

Macrocyclic Peptides: Chemistry and biology of stapled and depsipeptides

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ABSTRACT

Macrocyclic peptides have been identified as key backbones in several biologically active compounds. They have been considered as great inspiration in the development of novel cyclic scaffolds in medicinal chemistry, notably in the introduction of α -helically constricted stapled peptides with the ability to mimic biologically relevant α -helices. DNA-binding transcription factors often bind their DNA promoter through an α -helix, making a parallel with stapled peptides as inhibitors. Despite this relevant feature, DNA-binding stapled peptides are highly unrepresented in the literature, as will be discussed here in a review. We also further expand this area of research with a study of DNA binding stapled peptide analogues with the goal of optimizing and investigating the DNA binding and antivirulence of an RpoN-based stapled peptide.

Cyclic depsipeptides are highly biologically active natural product molecules however their synthesis can be challenging with the presence of a macrolactone. Due to this complexity, solid phase peptide synthesis strategies have been utilized to access peptide intermediates that can be synthetically macrocyclized using solution phase or on-resin approaches via macrolactam or macrolactone formation. A representative number of total syntheses in the literature is reviewed. Furthermore, we describe here the chemical total synthesis and chemoenzymatic synthesis of seongsanamide E cyclic depsipeptide via thioesterase mediated macrolactonization.

Cyclic depsipeptides also play major roles in their producing organisms, notably siderophores capable of chelating and transporting iron. The biosynthesis of fungal siderophores is poorly explored, such as the iterative mechanism of oligomeric compound fusarinine C. We explore the synthesis of the previously never synthesized fusarinine C monomer to be utilized as a di-domain inhibitor of the adenylation-thiolation domains of the non-ribosomal peptide synthetase (NRPS) SidD.

DEDICATION

To my dad.

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List of Symbols, Nomenclature or Abbreviations

Ac ₂ O	Acetic anhydride
ACN	Acetonitrile
ATP	Adenosine triphosphate
Boc	Tert-butyloxycarbonyl
Boc ₂ O	Di-tert-butyl dicarbonate
BOP	Oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
BSA	Bovine serum albumin
CD	Circular dichroism
DBU	1,8-Diazabicyclo(5.4.0)undec-7-ene
DCC	N,N'-Dicyclohexylcarbodiimide
DCE	1,2-Dichloroethane
DCM	Dichloromethane
DIAD	Diisopropyl azodicarboxylate
DIBAL	Diisobutylaluminium hydride
DIC	N,N'-Diisopropylcarbodiimide
DIPEA	N,N'-Diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	N,N-Dimethyl formamide
DMSO	Dimethylsulfoxide
DMTMM·BF ₄	4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate
DTNB	Dithionitrobenzoic acid
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EMSA	Electrophoretic mobility shift assay
EtOAc	Ethyl acetate
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HBTU	<i>N,N,N',N'</i> -Tetramethyl- <i>O</i> -(1 <i>H</i> -benzotriazol-1-yl)uronium hexafluorophosphate
HFIP	1,1,1,3,3,3-Hexafluoroisopropanol
HMPB	4-(4-Hydroxymethyl-3-methoxyphenoxy)butyric acid
HOAt	1-Hydroxy-7-azabenzotriazole
HOBt	Hydroxybenzotriazole
HPLC	High pressure liquid chromatography
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Lysogeny broth
LCMS	Liquid chromatography mass spectrometry
MBHA	4-Methylbenzhydramine

MeOH	Methanol
MNBA	2-methyl-6-nitrobenzoic anhydride
NMM	N-Methylmorpholine
NMP	N-Methyl-2-pyrrolydone
NMR	Nuclear magnetic resonance
ONPG	ortho-Nitrophenyl- β -galactoside
PBS	Phosphate-buffered saline
PEG	Poly ethylene glycol
PPh ₃	Triphenylphosphine
pTsOH	para-Toluenesulfonic acid
PyAOP	(7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate
PyBOP	Benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
RCM	Ring closing metathesis
SPPS	Solid phase peptide synthesis
TBAF	Tetrabutylammoniumfluoride
TBS	Tert-butyldimethylsilane
tBu	Tert-Butyl
TEA	Triethylamine
TFA	Trifluoroacetic acid
TIPS	Triisopropylsilane

Chapter 1: Introduction

1.1 A brief history of peptide chemistry

Peptide chemistry emerged as an independent discipline in synthetic chemistry in 1882 with the efforts of Theodor Curtius to synthesize benzoyl-Gly-Gly-OH using the silver salt of glycine and benzoylchloride.¹ Key milestones in the evolution of peptide chemistry include the development of solid-phase peptide synthesis (SPPS) with the first successful tetrapeptide synthesis by Merrifield in 1963 on a benzyl chloride polystyrene solid support.² Following this, Merrifield introduced the tert-butyloxycarbonyl/benzyl (Boc/Bzl) protecting group strategy³ and reaction conditions were further optimized to introduce HF peptide cleavage, automation of synthesis and HPLC purification. SPPS was further revolutionized by the introduction of 9-fluorenylmethyloxycarbonyl/tert-butyl (Fmoc/tBu) coupling conditions in 1978⁴ following the introduction of the Fmoc protecting group in 1970.⁵ Different resin types were developed to enable varying C-terminal chemistry, including C-terminus acid yielding Wang resin in 1973 and amide yielding Rink resin in 1987⁶ (**Figure 1.1**).

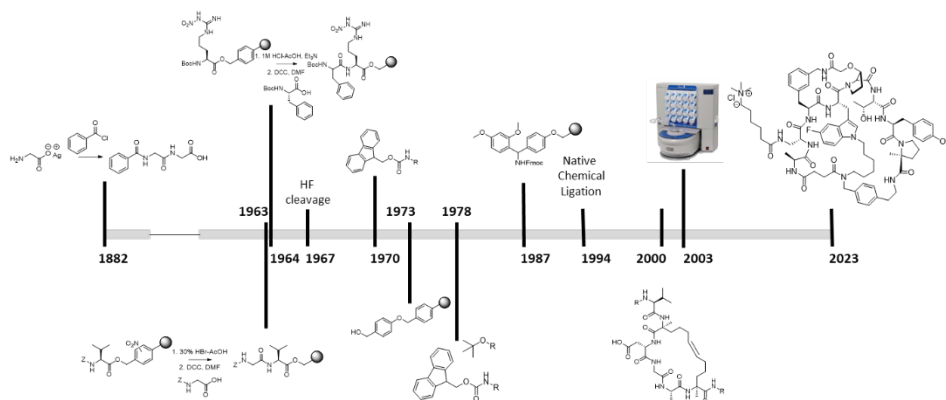


Figure 1.1 Timeline of key events in the development of peptide synthesis

A major advance in the field came with the development of native chemical ligation,⁷ which enabled for the first time the chemical synthesis of small folded protein. The development of stapled peptides enabled secondary structure such as α -helices to be stabilized in short peptide sequences making peptide based inhibitors of protein-protein interactions a viable target for development⁸. Today, complex peptides⁹ and small proteins are routinely synthesized in high yields on fully automated microwave-based SPPS systems. These systems have very short coupling times and can enable synthesis from small to large scale. Peptide chemistry has become an essential and standard practice in research labs, enabling the synthesis of medicinal chemistry libraries and the synthesis of natural products and their analogues. This introduction will further explore the use of SPPS for stapled peptides, depsipeptide natural products and their analogues.

1.2 Macrocyclic natural products as potent compounds and inspiration for cyclic scaffolds

Macrocyclic compounds are generally defined as compounds with a ring of at least 12 heavy atoms. Due to the presence of macrocycles in new therapeutics, the interest in macrocycles has exponentially increased since the 1980's.¹⁰ Macrocycles in natural products are functionally diverse, semirigid, preorganized structures that can impact biophysical properties including but not limited to solubility and membrane permeability,¹¹ target binding affinity and selectivity, and stability in biological environments. Naturally, these macrocycles are incorporated biosynthetically via cyclase enzymes, usually at the end of their biosynthetic pathway. Several isolated cyclic peptide natural products have been identified as biologically active¹² and are amongst the most potent medicinally relevant compounds to date including gramicidin S,¹³ an antibacterial compound discovered in 1942, griselimycin, an anti-tuberculosis compound and cyclosporin A, which is used as an immunosuppressive (**Figure 1.2**). Recognizing the importance

of macrocyclic natural products, chemists have embarked on the total syntheses of naturally occurring macrocyclic peptides over the last 30 years.

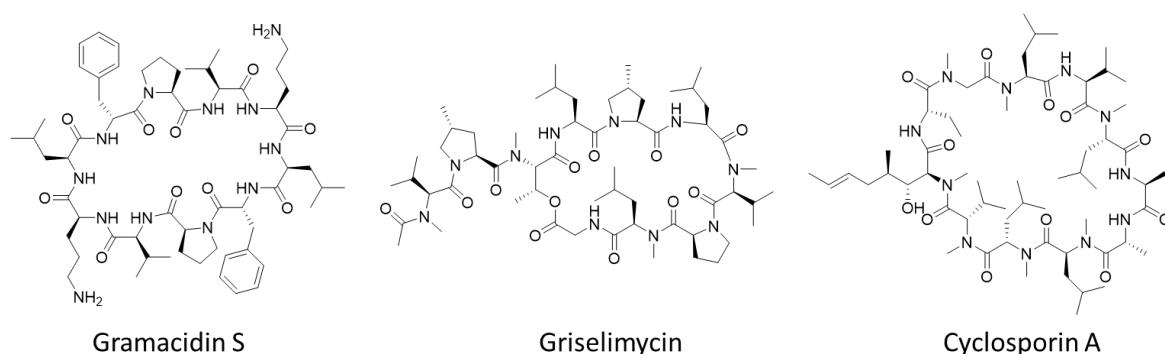


Figure 1.2 Macrocyclic natural product examples

Small molecules (< 1000 Da) have been a focus in drug design for years due to their general hydrophobicity, which enables membrane permeability and oral administration. This has been codified in the pharmaceutical industry's adoption of the Lipinski's Rule of Five for small^{14,15} molecule drug design. These compounds are typically limited to small binding pockets and some inhibitors can suffer from low target selectivity. On the other hand, macromolecules or biologics (5-150 kDa) such as antibodies exhibit high binding affinity and selectivity for their target however they must generally be administered intravenously. Cyclic peptides (500-2000 Da) are a middle ground, where the cyclic structure confers the biophysical properties needed to be a drug and the amino acid sidechains can make contact with several areas of a large target, ensuring high target selectivity for larger binding sites¹⁶. Despite residing in chemical space beyond the Rule of 5, there is a large focus on cyclic peptide natural products discovery as well as the design of important cyclic chemical scaffold (**Figure 1.3**).

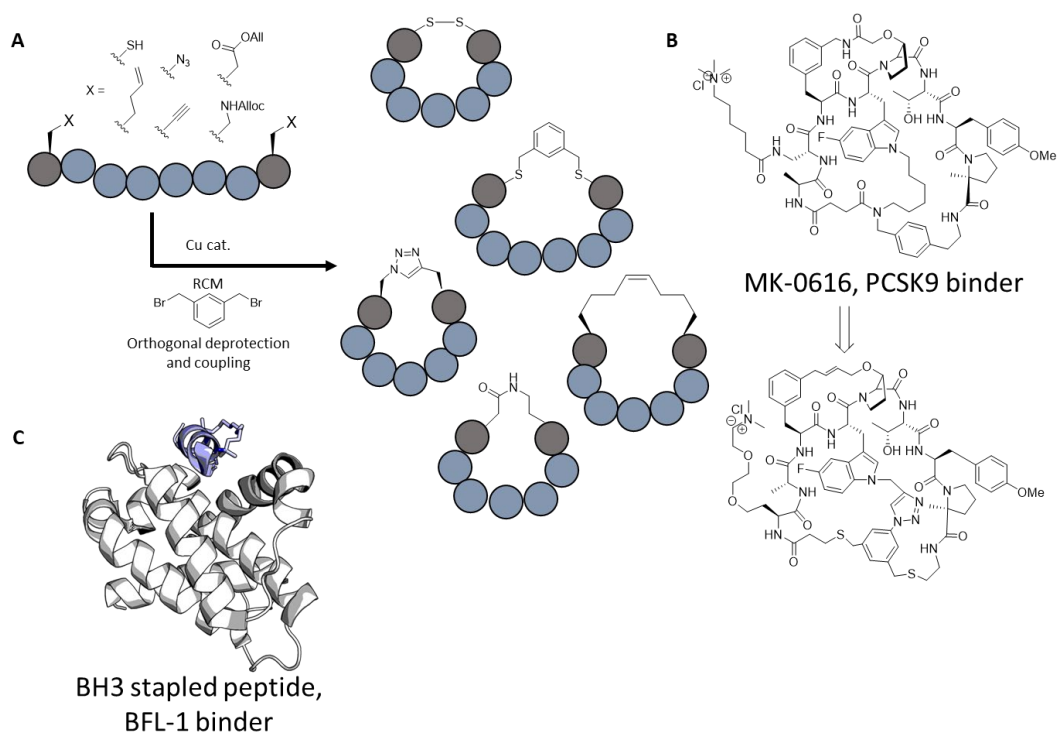


Figure 1.3 Cyclic peptides constrained by innovative synthetic strategies.

A. General cyclic peptide workflow. **B.** MK-0616 inhibitor, a potent synthetic cyclic peptide. **C.** Stapled peptide bound to its protein target.

The synthesis of cyclic peptides and cyclic peptide mimics has been inspired by natural products and has pushed researchers to develop innovative synthetic strategies to obtain these in easy, orthogonal manners with a variety of chemistries including SPPS (**Figure 1.3A**). Examples include orthogonal amino acid sidechains reaction, the reaction of two cysteine groups with a dibenzyl bromides fragment¹⁷, introducing azide containing amino acids that can react with alkyne residues¹⁸ and disulfide-rich peptide mimicking epitopes¹⁹. The recent synthesis of the MK-0616 PCSK9 inhibitor (**Figure 1.3B**) has explored several types of macrocyclization chemistry in this complex structure, over time eliminating key features to gain optimal binding.^{20,9} Maybe the most popular scaffold-based cyclization are stapled peptides (**Figure 1.3C**)²¹ which will be thoroughly reviewed in future paragraphs, chapter 2 and chapter 3.

When considering the tremendous efforts in natural product total synthesis, cyclic products are at the forefront of these challenges. This can be seen in the first total synthesis of erythromycin by Woodward *et al.* that attempted the cyclization step 17 times before finding a suitable route²². Macrocyclization chemistry is still a challenge with modern synthetic efforts, where cyclic peptides require clever cyclization strategies and each type of peptide has its own set of challenges. Notably for the context of this thesis, SPPS has been used to complete several natural product total syntheses, specifically in depsipeptide synthesis, which will be covered in chapters 4 and 5.

1.3 Stapled peptides are stabilized helical peptides *via* macrocyclization

Schafmeister *et al.* developed the first stapled peptide utilizing nonproteinogenic amino acids²³ that are incorporated into a peptide sequence and when covalently linked together²⁴, helically constrain an otherwise unorganized peptide sequence²⁵. Applying this technology to develop α -helical inhibitors based on α -helices involved in a target protein-protein interaction (PPI) yielded the first active stapled peptide that binds to the oncogene cofactor MDM2.^{8,26} Stapled peptides that inhibit PPI's have been a powerful tool for research groups to drug “undruggable” targets, highlighted by ALRN-6924, a HDM2M/p53 all-hydrocarbon stapled peptide, (clinicaltrials.gov identifier: NCT04022876), currently in clinical trials.

Peptides are known to have limitations as drug compounds, including membrane permeability,²⁷ proteolytic stability in blood plasma, low oral bioavailability, and low solubility²⁸. Stapled peptides can surmount these barriers by giving traditional peptides a more drug-like, small molecule profile through an organized α -helix structure in solution, causing intramolecular H-bonding from the backbone amides, in turn reducing polarity and increasing membrane permeability. One of the major benefits of this α -helical conformation disfavours binding to

protease active sites, with a preference for random coiled peptides.²⁹ Stapled peptides are a middle ground between drug classes, having characteristics typically associated with small molecules¹⁶ with large surface binding capabilities like biologics. Importantly, these linear peptide intermediates can be generated with standard Fmoc-SPPS followed by chemical cyclization, often using ring closing metathesis (RCM) (**Figure 1.4**).

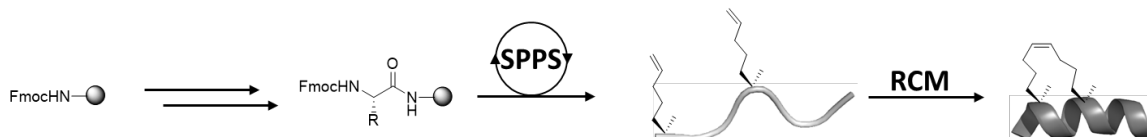


Figure 1.4 SPPS synthesis of all hydrocarbon stapled peptides

Stapled peptides are a popular chemical biology strategy when targeting PPI's³⁰⁻³³; however many protein-DNA interactions rely on α -helical contacts from the protein binding interface, such as is seen with many transcription factors binding DNA. A well defined α -helix typically makes significant stabilizing contacts to the major groove of DNA, imparting tight and sequence selective binding, yet there are only a few of examples to date that design and utilize stapled peptides as DNA binders. These are covered in depth in chapters 2 and 3.

1.4 Depsipeptides – Biosynthesis, classification and total synthesis

Natural product peptides can be classified based on their structural characteristics such as type of macrocycle. The large family of cyclic peptides can be divided into those whose rings are formed by disulfide or thioether bonds, such as the lanthipeptides, α -amide linkages (homodectic cyclic peptides), at least one non- α amide linkage (isopeptide), at least one ester linkage (depsipeptide) or a mixture of these linkages and bridged sidechains (multicyclic). Depsipeptides are macrolactones that are composed of either α , β , or higher order hydroxyl group esters that are

structurally diverse due to their biosynthetic machinery. Bacteria and fungi produce depsipeptides through large biosynthetic complexes, notably non-ribosomal peptide synthetases (NRPS), which are responsible for the synthesis of the linear peptide sequence and cyclization to generate the depsipeptide.

NRPS are modular enzyme complexes that are composed of three core catalytic domains, an adenylation (A) domain to select and activate an amino acid building block, a thiolation (T or PCP) domain to hold the growing peptide chain, and a condensation (C) domain that catalyzes the amidation (**Figure 1.5**). Within these basic functions, there are several enzymes that can add diversity along the way including epimerization,³⁴ oxidation,³⁵ methylation,³⁶ and many more. Each biosynthetic step is divided into modules where a set of reactions occur, to then be transferred to the next module to extend the peptide until completed and released. Further post-NRPS modifications, often called tailoring reactions, are possible such as methylation and oxidation often performed by a methyltransferase or a cytochrome P450 enzyme respectively.

Cyclic depsipeptides are cyclized via an internal hydroxyl nucleophile, catalyzed by thioesterases (TEs) (**Figure 1.5**) in bacteria and specialized C domains in fungi,³⁷ forming the final ester bond and releasing the product off the biosynthetic machinery. Nearly all depsipeptides are cyclic, containing one ester such as ecumicin or multi-ester such as enterobactin; however the linear asperphanemate is the only linear depsipeptide known to date³⁸ (**Figure 1.5**).

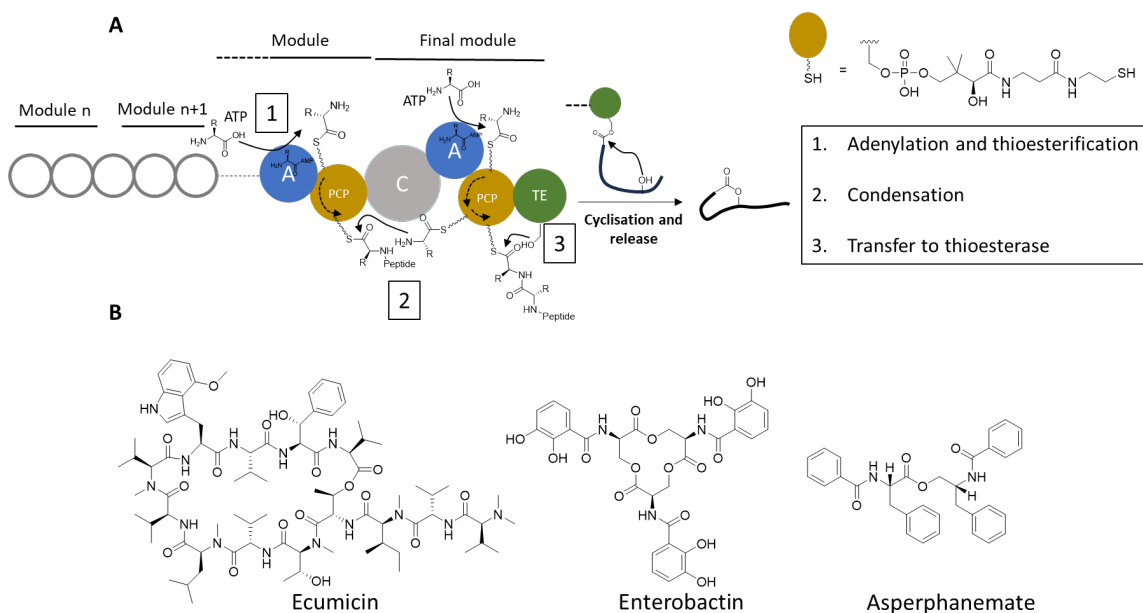


Figure 1.5 Biosynthesis of depsipeptides

A. General depsipeptide biosynthesis. **B.** Cyclic mono-, multi-ester containing depsipeptides and linear depsipeptide

Depsipeptides are used by their producing organisms as signalling molecules, for ion shuttling, notably as siderophores, and for interspecies competition. As such they exhibit a range of pharmacological activity including antibiotic, immunosupresant, surfactant, piscicide, anticancer, and antiviral among others conferred by their structural diversity, their cyclic nature, and for the presence of cyclic esters, which can further confer protease resistance.³⁹

The total synthesis of depsipeptides has been a source of interest to chemists for years due to their biological relevance and synthetic complexity. These non-ribosomally synthesized peptides (NRPs) contain proteogenic and non-proteogenic amino acids conferred to by the biosynthetic machinery. This is a commonly found challenge in NRPS natural products, such as the development of synthetic routes to (2*S*,3*R*,4*R*)-4,5-dihydroxy-isoleucine found in α -amanitin.⁴⁰ A constant challenge in depsipeptide synthesis is the generation of macrocyclic esters. These come with their own unique challenges. Synthetic strategies to generate macrolactones have been a rich

area of synthetic development since the introduction of the seminal Corey-Nicolaou⁴¹ and Yamaguchi macrolactonizations⁴². These reactions have been used in the synthesis of numerous macrolactone containing natural products;⁴³ however macrocyclic depsipeptides are often not suited for these harsh reaction conditions due to the epimerizable nature of the activated ester and the reactivity of amino acid functional groups⁴⁴. Several milder alternatives to the synthesis of macrolactones exist such as the Yonemitsu esterification⁴⁵, which utilizes catalytic amounts of DMAP, the mild Mitsunobu reaction⁴⁶, where attention to the alcohol nucleophile is important, or recently with the use of thioamides in conjunction with silver salts.⁴⁷ Alternatively, macrocyclization through lactamization allows for the introduction of linear esters utilizing mild methods that can greatly reduce the risk of epimerization.

With the combination of mild esterification chemistry and the high level of structural diversity gained from biosynthesis, solid-phase peptide synthesis strategies have been adapted to obtain these compounds, often with fully on-resin synthetic approaches allowing for the complete synthesis of a complex natural product with only a single HPLC purification. These synthetic strategies will be reviewed in chapter 4 and the cyclic depsipeptide seongsanamide E is chemically total synthesized and chemoenzymatically synthesized in chapter 5.

1.5 Depsipeptides – Chemoenzymatic strategies and substrate synthesis

The final enzymatic step in the biosynthesis of cyclic depsipeptides often involves the macrolactonization and release of the peptide. In NRPS and PKS biosynthetic pathways, this step is often performed by a TE,⁴⁸ an α/β -serine hydrolase that cyclizes a enzyme bound thioester substrate. In depsipeptide biosynthesis, the substrate is a peptidyl chain containing an internal alcohol nucleophile. TEs have been the source of substantial research interest due to their role in

the biosynthesis of potent natural products and their potential application as powerful biocatalysts to access otherwise synthetically challenging cyclic peptides.⁴⁹⁻⁵² For this reason, our group and several others have been interested in the biochemical characterization of TEs from new biosynthetically characterized pathways (**Figure 1.6**).

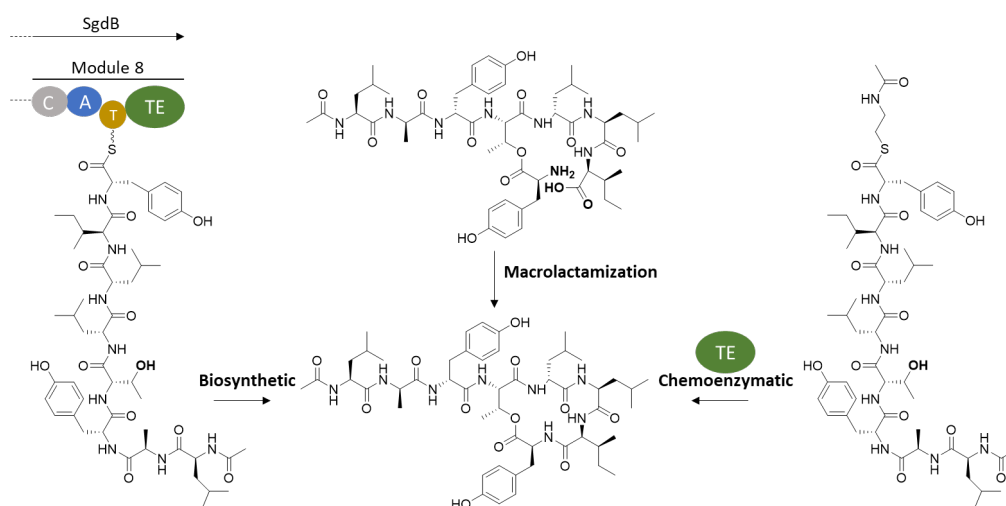


Figure 1.6 Total and chemoenzymatic synthesis of depsipeptide natural products

Characterization of a new TE domain typically involves the expression and purification of the excised TE from its biosynthetic machinery and the synthesis of substrates to test the activity and the efficiency of the enzyme. Typically, the natural product is chemically synthesized to confirm the enzymatic product, providing an enzymatic characterization, a natural product total synthesis and a chemoenzymatic synthesis in the same study (**Figure 1.6**). An example of this type of study will be covered in chapter 5 of this thesis. Modern peptide synthesis has been massively enabled by SPPS. It provides automated, high yielding routes to numerous complex peptides as is used throughout this thesis. Key aspects are reviewed below.

With the C-terminus anchored, C-terminal modifications of peptides, particularly C-terminal thioesters in Fmoc-SPPS, have been the subject of several impressive strategies such as safety catch⁵³⁻⁵⁵ and acyl shifts⁵⁶ custom resins, post synthetic modifications and alternative SPPS

modifications.⁵⁷ Thioester peptide synthesis has been developed mainly for native chemical ligation⁷ however the synthesis of short C-terminal thioester peptides are often required in chemoenzymatic synthesis of cyclic peptides, as seen in chapter 4.

1.6 Solid-phase peptide synthesis

SPPS predominantly synthesizes peptides from C- to N-terminus. The growing peptide is anchored by its C-terminus to a solid support and the N-terminus is deprotected and coupled with an incoming amino acid activated ester, growing the peptide chain by one residue. Protecting groups are essential for effective SPPS. The use of carbamate protecting groups on the α -amine of the incoming amino acid such as N-Boc and later N-Fmoc enabled effective masking of the α -amino reactivity and were readily deprotected without cleavage of the growing peptide from the stationary phase. These protecting groups have the added advantage of suppressing epimerization of the activated ester intermediate and the introduction of low epimerization coupling agents reduced unwanted oxazolone formation even more, enabling very high yielding couplings (**Figure 1.7**).

SPPS has been a rich area of research to optimize and diversify peptide synthesis. Boc-SPPS was the first widely used strategy due to the efficient solubilization of the resin-bound peptide in trifluoroacetic acid (TFA) during N-terminus deprotection, the exceptionally quick and high yielding Boc deprotection⁵⁸ and the synthesis of long and difficult peptides.⁵⁹ The major downfall of this approach is the utilization of dangerous anhydrous HF to perform the final peptide resin cleavage and sidechain deprotections.⁵⁷ To circumvent these problems, Fmoc-SPPS was explored as an alternative,⁶⁰ enabling base-mediated N-terminus deprotection and milder acidic conditions with TFA for final resin cleavage and sidechain protecting group removal.

Different types of Fmoc-SPPS solid supports exist, composed of a variety of linkers and matrices used for specific utilizations. The crosslinked-polystyrene matrix is the most prevalent due to a range of loading rates, high swelling in DMF or DCM and efficient reagent diffusion within the polymer matrix. Other types such as crosslinked polyamide- and PEG-based resins are more hydrophilic, allowing for lower loading rates and often higher efficiency for longer and tougher peptide sequences. Peptides are mainly C-terminally anchored through a covalent linker and there are several types, depending on the desired C-terminus following peptide cleavage. Some popular linkers yield C-terminal amides such as Rink amide, Pal and Sieber, often desired in medicinal chemistry to reduce overall peptide charge or C-terminal carboxylate including Wang, 2-chlorotrityl chloride and HMPB (**Figure 1.7**)⁶¹ often involved in peptide natural product total synthesis.

SPPS relies on high yielding individual steps to have the very best outcome. There has been over a century of peptide synthesis development, specifically in coupling reagent research including carbodiimides (DCC, DIC, EDC, etc.), phosphonium based (BOP, PyBOP, etc.), uronium/ammonium (HBTU, HATU, etc.) and many more⁶² (**Figure 1.7**). These combined with certain additives have been used to suppress epimerization to almost undetectable levels and tremendously high coupling efficiencies especially under microwave irradiation. There isn't an overall best coupling agent however some are preferred based on peptide sequence, such as N-methylated amide formation,⁶³ safety, such as Oxyma over benzotriazole HOBt and HOAt explosive properties,⁶⁴ and synthesis method, whether it is room temperature or microwave assisted SPPS.⁶⁵

Amino acid sidechain diversity requires functional protecting groups to permit orthogonal chemistry and efficient removal in peptide deprotection. These focus on stability during synthesis

and avoiding side reactions. Common SPPS side reactions include aspartimide formation during Asp coupling notably in an Asp-Gly sequence, δ -lactam formation during Arg coupling,⁶⁶ epimerization during Fmoc-His(Trt)-OH coupling and several more which have been highly documented.⁶⁷

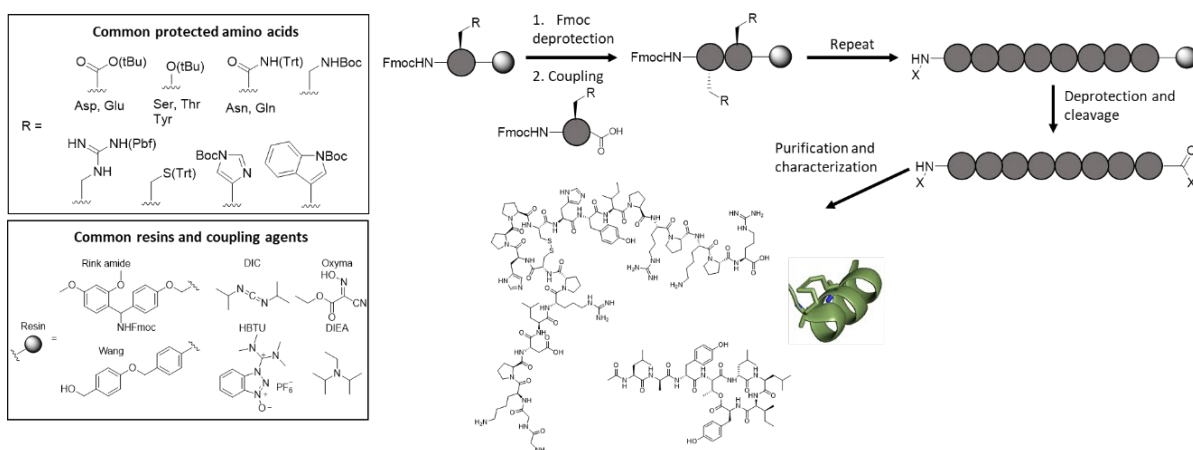


Figure 1.7 Solid phase peptide synthesis workflow

The fundamental concept of SPPS involving an insoluble solid support permits repetitive chemistry followed by simple washing before subsequent steps. Hand coupling in simple fritted vessels allows for effective peptide synthesis; however automated instruments have quickly improved peptide synthesis by relying on pumps and valves to conveniently perform the synthesis following a simple sequence programming.⁶⁸ Microwave irradiation has also been adopted to dramatically decrease reaction times and increase crude product yield.⁶⁹ The combination of automation and microwave irradiation has significantly facilitated peptide synthesis and is easily tunable for phosphopeptides, glycopeptides and several other types of peptides.⁶⁹ Flow systems have been developed to perform efficient automated peptide synthesis, accomplishing amide bond formation in 7 seconds and completion of an amino acid cycle in 40 seconds.⁷⁰

1.7 Summary and thesis objectives

In summary, natural product cyclic peptides have unique properties, making them an attractive target in chemical biology and medicinal chemistry. Stapled peptides have been widely used to selectively disrupt protein-protein interactions however they are infrequently used to disrupt protein-DNA binding. With the careful design of transcription factor-based stapled peptides, compounds can be rapidly identified to bind directly to a specific DNA promoter sequence. In chapter 2, the literature regarding DNA binding stapled peptides will be reviewed, highlighting the few examples to date. This work is published as a first author review article in ChemBioChem. In chapter 3, a previously identified RpoN inhibiting stapled peptide will be optimized through the synthesis of a library of stapled peptides and characterization of their biophysical and pharmacodynamic properties.⁷¹ This work was published as a first author manuscript in RSC Medicinal Chemistry.

Depsipeptides, an important subclass of cyclic natural product, are biosynthetically macrocyclized by a class of enzymes called TEs. Characterization of these TEs provides insight into the biosynthesis and helps identify biocatalysts for complex molecule syntheses. These studies require the synthesis of thioester activated substrates and the total synthesis of natural products themselves. In chapter 4, the strategies for synthesis of cyclic depsipeptides using SPPS will be reviewed, examining selected natural product syntheses from 2001-2023. This work is published in Organic & Biomolecular Chemistry as a first author review article. In chapter 5, the biosynthetic characterization of the seongsanamide E TE is described. Synthetic peptide substrates and synthetic natural products were used to accomplish the first seongsanamide E chemoenzymatic synthesis. This work is published as a co-first author manuscript in Organic Letters. In chapter 6, the synthesis of a unique cyclic depsipeptide fusarinine C will be investigated including the

synthetic efforts and future suggestions towards an inhibitor for the characterization of the adenylation domain of the NRPS SidD. Lastly in chapter 7, future directions are presented regarding future generations of RpoN stapled peptides, discussion on several ongoing cyclic depsipeptide projects and a short discussion on the total synthetic efforts of fusarinine C.

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Chapter 2: DNA binding stapled peptides - A review

2.1 Introduction

DNA-protein interfaces have high potential as inhibitor targets since they are often involved in the initial complex of transcription factor binding to initiate downstream transcription. This downstream transcription can lead to the expression of proteins involved in cancer cell survival¹ and bacterial virulence factors. There has been development of several DNA binding inhibitors such as small molecules, miniature proteins², and more recently stapled peptides that effectively inhibit these types of binding events. Transcription factors bind to DNA promoters through conserved DNA-binding motifs, such as leucine zippers, helix-turn-helix, zinc fingers and as well as others³. These motifs mainly bind DNA through an α -helix, making them promising candidates for the development of α -helical stapled peptides.

Despite this parallel, there are only a few DNA-binding stapled peptides. Due to the large surface area of a DNA-promoter interaction, small molecule DNA binders typically lack sequence specificity. However, stapled peptides are ideal candidates for major groove binding. Design of these inhibitors is highly similar to the more common protein-protein inhibiting stapled peptide⁴. Incorporating the native binding sequence of the α -helix to the inhibitor is key to maintain binding specificity and the stapling residues should be installed on the opposite face of the binding event. Insights from crystal structures and solution NMR structures of transcription factors binding to DNA are key when deciding peptide length and which residues to replace with stapling residues. The inhibitors can be readily synthesized using standard Fmoc solid phase peptide synthesis, incorporating the chosen residues for cyclization. With inhibitors in hand, *in vitro* binding assays, cellular permeability assays, microscopy and inhibition assays will help biochemically characterize and confirm the specific binding to the promoter.

This review looks at the DNA-binding stapled peptides in the literature up to 2023, highlighting their design similarities to the more popular protein-protein interaction inhibitors.

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2.3 Author contributions

A.R.P. and C.N.B. conceptualized the work, A.R.P. wrote the original draft and A.R.P. and C.N.B. edited and finalized the draft.

2.4 Copyright

Paquette, A. R. & Boddy, C. N. Double stranded DNA binding stapled peptides, an emerging tool for transcriptional regulation. *ChemBioChem*. (2023). Early view, DOI: <https://doi.org/10.1002/cbic.202300594>. Reproduced by permission of Chemistry Europe, John Wiley & Sons, inc.

2.5 Double stranded DNA binding stapled peptides, an emerging tool for transcriptional regulation

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Introduction

Stapled peptides are a powerful chemical biology strategy for selectively disrupting protein-protein interactions (PPI) on intracellular or secreted proteins^[1,2] through the use of short α -helices that mimic one of the PPI binding partners^[3,4]. Peptide stapling was pioneered by Schafmeister *et al.* in 2000's and involved constraining a short peptide's conformation by the addition of a covalent bridge or staple, locking it into an α -helical conformation^[5]. This led to the development of the first active stapled peptide, which bound the BH3 portion of MDM2^[6]. In addition to providing insight into biology, the disruption of PPIs by stapled peptides has led to the development of new therapeutic strategies including the first stapled peptide in clinical trials, ALRN-6924, a HDM2M/p53 all-hydrocarbon stapled peptide (clinicaltrials.gov identifier: NCT04022876).

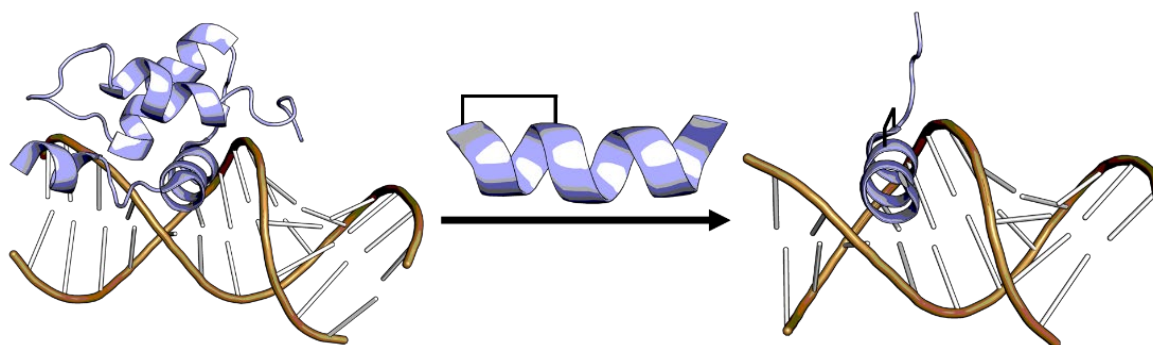


Figure 2.1 DNA binding stapled peptide fundamental design. (Generated from PDB: 2O8K)

Not surprisingly, a number of stapled peptides have been designed to target transcription factors (TFs) to modulate downstream gene regulation. These stapled peptides target TF PPIs, disrupting the transcriptional machinery complex and thus modulating transcription^[7]. TFs, other initiators, and repressors however all bind DNA directly and disrupting this binding will also modulate transcription^[8–10]. Sequence specific protein binding to double stranded DNA^[11], as is seen with TFs, is mediated by α -helices binding in the major groove of DNA. This presents an ideal opportunity for development of stapled peptides to disrupt TF-DNA binding^[12]. Surprisingly however, DNA-binding stapled peptides are not yet widely seen.

The successful design of a stapled peptide requires detailed knowledge of the binding interaction between the protein and its binding partner. Some recurring features of stapled peptide design are essential. Key binding residues must be maintained in the stapled peptide. The staple should not obscure the binding interface. Lastly, it is important to recognize the effects of the other residues along the peptide sequence^[13]. Medicinal chemistry approaches can be employed to optimize the peptide through library synthesis, including synthesis of staple walk libraries and residue substitution libraries. Both PPI's and DNA-protein binding interactions have been structurally characterized, either via high-resolution crystallography or NMR methods, yielding essential information on the target interaction to facilitate the design of these peptide drugs¹⁴.

This review will examine published DNA binding stapled peptides (**Fig. 2.1**) between 2002 and the present by comparing them with three representative modes of protein binding stapled peptides. The differences and similarities in binding are analyzed in-depth to identify key features for design, development, and optimization of DNA binding stapled peptides.

Stapled peptides

Short peptide sequences of less than 20-30 residues are unstructured in aqueous solution. The backbone hydrogen bonding interactions essential for secondary structure formation can be easily satisfied by water and thus do not provide sufficient energetic stabilization to overcome the entropic penalty of folding^[15]. The installation of a chemical crosslink or staple between residues can constrain the conformation of the peptide, enabling stable secondary structure elements, such as α -helices, to be maintained in short peptides in aqueous environments^[16,17]. While unstructured peptides are known to have limitations in a biological environment, including low cellular membrane permeability^[18], low proteolytic stability in blood plasma^[19], low oral bioavailability and low solubility^[20,21], the structured nature of stapled peptides can overcome these limitations. The rigidity and reduction in polarity and number of hydrogen bond donors and acceptors from burying the backbone amides in the α -helical core gives these peptides a more small molecule drug-like profile with respect to physicochemical and pharmacokinetic properties. Unlike small molecules, stapled peptides also provide a large surface area for binding, enabling them to interact selectively and with high affinity to large shallow binding cavities to which small molecules cannot bind effectively. As such, stapled peptides represent an ideal middle ground between small molecules and biologics for drug development.

A chemical staple can be any type of covalent linkage between any two natural or unnatural amino acid sidechains on a peptide. To reduce the possibility of side-reactions and optimize synthetic yields, chemical stapling strategies require the use of mild and orthogonal reactions. Peptides are generally synthesized following standard solid phase peptide synthesis (SPPS) protocols, incorporating the amino acids for the chosen type of chemical stapling^[22,23]. To access α -helical stapled peptides, the stapling residues are usually inserted on the same peptide face at the

i and $i+4$, i and $i+7$, i and $i+11$ and i and $i+3$ positions^[13] of a standard 3.6 residue per turn α -helix (3.6₁₃-helix). The multiple types of effective stapling strategies include lactamization^[24], Cu-catalyzed cycloaddition^[25], cysteine alkylation^[26,27] and many others^[28,29]. The all-hydrocarbon staple stands out amongst these strategies due to the efficiency and robustness^[6,17,30].

Stapled peptides that disrupt PPIs

Stapled peptides that can disrupt PPIs typically rely on a large number of hydrophobic interactions between interacting residues, with specificity driven through a few well-placed, desolvated hydrogen-bonds and salt bridges^[31], mimicking native protein-protein interactions^[31]. The staple often participates in generating stabilizing contacts with the target, particularly with the all-hydrocarbon staples, which provide a large hydrophobic surface for binding^[32] and solvent shielding^[33]. The following examples will examine specific stapled peptides, their design and their binding characteristics.

Amphipathic faces can drive high affinity and selective binding in BCL-2-based stapled peptides

Walensky *et al.* developed and applied the first all-hydrocarbon stapled peptide targeting the apoptotic controlling BCL-2 family of proteins. A series of stabilized α -helix of BCL-2 domains (SAHBs) were synthesized. These mimic the key interacting helix of the BH3 domain of BID, a pro-apoptotic protein with multiple roles in apoptosis signaling within the BCL-2 family^[34]. This helix binds a hydrophobic groove on the anti-apoptotic BCL-2 and BCL-XL and prevents them from binding BAK/BAX, ultimately activating apoptosis. Inserting stapling residues at i , $i+4$

positions opposing the binding face of the key amphipathic α -helix from BID generated a short peptide that retained helical structure, was protease resistance *in vitro* and *in vivo*, and bound BCL-XL with nanomolar affinity^[35].

Following this strategy, a number of high affinity stapled peptides based on amphipathic helices from BH3 domains have been generated to promote apoptosis. In particular Harvey *et al.* developed a high affinity all-hydrocarbon stapled peptide based on the NOXA BH3 domain with an *i, i+7* staple, binding to its target BFL-1, and were able to obtain a high resolution co-crystal structure of the SAHB stapled peptides bound to BFL-1^[36] (**Fig. 2.2A**). From the structure it is clear that the stapled peptide has two distinct faces. The electrostatic face establishes key hydrogen-bond interactions between stapled peptide residues Arg30, Asp34 and Asn37 and corresponding BFL-1 hydrogen-bond partners Glu80, Arg88 and Lys147 respectively (**Fig. 2.2B**). The hydrophobic face consisting of residues Leu29, Ile36 and Phe32 sits in the BFL-1 hydrophobic groove interacting with Val40, Val44, Val48 and Val90 residues^[36] (**Fig. 2.2C**). This amphipathicity further helps the peptide with solubility and cellular permeability^[37].

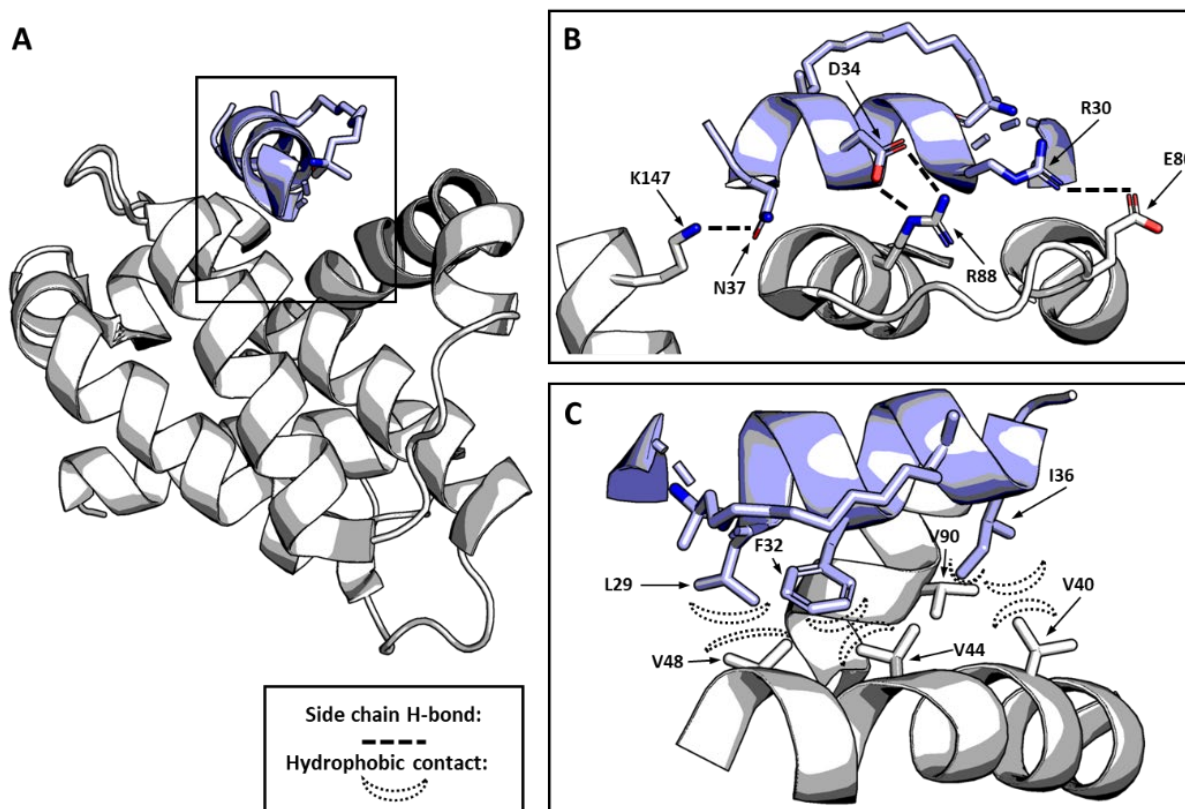


Figure 2.2 Amphipathic interaction of BCL-2 based stapled peptides.

A. SAHB stapled peptide bound to BFL-1. **B.** Hydrogen-bonding of electrostatic stapled peptide face. **C.** Hydrophobic face interactions of stapled peptide with BFL-1 hydrophobic groove (Generated from PDB: 5WHH)

Involvement of hydrophobic staple interactions in ER- β and p53 binding stapled peptides

Estrogen receptor (ER) activation plays a key role in a number of disease states, including breast cancer and osteoporosis. ER activation is mediated by cytosolic estrogen binding to the C-terminal domain, leading to homodimerization of the ER. Signalling is also dependent on coactivation of the ER through binding of an α -helix from coregulatory proteins to the ligand binding domain (LBD) of the ER. This helix is a short leucine rich motif, ILXXLL, where the leucine residues bind the hydrophobic surface of the ER and the helix dipole interacts with charged

residues in the ER that flank the N- and C-terminus of the helix. Philips *et al.* designed and synthesized all-hydrocarbon stapled peptides (**SP1 – SP6**) to bind the ER- β isoform (Er β) LBD with high affinity^[32]. A co-crystal X-ray structure of **SP1** and **SP2**, which contain an *i, i+4* staple, bound to the Er β LBD showed the peptide in the hydrophobic groove and the N-terminal Lys and C-terminal Asp of the helix interacting with the flanking charged residues of the Er β LBD. Surprisingly however the structures showed the staple binding to the hydrophobic groove rather than the conserved leucine residues. Thus in addition to constraining the conformations and perturbing the physicochemical properties of peptides, the hydrophobic all-hydrocarbon staple can contribute to target binding affinity, particularly for interactions with large hydrophobic surfaces.

Contributions of the staple to binding affinity have also been seen with stapled p53-based peptides. Transcription factor p53 plays an important regulatory role in cell development by activating apoptotic programs to block replication of faulty cells. The transcription factor is negatively regulated by binding of the E3 ubiquitin ligase MDM2, which is overexpressed in some cancer cells. p53 binding to MDM2 is mediated through Phe19, Leu22, Trp23, and Leu26 of the N-terminal α -helix of p53 interacting with well-defined hydrophobic surface pocket in MDM2^[38]. Based on this interaction, potent MDM2 binding stapled peptides including **SAH-p53-8**^[39] have been generated.

A co-crystal structure of the *i, i+7* stapled **SAH-p53-8** bound to the MDM2 hydrophobic pocket showed the expected interactions between the stapled peptide Phe19, Trp23 and Leu26 residues and MDM2/MDMX^[40]. The staple however impacted the binding mode of Trp23. Instead of forming a hydrophobic interaction, the Trp23 indole nitrogen hydrogen-bonded with MDM2 Leu54 backbone amide, which was protected from solvent by the staple position (**Fig. 2.3**). The aliphatic staple also participates in extensive hydrophobic interactions with Leu54, Phe55, Gly58

and Met62 of MDM2, significantly stabilizing the interaction (**Fig. 2.3**). These two examples clearly demonstrate the importance of the staple on binding mode and affinity and thus the importance of probing staple placement. Interestingly, staple participation in large hydrophobic interacting networks appears to be unique to stapled peptides that disrupt PPIs.

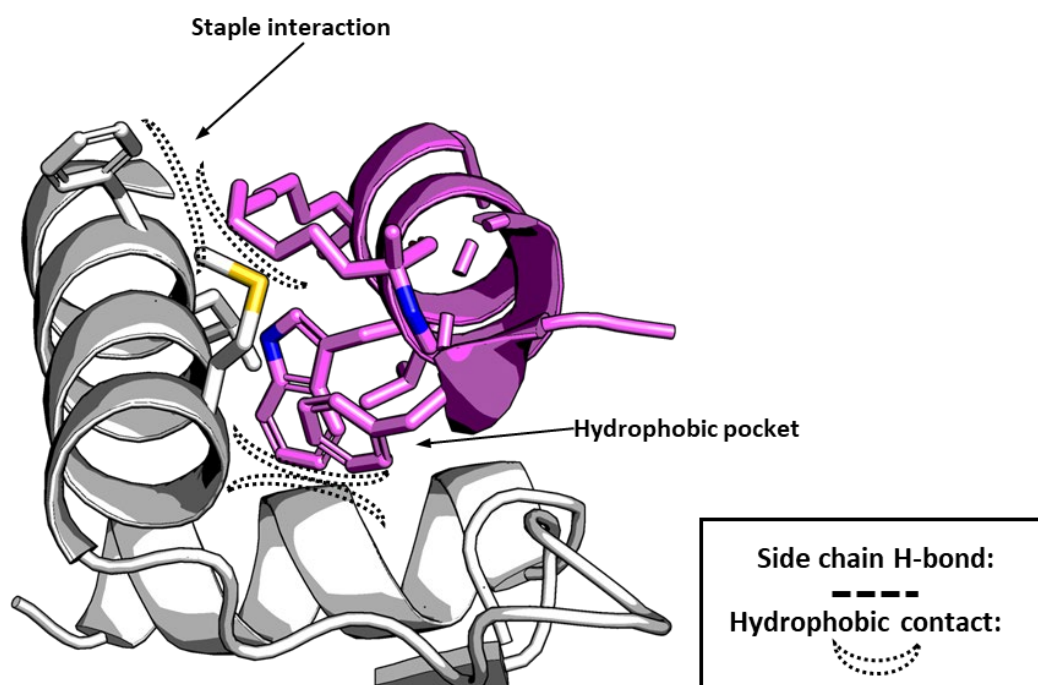


Figure 2.3 Staple participation of SAH-p53-8 binding to MDM2 with solvent shielding and extensive hydrophobic network. (Generated from PDB: 3V3B)

Additional stabilization of staple peptide by intramolecular interactions can boost affinity

Increased oncogene translation is a hallmark of some cancers and is mediated by eIF4E overexpression. eIF4E is a component of the eIF4F trimeric protein complex that is responsible for binding the 5' cap of mRNA in eukaryotic translation initiation^[41]. In addition to eIF4E, the complex contains the RNA helicase eIF4A and the scaffolding protein eIF4G. eIF4G binding to eIF4E is mediated by the α -helical eIF4G1 binding epitope, which binds a large planar binding region in eIF4E. In particular, Tyr4 from eIF4G forms a key hydrogen-bond with eIF4E. Arg6 generates a salt-bridge with eIF4E E132. Lastly Leu9, Leu10 and Leu12 all form hydrophobic interactions with eIF4E. Based on this interaction, Lama *et al.* synthesized and evaluated a series of stapled peptides based on the eIF4G interacting helix from eIF4E^[42]. One of the tightest binders was the *i, i+4* staple containing **sTIP-04 (Fig. 2.4A)**.

The high-resolution structure of **sTIP-04** complexed to eIF4E shows hydrophobic packing between the leucine residues and the α -helix binding site. The key electrostatic interactions are between stapled peptide Tyr4 phenol, Leu10 backbone and Arg6 and protein Pro38 backbone, Trp73 and Glu132 sidechains

respectively (**Fig. 2.4B**). The *i, i+4* all-hydrocarbon stapling residues offer a notable intramolecular interaction between Ser5 sidechain and neighboring Gln5, staple residue 7 and Arg6 backbone amides^[42,43] (**Fig. 2.4C**). These intramolecular interactions enhance the stapled peptide's stability, previously reported in eIF4G binding peptides, where helicity formed in the binding event can further stabilize the interaction^[44]. In general stapled peptides will benefit from these intramolecular stabilizing interactions, which further reduce the entropic cost of binding by

rigidifying the peptide and increasing the enthalpic stabilization of binding by more precise geometric alignment of the stabilizing interactions.

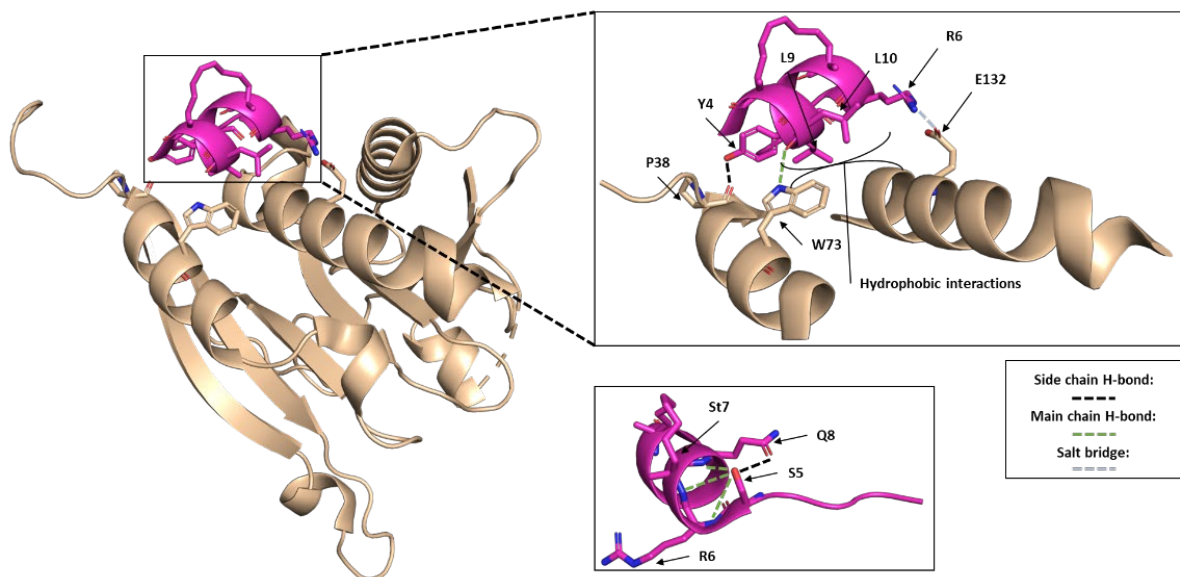


Figure 2.4 sTIP-04 peptides interacting with eIF4G, highlighting intramolecular sidechain-backbone interactions.

(Generated from PDB: 4BEA)

This analysis of stapled peptides that disrupt PPIs highlights a select few stapled peptides, representative of some high-level design features that will be compared to DNA-binding stapled peptides. For a more in-depth reviews of stapled peptides that disrupt PPIs please see these excellent review articles^[45–49].

Affinity and Selectivity in DNA-protein interactions

While there are a large number of proteins that bind DNA, many bind with little sequence specificity. For example histones are the most prevalent DNA binding protein in eukaryotes and

they bind DNA in a non-sequence-specific manner^[50], primarily relying on hydrogen bonding interactions from the histone to the phosphodiester bond of the DNA and salt bridges between Arg sidechains positioned in the DNA minor groove and the phosphodiester chain^[51]. Many other types of common DNA binding proteins and enzymes such as exonucleases, polymerases and transcription activator-like (TAL) effectors^[11,52–54] also bind DNA with little to no sequence specificity through similar types of mechanisms.

To selectively regulate gene transcription, TF must bind their cognate DNA promoter sequence-specifically. TFs rely on non-specific low affinity DNA binding interactions to associate with DNA before scanning the genome to identify their high affinity binding sequence^[55,56]. Electrostatic interactions between the negatively charged DNA and positively charged regions of the protein play a role in these non-specific, transient DNA-protein interactions. They are also mediated by direct and water-mediated hydrogen-bonding to the phosphate backbone and sugars and salt bridges between the DNA backbone and protein sidechains^[57].

Double stranded DNA in most DNA-protein interactions retains its right-handed B-DNA form with a major and minor groove. The major groove is the main binding site for sequence-specific protein binding interactions and these associations are driven by enthalpic contributions,^[58] including specific DNA base hydrogen bonding and hydrophobic Van der Waals interactions^[59] (**Fig. 2.5**). Within the major groove, nucleobase interactions involve the carbonyl oxygens and amino groups from the purine and pyrimidine residues (**Fig. 2.5A**). For example, N η and N ϵ of Arg can form bidentate hydrogen bonds with N7 and O6 of guanine (**Fig. 2.5B**). Similarly, Lys can make strong hydrogen bonds with both guanine or thymine. These three interactions constitute the most energetically favorable direct sidechain-base interactions^[60], though a large variety of additional direct and water mediated weaker hydrogen bonds occur between amino acid sidechains

and DNA bases^{[52],[61]}. In addition, hydrophobic interactions also have an important role in DNA binding. Selectivity is primarily generated via differentiating between the major groove exposed methyl of thymine (**Fig. 2.5C**) versus unsubstituted cytosine^[62].

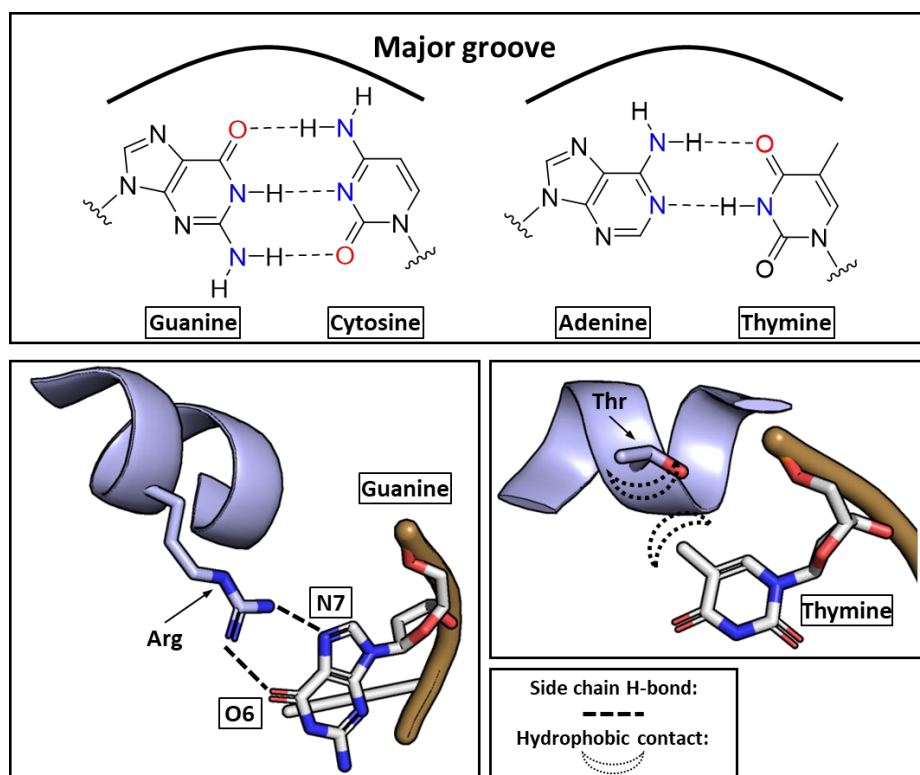


Figure 2.5 DNA major groove binding interface and examples of predominant helix-DNA H-bonding (Generated from PDB: 2O8K).

The α -helix is the most common directly interacting protein secondary structural feature for sequence specific DNA binding^[52]. This is consistent with the complimentary sizes of the α -helix and the major groove^[63]. DNA-binding helices can be found in many different motifs and folds including helix-turn-helix (HTH), leucine zipper^[64], helix-loop-helix (HLH), and the zinc finger^[52]. The HTH motif is the most common and is found in transcription factors across the tree of life. The second helix of this motif, the recognition helix, inserts into the major groove contacting 4-10 base pairs and the backbone. Combining multiple DNA binding elements enables

high affinity protein-DNA interactions as well as more specific binding by increasing the length of the recognition sequence. For example, dimeric TF that bind DNA via HTH motif typically recognize inverted sequence repeats, doubling the recognition length and increasing selectivity by orders of magnitude.

Designer peptides that selectively bind DNA

Small molecules that bind DNA tightly have been developed. For example, polyamides^[65–67] and intercalators^[68] can bind with high affinity, however they are often incapable of sequence-specific binding. To access small molecule-like compounds with sequence-specific binding, DNA-binding elements from proteins have been successfully mimicked. For example, the c-Myc/Max heterodimeric HLH zipper transcription factor, which is dysregulated in many cancers^[69], was used as the template to develop a selective DNA binding agent to inhibit c-Myc transcription. HLH DNA binding domains are thought to be unstructured to scan the genome and adopt a folded structure upon binding their cognate DNA sequence^[70,71]. Thus unstructured short linear peptides (15-20 residues) from the DNA binding domain of c-Myc and Max were covalently linked to a steroid scaffold and shown to have moderate μM affinity for the promoter DNA sequence^[72]. While not a high affinity interaction, this work demonstrates the design principle of mimicking the DNA binding α -helix to obtain sequence specific binding.

Providing a well folded mimic of the DNA binding domain can generate high affinity sequence-selective binders by eliminating the entropic penalty of helix folding. For example, Talanian *et al.* grafted the DNA interacting residues from the leucine zipper-based GCN4 TF onto the avian pancreatic polypeptide (aPP). aPP is a stably folded, short peptide consisting of an α -

helix stabilized by hydrophobic interactions with a type II polyproline helix. The corresponding 39 residue polypeptide generated a well folded mimic of GCN4 and was able to bind the consensus DNA sequence with high affinity^[73]. These examples clearly demonstrate the design principle that a folded, well-structured short peptide sequence mimicking the DNA binding domain from a sequence-selective DNA binding protein, will produce a high affinity sequence-specific DNA binding peptide.

DNA binding stapled peptides

Our understanding of the fundamental interactions that lead to high affinity sequence-specific DNA binding by proteins are well established and the most common DNA binding motifs are well described and characterized. Furthermore these motifs can be readily identified from primary sequence data. There is a clear need to disrupt sequence-specific DNA-protein interactions that drive TF factor binding and thus regulate transcription. There are limited chemical tools to do this selectively in cell culture and *in vivo*. It is thus surprising that the development of stapled peptides to disrupt TF-DNA binding has lagged. There are a plethora of stapled peptides to disrupt PPIs. The design of stapled peptides to disrupt DNA-protein interactions should follow a similar design strategy^[74] however there are only five examples of stapled peptides that disrupt DNA-protein interactions. Each of these five will be examined.

GCN4-based stapled peptides

The first reported stapled peptide based on a DNA binding domain was designed based on the GCN4 leucine zipper TF and reported in 2002^[75]. GCN4 is a well characterized TF in *S. cerevisiae*, with a mammalian homolog called AP-1. GCN4 acts as a master regulator for amino acid biosynthesis and is positively regulated in multiple stress conditions^[76]. The homodimeric TF binds 5'-TGAC-3' sequences (often called half-sites) on both strands of dsDNA^[77] (**Fig. 2.6A**). Hydrophobic packing plays a key role in binding this DNA sequence. For example the Thr236 methyl group interacting with Gua-32* ribose, Ala239 and Ala238 sidechain methyl groups pack against Thy-7 and Thy-33* methyl groups respectively, and a slight hydrophobic network is generated between Ser242 β -carbon and Thy-7 (**Fig. 2.6B**). Key hydrogen bonds that dictate binding selectivity are between GCN4 Arg243 and its half site Gua-32 and GCN4 Asn235 with Cyt-34*, Thy-7 and Ade-6 (**Fig. 2.6C**). In addition, these two amino acids also build extensive water binding networks^[78,79]. Lastly a number of charged and hydrogen bonding residues flanking the exposed edge of the major groove buried helix interact non-selectively with the phosphate backbone (**Fig. 2.6D**).

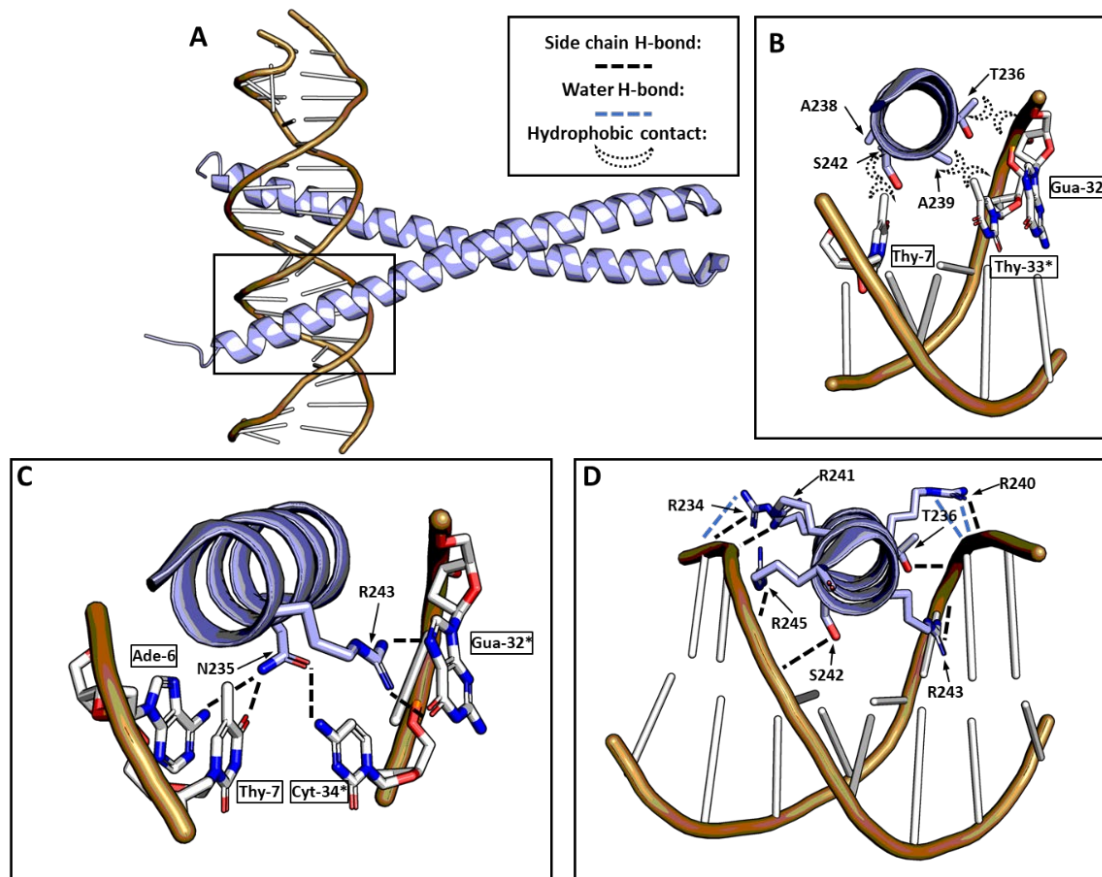


Figure 2.6 GCN4 leucine zipper interaction.

A. Homodimeric transcription factor binding to dsDNA. **B.** Hydrophobic packing to Thy-7 and Thy-33*. **C.** Sidechain hydrogen-bonding driving selectivity. **D.** Phosphate backbone hydrogen-bonding interactions. (Generated from PDB: 1YSA)

The first GCN4-based stapled peptide incorporated the 29-residue basic region of GCN4. In an attempt to staple the peptide into a helical conformation, the C-terminal aspartic acid sidechain carboxylate was lactamized with the sidechain $N\epsilon$ of Lysine in the $i-4$ position making an $i, i+4$ staple^[75]. The α -helical content of the stapled peptide was increased relative to the unstapled control as was the binding affinity to the half site. While the study showed a 1:1 stoichiometry of stapled peptide binding to DNA, it did not provide evidence of sequence-specific binding^[75]. In 2015, Iyer *et al.* used an alternative stapling strategy to generate a 23 amino acid

stapled GCN4-based peptide^[80]. The authors introduced two unique Cys residues at two different $i, i+4$ sites near the N-terminus of the sequence and in a third peptide at $i, i+7$ sites near the C-terminus. Alkylation of these Cys with different bis-ethylbromobenzene linkers generated five stapled GCN4-based peptides. Interestingly these stapled peptides showed limited helicity by CD in the absence of DNA, suggesting the staple does not restrict them into a helical conformation.

However, electromobility shift assays (EMSA) showed that the linear peptide did not bind DNA and that the two $i, i+4$ stapled peptides bound DNA at a lower concentration than the three $i, i+7$ stapled peptides. Binding affinities were not determined nor was sequence specificity evaluated. Also of note, the authors observed the formation of multiple bands in the EMSA assays with their $i, i+4$ stapled peptides, which they attribute to formation of 1:1 and 1:2 complexes of DNA to stapled peptide. Confocal microscopy confirms notable cellular uptake of stapled peptides inside RAW 264.7 mouse macrophages compared to the unstapled variant. The more helical $i, i+7$ stapled peptide showed the highest uptake, confirmed by flow cytometry.

Homeodomain Q50K-based stapled peptides

TFs that use HTH motif for DNA binding have also served as the inspiration for DNA binding stapled peptides. Many eukaryotic TFs use a HTH containing homeodomain to bind DNA^[81]. The 60 amino acid homeodomain is a highly conserved three helix bundle with helices 2 and 3 forming a HTH motif. Helix-3 is the DNA recognition helix making most of the contacts with the major groove. The flexible N-terminal arm interacts with the minor groove^[82] presumably displacing highly ordered water in an entropically driven interaction^[58]. The Q50K mutant of the *Drosophila* engrailed homeodomain binds the TAATCC sequence with high affinity and this

interaction has been well-characterized^[83,84] (**Fig. 2.7A**). A co-crystal structure of Q50K bound to DNA shows Lys50 is responsible for hydrogen-bonding with Thy-4, Gua-5* and Gua-6* (**Fig. 2.7B**). Additionally, Asn51 directly hydrogen bonds with Ade-3 as well as forming a water mediated contact with Ade-7*. Ile47 makes hydrophobic contacts with Thy-4 methyl group (**Fig. 2.7C**). These hydrogen bonding and hydrophobic interactions largely drive sequence specificity. The phosphate backbone is bound by nearly all residues with hydrogen bonding capabilities (**Fig. 2.7B**), stabilizing the α -helix further in the groove^[85].

In 2005, a DNA binding stabilized 18 residue homeodomain helix-3 was generated, retaining these key DNA interacting residues^[86]. Guerrero *et al.* used a photoisomerizable diazobenzene cross-linker staple between Cys residues inserted at positions i , $i+11$ on the non-binding face of the helix. In the more stable, relaxed state, the stapled peptide was shown to be helical by CD spectroscopy and bind its cognate DNA sequence with a $K_D = 7.5$ nM by fluorescence anisotropy. Excitation of the diazobenzene in the cross-linked form caused isomerization from *trans* to *cis*, disrupting the helical conformation and reducing DNA binding affinity by approximately 20 fold. Furthermore binding of the *trans*-diazobenzene stapled peptide was shown to be sequence-specific in their binding assay. The photocontrol of binding offers an intriguing method for temporal control of DNA binding. Cellular permeability and toxicity was not accessed.

Kajino *et al.* in 2011 synthesized a stapled peptide based on Q50K with two ornithine residues at positions i , $i+11$ and stapled with diacetylenic crosslink^[87]. In addition to a different staple, three of the bulky, outward facing, non-DNA contacting residues from the helix (Trp48, Phe49, Lys52) were replaced with Ala. CD spectroscopy showed stapling substantially increased the helical content of the peptide versus the unstapled sequence. The dissociation constants for

binding to its cognate DNA sequence was determined with a $K_D = 3 \pm 1$ nM in buffer supplemented with 300 mM NaCl. The cellular membrane permeability and toxicity was not evaluated in this study.

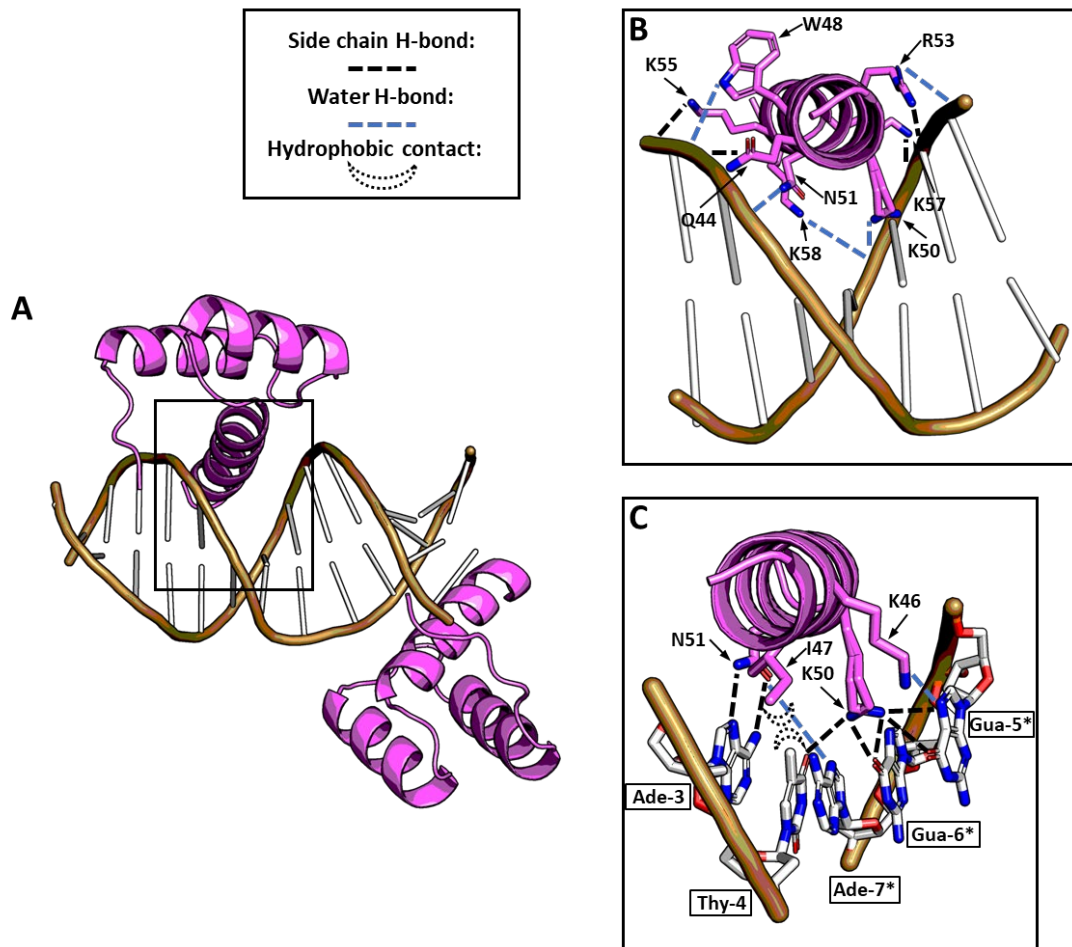


Figure 2.7 Homeodomain transcription factor.

A. HTH major groove helix binding. **B.** Helix-3 residues participating in phosphate backbone binding. **C.** Helix-3 residues performing direct hydrogen-bonding and hydrophobic interactions with the promoter nucleobases.

(Generated from PDB: 2HDD)

HNF-3 γ -based stapled peptides

Kajino *et al.* also generated a stapled α -helical peptide from the HTH motif of the hepatocyte nuclear factor 3-gamma (HNF-3 γ) FOX protein^[87]. The FOX (forkhead box) group of TFs are involved in cell growth, differentiation, and proliferation. They bind DNA via a 100 residue winged helix (wHTH) motif, which consists of two wings, three α -helices, and three β -strands^[88]. Helices 2 and 3 of the winged helix form a HTH structure with helix-3 functioning as the recognition helix for DNA binding (**Fig. 2.8A**). This α -helix binds the DNA major groove through direct sidechain hydrogen bonds between Asn165 and Ade-10 and His169 and Thy-8 (**Fig. 2.8B**). Water mediated hydrogen bonds between Phe173 and Asn174 and Gln-7 also add to the specificity of binding to its cognate DNA sequence, TAAGTCA^[89]. Interestingly, a magnesium ion is complexed to the C-terminus of helix-3 through the amide backbone of Leu171, Ser172, Asn174 and Phe177 (**Fig. 2.8C**). Due to the proximity of major groove to other secondary structure elements in HNF-3 γ , helix-3 has only a limited number of interactions with the phosphate backbone (**Fig. 2.8D**). These are from Arg162 and Ser172^[89].

Relying on this structural information, Kajino and coworkers designed a 16-residue stapled peptide. They relied on a different stapling strategy, opting for crosslinking between *i*, *i*+7 Orn residues with a isophthalate linker^[87]. Akin to their strategy for their Q50K stapled peptide, bulky and non-DNA-binding residues opposite to the DNA binding face of the stapled peptide were replaced with Ala. Lastly, an Arg repeat was added to the C-terminus generating a single stapled peptide, **hnf-1**. CD spectroscopy showed the stapled peptide to be substantially α -helical and to bind its cognate DNA sequence with a $K_D = 4$ nM, approximately 140-fold higher affinity interaction than with the unstapled control peptide. No evaluation of the sequence-specificity of the stapled peptide is reported.

