Abstract

Antibiotic resistance is a major threat to public health. Currently new multi-drug resistant bacterial strains are appearing faster than new antibiotics can enter the clinic. Resistance modifying agents can potentially potentiate whole families of antibiotics and are one way to combat antibiotic resistance. Previously, we synthesized a library of polycyclic indolines which were found to potentiate β-lactams in MRSA in vitro but were poor candidates for in vivo testing due to their physical properties. To improve their physical properties, we synthesized a small library of aza-tetracyclic indolines. The nitrogen was functionalized with various functional groups to determine which modifications are tolerated at that position. One methyl carbamate aza-tetracyclic indoline has improved physical properties compared to its lead compound, while still maintaining β-lactam potentiating activity in MRSA.

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1. Introduction

1.1 Antibiotic discovery, use, and resistance

Antibiotics are a cornerstone of modern medicine. Before their discovery, bacterial infections were treated with procedures such as rinses with aqueous bromine, blood-letting, and amputation, which were rarely effective and caused major risk for the patient.[1] Invasive procedures such as surgery and childbirth carried a significant risk of bacterial infection for which there was no effective treatment.[2]

In 1928, Alexander Fleming was culturing *Staphylococcus aureus* when he made a fortunate observation.[3] He noticed that a mold had colonized the plate and around the mold there was a perimeter in which no bacteria grew. Intrigued, he attempted to identify the substance that was inhibiting bacterial growth. With the help of an English research group, the substance was isolated in 1940.[4] This substance was later revealed to be a penicillin antibiotic, benzylpenicillin (penicillin G). Penicillin G was used to create analogues such as penicillin V and benzathine penicillin. These compounds are all part of the penicillin class of antibiotics. The penicillin class of antibiotics falls under the larger β-lactam class of antibiotics, which is named after the β-lactam moiety they share. β-lactam antibiotics are effective against a variety of bacteria, allowing them to treat a many of conditions. Additionally, β-lactam antibiotics rarely cause negative side effects in patients, earning them the title of "wonder drug".[5]

Penicillins gained widespread use in the 1940s providing improved patient care and the development of new medical procedures. However, by the mid-1950s resistance to penicillins was so widespread that many of the advancements and procedures developed with the use of them were threatened.[6] Bacteria gained resistance to penicillins through production of penicillinase (β-lactamase) enzymes, which hydrolyzes the core of penicillins and renders it inactive. In response, β-lactamase resistant antibiotics were developed.

Methicillin was deployed in 1960 as the first β-lactamase resistant β-lactam antibiotic. Steric hindrance protects methicillin from β-lactamases.[7] Because methicillin has a relatively bulky side chain, it is not easily bound by β-lactamases (Fig 1.1.1). Prompted by the success of methicillin, other β-lactamase resistant β-lactam antibiotics were developed such as oxacillin, which also has a relatively bulky side chain. However, the success of methicillin was short lived; two years after its deployment, methicillin resistant *Staphylococcus aureus* (MRSA) was observed in England, and in the United states in 1968.[8]

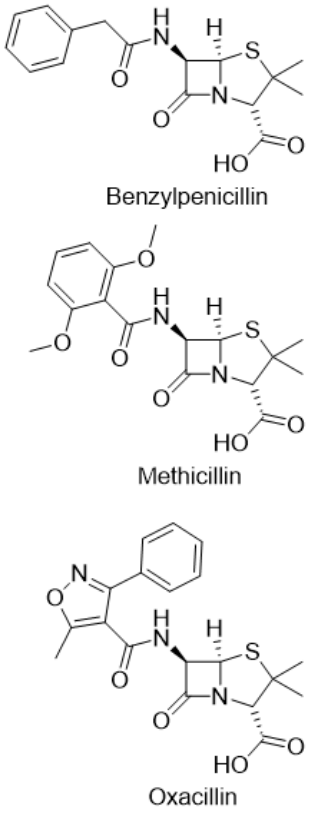


Figure 1.1.1 Methicillin and Oxacillin are protected from β-lactamase by the steric bulk of their side chains.

Methicillin resistance was not due to a novel β-lactamase. Rather, the drug target within MRSA changed. β-lactam antibiotics bind and inactivate a protein (PBP2) that catalyzes the cross linking of

peptidoglycan. Peptidoglycan cross-linking is a necessary process that protects the bacterial cell from osmotic forces. MRSA can detect the presence of β-lactam antibiotics and respond by expressing an alternative version of PBP2 called PBP2a. PBP2a has a low affinity for β-lactams and can function in the presence of them, granting *S aureus* which express PBP2a a greater chance of survival in the presence of β-lactam antibiotics.

Since the original discovery of penicillin G, many other β-lactams and antibiotics from other classes have been discovered. The time period from 1940-1970 is known as the "golden age of antibiotic discovery". During this time, antibiotic discovery was based on exploiting chemical warfare between microorganisms. Many antibiotic classes during this time were discovered by observing the growth inhibitory effects one species had on another on culture plates and then isolating the active compounds.[9] As follows, most antibiotic classes were discovered through natural sources.

As the easily accessible microorganisms were exploited, efforts turned away from natural sources to large combinatorial synthesis libraries. However, these libraries have seen limited success in terms of antibiotic discovery.[10] In the mid-1990s, the pharmaceutical company GlaxoSmithKline took advantage of the recent genomics revolution to identify 160 essential genes unique to bacteria. Having identified their targets, they screened an in-house library of 500,000 compounds for activity.[11] Despite the target-based approach and massive drug library this project returned no hits, underscoring the difficulty of antibiotic discovery through combinatorial synthesis and high-throughput screening.

Considering the difficulties of synthetic antibiotic discovery, some antibiotic discovery efforts have turned back to natural sources. New techniques to discover natural products are being developed, and are providing more lead compounds.[12,13,14] Despite this, the "antibiotic pipeline" is still running dry. This is concerning because the current model of fighting antibiotic resistant bacteria relies on using antibiotics to which that bacteria is not yet resistant. However, after an antibiotic is put into clinical use, resistance follows shortly after (Fig 1.1.2). With new strains of multi-drug resistant bacteria being observed more quickly than novel antibiotics are discovered, treatment options are becoming increasing limited.

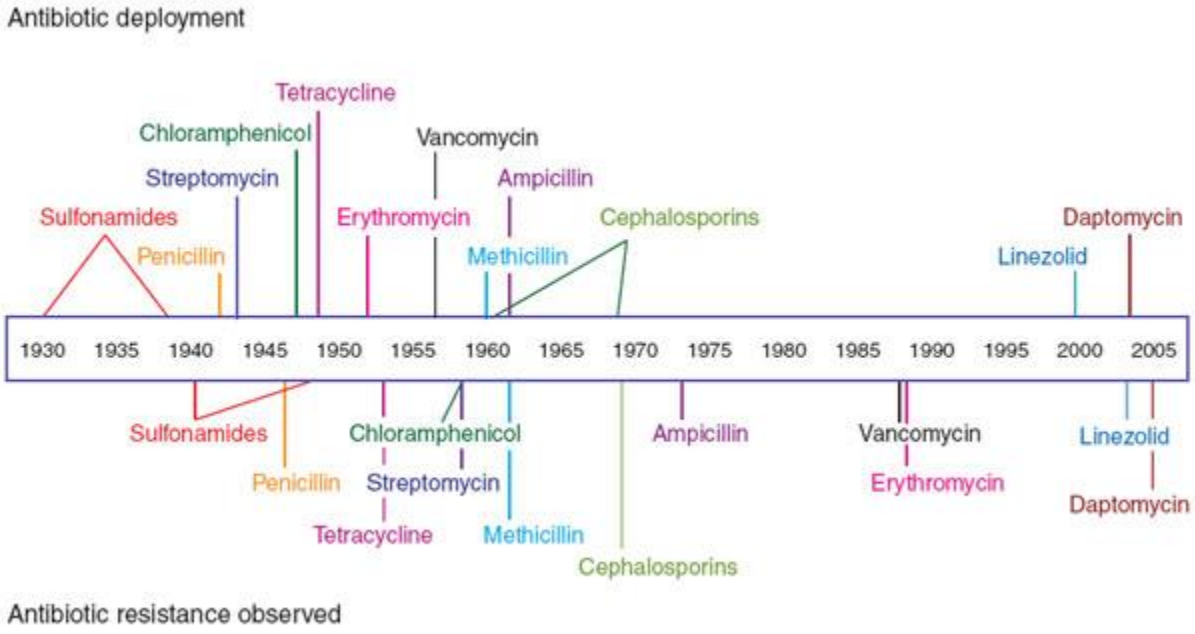


Figure 1.1.2 Antibiotics are listed above the timeline when they were first deployed, and again under the timeline when resistance was first observed.
Clatworthy E, Anne. "Timeline of antibiotic deployment and the evolution of antibiotic resistance." *Nature*, Emily Pierson, Deborah T Hung, Nature Chemical Biology, 20 August 2007, http://www.nature.com/nchembio/journal/v3/n9/fig_tab/nchembio.2007.24_F1.html

Antibiotics and antibiotic resistance are an expected consequence of natural selection. As mentioned previously, antibiotics exist in nature as the tools of microbial chemical warfare. One explanation for why antibiotics evolved is that they are a tool to eliminate competition, giving the organism that produces antibiotic compounds less competition and more resources. As a corollary, some mechanism of antibiotic resistance would eventually arise to allow that resistant organism access to more resources. Thus, antibiotics and antibiotic resistance are ubiquitous in nature because they are adaptations that help microorganisms survive.

The ubiquity of antibiotic resistance was expanded upon and formally proposed by Gerry Wright as "the resistome".[15] The resistome is a collection of all antibiotic resistance conferring genes. This collection includes genes found in human-pathogenic bacteria, non-human-pathogenic bacteria, and all "cryptic genes", which are genes that may confer resistance, but are not specifically transcribed when the cell is exposed to existing antibiotics. This collection of resistance genes is the result of billions of years of natural selection, and as a resulting collection is massive and diverse. Then, through horizontal gene transfer, the library of resistance genes can be spread throughout microbial populations. Thus for any given antibiotic, some mechanism of resistance likely already exists; it's just a matter of time before resistance is observed in a clinically relevant strain.

1.2 Mechanism of action of β-lactam antibiotics.

For gram positive bacteria such as *S aureus*, the outermost component of their outer membranes is a thick peptidoglycan layer. Peptidoglycan is polymer of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) subunits (Fig 1.2.1). A short

pentapeptide is attached to carbon 3 of NAM to form the Muramyl-peptide: NAM- L-Ala-D-Glu-L-Lys-D-Ala-D-Ala.[16] In the final step of peptidoglycan synthesis, penicillin binding proteins (PBPs) catalyze a transpeptidation reaction to link one pentapeptide to an adjacent pentapeptide. Once transpeptidations have occurred, fully cross linked peptidoglycan polymer resembles a mesh, and provides a very rigid protective layer against osmotic pressure.

