THE DEVELOPMENT OF DEOXNYBOMYCINS AS POTENT AND SELECTIVE ANTIBIOTICS AGAINST FLUOROQUINOLONE RESISTANT BACTERIA

BY

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THESIS

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Abstract

Antibiotic-resistant bacterial infections pose a real and increasing threat to public health and welfare. As bacteria continue to develop and spread resistance to currently used antibiotics it is of the upmost importance to develop new classes of antibiotics to combat them. Deoxnybyomycin (DNM), which has been previously found to be efficacious against fluoroquinolone resistant bacteria including *S. aureus*, is one such antimicrobial compound, and its further derivatization is reported herein.

Derivatives of DNM had been previously synthesized possessing alkyl substitutions at four different positions. Described in Chapter 2 is the synthesis of several new DNM based compounds containing either previously unexplored polar functionality or new modifications to the five-membered ring. These compounds were tested against fluoroquinolone resistant and wild-type *S. aureus*, and the derivatives based on modifying the five-membered ring were found to maintain strong activity and selectivity against fluoroquinolone resistant bacteria possessing a S84L mutation in GyrA. The polar compounds were found to be less active against fluoroquinolone resistant *S. aureus*, but at least one was found to maintain a strong difference in selectivity.

Acknowledgements

There are many people I would like to thank for supporting me throughout my time at Illinois. First and foremost I would like to thank my adviser Paul Hergenrother for his continued encouragement, advice and leadership. He created the Hergenrother research group in which I have enjoyed the past several years, and provided the funding, resources, and environment in which I have researched.

Next I would like to thank my Dr. Elizabeth Parkinson, who mentored me throughout my time in the lab. Her advice, leadership and encouragement allowed me to achieve as much as I did, and I am unendingly grateful to have been able to receive her guidance.

I would like to thank my family who have never ceased to support me unconditionally no matter what I went through. Their encouragement through good times and bad along with several trips up to Champaign to make sure I was still eating healthy brought me more peace than they could know. Without them I would not be here today.

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Table of Contents

Chapter 1. Introduction

1.1 Antibiotic usage before the modern era

The modern antibiotic era is often thought to begin with the discovery of penicillin by Alexander Fleming in 1928.[1,2] However, human exposure to antibiotics goes back at least as far as 350-550CE as traces of tetracycline likely high enough to have a protective effect have been found in human bone samples with tissue sample suggesting low rates of infection.[3,4] Additionally, antibiotics and antibiotic containing compounds have been utilized in traditional Chinese medicine, most famously artemisinin,[5] but other compounds have been discovered in different herb mixtures.[6] The use of antibiotics in humans ultimately pales in comparison to the production and use of antibiotics in the terrestrial and marine biomes from which many current antibiotics are derived. Cyanobacterial communities producing antibacterials have been dated back as far as 3.5 billion years.[7]

1.1.1 Ancient bacterial resistance mechanisms

As a result of the extensive history of antimicrobial use in the natural world, methods of resistance have evolved with all bacteria possessing genes capable of responding to foreign small molecules.[8] As a result, the bacterial resistome is ancient and vast and serves as a large reservoir to facilitate gene transfer and eventual resistance to the use of antibiotics in the present era with many non-pathogenic species carrying extensively drug resistance conferring genes.[9] These bacteria can facilitate the maintenance and spread of bacterial resistance genes. Plasmids encoding for the production of β-lactamases, which provide resistance against antibiotics such as penicillin and cephalosporin, have been found to be nearly 100 million years old.[10]

1.2 The threat of antibiotic resistance to human health

Since the beginning of the modern antibiotic era bacteria resistance has eventually emerged to all major classes of antibiotics.[11] This has resulted in an epidemic in which each year roughly 25,000 EU and 23,000 US residents die as a result of infection from multidrug resistant bacteria.[12,13] Unfortunately, human antibiotic consumption is strongly correlated with an increase in antibiotic resistance, leading to the scenario in which antibiotics eventually render themselves obsolete.[14] As a result, the need to develop new antibiotics and, in particular, new antibiotics with orthogonal modes of action to current agents, is high and constantly increasing.

Resistance mechanisms are often described in terms of “target” or “bullet”.[2] The antibiotic, the bullet, can be modified to render it inactive, destroyed by enzymes, removed via efflux pumps or prevented from entering the cell at all through manipulation of the cell membrane. The target of the antibiotic can be modified to be insensitive to the antibiotic, replaced by another protein capable of fulfilling its function or otherwise protected to prevent the antibiotic from accessing it. Bacteria that are able to acquire these methods of resistance through either gene transfer or mutation are capable of resisting antibiotics and passing these methods on to other bacteria thus increasing the prevalence of resistance mechanisms in nature.[15]

1.3 Deoxynybomycin as an antibiotic for fluoroquinolone resistant bacteria

Nybomycin (NM) was first identified and isolated from a streptomycete Missouri soil sample and wasfound to be active against *S. aureus*, *M. smegmatis*, and *Bacillus* species.[16,17] During its chemical synthesis the compound deoxynybomycin (DNM,

Figure 1.1) was synthesized as an intermediate and eventually shown to be a natural product itself.[18,19] Recently, DNM was recently shown to target fluoroquinolone resistant (FQR) MRSA by inhibiting the mutant DNA gyrase A,[20,21] and was considerably more selective for the FQR *S. aureus* than the wild type, prompting an investigation into a possible new class of antibiotics that target resistance mechanisms. Until recently DNM lacked a practical synthetic route and issues with its low solubility confounded efforts to explore it further. In chapter 2 we will demonstrate how new derivatives with greater solubility have been synthesized addressing this low solubility.[20]

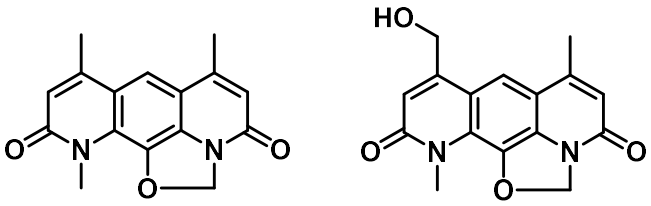

Figure 1.1. Structures of Deoxynybomycin and Nybomycin

1.3.1 Deoxnybomycin and floruoqinolone resistance

It is perhaps unsurprising that resistance to many natural product or semisynthetic antibiotics could develop in pathogenic bacteria due to the heavy selective pressure from the clinical use of antibiotics. These bacteria are able to draw not just on their own ability to evade antibiotics, but the bacterial resistome as well. Other bacteria may have evolved methods of evading or neutralizing the antibiotics which can be passed horizontally, by passing and receiving plasmids containing resistant genes, and then spread vertically to their daughter cells. Most clinically approved antibiotics target only a few pathways, such as folate, and cell wall

biosynthesis, as protein synthesis in the ribosome.[19,22] Between selective pressure and horizontal gene transfer, bacteria can develop or acquire resistance to natural product antibiotics that attack these highly conserved bacterial pathways given time and exposure. It is more perplexing to consider the case of synthetic antibiotics such as fluoroquinolones, which are currently the only clinically approved antibiotics that attack DNA replication. Being entirely synthetic, one might expect that resistance to fluoroquinolones would be slower to develop due to a lack of ready-made defenses present in the bacterial genome, but bacteria are able to respond to fluoroquinolones through both adaptation of cellular responses as well as acquiring low level resistance mechanisms through co-opting other cellular response pathways. The qnr genes are an important example of how bacteria can respond to synthetic antibiotics. QnrA and qnrS, thought to originate from the marine algae *Shewanella algae*, aids bacterial replication by easing DNA relaxation ahead of the replication fork which also gives a protective effect against flouroquinolones.[23] Plasmids carrying these genes are increasingly found in cases of low level fluoroquinolone resistant pathogens and it has been shown that they greatly increase the likelihood of developing high level resistance.

An equally pressing question: why do the wild-type *S. aureus* display considerable resistance to the natural product DNM, which targets DNA replication, when the fluoroquinolone resistant strains possessing gyrase A mutations of S84L, as in the majority of MRSA cases, are highly susceptible?[20,21] This same serine mutation is known across a number of FQR bacterial species and is consistently associated with resistance to the synthetic antibiotic fluoroquinolones.[20] It has even been shown that cycling DNM and ciprofloxacin exposure can result in resistance and reversion of that

resistance back to wild type suggesting that the key difference between the two strains hinges on this serine.[20] In other words, why is a natural product antibiotic like DNM mostly ineffective against wild-type bacteria, but highly potent against bacteria that have undergone this serine mutation to resist fluoroquinolones?

Though DNM has been shown to inhibit the mutant DNA gyrase A, the exact nature of its complex with bacterial DNA and proteins remains unknown as no crystal structure has been reported. It is, however, believed that the presence of this serine 84 allows for fluoroquinolones to function by helping forming a Mn^{2+} water bridging interaction between the keto acid motif of fluoroquinolones and the serine, thus stabilizing the cleavage complex and giving fluoroquinolones their potency (Figure 1.2).[24,25] Based upon DNM's lack of strong potency against bacteria possessing this serine, and its status as a natural product, it is possible that the large degree of conservation of the serine across many bacterial species is itself a resistance mechanism against DNM and NM which the fluoroquinolones such as ciprofloxacin are exploiting for their mode of action. The mutation of the serine to a leucine or isoleucine, which has been shown to be achievable with a single point mutation, is then a reversion or a mutation along a previous pathway to avoid the fully synthetic fluoroquinolones.

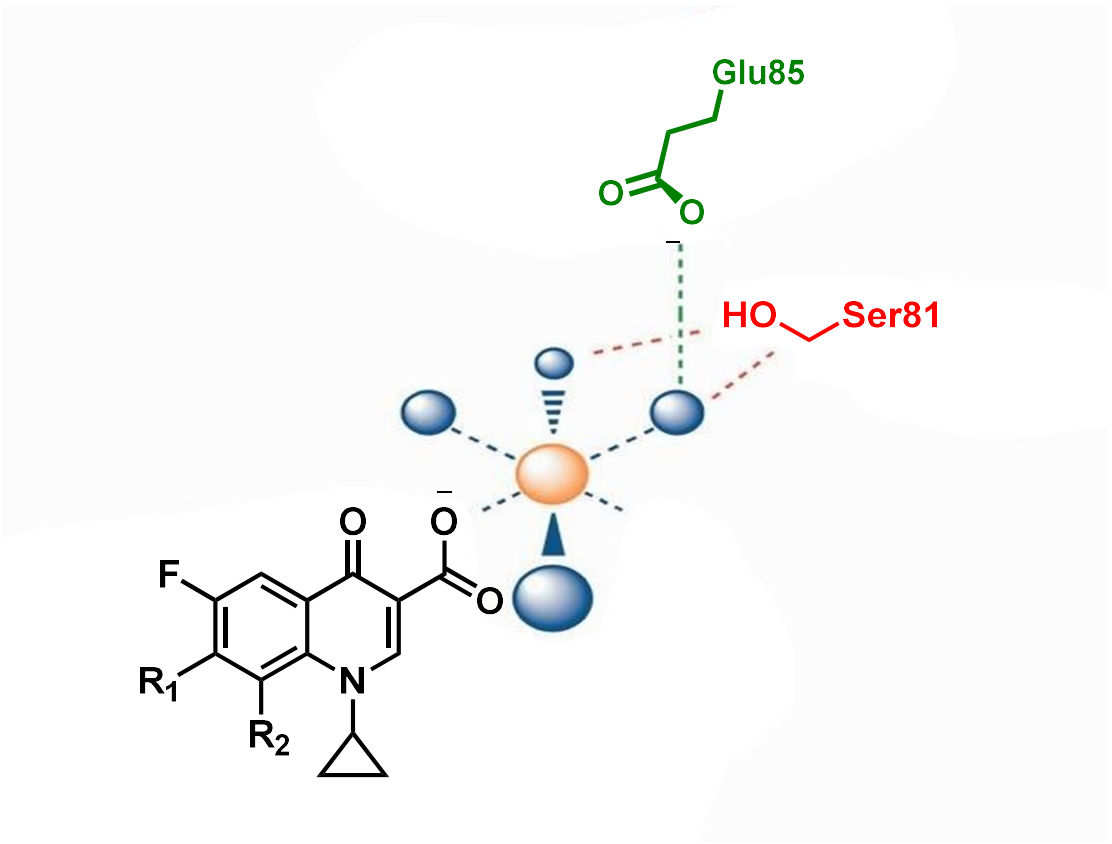

Figure 1.2 Keto-acid motif of fluoroquinolones chelates a Mn^{2+} ion (pink circle) forming a water (blue circles) bridge to the serine residue in Gyr A of *B. anthracis*. Mutation of Ser81 disrupts this interaction. Figure adapted from Wohlkonig et al.[26]

1.4 Summary

Antibiotic resistance is a recognized and still growing problem of immense clinical relevance. The need to discover and develop new antibiotics is obvious, and the DNM platform demonstrates a method of subverting the usual bacterial resistance mechanisms to target FQR bacteria. Because it targets the mutation that confers resistance to the synthetic fluoroquinolones, resistance to DNM typically restores susceptibility to fluoroquinolones thus preventing the generation of new more highly resistant bacterial strains. The difficulty in accessing DNM derivatives synthetically and

its lack of potency against the fluoroquinolone sensitive *S. aureus*, as well as against gram negative pathogens has largely inhibited its exploration as an antibiotic.

