Solid-Phase Total Synthesis of Dehydrotryptophan-Bearing Cyclic Peptides Tunicyclin B, Sclerotide A, CDA3a and CDA4a using a Protected β- Hydroxytryptophan Building Block

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including

any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Abstract

The non-proteinogenic amino acid Z-dehydrotryptophan can be found in a several naturally occurring peptide. Amongst them are cyclic peptides such as tunicyclin B, the antifungal agents keramamide F and sclerotide A, as well as the antibiotic telomycin and the calcium-dependent antibiotics (CDAs). Despite there being numerous means of accessing simple Z-dehydrotryptophan building blocks, it was only recently that the first synthesis of a Z-dehydrotryptophan peptide containing more than two amino acids was reported. However, the synthetic method reported in this study is labor-intensive and not practical for rapid synthesis of Z-dehydrotryptophan containing peptides.

In Chapter 2, a new and highly efficient approach to the synthesis of Z-dehydrotryptophan peptides is initially described. This approach uses Fmoc-β-HOTrp(Boc)(TBS)-OH as a building block, which is readily prepared in high yield and incorporated into peptides using solid-phase Fmoc chemistry. The TBS-protected indolic alcohol eliminates during global deprotection/resin cleavage to give Z-dehydrotryptophan peptides exclusively as the thermodynamically favored Z-isomer. This approach was applied to the solid-phase synthesis of tunicyclin B and sclerotide A which were prepared in 28 % and 24 % overall yields respectively.

In Chapter 3, this method was used in the solid-phase synthesis of more challenging targets which included biologically active CDA3a and CDA4a which were prepared in 6 % and 8.5 % overall yields respectively. Furthermore, using a similar solid-phase approach, two CDA analogs, which contain either Trp or Kyn in place of the Z-dehydrotryptophan of CDA4a, were prepared. The antibacterial potencies of CDA3a, CDA4a and these CDA analogs were assessed in

order to assemble a preliminary structure activity relation (SAR) study. Finally, early biological studies were conducted on Z-dehydrotryptophan containing CDA4a in order to begin to decipher the CDA's biological mechanism of action (MoA).

Chapter 4 offers general conclusions about the work within this thesis. Furthermore, the chapter presents recommendations for future work to be conducted in a continuation of the findings outlined within this thesis. They include pursuing the synthesis of other, complex Z-dehydrotryptophan containing peptides as well as preparing further CDA analogs in order to conduct a thorough SAR study on this class of compounds.

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List of Abbreviations

2′-Cl-Trt	2′-chlorotrityl polystyrene resin
°C	degrees Celsius
K	kelvin
2-MP	2-methylpiperidine
4-MP	4-methylpiperidine
Ac	acetyl
AcOH	acetic acid
Ac₂O	acetic anhydride
Ala	alanine
Alloc	allyloxycarbonyl
Ant	anthranilic acid
Asn	asparagine
Asp	aspartate
aq	aqueous
Bn	benzyl
Bz	benzoyl
BOP	(benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate
cat.	catalytic/catalyst
Cbz	benzyloxycarbonyl
CDA	calcium-dependent antibiotic
CHO	formyl
cLPA	cyclic-lipodepsipeptide antibiotic
δ	chemical shift (ppm)
DABCO	1,4-diazabicyclo[2.2.2]octane
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DIAD	diisopropyl azodicarboxylate
DIC	*N,N′*-diisopropylcarbodiimide
DIBAL	diisobutylaluminum hydride
DIPEA	*N,N*-diisopropylethylamine
DKP	diketopiperazine
DMAP	*N,N*-dimethyl-4-aminopyridine
DMBA	*N,N′*-dimethylbarbituric acid
dmso	dimethylsulfoxide

DMF	*N,N*-dimethylformamide
DME	1,2-dimethoxyethane
DOPC	dioleoylphosphatidylcholine
DOPG	dioleoylphosphatidylglycerol
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
Et₃N	triethylamine
equiv	equivalents
Fmoc	fluorenylmethoxycarbonyl
Glu	glutamate
Gly	glycine
HCTU	2-(6-Chloro-1-H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate
HOAsn	hydroxyasparagine
HOAt	1-hydroxy-7-azabenzotriazole
HOBt	hydroxybenzotriazole
HPLC	high performance liquid chromatography
HOPhGly	hydroxyphenylglycine
im.	imidazole
ⁱPr	isopropyl
Kyn	kynurenine
KDA	potasium diisopropylamide
LDA	lithium diisopropylamide
LRMS	low-resolution mass spectrometry
Leu	leucine
Lys	lysine
Me	methyl
MeCN	acetonitrile
MeGlu	L-*threo*-3-methylglutamate
NMR	nuclear magnetic resonance
NRPS	non-ribosomal peptide synthetase
Orn	ornithine
pyr.	pyridine
PNB	*p*-nitrobenzoate
PPh₃	triphenylphosphine
Ph	phenyl
Phe	phenylalanine
PhMe	toluene
POAsn	phoshohydroxyasparagine
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate

PyAOP	(7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate		^{t}Bu	*tert*-butyl
TEMPO	(2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl		Tf	trifluoromethanesulfonyl
			TFA	trifluoroacetic acid
			THF	tetrahydrofuran
quant.	quantitative yield			
R	undefined substituent		Thr	threonine
RP	reversed-phase		TIPS	triisopropylsilane
sat.	saturated			
			TMS	trimethylsilyl
Ser	serine			
SAR	structure-activity relationship		Trp	tryptophan
sat.	saturated		Trt	trityl
Ser	serine			
SPPS	solid-phase peptide synthesis		Ts	tosyl
quant.	quantitative yield		(*p*)-TsOH	*para*-toluenesulfonic acid
TBAF	tetrabutylammonium fluoride			
TBS	*tert*-butyldimethylsilyl		Val	valine

List of Schemes

Discovery consists of seeing what everybody
has seen and thinking what nobody has
thought.

-Albert Szent-Györgyi

List of Publications

Diamandas, M.; Moreira, R.; Taylor, S. D. Solid-Phase Total Synthesis of Dehydrotryptophan-Bearing Cyclic Peptides Tunicyclin B, Sclerotide A, CDA3a, and CDA4a Using a Protected β-Hydroxytryptophan Building Block. *Org. Lett.* **2021**. https://doi.org/10.1021/acs.orglett.1c00717.

Moreira, R.; **Diamandas, M.**; Taylor, S. D. Synthesis of Fmoc-Protected Amino Alcohols via the Sharpless Asymmetric Aminohydroxylation Reaction Using FmocNHCl as the Nitrogen Source. *J. Org. Chem.* **2019**, *84* (23), 15476–15485. https://doi.org/10.1021/acs.joc.9b02491.

Chapter 1

General Introduction

Amino acids play a critical role in numerous biological functions such as cellular metabolism and are the building blocks of proteins. They also serve as metabolic intermediates for certain neurotransmitters, hormones, and other important biological molecules. For example, the amino acid phenylalanine is a precursor to the secretory hormone adrenaline whilst the amino acids cysteine and methionine are key intermediates in the biosynthesis of biologically important sulfur-containing molecules such as taurine and glutathione.[1] Phenylalanine, cysteine and methionine are examples of the 20 so-called 'proteinogenic amino acids' which are encoded directly in the genetic material of biological organisms. The amino acids selenocysteine and pyrrolysine have been acknowledged as the 21st and 22nd proteinogenic amino acids expanding the number of proteinogenic amino acids beyond the original 20.[2] It is remarkable that there is such a small number of these proteinogenic amino acids, given that in 1953 Milner and Urey demonstrated that under conditions developed to emulate those of primordial Earth—by combining ammonia, hydrogen, methane, water vapor and electrical energy—well beyond 20 amino acids were produced.[3] Likewise, in 1969, researchers discovered numerous unique amino acids within a meteorite that had been found in Australia.[4] As a result, it is clear that the proteinogenic amino acids we know of today were selected as a result of billions of years of evolution and it is perhaps unsurprising that there exists hundreds of so-called non-proteinogenic amino acids in nature. These non-proteinogenic amino acids are typically formed as a result of

post-translational modifications of the naturally encoded proteinogenic ones.[5] The non-proteinogenic amino acids are wide ranging and extremely structurally diverse.

Perhaps one of the more interesting non-proteinogenic amino acids are the α,β-dehydroamino acids (Figure 1.1). These amino acids play an important role in nature as biosynthetic intermediates for other non-essential amino acids and also confer unique structural properties to the peptides they are found in.

Figure 1.1. General structure of trisubstituted α,β-dehydroamino acids.

1.1   α,β-dehydroamino acids

α,β-dehydroamino acids are non-encoded, non-proteinogenic amino acids primarily found in bacteria while sometimes found in fungi, marine invertebrates, and higher plants. The name α,β-dehydroamino acid implies a degree of unsaturation between the alpha-carbon and the beta-carbon (Figure 1.1, shown in blue) of the amino acid. α,β-dehydroamino acids are often referred to as simply dehydroaminoacids or given the prefix delta (Δ) in place of the term dehydro. Dehydroamino acids are given the general chemical structure as depicted in Figure 1.1 either existing as the Z-isomer (shown in red) or the E-isomer (shown in green). The two isomers are often given the suffix denotation Δ^Z or Δ^E for either the Z-isomer or the E-isomer respectively. For example, the amino acid Z-dehydrophenylalanine can also be referred to as Δ^ZPhe . In most peptides, the Z-dehydroamino acid is thermodynamically favored and predominates in nature; however, several peptides have been found to containing E-dehydroamino acid residues as well.

1.1.1 Biophysical properties of dehydroamino acids and dehydroamino acid peptides

The unique unsaturation of dehydroamino acid residues dramatically impacts the conformational properties of the amino acid itself as well as the conformation of the peptide in which the residue is found.[6] The bonds between the alpha- and beta-carbon in dehydroamino acids are shorter while the bond angles are larger when compared to saturated sp^3 amino acids. Similarly, the unsaturation constricts the topology of the side chain substituent which limits it to exist in one of two isomeric forms, either Z or E. The α,β-double bond lies co-planar to the flanking amide group in dehydroamino acids. This co-planarity permits π-electron conjugation which can be considered as a stabilizing force of selected conformations.[6] The study of the conformational impacts of dehydroamino acids has been primarily conducted on ΔPhe containing peptides while some have been conducted on dehydroalanine (ΔAla) containing peptides. Crystal structures obtained of ΔPhe containing peptides revealed that the ΔPhe residue promotes the formation of β-turns in short sequences and helical conformations in larger peptides, as well as in peptides containing more than one dehydro residue.[7] Studies of ΔPhe containing peptides in solution revealed similar results. Like in the solid state, short ΔPhe peptides in solution typically adopt conformations of the β-turn type while larger ΔPhe peptides are helical in nature.[7] In contrast, in the solid-phase and in solution, the ΔAla residue in ΔAla-containing peptides adopts a roughly planar conformation and induces an inverse γ-turn in the preceding amino acid residue.[7-9] It is thought that incorporation of dehydroamino acids also offer some protection against enzymatic degradation. English and Stammer revealed that a $Δ^Z$Phe containing pentapeptide was able to withstand enzymatic degradation by both hydrolytic enzymes chymotrypsin and thermolysin. In

contrast, the corresponding saturated Phe containing pentapeptide was found to be rapidly digested.[10] Furthermore, it has been suggested that dehydroamino acids show an enhanced ability to bind metal ions. This phenomenon has been explained by the unique amide nitrogen of the dehydroamino acid.[11, 12] Even at a relatively low pH, metal ions such as Zn^{II} and Co^{II} are capable of forming strong complex species with the dehydroamino acid's enamide nitrogen.[12] Brasun and coworkers also found that the side chain steric bulk and the E/Z-isomeric form of the dehydroamino acid greatly impact its ability to bind certain Cu^{II} species.[12] The bio-physical properties of dehydroamino acids are indeed unique and provides insight into why these unsaturated amino acid residues are so abundant in nature.

1.1.2 Chemical synthesis of dehydroamino acids

In addition to the interesting biophysical properties of dehydroamino acids and dehydro amino acid peptides, the chemical reactivity and the chemical synthesis of dehydroamino acids have been the focus of numerous studies over the past several decades as well. Dehydroamino acids are unique in that they are particularly prone to Michael addition, Z/E isomerization, hydrogenation, and cycloaddition reactions.[6] Dehydroamino acids cannot be easily incorporated into peptides through conventional peptide synthesis techniques. The reduced nucleophilicity of a dehydroamino acid's enamide nitrogen prevents effective peptide elongation in the C to N direction. As a result, carbamate (such as Fmoc or Boc) protected dehydroamino acids are not particularly useful building blocks in peptide synthesis.[13] Dehydroamino acids are usually generated after the peptide sequence has been assembled. The chemical synthesis of dehydroamino acids is a very well-studied field and numerous methods have been described to

prepare them. Several of these methods have been used in the synthesis of dehydroamino acid containing peptides. Some of the ways of accessing dehydroamino acids are described below.

The Erlenmeyer synthesis

The classical method of preparing dehydroamino acid residues is via the Erlenmeyer synthesis (Scheme 1.1). This reaction, first described in 1893 by Erlenmeyer, involves a condensation of an aldehyde and acetylglycine in the presence of acetic anhydride and sodium acetate.[14] The intermediate Erlenmeyer azlactone can be opened either with alcohols, water or the α-NH$_2$ of amino acids/peptides (R$_3$) to generate the corresponding dehydroamino esters, dehydroamino acids or dehydroamino acid peptides. The Erlenmeyer reaction typically gives the dehydro product solely in its Z-isomeric form. An example of an application of the Erlenmeyer synthesis is by Humphrey and coworkers, who used it as a key step in the synthesis of L-*m*-tyrosine on a process scale (Scheme 1.2).[15] Over the years, researchers have developed alternative conditions for the Erlenmeyer reaction that allow for improved azlactone yields with aliphatic aldehydes or to allow for the application of a continuous-flow method.[16, 17]
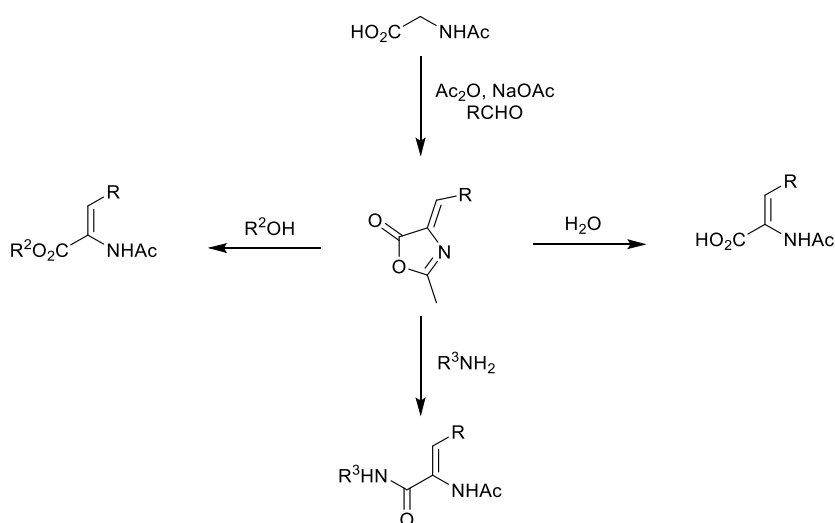
Scheme 1.1. General approach of the Erlenmeyer synthesis.
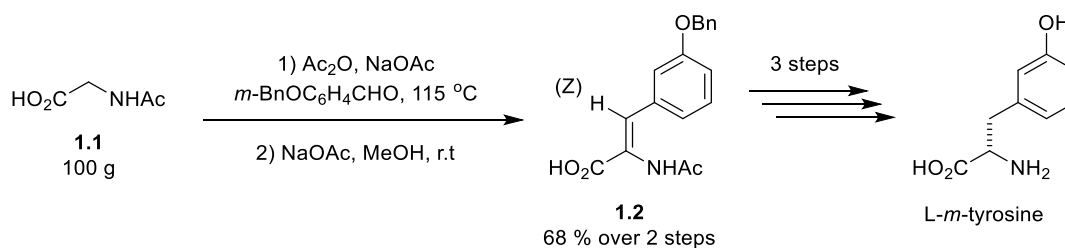
Scheme 1.2. Process scale synthesis of L-*m*-tyrosine via Erlenmeyer synthesis.[15]

Elimination of β-hydroxy, seleno or thio-α-amino acid

Perhaps one of the more well-known methods of preparing dehydroamino acids is by the elimination of an activated β-hydroxyl group from a β-hydroxy-α-amino acid. This method has been historically used in the synthesis of dehydroalanine and dehydrothreonine from serine and threonine respectively.[18] Activation of the hydroxyl group and subsequent elimination has been achieved by a multitude of different reagents and conditions; such as dichloroacetyl chloride/triethylamine, tosyl anhydride/1,4-diazabicyclo[2.2.2]octane (DABCO), triphenylphosphine/diethyl azodicarboxylate (DEAD), diethylaminosulfur trifluoride (DAST)/*N,N*-diisopropylethylamine (DIPEA), tosyl chloride and carbodiimides—such as 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl)—with copper(I) or copper (II) chloride (Scheme 1.3).[19-23] In many scenarios, the Z-dehydroamino acid is the dominant product irrespective of which stereoisomer of the starting material that is used. This is a result of the thermodynamic preference of the Z-dehydroamino acid and that the E- and Z-isomers are likely capable of interconverting under mildly basic reaction conditions.[24]
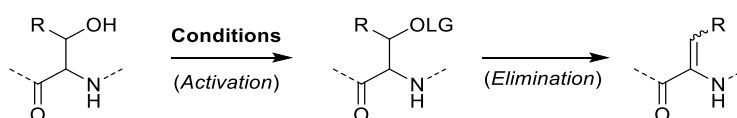
Scheme 1.3. General approach to dehydroamino acids from the β-hydroxy-α-amino acid.

Under certain conditions, the stereochemistry of the dehydroamino acid can be controlled depending on whether the *threo-* or *erythro*-β-hydroxy-α-amino acid starting material is utilized. This stereoselective elimination has been best achieved either by activation with thionyl chloride (SOCl₂) and subsequent *anti*-elimination of the resulting cyclic sulfamidite with excess triethylamine at low temperatures in dichloromethane (Scheme 1.4), or by activation and resulting *syn*-elimination using EDC with CuCl₂ in toluene (PhMe) at 80 °C (Scheme 1.5).[24, 25] Both of these approaches have been used on preassembled β-hydroxy amino acid containing peptides in order to generate the resulting dehydroamino acid peptide. However, the a*nti*-elimination of *erythro*-β-hydroxy-α-amino acid starting materials—like those in Scheme 1.4—rarely produce the E-isomer with complete selectivity as there is competing E/Z isomerization leading to formation of small quantities of the thermodynamically favored Z-isomer. Likewise, in the case of the EDC/CuCl₂ *syn*-elimination, if the reaction is left for a prolonged period of time (i.e 30 min
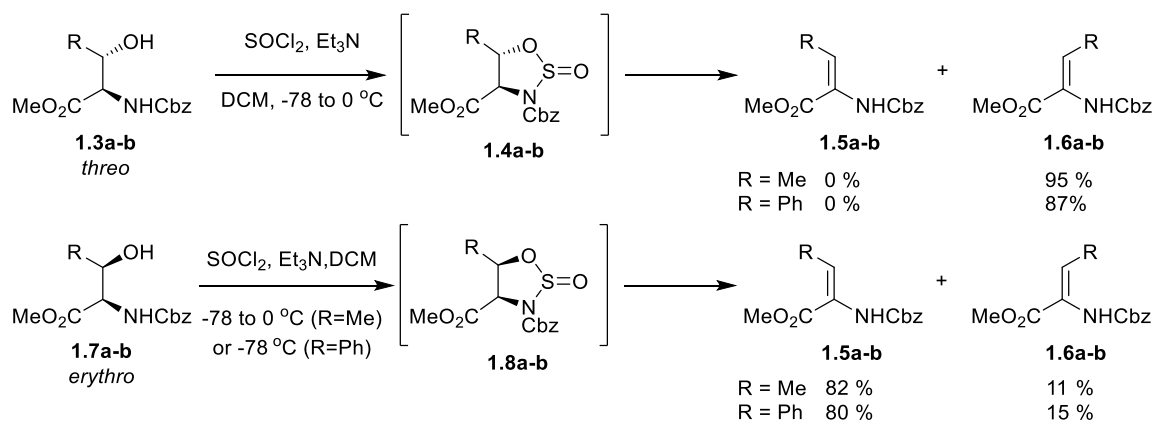
Scheme 1.4. Stereoselective *anti*-elimination of β-hydroxy amino acids using SOCl₂/Et₃N.[25]
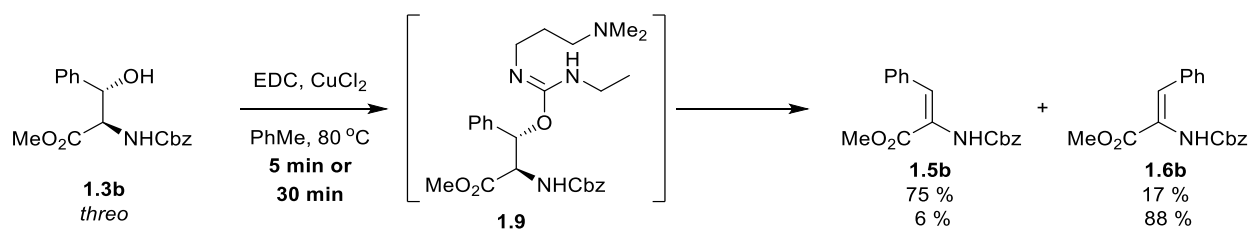
Scheme 1.5. Stereoselective *syn*-elimination of β-hydroxy amino acids using EDC/CuCl$_2$.[24]

rather than 5 min as seen in Scheme 1.5), then the E-selectivity of the *syn*-elimination is eroded.

Remarkably, this is one of the few elimination reactions of a β-hydroxy-α-amino acid that has

been successfully used both in solution and the solid-phase to prepare dehydroamino acid

containing peptides (See section 1.3 for a description of solid-phase peptide synthesis).[13]

Similar activation and elimination reactions have been described to prepare

dehydroamino acids starting from β-thio- and β-seleno-amino acids (Scheme 1.6).[18, 26] This

process involves the oxidation of the thio- or seleno-amino acid to the corresponding sulfoxide

or selenoxides, which then undergoes thermolysis to stereoselectivity produce the

dehydroamino acid product.[18] The elimination of the intermediate sulfoxides and selenoxides

proceeds in a *syn-* manner such that the *erythro*-selenoxide amino acid generates the Z-

dehydroamino acid and the *threo*-selenoxide amino acid generates the E-dehydroamino acid.[26]

Historically, this method has been only applied in the preparation of quite simple amino acids

such as dehydroalanine and dehydrothreonine. Likewise, it has been used as a late-stage

functionalization tool to make simple dehydroamino acid peptides and, in one example, has been

used on a resin-bound peptide.[27]
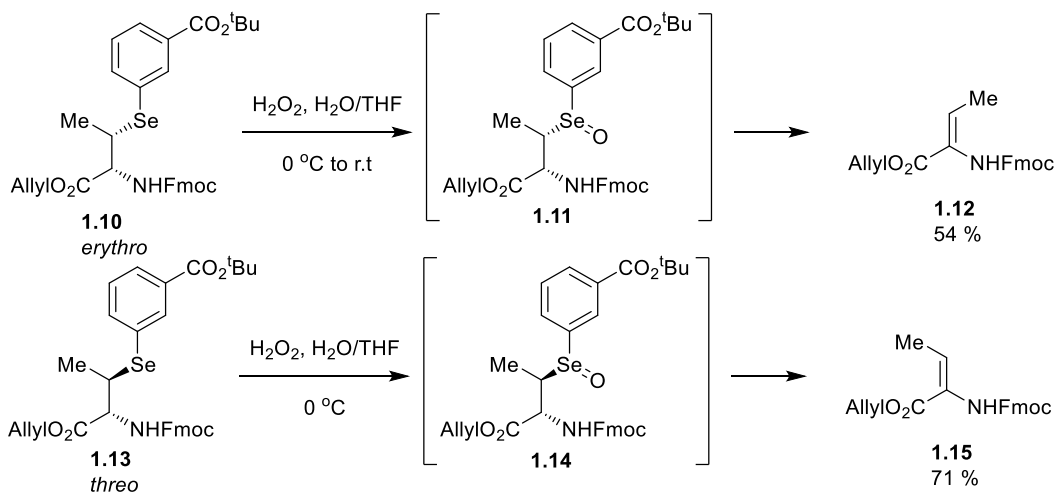
Scheme 1.6. Stereoselective *syn*-elimination of β-seleno-α-amino acids using H_2O_2.[26]

Horner-Wadsworth-Emmons and Wittig type olefination reactions

There are many examples in the literature in which the Horner-Wadsworth-Emmons (HWE) and Wittig type reactions have been used to prepare dehydroamino acids. One of the earliest examples was reported by Schmidt and coworkers, who reacted alkyl and aryl aldehydes with protected 2-amino-2(dimethoxyphosphoryl)acetates in the presences of DBU as the base (Scheme 1.7). They found that the best Z-selectivity and yields were achieved using DBU as the base rather than other bases such as KO^{t}Bu or DBU with LiCl as an additive. The reaction with aryl aldehydes required slightly elevated temperatures (r.t rather than -20 °C to r.t).[28]
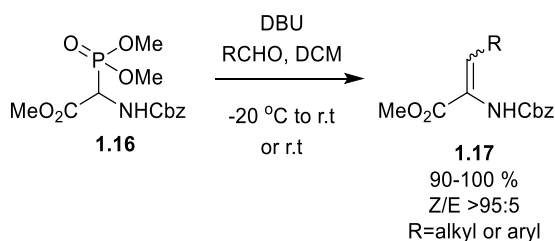
Scheme 1.7. The synthesis of dehydroamino acids through a HWE reaction.[28]

In 2004, Yosuke and coworkers described a method of preparing dehydroamino acids through a Wittig type reaction of α-tosylglycine species **1.18** with tributylphosphine (Bu$_3$P), DBU and alkyl/aryl aldehydes. The reaction was found to proceed via an intermediate phosphonium ylide. Yosuke and coworkers were successful in applying this method to prepare Z-dehydroamino acid dipeptides **1.19** in moderate to excellent yields (Scheme 1.8).[29]
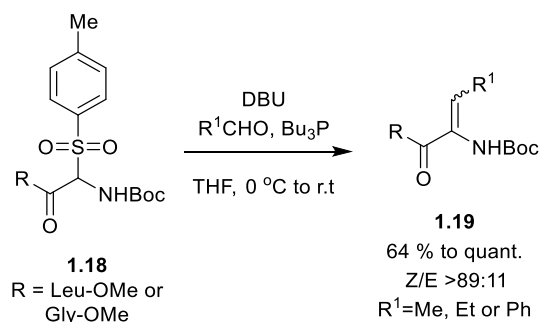
Scheme 1.8. A Wittig type reaction used to make dehydroamino dipeptides by Yosuke et al.[29]

Condensation with nitroalkanes

Yosuke and coworkers described a method for preparing bulky, tetrasubstituted dehydroamino acids. Condensation of an α-tosylglycine **1.20** with a nitro alkane in the presence of KO^{t}Bu generates an intermediate nitro compound **1.21** which is then treated with DBU to eliminate an equivalent of NO$_2^-$ and generate the resulting dehydroamino acid **1.22** (Scheme 1.9).[29] They found that this two-step transformation proceeded in moderate yields and with good Z-selectivity.[29]
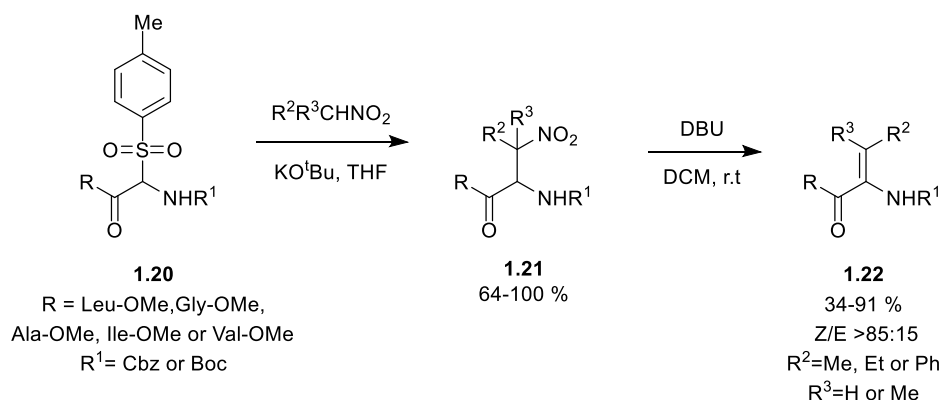
Scheme 1.9. Dehydroamino acids by a condensation reaction by Yosuke et al.[29]

One of the more challenging dehydroamino acids to prepare is dehydrotryptophan. The synthesis of dehydrotryptophan and dehydrotryptophan derivatives has been described using many of the transformations previously outlined in section 1.1.2—with varying degrees of success—as well as a by handful of other more substrate specific methods.

1.2 Dehydrotryptophan and dehydrotryptophan derivatives

Dehydrotryptophan and dehydrotryptophan derivatives exist in a handful of naturally occurring peptides (Figure 1.2).[30] All of these dehydrotryptophan-containing peptides are cyclic and are produced by several different fungal and bacterial strains. In nature, dehydrotryptophan species are produced as a result of direct enzymatic oxidation of L-tryptophan moieties once incorporated into a peptide. For example, the biosynthesis of dehydrotryptophan is achieved by L-tryptophan 2',3'-oxidase in *Chromobacterium violaceum* and by tryptophan side chain oxidase in *Pseudomonas*.[31, 32] L-Tryptophan 2',3'-oxidase is extremely stereoselective in its oxidation as only Z-dehydrotryptophan is produced via direct dehydrogenation of L-tryptophan residues. In

contrast, the enzyme tryptophan side chain oxidase produces a mixture of E- and Z-dehydrotryptophan species.[30] Only Z-dehydrotryptophan has been found in naturally occurring peptides to date with the cyclic peptide sclerotide B as the sole exception. Sclerotide B—discovered by Zhu and coworkers in 2009—was found to contain E-dehydrotryptophan rather than the Z-dehydrotryptophan of sclerotide A (Figure 1.2).[33] However, the researchers suggested that the thermodynamically favored sclerotide A was likely the naturally occurring material and that sclerotide B was likely formed upon photoisomerization of sclerotide A during the researchers' initial fermentation and isolation steps. [33]
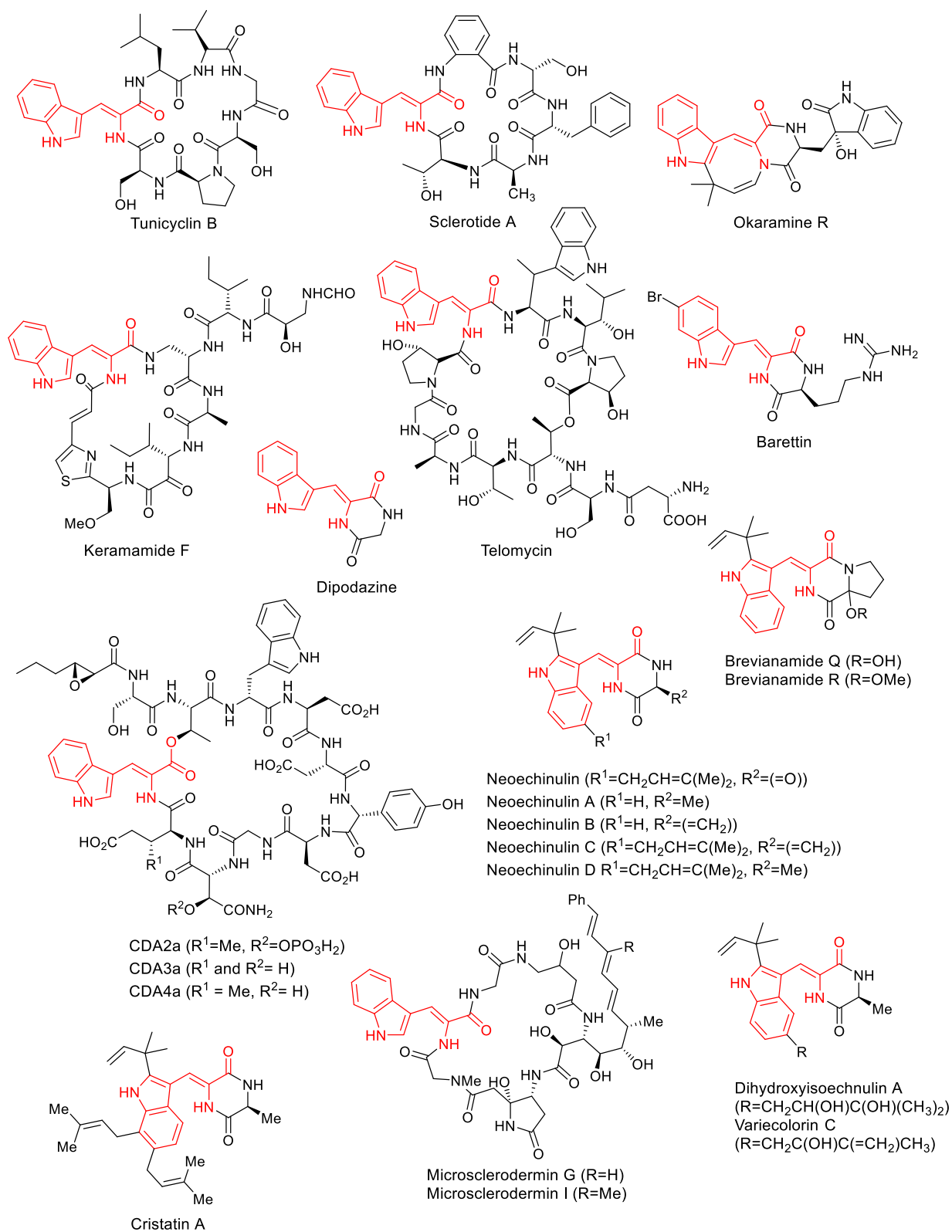
Figure 1.2. Natural dehydrotryptophan containing peptides.

1.2.1 The chemical synthesis of dehydrotryptophan

As previously mentioned, there exist several ways of chemically synthesizing dehydrotryptophan and dehydrotryptophan derivatives, many of which are based on methods described in section 1.1.2. However, many of these transformations come with limitations such as varying yields as well as limited substrate scopes. These transformations are discussed below.

Dehydrotryptophan derivatives using the Erlenmeyer synthesis

The Erlenmeyer synthesis was the first method used to prepare dehydrotryptophan residues. The Erlenmeyer synthesis of dehydrotryptophan species was used over a century ago as a key reaction in the first chemical synthesis of the amino acid tryptophan.[34] The Erlenmeyer synthesis has been used since then to prepare dehydrotryptophan moieties, but with varying yields. For example, Oba and coworkers were able to obtain a N^{α}-acylated dehydrotryptophan **1.24** in good yield (Scheme 1.10); whereas, in earlier work, yields of 20% and 26% were obtained by Kirby et al. and Skrabl et al. respectively in the synthesis of a very similar indole-*N*-protected acyl-dehydrotryptophan **1.26** (Scheme 1.11). [35-37] In both scenarios, the Z-dehydrotryptophan was formed exclusively.
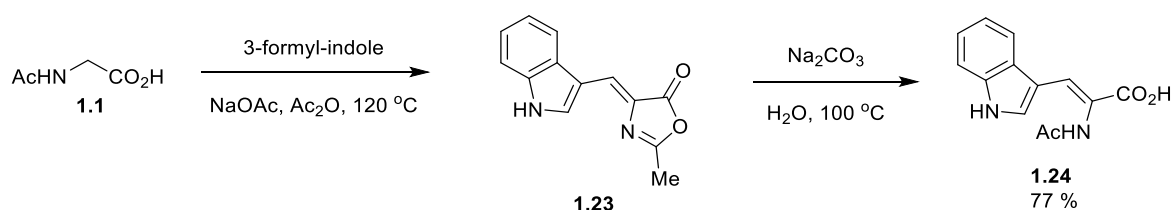
Scheme 1.10. Z-dehydrotryptophan compounds through Erlenmeyer synthesis by Oba et al.[35]
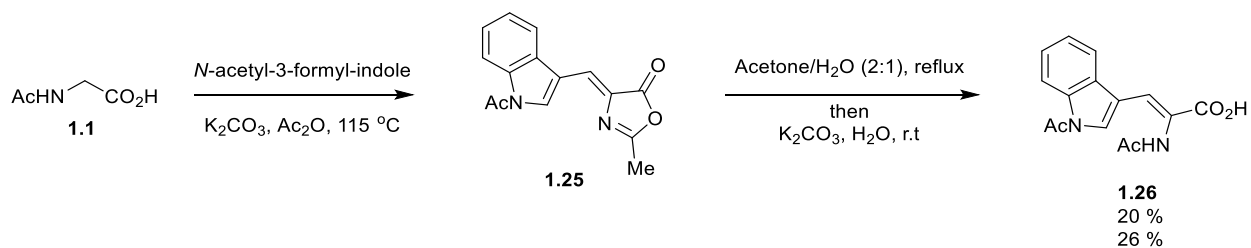
Scheme 1.11. Synthesis of a Z-dehydrotryptophan derivative via an Erlenmeyer reaction.[36, 37]

The variable and often poor yields of the classical Erlenmeyer synthesis in preparing dehydrotryptophan derivatives have been attributed to the significant contribution of the indolium form (**1.27b**) of 3-formyl indole, which dramatically reduces the aldehyde's inherent electrophilicity (Scheme 1.12.).[38] Moriya and coworkers developed an improved method which
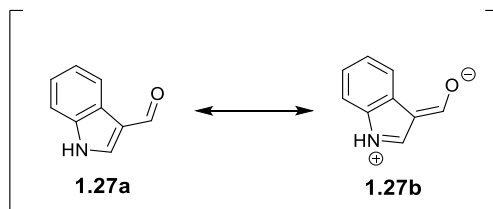
Scheme 1.12. An explanation for the reduced electrophilicity of 3-formylindole species.

uses a 3-(aminomethylene)-3H-indole derivative **1.28** was used as a 1,4-dipolar synthon of the traditionally used indolic aldehyde, 3-formylindole, to prepare protected dehydrotryptophan derivatives almost exclusively as the Z-isomer (Scheme 1.13). [38]
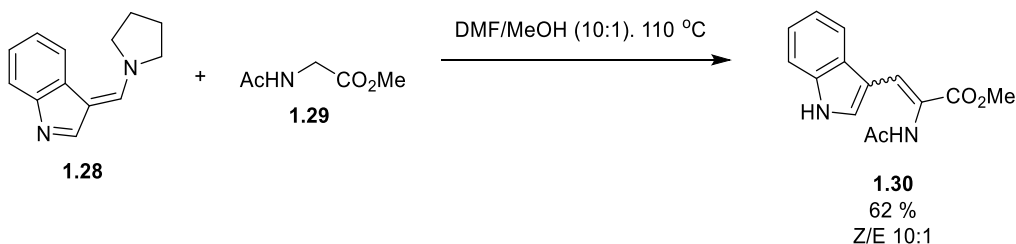
Scheme 1.13. Alternative route to dehydrotryptophan species by Moriya et al.[38]

In 2009, Barros and coworkers utilized the Erlenmeyer synthesis to prepare a dehydrotryptophan-containing compound **1.34** as a potential inhibitor of Hepatitis C serine protease.[39] They were successful in opening the intermediate indole-azlactone **1.32** with the primary amine of a bis-tetrahydrofuran species **1.33** in order to get the corresponding ring-opened Z-dehydrotryptophan species **1.34** (Scheme 1.14), although the final azlactone ring opening only proceeded in a 55 % yield.[39] This azlactone opening is the only example where an indolic-azlactone was opened with an amine more complex than a simple alkyl amine.[39]
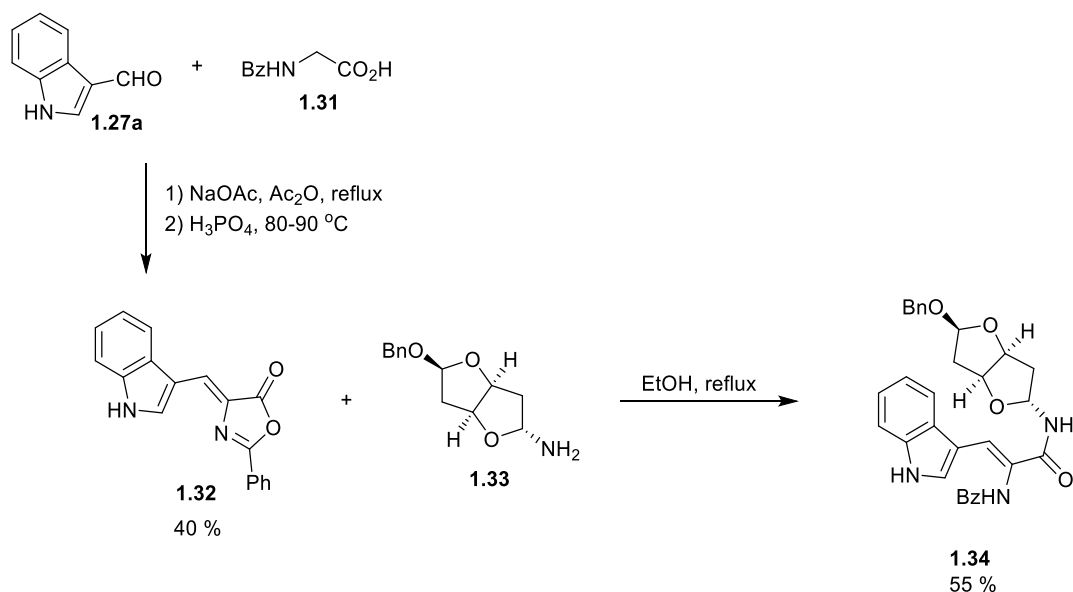
Scheme 1.14. Z-dehydrotryptophan compounds through Erlenmeyer synthesis by Barros et al.[39]

Dehydrotryptophan derivatives by acid-catalyzed elimination of water from β-hydroxytryptophan

The synthesis of dehydrotryptophan and dehydrotryptophan-like compounds by subjecting a β-hydroxytryptophan derivative to acidic conditions has been described several

times. This method has been mainly used to make relatively small dehydrotryptophan derivatives or simple dehydrotryptophan-containing dipeptides.

In 1978, Noda and coworkers—when studying the dehydrotryptophan forming enzyme tryptophan side chain oxidase—found that, at an elevated pH, the main oxidative product of this enzyme was β-hydroxy-tryptophan rather than dehydrotryptophan. The researchers found that the β-hydroxytryptophan residue could be converted to the corresponding dehydro compound via an acid-catalyzed dehydration when the pH was 5.5 or less.[32]

In 1990 by O'Sullivan and coworkers demonstrated that subjecting Janthinocin A, a naturally-occurring cyclic peptide antibiotic which contains a β-hydroxytryptophan residue, to mild aqueous acid results in elimination of the hydroxyl group and the formation of the corresponding Z-dehydrotryptophan-containing peptide, Janthinocin C (Scheme 1.5) [40]
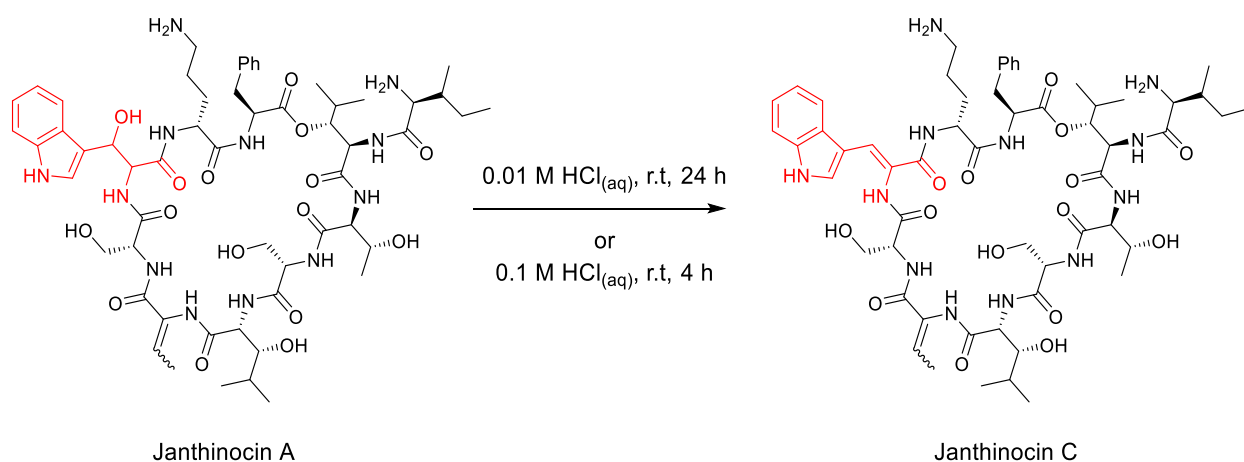
Scheme 1.15. Janthinocin A can be dehydrated to give Janthinocin C under acidic conditions as demonstrated by Johnson et al.[40]

In 2008, Kuramochi and coworkers demonstrated that a β-hydroxytryptophan dipeptide **1.35** could be used in the synthesis of Z-dehydrotryptophan-containing cyclic dipeptide

neoechinulin A—a potent antioxidant and radical scavenger. [41] They found that a diastereomeric mixture of a β-hydroxytryptophan-containing dipeptide **1.35** could be converted to the corresponding Z-dehydrotryptophan-containing dipeptide upon exposure to acid **1.36** (Scheme 1.16).[41] The dehydration was found to take place more slowly than removal of the acid-labile indole protecting group. Nevertheless, the deprotected indole intermediate **1.37** was easily converted to the desired product **1.36** upon prolonged exposure to 1 M HCl$_{(aq)}$ in ethanol. After further manipulation, the researchers were successful in synthesizing (-)-neoechinulin A. Kuramochi and coworkers postulated that the reaction likely proceeds via initial direct elimination of water from the β-hydroxytryptophan dipeptide intermediate—generating a stabilized indolic cation—followed by stereoselective elimination to exclusively generate the thermodynamically favored Z-enamide. [41]
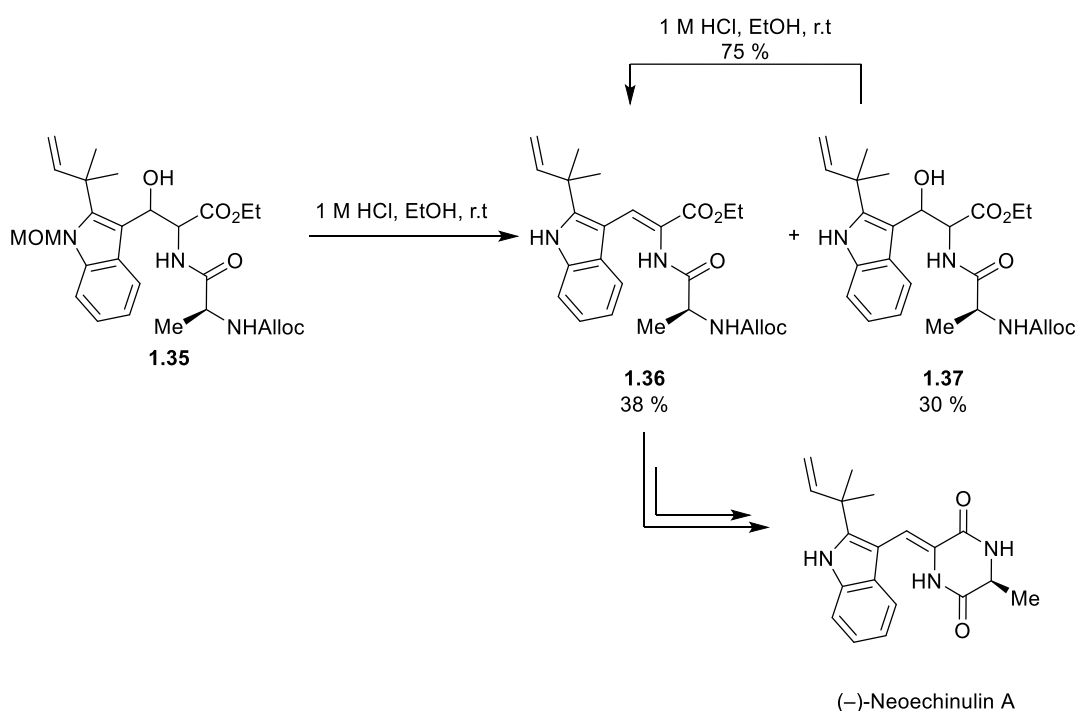
Scheme 1.16. The synthesis of (-)-neoechinulin A by Kuramochi et al.[41]

A similar dehydration was described by Liu and coworkers in 2019. The dehydration of a functionalized β-hydroxytryptophan **1.38** species was used to generate the corresponding dehydrotryptophan compound **1.39** as a key intermediate in the synthesis of speradine C (Scheme 1.17).[42] The dehydrotryptophan was made by treating a diastereomeric mixture of β-hydroxytryptophan derivative **1.38** with catalytic amounts of bis(trifluoromethane)sulfonimide (Tf$_2$NH). The dehydro compound was not isolated and was allowed to react further in a cascade cyclization reaction to form the resulting tetracycle **1.40**.
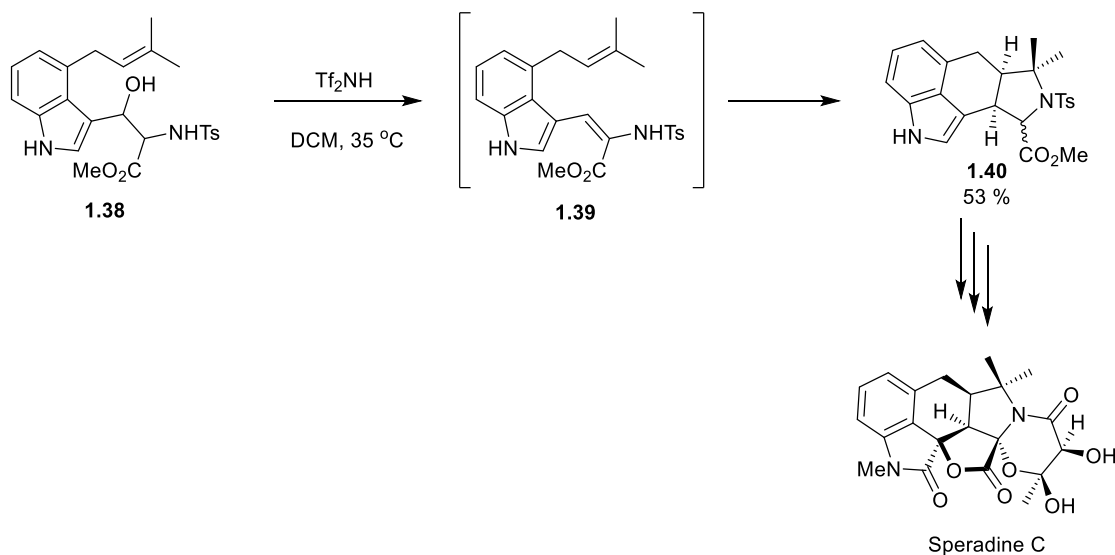
Scheme 1.17. The synthesis of speradine C by Liu et al.[42]

In 2005, Baron and coworkers utilized the dehydration of β-hydroxytryptophan-like species to prepare a library of selective glycine-site *N*-methyl-D-aspartate (NMDA) receptor antagonists.[43] The researchers found that diastereomeric mixtures of β-hydroxy- and β-trimethyl silyl (TMS) ether compounds **1.41a-b** could be converted to the corresponding dehydrotryptophan-like species **1.42** upon the addition of either *para*-toluenesulfonic acid (*p*TsOH) or trifluoromethanesulfonic anhydride (Tf$_2$O) in high yields (Scheme 1.18). Baron and

coworkers also found that, in one scenario, attempted dehydration of an intermediate TMS ether resulted in a quantitative retro-aldol reaction when trifluoracetic acid (TFA) was used as the source of acid. This side reaction was not observed when the dehydration was elicited using the pTsOH/Tf$_2$O conditions.[43]

Scheme 1.18. The acid-initiated dehydration of β-hydroxytryptophan-like derivatives by Barron et al.[43]

Dehydrotryptophan derivatives by base-promoted elimination of an activated hydroxyl group from β-hydroxytryptophan

Several examples of activation and subsequent base-promoted elimination of β-hydroxy-tryptophan species have been developed.[44-46] Many of these transformations are similar in that they are achieved by activation with methanesulfonyl chloride (MsCl)/Et$_3$N followed by elimination with DBU.[44-46] For example, Frebault and coworkers in 2009 prepared a β-hydroxytryptophan-like motif **1.45** via an aldol condensation which was then converted to the mesylate using MsCl with NEt$_3$. Upon treatment with DBU, the intermediate mesylate then underwent an elimination reaction to give the dehydrotryptophan derivative **1.46**. The resulting unsaturated ester **1.46** was hydrolyzed to form the corresponding unsaturated acid **1.47** as a 3:1