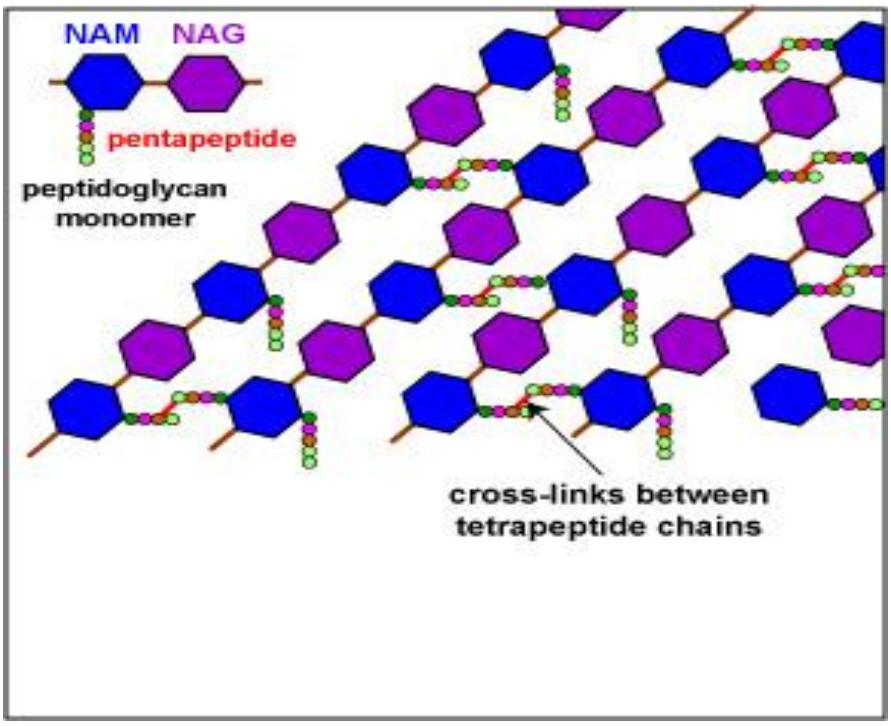


Figure 1.2.1 A peptidoglycan monomer (top left) and cross linked peptidoglycan.
Doc Kaiser. Doc Kaiser's microbiology website. Community college of Baltimore County. August 2011 http://faculty.ccbcmd.edu/courses/bio141/lecguide/unit1/prostruct/u1fig8_ec.html

The transpeptidation catalyzed by PBPs is inhibited by β-lactams antibiotics. A defining feature of β-lactam antibiotics is a β-lactam ring fused with a five or six membered ring (Fig 1.2.2). Tipper and Strominger were the first to suggest that the β-lactam core is a structural analog to the substrate of PBPs.[17] Indeed, the terminal R-D-Ala-D-Ala dipeptide can adopt a conformation such that its amide bond is analogous to the amide bond of the β-lactam (Fig 1.2.2A). Based on the Tipper-Strominger hypothesis, PBPs will "recognize" β-lactam antibiotics

as substrate, however after binding a β-lactam the enzyme is irreversibly acylated and unable to catalyze further transpeptidations.

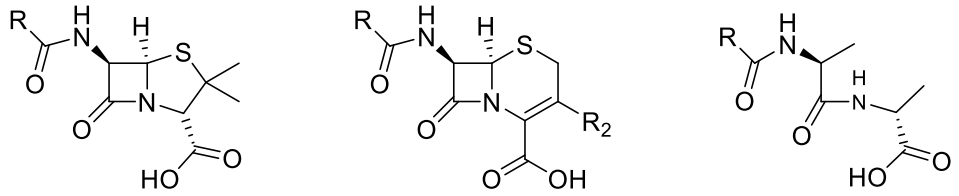


Figure 1.2.2 Generic penams and cephalosporins, two sub-classes of β-lactams. **A)** A terminal D-Ala-D-Ala dipeptide in a conformation that is a structural mimic to the β-lactam core.

Inhibition of PBPs creates a significant problem for a bacterial cell. Because peptidoglycan is a very rigid molecule, it must be broken down by bacterial autolysins for the cell to grow and divide. During growth and division, PBPs and autolysins perform dynamic roles to cross-link and hydrolyze pentapeptides where needed. However, if PBP activity is inhibited but there is autolysin activity, the cell will eventually lyse due to osmotic pressure.

1.3 Methicillin Resistant *Staphylococcus aureus* and mechanisms of β-lactam resistance in MRSA.

MRSA is an abbreviation for Methicillin-Resistant *Staphylococcus aureus.* While methicillin is rarely in clinical use, the mechanism by which *S aureus* becomes resistant to methicillin also applies to many other β-lactam antibiotics. Therefore, the term MRSA has remained as a descriptor of any *S aureus* strain that is resistant to many β-lactam antibiotics.

MRSA is a major threat to public health. Infections from antibiotic resistant bacteria in general are more likely to occur in hospitals due to the close quarters setting filled with vulnerable patients, use of invasive procedures, and frequent use of antibiotics. MRSA in particular is a leading cause of healthcare-associated (HA) infections. In the United States in 2013, MRSA was responsible for approximately 80,000 infections and 11,000 deaths, which is just under half of all deaths caused by drug resistant bacteria in that year.[18]

MRSA is resistant to β-lactam antibiotics through two primary mechanisms: expression of β-lactamase and expression of an alternative PBP, PBP2a. MRSA strains with efflux pumps and thickened peptidoglycan are observed. However, because β-lactams target extracellular processes, reducing the concentration of β-lactam in the cytoplasm would have little effect on PBP inhibition and are generally not significant mechanisms of β-lactam resistance.

β-lactamases are a family of enzymes that can hydrolyze the β-lactam moiety on β-lactam antibiotics. When expressed, β-lactamases keep antibiotics below a cytotoxic concentrations allowing cells to grow in the presence of antibiotics. The β-lactamase expression is controlled by the *bla* operon. The *bla* operon codes for β-lactamase, *blaZ*, and also two regulatory proteins: blaI (inhibitor of *bla*) and blaR1 (activator of *bla*).[19] blaI inhibits *blaZ* expression by binding to the promoter as a blaI dimer. blaR1 exists as a transmembrane protein; when its extracellular domain binds a β-lactam, the intracellular domain is released and degrades blaI, allowing expression of *blaZ*.[20]

PBP2a is a transpeptidase with low affinity for β-lactams because it exhibits allostery. The active site of PBP2a remains in a closed conformation until its allosteric site binds to a NAM molecule.[21,22] Then, the active site adopts an open conformation, allowing the enzyme to briefly catalyze transpeptidations. This mode of action places the enzyme in close proximity of its substrate before the active site is opened, greatly reducing its affinity for β-lactam antibiotics. The β-lactam antibiotic ceftaroline can bind the allosteric site of PBP2a, leaving the active site vulnerable to inactivation by another β-lactam molecule.[23] However, mutations to PBP2a have caused ceftaroline resistance in several clinical isolates of MRSA.[24]

PBP2a is coded for by the *mecA* gene, which is located on the *mec* operon. In addition to *mecA*, this operon also codes for two regulatory proteins, mecI (inhibitor of *mec*) and mecR1 (a transmembrane protein and activator of *mec).* mecI and mecR1 function analogously to blaI and blaR1; upon binding a β-lactam, the intracellular mecR1 domain is released to degrade mecI and allow *mecA* transcription.

The *mec* and *bla* pathways are similar in both their components and order of events. In addition to having similar functions, mecI and blaI also have homology in amino acid sequence.[25] Following this, blaI is observed to regulate both *mec* and *bla* operons in some clinical MRSA strains.[26]

Possessing *mecA* alone is not enough to create many clinically observed MRSA phenotypes. mecR1 mediated *mecA* expression and then subsequent localization of PBP2a to the cell surface is observed to take several hours after initial exposure to β-lactam antibiotics, during

which the cells are still phenotypically vulnerable.[27] blaR1 mediated *blaZ* induction is relatively faster, requiring minutes before functional beta lactamase is observed.[28,29] However, *mec* operons which code for non-functional mecI have been observed.[30] In these cases, *mec* is inhibited by blaI and activated by blaR1. In this regulatory configuration, *mecA* expression is observed to be quicker.[19]

A body information supports that altered PBPs and β-lactamases are the direct mechanisms of β-lactam resistance. However, information also exists that suggests these two mechanisms are supported by a number of auxiliary factors. In a gene knockout experiment conducted on the MRSA "COL" strain, it was observed that 70 separate genes could be disrupted to reduce levels of antibiotic resistance.[31] An understanding of PBPs and β-lactamases may therefore only scratch the surface in terms of the mechanisms of β-lactam resistance.

One example of an auxiliary factor in β-lactam antibiotic resistance is the wall teichoic acid (WTA) synthesis pathway. One enzyme in the WTA synthesis pathway, tarO, is necessary for β-lactam antibiotic resistance in MRSA. tarO can be inhibited with tunicamycin. When MRSA strains are treated with tunicamycin, they're observed to be sensitized to β-lactam antibiotics.[32] One possible explanation proposed by the authors is that tarO is necessary for proper PBP2a localization.

1.4 Resistance modifying agents for the treatment of MRSA.

Resistance modifying agents (RMAs) are molecules that restore antibiotic potency in antibiotic resistant bacteria. RMAs generally target genes or gene products that produce resistant phenotypes, inhibiting that resistance pathway.

RMAs are a promising solution to the problem of MRSA. Because RMAs target a resistance mechanism, they potentially potentiate all antibiotics that mechanism acted on. This can extend the lifetime of current antibiotics which have been optimized for mass production and have well studied toxicology profiles. Additionally, while antibiotics target essential genes, RMAs target non-essential genes. Therefore, there is pressure to develop resistance to an RMA than an antibiotic. While a new antibiotic introduces one more treatment option, an RMA introduces multiple.

Several RMAs for MRSA are currently being researched. One example is a clerodane diterpene compound (CD) isolated from the leaves of the False Ashoka (*Polyalthia longifolia)* tree (Fig 1.4.1A). This compound was discovered to potentiate fluoroquinolones up to 16 fold against MRSA.[33] CD is an inhibitor of the MRSA multi-drug efflux pump, norA. Because norA prevents a therapeutic dose of fluoroquinolones from accumulating inside the cell, norA inhibition by CD has a fluroquinolone re-sensitizing effects against MRSA.

Another RMA currently being researched is Tunicamycin (Fig 1.4.1B). Tunicamycin is an inhibitor of tarO, one of the early-stage enzymes involved in wall teichoic acid (WTA)

synthesis.[34] While WTA is not necessary for the cell to survive, inhibition of WTA synthesis by tunicamycin has been shown to act synergistically with β-lactams in MRSA.[35] The authors note that cells treated with tunicamycin showed irregularity in peptidoglycan structure and irregular cell division. Based on this, WTA synthesis and peptidoglycan are possibly share some commonality in the early steps of their syntheses such that inhibiting WTA synthesis also disrupts the synthesis of peptidoglycan.

Another RMA candidate is epigallocatechin gallate (EGCg), a flavonoid found in green tea (Fig 1.4.1C). EGCg potentiates the carbapenem subclass of β-lactams in several clinical isolates of MRSA.[36] The synergistic effect between EGCg and carbapenems is thought to be because EGCg interferes with the localization of PBP2a to the cell wall.[37]

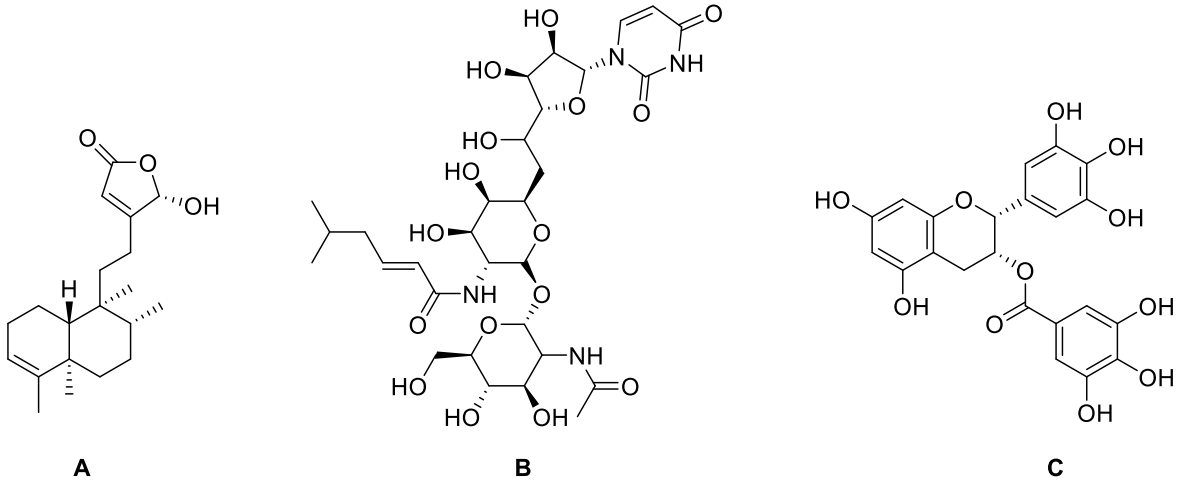


Figure 1.4.1 Three resistance modifying agents in MRSA. **A)** A clerodane diterpene that re-sensitizes MRSA to fluoroquinolones by inhibiting the norA multidrug efflux pump. **B)**

Tunicamycin, an inhibitor of teichoic acid synthesis potentiates oxacillin 125 fold in MRSA **C)** epigallocatechin gallate is thought to potentiates carbapenems in MRSA by causing mislocalization of PBP2a.