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Chapter 2. Synthesizing Deoxynybomycin derivatives for activity against FQR gram positive and gram negative pathogens

Portions of this chapter are reprinted with permission form Parkinson, E. I.; Bair, J. S.; Nakamura, B.; Lee, H. Y.; Kuttab, H. I.; Southgate, E. H.; Lezmi, S. Lau, G. W.; Hergenrother, P. J. *Nat. Commun*. **2015**, *6*, 6947. Copyright 2015 Nature Publishing Group. Contributions of others are noted when applicable.

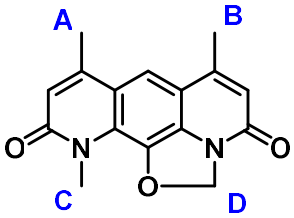
2.1 Previous advances in deoxynybomycin synthesis

When DNM was reported to possess activity against FQR *S. aureus* as well as *M. smegmatis* and *Bacillus* species there was still no practical synthetic route to produce significant quantities and new derivatives of DNM.[1] Efforts by Dr. Elizabeth Parkinson, a previous graduate student in the Hergenrother group, resulted in a modular synthetic route that allowed access to several DNM derivatives with simple alkyl substitutions at four positions on the core scaffold by using a late stage intermediate in a previously developed synthesis of deoxynyboquinone by Dr. Joseph Bair.[2] Fourteen derivatives of DNM were synthesized in 7 steps with an 11% overall yield.

2.1.1 Goals in synthesizing new DNM derivatives

Despite many of these compounds showing increased solubility (3-13 fold in pH 7.4 buffered saline compared to the parent DNM) with comparable potency against FQR *S. aureus*,[2] it was thought that solubility and by extension pharmacokinetic properties could be improved further with the synthesis of DNM derivatives containing polar functionality. It was hypothesized that the synthetic route could be modified to carry protected functional groups, to be unmasked as the desired derivatives. Additionally, we

hypothesized that some polar derivatives could display greater efficacy against FQR bacteria expressing alternative serine mutations to polar residues such as arginine, against which DNM has reduced potency. Eventually it became clear that further functionalization at the methylene bridge of the five member ring (site D in Figure 2.1) could allow access to further derivatives with increases in solubility, and this became a new site of derivatization.



Deoxynybomycin
DNM

Figure 2.1 Structure of deoxnybomycin, with primary sites of derivatization labeled A-D.

2.2 Synthesis of nybomycin and DNM derivatives containing polar functionality

Building on work previously performed in the Hergenrother research group, several DNM derivatives were synthesized by making modifications to the previous route to carry methyl protected alcohols which would then be unmasked and transformed into the desired functional groups (Figure 2.2).[2] The route involved the synthesis of two vinyl iodo-amide cross coupling partners carrying the derivatives' modifications, and their use in a mixed Suzuki cross coupling with a symmetrical aryl boronate. The mixed cross coupling produced both symmetrically and non symmetrically substituted products, and the desired cross coupling product was isolated. Subsequently, a palladium-catalyzed Buchwald-Hartwig ring closing was used

to generate the diazanthracene which was then unmasked via HBr to reveal the dianthracenol. A methylene bridge was then inserted with dibromomethane to give the final DNM derivatives as previously described by Hergenrother and coworkers (Scheme 2.1).[2,3]

This route was followed closely, but modified by the addition of protected functional groups which could be carried inert through the route and deprotected at the end to yield both a polar final product as well as a functional handle which could then be used to access new polar functionality. Nybomycin, as well as its amine variant (**1**, see Figure 2.2) were selected as targets as nybomycin could be compared to literature values, and **2** and **3** were selected do to their similarities to a DNM variant **4** possessing an alkyl chain whose activity was known to be comparable to DNM.[2]

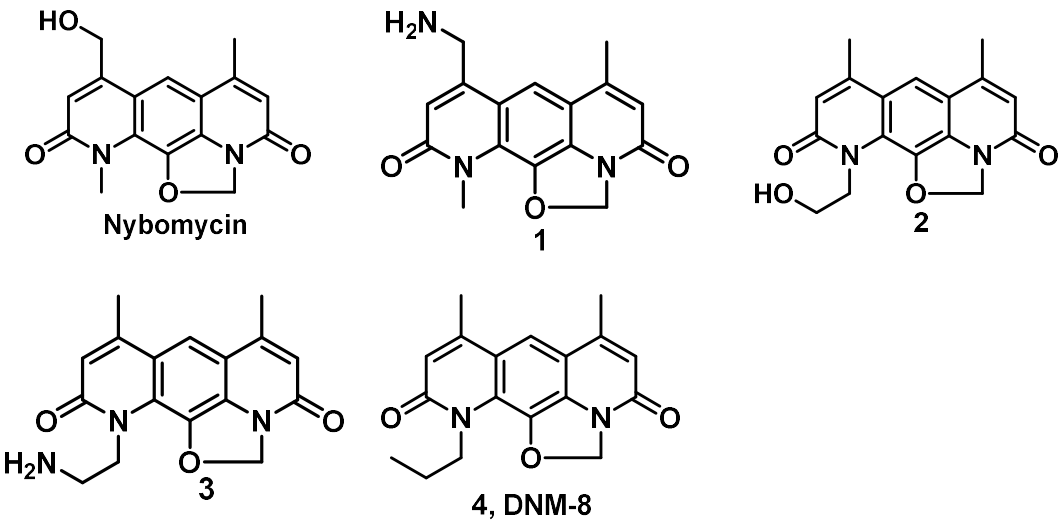
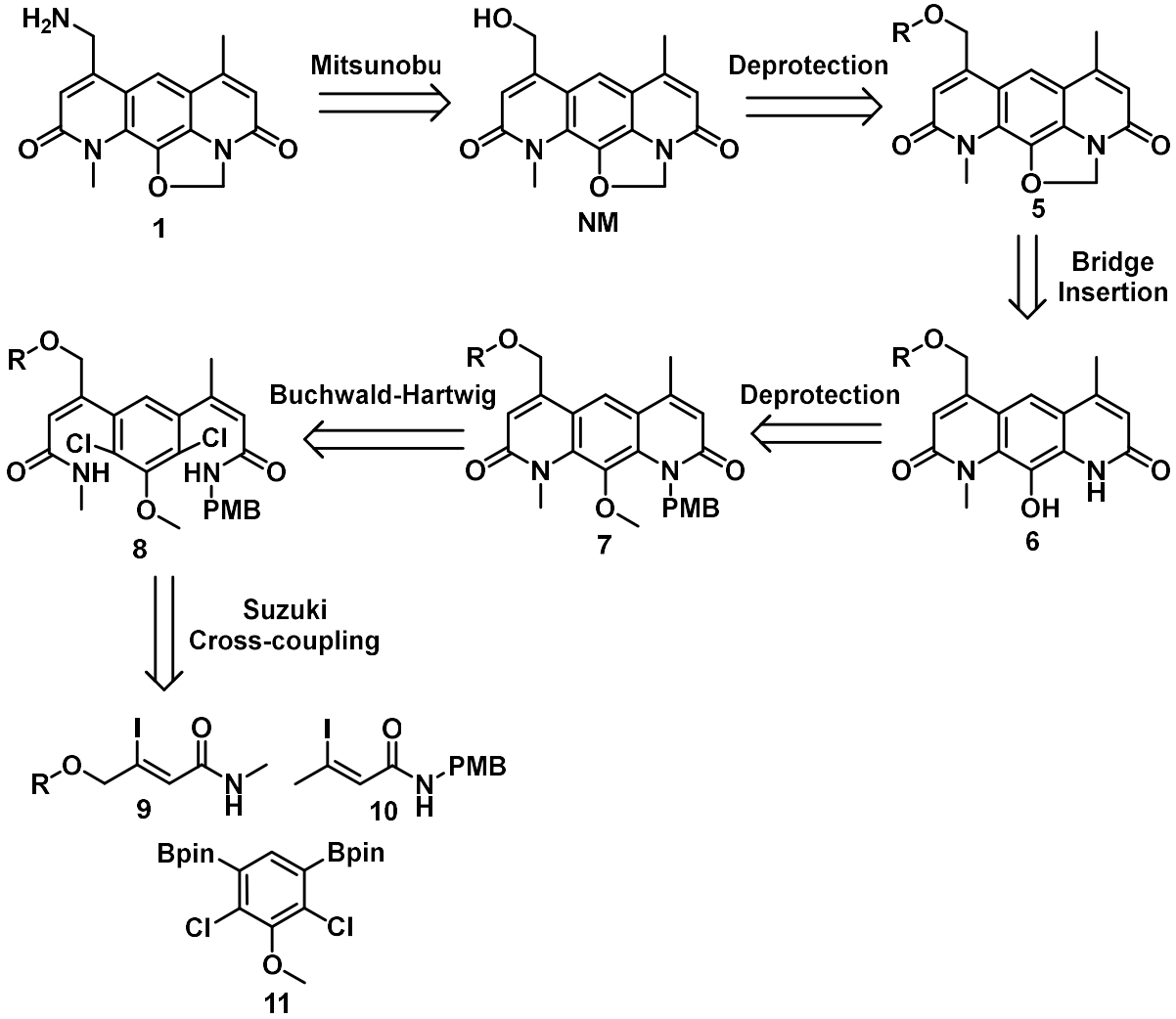


Figure 2.2 Targets for synthesis containing polar functionality. DNM-8, **4** originally synthesized by Dr. Elizabeth Parkinson.

2.2.1 Proposed route for synthesis of DNM derivatives with polar functionality

Adapting the previous synthesis it was hypothesized that protected functional groups could be carried through the synthetic route to be unmasked following diazanthracene formation (Scheme 2.1). Full experimental details including tabulated spectra can be found in section 2.5.

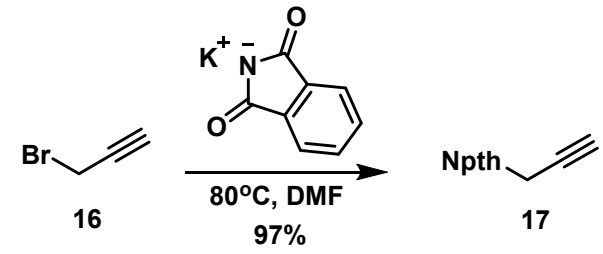


Scheme 2.1 Proposed synthesis of NM and **1** via adaptation of DNM synthetic route using cross coupling partners with protected polar functional groups.

