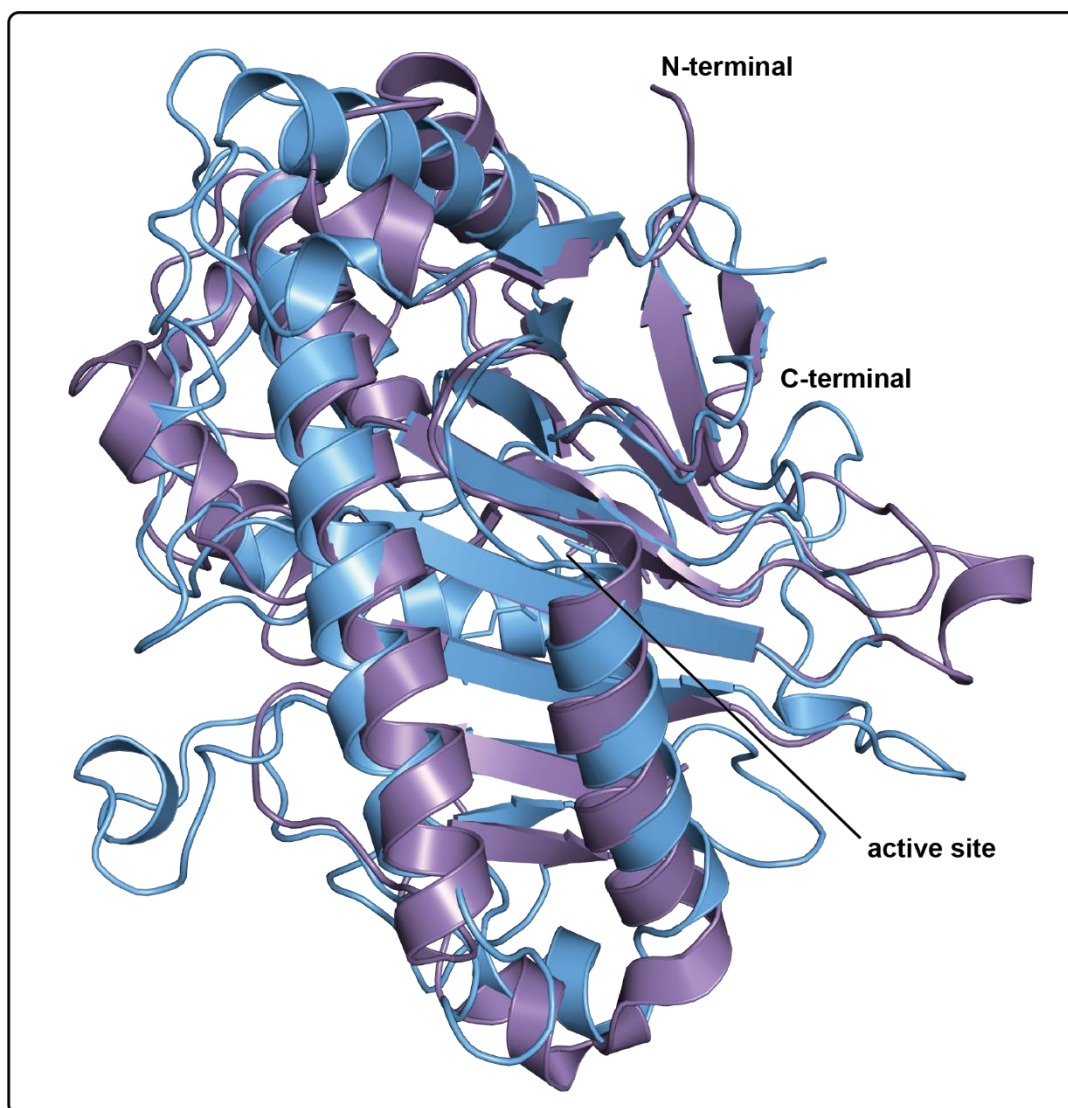


Figure 4.8 | Crystals produced from broad screen of DAOCS/DACS-Zn(II)-2OG-penN co-crystallisation attempts. A Crystal Screen condition A3. **B** SaltRx, condition E1. Specific crystallisation conditions are given in main text (**above**).

4.1.9 Cross-seeding of ICM DAOCS and DAOCS/DACS with IPNS crystals

As mentioned previously, seeding has been used extensively to produce well-ordered crystals capable of diffraction. This has been explored in the context of self-seeding (**Section 4.1.6**), which uses microcrystal seeds of the same protein. Cross-seeding, where microcrystal seeds of a closely related enzyme are used to facilitate crystallisation of the target enzyme, was also explored. Heterologous proteins can include mutants of the same enzyme^{218, 219} or structurally homologous enzymes from different organisms.^{217, 220, 221} ICM DAOCS and DAOCS/DACS belong to the 2OG oxygenase structural superfamily²⁹ and display structural homology with other members of this enzyme family, some of which are more amenable to crystallisation. IPNS was selected as the structurally related enzyme from the 2OG oxygenase superfamily with which to perform cross-seeding experiments with ICM DAOCS and DAOCS/DACS. IPNS catalyses the formation of isopenicillin N, the biosynthetic precursor to all penicillin and cephalosporin antibiotics.^{46, 47} Whilst overall sequence homology between IPNS and DAOCS/DACS is only 11%,⁵⁶ they share high structural homology, both possessing the double-stranded β -helix (DSBH) topology (Figure 4.9).²⁹

IPNS has previously proved very amenable to both static²²²⁻²²⁵ and time-resolved^{155, 226} crystallography.



■ = DAOCS

■ = IPNS

Figure 4.9 | Superimposition of IPNS (blue, PDB 1BLZ²²⁷) and DAOC (lavender, PDB 1UO9⁴³) ribbon structures demonstrating the high structural homology between the two enzymes. Superimposition was performed with WinCoot.²²⁸

Cross-seeding screening with IPNS microcrystalline seeds were performed with ICM DAOCS and DAOCS/DACS. A solution of enzyme (ICM DAOCS or DAOCS/DACS, 0.75 mM), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (2 mM), $2\text{OG} \cdot 2\text{Na}$ (4 mM) and DAOC (8 mM) was plated on JCSG+, Morpheus and BSG broad screens supplemented with IPNS seed stocks (kindly provided by Dr Patrick Rabe). DAOC was preferred to penG as it is more stable and therefore less prone to degradation. Plates were stored at 22 °C.

Several crystals of differing morphology were produced in both ICM DAOCS and DAOCS/DACS screens across a range of growth conditions. A collection of the most promising crystals is given in Figure 4.10. Crystals were harvested and data was collected on the microfocus beamline (Diamond Light Source, UK). However, as with previous data collections, no protein structures could be obtained from the harvested crystals.

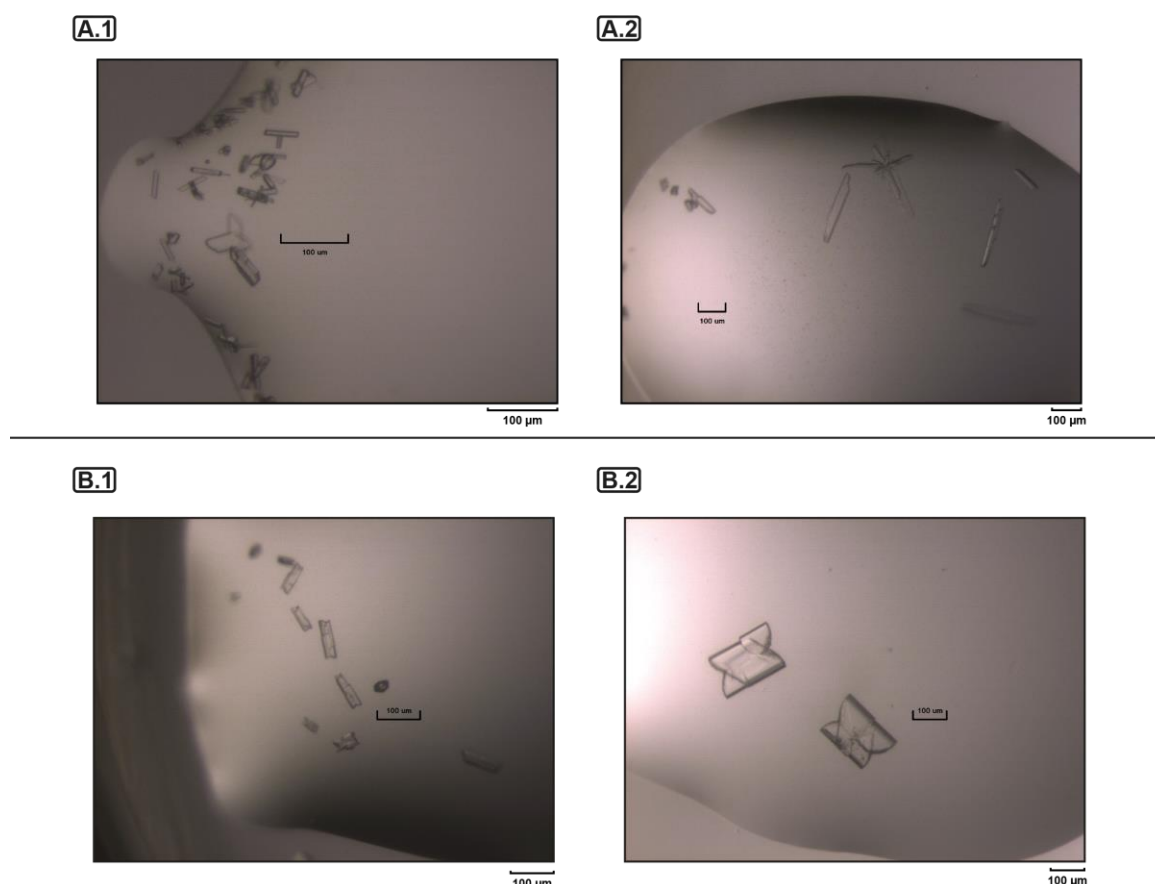


Figure 4.10 | Crystals produced from cross-seeding experiments with microcrystalline seeds of IPNS. Panel A crystals from ICM DAOCS-Mn(II)-2OG-DAOC co-crystallisation experiments. **A.1** Morpheus A1 (0.06 M divalents (0.3 M magnesium chloride hexahydrate, 0.3 M calcium chloride dihydrate), 0.1 M buffer system 1 (1.0 M Imidazole; MES monohydrate, pH 6.5), 30% v/v precipitant mix 1 (40% v/v PEG 500 MME; 20% w/v PEG 20000). **A.2** Morpheus A4 (0.06 M divalents (0.3 M magnesium chloride hexahydrate, 0.3 M calcium chloride dihydrate), 0.1 M buffer system 1 (1.0 M Imidazole; MES monohydrate, pH 6.5), 37.5% precipitant mix 4 (25% v/v MPD, 25% PEG 1000, 25% w/v PEG 3350). **Panel B** crystals from DAOCS/DACS-Mn-2OG-DAOC co-crystallisation experiments. **B.1** BCS H1 (0.04 M calcium chloride dihydrate, 0.04 M sodium formate, 0.1 M Tris, pH 8.0, 25% v/v PEG smear low. **B.2** JCSG+ E11 (0.16 M calcium acetate hydrate, 0.08 M sodium cacodylate, pH 6.5, 14.4% w/v PEG 8000).

4.1.10 Discussion and future work

Crystal structures of DAOCS in complex with (co)substrate and products have been purported to show overlapping binding sites of penicillin and 2OG. Based on this observation, a novel mechanism was proposed in which formation of the active ferryl species occurred before penicillin

N binding.⁴³ This so called “ping-pong” mechanism was unprecedented for 2OG oxygenases. Subsequent pre-steady state kinetics studies on DAOCS cast doubt on this “ping-pong” mechanism, and have encouraged a re-evaluation of the crystal structures of DAOCS. Structures of DAOCS-penicillin complexes were produced using penG, rather than the wild-type substrate penicillin N. PenG displayed multiple binding modes in the active site of DAOCS, and it was unclear which mode, if any, was catalytically productive.

Apo-DAOCS crystallised as a trimer, with the active site of each monomer partially blocked by the C-terminal arm of the adjacent monomer. This hindered attempts to soak (co)substrates into the active site, and may have encouraged penG into aberrant binding modes. The addition of an N-terminal His₆-tag to DAOCS yielded a new crystal form that did not trimerize. With this new crystal form, product structures of DAOCS in complex with DAOC were produced that claimed to support the “ping-pong” mechanism. However, these structures were of low resolution (3 Å) and therefore inconclusive.

In an effort to reconcile the crystallographic and kinetic data, new crystal structures of DAOCS were sought. During this work, a DAOCS variant (ICM DAOCS) in complex with penG was screened across crystallisation conditions in the hope of obtaining protein crystals. ICM DAOCS displays improved activity towards penG compared to wild-type DAOCS. It was envisioned that catalytically productive binding modes of penG were more likely to be achieved in the active site of a highly active variant than the wild-type enzyme. In addition, rather than producing enzyme-substrate complexes by soaking ligands into apo-crystals, as was done previously, enzyme-substrate complexes were prepared in solution prior to the attempted crystallisation. Initial broad screening experiments with ICM DAOCS produced microcrystals which were used as crystal seeds, supplementation of which into additional ICM DAOCS broad screens afforded crystals of new morphology and increased size. However, the needle-shaped crystals that were produced did not diffract, likely due to their small size. It is possible that further optimisation of seed stocks and

growth conditions could produce larger crystals of new morphologies that are more amenable to diffraction.

Of the three primary cephalosporin biosynthesis enzymes: DAOCS/DACS, DAOCS, and DACS, only crystal structures of DAOCS have been reported. New recombinant expression methods were developed for DAOCS/DACS (**Chapter 2**) and DACS (**Section 4.2.2**), and both enzymes can now be produced in large quantities and high purity. Crystallisation attempts initially focused on DAOCS/DACS and employed the co-crystallisation approach that was used for ICM DAOCS. Macrocrystals were observed across a range of crystallisation conditions, but all failed to diffract during data collection.

Seeding can expedite the time taken for crystals to form within droplets, which can typically take weeks for wild-type DAOCS.^{147, 195} Given the hydrolysable nature of penicillin N and penG, especially at non-neutral pH,²¹⁶ reducing the crystallisation time is especially important to prevent substrate degradation. Moreover, seeding can lead to the production of different crystal morphologies, which might be a means of avoiding trimerization of DAOCS that was previously observed. A self-seeding crystallisation method had not previously been reported with DAOCS, and was therefore explored with the variant ICM DAOCS. Self-seeding of ICM DAOCS microcrystals produced macrocrystals of needle morphology. Further seeding experiments with different ICM DAOCS seed stocks and growth conditions could produce new crystal morphologies which readily diffract.

When self-seeding failed to produce diffractable crystals of ICM DAOCS and DAOCS/DACS, cross-seeding was explored. Cross-seeding can be used to produce crystals when more traditional crystallisation techniques fail.²¹⁷ The more homologous the seed and target protein structures are, the better the chances of producing crystals. The 2OG oxygenase superfamily to which ICM DAOCS and DAOCS/DACS belong contains many structurally similar enzymes for which crystal structures exist. As such, ICM DAOCS and DAOCS/DACS make excellent targets for cross-seeding

experiments. IPNS was selected as the homologous protein with which to explore cross-seeding with both enzymes. When ICM DAOCS and DAOCS/DACS broad screens were supplemented with IPNS seeds, many macrocrystals were produced, however they were subsequently identified as salt crystals. These salt crystals were likely to be manganese(II) sulfate, formed by the combination of manganese chloride, used as an Fe(II) analogue, and lithium sulfate, which was present in the IPNS seed stock buffer. To overcome this, alternative Fe(II) analogues could be explored such as: Cd(II), Co(II) and Zn(II). Alternatively, the native substrate Fe(II) could be used under anaerobic conditions. T_M shift assays would enable the stability of the enzymes in the presence of metal ion analogues to be studied, and may help find more favourable metal ions.

Whilst penG has been the preferred substrate when generating crystals of DAOCS, it displays several binding modes within the enzyme's active site which makes analysis of the catalytic relevance of each binding mode more challenging. Producing enzyme-substrate complexes with penicillin N is more likely to yield structures that are representative of the substrate binding that occurs during catalysis. However, the use of penicillin N has been limited due to its relative scarcity compared to penG, as well its tendency to degrade more easily. The wild-type product DAOC is more readily available than penicillin N and is more stable, hence its use in cross-seeding experiments with IPNS. Alternatively, the exomethylene cepham (Figure 4.11) may provide an alternative to penG, penicillin N, and DAOC. As stated with DAOC, cephams are more stable than penams and therefore less prone to degradation. Moreover, the exomethylene cepham possesses a chiral centre at the C-4 position, akin to the C-3 position of penicillin N and penG, thereby containing structural features of both the substrate and products, unlike DAOC.

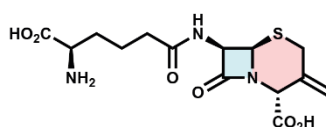


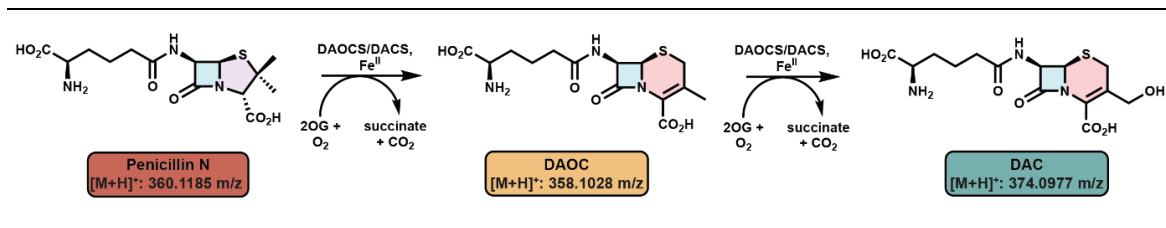
Figure 4.11 | The exomethylene cepham substrate analogue.

4.2 Potential β -lactamase contamination of DAOCS/DACS and DACS

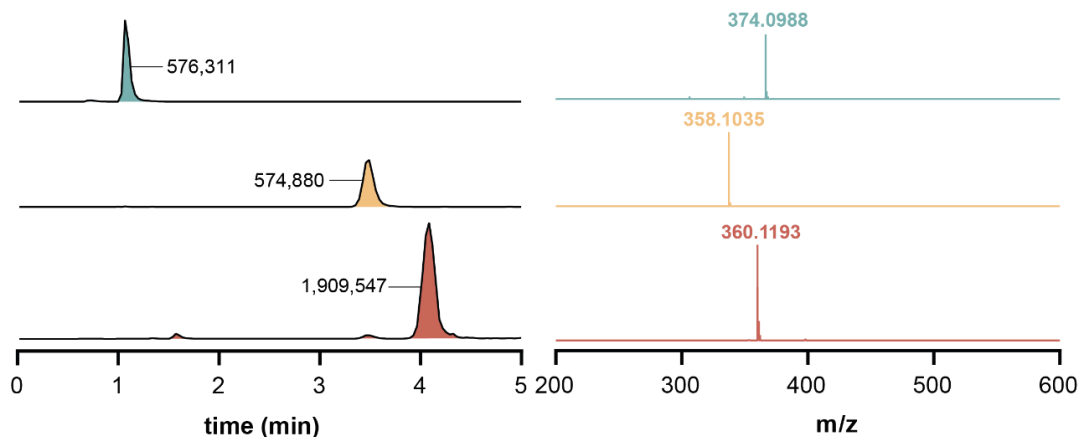
4.2.1 DAOCS/DACS

Initial batches of DAOCS/DACS were produced using pCold-derived vectors that utilised an ampicillin resistance selection marker. This resistance is conferred through the production of TEM-1, a serine β -lactamase.¹⁵³ There was concern that TEM-1 might contaminate DAOCS/DACS, potentially compromising its biochemical characterisation. Incubations of DAOCS/DACS with nitrocefin had indicated the presence of a β -lactamase (**Section 2.6**). To explore this matter further, DAOCS/DACS was incubated with penicillin N in the presence of two β -lactamase inhibitors (BLIs): clavulanic acid and avibactam. Clavulanic acid has a β -lactam structure and is effective against class A and C β -lactamases.¹⁵⁷ Avibactam is a non- β -lactam BLI that displays activity against class A, C, and D β -lactamase.¹⁵⁸ Since TEM-1 is class A β -lactamase,²²⁹ both BLIs are capable of its inhibition.

Initially, penicillin N was incubated with DAOCS/DACS under established assay conditions¹⁰⁶ with and without clavulanic acid (0.5 mM). The quenched assays were analysed by LC-MS (Figure 4.12). Turnover and the ratio of the DAOC and DAC products, as measured by peak integrals of mass-extracted chromatograms, were the same with and without clavulanic acid added to the incubations.

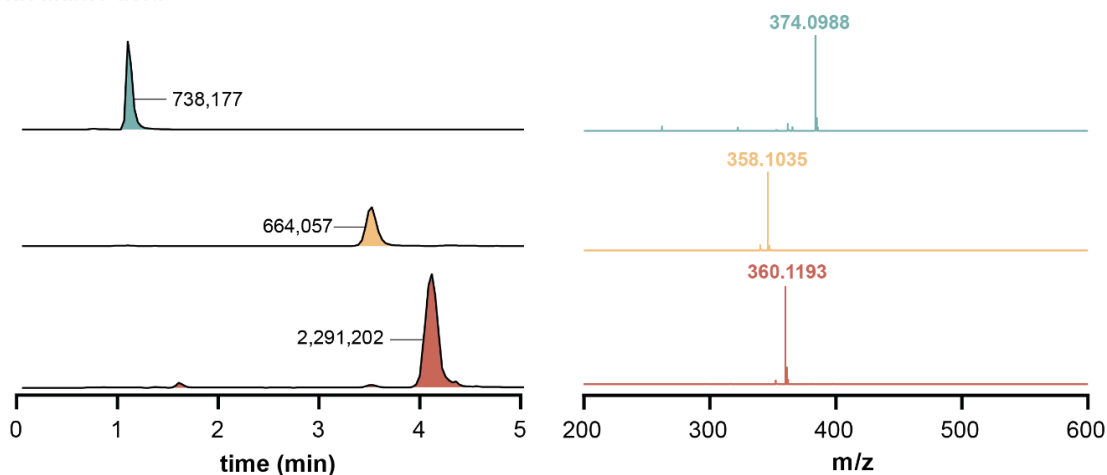


– clavulanic acid



Turnover = 38%
 DAC:DAOC = 1:1

+ clavulanic acid



Turnover = 38%
 DAC:DAOC = 1.1:1

Figure 4.12 | Comparison of penicillin N turnover in DAOCS/DACS incubations with and without clavulanic acid. LHS Mass-extracted chromatograms for: penicillin N (red, 360.1), DAOC (yellow, 358.1), and DAC (green, 374.1). Peak integrals are labelled. RHS Mass spectra for the labelled peaks. **Top** Results for incubations without clavulanic acid. **Bottom** Clavulanic acid incubation results. In each case, turnover and DAC:DAOC ratio are given. Assay conditions: DAOCS/DACS (0.02 mM), DTT (1.68 mM), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.045 mM), L-ascorbic acid (0.83 mM), ammonium sulfate (42 mM), α -ketoglutaric acid (0.85 mM), penicillin N (1 mM).

The impact of avibactam on the conversion of penicillin N by DAOCS/DACS was then assessed.

Incubations were performed using assay conditions adapted from DAOCS incubations.⁴⁴ Full turnover of penicillin N to DAC was observed in the presence and absence of avibactam (Figure

4.13, **A**). Incubations were also repeated without the addition of 2OG (α -ketoglutarate). Under these conditions, DAOCS/DACS was inactive so any additional penicillin N degradation without avibactam would be indicative of β -lactamase activity. Integration of the penicillin N peaks revealed the amount remaining was approximately the same in both assays (Figure 4.13, **B**).

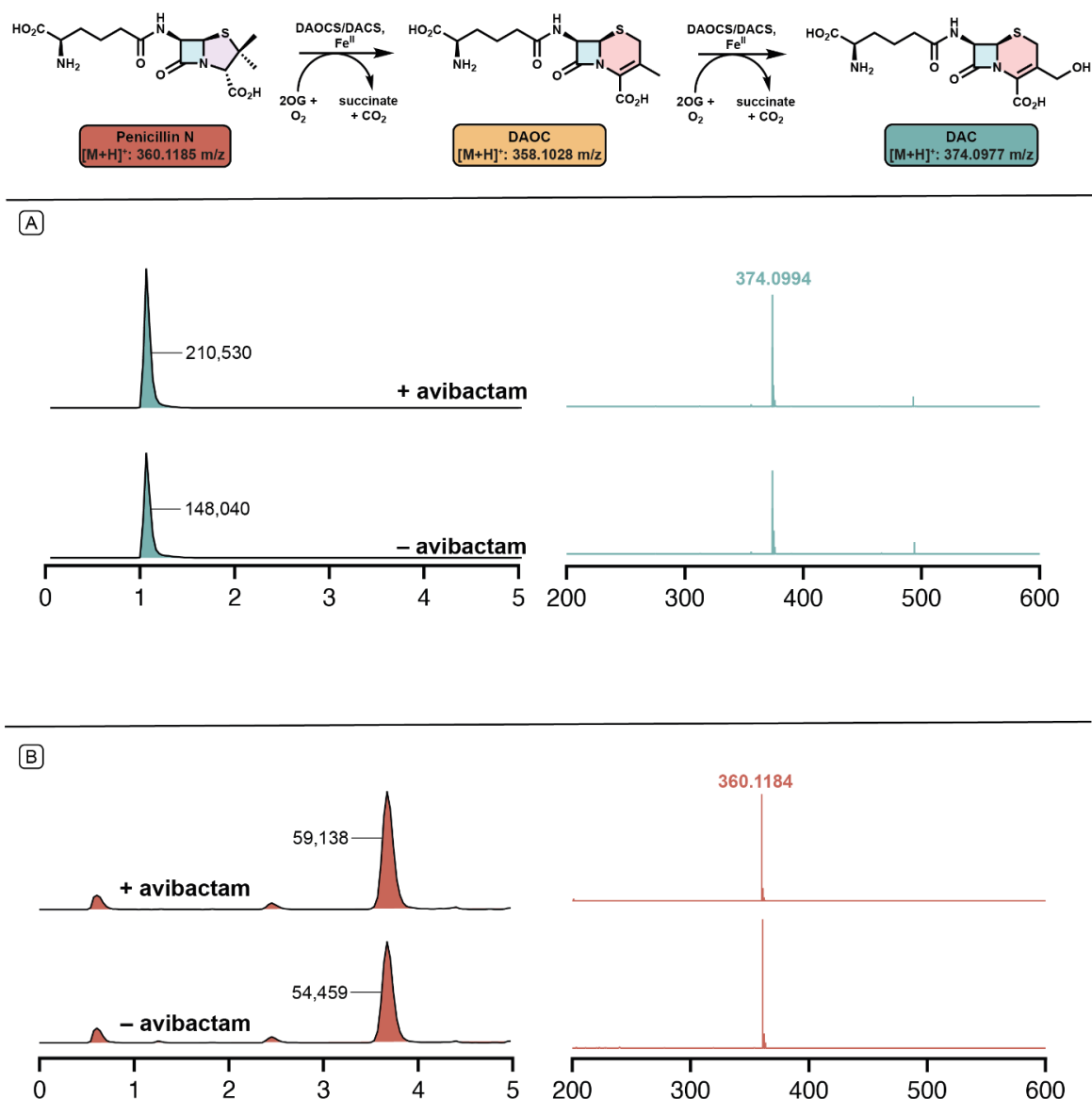


Figure 4.13 | LC-MS analysis of the role of avibactam in penicillin N incubations with DAOCS/DACS. LHS Mass-extracted chromatograms for DAC (green, 374.1) and penicillin N (red, 360.1). Mass spectra of the highlighted peaks are given on the RHS. **A** Full conversion of penicillin N to DAC was observed in both the presence and absence of avibactam. **B** Incubations of penicillin N and DAOCS/DACS in the absence of 2OG. Similar levels of penicillin N remained in both + and – avibactam assays, suggesting no β -lactamase contamination. Assay conditions: DAOCS/DACS (0.02 mM), $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ (0.2 mM), sodium-L-ascorbate (0.4 mM), α -ketoglutarate disodium salt (2 mM), penN (1 mM).

4.2.2 DACS

Like DAOCS/DACS, the hydroxylase enzyme DACS was produced from a pCold-derived construct that utilised an AmpR selection marker. The DACS gene (*cefF*) was expressed using a pCold-derived vector and purified by HisTrap affinity and size-exclusion chromatography (Figure 4.14).

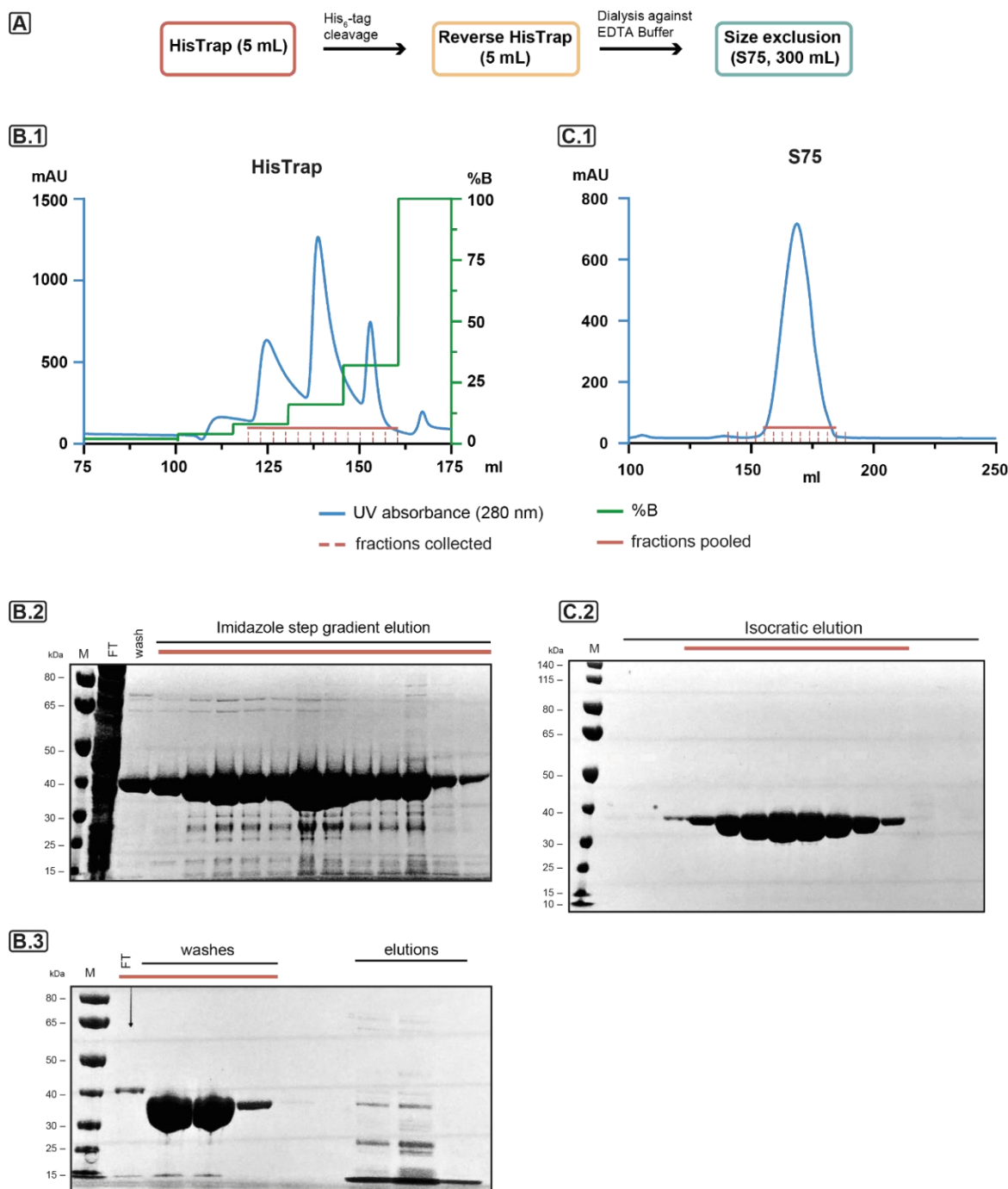


Figure 4.14 | Overview of the FPLC purification of DACS. Panel A Purification workflow. Panel B HisTrap purification columns, including the elution profile for the first HisTrap column (B.1) and its corresponding SDS-PAGE gel (B.2). The SDS-PAGE of HisTrap column after His₆-tag cleavage is given in B.3. Panel C Size-exclusion chromatography, with the elution profile (C.1) and SDS-PAGE gel (C.2).

