Biosynthesis and Semi-synthesis of Kibdelomycin

Drew University College of Liberal Arts

A Thesis in Biochemistry and Molecular Biology

By

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Preface and Acknowledgment

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Abstract

Kibdelomycin is a broad-spectrum antibiotic isolated from *Kibdelosporangium banguiense* and was discovered by a very sophisticated *Staphylococcus aureus* Fitness Test (SaFT) assay at Merck that inhibited topoisomerase II activity. About the same time, amycolamicin, an identical compound produced by *Amycolatopsis* sp. MK575-fF5, was reported with the similar activity but was discovered using a standard antibiotic assay. Kibdelomycin inhibits bacterial growth by potently inhibiting topoisomerase II, specifically, DNA gyrase B and ParE. Kibdelomycin showed broad-spectrum gram-positive antibacterial activity but only modest potency against gram-negative ESKAPE pathogens, despite potent inhibition of *E. coli* gyrase B activity (IC_{50} 60 nM), indicating a lack of target access affected by entry/efflux instead of a lack of target engagement. Kibdelomycin showed no cross-resistance to known antibiotics including quinolones which inhibit gyrase, indicating its high potential as a new antibiotic. X-ray crystal structure analysis bound to *S. aureus* GyrB and ParE showed unique U-shaped binding with multi-point contacts helping to attain low frequency of resistance. This structure provides an opportunity for structure-guided chemical modification to improve its gram-negative antibacterial profile. We focused on three independent groups of studies to improve upon understanding kibdelomycin with the goal of discovering a broad-spectrum antibacterial compound. These studies include the biosynthesis of kibdelomycin, isolation and structure elucidation of and biological evaluation of new congeners of kibdelomycin, and synthetic modification of kibdelomycin and determination of minimum structural requirement for antibacterial activity. Biosynthesis studies confirmed the direct incorporation of pyruvates, valine, and proline which should benefit in designing potentially tailored metabolites. A new approach of repeated batch fermentation led to enhanced production of trace metabolites leading to discovery and characterization of two new kibdelomycin congeners A-1 and A-2 with modest activity. Semi-synthetic studies of kibdelomycin and related simple

pyrrole amides show that the full molecular framework is essential for strong activity, as suggested by static X-ray data. However, these studies do suggest that modifications to the topmost sugar of kibdelomycin may yield improved properties.

Chapter 1: Review of global antibiotic resistance crisis and kibdelomycin

Antibiotics are life-saving medications produced either by bacteria or fungi, or made synthetically that can kill or inhibit the growth of competing microbial species. The initial breakthroughs of antibiotic discovery came from Alexander Fleming and his discovery of penicillin, as well as soil bacteria screening/isolation techniques developed by Selman Waksman in the 1940s to develop the antibiotic streptomycin.[1] The combination of techniques used in penicillin discovery from a fungus, *Penicillium notatum* using a petri dish agar diffusion-based *Staphylococcus aureus* assay with soil bacterial isolation technique allowed for discovery of many antibiotics. These discoveries transformed modern medicine and changed the drug discovery research permanently. Many companies within the pharmaceutical industry performed antibiotic discovery campaigns based on Waksman's original screening approach, testing actinomycetes from soil samples using petri dish agar diffusion assays to detect antibiotic compounds against pathogens. This led to what is known as the "golden era" of antibiotic discovery, leading to the development of many newly discovered compounds from actinomycetes as clinically effective approved antibiotics. From the 1940s to 1960s (termed as Golden Era), a number of compounds, including aminoglycosides, tetracyclines, b-lactams, chloramphenicols, and macrolides, were discovered (Figure 1) and implemented into patient healthcare, resulting in a decrease in average deaths from bacteria as well as increased life-expectancy.[2] Only a few compounds were discovered, approved and implemented in the healthcare system post 1960's (Figure 1).[1]

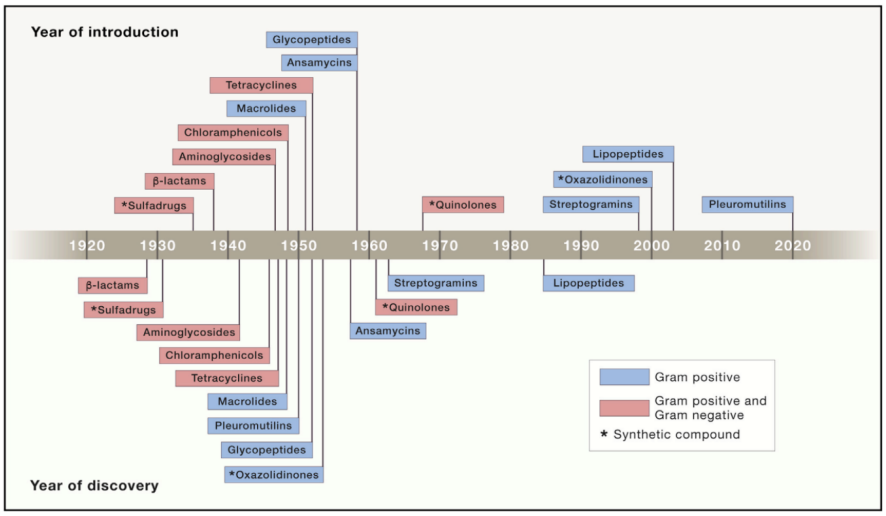


Figure 1. The timeline of antibiotic discovery (adapted from Lewis, K. *Cell* 2020)[1].

One of the drawbacks of antibiotics is the ability of the bacteria to eventually become resistant to the antibiotics themselves in contrast to non-antibiotic drugs. Antibiotic resistance remains one of the largest challenges within healthcare, as all clinically used antibiotics discovered in the past 80 years have become ineffective due to resistant to one or more bacteria including showing pan resistance.[3] Antibiotic resistance was first noted almost immediately after introduction of the first antibiotics in clinic. The effect of resistance was less pronounced during the decades post "Golden Era" due to incremental improvements by synthetic chemistry of the approved antibiotic classes. However, that approach is producing diminishing returns causing significant new challenges to pharmaceutical companies for discovering new antibiotics (Figure 2).[4]

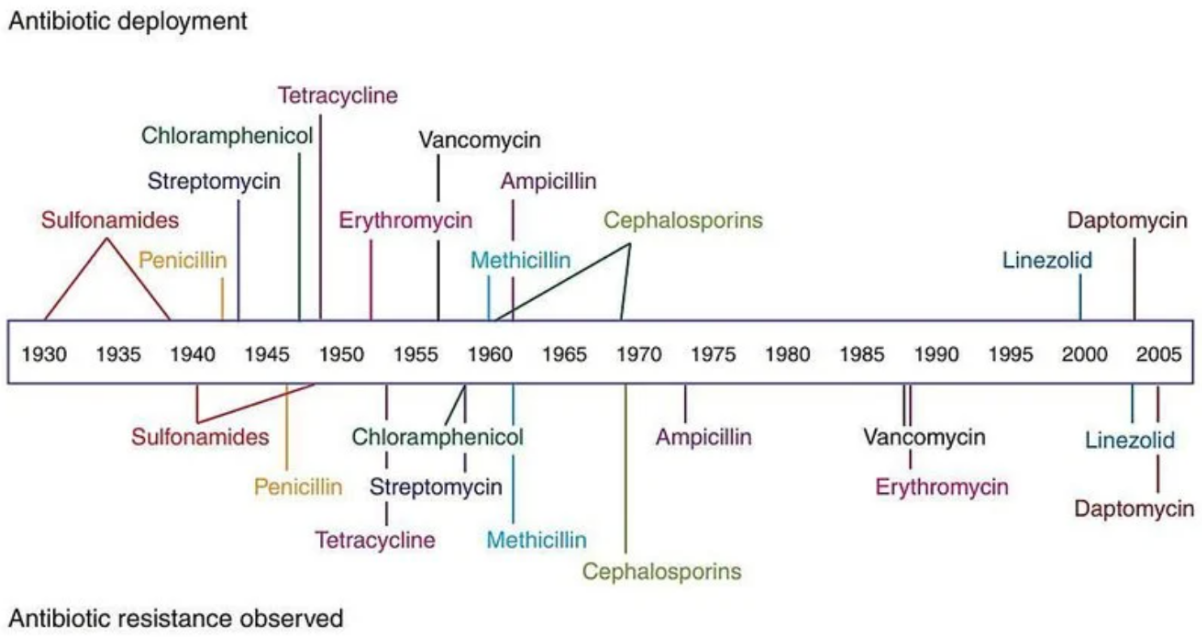


Figure 2: Timeline of antibiotic usage compared and timeline of first antibiotic resistance to respective antibiotics (adapted from Clatworthy et al. *Nat. Chem. Biol.* 2007)[4].

One of the many challenges of antibiotic resistance comes from intrinsic resistance, where the antibiotic cannot interact with genes/proteins present in the organism that are fundamental for the organism's genome to function. This, along with acquired resistance via mutations due to selective pressure, results in many organisms evading antibiotics and becoming a resistant bacterium. Gram-negative bacteria are generally insensitive to many antibiotics due to existence of extra outer membrane consisting of a separate lipopolysaccharide layer. Acquired resistance occurs by the cell's ability to modify internal components of the cell, either via modification of protein or changing chemical properties or creating a physical barrier (Figure 3).[5]

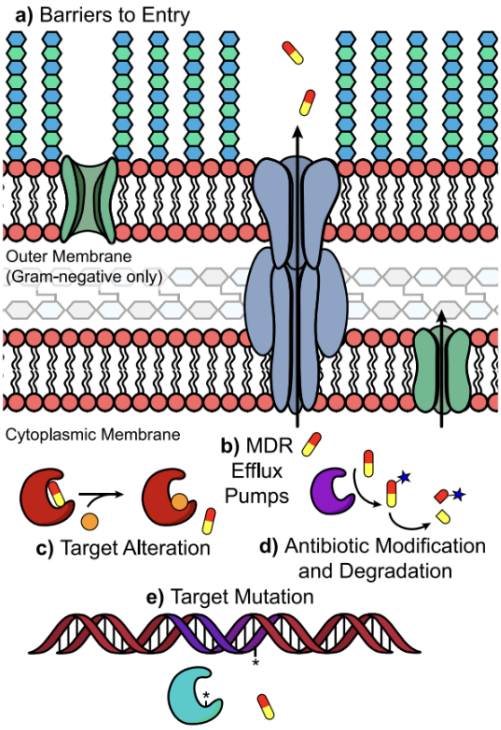


Figure 3: Various cellular processes for antibiotic resistance (adapted from Hobson et al *Chem. Rev.* 2021)[5].

Antibacterial resistance continues to be burden both to healthcare and financially with increasing rates of death. In 2021, it was estimated that 4.71 million deaths were associated with bacterial antimicrobial resistance, with trends of death drastically increasing specifically from methicillin-resistant *Staphylococcus aureus* (MRSA) and many gram-negative bacteria.[5] By 2050, this death numbers associated with antimicrobial resistance are predicted to increase to 8.22 million. Similarly, as described by Jim O'Neill of the Review on Antimicrobial Resistance (RAR), titled "Tackling Drug-Resistant Infections Globally: Final Report and Recommendations," by 2050 an estimated economic burden of 100 trillion USD would result from antimicrobial resistance due to extended hospital visits, lost wages by family members, and the decreased income from the inability to perform common surgical medical procedures requiring antibiotics.[6] With continued successful efforts to fight against these severe bacterial infections, about 92 million deaths could be prevented by 2050. These efforts include improved access to antibiotics, discovering novel

drugs targeting important bacteria like MRSA, improved treatment of *Clostridioides difficile,* and gram-negative bacteria including *Acinetobacter baumannii*.

While bacterial resistance can be controlled by prudent and restricted usage of current antibiotics, it cannot be solved unless new classes of antibiotics are discovered and introduced in clinical practice. Most currently used antibiotics originally came from naturally occurring sources, with a large number of those original compounds were chemically modified leading to incrementally improved efficacious antibiotics.[7] With these modified antibiotics, certain properties of the drugs were improved often by introduction of additional binding targets, but the predominant mode of resistance did not change. However, additional improvements to existing chemical scaffolds continue to be challenging, time-consuming and having diminishing returns, and unaltering bacteria's natural ability to become resistant to such existing chemical scaffolds. This grim result leads to only two options to abet antibiotic resistance: (1) discover and develop new antibiotic scaffolds that bind differently to the targets that interact with to previously discovered antibiotics or better yet, (2) discover and develop new antibiotics with novel modes of action.

One of such antibiotics recently discovered in the search for novel antibiotics with a novel mode of action was kibdelomycin.[8] It was discovered by the Merck team from a soil bacterium collected from the forest of Pama in the plateau of Bangui, Central African Republic.[8] One of the keys to this discovery was the usage of a chemical-genetic profiling technique called the *Staphylococcus aureu*s Fitness Test (SaFT).[9] SaFT was an innovative and powerful biological profiling screening assay used to differentiate the activity of crude extracts based on mechanism of action. The approach to the SaFT assay is to use a target-based whole cell screening approach that uses inducible antisense to down regulate the expression of individual specific gene targets in *S. aureus*, in an array format, simultaneously testing multiple targets of the Single strain antisense

assay, the precursor of SaFT assay, and was used successfully for screening of novel target-based whole cell active antibiotics such as fatty acid synthesis inhibitors, platensimycin and platencin[10-19] along with many other targets.[17-22] By doing this screening approach one target at a time, the screening results were highly successful, but screening essential targets individually would have required over 250 screens which would be highly resource intensive, expensive, and time consuming. A new innovative approach was created to have a SaFT assay consisting of 245 inducible antisense RNA strains for reduced expression of target genes essential for cell growth. When using a crude extract, the down regulation of target genes leads to differential growth sensitivity of cells to the compounds found in the extracts, resulting in the inhibition of targeted cell growth gene products. These strains were then combined and grown for about 20 population doublings in the presence of the test extracts and compounds, leading to the antisense strains either being depleted or enriched in various growth factors. Multiplex PCR, capillary electrophoresis and gene fragment analysis could then be used to compare the amount of RNA in the strains at the end of the population doublings with mock (DMSO-media) treated controls to generate characteristic antisense-induced strain sensitivity (AISS) profiles that suggest the compounds within the extracts whole cell mechanism of action (MOA). A compound's MOA can be deduced from the AISS profile directly or can be compared to a database of AISS profiles of known inhibitors[3]. Both approaches are useful for the discovery and differentiation of new compounds from extracts by indicating preliminary MOA information and providing comparative information for the elimination of known natural products already discovered. This analysis via the SaFT AISS assay could allow for the pairing of putative-targets with an extract and allowed for prioritization based on the potential mechanism, which was valuable for the drug industry and patients as new classes of compounds were key for future development.

The extract that contained kibdelomycin showed an AISS profile with strong depletions of *gyrB-AS* and *parE/C-AS* and weak depletions of *gyrA-AS* along with subtle differences in a few other antisense sensitized *S. aureus* test strains comparable to a profile displayed by novobiocin, a well-known bacterial DNA topoisomerase II inhibitor with minor resemblance of AISS to quinolones (Figure 4).[8] LCMS analysis of the crude extract did not match with data of novobiocin and other known DNA topoisomerase inhibitors. Additionally, the AISS profile of purified kibdelomycin, extracted via high-resolution liquid chromatography mass spectrometry (HRLCMS), was shown to be identical to the crude extract AISS SaFT profile (Figure 4).[8] This confirmed that the isolated compound was the same compound that showed the AISS profile in the extract while continuing to show the potential of being a novel class of DNA gyrase B inhibitor, representing option (1) of proposed discovery paradigm that is novel compound with existing gyrase inhibitory mechanism but completely different binding mode (vide infra).

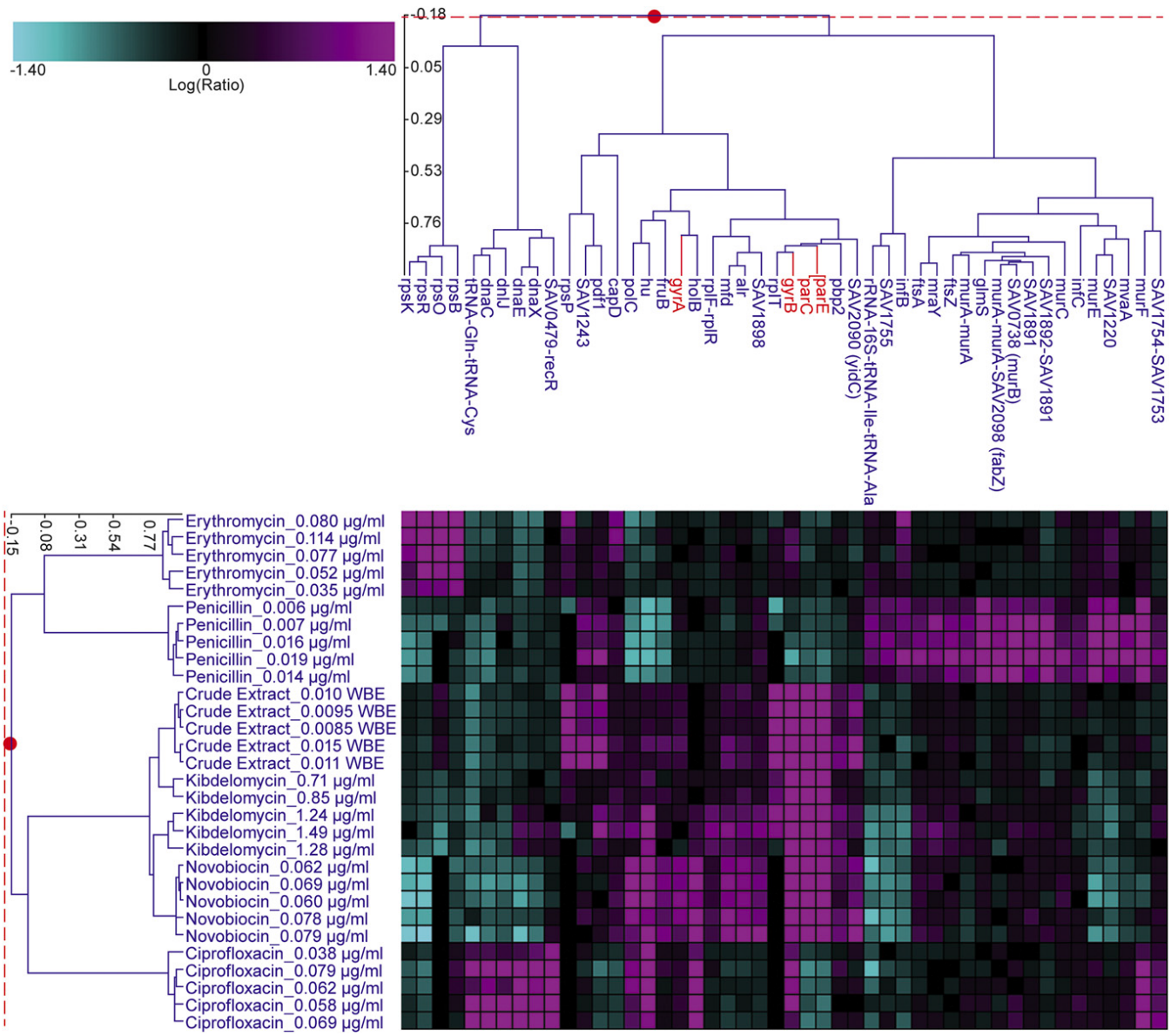


Figure 4. Heat map of two-Dimensional cluster analysis of *S. aureus* Fitness Test profiles for the crude extract, kibdelomycin, and four compounds of known mechanism of action (adapted from Phillips, Singh et al *Chem. Biol*. 2011)[8].

Kibdelomycin (MW 938) (Figure 5) is a hexacyclic polyketide-peptide hybrid natural product consisting of a central 4-hydroxy decalin tetramic acid core connected to a fully substituted pyranosyl sugar (6-methyl-2-methoxy-3-acetoxy-4-carbamoyl-epiallose) by an *N*- glycosidic linkage in the northern hemisphere and to a deoxy sugar (3-aminoethyl-2,6-dideoxy pyranose) by *O*-glycosidic linkage at C-4 of the decalin in the southern hemisphere, terminating with an amide

bond between the amino ethyl group with 3,4-dichloro-5-methyl-pyrrole-2- carboxylic acid (Figure 5). The highly complex chemical structure of kibdelomycin was elucidated by extensive application of 2D-NMR and high-resolution mass spectrometry techniques.[8] The tautomerization of the tetramic acid unit presented significant challenges in the collection and interpretation of NMR spectral data and could be solved only by collection of NMR data rapidly as well as in multiple solvents. The absolute configuration is based on the 2.3 Å X-ray crystal structure of kibdelomycin bound to GyrB and ParE enzymes.[23] Kibdelomycin A (MW 924) (Figure 5), a minor des-methylated congener, was also isolated from the same extract.[24]

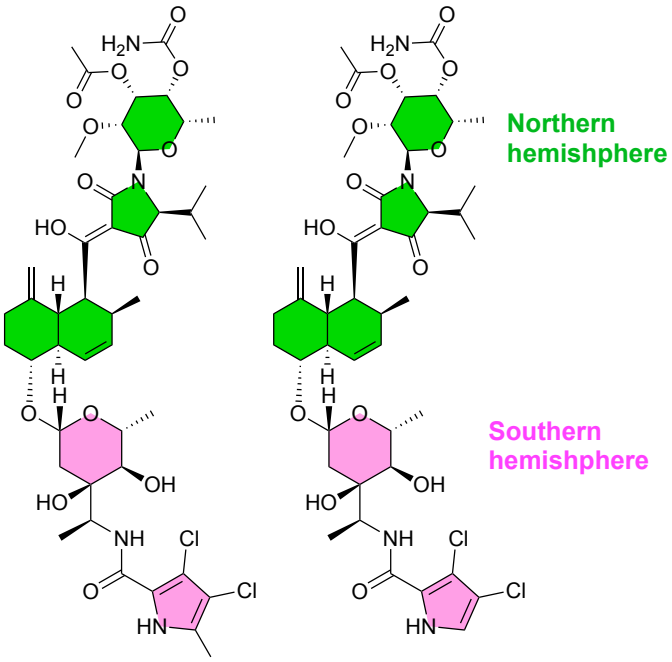


Figure 5: Structures of kibdelomycin and kibdelomycin A.

Kibdelomycin is a selective inhibitor of DNA synthesis and type II bacterial topoisomerase. Kibdelomycin selectively and potently inhibited DNA synthesis in a macromolecular synthesis assay with IC_{50} value of 0.03 µg/mL (0.03 µM), with more than a 100-fold selective difference for

inhibition of DNA synthesis as compared to inhibition of RNA, protein, peptidoglycan, and phospholipid synthesis. It is a potent inhibitor of DNA supercoiling in *E. coli* with an IC_{50} of 0.06 μM and a poor inhibitor of *E. coli* DNA topoisomerase IV decatenation with an IC_{50} of 29 μM. Kibdelomycin inhibited *S. aureus* DNA gyrase supercoiling and DNA topoisomerase IV decatenation activity more potently exhibiting IC_{50} values of 0.009 and 0.5 μM, respectively. It inhibits the *E. coli* gyrase and topoisomerase IV ATPase enzyme activity with IC_{50} values of 0.011 and 0.90 μM, respectively. The enzyme inhibitory profile of kibdelomycin is similar to novobiocin, with both compounds showing better inhibition against DNA gyrase than topoisomerase IV (Table 1).[25] The inhibition profile of aminocoumarins (Clorobiocin and Coumermycin A1) trends similarly. They showed significantly more potent gyrase inhibition than topoisomerase IV (Table 1).[26] Cyclothialidine is a potent inhibitor of *E. coli* supercoiling activity (Table 1).[26]

Table 1. Enzyme inhibitory activities (IC50, mM) of kibdelomycin and known natural gyrase inhibitors

Assay	Kibdelomycin[b]	Kibdelomycin A[c]	Novobiocin[d]	Clorobiocin[d]	Coumermycin A1[d]	Cyclothialidine[d]
E. coli gyrase (supercoiling)	0.06	0.05	0.50	0.03	0.03	0.05
E. coli topoIV (decatenation)	29	29	10.0	3	5	ND
S. aureus gyrase (supercoiling)	0.009	0.4	<0.004	0.006	0.006	ND
S. aureus topoIV (decatenation)	0.50	5	35.0	10	100	ND
E. coli gyrase (ATPase)	0.011	0.009	0.023	ND	ND	ND
E. coli topoIV (ATPase)	0.90	6.4	0.45	ND	ND	ND

Table 1 adapted from Singh, S. B., *Bioorg. Med. Chem*. 2016[25]

Kibdelomycin is also a potent and broad-spectrum antibiotic against aerobic bacteria.[8,25,27] Minimum inhibitory concentration (MIC) of kibdelomycin and other known gyrase inhibitors are listed in Table 2 against a selected group of important bacterial strains. MIC is defined as the minimum inhibitory concentration of a compound that shows no visible growth of a microorganism after incubation. MIC_{50} and MIC_{90} are defined as the minimum inhibitory concentrations of a compound that inhibits the growth of either 50% or 90% of each organism respectively. By using a larger panel of clinical isolates, it provides confirmation that an antibiotic

would be effective against organisms present in a large patient population. One strain of particular importance is its strong antibacterial activity against *S. aureus* including MRSA strains with MIC values of 0.25 μg/mL (Table 2).[25] A broader spectrum of *S. aureus* strains were then tested with kibdelomycin, showing MIC_{50} values of 1 μg/mL and MIC_{90} of 2 μg/mL (Table 2). Kibdelomycin similarly inhibited other important gram-positive pathogens *Streptococcus pneumoniae, Enterococcus faecium* and *E. faecalis* with MIC/MIC 90 values of 0.25/2, 1/4 and 2/2 μg/mL, respectively. It also showed excellent MIC/MIC_{90} values against gram-negative pathogens *Moraxella catarrhalis* (0.25/0.50), *Haemophilus influenzae* (2/4), and *Acinetobacter baumannii* (MIC value of a laboratory strain 2 μg/mL, MIC_{90} against clinical strains 0.125 μg/mL)[24]. Kibdelomycin, however, did not show potent activity against other gram-negative pathogens such as *E. coli* and *Pseudomonas aeruginosa, more likely due* to lack of cell penetration and less likely due to efflux.[25] The MIC of kibdelomycin was also significantly affected by 50% human serum, a 256-fold MIC increase, but not 2.5% lysed horse blood serum. The high serum binding and lack of effectiveness against gram-negative bacteria of kibdelomycin posed some of the initial challenges for *in vivo* efficacy as a systemic agent.

