Total Synthesis of Substrate Analogue Inhibitors:
N-acetyl Nocardicin G Aldehyde

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1.0 Investigation of NocTE hydrolase/epimerase dual function
1.1 Background

Nonribosomal peptide synthetases (NRPSs) are messenger RNA independent, multi-modular protein biocatalysts that perform regio- and stereo-specific syntheses of a diverse array of medicinally active nonribosomal peptides (NRPs). Unlike ribosomes, the monomer substrates for NRPSs are not limited to the 20 proteinogenic amino acids, but includes around 500 nonproteinogenic amino acids, fatty acids, and \(\alpha\)-hydroxy acids, which is reflected in the structural and functional diversity of the NRP products.\(^1\) The biosynthesis occurs through condensation reactions in an assembly line fashion that occur through single building block carrying modules until the polypeptide product reaches a termination domain and is released by hydrolysis, cyclization, or reduction of the C-terminus carboxyl by NADPH dependent reduction domains. Bioactive NRPs include important antibiotics vancomycin, surfactin, cyclosporin A, and \(\beta\)-lactam containing penicillins, cephalosporins, and nocardicins in addition to other medically relevant compounds (Figure 1).

![Figure 1: Structural diversity of bioactive NRPS products.](image)

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NRPS modules are composed of three ubiquitous catalytic domains: activating adenylation (A), peptide carrier protein (PCP), and condensation (C) domains responsible for oligomerization.\(^2\) The final module in NRPSs also contains a thioesterase (TE) domain responsible for releasing the NRP product by hydrolysis or macrocyclization. Additional catalytic domains include, but are not limited to, epimerization, \(N\)-methylation, and \(N\)-formylation domains.

Of particular interest has been the biosynthesis of the first discovered monocyclic \(\beta\)-lactam, nocardicin A. The last structural variant to be discovered, the monocyclic \(\beta\)-lactams have been found to display some resistance to \(\beta\)-lactamases because of their increased ring stability due to greater flexibility compared to the more rigid bicyclic \(\beta\)-lactam structures, as well as lower enzyme affinity for the monocyclic backbone (Figure 2).\(^3\)

![Figure 2: Representative members of the monobactam antibiotic family.](image)

Nocardicin A is isolated from *Nocardia uniformis* ssp. *tsuyamanensis* and is active against Gram-negative bacteria.\(^4,5\) The discovery of the biosynthetic gene cluster in 2004 suggested that the peptide core of the monobactam antibiotic is synthesized by the five module NRPS pair NocA and NocB, though several key details of the biosynthesis remained obscured.\(^6\) Further experiments showed that the immediate product from the NocA/B NRPS is the \(\beta\)-lactam
containing pro-nocardin G, L-pHPG-L-Arg-D-pHPG-L-Ser-D-pHPG, which is contrary to the observed tripeptide nocardicin A precursor, nocardicin G (D-pHPG-L-Ser-L-pHPG), where pHPG is para-hydroxyphenylglycine (Figure 3). The presence of the $\beta$-lactam ring in the NRPS product indicated the formation of the ring must occur during the installment of the C-terminus pHPG amino acid in module 5 of NocB.

Figure 3: Abridged biosynthesis of monobactam nocardicin A.

The discrepancy between the NocA/B product and nocardicin G suggests that the L-pHPG-L-Arg dipeptide is removed by a proteolytic event in trans after being synthesized by the NRPS. The D-pHPG stereochemistry of the third residue is established by an embedded epimerization domain in module 3, while the epimerization of the C-terminal pHPG was recently shown to be carried out by a novel dual catalytic TE domain in NocB (NocTE) prior to hydrolysis. (Figure 4)
Figure 4: Dual catalytic function of NocTE.

Kinetic experiments demonstrated that the C-terminal pHPG epimerization occurs as a peptidyl-O-TE intermediate, and not as the peptidyl-S-PCP thioester intermediate. Through the synthesis of potential NocTE substrates, it was found that prior β-lactam formation was necessary for NocTE epimerization and release. The stringent requisite of β-lactam ring formation prior to NocTE activity establishes the thioesterase as a downstream “gatekeeper” in the assembly of the pentapeptide in the NRPS. It was recently discovered that the histidine rich module 5 condensation domain was responsible for the formation of the four-membered ring. The thioesterase holds intermediates upstream until the β-lactam ring is formed and then quickly epimerizes and releases the pentapeptide β-lactam, with kinetic experiments finding a 14 fold preference by NocTE for the D-pHPG stereocenter at the C-terminus for hydrolysis.

TE domains are 25-35 kDa structures with homology to fatty acyl thioesterases. Solved TE structures have identified a common α/β-fold and a Asp-His-Ser catalytic triad, where a His residue, activated by an Asp residue, acts as a general base for a catalytic Ser residue nucleophile. Crystallization and structure determination of NocTE revealed a globular protein of approximately 40 x 40 x 40 Å³ with the expected α/β architecture containing the Asp-His-Ser active site triad (Ser1779, His1901, and Asp1806) as well as an additional close proximity His
residue. The epimerase function of NocTE was hypothesized to be facilitated by this “extra” His residue, but mutational analysis revealed the epimerase activity was conserved. Mutational analysis of the active site residues was inconclusive with respect to finding the residue responsible for the epimerization activity, though earlier kinetic data suggests epimerization is carried out within the active site of the thioesterase. It therefore follows that the triad His residue (His1901) is most likely responsible for both activating the Ser residue for the formation of the acyl-\(O\)-Ser-TE intermediate as well as catalyzing the epimerization of the peptidyl-\(O\)-TE substrate (Figure 5).

![Figure 5: Proposed epimerization mechanism of the peptidyl-\(O\)-Ser-TE intermediate by His1901.](image)

The epimerization function of NocTE is unprecedented in TE domains. While several X-ray crystal and NMR studies have been performed on some NRPS domains and modules, the structures have not been crystallized with a bound substrate, limiting the postulation of enzymatic mechanisms and their participating catalytic residues to speculation by residue mutation activity assays and substrate/enzyme docking models.\(^\text{10}\) Additionally, the NocTE structure was solved in the open conformation, and the lid believed to be responsible for water
exclusion from the active site is unresolved. The current research effort is to synthesize a suitable substrate mimic in order to obtain a substrate bound NocTE structure, which would provide invaluable structural and mechanistic information not only in regards to the epimerization catalytic function, but also for the structural basis of the critical NocTE $\beta$-lactam substrate discrimination.