To evaluate potential TEM-1 contamination, DACS was combined with the expandase enzyme DAOCS, produced as previously described.⁴⁴ The two enzymes were incubated with penicillin N with and without clavulanic acid. Assays were monitored by ¹H NMR (Figure 4.15). In both the absence (B) and presence (C) of clavulanic acid, turnover of penicillin N was approximately 37% and the ratio of DAOC:DAC was 1:1. No β -lactam degradation products were observable in the spectra (Figure 4.15).

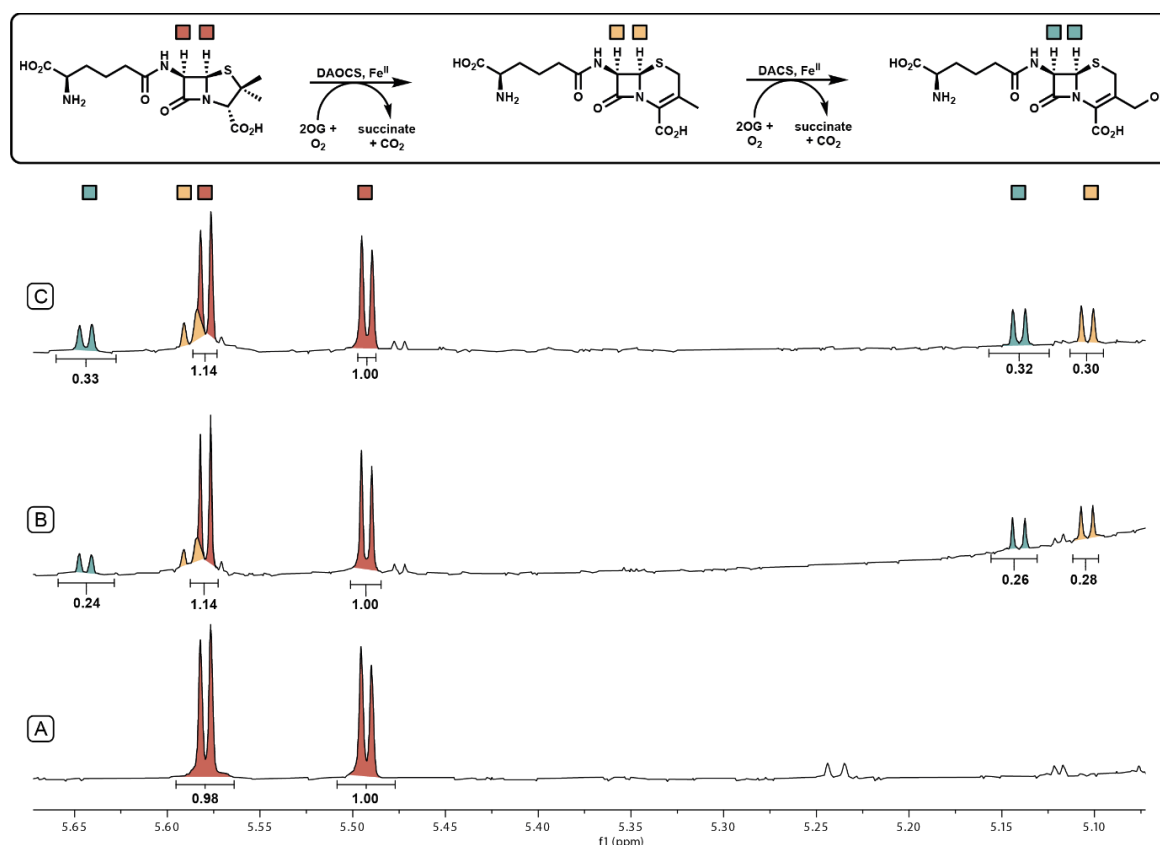


Figure 4.15 | Stacked ¹H NMR spectra showing that addition of clavulanic acid had no effect on assay outcomes when DAOCS and DACS were incubated with penicillin N. A No enzyme control. **B** DAOCS + DACS + penN without clavulanic acid. **C** DAOCS + DACS + penN with clavulanic acid. Assay conditions: DAOCS (0.02 mM), DACS (0.02 mM), DTT (1 mM), (NH₄)₂Fe(SO₄)₂·6H₂O (0.1 mM), sodium-L-ascorbate (1 mM), α -ketoglutaric acid (1 mM), penN (1 mM).

4.2.3 Discussion

Through the expression of DAOCS/DACS and DACS from pCold-derived constructs, the target proteins could be isolated in larger yields and higher purities compared to previous expression protocols employing pET-derived constructs. However, one potential disadvantage of using pCold vectors was their ampicillin resistance selection marker, with resistance conferred by the expression of TEM-1, a serine- β -lactamase capable of hydrolysing penicillins and cephalosporins.

Contamination of DAOCS/DACS or DACS with TEM-1 would potentially undermine biochemical characterisation of these enzymes. β -Lactamase activity had been detected during incubations of DAOCS/DACS with the nitrocefin substrate (**Section 2.6**). However, the nitrocefin assay for β -lactamase detection is error-prone. In order to directly evaluate the presence of TEM-1 in DAOCS/DACS and DACS, the enzymes were incubated with their respective substrates in the presence of a β -lactamase inhibitor (BLI), either clavulanic acid or avibactam. These incubations demonstrated that the addition of a BLI had a negligible impact on substrate turnover or degradation, suggesting β -lactamase contamination was not an issue. Ultimately, the decision was taken to substitute the ampicillin selection marker for kanamycin resistance in the DAOCS/DACS and DACS pCold-constructs. TEM-1 contamination might be batch specific, and may occur in the future if the protein purification protocols became sub-optimal. Facile incorporation of the kanamycin resistance cassette in the construct was achieved using Gibson Assembly (**Section 2.6**).

Penicillin N incubations with DAOCS/DACS that were performed using established conditions for DAOCS/DACS (Table 4.1, **Condition A**)¹⁰⁶ yielded only 39% turnover of penicillin N to produced DAOC and DAC in an $\approx 1:1$ ratio. When the same incubations were performed under assay conditions optimised for DAOCS (Table 4.1, **Condition B**),⁴⁴ full conversion of penicillin N to DAC was observed. Interestingly, Condition B did not contain DTT, which was considered to be essential for activity with previous forms of DAOCS/DACS.^{80, 106} DTT is added to assays to maintain the proteins in a reduced, and therefore active state.²³⁰ This could suggest that DAOCS/DACS produced for these studies is more stable than previous preparations of the enzyme. Indeed, the DAOCS/DACS used originally with condition A was re-folded from insoluble aggregates using urea.¹⁰⁶ This process could have made the enzyme inherently unstable, necessitating the use of DTT to retain activity. Recombinant DAOCS/DACS used in this study was produced in a soluble form and so likely demonstrated improved stability and therefore did not require DTT. Thus, the updated recombinant expression methodologies permit the use of simplified assay conditions

whilst still retaining high enzyme activity that was capable of complete conversion of penicillin N to DAC.

Table 4.1 | A comparison of assay conditions established for DAOCS/DACS in 1990 (condition A) and for DAOCS in 2014 (condition B).

Condition A		Condition B	
Component	Conc / mM	Component	Conc / mM
DAOCS/DACS	0.02	DAOCS/DACS	0.02
DTT	1.68	(NH ₄) ₂ Fe(SO ₄) ₂ ·6H ₂ O	0.2
FeSO ₄ ·7H ₂ O	0.045	Sodium-L-ascorbate	0.4
L-Ascorbic acid	0.83	α-Ketoglutarate disodium salt	2
Ammonium sulfate	42	Penicillin N	1
α-Ketoglutaric acid	0.85		
Penicillin N	1		

TEM-1 contamination was assessed using LC-MS and ¹H NMR analytical methods. In all ¹H NMR spectra, each β-lactam component possesses two doublets with J-values ≈4–5 Hz in the β-lactam region (δ 4.5–6 ppm). The region is very close to the water peak and so water suppression methods were used to prevent oversaturation of the receiver, which can reduce the signal:noise ratio. Of the two water suppression methods that were employed, excitation sculpting and 1-D NOESY pre-saturation, the latter was preferred as the suppression peak was narrower and thus there was less reduction in the intensity of the adjacent β-lactam peaks. In the LC-MS analysis, the retention times for β-lactam analytes can vary substantially. For example, on a standard C₁₈ column, the G-side chain penicillins and cephalosporins elute at 20–25 min,¹²⁵ whereas this work has demonstrated that natural β-lactams containing the D-α-aminoadipoyl side chain elute at 0–5 min. This means new elution methods must be established for each new substrate. As such, LC-MS analysis is not ideally suited for monitoring initial enzyme conversion of new substrate analogues, such as the 3-vinyl cephem (**Chapter 3**), where product elution times are not immediately clear. There is some evidence that fragmentation of penams can occur under the acidic conditions employed to produce protonated species during ESI LC-MS (e.g. 0.1% formic

acid).²³¹ This process could potentially undermine the peak integration method used to determine substrate turnover. However, any fragmentation would be consistent across all assays and therefore would not impact comparisons between them.

Ultimately, both ^1H NMR and LC-MS methods complement each other and are useful in different contexts. The general application of a single water suppression protocol to all β -lactam assays makes ^1H NMR the preferred analytical method for initial screening of turnover with a new substrate. Once an effective elution method has been established for a specific assay, LC-MS provides clear benefits as an analytical method, especially for time-course studies, since it requires lower concentrations of analytes and is higher throughput compared to ^1H NMR.

4.3 ^{18}O incorporation into DAC

It has been demonstrated previously that the hydroxyl group oxygen atom of the natural hydroxylated product DAC is derived from both molecular oxygen and water. Incubations of the natural substrate penicillin N with DAOCS/DACS under an $^{18}\text{O}_2$ atmosphere gave the hydroxylated product DAC with ^{18}O -incorporation values of $\approx 50\%$.^{92, 93, 179, 232} When these incubations were repeated using H_2^{18}O , $\approx 50\%$ incorporation of labelled oxygen was also observed.¹⁷⁹ An oxygen exchange mechanism with water from the medium was proposed to account for this, with exchange anticipated to occur between an enzyme-bound intermediate formed after the Fe(IV)=O species. This notion was based on incubations of $[4\text{-}^2\text{H}]$ exomethylene cepham with DAOCS/DACS, which gave two products: DAC and a spiro-epoxide.¹¹² Whilst there was just 50% incorporation of $^{18}\text{O}_2$ into DAC, the epoxide oxygen of the spiro-epoxide was derived exclusively from molecular oxygen. Incubations of the 3-vinyl cephem substrate analogue with DAOCS/DACS under an $^{18}\text{O}_2$ atmosphere had revealed 100% incorporation of the ^{18}O -label into the products (**Section 3.6.1**). Based on this result, it was proposed that conversion of the 3-vinyl cephem by DAOCS/DACS did not proceed via the same mechanism as the wild-type substrates penicillin N and DAOC. In order

to validate this hypothesis, it was decided to repeat the ^{18}O incubations of the wild-type substrates with DAOCS/DACS to confirm the previous observations.

Initially, penicillin N and DAOC were incubated with DAOCS/DACS under an $^{18}\text{O}_2$ atmosphere. DAOC was also incubated with the hydroxylase enzyme DACS under the same $^{18}\text{O}_2$ atmosphere. Incubations were run for 2 h after which the assay was quenched. Care was taken to ensure that the enzyme was fully quenched before the assays were exposed to the normal atmosphere. LC-MS analysis of penicillin N and DAOC incubations with DAOCS/DACS revealed full conversion of both substrates to DAC, which displayed $\approx 50\%$ incorporation of the ^{18}O label into the hydroxyl group (Figure 4.16, A–C). The same result was observed in the incubations of DAOC with DACS (Figure 4.16, D).

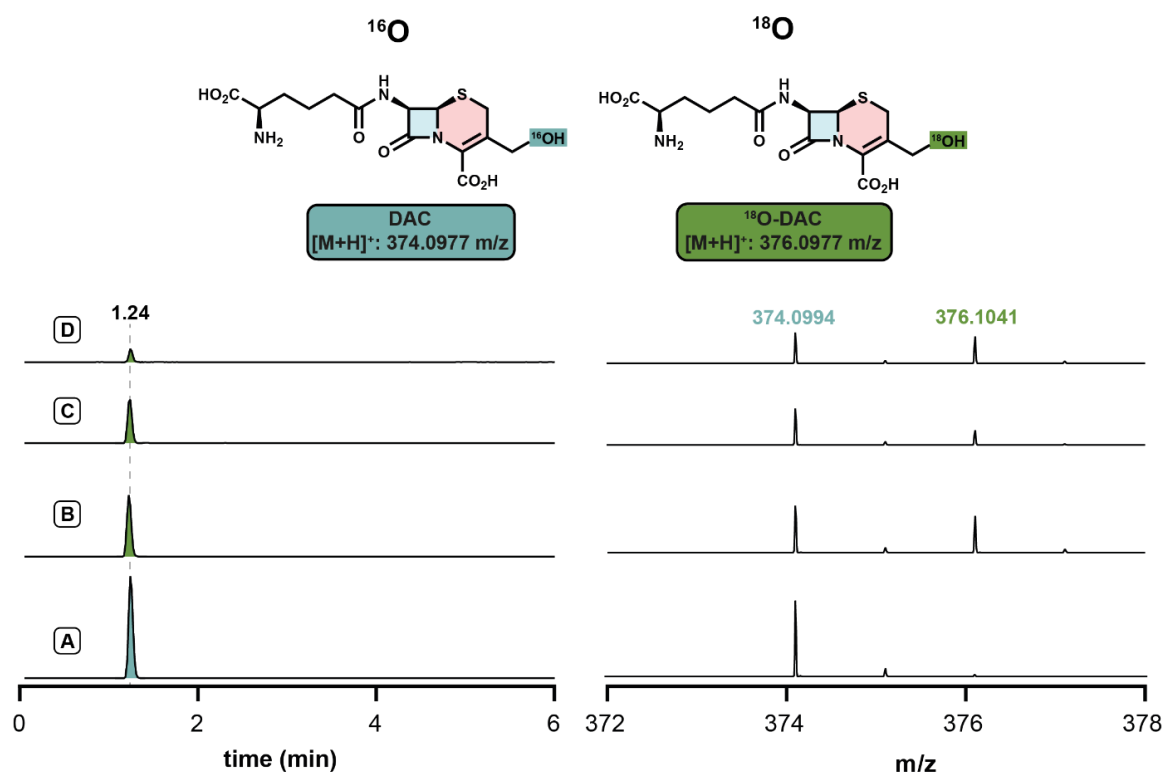


Figure 4.16 | Incorporation of the ^{18}O -label from $^{18}\text{O}_2$ into DAC. LHS Mass-Extracted chromatograms for DAC. RHS Mass spectra at 1.24 min. **A** DAOCS/DACS + penN in $^{16}\text{O}_2$ (control) in H_2^{16}O buffer. **B** DAOCS/DACS + penN ($^{18}\text{O}_2$) in H_2^{16}O buffer. **C** DAOCS/DACS + DAOC ($^{18}\text{O}_2$) in H_2^{16}O buffer. **D** DACS + DAOC ($^{18}\text{O}_2$) in H_2^{16}O buffer.

The incubations were then repeated using H_2^{18}O . As with $^{18}\text{O}_2$, approximately 50% incorporation of one labelled oxygen atom into DAC was observed (Figure 4.17). The results obtained here

corroborate previous work, and support the proposal of exchange between an enzyme intermediate and water during the catalytic cycle.¹⁷⁹

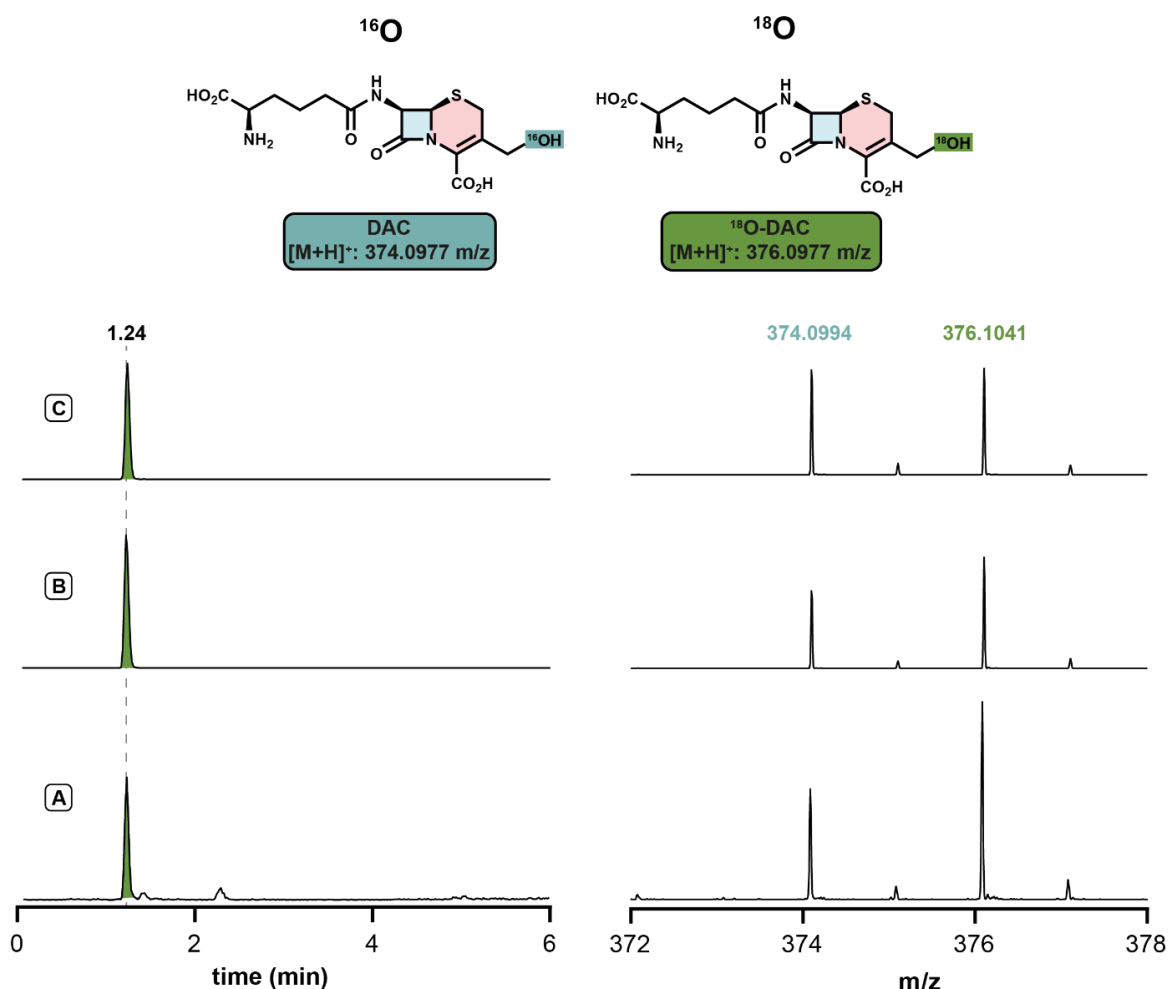
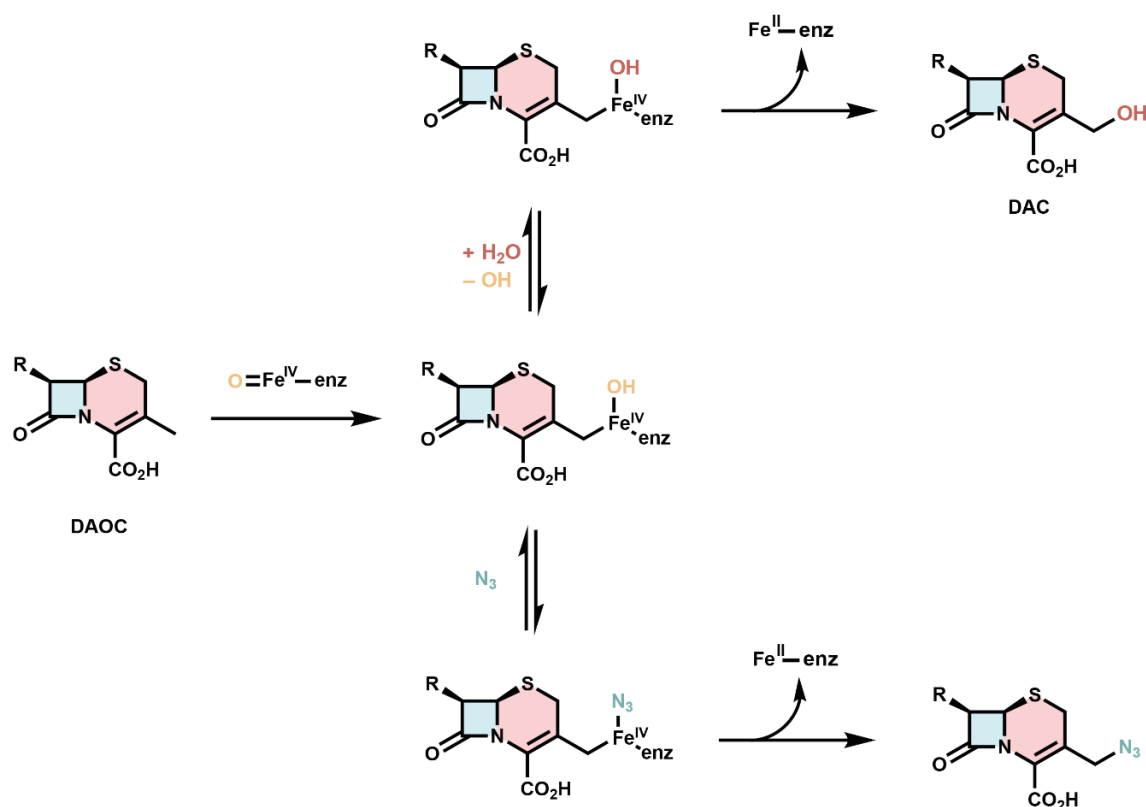


Figure 4.17 | Incorporation of the ^{18}O -label from H_2^{18}O into DAC. LHS Mass-extracted chromatograms for DAC. RHS Mass spectra at 1.24 min. **A** DAOCS/DACS + penN in H_2^{18}O under an $^{16}\text{O}_2$ atmosphere. **B** DAOCS/DACS + DAOC in H_2^{18}O . **C** DACS + DAOC in H_2^{18}O .

The evidence for exchange between water and an enzyme-bound intermediate raised the intriguing question of whether this exchange mechanism could be exploited to insert other nucleophiles, such as azide, into the cephem products (Scheme 4.1).



R' = D-a-aminoadipoyl

Scheme 4.1 | Proposed oxygen exchange mechanism and its potential exploitation to incorporate the azide functional group into cephem scaffolds. The figure has been adapted from literature.¹⁷⁹

To explore this, DAOC was incubated with DAOCS/DACS and DACS in the presence of sodium azide. Incubations were assessed by ¹H NMR (Figure 4.18). Initially, control incubations without azide were performed and full conversion of DAOC to DAC by DAOCS/DACS and DACS was observed (Figure 4.18, **B** and **D**). When these assays were repeated with azide, DAC was the only observable product with no additional β-lactam peaks visible (**C** and **E**). This suggests that azide insertion had not occurred.

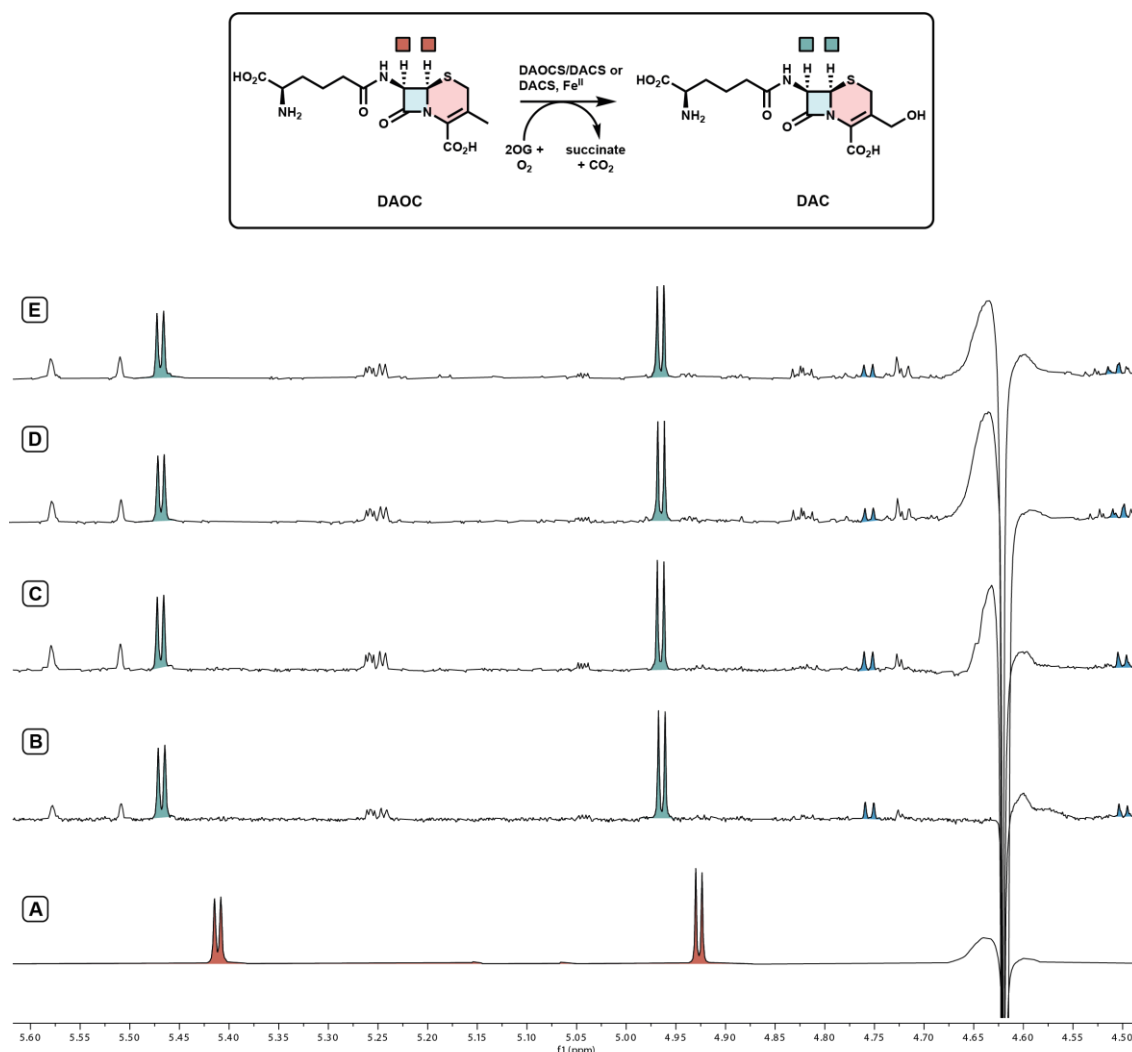


Figure 4.18 | DAOC incubations with DACS or DAOCS/DACS in the presence of azide. **A** DAOC with no enzyme. **B** DACS + DAOC. **C** DACS + DAOC + N_3 . **D** DAOCS/DACS + DAOC. **E** DAOCS/DACS + DAOC + N_3 . Assay conditions: DACS (0.02 mM), $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (0.2 mM), sodium-L-ascorbate (0.4 mM), α -ketoglutarate disodium salt (2 mM), DAOC (2 mM), $\pm$ sodium azide (0.5 mM)

4.3.1 Discussion

During the conversion of the substrate analogue 3-vinyl cephem by DAOCS/DACS into a cephem hydroxy azide product, ^{18}O -labelling experiments revealed that the hydroxyl group of the product was derived exclusively from molecular oxygen (**Chapter 3**). This result necessitated a re-examination of ^{18}O incorporation into the natural substrate of DAOCS/DACS and DACS; previous results had demonstrated both O_2 and H_2O as sources of the hydroxyl group oxygen atom of DAC. The results obtained here corroborate previous observations and implicate an oxygen-exchange mechanism in which the hydroxyl group of an enzyme-bound intermediate, derived from O_2 , can exchange with water. These results suggest that natural hydroxylase activity that affords DAC

proceeds via a different mechanism from the conversion of the 3-vinyl cephem substrate analogue. Discussions on the mechanism of 3-vinyl cephem conversion are made in **Chapter 3**. Efforts to utilise the oxygen-exchange mechanism to insert the azide nucleophile into the natural substrate DAOC were not successful. This could be an issue of concentration; azide was present in solution at 0.5 mM, whereas water is at 55.5 M. Increasing the azide concentration might yield more positive results.

During incubations of DAOC with DAOCS/DACS and DACS, in addition to DAC, several new peaks were observed in the β -lactam region of the ^1H NMR spectra (Figure 4.19). In these assays, DAOC is completely consumed and so no starting material peaks are present in the ^1H NMR spectra (Figure 4.19, **B**). Oxidation of DAC to a cephem aldehyde by DAOCS/DACS has been observed previously¹⁰⁴ with the resultant aldehyde undergoing facile β -lactam cleavage to give the 3-formyl cephalosporoate.^{66, 104, 233} The doublets either side of the water peak in Figure 4.19 (δ 4.50 and 4.75 ppm, **brown**) corresponded well to this 3-formyl cephalosporoate species.⁶⁶

The other peaks in the β -lactam region (Figure 4.19, **peaks 1–6, yellow**) remain unidentified. One set of peaks could potentially correspond to the intact cephem aldehyde, whose unstable nature has prevented its previous characterisation. 2-D NMR experiments (TOCSY, COSY) would help clarify if any of the peaks **1–6** are coupled to each other, and therefore belong to the same molecule. HSQC and HMBC experiments would also permit further characterisation of these potential β -lactam species. Product formation could be monitored in real-time using PROJECT-CPMG ^1H NMR methods.