Table 2. In vitro antibacterial activity MIC (μg/mL) of kibdelomycin and comparators

Strain	Phenotype	Kibdelomycin	Novobiocin	Clorobiocin	Coumermycin A1	Cyclothialidine	Levofloxacin
Gram-positive							
Staphylococcus aureus	WT	0.25	0.25	<0.06	0.0012	>128	0.125
S. aureus + 50% *human serum*	WT	64	16	ND	ND	ND	ND
Staphylococcus aureus	MRSA	0.25	0.06	ND	ND	ND	32
Streptococcus pneumoniae + 2.5% *lysed horse blood*	WT	0.25	1	2	0.78	ND	1
Enterococcus faecium	Van^R	1	ND	ND	ND	ND	4
Enterococcus faecalis	WT	2	32	ND	ND	ND	ND
Clostridium difficile	WT	0.125	ND	ND	ND	ND	ND
Gram-negative							
Moraxella catarrhalis	WT	0.25	>32	ND	ND	ND	1
Haemophilus influenzae	WT	2	0.12	ND	ND	ND	<0.015
Escherichia coli	WT	>64	>32	ND	6.25	>128	0.031
Pseudomonas aeruginosa	WT	>16	100	ND	12.5	ND	0.5
Acinetobacter baumannii	WT	2	ND	ND	ND	ND	0.125
Bacteroides spp.	WT	>32	ND	ND	ND	ND	ND

Table 2 adapted from Singh, S. B., *Bioorg. Med. Chem*. 2016[25]

The X-ray co-crystal structure of kibdelomycin bound to *N*-terminal domains of *S. aureus* gyrase B (GyrB, 24 kDa) and topoisomerase IV (ParE, 24 and 43 kDa) showed a highly unique "dual-arm", U-shape binding mode in which a large part of the kibdelomycin structure showed hydrophobic and polar interactions with the backbone residues of GyrB and ParE as shown in Figure 3.[23] The pyrrolamide moiety of kibdelomycin present in the southern end of the molecule penetrates deeply into the hydrophobic pocket ATP binding site. The dichloro pyrrole ring is surrounded by a large number of hydrophobic amino acids in both enzymes, showing a strong connection via Van der Waals interactions. The methyl group of the pyrrole is situated in a small pocket formed by hydrophobic amino acids Val and Ile. The two polar groups of the pyrrolamide are engaged with a series of amino acids either directly or through water mediated hydrogen bonding (Figure 6). The hydroxyl group of dihydroxy-tetrahydropyran sugar is directly engaged in hydrogen bonding with Glu-53 (ParE) and Glu-58 (GyrB) as well as engaged in water-mediated hydrogen bonding with Arg-79 (ParE) and Arg-84 (GyrB). A series of hydrophobic residues surround the central decalin unit and provide binding energy through van der-Waals interactions. The highly polar tetramic acid unit tightly anchors kibdelomycin to both enzymes by strong polar interactions with His-86 (ParE) and Gln-91 (GyrB) as well as Gly-103/109. The isopropyl group of the tetramic acid gains energy by hydrophobic interactions from Leu-100/105 and Val-99/104. The northern most part of kibdelomycin appears to be exposed mostly to solvent except for apparent interactions of His-101/107 with the carbamate group.

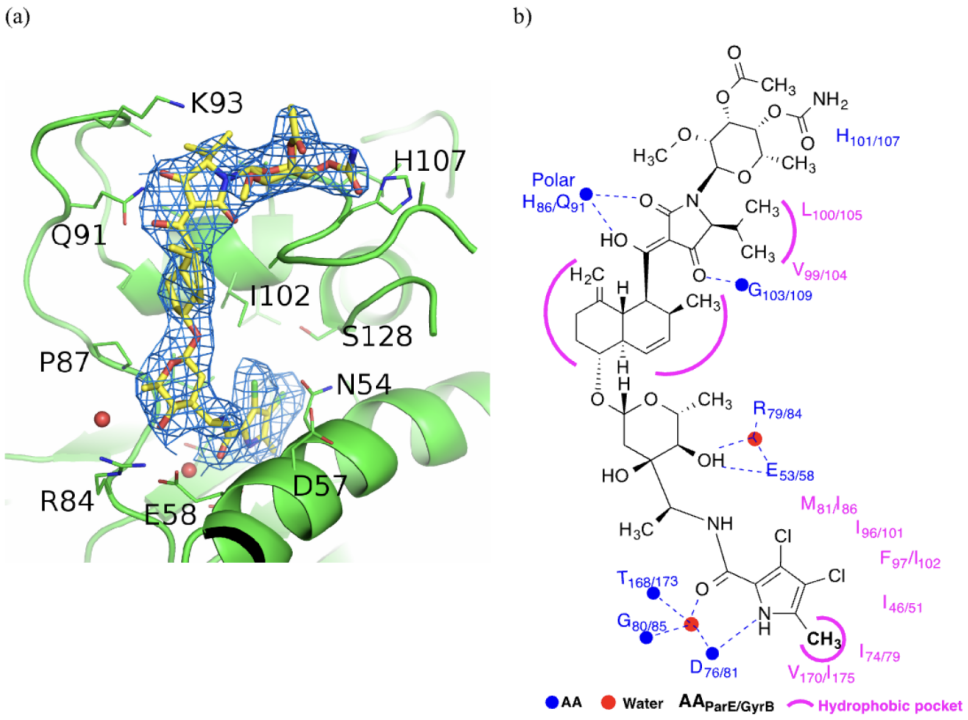


Figure 6. Co-crystal structure of 24 kDa *S. aureus* GyrB-kibdelomycin. (a) Overall binding mode of kibdelomycin showing electron density map of kibdelomycin and (b) schematic representation of the hydrophobic and hydrophilic interactions engaging amino acids of ParE protein (the first number) and GyrB protein (the second number), (adapted from Lu et al, *ACS Chem. Biol.* 2014)[23].

The antibacterial activity of analogs, kibdelomycin acetate and tetrahydro-*bis*-dechloro kibdelomycin (Figure 7) along with congener kibdelomycin A (Figure 5) provided initial insight into the structure function relationship, which has been supported by X-ray co-crystal studies.[24] Kibdelomycin A, the des-methyl congener of kibdelomycin, showed more than 8-fold MIC diminution due to the loss of the binding energy of the methyl group from the hydrophobic interactions provided by the small hydrophobic pocket. Kibdelomycin acetate showed similar results, confirming the role of the strong hydrogen bond in the hydroxy group observed in the initial crystal structures. The tetrahydro-*bis*-dechloro kibdelomycin showed an even greater (>32-fold) reduction in the MIC confirming the critical role-played by the large hydrophobic chlorines attached to pyrrole buried in the deep hydrophobic pocket. From these initial studies, structure-

guided semisynthesis could be potentially implemented to improve antibacterial potency, enhancing the antibacterial spectrum including gram-negative activity.

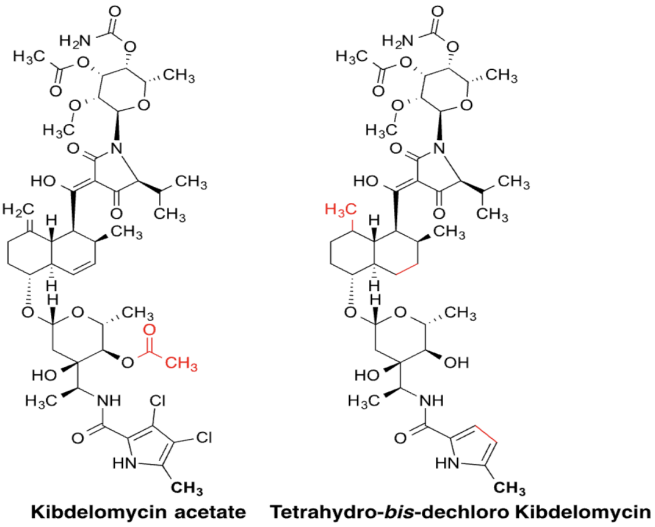


Figure 7. Semisynthetic analogs of kibdelomycin.

At the same time of the discovery of kibdelomycin (KBD), another molecule called amycolamicin (AMM) was also discovered from the soil actinomycete, *Amycolatopsis* sp. MK575-fF5 in 2009.[28] (Figure 8) Amycolamicin displayed essentially similar antibacterial activities as reported for kibdelomycin.[29]

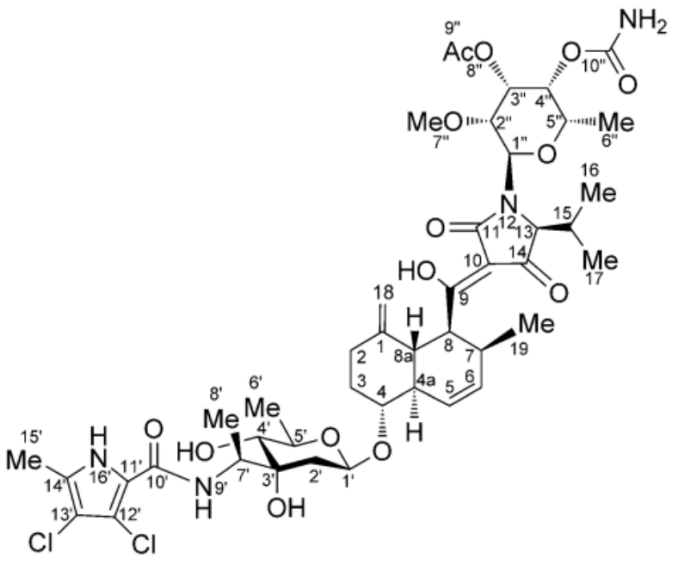


Figure 8. Structure of amycolamicin.

AMM was initially described as related to kibdelomycin, decatromicin B, and pyrrolosporin A based on their common pyrrolamide-sugar-decalin ring scaffold, leading to a classification of new antibacterial agents that specifically target and inhibit DNA gyrase and DNA synthesis. When both structures were initially proposed, they had nearly identical structures with variation in the absolute configuration of the amino sugar residue and differing specific rotation. These differences were reconciled after the first total synthesis in 2021 (Figure 9).[30]

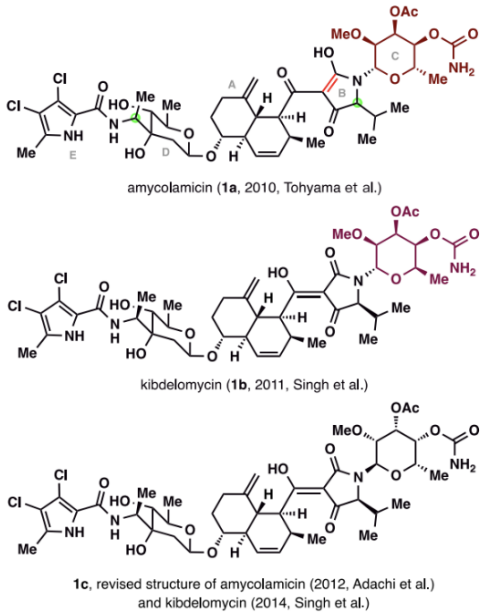
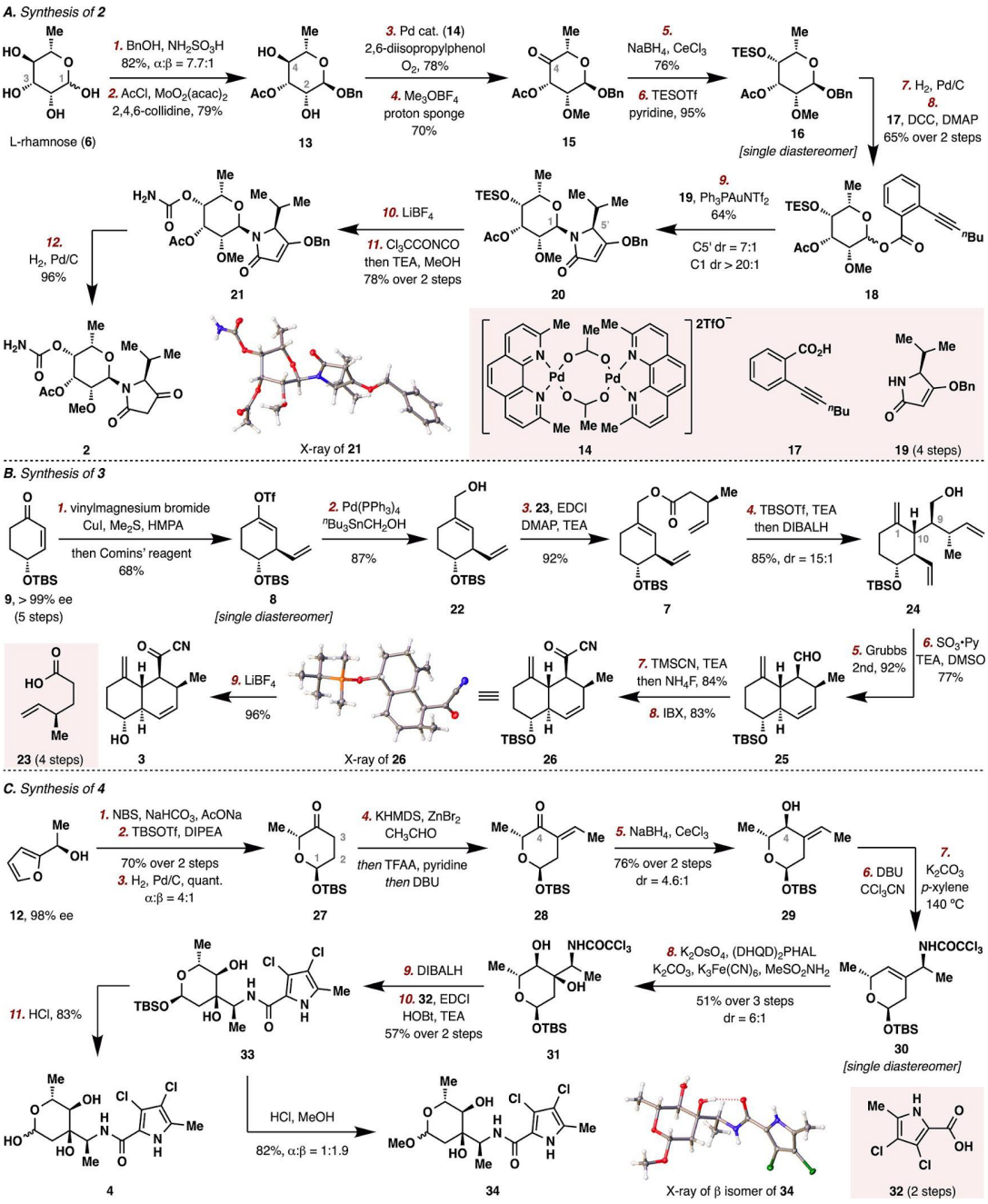
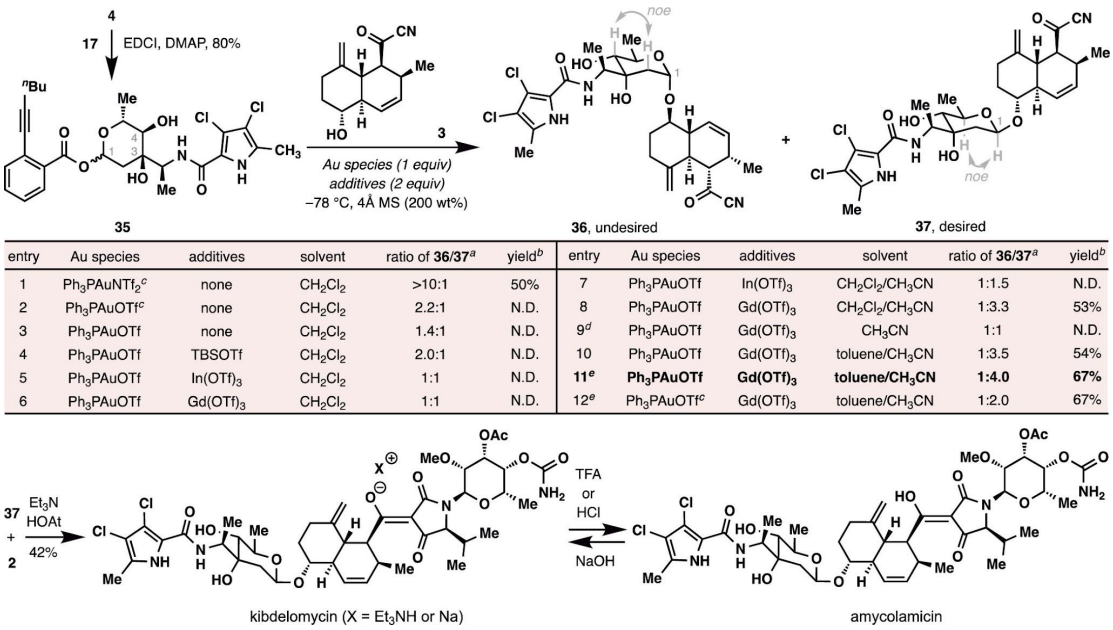


Figure 9. Review of the proposed and corrected structures of kibdelomycin and amycolamicin. The colored atoms or colored portion of the molecule show where subsequent revision of configuration was made. (adapted from yang et al *JACS* 2021)[30].

Both structures had distinct NMR spectra, leading to initial questions about whether the structures were the same molecule. After a modular three fragment total synthesis of amycolamicin and kibdelomycin (Figure 10), it was discovered that the differences lie in differing acid-base counter ion. Kibdelomycin was isolated as sodium salt whereas amycolamicin was reported as a free acid.[30]


A. Synthesis of 2
L-rhamnose (6)
1. BnOH, NH2SO3H
82%, α:β = 7.7:1
2. AcCl, MoO2(acac)2
2,4,6-collidine, 79%
13
3. Pd cat. (14)
2,6-diisopropylphenol
O2, 78%
4. Me3OBF4
proton sponge
70%
15
5. NaBH4, CeCl3
76%
6. TESOTf
pyridine, 95%
16
[single diastereomer]
7. H2, Pd/C
8. 17, DCC, DMAP
65% over 2 steps
18
9. 19, Ph3PAuNTf2
64%
C5' dr = 7:1
C1 dr > 20:1
20
10. LiBF4
11. Cl3CCONCO
then TEA, MeOH
78% over 2 steps
21
12. H2, Pd/C
96%
2
X-ray of 21
14
17
19 (4 steps)
B. Synthesis of 3
9, > 99% ee
(5 steps)
1. vinylmagnesium bromide
CuI, Me2S, HMPA
then Comins' reagent
68%
8
[single diastereomer]
2. Pd(PPh3)4
nBu3SnCH2OH
87%
22
3. 23, EDCI
DMAP, TEA
92%
7
4. TBSOTf, TEA
then DIBALH
85%, dr = 15:1
24
5. Grubbs
2nd, 92%
6. SO3•Py
TEA, DMSO
77%
25
7. TMSCN, TEA
then NH4F, 84%
8. IBX, 83%
26
X-ray of 26
9. LiBF4
96%
3
23 (4 steps)
C. Synthesis of 4
12, 98% ee
1. NBS, NaHCO3, AcONa
2. TBSOTf, DIPEA
70% over 2 steps
3. H2, Pd/C, quant.
α:β = 4:1
27
4. KHMDS, ZnBr2
CH3CHO
then TFAA, pyridine
then DBU
28
5. NaBH4, CeCl3
76% over 2 steps
dr = 4.6:1
29
6. DBU
CCl3CN
7. K2CO3
p-xylene
140 °C
30
[single diastereomer]
8. K2OsO4, (DHQD)2PHAL
K2CO3, K3Fe(CN)6, MeSO2NH2
51% over 3 steps
dr = 6:1
31
9. DIBALH
10. 32, EDCI
HOBt, TEA
57% over 2 steps
33
11. HCl, 83%
4
HCl, MeOH
82%, α:β = 1:1.9
34
X-ray of β isomer of 34
32 (2 steps)



entry	Au species	additives	solvent	ratio of **36/37**[a]	yield[b]
1	$Ph_3PAuNTf_2$[c]	none	CH_2Cl_2	>10:1	50%
2	$Ph_3PAuOTf$[c]	none	CH_2Cl_2	2.2:1	N.D.
3	$Ph_3PAuOTf$	none	CH_2Cl_2	1.4:1	N.D.
4	$Ph_3PAuOTf$	TBSOTf	CH_2Cl_2	2.0:1	N.D.
5	$Ph_3PAuOTf$	$In(OTf)_3$	CH_2Cl_2	1:1	N.D.
6	$Ph_3PAuOTf$	$Gd(OTf)_3$	CH_2Cl_2	1:1	N.D.
7	$Ph_3PAuOTf$	$In(OTf)_3$	CH_2Cl_2/CH_3CN	1:1.5	N.D.
8	$Ph_3PAuOTf$	$Gd(OTf)_3$	CH_2Cl_2/CH_3CN	1:3.3	53%
9[d]	$Ph_3PAuOTf$	$Gd(OTf)_3$	CH_3CN	1:1	N.D.
10	$Ph_3PAuOTf$	$Gd(OTf)_3$	toluene/CH_3CN	1:3.5	54%
11[e]	**$Ph_3PAuOTf$**	**$Gd(OTf)_3$**	**toluene/CH_3CN**	**1:4.0**	**67%**
12[e]	$Ph_3PAuOTf$[c]	$Gd(OTf)_3$	toluene/CH_3CN	1:2.0	67%

Figure 10. The first total synthesis of kibdelomycin and amycolamicin (adapted from yang et al *JACS* 2021)[30].

The last step of the synthesis required a base catalyzed condensation step of intermediates **2** and **37** to create tetramic acid ring required reaction (Figure 10) with triethyl amine (TEA) to produce final product isolated as a TEA salt. The ^{1}H NMR spectrum of the TEA salt of the final product in deuterated methanol looked very similar to the published spectrum of naturally occurring kibdelomycin-sodium salt in the same solvent and desalted free acid (protonated) form of the product in deuterated chloroform looked very similar to the published spectrum of naturally occurring amycolamicin in the same solvent. The collection of ^{1}H NMR spectrum of the synthetic material as a TEA salt was fortuitous which helped in deciphering the differences of the NMR spectra of the two compounds leading to the conclusion that kibdelomycin is the sodium salt form of amycolamicin, conversely the amycolamicin is a free acid form of kibdelomycin-Na, indicating that they were identical.[30] They firmly confirmed the identity of the free acid of the synthetic material with the an authentic sample of natural amycolamicin by comparison of ^{1}H and ^{13}C NMR spectra and specific rotations. Ultimately, the team converted the synthetic product to a sodium

salt and likewise compared its NMR spectra with published NMR spectra and specific rotation of kibdelomycin-Na. Thus confirming that kibdelomycin and amycolamicin were one and the same.

In summary, kibdelomycin is a broad-spectrum antibiotic and was isolated from *Kibdelosporangium banguiense* by a very sophisticated a *Staphylococcus aureus* Fitness Test (SaFT) assay at Merck that inhibited topoisomerase II activity.[8,25,31] About the same time, amycolamicin an identical compound produced by *Amycolatopsis* sp. MK575-fF5 was reported with the similar activity but was discovered using standard antibiotic assay.[28,29] Kibdelomycin inhibits bacterial growth by potently inhibiting topoisomerase II, specifically, DNA gyrase B and ParE. Kibdelomycin showed broad-spectrum gram-positive antibacterial activity but only modest potency against gram-negative ESKAPE pathogens, despite potent inhibition of *E. coli* gyrase B (IC_{50} 60 nM),[8,27,32] indicating a lack of target access affected by entry ruled out earlier instead of a lack of target engagement.[32] Kibdelomycin showed no cross-resistance to known antibiotics including quinolones which inhibit gyrase, indicating its high potential as a new antibiotic.[32] X-ray crystal structure analysis bound to *S. aureus* GyrB and ParE showed unique U-shaped binding with multi-point contacts helping to attain low frequency of resistance.[23] This structure provides an opportunity for structure-guided chemical modification to improve its gram-negative antibacterial profile.[23]

With such a large potential to meet unmet need, discovery of kibdelomycin rightfully drew a lot of attention and became focus of current project at Drew. The team at Drew has the overarching goal to improve its activity against gram-negative bacteria by developing an analog as a broad-spectrum antibiotic. As has been reviewed here, this molecule is very complex and highly challenging for chemical transformation and requires a lot of material and synthetic expertise. The Drew team had made a significant progress to improve production, purification,

understanding the structure features and physical properties and synthetic modification of kibdelomycin, enabling me to work on other dimensions of the project.[33] To achieve the overall goal, the project has been divided in many sub-goals, all laying foundation for the achieving the final goal to discover a derivative with improved properties that can be developed as a broad-spectrum antibiotic. This thesis covers a small but foundational part of the overall goal.

The thesis has three, related but independent, sub-goals presented in three chapters.

(1) Biosynthesis of kibdelomycin.

Goal: To understand precursors needed by the cells to make kibdelomycin enabling to design and feed new intermediates to make new molecules and determine activities

(2) Isolation, structure elucidation of and biological evaluation of new congeners of kibdelomycin.

Goal: To discover new minor compounds from the extract, determine their antibacterial activity, define structure activity relationship compared to kibdelomycin and understand their role in biosynthetic pathway

(3) Synthetic modification of kibdelomycin and determination of minimum structural requirement for antibacterial activity.