While there is an ongoing effort to discover RMAs, currently the only clinically approved RMAs are β-lactamase inhibitors, which are used to treat β-lactamase producing bacteria.[38] (Fig 1.4.2). These compounds acylate β-lactamase and form a very stable enzyme-substrate complex, leaving the enzyme inactive.

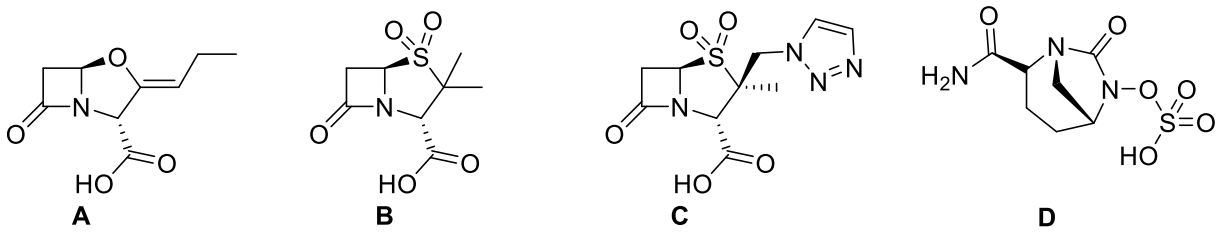


Figure 1.4.2 Clinically used β-lactamase inhibitors. **A)** Clavulanic acid. **B)** Sulbactam **C)** Tazobactam. **D)** Avibactam, a newer inhibitor approved in 2015. **A, B, C** share a similar mechanism of action. They form stable "bridges" between two serines in the β-lactamase active site.[39]

Resistance to current β-lactamase inhibitors is not common, despite the popularity of the combinatorial therapies that utilize β-lactamase inhibitors.[40] This may be due to the similarity between the inhibitor and substrate. A mutation in β-lactamase that still allows the enzyme to bind β-lactams while not binding β-lactamase inhibitors would be less likely to occur. Unfortunately, β-lactamase inhibitors themselves are generally not useful in treating MRSA infections because the MRSA phenotype is primarily due to PBP2a.

2. Prior Wang group research

2.1 Synthesis of polycyclic indole alkaloids and their β-lactam re-sensitizing activity in MRSA

Indole alkaloids are a diverse group of plant-based compounds that contain the indole moiety (Fig 2.1.1A). Many indole alkaloids have physiological effects, such as the chemotherapy drugs vinblastine and vincristine, the psychedelic psilocybin, and the antihypertensive ajmalicine. Indole itself is a component of many biomolecules, such as serotonin and tryptophan. Additionally, indole is a byproduct of bacterial tryptophan metabolism, and further metabolites of indole have roles in inducing virulence and drug resistance.[41]

Because of the massive range of physiological effects indole compounds have, the Wang group developed a one-pot procedure for the synthesis of functionalized alkynyl indoles (Fig 2.1.1C).[42] Then, using a previously described gold catalyzed tandem cyclization, the alkynyl indoles are transformed into a tetracyclic indolines (2.1.1D).

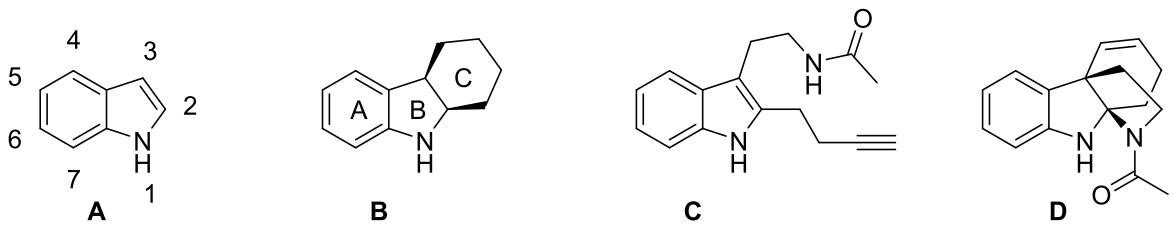
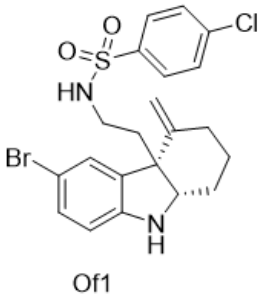


Figure 2.1.1 A) indole with atoms numbered **B)** A generic tricyclic indoline with rings labelled **C)** An alkynyl indole synthesized via the one-pot procedure **D)** A tetracyclic indoline, synthesized through gold catalyzed tandem cyclization of **C.**

Using this same procedure, a library of tricyclic and tetracyclic indolines was assembled.[43] This compound library was then screened for their ability to potentiate



Of1

Figure 2.1.2 A screen of the initial indoline library reveals Of1 selectively re-potentiates β-lactams in MRSA

methicillin in American Type Culture Association (ATCC) MRSA strain BAA-044, which is a strain resistant to many β-lactams.[44] One compound, Of1 (Fig 2.1.2), was found to re-sensitize MRSA to β-lactams, but not other classes of antibiotics (Table 2.1.1). Of1 was not observed to have any antibiotic activity itself.

Table 2.1.1 Of1 potentiates β-lactams in MRSA. The minimum re-sensitizing concentration of Of1 is dependent on the antibiotic.

Compound	MIC (μg/mL)	MIC (+Of1)* (μg/mL)	Fold of potentiation	Sensitive range† (μg/mL)
Of1	>128	—	—	—
Methicillin	128	8	16	≤8
Oxacillin	64	0.5	128	≤2
Amox/clav	32/16	4/2	8	≤4/2
Meropenem	4	0.25	16	≤4
Imipenem	8	1	8	≤4
Cephalexin	256	16	16	≤8
Cefazolin	128	4	32	≤8
Rifampicin	2	1	2	≤1
Tetracycline	64	64	1	≤4
Ciprofloxacin	8	8	1	≤1
Azithromycin	>256	>256	—	≤2
Erythromycin	>256	>256	—	≤0.5
Clindamycin	>256	>256	—	≤0.5
Streptomycin	>256	>256	—	—
Vancomycin	1	1	1	≤2
Linezolid	2	2	1	≤4

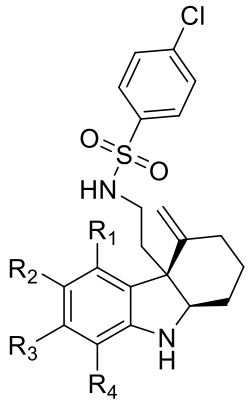
* MIC measured with 20 μM Of1
† Values obtained from the Clinical and Laboratory Standards Institute (CLSI)[45]

2.2 Structure Activity Relationship studies of Of1

Using Of1 as a lead compound, a structure activity relationship (SAR) study was done to first determine which parts of the molecule were critical to its function, and then determine what changes could be made to make the molecule more potent. First, multiple analogues of Of1 were synthesized with different substitution patterns on the indoline aromatic ring. Their minimum re-sensitizing concentrations (MRC) for various β-lactams were assessed.[46]

There were several notable observations made during this SAR study (Table 2.2.1). Modification of the indoline or sulfonamide nitrogens resulted in a complete loss of activity. A halogen at R_2 (indoline five position) is necessary for activity; a bromo substitution gives most potent compound, chloro will give slightly diminished activity but decreased mammalian toxicity. Further experimentation with halogen substitution revealed that maintaining the bromine at R_2 while adding a fluorine at R_4 gives slightly improved activity and decreased mammalian toxicity. Chlorines at R_2 and R_4 maintains the same activity as Of1 with slightly decreased mammalian toxicity. Halogen substitutions are best, but a methyl group at R_2 is tolerated with slight loss of activity.

Table 2.2.1 Hit compounds discovered from SAR study of indoline aromatic ring substitution. Lead compound is bold faced.

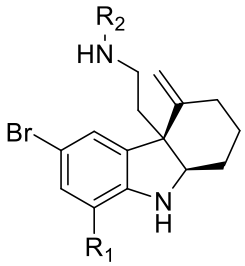


Entry	Compound	R_1	R_2	R_3	R_4	Amox/clav[ad]	Cefazolin[ad]	Meropenem[bd]	GI_{50}[c]
1	**Of1**	**H**	**Br**	**H**	**H**	**4**	**4**	**4**	**17.1**
2	6a	H	Cl	H	H	8	4	8	35
3	6k	H	Cl	H	Cl	4	4	4	13.6
4	6l	H	Br	H	F	2	4	4	18.1

[a] MRSA ATCC BAA-044. [b] MRSA ATCC 33592. [c] HeLa cells. [d]MRC
All units µg/ml.

Next, substitution of the side chain was assessed. The bromine was kept at the indoline five position of all analogues, while fluorine was added to some analogues at the indoline seven position (Table 2.2.2). The sulfonamide phenyl was found to be necessary for activity; when it was exchanged for a pyridine activity was lost. For the sulfonamide phenyl, substitution is required at the para position (relative to the sulfonamide) for activity. When R_1 is Hydrogen, a para fluoro or methyl substitution on the sulfonamide phenyl eliminates activity. But, these changes are tolerated when R_1 is fluoro. Substitutions may be made to the meta position of the phenyl ring if the para substitution is maintained.

Table 2.2.2 Selected active compounds from SAR study of the indoline side chain. Lead compounds are bold faced.



Entry	Compound	R_1	R_2	Amox/clav[ad]	Cefazolin[ad]	Meropenem[bd]	GI_{50}[c]
1	**Of1**	**H**	**SO_2Ph^pCl**	**4**	**4**	**4**	**17.1**
2	12g	H	SO_2Ph^pBr	4	4	8	40
3	12h	H	SO_2Ph^pI	4	4	32	33
4	12i	H	$SO_2Ph^{3,4}Cl$	4	2	4	12.8
5	13a	F	SO_2Ph^pOMe	8	4	8	49
6	13b	F	SO_2Ph^pMe	4	4	4	22
7	**6i**	**F**	**SO_2Ph^pCl**	**2**	**4**	**4**	**18.1**
8	13d	F	SO_2Ph^pBr	1	1	1	22
9	13e	F	SO_2Ph^p I	4	2	4	19.6
10	13f	F	$SO_2Ph^{3,4}Cl$	4	4	4	31
11	13h	F	SO_2Ph^pCN	8	4	4	17

[a] MRSA ATCC BAA-044. [b] MRSA ATCC 33592. [c] HeLa cells. [d] MRC. All units µg/ml

The Of1 SAR study established trends for what changes could be made to the Of1 core while still maintaining activity. In summary, the aromatic rings are tolerant to some modification. The side chain phenyl requires a para substitution for activity, and the indoline aromatic ring requires a substitution at the 5 indoline

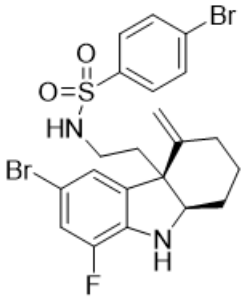


Figure 2.2.1 Compound 13d, the most potent analogue of Of1 discovered during SAR of Of1

position. However, neither nitrogen on the molecule is tolerant of further changes. The 5-Bromo-7-Fluoro substitution pattern on the indoline aromatic system gives the most potent compounds. Compound 13d (Fig 1.6.3) was the most potent compound discovered in this series of SAR investigations.