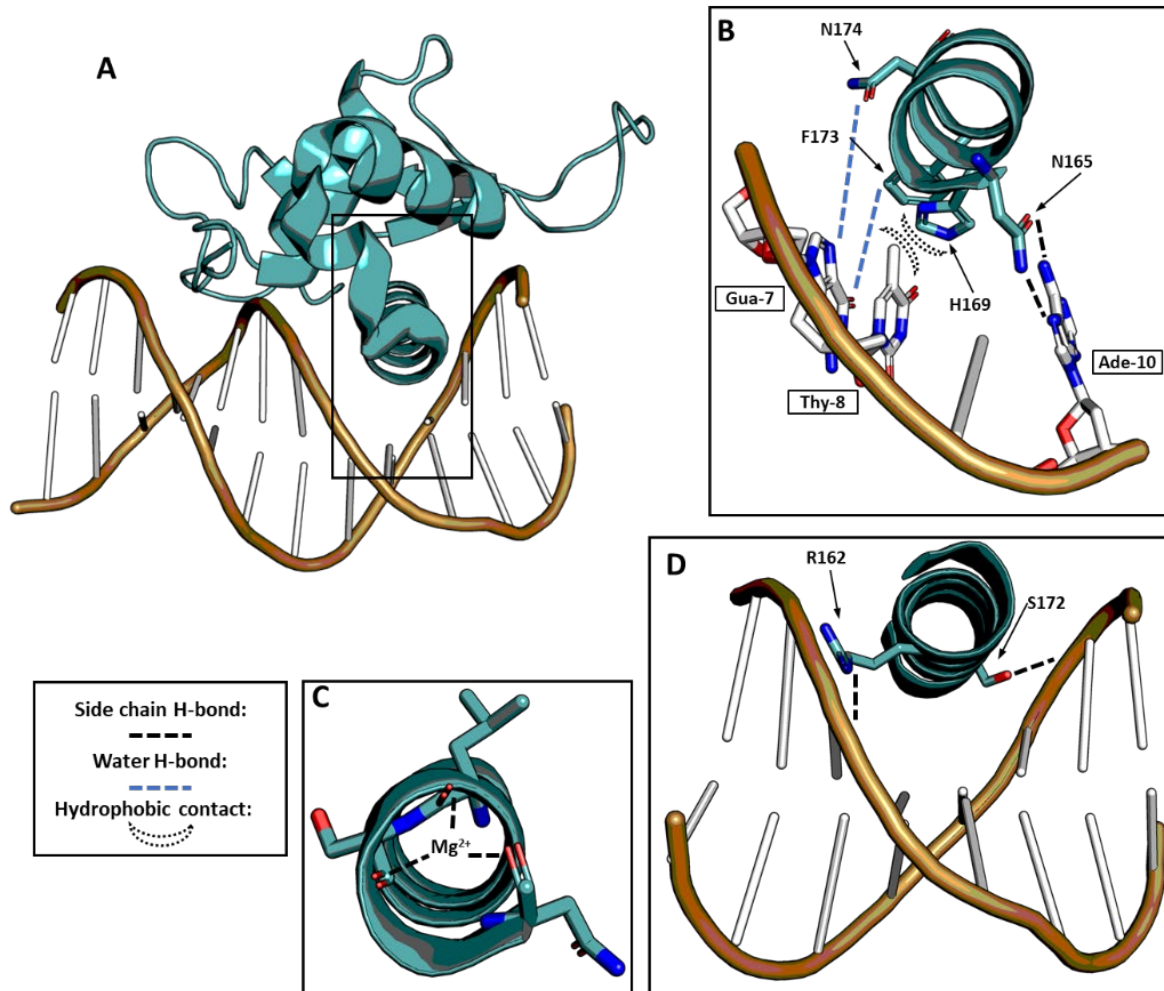


Figure 2.8 HNF-3 γ fox protein binding DNA with the forkhead box.

A. DNA binding through a wHTH motif. **B.** Helix-3 sidechain hydrogen-bonding with DNA nucleobases. **C.** Magnesium ion complexed to helix-3 C-terminus. **D.** Limited sidechain hydrogen-bonding to DNA phosphate backbone. (Generated from PDB: 1VTN)

Nrf2-based stapled peptides

In 2022, stapled peptides were used to inhibit the DNA-protein interaction of the TF Nrf2 (nuclear factor erythroid 2-related factor 2)^[90]. Nrf-2 binds DNA using a leucine zipper motif and is responsible for mediating the transcription of genes involved in protein degradation and in the

regulation of inflammatory responses^[91,92]. As it is over activated in tumour cells, leading to tumour progression and metastasis, it is an attractive therapeutic target. However, the disordered structure of leucine zippers when not bound to DNA^[91,92] makes design of small molecule inhibitors challenging. Disruption of Nrf2-DNA interaction with a stapled peptide is thus an appealing approach to Nrf2 inhibition.

Unlike the previous examples, no high-resolution structural data was available for the binding of Nrf2 to its cognate DNA sequence when these stapled peptides were designed. The authors relied on a homologous TF Skn1 crystal structure where it is clear from the primary sequence which residues form the recognition helix that binds the major groove^[93]. Since the publication of these stapled peptides a structure of Nrf2 bound to DNA was published (**Fig. 2.9A**)^[94]. It shows the α -helix to be highly cationic with five Arg and one Lys making contacts with the phosphodiester backbone (**Fig. 2.9C**). Selectivity is mediated through Asn507 hydrogen bonding to Thy-5 and Cyt-6* and via a water mediated hydrogen bond to Ade-4. Ala511 and Ala510 pack against the methyls of Thy-5 and Thy-7* (**Fig 2.9B**). This is an unusual interaction on two fronts. First there are a small number of lower strength, base specific hydrogen bonding interactions. Second while there are two significant hydrophobic interactions, these are mediated through the Ala side chains rather than the more common and more exposed β -methyl of Thr.

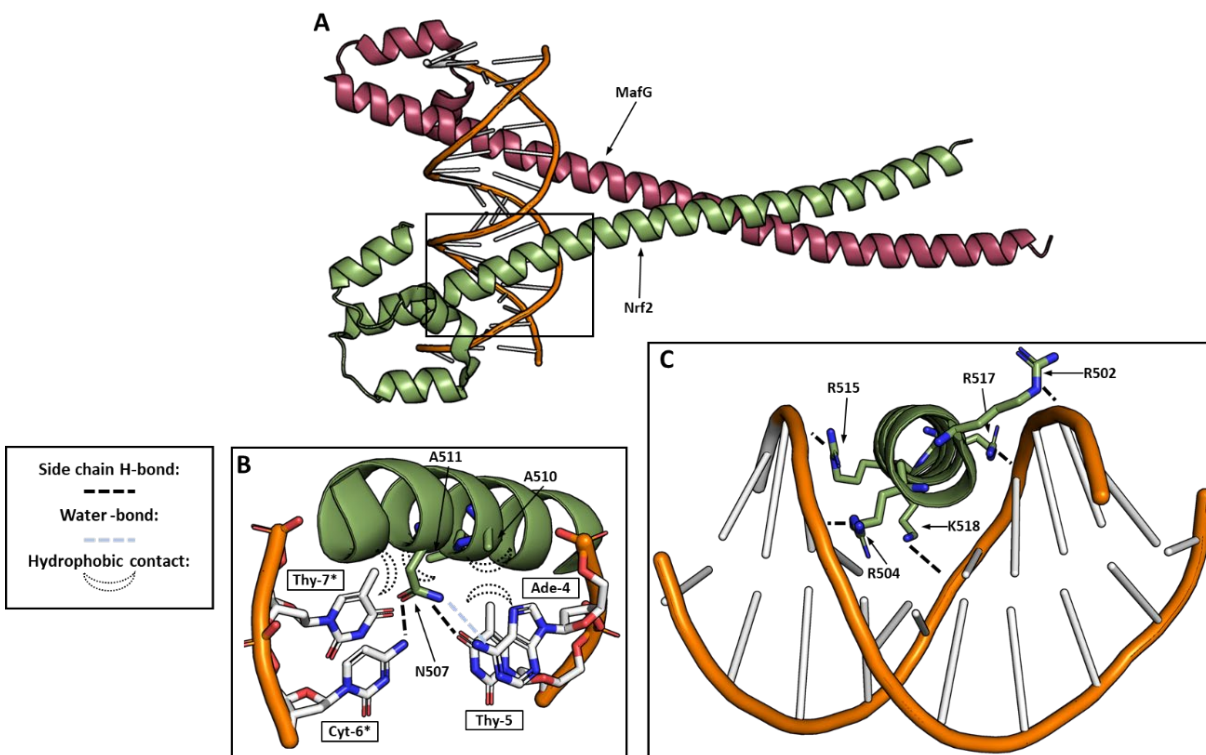


Figure 2.9 Nrf2-MafG heterodimer binding DNA.

A. DNA binding through a leucine zipper. **B.** Direct nucleobase interactions. **C.** Sidechain H-bonding to DNA phosphate backbone. (Generated from PDB: 7X5E)

Using a homology model of Nrf2-DNA binding, Wiedemann *et al.* designed and synthesized four stapled peptides based on the major-groove binding helix with varying all-hydrocarbon stapling at the i and $i+3$, i and $i+4$, i and $i+7$ positions^[90]. The peptides were evaluated for their helical content using CD, which showed them to be helical, and the DNA binding affinities were probed using fluorescence polarization with the FAM-labelled Nrf2 ds-DNA sequence. Further evidence of DNA binding was evaluated with EMSA. Three of the four peptides showed 1-2 μM binding affinities, which were approximately 5-fold higher affinity than the unstapled sequence. One of these stapled peptides was able to induce a DNA mobility shift at lower concentrations than the unstapled peptide in the EMSA. Importantly, the stapled peptides also

exhibited binding to the randomized negative control DNA sequences in the fluorescence polarization assay. These data suggest that the selectivity of these DNA binding stapled peptides maybe be limited. This is perhaps not surprising based on the atypical elements of selectivity observed in the interaction between the Nfr2 α -helix and its cognate DNA sequence. Their fluorescently labelled lead stapled peptide was permeable to fixed HeLa cells and displayed nuclei enrichment compared to the linear unstapled peptide based on their fluorescence microscopy images. The peptide was further confirmed to be permeable to living HeLa cells and localized to the cytosol and nuclei without disrupting the membrane, confirmed by the impermeability of propidium iodide dye.

RpoN-based stapled peptides

The most comprehensive set of DNA binding stapled peptides have been generated to inhibit the binding of the bacterial alternative sigma factor, RpoN, from its cognate promoter DNA sequence^[95,96]. Bacterial transcription is controlled by sigma factors, which are responsible for binding both RNA polymerase (RNAP) and DNA during transcriptional initiation. Sigma factor σ^{54} interacts with bacterial DNA through a quasi winged-HTH motif, with the RpoN box helix-3 as the recognition helix (**Fig. 2.10A**). A high-resolution NMR structure of σ^{54} binding its cognate DNA sequence shows multiple residues responsible for specific nucleobase bonding. Multiple other residues containing hydroxyl or amine sidechains participate in DNA backbone interactions, helping the base-binding amino acids to be positioned properly (**Fig. 2.10B**). Direct hydrogen bonds are observed between Arg379, Arg378 and Lys383 and Gua-26*. Arg379 also binds directly to Gua-25* and Lys383 to Thy-23 (**Fig. 2.10C**). An important binding aspect of this interaction is the packing between Arg379 with Gua-24 and Thr380 with Thy-23 (**Fig. 2.10D**)^[97].

Payne *et al.* in 2018 mimicked the RpoN box with a stapled peptide using α,α -disubstituted terminal olefin unnatural amino acids to create four all-hydrocarbon stapled peptides that were capable of selectively binding the -24 glnAP2 promoter^[95]. Two $i, i+4$ and two $i, i+7$ stapled 15 residue peptides were synthesis based on *Aquifex aeolicus* RpoN box. In addition, the native Met residue in the sequence was replaces with norleucine, to limit unwanted side reactions during crosslinking (ring closing metathesis)^[98]. All four were found to be substantially helical by CD spectroscopy. Using a fluorescence polarization assay, the binding affinity of three of the four stapled peptides was shown to be 1 μ M, 40-fold higher affinity than binding of the unstapled peptide. None of the stapled peptides bound to a scrambled DNA sequence, suggesting sequence-specificity of binding. Lead stapled peptides were shown to protect the promoter DNA sequence from nuclease digestion in a foot printing assay and to bind the promoter DNA in an EMSA assay.

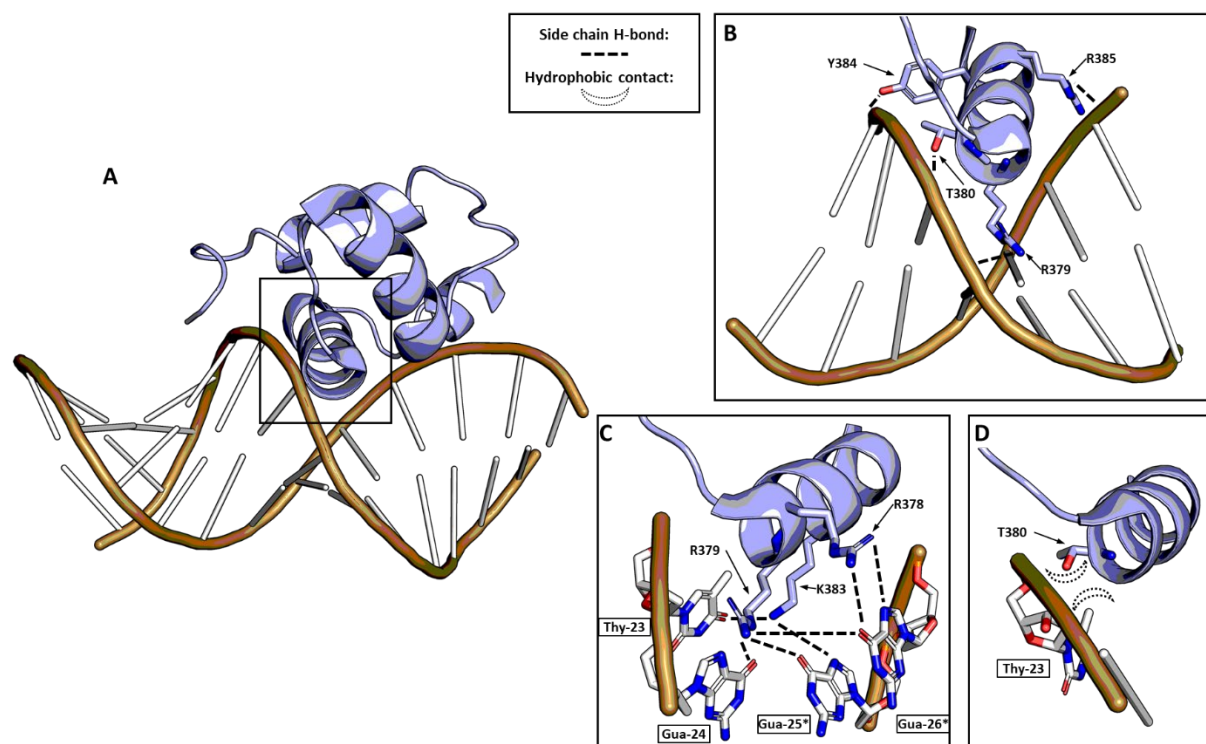


Figure 2.10 N-terminal portion of $\sigma 54$ containing the RpoN box binding dsDNA.

A. Quasi winged HTH motif binding DNA. **B.** Phosphate backbone hydrogen-bonding. **C.** Direct helix-3 hydrogen-bonding and water-mediated interactions to specific nucleobases. **D.** Hydrophobic interactions of Thr β -methyl and Thy-121 methyl (Generated from PDB: 2O8K)

The lead RpoN based stapled peptide was then tested for its ability to inhibit RpoN mediated transcription in the Gram-negative bacteria *Escherichia coli*^[96]. Using fluorescently labelled stapled peptides, the peptides were shown to cross bacterial membranes in an ATP independent manner and using RNA-seq analysis followed by gene set enrichment analysis the lead stapled peptide was found to selectively inhibit transcription of genes under direct RpoN-mediated transcription versus genes under direct RpoH-mediated transcription. These data provide strong evidence for the ability of DNA-binding stapled peptides to bind DNA sequence selectively both in biochemical assays and in more demanding cell-based assays.

Following this, a library of 33 stapled peptides based on the lead stapled peptide from the 2018 study were generated. The library contained analogs where direct DNA binding residues were exchanged, where non-binding residues were modified, and where the length of the peptide was modified^[96]. As the lead peptide could inhibit RpoN-mediated transcription in *E. coli*, an *E. coli* cell-based assay was used to screen the library for analogs that inhibited RpoN transaction on par or better than the lead compound. A counter screen was then used to confirm that active compounds did not inhibit transcription non-selectively. This provided 6 stapled peptides that were characterized by CD spectroscopy and for helicity and in a fluorescence polarization assay for binding affinity. Four analogs bound with 3-fold enhanced affinity as compared to the lead stapled peptide.

The higher affinity binding stapled peptides had a variety of modifications leading to their enhanced binding. Specific DNA binding residue such as Arg and Lys could be replaced with Lys and Arg respectively. Presumably this swap maintains sufficient hydrogen-bonding capabilities in the Arg to Lys swap even with elimination of the native bidentate interaction, and in the Lys to Arg swap in light of the additional bidentate capabilities. An Ala to Gly substitution in the middle of the peptide sequence presumably enhances peptide flexibility within the DNA major groove and the staple walk peptide towards the C-terminus likely has optimally placed the stapling position. The C-terminus truncation of a Leu residue also lead to tighter binding^[96]. This library demonstrates the importance of conserving hydrogen bonding amino acid sidechains that interact with nucleobases and highlights the capability of Lys to replace Arg without disrupting important salt bridges. The staple walk data reveals the importance of having an outward facing staple from the interaction. This is somewhat counter intuitive since the staple is highly hydrophobic and is

solvent exposed in the outward facing direction, however in this location the outward facing staple does not disrupt the interaction with DNA.

These lead peptides did not disrupt gram-negative bacterial membranes and displayed low hemolysis facing human red blood cells. They do display bacterial membrane permeability as they can affect transcription in the *E.coli* cell-based GFP assay and affect the anti-virulence of *Pseudomonas aeruginosa* in a *Galleria mellonella* model.

Comparison of protein binding and DNA binding stapled peptides

DNA-binding stapled peptides can be designed following the major features found in PPI inhibiting stapled peptides. PPI stapled peptides are often amphipathic due to the native protein-protein interactions containing major hydrophobic grooves and polar contacts performing hydrogen bonding with the protein backbone. Compared to PPI disrupting stapled peptides, DNA-binding peptides are highly charged due to the presence of key hydrogen bonding residues, Arg and Lys, to the negatively charged DNA. Unlike stapled peptides that disrupt PPI, the staple does not appear to make stabilizing interactions with the DNA and is highly solvent exposed. This suggest that further modification of the staple may improve performance of these binders.

Trends in DNA binding stapled peptides

While there are limited examples of stapled peptides binding DNA, a few key observations can be made. For example two of the five stapled peptides are based on leucine zipper-DNA interactions (GCN4, Nrf2). The remaining three are based on HTH (homeodomain Q50K, HNF-

3 γ , RpoN). Unlike HTH, leucine zippers are unstructured prior to DNA binding^[99], suggesting the unfolded and folded conformation of the helix are energetically comparable. As such, the expectation is that stapled and non-stapled peptide sequences should bind with comparable affinity. This is born out in the Nrf2-based stapled peptides where there is a five fold difference in affinity between the stapled and unstapled peptide control^[90]. HTHs on the other hand are well folded prior to binding. As such peptides derived from these motifs show a significant increase in affinity upon stapling. For example the RpoN unstapled peptide does not bind DNA appreciably even at 40 μ M where as the stapled derivatives bind with dissociation constants of approximately 1 μ M, representing at least a 40 fold increase in binding affinity^[95]. This observation however does not rule out leucine zippers as candidates for design of stapled peptides since the stapling significantly improves the physicochemical and pharmacokinetic properties of the peptide sequence.

In general, stapled peptides derived from HTH elements appear to show higher affinity for their targets. Both the GCN4 and Nrf2 leucine zipper derived stapled peptides show binding affinities, where reported, in the low μ M range^[75,90]. The HTH derive stapled peptides typically show low nM dissociation constants, such as with the HNF-3 γ -derived stapled peptide (K_D = 4 nM) and the Q50K-derived stapled peptide (K_D = 7.5 nM)^[87], though the RpoN-derived stapled peptide is an outlier with a high nM affinity (K_D = 366 nM)^[96].

A particular need in the field is rigorous evaluation of sequence specific DNA binding. The ability to bind specific DNA sequences is what provides stapled peptides with the key functionality for modulating biological systems. With the exception of RpoN-based stapled peptides, most stapled peptide evaluation is limited to characterizing *in vitro* binding to a scrambled DNA sequence^[96]. The work with RpoN-based stapled peptides shows that they can

bind specifically to DNA and inhibit protein-DNA binding interaction *in vivo*. While the RpoN-based stapled peptides displayed specificity sufficient for modifying biological function in bacteria, it is likely that additional selectivity will be needed to modulate biological function in eukaryotes due to their much larger genomes. While a number of solutions are possible to address this expanded selectivity issue, the most straight forward is linking multiple stapled peptides together to bind additional points of contact in the major groove, much like dimeric TF do.

RNA and Non-canonical DNA stapled peptides

There are a number of other nucleic acid-based structures in cells that can be bound by α -helices and are thus ripe for the development of stapled peptides. While dsDNA is the most common DNA motif in cells, in guanine rich DNA sequences, G-quadruplexes (G4s) can form and have been shown to be fundamental features of the genome. It is thus not surprising that a number of proteins have been shown to bind and stabilize G4 structures^[100]. For example, the Bovine DHX36 helicase binds and unfolds a G4 structure formed in the promoter region of the c-MYC oncogene^[101]. A series of hydrocarbon stapled peptides based on the DNA binding α -helix in DHX36 were synthesized and shown to bind to this G4sequence. However no selectivity was observed versus ssDNA and dsDNA^[102]. More work in developing G4 selective binding stapled peptides is thus needed.

In addition to non-canonical DNA structures, there are a significant number of structured RNAs in the cell, including linear (mRNA), double stranded (dsRNA), and stem-loop structures^[103]. Taking advantage of the *Tomato aspermy cucumovirus* (TAV) plant virus protein TAV2B, which binds to dsRNA involved in plant RNA silencing, Kuepper *et al.* developed a

hydrocarbon stapled peptide that selectively bound dsRNA over ssRNA, ssDNA, and dsDNA in a sequence specific manner^[104]. While these two examples are not a comprehensive review of stapled peptides that bind to non-dsDNA targets, they highlight the potential of stapled peptides in this area.

Conclusion

An indication of the utility of a biochemical tool can be gathered from its application scope. In the case of stapled peptides, being able to bind large biomolecule surfaces with limited features, such as protein surfaces or the major groove of dsDNA, in a complex biological environment demonstrates its enormous scope and potential. While stapled peptides that disrupt protein-protein interactions have made their mark since the early 2000's, stapled peptides that selectively disrupt DNA-protein interactions are still rare. While there are a few examples of high affinity DNA-binding stapled peptides and fewer showing high sequence specificity, there do seem to be clear trends. HTH-based stapled peptides tend to have greatly increased binding affinity compared to unstapled peptides, while the unstapled and stapled form of leucine zipper-based peptides bind with more comparable affinity. HTH-based stapled peptides also generally show greater affinity, binding DNA with low nM dissociation constants while leucine zipper based stapled peptides bind with low μ M affinity. These trends can be drawn back to the natural folded state of these proteins prior to binding DNA, where HTH are well folded and leucine zippers are unfolded before binding. The potential to generate potent inhibitors of transcription is enormous and once realized will be disruptive to both understanding biology and developing new pharmacological agents.

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Chapter 3: RpoN-Based stapled peptides with improved DNA binding suppress *Pseudomonas aeruginosa* virulence

3.1 Introduction

Bacterial transcription is controlled by sigma factors that bind RNA polymerase (RNAP) and DNA during initiation to form the RNAP holoenzyme. Sigma factor σ^{54} has been shown to control the downstream expression of virulence proteins implicated in bacterial biofilm formation, motility and quorum sensing¹. σ^{54} interacts with bacterial DNA through a quasi winged-HTH (helix-turn-helix) motif, with the RpoN box helix-3 as the dominant DNA binding portion². Payne & *al.* mimicked the native RpoN box with a stapled peptide using α,α -disubstituted terminal olefin unnatural amino acids to create an all-hydrocarbon stapled peptide, **SP σ 54-2**, which is capable of selectively binding the -24 glnAP2 promoter³. Based on the NMR structure of σ^{54} N-terminus binding DNA², multiple residues were determined to be responsible for specific nucleobase binding and phosphate backbone binding. The protein binds DNA specifically with H-bonding residues Arg379, Arg378 and Lys383 with their respective nucleobase. Water mediated H-bonding is also an important binding aspect of this interaction with Arg379 binding with G-24n and Thr380 binding with T-23t. Multiple other residues containing hydroxyl or amine sidechains participate in DNA backbone interactions, helping the base-binding amino acids to be positioned properly⁴.

Following up on this study, our group has optimized this stapled peptide through a medicinal chemistry approach, looking at the DNA binding residues, the non-binding residues and the length of the peptide⁵. A library of over 30 stapled peptides were synthesized. Four were determined to have a 2-fold binding affinity greater than **SP σ 54-2** (**Figure 3.1**). The top compounds had a variety of modifications leading to their enhanced binding association, notably binding residues Arg to Lys and Lys to Arg, flexibility enhancing peptide Ala to Gly and a staple walk peptide towards the

C-terminus. The C-terminus truncation of a Leu residue, led to lowered hemolytic activity and tighter DNA binding⁶. This library demonstrates the importance of H-bonding amino acid sidechains to be conserved at the nucleotide binding residues but highlights the capability of Arg replacement by Lys, where important salt bridges can still be maintained. The staple walk compounds reveal the importance of having an outward facing staple from the interaction. These findings will help in the development of other DNA-protein interactions as potential therapeutic targets rather than rely solely on protein-protein interaction for novel stapled peptide targets.

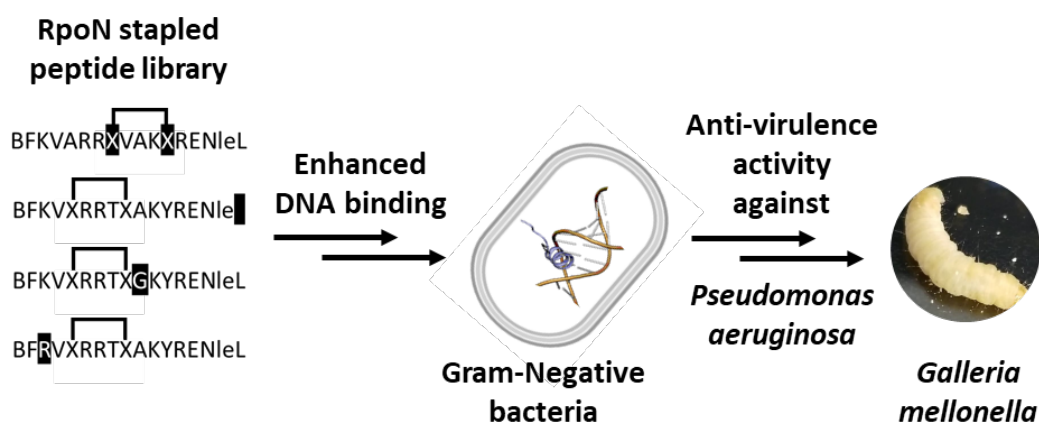


Figure 3.1 Optimized DNA binding stapled peptides shows anti-virulence activity in *Pseudomonas aeruginosa*

This library of stapled peptides was synthesized using standard Fmoc-SPPS and screened using whole cell *E.coli* GFP transcriptional reporter assays. The native promoter was used to confirm stapled peptide binding in a cellular environment and a constitutive promoter was used to confirm promoter binding specificity. The top hits were further characterized for *in vitro* DNA-binding via a fluorescence polarization assay, hemolysis assay for human red blood cell toxicity, minimal inhibitory concentration assays and bacterial lysis assays. The best compounds were tested in a preliminary *in vivo* model using *Galleria mellonella* to support anti-virulent activity with *P. aeruginosa* PA01 infection and co-injected peptide.

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3.3 Author contributions

C. N. B., F. B., T.-F. M., S. G., and D. N. conceived of the study. A. R. P. and S. R. P. synthesized all the peptides. J. T. B.-H. generated the $\sigma 70$ transcriptional reporter system. A. R. P. collected the cell-based screen data, purified lead peptides, collected biophysical data and performed *in vivo* evaluation of stapled peptide activity. A. R. P. and M. G. D. collected the MIC data. G. A. M. collected haemolysis data. A. R. P. and A. K. evaluated membrane permeability. C. N. B., F. B., and D. N. supervised and managed the study. All authors contributed to analysing data and writing and editing the manuscript.

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3.5 RpoN-based stapled peptides with improved DNA binding suppress *Pseudomonas aeruginosa* virulence

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Stapled peptides have the ability to mimic α -helices involved in protein binding and have proved to be effective pharmacological agents for disrupting protein-protein interactions. DNA-binding proteins such as transcription factors bind their cognate DNA sequences via an α -helix interacting with the major groove of DNA. We previously developed a stapled peptide based on the bacterial alternative sigma factor RpoN capable of binding the RpoN DNA promoter sequence and inhibiting RpoN-mediated expression in *Escherichia coli*. We have elucidated a structure-activity relationship for DNA binding by this stapled peptide, improving DNA binding affinity

constants in the high nM range. Lead peptides were shown to have low toxicity as determined by their low hemolytic activity at 100 μ M and were shown to have anti-virulence activity in a *Galleria mellonella* model of *Pseudomonas aeruginosa* infection. These findings support further preclinical development of stapled peptides as antivirulence agents targeting *P. aeruginosa*.

Introduction

The ability to perturb the interaction between a transcription factor and its cognate DNA sequence with selectivity has the potential to impact a vast array of therapeutic areas. While the identification of potent and selective DNA binding small molecules that block transcription factor binding has been an intensely researched area,¹⁻² the medicinal chemistry community has yet to develop general and systematic strategies for disrupting the binding of a specific transcription factor of interest.³ Because transcription factor-DNA binding is mediated primarily by an α -helix that selectively binds to the major groove of DNA⁴⁻⁵, stapled peptides, short oligopeptides locked into a helical conformation by crosslinking of two or more side-chains, have enormous potential to mimic these crucial DNA binding α -helices.⁶⁻⁷ Hydrocarbon stapled peptides where the crosslink is generated via ring closing metathesis of side-chain alkenes have proved effective at disrupting protein-protein interactions *in vitro* and *in vivo* and have led to the development of a clinical stapled peptide candidate targeting p53 binding to MDM2 and MDMX (ALRN-6924, clinicaltrials.gov identifier: NCT04022876).⁸⁻⁹

The alternative bacterial sigma factor RpoN and its cognate promoter DNA sequence are highly conserved across Gram-negative bacteria, including pathogens such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Acinetobacter baumannii*. RpoN was originally discovered as the regulator of the nitrogen response,¹⁰ however, it functions broadly, affecting nutrient uptake,¹¹

motility,¹²⁻¹³ quorum sensing,^{14,15} and biofilm formation.¹⁶ RpoN plays an important role in the virulence of many pathogens¹⁷⁻¹⁸ and inhibition of the RpoN regulon has been shown to reduce virulence in Gram-negative pathogens.¹⁹⁻²⁰ As such, a hydrocarbon stapled peptide that disrupts RpoN-DNA binding has the potential to be a broadly applicable anti-virulence agent for use against Gram-negative pathogens.

We have previously developed a hydrocarbon stapled peptide, **2**, based on the key α -helix of the DNA binding domain of RpoN, which binds the major groove of the conserved RpoN promoter DNA sequence.⁷ **2** is able to cross Gram-negative membranes in an ATP-independent manner, enabling it to access its DNA target sequence. It binds the RpoN promoter DNA selectively, as determined by fluorescence polarization, and can competitively displace the DNA binding domain of RpoN from its promoter DNA sequence, as determined by electrophoretic mobility shift. Lastly it can selectively suppress RpoN controlled gene expression in *Escherichia coli*, as determined by RNA-Seq and gene set enrichment analysis.⁷

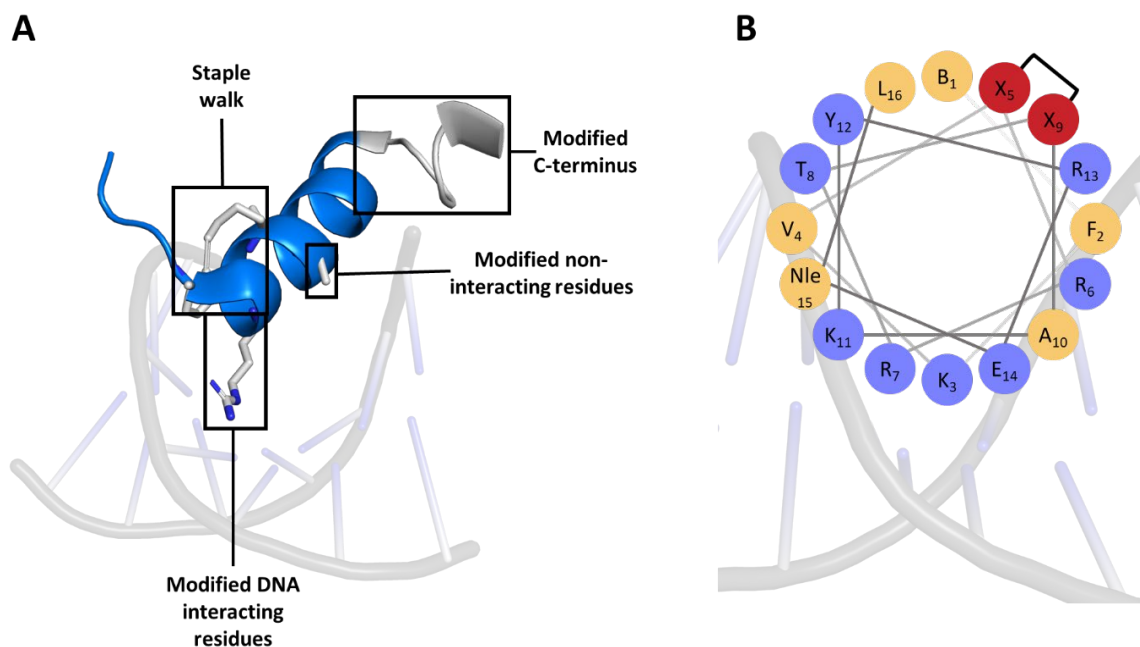


Figure 3.2 The model for stapled peptide binding to DNA informs library design.

A. A model of the RpoN stapled peptide binding to DNA major groove *glnAP2* promoter, based on PDB 2O8K, highlights the design modifications used in this library. B. Helical wheel of the stapled peptide sitting in the major groove, based on PDB 2O8K.

In this study we show that our original 16-residue lead stapled peptide **2** can be optimized for DNA binding and reduced toxicity, and that optimized peptides can delay *P. aeruginosa* infections in an *in vivo* animal model. **2** was directly adapted from residues 338–398 of *Aquifex aeolicus* RpoN. The DNA-binding domains of the protein binds the -24 site of the RpoN promoter with a ten-fold greater affinity than **2** (DNA binding domain $K_d = 109$ nM; **2** $K_d = 1.14 \pm 0.09$ μ M).²¹ This suggests that the binding of **2** can be further improved. We set out to collect structure-activity relationship information by synthesizing and evaluating 33 analogues with modification of residues, overall peptide length, and the location of the staple. As previous work on **2** had focused primarily on its ability to bind DNA and negatively regulate the RpoN regulon in Gram-negative bacteria, we also set out to investigate the toxicity of **2** and its most potent analogues as

measured by hemolysis^{22,23} and their ability to inhibit virulence in an animal model of *P. aeruginosa* infection. Our work shows that our most advanced stapled peptides bind better than lead peptide **2**, show very low hemolysis, and can delay infection by *P. aeruginosa* in an animal model.

Results and discussion

Design of analogues

A library of 33 stapled peptides was created to map out the impact of multiple design features on the ability of stapled peptides to inhibit RpoN-mediated transcription in Gram-negative bacteria. Twenty-eight analogues of the lead (*i, i+4*) stapled peptide **2** were synthesized as well as five of the lead (*i, i+7*) stapled peptide **3**.⁷ The first half of our library focused on optimizing the placement of the hydrocarbon staple and its length (Fig 3.2). Our first approach to optimize placement was to walk the position of the (*i, i+4*) staple over the first two turns of the N-terminus of the helix (**5-10**). In addition, we moved the (*i, i+7*) staple from the N-terminus, as found in **3**, to residue 4 (**11**). To evaluate length, residues were deleted from the N- and C-termini (**12-15**). The C-terminus of the peptide in the native RpoN protein contains a short six residue loop that reaches across and binds in the minor groove, further stabilizing RpoN binding to DNA (Fig 3.2).²¹ To evaluate the role of this feature, we extended the C-terminus of the stapled peptide by six residues (**16**).

The second half of the library focused on mutation of residues in the 16-residue core of **2** and **3**. The design of these analogues was informed by the NMR structure of the C-terminal domain of RpoN bound to the *glnAP2* promoter DNA sequence.^{24,21} This structure shows that the α -helix binds the major groove of the -24 promoter sequence 5'-TGGCACG-3', with its main contacts to

the GG of the 5'-3' strand and T of the 3'-5' strand. Extrapolating these structural data to **2**, Arg6, Arg7, and Lys11 are thus predicted to act as H-bond donors to the guanine oxygens (Fig S3). Lys11 is expected to H-bond with the thymine major groove oxygen. In addition, the methyl group of the same thymine is predicted to interact with Thr8 via a van der Waals interaction. Finally, several residues in the sequence are predicted to interact with the negatively charged phosphate backbone, including Arg6, Arg7, Thr8, Lys11, Tyr12, and Arg13 (Fig S3).

To optimize binding to DNA, basic and H-bond donor amino acids were screened (Fig 3.2). The essential arginines at positions 6 and 7 were exchanged for lysines and ornithines (**17-20**), retaining the H-bond donor ability at the location. Similarly, Lys11 was substituted for an arginine (**21**) to enhance contacts with a thymine. The hydroxyl-containing residues Thr8 and Tyr12, which interact with the phosphate backbone were converted to Ser in both **2** and **3**, the (*i*, *i*+7) stapled peptides (**22-25**), maintaining their ability to interact with the backbone. In addition, Tyr12 was converted to Thr (**26, 27**) in both the (*i*, *i*+4) and (*i*, *i*+7) peptides.

Hydrophobic residues in the stapled peptide not involved in direct DNA binding originated from the primary sequence of full-length RpoN. In the context of the full-length protein these residues play roles in folding, stability, and function of full-length protein. As the stapled peptide is only a single structural element from RpoN, these residues will be differentially exposed as compared to the full-length protein and may be tolerant to modification and optimization. To optimize these residues in our stapled peptide, Phe2 was converted to Trp (**28**) to test the impact of increasing surface area and electrostatic potential,²⁵⁻²⁶ Val4 was converted to Phe and Ala (**29, 30**) to reduce the destabilizing effect of the β -branch on the helix,²⁷ Ala10 was converted to Gly (**31**) to add flexibility into the helix. While Gly is typically thought of as a helix breaker,²⁸ the staple is expected to enforce helicity. Thus, Gly in the middle of the helix is expected to increase

flexibility, akin to what is seen in transmembrane helices.^{29,30} Conversely, Val4 and Ala10 were swapped (**32**) to determine whether rigidity in the middle of the peptide might enhance binding. In addition, the exposed charged Lys3 was converted to Arg and Orn (**33**, **34**)

A set of three stapled peptides (**35-37**) where multiple residues were modified was designed to enhance the amphipathic character of the peptide by reinforcing the hydrophilic DNA-binding face and connecting hydrophobic networks on the exposed face. Behind this design was the complex relationship between hydrophobicity networks and overall charge with cellular uptake, binding, and unwanted hemolytic activity.³¹⁻³² The partially exposed Thr8 and fully exposed Tyr12 were both converted to the non-polar residues, Leu, Val, or Phe. To increase the overall positive charge, Ala10, which is positioned in the major groove, and Glu14, which faces the phosphate backbone, were simultaneously converted to Lys.

Lastly, the enantiomer of our lead peptide **2** (**D-2**) was designed to test the enantio-specificity of the biological activity of **2**. **D-2** will confirm the mechanism of specific DNA binding interactions as opposed to non-specific DNA interaction or interactions with the membrane.

Synthesis of analogues

All L-configured library members (**2-37**) were readily synthesized using automated Fmoc-based solid phase peptide synthesis (SPPS). The deprotected N-termini were acetylated and the linear resin bound peptides were subjected to on-resin ring-closing metathesis with Grubbs-I Catalyst. Cleavage of the stapled peptide was followed by ether precipitation and resuspension in water, followed by lyophilisation to give the crude stapled peptides. The structures of all peptides were confirmed by LCMS analysis of the crude peptide and shown to be > 50 % pure, with the

majority being 60-90% pure (Fig S1-S2). D-**2** was synthesized by manual Fmoc solid phase peptide synthesis. It was end-capped, stapled and cleaved from the resin to generate crude enantiomeric stapled peptide (Fig S1).

Initial screen of crude stapled peptides

A cell-based screening strategy was developed and used to identify compounds that were able to inhibit RpoN-mediated transcription. The minimum inhibitory concentration of peptide **2** was determined using the microtiter broth dilution method³³ to determine if the stapled peptides were likely to inhibit cell growth in the screening assay. Peptide **2** did not inhibit wild type *E. coli* or *E. coli* XL1Blue growth at up to 32 µg/mL (> 150 µM, Table S2). Therefore *E. coli* XL1-Blue were transformed with a plasmid containing the reporter gene encoding GFP under the control of the well characterized *E. coli* RpoN promoter *glnAp2* (Fig S4).³⁴⁻³⁵ This *glnAp2-gfp* construct has been previously used in genome-wide cell-based screens examining RpoN function³⁶ and is known to be induced by RpoN under low nitrogen conditions. Furthermore, we have previously shown that **2** binds to the *glnAp2* promoter *in vitro* and *in vivo* with high affinity⁷, indicating that **2** should be able to inhibit GFP expression in this system. Transformants were grown in M9 minimal media with 40 µM NH₄Cl. This low nitrogen level is known to induce RpoN-mediated transcription from *glnAp2* controlled genes.^{7,37} In the absence of RpoN inhibition, significant levels of GFP are readily observed. Addition of 10 µM **2**, which binds *glnAp2* with a $K_d = 1.14 \pm 0.09 \mu\text{M}$ ⁷ leads to a statistically significant $75 \pm 16 \%$ decrease of the normalized GFP response as compared to the PBS vehicle control ($p = 4 \times 10^{-9}$, $n = 12$, independent, two-tailed *t*-test, Fig 3.2). Treatment with a lower concentration of **2** (2.5 µM) led to a statistically insignificant decrease in GFP levels (Table S3). Additionally, treatment with wild-type linear **2** (WT, 10 µM) showed no statistically

significant change in GFP expression. Furthermore, treatment with the enantiomer of **2**, D-**2** (10 μ M), also produced no significant change in GFP expression, ruling out non-specific interactions as the source of the inhibition. Based on these data, this new cell-based assay shows reproducible, statistically significant, specific inhibition of RpoN-mediated transcription in the presence of a stapled peptide known to bind RpoN promoters.

All 33 crude stapled peptides were evaluated at 10 μ M in this cell-based assay (Fig 3.3, Table S3). Sixteen of 33 peptides showed a statistically significant decrease in GFP expression versus the PBS control ($p < 0.05$, $n = 3$, Fig 3.3). Peptides **10**, **14**, **17**, **18**, **31** and **33** showed greater mean inhibition at 10 μ M than **2**.

The library was further screened to rule out nonselective inhibition of transcription. This counter screen utilized *E. coli* XL1-Blue transformed with a GFP expression vector under the control of the *lacUV5* promoter. The *lacUV5* promoter is regulated by the house keeping sigma factor σ^{70} in *E. coli*³⁸. Transformants were cultivated under identical conditions to the initial screen (low nitrogen M9 media) and induced with IPTG (1 mM) to relieve LacI repression of the *lacUV5* promoter. The vehicle control treatment showed high levels of GFP expression, indicating σ^{70} -mediated transcription of GFP had occurred. To evaluate the impact of stapled peptides on non-RpoN mediated transcription, transformants were treated with peptides **2-37** at 10 μ M. Most displayed high levels of GFP expression, comparable to the vehicle control (Fig S6). In particular peptides **10**, **14**, **17**, **18**, **31** and **33**, which showed significant inhibition of RpoN-mediated GFP expression, did not inhibit σ^{70} -mediated expression of GFP, indicative of selective transcriptional inhibition.

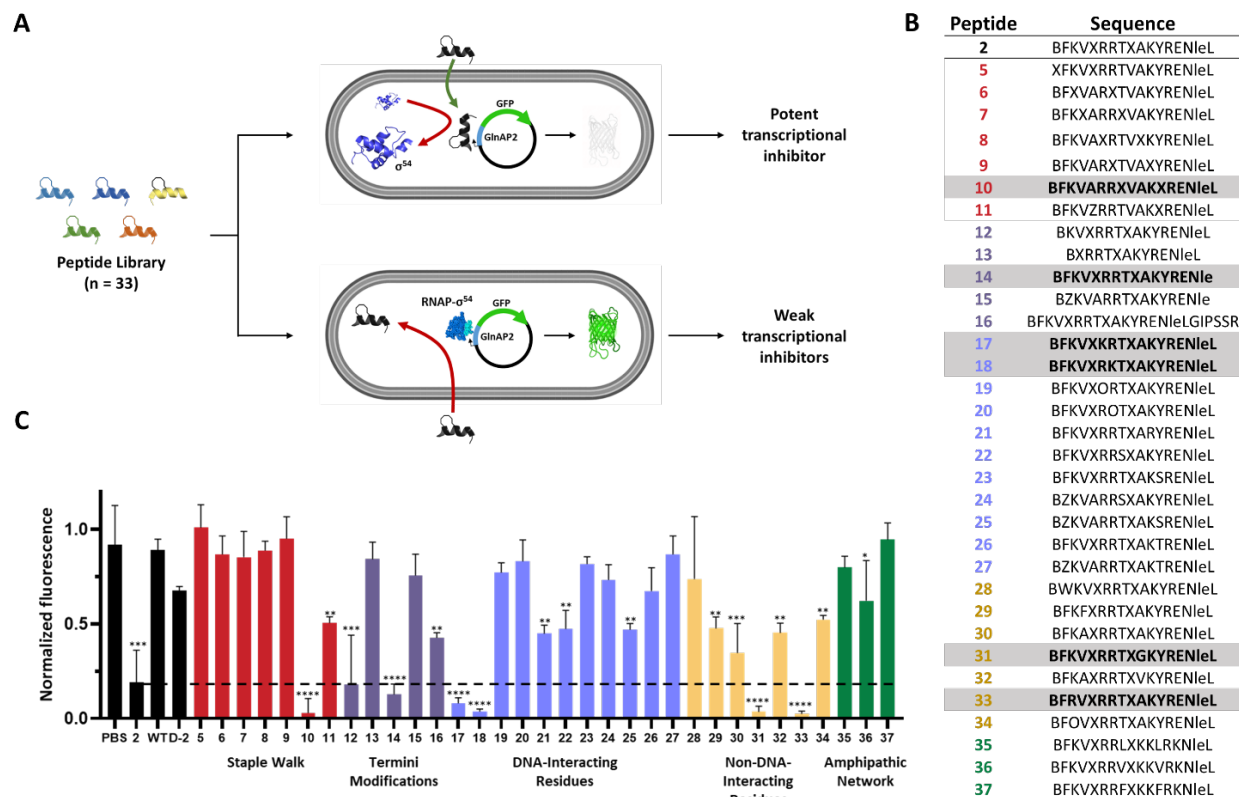


Figure 3.3 An *E. coli* cell-based assay was used to evaluate all library members.

A. Cell-based screening assay workflow for peptide library, indicating weak transcriptional inhibitors or potent transcriptional inhibitors. B. Stapled peptide library sequence with lead peptides highlighted in grey (additional details on the sequences and the structures are given in Table S1). C. Normalized fluorescence graph subdivided in Staple walk (●), Termini Modifications (●), DNA-Interacting Residues (●), Non-DNA-Interacting Residues (●) and Amphipathic Network (●), peptide groups. All peptides were tested at 10 μ M. Data are represented as $n = 3$ (except for PBS and 2 where $n = 12$), means $\pm$ STD. Fluorescence is normalized to untreated samples. The dotted line is set at the mean value of 2. Statistical significance is determined by an independent two-tailed *t*-test compared to the PBS control. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$, **** $p \leq 0.0001$

Examination of the screen and counter screen data from the staple walk library (5-11) shows staple placement has a significant impact on activity. Based on the binding model to the *glnAp2* promoter, the staples of 6, 7, 8, and 9 are expected to disrupt the peptide-DNA binding interface and abolish inhibition. This is consistent with the data, which show no inhibition of GFP

expression at 10 μ M for **6-9**. Peptide **10**, in which the staple is placed a single residue before the staple placement in **2**, shows potent inhibition of GFP expression, as predicted from the structural model. In addition this is clearly selective inhibition of RpoN-mediated transcription as **10** does not inhibit σ^{70} -mediated transcription significantly (Fig 3.4A and S7). While **5** is predicted to be active based on the model, it does not show inhibitory activity. Because this cell-based assay does not only test the ability of the peptide to inhibit RpoN function by binding DNA, but also tests properties such as stapled peptide cell penetration and intracellular stability among others, we cannot unambiguously rule out other factors contributing to the lack of activity of **5**. However, the lack of activity regardless of mechanism eliminates a peptide from further consideration. Peptide **11**, which possesses an (*i*, *i*+7) staple at the N-terminus, was predicted to function but displayed only modest inhibitory activity. Thus, from the staple walk library, **2** and **10** proved to be the most active compounds and were carried forward for further characterization.

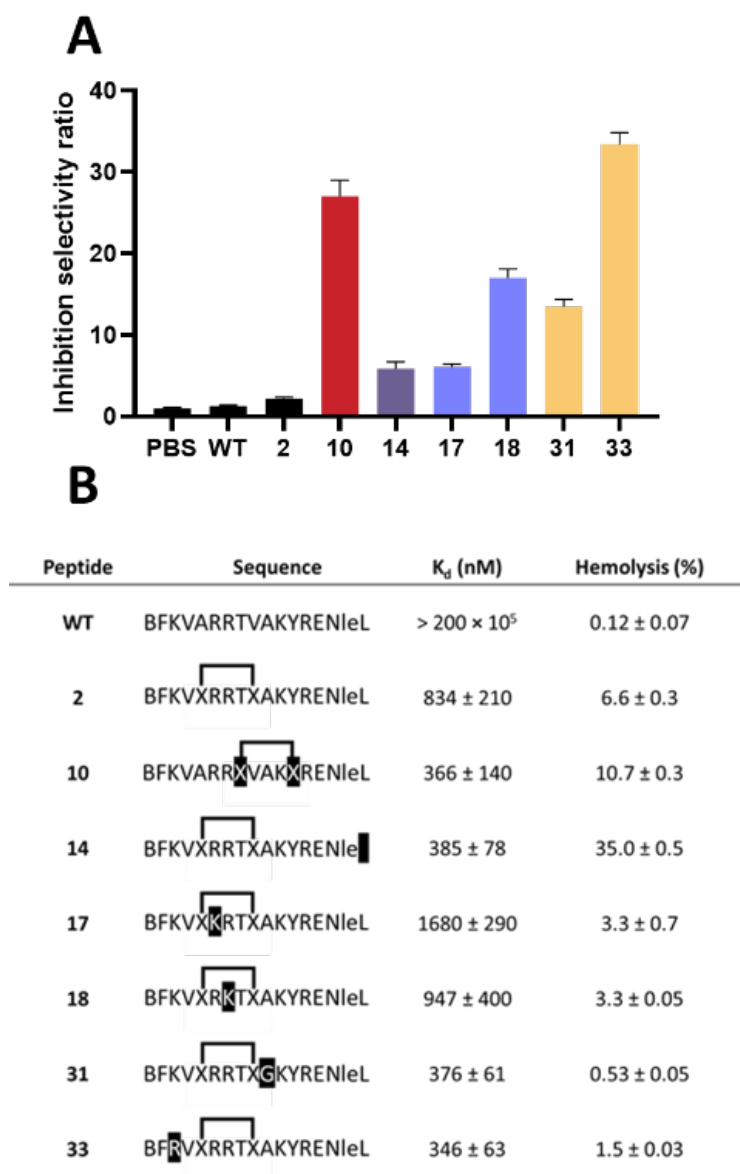


Figure 3.4 Stapled peptide selectivity, binding affinity, and Hemolysis.

(a) Lead stapled peptides selectivity inhibit RpoN-mediated transcription. Bar graph showing the ratio of the normalized GFP expression from the *lacUV5* and *glnAp2* promoter screens. (b) Binding affinity (K_d , nM) determined through Fluorescence Polarization Assay (FPA) and Hemolysis (%) with 100 μ M stapled peptide against human red Blood Cells (HBC). Highlighted in black are the changes made from **2**.

Truncations of **2** by a single residue at either the N- or C-terminus led to potent inhibitors (**12** & **14**). Elimination of N-terminal Phe2 of (**12**) did not affect the inhibitory activity in a significant manner while removal of the N-terminal Phe2 and Lys3 abolished inhibitor activity (**13**). While Lys3 is not predicted to make specific contacts with the DNA, these data suggest it may play a role in providing non-specific interactions with the DNA. Alternatively, the charge from Lys3 may play a role in enabling cell permeability as has been documented for antimicrobial peptides.^{39,40} Surprisingly, the C-terminal single residue truncation on the (*i, i+7*) peptide (**15**) was inactive. Lastly, the addition of six residues to the C-terminus (**16**) that are known to make additional contacts with the minor groove,²¹ failed to improve inhibition. Stapled peptides **17-28** contain modified DNA-binding residues. Replacing central binding residues Arg6 and Arg7 with Lys in **17** and **18** respectively increase the inhibition of RpoN-mediated GFP expression compared to **2** without inhibiting σ^{70} -mediate transcription (Fig 3.4A and S7). The Orn derivatives **19** and **20** on the other hand show no significant inhibition. This is a particularly interesting result as examination of the Pfam representative proteome 15 alignment for the RpoN DNA binding domain shows these two Arg residues are universally conserved in the full length RpoN protein.⁴¹ However, in the stapled peptide context, activity can be improved via their conversion to Lys. Lys11 H-bonds with guanine and thymine of the RpoN promoter. Its replacement with Arg in peptide **21**, generates an active inhibitor, although with weaker inhibitory activity than **2**. Peptides **22-27**, in which the hydroxyl group containing residues Thr8 and Tyr12 that interact with the phosphate backbone interactions have been modified, show minimal-to-no inhibition of GFP expression. Thus, modification of direct DNA-binding residues yields two peptide candidates **17** and **18** with superior inhibition activity versus **2**.

Modification of residues that do not directly contact promoter DNA (**28-34**) was predicted to improve the inhibitory properties of the stapled peptide significantly, since these residues in the primary sequence are buried in the hydrophobic core of the protein and are evolutionarily selected for their ability to generate the overall tertiary structure and function of RpoN⁴. In the stapled peptide, however, they are surface-exposed. Their modification may therefore have a major impact on the physicochemical properties and DNA binding ability of the compounds. Indeed, most of these modifications retained inhibitory activity (**29-34**). Interestingly, the conversion of Ala10 to Gly (**31**) and Lys3 to Arg (**33**) showed superior inhibition to **2** with **33** showing high selectivity for inhibition of RpoN-mediated transcription of GFP versus σ^{70} -mediated transcription (Fig 3.4A and S7). Stapled peptide **31** is particularly interesting as the Ala to Gly substitution likely yields a more flexible helix, suggesting that its increased inhibitory activity may be due to improved DNA binding. Based on these results, **31** and **33** from the non-DNA contacting residue library were selected for further characterization due to their superior inhibition and selectivity as compared to **2**.

None of the peptides generated in the final small library (**35-37**) evaluating contribution of hydrophobic and hydrophilic networks showed statistically significant inhibition of GFP expression as compared to the PBS control. None of these compounds were carried on for further analysis.

Purification and Biophysical analysis of lead compounds

Lead compounds **10**, **14**, **17**, **18**, **31** and **33** all displayed increased inhibition of RpoN-mediated transcription relative to **2** in the cell-based GFP expression assay (Fig 3.3C) and showed selectivity for inhibition of RpoN-mediated transcription over σ^{70} -mediated transcription (Fig

3.4A). These compounds were thus purified to homogeneity by preparative reverse phase HPLC for further biophysical characterization (Table S1, Fig S1). In addition, **2** and D-**2** were purified to provide key controls. The helicity of the lead stapled peptides was confirmed by circular dichroism (CD) spectroscopy (Fig S5). Spectrum of the unstapled WT peptide were consistent with a random coil conformation.

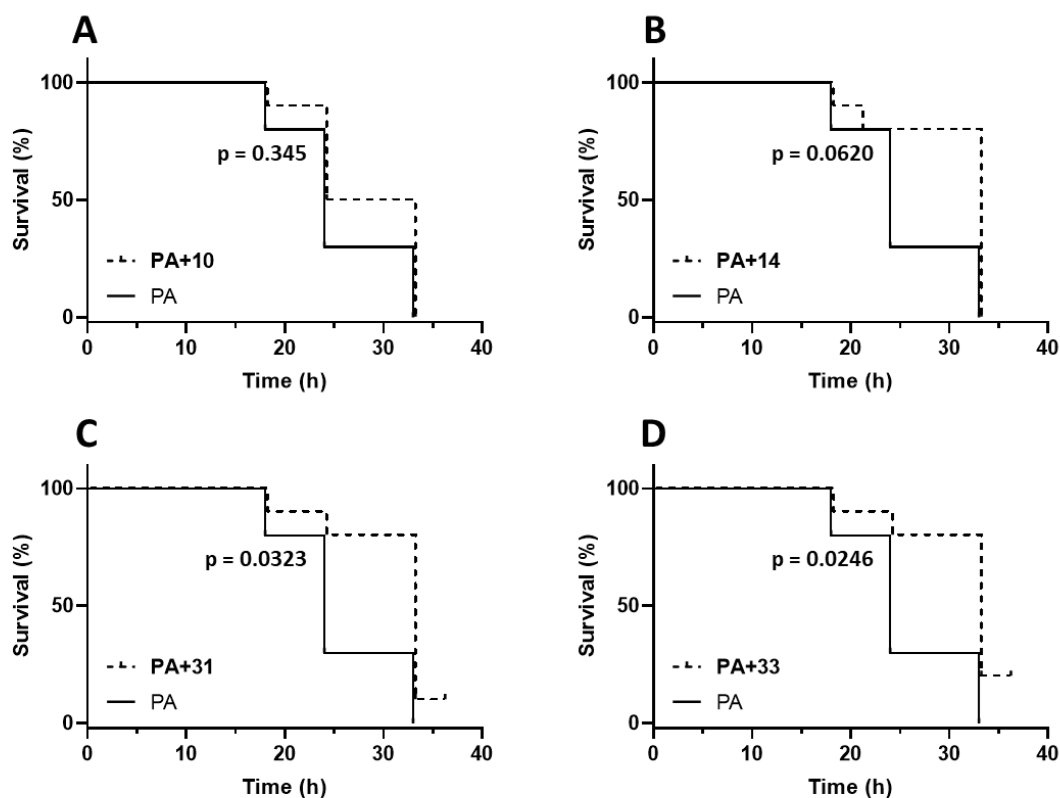


Figure 3.5 *In vivo* wax worm model of *P. aeruginosa* infection.

Log-rank Mantel-Cox test survival (%) of wax worms infected with *P. aeruginosa* (PA) and treated with stapled peptide or PBS vehicle control at 30°C: A. Stapled peptide **10**, B. Stapled peptide **14**, C. Stapled peptide **31**, D.

Stapled peptide **33**

The CD spectrum of D-**2** showed the expected spectrum of an enantiomer of **2** with dual absorption maxima at 222 nm and 208 nm. Thus, CD analysis confirms that **2**, D-**2**, **10**, **14**, **17**, **18**, **31** and **33** adopt a predominantly helical conformation.

DNA-binding affinity was determined using a fluorescence polarization assay (FPA) between lead compounds and fluorescein labelled double stranded DNA *glnAP2* promoter oligomer.⁷ The forward strand is labelled with fluorescein at the 5' position and the reverse strand is unlabelled. Dissociation constants, K_d , were obtained by fitting the polarization data at varying stapled peptide concentrations to a four-parameter variable slope model (Fig S6). The K_d value obtained for **2** ($K_d = 0.83 \pm 0.21 \mu\text{M}$) was comparable to that previously reported using a similar assay ($K_d = 1.14 \pm 0.09 \mu\text{M}$).⁷ The addition of non-ionic detergents including Tween-20 and Triton X-100 did not abolish the change in polarization from the addition of **2**, indicating that the observed response was not due to peptide aggregation. Also consistent with previously reported data, the WT unstapled sequence did not bind the DNA. Furthermore the enantiomer D-**2** showed no binding to DNA at the concentrations tested, also consistent with the proposed binding model of **2** with the major groove. Lastly, **2** did not bind to a scrambled DNA sequence, confirming the sequence selectivity of stapled peptide binding.

The dissociation constants for **10**, **14**, **17**, **18**, **31**, and **33** were found to range from the low μM to high nM (Fig 3.4B, Fig S6). Stapled peptides **17** and **18**, despite exhibiting greater inhibition than **2** in the cell-based assay proved to be weaker or comparable binders, respectively, to **2** *in vitro*. Stapled peptides **10**, **14**, **31** and **33** all showed more potent binding to DNA than **2**. This is particularly surprising for **10** as Thr8 and Tyr12, two residues that are highly conserved in bacterial RpoN sequences and known to play a role in binding DNA have been replaced with staple residues. As the staple in many stapled peptide-protein complexes can make stabilizing contacts with the

protein target,^{9,42} it is possible that the staple in **10** is providing added stabilization to the DNA binding.

Lead compounds do not cause hemolysis

The interaction of helical peptides with cellular membranes derives from a combination of size, residue composition, overall charge, secondary structure, hydrophobicity, and amphiphilic character.^{43,44} With hydrocarbon stapled α -helical peptides, excess hydrophobicity, and positive charge correlate with membrane lysis.⁴⁵ To evaluate the lytic potential of our lead stapled peptides, we examined their ability to lyse human red blood cells (hemolysis), an assay that serves as an *in vitro* surrogate for nonspecific membrane-based mammalian cell toxicity.^{46,47} Stapled peptide **2** showed low levels of hemolysis, < 10 % (Fig 3.4B, Fig S7), at 100 μ M ($\approx$ 200 μ g/mL), which is comparable to a lead antimicrobial stapled peptide that was shown to be well-tolerated in a mouse model when dosed twice daily at 5 mg/kg intravenously.⁴⁷ As expected, the WT unstapled peptide, which lacks α -helical structure, showed significantly lower levels of hemolysis ($p = 0.0008$, two-tailed t-test). Stapled peptide **10**, with its varied staple location compared to **2**, showed a statistically significant increase in hemolysis ($p = 0.004$, two-tailed t-test), consistent with the observations that staple placement has a significant impact on hemolysis, especially when it expands the hydrophobic face of the stapled peptide.⁴⁷ Stapled peptide **17**, **18**, **31**, and **33** all exhibited a statistically significant decrease in hemolysis ($p < 0.05$ two-tailed t-test) compared to **2**. Peptide **31**, which has a central flexible glycine residue, showed the lowest level of hemolysis at only 0.53 ± 0.05 % hemolysis at 100 μ M ($\approx$ 200 μ g/mL) of **31**. **14**, which has a single residue truncation as compared to **2**, showed very high levels of hemolysis. This result highlights the need to measure hemolysis for all advanced stapled peptide candidates, even those that have

conservative modifications. Consistent with the low levels of membrane lysis of red blood cells, the lead stapled peptides also did not meaningfully increase permeability of the inner membrane of *E. coli* ML35 as measured using the established ONPG hydrolysis assay (Fig. S4)⁴⁸.

***In vivo* activity in a Wax Worm *P. aeruginosa* infection model.**

The tightest binding stapled peptides, **10**, **14**, **31** and **33**, were then tested in an *in vivo* *Galleria mellonella* (wax worm) model for systemic *P. aeruginosa* infection. *G. mellonella* provides an alternative model to study Gram-positive and Gram-negative bacterial infections.⁴⁹ Like all insects, it lacks an adaptive immune response, yet it possesses an innate immune response that shows remarkable similarities with that of vertebrates,⁵⁰ making it ideal for initial evaluation of anti-virulence properties of our lead stapled peptides.

G. mellonella is highly sensitive to *P. aeruginosa* infection.⁵¹ Administration of 5 CFU of *P. aeruginosa* via intrahemocoelic injection through the last left pro-leg⁵² is sufficient to kill over 50% of the individuals (n = 10) in 24 h at 30°C (Fig 3.5). Administration of **2**, **10**, **14**, **31**, and **33** at 10 mg/kg in PBS via intrahemocoelic injection did not lead to death of any individuals (n= 10) over 24 h at 30 °C (Fig 3.5, Fig S8). Administration of peptide immediately following infection *P. aeruginosa* enhanced survival at 24 h for all lead peptides (Fig 3.5). Stapled peptides **31** and **33** showed a statistically significant enhancement of survival according to a log-rank Mantel-Cox test, with 80% of individuals surviving at the 24 h time point and 10% and 20% of individuals surviving, respectively, at 36 h post infection. In all other cases none of the infected individuals survive past 36 h irrespective of treatment. As none of the lead peptides were able to inhibit the growth of *P. aeruginosa* (MIC > 32 µg/mL, Table S2), the enhanced survival is not due to antibiotic activity but rather likely due to the antivirulence activity of inhibition of the RpoN regulon. These results

compare favorably with the highly promising anti-virulence compound LED209, which inhibits QseC, a sensor kinase that controls virulence gene expression in *Salmonella typhimurium*,⁵² and delays death in a mouse model of *S. typhimurium* infection without curing the infection.⁵³ Based on these data, **31** and **33** are the most promising lead compounds, having shown tight binding to RpoN DNA promoter sequence, low general toxicity to mammalian cells and efficacy in delaying infection of *P. aeruginosa* in an animal infection model.

Conclusions

We have successfully improved our DNA-binding RpoN-based hydrocarbon stapled peptide through synthesis and evaluation of a 33-membered peptide library. This library has yielded lead compounds that are α -helical, bind the *glnAP2* promoter with high affinity, exhibit low hemolytic activity, and show anti-virulence efficacy by delaying *P. aeruginosa* infection in *G. mellonella*. The library provided the first structure-activity relationship for RpoN-based hydrocarbon stapled peptides that inhibit RpoN-mediated transcription. The subset of staple walk analogues shows that staple placement opposite the DNA-binding face is essential for activity. Modification of the stapled peptide length or DNA binding residues did not significantly improve overall performance. Analogues with these modifications showed either decreased affinity, increased toxicity, or were lacking efficacy. The most promising analogues were **31** and **33** where the non-DNA contacting residues were modified, yielding the two highest affinity and lowest toxicity compounds. These two compounds were the only stapled peptides that showed statistically significant efficacy in our *in vivo* model. This work clearly shows that these unique DNA-binding stapled peptides can be optimized to improve their pharmacological properties and that they can demonstrate efficacy in delaying infection in an animal model of infection.

In general, a significant challenge in the development of anti-virulence compounds is the identification of relevant preclinical assays to define success.^{54,55} Immediate bacterial death is not expected with anti-virulence compounds. Clearance of the infection can occur but requires host immunity and may take up to several days. Surrogate outcomes such as reduction in colony forming units or delayed onset of disease severity among others are often evaluated. In addition, unlike antibiotic development, there are no validated animal models that are predictive for clinical outcome.^{56,57} The use of the invertebrate *G. mellonella* model is considered ideal since the model is highly sensitive to *P. aeruginosa* infection, enabling evaluation of the compounds. While this work makes a major contribution by demonstrating the efficacy of our lead compounds in delaying death from *P. aeruginosa* infection in this model, all individuals eventually succumbed to their infection. Thus, further *in vivo* models will likely require combination of our anti-virulence stapled peptides with a direct-acting antibacterial therapeutic, as this has been shown to synergize in some cases⁵⁸ and can lead to clearance of the infection from the host.

Experimental Methods

Materials

Fmoc-protected amino acids for peptide synthesis were purchased from GL Biochem (Shanghai) Ltd. (Shanghai, China), Combi-Blocks, Inc (San Diego, CA) and Fmoc-(S)-2-(4-pentenyl)alanine-OH was purchased from Nagase & Co., LTD. (Kyoto, Japan). MBHA rink amide resin (100-200 mesh, 0.4-0.6 mequiv/g), *N,N,N',N'*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) were purchased from GL Biochem (Shanghai) Ltd. *N,N*-Diisopropylethylamine (DIPEA) was purchased from Combi-Blocks, acetic anhydride (Ac₂O), piperidine, triisopropylsilane (TIPS) and trifluoroacetic acid (TFA) were purchased from Sigma-

Aldrich (St. Louis, MO). Benzyldiene-bis(tricyclohexylphosphine)dichlororuthenium (Grubb's I catalyst) was purchased from Combi-Blocks. *N,N*-Dimethylformamide (DMF), *N*-methyl-2-pyrrolidone (NMP), 1,2 -dichloroethane (DCE) and dichloromethane (DCM) were purchased from MiliporeSigma. All *D*-configuration Fmoc-protected amino acids were purchased from Combi-Blocks, Inc or Sigma-Aldrich, VWR (West Chester, PA) 5'-Fluorescein labelled and unlabeled forward and reverse *glnAP2* 30 base DNA oligos were purchased from Integrated DNA Technologies (Coralville, IA) and bovine serum albumin (BSA) was purchased from Sigma-Aldrich. All solvents and other chemical reagents were obtained from Sigma-Aldrich, VWR or Fisher Scientific (Waltham, MA) and were used without further purification unless noted otherwise.

Solid Phase Peptide Synthesis

Automated peptide synthesis: Peptides were synthesized using standard automated microwave Fmoc-based SPPS procedures on a CEM Liberty Blue (Charlotte, NC) except D-2. MBHA rink amide resin (0.05 mmol scale) was swelled in DMF for 20 minutes followed by Fmoc deprotection with 20% piperidine in DMF for 5 minutes at 75°C. The first Fmoc amino acid (0.5 M) was loaded on the resin with HBTU (0.5 M) in DMF and DIPEA (2 M) in NMP, reacting for 20 minutes at 75°C followed by Fmoc deprotection with 20% piperidine in DMF, reacting for 5 minutes at 75°C. The following Fmoc protected amino acids (0.5 M) were coupled using HBTU (0.5 M) in DMF and DIPEA (2 M) in NMP, and deprotected with 20% piperidine in DMF following the same reactional conditions. Following final Fmoc deprotection, the N-terminus was acetylated using 20% Ac₂O in DMF and DIPEA (2 M) in NMP, reacting for 20 minutes at 75°C. All Fmoc-Arg-OH and amino acids coupled following the stapling residues Fmoc-(S)-2-(4-

pentenyl)alanine-OH were double coupled (0.5 M x 2) at 75°C. Ring closing metathesis (RCM) was performed using Grubb's I catalyst (0.4 eq) in DCE, reacting for 1h at 75°C. DMF and DCM washes were performed following each coupling, deprotection, acetylation and RCM step.

Manual peptide synthesis: D-2 that was synthesized manually, starting with a 1h swell of MBHA rink amide resin (0.05 mmol scale) in DMF (5 mL) followed by resin Fmoc deprotection for 3 x 5 minutes with fresh 20% piperidine in DMF under nitrogen bubbling resin in a fritted peptide hand shaker. All amino acids (0.5 M) were preactivated with HBTU (0.5 M) in DMF and DIPEA (2 M) in NMP and stirred for 5 minutes in a separate vial. The activated mixture was added to the resin, and nitrogen was bubbled into the solution for 1h at room temperature. The Fmoc amino groups were deprotected with 20% piperidine in DMF, reacting for 3 x 5 minutes under nitrogen bubbling. Following final Fmoc-deprotection, the N-terminus was acetylated using 20% Ac₂O in DMF and DIPEA (2 M) in NMP, reacting for 3h at room temperature under nitrogen bubbling. RCM was performed using Grubb's I catalyst (0.4 eq) in DCE, reacting overnight at room temperature under nitrogen with a subsequent addition of 0.4 eq of Grubb's I catalyst in DCE for 6 h at room temperature. DMF and DCM washes were performed following each coupling, deprotection, acetylation and RCM step.

Peptide workup and purification: All peptides were transferred to a fritted peptide hand shaker to wash out excess solvents with DMF (5mL), DCM (5 mL) and DMF (5 mL). Full peptide deprotection and cleavage from the solid support was performed using a TFA:TIPS:H₂O (95 : 2.5 : 2.5) cocktail, mixed by shaking for 2h. The peptide was isolated from the mixture following cold diethyl ether (10 x volume of TFA) precipitation and centrifugation at 25,000 x g for 20 minutes.

The supernatant was decanted, and the precipitate was resuspended in H₂O to be lyophilized to a white to an off white powder. Peptides were further characterized by LCMS and used without purification in the whole cell *E. coli* GFP assay. Purification of hits was performed on a 1100 Series Agilent HPLC with fraction collector and DAD at 274 nm on a Thermo C18 column. A linear gradient of 5-95% (0.1% TFA H₂O- 0.1% TFA MeOH) over 20 minutes was used.

Whole cell *E. coli* RpoN GFP screen

E. coli XL-1 Blue cells were transformed with pMK-RQ encoding GFP under the control of the *glnAp2* RpoN promoter (Lot. No. 2735627, Invitrogen GeneArt Gene Synthesis, Waltham, MA, Fig S4) using standard protocols. Plasmids were mini prepped with E.Z.N.A. Plasmid DNA Mini Kit 1 (Lot: D69431125-10, Omega Bio-Tek, Norcross, GA) and confirmed through sequencing. Transformed *E. coli* was grown to a dense culture overnight in LB + Kan broth followed by a dilution to an OD₆₀₀ of 0.075 in M9 minimal nitrogen media (40 μ M NH₄Cl). Diluted bacteria in M9 media was added to black opaque 96-well plates and peptides were added from a 10 mM stock in water to a final concentration of 2.5 μ M or 10 μ M in triplicate. The plates were incubated at 37 °C for 4 h and GFP fluorescence emission at 510 nm was detected on a Synergy H1 Hybrid Multi-Mode Reader (Biotek, Winooski, VT). Fluorescence change was normalized relative to the untreated control sample. Samples were identified as possible candidates if they displayed a significant fluorescence level compared to **2** at 10 μ M (Fig. 3.3).

Whole cell *E. coli* GFP constitutive promoter backscreen

Cloning: GFP coding sequence was PCR amplified from pMK-RQ encoding GFP under the *glnAp2* promoter. PCR reactions were set in triplicate and contained the following: Q5 Reaction Buffer (1X), GFP_fwd and GFP_rev primers (Table S2, both 0.5 μ M), dNTPs (200 μ M, NEB #N0447), template DNA (1 ng), Q5 High-Fidelity Polymerase (0.5 units, NEB #M0491). A negative control was prepared similarly as described above without the addition of polymerase. PCR was performed as per the manufacturer's recommended conditions with an annealing temperature of 65°C and extension time of 25 seconds. PCR was confirmed via gel electrophoresis and GFP amplicons purified using an E.Z.N.A. Cycle-Pure Kit (Omega Bio-tek #D6492). GFP amplicon was cloned using standard techniques in between EcoRI and XbaI sites of a pUC18 backbone vector generating the pAP01 construct. Construct was verified by sanger sequencing with pUC18_seq_fwd and pUC18_seq_rev primers.

Screen: *E. coli* XL-1 Blue cells were transformed with pUC18 construct using standard protocols. Transformed *E. coli* was grown to a dense culture overnight in LB + Amp broth followed by a dilution to an OD₆₀₀ of 0.075 in M9 minimal nitrogen media (40 μ M NH₄Cl). Diluted bacteria in M9 media was added to black opaque 96-well plates and peptides were added from a 1 mM stock in water to a final concentration of 10 μ M in triplicate. The plates were incubated at 37 °C for 4 h and GFP fluorescence emission at 510 nm was detected on a Synergy H1 Hybrid Multi-Mode Reader (Biotek, Winooski, VT). Fluorescence change was normalized relative to the untreated control sample.

Minimal Inhibitory Concentration (MIC)

Bacterial strains were plated on LB plates and grown overnight and refrigerated before use. Peptide stocks were prepared to 64 μ g/mL from a 1 mM water stock. Mueller Hinton (MH) broth

was added to clear 96-welled plates with the first wells containing 32 $\mu\text{g/mL}$ compound in triplicate. The wells were diluted in series to 0.125 $\mu\text{g/mL}$, including only compound and only bacteria controls in final wells. Bacteria was prepared in MH broth to an $\text{OD}_{600} = 0.070$ and added to wells except for the no bacteria control. The plates were incubated in an oven at 37°C overnight and OD_{600} values of the plates were obtained on a Synergy H1 Hybrid Multi-Mode Reader (Biotek, Winooski, VT).

Inner Membrane Permeability assay (ONPG β -galactosidase)

E. coli ML35 was obtained from Prof. Brown's group (McMaster University). Cells were grown to mid-log phase ($\text{OD}_{600} = 0.4$) and spun down. The cell pellet was washed with PBS and the cells were resuspended in LB media and diluted to an OD_{600} 0.2. The suspension was pipetted into a 96-welled plate where peptides (10 μM) and vehicle control (PBS) were added to the appropriate well. BPer reagent was used as the negative control where the cell pellet was resuspended in PBS and diluted to an $\text{OD}_{600} = 0.2$ and spun back down. To this pellet was added 50% BPer solution. ONPG (1.5 mM) was added from a concentrated stock in water to all wells and the plate was incubated at 37°C for 1h. The plate was visualized on a Synergy H1 Hybrid Multi-Mode Reader (Biotek, Winooski, VT) and analyzed in GraphPad Prism 8.

Circular Dichroism (CD)

Peptides were dissolved in purified water to concentrations ranging from 20 to 40 μM . Spectra were obtained on a Jasco circular dichroism spectrometer, Model 420 (Easton, MD) at 25°C. The spectra were collected using a 1 cm path length quartz cuvette (Hellma Analytics, Germany) with the following measurement parameters: wavelength, 240-190 nm; step resolution,

0.50 nm; averaging time, 5.0 sec per step. Spectra were processed and converted to mean residue molar ellipticity using the cuvette path length (1 cm), the measured concentration, and the number of amino acids in the peptide (cross-linking amino acids and β -alanine were included as amino acids in this count). Spectra were combined and analyzed in GraphPad Prism 8.

Fluorescence Polarization Assay (FPA)

FP assays were performed to determine the dissociation constants for the peptide-DNA interactions.⁵⁹ Double stranded (ds) DNA is composed of a FITC-labelled forward single-stranded (ss) oligonucleotide and its un-labelled complementary oligonucleotide (Fig S6A), annealed together with a 2 min 95 °C incubation and a 1 h slow return to 24°C in annealing buffer (10 mM Tris pH 7.5, 50 mM NaCl and 1 mM EDTA). FITC-ds DNA strands (5.625 nM) were incubated with serially diluted concentrations of peptide (from 20 μ M) with peptide water stocks at 25 °C for 10 min in buffer (100 μ M Tris-HCl pH 7.5, 0.1 mg/mL BSA) in a black, opaque 96-well plate. The fluorescence polarization was measured at equilibrium on a Synergy H1 Hybrid Multi-Mode Reader (Biotek, Winooski, VT) and analyzed in GraphPad Prism 8.

Hemolytic activity

All experiments were performed in accordance with the Guidelines of the “Regulatory Framework in Health Research at the McGill University Health Centre”, and Experiments were approved by the ethics committee at the McGill University Health Centre institutional REB. Informed consents were obtained from human participants of this study. Human blood from a healthy volunteer was collected according to the McGill University Health Centre institutional REB approved protocol and prepared according to the Oddo and Hansen protocol²³ with minor

modifications (1 mL of RBC were prepared in a final volume of 10 mL PBS without further dilution and shaking was used to ensure consistent mixing). Peptides were diluted to 400 μ M in PBS from 1 mM stock in water. 1 % Triton X-100 was used as the positive control. Peptides were added to freshly prepared RBC in a clear 96 well plate and sealed with a plastic film to prevent evaporation and incubated at 37 °C for 1 hour. The plates were centrifuged for 10 mins at RT at $1000 \times g$ and the supernatant was removed. A 10 μ L aliquot from the supernatant was added to 90 μ L of PBS, diluted 10-fold, prior to measuring absorbance at 414 nm. % hemolysis was calculated = (abs sample – abs neg average)/(abs pos average – abs neg average) $\times$ 100%. The triplicate data was analyzed in GraphPad Prism 8.