Z/E mixture of isomers (Scheme 1.19). They used this dehydro intermediate **1.47** for the synthesis of ent-malbrancheamide B.[44]
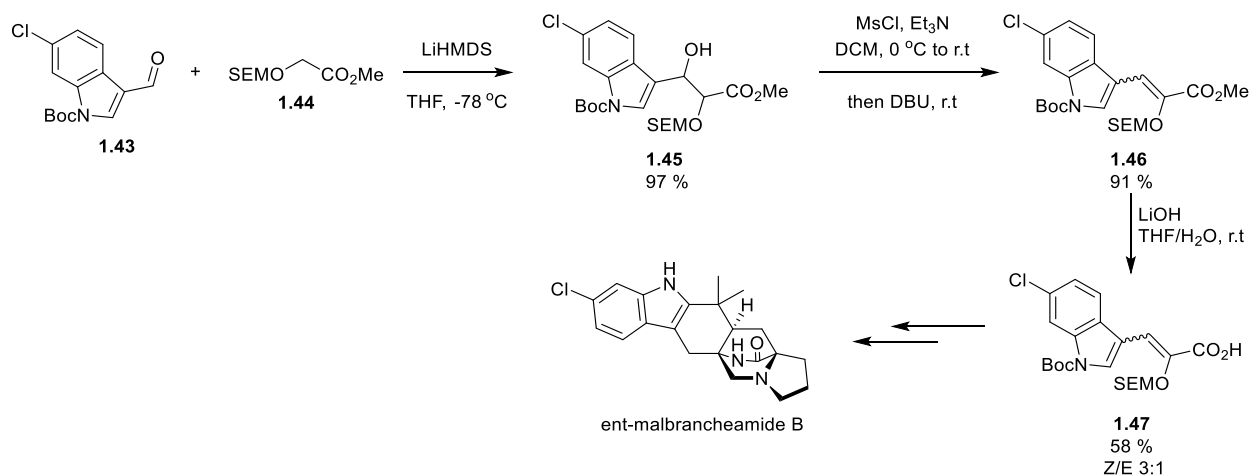
Scheme 1.19. The synthesis of ent-malbrancheamide B by Frebault et al.[44]

In 2009, Kometani and coworkers were able to prepare dehydrotryptophan derivatives **1.50** via elimination of a *cis*-oxazolidinone intermediate **1.49**.[47] One of the main features of this method is the stereoselective formation of the *cis*-oxazolidinone intermediate **1.49** and resulting *anti*-elimination—using LiHMDS—to generate the E-dehydrotryptophan **1.50** as the major product (Scheme 1.20). Interestingly, the researchers also noted that the E-dehydrotryptophan **1.50** could be isomerized to the thermodynamically favored Z-dehydrotryptophan **1.51** upon the addition of iodine (I$_2$).[47]
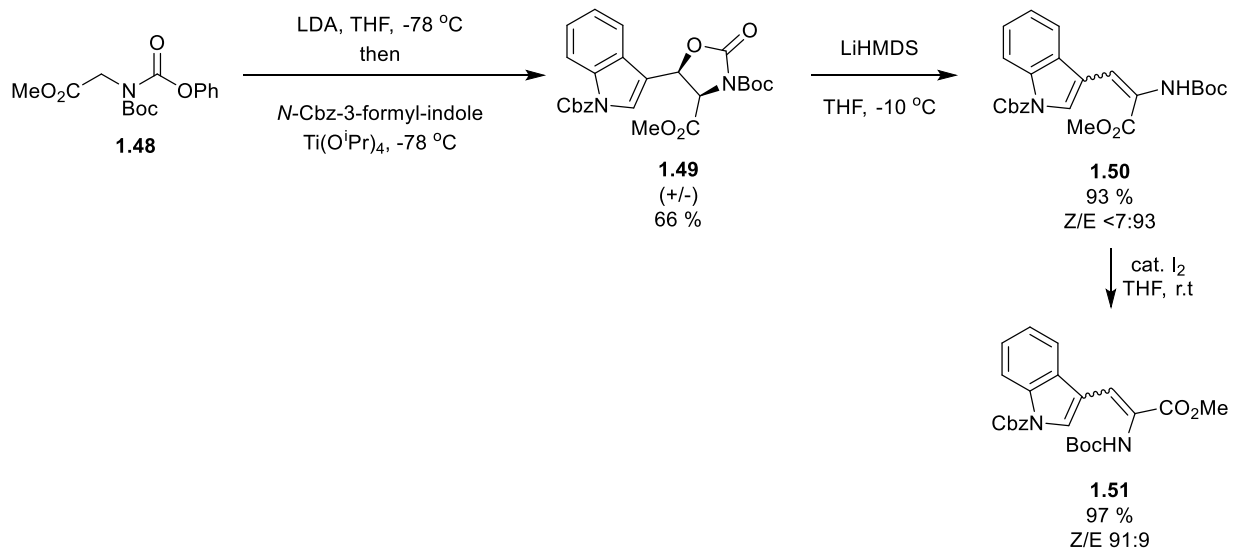
Scheme 1.20. The synthesis of dehydrotryptophan derivatives via elimination of a *cis*-oxazolidinone intermediate by Kometani et al.[47]

Dehydrotryptophan derivatives by HWE and Wittig type reactions

The use of the HWE reaction to make dehydrotryptophan derivatives has been reported a handful of times. Shin et al. were able to prepare several *N*-indole-protected Z-dehydrotryptophan compounds **1.53** using a HWE reaction in moderate to good yields (Scheme 1.21).[48]
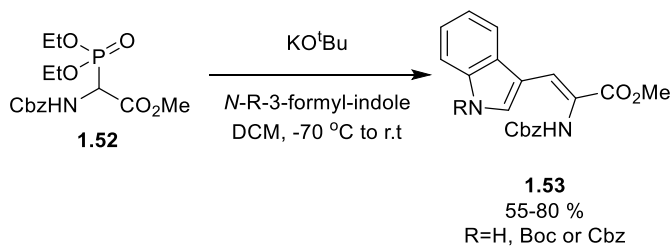
Scheme 1.21. The synthesis of dehydrotryptophan derivatives by a HWE reaction by Shin et al. [48]

In 1995, Sowinski and Toogood utilized an HWE reaction of a thiazolidine-containing dimethylphophsonoglycinate residue **1.56** to make the corresponding dehydrotryptophan fragment **1.57** in their attempted synthesis of the peptide keramamide F (Scheme 1.22.).[49] This two-step procedure only proceeded in a 15 % overall yield. The researchers had originally found that the α-NH$_2$ of a dehydrotryptophan residue was an ineffective nucleophile in amide forming coupling reactions—resulting in a comparably poor yield.[49] Sowinski and Toogood were unable to make keramamide F. Instead, they prepared keramamide J, which contains an L-tryptophan residue in place of the Z-dehydrotryptophan. [50]
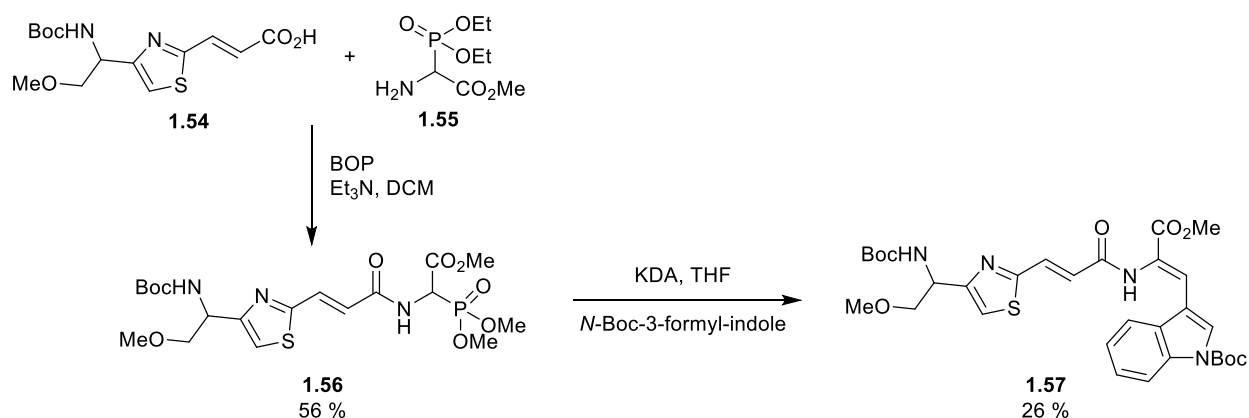
Scheme 1.22. The synthesis of a dehydrotryptophan fragment of keramamide F by Sowinski and Toogood.[49]

In 2004, Johnson and coworkers—in the synthesis of the peptide barettin—were able to construct the Z-dehydrotryptophan dipeptide core **1.59** of their target through a HWE reaction of the corresponding diethylphosphonodipeptide **1.58** which proceeded in a 55 % yield (Scheme 1.23). The Z-dehydrotryptophan containing dipeptide **1.59** was then converted to barettin through two further steps.[51]
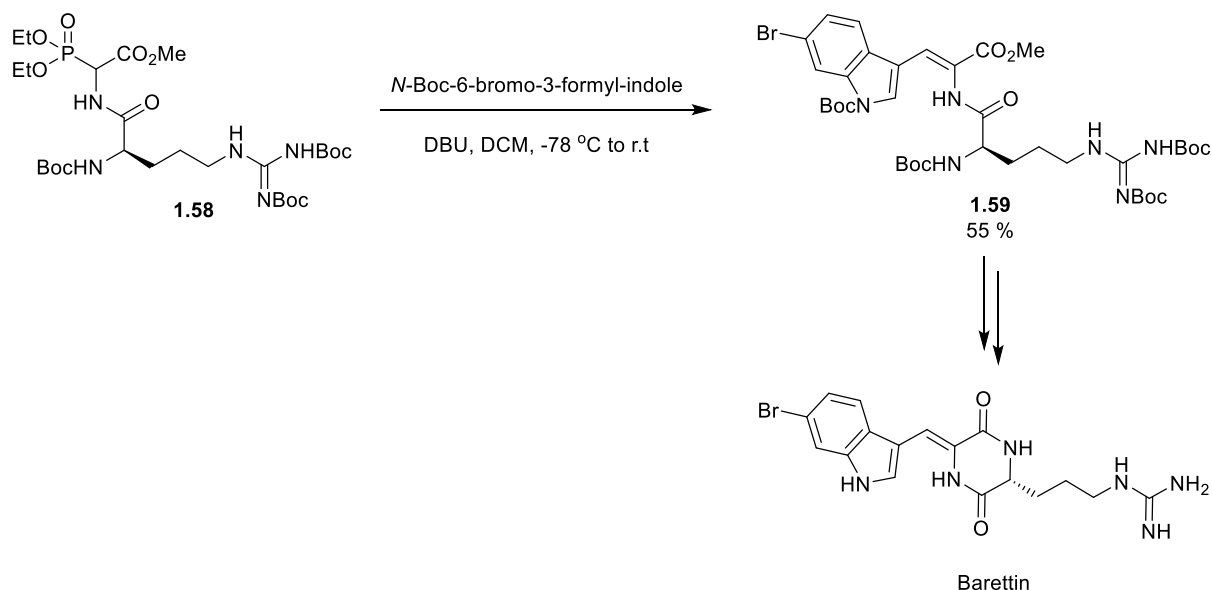
Scheme 1.23. The synthesis of barettin by Johnson et al.[51]

In 2002, Kimura and coworkers described a method to access an array of dehydroamino acid derivatives, including dehydrotryptophan derivatives, by reacting protected α-tosylglycine derivatives **1.60** with tributylphosphine (Bu₃P) and alkyl/aryl aldehydes. They used this method to prepare protected Z-dehydrotryptophan derivatives **1.61** in fair yields; however, the reaction was found to take 67 h to reach completion and required 3 equivalents of the α-tosylglycinate **1.60** (Scheme 1.24).[52]
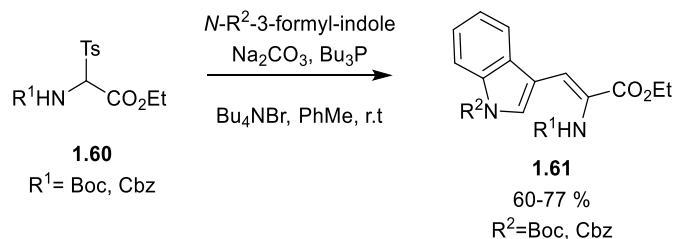
Scheme 1.24. A Wittig type reaction used to make dehydrotryptophan species by Kimura et al. [52]

Dehydrotryptophan derivatives by direct oxidation of tryptophan

Relatively recently, it was discovered that dehydrotryptophan residues could be prepared by direct oxidation of the corresponding tryptophan moiety using nitrosobenzene (PhNO) in the presence of the Lewis acid zirconium (IV) chloride (ZrCl$_4$).[53] This unique transformation was originally discovered by Baran and coworkers in 2006.[53] Baran et al. found that a variety of protected tryptophan analogs could be directly and exclusively converted to the corresponding Z-dehydrotryptophan species under mild oxidizing conditions with PhNO/ZrCl$_4$ in either DCM or PhMe (Scheme 1.25).[53] However, the yields varied greatly depending on the nature of the α-NH$_2$ protecting group, finding best success with phthalimide (Phth) and methyl carbamate (CO$_2$Me) protected tryptophan methyl esters (**1.62a,e**) as well as tryptophan-containing diketopiperazine (DKP) compounds (**1.64a-b**).[53] Due to the nature of the transformation, the indole had to remain unprotected. The researchers proposed that the reaction likely proceeds as follows: Lewis acid-activated nitrosobenzene is initially attacked by the enamine of tryptophan's indole group leading to an *O*-linked nitrosobenzene derivative (**1.66a**) at the 3 position of the indole, which is then followed by fragmentation (**1.66b**) and immediate tautomerization to form the desired Z-dehydrotryptophan product (**1.63a**) as well as phenylhydroxyamine (Scheme 1.26).[53] They implemented this oxidative transformation in the synthesis of the highly complex natural products, avrainvillamide and the stephacidins.[53]
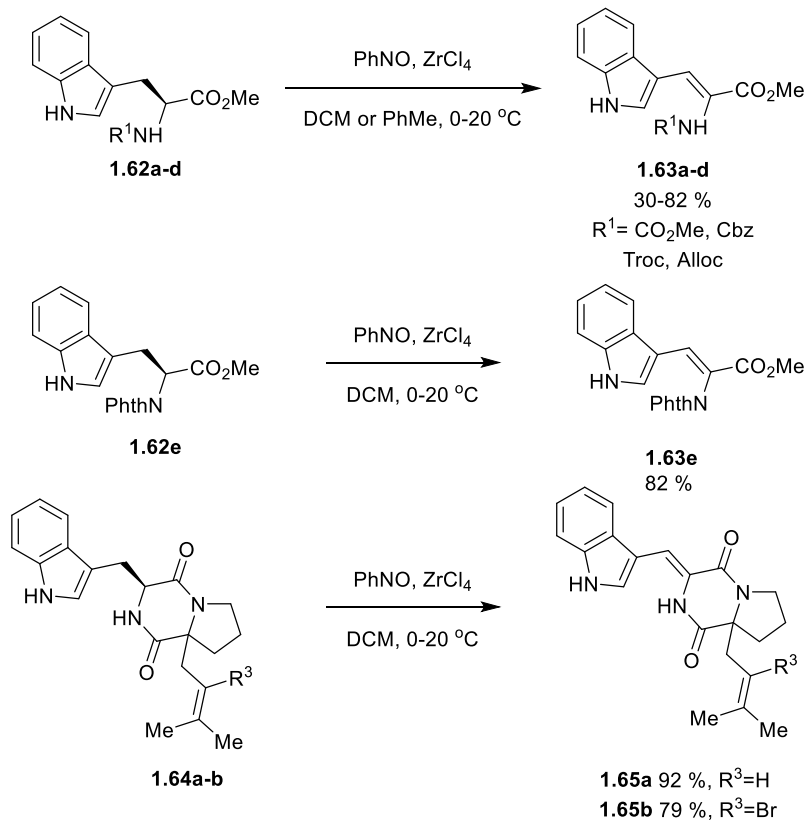
Scheme 1.25. The synthesis of dehydrotryptophan species by direct oxidation of Trp

compounds using PhNO/ZrCl$_4$ by Baran et al.[53]
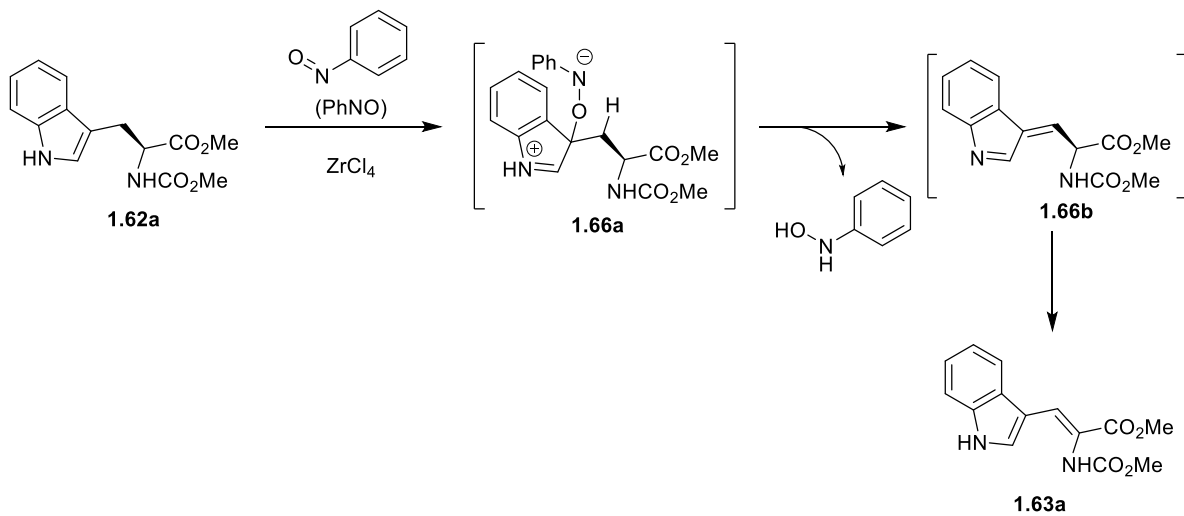
Scheme 1.26. A mechanism for the direct oxidation of Trp compounds using PhNO/ZrCl$_4$.[53]

In 2011, Dai and coworkers used the direct oxidation method developed by Baran and coworkers in the synthesis of isoechinulin A and variecolorin C (Scheme 1.27).[54] Isoechinulin A and variecolorin C were both isolated from the *Aspergillius* species and display radical scavenging activity, ultraviolet-A protecting activity, immunosuppressive activity, and antibacterial activity.
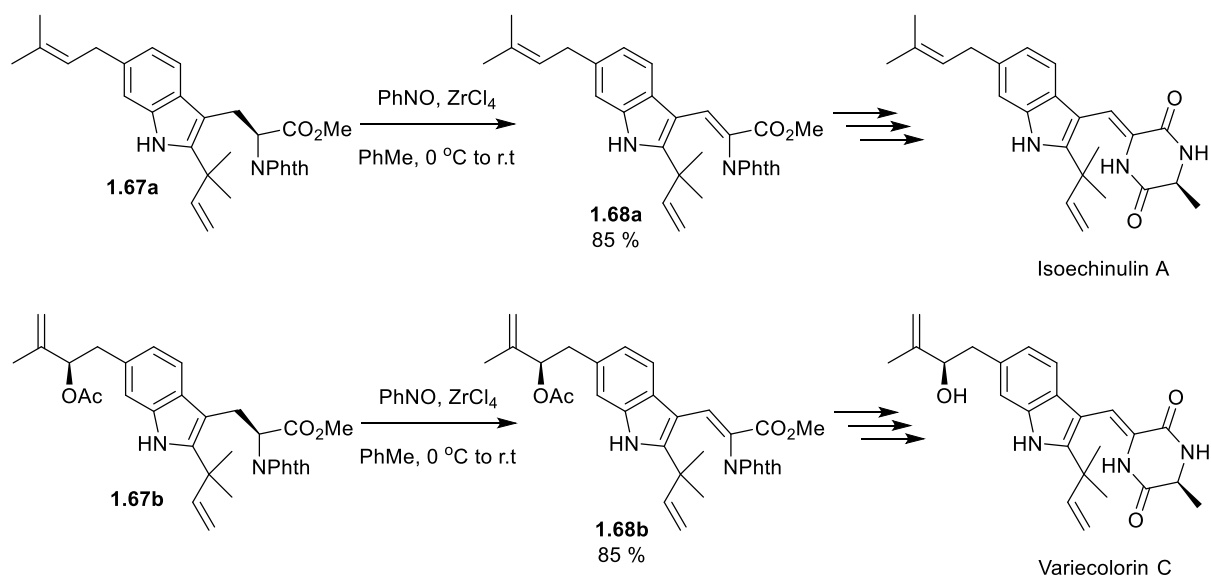
Scheme 1.27. The synthesis of isoechinulin A and variecolorin C by Dai et al. [54]

Until recently, the synthesis of a peptide that containing dehydrotryptophan consisting of more than two amino acids had not been reported. In 2020, Chen and coworkers described the synthesis of the antibiotic CDA3a (the CDAs are discussed in detail in Chapter 3).[55] In their synthesis, the researchers prepared the Z-dehydrotryptophan residue through direct oxidation of a Phth-protected tryptophan-containing dipeptide **1.69** using PhNO/ZrCl$_4$, which was converted to Z-dehydrotryptophan containing tripeptide **1.71** after further manipulation (Scheme 1.28).[55] They used this tripeptide as a building block for the solid-phase synthesis of the cyclic core of CDA3a (see chapter 3 for more details). They had originally attempted two other

routes to this tripeptide. The first involved the direct oxidation of the corresponding Trp-containing tripeptide **1.73** under various conditions, and the second involved esterification of the Z- dehydrotryptophan dipeptide **1.74** with Alloc-Thr-OMe (Scheme 1.29); however, in both

1.69
67 % over 2 steps

1.70
85 %

1.72
86 %
(16 % overall)
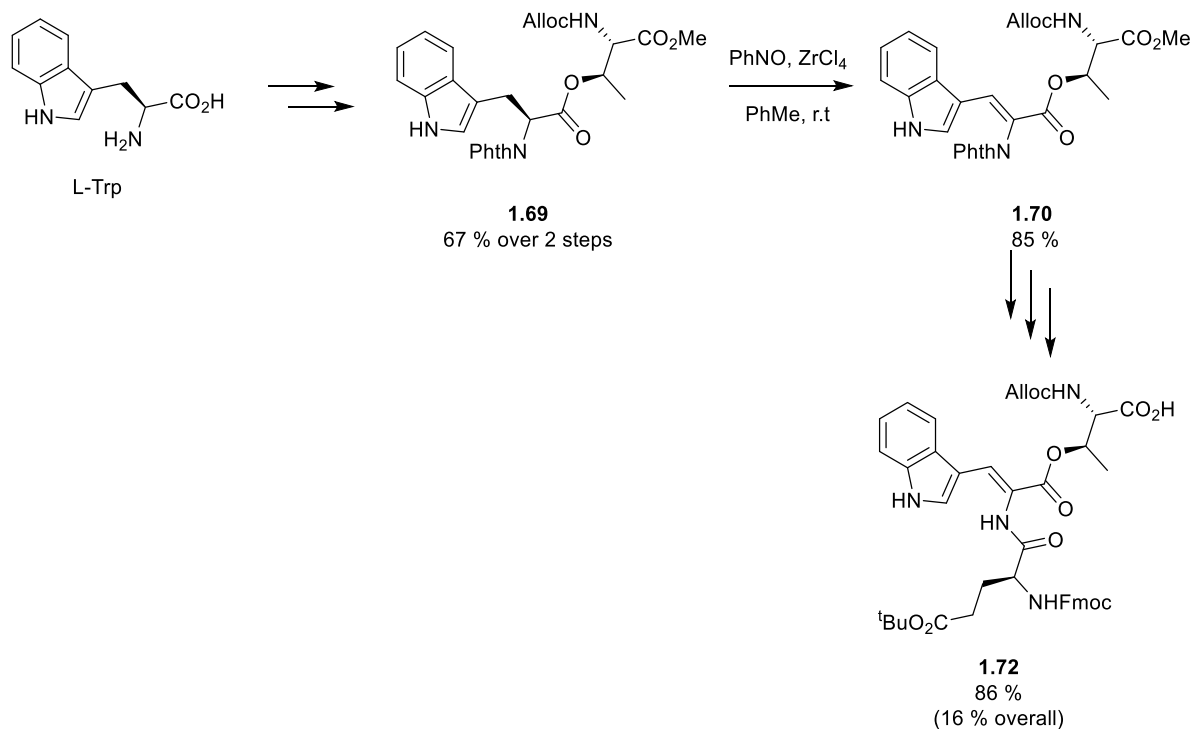
Scheme 1.28. The synthesis of Z-dehydrotryptophan containing **1.72** described by Chen et al.[55]

scenarios, the final steps were unsuccessful.[55] The researchers postulated that the esterification of the Z-dehydrotryptophan carboxylic acid had failed as a result of the residue's unsaturation leading to greatly reduced reactivity.[55]
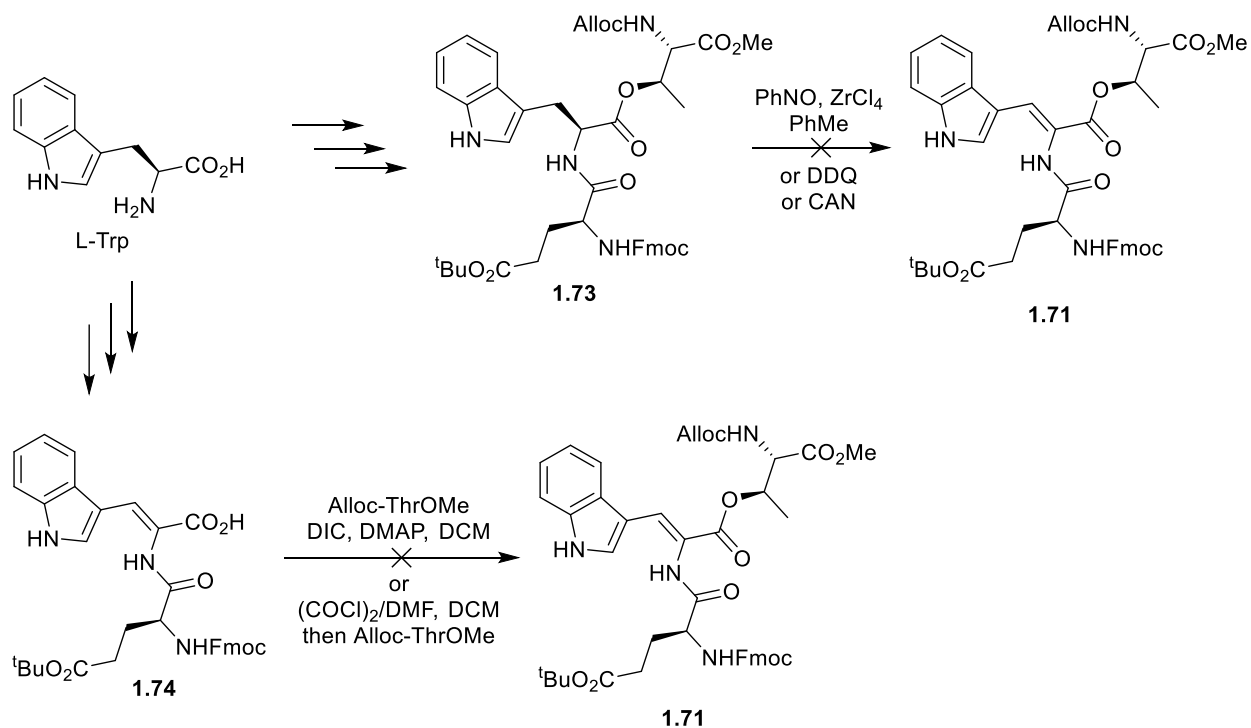
Scheme 1.29. Failed attempts of making a Z-dehydrotryptophan tripeptide **1.71** by Chen at al.[55]

Dehydrotryptophan derivatives by direct dehydration of a *N*-hydroxy tryptophan derivative

The synthesis of dehydrotryptophan compounds through base-mediated dehydration of a *N*-hydroxy tryptophan species has only been reported once. In 1990, Hermkens and coworkers reported the base-catalyzed dehydration of a protected *N*-hydroxy tryptophan **1.75** using either KO^tBu in DMSO or NaH in DME. The yield of the transformation was only reported using KO^tBu in DMSO (Scheme 1.30).[56] Interestingly, this transformation was actually undesired as the researchers had intended on alkylating the *N*-hydroxyl group of **1.75** with the 4-bromo-1,1-dimethoxybutane.[56]
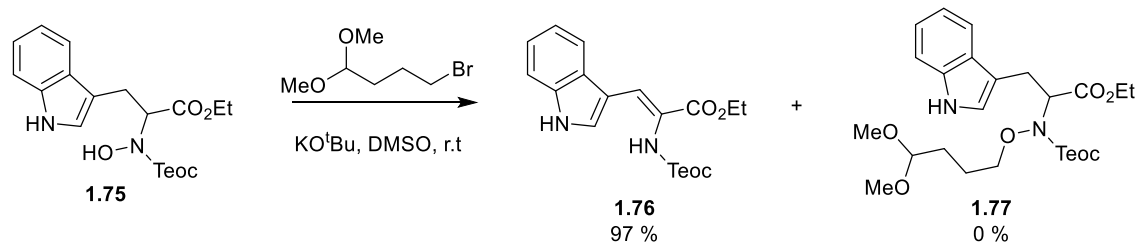
Scheme 1.30. The synthesis of dehydrotryptophan derivatives via dehydration of a *N*-hydroxytryptophan derivative described by Hermkens et al.[56]

Dehydrotryptophan derivatives by palladium and copper cross coupling reactions

Palladium-catalyzed and copper-catalyzed cross coupling reactions of vinyl-halides have been used to prepare both tri- and tetrasubstituted dehydroamino acids including the stereoselective synthesis of Z-dehydrotryptophan motifs.[30, 57] A palladium-catalyzed Heck reaction to form a Z-dehydrotryptophan species **1.80** was originally reported by Harrington and Hegedus in 1984 (Scheme 1.31).[58] The reaction proceeded with a yield of only 47 % , which was attributed to competing polymerization of the unsaturated compounds. Shortly thereafter, they were able to slightly improve upon their original method by reducing the reaction time to 5 h from 45 h (Scheme 1.31).[58, 59] In 1990, Merlic and coworkers further improved on these palladium-catalyzed Heck reactions through the addition of lithium chloride (LiCl). Remarkably, the addition of LiCl improved the yield of this reaction to 77 % from 40 % while using heterogeneous Pd/C (Scheme 1.32).[60] The researchers suggested the improved yields were attributed to the LiCl allowing the formation of the more reactive palladium-chloride intermediate. [60]
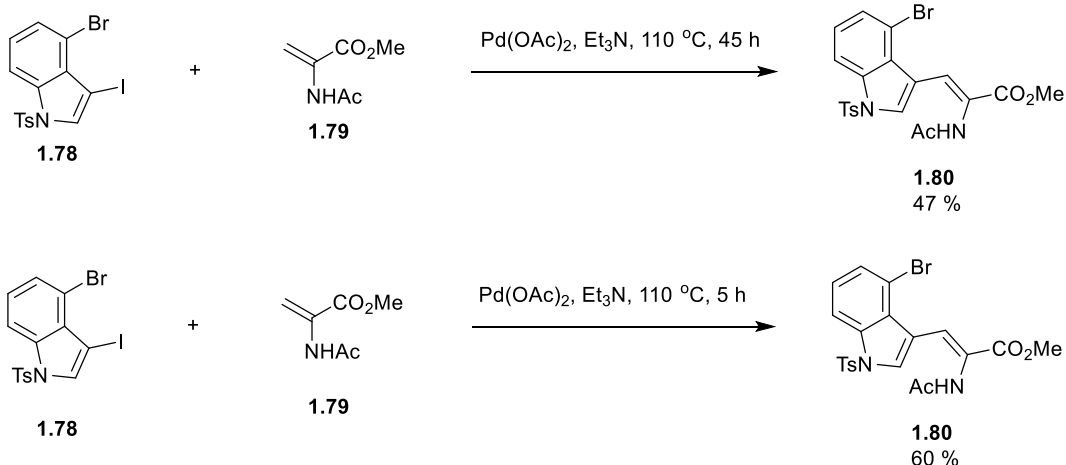
Scheme 1.31. The synthesis of dehydrotryptophan derivatives via a palladium-catalyzed cross coupling reaction described by Hegedus and coworkers.[58, 59]
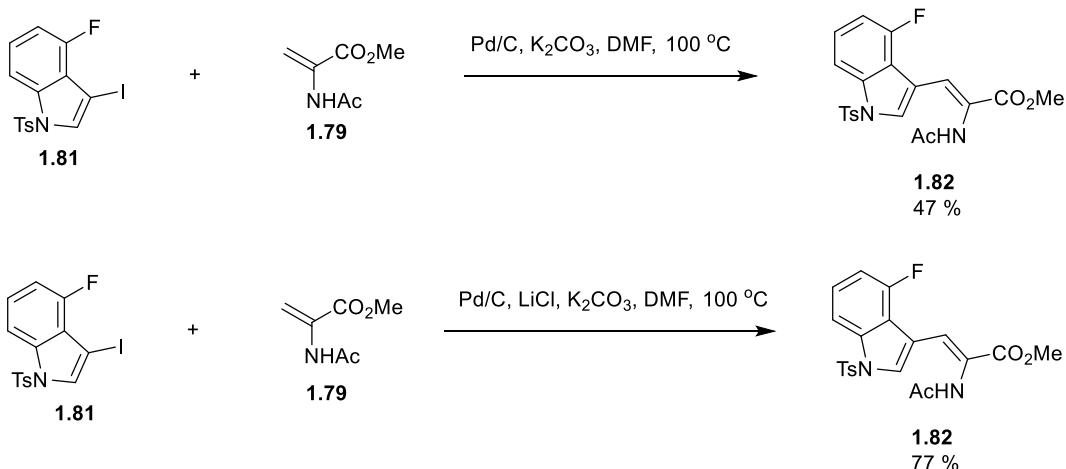
Scheme 1.32. The synthesis of dehydrotryptophan derivatives via heterogeneous palladium cross coupling described by Merlic et al.[60]

Similar methods, this time involving direct C-H vinylation, were implemented by Yokayama et al. Their original report in 1994 involved vinylation of indole derivatives **1.83** in the presence of a stoichiometric amount of palladium (II) chloride (Scheme 1.33).[61] In 1995, a modified procedure utilizing palladium (II) acetate, NaHCO$_3$ and the additive chloranil was

reported by the same group (Scheme 1.34).[62] This modified method improved the yields of the

desired Z-dehydrotryptophan species **1.87** significantly. They suggested that the chloranil aided

in oxidizing Pd(0) to Pd(II) although they could not verify this hypothesis.[62]
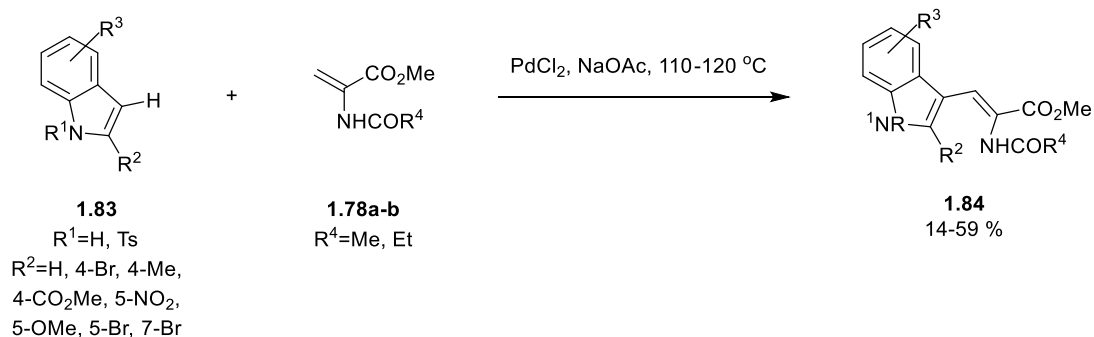
Scheme 1.33. The synthesis of dehydrotryptophan derivatives via direct C-H vinylation

described by Yokayama et al.[61]
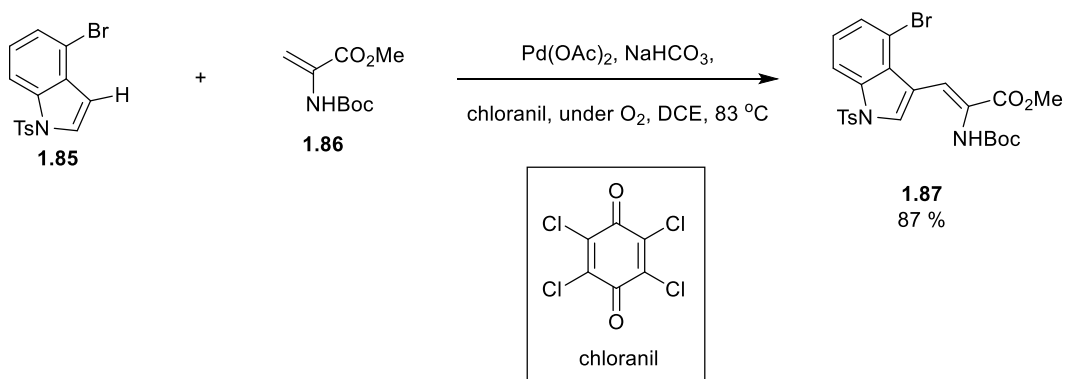
Scheme 1.34. An improved synthesis of dehydrotryptophan derivatives via direct C-H vinylation

as described by Yokayama et al.[62]

Dehydrotryptophan containing dipeptides have been successfully constructed by means

of a copper-catalyzed amidation of vinyl iodides and bromides.[63, 64] For example, in 2008,

Stanovnik et al. demonstrated that a copper (I)-catalyzed amidation of vinyl iodide **1.88** could be

used to prepare proline-dehydrotryptophan dipeptide **1.90**.[64] The indole containing vinyl iodide

1.88 was reacted with *N*-Boc-protected prolinamide **1.89** in the presence of copper (I) iodide,

cesium carbonate and an amine ligand which proceeded in a 57 % yield (Scheme 1.35).[64]
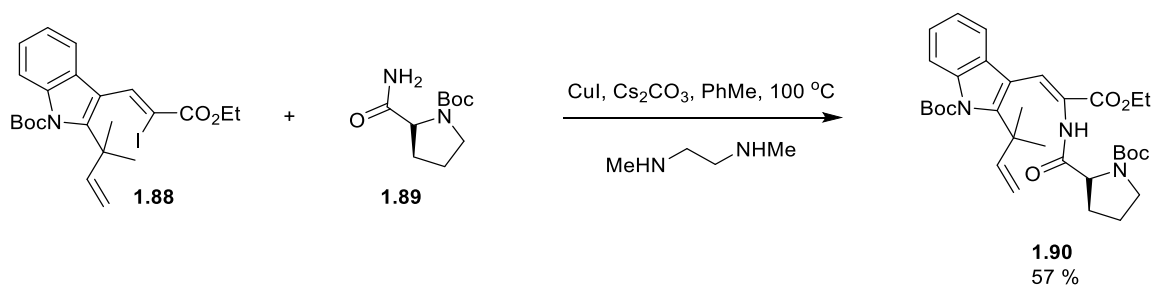
Scheme 1.35. The synthesis of dehydrotryptophan derivatives via copper (I) catalyzed

amidation as described by Stanovnik et al.[64]

From the above discussion, it is clear that there are many different methods of making

dehydrotryptophan derivatives and simple dehydrotryptophan-containing peptides; however,

none of these methods have been used to make complex dehydrotryptophan-containing

peptides using entirely solid-phase approaches, which is the most rapid and effective means for

making peptides.

1.3   Overall objectives and thesis overview

The overall objective of the work described in this thesis was to develop an entirely Fmoc

solid phase-peptide synthesis (SPPS) approach to making complex Z-dehydrotryptophan-

containing peptides.

Fmoc SPPS typically begins with attachment of a N^{α}-Fmoc-protected amino acid to an

insoluble polymer/solid support, often through a C-terminus or side chain carboxylate (Scheme

1.36—Step 1).[65] In every step, the Fmoc-protected amino acid being coupled to the resin/growing resin-bound peptide is used in excess to insure reaction completeness. Upon reaction completion, the resin with the newly bound Fmoc-protected amino is filtered and rinsed to remove any excess amino acid and coupling reagent starting material. This filtration and rinsing is conducted in all steps.[65] The second step (Scheme 1.36—step 2) involves the removal of the N^α-Fmoc protecting group using mild base, such as 20 % piperidine in N,N-dimethylformamide (DMF). The newly liberated amino group is coupled to the activated C-terminus of a subsequent N^α-Fmoc-protected amino acid (Scheme 1.36—step 3). Common coupling reagents used to activate a C-terminus carboxylate include several carbodiimides as well as numerous uronium salts. It is essential that amino acid side chains possess protecting groups that are orthogonal (e.g. removed with acid or fluoride) to the Fmoc group such that they are not inadvertently removed during base mediated Fmoc removal. The previous steps (Scheme 1.36—step 2 and step 3) are repeated to elongate the resin bound peptide.[65] Upon completion of the desired peptide sequence, the resin bound peptide is cleaved from the solid support and liberated of all acid labile amino acid side chain protecting groups upon treatment with acid, such as TFA. Typically, the full-length peptide is finally purified by reversed-phase high performance liquid chromatography (RP-HPLC).
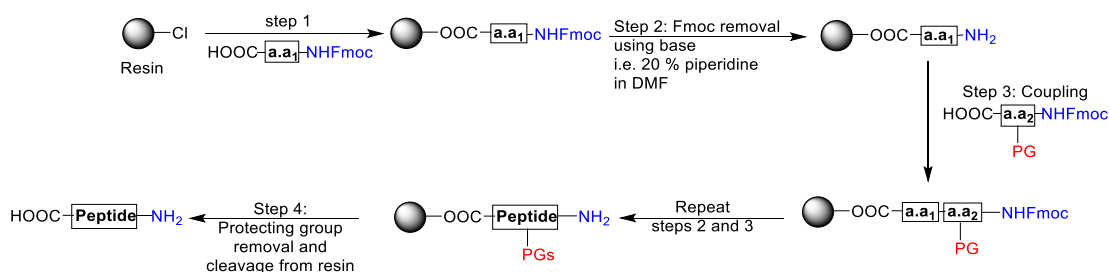
Scheme 1.36. Schematic depiction of Fmoc SPPS.

Fmoc protected dehydroamino acids cannot be used to make dehydroamino acid containing peptides using a standard Fmoc SPPS protocol because, as mentioned earlier, the reduced reactivity of the enamide nitrogen prevents it from efficiently forming amide bonds under standard coupling procedures.[13] As a result, a building block such a Fmoc-Z-dehydrotryptophan-OH would surely be ineffective in preparing Z-dehydrotryptophan containing peptides. However, β-hydroxytryptophan species are especially prone to dehydration under acidic conditions—as shown throughout section 1.2. This facile dehydration of β-hydroxytryptophan is perhaps one of the most unique features of this amino acid and, it would seem, has been overlooked in efforts to make dehydrotryptophan-containing peptides. We wished to exploit this characteristic in order to make complex Z-dehydrotryptophan-containing peptides using an entirely SPPS approach.

Chapter 2 outlines the development of a novel β-hydroxytryptophan building block that was developed for Fmoc SPPS of Z-dehydrotryptophan-containing peptides. The utility of this building block was demonstrated in the first ever synthesis of two small Z-dehydrotryptophan containing cyclic peptides, namely, tunicyclin B and sclerotide A, entirely through Fmoc SPPS.

In *Chapter 3*, we demonstrate that the methodology described in Chapter 2 can be used to make very complex Z-dehydrotryptophan-containing peptides by making the cyclic lipodepsipeptide antibiotics CDA3a and CDA4a. Analogs of CDA3a and CDA4a were also prepared, giving some insight into the structure activity relationship (SAR) of the CDAs. Preliminary biological studies with the synthetically prepared CDAs are also presented.

Chapter 4 provides a general conclusion to the thesis and offers insight into future work

that should be done to elaborate on the findings within this report.

Chapter 2

Development of a Novel β-hydroxy Tryptophan Building Block used in the Fmoc SPPS Synthesis of Z-dehydrotryptophan containing Tunicyclin B and Sclerotide A

2.1 Introduction

β-hydroxytryptophan has never been explored as a potential building block for the synthesis of complex Z-dehydrotryptophan-containing peptides. The difficulties of preparing β-hydroxytryptophan and β-hydroxytryptophan peptides are twofold. Firstly, β-hydroxytryptophan is extremely prone to undergoing a retro-aldol reaction when exposed to basic conditions and secondly, as highlighted in chapter 1, it is extremely sensitive to acid and cannot tolerate even slightly acidic conditions. In chapter 1, it was shown that the latter property has been exploited in forming Z-dehydrotryptophan and Z-dehydrotryptophan-like compounds from the β-hydroxytryptophan precursors. The former property—the retro-aldol reaction of β-hydroxytryptophan species—has been well documented in several publications. In 2004, Sugiyama and coworkers prepared an epoxidated β-hydroxytryptophan fragment **2.2** via a Sharpless asymmetric amino hydroxylation (SAAH) reaction in a 36 % yield (Scheme 2.1).[66] The researchers attributed the poor yield of the β-hydroxytryptophan species **2.2** to a retro-aldol

reaction occurring under the basic conditions required to elicit the SAAH transformation. This retro-aldol produced the corresponding indole-aldehyde **2.3** and protected glycine **2.4** (Scheme 2.2).[66]
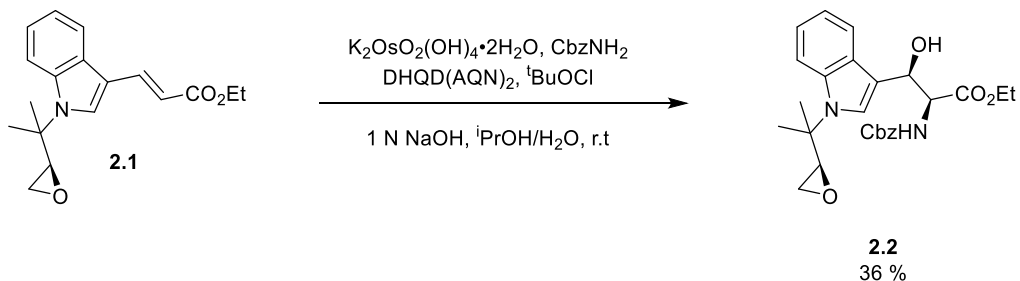
Scheme 2.1. The synthesis of **2.2** described by Sugiyama et al.[66]
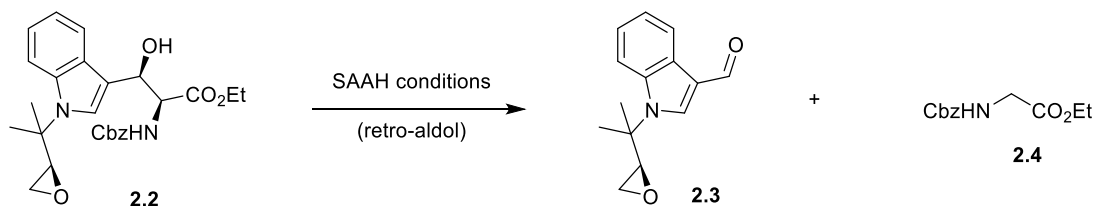
Scheme 2.2. The retro-aldol reaction of **2.2** under the basic SAAH conditions described by Sugiyama et al.[66]

A similar result was described by Koketsu and coworkers in 2006. The researchers found that hydrolysis of the methyl ester (conditions not specified) of β-hydroxytryptophan species **2.6** resulted in both epimerization and a retro-aldol reaction. The researchers ultimately resorted to using Amano protease P to generate the desired unprotected amino acid **2.7** (Scheme 2.3).[67]
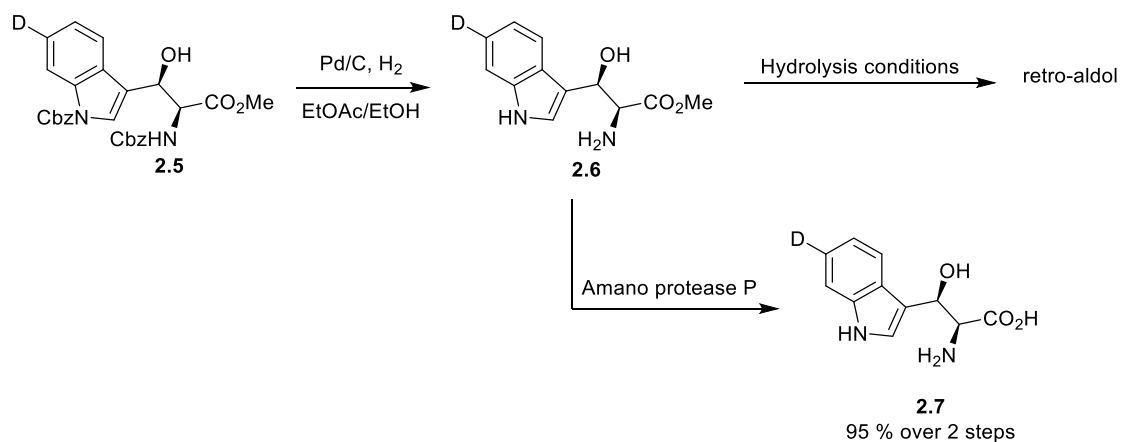
Scheme 2.3. The synthesis of **2.7** using enzymatic hydrolysis described by Koketsu et al.[67]

In 2013, during the total synthesis of (+)-bionectins A and C, Coste and coworkers also reported on the propensity of a β-hydroxytryptophan species to undergo a retro-aldol reaction. They found that the β-hydroxytryptophan derivative **2.10**, made through an asymmetric aldol reaction, had to be immediately protected as the silyl ether to avoid any retro-aldol degradation (Scheme 2.4).[68] The silylated β-hydroxytryptophan derivative **2.11** was stable enough to permit further manipulation.
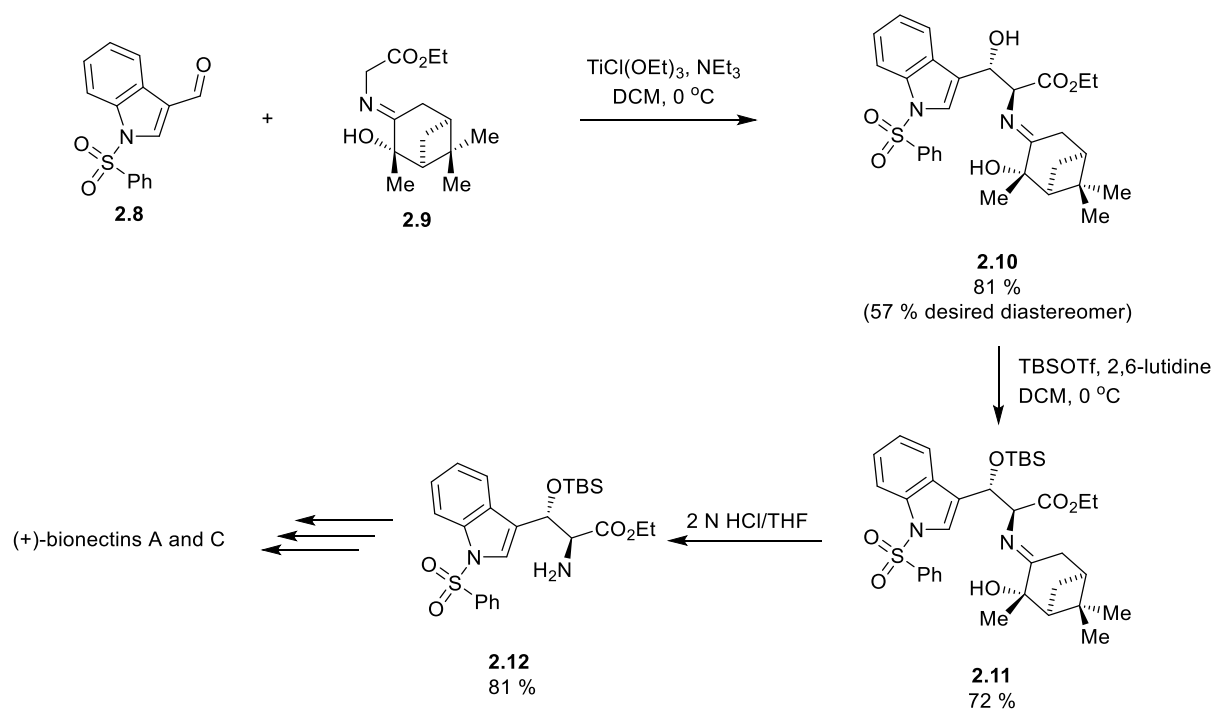
Scheme 2.4. The synthesis of a TBS-protected β-hydroxytryptophan building block **2.12** in the total synthesis of (+)-bionectins A and C described by Coste et al.[68]