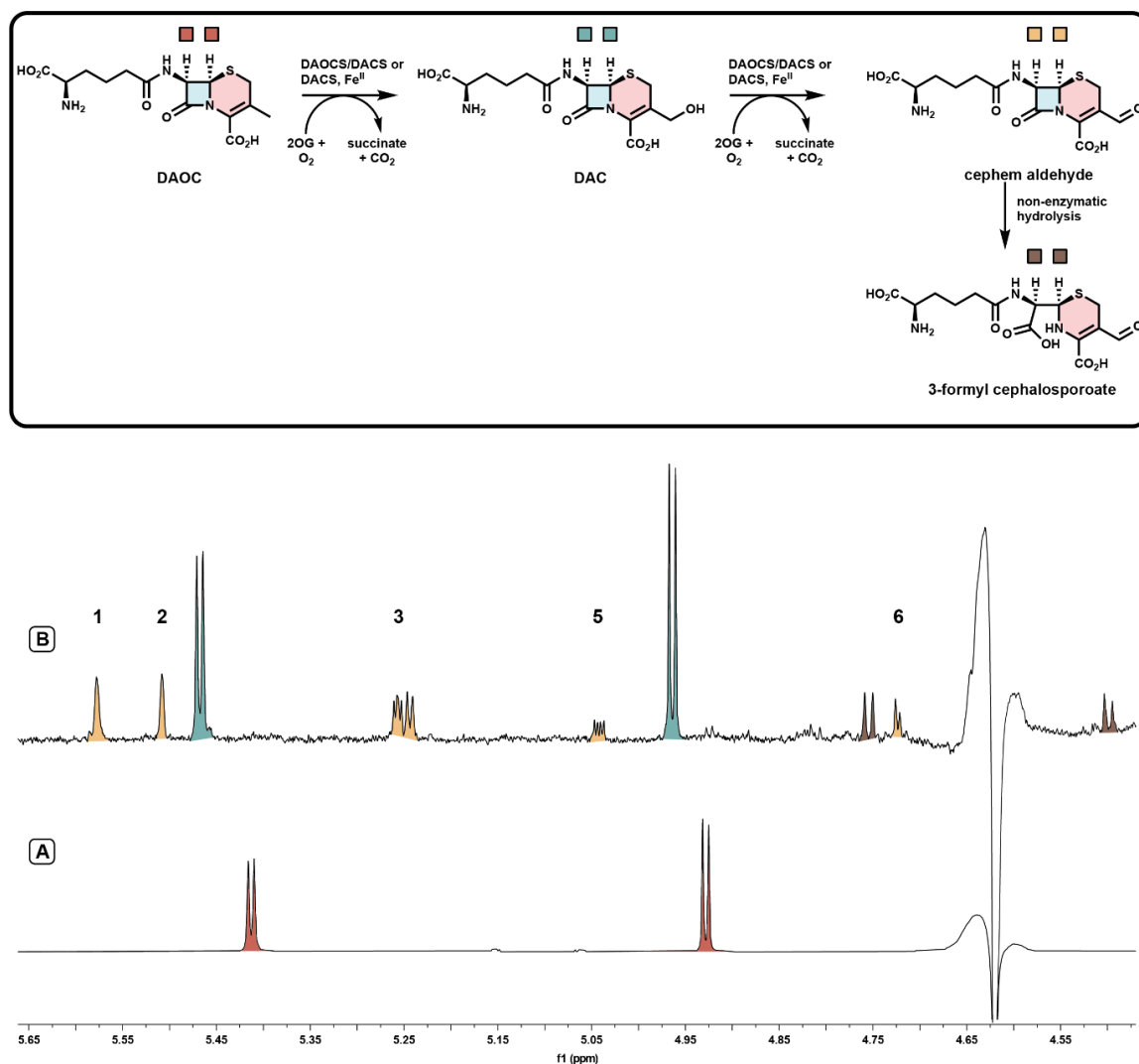


Figure 4.19 ¹H NMR spectrum of DAOC incubations with DACS, revealing the formation of several minor peaks in the β-lactam region. **A** DAOC without enzyme. **B** DAOC incubation with DACS. Assay conditions: DACS (0.02 mM), (NH₄)₂Fe(SO₄)₂·6H₂O (0.2 mM), sodium-L-ascorbate (0.4 mM), α-ketoglutarate disodium salt (2 mM), DAOC (2 mM).

4.4 Conclusions

Attempts to crystallise ICM DAOCS and DAOCS/DACS (co)substrate complexes failed to produce structures of either protein. Co-crystallisation, in which enzyme-substrate complexes were formed in solution prior to crystallisation, demonstrated promise; many microcrystals were produced across a range of crystallisation conditions for complexes of both ICM DAOCS and DAOCS/DACS. Seeding small-crystals of ICM DAOCS into further broad screens with ICM DAOCS produced crystals of needle-morphology. However, the crystals were of small-size (<50 μm) and failed to diffract during data collection. Importantly, crystals for both enzymes appeared after a few days' growth, where previously DAOCS crystals required weeks to grow. Expediting crystal formation is important when crystallising complexes with penicillin substrates and opens up the possibility of

co-crystallising the ICM DAOCS and DAOCS/DACS with penicillin N. Optimisation of the crystal growth conditions increased the size of these needle-clusters, but not enough to be capable of diffraction. In the optimisation screen, the size of needle-crystals increased as the concentrations of calcium chloride and MPD were reduced. However, crystal formation unexpectedly ceased below a concentration threshold. Self- and cross-seeding approaches to the crystallisation of ICM DAOCS and DAOCS/DACS produced new crystal morphologies with a further reduction in growth times. Prolific formation of salt crystals was observed during cross-seeding experiments with IPNS. This might be overcome by changing the buffer in which the IPNS seeds were prepared, as well as the Fe(II) analogue used for aerobic co-crystallisation experiments.

Incubations of DAOCS/DACS and DACS produced using pCold-derived constructs showed no signs of β -lactamase contamination by ^1H NMR or LC-MS analysis. DAOCS/DACS produced recombinantly during the course of this work had less stringent requirements for activity than DAOCS/DACS produced previously. This was likely due to the soluble expression of DAOCS/DACS from its pCold-construct, whereas previous forms of DAOCS/DACS were produced in inclusion bodies and had to be re-folded using urea. The re-folding process may have made the protein less stable. ^1H NMR has been established as the preferred method for initial screenings of enzyme activity, especially with new or unnatural β -lactam substrates. In the context of substrate analogues, LC-MS can provide ancillary analysis once substrate conversion has been established by ^1H NMR.

Using ^{18}O -labelling experiments, the hydroxyl group oxygen atom of DAC was shown to be derived from both molecular oxygen and water. This replicates previous ^{18}O experiments and supports the existence of an exchange mechanism between an enzyme-bound intermediate and water. Efforts to utilise this exchange mechanism to perform azidation were unsuccessful, but increasing the concentration of azide might yield different results.

Chapter 5

Summary and conclusions

5 Summary and conclusions

5.1 Production of DAOCS/DACS and DACS using pCold vectors

For the first time, DAOCS/DACS and DACS were produced in His₆-tagged forms using pCold-derived expression vectors, enabling their facile purification by IMAC. A two-step FPLC purification procedure employing IMAC and SEC afforded DAOCS/DACS and DACS in high purity (>90% by SDS PAGE). This procedure marks a significant improvement in time efficiency and applicability over previous three-column FPLC protocols implementing ion-exchange and gel-filtration chromatography.^{80, 125, 126} Moreover, the concentrating effect of affinity chromatography enables large volumes of clarified cell lysates to be processed conveniently. As such, this protocol should be readily scalable to hundreds of litres of cultures. Efficient cleavage of the N-terminal His₆-tags was achieved through the use of the HRV3C protease,²³⁴ and the activity of DAOCS/DACS and DACS was retained after the cleavage. Mononuclear ferrous enzymes are O₂-sensitive and degrade under aerobic conditions.²³⁵ With the new expression systems, purification times were shortened, minimising oxygen exposure and reducing degradation of the active proteins.

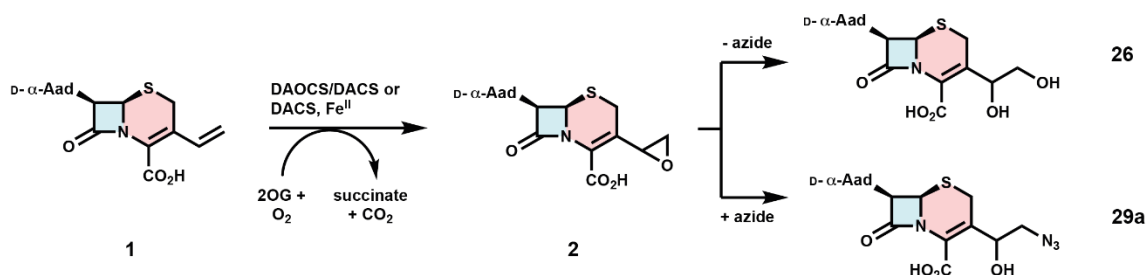
Expression of the *cefEF* and *cefF* genes using pCold-derived constructs produced soluble DAOCS/DACS and DACS that demonstrated apparently improved stability compared to the previously purified forms of these enzymes; both enzymes were highly active, as shown by assays with their natural substrates penicillin N and DAOC, even after several freeze-thaw cycles, and did not require the addition of DTT for activity. This improved stability enabled assay conditions to be simplified which will permit more facile screening of additional substrate analogues.

5.2 Hydroxylase catalysed azidation

Incubations of the 3-vinyl cephem substrate analogue (**1**) with DAOCS/DACS produced the diol (**26**), as shown by NMR and MS assays. When 0.5 mM sodium azide was added to the incubation, a major new product was observed which was characterised as a cephem hydroxy azide (**29a**). The diol (**26**) was obtained as a minor product. When the incubations were performed using 5 mM

azide, the cephem hydroxy azide **29a** was the sole observed product (Scheme 5.1). In the original work leading to the identification of the product, now assigned as the hydroxy azide (**29a**), the azide ion was likely used as a biocide and its presence in the assay was not known to the experimentalist.

Based on the activity of DAOCS/DACS towards cepham analogues that bear a structural resemblance to the 3-vinyl cephem, it was proposed that the diol and hydroxy azide were formed from a common epoxide intermediate (**2**) through ring-opening with the appropriate nucleophile. The formation of the hydroxy azide proceeds with apparent regioselectivity; (**29a**) was isolated as its two C3' epimeric diastereomers, with no evidence for the formation of the regioisomer (**29b**) being accrued. One diastereomer of **29a** was produced in excess of the other, the exact ratio of the diastereomers produced and their C3' stereochemistries are yet to be assigned.

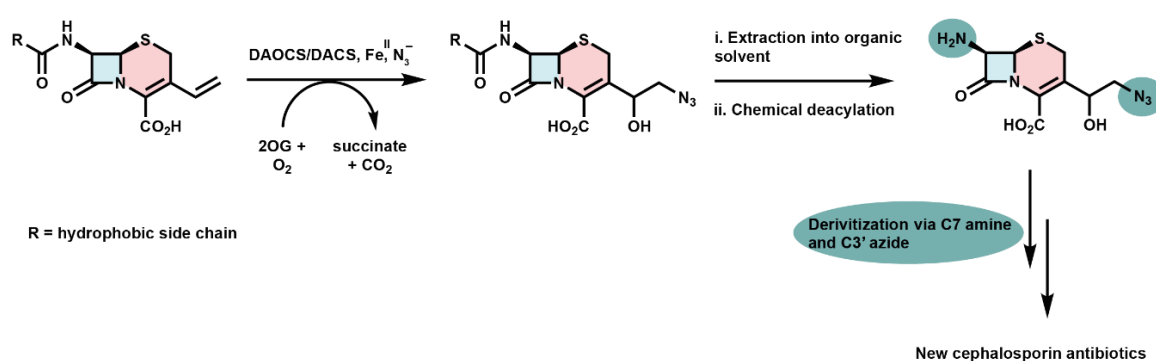


Scheme 5.1 | Conversion of the 3-vinyl cephem substrate analogue (1**) to either the diol (**26**) in the absence of azide or the cephem hydroxy azide (**29a**) in the presence of (5 mM) azide.** Formation of **26** and **29a** is proposed to proceed via the common intermediate (**2**).

These results reflect the first reported enzyme-catalysed incorporation of the azide functional group into a cephalosporin structure. Based on the evidence described herein, the azidation step is proposed to be enzyme catalysed. Whilst other 2OG oxygenases, such as the halogenase SyrB2, have previously been shown to catalyse azidation,¹⁷⁸ DAOCS/DACS-catalysed azide insertion would mark the first time this has been achieved in a 2OG oxygenase containing the typical 2-His-1-carboxylate facial triad. Thus, the capacity for 2OG oxygenases to perform azidation could be more general than previously thought. It may therefore be possible to engineer other 2OG oxygenases to catalyse insertion of an azide group into their substrates. Given the diverse range

of substrates that 2OG oxygenases act on, there is potential for the azide functionality to be incorporated by 2OG oxygenases into other small molecules and a range of biopolymers, including proteins, nucleic acids, and lipids.

As described in **Chapter 1**, DAOCS/DACS catalyses medically-important biosynthetic steps; the production of cephalosporin antibiotics relies on the enzyme-catalysed ring-expansion of penicillins before the resultant cephalosporins are chemically deacylated and derivatised. Currently, the deacylated form of DAOC (7-ADCA) is a key pharmaceutical intermediate in the semi-synthetic production of cephalosporin antibiotics.⁸¹ 7-ADCA is produced by the enzymatic ring-expansion of penicillin V or G by engineered forms of DAOCS. The product, a hydrophobic DAOC analogue, is then extracted into an organic solvent and chemically deacylated to give 7-ADCA.²³⁶ The production of cephem hydroxy azides has substantial potential for the industrial development of new, currently inaccessible, cephalosporin antibiotics. By analogy to 7-ADCA, the deacylated cephem hydroxy azide nucleus could serve as a novel pharmaceutical intermediate with new functional groups installed via click chemistry at the azide.¹⁹⁰ This would enable access to functional groups that are not available through current derivatisation methods. The engineering of DAOCS/DACS to efficiently convert 3-vinyl cephem substrate analogues possessing hydrophobic side chains, which are prepared via one-step from commercially available precursors, would produce cephem hydroxy azides that are readily extractable into organic solvent and therefore are more industrially relevant (Scheme 5.2).



Scheme 5.2 | Potential workflow for the production of new cephalosporin antibiotics via the industrial application of cephem hydroxy azide formation. 3-Vinyl cephem analogues possessing hydrophobic side chains could be converted to hydrophobic cephem hydroxy azides that could be extracted into organic solvent and chemically de-acylated. The resultant hydroxy azide core could be derivatized via click chemistry to produce libraries of potential cephalosporin antibiotics.

5.3 The consensus hydroxylase mechanism: in need of revision?

Currently, it is recognised that the hydroxylation of DAOC to DAC by DAOCS/DACS proceeds via a modified form of the consensus hydroxylation mechanism that is the archetypal reaction catalysed by 2OG oxygenases.⁴⁶ However, mounting evidence suggests that the hydroxylation mechanism of DAOCS/DACS (and possibly other 2OG oxygenases) may not follow the currently established superfamily mechanism and may require revision.

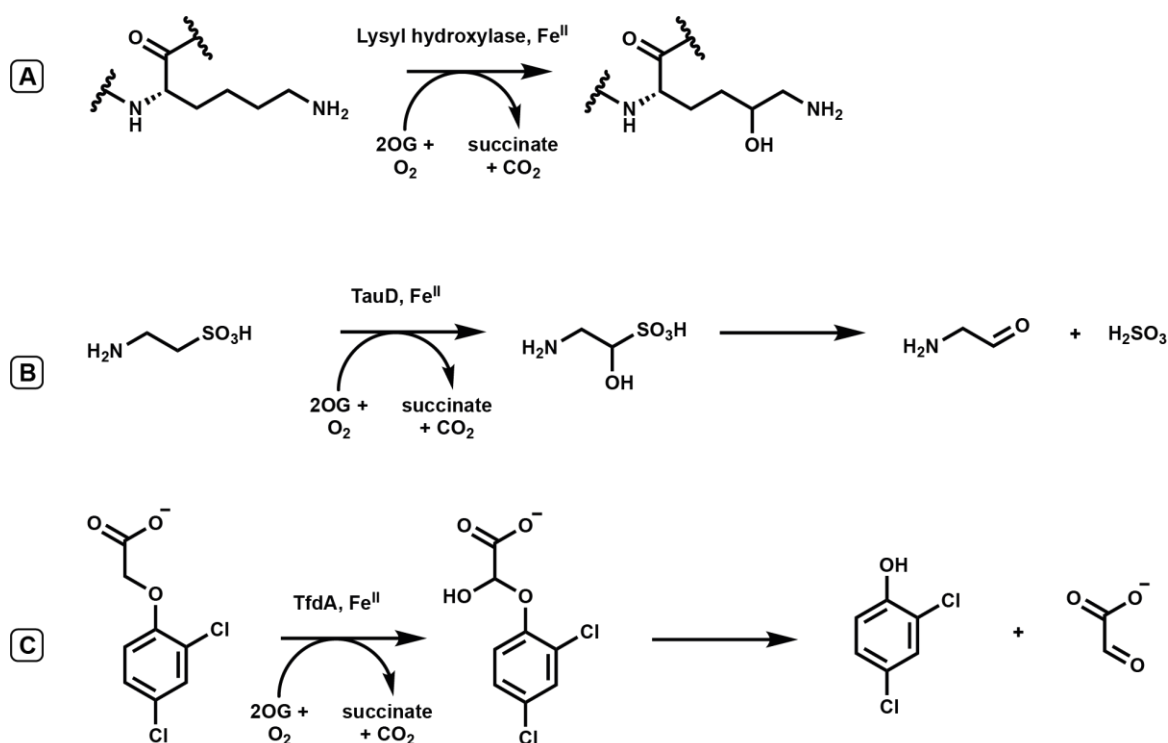
Investigations into the hydroxylase activity of DAOCS/DACS using ¹⁸O-labelling experiments have revealed interesting mechanistic features. Incubations of DAOCS/DACS with the natural substrates penicillin N and DAOC using ¹⁸O₂ or H₂¹⁸O showed incorporation from both labelled sources into the hydroxyl group oxygen atom of DAC and the shunt metabolite 3β-hydroxy cepham.^{92, 179}

In addition to DAOCS/DACS and DACS, ¹⁸O-incorporation experiments have identified six Fe-dependent oxygenases where catalysis involves sub-stoichiometric incorporation of an oxygen atom from dioxygen (O₂) into their hydroxylated products (Table 5.1). Of the enzymes listed below, four are 2OG dependent oxygenases: clavaminic acid synthase (CAS), procollagen lysyl hydroxylase, taurine dioxygenase (TauD), and 2,4-dichlorophenoxyacetate oxygenase (TfdA). These enzymes have been shown to manifest partial incorporation of an oxygen atom from water into their hydroxylated products. This feature is apparently atypical of 2OG dependent hydroxylases, which normally exclusively utilise dioxygen as the source of the hydroxyl group

oxygen.²³⁷ An overview of the reactions catalysed by procollagen lysyl hydroxylase, TauD, and TfdA are given below (Scheme 5.3). In the five cases where partial incorporation of water into products has been demonstrated (DAOCS/DACS, CAS, lysyl hydroxylase, TauD, and TfdA), stoichiometric incorporation of an oxygen atom from dioxygen into succinate was also observed. The latter observation suggests that the Fe(IV)=O intermediate, whose formation is coupled to succinate production, is derived solely from dioxygen in all 2OG oxygenases.^{232, 237}

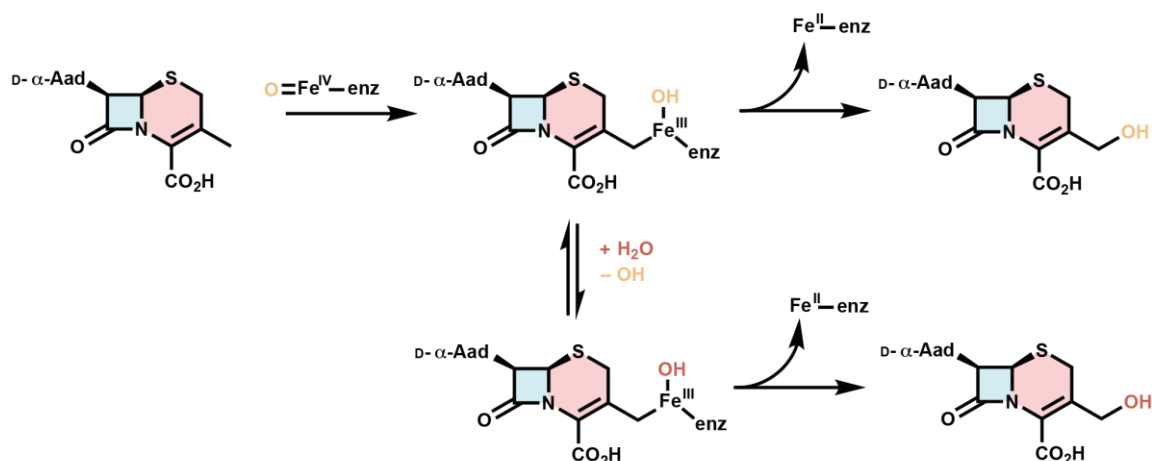
Table 5.1 | Enzymes in which sub-stoichiometric incorporation of $^{18}\text{O}_2$ into the hydroxylated products was observed. This implies an oxygen-exchange mechanism is occurring, in which an oxygen atom from water is incorporated into the hydroxylated product(s).

#	Enzyme	Role
1	DAOCS/DACS; DAOCS; DACS ¹⁷⁹	Hydroxylation of DAOC to DAC during cephalosporin biosynthesis
2	Clavaminic acid synthase (CAS) ²³⁸	Trifunctional bacterial enzyme catalysing the formation of clavaminic acid
3	Lysyl hydroxylase ²³⁹	Conversion of lysine to hydroxylysine in humans
4	Taurine dioxygenase (TauD) ²⁴⁰	Hydroxylates taurine which induces the liberation of sulfite
5	2,4-dichlorophenoxyacetate oxygenase (TfdA) ²⁴¹	Catalyses the first step in the biodegradation of the herbicide 2,4-dichlorophenoxyacetate.
6	p-Hydroxyphenylpyruvate hydroxylase (HPPD) ²⁴²	Conversion of 4-hydroxyphenylpyruvate into homogentisate during the catabolism of tyrosine
7	α-Ketoisocaproate oxygenase ²⁴³	Decarboxylation and hydroxylation of α -ketoisocaproate to β -hydroxyisovalerate



Scheme 5.3 | Three examples of reactions catalysed by 2OG dependent hydroxylases in which an oxygen-exchange mechanism is proposed. A Procollagen lysyl hydroxylase. **B** Taurine dioxygenase (TauD). **C** 2,4-Dichlorophenoxyacetate oxygenase (TfdA). In the cases of **B** and **C**, which both involve self-hydroxylation, it is likely that exchange occurs with a long-lived intermediate.²³⁷ This cannot occur in **A** or during CAS catalysed reactions (Scheme 5.6).

It has previously been proposed that the partial incorporation of oxygen from water into the hydroxylated products of 2OG oxygenase catalysed reactions occurs through exchange with water from the solvent, which binds to the active site Fe ion at the Fe(III)–OH intermediate stage in catalysis.²³⁷ An example of this exchange mechanism in the context of DAC formation is given below (Scheme 5.4).¹¹²



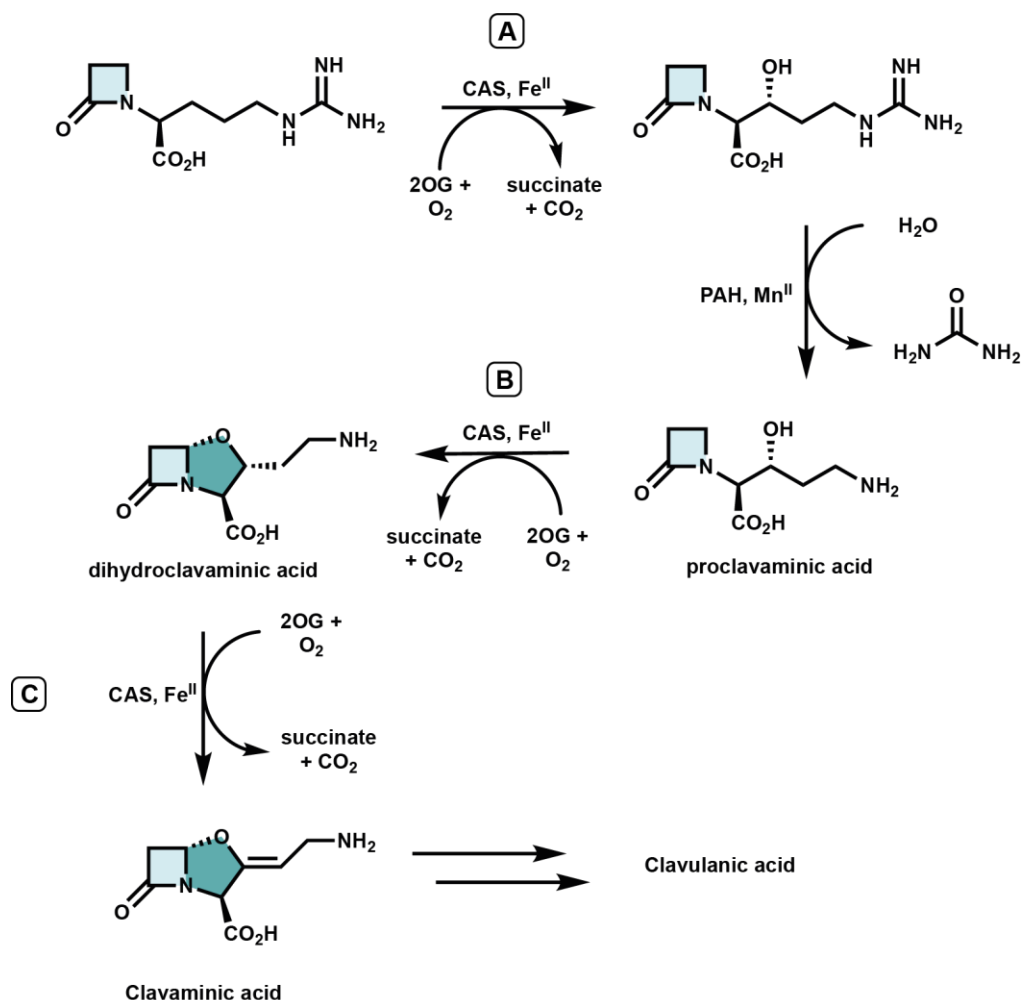
Oxygen derived from dioxygen

Oxygen derived from water

Scheme 5.4 | Exchange mechanism between an enzyme bound intermediate and water from the solvent, which has previously been proposed to account for the incorporation of water into DAC.¹⁷⁹ After initial hydrogen abstraction by the Fe(IV)=O intermediate, the hydroxyl group of the resultant Fe(III)-OH intermediate can exchange with water before it is incorporated into the product to give DAC.

An alternative hypothesis is that a water molecule remains bound to the Fe-centre throughout the catalytic cycle. Precedents for this proposal come from modelling²⁴⁴ and time resolved crystallographic work (unpublished results, G. Fiorini, Schofield Group) on the human hypoxia inducible factor prolyl hydroxylase PHD2. The latter provides experimental evidence for the presence of an Fe-bound water throughout the catalytic cycle. In such a system, the reactive intermediate would be an HO-Fe(IV)=O, rather than a Fe(IV)=O species. The involvement of a second Fe-coordinated oxygen atom may explain the catalytic plasticity of some 2OG oxygenases such as DAOCS/DACS. In the case of multifunctional enzymes, such as DAOCS/DACS and clavaminic acid synthase (CAS), the HO-Fe(IV)=O intermediate may remove the need for substantial conformational changes in order to reorientate the substrate molecule to perform a second oxidative reaction at a spatially distant site from where the initial hydrogen abstraction occurs. Further evidence for the viability of an HO-Fe(IV)=O intermediate comes from structural work on the 2OG dependent oxygenase AspH which catalyses protein hydroxylation; instead of the normal HXD/E...H Fe-coordinating triad, AspH only employs two histidine ligands to ligate the Fe-centre.²⁴⁵

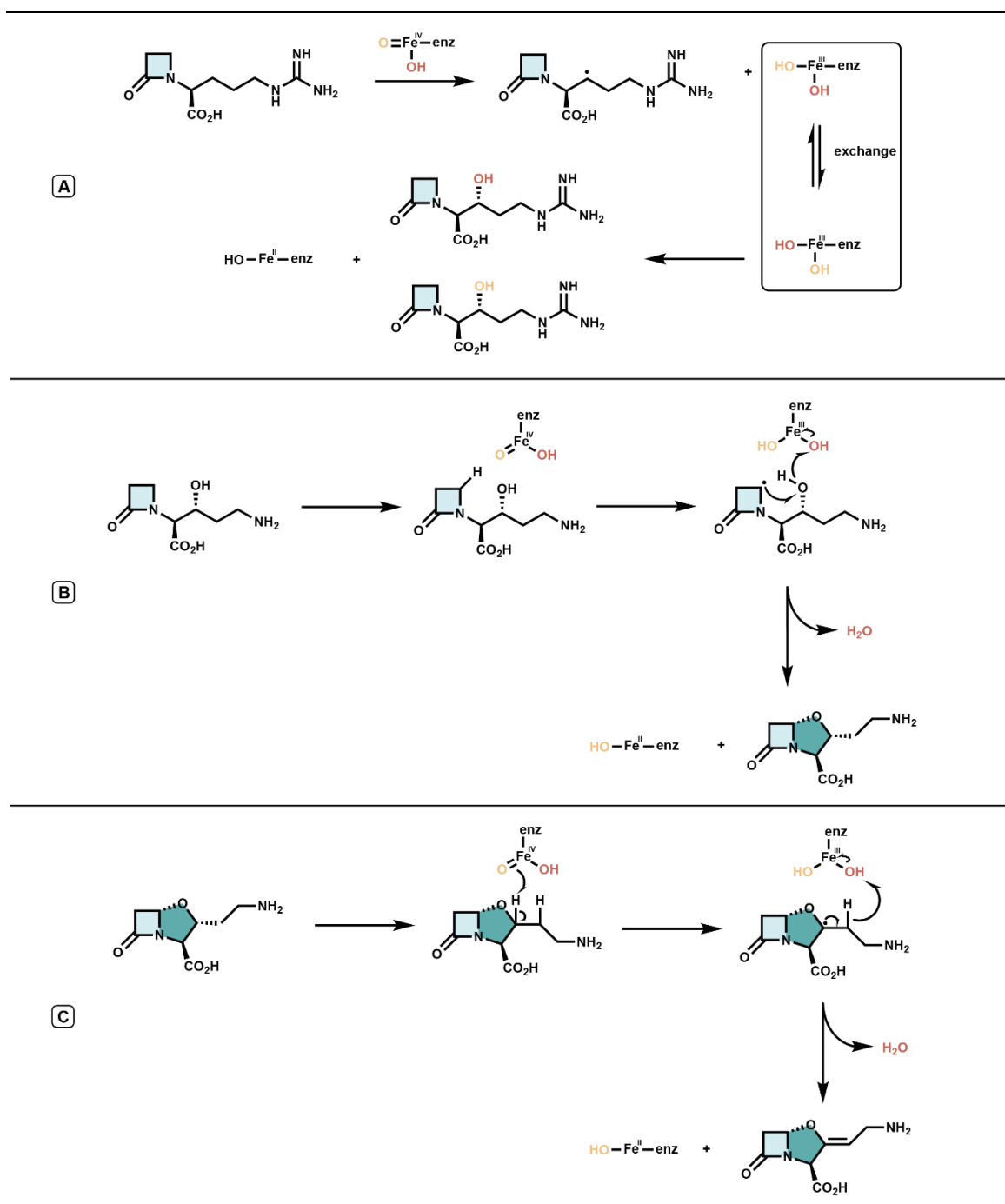
A role for the HO-Fe(IV)=O species is explored in the context of clavaminic acid synthase (CAS). CAS catalyses three reactions during the biosynthesis of clavulanic acid. Each CAS-catalysed reaction is coupled to the conversion of 2OG and O₂ to succinate and CO₂ (Scheme 5.5).