In vivo Galleria mellonella survival assay

Galleria mellonella wax moth larvae (125-200 mg) were purchased from Critters Corners (Ottawa, ON). Larvae were handled and treated according to the methods of Harding et al.⁶⁰ The larvae were stored at room temperature for 24-48 h in wood chip bedding and dead larvae were removed from the container. When ready, the larvae were injected with 2.5 μ L of all solutions using a 26s gauge 10 μ L Hamilton syringe. The compounds dissolved in PBS buffer were injected intrahemocoelically through the last right pro-leg to give a final concentration of 10 mg/kg. *P. aeruginosa* (PA01) from a dense overnight culture was diluted down to 10^3 CFU/mL with PBS buffer and used to infect the wax worms by intrahemocoelic injection through the last left pro-leg. The vehicle and injection controls were performed with PBS buffer. Once inoculated, larvae were placed in covered empty petri dishes and incubated in a 30 °C oven for 36 hours. Survival count was verified every 4 hours and dead larvae were removed from the petri dish with tweezers. The

data were analyzed using GraphPad Prism 8 to test with log-rank Mantel-Cox estimate plots to determine survival significance.

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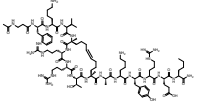
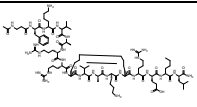
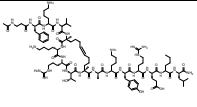
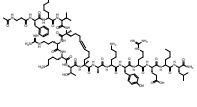
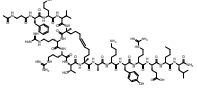
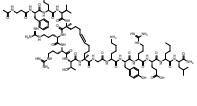
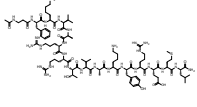
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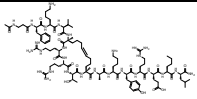
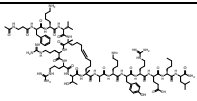
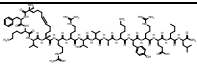
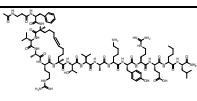
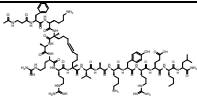
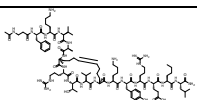
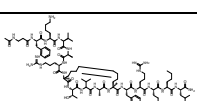
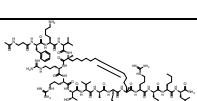
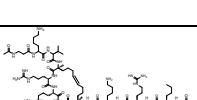
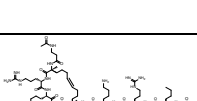
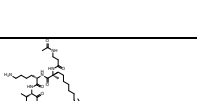
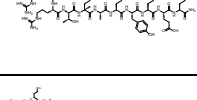
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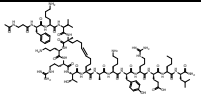
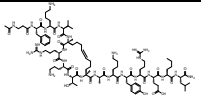
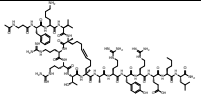
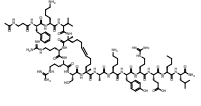
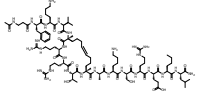
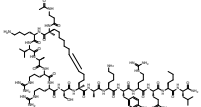
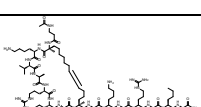
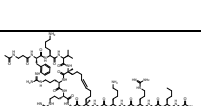
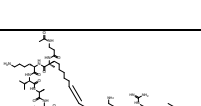
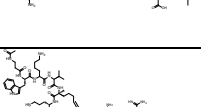
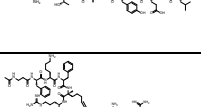
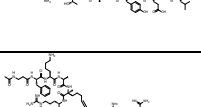
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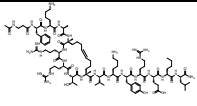
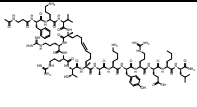
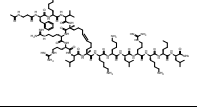
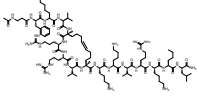
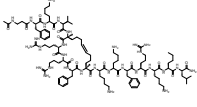
3.6 Supplementary Information

Table S1. Lead peptide purity and peptide library. Peptide sequence, structure, molecular weight (MW), calculated mass $[M+3H]^{3+}$ and observed masses $[M+3H]^{3+}$ are reported. All peptides are N-terminally acetylated and contain C-terminus amide. B: β -alanine, X: (S)-2-4-(pentenyl)alanine, Z: (R)-2-(7-octenyl)alanine, Nle: Norleucine, O: Ornithine

	New	Purity (%)	Sequence	Structure	Exact mass	Mass calc.	Mass obs.
σ 54-10	14	99.61	BFKVXRRTXAKYRENle		1928.15	643.72	643.90
σ 54-40	10	95.13	BFKVARRXVAKXRENleL		1947.23	650.08	650.60
σ 54-20	17	96.81	BFKVXKRTXAKYRENleL		2013.13	672.04	672.50
σ 54-21	18	95.75	BFKVXRRTXAKYRENleL		2013.13	672.04	672.55
σ 54-23	33	95.03	BFRVXRRTXAKYRENleL		2070.53	691.84	691.25
σ 54-27	31	97.03	BFKVXRRTXGKYRENleL		2027.22	676.74	677.20
			Sequence	Structure	Exact mass	Mass calc.	Mass obs.
σ 54-WT	WT		BFKVARRTVAKYREML		1979.13	660.71	661.20

σ54-2	2	BFKVXRRTXAKYRENIeL		2041.23	681.41	682.00
	D-2	D-(BFKVXRRTXAKYRENIeL)		2041.23	681.41	681.80
	3*	BZKVARRTXAKYRENIeL				
σ54-35	5	XFKVXRRTVAKYRENIeL		2027.25	676.75	676.75
σ54-36	6	BFXVARXTVAKYRENIeL		1927.14	643.38	643.90
σ54-37	7	BFKXARRXVAKYRENIeL		2011.22	671.41	671.85
σ54-38	8	BFKVAXRTVXKYRENIeL		1984.20	662.40	662.85
σ54-39	9	BFKVARXTVAXYRENIeL		1927.14	643.38	643.90
σ54-48	11	BFKVZRRTVAKXRENIeL		2019.28	674.09	674.55
σ54-7	12	BKVXRRTXAKYRENIeL		1894.16	632.39	633.00
σ54-9	13	BXRRTXAKYRENIeL		1667.00	556.67	557.00
σ54-15	15	BZKVARRTXAKYRENIe		1894.16	632.39	632.90
σ54-91	16	BFKVXRRTXAKYRENIeLGIPSSR		2638.55	880.52	[M+4H] ⁴⁺ 661.05

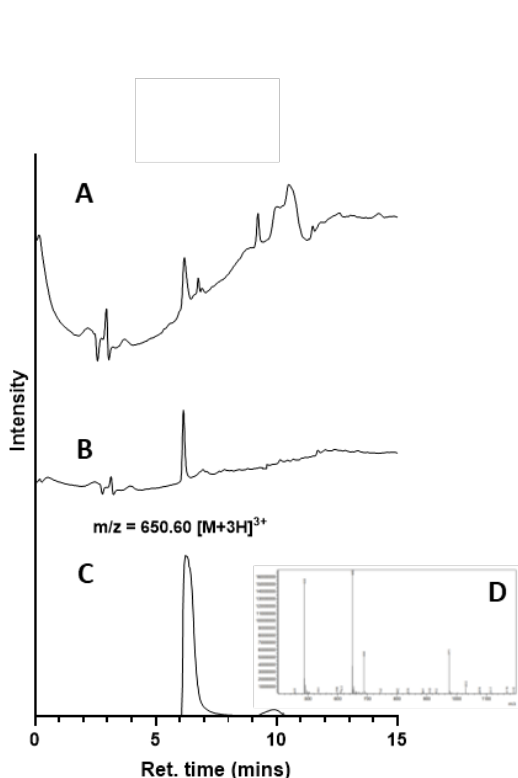
σ54-79	19	BFKVXORTXAKYRENIeL		1999.21	667.40	667.85
σ54-80	20	BFKVXROTXAKYRENIeL		1999.21	667.40	667.90
σ54-24	21	BFKVXRRTXARYRENIeL		2069.24	691.18	691.20
σ54-53	22	BFKVXRRSXAKYRENIeL		2027.22	676.74	676.90
σ54-54	23	BFKVXRRTXAKSRENIeL		1965.20	656.07	656.50
σ54-55	24	BZKVARRSXAKYRENIeL		1993.23	660.41	660.80
σ54-56	25	BZKVARRTXAKSRENIeL		1931.22	644.74	645.10
σ54-57	26	BFKVXRRTXAKTRENIeL		1979.22	660.74	661.15
σ54-58	27	BZKVARRTXAKTRENIeL		1945.23	649.41	649.80
σ54-25	28	BWKVXRRTXAKYRENIeL		2080.24	694.41	695.00
σ54-26	29	BFKFXRRTXAKYRENIeL		2089.23	697.41	697.95
σ54-99	30	BFKAXRRTXAKYRENIeL		2013.2	672.07	672.50

σ 54-98	32	BFKAXRRTXVKYRENIeL		2041.23	681.41	681.00
σ 54-78	34	BFOVXRRTXAKYRENIeL		2027.22	676.74	677.20
σ 54-100	35	BFKVXRRLLKKLRKNIeL		2045.42	682.81	681.85
σ 54-102	36	BFKVXRRVXKKVRKNIeL		2031.37	678.12	678.55
σ 54-103	37	BFKVXRRFXKKFRKNIeL		2127.37	710.12	711.2

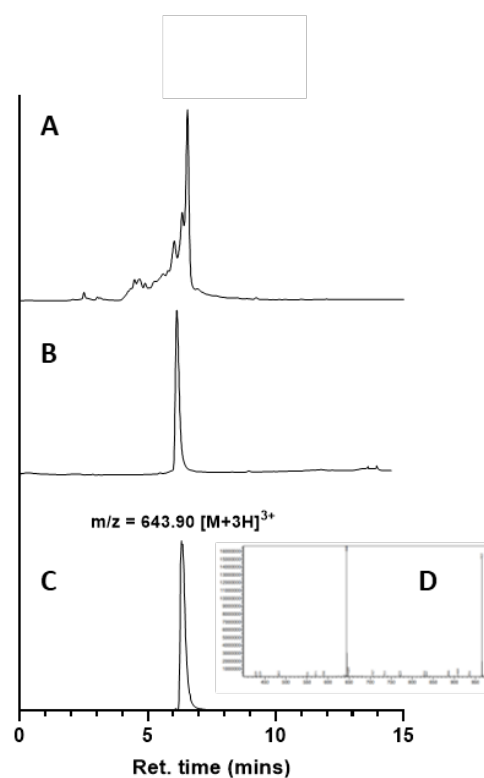
*Peptide 3 was used as a reference sequence for $i, i+7$ constructs³.

Figures S1. HPLC purified peptides 10, 14, 17, 18, 31, 33, WT, 2 and D-2 and LCMS traces.

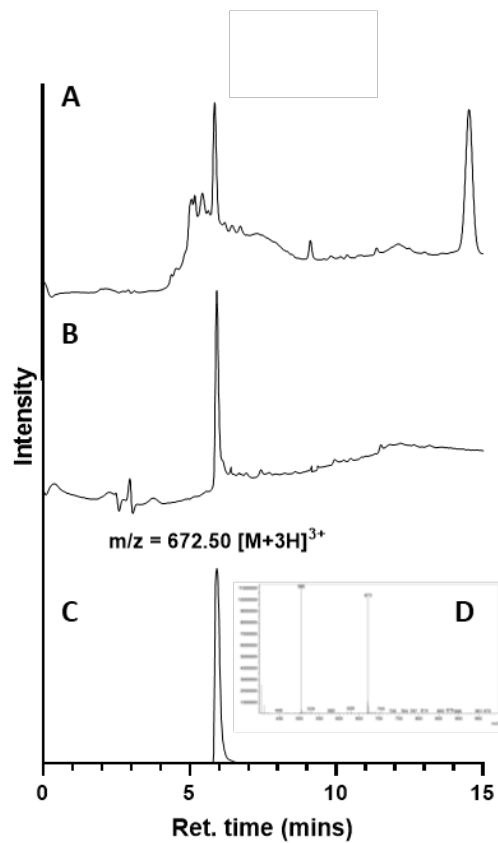
A. Crude chromatogram traces (absorbance at 274 nm). **B.** Extracted ion chromatogram (EIC). **C.** Purified chromatogram trace (absorbance at 274 nm). **D.** ESI+/MS spectra. Data were collected using a 2020-LCMS Shimadzu instrument (ESI+ and UV-Vis detection at 274 nm or 210 nm) with a Prontosil C18 analytical column. A linear gradient of 5-95% (0.05% formic acid H₂O- 0.05% formic acid MeOH) over 20 minutes was used for analytical runs. Purification was performed on a 1100 Series Agilent HPLC with fraction collector and DAD at 274 nm on a Thermo C18 column. A linear gradient of 5-95% (0.1% TFA H₂O- 0.1% TFA MeOH) over 20 minutes was used.



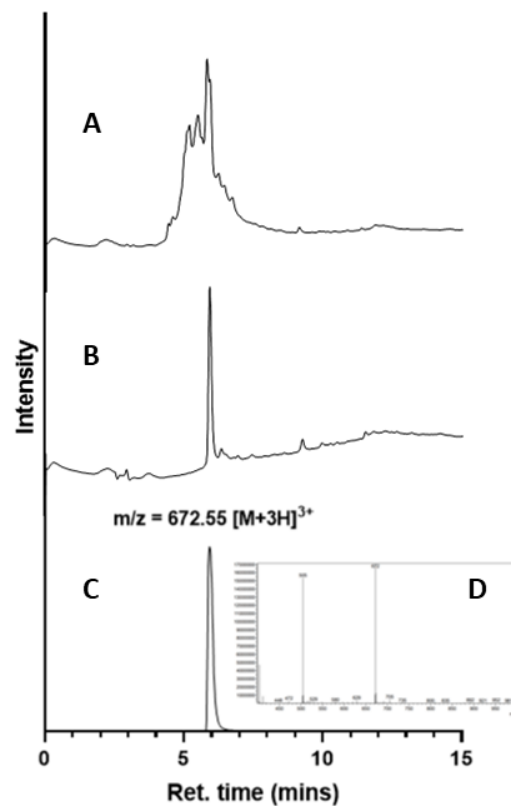
Analysis of peptide 10 – A. Crude LCMS trace detected at 274 nm B. Purified LCMS trace detected at 274 nm C. Purified LCMS trace with extracted ion chromatogram, $m/z = 650.60$ D. ESI/MS of peptide 10



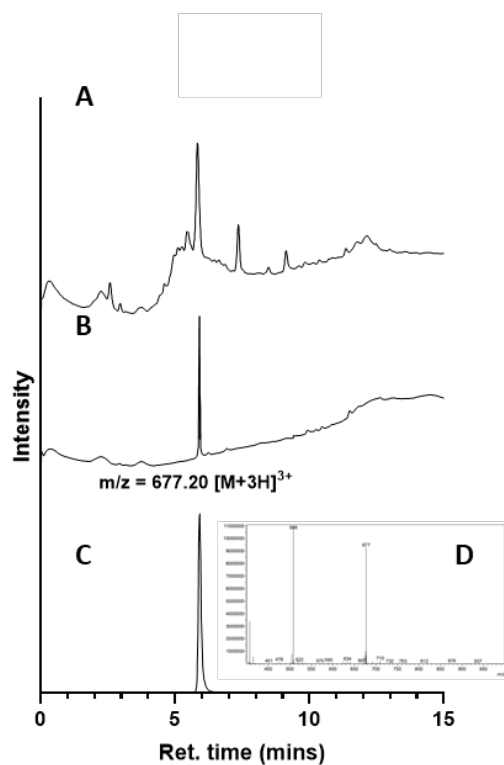
Analysis of peptide 14 – A. Crude LCMS trace detected at 274 nm B. Purified LCMS trace detected at 274 nm C. Purified LCMS trace with extracted ion chromatogram, $m/z = 643.90$ D. ESI/MS of peptide 14



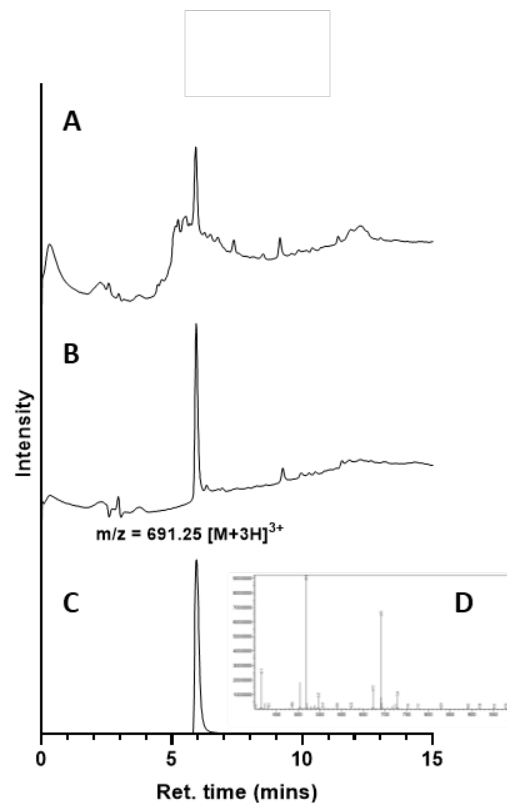
Analysis of peptide 17 – A. Crude LCMS trace detected at 274 nm B. Purified LCMS trace detected at 274 nm C. Purified LCMS trace with extracted ion chromatogram, $m/z = 672.50$ D. ESI/MS of peptide 17



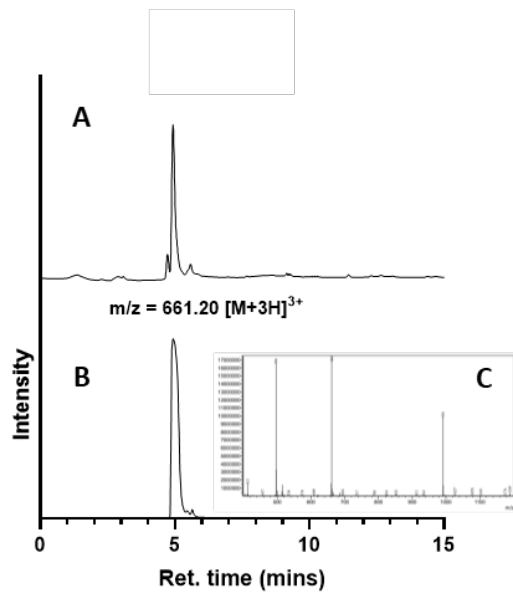
Analysis of peptide 18 – A. Crude LCMS trace detected at 274 nm B. Purified LCMS trace detected at 274 nm C. Purified LCMS trace with extracted ion chromatogram, $m/z = 672.55$ D. ESI/MS of peptide 18



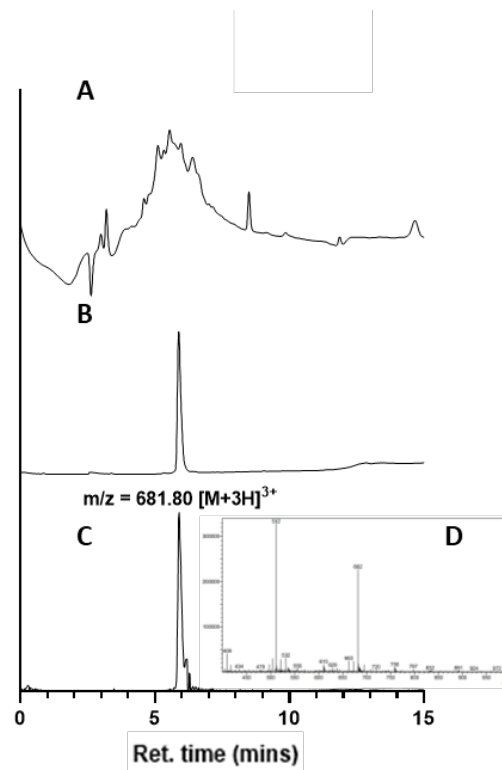
Analysis of peptide 31 – A. Crude LCMS trace detected at 274 nm B. Purified LCMS trace detected at 274 nm C. Purified LCMS trace with extracted ion chromatogram, $m/z = 677.20$ D. ESI/MS of peptide 31



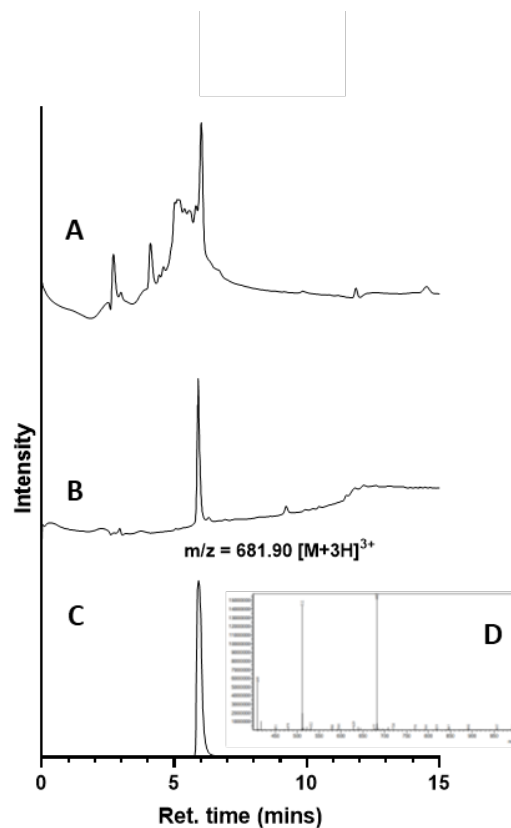
Analysis of peptide 33 – A. Crude LCMS trace detected at 274 nm B. Purified LCMS trace detected at 274 nm C. Purified LCMS trace with extracted ion chromatogram, $m/z = 691.25$ D. ESI/MS of peptide 33



Analysis of WT peptide – A. Pure LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 661.20$ D. ESI/MS of WT peptide

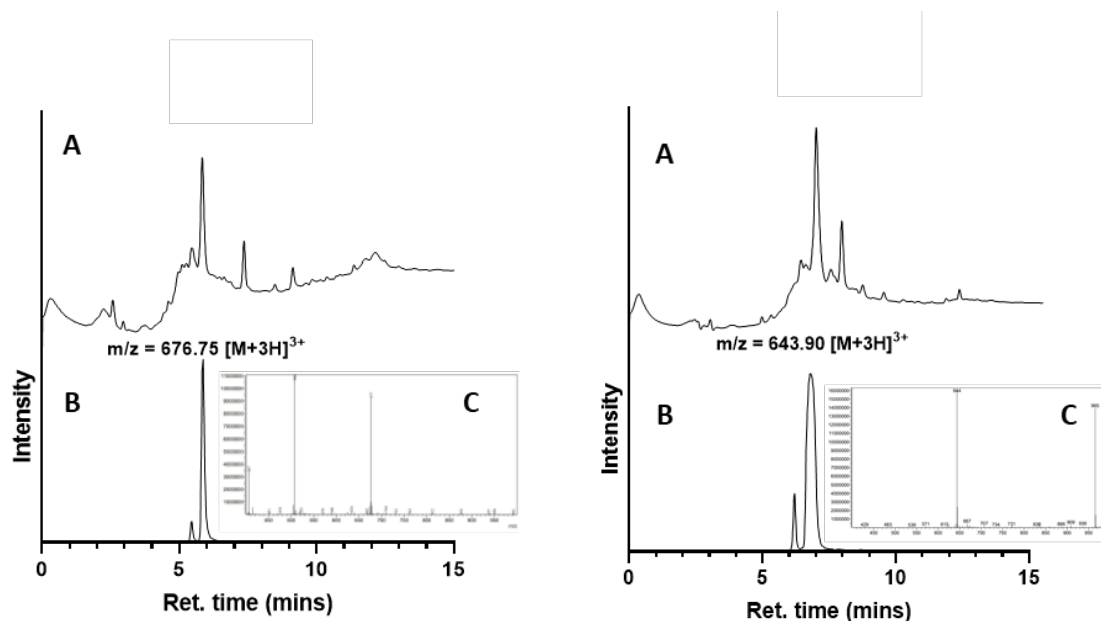


Analysis of peptide D-2 – A. Crude LCMS trace detected at 274 nm B. Purified LCMS trace detected at 274 nm C. Purified LCMS trace with extracted ion chromatogram, $m/z = 681.80$ D. ESI/MS of peptide D-2



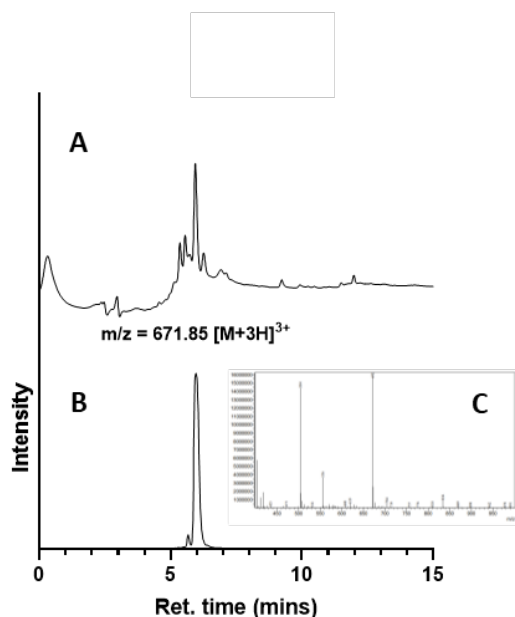
Analysis of peptide 2 – A. Crude LCMS trace detected at 274 nm B. Purified LCMS trace detected at 274 nm C. Purified LCMS trace with extracted ion chromatogram, m/z = 682.00 D. ESI/MS of peptide 2

Figures S2. Crude peptides LCMS traces. **A.** Crude chromatogram traces (Absorbance at 274 nm). **B.** Extracted ion chromatogram (EIC). **C.** ESI+/MS spectra. Spectra were obtained on a 2020-LCMS Shimadzu instrument (ESI+ and 274 nm detection) with a Prontosil C18 analytical column. A linear gradient of 5-95% (0.05% formic acid H₂O- 0.05% formic acid MeCN) over 20 minutes was used.

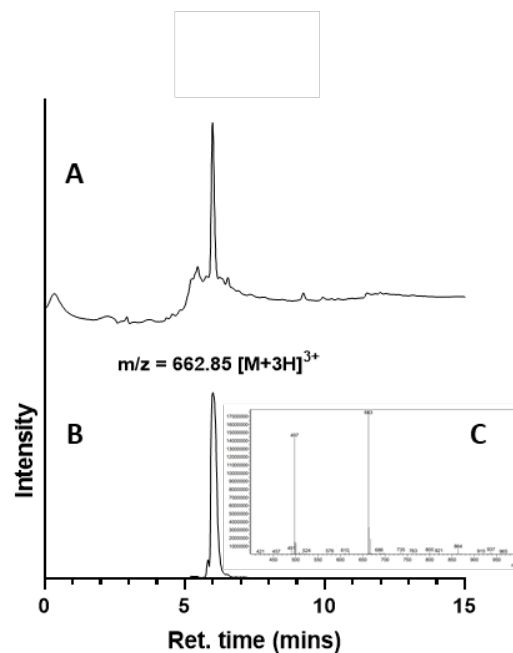


Analysis of peptide 5 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 676.75$ D. ESI/MS of peptide 5

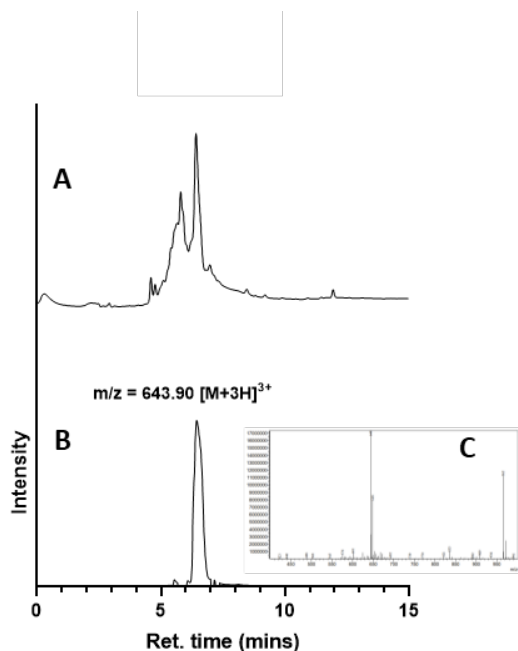
Analysis of peptide 6 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 643.90$ D. ESI/MS of peptide 6



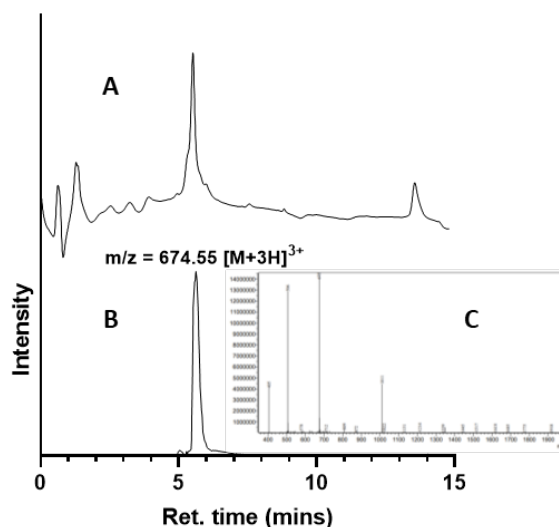
Analysis of peptide 7 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 671.85$ D. ESI/MS of peptide 7



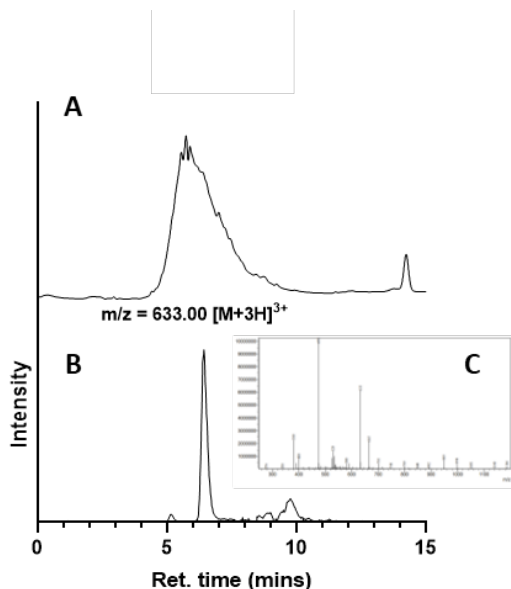
Analysis of peptide 8 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 662.85$ D. ESI/MS of peptide 8



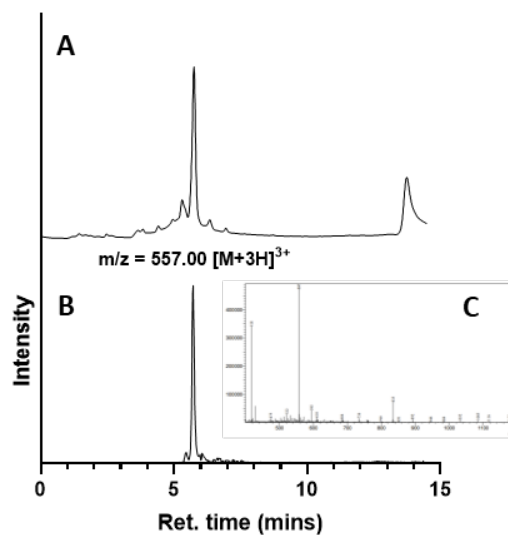
Analysis of peptide 9 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 643.90$ D. ESI/MS of peptide 9



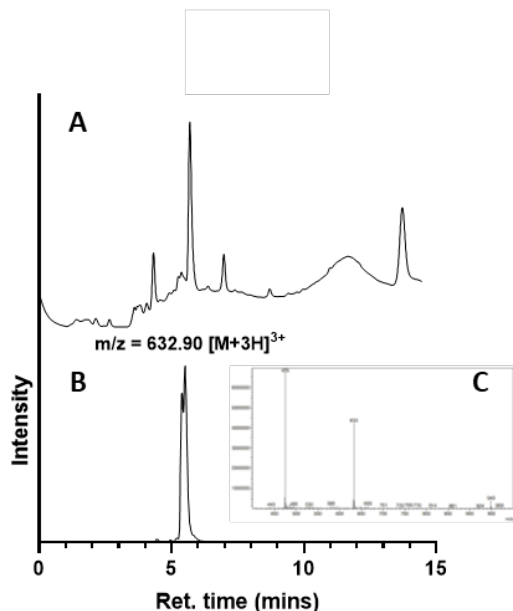
Analysis of peptide 11 – A. Crude LCMS trace detected at 210 nm B. LCMS trace with extracted ion chromatogram, $m/z = 674.55$ D. ESI/MS of peptide 11



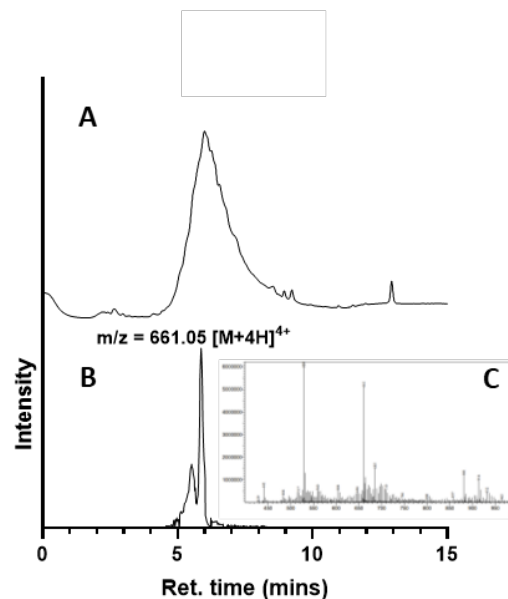
Analysis of peptide 12 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 633.00$ D. ESI/MS of peptide 12



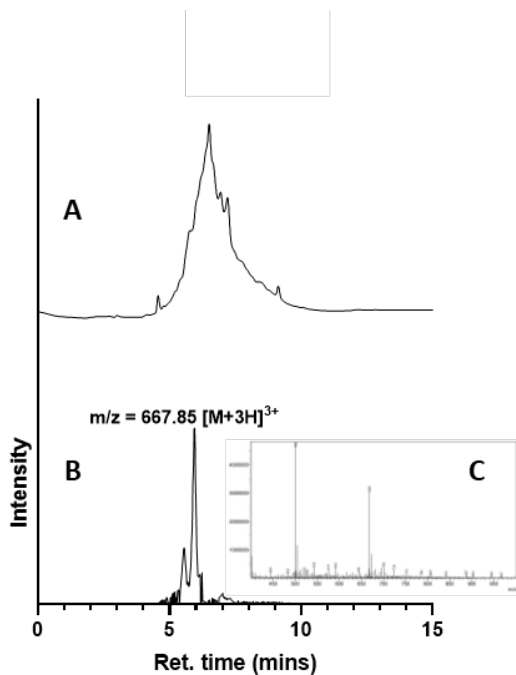
Analysis of peptide 13 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 557.00$ D. ESI/MS of peptide 13



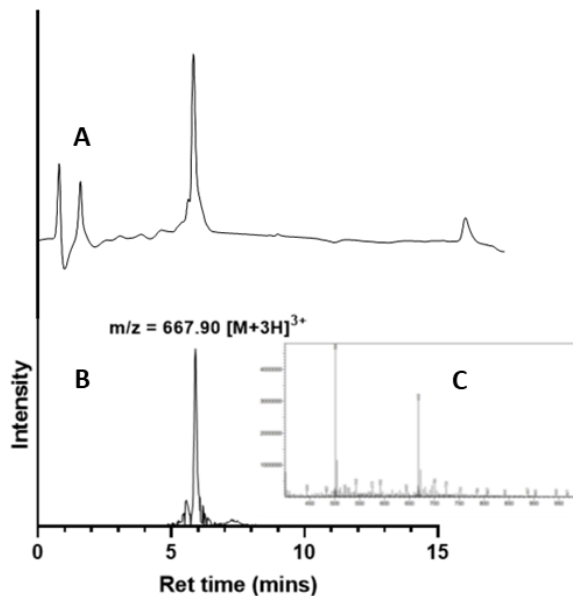
Analysis of peptide 15 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 632.90$ D. ESI/MS of peptide 15



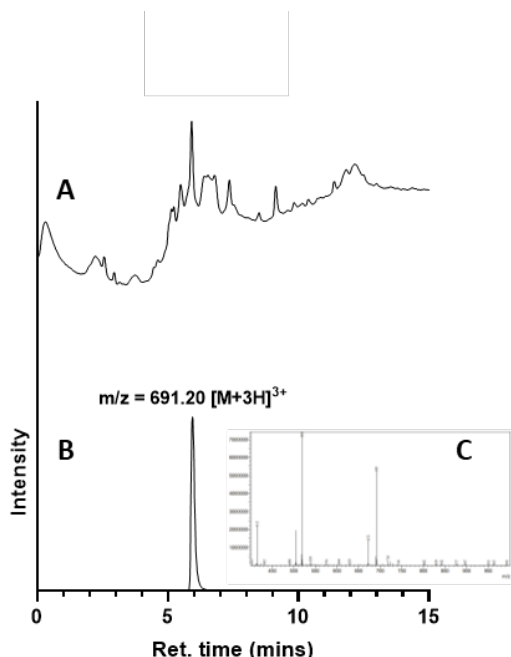
Analysis of peptide 16 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 661.05$ D. ESI/MS of peptide 16



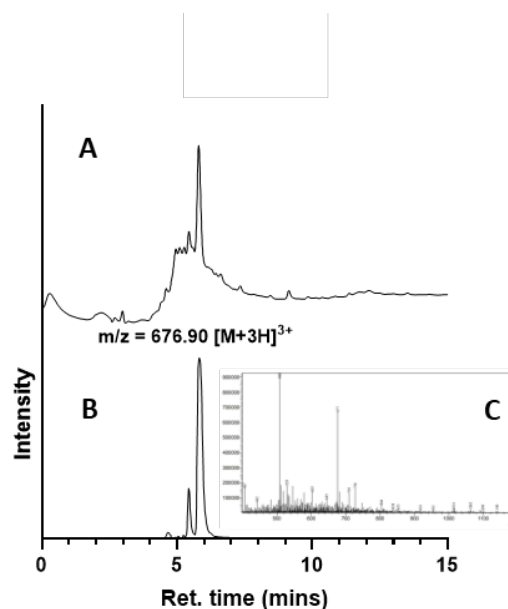
Analysis of peptide 19 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 667.85$ D. ESI/MS of peptide 19



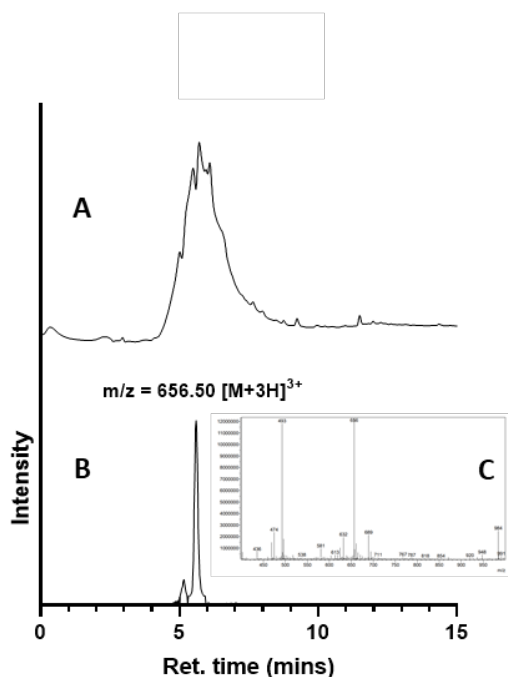
Analysis of peptide 20 – A. Crude LCMS trace detected at 210 nm B. LCMS trace with extracted ion chromatogram, $m/z = 667.90$ D. ESI/MS of peptide 20



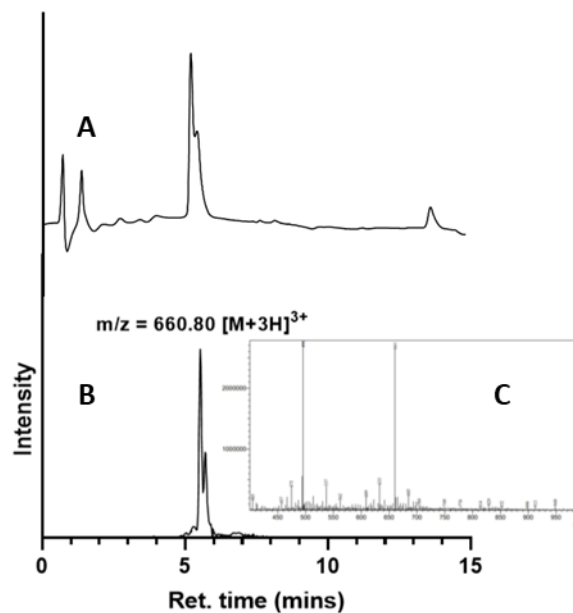
Analysis of peptide 21 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 691.20$ D. ESI/MS of peptide 21



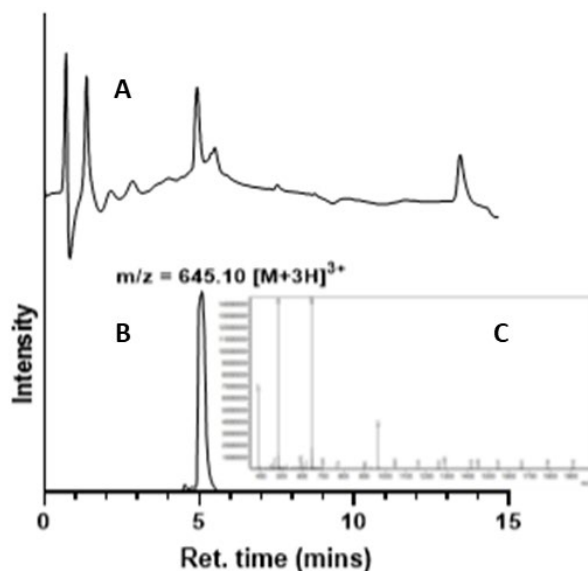
Analysis of peptide 22 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 676.90$ D. ESI/MS of peptide 22



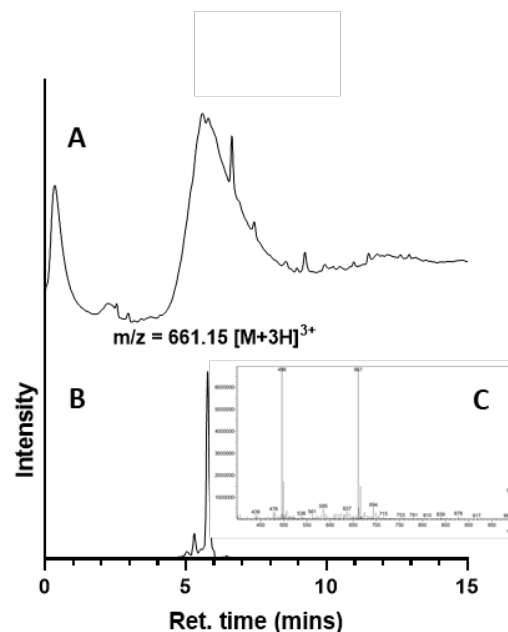
Analysis of peptide 23 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 656.50$ D. ESI/MS of peptide 23



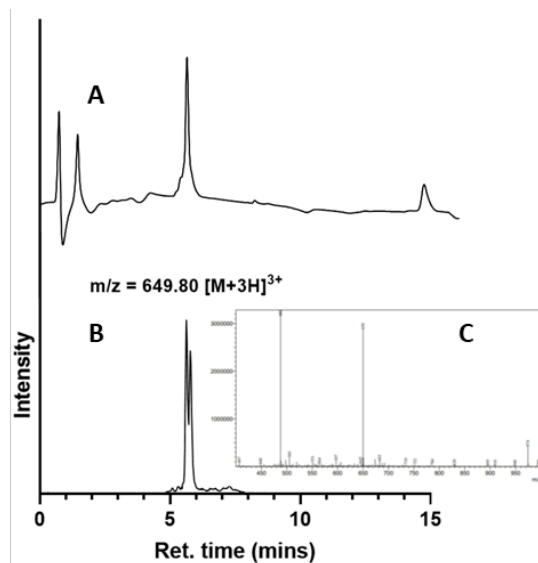
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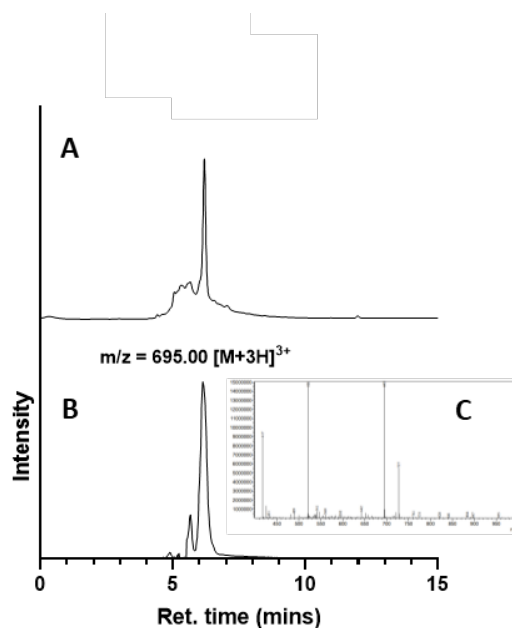
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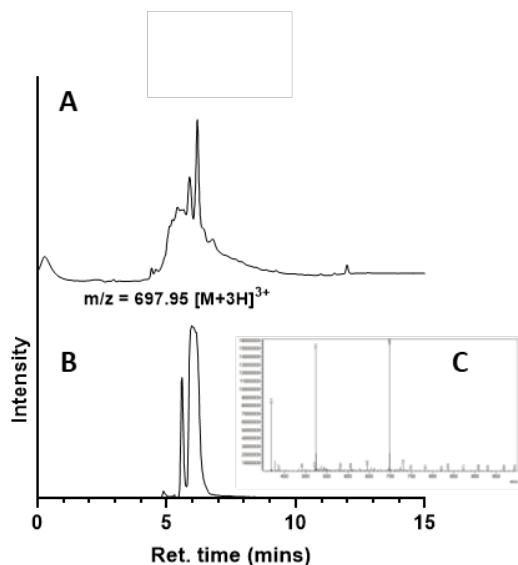
Analysis of peptide 26 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 661.15$ D. ESI/MS of peptide 26



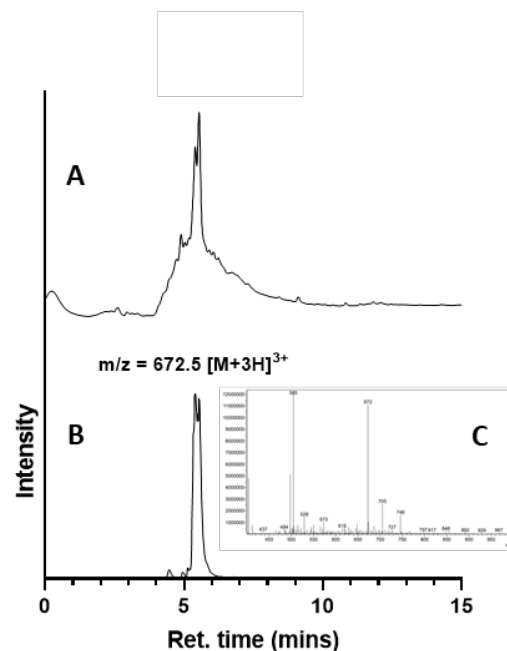
Analysis of peptide 27 – A. Crude LCMS trace detected at 210 nm B. LCMS trace with extracted ion chromatogram, $m/z = 649.80$ D. ESI/MS of peptide 27



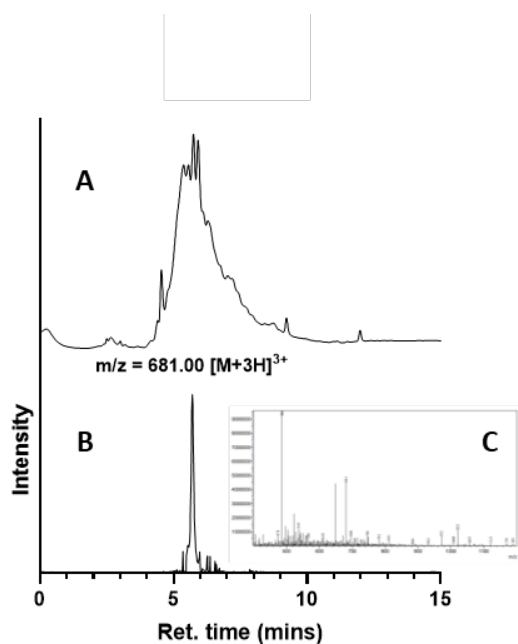
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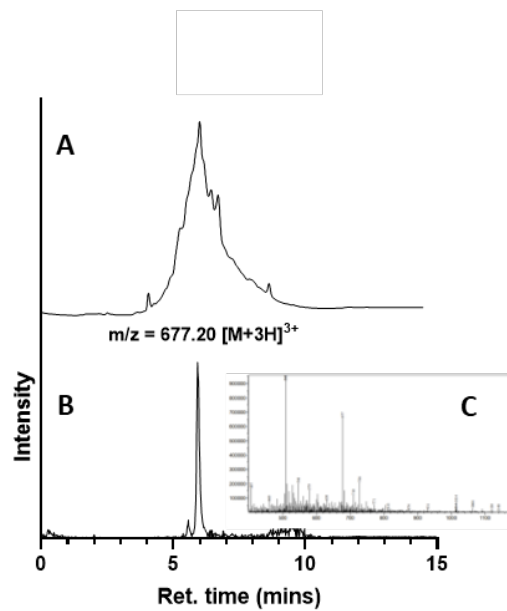
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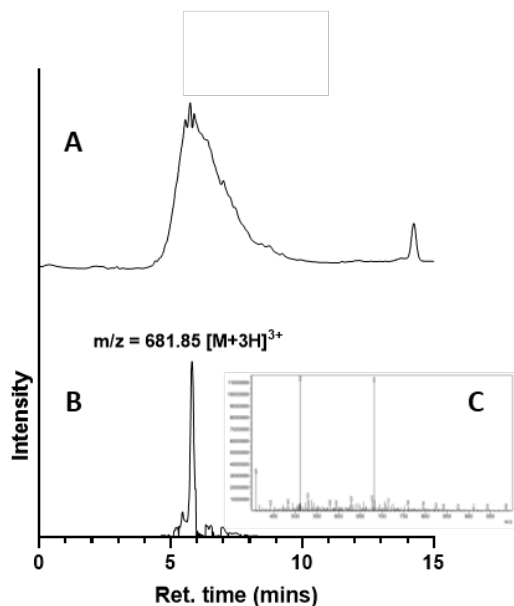
Analysis of peptide 30 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 672.50$ D. ESI/MS of peptide 30



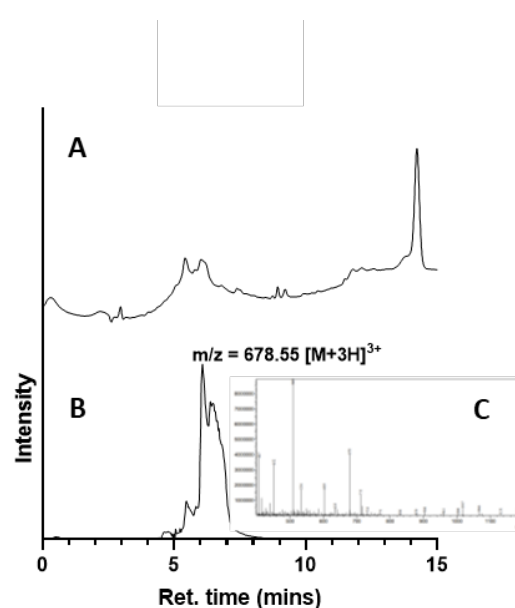
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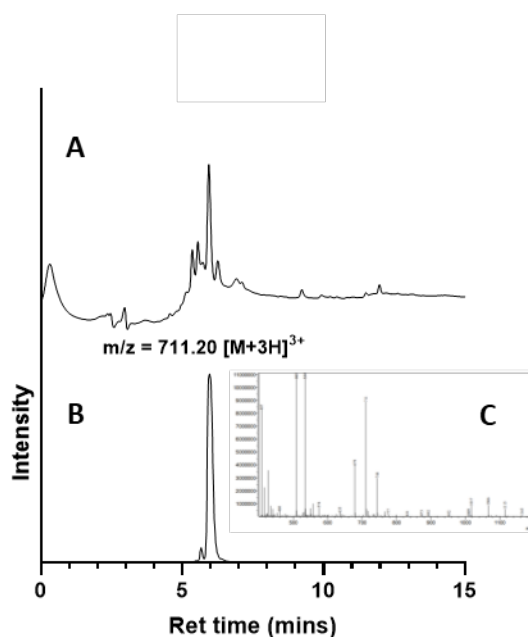
Analysis of peptide 34 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 677.20$ D. ESI/MS of peptide 34



Analysis of peptide 35 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 681.85$ D. ESI/MS of peptide 35



Analysis of peptide 36 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 678.55$ D. ESI/MS of peptide 36



Analysis of peptide 37 – A. Crude LCMS trace detected at 274 nm B. LCMS trace with extracted ion chromatogram, $m/z = 711.20$ D. ESI/MS of peptide 37

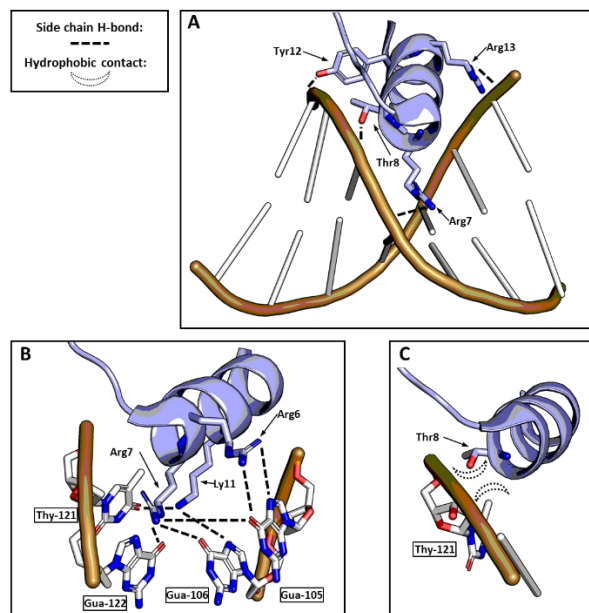


Figure S3. σ^{54} -24 promoter recognition model. **A.** RpoN helix with DNA phosphate binding residues Arg7, Thr8, Tyr12 and Arg13. **B.** RpoN helix with nucleobase H-bonding residues Arg6, Arg7 and Lys11. **C.** RpoN helix with hydrophobic interaction of Thr 8. Based on PDB: 2O8K

Table S2. Minimal Inhibitory concentration (MIC). Peptide **WT, 2, 10, 14, 17, 18, 31** and **33** MIC against *Escherichia coli* (XL1Blue and BW25113) and *Pseudomonas aeruginosa* (PA01) starting from 32 $\mu\text{g/mL}$.

Bacterial species	MIC ($\mu\text{g/mL}$)							
	WT	2	10	14	17	18	31	33
XL1Blu	>32	>32	32	>32	>32	>32	16	32
BW25113	>32	>32	32	>32	>32	>32	32	32
PA01	>32	>32	>32	>32	>32	>32	>32	>32

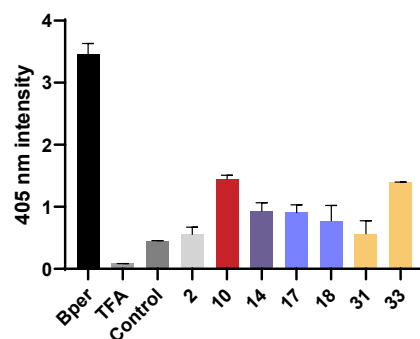


Figure S4. Inner membrane permeability assay. Peptide WT, 2, 10, 14, 17, 18, 31 and 33 permeability. 0.1% TFA was used as positive control, control conditions is 1.5 mM ONPG and BPer treatment as negative control

>PglAP2-GFP

CGGAATTCGCGGCCGCTTCTAGAGACATCCTCCGCAAACAAGTATTGCAGAGTCCCT
 TTGTGATCGCTTTCACGGAGCATAAAAAGGGTTATCCATCATGAGATGCACCAACAT
 GGTGCTTAATGTTTCCATTGAAGCACTATATTGGTGCAACATTCACATCGTGGTGCA
 GCCCTTTTGCACGATGGTGCGCATGATAACGCCTTTTAGGGGCAATTTAAAAGTTGG
CACAGATTTCGCTTTATCTTTTAAATACTAGGATTAAAGAGGAGAAATACTAGATGC
 GTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTG
 ATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATAC
 GGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCA
 ACACTTGTCACTACTTTCGGTTATGGTGTTCAATGCTTTGCGAGATACCCAGATCATA
 TGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGA
 ACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAA
 GGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGA
 AACATTCTTGGACACAAATTGGAATACAACATACTCACACAATGTATACATCATG

GCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGA
AGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATG
GCCCTGTCCTTTTACCAGACAACCATTACCTGTCCACACAATCTGCCCTTTCGAAAG
ATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTGAGTTTGTAACAGCTGCTGGGA
TTACACATGGCATGGATGAACTATACAAAT***TAA***TAATACTAGAGCCAGGCATCAAAT
AAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGT
GAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTCTGCGTTTAT
ATACTAGTAGCGGCCGCTGCAGGGTACCCC

Figure S5. Pgl_{AP2}-30 GFP construct sequence. Fasta format sequence of the synthetic construct used for the whole cell assay to evaluate stapled peptide inhibition of RpoN-mediated transcription. The construct encodes GFP (start and stop codons in bold italics) under the control of the *glnAP2* promoter (italicized with the -24 and -12 sites underlined).

Table S3. Primer sequences used for GFP cloning. Oligos were designed using standard primer design guidelines and ordered from Integrated DNA Technologies with standard desalting. All oligos are shown 5' to 3' direction. Restriction sites are underlined in the appropriate primers.

Oligo name	Sequence
GFP_fwd	TCAT <u>GAATTCA</u> ATGCGTAAAGGAGAAGAACTTTTC
GFP_rev	GATCGT <u>CTAGAT</u> TATTTGTATAGTTCATCCATGCCATG
pUC18_seq_fwd	GCACGACAGGTTTCCCGACTG
pUC18_seq_rev	CGGGCCTCTTCGCTATTACGC

Table S4. GFP assay fluorescence results for all peptides. Peptides were tested at 10 μ M and 2.5 μ M in M9 40 μ M NH_4Cl content. Data are represented as $n = 3$ (except for PBS and **2** where $n = 12$), means $\pm$ STD. Fluorescence is normalized to untreated samples.

Peptide	Sequence	Net Fluorescence Change (GFP)	
		2.5 μ M	10 μ M
2	BFKVXRRTXAKYRENIeL	0.935 ± 0.0656	0.246 ± 0.160
WT	BFKVARRTVAKYREML	0.888 ± 0.0479	0.892 ± 0.0468
D-2	D-(BFKVXRRTXAKYRENIeL)	0.916 ± 0.0415	0.677 ± 0.0178
5	XFKVXRRTVAKYRENIeL	1.19 ± 0.0758	1.009 ± 0.122
6	BFXVARXTVAKYRENIeL	1.13 ± 0.0403	0.831 ± 0.115
7	BFKXARRXVAKYRENIeL	0.970 ± 0.0386	0.854 ± 0.136
8	BFKVAXRTVXKYRENIeL	1.09 ± 0.219	0.888 ± 0.503
9	BFKVARXTVAXYRENIeL	1.10 ± 0.141	0.951 ± 0.117
10	BFKVARRXVAKXRENIeL	0.577 ± 0.0908	0.0298 ± 0.0768
11	BFKVZRRTVAKXRENIeL	0.4901 ± 0.0406	0.505 ± 0.0330
12	BKVXRRTXAKYRENIeL	0.962 ± 0.0819	0.947 ± 0.0883
13	BXRRTXAKYRENIeL	0.919 ± 0.888	0.843 ± 0.0902
14	BFKVXRRTXAKYRENIe	0.885 ± 0.114	0.0288 ± 0.0343
15	BZKVARRTXAKYRENIe	0.963 ± 0.0652	0.757 ± 0.113
16	BFKVXRRTXAKYRENIeLGIPSSR	0.894 ± 0.0628	0.427 ± 0.0278
17	BFKVXKRTXAKYRENIeL	0.943 ± 0.0699	0.0801 ± 0.0308
18	BFKVXRKTXAKYRENIeL	1.008 ± 0.00746	0.0374 ± 0.0129
19	BFKVXORTXAKYRENIeL	0.519 ± 0.0375	0.523 ± 0.0224
20	BFKVXROTXAKYRENIeL	0.909 ± 0.155	0.773 ± 0.0516
21	BFKVXRRTXARYRENIeL	0.935 ± 0.0113	0.4493 ± 0.0446
22	BFKVXRRSXAKYRENIeL	1.02 ± 0.0916	0.475 ± 0.0976
23	BFKVXRRTXAKSRENIeL	0.801 ± 0.0990	0.818 ± 0.0371
24	BZKVARRSXAKYRENIeL	0.850 ± 0.476	0.733 ± 0.0815
25	BZKVARRTXAKSRENIeL	0.529 ± 0.0398	0.471 ± 0.0321
26	BFKVXRRTXAKTRENIeL	0.999 ± 0.0911	0.675 ± 0.124
27	BZKVARRTXAKTRENIeL	0.883 ± 0.113	0.813 ± 0.170
28	BWKVXRRTXAKYRENIeL	0.9201 ± 0.0142	0.179 ± 0.262
29	BFKFXRRTXAKYRENIeL	1.006 ± 0.0646	0.739 ± 0.330
30	BFKAXRRTXAKYRENIeL	0.520 ± 0.0126	0.454 ± 0.0508
31	BFKVXRRTXGKYRENIeL	0.451 ± 0.0839	0.0358 ± 0.0292
32	BFKAXRRTXVKYRENIeL	0.919 ± 0.0740	0.477 ± 0.0615
33	BFRVXRRTXAKYRENIeL	0.700 ± 0.657	0.0247 ± 0.0152
34	BFOVXRRTXAKYRENIeL	1.11 ± 0.140	0.872 ± 0.0590
35	BFKVXRRLXKKLRKNIeL	0.926 ± 0.0676	0.346 ± 0.156
36	BFKVXRRVXKKVRKNIeL	0.998 ± 0.0802	0.800 ± 0.0587
37	BFKVXRRFXKKFRKNIeL	0.743 ± 0.00807	0.623 ± 0.212

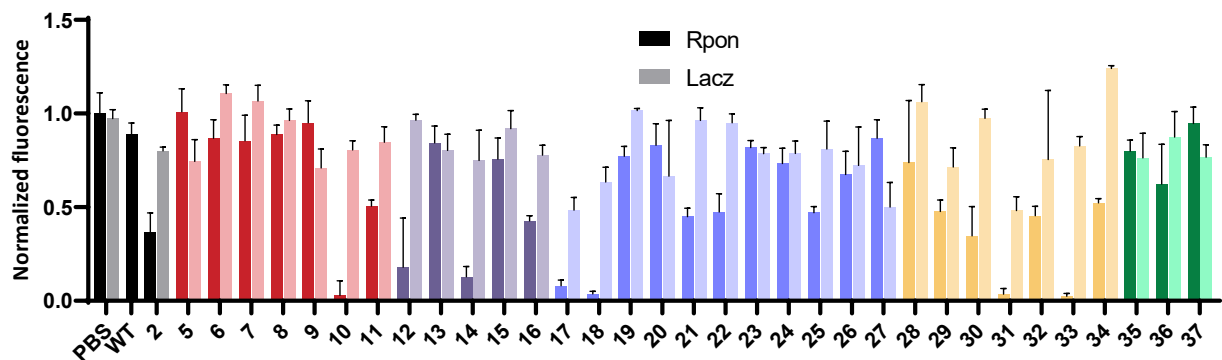


Figure S6. Constitutive promoter GFP and RpoN-GFP results. Library screen of RpoN-*glnAp2* promoter (black or dark colored) and constitutive *lacUV5* promoter (grey or light colored) GFP screen. Means $\pm$ STD, normalized to vehicle control

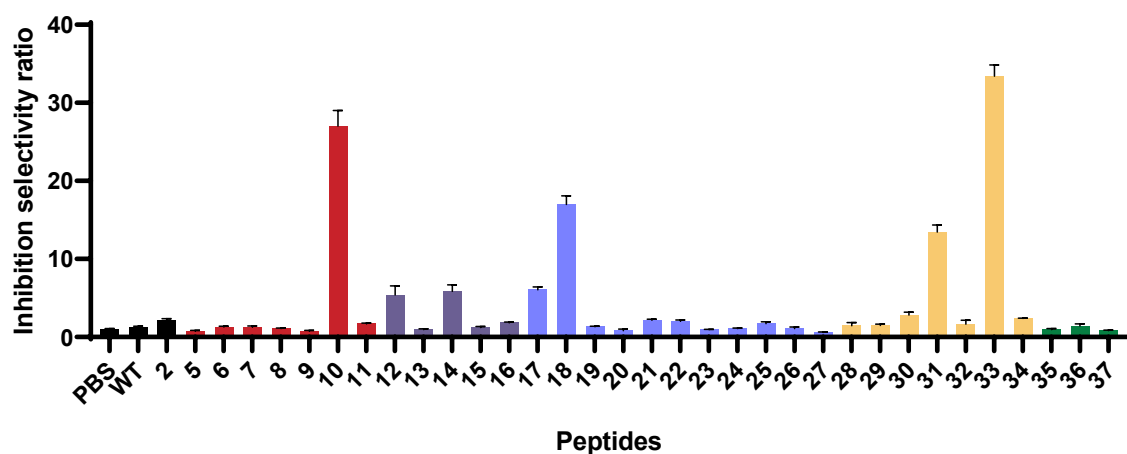


Figure S7. Inhibitory selectivity ratio. Ratio of GFP expression results of peptides 2-37 of RpoN-*glnAp2* promoter and counter-screen *lacUV5* promoter GFP screen. Means $\pm$ STD, normalized to vehicle control

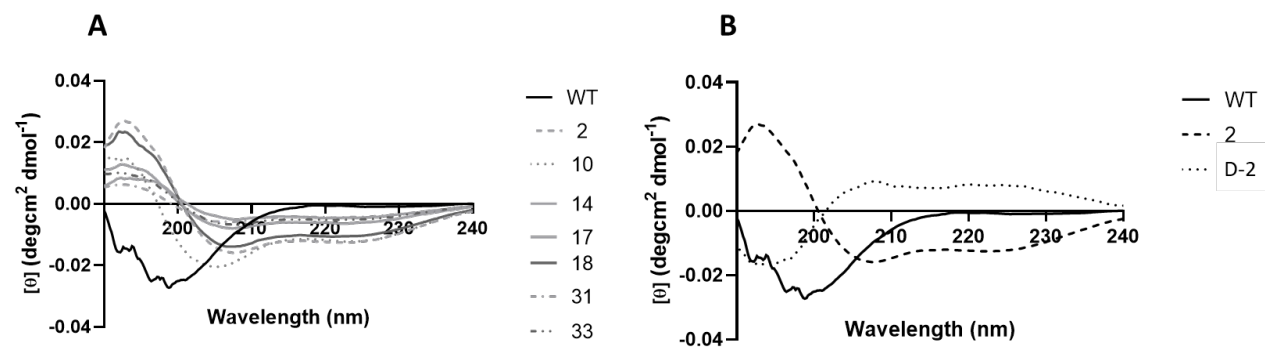


Figure S8. Circular Dichroism (CD). A. CD of purified peptides 10, 14, 17, 18, 31 and 33. B. CD of controls WT, 2, D-2.

A

Name	Sequence (5' -> 3')
FITC <i>glnAP2</i> 30mer Forward	/5FluorT/AGTTGGCACAGATTTTCGCTTTATCTTTTT
FITC <i>glnAP2</i> 30mer Reverse	/5FluorT/AAAAAAGATAAAGCGAAATCTGTGCCAACT
FITC <i>glnAP2</i> 30mer-scrambled Forward	/5FluorT/GTGCTATTATTCCGTTATCGTTGTTACTAT
FITC <i>glnAP2</i> 30mer-scrambled Reverse	/5FluorT/ATAGTAACAACGATAACGGAATAATAGCAC

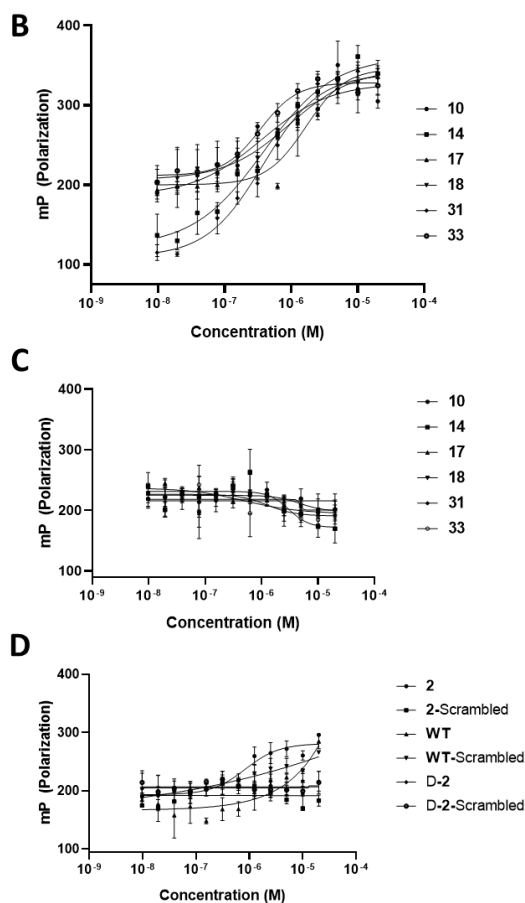


Figure S9. Fluorescence polarization assay (FPA) binding of purified peptides. Binding of serially diluted (from 20 mM) peptides incubated with DNA (5.625 nM), reporting binding affinity (K_d) **A.** *glnAP2* and scrambled forward and reverse fluorescein labelled DNA oligos name and sequence. **B.** Binding curves of peptides with *glnAP2* promoter. **C.** Binding curves of peptides with scrambled DNA oligo. **D.** Controls WT, 2 and D-2 binding to *glnAP2* promoter and scrambled DNA sequence.

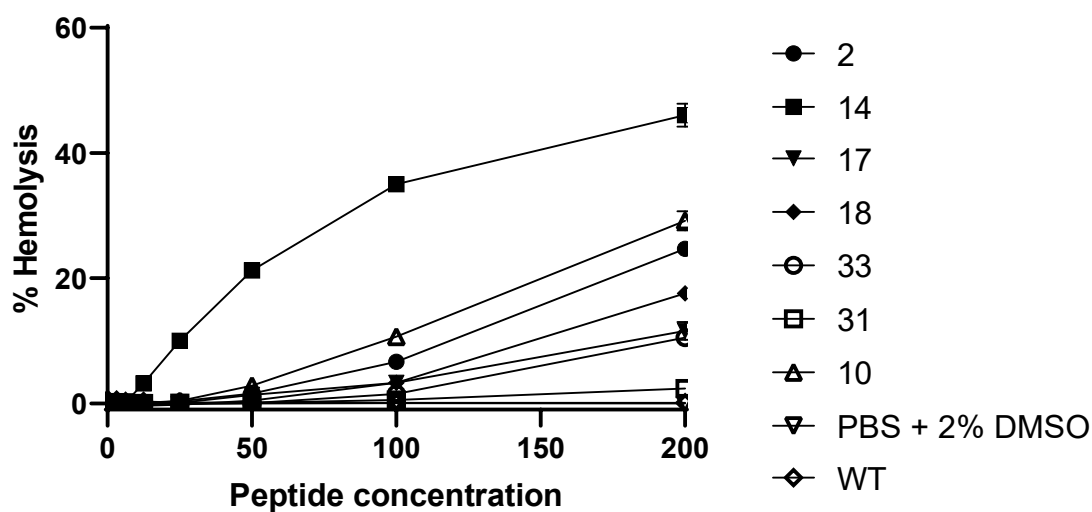


Figure S10. Hemolysis data up to 200 μ M in human red blood cells. Purified peptides, 2 and WT % hemolysis.

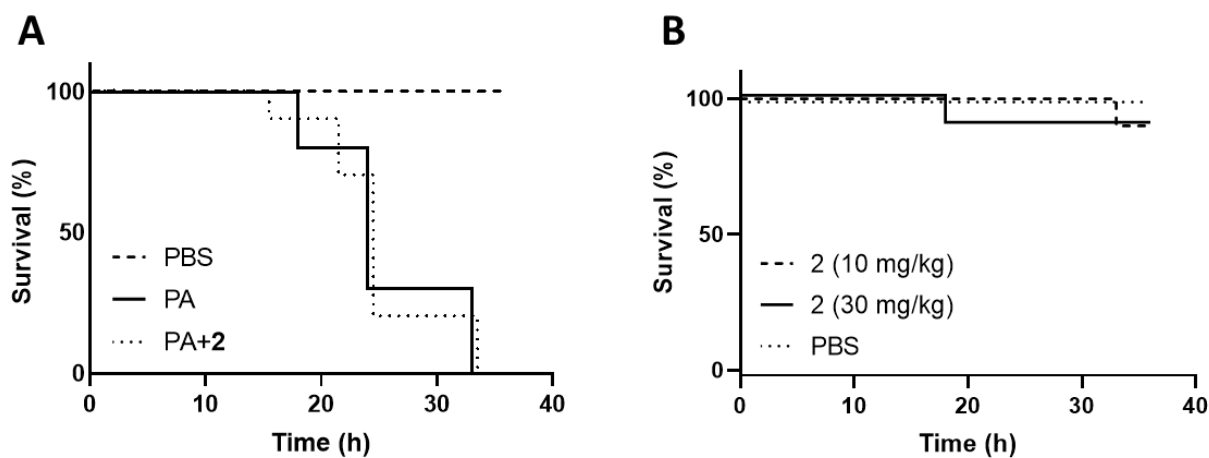


Figure S11. *In vivo* *G. mellonella* supplemental data. A. PBS vehicle control, *Pseudomonas aeruginosa* (PA) and PA + 2. B. 2 (10 mg/kg), 2 (30 mg/kg) and PBS vehicle control.

References

1. Payne SR, Pau DI, Whiting AL, et al. Inhibition of Bacterial Gene Transcription with an RpoN-Based Stapled Peptide. *Cell Chem Biol.* 2018;25(9):1059-1066.e4.
doi:10.1016/j.chembiol.2018.05.007

Chapter 4: Macrocyclization strategies for the total synthesis of cyclic depsipeptides

4.1 Introduction

The synthesis of cyclic depsipeptides has interested chemists over the last 20 years due to their potential as biologically relevant compounds and for their interesting and vast structural diversity. This subclass of nonribosomally synthesized peptides (NRPs) is characterized by containing at least one macrolactone bond within the macrocycle ring. These esters are produced by biosynthetic enzymes, including the macrocyclizing enzyme thioesterase (TE). Macrolactones have been a notorious synthetic challenge due to the lowered nucleophilicity of the alcohol nucleophile and the increased chance of C-terminal epimerization due to harsh activation conditions. This is especially the case for depsipeptides where peptide C-termini are readily epimerizable under standard macrolactone conditions (Corey-Nicolau, Yamaguchi, etc.¹), requiring milder cyclizing conditions.

With the development of SPPS, chemists have undertaken challenging peptide syntheses that would be otherwise very lengthy using standard solution phase peptide chemistry. This has been applied to several cyclic depsipeptide syntheses, accomplishing complex linear peptide synthesis and establishing clever macrocyclizing strategies.

This review explores some examples of depsipeptide chemistry², focusing on total syntheses accomplished mostly on-resin with different macrocyclization strategies. The dominant strategy is to select an amide within the macrocycle to be established as the macrocyclizing point, synthesizing the ester using milder esterification conditions on a linear peptide substrate. We first discuss solution phase macrolactamization from branched peptides synthesized on solid support and cleaved under mild conditions enabling retention of sidechain protecting groups. We then

examined syntheses taking advantage of the peptide sequences with a functionalizable sidechain that can be used as the solid support anchoring position. With the use of orthogonal protecting group schemes, this allows for complete cyclic depsipeptide syntheses on-resin, requiring one final peptide purification. Several depsipeptides are synthesized through macrolactonization approaches using strategic lactonization positions and mild esterification conditions. Lastly we highlight select chemoenzymatic syntheses that use cyclizing enzymes as biocatalysts to perform otherwise challenging macrolactonizations.

4.2 References

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2. Hamada, Y. & Shioiri, T. Recent Progress of the Synthetic Studies of Biologically Active Marine Cyclic Peptides and Depsipeptides. *Chem. Rev.* **105**, 4441–4482 (2005).

4.3 Author contributions

A.R.P. and C.N.B. conceptualized the work, A.R.P. wrote the original draft and A.R.P. and C.N.B. edited and finalized the draft.

4.4 Copyright

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4.5 Macrocyclization strategies for the total synthesis of cyclic depsipeptides

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Abstract

Cyclic depsipeptides are an important class of peptide natural products that are defined by the presence of ester and amide bonds within the macrocycle. The structural diversity of depsipeptides has required the development of a broad range of synthetic strategies to access these biologically active compounds. Solid phase peptide synthesis (SPPS) strategies have been an invaluable tool in their synthesis. The key aspect of their synthesis is the macrocyclization strategy. Three main strategies are used, solution phase macrolactamization of acyclic ester containing peptide, on-resin macrolactamization of a sidechain-anchored peptide, and the solution phase macrolactonization of a linear peptide. Additionally, biocatalysts have been used to produce these compounds in a regio- and chemo-selective manner. Each compound offers unique challenges, requiring careful synthetic design to avoid undesirable side reactivity or unwanted epimerization during the esterification and macrocyclizing steps. This focused review analyzes these three strategies for cyclic depsipeptide natural product total synthesis with selected examples from the literature between 2001-2023.

Introduction

Depsipeptide natural products are a class of peptides that exhibit a range of pharmacological activity¹ including antibiotic, immunosuppressant, surfactants, piscicides,

anticancer, and antiviral activity among others². These peptides are produced by bacteria, fungi and marine organisms³ and serve several functions such as signaling molecules involved in intra- and interspecies communication⁴, transporting cations⁵, inhibiting growth of competing organisms and modulating host defense mechanisms². They are produced by non-ribosomal peptide synthetase (NRPS)^{6,7} containing biosynthetic pathways, which are able to generate remarkably structurally diverse peptides, including cyclic and linear peptides, those containing proteinogenic and non-proteinogenic amino acids, and those with a vast array of possible chemical modifications including epimerization, methylation, oxidation, and others. Their biosynthesis has been expertly reviewed recently².