2.3 Synthesis and SAR studies of aza-tricyclic indolines.

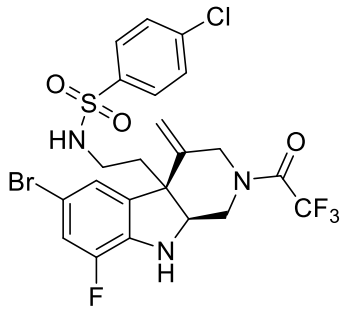
The Of1 SAR studies optimized the molecule to act in vitro. However, in vivo, drug molecules are must do many more things, such as absorb into the body and be metabolically stable. A compound's ability to do these things can be approximated by that compound's clogD measurement. This is a measure of that molecule's lipophilicity, where higher clogD values indicate a more lipophilic molecule.

All of the hit compounds from the Of1 series are very lipophilic molecules. Adequate lipophilicity is a necessary property for any drug that must pass through plasma membranes, such as an orally administered drug which must pass through the plasma membranes of enterocytes before entering the blood stream. However, a molecule that is too lipophilic stands to become "stuck" within the plasma membrane. Conversely, a molecule that lacks lipophilic character will not diffuse across the plasma membrane and would likely need to be injected into blood or some body cavity. Thus for an orally administered drug, lipophilicity must lie in some "goldilocks zone" so that the molecule can diffuse into a plasma membrane, but will also diffuse out to ensure optimal distribution throughout the body.

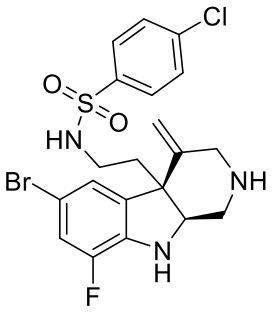
Additionally, highly lipophilic compounds are more likely to have unfavorable interactions with serum protein, and more likely to cause cytotoxicity.[47] Furthermore, compounds with high clogD are generally metabolically unstable because they share these physical properties with the native substrate of the Cytochrome P450 (CYP450) family of enzymes and are therefore easily metabolized.[48] Of1 and 13d are relatively lipophilic molecules, with $ClogD_{7.4}$ of 4.8 and 5.1, respectively. The stability of Of1 was tested with CYP450, and was found to be nearly fully metabolized after half an hour (data not published).

As follows, the next series of analogues was focused on improving the physical properties of the Of1 series to optimize their pharmacokinetics (absorption, metabolism, distribution). Using an adapted synthetic pathway, a series of tricyclic indolines were synthesized in which a carbon of the C ring was replaced with a nitrogen (to make an "aza-compound" is to replace a carbon atom in that compound with a nitrogen atom).[49] The C ring nitrogen first functionalized to see if that position is tolerant of modification, and what types of functional groups would be accepted at that position. Following that, the C ring nitrogen was used to further optimize the physical properties of the molecule (Table 2.2.1). The addition of a guanidine at the C ring nitrogen produced a compound with low lipophilicity ($ClogD_{7.4}$ = 0.54), low mammalian toxicity, and still maintained the ability to potentiate β-lactam antibiotics; in fact, the guanidine compound is a more potent RMA than Of1. This compound was also found to have bacteriostatic effects in MRSA.

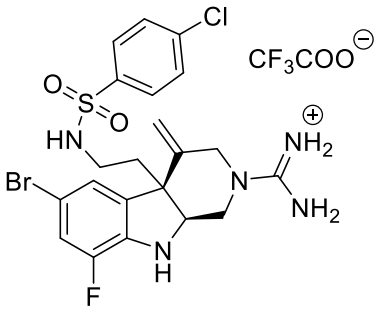
Table 2.2.1 Hit compound from the Aza-Of1 series.



11pb



12pb



13pb

Entry	Compound	Cefazolin[a]	Amox/Clav[a]	MIC[a]	GI_{50}[b]	$ClogD_{7.4}$
1	Of1	4	4	>128	17	4.8
2	13d	1	1	NT	22	5.1
3	11pb	4	4	>32	19	4.13
4	12pb	4	4	8	6.7	2.0
5	13pb	2	2	8	40	0.74

[a] MRC MRSA ATCC BAA-044, µg/ml. [b] HeLa cells, µg/ml.

Compound 13pb is an improvement to Of1 and 13d in terms of physical properties. The $clogD_{7.4}$ of 13pb is significantly lower than both Of1 and 13d. These properties would likely improve the metabolic stability and distribution of the compound, but the very polar and charged guanidine group on 13pb makes oral administration unviable because it has too much polar area to enter a lipid layer.[50] Therefore, to test its pharmacokinetic properties in vivo, 13pb was injected intraperitoneally into mice. [41] A maximum concentration of 8µg/ml was achieved after half an hour, and the biological half-life of 13pb was observed to be 2.5 hours. These injections were well tolerated, with only minor clinical observations.

The aqueous solubility of Of1 and 13pb was also quantified. Of1 reaches its aqueous saturation concentration at 0.86 µg/ml, which is less than its effective concentration in MRSA.

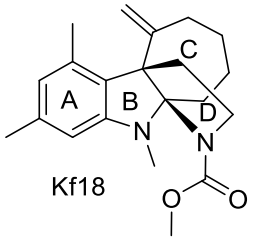
This alone renders Of1 non-viable as a drug, because regardless of how it is administered it will never reach a therapeutic dose in the body. However, 13pb has a saturation concentration of 587 μg/ml which is orders of magnitude greater than its effective concentration.[41]

These findings underscore the potential of the aza-C ring: the nitrogen can be functionalized to fine tune the physical and chemical properties of the molecule. Through functionalization of the aza-C ring, the pharmacokinetic properties of the molecule can be optimized. Compared to Of1, 13pb has increased activity, metabolic stability, and water solubility, with decreased mammalian toxicity.

2.4 Synthesis and SAR studies of tetracyclic indolines.

Along with tricyclic indolines, tetracyclic indolines were also explored. Compound Kf18 is a tetracyclic indoline that was part of the original library along with Of1. Kf18 also selectively potentiates β-lactam antibiotics in MRSA (Table 2.4.1). There are a few significant changes that separate Kf18 from Of1. Along with an additional D ring, the Kf18 C ring is seven membered, the indoline nitrogen is methylated, and the substitution on the indoline aromatic ring are methyl groups.

Table 2.4.1 Hit compound Kf18 selectively potentiates β-lactams in MRSA. Rings are labelled.



Kf18

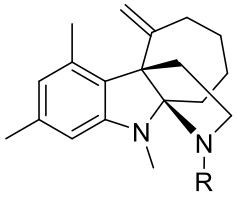
Antibiotic	MIC[a]	MIC + Kf18[ab]	Fold potentiation
Methicillin	128	8	16
Amox/Clav	32	4	8
Cefazolin	128	8	16
Meropenem	32	2	16
Oxacillin	64	8	8
Tetracycline	32	16	2
Ciprofloxacin	8	4	2
Erythromycin	128	128	1

[a] MRSA ATCC BAA-044. [b]MIC determined with 20 μg/ml Kf18. MIC units are μg/ml

Using Kf18 as a lead compound, a library of tetracyclic indolines was synthesized for further SAR studies.[51] First, the substitution on the D ring nitrogen was assessed.[42] Other carbamate groups were installed in place of the methyl carbamate (Table 2.4.2). Then, to investigate of the necessity of the carbamate moiety, analogues were made replacing the carbamate with amides, ureas, guanidines, and alkyl groups. Additionally, the para-bromosulfonamide group, identified as a key component to the hit compounds of the tricyclic indolines, was used. The sulfonamide and urea groups eliminated activity. Guanidine, amide, and alkyl groups led to less potent RMAs with similar mammalian toxicity to Kf18. Carbamate substitutions at the D ring nitrogen were found to produce the most potent compounds. Of the carbamate substitutions, ethyl carbamate performed well, giving hit compound 6a. 6a is more

potent and less toxic to mammalian cells relative to Kf18. However, larger alkyl groups on the carbamate are not correlated with increased potency; expanding the carbamate to a *tert*-butyl carbamate led to a less potent compound than Kf18. Of all compounds tested, none had bacteriostatic effects.

Table 2.4.2 Selected compounds from Kf18 SAR study of the D ring nitrogen. Lead compound is bold face.



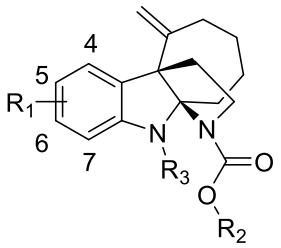
Entry	Compound	R	Amox/clav[ad]	Cefazolin[bd]	MIC	GI_{50}[c]
1	**Kf18**	**COOMe**	**16**	**16**	**>64**	**20**
2	6a	COOEt	2	2	>64	61
3	6c	COO^tBu	4	8	>64	47
4	6d	$COCH_3$	32	32	>64	15
5	6g	SO_2Ph^pCl	>64	>64	>64	>64
6	6i	CONHEt	>64	>64	>64	>64
7	6k	$C(NH)NH_2$	32	16	>64	19
8	6l	Me	16	8	>64	20
9	6m	Et	32	8	>64	18

[a] MRC in MRSA NRS-100. [b] MRC in MRSA ATCC BAA-044. [c] HeLa cells. [d] MRC. All units are µg/ml

Using 6a and Kf18 as lead compounds, further SAR studies were done on the indoline nitrogen and aromatic indoline ring (Table 2.4.3). C ring size (six vs seven membered) was also assessed, but all with all other modifications being the same, compounds with seven membered rings were more potent RMAs than compounds with six membered rings (data not shown).

It was concluded that the 4,6-dimethyl is the best substitution pattern on the indoline aromatic ring. 5-methyl and 5-chloro substitutions give less potent compounds, but these were observed to be less toxic. Additionally, loss of the methyl group at the indoline nitrogen leads so a slight loss of activity.

Table 2.4.3 Selected compounds from SAR at the indoline aromatic ring and indoline nitrogen. Lead compounds are bold faced.



Entry	Compound	R_1	R_2	R_3	Amox/Clav[bd]	Cefazolin[ad]	MIC[a]	GI_{50}[c]
1	**Kf18**	**4,6-diMe**	**Me**	**Me**	**16**	**16**	**>64**	**20**
2	**6a**	**4,6-diMe**	**Et**	**Me**	**2**	**2**	**>64**	**61**
3	13a	4,6-diMe	Me	Et	4	2	>64	30
4	11	4,6-diMe	Me	H	8	4	>64	28
5	13d	5-Me	Me	Me	4	8	>64	27
6	13e	5-Cl	Me	Me	4	8	>64	53
7	13f	5-F	Et	Me	>64	>64	>64	>64
8	13g	5-F-	Et	Me	>64	>64	>64	>64

[a] MRSA NRS-100. [b] MRSA ATCC BAA-44. [c] HeLa cells. [d] MRC. Units are in µg/mL

2.5 Summary of SAR studies

The tricyclic series was first optimized for activity in vitro. It was found that the 5-bromo and 5-bromo-7-fluoro substitution pattern on the indoline aromatic ring produced the most potent compounds. The phenyl ring on the sulfonamide is necessary for activity, and a para-bromo or

para-chloro substitution on the sulfonamide phenyl provides the most potent compounds. The indoline and tryptamine nitrogen is not tolerant of modification in these compounds. 13d was discovered as the hit compound during these experiments. While potent in vitro, 13d lacked the physical properties to be useful in vivo due to its high lipophilicity and low polar surface area.