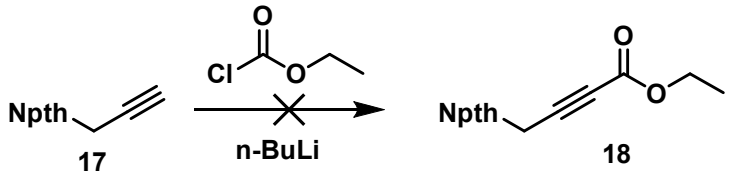
A number of protecting groups were utilized in an attempt to carry polar functionality through the route. As the route hinged on a Pd-mediated Suzuki cross coupling carried out in the presence of base and water it was necessary to use functional groups that were stable to basic and aqueous conditions. Most challenging, the protecting group or the polar functionality it masked would have to be stable through the harsh 48% HBr or BBr_3 as a result of the need to deprotect the aryl methyl ether. Finally, protecting groups carrying polar functionality to the A or B position (see Figure 2.1) would need to be stable to n-BuLi as well as refluxing acetic acid used during the synthesis of the iodoamide coupling partners.

2.2.2 Synthesizing cross coupling partners with masked polar functional groups

To optimize step economy, both primary amines and alcohols were intended to serve as starting materials for the synthesis of the iodoamide coupling partners. As it was expected that the amine would need to be completely protected for the addition of ethyl chloroformate, a pthallamide protecting group was chosen due its relative stability and the ability to deprotect it orthogonally later on. **17** was formed in near quantitative yield through the reaction of commercially available **16** with potassium pthalimide. Ultimately this line of inquiry was abandoned due to immediate polymerization upon exposing the pthalimide protected amine to n-BuLi at any concentration or temperature (Scheme 2.2).



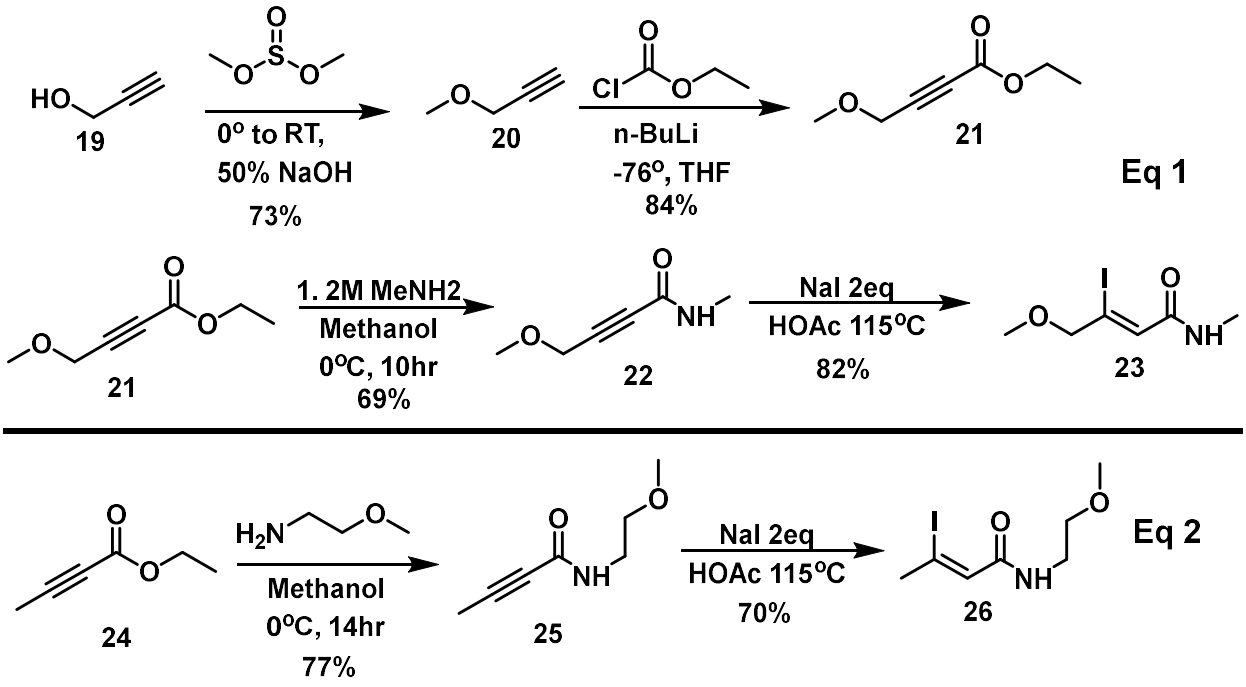
Eq. 1



Eq. 2

Scheme 2.2 Attempts to carry a primary amine with a protecting group as a trisubstituted amine were unsuccessful as polymerization stymies attempts to make **16**.

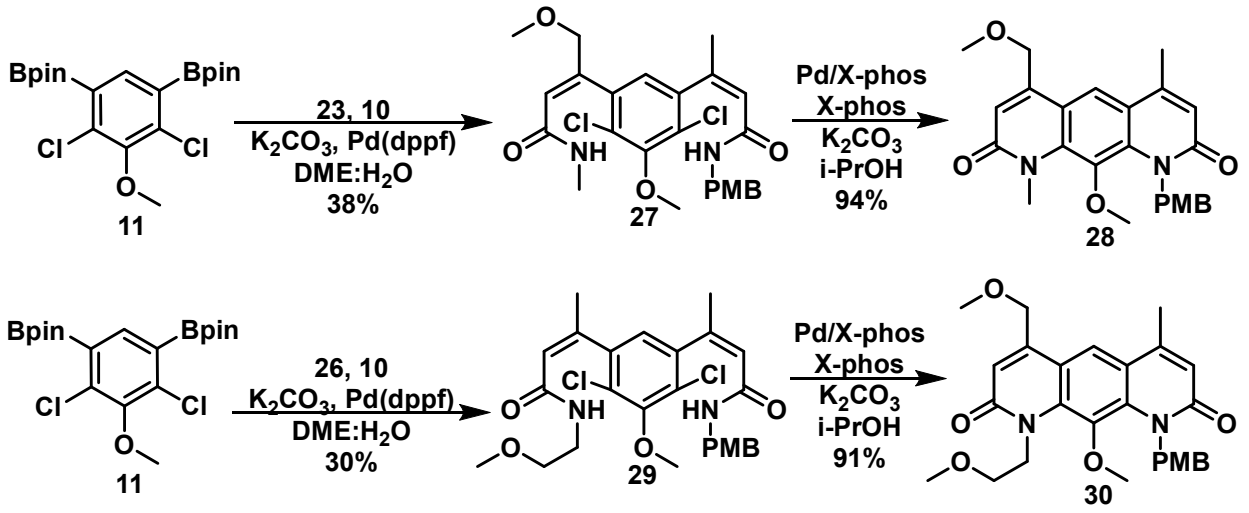
After the primary amine showed difficulties in achieving the intended results, a primary alcohol was chosen as the protected functionality to carry through the synthetic route as it could be easily installed, masked, and starting materials containing them were cheap and abundant. **19** was chosen as a starting point due to its commercial availability, and **20** was synthesized via reaction with dimethyl sulfate. The methyl protected propargyl ether was deprotonated with n-BuLi and reacted with ethyl chloroformate to generate the protected ester **21**. Amide **22** was synthesized from **21** through the addition of 2M methylamine. **23** was synthesized from **22** through refluxing acetic acid in the presence of NaI (Scheme 2.3).[2] Cross coupling partner **26** was synthesized from commercially available **24** by analogous reactions with methoxyethylamine and sodium iodide. See section 2.4 for full synthetic protocols.



Scheme 2.3 Synthesis of methyl protected iodoamides **23** and **26** for use as coupling partners

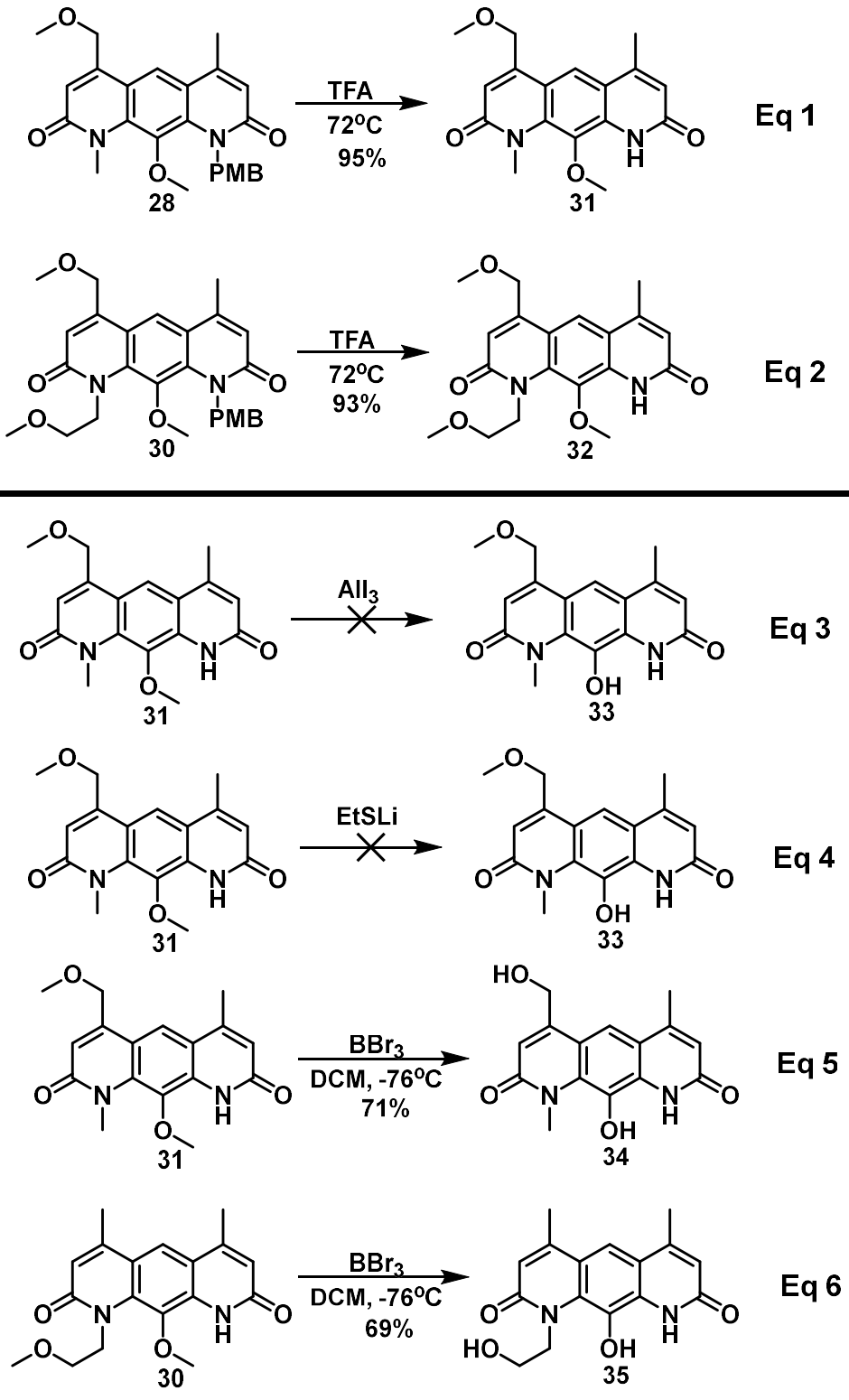
2.2.3 Synthesizing polar DNM derivatives from cross coupling partners

After obtaining cross coupling partners **23** and **26**, a mixed cross coupling was carried out according to the previously established protocols with symmetrical aryl boronate **11** and **10** resulting in **27** and **29** in 30-38% yields as well as small amounts of the symmetrically coupled products (Scheme 2.4).[3] The compounds were subjected to the palladium catalyzed ring closing reaction to give **28** and **30** in over 90% yields.