In 2015, Barbie and Kazmaier were able to prepare the β-hydroxytryptophan-containing cyclic peptide cyclomarin A using a similar silylated β-hydroxytryptophan building block **2.17**. The building block was made from the protected serinal **2.14** which was reacted with the indolic zincate species **2.13** to form the corresponding β-hydroxytryptonol **2.15** which was then silylated with *tert*-butyldimethylsilyl trifluoromethanesulfonate (TBSOTf) to give **2.16**. The researchers were then able to remove the primary TBS group and oxidize the corresponding alcohol which produced their desired Alloc-protected amino acid building block **2.17** (Scheme 2.5).[69]
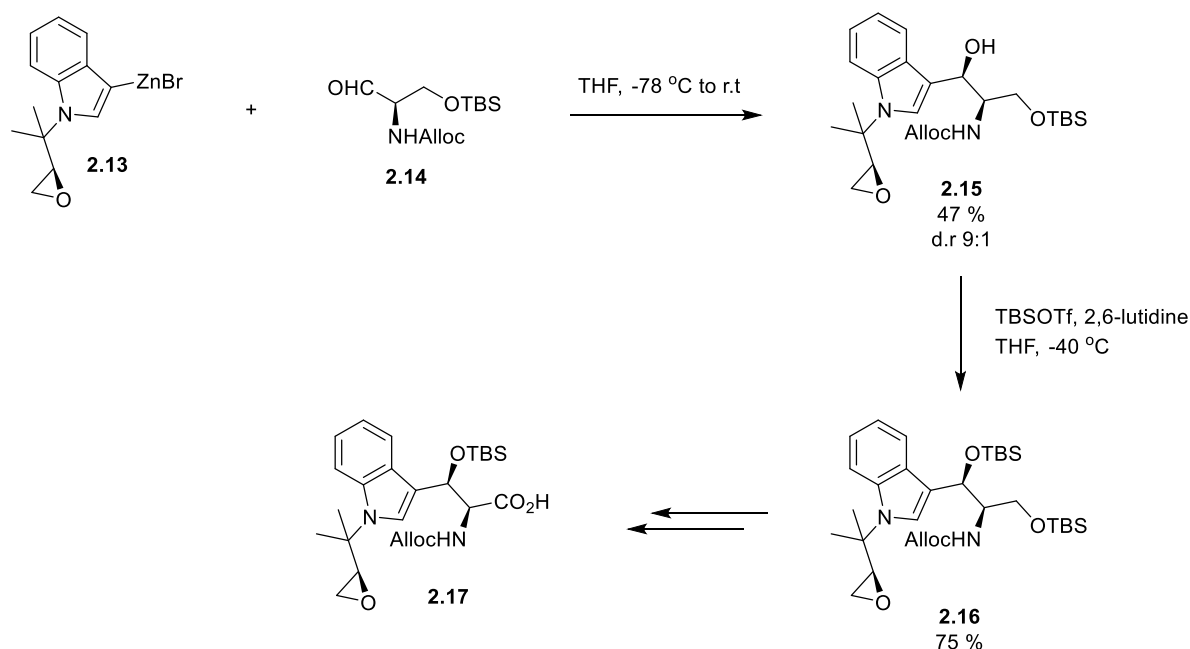
Scheme 2.5. The synthesis of **2.17** used in the synthesis of cyclomarin A described by Barbie and Kazmaier.[69]

Interestingly, during earlier preliminary studies in 2015, Barbie and Kazmaier described the synthesis of a similar *N*-Boc-protected building block which they had originally aimed to use during the synthesis of cyclomarin A. However, they reported—without providing details—that the Boc group could not be removed in the peptide without several side reactions. Initial attempts to cleave the primary TBS ether in the presence of the secondary one under acidic conditions failed or resulted in decomposition of intermediate **2.18**. They discovered that treating the doubly silylated β-hydroxytryptonol **2.18** with catalytic pyridinium *p*-toluenesulfonate (PPTS) in methanol afforded the corresponding secondary methyl ether **2.20** as a 1:1 mixture of diastereomers (Scheme 2.6A).[70] When water was used as the solvent, they found that a deprotected alcohol product **2.21** was obtained in 71 % yield, however, it was not the desired primary alcohol but rather the secondary one as a mixture of diastereomers (Scheme

2.6B).[70] The researchers explained that these side reactions were the result of a well-stabilized indolic cation **2.19** that was being captured by either methanol or water (Scheme 2.6A & B).[70] Eventually, the desired TBS removal was achieved by using ammonium fluoride (NH_4F) in methanol.
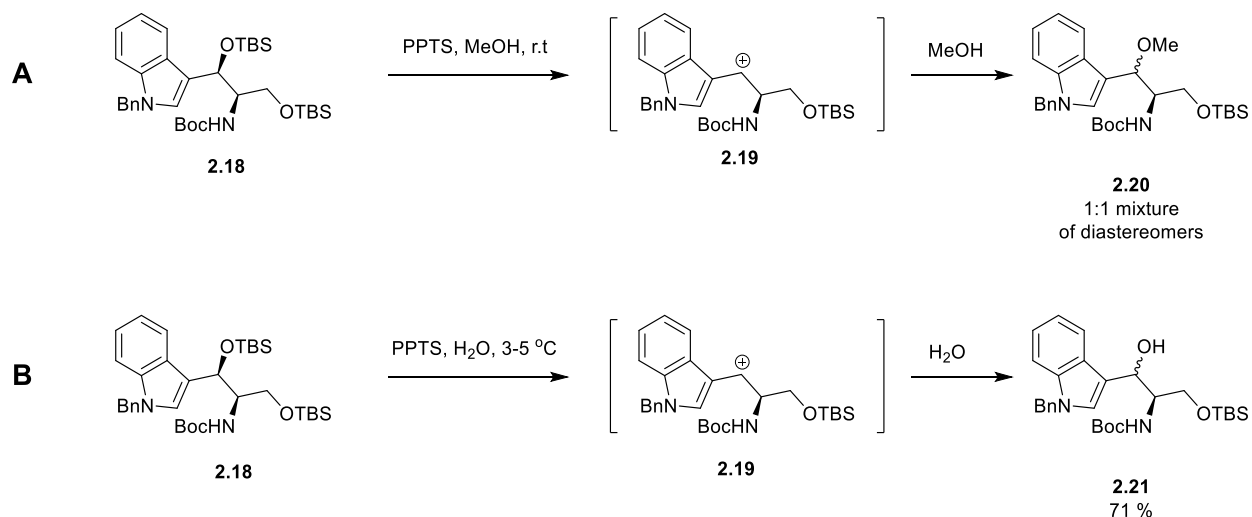
Scheme 2.6. The generation of indolic cations from **2.18** observed by Barbie and Kazmaier.[70]

2.2 Objectives

The findings of Barbie and Kazmaier's suggest that indolic cations can be easily generated directly from the corresponding silyl ether under acidic conditions. As previously mentioned, similar indolic cations generated from non-silylated β-hydroxytryptophan compounds have been shown to undergo proton abstraction to generate the corresponding Z-dehydrotryptophan species. We believed a similar reaction could be achieved from a silylated β-hydroxytryptophan residue under acidic conditions to form Z-dehydrotryptophan residues. A silylated β-hydroxytryptophan motif would also prevent a retro-aldol reaction, thus making it applicable in Fmoc SPPS, as it could withstand basic Fmoc removal conditions without fragmenting. This

process would involve preparing a N^α-Fmoc Trp building block with a suitably protected indolic alcohol, such as TBS ether/Fmoc protected β-hydroxytryptophan **2.22** (Scheme 2.7), that could be incorporated into a peptide using standard Fmoc SPPS. Upon cleavage from the resin and global deprotection of the peptide under standard acidic conditions, the TBS-protected indolic alcohol would eliminate generating the resulting stabilized indolic cation and, upon proton abstraction, would give the corresponding dehydrotryptophan containing peptide as the thermodynamically favored Z-isomer (Scheme 2.7).
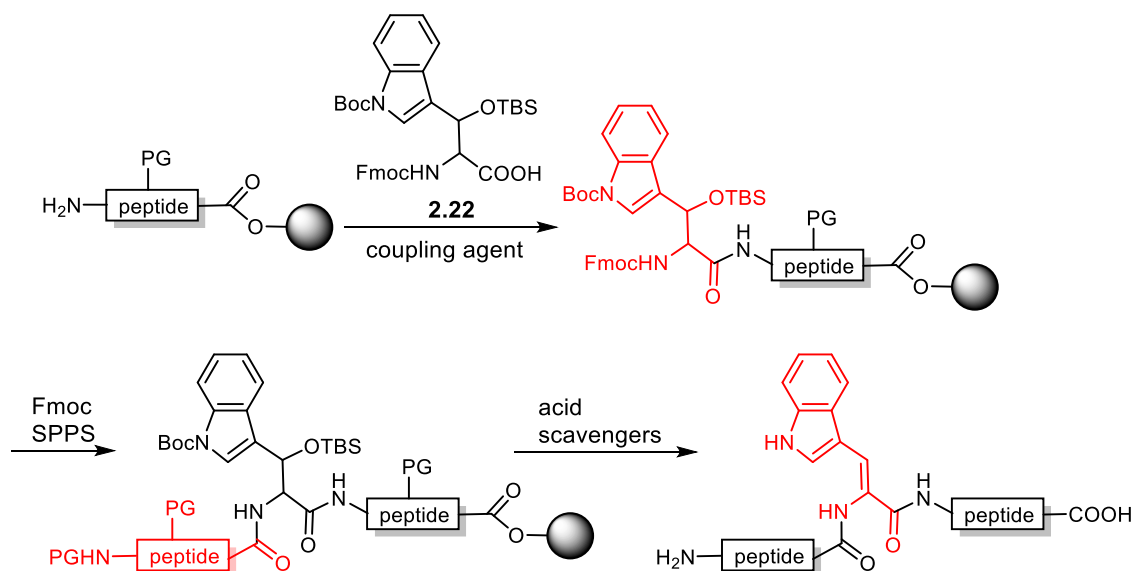
Scheme 2.7. Proposed route to Z-dehydrotryptophan-containing peptides.

The objective of the work described in this chapter was to demonstrate that dehydrotryptophan-bearing peptides can be prepared using the approach outlined in Scheme 2.7.

2.3 Results and Discussion

2.3.1 Synthesis of Fmoc-β-HOTrp(Boc)(TBS)-OH—a protected β-hydroxytryptophan building block

The first step was to prepare building block **2.22**. Perhaps the most facile way of preparing β-hydroxytryptophan—which could then be TBS-protected—is via an aldol reaction. Luckily, in the approach depicted in Scheme 2.7, the stereochemistry of the protected β-hydroxytryptophan is not important as, under the acidic conditions to generate the indolic cation, only the thermodynamically favored Z-isomeric product should be formed as a result of either stereochemical elimination of the intermediate indolic cation or by E-/Z-isomerization of the enamide under the acidic conditions. Thus, building block **2.22** was prepared as a mixture of diastereomers (Scheme 2.8). We devised a synthesis of **2.22** beginning with an aldol reaction between commercially available Cbz-Gly-OMe and Boc-indole-3-carboxaldehyde using LDA and ZnCl$_2$ in THF at -78 °C. The addition of ZnCl$_2$ greatly improved the yield as the intermediate glycine zincate species remained soluble even at -78 °C whereas the dilithiated glycinate (when ZnCl$_2$ is not added) precipitated from solution. The resulting crude alcohol **2.23** was treated with TBSOTf with DIPEA to generate the TBS-protected product **2.24**, which was immediately hydrolyzed, using aq. LiOH in THF, to give **2.25** as a mixture of diastereomers in 78 % yield over 3 steps. Next, **2.25** was subjected to palladium-catalyzed hydrogenolysis to remove the Cbz group followed by Fmoc protection of the α-amino group using FmocOSuc under Schotten-Baumann conditions to give **2.22** as a mixture of diastereomers in 97 % over 2 steps. The simplicity of this synthesis allowed for bench stable amino acid **2.22** to be rapidly and easily prepared on a multigram scale.
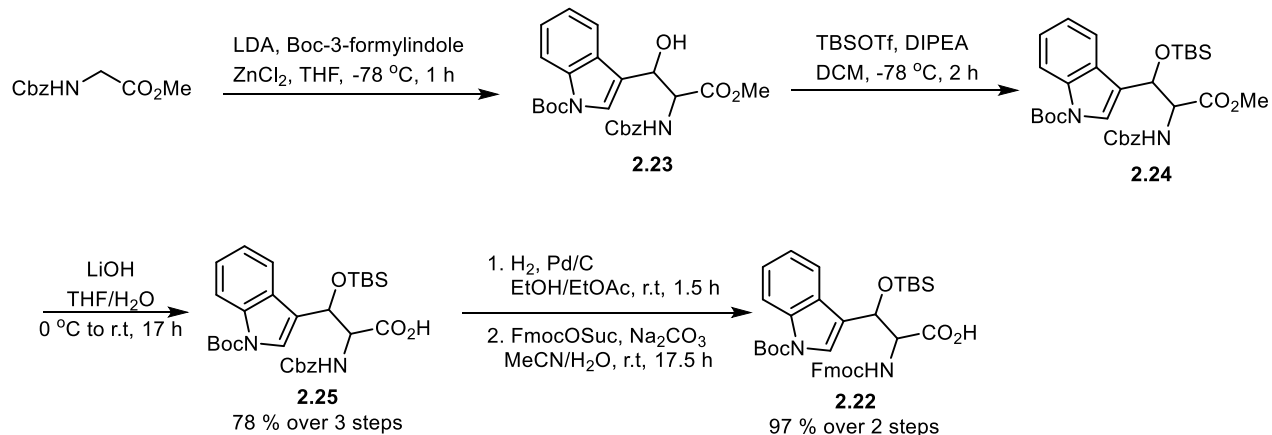
Scheme 2.8. The synthesis of building block **2.22**.

With the building block **2.22** in hand, we next aimed to see if this building block could be used to prepare Z-dehydrotryptophan containing peptides using an Fmoc SPPS approach as depicted by Scheme 2.7.

2.3.2. The synthesis of tunicyclin B

We decided to first test out our approach to Z-dehydrotryptophan peptides by attempting the synthesis of a relatively simple target, tunicyclin B. The tunicyclins are a family of cyclic peptides that were originally derived from the medicinal herb *Psammosilene tunicoides* (Figure 2.1).[71] Tunicylin A, which was the first of the tunicyclins to be discovered in 2009 by Tian et al., was found to have a unique substituted pyrrolopyrazinone ring embedded within its cyclic peptide core.[72] The remaining tunicyclins (B-D) were discovered in 2010, also by Tian and coworkers.[73] Tian et al. revealed that tunicyclin D showed potent antifungal activity. In contrast, the researchers were unable to determine the biological function of tunicylin A-C. Structurally, tunicyclin B was found to contain a Z-dehydrotryptophan residue as part of its heptapeptide cyclic core.[73] Tian and coworkers initially identified the presence of the Z-dehydrotryptophan residue

by its unique maximum absorption at 338 nm in methanol which was consistent with other dehydrotryptophan containing peptides such as keramamide F. The researchers assigned the stereochemistry of the residue to be Z based on the remarkably downfield chemical shift of its olefinic proton (δ_H 8.82) and by ROESY correlations between aromatic Z-dehydrotryptophan protons and the α-proton of the adjacent serine residue. [73]

Tunicyclin A

Tunicyclin B

Tunicyclin C
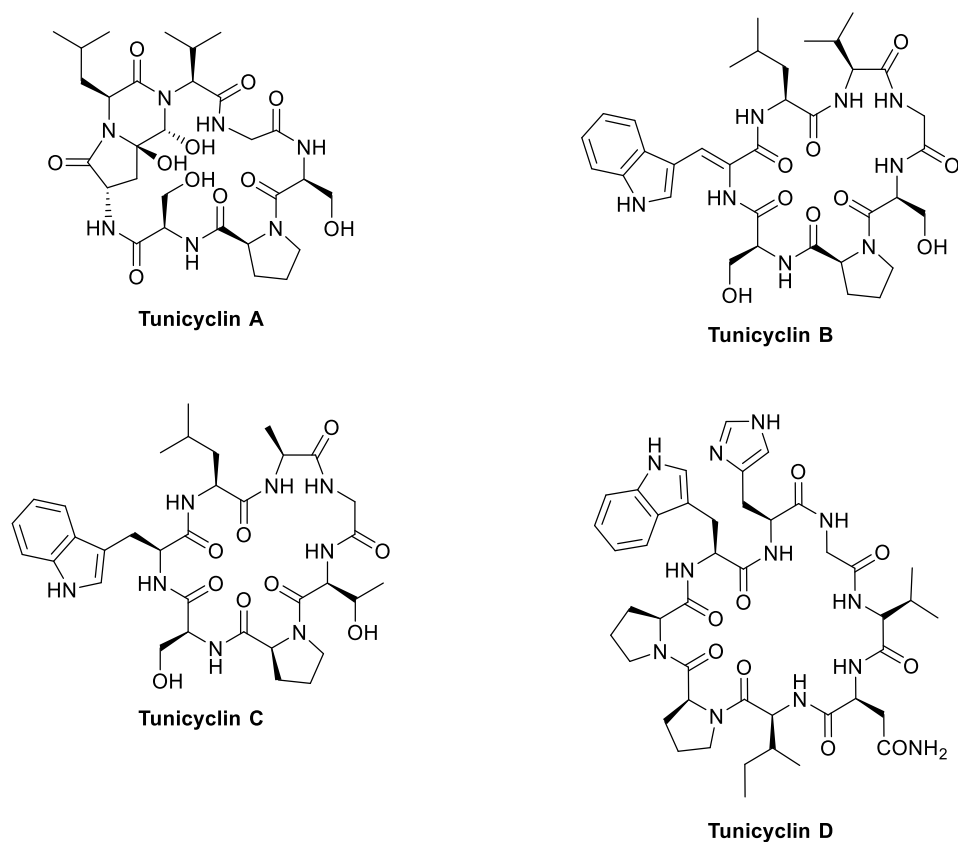
Tunicyclin D

Figure 2.1. Structure of the tunicyclins.[72, 73]

In 2011, Kaur and coworkers were able to successfully prepare tunicyclin C and tunicyclin D using an Fmoc SPPS approach.[71] The researchers utilized the C-terminus achiral glycine residue in both peptides as the resin anchoring point and the eventual off-resin cyclization point.[71] In their synthesis—as well as most peptide syntheses—glycine residues are the ideal cyclization and

resin attachment point as this C-terminus glycine minimizes steric interactions at the cyclization site during macrocyclization while also preventing any opportunity for epimerization during this final step. Using this approach, the researchers isolated tunicylin C and D in 55 % and 40 % overall yields respectively (Scheme 2.9).[71] They found that the macrocyclizations proceeded rapidly likely as a result of the turn-inducing proline residue found in both peptides **2.26** and **2.27**. A similar synthesis of tunicyclin D was reported in 2012 by Guo which used a Fmoc SPPS approach as well. [74] In contrast, the chemical synthesis of Z-dehydrotryptophan containing tunicyclin B had yet to be described. We believed tunicyclin B would be the ideal peptide to test our new approach to making Z-dehydrotryptophan-peptides as it is relatively simple and contains commercially available amino acids (apart from the Z-dehydrotryptophan residue). We dissected the peptide into two key fragments and the synthesis into two key-steps (Scheme 2.10). Firstly, we envisioned that the Z-dehydrotryptophan residue could be generated by an acid-mediated elimination of the proceeding TBS-protected β-HOTrp containing peptide **2.28** during final acidic global deprotection. This TBS protected β-HOTrp containing peptide would be constructed via Fmoc SPPS using amino acid **2.22** as a building block. We were hopeful that standard TFA based resin cleavage/protecting group removal conditions would be sufficient to generate the desired Z-dehydrotryptophan containing peptide. Secondly, much like the syntheses of tunicylin C and D, the achiral Fmoc-glycine residue was selected as the C-terminus resin attachment point and the site for an off-resin macrocyclization.
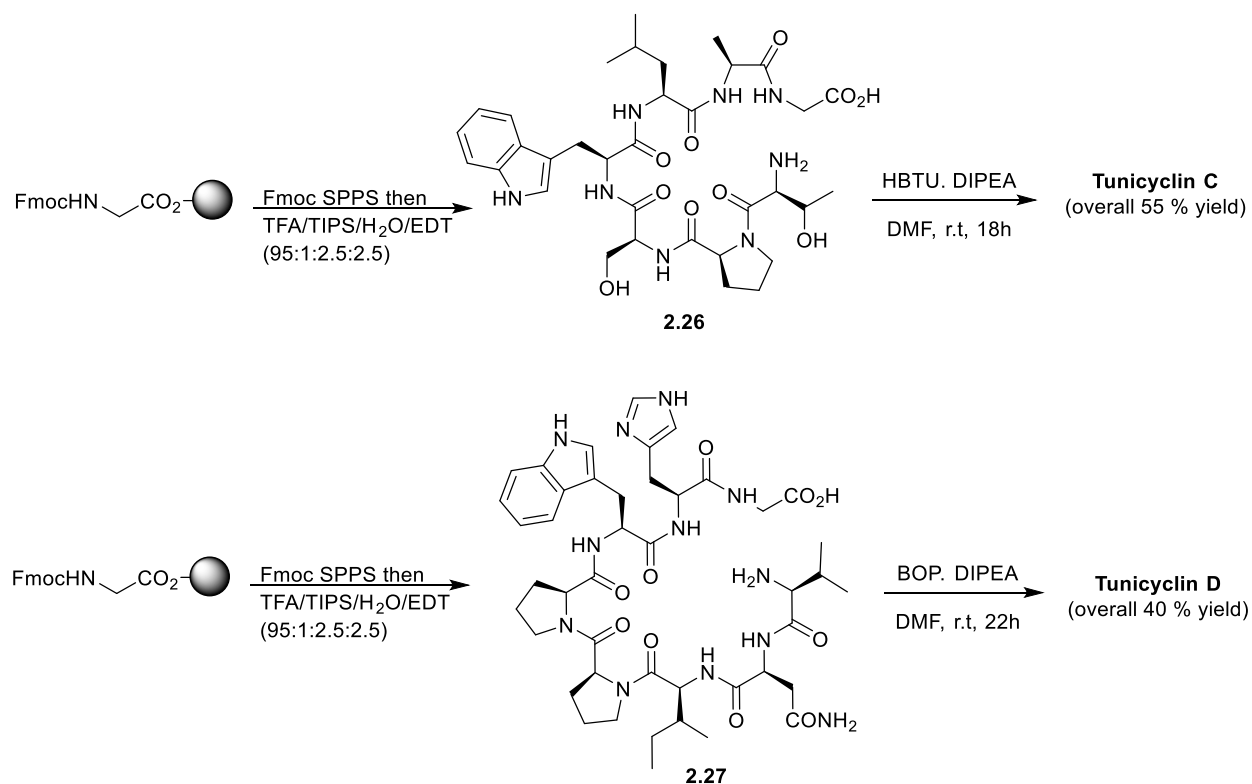
Scheme 2.9. Synthesis of tunicyclin C and D by Kaur et al.[71]
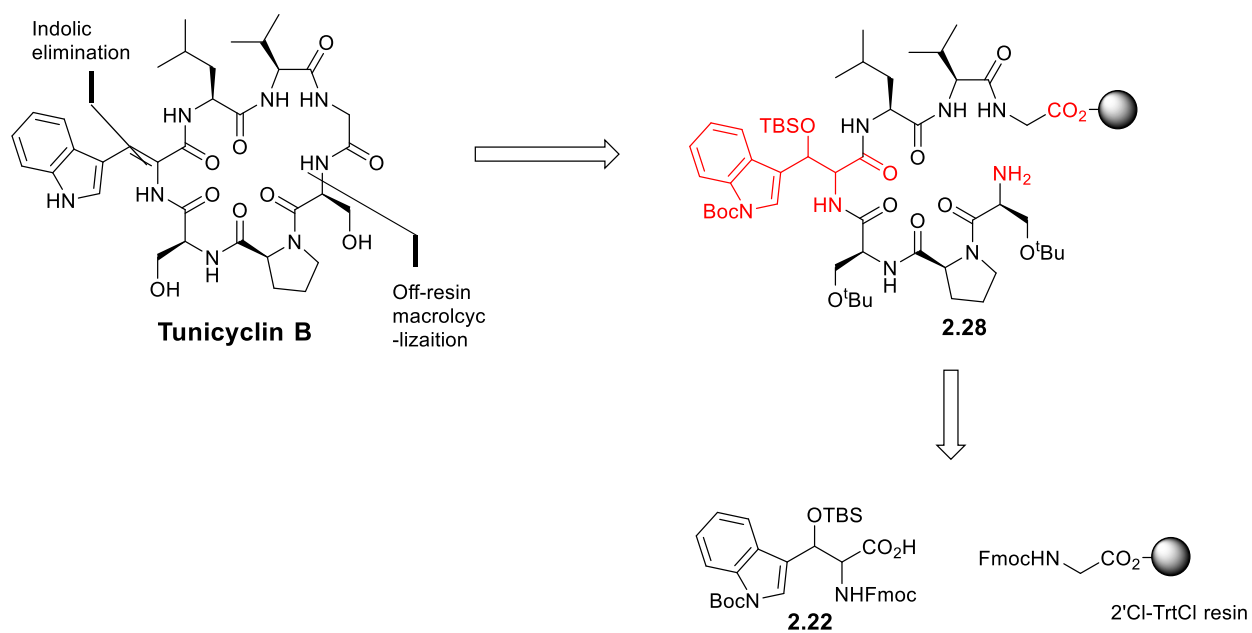
Scheme 2.10. Retrosynthesis of tunicyclin B.

The synthesis of tunicyclin B began by loading Fmoc-Gly-OH on the 2'-Cl-TrtCl resin through its C-terminus carboxylate (Scheme 2.11). This was done by treating the resin the Fmoc-Gly-OH with DIPEA in dry DCM through an S_N1 type reaction between the carboxylate of glycine and trityl cation generated from the 2'-Cl-TrtCl resin. Through typical Fmoc SPPS protocols, using 10 % 4-methylpiperidine (4-MP) for Fmoc removal and DIC/HOBt as coupling agents, the Val and Leu residues were incorporated giving resin bound tripeptide **2.29**. The Fmoc group of the Leu residue was removed and **2.22** was successfully and easily incorporated using DIC/HOBt in DMF to give **2.30**.The remaining amino acids were installed using standard Fmoc SPPS to produce resin-bound linear peptide **2.28**. The Fmoc group of the final Ser residue was removed and the peptide was subjected to global deprotection using TFA/H_2O (95:5) at room temperature. We found that the diastereomeric peptide intermediate had been quantitatively converted to a single peak by HPLC whose mass corresponded to that of the Z-dehydrotryptophan containing peptide (see Appendix A). The peptide also strongly absorbed at 339 nm reaffirming that our indolic elimination approach had been successful. Interestingly, if the popular carbocation scavenger triisopropylsilane (TIPS) was added to the TFA/H_2O cleavage cocktail, the Z-dehydrotryptophan was completely reduced to the corresponding tryptophan. This reaction was also recently reported when TIPS was added to a Z-dehydrotryptophan-containing peptide.[55] Finally, off-resin macrocyclization of the cleaved linear peptide proceeded rapidly—presumably a result of the turn inducing Pro and Z-dehydrotryptophan residues—with PyOAP/HOAt/2,4,6-colidine in DMF which, upon reversed-phase HPLC purification, produced tunicyclin B in a 28 % overall yield (based on resin loading). The NMR and HRMS spectra of tunicyclin B closely matched with those reported in the literature (See Appendix A).[73]

FmocHN⏤CO₂⏤◯

2'-Cl-TrtCl

→ Fmoc SPPS →

FmocHN ... NH HN ⏤CO₂⏤◯

2.29

→ **2.22** DIC, HOBT DMF, 4h →

TBSO, HN, NH HN ⏤CO₂⏤◯ BocN NHFmoc

2.30

→ Fmoc SPPS →

TBSO HN NH HN⏤CO₂⏤◯ BocN HN NH₂ OᵗBu OᵗBu

2.28

→ 1 TFA/H₂O (95:5), r.t, 75 min
2) PyOAP, HOAt, 2,4,6-collidine DMF, r.t, 20 h →

HN HN NH HN OH OH

Tunicyclin B
overall 28 % yield
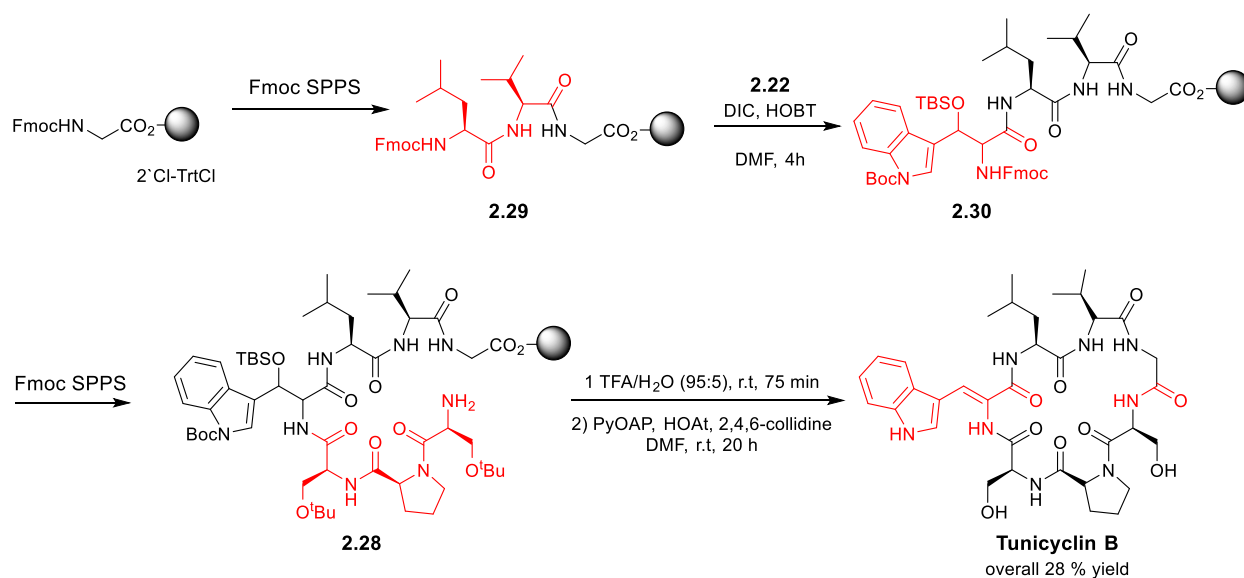
Scheme 2.11. The synthesis of tunicyclin B.

2.3.3 The synthesis of sclerotide A

Although we demonstrated that our methodology could be used to prepare tunicylin B, this peptide is a relatively simple one. Therefore, we decided to evaluate our methodology on the slightly more challenging target sclerotide A. In 2009, Zheng and coworkers isolated two novel hexapeptide compounds from *Apergillus scletotiorum*, a marine-derived halotolerant fungus, found in the Putian Sea off the coast of China. Of the two peptides, the major metabolite was sclerotide A and the minor metabolite was sclerotide B (structures shown in Figure 2.2).[33] As briefly discussed in Chapter 1, sclerotide A and B were both found to be structurally identical except for the stereochemistry of the dehydrotryptophan residue which was determined to be Z- in sclerotide A and E- in sclerotide B. The two peptides were also found to contain two uncommon D-amino acids, D-Phe and D-Ser, as well as the unusual amino acid anthranilic acid (Ant). Both compounds were found to show moderate antifungal activity while sclerotide B also showed weak cytotoxic and antibacterial activity. It was found that the two isomeric forms of

50

dehydrotryptophan could rapidly interconvert when exposed to light with the Z-isomer in sclerotide A dominating.[33] The researchers suggested that only sclerotide A is produced by *A. sclerotiorum*, and that sclerotide B was probably generated during fermentation and isolation steps as a result of this photoreaction.[33]
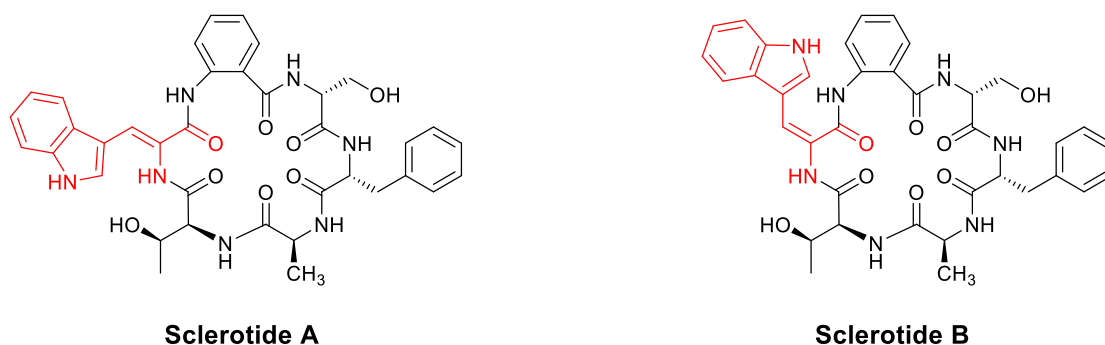
Figure 2.2. The structures of sclerotide A and B from *Apergillus scletotiorum*.[33]

Unlike tunicyclin B, sclerotide A contains no achiral glycine residue. Resin attachment to the C-terminus of the Ant residue would likely prove unsuccessful as these residues often result in extremely low resin loading and poor macrocyclization efficiency as a result of the steric hindrance that surrounds the Ant carboxylate.[75] Similarly, cyclizing onto the C-terminus of amino acid **2.22** from the NH$_2$ of the Ant would likely prove to be difficult, due to the reduced nucleophilicity of the aniline NH$_2$ when compared to other amino acids. However, Ant containing hexapeptides have been made through cyclization onto the C-terminus carboxylate of an Ala residue with no reported epimerization.[76] Therefore, we adopted a similar approach to the synthesis of sclerotide A with the C-terminus carboxylate of Ala as the resin attachment point and site of macrocyclization. We anticipated that one of the major challenges in the synthesis of sclerotide A was going to be the coupling of amino acid **2.22** onto the aniline NH$_2$ of Ant. Therefore, we devised two parallel approaches; one where the Ant/**2.22** amide bond was to be

prepared in solution—to form the corresponding Fmoc protected dipeptide **2.33** (Scheme 2.11A), and the other where the Ant/**2.22** amide bond was to be constructed on the resin bound peptide from the proceeding Fmoc-Ant-OH residue (**2.32**) (Scheme 2.11B). In either approach, the synthesis would also involve acid-mediated resin cleavage/protecting group removal and Z-dehydrotryptophan formation from a TBS protected β-HOTrp containing peptide **2.31** (made from Fmoc SPPS using amino acid **2.22**) as a key step. It was reported in 2014 by Masuda et al. that the solid-phase amidation of Ant residues could be achieved via an in situ generated amino acyl chloride using triphosgene.[76] However, triphosgene is extremely toxic so we first planned to attempt the hybrid solution-phase dipeptide/Fmoc SPPS approach to sclerotide A.
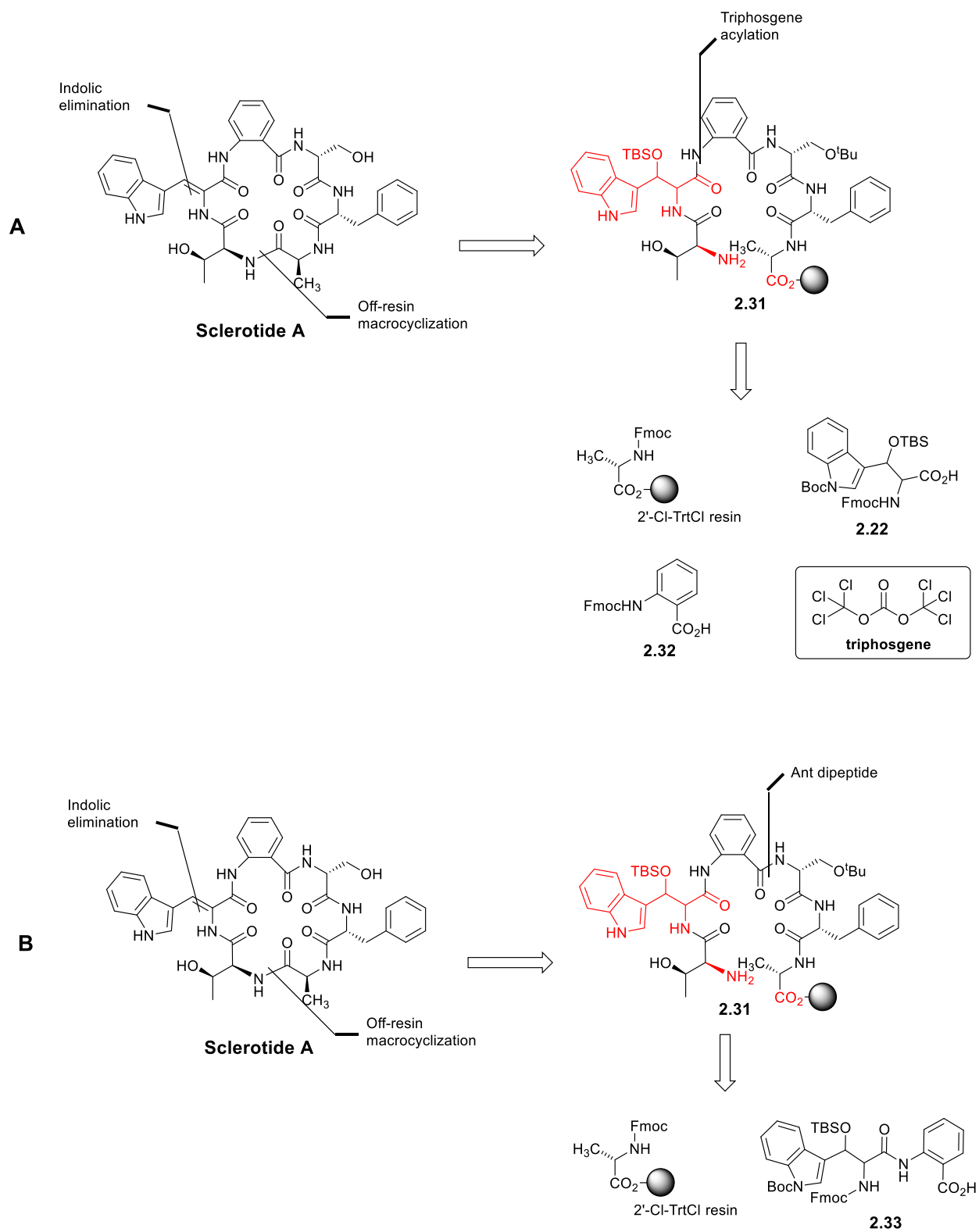
Scheme 2.12. Retrosynthesis of sclerotide A.

Ant containing dipeptides **2.34** are prone to benzoxazinone formation upon activation of the aryl carboxylate. A general depiction of this reaction is shown in Scheme 2.13.[75] These benzoxazinone species **2.35** have been shown to rapidly form and are often quite unreactive towards nucleophilic attack (shown in blue),[75] although conflicting reports suggest that Ant-containing dipeptides can react with the secondary amine of Pro residues to generate the resulting amide compounds **2.36** in good yields.[77] So we were hopeful that the α-NH$_2$ of the D-Ser residue could be sufficiently nucleophilic to react with activated **2.33** and produce the resulting desired product.
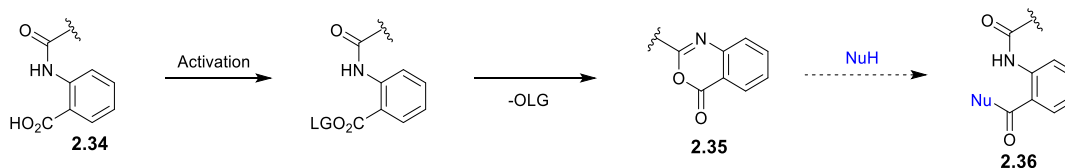
Scheme 2.13. Proposed nucleophilic opening of an intermediate benzoxazinone species **2.35**.

It has been shown that unprotected Ant can be coupled to the carboxylate of protected cysteine using *N,N'*-carbonyldiimidazole (CDI) in a good yield.[78] Similarly, Ant residues have been acylated using acyl fluorides of the corresponding amino acids.[79] However, attempts to couple unprotected Ant to **2.22** using DIC/HOBt/DMAP in DCM, CDI in DMF or SOCl$_2$ in either DCM or MeCN resulted in no formation of dipeptide **2.33** but instead resulted in recovery of starting material and the formation of unidentifiable by-products (Scheme 2.14).
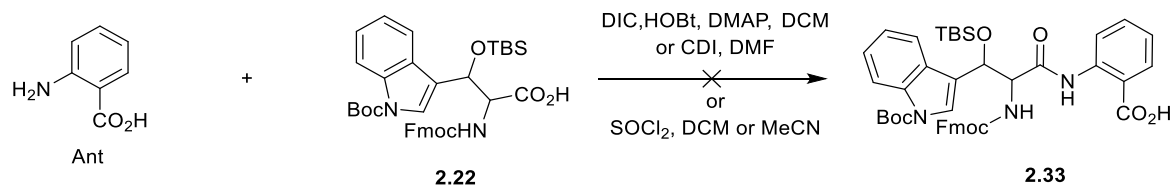
Scheme 2.14. Failed attempts to prepare dipeptide **2.33**.

We suspected the above coupling reactions failed because the Ant could not be fully solubilized in DCM, MeCN or even DMF. This prompted us to devise a modified approach to dipeptide **2.33**. We believed that a COOH-protected Ant residue would be more soluble in common coupling reaction solvents (such as DCM and DMF), and the reaction would proceed with less by-products. So, we devised a one-pot procedure in which the Ant carboxylate was protected as the TMS ester—generated in situ using trimethylsilyl chloride (TMSCl)—and then the aniline NH_2 was acylated from the HOBt ester of **2.22** in the presence of catalytic DMAP. The TMS ester was hydrolyzed upon acidic workup and was therefore never isolated. This procedure gave compound **2.33** in an 83 % yield (Scheme 2.15).
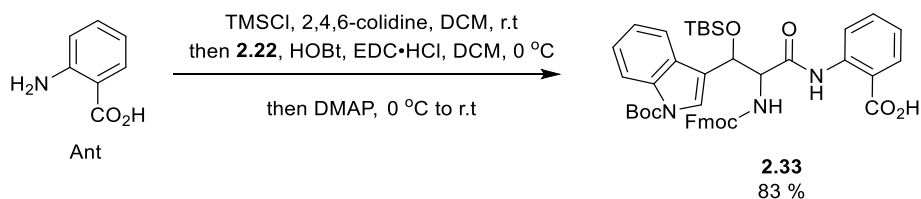
Scheme 2.15. The synthesis of **2.33**.

With all the required building blocks to make sclerotide A in hand, we began to assemble the peptide using Fmoc SPPS (Scheme 2.16). 2′-Cl-TrtCl resin, loaded with Fmoc-Ala-OH, was subjected to standard Fmoc SPPS resulting in tripeptide **2.37**. Coupling of **2.33** using DIC/HOBt in DMF resulted in complete consumption of tripeptide **2.37**. Cleavage/deprotection of a small amount of the resulting peptide from the resin using TFA/H_2O followed by HPLC-MS analysis revealed that the desired pentapeptide was not formed, instead, tetrapeptide **2.38** was generated. This fragmented peptide could be elongated by coupling on the subsequent Thr residue. Analysis of this reaction showed that the Thr had been coupled to **2.38** to form **2.39**. To

the best of our knowledge, such a fragmentation reaction has not been reported in the literature.

A possible mechanism for this fragmentation reaction is given in Scheme 2.17. Activation of the C-terminus carboxylate of **2.33** results in rapid benzoxazinone formation **2.40** which is then attacked by the α-NH$_2$ of tripeptide **2.37** to form a tetrahedral intermediate **2.41**. The next step may involve protonation of the aniline nitrogen (intermediate **2.42**) and subsequent collapse of the tetrahedral intermediate (intermediate **2.43**) which, after attack at the aryl ester with either water found in DMF or -OBt, would produce the corresponding fragmented tetrapeptide **2.38**. Acidic cleavage and deprotection using TFA/H$_2$O, would give rise to the observed cleaved/Z-dehydrotryptophan peptide derivative of resin bound **2.38** detected by HPLC-MS. Suspecting that HOBt was involved in producing the fragmented **2.33**, we attempted the same coupling in the absence of HOBt. Unfortunately, this did not suppress the formation of **2.38**.
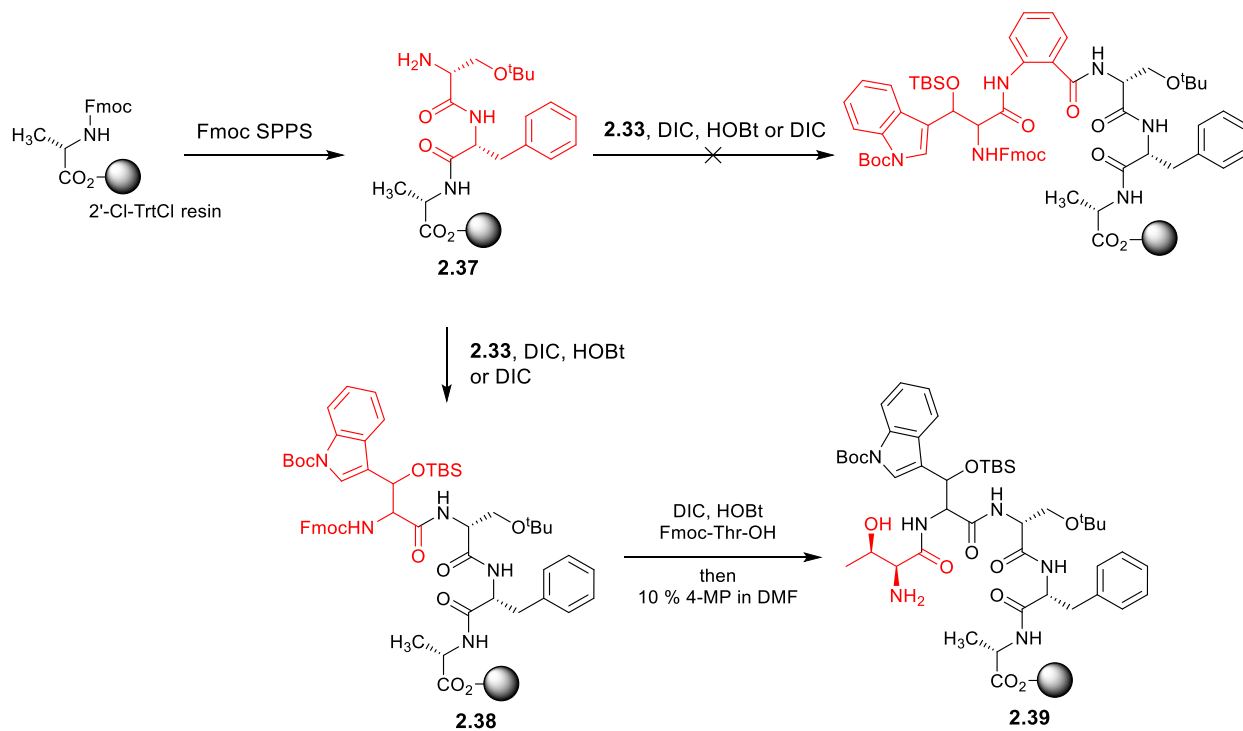
Scheme 2.16. Attempts to couple dipeptide **2.33** during Fmoc SPPS of sclerotide A.
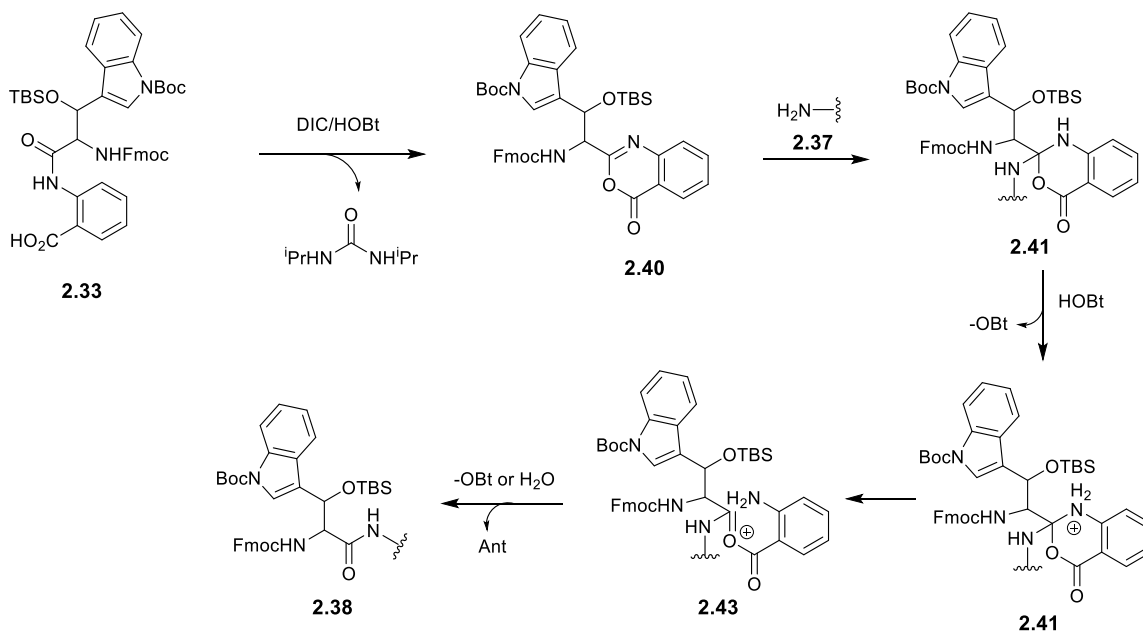
Scheme 2.17. Proposed mechanism of the side reaction observed when coupling dipeptide **2.33.**

We decided to abandon this approach using dipeptide **2.33** and attempt the alternative approach outlined in Scheme 2.12A (involving the key triphosgene coupling step). In the same way as before, 2′-Cl-TrtCl resin, loaded with Fmoc-Ala-OH, was subjected to standard Fmoc SPPS resulting in tripeptide **2.37** (Scheme 2.18). Fmoc-Ant-OH (**2.32**) was coupled using DIC/HOBt over 18.5 h and the Fmoc group was removed using 10 % 4-MP/DMF which gave peptide **2.44**. Next, **2.44** was coupled to **2.22** using triphosgene/2,4,6-collidine. Interestingly, this coupling step was difficult to monitor as we found that neither the desired peptide **2.45** nor the starting material **2.44** could be detected by HPLC-MS analysis after acidic cleavage. Instead, only dehydrotryptophan containing benzoxazinone **2.46** could be observed—likely formed as a result of TFA promoted lysis of the Ant-Ser amide bond (Scheme 2.19). Others have reported similar

Ant amide bond fragmentations in the presence of strong acid, suggesting that these reactions proceed via an acid-promoted activation of the amide followed by an intramolecular cyclization mechanism.[80-82] Fortunately, we found that if the next amino acid was coupled (Thr), then only small amounts of the benzoxazinone fragment was detected. Upon analyzing this elongated peptide (where the Thr has been attached) by HPLC-MS, we determined that the reaction of peptide **2.44** and **2.22** using triphosgene/2,4,6-collidine was sluggish and required 19 h to be completely converted into peptide **2.45**. Peptide **2.45** was then elongated to the Thr residue using Fmoc SPPS to give **2.47**. Peptide **2.47** was subjected to TFA/H_2O (95:5) to cleave the peptide from the resin and remove any protecting groups and generate the Z-dehydrotryptophan residue. This step took some optimization as we found that the formation of the Z-dehydrotryptophan took longer than the time that is typically required for cleaving a peptide from the 2'-Cl-Trt resin and side chain deprotection. If the reaction was left too long, then the Ant-Ser amide bond was found to significantly fragment. We settled on a 3 h treatment with TFA/H_2O (95:5) at room temperature. With this reaction time, nearly all of the Z-dehydrotryptophan was generated and only a small amount of the fragmented Ant-Ser amide bond compound was observed. Finally, the crude linear peptide was cyclized using PyOAP/HOAt/2,4,6-colidine in DMF. This cyclization was found to go completion after 16 h, and, after reversed-phase HPLC purification, sclerotide A was isolated in a 24 % yield. Noteworthy is that no sclerotide B was detected. The NMR spectra matched closely with those reported in the literature (see Appendix A).[33]
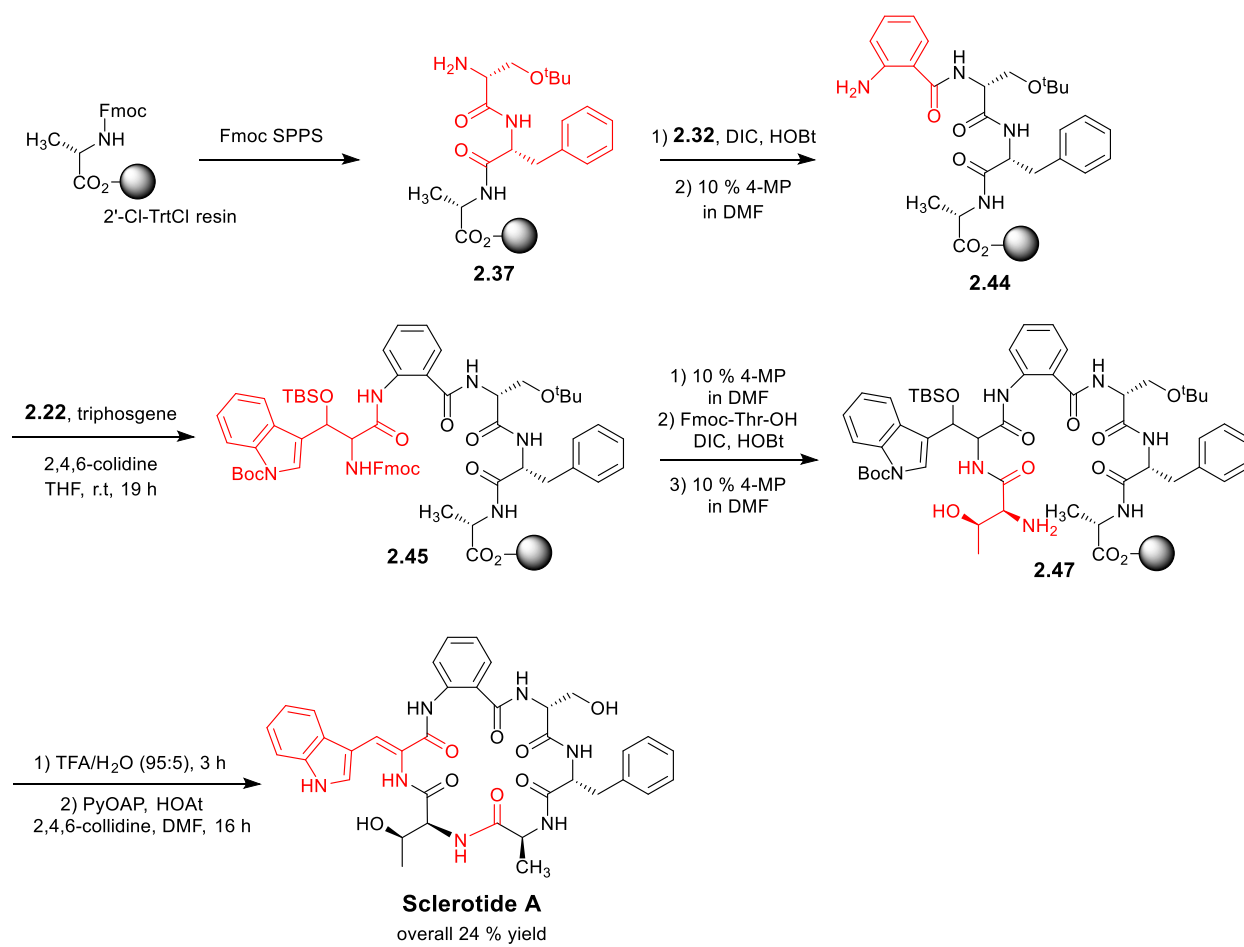
Scheme 2.18. The synthesis of sclerotide A.
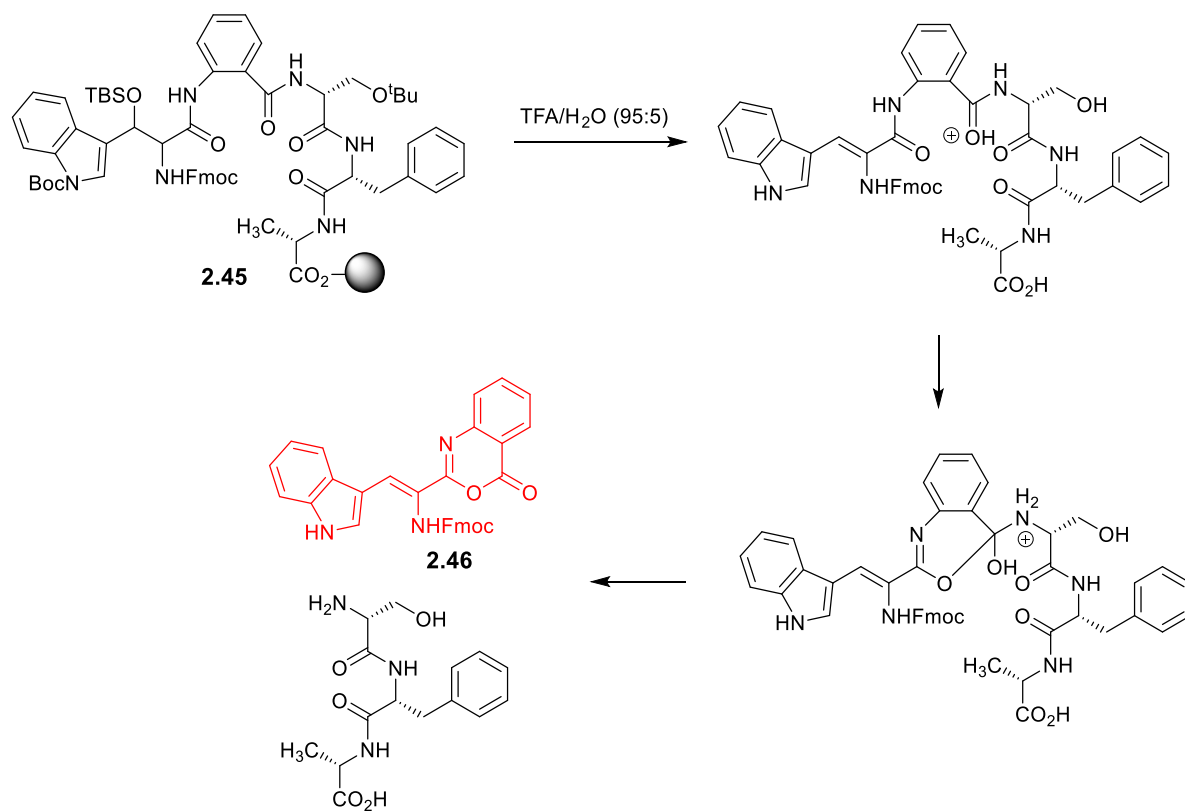
Scheme 2.19. The fragmentation of **2.45** during TFA/H₂O deprotection producing **2.46**.