Goal: To synthesize derivatives by selective chemical modification, determine antibacterial activity, define structure activity relationship and understand their role in biosynthetic pathway

Chapter 2: Biosynthesis of kibdelomycin

Goal: To understand precursors needed by the cells to make kibdelomycin enabling to design and feed new intermediates to make new molecules and determine activities

Biosynthesis is the study of natural product synthesis inside the cells starting from small precursor molecules catalyzed by biosynthetic enzymes. There are two ways to understand biosynthesis, both are equally important. The traditional approach involves feeding ^{13}C-labeled potential precursors to the producing organism during fermentation, isolating the compound of interest, analyzing the compound by ^{13}C NMR and measuring precursor incorporation by differential ^{13}C NMR analysis. The second approach involves whole genome sequencing and biosynthetic gene cluster analysis. Understanding biosynthesis will help identify early and late precursors involved in assembling kibdelomycin in cells. As a result, by substituting alternate precursors, it is possible to generate new congeners helping to achieve the overall goal, new kibdelomycin analogs. For kibdelomycin, both approaches were applied. My work focused on the precursor directed biosynthesis, summarized in this chapter.

Results and Discussion

Kibdelomycin (Figure 1) is composed of four structural units. Fully substituted epiallose (A) connected by a amino glycosidic bond to tetramic acid (B) which in turn makes glycosidic linkage to amycolose (C) terminating with an amide bond connected to pyrrole unit (D). Retro-biosynthetic analysis indicates that sugars A and C would be derived from glucose, tetramic acid (B) would be derived from acetate and valine by polyketide synthase (PKS) and non-ribosomal peptide synthase (NRPS) pathway and pyrrole (D) from proline (Figure 2).

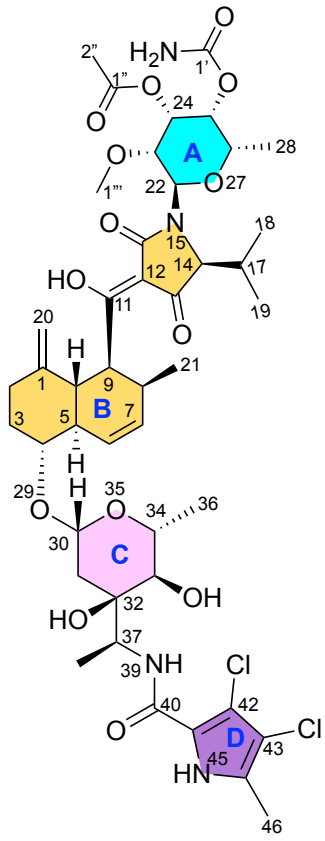


Figure 1. Kibdelomycin with designated structural fragment units.

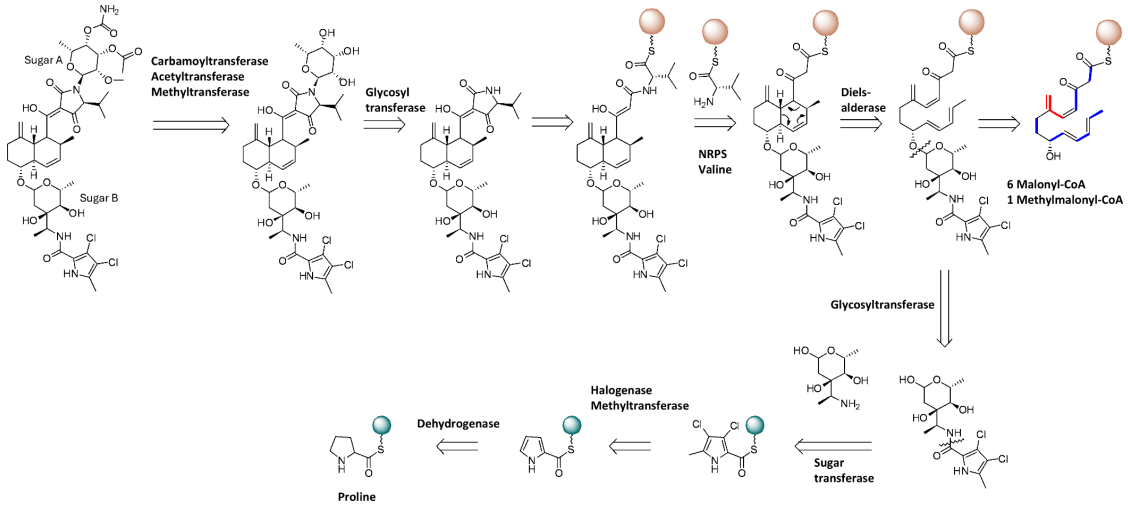


Figure 2. Retro-biosynthetic analysis of kibdelomycin (adapted from Sanchez-Hidalgo, Singh et al, *J. Nat. Prod*, 2025)[33].

Fermentation

Kibdelosporangium banguiesne CA-240109F is typically grown in ISM-3 seed media for four days and the seed culture was used to inoculate the production media which was grown for 14 days (Figure 3). Kibdelomycin was extracted from the production culture by shaking with 1:1 methyl ethyl ketone (MEK) for several hours and analyzed by reversed phase high performance liquid chromatography and mass spectrometry (HPLC-MS, LCMS) (Figure 3). LCMS shows a major (7.2 min) and a minor peak (6.4 min) by ultraviolet detection. The mass spectral analysis of the peak at 7.2 min shows a mass over charge (m/z) at 938 as [M+H] and consistent with kibdelomycin (Figure 3) and the minor peak eluted at 6.4 min showing mass over charge ratio (m/z) 924 as [M+H] for des-methylated kibdelomycin A (MS data not shown, see chapter 1 for structure).

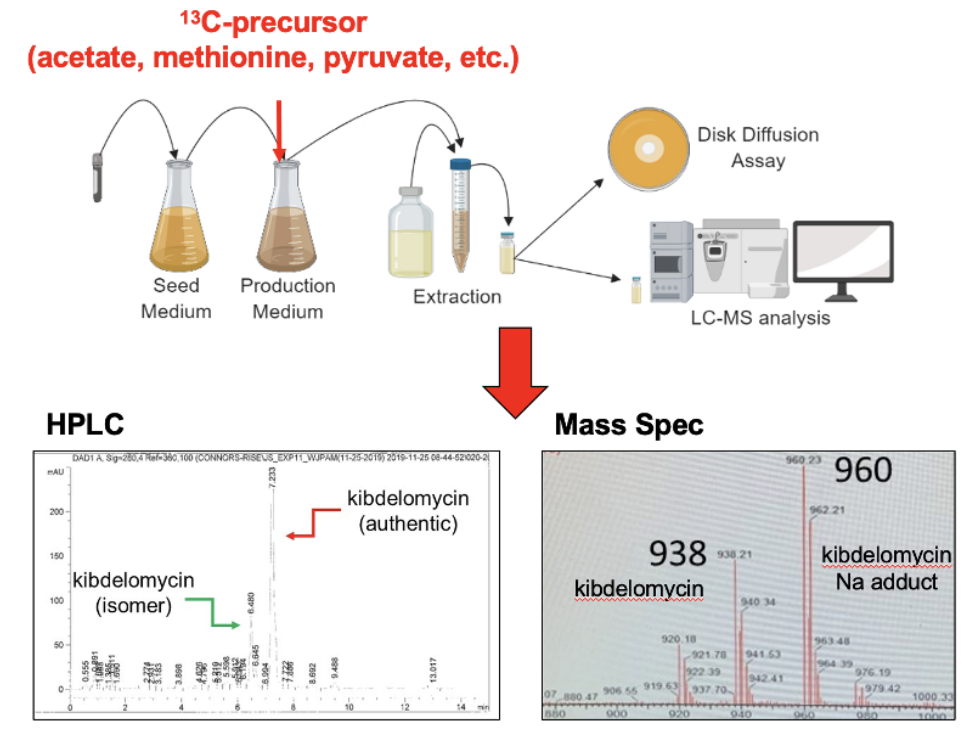


Figure 3. Fermentation procedure and HPLC-MS analysis of the MEK extract showing production of kibdelomycin.

Purification

The MEK extract was concentrated, and the resulting aqueous solution was washed with hexane and then extracted with ethyl acetate. The latter extract was successively washed with 10% aqueous citric acid, water, 10% aqueous sodium bicarbonate and concentrated. The ethyl acetate extract was successively chromatographed on silica gel, preparative reversed phase high performance liquid chromatography (HPLC) followed by solid phase extraction on XAD-16. A flow chart of the chromatographic purification of sodium salt of kibdelomycin is shown in Figure 4.

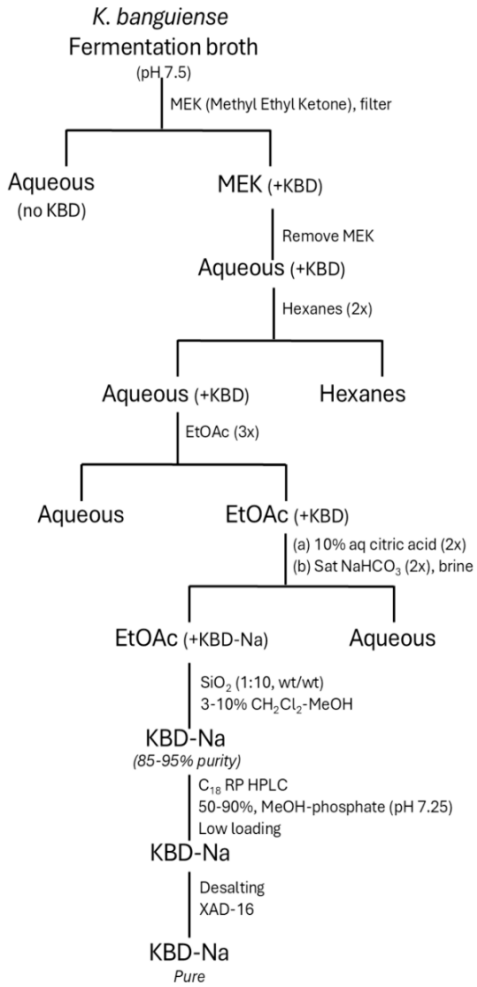


Figure 4. Flow chart of purification of kibdelomycin sodium salt (adapted from Sanchez-Hidalgo, Singh et al, *J. Nat. Prod*, 2025)[33].

^{13}C feeding experiments

Based on retro-biosynthesis (Figure 2), we selected and performed labeling experiments utilizing ten specifically labeled precursors. They were [$^{13}C_1$,$^{13}C_2$ and $^{13}C_{1,2}$]-acetate, [$^{13}C_2$ and $^{13}C_3$]-propionate, $^{13}C_1$-L-proline, $^{13}C_1$-L-valine, $^{13}CH_3$-L-methionine, [$^{13}C_2$- and $^{13}C_3$]-pyruvate. These labeled precursors were directly fed in the production medium of the fermentation using overproducing strain *K. banguiense* CA-240109F. Specifically ^{13}C -labeled precursors were fed to the fermentation at day 4 when the production has just started and then at days 7 and 10. Four days later kibdelomycin was extracted at day 14 and purified as described in the flow chart (Figure 4).

[$^{13}C_1$, $^{13}C_2$ and $^{13}C_{1,2}$]-acetate labeling results

^{13}C- NMR spectral analysis (Figure 5) of kibdelomycin derived from the three labeled acetates showed that all carbons of the tetramic acid portion of the molecule were derived from acetate (Figure 6). $^{13}C_2$ acetate labeled six alternating carbons starting with the beginning acetate-derived methyl C-21, followed by five malonate-derived carbons C-7, C-5, C-3, C-9, and C-12. $^{13}C_1$ acetate labeled the remaining alternate carbons C-8, C-6, C-4, C-2, C-11, and C-16. These were also observed with the feeding and labeling of $^{13}C_{1,2}$ which showed direct incorporation of carbons C-8-C-21, C-6-C-7, C-4-C-5, C-2-C-3, C-9-C-11, and C-12-C-16. Additional labeling of the tetramic acid moiety was observed from $^{13}C_2$ acetate from carbons C-1, C-10, and C-12, with C-10 also being labeled by $^{13}C_1$ acetate. This extender unit of the tetramic acid was initially expected to be derived from methylmalonate using a propionate precursor or malonate followed by a methylation by S-adenosyl methionine (SAM). After further analysis, it has been proposed that these carbons were derived from a two-step process involving the citric acid cycle and a rearrangement of succinyl-CoA to (2R)-methylmalonate. This reaction was done via the catalyzation by methylmalonyl-CoA mutase (MCM) and a subsequent racemization to (2S)-

methylmalonate catalyzed by methylmalonyl-CoA racemase (MCR). The pathway is shown below in Figure 7.

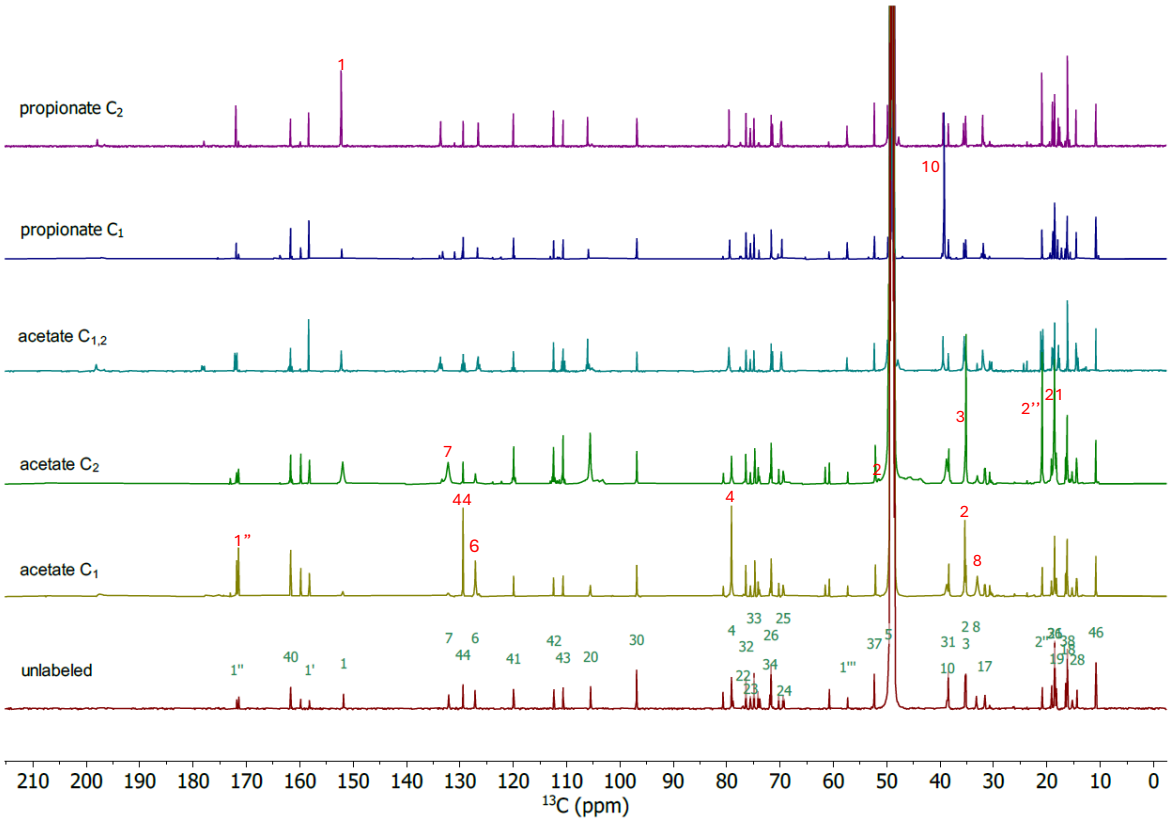


Figure 5. Overlaid ^{13}C NMR Spectra (150.89 MHz, CD_3OD, 298.0 K) of unlabeled and labeled kibdelomycin derived from three ^{13}C labelled acetates and the two ^{13}C-labelled propionates. The red numbers on spectra show enhancement of those specific carbon numbers in kibdelomycin.

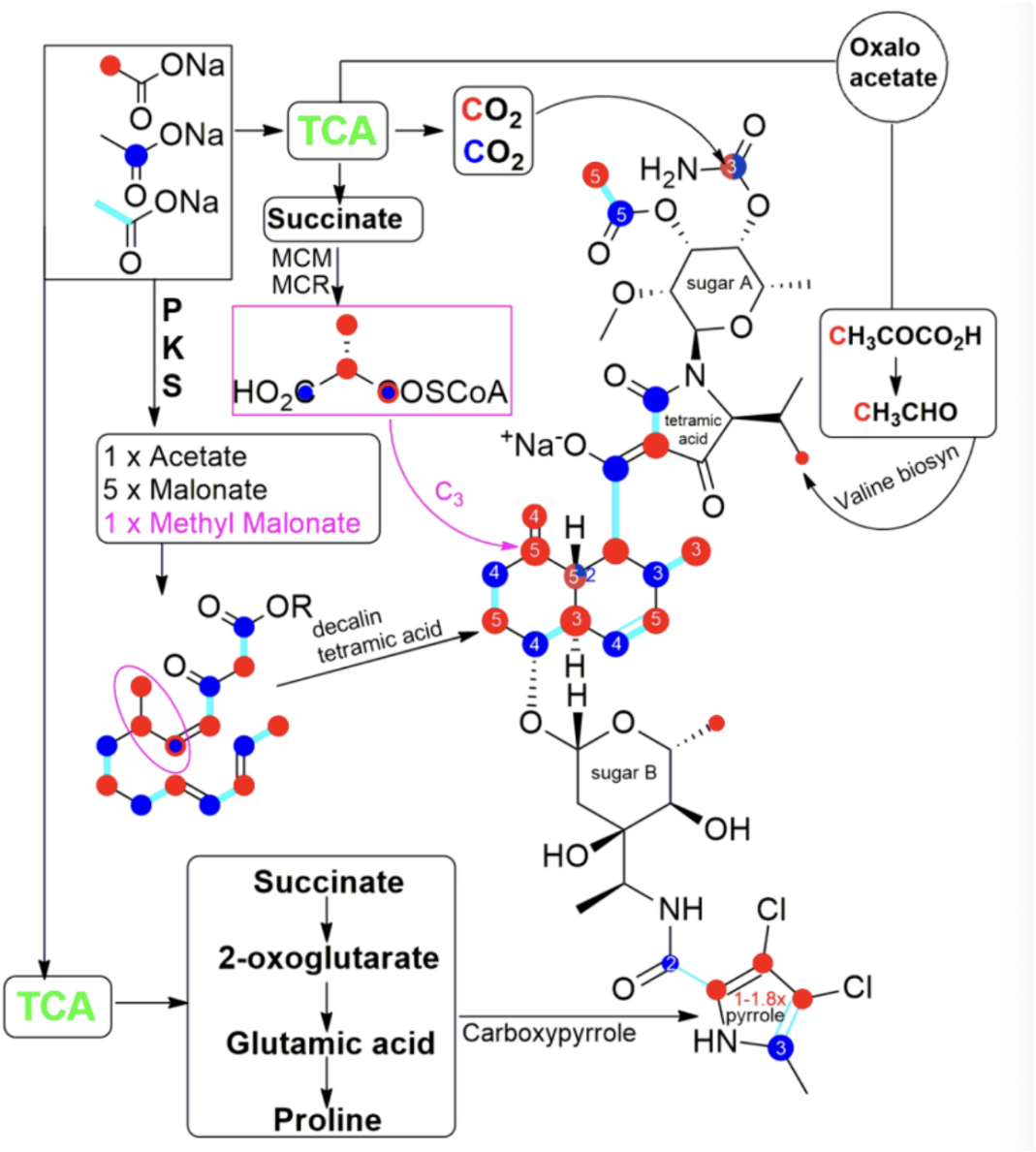


Figure 6. Labeling pattern of kibdelomycin from feeding of [$^{13}C_1$,$^{13}C_2$ and $^{13}C_{1,2}$]-sodium acetate determined by differential ^{13}C NMR (150 MHz) analysis. Numbers in circle represent percent incorporation. Lack of numbers denotes that percentage could not be determined due to exchange broadening. Red filled circle represents labelling from C-2 carbon of the C-2 ^{13}C labelled acetate and blue filled circle represents labelling from C-1 carbon of the C-1 ^{13}C labelled acetate, blue-green thick bond represents intact incorporation of C-1 and C-2 doubly ^{13}C labelled acetate. (adapted from Sanchez-Hidalgo, Singh et al, *J. Nat. Prod*, 2025) [33].

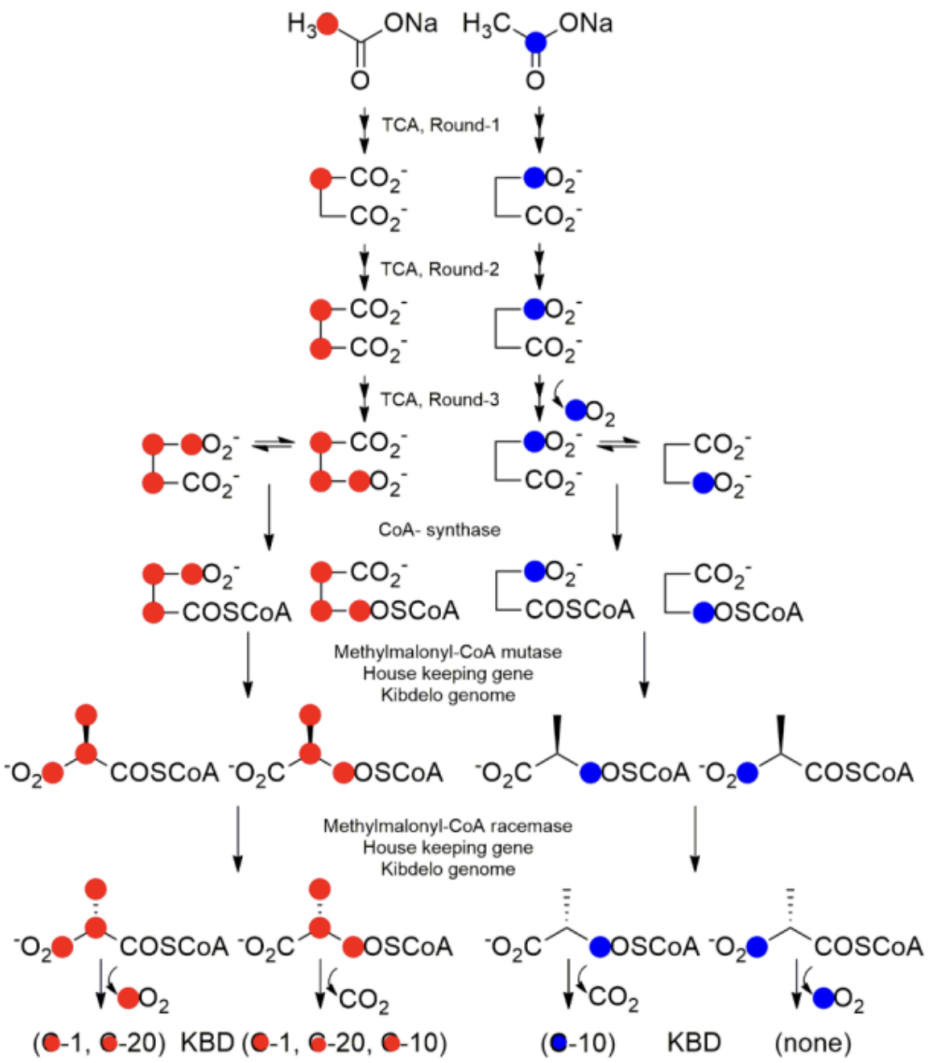


Figure 7. Labeled acetate incorporation in (3*S*)-methylmalonyl-CoA and kibdelomycin (KBD) at C-1, C-10 and C-20. Same color scheme as detailed in legend of Figure 6. (adapted from Sanchez-Hidalgo, Singh et al, *J. Nat. Prod*, 2025) [33].

Labeling patterns throughout the molecule are also consistent with the citric acid cycle pathway. The C-19 methyl carbon of valine showed weak labeling from $^{13}C_2$ acetate, derived from putative acetaldehyde and pyruvate via oxaloacetate. Carbamate carbonyl C-1' was labeled from both acetates from CO_2 derived from the TCA cycle. Additional labeling of C-1" and C-2" of the acetate group at C-24 of sugar A showed strong labeling of $^{13}C_1$ and $^{13}C_2$ acetates, with support

from $^{13}C_{1,2}$ acetate. The lower portion of the molecule shows further labeling of dichloro-carboxy-pyrrole unit, with C-42, C-42, and C-43 labeled with $^{13}C_2$ acetate and C-40 and C-44 labeled by $^{13}C_1$ acetate. The labeling of the pyrrole unit was derived from the active TCA cycle involving succinate-2-oxoglutarate-glutamic acid-proline, which is then converted to pyrrole. This observation was confirmed by incorporation of $^{13}C_{1,2}$ acetate labeling at C-40-C-41 and C-43-C-44 from symmetrical succinate.

[$^{13}C_1$ and $^{13}C_2$]-propionate labeling results

$^{13}C_1$ propionate and $^{13}C_2$ propionate strongly labeled C-10 and C-1 respectively via the methyl malonate pathway (Figure 8). This was expected as methyl malonate predicted monomer incorporated by the acyl transferase domain present in the second polyketide synthase of the biosynthetic gene cluster. It was interesting to discover that the microorganism utilizes both acetate via succinate and propionate for the methyl malonate precursor. While unable to validate the biosynthetic gene cluster using all established protocols, the incorporation of ^{13}C acetate and propionate provides evidence for the polyketide synthesized by genes *kbd29-kbd30* which strongly supports the biosynthetic gene cluster analyzed (see later).