1.2 Peptide Aldehydes as Substrate Analogue Inhibitors

The design of a suitable substrate analogue inhibitor for NocTE must draw inspiration from the enzyme’s mechanism of action. The NocTE follows a classic serine protease mechanism. The $\beta$-lactam pentapeptide is transferred from the PCP domain to the TE as the peptidyl-$O$-Ser-TE intermediate. The His1901 residue then activates a molecule of water for the hydrolysis of the peptide-TE oxyester, leading to the release of pro-nocardicin G. The hydrolysis of the oxyester intermediate goes through an anionic tetrahedral intermediate that is transiently stabilized by the oxyanion hole expected to be formed by the backbone amide group of the Phe1780 residue. The collapse of the tetrahedral intermediate regenerates the active site Ser1779 and releases the peptide (Figure 6).

Figure 6: NocTE hydrolysis mechanism of the oxyester post-epimerization by Ser1779 and His1901. Asp1806 residue not shown.
A suitable substrate analogue inhibitor must adequately mimic the native substrate structure in order to be accepted by the active site and provide an accurate representation of the residue orientations relative to the substrate. Additionally, in order to obtain a co-crystal, the inhibitor must be sufficiently stabilized in the active site, in order to circumvent processing and release by the enzyme. After taking these factors into consideration, the peptide aldehydes N-acetyl nocardicin G aldehyde and N-acetyl epi-nocardicin G aldehyde were chosen as potential NocTE co-crystallization substrates (Figure 6).

Figure 6: Potential peptide aldehyde substrate analogue inhibitors for NocTE.

Peptide aldehydes have been of recent interest to the scientific community since their discovered inhibitory activity against serine and cysteine proteases such as trypsin, plasmin, and papain. The inhibitory potency of peptide aldehydes arises from the formation of an oxyanion hole stabilized tetrahedral hemiacetal intermediate formed upon nucleophilic attack by the catalytic serine. The aldehyde is trapped in the active site because the hemiacetal intermediate mimics the hydrolysis transition state of the natural substrate. The tetrahedral intermediate, however, is not a productive collapsible intermediate because it lacks a stable leaving group (Figure 7). There are several literature examples of peptide aldehyde transition state analogs co-crystallized whilst bound at the active site as tetrahedral intermediates. Additional inhibitors which form reversible tetrahedral adducts with serine proteases include trifluoromethyl ketones.
and boronic acids. The aldehyde was chosen due to its relative synthetic facility and greatest resemblance to the natural substrate carboxyl.

![Figure 7: Trapping of the peptide aldehyde inhibitor in the active site as the tetrahedral intermediate hemiacetal.](image)

The acetylation of the N-terminal pHPG was strategically chosen to mimic the extension of the peptide backbone of the nocardicin G aldehyde tripeptide to the natural pentapeptide substrate. Competition experiments have demonstrated that the NocTE shows a 20 fold preference for the pentapeptide N-acetylcysteamine (SNAC) thioester over the tripeptide SNAC thioester although the tripeptide is still epimerized and hydrolyzed. The pentapeptide aldehyde may be a future synthetic target because it is postulated that the presence of hydrogen bonding and charge-charge interactions with the N-terminus dipeptide (L-pHPG-L-Arg) and the lid region is involved in creating a preference for the longer β-lactam peptide substrate. A crystal structure of NocTE with the transition state analog in the active site will answer important questions regarding the structural specificity and mechanism of the dual function NocTE in the biosynthesis of nocardicin A.

2.0 Synthesis of N-acetyl nocardicin G aldehyde

The synthesis of peptide aldehydes can be realized by following two possible routes, with the establishment of the aldehyde moiety post-peptide elongation, or with the creation and
protection of the aldehyde as the monomer followed by peptide elongation. Both routes were investigated during the synthesis of N-acetyl nocardicin G aldehyde. The aldehyde moiety would be protected as the acetal in order to mask the reactivity of its electrophilic carbonyl, and deprotected as the last step of the synthesis. One major concern of peptide aldehyde product is the low pKa of the α-position, which could lead to rapid racemization.

2.1 Aldehyde Installation and Protection Post-β-lactam Formation

Synthesis of the N-acetyl nocardicin G aldehyde was envisioned retrosynthetically from the N-protected β-lactam containing aminocardicinate (4) and requires the aldehyde to be installed after the formation of the β-lactam ring (Figure 8).

Figure 8: Retrosynthesis of N-acetyl nocardicin G.

The synthesis of 4 was reported by Gaudelli from D-pHPG. The synthetic scheme for the preparation of 4 is presented in Scheme 1. The preparation of 3 from 4 was found to be unsuccessful after varied reagents and conditions were attempted. The treatment of 4 with diisobutylaluminum hydride (DIBAL-H) at -78°C yielded no observable reaction by TLC (40% EtOAc/Hex), even after additional equivalents were added and the reaction performed at room temperature. Rather than attempting to halt the reduction of the carboxyl at the aldehyde stage, it was decided to try reducing the carboxylic acid to the primary alcohol followed by a controlled oxidation to the aldehyde. Unfortunately, the treatment of 4 with lithium aluminum hydride
(LAH) did not yield the alcohol product, despite additional equivalents to account for the consumption by the acid and higher reaction temperatures.

Scheme 1: Preparation of intermediate 4.

The absent reactivity of the free acid 4 is likely rooted in the electron rich carboxylate, carbonyl carbon is less susceptible to nucleophilic hydrides, though the direct reduction of aliphatic and aromatic carboxylic acids is well established in the literature.\textsuperscript{16,17} After the failure to reduce the free acid 4, the reduction of the methyl ester 11 was attempted (Scheme 2).

Scheme 2: Attempted reductions of methyl ester 11.
The methyl ester 11 was quantitatively prepared by treatment of 4 with freshly prepared diazomethane in diethyl ether. Diazomethane is obtained as a yellow ethereal solution by the base hydrolysis of Diazald\textsuperscript{TM} with KOH and co-distillation with ether (FBD-I-258). The methyl ester 11 was then subjected to DIBAL-H reduction in an attempt to obtain the aldehyde 3 directly, and LiAlH\textsubscript{4} reduction to obtain the primary alcohol 12, which would then be mildly oxidized to 3. Neither route using methyl ester 11 was successful, with starting material recovered from both attempted reductions.