2.4 Experimental Section

General experimental method

All reagents used for peptide synthesis were obtained from commercial sources including coupling reagents, resins, and Fmoc amino acids unless stated otherwise. ACS grade *N,N'*-dimethylformamide (DMF), 4-methylpiperidine (4-MP), 2-methylpiperidine (2-MP) and TFA were purchased from commercial suppliers and used without further purification. CH_2Cl_2 (DCM) was distilled from calcium hydride under nitrogen. THF was distilled from sodium metal and benzophenone under nitrogen. Peptide synthesis was performed manually using a rotary mixer for agitation. Peptide syntheses were monitored by treating small aliquots of resin with 95:5 TFA/H_2O for 75 min, removing the solvent by a N_2 stream, redissolving the peptide in 1:1 MeCN/H_2O, and analyzing by RP-HPLC and LRMS using a linear ion trap mass spectrometer.

Chemical shifts (δ) for ^1H NMR spectra run in $CDCl_3$ are reported in ppm relative to the standard TMS. Chemical shifts for ^{13}C NMR spectra run in $CDCl_3$ are reported in ppm relative to the solvent residual carbon (δ 77.16 for central peak). Chemical shifts (δ) for ^1H & ^{13}C NMR spectra run in pyridine-d_5 are reported relative to the residual solvent peaks at 8.71 and 135.5 respectively. Chemical shifts (δ) for ^1H & ^{13}C NMR spectra run in DMSO-d_6 are reported relative to the residual solvent peaks at 2.50 and 39.52 respectively.

Analytical HPLC was accomplished with a reversed-phase C18 column (10 μm, 250 mm × 4.6 mm, 1 mL/min flow rate). Peptides were purified by reversed-phase semipreparative HPLC using a C18 column (10 μm, 150 mm × 20 mm, 10 mL/min flow rate). High-resolution positive ion electrospray (ESI+) mass spectra were obtained using a hybrid quadrupole-orbitrap mass spectrometer, dissolving samples in 1:1 MeOH/H_2O + 0.1% formic acid.

Resin loading was estimated using a procedure described by Gude at al.[83]

2.4.1 Experimental procedures

Compound 2.25. To a solution of LDA (nBuLi; 8.2 mL, 2.5 M, 20.5 mmol, 5.0 equiv DIPA; 2.9 mL, 20.5 mmol, 5.0 equiv) in THF (23 mL), at -78 °C, was added dropwise a solution of Cbz-Gly-OMe (2.2 g, 9.9 mmol, 2.4 equiv) in THF (10 mL). The mixture was stirred at − 78 °C for 10 min before a solution of $ZnCl_2$ (1.5 g, 11.0 mmol, 2.6 equiv), dried by melting under vacuum, in THF (10 mL) was added. The resulting mixture was stirred vigorously at -78 °C for 10 min and then a solution of Boc-3-formylindole (1.0 g, 4.1 mmol) in THF (6.0 mL) was added. The reaction mixture was allowed to stir at -78 °C for 1 h before it was warmed to 0 °C and quenched with ice cold 0.1 M HCl (50 mL). The aqueous phase was extracted with Et_2O (100 mL x 3). The combined organic layers were washed with brine (50 mL), dried over $MgSO_4,$ and evaporated. The crude material was passed through a short silica gel plug to give an inseparable mixture of desired product **2.23** and Cbz-Gly-OMe. This mixture was then immediately taken up in DCM (20 mL) and cooled to -78 °C. DIPEA was added (1.4 mL, 8.2 mmol, 2.0 equiv) followed by TBSOTf (1.4 mL, 6.2 mmol, 1.5 equiv). The resulting mixture was stirred at − 78 °C for 2 h, then it was warmed to 0 °C and sat. NH_4Cl (50 mL) was added. The residue was extracted with DCM (100 mL x 3). The resulting combined organic extracts were washed with brine (100 mL), dried over $MgSO_4$, evaporated and

passed through a short silica gel column to give crude **2.24**. The resulting material was dissolved in 3:1 THF/H$_2$O (40 mL) and cooled to 0 °C. The mixture was then treated with LiOH (8.2 mL, 1.0 M, 2.0 equiv) and then warmed to room temperature and stirred for 17 h. The reaction mixture was then carefully acidified with 1 M HCl (pH ca. 2) and then extracted with EtOAc (100 mL x 3). The combined organic extracts were dried over MgSO$_4$, evaporated, and purified by FC (40 % EtOAc/58 % hexanes/2 % AcOH) to give **2.25** (inseparable mixture of diastereomers) as an amorphous white foam (1.8 g, 78 % over 3 steps). ^{1}H NMR (300 MHz, CDCl$_3$, δ): 9.82 (1H, br. s), 8.31-8.08 (1H, m), 7.94 -7.47 (2H, m), 7.44-7.10 (7H, m), 5.74-5.60 (1H, m), 5.48-5.06 (2H, m), 5.02-4.61 (2H, m), 1.70-1.63 (9H, m), 0.91 (9H,s), 0.13 - -0.08 (6H, m). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): 175.2, 173.6, 156.3, 155.8, 149.7, 149.6, 136.2, 136.1, 135.7, 128.6, 128.5, 128.2, 128.2, 128.1, 127.9, 127.4, 124.7, 124.6, 123.9, 122.8, 120.4, 120.3, 120.3, 119.3, 115.3, 115.3, 83.8, 70.6, 69.3, 67.2, 59.9, 59.6, 28.2, 25.7, 25.6, 18.2, 18.0 -3.7, -4.7, -4.9, -5.3, -5.4. HRMS (ESI+) m/z: [M + Na]$^+$ calcd for C$_{30}$H$_{40}$N$_2$O$_7$SiNa, 591.2497; found, 591.2504.

Compound 2.22. To a solution of compound **2.25** (2.8 g, 5.0 mmol) in 2:3 EtOH/EtOAc (230 mL) was added 5 % Pd/C (1.7 g) before the vessel was purged with N$_2$ gas. The flask was then charged with hydrogen gas and stirred for 1.5 h at room temperature before being diluted with EtOAc and filtered through Celite. The residue was washed several times with EtOAc/EtOH and then the resulting pooled filtrates were evaporated in vacuo. The resulting material was dissolved in 3:1 MeCN/10 % NaCO$_3$ $_{(aq)}$ (50 mL) before a solution of FmocOSuc (1.67 g, 5.0 mmol, 1.0 equiv) in MeCN (14 mL) was added at room temperature. The resulting mixture was stirred for 17.5 h at room before the mixture was acidified with 1 M HCl (pH ca. 2) and extracted with DCM (150 mL x 4). The combined organic extracts were dried over MgSO$_4$, filtered, evaporated, and then purified by FC (20 % EtOAc/78 % hexanes/2 % AcOH) to give **2.22** (inseparable mixture of diastereomers) as an off white, amorphous solid (3.2 g, 97 % over 2 steps). ^{1}H NMR (300 MHz, CDCl$_3$, δ): 10.71 (1H, br. s), 8.29-8.09 (1H, m), 7.99 -7. 27 (2H, m), 7.71-7. 06 (7H, m), 5.86-5.36 (2H, m), 5.00-4.71 (1H, m), 4.67-4.41 (1H, m), 4.37-4.00 (2H, m), 1.74-1.60 (9H, m), 1.05-0.90 (9H,m), 0.2 - -0.04 (6H, m). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$):174.9, 173.7, 156.2, 155.8, 149.5, 143.8, 143.8, 131.3, 131.2, 135.7, 128.0, 127.8, 127.7, 127.1, 125.1, 124.8, 123.8, 122.9, 120.1, 120.0, 119.9, 119.3, 115.5, 115.4, 84.0, 70.6, 69.1, 67.4, 59.9, 59.6, 47.1, 47.0, 28.2, 28.2, 25.7, 18.2, -4.7, -4.8, -5.2, -5.4. . HRMS (ESI+) m/z: [M + Na]$^+$ calcd for C$_{30}$H$_{40}$N$_2$O$_7$SiNa, 679.2810; found, 679.2834.
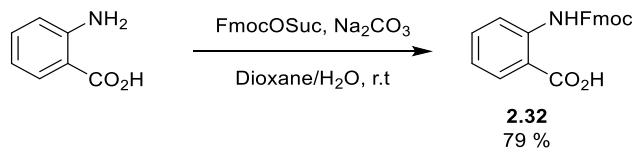
Scheme 2.20. Synthesis of Fmoc-Ant-OH (**2.32**)

Compound 2.32.[84] A solution of anthranilic acid (2.0 g, 14.6 mmol, 1.0 equiv) in a mixture dioxane (40 mL) and 10 % Na$_2$CO$_3$ $_{(aq)}$ (30 mL) at room temperature was treated with a solution of FmocOSuc (5.2 g, 15.3 mmol, 1.05 equiv) in dioxane (20 mL). A white precipitate began to form upon the final addition of the FmocOSuc. The resulting precipitous mixture was stirred at room temperature for 18 h before it was diluted with EtOAc (100 mL) and then acidified (pH ca. 2) with 1 M HCl (75 mL). The resulting organic phase was separated and the aqueous layer was extracted

with EtOAc (75 mL x 3). Combined organic extracts were washed with brine (100 mL), dried (MgSO$_4$), filtered and evaporated in vacuo. The resulting white solid was resuspended in boiling EtOAC (100 mL) and allowed to cool to room temperature. Once the mixture had reached room temperature, hexanes were added until the point of precipitation. The mixture was cooled further to 0 °C and then the resulting white precipitate was filtered. The precipitate was rinsed with hexanes and dried in vacuo to give **2.32** (4.1 g, 79 %) as a fluffy white solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 10.81 (1H, s), 8.17 (1H, d, J = 7.5 Hz), 7.99 (1H, d, J = 8.0 Hz), 7.91 (2H, d, J = 7.5 Hz), 7.69 (2H, d, J = 7.5 Hz), 7.57 (1H, t, J = 7.8 Hz), 7.43 (2H, t, J = 7.7 Hz), 7.34 (2H, t, J = 7.7 Hz), 7.11 (1H, t, J = 7.8 Hz), 4.50 (2H, d, J = 7.8 Hz), 4.36 (1H, t, J = 7.2 Hz). ^{13}C{^{1}H} NMR (75 MHz, DMSO-d_6): δ 170.2, 153.2, 144.1, 141.3, 141.3, 134.7, 131.7, 128.2, 127.6, 125.5, 122.5, 120.7, 118.9, 116.2, 66.8, 46.9. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{22}$H$_{18}$NO$_4$, 360.1230; found, 360.1215.

Compound 2.33. A suspension of anthranilic (170.5 mg, 1.245 mmol, 2.0 equiv) in dry DCM (6.0 mL) at 0 °C was treated with 2,4,6-collidine (0.27 mL, 2.079 mmol, 3.3 equiv) followed by TMSCl (0.16 mL, 1.2666 mmol, 2.0 equiv). The mixture was then removed from the ice bath. In a separate flask, a solution of **2.22** (410.7 mg, 0.6258 mmol, 1.0 equiv) in dry DCM (2.0 mL) was added HOBt (96 mg, 0.6275 mmol, 1.0 equiv) and EDC•HCl (120 mg, 0.6283 mmol, 1.0 equiv). Both mixtures were stirred for 20 min before the solution of the HOBt ester was added to the solution containing the anthranilic acid at 0 °C. This mixture was stirred for 5 min at 0 °C before DMAP (78 mg, 0.6393 mmol, 1.0 equiv) was added. The resulting mixture was stirred at r.t for 4 h. It was then acidified with 1 M HCl (pH ca. 2) and extracted with DCM (10 mL x 4). The combined organic extracts were dried over MgSO$_4$, filtered, evaporated, and then purified by FC (25 % EtOAc/74 % hexanes/1 % AcOH) to give **2.33** (inseparable mixture of diastereomers & 1:1 mixture of rotamers) as an off white, amorphous solid (405.0 mg, 83 %).^{1}H NMR (300 MHz, CDCl$_3$, δ): 11.84 (1H, s), 11.74 (1H, s), 10.95 (1H, s), 8.81 (1H, m), 8.11 (2H, m), 7.58 (9H, m), 7.13 (5H, m), 6.00 (2H, m), 4.76 (1H, m), 4.27 (3H, m), 1.61 (9H, m). 0.88 (9H, m), 0.025 (6H, m). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): 172.2, 171.8, 171.5, 169.4, 168.0, 156.7, 156.4, 153.1, 149.5, 143.8, 143.2, 141.2, 135.8, 135.4, 132.0, 131.8, 130.9, 128.7, 128.3, 137.7, 127.1, 125.2, 124.7, 127.1, 125.2, 124.7, 124.5, 123.8, 123.6, 123.2, 123.0, 121.2, 121.0, 120.4, 119.9, 119.5, 118.9, 116.9, 115.5, 115.3, 114.5, 83.9, 77.3, 69.3. 69.1, 67.9, 67.6, 62.4, 62.1, 47.1, 46.6, 28.2, 28.1, 25.8, 25.6, 25.5, 18.1, -4.8, -4.9, -5.2, -5.4. HRMS (ESI+) m/z: [M + Na]$^+$ calcd for C$_{30}$H$_{40}$N$_2$O$_7$SiNa, 679.2810; found, 679.2834.

Fmoc Solid Phase Synthesis (SPPS) of Tunicyclin B

The SPPS synthesis was performed manually beginning from 2'-Cl-TrtCl polystyrene resin (theoretical substitution = 1.5 mmol/g, 33.4 mg, 0.2 mmol, 1 equiv). The 2'-Cl-TrtCl polystyrene resin was preactivated in dry DCM (20 mL) with SOCl$_2$ (26 µL, 3.6 equiv) and pyridine (58 µL, 7.2 equiv) under reflux for 2 h. The resin was then transferred to a disposable peptide cartridge and rinsed with dry DCM, followed by loading with Fmoc-Gly-OH (4.0 equiv) and DIPEA (8.0 equiv) in dry DCM (2.0 mL) (2 x 2 h). The resin was capped with 17:2:1 DCM/MeOH/DIPEA (3 × 10 min), and the loading efficiency was determined to be 0.72 mmol/g. All Fmoc-amino acids (4 equiv) were activated for 5 min using HOBt (4 equiv) and DIC (4 equiv) in DMF (2.0 mL) and then coupled for 4 h. All Fmoc groups were removed using 10 % 4-methylpiperidine in DMF (2.0 mL) (1 x 10

min, 1 x 5 min). The global deprotection/indolic dehydration was executed by treating the resin bound peptide **2.28** with 95:5 TFA/H$_2$O (2.5 mL) for 75 min. The cartridge was drained and the resin was rinsed with 95:5 TFA/H$_2$O (2 x 1.0 mL). The cleavage cocktail was condensed under a stream of N$_2$ to remove the TFA and then precipitated with pre-chilled Et$_2$O (-78 °C). The suspension was centrifuged and the Et$_2$O was carefully decanted. The peptide was then taken up in DMF (100 mL) and cyclized with PyAOP (5 equiv), HOAt (5 equiv), and 2,4,6-collidine (10 equiv) for 20 h. The solvent was removed by high-vacuum rotary evaporation, and the crude solid was purified by preparative RP-HPLC employing a gradient of 90 % H$_2$O (0.1 % TFA)/10 % MeCN to 82 % H$_2$O (0.1 % TFA)/18 % MeCN over 10 min then from 82 % H$_2$O (0.1 % TFA)/18 % MeCN to 55 % H$_2$O (0.1 % TFA)/45 % MeCN. Fractions containing **Tunicyclin B** were pooled and lyophilized giving **Tunicyclin B** as a pale-yellow powder (19.3 mg, 28 % based on resin loading), judged to be >95 % pure by analytical RP-HPLC (Appendix A). HRMS (ESI+) m/z: [M + H]$^+$ calcd for C$_{35}$H$_{49}$N$_8$O$_9$, 725.3617; found, 725.3627.

Fmoc SPPS Synthesis of Sclerotide A

Resin Loading

The SPPS synthesis was performed manually beginning from 2'-Cl-TrtCl polystyrene resin (theoretical substitution = 1.5 mmol/g, 33.4 mg, 0.2 mmol, 1 equiv). 2'-Cl-TrtCl polystyrene resin, in a disposable peptide cartridge, was swollen in anhydrous DCM (5 mL) before it was drained and then treated with a solution of Fmoc-Ala-OH (4.0 equiv) and DIPEA (8.0 equiv) in dry DCM (5 mL) for 6 h. The cartridge was drained, and the resin was capped with 17:2:1 DCM/MeOH/DIPEA (3 × 10 min), and the loading efficiency was determined to be 0.36 mmol/g.

Fmoc Deprotections

All Fmoc deprotections were accomplished with 10 % 4-MP/DMF (2.0 mL) (1 x 10 min, 1 x 5 min). After each Fmoc deprotection, the resin was washed with DMF (3 x 1.0 mL).

Procedure for the coupling of Fmoc-Ant-OH (2.32)

Fmoc-Ant-OH was coupled using a procedure described by Masuda et al.[84] A solution of Fmoc-Ant-OH (**2.32**) (3.0 equiv), HOBt (4.5 equiv.) and DIC (3.0 equiv) in DMF (2.0 mL) was added to the peptide. The cartridge was gently agitated for 18.5 h before it was drained and washed with DMF (5 x 1.0 mL).

Procedure for the coupling of Fmoc-β-HOTrp(Boc)(TBS)-OH (2.22)

A solution of **2.22** (4.0 equiv) and triphosgene (1.33 equiv) in dry THF (1.5 mL) was added 2,4,6-colidine (10 equiv). Upon the addition of the 2,4,6-collidine a suspension immediately formed. This suspension was gently shaken for 1 minute before it was added to the peptide (previously washed with dry THF x 3). The reaction was gently agitated for 19.5 h before it was carefully drained. The peptide was then rinsed with THF (1.0 mL x 2), DMF (1.0 mL x 3), 3:1 THF/H$_2$O (1.0 mL x 3), MeOH (1.0 mL x 3) and then DMF (1.0 mL x 3).

Procedure for the coupling of all other Fmoc amino acids

A solution of Fmoc amino acid (4.0 equiv) in DMF (2.0 mL) was treated with DIC (4.0 equiv) and HOBt (4.0 equiv). The mixture was incubated for 5 min before it was added to the peptide. The mixture was then gently agitated for 4 h. The cartridge was drained and the resin was washed with DMF (1.0 mL x 5).

Procedure for acidic cleavage and off-resin cyclization

The resin bound peptide **2.47** with 95:5 TFA/H_2O (2.5 mL) for 3 h in peptide cartridge wrapped with foil. The cartridge was drained and the resin was rinsed with 95:5 TFA/H_2O (3 x 1.0 mL). The cleavage cocktail was condensed under a stream of N_2 to remove most of the TFA. The reaming TFA was removed by evaporating the residue from toluene. The peptide was then taken up in DMF (50 mL) and cyclized with PyAOP (5 equiv), HOAt (5 equiv), and 2,4,6-collidine (10 equiv) for 16.5 h in a foil wrapped flask. The solvent was removed by high-vacuum rotary evaporation. The resulting crude material was dissolved in EtOAc (100 mL) and washed with 1 M HCl (25 mL). The organic phase was separated and the resulting aqueous phase was extracted with EtOAc (30 mL x 3). Combined organic extracts were then washed with sat. $NaHCO_3$ (50 mL) followed by brine (50 mL). The organic residue was then dried ($MgSO_4$), filtered and evaporated in vacuo. The crude yellow solid was then taken up in 1:1 MeCN/H_2O (5 mL) and purified by preparative RP-HPLC employing a gradient of 82 % H_2O (0.1 % TFA)/18 % MeCN to 48 % H_2O (0.1 % TFA)/52 % MeCN over 75 min. Fractions containing **Sclerotide A** were pooled and lyophilized giving **Sclerotide A** as a pale-yellow powder (8.5 mg, 24 % based on resin loading), judged to be >95 % pure by analytical RP-HPLC (Appendix A). HRMS (ESI+) m/z: [M + H]$^+$ calcd for $C_{37}H_{40}N_7O_8$, 710.2933; found, 710.2938.

Chapter 3

Solid-Phase Total Synthesis of CDA3a and CDA4a and Analogs

3.1 Introduction

The calcium-dependent antibiotics (CDAs) are one of the most complex Z-dehydrotryptophan containing peptides (the structures of the CDAs are shown in section 3.3.1). The CDAs are a structurally diverse group of bio-active molecules which belong to a larger class of compounds, the calcium-dependent cyclic lipopeptide antibiotics (cLPAs), which includes other biologically active compounds such as friulimycins, the A54145 group, the glycinocins, the laspartomycins, daptomycin, as well as the recently discovered taromycins, malicidins and cadasides.[85] The antibacterial profile of many of these compounds have been elucidated but by far the most important member of this family of antibiotics is daptomycin, as it is the only calcium-dependent cLPA in clinical use. In contrast, up until 2020, nothing was known about the antibacterial potency of any of the CDA members. As the focus of this chapter is on one group within the calcium-dependent cLPA family of antibiotics, the CDAs, and since daptomycin is one of the most studied and well-characterized of all of the calcium-dependent cLPAs, daptomycin is discussed in more detail below.

3.2 An Overview of the cLPA Daptomycin

Daptomycin (Figure 3.1) was first isolated from the fermentation broth of the soil dwelling bacterium *Streptomyces roseosporus* in the early 1980's by researchers at Eli Lilly & Co. Studies revealed that it possessed impressive bactericidal properties against many serious Gram-positive bacteria, but only in the presence of calcium.[85] However, the drug exhibited musculoskeletal side effects that were noticed during Phase I studies. As a result, early investigations of the drug were halted until 1997 when Cubist Pharmaceuticals obtained the worldwide licensing rights to daptomycin.[86] Researchers revealed that the musculoskeletal side effects could be attenuated by altering the dosing regimen of the drug. Daptomycin—under the trade marked name Cubicin—reached the market in 2003.[86, 87] It is used to treat serious infections caused by Gram-positive organisms including methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant enterococci (VRE) infections.[85] While bacterial resistance to daptomycin resistance has been observed, it is still not widespread. Daptomycin is classified by the WHO as one of the highly important last resort antibiotics. However, it is not effective against Gram-negative bacteria—as is the case with many of the other cLPA members.
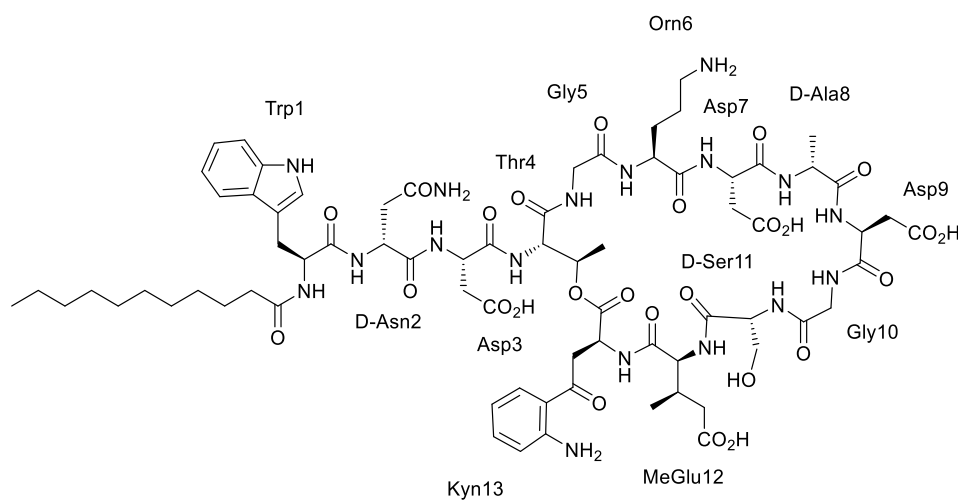
Figure 3.1. The chemical structure of daptomycin.

Daptomycin is a constituent of the A21978C group of antibiotics, which are all produced via nonribosomal peptide synthesis by *Streptomyces roseosporus.*[85] All members of the A21978C group consist of a 10 amino acid macrocyclic lactone to which is attached an exocyclic lipidated tripeptide.[85] The A21978C factors only differ in their lipid tails, with a *n*-decanoyl group in daptomycin.[85] All of the A21978C factors contain three D-amino acids; D-Asn at position 2 (D-Asn2), D-Ala at position 8 (D-Ala8), and D-Ser at position 11 (D-Ser11), and three unusual or uncommon amino acids; ornithine at position 6 (Orn6), kynurenine at position 13 (Kyn13) and L-*threo*-3-methylglutamate at position 12 (MeGlu12).[88] The macrocycle is closed by an ester (depsi) bond between the side chain of Thr4 and the α-COOH group of Kyn13.

Daptomycin exhibits optimal antibacterial activity at approximately 1 mM Ca^{2+}, which is similar to the physiological concentration of calcium ions in human serum. Studies have revealed that daptomycin binds to calcium ions—demonstrated through fluorescence experiments with phosphatidyl choline/phosphatidyl glycerol liposomes—and that residues 7-10 (Asp7-D-Ala8-Asp9-Gly10) are directly involved in calcium binding.[85, 89, 90] This DXDG calcium-binding motif is found in most, but not all calcium-dependent cLPAs.[85]

Despite its clinical importance, the precise mechanism by which daptomycin kills bacteria is yet to be fully understood. Since finding clinical relevance in the early 2000s, numerous studies have attempted to reveal the exact MoA of daptomycin, many with conflicting results and models.[85] The proposed models include calcium-dependent oligomerization of daptomycin monomers and phosphatidylglycerol (PG)-mediated membrane pore formation, disruption of membrane fluidity as well as inhibition of cell wall synthesis.[91]

The synthesis and development of daptomycin analogs with enhanced biological properties has been vigorously pursued in the last 15 years. Daptomycin and daptomycin derivatives have been prepared by several different means. For example, Cubist Pharmaceuticals have prepared a large number of daptomycin analogs via semisynthesis which involved appending different chemical groups to the side chain amino group of the Orn residue.[92, 93] None of these analogs made it to market. The obvious limitation of this semisynthetic approach is that it only allows for very few potential sites of modification. Daptomycin analogs have been prepared using a chemo-enzymatic approach as well. In this approach, SPPS is used to prepare a linear daptomycin analog having a thioester C-terminus. After removal from the solid support and HPLC purification, the resulting thioester peptide is cyclized using a thioesterase/cyclase. In 2004 and 2006 Grunewald et al. reported the synthesis and biological activity of nine daptomycin analogs using this approach.[94, 95] However, all of these analogs exhibited low biological activity. This chemoenzymatic approach to cLPA and daptomycin analog synthesis is extremely appealing, however, it does have some drawbacks. One is that the efficiency of cyclization reaction depends upon the sequence of the linear thioester peptide. Hence, the overall yields can be quite low. Also, the peptides must be purified by HPLC both before and after cyclization, which does not make it a very practical approach to making large numbers of analogs.

Daptomycin analogs have been prepared by combinatorial biosynthesis. In this approach, the enzymatic modules responsible for the incorporation of the amino acids are swapped with NRPS systems encoding similar, naturally occurring lipopeptides.[96] Cubist Pharmaceuticals used this approach to prepare about a dozen analogs of daptomycin, none of which exhibited activity that was superior to daptomycin.[97] A major shortcomings to this approach to daptomycin analog

synthesis is that it is dependent on the substrate specificity of the modules. Although some amino acid substitutions can be made, no dramatic changes are feasible. For example, Cubist Pharmaceuticals was unable to exchange *threo*-3MeGlu12 for anything other than Glu. It was also not possible to alter the stereochemistry of the residues.

More recently, daptomycin and daptomycin analogs have been prepared by chemical synthesis using SPPS. The first complete total synthesis of daptomycin was achieved in 2006 by researchers at Cubist Pharmaceuticals and is described in a vaguely written patent. The yield of this synthesis was omitted from this report.[98] The Li group reported the total synthesis of daptomycin in 2013 using a hybrid solution-phase/Fmoc SPPS approach.[99] The researchers were unable to form the crucial ester linkage using Fmoc-Kyn(CHO,Boc)-OH as a building block either in solution or on a solid-support. Hence, they resorted to a 12-step solution-phase synthesis of a branched tetrapeptide, during which formation of the ester linkage between Thr4 and Kyn13 was accomplished by ozonolysis of a suitably protected Trp residue. This tetrapeptide was used as a building block for the preparation of a linear daptomycin precursor using solid-phase methods. A solution-phase (off-resin) serine ligation procedure was used to achieve macrocyclization (Scheme 3.1).[99] This combination of solid- and solution-phase chemistry, along with the need for HPLC purification immediately before and after cyclization, made this approach very labour-intensive.[99] In 2015, the Taylor group executed the first totally solid-phase synthesis of daptomycin (Scheme 3.2).[100] The peptide was attached to the resin via the side chain of Asp9 in the form of an Asp-Gly dipeptide. The researchers were able to form the crucial ester bond between Kyn13 and Thr4 on a resin bound peptide that lacked residues 1, 2, 11 and 12 and the lipid tail, and contained an α-azido group at the N-terminus. After ester bond formation and

reduction of the terminal azido group to an amino group, α-azido acid building blocks were used to install residues 1 and 2 and lipid tail. Residues 11 and 12 were then installed followed by an on-resin cyclization between Gly10 and Ser11. This synthesis of daptomycin opened the door to the synthesis of many daptomycin analogs, some of which showed excellent biological activity such as a daptomycin derivative that contained Trp, Glu and Lys residues in place of the Kyn, MeGlu and Orn residues found in natural daptomycin.[101]
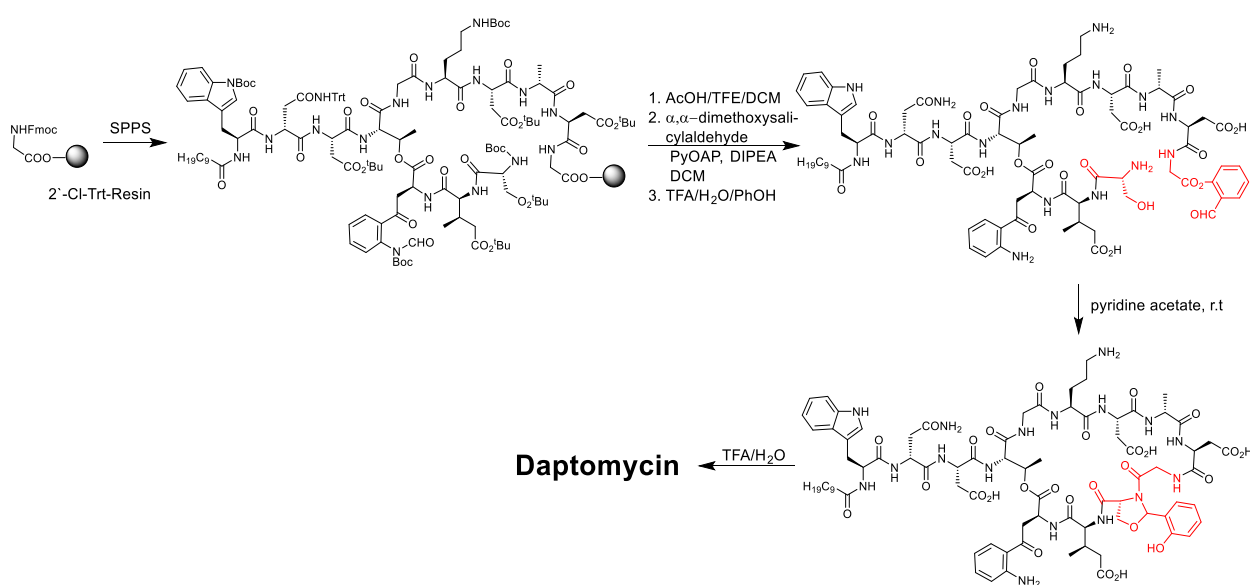
Scheme 3.1. Synthesis of daptomycin by the Li group using an off-resin serine ligation.[99]

Scheme 3.2. Entirely Fmoc SPPS approach by the Taylor group in the synthesis of daptomycin.[100]

Using solid-phase synthesis approaches, researchers have now made more than 80 daptomycin analogs. Most notable is a recent report by the Li group in 2020 who synthesized a

daptomycin analog with a *N*-methylated Kyn side chain.[102] This analog, named kynomycin, showed improved bactericidal properties compared to daptomycin and elicited bacterial death in MRSA, VRE as well as daptomycin-resistant bacteria.[102]

In recent years, much focus has been on the synthesis of other cLPAs besides daptomycin using Fmoc SPPS approaches. For example, both the Taylor group and the Li group have reported the synthesis of a closely related cLPA named A54145D.[103, 104] Both groups were able to access analogs of this peptide through Fmoc SPPS approaches as well.

3.3 The CDA class of compounds

3.3.1 Isolation, mechanism of action and structure of the CDAs

In 1978, researchers at Ely Lilly isolated a novel peptide antibiotic from the bacterial lysate of *Streptomyces coelicolor* that was found to inhibit the growth of *B. subtillis,* but only when the growth medium was supplemented with calcium ($CaCl_2$). As a result, this newly discovered antibiotic was given the name calcium-dependent antibiotic (CDA). No confusion stemmed from the name at the time as the calcium-dependent class of antibiotics, including daptomycin, had not yet been established. Through crude zone of inhibition assays, researchers deduced that it was at 16 mM calcium when the CDA was most active and that the CDA possessed a broad antimicrobial spectrum against Gram-positive bacteria.[105]

Mechanism of action (MoA) studies on the CDA suggest that CDA, in the presence of calcium, formed tri- and tetrameric pores in bacterial membranes and facilitate the conductance of monovalent cations through the membrane (like K^+ and Na^+). In these studies, researchers

subjected a model membrane, held under a steady voltage of 50 mV, in the presence of calcium ions to small amounts of CDA.[105] What the researchers observed was a discrete fluctuating behavior of the current passing through the membrane, an indication of the opening and closing of ionophoric channels.[105] It was speculated that the antibacterial properties of CDA were a result of the cell depolarization invoked by CDA's ionophoric channels, leading to cell membrane depolarization and eventual cell death.[105]

It would not be until 1997 that Kempter and coworkers, using various analytical and spectroscopic techniques, determined that the previously isolated CDA was in fact composed of several structurally different members that could be individually isolated. Kempter et al. were able to isolate four unique CDA members: CDA1b, CDA2b, CDA3b and CDA4b from *S. coelicolor*.[106] However, several years later in 2002, the Micklefield group determined that there existed seven unique CDA members: CDA1b, CDA2a, CDA2b, CDA3a, CDA3b, CDA4a and CDA4b (Figure 3.2).[107] The biological activity of the isolated individual members of the CDA group was not reported.

CDAx	R^{11}	R^{10}	R^9
CDA1b	H, H	H	OPO_3H_2
CDA2a	π-bond	CH_3	OPO_3H_2
CDA2b	H, H	CH_3	OPO_3H_2
CDA3a	π-bond	H	OH
CDA3b	H, H	H	OH
CDA4a	π-bond	CH_3	OH
CDA4b	H, H	CH_3	OH
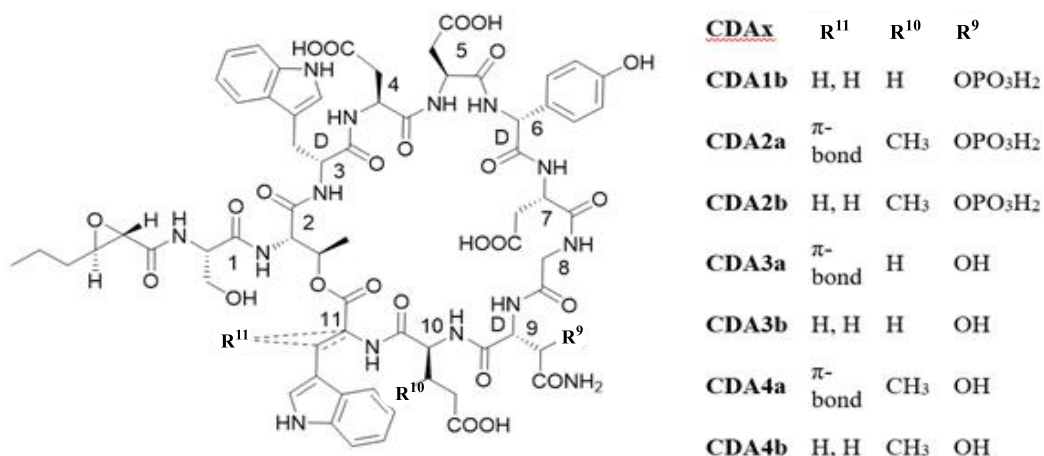
Figure 3.2. Members of the CDA family of antibiotics.[106, 107]

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All of the CDAs possess the same general structure with a 10 amino acid macrocycle, an exocyclic Ser residue and a (2S,3R)-2,3-epoxyhexanoic acyl tail.[108] The lactone is conjoined between the side chain of L-Thr2 and L-Trp11 in CDA1b, 2b, 3b and 4b. In CDA2a, 3a and 4a the L-Trp at position 11 is replaced with the nonproteinogenic Z-dehydrotryptophan. All of the CDAs contain the uncommon amino acids D-4-hydroxyphenylglycine (HOPhGly), at position 6, and D-tryptophan, at position 3. [108] The HOPhGly was shown to be the X amino acid in the calcium biding DXDG motif of the calcium-dependent cLPAs. Like with the Z-dehydrotryptophan residue, other uncommon amino acids were not retained between CDA members: D-*erythro*-3-phosphohydroxyasparagine 9 (POAsn) in CDA1b/CDA2b, L-*threo*-3-methyl glutamate 10 (MeGlu) in CDA2b/CDA3b, and D-*erythro*-3-hydroxyasparagine 9 (HOAsn) in CDA3b/CDA4b.[108] Interestingly, the CDAs are the only known natural product to contain the unusual POAsn residue.[106]

3.3.2 Biosynthetic pathway of the CDAs

Since the structural determination of all the CDAs in 2002, the majority of the work on these compounds, mostly done by the Micklefield group, has been to determine their biosynthetic pathway. Like daptomycin, the CDAs are biosynthesized by NRPS.[109] Three subunits of the NRP synthetases—*CdaPS1, CdaPS2* and *Cda*PS3—are involved in the synthesis of the antibiotics. These subunits possess a modular organization and are composed of repeating condensation (C), adenylation (A), and thiolation (T) domains.[109] These CAT domains facilitate the addition of amino acids to the growing peptide. Several modules are flanked by epimerization domains that are involved in the formation of D-configured amino acids, such as D-HOAsn.

Researchers have speculated that the biosynthetic pathway begins at the epoxidated lipid where it is bound to the *CdaPS1* subunit by a C' domain (Figure 3.3).[107, 109]
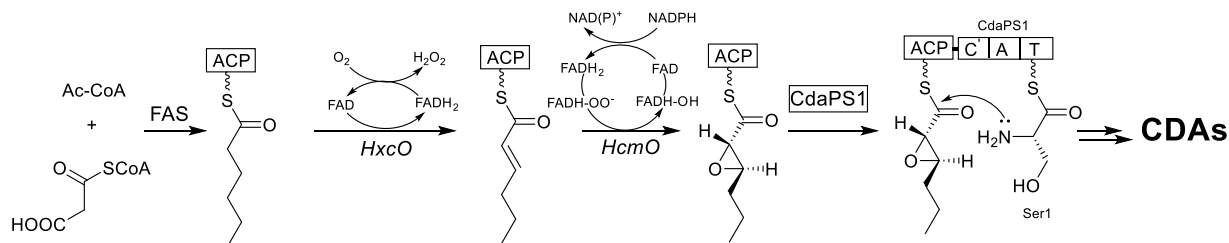
Figure 3.3. First steps in the biosynthesis of CDAs. The 2,3-epoxyhexanoyl fatty side chain, biosynthesized from Fatty Acid Synthetase (FAS), oxidase (*HxcO)* and monooxygenase (*HcmO*) enzymes, is transferred to *CdaPSI* enzyme of NRPS.[109]

Researchers have determined that the Z-dehydrotryptophan of the CDAs is likely produced through direct dehydrogenation of a Trp residue in a fashion similar to that of the enzyme L-tryptophan 2',3'-oxidase from *Chromobacterium violaceum*.[31] Similarly, the D-amino acids as well as the MeGlu residues are incorporated directly into the growing CDA peptide using epimerization and adenylation domains respectively.[109] The D-HOAsn is likely formed as a result of the oxidation of an Asn residue by a specific asparagine oxygenase (AsO). It is presumed that the D-HOAsn is phosphorylated by an encoded 3-hydroxyasparagine phosphotransferase.[109] The precise biological role of the POAsn is unknown. Some have speculated that the phosphorylation is a self-resistance mechanism of the CDAs in *S. coelicolor*.[107, 109] Remarkably, although all seven naturally occurring CDA members were isolated, their individual antimicrobial potencies were not deduced.

3.3.3 Biosynthetic and chemical synthesis of CDA analogs

Given that the CDA biosynthetic pathway had been well-elucidated, many researchers turned their attention towards the preparation of CDA analogs biosynthetically. In 2009, using combinatorial biosynthesis, the Micklefield group prepared 21 CDA analogs.[109] These analogs differed in the acyl tail as well as specific amino acid residues (Table 3.1). They were unable to incorporate fatty acids that were greater than 6 carbon atoms in length. As was the case with the naturally occurring CDA members, Micklefield did not determine the MICs for any of the 21 CDA analogs.[109]

Table 3.1. CDA analogs prepared by Micklefield and coworkers.[109]
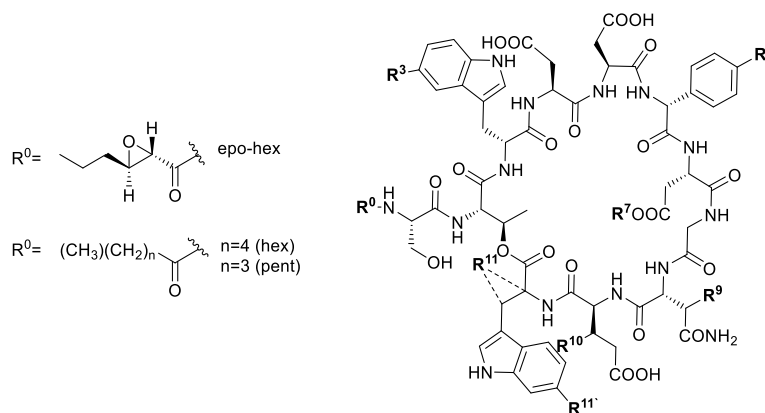
CDA	R^0	R^3	R^6	R^7	R^9	R^{10}	R^{11}	$R^{11'}$
CDA2d	epo-hex	H	H	OH	OPO_3H_2	Me	H, H	H
CDA2fa	epo-hex	H	F	OH	OPO_3H_2	Me	π-bond	H
CDAfb	epo-hex	H	F	OH	OPO_3H_2	Me	H, H	H
CDAbN7	epo-hex	H	OH	NH_2	OPO_3H_2	Me	H, H	H
CDA5b	epo-hex	H	OH	OH	H	H	H, H	H
CDA5a	epo-hex	H	OH	OH	H	H	π-bond	H
CDA6a	epo-hex	H	OH	OH	H	Me	π-bond	H
CF_3CDA3a	epo-hex	H	OH	OH	OH	CF_3	π-bond	H
CF_3CDA3b	epo-hex	H	OH	OH	OH	CF_3	H, H	H
EtCDA3b	epo-hex	H	OH	OH	OH	Et	H, H	H
(5HW)CDA4a	epo-hex	OH	OH	OH	OH	Me	π-bond	H
(5HW)CDA4a	epo-hex	H	OH	OH	OH	Me	π-bond	OH
$(5HW)_2$CDA4a	epo-hex	OH	OH	OH	OH	Me	π-bond	OH
$(5FW)_1$CDA4a	epo-hex	H	OH	OH	OH	Me	H, H	F
$(5FW)_1$CDA3b	epo-hex	H	OH	OH	OH	H	H, H	F
$(5FW)_2$CDA4b	epo-hex	F	OH	OH	OH	Me	H, H	F
$(5FW)_2$CDA3b	epo-hex	F	OH	OH	OH	H	H, H	F
hCDA3b	hex	H	OH	OH	OH	H	H, H	H
hCDA4a	hex	H	OH	OH	OH	Me	π-bond	H
hCDA4b	hex	H	OH	OH	OH	Me	H, H	H
hCDA4b	pent	H	OH	OH	OH	Me	H, H	H

The Marahiel group in 2004 prepared several CDA-like analogs using a chemoenzymatic approach (Table 3.2).[94] They utilized SPPS to prepare linear CDA-like C-terminus thioester analogs which were removed from the resin and cyclized using a CDA cyclase enzyme.[94] The HOAsn, MeGlu and HOPhGly residues were replaced with Asn, Glu and Phe respectively to make the syntheses easier. Some structural variation was achieved in these CDA analogs; however, the MICs for all of the analogs were not determined.