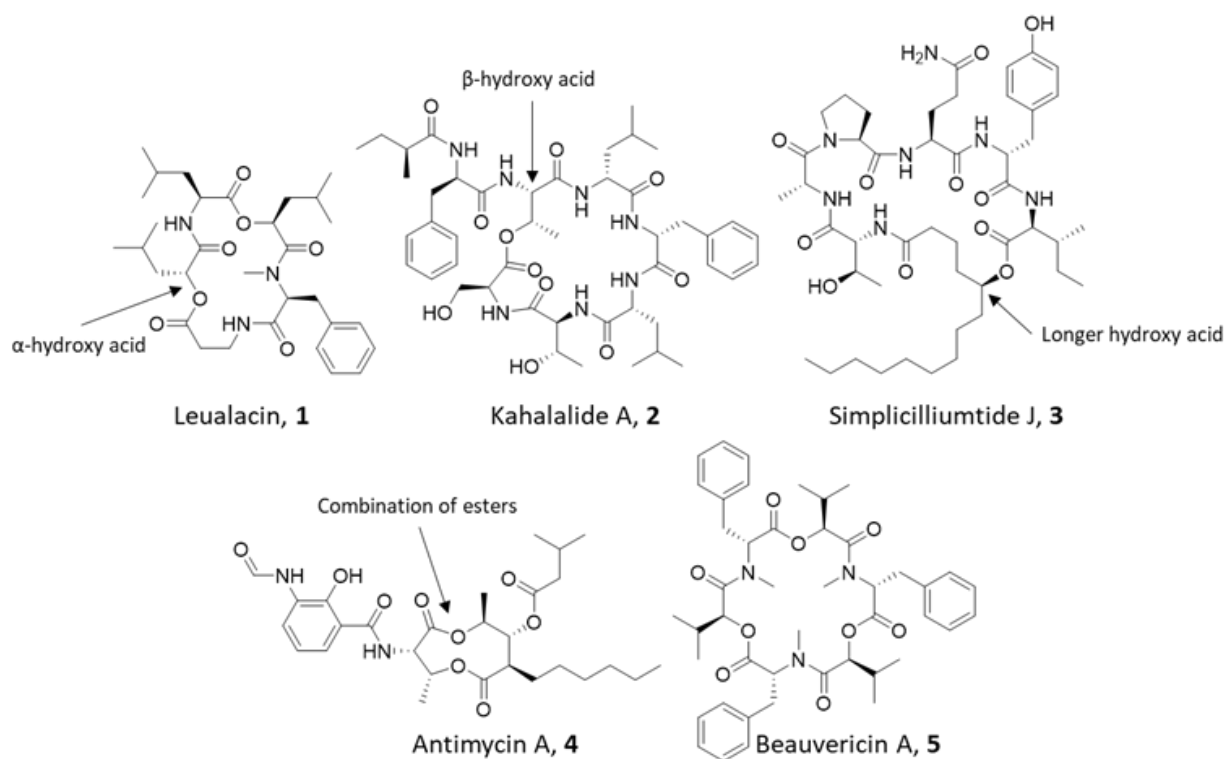


Figure 4.1 Examples of cyclic depsipeptides. Structurally classified by ester type, occurrence, and number.

Depsipeptides are distinguished by the presence of both ester and amide bonds⁸. The ester bond is typically found in a macrocycle, a feature at the core of these molecules' biological function⁹. The macrocycle confers rigidity to the peptide that increases protease resistance¹⁰, membrane permeability, organization in target binding events and ion binding capabilities. Depsipeptides have been structurally classified¹ by the number of ester bonds present (one or more) as well as the nature of the alcohol nucleophile participating in the ester bond, such as α -hydroxy acids (α -hydroxyisocaproic acid residue found in leualacin, **1**)¹¹, β -hydroxy acids (Thr amino acid found in kahalalide A, **2**), other hydroxy acids (δ -hydroxy acid found in simplicilliumtide J, **3**)¹² and combinations of these (antimycin A, **4**) (**Fig. 4.1**). Furthermore, they have also been biosynthetically classified² by the timing of the ester bond incorporation, the NRPS biosynthetic domain responsible for ester bond formation, by the ester alcohol origin, and whether they are produced in a single-pass manner such as kahalalide A, **2**, or in an iterative fashion generating peptide oligomers such as beauvericin, **5** (**Fig. 4.1**).

Accessing these compounds synthetically has been a challenge for chemists due to the structural diversity of the individual amino acid and hydroxy acid building blocks and the challenges of ring closure¹³, particularly via macrolactonization. Both solution-phase and solid-phase approaches as well as a combination of the two have been used to synthesize members of this class of compounds. In most modern syntheses, solid phase peptide synthesis (SPPS) is used to access the linear, cyclization precursor peptide, however clever strategies must be employed to introduce the macrolactone. This review will focus on the macrocyclizing chemistry with examples of depsipeptide total syntheses that are completed using SPPS between 2000 and 2023. In particular we examine the prevalence of generating macrocyclic depsipeptides through

macrolactamization, the use of different solid support anchoring approaches, the challenges of peptide macrolactonization, and briefly the use of biocatalysts for cyclic depsipeptide synthesis.

Cyclic depsipeptide synthesis using solid phase peptide synthesis

Depsipeptides vary widely in their amino acid sequence, frequently incorporating non-proteinogenic amino acids, and have macrolactones of varying residue size^{1,2}. This structural diversity is reflected in the diversity of strategies employed in the total syntheses of these molecules. While many cyclic depsipeptides have been synthesized via solution phase chemistry, such as largazole¹⁴, viequeamide A¹⁵, FR901375¹⁶ and FE399¹⁷, in this review we focus on recent syntheses that employ SPPS to generate advanced peptide precursors. We highlight three key strategies for their synthesis. In the first strategy, the C-terminal residue where macrolactamization will occur is linked to the stationary phase. Standard solid phase peptide synthesis methods, frequently with modern microwave (MW) assisted 9-fluorenylmethyloxycarbonyl (Fmoc)-based chemistry¹⁸, are used to quickly and efficiently synthesize the linear peptide. A late-stage esterification and cleavage from the resin generates a branched peptide that is macrolactamized and deprotected in solution phase. Typically macrocyclization is performed in high-dilution to suppress oligomerization and higher order cyclic products¹⁹. In the second strategy, on-resin macrocyclization is possible, which offers “*pseudo-dilution*”-like^{20,21} conditions to suppress oligomer formation. Recently, on-resin depsipeptide bond conditions and factors have been investigated in depth²². In this case the peptide is linked to the resin through side chain functional groups, enabling the C-terminus to be orthogonally protected and thus accessible. Standard SPPS followed by esterification is used to generate the cyclization precursor on resin, which can then have the N- and C-termini unmasked and cyclized on resin. Cleavage from the resin then produces

the natural product. In the third approach, the carboxylate involved in the macrolactone linkage is the attachment point for the peptide. Solid phase peptide synthesis generates the full-length linear peptide, which is cleaved from the resin and cyclized. This strategy typically requires highly specialized macrolactonization conditions for successful ring closure. Detailed examples and critical analysis of each strategy is presented below.

Macrolactamization: C-terminus anchored synthesis

The most common strategy for cyclic depsipeptide synthesis is macrolactamization at a strategic amide from a branched peptide sequence synthesized through a C-terminal anchor to the stationary phase. The general workflow enables the on-resin synthesis of the longest linear peptide sequence, esterification of the linear intermediate with the appropriate residue and finally a solution phase macrocyclization. A popular approach for the SPPS is the use of hyper acid sensitive resins such as 2-chlorotrityl chloride (2-Cl-Trt) resin^{23,24}. The peptide can be extended through standard SPPS conditions followed by a late-stage esterification on a free alcohol containing amino acid sidechain generating an on-resin linear protected ester peptide²⁵. The chosen amine involved in macrolactamization can be deprotected under orthogonal conditions and the C-terminus can be cleaved from the resin selectively under mild acidic conditions while maintaining acid labile protecting groups on the various amino acid sidechains²⁶. These branched ester intermediates can be macrolactamized in solution, yielding protected macrocyclic product that can be further deprotected and purified.

Kahalalide F, **9**, was synthesized in 2001 utilizing the strategy described above²⁷. This sequence presents several challenges with a dehydro amino acid within the macrocycle, an ester between two β -branched residues, and a highly hydrophobic sequence. In this case, the authors

completed the synthesis via two routes, with one yielding crude product with greater purity. The first route involved esterification of the complete linear hydrophobic sequence, which led to lower yields and reduced purity. Instead, a shorter tetrapeptide **6** was synthesized on 2-Cl-Trt resin. The *D-allo*Thr was left unprotected, providing the site for esterification with Alloc-Val-OH using Steiglich-like conditions with *N,N'*-diisopropylcarbodiimide (DIC) and 4-dimethylaminopyridine (DMAP), generating branched peptide **7**. Subsequent Fmoc-SPPS completed the N-terminal portion of the peptide followed by Alloc-Thr-OH and Alloc-Phe-OH coupling onto the esterified *D-allo*Thr. A β -elimination yielded the desired *Z*-configured dehydro residue, producing the on-resin branched ester intermediate **8**. Alloc deprotection of the Phe N-terminus led to an overall 92% yield as determined by amino acid analysis. The peptide was cleavage with 1% trifluoroacetic acid (TFA) in dichloromethane (DCM), revealing the free amine and acid, which was cyclized in solution under high dilution conditions with benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) and *N,N*-diisopropylethylamine (DIEA) in *N,N*-dimethylformamide (DMF) to generate the 6-residue macrocycle (**Fig 4.2A**). Complete deprotection afforded the desired depsipeptide natural product **9**²⁷ in an isolated 14% yield.

A similar strategy enabled the synthesis of polydiscamide B-D²⁸. Esterification was similarly accomplished between the free Thr β -hydroxy group of an on-resin pentapeptide and an Fmoc-*D*-Asp(OtBu)-OH using DIC and DMAP to yield the branched intermediate **10**. Fmoc deprotection and cleavage from the hyper acid sensitive 2-Cl-Trt resin using 30% 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) conditions²⁶, provided the free amine and acid for macrolactamization while retaining the remaining protecting groups. Treatment with 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU) and DIEA under high dilution conditions generated the 5-residue

macrocycle **11** (Fig. 4.2B) in a 12% isolated yield from the loaded resin. To complete the total syntheses, native chemical ligation was used to append the three different linear C-terminal thioesters to an N-terminal cysteine on the macrocyclic core, followed by oxidation of the cysteine residue to cysteic acid and final deprotection in 30-50% isolated yields of these final steps.²⁸

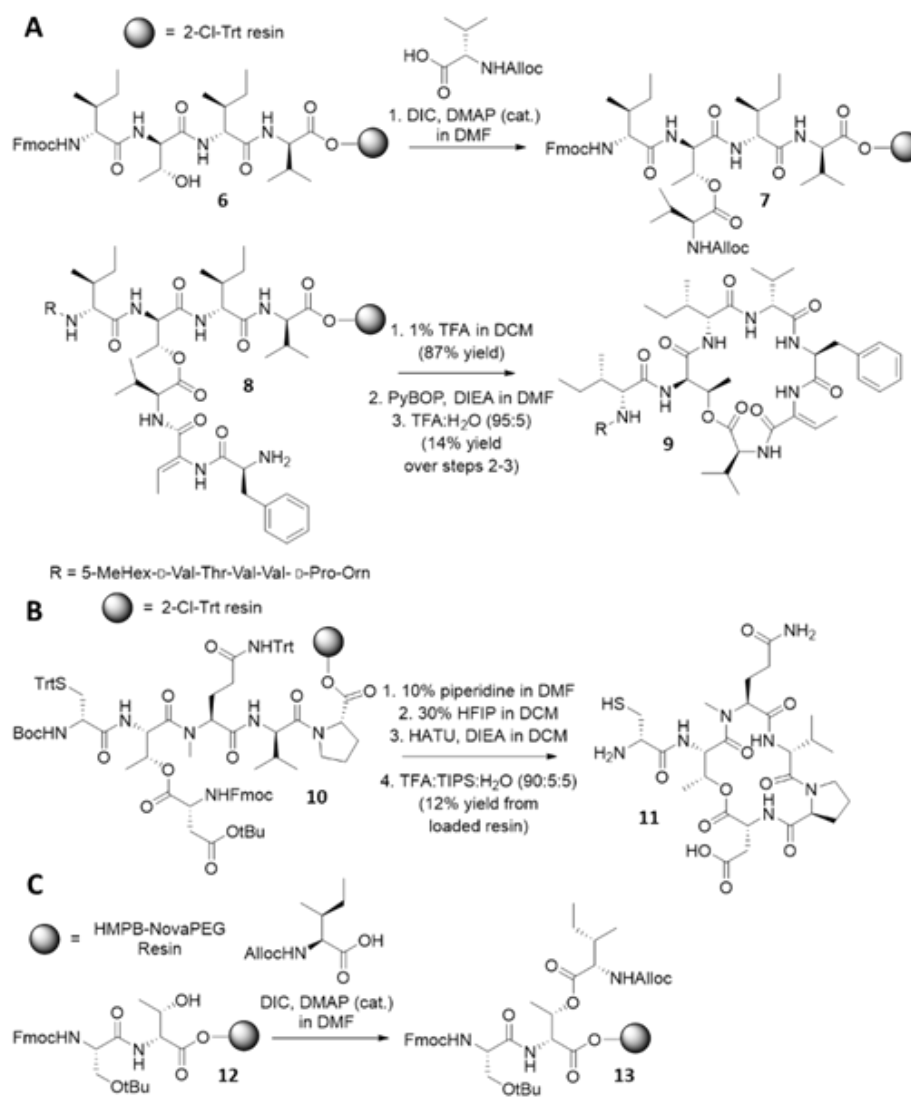


Figure 4.2 C-terminal resin loading strategies with solution phase macrolactamization.

A. Kahalalide F, **9**, esterification and macrocyclization **B.** Macrocyclization of the polydiscamides B-D core **C.** Challenging teixobactin esterification.

Teixobactin presented a unique problem²⁹. Esterification of the free alcohol of D-Thr loaded onto 2-Cl-Trt resin failed under all conditions evaluated. To reduce the steric effects from the bulky 2-Cl-Trt resin, d-Thr was loaded onto the 4-(4-hydroxymethyl-3-methoxyphenoxy)butyric acid (HMPB) NovaPEG resin, which has decreased steric bulk and increased swelling properties, and was extended with Fmoc-Ser(OtBu)-OH generating **12**. Esterification of the free alcohol with Alloc-Ile-OH under Steiglich-like conditions with DIC and DMAP readily generated the ester **13** (**Fig. 4.2C**). The peptide was completed in similar fashion as above, with peptide extension, N-terminus orthogonal deprotection, mild acidic peptide cleavage, and solution-phase macrolactamization to generate the 4-residue macrocycle, which was deprotected to yield teixobactin in a 3.3% yield over 24 linear steps²⁹.

The larger 10-residue cyclic depsipeptides ohmyungsamycin, **16**, deoxyecumicin, **17**, and ecumicin, **18**, were also synthesized following a similar strategy via esterification of a free hydroxyl group of an on-resin Thr with Fmoc-Val-OH³⁰. This was followed by extension of the Val branch by three residues using standard SPPS ending with a methylated N-terminal Trp derivative. Although this site for macrolactamization involved a challenging N-methylated N-terminus and significant steric hindrance from the β -branched C-terminus, it was hypothesized that pre-organization of the linear chain might be driven by the reported β -sheet conformation of ecumicin³¹. The C-terminus was released following 30% HFIP cleavage yielding branched peptide **14** in a 7-21% yield from loaded resin and macrolactamization was successfully accomplished under high dilution with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM·BF₄) and N-methylmorpholine (NMM) at 50 °C to overcome the challenging coupling with the secondary amine, yielding **15** (**Fig 4.3, A**). Final depsipeptides **16**, **17** and **18** were completed following deprotection with TFA:DCM:TIPS (49:49:2) and

purification³⁰, affording the natural products in 22-41% yield following macrolactamization and deprotection, with an overall 2-5% yield from the loaded resin.

Similarly, the small 6-residue formylated cyclic peptide szentiamide, **21**, was also synthesized incorporating a free Thr β -hydroxy group³², with the synthesis of linear 5-mer **19**. However esterification under standard Steiglich-like conditions was unsuccessful. Fortunately the modified Yamaguchi conditions with benzoyl chloride (BzCl), triethylamine (TEA) and DMAP with Fmoc-Trp-OH successfully installed the ester in branched intermediate **20**. The peptide was completed with Fmoc deprotection, 3% HFIP selective resin cleavage, and cyclization in solution under MW irradiation conditions using HATU and DIEA (**Fig. 4.3B**). Szentiamide, **21**, was obtained after deprotection and purification³² in a 14% yield from the loaded resin.

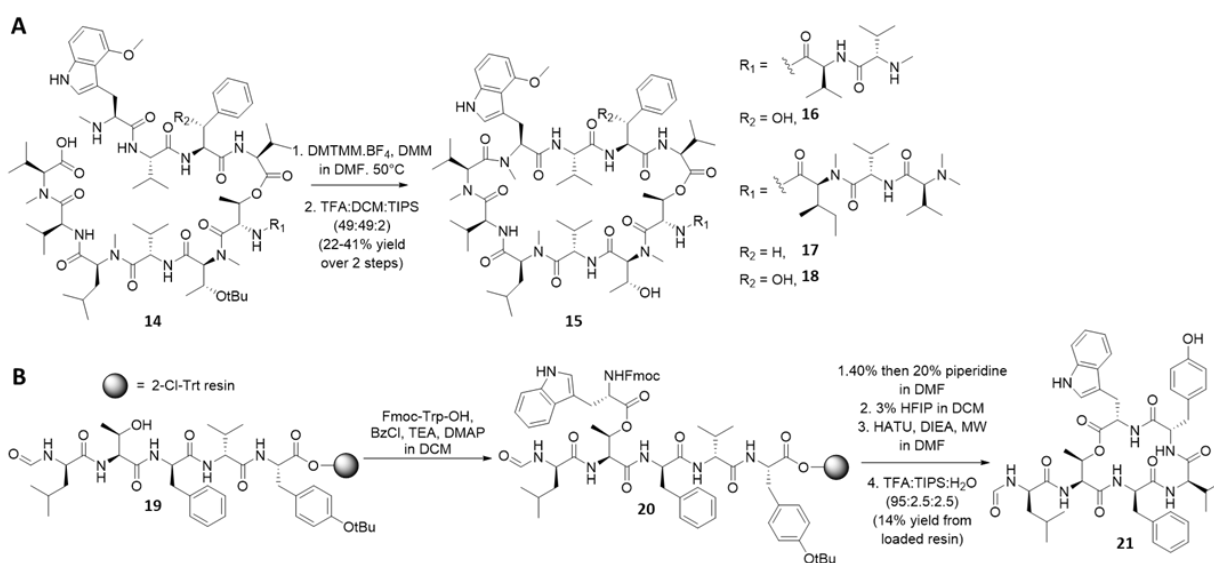


Figure 4.3 C-terminal resin loading strategies with solution phase macrolactamization.

A. Ohmyungsamycin A, **16**, deoxyecumicin, **17**, and ecumicin, **18**, macrolactamization with a secondary amine macrolactamization. **B.** Modified Yamaguchi esterification conditions and microwave assisted macrolactamization of szentiamide, **21**

Depsipeptides, such as Theonellapeptolide Id³³, **23**, have all hydrocarbon, inert sidechains thus the branched peptide can be fully deprotected and cleaved from the resin to generate the free amine and carboxylic acid ready for macrolactamization. Thus acyclic theonellapeptolide Id peptide was synthesized on 2-Cl-Trt solid support, incorporating a free Thr alcohol that was esterified with Steiglich-like conditions with Fmoc-D-*allo*-Melle-OH, and followed by final Fmoc deprotection to afford **22**. Cleavage from the resin and macrolactamization with PyBOP, 1-hydroxy-7-azabenzotriazole (HOAt) and 2,4,6-collidine under high dilution conditions in DMF generated the natural product **23**³³ in 26.8% yield from the loaded resin (**Fig. 4.4A**).

Similarly, pagoamide A, **25**, was synthesized on 2-Cl-Trt resin by incorporating Thr with a free alcohol that could be esterified with Boc-Phe-OH under Steiglich-like conditions³⁴. The peptide was further elongated, incorporating the N-terminal thiazole residues, and was cleaved and deprotected to generate the branched peptide **24**. Macrolactamization was accomplished with (7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) and DIEA, affording the depsipeptide **25**. Of note is that the macrolactamization occurred in the presence of two free Ser hydroxyl groups without competing macrolactonization³⁴ (**Fig. 4.4B**).

An alternative strategy is used to incorporate residues with esters to α -hydroxy acids. Unlike esters to the β -hydroxy group of Thr or Ser, the α -hydroxy ester can be installed by coupling of a 2-residue ester-linked building block. In the case of Ser and Thr, preinstalling the ester is not possible since additional coupling to the α -amino group require its deprotection in the presence of the ester, enabling an O-N intramolecular acyl shift^{35,36}. For example, the di- α -hydroxy ester containing depsipeptide YM-280193, **27**,³⁷ starts from a 2-Cl-Trt resin loaded β -hydroxy-Leu. The sequence is extended, including coupling of a 2-residue α -hydroxy ester containing N-Ac-Thr- α -hydroxy-Phe-OH. The free Thr alcohol was further esterified on-resin with Boc-N-Me-Thr(OMe)-

OH under symmetric anhydride conditions, without esterifying the sterically hindered alcohol on the β -hydroxy-Leu. Elimination of sidechain functionality generated the dehydropeptide **26**, which was cleaved from the resin and deprotected in a 16% yield from loaded resin. Finally macrolactamization occurred under treatment with HATU, HOAt, and DIEA, yielding a mixture of epimerized but separable products, to afford the desired depsipeptide **27** in a 27% yield (**Fig 4.4C**)³⁷.

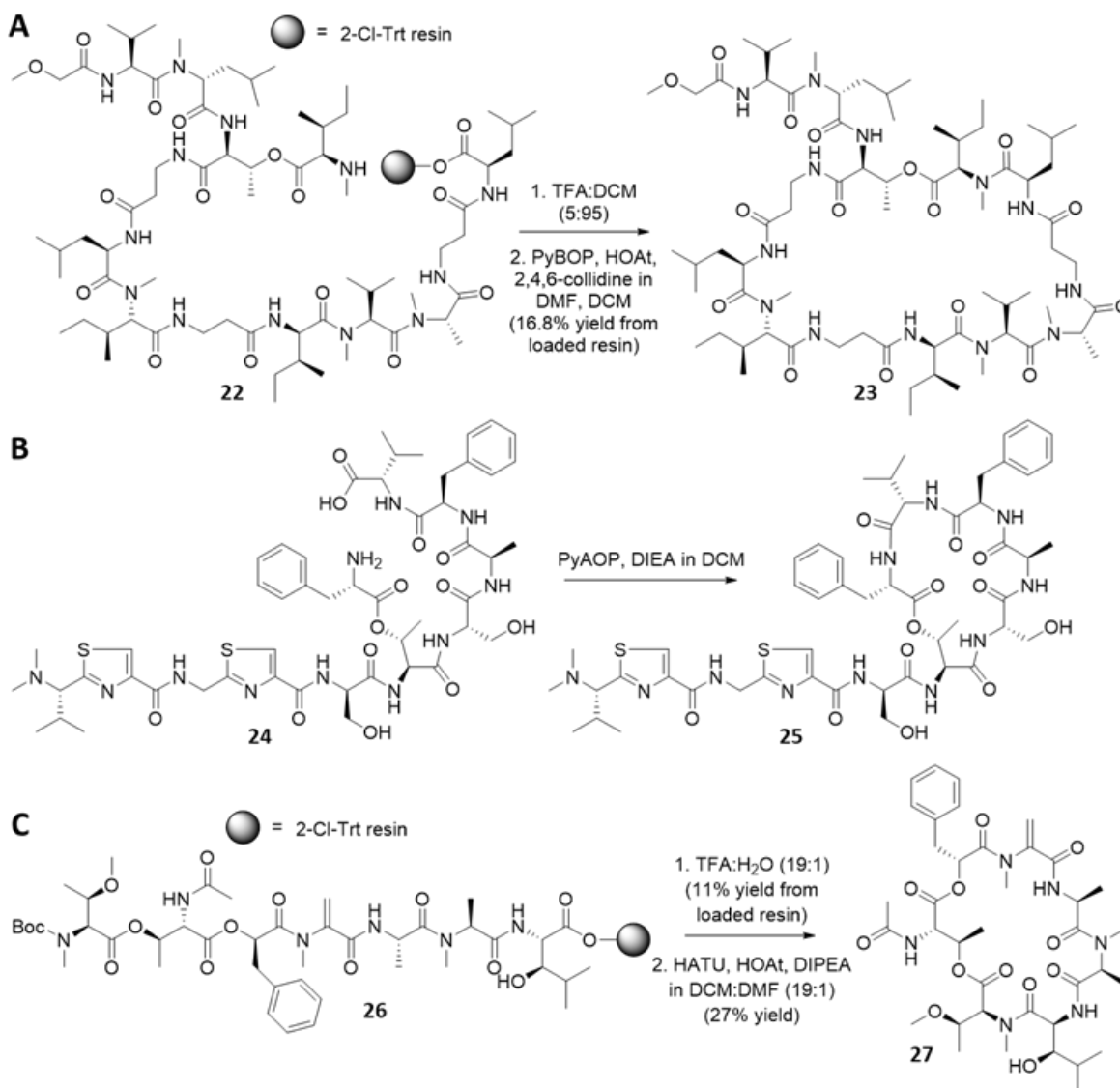


Figure 4.4 Strategies with solution phase macrolactamization of highly inert amino acid sidechains.

A. Highly N-methylated and hydrophobic sidechain synthesis of theonellapeptolide Id, **23**. **B.** Non-reactivity of pangoamide A, **25**, Ser hydroxyl groups **C.** Inertness of β -hydroxy leucine during YM-280193, **27**,
macrolactamization

These examples of C-terminally anchored synthesis all involve the formation of a branched linear depsipeptide via SPPS that is subsequently cleaved from the resin and macrolactamized in solution. The amide bond site of macrolactamization is often selected to limit branched peptide

synthesis, as seen in the syntheses of polydiscamides B-D²⁸, szentiamide³², theonellapeptolide Id³³ and pagoamide A³⁴. It is however not uncommon to perform several orthogonally protected couplings to generate a macrocyclization point further from the ester bond. This design choice is seen in challenging depsipeptide syntheses such as with kahalalide F²⁷, which contains a dehydro residue neighbouring the ester forming residue, or in the diversity-generating synthesis seen in the ohmyungsamycin A, deoxyecumicin and ecumicin synthesis,³⁰ where they share a similar macrocycle with differing N-terminal residues. In most cases, a linear peptide is generated on SPPS containing a free Thr or Ser sidechain, ready for esterification. While Steiglich-like conditions typically generate the ester effectively, in some cases esterification fails and alternative strategies need to be investigated. This is the case in the szentamide synthesis³². In the case of teixobactin, a change of resin was required to facilitate esterification²⁹. Most frequently 2-Cl-Trt resin is used for these syntheses as it enables mild cleavage, retaining side chain protecting groups, thus providing a branched peptide ready for solution phase macrolactamization under high dilution conditions. Coupling conditions typically use a combination of highly reactive, low epimerizing coupling agents such as PyBOP, PyAOP, HATU, HOAT and DMTMM·BF₄ and additives like DIEA, NMM and 2,4,6-collidine.

Macrolactamization: sidechain anchored synthesis

Resin attachment does not have to occur only via the C-terminal residue of the peptide. A number of sidechain functional groups can be used to anchor the peptide to the resin. Common side chains for resin attachment include Asp, Glu, Asn, Gln, Ser, Thr, and Tyr, though others can be used³⁸. It is optimal to select a residue for anchoring that is near the ester bond to limit esterification and branching reactions on resin.

This strategy has been utilized to complete the total synthesis of cyclic depsipeptides containing Asn residues such as callipeltin B³⁹ and halicylindramide A⁴⁰. Utilizing Sieber or Rink Amide resins respectively, the coupling of Fmoc-Asp-OAll yielded a sidechain linked resin, which upon cleavage from the resin generates an amide sidechain. Halicylindramide A total synthesis exemplifies this strategy⁴⁰. After anchoring through the side chain, the linear peptide was elongated to generate a hexapeptide **28**. On-resin esterification of Alloc-*N*-Me-Gly-OH onto an unprotected Thr alcohol was accomplished using a Steiglich-like approach with DIC and DMAP. The Alloc/Allyl protection strategy allows for orthogonal deprotection of the appropriate amine and carboxylic acid enabling on-resin macrolactamization with HATU, HOAt and NMM in DMF (**Fig. 4.5A**) to afford resin-bound macrocycle **29**. The peptide synthesis was completed and the full macrocyclic peptide was deprotected and cleaved with acidolysis in a 1.5% yield from loaded resin. The N-terminus was formylated in a 60% yield, affording the natural product.

Laterocidine was similarly synthesized⁴¹ by loading Fmoc-Asp-OAll onto Rink amide resin and an on-resin esterification of an unprotected Thr alcohol with Alloc-Gly-OH. This is followed by a C-terminus deprotection and coupling of the resin-bound Asn with Gly-Oall to afford **30**. Orthogonal Allyl/Alloc deprotection and on-resin macrolactamization between the amino Gly ester and a Gly C-terminus with benzotriazole-1-yl-

Oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) and DIEA conditions⁴¹ afforded **31** (**Fig. 4.5B**). The natural product was then completed via Fmoc-SPPS and cleaved from the resin, affording an isolated 2% yield from the loaded resin. The first total synthesis of laterocidine relied upon a solution-phase macrolactamization of the branched peptide⁴². Unlike solution phase, where competing oligomerization can occur and must be suppressed by lowering the cyclization precursor concentration, the on resin macrocyclization

cannot undergo oligomerization and benefits from the use of excess reagent and easy byproduct removal that is common to solid phase chemistry.

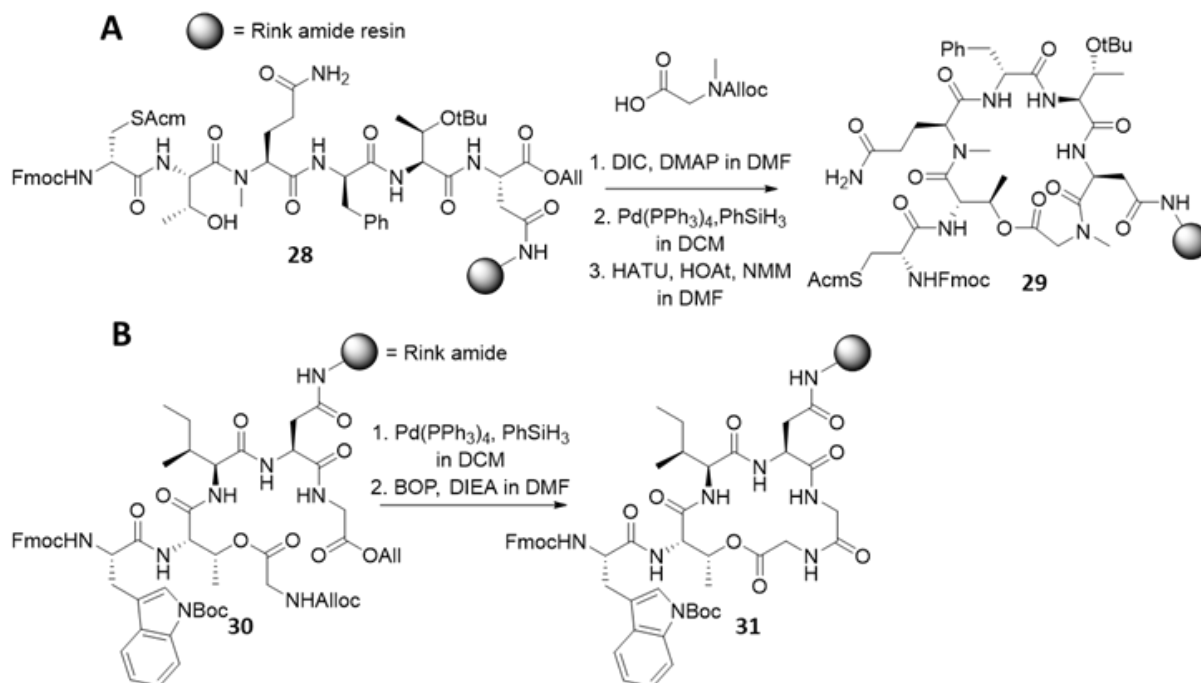


Figure 4.5 Asparagine sidechain anchored and on-resin macrolactamization examples.

A. Esterification, orthogonal deprotection and macrolactamization of halicyclindramide A macrocyclic core. **B.**

Orthogonal deprotection and macrolactamization of laterocidine macrocyclic core.

Alcohol sidechain containing amino acids Thr and Ser are also useful solid phase anchoring points as they can form acid labile ethers with 2-Cl-Trt resin or Wang resin. Ser residues are loaded onto 2-Cl-Trt resin with the N-terminus Fmoc and C-terminus Allyl protected. The allyl protection similarly enables late stage orthogonal deprotections for on resin macrolactamization. Several cyclic depsipeptides have been synthesized in this manner such as pseudodesmin A and WLIP Glu2 variant^{43,44}. For pseudodesmin A, the longest linear peptide sequence **32** was synthesized incorporating a free Thr sidechain that was esterified with Alloc-Ile-OH using Steiglich-like approach. From this ester, the orthogonal Alloc/Allyl protecting groups were removed, revealing

the amine and carboxylic acid. On-resin macrolactamization with HATU and HOAt generated the macrocycle **33**, which was deprotected and cleaved from the resin (**Fig. 4.6A**), affording an isolated 22% yield from the loaded resin. Similarly, orfamide A, a lipodepsipeptide, was synthesized by anchoring Fmoc-d-Ser-OAll through the sidechain to 2-Cl-Trt resin⁴⁵ (**Fig. 4.6B**). Synthesis of the linear chain proceeded under standard conditions and incorporating a dipeptide containing the ester, generating acyclic intermediate **34**. This is followed by orthogonal Alloc/Allyl deprotection of the resin-bound peptide that was macrolactamized to generate the 8-residue macrocycle **35**. To complete the synthesis, the Fmoc protected α -amino group of the ester containing d-*allo*Thr residue was unmasked, extended under Fmoc-SPPS conditions, deprotected and cleaved from the resin to generate orfamide A⁴⁵ in a 33-35% yield from resin loading (**Fig. 4.6B**). Interestingly, O-N migration⁴⁶ was not observed during this first Fmoc deprotection with 2% DBU/2% Piperidine in DMF or the subsequent coupling to afford **36**, presumably due to the constraints imposed by the macrocycle, which limit the ability of the α -amino group to attack the ester carbonyl.

Tyr can also be used to anchor onto solid phase resins, with the Tyr ether generated either via substitution chemistry with 2-Cl-Trt resin or through a Mitsunobu chemistry with Wang resin. The synthesis of coibamide A⁴⁷ used the phenol ether anchored 2-Cl-Trt resin and the linear peptide was synthesized, incorporating a free Thr hydroxyl, generating **37**. Esterification with Alloc-*N*-Me-Ala-OH, followed by Allyl/Alloc deprotection and on-resin macrolactamization under BOP and hydroxybenzotriazole (HOBt) conditions afforded macrocycle **38**⁴⁷ (**Fig. 4.6C**). The depsipeptide was completed following N-terminal extension, on-resin N-terminal dimethylation, deprotected, and cleaved from the resin to afford *O*-Desmethyl coibamide A in a 4.1% from resin loading.

In an atypical example of sidechain anchored depsipeptide synthesis, a SPPS strategy was developed to tether Phe residues through silylation to solid support by application of a Suzuki coupling⁴⁸. The hydrophobic pentapeptide sansalvamide A, **40**, was successfully synthesized by this approach. Here the peptide is synthesized in fragments, first the pre-synthesized amino- Leu- α -hydroxy isocaproate-*O*-allyl ester 2-mer is coupled onto the C-terminus of Phe. The peptide is then extended through the N-terminus using standard SPPS to add the final two residues, generating **39**. The amine and C-terminus are deprotected and the peptide is macrolactamized on-resin with *O*-(Benzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) and DIEA in N-methyl-2-pyrrolidone (NMP). Finally the macrocycle is cleaved from the resin under 50% TFA with 2% thioanisole over 36h to afford the natural product⁴⁸ in 67% yield from loaded resin (**Fig. 4.6D**).

Macrolactonization chemistry

The synthesis of depsipeptides through macrolactonisation is known to be challenging due to the lowered nucleophilicity of the alcohol versus the amine in macrolactamization. This decreases macrocyclization rates and enhances the possibility of unwanted epimerization during macrolactonization due to the longer lifetime of the activated esters at the C-terminus⁴⁹. To avoid this problem, depsipeptides are overwhelmingly macrolactamized. Yet despite this, there are some reported syntheses of cyclic depsipeptides through macrolactonization both in solution and on-resin.

The antimicrobial peptide LI-F04a was macrolactonized through the N-terminal Thr alcohol and the d-Ala C-terminus⁵⁰. The peptide was synthesized on 2-Cl-Trt resin and selectively cleaved under mild acidic conditions, yielding acyclic intermediate **41**. Standard Yamaguchi macrolactonization conditions were attempted but led to high levels of epimerization. However the use of modified Yonemitsu conditions led to the macrolactone with < 5% epimerization, yielding intermediate **42** in 58% yield⁵⁰ (**Fig. 4.7A**). In a more comprehensive evaluation of LI-F04a macrocyclization conditions, Corey-Nicolaou conditions led to the highest level of epimerization at the C-terminus while the Yonemitsu and the modified Yamaguchi macrolactonization with 2-methyl-6-nitrobenzoic anhydride (MNBA) led to the least with < 8% epimerization.⁵¹ The cyclic callipeltin B, **44**, was linearly synthesized on-resin, incorporating a free hydroxyl D-*allo*Thr and the sequence was cleaved under mild acidic conditions⁵². Surprisingly, the final coupling of dimethyl pyroglutamic acid (pDME) onto the α -amino group of D-*allo*Thr also esterified the unprotected β -hydroxyl group, requiring subsequent saponification and resin cleavage to afford peptide **43** from the resin. The seco-acid was then macrolactonized by treatment with a large excess of DIC (5 eq.) and DMAP (20 eq.) without detectable epimerization⁵² (**Fig. 4.7B**). Destruxin E has its lactone linkage between an α -hydroxy acid residue and a C-terminal β -Ala. The β -Ala residue removes concerns over epimerization of the activated ester during macrolactonization, thus this was an ideal candidate for a macrolactonization strategy. Treatment of the SPPS generated seco-acid **45** in solution phase with MNBA and the DMAP N-oxide DMAPO generated the desired macrolactone **46** in 44% yield⁵³ (**Fig. 4.7C**).

A number of atypical lactonization strategies have also been developed to access cyclic depsipeptides. Notable examples include thioester based lactonizations utilized in the synthesis of spiruchostatin⁵⁴ and S-to-S-to-O acyl transfers explored by Nagano *et al.*⁵⁵ as well as Mitsunobu

based macrolactonizations utilized in the synthesis of FR-901375⁵⁶ and the oligomeric cyclization of depsipeptides⁵⁷. Recently, ynamides were used for macrolactonization when traditional esterification conditions were unsuccessful in the total synthesis of metacridamides A and B⁵⁸. Another example was a Ag(I)-promoted conversion of thioamide peptide to an isoimide, which undergoes an acyl transfer to the β -hydroxyl group of Ser/Thr sidechain, leading to a ring expansion of 2 atoms was recently developed and the total synthesis of teixobactin, seongsanamide E and kahalalide B were accomplished in this manner, with around 30% yield for the macrocyclization step⁵⁹. While effective, this method does require the synthesis of the thioamide linkage for the Ser/Thr residue involved in macrolactonization. Brevicidine, a lipodepsipeptide with a 4-residue macrocycle was macrolactonized on-resin. The sidechain resin bound Fmoc-Ser-OAll allows for orthogonal deprotection, generating an on-resin peptide intermediate **47** that was C-terminally deprotected. On-resin Yonemitsu macrolactonization generated macrocycle **48** followed by further couplings and global cleavage and deprotection to afford the natural product in a 9% yield from the loaded resin⁴¹ (**Fig. 4.7D**). The first total synthesis of brevicidine utilized a solution-phase macrolactamization between the C-terminal Gly and ester-linked Ser N-terminus⁴².

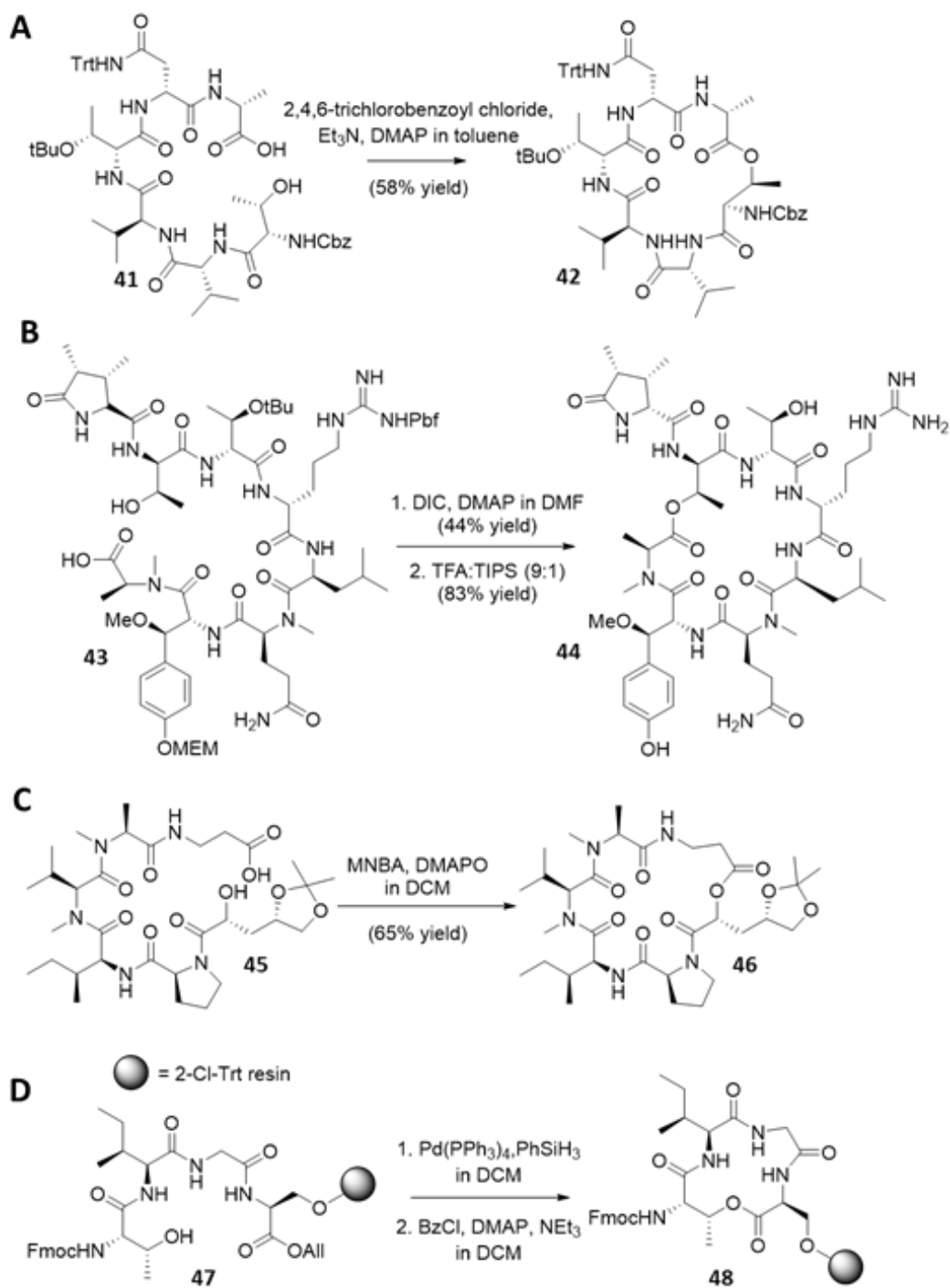


Figure 4.7 Macrolactonization chemistry examples.

A. Solution phase lactonization of LI-F04a **B.** Callipeltin B cyclization **C.** Destruxin macrolactonization before epoxidation **D.** Serine sidechain anchored, on-resin macrolactonization of brevicidine

Chemoenzymatic synthesis of depsipeptides

As seen above, macrolactonization chemistry can lead to lower coupling yields and increased C-terminal epimerization. Despite this chemical challenge, depsipeptides are produced by NRPS biosynthesis with an enzymatic macrolactonization step. These macrolactonizing enzymes thus present a unique opportunity to develop biocatalysts for this challenging synthetic step. The primary macrocyclizing enzyme from depsipeptide biosynthesis is the thioesterase (TE), a serine hydrolase that cyclizes a linear thioester activated peptide using an internal hydroxyl nucleophile. Recombinant purified TEs from nonribosomal depsipeptide biosynthesis have been used in a number of chemoenzymatic total syntheses. Accessing the thioester peptide is a significant challenge for this strategy in light of the incompatibility of thioesters and Fmoc SPPS. Typically the thioester peptides are accessed through synthesis of the linear peptide on hyper sensitive resins, such as 2-Cl-Trt resin followed by cleavage under mild acidic conditions to generate the free C-terminal acid with the remainder of the functional groups protected. The C-terminus can then be activated as thioesters, deprotected, and used with the enzyme to generate the macrolactone⁶⁰.

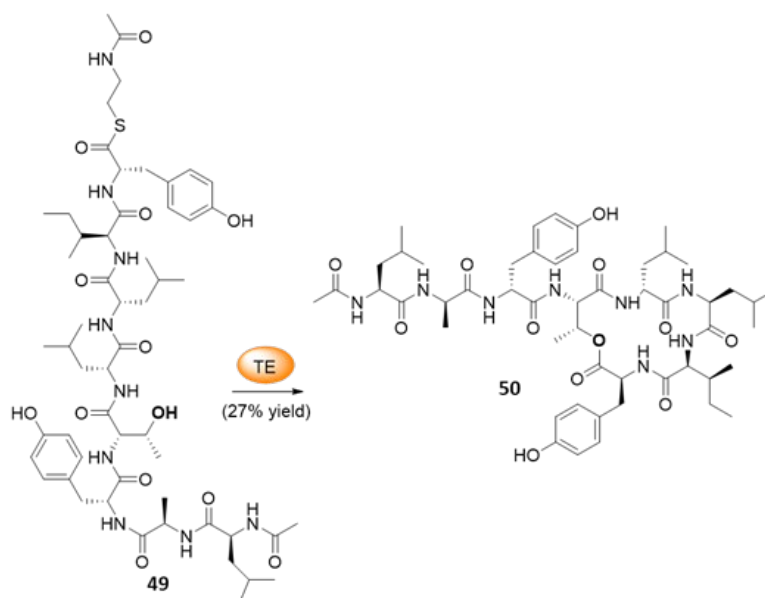


Figure 4.8 Thioesterase mediated macrolactonization of seongsanamide E, **44**.

A number of cyclic depsipeptide have been accessed using this strategy, including surfactin⁶¹, calcium dependent antibiotic⁶², streptogramin B⁶³, lysobactin⁶⁴, teixobactin⁶⁵, seongsanamide E⁶⁶, cereulide⁶⁷, valinomycin⁶⁸ and rhizomide A⁶⁹. Of particular note is the enzymatic macrocyclization of streptogramin B⁷⁰, which contains an ester bond between the carboxylate of a phenylglycine residue and the β -hydroxyl group of a Thr. Chemical lactonization would require formation of the activated ester of phenylglycine, a highly epimerizable residue and lead to a mixture of diastereomers. In the enzymatic TE-mediated macrolactonization, the thioester of phenylglycine undergoes in situ epimerization, however the enzyme only cyclizes the correct stereochemical configuration to generate the desired macrocycle, which is then configurationally stable under the reaction conditions⁷⁰.

As a final example, seongsanamide E, **50**, a monocyclic depsipeptide was chemoenzymatically synthesized using the recombinant purified TE from its biosynthesis and a linear thioester peptide substrate. The N-acetyl cysteamine thioester substrate **49** was synthesized using EDC and HOBt from the protected linear free acid, generated by standard SPPS on a 2-Cl-

Trt resin, and N-acetyl cysteamine. The thioester substrate was incubated with the TE and macrocycle **50** formation was readily detected without detectable epimerization in a 27% yield⁶⁶ (**Fig. 4.8**). As a direct comparison to chemical approaches, attempts to macrolactonize the seco-acid under Yonemitsu conditions were unsuccessful.

In addition to TEs for macrolactonization of depsipeptides, there is a recent exciting new enzymatic strategy to access cyclic depsipeptides. This approach relies on split intein technology⁷¹, which catalyzes the excision of an intervening peptide sequence flanked by the intein N- and C-terminal domains through macrolactamization. By incorporating non-canonical α -hydroxy-residues into the intervening sequence, cyclic depsipeptides can be generated via macrolactamization⁷². This approach is particularly exciting as it may enable directed evolution of cyclic depsipeptides with new functions.

Conclusions

While the synthesis of cyclic depsipeptides has been enabled by SPPS, macrocyclization continues to be challenging, particularly through lactonization. A number of viable strategies and methodologies to access these important compounds have been developed. The prevailing synthetic approach is esterification to generate a branched peptide that can be macrolactamized through a strategically selected amide, avoiding the challenging macrolactonization. Solid phase peptide synthesis has been an indispensable tool to generate these peptides precursors efficiently. An exciting advancement has been the utilization of amino acid sidechain functional groups for anchoring the peptide to the resin, enabling on-resin macrocyclization, avoiding additional purification steps and high dilution cyclization steps. Finally, the utilization of biocatalysts such as thioesterases can help perform challenging macrolactonizations from a linear C-terminal

thioester peptide, providing the macrocyclic product. This is especially useful for notoriously epimerizable C-terminus residues as these enzymes can be highly selective.

Conflicts of interest

There are no conflicts to declare.

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Chapter 5: Total and chemoenzymatic synthesis of seongsanamide E

5.1 Introduction

Peptide synthesis is greatly facilitated by solid phase peptide synthesis (SPPS) as previously discussed. Peptide chemists have established optimal routes to obtain various classes of peptides including depsipeptides, characterized by a cyclic ester. Biosynthetically, these peptides are cyclized via macrolactonization, chemistry that is mainly avoided by chemists due to the harsh conditions generally used that are often unsuitable for peptide compounds.

Previous work in the Boddy lab looked at thioesterases (TEs) as standalone biocatalysts that can perform macrolactonization of nonribosomally synthesized peptides (NRPs), including valinomycin¹ and cereulide². These peptides are oligomeric depsipeptides and the native excised purified TE was utilized to iteratively macrocyclize a linear thioester substrate. To further expand our understanding of biosynthetic cyclization chemistry and to obtain powerful macrocyclizing biocatalysts, it is important to investigate novel biosynthetic pathways. We sought to expand the biocatalytic toolbox by characterizing a novel TE.

The seongsanamides are a family of cyclic depsipeptide natural products. Seongsanamides A-D are bicyclic compounds whereas seongsanamide E is monocyclic and is presumably macrolactonized and released from the biosynthetic machinery via TE activity. This monocyclic precursor is further cyclized, forming a phenolic ether through P450 mediated oxidative coupling. These peptides were isolated from a South Korean sponge following their identification in an antiallergenic activity screen³, and several of these peptides were synthesized through a combination of synthetic strategies including solution and solid phase^{4,5,6}. The synthesis of the

macrocyclic core through macrolactonization proved to be challenging for these groups, and thus they mostly relied on macrolactamization (**Figure 5.1**), a concept covered in chapter 4.

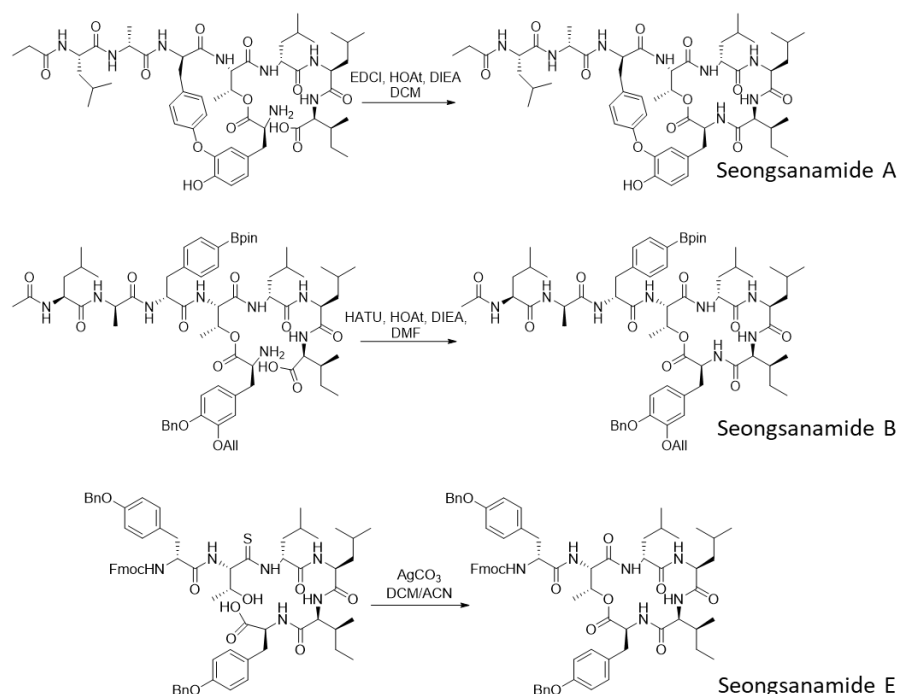


Figure 5.1 Macrocyclizing step of seongsanamide A, B and E

We saw this as an opportunity to utilize a TE to perform this challenging synthetic macrocyclization step, however C-terminal thioester peptide substrates were required.

C-terminal thioesters are particularly challenging with Fmoc-SPPS conditions as the thioester is not compatible with the amine bases used for Fmoc deprotection. Traditional solid phase access to C-terminal thioester peptides is accomplished with Boc-synthesis containing thioester linker resins, cleaved with anhydrous HF or through cleavage cocktail modifications⁷. Direct C-terminal modifications of peptides in Fmoc-SPPS have been the subject of several impressive strategies such as safety catch linkers^{8,9,10} and acyl shift linkers¹¹ (**Figure 5.2**) particularly for C-terminal thioesters used in native chemical ligation¹². A simple Fmoc-SPPS method for C-terminal thioesters of shorter peptides is possible with a combination of SPPS and

solution phase, where hyper acid-sensitive resins can be used to synthesize peptides that can be selectively cleaved under mild acidic conditions to then be thioesterified¹³. An on-resin approach can take advantage of the peptide sequence and link amino acid sidechains to the resins, allowing for on-resin C-terminal modification (**Figure 5.2**) and producing the correct amino acid sidechain following peptide cleavage and deprotection with the appropriate resin type.

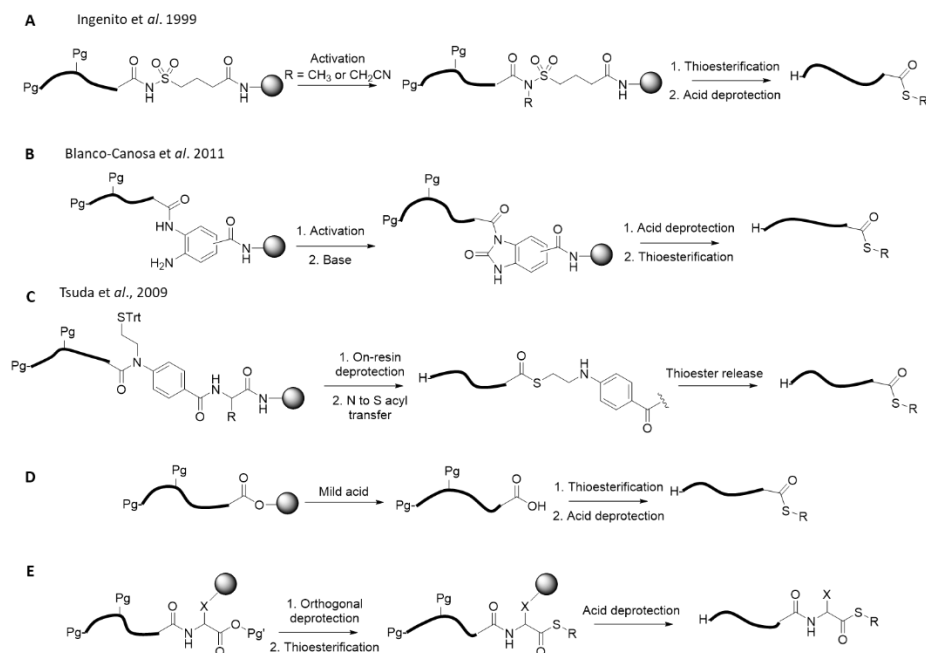


Figure 5.2 Thioester peptide yielding strategies

Seongsanamide E was chemoenzymatically synthesized using the excised, expressed and purified native TE and the thioester linear peptide substrate. Mostly SPPS was relied upon to generate a C-terminal N-acetyl cysteamine thioester substrate, a simplified natural thioester mimic, and a synthetic macrocycle standard, accomplishing a natural product total synthesis. The macrocycle was generated chemoenzymatically, yielding the product without detectable epimerization, however interesting hydrolysis side products were observed¹⁴.

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5.3 Author contributions

J.T.B.-H. and A.R.P. contributed equally to this work. J.T.B.-H. performed cloning, protein expression, purification, kinetic analysis, enzymatic assays, and LCMS analysis of the biochemical assays. A.R.P. synthesized and characterized all compounds and performed LCMS analysis of the

biochemical assays. A.Q.O. performed enzymatic assays and HPLC analysis of the biochemical assays. M.G.D. performed compound characterization.

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T. Brazeau-Henrie, J. [‡], R. Paquette, A. [‡], Q. O'Rourke, A., G. Darnowski, M. & N. Boddy, C. Total and Chemoenzymatic Synthesis of Seongsanamide E. *Org. Lett.* **24**, 6369-6373 (2022).
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5.5 Total and Chemoenzymatic Synthesis of Seongsanamide E

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and Christopher N. Boddy*

Abstract

The chemoenzymatic synthesis of the depsipeptide natural product seongsanamide E, **3** is described. Synthetic C-terminal N-acetylcysteamine thioester of the linear natural product, **1**, was macrolactonized by the excised recombinant purified seongsanamide thioesterase (Sgd-TE) domain, generating **3**. Sgd-TE also effects ring-opening of **3**. Chemical synthesis provided **3** through a macrolactamization strategy. This work confirms the biosynthesis of **3** and demonstrates the power of Sgd-TE as a biocatalyst. The seongsanamides (Sgds) are a family of cyclic non-ribosomal depsipeptides isolated from the marine sponge associated bacterial *Bacillus safensis* KCTC 12796BP¹. There are 6 known seongsanamides, A-F. A-E are N-acylated octapeptides with a lactone linkage between the terminal carboxylate of Tyr₈ and the sidechain hydroxyl of Thr₄. All five have the peptide sequence L-Leu-D-Ala-D-Tyr-L-Thr-D-Leu-L-Leu-L-Ile-L-Tyr. Sequencing of the *B. safensis* genome identified a non-ribosomal peptide synthetase (NRPS) gene cluster^{1,2} that encoded two NRPS genes possessing A domain selectivity³⁻⁶ consistent with these amino acids and E domains appropriately placed to generate the three D-configured residues.

Introduction

Based on this putative gene cluster, it is proposed that a linear NRPS-bound octapeptide is generated and macrocyclized seongsanamide E, **3**, is then released¹ by the C-terminal thioesterase (TE) domain (**Fig 5.3**)⁷. Ultimately **3** is proposed to be further cyclized by a P450⁸, forming a

biaryl ether linkage between Tyr₃ and Tyr₈ via oxidative phenol-coupling, generating seongsanamide B (**Fig S1**). In this study we characterized the ability of the TE to macrocyclize the N-acetylcysteamine (SNAC) derivative of the native linear peptide substrate. We show the recombinant purified excised Sgd-TE effectively forms **3**, experimentally confirming that the biosynthetic gene cluster identified by Kim *et al.*¹ is responsible for seongsanamide biosynthesis. We also show that chemical macrolactonization does not occur from the linear free acid substrate, but that authentic macrocycle can be synthesized via lactamization, successfully completing the first total synthesis of **3** and demonstrating the macrolactonizing power of the Sgd-TE. Lastly, we show that the TE can effect hydrolytic ring opening of **3**, a rare enzymatic activity only seen for the epothilone (Epo) TE⁹. Characterization of the excised Sgd-TE domain required access to an appropriate substrate, authentic standard of the natural product, and the recombinant purified TE domain. Recently, seongsanamide A¹⁰, B¹¹ and E¹² have been successfully synthesized using a combination of solution and solid-phase peptide synthesis. We envisioned a similar strategy to access the SNAC activated substrate **1** and the authentic natural product **3**.

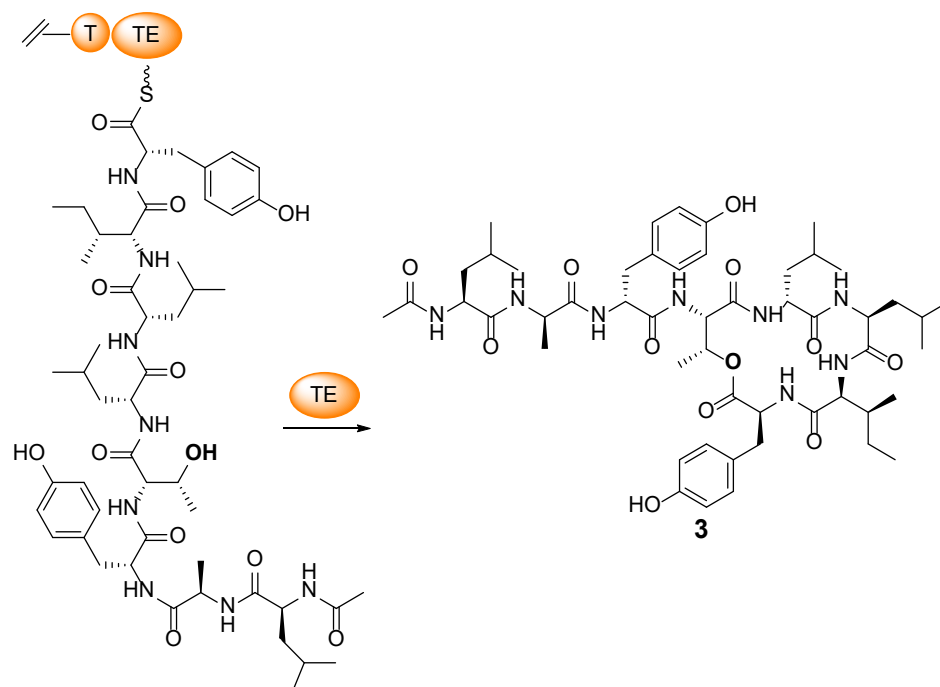
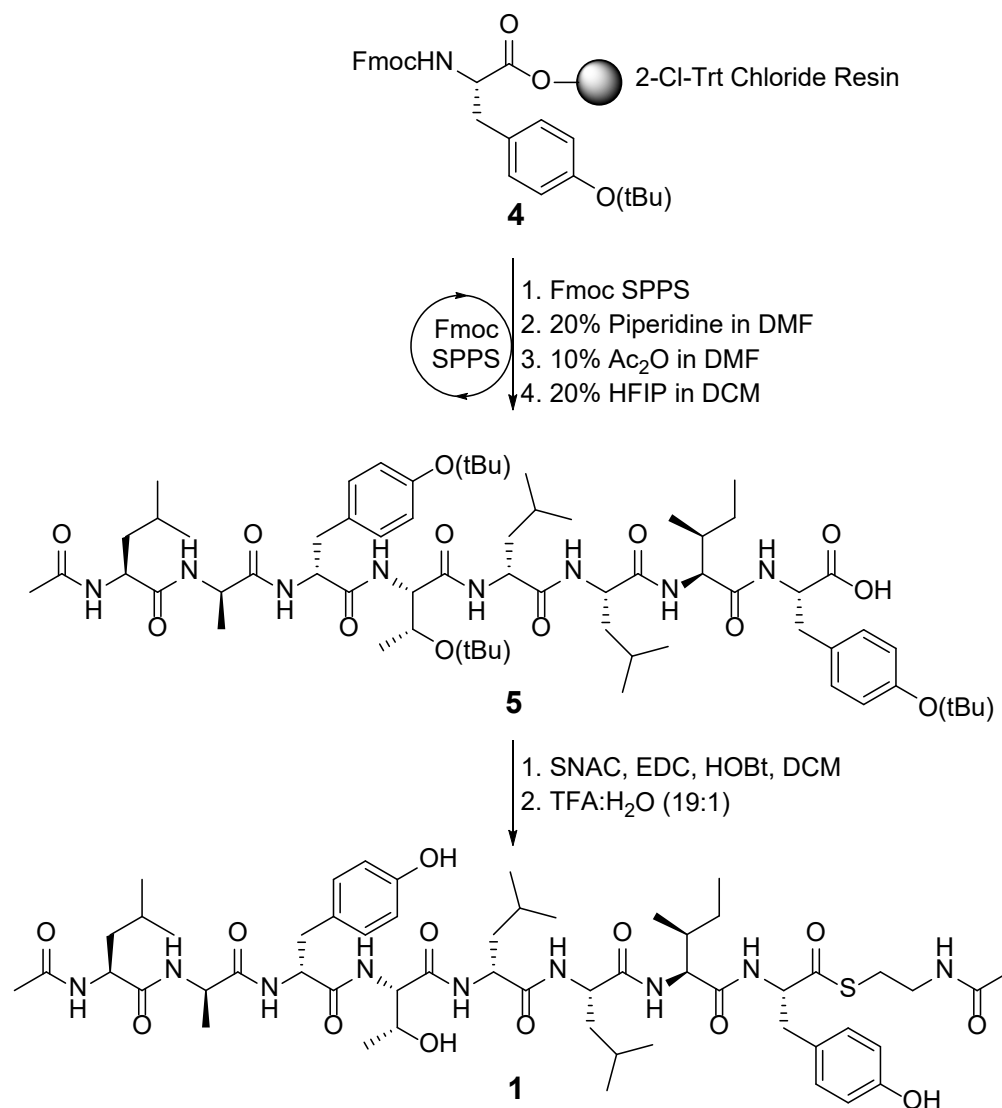


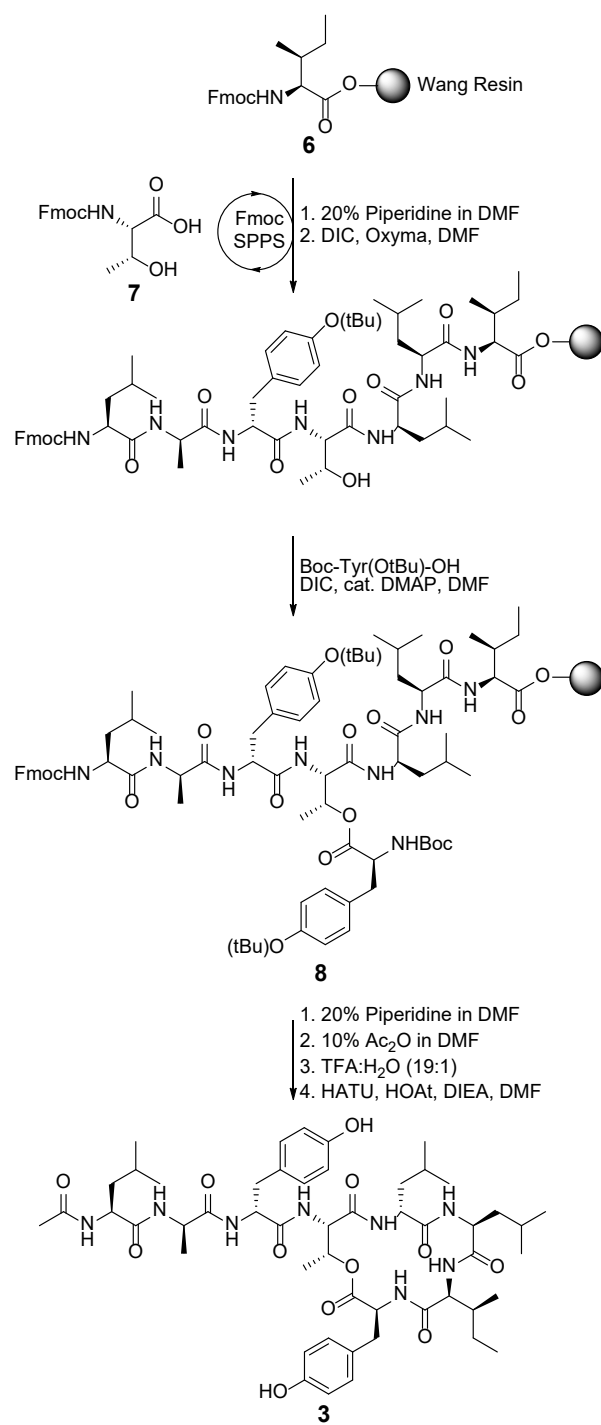
Figure 5.3 Seongsanamide E TE-mediated formation.

C-terminal thioester peptides like **1** are synthetically challenging¹³. While linear unactivated peptides can be routinely synthesized with automated Fmoc solid phase peptide synthesis (SPPS), C-terminal thioesters require specialized resins^{14,15} or synthetic modifications^{16,17}. In our hands, the most effect approach for accessing the peptidyl-SNAC thioesters, is to install the thioester using solution phase chemistry on a protected linear free acid peptide^{18,19}. Thus we generated the on resin linear protected peptide from hyper-sensitive 2-Cl-Trt Chloride resin²⁰ loaded with the C-terminal Fmoc-Tyr(OtBu)-OH **4**, which was subjected to SPPS under HBTU/DIEA conditions, subsequent N-terminal Fmoc-deprotection, and acetylation. Cleavage from the resin under mild acidic conditions yielded free acid **5**, which was activated as the SNAC thioester. The sidechain tBu protecting groups were removed with TFA and the substrate, **1**, was purified to > 95 % purity using RP-HPLC (65% yield from **5**).



Scheme 5.1 SPPS of linear SNAC substrate **1**

Synthesis of authentic **3** was not possible via a macrolactonization strategy. All attempts to generate macrocycle from linear free acid with unmasked Thr β -hydroxyl group failed²¹. We thus turned our attention to a common alternative approach for depsispetide cyclization,^{22–24} macrolactamization. We focused on cyclization through the Ile-Tyr amide bond. Wang resin loaded with Fmoc-Ile-OH **6** underwent SPPS with DIC/Oxyma Pure conditions to extend the linear peptide sequence, incorporating Fmoc-Thr-OH **7** with an unprotected β -alcohol.



Scheme 5.2 SPPS of seongsanamide E, **3**

The on-resin peptide ester **8** was formed through standard esterification conditions with Boc-Tyr(OtBu)-OH, followed by N-terminus deprotection and acetylation to complete the linear sequence. The peptide was fully deprotected and cleaved from the resin using TFA to afford the linear peptide ester. Macrolactamization under dilute HATU/HOAt coupling conditions generated **3**^{11,25}. Upon LCMS analysis, two peaks with the same mass were observed eluting close together. The isomers were separated by RP-HPLC and ¹H-NMR (**Fig S21**) confirmed the first eluting isomer was the natural product seongsanamide E, **3**. Coupling conditions known to reduce epimerization at the Ile α -carbon were screened though no difference in product distribution was observed²⁵. Regardless authentic **3** could be isolated in >95 % purity by RP-HPLC (32% yield from seco acid).

To obtain recombinant purified Sgd-TE, a synthetic DNA construct encoding the excised Sgd-TE from the *B. safensis* genome was generated¹. This gene was cloned into an *Escherichia coli* expression vector under the control of an inducible T7 promoter and fused to an N-terminal hexa-His tag. Sgd-TE was overexpressed and purified by metal-affinity chromatography to high purity (>95%, see **Fig S3**). Enzymatic assays with 1 mM of **1** and recombinant purified Sgd-TE (5 μ M) in phosphate buffer (50 mM, pH 7.4) and 7.5% *v/v* DMSO were performed at room temperature. Aliquots were taken at varying time points and analyzed by LCMS to evaluate the product distribution.

Incubation of **1** with Sgd-TE showed formation of the macrocycle **3**, confirming that the standalone TE is active. No formation of **3** was observed in the no enzyme or boiled enzyme control incubations. **3** was readily detected at the 30 min time point with Sgd-TE (**Fig 5.4B**). At 1.5 h, formation of the hydrolysis product **2** could be detected above background in the Sgd-TE incubation (**Fig 5.4C**) but not the control incubations. At 20 h, significantly more hydrolysis

product was detected in the Sgd-TE incubations (**Fig 5.4D**). Since both **3** and **2** are generated from the same peptidyl-TE intermediate, the relative product ratios for macrocyclization and hydrolysis should not change over the course of the reaction. The observation that hydrolysis increases with prolonged incubation thus suggested additional reactivity was occurring.

As the Epo-TE has been shown to effect ring opening of epothilone C⁹, we hypothesized that Sdg-TE could be affecting ring opening of **3**, ultimately leading to additional hydrolysis product **2**. To test this 1 mM of **3** was incubated with Sgd-TE (5 μ M) in phosphate buffer (50 mM, pH 7.4) and 7.5% v/v DMSO at room temperature. LCMS analysis showed formation of **2**, confirming that Sgd-TE can hydrolyze **3** (**Fig 5.4F and G**).

Sgd-TE consumption of **1** was kinetically characterized. Sgd-TE (5 μ M) was incubated with varying concentrations of **1** (0-750 μ M) in phosphate buffer (50 mM, pH 7.4) and 7.5% v/v DMSO. DTNB was also supplemented to each incubation to continuously track release of SNAC at 412 nm as substrate **1** is consumed. Incubations were performed for 1 h at room temperature.

Initial velocities were all linear over the time course of the incubations and less than 10% of **1** was consumed. Fitting these data to the Michaelis-Menten model showed Sgd-TE to be able to turn-over **1** in the presence of DTNB with a $k_{cat} = 0.24 \pm 0.02 \text{ min}^{-1}$ and a $K_M = 190 \pm 45.4 \text{ }\mu\text{M}$ ($k_{cat}/K_M = 21.0 \pm 5.3 \text{ M}^{-1} \text{ s}^{-1}$, Fig S5). These kinetic parameters are comparable to those obtained from other previously characterized NRPS TEs^{19,26–32}.

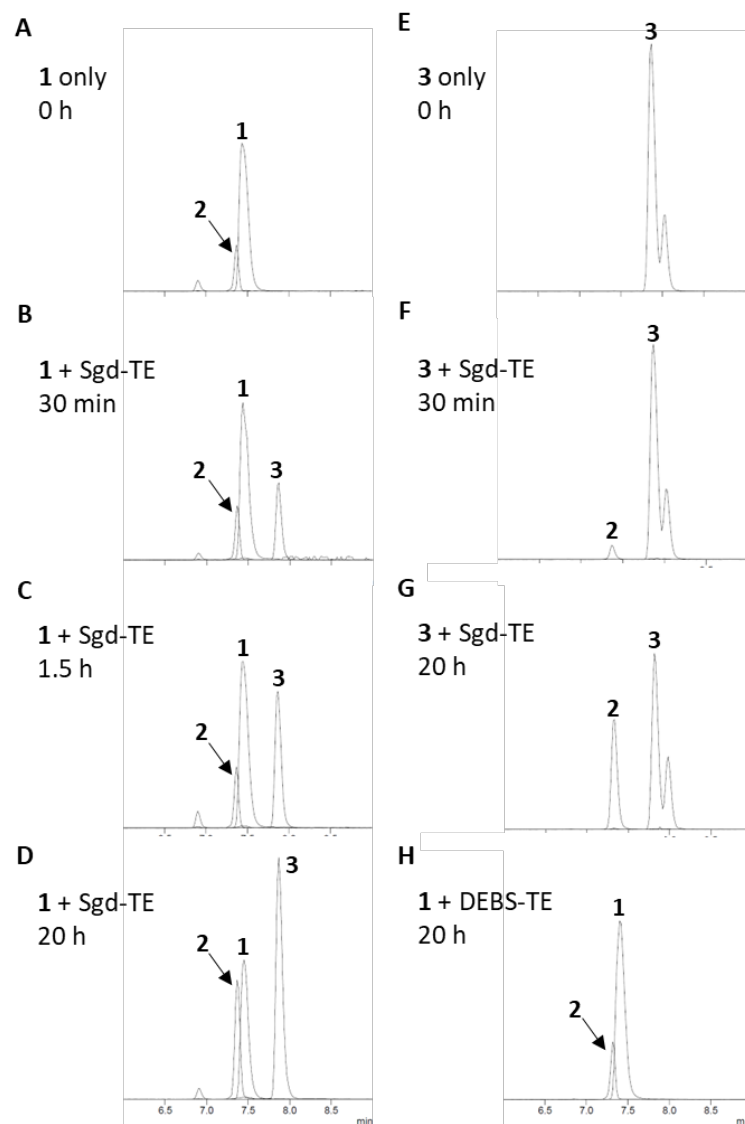


Figure 5.4 Extracted ion chromatograms of biochemical assays.

(A) Substrate **1** only control; boiled TE control showed a similar result (see Supporting Information). Sgd-TE assays after 0.5 hours (B), 1.5 hours (C) and 20 hours (D) of incubation with 1 mM of **1**. (E) Macrocycle **3** only control; boiled TE control showed a similar result (see Supporting Information). Sgd-TE assays after 0.5 hours (F) and 20 hours (G) with 1mM of **3**. (H) DEBS-TE assay after 20 hours of incubation with 1 mM of **1**; substrate **1** only and boiled TE controls showed similar results (see Supporting Information).

Macrocyclization is a demanding type of reactivity. Formation of a macrolactone from a thioester substrate does not occur spontaneously as demonstrated by the no enzyme and boiled

enzyme negative controls (**Fig S6 and S7**). Furthermore even substrate tolerant macrocyclizing TEs, like the DEBS-TE^{33–39}, are unable to macrocyclize **1** (**Fig 5.4H**). Thus the ability of the Sgd-TE to convert the SNAC activated analog of its native substrate **1** into the natural product **3** experimentally confirms that the gene cluster identified through bioinformatic analysis by Kim *et al.*¹ is responsible for biosynthesis of the seongsanamide family of natural products.

Sgd-TE also demonstrates potential as a potent biocatalyst. While we were ultimately able to perform the total synthesis of **3**, macrocyclization proved challenging. Macrolactonization was not possible under non-enzymatic conditions, a problem common in depsipeptide total syntheses^{11,40–42}. Ultimately we relied on macrolactamization to access authentic **3**, though the macrocyclization suffered from unwanted isomerization, presumably due to epimerization of the activated ester²⁵. Sgd-TE on the other hand readily generated the depsipeptide product through macrolactonization and did not suffer from epimerization issues, thus proving itself as a powerful biocatalyst and a promising alternative strategy for depsipeptide synthesis.

Lastly, we show that Sgd-TE can ring open its cognate natural product **3**. While the Epo-TE has been shown to ring open epothilone C⁹, other characterized TEs do not demonstrate this activity⁷. In the case of epothilone, the ester bond is sterically very accessible and is known to be highly sensitive to hydrolysis, a problem that limits the serum half life of epothilone in clinical settings^{43,44}. In the case of seongsanamide, the ester is far more hindered and thus more stable. Ring opening by Sgd-TE suggests a discrete binding of **3**, consistent with enzyme-substrate interactions specifically stabilizing a late transition state for macrocyclization, thus binding the product in a conformation that enables attack of the active site Ser on the lactam carbonyl. This is supported by the low Michaelis constant, which is atypical of macrocyclizing PKS TEs and more similar to macrocyclizing NRPS TEs^{28,45–47} which, at least in the case of the tyrocidine (Tyc) TE,

bind its substrate in a very product like conformation^{47–49}. Interestingly, product inhibition of macrocyclization might be anticipated from this model, however our kinetic characterization fits a Michaelis-Menten model without recourse to including a product inhibition step.

Conclusion

In summary, using SPPS we have completed the total synthesis of seongsanamide E **3** through a macrolactamization strategy. In a complementary biocatalytic strategy using recombinant purified Sgd-TE, we have effectively macrolactonized the SNAC activated biosynthetic precursor to **3**. This result confirms the involvement of the previously identified biosynthetic gene cluster in seongsanamide biosynthesis and shows the power of TEs in accessing macrocycles. Lastly we have shown that Sgd TE can effect ring opening of the natural product providing new insights into potential enzyme substrate interactions driving macrocyclization.

Acknowledgement

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<https://doi.org/10.1021/bi00593a016>.

5.6 Supplementary information

General synthetic protocols

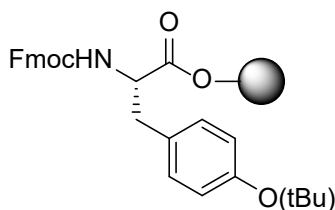
All solvents were purchased from Fisher Scientific. All reagents were purchased at the highest available purity and used without further purification. EDC.HCl, HATU, DIEA and Boc-Tyr(OtBu)-OH were purchased from Oakwood Chemical (Estill, SC, USA), all Fmoc protected amino acids and HOBt were purchased from GL Biochem (Shanghai, PRC), and all other chemicals were purchased from Sigma-Aldrich (Oakville, ON, Canada or Milwaukee, WI, USA). All reactions were carried out using dry solvents under a nitrogen atmosphere unless otherwise noted. Automated peptide synthesis was performed on a CEM Liberty Blue peptide synthesizer using microwave (MW) irradiation for all steps involving heating above ambient at a 0.1 mmol scale. NMR spectroscopy was performed with either a Bruker Avance II operating at 400 MHz for ^1H and 100 MHz for ^{13}C spectra or a Bruker Avance III with a cryoprobe operating at 600 MHz for ^1H and 150 MHz for ^{13}C spectra. LCMS analysis was carried out using a Shimadzu LC20A HPLC coupled with a Shimadzu LCMS2020 single quad mass spectrometer with an ESI source. Reverse-phase purification was carried out using an Agilent 1260 infinity and a Phenomenex Kinetex 5 μm XB-O18 100Å, 150 x 10.0 mm Semi-Preparative column. High-resolution mass spectroscopy (HRMS) was conducted on a Micromass Q-TOF I (John L. Holmes Mass Spectroscopy Facility, Ottawa, ON, Canada).