Scheme 5.5 | The three reactions catalysed by CAS during the biosynthesis of clavulanic acid. Note two of the reactions (A and B) are separated from each other by the action of proclavaminic amidino hydrolase (PAH).

The first of the three CAS catalysed reactions involves an apparently typical 2OG dependent hydroxylation (Scheme 5.5, A); this reaction is followed by PAH catalysed hydrolysis of the guanidine group of the product to give proclavaminic acid, which undergoes two subsequent 2OG dependent CAS-catalysed reactions – cyclisation (Scheme 5.5, B), then desaturation (Scheme 5.5, C) giving clavaminic acid. Although the involvement of HO-Fe(IV)=O species (wherein the water or hydroxide species is present throughout the catalytic cycle), rather than a Fe(IV)=O species would seem unnecessary for the typical CAS catalysed hydroxylation reaction (Scheme

5.5, **A**), it is potentially useful in both CAS-catalysed cyclisation and desaturation reactions (Scheme 5.5, **B** and **C**). Possible outline mechanisms involving the HO–Fe(IV)=O species in the three CAS-catalysed reactions are given below (Scheme 5.6). During the cyclisation (Scheme 5.6, **B**) and desaturation (Scheme 5.6, **C**) steps, the coordination of two oxygen atoms to the Fe-centre might enable these multi-step reactions to proceed with minimal conformational changes of the substrate. Note that the precise protonation states of the HO–Fe(IV)=O and HO–Fe(III)–OH intermediates are unknown.

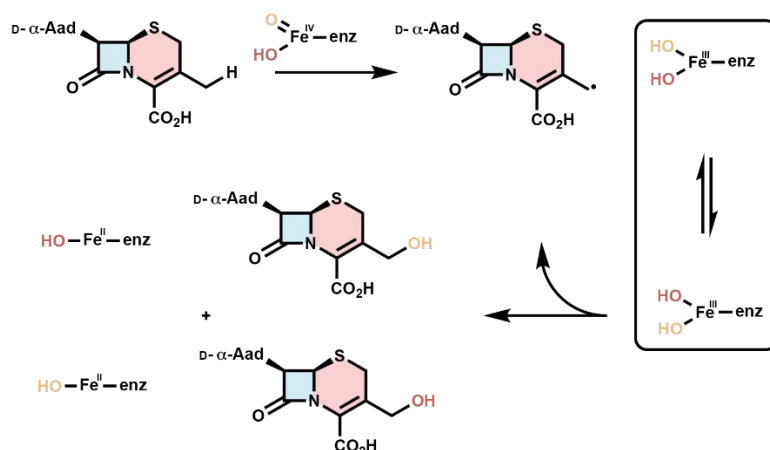


Oxygen derived from dioxygen

Oxygen derived from water

Scheme 5.6 | Proposed roles of the HO-Fe(IV)=O intermediate in CAS catalysis. A Hydroxylation mechanism. **B** Cyclisation. **C** Desaturation. In the cases of **B** and **C**, the water-derived hydroxyl group may be involved in the second oxidative step. Through the mechanism, it may be possible to avoid substantial conformation changes in the substrates. Note, there may be differences in the order of the steps in **B** and **C**. In **B** and **C**, the HO-Fe(III)-OH species could exchange such that the hydroxyl group derived from water remains bound to the Fe and the oxygen from O₂ forms water. For the sake of clarity, these possibilities are not shown.

An HO-Fe(IV)=O species may also be involved during the hydroxylation of DAOC to DAC. Formation of DAC proceeds with partial retention of stereochemistry.⁶⁹ This implies that hydroxylation occurs, at least sometimes, by direct insertion of the hydroxyl group into DAOC, rather than via the formation of a distinct 3'-methylene radical. At the same time, partial incorporation of an oxygen atom from water into DAC has also been established. Exchange of a ferryl-derived Fe(III)-OH species with water from the solvent may occur too slowly to account for the observed retention in stereochemistry, as the resultant 3'-methylene radical may rotate before the addition of the hydroxyl group. If the water was already bound to the Fe-centre, exchange could occur more quickly, which would account for both the partial incorporation of an oxygen atom from water as well as the partial retention of stereochemistry (Scheme 5.7). This mechanism is akin to the desaturation step catalysed by CAS (Scheme 5.6, C).



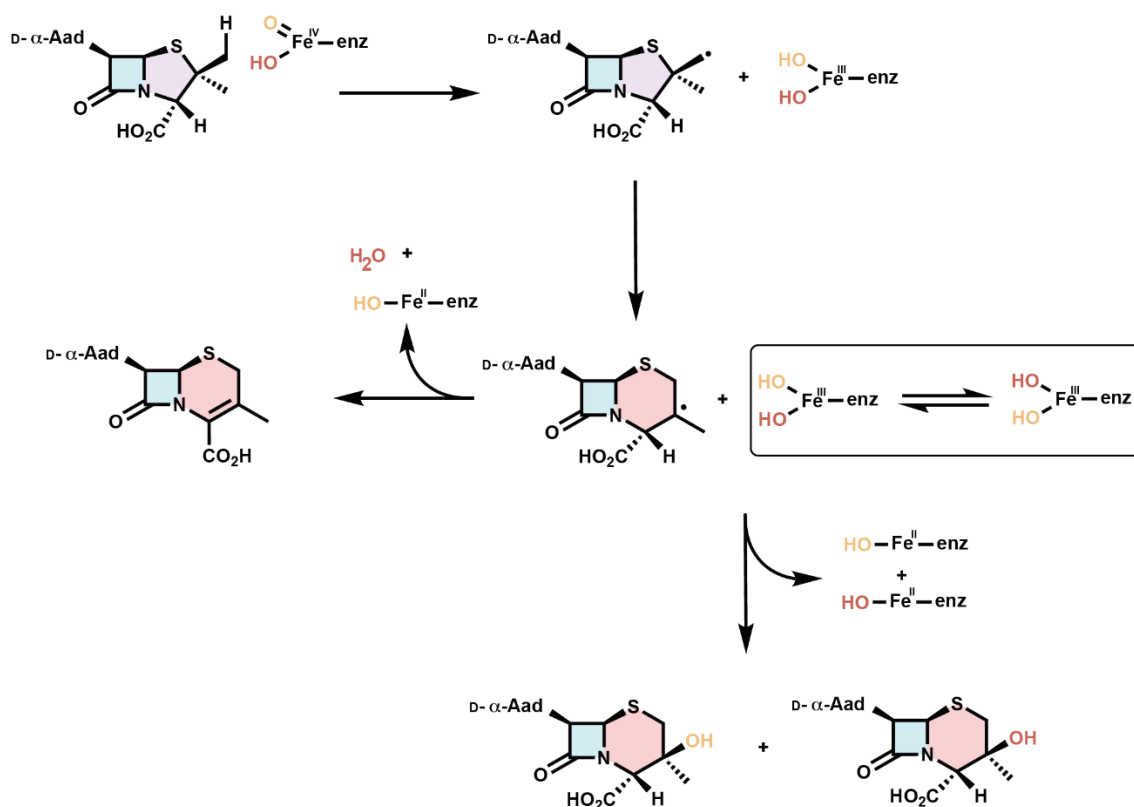
Oxygen derived from dioxygen

Oxygen derived from water

Scheme 5.7 | Possible role of an HO-Fe(IV)=O species in the 2OG oxygenase catalysed hydroxylation of DAOC to DAC. Initial hydrogen by the Fe(IV)=O species that is formed solely from dioxygen produces an HO-Fe(III)-OH species which can exchange (**box**) before insertion of a hydroxyl group into the 3'-methylene radical. Through this exchange mechanism, oxygen from both water (**red**) and dioxygen (**yellow**) can be incorporated into DAC.

The HO-Fe(IV)=O intermediate could also be implicated in the ring-expansion of penicillin N to DAOC. Recent modelling work has been performed on the ring-expansion reaction, based on crystal structures of DAOCS in complex with succinate (PDB ID: 1UO9).²⁰¹ The calculated lowest energy binding conformation of penicillin N to this DAOCS·Fe(II)·succinate structure was established computationally, and was determined to be a conformation in which penicillin N was bound to the Fe-centre via its C3 carboxylic acid group. This binding orientation of penicillin N differs from the previous mechanism, where penicillin N is bound to a hydrophobic pocket that is adjacent to the Fe-centre but does not coordinate directly to it.⁴⁴ Based on the above information, a ternary DAOCS·αKG·penicillin N complex was constructed and molecular modelling experiments were performed. This modelling work led to the proposal that the conformation of penicillin N was crucial in determining which methyl group, α- or β-, the first hydrogen atom was abstracted from. The conformation of the ring-expanded cepham radical was also vital in dictating whether the reaction proceeded via desaturation to give DAOC, or by hydroxyl rebound to give the 3β-hydroxy cepham shunt product.

Whether or not penicillin N coordinates to the iron during catalysis, an HO-Fe(IV)=O intermediate may play a role in the expansion reaction and in the formation of the 3β-hydroxy cepham shunt metabolite (Scheme 5.8). Initial abstraction of a hydrogen atom from the 2β-methyl group of penicillin N by an HO-Fe(IV)=O species would produce an HO-Fe(III)-OH species. Exchange of oxygen could occur either at the Fe(IV) or, maybe more likely, the Fe(III) stages. After rearrangement of the resultant 2β-methylene radical to the tertiary cepham radical, the hydroxyl group in the water derived position complexed to the Fe-centre could rebound onto the cepham radical to give the 3β-hydroxy cepham product. Alternatively, the hydroxyl group from water could enable desaturation to give the major cephem product. This proposal enables the oxidising intermediate to bridge between the two sites from which hydrogens must be abstracted.



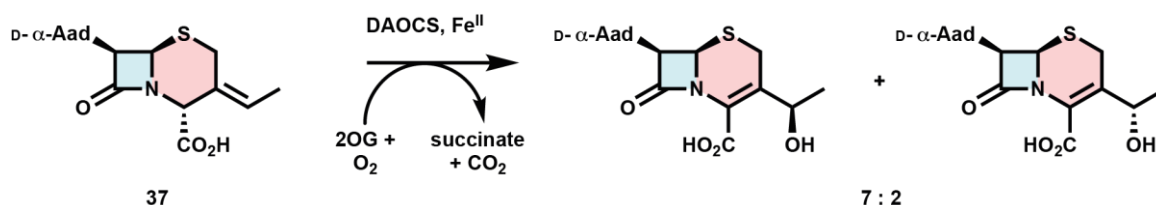
Oxygen derived from dioxygen

Oxygen derived from water

Scheme 5.8 | Purported role of an HO-Fe(IV)=O intermediate in the ring expansion of penicillin N to DAOC. The abstraction of a hydrogen atom from the 2 β -methyl group of penicillin N initiates ring-expansion to the tertiary cepham radical and produces a HO-Fe(III)-OH intermediate. The presence of two hydroxyl groups in this intermediate may allow it to bridge between the two sites of hydrogen abstraction. Exchange between the hydroxyl groups in the HO-Fe(III)-OH species may account for the incorporation of an oxygen atom from water into the 3 β -hydroxy cepham product.

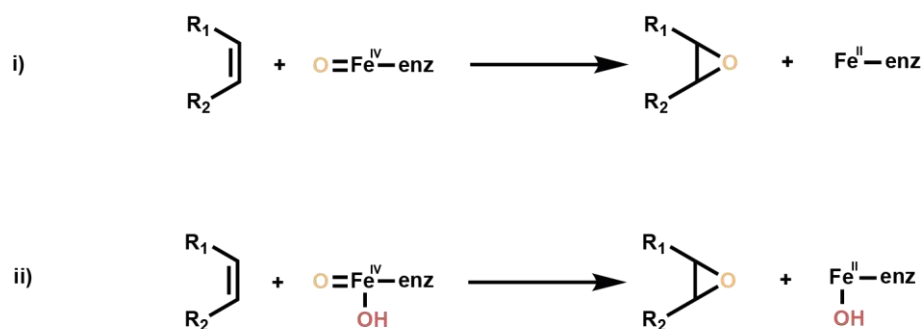
Interestingly, the source of oxygen that is incorporated into the product during oxygenation reactions catalysed by DAOCS/DACS manifests a substrate-dependence. By contrast to the wild-type hydroxylation of DAOC to DAC, 3-vinyl cephem incubations with DAOCS/DACS (**Chapter 3**) produced a cephem hydroxy azide (**29a**) in which the hydroxyl group oxygen atom was derived exclusively from molecular oxygen, with no incorporation from water. The cephem diol (**26**) produced from the 3-vinyl cephem is formed with incorporation of one oxygen atom from O₂ and one from H₂O, consistent with a mechanism involving epoxide ring opening. Incubations of the [4-²H]exomethylene cepham with DAOCS/DACS produced a spiro-epoxide product, the new oxygen of which was derived solely from dioxygen.¹⁷⁹ Epoxidase activity was also observed during

incubations of the Z-ethylidene cepham substrate (**37**) with DAOCS. Although the products in this incubation were two diastereomeric alcohols (Scheme 5.9), both hydroxyl group were derived almost completely from dioxygen, implying that the conversion of the **37** proceeds via the ‘epoxidase’ pathway, rather than via the wild-type hydroxylation mechanism.¹¹³



Scheme 5.9 | The conversion of the Z-ethylidene cepham substrate to two diastereomeric alcohols by DAOCS. Whilst no epoxide formation was detected during this incubation, the hydroxyl groups of the two alcohols are derived exclusively from molecular oxygen, implying that their formation proceeds via the ‘epoxidase’ pathway, rather than in the same manner that DAC is produced from DAOC.

Based on work described herein, it is proposed that DAOCS/DACS exhibits ‘epoxidase’ activity on the 3-vinyl cephem substrate analogue, but the structure of the resultant epoxide has not yet been confirmed by isolation. These observations are consistent with exchange occurring at the Fe(III) stage, rather than the Fe(IV) stage. Thus, 3’-epoxidation of unsaturated substrate analogues occurs by direct transfer of the ferryl oxygen from either an Fe(IV)=O or HO–Fe(IV)=O species (the ferryl oxygen of which is derived from dioxygen and which has not exchanged) to the alkene to give an epoxide (Scheme 5.10, i and ii). By contrast, hydroxylation occurs via initial hydrogen abstraction to give a carbon centred radical and an HO–Fe(III)–OH species, the oxygens of which can undergo partial exchange before reacting with the radical to produce an alcohol product with partial incorporation of water.

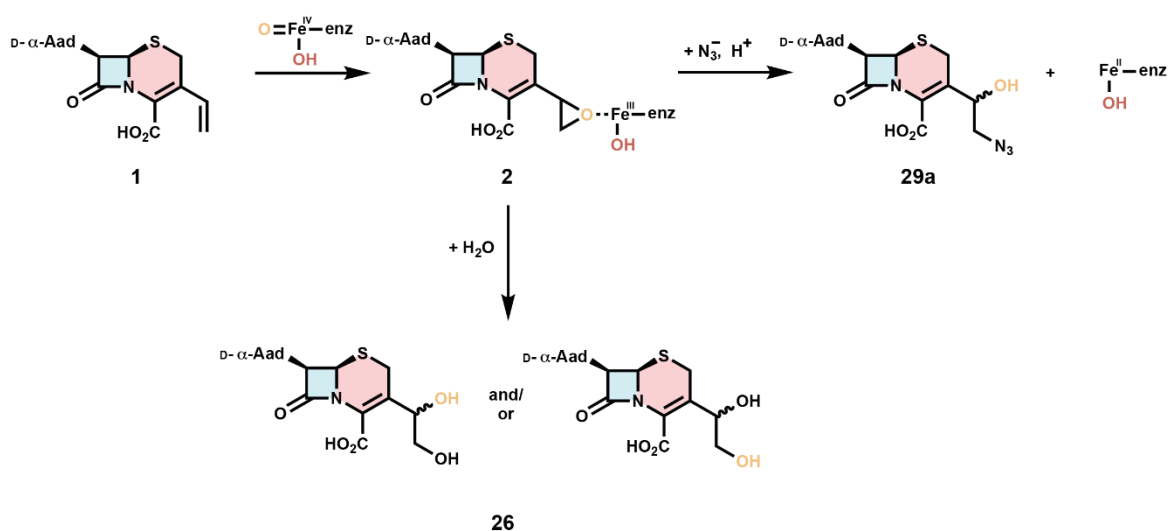


Oxygen derived from dioxygen

Oxygen derived from water

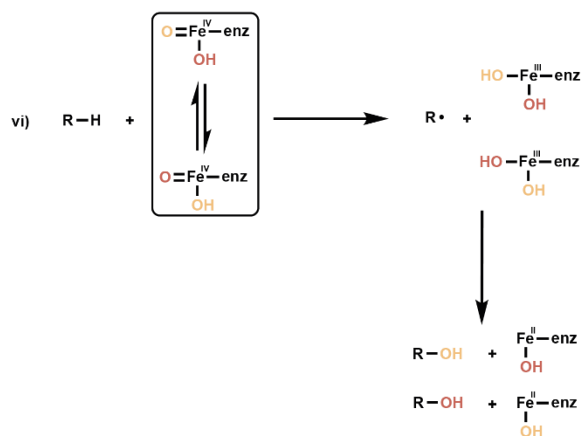
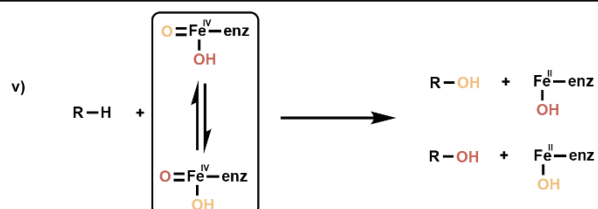
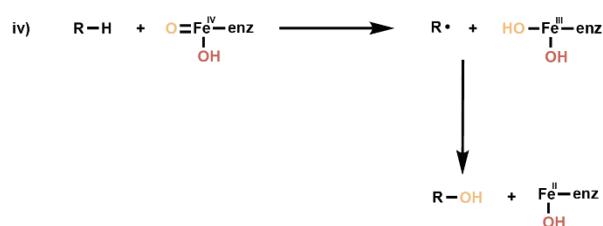
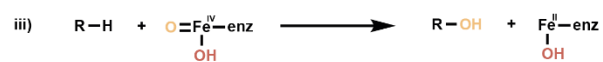
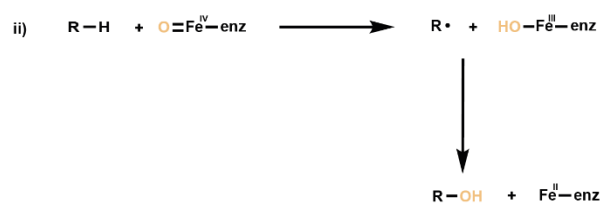
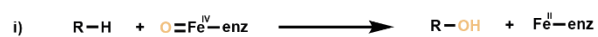
Scheme 5.10 | Options for 2OG oxygenase catalysed epoxidations. Note, oxygen from water is not incorporated in the epoxide product, so some of the options shown for hydroxylations in Scheme 5.12 cannot apply for epoxidation. Direct insertion of the ferryl oxygen atom – derived solely from dioxygen – can occur from either an Fe(IV)=O (i) or HO-Fe(IV)=O (ii) intermediate.

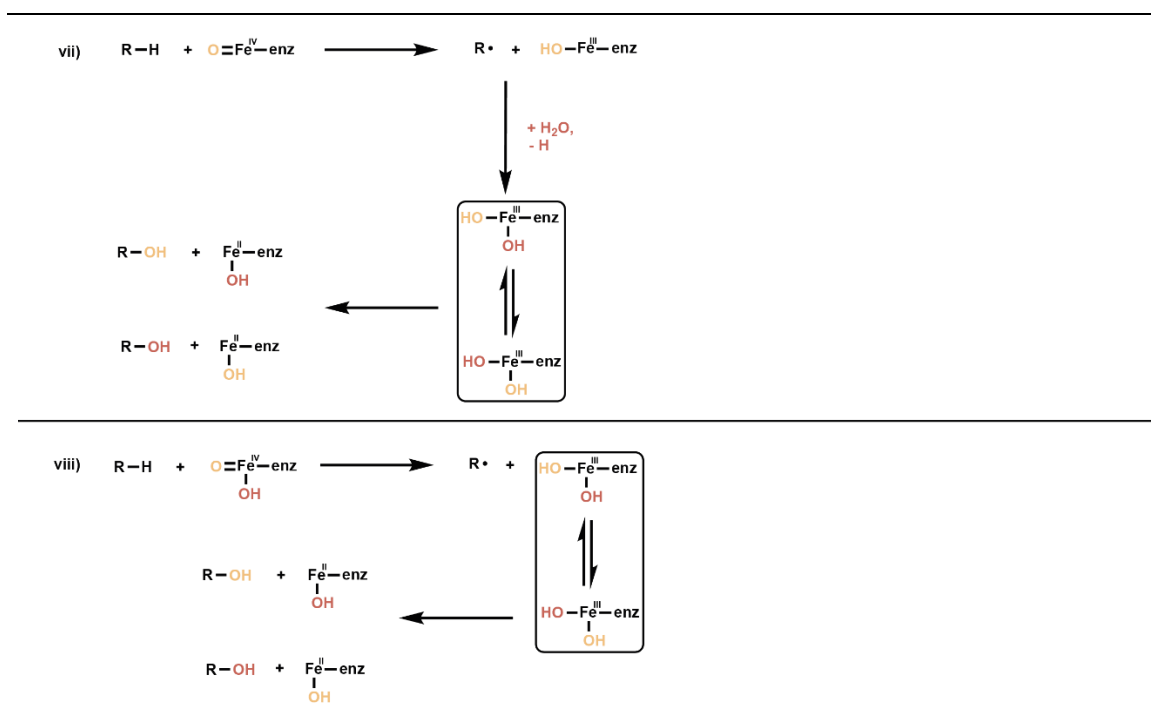
Formation of the diol (**26**) from the 3-vinyl cephem is a special case – initially an enzyme bound epoxide is formed by reaction with the ferryl oxygen; the epoxide then reacts with a water molecule to give the diol. An overview of the mechanisms for the formation of the hydroxy azide (**29a**) and the diol (**26**) from the 3-vinyl cephem (**1**) are given below (Scheme 5.11).



Scheme 5.11 | Mechanism for the formation of diol (26**) and hydroxy azide (**29a**) via an epoxide intermediate (**2**).** Note, the precise source of the water molecule giving rise to **26** is unknown. It may be an Fe-ligated water/hydroxide. Similarly, an Fe-N_3 intermediate may be involved in the formation of **29a**.

In summary, eight mechanistic options **(i–viii)** for 2OG-dependent oxygenase catalysed hydroxylation are described below (Scheme 5.12). These possibilities are not collectively exhaustive. For example, in option **vii**, the incoming water molecule may displace the Fe-bound hydroxyl group derived from dioxygen rather than binding in concert with it. Hydroxyl group displacement has been proposed to account for DAC formation (Scheme 5.4). In addition, it is not clear whether the water molecule coordinates to the Fe-centre as H₂O or as a hydroxyl ligand. In options **(i–iv)** there is no exchange and therefore no incorporation of water into the hydroxylated product. The current view is that these mechanisms are likely utilised by the majority of 2OG dependent hydroxylases. ¹⁸O incorporation results with the 3-vinyl cepham, [4-²H]exomethylene cepham and Z-ethylidene cepham argue against **(v)** or **(vi)**, which involve exchange at the Fe(IV)=O stage. Option **(vii)**, or the hydroxyl group displacement variation described above, has been proposed for DAOCS/DACS.¹¹² However, the evidence presented here suggests that **(viii)** is more likely to account for the partial incorporation of water into hydroxylated products of some 2OG oxygenase catalysed reactions, including hydroxylations as catalysed by DAOCS/DACS, DACS, and CAS.





Oxygen derived from dioxygen

Oxygen derived from water

Scheme 5.12 | Outline of eight mechanistic options for 2OG oxygenase dependent hydroxylase reactions. i – iv Mechanisms in which no exchange occurs, and therefore an oxygen atom from water is not incorporated into the hydroxylated product. This is the outcome most commonly observed with 2OG hydroxylases. **v** and **vi** Exchange occurs at the Fe(IV)=O stage and an oxygen atom from water is incorporated into the product by either a direct insertion mechanism (**v**) or via a radical intermediate (**vi**). These two mechanisms are considered unlikely as epoxidation of 3'-unsaturated substrates by DAOCS/DACS occurs with complete incorporation of an oxygen atom from O₂. **vii** and **viii** Exchange occurs at the Fe(III)-OH stage. Either a water molecule from solution binds to the Fe(III)-OH species (**vii**) or a hydroxyl group from water remains coordinated to the Fe-centre throughout and there is exchange within the HO-Fe(III)-OH species. Several pieces of independent evidence have suggested that a water molecule is bound to the Fe-centre throughout the catalytic cycle, therefore **viii** is considered more likely to be the mechanism by which an oxygen atom from water is incorporated into hydroxylated products. This list is not collectively exhaustive and other possibilities exist.

The overall findings appear to support the exceptionality of the reactions of DAOCS/DACS and DACS, as well as some other 2OG oxygenases, such as CAS. The role of an HO-Fe(IV)=O species can be most clearly seen during hydroxylation reactions, when water incorporation can be observed directly. However, it cannot be ruled out that HO-Fe(IV)=O and HO-Fe(III)-OH intermediates are more widely involved in 2OG oxygenase catalysis, as supported by recent studies on PHD2 and Asph.^{244, 245} Whilst extensive studies have rightly been performed on the enzymatically unique ring expansion of penicillin N to DAOC that occurs in the preceding step, the capacity of the hydroxylase activity of DAOCS/DACS to accommodate a range of ancillary

transformations, in particular epoxidation and azidation, validates these enzymes as worthy of further investigation, as is more generally the case for the 2OG oxygenase catalysed hydroxylations.

Chapter 6

Materials and Methods

6 Materials and methods

6.1 General Molecular Biology

6.1.1 Introduction

Materials (pipette tips, MQ, microcentrifuge tubes) were autoclaved (121 °C, 20 min) before use. All water used was purified with an Elix® Essential 10 UV Water Purification System equipped with a Progard® TS2 Pretreatment pack. The following materials were purchased from Thermo Fisher: 6× DNA Gel Loading Dye, GeneJET PCR Purification Kit, GeneJET Gel Extraction Kit, and a GeneRuler 1kb DNA ladder. Custom DNA oligonucleotide primers, synthetic genes, and SYBR Safe DNA gel stain (1000× stock in DMSO) were purchased from Invitrogen. XL-10 ultracompetent *E. coli* cells (Cat# 200317) were purchased from Agilent. Q5 Polymerase and dNTPs were purchased from NEB. DNA concentrations were determined on a NanoDrop™ One Microvolume UV-Vis Spectrophotometer.

6.1.2 Polymerase Chain Reaction (PCR)

PCR reactions were performed in standard thin wall 100 µL PCR tubes in a 3Prime thermocycler (TECHNE). A representative PCR mixture is given below in Table 6.1. Annealing temperatures were calculated using the NEB T_m calculator.

Table 6.1 | Representative PCR mixture for amplification of a gene insert.

Component	Concentration	Volume (µL)	Final concentration
Template DNA	Variable	0.6	2–5 ng/µL
Forward primer	10 µM	2.5	0.5 µM
Reverse primer	10 µM	2.5	0.5 µM
Q5 reaction buffer	5×	10	1×
dNTP mix	10 mM	1	200 µM
Q5 High GC enhancer	5×	10	1×
Q5 HF DNA Polymerase	-	0.5	-
Sterile MQ	-	22	-
Σ		50	

6.1.3 Colony PCR

Cells were harvested from culture (100 μL) by centrifugation (16,000 $\times g$, 2 min) and the supernatant was removed. The cell pellet was resuspended in sterile MQ (100 μL) and boiled (95 $^{\circ}\text{C}$, 10 min) to lyse cells. Cell debris was pelleted by centrifugation (16,000 $\times g$, 2 min). Culture supernatant (5 μL) was used in the colony PCR reaction mixture (Table 6.2). Forward and reverse primer stocks (100 μM) were diluted 10-fold in sterile MQ and prepared as a master mix. Components were added in descending order.

Table 6.2 | Colony PCR reaction set-up. Primer sequences are reported in Table 6.9.

Component (stock conc.)	Volume (μL)
Culture supernatant	5
Forward primer (10 μM)	1
Reverse primer (10 μM)	1
Sterile MQ	3
BioMix Red (2X)	10
Σ	20

A representative amplification method is given in Table 6.3.

Table 6.3 | Representative thermocycler settings for a colony PCR. These settings were used with DAOCS/DACS primers.

	Step	Temperature ($^{\circ}\text{C}$)	Time (sec)
30 cycles	Initial denaturation	98	120
	Denaturation	98	10
	Annealing	62	15
	Extension	70	120
	Final extension	70	300

6.1.4 Agarose Gel Electrophoresis

Buffer: TAE Buffer was prepared as a 50 $\times$ stock and diluted to 1 $\times$ before use (50 $\times$ stock: 2 M Trizma base, 1 M glacial acetic acid, 50 mM EDTA, pH $\approx$ 8.5).

Agarose (1% w/v) was dissolved in 1 $\times$ TAE Buffer (60 mL) by microwave irradiation for 1–2 min. SYBR[®] Safe DNA gel stain (6 μL) was added and mixed by gently swirling. The mixture was set in

gel casting trays. Samples were combined with 6× DNA Gel Loading Dye (5:1) and loaded onto the gel (max. 30 µL/well). A 1 kB DNA ladder (6 µL) was also loaded in a separate lane for reference. Gels were run at 100 V for 25 min in 1× TAE Buffer and visualised on a Bio-Rad ChemiDoc MP Universal Hood III ('SYBR Safe' method). Visualisation for bands destined to be excised was carried out under blue light to prevent DNA degradation.

6.2 Ligation Independent Cloning (LIC)

The LIC protocol²⁴⁶ below was used to subclone the DAOCS/DACS gene (*cefEF_v1*) and DACS gene (*cefF*) into pNIC28-Bsa4.

6.2.1 LIC insert preparation

The gene of interest was amplified by PCR using the commercial plasmid as a template, as described in **Section 6.1.2**. Primers for each gene are given in Table 6.4 and were designed to amplify the inserts with overhangs complementary to the pNIC28-Bsa4 plasmid in which the gene was to be inserted.

Table 6.4 | PCR primers for DAOCS/DACS (*cefEF_v1*) and (*cefF*) used to amplify genes out of their commercial plasmids for LIC.

Name	Sequence 5'–3'
DAOCS/DACS_fwd	TACTTCCAATCCATGACTTCTAAGGTACCAAGTTTCCG
DAOCS/DACS_rev	TATCCACCTTTACTGATCCTCGCGGGTTCCAG
DACS_fwd	TACTTCCAATCCATGCGCAGATACACCGGTTT
DACS_rev	TATCCACCTTTACTGTGCCTGCGGTTTCATTTTTGC

After amplification, the mixture was incubated with DpnI at 37 °C for 3 h to degrade template (methylated) DNA. Separation of DNA components was carried out on a 1% agarose gel and the insert bands were excised with Axygen® Gel Cutting Tips and isolated using a GeneJET Gel Purification Kit.