Table 3.2. Chemoenzymatically prepared CDA analogs prepare by the Marahiel group.[94]
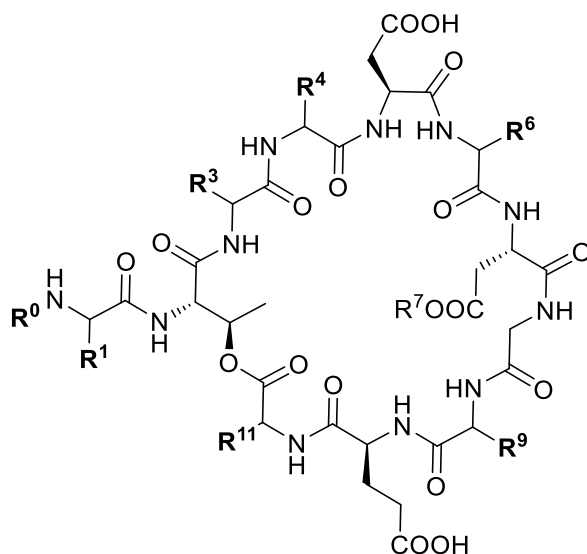
CDA	R0	R1	R3	R4	R6	R9	R11
AcCDA-G3	Ac	Ser	Gly	Asp	D-Phe	D-Asn	Trp
HexCDA-G3	Hexanoyl	Ser	Gly	Asp	D-Phe	D-Asn	Trp
AcCDA-04	Ac	Ser	D-Trp	Orn	D-Phe	D-Asn	Trp
HexCDA-04	Hexanoyl	Ser	D-Trp	Orn	D-Phe	D-Asn	Trp
AcCDA-DA6	Ac	Ser	D-Trp	Asp	D-Ala	D-Asn	Trp
HexCDA-DA6	Hexanoyl	Ser	D-Trp	Asp	D-Ala	D-Asn	Trp
AcCDA-DS9	Ac	Ser	D-Trp	Asp	D-Phe	D-Ser	Trp
HexCDA-Ds9	Hexanoyl	Ser	D-Trp	Asp	D-Phe	D-Ser	Trp
HexCDA-D1	Hexanoyl	Asp	D-Trp	Asp	D-Phe	D-Asn	Trp
HexCDAU11	Hexanoyl	Ser	D-Trp	Asp	D-Phe	D-Asn	Kyn

In 2020 Chen and coworkers reported the first chemical synthesis of CDA3a as well as several other non-naturally occurring CDA3A analogs that varied in their lipid tails (Scheme 3.3).[55]

Up until this point, as previously mentioned, Z-dehydrotryptophan residues had not been successfully installed into a peptide containing more than 2 amino acids via chemical synthesis. This residue is unquestionably the most challenging aspect of the synthesis of the CDAs. In their synthesis, Chen et al. opted not to attempt to incorporate the Z-dehydrotryptophan directly by Fmoc SPPS and instead chose to prepare a Z-dehydrotryptophan containing tripeptide **1.72** in solution that they would then couple to a resin bound peptide precursor through standard Fmoc SPPS techniques.[55] The synthesis of the Z-dehydrotryptophan residue in this tripeptide **1.72**, previously mentioned in chapter 1-section 1.2.1, was achieved through a direct oxidation of L-Trp using $PhNO/ZrCl_4$ which was followed by Phth removal, coupling of a glutamate (Glu) reside and deprotection of the C-terminus methyl ester of **1.71** (Scheme 3.4). However, the overall yield of the resulting tripeptide was low, which was mainly due to the low yields obtained during removal of the Phth from the Z-dehydrotryptophan dipeptide **1.70** and subsequent installation of the Glu residue. This tripeptide **1.72**, although prepared in a low overall yield, was successfully coupled to a 2'-Cl-Trt bound hexapeptide **3.1** using HATU/DIPEA in DMF which provided them with **3.2**. Hexapeptide **3.1** had been made using Fmoc SPPS from a resin bound Fmoc-Gly residue. The researchers noted that alternative coupling conditions had to be used (COMU/2,6-lutidine in DMF) to couple the HOPhGly residue as this amino acid was found to easily epimerize under other coupling conditions such as HATU/DIPEA. This observation had already been reported in 2017 by Liang et al. who determined that HOPhGly residues could be coupled using COMU in combination with 2,6-lutidine without any appreciable epimerization.[110] The turn-inducing nature of dehydroamino acids promotes the formation of DKP which is exacerbated during basic
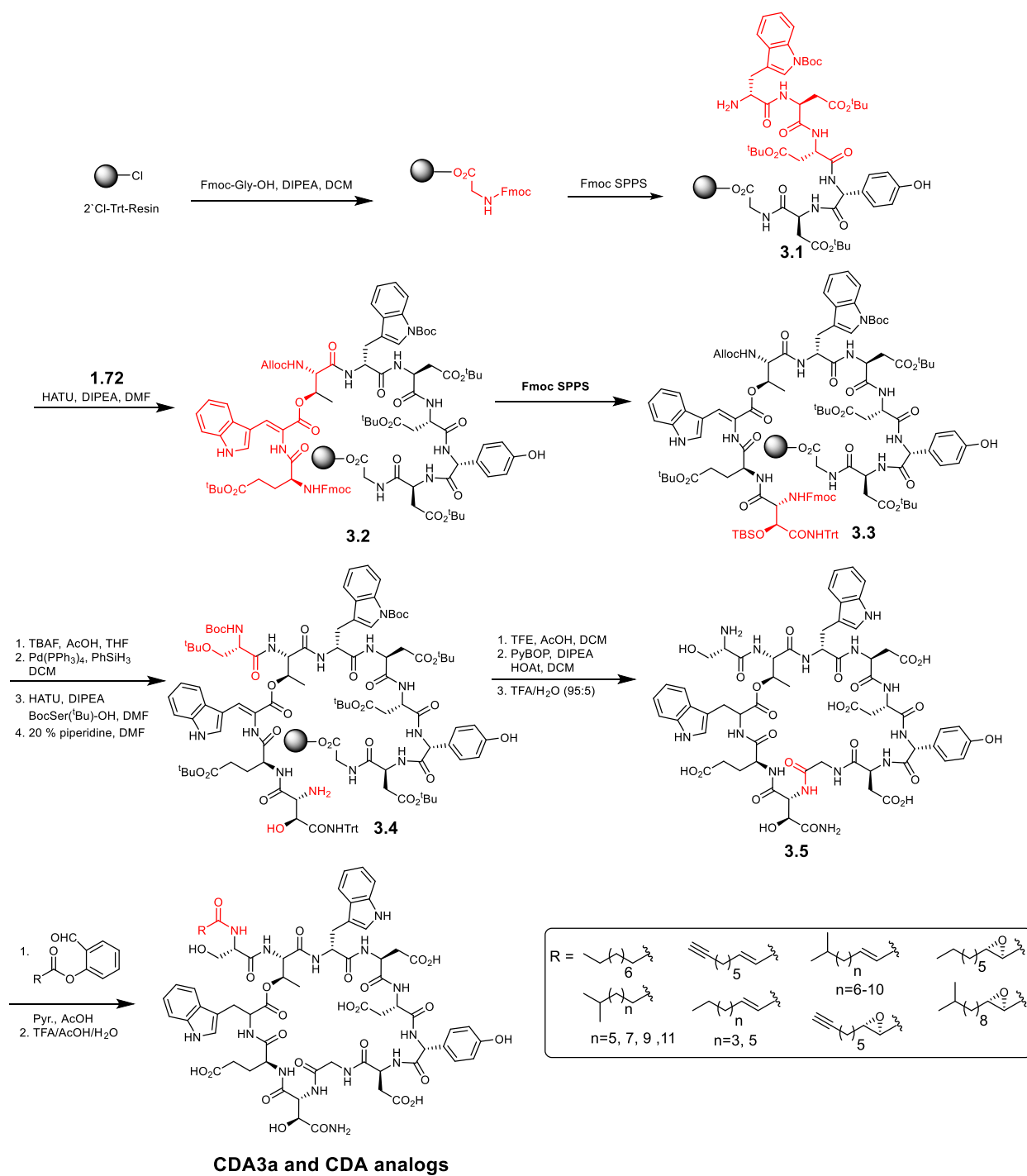
Scheme 3.3. The synthesis of CDA3a and CDA3a analogs by Chen et al.[55]
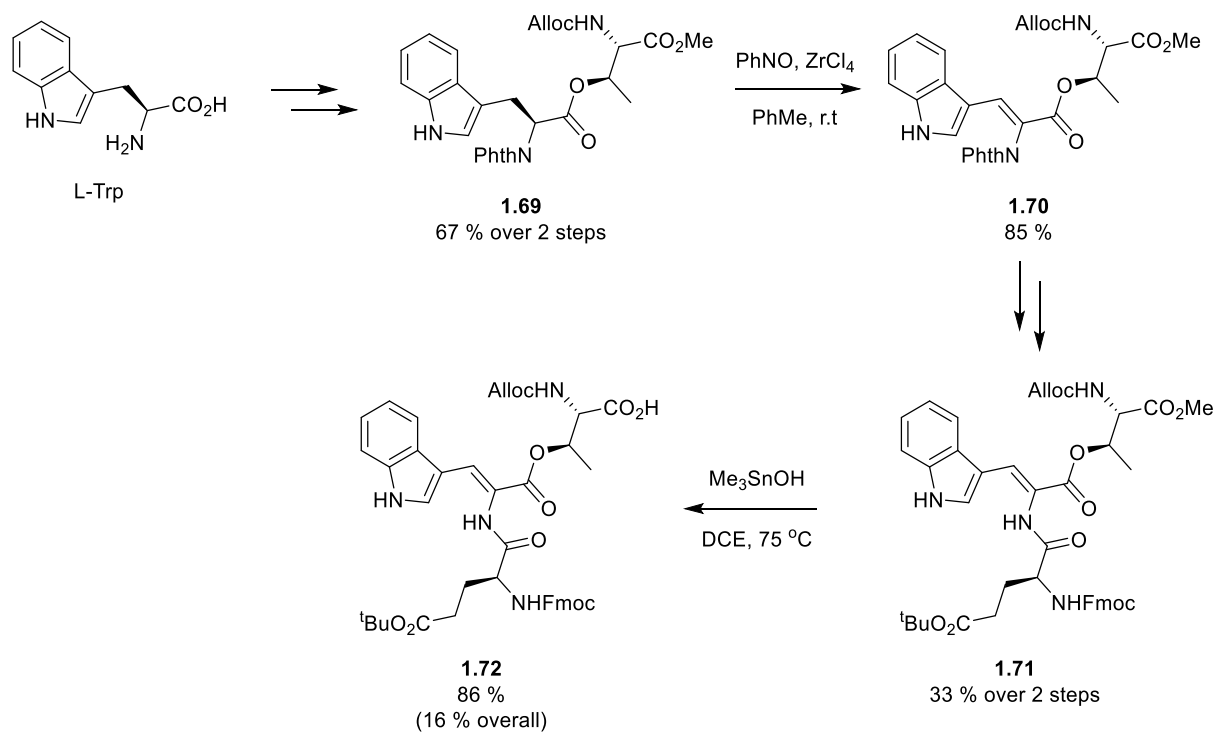
Scheme 3.4. The synthesis of tripeptide **1.72** by Chen et al.[55]

Fmoc deprotection. Thus, the researchers had to use extremely short (60 second) deprotection times with 20 % piperidine to remove the Fmoc group of peptide **3.2** in order to circumvent this potential DKP issue. From Z-dehydrotryptophan containing **3.2**, the researchers elongated the peptide to the *N*-terminus Fmoc-HOAsn(TBS) 9 (peptide **3.3**). They then removed the TBS ether of HOAsn (using TBAF) followed by the α-NH$_2$ Alloc protecting group of Thr2 (using a palladium catalyzed Tsuji-Trost deallylation) and then coupled Ser1 giving them peptide **3.4**. It was at this point that the researchers removed the Fmoc protecting group from HOAsn and preformed an off-resin cyclization followed by global deprotection using TFA/H$_2$O (95:5) to remove all acid labile protecting groups which gave them cyclic peptide **3.5**. This CDA peptide core **3.5** had to be purified by preparative-HPLC before a series of lipid tails were conjoined to the α-NH$_2$ of the Ser1 residue through a solution-phase (off-resin) serine ligation method. As mentioned previously,

this labor-intensive method had been previously used by the same group during the macrocyclization of daptomycin. They chose to introduce Ser1 and the lipid tails to the cyclic core using this serine ligation approach as they were concerned that the epoxide moiety would be completely or partially destroyed upon exposure to TFA during global deprotection/resin cleavage. Nevertheless, upon the second HPLC purification—following the solution-phase serine ligation step—the researchers were successful in preparing CDA3a as well as sixteen CDA3a analogs, each containing a different lipid tail, which were evaluated as antibiotics against a series of Gram-positive bacteria.[55] Surprisingly, these experiments were not conducted at 16 mM Ca^{+2}— the originally proposed optimal Ca^{+2} concentration of the CDAs—but rather they were done at 1.25 mM Ca^{+2}.[55, 105] A few of these synthetic CDAs showed potent activities while the naturally occurring CDA3a was essentially inactive at 1.25 mM Ca^{+2} (512 µg/mL). This SAR study indicated that the epoxide in the lipid tail was important for biological function and that the lipid tail's chain length as well as the nature of the substituent at the terminal position of the lipid tail's acyl chain were directly correlated to antibacterial potency.[55] Surprisingly, the researchers did not prepare any other CDA analogs, most notably MeGlu containing CDA4a. In daptomycin, replacement of the MeGlu for Glu has been shown to result in about a 7-fold loss in activity.[111] So, it is possible that such a phenomenon is the same in the CDAs where MeGlu-containing CDA4a is markedly more active than Glu containing CDA3a.

3.4   Objectives

We wished to demonstrate the utility of our new synthetic strategy for accessing Z-dehydrotryptophan-containing peptides by preparing CDA3a and CDA4a. We also aimed to

evaluate the antibacterial potency of these peptides at higher calcium concentration, such as 16 mM Ca^{+2}, as well as to develop other CDA analogs and conduct preliminary MoA studies on this class of compounds.

3.5   Results and discussion

3.5.1 The synthesis of CDA3a and CDA4a

Retrosynthetic analysis of CDA3a and CDA4a

The DXDG motif is often a common feature found in many of the calcium-dependent lipopeptide antibiotics. The glycine residue of this motif offers an ideal cyclization point as to avoid the possibility of epimerization. This achiral glycine within the DXDG motif was utilized as the cyclization point in the elegant synthesis of daptomycin performed by the Taylor group in 2015. Given that the CDAs all contain this Asp-Gly as part of the DXDG motif, a similar on-resin cyclization approach to prepare these compounds was used. (Scheme 3.5). In the synthesis of CDA3a and CDA3a analogs, Chen and coworkers forwent making a fully protected CDA3a due to apprehensions about the epoxide moiety's stability to acid. However, in 2013, Radzey et al. demonstrated that similar epoxides are in fact capable of withstanding 95:5 v/v TFA/H$_2$O.[112] So, we were confident that the global deprotection using TFA could be conducted on the full-length epoxide-containing peptide precursor **3.6**. The on-resin cyclization strategy could be applied to the synthesis of CDA3a and CDA4a. Therefore, a resin bound (2'-Cl-Trt) Asp(^{t}Bu)-GlyOAll dipeptide **3.7** was selected as starting point of the synthesis. Building block **2.22** was to be used as the Z-dehydrotryptophan precursor much the same as in the synthesis of tunicyclin B and

sclerotide A. The unusual amino acid, MeGlu (**3.8**), HOAsn (**3.9**), and HOPhGly (**3.10**), suitably protected for Fmoc SPPS, along with (2S,3R)-3-propyloxirane-2-carboxylic acid (**3.11**) would have to be synthesized as they are not commercially available.
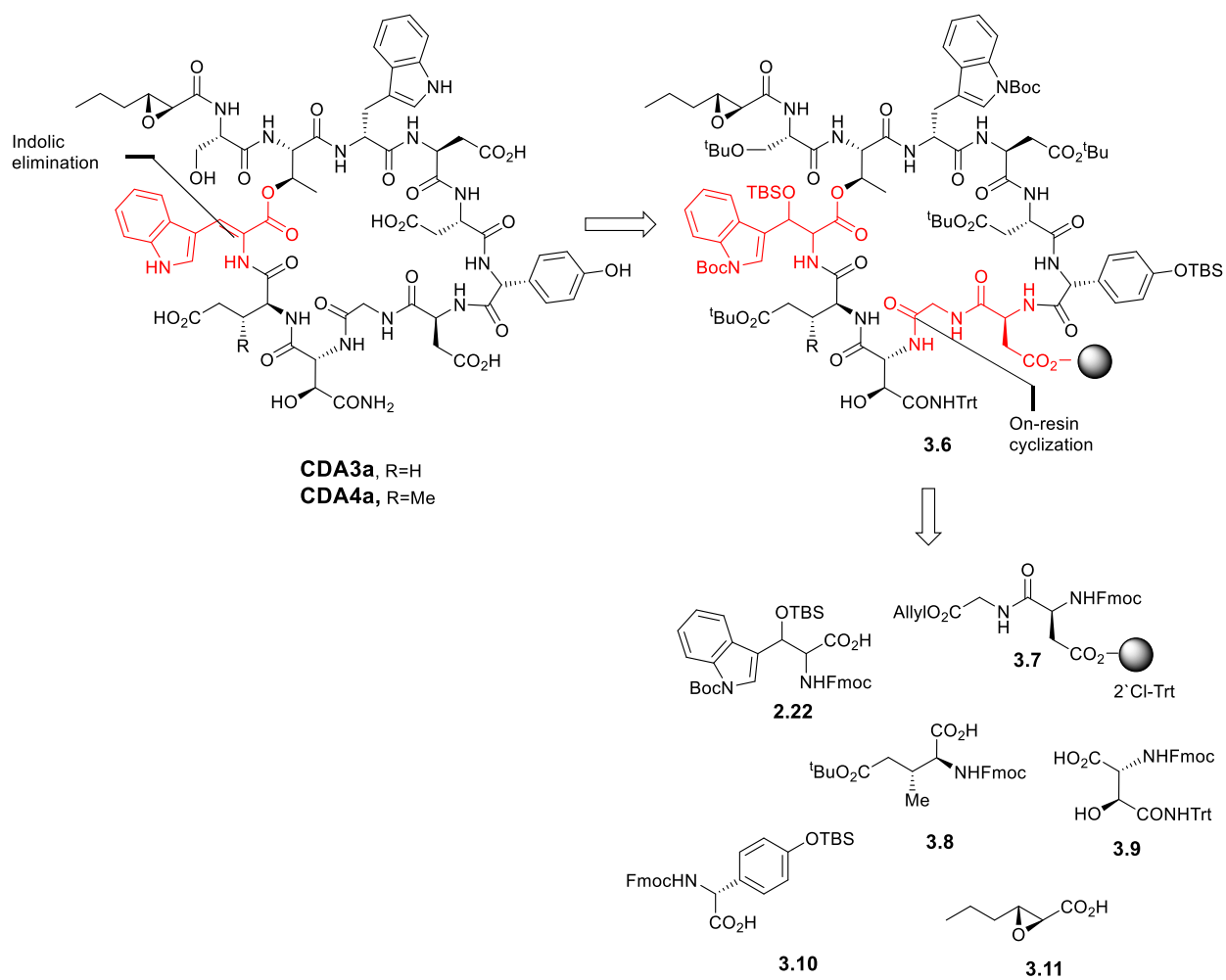
Scheme 3.5. Retrosynthetic analysis of CDA3a and CDA4a.

The synthesis of CDA3a and CDA4a building blocks

We began our synthesis of CDA3a and 4a with the synthesis of precursors **3.7**, **3.10** and **3.11**. The Fmoc-Asp-Gly-OAllyl dipeptide was made as previously described by the coupling of Gly-OAllyl tosylate salt **3.12** with FmocAsp(^{t}Bu)OH using EDC•HCl, HOBt and *N*-ethylmorpholine

in THF followed by TFA mediated removal of the side chain ^{t}Bu group of the Asp which produced

the desired peptide in 95 % yield over 2 steps (Scheme 3.6).[101]
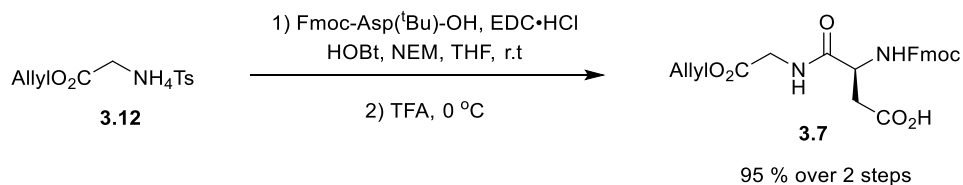
Scheme 3.6. The synthesis of the Fmoc-Asp-Gly-OAllyl dipeptide **3.7**.

The (2S,3R)-3-propyloxirane-2-carboxylic acid (**3.11**) was made beginning from *trans*-2-

hexen-1-ol which was subjected to a Sharpless asymmetric epoxidation using a procedure

adapted from that described by Regenye and coworkers (Scheme 3.7).[113]This epoxy alcohol **3.14**,

obtained in 60 % yield, was oxidized using TEMPO with NaOCl/NaClO$_2$ in MeCN/phosphate buffer

following a procedure described by Zhang et al.[114] This oxidation provided **3.11** in 61 % yield.
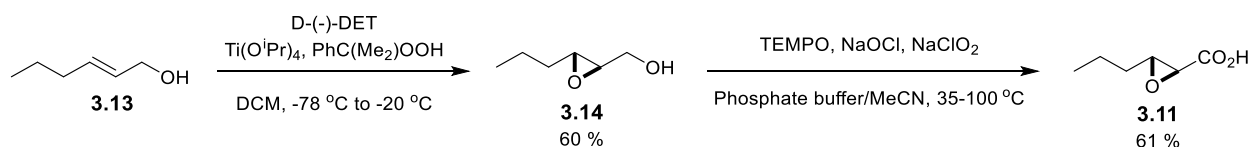
Scheme 3.7. The synthesis of (2S,3R)-3-propyloxirane-2-carboxylic acid **3.11**.

Amino acid **3.10** was made in 83 % yield by treating Fmoc-D-HOPhGly-OH with

TBSCl/imidazole in DMF(Scheme 3.8).

Next, we were tasked with making protected MeGlu **3.8** and protected HOAsn **3.9**. Compound

3.8 was graciously supplied by Ryan Moreira, a member of the Taylor group, who had made it

following a method he had recently described in 2020 (Scheme 3.9).[115] This method began with

acetonide formation of Fmoc-D-Ser-OMe using 2,2-dimethoxypropane with catalytic BF$_3$
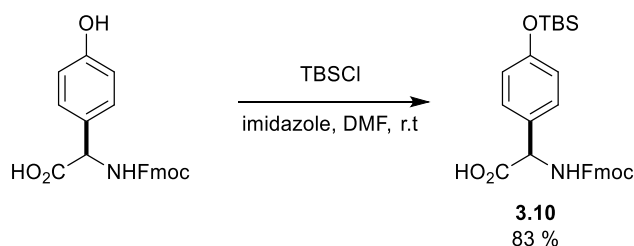
Scheme 3.8. The synthesis of building Fmoc-D-HOPhGly(TBS)-OH **3.10**.

diethyletherate in acetone. The ester of the resulting acetonide intermediate **3.16** was reduced using DIBAL while maintaining careful temperature control as to avoid over reduction of the aldehyde group. Both of these steps proceeded with excellent yields. The resulting Fmoc Garner's aldehyde **3.17** was subjected to a HWE reaction which produced the corresponding α, β-unsaturated ester **3.18** in excellent yield. Compound **3.18** was reacted with lithium dimethyl cuprate in the presence of TMSCl which provided **3.19** in near quantitative yield, and with essentially complete *threo* diastereoselectivity. The acetonide was opened using BiBr$_3$ in a mixture of H$_2$O and MeCN, and the resulting primary alcohol was oxidized using TEMPO/NaOCl/NaClO$_2$ in MeCN/phosphate buffer which provided **3.8** in 79 % over 2 steps.
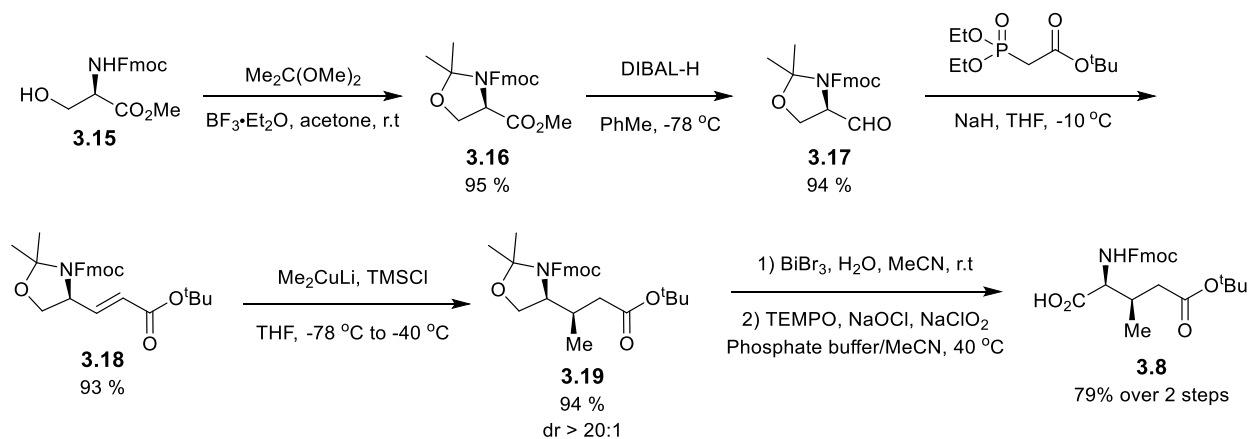
Scheme 3.9. The synthesis of Fmoc-MeGlu(^{t}Bu)-OH 3.8 performed by Ryan Moreira.[115]

We have previously described a method to access D-*threo*-HOAsn suitably protected for Fmoc SPPS.[115] A structurally similar amino acid, L-*erythro*-MeOAsp, was prepared during the synthesis of A54145D in which a Mitsunobu reaction was used to invert the stereochemistry of the β-hydroxy group from *threo*- to the desired *erythro*-configuration.[103] We anticipated a similar method could be used to prepare D-*erythro*-HOAsn **3.9**. The synthesis of this residue began with the Sharpless asymmetric aminohydroxylation (SAAH) reaction of alkene **3.20** following a literature procedure producing compound **3.21** (Scheme 3.10).[115] This chiral alcohol **3.21** was then subjected to a Mitsunobu reaction with p-nitrobenzoic acid/diisopropyl diazodicarboxylate (DIAD)/triphenyl phosphine to give **3.22** in 66 % yield. The p-nitrobenzoate group in **3.22** was removed via an azidolysis reaction utilizing sodium azide producing **3.23** in 92 % yield. The methyl ester was hydrolyzed using aqueous LiOH in THF/^{i}PrOH in the presence of $CaCl_2$. The corresponding crude acid was treated with BOP/HOBt/NH_4Cl/DIPEA in DMF which provided the resulting amidated product **3.24** in 71 % over 2 steps. Next, the secondary alcohol of intermediate **3.24** was protected as the TBS silyl ether using TBSCl/imidazole and DMAP in DCM at 0 °C which proceed in 72 % to give **3.25**. This step was conducted in order to aid in the purification of later intermediates as we suspected that some of these compounds lacking the TBS group would be very polar and difficult to purify by conventional flash chromatography. Thus, the PMB group in intermediate **3.25** was removed using BCl_3 with pentamethyl benzene giving compound **3.26** in an 83 % yield. The liberated primary alcohol of **3.26** was oxidized using TEMPO/NaOCl/$NaClO_2$, producing carboxylate **3.27** in 88 % yield, which was tritylated using trityl alcohol in the presence of acetic anhydride/H_2SO_4 in acetic acid. These conditions also resulted in the removal of the TBS group providing **3.9** in a 60 % yield.
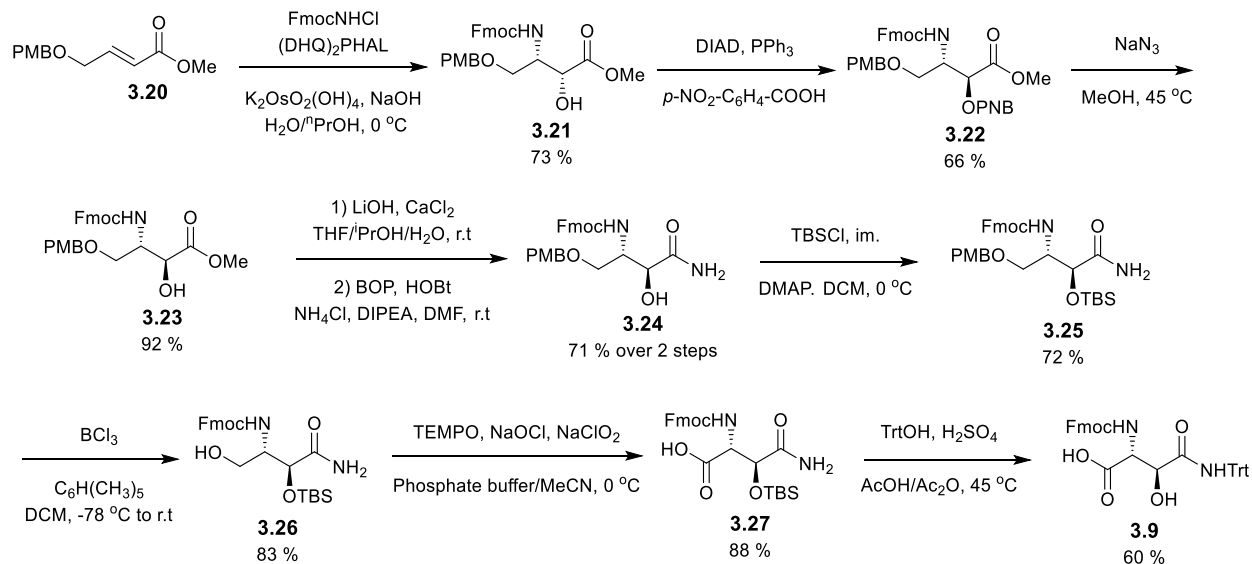
Scheme 3.10. The synthesis of Fmoc-D-*erythro*-HOAsn(Trt)-OH **3.9**.

Fmoc SPPS of CDA3a and CDA4a

With all of the building blocks in hand, we turned our attention towards the Fmoc SPPS of CDA3a and CDA4a (Scheme 3.11). 2'-Cl-Trt-Cl resin was loaded with the Fmoc-Asp-Gly-OAllyl dipeptide which provided the starting point to our synthesis in resin-bound dipeptide **3.7**. Next, HOPhGly **3.10** was coupled using the conditions described by Liang and coworkers utilizing COMU and 2,6-lutidine as coupling agents.[110] This reaction went smoothly, and no epimerization could be detected upon HPLC analysis of this peptide. This resin bound tripeptide **3.28** was elongated through Fmoc SPPS, producing peptide **3.29**, and then (2S,3R)-3-propyloxirane-2-carboxylic acid **3.11** was attached using DIC/HOBt as coupling agents which gave unbranched peptide **3.30**. DIC/DMAP in dry DCM was used to form the crucial depsi bond between peptide **3.30** and building block **2.22.** The reaction was sluggish using only 0.1 equivalents of catalytic DMAP. For DIC/DMAP on-resin esterifications, the amount of DMAP is typically kept to 10 % or less to avoid

epimerization of the activated amino acid. In this case, since the stereochemistry of amino acid

2.22 is not important, a higher DMAP loading could be used without consequence. Thus, using

0.5 equivalents of catalytic DMAP, the reaction between **3.30** and **2.22** went to completion after

24 h to give peptide **3.31**. Through Fmoc SPPS, now using 2-methylpiperidine (2-MP) rather than

4-MP for Fmoc deprotections, either MeGlu **3.8** or Glu were coupled, followed by HOAsn **3.9**

which produced peptide **3.32** and **3.33** respectively. 2-MP was used to remove the Fmoc groups,

to minimize the possibility of aminolysis of the ester bond. The allyl group and Fmoc group of

both peptides (**3.32** and **3.33**) were removed using catalytic Pd(PPh$_3$)$_4$/DMBA and 20 % 2-MP in

DMF
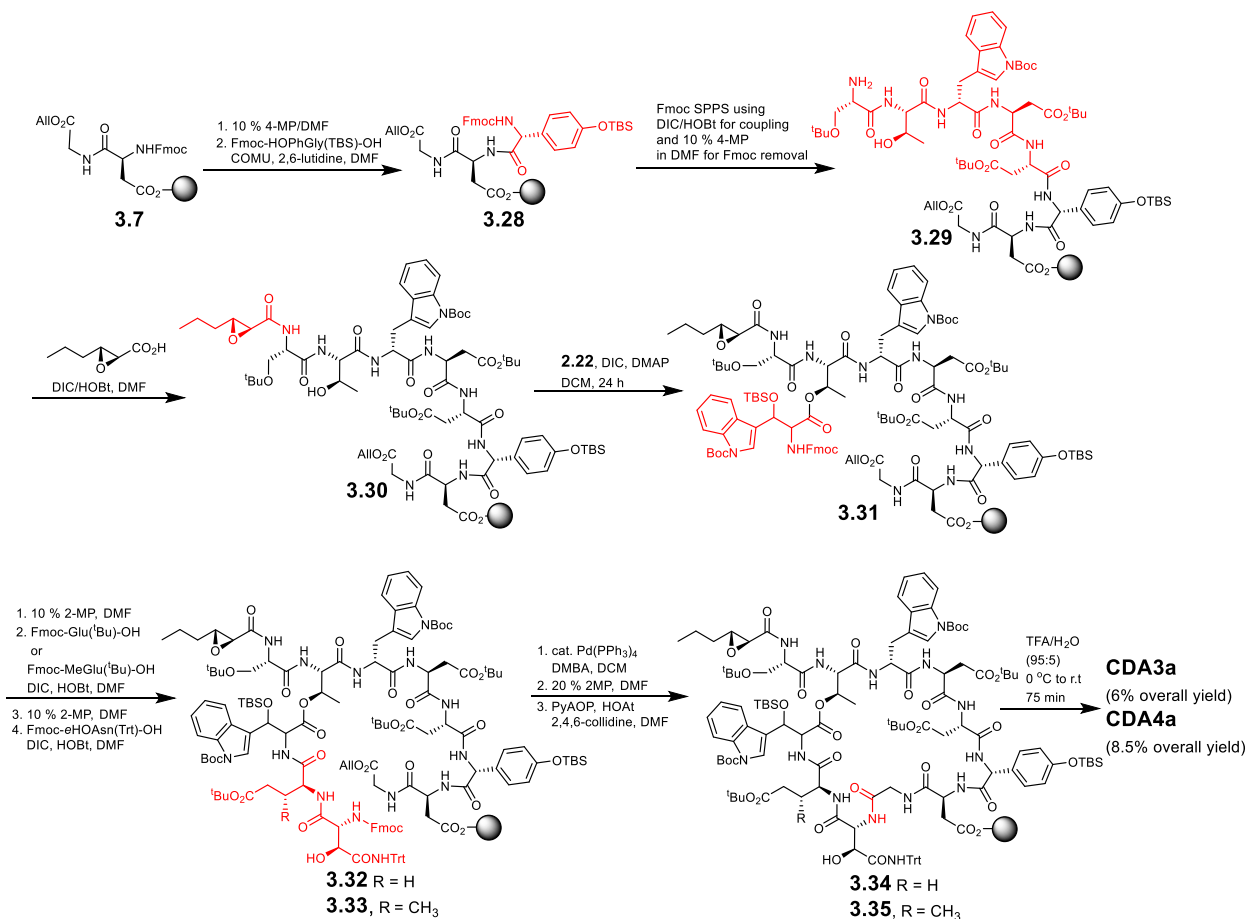
Scheme 3.11. The synthesis of CDA3a and CDA4a.

(10 min x 3) respectively, and then the peptides were subjected to on-resin cyclization using PyOAP/HOAt/2,4,6,-collidine in DMF producing cyclic **3.34** and **3.35**. We found that Fmoc removal from peptide **3.32/3.33** using 10 % 2-MP was excruciatingly slow, whereas with 20 %-2MP, the Fmoc group could be removed upon three 10-minute treatments. Both cyclized peptides, **3.34** and **3.35**, were poised for global deprotection, cleavage from the resin and Z-dehydrotryptophan formation. However, this step took some optimization as we found that the epoxide group was acid sensitive as expected. An array of cleavage/deprotection conditions were examined (Table 3.3). Initially, mixtures of TFA with different scavengers and additives were reacted with the peptide for 1 h at 0 °C to room temperature. However, reacting either TFA/DCM/*N*-Me-indole (70:28:2), TFA/anisole/thioanisole (90:5:5) or TFA/PhOH/*p*TsOH (95:2.5:2.5) with the peptide for 1h at 0 °C to room temperature produced complex mixtures that were not easily analyzed by HPLC-MS (entries 1-3). Using 95:5 TFA/H_2O at room temperature for 60 min gave a significant amount of the epoxide-hydrolyzed peptide (entry 4). Reducing the amount of water to 2% gave similar results (entry 5). In all of these reactions thus far, after removing all the TFA using a stream of condensed air, the cleaved peptides were incubated in 1:1 MeCN/H_2O overnight to hydrolyze the tryptophan CO_2 adducts. This proved to be problematic as when performing the reaction in the absence of water (9:1 TFA/PhMe), and incubating overnight under the aforementioned conditions, significant quantities of the product resulting from epoxide hydrolysis was detected (entry 6). This indicated that appreciable amounts of the desired epoxide was being hydrolyzed when incubated in the acidic MeCN/H_2O mixture overnight. Thus, subsequent reactions were incubated overnight in 1:1 MeCN/phosphate buffer at 0 °C to

hydrolyze the CO_2 adducts. Now performing the reaction in the absence of water (9:1 TFA/PhMe), and incubating overnight under the buffered conditions, resulted in no detectable epoxide hydrolysis, removal of the peptide from the resin and removal of all of the protecting groups with the exception of the TBS protecting group on HOPhGly6 (entry 7). Finally, we found that epoxide hydrolysis could be minimized by adding ice cold 95:5 TFA/H_2O to **3.34** or **3.35** which was allowed to react for 75 min at r.t, followed by incubation in 1:1 MeCN/phosphate buffer (entry 8). Interestingly, we found that the residue at position 10 (Glu or MeGlu) affected the amount of epoxide-hydrolyzed product obtained. The Glu analog gave ≈ 10 % hydrolyzed product whereas the MeGlu analog gave about 18 % epoxide hydrolyzed product. Nevertheless, with these cleavage conditions, CDA3a and CDA4a were obtained, after RP-HPLC purification, in 6 % and 8.5 % overall yield respectively.

Table 3.3. Optimization study of the acidic global deprotection in the synthesis of CDA4a.

entry	cleavage cocktail mixture	reaction time	temperature	result
1	TFA/DCM/N-Me indole (70:28:2) [a]	1 h	0 ºC-r.t	Decomposition/no identifiable product
2	TFA/anisole/thioanisole (90:5:5) [a]	1 h	0 ºC-r.t	Decomposition/no identifiable product
3	TFA/PhOH/TsOH•H_2O (95:2.5:2.5) [a]	1 h	0 ºC-r.t	Decomposition/no identifiable product
4	TFA/H_2O (95:5) [a]	1 h	0 ºC-r.t	~3:1 desired peptide: opened epoxide
5	TFA/H_2O (98:2) [a]	1 h	0 ºC-r.t	~2:1 desired peptide: opened epoxide
6	TFA/PhMe (90:10) [a]	1 h	0 ºC-r.t	~1:1 desired peptide: opened epoxide
7	TFA/PhMe (90:10 [b]	1 h	0 ºC-r.t	HOPhGly TBS not removed, no opened epoxide
8	TFA/H_2O (95:5) [b]	75 min[b]	0 ºC-r.t	~82:18 desired peptide: opened epoxide

3.5.2 Biological studies on CDA3a, 4a and synthesis of CDA analogs

With CDA3a and CDA4a in hand we conducted several preliminary biological studies. We first aimed to determine the MIC of both compounds. As mentioned in section 3.4, using a disc assay, Lakey et al. noted that a crude mixture of the CDAs was only active at Ca^{+2} concentrations of 16 mM or greater.[105] Using a microbroth dilution assay, we also found that CDA4a (10 µg/mL) required at least 16 mM Ca^{2+} for activity against *Bacillus subtilis* 1046. This Ca^{+2} concentration was used to deduce the MIC values of CDA3a and CDA4a. At 16 mM Ca^{+2}, the MIC values of CDA3a and 4a were 16 µg/mL and 8 µg/mL respectively (Table 3.4). Although not particularly potent, both compounds are significantly more active at this Ca^{+2} concentration compared to the MIC of CDA3a at 1.25 mM Ca^{+2} reported by Chen et al.[55] These results also indicate that the MeGlu in the CDAs augments their antibacterial potencies.

Table 3.4. MIC of CDA3 and CDA4a against *B. subtills* 1046 at 16 mM Ca^{+2}.

entry	peptide	MIC (µg/mL)
1	CDA3a	16
2	CDA4a	8

No one has demonstrated whether the CDAs bind to phosphatidylglycerol (PG) in the presence of Ca^{+2} in the same way as daptomycin. In 1985, Lakey and Ptak demonstrated that the changes in kynurenine fluorescence of daptomycin could be used to monitor Ca^{+2}-dependent membrane binding with PC containing liposomes.[117] The researchers noted a 10-fold fluorescence enhancement and a slight blue shift upon the addition of 50 mM Ca^{+2}. In 2004, Jung

and coworkers conducted similar experiments, but used PC/PG liposomes in addition to PC liposomes. Much like what Lakey and Ptak had reported in 1985, Jung and coworkers found that daptomycin incubated in the presence of PC liposomes showed no increase in fluorescence—compared to daptomycin itself in aqueous solution—until Ca^{+2} was added which resulted in a slight blue shift and a 5-fold increase in fluorescence.[118] If 1:1 PC/PG liposomes were used instead in the presence of Ca^{+2}, the researchers noted a larger blue shift which was accompanied with a 9-fold increase in fluorescence intensity.[118] The researchers concluded that daptomycin was able to bind to both PC and PC/PG liposomes only in the presence of Ca^{+2} and that daptomycin was able to insert more deeply into PC/PG liposomes compared to PC liposomes.[118] A similar result was reported in 2016, when Taylor et al. showed that the change in fluorescence of the kynurenine residue in daptomycin could be used to monitor Ca^{+2} dependent membrane binding. The researchers found that daptomycin, incubated with a model 1:1 PC/PG membrane, showed a rapid increase in fluorescence as the Ca^{+2} concentration approached 1 mM with little change before that point.[90] We believed similar fluorescence studies could be conducted on the CDAs using Z-dehydrotryptophan as the fluorophore. It has been reported by Micklefield et al. that the maximum absorbance of the Z-dehydrotryptophan residue in the CDAs occurs at 349 nm.[107] A excitation λ_{max} within this range is ideal as it does not overlap with the excitation λ_{max} of other aromatic amino acid residues such as Trp. Thus, using an excitation wavelength of 349 nm, we incubated 3 µM of CDA4a with 250 µM 1:1 DOPG/DOPC liposomes and titrated in Ca^{+2} while monitoring the dehydrotryptophan fluorescence from 375-550 nm (Figure 3.4). A small increase in fluorescence from 0 mM Ca^{+2} to 1 mM mM Ca^{+2} was observed At Ca^{+2} concentrations above 1 mM, we observed a large increase in fluorescence which did not increase significantly above 15

mM. This indicates Ca^{+2}-dependent binding of CDA4a to the PG/PC liposomes. This experiment was repeated using liposomes that contained only DOPC (Figure 3. 5). In comparison to the PC/PG liposomes, smaller changes in fluorescence were observed under increasing Ca^{+2}, with a significant increase in fluorescence only occurring only at relatively high Ca^{+2} concentrations (30 mM). The two sets of data can be directly compared through a fluorescence response curve (Figure 3.6), where the effect of PG on the interaction of the peptide to the liposome is clearly evident. (Figure 3.6-in green). These membrane binding studies suggest that a possible first step in the MoA of CDA4a, like daptomycin, is binding to the PG in the bacterial membranes in a Ca^{+2}-dependent manner.

Figure 3.4. CDA4a Membrane Binding (DOPC/DOPG)
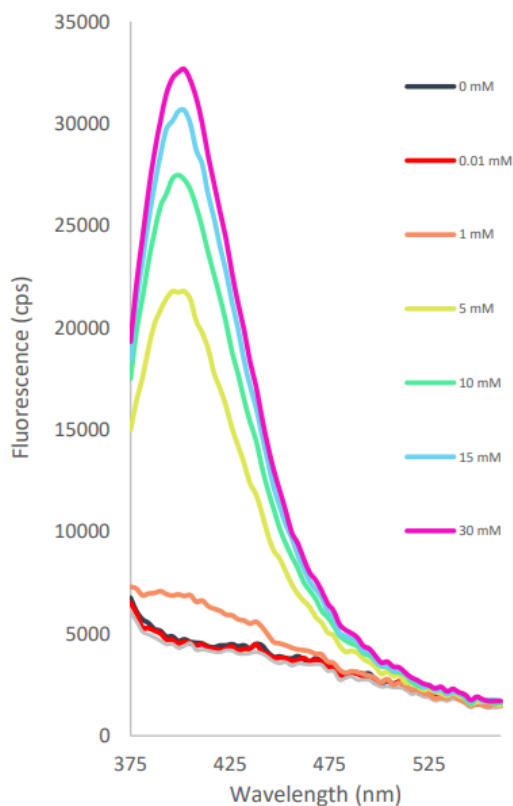
Figure 3.5. CDA4a Membrane Binding
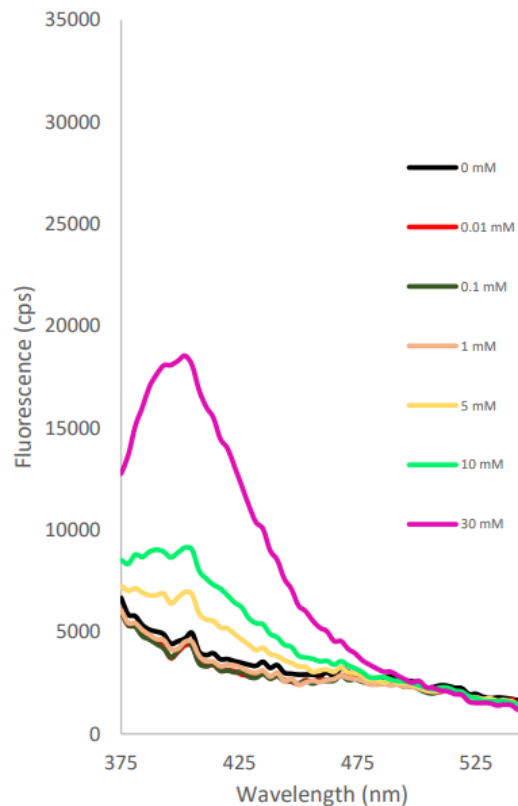
Figure 3.6 Fluorescence response of CDA4a (DOPG/DOPC & DOPC)
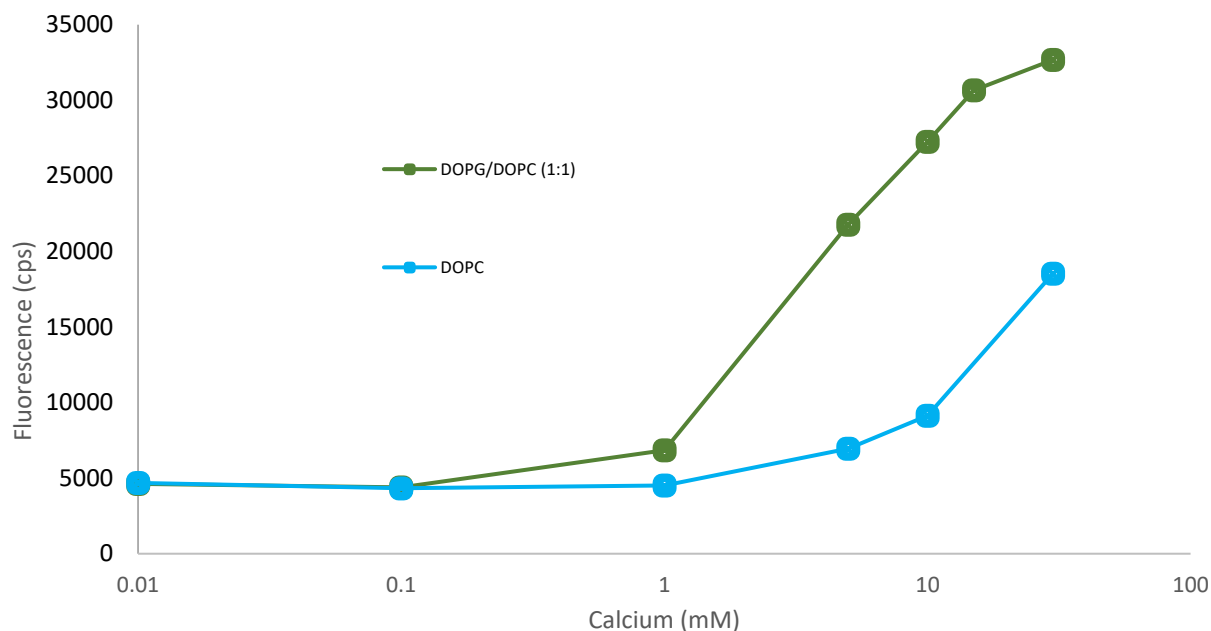
The success of our synthesis of CDA3a and 4a prompted us to develop other CDA analogs to see whether the Z-dehydrotryptophan residue was amenable to substitution. Several of the naturally occurring CDAs contain a Trp in place of a Z-dehydrotryptophan. Therefore, we prepared CDA4b which is structurally identical to CDA4a except that Z-dehydrotryptophan is replaced with a L-Trp. In addition to CDA4b, we also wished to prepare a CDA analog that possesses a Kyn residue in place of the Z-dehydrotryptophan, as Kyn is a more strongly fluorescent amino acid than Z-dehydrotryptophan. As a result, this Kyn containing analog could be very useful for further MoA studies as long as it retained its antibacterial properties. Both the Trp containing and Kyn containing CDA analogs were easily made using our Fmoc SPPS strategy to CDA3a/4a except that, during depsibond formation, either Fmoc-Trp(Boc)-OH or Fmoc-Kyn(Boc)-OH with only 0.1 equivalent of DMAP (as to avoid epimerization) was used (Scheme 3.12). Interestingly, we found that, even with 0.1 equivalents of DMAP during depsibond

formation with Fmoc-Kyn(Boc)-OH, noticeable epimerization of the Kyn residue occurred. Luckily, the desired CDA
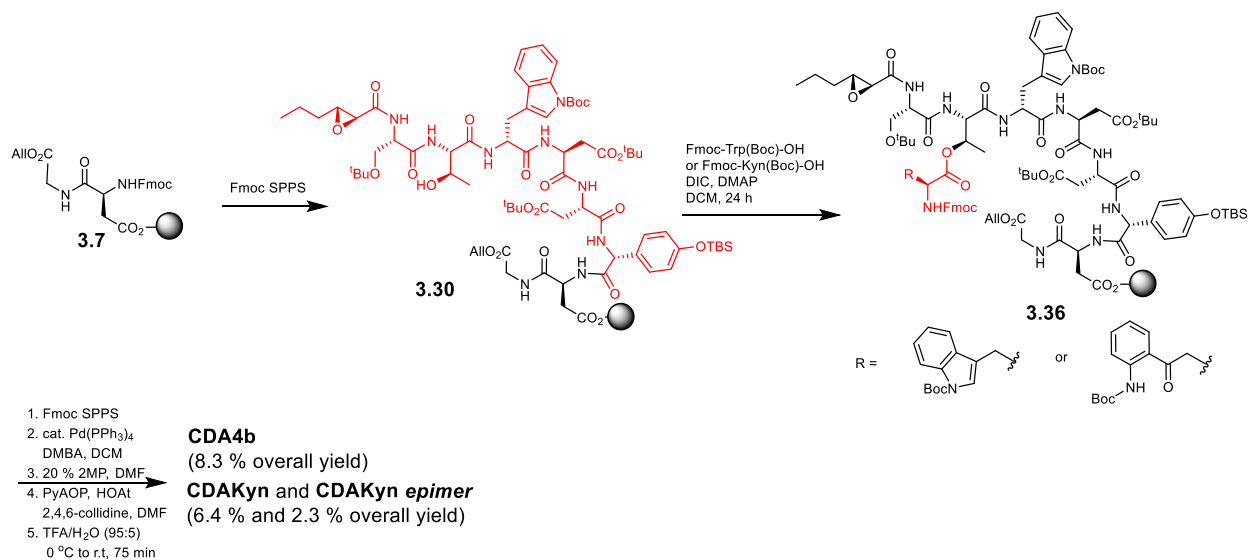
Scheme 3.12. The synthesis of CDA4b, CDAKyn and CDAKyn *epimer*.

-Kyn11 analog and the CDA-Kyn11 *epimer* could be separated during final RP-HPLC purification (See HPLC/NMR spectra in Appendix B). These two peptides were isolated in 6.4 % and 2.3 % overall yields respectively. In contrast, no epimer of CDA4b was observed and this peptide was easily obtained in an 8.3 % overall yield. The MICs of these three peptides were determined against *B. subtillis* 1046 at 16 mM Ca^{+2} (Table 3.5). To our surprise, CDA4b showed improved activity to both Z-dehydrotryptophan containing CDA3a and CDA4a with a MIC of 4 µg/mL. CDAKyn exhibited the same MIC (8 µg/mL) as CDA4a. In contrast, the CDAKyn *epimer* was inactive up to 32 µg/mL indicating that D-amino acids are not compatible at this position.

Table 3.5. MIC of CDA analogs against *B. subtills* 1046 at 16 mM Ca^{+2}.

entry	peptide	MIC (µg/mL)
1	CDA4b	4
2	CDAKyn	8
3	CDAKyn *epimer*	>32

3.6 Experimental section

General experimental method

All reagents used for peptide synthesis were obtained from commercial sources including coupling reagents, resins, and Fmoc amino acids unless stated otherwise. ACS grade, N,N'-dimethylformamide (DMF), 4-methylpiperidine (4-MP), 2-methylpiperidine (2-MP) and TFA were purchased from commercial suppliers and used without further purification. CH_2Cl_2 (DCM) was distilled from calcium hydride under nitrogen. THF was distilled from sodium metal and benzophenone under nitrogen. Peptide synthesis was performed manually using a rotary mixer for agitation. Peptide syntheses were monitored by treating small aliquots of resin with 95:5 TFA/H_2O for 75 min, removing the solvent by a N_2 stream, redissolving the peptide in 1:1 MeCN/H_2O, and analyzing by RP-HPLC and LRMS using a linear ion trap mass spectrometer.

Chemical shifts (δ) for ^1H NMR spectra run in $CDCl_3$ are reported in ppm relative to the standard TMS. Chemical shifts for ^{13}C NMR spectra run in $CDCl_3$ are reported in ppm relative to the solvent residual carbon (δ 77.16 for central peak). Chemical shifts (δ) for ^1H & ^{13}C NMR spectra run in DMSO-d_6 are reported relative to the residual solvent peaks at 2.50 and 39.52.

Analytical HPLC was accomplished with a reversed-phase C18 column (10 μm, 250 mm × 4.6 mm, 1 mL/min flow rate). Peptides were purified by reversed-phase semipreparative HPLC using a C18 column (10 μm, 150 mm × 20 mm, 10 mL/min flow rate). High-resolution positive ion electrospray (ESI+) mass spectra were obtained using a hybrid quadrupole-orbitrap mass spectrometer, dissolving samples in 1:1 MeOH/H_2O + 0.1% formic acid.