Table S1. Purity and expected/observed m/z of peptides used in this study.

Compounds	Molecular weight	Purity	Expected m/z	Observed m/z
SNAC-Sgd E 1	1112.40	99 %	1134.5895	1134.5885
Sgd E hydrolysis 2 *	1011.23	NA	NA	NA
Sgd E 3	993.21	99 %	1015.5301	1015.5301

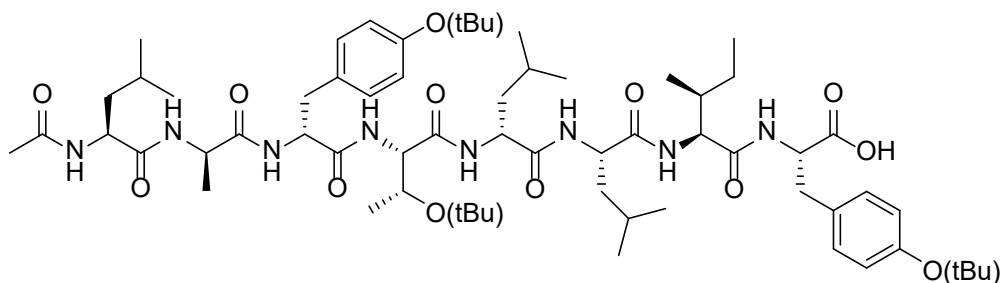
* **2** was not synthesized, detection only on low-res Shimadzu LCMS-2020 single quadrupole.

Sgd linear thioester **1**



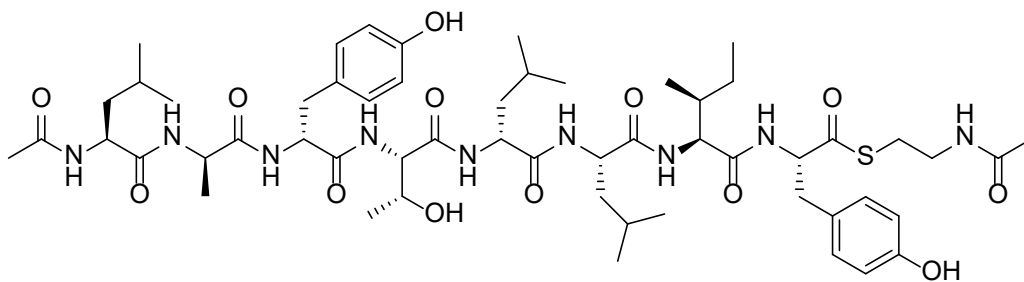
Fmoc-L-Tyr(tBu)-2-TrtCl resin (**4**). 2-Chlorotrityl chloride resin (300 mg, 200-400 mesh, 0.6 mmol/g) in a fritted peptide vessel was added DCM (5 mL), allowing the resin to swell at room temperature for 30 min. Fmoc-L-Tyr(tBu)-OH (230 mg, 0.5 mmol, 1 eq) and DIEA (350 μ L, 2 mmol, 4 eq) were added to the solution and the vessel was agitated uncapped for 20 seconds and the vessel was capped and agitated for 1.5 h. The solution was flushed and washed with DCM (10 mL x 4). The resin was added 10 mL capping solution, DCM:MeOH:DIEA (17 mL: 2 mL: 1 mL), and agitated for 30 min. The solution was flushed, washed with DCM (10 mL x 4) and transferred to a round bottom flask to be dried under reduced pressure. The loading rate was determined with 2% DBU solution and spectrometric analysis (Sigma-Aldrich, Fmoc Loading Test) to afford compound **4** (350 mg, 0.45 mmol/g).

Protected linear Sgd E. Automated peptide synthesis was performed on a CEM Liberty Blue peptide synthesizer at a 0.1 mmol scale. Dried Fmoc-L-Tyr(tBu)-2-TrtCl resin (220 mg) was placed in the reaction vessel followed by a 5-minute swell in DMF. Couplings were performed with Fmoc-amino acid (0.2 M in DMF), HBTU (0.2 M in DMF) and DIEA (1 M in NMP) at 50°C for 10 min and Fmoc deprotection were performed with 20% piperidine solution in DMF at 95°C for 2 min. N-terminal acetylation was done with 10% acetic anhydride in DMF at 50°C for 1 h. Once complete, the resin was transferred to a fritted vessel and flushed with DCM (5 mL x 3) and dried under reduced pressure to afford crude linear peptide loaded resin. The substrate was confirmed by LCMS and used without further characterization.



Protected linear Sgd E acid (**5**). To protected linear Sgd E peptide loaded resin (100 mg) was added 5 mL cleavage solution (20% HFIP in DCM) and agitated for 30 min. The solution was flushed into a round bottom flask and this was repeated twice. The organic solvents were removed under reduced pressure to afford a white solid and compound **5** (59 mg, 74% yield) was confirmed by LCMS and ¹H NMR. ¹H NMR (400 MHz, CDCl₃) δ 8.92 (d, J = 7.6 Hz, 1H), 8.68 (d, J = 7.8 Hz,

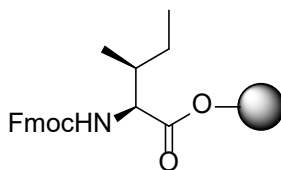
1H), 8.59 (d, J = 9.6 Hz, 1H), 8.19 (d, J = 5.4 Hz, 1H), 7.94 (d, J = 6.2 Hz, 1H), 7.88 (d, J = 8.9 Hz, 1H), 7.05 (dd, J = 9.0, 2.6 Hz, 3H), 6.98 (d, J = 8.3 Hz, 2H), 6.86 (s, 1H), 6.58 (d, J = 8.4 Hz, 2H), 6.50 (d, J = 10.1 Hz, 1H), 5.72 (t, J = 11.7 Hz, 1H), 5.06 (t, J = 9.6 Hz, 2H), 4.95 (t, J = 6.6 Hz, 1H), 4.87 (q, J = 6.1 Hz, 1H), 4.54 (q, J = 7.6 Hz, 1H), 4.25 (t, J = 10.9 Hz, 1H), 4.12 (t, J = 4.9 Hz, 1H), 3.62 (q, J = 5.1 Hz, 1H), 3.18 – 2.91 (m, 3H), 2.52 (t, J = 12.4 Hz, 1H), 1.73 (d, J = 7.9 Hz, 1H), 1.56 (t, J = 10.3 Hz, 5H), 1.38 (td, J = 12.2, 4.5 Hz, 2H), 1.32 – 1.25 (m, 2H), 1.23 (s, 9H), 1.20 (s, 9H), 1.15 (s, 9H), 0.84 (ddt, J = 12.2, 6.3, 3.9 Hz, 27H), 0.64 (t, J = 7.3 Hz, 3H), 0.29 (d, J = 6.3 Hz, 3H).



Sgd E N-acetylcysteamine thioester (**1**). Compound **5** (42 mg, 0.0365 mmol, 1 eq.) and HOBt (5.2 mg, 0.0383 mmol, 1.05 eq.) were dissolved in DCM (0.1 M) and stirred at 0°C for 10 min. EDC.HCl (6 mg, 0.0383 mmol, 1.05 eq.) was added to the stirring solution and maintained at 0°C for 30 mins. The solution was added N-acetylcysteamine (5.2 mg, 0.0438 mmol, 1.2 eq.) and was stirred at room temperature overnight. The solution was diluted with DCM (5 mL) and quenched with NaHCO₃ (aq.) (5 mL). The aqueous phase was extracted with DCM (3x 5 mL) and the organic phase was separated, washed with brine (5 mL), dried over MgSO₄ and removed under reduced pressure. The thioester protected peptide was purified by column chromatography (2.5-5% MeOH in DCM) to afford (12 mg, 26%). R_f (5% MeOH in DCM) = 0.55. The substrate was confirmed by LCMS and used without further characterization.

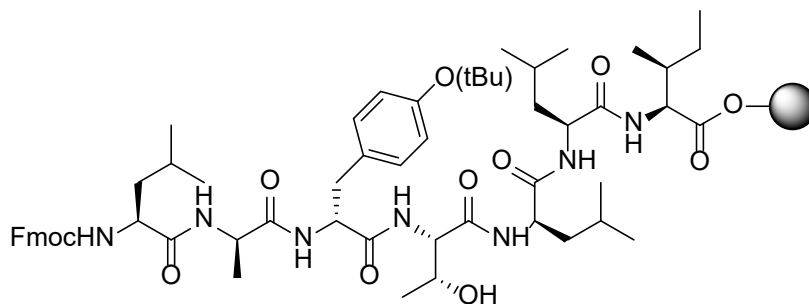
The purified protected thioester peptide was dissolved in 1 mL TFA:H₂O (19:1) and the solution was stirred at room temperature for 2 h or until the reaction was judged to be complete by TLC. The solution was precipitated out of cold ether and left in the -20°C freezer overnight. The solution was centrifuged and the supernatant was decanted and the residue was further dried by N₂ stream. The residue was purified by RP-HPLC to afford compound **1** (4.5 mg, 42%). ¹H NMR (600 MHz, MeOD) δ 7.08 (d, *J* = 8.5 Hz, 2H), 7.02 (d, *J* = 8.5 Hz, 2H), 6.73 – 6.67 (m, 4H), 4.70 (dd, *J* = 9.2, 5.4 Hz, 1H), 4.47 (ddd, *J* = 17.1, 9.3, 5.8 Hz, 2H), 4.39 (dd, *J* = 9.7, 4.9 Hz, 1H), 4.34 (dd, *J* = 8.5, 6.6 Hz, 1H), 4.25 (t, *J* = 7.2 Hz, 3H), 4.19 (dd, *J* = 6.5, 4.3 Hz, 1H), 4.01 (d, *J* = 4.3 Hz, 1H), 3.10 (dd, *J* = 14.4, 5.5 Hz, 1H), 3.07 – 3.01 (m, 1H), 3.02 – 2.98 (m, 4H), 2.85 (dd, *J* = 14.2, 9.2 Hz, 2H), 2.04 (s, 4H), 1.91 (s, 3H), 1.74 – 1.47 (m, 13H), 1.33 – 1.22 (m, 9H), 1.17 – 1.09 (m, 2H), 1.01 – 0.83 (m, 27H). HRMS (ESI⁺) exact mass calculated for C₅₅H₈₅N₉O₁₃SNa 1134.5895, found 1134.5885.

Sgd E macrocycle **3**

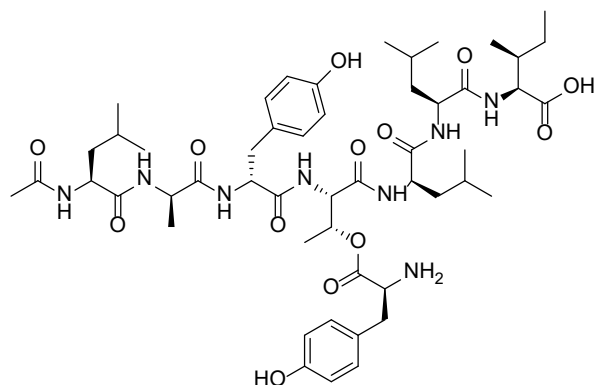


Fmoc-L-Ile-Wang resin (**6**). Wang resin (200 mg, 100-200 mesh, 1.2 mmol/g) in a fritted peptide vessel was added DMF (5 mL), allowing the resin to swell at room temperature for 30 min. Fmoc-L-Ile-OH (848 mg, 2.4 mmol, 10 eq) and DIC (188 µL, 1.2 mmol, 5 eq) were dissolved in DCM (5 mL) in a round bottom flask and stirred at 0°C for 20 min. The solvents were removed under reduced pressure and DMF (0.5 mL) and DMAP (3 mg, 0.024 mmol, 0.1 eq) were added to the amino acid anhydride mixture which was then transferred to the swelled resin. The solution was agitated for 1 h at room temperature then flushed, washed with DCM (10 mL x 2), DMF (10 mL x 2) and DCM (10 mL x 2) and transferred to a round bottom flask to be dried under reduced

pressure. The loading rate was determined with 2% DBU solution and spectrometric analysis (Sigma-Aldrich, Fmoc Loading Test) to afford compound **6** (250 mg, 0.49 mmol/g).



Linear Seongsanamide E precursor. Automated peptide synthesis was performed on a CEM Liberty Blue peptide synthesizer at a 0.1 mmol scale. Dried Fmoc-L-Ile-Wang resin (200 mg) was placed in the reaction vessel followed by a 5-minute swell in DMF. Couplings were performed with Fmoc-amino acids (0.2 M in DMF), DIC (0.2 M in DMF) and Oxyma Pure (1 M in DMF) at 95°C for 1.5 min and Fmoc deprotection were performed with 20% piperidine in DMF solution at 95°C for 2 min. The synthesis was paused directly following last Fmoc-L-Leu-OH and the resin was transferred to a fritted vessel and flushed with DCM (5 mL x 1).



Esterification (**8**). Boc-Tyr(tBu)-OH (1.38 g, 4.08 mmol, 10 eq) was dissolved in DCM (5 mL) and cooled to 0°C. DIC (638 μ L, 4.08 mmol, 10 eq) was added and the solution was stirred at room temperature for 30 min. Half the DCM was removed under N₂ stream and DMF (2.5 mL) was added and the solution was added to the resin in the fritted vessel followed by HOBt (mg,

Linear Seongsanamide E. The resin was transferred to the automated peptide synthesis reaction vessel and synthesis was resumed. N-terminal Fmoc deprotection was performed with 20% piperidine in DMF at 95°C for 2 min and acetylation was done with 10% acetic anhydride at 95°C for 1 h. The resin was removed from the instrument and placed in a fritted peptide vessel. The substrate was confirmed by LCMS and used without further characterization.

[illegible]

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removed under reduced pressure. The residue was purified by RP-HPLC to afford compound **3** (3.1 mg, 32%). ^1H NMR (600 MHz, DMSO) δ 9.20 (d, J = 3.8 Hz, 2H), 8.39 (d, J = 7.1 Hz, 1H), 8.14 (d, J = 8.9 Hz, 1H), 8.09 (d, J = 7.6 Hz, 1H), 8.06 – 8.00 (m, 2H), 7.95 (d, J = 5.3 Hz, 1H), 7.73 (d, J = 9.3 Hz, 1H), 7.25 (d, J = 7.2 Hz, 1H), 7.03 (d, J = 8.3 Hz, 3H), 6.91 (d, J = 8.2 Hz, 2H), 6.64 (dd, J = 13.8, 8.3 Hz, 4H), 5.02 (s, 1H), 4.48 – 4.37 (m, 2H), 4.26 (p, J = 7.6 Hz, 3H), 4.19 (dd, J = 8.7, 2.7 Hz, 1H), 4.01 (d, J = 15.6 Hz, 1H), 3.88 (t, J = 9.5 Hz, 1H), 2.96 (dd, J = 13.8, 4.9 Hz, 1H), 2.89 – 2.83 (m, 1H), 2.79 (d, J = 7.6 Hz, 2H), 1.75 (s, 4H), 1.64 – 1.51 (m, 2H), 1.50 – 1.43 (m, 1H), 1.41 (t, J = 7.1 Hz, 4H), 1.37 (s, 1H), 1.23 (s, 6H), 1.14 (d, J = 7.0 Hz, 4H), 1.02 (dd, J = 14.3, 7.2 Hz, 1H), 0.89 (d, J = 6.5 Hz, 3H), 0.87 (d, J = 6.6 Hz, 2H), 0.86 – 0.83 (m, 3H), 0.83 (d, J = 3.8 Hz, 7H), 0.80 (d, J = 6.8 Hz, 6H). HRMS (ESI+) exact mass calculated for $\text{C}_{51}\text{H}_{76}\text{N}_8\text{O}_{12}\text{Na}$ 1015.5301, found 1015.5301.

A

BSL056_13655 *BSL056_13660* *BSL056_13665*
SgdC SgdB SgdA

Non-Ribosomal Peptide Synthetase (NRPS) Cytochrome P450

10 kbp

B

BSL056_13665 - SgdA *BSL056_13660* - SgdB

Module 1 Module 2 Module 3 Module 4 Module 5 Module 6 Module 7 Module 8

L-Leu D-Ala D-Tyr L-Thr D-Leu L-Leu L-Ile L-Tyr

CATCATECATCATECATCATECATCATE

Chemical structures of the peptide intermediates and the final product are shown, along with the SgdC and SgdA enzymes.

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and tailoring genes have been identified beyond the cluster. (B) Biosynthesis of Sgd analogues and proposed formation of Sgd biaryl-ether analogues. Abbreviations; C, condensation domain; A, adenylation domain; T, thiolation or peptidyl carrier domain; E, epimerization domain; TE, thioesterase domain.

Cloning, expression and purification

Cloning and expression. A synthetic gene construct was designed using the seongsanamide (Sgd) TE coding sequence from *Bacillus safensis* genome² (CP018197.1 range 2,629,387 - 2,630,191) and codon optimized for *E.coli* (GeneArt, Thermo Fischer). Construct was cloned into NdeI and EcoRI linearized pET-28a(+) expression vector, generating pJBH19. Resulting construct was confirmed by Sanger sequencing (G  nome Quebec).

Expression vector pJBH19 was transformed into *E. coli* BL21(DE3) chemically competent cells for protein expression. A 400 mL flask containing LB media supplemented with 50 µg/mL of kanamycin was inoculated with 0.5 % v/v of a dense transformed overnight culture. Culture was grown at 37 °C with 200 rpm shaking to an OD₆₀₀ of 0.8. Protein expression was induced with the addition of isopropyl-  -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Culture was then incubated at 16 °C with 200 rpm shaking for 18 hours. DEBS-TE was expressed from the pRSG33 construct as previously described^{3,4}.