With the failure of the reduction of the methyl ester, a more reactive carboxyl derivative was prepared and subsequent reduction attempted (Scheme 3).

![Scheme 3: Attempted formation of 12 through the Weinreb amide 13.](image)

The Weinreb amide\textsuperscript{18} 13 was prepared by treating the free acid 4 with PyBOP to form the activated ester, followed by nucleophilic displacement with \textit{N,O}-dimethylhydroxylamine (FBD-I-239). The Weinreb amide was then treated with LAH, which led to numerous unidentified products, none stained positive for the aldehyde. The inability to successfully reduce 13 necessitated a novel synthetic strategy for obtaining the peptide aldehyde.
2.2 Aldehyde Installation and Protection Pre-β-lactam Formation

With the failure to obtain the aldehyde moiety in the presence of the β-lactam ring, the aldehyde functional group would need to be established and protected as the pHPG monomeric amino acetal prior to peptide elongation and β-lactam formation. The synthesis can be described in two parts, the first concerning the creation and protection of the D-pHPG aldehyde as the D-pHPG dimethyl acetal. A major challenge to the synthesis is maintaining stereocontrol of the acidic pHPG α position, especially once the aldehyde is formed. The second half of the synthesis involves the coupling of the amino dimethyl acetal with Ox protected L-serine followed by β-lactam formation, deprotection of the Ox group, coupling with N-acetyl-D-pHPG, and acetal deprotection to yield the desired N-acetyl nocardicin G aldehyde. The first synthetic route for the preparation of the D-pHPG dimethyl acetal is displayed in Scheme 4.

Scheme 4: Synthesis of D-pHPG dimethyl acetal 21.
The synthetic route from 21 to the target N-acetyl nocardicin G aldehyde is displayed in Scheme 5.

Scheme 5: Synthesis of 2 from the protected D-pHPG dimethyl acetal.

The pHPG phenol must be protected to prevent interference with the β-lactam forming Mitsunobu reaction. The formation of the D-pHPG benzyl ether (6) was effected without prior amine protection by first forming a five-membered cyclic copper chelate with the α-amino and carboxyl groups followed by treatment with benzyl bromide, as utilized by Gaudelli (FBD-II-052). While a useful reaction, the overall yields of 6 were unsatisfactory, typically between 40-50%. Considering the length of the synthesis, a low yielding first reaction greatly limits the flux of intermediates through the scheme. An improved order of transformations was therefore

Scheme 6: Improved synthesis of benzyl protected D-pHPG.
devised (Scheme 6).

Preparing the α-amino group Boc carbamate with prior to benzylation removes the necessity of the low yielding chelate protection. With the more nucleophilic amine capped, the benzyl bromide electrophile reacts with the more nucleophilic phenolate to provide the N-Boc-O-benzyl ether 14. The methyl ester can be efficiently prepared in large scale reactions (~20 g) by overnight reflux with dimethyl sulfate in alkaline acetone. Alternatively, 14 can be treated directly (one-pot) with diazomethane, after neutralization, to yield the desired methyl ester 15, advancing the synthesis of 22, (Scheme 7). In addition to the peptide aldehyde synthesis, the facile removal of the Boc group with 1:1 TFA/DCM yields 6, which can be used for other peptide syntheses.

Scheme 7: Improved synthetic scheme for 21.

The reduction of 15 with an equimolar quantity of LAH (1M in THF) proceeds smoothly at -78°C in 40 minutes (FBD-I-261, FBD-II-041). The reaction mixture is quenched slowly at 0°C using a saturated solution of Rochelle’s salt (potassium sodium tartrate, KNaC₄H₄O₆·4H₂O).
The quenched mixture forms a dense emulsion which clears up to provide a clear biphasic solution after overnight stirring at room temperature. The product is obtained with minor impurities (<5%) and was used without further purification.

While the swapping of the Boc group with Fmoc is inefficient, it was found that the Swern oxidation of 16 produces the Boc aldehyde as an oily solid, which is unstable to the acidic acetal forming conditions (FBD-I-275, FBD-I-278, FBD-I-280). The instability of the Boc aldehyde to acid is likely due to unfavorable kinetics, where the acid deprotection of the Boc group occurs more quickly than the acid catalyzed formation of the acetal. The reactive amino aldehyde would then quickly undergo acid catalyzed imine condensation. It was therefore necessary to install an acid stable protecting group to withstand the acetalization conditions, which could then be removed without disturbing the acetal for coupling with Ox-L-Ser. The base sensitive Fmoc (fluorenylmethyloxycarbonyl-) group was chosen over a hydrogenable group (i.e. Cbz) because the hydrogenolysis to free the amine would also deprotect the benzyl ether.

A synthetic scheme for the amino acetal 21 was attempted where the Fmoc group was installed on 6 instead of Boc. Interestingly, it was found that while the Fmoc protection of amino alcohol 17 readily occurs at 0°C, the Fmoc protection of amino acid 6 would only go to completion when the reaction is refluxed in 1:1 H₂O/dioxane, (FBD-I-282, FBD-II-028). Unfortunately, it was found that the N-Fmoc-O-benzyl-D-pHPG methyl ester does not undergo clean reduction to alcohol with LAH, with slight deprotection of the Fmoc group observed (FBD-II-030). After working with several Fmoc protected compounds, it has been observed that a small quantity of material deprotects if allowed to stir in solution for extended periods of time, as would be necessary for the de-emulsification of the LAH reduction. It was therefore decided
to install the Boc group in order to carry out the LAH reduction, and exchange with Fmoc through the amino alcohol 17 before performing the oxidation and acetalization.

The controlled oxidation of 18 to the aldehyde 19 establishes a particularly acidic α position owing to the electron withdrawing nature of the aldehyde and the capacity to delocalize charge over the phenyl ring. As a result, the nature of the oxidizing reagent was chosen to avoid acidic or basic conditions. The Dess-Martin periodinane (DMP) oxidant at 0°C in methylene chloride was chosen because of its neutral and mild oxidizing capacity. The oxidation of 18 with DMP proceeds smoothly in 45 minutes, after adding the oxidant at 0°C and allowing the mixture to warm to room temperature (FBD-II-017). It was found that several washes with saturated sodium thiosulfate (Na₂S₂O₃) is necessary to obtain a pure product. Inadequate washing results in the isolation of DMP byproducts (FBD-II-021). The oxidation yields the aldehyde 19 in sufficient purity to carry on without further purification.