Resin loading was estimated using a procedure described by Gude at al.[83]

Antibacterial activity of each synthetic antibiotic was determined using a broth dilution assay[119] in which overnight bacterial cultures are diluted approximately 1×10^6 CFU/mL based on the measured optical density at 600 nm (OD600). A series of twofold serial dilution in LB were preformed directly on a 96-well microplate which were then inoculated with equal volume of diluted bacterial culture. 96-well plates were then incubated for 24 h at 37 °C at which point the MIC was determined by the lowest peptide concentration at which there was no visible bacterial growth.

Fluorescent membrane experiments were conducted following the procedure described by Taylor et al. with an excitation wavelength set to of 349 nm.[90] The molar extinction coefficient of CDA4a at 349 nm was determined to be ε = 12,700 $M^{-1}cm^{-1}$

3.6.1 Experimental procedures

Fmoc-D-*erythro*-HOAsn(Trt)-OH (3.9)

Fmoc-D-*erythro*-HOAsn(Trt)-OH (**3.9**) was prepared using two different routes. The route outlined in Scheme 3.10 was initially used and was based upon our synthesis of Fmoc-D-*threo*-HOAsn(Trt)-OH.[103, 116] The route outlined in Scheme 3.13 was used for the large-scale synthesis of **3.9** which is based upon the procedures of Moeller et al. [120] and Guzman-Martinez.[121]

Compound 3.22. A solution of **3.21** [103] (2.70 g, 5.50 mmol), *p*-nitrobenzoic acid (1.38 g, 8.26 mmol, 1.5 equiv) and triphenylphosphine (2.17 g, 8.27 mmol, 1.5 equiv) in THF (28 mL), cooled to 0 °C, was treated with diisopropyl azodicarboxylate (1.6 mL, 8.15 mmol, 1.5 equiv). The resulting mixture was left to stir for 21 h at room temperature before being evaporated in vacuo. The crude material was recrystalized from ^{i}PrOH to give **3.22** as a fine pale-yellow, crystalline solid (2.32 g, 66 % yield). ^{1}H NMR (300 MHz, CDCl$_3$, δ): 8.20 (2H, d, J = 8.4 Hz), 8.05 (2H, d, J = 8.4 Hz), 7.75 (2H, d, J = 7.8 Hz), 7.58 (2H, m), 7.43-7.24 (4H, m), 7.2 (2H, d, J = 8.4 Hz), 6.78 (2H, d, J = 8.4 Hz) 5.43 7–5.30 (2H, m), 4.59–4.32 (5H, m), 3.78–3.50 (8H, m). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 171.0, 166.5, 162.3, 158.7, 153.6, 146.6, 144.2, 137.2, 133.9, 132.6, 132.06, 130.7, 130.0, 127.9, 126.4, 122.9, 116.7, 75.9, 75.5, 70.0, 58.0, 55.6, 54.1, 50.0. HRMS (ESI+) m/z: [M + H]$^+$ calcd for C$_{35}$H$_{33}$N$_2$O$_{10}$, 641.2130; found, 641.2129.

Compound 3.23. A suspension of **3.22** (2.76 g, 4.31 mmol) and NaN$_3$ (0.84 g, 12.93 mmol, 3.0 equiv) was stirred at 45 °C for 4h. The homogenous mixture was concentrated in vacuo and the crude material was purified by FC (10 % EtOAc/90 % hexanes), which provided **3.23** as a white solid (1.93 g, 92 % yield). ^{1}H NMR (300 MHz, CDCl$_3$, δ): 7.76 (2H, d, J = 6.9 Hz), 7.60 (2H, d, J = 6.9 Hz), 7.43–7.18 (6H, m), 6.86 (2H, d, J = 6.9 Hz), 5.50 (1H, d, J = 7.9 Hz), 4.48–4.13 (7H, m), 3.78 (3H, s), 3.63 (3H, s), 3.55 (2H, m), 3.37 (1H, d, J = 7.3 Hz). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 175.8, 162.3, 158.8, 146.7, 144.2, 132.5, 132.3, 130.6, 130.0, 127.9, 122.9,166.7, 76.1, 74.2, 70.9, 69.9, 58.1, 55.5, 55.3, 50.0. HRMS (ESI+) m/z: [M + H]$^+$ calcd for C$_{28}$H$_{30}$NO$_7$, 492.2017; found, 492.2017.

Compound 3.24. A cooled (0 °C, ice bath) suspension of **3.23** (1.12 g, 2.28 mmol) and pulverized CaCl$_2$ (4.10 g, 36.9 mmol, 16.4 mmol) in THF (16 mL)/^{i}PrOH (53 mL) was treated dropwise with ice-cold aq. LiOH (16.4 mL, 0.28 M, 2.0 equiv). The mixture was stirred at 0 °C for 15 min before additional ice-cold aq. LiOH (13.1 mL, 0.28 M, 1.6 equiv) was added dropwise. The resulting mixture was allowed to stir at room temperature for 90 min before it was acidified with 1 M HCl (pH ca. 2) and extracted with EtOAc (100 mL x 3). The combined extracts were washed with brine (100 mL), dried over MgSO$_4$, and evaporated in vacuo. The crude material was then taken up in DMF (46 mL) and reacted with benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP; 1.51 g, 3.42 mmol, 1.5 equiv) for 5 min at room temperature. Then, NH$_4$Cl (0.23 g, 4.30 mmol, 2.0 equiv) and DIPEA (1.1 mL, 6.30 mmol, 3.0 equiv) were added and the resulting suspension was stirred at room temperature for 2.5 h. The reaction mixture was then diluted with EtOAc (500 mL) and washed with 1 M HCl (400 mL), sat. NaHCO$_3$ (400 mL), and brine (300 mL x2). The organic layer was dried with MgSO$_4$, concentrated, and subjected to silica gel FC (66% EtOAc/31 % hexanes/2 % AcOH then 85 % EtOAc/13 % hexanes/2 % AcOH) to give **3.24** as an off-white solid (0.77 g, 71 % yield over 2 steps). ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.74 (2H, d, J = 6.9 Hz), 7.57 (2H, d, J = 7.3 Hz), 7.38 (2 H, t, J = 6.4 Hz), 7.28 (2 H, t, J = 7.3 Hz), 7.18 (2H, d, J = 7.9 Hz), 6.82 (2H, d, J = 7.9 Hz), 6.7 (1H, br. s), 5.98 (1H, br. s), 5.67 (1H, d, J = 7.7 Hz), 4.50–4.11 (8H, m), 3.87–3.55 (5H, m). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 177.4, 162.4, 159.9, 146.6, 144.2, 132.5, 132.08, 130. 6, 129.0, 128.0, 127.9, 122.9, 116.8, 76.7, 76.1, 72.0, 69.9, 58.1, 56.2, 50.0. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{27}$H$_{29}$O$_6$N$_2$, 477.2020; found, 477.2024.

Compound 3.25. A solution of **3.24** (0.91 g, 1.91 mmol) in DCM (19 mL), cooled to 0 °C, was treated with imidazole (0.65 g, 9.55 mmol, 5.0 equiv) followed by TBSCl (1.43 g, 9.49 mmol, 5.0 equiv). The mixture was stirred for 5 min at 0 °C before DMAP (24 mg, 0.20 mmol, 0.1 equiv) was

added. The resulting mixture was stirred at 0 °C for 3.5 h before being diluted with DCM (100 mL) and sat. NH$_4$Cl (100 mL), still while at 0 °C. The organic phase was separated and the aqueous phase was extracted with DCM (50 mL x 3). Combined organic extracts were then washed with brine (100 mL), dried over MgSO$_4$, concentrated in vacuo and then purified by FC (30 % EtOAc/70 % hexanes then 50 % EtOAc/50 % hexanes) to give **3.25** as a white amorphous solid (0.79 g, 71 % yield). ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.75 (2H, d, J = 7.7 Hz), 7.61 (2H, d, J = 6.5 Hz), 7.27–7.22 (6H, m), 6.85 (2H, d, J = 7.5 Hz), 6.54 (1H, br. s), 6.42 (1H, br. s), 5.28 (1H, m), 4.49–3.14 (7H, m), 3.86–3.51 (5H, m). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 177.5, 162.1, 158.9, 146.9, 146.8, 144.2, 132.8, 132.3, 130.6, 130.0, 128.1, 122.9, 116.7, 75.8, 75.5, 70.5, 69.8, 58.1, 56.9, 50.1, 26.6, 20.9, -2.3, -2.4. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{33}$H$_{43}$N$_2$O$_6$Si, 591.2885; found, 591.2868.

Compound 3.26. A solution of **3.25** (0.78 g, 1.32 mmol) and pentamethyl benzene (0.22 g, 1.48 mmol, 1.10 equiv), at -78 °C, was treated dropwise with BCl$_3$ (1.33 mL, 1 M in DCM, 3.0 equiv). Upon the addition of all the BCl$_3$, the reaction mixture was allowed to warm to 0 °C and stirred for 75 min at 0 °C. The reaction was quenched at 0 °C with sat. NaHCO$_3$ (20 mL) and the biphasic mixture was stirred at 0 °C for 10 min before being diluted with EtOAc. The organic phase was separated and the aqueous phase was extracted with EtOAc (75 mL x 3). Organic extracts were then washed with brine (100 mL), dried over MgSO$_4$, evaporated, and purified by FC (40 % EtOAc/58 % hexanes/2 % AcOH then 70 % EtOAc/28 % hexanes/2 % AcOH) to give **3.26** as an amorphous white solid (0.52 g, 83 % yield). ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.73 (2H, d, J = 7.1 Hz), 7.58 (2H, m), 7.43-7.21 (4H, m), 6.91 (1H, br. s), 6.67 (1H, br. s), 5.63 (1H, br. s), 4.50-4.26 (3H, m), 4.25-4.05 (2H, m), 3.87-3.89 (2H, m), 0.91 (9H, s), 0.09 (6H, s). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 178.4, 159.2, 146.8, 146.7, 144.2, 130.6, 129.9, 128.0, 122.9, 77.1, 69.8, 64.5, 58.4, 50.0, 28.6, 20.9, -2.3, -2.5. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{25}$H$_{35}$N$_2$O$_5$Si, 471.2310; found, 471.2310.

Compound 3.27. A solution of NaClO$_2$ (80 % w/w, 0.34 g, 2.97 mmol, 2.8 equiv) in phosphate buffer (0.67 N, pH=7, 10 mL) was added dropwise to a stirred mixture of **3.26** (0.50 g, 1.06 mmol) and TEMPO (0.25 g, 1.60 mmol, 1.50 equiv) in MeCN (15 mL) at 0 °C. NaOCl (6 % bleach, 0.90 mL, 0.73 mmol, 0.7 equiv) was added dropwise over 30 min, and the reaction mixture was allowed to stir at 0 °C for 3 h then quenched with sat. Na$_2$SO$_3$ (10 mL) and allowed to warm to room temperature. The reaction mixture was acidified with 12 M HCl (pH ca. 2), and the aqueous layer was extracted with DCM (75 mL x 3). The combined organic layer was dried with MgSO$_4$ and filtered. The crude material was then purified by FC (50 % EtOAc/48 % hexanes/2 % AcOH) to give **3.27** as an amorphous white solid (0.45 g, 88 % yield). ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.77 (2H, d, J = 7.6 Hz), 7.62 (2H, m), 7.45-7.28 (4H, m), 6.79 (1H, br. s), 5.73 (1H, br. s), 4.90 (1H, m), 4.79 (1H, s) 4.51-4.35 (2H, m), 4.29-4.20 (2H, m), 0.94 (9H, s), 0.17 (6H, s). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 178.8, 174.6, 158.8, 146.8, 146.6, 144.2, 130.6, 130.0, 128.0 122.9, 76.8, 70.2, 60.7, 50.0, 28.6, 20.9, -2.1, -2.4. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{25}$H$_{33}$N$_2$O$_6$Si, 485.2102 found, 485.2097.

Compound 3.9. H$_2$SO$_4$ (27 µL, 0.50 mmol, 0. 6 equiv) followed by Ac$_2$O (0.12 mL, 1.27 mmol) were added to suspension of TrtOH (2.15 g, 8.26 mmol, 10 equiv) in AcOH (2.7 mL) at 50 °C. The mixture was stirred at 50 °C for 10 min then **3.27** (0.40 g, 0.83 mmol) was added. The resulting mixture was stirred at 50 °C until no starting material remained. It was then cooled to 0 °C, diluted with EtOAc (50 mL) and carefully quenched with sat. NaHCO$_3$ (pH ca. 3). The mixture was then diluted with H$_2$O (50 mL) and extracted with EtOAc (50 mL x 3). Organic extracts were washed with brine

(50 mL), dried over MgSO$_4$, evaporated and purified by FC (30 % EtOAc/70 % hexanes then 60 % EtOAc/38 % hexanes/2 % AcOH) to give **3.9** as an amorphous white solid (0.30 g, 60 % yield). ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.32 (1H, s), 7.81-7.70 (2H, m), 7.64-7.54 (2H, m), 7.47-7.32 (2H, m), 7.31-7.09 (17H, m), 5.80 (1H, d, *J* = 6.7 Hz), 4.72-4.58 (2H, m), 5.53-5.35 (2H, m), 4.27-4.17 (1H, m). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 170.7, 169.6, 158.5, 144.0, 143.5, 143.4, 141.3, 128.6, 128.1, 127.9, 127.3, 127.1, 125.1, 120.1, 120.0, 74.4, 70.6, 68.0, 57.0, 47.0. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{38}$H$_{33}$N$_2$O$_6$, 613.2333 found, 613.2361.
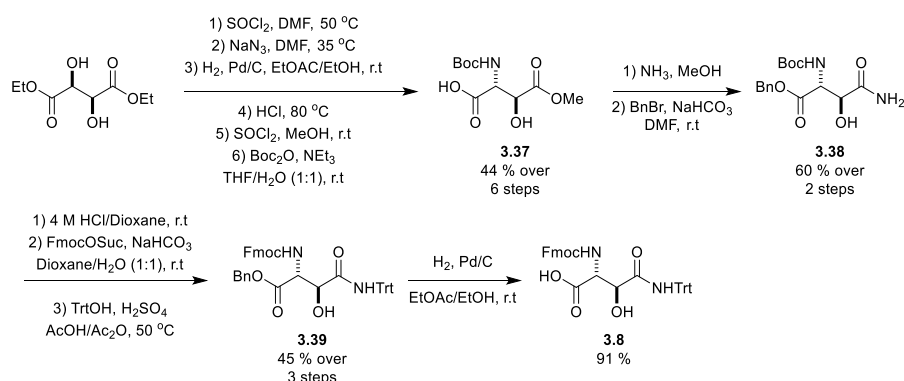
Scheme 3.13. Alternative/Scalable synthesis of Fmoc-D-*erythro*-HOAsn(Trt)-OH (**3.9**).

Compound 3.37. (−)-Diethyl D-tartrate (5.0 g, 24.25 mmol), cooled to 0 °C, was treated dropwise with SOCl$_2$ (2.6 mL, 36.37 mmol, 1.5 equiv) followed by DMF (40 µL, 0.48 mmol, 0.02 equiv). The mixture was warmed to room temperature and stirred for 100 min before being heated to 50 °C and stirred for an additional 110 min. Then, the mixture was evaporated, taken up in DMF (6.7 mL), and added to a suspension of NaN$_3$ (4.7 g, 72.30 mmol, 3.0 equiv) in DMF (7.5 mL) at 0 °C. The resulting suspension was heated to 35 °C and stirred for 19 h. It was then cooled to room temperature and aq. HCl (1.0 M)/EtOAc (1:1, 20 mL) was added. The mixture was stirred for 2 h at room temperature before being diluted with H$_2$O (200 mL) and extracted with EtOAc (150 mL x 5). The combined organic extracts were washed with brine (200 mL), dried over MgSO$_4$, and evaporated. The crude material was dissolved in EtOAc/EtOH (3:2, 500 mL) and 5 % Pd/C (1.2 g) was added before the vessel was purged with N$_2$ gas. The flask was then charged with hydrogen gas and stirred for 2.5 h at room temperature before being diluted with EtOAc and filtered through Celite. The residue was washed several times with EtOAc/EtOH and then the resulting pooled filtrates were evaporated in vacuo. The resulting material was dissolved in 6 M HCl (60 mL) and stirred at 80 °C for 21 h. The mixture was then evaporated in vacuo and azeotroped several time from toluene until all the water had been removed. The resulting material was dissolved in MeOH (70 mL) and treated with SOCl$_2$ (1.8 mL, 24.81 mmol, 1.02 equiv) at 0 °C. The mixture was left to stir at room temperature for 4 h before being evaporated. The crude material was taken up in THF/H$_2$O (1:1, 80 mL) and treated with NEt$_3$ (10.0 mL, 72 mmol, 3.0 equiv) followed by Boc$_2$O (5.82 g, 26.67 mmol, 1.1 equiv). The mixture was stirred at room temperature for 20 h before it was diluted with EtOAc (300 mL) and acidified with 1 M HCl (pH ca. 1). The organic phase was separated and the aqueous phase was extracted with EtOAc (150 ml x 3). Combined organic extracts were washed with brine (200 mL), dried over MgSO$_4$, evaporated and

purified by FC (65% hexanes/33 % EtOAc/1% AcOH then 23 % hexanes/75 % EtOAc/2 % AcOH) to give **3.37** as an off white, amorphous solid (2.81 g, 44 % yield over 6 steps). ^{1}H NMR (300 MHz, CDCl$_3$): δ 5.62 (2H, d, J = 7.4 Hz), 4.86 (2H, d, J = 7.4 Hz) 4.54 (1H, s), 3.82 (3H, s), 1.44 (9H, s). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 172.1, 155.8, 81.0, 72.1, 57.0, 53.2, 28.3.HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{10}$H$_{18}$N$_2$O$_7$, 264.1078 found, 264.1073.

Compound 3.38. Ammonia was bubbled through a solution of **3.37** (2.60 g, 9.88 mmol) in MeOH (40 mL), cooled to 0 ^{o}C, for 3 h. The reaction vessel was then sealed and the reaction was stirred at room temperature for 5 d. The reaction mixture was then evaporate in vacuo and the resulting material was taken up in DMF (51 mL) and cooled to 0 ^{o}C. NaHCO$_3$ (2.70 g, 32.14 mmol, 3.3 equiv) followed by BnBr (5.2 mL, 43.47 mmol, 4.4 equiv) were added. The mixture was then warmed to room temperature and stirred for 20 h before H$_2$O (100 mL) was added at 0 ^{o}C. The mixture was extracted with EtOAc (200 mL x 5). Combined organic extracts were washed with brine (300 mL), dried over MgSO$_4$, evaporated, and purified by FC (65 % hexanes/33 % EtOAc/2 % AcOH) to give **3.38** as a white solid (1.98 g, 60 % yield over 2 steps). ^{1}H NMR (300 MHz, CDCl$_3$): δ 7.69 (5H, m), 7.14 (1H, br. s) 6.28-6.10 (2H, m), 5.63-5.47 (2H, m), 5.08-4.92 (2H, m), 1.77 (9H, s). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 173.6, 168.1, 135.0, 128.6, 128.5, 128.4, 81.5, 73.7, 68.0, 57.4, 28.2. HRMS-(ESI+) m/z: [M + H]$^+$ calcd. for C$_{16}$H$_{23}$N$_2$O$_6$, 339.1551 found, 339.1558.

Compound 3.39. Intermediate **3.38** (1.93 g, 5.71 mmol) was treated with 4 M HCl in dioxane (50 mL) at 0 ^{o}C then warmed to room temperature and allowed to stir for 2 h. The mixture was evaporated and taken up in 1:1 dioxane/H$_2$O (1:1, 60 mL), cooled to 0 ^{o}C, and then treated with NaHCO$_3$ (2.40 g, 28.57 mmol, 5.0 equiv) followed by a solution of FmocOSuc (1.94 g, 5.71 mmol, 1.0 equiv) in dioxane (15 mL). The mixture was warmed to room temperature and the pH was adjusted to ~9 with sat. NaHCO$_3$. An additional portion of 1:1 dioxane/H$_2$O (10 mL) was added to the reaction mixture before it was left to stir at room temperature for 15 h. The mixture was the diluted with H$_2$O (100 mL) and then extracted with EtOAc (150 mL x 4). Combined organic extracts were washed with brine (150 mL), dried over MgSO$_4$ and evaporated. The resulting crude material was recrystalized from EtOH. In separate flask, to a suspension of TrtOH (14.8 g, 56.85 mmol, 10 equiv) in AcOH (14 mL) at 50 ^{o}C was added Ac$_2$O (1.3 mL, 13.75 mmol, 2.5 equiv) and H$_2$SO$_4$ (0.15 mL, 2.80 mmol, 0.5 equiv). The mixture was stirred at 50 ^{o}C for 10 min before the recrystalized material from the subsequent step was added. The reaction was stirred at 50 ^{o}C for 3.5 h before it was then cooled to 0 ^{o}C, diluted with EtOAc (50 mL) and carefully quenched with sat. NaHCO$_3$ (pH ca. 5). The mixture was then diluted with H$_2$O (50 mL) and extracted with EtOAc (50 mL x 3). The combined organic extracts were washed with brine (50 mL), dried over MgSO$_4$, evaporated and purified by FC (40 % EtOAc/59 % hexanes/1 % AcOH) to give **3.39** as an amorphous white solid (1.80 g, 45 % yield over 3 steps). ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.18 (1H, s), 7.83-7.70 (2H, m) 7.64-7.55 (2H, m), 7.49-7.14 (28H, m), 5.91 (1H, d, J = 6.0 Hz), 5.20 (2H, s), 4.75 (2H, m), 4.46 (2H, m), 4.23 (1H, t, J = 7.3 Hz). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 169.3, 167.7, 158.5, 144.4, 143.5, 143.4, 141.3, 128.6, 128.6, 128.0, 127.9, 127.2, 125.1, 120.1, 120.1, 75.0, 70.4, 68.0, 57.9, 47.0. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{45}$H$_{39}$N$_2$O$_6$, 703.2803; found, 703.2809.

Compound 3.8. Intermediate **3.39** (2.27 g, 3.23 mmol) was dissolved in EtOAc/EtOH (3:2, 20 mL) and 10 % Pd/C (0.2 g) was added before the vessel was purged with N$_2$ gas. The flask was then charged with hydrogen gas and stirred for 2.5 h at room temperature before being diluted with

EtOAc and filtered through Celite. The residue was washed several times with EtOAc/EtOH and then the resulting pooled filtrates were evaporated in vacuo. The crude material was purified by FC (40 % EtOAc/59 % hexanes/1 % AcOH then 60 % EtOAc/38 % hexanes/2 % AcOH) to give **3.8** as an amorphous white solid (1.80 g, 91 % yield). ^{1}H NMR (300 MHz, CDCl$_3$): δ 8.32 (1H, s), 7.81-7.70 (2H, m), 7.64-7.54 (2H, m), 7.47-7.32 (2H, m), 7.31-7.09 (17H, m), 5.80 (1H, d, J = 6.7 Hz), 4.72-4.58 (2H, m), 5.53-5.35 (2H, m), 4.27-4.17 (1H, m). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 170.7, 169.6, 158.5, 144.0, 143.5, 143.4, 141.3, 128.6, 128.1, 127.9, 127.3, 127.1, 125.1, 120.1, 120.0, 74.4, 70.6, 68.0, 57.0, 47.0. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{38}$H$_{33}$N$_2$O$_6$, 613.2333; found, 613.2361.

Fmoc-D-HOPhGly(TBS)-OH (3.10)

Compound 3.10. A solution of Fmoc-D-HOPhGly-OH (4.51 g, 11.6 mmol) in DMF (60 mL) at 0 ^{o}C was treated with imidazole (3.15 g, 46.3 mmol, 4.0 equiv) followed by TBSCl (4.37 g, 29.0 mmol, 2.5 equiv). The mixture was allowed to stir at room temperature for 18 h before it was diluted with Et$_2$O (400 mL) and washed with 0.1 M HCl (150 mL). The resulting aqueous phase was extracted with Et$_2$O (3x100 mL). Combined organic extracts were washed with brine (100 mL), dried (MgSO$_4$), filtered, evaporated, then purified by FC (20 % EtOAc/78 % hexanes/2 % AcOH). Residual AcOH was removed by evaporating the material from heptane several times to give **3.10** (4.84 g, 83 %) as an off white solid. ^{1}H NMR (300 MHz, CDCl$_3$): (1:1 mixture of rotamers) δ 11.8 (1H, br. s), 8.19 (0.5 H, d, J = 3.7 Hz), 7.84-7.73 (2H, m), 7.62 (0.5H, d, J = 5.0 Hz), 7.48-7.19 (7H, m), 6.90-6.80 (2H, m), 5.81 (0.5H, d, J = 6.7), 5.40 (0.5H, d, J = 6.7), 4.51-4.35 (2H, m), 4.29-4.08 (1H, m), 1.05 (9H, m), 0.25 (6H, m). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 173.7, 157.3, 155,8, 143.7, 143.3, 141.4, 130.0, 128.5, 128.3, 127.2, 125.1, 124.8, 120.6, 120.0, 67.9, 67.3, 59.8, 47.2, 46.9, 25.7, 18.2, -4.3. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_{29}$H$_{34}$NO$_5$Si, 504.2201; found, 504.2201.

(2S,3R)-3-propyloxirane-2-carboxylic acid (3.11)

Compound 3.14.[113] To a flame dried flash with 4Å powder sieves (500 mg, preactivated by flame drying under vacuum) and anhydrous DCM (40 mL) at room temperature was added Ti(O^{i}Pr)$_4$ (1.6 mL, 5.4 mmol, 0.54 equiv.) followed by D-(-)-DET (1.4 g, 6.4 mmol, 0.64 equiv.). The mixture was then cooled to – 78 ^{o}C. Then, *trans*-hexen-1-ol (1.0 g, 10.0 mmol) was added and this mixture was stirred for 10 min at – 78 ^{o}C before cumene hydroperoxide (technical grade 80 % w/w, 3.7 mL, 20 mmol, 2.0 equiv.) was added dropwise. The resulting mixture was then carefully transferred to a -20 ^{o}C freezer and incubated for 17 h. The mixture was then dumped into a flask containing NaOH dissolved in brine (1M, 50 mL) cooled to 0 ^{o}C. This mixture was stirred for 1 h at 0 ^{o}C in order to hydrolyze and remove all the D-(-)-DET from the organic phase. The biphasic mixture was then filtered through celite. The aqueous phase of the resulting filtrate was separated and extracted with DCM (40 mL x 3). The combined organic extracts were washed with brine (75 mL), dried (MgSO$_4$), filtered, evaporated in vacuo, and then purified by FC (20-30 % EtOAc/hexanes) to give **3.14** (0.70 g, 60 %) as a clear oil. ^{1}H NMR (300 MHz, CDCl$_3$): δ 3.90 (1H, d, J = 12.5 Hz), 3.61 (1H, d, J = 12.1 Hz), 2.99-2.88 (2H, m), 2.01 (1H, br. s), 1.62-1.41 (4H, m), 0.95 (3H, t, J = 7.3 Hz). ^{13}C{^{1}H} NMR (75 MHz, CDCl$_3$): δ 61.7, 58.4, 55.8, 33.6, 19.2, 13.9. HRMS (ESI+) m/z: [M + H]$^+$ calcd. for C$_6$H$_{13}$O$_2$, 117.0910; found, 117.0918.

Compound 3.11.[114] A solution of TEMPO (0.33 g, 2.1 mmol, 15 mol %) and **3.14** (1.6 g, 13.8 mmol, 1.0 equiv) in MeCN (80 mL) and phosphate buffer (pH 7.4, 64 mL) at 35 °C was treated with a solution of $NaClO_2$ (80 % w/w, 5.0 g, 44 mmol, 3.1 equiv) in H_2O (38 mL). Then a solution of NaOCl (6.15 % w/w, 1.0 mL, 1.1 mmol, 8 mol %) in H_2O (22 mL) was added dropwise over 15 min. The resulting mixture was heated to 100 °C and stirred for 3 h. Next, the mixture was cooled to room temperature and sat. NaS_2O_3 (50 mL) was added. The resulting solution was stirred for 10 min at room temperature before it was acidified (pH ca. 2) with 1 M HCl. The resulting mixure was extracted with EtOAc (100 mL x 3). Combined organic extracts were washed with brine (100 mL), dried ($MgSO_4$), filtered, evaporated in vacuo and then purified by FC (30 % EtOAc/70 % hexanes then 40 % EtOAc/59 % hexanes/1 % AcOH0 to give **3.11** (1.1 g, 61 %) as a clear oil. ^1H NMR (300 MHz, $CDCl_3$): δ 10.19 (1H, br. s), 3.42-3.16 (2H, m), 1.71-1.45 (4H, m), 0.97 (3H, t, J = 7.1 Hz). $^{13}C\{^1H\}$ NMR (75 MHz, $CDCl_3$): δ 174.9, 58.9, 52.5, 33.4, 19.0, 13.7. HRMS (ESI+) m/z: $[M + H]^+$ calcd. for $C_6H_{11}O_3$, 131.0703; found, 131.0711.

General procedures for the Fmoc SPPS synthesis of the CDAs

Resin loading

The SPPS synthesis was performed manually. 2'-Cl-TrtCl polystyrene resin (theoretical substitution = 1.5 mmol/g, 33.3 mg, 0.05 mmol, 1 equiv in the synthesis of CDA3a/CDA4a and 66.6 mg, 0.1 mmol, 1 equiv in the synthesis of CDA4b/CDAKyn) was preactivated in dry DCM (20 mL) with $SOCl_2$ (3.6 equiv) and pyridine (7.2 equiv) under reflux for 2 h. The resin was then transferred to a disposable peptide cartridge and rinsed with dry DCM, followed by loading with Fmoc-Asp-Gly-OAll (4.0 equiv) and DIPEA (8.0 equiv.) in dry DCM (2.0 mL) (2 x 2 h). The resin was capped with 17:2:1 DCM/MeOH/DIPEA (3 × 10 min), and the loading efficiency was determined to be 0.69 mmol/g in the synthesis of CDA3a/CDA4a and 0.47 mmol/g in the synthesis of CDA4b/CDAKyn.

Fmoc deprotections

All deprotections up to peptide **3.30** were accomplished with 10 % 4-MP/DMF (2.0 mL) (1 x 10 min, 1 x 5 min). Deprotections from peptide **3.31** onwards were done using 2-MP/DMF (2.0 mL, 2 x 5 min) except for peptides **3.32** and **3.33** which were deprotected using 20 % 2-MP/DMF (2.0 mL, 3 x 10 min). After each Fmoc deprotection, the resin was washed with DMF (3 x 1 .0 mL).

Procedure for the coupling of Fmoc-D-HOPhGly(TBS)-OH (3.10)

Fmoc-D-HOPhGly(TBS)-OH (**3.10**) (3.0 equiv), COMU (3.0 equiv) and 2,6-lutidine (4.0 equiv) in DMF (2.0 mL) was added to the peptide. The reaction was gently agitated for 1 h. The cartridge was drained, and this process was repeated. The resin was washed with DMF (5 x 1.0 mL).

Procedure for the coupling of Fmoc-D-*erythro*-HOAsn(Trt)-OH (3.9) and Fmoc-L-*threo*-MeGlu(^{t}Bu)-OH.

A solution of Fmoc amino acid (3.0 equiv) in DMF (2.0 mL) was treated with DIC (3.0 equiv) and HOBt (3.0 equiv). The mixture was incubated for 5 min before it was added to the peptide. The mixture was then gently agitated for 4 h. The cartridge was drained and the resin was washed with DMF (5 x 1.0 mL).

Procedure for the coupling of all other Fmoc amino acids and (2S,3R)-3-propyloxirane-2-carboxylic acid (3.11).

A solution of Fmoc amino acid or epoxy acid (4.0 equiv) in DMF (2.0 mL) was treated with DIC (4.0 equiv) and HOBt (4.0 equiv). The mixture was incubated for 5 min before it was added to the peptide. The mixture was then gently agitated for 4 h. The cartridge was drained and the resin was washed with DMF (1.0 mL x 5).

Procedure for esterification of peptide 14 with Fmoc-β-HOTrp(Boc)(TBS)-OH (2.22).

To a solution of amino acid **2.22** (10 equiv) in dry DCM (2.5 mL) was added DIC (10 equiv). The mixture was incubated for 45 min to allow formation of the symmetrical anhydride. The mixture was added to peptide **3.30** followed by DMAP (0.5 equiv). The rection was gently agitated for 24 h before the cartridge was drained and the resin was washed with DCM (3 x 1 .0 mL) then DMF (5 x 1.0 mL).

Procedure for esterification of peptide 3.30 with Fmoc-Trp(Boc)-OH or Fmoc-Kyn(Boc)-OH.

To a solution of amino acid (10 equiv) in dry DCM (2.5 mL) was added DIC (10 equiv). The mixture was incubated for 45 min to allow formation of the symmetrical anhydride. The mixture was added to peptide **3.30** followed by DMAP (0.1 equiv). The rection was gently agitated for 24 h before the cartridge was drained and the resin was washed with DCM (3 x 1 .0 mL) then DMF (5 x 1.0 mL).

Procedure for deallylation, on resin cyclization and global deprotection

The allyl group was removed by treatment with $Pd(PPh_3)_4$ (0.2 equiv) and DMBA (10 equiv) in 3:1 DCM/DMF (v/v) using a N_2 gas inlet for agitation. Unreacted palladium was quenched by rinsing the resin with 1% (m/v) sodium diethyldithiocarbamate trihydrate in DMF (5 x 3 min). The peptide was rinsed with DMF (3 x 1.0 mL) and then it was cyclized by two 1.5 h treatments of PyAOP (5 equiv), HOAt (5 equiv), and 2,4,6-collidine (10 equiv) in DMF (2 mL). The peptide was treated with ice cold 95:5 TFA/H_2O (2.0 mL) and reacted at room temperature for 75 min. The filtrate was collected and the resin was rinsed with 95:5 $TFA:H_2O$ (2 x 1.0 mL). Pooled cleavage cocktail was concentrated by the N_2 stream, and the peptide was precipitated by the addition of pre-chilled diethyl ether (-78 °C). The suspensions were centrifuged, and the pellets were resuspended in 1:1 phosphate buffer/MeCN and purified by preparative RP-HPLC:

CDA3a: A gradient of 82 % H_2O (0.1 % TFA) /18 % MeCN to 59 % H_2O (0.1 % TFA) /41 % MeCN over 90 min was employed. Fractions containing the desired product were pooled and concentrated under vacuum. The residue was resuspended in water, frozen, and lyophilized affording **CDA3a** (2.0 mg, 6.0 % based on resin loading), which was determined to be >95 % pure by analytical RP-HPLC (Appendix B). HRMS (ESI+) m/z: $[M + H]^+$ calcd for $C_{66}H_{77}N_{14}O_{26}$, 1481.5128; found 1481.5112.

CDA4a: A gradient of 82 % H_2O (0.1 % TFA)/18 % MeCN to 59 % H_2O (0.1 % TFA)/41 % MeCN (+0.1 % TFA) over 50 min was employed. Fractions containing the desired product were pooled and concentrated under vacuum. The residue was resuspended in water, frozen, and lyophilized affording **CDA4a** (2.9 mg, 8.5 % based on resin loading), which was determined to be >95 % pure

by analytical RP-HPLC (Appendix B). HRMS (ESI+) m/z: [M + H]$^+$ calcd for $C_{67}H_{79}N_{14}O_{26}$, 1495.5284; found 1494.5286.

CDA4b: A gradient of 82 % H_2O (0.1 % TFA)/18 % MeCN to 59 % H_2O (0.1 % TFA)/41 % MeCN (+0.1 % TFA) over 50 min was employed. Fractions containing the desired product were pooled and concentrated under vacuum. The residue was resuspended in water, frozen, and lyophilized affording **CDA4b** (3.9 mg, 8.3 % based on resin loading), which was determined to be >95 % pure by analytical RP-HPLC (Appendix B). HRMS (ESI+) m/z: [M + H]$^+$ calcd for $C_{67}H_{81}N_{14}O_{26}$, 1497.5441; found 1497.5432.

CDAKyn/CDAKyn epimer: A gradient of 82 % H_2O (0.1 % TFA)/18 % MeCN to 61 % H_2O (0.1 % TFA)/39 % MeCN (+0.1 % TFA) over 70 min was employed. Fractions containing the desired product were pooled and concentrated under vacuum. The residue was resuspended in water, frozen, and lyophilized affording **CDAKyn** (3.0 mg, 6.4 % based on resin loading) and **CDAKyn epimer** (1.1 mg, 2.3 %), which were determined to be >95 % pure by analytical RP-HPLC (Appendix B). HRMS (ESI+) m/z: [M + H]$^+$ calcd for $C_{66}H_{81}N_{14}O_{27}$, 1501.5390; found 1501.5388 for **CDAKyn** and HRMS (ESI+) m/z: [M + H]$^+$ calcd for $C_{66}H_{81}N_{14}O_{27}$, 1501.5390; found 1501.5398 for **CDAKyn epimer** .

Chapter 4

General Conclusions and Future work

In conclusion, we have developed a new method to easily access peptides containing the unusual amino acid Z-dehydrotryptophan using Fmoc- β-HOTrp(Boc)(TBS)-OH (**2.22**) as a key building block. This approach to Z-dehydrotryptophan-bearing peptides avoids the pitfalls of previous methods and greatly enhances the ease of preparing such peptides. We have demonstrated in **chapter 2** that this building block can be easily made in gram quantities, and can be incorporated into resin-bound peptides under typical Fmoc-SPPS conditions, and converted into Z-dehydrotryptophan upon acid-promoted global deprotection/resin cleavage as demonstrated by the synthesis of tunicylin B and sclerotide A. In **chapter 3** we demonstrated that this methodology can be used to make the complex Z-dehydrotryptophan-containing peptides CDA3a and CDA4a. We have found that CDA3a and CDA4a show moderate antibacterial activity at Ca^{+2} concentrations of 16 mM, and that the Z-dehydrotryptophan residue can be used as a fluorescent probe in membrane binding studies. Our membrane binding studies suggest that a possible first step in the MoA of CDA4a, and likely all the CDAs, is binding to the PG in the Gram-positive bacterial membranes. In **chapter 3**, we also used our new methodology to prepare CDA4b and CDAKyn. We show that CDA4b is more active than CDA4a or CDA3b, and CDAKyn has identical activity to CDA4a.

The findings in this thesis lay the groundwork for many future studies. Firstly, our methodology could be applied to other targets that have yet to be prepared by chemical

synthesis. For example, it could be used to prepare the extremely complex antibiotic telomycin and, as such, this should be pursued. Secondly, biological studies on the CDAs should continue so that the precise MoA of the CDAs can be elucidated. CDAKyn should prove to be extremely useful analog for these MoA studies as Kyn is more fluorescent than dehydrotryptophan. Lastly, CDA analogs should made in order to determine which amino acids are amenable to substitution. This information should prove to be useful for designing CDA analogs with improved activity and for MoA studies. It would be extremely interesting if the POAsn-containing CDAs could be made as the biological function of this amino acid within the CDAs is not yet understood.

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Appendences

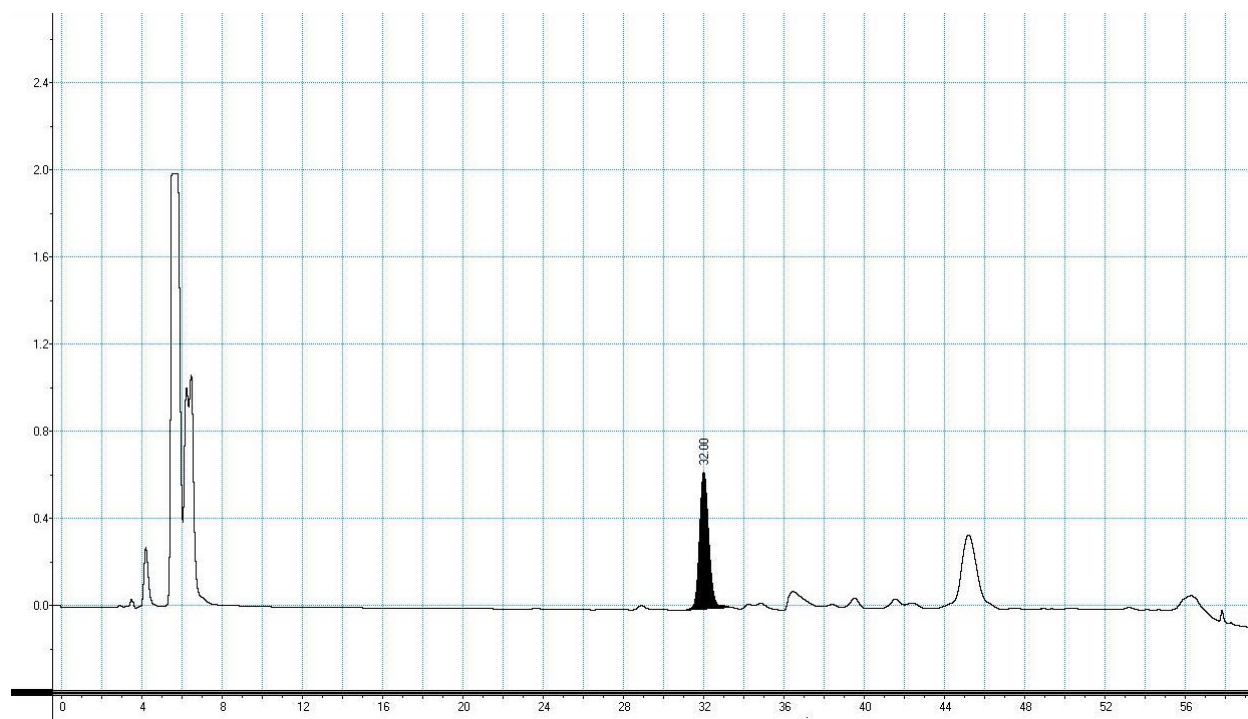# Spectral Data of New Compounds

Appendix A

—Chapter 2—

Figure A.1. HPLC chromatogram of crude Tunicyclin B after cleavage. 90-82 H_2O/MeCN+0.1 % TFA) in 10 min then 82-50 (H_2O/MeCN+0.1 % TFA) in 40 (λ=220 nm)
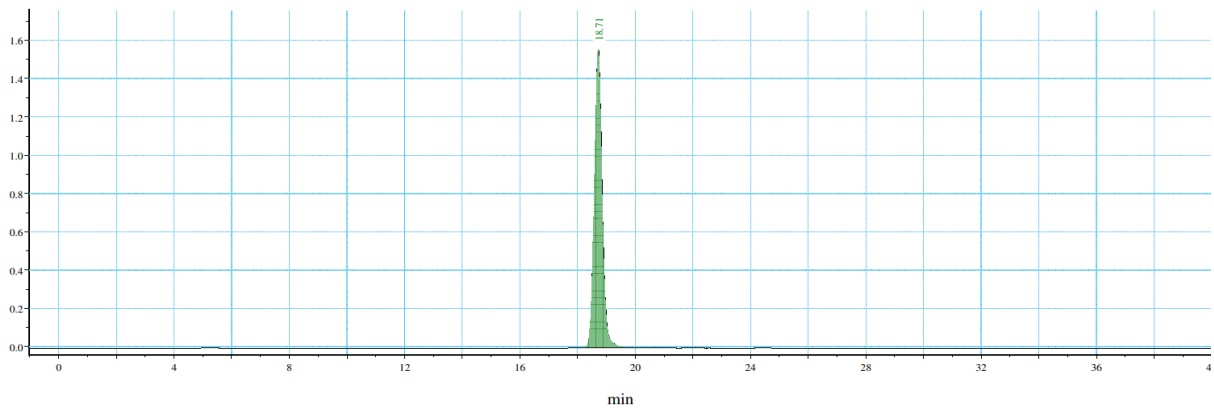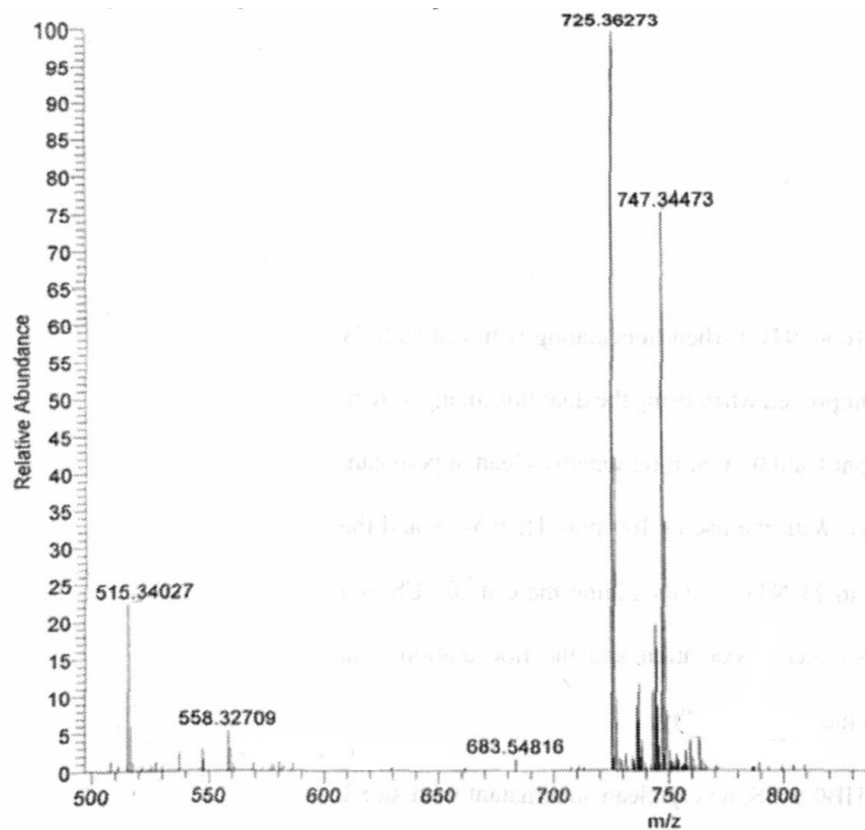
Figure A.2. HPLC chromatogram of pure synthetic Tunicyclin B. Gradient: 90-10 (H_2O/MeCN+0.1 % TFA) in 40 min (λ=220 nm)
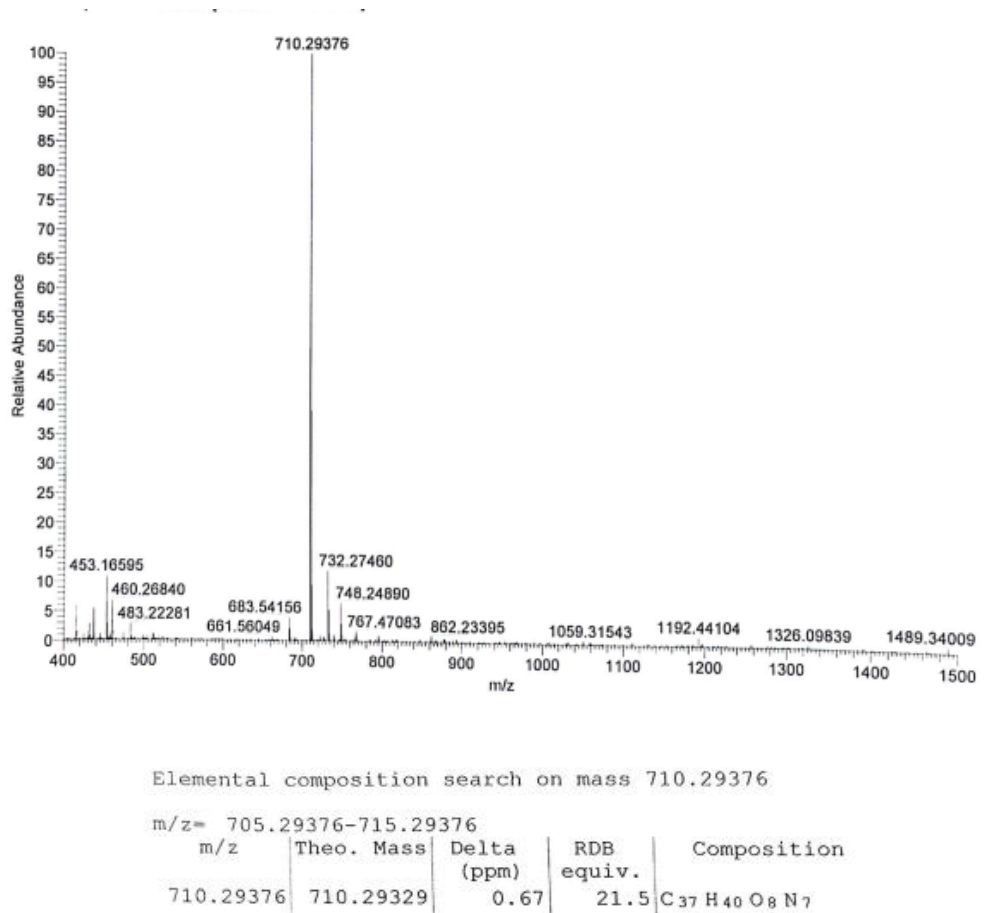