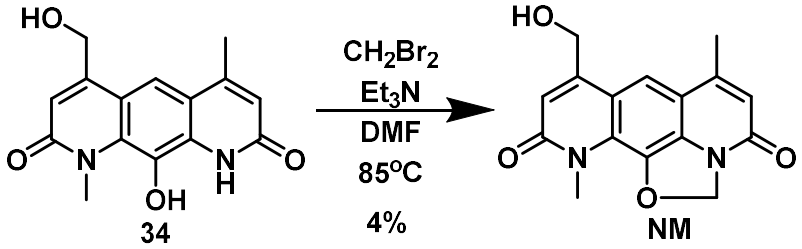
Scheme 2.4 Dianthracenes **28** and **30** synthesized from mixed cross couplings followed by palladium mediated ring closing.

Based upon Rineheart et al's synthesis of deoxnybomycin it was hypothesized that the use of HBr could result in the loss of the alcohol along with its methyl protecting group.[4,5] A two-step deprotection strategy was thus employed in which TFA was first used to deprotect the PMB group which resulted in **31** and **32**. Several agents were then used to attempt a selective demethylation of the phenol or methyl protected alkyl ether (Scheme 2.5).[3] Despite proving successful in the demethylation of simple anisole, neither thiol lithium salts nor aluminum and triiodide were able to deprotect either alcohol.[6,7] Ultimately BBr_3 was used to deprotect at both positions simultaneously resulting in the fully deprotected dianthracenes **34** and **35** (Scheme 2.5).



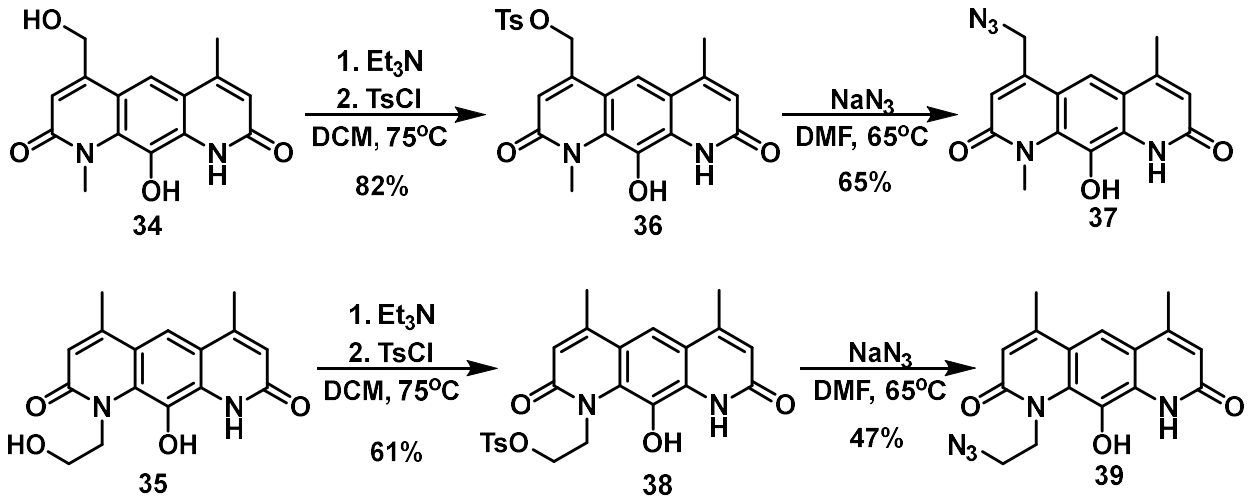
Scheme 2.5 Fully deprotected dianthracenes **34** and **35** synthesized from TFA and BBr_3.

With the alcohols now unmasked, the resultant dianthracenes were insoluble in most organic solvents except for DMF. The products could not be readily purified by chromatography. Furthermore, attempts to insert the methylene bridge resulted in non-selective alkylation that thwarted attempts to produce the polar products in good yields, though NM was prepared from **34** in very low yields (Scheme 2.6).



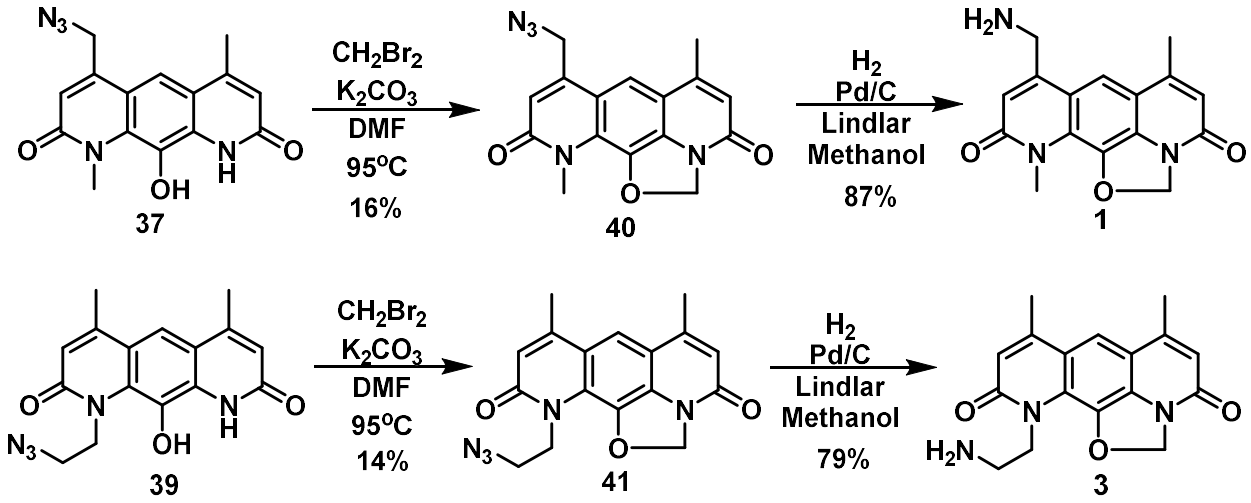
Scheme 2.6 Nybomycin synthesized in low yields from **34**.

Instead of attempting the bridge insertion and then transforming the alcohols to the desired amines, the alcohols were first tosylated to produce **36** and **38**, then displaced by excess sodium azide to generate **37** and **39** (Scheme 2.7). The Mitsunobu reaction was also successful in generating the azides **37** and **39**, but it also produced inseparable phosphine oxide contamination that ultimately rendered it unusable.



Scheme 2.7 Azides **37** and **39** generated from tosylation and displacement of primary alcohols **34** and **35**.

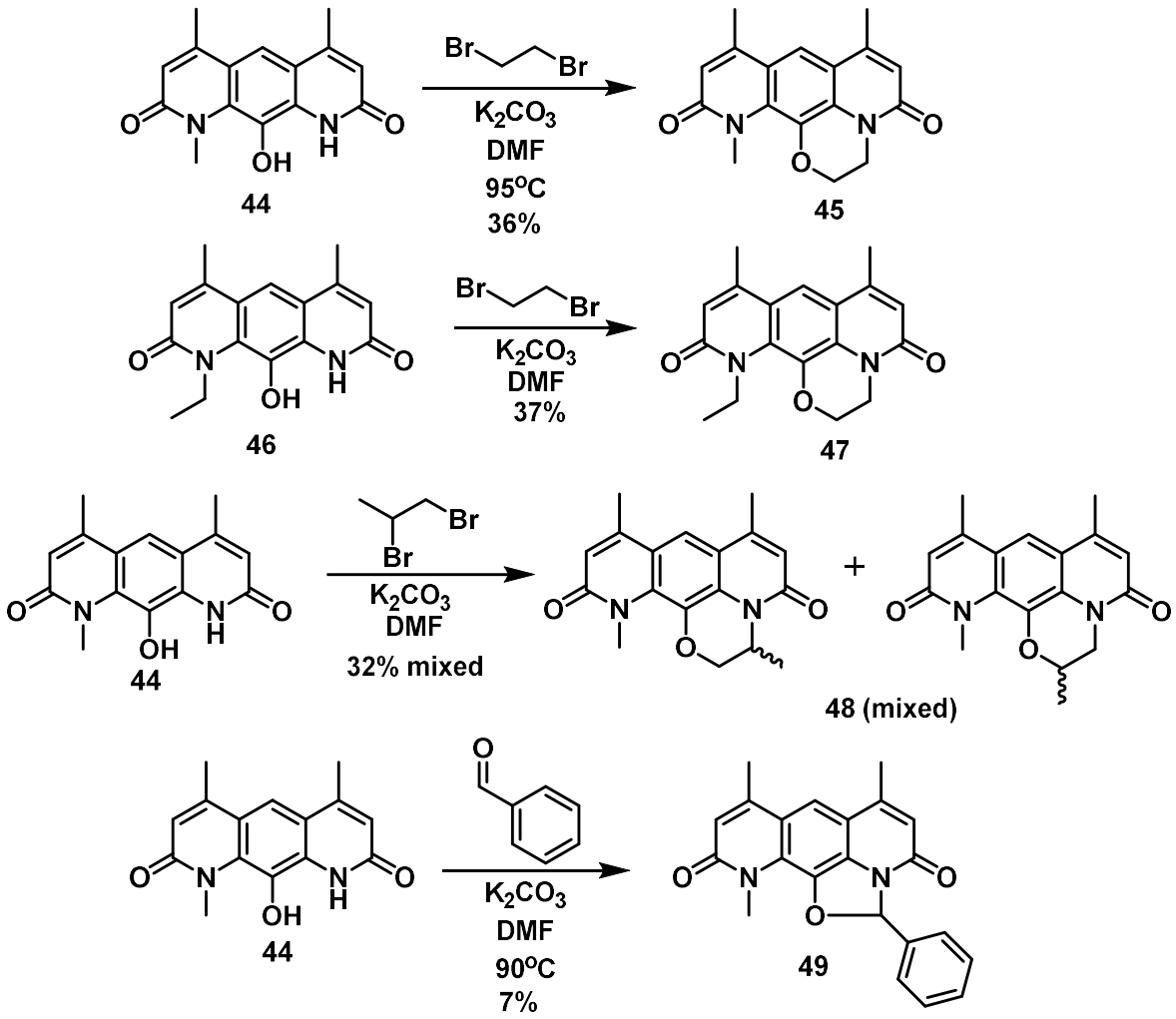
Finally, the methylene bridge could be inserted by dibromomethane addition to **37** and **39** to synthesize **40** and **41** which were much more soluble in organic compounds and comparatively easier to isolate (Scheme 2.8). Azides **40** and **41** were reduced to their amines **1** and **3** by Lindlar's catalyst with 1 atm hydrogen to avoid any issues with reducing the core ring system. In this manner, amines **1** and **3** were synthesized in 12 steps and 0.63% yield for **1** and 9 steps and 0.3% yield for **3**.



Scheme 2.8 Amines **1** and **3** were synthesized by reduction with Lindlar's catalyst.

Section 2.3 Synthesis of new DNM derivatives off the five-membered ring

Though positions 1-3 had been investigated with a variety of alkyl substitutions and position 4 has seen the addition of a methyl group, there were still opportunities to further the SAR at the five member ring (figure 2.1).[2] In order to investigate the effects of substitution at that position on antibacterial activity, three new derivatives were synthesized as simple variations on a 6 member ring from **44** and **46** whose synthesis was previously reported by the Hergenrother group.[2] Following the usual bridge insertion protocol, alkyl bromides were used to extend the five-membered ring. Additionally, derivative **49** containing a phenyl ring was synthesized using benzaldehyde to examine whether substitutions of this size and shape would be tolerated (Scheme 2.9).



Scheme 2.9 DNM derivatives from the five membered ring core were synthesized.