Cohesive ends were generated using LIC-qualified Q5 HF DNA polymerase (New England Biolabs). Reactions were prepared as described in Table 6.5 and incubated at 25 °C for 30 min. Enzyme inactivation was achieved by incubation at 75 °C for 20 min.

Table 6.5 | Reaction set-up to generate cohesive-ends in inserts and linearised vectors.

Component	Stock concentration	Volume (μL)
DNA	Varied	5
Q5 Reaction Buffer	5×	1
BSA	10 mg/mL	0.2
dNTP	25 mM	1
DTT	100 mM	0.5
Sterile MQ	N/A	2.05
Q5 HF DNA pol.	N/A	0.25
Σ		10

6.2.2 LIC vector preparation

A stock solution of the BsaI-treated pNIC28-Bsa4 vector was available in the laboratory and was used without further modifications.

6.2.3 Annealing and transformation

Q5 HF DNA polymerase-treated gene insert and vector were incubated together in a 2:1 v/v ratio (3 μL final volume) at 25 °C for 30 min then transformed into XL10-Gold ultracompetent *E. coli* cells (30 μL) as described in **Section 6.6.5** and grown overnight at 37 °C on 2TY agar plates containing kanamycin (50 μg/mL). Single colonies were used to inoculate 2TY media (10 mL) containing kanamycin (50 μg/mL) and grown at 37 °C and 250 rpm shaking for 6–8 h. Colony PCR (**Section 6.1.3**) was used to confirm the presence of the gene insert. Cells were harvested from culture (3 × 1.5 mL) by centrifugation (4,500 ×g, 5 min). Plasmids were purified from cell pellets using a GeneJET Plasmid Miniprep Kit and successful subcloning was confirmed by Sanger sequencing (TubeSeq, Eurofins Genomics).

6.3 Restriction-Ligation cloning

6.3.1 Materials

A modified pCold I (Addgene #41096)²⁴⁷ vector containing an IPNS insert was kindly gifted by Dr Patrick Rabe (University of Oxford). This vector contains an N-terminal His₆-HRC3C site in frame with Sall and NotI cloning sites. pET24a(+) was kindly donated by Dr Zhihong Zhang (University of Oxford). Materials were used as described in **Section 6.1.1**.

6.3.2 Subcloning into modified pCold I

Mod. pCold I and insert digestion: Mod. pCold I and the gene insert, either DACS (*cefF*) or DAOCS/DACS (*cefEF_v2*), were digested by overnight incubation at 37 °C with Sall and NotI-HF restriction enzymes (Table 6.6). DAOCS/DACS was received from Invitrogen in a pMA-RQ (AmpR) vector. DNA fragments were separated on a 1% agarose gel and desired bands were excised with Axygen® Gel Cutting Tips and isolated using a GeneJET Gel Purification Kit.

Table 6.6 | Representative restriction reaction set-up for the digestion of *cefEF_v2*. Similar reactions were also set up for the subcloning of the *cefF* gene into the modified pCold vector. Concentrations of linearised vector and gene insert were measuring using a NanoDrop™. Volumes required for 2 mg of DNA were calculated based on the concentration. Sterile MQ was added to make the reaction mixture up to 50 µL.

Component	Stock concentration	Volume (µL)	Final Concentration
<i>cefEF_v2</i> in pMA-RQ (2 mg)	260 ng/µL	7.70	40 ng/µL
CutSmart Buffer	2×	5	1×
Sterile MQ	-	34.3	-
Sall	-	2	-
NotI-HF	-	1	-
		Σ 50	

Ligation and transformation: Digested insert and linearised vector were combined in 3:1 and 7:1 molar ratios together with T4 DNA Ligase (Table 6.7) and incubated at 16 °C for 48 h. Masses of insert and linearised vectors were calculated based on sequence length using NeBioCalculator. The ligation mixture (5 µL) was heated (65 °C, 10 min) to inactivate the enzyme, and subsequently used to transform XL10-Gold ultracompetent cells (30 µL) as described in **Section 6.6.5**. The transformed cells were plated on 2TY agar plates containing ampicillin (50 µg/mL) and were

incubated overnight at 37 °C. Single colonies were used to inoculate 2TY media (10 mL) containing ampicillin (50 µg/mL) which were grown at 37 °C and 250 rpm shaking for 8–10 h. Colony PCR (Section 6.1.3) with insert specific primers (Table 6.4) was used to confirm the presence of the gene insert in selected colonies. Cells were harvested from culture (3 × 1.5 mL) by centrifugation (4,500 ×g, 5 min). Plasmids were purified from cell pellets using a GeneJET Plasmid Miniprep Kit and successful subcloning was confirmed by Sanger sequencing (TubeSeq, Eurofins Genomics).

Table 6.7 | Representative ligation reaction. Ligation reaction were set-up based on the used of 50 ng of linearised vector DNA. The example below is for the ligation of *cefEF_v2* into mod. pCold using a 3:1 molar ratio.

Component	Stock concentration	Volume (µL)	Final concentration
Linearised mod. pCold (50 ng)	16.9 ng/µL	3.78	3.2 ng/µL
<i>cefEF_v2</i>	8.7 ng/µL	3.25	1.6 ng/µL
T4 DNA Ligase Reaction Buffer	10×	2	1×
Sterile MQ	-	9.97	-
T4 DNA Ligase	-	1	-
		Σ	20

6.3.3 Subcloning into pET24a(+)

Digestion of DAOCS/DACS (*cefEF_v2*) gene insert and pET24a(+): pET24a(+) vector and *cefEF_v2* in pMA-RQ were digested by overnight incubation at 37 °C with NdeI and NotI-HF restriction enzyme (Table 6.8). DNA fragments were separated on a 1% agarose gel and the desired bands were excised with Axygen® Gel Cutting Tips and isolated using a GeneJET Gel Purification Kit.

Table 6.8 | Restriction mixture for the digestion of *cefEF_v2* and pET24a(+) with NdeI and NotI-HF.

Component	Volume (μL)
Mod. pCold or <i>cefF/cefEF_v2</i> (2 mg)	Varied
CutSmart Buffer (2×)	5
Sterile MQ	Varied
NdeI	2
NotI-HF	1
Σ 50	

Ligation and transformation: Digested *cefEF_v2* insert and linearised pET24a(+) were combined in 3:1 and 7:1 molar ratios together with T4 DNA Ligase and incubated at 16 °C for 48 h. Ligation mixture (5 µL) was heated (65 °C, 10 min) then transformed into XL10-Gold ultracompetent cells (30 µL) as described in **Section 6.6.5**. Transformants were plated on 2TY agar plates containing kanamycin (50 µg/mL) and incubated overnight at 37 °C. Colony PCR was used to confirm that selection colonies contained *cefEF_v2*. Plasmids were isolated from cultures and sequences as described in **Section 6.3.2**.

Primers sequences are given below in Table 6.9.

Table 6.9 | Primer sequences used for colony PCR reactions to confirm that isolated colonies contained the gene insert of interest.

Vector	Primer	Sequence (5'–3')
pCold_AC/pCold_kanR_AC	Forward	CCATATGACCAGCAAAGTTCCGG
	Reverse	GCGGCCGCTTAGGTTGC
pCold_DACS	Forward	TACTTCCAATCCATGCGCAGATACACCGGTTTC
	Reverse	TATCCACCTTTACTGTGCCTGCGGTTTCATTTTTTGC

A summary of constructs produced by restriction-ligation is given in Table 6.10.

Table 6.10 | List of constructs generated using restriction-ligation cloning

#	Construct	Restriction enzymes
1	pCold I_DD	NdeI; BamHI
2	pET24a(+)_DD	NdeI; BamHI
3	pET_AC	NdeI; NotI-HF
4	pCold_AC	Sall; NotI-HF
5	pET24a(+)_DACS	Sall; NotI
6	pCold_DACS	Sall; NotI-HF
7	pCold_nIDAACS	Sall; NotI

6.4 Gibson Assembly molecular cloning

The protocol was used to substitute the *ampR* selection marker of pCold_AC for a kanamycin resistance gene (*kanR*).

6.4.1 PCR amplification of dsDNA fragments

Two PCR reactions were set-up: one to generate the linearised pCold_AC vector without the ampicillin resistance gene, the other to amplify the kanamycin resistance gene insert (*kanR*) from pET24a(+). Primer sets (Table 6.11) were designed using the NEBuilder Assembly Tool to produce insert and vector fragments containing 20–40 bp overlapping end.

Table 6.11 | Primers for the amplification of target DNA. Primers were designed using the NEBuilder Assembly Tool.

Name	Sequence 5'–3'
pCold_AC_fwd	GTTTTCTAACTGTCAGACCAAGTTTAC
pCold_AC_rev	TATGGCTCATACTCTTCCTTTTCAATATTATTG
kanR_fwd	AAGGAAGAGTATGAGCCATATTCAACGG
kanR_rev	GGTCTGACAGTTAGAAAACTCATCGAGC

Reaction mixtures for the PCRs were set-up as described in Table 6.12.

Table 6.12 | Reaction set-up for Gibson Assembly PCR

Component	Volume (μL)
Template DNA (≈200 ng μL ⁻¹)	0.2
Sterile MQ	23.3
Forward primer (10 μM)	2.5
Reverse primer (10 μM)	2.5
Q5 Reaction Buffer (5X)	10
Q5 High GC Enhancer (5X)	10
dNTPs (10 mM)	1
Q5 HF DNA Polymerase	0.5
Σ	50

Cycling parameters for the reactions are given in Table 6.13. Successful amplification of the *kanR* fragment and linearised pCold sequences was confirmed by running an aliquot (5 μL) of each PCR on a 1% agarose gel. Once their identities had been confirmed, the PCR mixtures were used without further purification.

Table 6.13 | Cycling parameters for the first Gibson Assembly PCR.

		Temperature (°C)	Time (sec)
30 cycles	Initial denaturation	98	120
	Denaturation	98	10
	Annealing	57	15
	Extension	72	120
	Final extension	72	600

6.4.2 Gibson Assembly reaction and transformation

The appropriate insert (38 ng) and vector (100 ng) were incubated in a 2:1 molar ratio with the Gibson Assembly Master Mix as described in Table 6.14. The reaction mixture was incubated at 50 °C for 15 min, after which time 2 µL of the solution was used to transform NEB-5α competent cells (50 µL), as described in **Section 6.6.5**. Cells were plated on pre-warmed 2TY agar plates containing kanamycin (50 µg/mL) and incubated overnight at 37 °C. Colony PCR (**Section 6.1.3**) using *cefEF_v2* primers was carried out to confirm the selected colonies were pCold_kanR_AC, rather than the pET24a(+) vector used for the amplification of *kanR*. Plasmids were harvested from culture (3 × 1.5 mL) using a GeneJET Plasmid Miniprep Kit and successful cloning was confirmed by Sanger sequencing (TubeSeq, Eurofins Genomics). In addition, to confirm that the *ampR* gene had been removed, XL10-Gold ultracompetent cells were transformed with isolated plasmid as described in **Section 6.6.5**, and plated on pre-warmed 2TY agar plates containing ampicillin (50 µg/mL). No colonies were observed.

Table 6.14 | Gibson Assembly reaction for combining linear pCold_AC without *ampR* and *cefEF_v2*.

Component	Concentration	Volume (µL)
Linearised pCold_AC	429 ng/µL	0.23
<i>cefEF_v2</i> insert	435 ng/µL	0.10
Gibson Assembly Master Mix	2×	10
Sterile MQ	-	9.67
Σ		20

6.5 Summary of Constructs Used

Table 6.15 | Summary of constructs used to produce the proteins described herein, and their features.

Construct	Template vector	Gene insert	Protein produced	Tag	Resistance
pNIC_DD	pNIC28-Bsa4	<i>cefEF_v1</i>	DAOCS/DACS	His ₆ -TEV	Kan
pCold_DD	pCold I			His ₆ -Xa	Amp
pCold_AC	Mod. pCold I	<i>cefEF_v2</i>	DAOCS/DACS	His ₆ -HRV3C	Amp
pCold_kanR_AC	Mod. pCold I			His ₆ -HRV3C	Kan
pET_AC	pET24a(+)			Untagged	Kan
pET_DAOCS	pET24a(+)	<i>DAOCS</i>	DAOCS	Untagged	Kan
pET_ICM DAOCS	pET30a(+)	<i>ICM DAOCS</i>	ICM DAOCS	Untagged	Kan
pCold_DACS	Mod. pCold I	<i>DACS</i>	DACS	His ₆ -HRV3C	Amp

6.6 General Microbiology

6.6.1 Materials

Microbiology work was performed under sterile conditions in a laminar flow hood. Microcentrifuge tubes, pipette tips and solution (media and MQ) were autoclaved (121 °C, 20 min) prior to use. All water used was purified with an Elix® Essential 10 UV Water Purification System equipped with a Progard® TS2 Pretreatment pack. Components for the production of growth media were from Sigma-Aldrich. SOC Outgrowth Medium was purchased from NEB (Cat. #B9020), the components of which are given in Table 6.16. Antibiotics (kanamycin sulfate, ampicillin, chloramphenicol) were from Apollo Scientific. XL10-Gold ultracompetent cells were from Agilent (Cat. #200317), BL21(DE3) (Cat. #C2527) and Lemo21(DE3) (Cat. #C2528) competent cells were obtained from NEB. SDS PAGE was performed on NuPAGE® 4–12% Bis-Tris Gels (Cat. #NP032) and run in NuPAGE® MOPS SDS Running Buffer (20×, Cat. #NP0001), using PageRuler™ Prestained Protein Ladder (Cat. #26616, 10–180 kDa) as referenced (all from Thermo Fisher). InstantBlue™ Ultrafast Protein Stain (discontinued) was purchased from Sigma-Aldrich. QuickBlue Protein Stain was purchased from LubioScience. Mini-PROTEAN TGX Stain-Free Gels (Cat. #4568095 and #4568096 for 12- and 15-well comb gels respectively) were purchased from Bio-Rad along with Precision Plus Protein Unstained Standards (Cat. #1310363, 10–250 kDa) and 10X Tris/SDS/Glycine

buffer (Cat. #1610732). NuPAGE® LDS Sample Buffer (2×, Cat. #NP0007) was purchased from ThermoFisher.

Table 6.16 | SOC Outgrowth Medium, purchased from Sourced from New England BioLabs (NEB).

Component	Amount
Vegetable Peptone	2
Yeast Extract	0.5%
NaCl	10 mM
KCl	2.5 mM
MgCl ₂	10 mM
MgSO ₄	10 mM
Glucose	20 mM

6.6.2 2TY medium preparation

Freshly prepared growth media was used for each culture growth. In all cases, 2TY medium was used, its composition it given in Table 6.17.

Table 6.17 |Composition of 2TY media.

Component	Concentration (g/L)
Tryptone	16
Yeast extract	10
NaCl	5

6.6.3 Preparation of antibiotic stocks

1000× stock solutions of the required antibiotic were made up in either MQ water (kanamycin of ampicillin) or EtOH (chloramphenicol) and filter sterilized (0.20 µm) in a laminar flow hood. Stocks were stored at 4 °C overnight or at –20 °C for longer term.

6.6.4 SDS-PAGE

Sample preparation: Protein sample (10 µL) was combined with 2× SDS Loading Buffer (10 µL) and denatured at 95 °C for 5 min. The recipe for the loading buffer is given in Table 6.18.

Table 6.18 | SDS Loading Buffer (2×) mixture.

Component	Volume / μL
NuPAGE® LDS Sample Buffer (4×)	450
MQ	405
β -mercaptoethanol	45
Σ	900

Sample loading: 10 μL of sample or 6 μL of ladder was loaded per well.

Electrophoresis: Invitrogen NuPAGE™ 4–12% Bis-Tris Gels were run in an Invitrogen Mini Gel Tank at 180 V for 35 min using 1× NuPAGE® MOPS SDS Running Buffer (50 mM MOPS, 50 mM Tris base, 3.5 mM SDS, 1 mM EDTA free acid, pH 7.7). Bio-Rad Mini-PROTEAN TGX Stain-Free gels were run in a Bio-Rad Mini PROTEAN Tetra Cells at 300 V for 19 min using 1× Tris/SDS/Glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Constant voltage was supplied by a Bio-Rad PowerPac™ Basic.

Gel staining: 4–12% Bis-Tris gels were stained with InstantBlue™ Ultrafast Protein Stain or QuickBlue Protein Stain at RT for 2 h whilst being rotated on a 3D orbital shaker.

Gel imaging: Visualisation employed a Bio-Rad ChemiDoc MP Universal Hood III using Image Lab™ Software. For 4–12% Bis-Tris gels stained with InstantBlue or QuickBlue, the ‘Protein gels – Coomassie’ method was applied. For Mini-PROTEAN TGX Stain-Free gels, visualisation was achieved by applying the pre-set ‘Stain Free’ method set to ‘best sensitivity’.

6.6.5 Bacterial transformations

Preparation of agar plates: Agar (1.6% w/v) was suspended in 2TY medium (Section 6.6.2). Full dissolution was achieved by autoclaving (121 °C, 20 min). Prior to use, 2TY agar was liquified by microwave irradiation (1–2 min). The appropriate antibiotic was added after the solution was cool enough to touch.

Transformation: In a laminar flow hood, (ultra)competent *E. coli* cells (30–50 μ L) were combined with the desired vector: either full construct (1 μ L), ligation mixture (2 μ L), or PCR product (5 μ L) in a sterile 1.5 mL microfuge tube and mixed gently by flicking. The transformation mixture was incubated on ice for 5 min followed by heat shock (42 °C, 10–30 sec) according to the manufacturer's protocols: XL10 Gold, NEB 5 α , Lemo21(DE3) (30 sec); BL21 (DE3) (10 sec). The mixture was chilled on ice for 2 min, SOC medium (200–400 μ L) was added and the cells were recovered at 37 °C for 60 min with shaking. For intact constructs, cells were pelleted by centrifugation (3,500 $\times$ g, 5 min) in a benchtop centrifuge and the supernatant media (90 μ L) was removed. Cells were then resuspended by pipetting and 70 μ L of the mixture was plated on a 2TY agar plate containing the required antibiotic selection marker (50 μ g/mL). In all other cases the entire transformation mixture was plated. 2TY agar plates were incubated at 37 °C overnight to give single colonies. XL10-Gold ultracompetent cells and NEB 5 α competent cells were used for plasmid proliferation, BL21(DE3) and Lemo21(DE3) competent cells were used for protein production.

Antibiotic selection: When using Lemo21(DE3) cells, the 2TY agar was supplemented with chloramphenicol (30 μ g/mL) as a secondary antibiotic selection to maintain the pLysY plasmid containing the T7 lysozyme gene.

6.7 Recombinant Protein Production

6.7.1 Stationary phase expression trials in BL21(DE3)

A single colony of BL21(DE3) freshly transformed with the required plasmid was inoculated into 2TY media containing kanamycin (50 μ g/mL) and grown to stationary phase by incubation overnight (16 h) at 37 °C with shaking at 200 rpm. Overnight culture (1 mL/flask) was used to inoculate 100 mL of the same media and grown as before to an OD₆₀₀ = 0.6. Cultures were induced with IPTG (varied concentrations) across a range of growth conditions. When using pCold-derived constructs, cultures were placed at 4 °C for 30 min prior to the addition of IPTG. At the end of the

induction period, cells producing His₆-tagged proteins were harvested from culture (50 mL) by centrifugation (3,750 ×g, 10 min) and stored at –80 °C. For un-tagged proteins (DAOCS/DACS from pET24a(+) and ICM DAOCS), culture (10 µL) was mixed with 2× SDS Loading Buffer (1:1) and analysed by SDS-PAGE. A summary of constructs expressed using this protocol is given in Table 6.19.

Table 6.19 | List of constructs whose expression trials were carried out using the stationary phase protocols described in Section 6.7.1. A summary of the constructs used can be found in Table 6.15.

Construct
pNIC_DD
pCold_DD
pCold_AC
pCold_kanR_AC
pCold_DACS
pET_AC
pET_ICM DAOCS
pET_DAOCS

6.7.2 Log phase expression trials in BL21(DE3)

A single colony of BL21(DE3) cells that had been freshly transformed with pNIC_DD was inoculated into 2TY media (6 mL) containing kanamycin sulfate (50 µg/mL) and grown at 37 °C and 200 rpm shaking for 3 h. 2 mL of culture was used to inoculate 100 mL of the same media and grown as before to an OD₆₀₀ = 0.6 before expression was induced by the addition of IPTG across a range of growth conditions (see **Chapter 2** for specific growth conditions). Once the culture period was complete, the cells were harvested from culture (50 mL) by centrifugation (3,750 ×g, 10 min) and stored at –80 °C.

6.7.3 Expression trials of pNIC_DD in Lemo21(DE3) without L-rhamnose or glucose

A single colony of Lemo21(DE3) cells that had been freshly transformed with pNIC_DD was inoculated into 2TY media (2 mL) containing kanamycin and chloramphenicol (both 30 µg/mL) and grown for 5 h at 37 °C and 200 rpm shaking. 1 mL of culture was used to inoculate 100 mL of the

same media and grown as before to an $OD_{600} = 0.6$ before being induced with IPTG across a range of growth conditions. Cells from culture (50 mL) were harvested by centrifugation (3,750 $\times g$, 10 min) and stored at $-80\text{ }^{\circ}\text{C}$.

6.7.4 Expression trials in Lemo21(DE3) with L-rhamnose and glucose

A single colony of Lemo21(DE3) cells that had been freshly transformed with pNIC_DD was inoculated into 2TY media (2 mL) containing kanamycin and chloramphenicol (both 30 $\mu\text{g/mL}$). Another single colony of the same transformant was inoculated into the same media, supplemented with glucose (1% w/v). Cultures were grown for 2 h at $37\text{ }^{\circ}\text{C}$ and 200 rpm shaking before 200 μL was used to inoculate 10 mL of the same media containing L-rhamnose (0, 0.5, 1 or 2 mM). Main cultures were grown as before to an $OD_{600} = 0.4$ then cooled ($18\text{ }^{\circ}\text{C}$, 30 min) and induced with IPTG (0.4 mM) for 3 h at $18\text{ }^{\circ}\text{C}$ and 200 rpm shaking. Cells were harvested from culture (50 mL) by centrifugation (3,750 $\times g$, 10 min) and stored at $-80\text{ }^{\circ}\text{C}$.

6.7.5 Large-scale protein expression from pNIC_DD

A single colony of BL21(DE3) cells that had been freshly transformed with pNIC_DD was inoculated into 2TY media (100 mL) containing kanamycin (50 $\mu\text{g/mL}$) and grown at $37\text{ }^{\circ}\text{C}$ and 200 rpm shaking to an $OD_{600} = 0.4$. Culture (12 mL/flask) was used to inoculate $12 \times 600\text{ mL}$ of the same media and grown as before to an $OD_{600} = 0.4$ (4 h). Cultures were cooled at $18\text{ }^{\circ}\text{C}$ for 20 min and induced with IPTG (0.4 mM) at $18\text{ }^{\circ}\text{C}$ for 3 h. Cells were harvested by centrifugation (8,000 $\times g$, 10 min, $4\text{ }^{\circ}\text{C}$) and stored at $-80\text{ }^{\circ}\text{C}$.

6.7.6 Large-scale expression from pCold and pET constructs

A single colony of BL21(DE3) cells freshly transformed with the required construct was inoculated into 2TY media (100 mL) containing the appropriate antibiotic (50 $\mu\text{g/mL}$) and grown overnight at $37\text{ }^{\circ}\text{C}$ and shaking at 200 rpm. Overnight culture (6 mL/flask) was used to inoculate $24 \times 600\text{ mL}$ of the same media and grown as before to an $OD_{600} = 0.6$ (3 h), at which point cultures of cells harbouring pET vectors were induced with IPTG, whilst cultures of cells containing pCold plasmid were cooled at $4\text{ }^{\circ}\text{C}$ for 60 min prior to IPTG addition. At the end of the induction period, the cells

were harvested by centrifugation (8,000 ×g, 10 min, 4 °C) and pellets were stored at –80 °C. Specific induction and growth conditions for each pCold-derived construct is given in Table 6.20, and those for pET-derived constructs is given in Table 6.21.

Table 6.20 | Induction conditions for different pCold constructs. In all cases, starter cultures were grown at 37 °C and 200 rpm shaking overnight before being used to inoculate 24 × 600 mL of the same media (1:100 v/v) and grown as before to an OD₆₀₀ = 0.6. Cultures were cooled (4 °C, 60 min) then induced with IPTG.

Construct	Final IPTG conc (mM)	Post-induction temperature (°C)	Post-induction time (h)
pCold_AC	0.5	30	3
pCold_kanR_AC	0	15	24
pCold_DACS	0.1	15	24
pCold_nIDAOCS [§]	0.5	15	24

Table 6.21 | Induction conditions for pET-derived constructs. Starter cultures were grown at 37 °C and 200 rpm shaking overnight, before being used to inoculate 24 × 600 mL of the same media (1:100 v/v) and grown as before to an OD₆₀₀ = 0.6 then expression was induced.

Construct	Final IPTG conc (mM)	Post-induction temperature (°C)	Post-induction time (h)
pET_AC	0.4	25	3
pET_DAOCS	1	28	4
pET_ICM DAOCS	0.5	28	3

6.8 Batch method Nickel resin IMAC for expression trials

6.8.1 Materials and buffers

Buffer (HisTrap A and B) compositions are given in Table 6.22. All buffers were passed through 0.2 µm filters before use and stored at 4 °C. BugBuster® Protein Extraction Reagent (Cat. #70923) was purchased from Merck Millipore. Ni Sepharose High Performance resin (Code #17-5268-01) was purchased from Cytiva (previously GE Healthcare). Rotation was carried out on a Stuart SB3 Variable Speed Rotator.

[§] nIDAOCS not purified. Cell pellet currently stored at –80 °C.

Table 6.22 | Recipes for HisTrap A and B Buffers.

Buffer	HEPES (mM)	NaCl (mM)	Imidazole (mM)	pH
HisTrap A (binding)	50	500	20	7.5
HisTrap B (elution)	50	500	500	7.5

6.8.2 Chemical Lysis

Cell pellets (≈ 0.5 g) were resuspended in BugBuster® Protein Extraction Reagent (2.5 mL) by pipetting and vortexing. The resuspended pellet (1 mL) was transferred to a microfuge tube and mixed by rotation at RT (20 rpm, 20 min). Lysate was clarified by centrifugation (16,000 $\times g$, 20 min, 4 °C) and the supernatant was transferred to a microfuge tube containing the washed Ni resin. The remain cell pellet (insoluble fraction) was resuspended in HisTrap A Buffer (1 mL) then diluted 2-fold in further HisTrap A Buffer.

6.8.3 Preparation of Ni Sepharose High Performance resin

The Ni Sepharose High Performance resin (Cytiva, Code #17-5268-01) stored in EtOH (20% v/v) was resuspended by shaking and vortexing vigorously and transferred to a microfuge tube (200 μ L/lysate sample). Resin was pelleted (800 $\times g$, 1 min) and the supernatant was removed. The resin was then washed with HisTrap A Buffer (4 $\times$ 1 mL) and between each wash step the resin was pelleted (800 $\times g$, 1 min) and the supernatant removed. Following the final wash step, the resin was resuspended in HisTrap A Buffer (1:1) and distributed evenly among microfuge tubes. These tubes were centrifuged (800 $\times g$, 1 min) and the supernatant was removed. The Ni resin was then ready to be mixed with clarified lysate.

6.8.4 Purification Protocol

Clarified cell lysates (1 mL) were added to washed Ni resin (200 μ L) and the lysate-resin suspension was rotated (40 rpm, 60 min, 4 °C) to allow His₆-tagged protein to bind the Ni-resin. The resin was then settled by low-speed centrifugation (800 $\times g$, 1 min, 4 °C) and the supernatant (flowthrough) was carefully removed. The resin was washed (4 $\times$ 1 mL) with HisTrap A Buffer. Following each wash step, the resin was settled (800 $\times g$, 2 min, 4 °C) and the supernatant discarded. His₆-tagged

protein was eluted by the addition of HisTrap B Buffer (200 μ L). Samples were rotated (40 rpm, 4 °C, 15 min) and centrifuged (800 $\times$ g, 2 min, 4 °C). 10 μ L of the supernatant (elution fraction), along with 10 μ L of the insoluble fraction sample, were taken for SDS-PAGE analysis. SDS-PAGE analysis was carried out as described in **Section 6.6.4**.

6.9 Recombinant Protein Purification

6.9.1 Materials and general information

DNase I (grade II from bovine pancreas) was purchased from Merck. SIGMAFAST™ Protease Inhibitor Cocktail Tablets, EDTA-Free (Product #S8830) was purchased from Sigma-Aldrich. HisTrap HP columns (5 mL, Code #17-5247-01)) were purchased from Cytiva. Superdex 75/200 loose resins were also from Cytiva and packed into columns in-house. Centrifugal filters (10 and 30 kDa MWCO) were purchased from Amicon. BioDesignDialysis Tubing™ (3500 MWCO, 21.3 mm wet diameter, 3.57 mL/cm volume) was purchased from Fisher Scientific. TEV and HRV3C proteases were kindly donated by Dr Patrick Rabe. Pelleting of insoluble cell debris was carried out in an Avanti J-26S XP centrifuge (Beckman Coulter) using a JA 25.50 rotor.

Protein purifications were carried out at 4 °C using ÄKTA Pure FPLC systems. Buffers were passed through filters (0.2 μ m) and thoroughly de-gassed before use. The presence of target proteins in elution fractions was determined by SDS-PAGE analysis as described in **Section 6.6.4**.

6.9.2 Preparation of clarified lysates

Sonication: Cell pellets were resuspended in binding buffer (4 $\times$ v/w) supplemented with DNase I and a SIGMAFAST™ Protease Inhibitor Cocktail Tablet. Cell disruption by sonication was achieved on a Sonics Vibra-Cell™ VC 505 Ultrasonic Liquid Processor (60% amplitude, 3 sec on/off, 10 min). During the sonication, the mixture was gently swirled intermittently to help ensure the cells remained fully resuspended. The cell debris was pelleted by centrifugation (48,384 $\times$ g, 45 min, 4 °C) and clarified lysate was passed through a 0.45 μ m filter.

High pressure cell disruption: High pressure cell disruption was carried out on a Constant Systems CF1 Cell Disruptor employing a Julaba F1000 Compact Recirculation Cooler to maintain the temperature at 4 °C. Cell pellets were resuspended in binding buffer (4× v/w) supplemented with DNase I and a SIGMAFAST™ Protease Inhibitor Cocktail Tablet. The cell disruptor was washed with MQ (2 × 100 mL, 15,000 psi) and binding buffer (2 × 100 mL, 25,000 psi). Cell lysis was achieved via single pass disruption at 25,000 psi. The system was then washed with binding buffer (25 mL) at 25,000 psi which was pooled with the cell lysate. The system was then cleaned with MQ (2 × 100 mL) and 70% EtOH (2 × 100 mL) at 40,000 psi. Lysate was clarified by centrifugation (48,383 ×g, 45 min, 4 °C) and filtration (0.45 µm).

6.9.3 IMAC using a HisTrap HP column

Buffers: HEPES was preferred to Tris as a buffering system given its more stable pH profile across a range of temperatures. HisTrap A and B Buffers are the same as those described in Table 6.22. For batch method Ni resin purifications, stripping and charging buffer compositions are given in Table 6.23.