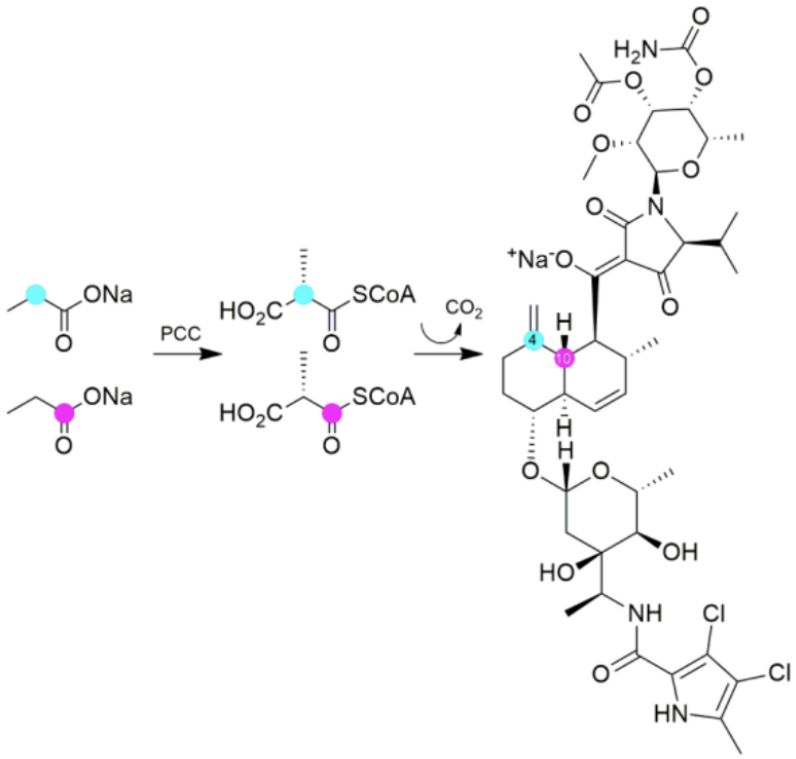


Figure 8. Labeling pattern of kibdelomycin from feeding of [$^{13}C_1$, and $^{13}C_2$]-sodium propionate determined by differential ^{13}C NMR (150 MHz) analysis. Numbers in circle represent percent incorporation. Blue-green filled circle represents labelling from C-2 carbon of the C-2 ^{13}C labelled propionate and purple filled circle represents labelling from C-1 carbon of the C-1 ^{13}C labelled propionate. (adapted from Sanchez-Hidalgo, Singh et al, J. Nat. Prod, 2025)[33].

$^{13}C_1$-proline and $^{13}C_1$-valine labeling results

$^{13}C_1$-proline exclusively labeled C-40 pyrrole amide ketone indicating direct role of proline in the biosynthesis of kibdelomycin (Figure 9, 10). Likewise, $^{13}C_1$-valine exclusively labeled ketone at C-13 associated with valine confirming direct and intact incorporation of valine residue in the tetramic acid (Figure 9, 10).

[$^{13}C_1$ and $^{13}C_2$]-pyruvate labeling results

The $^{13}C_1$-pyruvate exclusively delivered the ^{13}C-label to the C-38 methyl group and $^{13}C_2$-pyruvate labeled C-37 of unit ring C (Figure 9, 10) confirming origin of these carbons from

pyruvate as has been reported for the biosynthesis of amycolamicin.[28] These two acetate equivalent carbons are derived from pyruvate by thiamine pyrophosphate (TPP) dependent enzyme which transfers acetate to a ketone by a nucleophilic two carbon donor mechanism (Figure 9)

$^{13}C_{Me}$-methionine labeling results

The ^{13}C-methyl group of methionine showed strong incorporation to the methoxy carbon C-1''' and C-45 pyrrole methyl moieties, confirming SAM dependent origin of these groups (Figure 9,10).

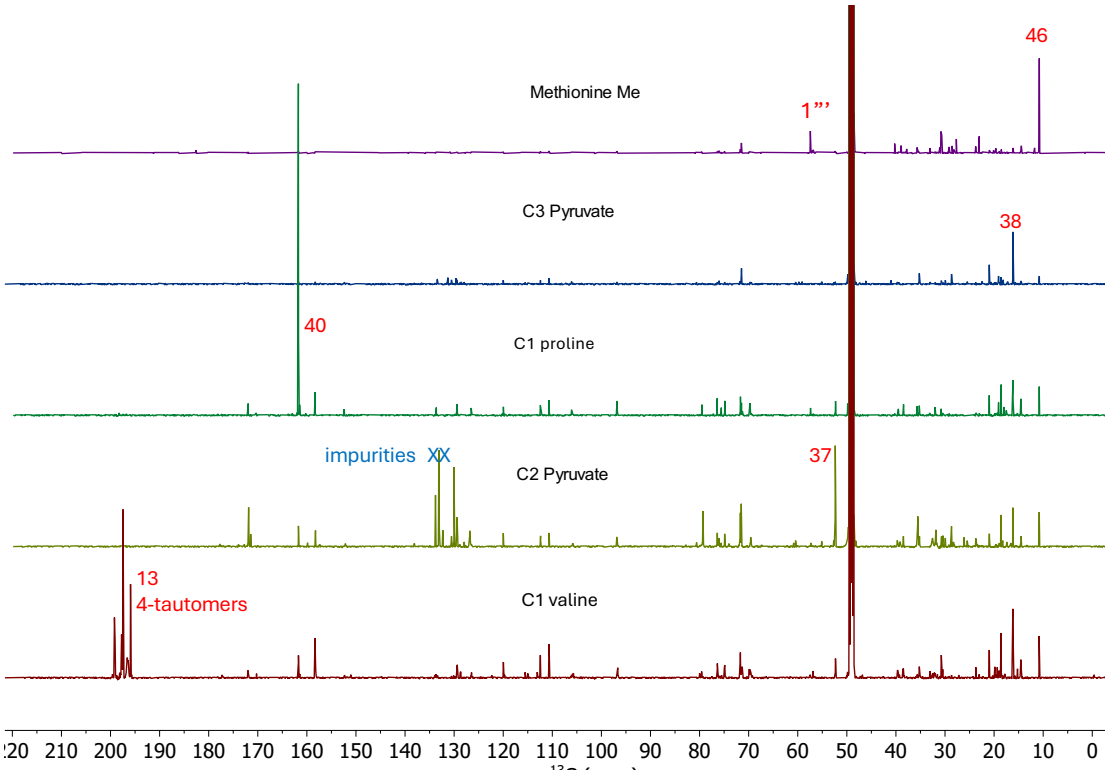


Figure 9. Overlaid ^{13}C NMR Spectra (150.89 MHz, CD_3OD, 298.0 K) of $^{13}C_1$-proline, $^{13}C_1$-valine, $^{13}C_1$ and $^{13}C_2$-pyruvates and $^{13}C_{Me}$-methionine. Interesting that the ^{13}C-NMR spectrum of kibdelomycin derived from $^{13}C_1$ labeled valine shows presence of the four strong sharp resonances at around d 200 ppm due to four tautomers of the valine derived carbonyl at position-13 of kibdelomycin. These four tautomeric ^{13}C-NMR resonances could not be observed in the unlabeled kibdelomycin spectrum due to significant suppression of resonances due exchange broadening. The red numbers on spectra show enhancement of those specific carbon numbers in kibdelomycin.

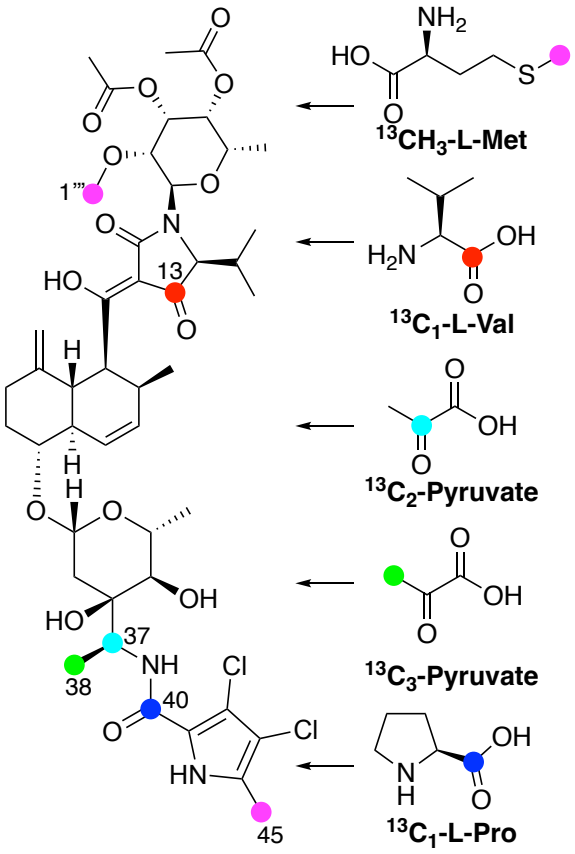


Figure 10. ^{13}C-Labeling of kibdelomycin from ^{13}C-labeled proline, valine, pyruvates and methionine.

These labeling pattern of kibdelomycin from all specifically labeled precursor discussed above strongly support the biosynthesis of kibdelomycin deduced from the biosynthetic gene cluster analysis shown in Figure 11 courtesy of our Medina collaborators at Spain.[33]

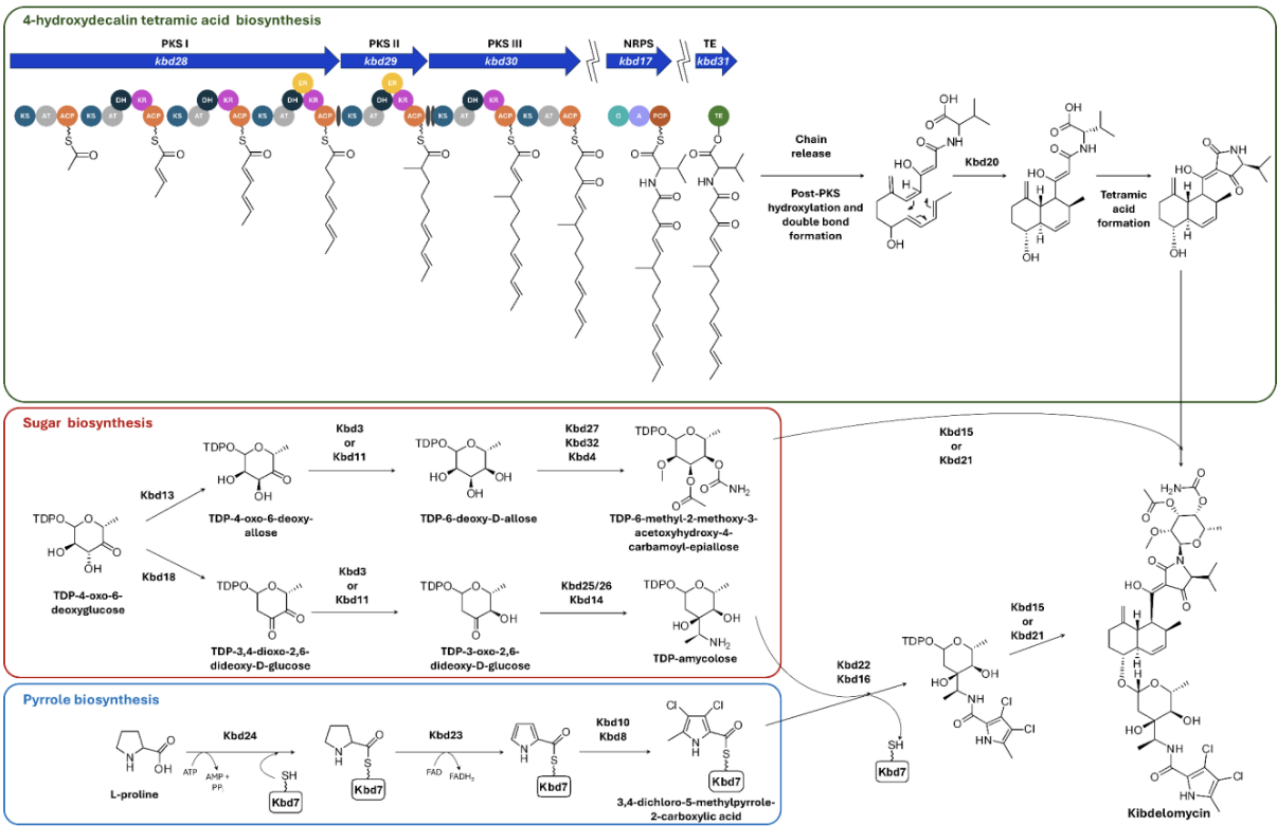


Figure 11. Kibdelomycin biosynthetic proposal (adapted from Sanchez-Hidalgo, Singh et al, *J. Nat. Prod.* 2025)[33].

Summary and conclusion

Labeling experiments from ten specifically ^{13}C labeled primary metabolite precursors established the origins of carbons of essentially all four structural units and firmly elucidated the biosynthetic pathway of kibdelomycin verified by biosynthetic gene cluster (BGC) analysis from genome sequencing experiments by Medina (Spain), a Drew collaborator and shown in Figure 11. The isotope labeling experiment played a major complementary role in elucidation of biosynthesis pathway of kibdelomycin due to lack of heterologous expression of BGC in another host using existing technology. More interestingly, strong intact incorporation of valine, proline, propionate, and pyruvate as well as methyl from methionine sets the stage for potential production of new kibdelomycins by new precursor directed biosynthesis. This fulfills our goal for learning about

direct incorporation of many of these precursors setting stage for feeding of different amino acids, substituted propionate, and pyruvates to potentially produce new kibdelomycin analogs.

Experimental

General Experimental Procedures

All chemicals and solvents were purchased either from Fisher Scientific and Sigma Aldrich. All microbial procedures were performed using good microbial practices and aseptic techniques. Streptomycin (100 μg/mL) or gentamycin (300 μg/mL) was used for selection of overproducing kibdelomycin mutant strain. Kibdelomycin and its derivatives were analyzed using HP1100 diode HPLC connected to a Waters Micromass mass spectrometer by all wavelength scanning mode for UV detection and positive and negative ion by electrospray ionization and simultaneous scanning mode for mass spectral detection covering m/z 50-1500 amu. Two reversed phase HPLC columns were used, a C_{18} (4.6 x 100 mm, Echelon column) or a Zorbax RX C-8 (4.6 x 250 mm) using a 11 or 25 min gradient of 5-90% or 30-90% acetonitrile-water mobile phase containing 0.1% formic acid in both mobile phases, at 1.5 mL/min. Preparative HPLC was performed on a Waters prep system on corresponding preparative columns 21 x 100 mm or 21x150 mm, eluting at 10 or 12 mL/min using gradients described with the isolation procedure.

NMR data collection and analysis

^{13}C NMR data collection was performed by Ryan D. Cohen at the Department of Analytical Research and Development at Merck & Co. in Rahway, New Jersey. All five of the ^{13}C labeled samples were acquired in CD_3OD with a 2 second relaxation delay on a Bruker 600 MHz NMR instrument. The samples were equipped with a 5 mm TCI CryoProbe™ system using 57,600 scans over 2 days of total acquisition. The integrated ^{13}C peaks were used for the calculation of percent incorporation. Normalization was done for each of the samples to reduce error, due to exchange

broadening and short relaxation delays, which was completed by diving the integral values of each carbon from the kibdelomycin samples with the the integration value of carbon 46. Carbon 46 was selected because of its likely origin being methylated by S-adenosylmethionine (SAM), which is unlikely to be affected by the feeding of labeled acetate and propionate. Percent incorporation was calculated by subtracting the normalized integral value from unlabeled kibdelomycin, then dividing the resultant integration value with the integral value of the unlabeled kibdelomycin sample again. Fold incorporation was then calculated by the percent enhancement divided by 100.

Production of kibdelomycin

To produce kibdelomycin, a spontaneous streptomycin-resistant strain was isolated at a concentration of 100 μg/ml of streptomycin from the original *K. banguiense* CA-240109 strain. The original strains only produced kibdelomycin at 5 mg/L, but when plated on ISP-2 agar plates containing 100 μg/ml streptomycin as well as additional media optimization, the new resistant strain CA-240109F (F) could produce 303 mg/L of kibdelomycin. The usage of this mutated strain was critical for the biosynthesis studies to supply the ample amount of kibdelomycin for ^{13}C NMR analysis. This strain was maintained in 20% glycerol vials stored at -78^{o}C until ready for use. The seed culture of the mutant strain was inoculated aseptically by transferring 1 ml of thawed F cells into a 125 ml Erlenmeyer flask containing 25 ml of seed medium with 100 μg/ml of streptomycin. The seed medium consists of the following: yeast extract (15 g/L); malt extract (10 g/L); $MgSO_4$ (0.50 g/L); ferric chloride (0.030 g/L); glucose (20 g/L) (ISM-3). This seed medium was prepared with distilled water and adjusted to pH 7.0 by adding NaOH before adding glucose. The seed culture was then incubated at 30^{o}C in a gyratory shaker (220 rpm) for 4 days prior to the inoculation of the production media. The production media: galactose (35 g/L); dextrin from corn, type 1 (10 g/L); bacto-soytone (5 g/L); (Difco); corn steep solid (2.5 g/L); $(NH_4)_2$ SO_4 (1.5 g/L); MOPS

(15.75 g/L) was prepared using distilled water, and the pH was adjusted to 7.4 using NaOH. The production media were capped in 2 L Erlenmeyer flasks containing 350 mL each of production media, then autoclaved at 250C for 25 minutes. The production medium was then inoculated using 25 mL of the seed growth medium containing the bacteria as well as streptomycin (100 μg/mL). The production medium was incubated at 30°C in a gyratory shaker (220 rpm) for 14 days prior to harvesting. For the feeding experiments containing [$^{13}C_1$], [$^{13}C_2$], or [$^{13}C_{1,2}$]-labeled acetates and [$^{13}C_1$] and [$^{13}C_2$]-labeled propionate, three aliquots of the precursors were fed on day 4 (0.5 g), day 7 (0.25 g), and day 10 (0.25 g). The cells were then harvested after an additional 4 days of fermentation (total 14 days) after the last aliquot was fed on day 10.

Isolation of unlabeled and ^{13}C-labeled kibdelomycin

Isolation of kibdelomycin was done in two separate ways, with the labeling of [$^{13}C_{1,2}$]-labeled acetate and [$^{13}C_1$] and [$^{13}C_2$]-labeled propionate being done in a more efficient manner. [$^{13}C_1$] and [$^{13}C_2$]-labeled acetate were isolated the following way. The culture broths (350 mL each) were extracted via whole broth extraction separately with methyl ethyl ketone (MEK) twice, resulting in 700 ml of MEK. The combined extract was concentrated to a 25 mL mostly aqueous solution, which was then washed with hexanes (25 mL) to remove nonpolar impurities, and extracted three times with ethyl acetate (EtOAc) (3 X 25 mL). The ethyl acetate was then concentrated to dryness, where it could be dissolved in CH_2Cl_2 and chromatographed on a 20 X 20 silica gel (500 uM) plate using a 90:8:1:1 ratio of CH_2Cl_2, MeOH, H_2O, and AcOH development solvent. Kibdelomycin band visualized under UV_{254} light and was scrapped off. Kibdelomycin was extracted from the silica gel powder with 80:20 EtOAc-MeOH (10 ml) by stirring for 10 min, filtered using a small, sintered glass funnel. The silica gel powder retained on the funnel was washed twice with 5 mL of the same solvent mixture generating 20 mL of combined filtrate

containing kibdelomycin which was diluted with EtOAc to make it 95:5 EtOAc-MeOH. This diluted mixture was washed with 20 mL each of H_2O and sodium phosphate buffer (7.25 pH, 0.25 mM). The EtOAc was then concentrated to dryness again and resuspended in a mixture of 800 uL of MeOH and 200 uL of sodium phosphate buffer. This mixture was injected into a preparative high performance liquid chromatography (HPLC) C_{18} column (20 X 150 mm) and eluted with a 60 minute 50-90% gradient of MeOH and 0.25 mM sodium phosphate buffer (pH 7.25) at a flow rate of 10 mL/minute. After analysis of fractions using liquid chromatography mass spectroscopy (LCMS), kibdelomycin was found to elute between 36 and 42 minutes for both samples. The fractions were then combined and concentrated to an aqueous solution before being extracted with EtOAc (3 X 25 mL). The EtOAc was then washed with brine and concentrated to dryness, resulting in purified kibdelomycin labeled from [$^{13}C_1$] and [$^{13}C_2$]-labeled acetate (4 and 6 mg, respectively).

[$^{13}C_{1,2}$]-labeled acetate, [$^{13}C_1$] and [$^{13}C_2$]-labeled propionate, $^{13}C_1$-proline, $^{13}C_1$-valine, $^{13}C_1$ and $^{13}C_2$-pyruvates and $^{13}C_{Me}$-methionine were extracted with MEK and processed as described above to give an ethyl acetate extract. This extract was successively washed twice with equal volume of 10% citric acid, H_2O, saturated aqueous $NaHCO_3$, and H_2O, with emulsion broken by addition of brine. The EtOAc extract containing sodium salt of kibdelomycin was concentrated to dryness and chromatographed on a 1.5 g SiO_2 column packed in a disposable pasture pipette using CH_2Cl_2. Crude kibdelomycin with trace of kibdelomycin A were dissolved in about 1 mL CH_2Cl_2 and charged slowly. The column was eluted with 10 mL each of 3, 5, 7, 10% step gradient of CH_2Cl_2 / methanol. Fractions were easily monitored using SiO_2 TLC plates using 92:8:1:1 ratio of CH_2Cl_2, MeOH, H_2O, and AcOH development solvent as eluent. Kibdelomycin eluted in 5-10% MeOH. The pooled kibdelomycin-Na fraction was concentrated to dryness and purified by reversed phase HPLC using pH 7.25 sodium phosphate buffer described above. Pooled HPLC

fractions consisting of kibdelomycin-Na were concentrated to remove methanol and desalted by slowly charging on ~1 mL XAD-16. After loading, the XAD-16 column was washed with about 10 mL water, 10% aqueous methanol, and 100% methanol. Kibdelomycin eluted in 100% methanol which was concentrated to dryness to give 6-8 mg of kibdelomycin labeled from [$^{13}C_{1,2}$]-labeled acetate, [$^{13}C_1$] and [$^{13}C_2$]-labeled propionate, $^{13}C_1$-proline, $^{13}C_1$-valine, $^{13}C_1$ and $^{13}C_2$-pyruvates and $^{13}C_{Me}$-methionine.

Chapter 3: Isolation, structure elucidation and biological evaluations of new congeners of kibdelomycin.

Goal: to discover new minor compounds from the extract, determine their antibacterial activity, define structure activity relationship compared to kibdelomycin and understand their any role in biosynthetic pathway

Discovery of kibdelomycin A-1 and A-2 via repeat batch fermentation experiments

During analysis of MEK extracts of *K. banguiesne* many trace congeners of kibdelomycin were observed, often only detectable by mass spectra in negative ion mode. We continue to be very interested to discover new congeners to address structure activity relationship to fulfil our overall goal. Unfortunately, when congeners are present in trace amounts it becomes challenging to isolate them unless we can scale-up the fermentation to 100's of liters. Of course, that is not an option at Drew. The other option was to find different fermentation conditions, such as different fermentation time and media to enhance production of these congeners. In the latter effort, we undertook an experiment using repeat batch fermentation which is popular in large scale commercial production of natural products but not for generation of new metabolites and congeners. This effort resulted in significantly enhanced production of two congeners which were isolated in mg quantities from a liter of fermentation batch. This chapter describes the details of the isolation, structure, and biological activities of two new congeners named as kibdelomycin A-1 and A-2 (Figure 1).

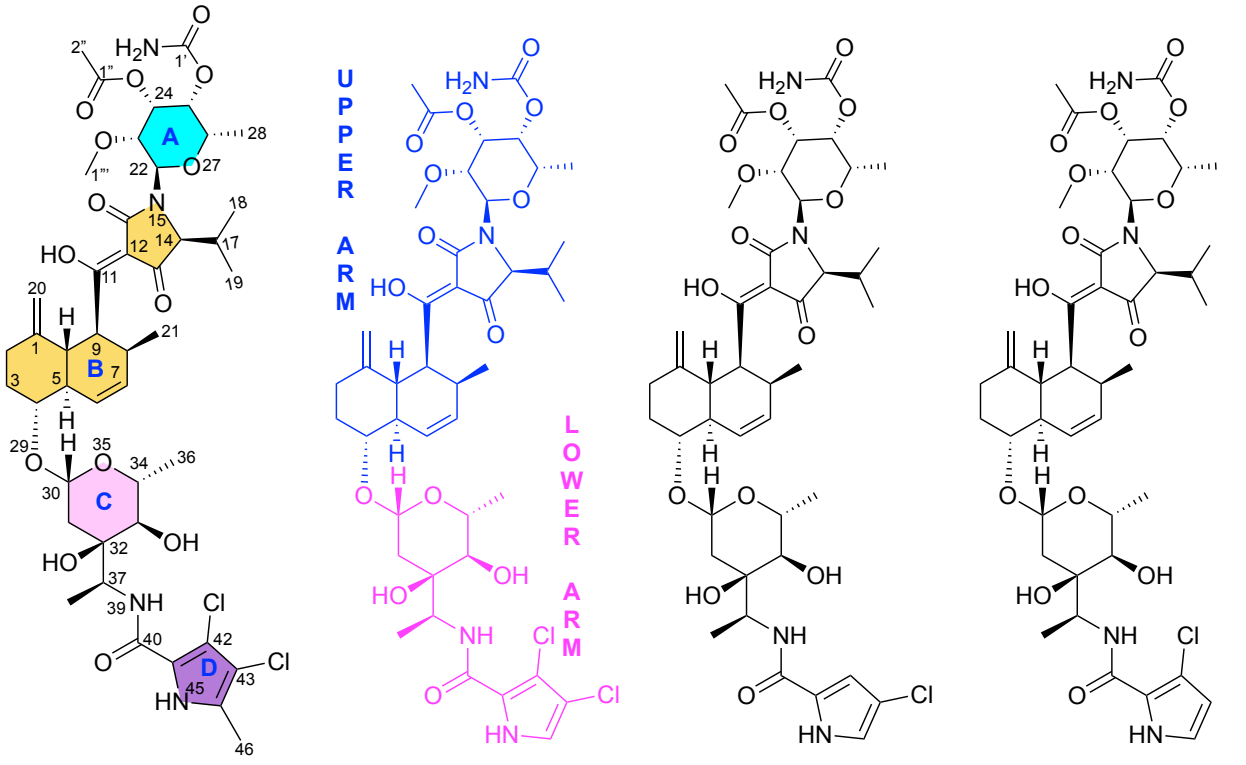


Figure 1. Structures of kibdelomycins (adapted from Fine, Singh et al, *Bioorg Med Chem Lett* 2026) [41].