The aza-tricyclic series was then synthesized to explore if the C ring is tolerant of modification, and if modifications at that position can be used to improve the pharmacokinetics of that molecule. Of all groups tested, a guanidine (compound 13pb) was found to be the most active while providing the physical properties desired for metabolic stability and optimal distribution. While these properties would likely hinder 13pb as an orally administered drug, it was injected intraperitoneally in mice with minimal clinical observations.

For the tetracyclic series, it was found that the 5,7-dimethyl substitution pattern on the indoline aromatic ring produced the most potent compounds. Analogues with six and seven membered C rings were tested, and all seven membered C ring analogues were more active than their respective six membered analogues. Of the D ring nitrogen substitutions, carbamates were the best, with ethyl carbamate producing the most potent compound; larger carbamates did not produce more active compounds.

3 Discussion

The tetracyclic series had been optimized for in vitro studies. However, the hit compounds were lacking the physical properties that would allow them to perform in vivo. Using

the aza-tricyclic project as a model, we planned to optimize the physical properties of the tetracyclic series. I would synthesize the aza-tetracyclic compounds to assess if the tetracyclic core can be modified while still remaining active.

We planned the structure of the indole core based on the most active compound from the tetracyclic series, 6a (Fig 3.1). However, the aza-tetracyclic series would first be made with a six membered C rings. The aza 7 membered C ring compounds are more synthetically difficult, so it would be more efficient to determine which functional groups are tolerated at the C ring, and then use those groups to functionalize the 7 membered C ring compounds. Following this, any hits from the aza-tetracyclic six membered C ring series would then be used as leads to synthesize a series with seven membered C rings.

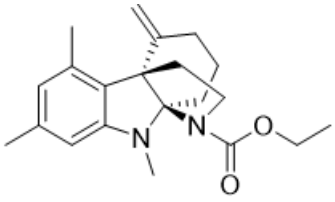


Figure 3.1 Compound 6a, the lead compound for the aza-tetracyclic series.

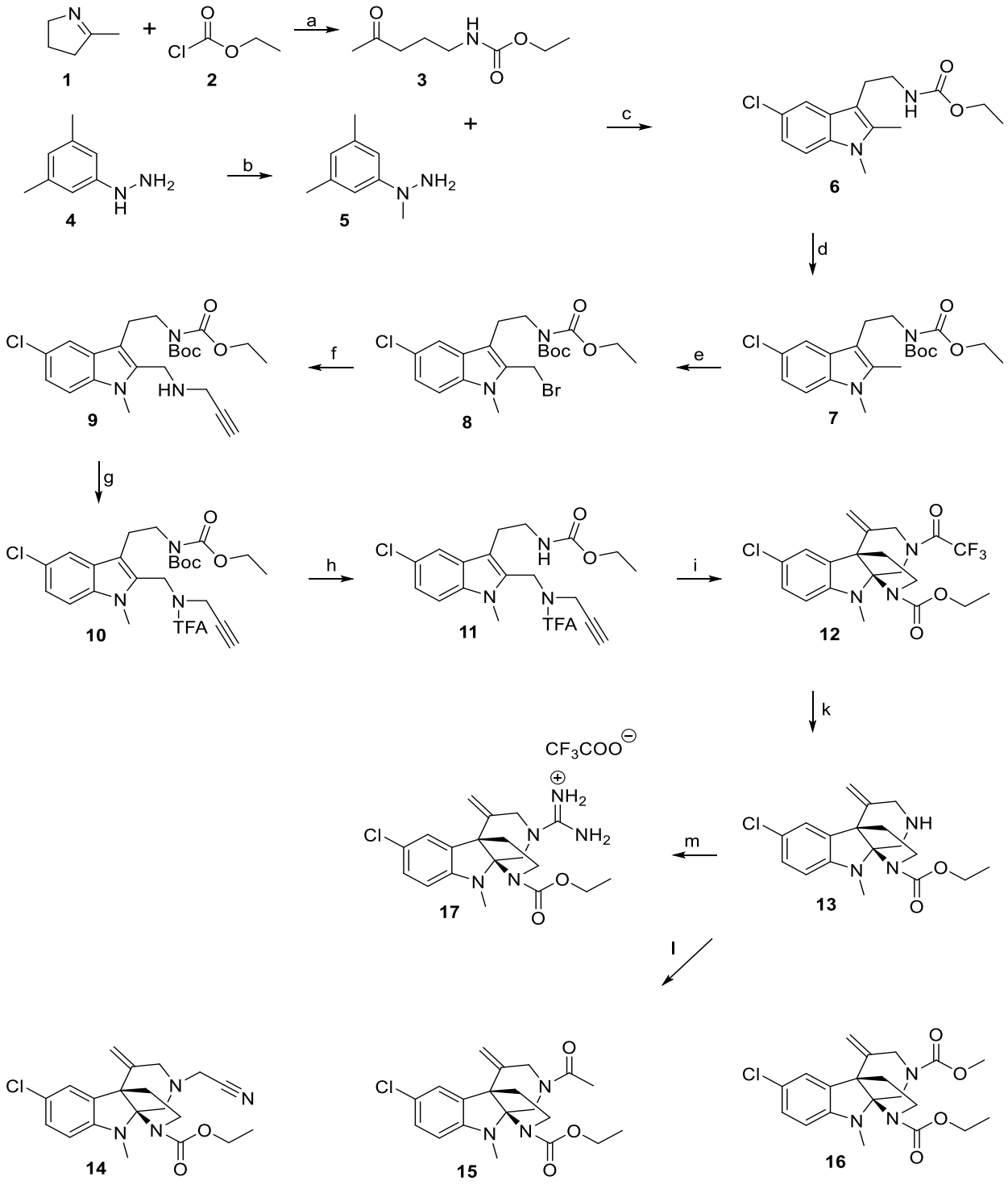
I began by synthesizing starting materials **3** and **5** (scheme 3.1). Commercially available imine **1** and chloroformate **2** were stirred with dimethylaminopyridine (DMAP) in acetonitrile (ACN) before adding excess aqueous hydrochloric acid to give ketone **3.** Phenyl hydrazine **4** was stirred in tetrahydrofuran (THF) with sodium bis-trimethylsilylamide (NaHMDS) before adding methyl iodide to give methylated phenylhydrazine **5**.

Next I synthesized the cyclization precursor **11**. **3** and **5** were combined in fischer indole conditions to give 2-methyl indole **6**, on which the tryptamine nitrogen is protected with a *tert*-butyoxycarbonyl (Boc) group to give **7**. **7** can be selectively bominated at the 2-methyl position

in the presence of other benzylic methyl groups using radical conditions described by Barbour *et al*. to get bromide **8**.[52] Mono-alkylation of 1-amino-3-propyne by **8** was obtained by running the reaction at -10 ^{o}C to yield alkynyl indole **9**, on which the newly added amine is protected by trifluoroacetic anhydride to give **10**.

While prior publications describe using a 4-nitrobenzenesulfonyl group to protect the propargylamine, I have found that the trifluoroacetamide group does not negatively impact the cyclization and is not affected by Boc deprotection conditions. Because deprotection of trifluoroacetamide can be achieved with milder conditions than deprotection of 4-nitrobenzenesulfonyl, I have elected to use trifluoroacetamide in this synthesis. From **10**, the Boc group is deprotected to give cyclization precursor **11.**

Cyclization precursor **11** was converted to aza-tetracyclic indoline **12** as a racemic mixture under conditions optimized by Barbour *et al.* I did not observe the addition of the methyl group at the indole nitrogen to have a negative impact on the cyclization. **12** was prepared for further functionalization by deprotecting the trifluoroacetamide group to give **13**. Analogues **14**, **15**, **16** were made by treating **13** with the corresponding chlorides (**15, 16**) or bromides (**14)**. Analogue **17** was made by treating **13** with 1,3-Di-Boc-2-(trifluoromethylsulfonyl)guanidine followed by deprotection of the Boc groups.



Scheme 3.1 Synthesis of **14, 15, 16, 17** from commercially available starting materials. Conditions and reagents: (a) ACN, DMAP, 2hr, then $HCl_{aq.}$ (b) THF, NaHMDS, 2hr, then MeI. (c) EtOH, TCT, 85°C, 16hr. (d) THF, DMAP, Boc_2O, 24hr. (e) CCl_4, NBS, BPO, 65°C, 30 min. (f) ACN, propargylamine, -10°C, 30 min. (g) DCM, TFA_2O, DMAP, 12hr. (h) DCM, TFA, 1hr. (i) 1,4 dioxane, $XPhosAuNTf_2$, 65°C, 1hr. (k) EtOH, $K_2CO_{3(aq)}$, 12hr. (m) DMF, 1,3-Di-Boc-2-(trifluoromethylsulfonyl)guanidine, 60°C, 12 hr; then MeOH, TFA, 1 hr. (l) ACN, R-Cl, DMAP, 12 hr.

Here, I would like to discuss why the analogues synthesized have 5-chloro substitutions at the indoline aromatic ring, whereas 6a has a 4,6-dimethyl substitution. The 4,6-dimethyl indole could not be selectively brominated at the 2-methyl position. An inseparable mixture of products was observed after running the reaction in standard conditions. I believe the aromatic system of this compound may have been too electron rich, allowing a competing electrophilic aromatic substitution reaction to occur. Therefore, a more electron deficient compound, the 5-chloro compound, was synthesized to overcome this barrier.

Compounds **12, 13, 14, 15, 16, 17** were then tested for β-lactam re-sensitizing activity in ATCC MRSA BAA-044 by Dr. Wei Wang following a procedure outlined by the Clinical and Lab Standards Institute (table 3.1).[53]

Table 3.1 Aza-tetracyclic indolines tested for MIC, MRC, and mammalian toxicity. Lead compound is bold faced.