The formation of the dimethyl acetal 20 is achieved by refluxing the aldehyde in freshly distilled methanol (distilled over 3Å molecular sieves), with an excess of trimethylorthoformate, and catalytic para-toluenesulfonic acid (TsOH) for at least three hours. Initial attempts to make the dimethyl acetal were difficult to monitor, as it was discovered that in a variety of TLC conditions, the aldehyde 19 and acetal 20 co-spot (FBD-II-020). In order to monitor the progress of the reaction, it is necessary to take small aliquots of the reaction mixture for ¹H-NMR. The ratio of the starting material (SM) and product benzyl methylene singlets as well as the disappearance of the downstream SM aldehydic singlet can be used to measure the extent of the reaction. Earlier experiments found that elevated reaction temperatures are necessary to push the reaction to completion. It was found that when the aldehyde was stirred with fresh methanol, trimethylorthoformate, and catalytic TsOH over molecular sieves at room temperature for 20
hours a 1:1 mixture of 19 and 20 was obtained (FBD-II-025). The acetal is obtained relatively pure after a standard aqueous workup and the amine is deprotected without further purification. The apparent co-spot occurring between the aldehyde and acetal raised concerns about the stability of the acetal to the mildly acidic silica-gel. These concerns were addressed by purifying the acetal by flash column chromatography (40% EtOAc/Hex) as well as by loading the acetal onto silica solvated with the TLC conditions overnight. In neither case was deprotection of the acetal observed. The Fmoc protected dimethyl acetal is deprotected to obtain the amino acetal 21 by room temperature treatment with a 20% piperidine/THF solution. The Fmoc deprotection by product is significantly non-polar compared to the free amino acetal. It is therefore possible to isolate 21 using a plug of silica and a gradient of eluents to first washout all the non-polar impurities. The byproduct impurities are eluted at the solvent front when using 50% EtOAc/Hex, with 21 hardly moving from the baseline. After elution of the byproducts, the polarity of the eluent is greatly increased to 10% MeOH/EtOAc in order to isolate the free amine ($R_\text{f} = 0.38$) in excellent purity as a clear oil (FBD-II-067). The free amine is only visible by UV when highly concentrated, ninhydrin stain (free amine stains yellow/red) was used for TLC analysis. The overall yield for the formation of 21 from D-pHPG is 20%.

The L-serine amine is protected by Sheehan’s 4,5-diphenyl-4-oxazolin-2-one (Ox) group. The bidendate protecting group was found by Gaudelli to eliminate competing aziridine formation during the $\beta$-lactam forming Mitsunobu reaction. The dioxolone, 27, is readily prepared by refluxing benzoin and carbonyldiimidazole (CDI) with dimethylaniline (DMA) in anhydrous benzene. The product readily crystallizes out of the solvent upon cooling as spiny white needles (FBD-II-046). L-serine is then treated with tetramethylammonium hydroxide
before reacting with 27. The intermediate is then dehydrated in neat trifluoroacetic acid (TFA) to afford the N-protected amino acid (Scheme 8).

![Scheme 8: Preparation of Ox protecting group and protection of L-serine.](image)

The Ox-L-Ser 8 is then coupled to amino acetal 21 using PyBOP and Hünig’s base (diisopropylethylamine) to afford the dipeptide 22. In early experiments, it was observed that the peptide coupling produced an appreciable mixture of two diastereomers, though it was unclear which stereocenter had been epimerized. The observation raised concerns about the lability of the pHPG α proton, and the possibility that the amino acetal 21 was racemized during its synthesis. Conversely, it was possible that the serine stereocenter was compromised by the influence of the electron-withdrawing PyBOP active ester intermediate. In order to address the latter possibility, the half-life of the active ester was minimized by dissolving Ox-L-serine and the amino acetal in the same reaction vessel, adding PyBOP, and lastly initiating the reaction with Hünig’s base. The modification to the original coupling procedure (which generated the active ester in a separate flask before the amine was added) greatly reduced the extent of epimerization, which suggests it was indeed the serine stereocenter being epimerized (FBD-II-071). A more important conclusion can be drawn from the experiment, the pHPG stereocenter was retained during the synthesis of the amino acetal. Of empirical note is the crystalline and insoluble nature of the dipeptide, which frequently crashes out as a white solid during work up or precipitates in the test tube fractions during column chromatography (60% EtOAc/Hex). If a
mixture of diastereomers is obtained, the desired L,D dipeptide stereoisomer can be isolated as a white solid by recrystallization from hot ethyl acetate and hexanes.

The mechanism and intermediates in the formation of the $\beta$-lactam ring by nucleophilic displacement of the activated serine hydroxyl by the amide nitrogen has been extensively investigated for serine, pHPG dipeptides with a carboxyl C-terminus (methyl ester and tert-butyl esters) and bidentate serine nitrogen protecting group.$^{19,20,21}$ It was found that ring closure with stereocenter retention could be most efficiently realized through a modified Mitsunobu reaction using triethyl phosphite [P(OEt)$_3$] and DEAD reagent, with the important exclusion of light.$^7$ Experimentally, it was found that efficient $\beta$-lactam ring formation for the Ox protected dipeptide acetal 22 required overnight reaction times with a concentration (relative to the dipeptide) of 0.025 M (FBD-II-081). When the reaction was run at higher concentrations (0.15 M) multiple products were formed, as observed using TLC (60% EtOAc/Hex, FBD-II-075). An additional attempt to increase the reaction rate through the use of a more reactive alkyl phosphine, i.e. triethyl phosphine (PEt$_3$), also resulted in a myriad of side products (FBD-II-077). The intramolecular cyclization is a sensitive reaction that can be further optimized in the future for the specific acetal substrate. It was experimentally found that a 60% ethyl acetate/hexanes eluent mixture is appropriate for TLC monitoring, but results in crystallization of the desired product in silica during attempted flash chromatography. Chromatographic purification of 23 should be carried out using a 5% v/v mixture of acetone and methylene chloride.