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Elemental composition search on mass 725.36273

m/z= 720.36273-730.36273
     m/z      Theo. Mass  Delta    RDB       Composition
                          (ppm)    equiv.
  725.36273   725.36170   1.42     15.5  C 35 H 49 O 9 N 8
```

Figure A.3. ESI+HRMS of pure Tunicyclin B. [M+H]$^+$ at m/z=725.36273 and [M+Na]$^+$ at m/z=747.34473.
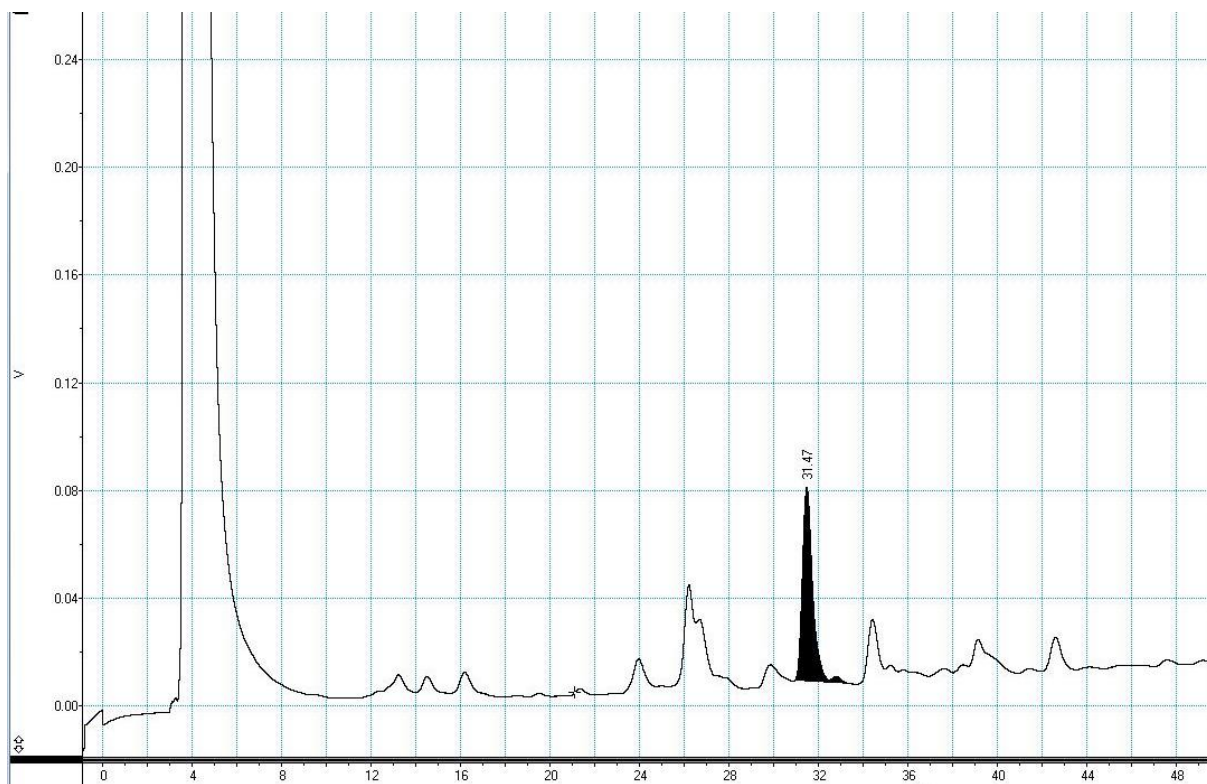
Figure A.4. HPLC chromatogram of crude Sclerotide A after cleavage. 82-59 H_2O/MeCN+0.1 % TFA) in 50 min (λ=220 nm)
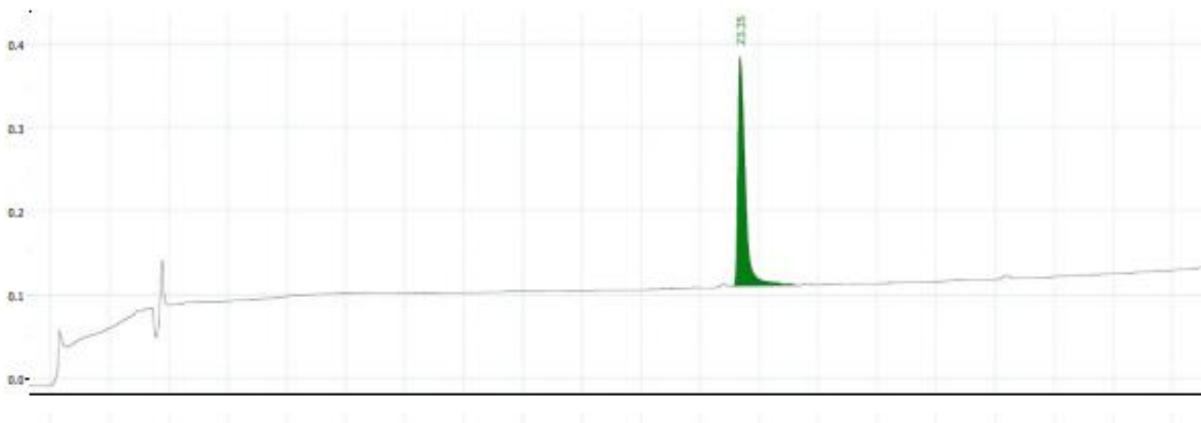
Figure A.5. HPLC chromatogram of purified synthetic Sclerotide A. Gradient: 90-10 (H_2O/MeCN+0.1 % TFA) in 40 min (λ=220 nm)

Elemental composition search on mass 710.29376

m/z= 705.29376-715.29376

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
710.29376	710.29329	0.67	21.5	$C_{37} H_{40} O_8 N_7$

Figure A.6. ESI+HRMS of pure Sclerotide A. [M+H]$^+$ at m/z=710.29376, [M+Na]$^+$ at m/z=732.27460 and [M+K]$^+$ at m/z=748.24890 .
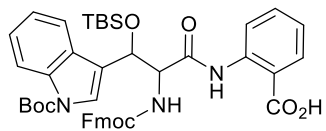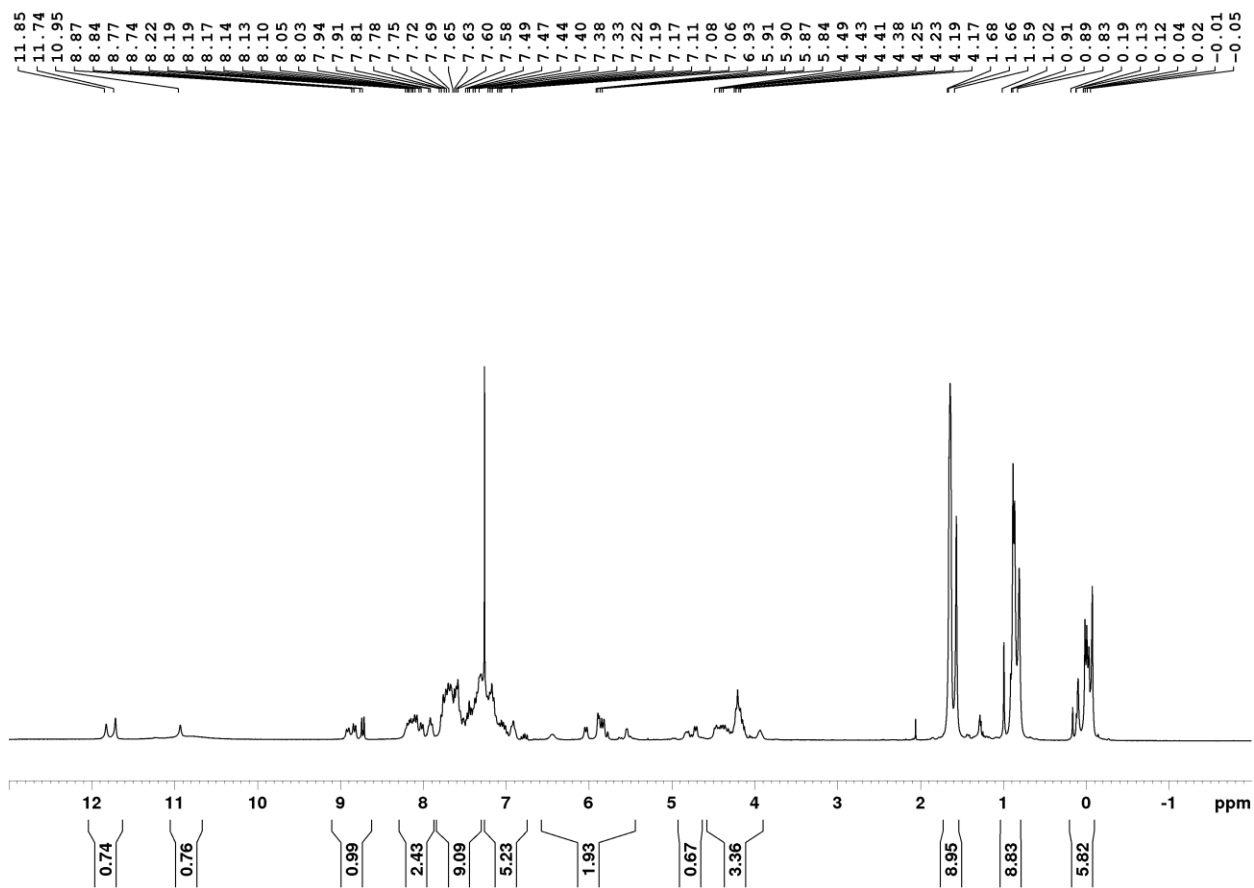
Figure A.7. ^{1}H-NMR spectrum of **2.33** (300 MHz, CDCl$_3$)
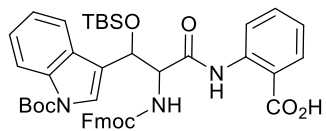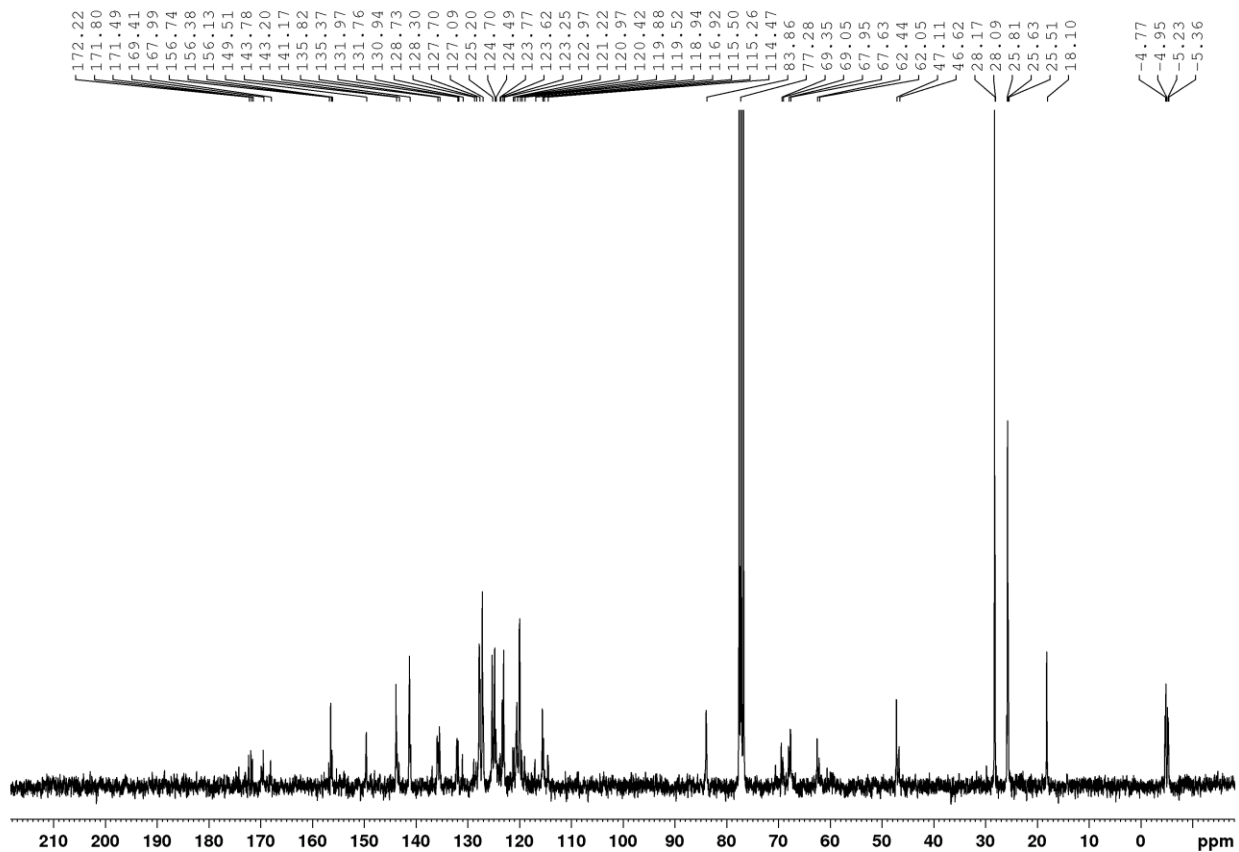
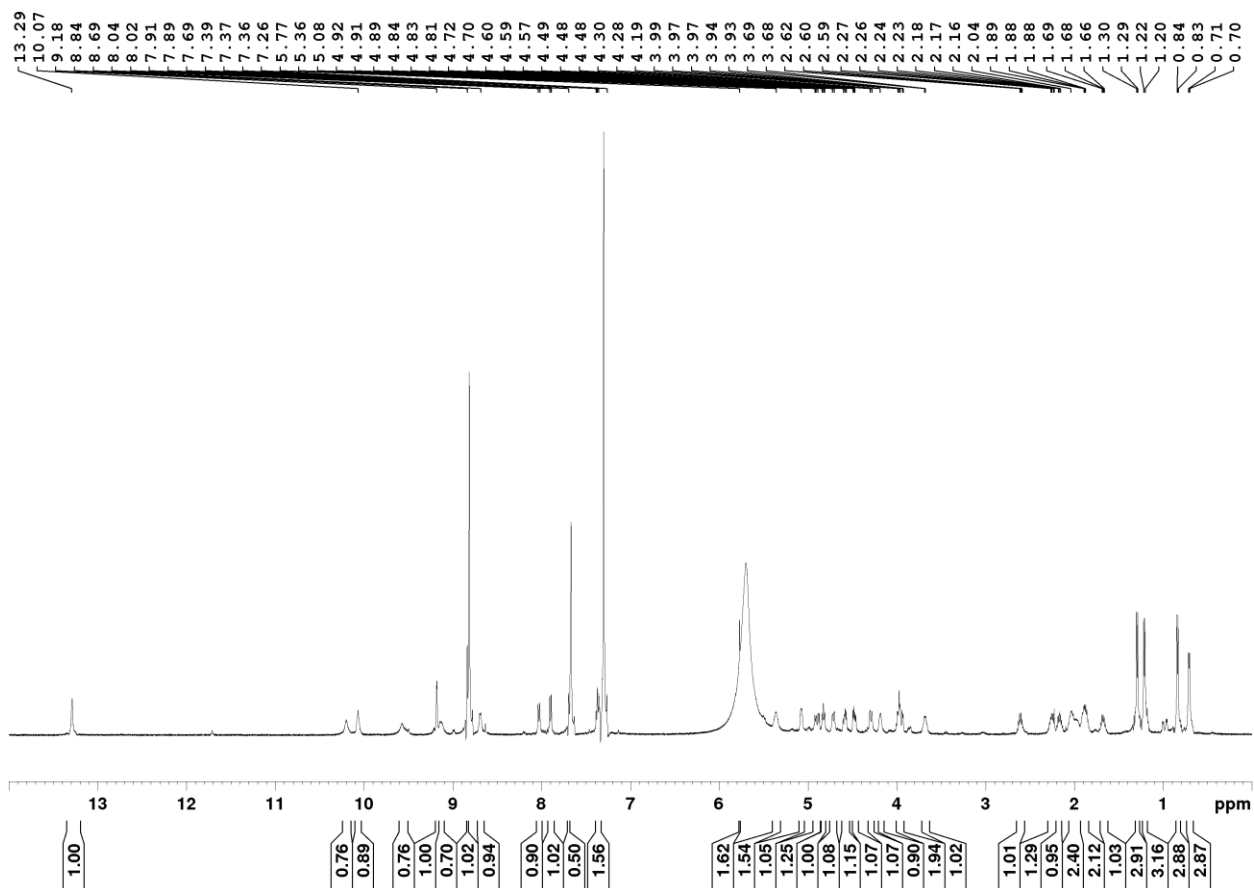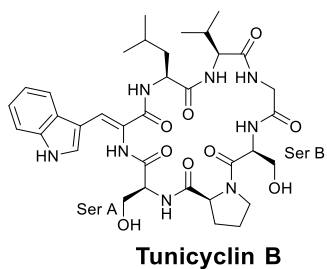Figure A.8. ^{13}C-NMR spectrum of **2.33** (75 MHz, CDCl$_3$)

Tunicyclin B

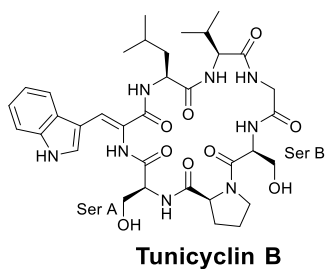Figure A.9. ¹H-NMR spectrum of Tunicyclin B (500 MHz, pyridine-d_5) at 300 K

Tunicyclin B

Figure A.10. ^{1}H-COSY spectrum of Tunicyclin B (500 MHz, pyridine-d_5) at 300 K
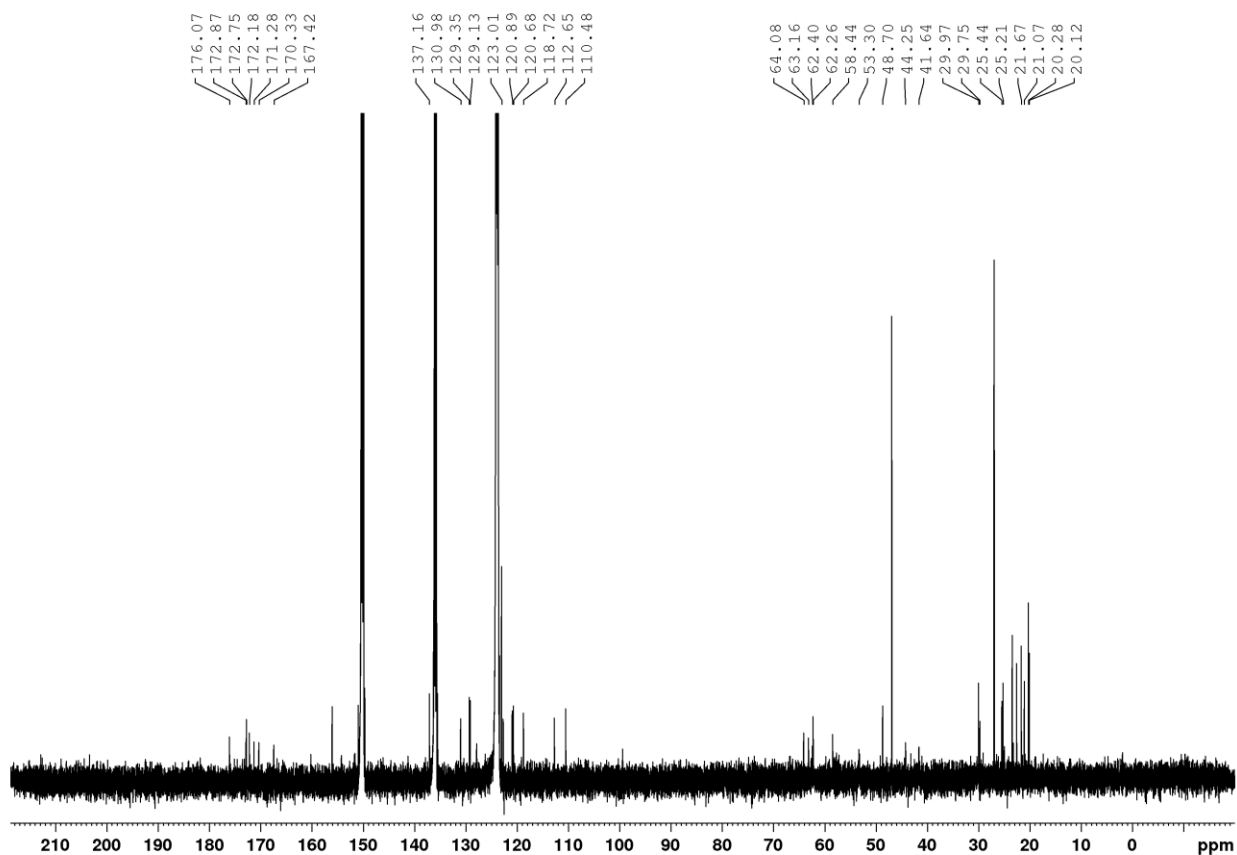
Tunicyclin B

Figure A.11 ^{13}C spectrum of Tunicyclin B. (125 MHz, pyridine-d_5) at 300 K

Tunicyclin B

Figure A.12. ^{1}H-^{13}C HSQC spectrum of tunicyclin B (500 MHz, pyridine-d_5) at 300 K.

Table A.1. Chemical Shift Assignments for Tunicyclin B
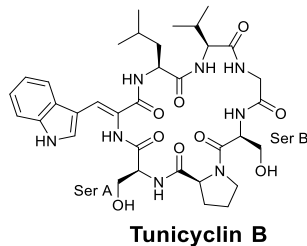
Tunicyclin B

	Natural Tunicyclin B [73]			Synthetic Tunicyclin B		
residue	δH (multiplicity, *J* value(s))	δC	residue	δH (multiplicity, *J* value(s))	δC	
Pro			*Pro*			
CO		172.5	CO		172.9	
α	4.82 (dd, 8.0, 6.0)	62.0	α	4.82 (dd, 7.1)	63.2	
βa	2.22 (m)	29.4	βa	2.27 (m)	29.8	
βb	2.16 (m)		βb	2.19 (m)		
γa	1.83 (m)	25.3	γa	1.86 (m)	26.1	
γb	1.63 (m)		γb	1.68 (m)		
δa	3.95 (m)	48.5	δa	3.98 (m)	48.7	
δb	3.62 (m)		δb	3.68 (m)		
Ser a			**Ser a**			
NH	8.68 (s)		NH	8.68 (br. s)		
CO		171.1	CO		171.3	
α	5.09 (s)	58.2	α	5.10 (br. s)	58.4	
βa	4.70 (d, 10.0)	63.0	βa	4.72 (d, 9.9)	63.2	
βb	4.30 (d, 10.0)		βb	4.20 (d, 9.9)		
ΔZ-Trp			**ΔZ-Trp**			
NH			NH			
CO		167.1	CO		167.4	
α		122.7	α		123.1	
β	8.82 (s)	127.7	β	8.85 (s)	129.1	
1′ NH	13.25 (s)		1′ NH	13.29(s)		
2′ CH	9.15 (s)	130.6	2′ CH	9.18 (s)	131.0	
3′ C		110.4	3′ C		110.5	
3a′ C		129.2	3a′ C		129.3	
4′ CH	7.91 (d, 8.0)	118.6	4′ CH	7.91 (d, 8,0)	118.7	*
5′ CH	7.30 (t, 8.0)	120.8	5′ CH	7.28	120.7	*
6′ CH	7.36 (t, 8.0)	122.6	6′ CH	7.37 (t, 8.0)	120.9	*
7′ CH	7.67 (d, 8.0)	112.5	7′ CH	7.68	112.7	*
7a′ CH		137.2	7a′ CH		137.2	
Leu			**Leu**			
NH	8.04 (d, 9.0)		NH	8.03 (d, 8.7)		
CO		176.0	CO		176.1	

α	5.34 (m)	52.9	α	5.36 (m)	53.3	
βa	1.98 (m)	41.5	βa	1.98 (m)	41.5	
βb	1.88 (m)		βb	1.89 (m)		
γ	2.02 (m)	25.0	γ	2.04 (m)	25.2	
δa	0.83 (3H, d, 6.0)	23.2	δa	0.84 (d, 6.0)	24.0	
δb	0.69 (3H, d, 6.0)	21.5	δb	0.71 (d, 6.0)	22.0	
Val			**Val**			
CO		172.5	CO		172.8	
NH	10.15 (s)		NH	10.21 (s)		
α	4.20 (s)	62.1	α	4.19 (br. s)	62.4	
β	2.60 (m)	29.8	β	2.62 (m)	30.7	
γa	1.27 (3H, d, 7.0)	20.1	γa	1.30 (d, 7.0)	20.3	
γb	1.18 (3H, d, 7.0)	19.9	γb	1.22 (d, 7.0)	20.1	
Gly			**Gly**			
NH	10.11 (s)		NH	10.08 (br. s)		
CO		170.1	CO		170.3	
α a	4.89 (dd, 17.0, 7.0)	44.0	α a	4.90 (dd, 6.8, 17.0)	44.3	
α b	3.95 (dd, 17.0, 7.0)		α b	3.95 (m)		
Ser b			**Ser b**			
NH	9.10 (s)		NH	9.14 (s)		
CO		171.9	CO		172.1	
α	5.66 (d, 6.0)	53.2	α	5.68	53.3	*
βa	4.56 (dd, 11.0, 6.0)	64.0	βa	4.59 (dd, 11.0, 6.0)	64.1	
βb	4.49 (dd, 11.0, 6.0)		βb	4.48 (dd, 11.0, 6.0)		

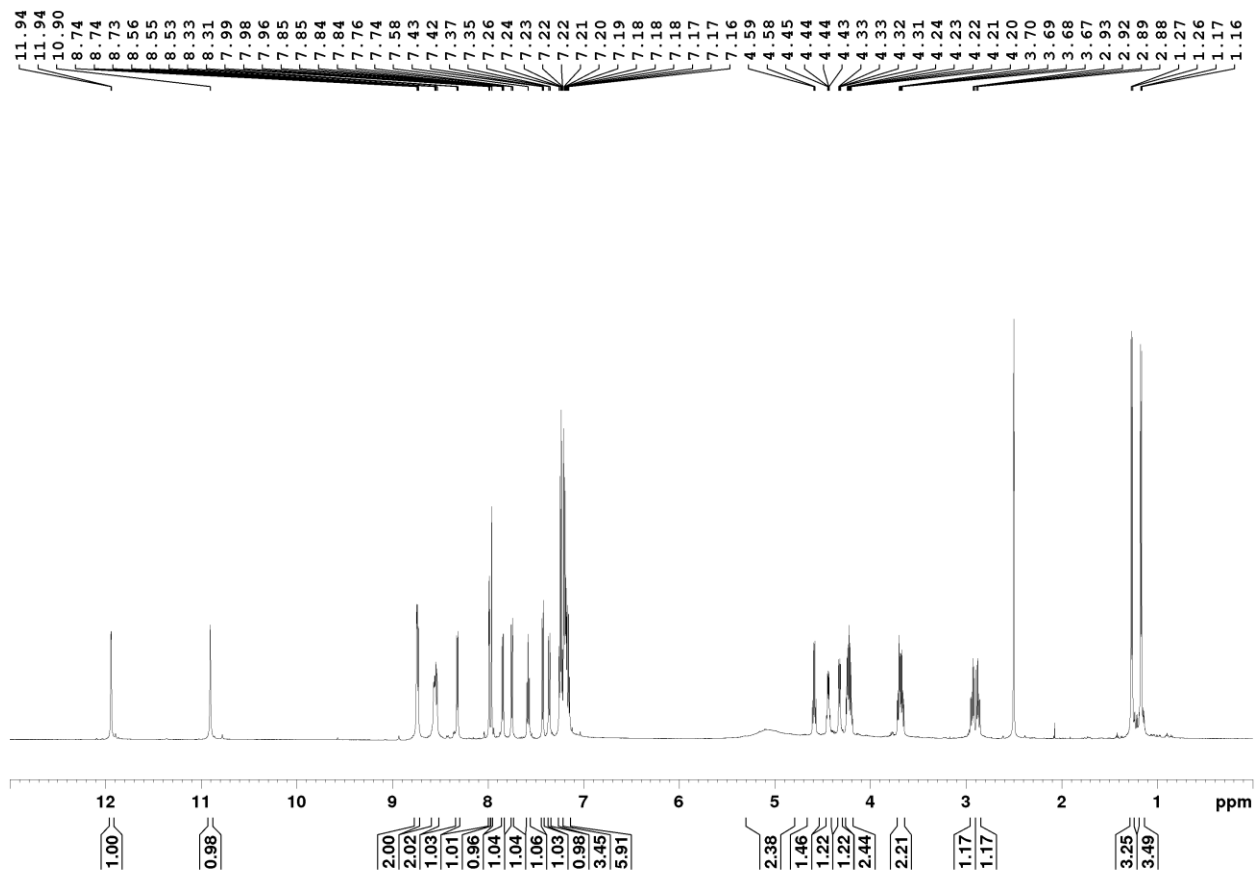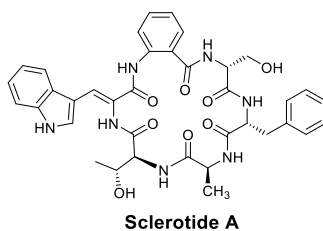* Indicates overlap with 1 or more other peaks/d5-pyridine peaks

Sclerotide A

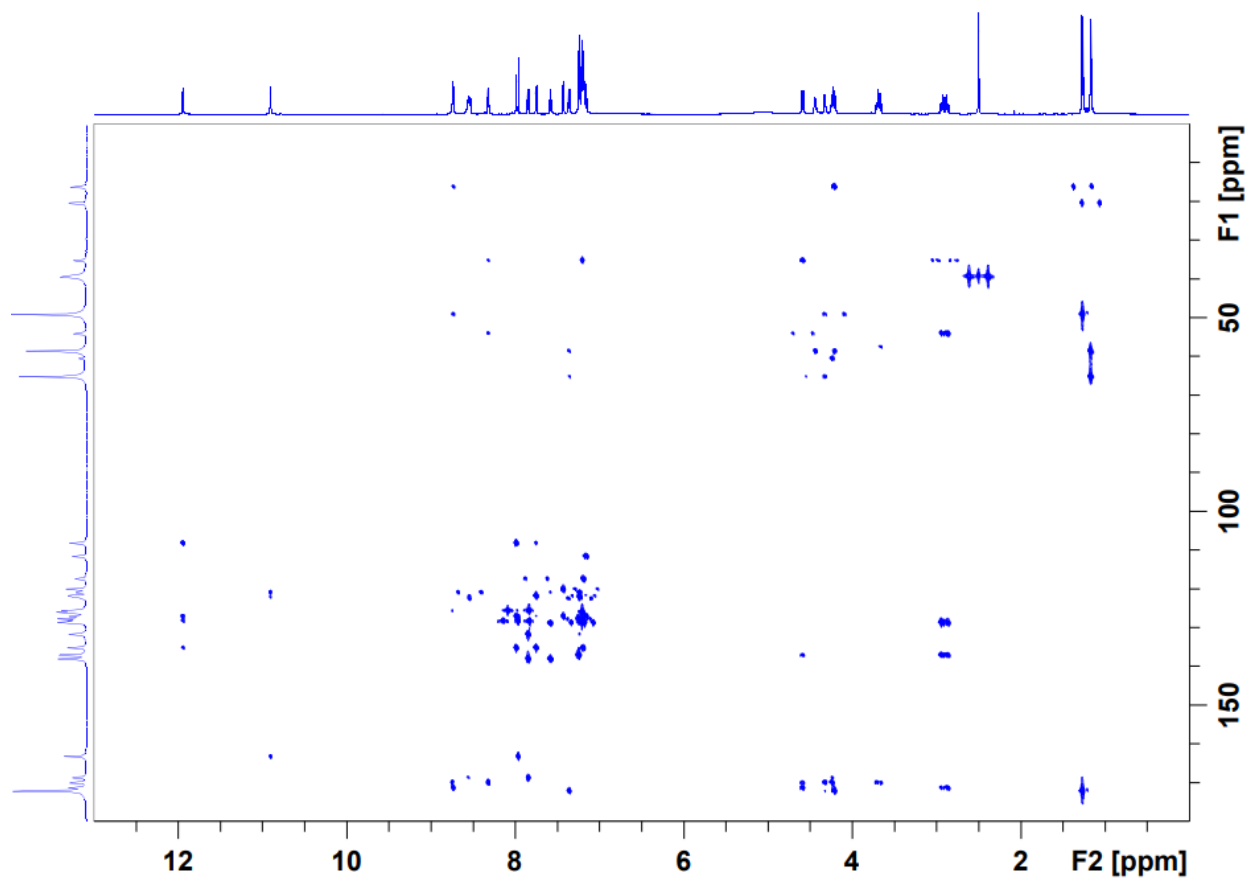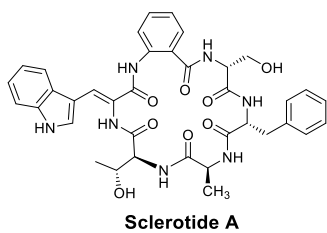Figure A.13. ^{1}H-spectrum of Sclerotide A (600 MHz, DMSO-d_6) at 298 K.

Figure A.14. ^{1}H-COSY spectrum of Sclerotide A (600 MHz, DMSO-d_6) at 298 K.

Figure A.15. ^{1}H-NOESY spectrum of Sclerotide A (600 MHz, DMSO-d_6) at 298 K

Figure A.16. ^{1}H-^{13}C-HSQC spectrum of Sclerotide A (600 MHz, DMSO-d_6) at 298 K

Sclerotide A

Figure A.17. ^{1}H-^{13}C-HMBC spectrum of Sclerotide A (600 MHz, DMSO-d_6) at 298 K

135

Table A.2. Chemical Shift Assignments for Sclerotide A
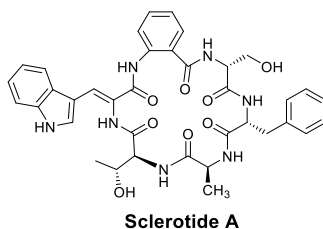
Sclerotide A

	Natural Sclerotide A [33]				Synthetic Sclerotide A	
residue	δH (multiplicity, *J* value(s))	δC		residue	δH (multiplicity, *J* value(s))	δC
Thr				**Thr**		
CO		170.3		CO		170.2
NH	7.37 (d, 8.2)			NH	7.36 (d, 8.2)	
α	4.43, m	65.6		α	4.44, m	65.4
β	4.31 (dd, 3.2, 8.7)	59.0		β	4.32 (dd, 3.2, 8.8)	58.8
γ	1.16 (d, 6.4)	25.3		γ	1.17 (d, 6.4)	20.7
OH	5.14 (br. s)			OH	5.08 (br. s)	
Ala				**Ala**		
NH	8.77 (d, 6.4)			NH	8.74 (d, 6.4)	
CO		172.6		CO		172.3
α	4.20 (dq, 6.3, 7.3)	49.6		α	4.21 (m)*	49.4
β	1.26 (d, 7.3)	16.6		β	1.27 (d, 7.3)	16.5
D-Phe				**D-Phe**		
NH	8.37 (d, 7.3)			NH	8.32 (d, 7.0)	
CO		171.7		CO		171.3
α	4.57 ("q" like, 7.6)	54.5		α	4.59 ("q" like, 7.5)	54.2
βa	2.93 (dd, 7.3, 13.7)	35.6		βa	2.93 (dd, 7.5, 13.8)	35.5
βb	2.87 (dd, 8.2, 13.7)			βb	2.87 (dd, 8.2, 13.8)	
1' C		137.5		1' C		137.2
2' CH	7.20 (d, 7.8)	129.1		2' CH	7.20 (m) *	129.0
3' CH	7.24 (t, 7.3)	128.1		3' CH	7.24 (t, 7.3)	127.9
4' CH	7.17 (t, 7.8)	126.4		4' CH	7.16 (m) *	126.3
5' CH	7.24 (t, 7.3)	128.1		5' CH	7.24 (t, 7.3)	127.9
6' CH	7.20 (d, 7.3)	129.1		6' CH	7.20 (m) *	129.0
D-Ser				**D-Ser**		
NH	8.62 (br. s)			NH	8.56 (d, 5.8) *	
CO		170.4		CO		170.0
α	4.23 ("q" like, 7.0)	58.0		α	4.24 (m) *	57.8
β	3.67 (m)	60.9		β	3.67 (m)	60.7
OH	5.15 (br. s)			OH	5.08 (br. s)	
Ant				**Ant**		
CO		169.0		CO		168.7
NH	10.92 (s)			NH	10.90 (s)	

1' C		122.0	1' C		122.3
2' CH	7.85 (dd, 1.4, 7.8)	129.2	2' CH	7.84 (dd, 1.1, 8.0)	128.9
3' CH	7.20 (dt, 1.1, 7.8)	122.9	3' CH	7.20 (m) *	122.6
4' CH	7.58 (ddd, 1.4, 7.8, 8.2)	132.1	4' CH	7.58 (ddd, 1.2, 7.7, 8.5)	131.9
5' CH	8.53 (d, 8.2)	121.1	5' CH	8.54 (d, 8.3) *	121.0
6' C		138.4	6' C		137.9
ΔZ-Trp			**ΔZ-Trp**		
NH	8.76 (s)		NH	8.75 (s)	
CO		163.6	CO		163.3
α		122.5	α		122.5
β	7.96 (s)	126.0	β	7.96 (s)	125.8
1' NH	11.96 (s)		1' NH	11.94 (d, 2.1)	
2' CH	7.98 (d, 2.3)	128.7	2' CH	7.99 (d, 2.7)	128.5
3' C		108.6	3' C		108.2
3a' C		127.4	3a' C		127.1
4' CH	7.75 (d, 7.8)	117.7	4' CH	7.75 (d, 7.7)	117.6
5' CH	7.16 (dt, 1.3, 7.8)	120.4	5' CH	7.16 (dt, 1.0, 8.1) *	120.3
6' CH	7.18 (dt, 1.4, 7.8)	122.2	6' CH	7.18 (m) *	121.9
7' CH	7.42 (d, 7.8)	112.0	7' CH	7.43 (d, 7.8)	111.9
7a' C		135.6	7a' C		135.2

* Indicates overlap with 1 or more other peaks/*d*6-DMSO peaks.

Appendix B
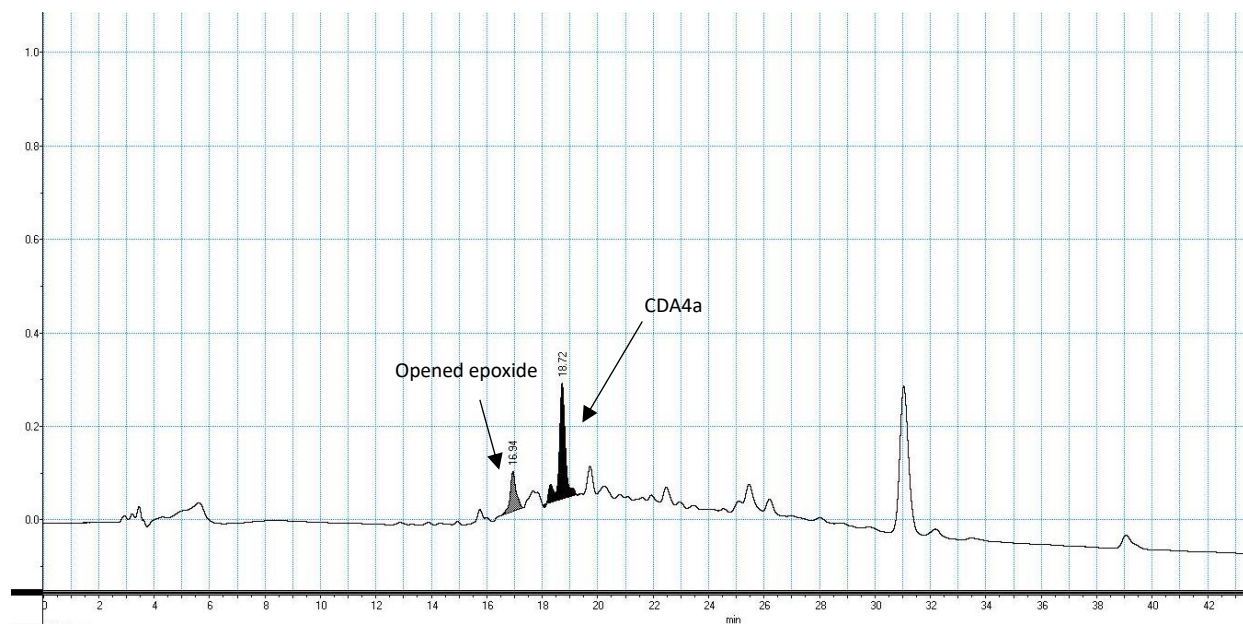
Spectral Data of New Compounds

—Chapter 3—
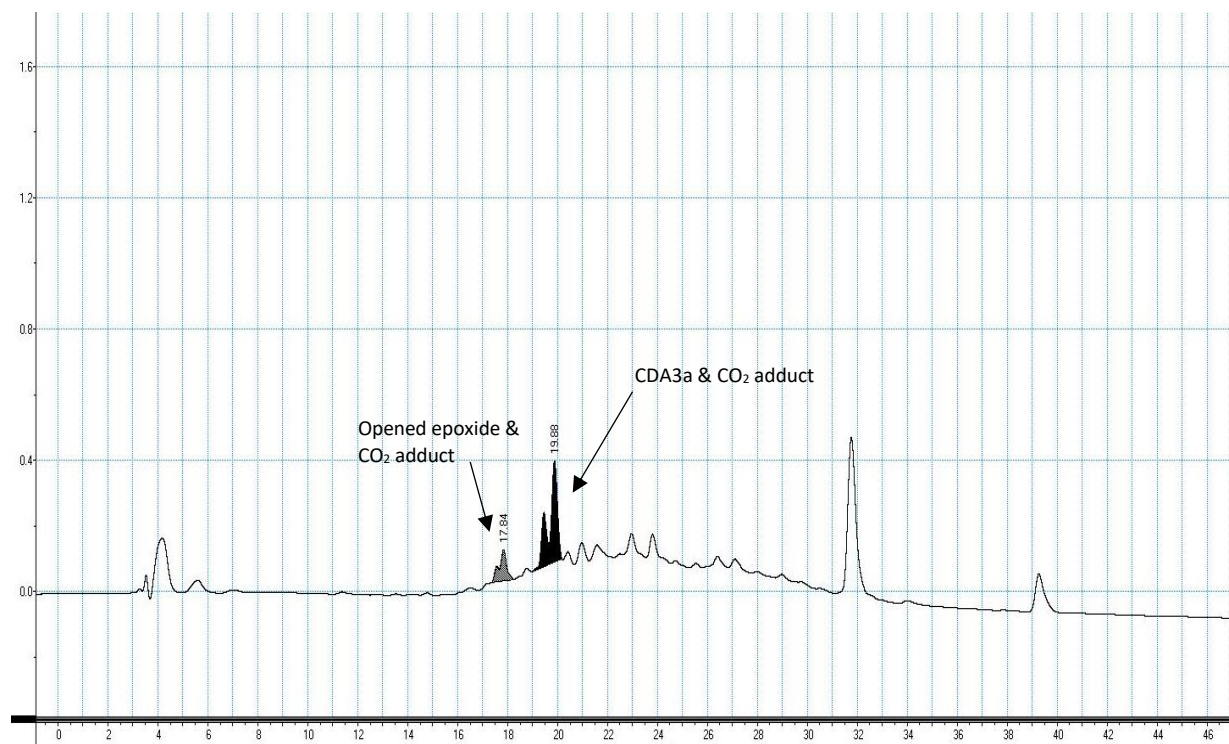
Figure B.1. HPLC chromatogram of crude CDA3a after cleavage. 90-10 (H_2O/MeCN+0.1 % TFA) in 40 min (λ=220 nm)
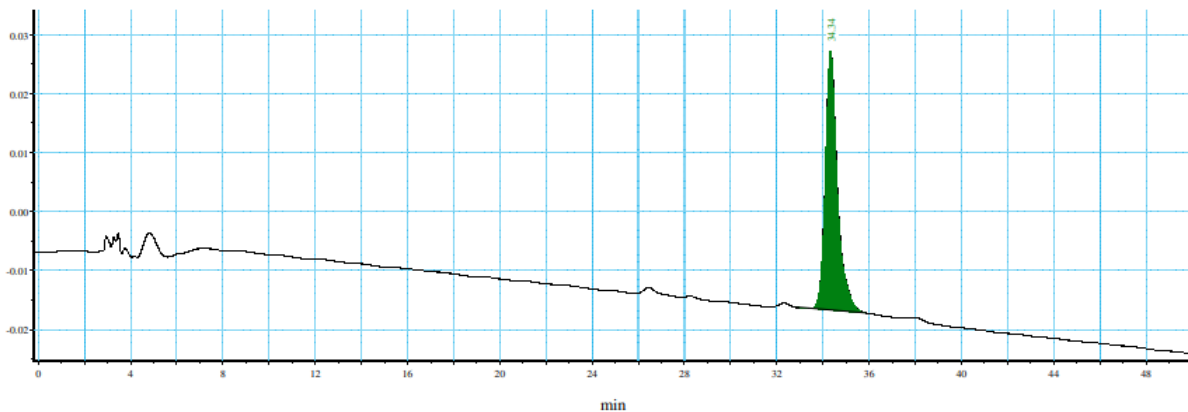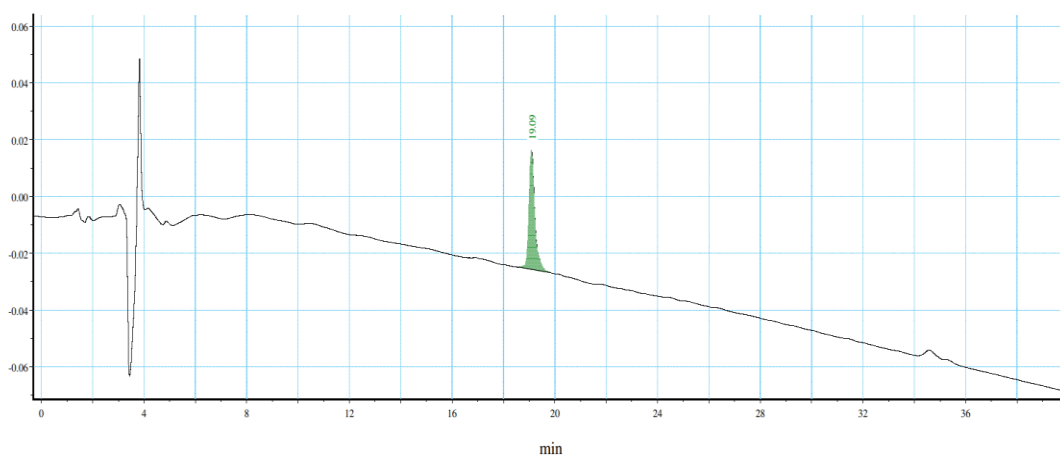
Figure B.2. HPLC chromatogram of purified synthetic CDA3a. Gradient: 82-59 (H_2O/MeCN+0.1 % TFA) in 50 min (λ=220 nm)
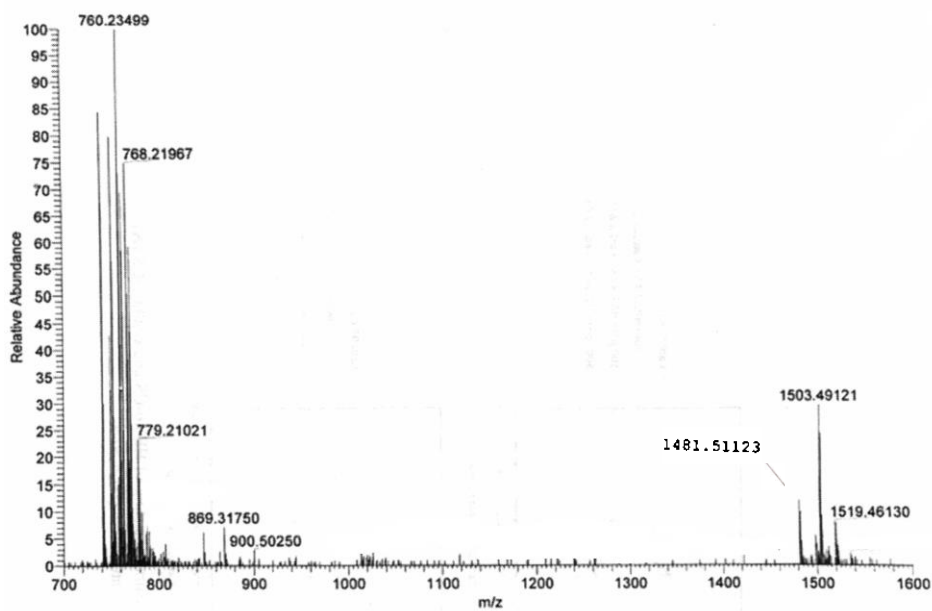