Repeat batch fermentation

Production of antibiotics and fine chemicals by fermentation occurs by batch, fed-batch, or continuous culturing. Key parameters to assess the efficacy of fermentation are titer (gram/liter), yield (gram product/gram carbon source), and productivity (gram/liter•time). One approach to improve productivity is to reduce the time between individual fermentation cycles by a process referred to as repeat fermentation (also referred to as repeated or repetitive fermentation). Repeated fermentation is where all or a part of the cells from a completed fermentation process is added to fresh medium for the next round of fermentation. Thus, the time of initiating a new fermentation cycle is dramatically reduced since cleaning, set-up, and

sterilization of the fermentation apparatus, preparation of the inoculum, etc. is reduced or eliminated, resulting in improved productivity. Moreover, repeat fermentation often leads to increased cell density, possibly improving product titer and yield. Repeat fermentation processes have been applied for the production of several finished products such as antibiotics thienamycin and cephalosporin[34] and natamycin;[35] fine chemicals L-lactic acid,[36,37] hyaluronic acid,[38] and L-tyrosine[39] as well as other important fermentation products.[40] However, to the best of our knowledge, the use of repeat batch fermentation has not been described for the production and isolation of new congeners and intermediate compounds in the biosynthetic pathway for antibiotics or other fine chemicals. (Passage extracted from our manuscript [41]).

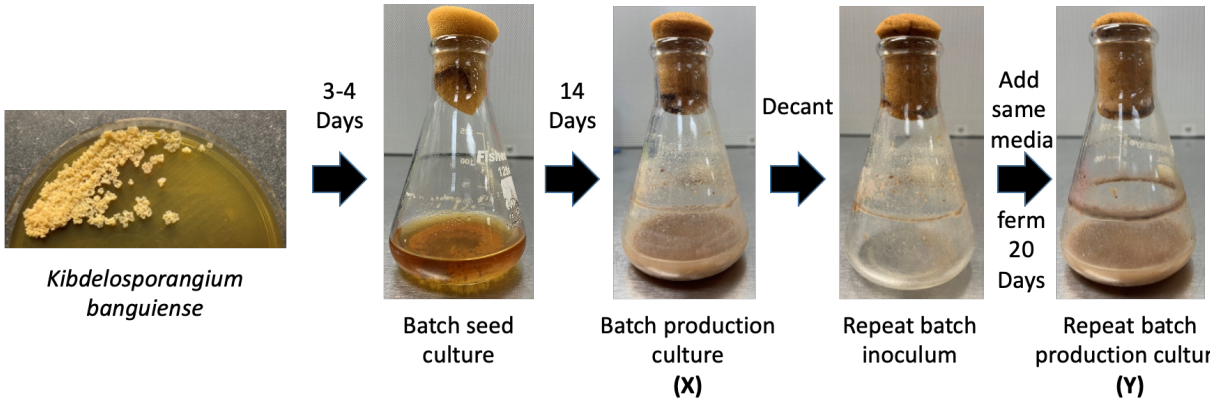


Figure 2. Schematic of repeat batch fermentation for new kibdelomycin congener production (adapted from Fine, Singh et al, *Bioorg Med Chem Lett* 2026) [41].

Fermentation and purification of new congeners

For new application of the repeat batch fermentation for this project, *Kibdelosporangium banguiense* CA240109F16 was grown in ISM-3 seed media for four days, followed by production media (described in chapter 2) for 20 days at 220 rpm at 30°C. Following the original batch culture fermentation (Figure 2, X), the 1.2 L of broth was harvested by transferring the

content to another flask, then to the empty fermentation flask containing the residual mycelia sticking to the sides of the flask, fresh production media was added and the culture grew again for 20 days under identical conditions (Figure 2, Y). Both X and Y batches were extracted using methyl ethyl ketone (MEK), then analyzed using LCMS at absorbance at $\boldsymbol{\lambda}_{278}$. The LCMS graphs (Figure 3) were then used to calculate the metabolic ratios of each of the products, with kibdelomycin (1) being predominant in both fermentation batches and kibdelomycin A (2) increasing in production in the repeat batch. The two new metabolites, kibdelomycin A-1 (3) and kibdelomycin A-2 (4) were found in the repeat batch fermentation at a ratio of 3.7% and 4.7% respectively after relative calculations. Table (1) presents the relative ratios of kibdelomycin, and congeners produced from the original and repeat batch fermentations. Trace amounts of kibdelomycin A-1 and A-2 were only found in the first fermentation extract after extracted single ion search of molecular *m/z* 889 in the negative ionization mode of the ESI-MS.

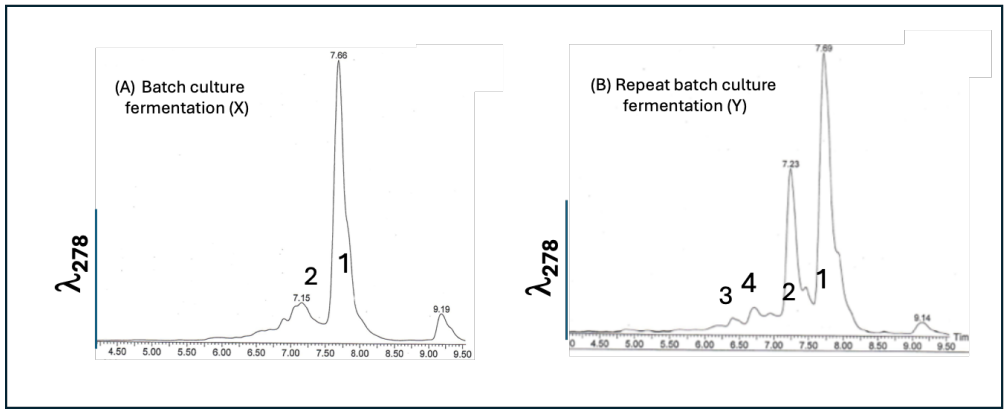


Figure 3: HPLC chromatograms of MEK extracts of whole broth of the batch culture fermentation (X) and the repeated batch culture fermentation (Y). (**1**) kibdelomycin, (**2**) kibdelomycin A, (**3**) kibdelomycin A-1, (**4**) kibdelomycin A-2 (adapted from Fine, Singh et al, *Bioorg Med Chem Lett* 2026) [41].

Table 1: Relative ratios of kibdelomycin and congeners produced by original batch fermentation (1st) and repeat batch (2nd) fermentations (adapted from Fine, Singh et al, *Bioorg Med Chem Lett* 2026) [41].

Kibdelomycins	Original (1st) fermentation		Original (2nd) repeat fermentation	
#	AUC (λ_{278})	Ratio (%)	AUC (λ_{278})	Ratio (%)
Kibdelomycin (1)	70541	90.4	166532	59.5
Kibdelomycin A (2)	7500	9.6	89903	32.1
Kibdelomycin A-1 (3)	Trace	--	10500	3.7
Kibdelomycin A-2 (4)	Trace	--	13185	4.7

After confirmation of the two congeners, the ethyl acetate extract of concentrated MEK from the repeat batch fermentation was chromatographed on silica gel, followed by reversed-phase C_{18} chromatography using an acetonitrile-water gradient. The earlier eluting fractions containing the two compounds were then re-chromatographed on the previously described C_{18} column using a methanol-sodium phosphate buffer (pH 7.25) gradient as applied to kibdelomycin to form Na salt forms of the molecule for stabilization. The phosphate buffer was finally removed using solid phase extraction on XAD-16, resulting in Na salts of kibdelomycin A-1 and A-2, 2.5 mg each.

Structure elucidation of kibdelomycin A-1 (3) and A-2 (4)

Analysis of LCMS diode array spectra showed that kibdelomycin A-1 and A-2 demonstrated a UV spectrum (λmax 250, 276 nm) in acidic environments, like kibdelomycin and kibdelomycin A. For kibdelomycin A-1, liquid chromatography-electrospray ionization-mass

spectrometry (LCESIMS) showed parent ions at m/z 891 $[M+H]^+$ and 889 $[M+H]^-$ simultaneously recorded in positive and negative ionization modes. High resolution-electrospray ionization-mass spectrometry (HRESIMS) was also used and produced a protonated parent ion at m/z 891.3796 $[M+H]^+$ which is consistent with the molecular formula $C_{43}H_{59}ClN_4O_{14}$ (calculated for M+H, 891.3789), which is one chlorine less than kibdelomycin A. Fragmentation also played a critical role in characterizing the new congeners of kibdelomycin. When kibdelomycin is performed in positive ion ESI, the mass spectrum exhibits two major fragments, the upper half of the molecule (Upper arm, northern hemisphere) and the lower half of the molecule due to cleavage of the glycosidic linkage (O-29 and C-30) between the tetramic acid and sugar C group. Kibdelomycin A-1 in the positive ion ESI exhibited fragmented ions at m/z 591 and m/z 283 (Figure 4). The m/z 591 fragment of the molecule is consistent with the upper arm of kibdelomycin and suggests that the structure of kibdelomycin A-1 is identical to that of kibdelomycin and kibdelomycin A. The m/z 283 fragment indicates a loss of a single chlorine atom from the bottom half of the molecule.

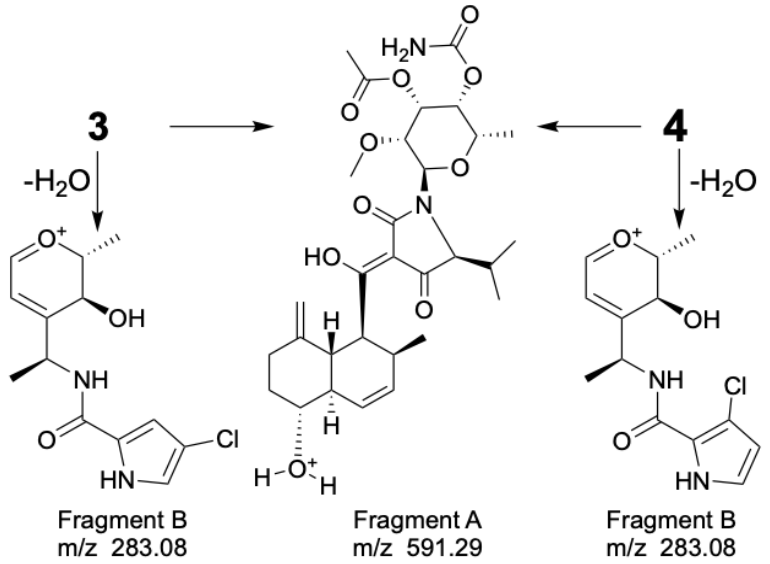


Figure 4. ESI^+ mass spectral fragmentation of kibdelomycins A-1 (**3**) and A-2 (**4**) (adapted from Fine, Singh et al, *Bioorg Med Chem Lett* 2026) [41].

Kibdelomycin A-2 was isomeric to kibdelomycin A-1, exhibiting similar UV (λ_{max} 250, 276 nm) and LCESIMS (ES^+ m/z 891 and ES^- m/z 889) and ES+ fragment ions at m/z 591 and m/z 283 along with molecular formula $C_{43}H_{59}ClN_4O_{14}$ from HRESIMS (observed 891.3797, calculated 891.3789). These two compounds only slightly differed from one another based on the location of the singular chlorine atom within the pyrrole ring. High resolution NMR analysis was performed by Drs Ryan D. Cohen and Sheo Singh[41] and was valuable to identify the difference in the two congeners based on the coupling within the pyrrole ring. Additional ^1H NMR studies were done comparing kibdelomycin A-1 and A-2 to authentic samples of methyl chloro-1H-pyrrole-2-carboxylate with the chloro substituent at the 3-, 4-, or 5- positions. The proton-proton coupling constants agree with the spectral data for methyl 4-chloro-1H-pyrrole-2-carboxylate matching kibdelomycin A-1 and methyl 3-chloro-1H-pyrrole-2-carboxylate consistent with kibdelomycin A-2 (Figure 5).

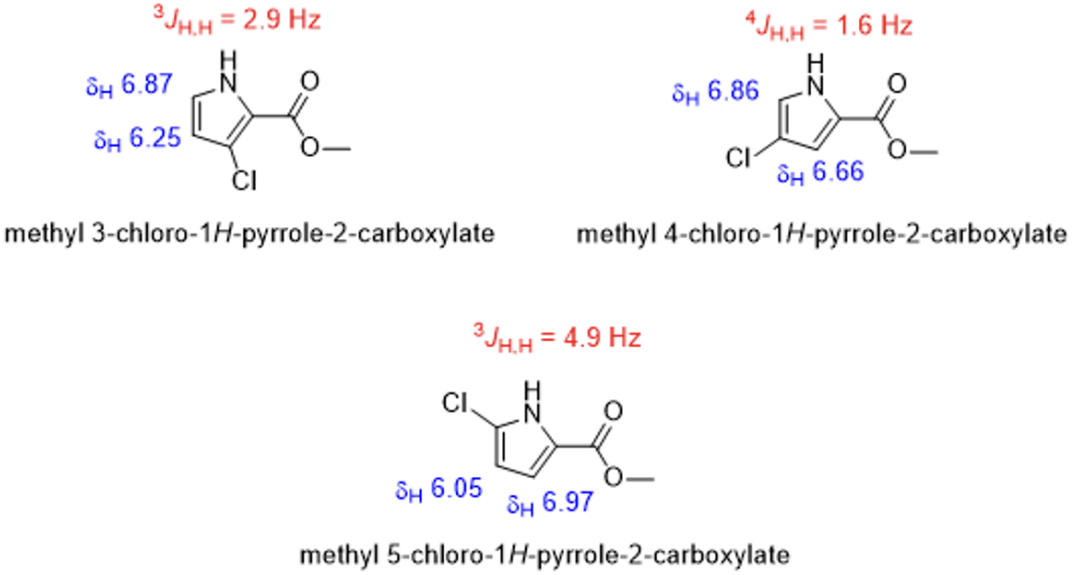


Figure 5: ^1H NMR data (ACD database, $CDCl_3$) for similarly substituted pyrrole ring systems as **3** and **4** (adapted from Fine, Singh et al, *Bioorg Med Chem Lett* 2026) [41].

Biological evaluation

Antibacterial evaluation of the two new congeners, as well as kibdelomycin and kibdelomycin A, were done using minimum inhibitory concentration assays to test the antibacterial activity of the four compounds (Table 2). Each compound was tested against *S. aureus* and *E. coli* BL21. Kibdelomycin inhibited *S. aureus* with the lowest MIC value of 0.25 μg/ml, which is within the range of reported MIC activities of 0.25-1 μg/ml, and is also consistent with the reported value of DNA gyrase B potency (IC_{50} 9 nM). Kibdelomycin A showed the second lowest MIC of 2 μg/mL, which is an 8-fold lower MIC compared to kibdelomycin and 16-fold lower reported gyrase B inhibitory IC_{50} value (400 nM). For *E. coli,* kibdelomycin showed an MIC of 64 μg/ml, which is within the range of reported values, and kibdelomycin A showed an MIC of 128 μg/ml, both significantly higher than *S. aureus* due to cell penetration problems. Kibdelomycin A-1 showed an MIC of 16 μg/ml and kibdelomycin A-2 showed an MIC of 32 μg/ml against *S. aureus*, both of which are significantly higher than kibdelomycin and kibdelomycin A. This indicates that the loss of the single chlorine atom in either location has a detrimental effect on the MIC activity of the new congeners compared to kibdelomycin A. Similarly, kibdelomycin A-1 and kibdelomycin A-2 both showed MIC values of >64 μg/mL for *E. coli*, indicating that the loss of the methyl or both methyl and chlorine groups have an impact on *E. coli* activity based on similar cell penetration problems.

Table 2. Minimum inhibitory of concentration (MIC) of kibdelomycin and its derivatives against selected bacterial strains.

Compounds	*S. aureus* (μg/mL)	*E. coli* BL21 (μg/mL)
Kibdelomycin	0.25	64
Kibdelomycin A	2	128
Kibdelomycin A-1	16	>64
Kibdelomycin A-2	32	>64

In conclusion, in a novel application of repeat batch fermentation of *K. banguiense* led to discovery of two new mono-chloro (**3** and **4**) biosynthetic analogs of kibdelomycin. These derivatives contribute to understanding the structure-activity relationship around the pyrrole ring. It remains undetermined whether these compounds are shunt metabolites, direct intermediates in kibdelomycin biosynthesis, or indirect intermediates. Current evidence suggests that halogenation by Kbd10 enzyme of kibdelomycin BGC (Chapter 2, Figure 11) involves a non-selective stepwise chlorination process leading to the formation of the dichloro-pyrrole ring. The *E. coli* MIC values for these compounds were within two-fold of kibdelomycin; however, the MIC was 64- to 128-fold less potent against *S. aureus*, highlighting the role of the hydrophobic interaction of the pyrrole methyl group in the DNA gyrase binding pocket in *S. aureus*.
The discovery of these two new mono-chloro compounds provided insight for the first time the role of both chlorines in the enhanced potency of kibdelomycin and provided insight that halogenase enzyme in not regio selective, helping in addressing our goal.

Experimental

General experimental procedure: See chapter 2.

Production of kibdelomycin

To produce kibdelomycin and new compounds, *K. banguiense* CA240109F (F strain) was used for spontaneous mutant selection on agar plate at a 300 mg/L gentamycin. This led to isolation of a surviving dual (streptomycin and gentamycin) resistant strain CA240109F16 (F16 strain), that produced enhanced levels of kibdelomycin. The seed culture of F16 strain was inoculated aseptically by transferring 1 ml of thawed F16 cells into 4 total 125 ml Erlenmeyer flask containing 25 ml of seed medium with 100 μg/ml of streptomycin and 300 mg/mL of gentamycin. The seed medium consists of the following: yeast extract (15 g/L); malt extract (10 g/L); $MgSO_4$ (0.50 g/L); ferric chloride (0.030 g/L); glucose (20 g/L) (ISM-3). This seed medium was prepared with distilled water and adjusted to pH 7.0 by adding NaOH before adding glucose. The seed culture was then incubated at 30^{o}C in a gyratory shaker (220 rpm) for 4 days prior to the inoculation of the production media. The production media: galactose (35 g/L); dextrin from corn, type 1 (10 g/L); bacto-soytone (5 g/L); (Difco); corn steep solid (2.5 g/L); $(NH_4)_2$ SO_4 (1.5 g/L); MOPS (15.75 g/L) was prepared using distilled water, and the pH was adjusted to 7.4 using NaOH. The production media were capped in 2 L Erlenmeyer flasks containing 350 mL each of production media, then autoclaved at 250^{o}C for 25 minutes. The production medium was then inoculated using 25 mL of the seed growth medium containing the bacteria as well as streptomycin (100 μg/mL) and gentamycin (300 mg/mL) . The production medium was incubated at 30^{o}C in a gyratory shaker (220 rpm) for 18 days prior to harvesting by pouring the liquid broth in large beaker for extraction of kibdelomycin (batch 1) which was not used for this study. Following the initial harvest, residual biomass stuck to the walls of the 2 L Erlenmeyer flask were not removed. For repeat batch fermentation, to these four flasks were added 300 mL of the same AMM production media as described above and flasks were transferred to the same gyratory shaker and incubated again by shaking at 30^{o}C at 200 rpm for 20 days and then harvested (batch 2).

Isolation and purification

The culture broths (4 flask of 350 mL each) after centrifugation were extracted separately with methyl ethyl ketone (MEK). Cells were extracted two times with 500 mL each, resulting in 1L of MEK extract. The supernatant (1.3L) was washed with hexane (2 x 250 mL) followed extraction with ethyl acetate (2 x 500 mL). The resulting extract was concentrated under reduced pressure to afford 230 mg of solid fraction A. MEK extract of cells was concentrated to yield aqueous solution (250 mL) which was washed with hexane (2 x 150 mL) and extracted with ethyl acetate (3 x 150 mL) and concentrated to dryness to yield 420 mg of an oily fraction B. These fractions A and B were combined due to similar LCMS profile. The combined fractions were dissolved in 1 mL ethyl acetate-methanol (3:1), pre-adsorbed on 2-3 g silica gel, solvents removed on rotary evaporator and loaded on to 9 g silica gel column (12 x 1.2 cm) packed in methylene chloride. The column was washed with 250 mL methylene chloride followed by elution with step gradient of methanol (3%, 5%, 8%, 10%, and 15%) in methylene chloride. 60- 100 mL fractions were collected. Kibdelomycin and minors eluted in 5-10% methanol fraction which were combined and concentrated to give 180 mg powder. This sample was chromatographed in four equal runs on reversed phase prep HPLC on Echelon column using 50 min 30-90% linear gradient of acetonitrile-water at 12 mL/min. Due to broad separation, samples were analyzed by LCMS and early fractions enriched with mono-chloro compounds along with kibdelomycin and kibdelomycin A were combined. They were concentrated to 100 mL aqueous and extracted with ethyl acetate (2 x 100 mL). Ethyl acetate was removed by concentration to yield 22 mg of fraction. This fraction was purified by reversed phase HPLC using MeOH and pH 7.25 sodium phosphate buffer 45 min 55-90% gradient at 10 mL/min flow rate. Pooled HPLC fractions consisting of sodium salts of kibdelomycin A-1 and A-2 were concentrated to remove methanol, extracted with EtOAc and washed with water and brine. Ethyl acetate extract was dried ($MgSO_4$) and concentrated to dryness

followed by dissolving in methanol, transfer two tubes and removal by methanol by blowing nitrogen gave 1.2 mg of each A-1 and A-2 as white powders.

MIC assay

The purpose of the *in vitro* MIC assay is to determine the lowest concentration of an antimicrobial compounds required to completely kill microorganisms in vitro. To do this experiment, we used a 4x6, 24 well cell culture plate with a growth area of 1.9 cm^2 and a working volume of 3.5 ml. For this testing we oriented plates in four rows and six columns; rows labeled A-D and columns labeled 1-6. Sigma LB broth tablets (Lennox L broth) were used throughout all MIC experiments to support bacterial growth. *S. aureus* and *E. coli* BL21 were the two test sensitive strains used to test the activity of each test compound.

All wells contained 1 mL of LB media, except for the starting well of the test, which contained 2 mL of media starting with the highest concentration of the test compound. The test compound was dissolved in dimethyl sulfoxide (DMSO) at a minimum of 1 mg/mL concentration. The well containing 2 mL of LB media had an amount of LB media removed and replaced with the test compound in DMSO to have a consistent volume of 2 mL. The concentration of DMSO in a well was maintained less than 10% in each well. 1:1 serial dilution then took place across wells. When the number of dilutions succeeded 6 wells, the dilution series continued onto the second row following. Depending on the number of dilutions done, either negative controls were tested on each plate in rows 2 and 4, columns 5 and 6, or if the number of experiments exceeded well count, then negative controls were completed on a single plate once during the experiment. After the dilution series, microorganisms (5 μL) are added to each of the wells containing test compound. Kibdelomycin was used as a positive control test compound to see if the cells can be killed at the expected MIC concentrations (32 μg/mL in *E. coli* and 0.25 μg/mL in *S. aureus*). As a negative

control, one well contains just LB broth, cells, and the highest volume of DMSO added to test DMSO toxicity against the microorganisms. A second negative control well contains LB alone to test for contamination of the broth used. An example plate template is shown below with kibdelomycin as a positive control (Figure 6). After plates were populated, they were incubated at 32 °C for 24 h and MIC was determined as the last well of the dilution series that shows clear liquid and no turbidity due to bacterial growth, as exemplified by MIC reading of Kibdelomycin (Figure 7).

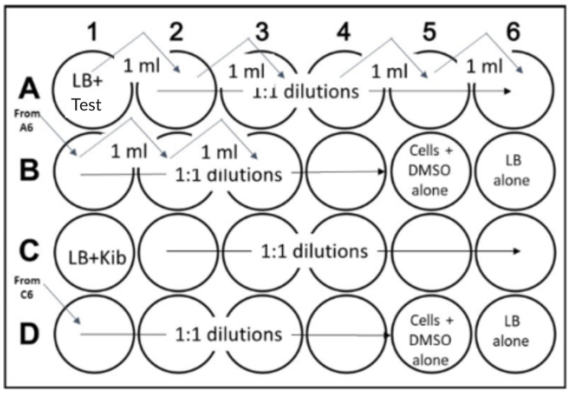


Figure 6. Plate map and setup of MIC measurement.

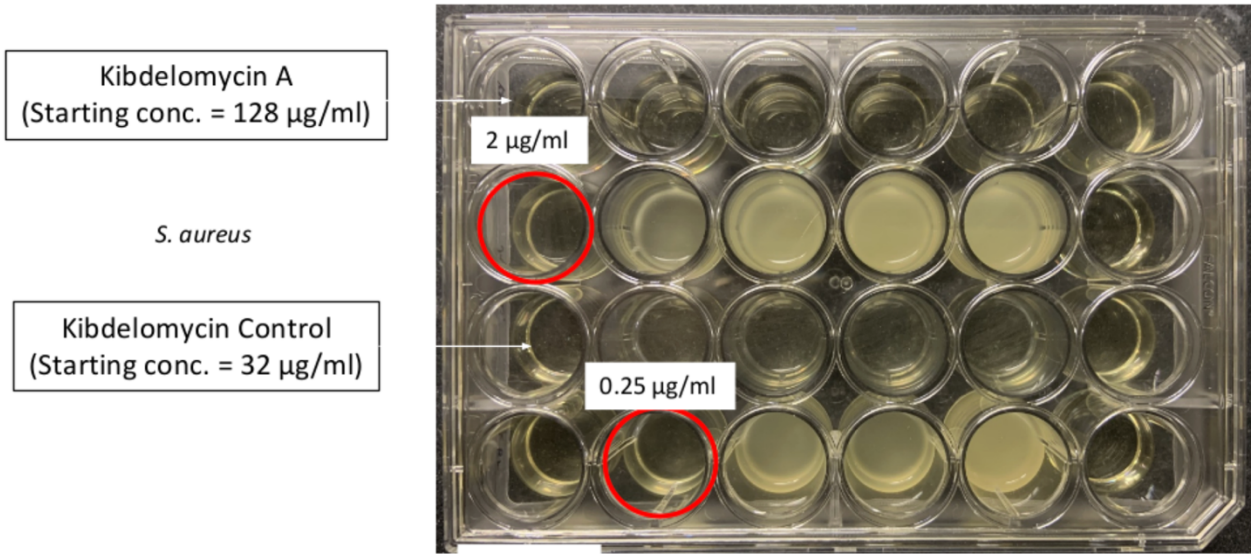


Figure 7. A plate after incubation and showing of MIC of kibdelomycin.