The reductive deprotection of benzyl and Ox groups was surprisingly tedious to optimize, considering the structural similarity of 22 to Gaudelli’s $N$-Ox- $O$-Bn-$t$-butyl nocardicinate. The acetal nocardicinate 22 is readily solubilized using minimal (<10 mL) non-fresh THF in a pressure flask. A catalytic (50% by weight) quantity of Pearlman’s catalyst is added, and the
flask is pressurized with 50 atm of H$_2$ for 48 hours. When an excess of Pd(OH)$_2$/C was used, adsorption to the activated charcoal made the recovery of product impossible, even after extensive filtration through Celite. Use of too little catalyst results in the partial hydrogenation of 22, with removal of the benzyl ether, but retention of the Ox group. The success of the hydrogenation can be qualitatively determined using TLC (50% EtOAc/Hex). The partial hydrogenation product can be detected as a bright blue spot ($R_f = 0.4$), while the bibenzyl byproduct from the reduction of the Ox group appears at the solvent front. The completely hydrogenated product, 23, is detected by staining with freshly prepared ninhydrin stain. Because of the acid labile acetal, several unsuccessful attempts were made to isolate 23 as the free amine. The attempts included trituration of bibenzyl using hexane, aqueous base extraction, and non-aqueous work up using methanol and hexane. After failing to isolate the free amine, 23 was ultimately isolated as the hydrochloride salt.$^7$ Because of the acid sensitive acetal moiety, the ether and acidic water are cooled in an ice bath prior to use. After extraction, the aqueous layer is quickly frozen with liquid nitrogen to further prevent degradation and prepare for lyophilization. In order to minimize potential complications with HCl degradation of the product, the acetate salt was isolated using a 0.1% aqueous solution of HOAc, followed by lyophilization. The acetate salt yielded a white powder, which was surprisingly deliquescent.

The formation of the D,L,D β-lactam tripeptide was envisioned by coupling the amino nocardicinate acetal 23 with N-acetyl-D-pHPG (25) through an in situ mixed anhydride with catalytic N-methyl morpholine, as reported by Nayler, and used by Gaudelli.$^{22,7}$ It was unsurprisingly observed that D-pHPG can be selectively N-acetylated in the presence of its free phenol if base equivalents are carefully controlled. Use of 2.1 equivalents of sodium bicarbonate and 1.1 equivalents of acetic anhydride readily provides the desired product (FBD-II-097). In
contrast, using the same equivalents of electrophile, but performing the reaction in aqueous saturated NaHCO₃, yields a 1:1 mixture of mono and di-acetylated products, regardless if the reaction is carried out at room temperature or 0°C.

Unfortunately, the peptide coupling between N-acetyl-D-pHPG and 23 failed, instead producing one major unknown by-product determined by ¹H-NMR to contain the pHPG core, as well as acetyl and isobutyl proton signatures. In order to troubleshoot the reaction, several control experiments were carried out. The coupling between N-acetyl-D-pHPG and the t-butyl ester analogue of 23 was attempted next, because the t-butyl nocardicinate had been previously successfully coupled to N-Boc-D-pHPG by Salituro. The reaction was unsuccessful, with the same unidentified by-product being the major compound produced. Three additional experiments were carried out, where the reactivity of N-acetyl-D-pHPG was compared to carbamates N-Boc-D-pHPG and N-Cbz-D-pHPG by attempting to couple with readily available O-Bn-L-Ser. While both carbamates successfully reacted to give the respective dipeptide, the reaction between N-acetyl-D-pHPG and O-Bn-L-Ser produced the same side-product as before, suggesting the identity of the amine donor was irrelevant to the undesired side reaction. In order to circumvent the peculiar reactivity of N-acetyl-D-pHPG, a modified scheme where N-Cbz-D-pHPG is coupled to 23, hydrogenated, and then selectively N-acetylated is proposed, (Scheme 9).
Scheme 9: Revised synthesis of 2, circumventing the coupling reaction with N-acetyl-D-pHPG.

The identity of the unknown compound was established through extensive $^1$H-NMR, $^{13}$C-NMR, HMBC, FT-IR, EI, and FAB mass spectrometry experiments. The reaction of N-acetyl-D-pHPG with isobutylchloroformate in the presence of N-methylmorpholine and 2,6-lutidine is depicted in Scheme 10.

Scheme 10: Identification of unknown product from the attempted coupling of N-acetyl-D-pHPG.

A proposed mechanism for the formation of the oxazole 31 is depicted in Scheme 11.
Scheme 11: Proposed mechanism for the formation of pHPG oxazole 31.

Mechanistically, the key reactivity difference between the acetamide and carbamate groups must be the nucleophilicity of their carbonyl oxygens. In order to investigate the predicted electronic properties, orbital calculations were carried out on Spartan at the B3LYP/6-31G* on methyl acetamide and N-methylmethylcarbamate model compounds, Figure 9.

Figure 9: Molecular orbital calculations for methyl acetamide and N-methylmethylcarbamate. Mesh diagram of highest occupied molecular orbital (HOMO) displayed.
Inspection of the electron distribution of the highest occupied molecular orbitals revealed that although the N-methylmethyl carbamate has a higher negative charge density, as expected from the additional charge donation from the carbamate oxygen, the HOMO orbital coefficient is greater for the nitrogen rather than oxygen atom, as depicted by the larger orbital lobe centered on nitrogen in Figure 9. In contrast, the orbital coefficient for the HOMO of methyl acetamide is larger for oxygen, suggesting greater nucleophilicity. Although these preliminary calculations are not a definitive justification for the peculiar reactivity of the isobutyl/N-acetyl-pHPG mixed anhydride, they provide an interesting perspective on possible orbital explanations. The serendipitously discovered reaction bears semblance to the Dakin-West reaction, where an amino acid is converted into a keto-amide through an oxazole/azlactone.\(^{23}\) The reaction differs, however, because acylation of the azlactone oxygen rather than the ring carbon by an additional equivalent of isobutylchloroformate occurs preferentially to give the oxazole carbonate.

Due to the reported failure of modern coupling methods by Salituro and Gaudelli to join N-Boc-pHPG and protected nocardicinate, as well as the specific incompatibility of N-acetyl-pHPG with the mixed anhydride protocol, it became necessary to perform the coupling between nocardicinate acetal\(^{24}\) and the Cbz-pHPG carbamate, Scheme 9. The coupling reaction was carried out using identical conditions attempted for N-acetyl-pHPG, and yielded the desired Cbz-nocardicin G acetal\(^ {29}\). Interestingly, compound\(^ {29}\) displayed unexpectedly high polarity, requiring TLC eluent conditions of 10% MeOH/CHCl\(_3\) in order to be detected, whereas other tripeptides were efficiently eluted using 60% EtOAc/Hexanes.