```
Elemental composition search on mass 1481.51123

m/z= 1476.51123-1486.51123
    m/z    | Theo. Mass | Delta  | RDB   | Composition
           |            | (ppm)  | equiv.|
1481.51123 | 1481.51279 | -1.06  | 35.5  | C 66 H 77 O 26 N 14
```

Figure B.3. ESI+HRMS of pure CDA3a. [M+H]+ at m/z=1481.51123, [M+Na]+ at m/z=1503.49121, [M+K]+ at mz=1519.46130. [M+2H]+2 at m/z=741.26204. [M+H+Na]+2 at m/z=752.25100. [M+H+K]+2 at m/z=760.23499. [M+NH4+K]+2 at m/z=768.21967. [M+2K]+2 at m/z=779.21021.

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Figure B.4. HPLC chromatogram of crude CDA4a after cleavage. 90-10 (H_2O/MeCN+0.1 % TFA) in 40 min (λ=220 nm)
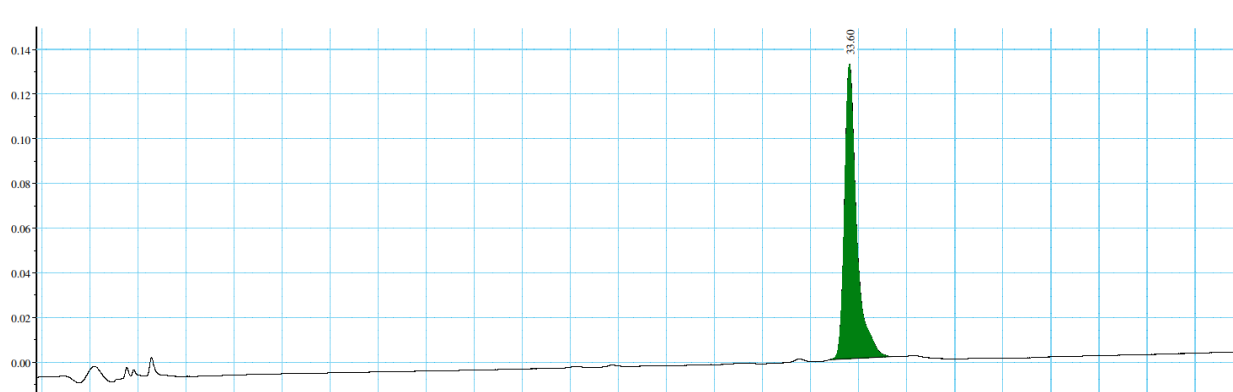
Figure B.6. HPLC chromatogram of purified synthetic CDA4a. Gradient: 90-10 (H_2O/MeCN+0.1 % TFA) in 40 min (λ=220 nm)
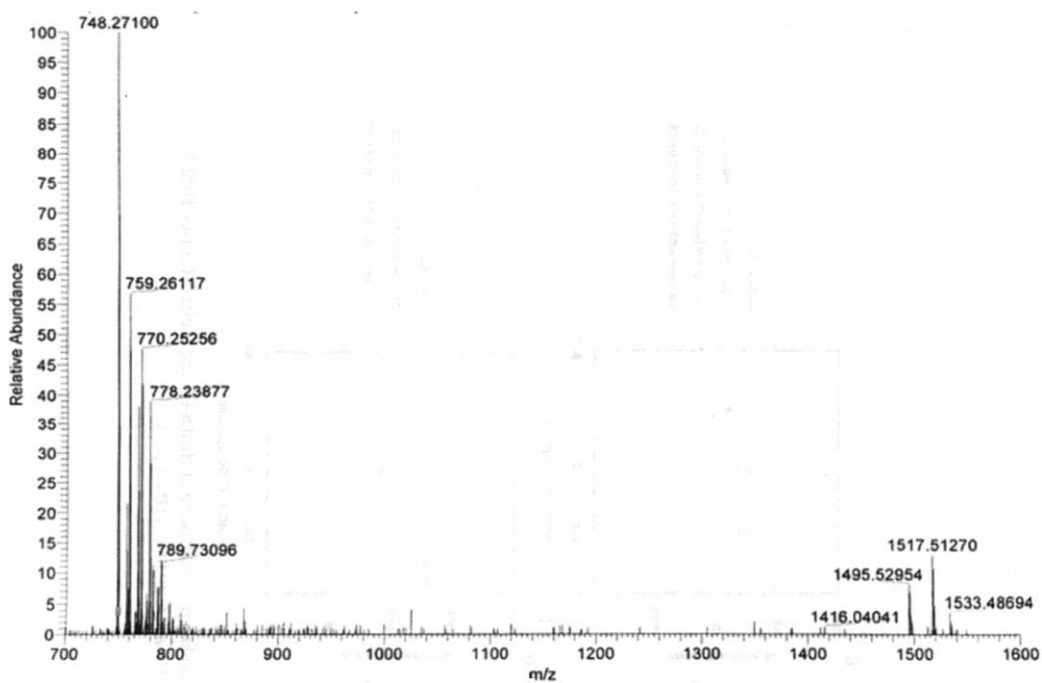
Elemental composition search on mass 1495.52954

m/z= 1490.52954-1500.52954

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
1495.52954	1495.52844	0.73	35.5	$C_{67} H_{79} O_{26} N_{14}$

Figure B.7. ESI+HRMS of pure CDA4a. [M+H]+ at m/z=1495.52954, [M+Na]+ at m/z=1517.51270, [M+K]+ at mz=1533.48694. [M+2H]+2 at m/z=748.27100. [M+H+Na]+2 at m/z=759.26117. [M+2Na]+2 at m/z=770.25256. [M+Na+K]+2 at m/z=778.23877.
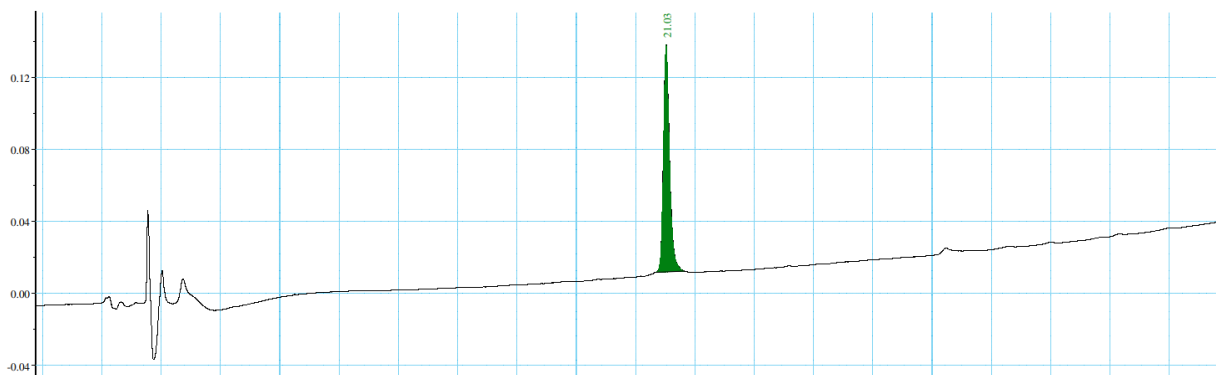
Figure B.8. HPLC chromatogram of purified synthetic CDA4b. Gradient: 90-10 (H_2O/MeCN+0.1 % TFA) in 40 min (λ=220 nm)
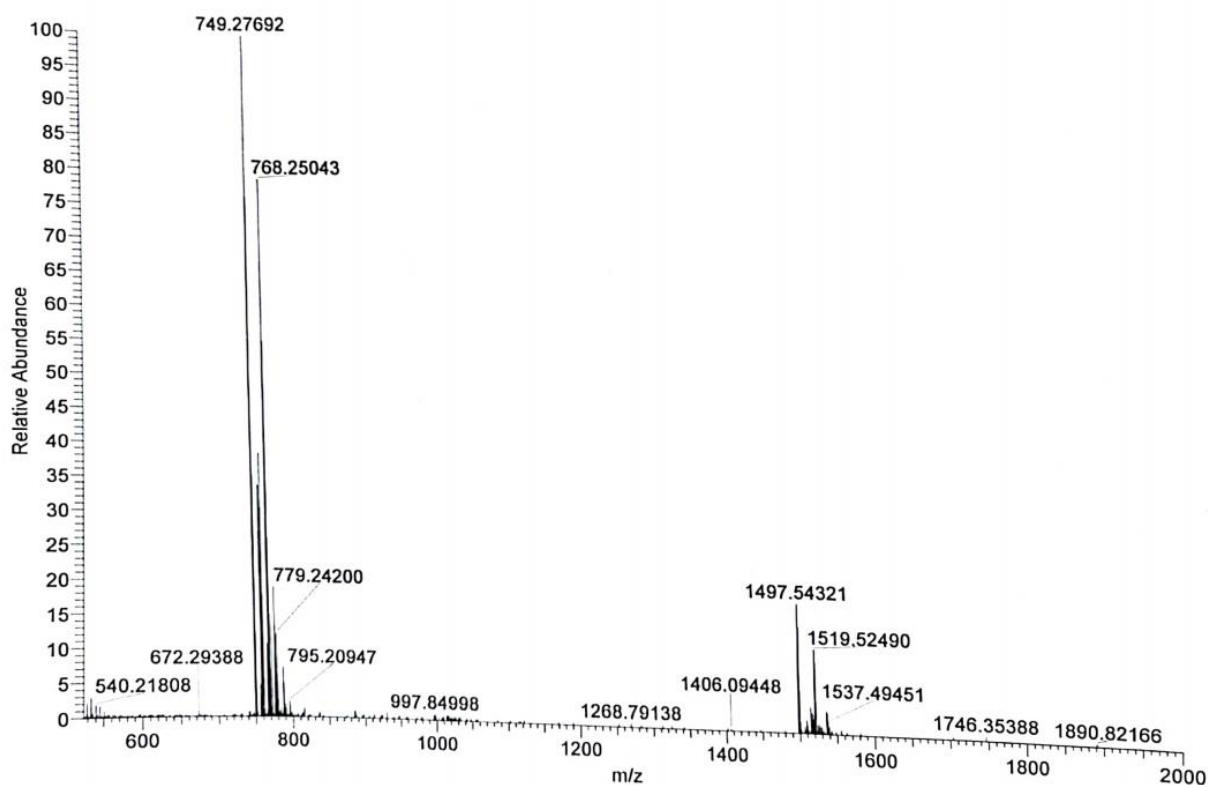
m/z= 1492.54321-1502.54321

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
1497.54321	1497.54409	-0.59	34.5	$C_{67} H_{81} O_{26} N_{14}$

Figure B.9. ESI+HRMS of pure CDA4b. [M+H]$^+$ at m/z=1497.54321, [M+Na]$^+$ at m/z=1519.63590, [M+Na+H_2O]$^+$ at m/z=1537.49451, [M+2H]$^{+2}$ at m/z=749.27692. [M+H+NH$_4$]$^{+2}$ at m/z=768.25043. [M+2Na]$^{+2}$ at m/z=770.25256. [M+Na+K]$^{+2}$ at m/z=779.779.24200.
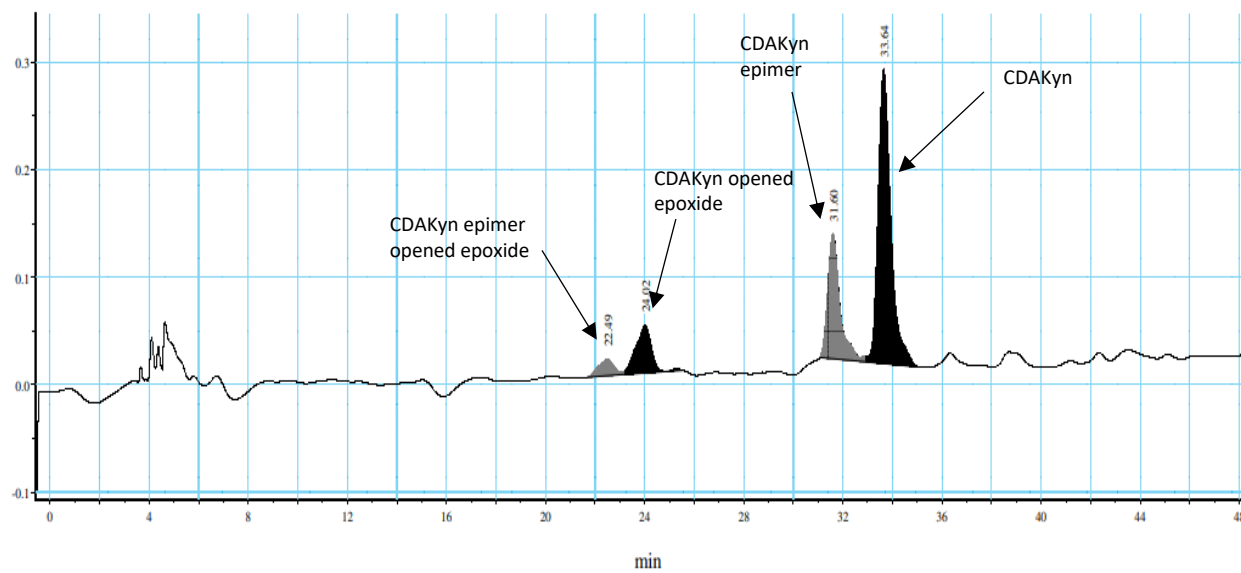
Scheme B.10. HPLC chromatogram of crude CDAKyn after cleavage. 82-59 (H_2O/MeCN+0.1 % TFA) in 50 min (λ=220 nm)
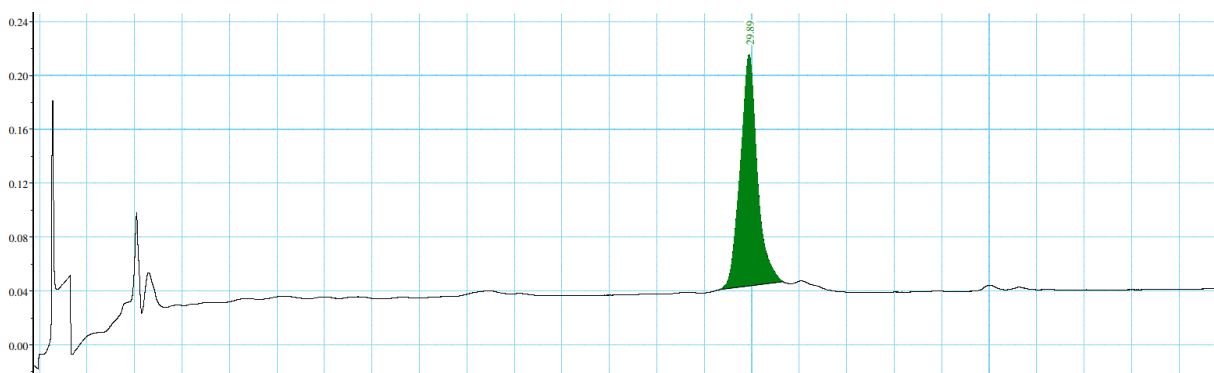
Figure B.11. HPLC chromatogram of purified synthetic CDAKyn. Gradient: 82-59 (H_2O/MeCN+0.1 % TFA) in 50 min (λ=220 nm)
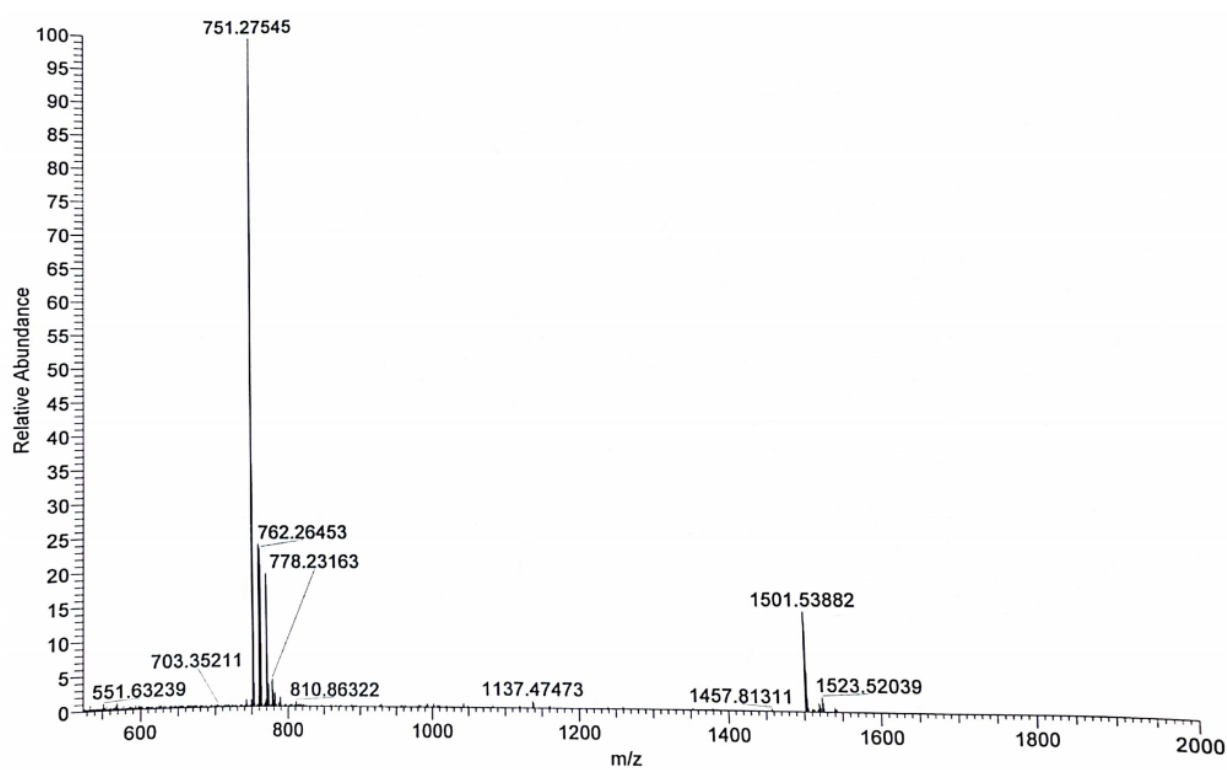
Elemental composition search on mass 1501.53882

m/z= 1496.53882–1506.53882

m/z	Theo. Mass	Delta (ppm)	RDB equiv.	Composition
1501.53882	1501.53901	-0.13	33.5	$C_{66} H_{81} O_{27} N_{14}$

Figure B.12. ESI+HRMS of pure CDAKyn. [M+H]$^+$ at m/z=1501.53882, [M+Na]$^+$ at m/z=1523.52039, [M+2H]$^{+2}$ at m/z=751.27545. [M+H+Na]$^{+2}$ at m/z=762.26453. [M+2Na]$^{+2}$ at m/z=770.25256. [M+NH$_4$+K]$^{+2}$ at m/z=778.23163.
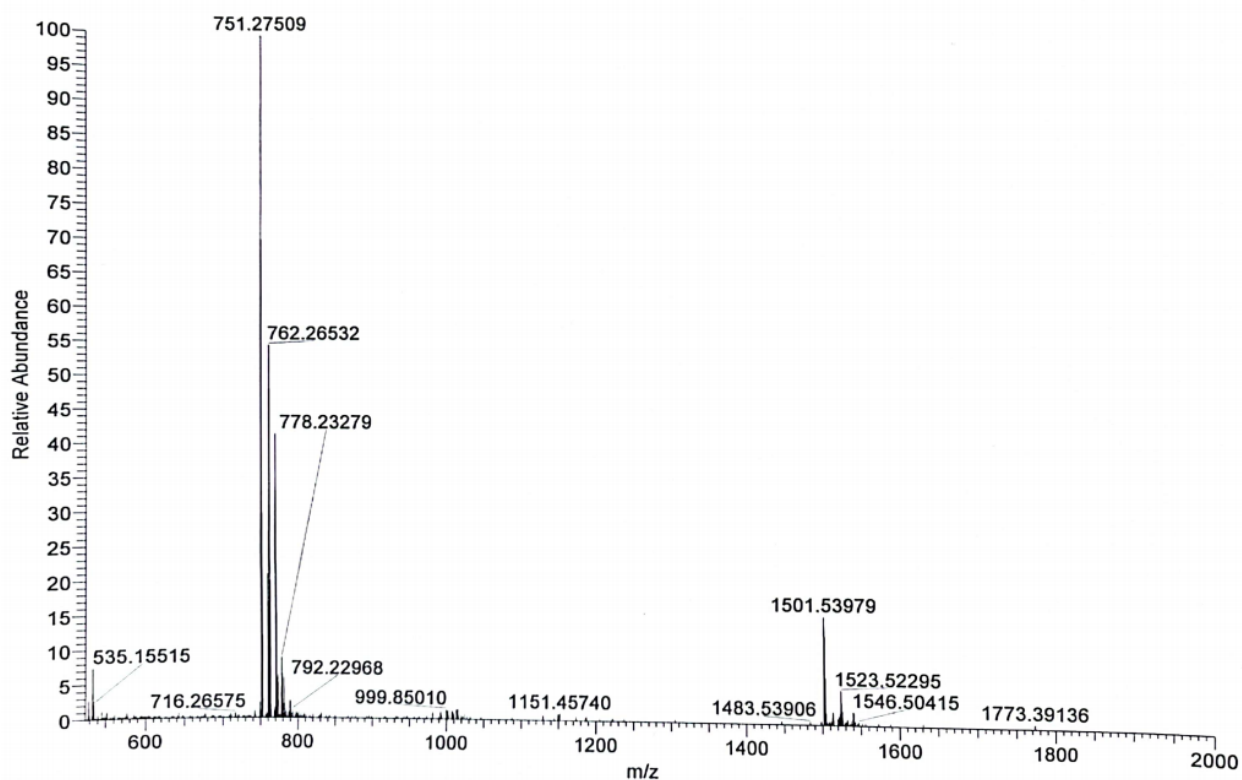
145

Figure B.13. HPLC chromatogram of purified synthetic CDAKyn epimer. Gradient: 82-59 (H_2O/MeCN+0.1 % TFA) in 50 min (λ=220 nm)


```
m/z= 1496.53979-1506.53979
       m/z     | Theo. Mass | Delta  | RDB    | Composition
               |            | (mmu)  | equiv. |
   1501.53979 | 1501.53901 |  0.78  |  33.5  | C₆₆ H₈₁ O₂₇ N₁₄
```

Figure B.14. ESI+HRMS of pure CDAKyn epimer. $[M+H]^+$ at m/z=1501.53979, $[M+Na]^+$ at m/z=1523.52295, $[M+2H]^{+2}$ at m/z=751.27509. $[M+H+Na]^{+2}$ at m/z=762.26532. $[M+NH_4+K]^{+2}$ at m/z=778.23279.
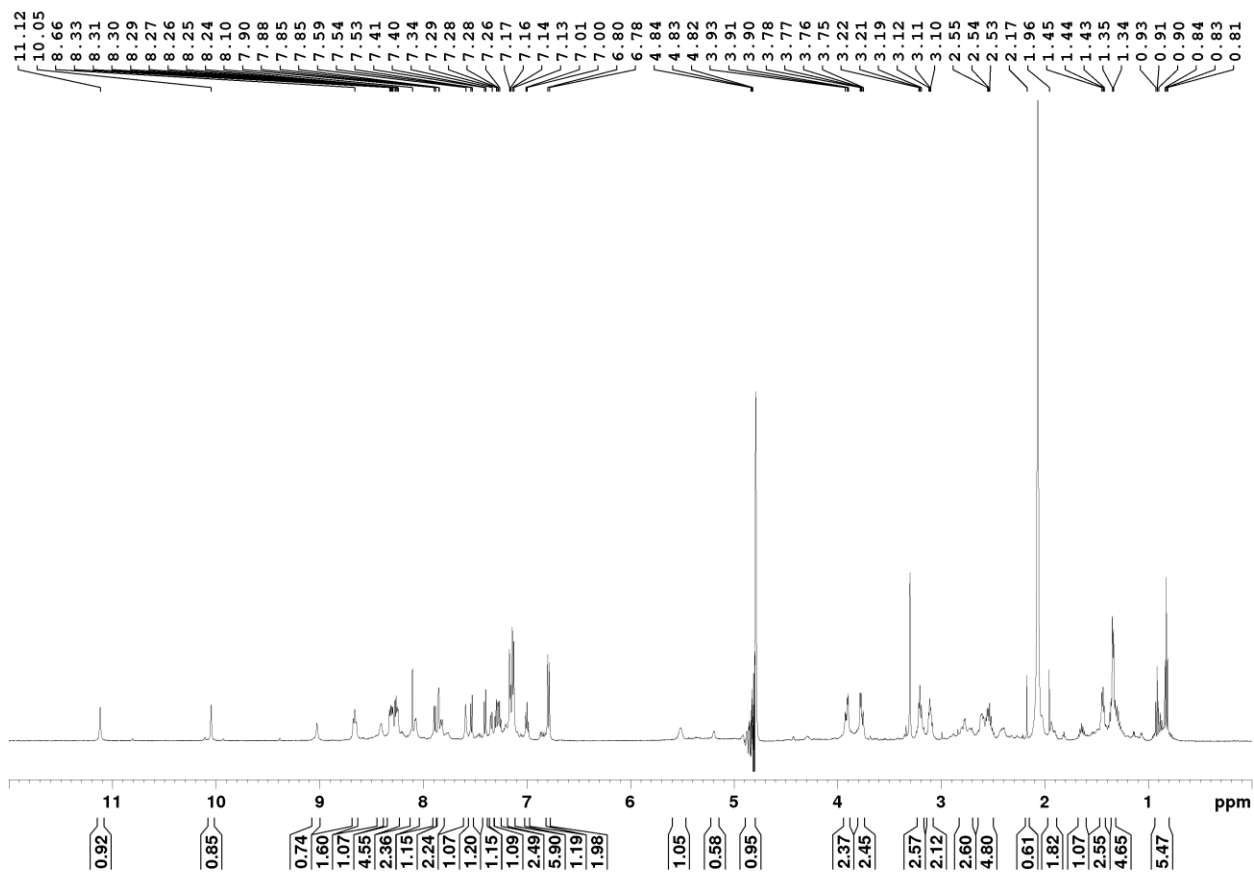
146

Figure B.15. ¹H-NMR spectrum of CDA3a (600 MHz, 9:1 H_2O/D_2O with 1 % AcOH) at 298 K

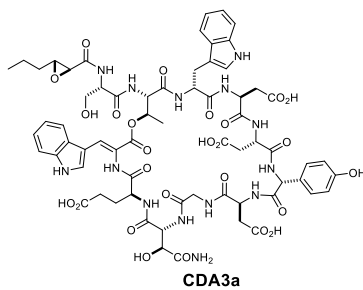Figure B.16. ^{1}H-TOSCY spectrum of CDA3a (600 MHz, 9:1 H_2O/D_2O with 1 % AcOH) at 298 K

Figure B.17. ^{1}H-NOESY spectrum of CDA3a (600 MHz, 9:1 H_2O/D_2O with 1 % AcOH) at 298 K
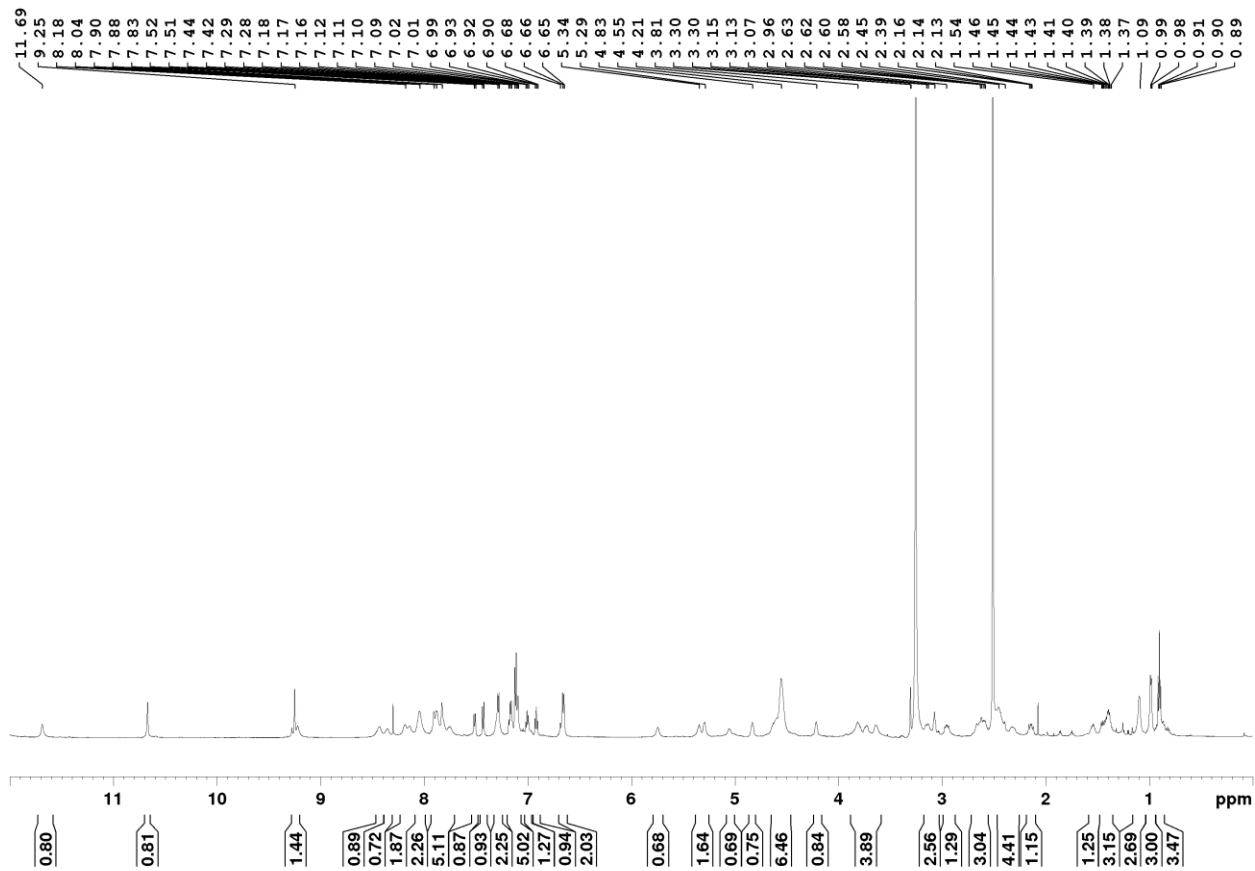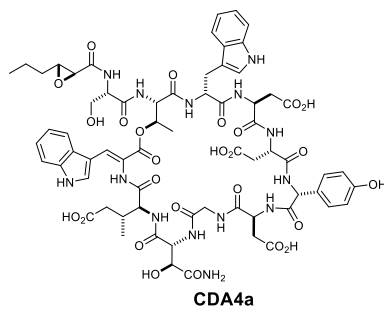
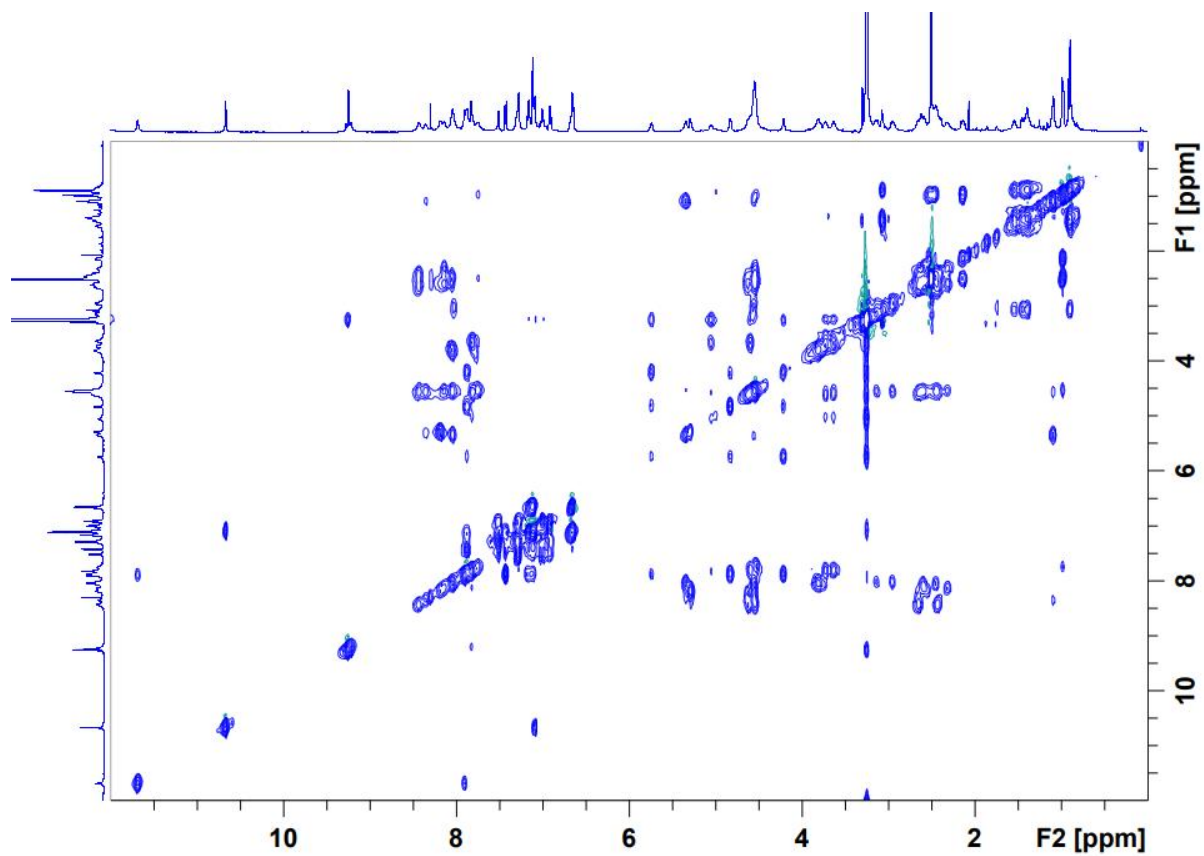Figure B.18. ^{1}H-NMR spectrum of CDA4a (600 MHz, DMSO-d_6) at 315 K
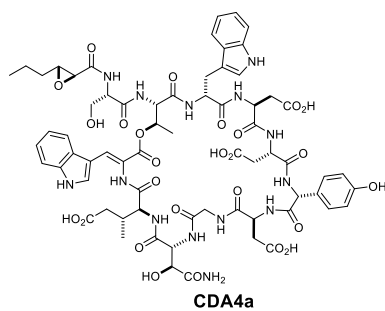
Figure B.19. ^{1}H-TOSCY spectrum of CDA4a (600 MHz, DMSO-d_6) at 315 K

Figure B.20. ^{1}H-NOESY spectrum of CDA4a (600 MHz, DMSO-d_6) at 315 K
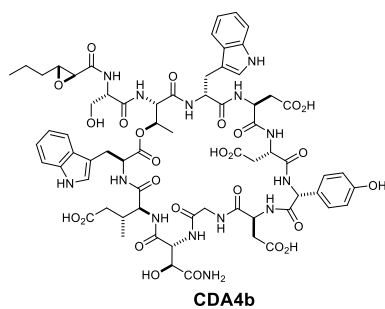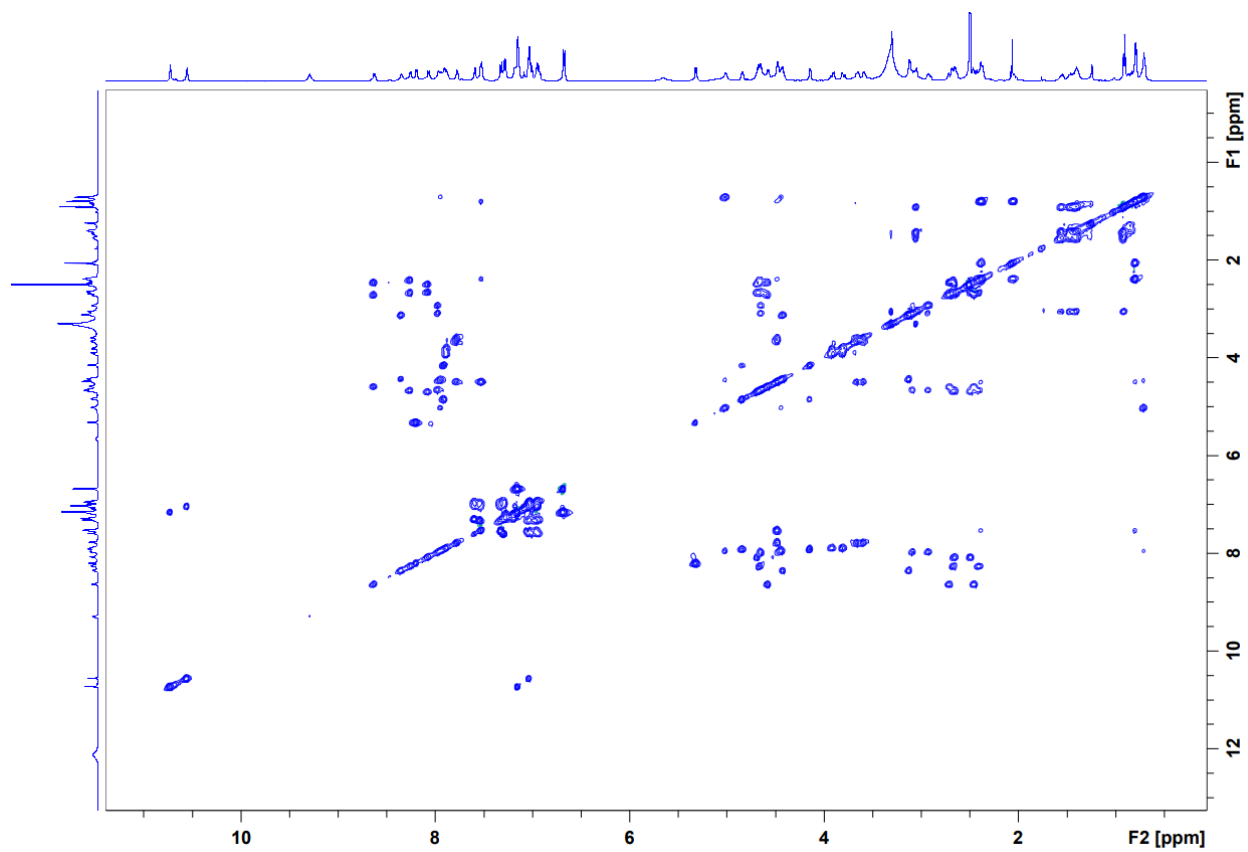
Figure B.21. ^{1}H-^{13}C HSQC spectrum of CDA4a (600 MHz, DMSO-d_6) at 315 K

Table B.1. Chemical Shift Assignments for CDA4a.

residue		CDA2a (Micklefield et al.) [107] δH (multiplicity, J value(s))	δC	residue		CDA4a (this work) δH (multiplicity, J value(s))	δC
Eph	2	3.36 (d, 2.0)	53.9	**Eph**	2	3.32 (m)*	53.8
	3	3.15 (td, 5.5, 2.0)	57.2		3	3.10 (m)*	57.3
	4	1.52, 1.46 (m)	32.6		4	1.54, 1.46 (m)*	32.5
	5	1.38 (m)	18.3		5	1.40 (m)*	18.2
	6	0.87 (t, 7.0)	13.4		6	0.91 (t, 7.0)	13.2
Ser (1)	NH	n.a	---	**Ser (1)**	NH	8.06*	---
	α	4.66	54.2		α	4.63	54.1
	β	3.83, 3.79	60.8		β	3.75, 3.66	61.2
Thr (2)	NH	n.a	---	**Thr (2)**	NH	8.07*	---
	α	4.53	56.2		α	4.55	56.3
	β	5.38	69.8		β	5.37	69.5
	γ	1.09	16.4		γ	1.11	16.1
Trp (3)	NH	n.a	---	**Trp (3)**	NH	8.0*	---
	α	4.37	54.4		α	4.54	53.6
	β	3.18, 2.91	26.3		β	3.16, 2.96	27.5
	1	10.67	---		1	10.67	---
	2	7.10	123.4		2	7.10	1.23.4
	3	---	109.9		3	---	n.d
	4	7.51 (d, 8.0)	117.7		4	7.53 (d, 8.2)	117.8
	5	6.96 (t, 8.0)	117.8		5	6.93 (t, 8.2)	117.7
	6	7.04 (t, 8.0)	120.3		6	7.05 (t, 8.0)	120.3
	7	7.31 (d, 8.0)	110.8		7	7.30 (d, 8.0)	110.7
	8	---	135.8		8	---	n.d
	9	---	126.9		9	---	n.d
Asp (4 or 5)	NH	n.a	---	**Asp (4 or 5)**	NH	8.44	
	α	4.43	49.7		α	4.55	49.3*
	β	2.52, 2.22	34.7		β	2.60, 2.31*	35.7*
Asp (5 or 4)	NH	n.a	---	**Asp (5 or 4)**	NH	8.14*	---
	α	4.61	49.0		α	4.64	49.0*
	β	2.66, 2.54	35.8		β	2.66*	35.6*
HOPhGly (6)	NH	n.a	---	**HOPhGly (6)**	NH	8.20*	---
	α	5.21 (d, 6.5)	56.5		α	5.30 (m)	55.5
	1	---	127.2		1	---	n.d
	2	7.17	127.2		2	7.17*	127.7
	3	6.69 (d, 8.85)	114.6		3	6.68	114.5
	4	---	156.6		4	---	n.d
Asp (7)	NH	n.a	---	**Asp (7)**	NH	8.05	---
	α	4.81	49.4		α	4.67	49.2*
	β	2.75, 2.53	35.1		β	2.66, 2.46*	35.4*
Gly (8)	NH	n.a	---	**Gly (8)**	NH	7.82*	---
	α	3.73	42.7		α	3.83	42.1
POAsn (9)	NH	n.a	---	**HOAsn (9)**	NH	7.75*	---
	α	5.05	55.2		α	4.87	55.4
	β	4.43	75.2		β	4.23	71.3
MeGlu (10)	NH	n.a	---	**MeGlu (10)**	NH	8.04*	---
	α	4.58	56.1		α	4.58*	55.7
	β	2.38	32.9		β	2.52*	31.5

	β-Me	0.95 (d, 7.0)	14.8		β -Me	0.99 (d, 6.6)	14.5
	γ	2.55, 2.08	37.1		γ	2.49*, 2.15	37.4
ΔTrp (11)	NH	n.a	---	**ΔTrp (11)**	NH	n.a	---
	α	---	n.o		α	---	n.d
	β	8.00	129.1		β	7.93	128.8
	1	n.a	---		1	n.a	---
	2	7.76	130.2		2	7.84	127.4
	3	---	107.9		3	---	n.d
	4	8.03	118.2		4	7.93	118.2
	5	7.15	119.4		5	7.13*	119.7
	6	7.18	121.3		6	7.19	121.5
	7	7.41 (d, 8.0)	111.5		7	7.45 (d, 8.0)	111.3
	8	---	135.2		8	---	n.d
	9	---	127.2		9	---	n.d

* Indicates overlap with 1 or more other peaks/*d*6-DMSO peaks.
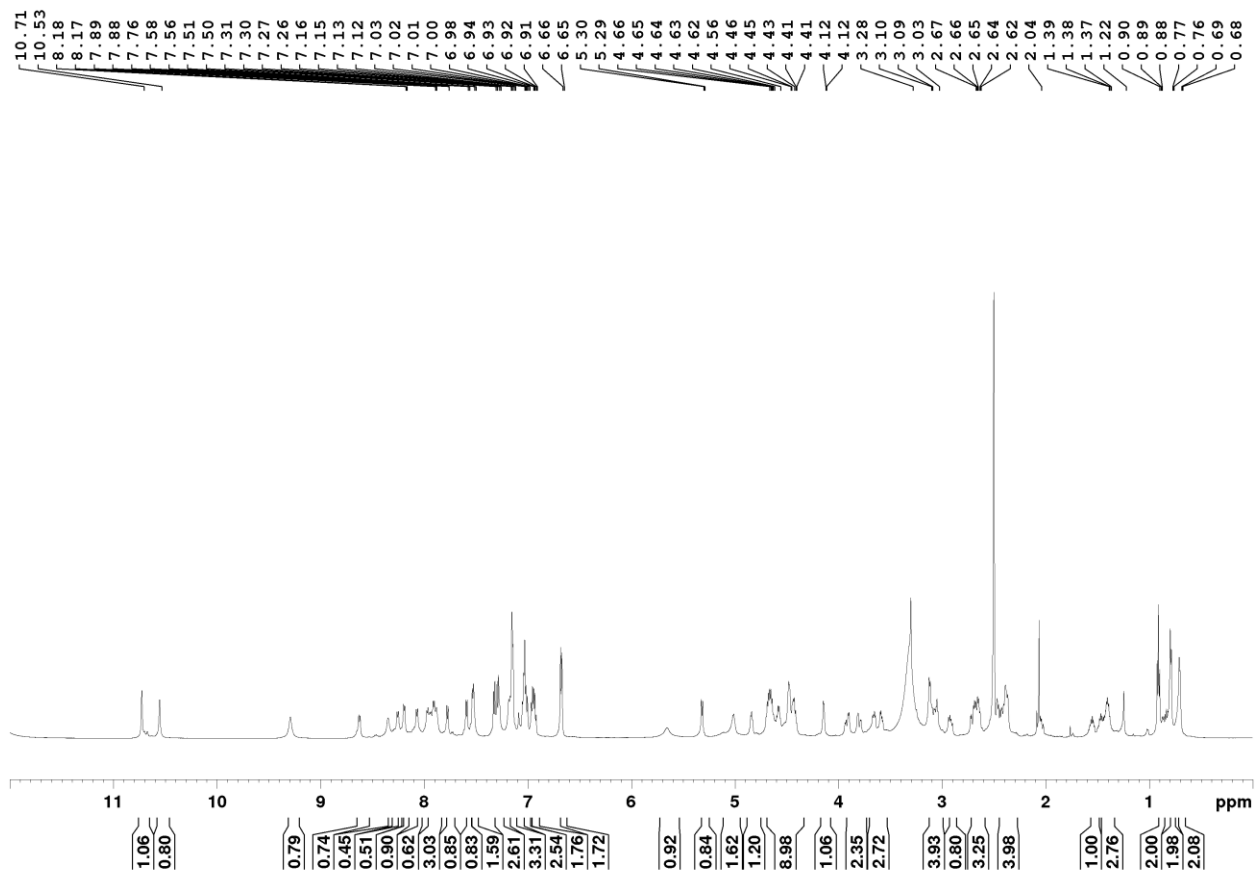
Figure B.22. ^{1}H-NMR spectrum of CDA4b (600 MHz, DMSO-d_6) at 315 K
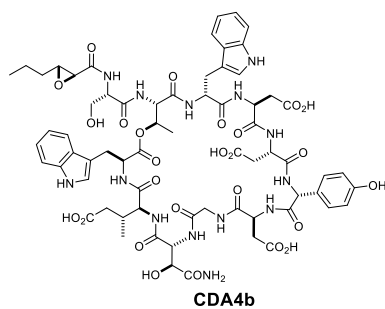
Figure B.23. ^{1}H-TOSCY spectrum of CDA4b (600 MHz, DMSO-d_6) at 315 K

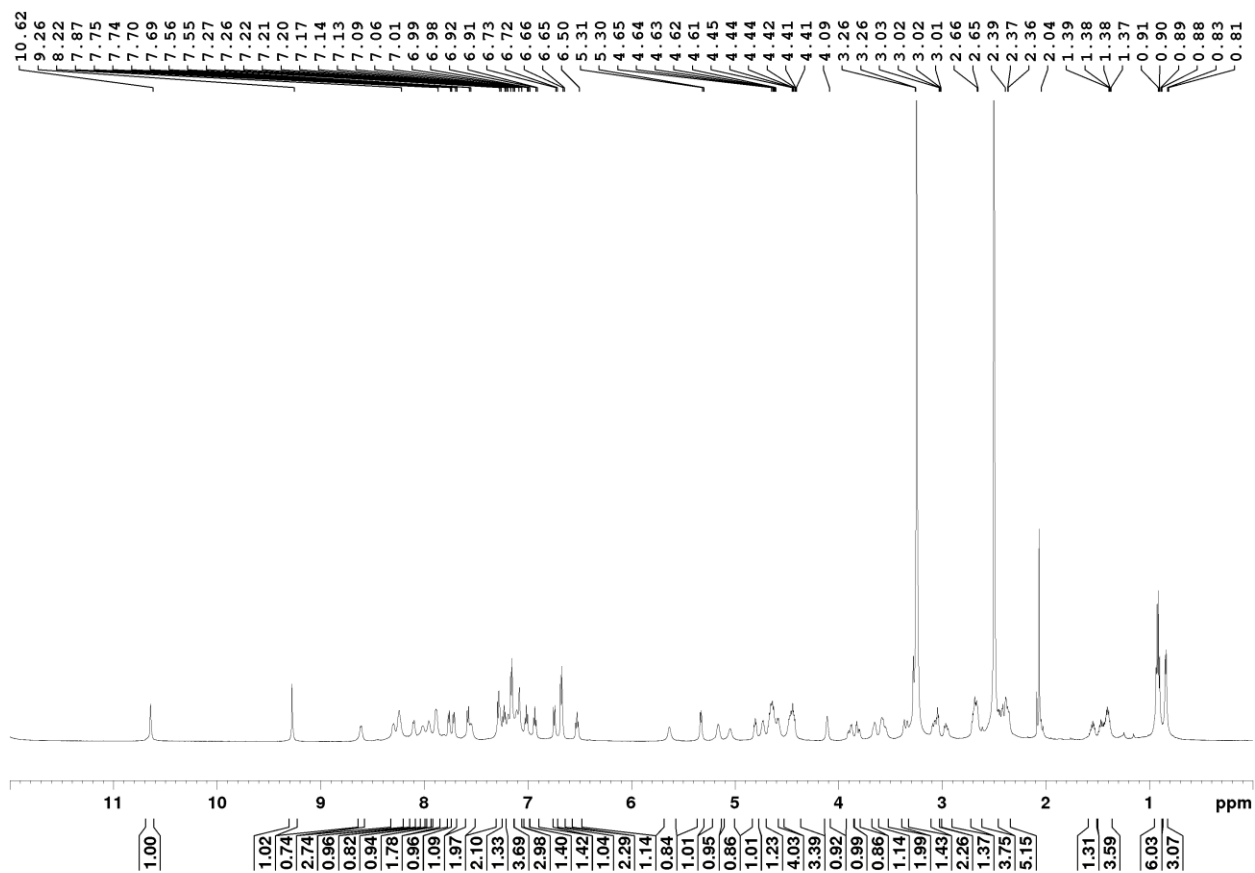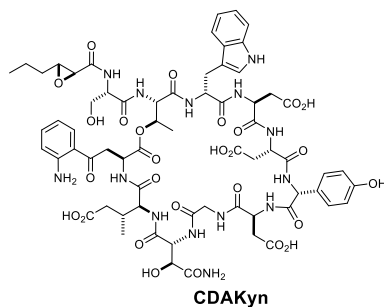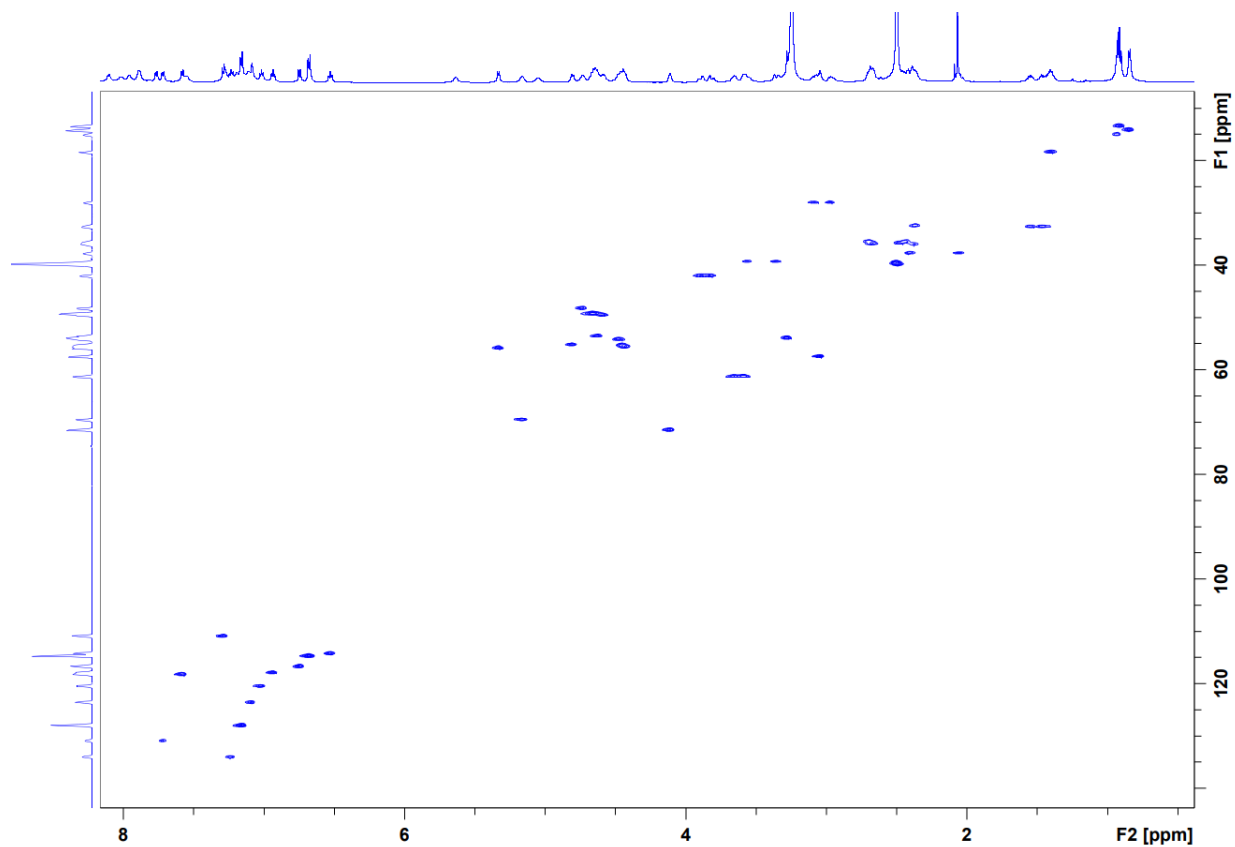Figure B.24. ^{1}H-NOESY spectrum of CDA4b (600 MHz, DMSO-d_6) at 315 K

Figure B.25. ^{1}H-^{13}C HSQC spectrum of CDA4b (600 MHz, DMSO-d_6) at 315 K

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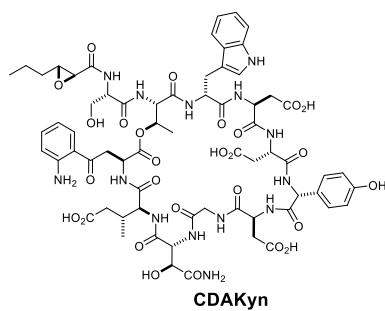Figure B.26. ^{1}H-NMR spectrum of CDAKyn (600 MHz, DMSO-d_6) at 315 K

Figure B.27. ^{1}H-TOSCY spectrum of CDAKyn (600 MHz, DMSO-d_6) at 315 K

Figure B.28. ^{1}H-NOESY spectrum of CDAKyn (600 MHz, DMSO-d_6) at 315 K

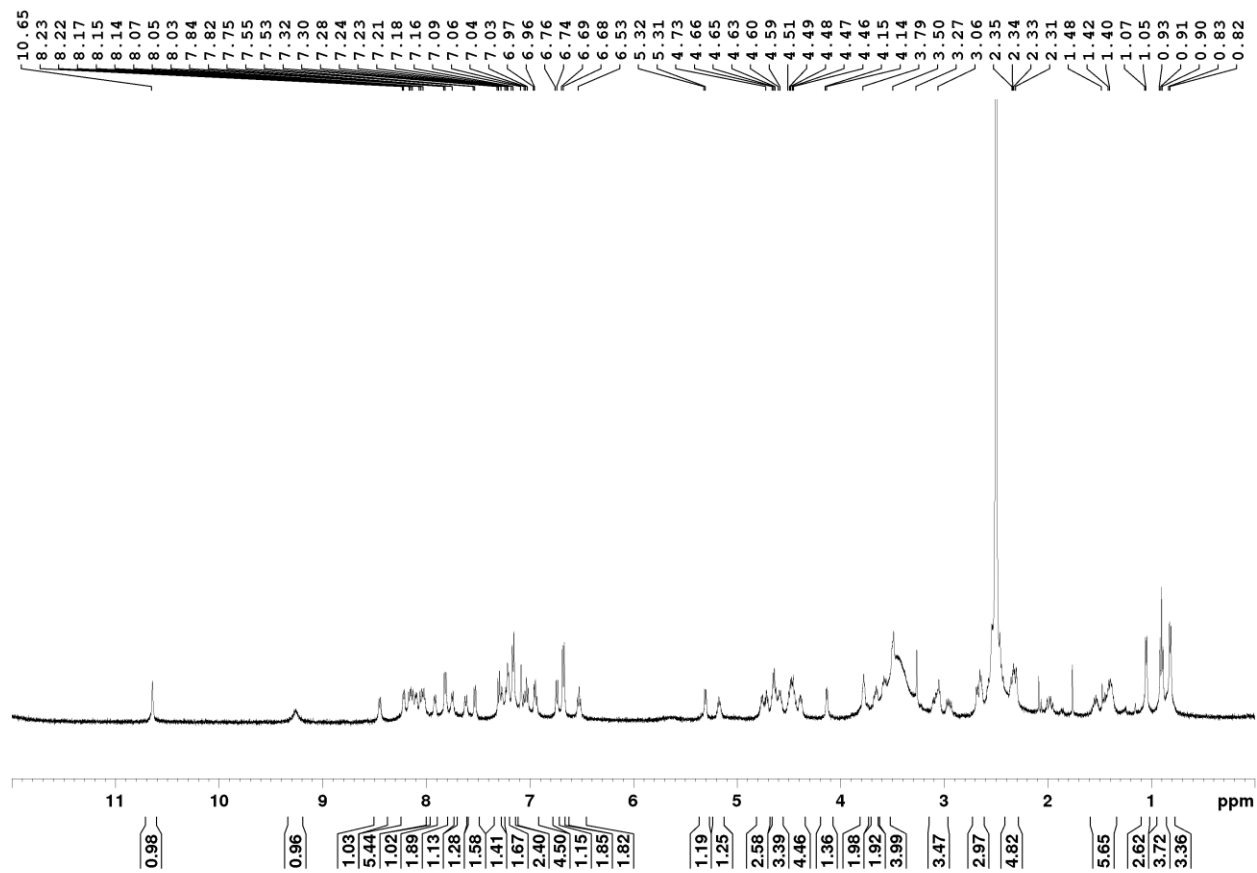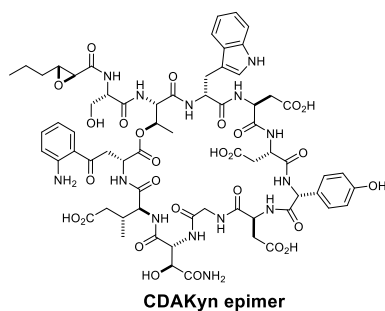Figure B.29. ^{1}H-^{13}C HSQC spectrum of CDAKyn (600 MHz, DMSO-d_6) at 315 K

CDAKyn epimer

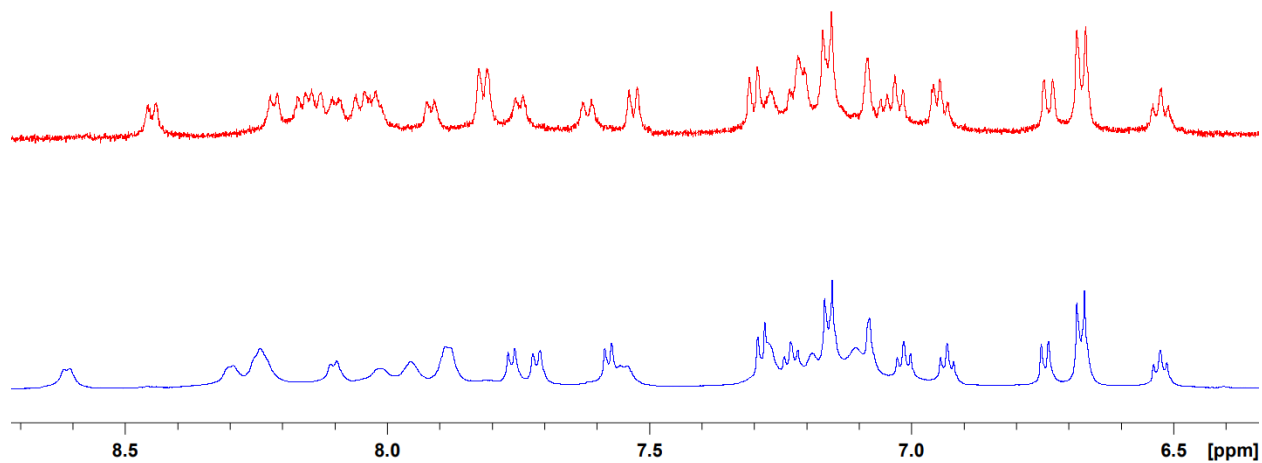Figure B.30. ^{1}H-NMR spectrum of CDAKyn epimer (500 MHz, DMSO-d_6) at 315 K

Figure B.31. The ^{1}H-NMR amide regions of CDAKyn (blue, 600 mHz) and CDAKyn epimer (red, 500 MHz) in DMSO-d_6 at 315 K