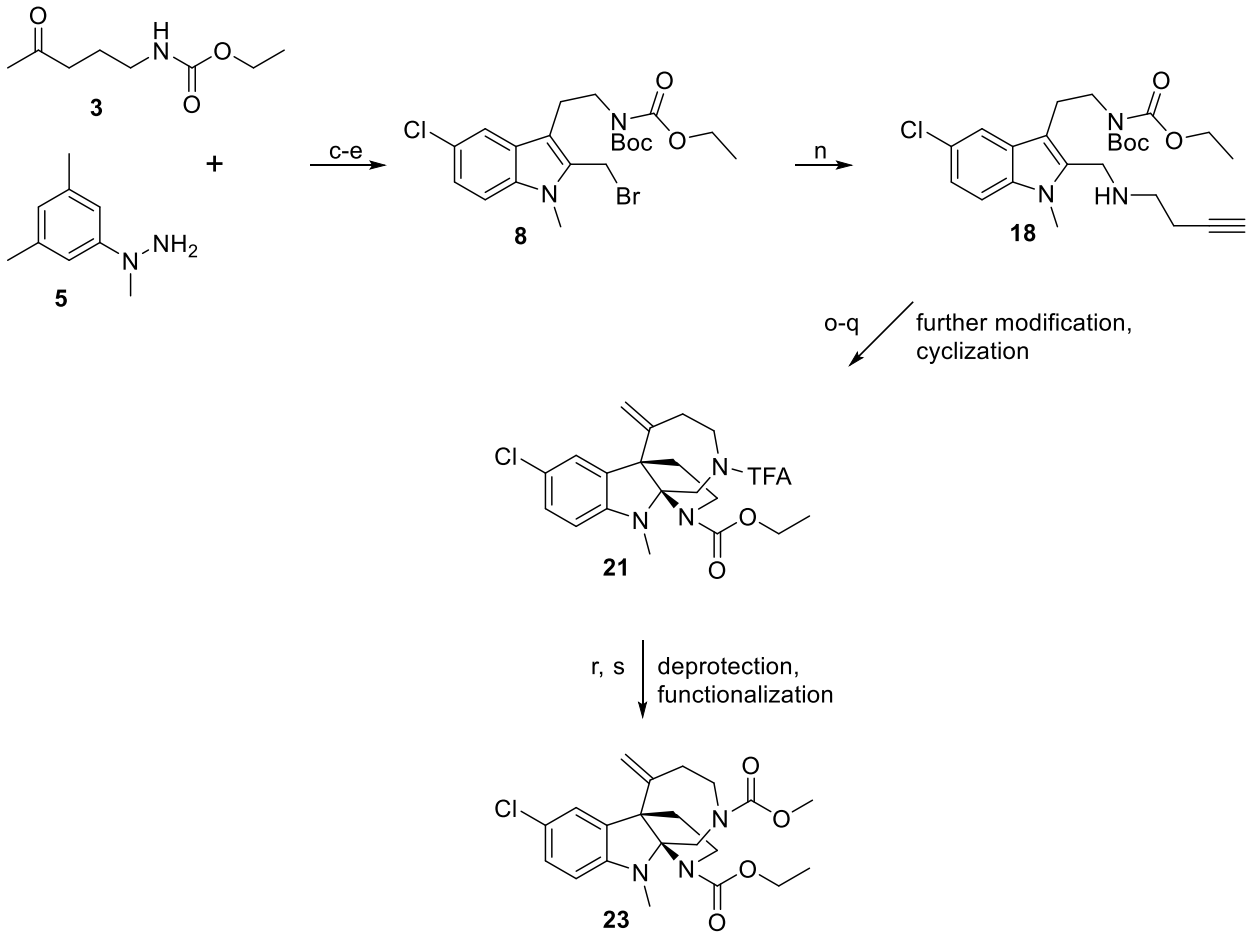
Entry	Compound	MIC[a]	Cefazolin[ab]	Amox/Clav[ab]	GI_{50}[ac]	$ClogD_{7.4}$	PSA[d]
1	**6a**	**>64**	**2**	**2**	**61**	**5.21**	**32.78**
2	12	>64	>64	>64	N/T	3.78	53.09
3	13	>64	64	64	25.4	2.25	44.81
4	14	>64	>64	>64	N/T	2.95	59.81
5	15	>64	>64	>64	N/T	2.64	53.09
6	16	>64	16	16	24.8	3.26	62.32
7	17	>64	64	64	51.5	0.12	87.63

[a] μg/ml. [b] MRC in ATCC BAA-044. [c] HeLa cells. [d] Angstroms. N/T: not tested

Unlike the tricyclic series, the tetracyclic series seems less tolerant to modification at the C ring. Groups from the hits of the aza-tricyclic series were tried, such as trifluoroacetamide and

guanidine, which yielded no active compounds. Other groups were tried that would produce a compound with a clog$D_{7.4}$ from ~2.5 – 3.5, but all of these were inactive except for the methyl carbamate analogue, **14**. Compared to **6a**, **14** shows decreased activity for cefazolin and amox/clav, as well as increased mammalian toxicity. However, **14** has improved physical properties compared to **6a** and would therefore be a better candidate to test in vivo.
Because seven membered C rings were more active in the tetracyclic series, I went forward with the synthesis of a seven membered C ring analogue of **14** (scheme 5.2).

I first synthesized brominated indole **8**, which was then used to mono-alkylate 1-amino-3-butyne at -10 ^{o}C to give **18**. Further modification of **18** yields the cyclization precursor which is cyclized to give **21.** Deprotection of the trifluoroacetamide gives **22**, which is treated with methyl chloroformate to give **23**.



Scheme 5.2 (c) EtOH, TCT, 85ºC, 16hr. (d) THF, DMAP, Boc_2O, 24hr. (e) CCl_4, NBS, BPO, 65ºC, 30 min. (n) ACN, 1-amino-3-butyne, -10ºC, 30 min. (o) DCM, TFA_2O, DMAP, 12hr. (p) DCM, TFA, 1hr. (q) 1,4 dioxane, $XPhosAuNTf_2$, 65ºC, 1hr. (r) EtOH, $K_2CO_{3(aq)}$, 12hr. (s) ACN, DMAP, Methyl Chloroformate, 4hr.

21 and **23** were then tested for β-lactam antibiotic potentiating activity as well as mammalian toxicity following procedures described by the CLSI (Table 3.2).

Table 3.2 seven membered C ring aza-tetracyclic indolines tested for MIC, MRC, and mammalian toxicity. Lead compound is bold faced.

Entry	Compound	MIC[a]	Cefazolin[ab]	Amox/Clav[ab]	GI_{50}[ac]	$ClogD_{7.4}$
1	**6a**	**>64**	**2**	**2**	**61**	**5.21**
2	16	>64	16	16	24.8	3.26
3	21	>64	>64	>64	7.2	4.06
4	23	>64	16	16	42.3	3.55

[a] μg/ml. [b] MRC in ATCC BAA-044. [c] HeLa cells.

We found that **23** had the same activity as **14**, its 6 member C ring analogue (table 5.2). This goes against the trend observed in the tetracyclic series, in which seven membered C ring analogues were more active than the six membered C ring analogues. As mentioned previously, the C ring in the tetracyclic series seems less tolerant of modification. It's possible that the added steric bulk at the C ring hinders how these molecules bind their target leading to the observed deviation from the trend.

Although **23** is a less potent RMA than 6a, it is an improvement in terms of physical properties. 6a has similar physical properties to Of1, i.e. high $clogD_{7.4}$. As follows, the poor pharmacokinetics that prevent Of1 from being a successful drug in vivo would also hinder 6a. **23** has a lower clogD compared to 6a. These properties are likely to decrease the affinity of CYP450 enzymes for **23**, and to facilitate faster diffusion across membranes.

4 Outlook

Interestingly, many of the functional groups from hit compounds in the aza-tricyclic series (guanidine, trifluoroacetamide, hydrogen) abolished activity on the aza-tetracyclic compounds. Furthermore, modification at the indoline nitrogen on the tricyclic series abolished activity, but improves activity in the tetracyclic series. It's also noteworthy that no tetracyclic compounds have bacteriostatic activity, and groups which confer bacteriostatic activity to the tricyclic series do not have the same effect in the tetracyclic series. For example, Of1 has no observable bacteriostatic effect, but the addition of a guanidine to the C ring (compound 13pb) produces a bacteriostatic compound (8µg/mL). Addition of guanidine to the tetracyclic series (**17)** does not produce a bacteriostatic compound.

Based on these observations, I propose that the tricyclic and tetracyclic series may be binding to different targets within bacterial cells. The additional D ring is a significant difference and greatly alters the three-dimensional shapes of these molecules. It's possible that a large structural change such as an additional bridged ring could impact how the molecule interacts with the components in a biological system. Following from this idea, functional groups on the tricyclic series which are conducive to target binding may hinder target binding on the tetracyclic series.

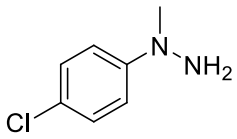
To test this hypothesis, I propose the synthesis of a further aza-tricyclic indoline series that are direct analogues of the aza-tetracyclic library. This would remove other structural variables between the two series such as indoline aromatic ring substitution and tryptamine nitrogen functionalization, and allow us to directly test how the D ring impacts activity.

Additionally, carbamate modifications were tolerated on the tetracyclic scaffold. Logically, more carbamate analogues could be synthesized with varying alkyl substitutions. It would be interesting to see how larger alkyl groups such as ethyl or isopropyl groups may impact activity.

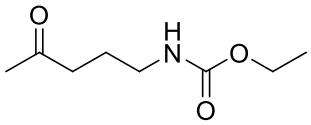
5. Summary and conclusions

New strains of multi-drug resistant bacteria are observed faster than new antibiotics can enter the clinic. Our lab have discovered two structurally distinct classes of polycyclic indolines that both potentiate β-lactam antibiotics in vitro against MRSA. These classes were optimized to perform in vitro, but lacked the physical properties to perform in vitro. Aza-tricyclic indolines were synthesized to improve the physical properties of the tricyclic series with great success. With this impetus, I synthesized a small library of aza-tetracyclic indolines to improve the physical properties of the tetracyclic series. I discovered two molecules with activity that have improved physical properties compared to its lead compound and is therefore a better candidate for in vivo testing. This molecule also demonstrates the utility of the aza-indoline scaffold. The nitrogen can be easily functionalized to produce a variety of molecules with different physical properties.

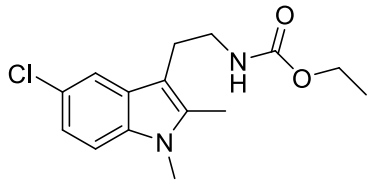
6. Experimental



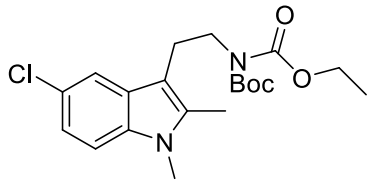
Synthesis of **5**: To a stirring solution of tetrahydrofuran (53ml) at 0°C, 4 (2.26g, 15.8mmol) was added. To this mixture, a 1M solution of NaHMDS (17ml, 17mmol) was added. The reaction was then placed under argon and allowed to stir at room temperature for two hours. Afterwards Methyl Iodide (0.94ml, 15.8mmol) was added at 0°C. The reaction was allowed to stir overnight at room temperature. The reaction was then concentrated at reduced pressure and reconstituted with ethyl acetate. The organic layer was washed with water, dried with sodium sulfate, and concentrated at reduced pressure. The residue as purified by silica gel column chromatography (hexanes:ethyl acetate = 8:2) to afford **5** as a white/light pink solid (1.61g, 10.3mmol, 66%). Rf = 0.2 (hexanes:ethyl acetate = 8:2). 1H NMR (300 MHz, Chloroform-d) δ 7.23 – 7.15 (m, 2H), 6.97 – 6.89 (m, 2H), 3.78 – 3.56 (m, 2H), 3.08 (s, 3H). IR (cm^{-1}): 2843, 2698, 2363, 1599, 1499.



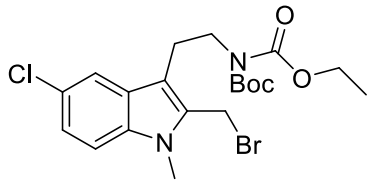
Synthesis of **3**: To a stirring solution of 4-dimethylaminopyridine (4.398g, 36mmol) in Acetonitrile (100mL) at 0°C, chloroformate **2** (3.906g, 36mmol) was added. This solution was stirred at room temperature for 15 minutes before imine **1** was added at 0°C. This solution was stirred at room temperature for two hours before excess aqueous HCl was added. The reaction then stirred overnight under argon before being extracted five times with DCM. The organic layers were combined and concentrated at reduced pressure and the residue was purified by silica gel column chromatography (hexanes:ethyl acetate = 7:3) to afford **3** as a yellow oil (4.527g, 26.1mmol, 87%). R_f = 0.3 (hexanes:ethyl acetate = 7:3) ^{1}H NMR (300 MHz, Chloroform-*d*) δ 4.73 (s, 1H), 4.19 – 3.97 (m, 2H), 3.25 – 3.01 (m, 2H), 2.56 – 2.41 (m, 2H), 2.15 (t, *J* = 0.5 Hz, 3H), 1.77 (p, *J* = 7.0 Hz, 2H), 1.29 – 1.16 (m, 3H). IR (cm^{-1}): 3342, 2936, 1696.