The removal of the Cbz protecting group was ineffective using Pd(OH)\(_2\)/C under 1 atm of H\(_2\). Overnight shaking at 50 psi of H\(_2\), however, successfully deprotected the tripeptide β-lactam, monitored by ninhydrin stain. Unfortunately, although the desired nocardicin G acetal free amine
was formed, a significant quantity of organic impurities was observed by $^1$H-NMR. It is possible the unknown species originated from contaminated Celite, through which the crude hydrogenated mixture was filtered, in order to remove the palladium catalyst. The free amine nocardicin G acetal was far too polar to purify using conventional silica chromatography. Instead, compound 30 was obtained through HPLC purification on a reverse phase prep-column, eluting at 23.2 minutes with a strong pHPG chromophore at 230 nm. The identity of 30 was confirmed by LCMS and $^1$H-NMR.

With the purified nocardicin G acetal isolated, the N-terminal amine was carefully selectively acetylated in the presence of two free pHPG phenols using freshly distilled acetic anhydride and freshly prepared sodium bicarbonate solution, by exploiting the greater amine reactivity. With the successful synthesis of acetyl nocardicin G acetal, the remaining transformation is the acid catalyzed deprotection of the dimethyl acetal to yield the free aldehyde 2.

3.0 Future Goals and Acknowledgements:

Ongoing work on the NocTE project includes the completion of the stereoselective N-acetyl nocardicin G aldehyde total synthesis, as well as the biochemical and crystallographic experiments made accessible by the successful acquisition of the peptide substrate analogue inhibitor. Obstacles and setbacks, as encountered in any scientific and chemical endeavor, were frequent. Although superficially discouraging and frustrating, they provide, in my opinion, the truest essence of research and opportunity for discovery and scholarship. I humbly express gratitude to my mentors, particularly Professor Craig A. Townsend, Dr. Eric Hill, Professor Chris J. Falzone, and Darcie Long, whose advice and experience was the *sine qua non* of the preceding work.
4.0 Experimental

4.1 Synthesis—complete chemical characterization is withheld anticipating manuscript preparation.

**N-Boc-O-Bn-D-pHPG (14).** To a 250 mL round bottom flask was added NaOH (3.0 g, 76 mmol) and 100 mL of 1:1 H2O (0.75 M) at room temperature, open to air. D-pHPG (6.0 g, 36 mmol) was dissolved in the alkaline solution, producing a light yellow color. The solution was added Boc₂O (8.61 g, 39 mmol) as a solid, at once. The mixture was stirred at room temperature until completion N-Boc-D-pHPG (40 minutes), monitored by TLC (40% EtOAc/Hex + 1% AcOH). To the alkaline solution of N-Boc-D-pHPG was added additional NaOH (3.0 g, 76 mmol), before benzyl bromide (4.70 mL, 39.4 mmol) was added dropwise at room temperature open to air. The reaction was allowed to stir to completion for 20 hours (40% EtOAc/Hex + 1% AcOH). The alkaline reaction mixture was extracted once with Et₂O (100 mL) before the aqueous layer was acidified to pH = 2 using 1M HCl, resulting in a white suspension, which was immediately extracted with EtOAc (200 mL). The organic layer was dried over anhydrous Na₂SO₄ and concentrated in vacuo to yield the N-Boc and benzyl ether protected D-pHPG in excellent yield and purity as a white foamy solid (10.72 g, 83%).

**N-Boc-O-Bn-D-pHPG methyl ester (15).** The protected free acid 14 (6.0 g, 16.8 mmol) was dissolved in non-fresh THF (100 mL) at room temperature, open to air. Freshly prepared diazomethane (FBD-I-258) by base hydrolysis of Diazald was added slowly until the yellow color persisted and completion was confirmed by TLC (40% EtOAc/Hex). Excess CH₂N₂ was quenched by adding acetic acid dropwise until effervescence and the yellow color disappeared. The THF was removed in vacuo, and the resultant oil was taken up in EtOAc (100 mL) before being washed once with saturated NaHCO₃ (200 mL). The organic layer was dried over Na₂SO₄.
and concentrated in vacuo to yield the methyl ester product as a white solid in excellent purity (5.66 g, 91%).

**N-Boc-O-Bn-D-pHPG alcohol (16).** To a flame dried 250 mL round bottom flask attached to an addition funnel was dissolved methyl ester 15 (7.66 g, 20.62 mmol) in freshly distilled THF (100 mL) at room temperature, under argon. The clear solution was cooled to -78°C before LiAlH₄ (1M in THF, 23 mL, 23 mmol) was added dropwise causing slight effervescence. The reaction mixture was stirred at -78°C for 5 minutes before being allowed to warm to room temperature until completion (1.25 hours, 30% EtOAc/Hex). The reaction mixture was then slowly poured into an ice cold saturated solution of Rochelle’s salt (sodium potassium tartrate, 200 mL) and stirred overnight at room temperature in order to break up alum in emulsions. The aqueous layer was then extracted with EtOAc (2 x 100 mL), dried over Na₂SO₄, and concentrated in vacuo to yield the desired alcohol as a pure flaky white solid (6.63 g, 94%).

**O-Bn-D-pHPG amino alcohol (17).** To a 250 mL round bottom flask was added Boc protected D-pHPG alcohol 16 (4.40 g, 12.81 mmol) and 1:1 TFA/DCM (25 mL) resulting in a dark red/brown solution. The solution was stirred at room temperature for 30 minutes (monitored by disappearance of starting material using 40% EtOAc/Hex TLC) before being concentrated in vacuo to an oil. The oil was taken up with EtOAc (100 mL) and slowly quenched with saturated NaHCO₃ until effervescence ceased. The aqueous layer was extracted with EtOAc (50 mL) and the combined organic layers were dried over Na₂SO₄, and concentrated in vacuo to yield the desired amino alcohol as an off-white/tan solid (3.10 g, 99%).