Preparation of test compounds for MIC testing

***S. aureus* test dilutions**:

Kibdelomycin was dissolved in DMSO to a starting concentration of 1 mg/mL. 64 µL of kibdelomycin was added to have a starting concentration of 32 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Kibdelomycin A was dissolved in DMSO to a starting concentration of 2 mg/mL. 128 µL of kibdelomycin A was added to have a starting concentration of 128 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Kibdelomycin A-1 and A-2 each were dissolved in DMSO to a starting concentration of 1 mg/mL. 64 µL of eachs was added to have a starting concentration of 32 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

***E. coli* test dilutions**:

Kibdelomycin was dissolved in DMSO to a starting concentration of 1 mg/mL. 128 µL of kibdelomycin was added to have a starting concentration of 64 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Kibdelomycin A was dissolved in DMSO to a starting concentration of 2 mg/mL. 128 µL of kibdelomycin A was added to have a starting concentration of 128 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Kibdelomycin A-1 and A-2 each were dissolved in DMSO to a starting concentration of 1 mg/mL. 128 µL of each was added to have a starting concentration of 64 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Chapter 4. Synthetic modification of kibdelomycin and determination of minimum structural requirement for antibacterial activity.

Goal: To synthesize derivatives by selective chemical modification, determine antibacterial activity, define structure activity relationship and understand their role in biosynthetic pathway

The structure of kibdelomycin consists of four distinct structural features A-D with many functional groups that bind to gyrase enzyme for potent activity. To address our goal, in this chapter we envisage to determine minimum structural requirement for antibacterial activity. To address, this we tested two groups of molecules. (1) Selective modification of kibdelomycin and (2) Acquisition and amidation of commercially available pyrrole-2-carboxylic acids.

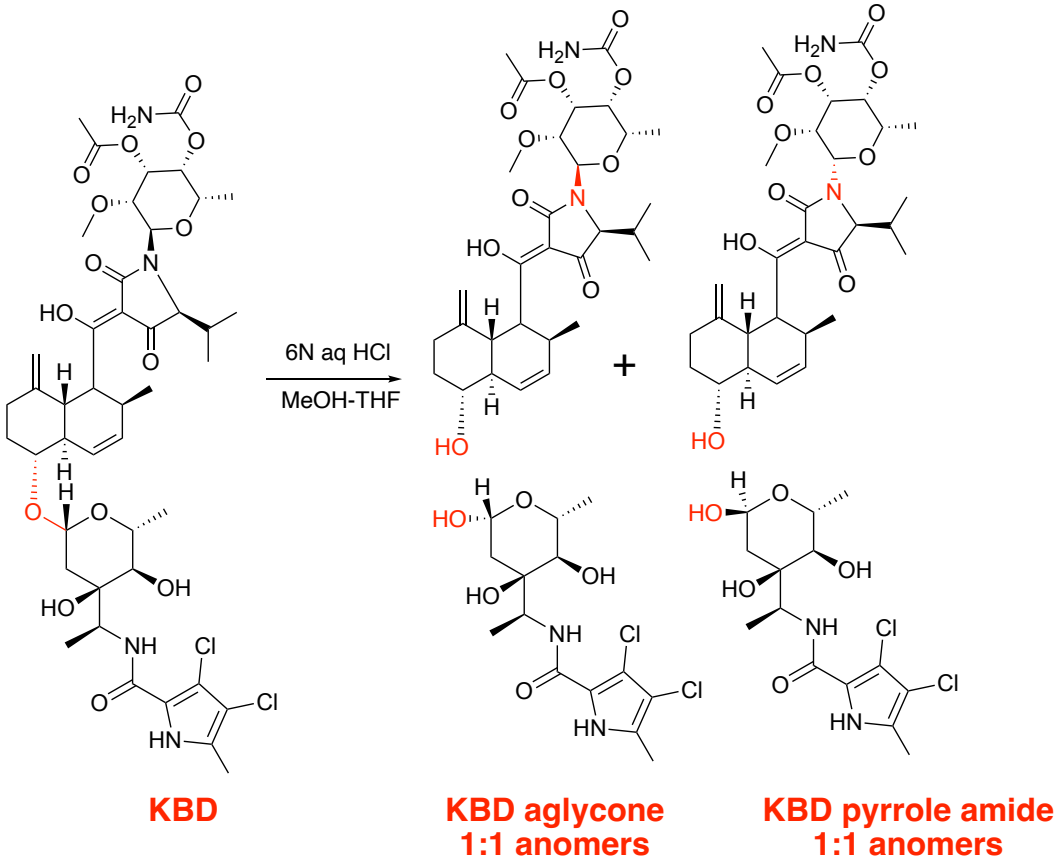
Selective modification of kibdelomycin.

Kibdelomycin is highly complex and fragile molecule. Any reaction must be done in very carefully. We performed three reactions highlighted in schemes 1-3.

Acidic hydrolysis of kibdelomycin.

Kibdelomycin was hydrolyzed with 4N aqueous hydrochloric acid in a 1:1 mixture of tetrahydrofuran and methanol at room temperature (Scheme 1). The hydrolysis selectively cleaved the glycosidic bond splitting the molecule in two halves with anomerization. The two halves were purified by reversed phase HPLC to afford two fractions consisting of anomeric mixtures of the upper half called here KBD aglycone and the anomeric mixtures of the lower half called KBD pyrrole amide. HPLC MS analysis indicated that the ratio of anomers in both samples was approximately 1:1. ESIMS analysis of both anomers eluting about 0.5 min apart showed a molecular ion at m/z 591 $(M+H)^+$ in positive ion mode and a complementary molecular ion at m/z 589 $(M-H)^-$ in the negative ion mode for the both anomers eluting about a consistent with the upper

half aglycone. Likewise, the fraction containing two anomeric mixtures of the lower half also eluting about 0.5 min apart showed a molecular ion at m/z 367 $(M+H)^+$ in positive ion mode and m/z 365 $(M-H)^-$ in negative ion mode consistent with the structure of the KBD pyrrole amide. The structures were confirmed by NMR analysis by Dr. Sheo Singh.

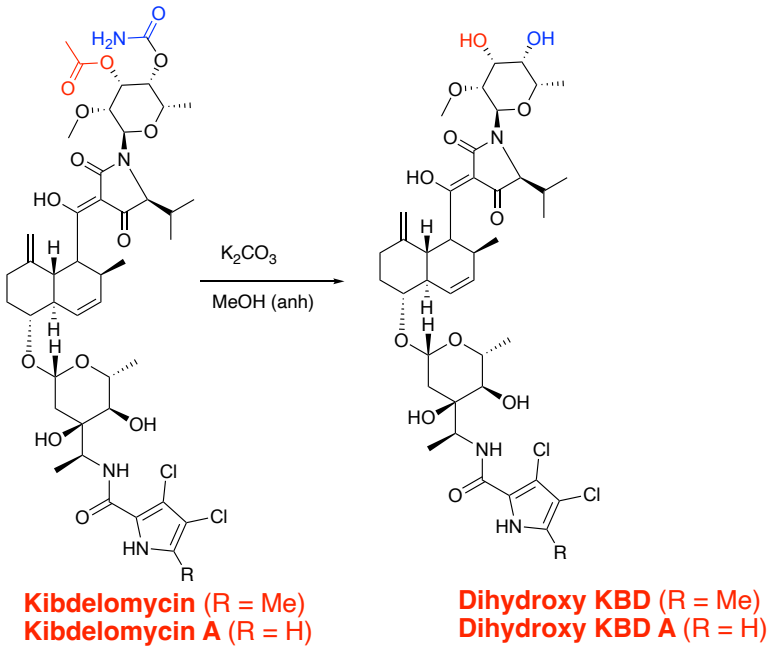


Scheme 1. Acidic hydrolysis of kibdelomycin.

Methanolysis of kibdelomycin and Kibdelomycin A

Treatment of a 4:1 mixture of kibdelomycin and kibdelomycin A with a saturated solution of potassium carbonate (K_2CO_3) in anhydrous methanol (MeOH) at 55°C for 60 minutes led to removal of the acetate and carbamate groups of the ring A, the top portion of the sugar residue (Scheme 2). The reaction cleanly produced a mixture of two dihydroxy derivatives of KBD and

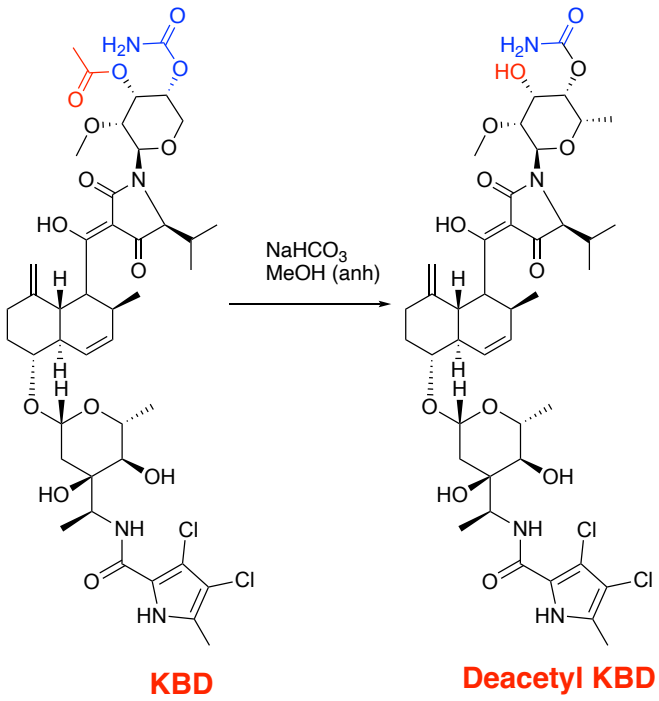
KBD A, which was purified by preparative HPLC. LCMS analysis of the dihydroxy KBD showed a molecular ion at m/z 854 $(M+H)^+$ and m/z 852 $(M-H)^-$ consistent with the loss of 85 amu accounting for loss of the acetyl and primary carbamate group. Similarly, the LCMS analysis of dihydroxy KBD A showed a molecular ion at m/z 840 $(M+H)^+$ and m/z 838 $(M-H)^-$ consistent with the loss of 85 amu accounting for loss of the acetyl and primary carbamate group. These structures were confirmed by NMR analysis by Dr. Sheo Singh.



Scheme 2. Methanolysis of kibdelomycin (KBD) and kibdelomycin A (KBD A).

Selective methanolysis of kibdelomycin

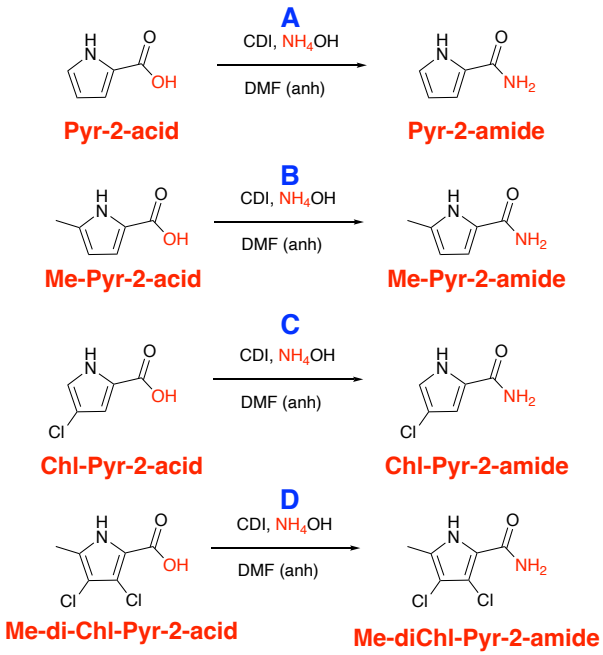
Kibdelomycin was treated with saturated solution of sodium bicarbonate in anhydrous methanol for 4 days at 45 ºC, then 60ºC for an additional hour. This led to selective methanolysis of the acetate group. Purification by preparative HPLC gave deacetylated KBD, which showed molecular ions in positive and negative ionization mode at m/z 897 (M+H)+ and m/z 895 $(M-H)^-$, respectively, consistent with the loss of the acetate group, which was confirmed by NMR analysis by Dr. Sheo Singh.



Scheme 3. Selective methanolysis of kibdelomycin.

Amidation of pyrrole-2-carboxylic acids

Four of the eight available pyrrole-2-carboxylic acid molecules relevant for kibdelomycin studies were acquired from commercial vendors. 5-Methyl-3,4-dichloro-pyrrole-2-carboxylic acid (Table 1, Scheme 4) and 4-chloro-pyrrole-2-carboxylic acid (Table 1, scheme 4) constitute pyrrole unit D in kibdelomycin and kibdelomycin A-1. Since they are present in kibdelomycin and its derivatives as amides, it was important to convert them into simple amides for structure activity studies, as the cell penetration properties of carboxylic acids are very different from the corresponding amides. Therefore, each compound was separately reacted with 1,1'-carbonyldiimidazole (CDI) in anhydrous dimethyl formamide (DMF) followed by addition of excess ammonium hydroxide leading to formation of respective primary amides listed in scheme 4 (A-D).[42] The amides eluted earlier than corresponding acids. The mass spectrum of each amide was one atomic mass unit (amu) less than corresponding acids.



Scheme 4. Amidation of pyrrole 2-carboxylic acids.

Table 1. ESI MS results of pyrrole acids amides.

Full name of compounds	Abbreviated name (Scheme 4)	Ion MS Spectra Title	Positive ion m/z $(M+H)^+$	Negative ion m/z $(M-H)^-$
Pyrrole-2-carboxylic acid	Pyr-2-acid	JRF-1-81-5	111	109
Pyrrole-2-carboxamide	Pyr-2-amide	JRF-1-81-1	110	108
5-Methyl pyrrole-2-carboxylic acid	Me-pyr-2-acid	JRF-1-81-6	126	124
5-Methyl pyrrole-2-carboxamide	Me-pyr-2-amide	JRF-1-81-2	125	123
4-Chloro-pyrrole-2-carboxylic acid	Chl-Pyr-2-acid	JRF-1-81-7	146	144
4-Chloro-pyrrole-2-carboxamide	Chl-Pyr-2-amide	JRF-1-81-3	145	143
5-Methyl-3,4-dichloro-pyrrole-2-carboxylic acid	Me-dichl-pyr-2-acid	JRF-1-81-8	194	192
5-Methyl-3,4-dichloro-pyrrole-2-carboxamide	Me-dichl-pyr-2-amide	JRF-1-81-4	193	191

Antibacterial activity

All the compounds were tested in minimum inhibitory concentration assay and were compared with kibdelomycin or kibdelomycin A. Kibdelomycin and kibdelomycin A was used control for each experiment. Dihydroxy KBD and deacetyl KBD each exhibited MIC of 1 μg/mL, a fourfold loss of activity compared to parent KBD whereas Dihydroxy KBD A retained the MIC values of its parent KBD-A. The KBD aglycone and KBD pyrrole amide did not show any activity at 64 and 32 μg/mL, the highest concentration tested (Table 2). Likewise, the pyrrole-2-carboxylic acids and corresponding amides show no activity at 250 μg/mL, except for the 3,4-dichloro-5-methyl-pyrrole-caboxamide which showed MIC of 256 μg/mL, also practically inactive. These results suggest all four rings of the molecule is critical for the activity with some tolerance on the substitutions in the pyrrole ring (e.g., A-1 and A-2, chapter 3) in the lower arm (southern hemisphere) and the sugar ring of the upper arm (northern hemisphere). Though none of the substitutions improve activity.

Table 2. Minimum inhibitory of concentration (MIC) of kibdelomycin, pyrrole derivatives, and synthetic derivatives against *S. aureus*.

Compounds	*S. aureus* (μg/mL)
Kibdelomycin	0.25
Kibdelomycin A	2
Pyrrole-2-carboxylic acid	>256
Pyrrole-2-carboxamide	>256
5-Methyl pyrrole-2-carboxylic acid	>256
5-Methyl pyrrole-2-carboxamide	>256
4-Chloro-pyrrole-2-carboxylic acid	>256
4-Chloro-pyrrole-2-carboxamide	>256
5-Methyl-3,4-dichloro-pyrrole-2-carboxylic acid	256
5-Methyl-3,4-dichloro-pyrrole-2-carboxamide	>256
KBD aglycone	>64
KBD pyrrole amide	>32
Dihydroxy KBD	1
Dihydroxy KBD A	2
Deacetyl KBD	1

In conclusion, I selectively modified kibdelomycin and kibdelomycin A and produced five compounds as well as prepared four amide derivatives, two of them new. I tested these compounds for their MIC activity against *S. aureus*. From X-ray crystal structure analysis, it is known that all four structural units A-D of kibdelomycin required for the binding to gyrase enzyme in a U-shape

binding conformation and likely needed for potent activity. The lack of the MIC activity of the acid hydrolyzed products and pyrrole-2-carboxylic acids, and amides confirmed the importance of four connected rings and groups around them. The removal of the acetate and that carbamate retained most of the activity as they appear to be exposed to solvent and don't show any binding to gyrase. This observation set stage to further modification of kibdelomycin for potential improvement of activity and physical property. The conclusion derived from the compounds prepared in this chapter strongly help fulfilment of the goal set out in this chapter.

Experimental.

For general experimental procedure: See chapter 2.

Acidic hydrolysis of kibdelomycin

Kibdelomycin (17.8 mg) was added to 2 ml of tetrahydrofuran (THF), stirred at room temperature for 10 minute followed by addition of 4M HCl (4 mL) and stirring at room temperature for 45 minutes. Precipitation was observed which was dissolved by addition of 1 ml methanol and continued stirring for 1h. Ethyl acetate 20 mL was added and washed with water and brine. The solution was concentrated to dryness, then dissolved in 3 ml of methanol and 1 ml of H_2O to prepare for 2 injections into the prep-HPLC using a C_8 column (21.2 X 250 mm) and eluted with a 60 minute 5-20-90% gradient of acetonitrile-water at a flow rate of 10 mL/minute. After LCMS analysis, fractions 28-31 were combined and concentrated to provide 3.1 mg of kibdelomycin pyrrole amide, and fractions 35-36 were combined and concentrated to provide 1.1 mg of kibdelomycin aglycone.

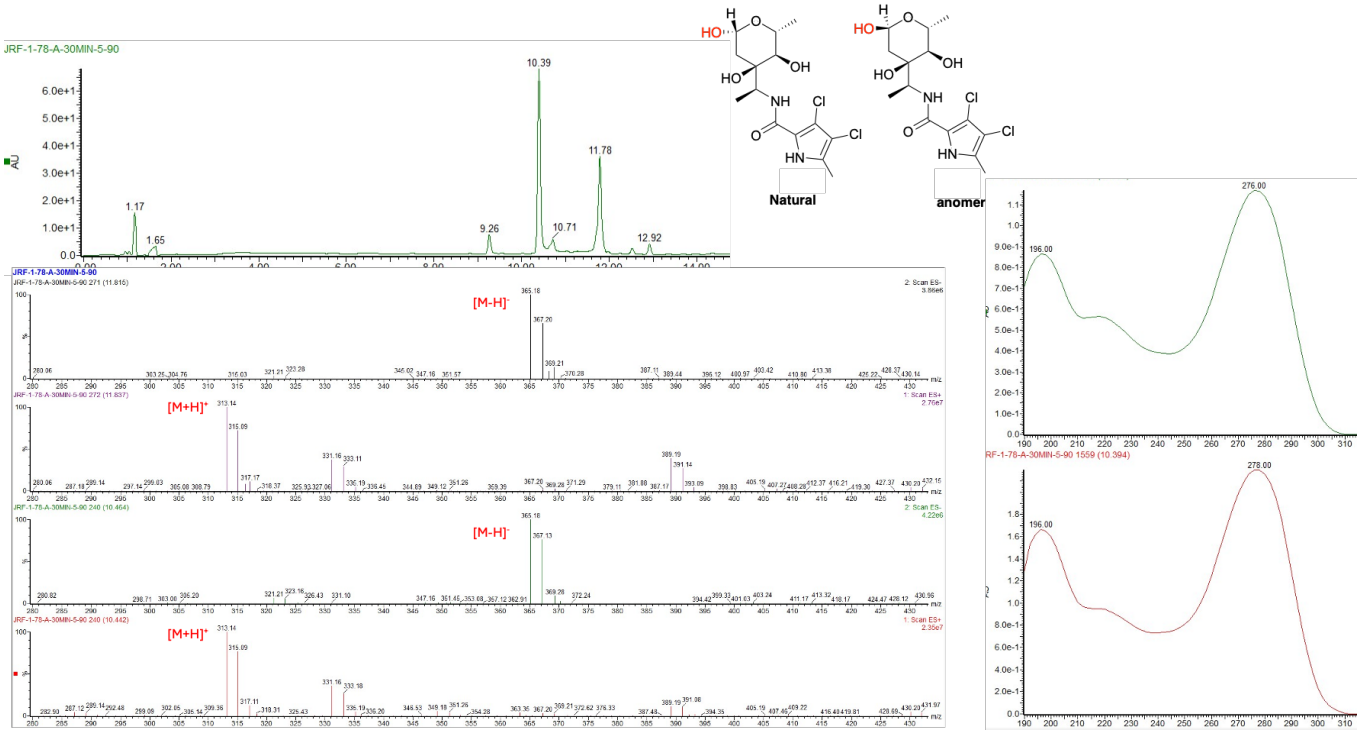


Figure 1: Chromatogram, Mass Spectroscopy, and UV of kibdelomycin pyrrole amide.

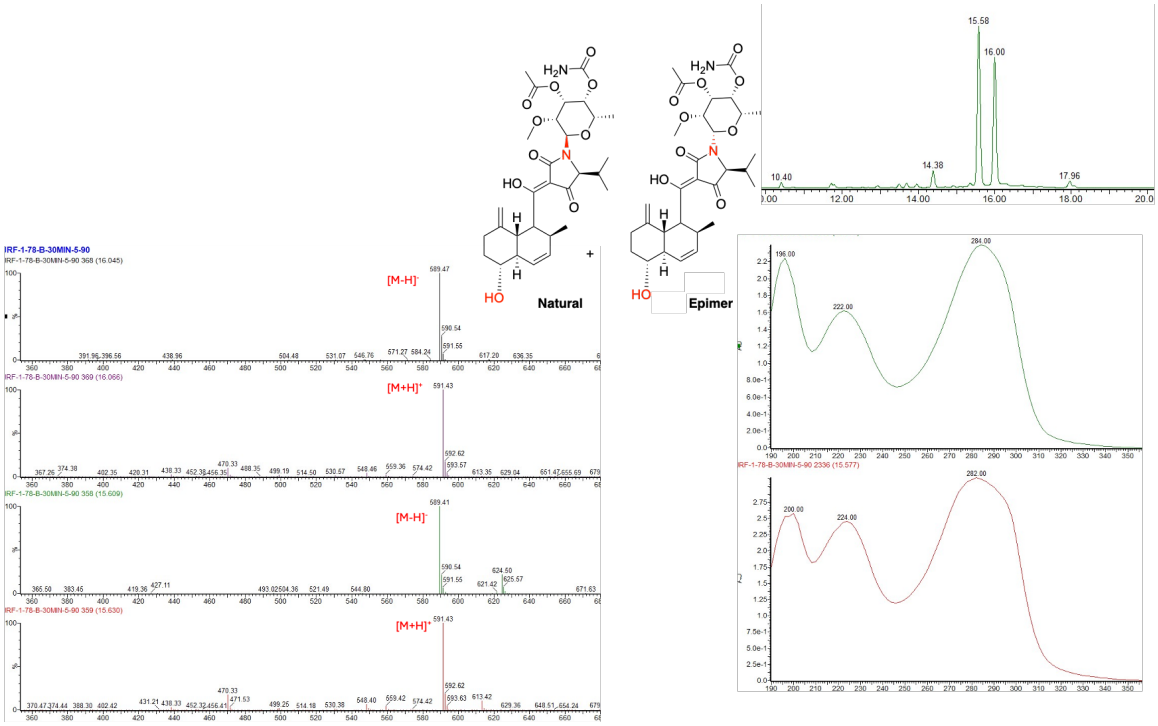


Figure 2: Chromatogram, Mass Spectroscopy, and UV of kibdelomycin aglycone.

Methanolysis of kibdelomycin and Kibdelomycin A

To a solution of a 4:1 mixture of kibdelomycin and kibdelomycin A (38.9 mg) anhydrous methanol (2 mL) in a round bottom flask was added K_2CO_3 (219 mg). The mixture was stirred at 55 ^oC for 60 minutes, the reaction was removed from heat and worked up using ethyl acetate and an H_2O wash. The reaction mixture was dissolved in 8 mL of methanol and 2 mL of sodium phosphate buffer (pH 7.25) before being injected into four separate runs into the preparative high performance liquid chromatography (HPLC) C_{18} column (20 X 150 mm) and eluted with a 60 minute 50-90% gradient of MeOH and 0.25 mM sodium phosphate buffer (pH 7.25) at a flow rate of 10 mL/minute. Dihydroxy kibdelomycin A eluted first from each run, resulting in the collection of fractions 19-20 and dihydroxy kibdelomycin eluted in fractions 37-39. Each pooled fractions were concentrated under reduced pressure and then desalted on one mL XAD-16. These from run 1 was combined and concentrated one mL aqueous. Each fractions were separately desalted on one mL XAD-16 column by directly charging and washing with 10 mL water, 10 mL 10% aqueous methanol and eluting with 100% methanol. Methanol was removed from the fractions of the two XAD-16 columns to give of dihydroxy kibdelomycin A (1.2 mg) and dihydroxy kibdelomycin (12.2 mg) as powders.