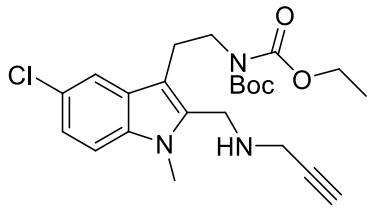
Synthesis of **6**: In a sealed tube, **3** (716mg, 3.71mmol) and **5** (706.9mg, 4.08mmol) are combined along with cyanuric chloride (1.364g, 7.4mmol) in ethanol (13ml). The reaction is placed under argon and heated at 85ºC overnight. The reaction is then concentrated under reduced pressure before being reconstituted with ethyl acetate. The organic layer is washed three times with a saturated bicarbonate solution and dried with sodium sulfate before being concentrated under reduced pressure. The residue is purified using silica gel column chromatography (hexanes:ethyl acetate = 7:3) to give **6** as a pale yellow oil (886mg, 3mmol, 81%). R_f = 0.3 (hexanes:ethyl acetate = 7:3).^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.45 (dd, *J* = 1.7, 0.9 Hz, 1H), 7.05 (t, *J* = 1.4 Hz, 2H), 4.97 (t, *J* = 6.0 Hz, 1H), 4.11 (q, *J* = 7.1 Hz, 2H), 3.54 (s, 3H), 3.31 (q, *J* = 6.7 Hz, 2H), 2.85 (t, *J* = 7.0 Hz, 2H), 2.31 (s, 3H), 1.23 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 156.79, 135.44, 135.00, 128.74, 124.53, 120.64, 117.14, 109.61, 107.56, 60.63, 41.62, 29.59, 24.78, 14.73, 10.20. IR (cm^{-1}): 3201, 2933, 1715, 1603, 1525, 1380, 1231.



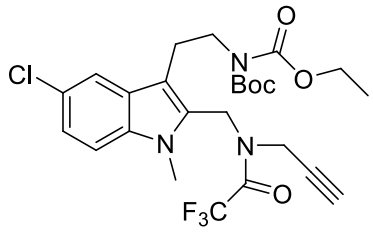
Synthesis of **7**: To a stirring solution of **6** (880mg, 2.95mmol) in THF (12ml), di-*tert*-butyl dicarbonate (3.11g, 14.5mmol) is added. The reaction is allowed to stir overnight at room temperature. The reaction is then concentrated under reduced pressure and reconstituted in ethyl acetate. The organic layer is washed three times with a saturated bicarbonate solution and dried with sodium sulfate before being concentrated under reduced pressure. The residue is purified using silica gel column chromatography (hexanes:ethy acetate 8.5:1.5) to afford **7** as a waxy yellow oil (1.251g, 3.17mmol, 89%). R_f = 0.5 (hexanes:ethyl acetate = 7:3). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.51 (dd, *J* = 1.9, 0.7 Hz, 1H), 7.12 – 6.97 (m, 2H), 4.18 (q, *J* = 7.1 Hz, 2H), 3.83 – 3.71 (m, 2H), 3.57 (s, 3H), 3.03 – 2.87 (m, 2H), 2.33 (s, 3H), 1.51 – 1.40 (m, 9H), 1.28 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, Chloroform-*d*) δ 135.44, 134.98, 128.85, 124.58, 120.65, 117.20, 109.50, 107.29, 82.47, 62.63, 46.72, 29.60, 27.94, 23.81, 14.23, 10.11. IR (cm^{-1}): 2981, 1748, 1480, 1372, 1152.



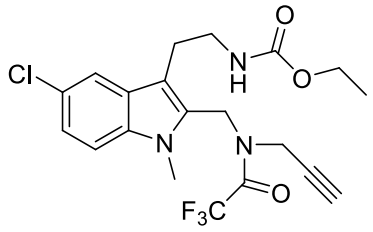
Synthesis of **8**: To a stirring solution of **7** (216.6mg, 0.549mmol) in CCl_4 (2ml), N-Bromosuccinimide (107.1mg, 0.604mmol) and Benzoyl peroxide (26.40mg, 0.109mmol) are added. The reaction is then placed under argon and stirred for 30 minutes at 65°C. The reaction is then filtered through a plug of celite and then concentrated under reduced pressure. The product is not purified further. Yield is assumed to be quantitative and **8** is used directly in the next step.



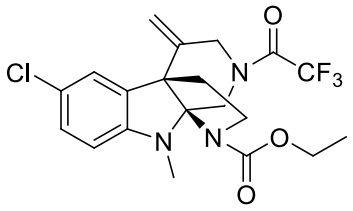
Synthesis of **9**: To a stirring solution of propargylamine (379.4mg, 5.49mmol) in acetonitrile (.549ml) at -10°C a solution of **8** (260.11mg, .549mmol) in acetonitrile (5.49ml) is added dropwise. This reaction is stirred for 30 minutes under argon at -10°C. The reaction is then diluted with ethyl acetate and washed three times with a saturated bicarbonate solution and dried with sodium sulfate. The organic layer is concentrated under reduced pressure and the residue is purified with silica gel column chromatography (hexanes:ethyl acetate = 1:1) to afford **9** as a pale yellow oil (172.5mg, 0.385mmol, 70% two steps). R_f = 0.1 (hexanes:ethyl acetate = 7:3). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.75 (dd, *J* = 2.0, 0.7 Hz, 1H), 7.39 – 7.27 (m, 2H), 4.23 (q, *J* = 7.1 Hz, 2H), 4.03 (s, 3H), 3.94 – 3.86 (m, 2H), 3.40 – 3.28 (m, 2H), 1.48 (s, 9H), 1.31 (t, *J* = 7.1 Hz, 3H). IR (cm^{-1}): 3305, 2933, 2366, 1748, 1480, 1372, 1156, 1111.



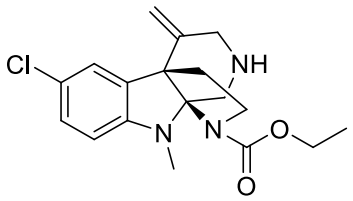
Synthesis of **10**: To a stirring solution of **9** (192.8mg, 0.42mmol) in DCM (1.4ml), trifluoroacetic anhydride (96.6mg, 0.46mmol) and triethyl amine (46.5mg, .46mmol) are added. The reaction is allowed to stir overnight under argon, and then washed with a saturated bicarbonate solution and then dried with sodium sulfate. The organic layer is concentrated and purified with silica gel column chromatography (hexanes:ethyl acetate = 8:2) to give **10** as a pale yellow solid (157mg, 0.28mmol, 67%). R_f = 0.5 (hexanes:ethyl acetate = 7:3). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.62 (t, *J* = 1.3 Hz, 1H), 7.18 (d, *J* = 1.3 Hz, 2H), 5.02 (s, 2H), 4.19 (q, *J* = 7.1 Hz, 2H), 3.98 (d, *J* = 2.4 Hz, 2H), 3.88 – 3.75 (m, 2H), 3.56 (s, 3H), 3.18 – 3.05 (m, 2H), 2.36 – 2.25 (m, 1H), 1.47 (s, 9H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.45, 156.97, 156.48, 155.99, 153.98, 152.12, 135.99, 130.02, 128.18, 125.48, 123.15, 118.63, 113.95, 110.47, 82.92, 76.74, 76.71, 73.79, 62.96, 47.01, 38.19, 30.24, 28.04, 23.68, 14.33. ^{19}F NMR (282 MHz, $CDCl_3$) δ -68.14, -68.26, -68.43, -68.60, -68.63, -68.80, -68.84, -68.92, -68.93, -68.98, -69.06, -69.09, -69.12, -69.27, -69.62, -69.76, -70.45, -75.75, -75.80, -76.00, -79.04, -92.89. IR (cm^{-1}): 3264, 2936, 2985, 2363, 2128, 1793, 1948, 1696, 1149. ESI Mass=543.17 *m/z* calcd for $C_{25}H_{29}ClF_3N_3O_5$ $[M+Na]^+$ 566.1 Found 566.1. MP 150-151C



Synthesis of **11**: To a stirring solution of **10** (157mg, 0.28mmol) in DCM (4ml) at 0ºC, a 1:1 mixture of trifluoroacetic acid (3.192g, 28mmol) in DCM (2.14ml) is added. The reaction is allowed to stir for two hours at room temperature before washing with a saturated bicarbonate solution. The reaction is then dried with sodium sulfate and concentrated under reduced pressure. The resulting residue is purified with silica gel column chromatography (hexanes:ethyl acetate = 7:3) to give **11** (64.3mg, 79mg, 50%) as a pale yellow oil. R_f = 0.3 (hexanes:ethyl acetate = 7:3). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.57 (t, *J* = 1.3 Hz, 1H), 7.20 (d, *J* = 1.2 Hz, 2H), 5.01 (s, 2H), 4.75 (d, *J* = 7.5 Hz, 1H), 4.11 (q, *J* = 7.1 Hz, 2H), 3.98 (d, *J* = 2.4 Hz, 2H), 3.59 (s, 3H), 3.41 (q, *J* = 6.8 Hz, 2H), 3.05 (t, *J* = 6.9 Hz, 2H), 2.36 (d, *J* = 2.5 Hz, 1H), 1.24 (d, *J* = 7.0 Hz, 3H).^{13}C NMR (75 MHz, $CDCl_3$) δ 156.92, 156.59, 135.97, 129.95, 127.87, 125.50, 123.21, 118.55, 114.08, 110.48, 77.45, 77.23, 77.02, 76.60, 73.92, 60.86, 41.70, 38.04, 34.92, 30.20, 29.70, 24.84, 14.64. ^{19}F NMR (282 MHz, $CDCl_3$) δ -68.17, -68.24, -68.61, -68.82, -68.89, -68.91, -68.96, -69.04, -69.11, -69.60, -142.03. IR (cm^{-1}): 3298, 2936, 2128, 1692, 1480, 1212, 1145.

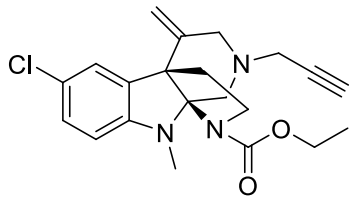


Synthesis of **12**: To a solution of **11** (204.1mg, .46mmol) in anhydrous 1,4 dioxane (9.2ml), $XPhosAuNTf_2$ (22.7mg, .023mmol) is added. The reaction is placed under argon and then stirred for half an hour at 65°C before being concentrated under reduced pressure. The residue is purified using silica gel column chromatography (hexanes:ethyl acetate = 8:2) to give tetracyclic indoline **12** as a clear oil (194.1mg, 0.44mmol, 95%). R_f = 0.4 (hexanes:ethyl acetate = 7:3). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.08 (ddd, *J* = 8.3, 5.4, 2.2 Hz, 1H), 6.97 – 6.79 (m, 1H), 6.27 (dd, *J* = 12.2, 8.4 Hz, 1H), 5.30 – 5.22 (m, 2H), 4.33 – 4.11 (m, 2H), 4.11 (s, 2H), 3.87 – 2.81 (m, 6H), 2.46 – 2.20 (m, 2H), 1.29 – 1.18 (m, 3H). ^{19}F NMR (282 MHz, $CDCl_3$) δ -68.21, -68.30, -68.59, -68.64, -68.83, -68.97, -69.02, -69.09, -69.16, -69.55, -69.61, -69.65, -70.41, -70.54, -70.80, -70.87, -70.94, -71.43, -71.58, -78.77. IR (cm^{-1}): 2936, 284, 1692, 107, 1495, 1383, 1197, 1145. ESI: mass=443.12 *m/z* calcd for $C_{20}H_{21}ClF_3N_3O_3Na$ $[M+Na]^+$ 466.1 found 466.0