**N-Fmoc-O-Bn-D-pHPG alcohol (18).** To a 250 mL round bottom flask containing the amino alcohol 17 (2.75 g, 11.30 mmol) was added 1:1 H₂O/1,4-dioxane (100 mL) at room temperature,
open to air. To the solution was added NaHCO₃ (1.90 g, 22.6 mmol) before the mixture was cooled to 0°C and Fmoc-Cl (3.22 g, 12.4 mmol) was added at once. The reaction mixture was stirred at 0°C for 45 minutes before being diluted with distilled H₂O, extracted with EtOAc (2 x 50 mL), dried over Na₂SO₄, and concentrated **in vacuo** to yield an off-white paste, consisting of desired product and hydrolyzed Fmoc-Cl. The desired product was purified using flash column chromatography (40% EtOAc/Hex) as a white solid (4.2 g, 80%). The crude product can alternatively be used without further purification.

**N-Fmoc-O-Bn-D-pHPG aldehyde (19).** To a 250 mL round bottom flask was added alcohol 18 (1.58 g, 3.4 mmol) and non-fresh methylene chloride (50 mL) at room temperature, open to air. The solution was cooled to 0°C before Dess-Martin periodinane (DMP, 2.16 g, 5.10 mmol) was added at once. The reaction mixture was allowed to warm to room temperature for 45 minutes (monitored using 40% EtOAc/Hex) before being quenched with saturated Na₂S₂O₃ and NaHCO₃. The quenched reaction was extracted with CH₂Cl₂ (2 x 50 mL) and washed twice more with Na₂S₂O₃ and NaHCO₃ in order to removed DMP byproducts. The organics were dried over Na₂SO₄, and concentrated **in vacuo** to yield the aldehyde in excellent purity as a foamy white solid (1.47 g, 94%).

**O-Bn-D-pHPG dimethyl acetal (20).** To a 250 mL round bottom flask was dissolved protected aldehyde 19 (5.84 g, 12.6 mmol) in freshly distilled MeOH (50 mL) and trimethylorthoformate (50 mL) before a catalytic amount of tosic acid monohydrate (0.5 g, 2.6 mmol) was added. The reaction mixture was refluxed for 3 hours before being allowed to cool to room temperature, concentrated **in vacuo** to an oil, and re-dissolved in EtOAc. The organic layer was washed with saturated NaHCO₃ before being dried over Na₂SO₄ and concentrated **in vacuo** to yield the
dimethyl acetal as a white solid in excellent purity (6.3 g, 98%). The product and starting material aldehyde co-spot on TLC (40% EtOAc/Hex). In order to monitor the progress of the reaction, a small aliquot of the mixture (< 5 mL) is removed for crude \(^1\)H-NMR analysis, where absence of the aldehydic peak signifies complete formation of the acetal.

**O-Bn-D-pHPG amino acetal (21).** To a 250 mL round bottom flask containing 20 (3.87 g, 7.6 mmol) was added non-fresh THF (40 mL) at room temperature, open to air. To the solution was added piperidine (10 mL) resulting in a darkening of the solution. The deprotection in 20% piperidine/THF was monitored by the disappearance of starting material using TLC (40% EtOAc/Hex) and was complete after 15 minutes at room temperature. The reaction mixture was concentrated in vacuo to an orange oil before being azeotroped with toluene (3 x 100 mL), resulting in an off-white/orange solid. The desired amino acetal product was isolated using a plug of silica gel and 50% EtOAc/Hex to elute the high Rf Fmoc byproducts, followed by 10% MeOH/EtOAc to elute the free amino acetal as a clear oil (1.30 g, 60%). It is likely that heavy streaking of the amine occurs, which results in lower isolation.

**N-Ox-L-seryl-D-pHPG acetal (22).** To a 250 mL round bottom flask containing 21 (1.0 g, 3.5 mmol) was added anhydrous DMF (60 mL) at room temperature, under argon. To the solution was added solid 8 (1.14 g, 3.50 mmol) before cooling to 0°C and dissolving PyBOP (2.19 g, 4.20 mmol), resulting in a yellowing of the mixture. After 5 minutes at 0°C, DIPEA (1.22 mL, 7.00 mmol) was added dropwise. The reaction mixture was stirred at 0°C for 20 minutes and then allowed to warm to room temperature for 3 hours before being quenched with saturated NH\(_4\)Cl (100 mL) and EtOAc (50 mL). The aqueous was extracted with EtOAc (50 mL) before the combined organics were washed again with NH\(_4\)Cl (100 mL), twice with NaHCO\(_3\) (2 x 100 mL),
and once with NaCl (100 mL) before being concentrated *in vacuo* to yield a white solid. The desired dipeptide was isolated *via* flash-column chromatography (60% EtOAc/Hex) as a white solid (1.356 g, 65%). **Note:** After concentrating the organic layers *in vacuo* to a white solid, it is possible to triturate the crude product with cold EtOAc, and isolate the pure product using vacuum filtration, as it is sometimes difficult to completely dissolve the crude solid in a reasonable volume of solvent for chromatography. Additionally, if diastereomers are isolated, the desired isomer can be obtained through recrystallization using EtOAc/Hex.

**(-)-3-N-Ox-nocardicinate dimethyl acetal (23).** To a flame dried 250 mL round bottom flask was added 22 (1.74 g, 2.93 mmol) and freshly distilled THF (120 mL) at room temperature, under argon. The flask was covered with aluminium foil before DEAD (0.55 mL, 3.52 mmol) was added to the clear solution. The reaction mixture was stirred for 6.5 hours before being quenched with saturated NH₄Cl. The quenched reaction was extracted with EtOAc (2 x 50 mL), washed once with brine, dried over Na₂SO₄, and concentrated *in vacuo* to yield an oily off white solid. Purification using flash-column chromatography (5% acetone/DCM) yielded the optically pure desired product as a white foamy solid (1.2 g, 75%).

**(-)-3-aminonocardicinate dimethyl acetal hydrochloride (24).** Ox protected β-lactam dipeptide 23 (0.93 g, 1.6 mmol) was dissolved in a minimal volume of non-fresh THF (< 5 mL) in a 250 mL Parr pressure flask. To the clear solution was added a catalytic quantity of Pearlman’s catalyst (0.47 g, 20% Pd(OH)₂/C), before the reaction mixture was hydrogenated at 50 atm for 48 hours at room temperature. The reaction mixture was filtered over Celite, which was then washed with THF (3 x 10 mL), before being concentrated to an oil *in vacuo*. The oil was partitioned in a biphasic mixture of Et₂O (9mL) and acidic water (3 mL, 150 μL 12 M HCl,
The aqueous layer was further extracted with ether (2 x 9 mL) before being frozen using liquid nitrogen and lyophilized overnight to yield the hydrochloride salt 24, with slight impurities.