Maintenance: HisTrap columns were stripped and recharged before every purification. The protocol is as follows: MQ (5 CV), stripping buffer (5 CV), MQ (5 CV), charging buffer (5 CV), HisTrap A (5 CV), HisTrap B (5 CV, to clear unbound Ni²⁺), and HisTrap A (5 CV). Stripping and recharging buffer compositions are given in Table 6.23.

Table 6.23 | Composition of stripping and charging buffers for HisTrap columns

Buffer	Component	Concentration (mM)
Stripping	HEPES	50
	NaCl	500
	EDTA	100
Charging	NiSO ₄	100

Purification: HisTrap A Buffer = A; HisTrap B Buffer = B. The clarified lysates were loaded onto a 5 mL HisTrap HP column (1–1.5 mL/min) that had been re-equilibrated in HisTrap A buffer (5 CV).

When purifying cells lysates from 24 × 600 mL of culture, 2 × 5 mL HisTrap columns were connected in series. The column was washed (2% B, 10 CV, 1.5 mL/min, before His₆-tagged protein was eluted with an imidazole step gradient: 4% B (6 CV); 8% B (9 CV); 32% B (10 CV); 100% B (6 CV). Fractions were assessed by SDS-PAGE and those containing the target protein were pooled.

6.9.4 His₆-tag cleavage and reverse HisTrap IMAC

His₆-tag cleavage was achieved using TEV protease (pNIC_DD) or HRV3C protease (pCold_(kanR)_AC, pCold_DACS). The concentration of target protein from pooled HisTrap fractions was measured on a NanoDrop™ One Microvolume UV-Vis Spectrophotometer using the protein's MW and extinction coefficient. These data were used to calculate the mass in mg of protein. Protease was added such that the ratio of protease to target protein was 1:100 w/w. The mixture was added to dialysis tubing and dialysed overnight against low concentration imidazole buffer (50 mM HEPES, 500 mM NaCl, 10 mM imidazole, pH 7.5, 2 L). A sample (10 µL) of cleavage mixture was set aside for SDS-PAGE analysis, the remaining mixture was loaded onto a 5 mL HisTrap HP column (1 mL/min) that had been pre-equilibrated with HisTrap A Buffer (5 CV) and the flowthrough containing the un-tagged target protein was collected. The column was washed with HisTrap A buffer (5 CV, 1 mL/min) and eluted with HisTrap B Buffer, 3 CV, 1 mL/min). Fractions were analysed by SDS-PAGE and those containing un-tagged target protein (typically flowthrough and washes 1–2) were pooled.

6.9.5 Anion-exchange chromatography

Buffers: Buffer compositions used are given in Table 6.24. All buffers were thoroughly degassed before use. DTT was added to the buffers immediately prior to use to limit its oxidation during buffer storage.

Table 6.24 | Composition of buffers used for anion-exchange chromatography.

Buffer	HEPES (mM)	DTT (mM)	EDTA (mM)	NaCl (mM)	pH
Buffer A	50	1	1	-	8.0
Buffer B	50	1	1	500	8.0

Maintenance: A QSepharose FastFlow (FF) column (50 mL) was washed prior to use with the following protocol: MQ (5 CV); NaOH (1 M, 5 CV); MQ (5 CV); NaCl (1 M, 5 CV); Buffer A (5 CV). All steps were performed at 4 mL/min.

Purification: The clarified cell lysates were loaded onto a 50 mL QSepharose FF column (1 mL/min) pre-equilibrated with Buffer A. The column was washed with Buffer (10 CV, 4 mL/min) then eluted with a NaCl gradient (0–500 mM, 15 CV, 4 mL/min). Collected fractions were analysed by SDS-PAGE and those containing the target protein were pooled.

6.9.6 Preparation of apoenzyme

Following reverse HisTrap purification, the pooled fractions were loaded into dialysis tubing and buffer exchanged against chelation buffer (25 mM HEPES, 30 mM EDTA, 5 mM 1,10-phenanthroline, pH 7.5) 2 × 1 L buffer exchanges were performed, with buffer refreshed after 3 h.

6.9.7 Size-exclusion chromatography (SEC)

Buffers: SEC Buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) was stirred in the presence of Chelex 100 resin (BioRad) for 1 h to remove divalent metals. The mixture was then filtered (0.2 µm) and thoroughly degassed before being stored at 4 °C prior to use. All other buffers were also thoroughly degassed prior to use.

Maintenance: Prior to use, a Superdex S75 column (300 mL) was washed with MQ water (1 CV, 1 mL/min), NaOH (1 M, 1 CV, 1 mL/min) then MQ water (1 CV, 1 mL/min) before equilibration with SEC Buffer (1 CV, 1 mL/min).

Purification: Following anion-exchange or reverse HisTrap (affinity) chromatography and dialysis against chelation buffer, protein was concentrated in a 10 kDa MWCO centrifugal filter (4,000 ×g, 4 °C) to ≈6 mL (2% of total column volume). The protein sample was loaded via a 10 mL Superloop™ (0.5 mL/min) pre-equilibrated in SEC Buffer and eluted with isocratic flow of SEC Buffer (1 mL/min). Fractions showing absorbance at 280nm were analysed by SDS-PAGE and those containing sufficient amounts of the target protein as judged by SD-PAGE analysis were pooled.

6.9.8 Protein concentration and storage

Chosen fractions containing the target protein were pooled and concentrated using a 10 kDa MWCO centrifugal filter (Fisher Scientific) (4,000 ×g, 4 °C) to the required concentration as determining by evaluation on a NanoDrop™ One Microvolume UV-Vis Spectrophotometer. Protein was aliquoted (50/200 µL) into 0.5 mL microfuge tubes, flash-frozen in liquid nitrogen and stored at –80 °C. A summary of the purification workflows for all constructs described herein is presented in Table 6.25.

Table 6.25 | Summary of purification workflows for DAOCS/DACS, DACS, DAOCS, and ICM DAOCS produced from different constructs.

Protein	Construct	Purification workflow
DAOCS/DACS	pCold_(kanR)_AC	HisTrap His ₆ -cleavage (HRV3C) Reverse HisTrap Dialysis SEC
	pET_AC	Anion-exchange Dialysis SEC
DACS	pCold_DACS	HisTrap His ₆ -cleavage (HRV3C) Reverse HisTrap Dialysis SEC
DAOCS	pET_DAOCS	Anion-exchange SEC
ICM DAOCS	pET_ICM DAOCS	Anion-exchange Dialysis SEC

6.10 General procedure for incubations with enzymes

Assays (100 µL scale) were carried out in 1.5 mL microfuge tubes. The enzyme solution was buffer-exchanged into assay buffer (50 mM Tris-*d*₁₁/Tris/HEPES/NH₄HCO₃, pH 7.5) using a Micro Bio-Spin 6 column (Bio-Rad) according to the manufacturer's protocols. Cofactors were prepared as 50× stocks in MQ purified water. (NH₄)Fe(SO₄)₂·7H₂O and FeSO₄·7H₂O were prepared as 500× stocks in 20 mM HCl then diluted 10-fold in MQ. All cofactors used in the assays were combined in a master mix. The buffer-exchanged enzyme was pre-incubated with the master mix at 30 °C and 300 rpm

for 2 min. The β -lactam substrate solution (1 mM final concentration) was then added and the assays were incubated at 30 °C and 300 rpm shaking for 2 h in a Thermomixer-Mixer HC (Starlab™, Art. #S8012-0000). At the end of the incubation period, assays were quenched by the addition of 1,10-phenanthroline in MeCN (2.5 mM, 200 μ L). Samples were vortexed thoroughly to ensure the precipitation of all enzyme, then centrifuged (16,000 $\times$ g, 5 min). The supernatant was flash-frozen in liquid-nitrogen and lyophilised overnight.

6.10.1 Optimised assays conditions

Incubations with penicillin N or DAOC, conditions: 20 μ M enzyme (DAOCS, DACS or DAOCS/DACS), 0.2 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.4 mM sodium-L-ascorbate, 2 mM α -ketoglutarate disodium salt, 2 mM substrate (penicillin N or DAOC).

In cases when DAOCS and DACS were combined in one assay, both enzymes were added at 20 μ M.

Incubations with penicillin G, conditions: 0.2 mM enzyme (ICM DAOCS or DAOCS/DACS), 0.6 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 2.4 mM sodium-L-ascorbate, 8 mM α -ketoglutarate disodium salt, 2 mM penicillin G.

Incubations with exomethylene cepham G, conditions: 0.2 mM enzyme (ICM DAOCS or DAOCS/DACS), 0.6 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 2.4 mM sodium-L-ascorbate, 4 mM α -ketoglutarate disodium salt, 0.5 mM sodium azide, 1 mM exomethylene cepham G.

DAOCS/DACS incubations with 3-vinyl cephem under the original 1990 conditions:¹⁰⁶ 100 μ M DAOCS/DACS, 1.68 mM dithiothreitol (DTT), 0.83 mM L-ascorbic acid, 45 μ M iron(II) sulfate heptahydrate, 0.85 mM α -ketoglutaric acid, 42 mM ammonium sulfate, 1 mM 3-vinyl cephem.

DAOCS/DACS incubation with 3-vinyl cephem; optimised conditions: 0.2 mM DAOCS/DACS, 0.6 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 2.4 mM sodium-L-ascorbate, 4 mM α -ketoglutarate disodium salt, 1 mM 3-vinyl cephem ($\pm$ 0.5/5 mM sodium azide)

When sodium azide was added to assays it was typically at 0.5 mM. At higher concentrations (5 mM), only 0.1 mM DAOCS/DACS was required for full turnover (all other conditions that same).

6.10.2 Large-scale incubations of 3-vinyl cephem and DAOCS/DACS

In total, 2.5 mg of 3-vinyl cephem substrate was converted, split across 2×1 mg and 1×0.5 mg assays. Assay conditions were the same as those described above (**Section 6.10.1**): 0.2 mM DAOCS/DACS, 0.6 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 2.4 mM sodium-L-ascorbate, 4 mM α -ketoglutarate disodium salt, 0.5 mM sodium azide, 1 mM 3-vinyl cephem; 50 mM Tris- d_{11} (pH 7.4). Each of the three assays was performed in a 50 mL Falcon Tubes and incubated in a New Brunswick Innova 44 Incubator Shaker (30 °C, 180 rpm). Cofactor and cosubstrate solutions were prepared as a master mix. Oxygen was bubbled through the master mix and buffer solutions before their addition to the incubation mixture. Assays were monitored by LC-MS (see below). Once complete consumption of the 3-vinyl cephem was observed by LC-MS, the assays were quenched with 1,10-phenanthroline in MeCN (2.5 mM, $2 \times$ assay volume). The quenched assays were centrifuged (4,200 $\times g$, 5 min) and the supernatant was lyophilised overnight. Purification by HPLC (Water system, monitoring at λ 280 nm) on a C_{18} column (SunFire, 5 μm , 150×10 mm id) eluting with 10 mM ammonium bicarbonate and 2% MeCN, gave the cephem hydroxy azide (retention time: 15.0 min).

Real-time monitoring by LC-MS An assay aliquot (10 μL) was quenched with 1,10-phenanthroline in MeCN (2.5 mM, 20 μL) The precipitated protein was removed by centrifugation (16,000 $\times g$, 2 min) and the supernatant (10 μL) was diluted in MilliQ water (190 μL) ready for LC-MS analysis.

6.10.3 ^{18}O experiments

Assays were performed on a 100 μL scale in 50 mM Tris- d_{11} (pH 7.4). Assays were prepared anaerobically as described below in 0.5 mL microcentrifuge tubes. For the H_2O^{18} experiments, 50 mM Tris- d_{11} (pH 7.4) buffer was prepared in H_2O^{18} water which was used to make cofactor and

substrate stock solutions. DAOCS/DACS was also buffer-exchanged into this buffer. For $^{16}\text{O}_2$ and $^{18}\text{O}_2$ assays in H_2O^{16} , normal 50mM Tris- d_{11} (pH 7.4) buffer was used.

Microcentrifuge tubes were placed in a two-necked pear-shaped flask. One neck of the flask was sealed with a septum cap and the other neck was fitted with a 3 mm J Young adapter. Reactions were initiated by oxygen exposure (either $^{16}\text{O}_2$ or $^{18}\text{O}_2$) and the assays were incubated at 30 °C and 150 rpm for 2 h. Assays were quenched with 2.5 mM phenanthroline in MeCN (200 μL) added via the septum cap. Once quenched, microcentrifuge tubes were removed from the pear-shaped flasks and centrifuged (14,000 $\times g$, 2 min). The supernatant was frozen in LN_2 and lyophilised overnight.

DAOCS/DACS incubations with 3-vinyl cephem, conditions optimised herein: 0.1 mM DAOCS/DACS, 0.6 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 2.4 mM sodium-L-ascorbate, 4 mM α -ketoglutarate disodium salt, 1 mM 3-vinyl cephem ($\pm$ 0.5/5 mM sodium azide).

6.10.4 ^1H NMR and LC-MS analysis

Lyophilised assays were re-dissolved in D_2O (165 μL). LC-MS samples were prepared by diluting 5 μL of sample in 195 μL of MQ. The remaining 160 μL was used for ^1H NMR analysis.

^1H NMR analyses: A sample (160 μL) was transferred to a three-millimetre MATCH tube using a Gel Loading Tip in 2 aliquots. Liquid was moved to the bottom of the tube by flicking and bubbles were removed on a hand-powered centrifuge. ^1H NMR spectra were recorded using a Bruker AVIII 700 MHz NMR spectrometer equipped with an inverse 5 mm $\text{TCI}^1\text{H}/^{13}\text{C}/^{15}\text{N}$ cryoprobe optimised for ^1H observation. Typical spectra settings are as follows (NS: 256, relaxation delay: 2 s). Water suppression was achieved by the 1D NOESY pre-saturation method. The appearance/disappearance of apparent doublet peaks ($J \approx 4.5$ Hz) within the 5–6 ppm range was analysed. Turnover was calculated using peak integration values determined on MestReNova.

LC-MS analysis: High-performance liquid chromatography (HPLC)-grade solvents (water, acetonitrile, formic acid) were purchased from Sigma-Aldrich. LC-MS analyses were carried out using an Acquity UPLC system coupled to a Xevo G2-XS or G2-S Q-TOF mass spectrometer.

Samples were injected (1–2 μ L) using an Agilent 1290 series autosampler onto an ACE Equivalence 3 C18 column (50 $\times$ 2.1 mm, Avantor™). Elution was achieved at 0.25 mL/min using H₂O and MeCN as solvents, both supplemented with formic acid (0.1% v/v). The elution gradient was as follows:

Method 1: 1% (1–3 min), 1–25% (3–4 min), 25–95% (4–5 min), 95% (5–5.70 min), 95–1% (5.70–6.10 min), 1% (6.10–7.50 min).

Method 2: 1% (1–3 min), 1–25% (3–5 min), 25–95% (5–6 min), 95% (6–6.70 min), 95–1% (6.70–7.10 min), 1% (7.10–8.50 min).

Method 3: 1% (1–3 min), 1–35% (3–4 min), 35–95% (4–5 min), 95% (5–5.70 min), 95–1% (5.70–6.10 min), 1% (6.10–7.50 min).

Method selection depended on the exact elution times on penicillin N and DAOC, which were observed to drift by up to 1 min.

Raw files were processed using MassLynx MS software (Waters). Data (chromatograms and mass spectra) were ‘copied as lists’ into GraphPad Prism 9 for preparation of figures. In order to calculate turnover, peak integrations were performed on mass-extracted chromatograms for starting material and products using MassLynx MS software.

6.11 Protein LC-MS

This method was used to analyse proteins following FPLC purification to confirm their purity and that their observed masses were consistent with the predicted masses.

6.11.1 Materials

High-performance liquid chromatography (HPLC)-grade solvents (water, acetonitrile, formic acid) were purchased from Sigma-Aldrich. LC-MS analyses were carried out using an Acquity UPLC system coupled to a Xevo G2-XS or G2-S Q-TOF mass spectrometer.

6.11.2 Method

Protein samples (200 μ L, 0.5 μ M), prepared in MQ and stored at 4 °C, were injected (2 μ L) using an Agilent 1290 series autosampler onto a ProSwift RP-4H column (50 $\times$ 1 mm). Proteins were eluted at 0.25 mL/min using H₂O and MeCN as solvents, both supplemented with formic acid (0.1% v/v). The elution gradient was as follows: 5–95% (0–10 min), 95% (10–12 min), 95–5% (13–15 min), 5% (13–15 min). Raw files were processed using MassLynx MS software (Waters). Protein mass spectra was obtained by applying the Maximum Entropy Deconvolution algorithm ('MaxEnt 1') to the mass spectrum. Data (chromatograms and mass spectra) were transferred into GraphPad Prism 9 for preparation of figures.

6.12 Carr-Purcell-Meiboom-Gill (CPMG) NMR experiments

NMR analysis was performed by applying the PROJECT-CPMG sequence¹⁸⁷ with the following experimental parameters: total echo time, 40 ms, relaxation delay 2 s. 1D NOESY pre-saturation was used to achieve water suppression. Spectra were generated using a Bruker Avance III HD Console 950 MHz spectrometer equipped with a 5 mm TCI cryoprobe, also from Bruker. The magnets were supplied by Oxford Instruments Co.

Assay solutions (180 μ L final volume, 10% D₂O) were prepared aerobically in a 1.5 mL microcentrifuge tube with the following concentrations: 0.1 mM DAOCS/DACS, 0.3 mM (NH₄)Fe(SO₄)₂·7H₂O, 2.4 mM sodium-L-ascorbate, 4 mM α -ketoglutarate disodium salt, 1 mM 3-vinyl cephem. Substrate turnover was stimulated by bubbling oxygen through the assay solution. Intermittently, the solution was centrifuged briefly to remove bubbles. After $\approx$ 1 min of oxygen

exposure, the sample was centrifuged one last time and transferred to a 3 mm NMR tube ready for analysis.

6.13 UV-Vis spectroscopy

6.13.1 Materials

Samples for UV-Vis spectroscopy were prepared under anaerobic conditions in a glovebox (Belle Technologies ($O_2 < 2\text{ppm}$)). All buffers and compounds were similarly prepared to exclude dioxygen. UV-Vis spectra were obtained for the apoenzyme and after the addition of each component. Measurements were taken in an Agilent Ultra-micro cuvette ($2.5 \times 2\text{ mm}$ aperture, 10 mm pathlength, 70 μL fill volume, z height 15 mm).

Spectra were collected using an Agilent Cary 3500 UV-Vis Compact Peltier machine. Components were added to the sample inside the glovebox. The cuvette was stoppered with a Suba-Seal (Sigma) to prevent oxygen exposure of the sample when it was removed from the glovebox for measurement. The enzyme solutions were freshly buffer exchanged into 50 mM HEPES (pH 7.5) using a PD Spintrap™ G25 (Cytiva, 28-9180-04) according to the manufacturer's protocol. The components were added in the order they are listed.

6.13.2 Cofactor concentrations for each complex

DAOCS/DACS | Fe | ascorbate | NaN_3 | 3-VC: Samples (90 μL total volume) contained 0.3 mM DAOCS/DACS, 0.32 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 4.2 mM succinic acid, 67.2 mM sodium azide, 0.6 mM 3-vinyl cephem in 50 mM HEPES (pH 7.5).

DAOCS/DACS | Fe | 2OG | NaN_3 | 3-VC: Samples (90 μL total volume) contained 0.3 mM DAOCS/DACS, 0.3 mM $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 3.75 mM 2-oxoglutarate disodium salt, 60 mM sodium azide, 1.5 mM 3-vinyl cephem in 50 mM HEPES (pH 7.5).

6.14 Crystallography

6.14.1 Materials

Crystal screens were carried out in SwisSci 3 Lens low-profile (LP) 96-well sitting drop plates. Plates were sealed with StartSeal Advances Polyolefin Film (STARLAB, Lot #2020-04-SW). Crystallisation drops were set-up using a Mosquito® Xtal3 machine (SPT Labtech). 8-well 5 µL and 2 µL micro-reservoir strips for use with the Mosquito® machine were purchased from SPT Labtech. Broad crystallisation screens were purchased from the relevant commercial suppliers. The broad screen mixtures were transferred to SwisSci LP plates using an VIAFLO 384 Handheld Electronic Pipette (Integra).

6.14.2 ICM DAOCS broad screen set-up protocols

A solution of ICM DAOCS (various concentrations) in storage buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) was thawed on ice (55 µL/crystal plate). To this solution were added MnCl₂·4H₂O, α-ketoglutarate disodium salt and penicillin G. Final concentrations for each attempted screening round are given in Table 6.26. After the addition of each component, the solution was mixed well by pipetting. After the addition of penicillin G sodium salt, mixtures were incubated on ice (20 min) then centrifuged (21,000 ×g, 10 min, 4 °C). The supernatant was transferred to crystal broad screen mixtures on SwisSci LP 96-well plates in 2:1, 1:1, 1:2 ratios of protein/well solution using a Mosquito® Xtal3 machine. The plates were sealed and stored at room temperature (approx. 22 °C) and observed from day 2 onwards.

Table 6.26 | Conditions for ICM DAOCS broad screen experiments.

Round	Components (concentrations)
1	ICM DAOCS (0.7 mM): Mn (2 mM): 2OG (4 mM): PenG (8 mM)
2	ICM DAOCS (0.7 mM): Mn (2 mM): 2OG (4 mM): PenG (8 mM)
3	ICM DAOCS (0.35 mM): Mn (1 mM): 2OG (2 mM): PenG (2 mM)

Table 6.27 | Crystal broad screens used for ICM DAOCS and their commercial suppliers.

Name	Supplier
NeXtal Cation	NeXtal
NeXtal Anion	NeXtal
JCSG Plus	Molecular Dimensions
Pact Premier	Molecular Dimensions
Index 1 + 2	Hampton Research
Matrix 1 + 2	Hampton Research
BCS	Molecular Dimensions
Salt Rx	Hampton Research
SG1	Molecular Dimensions
LMB	Molecular Dimensions
Morpheus	Molecular Dimension

6.14.3 DAOCS/DACS broad screen protocols

A solution of DAOCS/DACS (15 or 20 mg/mL) in storage buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) were thawed on ice (55 µL/crystal plates). To the solution was added the following: ZnSO₄·7H₂O (50 mM), 2-oxoglutaric acid (100 mM), penicillin N (100 mM). Final concentrations: 2.5 mM Zn, 10 mM 2OG, 5 mM penicillin N. The solution was mixed thoroughly by pipetting then incubated on ice (20 min). Samples were centrifuged (21,000 ×g, 10 min, 4 °C) and then plated on 96-well plates containing broad screen conditions. Plates were sealed and stored at either room temperature (approx. 22 °C) or at 4 °C.

6.14.4 Preparation of seed stocks

Well solution (5 µL) was added to the drop containing crystals to be used for microseeding. The resuspended crystals were transferred to a 1.5 mL microcentrifuge tube containing well solution (65 µL) and a Seed Bead (Hampton Research). The microcentrifuge tube was vortexed for 90 sec then chilled on ice, ready for use. For longer term storage, the seed stock was aliquoted into 0.5 mL microcentrifuge tubes and stored at –80 °C.

6.14.5 Broad screen plate set-up with IPNS cross-seeding

A solution of ICM DAOCS (12 mg/mL, 0.35 mM) or DAOCS/DACS (10 mg/mL, 0.27 mM) in storage buffer (50 mM HEPES, 100 mM NaCl, pH 7.5) was thawed on ice (55 μ L/crystal plate). To this enzyme solution was added $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (100 $\times$), α -ketoglutarate disodium salt (100 $\times$) and DAOC (25 $\times$). Final concentrations: $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (2 mM), α -ketoglutarate (4 mM), DAOC (4 mM). After the addition of each component, the solution was mixed thoroughly by pipetting to minimize precipitation. The solution was incubated on ice (20 min) then centrifuged (21,000 $\times g$, 10 min, 4 $^{\circ}\text{C}$). Simultaneously, IPNS crystal seed stock, kindly donated by Dr Patrick Rabe, was diluted 1000 $\times$ in its storage buffer (1.7 M Li_2SO_4 , 0.1 M Tris, pH 8.3). The protein solution and the IPNS seeds were combined in 96-well plates containing broad screen conditions in 2:1, 1:1, 1:2 ratios of protein/well solution using a Mosquito[®] Xtal3. The plates were then sealed and stored at room temperature (approx. 22 $^{\circ}\text{C}$). Plates were observed from day 2 onwards.

Table 6.28 | Commercial broad screens used for IPNS cross-seeding experiments.

Name	Supplier
NeXtal Cation	NeXtal
NeXtal Anion	NeXtal
JCSG Plus	Molecular Dimensions
Pact Premier	Molecular Dimensions
Salt Rx	Hampton Research
BCS	Molecular Dimensions
Morpheus	Molecular Dimensions

Chapter 7

Synthetic methods

7 Synthetic methods

7.1 General information

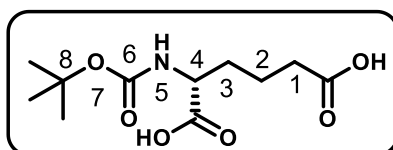
Commercial reagents and solvents were from Merck, Alfa Aesar (Karlsruhe, Germany), Sigma-Aldrich (Dorset, UK), Acros Chemicals (Loughborough, UK) or Flourochem (Glossop, UK) and were used as received. Air- and moisture-sensitive transformations were performed under a positive pressure of argon in oven-dried glassware. Dried solvents were from Aldrich (Dorset, UK). TLC monitoring of reactions was performed on aluminium sheets coated with Merck Kieselgel 60 F254 (230–400 mesh) silica. TLC plates were visualised under ultra-violet light (254 nm) and/or stained with ninhydrin or permanganate. Flash column chromatography was performed using a Biotage® Isolera systems in combination with a Sfär C₁₈ HC Duo cartridge (5 g, 10 g, 25 g, 50 g, or 100 g). HPLC purifications were performed on a Shimadzu HPLC machine furnished with a CBM-20A control module, two LC20-AR pumps, and an SPD20-A UV/Vis detector. The HPLC machine was equipped with a Waters SunFire C₁₈ Prep column (5 μ M, 150 $\times$ 10 mm i.d.).

¹H and ¹³C NMR spectra were obtained using Bruker AVIII 700 MHz or Bruker AV 400 MHz spectrometers in CDCl₃, DMSO-*d*₆, or D₂O. Residual solvent peaks were used as internal standards. Chemical shifts are reported in ppm from SiMe₄ with resonances recorded as singlet (s), doublet (d), triplet (t), quarter (q) and multiplet (m). Coupling constants (*J*) are reported in Hz. Electrospray Ionisation (ESI) Mass Spectrometry (MS) data (*m/z*) were obtained using an Agilent 1200 series LC machine and an Agilent 6120 Quadrupole MS. High resolution MS data was obtained using a Bruker MicroTOF instrument with an ESI source and Time of Flight (TOF) analyser. NMR spectra were process using MestReNova software.

The specific rotations of chiral molecules were measured using a Schmidt-Haensch UniPol polarimeter. Melting points were assessed using a Stuart® SMP40 automatic melting point instrument. Infrared spectra were obtained using a Bruker Tensor27 FTIR spectrometer.

7.2 Synthesis of the 3-Vinyl cephem

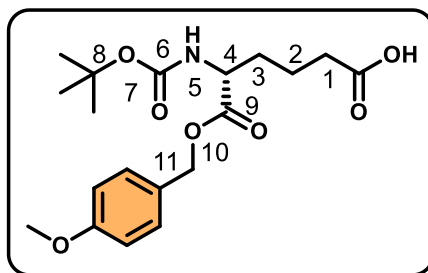
7.2.1 (*R*)-2-((*tert*-butoxycarbonyl)amino)hexanedioic acid



The product was prepared according to the reported procedure.¹⁶³ (*R*)-2-Aminohexanedioic acid (2.5 g; 15.5 mmol) and NaOH (1.6 g; 38.8 mmol) were dissolved in a 1:1 v/v mixture of H₂O/*t*-BuOH (15 mL). Di-*tert*-butyl dicarbonate (3.2 g; 14.7 mmol) was added and the solution was stirred at 25 °C for 16 h. The reaction mixture was then acidified to pH 3 using 1 M aq HCl before EtOAc (10 mL) was added. The layers were separated. The aqueous layer was extracted with ethyl acetate (3 × 30 mL). The combined organic extracts were washed with brine (50 mL), dried (MgSO₄), filtered, and concentrated *in vacuo* to afford the desired product as a white crystalline solid (3.6 g; 89%).

$[\alpha]_D^{25} + 0.35$ (c. 1 in MeOH) (lit.,²⁶ +10.1); $\nu_{\max}$ / cm⁻¹: 3342 (N-H), 2938 (broad, O-H), 1697 (C=O); ¹H NMR (400 MHz, DMSO-*d*₆) δ 12.24 (s, 2H, CO₂H), 7.06 (d, *J* = 8.0 Hz, 1H, N5-H), 3.91–3.79 (m, 1H, C4-H), 2.26–2.11 (m, 2H, C1-H₂), 1.72–1.60 (m, 1H, C2-H), 1.60–1.46 (m, 3H, C2/3-H), 1.37 (s, 9H, (CH₃)₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 174.64 (CO₂H), 174.49 (CO₂H), 156.04 (urethane-CO), 78.45 ((CH₃)₃C-8), 53.65 (C4), 33.62 (C1), 30.65 (C3), 28.68 (CH₃)₃, 21.71 (C2); ESI-HRMS [M+Na]⁺ calculated for [C₁₁H₁₉N₁NaO₆]⁺: 284.11046, found: 284.11050.

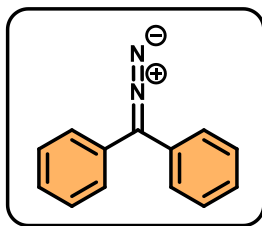
7.2.2 (R)-5-((*tert*-butoxycarbonyl)amino)-6-((4-methoxybenzyl)oxy)-6-oxohexanoic acid



The product was prepared according to the reported procedure.¹⁶⁴ **7.2.1** (3.6 g; 13.7 mmol) was transferred into an oven-dried round-bottomed flask and the headspace was purged with N₂. Anhydrous DMF (10 mL) was then added and the reaction mixture was stirred until **7.2.1** had completely dissolved. Triethylamine (2.0 mL; 14.9 mmol) and 4-methoxybenzyl chloride (2.0 mL; 15.1 mmol) were added and the resultant solution was left stirring at 36 °C for 12 h. Next, ethyl acetate (50 mL) was added and the organic phase was washed with a 1:1 mixture of H₂O/NH₄Cl (6 × 50 mL), dried (Na₂SO₄) and filtered. Volatiles were then removed *in vacuo* to give a crude product as a pale-yellow solid, which was purified by flash chromatography [SiO₂, Buffer A: ethyl acetate (1% formic acid) Buffer B: *n*-hexane; 2 CV 0%, 20 CV 0-30%, 2 CV 30%]. The desired product **7.2.2** was isolated as a colourless oil (2.2 g, 42%), which crystallised as a white solid on standing over several days at -20 °C.