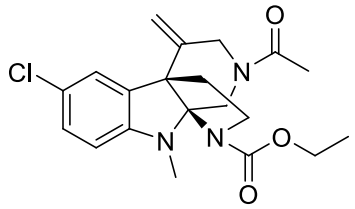


Synthesis of **13**: To a stirring solution of **12 (**205mg, 0.462mmol) in ethanol (1.5ml), potassium carbonate (127mg, 0.92mmol) dissolved in minimal water is added. The reaction is stirred overnight under argon and then concentrated. The residue is reconstituted using ethyl acetate, washed with a saturated solution of bicarbonate in water, and then dried with sodium sulfate. The organic layer is concentrated under reduced pressure and the residue is purified using silica gel column chromatography (DCM:Methanol:triethylamine = 95:4.9:0.1) to yield **13** as a pale yellow oil. R_f = 0.1 (DCM:Methanol = 95:5). ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.07 (dd, *J* = 8.3, 2.2 Hz, 1H), 6.95 (d, *J* = 2.1 Hz, 1H), 6.31 (d, *J* = 8.3 Hz, 1H), 5.01 (d, *J* = 4.8 Hz, 2H), 4.25 – 3.98 (m, 2H), 3.69 (dd, *J* = 30.9, 12.9 Hz, 2H), 3.48 – 3.26 (m, 3H), 3.26 – 3.10 (m, 1H), 3.05 – 2.80 (m, 4H), 2.32 (dt, *J* = 12.9, 8.2 Hz, 1H), 2.08 (ddd, *J* = 12.7, 6.3, 4.2 Hz, 1H), 1.27 (t, *J* = 7.3 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.00, 148.98, 145.00, 132.22, 128.44, 123.33, 112.05, 107.33, 90.72, 60.99, 59.51, 49.80, 47.14, 46.80, 45.81, 33.24, 30.49, 14.55, 9.20. IR (cm^{-1}): 3055, 2981, 2892, 2132, 1696, 1603, 1380. ESI: mass=347.14 *m/z* calcd for $C_{18}H_{23}ClN_3O_2$ $[M+H]^+$ 348.1 found 348.1

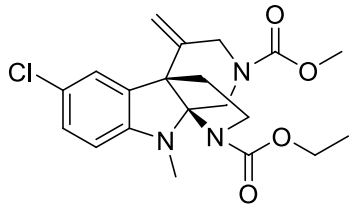
General procedure for the synthesis of **14, 15, 16**: To a solution of **13** (10mg, 0.029mmol) in DCM (0.1ml), the corresponding chloride (**15, 16**) or bromide (**14**) is added (0.032mmol) along with triethylamine (0.032mmol). The reaction to stirs overnight under argon and then is washed with a saturated solution of bicarbonate and dried with sodium sulfate. The organic layer is concentrated under reduced pressure and then the residue is purified using silica gel column chromatography to give **14, 15, 16**.



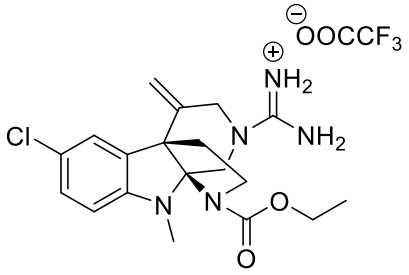
^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.12 (dd, *J* = 8.3, 2.2 Hz, 1H), 6.98 (d, *J* = 2.2 Hz, 1H), 6.34 (d, *J* = 8.4 Hz, 1H), 5.19 (d, *J* = 14.0 Hz, 2H), 4.11 (q, *J* = 7.2 Hz, 2H), 3.79 – 3.54 (m, 4H), 3.45 – 3.16 (m, 4H), 2.97 (s, 3H), 2.09 (dt, *J* = 12.4, 6.2 Hz, 2H). Note: triplet 3H should be present at 1.25ppm but was not observed in spectra due to solvent impurity. IR (cm^{-1}): 2925, 2854, 2337, 2165, 2188, 1704, 1383. ESI: Mass=386.15 *m/z* calcd for $C_{20}H_{24}ClN_4O_2$ $[M+H]^+$ 387.1 found 387.1



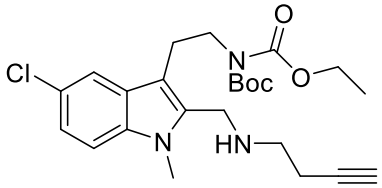
^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.06 (ddd, *J* = 8.9, 8.3, 2.2 Hz, 1H), 6.89 (dd, *J* = 19.9, 2.1 Hz, 1H), 6.24 (dd, *J* = 12.4, 8.4 Hz, 1H), 5.27 – 5.03 (m, 2H), 4.47 – 4.22 (m, 2H), 4.21 – 4.05 (m, 2H), 3.99 – 3.60 (m, 3H), 3.10 (ddt, *J* = 16.9, 11.2, 5.9 Hz, 1H), 2.96 (s, 3H), 2.38 – 2.19 (m, 1H), 2.14 (s, 3H), 1.89 (s, 1H), 1.31 – 1.19 (m, 3H). IR (cm^{-1}): 2925, 2854, 2363, 1715, 1465. ESI: mass = 389.15 *m/z* calcd for $C_{20}H_{25}ClN_3O_3$ $[M+H]^+$ 390.1 found 390.1



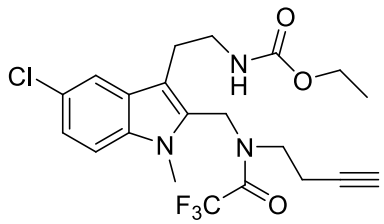
^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.04 (dd, *J* = 8.4, 2.2 Hz, 1H), 6.88 (s, 1H), 6.23 (d, *J* = 8.4 Hz, 1H), 5.16 (d, *J* = 7.6 Hz, 2H), 4.36 – 3.80 (m, 5H), 3.78 – 3.56 (m, 4H), 3.15 – 2.86 (m, 4H), 2.24 (dd, *J* = 20.9, 10.6 Hz, 2H) Note: triplet 3H should be present at 1.25ppm but was not observed in spectra due to solvent impurity. IR (cm^{-1}): 2925, 2854, 2363, 2344, 1707,1454. ESI: Mass=405.15 m/z calcd for $C_{20}H_{25}ClN_3O_4$ $[M+H]^+$ 406.1 found 406.1



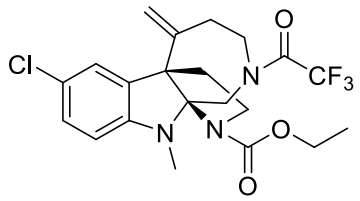
Synthesis of **17**: To a solution of **13** (10mg, 0.029mmol) in DMF (0.5ml), 1,3-Di-Boc-2-(trifluoromethylsulfonyl)guanidine (17mg, 0.044mmol) was added. This reaction was heated at 85ºC overnight and concentrated under reduced pressure. The residue was purified using silica gel column chromatography (DCM:Methanol = 98:2) to give the di-boc guanidine tetracyclic indoline. This indoline was then dissolved into DCM (0.5ml) and trifluoracetic acid was added (0.5ml). The reaction was allowed to stir for 24 hours before concentrating under reduced atmosphere to give **17** as a TFA salt. ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.17 (dt, *J* = 8.4, 2.3 Hz, 1H), 7.09 – 7.00 (m, 1H), 6.36 (d, *J* = 8.4 Hz, 1H), 5.31 (d, *J* = 57.5 Hz, 2H), 5.02 (d, *J* = 14.7 Hz, 1H), 4.46 – 4.03 (m, 4H), 3.94 – 2.98 (m, 4H), 2.90 (d, *J* = 5.3 Hz, 3H). IR (cm^{-1}): 3338, 2925, 2095, 2117, 1674, 1607, 1495, 1204. ESI: Mass=389.16 *m/z* calcd for $C_{19}H_{25}ClN_5O_2$ $[M+H]^+$ 390.1 found 390.1



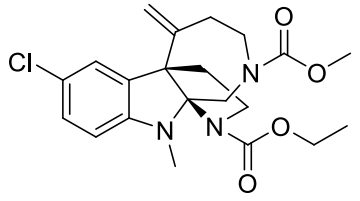
19 ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.56 (dd, *J* = 1.9, 0.7 Hz, 1H), 7.21 – 7.04 (m, 2H), 4.20 (q, *J* = 7.1 Hz, 2H), 3.92 (s, 2H), 3.83 – 3.77 (m, 2H), 3.76 (d, *J* = 2.2 Hz, 3H), 3.04 – 2.96 (m, 2H), 2.82 (t, *J* = 6.6 Hz, 2H), 2.40 (td, *J* = 6.6, 2.6 Hz, 2H), 2.01 – 1.94 (m, 1H), 1.44 (s, 9H), 1.29 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 153.94, 152.06, 146.81, 136.48, 135.49, 128.39, 124.67, 121.69, 117.95, 109.90, 109.23, 82.64, 82.49, 77.49, 77.27, 77.07, 76.65, 69.53, 69.03, 68.85, 62.74, 59.39, 54.27, 47.54, 47.07, 42.51, 31.58, 30.06, 29.70, 27.94, 27.63, 23.76, 19.57, 17.21, 14.26.



20 ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.56 (t, *J* = 1.3 Hz, 1H), 7.24 – 7.17 (m, 2H), 4.97 (d, *J* = 19.1 Hz, 2H), 4.83 (t, *J* = 6.5 Hz, 1H), 4.12 (q, *J* = 7.0 Hz, 2H), 3.61 (d, *J* = 17.5 Hz, 3H), 3.48 – 3.27 (m, 4H), 3.03 (t, *J* = 7.1 Hz, 2H), 2.41 (qd, *J* = 8.9, 8.2, 2.6 Hz, 2H), 1.31 – 1.23 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 157.46, 156.99, 156.71, 135.95, 130.88, 129.27, 127.81, 125.66, 125.52, 123.41, 123.17, 118.60, 118.43, 118.32, 114.51, 113.32, 110.53, 110.41, 79.14, 77.46, 77.24, 77.04, 76.62, 71.70, 70.74, 60.91, 43.64, 43.29, 41.80, 38.68, 30.29, 29.69, 24.93, 18.63, 16.16, 14.66.



21 ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.11 (dd, *J* = 8.3, 2.2 Hz, 1H), 6.84 (d, *J* = 2.2 Hz, 1H), 6.34 (d, *J* = 8.3 Hz, 1H), 5.12 (d, *J* = 28.2 Hz, 2H), 4.06 (d, *J* = 70.7 Hz, 4H), 3.86 – 2.79 (m, 10H), 2.17 (s, 1H), 1.97 (t, *J* = 12.9 Hz, 1H). Note: Triplet 3H should be present at 1.25ppm but was not visible due to solvent impurity. IR (cm^{-1}): 2929, 2858, 2363, 2344, 1707, 1495. ESI: Mass=457.14 *m/z* calcd for $C_{21}H_{24}ClF_3N_3O_3$ $[M+H]^+$ 458.1 found 458.1



23 ^{1}H NMR (300 MHz, Chloroform-*d*) δ 7.09 (dd, *J* = 8.3, 2.2 Hz, 1H), 6.83 (d, *J* = 2.1 Hz, 1H), 6.31 (d, *J* = 8.3 Hz, 1H), 5.01 (s, 2H), 4.11 (s, 5H), 3.76 (s, 4H), 3.38 (d, *J* = 3.3 Hz, 3H), 3.23 – 2.87 (m, 6H). Note: Triple 3H should be present at ~1.25ppm but is not present due to solvent impurity. IR (cm^{-1}): 2929, 2858, 2363, 2344, 1711, 1454, 1380. ESI: Mass=419.16 *m/z* calcd for $C_{21}H_{27}ClN_3O_4$ $[M+H]^+$ 420.1 found 420.1

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