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>Sgd-TE
MGSSHHHHHHSSGLVPRGSHMKKKDKTEKNLELI IKLRES DGAPFFFIHPASGNTLCYME LAKLLDNACS
LYGINFPYVEDRSMENIRLEDLSRLYLKKIKEIQPKGPYMLGGWSIGGLFAYEIANQLIEQGEEVSLLSL
IDTAFPRAEYILSKEESI IQVLNSISAH LILDPNKEKEIAEVTFKNLINS PKIRQMLPPGLEVEDIKTLS
NIFHANTKVS AEYKIEKQYRGLIYFEAQEGTRLGNKWSSYLEGDLKNIQVPGKHAEMMDSPSVNI IANKL
KEEILSATYI-

```

Figure S2. Sgd TE translated protein sequence from pJBH19 construct. N-terminal hexa His tag used for purification is bolded.

Protein purification. Entire procedure was performed at 4 °C and all plasticware used were pre-chilled. Cells were harvested by centrifugation at 4,000 g and resuspended in 10 mL of lysis buffer (100 mM sodium phosphate, 300 mM NaCl, 10% w/v glycerol, 1 mg/mL lysozyme, 1 µg/mL of pepstatin A and 1 µg/mL leupeptin, pH 8.0). Cells were lysed by sonication on ice and cell debris removed by centrifugation at 12,000 g. Resulting lysate was transferred to a 15 mL falcon tube and incubated with 400 µL of Ni-NTA resin (#30210, QIAGEN) on a rocking platform shaker for 1.5 hours. Lysate was then loaded into a gravity flow column and resin allowed to settle by gravity. Purification buffer (100 mM Tris, 300 mM NaCl, pH 7.4) was used at the indicated imidazole concentrations for resin washing and protein elution. Resin was first washed once with 0 mM imidazole and once more with 20 mM imidazole. Protein was eluted twice with 100 mM imidazole and then twice more with 250 mM imidazole. Protein collected during elution was concentrated and the buffer exchanged for dialysis buffer (100 mM Tris, 300 mM NaCl, pH 7.4) by centrifugation at 4,000 g using an Amicon Ultra-15 10 kDa MWCO tube (#UFC9010, Millipore). An equal volume of 60% w/v glycerol was added to protein concentrate, flash frozen in liquid nitrogen then stored at -80 °C. Protein concentration was determined by Bradford assay (#5000006, Bio-rad). Approximately 4.7 mg of purified protein was obtained per liter of cell culture. DEBS-TE was purified as previously described⁴.

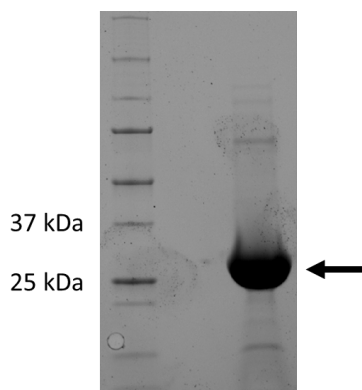


Figure S3. SDS-PAGE analysis of purified recombinant Sgd-TE. Expected molecular weight of protein is approx. 33 kDa. Left lane corresponds to Precision Plus Protein Unstained Protein Standards (#1610363, Bio-rad), right lane corresponds to the purified sample (loaded ~33 μ g).

Enzymatic assays

Michaelis-Menten kinetic measurements. Continuous enzymatic assays using DTNB (#D8130, Sigma-Aldrich) to track consumption of SNAC coupled substrate was performed in 96-well plates (50 mM phosphate buffer pH 7.4, 7.5% *v/v* DMSO, 4% *v/v* DTNB, 5 μ M Sgd-TE) and varying concentrations of substrate (0 to 750 μ M) in triplicate. A 2X substrate master mix (containing phosphate buffer, DMSO and substrate) and Sgd-TE master mix (containing phosphate buffer, DTNB and Sgd-TE) were prepared separately and arrayed into a 96-well plate. Sgd-TE master mix was mixed into each substrate master mix well and absorbance measurements were taken every 15 seconds for 1 hour at 412 nm and room temperature in a microplate reader. An SNAC standard curve (0 to 100 μ M) was generated to convert absorbance readings into concentrations (μ M). Initial velocities were plotted against substrate concentration and the data fitted to the Michaelis-Menten model using GraphPad Prism 8.

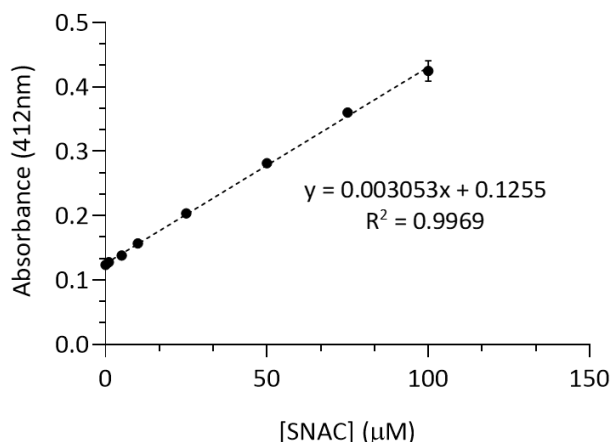


Figure S4. Single absorbance measurements at 412 nm were taken in a 96-well plate. Mixtures containing varying concentrations of SNAC (0 to 100 μM) were prepared in triplicate using enzymatic assay buffer conditions. Absorbance readings were plotted against SNAC concentration to yield the above SNAC standard curve.

Time-course assay and macrocycle ring opening assay. Sgd-TE was dialysed with dialysis buffer (5 spins, 14,000 g, 15 minutes, 4 $^{\circ}\text{C}$) to remove glycerol using Amicon Ultra-0.5 10 kDa MWCO tubes (#UFC5010, Millipore). Assays were carried out in eppendorf tubes in a 60 μL final reaction volume (50 mM phosphate buffer pH 7.4, 7.5% v/v DMSO, 1 mM substrate, 5 μM Sgd-TE). A substrate and negative control were prepared similarly as described above with the addition of water and heat denatured Sgd-TE (5 μM) in place of functional Sgd-TE respectively. A set of assays was prepared using recombinantly purified DEBS-TE, substrate only and heat denatured TE controls. Macrocycle ring opening assays were prepared similarly as described above except with 0.5 mM macrocycle authentic standard in place of substrate. Reactions were left to incubate on the benchtop at room temperature for turnover.

Aliquots at time points 0, 0.5, 1.5, 3 and 20 hours were taken and quenched in 3 volumes of LCMS Optima grade acetonitrile (MeCN) supplemented with 0.05% v/v LCMS Optima grade formic acid (FA). DEBS-TE assays were quenched after 20 hours only. Quenched aliquots were left on the benchtop for 15 minutes, then centrifuged at max speed for 10 minutes at room temperature before analysis by LCMS (Shimadzu Prominence UFLC system with SPD-20A Prominence UV/VIS Detector, LCMS-2020 single quadrupole MS). Chromatographic separations were done using an Agilent Eclipse Plus C18 (3.5-micron, 150×4.6 mm) reverse phase column in ESI+ mode with a flow rate of 1 mL/min. The mobile phase was composed of LCMS Optima grade H₂O (A) and MeCN (B) both supplemented with 0.05% v/v FA. Samples were monitored at 210 and 254 nm with an elution gradient of 10% B to 95% B over 15 minutes.

Michaelis-Menten kinetic data fit

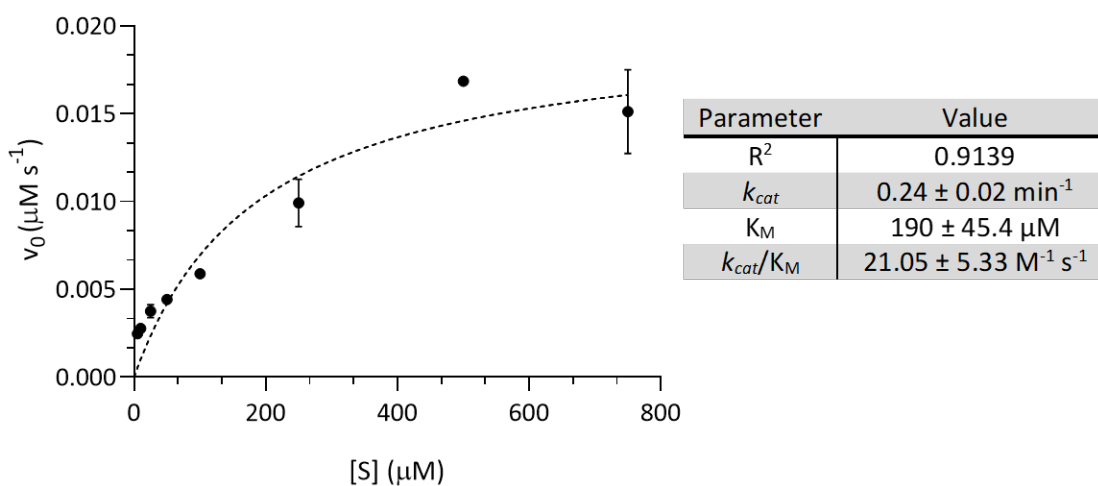


Figure S5. Initial velocities ($\mu\text{M}\cdot\text{s}^{-1}$) were plotted against substrate concentration (μM). The data was then fitted to the Michaelis-Menten model (dashed curve), parameters of the fit are displayed in the table.

Examples of time-course chromatograms and mass spectra

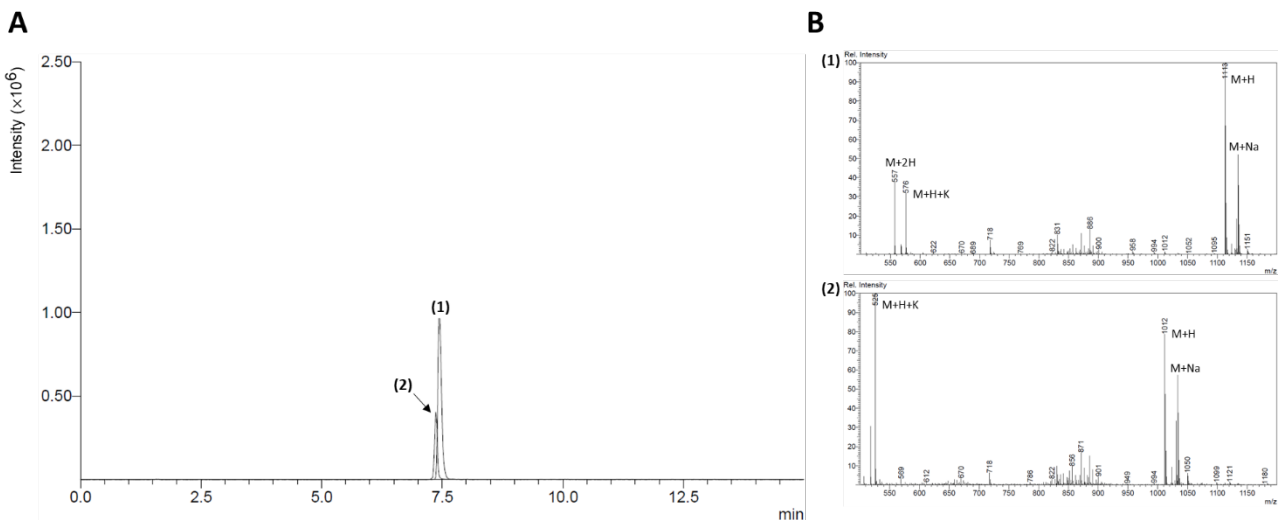


Figure S6. (A) Extracted ion chromatogram for substrate only control assay after 20 hours of incubation. (B) Mass spectra for substrate (1) and hydrolysis product (2), mass adducts are identified in each spectra.

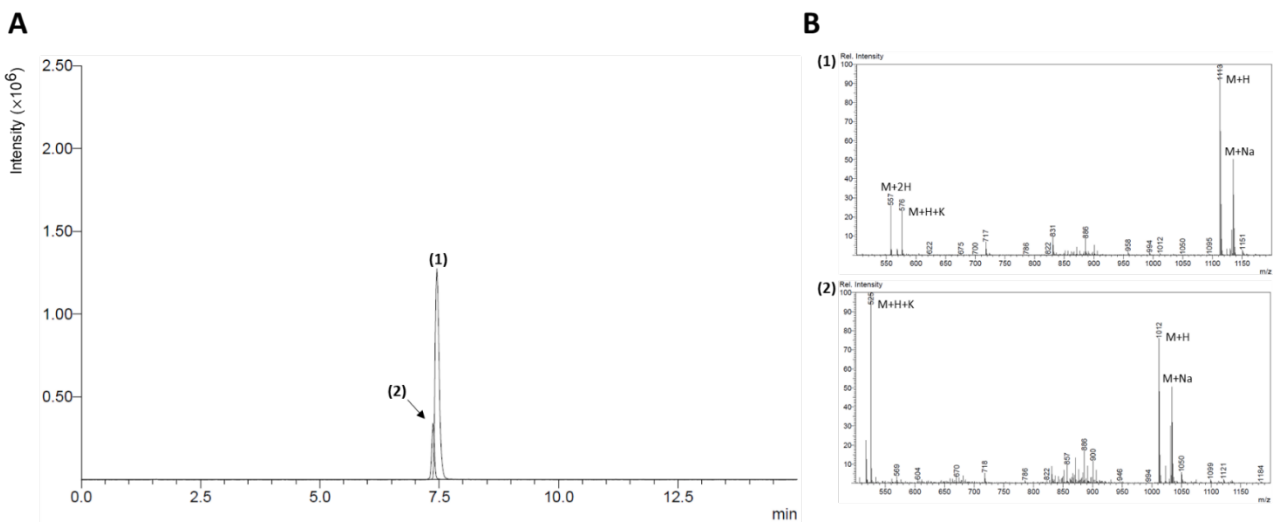


Figure S7. (A) Extracted ion chromatogram for boiled Sgd-TE control assay after 20 hours of incubation. (B) Mass spectra for substrate (1) and hydrolysis product (2), mass adducts are identified in each spectra

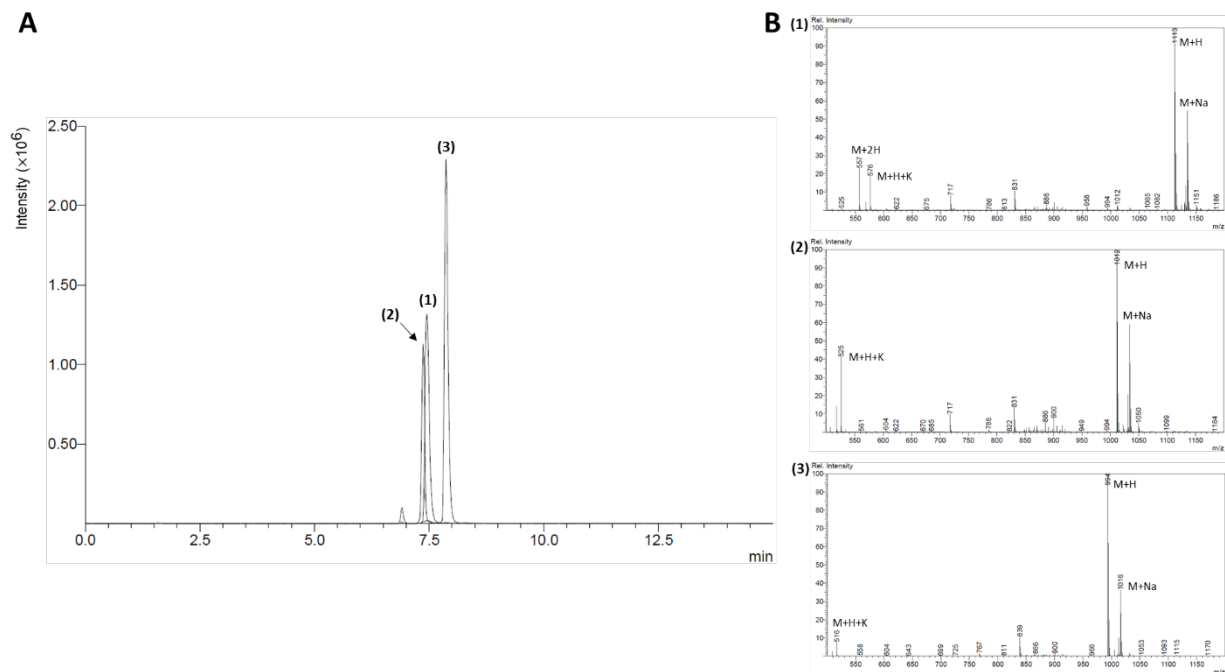


Figure S8. (A) Extracted ion chromatogram for Sgd-TE assay after 20 hours of incubation. (B) Mass spectra for substrate (1), hydrolysis (2) and macrocyclic (3) products, mass adducts are identified in each spectra.

Examples of DEBS assay chromatograms and mass spectra

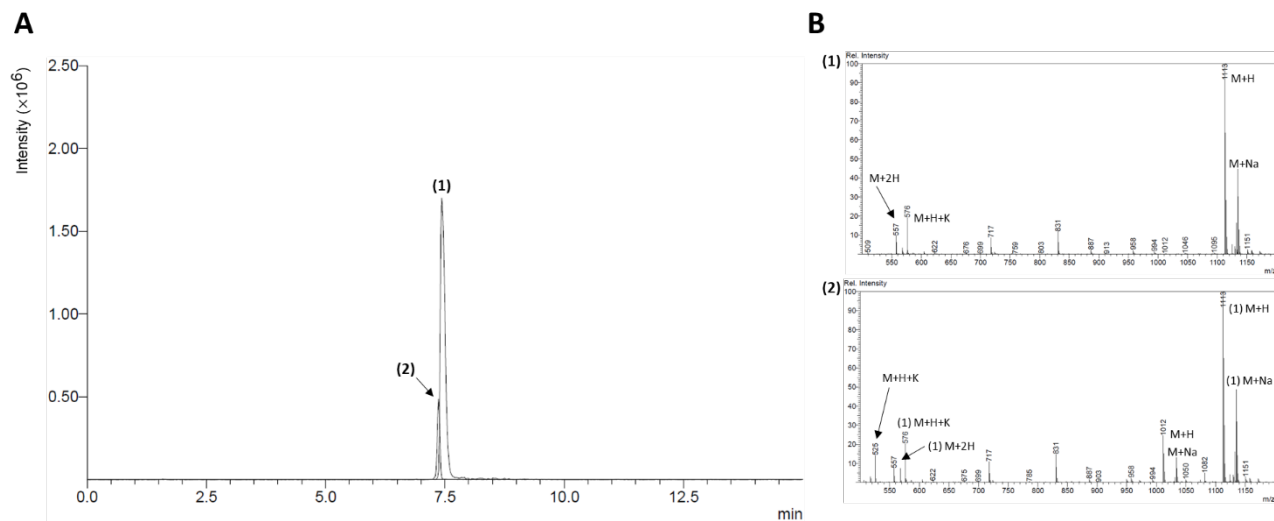


Figure S9. (A) Extracted ion chromatogram for substrate only control assay after 20 hours of incubation. (B) Mass spectra for substrate (1) and hydrolysis product (2), mass adducts are identified in each spectra.

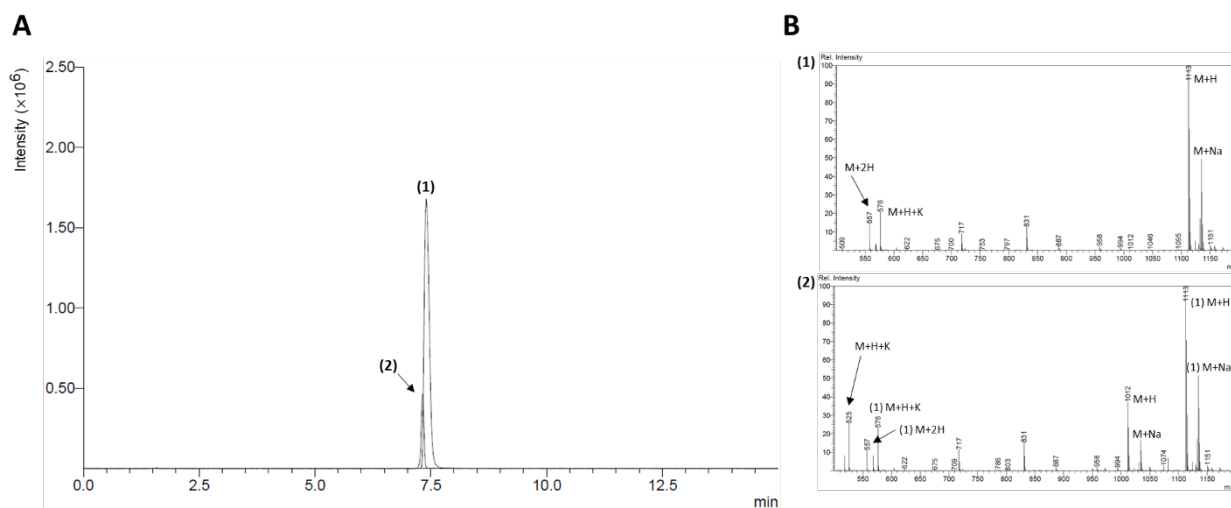


Figure S10. (A) Extracted ion chromatogram for boiled DEBS-TE control assay after 20 hours of incubation. (B) Mass spectra for substrate (1) and hydrolysis product (2), mass adducts are identified in each spectra.

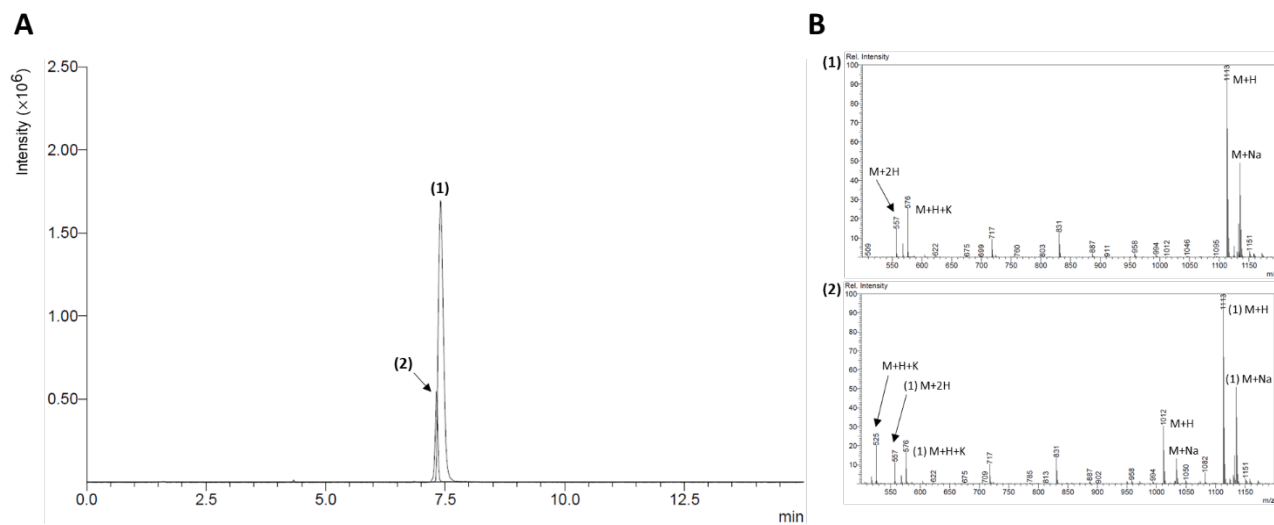


Figure S11. (A) Extracted ion chromatogram for DEBS-TE assay after 20 hours of incubation. (B) Mass spectra for substrate (1) and hydrolysis product (2), mass adducts are identified in each spectra. Substrate is present in hydrolysis mass spectra from co-elution during chromatographic separation (shown as (1) adducts).

Examples of macrocycle ring opening chromatograms and mass spectra

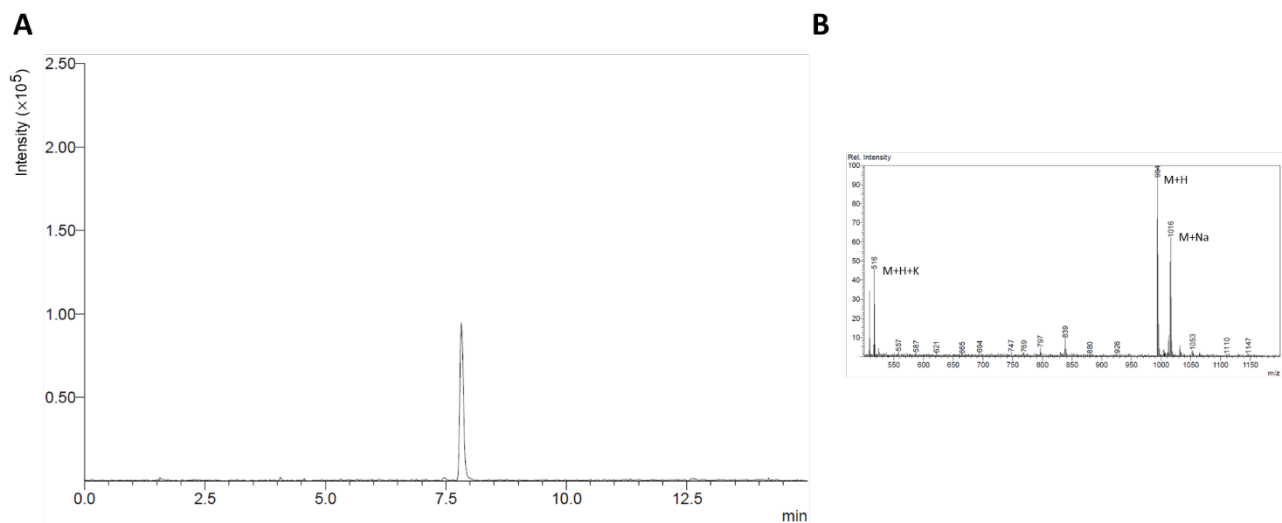


Figure S12. (A) Extracted ion chromatogram for macrocycle only control ring-opening assay (0.5 mM macrocycle) after 20 hours of incubation. (B) Mass spectra for macrocycle, mass adducts are identified in the spectra.

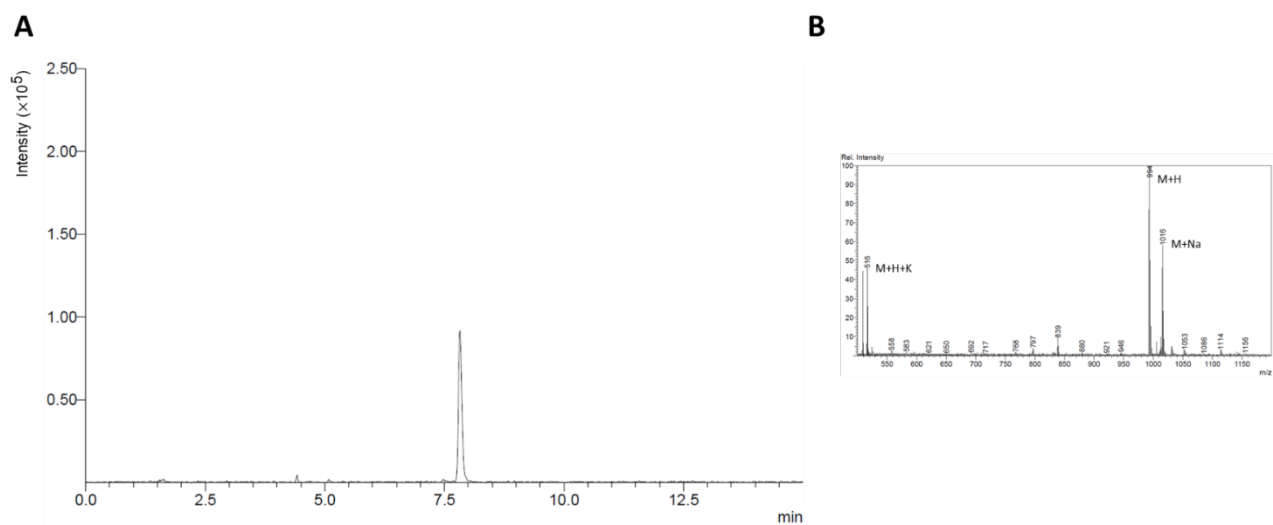


Figure S13. (A) Extracted ion chromatogram for boiled Sgd-TE control ring-opening assay (0.5 mM macrocycle) after 20 hours of incubation. (B) Mass spectra for macrocycle, mass adducts are identified in the spectra.

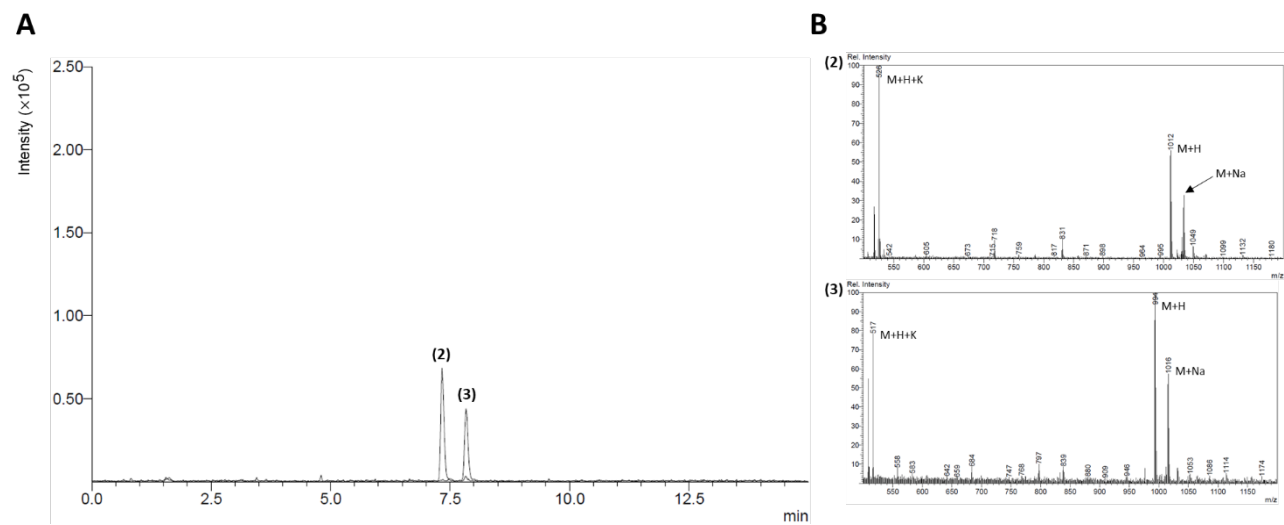


Figure S14. (A) Extracted ion chromatogram for Sgd-TE ring-opening assay (0.5 mM macrocycle) after 20 hours of incubation. (B) Mass spectra for macrocycle (3) and hydrolysis (2) product, mass adducts are identified in each spectra.

Example LCMS UV spectra

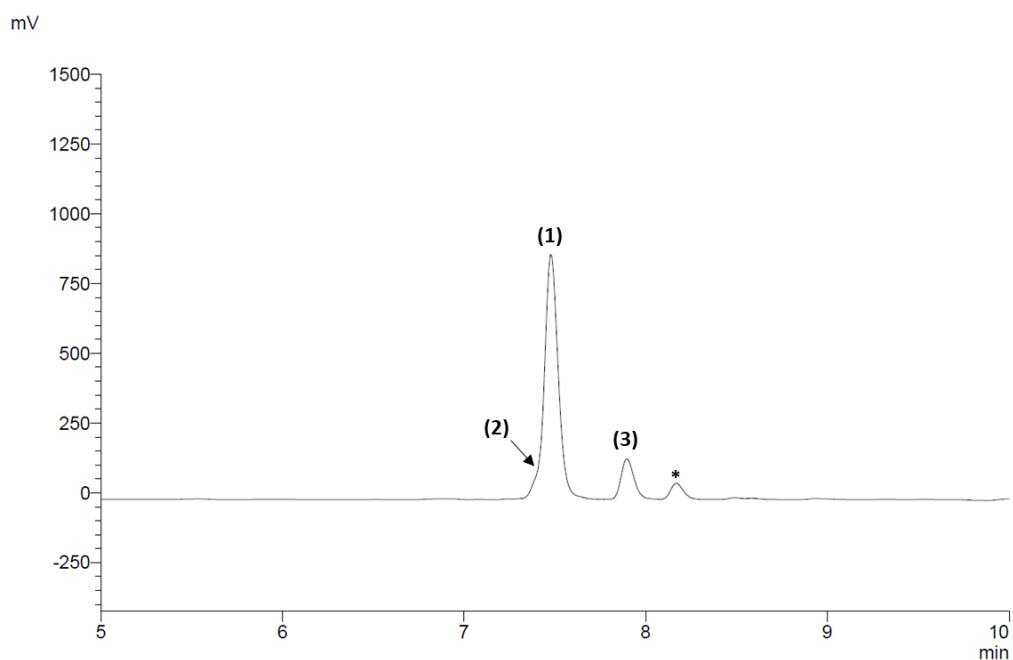


Figure S15. LCMS UV spectra obtained at 210 nm of Sgd-TE assay after 3 hours of incubation.

Peaks corresponding to the substrate (1), hydrolysis (2) and macrocycle (3) products are identified. Both hydrolysis and substrate peaks overlap leading to a small hydrolysis shoulder peak as shown. Peak labeled with an asterisk is an injector peak

Sgd-TE assay and ring-opening product ratios

Table S2. Calculated product ratios of enzymatic assays. Extracted ion chromatograms were used, integration of each peak corresponding to macrocycle (m/z 994), hydrolysis (m/z 1012) and substrate (m/z 1113) was done to obtain AUCs. AUCs were then used to calculate product ratios (as shown below).

Time (hours)	1 mM substrate 1		0.5 mM macrocycle 3
	Hydrolysis/Substrate	Macrocycle/Hydrolysis	Hydrolysis/Macrocycle
0	0.16193	N/A	N/A
0.5	0.18765	1.76690	0.02805
1.5	0.19552	3.07404	0.09162
3	0.24973	3.43211	0.16143
20	0.60648	2.45733	1.53637

Selected NMR, mass spectra and UV traces

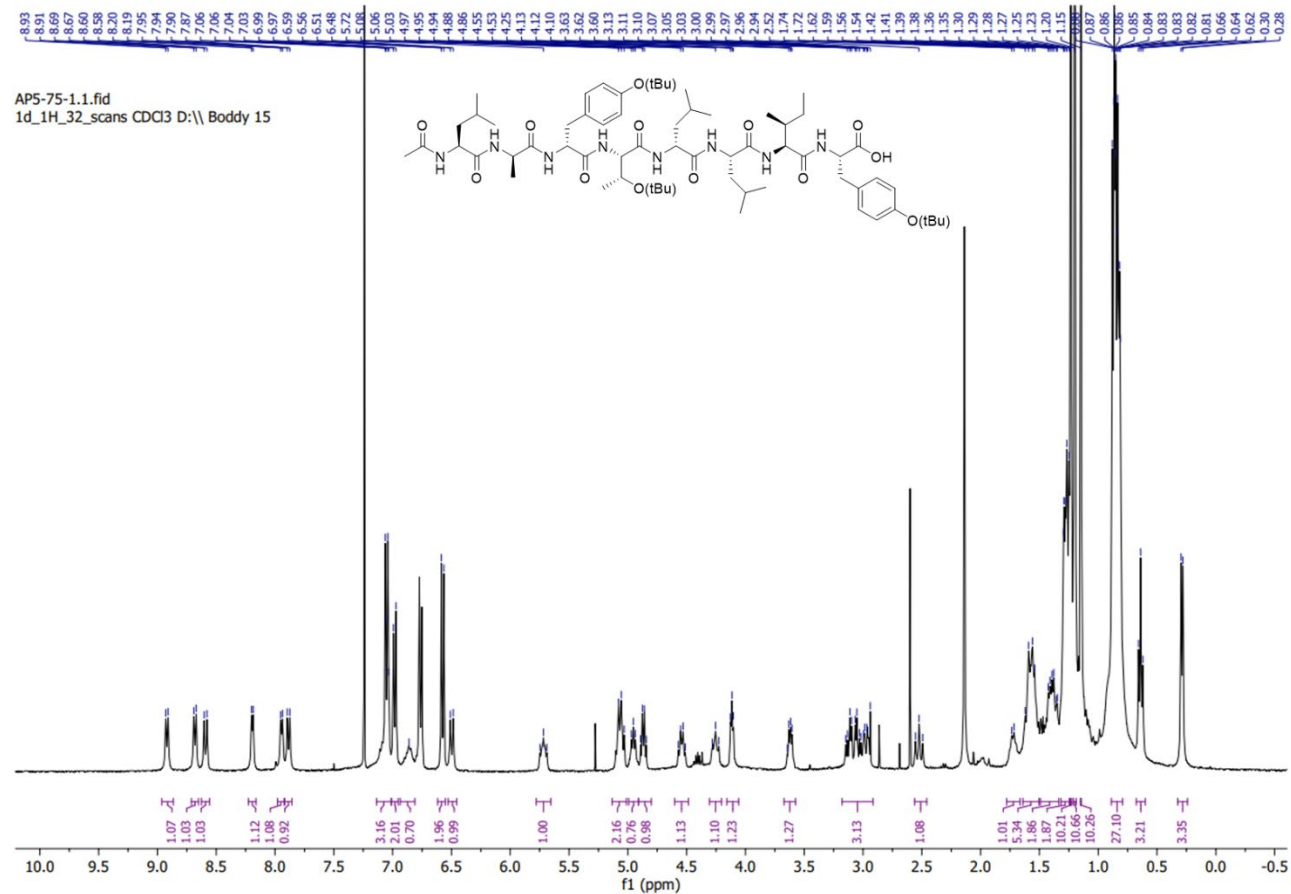


Figure S16. ^1H NMR spectrum of compound **5** in CDCl_3 .

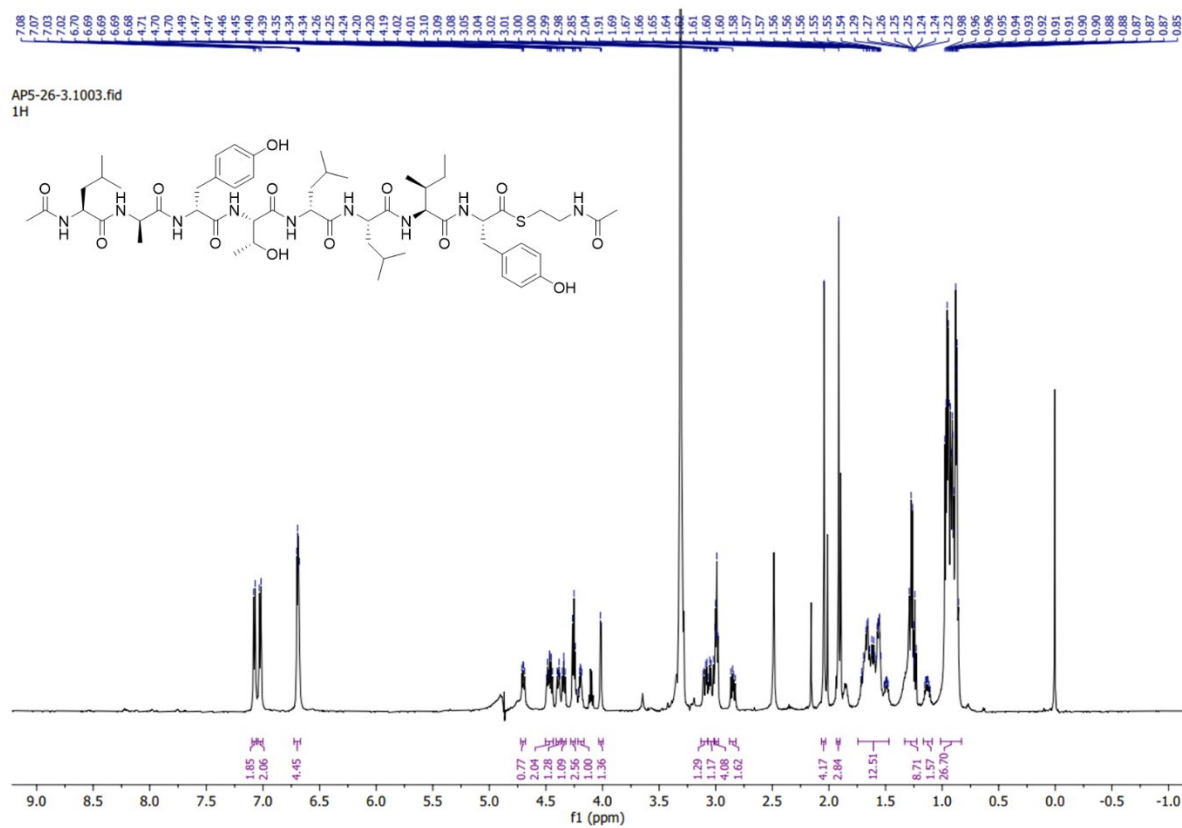
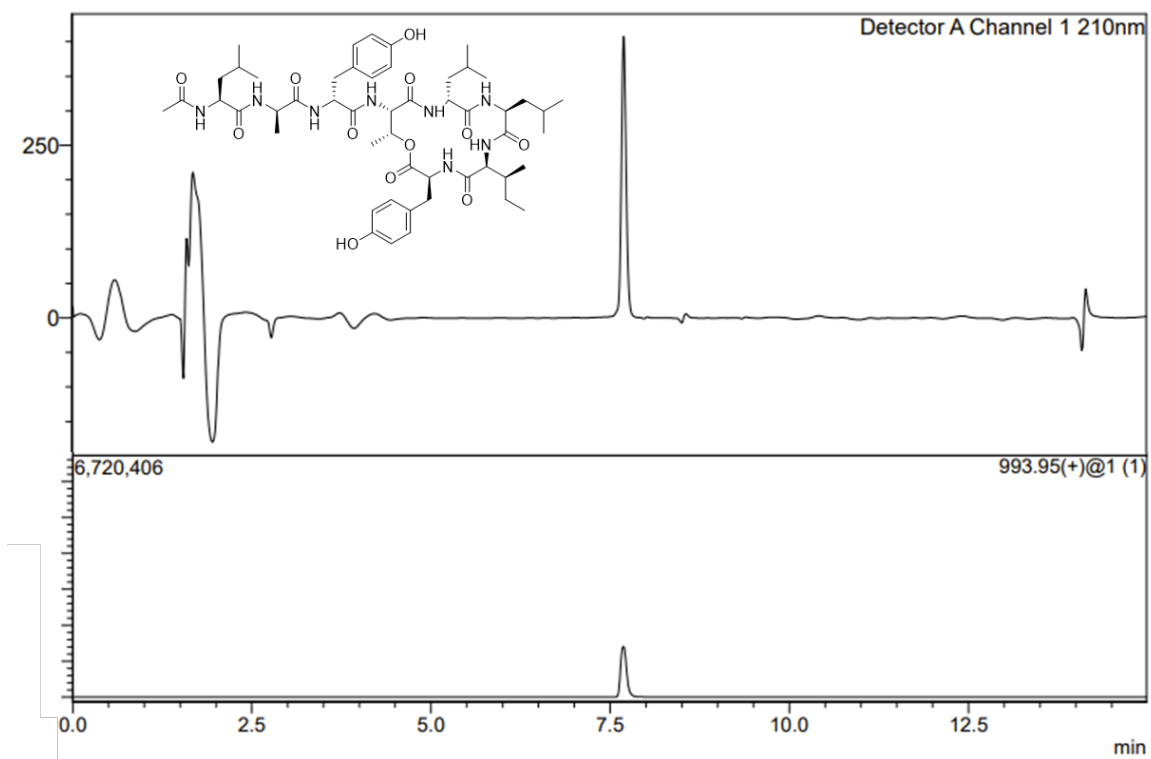


Figure S18. ^1H NMR spectrum of compound **1** in MeOD- D_4 .



R.Time:7.867(Scan#:473)
 MassPeaks:906
 Segment 1 - Event 1
 ?

MS Spectrum

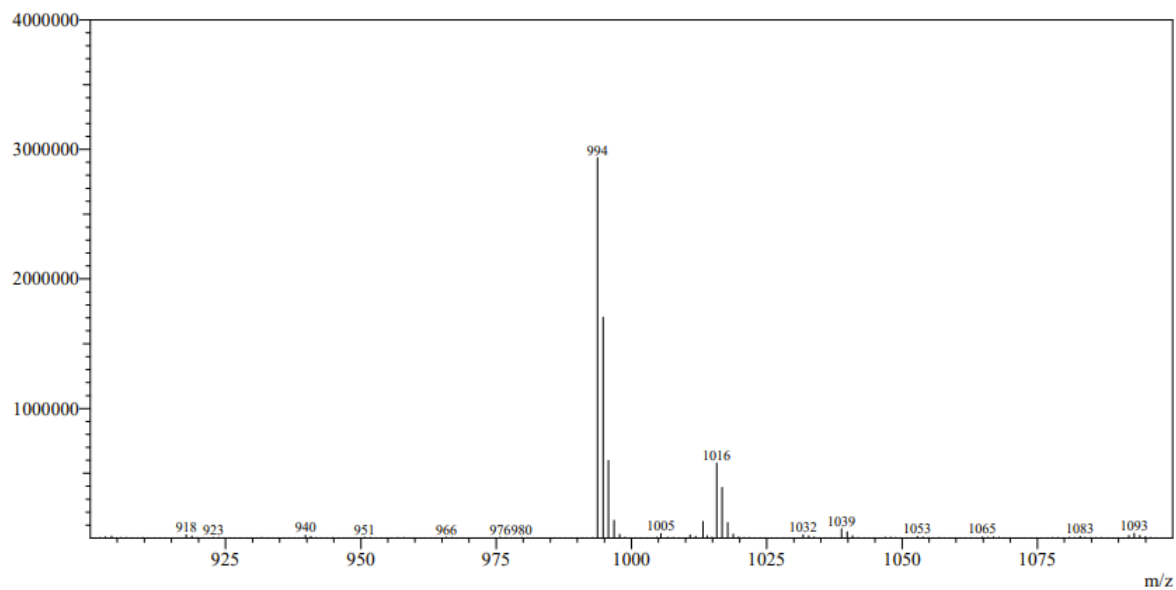


Figure S19. LCMS trace of compound **3** including 210 nm UV trace, extracted ion chromatogram and M+1 and M+Na mass spectrum.

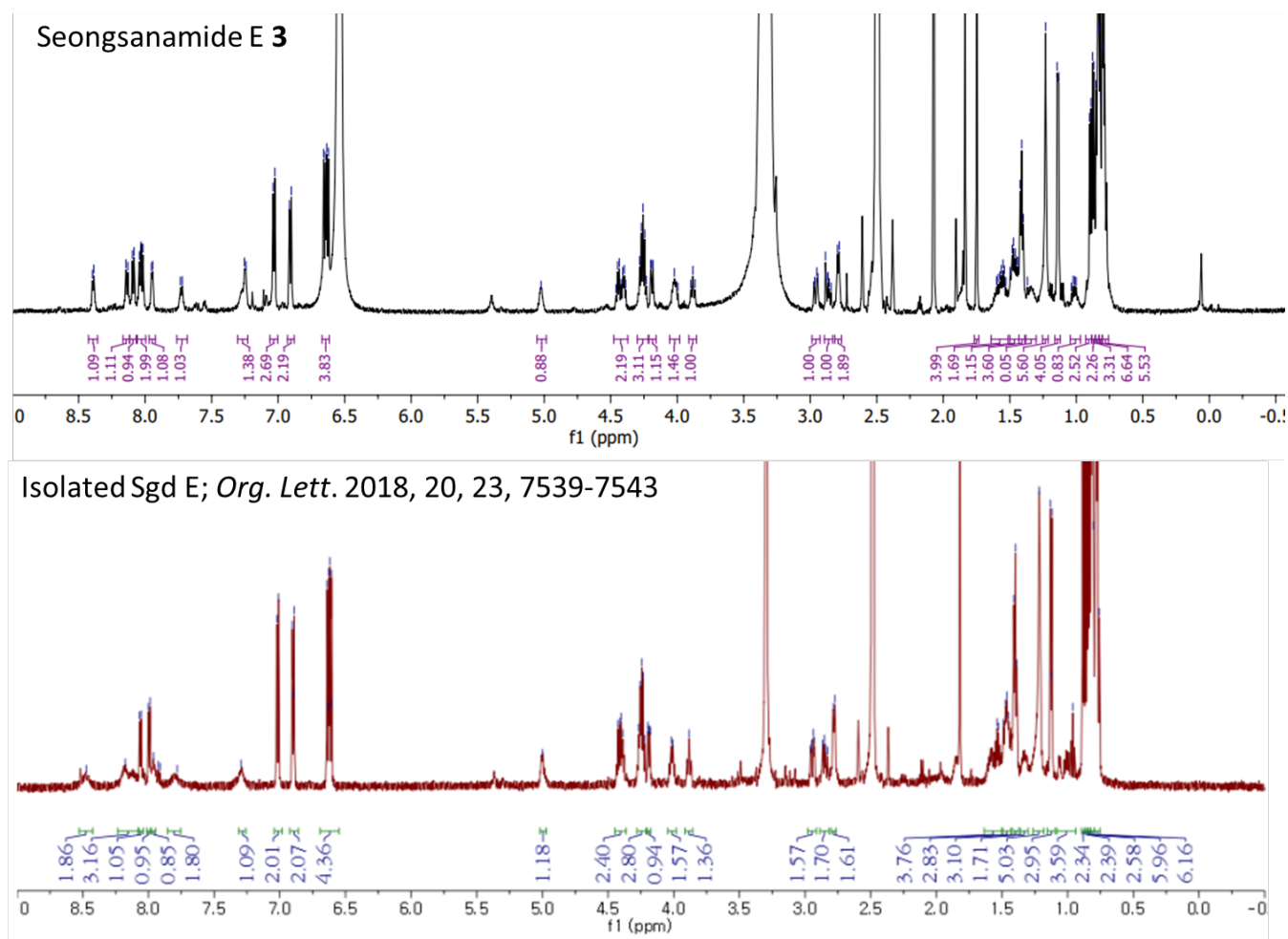


Figure S21. ^1H NMR comparison of Seongsanamide E with isolated material².

References

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Chapter 6: Synthetic efforts towards a fusarinine C di-domain inhibitor

6.1 Introduction

6.1.1 Brief introduction to siderophore biosynthesis and function

Siderophores make up an important proportion of nonribosomally synthesized peptides (NRPs) and have essential natural roles involved in iron chelation, storage and transport between intra- and extracellular environments. Several siderophores such as fusarinine C, **6.1**, triacetylfusarinine C, **6.2**, enterobactin, **6.3** and vacidobactin A¹, **6.4** are cyclic depsipeptides with the presence of at least one ester bond (**Figure 6.1**). Siderophores chelate ferric (Fe^{3+}) iron forms once exported into the cytosol. Once complexed, the siderophores are imported into the cell through different types of transport mechanism² where the ferric form is reduced to its ferrous form (Fe^{2+}) by an iron reductase enzyme to then be utilized in a range of processes such as electron transport, amino acid biosynthesis, glycolysis, and many more^{3,4}. There are four main classes of siderophores categorized by their chelating functional groups, including hydroxamate, catecholate, polycarboxylate and mixed types. Several of these compounds are cyclic to help with the thermodynamically favored hexadentate iron coordination⁵. These compounds are biosynthesized by the organism in times of iron-deficiency by two main pathways, nonribosomal peptide synthetases (NRPS) or NRPS-independent siderophore synthetases (NIS)⁶.

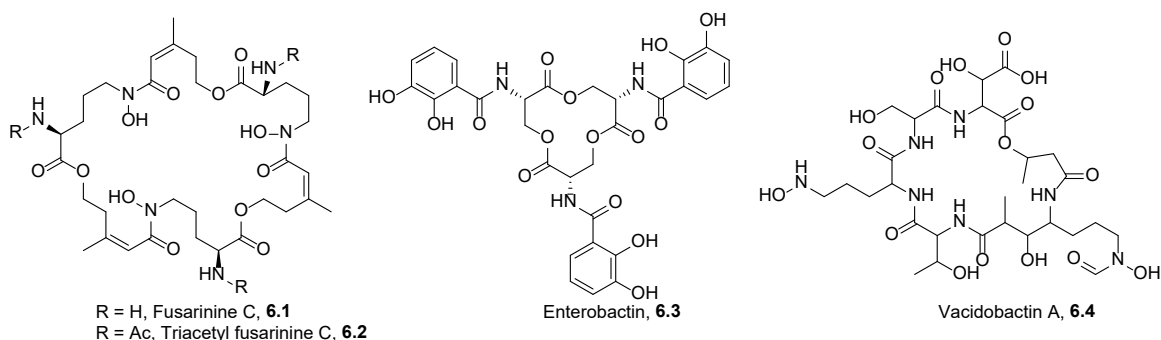


Figure 6.1 Examples of NRPS siderophores

Fusarinine C, **6.1**, triacetyl fusarinine C, **6.2**, enterobactin, **6.3**, and vacidobactin A, **6.4**

Due to their interesting scavenging roles and involvement in the virulence of pathogenic organisms, they have been subject to research spanning the discovery of novel siderophores¹, the investigation of their biosynthesis, and their chemical synthesis.

6.1.2 Fusarinine C function, biosynthesis and production in *Aspergillus fumigatus*

Aspergillus fumigatus is a saprophyte found in soil and has become the most common airborne pathogenic fungus, which can cause disease in immune-compromised patients⁷. This mold utilizes several iron uptake systems, notably siderophore-assisted mobilization of iron. The production and excretion of fusarinine C (**6.1**, FsC) and triacetyl fusarinine C (**6.2**, TAFC) is essential for iron chelation and the siderophore-iron complex can be utilized by reductive iron assimilation after being taken up by transporters⁸. The organism also uses two other siderophores for intracellular transport such as ferrichrocin for hyphal iron storage and hydroxyferricrocin for conidial iron storage³. Iron levels are essential for *A. fumigatus* virulence in different host animals or other fungal species. Siderophore transport has only been recently characterized in *A. fumigatus*, where from seven potential siderophore-iron transporters, five of them are found to be essential.

Interestingly, MirB but not MirC is essential for virulence. MirB mediates TAFC iron uptake, giving TAFC a more dominant role during infection compared to FsC which is transported by MirC⁹.

Structural diversity in siderophores is complex and vast as is common among all natural product classes and is conferred by its biosynthetic machinery. For FsC, TAFC, and several other siderophores, a core amino acid building block required for their biosynthesis is the non-proteogenic amino acid ornithine. Ornithine, **6.6**, is produced via glutamate catabolism pathways, degradation of arginine, **6.5**, or from citrulline production in the mitochondria.

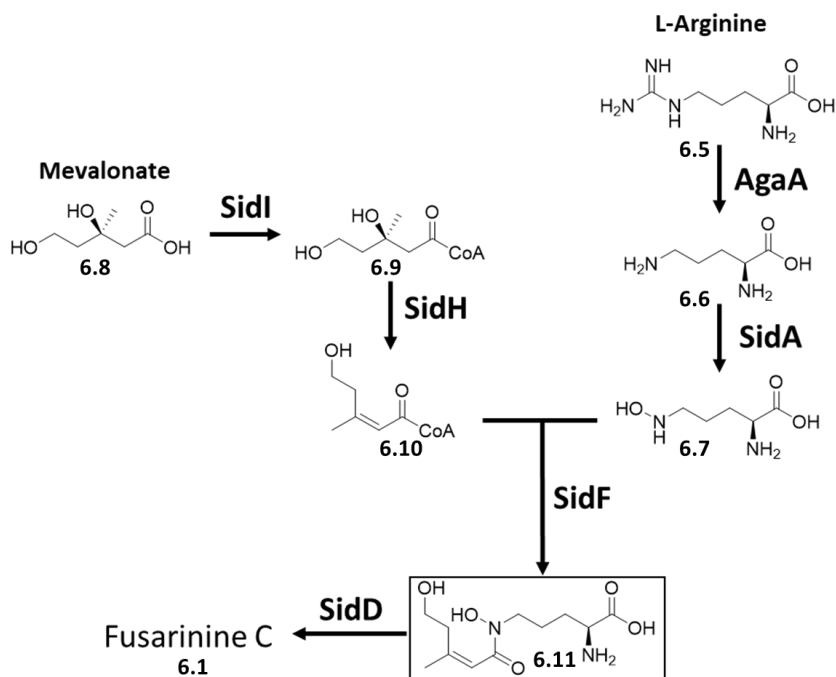


Figure 6.2 Fusarinine C, *cis*-anhydromevalonate and N⁵-hydroxyornithine biosynthesis

In *A. fumigatus*, ornithine is then hydroxylated by L-ornithine-N⁵-monooxygenases SidA in the FsC/TAFC pathways, producing N⁵-hydroxyornithine, **6.7** (**Figure 6.2**). N⁵-hydroxyornithine **6.7** can undergo several types of modifications in other biosynthetic pathways that will not be covered here such as acetylation via acetyl-CoA and N⁵-transacetylases SidL¹⁰ in

ferricrocin biosynthesis and formylation by L -N⁵-hydroxyornithine-formyltransferase (CchA) in coelichelin biosynthesis¹¹. In fusarinine C biosynthesis, N⁵-hydroxyornithine is acylated with anhydromevalonate (**Figure 6.2**). Several siderophores are modified with *E*-anhydromevalonate commonly known as *trans*-anhydromevalonate **6.10**, generated via activation with CoA followed by dehydration of mevalonate **6.8**. FsC and TAFC utilizes SidI for CoA activation forming **6.9** followed by SidH mediated dehydration, yielding *Z*-anhydromevalonate, commonly known as *cis*-anhydromevalonate **6.10** (**Figure 6.2**). Fsc, TAFC and ferrirhodin, **6.17**, (**Figure 6.3**) are the only known siderophores which incorporate the *Z*-configured building block. The incorporation of *trans*-anhydromevalonate is found in dimerumic acid, **6.12**, coprogen, **6.13**, neocoprogen, **6.14**, isoneocoprogen, **6.15**, and B-dimethyl coprogen, **6.16** (**Figure 6.3**). N⁵-hydroxyornithine and activated anhydromevalonate units are coupled via transacylase SidF to give the *cis*-anhydromevalonate N⁵-hydroxyornithine monomer (*cis*-AMHO, **6.11**)¹², (**Figure 6.2**).

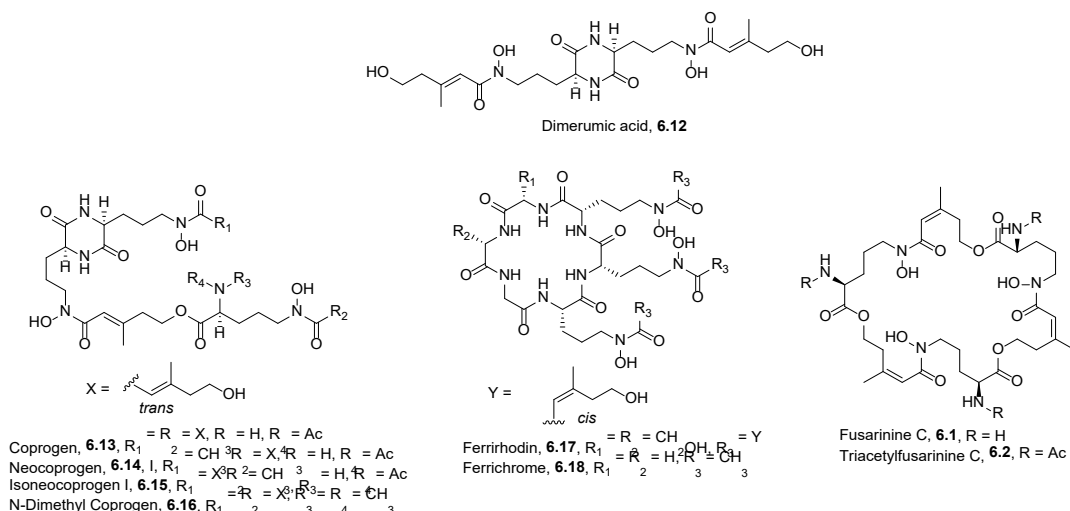


Figure 6.3 Anhydromevalonate and N⁵-hydroxyornithine containing siderophores

The assembly of siderophores by fungal nonribosomal peptide synthetases (NRPSs) has been largely uncharacterized until recently when Hai *et al.* characterized the fusarinine C NRPS, SidD¹³. This bimodular NRPS is responsible for building the extracellular siderophore in an

iterative mechanism. The iterative adenylation (A) and condensation (C) domain load and condense a large amino acid building block, *cis*-AMHO, **6.11**, until a linear trimer forms that is cyclized and released by the chain-releasing (C_T) domain (**Figure 6.4**). The utilization of the A domain to add repeat units of the monomer is a notable characteristic of fungal siderophore-producing NRPS. A degenerate A domain (dA) is predicted to be catalytically inactive in SidD, likely vestiges of evolution playing a structural modular assembly role¹³.

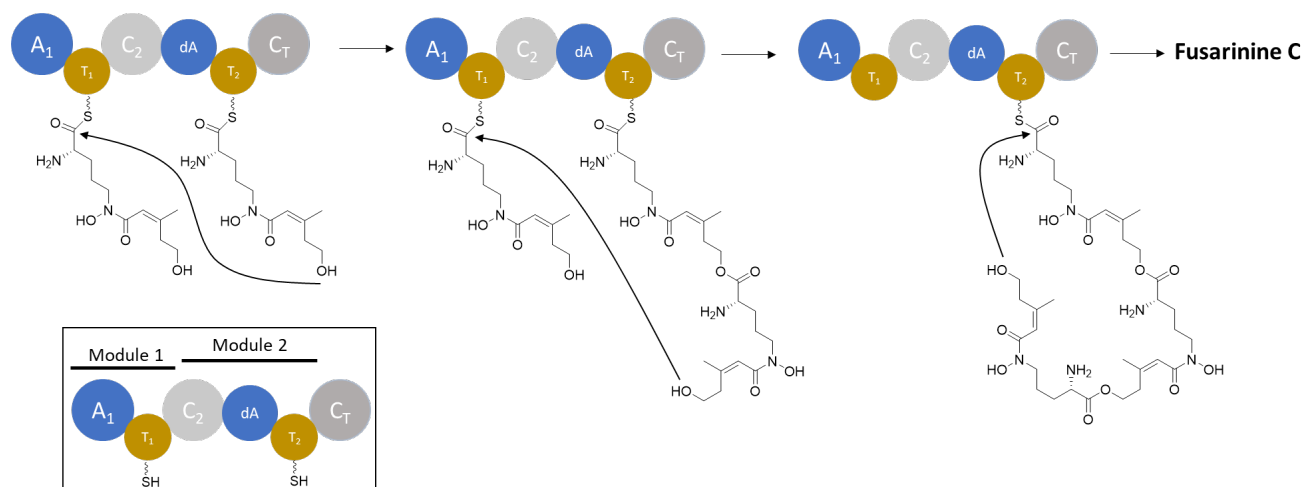


Figure 6.4 Fusarinine C assembly with NRPS SidD

6.1.3 Fusarinine C synthetic background

Siderophores have been interesting synthetic targets for the last several decades since these natural products possess interesting biological applications. Our collaborative research project with the Schmeing Lab at McGill University required the synthesis of a fusarinine C analogue to investigate its biosynthesis. Fusarinine C and TAFC have not previously been total synthesized, making this a challenging synthetic project. To add to the challenge, the synthesis of N-hydroxy amides can be notoriously challenging and the acylation of the N⁵-hydroxyornithine with the *cis*-anhydromevalonate portion has never been reported.

Several acetylated hydroxamate based natural products have been synthesized previously using classical *O*-benzoyl and benzyl protection strategies with simple acylation with acetyl chloride¹⁴. This subject is covered in several reviews, notably by Marvin Miller who investigated the synthesis of a number of siderophores starting in the 1980's^{15,16}. There are very few examples of unsaturated N-acylated natural product syntheses. as the most notable example is nannochelin A, **6.19**,^{17,18} which is a N⁶-hydroxylysine containing product natural product with an *N*-trans cinnamic amide (**Figure 6.5**).

To date, there have been no reported syntheses of fusarinine C. Substrates for characterizing SidD and FsC biosynthesis were generated through strategies involving direct modification of the natural product by gentle hydrolysis¹³ of the cyclic product to access the monomer¹³. Interestingly, the only anhydromevalonate containing natural product to have been synthesized is dimerumic acid, **6.12**, in 1974 by Widmer *et al.* which is composed of *trans*-anhydromevalonate and the N⁵-hydroxyornithine diketopiperazine¹⁹ (**Figure 6.2**). In 2010, Bertrand *et al.* synthesized a protected *trans*-AMHO monomer **6.20** in low final coupling yields (4%)²⁰, with a benzoyl protected hydroxy ornithine and an acyl chloride *trans*-anhydromevalonate (**Figure 6.5**), present in dimerumic acid and copragen B, both produced by pathogenic fungus *Scedosporium apiospermum*.

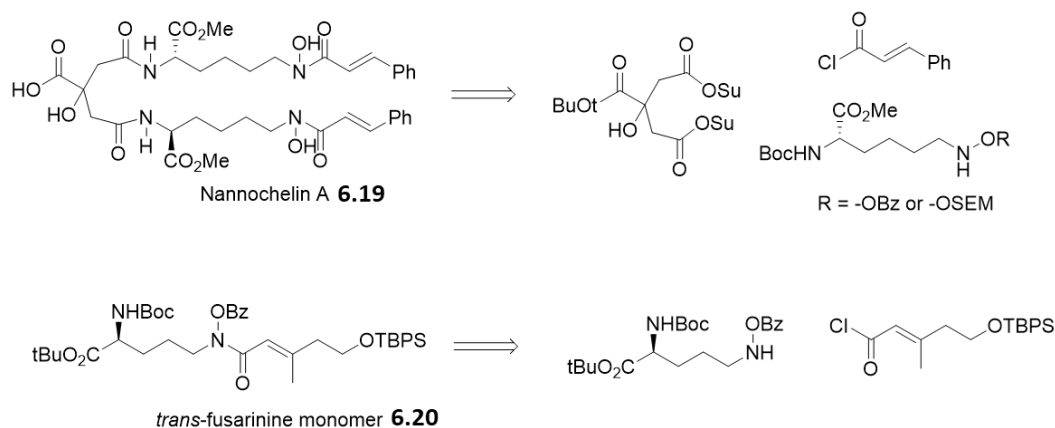


Figure 6.5 Retrosynthetic analysis of the synthesis of nannochelin A and *trans*-fusarinine monomer

FsC *N*-hydroxy amide is particularly challenging due to the presence of the *cis*-anhydromevalonate where the choice of *O*-protected hydroxylamine protecting group is crucial, seen by Bertrand *et al.*²⁰ low coupling yield. The monomer will be sensitive to the deprotection method, where the *cis*-anhydromevalonyl moiety of FsC is sensitive to acidic conditions, possibly elimination mevalonyl lactone (**Figure 6.12**)²¹. Similarly, the synthesis of the *cis*-anhydromevalonate could possibly interconvert under hydrolysis conditions or generate the lactone product under activation chemistry. The prevailing method to access these are through coupling approaches between an *O*-protected hydroxylamine hydrochloride salt and a carboxylic acid where upon activation *via* acyl chloride or coupling agent can yield the desired product.

The fusarinine C synthetic challenges lie in the synthesis of a *cis*-anhydromevalonate building block, the formation of the hydroxamic amide, and the stability of the monomer under basic and acidic reaction conditions during coupling and deprotection steps.

6.1.4 A domain vinyl sulfonamide inhibitors

The interest in the iterative SidD A domain from the biosynthesis of fusarinine C focuses on characterizing the mechanism for interaction of the thiolation (T) domain and the A domain and the subsequent acylation of the T domain. T domains or NRPS peptidyl carrier proteins (PCP) form thioesters with the terminal thiol of the phosphopantetheine (Ppant) prosthetic group, which is covalently linked to the side chain of a Ser residue in the T domain. The incoming activated ester for acylation of the phosphopantetheinyl group is the AMP-ester of the building block, which is generated by the A domain from the free acid of the building block and ATP (**Figure 6.6A**).²² It has been shown that NRPS A domains undergo large conformational changes, with motifs rotating 140° between the adenylation stage and the thioesterification stage^{23,24}. Due to the flexibility of the A and T domains relative to each other, characterizing their interaction with high resolution structural data has been particularly challenging.

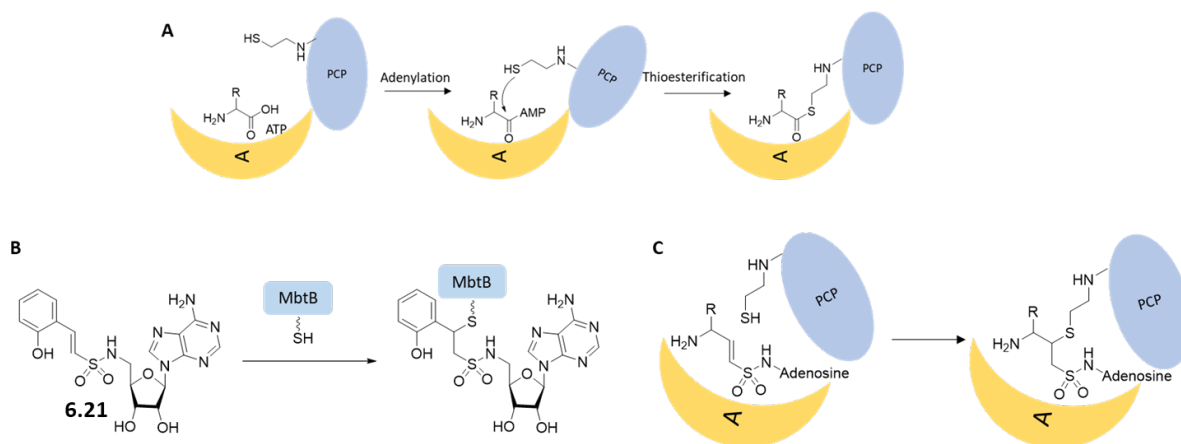


Figure 6.6 Vinyl sulfonamides as di-domain inhibitors

A. A domain adenylation and thioesterification activity. B. The first vinyl sulfonamide inhibitor modified by the thiolation domain. C. Covalent mechanism of thiol Micheal acceptor addition

One solution to high resolution structural characterization of the A-T interaction has been to trap the thioesterification step with a mimic of the activated AMP ester. The A domain has high

affinity for the covalently linked adenosine group to the T domain, which will in turn trap the A-T domains together in their native thioesterification conformation (**Figure 6.6C**). Qiao *et al.* designed and synthesized the first mechanism-based T inhibitor, **6.21**, which when bound to the A domain, is attacked by the Ppant terminal thiol of protein-domain, generating a thioether linkage to the T domain which is observable via MS/MS²⁵ (**Figure 6.6B**). Later, Gulick's group used similar inhibitors to capture the crystal structure of an A-T di-domain from the fusion protein EntE-B, revealing intermolecular interactions of the adenylation domain during thioesterification²⁶. Similarly, a crystal structure was obtained of a trapped di-domain of the natural two-domain protein PA1221, showing another large adenylation domain change in conformation²⁷.

These studies utilized inhibitors to trap the Ppant thiol to lock that transient conformation long enough for structure elucidation (**Figure 6.6C**) by mass spectrometry or high resolution structural methods. These inhibitors contained an adenosine vinyl sulfonamide linked to the side chain of the native building block, mimicking the native AMP-ester intermediate. The Ppant thiol covalently attacks the vinyl sulfonamide Michael acceptor, which is located approximately in the same place as the activates ester carbonyl would be in the native intermediate. These compounds are thus powerful trapping agents for bringing A and T domains together. Our collaborators at McGill University required a fusarinine C monomer adenosine vinyl sulfonamide inhibitor to capture the structural conformation of the SidD A and protein domain interaction (**Figure 6.7**). In this chapter, the synthetic efforts and future prospects towards this substrate will be described.

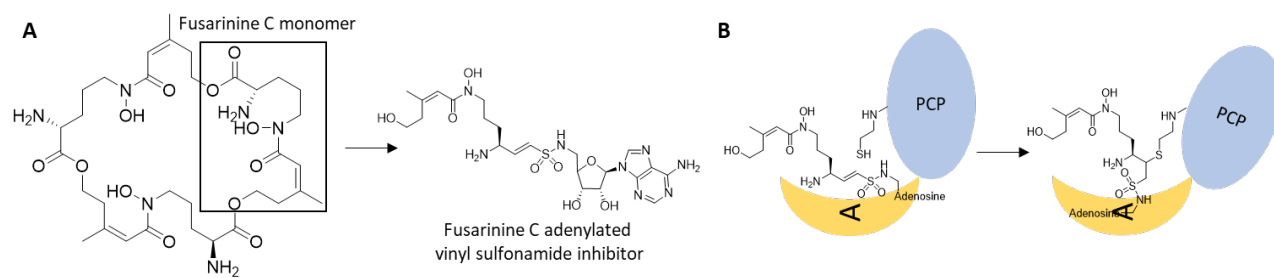


Figure 6.7 Fusarinine C based di-domain inhibitor.

A. Fusarinine C monomer and structure of the adenylated vinyl sulfonamide inhibitor. B. Proposed mechanism of action and the formation of a linked di-domain

6.2 Results and discussion

The general retrosynthetic analysis of the penultimate adenosine vinyl sulfonamide **6.28** is described in **Figure 6.8**. A Horner-Wadsworth-Emmons (HWE) olefination with an aldehyde of the monomer **6.26** (**Figure 6.8**) with phosphonate carbanion sulfonamide reagent will provide the vinyl sulfonamide intermediate. A Mitsunobu coupling between protected adenosine **6.29** and the vinyl sulfonamide will provide the protected adenylated vinyl sulfonamide. This was the initial goal of the synthesis; however complications along the way has pushed the synthesis in other directions using compound **6.27** (**Figure 6.9**).

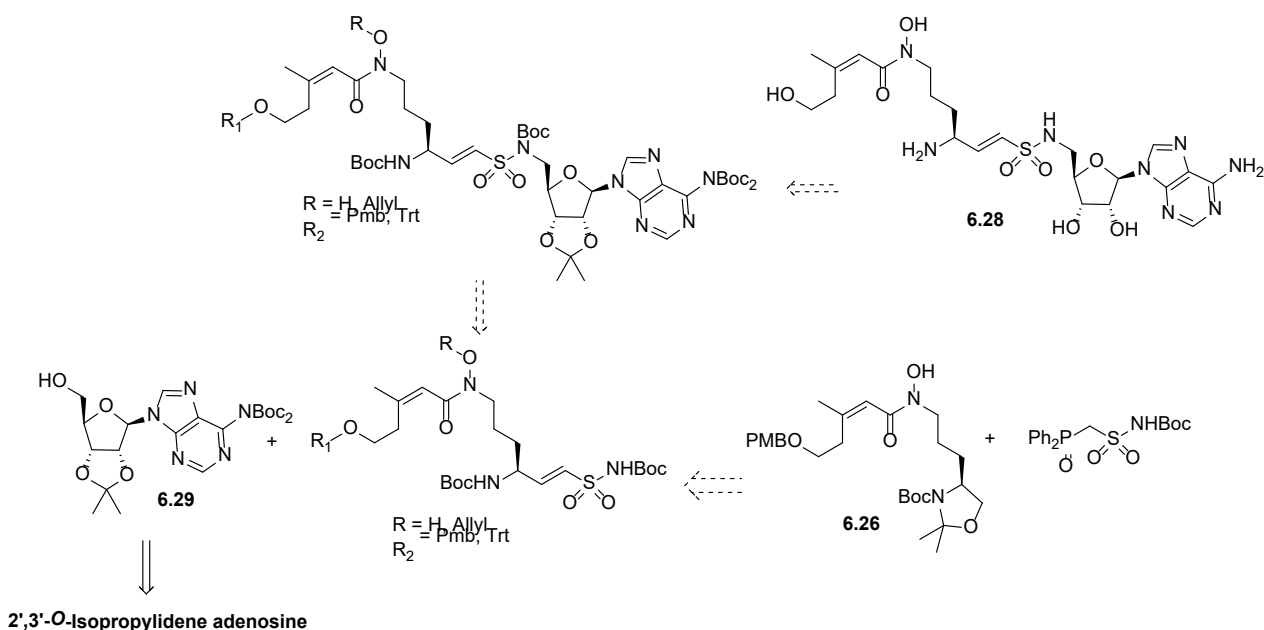


Figure 6.8 General retrosynthetic analysis of a fusarinine C adenylated vinyl sulfonamide inhibitor

The retrosynthetic analysis in **Figure 6.9** provides the general synthetic strategies used to access the protected N⁵-hydroxyornithine **6.24** and **6.25** as well as the fusarinine C monomer aldehyde precursors **6.26** and **6.27** required for vinyl sulfonamide substrate. The protected *cis*-anhydro-mevalonate **6.22** and **6.23** will be synthesized from 3-butyne-1-ol and be used for both monomers. The monomer carboxylate will be synthesized from (*S*)-pyroglutamic acid to access an

orthogonally protected N⁵-hydroxyornithine **6.24** and **6.25**, encountering coupling and deprotection issues. The monomer aldehyde will be synthesized in several ways from glutamic acid to generate **6.27** or protected monomer from **6.26**.

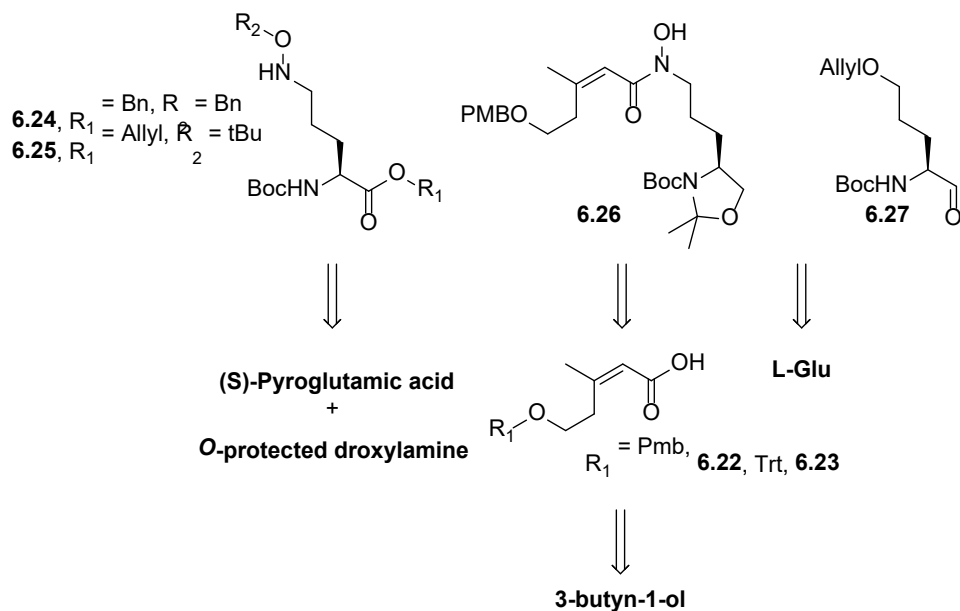


Figure 6.9 Retrosynthetic analysis of fusarinine C amino acid and monomer building blocks

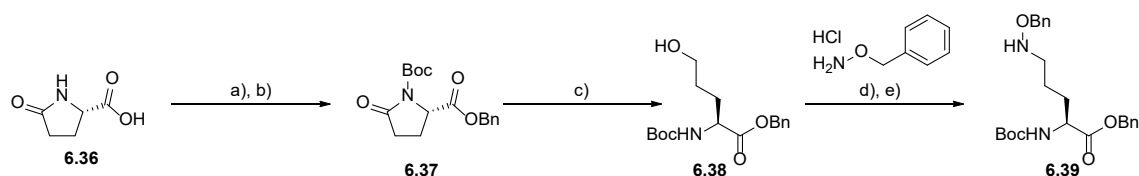
6.2.1 Towards the synthesis of fusarinine C monomer carboxylate

The synthesis of the fusarinine C monomer carboxylate served as a platform for screening conditions to access this siderophore monomer core. Ultimately, an amino aldehyde building block will be discussed, as it will be essential for the synthesis of the vinyl sulfonamide inhibitor.

The N⁵-hydroxy ornithine intermediate required careful attention to the protecting group scheme. After several different path, the best route to access orthogonally protected 5-hydroxynorvaline amino acids is from commercially available (*S*)-pyroglutamic acid in 3 steps.

The carboxylate was benzylated or allylated and the amide nitrogen was Boc protected using DMAP/Boc₂O conditions, generating **6.37** (Scheme 6.1) and **6.41** (Scheme 6.2)

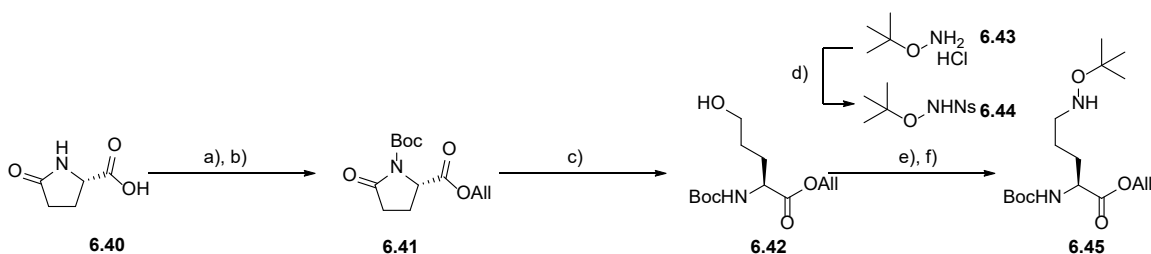
respectively. The protected intermediates were reductively ring opened to the free 5-hydroxynorvaline **6.38** (Scheme 6.1) and **6.42** (Scheme 6.2). Following this, the alcohol **6.38** could be oxidized to the aldehyde under Parikh-Doering oxidation conditions and reductively aminated with protected hydroxylamine in the same pot²⁸ to afford **6.39**. The first attempt (Scheme 6.1) utilized *O*-benzyloxyamine due to its overwhelming utilization in hydroxamic acid synthesis. However this protecting group strategy is not compatible with the α,β -unsaturated hydroxamic amide of the desired monomer, since its traditional removal requires hydrogenation conditions likely to reduce the double bond as well.



Scheme 6.1 Conditions for the synthesis of **6.39**.

a) Benzyl alcohol, SOCl_2 , 0°C to rt, 18 h; b) TEA, DMAP, Boc_2O , DCM, 0°C to rt, 18 h; c) NaBH_4 , $\text{THF}:\text{H}_2\text{O}$, 0°C to rt, 2 h; d) $\text{SO}_3\cdot\text{Pyr}$ complex, DIEA, DMSO, DCM, -15°C to rt, 2.5 h; e) NaCNBH_3 , HCl, EtOH, rt, 1.5 h, 76% yield

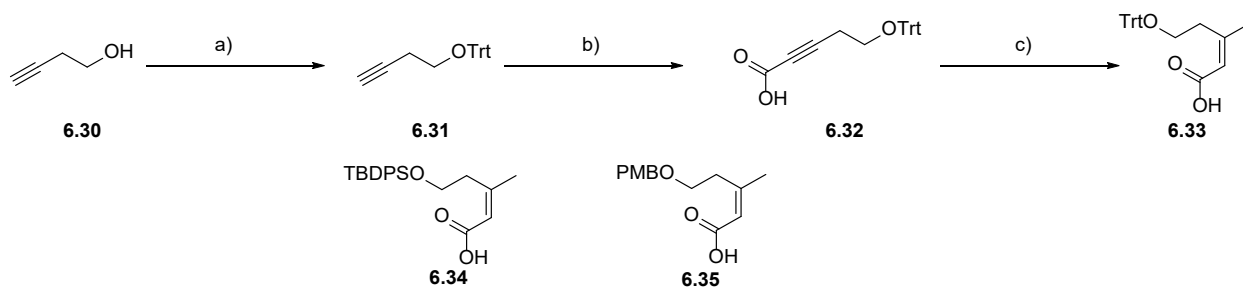
The successful synthesis of the benzyl protected hydroxylamine derivative (Scheme 6.1), provided a route to access various *O*-protected hydroxylamines. These included the *tert*-butyl group as it could be easily removed under mild acidic conditions. Unfortunately using *tert*-butyl protected hydroxylamine **6.43** in a synthetic route like Scheme 6.1 led to poor reductive amination yields. To overcome this problem, the nosylated *O*-*t*-butyl-hydroxylamine **6.44**, was coupled directly to the alcohol **6.42** under Mitsunobu reaction conditions. This was followed by a thiol-based removal of the nosyl group to generate hydroxylamine **6.45**.



Scheme 6.2 Conditions for the synthesis of **6.45**.

- a) Allyl alcohol, p-TsOH.H₂O, Toluene, reflux, 3 h, 53% yield; b) TEA, DMAP, Boc₂O, DCM, rt, 18 h, 78% yield;
 c) NaBH₄, THF:H₂O, 0°C to rt, 2 h, 34% yield; d) TEA, 2-nosyl-chloride, -5°C to rt, 18 h, 99% yield; e) PPh₃,
 DIAD, THF, 0°C to 40°C, 18 h; f) DBU, Mercaptoethanol, DMF, 3 h, 79% yield over 2 steps

The key protected *cis*-anhydromevalonate **6.33** was synthesized in 3 steps from the commercially available 3-butyn-1-ol. The alcohol was protected with a suitable protecting group, in this case a trityl group, followed by carboxylation of the alkyne. The carboxylate could then undergo a 1,4-addition under Gillman reagent conditions with MeLi and Cu_(I), generating the exclusive *cis*-configured product in moderate yields (**Scheme 6.3**)^{29,30}. This type of reaction is generally performed on ester alkyne³¹, without any examples of methylation of a carboxylic acid alkyne like **6.32**. Several alcohol protecting groups were explored based on their compatibility with future reaction conditions and deprotection strategies, generating *cis*-anhydromevalonates **6.34** and **6.35**.



Scheme 6.3 Conditions for the synthesis of **6.33** and structures of **6.34** and **6.35**

a) Trityl chloride, pyridine, DCM, 0°C to rt, 18 h, 77% yield; b) n-BuLi, CO₂ (s), THF, -78°C to -30°C to rt, 2 h, 85% yield; c) CuI₍₁₎, MeLi, THF, -5°C to -78°C to -15°C, 93% yield

Preliminary coupling attempts between **6.39** and early benzyl protected *cis*-anhydromevalonate led to the first protected fusarinine C monomer **6.46**, with *N*-hydroxy amide formation through coupling approaches as detected via LCMS. However, as predicted, testing hydrogenolysis of the benzyl protecting groups led to the reduction of the α,β -unsaturated hydroxamic amide as well as major degradation products. With the requirement of orthogonal protecting group strategies to access the target molecule, **6.46** was used as a model system. A similar HATU/DIEA couplings was attempted with the *O*-*tert*-butyl protected **6.45** however very low coupling yields were observed, most likely due to the steric effect of the *tert*-butyl group (**Figure 6.10**). These results led to redesigning the synthetic approach.

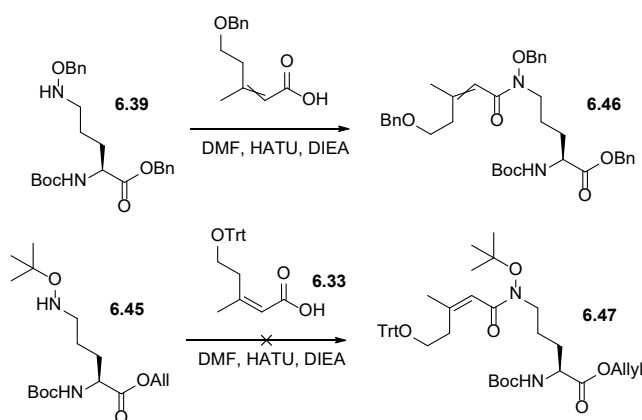


Figure 6.10 Successful model system coupling with benzyl protected derivative **6.39** and failed coupling with *tert*-butyl protected **6.45**

6.2.2 Towards the synthesis of fusarinine C monomer aldehyde

The amino aldehyde monomer was required for the synthesis of the vinyl sulfonamide inhibitors. This protected monomer could be generated using a series of alternative building blocks based on to the carboxylate monomer of fusarinine C.

A new coupling scheme was established to circumvent coupling issues seen with the carboxylate monomer and to utilize different protecting group schemes. An oxidation of the alcohol of a glutamic acid derivative should permit access to the desired amino aldehyde building block **6.57** (Figure 6.11). A key 5-hydroxynorvaline primary alcohol derivative **6.52** can be synthesized from glutamic acid **6.50**. **6.52** will allow access to monomer aldehydes through a Mitsunobu reaction, similar to the preparation of **6.45** with protected *cis*-anhydromevalonyl hydroxamic amide **6.53**.

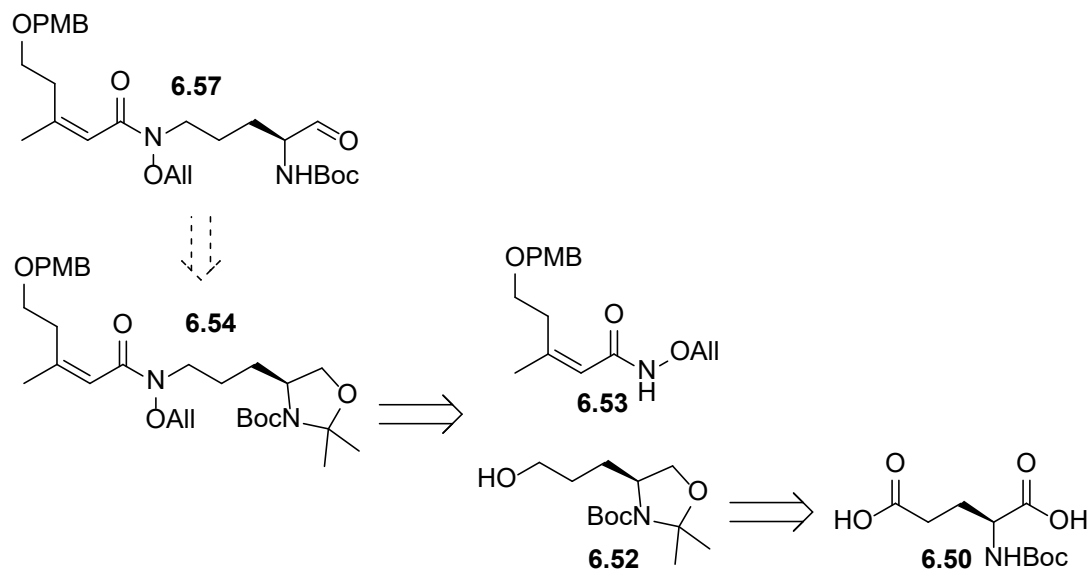
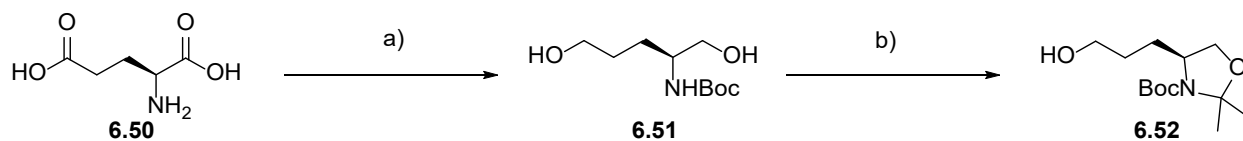


Figure 6.11 Amino aldehyde building block retrosynthesis

The amino aldehyde building block synthesis could be accomplished from glutamic acid **6.50** in 4 steps, with di-methyl esterification, Boc protection and ester reduction in one pot, affording protected (2*S*)-aminopentan-1,5-diol **6.51** in good yields. From there, the δ alcohol could

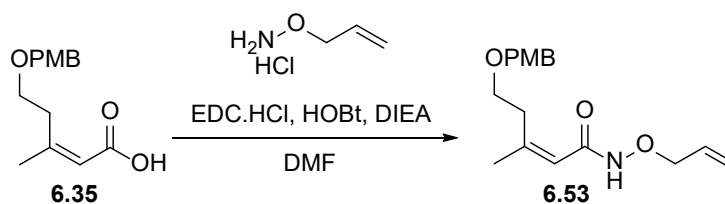
be selectively accessed with an oxazolidine protection between the NH carbamate and the alcohol to obtain the Boc protected oxazolidine **6.52** as a mixture of rotamers³² (**Scheme 6.4**).



Scheme 6.4 Conditions for the synthesis of **6.52**

a) TMSCl, MeOH, 0°C to rt, 18 h; TEA, Boc₂O, rt, 24 h; NaBH₄, THF, H₂O, 0°C to rt, 24 h, 61% yield; b) 2,2-dimethoxypropane, p-TsOH.H₂O, DCM, rt, 1.5 h, 69% yield

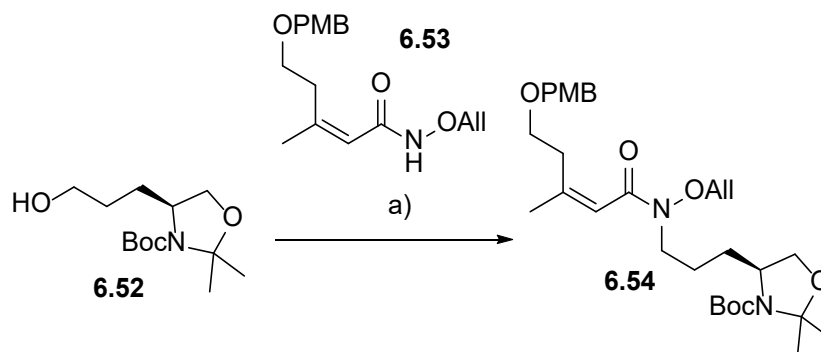
The protected *cis*-anhydromevalonyl hydroxamic amide **6.53** was synthesized in 1 step from **6.35** following a protected hydroxyl amine standard coupling, giving the required nucleophile for the Mitsunobu reaction.



Scheme 6.5 Conditions for the synthesis of **6.53**

a) EDC, HOBT, DIEA, DMF, rt, 18 h, 41% yield

With both hydroxamic amide **6.53** and alcohol **6.52** in hand, coupling was achieved using Mitsunobu conditions using DIAD and PPh₃ affording **6.54** (**Scheme 6.6**). This approach has been the most efficient for the generation of the protected hydroxamic acids and is the coupling method of choice to build these monomers. Product separation from the DIAD byproduct however can sometimes be a problem depending on the choice of protecting groups, such as was the case with the synthesis of **6.54**.



Scheme 6.6 Conditions for the synthesis of **6.54**

a) PPh_3 , DIAD, THF, 0°C to rt, 18 h

A general problem involving the fusarinine C monomer is its acid sensitivity and formation of lactone by product. This has been previously observed in fusarinine C¹³. In fact it was the original method to confirm the *Z* conformation of the anhydromevalonate²¹. We anticipated the protection of the terminal alcohol would avoid this issue entirely; however degradation was often observed, especially when the hydroxamic amide was protected (**Figure 6.12**).

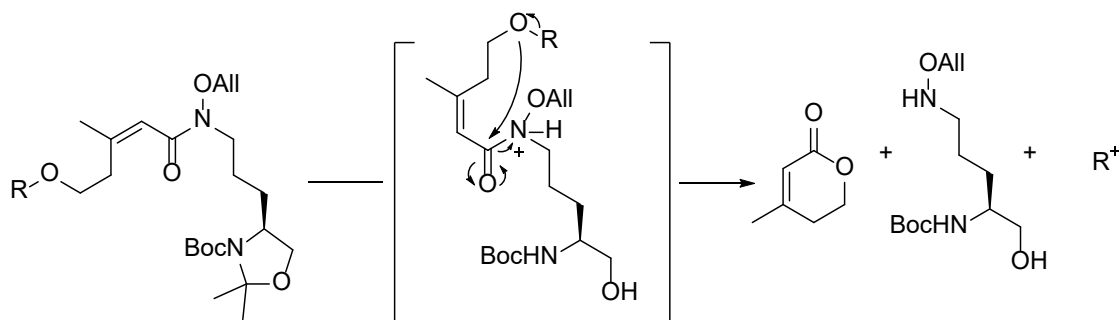
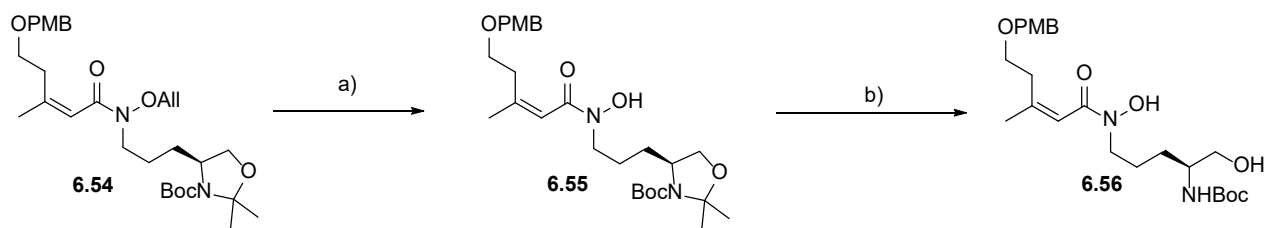


Figure 6.12 Acidolysis degradation of monomer to mevalonyl lactone and hydroxy ornithine derivative

Several protected monomers were synthesized in attempts to circumvent the product degradation following acidolysis, hydrogenation, or hydrolysis. The most promising route utilized compound **6.54**, where the most suitable order of attack started with the allyl deprotection affording **6.55**, allowing for mild acidolysis of the oxazolidinone with minimal elimination product,

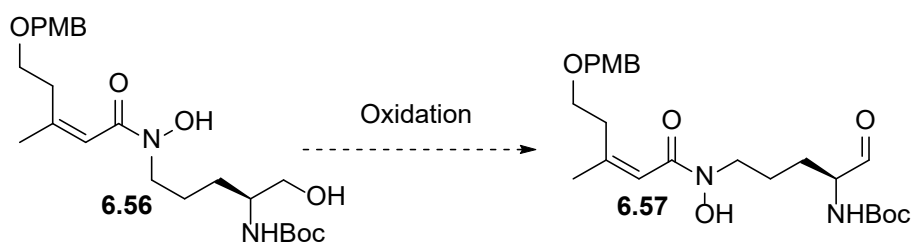
however this two-step scheme led to very low yield of **6.56** and formation of **6.56** was only confirmed by LCMS analysis (**Scheme 6.7**).



Scheme 6.7 Conditions for the synthesis of **6.56**

a) Pd(OAc)₂, PPh₃, formic acid, triethylamine, ACN:H₂O, 50°C to rt, 3 h; b) p-TsOH.H₂O, MeOH, rt, 30 min

With alcohol **6.56** in hand, the first synthetic route to the adenylated vinyl sulfonamide monomer was anticipated through an olefination, using aldehyde **6.57**. This aldehyde proved to be challenging due to limited access of compound **6.56** for condition screening and to the unsuccessful oxidation attempts, including Parikh-Doering and Dess-Martin periodinane oxidation. Due to the limited success, an alternate synthetic route was attempted.



Scheme 6.8 Fusarinine C monomer β -alcohol oxidation attempts

6.2.3 Towards the synthesis of adenylated vinyl sulfonamide inhibitors

With the challenge using the full fusarinine C monomer including the lactone byproduct formation (**Figure 6.12**) and the failed aldehyde formation (**Scheme 6.8**), an alternative and promising route was attempted. This involved the synthesis of allyl protected aldehyde **6.27** and

the synthesis of the protected 5-hydroxynorvaline adenylated vinyl sulfonamide **6.59**. This product could be synthesized from glutamic acid to generate the amino aldehyde **6.27**, which can be olefinated to afford **6.58** and adenylated with **6.29** to synthesize adenylated vinyl sulfonamide **6.59** (Figure 6.13).

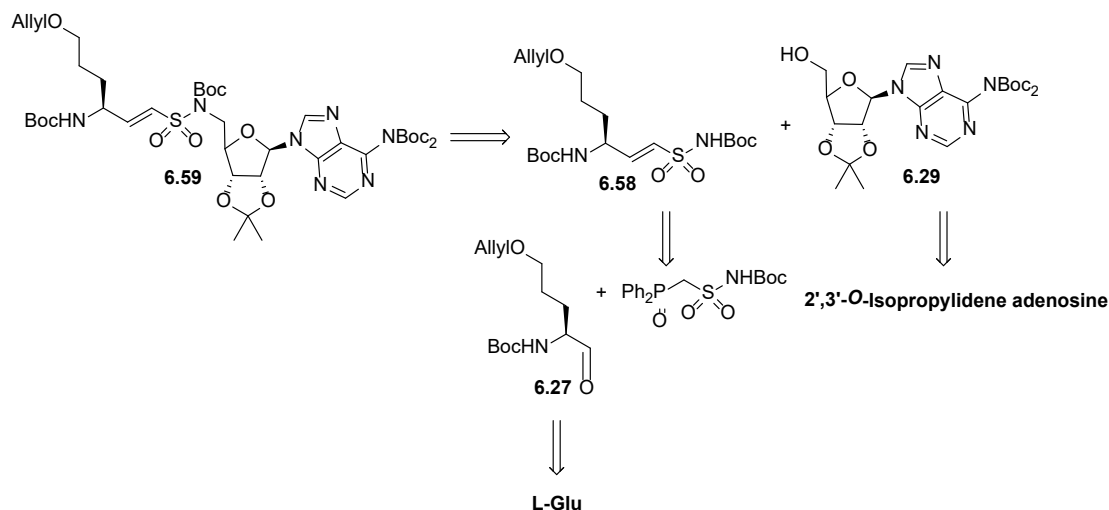
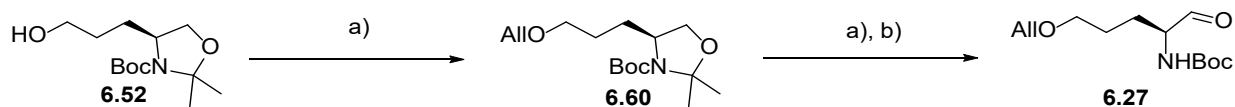


Figure 6.13 Adenylated vinyl sulfonamide inhibitor retrosynthesis

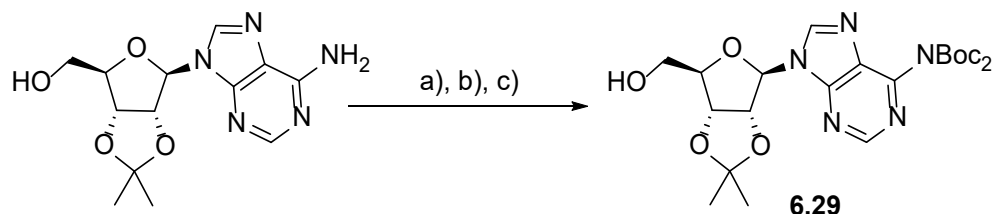
The key alcohol intermediate **6.52**, synthesized in **Scheme 6.4** from glutamic acid, was allylated to afford **6.60** and the oxazolidine was removed under mild acidic conditions followed by the oxidation of the β -alcohol to the aldehyde under Parikh-Doering conditions to afford compound **6.27** (**Scheme 6.9**).



Scheme 6.9 Conditions for the synthesis of **6.27**

a) NaH, DMF, 0°C to rt, 18 h, 86% yield; b) pTsOH.H₂O, MeOH, rt, 30 min, quant. yield; c) SO₃.Pyr, TEA, DMSO, DCM, -10°C to rt, 2 h, 80% yield

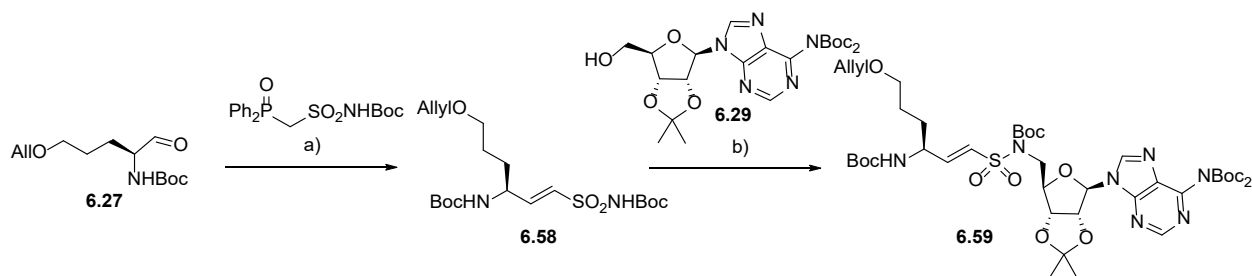
A protected adenosine building block **6.29** was required to adenylate the sulfonamide and was synthesized from commercially available 2',3'-*O*-isopropylidene adenosine in 3 steps.³⁸ The alcohol was protected with a TBS group to allow for the di-Boc protection of the amine followed by TBS deprotection under TBAF conditions, affording compound **6.29** (Scheme 6.10).



Scheme 6.10 Conditions for the synthesis of **6.29**

a) TBS chloride, Imidazole, DMF, 0°C to rt, 18 h; b) Boc₂O, DMAP, DCM, 0°C to rt, 18 h; c) TBAF, THF, rt, 18 h, 95% yield

With aldehyde **6.27** and protected adenosine **6.29** in hand, the adenylated vinyl sulfonamide was synthesized with commercially available Boc-sulfonamide phosphonate in 2 steps (Scheme 6.11). The aldehyde **6.27** underwent a Horner-Wadsworth-Emmons olefination³³ to afford vinyl sulfonamide **6.58** followed by a Mitsunobu coupling with protected adenosine **6.29**, providing **6.59**.



Scheme 6.11 Conditions for the synthesis of **6.59**

a) LiHMDS, DMF, THF, -78°C to rt, 68% yield; b) PPh₃, DIAD, THF, 0°C to rt

Unfortunately, attempts to deprotect the allyl group for subsequent Mitsunobu coupling with protected *cis*-anhydromevalonyl hydroxamic amide **6.61** were unsuccessful (**Figure 6.14**), furnishing only a mono-Boc deprotection product, confirmed by LCMS analysis. Limited material made optimization a challenge.

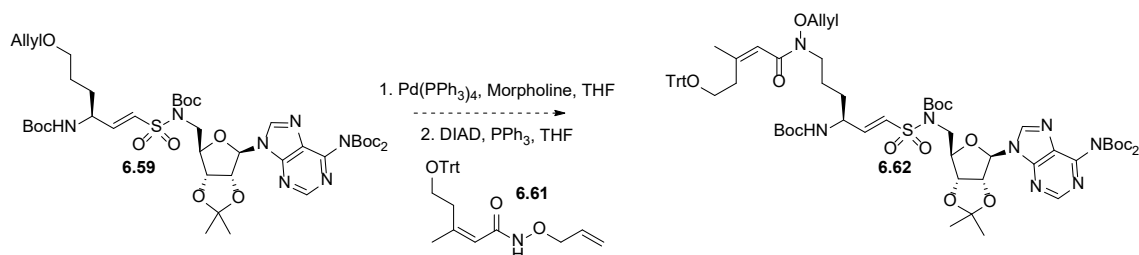
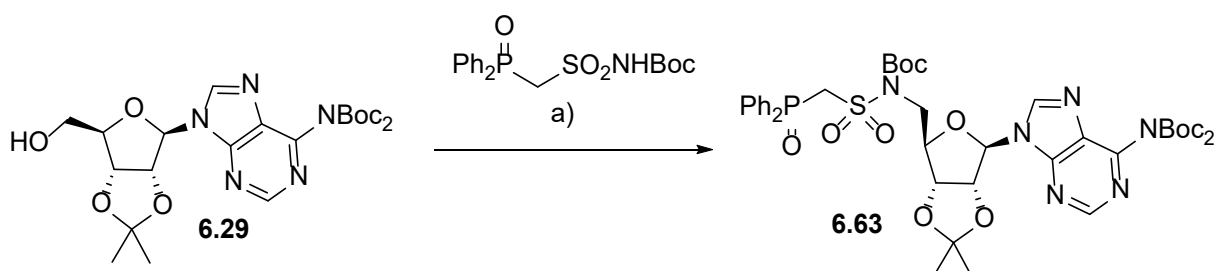


Figure 6.14 Proposed synthesis of fusarinine C monomer on the protected vinyl sulfonamide

With future reaction optimization in mind, simplification of the vinyl sulfonamide synthesis is an important aspect to consider. The synthesis of adenosine sulfonamide **6.63** could help the synthesis by limiting the coupling steps on the sensitive vinyl sulfonamide products (**Figure 6.14**) and could be applied to sensitive aldehydes such as the fusarinine C monomer aldehyde **6.57** (**Scheme 6.8**). The phosphonate could be generated in 1 step through a Mitsunobu coupling between the sulfonamide and the protected adenosine **6.29** (**Scheme 6.12**).



Scheme 6.12 Conditions for the synthesis of **6.63**

a) PPh_3 , DIAD, THF, 0°C to rt

6.3 Summary and outlook

A separate vinyl sulfonamide substrate with an ornithine monomer will also be required as an inhibitor and can act as a model system for the more complex inhibitor. Due to the potential 6-membered ring formation between the aldehyde and the δ -carbamate NH^{34} , a di-Boc amino aldehyde derivative was synthesized in 4 steps. Commercially available HCl salt of ornithine can be first methyl esterified followed by Boc protection in TEA/MeOH conditions then further di-Boc protected under DMAP/DCM conditions. The aldehyde is then obtained following careful DIBAL-H reduction of the methyl ester (**Figure 6.15**).

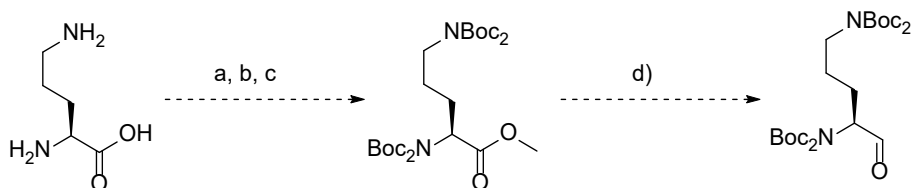


Figure 6.15 Conditions for the synthesis of protected ornithine aldehyde

a) SOCl_2 , MeOH, 0°C to 60°C , 3 h; b) Boc_2O , TEA, MeOH, 0°C to rt, 18 h; c) Boc_2O , DMAP, DCM, 48 h; d) DIBAL-H, DCM, -78°C , 3 h

The olefination with the ornithine aldehyde could be possible with the adenylated sulfonamide phosphonate **6.24** and a global deprotection could afford this simplified inhibitor (**Figure 6.16**).

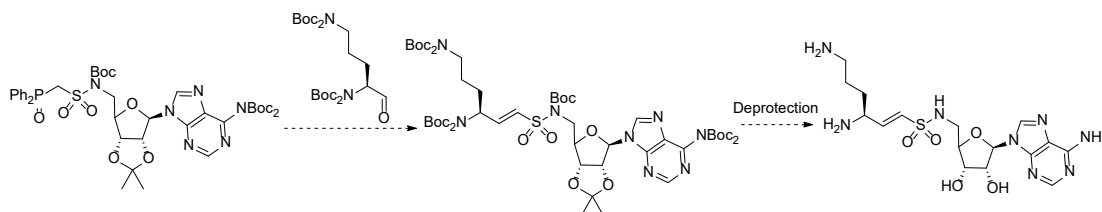


Figure 6.16 Synthesis of ornithine adenylated vinyl sulfonamide

Although the synthetic groundwork is set for a protected *cis*-anhydromevalonate hydroxyornithine, there is still optimization required to access the title adenylated vinyl sulfonamide monomer of fusarinine C. The access of an amino aldehyde building block is key to perform the olefination, which seems to be the limiting step. Optimization of the monomer **6.56** oxidation conditions (**Scheme 6.8**) is a feasible route since the olefination with the adenylated sulfonamide **6.63** (**Scheme 6.13**) might be the key to the protected final product.

With the success of adenylated vinyl sulfonamide **6.59** (**Scheme 6.11**), there is precedence to optimize building the monomer once olefinated; however removal of protecting groups orthogonally can be challenging at these final pivotal steps (**Figure 6.14**). Alternative building blocks could be synthesized with an O-TBS protection which could undergo orthogonal dilute HF mediated deprotection conditions. (**Figure 6.17**)

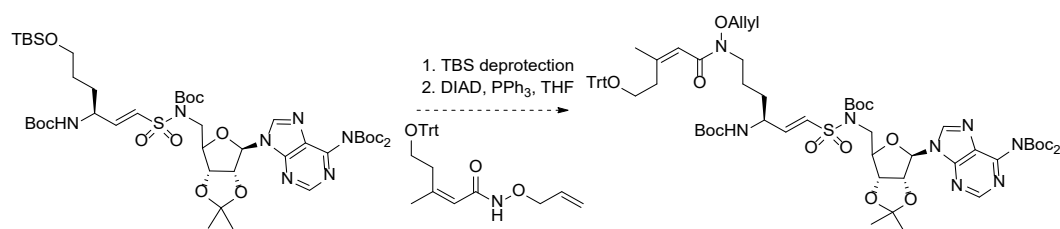


Figure 6.17 Alternative protecting groups to circumvent allyl deprotection

In a final suggestion, olefination with an aldehyde alcohol could circumvent the need of deprotection altogether, allowing for direct Mitsunobu coupling with *cis*-anhydromevalonyl hydroxamic amide **6.53** (**Figure 6.18**).

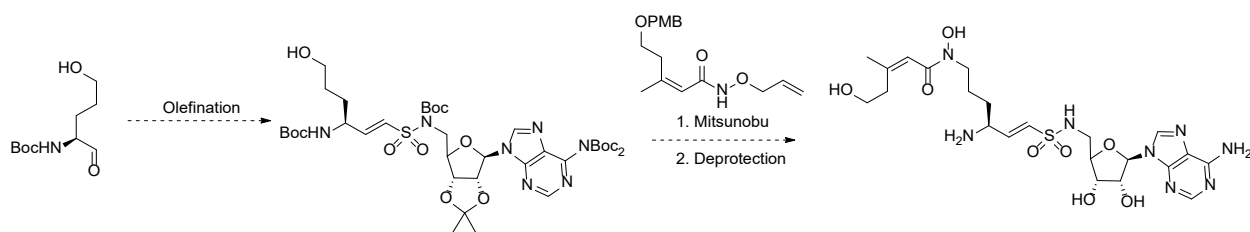


Figure 6.18 Aldehyde alcohol adenylated vinyl sulfonamide synthetic approach

The aldehyde alcohol would be in equilibrium with the lactol product however there is precedence for this type of reaction to eventually yield olefinated product³⁵. The synthesis of the aldehyde alcohol could be accomplished in 5 steps from glutamic acid (**Figure 6.14**)³⁶.

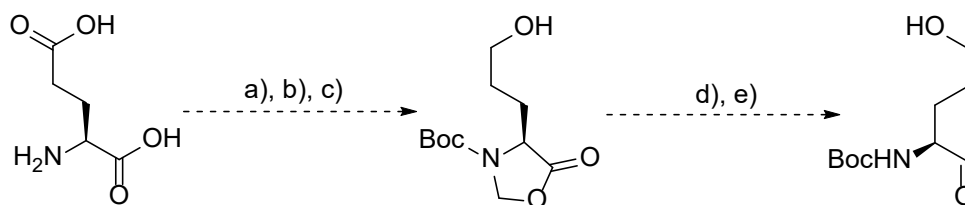


Figure 6.19 Proposed synthesis of an aldehyde alcohol building block