Section 2.4 Evaluating new derivatives for antibiotic activity against wild type and FQR *S. aureus*

The DNM derivatives newly synthesized above were tested for activity against wild type (ATCC 29213) and FQR (NRS3) *S. aureus* using 3 repetitions of the standard microbroth dilution assay to determine MIC (Figure 2.3). These two strains display the critical S84 and S84L residues that confer fluoroquinolone resistance and DNM sensitivity (see chapter 1). The activity of several DNM derivatives has already been

reported against these strains.[2] The susceptibility of 29213 and NRS3 was determined for the newly synthesized derivatives (table 2.1).

All of the derivatives made off the five-membered ring maintained both their strong selectivity for the FQR strain (4**48** is 246 times more potent against FQR *S. aureus)* as well as their general efficacy with the exception of **49**. The lack of potency suggested that the steric bulk of the phenyl ring of **49** may be too large, and it may be inhibiting the usual mode of binding. In contrast, **4**, **45**, **47** and **48** all retained potency just short of the parent compound DNM, while being soluble in this assay at higher concentrations in DMSO.

The polar derivatives including amines **1** and **3** as well as azides **40** and **41** were unfortunately much less potent against FQR *S. aureus* though at least one maintained a strong (16fold) preference for the FQR strain which suggested an engagement with its target. All derivatives were soluble at concentrations of at least 0.8 mg/mL in DMSO.

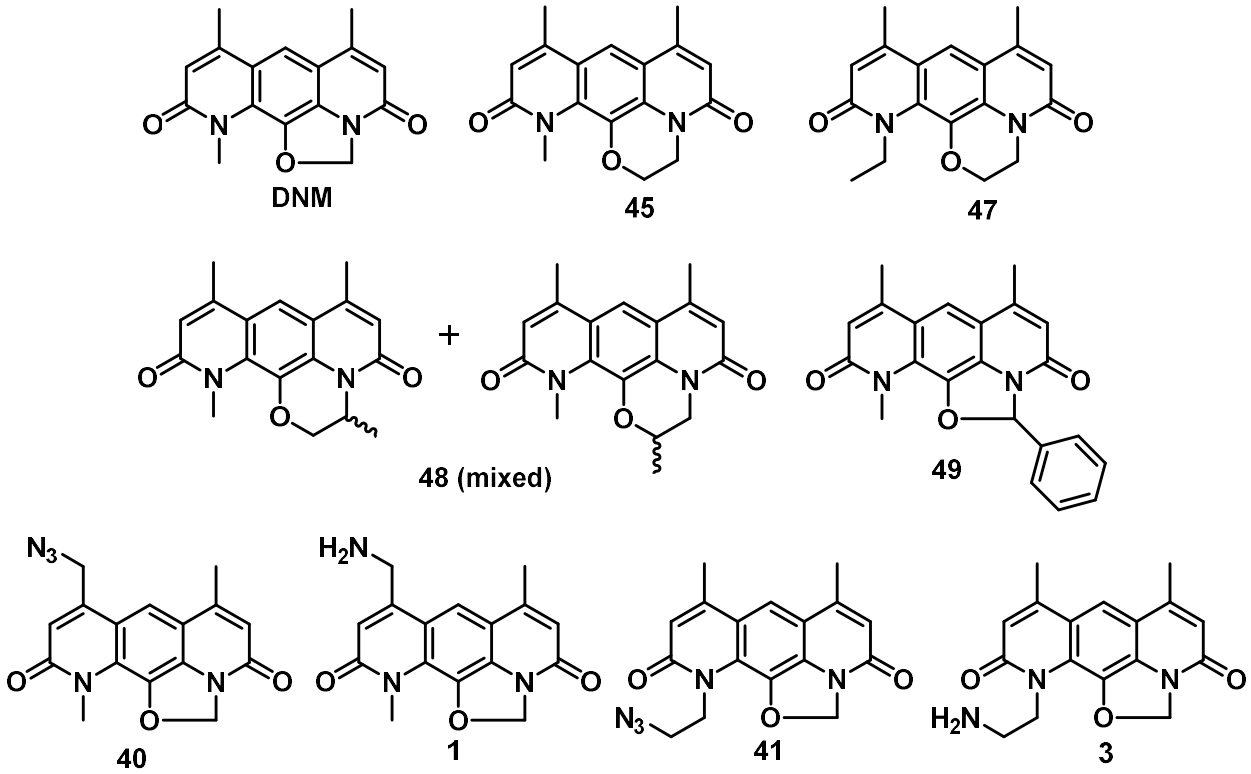


Figure 2.3 Newly synthesized DNM derivatives including amines and derivations from the five-membered ring

Table 2.1 Sensitivity of wild type and fluoroquinolone resistant *S. aureus* with DNM, CIP, and DNM derivatives

Strain	DNM MIC µg/mL	CIP MIC µg/mL	45 MIC µg/mL	47 MIC µg/mL	48 MIC µg/mL	49 MIC µg/mL	40 MIC µg/mL	1 MIC µg/mL	41 MIC µg/mL	3 MIC µg/mL
29213	>1	0.5	4	16	16	>64	16	32	32	16
NRS3	0.03	>64	0.062	0.125	0.062	>64	8	16	32	1

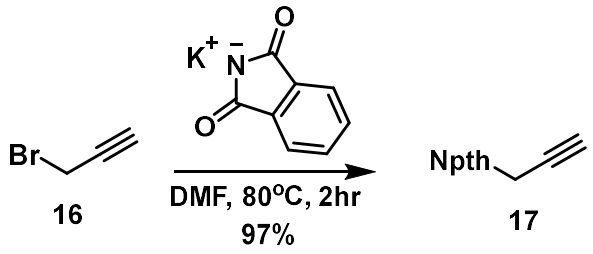
MIC's determined against fluoroquinolone sensitive (29213) and FWR (NRS3) *S. aureus* strains by microdilution broth method (see Section 2.6). 40, 1, 41, and 3 were tested by Dr. Elizabeth Parkinson.

Section 2.5 Chemistry materials and methods

General chemical reagents were purchased from Sigma Aldrich and used without further purification except where indicated. Palladium catalysts and ligands were purchased from Strem Chemicals Inc. (Newburyport, MA). Ethyl 2-butynoate was purchased from GFS Chemicals (Powell, OH) and bis-pinacolboronate was purchased from Frontier Scientific (Logan, UT). In some cases vinyl iodoamide **10** and aryl boronate **11** were obtained from Wu Xi. Solvents were dried by passage through columns packed with activated alumina (THF, CH_2Cl_2, diethyl ether) or activated molecular sieves (DMSO). Reactions involving n-BuLi or LDA were performed using standard Schlenk techniques under argon.

^{1}H-NMR and ^{13}C spectra were recorded on Varian Unity spectrometers at 500 MHz and 125MHz respectively. Spectra generated from a solution of $CDCl_3$ were referenced to residual chloroform (^{1}H: δ 7.26 ppm, ^{13}C: δ 77.16 ppm). Spectra generated in mixtures of $CDCl_3$ and CD_3OD were referenced to C*D*$_3$OD (^{1}H: δ 3.31 ppm, ^{13}C: δ 49.0 ppm).

Section 2.5.1 Synthetic protocols and characterizations

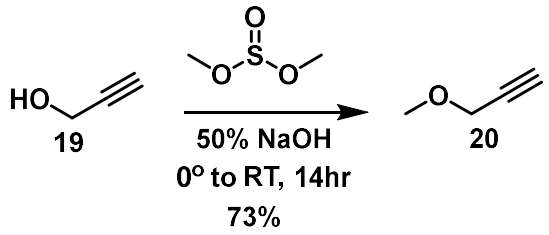


To an oven dried flask with stir bar charged with **16** (0.93g, 5mmol) under argon was added 5mL dry DMF by syringe. Propargyl bromide was added (0.72 mL, 6.4mmol) and the mixture was heated to 80°C in an oil bath. The reaction was quenched two

hours later with the addition of DI water cooled in an ice bath resulting in a white precipitate that was isolated by filtration and dried under vacuum. The remaining solution was extracted twice with 10mL EtOAc and dried with $MgSO_4$ before being filtered and dried under vacuum. Product **17** was a pure white solid obtained in 97% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.89 (dd, *J* = 1.0 Hz, 2H), 7.74 (d, *J* = 1.0 Hz, 2H), 4.46 (d, J=1Hz, 2H), 2.23 (t, J= 1Hz, 1H)

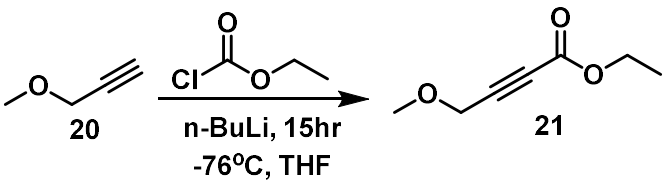
^{13}C NMR (125 MHz, $CDCl_3$): δ 168.2, 132.1, 131, 123.8, 78, 73.2, 30.5



To a flask with stir bar was added **19** (3.5mL, 59.2mmol) and 50% NaOH (4.33mL). The solution was cooled to 0°C on ice and then dimethyl sulfate (3.36mL, 35.12mmol) was added. The solution was prevented from reaching 50°C, and stirred for 14 hours. The solution was then distilled, giving the product at 81°C as a clear oil with 73% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.1 (d, *J* = 1.0 Hz, 2H), 3.39 (s, 3H), 2.44 (t, J=1Hz, 1H)

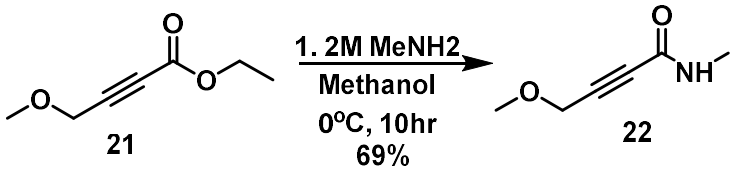
^{13}C NMR (125 MHz, $CDCl_3$): δ 78.7, 76.4, 59.6, 58.1



To an oven dried flask containing a magnetic stir bar and flushed with argon was added recently distilled **20** (0.5mL, 5.92mmol) and THF (6mL). The solution was cooled using a dry ice IPA bath, and n-BuLi was added dropwise (4.1mL, 6.52mmol). After 30 minutes, ethyl chloroformate was added dropwise (0.7mL, 7.3mmol) and the solution was allowed to stir overnight gradually warming to 0°C. After 15 hours water was added and the mixture was extracted with ethyl acetate 5mL three times. The ethyl acetate was brined, dried under $MgSO_4$ and evaporated under reduced pressure down to an oil. The oil was purified by column chromatography using 9:1 hexanes to ethyl acetate with an R_f of 0.2. The product was a clear oil obtained in 84% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 4.24 (q, *J* = 7.0 Hz, 2H), 4.22 (s, 2H), 3.41 (s, 3H), 1.31 (t, J=7Hz, 3H)

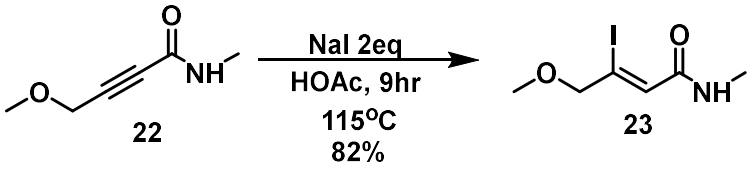
^{13}C NMR (125 MHz, $CDCl_3$): δ 161.2, 89.3, 82, 62.1, 59.7, 58.1, 13.2



To a 7mL vial with stirbar containing **21** (2g, 14mmol) was added methanol (5.55mL) and the solution was cooled in an ice water bath. The amine was added (8.4mL), and the solution was stirred overnight. The solution was evaporated directly from the vial under reduced pressure after 10hr, and the residue was separated by silica gel chromatography (9:1 hexanes:ethyl acetate to 1:1 hexanes: ethyl acetate) to give the product as a clear oil in 69% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 5.75 (bs, 1H, major rotomer NH), 5.56 (bs, 1H, minor rotomer NH), 4.22 (s, 2H), 3.41 (s, 3H), 2.98 (d, 3H, J=5Hz, minor rotomer NCH_3) 2.81(d, 3H, J=5Hz, major rotomer NCH_3)