Mp: 80–86 °C (from CH₂Cl₂ DCM); $[\alpha]_D^{25} + 0.43$ (c. 1.8, DCM); $\nu_{\max} / \text{cm}^{-1}$: 2975 (broad, O-H), 1711 (C=O, ester), 1613 (C=O, acid); ¹H NMR (700 MHz, DMSO-*d*₆) δ 8.48 (s, 1H, N5-H), 7.74 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.34 (d, *J* = 8.5 Hz, 2H, Ar-H), 6.43 (s, 1H, CO₂H), 5.66–5.47 (m, 2H, C11-H₂), 4.85–4.72 (m, 1H, C4-H), 4.26 (s, 3H, O-CH₃), 2.84–2.74 (m, 2H, C1-H₂), 2.37–2.23 (m, 1H, C2-H), 2.22–2.06 (m, 3H, C2/3-H), 1.88 (s, 9H, (CH₃)₃); ¹³C NMR (126 MHz, CDCl₃) δ 178.00 (CO₂H), 172.51 (CO₂H), 159.80 (*para*-C), 155.44 (urethane-CO), 130.19 (*ortho*-C), 127.44 (*ipso*-C), 114.00 (*meta*-C), 80.05 ((CH₃)₃C), 67.06 (C11), 55.30 (OCH₃), 53.20 (C4), 33.18 (C1), 31.94 (C3), 28.32 ((CH₃)₃), 20.40 (C2); ESI-HRMS [M+Na]⁺ calculated for [C₁₉H₂₇NNaO₇]⁺: 404.16797, found: 404.16852.

7.2.3 Diphenyldiazomethane



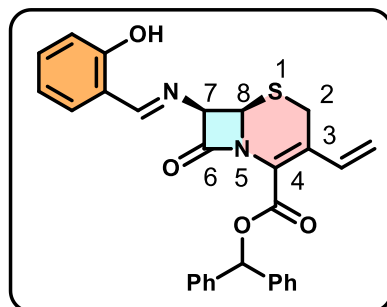
7.2.3.1 Method A

The product was prepared according to the reported procedure.¹¹⁵ To a suspension of mercury(II) oxide (13.8 g; 63.7 mmol) in Et₂O (100 mL) was added Na₂SO₄ (5.79 g; 40.8 mmol) and a saturated solution of KOH in ethanol (2 mL). Next, benzophenone hydrazone (5 g; 25.5 mmol) was added, and the colour changed from orange to dark brown. The reaction vessel was completely covered in aluminium foil and left stirring for 90 min at room temperature. Precipitate was then removed by filtration and the filtrate concentrated *in vacuo* to give **7.2.3** (4.3 g; 86%) as a purple oil, which crystallised upon standing overnight at –20 °C.

7.2.3.2 Method B

The product was prepared according to the previously reported procedure.¹⁷¹ Benzophenone hydrazone (5 g; 25 mmol) and MgSO₄ (2.5 g) were dissolved in CH₂Cl₂ DCM (64 mL), and the resultant solution was cooled to 0 °C. MnO₂ (4.76 g; 54 mmol) was then added, and the reaction mixture was warmed up to 25 °C and stirred overnight. The precipitate was removed by filtration and the filtrate concentrated *in vacuo* to afford **7.2.3** (4.99 g; quant.) as a purple crystalline solid which was stored in the dark, under argon at –20 °C and used without further purification.

7.2.4 Benzhydryl (7*R*)-7-(((*E*)-2-hydroxybenzylidene)amino)-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate



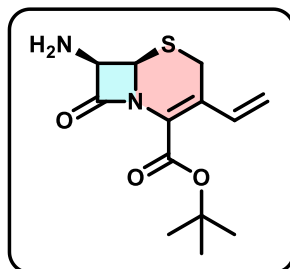
The product was prepared according to the reported procedure.¹⁶² To a stirred suspension of 7-amino-3-vinyl-3-cephem-4-carboxylic acid (7-AVCA) (200 mg; 0.89 mmol) in a 1:2 mixture of MeOH/H₂O (5 mL) was added 2-hydroxybenzaldehyde (140 mg; 1.2 mmol). The reaction mixture was then stirred at 25 °C for 1 h, before it was acidified to pH 4–5 with 1 M aq. HCl. Diphenyldiazomethane **7.2.3** (206 mg; 1.1 mmol) was dissolved in ethyl acetate (2.5 mL) and added to the reaction mixture, which was then left stirring at 25 °C for 1 h. Ethyl acetate (15 mL) was added and the layers were separated. The organic phase was washed with brine (15 mL), dried (MgSO₄), filtered and solvent removed *in vacuo* to give a crude product as a brown solid. Purification by flash chromatography [SiO₂, Buffer A: ethyl acetate (1% FA) Buffer B: *n*-hexane; 3 CV 0%, 20 CV 0–25%, 3 CV 25%] afforded **7.2.4** as a white solid (42 mg, 10%).

Mp: 174.7–177.5 °C (from DCM)(lit., 146–149 °C¹⁰⁶; lit., 180.5–182 °C¹⁶²); $[\alpha]_D^{25}$ -22.4 (c. 0.97, CDCl₃) (lit., -218.9)**; $\nu_{\max}$ / cm⁻¹: 3061 (C-H, ar.), 1779 (C=O, β -lactam), 1721 (C=O, ester), 1624 (C=C); ¹H NMR (400 MHz, CDCl₃) δ 8.65 (d, *J* = 1.5 Hz, 1H, -OH), 7.52–7.27 (m, 13H, aromatic-CH), 7.10–6.81 (m, 4H, aromatic-CH, (Ph)₂CH and HC=N, CH=CH₂), 5.47 (d, *J* = 17.5 Hz, 1H, *trans*-CH=CH₂), 5.36 (d, *J* = 5.5 Hz, 1H, C7-H), 5.28 (d, *J* = 11.0 Hz, 1H, *cis*-CH=CH₂), 5.19 (d, *J* = 5.5 Hz, 1H, C6-H), 3.67, 3.56 (ABq, *J* = 17.5 Hz, 2H, C2-H₂); ¹³C NMR (126 MHz, CDCl₃) δ 169.14 (C4'-CO₂H), 163.11 (C6O), 161.40 (C=N), 161.02 (*ar. ortho*-C-OH), 139.54, 139.34, 133.70, 132.61, 132.15, 128.70, 127.93, 127.26, 124.52, 119.25, 118.63, 117.49 (*aromatic*-C and *alkene*-C), 118.06 (CH=CH₂), 79.58 (CHPh₂), 74.11

** Literature measurement taken at 0 °C.

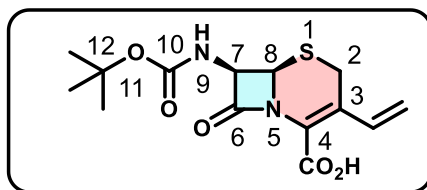
(C7), 57.54 (C6), 24.55 (C2); ESI-HRMS $[M+Na]^+$ calculated for $[C_{29}H_{24}N_2NaO_4S]^+$: 519.13490, found: 519.13507.

7.2.5 *tert*-Butyl (7*R*)-7-amino-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate



The desired product was prepared according to a modified form of the previously reported procedure.¹⁶⁸ 7-AVCA (100 mg, 0.44 mmol) was suspended in *tert*-butyl acetate (*t*-BuOAc) (2.2 mL, 16.32 mmol) and the reaction vessel was flushed with N₂ while the mixture was cooled to 0 °C in an ice-bath. Next, Triflic acid (TfOH) (39.1 µL, 0.44 mmol) was added dropwise over 2 min using a syringe. The reaction mixture was then allowed to warm-up to room temperature and stirred for 4 h. The reaction mixture was then quenched by slowly pouring it into a mixture of ethyl acetate (1.2 mL) and 3 M aq. NaOH (2.4 mL) under salt-ice bath cooling. The organic phase was isolated from the reaction mixture. The organic phase was washed with water (5 mL) and brine (5 mL). The combined organic extracts were dried (Na₂SO₄), filtered, then concentrated *in vacuo* to give a yellow solid. ¹H NMR analysis of the crude product revealed the loss of the vinyl group proton signals. The product was discharged and alternative synthetic methods were explored.

7.2.6 (7*R*)-7-((*tert*-butoxycarbonyl)amino)-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid



7.2.6.1 Method A

The reported procedure was followed.²⁴⁸ 7-AVCA (200 mg; 0.89 mmol) was suspended in saturated NaHCO₃ (10 mL). Di-*tert*-butyl dicarbonate (289 mg; 1.3 mmol) dissolved in dioxane (10 mL) was then added dropwise over 15 min, after which the reaction mixture was left stirring at 25 °C for 16 h. H₂O (15 mL) was added and the layers were separated. The aqueous layer was washed with ethyl acetate (3 × 15 mL). Acidification of the aqueous phase to pH 2 with 1 M aq. HCl gave a cloudy white solution, which was extracted with ethyl acetate (3 × 15 mL). The combined organic phases were dried (MgSO₄), filtered, then concentrated *in vacuo* to afford the desired product as a brown crystalline solid (94 mg; 33%).

$[\alpha]_D^{25}$ -1.70 (c. 2.4, CH₂Cl₂); $\nu_{\max}$ / cm⁻¹: 2976 (broad, O-H), 1774 (C=O, β -lactam), 1706 (C=O, acid), 1526 (C=C). ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.02 (d, *J* = 9.0 Hz, 1H, N9-H), 6.92 (dd, *J* = 17.5, 11.0 Hz, 1H, CH=CH₂), 5.59 (d, *J* = 17.5 Hz, 1H, *trans*-CH=CH₂), 5.45 (dd, *J* = 9.0, 5.0 Hz, 1H, C7-H), 5.31 (d, *J* = 11.5 Hz, 1H, *cis*-CH=CH₂), 5.09 (d, *J* = 5.0 Hz, 1H, C6-H), 3.82, 3.54 (ABq, *J* = 17.5 Hz, 2H, C2-H₂), 1.40 (s, 9H, (CH₃)₃); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 164.58 (C4 (CO₂H)), 163.23 (C6O), 155.07 (C10O), 132.00 (C4), 125.48 (CH=CH₂), 124.81 (C3), 117.20 (CH=CH₂), 79.13 ((CH₃)₃C), 60.84 (C7), 58.12 (C6), 28.02 ((CH₃)₃), 23.08 (C2); ESI-HRMS [M+Na]⁺ calculated for [C₁₄H₁₈N₂NaO₅S]⁺: 349.08286, found: 349.08307.

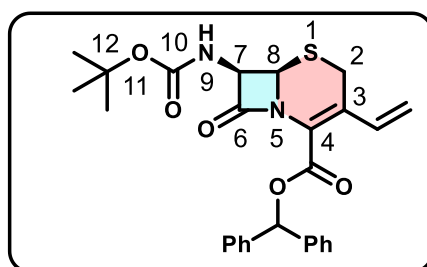
7.2.6.2 Method B

The desired product was prepared according to the previously reported procedure.²⁴⁹ In an oven-dried round-bottomed flask, 7-AVCA (1.00 g; 4.44 mmol) was suspended in MeOH (160 mL) and the headspace of the reaction vessel was purged with N₂ for 15 min. Triethylamine (928 μ L; 6.66

mmol) was then added dropwise until 7-AVCA was completely dissolved, giving a pale yellow solution. Next, di-*tert*-butyl dicarbonate (1.45 g; 6.66 mmol) dissolved in MeOH (35 mL) was added dropwise over 10 min via a pressure-equalising dropping funnel. The resultant solution was left stirring at 25 °C for 16 h before the total volume of the reaction mixture was reduced to approx. 50 mL *in vacuo*, and the resultant mixture was diluted with H₂O (150 mL). The reaction mixture was washed with ethyl acetate (3 × 100 mL) and acidified to pH 1-2 with 4 M HCl resulting in the formation of an abundant white precipitate. The aqueous phase was extracted with ethyl acetate (3 × 100 mL) and the combined organic phases were then washed with brine (100 mL) dried (MgSO₄), then concentrated *in vacuo* to give **7.2.6** as a yellow foam (1.16 g; 80%).

The analytical data obtained were analogous to the set reported for Method A (**7.2.6.1**)

7.2.7 Benzhydryl (7*R*)-7-((*tert*-butoxycarbonyl)amino)-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate

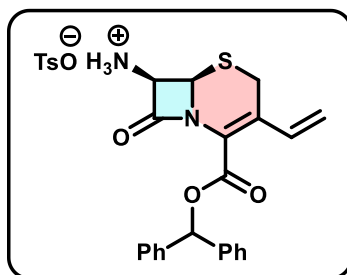


The reported procedure was followed.¹⁷⁰ **7.2.6** (1.12 g, 3.43 mmol) was dissolved in acetonitrile (50 mL). Diphenyldiazomethane **7.2.3** (668 mg, 3.43 mmol) was added until a faint pink colour persisted, at which point the esterification of the C-4 carboxylate was considered to be complete. The volatiles were removed *in vacuo* and the crude product was purified using flash chromatography [SiO₂, Buffer A: *c*-hexane, Buffer B: ethyl acetate; 2 CV 0%, 25 CV 0-20%, 2 CV 20%] to afford the desired product as a white solid (690 mg, 40%).

Mp: 160.8-165 °C (from CH₂Cl₂/DCM); $[\alpha]_D^{25}$ -5.10 (c. 1.0, CDCl₃); $\nu_{\max}$ / cm⁻¹: 3031 (C-H, ar.), 1782 (C=O, β -lactam), 1718 (C=O, ester), 1522 (C=C). ¹H NMR (400 MHz, CDCl₃) δ 7.46–7.26 (m, 10H, *aromatic-CH*), 7.01 (dd, *J* = 17.5, 11.0 Hz, 1H, *CH=CH*₂), 6.98 (s, 1H, *Ph*₂*CH*), 5.64 (dd, *J* = 9.5, 5.0

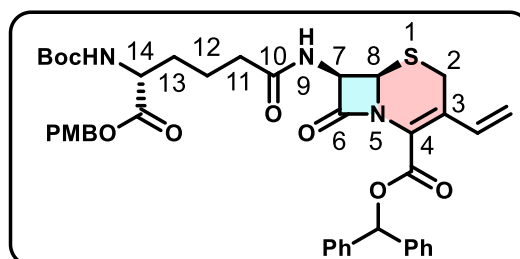
Hz, 1H, C7-H), 5.46 (d, $J = 17.5$ Hz, 1H, *trans*-CH=CH₂), 5.28 (d, $J = 11.0$ Hz, 1H, *cis*-CH=CH₂), 5.01 (d, $J = 5.0$ Hz, 1H, C6-H), 3.73, 3.48 (ABq, $J = 17.9$ Hz, 2H, C2-H₂), 1.46 (s, 9H, (CH₃)₃); ¹³C NMR (126 MHz, CDCl₃) δ 165.45 (C4' (CO₂H)), 161.25 (C6O), 154.60 (urethane-CO), 139.42 (C4) 139.14 (*ipso*-C), 132.03 (CH=CH₂), 128.51 (*ortho/meta/para*-C), 127.10 (*ortho/meta/para*-C) 124.33 (*ortho/meta/para*-C), 117.82 (CH=CH₂), 81.25 (CHPh₂), 79.43 ((CH₃)₃C12), 60.99 (C7), 57.97 (C6), 28.22 ((CH₃)₃), 24.13 (C2); ESI-HRMS [M+Na]⁺ calculated for [C₂₇H₂₈N₂NaO₅S]⁺: 515.16352, found: 515.16107.

7.2.8 (7R)-2-((benzhydryloxy)carbonyl)-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-en-7-aminium 4-methylbenzenesulfonate



Following the reported procedure,¹⁷⁰ **7.2.7** (300 mg, 0.61 mmol) was dissolved in anhydrous THF (1.2 mL). *para*-Toluenesulfonic acid monohydrate (116 mg, 0.61 mmol) was then added and the resultant solution was stirred for 15 min. The volatiles were removed *in vacuo* to afford a brown oil, which was re-dissolved in anhydrous THF (1.2 mL) with the aid of sonication. The “cycle” of concentration and re-dissolution (using an ultrasonic bath) was repeated 8 times over 3 h until no starting material was observed by TLC. Through the process, the solution gradually turned from pale yellow to orange, and a brown foam was formed when solvent was removed. Once the reaction was deemed complete by TLC analysis, the tosylammonium salt **7.2.8** was used immediately in the next step without further purification.

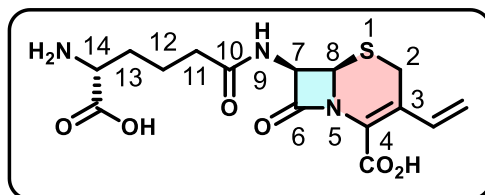
7.2.9 Benzhydryl (7*R*)-7-((*R*)-5-((*tert*-butoxycarbonyl)amino)-6-((4-methoxybenzyl)oxy)-6-oxohexanamido)-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate



To an oven-dried round-bottomed flask were added **7.2.2** (183 mg, 0.48 mmol), **7.2.8** (270 mg, 0.48 mmol), EEDQ (150 mg, 0.48 mmol) and triethylamine (134 μ L, 0.96 mmol). The flask was purged with argon before anhydrous CH_2Cl_2 (4 mL) was added. The resulting solution was then stirred under argon at 25 $^\circ\text{C}$ for 18 h. Volatiles were removed *in vacuo* and the crude product thus obtained was then purified by flash chromatography [SiO_2 , Buffer A: *n*-hexane, Buffer B: ethyl acetate; 2 CV 0%, 30 CV 0-50%, 4 CV 50-100%], to afford **7.2.9** a white solid (165 mg, 46%).

$[\alpha]_D^{25}$ -0.90 (c. 0.73, CDCl_3); ν_{max} / cm^{-1} : 3040 (C-H, ar.), 1773 (C=O, β -lactam), 1704 (C=O, ester), 1516 (C=C); ^1H NMR (400 MHz, CDCl_3) δ 7.38–7.16 (m, 14H, *aromatic-CH*), 6.91 (s, 1H, Ph_2CH), 6.26 (d, J = 8.0 Hz, 1H, N9-H), 5.73 (dd, J = 8.6, 5.0 Hz, 1H, $\text{CH}=\text{CH}_2$), 5.38 (d, J = 17.5 Hz, 1H, *trans*- $\text{CH}=\text{CH}_2$), 5.21 (d, J = 11.0 Hz, 1H, *cis*- $\text{CH}=\text{CH}_2$), 5.11–4.99 (m, 3H, C7-H and $\text{PMB-CO}_2\text{CH}_2$), 4.94 (d, J = 5.0 Hz, 1H, C6-H), 4.24 (br. s, 2H, C14-H and C14'-NH), 3.73 (s, 3H, PMB-OCH_3), 3.58, 3.44 (ABq, J = 17.5 Hz, 2H, C2-H), 2.36–2.07 (m, 3H, C11-H₂ and C13-H), 1.81–1.72 (m, 1H, C13-H), 1.68–1.60 (m, 2H, C12-H₂), 1.34 (s, 9H, $(\text{CH}_3)_3$); ^{13}C NMR (126 MHz, $\text{Chloroform-}d$) δ 175.20, 172.46, 164.69, 161.16, 159.81, 155.45 (5 C=O and *PMB para-C*), 139.39, 139.11, 131.95, 130.26, 128.58, 128.20, 127.75, 127.10, 124.38, 117.87, 114.02 (*aromatic-C* and *alkene-C*), 79.45, 67.10, 59.39, 57.66, 55.32, 35.11, 32.76, 32.28, 29.73, 28.35, 24.14, 21.42 (*aliphatic-C*); ESI-HRMS $[\text{M}+\text{Na}]^+$ calculated for $[\text{C}_{41}\text{H}_{45}\text{N}_3\text{NaO}_9\text{S}]^+$: 778.27687, found: 778.27692.

7.2.10 (7*R*)-7-((*R*)-5-Amino-5-carboxypentanamido)-8-oxo-3-vinyl-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid



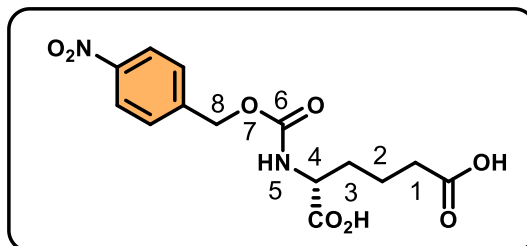
The desired product was prepared according to a modified form of the reported procedure.¹⁷³ To a round-bottomed flask was added **7.2.9** (50 mg, 66 μ mol). The flask was purged with N₂ before anhydrous CH₂Cl₂ (10 mL) was added to give a clear solution. Next, trifluoroacetic acid (9.4 mL) was added dropwise over 15 min, during which time the solution first turned pink, before developing into a deep purple colour. After approximately 1 h, all starting material was consumed as judged by TLC and LC-MS. The volatiles were removed *in vacuo*, and the crude product thus obtained was purified by preparatory-HPLC. The product-containing fraction were combined and lyophilised to afford and **7.2.10** as white fluffy solid (5.9 mg, 25%).

¹H NMR (600 MHz, D₂O) δ 6.84 (dd, J = 17.5, 11.0 Hz, 1H, CH=CH₂), 5.66 (d, J = 4.5 Hz, 1H, C7-H), 5.52 (d, J = 17.5 Hz, 1H, *trans*-CH=CH₂), 5.34 (d, J = 11.0 Hz, 1H, *cis*-CH=CH₂), 5.20 (d, J = 5.0 Hz, 1H, C6-H), 3.91–3.86 (m, 1H, C14-H), 3.77, 3.66 (ABq, J = 17.5 Hz, 2H, C2-H), 2.51–2.41 (m, 2H, C11-H₂), 2.05–1.88 (m, 2H, C13-H₂), 1.88–1.69 (m, 2H, C12-H₂); ¹³C NMR (151 MHz, Deuterium Oxide) δ 176.56 (C10=O), 173.64 (C12 (CO₂H)), 168.68 (C4' (CO₂H)), 164.89 (C6=O), 132.04 (CH=CH₂), 128.70 (C4), 120.79 (C3), 116.14 (CH=CH₂), 59.12 (C7), 57.42 (C6), 53.94 (C14), 34.57 (C11), 29.63 (C13), 23.14 (C2), 20.87 (C12). ESI-HRMS [M+H]⁺ calculated for [C₁₅H₂₀N₃O₆S]⁺: 370.1073, found: 370.1057.

7.3 Synthesis of Penicillin N

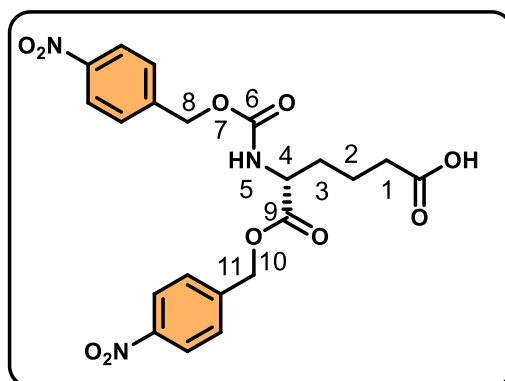
The synthetic route to penicillin N was taken from literature,⁴⁴ and used without further modification.

7.3.1 *N*-4-Nitrobenzyloxycarbonyl-(*R*)- α -aminoadipic acid

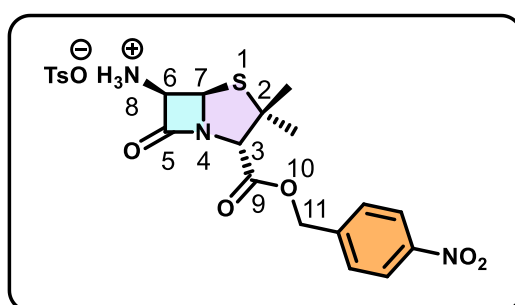


The desired product was prepared according to the reported procedure.^{250, 251} (*R*)- α -Aminoadipic acid (500 mg, 3.1 mmol) was suspended in 2 M aq NaOH (3.1 mL, 6.2 mmol) and dioxane (1.3 mL) and stirred vigorously at 0 °C. To the resultant solution, *p*-nitrobenzyl chloroformate (936 mg, 4.3 mmol) in dioxane (5 mL) and 2M NaOH (3.1 mL, 6.2 mmol) were added simultaneously dropwise over 30 min. Upon the complete addition, the solution was warmed to room temperature and left stirring for 30 min. The reaction mixture was then filtered and the precipitate was washed with water (10 mL). The aqueous phase was washed with ethyl acetate (25 mL) before it was acidified to pH 3 with 1 M aq HCl and extracted with ethyl acetate (3 x 25 mL). The combined organic layers were washed with brine (25 mL), dried (MgSO₄) and concentrated *in vacuo* to give **7.3.1** as a white solid (664 mg, 63%).

¹H NMR (400 MHz, DMSO-*d*₆) δ 8.23 (dd, *J* = 9.1, 2.3 Hz, 2H, *Ar*-H), 7.75 (d, *J* = 8.0 Hz, 1H, N5-H), 7.64–7.55 (m, 2H, *Ar*-H), 5.19 (s, 2H, C8-H₂), 4.07–3.90 (m, 1H, C4-H), 2.24–2.17 (m, 2H, C1-H₂), 1.81–1.68 (m, 1H, C3-H), 1.67–1.48 (m, 3H, C2-H₂ and C3-H). The analytical data matched those previously reported.²⁵¹

7.3.2 α -*p*-Nitrobenzyl-*N*-*p*-nitrobenzyloxycarbonyl-(*R*)- α -aminoadipic acid

The reported procedure was followed.^{250, 251} **7.3.1** (664 mg, 1.95 mmol) was dissolved in *N,N*-dimethylformamide (7.5 mL) and heated to 80 °C. Dicyclohexylamine (0.39 mL, 1.95 mmol) was then added and the resulting solution was stirred for 5 min at 80 °C before *p*-nitrobenzylbromide (422 mg, 1.95 mmol) in *N,N*-dimethylformamide (2 mL) was added in a single portion, resulting in the formation of a precipitate. The reaction mixture was cooled to room temperature before ethyl acetate (25 mL) was added. The precipitate formed was removed by filtration and the residue was washed with ethyl acetate. The filtrate was washed with water (25 mL) and brine (25 mL), dried (MgSO_4), then concentrated *in vacuo* to give **7.3.2** as a white solid (crude mass 680 mg), which was used directly in the next step without further purification.

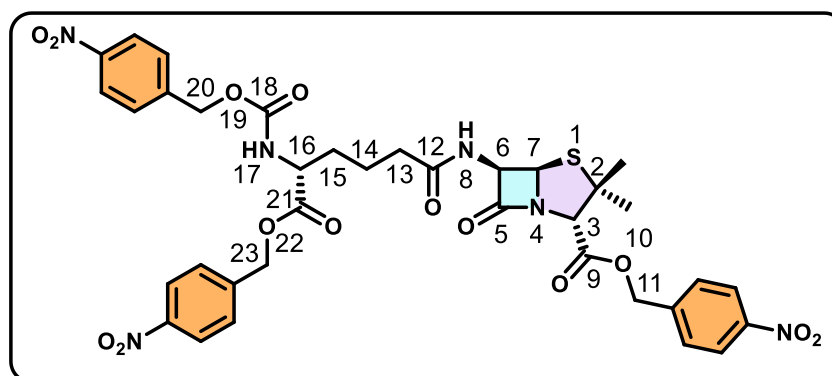
7.3.3 6-Aminopencillanic acid benzyl ester *p*-toluenesulfonic acid salt

The reported procedure was followed.²⁵² 6-APA (2 g, 9.25 mmol) was dissolved in acetone (4 mL) with stirring at 0 °C. Triethylamine (1.4 mL, 10.1 mmol) was then added and the resultant solution was stirred for 30 min. Next, *p*-nitrobenzyl bromide (2.2 g, 10.2 mmol) in acetone (9 mL) was added dropwise and the reaction mixture was stirred for 4 h at 0 °C, before it was poured into anhydrous diethyl ether (30 mL). Precipitate formed was removed by filtration, washing with

anhydrous diethyl ether (5 mL). The filtrate was washed with saturated aqueous NaHCO_3 (30 mL) and water (30 mL). The organic phase was treated with *p*-toluenesulfonic acid (1.9 g, 10 mmol) in acetone (7 mL) upon which precipitate was formed. The precipitate was collected by filtration and dried *in vacuo* to afford **7.3.1** as a white solid (3.00 g, 62%).

^1H NMR (400 MHz, DMSO) δ 8.30–8.21 (m, 2H, Ar-H), 7.71 (d, J = 8.6 Hz, 2H, Ar-H), 7.48 (d, J = 7.7 Hz, 2H, Ar-H), 7.11 (d, J = 7.7 Hz, 2H, Ar-H), 5.55 (d, J = 4.3 Hz, 1H, C6-H), 5.37 (s, 2H, C11-H₂), 5.12 (d, J = 4.3 Hz, 1H, C7-H), 4.66 (d, J = 1.0 Hz, 1H, C3-H), 2.29 (s, 3H, *p*-TsOH *ortho*-CH₃), 1.64 (s, 3H, C2'-CH₃), 1.43 (s, 3H, C2'-CH₃). The analytical data matched those previously reported.

7.3.4 *n*-Butyl-(*R*)-*p*-nitrobenzyloxycarbonyl-*p*-nitrobenzyloxycarbonylamino penicillin *p*-nitrobenzyl ester



Step 1: Free base formation

The reported procedure was followed.^{250, 251} **7.3.3** (1.45 g, 2.78 mmol) was dissolved in ethyl acetate (50 mL) and washed with sat. aqueous NaHCO_3 , water (50 mL) and brine (50 mL). The combined organic phases were dried (MgSO_4), filtered, then concentrated *in vacuo* to afford 6-aminopenicillanic acid benzyl ester as a free base (720 mg).

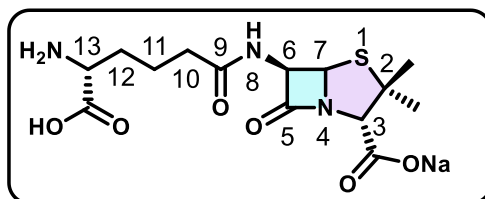
Step 2: amide coupling

To the free base from Step 1 (720 mg, 2.05 mmol) dissolved in CH_2Cl_2 (20 mL) were added **7.3.2** (973 mg, 2.05 mmol) and 2-ethoxy-*N*-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 507 mg, 2.05 mmol). The resultant solution was stirred at room temperature for 16 h before solvent was removed *in vacuo*. The solid residue thus obtained was re-dissolved in ethyl acetate (25 mL) and

the organic phase was washed with 1 M aq HCl (25 mL), sat. aqueous NaHCO₃ (25 mL), water (25 mL) and brine (25 mL). The combined organic extracts were then dried (MgSO₄), then concentrated *in vacuo*. The crude product thus obtained was purified using flash column chromatography [SiO₂, Buffer A: DCM Buffer B: EtOAc; 3 CV 0%, 30 CV 0-30%], to afford **7.3.4** as an off-white solid (677 mg, 41%).