Future efforts should also be focused on developing building blocks capable of also participating in the total synthesis of fusarinine C. This synthetic achievement would be the first synthesis of this siderophore, opening the door for the first total synthesis of triacetyl fusarinine C and ferrirhodin, both *cis*-anhydromevalonate containing natural products.

6.4 Experimental section

All reactions were performed under inert N₂ atmosphere unless otherwise stated.

6.4.1 Synthetic methods and characterization

3-butyn-1-O-Trt (6.31). 3-butyn-1-ol (1g, 12.25 mmol), trityl chloride (4.15 g, 15.00 mmol, 1.2 eq.) and pyridine (1 mL, 12.41 mmol, 1.01 eq.) were dissolved in DCM (35 mL, 0.35 M) and the mixture was cooled to 0°C. Powdered 4Å sieves (10g) were added and the solution was stirred overnight at room temperature. The mixture was filtered and the volatiles were removed under reduced pressure. The residue was purified by column chromatography (20% EtOAc in hexanes) to afford 2.95 g of **6.31** (77% yield) as a white powder. ¹H NMR (400 MHz, CDCl₃) δ 7.56 – 7.52 (m, 6H), 7.38 – 7.27 (m, 9H), 3.30 (t, *J* = 7.0 Hz, 2H), 2.54 (td, *J* = 7.0, 2.7 Hz, 2H), 2.03 (t, *J* = 2.7 Hz, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 144.1, 128.7, 127.9, 127.1, 86.7, 81.8, 69.4, 62.0, 20.1.

3-butyn-1-O-Trt carboxylate (6.32). **6.31** (1.24 g, 3.95 mmol) was dissolved in dry THF (8 mL, 0.5 M) and cooled to -78°C. *n*-BuLi (1.6 M in hexanes, 3.7 mL) was added dropwise to the stirring solution and was maintained at this temperature for 30 mins under N₂ atmosphere. The solution was warmed to -30°C and crushed CO₂ (s) was added portion wise over 5 min and the solution was back purged with N₂ and stirred at this temperature for 30 min. The solution was warmed to room temperature and stirred for 1 h, quenched with sat. sol. NH₄Cl (20 mL), dilute with EtOAc (30 mL) and the two phases were separated. The organic phase was extracted with EtOAc (30 mL x 2) and the combined organics were washed with brine, dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was purified by column chromatography (15%-30% EtOAc in hexanes) to afford 1.2 g of **6.32** (85% yield) as a white solid. ¹H NMR (400 MHz, CDCl₃)

δ 11.09 (s, 1H), 7.55 – 7.46 (m, 6H), 7.38 – 7.26 (m, 9H), 3.32 (t, J = 6.7 Hz, 2H), 2.64 (t, J = 6.6 Hz, 2H). ^{13}C NMR (101 MHz, CDCl_3) δ 158.4, 143.7, 128.7, 128.7, 128.0, 127.2, 90.0, 87.0, 73.5, 60.9, 20.5.

O-Trityl-*cis*-anhydromevalonate (6.33). CuI (I) (159 mg, 0.833 mmol, 2.3 eq.) was added to dry THF (750 μL , 0.46 M) and cooled to -5°C followed by the addition of MeLi, LiBr in ether solution (1.1 mL, 1.5 M, 4.6 eq.) and the suspension was stirred until the solution became clear (approx. 20 mins). The solution was cooled to -78°C and **6.32** (130 mg, 0.362 mmol) in dry THF (164 μL , 2.2 M) was added dropwise and stirred at this temperature for 1.5 h. The solution was warmed to -15°C , stirred for an additional 5 h and slowly poured into a 0°C 1M HCl solution. The mixture was extracted with EtOAc (20 mL x 5), washed with brine, dried over MgSO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography (10%-30% EtOAc in hexanes) to afford 125 mg of **6.33** (93% yield) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 11.36 (s, 1H), 7.47 – 7.37 (m, 6H), 7.32 – 7.19 (m, 9H), 5.76 (s, 1H), 3.27 (t, J = 6.5 Hz, 2H), 2.96 (t, J = 6.5 Hz, 2H), 1.92 (d, J = 1.5 Hz, 3H). ^{13}C NMR (101 MHz, CDCl_3) δ 170.9, 160.8, 144.1, 128.7, 127.8, 126.9, 117.1, 86.9, 62.3, 34.0, 26.4.

O-TBDPS-*cis*-anhydromevalonate (6.34). Following methods for the preparation of **6.33**, from 3-butyn-1-O-TBDPS carboxylate, to afford 900 mg of **6.34** (94% yield). ^1H NMR (400 MHz, CDCl_3) δ 7.68 – 7.65 (m, 4H), 7.42 – 7.37 (m, 6H), 5.78 (d, J = 1.4 Hz, 1H), 3.83 (t, J = 6.3 Hz, 2H), 2.90 (t, J = 6.3 Hz, 2H), 1.96 (d, J = 1.4 Hz, 3H), 1.05 (s, 13H). ^{13}C NMR (101 MHz, CDCl_3) δ 171.1, 160.3, 135.6, 133.5, 129.7, 127.7, 127.7, 117.3, 62.7, 36.5, 26.8, 26.5, 19.2.

O-PMB-*cis*-anhydromevalonate (6.35). Following methods for the preparation of **6.33**, from 3-butyn-1-O-PMB carboxylate, to afford 420 mg of **6.35** (54% yield). ^1H NMR (600 MHz, CDCl_3) δ 7.28 (dd, $J = 8.8, 0.7$ Hz, 2H), 6.90 (d, $J = 8.7$ Hz, 2H), 5.79 (dd, $J = 1.4, 0.7$ Hz, 1H), 4.49 (s, 2H), 3.82 (s, 3H), 3.66 (t, $J = 6.6$ Hz, 2H), 2.97 (t, $J = 6.6$ Hz, 2H), 1.99 (s, 3H). ^{13}C NMR (151 MHz, CDCl_3) δ 171.4, 160.2, 159.2, 130.3, 129.3, 117.3, 113.8, 72.5, 68.4, 55.3, 33.9, 26.3.

(*S*)-*N*-Boc-5-hydroxynorvaline-carboxybenzyl-O(Benzyl)-amine (6.39). **6.38** (1 g, 3.1 mmol) and DIEA (1.24 mL, 7.13 mmol, 2.3 eq.) were dissolved in DCM (8.8 mL, 0.35 M) and dry DMSO (overnight over 3Å sieves) (4.8 mL) and the stirring solution was cooled to -15°C . Sulfur trioxide pyridine complex (1.13 g, 7.13 mmol, 2.3 eq.) was dissolved in dry DMSO (4.8 mL) and the clear solution was added dropwise to the stirring solution. The mixture was stirred for 1h then at room temperature for 30 min. EtOH (3.8 mL) was added to the solution and was stirred for an additional 5 min at room temperature. DIEA (609 μL) was added followed by O-Benzylhydroxylamine.HCl (559 mg, 3.5 mmol, 1.13 eq.) which was stirred at room temperature for 2 h. The solution was diluted with Et_2O (60 mL) and the mixture was washed with H_2O (40 mL), sat. aq. 1M HCl (40 mL), sat. aq. NaHCO_3 (40 mL), brine and the organic fraction was dried over MgSO_4 and the solvent was removed under reduced pressure. A portion of the oxime residue (420 mg, 1.07 mmol) was redissolved in EtOH (10 mL, 0.1 M) followed by the addition of conc. HCl (97 μL) and NaCNBH_3 (44 mg, 0.706 mmol, 0.66 eq.). The mixture was stirred at room temperature for 1.5 h, diluted with EtOAc (10 mL) and water (10 mL), washed with brine and the organic fraction was dried over MgSO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography (20%-40% EtOAc in hexanes) to afford 350 mg of **6.39** (76% yield) as a clear oil. ^1H NMR (400 MHz, CDCl_3) δ 7.40 – 7.27 (m, 12H), 5.16 (q, $J = 12.1$ Hz, 4H), 4.67

(s, 2H), 4.35 (td, $J = 7.9, 5.0$ Hz, 1H), 2.90 (t, $J = 6.8$ Hz, 2H), 1.86 (dq, $J = 14.9, 5.2$ Hz, 1H), 1.76 – 1.64 (m, 1H), 1.54 (dq, $J = 9.6, 6.7$ Hz, 2H), 1.44 (s, 11H). ^{13}C NMR (101 MHz, CDCl_3) δ 172.6, 155.4, 137.9, 135.4, 128.6, 128.5, 128.4, 128.3, 127.9, 79.9, 76.3, 67.0, 53.4, 51.4, 30.3, 28.4, 23.2.

(S)-Pyroglutamic-O-Allyl. (S)-Pyroglutamic acid (2 g, 15.5 mmol, 1 eq.) was dissolved in toluene (62 mL, 0.25 M) and allyl alcohol (4.2 mL, 62.0 mmol, 4 eq.) and pTsOH.H₂O (147 mg, 0.05 eq.) were added to the stirring solution. The mixture was refluxed for 3 h then cooled to room temperature. The solution was quenched with sat. aq. NaHCO_3 (40 mL), the volatiles were removed under reduced pressure and the residue was resuspended in DCM (20 mL). The solution was separated, the aqueous layer extracted with DCM (20 mL x 2) and the combined organic fractions were washed with brine, dried over MgSO_4 and the solvent was removed under reduced pressure. The product was used without further purification to afford 1.4 g (53% yield) as a white powder. Characterization is consistent with values reported in the literature²⁸. ^1H NMR (400 MHz, CDCl_3) δ 7.42 (s, 1H), 5.81 (ddt, $J = 16.5, 10.4, 5.8$ Hz, 1H), 5.28 – 5.20 (m, 1H), 5.16 (dd, $J = 10.5, 1.3$ Hz, 1H), 4.55 (d, $J = 5.8$ Hz, 2H), 4.19 (dd, $J = 8.8, 4.9$ Hz, 1H), 2.44 – 2.31 (m, 1H), 2.31 – 2.17 (m, 2H), 2.10 (dddd, $J = 13.0, 11.4, 8.1, 4.9$ Hz, 1H). ^{13}C NMR (101 MHz, CDCl_3) δ 178.5, 171.9, 131.4, 118.9, 65.9, 55.6, 29.3, 24.8.

(S)-Boc-Pyroglutamic-O-Allyl (6.41). (S)-Pyroglutamic-O-Allyl (883 mg, 5.22 mmol) was dissolved in DCM (22 mL, 0.24 M) and triethylamine (800 μL , 5.74 mmol, 1.1 eq.) and DMAP (64 mg, 0.522 mmol, 0.1 eq.) were added and the stirring solution was cooled to 0°C. Boc_2O (1.25 g, 5.74 mmol, 1.1 eq.) was added and the mixture was stirred overnight at room temperature. The

solution was diluted with DCM (30 mL) and washed with sat. aq. NH_4Cl (30 mL), brine, dried over MgSO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography (33% EtOAc in hexanes) to afford 1.1 g of **6.41** (78% yield) as a colourless oil. Characterization is consistent with values reported in the literature²⁸. ^1H NMR (400 MHz, CDCl_3) δ 5.72 (ddt, $J = 17.3, 10.4, 5.8$ Hz, 1H), 5.16 (dq, $J = 17.2, 1.5$ Hz, 1H), 5.08 (dq, $J = 10.5, 1.2$ Hz, 1H), 4.48 (t, $J = 1.4$ Hz, 1H), 4.47 – 4.42 (m, 2H), 2.49 – 2.11 (m, 3H), 1.85 (ddt, $J = 13.0, 9.5, 3.2$ Hz, 1H), 1.28 (s, 10H). ^{13}C NMR (101 MHz, CDCl_3) δ 173.2, 170.9, 149.0, 131.2, 119.1, 83.2, 65.9, 58.8, 30.9, 27.7, 21.3.

(S)-N-Boc-5-hydroxynorvaline-carboxyallyl (6.42). **6.41** (742 mg, 2.76 mmol) was dissolved in THF (4.6 mL, 0.6 M) and water (885 μL) and the solution was cooled to 0°C . NaBH_4 (157 mg, 4.14 mmol, 1.5 eq.) was added in one portion and the reaction was stirred at this temperature for 1h and at room temperature for 1h. The volatiles were removed under reduced pressure and the residue was resuspended in EtOAc (20 mL), washed with brine, dried over MgSO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography (40%-60% EtOAc in hexanes) to afford 260 mg of **6.42** (34% yield) as a colourless oil. Characterization is consistent with values reported in the literature. ^1H NMR (400 MHz, CDCl_3) δ 5.90 – 5.75 (m, 1H), 5.36 (d, $J = 8.3$ Hz, 1H), 5.25 (dd, $J = 17.3, 1.5$ Hz, 1H), 5.20 – 5.11 (m, 1H), 4.61 – 4.47 (m, 2H), 4.22 (q, $J = 7.1$ Hz, 1H), 3.55 (t, $J = 6.2$ Hz, 2H), 2.96 (s, 1H), 1.82 (p, $J = 6.0$ Hz, 1H), 1.65 (dt, $J = 13.4, 7.7$ Hz, 1H), 1.54 (p, $J = 6.8$ Hz, 2H), 1.35 (s, 10H). ^{13}C NMR (101 MHz, CDCl_3) δ 172.6, 155.6, 131.6, 118.7, 79.8, 65.8, 61.7, 53.3, 29.2, 28.3, 28.3.

Nosyl-N,O-*tert*-butyl (6.44). *O-tert*-butyl hydroxylamine (361 mg, 2.87 mmol) was dissolved in chloroform (5.7 mL, 0.5 M) and cooled to -5 °C under inert atmosphere. Triethylamine (781 µL, 5.74 mmol, 2 eq.) was added dropwise followed by the slow addition over 1.5 h of 2-nosyl-chloride (637 mg, 2.87 mmol, 1 eq.) solution in chloroform (3.6 mL, 0.8 M) at this temperature. The resulting solution was stirred for another 2 h at this temperature then at room temperature overnight. The mixture was washed with H₂O (15 mL), aq. 1M HCl solution (15 mL x 2), sat. aq. NaHCO₃ (15 mL), brine and the organic fraction was dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was recrystallized from cold EtOH, affording 785 mg of **6.44** (99% yield) as clear crystals. ¹H NMR (400 MHz, CDCl₃) δ 8.19 – 8.14 (m, 1H), 7.89 – 7.84 (m, 1H), 7.81 – 7.77 (m, 2H), 7.60 (s, 1H), 1.27 (s, 9H).

(*S*)-*N*-Boc-5-hydroxynorvaline-carboxyallyl-O(*t*Bu)-amine (6.45). **6.42** (100 mg, 0.354 mmol), **6.44** (111 mg, 0.354 mmol, 1 eq.) and PPh₃ (278 mg, 1.06 mmol, 3 eq.) were dissolved in dry THF (708 µL, 0.5 M) and stirred at room temperature for 15 min. The solution was cooled to 0°C and DIAD (209 µL, 1.06 mmol, 3 eq.) was added dropwise and the solution was stirred at this temperature for 30 min. The solution was heated to 40°C and was stirred overnight. The volatiles were removed under reduced pressure and the residue was diluted with EtO₂ (10 mL) and the mixture was cooled to 0°C until precipitate was formed. The mixture was filtered and the filtrate was collected, solvent removed under reduced pressure and the residue was purified by column chromatography (20% EtOAc in Hexanes) to afford 175 mg (93% yield). The residue (175 mg, 0.33 mmol) and DBU (50 µL, 0.33 mmol, 1 eq.) were dissolved in DMF (446 µL, 0.74 M). Mercaptoetanol (23 µL, 0.33 mmol, 1 eq.) was added and solution was stirred at room temperature for 3 h. The volatiles were removed under reduced pressure and H₂O (5 mL) was added and the

mixture was extracted with DCM (5 mL x 3). The organic fractions were combined and the solvent was removed under reduced pressure and the residue was purified by column chromatography (33% EtOAc in hexanes) to afford 90 mg of **6.45** (79% yield) as a colourless oil. ¹H NMR (400 MHz, CDCl₃) δ 5.89 (ddt, *J* = 17.2, 10.4, 5.8 Hz, 1H), 5.38 – 5.28 (m, 1H), 5.24 (dq, *J* = 10.4, 1.3 Hz, 1H), 5.16 (d, *J* = 8.4 Hz, 1H), 4.85 (s, 1H), 4.62 (tt, *J* = 5.7, 1.4 Hz, 2H), 4.31 (q, *J* = 7.9 Hz, 1H), 2.85 (t, *J* = 6.8 Hz, 2H), 1.87 (dq, *J* = 14.6, 8.3 Hz, 1H), 1.77 – 1.62 (m, 1H), 1.61 – 1.47 (m, 2H), 1.43 (s, 9H), 1.15 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 172.5, 155.4, 131.7, 118.7, 79.8, 76.8, 65.8, 53.4, 52.3, 30.6, 28.3, 26.8, 23.3.

Crude benzyl protected monomer (6.46). Early benzyl protected mixed *cis/trans* anhydromevalonate (15 mg, 0.066 mmol, 1 eq.) and HATU (38 mg, 0.10 mmol, 1.5 eq.) were dissolved in dry DMF (132 μL, 0.5 M) and stirred at 0°C for 30 mins. **6.39** (30 mg, 0.07 mmol, 1.05 eq.) was added at this temperature for 30 min. The solution was stirred at room temperature for 2.5 h. The solution was diluted with EtOAc (1 mL), washed with brine (2 mL) and the organic fractions were collected and the solvent were removed under reduced pressure. The residue was directly characterized by low-resolution LCMS, showing the formation of product (*m/z* = 631.40) with a mixture of diastereomer, presumably from the mixed *cis/trans* protected anhydromevalonate.

(*S*)-*N*-Boc-diolorvaline (6.51). Glutamic acid (10 g, 67.9 mmol) was dissolved in dry MeOH (113 mL, 0.6 M) and the stirring solution was cooled to 0°C. Chlorotrimethylsilane (37 mL, 298.76 mmol, 4.4 eq.) was distilled and added dropwise and the solution was stirred at room temperature overnight.

Triethylamine (62.4 mL, 442.35 mmol, 6.5 eq.) and Boc₂O (16.3 g, 74.69 mmol, 1.1 eq.) were added and the mixture was stirred at room temperature for 24 h. The volatiles were then removed under reduced pressure and the residue was dissolved in H₂O (80 mL) and extracted with EtOAc (60 mL x 3). The combined organics were washed with H₂O (80 mL), brine and dried over MgSO₄ and the solvent was removed under reduced pressure.

The crude product was redissolved in THF:MeOH (5:1, 200 mL, 0.34 M) and the stirring solution was cooled to 0°C. NaBH₄ (10.3 g, 271.6 mmol, 4 eq.) was added in portion and the solution was stirred at room temperature for 24 h. The solvents were removed under reduced pressure and the residue was dissolved in H₂O (80 mL) and the solution was acidified to pH 6-7 with a citric acid and then extracted with EtOAc (60 mL x 3), washed with brine and dried over MgSO₄. The solvent was removed under reduced pressure and the residue was purified by column chromatography (5% MeOH in DCM) to afford 9.1 g of **6.51** (61% yield over 3 steps) as a white solid. Characterization is consistent with values reported in the literature³⁷. ¹H NMR (400 MHz, CDCl₃) δ 5.09 (s, 1H), 3.71 – 3.49 (m, 5H), 3.30 (s, 3H), 1.73 – 1.54 (m, 3H), 1.52 – 1.44 (m, 1H), 1.42 (s, 8H).

(S)-N-Boc-2,2-dimethyloxazolidine-5-hydroxynovaline (6.52). **6.51** (2 g, 9.12 mmol), 2,2-dimethoxypropane (17 mL, 136.8 mmol, 15 eq) and p-TsOH.H₂O (153 mg, 0.912 mmol, 0.1 eq.) were dissolved in DCM (20 mL, 0.44 M) and the solution was stirred at room temperature for 18h. The solution was quenched with sat. aq. NaHCO₃ (30 mL), extracted with DCM (20 mL x 2), dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was purified by column chromatography (5%-25% EtOAc in hexanes) to afford 1.62 g of **6.52** (69% yield) as a colorless oil (two rotamers)³². ¹H NMR (400 MHz, CDCl₃) δ 3.92 (dd, *J* = 9.1, 4.8 Hz, 1H), 3.82

– 3.52 (m, 4H), 1.81 (d, $J = 11.0$ Hz, 1H), 1.73 – 1.50 (m, 7H), 1.49 – 1.40 (m, 12H). ^{13}C NMR (151 MHz, CDCl_3) δ 152.5, 151.8, 93.7, 93.3, 80.3, 79.6, 67.1, 67.0, 62.7, 62.3, 57.1, 30.0, 29.9, 29.6, 29.4, 28.9, 28.7, 28.4, 28.4, 28.0, 27.6, 26.8, 24.5, 23.2.

***O*-PMB-*cis*-mevalonyl-*O*-Allyl-hydroxyamide (6.53).** **6.35** (335 mg, 1.34 mmol), EDC.HCl (335 mg, 1.74 mmol, 1.3 eq.) and HOBt (235 mmol, 1.74 mmol, 1.3 eq.) were dissolved in DMF (6.7 mL, 0.2 M) and the solution was stirred at 0°C for 10 mins. *O*-allyl hydroxylamine.HCl (160 mg, 1.47 mmol, 1.1 eq.) and DIEA (700 μL , 4.02 mmol, 3 eq.) were dissolved in DMF (2 mL) and the clear solution was added dropwise to the stirring solution. The solution was stirred at room temperature overnight and the resulting mixture was diluted with EtOAc (20 mL), washed with sat. aq. NH_4Cl (20 mL), sat. aq. NaHCO_3 (20 mL), brine and the organic fraction was dried over MgSO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography (50% EtOAc in hexanes) to afford 168 mg of **6.53** (41% yield) as a white powder. ^1H NMR (600 MHz, CDCl_3) δ 10.00 (s, 1H), 7.26 – 7.19 (m, 2H), 6.87 (d, $J = 8.6$ Hz, 2H), 5.85 (ddt, $J = 16.8, 10.3, 6.3$ Hz, 1H), 5.66 (s, 1H), 5.28 – 5.17 (m, 2H), 4.46 (s, 2H), 4.21 (d, $J = 6.3$ Hz, 2H), 3.79 (s, 3H), 3.63 (dd, $J = 6.2, 5.2$ Hz, 2H), 2.57 (s, 2H), 1.79 (s, 3H). ^{13}C NMR (151 MHz, CDCl_3) δ 164.6, 159.5, 145.7, 132.7, 129.6, 129.4, 120.7, 119.9, 113.9, 77.0, 73.1, 67.2, 55.3, 34.4, 23.8.

Coupled monomer for aldehyde (6.54). **6.52** (43 mg, 0.14 mmol), **6.53** (42 mg, 0.16 mmol, 1.1 eq.) and PPh_3 (63 mg, 0.24 mmol, 1.7 eq.) were dissolved in dry THF (705 μL , 0.2 M) and stirred at room temperature for 15 min. The solution was cooled to 0°C and DIAD (47 μL , 0.24 mmol, 1.7 eq.) was added dropwise and the solution was stirred at room overnight. The solvent was

removed under reduced pressure and the residue was purified by column chromatography (20% EtOAc in hexanes) to afford 90 mg of **6.54** as a colorless oil (two rotamers), inseparable from DIAD by-product (1:2.5, product:DIAD product) in red. ^1H NMR (600 MHz, CDCl_3) δ 7.21 (d, J = 8.2 Hz, 2H), 6.86 – 6.78 (m, 2H), **6.64 (s, 4H)**, 5.97 (ddt, J = 16.4, 11.0, 5.1 Hz, 1H), 5.56 (s, 1H), 5.24 (dt, J = 17.3, 1.6 Hz, 1H), 5.14 (d, J = 10.5 Hz, 1H), **5.00 – 4.81 (m, 6H)**, 4.44 – 4.34 (m, 4H), 4.01 – 3.90 (m, 2H), 3.90 – 3.83 (m, 1H), 3.76 (d, J = 1.5 Hz, 4H), 3.67 (d, J = 8.7 Hz, 1H), 3.54 (d, J = 6.7 Hz, 2H), 2.59 (d, J = 7.1 Hz, 2H), 1.86 – 1.80 (m, 3H), 1.79 – 1.65 (m, 1H), 1.64 – 1.48 (m, 6H), 1.43 (d, J = 11.8 Hz, 13H), **1.30 – 1.12 (m, 37H)**. ^{13}C NMR (151 MHz, CDCl_3) δ 159.1, **156.5**, 147.1, 134.7, 130.5, 129.2, 117.3, 114.9, 113.7, 93.7, 93.3, 80.0, 79.5, 75.0, 72.4, 72.3, **69.9**, 69.1, 69.0, 68.5, 66.9, 66.6, 57.2, 57.0, 55.2, 34.1, 34.0, 29.9, 29.0, 28.4, 28.4, 27.5, 26.7, 26.6, 24.4, 24.3, 23.1, **21.9**, 21.9, 21.6.

Allyl deprotected monomer for aldehyde (6.55). **6.54** (150 mg, 0.274 mmol) was dissolved in ACN:H₂O (2.2 mL:548 μL , 0.125 M:0.5 M), followed by the addition of $\text{Pd}(\text{OAc})_2$ (7 mg, 0.0274 mmol, 0.1 eq.), PPh_3 (29 mg, 0.110 mmol, 0.4 eq.), formic acid (87 μL , 2.3 mmol, 8.7 eq.) and triethylamine (313 μL , 2.3 mmol, 8.7 eq.). The mixture was stirred at 50°C for 2 h then at room temperature for 1 h. The solution was diluted with EtOAc (5 mL) and washed with H₂O (5 mL x 2) and brine and the organics were dried over MgSO_4 and the solvent was removed under reduced pressure and the residue was purified by column chromatography (30% EtOAc in hexanes) to afford 56 mg of **6.55** as a colorless oil (two rotamers), inseparable from DIAD by-product (1:4.5, product:DIAD product) in red. ^1H NMR (600 MHz, CDCl_3) δ 7.23 (d, J = 8.2 Hz, 2H), 6.85 (d, J = 8.7 Hz, 2H), **6.58 (s, 8H)**, 6.12 (s, 1H), **5.04 – 4.88 (m, 9H)**, 4.41 (s, 2H), 3.96 (s, 2H), 3.88 (d, J = 7.6 Hz, 2H), 3.79 (d, J = 1.0 Hz, 4H), 3.69 (d, J = 7.7 Hz, 1H), 3.55 (t, J = 7.0 Hz, 2H), 2.64

(t, $J = 6.7$ Hz, 2H), 1.91 (s, 3H), 1.83 – 1.60 (m, 5H), 1.58 – 1.50 (m, 5H), 1.48 – 1.41 (m, 17H), 1.25 (dd, $J = 6.4, 1.2$ Hz, 60H). ^{13}C NMR (151 MHz, CDCl_3) δ 159.1, 156.4, 129.1, 113.7, 112.0, 72.5, 70.0, 66.7, 55.2, 34.6, 28.4, 28.3, 27.5, 25.7, 24.4, 21.9.

Oxazolidine deprotection of monomer for aldehyde (6.56). 6.55 (50 mg, 0.1 mmol) and p-TsOH.H₂O (1.9 mg, 0.01 mmol, 0.1 eq.) were dissolved in MeOH (1 mL, 0.1 M) and the solution was stirred at room temperature for 30 mins. The solution was quenched with sat. aq. NaHCO₃ (30 mL) and the volatiles were removed under reduced pressure. The resulting mixture was extracted with EtOAc (20 mL, x 3), dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was directly characterized by low-resolution LCMS, confirming small amounts of product 6.56 ($M+1 = 467.40$).

***N*-Boc-2,2,-dimethyloxazolidine-*O*-allyl-5-hydroxynovaline (6.60).** 6.52 (804 mg, 3.1 mmol) was dissolved in DMF (6.2 mL, 0.5 M) and the stirring solution was cooled to 0°C. NaH 60% disp. in oil (150 mg, 3.41 mmol, 1.1 eq) was added in one portion and the solution was stirred at this temperature for 10 mins. Allyl bromide (295 μL , 3.41 mmol, 1.1 eq.) was added dropwise and the solution was stirred overnight at room temperature. Sat. aq. NH₄Cl (15 mL) was added and the mixture was extracted with EtOAc (20 mL x 3). The combine organic fractions were washed with brine (20 mL x 3), dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was purified by column chromatography (20% EtOAc in hexanes) to afford 800 mg of 6.60 (86% yield) as a colorless oil (two rotamers). ^1H NMR (400 MHz, CDCl_3) δ 5.85 (ddt, $J = 17.3, 10.8, 5.6$ Hz, 1H), 5.20 (dd, $J = 17.4, 2.0$ Hz, 1H), 5.10 (d, $J = 10.4$ Hz, 1H), 3.95 – 3.81 (m, 4H), 3.78 – 3.62 (m, 2H), 3.38 (t, $J = 6.4$ Hz, 2H), 1.80 – 1.61 (m, 1H), 1.60 – 1.46 (m, 7H), 1.42

(d, $J = 5.1$ Hz, 13H). ^{13}C NMR (101 MHz, CDCl_3) δ 152.1, 151.7, 134.9, 116.6, 93.6, 93.1, 79.8, 79.3, 71.7, 70.0, 67.0, 66.6, 57.4, 57.2, 30.4, 29.5, 28.4, 27.5, 26.7, 26.4, 24.5, 23.2.

***N*-Boc-*O*-allyl-5-hydroxynovaline-carboxyaldehyde. (6.27).** **6.60** (800 mg, 2.67 mmol) and *p*-TsOH.H₂O (51 mg, 0.267 mmol, 0.1 eq.) were dissolved in MeOH (22 mL, 0.12 M) and the solution was stirred at room temperature for 30 mins. The solution was quenched with sat. aq. NaHCO₃ (30 mL) and the volatiles were removed under reduced pressure. The resulting mixture was extracted with EtOAc (20 mL, x 3), dried over MgSO₄ and the solvent was removed under reduced pressure. The residue was purified by column chromatography (30-40% EtOAc in hexanes) to afford 693 mg of deprotected alcohol (quant. yield) as a white solid. ^1H NMR (400 MHz, CDCl_3) δ 5.87 (ddt, $J = 17.2, 10.4, 5.7$ Hz, 1H), 5.23 (dq, $J = 17.2, 1.7$ Hz, 1H), 5.17 – 5.10 (m, 1H), 5.00 – 4.88 (m, 1H), 3.93 (dt, $J = 5.7, 1.4$ Hz, 2H), 3.65 – 3.48 (m, 3H), 3.41 (t, $J = 6.0$ Hz, 2H), 3.08 (qd, $J = 7.3, 4.7$ Hz, 1H), 1.61 (pd, $J = 8.2, 4.6$ Hz, 3H), 1.47 (dd, $J = 10.0, 3.8$ Hz, 1H), 1.40 (s, 9H). ^{13}C NMR (101 MHz, CDCl_3) δ 156.5, 134.7, 117.0, 79.4, 71.8, 69.9, 65.6, 52.5, 28.3, 28.1, 26.1.

Deprotected alcohol (693 mg, 2.67 mmol) and triethylamine (3.6 mL, 26.7 mmol, 10 eq.) were dissolved in DCM (43 mL, 0.061 M) and dry DMSO (overnight over 3Å sieves) (7.3 mL, 0.365 M) and the stirring solution was cooled to -10°C under inert atmosphere. Sulfur trioxide pyridine complex (3.4 g, 21.36 mmol, 8 eq.) was dissolved in dry DMSO (7.3 mL) and the clear solution was added dropwise to the stirring solution. The mixture was stirred at this temperature for 1 h and at room temperature for 1h. The solution was diluted with EtOAc (60 mL) and the mixture was washed with sat. aq. NH₄Cl (120 mL), sat. aq. NaHCO₃ (120 mL), brine (120 mL) and the organic fraction was dried over MgSO₄ and the solvent was removed under reduced

pressure. The residue was purified by column chromatography (12%-25% EtOAc in hexanes) to afford 550 mg of **6.27** (80% yield) as a clear colourless oil. ¹H NMR (400 MHz, CDCl₃) δ 9.54 (s, 1H), 5.87 (ddt, *J* = 17.3, 10.4, 5.7 Hz, 1H), 5.36 – 5.29 (m, 1H), 5.24 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.15 (dq, *J* = 10.4, 1.4 Hz, 1H), 4.18 (t, *J* = 6.9 Hz, 1H), 3.92 (dt, *J* = 5.7, 1.4 Hz, 2H), 3.42 (td, *J* = 5.9, 1.5 Hz, 2H), 1.94 (td, *J* = 13.2, 6.3 Hz, 1H), 1.75 – 1.60 (m, 3H), 1.42 (s, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 200.1, 155.6, 134.5, 117.0, 79.9, 71.8, 69.4, 59.4, 28.2, 26.0, 25.3.

Acetal di-boc adenosine (6.29). Di-boc protected O-TBS product was synthesized from 2',3'-O-Isopropylidene adenosine in 2 steps following literature procedures³⁸. TBS deprotection was performed. Product (1.16 g, 1.87 mmol) was dissolved in THF (11 mL) and 1M TBAF solution (2.8 mL, 2.8 mmol) was added dropwise and the solution was stirred at room temperature overnight. The volatiles were removed under reduced pressure and the residue was purified by column chromatography (33-66% EtOAc in Hexanes) to afford 903 mg (95% yield) of **6.29** as a white solid. Characterization is consistent with values reported in the literature³⁸. ¹H NMR (600 MHz, CDCl₃) δ 8.80 (s, 1H), 8.18 (s, 1H), 5.96 (d, *J* = 4.6 Hz, 1H), 5.31 (s, 1H), 5.17 (dd, *J* = 5.9, 4.6 Hz, 1H), 5.07 (dd, *J* = 5.9, 1.5 Hz, 1H), 4.50 (d, *J* = 1.8 Hz, 1H), 3.93 (dd, *J* = 12.6, 1.9 Hz, 1H), 3.77 (d, *J* = 12.6 Hz, 1H), 1.61 (s, 3H), 1.42 (s, 19H).

HWE product (6.58). tert-butyl ((diphenylphosphoryl)methyl)sulfonylcarbamate (246 mg, 0.622 mmol, 2 eq.) was dissolved in dry DMF:THF (1:3, 2.5 mL, 0.125 M) and the stirring mixture was cooled to -78°C. LiHMDS 0.65 M (1.9 mL, 1.24 mmol, 4 eq.) was added dropwise over 15 min and the mixture was stirred at this temperature for 15 more minutes. **6.27** (40 mg, 0.311 mmol) in dry THF (622 μL, 0.5 M) was added to the solution dropwise over 15 mins and the mixture and

was stirred overnight at room temperature. The volatiles were removed under reduced pressure and the residue was taken up in H₂O (10 mL) and the pH adjusted to ~ 3 with aq, 6M HCl. The mixture was extracted with EtOAc (15 mL x 3), washed with H₂O (10 mL) and brine (10 mL) and the combined organic fractions were dried over MgSO₄ and removed under reduced pressure. The residue was purified by column chromatography (20%-30% EtOAc in hexanes) to afford 92 mg of **6.58** (68% yield) as a clear colourless oil. ¹H NMR (400 MHz, CDCl₃) δ 7.18 (s, 1H), 6.86 (dd, *J* = 15.1, 4.9 Hz, 1H), 6.55 (d, *J* = 15.0 Hz, 1H), 5.88 (ddt, *J* = 17.3, 10.4, 5.7 Hz, 1H), 5.25 (dq, *J* = 17.2, 1.6 Hz, 1H), 5.16 (dq, *J* = 10.4, 1.3 Hz, 1H), 4.90 (d, *J* = 8.5 Hz, 1H), 4.39 (s, 1H), 3.94 (dt, *J* = 5.7, 1.4 Hz, 2H), 3.42 (t, *J* = 5.6 Hz, 2H), 1.74 – 1.56 (m, 4H), 1.46 (s, 8H), 1.41 (s, 8H).

Adenylated vinyl sulfonamide (6.59). **6.58** (41 mg, 0.094 mmol), **6.29** (53 mg, 0.104, 1.1 mmol) and PPh₃ (42 mg, 0.16 mmol, 1.7 eq.) were dissolved in dry THF (723 μL, 0.13 M) and the stirring solution was cooled to 0°C. A DIAD solution (32 μL, 0.16 mmol, 1.7 eq.) in dry THF (723 μL) was added dropwise over 1h. and the resulting mixture was stirred overnight at room temperature. The mixture was filtered over a short silica pad and the filtrate was collected and the volatiles were removed under reduced pressure. The residue was purified by column chromatography (20%-30% EtOAc in hexanes) to afford 60 mg of **6.59** (69% yield) as a clear colourless oil. ¹H NMR (600 MHz, CDCl₃) δ 8.87 (s, 1H), 8.23 (s, 1H), 6.62 – 6.40 (m, 2H), 6.17 (d, *J* = 2.0 Hz, 1H), 5.94 – 5.85 (m, 1H), 5.46 (ddd, *J* = 10.3, 6.3, 1.9 Hz, 1H), 5.26 (dt, *J* = 17.2, 1.6 Hz, 1H), 5.21 – 5.15 (m, 1H), 5.13 (dd, *J* = 6.7, 3.5 Hz, 1H), 4.88 (d, *J* = 8.8 Hz, 1H), 4.47 (td, *J* = 6.8, 3.5 Hz, 1H), 4.31 (s, 1H), 3.99 – 3.94 (m, 4H), 3.46 – 3.39 (m, 2H), 1.68 – 1.63 (m, 4H), 1.61 (s, 3H), 1.45 (s, 9H), 1.44 (s, 21H), 1.43 (d, *J* = 4.4 Hz, 10H), 1.39 (s, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 154.9, 152.4, 152.1, 150.8, 150.5, 150.5, 150.4, 147.0, 144.3, 134.6, 129.5, 128.0, 117.1, 114.6, 114.6, 90.6,

90.6, 85.5, 85.4, 84.9, 84.4, 84.3, 83.8, 83.8, 82.4, 79.8, 71.9, 69.4, 50.6, 47.5, 31.1, 28.3, 27.9, 27.9, 27.8, 27.8, 27.1, 27.1, 25.8, 25.3, 25.3.

Sulfonamide adenosine (6.63). tert-butyl ((diphenylphosphoryl) methyl) sulfonylcarbamate (50 mg, 0.127 mmol), **6.29** (71 mg, 0.139 mmol, 1.1 eq.) and PPh₃ (57 mg, 0.216 mmol, 1.7 eq.) were dissolved in dry THF (635 μ L, 0.2 M) and the stirring solution was cooled to 0°C. A DIAD solution (343 μ L, 0.216 mmol, 1.7 eq.) in dry THF (800 μ L) was added dropwise over 1h. and the resulting mixture was stirred overnight at room temperature. The mixture was filtered over a short silica pad and the filtrate was collected and the volatiles were removed under reduced pressure. The residue was purified by column chromatography (40% EtOAc in hexanes) to afford 145 mg of **6.63** as a clear oil, inseparable from excess PPh₃ and PPh₃ oxide by-product (1:3.2, product:PPh₃ product). ¹H NMR (600 MHz, CDCl₃) δ 8.75 (d, J = 0.6 Hz, 1H), 8.17 (s, 1H), 7.77 – 7.70 (m, 5H), **7.70 – 7.64 (m, 21H)**, 7.61 – 7.55 (m, 5H), **7.55 – 7.53 (m, 6H)**, 7.52 – 7.48 (m, 5H), **7.48 (m, 21H)**, 6.14 (d, J = 2.2 Hz, 1H), 5.27 (dd, J = 6.4, 2.3 Hz, 1H), 5.06 (dd, J = 6.4, 3.8 Hz, 1H), 4.65 (dd, J = 15.2, 9.7 Hz, 1H), 4.56 (dd, J = 15.2, 9.4 Hz, 1H), 4.45 (td, J = 6.5, 3.8 Hz, 1H), 3.99 (dd, J = 15.2, 6.2 Hz, 1H), 3.87 (dd, J = 15.2, 7.0 Hz, 1H), 1.60 (s, 3H), 1.51 – 1.49 (m, 11H), 1.45 (d, J = 0.8 Hz, 22H), 1.36 (s, 3H). ¹³C NMR (151 MHz, CDCl₃) δ 152.4, 152.1, 151.2, 150.4, 150.4, 143.7, 132.8, 132.8, 132.1, **132.1, 132.0, 131.9, 131.9**, 131.1, 131.0, 131.0, 131.0, 128.9, 128.9, **128.5, 128.4**, 114.9, 105.6, 90.3, 85.5, 85.1, 84.4, 83.8, 82.0, 48.6, 27.9, 27.8, 27.2, 25.4.

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Chapter 7: Conclusions and Future Directions

7.1 Overview

The summary presented in Chapter 1 established the relevance and importance of cyclic peptides, a concept that is well established in research. This is highlighted within the relevant fields of peptide chemistry, cyclic peptide research, depsipeptide synthesis and chemoenzymatic synthesis. The first focus of my thesis is on the well recognised synthetic cyclic stapled peptides. Chapter 2 reviews the concept of stapled peptides and emphasizes the lack of DNA-binding stapled peptides in the field. This comprehensive review of DNA binding stapled peptides highlights the potential of this novel types of peptide inhibitor of transcription. Chapter 3 discusses my research efforts to optimize an anti-virulent DNA binding stapled peptide. Synthetic macrocyclic peptides have been a persistent challenge to synthesize. Notable examples are cyclic depsipeptides, which contain a macrolactone, where multiple approaches have been developed to tackle their synthesis. In chapter 4, the solid phase peptide synthesis strategies of cyclic depsipeptides cyclization are reviewed while in chapter 5, my research on the chemoenzymatic synthesis of a cyclic depsipeptide seongsanamide E is described. This study highlights the power of thioesterases as a biocatalyst for generating difficult macrolactone bonds. Chapter 6 looks at a different step in depsipeptide biosynthesis and describes my synthetic efforts towards a di-domain inhibitor of a never synthesized siderophore fusarinine C to study its iterative assembly.

7.2 Perspective on DNA binding stapled peptides

Stapled peptides are established as an effective method to generate protein-protein interaction inhibitors, an otherwise “undruggable” target. While these are important interactions to perturb in

medicinal chemistry, a less explored though equally important area of inhibition is at the interface of protein and DNA. Often the challenge of DNA binding molecules is the membrane permeability and DNA sequence specificity, both of which are possible with stapled peptides. The structured nature of the α -helical mimic of the native DNA binding motif is at the core of binding to these large DNA surface areas selectively, something that is rarely possible with small molecules. There are many transcription factors involved in key pathways that could be inhibited with these types of stapled peptides, leaving an interesting area of inhibitor development, as reviewed in chapter 2.

Particularly for these RpoN based stapled peptides developed in Chapter 3, their properties have potential of exhibiting novel antibiotic mechanisms of actions due to their anti-virulent activities. Further studies are required to explore this area, including the impact of our lead stapled peptides on biofilm formation and motility assays with Gram-negative bacteria. Anti-virulent compounds have gained interest as these types of compounds can avoid imposing strong selective pressure that favours resistance development in bacteria¹. Synergistic assays with known antibiotics could help show the importance of anti-virulent compounds. This is particularly true in *in vivo* animal models. Including the anti-virulence stapled peptide to limit the spread of infection and a known clinical antibiotic to knock down the infective bacteria would produce a far more effective treatment than anti-virulence stapled peptide alone.

This first-generation peptide library goal was to have a diverse set of modifications that look at different aspects of the peptide such as staple placement, C-terminus length, DNA-binding residues, etc. A “second generation” library could expand these areas including the completion of the “staple walk”, the utilization of more non-proteogenic amino acids and the combination of previous lead substitutions. Future stapled peptides could explore staple participation where the

peptide staple can be modified and functionalized² to reduce the outward facing hydrophobicity of the staple, as seen in some protein-protein inhibitor peptides³.

Specifically for $\sigma 54$ binding and our RpoN mimicking stapled peptide, the major DNA interaction portion is to the -24 region of the promoter in the DNA major groove. A minor groove interaction seen outside of the RpoN box was briefly investigated with the extension of the peptide and the synthesis of a larger peptide including an extra 6 residues (-GIPSSR). This peptide did not exhibit substantial promoter binding in our study, possibly due to largely unstructured random coil sequence. This C-terminal region includes a turn and a half of an α -helix, highlighting the possible requirement of a secondary staple in the C-terminus to maintain helicity. It was pointed out that this minor groove interaction was key for DNA binding⁴, including some potential nucleobase H-bonding between a Ser and Arg to a Thymine, making this a particularly interesting target (**Figure 7.1**).

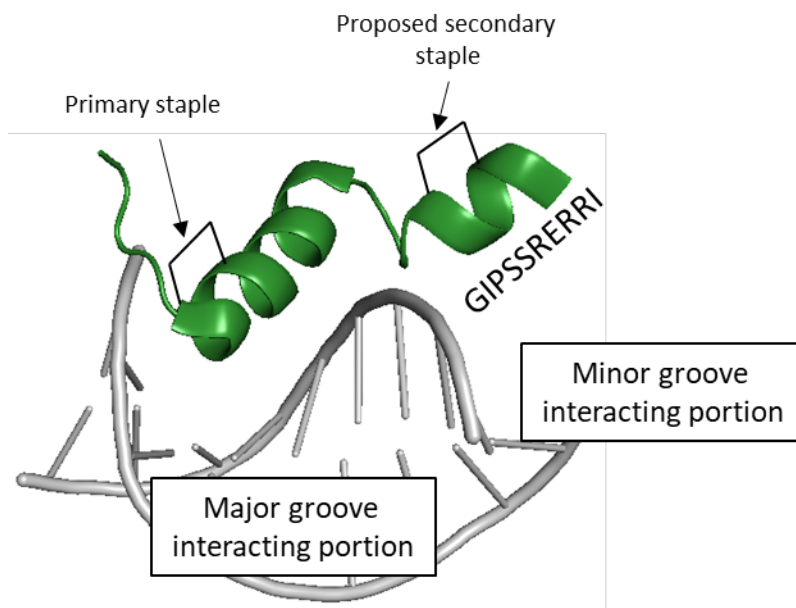


Figure 7.1 Proposed secondary staple for minor groove binding

7.3 Future of depsipeptide biosynthesis

Depsipeptides remain an interesting class of peptide natural products, which exhibit potent biological activities due to their cyclic structure and presence of ester bonds. Studying their biosynthesis has helped answer fundamental enzyme function questions and helped elucidate the biological strategies for the synthesis of depsipeptides. Biochemically characterizing novel cyclizing enzymes such as the seongsanamide TE in chapter 5 can help us better understand the key final step of these peptides and supports the discovery of novel biocatalysts. We have shown that the seongsanamide TE can recognize a linear thioester peptide mimic and convert it to the monocyclic seongsanamide E without epimerization. This is in contrast to the chemical strategies which, cannot macrolactonize the peptide and can only macrolactamize the molecule with epimerization. This enzyme was also found to bind the natural product and hydrolyze it with a water molecule to generate the hydrolyzed linear product, providing interesting insight into the mechanism of the TE-mediated macrocyclization.

While seongsanamide E has previously been synthesized⁵, we showed that it could be readily synthesized by a combination of SPPS and solution-phase chemistry through a macrolactamization approach. This type of approach has been utilized in the synthesis of several cyclic depsipeptides, as was reviewed in chapter 4.

This has been the basis for the synthesis of other peptides in our group, notably for the use in a chemoenzymatic synthesis and novel TE characterization⁶. Rhizomide A was discovered in 2018 from a cryptic biosynthetic gene cluster analysis in Gram-negative Burkholderiales strains, provided by a single module with a final cyclization and release via a thioesterase⁷. While this compound exhibited very weak antimicrobial and anti-tumour activity, it had not yet been synthesized. The peptide is composed of a 6-amino acid macrocycle that is lactonized through a

Thr β -alcohol and Val C-terminus and contains a variety of D and L configured amino acids. This peptide was synthesized by Corinne Lepetit, a visiting Master's student from France and she completed the first total synthesis of this compound. The linear thioester substrate was synthesized following the approach described in Chapter 5 and used for the total chemoenzymatic synthesis of rhizomide A and to characterize the rhizomide TE, which displayed kinetically slow macrolactonization activity.

Gacamide A is lipodepsipeptide that was isolated in 2018 from *Pseudomonas fluorescens* Pfo-1 and exhibited significant swarming and surfactant activity⁸. The biosynthetic pathway contains two thioesterases with unknown roles and activity. To address this question we set out to use the strategy developed for seonsanamide E to undertake a novel total synthesis of gacamide A and to characterize the biosynthesis of the macrocycle by the di-TE domain. This challenging synthesis required the synthesis of an (*R*)- β -hydroxy decanoic acid fragment for N-terminal acylation, limiting aspartimide formation from an Asp residue, addressing the physicochemical impact of highly hydrophobic amino acid sidechains, and alternating amino acid stereochemistry⁹. A fully on-resin synthetic strategy from a sidechain *O*-serine linked resin allowed access of small amounts of macrocyclic final product to be synthesized. The product had terrible solubility and exhibited possible aggregation in the final coupling steps. This low yield could be addressed in further work looking at lowering the resin loading or using PEG-based resins. The poor product solubility however will remain a significant problem.

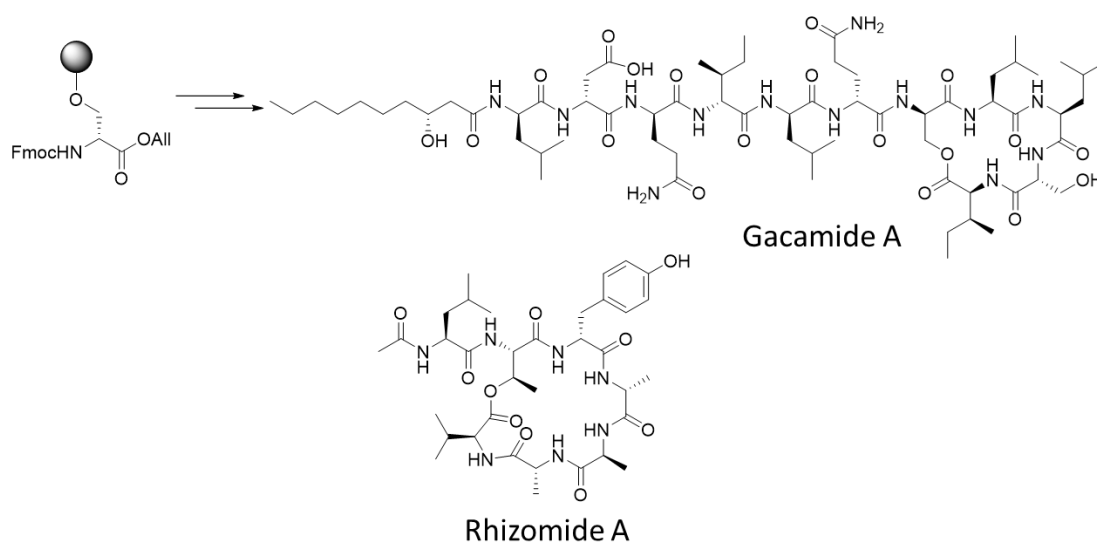


Figure 7.2 Rhizomide A and gacamide A cyclic depsipeptides

Similar strategies were undertaken to synthesize the linear gacamide A C-terminal thioester, where the *O*-linked serine resin allowed to synthesize the thioester peptide fully on resin including a thioesterification step. This is an ideal situation where the peptide sequence can be utilized to complete the substrate synthesis, simplifying Fmoc-SPPS thioester peptides. Work is currently underway to biochemically characterize the ability of both of the TE domains to macrocyclize this ring system. Preliminary data show the first TE is sufficient for macrocycle formation.

7.4 The total synthesis of fusarinine C

The cyclic depsipeptide siderophore fusarinine C is biosynthesized under an iterative mechanism, as discussed in chapter 6. Efforts towards the synthesis of inhibitors capable of probing its biosynthesis are described, focusing on the synthesis of a fusarinine C monomer. There has never been a total synthesis of fusarinine C, making the synthesis of a biosynthetic inhibitor that much more challenging. During the inhibitor synthesis, the total synthesis has been a side focus during the time of this project. Fusarinine C monomers were synthesized to access both the

aldehyde required for the inhibitor and the carboxylate for future ester coupling involved in a total synthesis.

This cyclic siderophore contains three esters between three monomers. Conceptually, orthogonally protected monomers at respective alcohol/C-terminus with sequential esterifications could form the product; however this approach seemed tedious with the already synthetically challenging nature of the hydroxamic amide monomer.

An alternative approach involves an interesting oligomerizing Mitsunobu reaction setup. This approach has been used for other multimeric macrocycles where the researchers generated macrocycles with varying numbers of monomers in them using excess Mitsunobu reagents and a carboxylic acid/alcohol monomer¹⁰. With some success at forming macrocycles, and some limited ring size control with the use of different salts, this seemed like an interesting method. The Mitsunobu reaction has been important in the synthesis of the hydroxamic amide of the protected fusarinine C monomer in chapter 6. A redesigned fusarinine C “ester-monomer” containing an alcohol and a protected *N*-hydroxy amide could potentially setup an oligomeric macrocyclization (**Figure 7.3**).

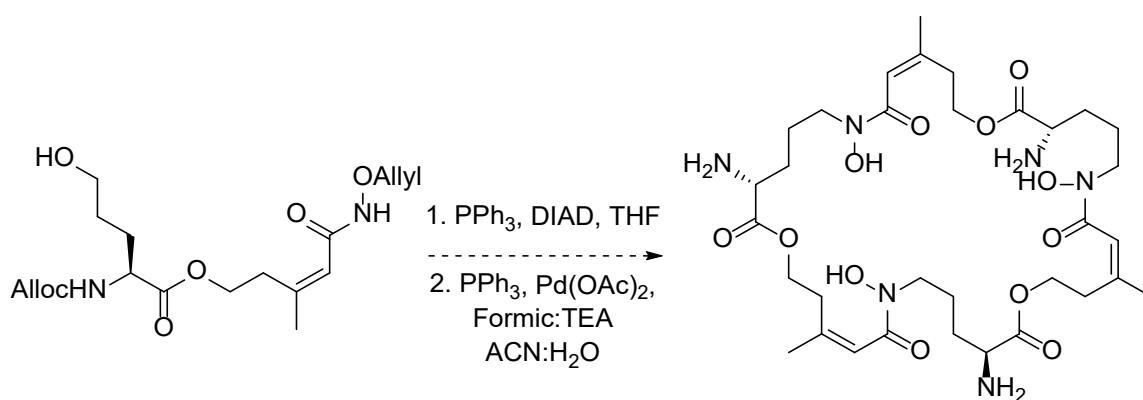


Figure 7.3 Oligomerization Mitsunobu macrocyclization of fusarinine C total synthesis

This total synthesis route may ultimately be the ideal strategy to access fusarinin C. Synthesis of sufficient material to screen reaction conditions and salts involved in trimer templating, such as iron, may enable selective formation of the trimeric natural product macrocycle. The final product is a very potent siderophore capable of binding iron in a hexadentate form. While this work has provided a potential route to the natural product, we ultimately focused on the synthesis of the key biosynthetic inhibitor for our collaborators. Thus the total synthesis remains an unachieved effort.

7.5 Conclusion

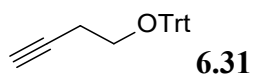