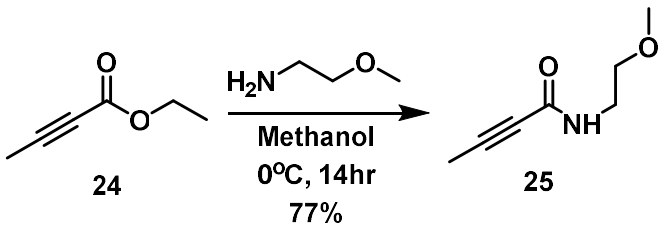
^{13}C NMR (125 MHz, $CDCl_3$): δ 154.2 (minor), 153.6 (major), 89.3, 82, 61.8 (minor), 59.9 (major), 59.7, 58.1



Alkynyl amide **22**, (1.01g, 7.9mmol), NaI (16mmol) and glacial acetic acid (1mL, 17.5mmol) were combined in a 1dram vial. The vial was sealed and heated to 115°C for 9hr. The deep red mixture was diluted with water (8mL) and CH_2Cl_2 (27mL) and treated with $NaHSO_3$ until colorless. The mixture was then neutralized with $NaHCO_3$. The mixture was partitioned and the aqueous layer was extracted with CH_2Cl_2 three times with 15mL. The combined organic fractions were brined, dried with $MgSO_4$ and evaporated to yield the desired iodoamide **23** as a white-yellow solid in 82% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.67 (s, 1H, vinyl CH), 5.74 (bs, 1H), 4.13 (s, 2H), 3.4 (s, 3H), 2.91 (d, *J* = 1.0 Hz, 3H)

^{13}C NMR (125 MHz, $CDCl_3$): δ 166.2, 129.8, 104.5, 82.8, 59.2, 23.8

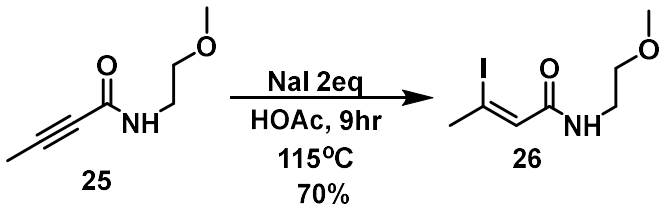


To a 7mL vial with stirbar containing **24** (1g, 8.9mmol) was added methanol (8.9mL) and the solution was cooled in an ice water bath. The amine was added

(10.68mmol), and the solution was stirred overnight. The solution was evaporated directly from the vial under reduced pressure after 10hr, and the residue was separated by silica gel chromatography (9:1 hexanes:ethyl acetate to 1:1 hexanes: ethyl acetate) to give the product as a clear oil in 77% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 6.12 (bs, 1H, NH), 3.57 (m, 2H), 3.46 (m, 2H), 3.36 (s, 3H), 1.94 (s, 3H)

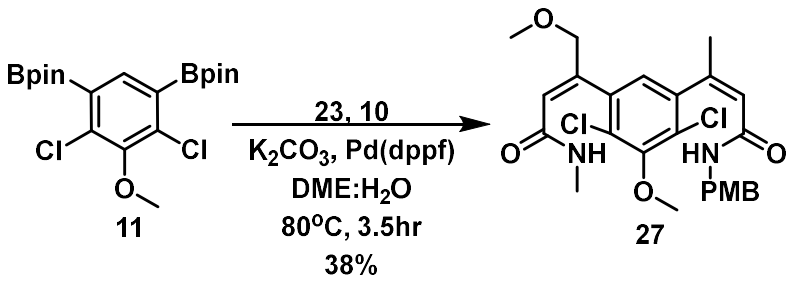
^{13}C NMR (125 MHz, $CDCl_3$): 145.2, 83.6, 75.03, 70.98, 59.03, 39.6, 3.91



Alkynyl amide **25**, (2.07g, 14.66mmol), NaI (29.3mmol) and glacial acetic acid (1.7mL, 29.75mmol) were combined in a 1dram vial. The vial was sealed and heated to 115°C for 9hr. The deep red mixture was diluted with water (15mL) and CH_2Cl_2 (55mL) and treated with $NaHSO_3$ until colorless. The mixture was then neutralized with $NaHCO_3$. The mixture was partitioned and the aqueous layer was extracted with CH_2Cl_2 three times with 30mL. The combined organic fractions were brined, dried with $MgSO_4$ and evaporated to yield the desired iodoamide **26** as a white solid in 70% yield.

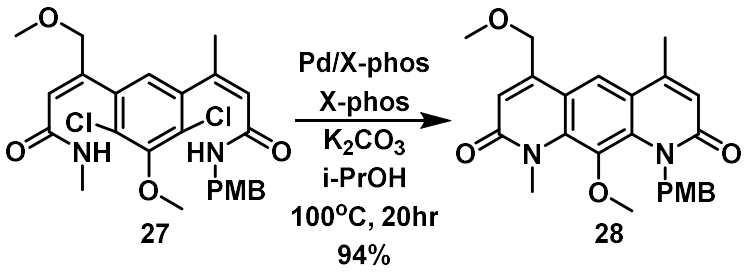
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.3 (bs, 1H, NH), 7.1 (s, 1H), 3.51 (m, 2H), 3.38 (m, 2H), 3.42 (s, 3H), 2.02 (s, 3H)

^{13}C NMR (125 MHz, $CDCl_3$): δ 145.2, 130.5, 116.4, 71.6, 59.0, 39.6, 31.8



To a flask with a stir bar was added **11** (1.4mmol), $PdCl_2$(dppf) (20mol%, 0.28mmol), K_2CO_3 (8.4mmol) and both desired iodoamides **23** (2.1mmol) and **10** (1.82mmol). The flask was evacuated and backfilled three times with argon. Water (3mL) and DME (27mL) were degassed by sparging 45 minutes with argon and added by syringe. The flask was plunged into an oil bath at 80°C for 3.5hr. The mixture was poured into a seperatory funnel and diluted with water (15mL). The mixture was partitioned and the aqueous layer was extracted with EtOAc (3x 30mL). The organic fractions were combined, washed with brine, and evaporated to a red foamy solid. The crude solid was dissolved in approximately 3mL CH_2Cl_2 and sepereated by silica gel chromatography (100:0 to 70:30 to 30:700 to 0:100 hexanes:ethyl acetate). The purity of the resultant product was variable and was subjected to the intramolecular amidation without further purification but 38% presents a typical yield of an off white foam.

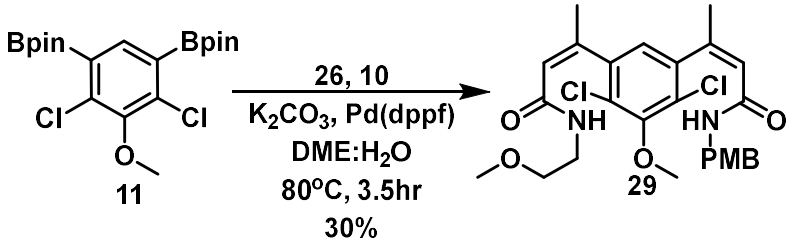
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.1 (s, 1H, anisole CH), 7.01 (d, 2H, J=8.5Hz, PMB CH), 6.62 (d, 2H, J=8.5Hz, PMB CH), 6.35 (bs, 1H, NH), 6.3 (bs, 1H, NH), 5.89 (s, 1H, vinyl CH), 5.63 (s, 1H, vinyl CH), 5.3 (bd, 2H, benzyl CH_2), 4.42 (s, 2H), 3.72 (s, 3H), 3.68 (s, 3H), 3.65 (s, 3H), 2.46 (d, 3H, J=1.0Hz)



In a schlenk flask the diamide starting material **27** (0.6mmol) was combined with K_2CO_3 (3.6mmol), Pd/X-phos admix (10mol%) and argon sparged isopropanol (20mL). The mixture was heated to 100°C and monitored by NMR of aliquots. Insoluble materials were removed by filtration through celite and rinsed with CH_2Cl_2. The filtrate was evaporated to give **28** as a white foam or solid. The product varied in purity but was typically used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.72 (s, 1H, anisole CH), 7.09 (d, 2H, J=8.5Hz, PMB CH), 6.73 (d, 2H, J=8.5Hz, PMB CH), 6.78 (s, 1H, vinyl CH), 6.52 (d, 1H, J=1.0Hz, vinyl CH), 5.3 (bd, 2H, benzyl CH_2), 4.42 (s, 2H), 3.72 (s, 3H), 3.68 (s, 3H), 3.65 (s, 3H), 2.46 (d, 3H, J=1.0Hz)

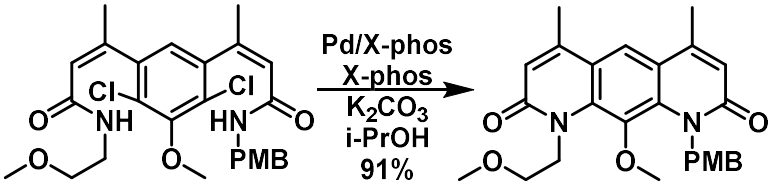
^{13}C NMR (125 MHz, $CDCl_3$): 164.86, 164.84, 147.6, 146.2, 137.0, 136.8, 129.8, 128.5, 120.3, 120.27, 120.2, 119.1, 113.6, 71.16, 62.5, 58.81, 55.3, 36.3, 20.04



To a flask with a stir bar was added **11** (1.4mmol), $PdCl_2$(dppf) (20mol%, 0.28mmol), K_2CO_3 (8.4mmol) and both desired iodoamides **26** (2.1mmol) and **10** (1.82mmol). The flask was evacuated and backfilled three times with argon. Water

(3mL) and DME (27mL) were degassed by sparging 45 minutes with argon and added by syringe. The flask was plunged into an oil bath at 80°C for 3.5hr. The mixture was poured into a seperatory funnel and diluted with water (15mL). The mixture was partitioned and the aqueous layer was extracted with EtOAc (3x 30mL). The organic fractions were combined, washed with brine, and evaporated to a red foamy solid. The crude solid was dissolved in approximately 3mL CH_2Cl_2 and separated by silica gel chromatography (100:0 to 70:30 to 30:700 to 0:100 hexanes:ethylacetate). The purity of the resultant product was variable and was subjected to the intramolecular amidation without further purification but 30% presents a typical yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.1 (s, 1H, anisole CH), 7.01 (d, 2H, J=8.5Hz, PMB CH), 6.62 (d, 2H, J=8.5Hz, PMB CH), 6.35 (bs, 1H, NH), 6.3 (bs, 1H, NH), 5.99 (d, 1H, J=1Hz, vinyl CH), 5.91 (d, 1H, J=1Hz, vinyl CH), 5.3 (bd, 2H, benzyl CH_2), 3.68 (s, 3H), 3.62 (m, 2H), 3.48 (s, 3H), 3.40 (m, 2H), 2.65 (d, 3H J=1Hz), 2.46 (d, 3H, J=1.0Hz)