¹H NMR (400 MHz, CDCl₃) δ 8.29–8.17 (m, 6H, *Ar-H*), 7.58–7.47 (m, 6H, *Ar-H*), 6.13 (d, *J* = 8.9 Hz, 1H, N17-*H*), 5.69 (dd, *J* = 8.9, 4.2 Hz, 1H, C6-*H*), 5.56 (d, *J* = 8.1 Hz, 1H, N8-*H*), 5.53 (d, *J* = 4.3 Hz, 1H, C7-*H*), 5.32–5.24 (m, 4H, C20-*H*₂ and C23-*H*₂), 5.20 (s, 2H, C11-*H*₂), 4.48 (s, 1H, C3-*H*), 4.45–4.39 (m, 1H, C13-*H*), 2.37–2.22 (m, 2H, C10-*H*₂), 2.00–1.67 (m, 4H, C14-*H*₂ and C15-*H*₂), 1.63 (s, 3H, C2'-*CH*₃), 1.43 (s, 3H, C2'-*CH*₃). The analytical data matched those previously reported.²⁵¹

7.3.5 Penicillin N monosodium salt



The reported procedure was followed.⁴⁴ **7.3.4** (100 mg, 0.12 mmol) and NaHCO₃ (10.4 mg, 0.12 mmol) were dissolved in water (10 mL) and tetrahydrofuran (10 mL). To the resultant solution was added 10% Pd-C (100 mg, 0.12 mmol) was added and the reaction flask was evacuated and purged with hydrogen 5 times, before being left to stir under hydrogen atmosphere for 3 h. The reaction mixture was then cooled to 0 °C and filtered through a Celite pad. Tetrahydrofuran was then removed *in vacuo* and the remaining aqueous solution was washed with ether (2 × 10 mL) before being frozen in liquid nitrogen and lyophilised. Purification by reverse-phase HPLC (eluting with 10 mM NH₄HCO₃/acetonitrile, 98:2). afforded **7.3.5** as a white solid (14 mg, 30%).

¹H NMR (400 MHz, D₂O) δ 5.57 (d, *J* = 3.9 Hz, 1H, C6-*H*), 5.48 (d, *J* = 3.9 Hz, 1H, C7-*H*), 4.25 (s, 1H, C4-*H*), 3.73 (dd, *J* = 6.7, 5.4 Hz, 1H, C13-*H*), 2.45–2.37 (m, 2H, C10-*H*₂), 1.98–1.83 (m, 2H, C12-*H*₂),

1.83–1.66 (m, 2H, C11-**H**₂), 1.65 (s, 3H, C2'-**CH**₃), 1.53 (s, 3H, C2'-**CH**₃). ESI-HRMS [M+H]⁺ calculated for [C₁₄H₂₂N₃O₆S]⁺: 360.4048, found: 360.1259. The analytical data matched those previously reported.

Chapter 8

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Appendix 1

Protein construct information

9 Appendix 1: Protein construct information

9.1 DAOCS/DACS

9.1.1 pCold_(kanR)_AC

The pCold_(kanR)_AC construct encodes for the following sequence:

MNHKVVHHHHHHIEGRHMELGTLLEGSEFLEVLQ[▼]GPVDHMTSKVPVFRLLDLKSGKVLTELAEAVTTKGIF
YLTESGLVDDDDHTSARETCVDFFKNGSEEEKRAVTLADRNARRGFSALEWESTAVVTETGKYSDYSTCYSM
GIGGNLFPNNGFEDVWQDYFDRMYGAAKDVARAVLNSVGAPLAGEDIDDFVECDPLLRLRYFPEVPEDRV
AEEELRMGPHYDLSTITLVHQTACANGFVSLQCEVDGEFVDLPTLPGAMVVFCGAVGTLATGGKVKAPK
HRVKSPGRDQRVGSSRTSSVFFLRPKPDFSFNVQQSREWGFNVRIPSERTTFREWLGGNYVNMRRDKPA
AAEAAVPAAAPVSTAAPIAT

Predicted mass: 40,934 Da (with His₆-tag); 36,985 Da (cleaved protein)

ε / 1000: ; 35.785 M⁻¹ cm⁻¹ (both uncleaved and cleaved protein) (assuming all pairs of Cys residues form cystines)

9.1.2 pET_AC

The pET_AC construct encodes for the following sequence:

MTSKVPVFRLLDLKSGKVLTELAEAVTTKGIFYLTESGLVDDDDHTSARETCVDFFKNGSEEEKRAVTLADRN
ARRGFSALEWESTAVVTETGKYSDYSTCYSMIGGNLFPNNGFEDVWQDYFDRMYGAAKDVARAVLNSV
GAPLAGEDIDDFVECDPLLRLRYFPEVPEDRVAEEELRMGPHYDLSTITLVHQTACANGFVSLQCEVDGEF
VDLPTLPGAMVVFCGAVGTLATGGKVKAPKHRVKSPGRDQRVGSSRTSSVFFLRPKPDFSFNVQQSREW
GFNVRIPSERTTFREWLGGNYVNMRRDKPAAAEAAVPAAAPVSTAAPIAT

Predicted mass: 36,479 Da

ε / 1000: 35.785 M⁻¹ cm⁻¹ (assuming all pairs of Cys residues form cystines)

9.2 DACS

9.2.1 pCold_cefF

The pCold_cefF construct encodes for the following sequence:

MNHKVVHHHHHHIEGRHMELGTLLEGSEFLEVLQ[▼]GPVDADTPVPIFNLAALREGADQEKFRECVTGMGVF
YLTGYGAGDKDHRLATDTAMDDFANGTEAEKAAVTTDVPTMRRGYSALEAESTAQVTRTGSYTDYSMSFS
MGISGNVFPSPFEFRVWTEYFDKLYAAAQETARLVLTASGGYDAEIVGSLDELLDADPVLRLRYFPEVPEHR
SAEHEPRRMAPHYDLSIITFIHQTPCANGFVSLQAEIGGELVSLPVVEDAVVVMCGAMAPLATQGALPAPR

HHVRSPGAGMREGSDRTSSVFFLRPTTDFSFSAKARSYGLAVDLDMETATFGDWIGTNYVTMHAKNEP
QA

Predicted mass: 38,714 kDa (with His₆-tag); 33,785 Da (cleaved protein)

ϵ / **1000**: 29.005 M⁻¹ cm⁻¹ (both uncleaved and cleaved protein) (assuming all pairs of Cys residues form cystines)

9.3 DAOCS

9.3.1 pET24_cefE

The pET24_cefE construct encodes for the following sequence:

MDTTVPTFSLAELQQGLHQDEFRRCLRDKGLFYLTDCGLTDTLKSADIVIDFFEHGSEAEKRAVTSPVPT
MRRGFTGLESESTAQITNTGSYSDYSMCYSMGADNLFP SGDFERIWTQYFDRQYTASRAVAREVLRATG
TEPDGGVEAFLDCEPLLRFRYPQVPEHRSAAEQPLRMAPHYDLSMVTLIQQTTPCANGFVSLQAEVGGAF
DLPYRPAVLVFCGAIAITLVTTGGQVKAPRHHVAAPRRDQIAGSSRTSSVFFLRPNADFTFSVPLARECGFDV
SLDGETATFQDWIGGNYVNIRRTSKA

Predicted mass: 34, 555 Da

ϵ / **1000**: 26.275 M⁻¹ cm⁻¹ (assuming all pairs of Cys residues form cystines)

9.4 ICM DAOCS

9.4.1 pET30_ICM DAOCS

The pET30_ICM DAOCS construct encodes for the following sequence:

MDTTVPTFSLAELQQGLHQDEFRRCLRDKGLFYLTDCGLTDTLKSADLVIDFFEHGSEAEKRAVTSPVPTT
RRGFTGLESESTAQITNTGSYSDYSMCYSMGADNLFP SGDFERIWTQYFDRQYTASRAVAREVLRATGTE
PDGGVEAFLDYEP LLRFRYPQVPEHRSAAEQPLRMAPH HDLSMVTLIQQTTPCANGFVSLQAEVGGAFVD
LPYRPAVLVFCGAIAITLVTTGGQVKAPRHHVAAPRRDQIAGSSRTSSVFFLRPNADFTFSIPLAREYGF DVSL
DGETATFQDWIGGNYVNMRRRTSKA

Mutated residues are highlight in red.

Predicted mass: 34,650 Da

ϵ / **1000**: 27.640 M⁻¹ cm⁻¹ (assuming all pairs of Cys residues form cystines)

Appendix 2

3-vinyl cephem incubations

10 Appendix 2: 3-vinyl cephem incubations

10.1 NMR Data for the major hydroxy azide cephem product

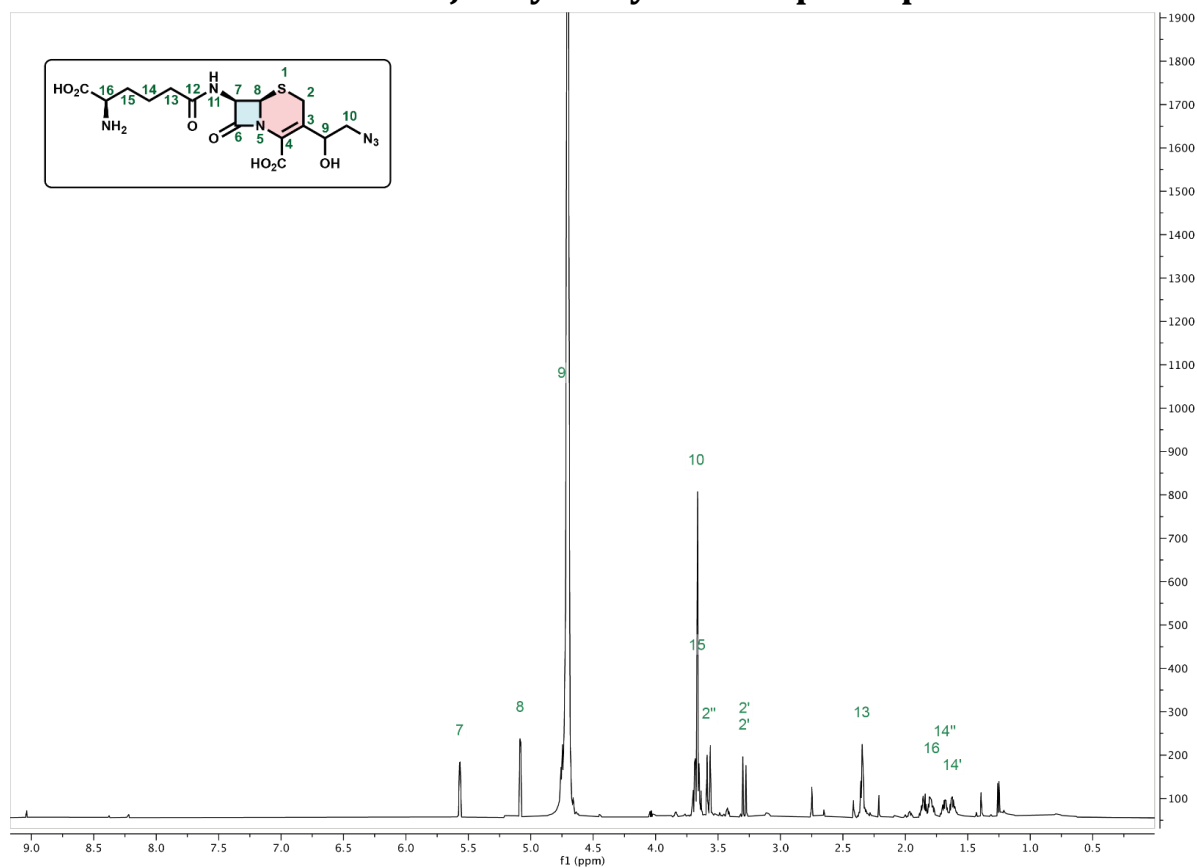


Figure 10.1 | ^1H NMR spectrum for the hydroxy azide product.

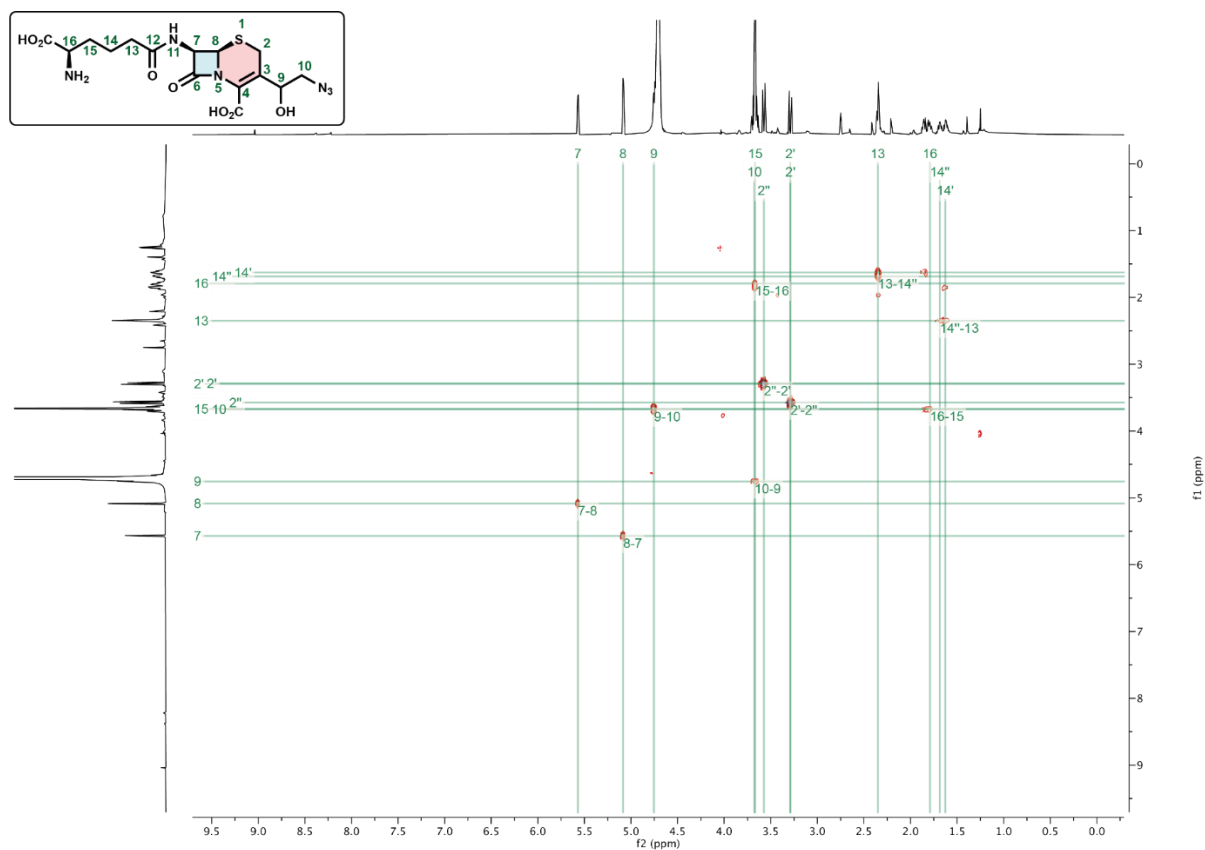


Figure 10.2 | ^1H - ^1H COSY spectrum for the hydroxy azide product.

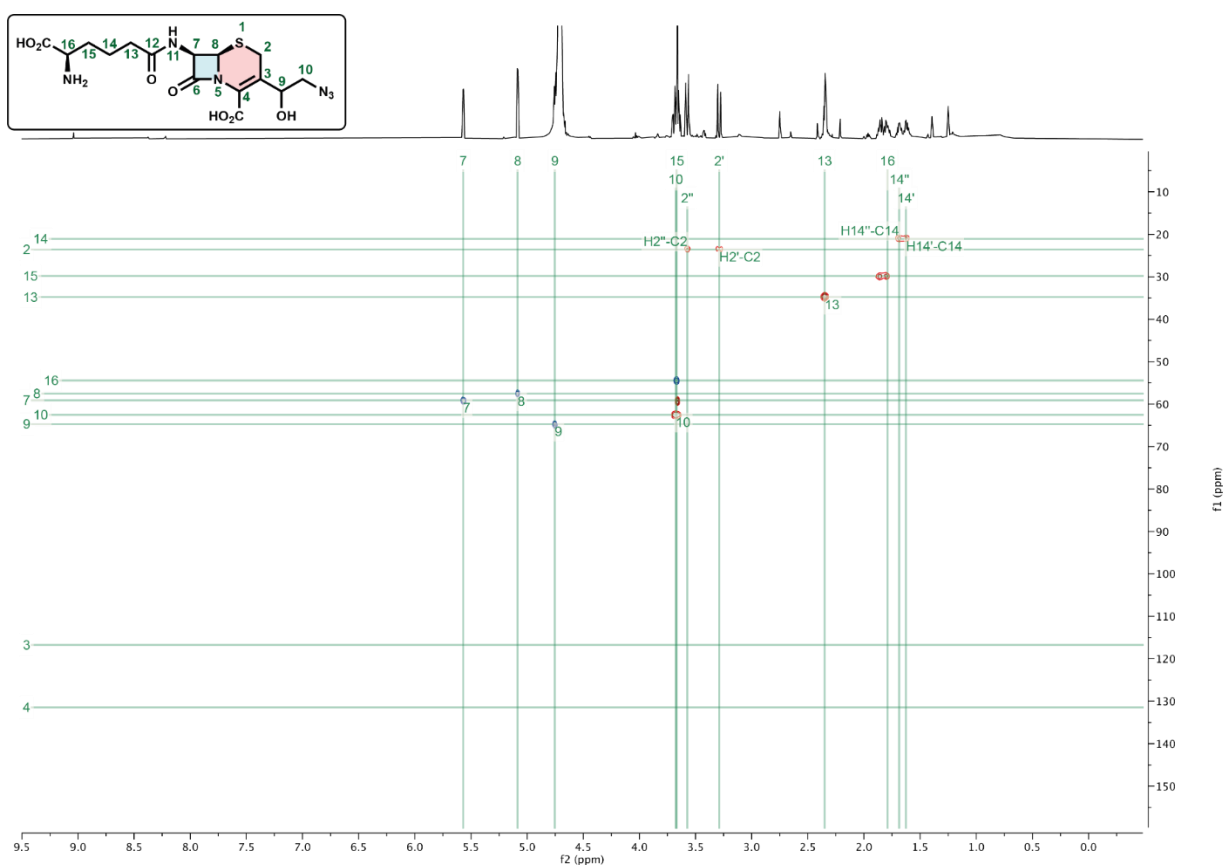


Figure 10.3 | ^1H - ^{13}C HSQC spectrum for the hydroxy azide product.

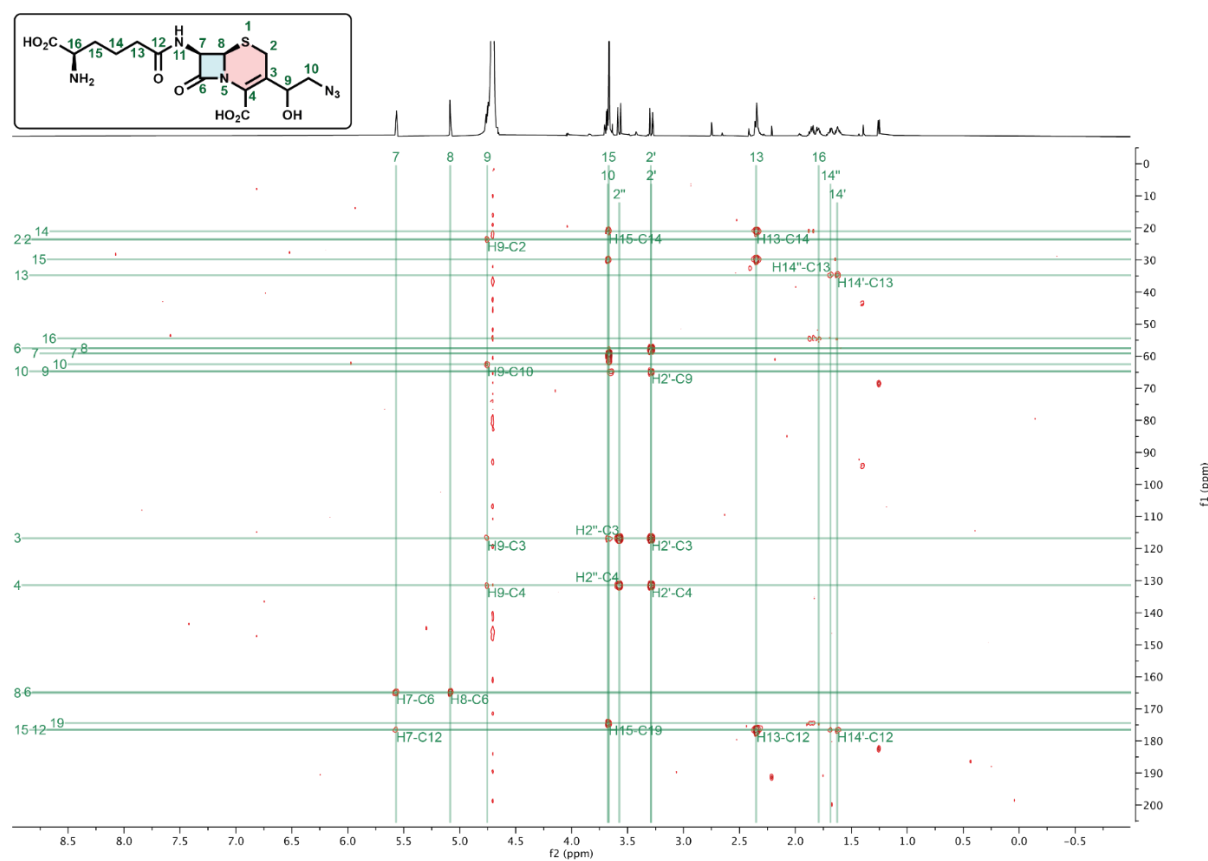


Figure 10.4 | ^1H - ^{13}C HMBC spectrum for the hydroxy azide product.

10.2 NMR data for the minor hydroxy azide cephem product

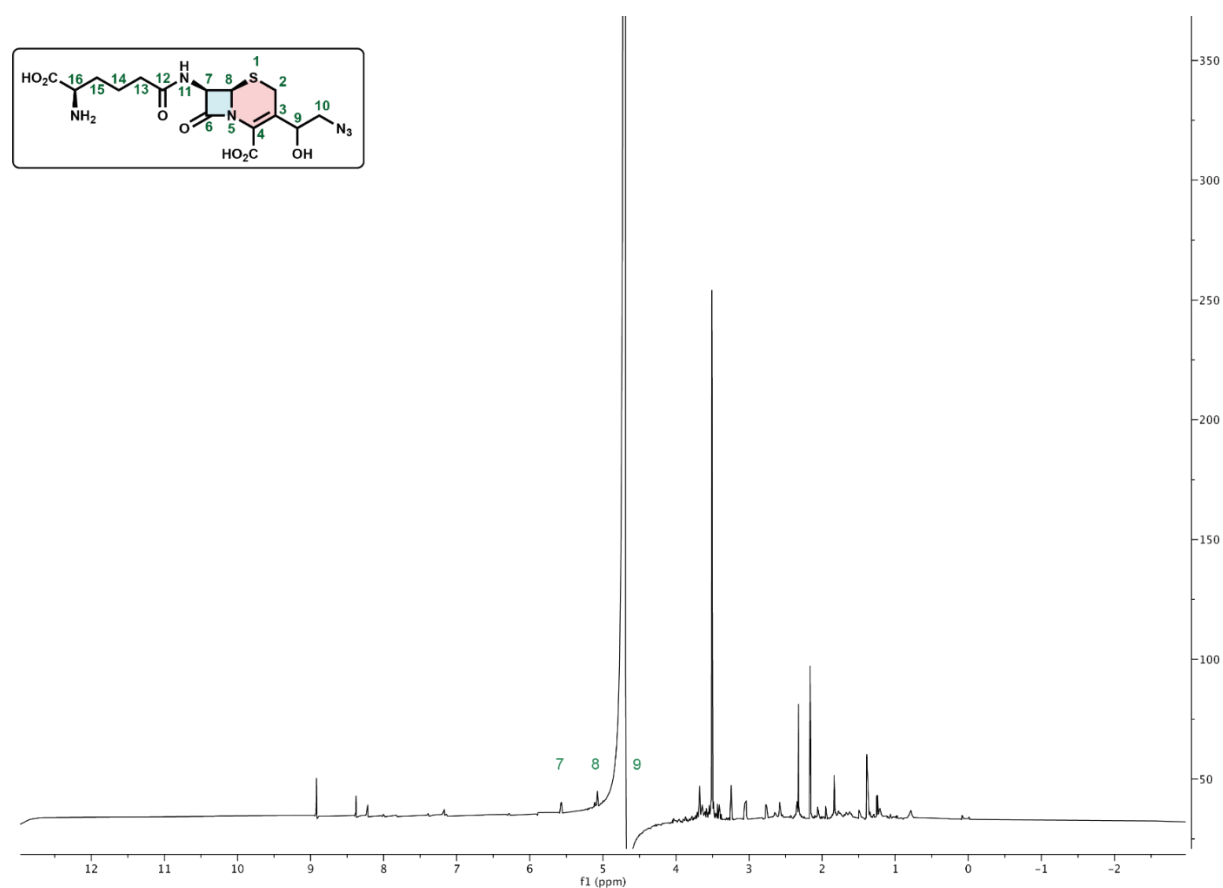


Figure 10.5 | ^1H NMR spectra for the minor hydroxy azide cephem product.

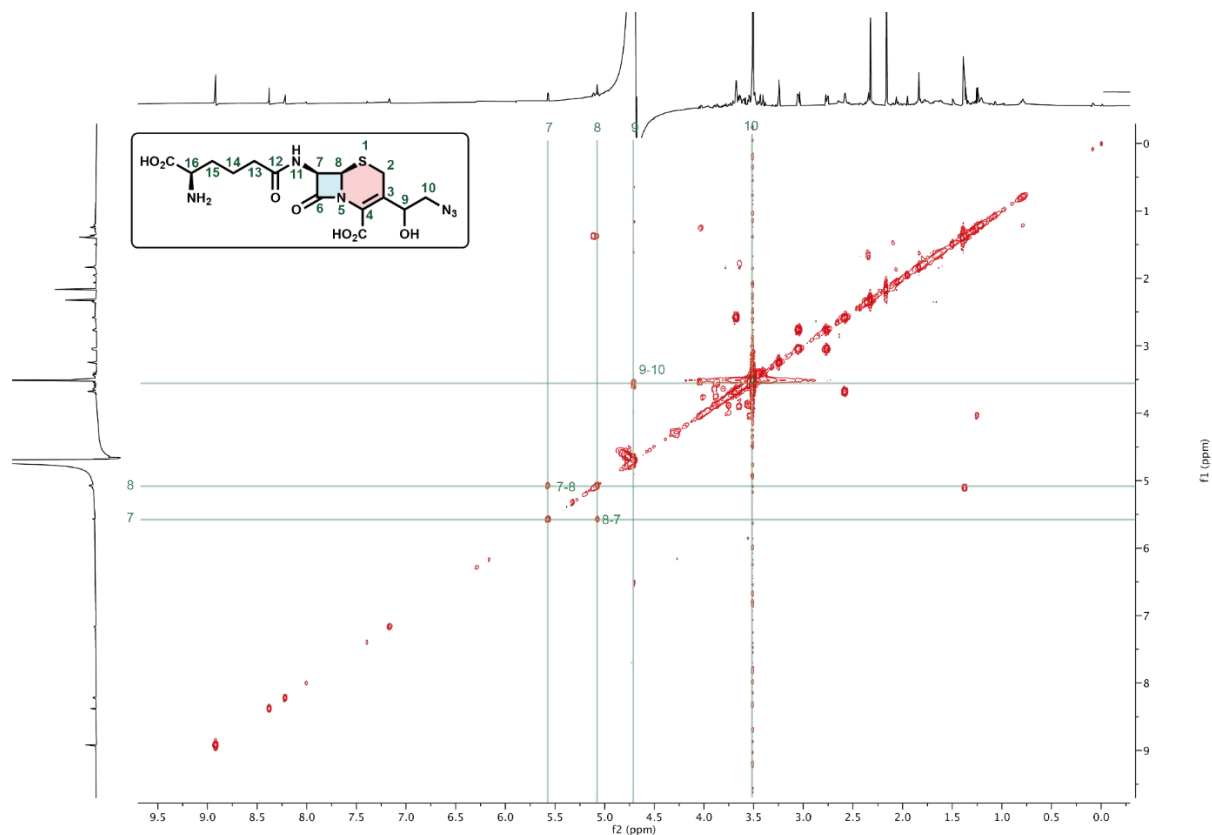


Figure 10.6 | Minor hydroxy azide cephem ^1H - ^1H COSY spectrum.

10.3 Epoxide analogue ¹H NMR spectra

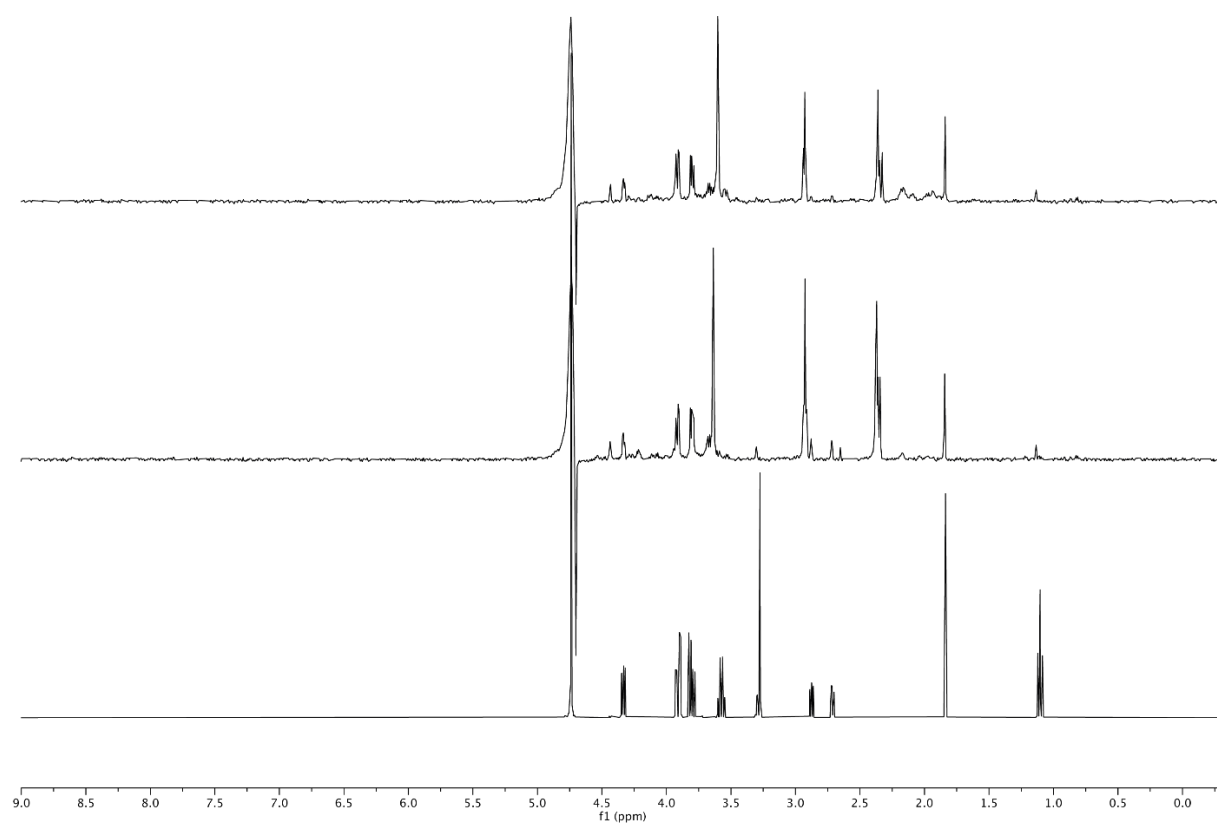


Figure 10.8 | Stacked ¹H NMR spectra of epoxide analogue incubations under assay conditions. Bottom Epoxide reference spectrum. **Middle** Assay without azide. **Top** Assay with azide. The structure of the epoxide analogue is given in **Figure 3.29**