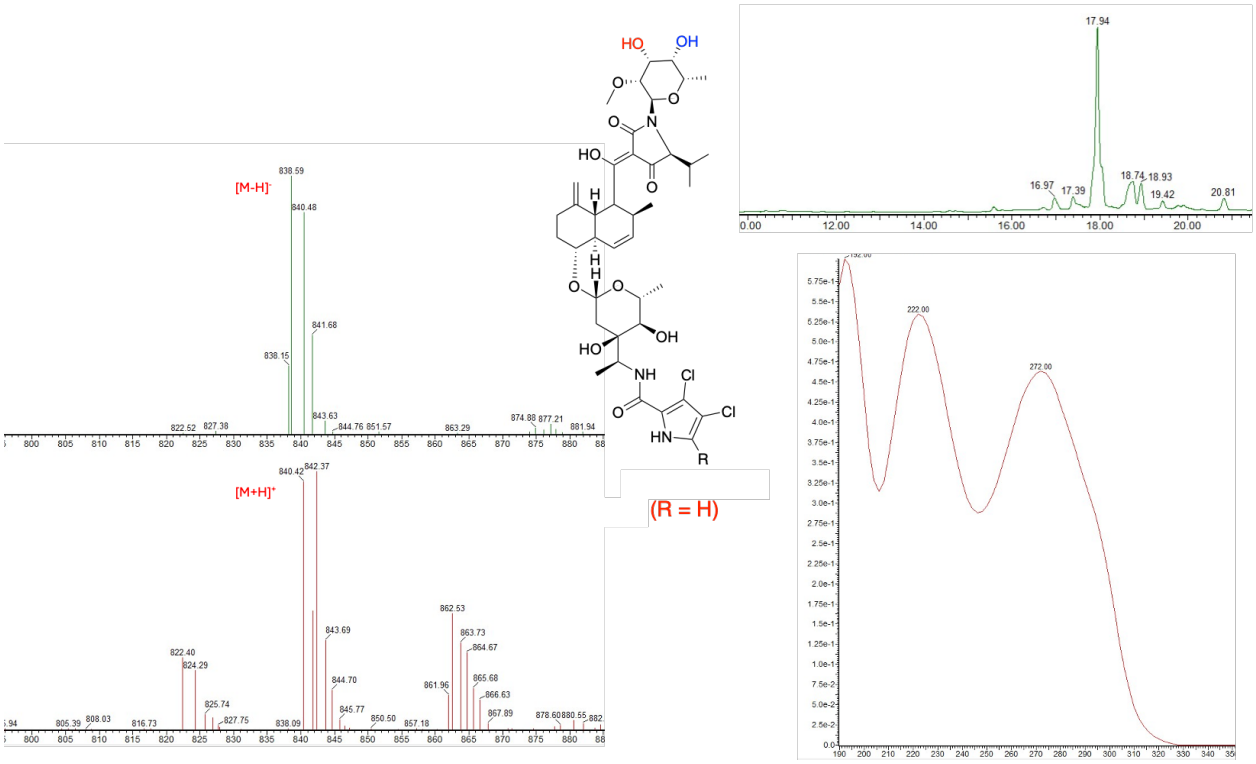


Figure 3: Chromatogram, Mass Spectroscopy, and UV of dihydroxy kibdelomycin A.

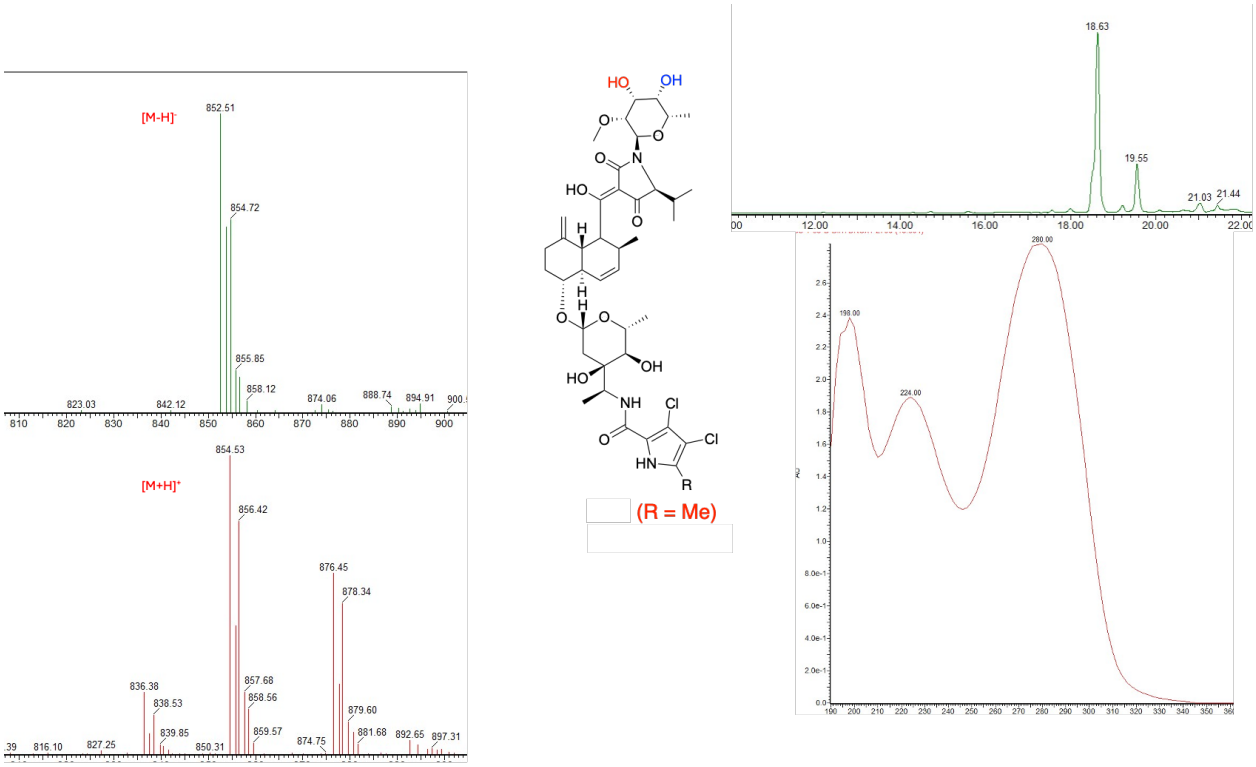


Figure 4: Chromatogram, Mass Spectroscopy, and UV of dihydroxy kibdelomycin.

Selective methanolysis of kibdelomycin

Kibdelomycin (20 mg) in a round bottom was dissolved in 10 ml of anhydrous methanol and 200 mg of $NaHCO_3$ was added under nitrogen. The mixture was heated at 45 ºC for 4 days followed by at 60 ºC for an hour. Ethyl acetate was added to the mixture and washed with brine and dried over $MgSO_4$. Ethyl acetate extract was concentrated to dryness and the dissolved in 4.5 ml of methanol and 1.5 ml of 0.25 mM sodium phosphate buffer (pH 7.25). This mixture was chromatographed in three separate runs using the prep HPLC on a C_{18} column (20 X 150 mm) and eluted with a 60 minute 30-90% gradient of MeOH and 0.25 mM sodium phosphate buffer (pH 7.25) at a flow rate of 10 mL/minute. Fractions 18-22 were pooled and concentrated to aqueous and then desalted by slowly charging on ~1 mL XAD-16. After the charge, the XAD-16 column was washed with about 10 mL water, 10% aqueous methanol, and 100% methanol. Deacetyl kibdelomycin eluted in 100% methanol which was concentrated to dryness give 6 mg powder. This compound appeared to show instability (see chromatogram).

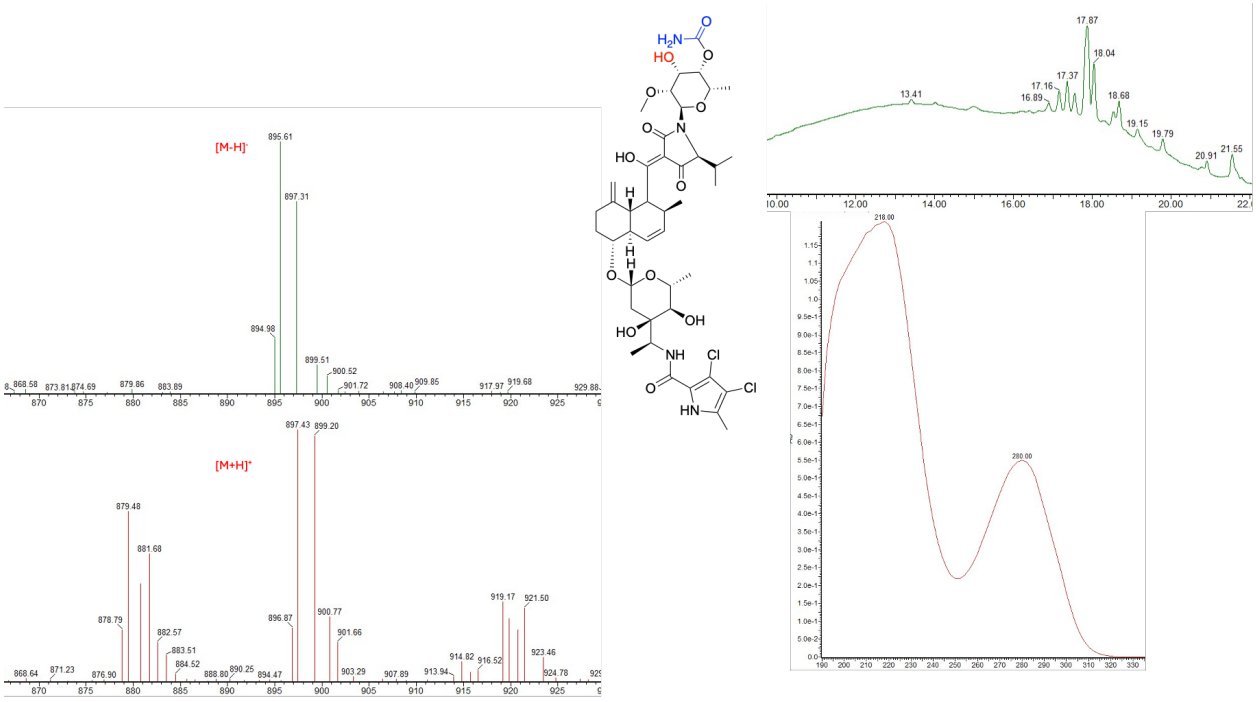


Figure 5: Chromatogram, Mass Spectroscopy, and UV of deacetyl kibdelomycin.

Amidation of pyrrole carboxylic acids.

Pyrrole-2-carboxyamide

Pyrrole-2-carboxylic acid (50 mg) was dissolved of anhydrous Dimethylformamide (3 mL). The reaction was cooled to 0 ^{o}C before adding 2 equivalents of 1,1′-Carbonyldiimidazole (CDI). The reaction mixture was stirred for 1 hour, then 1 ml of ammonium hydroxide (NH_4OH) was added and stirred at room temperature overnight. The reaction was terminated addition of H_2O, followed by an extraction with ethyl acetate, washed with water and dried over sodium sulfate and filtered. The filtrate was concentrated to dryness and dissolved in 0.5 mL DMF and 1 mL water, injected directly to preparative-HPLC C_8 column (21.2 X 250 mm) and eluted with a 60 minute 5-10-90% gradient of acetonitrile-water containing 0.1% TFA at a flow rate of 10 mL/minute. After analysis using LCMS, fractions 3 through 20 were combined and concentrated to dryness, resulting in 45.5 mg of material as a powder.

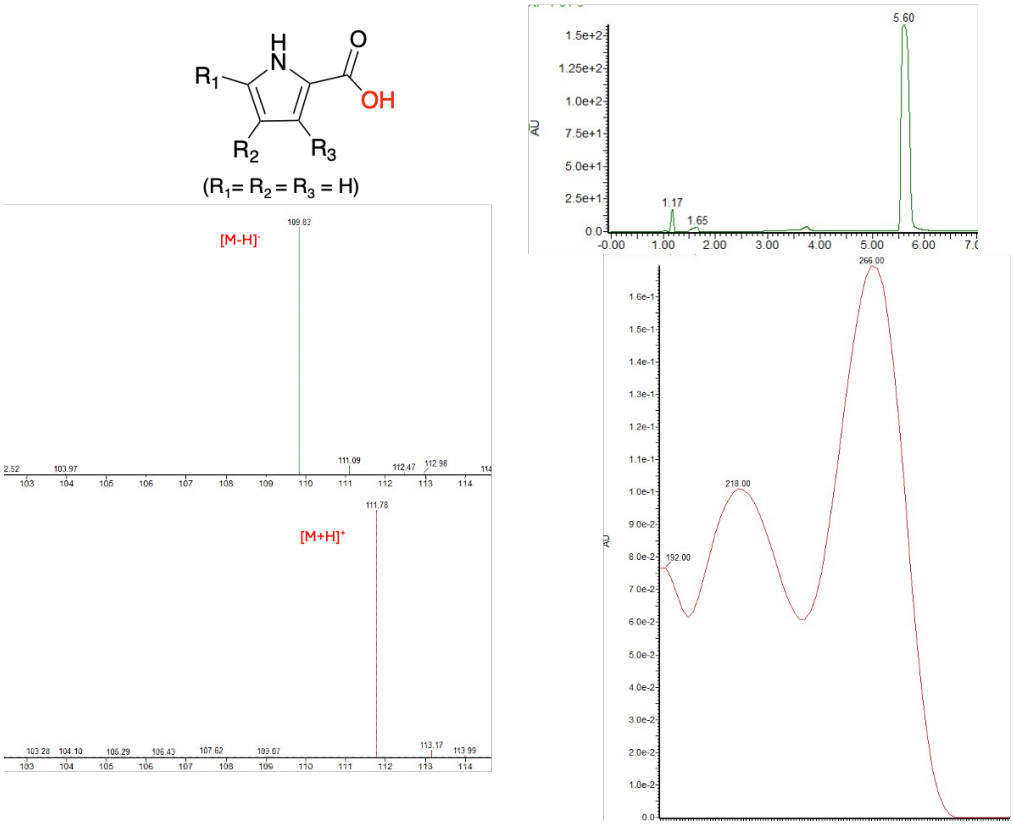


Figure 6: Chromatogram, Mass Spectroscopy, and UV of Pyrrole-2-carboxylic acid.

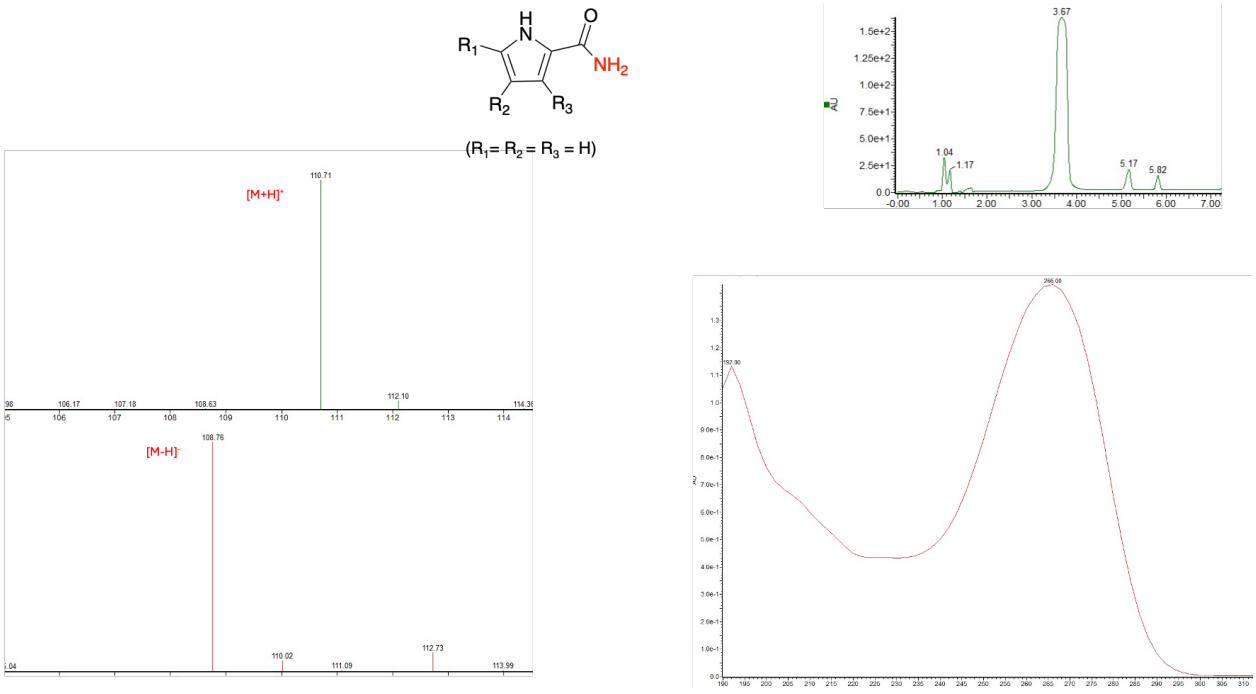


Figure 7: Chromatogram, Mass Spectroscopy, and UV of Pyrrole-2-carboxamide.

LCMS retention times decreased from carboxylic acid to carboxamide, with Pyrrole-2-carboxylic acid elucidating at 5.7 minutes and Pyrrole-2-carboxamide elucidating at 3.7 minutes. Each structure was further characterized by ^{1}H NMR and ^{13}C NMR analysis.

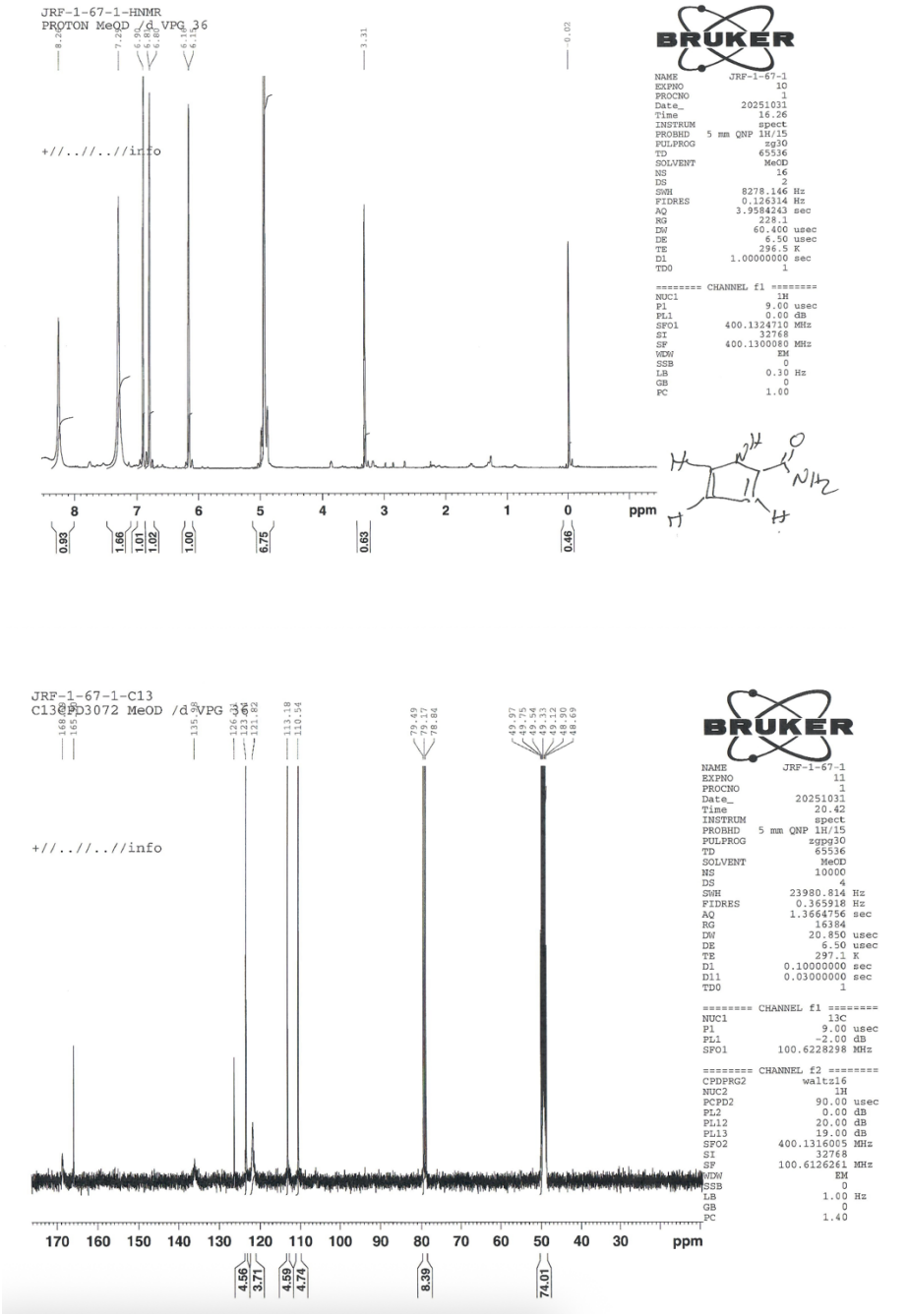


Figure 8: NMR Spectra of Pyrrole-2-carboxamide.

5-methyl-1H-Pyrrole-2-carboxamide, 4-chloro-1H-Pyrrole-2-carboxamide, and 3,4-dichloro-5-methyl-1H-Pyrrole-2-carboxamide

For the synthesis of these compounds, each corresponding carboxylic acid (20 mg) was dissolved in 0.5 ml of anhydrous DMF then cooled to 0 ^{o}C. Carbonyl diimidazole (CDI, 2 eq) was added after cooling, then stirred for 1 hour. NH_4OH (0.5 ml) were added to the reaction mixture then stirred at room temperature overnight. LCMS analysis showed exclusive product formation. After overnight stirring, 0.5 ml of H_2O was added to terminate the reaction, then stirred for 10 minutes. This reaction mixture was then directly injected into the preparative HPLC using a C_8 column (21.2 X 250 mm) and eluted with a 60 minute 5-20-90% gradient of acetonitrile-water containing 0.1% TFA, at a flow rate of 10 mL/minute. Fractions 6 through 24 were analyzed via LCMS, fractions containing product were pooled, concentrated under vacuum and transfer in a vial using methanol, methanol was removed by blowing nitrogen to give 5-methyl-1H-Pyrrole-2-carboxamide (18 mg, 90.7%), similar pooling and concentration of fractions 12-25 from 4-chloro acid gave 4-chloro-1H-Pyrrole-2-carboxyamide (18 mg, 90.6%) , and fractions 27-40 gave 3,4-dichloro-5-methyl-1H-Pyrrole-2-carboxyamide (18.0 mg, 91.%), each as a powder.

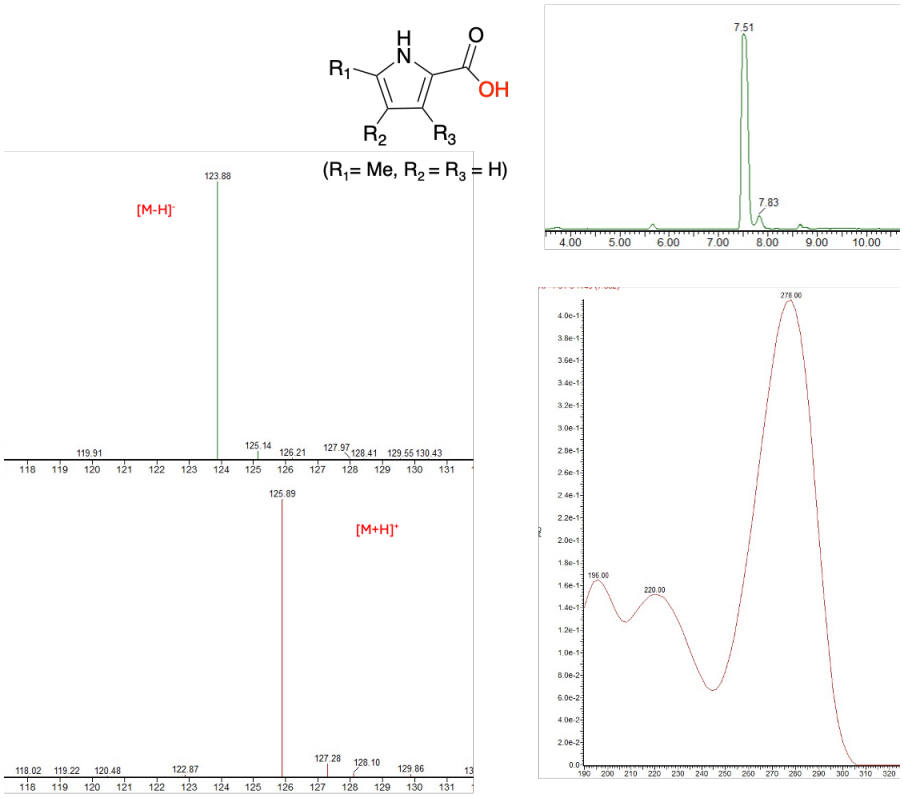


Figure 9: Chromatogram, Mass Spectroscopy, and UV of 5-methyl-1H-Pyrrole-2-carboxlic acid.