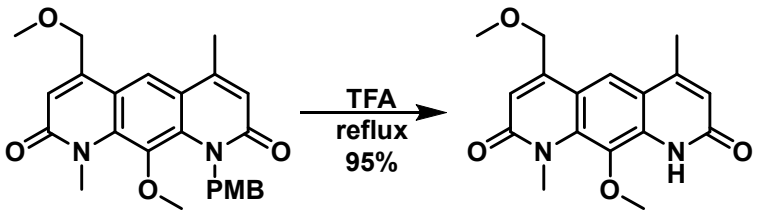


29 30

In a schlenk flask the diamide starting material **29** (0.4mmol) was combined with K_2CO_3 (2.4mmol), Pd/X-phos admix (10mol%) and argon sparged isopropanol (10mL). The mixture was heated to 100°C and monitored by NMR of aliquots. Insoluble materials were removed by filtration through celite and rinsed with CH_2Cl_2. The filtrate was evaporated to give **30** as a white foam or solid. The product varied in purity but was typically used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.6 (s, 1H, anisole CH), 7.01 (d, 2H, J=8.5Hz, PMB CH), 6.62 (d, 2H, J=8.5Hz, PMB CH), 6.52 (d, 1H, J=1Hz, vinyl CH), 6.39 (d, 1H, J=1Hz, vinyl CH), 5.3 (bd, 2H, benzyl CH_2), 3.68 (s, 3H), 3.62 (m, 2H), 3.48 (s, 3H), 3.40 (m, 2H), 2.65 (d, 3H J=1Hz), 2.46 (d, 3H, J=1.0Hz)

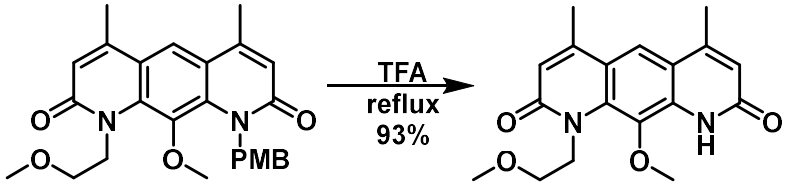
^{13}C NMR (125 MHz, $CDCl_3$): δ 162.3, 159.87, 146.3, 145.8, 137.7, 136.43, 128.8, 127.2, 120.9, 120.16, 120.11, 119.1, 113.6, 71.16, 70.8, 62.5, 59.6, 55.3, 43.2, 20.04



28 31

28 was evaporated into a flask (0.2mmol). TFA was added (3mL) and the solution was heated to reflux for 1.5 hours. The solvent was evaporated and the residue was purified by silica gel chromatography to give **31** as an off white solid in 95% yield.

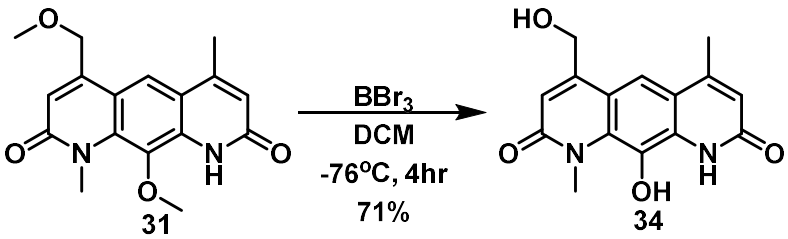
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.73 (s, 1H), 6.62 (s, 1H, vinyl CH), 6.41 (s, 1H, vinyl CH), 4.6 (s, 2H), 3.85 (s, 3H), 3.39 (s, 3H), 2.44 (s, 3H)



30 32

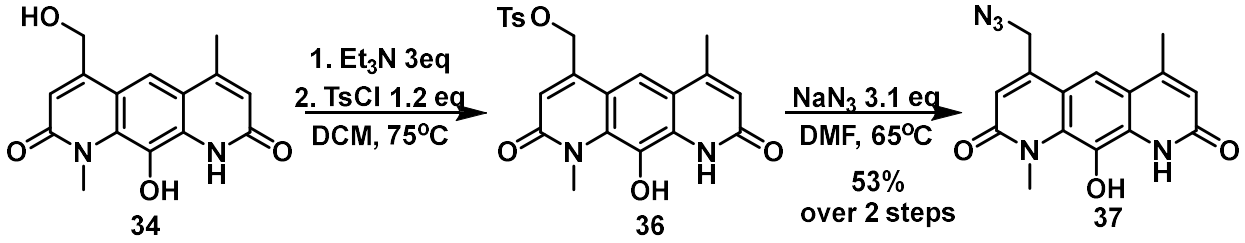
30 was evaporated into a flask (0.2mmol). TFA was added (3mL) and the solution was heated to reflux for 1.5 hours. The solvent was evaporated and the residue was purified by silica gel chromatography to give **32** as an off white solid in 93% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.69 (s, 1H), 6.52 (d, 1H, J=1Hz, vinyl CH), 6.39 (d, 1H, J=1Hz, vinyl CH), 3.68 (s, 3H), 3.62 (m, 2H), 3.40 (m, 2H), 2.65 (d, 3H J=1Hz), 2.46 (d, 3H, J=1.0Hz)



31 (20mg 0.06mmol) was charged into a dried flask with a magnetic stirrer under argon. Dried DCM (1mL) was added by syringe. The solution was chilled with a dry ice IPA bath, and BBr_3 (0.636mmol) was added dropwise. The solution was allowed to stir for 4 hours and monitored by TLC until starting material was consumed. The reaction was neutralized with concentrated $NaHCO_3$, and the solvents were evaporated to give the product **34** as a brown-red solid. The solid was not completely pure, but purity was increased by washing with hexanes and **34** used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.81 (s, 1H), 6.68 (s, 1H, vinyl CH), 6.41 (s, 1H, vinyl CH), 4.4 (s, 2H), 3.12 (s, 3H) 2.44 (s, 3H)

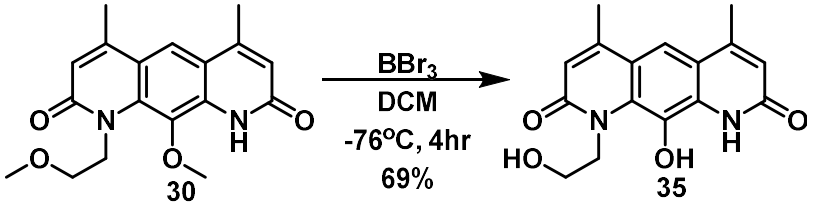


34 (110mg, 0.384mmol) was placed in a flask with 20mL DCM, and Et_3N was added (1.152mmol). Tosyl chloride was added by portion (90mg, 0.46mmol) and the solution was heated while stirring to 75°C in an oil bath. Reaction was monitored by

TLC and after the starting material was consumed the solvents were evaporated. After thoroughly removing all DCM, DMF was added with NaN_3 (1.19mmol), and the reaction was allowed to stir at 65 degrees overnight. Water was added to quench the reaction and the solvents were evaporated to give **37** impure as a black solid.

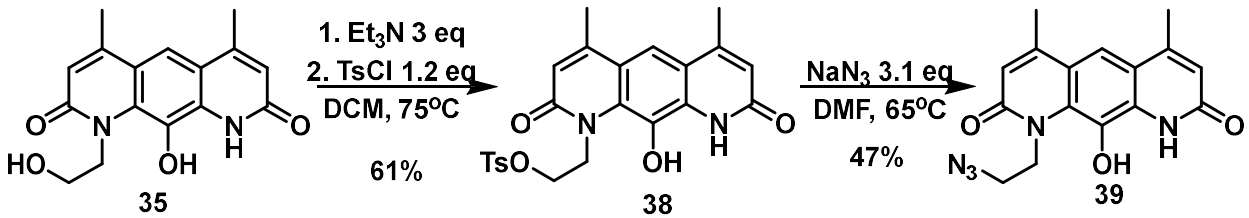
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.81 (s, 1H), 6.68 (s, 1H, vinyl CH), 6.41 (s, 1H, vinyl CH), 3.9 (s, 2H), 3.12 (s, 3H) 2.44 (s, 3H)

HRMS (m/z, ESI-TOF): $(M+H)^+$ calcd for $C_{15}H_{13}N_5O_3$, 311.1709; found, 311.1714.



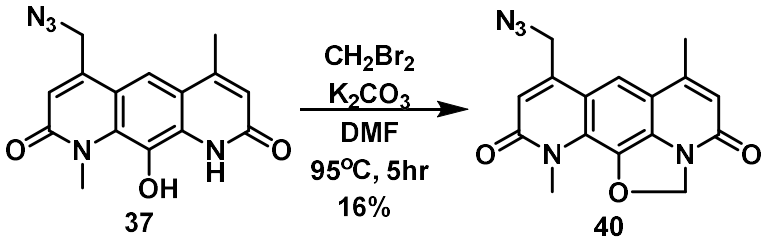
30 (20mg 0.06mmol) was charged into a dried flask with a magnetic stirrer under argon. Dried DCM (1.5mL) was added by syringe. The solution was chilled with a dry ice IPA bath, and BBr_3 (0.65mmol) was added dropwise. The solution was allowed to stir for 4 hours and monitored by TLC until starting material was consumed. The reaction was neutralized with concentrated $NaHCO_3$, and the solvents were evaporated to give the product **35** as a brown-red solid. The solid was not completely pure, but purity was increased by washing with hexanes and **35** used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.71 (s, 1H), 6.52 (d, 1H, J=1Hz, vinyl CH), 6.39 (d, 1H, J=1Hz, vinyl CH), 3.59 (m, 2H), 3.32 (m, 2H), 2.58 (d, 3H J=1Hz), 2.46 (d, 3H, J=1.0Hz)



35 (65 mg, 0.225mmol) was placed in a flask with 10mL DCM, and Et_3N was added (0.68mmol). Tosyl chloride was added by portion (50mg, 0.27mmol) and the solution was heated while stirring to 75°C in an oil bath. Reaction was monitored by TLC and after the starting material was consumed the solvents were evaporated. After thoroughly removing all DCM, DMF was added with NaN_3 (0.681mmol), and the reaction was allowed to stir at 65 degrees overnight. Water was added to quench the reaction and the solvents were evaporated to give **39** impure as a black solid.

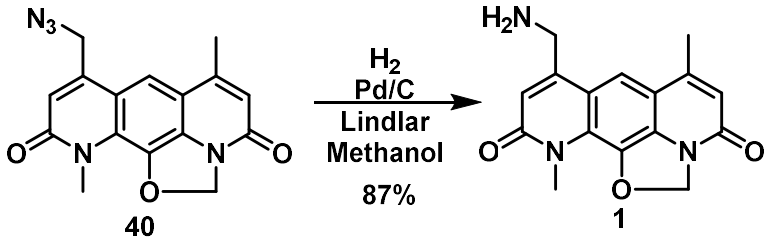
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.71 (s, 1H), 6.52 (d, 1H, J=1Hz, vinyl CH), 6.39 (d, 1H, J=1Hz, vinyl CH), 3.16 (m, 2H), 3.1 (m, 2H), 2.58 (d, 3H J=1Hz), 2.46 (d, 3H, J=1.0Hz)
HRMS (m/z, ESI-TOF): $(M+H)^+$ calcd for $C_{16}H_{15}N_5O_3$, 325.122; found, 325.1227.



In a flask containing a magnetic stir bar **37** (8mg) was combined with K_2CO_3 (21mg) and dissolved in DMF (2mL). CH_2Br_2 (1mL) was added and the solution was heated to 95°C for 5hr and monitored by TLC (10%MeOH in EtOAc) for the disappearance of starting material. DMF was evaporated, and the residue was dissolved in EtOAc and methanol and filtered. The residue was evaporated to a brown solid, and isolated by silica gel chromatography (8%MeOH in EtoAc) to give **40** as an off white solid in 16% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.81 (s, 1H), 6.68 (s, 1H, vinyl CH), 6.41 (s, 1H, vinyl CH), 6.38 (s, 2H), 3.9 (s, 2H), 3.12 (s, 3H) 2.44 (s, 3H)

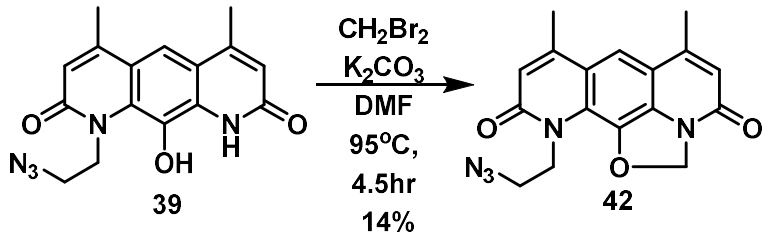
HRMS (m/z, ESI-TOF): $(M+H)^+$ calcd for $C_{16}H_{13}N_5O_3$, 323.8965; found, 323.8974.