Overall, cyclic peptide chemistry and biochemistry were explored in various forms from stapled peptides, cyclic peptide natural products synthesis, and biosynthetic enzyme characterization. Exploring a DNA binding stapled peptide library has produced compounds with enhanced binding capabilities and improved anti-virulence activities. Furthermore, the synthesis and chemoenzymatic synthesis of cyclic depsipeptide seongsanamide E was performed, requiring peptide analogues as substrates for the characterization of a new thioesterase capable of macrolactonization. This has allowed the establishment of a chemoenzymatic workflow for the utilization of SPPS to access new cyclic depsipeptides and their analogues for similar projects. The biosynthesis of cyclic depsipeptides can be even further investigated with the synthesis of enzymatic inhibitors such as the fusarinine C monomer adenylated vinyl sulfonamide. Exploration of new types of chemistry and the development of novel synthetic routes has laid the groundwork for the completion of the synthetic inhibitor and the start of the first fusarinine C total synthesis.

7.6 References

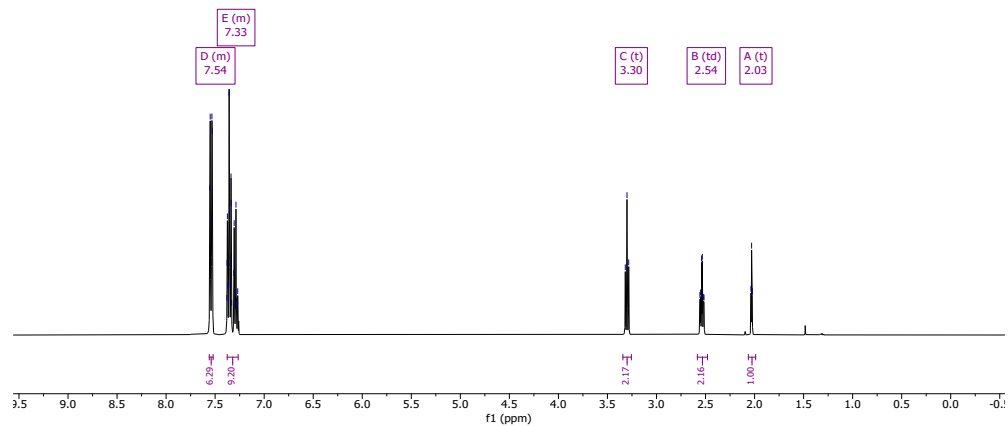
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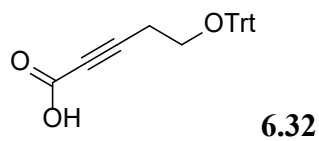
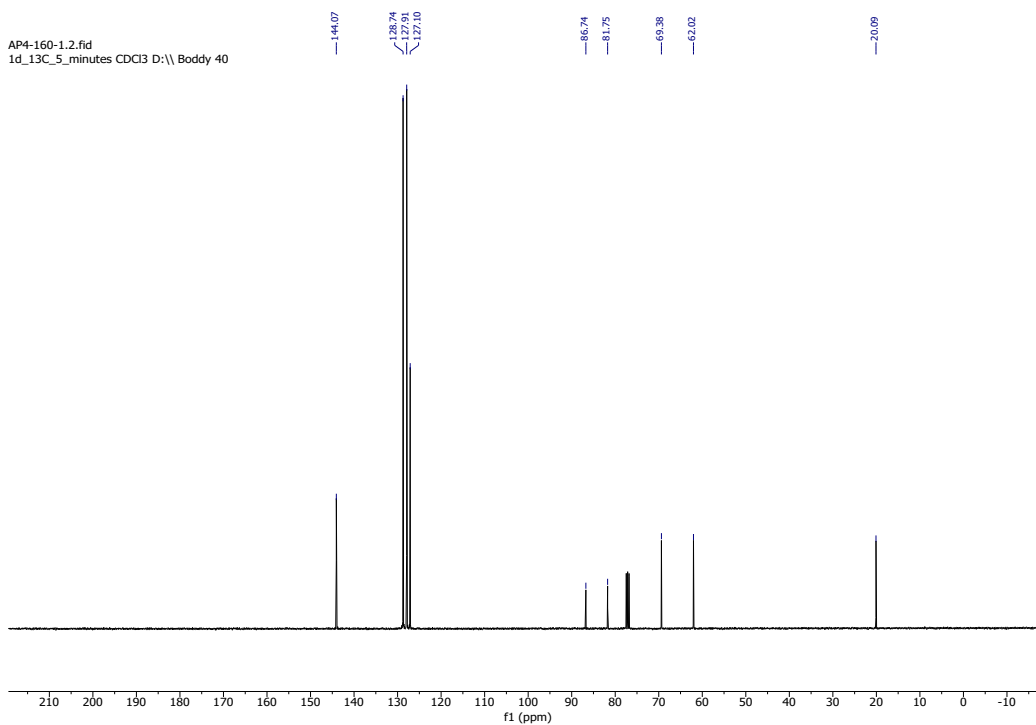
Appendix 1 – Chapter 6 characterization



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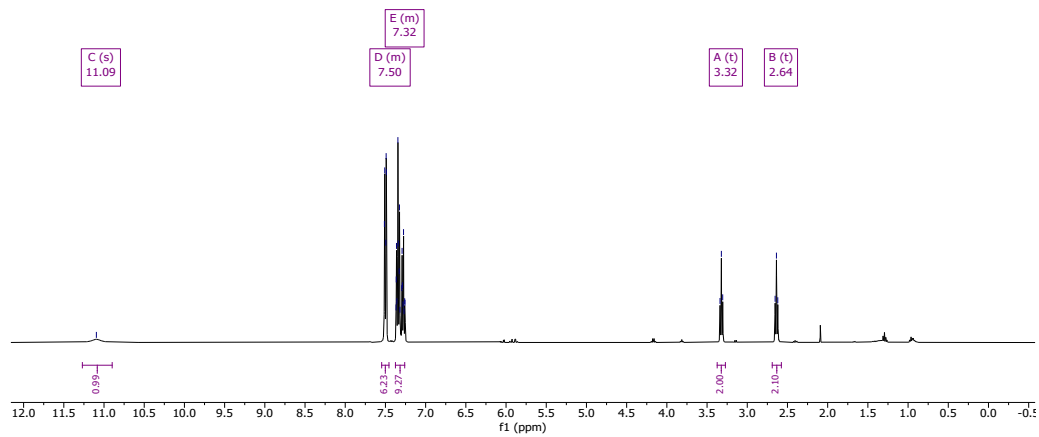


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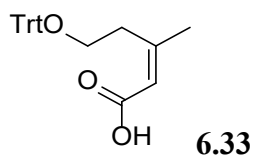
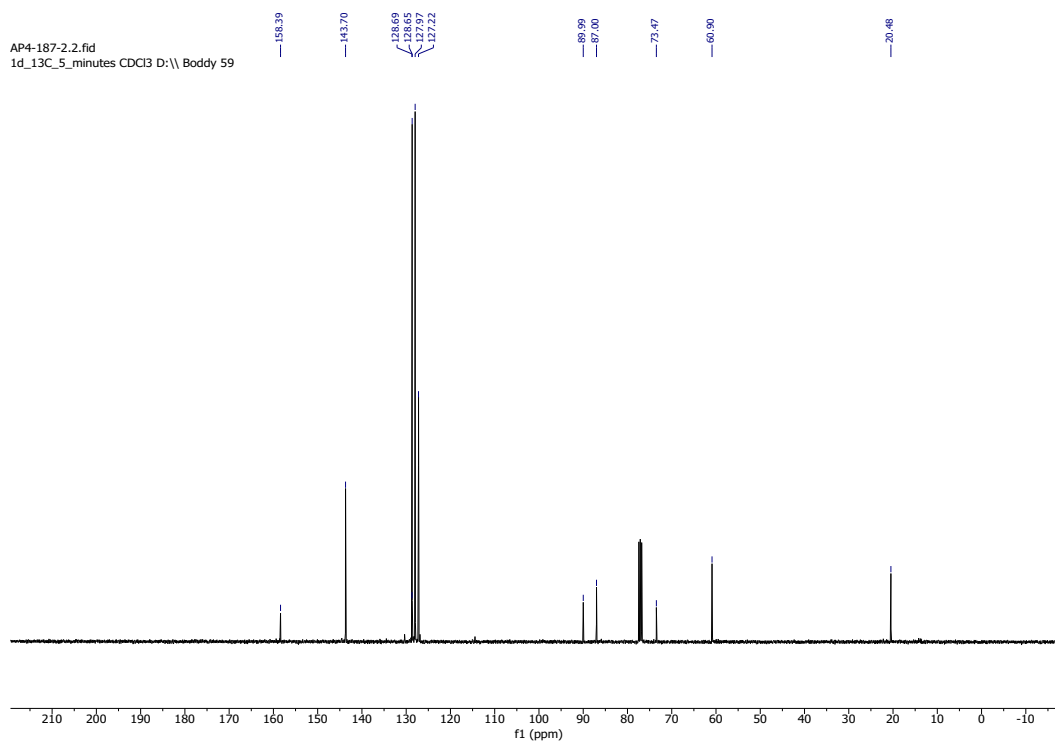


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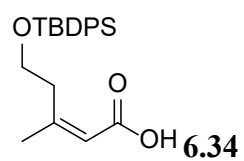
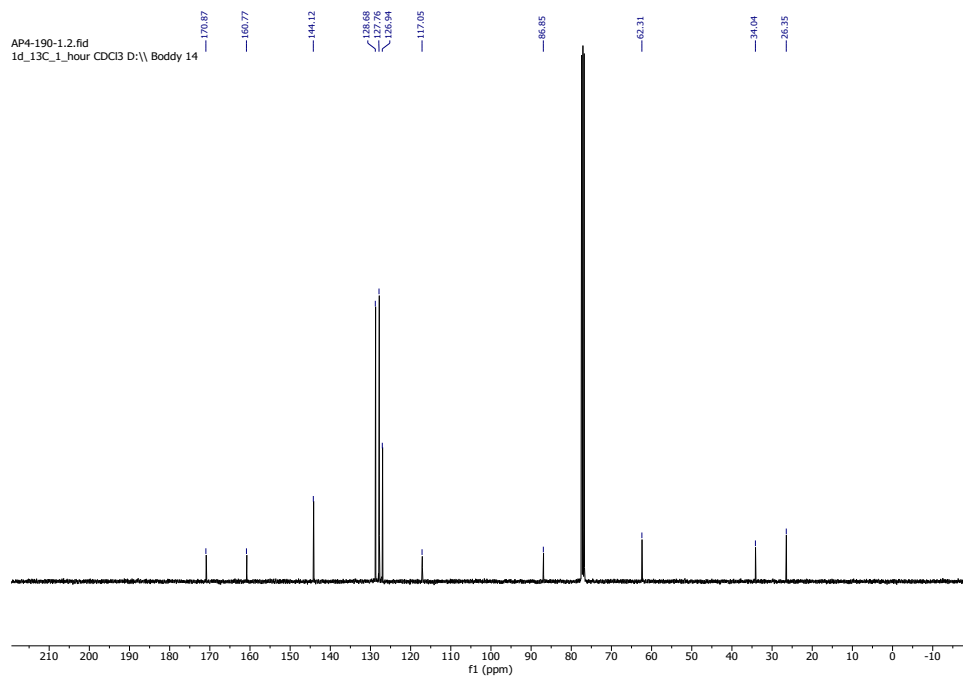
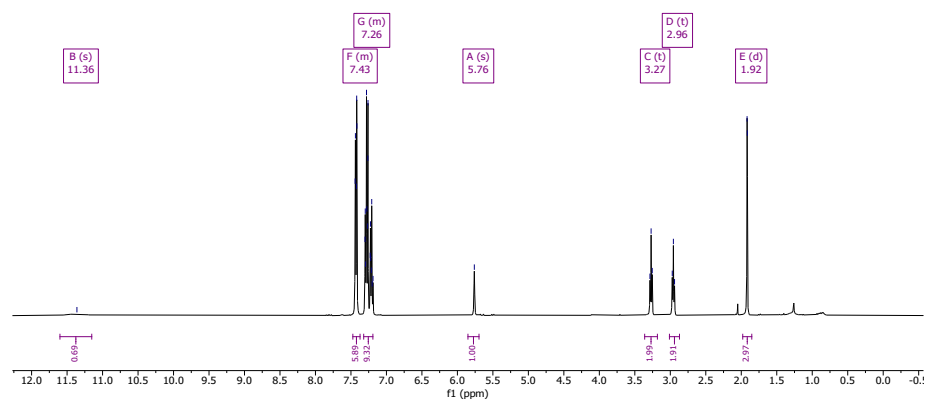
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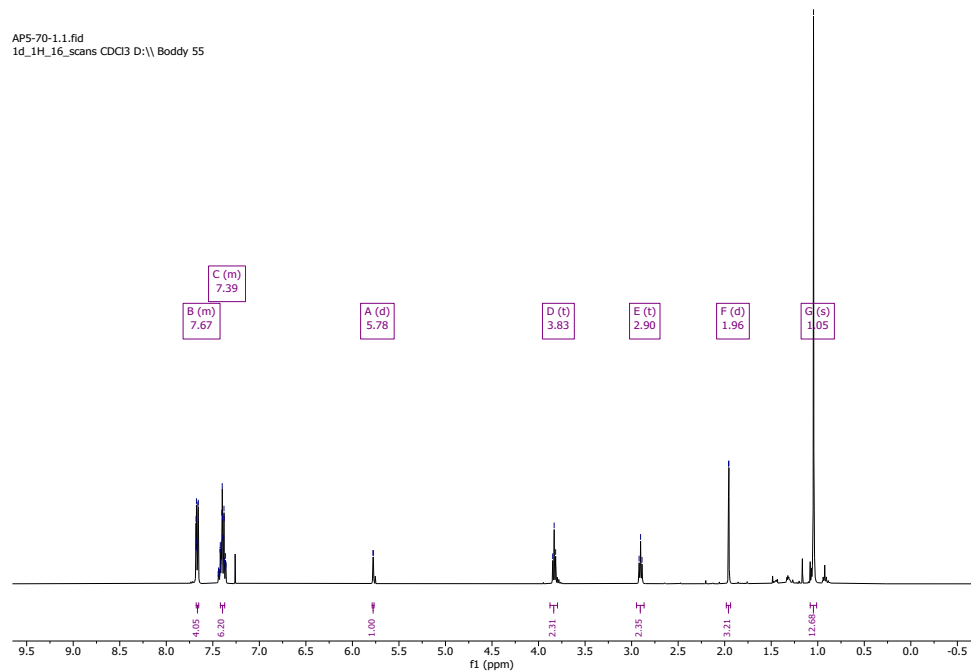
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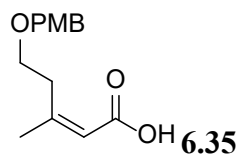
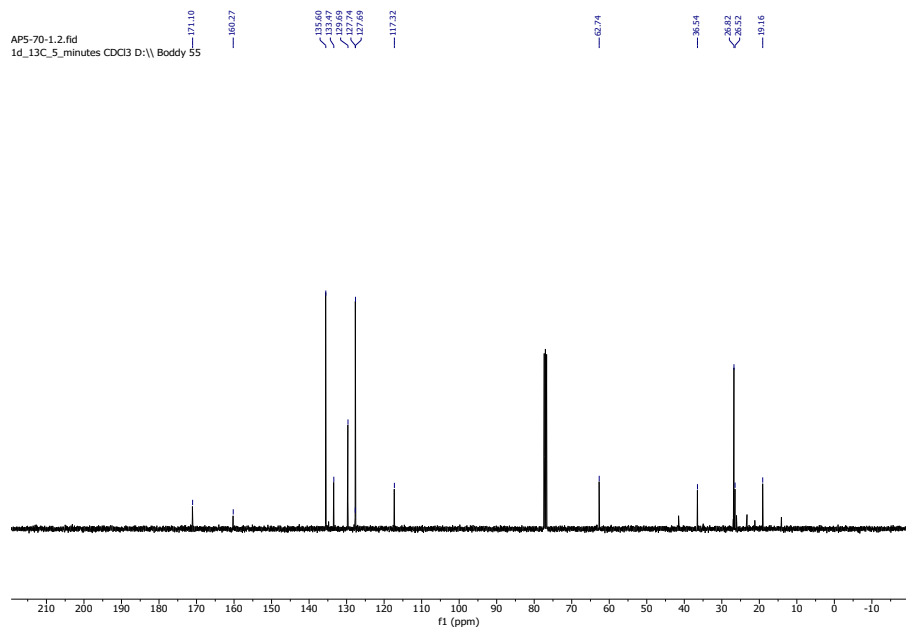
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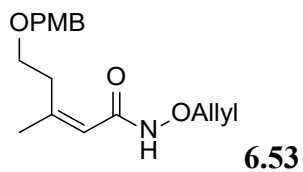
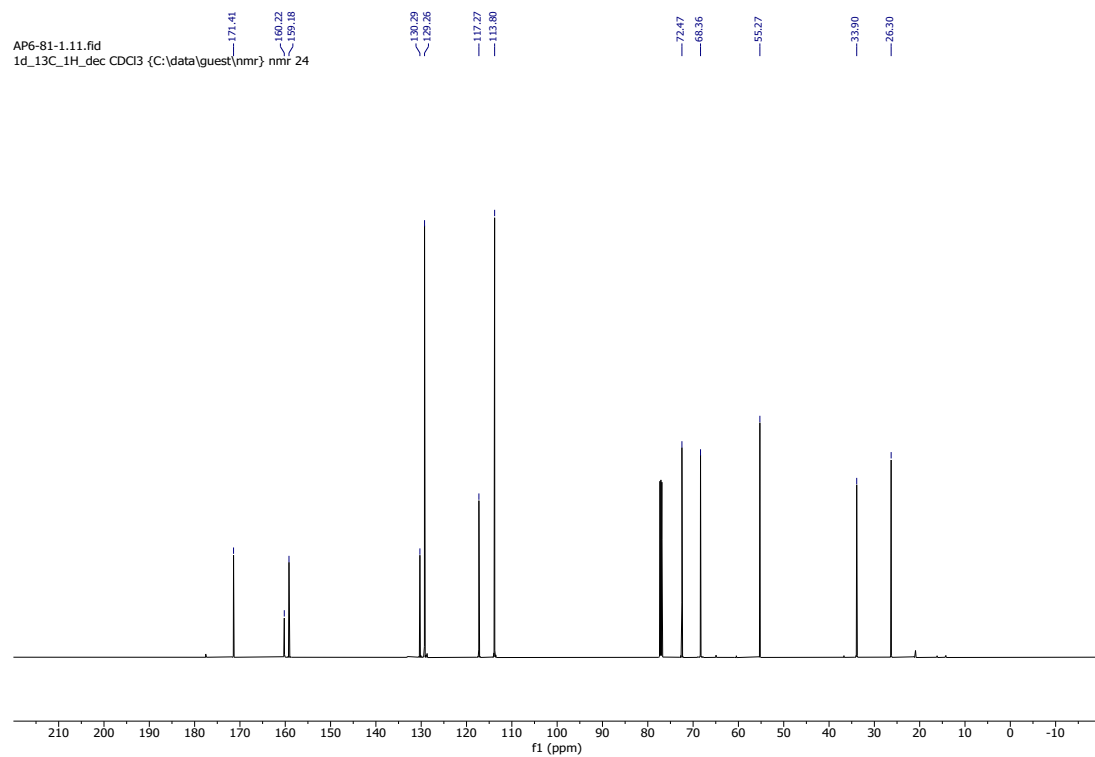
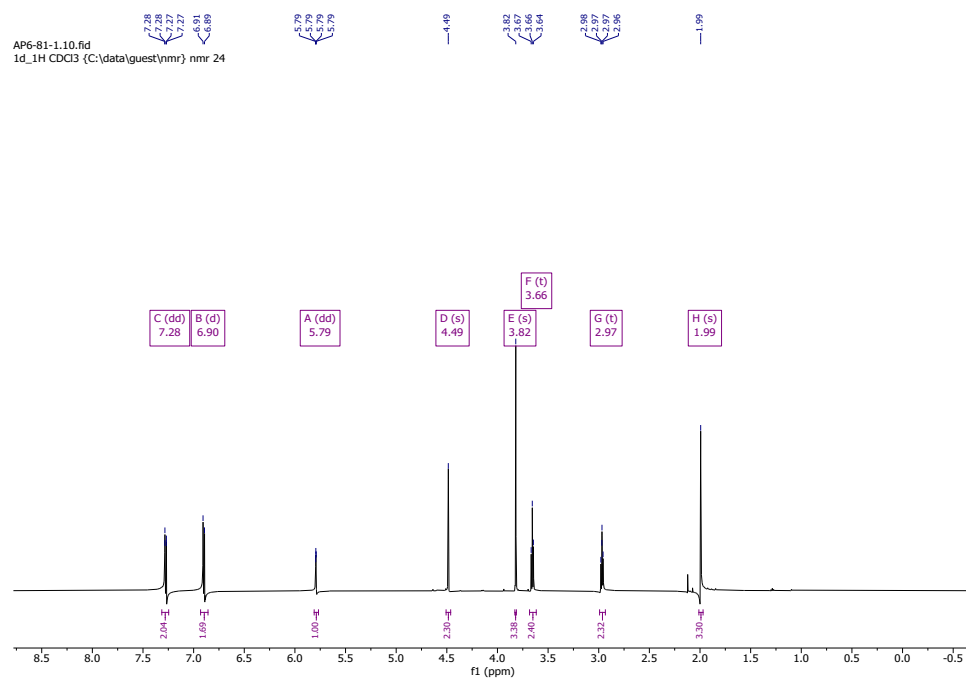


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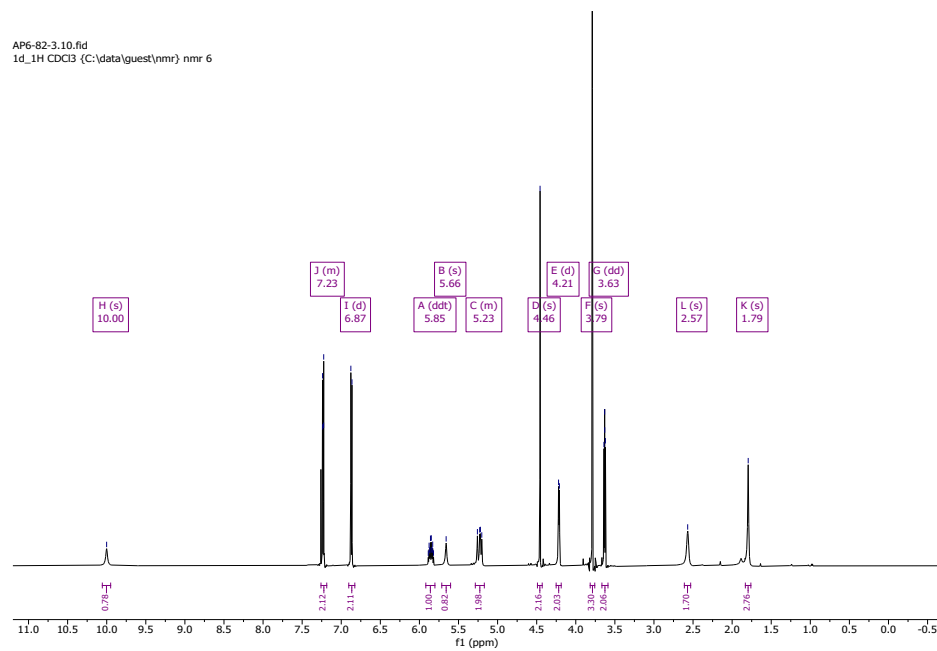


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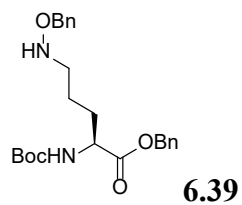
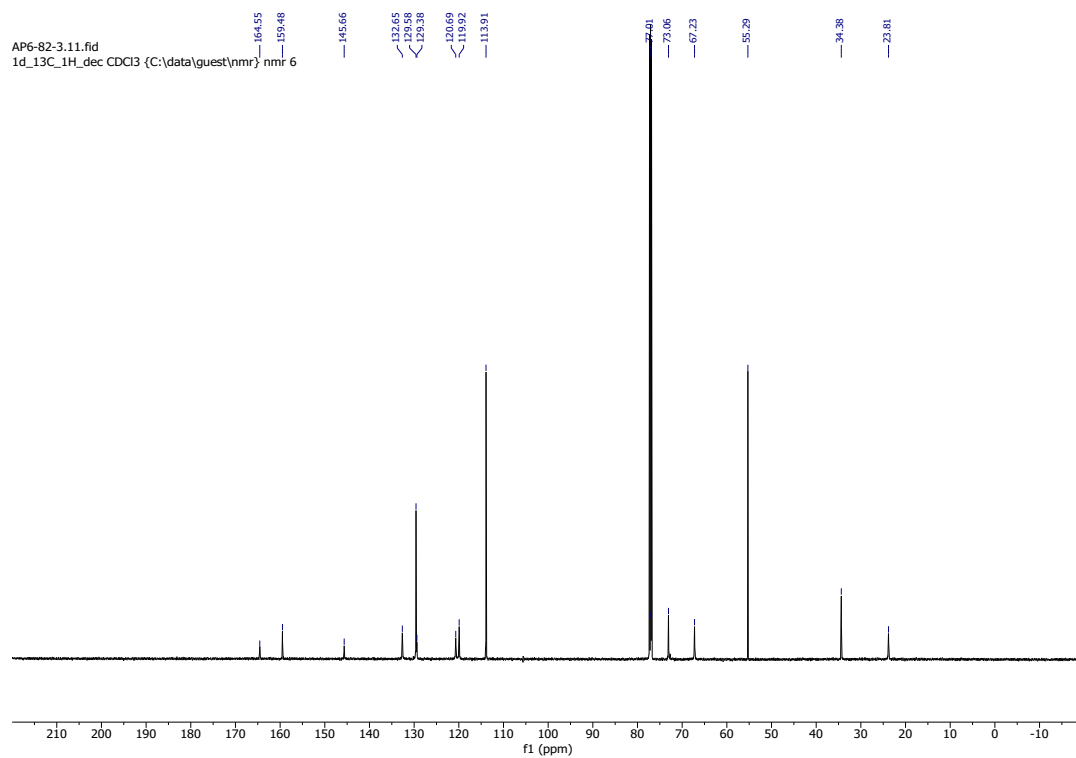


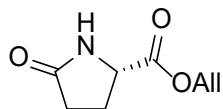
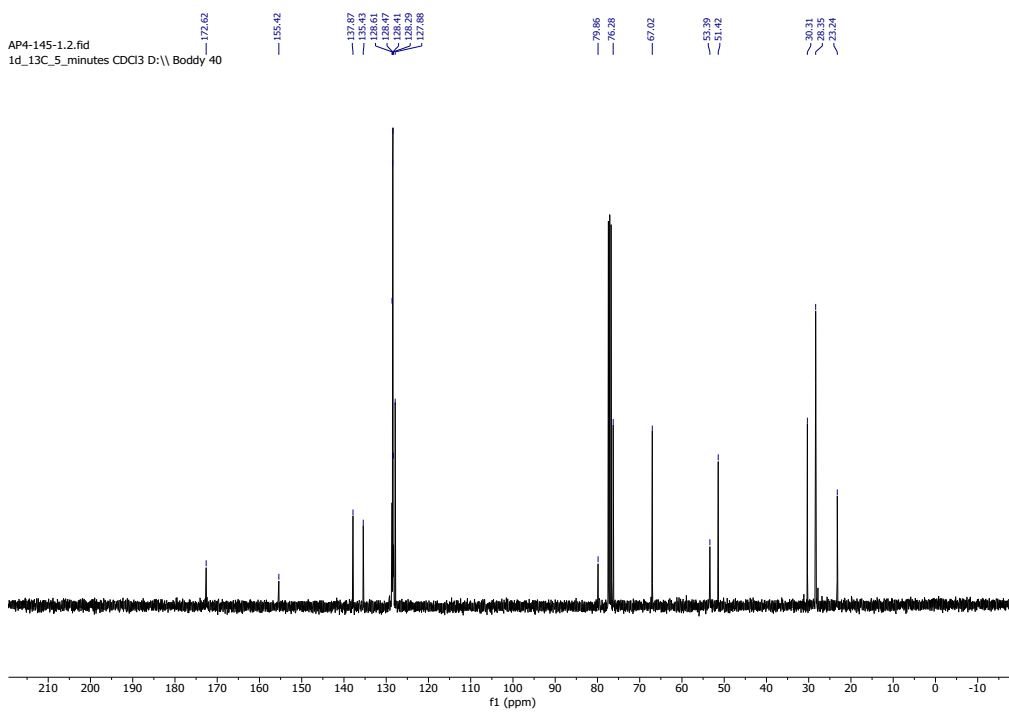
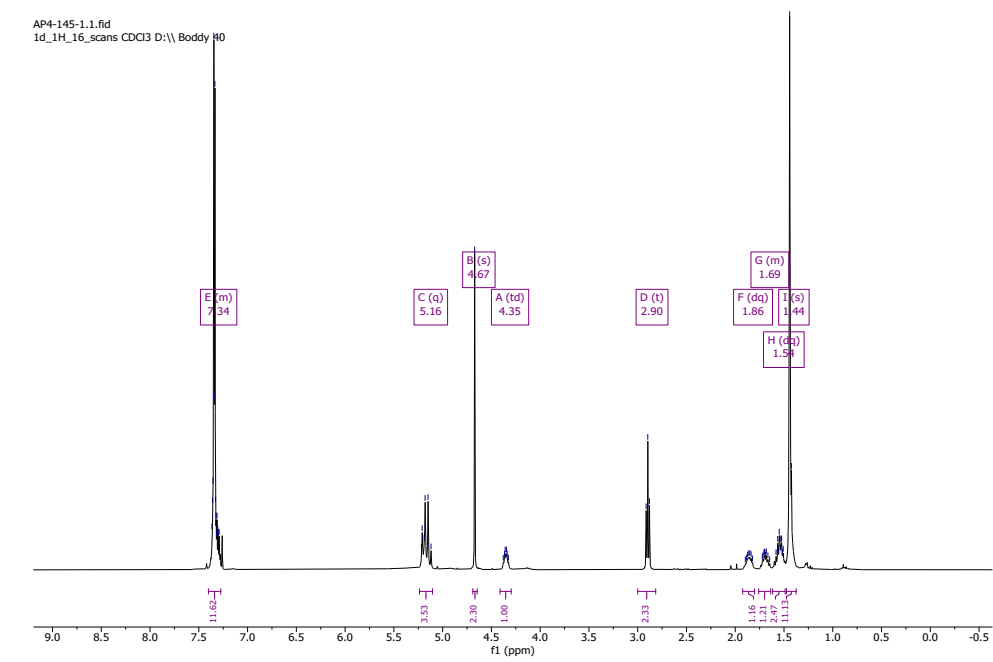


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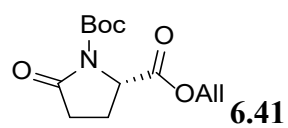
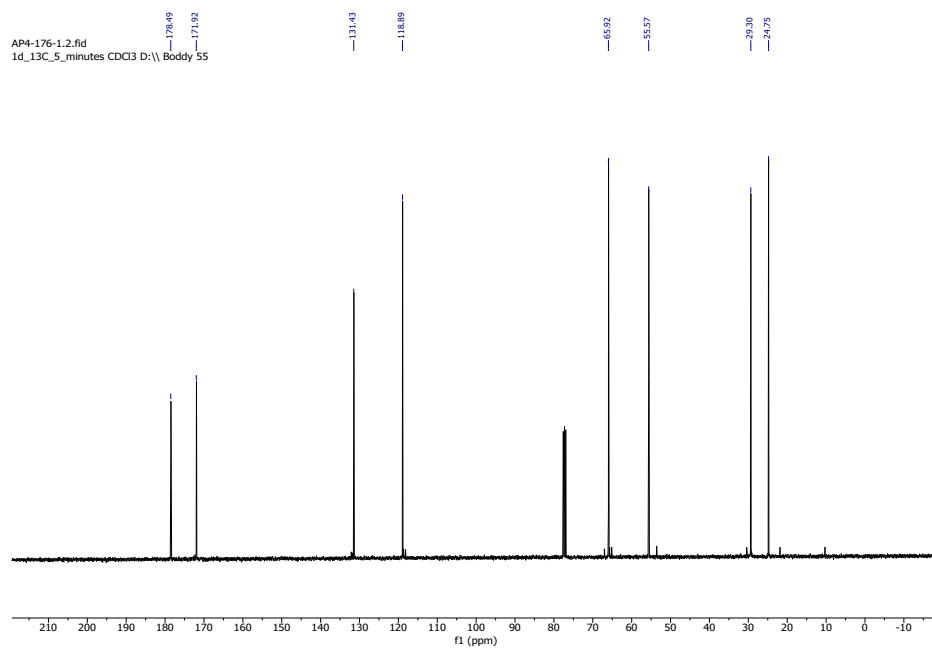
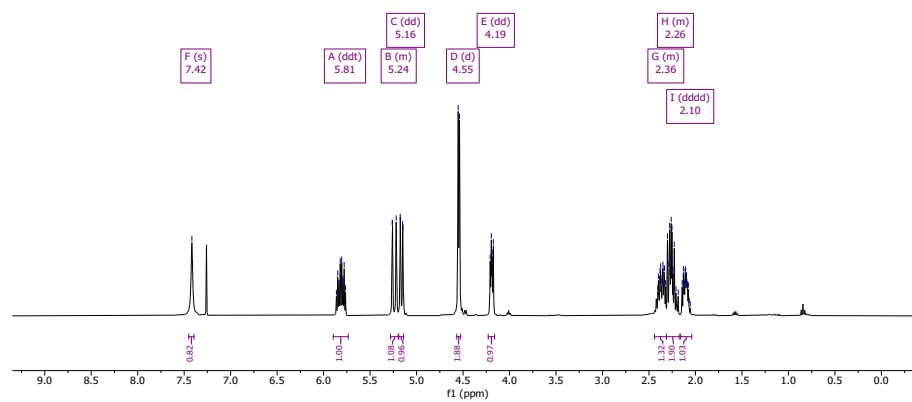


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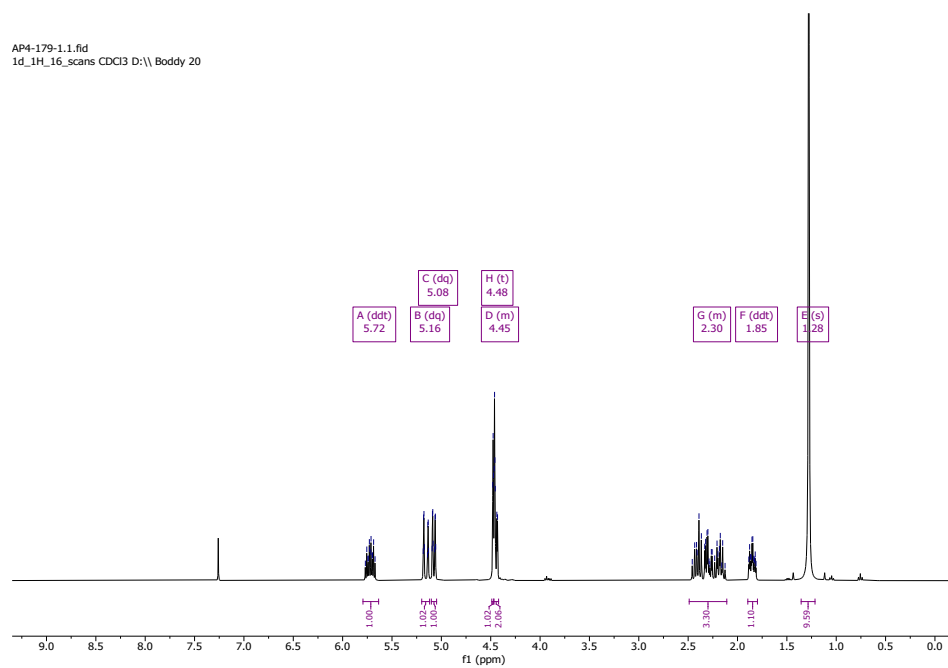




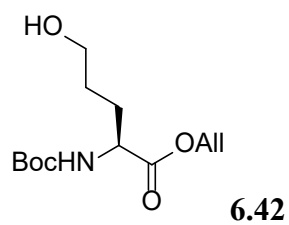
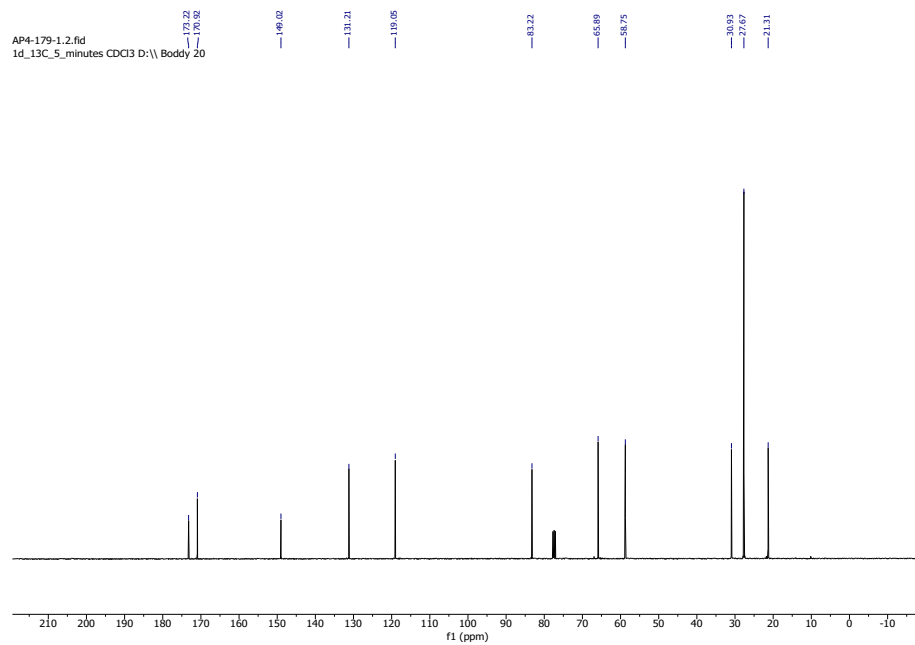
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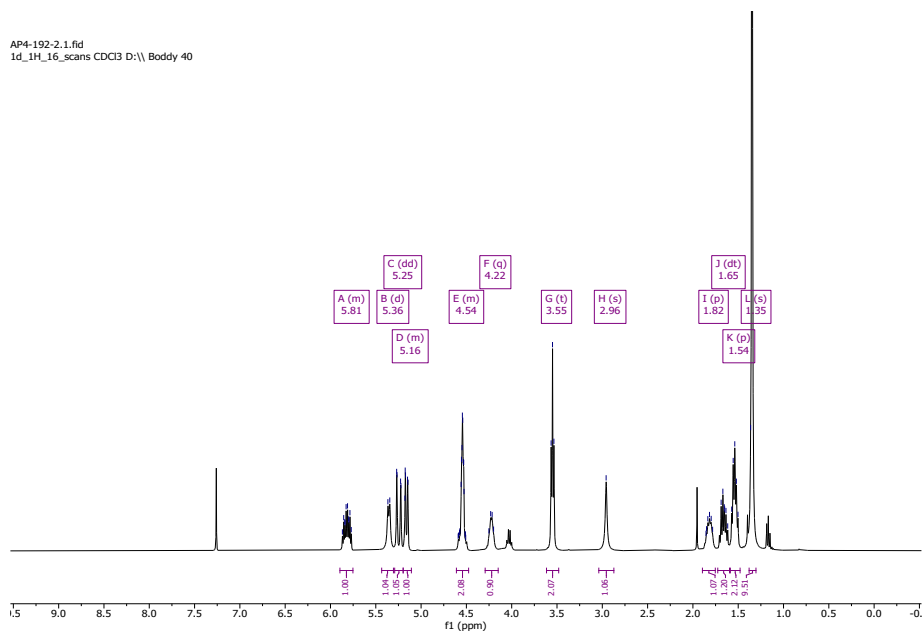
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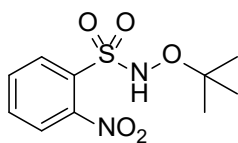
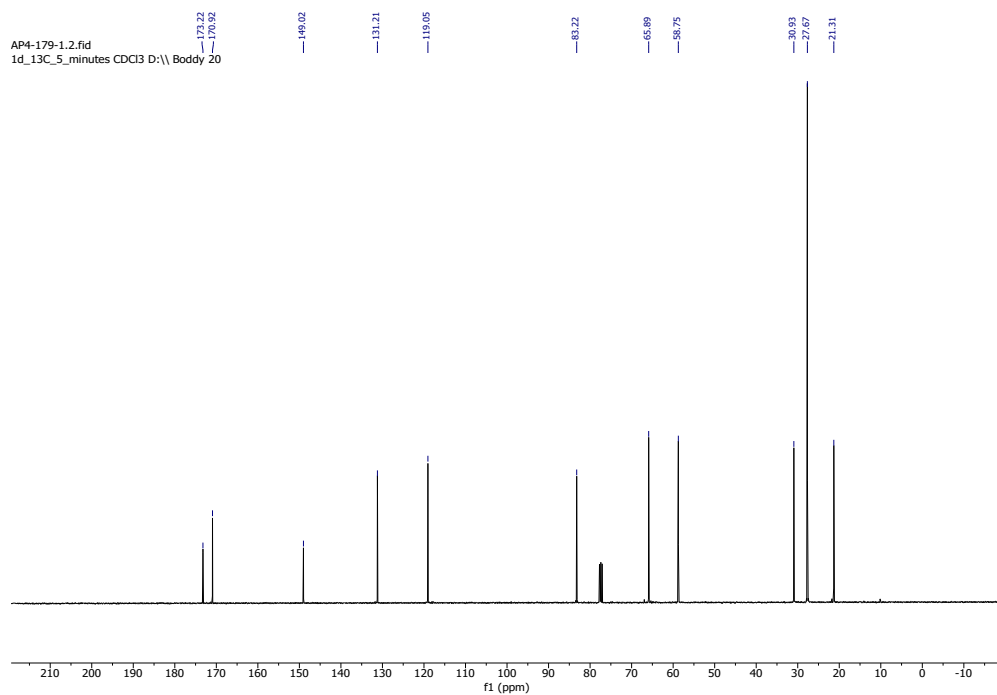
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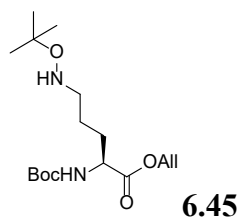
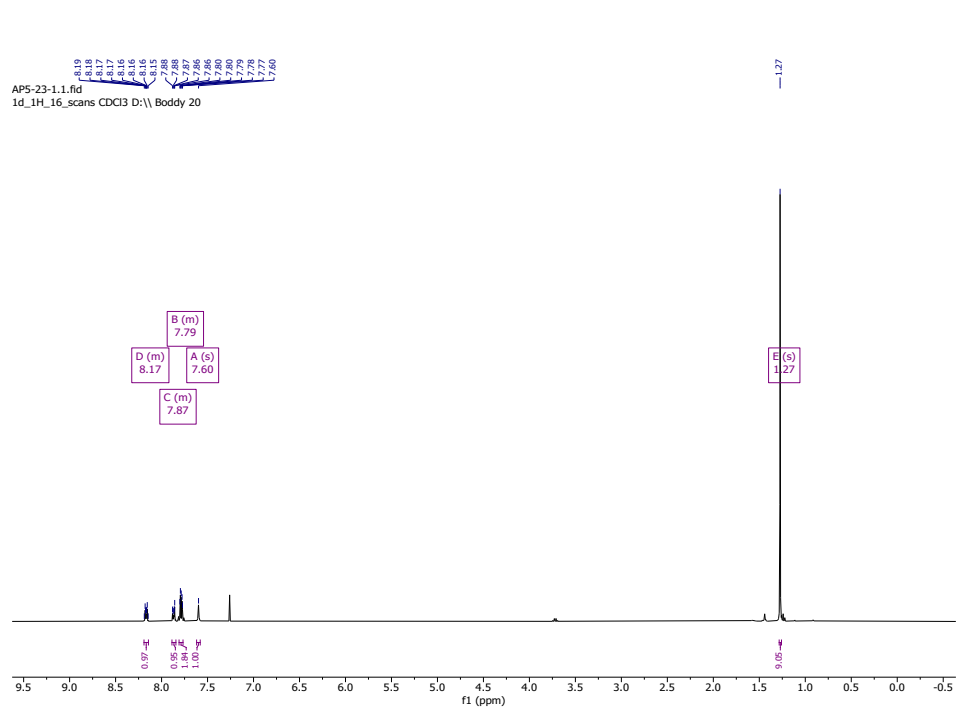
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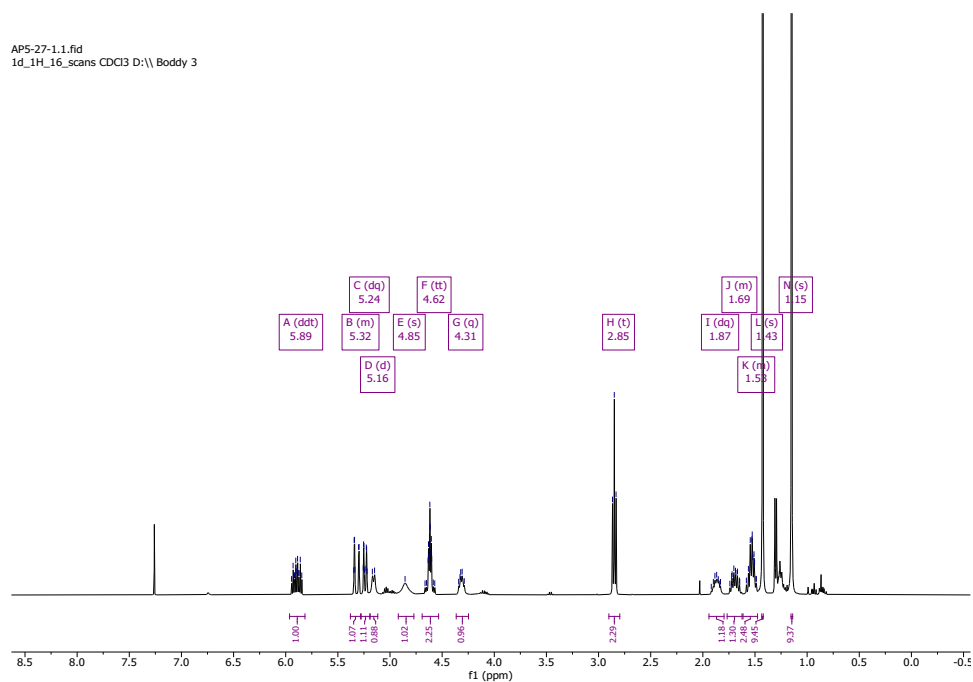
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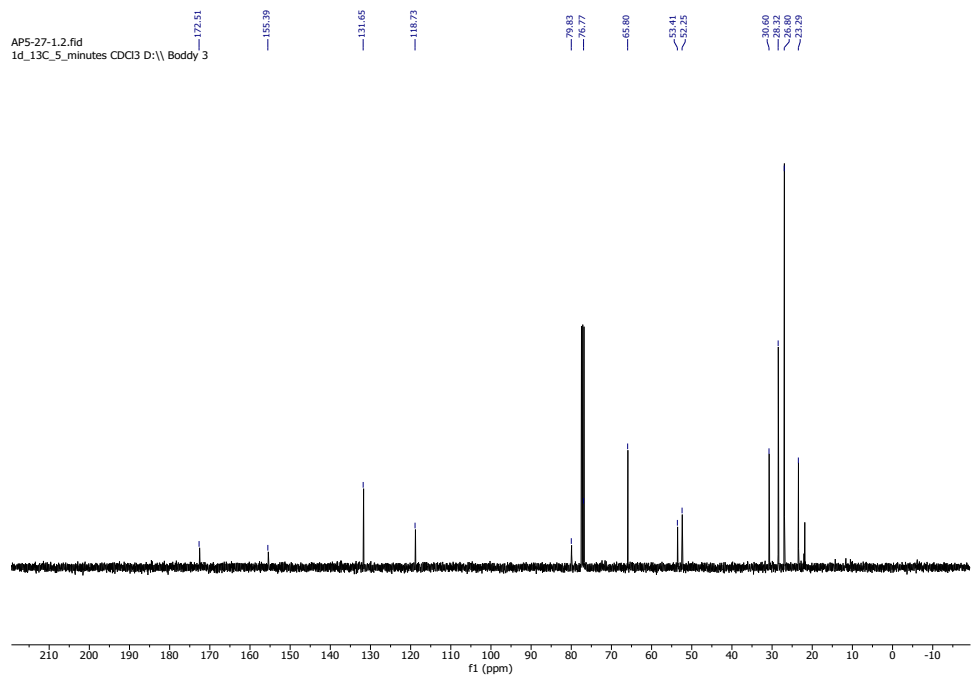
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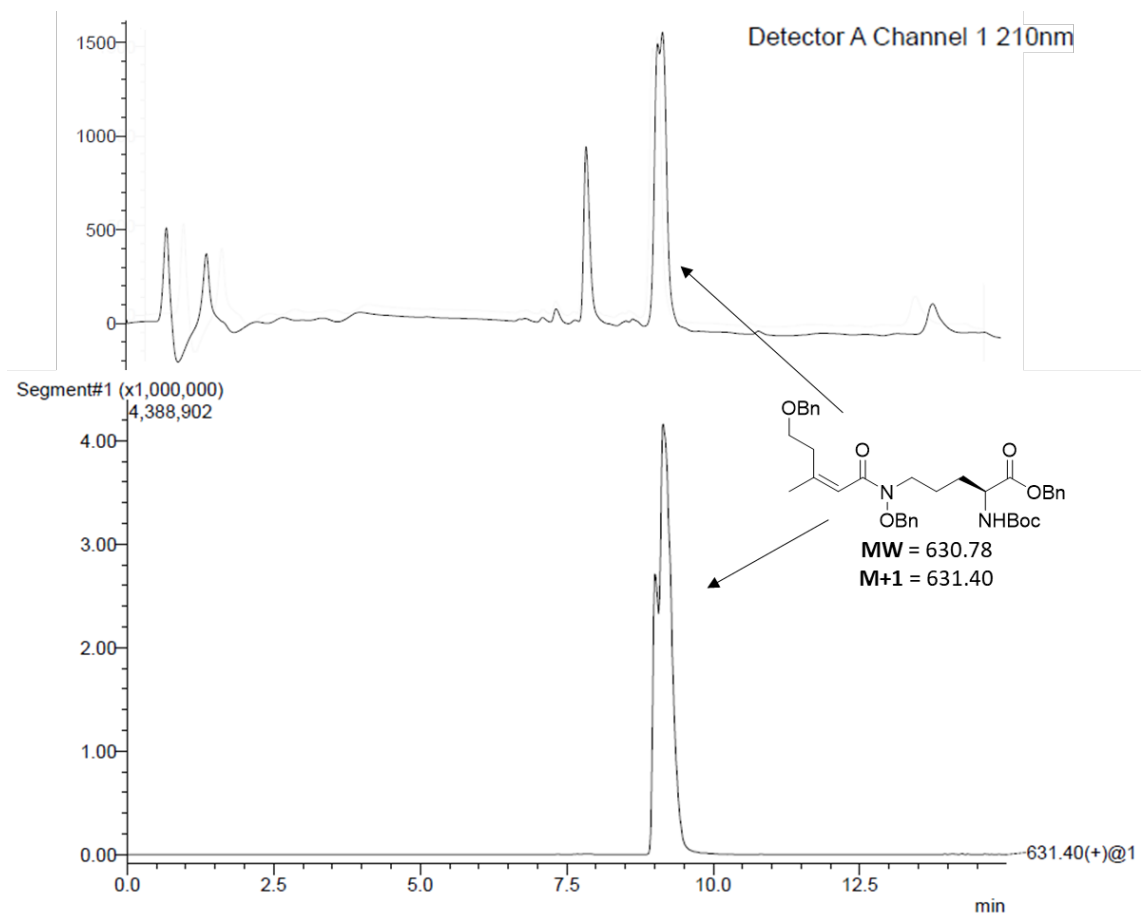
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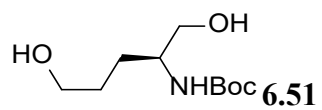


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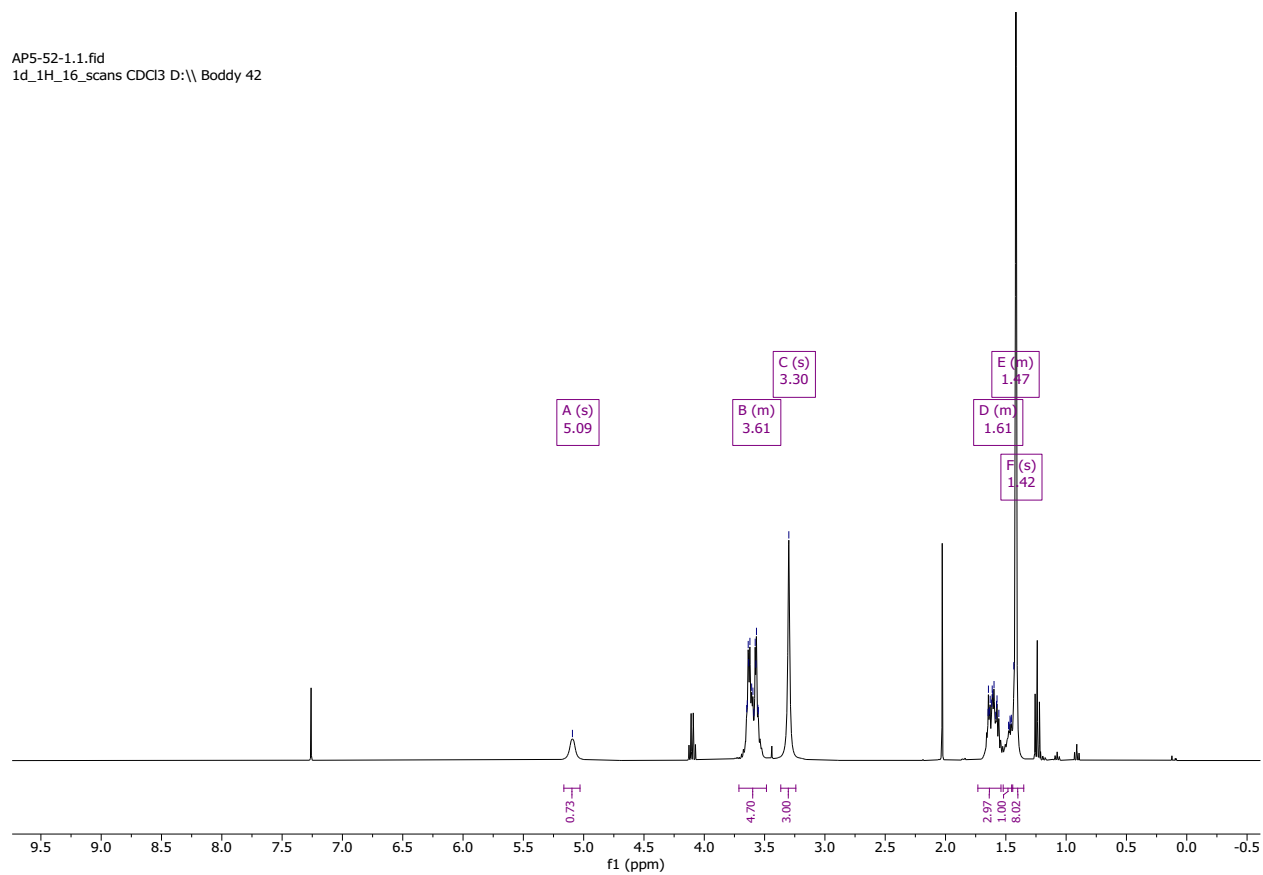


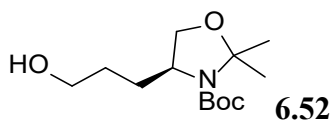
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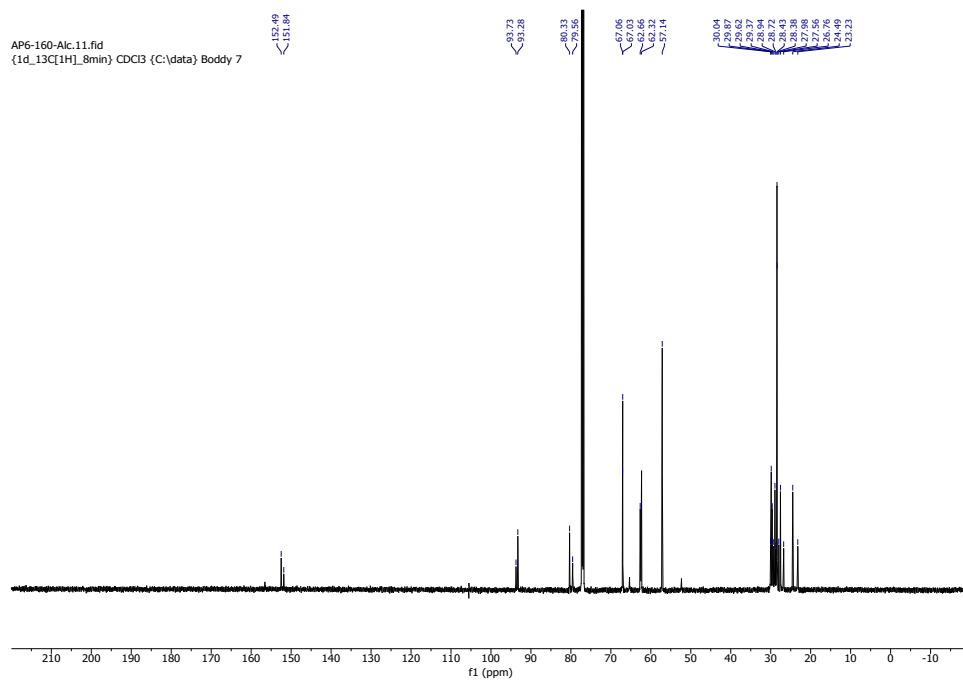
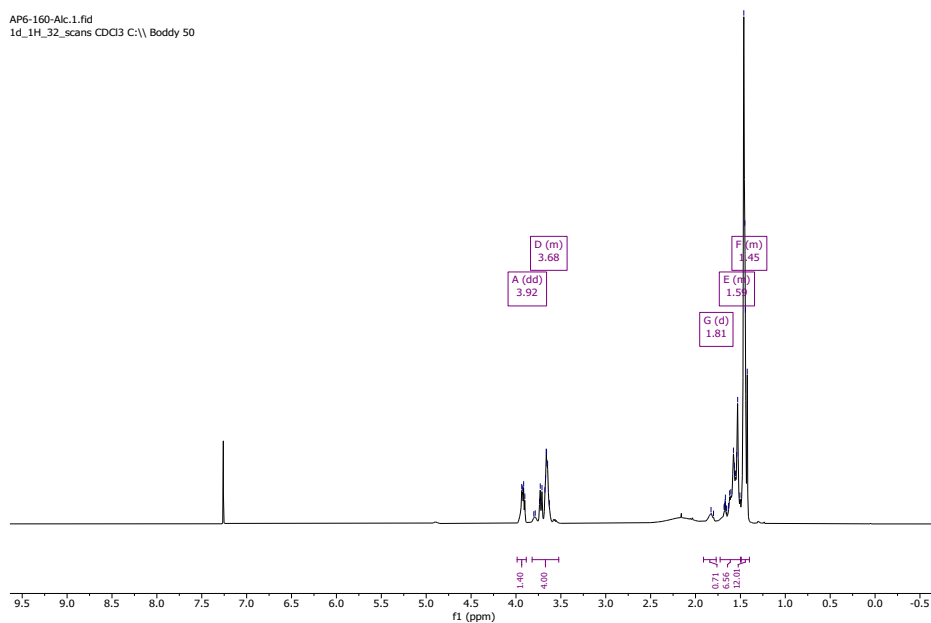


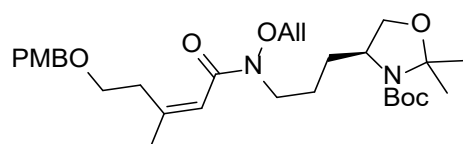
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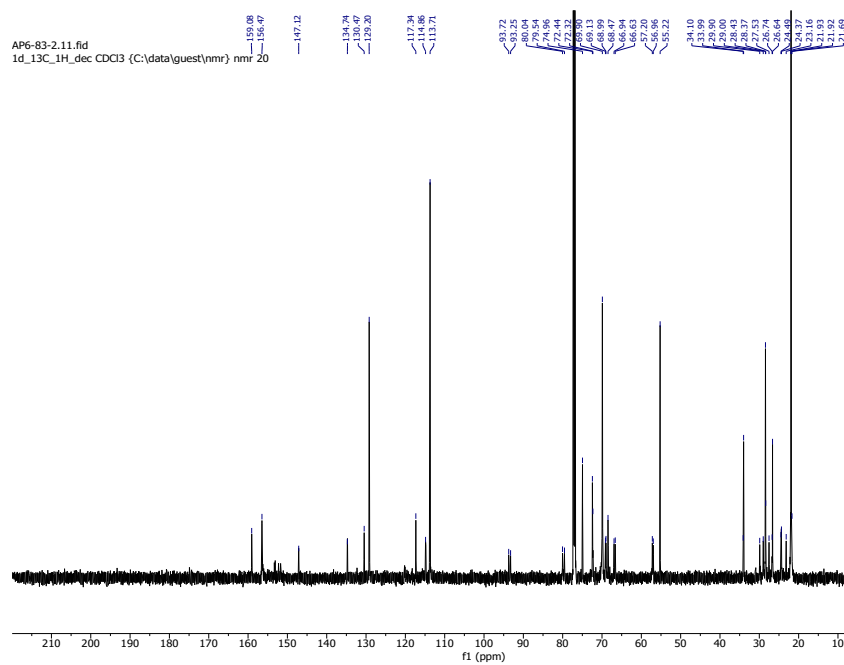
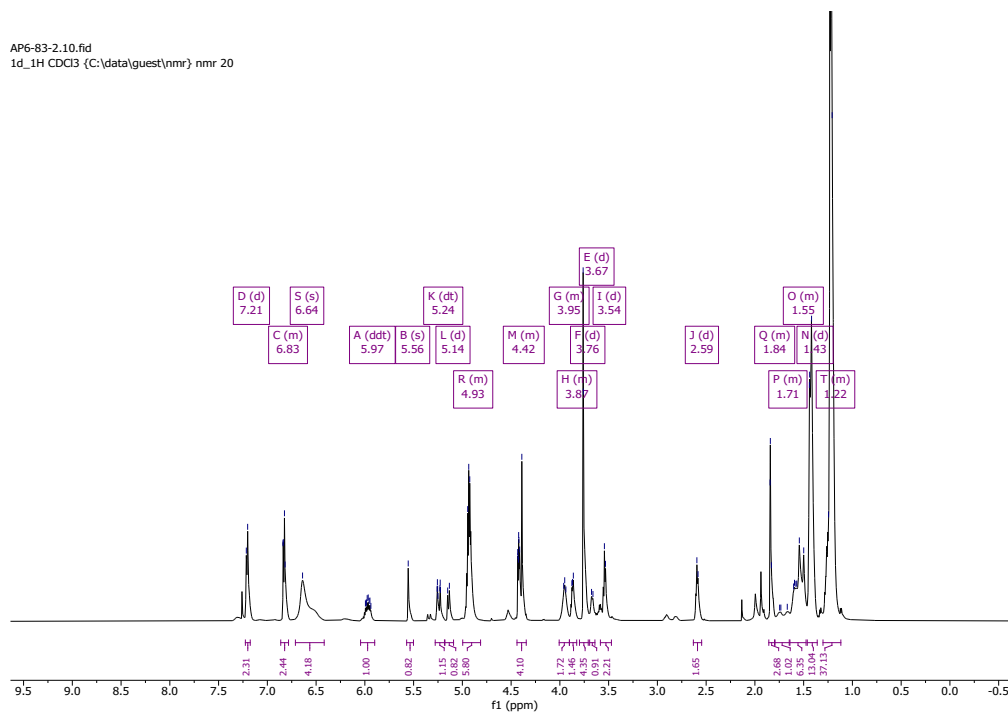
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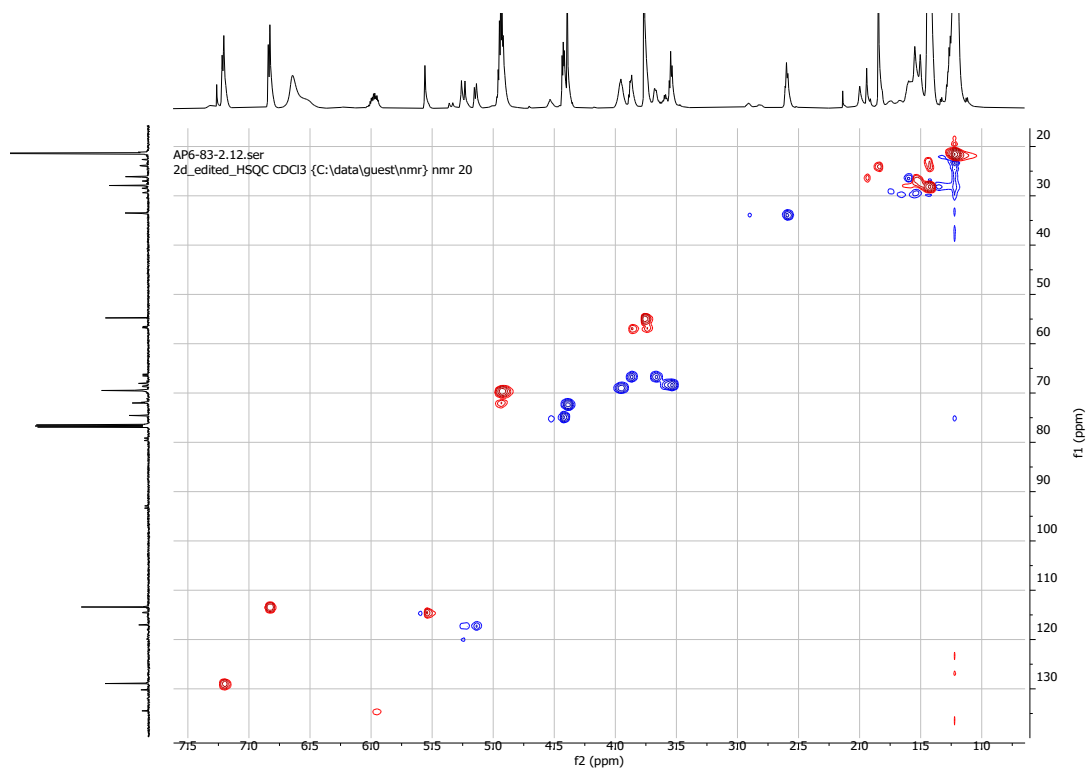


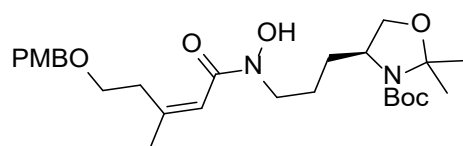


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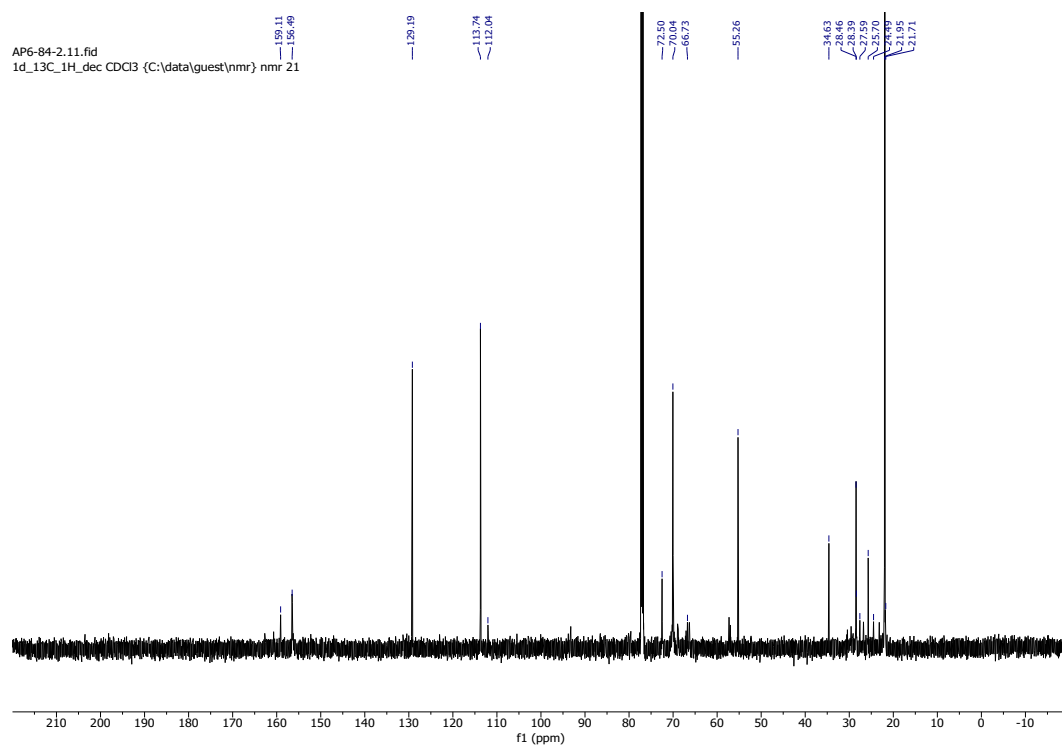
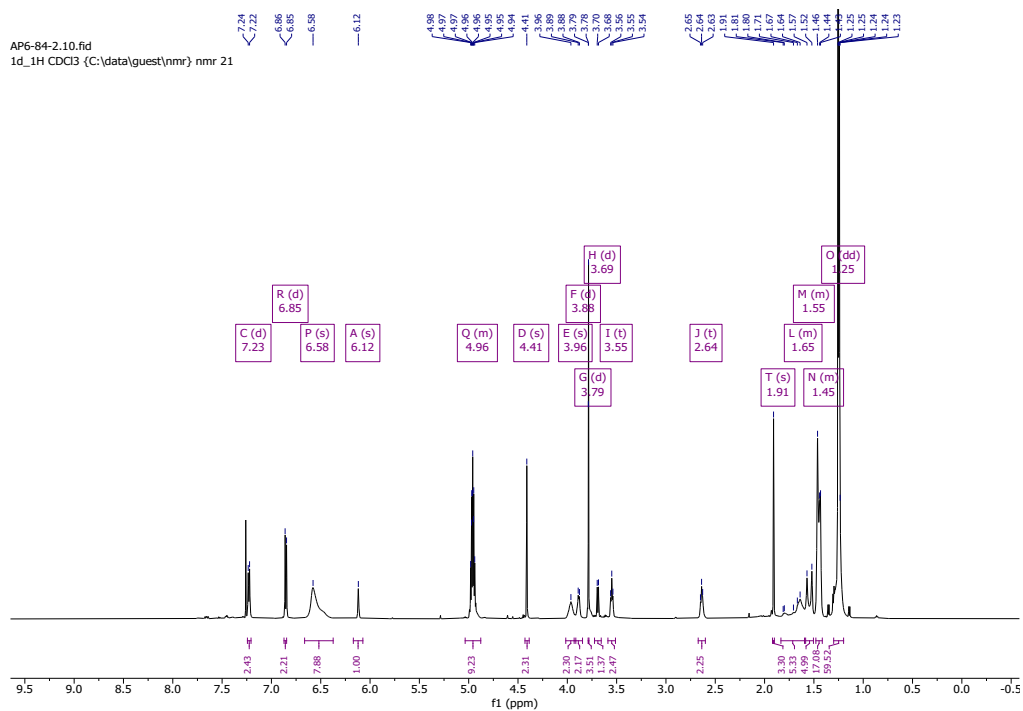
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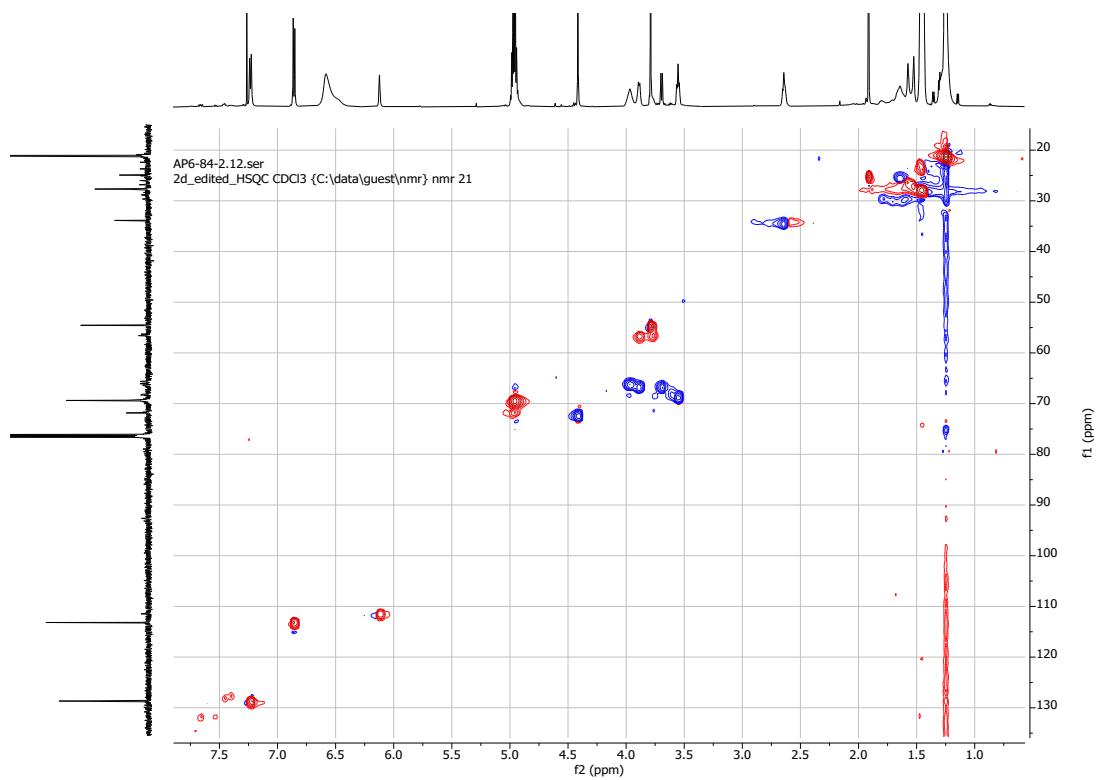




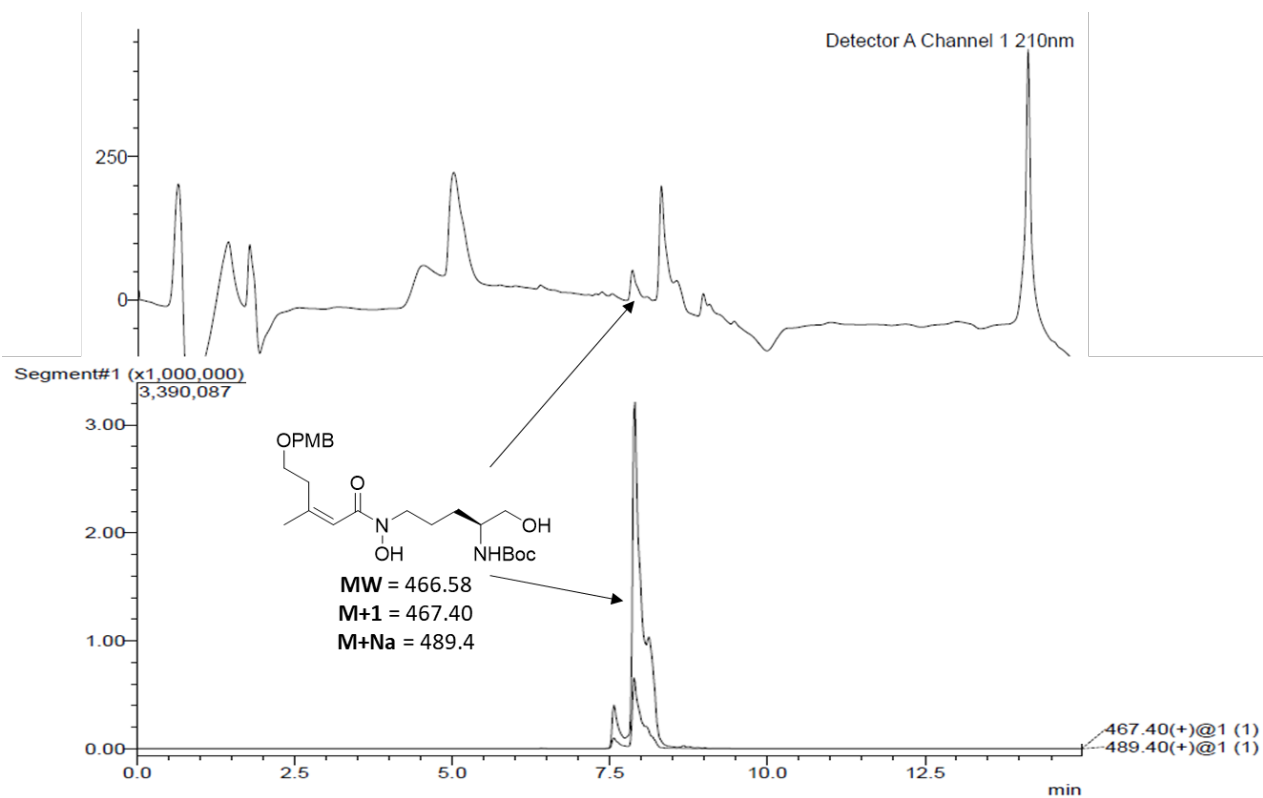


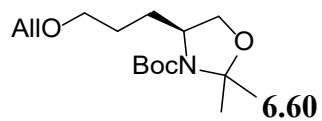
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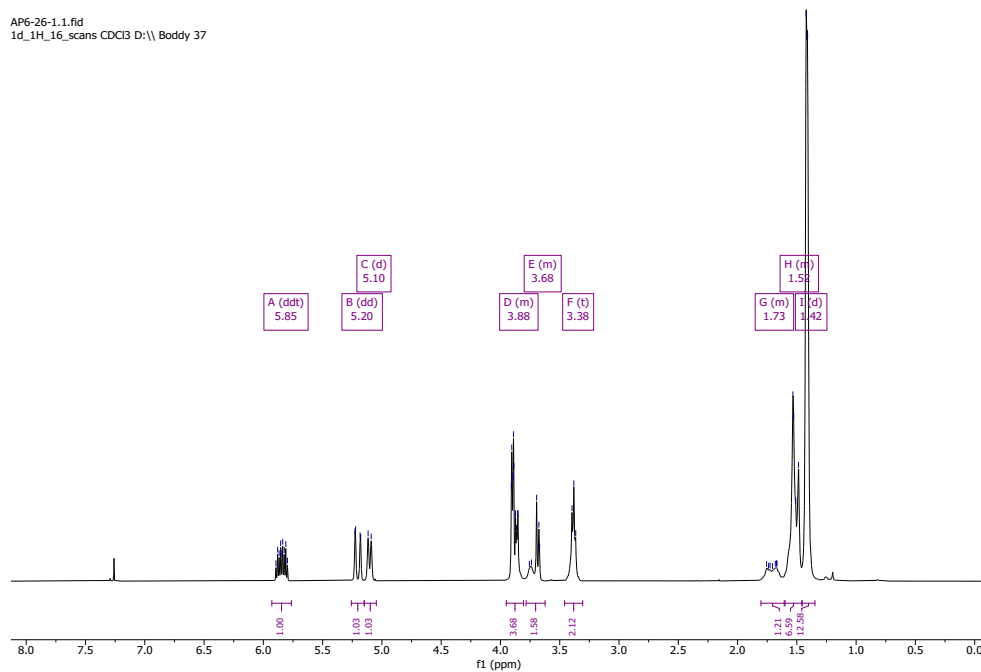


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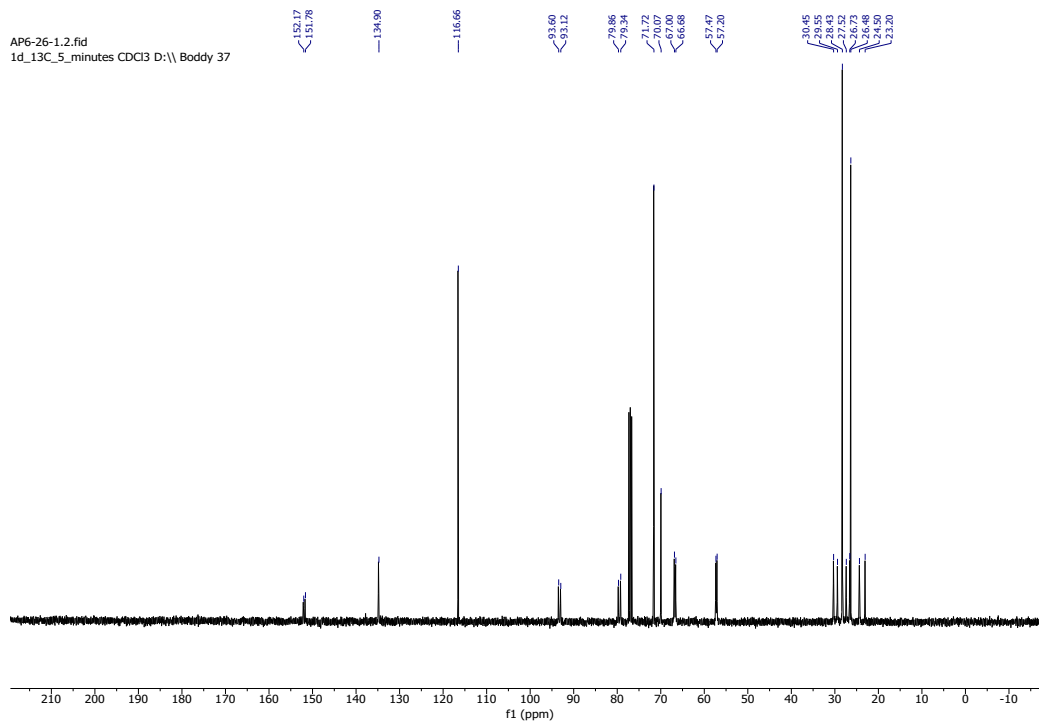


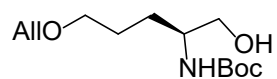


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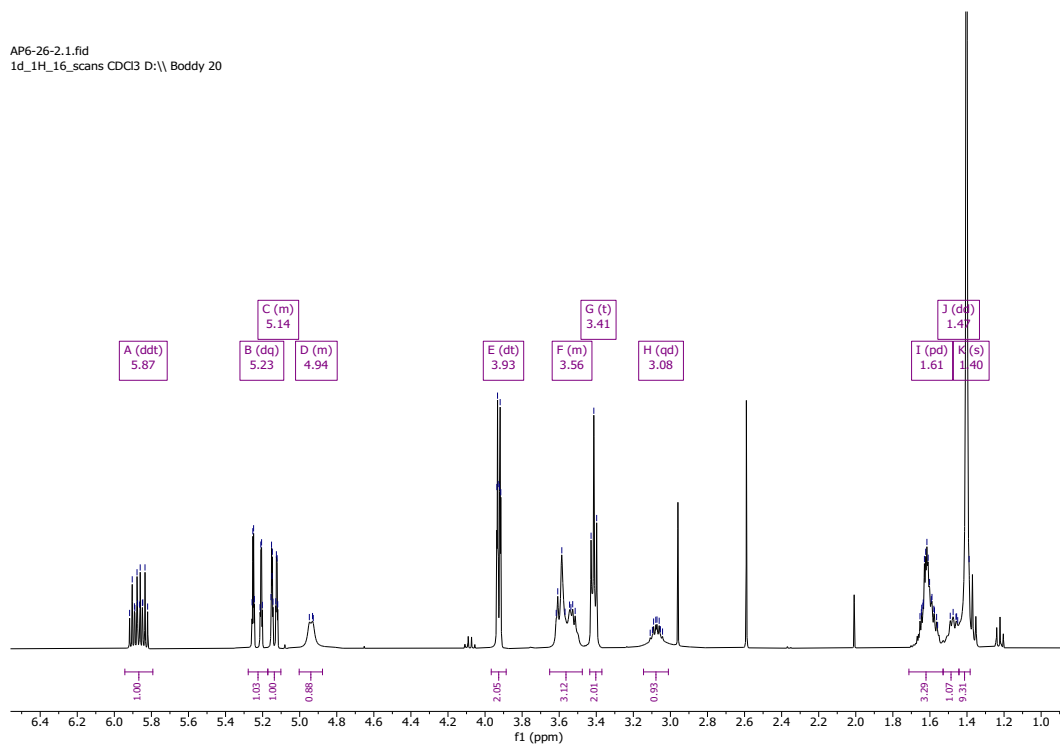


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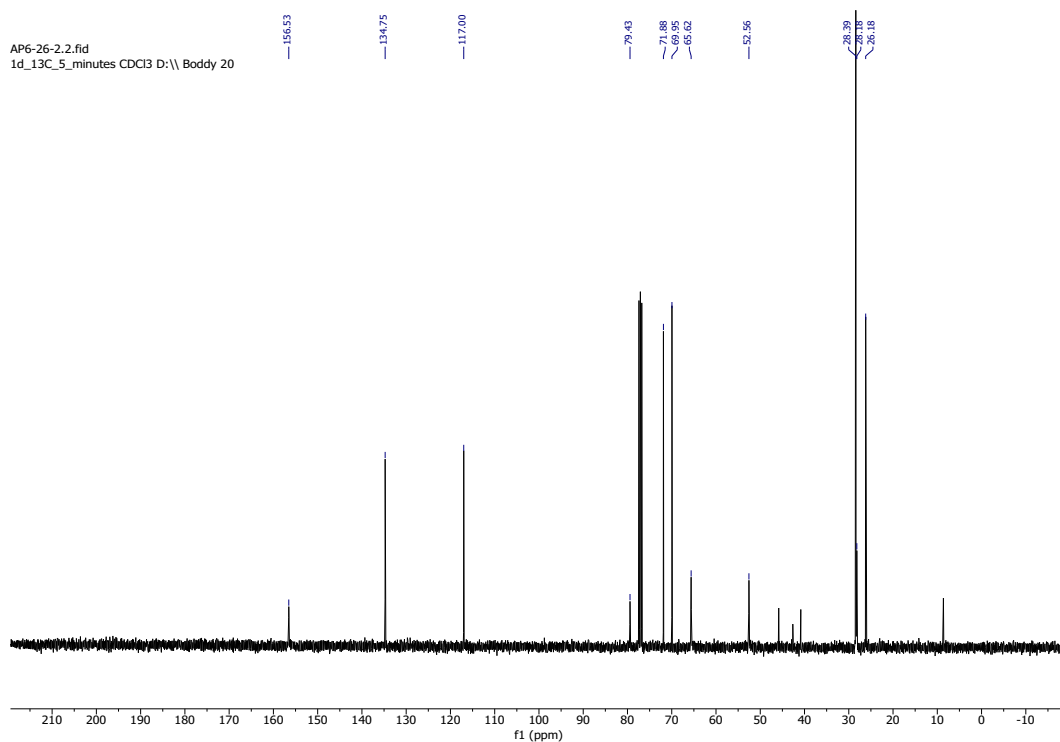


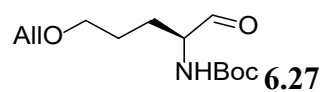


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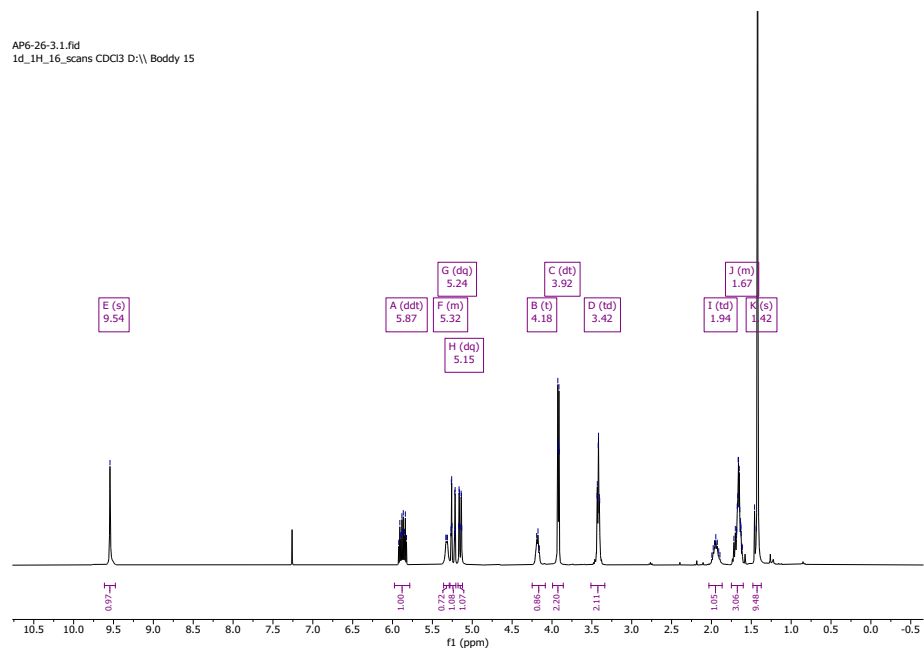


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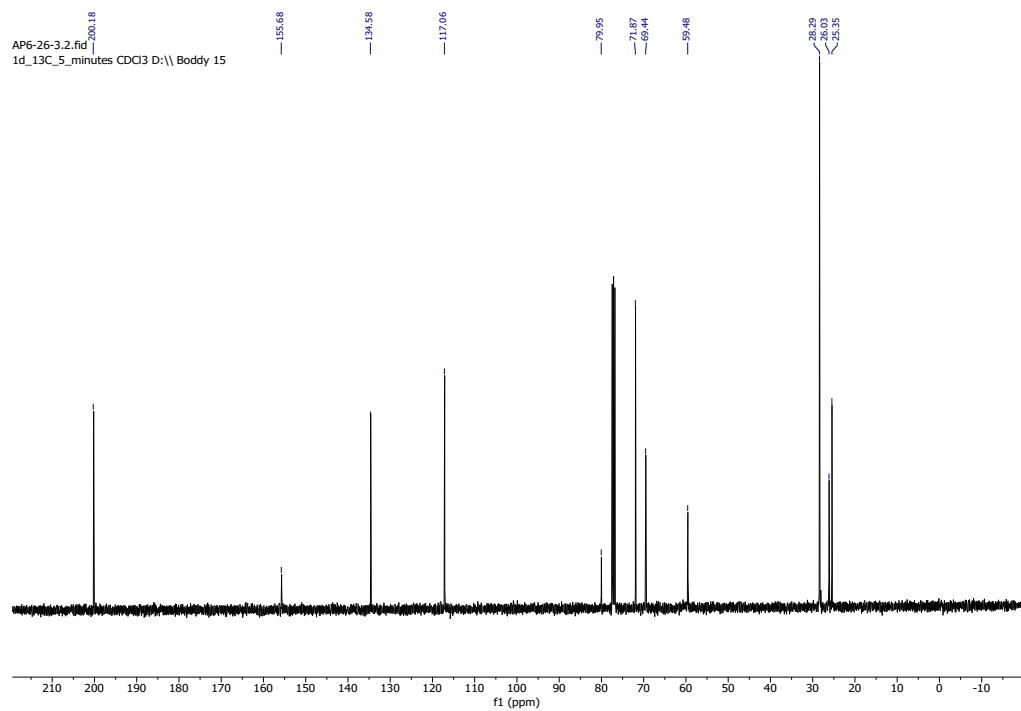


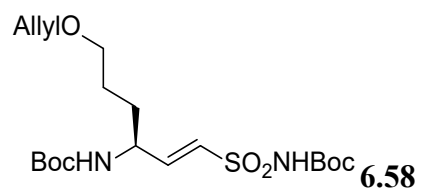


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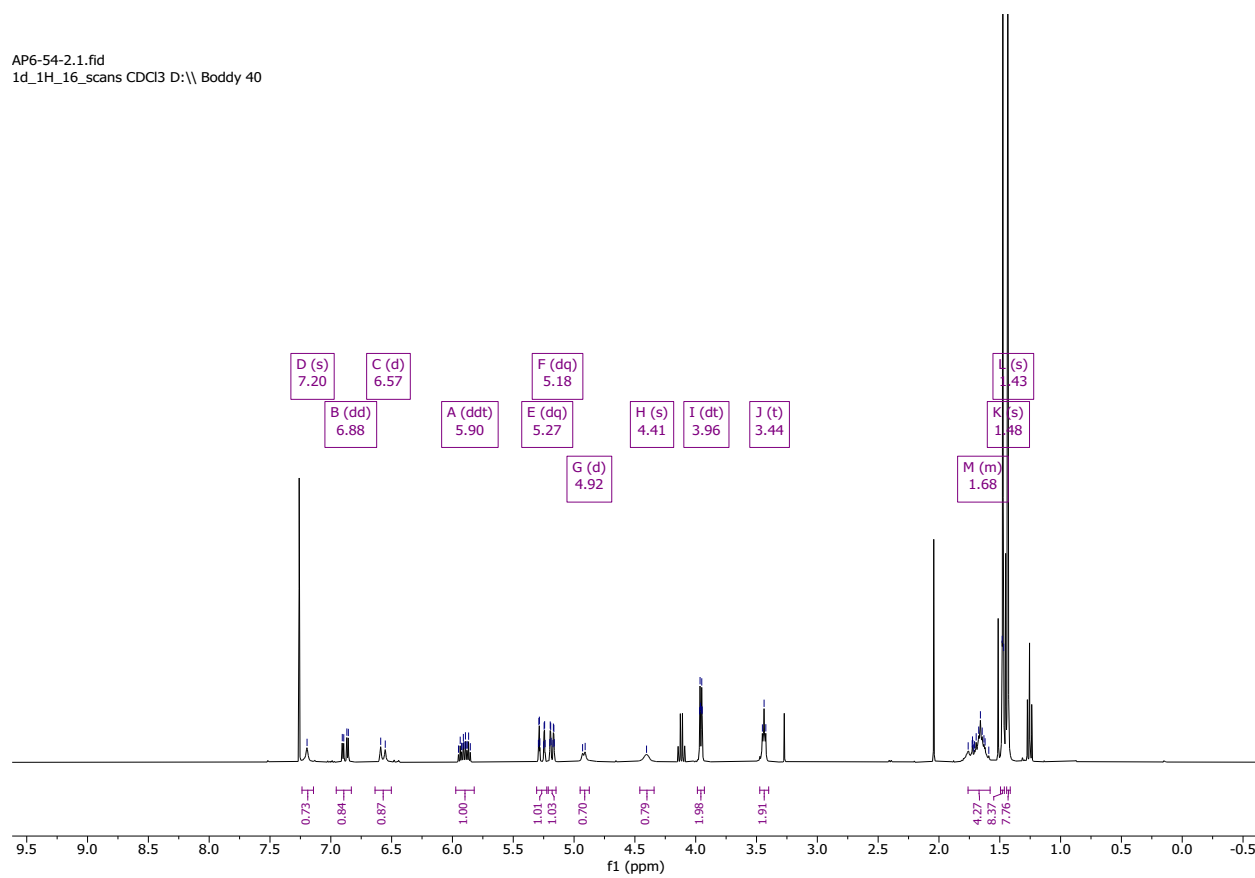


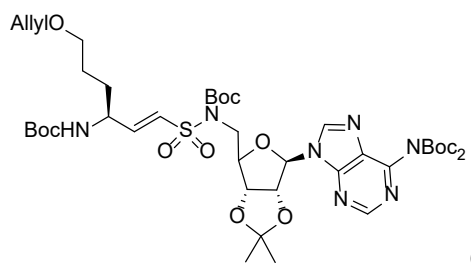
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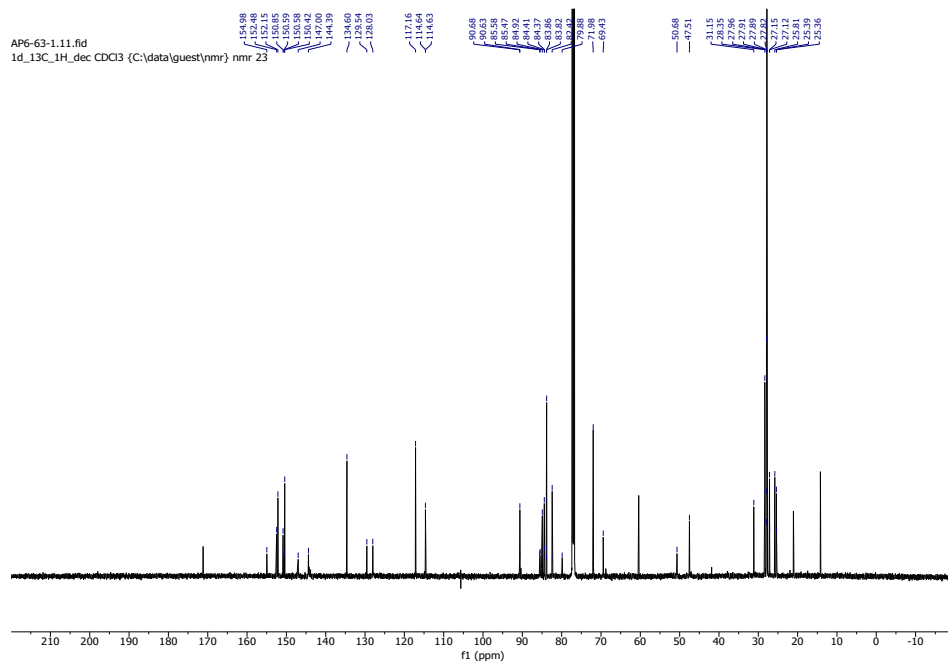
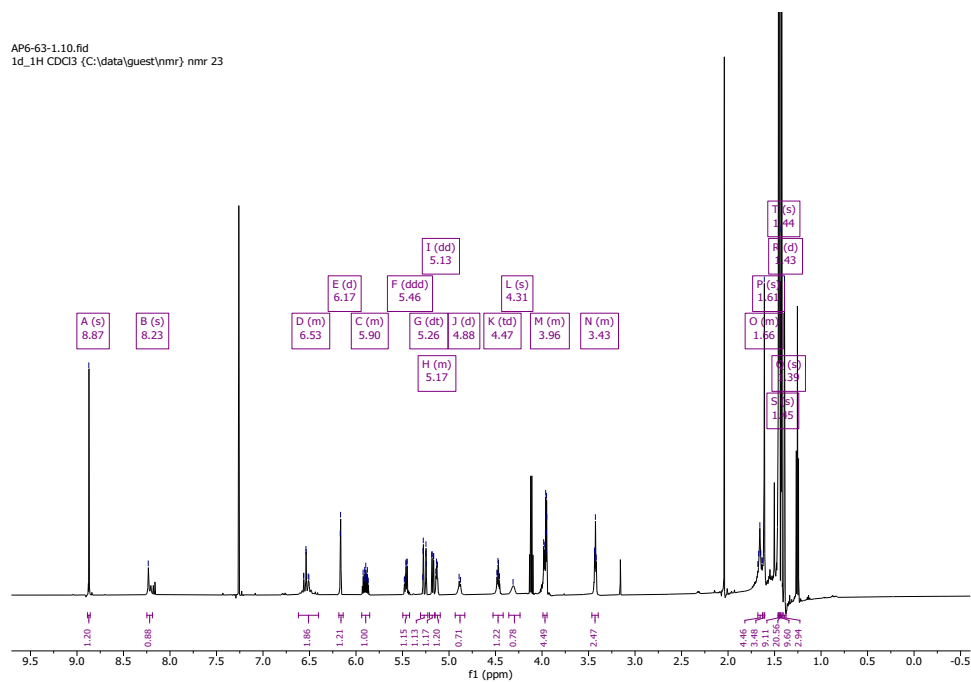
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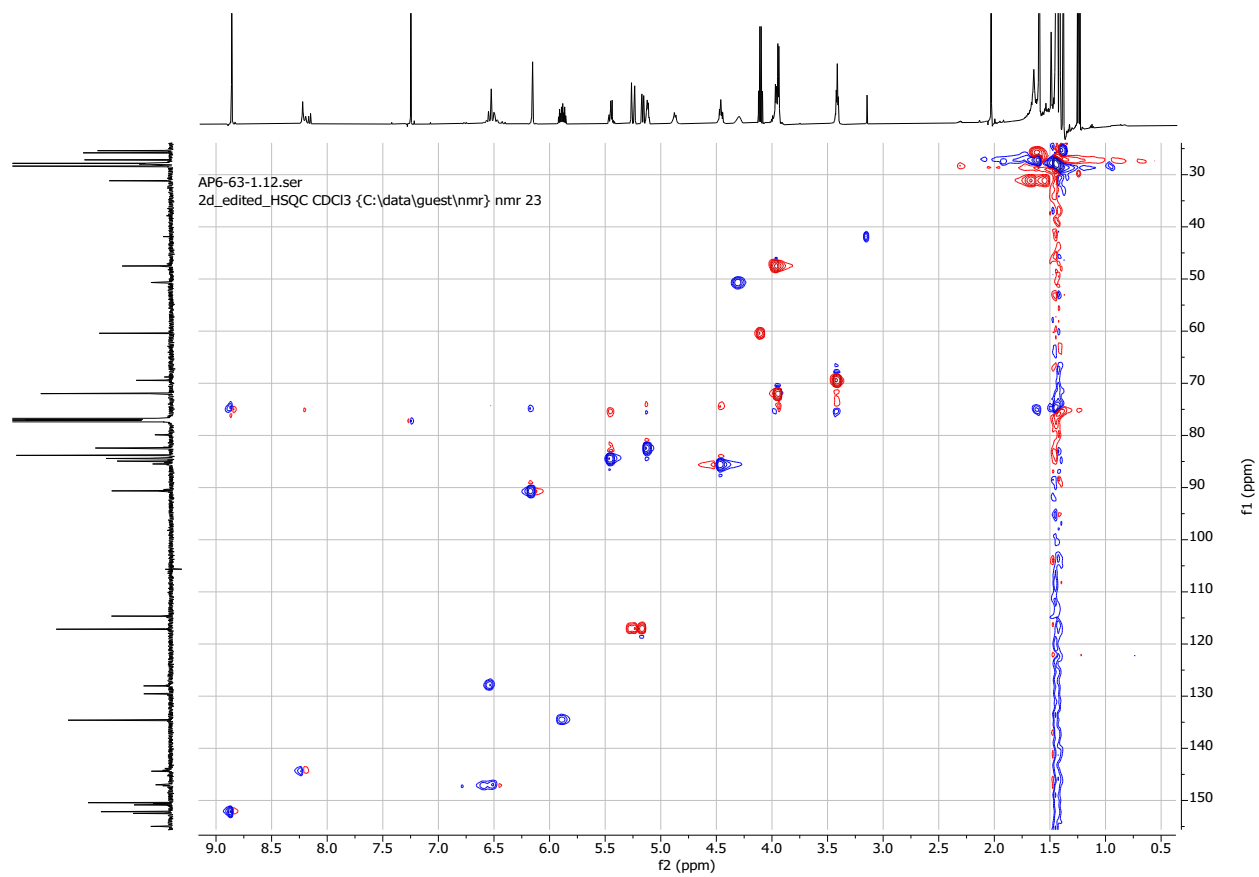


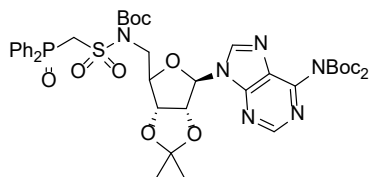


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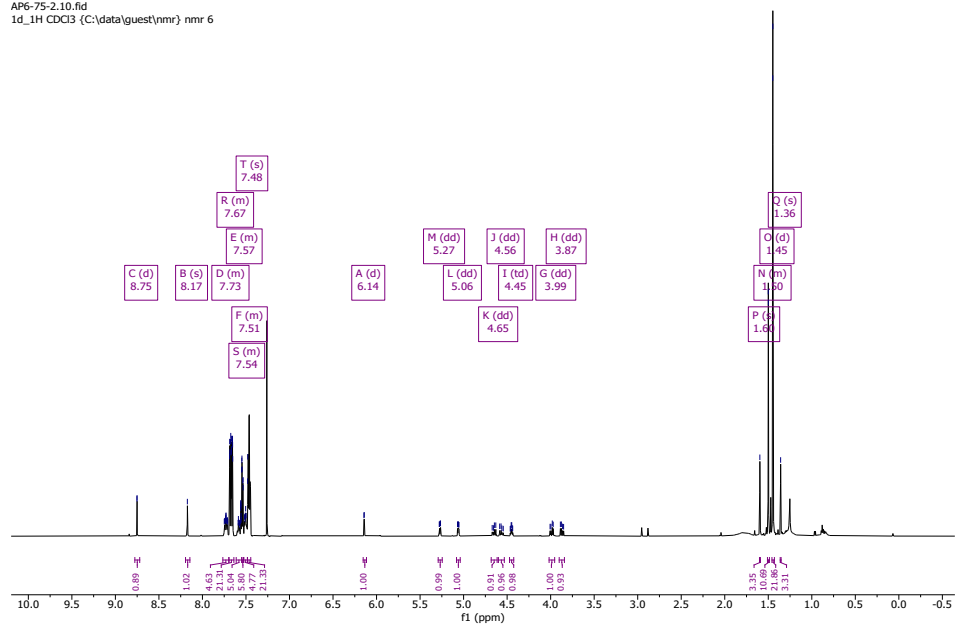




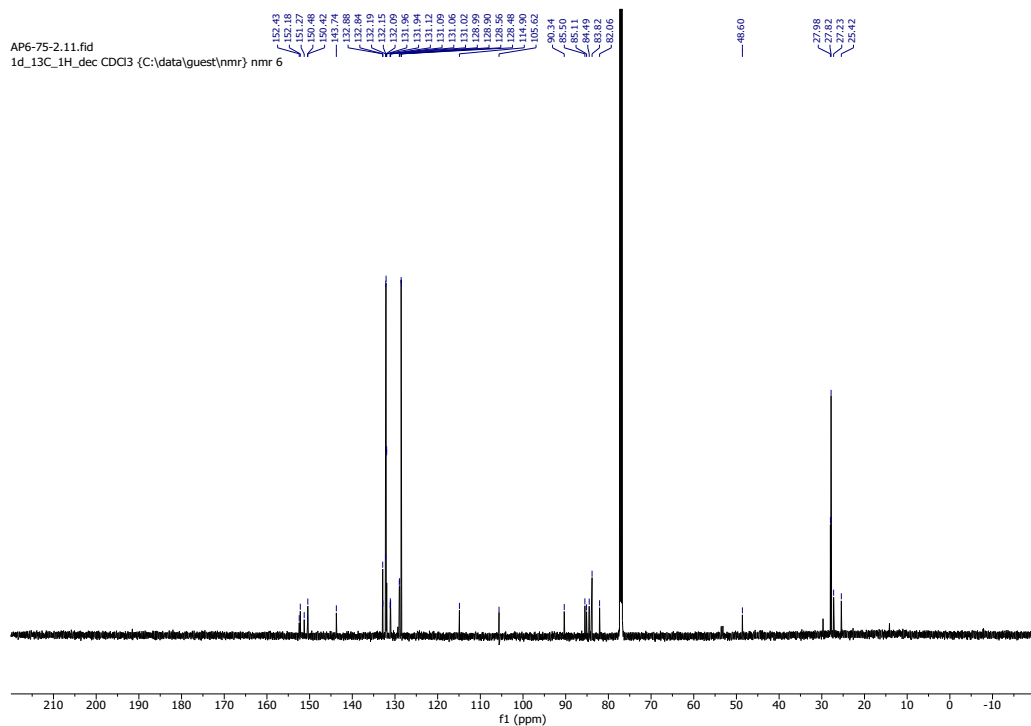


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Appendix 2 – Copyright information

Chapter 2

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Article Title	Double stranded DNA binding stapled peptides, an emerging tool for transcriptional regulation.	Rightsholder	John Wiley & Sons - Books
		Publication Type	e-Journal
		URL	http://www3.interscience.wiley.com/cgi-bin/jhome/72510898
Author/Editor	EU ChemSoc., Gesellschaft Österreichischer Chemiker., Société royale de chimie (Belgium), Koninklijke Vlaamse Chemische Vereniging., Česká společnost chemická., Société française de chimie., Gesellschaft Deutscher Chemiker., Henōsis Hellēnōn Chēmikōn., Magyar Kémikusok Egyesülete., Società chimica italiana., Koninklijke Nederlandse Chemische Vereniging., Polskie Towarzystwo Chemiczne., Sociedade Portuguesa de Química., Real Sociedad Española de Química., Svenska kemistsamfundet.		
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Title	Double Stranded DNA Binding Stapled Peptides: An Emerging Tool for Transcriptional Regulation	Institution Name	University of Ottawa
		Expected Presentation Date	2023-11-06
Instructor Name	Christopher N. Boddy		

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Title, Description or Numeric Reference of the Portion(s)	ChemBioChem	Title of the Article / Chapter the Portion Is From	Double stranded DNA binding stapled peptides, an emerging tool for transcriptional regulation.
Editor of Portion(s)	Paquette, André R; Boddy, Christopher	Author of Portion(s)	Paquette, André R; Boddy, Christopher
Volume / Edition	N/A	Publication Date of Portion	2023-09-26
Page or Page Range of Portion	1-13		

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C) Posting e-reserves, course management systems, e-coursepacks or other academic distribution for audiovisual content, which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorizations: (i) to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above; (ii) to display and perform the requested material to such members of such class in the physical classroom or remotely by means of streaming media or other video formats; and (iii) to “clip” or reformat the requested material for purposes of time or content management or ease of delivery, provided that such “clipping” or reformatting does not alter the underlying editorial content or meaning of the requested material and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms. Unless expressly set forth in the relevant Order Confirmation, the License does not authorize any other form of manipulation, alteration or editing of the requested material.

ii) Unless expressly set forth in the relevant Order Confirmation, no License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied or, in the case of Works subject to Sections 14(b)(1)(B) or (C) above, as described in such Sections) (ii) permit “publishing ventures” where any particular course materials would be systematically marketed at multiple institutions.

iii) Subject to any further limitations determined in the Rightsholder Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the electronic course content pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular materials, whether photocopied or electronic, at more than one institution of learning;

E) electronic access to material which is the subject of an electronic-use permission must be limited by means of electronic password, student identification or other control permitting access solely to students and instructors in the class;

F) User must ensure (through use of an electronic cover page or other appropriate means) that any person, upon gaining electronic access to the material, which is the subject of a permission, shall see:

- a proper copyright notice, identifying the Rightsholder in whose name CCC has granted permission,
- a statement to the effect that such copy was made pursuant to permission,
- a statement identifying the class to which the material applies and notifying the reader that the material has been made available electronically solely for use in the class, and
- a statement to the effect that the material may not be further distributed to any person outside the class, whether by copying or by transmission and whether electronically or in paper form, and User must also ensure that such cover page or other means will print out in the event that the person accessing the material chooses to print out the material or any part thereof.

G) any permission granted shall expire at the end of the class and, absent some other form of authorization, User is thereupon required to delete the applicable material from any electronic storage or to block electronic

access to the applicable material.

iv) Uses of separate portions of a Work, even if they are to be included in the same course material or the same university or college class, require separate permissions under the electronic course content pay-per-use Service. Unless otherwise provided in the Order Confirmation, any grant of rights to User is limited to use completed no later than the end of the academic term (or analogous period) as to which any particular permission is granted.

v) Books and Records; Right to Audit. As to each permission granted under the electronic course content Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any electronic copies used by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this license for any reason.

c) Pay-Per-Use Permissions for Certain Reproductions (Academic photocopies for library reserves and interlibrary loan reporting) (Non-academic internal/external business uses and commercial document delivery). The License expressly excludes the uses listed in Section (c)(i)-(v) below (which must be subject to separate license from the applicable Rightsholder) for: academic photocopies for library reserves and interlibrary loan reporting; and non-academic internal/external business uses and commercial document delivery.

i) electronic storage of any reproduction (whether in plain-text, PDF, or any other format) other than on a transitory basis;

ii) the input of Works or reproductions thereof into any computerized database;

iii) reproduction of an entire Work (cover-to-cover copying) except where the Work is a single article;

iv) reproduction for resale to anyone other than a specific customer of User;

v) republication in any different form. Please obtain authorizations for these uses through other CCC services or directly from the rightsholder.

Any license granted is further limited as set forth in any restrictions included in the Order Confirmation and/or in these Terms.

d) Electronic Reproductions in Online Environments (Non-Academic-email, intranet, internet and extranet). For "electronic reproductions", which generally includes e-mail use (including instant messaging or other electronic transmission to a defined group of recipients) or posting on an intranet, extranet or Intranet site (including any display or performance incidental thereto), the following additional terms apply:

i) Unless otherwise set forth in the Order Confirmation, the License is limited to use completed within 30 days for any use on the Internet, 60 days for any use on an intranet or extranet and one year for any other use, all as measured from the "republishing date" as identified in the Order Confirmation, if any, and otherwise from the date of the Order Confirmation.

ii) User may not make or permit any alterations to the Work, unless expressly set forth in the Order Confirmation (after request by User and approval by Rightsholder); provided, however, that a Work consisting of photographs or other still images not embedded in text may, if necessary, be resized, reformatted or have its resolution modified without additional express permission, and a Work consisting of audiovisual content may, if necessary, be "clipped" or reformatted for purposes of time or content management or ease of delivery (provided that any such resizing, reformatting, resolution modification or "clipping" does not alter the underlying editorial content or meaning of the Work used, and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular License described in the Order Confirmation and the Terms.

15) Miscellaneous.

a) User acknowledges that CCC may, from time to time, make changes or additions to the Service or to the Terms, and that Rightsholder may make changes or additions to the Rightsholder Terms. Such updated Terms will replace the

prior terms and conditions in the order workflow and shall be effective as to any subsequent Licenses but shall not apply to Licenses already granted and paid for under a prior set of terms.

b) Use of User-related information collected through the Service is governed by CCC's privacy policy, available online at www.copyright.com/about/privacy-policy/.

c) The License is personal to User. Therefore, User may not assign or transfer to any other person (whether a natural person or an organization of any kind) the License or any rights granted thereunder; provided, however, that, where applicable, User may assign such License in its entirety on written notice to CCC in the event of a transfer of all or substantially all of User's rights in any new material which includes the Work(s) licensed under this Service.

d) No amendment or waiver of any Terms is binding unless set forth in writing and signed by the appropriate parties, including, where applicable, the Rightsholder. The Rightsholder and CCC hereby object to any terms contained in any writing prepared by or on behalf of the User or its principals, employees, agents or affiliates and purporting to govern or otherwise relate to the License described in the Order Confirmation, which terms are in any way inconsistent with any Terms set forth in the Order Confirmation, and/or in CCC's standard operating procedures, whether such writing is prepared prior to, simultaneously with or subsequent to the Order Confirmation, and whether such writing appears on a copy of the Order Confirmation or in a separate instrument.

e) The License described in the Order Confirmation shall be governed by and construed under the law of the State of New York, USA, without regard to the principles thereof of conflicts of law. Any case, controversy, suit, action, or proceeding arising out of, in connection with, or related to such License shall be brought, at CCC's sole discretion, in any federal or state court located in the County of New York, State of New York, USA, or in any federal or state court whose geographical jurisdiction covers the location of the Rightsholder set forth in the Order Confirmation. The parties expressly submit to the personal jurisdiction and venue of each such federal or state court.

Last updated October 2022

Chapter 3

6/12/23, 3:41 PM

Manage Account



Order Number: 1361514

Order Date: 02 Jun 2023

Payment Information

André Paquette

Payment method: Invoice

Billing Address:

André Paquette
University of Ottawa
75 Laurier Ave E
Ottawa, ON K1N 6N5
Canada

Customer Location:

André Paquette
University of Ottawa
75 Laurier Ave E
Ottawa, ON K1N 6N5
Canada

Order Details

1. RSC Medicinal Chemistry

Article: RpoN-based stapled peptides with improved DNA binding suppress Pseudomonas aeruginosa virulence

Billing Status:
Open

Order License ID	1361514-1	Type of Use	Republish in a thesis/dissertation
Order detail status	Completed	Publisher	Royal Society of Chemistry
ISSN	2632-8682	Portion	Chapter/article
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LICENSED CONTENT

Publication Title	RSC Medicinal Chemistry	Publication Type	Journal
Article Title	RpoN-based stapled peptides with improved DNA binding suppress Pseudomonas aeruginosa virulence	Start Page	445
		End Page	455
		Issue	4
Date	01/01/2020	Volume	13
Country	United Kingdom of Great Britain and Northern Ireland		
Rightsholder	Royal Society of Chemistry		

REQUEST DETAILS

Portion Type	Chapter/article	Rights Requested	Main product
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1/7

Title	MACROCYCLIC PEPTIDES: CHEMISTRY AND BIOLOGY OF STAPLED AND DEPSIPEPTIDES	Institution Name	University of Ottawa
Instructor Name	Dr. Christopher N. Boddy	Expected Presentation Date	2023-07-28

ADDITIONAL DETAILS

**The Requesting
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REQUESTED CONTENT DETAILS

Title, Description or Numeric Reference of the Portion(s)	RSC Medicinal Chemistry	Title of the Article/Chapter the Portion Is From	RpoN-based stapled peptides with improved DNA binding supress Pseudomonas aeruginosa virulence
Editor of Portion(s)	Paquette, Andre; Payne, Sterling; McKay, Geoffrey; Brazeau-Henrie, Jordan; Darnowski, Michael; Kammili, Anitha; Bernal, Federico; Mah, Thien-Fah; Gruenheid, Samantha; Nguyen, Dao; Boddy, Christopher Noyce	Author of Portion(s)	Paquette, Andre; Payne, Sterling; McKay, Geoffrey; Brazeau-Henrie, Jordan; Darnowski, Michael; Kammili, Anitha; Bernal, Federico; Mah, Thien-Fah; Gruenheid, Samantha; Nguyen, Dao; Boddy, Christopher Noyce
Volume / Edition	13	Issue, if Republishing an Article From a Serial	4
Page or Page Range of Portion	445-455	Publication Date of Portion	2022-04-20

Total Items: 1

Subtotal: 0.00 CAD

Order Total: 0.00 CAD**Marketplace Permissions General Terms and Conditions**

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1) **Definitions.** For purposes of these General Terms, the following definitions apply:

"License" is the licensed use the User obtains via the Marketplace platform in a particular licensing transaction, as set forth in the Order Confirmation.

"Order Confirmation" is the confirmation CCC provides to the User at the conclusion of each Marketplace transaction. "Order Confirmation Terms" are additional terms set forth on specific Order Confirmations not set forth in the General Terms that can include terms applicable to a particular CCC transactional licensing service and/or any Rightsholder-specific terms.

"Rightsholder(s)" are the holders of copyright rights in the Works for which a User obtains licenses via the Marketplace platform, which are displayed on specific Order Confirmations.

"Terms" means the terms and conditions set forth in these General Terms and any additional Order Confirmation Terms collectively.

"User" or "you" is the person or entity making the use granted under the relevant License. Where the person accepting the Terms on behalf of a User is a freelancer or other third party who the User authorized to accept the General Terms on the User's behalf, such person shall be deemed jointly a User for purposes of such Terms.

"Work(s)" are the copyright protected works described in relevant Order Confirmations.

2) **Description of Service.** CCC's Marketplace enables Users to obtain Licenses to use one or more Works in accordance with all relevant Terms. CCC grants Licenses as an agent on behalf of the copyright rightsholder identified in the relevant Order Confirmation.

3) Applicability of Terms. The Terms govern User's use of Works in connection with the relevant License. In the event of any conflict between General Terms and Order Confirmation Terms, the latter shall govern. User acknowledges that Rightsholders have complete discretion whether to grant any permission, and whether to place any limitations on any grant, and that CCC has no right to supersede or to modify any such discretionary act by a Rightsholder.

4) Representations; Acceptance. By using the Service, User represents and warrants that User has been duly authorized by the User to accept, and hereby does accept, all Terms.

5) Scope of License; Limitations and Obligations. All Works and all rights therein, including copyright rights, remain the sole and exclusive property of the Rightsholder. The License provides only those rights expressly set forth in the terms and conveys no other rights in any Works

6) General Payment Terms. User may pay at time of checkout by credit card or choose to be invoiced. If the User chooses to be invoiced, the User shall: (i) remit payments in the manner identified on specific invoices, (ii) unless otherwise specifically stated in an Order Confirmation or separate written agreement, Users shall remit payments upon receipt of the relevant invoice from CCC, either by delivery or notification of availability of the invoice via the Marketplace platform, and (iii) if the User does not pay the invoice within 30 days of receipt, the User may incur a service charge of 1.5% per month or the maximum rate allowed by applicable law, whichever is less. While User may exercise the rights in the License immediately upon receiving the Order Confirmation, the License is automatically revoked and is null and void, as if it had never been issued, if CCC does not receive complete payment on a timely basis.

7) General Limits on Use. Unless otherwise provided in the Order Confirmation, any grant of rights to User (i) involves only the rights set forth in the Terms and does not include subsequent or additional uses, (ii) is non-exclusive and non-transferable, and (iii) is subject to any and all limitations and restrictions (such as, but not limited to, limitations on duration of use or circulation) included in the Terms. Upon completion of the licensed use as set forth in the Order Confirmation, User shall either secure a new permission for further use of the Work(s) or immediately cease any new use of the Work(s) and shall render inaccessible (such as by deleting or by removing or severing links or other locators) any further copies of the Work. User may only make alterations to the Work if and as expressly set forth in the Order Confirmation. No Work may be used in any way that is unlawful, including without limitation if such use would violate applicable sanctions laws or regulations, would be defamatory, violate the rights of third parties (including such third parties' rights of copyright, privacy, publicity, or other tangible or intangible property), or is otherwise illegal, sexually explicit, or obscene. In addition, User may not conjoin a Work with any other material that may result in damage to the reputation of the Rightsholder. Any unlawful use will render any licenses hereunder null and void. User agrees to inform CCC if it becomes aware of any infringement of any rights in a Work and to cooperate with any reasonable request of CCC or the Rightsholder in connection therewith.

8) Third Party Materials. In the event that the material for which a License is sought includes third party materials (such as photographs, illustrations, graphs, inserts and similar materials) that are identified in such material as having been used by permission (or a similar indicator), User is responsible for identifying, and seeking separate licenses (under this Service, if available, or otherwise) for any of such third party materials; without a separate license, User may not use such third party materials via the License.

9) Copyright Notice. Use of proper copyright notice for a Work is required as a condition of any License granted under the Service. Unless otherwise provided in the Order Confirmation, a proper copyright notice will read substantially as follows: "Used with permission of [Rightsholder's name], from [Work's title, author, volume, edition number and year of copyright]; permission conveyed through Copyright Clearance Center, Inc." Such notice must be provided in a reasonably legible font size and must be placed either on a cover page or in another location that any person, upon gaining access to the material which is the subject of a permission, shall see, or in the case of republication Licenses, immediately adjacent to the Work as used (for example, as part of a by-line or footnote) or in the place where substantially all other credits or notices for the new work containing the republished Work are located. Failure to include the required notice results in loss to the Rightsholder and CCC, and the User shall be liable to pay liquidated damages for each such failure equal to twice the use fee specified in the Order Confirmation, in addition to the use fee itself and any other fees and charges specified.

10) Indemnity. User hereby indemnifies and agrees to defend the Rightsholder and CCC, and their respective employees and directors, against all claims, liability, damages, costs, and expenses, including legal fees and expenses, arising out of any use of a Work beyond the scope of the rights granted herein and in the Order Confirmation, or any use of a Work which has been altered in any unauthorized way by User, including claims of defamation or infringement of rights of copyright, publicity, privacy, or other tangible or intangible property.

11) Limitation of Liability. UNDER NO CIRCUMSTANCES WILL CCC OR THE RIGHTSHOLDER BE LIABLE FOR ANY DIRECT, INDIRECT, CONSEQUENTIAL, OR INCIDENTAL DAMAGES (INCLUDING WITHOUT LIMITATION DAMAGES FOR LOSS OF BUSINESS PROFITS OR INFORMATION, OR FOR BUSINESS INTERRUPTION) ARISING OUT OF THE USE OR INABILITY TO USE A WORK, EVEN IF ONE OR BOTH OF THEM HAS BEEN ADVISED OF THE POSSIBILITY OF SUCH DAMAGES. In any event, the total liability of the Rightsholder and CCC (including their respective employees and directors) shall not exceed the total amount actually paid by User for the relevant License. User assumes full liability for the actions and omissions of its principals, employees, agents, affiliates, successors, and assigns.

12) Limited Warranties. THE WORK(S) AND RIGHT(S) ARE PROVIDED "AS IS." CCC HAS THE RIGHT TO GRANT TO USER THE RIGHTS GRANTED IN THE ORDER CONFIRMATION DOCUMENT. CCC AND THE RIGHTSHOLDER DISCLAIM ALL OTHER WARRANTIES RELATING TO THE WORK(S) AND RIGHT(S), EITHER EXPRESS OR IMPLIED, INCLUDING WITHOUT LIMITATION IMPLIED WARRANTIES OF

MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE. ADDITIONAL RIGHTS MAY BE REQUIRED TO USE ILLUSTRATIONS, GRAPHS, PHOTOGRAPHS, ABSTRACTS, INSERTS, OR OTHER PORTIONS OF THE WORK (AS OPPOSED TO THE ENTIRE WORK) IN A MANNER CONTEMPLATED BY USER; USER UNDERSTANDS AND AGREES THAT NEITHER CCC NOR THE RIGHTSHOLDER MAY HAVE SUCH ADDITIONAL RIGHTS TO GRANT.

13) Effect of Breach. Any failure by User to pay any amount when due, or any use by User of a Work beyond the scope of the License set forth in the Order Confirmation and/or the Terms, shall be a material breach of such License. Any breach not cured within 10 days of written notice thereof shall result in immediate termination of such License without further notice. Any unauthorized (but licensable) use of a Work that is terminated immediately upon notice thereof may be liquidated by payment of the Rightsholder's ordinary license price therefor; any unauthorized (and unlicensable) use that is not terminated immediately for any reason (including, for example, because materials containing the Work cannot reasonably be recalled) will be subject to all remedies available at law or in equity, but in no event to a payment of less than three times the Rightsholder's ordinary license price for the most closely analogous licensable use plus Rightsholder's and/or CCC's costs and expenses incurred in collecting such payment.

14) Additional Terms for Specific Products and Services. If a User is making one of the uses described in this Section 14, the additional terms and conditions apply:

a) Print Uses of Academic Course Content and Materials (photocopies for academic coursepacks or classroom handouts). For photocopies for academic coursepacks or classroom handouts the following additional terms apply:

i) The copies and anthologies created under this License may be made and assembled by faculty members individually or at their request by on-campus bookstores or copy centers, or by off-campus copy shops and other similar entities.

ii) No License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied) (ii) permit "publishing ventures" where any particular anthology would be systematically marketed at multiple institutions.

iii) Subject to any Publisher Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the academic pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to no more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular anthology, whether photocopied or electronic, at more than one institution of learning;

E) in the case of a photocopy permission, no materials may be entered into electronic memory by User except in order to produce an identical copy of a Work before or during the academic term (or analogous period) as to which any particular permission is granted. In the event that User shall choose to retain materials that are the subject of a photocopy permission in electronic memory for purposes of producing identical copies more than one day after such retention (but still within the scope of any permission granted), User must notify CCC of such fact in the applicable permission request and such retention shall constitute one copy actually sold for purposes of calculating permission fees due; and

F) any permission granted shall expire at the end of the class. No permission granted shall in any way include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied).

iv) **Books and Records; Right to Audit.** As to each permission granted under the academic pay-per-use Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any photocopies sold or by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this License for any reason.

b) Digital Pay-Per-Uses of Academic Course Content and Materials (e-coursepacks, electronic reserves, learning management systems, academic institution intranets). For uses in e-coursepacks, posts in electronic reserves, posts in learning management systems, or posts on academic institution intranets, the following additional terms apply:

i) The pay-per-uses subject to this Section 14(b) include:

A) **Posting e-reserves, course management systems, e-coursepacks for text-based content**, which grants authorizations to import requested material in electronic format, and allows electronic access to this material to members of a designated college or university class, under the direction of an instructor designated by the college or university, accessible only under appropriate electronic controls (e.g., password);

B) **Posting e-reserves, course management systems, e-coursepacks for material consisting of photographs or other still images not embedded in text**, which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorization: to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above, including any necessary resizing, reformatting or modification of the resolution of such requested material (provided that such modification does not alter the underlying editorial content or meaning of the requested material, and provided that the resulting modified content is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms), but not including any other form of manipulation, alteration or editing of the requested material;

C) **Posting e-reserves, course management systems, e-coursepacks or other academic distribution for audiovisual content**, which grants not only the authorizations described in Section 14(b)(i)(A) above, but also the following authorizations: (i) to include the requested material in course materials for use consistent with Section 14(b)(i)(A) above; (ii) to display and perform the requested material to such members of such class in the physical classroom or remotely by means of streaming media or other video formats; and (iii) to "clip" or reformat the requested material for purposes of time or content management or ease of delivery, provided that such "clipping" or reformatting does not alter the underlying editorial content or meaning of the requested material and that the resulting material is used solely within the scope of, and in a manner consistent with, the particular authorization described in the Order Confirmation and the Terms. Unless expressly set forth in the relevant Order Confirmation, the License does not authorize any other form of manipulation, alteration or editing of the requested material.

ii) Unless expressly set forth in the relevant Order Confirmation, no License granted shall in any way: (i) include any right by User to create a substantively non-identical copy of the Work or to edit or in any other way modify the Work (except by means of deleting material immediately preceding or following the entire portion of the Work copied or, in the case of Works subject to Sections 14(b)(1)(B) or (C) above, as described in such Sections) (ii) permit "publishing ventures" where any particular course materials would be systematically marketed at multiple institutions.

iii) Subject to any further limitations determined in the Rightsholder Terms (and notwithstanding any apparent contradiction in the Order Confirmation arising from data provided by User), any use authorized under the electronic course content pay-per-use service is limited as follows:

A) any License granted shall apply to only one class (bearing a unique identifier as assigned by the institution, and thereby including all sections or other subparts of the class) at one institution;

B) use is limited to not more than 25% of the text of a book or of the items in a published collection of essays, poems or articles;

C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular materials, whether photocopied or electronic, at more than one institution of learning;

E) electronic access to material which is the subject of an electronic-use permission must be limited by means of electronic password, student identification or other control permitting access solely to students and instructors in the class;

F) User must ensure (through use of an electronic cover page or other appropriate means) that any person, upon gaining electronic access to the material, which is the subject of a permission, shall see:

- a proper copyright notice, identifying the Rightsholder in whose name CCC has granted permission,
- a statement to the effect that such copy was made pursuant to permission,
- a statement identifying the class to which the material applies and notifying the reader that the material has been made available electronically solely for use in the class, and
- a statement to the effect that the material may not be further distributed to any person outside the class, whether by copying or by transmission and whether electronically or in paper form, and User must also ensure that such cover page or other means will print out in the event that the person accessing the material chooses to print out the material or any part thereof.

G) any permission granted shall expire at the end of the class and, absent some other form of authorization, User is thereupon required to delete the applicable material from any electronic storage or to block electronic access to the applicable material.

iv) Uses of separate portions of a Work, even if they are to be included in the same course material or the same university or college class, require separate permissions under the electronic course content pay-per-use Service. Unless otherwise provided in the Order Confirmation, any grant of rights to User is limited to use completed no later than the end of the academic term (or analogous period) as to which any particular permission is granted.

v) Books and Records; Right to Audit. As to each permission granted under the electronic course content Service, User shall maintain for at least four full calendar years books and records sufficient for CCC to determine the numbers of copies made by User under such permission. CCC and any representatives it may designate shall have the right to audit such books and records at any time during User's ordinary business hours, upon two days' prior notice. If any such audit shall determine that User shall have underpaid for, or underreported, any electronic copies used by three percent (3%) or more, then User shall bear all the costs of any such audit; otherwise, CCC shall bear the costs of any such audit. Any amount determined by such audit to have been underpaid by User shall immediately be paid to CCC by User, together with interest thereon at the rate of 10% per annum from the date such amount was originally due. The provisions of this paragraph shall survive the termination of this license for any reason.

c) Pay-Per-Use Permissions for Certain Reproductions (Academic photocopies for library reserves and interlibrary loan reporting) (Non-academic internal/external business uses and commercial document delivery). The License expressly excludes the uses listed in Section (c)(i)-(v) below (which must be subject to separate license from the applicable Rightsholder) for: academic photocopies for library reserves and interlibrary loan reporting; and non-academic internal/external business uses and commercial document delivery.

- i) electronic storage of any reproduction (whether in plain-text, PDF, or any other format) other than on a transitory basis;
- ii) the input of Works or reproductions thereof into any computerized database;
- iii) reproduction of an entire Work (cover-to-cover copying) except where the Work is a single article;
- iv) reproduction for resale to anyone other than a specific customer of User;
- v) republication in any different form. Please obtain authorizations for these uses through other CCC services or directly from the rightsholder.

Any license granted is further limited as set forth in any restrictions included in the Order Confirmation and/or in these Terms.

d) Electronic Reproductions in Online Environments (Non-Academic-email, intranet, internet and extranet). For "electronic reproductions", which generally includes e-mail use (including instant messaging or other electronic transmission to a defined group of recipients) or posting on an intranet, extranet or Intranet site (including any display or performance incidental thereto), the following additional terms apply:

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Last updated October 2022

Chapter 4

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Publication Title	Organic & biomolecular chemistry	Country	United Kingdom of Great Britain and Northern Ireland
Article Title	Macrocyclization strategies for the total synthesis of cyclic depsipeptides	Rightsholder	Royal Society of Chemistry
Author/Editor	Royal Society of Chemistry (Great Britain)	Publication Type	e-Journal
Date	01/01/2003	URL	http://www.rsc.org/is/journals/current/obc/obcpub.htm
Language	English		

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Title	Macrocyclization strategies for the total synthesis of cyclic depsipeptides	Institution Name	University of Ottawa
Instructor Name	Christopher N. Boddy	Expected Presentation Date	2023-11-06

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Title, Description or Numeric Reference of the Portion(s)	Organic & Biomolecular Chemistry	Title of the Article / Chapter the Portion Is From	Macrocyclization strategies for the total synthesis of cyclic depsipeptides
Editor of Portion(s)	Paquette, Andre; Boddy, Christopher Noyce	Author of Portion(s)	Paquette, Andre; Boddy, Christopher Noyce
Volume / Edition	N/A	Issue, if Republishing an Article From a Serial	N/A
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C) use is limited to no more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

D) no User may sell or distribute any particular anthology, whether photocopied or electronic, at more than one institution of learning;

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C) use is limited to not more than the greater of (a) 25% of the text of an issue of a journal or other periodical or (b) two articles from such an issue;

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15) Miscellaneous.

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Last updated October 2022

Chapter 5



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Total and Chemoenzymatic Synthesis of Seongsanamide E

Author: Jordan T. Brazeau-Henrie, André R. Paquette, Allison Q. O'Rourke, et al

Publication: Organic Letters

Publisher: American Chemical Society

Date: Sep 1, 2022

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