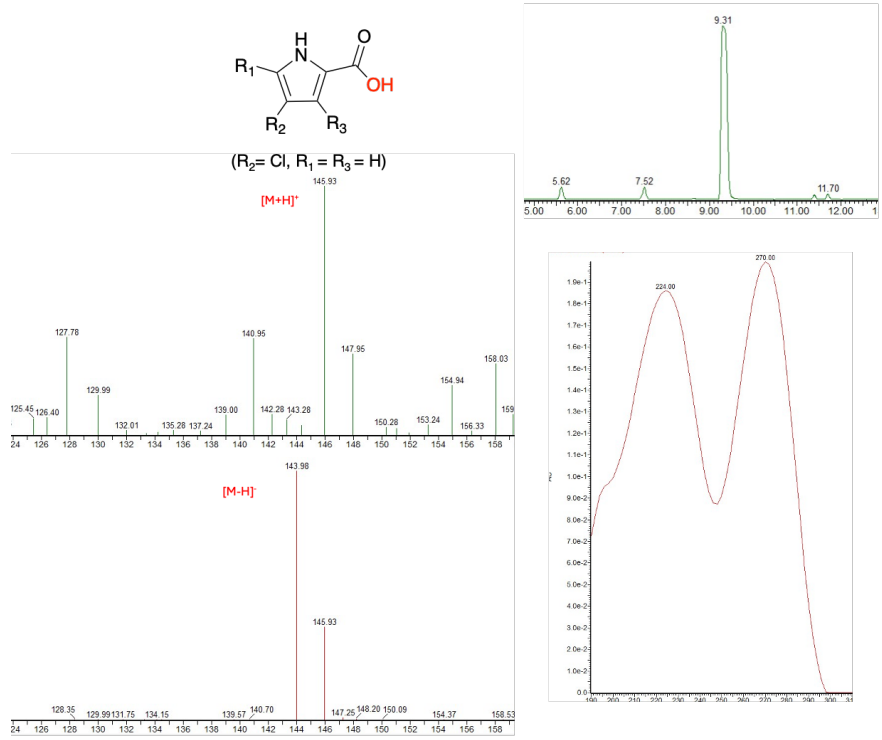


Figure 10: Chromatogram, Mass Spectroscopy, and UV of 4-chloro-1H-Pyrrole-2-carboxylic acid.

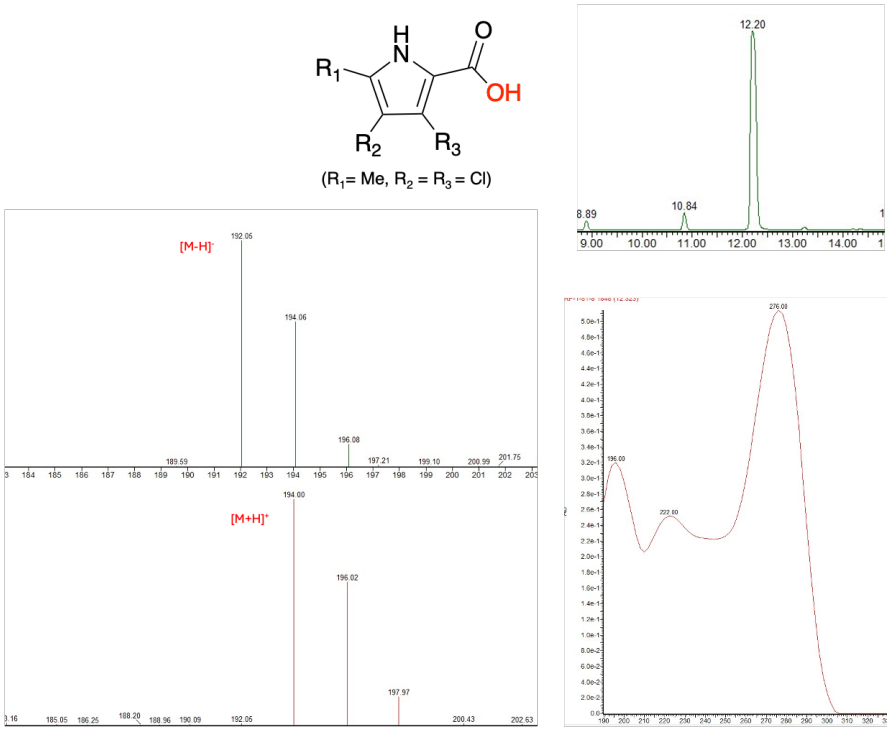


Figure 11: Chromatogram, Mass Spectroscopy, and UV of 3,4-dichloro-5-methyl-1H-Pyrrole-2-carboxylic acid.

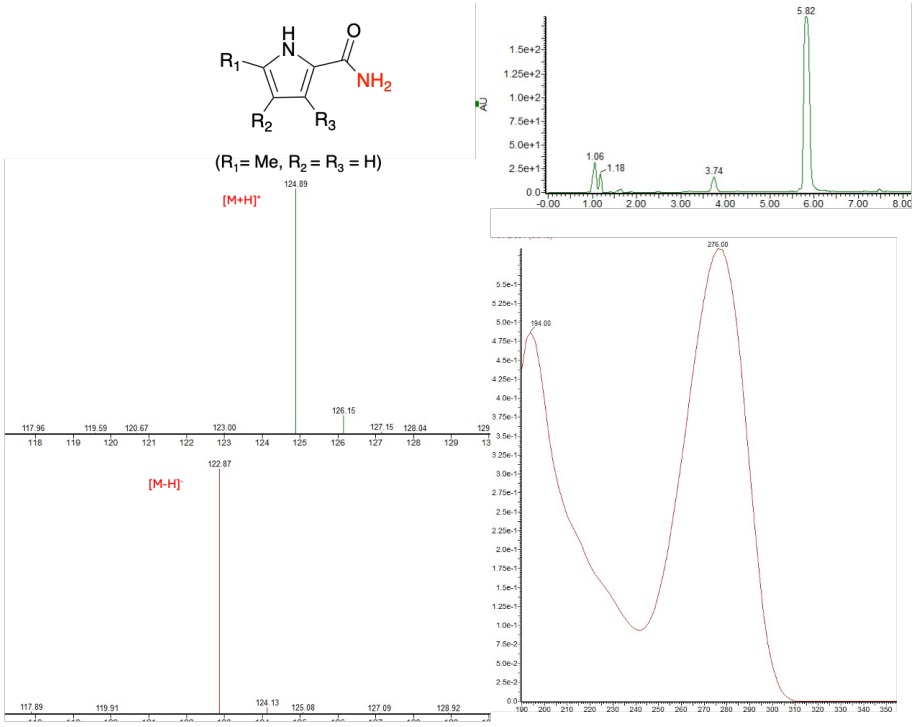


Figure 12: Chromatogram, Mass Spectroscopy, and UV of 5-methyl-1H-Pyrrole-2-carboxamide.

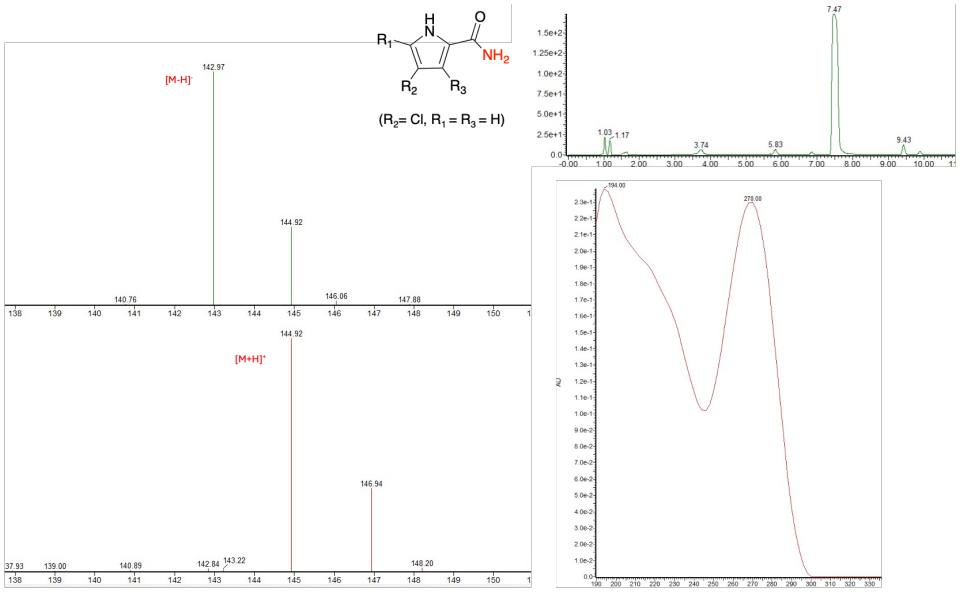


Figure 13: Chromatogram, Mass Spectroscopy, and UV of 4-chloro-1H-Pyrrole-2-carboxamide.

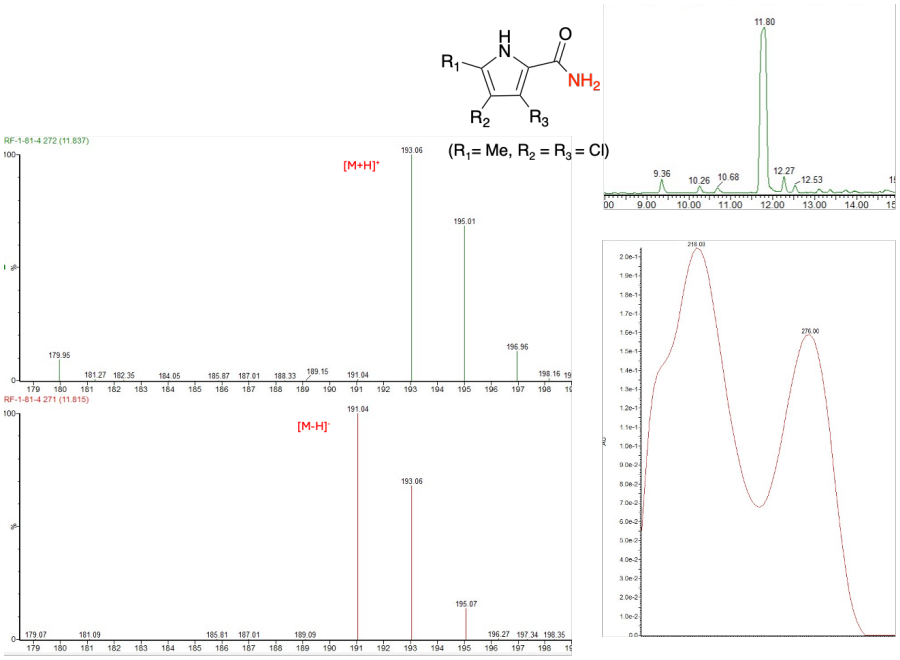


Figure 14: Chromatogram, Mass Spectroscopy, and UV of 3,4-dichloro-5-methyl-1H-Pyrrole-2-carboxamide.

LCMS characterization of the initial carboxylic acids elucidated later than carboxamides. 5-methyl-1H-Pyrrole-2-carboxamide elucidated at 5.9 minutes compared to starting material elucidating at 7.6 minutes. 4-chloro-1H-Pyrrole-2-carboxyamide elucidated at 7.5 minutes compared to starting material elucidating at 9.4 minutes. 3,4-dichloro-5-methyl-1H-Pyrrole-2-carboxyamide elucidated at 11.9 minutes compared to starting material elucidating at 12.3 minutes. Each structure was further characterized by ^{1}H NMR and C^{13} NMR.

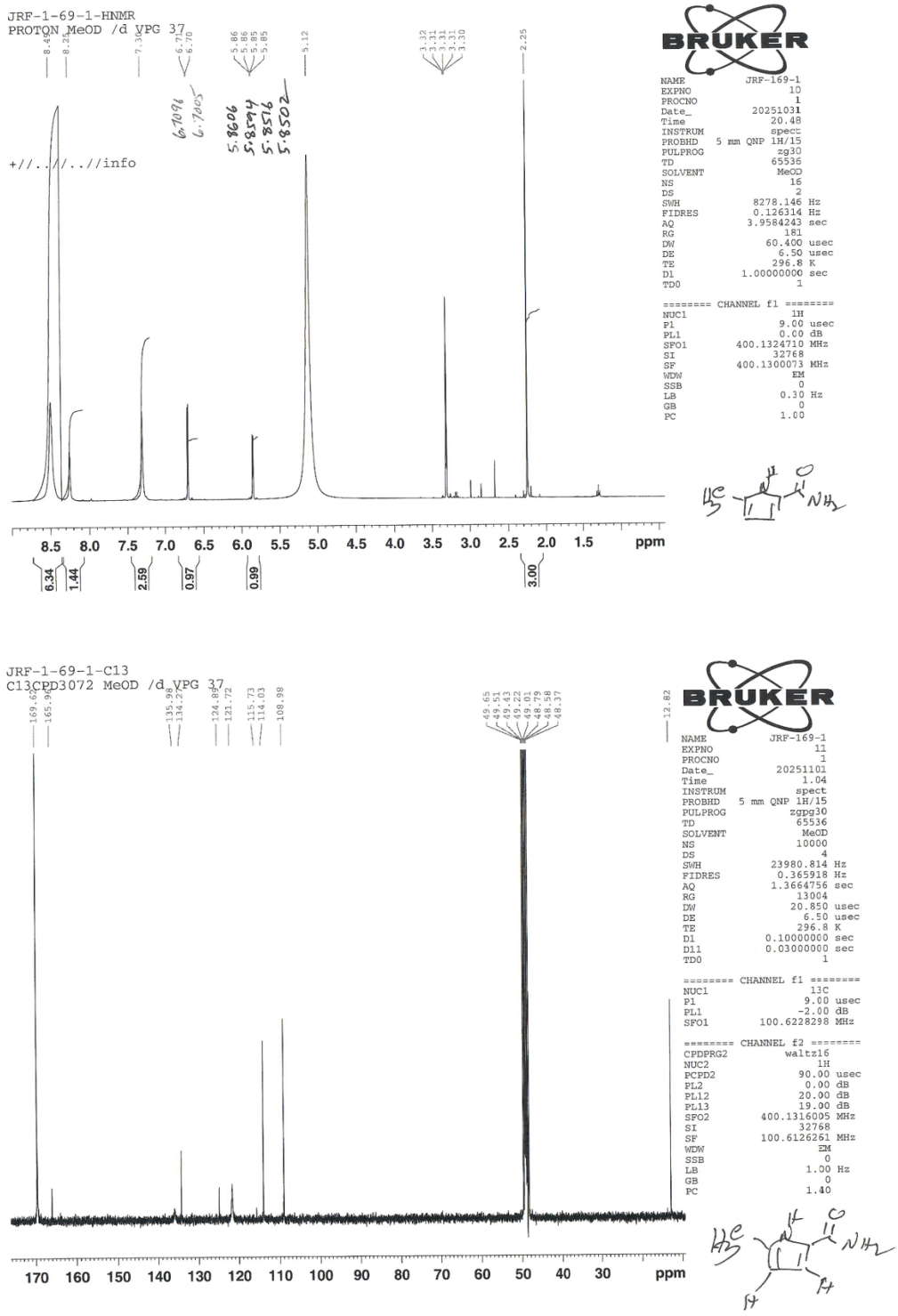


Figure 15: NMR spectra of 5-methyl-1H-Pyrrole-2-carboxamide.

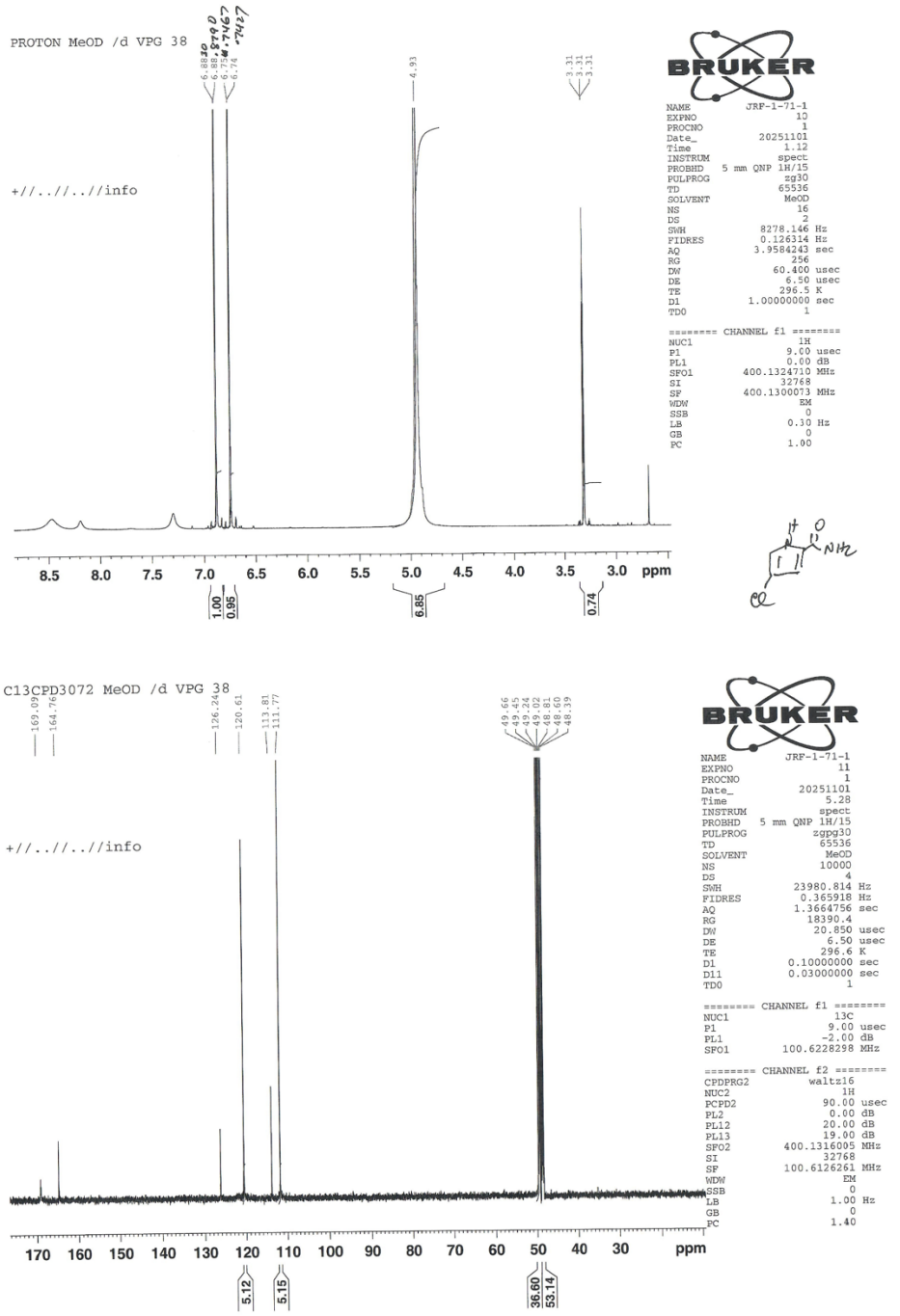


Figure 16: NMR spectra of 4-chloro-1H-Pyrrole-2-carboxamide.

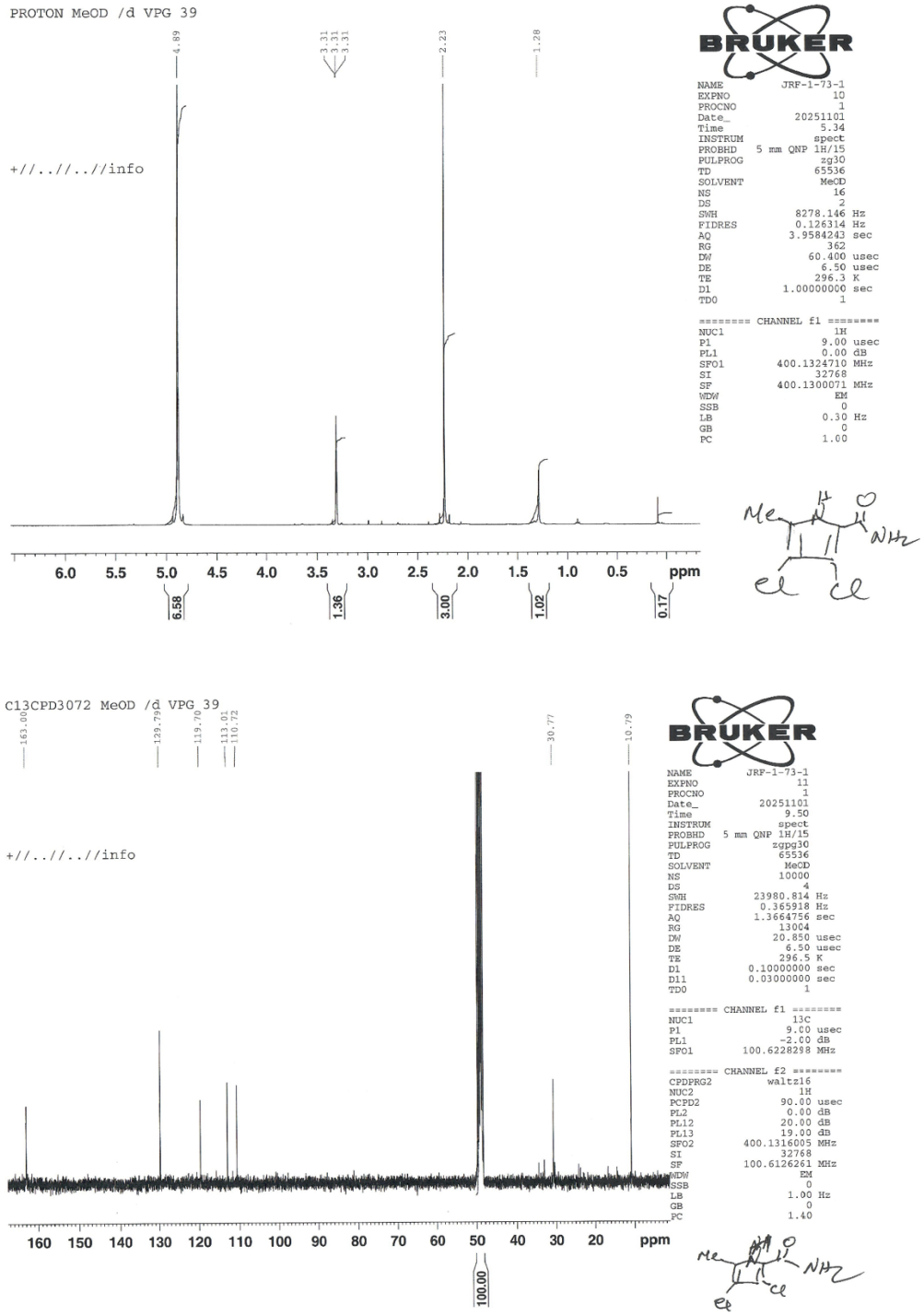


Figure 17: NMR Spectra of 3,4-dichloro-5-methyl-1H-Pyrrole-2-carboxamide.

MIC Assay

For MIC assay procedure, see chapter 3.

Preparation of test compounds for MIC testing

***S. aureus* test dilutions**:

Kibdelomycin was dissolved in DMSO to a starting concentration of 1 mg/mL. 64 µL of kibdelomycin was added to have a starting concentration of 32 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Kibdelomycin A was dissolved in DMSO to a starting concentration of 2 mg/mL. 128 µL of kibdelomycin A was added to have a starting concentration of 128 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

1H-Pyrrole-2-carboxyamide**,** 5-methyl-1H-Pyrrole-2-carboxamide, 4-chloro-1H-Pyrrole-2-carboxyamide, 3,4-dichloro-5-methyl-1H-Pyrrole-2-carboxyamide, 1H-Pyrrole-2-carboxylic acid, 5-methyl-1H-Pyrrole-2-carboxylic acid, 4-chloro-1H-Pyrrole-2-carboxylic acid, 3,4-dichloro-5-methyl-1H-Pyrrole-2-carboxylic acid were all dissolved in DMSO to a starting concentration of 50 mg/mL. 10 µL of each compound was added to have a starting concentration of 256 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Kibdelomycin aglycone was dissolved in DMSO to a starting concentration of 1 mg/mL. 128 µL of kibdelomycin aglycone was added to have a starting concentration of 64 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Kibdelomycin pyrrole amide was dissolved in DMSO to a starting concentration of 1 mg/mL. 128 µL of kibdelomycin pyrrole amide was added to have a starting concentration of 64 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Dihydroxy kibdelomycin, dihydroxy kibdelomycin A and deacetyl kibdelomycin were each dissolved in DMSO to a starting concentration of 1 mg/mL. 128 µL of dihydroxy kibdelomycin was added to have a starting concentration of 64 µg/mL. The compound was diluted 9 times in the proceeding wells containing 1 mL of LB excluding the first well. One mL was removed from the last well in the dilution series and discarded to have a final volume of 1 mL.

Final Thesis Conclusion

The work described in this thesis covers a wide range of topics within the broad field of natural products research and the discipline of chemical biology focusing on very important new antibiotic, kibdelomycin. The overall goal of the kibdelomycin program is to discover an analog with potency, antibiotic spectrum and physical property improvement that can be advanced for clinical development. This thesis work delves into the early discovery aspect of the overall goal. The studies include biosynthesis, new compound discovery, and synthetic modification and antibacterial evaluations. Biosynthetic studies confirmed direct incorporation of pyruvate, valine and proline providing insight for potential substitution of these precursors with other amino acids, modified amino acids and substituted pyruvates for potential production of new tailored natural product congeners by the cells. To make new congeners in the cells, we applied a new repeat batch fermentation to enhance production of trace metabolites that can be isolated. This method can be further extended to discover additional compounds in the kibdelomycin fermentation or applied to other fermentations products. This method in combination with precursor directed biosynthesis of new compounds could prove beneficial in the discovery of a wide range of congeners. Finally, the semi-synthesis work of this thesis helped us to learn the minimal structural requirement for the activity of kibdelomycin class and confirmed that the entire molecular assembly was needed for activity as was predicted static X-ray structure. Collectively these studies allow us to explore new natural product discoveries and at the same time, help us to narrow our focus on critical areas of the molecule for SAR studies to accelerate efforts to accomplish overall goal for discovery of new antibiotics from kibdelomycin class with a new mode of action with low resistance potential.

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