40 was placed in a 7mL vial along with 1mol% Lindlar's catalyst and 1mL methanol under an atmosphere of Hydrogen. The reaction was allowed to stir at room temperature for 4 hours. More methanol was added, and the solution was filtered. The solution was evaporated under reduced pressure and purified by silica gel chromatography with 5% MeOH in DCM to give **1** as a tan solid.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.81 (s, 1H), 6.68 (s, 1H, vinyl CH), 6.41 (s, 1H, vinyl CH), 6.38 (s, 2H), 3.86 (m, 2H), 3.12 (s, 3H) 2.44 (s, 3H), 2.1 (bs, 2H)

HRMS (m/z, ESI-TOF): $(M+H)^+$ calcd for $C_{16}H_{15}N_3O_3$, 297.1175; found, 297.1179.

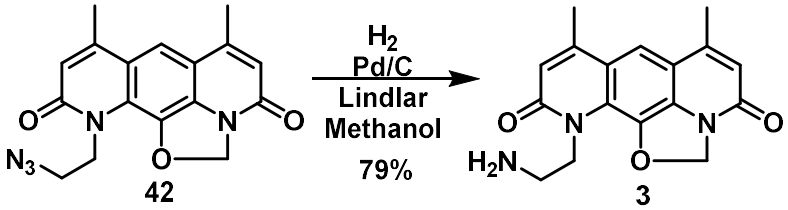


In a flask containing a magnetic stir bar **39** (4mg) was combined with K_2CO_3 (12mg) and dissolved in DMF (1mL). CH_2Br_2 (0.5mL) was added and the solution was heated to 95°C for 5hr and monitored by TLC (10%MeOH in EtOAc) for the disappearance of starting material. DMF was evaporated, and the residue was dissolved in EtOAc and methanol and filtered. The residue was evaporated to a brown

solid, and isolated by silica gel chromatography (8%MeOH in EtoAc) to give **42** as an off white solid in 14% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.71 (s, 1H), 6.52 (d, 1H, J=1Hz, vinyl CH), 6.39 (d, 1H, J=1Hz, vinyl CH), 6.24 (s, 2H) 3.16 (m, 2H), 3.1 (m, 2H), 2.58 (d, 3H J=1Hz), 2.46 (d, 3H, J=1.0Hz)

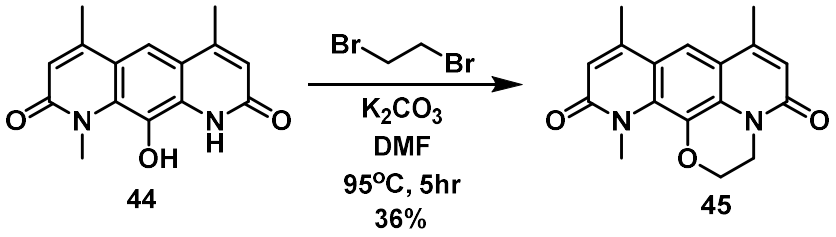
HRMS (m/z, ESI-TOF): $(M+H)^+$ calcd for $C_{17}H_{15}N_5O_3$, 337.1208; found, 337.1209.



42 was placed in a 7mL vial along with 1mol% Lindlar's catalyst and 1mL methanol under an atmosphere of Hydrogen. The reaction was allowed to stir at room temperature for 4 hours. More methanol was added, and the solution was filtered. The solution was evaporated under reduced pressure and purified by silica gel chromatography with 5% MeOH in DCM to give **3** as a gray/offwhite powder.

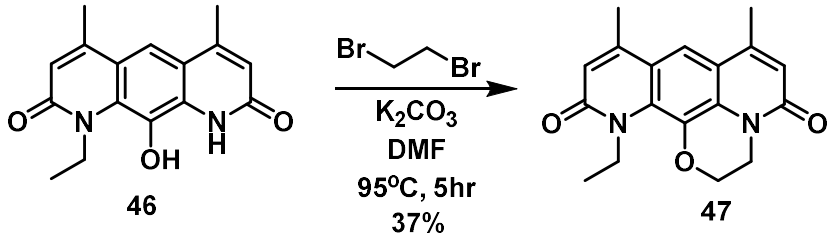
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.71 (s, 1H), 6.52 (d, 1H, J=1Hz, vinyl CH), 6.39 (d, 1H, J=1Hz, vinyl CH), 6.24 (s, 2H) 3.16 (m, 2H), 3.1 (m, 2H), 2.58 (d, 3H J=1Hz), 2.46 (d, 3H, J=1.0Hz), 2.05 (bs, 2H)

HRMS (m/z, ESI-TOF): $(M+H)^+$ calcd for $C_{17}H_{17}N_3O_3$, 311.1307; found, 339.1309.



In a flask containing a magnetic stir bar **44** (10mg) was combined with K_2CO_3 (30mg) and dissolved in DMF (2mL). 1,2-Dibromoethane (1mL) was added and the solution was heated to 95°C for 5hr and monitored by TLC (10%MeOH in EtOAc) for the disappearance of starting material. DMF was evaporated, and the residue was dissolved in EtOAc and methanol and filtered. The residue was evaporated to a brown solid, and isolated by silica gel chromatography (8%MeOH in EtoAc) to give **45** as an off white solid in 36% yield.

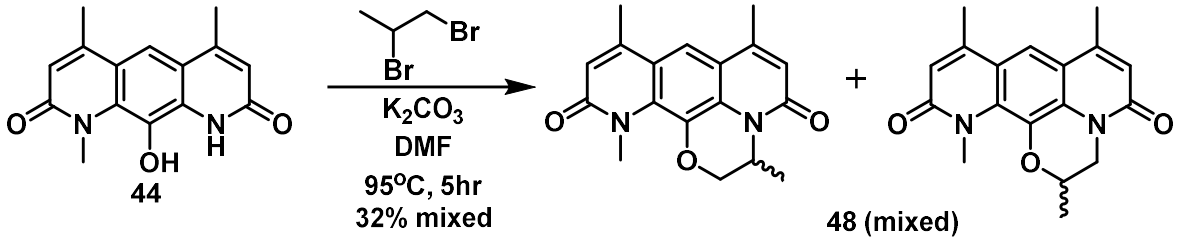
^{1}H NMR (500 MHz, $CDCl_3$): δ 7.55 (s, 1H), 6.49 (d, *J* = 1.0 Hz, 1H), 6.47 (d, *J* = 1.0 Hz, 1H), 4.86 (d, J=1Hz, 2H), 4.39 (d, J=1Hz, 2H), 3.92 (s, 3H), 2.54 (d, *J* = 1.0 Hz, 3H), 2.52 (d, *J* = 1.0 Hz, 3H);



In a flask containing a magnetic stir bar **46** (10mg) was combined with K_2CO_3 (30mg) and dissolved in DMF (2mL). 1,2-Dibromoethane (1mL) was added and the solution was heated to 95°C for 5hr and monitored by TLC (10%MeOH in EtOAc) for the disappearance of starting material. DMF was evaporated, and the residue was dissolved in EtOAc and methanol and filtered. The residue was evaporated to a tan

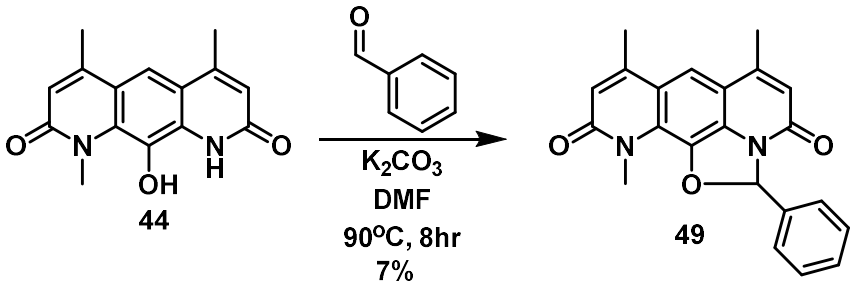
solid, and isolated by silica gel chromatography (8%MeOH in EtoAc) to give **47** as an off white solid in 37% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.55 (s, 1H), 6.49 (d, *J* = 1.0 Hz, 1H), 6.47 (d, *J* = 1.0 Hz, 1H), 4.86 (d, J=1Hz, 2H), 4.39 (d, J=1Hz, 2H), 3.99 (d, 2H, J=7Hz), 2.54 (d, *J* = 1.0 Hz, 3H), 2.52 (d, *J* = 1.0 Hz, 3H); 1.36 (t, 3H, J=7Hz)



In a flask containing a magnetic stir bar **44** (10mg) was combined with K_2CO_3 (30mg) and dissolved in DMF (2mL). 1,2-Dibromopropane (1mL) was added and the solution was heated to 95°C for 5hr and monitored by TLC (10%MeOH in EtOAc) for the disappearance of starting material. DMF was evaporated, and the residue was dissolved in EtOAc and methanol and filtered. The residue was evaporated to a brown solid, and isolated by silica gel chromatography (8%MeOH in EtoAc) to give the products labeled **48** as a mixture of regioisomers and stereoisomers. Products could not be separated.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.55 (s, 1H), 6.49 (d, *J* = 1.0 Hz, 1H), 6.47 (d, *J* = 1.0 Hz, 1H), 4.91 (m, 1H major isomer), 4.86(m, 1H minor isomer) 4.43, (m, 1H major isomer) 4.39 (m, 1H minor isomer), 3.92 (s, 3H), 2.54 (d, *J* = 1.0 Hz, 3H), 2.52 (d, *J* = 1.0 Hz, 3H);



In a flask containing a magnetic stir bar **44** (20mg) was combined with K_2CO_3 (30mg) and dissolved in DMF (4mL). Benzaldehyde (60mg) was added and the solution was heated to 90°C for 8hr and monitored by TLC (10%MeOH in EtOAc) for the disappearance of starting material. DMF was evaporated, and the residue was dissolved in EtOAc and methanol and filtered. The residue was evaporated to a brown solid, and isolated by silica gel chromatography (8%MeOH in EtoAc) to give **49** as an off white solid in 7% yield.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.55 (s, 1H), 7.27 (d, 2H, J=7Hz), 7.25 (m, 2H), 7.19 (d, 1H), 7.07 (s, 1H6.49 (d, *J* = 1.0 Hz, 1H), 6.47 (d, *J* = 1.0 Hz, 1H), 3.92 (s, 3H), 2.54 (d, *J* = 1.0 Hz, 3H), 2.52 (d, *J* = 1.0 Hz, 3H)

Section 2.6 Antibiotic susceptibility tests

Antibiotic susceptibility for all bacterial strains was performed in triplicate using the microdilution broth method as outlined by the Clinical and Laboratory Standards Institute.[8] MH broth was used. 2μL of a 50X compound stock in DMSO or water was added to the wells of a 96-well round well plate. Live and dead controls received 2μL of vehicle. 88μL of MH broth were added to the wells except the dead controls which received 98μL. 100μL of an overnight culture of bacteria were then added to 10mL of MH broth and grown until the inoculates reached a turbidity between $1*10^7$ and $2*10^8$ cfu/mL based on a previously established calibration curve. The culture was diluted to

$5*10^6$ cfu/mL and 10µL of this solution was added to all but the dead control wells to reach a final concentration of $5*10^5$ cfu/mL and 100 µL in each well. The plates were incubated at 37°C for 16-20 hours and read on a Molecular Devices SpectraMax Plus 384 Microplate reader at λ=600nm. MIC values were defined by the lowest concentration to result in >90% growth inhibition.

2.7 References

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