N-acetyl-D-pHPG (25): To a 250 mL round bottom flask containing 1:1 THF/H₂O (120 mL) was added D-pHPG (6.0 g, 35.9 mmol) and NaHCO₃ (6.0 g, 71.8 mmol) at room temperature, open to air. To the suspension was added acetic anhydride (3.73 mL, 39.5 mmol) dropwise, resulting in effervescence. The reaction mixture was stirred at room temperature for 45 minutes, during which the suspension cleared. The reaction was then diluted with EtOAc and acidified to pH ~ 1 with 1M HCl. The aqueous layer was extracted with EtOAc (3 x 100 mL), with the combined organics then washed with brine (100 mL), dried over Na₂SO₄, and concentrated in vacuo to yield the desired product as a pure white solid (6.07 g, 81%).

N-acetyl-(-)-nocardicin G dimethyl acetal (26): To a flame dried 2 mL pear shaped flask was added lyophilized nocardicin G acetal 30 (5.4 mg, 0.013 mmol) and freshly distilled THF (260 μL) at room temperature, under argon. To the clear solution was added freshly prepared aqueous NaHCO₃ (260 μL, 0.05 M stock, 0.013 mmol) and freshly distilled acetic anhydride (130 μL, 0.1 M stock, 0.013 mmol). The reaction mixture was allowed to stir at room temperature for 1.5 hours, whereby monitoring by TLC (10% MeOH/CHCl₃) and ninhydrin stain showed complete consumption of the baseline starting material. The reaction mixture was then frozen and lyophilized prior to analysis by LCMS.

4,5-diphenyl-4-oxazolin-2-one (27): To a flame dried 1 L round bottom flask fitted with a reflux condenser was added benzoin (40 g, 188 mmol), anhydrous benzene (400 mL), 1,1-CDI (36.7 g, 226 mmol), and N,N-dimethylaniline (24 mL, 188 mmol) at room temperature, under
argon. The reaction mixture was heated to reflux for 1 hour and 40 minutes before being cooled to room temperature, concentrated in vacuo to an oil, and redissolved in methylene chloride (100 mL). The organic layer was washed with 1M HCl (3 x 100 mL), causing a white precipitate/suspension to briefly form before clearing, distilled H₂O (2 x 200 mL), and saturated NaCl (100 mL) before being dried over Na₂SO₄ and concentrated to an oil. The desired product began to crystallize out of the oil at room temperature, and was placed in the refrigerator overnight to complete crystallization. The crystals were collected and washed with EtOH to yield pure product as long, thick white needles (44.43 g, 99%).

**N-Cbz-D-pHPG (28):** In a 250 mL round bottom flask was dissolved Na₂CO₃ (4.0 g, 37.7 mmol) in distilled water (40 mL, 10% Na₂CO₃ solution) at room temperature, open to air, before D-pHPG (3.0 g, 18 mmol) was added. The solution was cooled to 0°C before Cbz-Cl (2.83 mL, 19.8 mmol) dissolved in dioxane (30 mL) was added at once. The reaction mixture was stirred at 0°C for 30 minutes and at room temperature for 1 hour. The dioxane was removed in vacuo and the aqueous poured into ice water (100 mL) before being extracted twice with EtOAc (2 x 50 mL). The aqueous was then acidified to pH ~ 2 with 1M HCl before being extracted with EtOAc (100 mL), dried over Na₂SO₄, and concentrated in vacuo to yield the desired product as a pure white solid (5.08 g, 94%).

**N-Cbz-(-)-nocardicin G dimethyl acetal (29):** To a flame fried 10 mL round bound flask was added N-Cbz-D-pHPG (0.25 g, 0.825 mmol) and freshly distilled acetone (1.8 mL), yielding a clear solution at room temperature, under argon. To the solution was added 2,6-lutidine (90 μL, 0.77 mmol) and N-methylmorpholine (18 μL, 0.165 mmol). The reaction mixture was then cooled to -50°C before isobutylchloroformate (100 μL, 0.77 mmol), dissolved in freshly distilled
acetone (500 μL), was added dropwise, resulting in a clear solution. The reaction mixture was allowed to warm from -50 to -30°C over 30 minutes, during which a white precipitate forms, indicating successful formation of the mixed anhydride. The mixture was then placed in a 0°C icebath for 20 minutes, during which the β-lactam reagent is prepared for addition. Free amine nocardinicinate 24 was dissolved in anhydrous dimethylformamide (1 mL) in a flame dried 5 mL round bottom flask at room temperature, under argon, before being cooled to 0°C and charged with 2,6-lutidine (90 μL, 0.77 mmol). The cold, basified β-lactam was then added dropwise to the mixed anhydride reaction mixture at 0°C. The combined reaction mixture was stirred at 0°C for 30 minutes, then allowed to warm to room temperature over 3 hours. The reaction was diluted with EtOAc, and washed with ammonium chloride (2 x 25 mL), sodium bicarbonate (2 x 25 mL), and brine (1 x 25 mL) before being dried over anhydrous Na₂SO₄ and concentrated in vacuo to yield the impure desired tripeptide as an off-white foam. The progress of the reaction was monitored by TLC (10% MeOH/CHCl₃). The product was isolated as a white foam via flash column chromatography (10% MeOH/CHCl₃).

(-)-Nocardicin G dimethyl acetal (30): To the 25 mL round bottom flask containing 29 (0.036 g, 0.065 mmol) was added non-fresh MeOH (5 mL) at room temperature, open to air. The solution was transferred to a hydrogenation pressure flask before 0.02 g of Pearlman’s catalyst was added. The reaction mixture was hydrogenated overnight at 50 psi, before being filtered through Celite, and concentrated in vacuo to yield an off-white solid. The reaction can be followed by the disappearance of starting material by TLC (10% MeOH/CHCl₃), and the appearance of a free amine spot with ninhydrin stain. The desired product was purified by reverse phase HPLC and lyophilized to yield a white solid. Retention time: 23.2 minutes, strong chromophore at 230 nm UV.
5.0 References:


