



# Discovery and Synthesis of Novobiocin Derivatives That Stimulate the Lipopolysaccharide Transporter and Synergize With Polymyxins

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Discovery and synthesis of novobiocin derivatives that stimulate the  
lipopolysaccharide transporter and synergize with polymyxins

A dissertation presented

by

Michael Mandler

to

The Department of Chemistry and Chemical Biology

in partial fulfillment of the requirements  
for the degree of  
Doctor of Philosophy  
in the subject of  
Chemistry

Harvard University  
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**Discovery and synthesis of novobiocin derivatives that stimulate the lipopolysaccharide transporter and synergize with polymyxins****Abstract**

Gram-negative bacterial infections are a worldwide problem and antibiotics capable of clearing such infections are an unmet clinical need. The polymyxins are highly potent drugs against Gram-negative bacteria but suffer from dose-limiting toxicity. Although the exact mechanism for polymyxin activity is unclear, it is known that polymyxin binds lipopolysaccharide. Lipopolysaccharide is a complex glycolipid that is synthesized inside the cell and transported through three cellular compartments to the cell surface by the seven essential lipopolysaccharide transport (Lpt) proteins. We discovered that novobiocin, a gyrase inhibitor, also binds the inner membrane Lpt complex LptB<sub>2</sub>FGC and stimulates lipopolysaccharide transport. The central finding of this dissertation is that stimulation of lipopolysaccharide transport by novobiocin derivatives sensitize bacteria to the polymyxins. The thesis presents preliminary evidence that polymyxin therapy can be safer when used in combination with a novobiocin derivative.

We demonstrated that novobiocin derivatives that bind LptB<sub>2</sub>FGC and stimulate lipopolysaccharide transport can synergize with polymyxin B independent of gyrase inhibition. In Chapter 2, a semi-synthetic route was used to modify the novobiocin scaffold at the sugar and benzamide positions. An NMR binding assay was combined with a previously published cellular assay for measuring lipopolysaccharide transport to assess the ability of novobiocin derivatives to maintain their target engagement on LptB<sub>2</sub>FGC. In Chapter 3, we showed that novobiocin derivatives that maintained their ability to stimulate lipopolysaccharide transport synergized with polymyxin. By adding these two drugs together in a thigh infection model, we demonstrated that combination therapy is a therapeutically viable strategy for making polymyxin efficacious at a lower dose and therefore safer. Previously published work suggests that active lipopolysaccharide

transport is important in the mechanism of action of the polymyxin antibiotics and ongoing work suggests that polymyxin acts at the inner membrane. In Chapter 4, we tethered novobiocin and polymyxin to leverage the co-localization of these molecules and their targets in the inner membrane, and we describe an efficient strategy to synthesize chimeric molecules in a modular fashion. This dissertation validates a novel therapeutic modality and describes the development of new chemical matter to treat Gram-negative infections by targeting active lipopolysaccharide transport.

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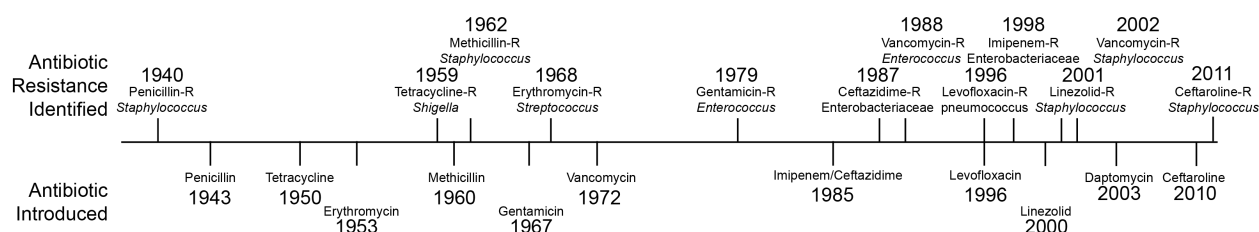
This dissertation is dedicated to my parents Raúl and Sylvia

## **Chapter 1: Introduction**



## 1.1. The antibiotic resistance problem

Antibiotic-resistant bacteria pose a serious threat to public health in both developed and developing parts of the world. The intrinsically fast doubling time of bacteria (e.g. ~20 minutes for *E. coli*) and antibiotic selection pressure promote the survival of antibiotic-resistant mutants with higher fitness. In fact,  $\beta$ -lactam resistance was observed before penicillin was even introduced to human use.<sup>1,2</sup> Antibiotic resistance has arisen against every antibiotic brought forward to the clinic (Figure 1.1). In 2016, the World Health Organization (WHO) investigated dangerous antibiotic-resistant species and created a priority list. The results revealed that emphasis on drug discovery should be placed on Gram-negative bacteria, especially those responsible for community-acquired infections.<sup>3</sup> The quinolones, which were discovered in the 1960s, were the last major class of antibiotics active against Gram-negatives. Besides posing health threats, antibiotic resistant bacteria hamper economic systems, with an estimated burden on the U.S. economy of nearly \$20 billion each year due to health care system costs.<sup>4</sup> Despite the increasing clinical need for new Gram-negative agents, pharmaceutical industries are sadly moving away from the antibiotic sector in part due to lack of commercial incentives and the scarcity of success.<sup>5</sup>



**Figure 1.1. Timeline of antibiotic introduction to widespread human use versus the discovery of antibiotic resistance.**

Bacterial resistance has been observed against every antibiotic ever introduced.<sup>6</sup> Timeline is not drawn to scale.

Clearly, more effective antibiotic stewardship is needed, and the Red Queen hypothesis<sup>7,8</sup> suggests that we will need to continuously develop (or find) new antibiotics to keep up with the inevitable resistance problem. One school of thought advocates for development of analogues based on already existing classes of antibiotics, with the benefit of already possessing an

understanding of the underlying biology behind the mechanism of action. This has been a tried-and-tested strategy. Hundreds of analogues of each class of antibiotic, including the penicillins, quinolones, tetracyclines, have been prepared with improvements in antibiotic activity or pharmacology. Some have been clinically successful; for example, ceftaroline<sup>9</sup> and ceftobiprole<sup>10</sup> are “fifth-generation” cephalosporins, and were developed within the past twenty years. The main counterargument to this approach is the following: Chemists will run out of structural modifications of given classes.<sup>1</sup> Thus, antibiotics that can hit novel targets are highly desirable, especially those that can be used to kill Gram-negative bacteria.

## **1.2. Combination therapy as a new avenue for antibacterial drug discovery**

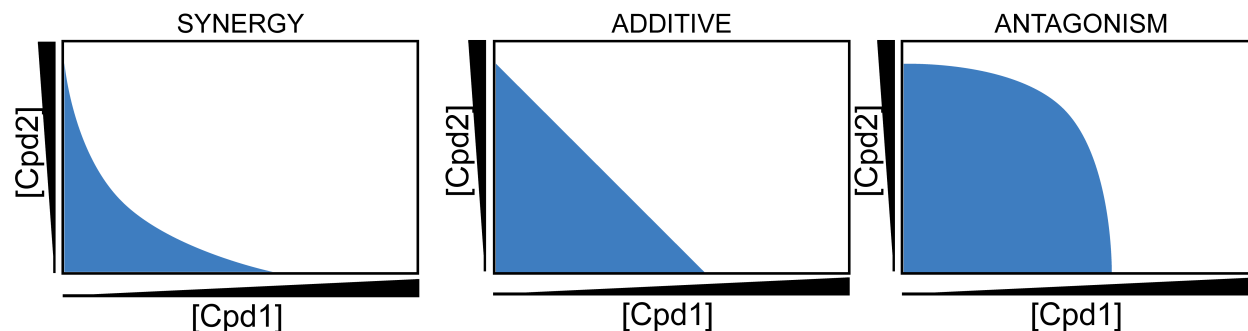
Combination therapies have not been the traditional approach to treating bacterial infections. Antibacterial drug discovery efforts have focused on monotherapy due to 1) a historical record of single-agent successes in the clinic 2) the fact that clinical trials are simpler when one compound is the subject of investigation 3) the reason that clinicians prefer single agents for simpler dosing regimens.<sup>11</sup> Nevertheless drug combinations are the standard line of treatment for viral diseases (e.g. HIV-1<sup>12</sup>), cancer,<sup>13</sup> and many other disease areas. A combination of molecules that hit multiple targets reduces the likelihood of resistance.<sup>14</sup> In theory this is because the probability of multiple resistance mutations conferring survival is lower than one occurring. Rifampicin (RNA polymerase inhibitor), when combined with tobramycin (protein synthesis inhibitor) and ticarcillin (a cell-wall inhibitor), was shown to be active against a highly resistant strain of *P. aeruginosa*.<sup>15</sup> It could be that the drugs act independently of each other, or perhaps the antibiotics are improving or worsening the effects of the other(s). Several possible scenarios for drug combinations exist.

**Table 1.1. Terminology for combination therapies.**

Specialized definitions exist for the combination of two agents against a biological system.<sup>16</sup>

|                         | Both compounds are active alone     | Only one compound is active | Neither compound is active |
|-------------------------|-------------------------------------|-----------------------------|----------------------------|
| Combination is better   | Loewe/Bliss synergism               | synergism                   | coalism                    |
| Combination is expected | Loewe additivity/Bliss independence | inertism                    | inertism                   |
| Combination is worse    | Loewe/Bliss antagonism              | antagonism                  |                            |

The definitions in Table 1.1 summarize the labels given to the effects of antibiotic drug combinations. In order to detect antibiotic synergism, checkerboard assays (isobolograms) are performed, in which compound gradients are established on both dimensions of a plate. The fractional inhibitory concentration (FIC) is then calculated as the minimal inhibitory concentration (MIC) of the compound acting in combination divided by the MIC of the compound in monotherapy.<sup>17</sup>

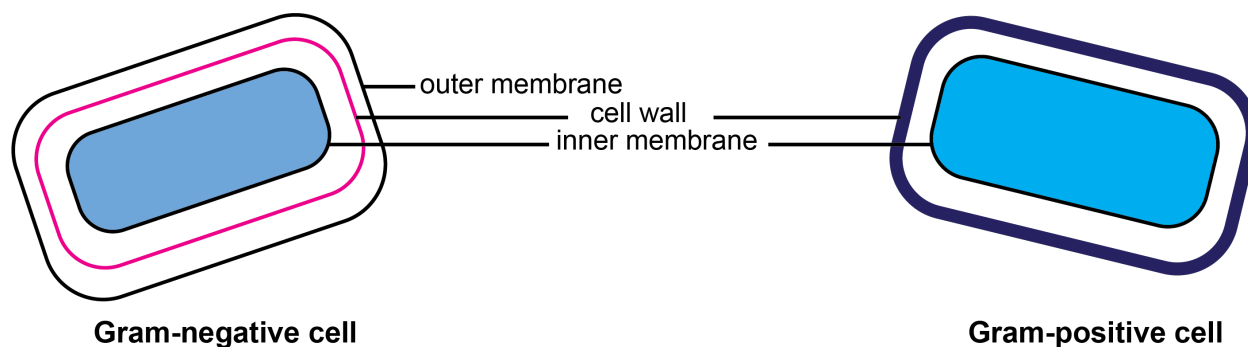
**Figure 1.2. Antibiotic checkerboard cases.**

Examples of checkerboard patterns for the combination of two compounds. The blue area represents bacterial growth and the white represents killing.

### 1.3. The Gram-stain and the bacterial cell envelope

Gram-negative bacteria were mentioned above as being especially resistant to many antibiotics. The major difference between Gram-negative and Gram-positive bacteria is the structure of their cell envelopes. When bacteria are treated with crystal violet, (tris(4-

(dimethylamino)phenyl)methylium chloride), those that retain the purple stain are referred to as Gram-positive. Those that do not retain this stain and are counter-stained pink with safranin are the Gram-negative bacteria. The Gram stain was discovered by accident in the 1880s by Christian Gram, a Danish bacteriologist working in Berlin.<sup>18, 19</sup> While the stain is not perfectly selective for bacteria (yeasts, molds, and other eukaryotes are stained purple), and while some bacteria cannot be classified by it (e.g. *Mycobacterium tuberculosis*), it is often the first test used to characterize a bacterial organism. The reason for this differential staining is that Gram-positive and Gram-negative bacteria possess different cell envelope structures.



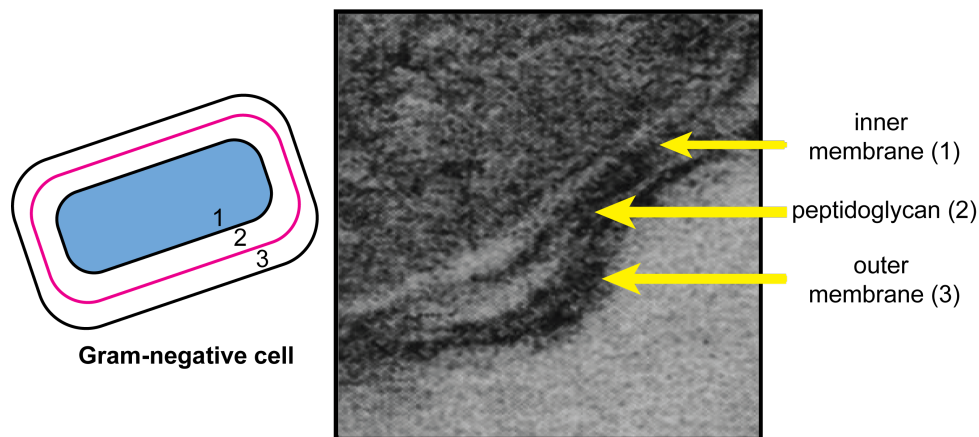
**Figure 1.3. The Gram stain distinguishes two major classes of bacteria.**

The major defining characteristic of Gram-negative bacteria is the presence of an outer membrane. Gram-positive bacteria have a much thicker cell wall, which stains with crystal violet.

Gram-positive bacteria possess a thick peptidoglycan layer (cell wall) surrounding their plasma membrane, while Gram-negatives possess a much thinner peptidoglycan (Figure 1.3).<sup>20</sup> The peptidoglycan layer is an important component of the cell envelope comprised of alternating units of *N*-acetylglucosamine and *N*-acetylmuramic acid (NAG-NAM) sugars that are crosslinked into a mesh via the NAM units (Figure 1.5). This structure helps bacteria maintain their shape and is the reason cells do not lyse in distilled water.<sup>21</sup> The Gram-positive peptidoglycan layer absorbs and binds the crystal violet stain, which does not wash out with decolorizing agent. The thin Gram-negative peptidoglycan is not stained by crystal violet but is counter-stained by safranin.

Advances in electron microscopy technology during the 20<sup>th</sup> century allowed for the visualization of the compartments of the bacterial cell envelope.<sup>22</sup> These findings drove efforts to

isolate the different compartments of the Gram-negative cell envelope. In 1964, Bladen and Mergenhagen demonstrated the presence of an outer membrane in Gram-negative *Veillonella* cells, which could be extracted with phenol/water while leaving the solid membrane (peptidoglycan) intact (Figure 1.4).<sup>23</sup> In 1968, Miura and Mizushima separated the outer and inner membranes of *E. coli* K12 by density gradient centrifugation.<sup>24</sup> In 1972, Osborn et al. isolated the cytoplasmic and outer membrane fractions from *S. typhimurium* using a modified version of the earlier procedures.<sup>25</sup> The outer membrane, found in Gram-negative bacteria, mitochondria, and chloroplasts, remains the subject of extensive investigation today.



**Figure 1.4. The Gram-negative cell envelope.**

Electron microscopy image of the Gram-negative cell envelope and discovery of an outer membrane.<sup>23</sup>

#### 1.4. The Gram-negative outer membrane

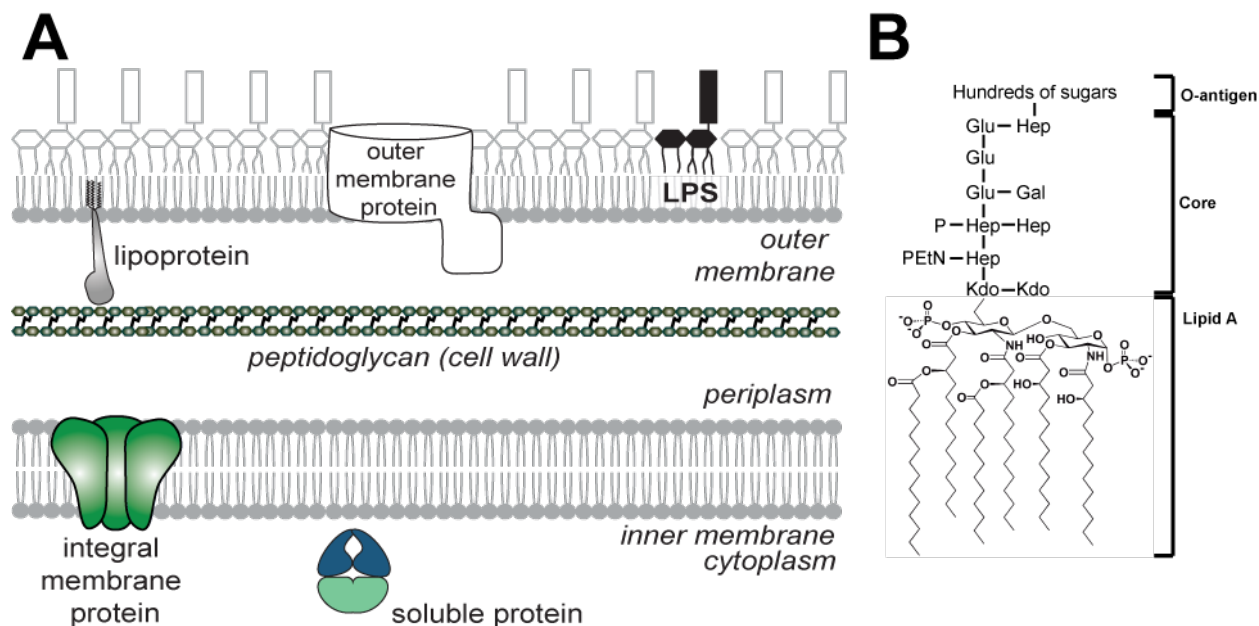
The Gram-negative outer membrane is an asymmetric lipid bilayer (Figure 1.5A) consisting of lipopolysaccharide (LPS, endotoxin) on the outer leaflet and phospholipids on the inner leaflet. In contrast, the inner membrane is comprised of a simpler symmetric lipid bilayer of phospholipids. The role of the outer membrane is thought to be that of a protective barrier, preventing the passage of bile salts and many antibiotics that kill Gram-positive bacteria easily.<sup>21</sup> In addition to lipids and glycolipids, the outer membrane contains proteins known as outer membrane proteins (OMPs). Few enzymes exist in the outer membrane (e.g. PldA<sup>26</sup>, OmpT<sup>27</sup>, and PagP<sup>28</sup>).<sup>21</sup> Most OMPs that reside within it are integral  $\beta$ -barrel proteins or membrane-

anchored lipoproteins (Figure 1.5A).<sup>29, 30</sup> These proteins serve important roles such as conduits for nutrients and toxins to pass through the membrane (e.g. LamB, TolC) or proteins that stabilize the cell wall (e.g. Lpp). In theory, OMPs would be ideal targets for antibiotics, since a small molecule or antibody would not need to cross a single membrane bilayer in order to reach its target. However, the lipopolysaccharide layer, which covers the cell surface, surrounds and protects OMPs from incoming xenobiotics. For example, a recent monoclonal antibody targeting a major *E. coli* OMP was only effective against strains of *E. coli* expressing truncated lipopolysaccharide molecules.<sup>31</sup> The lipopolysaccharide layer is a formidable permeability barrier and has been viewed as an important antibiotic target.

### **1.5. The structure and function of lipopolysaccharide**

Lipopolysaccharide (LPS) is a complex glycolipid covering the cell surface of Gram-negative bacteria (Figure 1.5A). The structure of lipopolysaccharide (LPS) is unique to each strain of Gram-negative bacteria, but usually contains three domains: the Lipid A moiety, the core sugars, and the O-antigen (Figure 1.5B). Lipid A is a hexaacylated diglucosamine diphosphate, which is conserved among many types of LPS molecules. Lipid A is responsible for the immunogenic activity in mammals, and for this reason LPS is known as endotoxin.<sup>32</sup> The negatively-charged phosphates, which are stabilized by the presence of  $Mg^{2+}$  ions on the cell surface,<sup>21</sup> create a polyelectrolyte barrier that impedes the diffusion of many nonpolar molecules across the membrane. The many hydrophilic core sugars attached to LPS also contribute to the deflection of hydrophobic molecules. As the same time, the extensively nonpolar portions regions on the Lipid A moiety prevent the passage of polar molecules. Therefore, as a large amphiphilic molecule, LPS confers complementary selectivities against both polar and nonpolar xenobiotics. The presence of O-antigen is often implicated in virulence and is the basis for the designation of rough (non-virulent) and smooth (virulent) strains.<sup>33</sup> Most Gram-negative organisms require LPS molecules for viability, but notable exceptions exist.<sup>34, 35</sup> Strains that lack LPS display an increased

sensitivity to most antibiotics; therefore, the LPS has been implicated as a defense against antibiotics and toxic xenobiotics.<sup>36</sup>

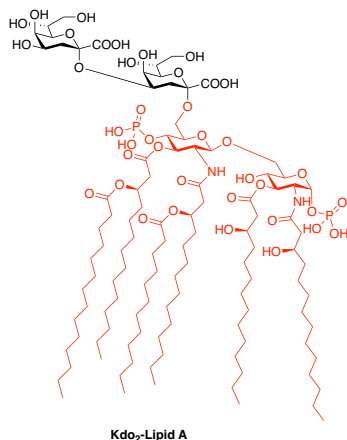


**Figure 1.5. The structure of the Gram-negative cell envelope and lipopolysaccharide.**

A) The Gram-negative cell envelope contains four layers: the cytoplasm, inner membrane, peptidoglycan, and outer membrane, and various types of proteins occupy these layers. B) LPS is a complex hexaacylated diglucosamine diphosphate decorated with over hundreds of sugars and is essential in most Gram-negative bacteria.

## 1.6. Lipopolysaccharide biosynthesis

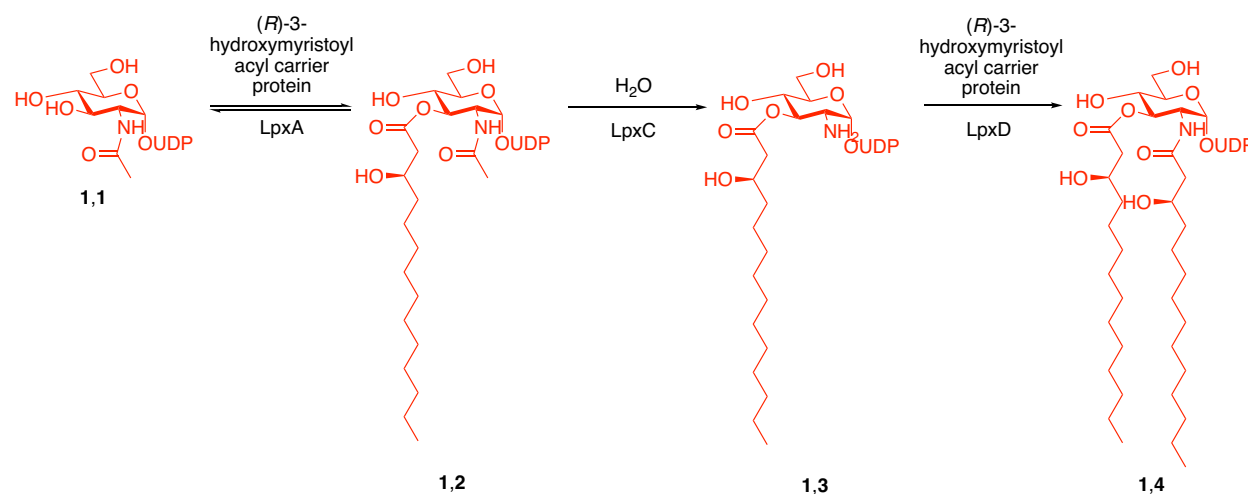
Gram-negative bacteria synthesize Kdo<sub>2</sub>-lipid A in a process known as the Raetz pathway.<sup>32, 37</sup> The structure of Lipid A is complex and can vary slightly depending on the bacterial species; however, the general structure produced by *E. coli* is shown in Figure 1.6.



### Figure 1.6. The structure of Kdo<sub>2</sub>-Lipid A.

Chemical structure of Kdo<sub>2</sub>-Lipid A from *E. coli*.<sup>32</sup> The hexaacylated diposphorylated diglucosamine (Lipid A) is highlighted in red. The 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) sugars are depicted in black.

The initial steps of Kdo<sub>2</sub>-lipid A synthesis occur in the cytoplasm. In the first step, which is thermodynamically unfavorable and reversible, uridine diphosphate *N*-acetylglucosamine (UDP-GlcNAc) **1,1** is transesterified by a thioester donor (*R*)-3-hydroxymyristoyl acyl carrier protein (ACP) and the reaction is catalyzed by LpxA (Figure 1.7).<sup>38-41</sup>

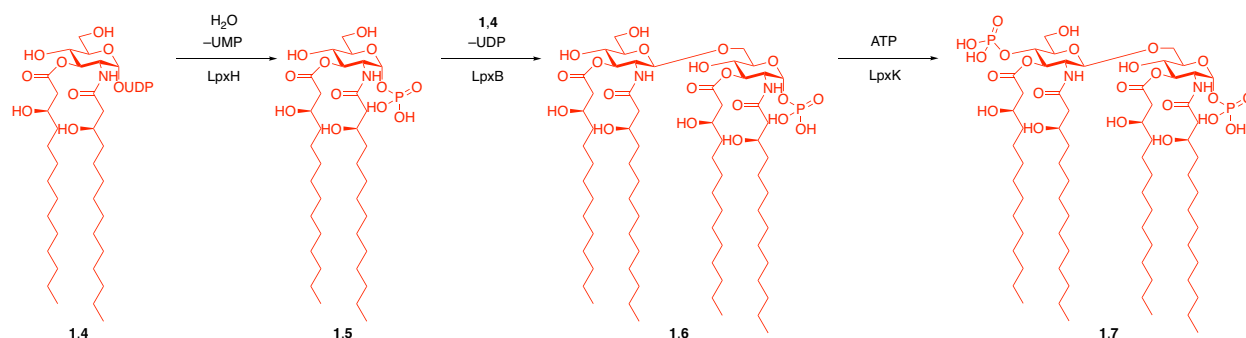


### Figure 1.7. The first three steps of LPS biosynthesis.

UDP-GlcNAc **1,1** is acylated at O3 by (*R*)-3-hydroxymyristoyl acyl carrier protein, whose 14-carbon tail is recognized by LpxA. The Zn<sup>2+</sup>-dependent enzyme LpxC catalyzes the first irreversible step of Kdo<sub>2</sub>-Lipid A synthesis, the amide bond hydrolysis of **1,2**. The LpxD enzyme catalyzes the amide bond formation reaction between the glucosamine **1,3** and 3-hydroxymyristoyl ACP.

In the next step, the metalloprotein LpxC deacetylates **1,2** to expose the free glucosamine. This step is irreversible and is referred to as the first committed step of the biosynthesis.<sup>42, 43</sup> LpxD adds another (*R*)-3-hydroxymyristoyl group to **1,3**, like LpxA, but this time to form a peptide bond on the amine residue (Figure 1.7).<sup>44, 45</sup> LpxH catalyzes the hydrolysis of the pyrophosphate in **1,4**, leading to monophosphate **1,5** (also known as Lipid X).<sup>46, 47</sup> Subsequently, Lipid X and **1,4** are coupled in a glycosylation reaction, catalyzed by LpxB<sup>48, 49</sup>, to form a β, 1'→6-linked disaccharide (Figure 1.8).<sup>49</sup>

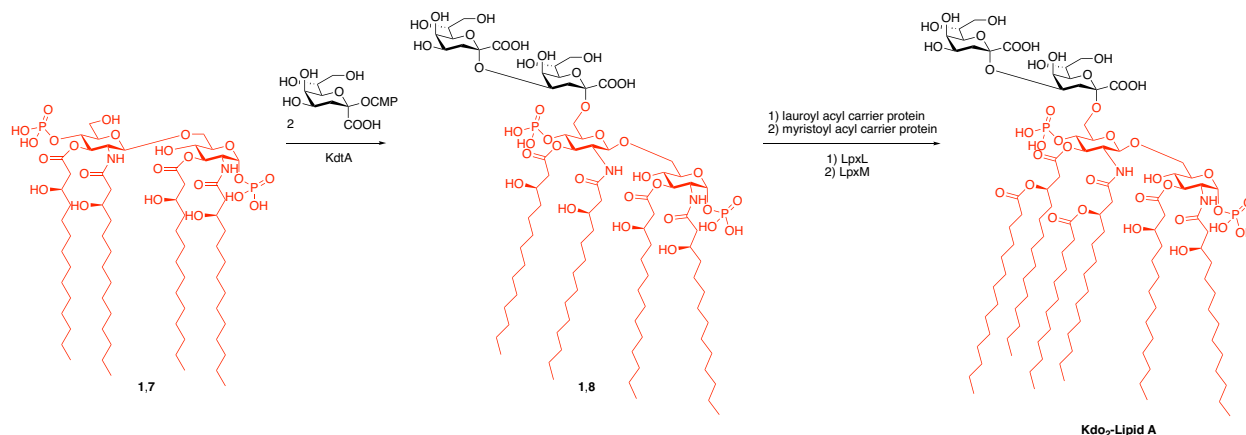




**Figure 1.8. LpxH, LpxB, and LpxK catalyze the formation of Lipid IV<sub>A</sub>.**

The LpxH enzyme hydrolyzes the pyrophosphate linkage of the UDP in **1,4** to leave behind the monophosphate ester **1,5** (Lipid X). **1,5** acts as the nucleophile in the next step towards another molecule of **1,4**; this reaction is catalyzed by LpxB and yields disaccharide **1,6**. The LpxK kinase phosphorylates the 4' sugar hydroxyl of **1,6** in a reaction utilizing ATP as the substrate to yield Lipid IV<sub>A</sub> **1,7**.

LpxK catalyzes the phosphorylation of **1,6** by ATP<sup>50</sup> and completes the synthesis of Lipid IV<sub>A</sub> **1,7** (Figure 1.8). The 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) sugars are appended on **1,7** by the enzyme KdtA<sup>51-53</sup> using the glycosyl donor Kdo-CMP (Figure 1.9).<sup>54-56</sup>



**Figure 1.9. KdtA, LpxL, and LpxM complete Kdo<sub>2</sub>-Lipid A.**

KdtA appends two Kdo units on the 6' sugar hydroxyl of **1,6**. LpxL adds a 12-carbon hydrocarbon chain to the hydroxyl group of the 2'-N-linked hydroxymyristoyl chain. LpxM adds a 14-carbon hydrocarbon chain to the hydroxyl group of the 3'-O-linked hydroxymyristoyl chain.

Finally, LpxL and LpxM append fully saturated hydrocarbon chains (of slightly different lengths) to the hydroxyl groups of the (*R*)-3-hydroxymyristoyl chains hanging from the glucosamine sugars (Figure 1.9).<sup>57, 58</sup> The result is the hexaacylated disaccharide diphosphate connected to two Kdo

sugars, **Kdo<sub>2</sub>-Lipid A**. LPS biosynthesis begins in the cytoplasm, but the **Kdo<sub>2</sub>-Lipid A** is finished on the monolayer of the inner membrane facing the cytoplasm (i.e. the inner leaflet of the inner membrane).<sup>32</sup> In order to begin its journey to the outer leaflet of the outer membrane, the LPS must first be flipped to the outer leaflet of the inner membrane. Before continuing to describe the journey of LPS, which is covered in Section 1.11, I wish to briefly outline other important membrane components, such as the ubiquitous phospholipids.

### 1.7. The structure and function of phospholipids

Gram-negative bacteria possess phospholipids on both leaflets of the inner membrane and on the inner leaflet of the outer membrane. These phospholipids encompass a wide diversity of molecules, whose synthesis and levels are tightly regulated in order to maintain homeostasis. The majority of bacterial phospholipids resemble the glycerophospholipids, which contain a glycerol backbone, a negatively charged phosphate, and two fatty acid tails. Phosphatidylglycerol is one such glycerophospholipid that is produced by *E. coli*. A condensation reaction between two molecules of phosphatidylglycerol produces cardiolipin, which is another component of the phospholipid layer.<sup>21, 59</sup> Phosphatidylglycerol and cardiolipin are known as acidic phospholipids due to their anionic phosphate residues. Phosphatidylethanolamine, another major *E. coli* lipid, contains a positively charged ammonium functionality that renders the molecule zwitterionic (neutral). The inner and outer membrane contain the same phosphatidylglycerol and cardiolipin molecules; however, the distributions are significantly different. In a phospholipid fractionation study utilizing *Caulobacter crescentus*, the outer membrane was found to possess more phosphatidylglycerol but less cardiolipin than the inner membrane.<sup>60</sup> Bacteria attempt to regulate the proportion of neutral and charged phospholipids in order to ensure the correct folding of membrane proteins<sup>61, 62</sup>, proper transport of molecules across the membrane<sup>63</sup>, and the timing of division processes.<sup>59, 64, 65</sup> Membrane phospholipids provide a selectively-permeable skin for bacteria, preventing the entry of toxins and the loss of essential molecules (e.g. ATP) from the

cell. Proteins are embedded in membrane phospholipid bilayers and serve a wide array of functions, such as antibiotic efflux<sup>66</sup>, nutrient/ion transport<sup>67, 68</sup>, and environmental sensors.<sup>69</sup> In order to properly assemble and compartmentalize membrane proteins, bacteria have evolved strategies to secrete proteins translated by ribosomes in the cytoplasm to their final destination.

### **1.8. Membrane protein translocation pathways**

Bacteria synthesize proteins that are either destined for the cytoplasm (soluble proteins) or are meant to be secreted to another compartment. Bacteria lack a nucleus, and the genetic material (DNA) containing the instructions for protein synthesis resides in the cytoplasm, which is transcribed into messenger RNA (mRNA) by RNA polymerase.<sup>20, 70</sup> Proteins are translated from the mRNA template in the cytoplasm, and those destined for secretion to the outer membrane possess an N-terminal signal sequence. This sequence consists of a few positively charged residues (N domain), followed by hydrophobic residues (H domain), and ending with polar residues (C domain).<sup>71, 72</sup> This post-translational translocation involves recognition of the signal sequence by SecB, a cytoplasmic chaperone, and the pre-protein is brought to the SecA-SecYEG complex. SecA hydrolyzes ATP and the protein passes through the SecYEG channel; then, the signal sequence is cleaved by a signal peptidase.<sup>71</sup> An early reconstitution showed that only SecYE is sufficient for SecA-dependent protein translocation; SecDF are not required, but deletions in these proteins render cells cold-sensitive.<sup>73, 74</sup> Proteins destined for secretion to the inner membrane may use either the Sec and/or YidC system. YidC is an essential membrane protein that is necessary for the membrane insertion of Sec-independent proteins.<sup>75</sup> It is thought that YidC also forms a complex with Sec and helps make Sec-dependent translocation more efficient.<sup>76</sup>

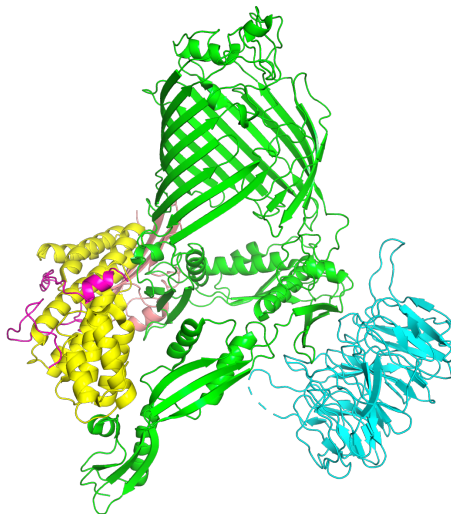
### 1.9. Lipoproteins and their transport

Bacterial lipoproteins are proteins modified with a lipid anchor (e.g. palmitate) and are located on both the inner and outer membrane. These are the most abundant post-translationally modified secretory proteins<sup>77</sup>, and the most abundant protein in *E. coli* is the outer-membrane anchored Braun's lipoprotein Lpp, a fraction of which is connected to the peptidoglycan layer.<sup>21, 78, 79</sup> Proteins destined to become lipoproteins possess an N-terminal signal sequence, which is cleaved to reveal a cysteine residue<sup>80-82</sup> that becomes the point of attachment of the mature protein to the acyl groups. After translation, the lipoprotein signal sequence (lipobox) triggers export of a lipoprotein precursor from the cytoplasm.<sup>83, 84</sup> Lipoproteins traverse through the inner membrane through the aforementioned Sec system in *E. coli*<sup>79</sup>, but in other organisms this occurs by the Tat translocon.<sup>85, 86</sup> The signal sequence, anchors the protein in the inner membrane while a transferase enzyme Lgt<sup>29</sup> adds a diacylglyceride to the side chain of the N-terminal cysteine residue. Then, a signal peptidase LspA<sup>87, 88</sup> cleaves the signal sequence, and further acylation on the glyceryl moiety is performed by a transacylase (LntA).<sup>89, 90</sup> The N-terminal cysteine residue of the mature protein is acylated with a lipid in the periplasm and is then sorted to either the inner or outer membrane. Lipoproteins destined for export to the outer membrane follow the localization of lipoproteins (Lol) pathway.<sup>91</sup> However, if a prolipoprotein possesses a Lol avoidance signal (i.e. the +2 rule)<sup>92</sup>, it will be retained in the inner membrane. The Lol pathway includes an inner membrane ATP-binding cassette (ABC)-type complex (LolCDE) that hydrolyzes ATP from the cytoplasm to harness energy for the extraction of the lipoprotein from the inner membrane.<sup>93</sup> The lipoprotein is maintained soluble in the periplasm by association with the carrier LolA<sup>94, 95</sup> and is ultimately delivered to the outer membrane acceptor protein LolB.<sup>96</sup>

### 1.10. Outer membrane $\beta$ -barrel biogenesis

Outer membrane proteins (OMPs) in Gram-negative bacteria take the form of  $\beta$ -barrels, with a hydrophilic interior and a hydrophobic exterior facing the membrane. Many OMPs are

porins<sup>97</sup> that serve as gates to allow the diffusion of small molecules into the cell. For example, the LamB porin,<sup>98, 99</sup> is selective for maltodextrins. The  $\beta$ -barrel assembly machinery (Bam) complex is responsible for folding the immature OMP barrel precursors from the periplasm and inserting the final folded barrels into the outer membrane. The five-protein  $\beta$ -barrel assembly machinery (Bam) complex was discovered by careful genetic screening of the mechanisms of action of chlorobiphenyl vancomycin and moenomycin.<sup>100, 101</sup> When a strain with a well-known permeability defect (*imp4213*)<sup>102</sup> was exposed to these antibiotics, *yfgL* mutants were isolated.<sup>100</sup> The YfgL protein (BamB) was then found to be part of a multiprotein complex required for OMP assembly.<sup>101</sup> The complex containing YaeT, YfgL, NlpB, YfiO, and SmpA was renamed to BamA-BamE, respectively.<sup>103, 104</sup> BamA is an 88-kDa  $\beta$ -barrel protein located on the outer membrane and contains five associated polypeptide transport-associated (POTRA) domains that extend to the periplasm. These POTRA domains are known to scaffold the BamB-BamE proteins in the complex.<sup>105</sup> Among the five proteins, only BamA and BamD are essential in *E. coli*.<sup>106, 107</sup> The activity of the Bam complex was reconstituted *in vitro*<sup>108, 109</sup> in 2010 and full structures of the Bam complex were made available in the latter half of the decade (Figure 1.10).<sup>110, 111</sup>



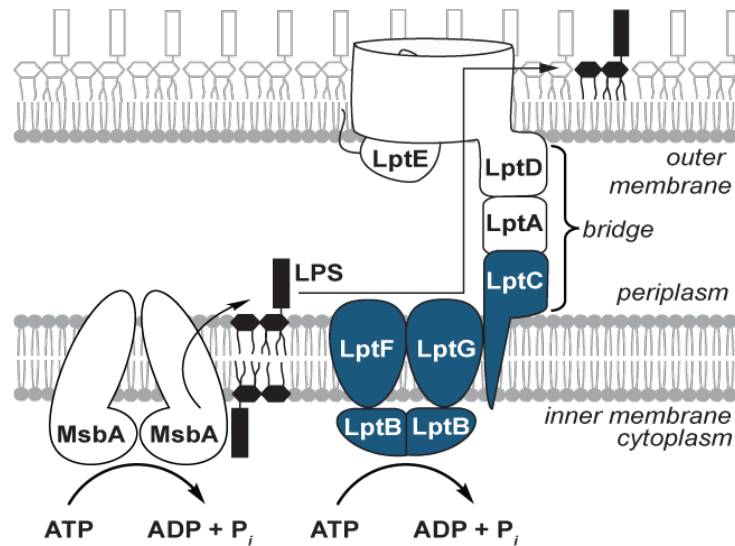
**Figure 1.10. The Bam complex.**

Crystal structure of the BamABCDE complex.<sup>110</sup> BamA (YaeT) in green, BamB (YfgL) in cyan, BamC (NlpB) in magenta, BamD (YfiO) in yellow, BamE (SmpA) in salmon. PDB accession code 5D0O.

### 1.11. The transport of lipopolysaccharide to the outer membrane

Following the final steps of biosynthesis on the inner membrane, LPS is translocated by the MsbA flippase from the inner to the outer leaflet of the inner membrane (Figure 1.11). MsbA is an essential ABC transporter that is conserved among Gram-negatives.<sup>112, 113</sup> For this reason, MsbA has been extensively investigated as a potential antibiotic target, as inhibition of MsbA disrupts Gram-negative outer membrane assembly<sup>114, 115</sup>, which would kill cells or sensitize them to other antibiotics. Several high-resolution structures of MsbA have been reported<sup>116, 117</sup>; the most recent ones to 4.2 Å (*E. coli*)<sup>118</sup> by cryo-EM, 2.9 Å (*E. coli*)<sup>119</sup> and 2.8 Å (*S. typhimurium*)<sup>120</sup> by X-ray crystallography. Both ATPase activity<sup>121-123</sup> and lipid flipping activities<sup>124</sup> have been reconstituted *in vitro*. After LPS is flipped across the inner membrane, it is delivered to the cell surface by the lipopolysaccharide transport pathway.

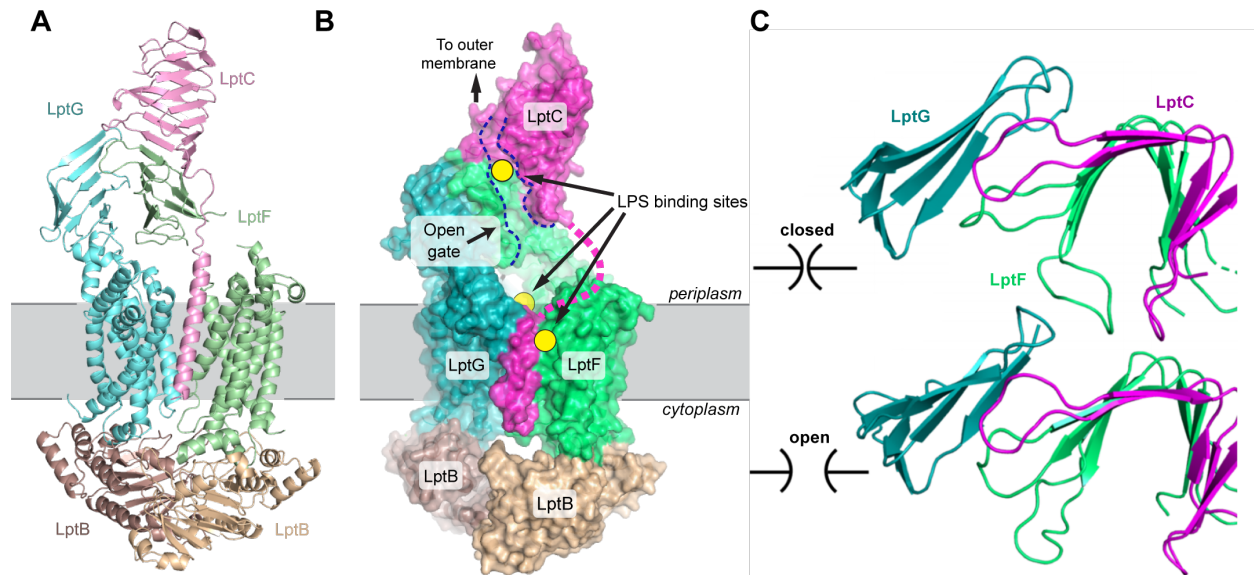
The lipopolysaccharide transport (Lpt) system is a set of seven essential proteins LptA-LptG in Gram-negative bacteria that spans four cellular compartments, the cytoplasm, inner membrane, periplasm, and outer membrane (Figure 1.11).<sup>125</sup> The inner membrane complex LptB<sub>2</sub>FGC is an ABC transporter whose function is to extract LPS from the inner membrane and begin its transport through the periplasm. LptB<sup>126, 127</sup>, the nucleotide binding domain, is the only enzyme in the Lpt pathway and hydrolyzes ATP in the cytoplasm, coupling the energy harnessed to the movement of the transmembrane proteins LptF and LptG.<sup>128</sup> Structural evidence for the basis of LPS extraction from the inner membrane has occurred relatively recently, with X-ray crystal structures released in 2017 for LptB<sub>2</sub>FG to 3.5 Å in *P. aeruginosa*<sup>129</sup> and 3.7 Å in *K. pneumoniae*.<sup>130</sup> Full structures of the LptB<sub>2</sub>FGC were reported by crystallography for *V. cholerae* and *E. cloacae* to 2.9 Å and 3.2 Å respectively (Figure 1.12A-B)<sup>131</sup>, and by cryo-EM for *E. coli* to 4.2 Å.<sup>132</sup>



**Figure 1.11. LPS is flipped and translocated to the outer leaflet of the outer membrane by MsbA and the Lpt bridge.**

The ABC transporter MsbA couples ATP hydrolysis to the flipping of LPS from the inner to the outer leaflet of the inner membrane. LPS is then extracted from the inner membrane by the ABC transporter LptB<sub>2</sub>FGC, which is connected to the outer membrane complex LptDE by a periplasmic bridge (LptA). LPS exits the LptD protein from a crenel in the  $\beta$ -barrel to the cell surface. The peptidoglycan layer is omitted for clarity.

UV-photocrosslinking studies were conducted *in vivo* to determine residues in LptB<sub>2</sub>FGC that contacted LPS during cellular growth. Positions that crosslinked LPS are labeled in highlighted in Figure 1.12B. An interesting contrast between the LptB<sub>2</sub>FGC crystal structures from *V. cholerae* and *E. cloacae* was that the former appeared to crystallize in a conformation in which loops from LptF and LptC closed the path for LPS transport, whereas the loops in the latter were open. A model was proposed in which a gate controls the passage of LPS through LptF and LptC towards the periplasm (Figure 1.12B-C). The structural and biochemical studies on LptB<sub>2</sub>FGC have mapped the pathway that LPS molecules take on their way to the periplasmic LptA protein.



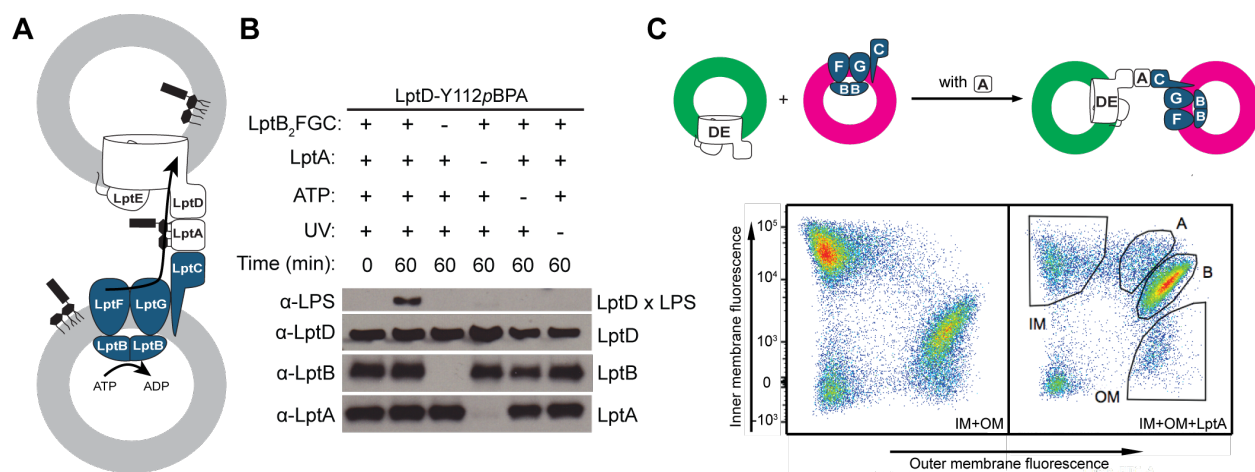
**Figure 1.12. Structural basis for LPS extraction and translocation from the inner membrane.**

A) The X-ray crystal structure of the ABC transporter LptB<sub>2</sub>FGC from *V. cholerae*. B) Surface representation of *E. cloacae* LptB<sub>2</sub>FGC in the gate-open conformation. Sites where LPS was shown to crosslink the protein complex are shown in yellow. C) Comparison between gate-open (*E. cloacae*) and gate-closed (*V. cholerae*) structures. Figure adapted from ref<sup>131</sup> (Dr. Tristan Owens).

After LPS is extracted from the inner membrane, it is transferred to the periplasmic LptA protein.<sup>133, 134</sup> LptA has been shown to interact specifically with the lipid A component in LPS and contains  $\beta$ -jellyroll motifs that encapsulate and shuttle the large hydrophobic molecule through the aqueous periplasm.<sup>135-137</sup> LptA has been observed to oligomerize<sup>138, 139</sup>, and it is unclear how many LptA proteins are involved in a transenvelope Lpt complex.<sup>140</sup> Upon reaching the C-terminus of LptA, LPS is transferred to the N-terminus of LptD, which shares the same  $\beta$ -jellyroll (OstA) structural subfamily.<sup>140</sup> LptD<sup>102</sup> is a 26-stranded  $\beta$ -barrel in the outer membrane whose proper folding is templated by its plug LptE.<sup>141-143</sup> Structural studies of the LptD/E protein complex reveal the presence of a lateral gate in LptD, from which LPS can pass from the lumen of the barrel into the outer membrane.<sup>144-147</sup> Interestingly, LptE bears structural similarity to the LPS-binding proteins from *Limulus* (horseshoe crab) and binds LPS to catalyze its assembly on the outer membrane.<sup>148</sup>



Two models have been proposed for LPS transport: a periplasmic chaperone model and a bridge model. In the former model, soluble LptA acts as a periplasmic shuttle of LPS between the inner and outer membranes, similar to the way the Lol system works. The bridge model was proposed during early biochemical studies involving sucrose gradient fractionation and pull down experiments using His-tagged inner membrane Lpt components.<sup>149</sup> To rule out a model involving LptA as a soluble periplasmic shuttle, the outer and inner membrane Lpt complexes were reconstituted in separately colored fluorescent proteoliposomes. Co-localization of these proteoliposomes was observed by fluorescence microscopy in an LptA-dependent fashion. Furthermore, complexes containing both inner and outer membrane proteoliposomes bridged by LptA could be isolated by fluorescence activated cell sorting (FACS) (Figure 1.13).<sup>150</sup>



**Figure 1.13. Reconstitution of LPS transport.**

A) Schematic of LPS reconstitution. B) The presence of seven Lpt proteins and ATP are required to observe a UV- and time-dependent crosslink between LptD Y112 and LPS. C) Schematic of LPS reconstitution with fluorescently labeled outer and inner membrane proteoliposomes. A new population 'B' is detected when LptA is added. Figure adapted from ref<sup>150</sup> (Dr. David Sherman; Dr. Ran Xie; Becca Taylor; Dr. Alex George).

## 1.12. Targeting lipopolysaccharide biogenesis

Since LPS is required for proper viability of most Gram-negative cells, the LPS biosynthesis and transport proteins have been investigated as potential antibiotic targets. The closest effort to a commercially successful antibiotic has been the development of an LpxC

inhibitor.<sup>151</sup> Several of these LPS biosynthesis inhibitors were synthesized by various companies and a molecule developed by Achaogen was brought into clinical trials (ACHN-975); however, there has been no success, due to dose-limiting toxicity.<sup>152, 153</sup> The zinc-chelating hydroxamate group, which is common to most LpxC inhibitors, may present a safety liability. Genentech discovered quinoline inhibitors of MsbA with single-digit  $\mu\text{g/mL}$  potencies against wildtype *E. coli* and *K. pneumoniae*<sup>119</sup>, but the translational future of these inhibitors is unclear. The Lpt proteins, which transport LPS through the periplasm, have attracted significant attention as novel antibiotic targets. Inhibition of the essential LPS transport process is known to impair cellular viability and trigger the bacterial stress response.<sup>154</sup>

The polymyxins, which will be discussed in later sections of this dissertation, target LPS directly and offer a different modality for targeting LPS biogenesis than inhibiting protein function. Polymyxins, which are cationic cyclic lipopeptides, are known to bind LPS in a stereospecific fashion<sup>155</sup> and inhibit the growth of many pathogenic Gram-negative bacteria at single-digit or lower  $\mu\text{g/mL}$  minimal inhibitory concentrations. The mechanism for why the polymyxins exhibit a lethal effect on bacteria is not well-understood, as some polymyxin derivatives (e.g. polymyxin B nonapeptide) can permeabilize the outer membrane without a lethal effect.<sup>156</sup> The membrane permeabilizing property of the polymyxins has encouraged combination therapies of polymyxin with antibiotics that are normally ineffective against recalcitrant Gram-negatives.

### 1.13. Goals of this work

In this dissertation, I will outline the discovery that the small molecule antibiotic novobiocin interacts with LptB, the only enzyme in the Lpt complex, and affects LPS transport (Chapter 2). Next, I will describe our studies on the *noncovalent* combination of novobiocin and polymyxin B with our understanding that novobiocin binds LptB (Chapter 3). I will outline our effort to create *covalent* combinations of novobiocin and polymyxin (i.e. chimeric novobiocin-colistin molecules; Chapter 4). Finally, I will present my conclusion and aims for the future (Chapter 5).

## **Chapter 2: Characterization of a small molecule binding the inner membrane lipopolysaccharide transporter**

This chapter is adapted/reproduced from:

May, J.M.\*; Owens, T.W.\*; Mandler, M.D.\*; Simpson, B.W.\*; Lazarus, M.B.; Okuda, S.; Sherman, D.J.; Davis, R.M.; Massefski, W.; Ruiz, N.; Kahne, D. "The antibiotic novobiocin binds and activates the ATPase that powers lipopolysaccharide transport" *J. Am. Chem. Soc.* **2017**, *139*, 17221-17224.

## 2.1. Introduction

As discussed in Chapter 1 of this thesis, an antibiotic that targets the lipopolysaccharide transport (Lpt) system would be of great interest since Lpt is highly conserved and essential among Gram-negative bacterial species. By inhibiting or otherwise perturbing lipopolysaccharide (LPS) transport, cells could die or become highly sensitive to other antibiotics.<sup>157-159</sup> Currently, there are no FDA-approved antibiotics that target the Lpt proteins; however, certain small molecules and peptides have been thought to engage with these proteins and are currently being investigated. For example, the Robinson group (University of Zurich) showed that the insect antimicrobial peptide thanatin<sup>160</sup> (Figure 2.1) binds LptA in the periplasm.<sup>161</sup>

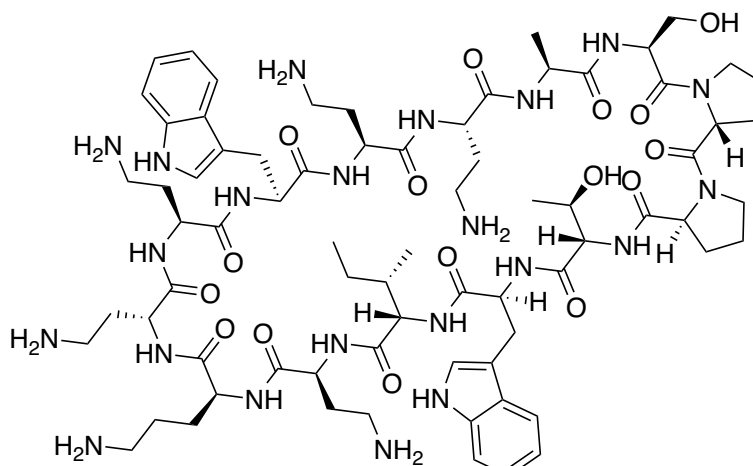


**Figure 2.1. Solution NMR structure of thanatin bound to LptA.**

Thanatin (colored in orange) is a 21 amino acid peptide containing an internal disulfide bond. LptA is shown in green.<sup>161</sup> PDB accession code 6GD5.

The same group has designed and studied other cyclic antimicrobial peptides such as murepavadin (POL-7080)<sup>162</sup>, which reportedly targets LptD (Figure 2.2). A recent study by Deborah Hung has shown that LPS modifications, similar to those produced during polymyxin therapy, ablate POL-7080 cell surface binding.<sup>163</sup> Currently this molecule is being developed by

Polyphor and is in Phase III clinical trials for *Pseudomonas* infections.<sup>164</sup> While the treatment of *Pseudomonas* infections is of high clinical interest, POL-7080 is a narrow spectrum antibiotic and is not active against other Gram-negative bacteria that also contain LptD. For this reason, it would be useful to discover antibiotics or combinations that had activity across different genera.

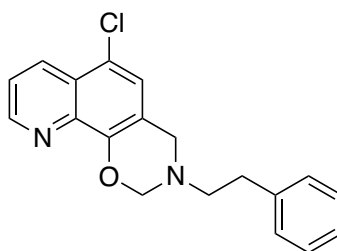


**Murepavadin (POL-7080)**

**Figure 2.2. Structure of Murepavadin which reportedly binds LptD.**

Currently POL-7080 is in Phase III clinical trials for treatment of *Pseudomonas* infections.

In April 2019, the small molecule IMB-881 (Figure 2.3) was reported to block the interaction between LptA and LptC, preventing LPS transport.<sup>165</sup> The compound was discovered from a yeast two-hybrid system that monitored the association between LptA and LptC, and was reported to block association between these Lpt proteins, possess antibacterial activity, and accumulate membrane material in the periplasm.<sup>165</sup> Since this compound was recently discovered, its safety and performance as a Gram-negative antibiotic agent have yet to be assessed.

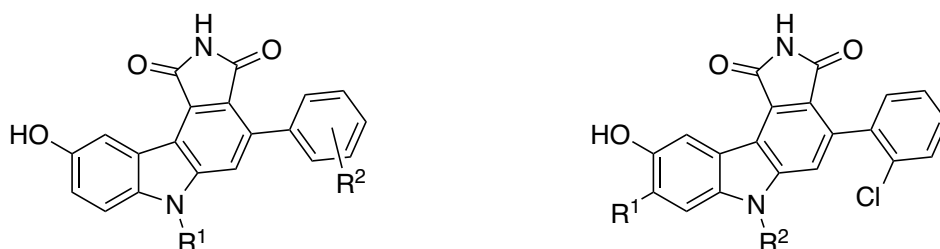


**IMB-881**

**Figure 2.3. Structure of IMB-881, a putative LptA/LptC inhibitor.**

IMB-881 displays activity against clinical carbapenem-resistant *E. coli* strains, clinical carbapenem-resistant *E. cloacae* strains, and a clinical isolate of carbapenem-resistant *K. pneumoniae*.

Compounds that inhibit the inner membrane LptB<sub>2</sub>FGC complex *in vitro* were discovered by our group by screening for inhibitors of ATPase activity.<sup>157, 166</sup> A collection of compounds from kinase inhibitor libraries were found to inhibit LptB and the full LptB<sub>2</sub>FGC complex (Figure 2.4); however, these compounds do not appear to be cell permeable and consequently have no activity against wildtype *E. coli*.



**Figure 2.4. Structure classes of putative LptB<sub>2</sub>FGC inhibitors.**

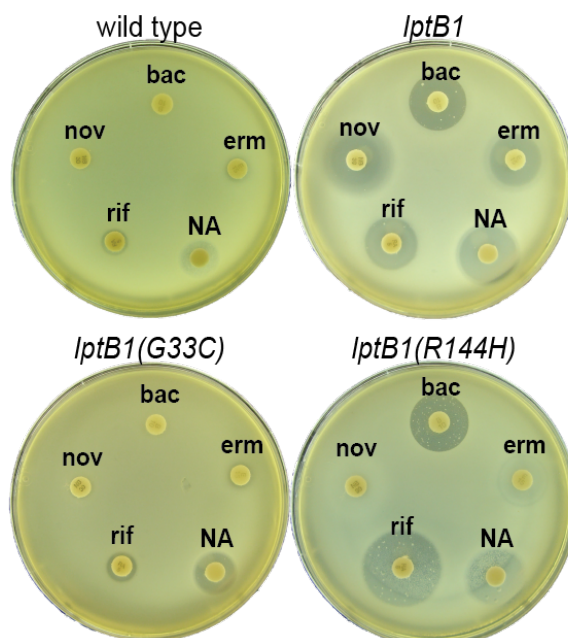
Hits were identified by screening for ATPase inhibition<sup>166</sup> of LptB<sub>2</sub>FGC (in detergent micelles).

Shortly after publishing the results of these screening efforts, Prof. Natividad (Natacha) Ruiz at the Ohio State University found genetic evidence that the small molecule novobiocin interacts with LptB. Novobiocin, as mentioned before, is an antibiotic that is widely recognized to bind and inhibit the GyrB subunit of DNA gyrase.<sup>167-171</sup> and other related DNA-binding targets such as topoisomerase IV.<sup>172-174</sup> Together with several students and postdocs in the lab, I have shown that novobiocin stimulates LPS transport in reconstituted systems. Janine May was the first to obtain structural evidence for the interaction. I joined the lab at a time when we were interested in the moieties that were responsible for Lpt binding and activity, and my task was to modify novobiocin for the purposes of improving its activity on Lpt and better elucidating its function. This section will discuss our evidence that novobiocin interacts with LptB, the chemistry used to synthesize novobiocin analogs, and the development of methods to characterize novobiocin-LptB interactions.

## 2.2. Results

### 2.2.1. Identification of an LptB mutant sensitive to a wide range of antibiotics

In the course of investigating the functional role of the C-terminus of LptB, which plays important roles in dimerization and turnover of the enzyme<sup>175, 176</sup>, Professor Natacha Ruiz (The Ohio State University) serendipitously prepared a mutant gene, *lptB1*, in which a frameshift mutation led to the creation of a 34-residue sequence (ISPSPSPYGSGPEAKCRLELLRSSYSLESIGTSE) after the last amino acid of LptB instead of a desired affinity tag.<sup>177</sup> When the wildtype copy of *lptB* in *E. coli* NR754 (MC4100 *ara*<sup>+</sup>) was replaced with the *lptB1* gene, the resulting haploid strain (NR1768) was found to be sensitive to a wide range of antibiotics (Table 2.1; Figure 2.5).



**Figure 2.5. *lptB1* is hypersensitive to a panel of antibiotics.**

The zone of inhibition is related to the sensitivity of the strain towards a particular antibiotic. The antibiotic sensitivity profiles of the intragenic suppressors to *lptB1*: *lptB1*(G33C) and *lptB1*(R144H) were compared to those of wild type and *lptB1* *E. coli*. Novobiocin selectively suppressed *lptB1*(R144H).

**Table 2.1. Antibiotic sensitivity measurements of *lptB* mutants by disc-diffusion.**

Lawns of bacteria were grown in the presence of disks containing novobiocin (nov), bacitracin (bac), erythromycin (erm), rifampicin (rif), and nalidixic acid (NA). The diameter (in mm) of the zone of total or

partial (in parenthesis) growth inhibition around the disks was measured as an indicator of antibiotic susceptibility. The *lptB1* allele encodes a defective LptB ATPase that renders the strain generally susceptible to antibiotics when compared to the wild type. Additional intragenic suppressor mutations in *lptB1* can decrease sensitivity to all antibiotics [*lptB1*(G33C)] or to novobiocin only [*lptB1*(R144H)].

|                      | Zone of inhibition (in mm) |            |              |            |                |
|----------------------|----------------------------|------------|--------------|------------|----------------|
|                      | Bacitracin                 | Novobiocin | Erythromycin | Rifampicin | Nalidixic acid |
| wild type            | <6                         | <6         | (8)          | 8(9)       | 11(14)         |
| <i>lptB1</i>         | 17                         | 14(24)     | 16(21)       | 17         | 18(21)         |
| <i>lptB1</i> (G33C)  | (8)                        | <6         | (11)         | 9          | 12(14)         |
| <i>lptB1</i> (R144H) | 17                         | <6         | (16)         | 24         | 17(20)         |

### 2.2.2. Identification of suppressors to *lptB1* reveal a special role of novobiocin

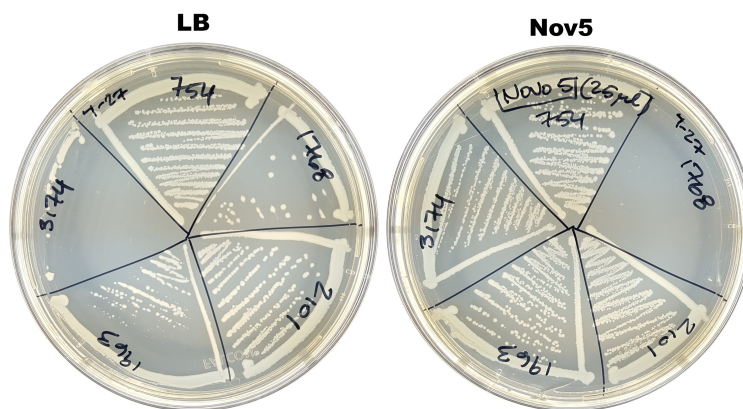
After “breaking” the LptB protein by replacing the *lptB* gene with *lptB1*, Natacha isolated mutants of bacteria that attempted to fix the permeability defect caused by the faulty LptB protein. Two intragenic suppressors arose to *lptB1*: *lptB1*(G33C) and *lptB1*(R144H). The former appeared to confer a general improvement in resistance towards bacitracin, novobiocin, erythromycin, rifampicin, and nalidixic acid whereas *lptB1*(R144H) appeared to selectively confer resistance towards novobiocin (Table 2.1).

### 2.2.3. Novobiocin suppresses growth defects caused by *lptB*(R144H)

In the course of studying the effects of novobiocin on the *lptB1*(R144H) mutation, the Ruiz laboratory prepared the *lptB*(R144H) *E. coli* strain with an otherwise wildtype background. The strain was found to have severe growth defects in rich media (e.g. LB Lennox) but could thrive when grown in the presence of 5 µg/mL novobiocin or minimal media. When plated on solid LB Lennox agar, the *lptB*(R144H) strain exhibited plating defects that could be suppressed by novobiocin. In fact, it appeared as though the strain required novobiocin to live on LB (Figure 2.6). We wondered whether novobiocin-mediated suppression was due to effects on gyrase, since slowing down the division rate can sometimes suppress mutations.<sup>178</sup> While nalidixic acid, a gyrase inhibitor, could not suppress *lptB*(R144H), a direct interaction between novobiocin and Lpt



was still unclear. Experiments designed to probe the role of novobiocin gyrase inhibition on Lpt binding and activity will be addressed in the next chapter of this thesis using a synthetic approach.

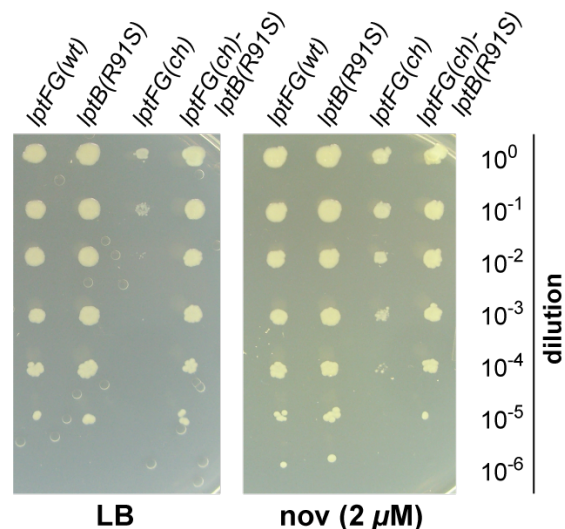


**Figure 2.6. Novobiocin suppresses the solid *lptB(R144H)* plating defect.**

The left plate was prepared with LB agar and the plate on the right with LB + 5  $\mu$ g/mL novobiocin. Clockwise from 12 o'clock: NR754 (wildtype *E. coli*), NR1768 (*lptB1*), NR2101 (NR754  $\Delta$ *lptB tet2* + *pET23/42 lptB*), NR1963 (NR754 *lptB1(R144H)::kan*), NR3174 (NR754  $\Delta$ *lptB tet2* + *pET23/42 lptB(R144H)*). This experiment was performed by the Ruiz laboratory.

#### 2.2.4. Novobiocin suppresses mutations in *lptF* and *lptG*

The Ruiz laboratory prepared a set of mutations in portions of LptF and LptG known to interact biochemically with LptB; it is noteworthy that at the time (prior to 2017) there were no published crystal structures of the LptB<sub>2</sub>FG or LptB<sub>2</sub>FGC complexes, and major evidence was drawn from genetics and biochemistry.<sup>176</sup> The coupling helices in LptF and LptG that connect in the groove regions of the LptB homodimer each contain a conserved glutamate, LptF(E84) and LptG(E88); mutations of these important glutamates to alanine results in death for the cell.<sup>176, 177</sup> The *lptF(E84A)lptG(E88A)* strain will hereafter be termed *lptFG(ch)*, *ch* = coupling helix defects. While the *lptFG(ch)* strain could not grow on LB agar, it was suppressed by plating on LB + 2  $\mu$ M novobiocin (Figure 2.7). Therefore, the *lptFG(ch)* strain behaved very similar to the *lptB(R144H)* strain. An intragenic suppressor was raised to *lptFG(ch)*, and the mutation was mapped to an arginine to serine mutation at R91 of LptB, *lptB(R91S)*, which is in the groove of LptB. Hence, defects caused by the *lptFG(ch)* mutation could be rescued by *lptB(R91S)* or novobiocin. This conclusion led us to wonder whether novobiocin were interacting near this groove.

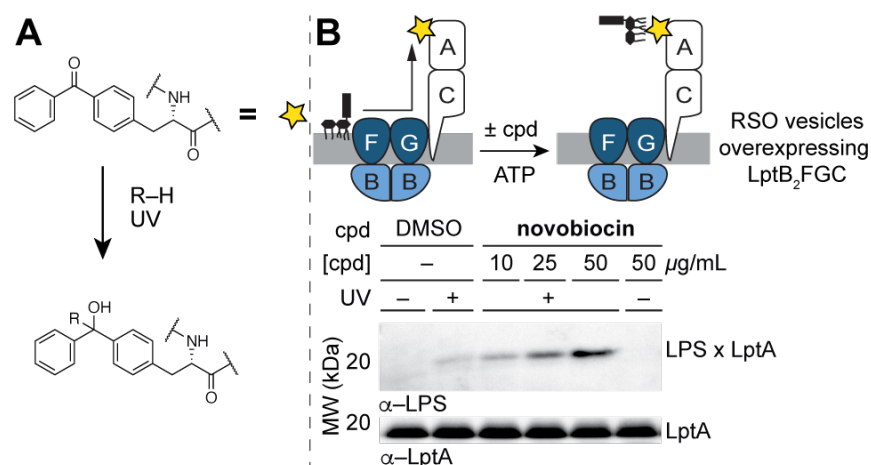


**Figure 2.7. Novobiocin rescues LPS transport phenotypes impaired by mutations in the coupling helices of LptF and LptG.**

Comparison of fitness on solid media for *lptFG(wt)*, *lptB(R91S)*, coupling helix mutant *lptFG(ch)*, and *lptFG(ch)-lptB(R91S)*. Overnight cultures grown in minimal medium were serially diluted ten-fold and spotted onto LB agar plates with and without compounds. Novobiocin rescued growth defects of the coupling helix mutant *lptFG(ch)* similarly to the genetic suppressor *lptFG(ch)-lptB(R91S)*. This experiment was performed by the Ruiz laboratory.

## 2.2.5. Novobiocin stimulates LPS transport

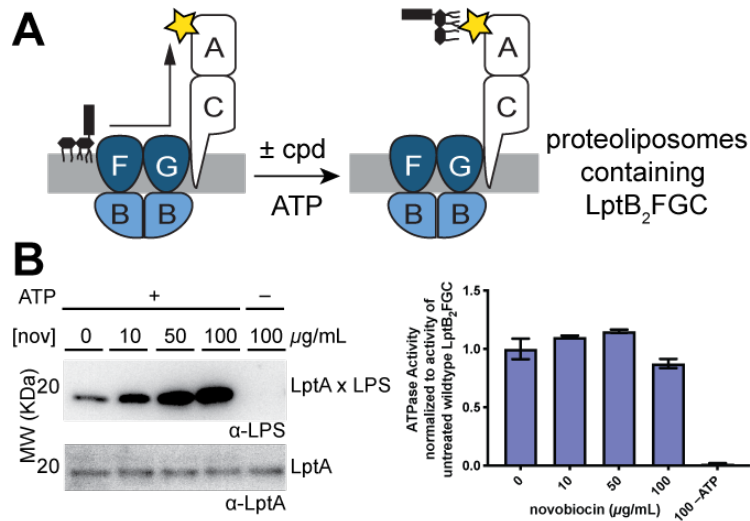
To determine if novobiocin has an effect on Lpt activity, we turned to a UV-photocrosslinking approach useful for measuring LPS flux through the complex.<sup>133, 179</sup> *E. coli* cells overexpressing untagged LptB<sub>2</sub>FGC complexes were stripped of their outer membrane with EDTA and their peptidoglycan digested with lysozyme to form spheroplasts, which were subsequently converted into right-side-out (RSO) vesicles<sup>133</sup> that possess the same proteinaceous lipid bilayer found in the bacterial inner membrane. In a reaction system possessing ATP/MgCl<sub>2</sub>, the RSOs containing overexpressed LptB<sub>2</sub>FGC complexes were mixed with purified LptA(I36pBPA) (pBPA = *p*-benzoylphenylalanine, an amino acid residue that upon UV irradiation can form covalent bonds by hydrogen atom abstraction via a triplet diradical intermediate<sup>180</sup>). With wildtype LptB<sub>2</sub>FGC, LPS is transported to LptA with the addition of ATP. As time passes, LPS is extracted from the inner membrane and accumulates on LptA, and we can visualize this interaction using the *in vivo* photocrosslinking.



**Figure 2.8. Schematic of right-side-out (RSO) vesicle experiment.**

Novobiocin stimulated LPS transport in a dose-dependent fashion. A) Scheme of protein-incorporated pBPA performing a C-H insertion reaction upon UV irradiation. B) RSO vesicle LPS release assay performed by incubating RSO vesicles containing overexpressed LptB<sub>2</sub>FGC with LptA(I36pBPA) and novobiocin for 60 minutes of incubation at 30 °C. Samples were crosslinked by UV irradiation at 365 nm and immunoblotted by Western blot to detect LPS-LptA crosslinking (see Materials and Methods section for full details).

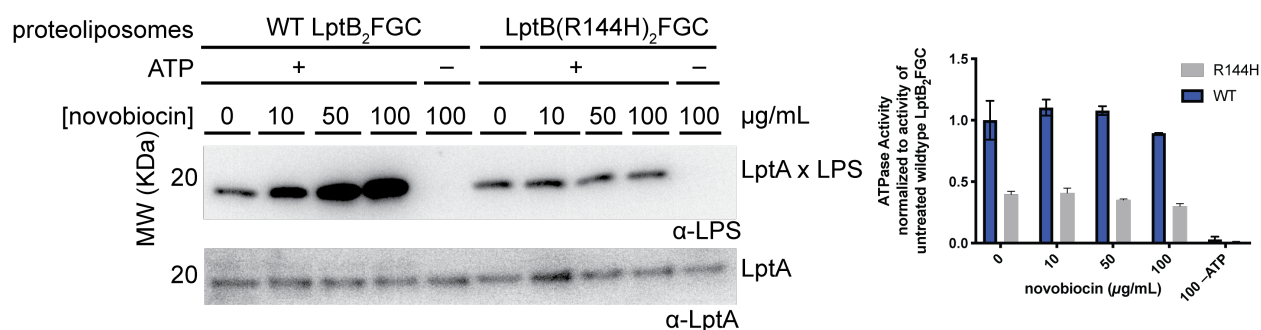
Novobiocin appeared to stimulate LPS transport in a dose-dependent fashion, as measured by the intensity of LPS crosslinked to LptA(I36pBPA) in the α-LPS blot (Figure 2.8). To ensure that the effect was specific to Lpt and was not the result of other potential targets of novobiocin on the bacterial inner membrane, we reconstituted LptB<sub>2</sub>FGC into liposomes containing LPS and performed a similar reaction with added LptA(I36pBPA) and novobiocin (Figure 2.9A). Like in the RSO experiment, novobiocin appeared to activate the LptB<sub>2</sub>FGC complex by stimulating LPS transport. With a purified system, it is possible to measure the ATPase activity using a colorimetric assay sensitive to the presence of the inorganic phosphate ion  $P_i$ ,<sup>181</sup> and it was found that novobiocin did not alter the amount of ATP hydrolyzed after 60 minutes (Figure 2.9B). We concluded that novobiocin likely accelerates an ATP hydrolysis-independent step in LPS transport.



**Figure 2.9. Schematic of *in vitro* reconstitution of LPS transport with proteoliposomes.**

A) Schematic of proteoliposome LPS release assay. Purified LptB<sub>2</sub>FGC and Ra-LPS was incorporated into liposomes via the detergent-dilution method.<sup>150</sup> B) Novobiocin stimulated LPS transport in a dose-dependent fashion as measured by UV crosslinking to LptA(I36pBPA) after 60 min of incubation at 30 °C (see experimental section for full details). An ATPase assay revealed no significant change in endpoint ATP hydrolysis after 60 min with [ATP]<sub>i</sub> = 5 mM.

Since the *lptB(R144H)* mutation appears to be related to novobiocin, we wondered how novobiocin would affect LPS transport in Lpt complexes containing this mutation. The effect of the novobiocin on the activity of the LptB(R144H)<sub>2</sub>FGC complex was investigated in the same purified proteoliposome system. As observed by others,<sup>182</sup> LptB(R144H)<sub>2</sub>FGC possessed weaker LPS transport activity relative to the wildtype system, as well as a defect in ATP hydrolysis. Karanbir Pahil of the Kahne group and Brent Simpson of the Ruiz group have investigated the effects of the *lptB(R144H)* mutation and the *lptB(F239A)* mutation, which are both deleterious, but together suppress each other.<sup>183</sup> Novobiocin did little to alter the LPS release activity of LptB(R144H)<sub>2</sub>FGC (Figure 2.10).

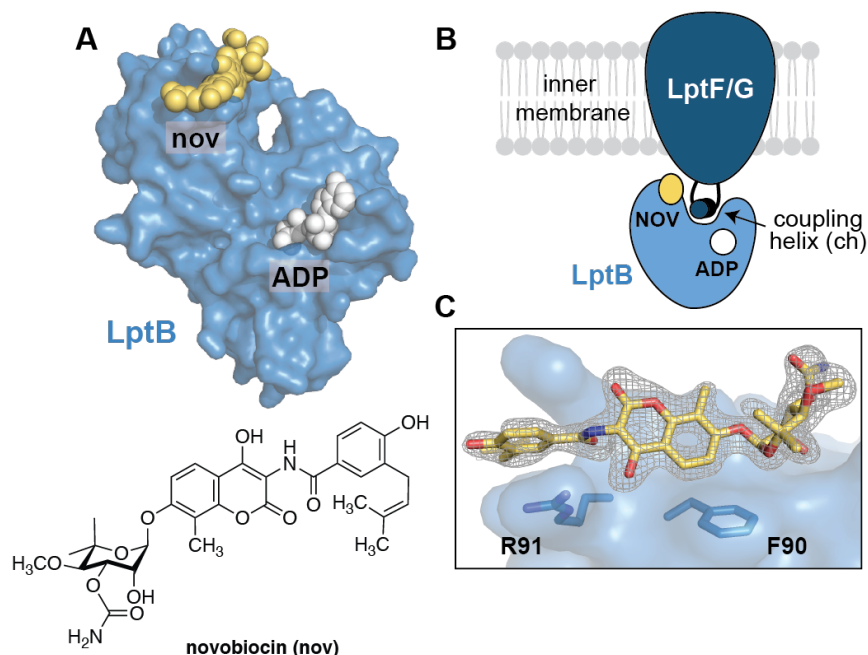


**Figure 2.10. Effect of novobiocin on LPS release and ATPase activity by wildtype LptB(R144H)<sub>2</sub>FGC.**

Purified *E. coli* wildtype LptB<sub>2</sub>FGC and LptB(R144H)<sub>2</sub>GC proteins were incorporated into liposomes as in Figure 2.7. Novobiocin effected a dose-dependent LPS transport stimulation in proteoliposomes containing wildtype complexes, but did not appear to stimulate complexes containing the R144H mutation. Novobiocin did not appear to affect ATPase activity of either complex.

## 2.2.6. Novobiocin was co-crystallized with LptB and was found in the groove

Following the biochemical and genetic experiments that posited a direct interaction between LptB and novobiocin, Janine May crystallized LptB<sup>177</sup> following similar conditions to those that David Sherman used earlier to crystallize LptB-His<sub>8</sub> in the ADP/Mg<sup>2+</sup> bound form.<sup>175</sup> Once Janine successfully reproduced the crystallization conditions, she soaked novobiocin into the crystals, obtained a 2Å dataset, (Figure 2.11) and solved the structure with Michael Lazarus. The structure unambiguously revealed the presence of a novobiocin molecule located in the groove region of LptB, where the coupling helices of LptF and LptG were predicted to interact with LptB.



**Figure 2.11. Novobiocin binds LptB in the groove region.**

A) A 2.0Å crystal structure of LptB complexed with novobiocin was obtained by soaking an LptB crystal grown in the presence of ADP/MgCl<sub>2</sub>. B) Novobiocin bound in the groove region near where the coupling helices of LptF and LptG interact with LptB. C) F<sub>o</sub>-F<sub>c</sub> omit map contoured at 3σ revealed clear density for novobiocin.

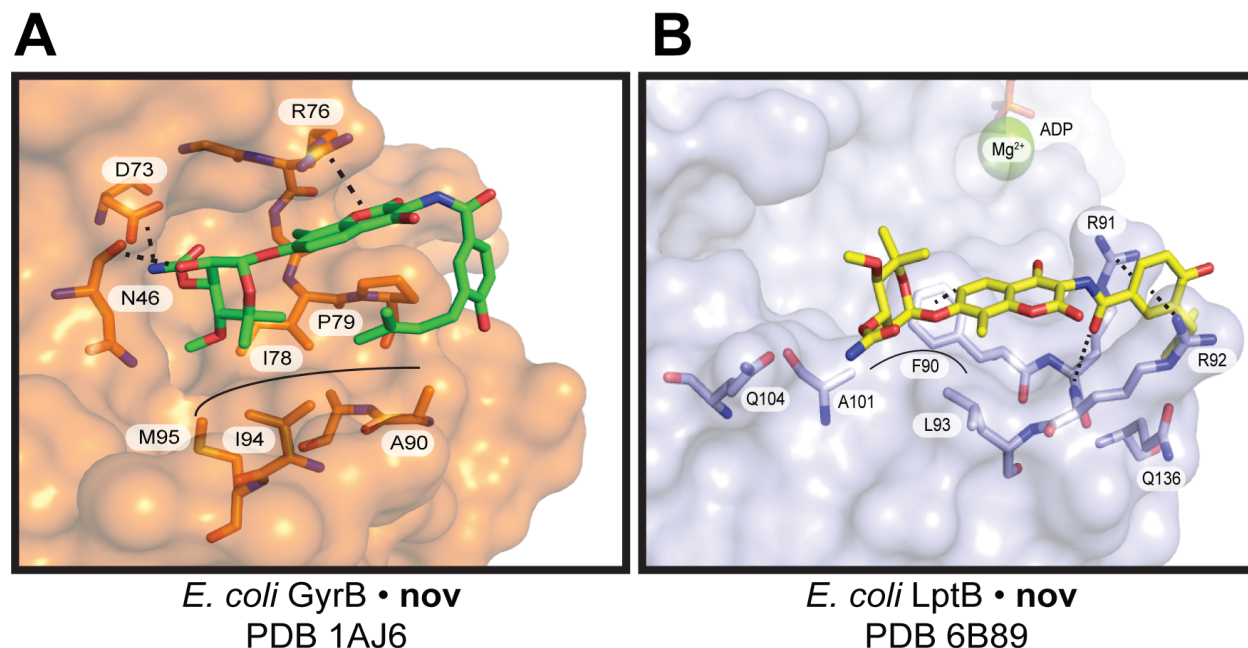
Unlike prior structures of novobiocin bound to gyrase<sup>184-186</sup> in which the benzamide is in the *cis* conformation, the LptB•novobiocin structure revealed a different binding mode, with the novobiocin benzamide in the *trans* conformation (Figure 2.12). Furthermore, unlike novobiocin binding to gyrase<sup>184-186</sup>, topoisomerase IV<sup>174, 185</sup>, and the C-terminus of Hsp90<sup>187</sup>, novobiocin binding to Lpt did not overlap with the ATP binding site. This suggested the possibility of an allosteric modulation to the enzymatic activity of LptB, perhaps by altering the coupling of transmembrane LptF and LptG helix movement with nucleotide hydrolysis by LptB.

### 2.2.7. Decoupling the gyrase activity of novobiocin by chemical modification

We wanted to understand which portions of novobiocin were important for Lpt binding and which were important for gyrase binding.<sup>170, 171</sup> We compared the X-ray crystal structures of novobiocin bound to the N-terminal 24 kDa fragment of GyrB and novobiocin bound to LptB, and



we reasoned that both benzamide and L-noviose moieties made important contacts in binding to gyrase (Figure 2.12); however, it seemed easier for us to initially modify the benzamide rather than try to functionalize the sugar. The goal of synthetic modification would be to reduce gyrase binding in order to better study the effects on Lpt.



**Figure 2.12. Comparison of novobiocin binding GyrB and LptB.**

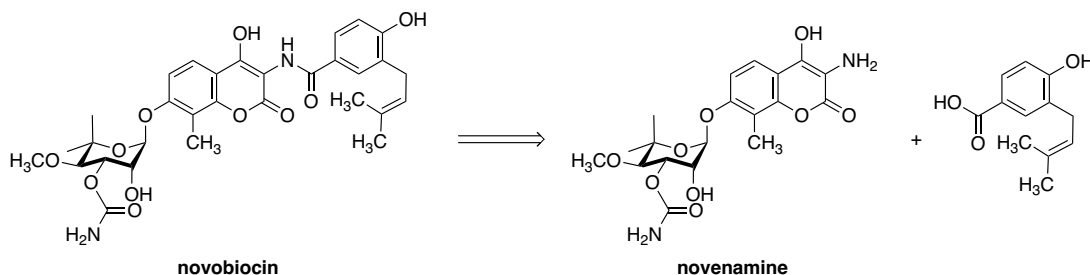
A) The ATP binding site in gyrase is occluded by the competitive inhibitor novobiocin<sup>184</sup> (note, there is no nucleotide present in 1AJ6). The L-noviose sugar appears to make critical interactions important for binding to gyrase and novobiocin is present in the *cis*-amide conformation. B) Novobiocin bound to LptB<sup>177</sup> does not overlap with the nucleotide binding site.

Examination of the chemical structure of novobiocin<sup>188</sup> and methods in the literature to modify the scaffold<sup>189-194</sup> suggested that late-stage functionalization would be the most ideal route for us to make the modifications we wanted. The alternative strategy would be either total synthesis of aminocoumarin derivatives or mutasynthesis. The total synthesis of aminocoumarin antibiotics is possible<sup>195, 196</sup>; however, these approaches either require novobiocin as the starting material (i.e. to obtain the L-noviose sugar for the synthesis of coumermycin A1 and derivatives) coupled with many atom uneconomical steps. The total, asymmetric synthesis of the (+)-L-noviose sugar alone required fifteen steps, and was achieved by Ogasawara's group in 2000.<sup>197</sup> Asymmetric syntheses

of (–)-D-noviose have also been published<sup>198, 199</sup>, but accessing the starting materials for the correct enantiomer are prohibitively expensive. Mutasynthesis of aminocoumarins was another option,<sup>200-202</sup> but we reasoned that this approach would 1) severely limit the diversification of the scaffold and 2) might require the engineering of several biosynthetic enzymes from the producer *Streptomyces niveus*<sup>203</sup> or *Streptomyces spheroides*.<sup>204</sup> In selecting late-stage functionalization as our approach, we could start with an inexpensive, yet complex molecule with most atoms in the proper place. The challenge of such an approach is to find conditions for achieving transformations that occur selectively, without functional group interference or destruction of sensitive moieties. The benzamide was selected as a fragment of novobiocin that could be swapped easily for other groups.

### 2.2.8. Initial chemical degradation of novobiocin

To generate a versatile scaffold for late stage functionalization, I explored different methods to chemically degrade novobiocin. A route for the chemical substitution of the novobiocin benzamide involved the use of novobiocin sodium (Sigma-Aldrich, \$30/g ca. 2016) as the starting material (Figure 2.13 and 2.14).



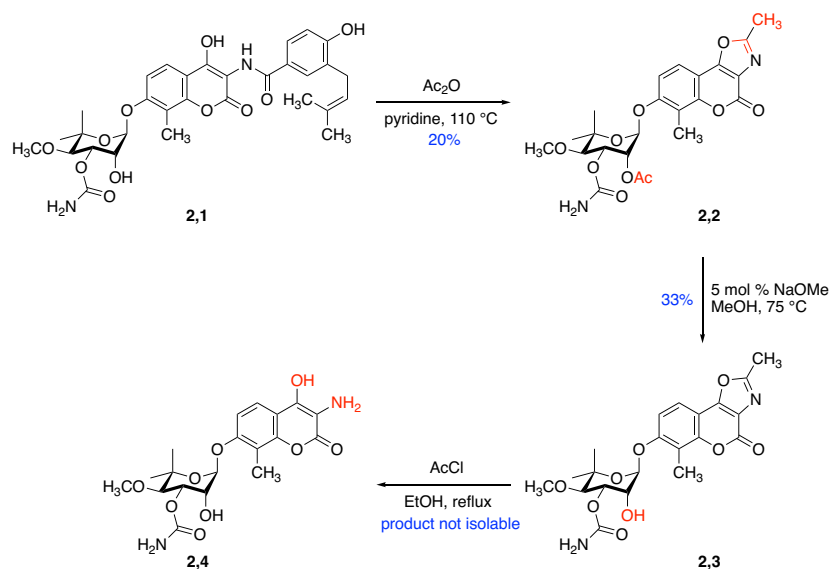
**Figure 2.13. Selective amide bond cleavage leads directly to novenamine.**

Novenamine is a versatile nucleophilic fragment of novobiocin, which could be acylated with a wide variety of electrophiles to generate derivatives. We envisioned utilizing novobiocin as a starting material because it was relatively inexpensive (~\$30/g in 2016). The forward cleavage of the amide bond in novobiocin was not immediately trivial; other acid and base-sensitive functional groups precluded this transformation.

A 2013 paper<sup>205</sup> described the formal hydrolysis of the benzamide moiety via the intermediacy of an oxazole **2,2** generated from treatment of **2,1** with acetic anhydride and pyridine under refluxing



conditions. Hinman et al. were the first to observe the conversion of novobiocin to **2,2**,<sup>206</sup> and since 1956, others have considered the oxazole route to generate key intermediates for aminocoumarin derivative synthesis.<sup>190, 192</sup> Unintended acetylation of the axial sugar hydroxyl occurred during this initial step, and **2,2** was saponified with NaOMe in MeOH to afford **3** in 33% yield (Figure 2.14). The low isolated yield for this step stems from the base lability of the L-noviose sugar, which can be deprotonated at O2 and attack the anomeric carbon, displacing a phenoxide leaving group. The oxazole in **2,3** was then opened by treatment with acetyl chloride in refluxing ethanol to yield novenamamine **2,4**. Unfortunately, **2,4** was not amenable to silica gel column chromatography, as the highly electron-rich aniline ring system is extremely prone to oxidation; **2,4** in the free base form (NH<sub>2</sub>) should be handled in an anoxic environment to prevent oxidation, which occurs within seconds upon exposure to air to form a pink product. Since it did not appear that this route would provide the quantities of **4** that we envisioned needing for our experiments, I searched the research and patent literature for alternative methods.

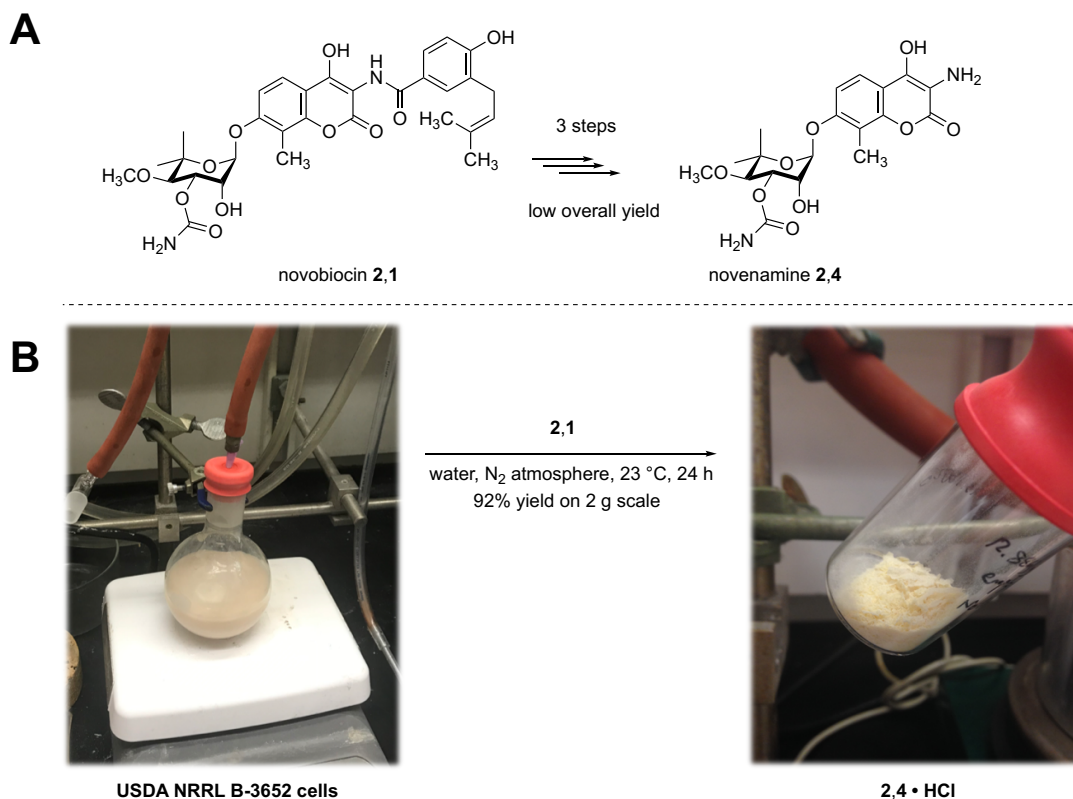


**Figure 2.14. Novobiocin benzamide cleavage by oxazole formation.**

The chemical route<sup>205</sup> for the degradation of novobiocin **2,1** to novenamamine **2,4** was plagued by low yields and harsh reagents/conditions (**2,1** to **2,2** to **2,3** to **2,4**). A direct conversion (**2,1** to **2,4**) was desired.

### 2.2.9. Biocatalytic benzamide hydrolysis

After several failed attempts to convert **2,1** to **2,4**, I found a United States Patent from The Upjohn Company filed in 1971<sup>207</sup> and an accompanying publication.<sup>193</sup> According to the authors, **2,1** could be directly converted into **2,4** by culture containing *Arthrobacter* sp. culture (USDA NRRL B-3652), which expresses an amidase capable of recognizing novobiocin as a substrate. The process did not require cell lysis or purification of the enzyme; rather, a heavy cell suspension in distilled water could be mixed with an aqueous solution of novobiocin sodium under a N<sub>2</sub> atmosphere for 16-24 h to afford nearly quantitative yields of **2,4**. I ordered the bacterial strain from the U.S. Department of Agriculture (USDA) in Peoria, IL, and after culturing a clonal population of the novobiocin-cleaving bacterium, I adapted this process to work in 250 mL round bottom flasks (Figure 2.15). Dr. Vadim Baidin and I were interested in this enzyme, and we sequenced the organism and found that it was a member of the *Comamonas* genus, not *Arthrobacter* (Appendix).



**Figure 2.15. Setup for the bioenzymatic cleavage of novobiocin.**

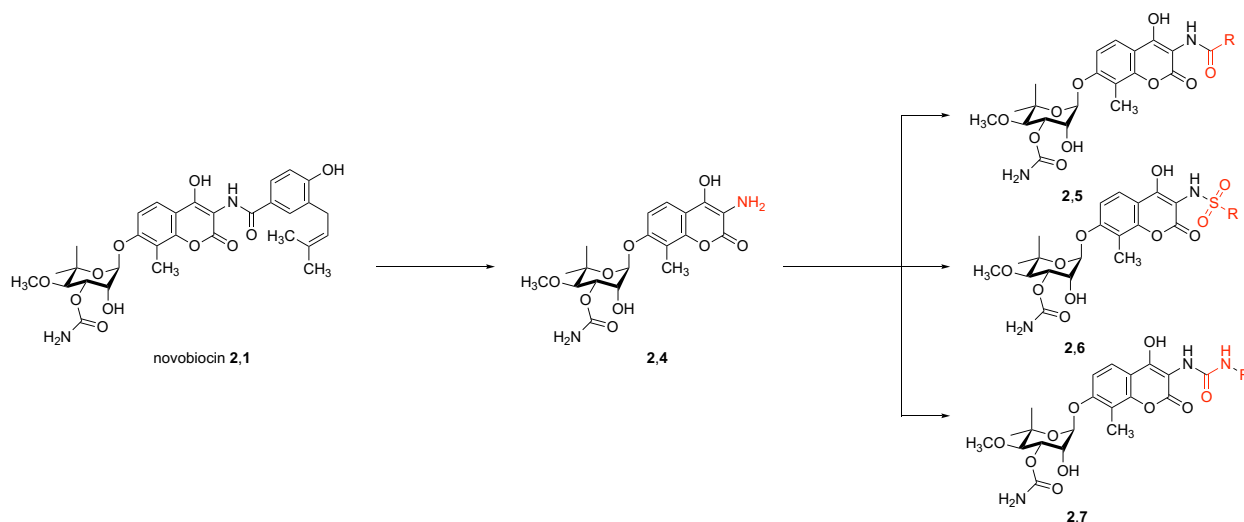
A) Benzamide hydrolysis by chemical means was step-intensive and suffered from low overall yield. B) Biocatalytic hydrolysis of novobiocin **2,1** to novenamine **2,4** was performed at multigram scales, using water as the only solvent and proceeded to near completion at room temperature in neutral water.

The enzymatic cleavage of novobiocin<sup>193</sup> could be performed on multigram scale using a heavy cell suspension of NRRL B-3652. Following an overnight culture and growth in NZ-amine-B media<sup>207</sup> for 1-2 days at 28 °C, cells were harvested and then resuspended in a minimal volume of distilled water to make a heavy cell suspension. An equal volume (e.g. 50 mL) of heavy cell suspension was mixed with 40 mg/mL novobiocin sodium (2 g, 50 mL total volume in H<sub>2</sub>O), and stirred at 23 °C, under a N<sub>2</sub> atmosphere for 16-24 h. The reaction was quenched with 1 M HCl to form the air-stable novenamine•HCl. Cell debris was removed by centrifugation at 10,000 g and the supernatant was extracted with an equal volume of *n*-butyl acetate to remove the benzoic acid byproduct and unreacted novobiocin. The aqueous layer was concentrated under reduced pressure to afford novenamine•HCl (**2,4** • HCl) as a yellow solid (1.33 g, 92% yield).

**2.2.10. Synthesis of novobiocin derivatives with reduced gyrase activity**

We were interested in knowing if the effect that novobiocin has on Lpt is due to gyrase binding. To address this question, we turned to a synthetic decoupling approach. With the ability to generate novenamine **2,4** in multigram quantities, I began by acylating commercially available carboxylic acid, sulfonic acid, and isocyanate fragments to generate a small library of benzamide derivatives (Figure 2.16). A full list of the novobiocin derivatives appear in the Appendix. I tested gyrase inhibitory activity by a DNA supercoiling assay using commercial gyrase assay kits from Inspiralis. In this assay, a bacterial gyrase is incubated with relaxed pBR322 DNA, ATP-containing buffer, and novobiocin derivative. The reaction mixture is incubated at 37 °C for 30 minutes and then quenched with STEB buffer. The DNA is then subject to agarose gel electrophoresis and stained with intercalator post-electrophoresis (the dye affects the differential electrophoresis of

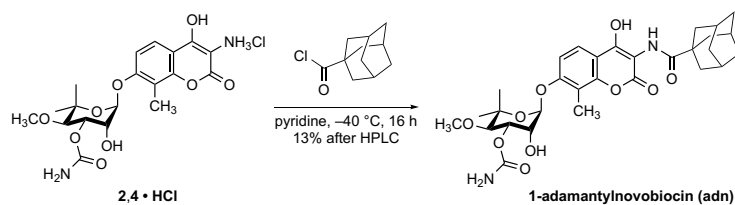
topoisomers). Relaxed topoisomers of DNA are less compact and therefore migrate more slowly through the gel than supercoiled topoisomers.

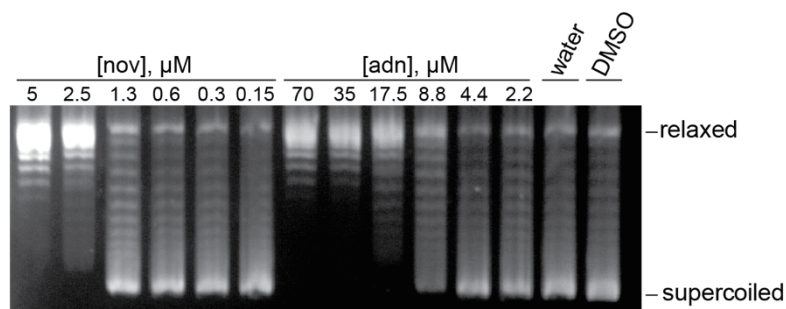


**Figure 2.16. Synthesis of novobiocin benzamide derivatives.**

Novenamamine **2,4** (1.3 equiv.) was sparged with N<sub>2</sub> in a 2-dram vial equipped with a septum cap and vent needle for 5 minutes. Then, pyridine (0.1 M) was added and the electrophile was added as a solution in pyridine. The mixture was stirred at 23 °C for 5 minutes. The vent needle was removed, and the mixture was allowed to stir for 4-24 h at 23 °C. Compounds could be purified by flash column chromatography with silica gel and eluting with 0-10% MeOH in DCM. Acyl fluorides gave higher yields of the coupling product than acyl chlorides.

The 1-adamantynovobiocin derivative (**adn**) was among the first thirty novobiocin derivatives synthesized, due to the commercial availability of the 1-adamanatanecarboxylic acid. The reason this group was chosen was to see if the adamantyl group could ablate the *cis/trans* peptide bond isomerism needed for novobiocin inhibitory activity against gyrase. The **adn** derivative was found to be about five-fold less active against *E. coli* gyrase compared to novobiocin (**nov**), with apparent IC<sub>50</sub>s of 13 and 1.9 μM, respectively (Figure 2.17).





**Figure 2.17. Synthesis of 1-adamantylnovobiocin and gyrase activity.**

The 1-adamantylnovobiocin (**adn**) derivative possesses weaker inhibitory activity against DNA gyrase compared to novobiocin (**nov**).

I sent our novobiocin derivative that is less potent against DNA gyrase to Natacha Ruiz's lab for further testing. Two additional compounds, cyclopropylnovobiocin and novenamine • HCl (**2,4 • HCl**) were also sent. There, a chemical conditionality<sup>100</sup> was observed in terms of differential effects on a variety of *E. coli* mutants (Figure 2.18).

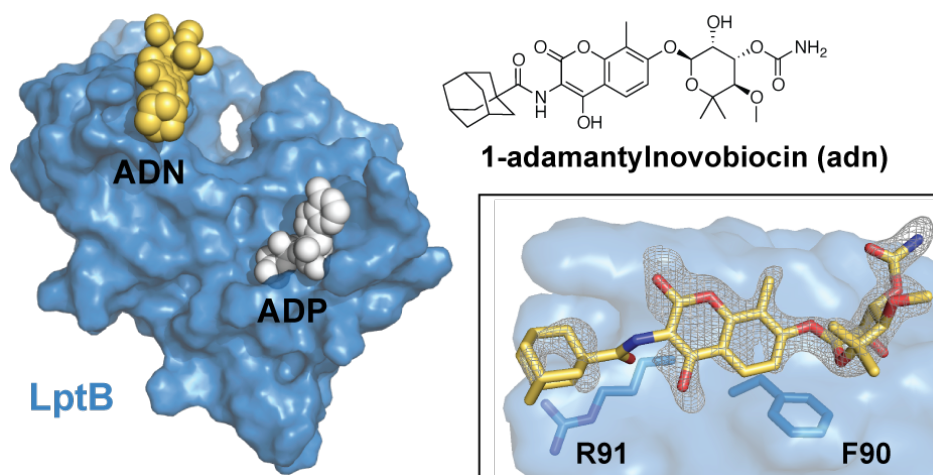


**Figure 2.18. Effects of novobiocin and 1-adamantylnovobiocin on Ruiz lab *E. coli* *lpt* mutants.**

NR754 (wildtype *E. coli*), NR1768 (*lptB1*), NR2101 (NR754  $\Delta lptB tet2 + pET23/42 lptB$ ), NR1963 (NR754 *lptB1(R144H)::kan*), NR3174 (NR754  $\Delta lptB tet2 + pET23/42 lptB(R144H)$ ). This experiment was performed by the Ruiz laboratory.

Novobiocin (5  $\mu\text{g/mL}$ ) suppressed *lptB(R144H)* but killed *lptB1*; 1-adamantylnovobiocin (5  $\mu\text{g/mL}$ ) suppressed *lptB(R144H)* but did not kill *lptB1*. Thus, the 1-adamantylnovobiocin derivative can rescue LptB associated growth defects independent of its ability to modulate gyrase activity. To confirm this, I co-crystallized LptB with 1-adamantylnovobiocin and obtained a co-complex to 1.95

Å resolution in collaboration with Tristan Owens and Michael Lazarus (Figure 2.19). This provided structural evidence that the artificial novobiocin analog could still bind LptB.

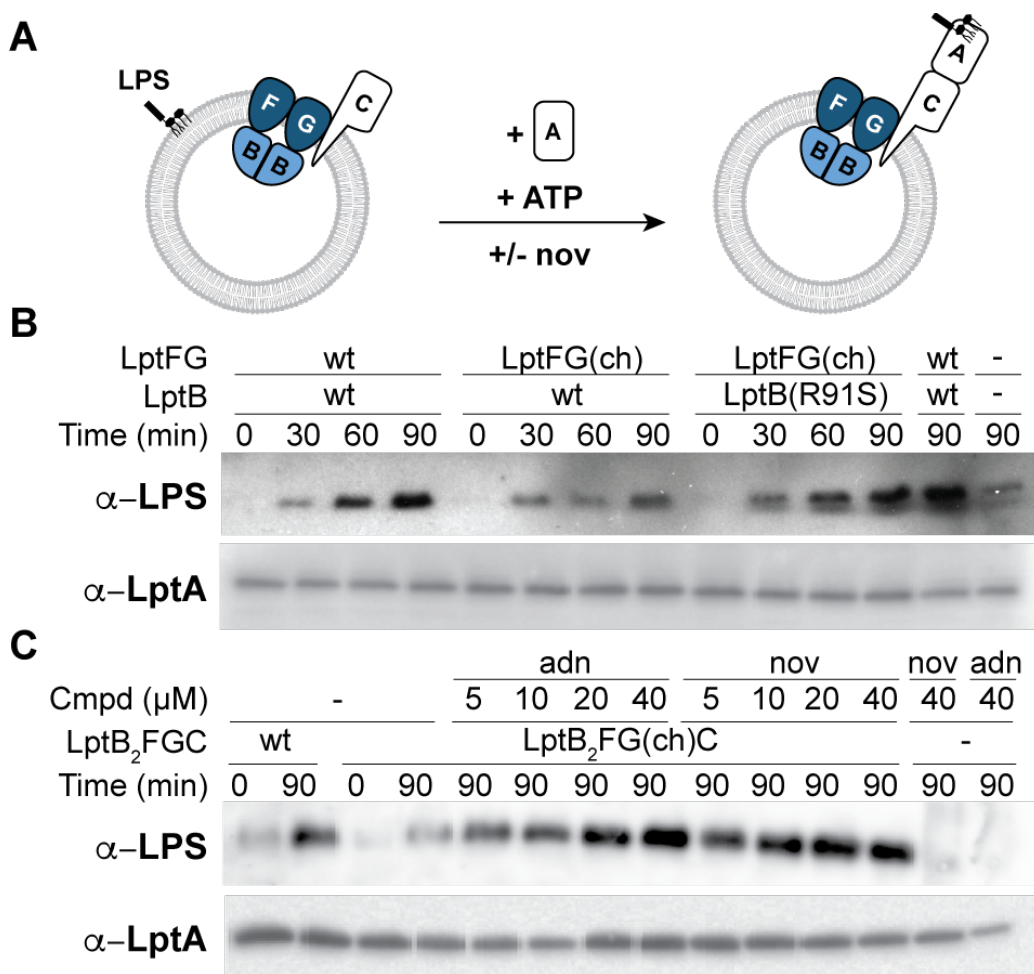


**Figure 2.19. Co-complex of 1-adamantylnovobiocin and LptB.**

Crystals were grown by vapor diffusion in hanging drops at room temperature. 1  $\mu$ L protein solution was mixed with 1  $\mu$ L reservoir solution consisting of 100 mM MES (pH 6.5), 30% (wt/vol) PEG 4000, and 1.3 mM 1-adamantylnovobiocin solution (final droplet [DMSO] = 7%). Crystals were flash-frozen in cryoprotectant containing 100 mM MES (pH 6.5), 33% PEG 4000, 24% glycerol, and 2.5 mM 1-adamantylnovobiocin. PDB code 6B8B.

This structure revealed that 1-adamantylnovobiocin binds in the same spot as novobiocin. There were very few differences between the LptB•**nov** and LptB•**adn** structures besides movement of the arginine residues (R91 and R92) to accommodate the adamantane cage. Clearly the cation- $\pi$  interaction between the novobiocin benzamide is not necessary for aminocoumarin binding to LptB. We set out to probe biochemical effects of novobiocin binding to LptB<sub>2</sub>FGC using our *in vitro* right-side-out vesicle assay for measuring LPS transport to LptA(I36pBPA). Tristan Owens prepared right-side-out vesicles containing overexpressed LptB<sub>2</sub>FGC, LptB<sub>2</sub>FG(ch)C, and LptB(R91S)<sub>2</sub>FG(ch)C. We found that vesicles containing LptB<sub>2</sub>FG(ch)C were relatively defective in LPS transport compared to wildtype complexes, but the defect could be suppressed by LptB(R91S) (Figure 2.20). Moreover, both the aminocoumarins 1-adamantylnovobiocin (**adn**) and novobiocin (**nov**) could phenocopy the effect of the LptB(R91S) suppressor and rescue LPS transport to wildtype levels (Figure 2.20).





**Figure 2.20. Aminocoumarins rescue LPS transport phenotypes impaired by mutations in the coupling helices of LptF and LptG.**

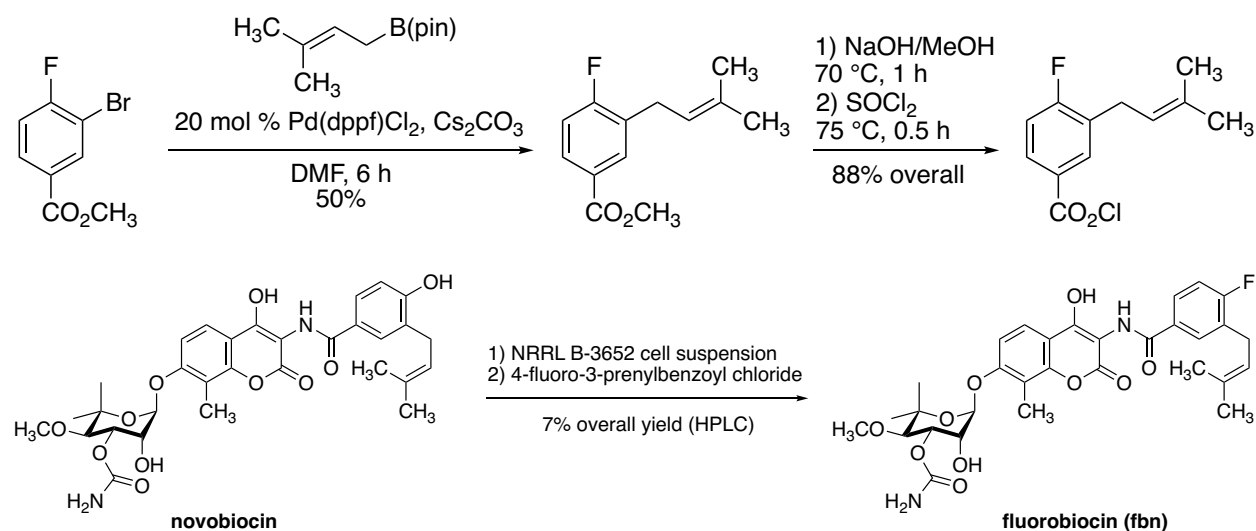
A) Schematic of right-side-out vesicle assay. LptA(I36pBPA) was added to right-side-out vesicles containing overexpressed LptB<sub>2</sub>FGC mutants and the mixture was incubated with ATP/Mg<sup>2+</sup> and aminocoumarins or vehicle at 30 °C. Samples were taken out at various timepoints for photocrosslinking at 365 nm to crosslink LPS to LptA(I36pBPA). B) The coupling helix mutants LptF(E84A) and LptG(E88A), taken together = LptFG(ch), form a transporter that is defective in LPS transport, but can be suppressed by LptB(R91S). LptC was present in these complexes. C) Suppression of LptB<sub>2</sub>FGC(ch)C can also be accomplished by the aminocoumarins novobiocin (**nov**) and 1-adamantynovobiocin (**adn**). The biochemical experiments were performed by Dr. Tristan Owens.

We did not have a quantitative method for measuring binding affinities of novobiocin derivatives to LptB and our next objective was to develop a tool we could use to quantify our compounds.

### 2.2.11. Development of a <sup>19</sup>F NMR binding assay for measuring LptB binding

The relative absence of fluorine in biomolecules coupled with the inherent sensitivity of the <sup>19</sup>F nucleus to NMR spectroscopy makes fluorine NMR an attractive technique for probing

biological systems.<sup>208-212</sup> After speaking to Dr. Greg Heffron, I was led to Dr. Walter Massefski (then the director of the Dana Farber Cancer Institute NMR core) who had the relevant expertise. He suggested that we perform a titration experiment in which the concentration of a fluorinated novobiocin derivative was maintained constant while the protein concentration [LptB] was increased. I synthesized the fluorinated novobiocin derivative fluorobiocin (**fbn**) by deconstruction/reconstruction of novobiocin (Figure 2.21).

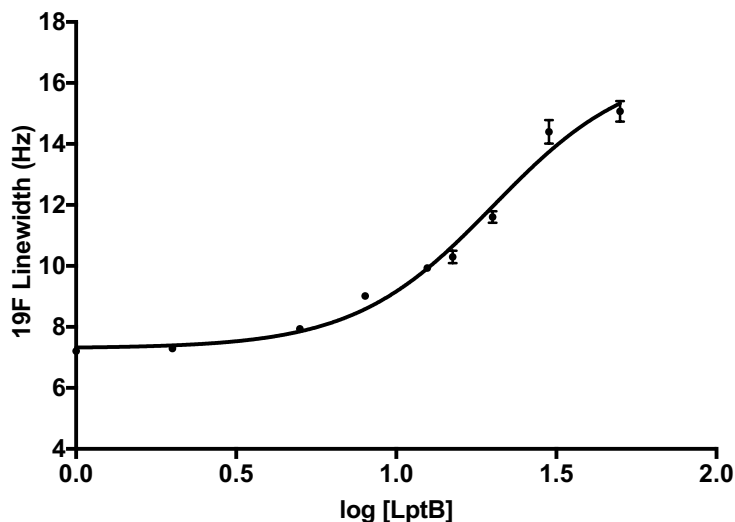


**Figure 2.21. Synthesis of fluorobiocin.**

Fluorobiocin (**fbn**) was prepared from enzymatic degradation of novobiocin to novenaminate followed by peptide coupling with the prenylated benzoic acid derivative. Experimental details in methods section.

LptB was purified in 50 mM Tris, pH 8.0 buffer containing 150 mM NaCl and ADP/ $\text{Mg}^{2+}$  was added at physiologically relevant concentrations (5 mM). Fluorobiocin (50  $\mu\text{M}$ ) was added to solutions of increasing protein concentration, and 1D  $^{19}\text{F}$  NMR spectra were recorded at 298.1 K. The data (Figure 2.22) reveal that fluorobiocin was a low micromolar binder, with a measured  $K_D$  of  $20 \pm 9$   $\mu\text{M}$ .





**Figure 2.22. Fluorobiocin-LptB binding assay.**

The  $^{19}\text{F}$  NMR linewidth of fluorobiocin (**fbn**) was plotted vs.  $\log[\text{LptB}]$ . Data were analyzed with GraphPad Prism 7 software using a nonlinear fit,  $\log(\text{agonist})$  vs. response with an  $R^2 = 0.98$ . Error bars represent the standard error of the mean ( $\pm\text{SEM}$ ). The  $K_D$  was calculated to be  $20 \pm 9 \mu\text{M}$ .

## 2.3. Discussion

This chapter describes the discovery that the natural product antibiotic novobiocin binds LptB. We used genetic, biochemical, and crystallographic tools to establish that novobiocin interacts with LptB at the groove region, at the interface with the LptF and LptG binding sites. Furthermore, we showed that novobiocin has a stimulatory effect on LPS transport. This was shown by *in vitro* photocrosslinking in liposomes containing purified LptB<sub>2</sub>FGC + LptA(I36pBPA) and in right-side-out vesicles containing overexpressed LptB<sub>2</sub>FGC + LptA(I36pBPA). A novobiocin derivative (1-adamantylnovobiocin) with less inhibitory activity on gyrase was shown to retain binding and stimulatory activity on LptB. This showed that novobiocin might engage Lpt in a manner that is not dependent on gyrase inhibition. Finally, a fluorine-19 NMR binding experiment was performed using  $^{19}\text{F}$ -labeled novobiocin (fluorobiocin) to probe the binding affinity for LptB.

The mechanism behind novobiocin-induced stimulation of LPS transport is still subject to investigation. Since publication of the initial discovery, we have shown that in whole *E. coli* cells overexpressing LptB<sub>2</sub>FGC(T47pBPA), LptB<sub>2</sub>FGC(G21pBPA), LptB<sub>2</sub>FGC(M19pBPA), novobiocin

stimulates LPS accumulation in these positions. Because LPS partitioning into the cavity of LptB<sub>2</sub>FGC (where LptC residues M19 and G21 are located) is thought to be ATP-independent,<sup>131</sup> we believe that novobiocin lowers the kinetic barrier for LPS entering the cavity of the transporter from the membrane.

We were enthusiastic to focus on novobiocin because it is a small molecule that has previously been used in humans; therefore, its physical properties are already drug-like (unlike many high throughput screening hits, for example). The results described in this chapter led us to synthesize novobiocin derivatives, which are described in Appendix. One of our reasons for doing so was to see if a novobiocin analog with better activity against Gram-negative bacteria could be discovered. Presumably a novobiocin derivative that perturbs Lpt, which is absent in Gram-positives, might be identified as more active against Gram-negatives and less so against Gram-positives.

As novobiocin did not inhibit LPS transport, but had the opposite effect, we wondered if we could leverage LPS transport stimulation to kill Gram-negative bacteria by synergy with the polymyxin family of antibiotics. The polymyxins, including colistin (polymyxin E) and polymyxin B, are extremely potent antibiotics against Gram-negative bacteria and function by binding LPS. The subsequent chapter of this dissertation will be devoted to studying the combination of novobiocin and novobiocin derivatives with polymyxin in an effort to elucidate novel mechanisms of action of synergy.

## 2.4. Materials and Methods

### 2.4.1. Strains, plasmids, and primers used

*E. coli* strain NovaBlue [*endA1 hsdR17* ( $r_{K12}^- m_{K12}^+$ ) *supE44 thi-1 recA1 gyrA96 relA1 lac F'* [*proA<sup>+</sup>B<sup>+</sup>lacI<sup>q</sup>ZΔM15::Tn10*] (*Tet<sup>R</sup>*)] from Novagen was used for plasmid manipulations. *E. coli* strain KRX [*F'*, *traD36, ΔompP, proA<sup>+</sup>B<sup>+</sup>, lacI<sup>q</sup>, Δ(lacZ)M15 ΔompT, endA1, recA1, gyrA96*

(*Nal<sup>r</sup>*), *thi-1*, *hsdR17* (*rK<sup>-</sup>*, *mK<sup>+</sup>*), *e14<sup>-</sup>* (*McrA<sup>-</sup>*), *relA1*, *supE44*,  $\Delta$ (*lac-proAB*),  $\Delta$ (*rhaBAD*)::T7 RNA polymerase] from Promega was used for protein purification. *E. coli* strain BL-21( $\lambda$ DE3) [*F- ompT gal dcm lon hsdSB(rB<sup>-</sup>, mB<sup>-</sup>)*  $\lambda$ (DE3)] from Novagen was used for right-side-out vesicle preparation.

The *lptB1* mutant was isolated serendipitously while attempting to add the codons for a C-terminal sequence containing a poly-histidine tag immediately upstream of the stop codon of chromosomal *lptB*. Primers 5HisTagP1 and P2 were used to amplify a kanamycin-resistance cassette using pKD4<sup>213</sup> as a template in PCR. The resulting amplified sequence was then used as a template in another PCR using the 5LptBhis and 3LptBP2 primers. The resulting PCR product was flanked by regions of homology to the site of insertion in the *lptB* chromosomal locus. Insertion of the recombinant DNA into strain DY378 was accomplished using recombineering and selecting for kanamycin resistance.<sup>214</sup> The *lptB* locus was then amplified from kanamycin-resistant recombinants using primers 5LptB77Up and 3LptBDOWN in PCR. Sanger DNA sequencing of the resulting PCR product from one of the recombinants revealed that a 5-bp deletion had occurred immediately after the last codon of wild-type *lptB* and within the sequence encoding the tag. This deletion resulted in a frameshift that causes the addition of a 34-residue sequence (ISPSPSPYGSGPEAKCRLELLRSSYSLESIGTSE) after the last amino acid of LptB instead of the desired tag. The mutant allele, which was named *lptB1*, was then introduced into strain NR754 using P1vir transduction and selecting for kanamycin-resistant transductants. Analysis of transductants demonstrated that *lptB1* confers sensitivity to a wide range of antibiotics. One such transductant, NR1768, was used in this study.

The plasmids used in this chapter are listed below. Point mutations in pCDFduet-LptB-LptFG were introduced by site-directed mutagenesis using the oligonucleotides listed in Table S4. PCR amplification was performed with KOD Hot Start DNA polymerase from Novagen. Restriction enzyme DpnI was purchased from New England Biolabs. All other materials were purchased from Sigma Aldrich unless noted otherwise.

**Table 2.2. Strains used in this chapter.**

| <b>Name</b> | <b>Description</b>                                                                           | <b>Ref.</b> |
|-------------|----------------------------------------------------------------------------------------------|-------------|
| NR754       | MC4100 <i>ara</i> <sup>+</sup>                                                               | 215         |
| NR1250      | NR754 $\Delta$ <i>tolC::frt</i>                                                              | This study  |
| NR1768      | NR754 <i>lptB1-kan</i>                                                                       | This study  |
| NR1962      | NR754 <i>lptB1/G33C-kan</i>                                                                  | This study  |
| NR1963      | NR754 <i>lptB1/R144H-kan</i>                                                                 | This study  |
| NR2761      | NR754 $\Delta$ <i>lptFG::frt</i><br>(pBAD18LptFG3)                                           | 216         |
| NR3327      | NR754 $\Delta$ <i>lptFG::frt</i><br>(pBAD18LptFG3/LptFE84A/LptGE88A)                         | 216         |
| NR3602      | NR754 <i>tet2 lptBR91S</i>                                                                   | 216         |
| NR4127      | NR754 <i>tet2 lptBR91S</i><br>$\Delta$ <i>lptFG::frt</i><br>(pBAD18LptFG3/LptFE84A/LptGE88A) | 216         |

**Table 2.3. Plasmids used in this chapter.**

| <b>Name</b>                    | <b>Description</b>                                                                          | <b>Ref.</b> |
|--------------------------------|---------------------------------------------------------------------------------------------|-------------|
| pET22/42-LptB-His <sub>8</sub> | Encodes LptB with a C-terminal His <sub>8</sub> tag                                         | 217         |
| pBAD/HisA-LptC                 | Encodes LptC for the RSO vesicle assay                                                      | 218         |
| pCDFduet-LptB-LptFG            | Encodes LptB and LptFG for the RSO vesicle assay                                            | 218         |
| pET22b-LptA(I36Am)-His         | LptA with amber codon (TAG) for substitution with pBPA                                      | 218         |
| pSup-BpaRS-6TRN                | Encodes amino-acyl tRNA synthetase as well as tRNAs for incorporation of pBPA at TAG codons | 219         |

**Table 2.4. Oligonucleotides used in this chapter.**

| <b>Name</b> | <b>Sequence (5' to 3')</b>                                                                                                    |
|-------------|-------------------------------------------------------------------------------------------------------------------------------|
| 5HisTagP1   | AGA GGA TCT CAC CAT CAC CAT CAC CAT ACG GAT CCG<br>GCC CTG AGG CTA AGT GTA GGC TGG AGC TGC TTC                                |
| P2          | CAT ATG AAT ATC CTC CTT A                                                                                                     |
| 5lptBhis    | CGG CAC GCC TAC AGA AAT CTT ACA AGA CGA ACA CGT<br>TAA GCG TGT ATA CCT TGG GGA AGA CTT CAG ACT CAG AGG<br>ATC TCA CCA TCA CCA |
| 3lptBP2     | CTG AGT TGC AAA CCT TGC TTC ATG TTC AGA ATC GTA<br>CTC TCC TGC TAA AAC GTC GCA AAC TTC TAC CCT ACA TAT<br>GAA TAT CCT CCT TA  |
| 5LptB77UP   | GTT CTG GTG CCG TCG CAG                                                                                                       |
| 3LptB50down | CAT GTT CAG AAT CGT ACT CTC CTG C                                                                                             |
| LptF_E84A_f | GGC AAA CTG TAT ACC GAA AGT GCG ATT ACG GTA ATG<br>CAT GCC TGC GGC C                                                          |
| LptF_E84A_r | GGC CGC AGG CAT GCA TTA CCG TAA TCG CAC TTT CGG<br>TAT ACA GTT TGC C                                                          |
| LptG_E88A_f | GGG ATG CTG GCG CAG CGC AGC GCG CTG GTG GTG<br>ATG CAG GCT TCT GG                                                             |

|             |                                                                        |
|-------------|------------------------------------------------------------------------|
| LptG_E88A_r | CCA GAA GCC TGC ATC ACC ACC AGC GCG CTG CGC<br>TGC GCC AGC ATC CC      |
| LptB_R91S_f | CTG CCA CAG GAA GCC TCC ATT TTC AGC CGC CTC AGC<br>GTT TAC GAT AAC CTG |
| LptB_R91S_r | CAG GTT ATC GTA AAC GCT GAG GCG GCT GAA AAT GGA<br>GGC TTC CTG TGG CAG |

#### 2.4.2. Disk diffusion and efficiency of plating assay

Disk diffusion assays were performed as described in ref.<sup>175</sup>. Premade Sensi-Disc (BD-Beckton, Dickinson and Company) were used for bacitracin 10IU, erythromycin 15  $\mu$ g, novobiocin 30  $\mu$ g, and rifampin 5  $\mu$ g. When needed, disks of nalidixic acid (10  $\mu$ g if unnoted or 5  $\mu$ g when noted), novobiocin (5  $\mu$ g) and 1-adamantylnovobiocin (5  $\mu$ g) were prepared by soaking into sterile 7 mm disks hole-punched from Whatman grade:17chr chromatography paper. For assessing the effect of novobiocin on outer membrane permeability, disk diffusion assays were performed with LB Miller plates (1.5% agar) and LB Miller top agar (0.75% agar) either without novobiocin or containing 0.2, 2, and 20  $\mu$ M.

Efficiency of plating assays were performed on indicated media using the method described in ref.<sup>220</sup>

#### 2.4.3. LptB-His overexpression and purification for crystallography

LptB-His (full-length LptB with a C-terminal His<sub>8</sub> tag) was purified as described in ref.<sup>221</sup> with minor modifications. Overnight cultures of KRX cells (Promega) transformed with plasmid pET22/42-LptB-His<sub>8</sub> were diluted 100X into LB Miller media containing 50  $\mu$ g/mL carbenicillin. Cultures were grown at 37 °C, 220 rpm to OD 0.8, at which point the temperature was reduced to 16 °C. Following 30 min of shaking at 16 °C, overexpression was induced with 0.2% L-rhamnose monohydrate. Cultures were grown at 16 °C, 220 rpm for 14 h. Cells were harvested by centrifugation at 5000 g, 4 °C for 20 min. Pellet was resuspended in Buffer A: Tris-buffered saline (TBS; 20 mM Tris [pH 8.0], 150 mM NaCl), 20% (vol/vol) glycerol, and 0.5 mM Tris(3-hydroxypropyl)phosphine (THP: EMD Milipore). To facilitate lysis, 0.5 mM phenylmethylsulfonyl

fluoride (PMSF), 50  $\mu\text{g/mL}$  lysozyme, and 50  $\mu\text{g/mL}$  DNase I were added to the cell suspension. Cells were lysed 3x through a high-pressure cell disruptor. Unbroken cells were removed by centrifugation at 6000  $g$ , 4  $^{\circ}\text{C}$  for 10 min. To pellet membranes, cell lysate was centrifuged at 100,000  $g$ , 4  $^{\circ}\text{C}$  for 30 min. Membranes were discarded and 10 mM imidazole was added to the supernatant.

In preparation for nickel affinity chromatography, Ni-NTA Superflow resin (Qiagen) was washed with water and equilibrated with Buffer A supplemented with 10 mM imidazole. Cell lysate supernatant was incubated with equilibrated Ni-NTA resin at 4  $^{\circ}\text{C}$  for 1 h with gentle rocking. Following incubation, flow-through was removed and resin was washed with 20 column volumes of Buffer A with 20 mM imidazole. Protein was eluted in one batch with 2.7 column volumes of Buffer A with 200 mM imidazole. Eluate was concentrated in a 10-kDa molecular weight cut-off (MWCO) centrifugation filter (Amicon; Millipore) to  $\sim 50$  mg/mL and flash frozen.

Protein was further purified by size exclusion chromatography on Superdex 200 10/30 GL column in Buffer A. Fractions containing protein were pooled and concentrated in a 10-kDa MWCO centrifugation filter to  $\sim 50$  mg/mL. Protein aliquots were flash-frozen and stored at  $-80^{\circ}\text{C}$ . Protein concentration was measured using the Biorad DC protein assay.

#### **2.4.4. LptB-His crystallization and novobiocin soak**

LptB-His was crystallized using conditions described in ref.<sup>221</sup>. Purified LptB-His was diluted into Buffer A to a concentration of 20 mg/mL. The 20 mg/mL stock was diluted 2X into 1X TBS, pH 8.0, yielding a final protein concentration of 10 mg/mL and glycerol concentration of 10%. This solution was incubated with 2.5 mM ATP and 2.5 mM  $\text{MgCl}_2$  for 1 h on ice before setting up drops. Crystals were grown by vapor diffusion in hanging drops at room temperature. 1  $\mu\text{L}$  protein solution was mixed with 1  $\mu\text{L}$  reservoir solution consisting of 100 mM MES (pH 6.5) and 30% (wt/vol) PEG 4000. As observed in ref.<sup>175</sup>, flat, triangular crystals appeared after several days. For the novobiocin soaks, crystals were transferred to 2  $\mu\text{L}$  drops of reservoir solution (100 mM MES

(pH 6.5), 30% PEG 4000) containing 2.5 mM novobiocin sodium salt. Crystals were soaked for ~90 min at room temperature and then flash-frozen in cryoprotectant containing 100 mM MES (pH 6.5), 33% PEG 4000, 24% glycerol, and 2.5 mM novobiocin.

#### **2.4.5. LptB-His co-crystallization with 1-adamantylnovobiocin**

LptB-His was crystallized using conditions described above. The 20 mg/mL stock was diluted 2X into 1X TBS, pH 8.0, yielding a final protein concentration of 10 mg/mL and glycerol concentration of 10%. This solution was incubated with 2.5 mM ATP, 2.5 mM MgCl<sub>2</sub> for 1 h on ice before setting up drops.

Crystals were grown by vapor diffusion in hanging drops at room temperature. 1  $\mu$ L protein solution was mixed with 1  $\mu$ L reservoir solution consisting of 100 mM MES (pH 6.5), 30% (wt/vol) PEG 4000, and 1.3 mM 1-adamantylnovobiocin solution (final droplet [DMSO] = 7%). As observed in ref.<sup>221</sup>, flat, triangular crystals appeared after several days. Crystals were flash-frozen in cryoprotectant containing 100 mM MES (pH 6.5), 33% PEG 4000, 24% glycerol, and 2.5 mM 1-adamantylnovobiocin.

#### **2.4.6. Crystallography data collection**

The X-ray diffraction data for the LptB-ADP-NOV and LptB-ADP-ADN crystals were collected at 0.97918 Å at beamline 24-ID-E of the Advanced Photon Source at Argonne National Laboratory. LptB-ADP-NOV and LptB-ADP-ADN belong to the space group C121.

#### **2.4.7. Crystallography data processing and structure determination**

The LptB-ADP-NOV dataset was indexed and integrated using iMosflm<sup>222</sup> and scaled using the CCP4<sup>223</sup> program AIMLESS<sup>224</sup>. The structure was solved by molecular replacement with Phaser<sup>225</sup> using the complete LptB-ADP structure from ref.<sup>221</sup> as a search model (PDB: 4P32). Initial rounds of refinement in Phenix<sup>226-228</sup> were performed with rigid body refinement, simulated

annealing, and ADP (atomic displacement parameter or *B*-factor) refinement, yielding a model with  $R_{\text{free}}$  and  $R_{\text{work}}$  values of 29.9% and 25.0%, respectively. This model contained clear unassigned density at the LptB dimer interface.

Following manual placement of novobiocin into the unassigned density in COOT<sup>229</sup>, the model was further refined in Phenix with cycles of minimization, simulated annealing, and ADP refinement, interspersed with manual editing in COOT. Waters and magnesium ions were placed, and the refinement was completed using cycles of minimization, ADP refinement, and translation/libration/screw (TLS) refinement with TLS parameters from the TLS motion determination server.<sup>230</sup> Ligand restraints were generated using the Mogul geometry optimization in eLBOW<sup>231</sup>, and coordination sphere restraints were generated with ReadySet.<sup>227</sup> The  $R_{\text{free}}$  and  $R_{\text{work}}$  values for the final LptB-ADP-NOV structure are 22.0% and 17.9%, respectively.

The LptB-ADP-ADN dataset was processed with the same procedure as the LptB-ADP-NOV dataset, except that an adamantyl group was modeled in to fit the electron density. The  $R_{\text{free}}$  and  $R_{\text{work}}$  values for the final LptB-ADP-ADN structure are 22.4% and 18.0%, respectively. Much of the software used in this project was installed and configured by SBGrid.<sup>232</sup> Figures were prepared using PyMOL.<sup>233</sup>



**Table 2.5. Data collection and refinement statistics.**

| Data Set                                                   | LptB-ADP-NOV           | LptB-ADP-ADN           |
|------------------------------------------------------------|------------------------|------------------------|
| Space group                                                | C121                   | C121                   |
| Unit cell                                                  |                        |                        |
| Dimensions (a, b, c), Å                                    | 190.32, 35.10, 63.05   | 104.03, 34.78, 62.71   |
| Angles ( $\alpha$ , $\beta$ , $\gamma$ ), °                | 90.00, 91.52, 90.00    | 90.00, 101.38, 90.00   |
| Data collection*                                           |                        |                        |
| Wavelength, Å                                              | 0.97918                | 0.97918                |
| Resolution range, Å                                        | 63.03-2.00 (2.05-2.00) | 43.71-1.95 (2.02-1.95) |
| <i>R</i> <sub>merge</sub>                                  | 0.128 (0.622)          | 0.068 (0.587)          |
| Completeness, %                                            | 99.4 (99.2)            | 99.2 (98.9)            |
| Mean <i>I</i> / $\sigma$ ( <i>I</i> )                      | 8.7 (2.1)              | 9.56 (1.95)            |
| Unique reflections                                         | 28,485                 | 16,230                 |
| Multiplicity                                               | 3.5 (3.5)              | 1.9 (1.9)              |
| Refinement*                                                |                        |                        |
| <i>R</i> <sub>work</sub> , %/ <i>R</i> <sub>free</sub> , % | 17.94/22.03            | 18.01/22.36            |
| No. of LptB molecules per asymmetrical unit                | 2                      | 1                      |
| No. of modeled LptB residues per chain                     | 234 (A)/226 (B)        | 233 (A)                |
| No. of water molecules                                     | 101                    | 105                    |
| No. of ions                                                | 2                      | 1                      |
| Average <i>B</i> -factor, Å <sup>2</sup>                   |                        |                        |
| Protein                                                    | 20.61                  | 33.98                  |
| Ligands                                                    | 18.26                  | 43.42                  |
| Solvent                                                    | 17.76                  | 37.10                  |
| Ramachandran plot                                          |                        |                        |
| Favored, %                                                 | 98.9                   | 98.27                  |
| Disallowed, %                                              | 0                      | 0                      |
| rmsd from ideal geometry                                   |                        |                        |
| Bond lengths, Å                                            | 0.007                  | 0.004                  |
| Bond angles, °                                             | 0.747                  | 0.72                   |

\*Values in parentheses are for the shell with the highest resolution.

#### 2.4.8. Minimum inhibitory concentration assay

Strains were grown overnight in LB Miller broth shaking at 220 rpm at 37 °C. Overnight cultures were diluted 1:1,000 (v/v) in LB Miller and 100  $\mu$ L of diluted culture were added to each well of a 96-well plate. To the first well of each row (column no. 1) of this plate, an additional 100  $\mu$ L of mixed diluted culture and test compound were added to a final concentration of 500  $\mu$ M. A 100- $\mu$ L aliquot was serially transferred across rows starting from column no. 1 to column no. 11. Wells in column no. 12 did not receive any test compound. After incubating the plate at 37 °C overnight (~16 h), turbidity (OD<sub>600</sub>) was measured using a Spectramax 384 Plus plate reader

(Molecular Devices) or an xMark™ microplate spectrophotometer (Bio Rad). The minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound needed to completely inhibit bacterial growth.

#### **2.4.9. Gyrase activity assay**

The *Escherichia coli* gyrase supercoiling inhibition assay kit was purchased from the Inspiralis company (Norwich, UK). The assay was conducted according to the provided instructions. 1 U of DNA gyrase was incubated with 0.5  $\mu$ g of relaxed pBR322, and the stated compound concentration, in a reaction volume of 30  $\mu$ L at 37 °C for 30 minutes in Assay Buffer (35 mM Tris•HCl, pH 7.5; 24 mM KCl; 4 mM MgCl<sub>2</sub>, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% (w/v) glycerol, 0.1 mg/mL albumin). The supercoiling reactions were quenched by the addition of 30  $\mu$ L of STEB buffer (40% (w/v) sucrose, 100 mM Tris•HCl pH 8, 10 mM EDTA, 0.5 mg/mL bromophenol blue) and 30  $\mu$ L of chloroform/isoamyl alcohol (v/v, 24:1). Samples were vortexed for 5 minutes and centrifuged at max speed for 1 minute. 20  $\mu$ L of the upper aqueous layer were loaded on a 1 % (w/v) agarose gel free of DNA intercalator and run at 85 V for 2 h. The gel was then stained with DNA intercalator and visualized with an Azure imaging system.

#### **2.4.10. Right-side out vesicle preparation**

Right-side out (RSO) vesicles were prepared as described in ref.<sup>218</sup> with minor modifications. BL-21( $\lambda$ DE3) *E. coli* were transformed with plasmid pBAD18HisA-LptC along with one of pCDFduet (empty vector), pCDFduet-LptB-LptFG (encoding wild-type *E. coli* proteins), pCDFduet-LptB-LptFG(ch) (encoding wild-type LptB and LptF(E84A)LptG(E88A)) or pCDFduet-LptB(R91S)-LptFG. Overnight cultures were diluted 1:100 into 50 mL of LB Miller media containing 50  $\mu$ g/mL carbenicillin and 50  $\mu$ g/mL spectinomycin and grown at 24 °C to OD<sub>600</sub> ~ 1. Growth temperature was then increased to 37 °C and expression induced by addition of 0.02% arabinose and 10  $\mu$ M Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). After two hours, cells were

pelleted, resuspended in 5 mL 50 mM Tris-HCl (pH 7.4) 250 mM sucrose, 300  $\mu$ g/mL lysozyme and 150  $\mu$ g/mL DNaseI, and converted to spheroplasts by drop-wise addition of 5 mL buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM sucrose and 3 mM EDTA followed by incubation on ice for 30 minutes.

To convert spheroplasts to RSO-vesicles, spheroplasts were pelleted, 6000 *g* x 10 minutes, resuspended in 5 mL lysis buffer (20 mM Tris (pH 8), 150 mM NaCl, 0.1 mM EDTA, 5 mM MgCl<sub>2</sub>, and 5 mM sodium-ATP (pH ~7)), pelleted at 10,000 *g* x 15 minutes, and resuspended in the supernatant to complete lysis. RSO-vesicles were collected by centrifugation at 200,000 *g* x 30 minutes and resuspended in 1 mL of 10% v/v glycerol, 20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM MgCl<sub>2</sub>, and 5 mM sodium-ATP (pH ~7). Total protein concentration in RSO-vesicle samples was determined by DC-protein assay (BioRad), and samples were either used immediately in LPS release assays or flash-frozen and stored at -80 °C.

#### **2.4.11. Purification of LptA(I36pBPA)**

LptA\* was purified as described in ref.<sup>218</sup> with minor modifications. BL-21( $\lambda$ DE3) *E. coli* containing pSup-BpaRS-6TRN and pET22b-LptA(I36Am) were grown to OD<sub>600</sub> ~ 0.6 at 37 °C in 1.5 L LB Miller media containing 50  $\mu$ g/mL carbenicillin, 30  $\mu$ g/mL chloramphenicol and 0.7 mM *para*-benzoylphenylalanine (pBPA, BaChem), and induced for 2 hours with 50  $\mu$ M IPTG. These cells were pelleted, converted to spheroplasts as described above, and the spheroplasts pelleted at 6000 *g* x 10 minutes. Supernatant from the spheroplasts was collected and supplemented with 1 mM PMSF and 10 mM imidazole, and clarified by ultracentrifugation at 100,000 *g* x 30 minutes. Supernatant was applied twice to Ni-NTA resin, washed with 2 x 20 column volumes of wash buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 10% v/v glycerol) with 20 mM imidazole, and eluted with 2 x 2.5 column volumes of wash buffer with 200 mM imidazole. Eluate was concentrated to ~1 mg/mL using an Amicon 10 kDa cut-off Amicon centrifugal filter (Millipore), aliquoted, and stored at -80 °C.

#### 2.4.12. LPS-release assay

The *in vitro* LPS-transport experiments in Figures 4b, 4c, and S8 were set-up as described in ref.<sup>218</sup> with some modifications. RSO-vesicles (50  $\mu$ g total protein) were diluted into 100  $\mu$ L reaction buffer (10% v/v glycerol, 20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM sodium-ATP, 5 mM  $\text{MgCl}_2$ ), and incubated on ice with novobiocin, 1-adamantylnovobiocin, or no compound for 15 minutes (50X compound stocks in water were used for each concentration). To start the assay, 3  $\mu$ g of LptA\* was added to each sample, with each time-point its own 100  $\mu$ L sample, and samples were then incubated at 30 °C for the stated time. To cross-link LptA\* to LPS, samples were transferred to a 96-well plate and irradiated with 365 nm UV-light for 5 minutes. Cross-linked samples were then mixed 1:1 with 2X SDS loading dye (100 mM Tris-HCl pH 6.8, 4% w/v SDS, 0.05% w/v bromothymol blue, 20% glycerol) with 5%  $\beta$ -mercaptoethanol, and boiled for 10 minutes.

Immunoblotting was used to assess LptA\*-LPS levels in each sample. Boiled samples were run on home-made 4%/15% polyacrylamide stacking gels at 0.02 A constant-current until the 15 kDa ladder band (BioRad Precision Plus all Blue standards) had run out of the gel, transferred to PVDF, and immunoblotted with mouse anti-LPS core antiserum (HyCult Biotechnology) followed by sheep-anti-mouse IgG horseradish peroxidase conjugate (GE Healthcare). LptA levels were assessed similarly, using rabbit anti-LptA antiserum<sup>234</sup> followed by donkey anti-rabbit horseradish peroxidase conjugate (GE Amersham). ECL Prime Western Blotting Detection Reagent (GE Amersham) was used to visualize antibody-label bands in conjunction with an Azure c400 imaging system (Azure Biosystems).

To assess the expression levels of inner-membrane Lpt components in the different RSO preparations, samples from LPS release assays were also subject to SDS-PAGE and immunoblotting as described above. Rabbit anti-LptC<sup>234</sup>, rabbit anti-LptF<sup>235</sup>, or rabbit anti-LptB<sup>221</sup>

antisera were used to label Lpt proteins on PVDF and in turn detected and imaged as described for LptA.

#### 2.4.13. Fbn–LptB binding assay

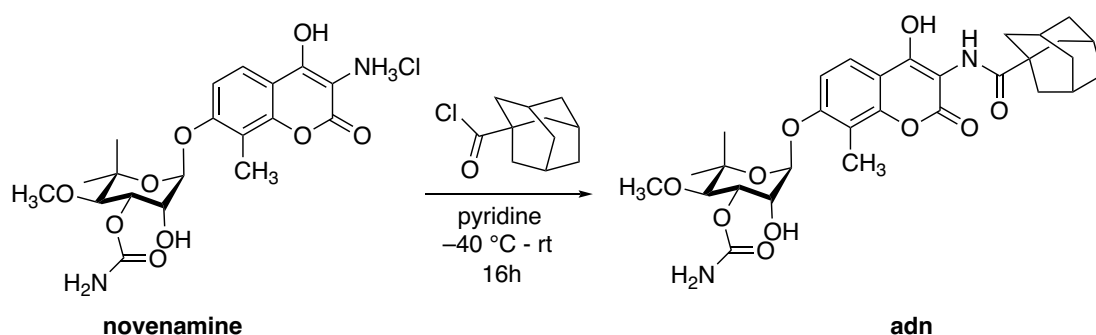
5 mL of the buffered **Fbn** ligand solution was prepared by mixing 200  $\mu$ L of 25X TBS pH 8.0, 125  $\mu$ L 200 mM ADP pH 7.0, 125  $\mu$ L 200 mM  $\text{MgCl}_2$ , 4.43  $\mu$ L of 56.5 mM **Fbn**, 4.55 mL of  $\text{D}_2\text{O}$ . Final [Tris-Cl] = 50 mM, [NaCl] = 150 mM, [ADP] = 5 mM, [ $\text{MgCl}_2$ ] = 5 mM, [**Fbn**] = 50  $\mu$ M. In ten 1.5 mL Eppendorf tubes, on ice, the corresponding volume of stock LptB-His protein was added to the buffered **Fbn** solution. The tubes were vortexed briefly, the solutions transferred to Bruker Biospin 5.0x103.5 mm NMR tubes (for SampleJet) and stored at +4 °C until acquisition. Data were acquired using a Bruker 500 MHz Avance III with multi-nuclear smart probe operating at a frequency of 470.5453180 MHz for  $^{19}\text{F}$  and 500.1320005 MHz for  $^1\text{H}$ . A standard fluorine 1D pulse program with proton decoupling was used; temperature (T) = 298.1 K, number of scans (NS) = 3072, relaxation delay (D1) = 0.75 s, pulse angle = 70 °. After acquisition, FIDs were Fourier transformed, phased, and processed with the exponential multiplication window function (LB = 3.00 Hz) in MestReNova MNova v10. The  $^{19}\text{F}$  linewidths were plotted against log [LptB] in GraphPad Prism 7 software using the nonlinear fit log(agonist) vs. response model, variable slope (four parameters).

#### 2.4.13. General synthetic methods

Unless otherwise noted, all reactions were performed under anhydrous  $\text{N}_2$ . Solvents were purchased in Sure-Seal bottles from Sigma-Aldrich and used without further purification. Thin layer chromatography (TLC) was carried out using EM Science silica gel 60 F254 plates; the developed plate was analyzed by UV lamp (254 nm). Column chromatography was performed with the Teledyne-Isco CombiFlash R<sub>f</sub>200 system using pre-packed silica gel column cartridges and hexanes/ethyl acetate or dichloromethane/methanol as the solvent system.  $^1\text{H}$  NMR and  $^{13}\text{C}$

NMR spectra were recorded in DMSO- $d_6$  or  $CDCl_3$  on a Varian Inova-500 MHz spectrometer unless otherwise noted. Chemical shifts are reported in ppm with the residual solvent signal as the reference and coupling constants ( $J$ ) are given in hertz. The peak information is described as: br = broad singlet, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. The molar ratio of isolated imine geometric isomers was determined by NMR spectroscopy. High-resolution mass spectra (HRMS) were performed on a Bruker microTOFII ESI LCMS system mass spectrometer using sodium formate as the standard.

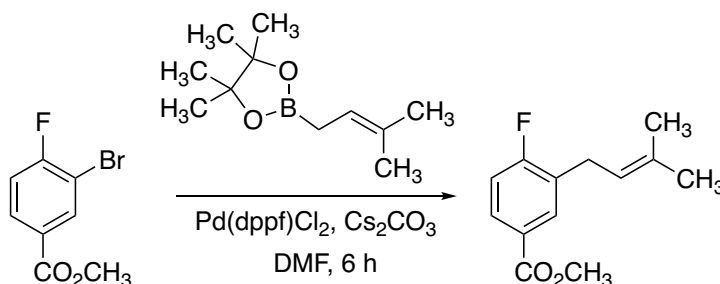
#### 2.4.14. Synthesis of 1-admantynovobiocin (adn)



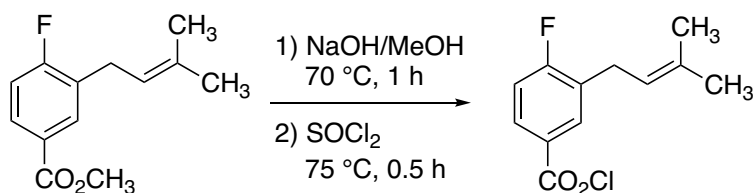
Novenamine hydrochloride (40 mg, 0.09 mmol, 1.0 eq.) and a magnetic stirbar were added to a 25 mL conical flask and sealed with a rubber septum. A vent needle was inserted and the flask was sparged with  $N_2$  for 5 minutes (**free novenamine begins to oxidize upon exposure to  $O_2$ !**). 2 mL of pyridine were added under positive  $N_2$  pressure and the flask was cooled to  $-40$  °C in a dry ice/acetonitrile bath. 1-adamantanecarbonyl chloride (17.2 mg, 0.09 mmol, 1.0 eq.) was dissolved in 0.5 mL of pyridine and added to the stirring novenamine solution. The solution was stirred for 16 h while allowing the bath to warm to room temperature. The reaction mixture was concentrated by rotary evaporation and then subjected to flash column chromatography with DCM/MeOH (10 mg, 16% isolated yield). The product was further purified by reversed-phase column chromatography with a Zorbax C18 column with acetonitrile/water as the eluent (90%

MeCN to 100% MeCN over the course of 15 minutes), and the final product was obtained as a white solid (6.7 mg, 13% isolated yield).

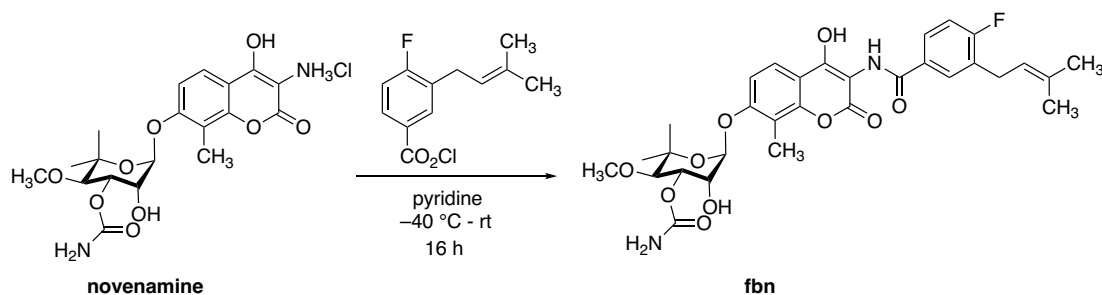
#### 2.4.15. Synthesis of fluorobiocin (fbn)



Methyl 3-bromo-4-fluorobenzoate (500 mg, 2.1 mmol, 1.0 eq.), palladium catalyst (314 mg, 0.43 mmol, 20 mol %), Cs<sub>2</sub>CO<sub>3</sub> (1.4 g, 4.3 mmol, 2.0 eq.) were charged in a 100 mL round bottom flask. 20 mL of anhydrous DMF and a magnetic stirbar were added and the flask was sealed with a rubber septum. A vent needle was inserted and the flask was sparged with N<sub>2</sub> for 5 minutes. 3-methyl-2-butenylboronic acid pinacol ester (951  $\mu$ L, 4.3 mmol, 2.0 eq.) was added and the reaction was heated to 90 °C under positive N<sub>2</sub> pressure for 6 h. The reaction mixture was cooled to room temperature and filtered through a pad of Celite rinsed with EtOAc. 100 mL of water were added and the product was extracted by washing with 3 x 50 mL ethyl acetate. The organic layer was washed several times with water, saturated sodium chloride, and then dried over sodium sulfate. Following filtration, the extract was concentrated by rotary evaporation and subjected to flash column chromatography (hexanes/EtOAc) to afford methyl 4-fluoro-3-(3-methylbut-2-en-1-yl)benzoate as an oil (50% isolated yield), which was used immediately in the next step.



Methyl 4-fluoro-3-(3-methylbut-2-en-1-yl)benzoate (220 mg, 1 mmol) was added to a 15 mL scintillation vial equipped with a magnetic stirbar. 2 mL of 1M NaOH in methanol and a few drops of water were added to the vial. The reaction was heated to 70 °C for 1 h. The carboxylate product precipitated upon completion of the reaction, and the mixture was concentrated by rotary evaporation. The carboxylate salt was protonated by the dropwise addition of 1M HCl. Ethyl acetate (5 mL) and water (5 mL) were added and the acid product was extracted from the organic layer. The extract was dried over sodium sulfate, filtered, and concentrated by rotary evaporation. The carboxylic acid product was a white solid and was used without further purification. Thionyl chloride (700  $\mu$ L, 14 eq.) was added and the mixture was heated to 75 °C for 0.5 h. The leftover thionyl chloride was removed by rotary evaporation and 150 mg of a sticky orange gel (88% isolated yield).



Novenamine hydrochloride (118 mg, 0.26 mmol, 1.0 eq.) and a magnetic stirbar were added to a 25 mL conical flask and sealed with a rubber septum. A vent needle was inserted and the flask was sparged with N<sub>2</sub> for 5 minutes (**free novenamine begins to oxidize upon exposure to O<sub>2</sub>!**). 2 mL of pyridine were added under positive N<sub>2</sub> pressure and the flask was cooled to -40



°C in a dry ice/acetonitrile bath. 4-fluoro-3-prenylbenzoyl chloride (75 mg, 0.31 mmol, 1.2 eq.) was dissolved in 0.5 mL of pyridine and added to the stirring novenamine solution. The solution was stirred for 16 h while allowing the bath to warm to room temperature. The reaction mixture was concentrated by rotary evaporation and then subjected to flash column chromatography with DCM/MeOH. The product was further purified by reversed phase column chromatography with a Zorbax C18 column with acetonitrile/water as the eluent (90% MeCN to 100% MeCN over the course of 15 minutes), 12.7 mg (8% isolated yield after semi-preparative HPLC).

### **Chapter 3: A gyrase-independent model for the synergy between novobiocin and polymyxin**

This chapter is adapted/reproduced from:

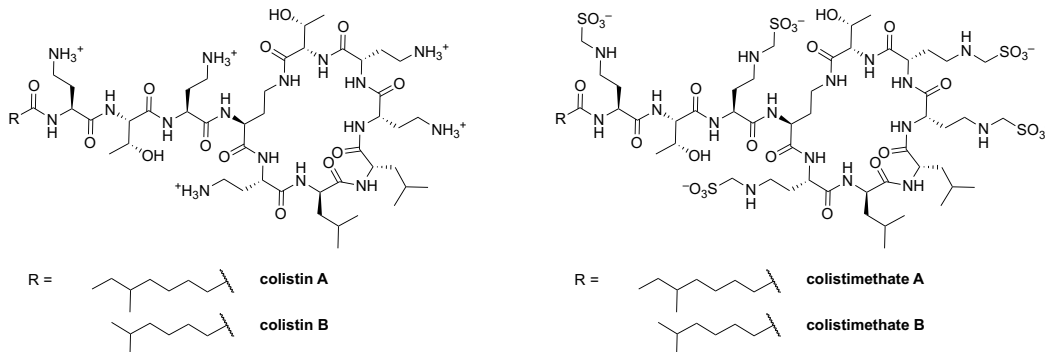
Mandler, M.D.\*; Baidin, V.\*; Lee, J.; Pahil, K.S.; Owens, T.W.; Kahne, D. "Novobiocin enhances polymyxin activity by stimulating lipopolysaccharide transport" *J. Am. Chem. Soc.* **2018**, *140*, 6749-6753,

### 3.1. Introduction

The rate of resistant Gram-negative pathogens has been increasing globally, presenting serious threats to the public health and patients in intensive care settings.<sup>236-240</sup> Among the ESKAPE pathogens<sup>241-243</sup> (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*) that have a propensity of “escaping” the action of many antibiotics, the last four—and the majority—are Gram-negative. Unfortunately, despite rising infection rates with antibiotic-resistant Gram-negative strains, the pipeline for new antibiotics has dried. At the time of writing, the last new class of antibiotics effective against Gram-negatives were the fluoroquinolones<sup>244</sup>, which were introduced in the 1960s.<sup>245</sup> Over the past several decades, last-resort antibiotics such as the polymyxins<sup>246, 247</sup> (in particular, polymyxin B and colistin) that were previously abandoned for safer alternatives, have been resurrected to tackle multidrug and extremely drug-resistant Gram-negative strains.<sup>248</sup> Recently, in the Gram-positive arena, daptomycin<sup>249, 250</sup> was successfully revived by Cubist pharmaceuticals from original efforts by Eli Lilly to place a drug on the market. Nevertheless, there is an unmet clinical need for new or repurposed antibiotics effective against resistant Gram-negative bacteria, such as the four mentioned above. The polymyxins have largely avoided clinical use due to the availability of safer alternatives. As bacteria have evolved resistance against those alternatives, the polymyxins are being reconsidered.

Polymyxins, including polymyxin B and polymyxin E (colistin) are cationic antimicrobial peptides that function by binding the anionic LPS layer coating most Gram-negative cells. Although they are highly selective for LPS, the polymyxins have well-established nephrotoxicity<sup>251, 252</sup> and neurotoxicity<sup>253</sup> that limits their therapeutic usefulness. They are known to accumulate in the liver, kidneys, brain, heart, muscle, and lung tissues.<sup>254</sup> Polymyxin E was once thought to be safer than polymyxin B, but when normalized for efficacy, this did not turn out to be the case.<sup>255, 256</sup> A major research investment has been made to curb the human toxicity of the polymyxins.

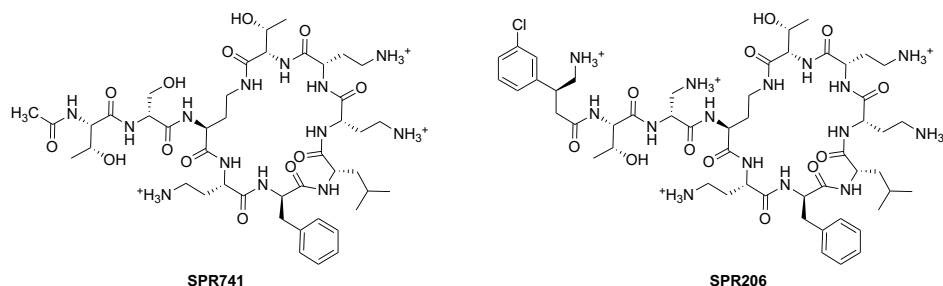
One such strategy has been to convert colistin into colistimethate sodium (Figure 3.1), a prodrug of the polymyxin, with reports claiming that colistimethate is less toxic than colistin<sup>257</sup> but that its potency is worse.<sup>258</sup>



**Figure 3.1. Chemical structures of colistins and the colistimethate prodrugs.**

Colistin is a mixture of compounds including colistin A and B that differ in the length of the fatty acid tail. Colistins possess five positively charged ammonium groups, while colistimethate prodrugs possess a cleavable sulfate moiety capping the diaminobutyric acid (Dab) amine side chain.

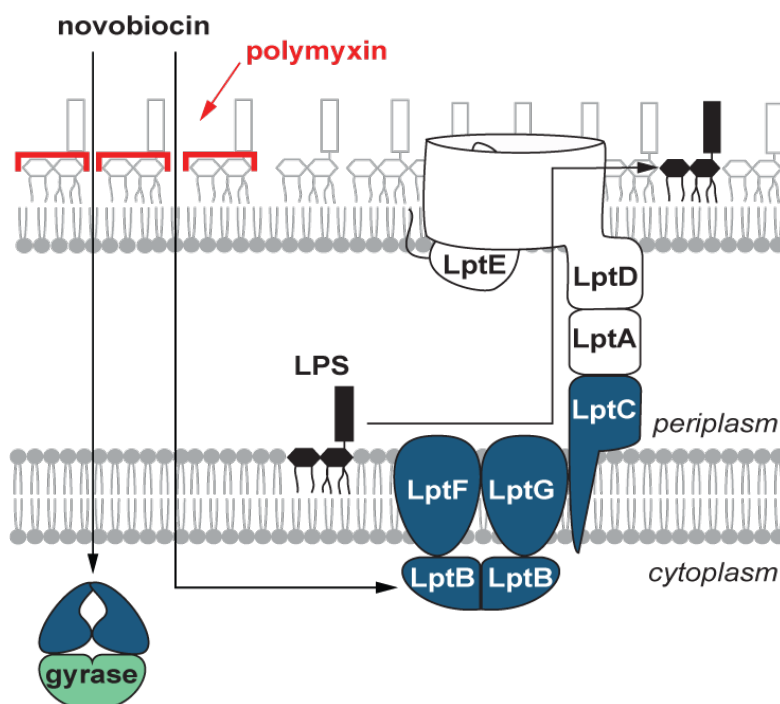
Another strategy has been to prepare novel chemical derivatives of polymyxin<sup>259</sup>, either by solid phase peptide synthesis or semi-synthesis from the natural material. Many papers have been published on novel polymyxin derivatives<sup>260-264</sup> and their improved antibacterial activities or safety profiles; however, to my knowledge, none have received FDA approval at the time of this writing. SPR741<sup>265-270</sup> is a polymyxin B derivative that has emerged as a lead potentiator candidate from Spero Therapeutics and has recently passed Phase I clinical studies. The next-generation analog, SPR206<sup>271</sup>, possesses antibiotic activity in monotherapy, including against *A. baumannii* and *P. aeruginosa*. The structures of these polymyxin analogs are given in Figure 3.2.



**Figure 3.2. Chemical structures of SPR741 and SPR206 polymyxin analogs.**

SPR741 is an unnatural polymyxin B derivative containing three Dab residues, a substitution of the fatty acid tail with an acetyl group, and a serine. SPR206 contains a *m*-chlorophenyl group and five Dab residues.

Synergistic combinations of polymyxins with other antibiotics<sup>272</sup> have been explored to increase the effectiveness and therapeutic window.<sup>273-275</sup> In these therapies, the polymyxins are combined with antibiotics<sup>276-284</sup>, some of which are only active against Gram-positive organisms, and the total antibacterial effect is greater than when the antibiotics are combined. Polymyxin disrupts the outer membrane and increases the permeability of large or otherwise poorly penetrable antibiotics so they can enter the cell and inhibit their targets. For example, the gyrase inhibitor novobiocin<sup>170</sup>, which has poor Gram-negative activity, synergizes with the polymyxins.<sup>285, 286</sup> Presumably a higher concentration of novobiocin will reach the cytoplasmic gyrase complex when combined with polymyxins that shear the outer membrane and increase small molecule permeability across the outer membrane.<sup>287</sup> However, our findings in Chapter 2 suggest that novobiocin binds and stimulates the LPS transporter. Because LPS is the target of the polymyxins, we wondered whether the effect of novobiocin on LptB<sub>2</sub>FGC could also contribute to observed synergy with the polymyxins (Figure 3.3). This section will describe my attempts to separate the activity of novobiocin against LptB<sub>2</sub>FGC and gyrase using a synthetic approach to demonstrate that a component of novobiocin-polymyxin synergy is gyrase independent. Moreover, I highlight that changes to the structure of novobiocin can influence LptB<sub>2</sub>FGC binding and LPS transport activity, and that these changes can affect synergy.



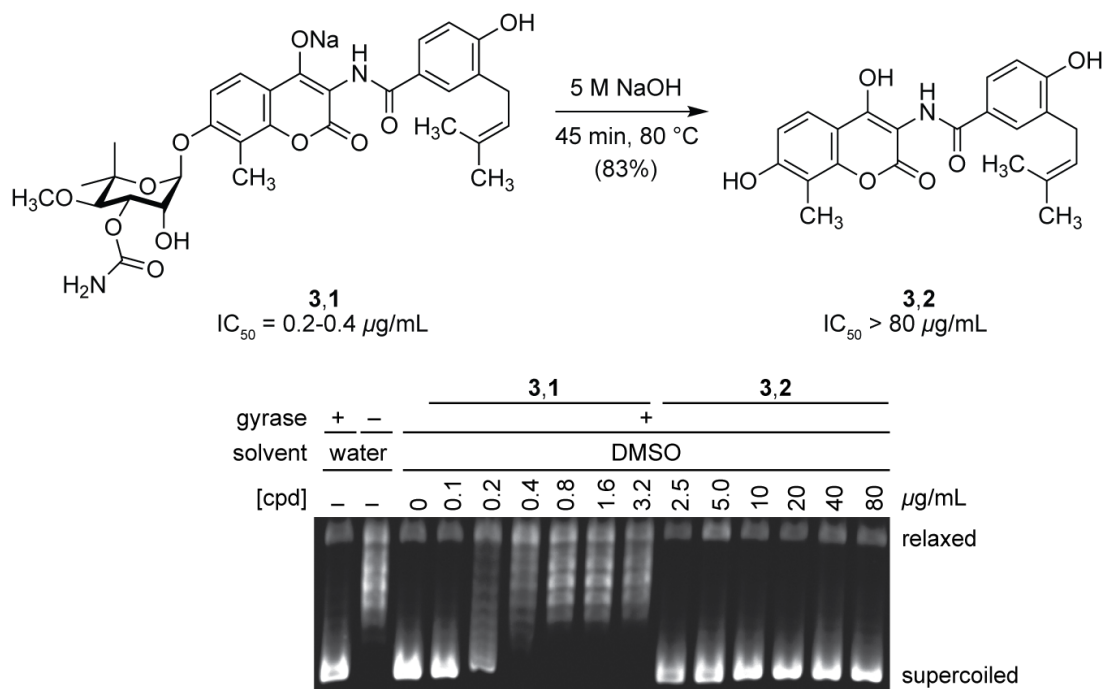
**Figure 3.3. Possible mechanisms of polymyxin/novobiocin synergy.**

Polymyxin binds the LPS on the outer membrane to disrupt the polyelectrolyte barrier and promote the uptake of novobiocin into the cell, where it can inhibit DNA gyrase. Novobiocin binds LptB<sub>2</sub>FGC, which stimulates LPS transport and provides more targets for polymyxin on the outer membrane or an active state of transport.

## 3.2. Results

### 3.2.1. Separating novobiocin gyrase inhibitory activity from Lpt stimulatory activity

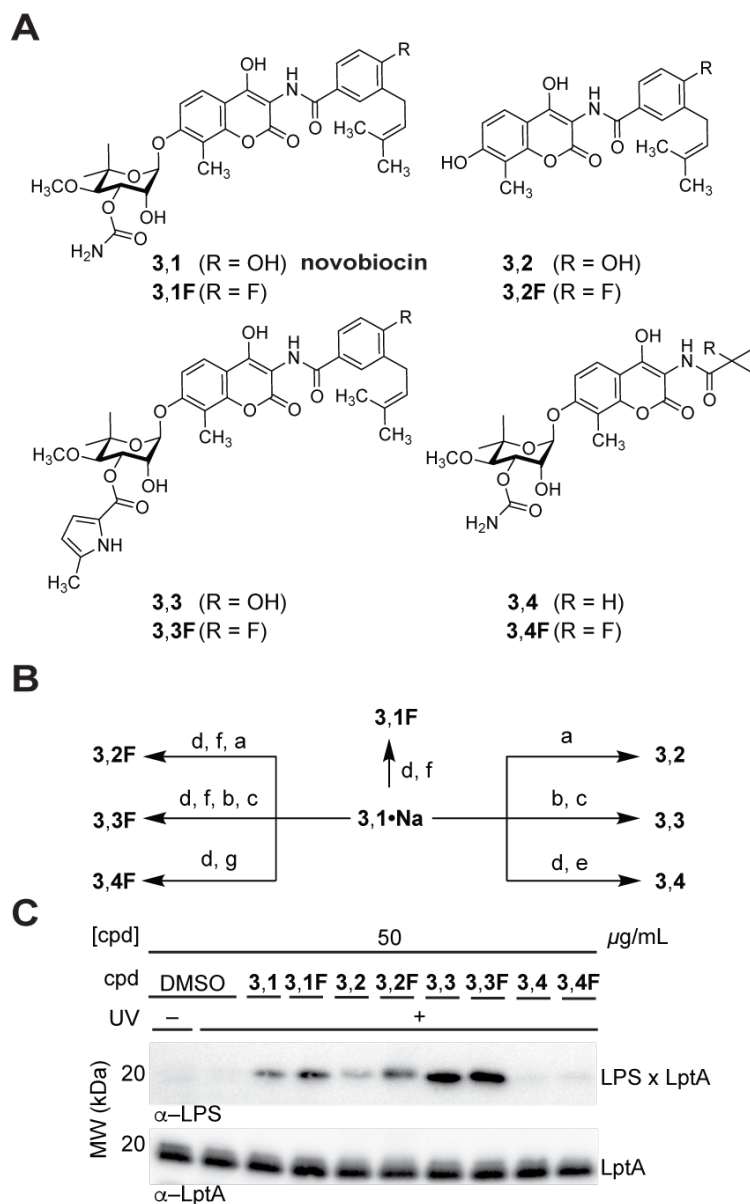
To study the effect of aminocoumarin stimulation on the lipopolysaccharide transporter, we began by fully deconvoluting the gyrase inhibitory effect of novobiocin. We reasoned that removing a moiety known to make important X-ray crystal contacts to GyrB<sup>288</sup> would ablate inhibitory activity. We synthesized a novobiocin analog **3,2** that lacks the L-noviose sugar of **3,1** (Figure 3.4). An *in vitro* DNA gyrase supercoiling assay, as described in Chapter 2, demonstrated that **3,2** was at least 260-fold less active than **3,1** against *Acinetobacter baumannii* gyrase (Figure 3.4). This organism was chosen as a model strain because it is considered a high priority strain according to the World Health Organization.<sup>289, 290</sup>



**Figure 3.4. Synthesis of novobiocin aglycone and activity on DNA gyrase supercoiling assay.**

Novobiocin aglycone **3,2** was synthesized by alkaline hydrolysis of commercially available novobiocin sodium **3,1**. The aglycone was tested alongside novobiocin for *A. baumannii* gyrase inhibitory activity by the DNA supercoiling assay. Compounds were incubated with purified *A. baumannii* gyrase, buffer, and relaxed pBR322 plasmid for 30 min at 37 °C. The supercoiling reaction was then quenched and the DNA topoisomers were separated by gel electrophoresis and stained.

After decoupling gyrase activity, we wondered whether **3,2** could bind the purified five-protein LPS transport complex LptB<sub>2</sub>FGC.<sup>133, 176, 291</sup> We developed a fluorine-19 NMR binding assay<sup>177</sup>, similar to the one described in Chapter 2, in which the fluorinated novobiocin analogs of **3,1** and **3,2** (**3,1F** and **3,2F**) were synthesized (Figure 3.5) and monitored by 1D <sup>19</sup>F NMR. Changes in the <sup>19</sup>F line widths of novobiocin derivatives were monitored in response to changing concentrations of purified protein complex. The fluorinated compounds were tested alongside their unlabeled counterparts to ensure that they retained a similar degree of LPS transport activation (Figure 3.5C).



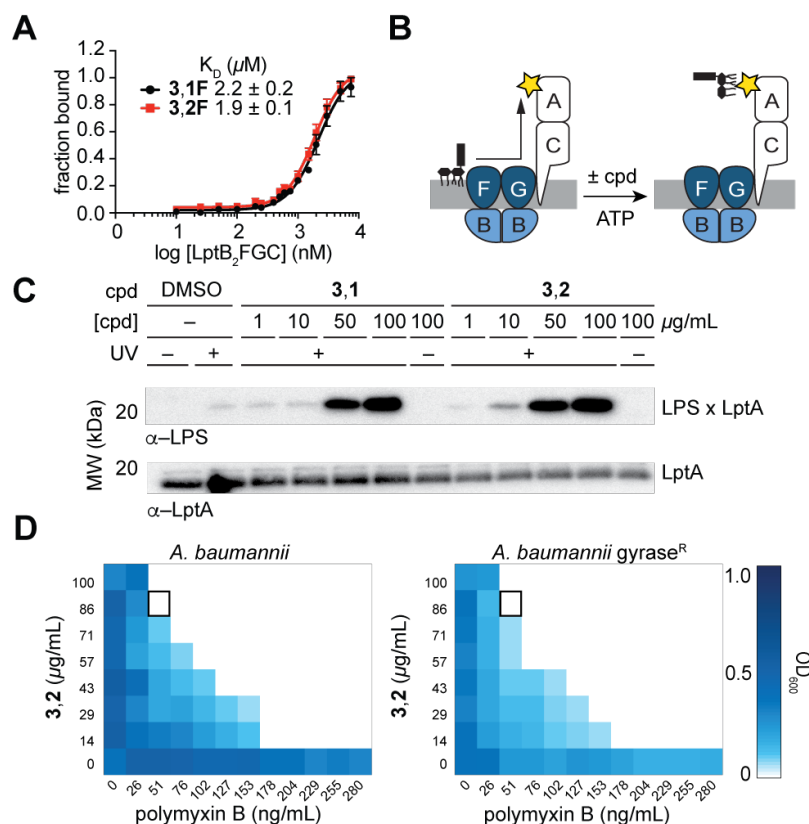
**Figure 3.5. Synthesis of novobiocin probes and their fluorinated analogs.**

(A) Fluorinated novobiocin derivatives used for  $^{19}\text{F}$  NMR binding assay. (B) Synthetic scheme for novobiocin derivatives. Conditions: a. 5 M NaOH/H<sub>2</sub>O, 80 °C, 45 min. b. 1 M NaOH/H<sub>2</sub>O, 50 °C, 45 min. c. 5-methyl-1H-pyrrole-2-carboxylic anhydride (1.5 eq.), 10 mol % Sc(OTf)<sub>3</sub>, MeCN, 23 °C, 3 h. d. NRRL-B-3652 heavy cell suspension, water, N<sub>2</sub> atmosphere, 16 h. e. cyclopropanecarbonyl chloride (0.8 eq.), pyridine, -40 °C, 16 h. f. 4-fluoro-3-prenylbenzoic acid (1.0 eq.), EDC•HCl (1.2 eq.), pyridine (10 eq.), DCM, 0 °C – 23 °C, 16 h. g. 1-fluorocyclopropane-1-carboxylic acid (0.7 eq.), EDC•HCl (1.0 eq.), pyridine (7 eq.), DCM, 0 °C – 23 °C, 16 h. C) LPS release assay comparing unfluorinated novobiocin derivatives to their corresponding fluorinated analogs.

We determined that the fluorinated analogs of both novobiocin (**3,1F**) and the aglycone (**3,2F**) bind LptB<sub>2</sub>FGC with single-digit micromolar affinity (Figure 3.6A; **3,1F** =  $2.2 \pm 0.2 \mu\text{M}$ ; **3,2F** =  $1.9$



$\pm 0.1 \mu\text{M}$ ). The fluorinated novobiocin aglycone **3,2F** maintained comparable binding for LptB<sub>2</sub>FGC. We wondered whether **3,2** possessed the same level of LPS transport stimulation activity as novobiocin. We used UV photocrosslinking tools,<sup>133</sup> described in Chapter 2, to show that novobiocin stimulates LPS transport<sup>177</sup> by accelerating the extraction of the glycolipid from the outer leaflet of the inner membrane and transport to LptA<sup>292</sup>, the periplasmic component of the membrane to membrane proteinaceous bridge.<sup>150, 293-296</sup> Using our LPS release assay in right-side-out vesicles (Figure 3.6B), we found that **3,2** stimulated LPS transport to LptA(I36pBPA) to a comparable degree as **3,1** (Figure 3.6C). Therefore, novobiocin aglycone **3,2**, which lacks gyrase activity, retains LptB<sub>2</sub>FGC binding and stimulatory activity on LPS transport. **3,2** lacks antibiotic activity up to 200  $\mu\text{g/mL}$  (Table 3.1). With this **3,2** that lacks *in vitro* gyrase activity, we were interested to determine if it could nevertheless synergize with polymyxin B. If so, this would suggest that stimulating LPS transport by binding LptB<sub>2</sub>FGC could be made lethal if combined with an agent that kills by binding LPS during transport. Compound **3,2** displayed synergy with polymyxin B against both wildtype *A. baumannii* 19606 and a novobiocin-resistant strain of *A. baumannii* that was isogenic except for a point mutation in the *gyrB* gene, *gyrB(R150C)*, which conferred >200-fold resistance to novobiocin (Figure 3.6D). The checkerboard assay represents growth, measured as OD<sub>600</sub> (ie. white = dead; blue = growth), and the axes correspond to two compound concentrations. Therefore, we have identified **3,2** as a molecule that can stimulate LPS transport but does not inhibit gyrase activity. The checkerboard assays imply that at least some portion of the synergy observed between novobiocin and polymyxin is independent of novobiocin's gyrase activity. Finally, these results establish that the sugar is important for gyrase inhibition and unnecessary for Lpt binding and stimulation. However, these results did not lead us to the conclusion that the sugar was unimportant for derivatization.



**Figure 3.6. Novobiocin and novobiocin aglycone bind LptB<sub>2</sub>FGC with similar affinity and stimulate LPS transport.**

(A) **3,1F** and **3,2F** both display similar binding affinities to LptB<sub>2</sub>FGC. Error bars represent the SEM (n = 3). (B) Schematic of LPS transport assay with right-side-out (RSO) vesicles overexpressing LptB<sub>2</sub>FGC and purified LptA(I36pBPA). (C) **2** retains activity on LptB<sub>2</sub>FGC. Compounds, ATP, and LptA(I36pBPA) were incubated with the vesicles for 1 h at 30 °C prior to UV irradiation at 365 nm for 5 min. LPS × LptA cross-links were detected by LPS immunoblots. (D) **3,2** synergizes with polymyxin B in a gyrase-independent fashion. Checkerboard broth microdilution assay against wild-type and gyrase-resistant *A. baumannii*. The black squares denote concentrations of **3,2** that reduce the polymyxin B concentration by at least five-fold. The data are representative of a minimum of two biological replicates. See Materials and Methods section for full experimental details.

**Table 3.1. Antibiotic sensitivity measurements of *lptB* mutants by disc-diffusion.**

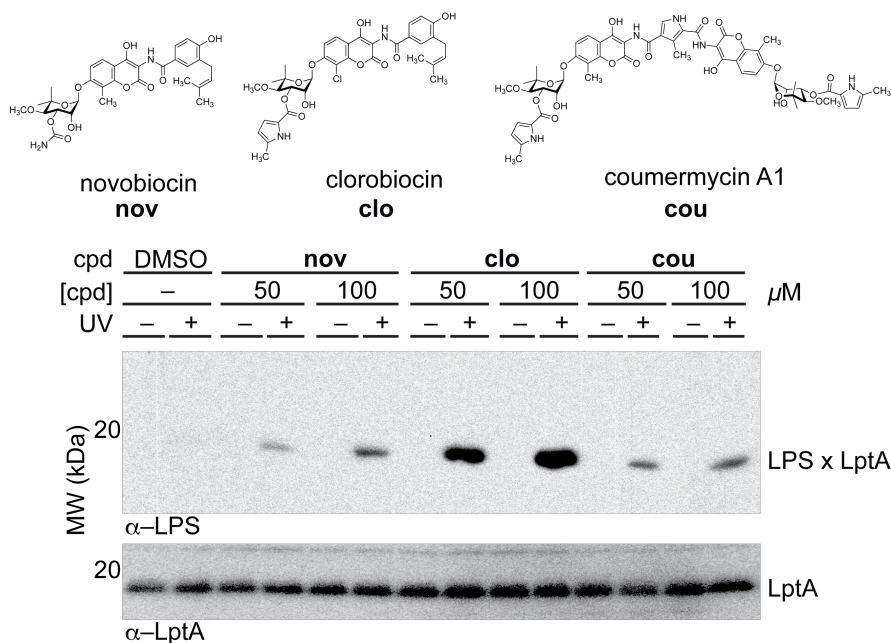
Minimal inhibitory concentrations (MIC) data are representative of two biological replicates and were determined using the microdilution broth method described by the Clinical and Laboratory Standards Institute<sup>297</sup> and reported as  $\mu$ g/mL.

| strain                       | 3,1 | 3,2  | 3,3  | 3,4  | 3,5  |
|------------------------------|-----|------|------|------|------|
| <i>S. aureus</i> RN4220      | 0.2 | >200 | 0.02 | 100  | 10   |
| <i>B. subtilis</i>           | 1   | 100  | 10   | >200 | 6    |
| <i>E. coli</i> 25922         | 80  | >200 | 20   | >200 | >200 |
| <i>E. coli</i> $\Delta$ tolC | 0.6 | 50   | 3    | 70   | >200 |
| <i>A. baumannii</i> 19606    | 1   | >200 | 0.3  | >200 | >200 |

|                                                   |      |      |       |      |      |
|---------------------------------------------------|------|------|-------|------|------|
| <i>A. baumannii</i> 19606 $\Delta 5^{298}$        | 0.01 | 50   | <0.01 | 50   | 2    |
| <i>A. baumannii</i> 19606 <i>gyrB<sup>R</sup></i> | >200 | >200 | 30    | >200 | >200 |

### 3.2.2. Identification of an aminocoumarin with improved LPS transport stimulation

If novobiocin/polymyxin synergy were (at least in part) dependent on stimulation of LPS transport by novobiocin, then novobiocin derivatives that bind and stimulate LptB<sub>2</sub>FGC better should synergize better with polymyxin. We screened the other naturally occurring aminocoumarins (coumermycin A1 and clorobiocin) and found that clorobiocin was exceptionally active at LPS transport stimulation (Figure 3.7). Clorobiocin was previously known to bind and inhibit gyrase with higher affinity than novobiocin.<sup>169, 202</sup> Hooper et al. measured the IC<sub>50</sub> of these three aminocoumarins against *E. coli* gyrase (novobiocin = 0.49  $\mu$ M, clorobiocin = 0.21  $\mu$ M, coumermycin A1 = 0.082-0.14).<sup>169</sup> Although coumermycin A1 was the most potent against gyrase, it did not stimulate LPS release as much as clorobiocin did. Therefore, we postulated that clorobiocin may have stronger binding to LptB<sub>2</sub>FGC than either novobiocin or coumermycin A1.

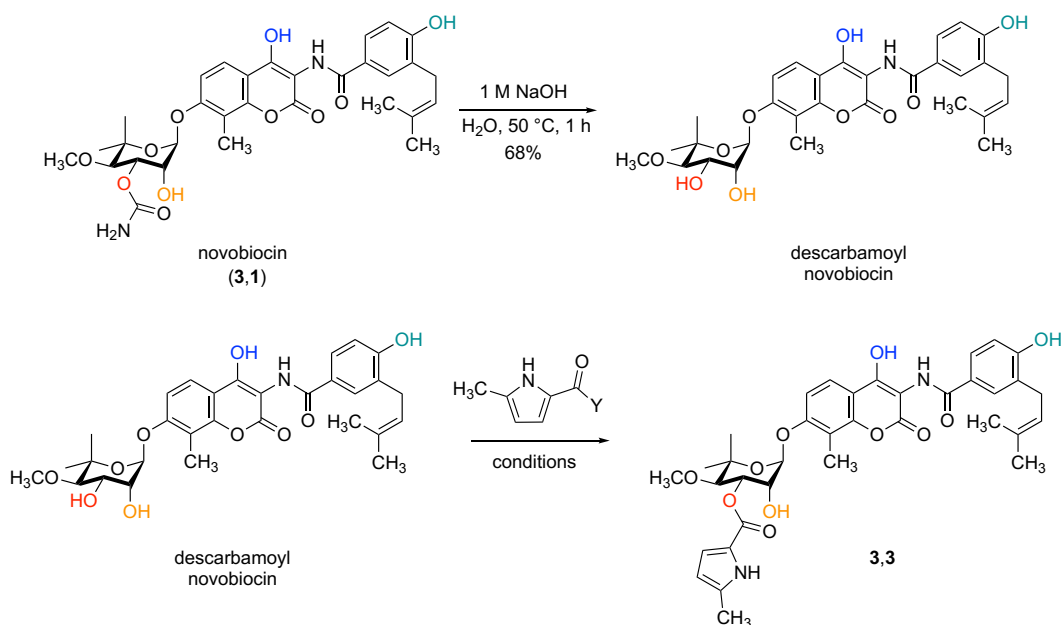


### Figure 3.7. LPS transport activity of naturally occurring aminocoumarins.

Structures of the three most common aminocoumarin antibiotics. An LPS release assay comparing the three natural aminocoumarins at two concentrations. The natural antibiotic clorobiocin, which differs from novobiocin by two substitutions (-CH<sub>3</sub> to -Cl; -CONH<sub>2</sub> to -CO-5-methyl-1*H*-pyrrole) significantly stimulates more LPS transport compared to the other two compounds.

Since clorobiocin is structurally identical to novobiocin except for two differences, we wondered which of those differences were responsible for this increase in activity. Either the 5-methyl-1*H*-pyrrole-2-carboxylic ester substitution or the chlorine substitution could be responsible. If we could determine the responsible moiety, then we could better understand what modifications might improve LptB<sub>2</sub>FGC binding and/or activity. To test this hypothesis, we took a synthetic approach and began looking for methods for the selective acylation of descarbamoyl novobiocin, which could be prepared from **3,1** by mild alkaline decarbamoylation (Figure 3.8), to yield the acylated derivative **3,3**. We did not replace the tolyl methyl group with a chlorine, since there was not a simple bond disconnection for late stage modification. This derivative would likely require the carbon-chlorine bond be installed at an early point in a total synthesis.

#### 3.2.3. Chemical acylation of descarbamoyl novobiocin



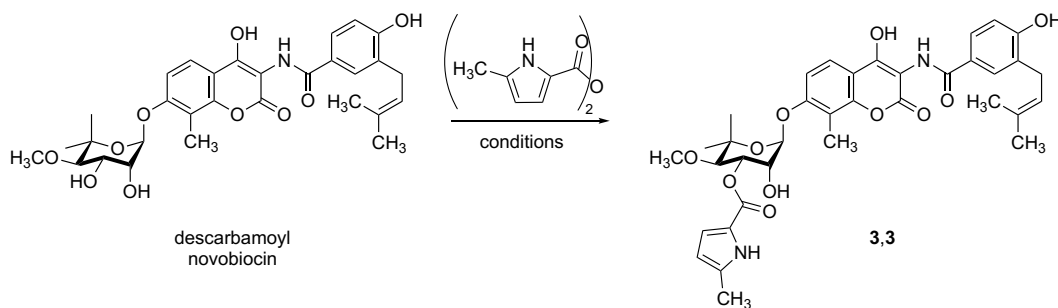
**Figure 3.8. Mild decarbamoylation of novobiocin and proposed acylation.**

Novobiocin can be converted to descarbamoyl novobiocin by gentle heating under mild alkaline conditions to avoid hydrolysis to the aglycone. Novobiocin sodium (5 g) was dissolved in 50 mL 1 M NaOH and stirred at 50 °C for 45-60 min. See Materials and Methods section for full experimental details.

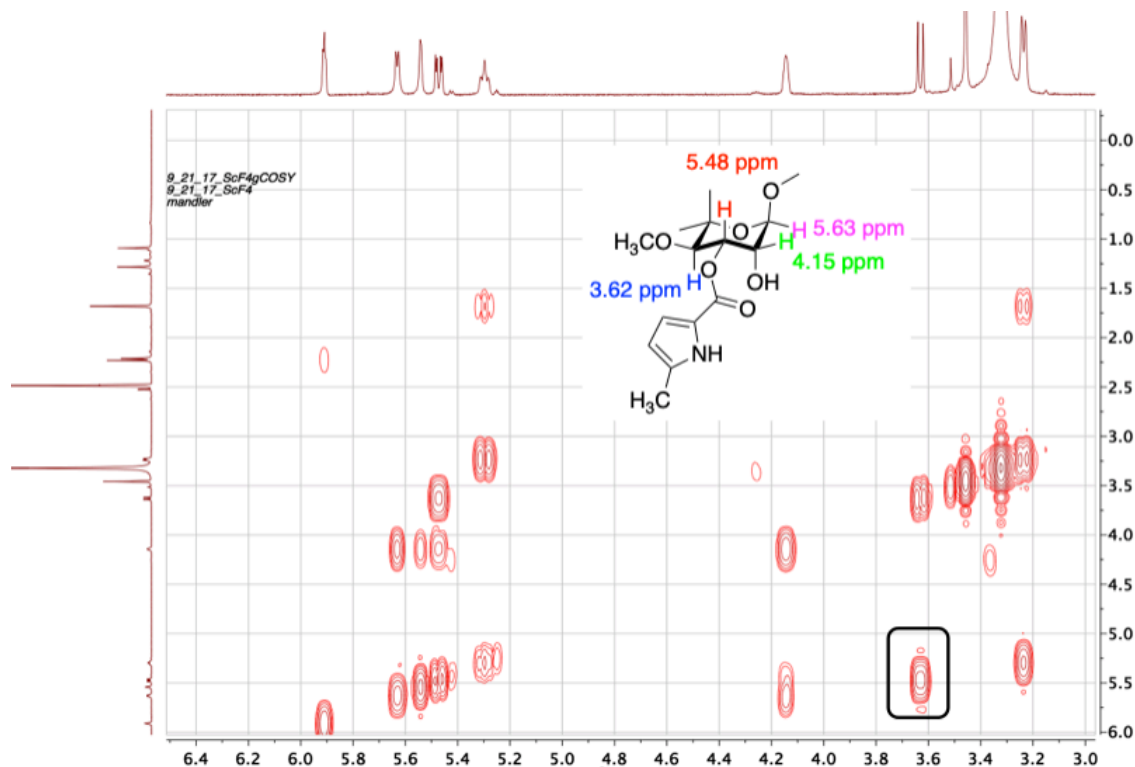
Multigram quantities of descarbamoyl novobiocin could be generated and purified by flash column chromatography; however, the selective acylation of the secondary alcohol on the noviose sugar did not seem trivial. Initially, the synthesis of 5-methyl-1*H*-pyrrole-2-carbonyl chloride was attempted by treatment of the acid with thionyl chloride at room temperature, but rapid polymerization was observed. The anhydride was chosen as an electrophile with attenuated reactivity. Treatment of the acid with EDCI or Mobashery's anhydride synthesis<sup>299</sup> (triphosgene + carboxylic acid + triethylamine) could be effectively employed, the latter being preferred due to the relative ease of purification of the anhydride. With a stable electrophile in hand, various conditions were attempted to effect the selective acylation on the aliphatic sugar alcohol over the aromatic phenolic hydroxyls. Basic conditions (Table 3.2; Entries 1,2) led to acylated products on the phenolic hydroxyl moiety, which was undesirable yet unsurprising.<sup>300</sup> Commercially available *Candida rugosa* lipase and porcine liver esterase (loading = 1 mg/mg of anhydride) failed to catalyze the reaction under the conditions in THF (Table 3.2; Entries 3,4). Next, we attempted to take advantage of the presence of the *syn*-diol for coordination, inspired by Taylor's precedents<sup>301, 302</sup>, albeit with the deliberate substitution of the usual acid chloride reagent with an anhydride. Unfortunately, diphenylborinic acid and DIPEA in acetonitrile failed to yield **3,3** (Table 3.2; Entry 5); hydrolysis of novobiocin to the aglycone **3,2** was observed. A method was needed that used anhydrides and alcohols as reaction partners, excluding methods that relied on basic conditions. A report by Procopiou<sup>303</sup> described a "fast, clean and efficient" esterification reaction between acid anhydrides and alcohols catalyzed by trimethylsilyl trifluoromethanesulfonate. When 5 mol % of TMSOTf was added to a 0 °C solution of descarbamoyl novobiocin and 5-methyl-1*H*-pyrrole-2-carboxylic anhydride in acetonitrile, the solution immediately turned yellow with concomitant precipitation. Analysis by LC/MS revealed a trace amount of a product corresponding to the

desired ester mass, but a significant amount of aglycone was formed (Table 3.2; Entry 6). Presumably the highly Lewis acidic TMSOTf was decomposing both the starting material and the product to the same aglycone. To mitigate this, the milder  $\text{Sc}(\text{OTf})_3$  catalyst was tested, according to the protocol of Yamamoto<sup>300</sup>, and the product was purified by reverse-phase HPLC for 1D  $^1\text{H}$  NMR and 2D COSY analysis (Table 3.2; Entry 7). The COSY experiment was selected to confirm proper assignments of sugar protons. When the equatorial alcohol of descarbamoyl novobiocin was esterified, the signal for the axial proton was shifted downfield by > 1 ppm to 5.48 ppm (Figure 3.9).

**Table 3.2. Screening conditions for regioselective acylation of descarbamoyl novobiocin.**



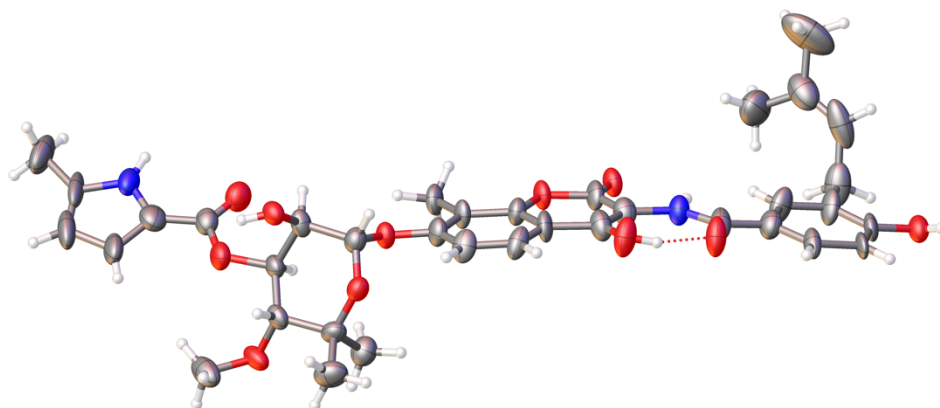
| Entry | catalyst<br>(mol %)                | solvent<br>(°C)     | additive<br>(equiv.) | yield<br>(%) |
|-------|------------------------------------|---------------------|----------------------|--------------|
| 1     | DMAP<br>(10)                       | pyridine<br>(23)    | --                   | 0            |
| 2     | --                                 | pyridine<br>(23)    | --                   | 0            |
| 3     | <i>Candida rugosa</i><br>lipase    | THF<br>(23)         | --                   | 0            |
| 4     | porcine liver<br>esterase          | THF<br>(23)         | --                   | 0            |
| 5     | diphenylborinic<br>acid<br>(10)    | MeCN<br>(23)        | DIPEA<br>(1.5)       | 0            |
| 6     | TMSOTf<br>(5)                      | MeCN<br>(0)         | --                   | trace        |
| 7     | <b>Sc(OTf)<sub>3</sub></b><br>(10) | <b>MeCN</b><br>(23) | --                   | <b>34</b>    |



**Figure 3.9. 2D COSY spectrum of the major product of the  $\text{Sc}(\text{OTf})_3$ -catalyzed reaction (**3,3**).**

The black rounded rectangle corresponds to the crosspeak between the doublet at 3.62 ppm and 5.48 ppm, suggesting acylation occurred at the desired position. Assignments of the four colored sugar protons of **3,3** are explicitly highlighted. The coumarin and benzamide moieties of **3,3** have been omitted for clarity.

In order to unambiguously confirm the regioselectivity of the acylation, attempts were made to crystallize **3,3**. Fortunately, one successful condition was found by recrystallization from a hot solution of ethyl acetate and water (Figure 3.10). Dr. Shao-Liang Zheng assisted with not only data collection, processing, and analysis, but also inspiration for obtaining better quality crystals.

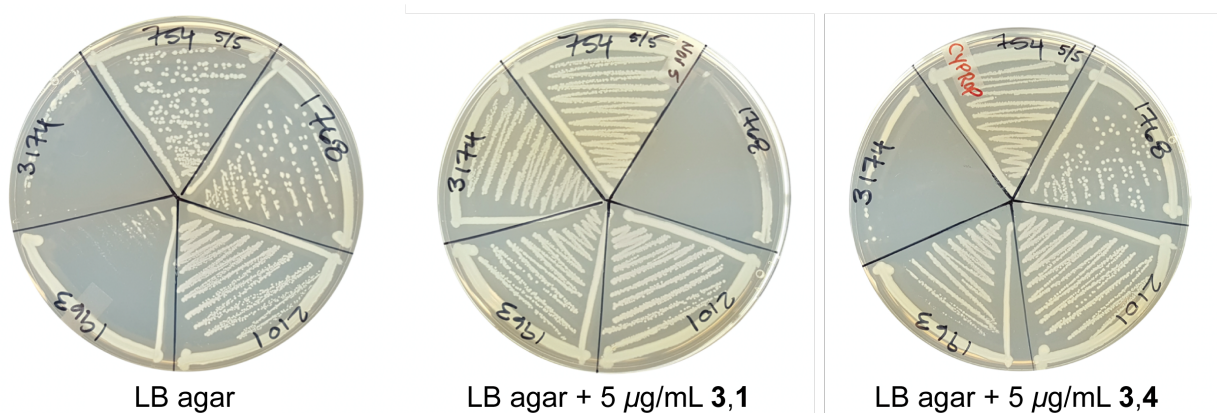


**Figure 3.10. X-ray crystal structure of 3,3.**

X-ray structure confirmed NMR assignments that the acylation occurred on the equatorial alcohol. X-ray structure details in Materials and Methods section.

### 3.2.4. Modulating LPS transport activity with novobiocin derivatives

We were interested in the modification of novobiocin to yield derivatives that could be both hyper- and hypoactive towards Lpt to gain insight about SAR. I previously synthesized a novobiocin derivative possessing a cyclopropyl group instead of the benzamide, cyclopropylnovobiocin (**3,4**). Cyclopropylnovobiocin was found to be unable to rescue cells from the lethal *lptB*(R144H) mutation (Figure 3.11).

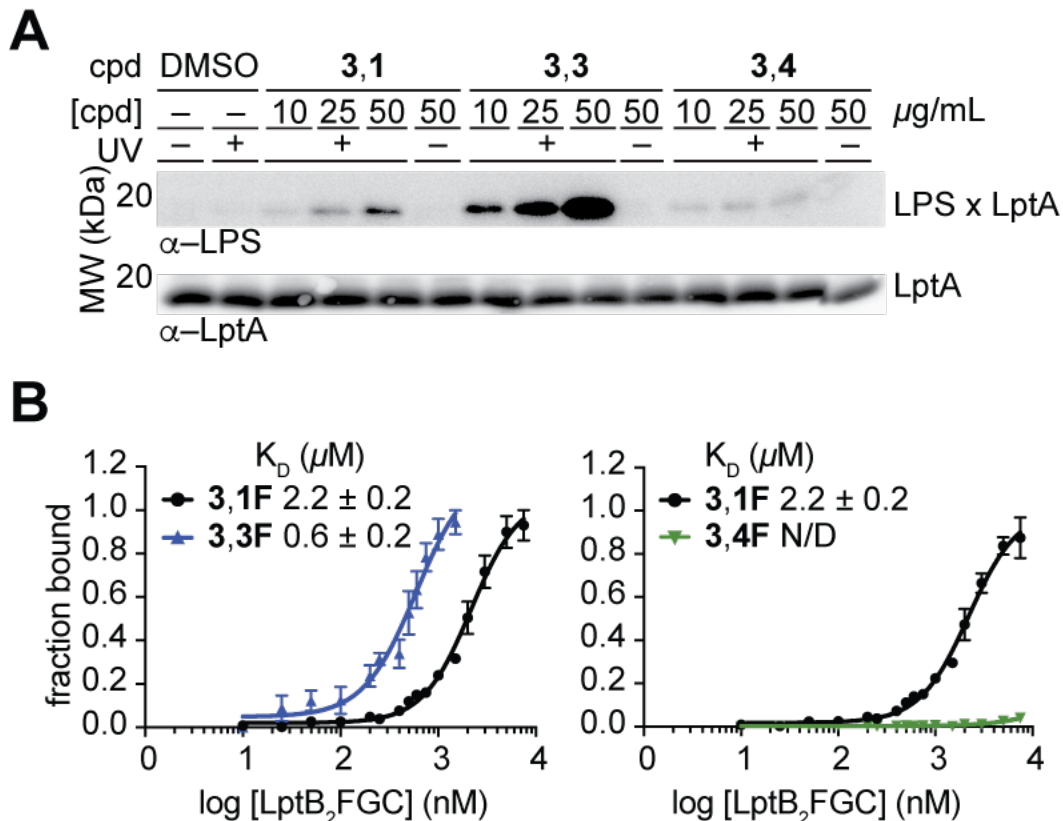


**Figure 3.11. Cyclopropyl novobiocin does not rescue *lptB*(R144H).**

NR754 (wildtype *E. coli*), NR1768 (*lptB1*), NR2101 (NR754  $\Delta$ *lptB tet2* + *pET23/42 lptB*), NR1963 (NR754 *lptB1*(R144H)::kan), NR3174 (NR754  $\Delta$ *lptB tet2* + *pET23/42 lptB*(R144H)). This experiment was performed by the Ruiz laboratory. Note how novobiocin (**3,1**) is able to rescue the slice labeled “3174” but cyclopropyl novobiocin (**3,4**) cannot.



The LPS release activity of **3,3** was compared, using a right-side-out vesicle assay, to that of novobiocin **3,1** and to cyclopropylnovobiocin **3,4** that lacked the ability to rescue *lptB(R144H)*. The results show that **3,3** is a novobiocin derivative with improved LPS transport stimulating activity (Figure 3.12A).



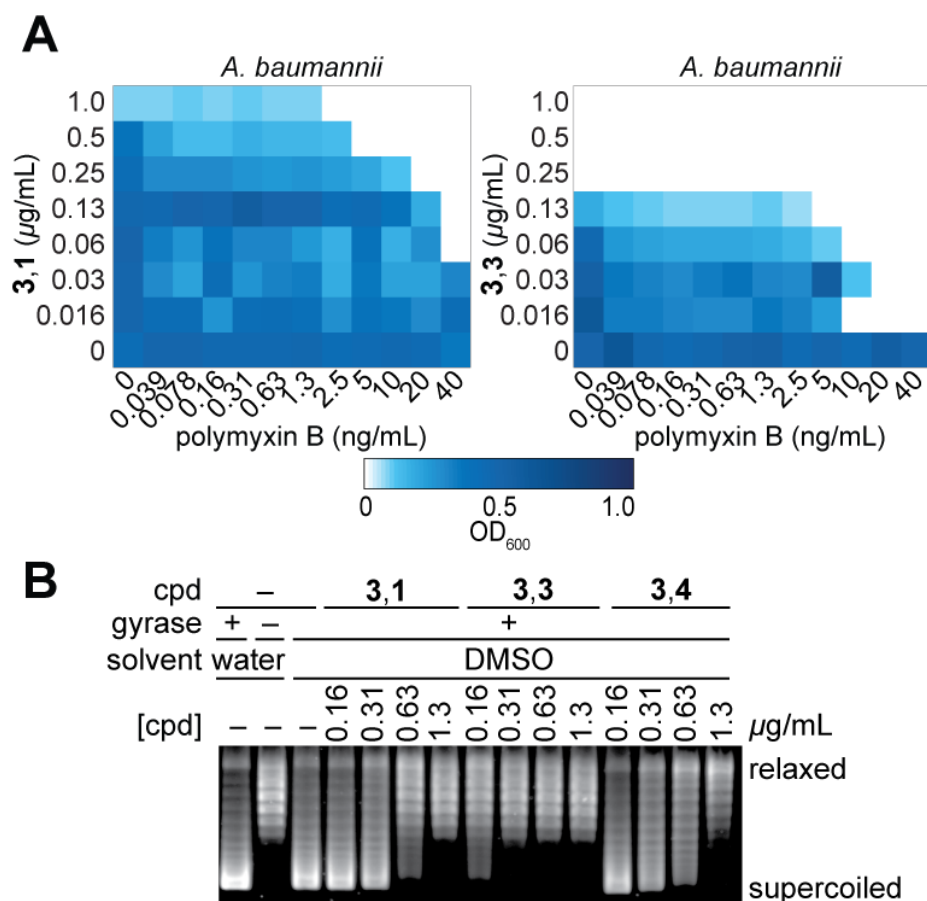
**Figure 3.12. LPS release activity and LptB<sub>2</sub>FGC binding of novobiocin (**3,1**) and synthetic derivatives.**

A) Right-side-out vesicle LPS release assay. Compound **3,3** stimulates more LPS release from the inner membrane Lpt complex to LptA than do compounds **3,1** and **3,4**. B) <sup>19</sup>F NMR binding assay demonstrating higher affinity binding of **3,3F** to LptB<sub>2</sub>FGC compared to **3,1F** and no binding from **3,4F**.

We were interested in measuring the affinity of **3,3** and **3,4** to the inner membrane LptB<sub>2</sub>FGC complex, and we synthesized the corresponding **3,3F** and **3,4F** (Figure 3.5) to utilize our NMR binding technique. **3,3F** showed nearly 4-fold tighter binding, while **3,4F** did not appear to bind the complex (Figure 3.12B). Given that **3,3** was likely stimulating more LPS transport by binding

LptB<sub>2</sub>FGC with higher affinity, we wondered whether it could potentiate polymyxin B activity better than **3,1** could. It is likely that the improved synergy of **3,3** with polymyxin B is a result of improved LPS stimulation and gyrase inhibition. After performing a checkerboard assay using *A. baumannii*, we found that **3,3** synergized with polymyxin B better than novobiocin **3,1** did (Figure 3.13A); however, **3,3** displayed improved activity against gyrase (Figure 3.13B).

### 3.2.5. Synergy of novobiocin derivatives with polymyxin B

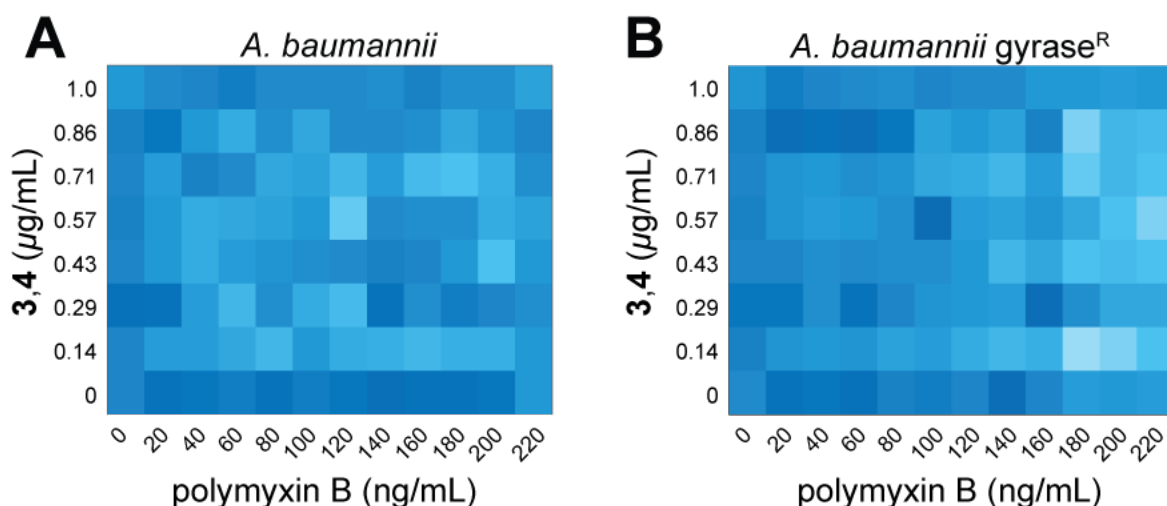


**Figure 3.13. Compound 3,3 synergy with polymyxin B involves a gyrase component.**

A) **3,3** potentiates polymyxin B activity greater than 8-fold than **3,1** does. B) **3,1** and **3,4** inhibit gyrase supercoiling at 0.63 µg/mL; **3,3** inhibits at 0.16 µg/mL.

**3,3** was able to potentiate the activity of polymyxin B against both gyrase-resistant and -sensitive strains, whereas **3,1** only showed synergy against the gyrase-sensitive strain at the

concentrations tested. These findings suggested that the LPS stimulatory activity plays an important role in the observed synergy; however, because **3,3** had gyrase activity, we were unsure what the boost in the synergy was the result of. We thought that a compound with similar *in vitro* gyrase activity but worse LPS transport activity might not synergize well with polymyxin. We found that **3,4**, which possessed *in vitro* gyrase activity but not LPS release activity, did not synergize with polymyxin B (Figures 3.12-3.14). However, these results were complicated by the fact that **3,4** was inactive against *A. baumannii* alone, suggesting permeability or other physical factors might be hampering its activity (Table 3.1). A compound with similar gyrase inhibitory activity but better LPS transport activity than **3,1** was needed.



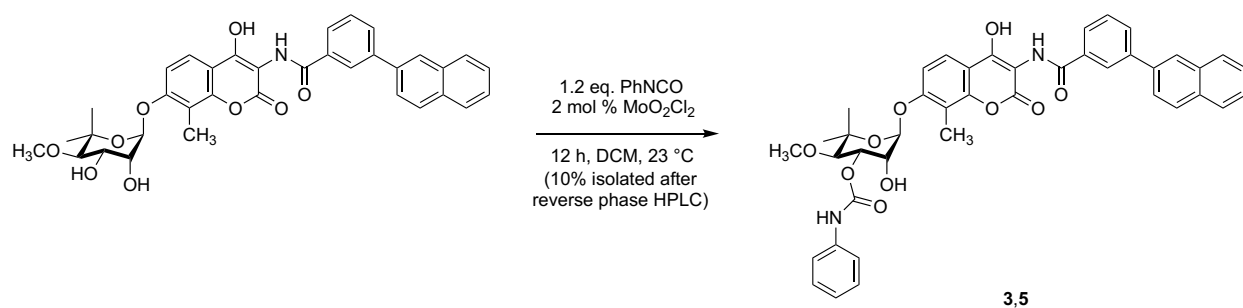
**Figure 3.14. Cyclopropylnovobiocin 3,4 does not synergize with polymyxin B.**

Cyclopropylnovobiocin **3,4**, which inhibits gyrase activity *in vitro*, but does not stimulate LPS release, did not synergize with polymyxin B against A) wildtype and B) gyrase-resistant *A. baumannii* at the concentrations shown. Note: lower concentrations of polymyxin B are effective at synergizing with **3,1**.

### 3.2.6. Chemical carbamoylation of descarbamoyl novobiocin and effect of compound **3,5** on LPS transport and gyrase activity

Our results suggested that the modifications on the sugar could improve novobiocin binding to Lpt and improved transport activity. Therefore, we explored further modifications at this position using urethane linkages (like the one present in novobiocin) to identify derivatives with improved LPS stimulation. The descarbamoyl novobiocin sugar could be modified with unnatural

urethane linkages. Treatment of descarbamoyl novobiocin with 1.2 equivalents of phenyl isocyanate at 23 °C for 2 hours in THF led to trace conversion of starting material and elevating the temperature to reflux did not promote significant product formation. A MoO<sub>2</sub>Cl<sub>2</sub>-catalyzed carbamoylation method<sup>304</sup> was found to work on descarbamoyl novobiocin, and various carbamate derivatives were synthesized (see Appendix). This method was applied to a semi-synthetic starting material (Figure 3.15), and compound **3,5** was synthesized as the major product using 2 mol % catalyst loading, mild temperature, and DCM as the solvent. These sugar derivatives typically needed to be purified by reverse-phase HPLC in order to separate undesired regioisomer of the carbamoylation (ie. on the axial hydroxyl). **3,5** was designed from observations that novobiocin derivatives containing an *N*-phenyl carbamate on the sugar or 3-biarylbenzamides were more active at LPS transport stimulation.

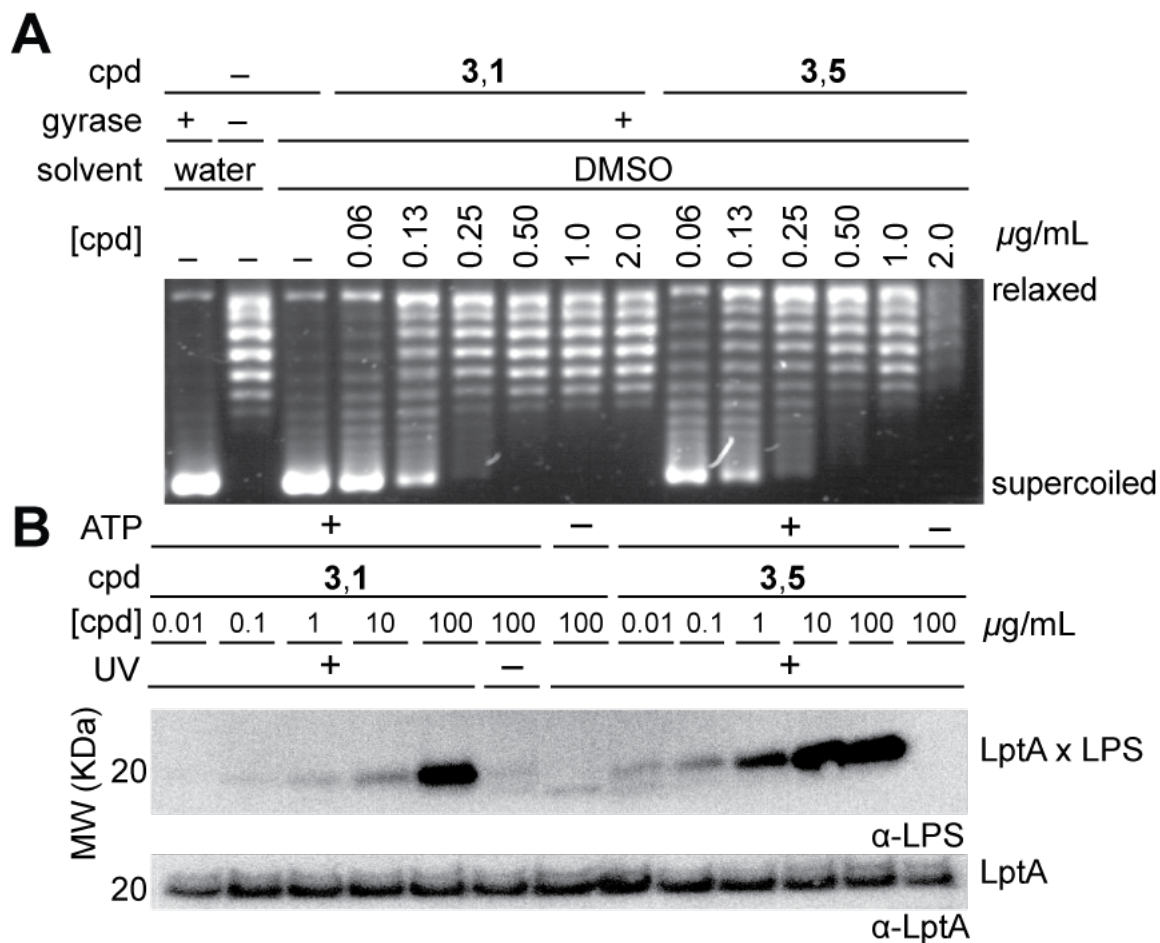


**Figure 3.15. Mo<sup>VI</sup>-catalyzed carbamoylation of descarbamoyl novobiocin.**

The sugar equatorial alcohol could be selectively modified using catalytic dichlorodioxomolybdenum and an isocyanate.

**3,5** was tested alongside **3,1** for inhibitory activity against *A. baumannii* gyrase and found to be approximately equipotent (Figure 3.16A). The LPS transport stimulatory activity for the two compounds showed that **3,5** was between 10-100-fold more active at stimulating LPS transport than **3,1** (Figure 3.16B). Taken together, these *in vitro* biochemical results showed that gyrase inhibition and LPS transport stimulatory features of aminocoumarins could be differentiated. It is likely that the substitution of a hydrogen bond donor on the primary carbamate of **3,1** for a phenyl in **3,5** reduces the affinity of **3,5** for gyrase, while the rigid biaryl benzamide improves gyrase

activity in a way that is nearly balanced; however, binding experiments were not conducted to test this hypothesis.



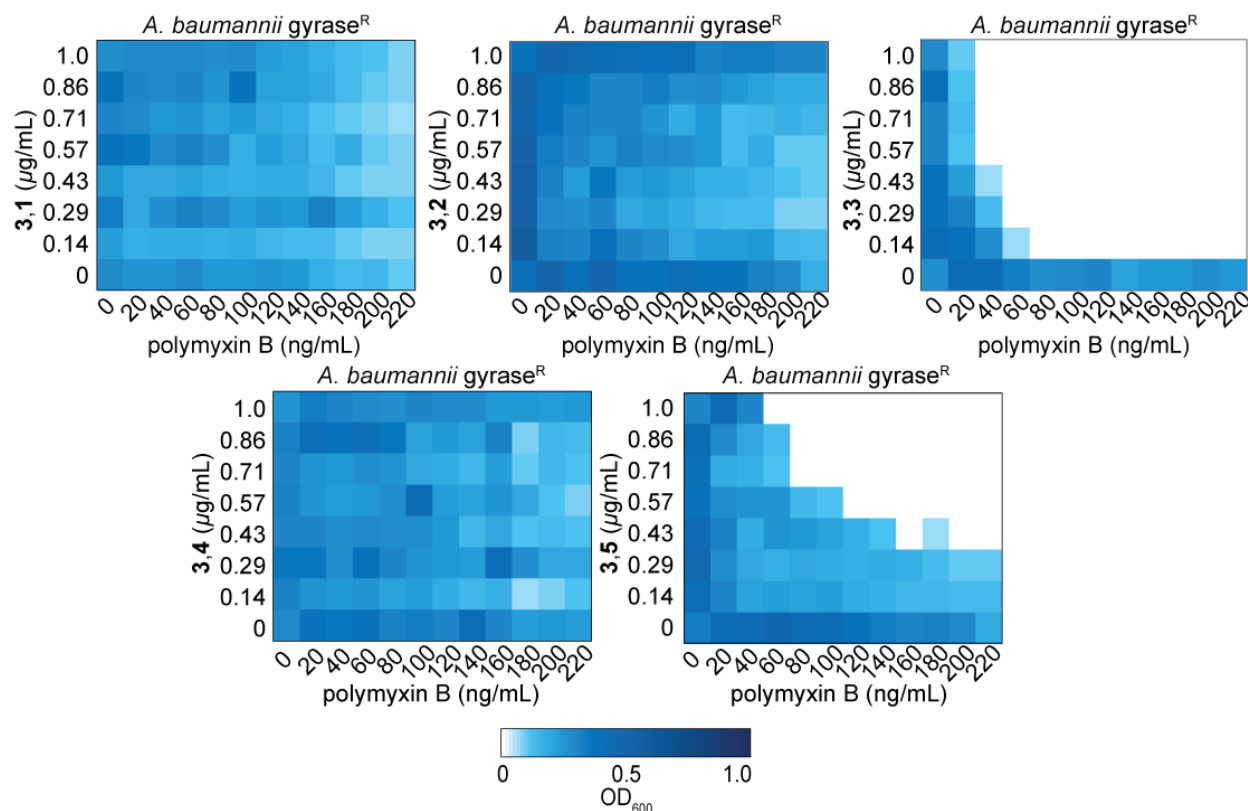
**Figure 3.16. Compound 3,5 inhibits gyrase activity as well as 3,1 but is more potent at LPS transport stimulation.**

A) 3,1 and 3,5 share *in vitro* activity against *A. baumannii* gyrase. B) 3,5 is between 10 and 100-fold more potent than 3,1 at stimulating LPS transport.

### 3.2.7. Synergy of 3,5 with polymyxin B

With a compound possessing a greater degree of LPS transport stimulation, we examined the ability of 3,5 to synergize with polymyxin B against gyrase-resistant *A. baumannii*. At the concentrations tested, 3,1, 3,2, 3,4 were ineffective at synergizing with polymyxin B against gyrase-resistant *A. baumannii* (Figure 3.17). 3,3, which has better LPS transport stimulation activity, synergized in a manner superior to 3,1 under the concentrations tested; however, this

compound also had more potent gyrase activity. **3,5**, with equal gyrase activity as **3,1** but 10-100x the LPS transport stimulatory activity, synergized better than **3,1**. Taken together, these results suggest that both gyrase inhibition and LPS transport play a role in the apparent synergy between aminocoumarins like novobiocin and the polymyxins.



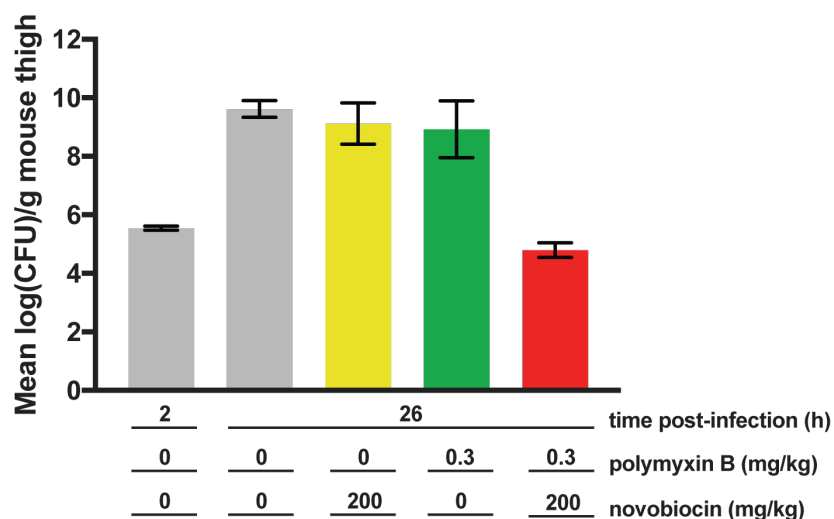
**Figure 3.17. Novobiocin induced LPS release enhances polymyxin-mediated killing.**

Checkerboard broth microdilution assay of **3,1**–**3,5** against gyrase-resistant *A. baumannii*. Both gyrase inhibition and LPS transport stimulation appear to play an important role in the synergy.

### 3.2.8. Synergy of novobiocin and polymyxin B in animals

We wondered whether the combination of novobiocin and polymyxin could be effective at clearing a Gram-negative bacterial infection in a murine thigh infection model. Both polymyxin B and novobiocin have been used clinically, but the combination of both agents has not been extensively investigated. We worried that the physical properties of novobiocin and polymyxin together might preclude their distribution to the active site of infection. We conducted a proof-of-concept murine thigh infection study involving neutropenic mice to determine if the combination

of novobiocin and polymyxin, administered subcutaneously, could reduce the colony forming units per gram of mouse thigh (CFU/g) in *E. coli* ATCC 25922-infected mice (Figure 3.18).



**Figure 3.18. Novobiocin and polymyxin B synergize *in vivo*.**

Female CD-1 mice (n=4 per group) were infected with an inoculum of *E. coli* into the rear thigh. 2 and 6 hours post infection, mice were subcutaneously injected with either polymyxin, novobiocin, or a combination of both. Mice were sacrificed at 26 hours and their rear thigh was excised, homogenized, and plated in serial dilutions to determine bacterial CFUs. This experiment was performed by NeoSome Life Sciences (Lexington, MA).

We found that the combination of novobiocin and polymyxin B, at doses that were each ineffective in monotherapy, effected a  $10^4$  reduction of CFU/g mouse thigh. The combination was superior to the untreated control or monotherapy groups. Therefore, novobiocin and polymyxin have potential for *in vivo* efficacy.

### 3.3. Discussion

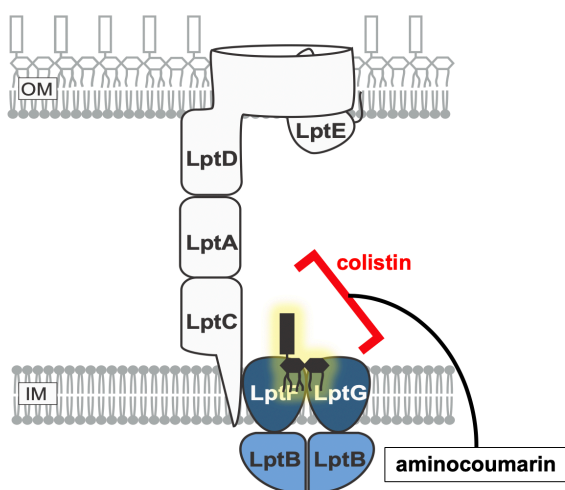
This chapter described the modification of novobiocin to yield unnatural aminocoumarin molecules that had different effects on both LptB<sub>2</sub>FGC and DNA gyrase. The goal was to see if the activity of aminocoumarins on LptB<sub>2</sub>FGC was dependent on gyrase inhibition. Upon chemical conversion of novobiocin **3,1** (gyrase IC<sub>50</sub> = 1.9  $\mu$ M) to the aglycone **3,2**, all DNA supercoiling inhibition is lost (gyrase IC<sub>50</sub> > 200  $\mu$ M). Despite this, **3,2** retains a similar degree of LPS transport stimulation compared to **3,1** as measured by the LPS release assay in right-side-out vesicles. The

fluorinated analogs **3,1F** ( $K_D = 2.2 \pm 0.2 \mu\text{M}$ ) and **3,2F** ( $K_D = 1.9 \pm 0.1 \mu\text{M}$ ) showed similar LptB<sub>2</sub>FGC binding as measured by <sup>19</sup>F NMR spectroscopy experiments in the low, single digit micromolar range. Therefore, novobiocin aglycone is a molecule that binds and activates the inner membrane lipopolysaccharide transport complex but does not inhibit DNA gyrase. Despite lacking antibiotic activity, the aglycone synergized with polymyxin B in a manner that did not depend on the presence of an aminocoumarin-resistant gyrase. Moreover, novobiocin derivatives with increased LPS transport stimulation (**3,3** and **3,5**) were synthesized and these molecules potentiated the activity of polymyxin B better than novobiocin did. A novobiocin derivative (**3,4**), which did not appear to bind LptB<sub>2</sub>FGC and stimulate LPS transport, did not synergize with polymyxin B. Hence the structure-activity relationships uncovered that although the sugar is dispensable for LptB<sub>2</sub>FGC binding and stimulation, modifications at this position can improve both of these properties. Substitution of the novobiocin benzamide moiety with a small, aliphatic cyclopropylcarboxamide (**3,4**) totally ablated LptB<sub>2</sub>FGC binding and stimulation. Therefore, modifications at both the noviose sugar and the benzamide can have a profound effect on LptB<sub>2</sub>FGC binding and LPS transport activity. Provided this insight, I synthesized novobiocin derivatives and screened their activities against the Gram-negative *ESKAPE* pathogens as well as some Gram-positive organisms (Appendix). The primary assay used was a minimal inhibitory concentration (MIC) assay, and subsequent compounds could be investigated by *in vitro* biochemical methods, such as the aforementioned LPS release assay or DNA supercoiling assay.

While the checkerboards in Figure 3.17 paint a picture of the combination antibiotic sensitivities of gyrase-resistant *A. baumannii*, they do not suggest a mechanism for how novobiocin and polymyxin synergize. In light of observations that an LpxC inhibitor antagonizes the activity of polymyxin,<sup>298</sup> we considered the role of LPS transport in the activity of polymyxin. Our evidence suggests that colistin is hyperactive when LPS transport is stimulated and hypoactive when LPS transport is inhibited.



We hypothesized that polymyxin might be binding LPS during active transport within LptB<sub>2</sub>FGC. If that were the case, a chimera between polymyxin and novobiocin could bind both Lpt and LPS concurrently. The proposed chimera would be significantly larger than 1000 Da, which is larger than most antibiotics active against Gram-negatives. However, because polymyxin can permeabilize the outer membrane, a chimera between polymyxin and novobiocin might be effective against Gram-negatives (Figure 3.19). The next chapter of this thesis will describe our path to design chimeric novobiocin-polymyxin molecules.



**Figure 3.19. Proposed chimera between an aminocoumarin and colistin.**

A synthetic chimera between an aminocoumarin similar to novobiocin and a polymyxin (e.g. colistin) could simultaneously bind LPS during an active state of transport and the novobiocin binding site in LptB<sub>2</sub>FGC.

## 3.4. Materials and Methods

### 3.4.1. *E. coli* strain used for right-side-out vesicle assay

*E. coli* strain NovaBlue [*endA1 hsdR17* (*r*<sub>K12</sub><sup>-</sup> *m*<sub>K12</sub><sup>+</sup>) *supE44 thi-1 recA1 gyrA96 relA1 lac F'* [*proA*<sup>+</sup> *B*<sup>+</sup> *lacI*<sup>q</sup>  $\Delta$ *M15::Tn10*] (*Tet*<sup>R</sup>)] from Novagen was used for plasmid manipulations. *E. coli* strain BL21( $\lambda$ DE3) [*F- ompT gal dcm lon hsdSB*(*rB*<sup>-</sup>, *mB*<sup>-</sup>)  $\lambda$ (*DE3*)] from Novagen was used for right-side-out (RSO) vesicle preparation. The plasmids used in this chapter are listed in Table

3.4.1. Restriction enzyme DpnI was purchased from New England Biolabs. All other materials were purchased from Sigma Aldrich unless noted otherwise.

**Table 3.3. Plasmids used in this study**

| Name                   | Description                                                                                        | Ref. |
|------------------------|----------------------------------------------------------------------------------------------------|------|
| pBAD/HisA-LptC         | Encodes LptC for the RSO vesicle assay                                                             | 133  |
| pCDFduet-LptB-LptFG    | Encodes LptB and LptFG for the RSO vesicle assay                                                   | 133  |
| pET22b-LptA(I36Am)-His | LptA with amber codon (TAG) for substitution with <i>pBPA</i>                                      | 133  |
| pSup-BpaRS-6TRN        | Encodes amino-acyl tRNA synthetase as well as tRNAs for incorporation of <i>pBPA</i> at TAG codons | 305  |

### 3.4.2. Purification of LptB<sub>2</sub>FGC

LptB<sub>2</sub>FGC-His was prepared as described<sup>176</sup> with minor modifications. Overnight cultures of C43(DE3) *E. coli* containing pCDFduet-LptB-LptFG and pET22/42-LptC-His<sub>7</sub> were diluted 1:100 into fresh LB supplemented with 50  $\mu$ g/mL carbenicillin and 50  $\mu$ g/mL spectinomycin, grown at 37 °C to OD<sub>600</sub> ~ 0.8, then cooled to 20 °C and grown to OD<sub>600</sub> ~ 1.0. Expression was then induced by addition of 200  $\mu$ M IPTG and carried out at 20 °C for 16 hours. Cells were harvested by centrifugation at 5,000 *g*, resuspended in lysis buffer (50 mM Tris pH 7.4, 300 mM NaCl) supplemented with 1 mM PMSF, 100  $\mu$ g/mL lysozyme, and 50  $\mu$ g/mL DNaseI, and homogenized with an IKA T18 basic UltraTurrax. Cells were then lysed by 3x passage through an EmulsiFlex-C3 cell disruptor at 15,000 psi, after which unbroken cells and cell debris were removed by centrifugation at 12,000 *g* for 10 minutes. Membranes were isolated from the lysate by centrifugation at 100,000 *g* for 1 hour, resuspended in solubilization buffer (20 mM Tris pH 7.4, 300 mM NaCl, 10% glycerol, 5 mM MgCl<sub>2</sub>), homogenized, supplemented with 1% w/v DDM (n-dodecyl- $\beta$ -D-maltopyranoside, Anatrace) 50  $\mu$ M PMSF, 15 mM imidazole, and 2 mM ATP, and rocked at 4 °C for 1 hour. Insoluble debris were removed by centrifugation at 100,000 *g* for 30 minutes, and the clarified supernatant was then rocked with Ni-NTA Superflow resin (Qiagen) at 4 °C. After 1 hour, the resin was drained, washed with affinity buffer (20 mM Tris pH 7.4, 300 mM NaCl, 10% glycerol, 0.01% DDM, 0.01% LMNG (lauryl maltose neopentyl glycol,

Anatrace)) containing 20 mM then 40 mM imidazole, and protein was eluted with affinity buffer containing 200 mM imidazole. This eluate was concentrated using a 100 kDa molecular weight cutoff Amicon Ultra centrifugal filter (Millipore) and applied to a Superdex 200 10/300GL column (GE Healthcare) with 20 mM Tris-HCl pH 7.4, 300 mM NaCl, 0.005% w/v LMNG, and 10% glycerol. Peak fractions from the size-exclusion chromatography step were pooled, concentrated to ~ 4 mg/mL again using a 100 kDa molecular weight cutoff Amicon filter, and then stored at 4 °C.

### 3.4.3. Gyrase activity assay

*Escherichia coli* and *Acinetobacter baumannii* gyrase supercoiling inhibition assay kits were purchased from the Inspiralis company (Norwich, UK). The assay was conducted according to the provided instructions. 1 U of DNA gyrase (as determined by a titration of gyrase dilution per kit instructions) was incubated with 0.5 µg of relaxed pBR322, and the stated compound concentration, in a reaction volume of 30 µL at 37 °C for 30 minutes in Assay Buffer (35 mM Tris•HCl, pH 7.5; 24 mM KCl; 4 mM MgCl<sub>2</sub>, 2 mM DTT, 1.8 mM spermidine, 1 mM ATP, 6.5% (w/v) glycerol, 0.1 mg/mL albumin). The supercoiling reactions were quenched by the addition of 30 µL of STEB buffer (40% (w/v) sucrose, 100 mM Tris•HCl pH 8, 10 mM EDTA, 0.5 mg/mL bromophenol blue) and 30 µL of chloroform/isoamyl alcohol (v/v, 24:1). Samples were vortexed for 5 minutes and centrifuged at max speed for 1 minute. 20 µL of the upper aqueous layer were loaded on a 1 % (w/v) agarose gel free of DNA intercalator and run at 85 V for 2 h. The gel was then stained with DNA intercalator and visualized with an Azure c400 imaging system (Azure Biosystems).

### 3.4.4. Right-side-out vesicle preparation

Right-side-out (RSO) vesicles were prepared as described previously by our group<sup>133, 177</sup> with minor modifications. BL21(λDE3) *E. coli* were transformed with plasmid pBAD18HisA-LptC

along with pCDFduet-LptB-LptFG (encoding wild-type *E. coli* proteins). Overnight cultures were diluted 1:100 into 50 mL of LB Miller media containing 50  $\mu\text{g/mL}$  carbenicillin and 50  $\mu\text{g/mL}$  spectinomycin and grown at 24 °C to  $\text{OD}_{600} \sim 1$ . Growth temperature was then increased to 37 °C and expression induced by addition of 0.02% L-arabinose and 10  $\mu\text{M}$  isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). After two hours, cells were pelleted, resuspended in 5 mL 50 mM Tris-HCl (pH 7.4) 250 mM sucrose, 300  $\mu\text{g/mL}$  lysozyme and 150  $\mu\text{g/mL}$  DNaseI, and converted to spheroplasts by dropwise addition of 5 mL buffer containing 50 mM Tris-HCl (pH 7.4), 250 mM sucrose and 3 mM EDTA followed by incubation on ice for 30 minutes.

To convert spheroplasts to RSO-vesicles, spheroplasts were pelleted, 6000  $g \times 10$  minutes, resuspended in 5 mL lysis buffer (20 mM Tris (pH 8), 150 mM NaCl, 0.1 mM EDTA, 5 mM  $\text{MgCl}_2$ , and 5 mM sodium-ATP (pH  $\sim 7$ )), pelleted at 10,000  $g \times 15$  minutes, and resuspended in the supernatant to complete lysis. RSO-vesicles were collected by centrifugation at 200,000  $g \times 30$  minutes and resuspended in 1 mL of 10% v/v glycerol, 20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM  $\text{MgCl}_2$ , and 5 mM sodium-ATP (pH  $\sim 7$ ). Total protein concentration in RSO-vesicle samples was determined by DC-protein assay (BioRad), and samples were either used immediately in LPS release assays or flash-frozen and stored at  $-80$  °C.

#### 3.4.5. Purification of LptA\*

LptA\* ((LptA(I36pBPA)) was purified as described<sup>133, 177</sup> with minor modifications. BL21( $\lambda$ DE3) *E. coli* containing pSup-BpaRS-6TRN and pET22b-LptA(I36Am) were grown to  $\text{OD}_{600} \sim 0.6$  at 37 °C in 1.5 L LB Miller media containing 50  $\mu\text{g/mL}$  carbenicillin, 30  $\mu\text{g/mL}$  chloramphenicol, and 0.7 mM *para*-benzoylphenylalanine (pBPA, BaChem), and induced for 2 hours with 50  $\mu\text{M}$  IPTG. These cells were pelleted, converted to spheroplasts as described above, and the spheroplasts pelleted at 6000  $g \times 10$  minutes. Supernatant from the spheroplasts was collected and supplemented with 1 mM PMSF and 10 mM imidazole and clarified by ultracentrifugation at 100,000  $g \times 30$  minutes. Supernatant was applied twice to Ni-NTA resin,

washed with 2 x 20 column volumes of wash buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 10% v/v glycerol) with 20 mM imidazole, and eluted with 2 x 2.5 column volumes of wash buffer with 200 mM imidazole. Eluate was concentrated to ~1 mg/mL using an Amicon 10 kDa cut-off Amicon centrifugal filter (Millipore), aliquoted, and stored at -80 °C.

#### **3.4.6. LPS-release assay**

The *in vitro* LPS-transport experiments were set-up as described<sup>177</sup> with some modifications. RSO-vesicles (50 µg total protein) were diluted into 100 µL reaction buffer (10% v/v glycerol, 20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM sodium-ATP, 5 mM MgCl<sub>2</sub>), and incubated on ice with novobiocin, novobiocin derivatives, or no compound for 15 minutes (50X compound stocks in water were used for each concentration). To start the assay, 3 µg of LptA\* was added to each sample, with each time-point its own 100 µL sample, and samples were then incubated at 30 °C for the stated time. To cross-link LptA\* to LPS, samples were transferred to a 96-well plate and irradiated with 365 nm UV-light for 5 minutes. Cross-linked samples were then mixed 1:1 with 2x SDS loading dye (100 mM Tris-HCl pH 6.8, 4% w/v SDS, 0.05% w/v bromothymol blue, 20% glycerol) with 5% β-mercaptoethanol, and boiled for 10 minutes.

Immunoblotting was used to assess LptA\*-LPS levels in each sample. Boiled samples were run on homemade 4%/15% polyacrylamide stacking gels at 0.02 A constant-current until the 15 kDa ladder band (BioRad Precision Plus all Blue standards) had run out of the gel, transferred to PVDF, and immunoblotted with mouse anti-LPS core antiserum (HyCult Biotechnology) followed by sheep-anti-mouse IgG horseradish peroxidase conjugate (GE Healthcare). LptA levels were assessed similarly, using rabbit anti-LptA antiserum<sup>234</sup> followed by donkey anti-rabbit horseradish peroxidase conjugate (GE Amersham). ECL Prime Western Blotting Detection Reagent (GE Amersham) was used to visualize antibody-label bands in conjunction with an Azure c400 imaging system (Azure Biosystems).

#### 3.4.7. $^{19}\text{F}$ NMR LptB<sub>2</sub>FGC binding assay

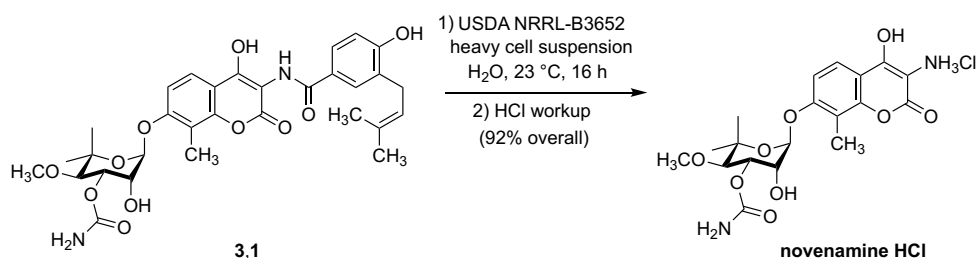
NMR buffer containing 0.005% (w/v) lauryl maltose neopentyl glycol (LMNG, Anatrace), 10% (v/v) glycerol, 300 mM NaCl, 20 mM Tris pH 7.4, 8 mM imidazole, and D<sub>2</sub>O was prepared. To a series of Eppendorf tubes, on ice, the NMR buffer was added, followed by the fluorinated novobiocin derivative, and purified LptB<sub>2</sub>FGC protein to a total volume of 200  $\mu\text{L}$ . The tubes were vortexed briefly, the solutions transferred to Bruker Biospin 3.0x103.5 mm SampleJet NMR tubes and stored at +6 °C until acquisition.  $^{19}\text{F}$  NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer equipped with a Prodigy liquid N<sub>2</sub> cooled cryoprobe. A standard fluorine 1D pulse program was used with the probe temperature set to 298.1 K. After acquisition, FIDs were Fourier transformed, phased, and processed with the exponential multiplication window function (LB = 10.00 Hz) in MestReNova MNova v10. The  $^{19}\text{F}$  linewidths were normalized, plotted against LptB<sub>2</sub>FGC concentration, and analyzed using the GraphPad Prism 7 software using the nonlinear fit log(agonist) vs. response model, variable slope (four parameters). Error bars represent the standard error of the mean (s.e.m) with n = 3.

#### 3.4.8. General synthetic methods

Unless otherwise noted, all reactions were performed under anhydrous N<sub>2</sub>. Solvents were purchased in Sure-Seal bottles from Sigma-Aldrich and used without further purification. Thin layer chromatography (TLC) was carried out using EM Science silica gel 60 F254 plates; the developed plate was analyzed by UV lamp (254 nm). Normal phase column chromatography was performed with the Teledyne-Isco CombiFlash R<sub>f</sub>200 system using pre-packed silica gel column cartridges and hexanes/ethyl acetate or dichloromethane/methanol as the solvent system. Reverse phase column chromatography was performed with the Teledyne-Isco CombiFlash R<sub>f</sub>200 system using pre-packed C<sub>18</sub>Aq columns and water/acetonitrile as the solvent system.  $^1\text{H}$  NMR and  $^{13}\text{C}$  NMR spectra were recorded in DMSO-*d*<sub>6</sub> on a Varian Inova-500 MHz spectrometer unless otherwise noted.  $^{19}\text{F}$  NMR spectra were recorded on a Bruker Avance II 600 MHz

spectrometer equipped with a Prodigy liquid N<sub>2</sub> cooled cryoprobe. Chemical shifts are reported in ppm with the residual solvent signal as the reference and coupling constants (*J*) are given in hertz. The peak information is described as: br = broad singlet, s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet, comp = composite of overlapping signals. High-resolution mass spectra (HRMS) were performed on a Bruker microTOFII ESI LCMS system mass spectrometer using sodium formate as the standard.

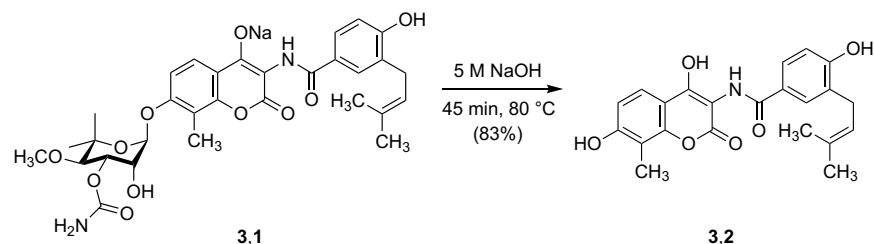
### 3.4.9. Synthesis of novenamine HCl



Novenamine HCl was prepared utilizing a modified procedure.<sup>193</sup> 600 mL of USDA NRRL-B3652 cells were grown for 24 h at 28 °C, 220 rpm. Cells were harvested by centrifugation at 5,200 g for 30 minutes, and the spent media was discarded. Using a minimal volume of distilled water, the cells were resuspended to generate 20 mL of a heavy cell suspension, which was transferred to a 100 mL round bottom flask equipped with a magnetic stir bar. The flask was fitted with a rubber septum and sparged with N<sub>2</sub>. The suspension was thoroughly stirred as 20 mL of a 20 mg/mL solution of novobiocin sodium (400 mg) was injected. The reaction was stirred for 16 h at room temperature. Once the reaction was complete, 1.2 mL of 2 M HCl were added while stirring under N<sub>2</sub> to convert novenamine to the air-stable anilinium form. Precipitation of soluble cellular material also took place. At this point, the septum was removed, and the reaction mixture was transferred to two 50 mL Falcon centrifuge tubes and centrifuged at 10,000 g for 20 min. The supernatant was decanted into two fresh 50 mL Falcon centrifuge tubes and 10 mL of *n*-butyl acetate were added to each centrifuge tube. The tubes were vortexed, centrifuged at 10,000 g for

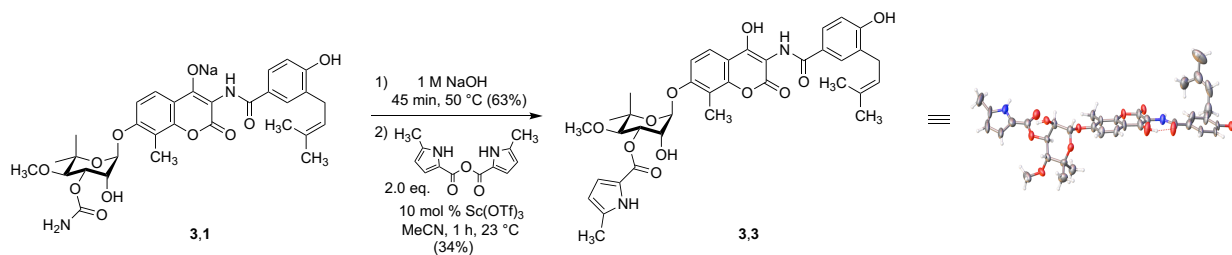
10 min in order to separate the organic layer, which was discarded. The *n*-butyl acetate extraction step was repeated two more times. Finally, the remaining aqueous layer was flash frozen and lyophilized to yield a pale-yellow powder (267 mg, 92%).

### 3.4.10. Synthesis of novobiocin aglycone (3,2)



To a 25 mL round bottom flask equipped with a magnetic stirbar was added 500 mg of **3,1** as the sodium salt and 5 mL of 5 M NaOH. The reaction mixture was capped with a septum to avoid evaporation of the solution and heated at 80 °C for 45 minutes. The mixture was then cooled to room temperature and neutralized with 5 M HCl, which precipitated **2** as an off-white solid. The suspension was solubilized and extracted with 3 x 20 mL of EtOAc. The organic layer was washed once with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, concentrated by rotary evaporation and purified by flash column chromatography using DCM/MeOH as the solvent system. Fractions containing the product (monitored by UV absorbance at 340 nm) were concentrated by rotary evaporation and hi-vaced to afford **3,2** as a yellow solid (523 mg, 83%).

### 3.4.11. Synthesis of (3,3)

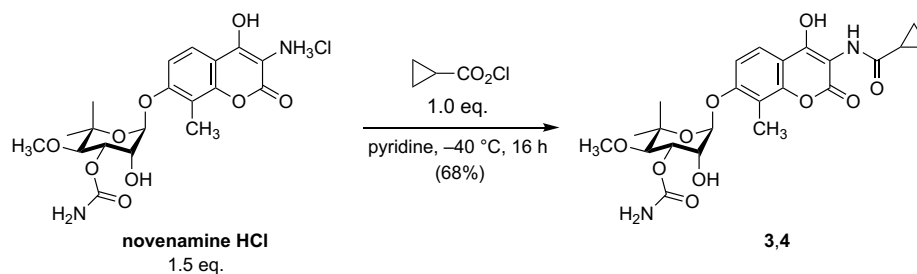




To a 250 mL round bottom flask equipped with a magnetic stirbar was added 5 g of **3,1** as the sodium salt and 50 mL of 1 M NaOH. The flask was capped with a septum to avoid evaporation of the solution and heated at 50 °C for a total reaction time of 45 minutes. The mixture was then cooled to 0 °C, neutralized with 2 M HCl, extracted with 3 x 50 mL DCM, and the organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> with heavy stirring for 30 min. The material was concentrated by rotary evaporation and then purified by flash column chromatography with DCM/MeOH as the solvent system and monitoring A<sub>340</sub>. The desired descarbamoyl novobiocin compound eluted after the aglycone **2**, but before unreacted starting material **3,1**. Fractions containing the product were concentrated and hi-vaced to afford descarbamoyl novobiocin as a pale-yellow solid (2.8 g, 63%), which was used in the next step.

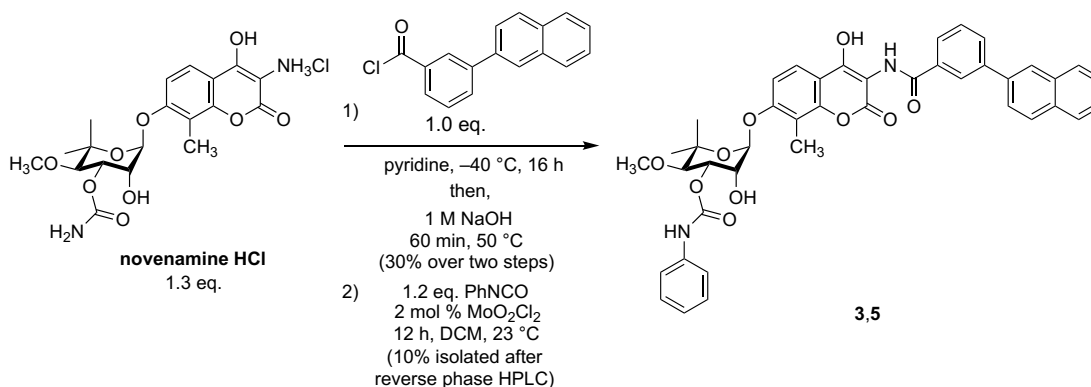
A 20 mL scintillation vial was charged with a magnetic stir bar, descarbamoyl novobiocin (100 mg, 0.18 mmol, 1.0 eq.), 5-methyl-1*H*-pyrrole-2-carboxylic anhydride<sup>196</sup> (81.5 mg, 0.35 mmol, 2.0 eq.), and 5 mL of anhydrous MeCN. The reaction mixture was stirred at room temperature for 5 min. Meanwhile, a 100 mg/mL solution of Sc(OTf)<sub>3</sub> dissolved in anhydrous MeCN was prepared and injected into the reaction mixture via Hamilton syringe (86 µL, 0.018 mmol, 0.1 eq.). The reaction mixture instantly turned a bright yellow color and was allowed to react for at least 1 h or until TLC indicated the reaction was complete. The mixture was then concentrated by rotary evaporation, redissolved in a minimal volume of DMSO, and subjected to reverse-phase column chromatography. Fractions containing the product were concentrated by rotary evaporation and hi-vaced to afford **3,3** as a tan solid (40 mg, 34%). To ensure that the major product was indeed the correct regioisomer, **3,3** was recrystallized from ethyl acetate and submitted for X-ray analysis (see X-ray structure of **3,3** below).

### 3.4.12. Synthesis of (3,4)



A 100 mL conical flask was charged with a magnetic stir bar and novenamine hydrochloride (660 mg, 1.44 mmol, 1.5 eq.). The flask was fitted with a rubber septum and a vent needle and sparged with N<sub>2</sub> for 10 minutes (novenamine is unstable towards molecular oxygen under basic conditions). While under N<sub>2</sub>, 7 mL of pyridine were added via syringe and the reaction mixture was stirred at −40 °C using a dry ice/acetonitrile bath. After 5 min of stirring at −40 °C, cyclopropanecarbonyl chloride (86.8 μL, 100 mg, 0.96 mmol, 1.0 eq.) was added via Hamilton syringe. The vent needle was removed after 10 minutes of stirring, and the reaction was allowed to warm to room temperature after 16 h while under N<sub>2</sub>. The reaction mixture was concentrated by rotary evaporation, resuspended in DCM, and subjected to normal phase silica gel column chromatography (DCM/MeOH). Fractions containing the product were concentrated and hi-vaced to afford **3,4** as a white solid (320 mg, 68%).

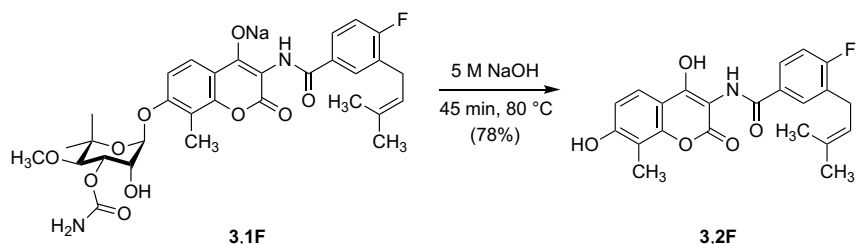
### 3.4.13. Synthesis of (3,5)



A 20-mL scintillation vial was charged with a magnetic stir bar, **novenamine hydrochloride** (160 mg, 0.35 mmol, 1.3 eq.), and solid 3-(naphthalen-2-yl)benzoyl chloride. The flask was fitted with a rubber septum and a vent needle and sparged with N<sub>2</sub> for 10 minutes (novenamine is unstable towards molecular oxygen under basic conditions). While under N<sub>2</sub>, 7 mL of pyridine were added via syringe and the reaction mixture was stirred at –40 °C using a dry ice/acetonitrile bath. The vent needle was removed after 10 minutes of stirring, and the reaction was allowed to warm to room temperature after 16 h while under N<sub>2</sub>. The reaction mixture was concentrated by rotary evaporation, resuspended in DCM, and subjected to an aqueous workup, in which the organic layer was subsequently evaporated and dissolved in 5 mL of 1 M NaOH. The solution was transferred to a clean 20 mL scintillation vial and heated at 50 °C for 60 minutes while sealed with a Teflon cap to prevent evaporation of the solution. The reaction was neutralized with 1 M HCl and subjected to an aqueous workup. The organic material was concentrated by rotary evaporation, resuspended in DCM, and subjected to normal phase silica gel column chromatography (DCM/MeOH) monitoring at 340 nm. Fractions containing the product were concentrated and hi-vaced before the final carbamoylation step.<sup>304</sup>

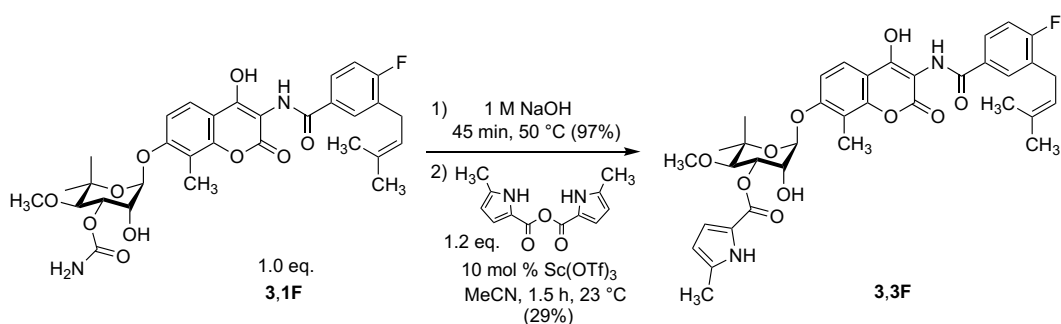
To the descarbamoylated material (48 mg, 0.08 mmol) was added solid MoO<sub>2</sub>Cl<sub>2</sub> (0.3 mg, 0.0016 mmol, 2 mol %), and 5 mL of anhydrous DCM were added. While stirring at room temperature (23 °C), PhNCO was delivered by Hamilton syringe (10 µL, 0.09 mmol, 1.2 eq.). The reaction was stirred for 12 h under positive N<sub>2</sub> pressure and monitored by TLC (DCM/MeOH). The solvent was subsequently removed by rotary evaporation and the material was subjected to normal phase silica gel column chromatography (DCM/MeOH) monitoring at 340 nm. Fractions containing the product were concentrated, hi-vaced, and further purified by reverse-phase HPLC with a Zorbax C18 column with acetonitrile/water as the eluent (80% MeCN to 100% MeCN over the course of 20 minutes). Fractions containing the product were concentrated by rotary evaporation and hi-vaced to yield **3,5** as a white solid (5.8 mg, 10%).

### 3.4.14. Synthesis of (3,2F)



In a 2-dram vial equipped with a magnetic stir bar, 20 mg of **3,1F** was dissolved in 5 M NaOH. The reaction mixture was capped to avoid evaporation of the solution and heated at 80 °C for a total reaction time of 45 minutes. The mixture was then cooled to room temperature and neutralized with 5 M HCl, which precipitated **3,2** as an off-white solid. The suspension was solubilized and extracted with 3 x 20 mL of DCM. The organic layer was washed once with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, concentrated by rotary evaporation and purified by flash column chromatography using DCM/MeOH as the solvent system. Fractions containing the product (monitored by UV absorbance at 340 nm) were concentrated by rotary evaporation and hi-vaced to afford **3,2F** as a yellow solid (10 mg, 78%).

### 3.4.15. Synthesis of (3,3F)

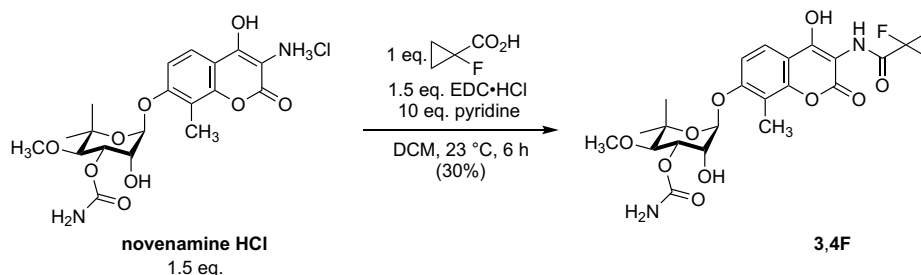


A 20 mL scintillation vial was charged with a magnetic stir bar and **3,1F** (69 mg, 0.11 mmol). The vial was capped with a cap to avoid evaporation of the solution and heated at 50 °C for a total reaction time of 45 minutes. The mixture was then cooled to 0 °C, neutralized with 2 M HCl, extracted with 3 x 50 mL DCM, and the organic layer was dried over Na<sub>2</sub>SO<sub>4</sub> with heavy

stirring for 30 min. The material was concentrated by rotary evaporation and hi-vaced to yield a white solid (62 mg, 97%) and used in the next step without further purification.

In a 2-dram vial equipped with a magnetic stir bar, the product from Step 1 (62 mg, 0.11 mmol, 1.0 eq.), 5-methyl-1*H*-pyrrole-2-carboxylic anhydride (30.7 mg, 0.13 mmol, 1.2 eq.), and 4 mL of anhydrous MeCN were stirred at room temperature. A 100 mg/mL solution of Sc(OTf)<sub>3</sub> dissolved in anhydrous MeCN was prepared and injected into the reaction mixture via Hamilton syringe (54  $\mu$ L, 0.01 mmol, 0.1 eq.). The reaction mixture instantly turned a bright yellow color and was allowed to react for 1.5 h or until TLC indicated the reaction was complete. The mixture was then concentrated by rotary evaporation, redissolved in a minimal volume of DMSO, and subjected to reverse-phase column chromatography. Fractions containing the product were concentrated by rotary evaporation and hi-vaced to afford **3,3F** as a white solid (25.17 mg, 29%).

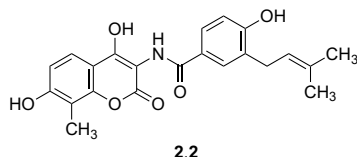
### 3.4.16. Synthesis of (3,4F)



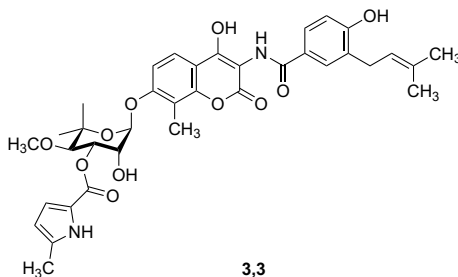
A 100 mL conical flask was charged with a magnetic stir bar, **novenamine HCl** (600 mg, 1.30 mmol, 1.5 eq.), 1-fluorocyclopropane-1-carboxylic acid (90 mg, 0.86 mmol, 1.0 eq.), EDC·HCl (250 mg, 1.30 mmol, 1.5 eq.). The flask was fitted with a rubber septum and a vent needle and sparged with N<sub>2</sub> for 10 minutes (novenamine is unstable towards molecular oxygen under basic conditions). While under N<sub>2</sub>, 15 mL of anhydrous DCM were added and 700  $\mu$ L of pyridine were added via syringe. The reaction mixture was stirred at 23 °C for 6 h. The vent needle was removed after 10 minutes of stirring. The reaction mixture was concentrated by rotary evaporation, resuspended in DCM, and subjected to normal phase silica gel column

chromatography (DCM/MeOH). Fractions containing the product were concentrated and hi-vaced to afford **3,4F** as a white solid (130 mg, 30%).

### 3.4.17. Compound characterization

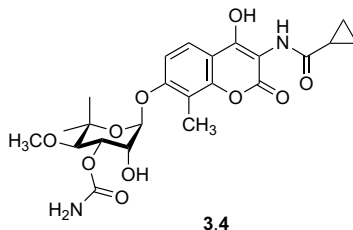


***N*-(4,7-dihydroxy-8-methyl-2-oxo-2*H*-chromen-3-yl)-4-hydroxy-3-(3-methylbut-2-en-1-yl)benzamide (2,2).** Yellow solid; TLC  $R_f$  = 0.66 (DCM);  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.9 (s, 1H), 10.4 (s, 1H), 10.1 (s, 1H), 9.2 (s, 1H), 7.8 – 7.7 (m, 2H), 7.6 (d,  $J$  = 8.7 Hz, 1H), 6.9 (t,  $J$  = 8.8 Hz, 2H), 5.3 (t,  $J$  = 7.3 Hz, 1H), 3.3 (d,  $J$  = 7.3 Hz, 2H), 2.2 (s, 3H), 1.7 (comp,  $J$  = 1.5 Hz, 6H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{DMSO}-d_6$ )  $\delta$  167.2, 161.4, 160.1, 159.6, 158.9, 151.8, 132.0, 130.4, 128.0, 127.8, 124.7, 123.1, 122.1, 114.8, 112.4, 111.0, 108.6, 101.0, 28.6, 26.1, 18.2, 8.6; HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{22}\text{H}_{20}\text{NO}_6$   $[\text{M}-\text{H}]^-$ , 394.1291 found: 394.1293.



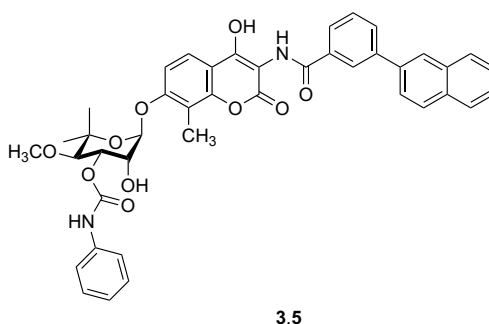
**(3*R*,4*S*,5*R*,6*R*)-5-hydroxy-6-((4-hydroxy-3-(4-hydroxy-3-(3-methylbut-2-en-1-yl)benzamido)-8-methyl-2-oxo-2*H*-chromen-7-yl)oxy)-3-methoxy-2,2-dimethyltetrahydro-2*H*-pyran-4-yl 5-methyl-1*H*-pyrrole-2-carboxylate (3,3).** Tan solid (white solid after recrystallization from EtOAc); TLC  $R_f$  = 0.3 (5% MeOH/DCM);  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.6 (s, 1H), 9.7 (s, 1H), 8.3 – 8.2 (m, 1H), 7.7 (s, 1H), 7.6 – 7.6 (comp, 2H), 6.9 (d,  $J$  = 8.8 Hz, 1H), 6.8 – 6.7 (comp, 2H), 5.9 (m, 1H), 5.6 – 5.5 (m, 1H), 5.5 – 5.4 (m, 2H), 5.3 (t,  $J$  = 7.7 Hz, 1H), 4.1 (s, 1H), 3.6 (d,  $J$  = 9.8 Hz, 1H), 3.4 (s, 3H), 3.2 (d,  $J$  = 7.6 Hz, 2H), 2.2 (s, 3H), 2.2 (s,

3H), 1.7 (comp, 6H), 1.3 (s, 3H), 1.1 (s, 3H);  $^{13}\text{C}$  NMR (101 MHz,  $\text{DMSO}-d_6$ )  $\delta$  170.5, 165.7, 162.8, 160.4, 157.6, 155.7, 152.5, 135.2, 131.6, 129.9, 127.3, 127.2, 127.0, 123.4, 123.1, 121.0, 118.6, 116.7, 114.5, 112.0, 108.9, 108.1, 98.9, 97.2, 81.3, 78.4, 71.0, 69.4, 61.5, 29.1, 28.6, 26.1, 23.4, 18.2, 13.3, 9.0; HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{36}\text{H}_{40}\text{N}_2\text{O}_{10}$   $[\text{M}-\text{H}]^-$ , 675.2554 found: 675.2571.



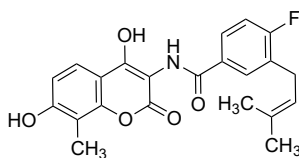
**(3R,4S,5R,6R)-6-((3-(cyclopropanecarboxamido)-4-hydroxy-8-methyl-2-oxo-2H-chromen-7-yl)oxy)-5-hydroxy-3-methoxy-2,2-dimethyltetrahydro-2H-pyran-4-yl carbamate (3,4).**

White solid; TLC  $R_f$  = 0.3 (5% MeOH/DCM);  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.6 (br, 1H), 9.9 (s, 1H), 7.7 (d,  $J$  = 8.9 Hz, 1H), 7.1 (d,  $J$  = 9.1 Hz, 1H), 6.6 (s, 2H), 5.6 (br, 1H), 5.5 (d,  $J$  = 2.4 Hz, 1H), 5.2 (dd,  $J$  = 9.9, 3.1 Hz, 1H), 4.1 (t,  $J$  = 2.8 Hz, 1H), 3.5 (comp, 4H), 2.2 (comp, 4H), 1.2 (s, 3H), 1.0 (s, 3H), 0.9 (comp, 4H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{DMSO}-d_6$ )  $\delta$  175.0, 160.1, 156.9, 156.4, 156.1, 150.0, 121.8, 112.7, 110.4, 110.2, 102.5, 98.6, 80.8, 78.2, 70.4, 68.8, 61.0, 28.5, 22.7, 13.8, 8.3, 8.2; HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{23}\text{H}_{27}\text{N}_2\text{O}_{10}$   $[\text{M}-\text{H}]^-$ , 491.1671 found: 491.1680.



**(3R,4S,5R,6R)-5-hydroxy-6-((4-hydroxy-8-methyl-3-(3-(naphthalen-2-yl)benzamido)-2-oxo-2H-chromen-7-yl)oxy)-3-methoxy-2,2-dimethyltetrahydro-2H-pyran-4-yl phenylcarbamate**

**(3,5)**. White solid; TLC  $R_f$  = 0.6 (10% MeOH/DCM);  $^1\text{H}$  NMR (500 MHz, DMSO- $d_6$ )  $\delta$  9.8 (s, 1H), 9.3 (s, 1H), 8.5 (s, 1H), 8.3 (s, 1H), 8.0 (t,  $J$  = 8.0 Hz, 2H), 8.0 (comp, 4H), 7.7 – 7.6 (m, 1H), 7.6 (comp, 6H), 7.3 (t,  $J$  = 7.7 Hz, 2H), 7.0 (q,  $J$  = 7.3 Hz, 2H), 5.7 (s, 1H), 5.5 (s, 1H), 5.3 (d,  $J$  = 9.8 Hz, 1H), 4.2 (s, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H);  $^{13}\text{C}$  NMR (126 MHz, DMSO- $d_6$ )  $\delta$  166.2, 163.5, 161.9, 156.6, 153.6, 151.8, 140.1, 139.6, 137.4, 135.8, 133.8, 132.8, 129.8, 129.2, 128.9, 128.7, 128.0, 127.5, 126.9, 126.7, 126.6, 125.9, 125.6, 122.9, 122.7, 118.8, 112.6, 109.2, 98.9, 96.0, 81.2, 78.5, 71.7, 69.2, 61.6, 29.1, 23.2, 8.9 (two resonances obscured due to spectral overlap); HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{42}\text{H}_{37}\text{N}_2\text{O}_{10}$   $[\text{M}-\text{H}]^-$ , 729.2448 found: 729.2457.

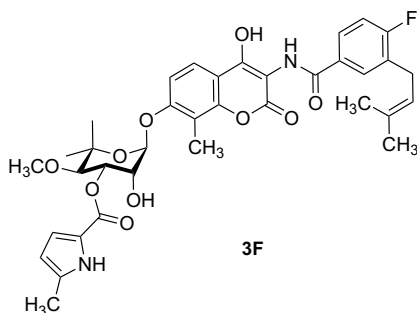


**3,2F**

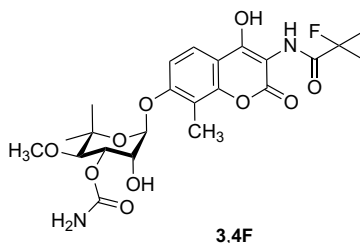
***N*-(4,7-dihydroxy-8-methyl-2-oxo-2*H*-chromen-3-yl)-4-fluoro-3-(3-methylbut-2-en-1-**

**yl)benzamide (3,2F)**. Off-white solid; TLC  $R_f$  = 0.3 (5% MeOH/DCM);  $^1\text{H}$  NMR (600 MHz, DMSO- $d_6$ )  $\delta$  11.9 (br, 1H), 10.4 (s, 1H), 9.3 (s, 1H), 7.9 (comp, 2H), 7.5 (d,  $J$  = 8.5 Hz, 1H), 7.2 (t,  $J$  = 9.0 Hz, 1H), 6.9 (d,  $J$  = 8.6 Hz, 1H), 5.3 (t,  $J$  = 6.5 Hz, 1H), 3.4 (m, 2H), 2.2 (s, 3H), 1.7 (comp, 6H);  $^{13}\text{C}$  NMR (151 MHz, DMSO- $d_6$ )  $\delta$  166.0, 163.5, 162.6 (d,  $J$  = 248.6 Hz), 161.5, 159.4, 152.0, 133.3, 131.1 (d,  $J$  = 5.9 Hz), 130.9, 128.4 (d,  $J$  = 9.4 Hz), 128.3 (d,  $J$  = 16.5 Hz), 122.1, 121.7, 115.4 (d,  $J$  = 23.0 Hz), 112.1, 110.8, 108.9, 99.9, 27.5, 26.0, 18.1, 8.6;  $^{19}\text{F}$  NMR (564 MHz, DMSO- $d_6$ )  $\delta$  -114.5; HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{22}\text{H}_{19}\text{FNO}_5$   $[\text{M}-\text{H}]^-$ , 396.1247 found: 396.1256.



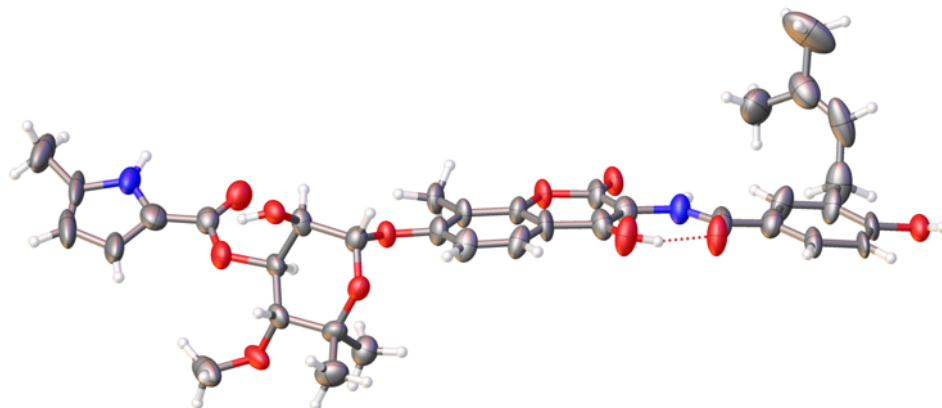


**(3*R*,4*S*,5*R*,6*R*)-6-((3-(4-fluoro-3-(3-methylbut-2-en-1-yl)benzamido)-4-hydroxy-8-methyl-2-oxo-2*H*-chromen-7-yl)oxy)-5-hydroxy-3-methoxy-2,2-dimethyltetrahydro-2*H*-pyran-4-yl 5-methyl-1*H*-pyrrole-2-carboxylate (3,3F).** Off-white solid; TLC  $R_f$  = 0.3 (5% MeOH/DCM);  $^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.1 (br, 1H), 11.6 (s, 1H), 9.4 (s, 1H), 7.9 (comp, 2H), 7.7 (d,  $J$  = 8.8 Hz, 1H), 7.3 (t,  $J$  = 9.2 Hz, 1H), 7.2 (d,  $J$  = 9.0 Hz, 1H), 6.8 (dd,  $J$  = 3.6, 2.5 Hz, 1H), 5.9 (t,  $J$  = 3.0 Hz, 1H), 5.7 (d,  $J$  = 5.0 Hz, 1H), 5.6 (d,  $J$  = 2.5 Hz, 1H), 5.5 (dd,  $J$  = 9.8, 3.1 Hz, 1H), 5.3 (t, 1H), 4.2 (m, 1H), 3.7 (d,  $J$  = 9.8 Hz, 1H), 3.5 (s, 3H), 3.4 (d,  $J$  = 7.3 Hz, 2H), 2.3 (comp,  $J$  = 4.3 Hz, 6H), 1.7 (comp, 6H), 1.3 (s, 3H), 1.1 (s, 3H);  $^{13}\text{C}$  NMR (126 MHz,  $\text{DMSO}-d_6$ )  $\delta$  166.0, 162.6 (d,  $J$  = 248.4 Hz), 161.2, 160.3, 157.4, 151.3, 135.2, 133.4, 131.1 (d,  $J$  = 5.7 Hz), 130.9, 128.5 (d,  $J$  = 9.5 Hz), 128.3 (d,  $J$  = 16.7 Hz; two signals obscured due to overlap), 122.4, 121.7, 120.9, 116.7, 115.4 (d,  $J$  = 22.3 Hz), 113.2, 110.3, 108.9, 100.9, 98.8, 81.1, 78.6, 70.8, 69.2, 61.5, 28.9, 27.5, 25.9, 23.3, 18.1, 13.2, 8.7.  $^{19}\text{F}$  NMR (471 MHz,  $\text{DMSO}-d_6$ )  $\delta$  -114.4; HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{36}\text{H}_{38}\text{FN}_2\text{O}_{10}$ , 677.2516 found 677.2500.



**(3*R*,4*S*,5*R*,6*R*)-6-((3-(1-fluorocyclopropane-1-carboxamido)-4-hydroxy-8-methyl-2-oxo-2*H*-chromen-7-yl)oxy)-5-hydroxy-3-methoxy-2,2-dimethyltetrahydro-2*H*-pyran-4-yl carbamate (3,4*F*).** White solid; TLC *R*<sub>f</sub> = 0.33 (10% MeOH/DCM); <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>) δ 12.1 (s, 1H), 9.2 (s, 1H), 7.7 (d, *J* = 8.9 Hz, 1H), 7.2 (d, *J* = 9.0 Hz, 1H), 6.8 – 6.4 (comp, 2H), 5.6 (d, *J* = 5.2 Hz, 1H), 5.5 (d, *J* = 2.5 Hz, 1H), 5.2 (dd, *J* = 9.8, 3.2 Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 – 3.4 (comp, 4H), 2.2 (s, 3H), 1.4 – 1.3 (m, 2H), 1.3 – 1.2 (comp, 5H), 1.0 (s, 3H); <sup>13</sup>C NMR (151 MHz, DMSO-*d*<sub>6</sub>) δ 169.9 (d, *J* = 21.1 Hz), 161.0, 160.9, 157.5, 156.7, 151.3, 122.4, 113.3, 110.5, 110.4, 100.2, 98.9, 81.2, 79.3, 78.6, 77.8, 70.8, 69.2, 61.4, 28.9, 23.2, 13.9 (d, *J* = 10.3 Hz), 8.7; <sup>19</sup>F NMR (471 MHz, DMSO-*d*<sub>6</sub>) δ -195.5; HRMS (ESI Neg) *m/z* calculated for C<sub>23</sub>H<sub>26</sub>FN<sub>2</sub>O<sub>10</sub> [M–H]<sup>–</sup>, 509.1571 found: 509.1585.

#### 3.4.18. X-ray crystal structure of (3,3)



**Figure 3.20. Crystal structure of (3,3).**

A crystal mounted on a diffractometer was collected data at 100 K. The intensities of the reflections were collected by means of a Bruker APEX DUO CCD diffractometer (CuK $\alpha$  radiation,  $\lambda$ =1.54178 Å), and equipped with an Oxford Cryosystems nitrogen flow apparatus. The collection method involved 1.0° scans in  $\omega$  at -30°, -55°, -80°, 30°, 55°, 80° and 115° in  $2\theta$ . Data integration down to 0.84 Å resolution was carried out using SAINT V8.37 A (Bruker diffractometer, 2015) with reflection spot size optimization. Absorption corrections were made with the program SADABS

(Bruker diffractometer, 2015). The structure was solved by the Intrinsic Phasing methods and refined by least-squares methods again  $F^2$  using SHELXT-2014<sup>306</sup> and SHELXL-2014<sup>307</sup> with OLEX 2 interface.<sup>308</sup> Non-hydrogen atoms were refined anisotropically, and hydrogen atoms were allowed to ride on the respective atoms. Crystal data as well as details of data collection and refinement are summarized in Table 3.4.2, geometric parameters are shown in Table 3.4.3 and Hydrogen-bond parameters are listed in Table 3.4.4. The ORTEP plots produced with SHELXL-2014 program, and the other drawings were produced with Accelrys DS Visualizer 2.0 (Accelrys, 2007).

**Table 3.4. X-ray Experimental details of 3,3.**

|                                                                            | Compound 3,3                                                   |
|----------------------------------------------------------------------------|----------------------------------------------------------------|
| Crystal data                                                               |                                                                |
| Chemical formula                                                           | C <sub>36</sub> H <sub>40</sub> N <sub>2</sub> O <sub>11</sub> |
| $M_r$                                                                      | 676.70                                                         |
| Crystal system, space group                                                | Orthorhombic, $P2_12_12_1$                                     |
| Temperature (K)                                                            | 100                                                            |
| $a, b, c$ (Å)                                                              | 7.1329 (2), 17.6817 (4), 35.8965 (8)                           |
| $V$ (Å <sup>3</sup> )                                                      | 4527.33 (19)                                                   |
| $Z$                                                                        | 4                                                              |
| Radiation type                                                             | Cu $K\alpha$                                                   |
| $\mu$ (mm <sup>-1</sup> )                                                  | 0.61                                                           |
| Crystal size (mm)                                                          | 0.16 × 0.10 × 0.08                                             |
| Data collection                                                            |                                                                |
| Diffractometer                                                             | Bruker D8 goniometer with CCD area detector                    |
| Absorption correction                                                      | Multi-scan<br><i>SADABS</i>                                    |
| $T_{\min}, T_{\max}$                                                       | 0.705, 0.806                                                   |
| No. of measured, independent and observed [ $I > 2\sigma(I)$ ] reflections | 45820, 7771, 7041                                              |
| $R_{\text{int}}$                                                           | 0.048                                                          |
| $(\sin \theta/\lambda)_{\text{max}}$ (Å <sup>-1</sup> )                    | 0.596                                                          |

|                                                             |                                                                                                                              |
|-------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------|
| Refinement                                                  |                                                                                                                              |
| $R[F^2 > 2\sigma(F^2)], wR(F^2), S$                         | 0.076, 0.219, 1.08                                                                                                           |
| No. of reflections                                          | 7771                                                                                                                         |
| No. of parameters                                           | 449                                                                                                                          |
| No. of restraints                                           | 4                                                                                                                            |
| H-atom treatment                                            | H-atom parameters constrained                                                                                                |
| $\Delta\rho_{\max}, \Delta\rho_{\min}$ (e Å <sup>-3</sup> ) | 0.30, -0.32                                                                                                                  |
| Absolute structure                                          | Flack x determined using 2638 quotients [(I+)-(I-)]/[(I+)+(I-)] (Parsons, Flack and Wagner, Acta Cryst. B69 (2013) 249-259). |
| Absolute structure parameter                                | 0.10 (8)                                                                                                                     |

Computer programs: *SAINT* 8.37A (Bruker-AXS, 2015), *SHELXT2014*<sup>306</sup> *SHELXL2014*<sup>307</sup>, Bruker *SHELXTL* (Sheldrick, 2015).

**Table 3.5. Geometric parameters (Å, °) of 3,3.**

|         |           |          |           |
|---------|-----------|----------|-----------|
| O1—C6   | 1.343 (8) | C12—H12C | 0.9800    |
| O1—C7   | 1.443 (6) | C13—H13A | 0.9800    |
| O2—C6   | 1.226 (7) | C13—H13B | 0.9800    |
| O3—C12  | 1.385 (7) | C13—H13C | 0.9800    |
| O3—C8   | 1.428 (7) | C14—H14A | 0.9800    |
| O4—C10  | 1.392 (7) | C14—H14B | 0.9800    |
| O4—C9   | 1.455 (7) | C14—H14C | 0.9800    |
| O5—C15  | 1.351 (6) | C15—C23  | 1.379 (7) |
| O5—C10  | 1.437 (6) | C15—C16  | 1.403 (8) |
| O6—C11  | 1.413 (6) | C16—C17  | 1.388 (8) |
| O6—H6   | 0.8400    | C16—H16  | 0.9500    |
| O7—C19  | 1.356 (8) | C17—C18  | 1.368 (8) |
| O7—H7   | 0.8400    | C17—H17  | 0.9500    |
| O8—C21  | 1.372 (6) | C18—C22  | 1.395 (8) |
| O8—C22  | 1.394 (6) | C18—C19  | 1.447 (7) |
| O9—C21  | 1.242 (7) | C19—C20  | 1.382 (8) |
| O10—C25 | 1.260 (9) | C20—C21  | 1.414 (9) |
| O11—C29 | 1.349 (6) | C22—C23  | 1.419 (7) |
| O11—H11 | 0.8400    | C23—C24  | 1.483 (8) |
| N2—C25  | 1.352 (8) | C24—H24A | 0.9800    |
| N2—C20  | 1.433 (7) | C24—H24B | 0.9800    |
| N2—H2   | 0.8800    | C24—H24C | 0.9800    |

|                |            |                   |            |
|----------------|------------|-------------------|------------|
| C1—C2          | 1.527 (13) | C25—C26           | 1.438 (8)  |
| C1—H1A         | 0.9800     | C26—C27           | 1.389 (9)  |
| C1—H1B         | 0.9800     | C26—C31           | 1.420 (9)  |
| C1—H1C         | 0.9800     | C27—C28           | 1.386 (7)  |
| N1—C2          | 1.379 (8)  | C27—H27           | 0.9500     |
| N1—C5          | 1.389 (9)  | C28—C29           | 1.449 (9)  |
| N1—H1          | 0.8800     | C28—H28           | 0.9500     |
| C2—C3          | 1.362 (13) | C29—C30           | 1.355 (8)  |
| C3—C4          | 1.389 (11) | C30—C31           | 1.379 (8)  |
| C3—H3          | 0.9500     | C30—C32           | 1.571 (10) |
| C4—C5          | 1.392 (10) | C31—H31           | 0.9500     |
| C4—H4          | 0.9500     | C32—C33           | 1.564 (15) |
| C5—C6          | 1.397 (9)  | C32—H32A          | 0.9900     |
| C7—C8          | 1.507 (7)  | C32—H32B          | 0.9900     |
| C7—C11         | 1.538 (7)  | C33—C34           | 1.312 (14) |
| C7—H7A         | 1.0000     | C33—H33           | 0.9500     |
| C8—C9          | 1.538 (8)  | C34—C35           | 1.470 (12) |
| C8—H8          | 1.0000     | C34—C36           | 1.513 (16) |
| C9—C14         | 1.509 (8)  | C35—H35A          | 0.9800     |
| C9—C13         | 1.510 (8)  | C35—H35B          | 0.9800     |
| C10—C11        | 1.518 (7)  | C35—H35C          | 0.9800     |
| C10—H10        | 1.0000     | C36—H36A          | 0.9800     |
| C11—H11A       | 1.0000     | C36—H36B          | 0.9800     |
| C12—H12A       | 0.9800     | C36—H36C          | 0.9800     |
| C12—H12B       | 0.9800     |                   |            |
|                |            |                   |            |
| C6—O1—C7       | 117.6 (4)  | H14A—C14—<br>H14C | 109.5      |
| C12—O3—C8      | 115.3 (4)  | H14B—C14—<br>H14C | 109.5      |
| C10—O4—C9      | 118.0 (4)  | O5—C15—C23        | 115.4 (5)  |
| C15—O5—<br>C10 | 119.5 (4)  | O5—C15—C16        | 121.8 (5)  |
| C11—O6—H6      | 109.5      | C23—C15—<br>C16   | 122.8 (5)  |
| C19—O7—H7      | 109.5      | C17—C16—<br>C15   | 118.2 (5)  |
| C21—O8—<br>C22 | 120.8 (4)  | C17—C16—<br>H16   | 120.9      |

|     |           |           |      |            |           |
|-----|-----------|-----------|------|------------|-----------|
| H11 | C29—O11—  | 109.5     | H16  | C15—C16—   | 120.9     |
| C20 | C25—N2—   | 131.2 (5) | C16  | C18—C17—   | 122.2 (5) |
|     | C25—N2—H2 | 114.4     | H17  | C18—C17—   | 118.9     |
|     | C20—N2—H2 | 114.4     | H17  | C16—C17—   | 118.9     |
|     | C2—C1—H1A | 109.5     | C22  | C17—C18—   | 118.1 (5) |
|     | C2—C1—H1B | 109.5     | C19  | C17—C18—   | 123.8 (5) |
| H1B | H1A—C1—   | 109.5     | C19  | C22—C18—   | 118.1 (5) |
|     | C2—C1—H1C | 109.5     |      | O7—C19—C20 | 126.0 (5) |
| H1C | H1A—C1—   | 109.5     |      | O7—C19—C18 | 114.5 (5) |
| H1C | H1B—C1—   | 109.5     | C18  | C20—C19—   | 119.5 (6) |
|     | C2—N1—C5  | 110.1 (7) | C21  | C19—C20—   | 120.8 (5) |
|     | C2—N1—H1  | 124.9     |      | C19—C20—N2 | 128.3 (5) |
|     | C5—N1—H1  | 124.9     |      | C21—C20—N2 | 110.9 (5) |
|     | C3—C2—N1  | 106.7 (7) |      | O9—C21—O8  | 114.4 (5) |
|     | C3—C2—C1  | 134.4 (7) |      | O9—C21—C20 | 126.0 (5) |
|     | N1—C2—C1  | 118.9 (9) |      | O8—C21—C20 | 119.6 (5) |
|     | C2—C3—C4  | 109.3 (7) |      | C18—C22—O8 | 121.1 (4) |
|     | C2—C3—H3  | 125.4     | C23  | C18—C22—   | 122.8 (5) |
|     | C4—C3—H3  | 125.4     |      | O8—C22—C23 | 116.1 (4) |
|     | C3—C4—C5  | 107.9 (8) | C22  | C15—C23—   | 116.0 (5) |
|     | C3—C4—H4  | 126.0     | C24  | C15—C23—   | 123.4 (4) |
|     | C5—C4—H4  | 126.0     | C24  | C22—C23—   | 120.5 (4) |
|     | N1—C5—C4  | 105.9 (6) | H24A | C23—C24—   | 109.5     |
|     | N1—C5—C6  | 122.5 (6) | H24B | C23—C24—   | 109.5     |
|     | C4—C5—C6  | 131.4 (7) | H24B | H24A—C24—  | 109.5     |

|                |           |                |           |
|----------------|-----------|----------------|-----------|
| O2—C6—O1       | 123.1 (5) | H24C C23—C24—  | 109.5     |
| O2—C6—C5       | 125.7 (6) | H24C H24A—C24— | 109.5     |
| O1—C6—C5       | 111.2 (6) | H24C H24B—C24— | 109.5     |
| O1—C7—C8       | 105.6 (4) | O10—C25—N2     | 121.3 (5) |
| O1—C7—C11      | 110.8 (4) | C26 O10—C25—   | 120.1 (6) |
| C8—C7—C11      | 109.8 (4) | N2—C25—C26     | 118.5 (6) |
| O1—C7—H7A      | 110.2     | C31 C27—C26—   | 117.3 (5) |
| C8—C7—H7A      | 110.2     | C25 C27—C26—   | 118.1 (6) |
| C11—C7—<br>H7A | 110.2     | C25 C31—C26—   | 124.5 (6) |
| O3—C8—C7       | 110.3 (5) | C26 C28—C27—   | 122.5 (6) |
| O3—C8—C9       | 107.4 (4) | H27 C28—C27—   | 118.8     |
| C7—C8—C9       | 112.1 (4) | H27 C26—C27—   | 118.8     |
| O3—C8—H8       | 109.0     | C29 C27—C28—   | 118.9 (5) |
| C7—C8—H8       | 109.0     | H28 C27—C28—   | 120.6     |
| C9—C8—H8       | 109.0     | H28 C29—C28—   | 120.6     |
| O4—C9—C14      | 103.7 (5) | C30 O11—C29—   | 120.8 (5) |
| O4—C9—C13      | 113.9 (5) | C28 O11—C29—   | 121.1 (5) |
| C14—C9—<br>C13 | 110.4 (5) | C28 C30—C29—   | 118.1 (5) |
| O4—C9—C8       | 108.3 (4) | C31 C29—C30—   | 122.8 (6) |
| C14—C9—C8      | 106.7 (5) | C32 C29—C30—   | 120.7 (5) |
| C13—C9—C8      | 113.2 (5) | C32 C31—C30—   | 116.4 (6) |
| O4—C10—O5      | 114.1 (4) | C26 C30—C31—   | 120.4 (5) |
| O4—C10—<br>C11 | 114.0 (4) | H31 C30—C31—   | 119.8     |

|      |           |           |      |           |            |
|------|-----------|-----------|------|-----------|------------|
| C11  | O5—C10—   | 105.7 (4) | H31  | C26—C31—  | 119.8      |
| H10  | O4—C10—   | 107.6     | C30  | C33—C32—  | 107.1 (6)  |
| H10  | O5—C10—   | 107.6     | H32A | C33—C32—  | 110.3      |
| H10  | C11—C10—  | 107.6     | H32A | C30—C32—  | 110.3      |
| C10  | O6—C11—   | 108.1 (4) | H32B | C33—C32—  | 110.3      |
|      | O6—C11—C7 | 111.1 (4) | H32B | C30—C32—  | 110.3      |
| C7   | C10—C11—  | 109.3 (4) | H32B | H32A—C32— | 108.6      |
| H11A | O6—C11—   | 109.4     | C32  | C34—C33—  | 124.4 (8)  |
| H11A | C10—C11—  | 109.4     | H33  | C34—C33—  | 117.8      |
| H11A | C7—C11—   | 109.4     | H33  | C32—C33—  | 117.8      |
| H12A | O3—C12—   | 109.5     | C35  | C33—C34—  | 124.6 (9)  |
| H12B | O3—C12—   | 109.5     | C36  | C33—C34—  | 120.7 (10) |
| H12B | H12A—C12— | 109.5     | C36  | C35—C34—  | 114.7 (10) |
| H12C | O3—C12—   | 109.5     | H35A | C34—C35—  | 109.5      |
| H12C | H12A—C12— | 109.5     | H35B | C34—C35—  | 109.5      |
| H12C | H12B—C12— | 109.5     | H35B | H35A—C35— | 109.5      |
| H13A | C9—C13—   | 109.5     | H35C | C34—C35—  | 109.5      |
| H13B | C9—C13—   | 109.5     | H35C | H35A—C35— | 109.5      |
| H13B | H13A—C13— | 109.5     | H35C | H35B—C35— | 109.5      |
| H13C | C9—C13—   | 109.5     | H36A | C34—C36—  | 109.5      |
| H13C | H13A—C13— | 109.5     | H36B | C34—C36—  | 109.5      |
| H13C | H13B—C13— | 109.5     | H36B | H36A—C36— | 109.5      |



|      |                  |            |      |                     |            |
|------|------------------|------------|------|---------------------|------------|
| H14A | C9—C14—          | 109.5      | H36C | C34—C36—            | 109.5      |
| H14B | C9—C14—          | 109.5      | H36C | H36A—C36—           | 109.5      |
| H14B | H14A—C14—        | 109.5      | H36C | H36B—C36—           | 109.5      |
| H14C | C9—C14—          | 109.5      |      |                     |            |
|      |                  |            |      |                     |            |
|      | C5—N1—<br>C2—C3  | 2.0 (8)    |      | C17—C18—<br>C19—C20 | 175.3 (6)  |
|      | C5—N1—<br>C2—C1  | -179.4 (7) |      | C22—C18—<br>C19—C20 | -1.4 (9)   |
|      | N1—C2—<br>C3—C4  | -3.4 (9)   |      | O7—C19—<br>C20—C21  | -173.7 (6) |
|      | C1—C2—<br>C3—C4  | 178.3 (8)  |      | C18—C19—<br>C20—C21 | 3.5 (9)    |
|      | C2—C3—<br>C4—C5  | 3.6 (9)    |      | O7—C19—<br>C20—N2   | 5.7 (11)   |
|      | C2—N1—<br>C5—C4  | 0.1 (8)    |      | C18—C19—<br>C20—N2  | -177.1 (6) |
|      | C2—N1—<br>C5—C6  | 175.4 (6)  |      | C25—N2—<br>C20—C19  | 2.6 (11)   |
|      | C3—C4—<br>C5—N1  | -2.2 (8)   |      | C25—N2—<br>C20—C21  | -177.9 (6) |
|      | C3—C4—<br>C5—C6  | -176.9 (7) |      | C22—O8—<br>C21—O9   | 176.1 (5)  |
|      | C7—O1—<br>C6—O2  | -4.7 (8)   |      | C22—O8—<br>C21—C20  | -1.7 (8)   |
|      | C7—O1—<br>C6—C5  | 175.7 (5)  |      | C19—C20—<br>C21—O9  | -179.5 (6) |
|      | N1—C5—<br>C6—O2  | -5.4 (10)  |      | N2—C20—<br>C21—O9   | 1.0 (9)    |
|      | C4—C5—<br>C6—O2  | 168.4 (7)  |      | C19—C20—<br>C21—O8  | -2.0 (9)   |
|      | N1—C5—<br>C6—O1  | 174.2 (6)  |      | N2—C20—<br>C21—O8   | 178.5 (5)  |
|      | C4—C5—<br>C6—O1  | -12.0 (10) |      | C17—C18—<br>C22—O8  | -179.1 (5) |
|      | C6—O1—<br>C7—C8  | -161.8 (5) |      | C19—C18—<br>C22—O8  | -2.2 (9)   |
|      | C6—O1—<br>C7—C11 | 79.3 (6)   |      | C17—C18—<br>C22—C23 | -0.9 (9)   |

|                    |            |                     |            |
|--------------------|------------|---------------------|------------|
| C12—O3—<br>C8—C7   | -99.7 (6)  | C19—C18—<br>C22—C23 | 176.0 (6)  |
| C12—O3—<br>C8—C9   | 137.8 (6)  | C21—O8—<br>C22—C18  | 3.8 (8)    |
| O1—C7—<br>C8—O3    | 63.8 (5)   | C21—O8—<br>C22—C23  | -174.5 (5) |
| C11—C7—<br>C8—O3   | -176.6 (4) | O5—C15—<br>C23—C22  | -178.6 (5) |
| O1—C7—<br>C8—C9    | -176.6 (4) | C16—C15—<br>C23—C22 | 1.2 (9)    |
| C11—C7—<br>C8—C9   | -57.0 (5)  | O5—C15—<br>C23—C24  | -0.1 (9)   |
| C10—O4—<br>C9—C14  | -165.2 (5) | C16—C15—<br>C23—C24 | 179.7 (6)  |
| C10—O4—<br>C9—C13  | 74.7 (6)   | C18—C22—<br>C23—C15 | 0.1 (9)    |
| C10—O4—<br>C9—C8   | -52.1 (5)  | O8—C22—<br>C23—C15  | 178.4 (5)  |
| O3—C8—<br>C9—O4    | 174.9 (4)  | C18—C22—<br>C23—C24 | -178.5 (6) |
| C7—C8—<br>C9—O4    | 53.6 (6)   | O8—C22—<br>C23—C24  | -0.2 (8)   |
| O3—C8—<br>C9—C14   | -74.0 (6)  | C20—N2—<br>C25—O10  | -5.8 (11)  |
| C7—C8—<br>C9—C14   | 164.7 (5)  | C20—N2—<br>C25—C26  | 172.2 (6)  |
| O3—C8—<br>C9—C13   | 47.6 (6)   | O10—C25—<br>C26—C27 | 4.4 (10)   |
| C7—C8—<br>C9—C13   | -73.6 (6)  | N2—C25—<br>C26—C27  | -173.6 (6) |
| C9—O4—<br>C10—O5   | -68.7 (6)  | O10—C25—<br>C26—C31 | -172.8 (7) |
| C9—O4—<br>C10—C11  | 52.9 (6)   | N2—C25—<br>C26—C31  | 9.2 (10)   |
| C15—O5—<br>C10—O4  | -74.1 (6)  | C31—C26—<br>C27—C28 | -2.4 (9)   |
| C15—O5—<br>C10—C11 | 159.8 (5)  | C25—C26—<br>C27—C28 | -179.9 (6) |
| O4—C10—<br>C11—O6  | 70.1 (6)   | C26—C27—<br>C28—C29 | 0.7 (9)    |
| O5—C10—<br>C11—O6  | -163.8 (4) | C27—C28—<br>C29—O11 | -179.5 (5) |
| O4—C10—<br>C11—C7  | -51.0 (6)  | C27—C28—<br>C29—C30 | 1.9 (8)    |

|                     |            |                     |            |
|---------------------|------------|---------------------|------------|
| O5—C10—<br>C11—C7   | 75.1 (5)   | O11—C29—<br>C30—C31 | 178.7 (6)  |
| O1—C7—<br>C11—O6    | 50.5 (6)   | C28—C29—<br>C30—C31 | -2.6 (10)  |
| C8—C7—<br>C11—O6    | -65.8 (5)  | O11—C29—<br>C30—C32 | -1.0 (10)  |
| O1—C7—<br>C11—C10   | 169.7 (4)  | C28—C29—<br>C30—C32 | 177.6 (7)  |
| C8—C7—<br>C11—C10   | 53.4 (5)   | C29—C30—<br>C31—C26 | 0.9 (11)   |
| C10—O5—<br>C15—C23  | -163.8 (5) | C32—C30—<br>C31—C26 | -179.4 (7) |
| C10—O5—<br>C15—C16  | 16.4 (8)   | C27—C26—<br>C31—C30 | 1.7 (10)   |
| O5—C15—<br>C16—C17  | 178.1 (6)  | C25—C26—<br>C31—C30 | 178.9 (7)  |
| C23—C15—<br>C16—C17 | -1.7 (10)  | C29—C30—<br>C32—C33 | -100.7 (8) |
| C15—C16—<br>C17—C18 | 0.8 (10)   | C31—C30—<br>C32—C33 | 79.6 (8)   |
| C16—C17—<br>C18—C22 | 0.5 (10)   | C30—C32—<br>C33—C34 | -107.8 (9) |
| C16—C17—<br>C18—C19 | -176.2 (6) | C32—C33—<br>C34—C35 | -2.6 (14)  |
| C17—C18—<br>C19—O7  | -7.2 (10)  | C32—C33—<br>C34—C36 | 175.7 (8)  |
| C22—C18—<br>C19—O7  | 176.1 (6)  |                     |            |

**Table 3.6. Hydrogen-bond parameters of 3,3.**

| $H \cdots A$                          | $D—H$<br>(Å) | $H \cdots A$<br>(Å) | $D \cdots A$<br>(Å) | $D—H \cdots A$ (°) |
|---------------------------------------|--------------|---------------------|---------------------|--------------------|
| O6—<br>H6 $\cdots$ O1                 | 0.84         | 2.33                | 2.802 (6)           | 115.9              |
| O6—<br>H6 $\cdots$ O9 <sup>i</sup>    | 0.84         | 2.53                | 2.814 (5)           | 100.8              |
| O7—<br>H7 $\cdots$ O10                | 0.84         | 1.63                | 2.449 (6)           | 162.6              |
| O7—<br>H7 $\cdots$ N2                 | 0.84         | 2.70                | 3.069 (7)           | 108.7              |
| O11—<br>H11 $\cdots$ O6 <sup>ii</sup> | 0.84         | 1.99                | 2.717 (5)           | 144.3              |

|                |      |      |           |       |
|----------------|------|------|-----------|-------|
| N2—<br>H2···O9 | 0.88 | 2.25 | 2.676 (6) | 109.4 |
|----------------|------|------|-----------|-------|

Symmetry code(s): (i)  $x-1/2, -y+3/2, -z+1$ ; (ii)  $-x+3/2, -y+1, z+1/2$ .

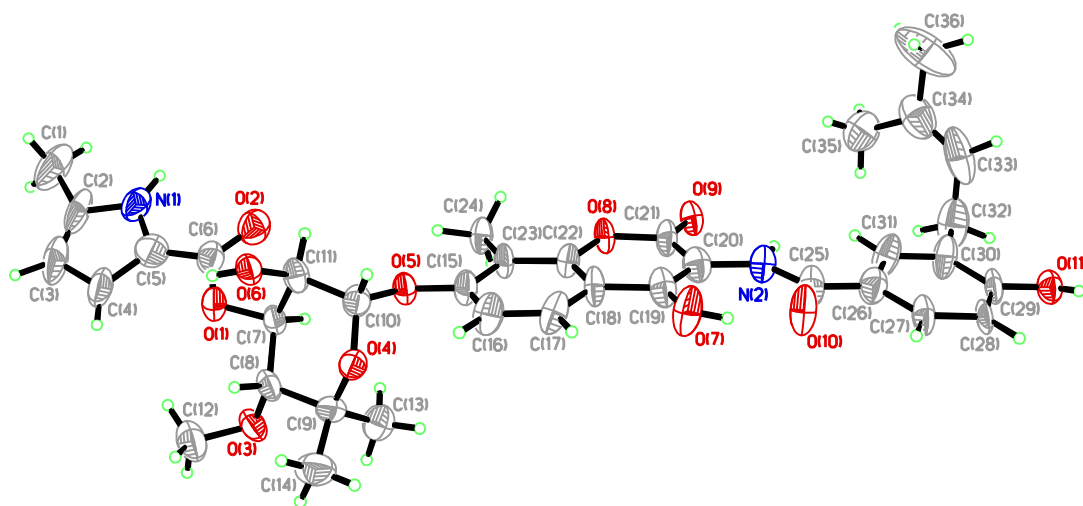


Figure 3.21. Perspective views of 3,3 showing 50% probability displacement.

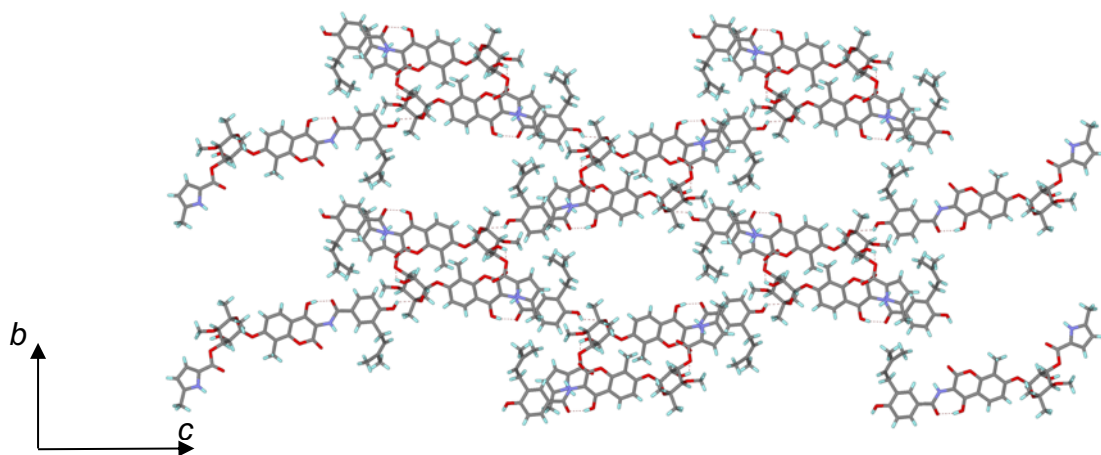


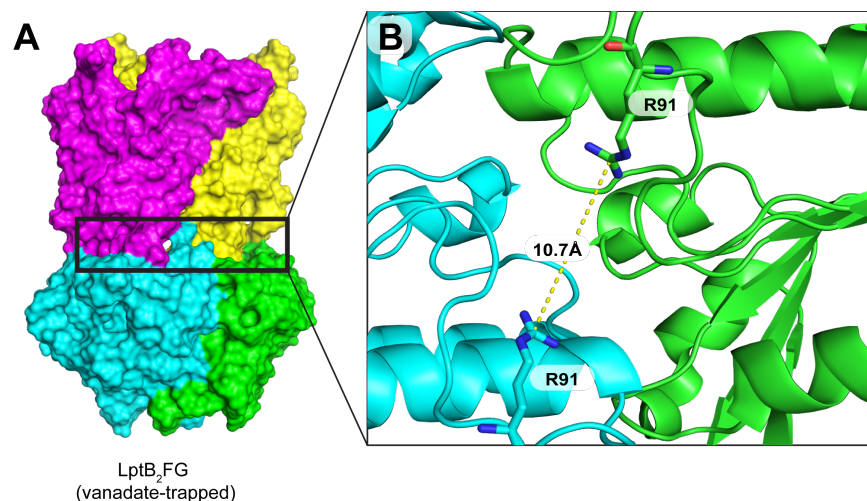
Figure 3.22. Three-dimensional supramolecular architecture of 3,3 viewed along the *a*-axis direction.

## **Chapter 4: Synthesis of novobiocin dimers and a novobiocin-polymyxin chimera**

## 4.1. Introduction

Our discovery that novobiocin binds the lipopolysaccharide (LPS) transporter LptB<sub>2</sub>FGC led us to wonder how we could design a better antibiotic. In the first half of this Chapter, I describe the synthesis of dimeric versions of novobiocin that were intended to inhibit LptB<sub>2</sub>FGC activity by binding each LptB domain. In the second half, I outline a semi-synthetic route to novobiocin-polymyxin chimeric molecules. The polymyxins are drugs of last resort due to their dose-limiting toxicity but possess potent activity against many resistant strains of Gram-negative bacteria. Strategies to increase their potency or effectiveness are an unmet clinical need. A novobiocin-polymyxin chimera would combine two antibiotics that have been successfully used in humans and could target LPS undergoing active transport, since novobiocin, polymyxin, LptB<sub>2</sub>FGC, and LPS would be co-localized at the inner membrane.

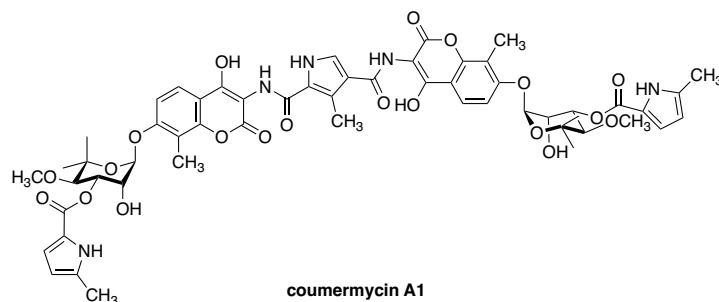
Recent structural elucidation of the inner membrane lipopolysaccharide transporter encouraged us to test the concept of dimeric novobiocin analogs. Crystal<sup>131</sup> and cryo-EM<sup>132</sup> structures of the full LptB<sub>2</sub>FGC complex, published in 2019, provided the first high resolution snapshots of LptB<sub>2</sub>FGC from *E. cloacae* and *V. cholerae* and mapped the pathway of unidirectional LPS transport. The cryo-EM structures of *E. coli* inner membrane Lpt complex illustrated the cycling between nucleotide-free and vanadate-trapped states. We examined the vanadate-trapped LptB<sub>2</sub>FG complex and found a groove where novobiocin might enter the complex (Figure 4.1A). We then compared the distances between novobiocin binding sites in LptB (residue R91) for both the nucleotide-free and vanadate-trapped structures. Upon inspection of the two structures, the vanadate-trapped structure appeared to better accommodate a novobiocin molecule than that of the nucleotide-free state and the inter-residue distance between novobiocin binding sites is 10.7Å (Figure 4.1B), a distance that could reasonably be spanned by a linker joining two novobiocin derivatives.



**Figure 4.1. Cryo-EM structure of the LptB<sub>2</sub>FG inner membrane complex (PDB 6MHU).**

A) Surface representation of LptB<sub>2</sub>FG. LptB homodimer is shown in green and cyan; LptF is shown in magenta; LptG is shown in yellow. The groove accommodating novobiocin is near the center of the rectangle. B) Close up view of residues R91 of both LptB chains, where novobiocin is present in the **nov**•LptB crystal structure. In the vanadate-trapped closed state (shown), the inter-residue distance is 10.7 Å, while in the nucleotide-free open state (not shown), the inter-residue distance is 26.6 Å.

Both covalent and noncovalent antibiotic dimers have significant roles in nature. For example, the natural product coumermycin A1<sup>309-311</sup> mentioned in earlier chapters is a dimeric, covalent aminocoumarin produced by *Streptomyces rishiriensis* (Figure 4.2).

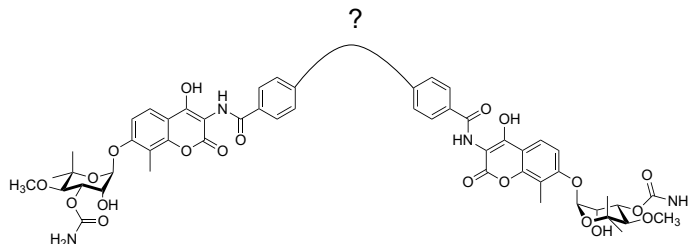


**Figure 4.2. Structure of coumermycin A1 from *Streptomyces rishiriensis*.**

Coumermycin A1 is a natural dimer comprised of two decorated noviose sugars and aminocoumarin cores separated by a 3-methyl-2,4-dicarboxamide-1H-pyrrole linker.

Noncovalent antibiotic dimers have also been described. For example, the glycopeptide antibiotics vancomycin<sup>312-315</sup> and ristocetin<sup>316</sup> are thought to homodimerize, which apparently enhances its antibiotic activity. Outside the scope of antibiotics, dimeric ligands have been studied

in the context of transcriptional regulation,<sup>317</sup> signal transduction,<sup>318</sup> and the cell cycle.<sup>319</sup> We hypothesized that dimeric aminocoumarins (Figure 4.3) with an ideal linker could bind both sites of LptB<sub>2</sub>FGC in the activated state and inhibit the complex by preventing nucleotide state cycling.



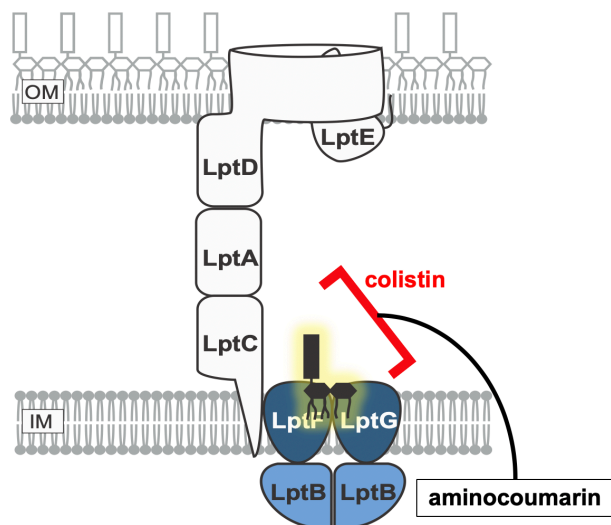
**Figure 4.3. Novobiocin dimer with undefined linker.**

The benzamide in novobiocin could be substituted with a linker to form homodimeric aminocoumarins similar to coumermycin A1.

The second objective of the chapter is the synthesis of a novobiocin-colistin chimera. With mounting evidence that polymyxin kills Gram-negative bacteria by interfering with lipopolysaccharide undergoing active biosynthesis or transport,<sup>298</sup> and that lipopolysaccharide transport agonists sensitize bacteria to the polymyxins<sup>320</sup>, we wondered if we could design a covalent combination of novobiocin and polymyxin E (colistin). Chimeric molecules, which were described as early as the late 1800s, are artificial compounds in which multiple functional components are assembled together to form an agent with a unique activity.<sup>321</sup> Our idea for a chimeric molecule came from our hypothesis that polymyxins bind LPS at the inner membrane during transport by the Lpt complex (Figure 4.4). Typically, molecules greater than 600 Da would not be very effective Gram-negative antibiotics due to poor membrane permeability at such high molecular weights. Nevertheless, a novobiocin-polymyxin chimera (MW > 1500 Da) could penetrate the Gram-negative outer membrane due to the permeabilizing property of the cationic polymyxin fragment. In 2019, Polyphor and Robinson described chimeric polymyxin molecules with murepavadin (POL-7080)<sup>322</sup>, the OMP-targeting antibiotic. They demonstrated that polymyxin chimeras can permeabilize both the inner and outer membranes and possess antibiotic



activity superior to the agents in monotherapy, while showing no lytic activity towards eukaryotic membranes.



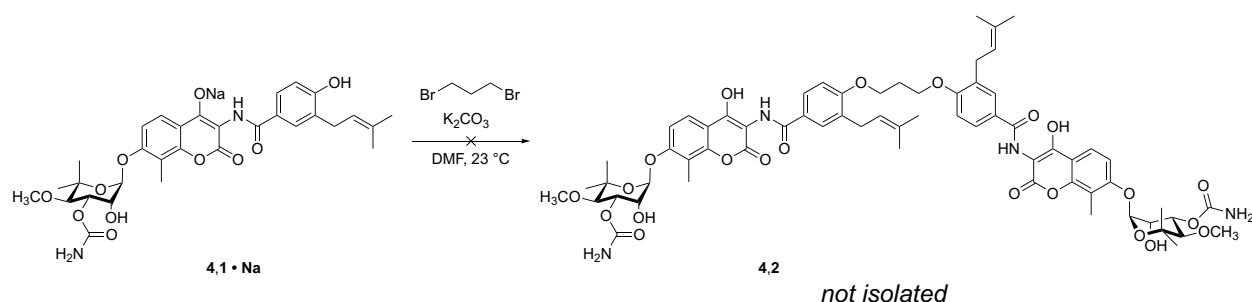
**Figure 4.4. Our proposed novobiocin-polymyxin chimera.**

An aminocoumarin similar to novobiocin will bind LptB<sub>2</sub>FGC and promote a hyperactive state of LPS transport. Polymyxin will bind the LPS in the inner membrane.

## 4.2. Results

### 4.2.1. Synthesis of alkane-tethered novobiocin dimers

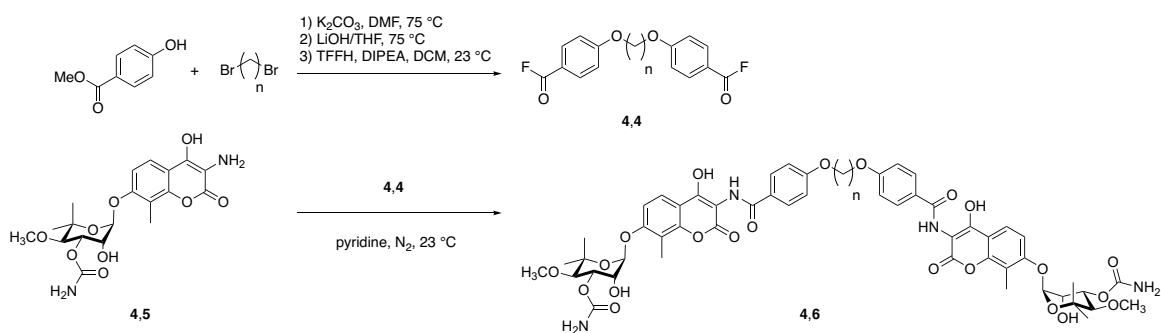
I reasoned that the easiest way to make novobiocin dimers would be to couple the existing phenol present in novobiocin with terminal dibromoalkanes. With multigram quantities of novobiocin sodium **4,1** • Na readily available, a Williamson etherification (Figure 4.5) was attempted by adding 1,3-dibromopropane and potassium carbonate base and stirring in DMF at 23 °C (higher temperatures were avoided due to problematic decarbamylation). Unfortunately, product **4,2** could not be formed in this manner.



**Figure 4.5. Williamson ether synthesis with simple dibromide linkers.**

Novobiocin sodium (Sigma-Aldrich), a dibromide linker, and inorganic base were stirred in anhydrous DMF at 23 °C, but the reaction was unsuccessful.

The remedy that I had to this problem was to build bifunctional bis-benzoic acid derivatives separated by alkane tethers (Figure 4.6). Activated linkers **4,4** could be assembled via Williamson etherification of inexpensive methyl 4-hydroxybenzoate and primary alkyl dibromides followed by saponification and activation with tetramethylfluoroformamidinium hexafluorophosphate (TFFH).<sup>323</sup> The acyl fluorides were observed to provide higher peptide coupling yields in the next step with the weakly nucleophilic novenamine **4,5** than acid chlorides or the combination of peptide coupling reagents (e.g. EDCI) and bis-carboxylic acids.

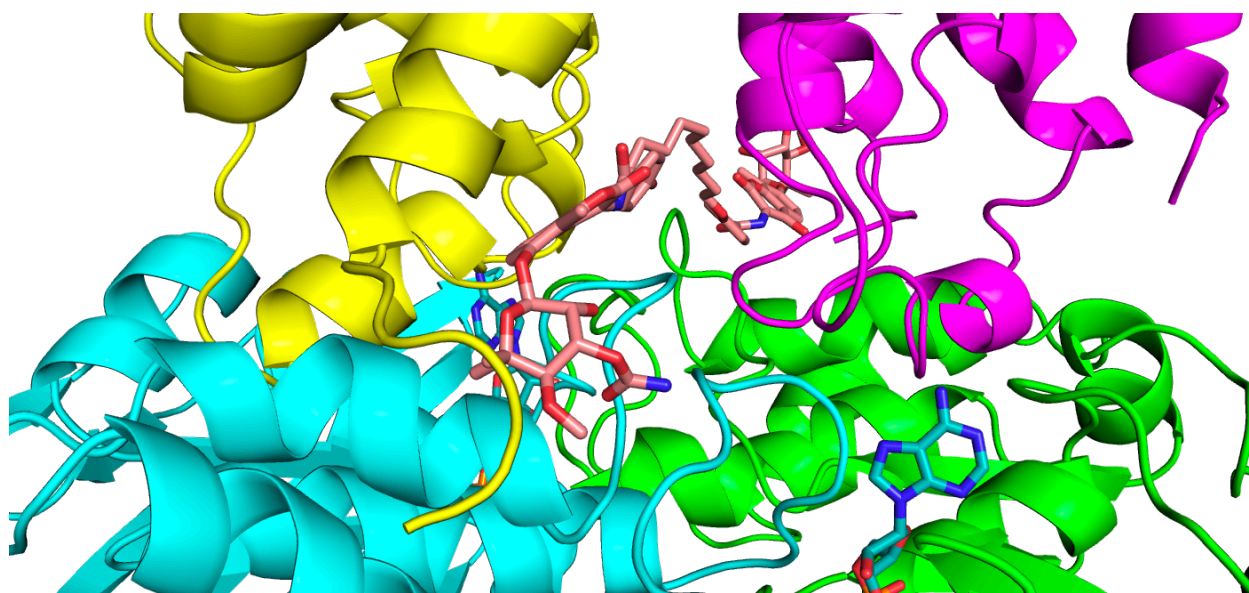


**Figure 4.6. Design of novobiocin alkane dimers.**

Novobiocin benzamide surrogates were designed for the synthesis of dimers **4,6** from a 4-hydroxybenzoate scaffold with fully saturated linkers ranging from  $n = 3$ -10, 12. Acyl fluorides were selected as the optimal coupling strategy.

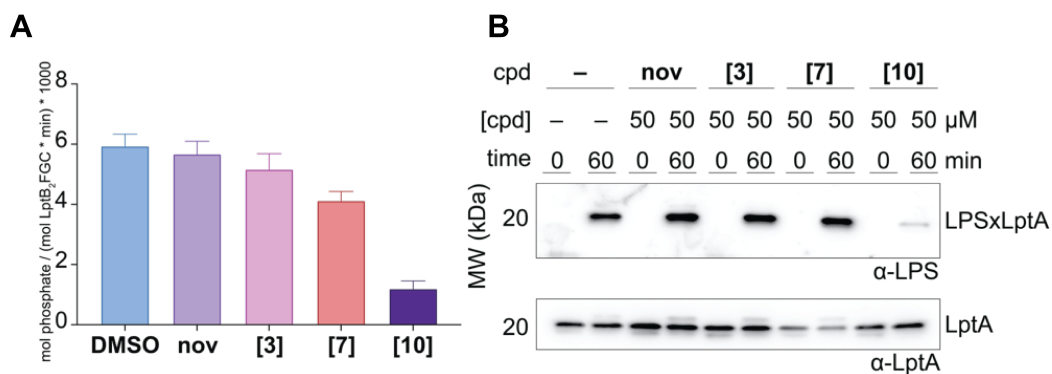
#### 4.2.2. Testing alkane novobiocin dimers in a proteoliposome reconstitution of LptB<sub>2</sub>FGC activity

Novobiocin dimers were modeled using the MOE software package (Chemical Computing Group; Figure 4.7), and the ideal linker length was predicted to be around 9.7 Å (10 carbons). A series of eight novobiocin dimers **4,6** (n = 3,4,5,6,7,8,9,10) were tested in an LPS transport reconstitution system in proteoliposomes (described in previous chapters). Initially, novobiocin (**nov**) and **4,6** (n = 3, 7, 10) were compared to a no-treatment control (Figure 4.8).



**Figure 4.7. MOE docking of 4,6 (n = 10) in the cavity of LptB<sub>2</sub>FGC.**

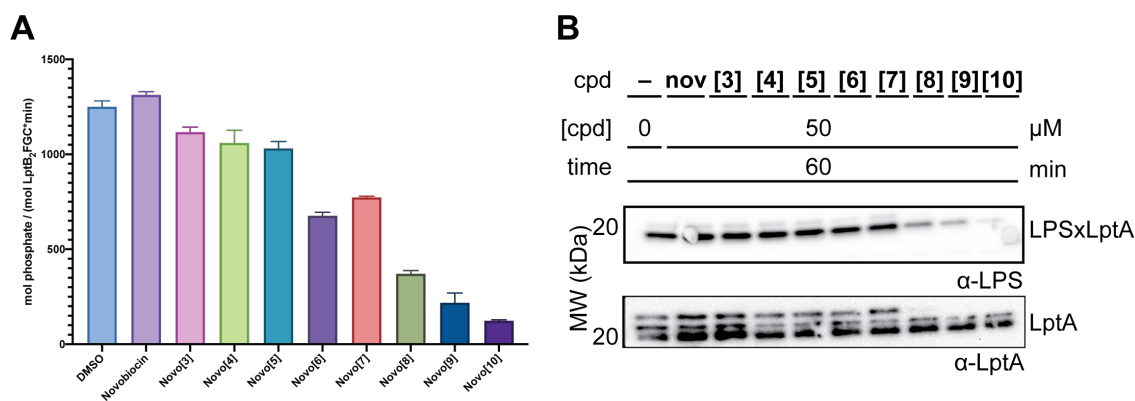
Novobiocin dimer **4,6** (n = 10) shown in pink, was docked using MOE to the LptB<sub>2</sub>FGC cavity using the vanadate-trapped nucleotide bound structure from ref.<sup>132</sup> LptB homodimer is shown in green and cyan; LptF is shown in magenta; LptG is shown in yellow.



#### Figure 4.8. Effect of novobiocin dimers on the proteoliposome LPS transport reconstitution.

Novobiocin dimers **4,6** show a linker length-dependence on inhibition of ATPase activity and LPS transport in proteoliposomes containing purified *E. coli* LptB<sub>2</sub>FGC and LPS. UV crosslinking was to added LptA(I36pBPA) after incubation at 30 °C, as done in previous chapters. A) ATPase assay ([ATP]<sub>i</sub> = 5 mM) revealed a significant decrease in hydrolysis activity by LptB<sub>2</sub>FGC upon treatment with 50 μM of **4,6** (n = 7, 10). B) LPS release assay showed a significant inhibition of LPS transport to LptA after 60 minutes upon treatment with 50 μM of **4,6** (n = 7, 10). These experiments were performed by Becca Taylor.

The results suggested that the novobiocin dimers were inhibiting LptB<sub>2</sub>FGC and that the inhibition was dependent on the length of the linker. The intermediate length novobiocin dimers were tested (Figure 4.9), and my results were consistent with Becca's in Figure 4.8.

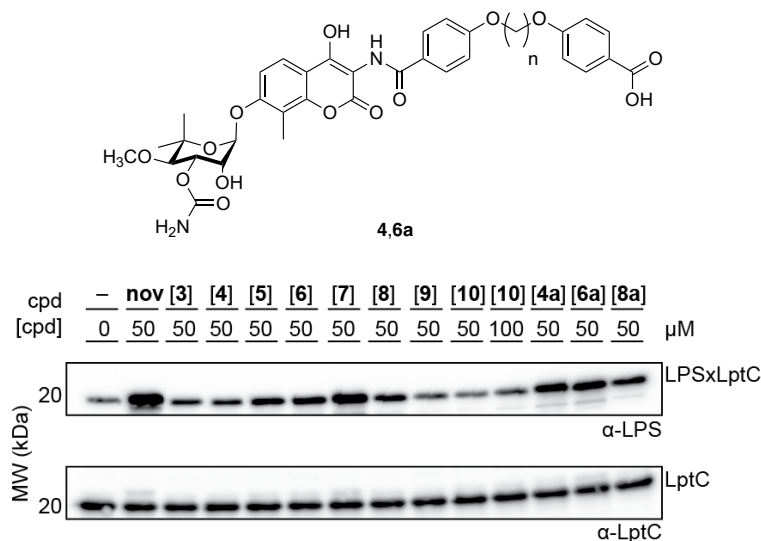


#### Figure 4.9. Effect of additional novobiocin dimers on LPS release in proteoliposomes.

Novobiocin dimers **4,6** (n = 3-10) show a linker length-dependence on inhibition LPS transport in proteoliposomes containing purified *E. coli* LptB<sub>2</sub>FGC and LPS. A) ATPase assay ([ATP]<sub>i</sub> = 5 mM) revealed a significant decrease in hydrolysis activity by LptB<sub>2</sub>FGC upon treatment with 50 μM of **4,6** (n = 3-10). B) LPS release assay showed a significant inhibition of LPS transport to LptA after incubation at 30 °C for 60 minutes upon treatment with 50 μM of **4,6** (n = 3-10). UV crosslinking was to added LptA(I36pBPA) after for 60 minutes.

#### 4.2.3. Effect of alkane novobiocin dimers on *in vivo* LPS release activity

We wondered whether novobiocin dimers **4,6**, which appeared to inhibit LPS transport and ATPase activity in proteoliposomes, had an effect on LPS transport to LptC *in vivo*. *E. coli* BL21(DE3) cells overexpressing LptB<sub>2</sub>FGC(T47pBPA)<sup>131, 133</sup> were treated with the series of **4,6** (n = 3-10) as well as the “novobiocin monomer acids” **4,6a** containing the linker but lacking the second novobiocin fragment (Figure 4.10).



**Figure 4.10. Effect of alkane novobiocin dimers on *in vivo* LPS transport to LptC(T47).**

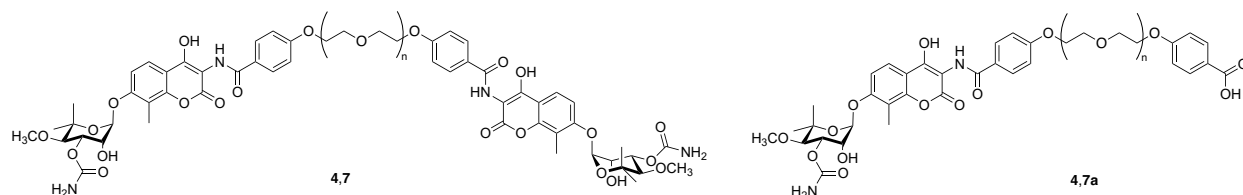
Top: Structure of “novobiocin monomer acids” **4,6a**. Bottom: Novobiocin dimers **4,6** ( $n = 3-10$ ) do not appear to inhibit LPS transport to LptC *in vivo*. In contrast, novobiocin, **4,6** ( $n = 7$ ), **4,6a** ( $n = 4,6,8$ ) appear to stimulate LPS release.

Neither the novobiocin alkane dimers **4,6**, nor the corresponding acids **4,6a** ( $n = 4,6,8$ ) inhibited LPS transport at the concentrations tested. We considered the possibility that these dimers were suffering from poor permeability properties and could not penetrate the outer membrane. Alternatively, we began to suspect that our results in Figures 4.8-4.9 were the result of **4,6** acting as bolaamphiphiles<sup>324-326</sup> and disrupting proteoliposomes without a specific interaction with the LptB<sub>2</sub>FGC protein component. We hypothesized that if the alkane linkers were replaced with more hydrophilic PEG linkers of comparable length and inhibition of LptB<sub>2</sub>FGC were still observed, then the interaction between the dimers and the protein complex was likely a specific one.

#### 4.2.4. Synthesis and effect of PEG-linked novobiocin dimers on a proteoliposome reconstitution of LptB<sub>2</sub>FGC activity

A series of PEG dibromides were purchased from Broadpharm (San Diego, CA) and the identical procedure was followed to synthesize PEG-linked novobiocin dimers **4,7** as in Figure 4.7. The corresponding monomeric PEG acids **4,7a** were also prepared, usually concurrently with

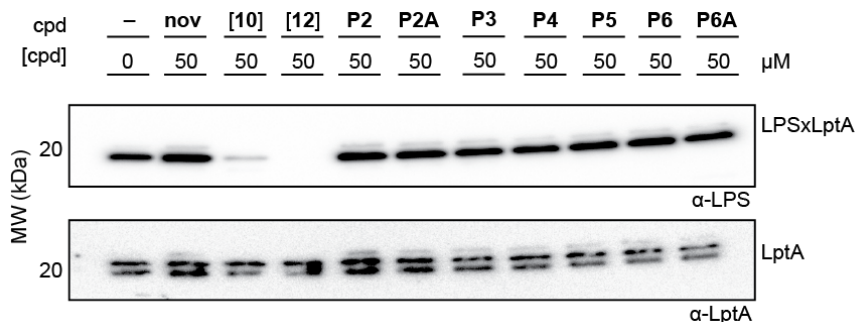
the dimers; that is, they were isolated as the minor product during the peptide coupling (Figure 4.11).



**Figure 4.11. Structure of PEG-linked novobiocin dimers **4,7** and monomer acids **4,7a**.**

Novobiocin PEG-linked dimers **4,7** and monomer acids **4,7a** were synthesized in the same fashion as the alkane dimers (Figure 4.7). The bis-benzoate PEG linkers were observed to have improved water solubility compared to the bis-benzoate alkane linkers.

Following synthesis, the PEG-linked novobiocin dimers were tested in the *in vitro* LPS release assay in proteoliposomes as done in Figures 4.8-4.9. In contrast to **4,6**, which again demonstrated LPS transport inhibition, the PEG-linked dimers **4,7** and acids **4,7a** did not show any sign of inhibition at the concentrations tested (Figure 4.12).



**Figure 4.12. PEG-linked novobiocin dimers **4,7** and monomer acids **4,7a** do not inhibit LPS transport in an *in vitro* proteoliposome reconstitution.**

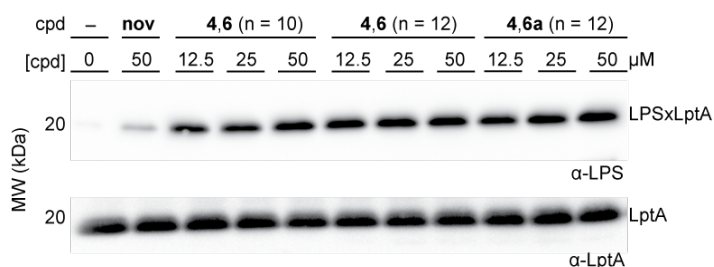
Novobiocin PEG-linked dimers **4,7** ( $n = 2-6$ ) and monomer acids **4,7a** ( $n = 2, 6$ ) are represented as 'P' and novobiocin alkane-linked dimers **4,6** ( $n = 10, 12$ ) were used as positive controls for inhibition of LPS transport in proteoliposomes to LptA(I36pBPA). The conditions for the reconstitution were identical to those in Figures 4.8-4.9.

These results could either suggest that 1) the PEG-linked novobiocin dimers were not acting as bolaamphiphiles and disrupting the proteoliposomes 2) the PEG-linked novobiocin dimers were not hydrophobic enough to span the hydrophobic cavity of LptB<sub>2</sub>FGC. Next, we wondered whether

the alkane-linked dimers demonstrated any effect on LPS release from right-side-out (RSO) vesicles overexpressing LptB<sub>2</sub>FGC to LptA(I36pBPA).

#### 4.2.5. Synthesis and effect of PEG-linked novobiocin dimers on an RSO vesicle reconstitution of LptB<sub>2</sub>FGC activity

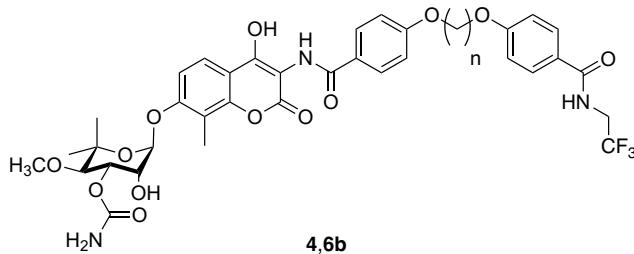
The same alkane-linked novobiocin dimers **4,6** (n = 10, 12) that inhibited LPS transport to LptA(I36pBPA) in proteoliposomes (Figures 4.8-4.9) were tested in an RSO vesicle reconstitution of LPS release to LptA; the acid **4,6a** (n = 12) was included in the experiment to probe the effect of the second novobiocin fragment on LPS release (Figure 4.13).



**Figure 4.13. Alkane-linked novobiocin dimers **4,6** and monomer acid **4,7a** (n = 12) do not inhibit LPS transport in an *in vitro* RSO vesicle reconstitution of LPS release.**

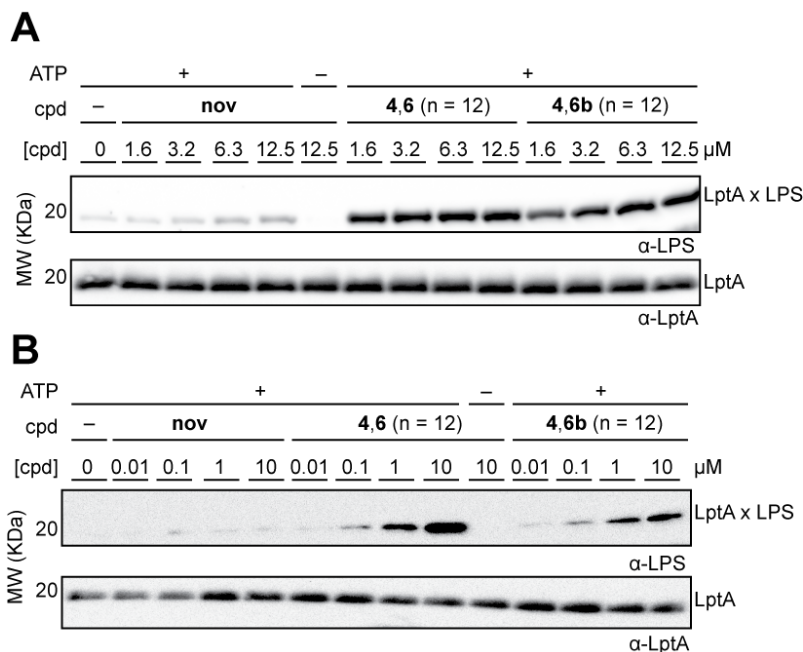
Novobiocin alkane-linked dimers **4,6** (n = 10, 12) and the monomer acid **4,6a** (n = 12) did not inhibit LPS transport in RSO vesicles, but instead stimulated LPS transport.

The results were unexpected: The alkane-linked dimers stimulated LPS release in the RSO vesicle assay, which was the opposite of what had been observed for the same molecules in the proteoliposome assay. In order to ensure that this effect was real, we reproduced the experiment with lower concentrations of novobiocin and **4,6** (n = 12). To see if the second novobiocin fragment were important, we prepared **4,6b** (n = 12), a monomeric novobiocin with a linker but containing a benzamide mimic on the opposite end that was more structurally conservative than a free carboxylic acid (Figures 4.14-4.15).



**Figure 4.14. Structure of novobiocin monomer trifluoroethylamide 4,6b.**

A novobiocin monomer with a twelve-carbon alkane linker ( $n = 12$ ) was synthesized to test the function of the linker missing the second novobiocin fragment. The bis-benzamide was designed to avoid having the free carboxylate as in **4,6a**, which could impart undesired ionic interactions. The trifluoromethyl group could be used in future  $^{19}\text{F}$  NMR studies, if desired.



**Figure 4.15. The alkane-linked novobiocin dimer 4,6 ( $n = 12$ ) and monomer 4,6b ( $n = 12$ ) stimulate LPS transport in RSO vesicles at lower concentrations than those needed by novobiocin for LPS transport stimulation.**

A) Novobiocin stimulates LPS transport in a dose-dependent fashion, but substantially less than comparable concentrations of alkane-linked dimers **4,6** ( $n = 12$ ) and the monomer **4,6b** ( $n = 12$ ). B) **4,6** ( $n = 12$ ) and the monomer **4,6b** ( $n = 12$ ) stimulated LPS transport at several orders of magnitude lower concentrations than novobiocin did. An ATP control was included to demonstrate that LPS release was dependent on ATP hydrolysis.

The subsequent experiments showed that the **4,6** dimer ( $n = 12$ ) and **4,6b** ( $n = 12$ ) were stimulating LPS release in RSO vesicles at concentrations orders of magnitude lower than what was observed with novobiocin. The effect did not appear to be dependent on a second novobiocin



fragment, as the monomeric **4,6b** significantly stimulated LPS transport, although having a dimer appeared to effect a higher degree of stimulation (Figure 4.15B). The interpretation for these results has not been elucidated; however, the data suggest that novobiocin + linkers causes the increased LPS transport seen in RSO vesicles.

#### 4.2.6. Antibiotic activities of novobiocin dimers

Novobiocin alkane- and PEG-linked dimers were tested for antibacterial activity in a standard minimal inhibitory concentration (MIC) assay.<sup>297</sup> The dimers lacked significant activity against wildtype *E. coli* ATCC 25922 (Table 4.1); it is possible that the dimers do not penetrate the outer membrane. Interestingly, however, **4,6** (n = 3) possessed weak micromolar activity comparable to that of novobiocin. We do not have an explanation for why this short dimer possessed antibiotic activity.

**Table 4.1. Minimal inhibitory concentrations (MIC) of novobiocin dimers**  
(16 hours, 37 °C).

| Compound            | <i>E. coli</i> 25922 MIC ( $\mu$ M) |
|---------------------|-------------------------------------|
| novobiocin          | 40                                  |
| <b>4,6</b> (n = 3)  | 44                                  |
| <b>4,6</b> (n = 4)  | >100                                |
| <b>4,6</b> (n = 5)  | >100                                |
| <b>4,6</b> (n = 6)  | >100                                |
| <b>4,6</b> (n = 7)  | >100                                |
| <b>4,6</b> (n = 8)  | >100                                |
| <b>4,6</b> (n = 9)  | >100                                |
| <b>4,6</b> (n = 10) | >100                                |
| <b>4,6</b> (n = 12) | >100                                |
| <b>4,7</b> (n = 2)  | >100                                |
| <b>4,7</b> (n = 3)  | >100                                |
| <b>4,7</b> (n = 4)  | >100                                |
| <b>4,7</b> (n = 5)  | >100                                |
| <b>4,7</b> (n = 6)  | >100                                |

#### 4.2.7. Concept of a novobiocin-polymyxin chimera

Our effort to build dimeric novobiocin molecules with the aim of inhibiting the LptB<sub>2</sub>FGC complex raised more questions than provided answers. Why were the dimers inhibiting LptB<sub>2</sub>FGC only in proteoliposomes but not in right-side-out vesicles? If so, was inhibition specific only to the concentration of complexes (ie. in proteoliposomes the [LptB<sub>2</sub>FGC] is presumably lower than that in vesicles). These questions turned out to be relatively difficult to answer. Instead, we turned to our recent data (Figure 4.15) that showed that novobiocin analogs with hydrophobic linkers were able to stimulate LPS transport at ~3 orders of magnitude lower than that was required by novobiocin. This suggested that novobiocin could be functionalized with a linker that might bridge not just another novobiocin molecule, but polymyxin, which we have investigated previously in combination with novobiocin.

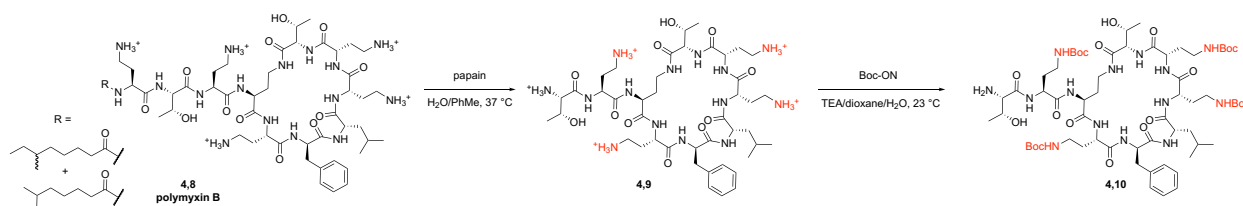
#### 4.2.8. Designing a synthetic route to a novobiocin-polymyxin chimera

We wondered if we could leverage our knowledge that novobiocin stimulates LptB<sub>2</sub>FGC for the purpose of creating a new and better antibiotic. Polymyxin B and colistin possess five positively charged diaminobutyric (Dab) acid residues, which imparts high water solubility, LPS binding, and membrane permeabilizing properties. We realized it was imperative to maintain these five Dab residues intact in a chimera; however, these residues would be the easiest to append functionality, and we initially considered *N*-alkylation as a viable means to attach an alkyne for subsequent click reactions to novobiocin azides. The azide-alkyne click reaction was chosen to link polymyxin and novobiocin because it could tolerate the five Dab residues. Moreover, an alkaline deprotection step (e.g. 20% piperidine in methanol) on a chimera containing a novobiocin moiety would hydrolyze the L-noviose sugar. The major Achilles heel to installing an alkyne on polymyxin is the fact that the five Dab amines are virtually chemically identical, and treatment of either molecule with 1.0 equivalent of an electrophile such as propargyl bromide and

a base would lead to a regiochemical mixture—a disaster! Instead, we turned to the literature, in which we discovered precedence for a route to selectively modify polymyxin at the N-terminus.

#### 4.2.9. N-terminal derivatives of polymyxin nonapeptide

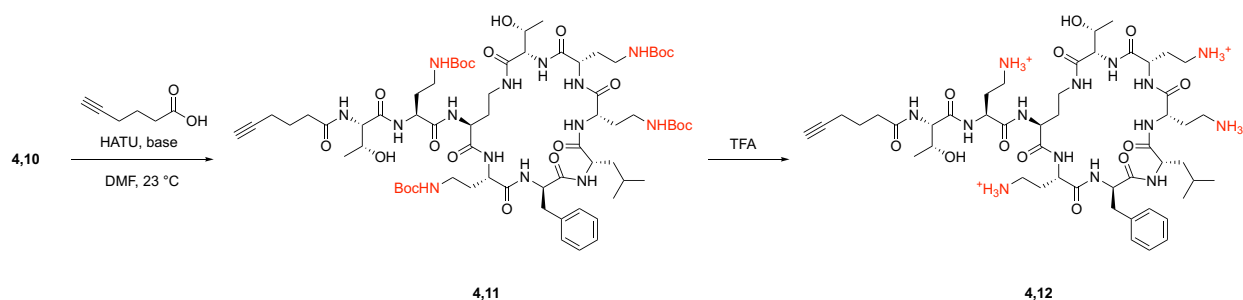
O'Dowd and coworkers<sup>327</sup> described a two-step 1) enzymatic hydrolysis of the fatty acid and 2) selective protection of the  $\gamma$ -Dab amines relative to the nascent terminal amine (Figure 4.16).



**Figure 4.16. Enzymatic cleavage of the polymyxin fatty acid and Dab side chain protection.**

A polymyxin with five Dab residues, such as polymyxin B **4,8**, is enzymatically cleaved with papain to yield polymyxin B nonapeptide **4,9** with four Dab residues. Selective protection of the Dab residues over the terminal amine to **4,10** can be accomplished by careful timing of the reaction as described in ref.<sup>327</sup>

Papain cleavage would remove a desired Dab residue, which was not atom economical, since the amine, if desired, would need to be reinstalled at a later stage of the synthesis. As a proof of concept, **4,10** could be coupled with an alkynoic acid to provide protected alkyne **4,11**, which could be deprotected with TFA to yield a polymyxin derivative containing handle suitable for copper-catalyzed click chemistry (Figure 4.17).<sup>328, 329</sup> In reality, isolation of a regiomERICALLY pure **4,10** was difficult and the reaction was difficult to control (ie. over-protection was observed).



**Figure 4.17. Proposed synthesis of alkynyl-functionalized polymyxin 4,12.**

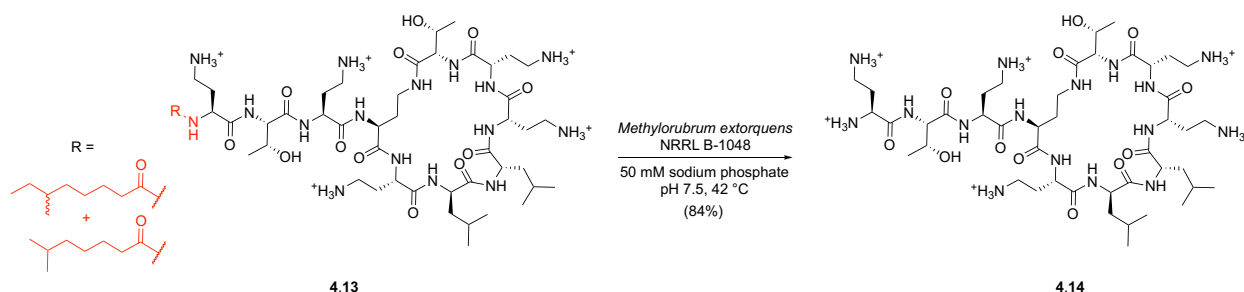
Selectively protected polymyxin nonapeptide **4,10** could be acylated with an alkyne-containing carboxylic acid or activated derivative to yield **4,11**, which could be deprotected with TFA to yield a polymyxin derivative **4,12** primed for a click reaction.

Further adding to the challenge was the observation that papain was not fully selective for the fatty acid amide and cleaved some peptide residues to yield a mixture of nonapeptide **4,9** and smaller compounds. An enzyme that were more selective for the fatty acid amide would be more ideal.

**4.2.10. Cultivation of *Methylobacterium extorquens* for polymyxin cleavage**

In the late 1980s, Yukio Kimura and co-workers (Mukogawa Women's University, Japan) reported the discovery of a polymyxin acylase from *Pseudomonas* sp. M-6-3, which could hydrolyze the fatty acyl groups of polymyxins without affecting the peptide portion.<sup>330-332</sup> Unfortunately, this organism could not be located at the USDA Agricultural Research Service (ARS) collection nor at the American Type Culture Collection (ATCC). In 1992, Kimura and co-workers described the isolation, purification, and characterization of a new enzyme from *Pseudomonas* sp. M-27, a carboxypeptidase G<sub>3</sub><sup>333</sup> that potentially had the same activity as the aforementioned polymyxin acylase. Further research into the *Pseudomonas* sp. M-27 strain in the literature revealed that in 1965, Anthony and Zatman had described the organism as being capable of subsisting on methanol as a sole carbon source.<sup>334</sup> *Pseudomonas* sp. M-27 turned out to be reclassified as *Methylobacterium extorquens* in 2002<sup>335</sup> and again moved into the genus *Methylobacterium* in 2018.<sup>336</sup> I was able to find and order *Methylobacterium extorquens* in the German Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) collection as well as the USDA ARS (NRRL B-1048). As expected, the strain was cultivated on methanol as the sole carbon source. For production of the deacylase enzyme, cultures of NRRL B-1048 were grown in a citrate medium at 30 °C for 3 days, harvested, and converted to an acetone-dried cell powder

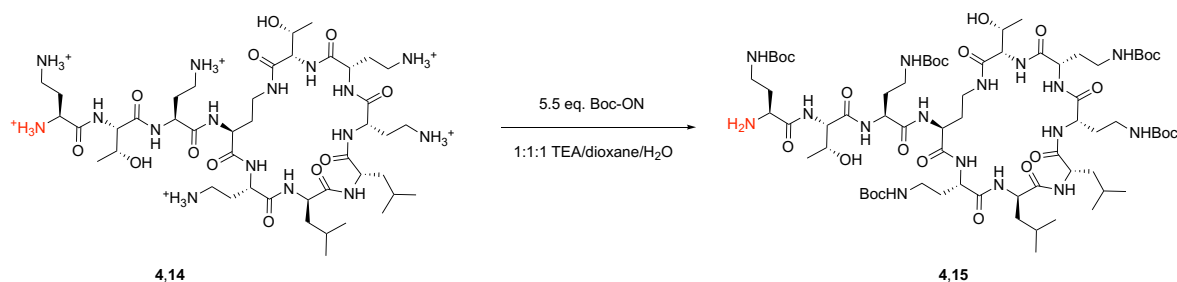
(see Materials and Methods). This cell powder could catalyze the hydrolysis of colistin **4,13** to deacylcolistin **4,14** (Figure 4.18).



**Figure 4.18. Hydrolysis of colistin fatty amide with *Methyloburum extorquens*.**

A dried cell powder prepared from *Methyloburum extorquens* cell culture<sup>332</sup> could effect the cleavage of colistin **4,13** to deacylcolistin **4,14** after overnight reaction time (>16 h) at 42 °C.

Unfortunately, when deacylcolistin **4,14** was treated with a slight excess of Boc-ON reagent to selectively protect the  $\gamma$ -Dab amines (Figure 4.19), the reaction was uncontrollable. There was little discrimination between the terminal amine and those of side chains, which was not entirely unexpected since they are both primary amines. At this point, I came to the understanding that the side chains of colistin needed to be protected in the first step.



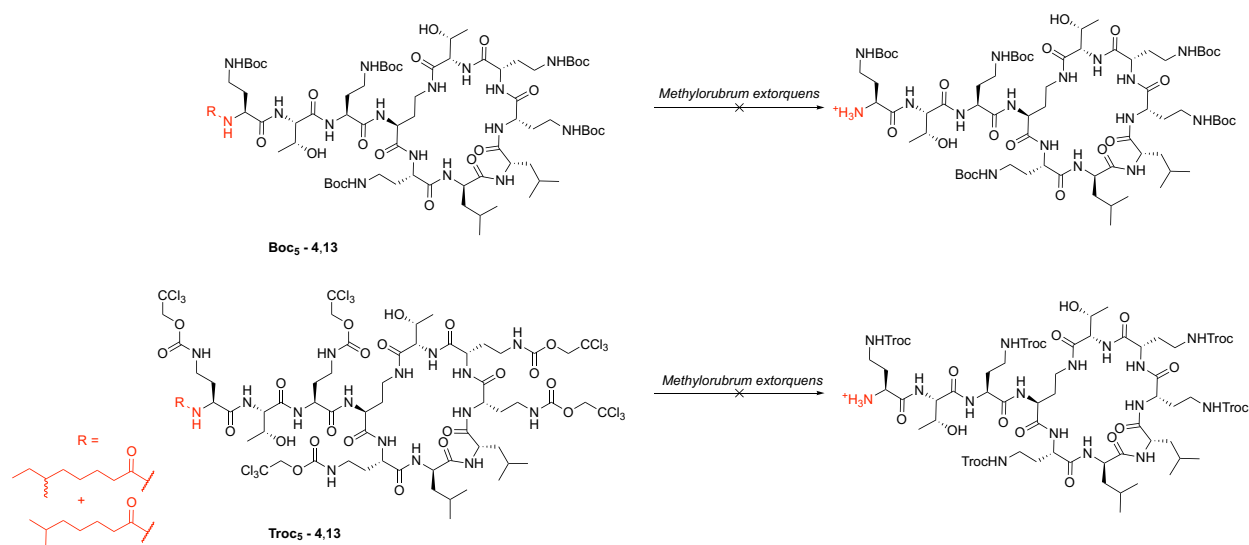
**Figure 4.19. Attempted selective protection of deacylcolistin **4,14** to **4,15**.**

Similar conditions for Boc protection were employed on **4,14** as described in Figure 4.15. The outcome of this reaction was a mixture of products, including over- and underprotected deacylcolistin, and the products of this reaction could not be separated by chromatography.

#### 4.2.11. Attempted deacylation of Boc<sub>5</sub>- and Troc<sub>5</sub>-colistin

The five  $\gamma$ -Dab amines of colistin **4,13** were fully protected with either Boc or Troc amino protecting groups.<sup>337</sup> **Boc<sub>5</sub> • 4,13** and **Troc<sub>5</sub> • 4,13** were sparingly soluble in water, and cosolvents of sodium phosphate buffer and THF, DMSO, or acetone were tested in order to dissolve them

along with the heavy cell suspension. No combination of solvents was found that promoted the hydrolysis of these protected colistins (Figure 4.20).



**Figure 4.20. Attempted hydrolysis of protected colistins.**

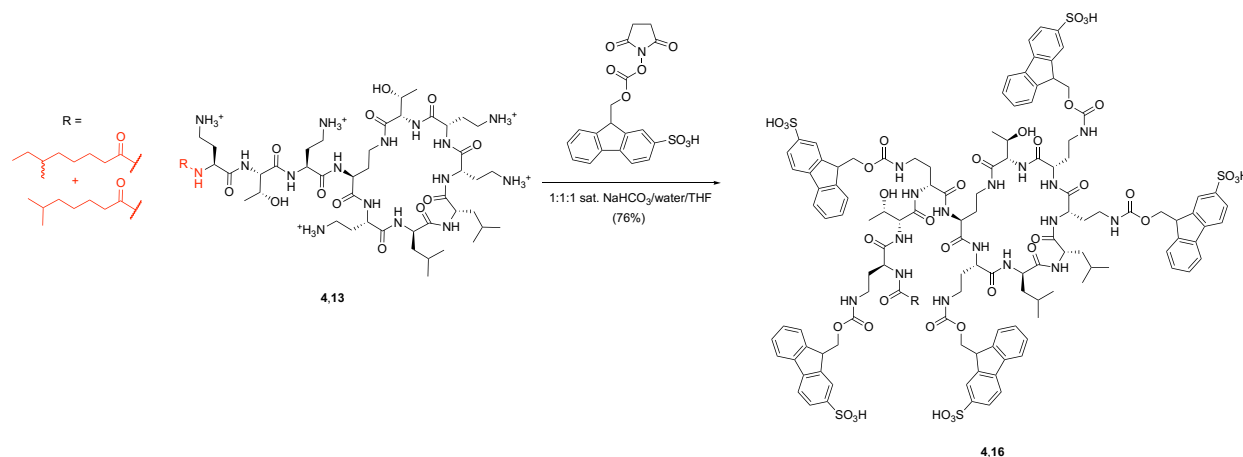
Penta-*tert*-butoxycarbonyl (Boc) and penta-2,2,2-trichloroethoxycarbonyl (Troc)-colistins were subjected to *Methyloburum extorquens* cell extracts as described earlier (Figure 4.18). Both reactions failed.

We did not find the reason for these reaction failures; however, we hypothesized that 1) the *Methyloburum extorquens* enzyme was inactivated by the presence of organic solvent, 2) the starting material was insoluble and inaccessible to the enzyme, or 3) the protected starting material was unrecognizable to the enzyme. It was conceivable that a different fatty amide hydrolase capable of recognizing the side chain of colistin could exist, and that we would need to obtain a different bacterial organism.

#### 4.2.12. Cleavage of protected colistin with *Actinoplanes utahensis* NRRL-12052

The Gram-positive bacterium *Actinoplanes utahensis*<sup>338</sup> (USDA NRRL-12052) was reported to hydrolyze the fatty amide of the antifungal lipopeptide echinocandin B.<sup>339</sup> This strain enabled the production of the drug daptomycin<sup>249, 340</sup> by Eli Lilly and subsequently Cubist Pharmaceuticals. BioSource Pharm, which was acquired by Cubist, utilized this organism for the synthesis of *N*-terminal polymyxin derivatives.<sup>341, 342</sup> In their process<sup>342</sup>, a water-soluble protecting

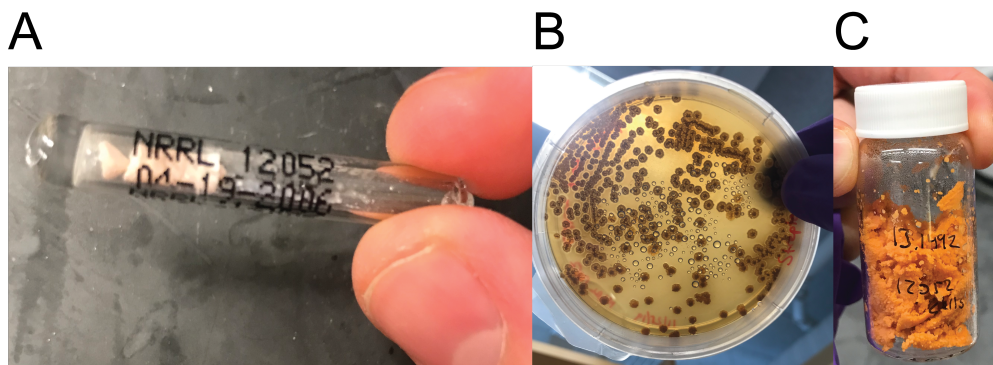
group (2-sulfo)-9-fluorenylmethoxycarbonyl (“sulfmoc”) <sup>343</sup> was first attached to the five  $\gamma$ -Dab amines (Figure 4.21). I found this reaction to be very efficient and reproducible when a syringe pump was employed to slowly deliver a solution of the NHS ester to a stirring mixture of colistin and aqueous sodium bicarbonate. The purification of **4,16** did not require chromatography, but instead involved protonation of the five anionic sulfates by lowering the pH < 1 with concentrated acid. The product **4,16** precipitated from the mother liquor as an oil, which upon trituration with EtOAc became a solid; isolated yields were routinely over 70%.



**Figure 4.21. Protection of colistin  $\gamma$ -Dab amines with water-soluble sulfmoc groups.**

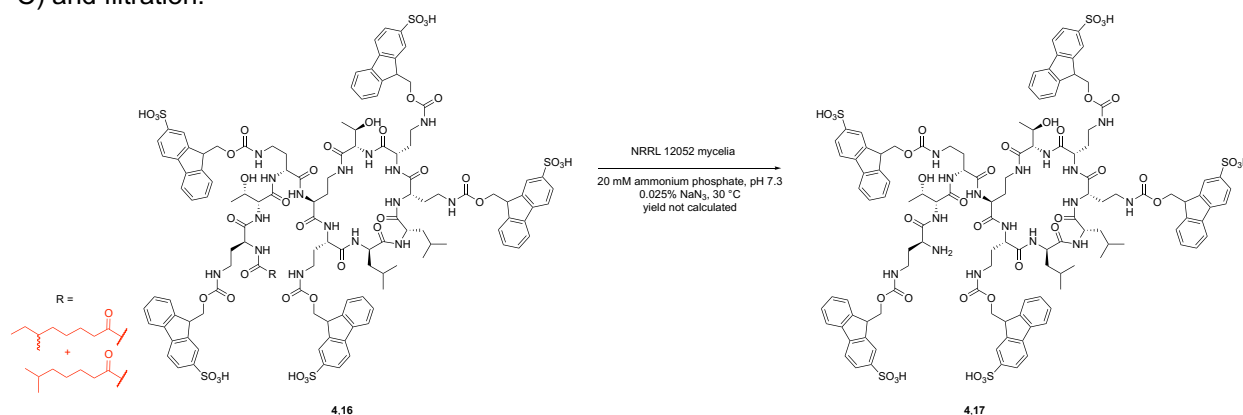
The sulfmoc protecting group was introduced by slow addition of the sulfofluorenylmethoxycarbonyl NHS ester to a solution of colistin sulfate **4,13** dissolved in an alkaline solution and THF. For experimental details see Materials and Methods section.

I obtained NRRL-12052 by contacting the USDA ARS and received a lyophilizate (Figure 4.22A), from which I isolated the strain (Figure 4.22B), grew liquid cultures, and harvested enzyme-containing mycelia (Figure 4.22C). These mycelia could be directly used for the deacylation of sulfmoc<sub>5</sub>colistin **4,16** without further enzymatic purification. The orange irregular spheres from 1.0 L of culture after 3 days of fermentation were mixed with **4,16** and dissolved in buffer (Figure 4.23).



**Figure 4.22. *Actinoplanes utahensis* NRRL-12052.**

A) Vacuum-dried culture. B) Mycelia on *Streptomyces* agar. C) Orange mycelia after cultivation (3 days, 28 °C) and filtration.



**Figure 4.23. Enzymatic hydrolysis of sulfmoc<sub>5</sub>colistin.**

Sulfmoc<sub>5</sub>colistin **4,16** was dissolved in an ammonium phosphate buffer at pH 7.3 (1-2 mg/mL). 0.025% sodium azide was added as a preservative and the reaction was shaken at 30 °C for 8-24 h. See Materials and Methods section for the modified procedure from ref.<sup>342</sup> It is imperative that analytical samples be filtered through 0.2 micron filters as NRRL-12052 mycelia will clog LC/MS systems.

Upon reaction completion, cells could be separated by centrifugation and frozen for later reuse.

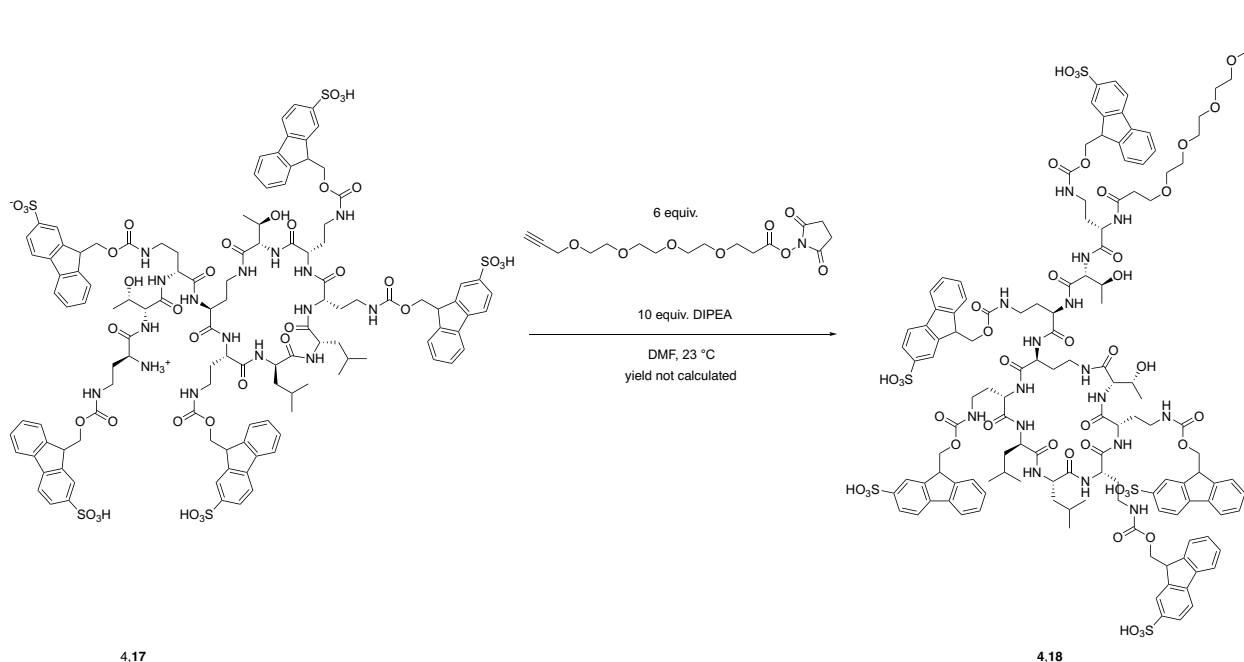
The product could be precipitated by lowering the pH of the solution to < 1 with concentrated HCl.

However, the product was found to be contaminated with leftover starting material **4,16**, as judged by flow injection analysis (FIA) mass spectrometry. Unfortunately, separation of **4,17** from **4,16** by reverse-phase HPLC was not successful. However, since FIA mass spectrometry is not quantitative, I could not calculate a yield for the step in Figure 4.23. A significant quantity of **4,16** remained in the next step (peptide coupling at the *N*-terminus), yet fortunately, **4,16** did not interfere with the reaction.



#### 4.2.13. N-acylation of deacylsulfmoc<sub>5</sub>colistin

With a mixture of **4,16** and **4,17**, I performed an experiment in which various equivalents of electrophile and base were needed to effect the full disappearance of the **4,17** signal from FIA mass spectrometry analysis. I found that 6 equivalents of an NHS ester and 10 equivalents of Hünig's base (Figure 4.24) were sufficient to clear the starting material signal within 3 hours. Concomitantly, a new signal emerged that corresponded to the product **4,18**. To quench the reaction mixture, which contained plenty of Hünig's base, its conjugate acid, leftover NHS ester, and DMF, I added water, ethyl acetate, and concentrated HCl. A triphasic mixture formed, which upon centrifugation, settled the product **4,18** as an oil at the bottom, leaving most impurities in the other two layers. Once again, the exact yield could not be determined, since **4,16** was carried forward.

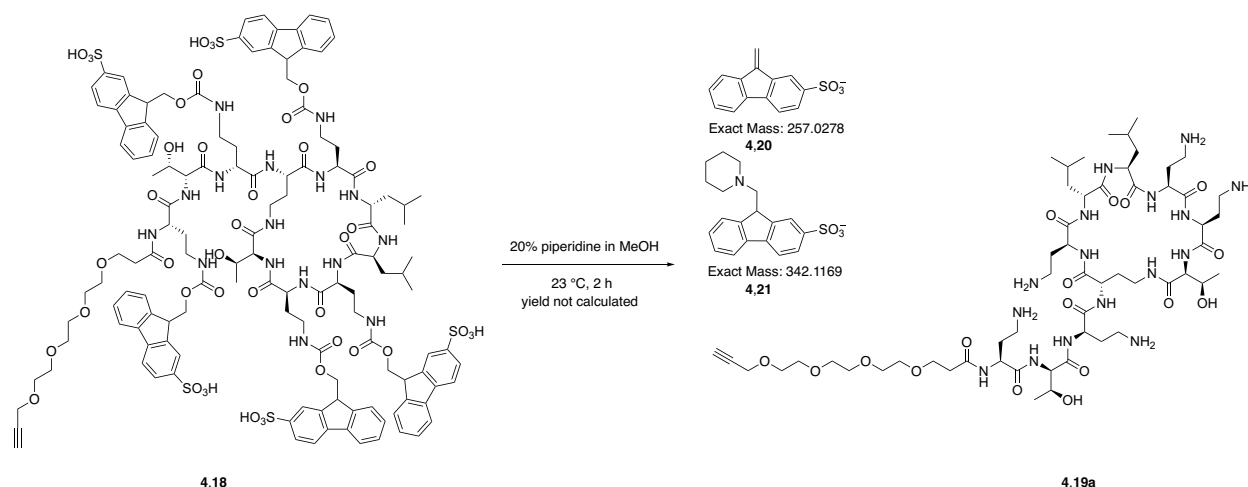


**Figure 4.24. Acylation of deacylsulfmoc<sub>5</sub>colistin 4,17.**

Deacylsulfmoc<sub>5</sub>colistin **4,17** was dissolved in DMF and excess NHS ester and Hünig's base were added. The reaction was shaken for 3 hours at 1000 rpm, 23 °C; conversion could be assessed by flow injection analysis mass spectrometry. See Materials and Methods section for full experimental details.

#### 4.2.14. Deprotection of 4,18

The crude precipitate from the previous step was dissolved in a 20% piperidine solution in methanol and stirred at 23 °C for 2 hours. The initially clear reaction mixture was observed to gradually turn turbid over this time, and towards the end was opaque. FIA mass spectrometry of the solution indicated the formation of **4,19a**, as well as byproducts sulfodibenzofulvene **4,20** and its piperidine adduct **4,21** (Figure 4.25). The conversion was not quantified, but no trace of **4,18** could be detected by FIA MS or LC/MS.



**Figure 4.25. Deprotection of 4,18.**

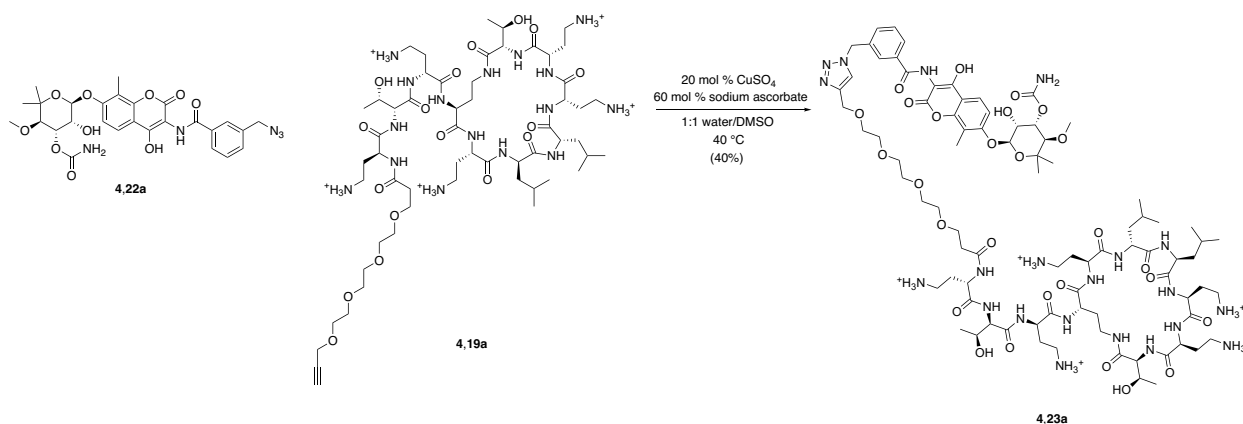
**4,18** was dissolved in 20% piperidine solution in methanol and stirred at 23 °C for 2 hours. During this time, a white precipitate formed and the *m/z* signals of **4,19a**, the sulfodibenzofulvene, and the sulfodibenzofulvene-piperidine adduct could be detected by FIA mass spectrometry. See Materials and Methods section for full experimental details.

A purification was desired for **4,19a**, since byproducts **4,20** and especially **4,21** could interfere with the subsequent Cu<sup>+</sup>-catalyzed click reaction. Evaporation of the piperidine and methanol followed by trituration with DCM, EtOAc, or ether did not remove **4,21**, which was highly water soluble, along with **4,19a**, at both high and low pH values. I utilized a size-exclusion desalting procedure with PD MidiTrap G-10 columns (GE Healthcare Life Science) that could remove molecules less than 700 Da. Upon evaporation of the methanolic piperidine solution, I dissolved the crude material in deionized water and passed the solution through a pre-equilibrated gravity

column and eluted with 3 x 0.6 mL water (see Materials and Methods section for full procedure). These first three fractions were collected in preweighed 1.5 mL Eppendorf tubes, which were subsequently lyophilized to snow-white powders of purified **4,19a**. Later fractions (which were not saved) were found to contain **4,21** by FIA MS and did not contain detectable **4,19a**. Therefore, size-exclusion chromatography with PD-10 columns was a good cleanup procedure for the deprotection step to ensure that **4,19a** was sufficiently pure for the click reaction. Finally, some colistin **4,13** was also detected, from the sulfmoc deprotection of **4,16**, which had been telescoped to this point. Fortunately, **4,13**, lacking either an azide or alkyne remained a spectator in the next reaction.

#### 4.2.15. Click reaction between colistin and novobiocin derivatives to chimeras **4,23**

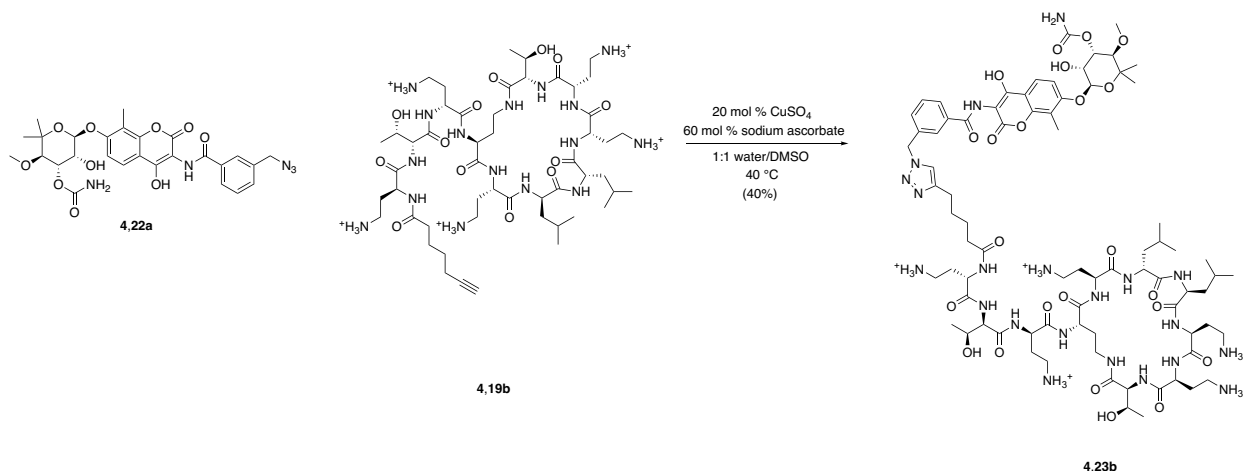
Standard procedures for the copper(I)-catalyzed azide-alkyne cycloaddition reaction<sup>344</sup> were found to couple **4,19a** with **4,22a** (Figure 4.26). There was no need to add a ligand such as THPTA, TBTA, or Hünig's base, and there was no need to use pre-formed copper(I) species. Upon reaction completion, the reaction mixture was quenched with DMSO, centrifuged to remove solids, and directly injected to a reverse-phase HPLC column for purification. The UV absorbance of the coumarin moiety ( $\lambda_{\text{max}} = 340 \text{ nm}$ ) in **4,23** was used to locate the retention time of the chimera during the HPLC purification.



**Figure 4.26. Click reaction between novobiocin 4,22a and colistin derivative 4,19a to yield chimeric 4,23a.**

In a 1.5 mL Eppendorf tube, **4,19a** was combined with **4,22a**, catalytic  $\text{CuSO}_{4(\text{aq})}$ . Sodium L-ascorbate $_{(\text{aq})}$  was added to initiate the reaction and the tube was capped to prevent additional  $\text{O}_2$  from quenching the catalytically active copper species. The reaction was incubated at 40 °C for a period of 12-24 h and quenched with DMSO. The mixture was then purified by reverse-phase HPLC. See Materials and Methods section for full experimental details.

Chimera **4,23b**, which possessed a hydrocarbon C7 linker was also synthesized (Figure 4.27).



**Figure 4.27. Click reaction between 4,22a and heptynyl-colistin 4,19b.**

In a 1.5 mL Eppendorf tube, **4,19b** was combined with **4,22a**, catalytic  $\text{CuSO}_{4(\text{aq})}$ . Sodium L-ascorbate $_{(\text{aq})}$  was added to initiate the reaction and the tube was capped to prevent additional  $\text{O}_2$  from quenching the catalytically active copper species. The reaction was incubated at 40 °C for a period of 12-24 h and quenched with DMSO. The mixture was then purified by reverse-phase HPLC.

#### 4.2.16. Testing the novobiocin-polymyxin chimeric molecules

HPLC purified chimeras **4,23** were compared with colistin **4,13** in a 16 hour minimal inhibitory concentration (MIC) assay<sup>297</sup> against *E. coli* 25922 (Table 4.2). The chimeras **4,23a** and **4,23b** possessed antibiotic activity against *E. coli*, although their performance is worse compared to the colistin control compound.

**Table 4.2. Minimal inhibitory concentrations (MIC) of novobiocin-polymyxin chimeras.** (16 hours, 37 °C).

| Compound               | <i>E. coli</i> 25922 MIC ( $\mu\text{M}$ ) |
|------------------------|--------------------------------------------|
| <b>4,13 (colistin)</b> | 0.25                                       |
| <b>4,23a</b>           | 7.0                                        |
| <b>4,23b</b>           | 3.7                                        |

Biochemical studies of the chimeras will need to be conducted (e.g. DNA supercoiling assay, LPS release assay, LptB<sub>2</sub>FGC ATPase assay) to elucidate specific mechanisms of action against protein targets.

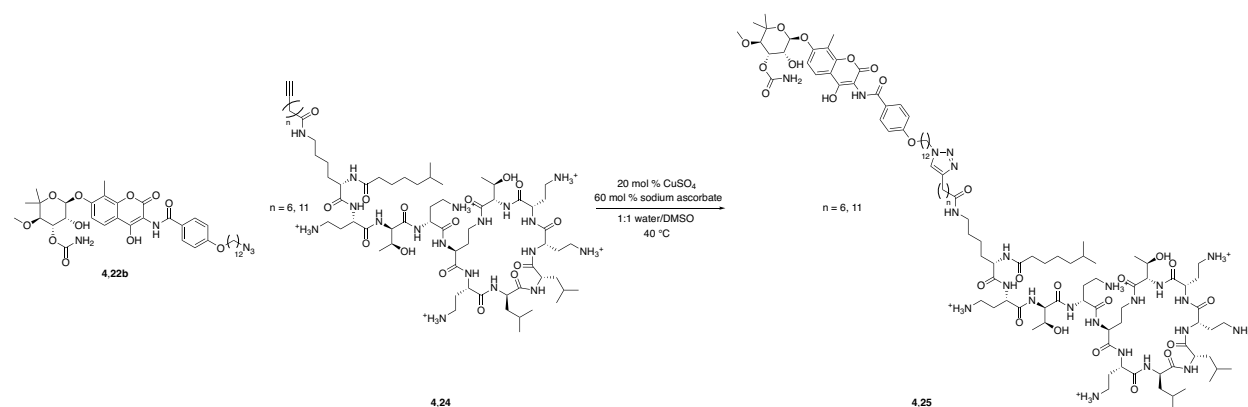
### 4.3. Discussion

This chapter began with the synthesis of novobiocin dimers and testing them in biochemical reconstitutions of LPS transport by LptB<sub>2</sub>FGC. Our results uncovered that novobiocin derivatives with hydrophobic linkers could dramatically stimulate LPS transport. These results coupled with our previous studies on antibiotic combinations<sup>320</sup> led us to propose the synthesis of a covalent combination of novobiocin and polymyxin. After many failed attempts at derivatizing polymyxin, a viable synthetic route to chimeric novobiocin-polymyxin molecules was outlined in the second half of the chapter. This bioenzymatic route, based on the route that BioSource Pharm<sup>342</sup> used to make semisynthetic colistin analogs, was found to be the most suitable for our purposes. Currently, the step involving the *Actinoplanes utahensis* NRRL-12052 deacylation of sulfmoc<sub>5</sub>colistin **4,16** remains a major bottleneck, since the hydrolase enzyme production by the producer is at native levels. If the enzyme (ECB deacylase<sup>345</sup>) were heterologously expressed, the efficiency of the conversion to deacylsulfmoc<sub>5</sub>colistin **4,17** could be dramatically improved, which would simplify the downstream purification procedure by eliminating telescoped byproducts. Alternatively, I plan on investigating whether the closely-related enzyme PvdQ<sup>346</sup> from *Pseudomonas aeruginosa* can hydrolyze the fatty acid amide of the polymyxins.

A novobiocin-polymyxin chimera could exhibit dramatic activity if the polymyxin interacts with LPS present in the cavity of LptB<sub>2</sub>FGC while the novobiocin binds LptB and sets the transporter in an activated state of LPS transport. With a viable synthetic route towards a chimera in hand, this project could become similar to that of a medicinal chemist's: various linker lengths will need to be explored empirically. Nevertheless, this does not mean we need to shoot in the

dark. With the knowledge of where LptB<sub>2</sub>FGC is accessible to polymyxin, we could design chimeras with the appropriate linker lengths to target both sites at once.

The chimeric molecules discussed in this Chapter, while lacking a mimic of the fatty acid tail in colistin, were the easiest to synthesize as a proof of concept. Pengxiang Shen and I are currently designing advanced linkers on both the colistin and novobiocin fragments with the aim of improving the activities of the chimeras (Figure 4.28).



**Figure 4.28. Future lysine-based chimeras 4,25.**

On the colistin **4,24**, the *N*-terminus of L-lysine will be acylated with 6-methylheptanoic acid and the side chain will be acylated with a terminal alkynoic acid ( $n = 6$  or  $11$ ). A novobiocin derivative **4,22b** will be prepared containing a 4-((12-azidododecyl)oxy)benzamide.

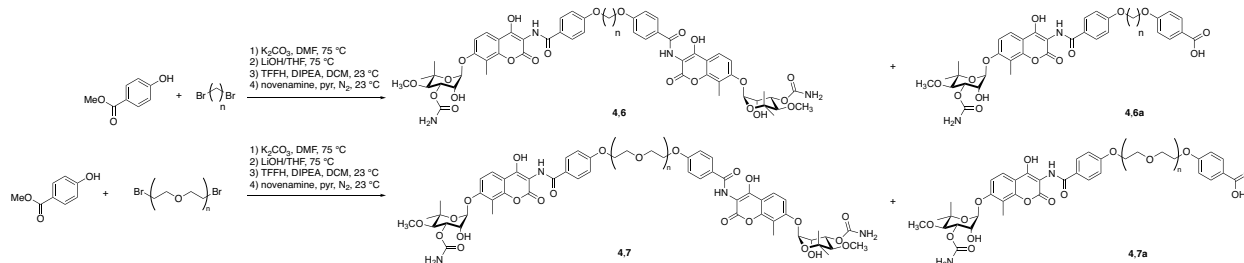
## 4.4. Materials and Methods

### 4.4.1. General synthetic methods

Unless otherwise noted, all reactions were performed under anhydrous N<sub>2</sub>. Solvents were purchased in Sure-Seal bottles from Sigma-Aldrich and used without further purification. Thin layer chromatography (TLC) was carried out using EM Science silica gel 60 F254 plates; the developed plate was analyzed by UV lamp (254 nm). Normal phase column chromatography was performed with the Teledyne-Isco CombiFlash R<sub>f</sub>200 system using pre-packed silica gel column cartridges and hexanes/ethyl acetate or dichloromethane/methanol as the solvent system. Reverse phase column chromatography was performed with the Teledyne-Isco CombiFlash R<sub>f</sub>

200 system using pre-packed C<sub>18</sub>Aq columns and water/acetonitrile as the solvent system. <sup>1</sup>H NMR and <sup>13</sup>C NMR spectra were recorded in DMSO-*d*<sub>6</sub> on a Varian Inova-500 MHz spectrometer unless otherwise noted. <sup>19</sup>F NMR spectra were recorded on a Bruker Avance II 600 MHz spectrometer equipped with a Prodigy liquid N<sub>2</sub> cooled cryoprobe. Chemical shifts are reported in ppm with the residual solvent signal as the reference and coupling constants (*J*) are given in hertz. The peak information is described as: br = broad singlet, s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet, comp = composite of overlapping signals. High-resolution mass spectra (HRMS) were performed on a Bruker Impact II q-TOF ESI LCMS system mass spectrometer using sodium formate as the standard.

#### 4.4.2. General synthesis of bis-benzoate alkane- and PEG-linked novobiocin dimers



Step 1) Methyl 4-hydroxybenzoate (2.5 equiv.), dibromide linker (1.0 equiv.), K<sub>2</sub>CO<sub>3</sub> (3.0 equiv.) were added to a 20 mL scintillation vial and DMF was added to a final [dibromide] of 0.2 M. The reaction mixture was stirred at 75 °C overnight and the bisester alkane-linked products precipitated as crystalline solids and could be filtered and triturated with cold EtOAc, 1M NaOH, and water. The bis-benzoate PEG-linked products were still in the DMF solution upon cooling. For these, the reaction mixture was quenched with equal volume 1M NaOH and extracted 3X with equal volumes of EtOAc. The organic layer was concentrated to give the bis-benzoate PEG-linked products.

Step 2) The bis-benzoate esters were saponified by dissolving them in 1:1 1M LiOH/THF for 3 h at 70 °C. Upon reaction completion, the reactions were neutralized with concentrated HCl

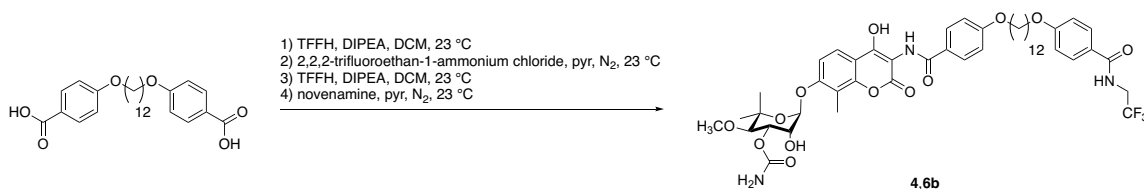
and the white solids were filtered, washed with distilled water, and dried under vacuum. Yields for Steps 1-2 (combined steps) for the alkane-linked bis-benzoic acids [n]: [3] = 16%, [4] = 55%, [5] = 46%, [6] = 11%, [7] = 8%, [8] = 70%, [9] = 27%, [10] = 61%. Yields for Steps 1-2 (combined steps) for the PEG-linked bis-benzoic acids [n]: [3] = 61%, [4] = 70%, [5] = 80%, [6] = 32%. Isolated yields should be excellent if no chromatography is used (initial purifications employed flash column chromatography which is unnecessary for the bis-benzoic acid linkers).

Step 3) The bis-benzoic acids were converted to their corresponding bis-benzoyl fluorides. Bis-benzoic acid (1.0 equiv.), TFFH (3.0 equiv.), and DCM ([bis-benzoic acid] = 0.1 M) were stirred in a 2-dram vial at room temperature. DIPEA (2.5 equiv.) was added and the reaction was stirred at room temperature for 1 h. An equal volume of water was added to quench the reaction and extract the water-soluble byproducts. The organic layer was washed with 3X equal volumes of water, dried with Na<sub>2</sub>SO<sub>4</sub>, and rotovapped extensively to afford the bis-benzoyl fluorides that were subsequently used without further purification.

Step 4) Novenamine hydrochloride (**4,5 • HCl**; 2.5 equiv.), whose preparation was described in Chapters 2-3 of this dissertation and bis-benzoyl fluoride (1.0 equiv.) were combined in a 1-dram glass vial equipped with a stir bar and septum cap. The cap was fitted with a vent needle and the mixture was sparged with N<sub>2</sub> for 5 minutes. Pyridine ([**4,5 • HCl**] = 0.1 M) was added to initiate the reaction (under N<sub>2</sub> pressure) and the vent needle was removed after 10 minutes of stirring. The mixture was stirred at room temperature for 24 h under nitrogen pressure. The next day, the reaction was concentrated by rotary evaporation and subjected to flash column chromatography (DCM/MeOH) monitoring the coumarin absorbance at 340 nm. Fractions containing the dimers or monomeric acids (byproduct) were concentrated by rotary evaporation.



#### 4.4.3. Synthesis of compound 4,6b (n = 12)



Steps 1-2) Bis-benzoic acid (1.0 equiv.), TFFH (1.1 equiv.), and DCM ([bis-benzoic acid] = 0.1 M) were stirred in a 2-dram vial at room temperature. DIPEA (1.1 equiv.) was added and the reaction was stirred at room temperature for 30 minutes. The solution went from clear to a white precipitate. 2,2,2-trifluoroethan-1-ammonium chloride (1.5 equiv.) and DIPEA (1.75 equiv.) were added. The reaction was stirred for 6 h total, concentrated by rotary evaporation, and subjected to flash column chromatography (DCM/MeOH). Fractions containing the benzoic acid-(CH<sub>2</sub>)<sub>12</sub>-benzoic amide product were concentrated by rotary evaporation.

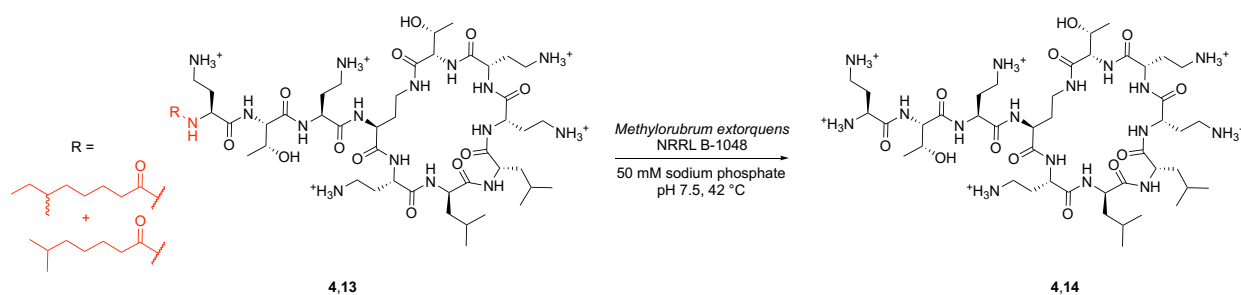
Steps 3-4) The benzoic acid-(CH<sub>2</sub>)<sub>12</sub>-benzoic amide compound (1.0 equiv.), TFFH (1.2 equiv.), and DCM (0.1 M) were stirred in a 2-dram vial at room temperature. DIPEA (2.5 equiv.) was added and the reaction was stirred at room temperature for 1 h. An equal volume of water was added to quench the reaction and extract the water-soluble byproducts. The organic layer was washed with 3X equal volumes of water, dried with Na<sub>2</sub>SO<sub>4</sub>, and rotovapped extensively to afford the benzoyl fluoride-(CH<sub>2</sub>)<sub>12</sub>-benzoic amide that was subsequently used without further purification. Novenamine hydrochloride (**4,5 • HCl**; 2.0 equiv.) and benzoyl fluoride-(CH<sub>2</sub>)<sub>12</sub>-benzoic amide (1.0 equiv.) were combined in a 1-dram glass vial equipped with a stir bar and septum cap. The cap was fitted with a vent needle and the mixture was sparged with N<sub>2</sub> for 5 minutes. Pyridine ([**4,5 • HCl**] = 0.1 M) was added to initiate the reaction (under N<sub>2</sub> pressure) and the vent needle was removed after 10 minutes of stirring. The mixture was stirred at room temperature for 24 h under nitrogen pressure. The next day, the reaction was concentrated by rotary evaporation and subjected to flash column chromatography (DCM/MeOH) monitoring the

coumarin absorbance at 340 nm. Fractions containing product were concentrated by rotary evaporation.

#### 4.4.4. Preparation of NRRL B-1048 cell powder

USDA NRRL B-1048 was grown in citrate medium (0.01% yeast extract, 1% sodium citrate, 0.2% ammonium sulfate, 0.1%  $K_2HPO_4$ , 0.05%  $MgSO_4 \cdot 7H_2O$ , 0.001%  $FeCl_3 \cdot 6H_2O$ , 0.001%  $CaCl_2$  at 30 °C, 250 rpm, for 3 days (4 x 15 mL cultures). By the third day, the OD > 1.0 and cells were harvested by centrifugation at 10,000 g x 10 min, 4 °C. The supernatant was discarded, and cells were washed with 10 mL of 50 mM sodium phosphate buffer, pH 7.5. The resuspended pellets were pooled and centrifuged at 10,000 g x 10 min, 4 °C, and the supernatant was discarded. The pellet was washed with 15 mL acetone at -20 °C and centrifuged at 10,000 g x 10 min, 4 °C and discarded. This step was repeated once and the pellet was dried and collected in a mason jar (yield = 50 mg per 60 mL cell culture).

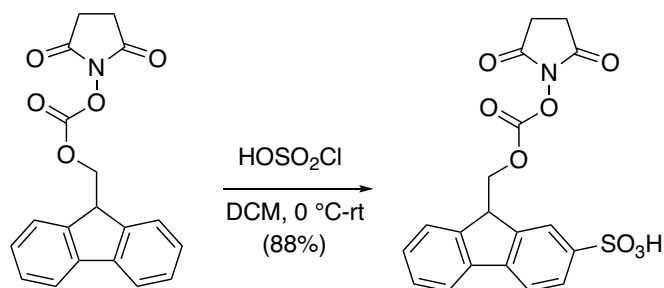
#### 4.4.5. Cleavage of colistin to deacylcolistin with NRRL B-1048 cell powder



USDA NRRL B-1048 dried cell powder or recently harvested cells were resuspended in a minimal volume of 50 mM sodium phosphate buffer, pH 7.5. A solution of colistin (2 g in 50 mL of 50 mM sodium phosphate buffer, pH 7.5) was mixed in a 150 mL plastic bottle with an equal volume of heavy cell suspension (final [colistin] = 10 mg/mL). The mixture was shaken at 200 rpm, 42 °C for 16 hours. The reaction mixture was analyzed by LC/MS for conversion. Upon reaction completion, the cells were centrifuged at 10,000 g x 10 min, 4 °C and the aqueous

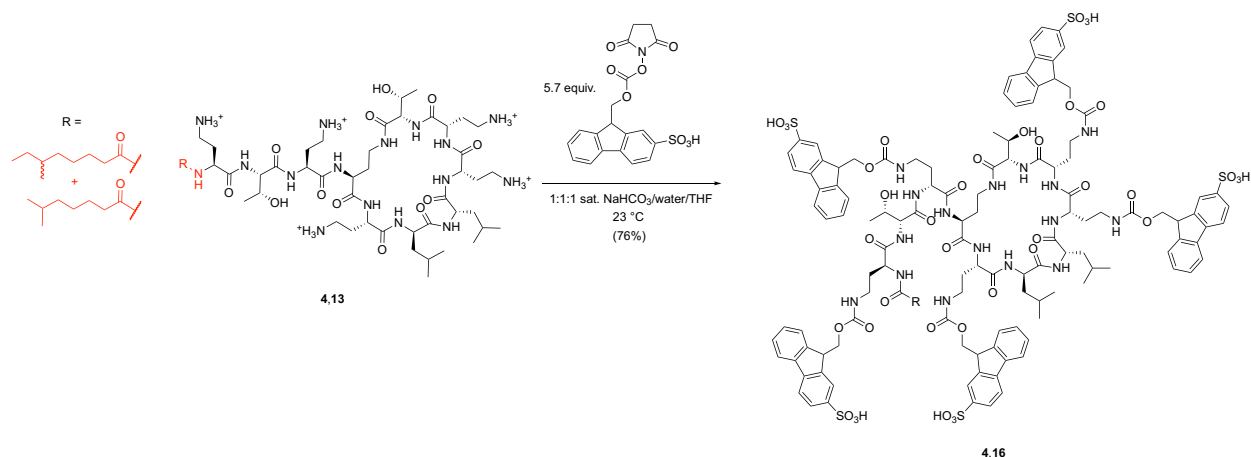
supernatant was discarded into a separatory funnel. The solution was adjusted to pH 1-2 using a few drops of concentrated hydrochloric acid to precipitate soluble extracellular proteins. The solution was centrifuged at 10,000 g x 10 min, 4 °C and the aqueous layer was extracted with an equal volume of EtOAc. Following another round of centrifugation at 10,000 g x 10 min, 4 °C, the organic layer was discarded and the aqueous layer was lyophilized to obtain the product as a yellow solid (1.6 g, 84%).

#### 4.4.6. Synthesis of sulfmoc NHS ester



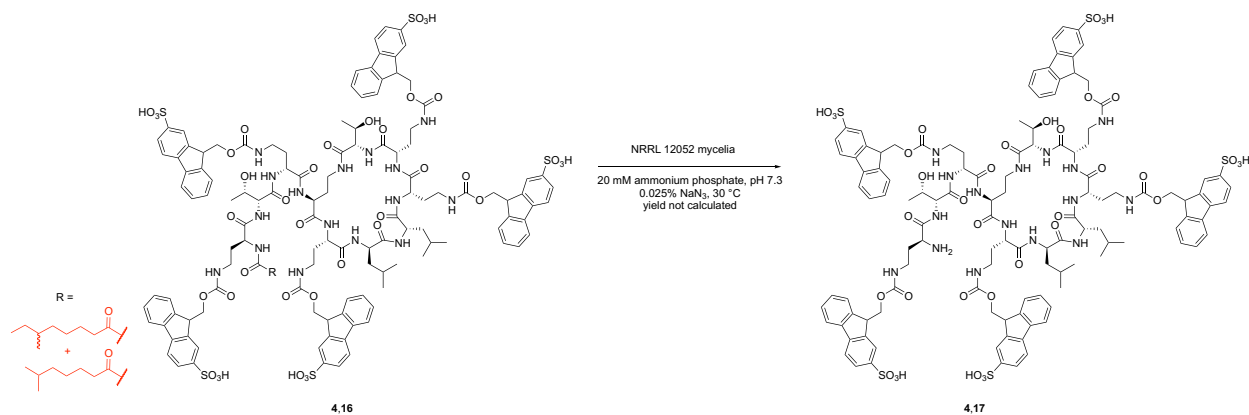
Original method modified from ref.<sup>343</sup> FmocOSu (8.89 mmol, 3.0 g) from Novabiochem (ensure that the starting material is a snow white pristine solid; do not use sticky or pale yellow material) was dissolved in ~ 40 mL DCM and cooled to 0 °C. Stirred for 5 min at 0 °C. Added a solution of chlorosulfonic acid (0.53 mL dissolved in 15 mL DCM). The solution turned slightly yellow during this addition. Stirred for 3 min at 0 °C and then warmed to room temperature for 2.5 h. Filtered white solid using vacuum filter fritted funnel and washed with 1:1 DCM/cyclohexane. Vacuum dried to obtain 3.3 g (89%) of white solid.

#### 4.4.7. Synthesis of sulfmoc<sub>5</sub>colistin 4,16



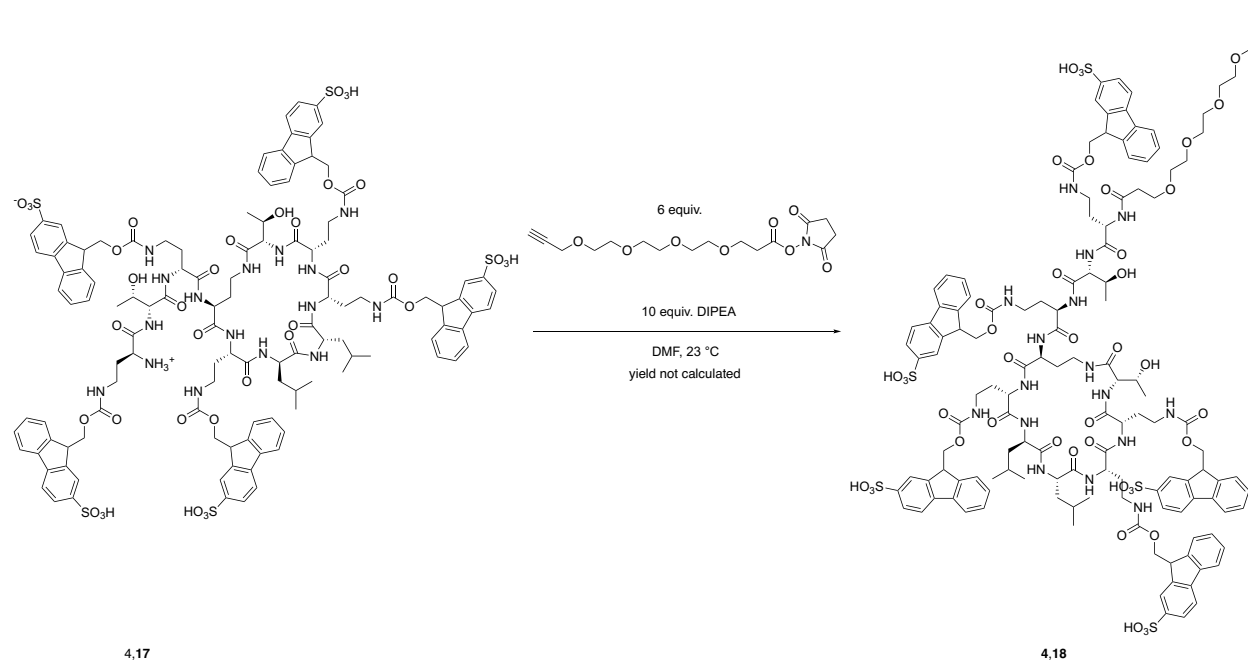
Method modified from ref.<sup>342</sup> Colistin sulfate (580 mg, 0.494 mmol, ChemImpex) was dissolved in 21 mL of 1:1:1 sat. sodium bicarbonate/H<sub>2</sub>O/THF in a 100 mL round bottom flask capped with a rubber septum and N<sub>2</sub> balloon vent. A solution of sulfmoc<sub>5</sub>NHS ester (1175 mg, 2.82 mmol) in 15 mL THF was added over the course of 3 h by a syringe pump (rate 5 mL/h) while stirring at 23 °C. Stirred for >16 h since beginning of injection (overnight at room temperature). Quenched with 30 mL H<sub>2</sub>O. Acidified with 12N HCl to pH 0.5; a white precipitate formed. Cooled for 1 h at 0 °C and then centrifuged suspension at 12,000 g x 10 min x 4 °C to separate layers. Discarded top aqueous layer by vacuum suction and redissolved the oil in a minimal volume of EtOH and rotovapped. Triturated solid with EtOAc and dried. Yield 1.01 g (76%) of a tan or off-white powder.

#### 4.4.8. Synthesis of deacylsulfmoc<sub>5</sub>colistin 4,17



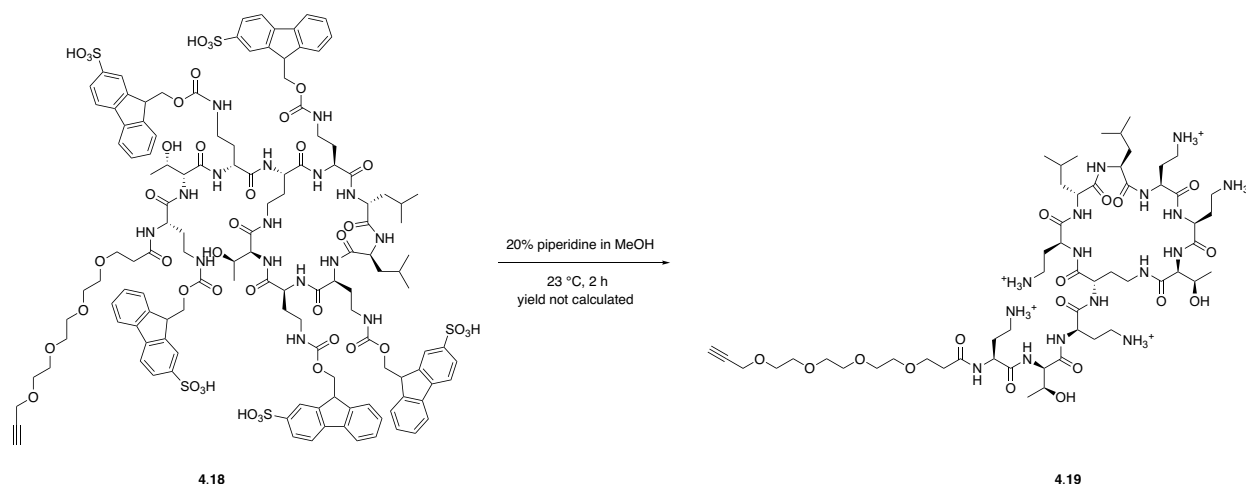
To a 50 mL Falcon tube was added 40 mg of sulfmoc<sub>5</sub>colistin, 100-500 mg of NRRL B-12052 orange mycelia (depending on quality of enzyme production), 40 mL 20 mM ammonium phosphate pH 7.3 + 0.025% NaN<sub>3</sub> was added and the mixture was shaken at 30 °C for 16-24 h. The cells were centrifuged at 12,000 g, 10 min, 4 °C and concentrated HCl was added to the supernatant until pH < 1.0 (warning, hydrazoic acid). White product precipitated and this material was recentrifuged to pellet it. The acidic supernatant was discarded and the pellet was redissolved in a minimal volume of MeOH and rotovapped. The recovered mass approximately equaled the mass of sulfmoc<sub>5</sub>colistin, but the yield could not be accurately determined since the material is contaminated with unreacted starting material, which could not be separated at this stage by HPLC.

#### 4.4.9. Synthesis of *N*-acylated sulfmoc<sub>5</sub>colistin 4,18



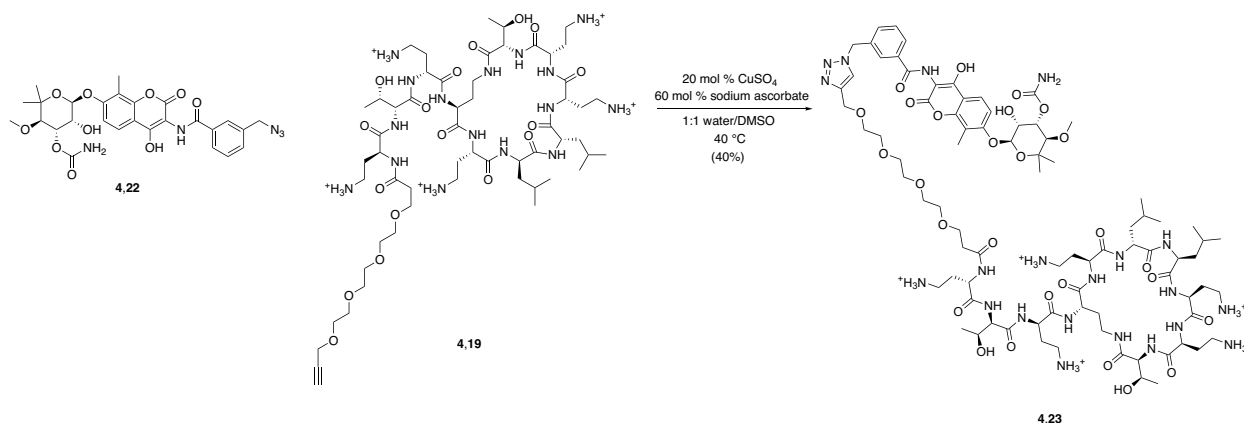
To a 2-dram vial was added crude deacylsulfmoc<sub>5</sub>colistin (13.7 mg, 0.005 mmol), 150  $\mu$ L DMF, and a solution of propargyl-PEG<sub>4</sub>-NHS ester in DMF (11.5 mg, 0.03 mmol, 6 equiv., 115  $\mu$ L of 100 mg/mL stock solution). The reaction was initiated by addition of DIPEA (6.96 mg, 0.05 mmol, 10 equiv., 9.4  $\mu$ L) and stirred vigorously for 2 h at room temperature. The mixture was transferred to a 15 mL Falcon centrifuge tube and 10 mL of water and 2 mL EtOAc were added. The aqueous layer was acidified to pH < 1 by the addition of drops of concentrated HCl. The mixture was vortexed and centrifuged at 10,000 g, 4 °C, 10 min, and both the aqueous and organic layers were discarded. The pellet was washed with EtOAc and resuspended. This suspension was re-centrifuged at 12,000 g, 4 °C, 10 min and the supernatant was discarded. The pellet was resuspended in MeOH and rotovapped to afford a brown solid product that was immediately used in the next step.

#### 4.4.10. Deprotection of *N*-acylated sulfmoc<sub>5</sub>colistin 4,18 to *N*-acylated colistin derivative 4,19



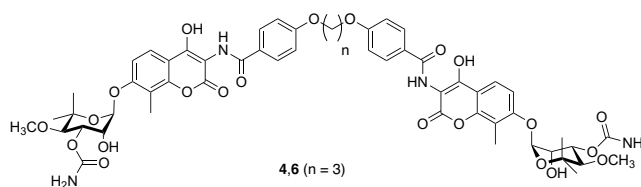
Protected propargyl-PEG<sub>4</sub>-colistin was dissolved in 1 mL of 20% piperidine in MeOH and stirred for 2 h at 23 °C. Over this time, the reaction mixture became cloudy. Once complete, the reaction was concentrated by rotary evaporation and the crude material was dissolved in 1.0 mL of water. 1.0 mL of crude reaction mixture was placed on the bed of an equilibrated PD MidiTrap G-10 column (GE) and once the solution entered the column an additional 0.7 mL water was added to the top. The column was eluted with 3 x 0.6 mL water into preweighed 1.5 mL Eppendorf tubes. The size-exclusion step was necessary to remove the sulfodibenzofulvene-piperidine adduct and other impurities. The tubes were flash frozen and lyophilized overnight to afford 3 mg (44%, over two steps) of desalted and deprotected material.

#### 4.4.11. Copper(I)-catalyzed azide-alkyne cycloaddition between **4,19** and **4,22**



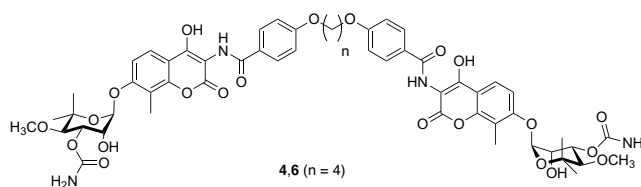
To an Eppendorf tube containing lyophilized propargyl-PEG<sub>4</sub>-colistin **4,19** (1.23 mg, 0.0008 mmol) was added a stock solution of novobiocin-azide derivative **4,22** (0.4 mg, 0.0007 mmol, 0.8 equiv., 4.0  $\mu\text{L}$  of 100 mg/mL solution in DMSO),  $\text{CuSO}_4$  (0.0002 mmol, 0.2 equiv., 2.7  $\mu\text{L}$  of a 10 mg/mL solution in  $\text{H}_2\text{O}$ ), DMSO was added (14  $\mu\text{L}$ ) to make the final DMSO:water ratio = 1. The reaction was initiated by the addition of sodium L-ascorbate (0.0005 mmol, 0.6 eq., 5.0  $\mu\text{L}$  of a 20 mg/mL solution in  $\text{H}_2\text{O}$ ). The reaction concentration with respect to the alkyne was approximately 24 mM. The reaction was shaken at 40 °C, 1000 rpm, for 14 h. Once the reaction was complete, it was centrifuged at 16.1 g x 1.0 min and the supernatant was injected into a reverse-phase C<sub>18</sub> HPLC Phenomenex Luna column (5  $\mu\text{M}$ , 100Å particle size) preequilibrated at 100%  $\text{H}_2\text{O}$  + 0.1% formic acid and eluted with a gradient of MeCN + 0.1% formic acid (0% to 80% over 20 min; 3 mL/min) and the product **4,23** was found between 13-17 min. Fractions containing the material were rotovapped and hi-vaced to afford the product as a white solid (0.64 mg, 40%).

#### 4.4.12. Characterization of alkane-linked novobiocin dimers **4,6**

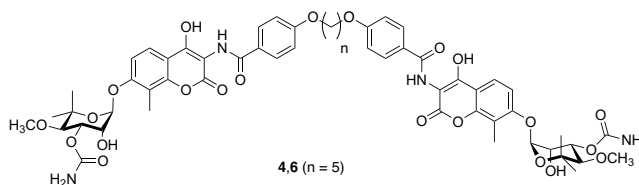




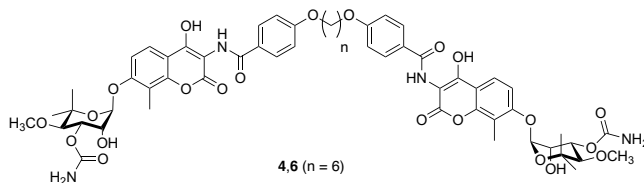
**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-(propane-1,3-diylbis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2,4-diyl) dicarbamate (4,6; n = 3).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.35 (s, 1H), 8.57 (s, 1H), 7.98 (comp, 2H), 7.89 (comp, 2H), 7.76 (comp, 2H), 7.67 – 7.58 (m, 1H), 7.45 – 7.30 (m, 1H), 7.16 (comp, 2H), 7.05 (comp, 5H), 6.63 (comp, 4H), 5.65 – 5.58 (m, 2H), 5.53 (dd, *J* = 7.2, 2.5 Hz, 2H), 5.15 (comp, 2H), 4.24 (comp, 5H), 4.08 (comp, 2H), 3.47 (comp, 8H), 2.22 (comp, 6H), 1.26 (comp, 6H), 1.05 (comp, 6H); HRMS (ESI Neg) *m/z* calculated for C<sub>55</sub>H<sub>59</sub>N<sub>4</sub>O<sub>22</sub> [M–H]<sup>–</sup>, 1127.3621 found: 1127.3630.



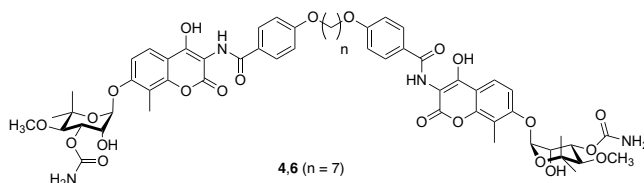
**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-(butane-1,4-diylbis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2,4-diyl) dicarbamate (4,6; n = 4).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.34 (s, 1H), 8.58 (s, 1H), 7.98 (comp, 2H), 7.82 (comp, 2H), 7.73 (comp, 2H), 7.38 (dd, *J* = 7.5, 5.5 Hz, 1H), 7.16 (dd, *J* = 9.2, 2.2 Hz, 2H), 7.06 (comp, 3H), 7.02 – 6.95 (m, 1H), 6.67 (comp, 4H), 5.59 (dd, *J* = 5.4, 2.7 Hz, 2H), 5.53 (dd, *J* = 7.2, 2.5 Hz, 2H), 5.23 – 5.08 (m, 2H), 4.20 – 4.02 (m, 7H), 3.49 – 3.45 (m, 8H), 2.21 (comp, 6H), 1.92 (comp, 4H), 1.26 (comp, 6H), 1.05 (comp, 6H); HRMS (ESI Neg) *m/z* calculated for C<sub>56</sub>H<sub>61</sub>N<sub>4</sub>O<sub>22</sub> [M–H]<sup>–</sup>, 1141.3785 found: 1141.3776.



**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-(pentane-1,5-diylbis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2,4-diyl)dicarbamate (4,6; n = 5).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.33 (s, 1H), 7.98 (d, *J* = 8.7 Hz, 3H), 7.90 – 7.76 (m, 1H), 7.73 (comp, 2H), 7.15 (d, *J* = 9.0 Hz, 2H), 7.05 (comp, 3H), 6.62 (comp, 4H), 5.59 (d, *J* = 5.1 Hz, 2H), 5.52 (dd, *J* = 6.1, 2.5 Hz, 2H), 5.19 – 5.11 (m, 2H), 4.15 – 4.01 (m, 6H), 3.47 (p, *J* = 3.4 Hz, 10H), 2.21 (comp, 6H), 1.83 (q, *J* = 6.7 Hz, 3H), 1.67 – 1.53 (m, 2H), 1.26 (comp, 7H), 1.15 (d, *J* = 6.2 Hz, 2H), 1.05 (comp, 6H); HRMS (ESI Neg) *m/z* calculated for C<sub>57</sub>H<sub>63</sub>N<sub>4</sub>O<sub>22</sub> [M–H]<sup>–</sup>, 1155.3939 found: 1155.3935.



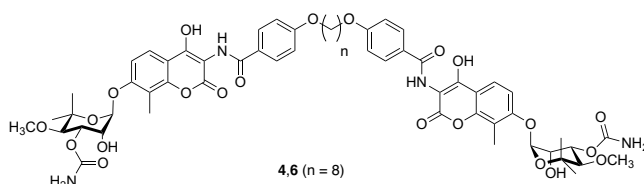
**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-(hexane-1,6-diylbis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2,4-diyl)dicarbamate (4,6; n = 6).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.30 (s, 1H), 7.97 (d, *J* = 8.4 Hz, 3H), 7.86 (comp, 2H), 7.72 (d, *J* = 8.9 Hz, 2H), 7.14 (dd, *J* = 9.2, 3.3 Hz, 2H), 7.00 (comp, 4H), 6.68 (comp, 4H), 5.56 (comp, 3H), 5.16 (dd, *J* = 9.8, 3.3 Hz, 2H), 4.07 (comp, 6H), 3.47 (comp, 8H), 2.21 (s, 6H), 1.79 (comp, 5H), 1.50 (comp, 5H), 1.27 (comp, 6H), 1.07 (comp, 6H), 0.90 – 0.77 (m, 1H); HRMS (ESI Neg) *m/z* calculated for C<sub>58</sub>H<sub>65</sub>N<sub>4</sub>O<sub>22</sub> [M–H]<sup>–</sup>, 1169.4096 found: 1169.4095.



**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-(heptane-1,7-diylbis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-**

**diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2,4-diyl)**

**dicarbamate (4,6; n = 7).**  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.55 (s, 1H), 9.32 (s, 1H), 7.97 (d,  $J$  = 8.4 Hz, 1H), 7.72 (dd,  $J$  = 9.0, 6.2 Hz, 2H), 7.15 (d,  $J$  = 9.0 Hz, 2H), 7.03 (d,  $J$  = 8.3 Hz, 1H), 6.62 (comp, 4H), 5.63 – 5.56 (m, 2H), 5.56 – 5.48 (m, 2H), 5.19 – 5.08 (m, 2H), 4.06 (comp, 5H), 3.47 (comp, 10H), 2.21 (comp, 6H), 1.76 (comp, 1H), 1.45 (comp, 3H), 1.26 (comp, 7H), 1.15 (comp, 4H), 1.05 (comp, 7H), 0.95 – 0.76 (m, 1H) five protons obscured by residual solvent signal; HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{59}\text{H}_{67}\text{N}_4\text{O}_{22}$   $[\text{M}-\text{H}]^-$ , 1183.4252 found: 1183.4254.

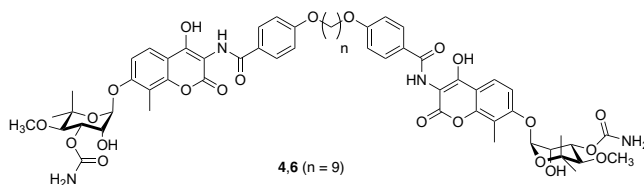


**(2R,2'R,3R,3'R,4S,4'S,5R,5'R)-((((4,4'-(octane-1,8-**

**diylbis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2H-chromene-3,7-**

**diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2,4-diyl)**

**dicarbamate (4,6; n = 8).**  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.34 (s, 1H), 7.97 (comp, 3H), 7.77 (comp, 3H), 7.16 (d,  $J$  = 9.1 Hz, 2H), 7.02 (comp, 4H), 6.63 (comp, 4H), 5.65 – 5.57 (m, 2H), 5.53 (dd,  $J$  = 6.8, 2.5 Hz, 2H), 5.19 – 5.11 (m, 2H), 4.07 (comp, 7H), 3.47 (comp, 9H), 3.17 (s, 2H), 2.21 (comp, 6H), 1.75 (comp, 3H), 1.40 (comp, 7H), 1.26 (comp, 6H), 1.15 (d,  $J$  = 6.2 Hz, 1H), 1.05 (comp, 6H); HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{60}\text{H}_{69}\text{N}_4\text{O}_{22}$   $[\text{M}-\text{H}]^-$ , 1197.4403 found: 1197.4408.

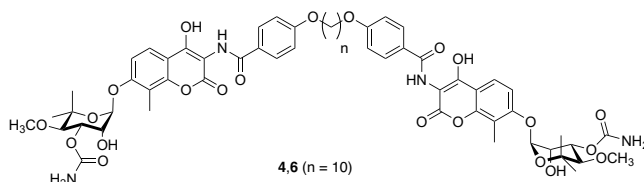


**(2R,2'R,3R,3'R,4S,4'S,5R,5'R)-((((4,4'-(nonane-1,9-**

**diylbis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2H-chromene-3,7-**

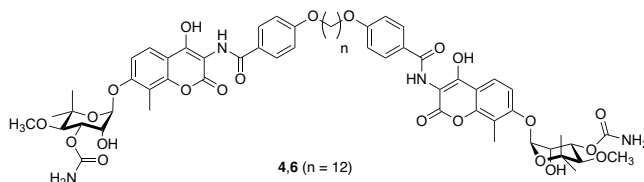
**diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2,4-diyl)**

**dicarbamate (4,6; n = 9).**  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.58 (s, 1H), 9.34 (s, 1H), 8.66 – 8.49 (m, 1H), 7.97 (d,  $J$  = 8.3 Hz, 2H), 7.91 – 7.78 (m, 1H), 7.73 (comp, 2H), 7.39 (t,  $J$  = 6.3 Hz, 1H), 7.16 (d,  $J$  = 9.0 Hz, 2H), 7.01 (comp, 3H), 6.62 (comp, 4H), 5.64 – 5.56 (m, 2H), 5.53 (d,  $J$  = 7.0 Hz, 2H), 5.15 (comp, 2H), 4.06 (comp, 6H), 3.47 (comp, 9H), 2.21 (comp, 7H), 1.73 (comp, 3H), 1.38 (comp, 7H), 1.26 (comp, 7H), 1.15 (d,  $J$  = 6.1 Hz, 2H), 1.05 (comp, 7H); HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{61}\text{H}_{71}\text{N}_4\text{O}_{22}$   $[\text{M}-\text{H}]^-$ , 1211.4565 found: 1211.4560.



**(2R,2'R,3R,3'R,4S,4'S,5R,5'R)-((((4,4'-(decane-1,10-diylbis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2H-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2,4-diyl)**

**dicarbamate (4,6; n = 10).**  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.31 (s, 1H), 7.97 (d,  $J$  = 8.4 Hz, 2H), 7.73 (t,  $J$  = 8.1 Hz, 2H), 7.15 (d,  $J$  = 9.0 Hz, 2H), 7.03 (d,  $J$  = 8.6 Hz, 2H), 6.65 (comp, 4H), 5.59 (dd,  $J$  = 5.3, 2.0 Hz, 2H), 5.52 (dd,  $J$  = 6.5, 2.5 Hz, 2H), 5.24 – 5.09 (m, 2H), 4.68 – 4.57 (m, 1H), 4.06 (comp, 6H), 3.47 (comp, 10H), 2.23 (comp, 7H), 1.73 (comp, 2H), 1.43 (comp, 3H), 1.33 (comp, 6H), 1.26 (comp, 8H), 1.15 (d,  $J$  = 6.2 Hz, 2H), 1.07 (comp, 9H), 0.95 – 0.78 (m, 1H); HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{62}\text{H}_{73}\text{N}_4\text{O}_{22}$   $[\text{M}-\text{H}]^-$ , 1225.4722 found: 1225.4717.

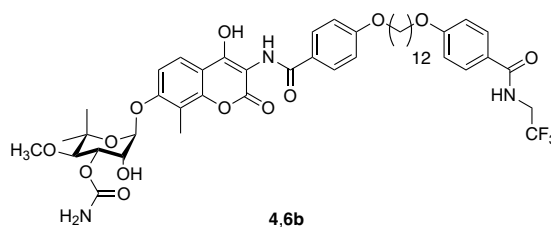


**(2R,2'R,3R,3'R,4S,4'S,5R,5'R)-((((4,4'-(dodecane-1,12-diylbis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2H-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2,4-diyl)**

**dicarbamate (4,6; n = 12).**  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.34 (s, 2H), 7.97 (d,  $J$  = 8.3 Hz, 4H),

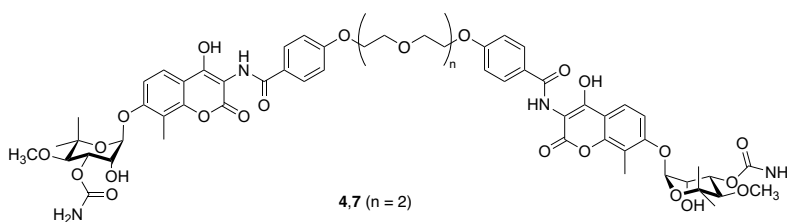
7.74 (d,  $J = 8.8$  Hz, 2H), 7.17 (d,  $J = 9.0$  Hz, 2H), 7.03 (d,  $J = 8.3$  Hz, 4H), 6.62 (comp, 4H), 5.60 (d,  $J = 5.1$  Hz, 2H), 5.56 – 5.48 (m, 2H), 5.16 (dd,  $J = 9.9, 3.1$  Hz, 2H), 4.06 (comp, 6H), 3.48 (comp, 8H), 2.23 (comp, 6H), 1.74 (comp, 4H), 1.43 (comp, 4H), 1.30 (comp, 20H), 1.06 (comp, 6H); HRMS (ESI Neg)  $m/z$  calculated for  $C_{64}H_{77}N_4O_{22}$   $[M-H]^-$ , 1253.5035 found: 1253.5037.

#### 4.4.13. Characterization of 4,6b

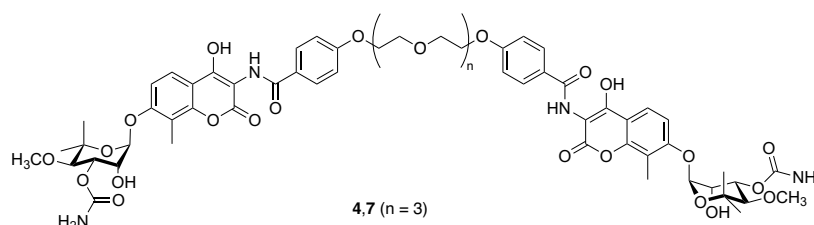


***z*(3*R*,4*S*,5*R*,6*R*)-5-hydroxy-6-((4-hydroxy-8-methyl-2-oxo-3-(4-((12-(4-((2,2,2-trifluoroethyl)carbamoyl)phenoxy)dodecyl)oxy)benzamido)-2*H*-chromen-7-yl)oxy)-3-methoxy-2,2-dimethyltetrahydro-2*H*-pyran-4-yl carbamate (4,6b,  $n = 12$ ).**  $^1H$  NMR (400 MHz,  $DMSO-d_6$ )  $\delta$  9.32 (s, 1H), 8.89 (t,  $J = 6.3$  Hz, 1H), 7.97 (d,  $J = 8.4$  Hz, 2H), 7.85 (d,  $J = 8.6$  Hz, 2H), 7.73 (d,  $J = 8.8$  Hz, 1H), 7.16 (d,  $J = 9.0$  Hz, 1H), 7.01 (t,  $J = 8.4$  Hz, 4H), 6.68 (comp, 2H), 5.59 (d,  $J = 5.2$  Hz, 1H), 5.53 (d,  $J = 2.5$  Hz, 1H), 5.16 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.04 (comp, 7H), 3.48 (comp, 4H), 2.69 (s, 1H), 2.22 (s, 3H), 1.72 (comp, 4H), 1.41 (comp, 4H), 1.29 (comp, 17H), 1.23 (s, 3H), 1.06 (s, 3H);  $^{19}F$  NMR (376 MHz,  $DMSO-d_6$ )  $\delta$  -70.36 (t,  $J = 9.9$  Hz); HRMS (ESI Neg)  $m/z$  calculated for  $C_{47}H_{57}F_3N_3O_{13}$   $[M-H]^-$ , 928.3843 found: 928.3852.

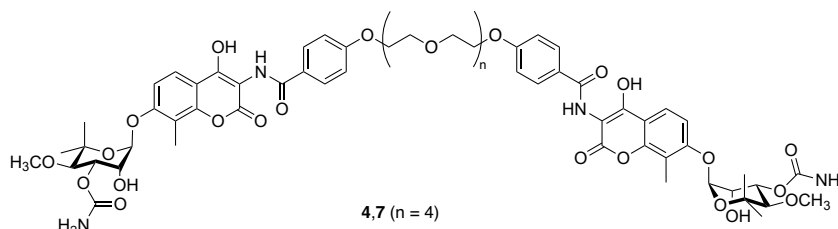
#### 4.4.14. Characterization of PEG-linked novobiocin dimers 4,7



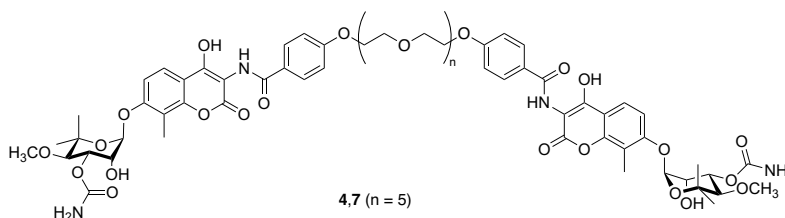
**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-(((ethane-1,2-diylbis(oxy)))bis(ethane-2,1-diyl)))bis(oxy)))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2,4-diyl) dicarbamate (4,7; *n* = 2).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.35 (s, 2H), 8.65 – 8.49 (m, 1H), 7.98 (d, *J* = 8.4 Hz, 4H), 7.74 (d, *J* = 8.9 Hz, 2H), 7.16 (d, *J* = 9.1 Hz, 2H), 7.06 (d, *J* = 8.5 Hz, 4H), 6.63 (comp, 4H), 5.59 (d, *J* = 5.2 Hz, 2H), 5.53 (d, *J* = 2.5 Hz, 2H), 5.16 (dd, *J* = 9.8, 3.1 Hz, 2H), 4.26 – 4.16 (m, 4H), 4.10 – 4.06 (m, 2H), 3.80 (comp, 4H), 3.64 (comp, 5H), 3.48 (comp, 8H), 2.22 (s, 6H), 1.27 (s, 6H), 1.05 (s, 6H); HRMS (ESI Neg) *m/z* calculated for C<sub>58</sub>H<sub>65</sub>N<sub>4</sub>O<sub>24</sub> [M–H]<sup>–</sup>, 1201.3994 found: 1201.3991.



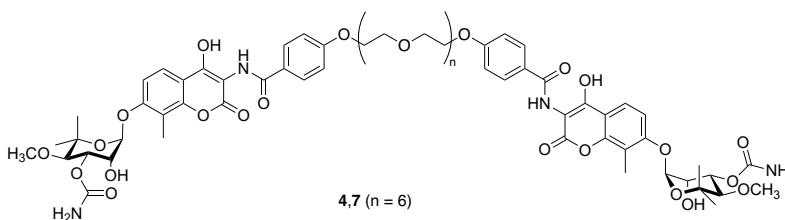
**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-(((oxybis(ethane-2,1-diyl))bis(oxy)))bis(ethane-2,1-diyl)))bis(oxy)))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2,4-diyl) dicarbamate (4,7; *n* = 3).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.32 (s, 1H), 8.57 (d, *J* = 4.8 Hz, 1H), 7.97 (d, *J* = 8.4 Hz, 2H), 7.91 – 7.68 (m, 4H), 7.38 (dd, *J* = 7.8, 5.2 Hz, 1H), 7.14 (comp, 2H), 7.01 (comp, 4H), 6.65 (comp, 4H), 5.59 (d, *J* = 5.1 Hz, 2H), 5.55 – 5.49 (m, 2H), 5.19 – 5.11 (m, 2H), 4.16 (comp, 4H), 4.07 (comp, 2H), 3.77 (comp, 4H), 3.59 (comp, 9H), 3.47 (comp, 10H), 2.21 (comp, 6H), 1.26 (comp, 6H), 1.15 (d, *J* = 6.2 Hz, 2H), 1.05 (comp, 6H); HRMS (ESI Neg) *m/z* calculated for C<sub>60</sub>H<sub>69</sub>N<sub>4</sub>O<sub>25</sub> [M–H]<sup>–</sup>, 1245.4256 found: 1245.4255.



**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-((3,6,9,12-tetraoxatetradecane-1,14-diyl))bis(oxy)))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2,4-diyl) dicarbamate (4,7; *n* = 4).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.28 (s, 1H), 8.57 (comp, 2H), 7.97 (comp, 2H), 7.74 (comp, 3H), 7.38 (comp, 2H), 7.13 (comp, 2H), 7.04 (comp, 3H), 6.64 (comp, 4H), 5.62 – 5.54 (m, 2H), 5.54 – 5.47 (m, 2H), 5.15 (dd, *J* = 9.8, 3.9 Hz, 2H), 4.23 – 4.02 (m, 6H), 3.77 (d, *J* = 5.8 Hz, 4H), 3.53 (comp, 22H), 2.21 (s, 6H), 1.26 (s, 6H), 1.17 – 1.10 (m, 2H), 1.05 (s, 6H) note integration at 3.53 ppm skewed by residual water peak; HRMS (ESI Neg) *m/z* calculated for C<sub>62</sub>H<sub>73</sub>N<sub>4</sub>O<sub>26</sub> [M–H]<sup>–</sup>, 1289.4519 found: 1289.4514.

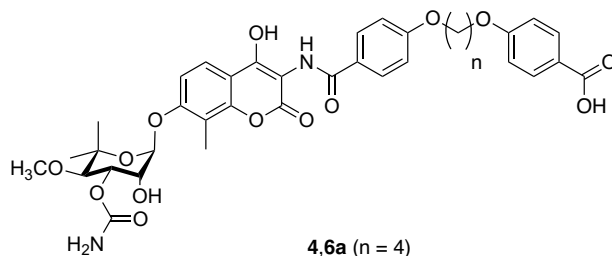


**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-((3,6,9,12,15-pentaoxaheptadecane-1,17-diyl))bis(oxy)))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2,4-diyl) dicarbamate (4,7; *n* = 5).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.33 (s, 2H), 7.97 (d, *J* = 8.4 Hz, 4H), 7.73 (d, *J* = 8.9 Hz, 2H), 7.15 (d, *J* = 9.0 Hz, 2H), 7.05 (d, *J* = 8.5 Hz, 4H), 6.67 (comp, 4H), 5.59 (d, *J* = 5.1 Hz, 2H), 5.53 (d, *J* = 2.5 Hz, 2H), 5.20 – 5.10 (m, 2H), 4.20 – 4.14 (m, 4H), 4.11 – 4.05 (m, 2H), 3.77 (comp, 4H), 3.60 (comp, 4H), 3.54 (comp, 13H), 3.48 (comp, 9H), 2.22 (s, 6H), 1.26 (s, 6H), 1.06 (s, 6H); HRMS (ESI Neg) *m/z* calculated for C<sub>64</sub>H<sub>77</sub>N<sub>4</sub>O<sub>27</sub> [M–H]<sup>–</sup>, 1333.4781 found: 1333.4772.



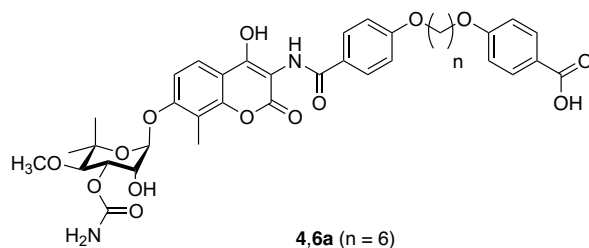
**(2*R*,2'*R*,3*R*,3'*R*,4*S*,4'*S*,5*R*,5'*R*)-((((4,4'-((3,6,9,12,15,18-hexaoxaicosane-1,20-diyl))bis(oxy))bis(benzoyl))bis(azanediyl))bis(4-hydroxy-8-methyl-2-oxo-2*H*-chromene-3,7-diyl))bis(oxy))bis(3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2,4-diyl) dicarbamate (4,7; n = 6).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.36 (s, 2H), 7.98 (d, *J* = 8.4 Hz, 3H), 7.85 (comp, 3H), 7.74 (d, *J* = 8.9 Hz, 2H), 7.17 (d, *J* = 9.1 Hz, 2H), 7.03 (comp, 7H), 6.64 (comp, 4H), 5.59 (d, *J* = 5.2 Hz, 2H), 5.54 (d, *J* = 2.5 Hz, 2H), 5.16 (dd, *J* = 9.8, 3.1 Hz, 2H), 4.13 (comp, 9H), 3.76 (comp, 7H), 3.59 (comp, 7H), 3.52 (comp, 29H), 3.47 (comp, 7H), 2.22 (s, 6H), 1.27 (s, 6H), 1.06 (s, 6H) integration skewed due to overabundance of PEG methylene signals at 3.52 ppm; HRMS (ESI Neg) *m/z* calculated for C<sub>66</sub>H<sub>81</sub>N<sub>4</sub>O<sub>28</sub> [M-H]<sup>-</sup>, 1377.5043 found: 1377.5036.

#### 4.4.15. Characterization of alkane-linked novobiocin monomer acids 4,6a

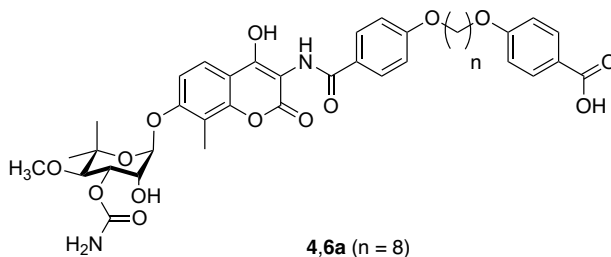


**4-(4-(4-((7-(((2*R*,3*R*,4*S*,5*R*)-4-(carbamoyloxy)-3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2-yl)oxy)-4-hydroxy-8-methyl-2-oxo-2*H*-chromen-3-yl)carbamoyl)phenoxy)butoxy)benzoic acid (4,6a; n = 4).** <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>) δ 9.32 (s, 1H), 8.57 (s, 1H), 7.97 (d, *J* = 8.7 Hz, 1H), 7.82 (comp, 4H), 7.45 – 7.32 (m, 1H), 7.15 (d, *J* = 8.9 Hz, 1H), 7.02 (comp, 4H), 6.60 (comp, 2H), 5.60 (d, *J* = 5.2 Hz, 1H), 5.56 – 5.48 (m, 1H), 5.16 (dd, *J* = 9.9, 3.3 Hz, 1H), 4.11 (comp, 5H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.90 (comp, 4H), 1.26 (s, 3H), 1.06 (s, 3H); HRMS (ESI Neg) *m/z* calculated for C<sub>37</sub>H<sub>39</sub>N<sub>2</sub>O<sub>14</sub> [M-H]<sup>-</sup>, 735.2407 found: 735.2405.

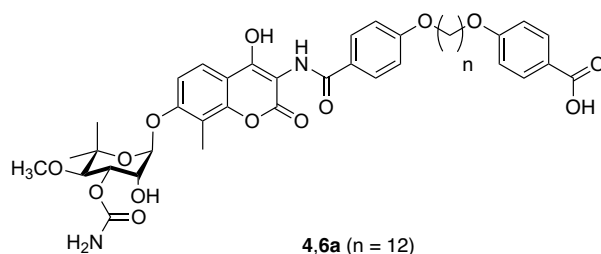




**4-((6-(4-((7-(((2R,3R,4S,5R)-4-(carbamoyloxy)-3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-4-hydroxy-8-methyl-2-oxo-2H-chromen-3-yl)carbamoyl)phenoxy)hexyl)oxy)benzoic acid (4,6a; n = 6).**  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.32 (d,  $J$  = 11.0 Hz, 1H), 7.98 (comp, 2H), 7.89 (comp, 2H), 7.75 (t,  $J$  = 11.1 Hz, 1H), 7.17 (t,  $J$  = 10.9 Hz, 1H), 7.03 (comp, 4H), 6.65 (comp, 2H), 5.54 (comp, 2H), 5.22 – 5.10 (m, 1H), 4.07 (comp, 5H), 3.48 (comp, 4H), 2.23 (comp, 4H), 1.78 (comp, 4H), 1.51 (comp, 5H), 1.28 (comp, 3H), 1.07 (comp, 3H); HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{39}\text{H}_{43}\text{N}_2\text{O}_{14}$   $[\text{M}-\text{H}]^-$ , 763.2720 found: 763.2720.

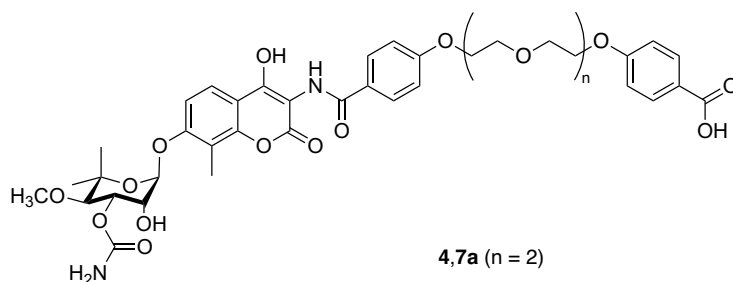


**4-((8-(4-((7-(((2R,3R,4S,5R)-4-(carbamoyloxy)-3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-4-hydroxy-8-methyl-2-oxo-2H-chromen-3-yl)carbamoyl)phenoxy)octyl)oxy)benzoic acid (4,6a; n = 8).**  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.33 (s, 1H), 7.97 (d,  $J$  = 8.3 Hz, 2H), 7.87 (d,  $J$  = 8.4 Hz, 2H), 7.74 (d,  $J$  = 8.8 Hz, 1H), 7.16 (d,  $J$  = 9.1 Hz, 1H), 7.02 (comp, 4H), 6.63 (comp, 2H), 5.60 (d,  $J$  = 5.1 Hz, 1H), 5.56 – 5.49 (m, 1H), 5.16 (dd,  $J$  = 9.9, 3.2 Hz, 1H), 4.05 (comp, 6H), 3.48 (comp, 4H), 2.22 (s, 3H), 1.74 (comp, 4H), 1.40 (comp, 9H), 1.27 (s, 3H), 1.06 (s, 3H); HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{41}\text{H}_{47}\text{N}_2\text{O}_{14}$   $[\text{M}-\text{H}]^-$ , 791.3033 found: 791.3034.



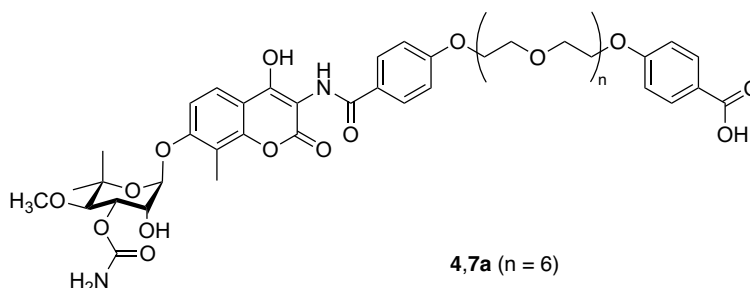
**4-((12-(4-((7-(((2*R*,3*R*,4*S*,5*R*)-4-(carbamoyloxy)-3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2-yl)oxy)-4-hydroxy-8-methyl-2-oxo-2*H*-chromen-3-yl)carbamoyl)phenoxy)dodecyl)oxy)benzoic acid (4,6a;  $n = 12$ ).**  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  9.11 (s, 1H), 7.95 (d,  $J = 8.3$  Hz, 2H), 7.86 (d,  $J = 8.1$  Hz, 2H), 7.70 (d,  $J = 8.8$  Hz, 1H), 7.09 (d,  $J = 8.9$  Hz, 1H), 6.99 (d,  $J = 8.3$  Hz, 4H), 6.62 (comp, 2H), 5.57 (d,  $J = 5.2$  Hz, 1H), 5.50 (s, 1H), 5.16 (d,  $J = 9.9$  Hz, 1H), 4.02 (comp, 5H), 3.47 (comp, 4H), 2.20 (s, 3H), 1.73 (comp, 4H), 1.41 (comp, 4H), 1.27 (comp, 15H), 1.08 (comp, 4H), 0.88 – 0.81 (m, 1H); HRMS (ESI Neg)  $m/z$  calculated for  $\text{C}_{45}\text{H}_{55}\text{N}_2\text{O}_{14}$   $[\text{M-H}]^-$ , 847.3659 found: 847.3660.

#### 4.4.16. Characterization of PEG-linked novobiocin monomer acids 4,7a



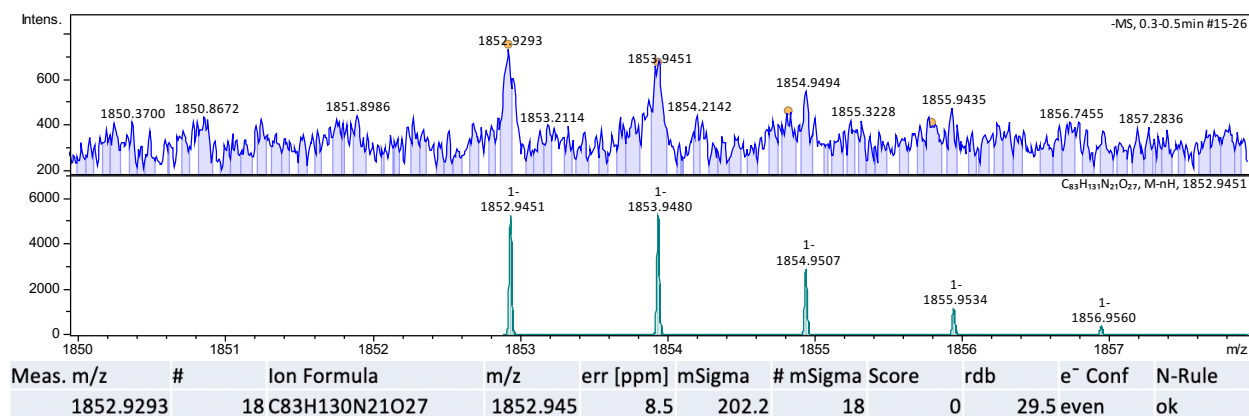
**4-(2-(2-(2-(4-((7-(((2*R*,3*R*,4*S*,5*R*)-4-(carbamoyloxy)-3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2*H*-pyran-2-yl)oxy)-4-hydroxy-8-methyl-2-oxo-2*H*-chromen-3-yl)carbamoyl)phenoxy)ethoxy)ethoxy)ethoxy)benzoic acid (4,7a;  $n = 2$ ).**  $^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.93 (s, 1H), 9.36 (s, 1H), 7.98 (d,  $J = 8.4$  Hz, 2H), 7.88 (d,  $J = 8.8$  Hz, 1H), 7.74 (d,  $J = 8.8$  Hz, 1H), 7.45 – 7.29 (m, 1H), 7.17 (d,  $J = 9.0$  Hz, 1H), 7.03 (comp, 4H), 6.64 (comp, 2H), 5.59 (d,  $J = 5.2$  Hz, 1H), 5.54 (d,  $J = 2.5$  Hz, 1H), 5.16 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.15 (comp, 5H),

3.78 (comp, 4H), 3.63 (comp, 4H), 3.48 (comp, 4H), 2.94 (s, 1H), 2.22 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H); HRMS (ESI Neg)  $m/z$  calculated for  $C_{39}H_{43}N_2O_{16}$   $[M-H]^-$ , 795.2618 found: 795.2616.

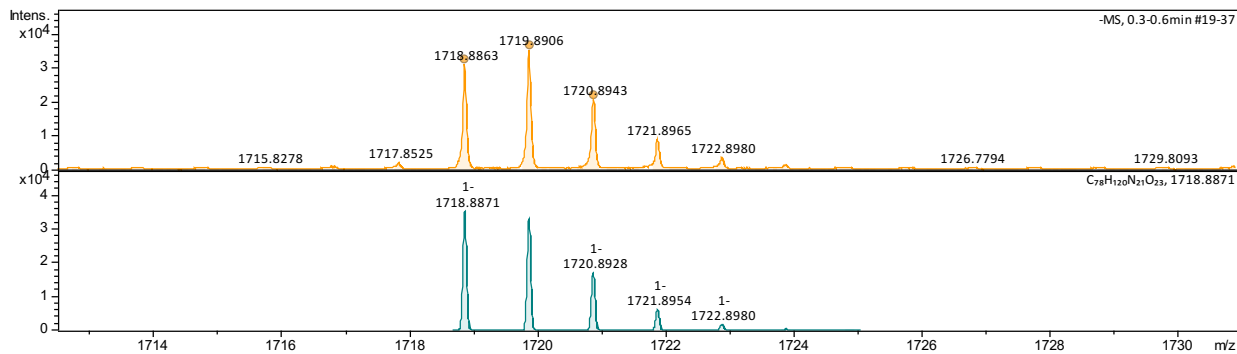


**4-((20-(4-((7-(((2R,3R,4S,5R)-4-(carbamoyloxy)-3-hydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-4-hydroxy-8-methyl-2-oxo-2H-chromen-3-yl)carbamoyl)phenoxy)-3,6,9,12,15,18-hexaoxaicosyl)oxy)benzoic acid (4,7a;  $n = 6$ ).**  $^1H$  NMR (400 MHz,  $DMSO-d_6$ )  $\delta$  11.92 (s, 1H), 9.36 (s, 1H), 7.98 (d,  $J = 8.4$  Hz, 2H), 7.86 (t,  $J = 8.5$  Hz, 4H), 7.74 (d,  $J = 8.9$  Hz, 1H), 7.17 (d,  $J = 9.1$  Hz, 1H), 7.04 (comp, 6H), 6.66 (comp, 2H), 5.59 (d,  $J = 5.2$  Hz, 1H), 5.54 (d,  $J = 2.5$  Hz, 1H), 5.16 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.16 (comp, 6H), 3.76 (comp, 6H), 3.52 (comp, 35H), 2.22 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H) note: integration of PEG methylene near 3.52 is higher than expected for this compound; HRMS (ESI Neg)  $m/z$  calculated for  $C_{47}H_{59}N_2O_{20}$   $[M-H]^-$ , 971.3667 found: 971.3665.

#### 4.4.17. Characterization of novobiocin-colistin chimera 4,23a by HRMS



#### 4.4.18. Characterization of novobiocin-colistin chimera 4,23b by HRMS



| Meas. m/z | # | Ion Formula   | m/z      | err [ppm] | mSigma | # mSigma | Score | rdb  | e <sup>-</sup> Conf | N-Rule |
|-----------|---|---------------|----------|-----------|--------|----------|-------|------|---------------------|--------|
| 1718.8871 | 1 | C78H120N21O23 | 1718.887 | 0         | 0.1    | 1        | 100   | 29.5 | even                | ok     |

In these high-resolution mass spectra, which were acquired in the ESI negative mode on a Bruker Impact II q-TOF instrument, the experimental data is on top (orange) and the predicted isotope pattern for the formula on the top right of the window is below (blue).

## **Chapter 5: Summary and Outlook**

## 5.1 Summary

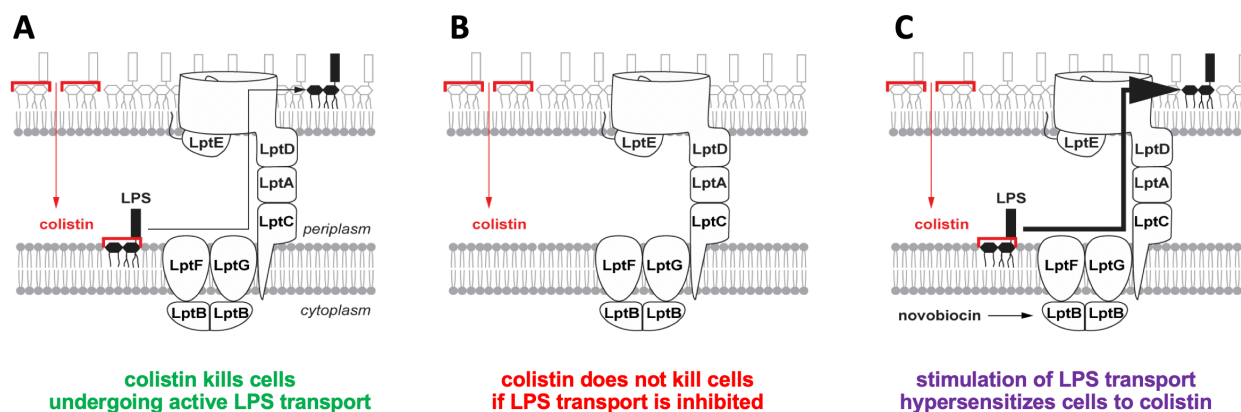
The spread of antibiotic-resistant bacteria around the world has prompted the need for new antibiotic agents or therapies. Most antibiotics that we have today were discovered during the 'Golden age' of antibiotic discovery, beginning with the production of penicillin in the 1940s to the synthetic quinolones in the 1960s.<sup>347</sup> As alluded to in the introduction, there will always be a need for new antibiotics, since bacteria have evolved resistance to every antibiotic that has ever been used by humans (and probably to many more antibiotics that have yet to be discovered). Walsh has argued that combination therapy, which has seen success in the anti-viral and oncology therapeutic areas, can help tackle the problem.<sup>347</sup> At the same time, we may begin to witness the resurgence of 'older' antibiotics, such as the polymyxins, which were previously withheld from clinical use due to the availability of safer and effective agents.

The polymyxins are very nephrotoxic by injection into the bloodstream (they are safe as topical agents), and currently there are various pharmaceutical companies and academic groups performing structural modifications of the polymyxin scaffold to widen its therapeutic window. Spero therapeutics has developed SPR206<sup>271</sup> and SPR741<sup>265</sup> (Chapter 3) for the treatment of multidrug-resistant Gram-negative nosocomial infections. There is also considerable interest in revisiting the mode of action of the polymyxins, with the goal of leveraging new mechanistic insights for designing more active agents.

The current understanding is that the polymyxins specifically recognize and bind lipopolysaccharide (LPS) molecules on the outer membrane. This interaction occurs in a stereospecific fashion and involves the cyclic peptide moiety.<sup>155</sup> This disrupts membrane permeability, as evidenced by the fact that several antibiotics, which are normally inactive against Gram-negative bacteria, are active (or more active) when combined with sublethal doses of the polymyxins. Membrane disruption is not thought to be sufficient for the bactericidal properties of polymyxins. Polymyxin derivatives lacking the fatty acyl tail, such as polymyxin B nonapeptide,

suffer a dramatic loss of antibiotic activity compared to the polymyxins, but retain similar outer membrane permeabilization properties.<sup>348-351</sup> What is perhaps less understood is the role of LPS transport on the activity of the polymyxins.

LPS transport may be central to the mechanism of action of the polymyxins (Figure 5.1A). Zhang et al. have demonstrated that the co-administration of an LpxC inhibitor antagonizes the activity of colistin against *A. baumannii* (Figure 5.1B).<sup>298</sup> *A. baumannii* strains that possess mutations in LPS biosynthesis (*lpxA*, *lpxC*, or *lpxD*), and therefore lack LPS on the cell surface, are resistant to colistin<sup>352</sup>. The wildtype strain, which possesses existing LPS on the cell surface, becomes more resistant to the polymyxins as LPS biosynthesis (and as a direct consequence, transport) is inhibited. My work supports this hypothesis in a complementary fashion. Novobiocin, which stimulates LPS transport activity *in vitro* by binding the LptB<sub>2</sub>FGC complex, enhances polymyxin activity (Figure 5.1C). The findings herein imply that the polymyxins may bind the LPS that is undergoing transport through the Lpt system.



**Figure 5.1. Model for the mechanism of action of colistin requiring active LPS transport.**

A) In cells undergoing active LPS transport, colistin binds actively transporting LPS at the inner membrane and is able to kill. B) In cells treated with an LpxC inhibitor, colistin loses its activity presumably because active LPS transport is inhibited. C) In cells treated with an aminocoumarin that stimulates LPS transport (e.g. **3,2**), more LPS in active transport is targeted by colistin, leading to improved synergy.

## **Appendix**



### A.1.1. Sequencing of the novobiocin hydrolase and reclassification of NRRL-B3652

Vadim Baidin and I were interested in the isolation, purification, and determination of the primary structure of the novobiocin hydrolytic enzyme. First, we extracted and isolated genomic DNA from the USDA NRRL B-3652 organism and submitted DNA fragments for whole genome sequencing at the Bauer Core at Harvard University. We were surprised to uncover that the bacterium was a member of the Gram-negative *Comamonas* genus, not the Gram-positive *Arthrobacter* genus that had been previously reported.<sup>193</sup> After elucidating the genus, we cultured the *Comamonas* strain and performed a biochemical fractionation experiment to isolate the active protein fractions responsible for cleaving novobiocin. Following a known procedure for the partial isolation of the enzyme<sup>353</sup>, we lysed the cells, treated the supernatant with ammonium sulfate (40%), resuspended the pellet in PBS buffer, and passed the solution through ion-exchange chromatography. Fractions from the ion-exchange chromatography were incubated with novobiocin in a 96-well plate; wells that turned brown (due to the air-mediated oxidation of novenaminate) indicated the presence of active enzyme. Vadim then subjected the appropriate column fractions to SDS-PAGE and excised candidate bands for mass spectrometry sequencing. Sequencing provided primary structure information for a putative hydrolase enzyme, with similarity to the feruloyl esterases. We were excited because in 2016, a *Science* paper described the discovery of a bacterium expressing a similar enzyme with the capability to degrade poly(ethylene terephthalate).<sup>354</sup> We reverse-translated the protein sequence to the DNA sequence contained within the *Comamonas* genome and the 5' to 3' DNA sequence corresponding to the novobiocin hydrolase is presented below (Figure A.1).

```
ATGTCACCCCCGAAGTCGATGCTCACTACCGCCGGCCTAAACGTGCGGGTACAGGAGCGCCGTGTCGTCGACACGGCGTGG
TCGCGATCGAGCTGCGTGCGGTGCGATGGTTATGAACTAGCCCCCTTCGCAGCGGGCTCGCACATTGACCTTGAAGTCCGGT
GCGTGACGCGCACAGTCACTTCATCGTGCGCCAGTACTCGTGTGCAACGACCCGGCCGAACACGATCGGTATGTGATCGGC
GTCGGCAGGGATGCCAACACGCGAGGCGGGTCGGCCTACCTGCACGACAGCCTCAAGGTTGGCGACGTTGTTACAGCCGG
CCTCGACTTTTTAGGCGATCTCACAACATCAACATTGCGCGGCCCAACGCCTGGCGGGCGCGCAAGGATTTCTTTTCCATTT
```

TCAAACATCATGAACACAAAGCATTTCAGAGACAACGAACACCATTTGCAGCCGCATTAGCCTGTCGTTTGGCACCGCGGCGGC  
 TGTTTTGGCGGCGTGACGGCATGTGGGGAGGGGGCGGAGGAGGCGCCTTCACCCCGATGCCGTACAGTAGTCAACTGTGA  
 GGCGCTCGTGGGCTATGCGATTCCGCAGAACATCATCGGCTTGCCACCTCAGGTGCTGTGGTCACGGCGGCCACTTTCAAG  
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 CCCGTTCTGTCGGAACGACCCCAAGCTGAACATGTTTACCTTCAACCCGACAACCTACAAAGTGCAAGTTCTTGCTGCGTCAGA  
 ATTGGTCGACTCGACGAACCCTGACCTCACGGCGTTCTTTCTACGTGGCGGGAAGCTCATTTTTCGAGAGAACACGGCGGATA  
 CTGCCCAAAGCGGAATGCTGGCCTCACCTATTACCAGAATGTGGTTTCAAGATGGGAAGGGATGTCGTCGACAAGTTCTTT  
 GCCACCTACACAGCGACAGGGTTGCCTCATACGTGCAACGGCGTGGCCGCGGGTACGCCGAATGCCCCCTCATATGGAATTC  
 CCGGAAGCTATGACTCGCTTGCACTAATCGATGAATGGGTGAGAAACGGCGTGGCACCAACCAACGTCGATCAGCTGACGCAG  
 AAGAGTCCGCTAGCACCGTTCCGCGTCTCGTCCACCAAGCCTATGTGCCGATACCCGCTCTACCCGCACTTCACGGGTGCGGC  
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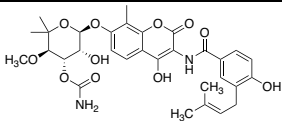
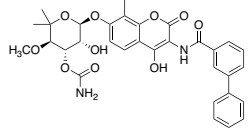
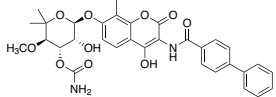
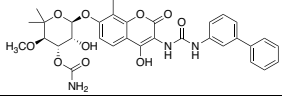
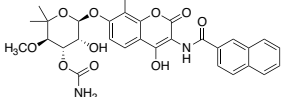
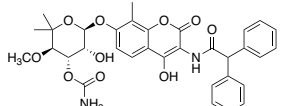
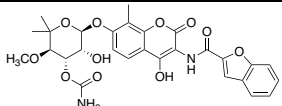
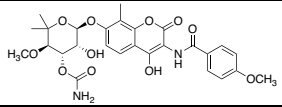
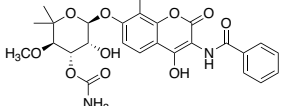
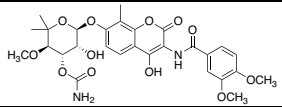
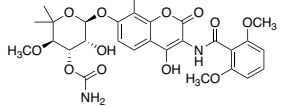
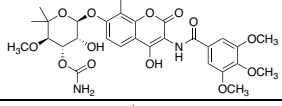
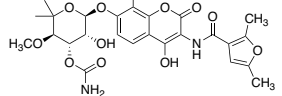
**Figure A.1. The DNA sequence of the *Comamonas novobiocin* hydrolase.**

The 5' to 3' sequence of the novobiocin hydrolase from the *Comamonas* species that catalyzes the hydrolysis of novobiocin to novenamine and 4-hydroxy-3-prenylbenzoic acid.

**A.1.2. Synthesis of novobiocin derivatives**

After utilizing the *Comamonas* enzyme to hydrolyze novobiocin to novenamine, I synthesized the following novobiocin derivatives (Table A.1) and tested their activities against wildtype Gram-negative and Gram-positive bacteria. Compounds with the designation “ACS” were synthesized in collaboration with Min Ge and Huaiqiu Wang at AceSys Pharmatech.

**Table A.1. Structures of novobiocin derivatives synthesized in Kahne Lab (MDM) or by collaboration with AceSys Pharmatech (ACS).**

| Cpd/Strain | MW      | Structure                                                                            |
|------------|---------|--------------------------------------------------------------------------------------|
| nov        | 612.632 |    |
| MDM1       | 604.612 |    |
| MDM2       | 604.612 |    |
| MDM3       | 620.635 |    |
| MDM4       | 578.574 |    |
| MDM5       | 618.639 |   |
| MDM6       | 568.535 |  |
| MDM7       | 558.540 |  |
| MDM8       | 528.514 |  |
| MDM9       | 588.566 |  |
| MDM10      | 588.566 |  |
| MDM11      | 618.592 |  |
| MDM12      | 546.529 |  |

|       |         |  |
|-------|---------|--|
| MDM13 | 492.461 |  |
| MDM14 | 586.253 |  |
| MDM15 | 517.491 |  |
| MDM16 | 567.551 |  |
| MDM17 | 567.551 |  |
| MDM18 | 594.577 |  |
| MDM19 | 618.639 |  |
| MDM20 | 632.666 |  |
| MDM21 | 610.634 |  |
| MDM22 | 613.235 |  |
| MDM23 | 649.609 |  |
| MDM24 | 647.681 |  |

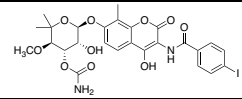
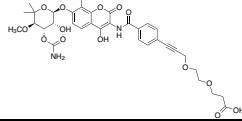
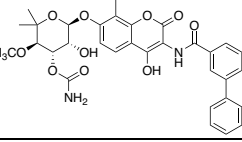
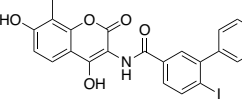
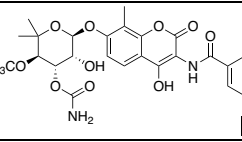
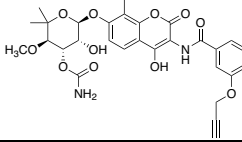
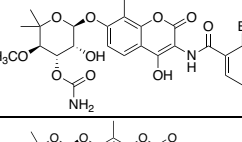
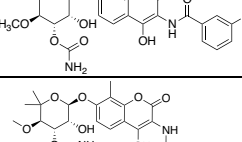
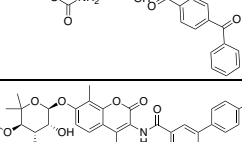
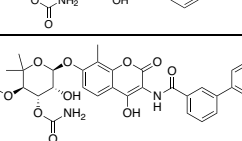
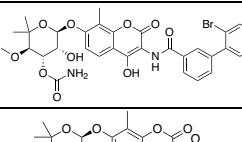
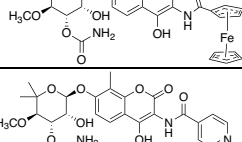
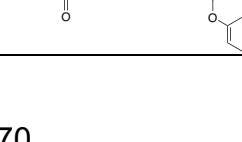
|       |         |  |
|-------|---------|--|
| MDM25 | 654.558 |  |
| MDM26 | 554.552 |  |
| MDM27 | 622.602 |  |
| MDM28 | 605.600 |  |
| MDM29 | 544.513 |  |
| MDM30 | 605.600 |  |
| MDM31 | 605.600 |  |
| MDM32 | 639.054 |  |
| MDM33 | 730.509 |  |
| MDM34 | 620.611 |  |
| MDM35 | 640.660 |  |
| MDM36 | 622.602 |  |

|       |         |  |
|-------|---------|--|
| MDM37 | 622.602 |  |
| MDM38 | 654.229 |  |
| MDM39 | 626.663 |  |
| MDM40 | 582.606 |  |
| MDM41 | 632.666 |  |
| MDM42 | 656.642 |  |
| MDM43 | 626.615 |  |
| MDM44 | 628.587 |  |
| MDM45 | 688.730 |  |
| MDM46 | 640.686 |  |
| MDM47 | 654.713 |  |
| MDM48 | 668.740 |  |

|       |         |  |
|-------|---------|--|
| MDM49 | 604.628 |  |
| MDM50 | 614.623 |  |
| MDM51 | 466.443 |  |
| MDM52 | 676.719 |  |
| MDM53 | 662.692 |  |
| MDM54 | 676.719 |  |
| MDM55 | 607.410 |  |
| MDM56 | 730.770 |  |
| MDM57 | 557.556 |  |
| MDM58 | 557.556 |  |
| MDM59 | 691.670 |  |
| MDM60 | 524.523 |  |
| MDM61 | 752.817 |  |
| MDM62 | 702.757 |  |

|       |         |  |
|-------|---------|--|
| MDM63 | 652.697 |  |
| MDM64 | 690.746 |  |
| MDM65 | 690.746 |  |
| MDM66 | 656.685 |  |
| MDM67 | 670.712 |  |
| MDM68 | 668.696 |  |
| MDM69 | 619.627 |  |
| MDM70 | 640.593 |  |
| MDM71 | 634.638 |  |
| MDM72 | 634.638 |  |
| MDM73 | 673.496 |  |
| MDM74 | 746.854 |  |
| MDM75 | 569.527 |  |



|       |         |                                                                                      |
|-------|---------|--------------------------------------------------------------------------------------|
| MDM76 | 654.411 |    |
| MDM77 | 698.678 |    |
| MDM78 | 730.509 |    |
| MDM79 | 513.288 |    |
| MDM80 | 552.540 |    |
| MDM81 | 582.560 |    |
| MDM82 | 607.410 |   |
| MDM83 | 607.410 |  |
| MDM84 | 632.622 |  |
| MDM85 | 683.508 |  |
| MDM86 | 683.508 |  |
| MDM87 | 683.508 |  |
| MDM88 | 636.435 |  |
| MDM89 | 621.599 |  |

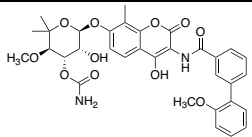
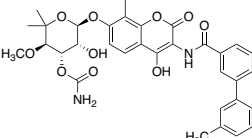
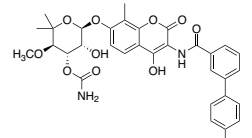
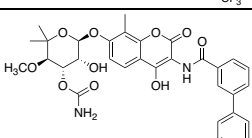
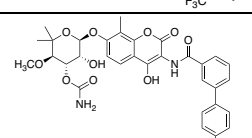
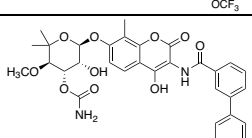
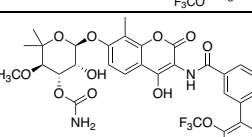
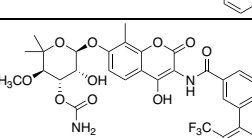
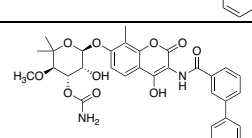
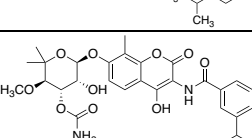
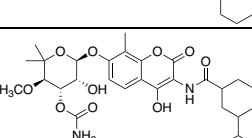
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|--------|---------|--|
| MDM90  | 656.041 |  |
| MDM91  | 621.599 |  |
| MDM92  | 650.637 |  |
| MDM93  | 688.609 |  |
| MDM94  | 645.621 |  |
| MDM95  | 655.053 |  |
| MDM96  | 652.609 |  |
| MDM97  | 655.053 |  |
| MDM98  | 655.053 |  |
| MDM99  | 634.638 |  |
| MDM100 | 676.719 |  |
| MDM101 | 638.601 |  |
| MDM102 | 618.595 |  |

|        |         |  |
|--------|---------|--|
| MDM103 | 688.609 |  |
| MDM104 | 746.508 |  |
| MDM105 | 665.608 |  |
| MDM106 | 638.601 |  |
| MDM107 | 634.638 |  |
| MDM108 | 650.637 |  |
| MDM109 | 644.633 |  |
| MDM110 | 699.507 |  |
| MDM111 | 675.651 |  |
| MDM112 | 649.653 |  |
| MDM113 | 656.592 |  |
| MDM114 | 597.577 |  |
| MDM115 | 572.330 |  |
| MDM116 | 655.053 |  |
| MDM117 | 652.628 |  |

|        |         |  |
|--------|---------|--|
| MDM118 | 673.043 |  |
| MDM119 | 610.616 |  |
| MDM120 | 645.621 |  |
| MDM121 | 663.611 |  |
| MDM122 | 664.427 |  |
| MDM123 | 686.670 |  |
| MDM124 | 686.670 |  |
| MDM125 | 698.696 |  |
| MDM126 | 616.620 |  |
| MDM127 | 638.601 |  |
| MDM128 | 696.709 |  |
| MDM129 | 624.574 |  |
| MDM130 | 652.628 |  |
| MDM131 | 677.663 |  |

|        |         |  |
|--------|---------|--|
| MDM132 | 652.628 |  |
| MDM133 | 636.610 |  |
| MDM134 | 650.637 |  |
| MDM135 | 664.664 |  |
| MDM136 | 693.764 |  |
| MDM137 | 745.603 |  |
| MDM138 | 677.703 |  |
| MDM139 | 684.698 |  |
| MDM140 | 646.649 |  |
| MDM141 | 730.690 |  |
| MDM142 | 662.648 |  |
| MDM143 | 648.665 |  |
| MDM144 | 603.581 |  |

|        |         |  |
|--------|---------|--|
| MDM145 | 617.608 |  |
| MDM146 | 981.918 |  |
| MDM147 | 564.544 |  |
| ACS1   | 604.612 |  |
| ACS2   | 610.660 |  |
| ACS3   | 542.541 |  |
| ACS4   | 556.568 |  |
| ACS5   | 620.611 |  |
| ACS6   | 634.638 |  |
| ACS7   | 634.638 |  |
| ACS8   | 618.639 |  |
| ACS9   | 646.693 |  |
| ACS10  | 646.693 |  |

|       |         |                                                                                      |
|-------|---------|--------------------------------------------------------------------------------------|
| ACS11 | 634.638 |    |
| ACS12 | 618.639 |    |
| ACS13 | 672.610 |    |
| ACS14 | 672.610 |    |
| ACS15 | 688.609 |    |
| ACS16 | 688.609 |   |
| ACS17 | 688.609 |  |
| ACS18 | 672.610 |  |
| ACS19 | 646.693 |  |
| ACS20 | 610.660 |  |
| ACS21 | 616.708 |  |

|           |         |  |
|-----------|---------|--|
| ACS22     | 649.609 |  |
| ACS23     | 619.627 |  |
| ACS24     | 816.860 |  |
| ACS25     | 636.610 |  |
| ACS27     | 618.639 |  |
| ACS28     | 581.578 |  |
| ACS28 HCl | 618.036 |  |
| ACS29     | 581.578 |  |
| ACS29 HCl | 618.036 |  |
| ACS30     | 581.578 |  |
| ACS31     | 680.710 |  |
| ACS32     | 628.634 |  |
| ACS33     | 632.666 |  |



|       |         |  |
|-------|---------|--|
| ACS34 | 620.611 |  |
| ACS35 | 638.601 |  |
| ACS36 | 620.611 |  |
| ACS37 | 654.600 |  |
| ACS38 | 619.627 |  |
| ACS39 | 619.627 |  |
| ACS40 | 620.611 |  |
| ACS41 | 620.611 |  |
| ACS42 | 619.627 |  |
| ACS43 | 614.564 |  |
| ACS44 | 615.592 |  |
| ACS45 | 670.671 |  |
| ACS46 | 670.671 |  |

|       |         |  |
|-------|---------|--|
| ACS47 | 636.610 |  |
| ACS48 | 654.600 |  |
| ACS49 | 686.670 |  |
| ACS50 | 654.600 |  |
| ACS51 | 686.670 |  |
| ACS52 | 636.610 |  |
| ACS53 | 680.751 |  |
| ACS54 | 680.751 |  |
| ACS55 | 654.713 |  |
| ACS56 | 694.778 |  |

### A.1.3. Testing novobiocin derivatives in monotherapy against Gram-negative bacteria

The novobiocin derivatives were screened in minimal inhibitory concentration (MIC) assays (Table A.2) against most of the *ESKAPE* pathogens.<sup>297</sup> Notably MDM107 possessed significantly better anti-*Klebsiella pneumoniae* and anti-*Escherichia coli* activity than novobiocin.

**Table A.2. Minimal inhibitory concentrations (MIC) of novobiocin derivatives against *ESKAPE* pathogens and *E. coli*.**

ATCC 35695 = MC4100; 25922 = wildtype *E. coli*; RN4220 = wildtype *S. aureus*; MW2 = methicillin-resistant *S. aureus*; 19606 = wildtype *A. baumannii*; 13047 = wildtype *E. cloacae*; 700721 = wildtype *K. pneumoniae*; 15692 = wildtype *P. aeruginosa* PAO1. MICs are reported in  $\mu\text{M}$  after incubation at 37 °C for 16 h with no shaking.

| Cpd/Strain | 35695 ara <sup>+</sup> | 25922 | RN4220 | MW2  | 19606 | 13047 | 700721 | 15692 |
|------------|------------------------|-------|--------|------|-------|-------|--------|-------|
| nov        | 59                     | 40    | 0.2    | 0.25 | 1.27  | 395   | 780    | >1000 |
| MDM1       | 26                     | 41    | 5      | 5    | >50   | >100  | >100   | >300  |
| MDM2       | >200                   | >200  | 21     | 21   | >50   | >100  | >100   | >300  |
| MDM3       | >200                   | >200  | 81     | 81   | >50   | >100  | >100   | >300  |
| MDM4       | >200                   | >200  | 22     | 22   | >50   | >100  | >100   | >300  |
| MDM5       | >200                   | >200  | >100   | 80   | >50   | >100  | >100   | >300  |
| MDM6       | >200                   | >200  | 88     | 88   | >50   | >100  | >100   | >300  |
| MDM7       | >200                   | >200  | 45     | 45   | >50   | >100  | >100   | >300  |
| MDM8       | >200                   | >200  | >100   | >100 | >50   | >100  | >100   | >300  |
| MDM9       | >200                   | >200  | 85     | 43   | >50   | >100  | >100   | >300  |
| MDM10      | >200                   | >200  | >100   | 85   | >50   | >100  | >100   | >300  |
| MDM11      | >200                   | >200  | >100   | >100 | >50   | >100  | >100   | >300  |
| MDM12      | >200                   | >200  | 91     | 91   | >50   | >100  | >100   | >300  |
| MDM13      | >200                   | >200  | >100   | >100 | >50   | >100  | >100   | >300  |
| MDM14      | >200                   | >200  | 43     | 21   | >50   | >100  | >100   | >300  |
| MDM15      | >200                   | >200  | >100   | >100 | >50   | >100  | >100   | >300  |
| MDM16      | >200                   | >200  | >100   | >100 | >50   | >100  | >100   | >300  |
| MDM17      | >200                   | >200  | 44     | 22   | >50   | >100  | >100   | >300  |
| MDM18      | >200                   | >200  | 42     | 42   | >50   | >100  | >100   | >300  |
| MDM19      | 26                     | 40    | 2.5    | 3    | 40    | >100  | >100   | >300  |
| MDM20      | 133                    | >200  | 10     | 10   | >50   | >100  | >100   | >300  |
| MDM21      | 89                     | 164   | 10     | 10   | >50   | >100  | >100   | >300  |
| MDM22      | >200                   | >200  | 82     | 82   | >50   | >100  | >100   | >300  |
| MDM23      | >200                   | 154   | 5      | 5    | >50   | >100  | >100   | >300  |
| MDM24      | >200                   | >200  | 0.96   | 0.96 | 19    | >100  | >100   | >300  |
| MDM25      | >200                   | >200  | >100   | >100 | >50   | >100  | >100   | >300  |
| MDM26      | >200                   | >200  | 45     | 23   | >50   | >100  | >100   | >300  |
| MDM27      | 26                     | 40    | 5      | 5    | >50   | >100  | 80     | >300  |
| MDM28      | >200                   | >200  | 83     | 83   | >50   | >100  | >100   | >300  |
| MDM29      | >200                   | >200  | 46     | 46   | >50   | >100  | >100   | >300  |
| MDM30      | >200                   | >200  | 41     | 41   | >50   | >100  | >100   | >300  |
| MDM31      | >200                   | >200  | 83     | 41   | >50   | >100  | >100   | >300  |
| MDM32      | 18                     | 20    | 1.96   | 0.98 | 39    | >100  | >100   | >300  |
| MDM33      | 59                     | 68    | 0.43   | 0.43 | 9     | >100  | >100   | >300  |
| MDM34      | 8                      | 10    | 40     | 20   | >50   | >100  | >100   | >300  |
| MDM35      | >200                   | >200  | >100   | >100 | >50   | >100  | >100   | >300  |
| MDM36      | 59                     | 80    | 10     | 10   | >50   | >100  | >100   | >300  |

|       |      |      |       |       |       |      |      |       |
|-------|------|------|-------|-------|-------|------|------|-------|
| MDM37 | 26   | 40   | 5     | 10    | >50   | >100 | >100 | >300  |
| MDM38 | >200 | 153  | 0.19  | 0.1   | 5     | >100 | >100 | >300  |
| MDM39 | >200 | 160  | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM40 | >200 | >200 | 11    | 11    | >50   | >100 | >100 | >300  |
| MDM41 | 40   | 79   | 0.99  | 0.99  | 20    | >100 | 79   | >300  |
| MDM42 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM43 | >200 | >200 | 40    | 40    | >50   | >100 | >100 | >300  |
| MDM44 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM45 | >200 | >200 | 36    | 36    | 9     | >100 | >100 | >300  |
| MDM46 | >200 | 156  | 2     | 2     | 10    | >100 | >100 | >300  |
| MDM47 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM48 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM49 | >200 | >200 | 83    | 41    | >50   | >100 | >100 | >300  |
| MDM50 | 20   | 20   | 2     | 1     | 41    | >100 | 81   | >300  |
| MDM51 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM52 | 74   | 18.5 | <0.01 | <0.01 | <0.11 | >100 | 37   | 18.47 |
| MDM53 | >200 | >200 | 0.5   | 0.24  | 2     | >100 | >100 | >300  |
| MDM54 | >200 | >200 | 74    | 74    | >50   | >100 | >100 | >300  |
| MDM55 | >200 | >200 | 82    | 82    | >50   | >100 | >100 | >300  |
| MDM56 | >200 | >200 | 9     | 9     | >50   | >100 | >100 | >300  |
| MDM57 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM58 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM59 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM60 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM61 | >200 | >200 | 33    | 33    | >50   | >100 | >100 | >300  |
| MDM62 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM63 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM64 | >200 | >200 | 2     | 0.5   | 5     | >100 | >100 | >300  |
| MDM65 | >200 | >200 | 5     | 5     | >50   | >100 | >100 | >300  |
| MDM66 | >200 | >200 | 38    | 19    | >50   | >100 | >100 | >300  |
| MDM67 | >200 | >200 | 19    | 19    | >50   | >100 | >100 | >300  |
| MDM68 | >200 | >200 | 19    | 9     | >50   | >100 | >100 | >300  |
| MDM69 | 80   | 161  | 10    | 10    | >50   | >100 | >100 | >300  |
| MDM70 | 39   | 20   | 5     | 5     | >50   | >100 | 78   | >300  |
| MDM71 | 158  | 160  | 10    | 10    | >50   | >100 | >100 | >300  |
| MDM72 | 10   | 5    | 10    | 10    | >50   | >100 | >100 | >300  |
| MDM73 | 37   | 37   | 0.5   | 0.2   | 9     | >100 | >100 | >300  |
| MDM74 | >200 | >200 | 17    | 34    | >50   | >100 | >100 | >300  |
| MDM75 | >200 | >200 | 88    | >100  | >50   | >100 | >100 | >300  |
| MDM76 | >200 | >200 | 38    | 38    | >50   | >100 | >100 | >300  |
| MDM77 | >200 | >200 | >100  | >100  | >50   | >100 | >100 | >300  |
| MDM78 | 9    | 34   | 4     | 4     | >50   | >100 | 34   | >300  |
| MDM79 | >200 | >200 | 97    | 97    | >50   | >100 | >100 | >300  |
| MDM80 | >200 | >200 | 90    | 90    | >50   | >100 | >100 | >300  |
| MDM81 | 86   | 172  | 86    | 86    | >50   | >100 | >100 | >300  |

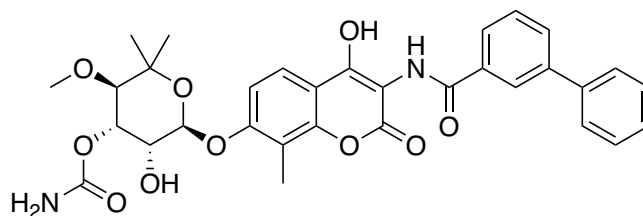
|        |      |      |      |      |      |      |      |      |
|--------|------|------|------|------|------|------|------|------|
| MDM82  | >200 | >200 | >100 | >100 | >50  | >100 | >100 | >300 |
| MDM83  | >200 | >200 | 82   | 82   | >50  | >100 | >100 | >300 |
| MDM84  | >200 | >200 | 40   | 40   | >50  | >100 | >100 | >300 |
| MDM85  | 18   | 18   | 0.9  | 0.9  | 18   | >100 | >100 | >300 |
| MDM86  | 18   | 18   | 0.7  | 0.5  | 18   | >100 | >100 | >300 |
| MDM87  | 18   | 18   | 9    | 5    | >50  | >100 | >100 | >300 |
| MDM88  | ND   | >200 | 79   | 79   | >50  | >100 | >100 | >300 |
| MDM89  | >100 | >200 | >100 | >100 | >50  | >100 | >100 | >300 |
| MDM90  | 150  | >200 | 10   | 10   | >50  | >100 | >100 | >300 |
| MDM91  | >200 | >200 | 20   | 20   | >50  | >100 | >100 | >300 |
| MDM92  | 5    | 38   | 19   | 19   | >50  | >100 | >100 | >300 |
| MDM93  | 160  | 150  | 9    | 9    | >50  | >100 | >100 | >300 |
| MDM94  | 150  | 150  | >100 | 77   | >50  | >100 | >100 | >300 |
| MDM95  | 5    | 38   | 10   | 10   | >50  | >100 | >100 | >300 |
| MDM96  | 5    | 38   | 19   | 19   | >50  | >100 | 38   | >300 |
| MDM97  | 38   | 76   | 10   | 19   | >50  | >100 | >100 | >300 |
| MDM98  | 79   | 76   | 10   | 5    | >50  | >100 | >100 | >300 |
| MDM99  | 18   | 39   | 10   | 5    | >50  | >100 | >100 | >300 |
| MDM100 | >200 | 148  | 2    | 2    | >50  | >100 | >100 | >300 |
| MDM101 | 5    | 3    | 20   | 20   | >50  | >100 | 20   | >300 |
| MDM102 | >100 | >200 | 10   | 10   | >50  | >100 | >100 | >300 |
| MDM103 | 4.5  | 9    | 9    | 9    | >50  | >100 | >100 | >300 |
| MDM104 | 17   | 33   | 2    | 2    | >50  | >100 | 38   | >300 |
| MDM105 | 75   | 75   | 38   | 38   | >50  | >100 | >100 | >300 |
| MDM106 | 2    | 5    | 10   | 10   | >50  | 79   | 20   | >300 |
| MDM107 | 2    | 5    | 5    | 5    | >50  | 77   | 10   | >300 |
| MDM108 | 10   | 10   | 19   | 19   | >50  | >100 | 19   | >300 |
| MDM109 | 39   | 19   | 5    | 5    | >50  | >100 | 71   | >300 |
| MDM110 | 5    | 18   | 5    | 5    | >50  | >100 | 20   | >300 |
| MDM111 | >200 | >200 | 19   | 19   | >50  | >100 | >100 | >300 |
| MDM112 | >200 | >200 | >100 | >100 | >50  | >100 | >100 | >300 |
| MDM113 | 19   | 19   | 19   | 19   | >50  | >100 | 76   | >300 |
| MDM114 | >200 | >200 | 84   | >100 | >50  | >100 | >100 | >300 |
| MDM115 | >200 | 175  | 11   | 22   | >50  | >100 | >100 | >300 |
| MDM116 | 5    | 10   | 2.39 | 5    | >50  | >100 | 76   | >300 |
| MDM117 | 5    | 10   | 2.39 | 2.39 | >50  | >100 | 38   | >300 |
| MDM118 | 5    | 9    | 2    | 5    | >50  | >100 | 37   | >300 |
| MDM119 | 164  | 164  | 10   | 10   | >50  | >100 | >100 | >300 |
| MDM120 | >200 | >200 | 77   | 77   | >50  | >100 | >100 | >300 |
| MDM121 | 151  | 151  | 38   | 38   | >50  | >100 | >100 | >300 |
| MDM122 | >200 | >200 | 75   | 75   | >100 | >100 | >100 | >300 |
| MDM123 | 9    | 36   | 18   | 18   | >100 | >100 | >100 | >300 |
| MDM124 | >200 | 73   | 9    | 9    | >100 | >100 | >100 | >300 |
| MDM125 | >200 | >200 | 72   | 72   | >100 | >100 | >100 | >300 |
| MDM126 | >200 | >200 | 41   | 81   | >100 | >100 | >100 | >300 |

|        |      |      |      |      |      |      |      |      |
|--------|------|------|------|------|------|------|------|------|
| MDM127 | 78   | 39   | 5    | 5    | 78   | >100 | >100 | >300 |
| MDM128 | >200 | >200 | 9    | 18   | 72   | >100 | >100 | >300 |
| MDM129 | 5    | 10   | 40   | 20   | >100 | 160  | >100 | >300 |
| MDM130 | 19   | 19   | <.78 | <.78 | 38   | >100 | 77   | >300 |
| MDM131 | >200 | >200 | 37   | 37   | >100 | >100 | >100 | 300  |
| MDM132 | ND   | 10   | ND   | ND   | >100 | >100 | 77   | >300 |
| MDM133 | ND   | 20   | ND   | ND   | 79   | >100 | >100 | >300 |
| MDM134 | ND   | >200 | ND   | ND   | >100 | >100 | >100 | >300 |
| MDM135 | ND   | >200 | ND   | ND   | >100 | >100 | >100 | >300 |
| MDM136 | ND   | >200 | ND   | ND   | >100 | >100 | >100 | >300 |
| MDM137 | ND   | >200 | ND   | ND   | >100 | >100 | >100 | >300 |
| MDM138 | ND   | >200 | ND   | ND   | >100 | >100 | >100 | >300 |
| MDM139 | ND   | 36.5 | ND   | ND   | 2    | >100 | >100 | >300 |
| MDM140 | ND   | >100 | ND   | ND   | 19   | ND   | >100 | ND   |
| MDM141 | ND   | 9    | ND   | ND   | >100 | ND   | 17   | ND   |
| MDM142 | ND   | >100 | ND   | ND   | >100 | ND   | >100 | ND   |
| MDM143 | ND   | >100 | ND   | ND   | 39   | ND   | >100 | ND   |
| MDM144 | ND   | >100 | ND   | ND   | ND   | ND   | ND   | ND   |
| MDM145 | ND   | >100 | ND   | ND   | ND   | ND   | ND   | ND   |
| MDM146 | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   |
| MDM147 | ND   | ND   | ND   | ND   | ND   | ND   | ND   | ND   |
| ACS1   | >200 | >200 | 41   | 38   | >50  | >100 | >100 | >300 |
| ACS2   | >200 | >200 | 20   | 20   | >50  | >100 | >100 | >300 |
| ACS3   | >200 | >200 | >100 | >100 | >50  | >100 | >100 | >300 |
| ACS4   | >200 | >200 | >100 | >100 | >50  | >100 | >100 | >300 |
| ACS5   | >200 | >200 | 45   | 40   | >50  | >100 | >100 | >300 |
| ACS6   | 89   | 79   | 5    | 3    | >50  | >100 | >100 | >300 |
| ACS7   | >200 | >200 | 5    | 3    | >50  | >100 | >100 | >300 |
| ACS8   | 26   | 40   | 20   | 10   | >50  | >100 | >100 | >300 |
| ACS9   | 89   | 77   | 0.77 | 0.48 | 19   | >100 | 77   | >300 |
| ACS10  | >200 | >200 | 10   | 5    | >50  | >100 | >100 | >300 |
| ACS11  | 40   | 39   | 19   | 10   | >50  | >100 | >100 | >300 |
| ACS12  | 26   | 81   | 1.6  | 1    | 40   | >100 | >100 | >300 |
| ACS13  | >200 | >200 | 1.5  | 0.93 | 37   | >100 | >100 | >300 |
| ACS14  | >200 | >200 | 0.74 | 0.46 | 37   | >100 | >100 | >300 |
| ACS15  | >200 | >200 | 1.5  | 0.9  | 18   | >100 | >100 | >300 |
| ACS16  | 89   | >200 | 0.4  | 0.2  | 18   | >100 | >100 | >300 |
| ACS17  | 40   | >200 | 9    | 5    | >50  | >100 | >100 | >300 |
| ACS18  | >200 | >200 | 9    | 5    | >50  | >100 | >100 | >300 |
| ACS19  | 59   | 77   | 0.4  | 0.2  | 10   | >100 | >100 | >300 |
| ACS20  | 59   | 82   | 5    | 3    | >50  | >100 | 82   | >300 |
| ACS21  | >200 | >200 | 10   | 5    | >50  | >100 | >100 | >300 |
| ACS22  | >200 | >200 | 77   | 39   | >50  | >100 | >100 | >300 |
| ACS23  | 59   | >200 | 81   | 40   | >50  | >100 | >100 | >300 |
| ACS24  | >200 | >200 | >100 | >100 | >50  | >100 | >100 | >300 |

|           |      |      |      |      |     |      |      |      |
|-----------|------|------|------|------|-----|------|------|------|
| ACS25     | 18   | 5    | 10   | 5    | >50 | >100 | 79   | >300 |
| ACS27     | 89   | 81   | 5    | 3    | >50 | >100 | >100 | >300 |
| ACS28     | >200 | >200 | 86   | 43   | >50 | >100 | >100 | >300 |
| ACS28 HCl | >200 | >200 | 81   | 41   | >50 | >100 | >100 | >300 |
| ACS29     | >200 | >200 | 86   | 43   | >50 | >100 | >100 | >300 |
| ACS29 HCl | >200 | >200 | >100 | 81   | >50 | >100 | >100 | >300 |
| ACS30     | >200 | >200 | >100 | >100 | >50 | >100 | >100 | >300 |
| ACS31     | >200 | >200 | 18   | 9    | >50 | >100 | >100 | >300 |
| ACS32     | 40   | 80   | 5    | 3    | 40  | >100 | >100 | >300 |
| ACS33     | 40   | 40   | 10   | 5    | >50 | >100 | >100 | >300 |
| ACS34     | 59   | 40   | 5    | 4    | 40  | >100 | >100 | >300 |
| ACS35     | 26   | 20   | 2    | 1    | 20  | >100 | 78   | >300 |
| ACS36     | 26   | 40   | 3    | 2.5  | 20  | >100 | >100 | >300 |
| ACS37     | 8    | 10   | 5    | 5    | >50 | 81   | 40   | >300 |
| ACS38     | 59   | 40   | 5    | 2.5  | >50 | >100 | >100 | >300 |
| ACS39     | 18   | 20   | 20   | 20   | >50 | >100 | 40   | >300 |
| ACS40     | 18   | 20   | 20   | 20   | >50 | >100 | 40   | >300 |
| ACS41     | 89   | 161  | >100 | 81   | >50 | >100 | >100 | >300 |
| ACS42     | 59   | 81   | 20   | 10   | >50 | >100 | >100 | >300 |
| ACS43     | >200 | >200 | >100 | >100 | >50 | >100 | >100 | >300 |
| ACS44     | >200 | >200 | >100 | >100 | >50 | >100 | >100 | >300 |
| ACS45     | 150  | >200 | 0.1  | 0.1  | 1   | >100 | >100 | >300 |
| ACS46     | 75   | >200 | 37   | 19   | >50 | >100 | >100 | >300 |
| ACS47     | >200 | >200 | 20   | 20   | >50 | >100 | >100 | >300 |
| ACS48     | 38   | 38   | 38   | 38   | >50 | >100 | >100 | >300 |
| ACS49     | >200 | 146  | 9    | 9    | 18  | >100 | 76   | >300 |
| ACS50     | >200 | 153  | 76   | 38   | >50 | >100 | >100 | >300 |
| ACS51     | >200 | >200 | 18   | 18   | 36  | >100 | >100 | >300 |
| ACS52     | >200 | >200 | 79   | 79   | >50 | >100 | >100 | >300 |
| ACS53     | >200 | >200 | 73   | 73   | 9   | >100 | >100 | >300 |
| ACS54     | >200 | >200 | >100 | >100 | >50 | >100 | >100 | >300 |
| ACS55     | >200 | >200 | 1.5  | 1.5  | 10  | >100 | >100 | >300 |
| ACS56     | >200 | >200 | >100 | >100 | >50 | >100 | >100 | >300 |

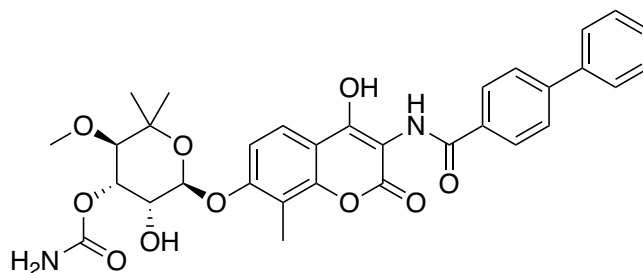
#### A.1.4. <sup>1</sup>H NMR characterization of novobiocin derivatives

<sup>1</sup>H NMR spectra were recorded in DMSO-*d*<sub>6</sub> on a JEOL 400, Varian Inova-500 or Varian Inova-600 MHz spectrometer unless otherwise noted. Chemical shifts are reported in ppm with the residual solvent signal as the reference and coupling constants (*J*) are given in hertz. The peak information is described as: s = singlet, d = doublet, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet, comp = composite of overlapping signals.



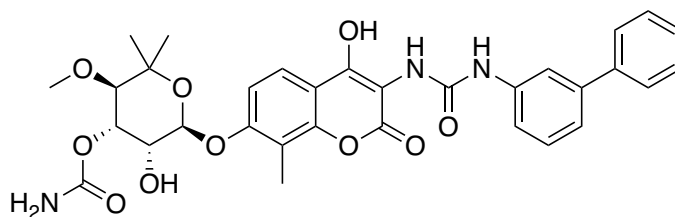
**MDM1**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.3 (s, 1H), 7.9 (d,  $J$  = 4.9 Hz, 1H), 7.8 (d,  $J$  = 7.8 Hz, 1H), 7.8 (d,  $J$  = 8.2 Hz, 2H), 7.7 (d,  $J$  = 8.8 Hz, 1H), 7.6 (t,  $J$  = 7.7 Hz, 1H), 7.5 (t,  $J$  = 7.8 Hz, 2H), 7.4 (t,  $J$  = 7.4 Hz, 1H), 7.0 (d,  $J$  = 8.7 Hz, 1H), 6.6 (comp, 2H), 5.6 (d,  $J$  = 5.2 Hz, 1H), 5.5 (s, 1H), 5.2 (dd,  $J$  = 9.9, 3.2 Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



**MDM2**

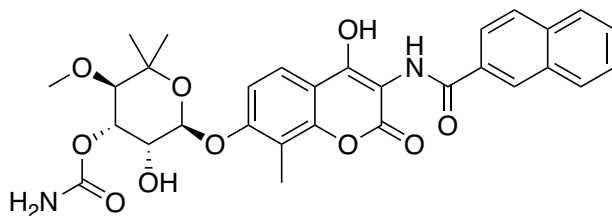
$^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.6 (s, 1H), 8.2 (s, 1H), 8.1 (d,  $J$  = 8.2 Hz, 2H), 7.8 (comp,  $J$  = 8.0, 4.0 Hz, 4H), 7.6 (d,  $J$  = 8.6 Hz, 1H), 7.5 (t,  $J$  = 7.6 Hz, 2H), 7.4 (t,  $J$  = 7.3 Hz, 1H), 6.9 (d,  $J$  = 8.8 Hz, 1H), 6.7 (comp, 2H), 5.5 (s, 1H), 5.4 (d,  $J$  = 2.5 Hz, 1H), 5.2 (dd,  $J$  = 9.7, 3.2 Hz, 1H), 4.0 (s, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



**MDM3**

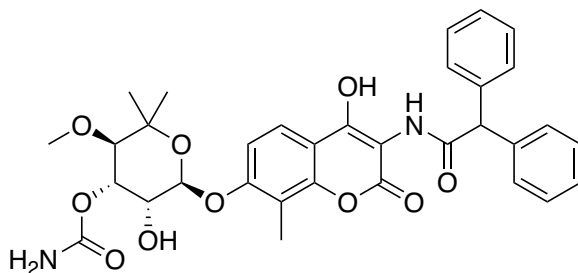


$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.1 (s, 1H), 7.7 (comp, 2H), 7.6 (comp, 2H), 7.5 (comp, 2H), 7.4 (comp, 3H), 7.2 (d,  $J = 7.5$  Hz, 1H), 7.0 (d,  $J = 8.8$  Hz, 1H), 6.6 (comp, 4H), 5.6 – 5.5 (m, 1H), 5.4 (d,  $J = 2.5$  Hz, 1H), 5.2 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.0 (s, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



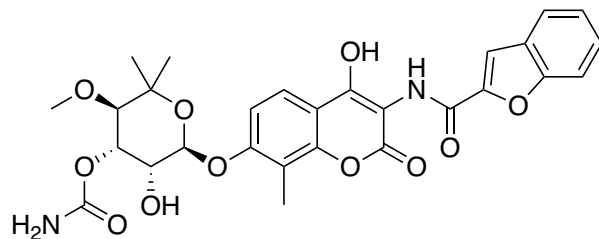
**MDM4**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.9 (s, 1H), 8.6 (s, 1H), 8.1 (s, 1H), 8.1 (d,  $J = 8.6$  Hz, 1H), 8.0 (d,  $J = 7.8$  Hz, 1H), 8.0 (comp, 2H), 7.6 (comp, 3H), 6.9 (d,  $J = 8.8$  Hz, 1H), 6.6 (comp, 2H), 5.6 (d,  $J = 5.1$  Hz, 1H), 5.4 (d,  $J = 2.5$  Hz, 1H), 5.2 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



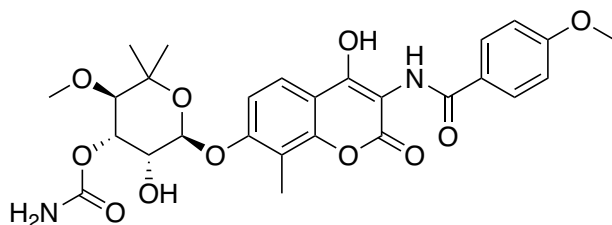
**MDM5**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.9 – 12.0 (m, 1H), 9.4 (s, 1H), 7.7 (d,  $J = 8.7$  Hz, 1H), 7.4 (comp, 4H), 7.3 (comp, 4H), 7.2 (comp, 2H), 7.1 – 7.0 (m, 1H), 6.6 (comp, 2H), 5.6 (d,  $J = 4.7$  Hz, 1H), 5.5 (d,  $J = 2.6$  Hz, 1H), 5.3 (s, 1H), 5.1 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.2 (s, 3H), 1.0 (s, 3H).



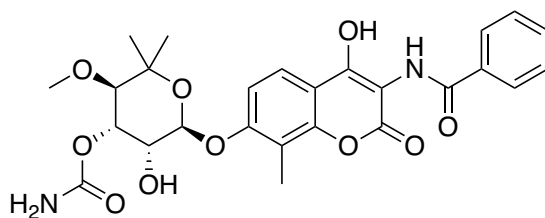
**MDM6**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.8 (s, 1H), 9.0 (s, 1H), 8.1 (s, 1H), 7.8 (d,  $J = 7.8$  Hz, 1H), 7.6 (comp, 2H), 7.4 (t,  $J = 7.6$  Hz, 1H), 7.3 (t,  $J = 7.6$  Hz, 1H), 6.9 (d,  $J = 8.8$  Hz, 1H), 6.6 (comp, 2H), 5.6 (d,  $J = 5.2$  Hz, 1H), 5.5 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



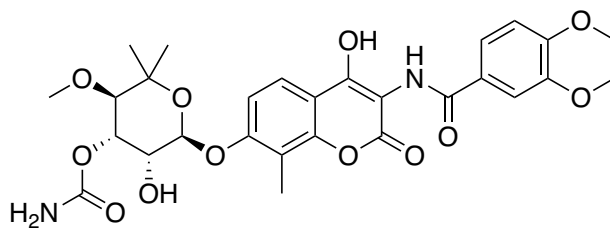
**MDM7**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.1 (s, 1H), 9.2 (s, 1H), 8.0 (d,  $J = 8.8$  Hz, 2H), 7.7 (d,  $J = 8.8$  Hz, 1H), 7.1 (d,  $J = 8.9$  Hz, 1H), 7.0 (d,  $J = 8.8$  Hz, 2H), 6.6 (d,  $J = 92.1$  Hz, 2H), 5.6 (d,  $J = 5.2$  Hz, 1H), 5.5 – 5.5 (m, 1H), 5.2 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.1 – 4.1 (m, 1H), 3.8 (s, 3H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



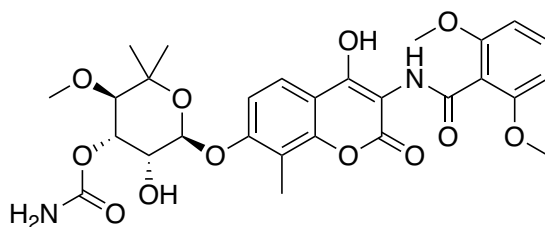
**MDM8**

NMR data unavailable



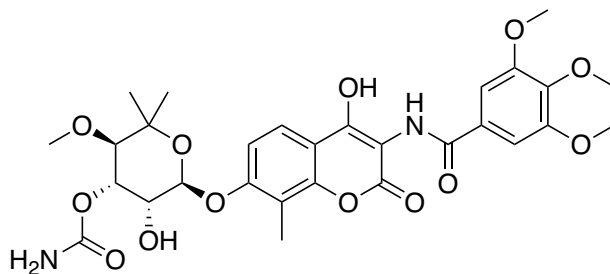
**MDM9**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.7 (s, 1H), 12.0 (s, 1H), 7.7 (d,  $J = 8.7$  Hz, 1H), 7.6 (d,  $J = 8.7$  Hz, 1H), 7.6 – 7.5 (m, 1H), 7.0 (d,  $J = 8.6$  Hz, 2H), 6.5 (s, 2H), 5.6 – 5.5 (m, 1H), 5.5 – 5.4 (m, 1H), 5.1 (d,  $J = 9.9$  Hz, 1H), 4.0 (s, 1H), 3.8 (comp, 6H), 3.5 (comp,  $J = 1.7$  Hz, 4H), 2.2 (s, 3H), 1.2 (s, 3H), 1.1 (s, 3H).



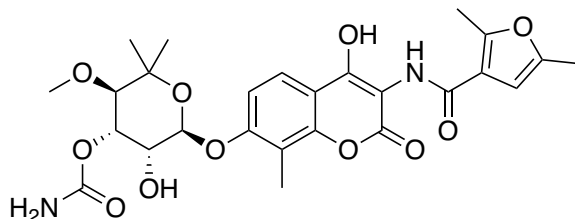
**MDM10**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.5 (s, 1H), 10.0 (s, 1H), 7.8 (d,  $J = 8.8$  Hz, 1H), 7.4 (t,  $J = 8.4$  Hz, 1H), 7.2 (d,  $J = 9.2$  Hz, 1H), 6.7 (comp,  $J = 8.4$  Hz, 4H), 5.6 (d,  $J = 4.4$  Hz, 1H), 5.5 (s, 1H), 5.2 (d,  $J = 10.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.8 (comp,  $J = 1.3$  Hz, 6H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



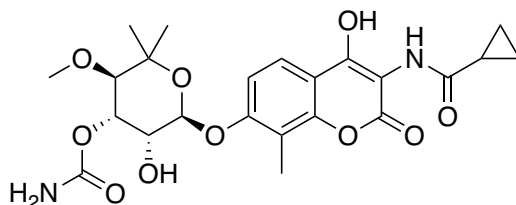
**MDM11**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.9 (s, 1H), 9.4 (s, 1H), 7.7 (d,  $J = 8.8$  Hz, 1H), 7.3 (s, 2H), 7.2 (d,  $J = 8.9$  Hz, 1H), 6.6 (comp, 2H), 5.6 (d,  $J = 5.2$  Hz, 1H), 5.6 – 5.5 (m, 1H), 5.2 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.9 (comp, 6H), 3.7 (s, 3H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



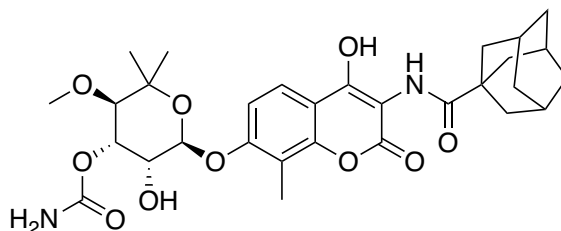
**MDM12**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.0 (s, 1H), 8.9 (s, 1H), 7.7 (d,  $J = 8.8$  Hz, 1H), 7.2 – 7.1 (m, 1H), 6.6 (s, 3H), 5.6 (d,  $J = 5.2$  Hz, 1H), 5.5 (d,  $J = 2.4$  Hz, 1H), 5.2 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.5 (s, 3H), 2.3 (s, 3H), 2.2 (s, 3H), 1.3 (s, 3H), 1.0 (s, 3H).



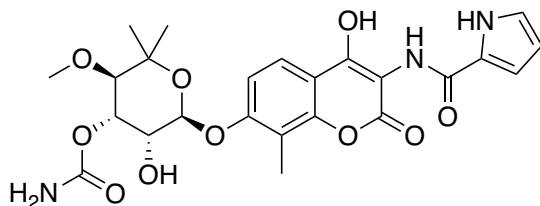
**MDM13**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.6 (s, 1H), 9.9 (s, 1H), 7.7 (d,  $J = 8.9$  Hz, 1H), 7.1 (d,  $J = 9.1$  Hz, 1H), 6.6 (s, 2H), 5.6 (s, 1H), 5.5 (d,  $J = 2.4$  Hz, 1H), 5.2 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.1 (t,  $J = 2.8$  Hz, 1H), 3.5 (comp, 4H), 2.2 (comp, 4H), 1.2 (s, 3H), 1.0 (s, 3H), 0.9 (comp, 4H).



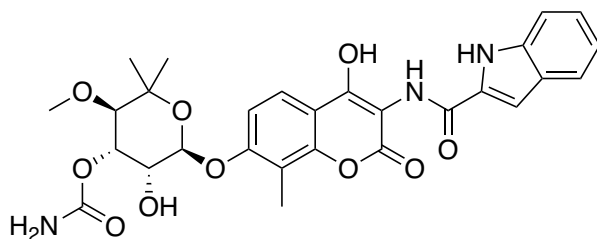
**MDM14**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.21 (s, 1H), 7.70 (d,  $J = 8.8$  Hz, 1H), 7.10 (d,  $J = 9.1$  Hz, 1H), 6.62 (d,  $J = 61.5$  Hz, 2H), 5.58 (s, 1H), 5.50 (d,  $J = 2.4$  Hz, 1H), 5.15 (dd,  $J = 9.7, 3.1$  Hz, 1H), 4.07 (d,  $J = 3.0$  Hz, 1H), 3.49 – 3.44 (comp, 4H), 3.23 (s, 1H), 2.19 (s, 3H), 1.99 – 1.91 (m, 3H), 1.90 – 1.84 (comp, 6H), 1.69 – 1.56 (comp, 6H), 1.25 (s, 3H), 1.04 (s, 3H).



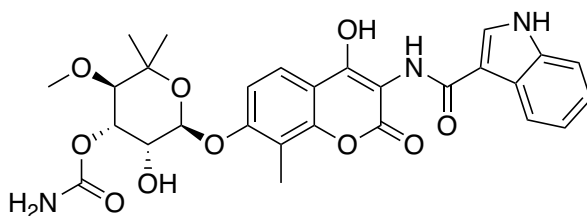
**MDM15**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.1 (s, 1H), 11.6 (s, 1H), 8.9 (s, 1H), 7.7 (d,  $J = 8.8$  Hz, 1H), 7.1 (s, 1H), 7.0 (s, 1H), 6.9 (s, 1H), 6.6 (comp, 2H), 6.2 – 6.1 (m, 1H), 5.6 (d,  $J = 5.1$  Hz, 1H), 5.5 – 5.5 (m, 1H), 5.2 (dd,  $J = 10.1, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



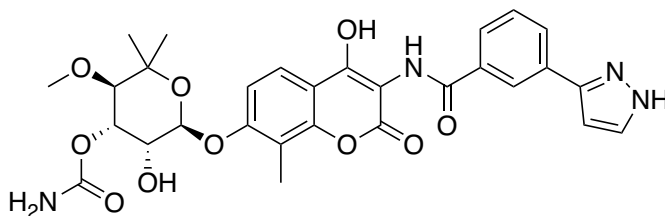
**MDM16**

NMR data unavailable



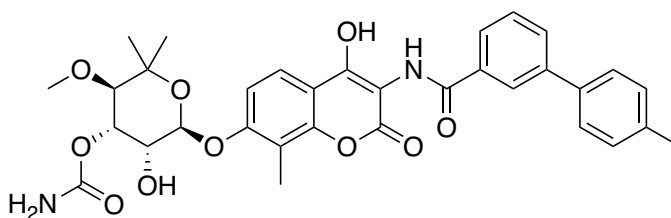
### MDM17

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.4 (s, 1H), 8.2 (comp, 2H), 8.1 (s, 1H), 7.6 (d,  $J = 8.7$  Hz, 1H), 7.4 (d,  $J = 8.1$  Hz, 1H), 7.1 (t,  $J = 7.6$  Hz, 1H), 7.1 – 7.0 (m, 1H), 6.9 (d,  $J = 8.7$  Hz, 1H), 6.6 (comp, 3H), 5.6 – 5.5 (m, 1H), 5.5 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.7, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



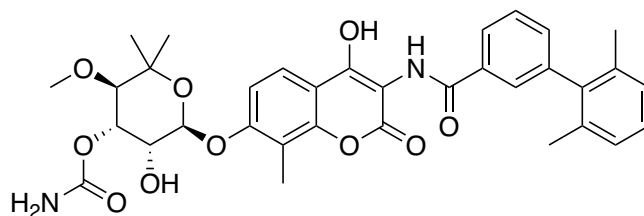
### MDM18

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  13.0 (s, 1H), 9.1 (s, 1H), 8.4 (s, 1H), 8.1 (s, 1H), 8.0 (s, 1H), 7.9 (d,  $J = 7.7$  Hz, 1H), 7.8 (s, 1H), 7.7 (d,  $J = 8.7$  Hz, 1H), 7.5 (t,  $J = 7.7$  Hz, 1H), 7.0 (s, 1H), 6.8 (s, 1H), 6.6 (comp, 2H), 5.6 (d,  $J = 5.1$  Hz, 1H), 5.5 (s, 1H), 5.2 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.1 (s, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



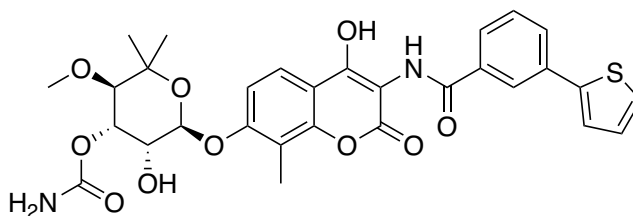
### MDM19

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.9 (s, 1H), 8.3 (s, 1H), 8.1 (s, 1H), 7.9 (d,  $J = 7.8$  Hz, 1H), 7.8 (d,  $J = 7.8$  Hz, 1H), 7.7 (d,  $J = 7.8$  Hz, 2H), 7.6 (d,  $J = 8.7$  Hz, 1H), 7.5 (t,  $J = 7.7$  Hz, 1H), 7.3 (d,  $J = 7.8$  Hz, 2H), 6.9 (d,  $J = 8.8$  Hz, 1H), 6.6 (comp, 2H), 5.5 – 5.5 (m, 1H), 5.5 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.4 (s, 3H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



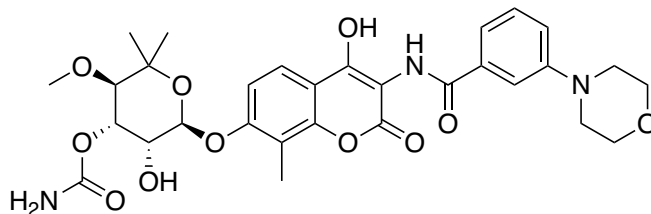
**MDM20**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.00 (s, 1H), 8.16 – 8.11 (m, 1H), 8.02 – 7.94 (m, 1H), 7.79 – 7.72 (m, 1H), 7.71 – 7.61 (m, 1H), 7.61 – 7.52 (m, 1H), 7.34 – 7.27 (m, 1H), 7.23 – 7.16 (m, 1H), 7.16 – 7.11 (m, 1H), 7.07 – 6.95 (m, 1H), 6.49 (comp, 2H), 5.48 (comp, 2H), 5.22 – 5.11 (m, 1H), 4.08 – 4.04 (m, 1H), 3.47 (comp, 4H), 2.18 (s, 3H), 2.00 (comp, 6H), 1.26 (s, 3H), 1.08 (s, 3H).



**MDM21**

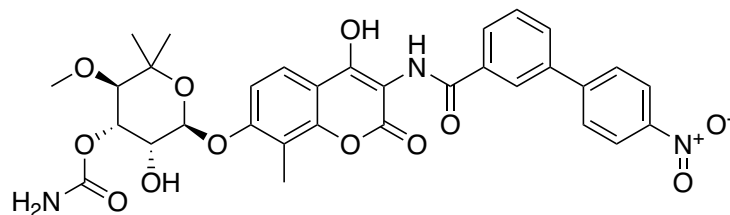
$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.8 (s, 1H), 8.3 (s, 1H), 8.1 (s, 1H), 7.9 (d,  $J = 7.7$  Hz, 1H), 7.8 (d,  $J = 7.8$  Hz, 1H), 7.6 (d,  $J = 3.9$  Hz, 2H), 7.6 (d,  $J = 5.0$  Hz, 1H), 7.5 (t,  $J = 7.7$  Hz, 1H), 7.2 (t,  $J = 4.4$  Hz, 1H), 6.9 (d,  $J = 8.8$  Hz, 1H), 6.7 – 6.5 (m, 2H), 5.5 (d,  $J = 4.9$  Hz, 1H), 5.4 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.7, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



**MDM22**

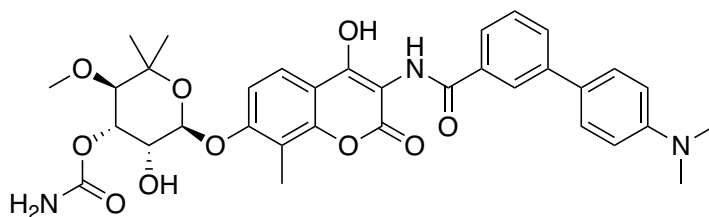
$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.7 (s, 1H), 8.1 (s, 1H), 7.6 (d,  $J = 8.6$  Hz, 1H), 7.5 (s, 1H), 7.4 (d,  $J = 7.7$  Hz, 1H), 7.3 (t,  $J = 7.9$  Hz, 1H), 7.1 (d,  $J = 8.2$  Hz, 1H), 6.9 (d,  $J = 8.9$  Hz, 1H), 6.6

(comp, 2H), 5.5 (d,  $J = 5.2$  Hz, 1H), 5.5 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.8, 3.0$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.8 (comp, 4H), 3.5 (comp, 4H), 3.2 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



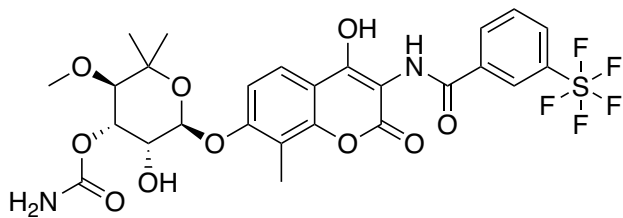
**MDM23**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  9.1 (s, 1H), 8.4 (s, 1H), 8.3 (d,  $J = 8.4$  Hz, 2H), 8.1 (s, 1H), 8.1 (d,  $J = 8.5$  Hz, 2H), 8.0 (d,  $J = 7.8$  Hz, 1H), 7.9 (d,  $J = 7.9$  Hz, 1H), 7.6 (comp, 2H), 6.9 (d,  $J = 8.9$  Hz, 1H), 6.5 (comp, 2H), 5.5 (d,  $J = 5.1$  Hz, 1H), 5.4 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.1 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



**MDM24**

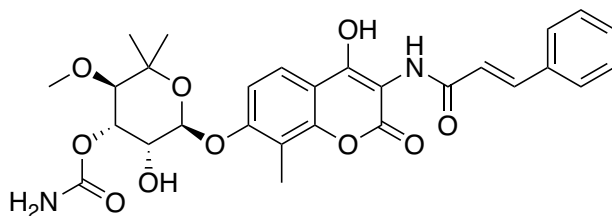
$^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  8.2 (s, 1H), 8.1 (s, 1H), 7.8 (d,  $J = 7.8$  Hz, 1H), 7.7 (d,  $J = 7.9$  Hz, 1H), 7.7 (comp, 4H), 7.5 (t,  $J = 7.7$  Hz, 1H), 7.0 – 7.0 (m, 1H), 6.8 (d,  $J = 8.9$  Hz, 2H), 6.5 (comp, 2H), 5.5 (d,  $J = 5.1$  Hz, 1H), 5.5 (s, 1H), 5.2 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 3.0 (comp, 6H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



**MDM25**

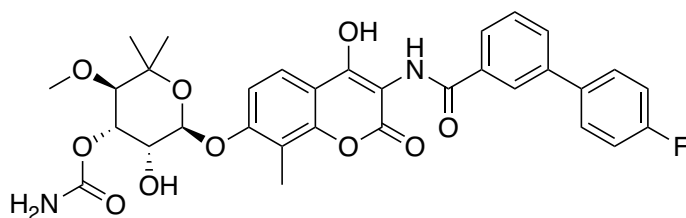


$^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  9.5 (s, 1H), 8.4 (s, 1H), 8.3 (d,  $J = 7.6$  Hz, 1H), 8.1 (d,  $J = 8.9$  Hz, 1H), 7.7 (t,  $J = 8.1$  Hz, 1H), 7.7 (d,  $J = 8.7$  Hz, 1H), 7.0 (s, 1H), 6.5 (s, 2H), 5.6 (d,  $J = 5.0$  Hz, 1H), 5.5 (s, 1H), 5.2 (comp, 2H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



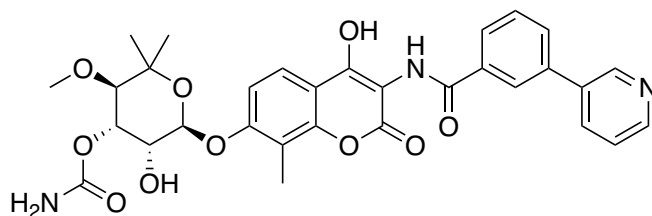
**MDM26**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  7.7 (d,  $J = 8.8$  Hz, 1H), 7.6 (d,  $J = 7.1$  Hz, 2H), 7.5 (d,  $J = 15.8$  Hz, 1H), 7.5 (comp, 2H), 7.4 – 7.4 (m, 1H), 7.1 (comp, 2H), 6.6 (comp, 4H), 5.6 (d,  $J = 5.2$  Hz, 1H), 5.5 (d,  $J = 2.5$  Hz, 1H), 5.2 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



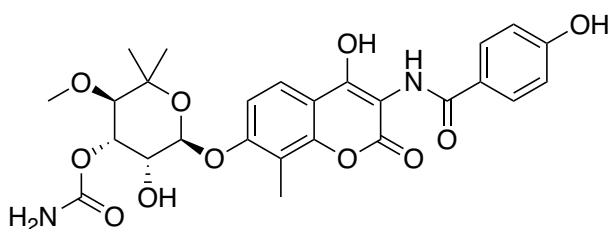
**MDM27**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  9.3 (s, 1H), 8.3 (d,  $J = 2.0$  Hz, 1H), 7.9 (d,  $J = 7.6$  Hz, 1H), 7.8 (dt,  $J = 11.9, 4.3$  Hz, 3H), 7.6 (d,  $J = 8.8$  Hz, 1H), 7.5 (t,  $J = 7.7$  Hz, 1H), 7.3 (comp, 3H), 7.0 (d,  $J = 8.8$  Hz, 1H), 6.7 (s, 2H), 5.6 (d,  $J = 5.1$  Hz, 1H), 5.5 (s, 1H), 5.2 (dd,  $J = 10.1, 3.2$  Hz, 1H), 4.1 (d,  $J = 4.5$  Hz, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



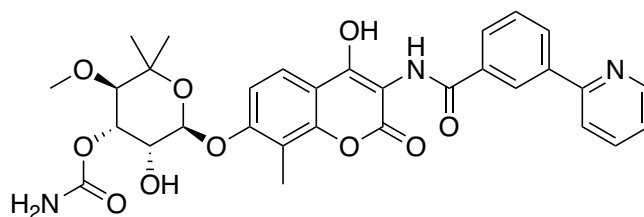
**MDM28**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.0 (s, 1H), 8.8 (s, 1H), 8.6 (d,  $J = 4.7$  Hz, 1H), 8.4 (s, 2H), 8.3 (s, 1H), 8.2 – 8.2 (m, 1H), 8.0 (d,  $J = 7.8$  Hz, 1H), 7.9 (d,  $J = 7.7$  Hz, 1H), 7.6 (d,  $J = 8.6$  Hz, 1H), 7.6 (t,  $J = 7.7$  Hz, 1H), 7.5 (dd,  $J = 8.0, 4.8$  Hz, 1H), 6.9 (d,  $J = 8.8$  Hz, 1H), 6.3 (s, 1H), 5.5 (s, 1H), 5.4 (d,  $J = 2.5$  Hz, 1H), 5.2 (dd,  $J = 9.7, 3.1$  Hz, 1H), 4.0 (s, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



**MDM29**

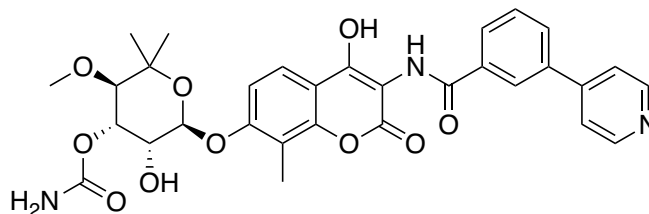
$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.9 (s, 1H), 8.7 (s, 1H), 7.8 (d,  $J = 8.4$  Hz, 2H), 7.6 (d,  $J = 8.8$  Hz, 1H), 7.0 (d,  $J = 8.9$  Hz, 1H), 6.8 (d,  $J = 8.3$  Hz, 2H), 6.5 (comp, 3H), 5.5 (d,  $J = 5.1$  Hz, 1H), 5.5 (d,  $J = 2.5$  Hz, 1H), 5.2 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



**MDM30**

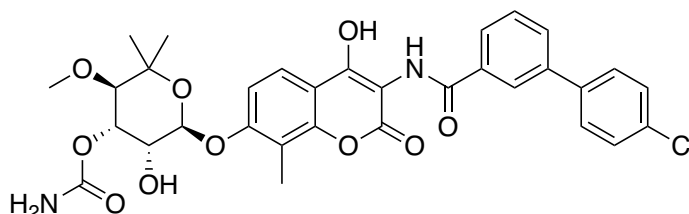
$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.9 (s, 1H), 8.7 (d,  $J = 4.7$  Hz, 1H), 8.7 (s, 1H), 8.2 (d,  $J = 7.8$  Hz, 1H), 8.1 (d,  $J = 8.0$  Hz, 1H), 8.0 (d,  $J = 7.7$  Hz, 1H), 7.9 (t,  $J = 7.7$  Hz, 1H), 7.6 (d,  $J = 8.6$  Hz,

1H), 7.6 (t,  $J = 7.7$  Hz, 1H), 7.4 (dd,  $J = 7.4, 4.8$  Hz, 1H), 6.9 (d,  $J = 8.8$  Hz, 1H), 6.5 (s, 3H), 5.5 (d,  $J = 5.0$  Hz, 1H), 5.4 (d,  $J = 2.5$  Hz, 1H), 5.2 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



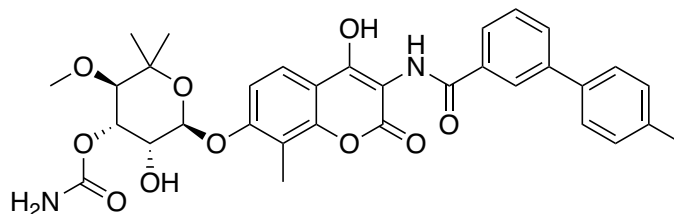
**MDM31**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.3 (s, 1H), 8.7 – 8.6 (m, 2H), 8.4 – 8.4 (m, 1H), 8.0 (d,  $J = 7.7$  Hz, 1H), 7.9 (d,  $J = 7.8$  Hz, 1H), 7.8 (d,  $J = 6.2$  Hz, 2H), 7.6 (comp, 2H), 7.0 (d,  $J = 8.7$  Hz, 1H), 6.5 (s, 2H), 5.6 (d,  $J = 5.1$  Hz, 1H), 5.4 (d,  $J = 2.6$  Hz, 1H), 5.2 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.1 (comp, 2H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



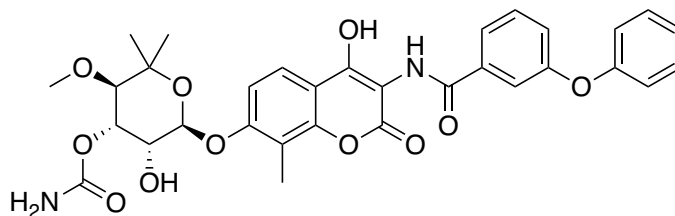
**MDM32**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.4 (s, 1H), 8.3 (s, 1H), 7.9 (d,  $J = 7.7$  Hz, 1H), 7.8 (comp, 3H), 7.6 (d,  $J = 8.8$  Hz, 1H), 7.5 (comp, 3H), 7.0 (d,  $J = 8.8$  Hz, 1H), 6.6 (comp, 3H), 5.6 (d,  $J = 5.2$  Hz, 1H), 5.5 – 5.5 (m, 1H), 5.2 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



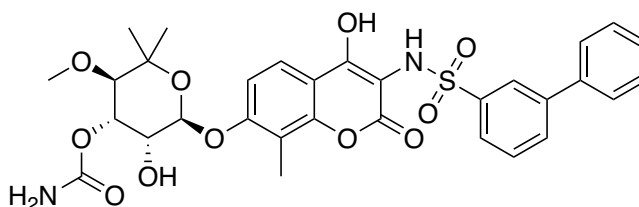
### MDM33

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  9.1 (s, 1H), 8.3 (s, 1H), 7.9 (d,  $J = 7.7$  Hz, 1H), 7.8 (d,  $J = 8.1$  Hz, 2H), 7.8 (d,  $J = 7.8$  Hz, 1H), 7.6 (comp, 3H), 7.5 (t,  $J = 7.7$  Hz, 1H), 6.9 (d,  $J = 8.8$  Hz, 1H), 6.5 (comp, 2H), 5.6 (d,  $J = 5.1$  Hz, 1H), 5.5 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.1 (s, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



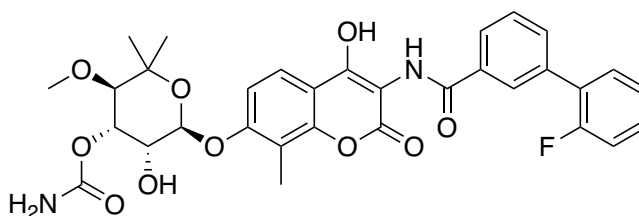
### MDM34

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  9.2 (s, 1H), 7.8 (d,  $J = 7.7$  Hz, 1H), 7.7 (d,  $J = 8.8$  Hz, 1H), 7.6 (d,  $J = 2.3$  Hz, 1H), 7.5 (t,  $J = 7.9$  Hz, 1H), 7.4 (comp, 2H), 7.2 (dt,  $J = 14.8, 7.8$  Hz, 2H), 7.0 (comp, 3H), 6.5 (s, 3H), 5.6 (d,  $J = 5.2$  Hz, 1H), 5.5 (s, 1H), 5.2 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp,  $J = 1.6$  Hz, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



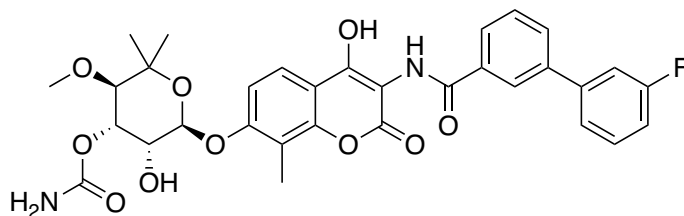
### MDM35

NMR data unavailable



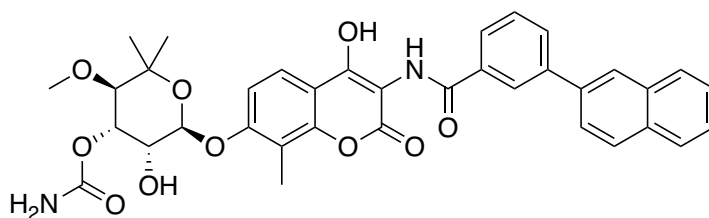
### MDM36

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.3 (s, 1H), 8.2 (s, 1H), 8.0 (d,  $J = 7.8$  Hz, 1H), 7.7 (d,  $J = 7.7$  Hz, 1H), 7.6 (d,  $J = 8.8$  Hz, 1H), 7.6 – 7.6 (m, 1H), 7.6 (t,  $J = 7.7$  Hz, 1H), 7.5 – 7.4 (m, 1H), 7.3 (comp, 2H), 7.0 (d,  $J = 8.9$  Hz, 1H), 6.5 (comp, 3H), 5.6 (d,  $J = 5.1$  Hz, 1H), 5.5 – 5.5 (m, 1H), 5.2 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.1 – 4.1 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



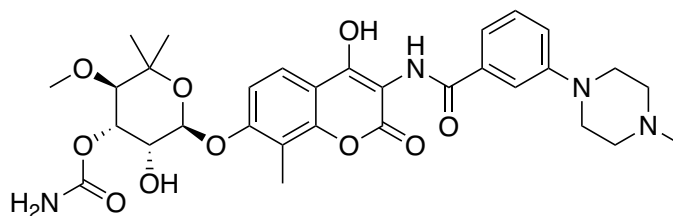
### MDM37

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.1 (s, 1H), 8.3 (s, 1H), 8.0 (d,  $J = 7.6$  Hz, 1H), 7.8 (d,  $J = 7.7$  Hz, 1H), 7.6 (comp, 3H), 7.5 (comp, 2H), 7.2 (td,  $J = 8.6, 2.5$  Hz, 1H), 7.0 (d,  $J = 8.7$  Hz, 1H), 6.5 (comp, 3H), 5.5 (d,  $J = 5.2$  Hz, 1H), 5.5 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



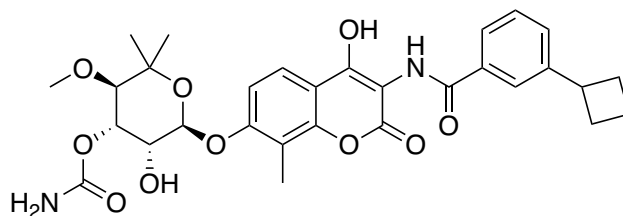
### MDM38

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.9 (s, 1H), 8.4 (s, 1H), 8.4 (s, 1H), 8.1 (s, 1H), 8.0 (comp, 2H), 8.0 (comp, 4H), 7.6 (d,  $J = 8.6$  Hz, 1H), 7.6 (comp, 3H), 6.9 (d,  $J = 8.7$  Hz, 1H), 6.5 (comp, 2H), 5.5 (d,  $J = 5.1$  Hz, 1H), 5.4 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.1 (d,  $J = 3.9$  Hz, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



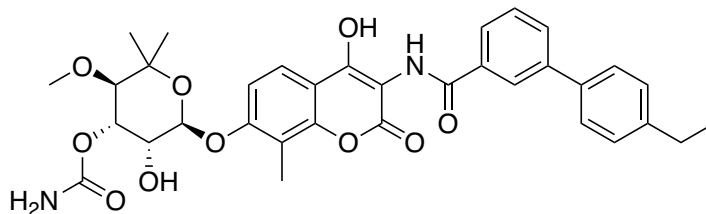
**MDM39**

NMR data unavailable



**MDM40**

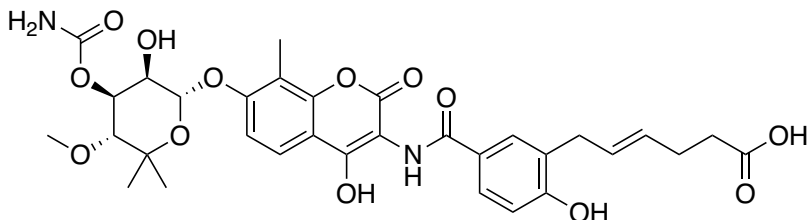
$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.1 (s, 1H), 7.9 (s, 1H), 7.8 (d,  $J = 6.7$  Hz, 1H), 7.6 (d,  $J = 8.7$  Hz, 1H), 7.3 (comp, 2H), 7.0 (d,  $J = 8.9$  Hz, 1H), 6.5 (comp, 2H), 5.6 (d,  $J = 5.1$  Hz, 1H), 5.5 – 5.5 (m, 1H), 5.2 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.6 (comp, 2H), 3.5 (comp, 4H), 2.3 (comp, 2H), 2.2 (s, 2H), 2.2 – 2.1 (m, 2H), 2.0 – 1.9 (m, 1H), 1.8 (dd,  $J = 10.7, 8.2$  Hz, 1H), 1.3 (s, 3H), 1.1 (s, 3H).



**MDM41**

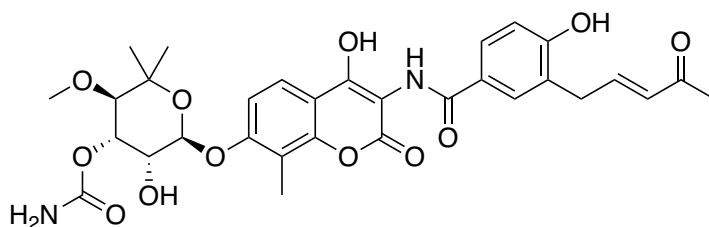
$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.0 (s, 1H), 8.3 (s, 1H), 7.9 (d,  $J = 7.7$  Hz, 1H), 7.8 (d,  $J = 7.7$  Hz, 1H), 7.7 (d,  $J = 7.9$  Hz, 2H), 7.6 (d,  $J = 8.7$  Hz, 1H), 7.5 (t,  $J = 7.7$  Hz, 1H), 7.3 (d,  $J = 7.9$  Hz,

2H), 6.9 (comp, 1H), 6.6 (d,  $J = 112.4$  Hz, 3H), 5.6 (d,  $J = 5.1$  Hz, 1H), 5.5 – 5.4 (m, 1H), 5.2 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 2.7 (q,  $J = 7.6$  Hz, 2H), 2.2 (s, 3H), 1.3 (s, 3H), 1.2 (comp, 3H), 1.1 (s, 3H).



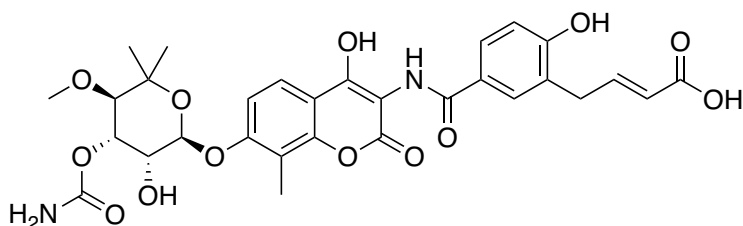
**MDM42**

NMR data unavailable



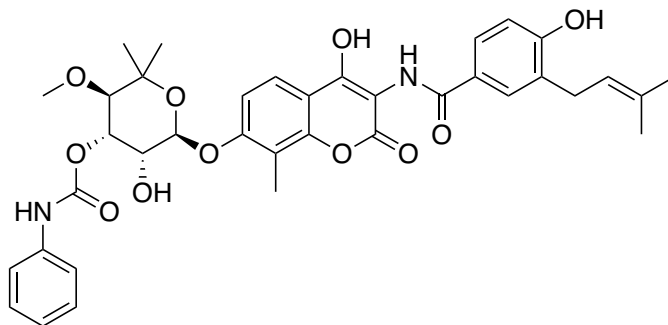
**MDM43**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.5 (s, 1H), 10.1 (s, 1H), 8.9 (s, 1H), 7.7 (comp, 2H), 7.7 (d,  $J = 8.8$  Hz, 1H), 7.0 (d,  $J = 8.9$  Hz, 1H), 7.0 (comp, 1H), 6.8 (d,  $J = 8.3$  Hz, 1H), 6.6 (comp, 2H), 6.0 (d,  $J = 15.8$  Hz, 1H), 5.6 (s, 1H), 5.5 (d,  $J = 2.5$  Hz, 1H), 5.2 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 6H), 2.2 (s, 6H), 1.3 (s, 3H), 1.1 (s, 3H).



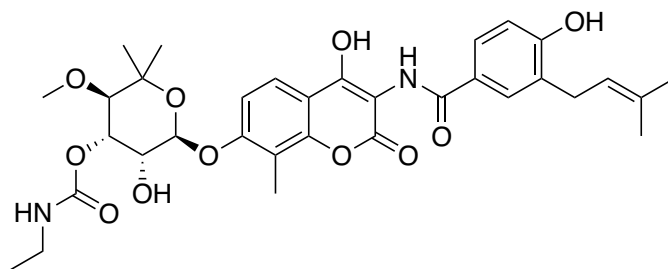
### MDM44

NMR data unavailable



### MDM45

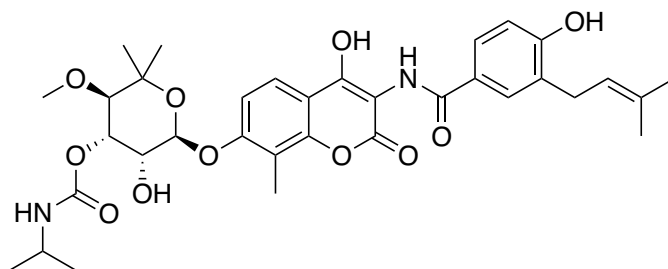
$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  10.0 (s, 1H), 9.8 (s, 1H), 9.1 (s, 1H), 7.7 (comp, 4H), 7.5 (comp, 2H), 7.3 (comp, 2H), 7.1 (d,  $J = 9.1$  Hz, 1H), 7.0 (t,  $J = 7.4$  Hz, 1H), 6.8 (d,  $J = 8.3$  Hz, 1H), 5.8 (d,  $J = 5.2$  Hz, 1H), 5.6 (d,  $J = 2.4$  Hz, 1H), 5.3 (comp, 2H), 4.2 – 4.2 (m, 1H), 3.6 (d,  $J = 9.9$  Hz, 1H), 3.5 (s, 3H), 3.3 (d,  $J = 7.4$  Hz, 2H), 2.2 (s, 3H), 1.7 (comp, 6H), 1.3 (s, 3H), 1.1 (s, 3H).



### MDM46

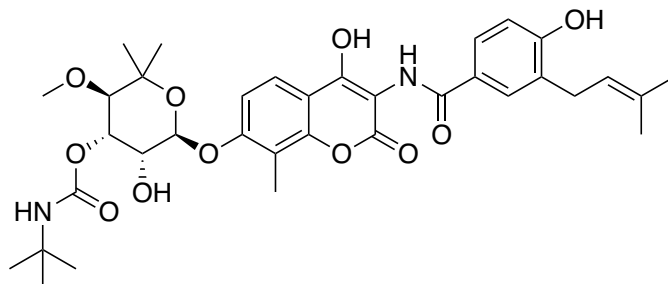
$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.8 (s, 1H), 8.4 (s, 1H), 7.7 (s, 1H), 7.6 (comp, 3H), 7.3 (t,  $J = 5.6$  Hz, 1H), 6.9 (s, 1H), 6.8 (d,  $J = 8.3$  Hz, 1H), 5.5 (s, 1H), 5.4 (s, 1H), 5.3 – 5.3 (m, 1H), 5.2 – 5.2 (m, 1H), 4.1 (s, 1H), 3.5 (comp, 4H), 3.3 (d,  $J = 7.5$  Hz, 2H), 3.1 (comp, 2H), 2.2 (s, 3H), 1.7 (comp, 6H), 1.3 (s, 3H), 1.1 (comp, 6H).





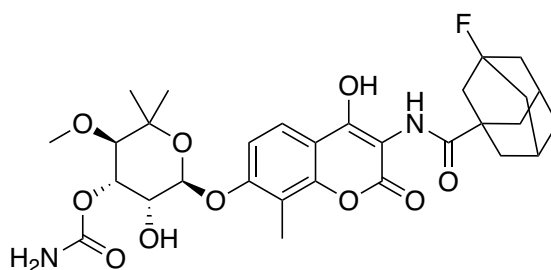
**MDM47**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.9 (s, 1H), 8.9 (s, 1H), 7.7 (comp, 3H), 7.2 (d,  $J$  = 7.8 Hz, 1H), 7.1 (d,  $J$  = 8.9 Hz, 1H), 6.8 (d,  $J$  = 8.3 Hz, 1H), 5.6 (d,  $J$  = 5.0 Hz, 1H), 5.5 (d,  $J$  = 2.5 Hz, 1H), 5.3 (t,  $J$  = 7.6 Hz, 1H), 5.2 – 5.2 (m, 1H), 4.1 (comp, 2H), 3.7 – 3.6 (m, 1H), 3.5 (comp, 4H), 3.2 (d,  $J$  = 7.3 Hz, 2H), 2.2 (s, 3H), 1.7 (comp, 6H), 1.3 (s, 3H), 1.1 (comp, 9H).



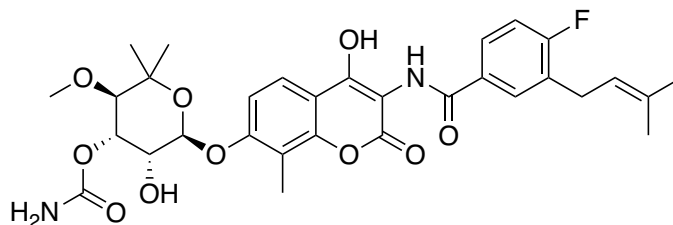
**MDM48**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.9 (s, 1H), 8.8 (s, 1H), 7.7 – 7.7 (m, 1H), 7.7 (comp,  $J$  = 8.5, 5.9 Hz, 2H), 7.0 (comp, 2H), 6.8 (d,  $J$  = 8.3 Hz, 1H), 5.5 (d,  $J$  = 5.0 Hz, 1H), 5.5 (d,  $J$  = 2.6 Hz, 1H), 5.3 (t,  $J$  = 7.4 Hz, 1H), 5.2 (dd,  $J$  = 9.6, 3.1 Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 (comp, 4H), 3.3 (d,  $J$  = 7.3 Hz, 2H), 2.2 (s, 3H), 1.7 (comp, 6H), 1.3 (comp, 12H), 1.1 (s, 3H).



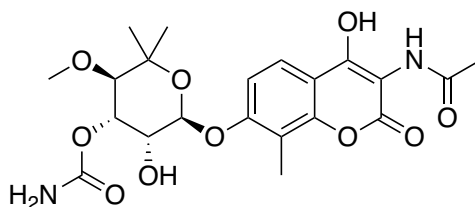
**MDM49**

NMR data unavailable



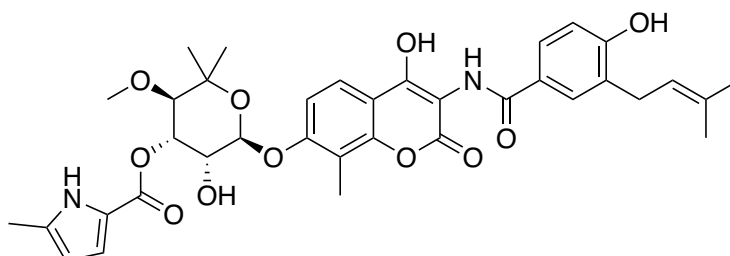
**MDM50**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.5 (br, 1H), 9.3 (s, 1H), 7.9 – 7.8 (comp, 2H), 7.6 (d,  $J$  = 8.8 Hz, 1H), 7.2 (t,  $J$  = 9.1 Hz, 1H), 7.1 (d,  $J$  = 8.9 Hz, 1H), 6.8 – 6.5 (comp, 2H), 5.6 (d,  $J$  = 5.2 Hz, 1H), 5.5 (d,  $J$  = 2.5 Hz, 1H), 5.3 (t,  $J$  = 7.5 Hz, 1H), 5.2 (dd,  $J$  = 9.9, 3.1 Hz, 1H), 4.1 – 4.0 (m, 1H), 3.5 – 3.5 (comp, 4H), 3.3 (d,  $J$  = 7.5 Hz, 2H), 2.2 (s, 3H), 1.7 – 1.7 (comp, 6H), 1.3 (s, 3H), 1.1 (s, 3H).



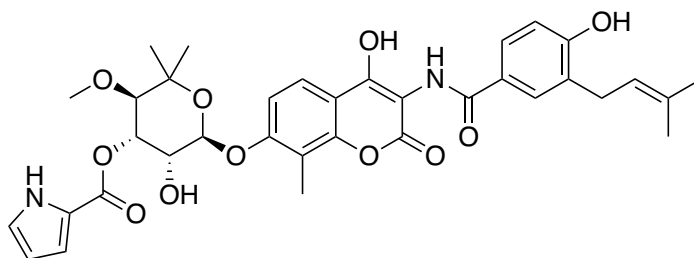
**MDM51**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.2 (s, 1H), 9.4 (s, 1H), 7.7 (d,  $J$  = 8.9 Hz, 1H), 7.1 (d,  $J$  = 9.0 Hz, 1H), 6.6 (comp,  $J$  = 114.2 Hz, 2H), 5.6 (d,  $J$  = 5.2 Hz, 1H), 5.5 (d,  $J$  = 2.5 Hz, 1H), 5.1 (dd,  $J$  = 9.9, 3.1 Hz, 1H), 4.1 – 4.1 (m, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 2.1 (s, 3H), 1.3 (s, 3H), 1.0 (s, 3H).



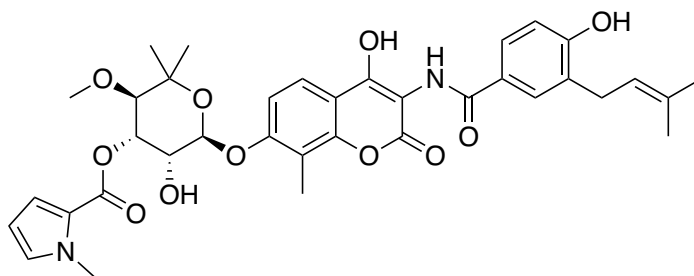
### MDM52

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.6 (s, 1H), 9.7 (s, 1H), 8.3 – 8.2 (m, 1H), 7.7 (s, 1H), 7.6 – 7.6 (comp, 2H), 6.9 (d,  $J = 8.8$  Hz, 1H), 6.8 – 6.7 (comp, 2H), 5.9 (m, 1H), 5.6 – 5.5 (m, 1H), 5.5 – 5.4 (m, 2H), 5.3 (t,  $J = 7.7$  Hz, 1H), 4.1 (s, 1H), 3.6 (d,  $J = 9.8$  Hz, 1H), 3.4 (s, 3H), 3.2 (d,  $J = 7.6$  Hz, 2H), 2.2 (s, 3H), 2.2 (s, 3H), 1.7 (comp, 6H), 1.3 (s, 3H), 1.1 (s, 3H)



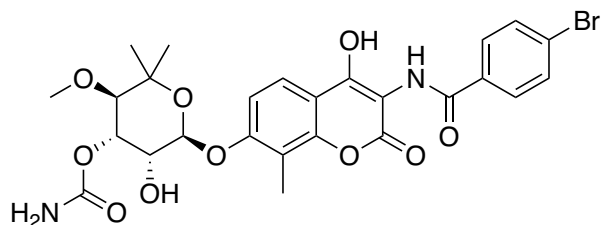
### MDM53

NMR data unavailable



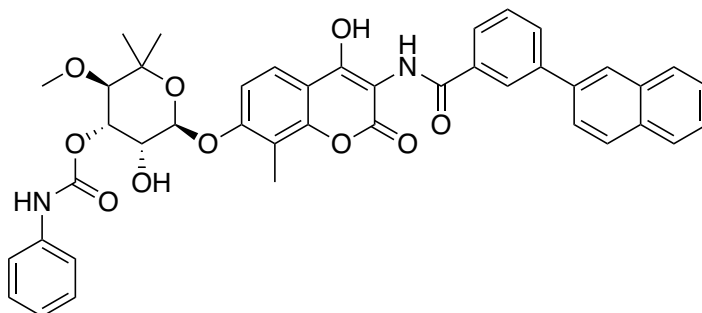
### MDM54

NMR data unavailable



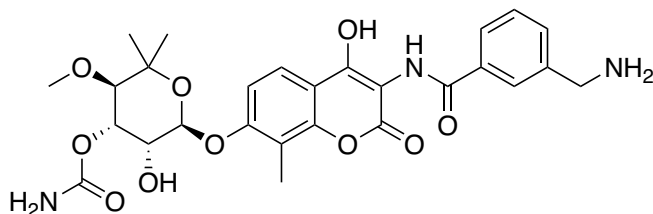
**MDM55**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.93 (s, 1H), 9.57 (s, 1H), 7.95 (comp, 2H), 7.75 (comp, 3H), 7.17 (d,  $J$  = 9.0 Hz, 1H), 6.60 (comp, 2H), 5.63 – 5.56 (m, 1H), 5.56 – 5.52 (m, 1H), 5.16 (dd,  $J$  = 9.8, 3.0 Hz, 1H), 4.08 (s, 1H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.27 (s, 3H), 1.05 (s, 3H).



**MDM56**

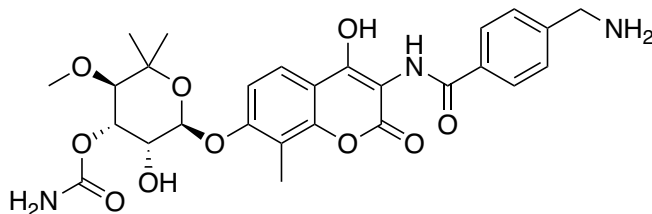
$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.8 (s, 1H), 9.3 (s, 1H), 8.5 (s, 1H), 8.3 (s, 1H), 8.0 (t,  $J$  = 8.0 Hz, 2H), 8.0 (comp, 4H), 7.7 – 7.6 (m, 1H), 7.6 (comp, 6H), 7.3 (t,  $J$  = 7.7 Hz, 2H), 7.0 (q,  $J$  = 7.3 Hz, 2H), 5.7 (s, 1H), 5.5 (s, 1H), 5.3 (d,  $J$  = 9.8 Hz, 1H), 4.2 (s, 1H), 3.5 (comp, 4H), 2.2 (s, 3H), 1.3 (s, 3H), 1.1 (s, 3H).



**MDM57**

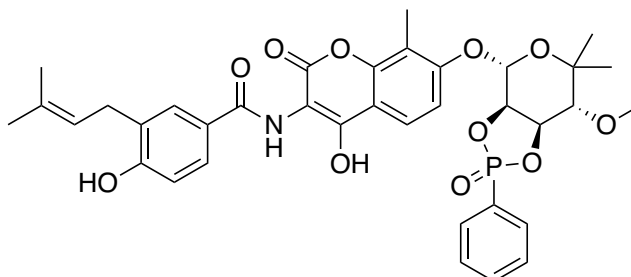
$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.50 (s, 1H), 8.23 (s, 2H), 8.04 (s, 1H), 7.95 (d,  $J$  = 7.6 Hz, 1H), 7.61 (d,  $J$  = 8.6 Hz, 1H), 7.53 (d,  $J$  = 7.7 Hz, 1H), 7.47 (t,  $J$  = 7.6 Hz, 1H), 6.88 (d,  $J$  = 8.8 Hz, 1H),

6.60 (comp, 2H), 5.50 (s, 1H), 5.44 – 5.40 (m, 1H), 5.16 (dd,  $J = 9.7, 3.1$  Hz, 1H), 4.03 (s, 3H), 3.45 (comp, 4H), 2.16 (s, 3H), 1.26 (s, 3H), 1.09 (s, 3H).



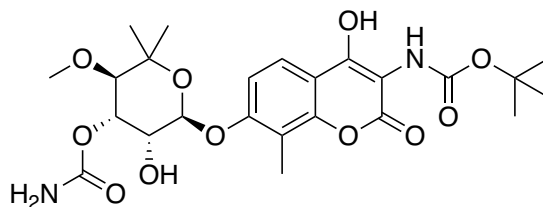
**MDM58**

NMR data unavailable



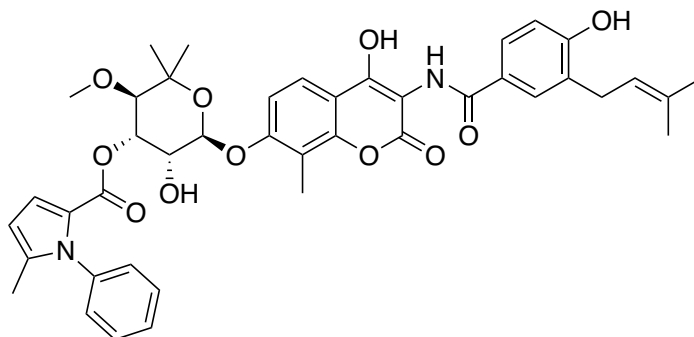
**MDM59**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  9.96 (s, 1H), 8.93 (s, 1H), 7.95 (comp, 2H), 7.71 (comp, 4H), 7.62 (comp, 2H), 7.15 (d,  $J = 9.0$  Hz, 1H), 6.82 (d,  $J = 8.3$  Hz, 1H), 6.53 (s, 1H), 6.04 (d,  $J = 3.7$  Hz, 1H), 5.30 (t,  $J = 7.3$  Hz, 1H), 5.20 (comp, 2H), 3.72 (d,  $J = 8.0$  Hz, 1H), 3.58 (s, 3H), 3.26 – 3.23 (m, 1H), 2.54 (s, 1H), 2.22 (s, 3H), 1.69 (comp, 6H), 1.38 (s, 3H), 1.17 (s, 3H).



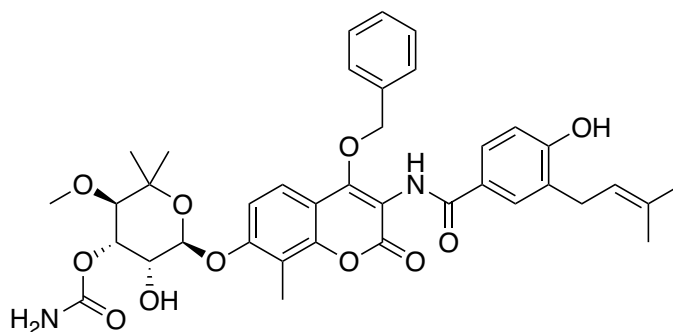
**MDM60**

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.79 (s, 1H), 7.87 (s, 1H), 7.69 (d,  $J$  = 9.4 Hz, 1H), 7.13 (d,  $J$  = 9.1 Hz, 1H), 6.61 (comp,  $J$  = 50.7 Hz, 2H), 5.58 (s, 1H), 5.53 – 5.48 (m, 1H), 5.14 (dd,  $J$  = 9.8, 3.0 Hz, 1H), 4.09 – 4.04 (m, 1H), 3.46 (comp, 4H), 2.19 (s, 3H), 1.43 (s, 9H), 1.26 (s, 3H), 1.04 (s, 3H).



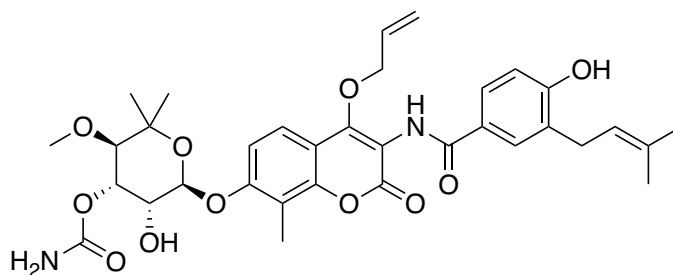
**MDM61**

NMR data unavailable



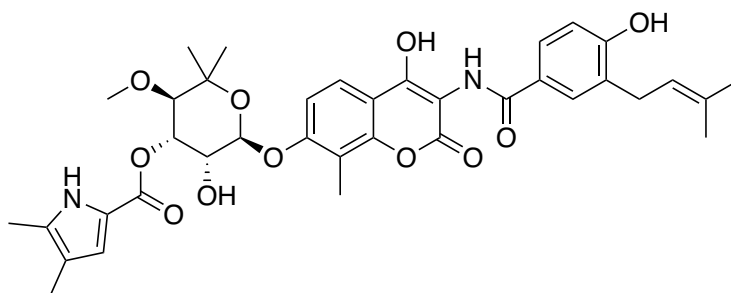
**MDM62**

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  10.16 (s, 1H), 9.66 (d,  $J$  = 2.5 Hz, 1H), 7.63 (comp, 3H), 7.07 (comp, 3H), 6.96 (comp, 3H), 6.86 (dd,  $J$  = 8.5, 2.0 Hz, 1H), 6.73 (comp, 2H), 5.65 – 5.56 (m, 1H), 5.49 (s, 1H), 5.33 – 5.22 (m, 1H), 5.07 (dd,  $J$  = 10.0, 3.0 Hz, 1H), 4.02 (s, 1H), 3.73 (t,  $J$  = 1.6 Hz, 1H), 3.45 (comp, 4H), 3.25 (d,  $J$  = 7.5 Hz, 2H), 2.54 (d,  $J$  = 2.0 Hz, 1H), 1.89 (d,  $J$  = 2.4 Hz, 3H), 1.68 (comp, 6H), 1.22 (s, 3H), 0.98 (d,  $J$  = 2.4 Hz, 3H).



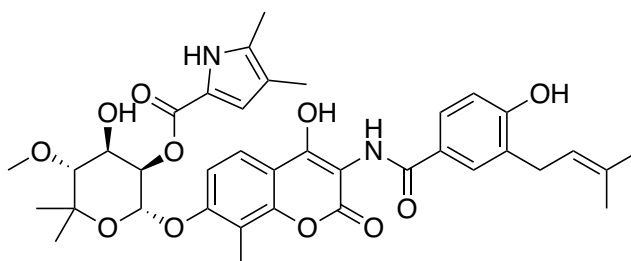
**MDM63**

NMR data unavailable



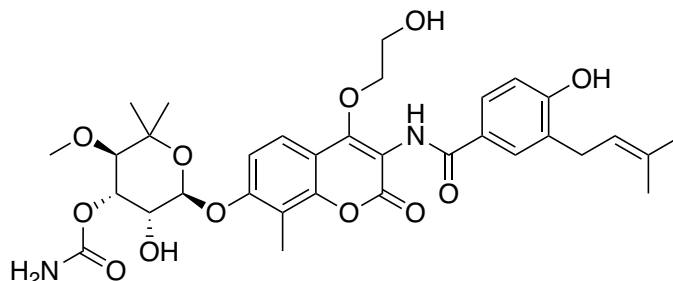
**MDM64**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.43 (s, 1H), 9.81 (s, 1H), 7.80 – 7.58 (m, 3H), 7.00 (s, 1H), 6.81 (d,  $J$  = 8.3 Hz, 1H), 6.64 (s, 1H), 6.52 (s, 2H), 5.57 (d,  $J$  = 4.9 Hz, 1H), 5.52 (s, 1H), 5.48 (d,  $J$  = 9.7 Hz, 1H), 5.37 – 5.24 (m, 1H), 4.13 (s, 1H), 3.62 (d,  $J$  = 9.8 Hz, 1H), 3.47 (s, 3H), 2.21 (s, 3H), 2.16 (s, 3H), 1.96 (s, 3H), 1.70 (s, 6H), 1.30 (s, 3H), 1.12 (s, 3H). (methylene protons obscured).



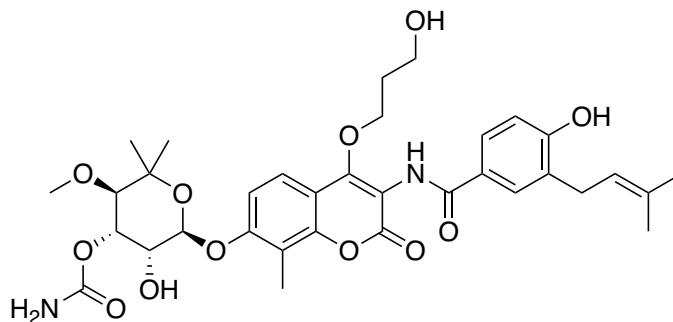
**MDM65**

NMR data unavailable



**MDM66**

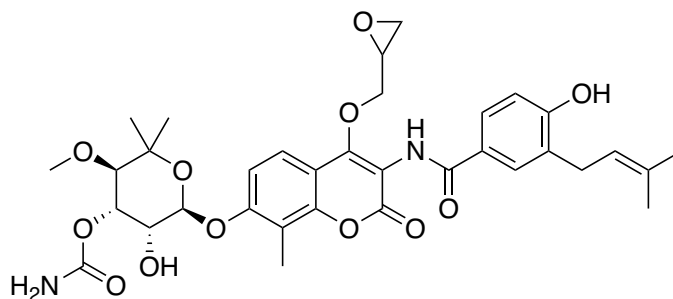
$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  10.07 (s, 1H), 9.44 (s, 1H), 7.82 (d,  $J$  = 9.0 Hz, 1H), 7.70 (comp, 2H), 7.17 (d,  $J$  = 9.1 Hz, 1H), 6.87 (d,  $J$  = 8.5 Hz, 1H), 6.59 (comp, 2H), 5.60 (d,  $J$  = 4.6 Hz, 1H), 5.57 – 5.54 (m, 1H), 5.35 – 5.27 (m, 1H), 5.15 (dd,  $J$  = 10.5, 2.3 Hz, 1H), 4.98 (t,  $J$  = 5.6 Hz, 1H), 4.36 (comp, 2H), 4.11 – 4.06 (m, 1H), 3.68 (comp, 2H), 3.48 (comp, 4H), 3.26 (comp, 2H), 2.22 (s, 3H), 1.72 – 1.66 (comp, 6H), 1.27 (s, 3H), 1.05 (s, 3H).



**MDM67**

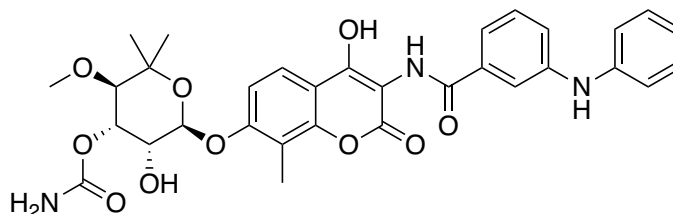
$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  10.08 (s, 1H), 9.45 (s, 1H), 7.72 (comp, 2H), 7.19 (s, 1H), 6.88 (comp, 2H), 6.69 (comp, 2H), 5.60 (comp, 3H), 5.35 – 5.28 (m, 1H), 5.16 (d,  $J$  = 11.2 Hz, 1H), 4.50 (comp, 2H), 4.08 (comp, 2H), 3.47 (comp, 8H), 3.29 – 3.24 (m, 1H), 2.22 (s, 3H), 2.04 – 1.99 (m, 1H), 1.86 (q,  $J$  = 5.8 Hz, 1H), 1.69 (comp, 4H), 1.27 (s, 3H), 1.05 (s, 3H).





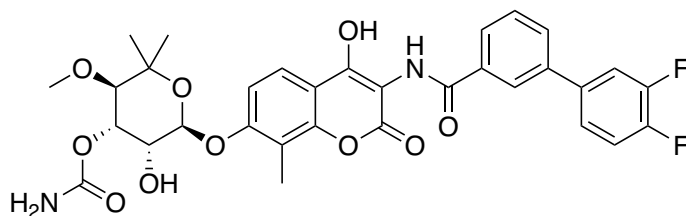
**MDM68**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  10.13 – 10.06 (m, 1H), 9.46 (s, 1H), 7.71 (comp, 3H), 7.20 (d,  $J$  = 9.1 Hz, 1H), 6.87 (d,  $J$  = 8.2 Hz, 1H), 6.63 (comp, 2H), 5.62 (d,  $J$  = 5.1 Hz, 1H), 5.55 (dd,  $J$  = 8.0, 2.6 Hz, 1H), 5.31 (t,  $J$  = 7.6 Hz, 1H), 5.19 – 5.11 (m, 1H), 4.64 (dd,  $J$  = 11.4, 2.6 Hz, 1H), 4.42 – 4.34 (m, 1H), 4.25 – 4.18 (m, 1H), 4.08 (d,  $J$  = 5.6 Hz, 1H), 3.47 (comp, 4H), 3.26 (comp, 2H), 2.82 – 2.78 (m, 1H), 2.69 – 2.66 (m, 1H), 2.22 (s, 3H), 1.70 (comp, 6H), 1.27 (s, 3H), 1.04 (s, 3H).



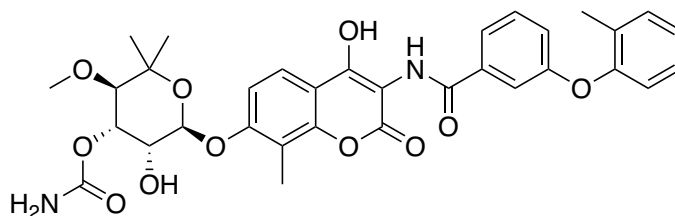
**MDM69**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.93 (s, 1H), 9.40 (s, 1H), 8.36 (s, 1H), 7.75 (d,  $J$  = 8.9 Hz, 1H), 7.71 – 7.66 (m, 1H), 7.48 (d,  $J$  = 7.7 Hz, 1H), 7.39 – 7.33 (m, 1H), 7.26 (comp, 3H), 7.15 (comp, 3H), 6.86 (t,  $J$  = 7.3 Hz, 1H), 6.62 (comp, 2H), 5.60 (s, 1H), 5.56 – 5.53 (m, 1H), 5.16 (dd,  $J$  = 10.6, 2.3 Hz, 1H), 4.13 – 4.06 (m, 1H), 3.48 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



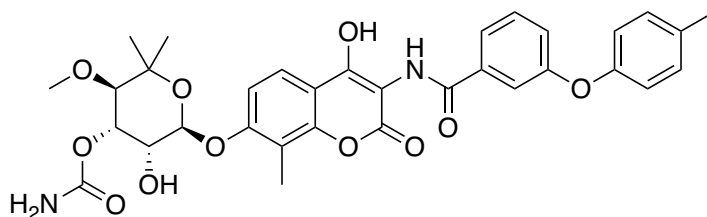
### MDM70

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.95 (s, 1H), 9.61 (s, 1H), 8.34 (s, 1H), 7.98 (d,  $J$  = 7.8 Hz, 1H), 7.91 (t,  $J$  = 8.2 Hz, 2H), 7.76 (d,  $J$  = 8.9 Hz, 1H), 7.62 (comp, 3H), 7.16 (d,  $J$  = 9.0 Hz, 1H), 6.68 (comp, 2H), 5.60 (d,  $J$  = 5.2 Hz, 1H), 5.54 (d,  $J$  = 2.4 Hz, 1H), 5.16 (dd,  $J$  = 9.9, 3.1 Hz, 1H), 4.12 – 4.06 (m, 1H), 3.48 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



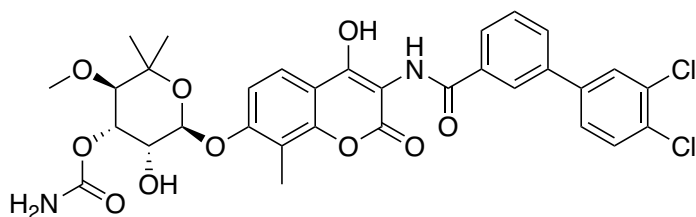
### MDM71

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  9.50 (s, 1H), 8.59 (d,  $J$  = 3.9 Hz, 1H), 7.83 – 7.71 (m, 2H), 7.50 (comp, 2H), 7.43 – 7.38 (m, 1H), 7.37 – 7.33 (m, 1H), 7.26 (comp, 1H), 7.15 (comp, 2H), 6.95 (dd,  $J$  = 8.0, 1.2 Hz, 1H), 6.62 (comp, 2H), 5.63 – 5.57 (m, 1H), 5.54 (d,  $J$  = 2.5 Hz, 1H), 5.16 (dd,  $J$  = 9.8, 3.2 Hz, 1H), 4.11 – 4.06 (m, 1H), 3.48 (comp, 4H), 2.21 (comp, 6H), 1.27 (s, 3H), 1.05 (s, 3H).



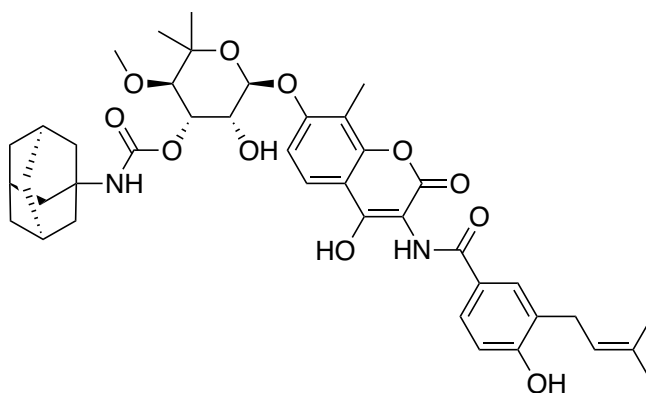
### MDM72

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.88 (s, 1H), 9.50 (s, 1H), 7.75 (comp, 2H), 7.61 – 7.56 (m, 1H), 7.51 (t,  $J = 7.9$  Hz, 1H), 7.20 (comp, 4H), 6.99 – 6.94 (comp, 2H), 6.67 (comp, 2H), 5.63 – 5.57 (m, 1H), 5.54 (d,  $J = 2.4$  Hz, 1H), 5.16 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.08 (s, 1H), 3.48 (comp, 4H), 2.30 (s, 3H), 2.22 (s, 3H), 1.27 (s, 3H), 1.05 (s, 3H).



**MDM73**

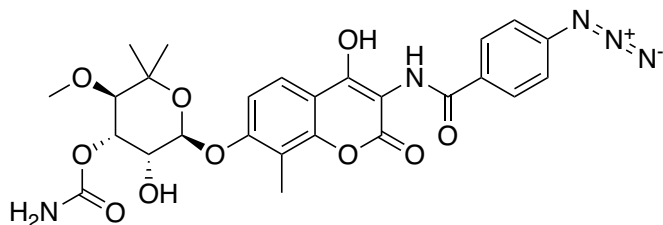
$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.92 (s, 1H), 9.63 (s, 1H), 8.38 (s, 1H), 8.15 – 8.05 (m, 1H), 7.98 (comp, 2H), 7.79 (comp, 3H), 7.63 (t,  $J = 7.7$  Hz, 1H), 7.17 (d,  $J = 9.0$  Hz, 1H), 6.58 (comp, 2H), 5.60 (d,  $J = 5.2$  Hz, 1H), 5.54 (d,  $J = 2.5$  Hz, 1H), 5.16 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.09 (s, 1H), 3.48 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



**MDM74**

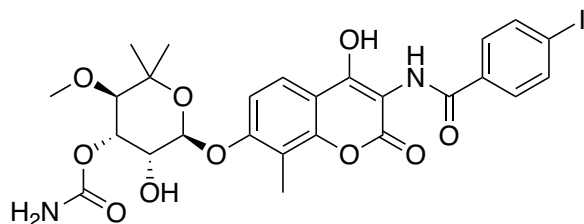
$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.82 (s, 1H), 8.46 (s, 1H), 7.70 – 7.67 (m, 1H), 7.64 (comp, 2H), 6.92 (comp, 2H), 6.79 (d,  $J = 8.3$  Hz, 1H), 5.51 (s, 1H), 5.44 (d,  $J = 2.6$  Hz, 1H), 5.31 (t,  $J = 7.4$  Hz, 1H), 5.18 (dd,  $J = 9.6, 3.1$  Hz, 1H), 4.01 (s, 1H), 3.44 (comp, 4H), 3.24 (d,  $J = 7.4$  Hz, 2H),

2.17 (s, 3H), 2.02 (comp, 4H), 1.90 (comp, 6H), 1.69 (comp, 6H), 1.62 (comp, 6H), 1.25 (s, 3H), 1.07 (s, 3H).



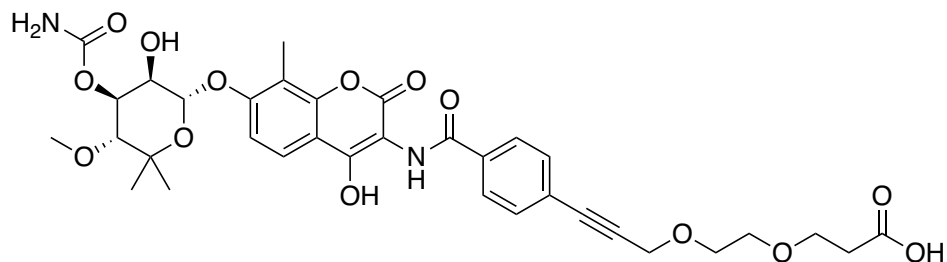
**MDM75**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.92 (s, 1H), 9.48 (s, 1H), 8.05 (comp, 2H), 7.75 (d,  $J$  = 8.8 Hz, 1H), 7.26 (comp, 2H), 7.16 (d,  $J$  = 9.0 Hz, 1H), 6.64 (comp, 2H), 5.59 (d,  $J$  = 5.2 Hz, 1H), 5.56 – 5.51 (m, 1H), 5.16 (dd,  $J$  = 9.9, 3.2 Hz, 1H), 4.12 – 4.05 (m, 1H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



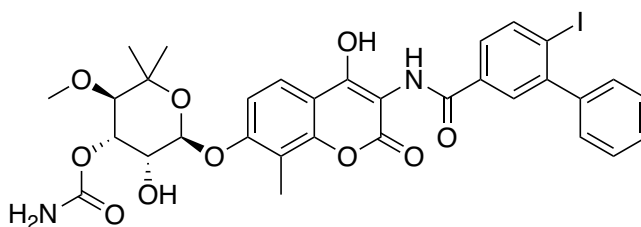
**MDM76**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.91 (s, 1H), 9.51 (s, 1H), 7.91 (comp, 2H), 7.76 (comp, 3H), 7.16 (d,  $J$  = 9.0 Hz, 1H), 6.68 (comp, 2H), 5.59 (d,  $J$  = 5.2 Hz, 1H), 5.56 – 5.50 (m, 1H), 5.16 (dd,  $J$  = 9.8, 3.1 Hz, 1H), 4.11 – 4.04 (m, 1H), 3.48 (comp, 4H), 2.22 (s, 3H), 1.27 (s, 3H), 1.05 (s, 3H).



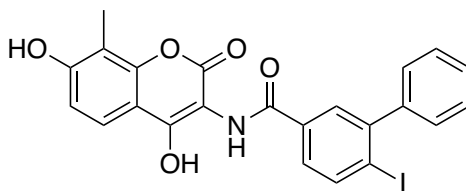
### MDM77

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.71 (s, 1H), 7.97 (d,  $J$  = 8.1 Hz, 1H), 7.62 (d,  $J$  = 8.5 Hz, 1H), 7.52 (d,  $J$  = 8.0 Hz, 1H), 6.89 (d,  $J$  = 8.8 Hz, 1H), 6.66 (comp, 2H), 5.49 (d,  $J$  = 5.2 Hz, 1H), 5.45 – 5.36 (m, 1H), 5.16 (dd,  $J$  = 9.9, 2.5 Hz, 1H), 4.43 (s, 2H), 4.07 – 4.02 (m, 1H), 3.63 (comp, 5H), 3.57 (comp, 3H), 3.45 (comp, 4H), 2.16 (s, 3H), 1.26 (s, 3H), 1.09 (comp, 6H).



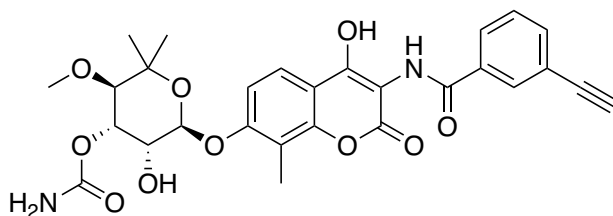
### MDM78

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.61 (s, 1H), 8.14 (d,  $J$  = 8.2 Hz, 1H), 7.97 (d,  $J$  = 2.1 Hz, 1H), 7.71 (comp, 2H), 7.45 (comp, 7H), 6.63 (comp, 2H), 5.59 (d,  $J$  = 5.2 Hz, 1H), 5.54 (d,  $J$  = 2.4 Hz, 1H), 5.15 (dd,  $J$  = 9.8, 3.1 Hz, 1H), 4.11 – 4.01 (m, 1H), 3.48 (comp, 4H), 2.22 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



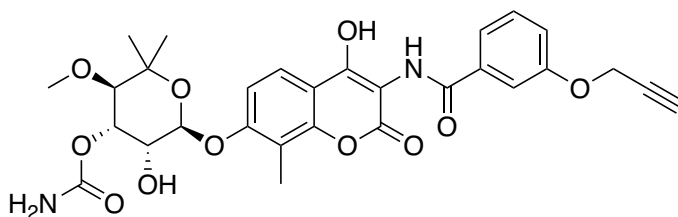
### MDM79

NMR data unavailable



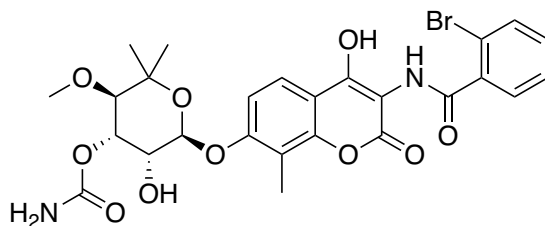
**MDM80**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.89 (s, 1H), 9.59 (s, 1H), 8.14 (s, 1H), 8.01 (d,  $J = 8.0$  Hz, 1H), 7.72 (comp, 2H), 7.55 (t,  $J = 7.8$  Hz, 1H), 7.17 (d,  $J = 9.1$  Hz, 1H), 6.66 (comp, 2H), 5.60 (d,  $J = 5.0$  Hz, 1H), 5.54 (d,  $J = 2.4$  Hz, 1H), 5.16 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.31 (s, 1H), 4.12 – 4.04 (m, 1H), 3.47 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.05 (s, 3H).



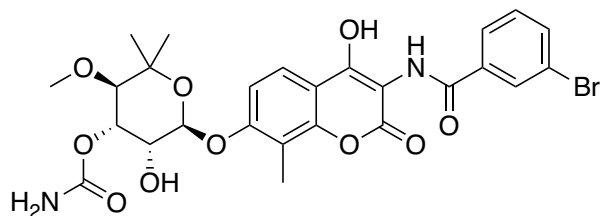
**MDM81**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.89 (s, 1H), 9.46 (s, 1H), 7.75 (d,  $J = 8.8$  Hz, 1H), 7.63 (comp, 2H), 7.46 (t,  $J = 8.1$  Hz, 1H), 7.20 (comp, 2H), 6.66 (comp, 2H), 5.60 (s, 1H), 5.54 (d,  $J = 2.5$  Hz, 1H), 5.16 (dd,  $J = 9.8, 3.2$  Hz, 1H), 4.88 (d,  $J = 2.4$  Hz, 2H), 4.08 (s, 1H), 3.59 (t,  $J = 2.4$  Hz, 1H), 3.48 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



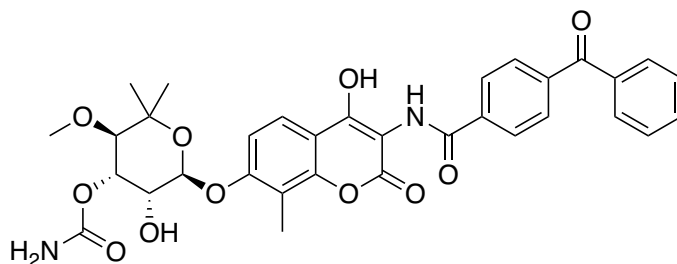
**MDM82**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.96 (s, 1H), 9.64 (s, 1H), 7.82 (comp, 2H), 7.70 (d,  $J = 7.8$  Hz, 1H), 7.56 – 7.46 (m, 1H), 7.46 – 7.38 (m, 1H), 7.18 (d,  $J = 9.0$  Hz, 1H), 6.63 (comp, 2H), 5.60 (s, 1H), 5.55 (d,  $J = 2.4$  Hz, 1H), 5.16 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.12 – 4.05 (m, 1H), 3.48 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.05 (s, 3H).



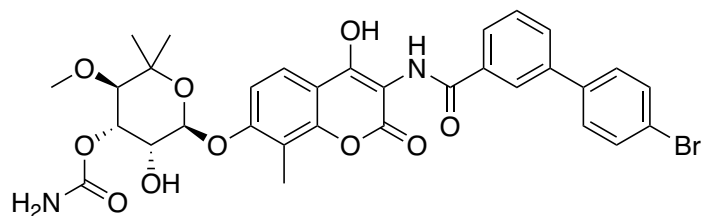
**MDM83**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.94 (s, 1H), 9.59 (s, 1H), 8.25 – 8.17 (m, 1H), 7.98 (d,  $J = 7.8$  Hz, 1H), 7.77 (comp, 2H), 7.49 (t,  $J = 7.9$  Hz, 1H), 7.17 (d,  $J = 8.9$  Hz, 1H), 6.62 (comp, 2H), 5.59 (d,  $J = 5.2$  Hz, 1H), 5.56 – 5.52 (m, 1H), 5.16 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.14 – 4.03 (m, 1H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



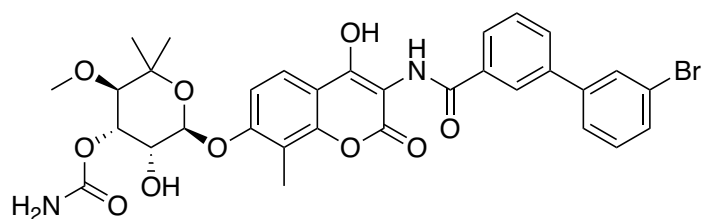
**MDM84**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.03 (s, 1H), 9.65 (s, 1H), 8.16 (comp, 2H), 7.85 (comp, 2H), 7.75 (comp, 4H), 7.60 (comp, 2H), 7.16 (d,  $J = 9.0$  Hz, 1H), 6.68 (comp, 2H), 5.60 (d,  $J = 5.2$  Hz, 1H), 5.54 (d,  $J = 2.4$  Hz, 1H), 5.16 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.12 – 4.06 (m, 1H), 3.47 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



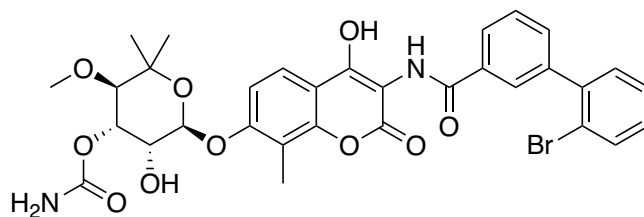
**MDM85**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.90 (s, 1H), 9.65 (s, 1H), 8.38 – 8.27 (m, 1H), 7.99 (d,  $J$  = 7.8 Hz, 1H), 7.91 (d,  $J$  = 7.8 Hz, 1H), 7.74 (comp, 5H), 7.62 (t,  $J$  = 7.8 Hz, 1H), 7.18 (d,  $J$  = 8.9 Hz, 1H), 6.62 (comp, 2H), 5.60 (d,  $J$  = 5.2 Hz, 1H), 5.57 – 5.53 (m, 1H), 5.16 (dd,  $J$  = 9.9, 3.0 Hz, 1H), 4.13 – 4.03 (m, 1H), 3.48 (comp, 4H), 2.24 (d,  $J$  = 1.8 Hz, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



**MDM86**

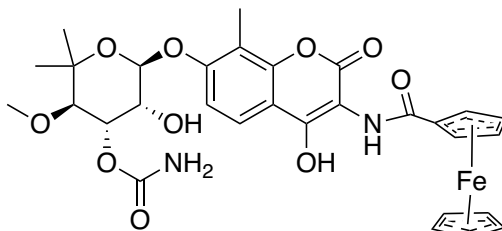
$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.65 (s, 1H), 8.36 (d,  $J$  = 2.1 Hz, 1H), 8.02 (d,  $J$  = 1.9 Hz, 1H), 7.95 (comp, 2H), 7.79 (comp, 2H), 7.62 (comp, 2H), 7.49 (comp, 1H), 7.18 (d,  $J$  = 9.0 Hz, 1H), 6.58 (comp, 2H), 5.61 (d,  $J$  = 5.2 Hz, 1H), 5.55 (d,  $J$  = 2.4 Hz, 1H), 5.16 (dd,  $J$  = 9.9, 3.1 Hz, 1H), 4.12 – 4.04 (m, 1H), 3.48 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



**MDM87**

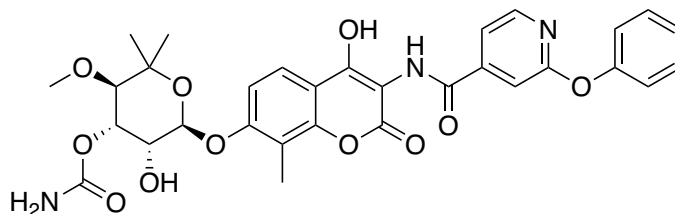


$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.90 (s, 1H), 9.57 (s, 1H), 8.04 (comp, 2H), 7.77 (comp, 3H), 7.62 (comp, 2H), 7.50 (comp, 3H), 7.37 (td,  $J = 7.6, 2.0$  Hz, 1H), 7.17 (d,  $J = 9.1$  Hz, 1H), 5.59 (s, 1H), 5.54 (d,  $J = 2.5$  Hz, 1H), 5.16 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.08 (s, 1H), 3.48 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



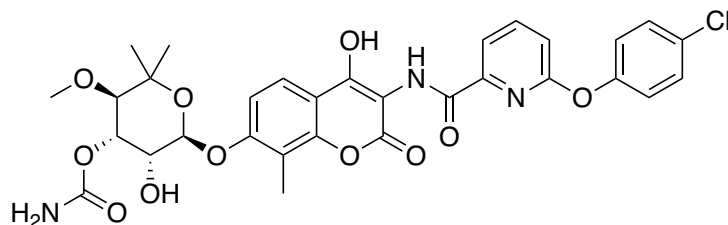
**MDM88**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.88 (s, 1H), 8.59 (s, 1H), 7.72 (d,  $J = 9.1$  Hz, 1H), 7.11 (s, 1H), 6.67 (comp, 2H), 5.57 (d,  $J = 5.2$  Hz, 1H), 5.51 (s, 1H), 5.16 (d,  $J = 9.6$  Hz, 1H), 4.94 (s, 2H), 4.41 (s, 2H), 4.33 (comp, 5H), 4.08 (s, 1H), 3.48 (comp, 4H), 2.22 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



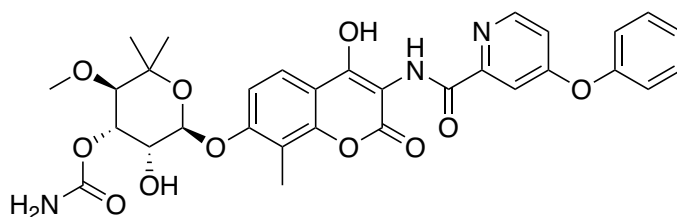
**MDM89**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  12.13 (s, 1H), 9.45 (s, 1H), 8.30 (d,  $J = 5.1$  Hz, 1H), 7.69 (d,  $J = 8.9$  Hz, 1H), 7.63 (d,  $J = 5.2$  Hz, 1H), 7.50 (s, 1H), 7.45 (comp, 2H), 7.24 (t,  $J = 7.4$  Hz, 1H), 7.17 (comp, 2H), 7.00 (d,  $J = 34.3$  Hz, 1H), 6.66 (comp, 2H), 5.55 (d,  $J = 5.1$  Hz, 1H), 5.49 (s, 1H), 5.16 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.11 – 4.03 (m, 1H), 3.46 (comp, 4H), 2.19 (s, 3H), 1.26 (s, 3H), 1.07 (s, 3H).



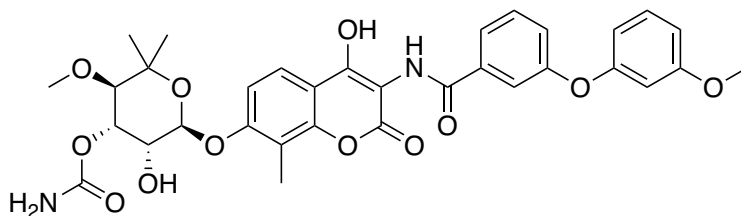
**MDM90**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.86 (s, 1H), 9.43 (s, 1H), 8.17 – 8.08 (m, 1H), 7.91 (d,  $J$  = 7.3 Hz, 1H), 7.72 (d,  $J$  = 9.0 Hz, 1H), 7.52 (comp, 2H), 7.32 (comp, 3H), 7.15 (d,  $J$  = 9.1 Hz, 1H), 6.63 (comp, 2H), 5.61 (d,  $J$  = 5.2 Hz, 1H), 5.52 (d,  $J$  = 2.5 Hz, 1H), 5.15 (dd,  $J$  = 9.9, 3.1 Hz, 1H), 4.07 (dt,  $J$  = 5.4, 2.8 Hz, 1H), 3.47 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H).



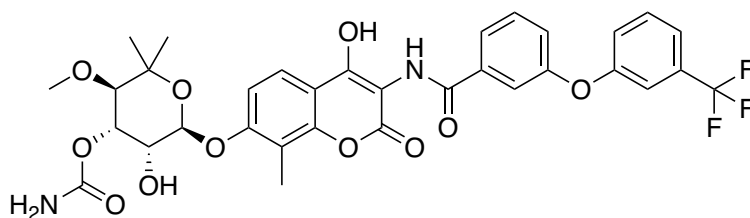
**MDM91**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.43 (s, 1H), 9.91 (s, 1H), 8.64 (d,  $J$  = 5.6 Hz, 1H), 7.73 (d,  $J$  = 8.9 Hz, 1H), 7.55 (comp,  $J$  = 7.9 Hz, 2H), 7.46 (d,  $J$  = 2.5 Hz, 1H), 7.37 (t,  $J$  = 7.4 Hz, 1H), 7.27 (comp, 3H), 7.16 (d,  $J$  = 9.0 Hz, 1H), 6.73 (comp, 2H), 5.61 (d,  $J$  = 5.2 Hz, 1H), 5.53 (d,  $J$  = 2.4 Hz, 1H), 5.15 (dd,  $J$  = 9.9, 3.2 Hz, 1H), 4.10 – 4.03 (m, 1H), 3.47 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H).



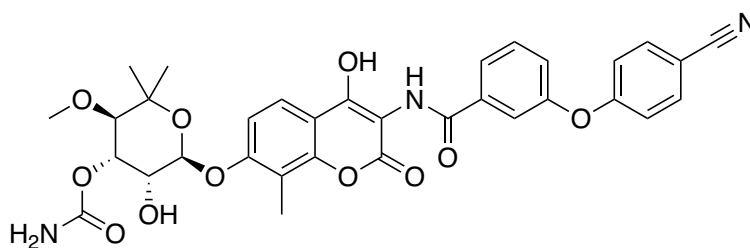
**MDM92**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.89 (s, 1H), 9.52 (s, 1H), 7.80 (d,  $J = 7.8$  Hz, 1H), 7.74 (d,  $J = 8.8$  Hz, 1H), 7.65 (d,  $J = 2.1$  Hz, 1H), 7.62 – 7.52 (m, 3H), 7.32 (t,  $J = 8.2$  Hz, 1H), 7.19 – 7.14 (m, 1H), 6.76 (dd,  $J = 8.2, 2.7$  Hz, 1H), 6.63 (t,  $J = 2.4$  Hz, 1H), 6.61 – 6.58 (m, 1H), 5.61 (d,  $J = 5.3$  Hz, 1H), 5.54 (d,  $J = 2.5$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.10 – 4.06 (m, 1H), 3.75 (s, 3H), 3.73 (d,  $J = 2.7$  Hz, 1H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



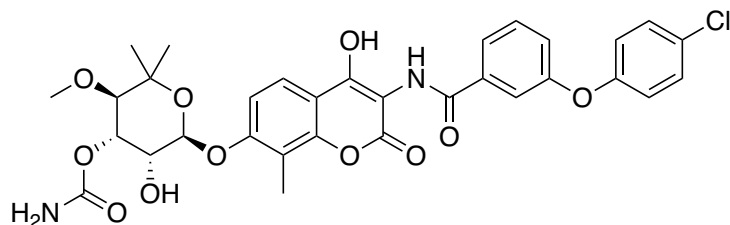
**MDM93**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.89 (s, 1H), 9.55 (s, 1H), 7.93 – 7.84 (m, 1H), 7.78 – 7.72 (m, 1H), 7.70 – 7.63 (m, 1H), 7.56 (comp, 3H), 7.36 (comp, 3H), 7.17 (d,  $J = 8.9$  Hz, 1H), 6.64 (comp, 2H), 5.62 (dd,  $J = 13.0, 5.2$  Hz, 1H), 5.54 (d,  $J = 2.4$  Hz, 1H), 5.15 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.08 (s, 1H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



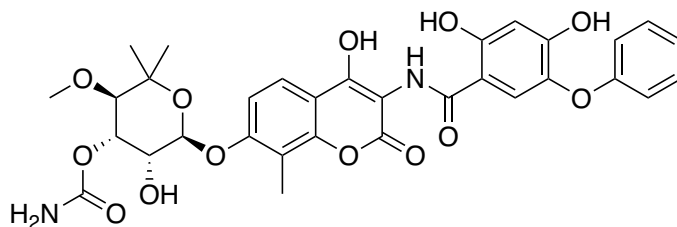
**MDM94**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.90 (s, 1H), 9.55 (s, 1H), 7.90 (comp,  $J = 10.6, 8.1$  Hz, 3H), 7.76 (comp, 2H), 7.63 (t,  $J = 7.9$  Hz, 1H), 7.42 – 7.38 (m, 1H), 7.17 (comp, 3H), 6.54 (comp, 2H), 5.60 (d,  $J = 5.2$  Hz, 1H), 5.53 (d,  $J = 2.4$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.11 – 4.04 (m, 1H), 3.48 (comp, 4H), 2.22 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



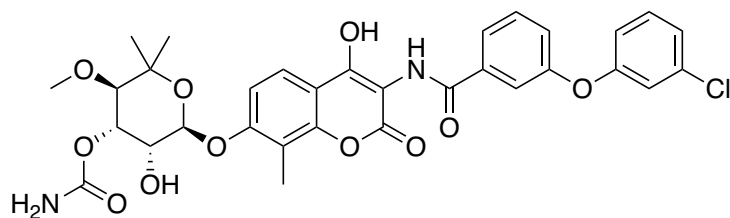
**MDM95**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.89 (s, 1H), 9.52 (s, 1H), 7.82 (d,  $J = 7.7$  Hz, 1H), 7.74 (d,  $J = 8.9$  Hz, 1H), 7.67 – 7.64 (m, 1H), 7.56 (t,  $J = 8.0$  Hz, 1H), 7.47 (comp, 2H), 7.29 (dd,  $J = 8.4, 2.5$  Hz, 1H), 7.16 (d,  $J = 9.0$  Hz, 1H), 7.09 (comp, 2H), 6.73 (comp, 2H), 5.61 (d,  $J = 5.2$  Hz, 1H), 5.53 (d,  $J = 2.4$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.10 – 4.06 (m, 1H), 3.48 (comp, 4H), 2.22 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



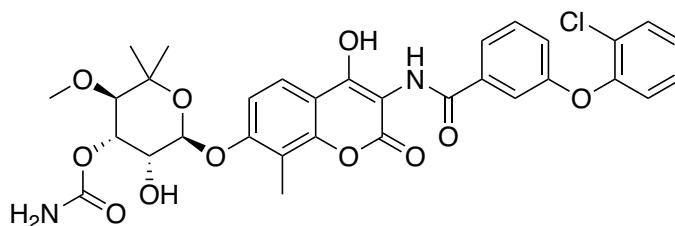
**MDM96**

$^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6$ )  $\delta$  13.69 (s, 1H), 12.39 (s, 1H), 10.63 (s, 1H), 7.80 – 7.61 (m, 2H), 7.31 (t,  $J = 7.9$  Hz, 2H), 7.17 (s, 1H), 7.01 (t,  $J = 7.3$  Hz, 1H), 6.86 (d,  $J = 8.1$  Hz, 2H), 6.57 (d,  $J = 42.7$  Hz, 3H), 5.60 (d,  $J = 5.2$  Hz, 1H), 5.52 (s, 1H), 5.15 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.10 – 4.03 (m, 1H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H).



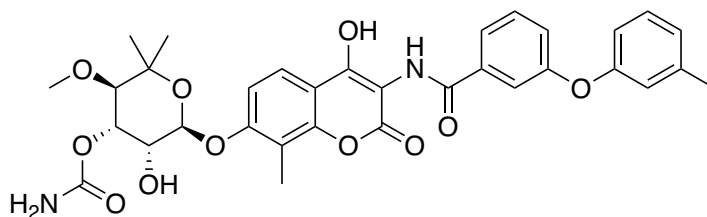
**MDM97**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.89 (s, 1H), 9.53 (s, 1H), 8.37 (s, 1H), 7.97 (dd,  $J$  = 14.6, 7.9 Hz, 1H), 7.85 (d,  $J$  = 7.9 Hz, 1H), 7.74 (d,  $J$  = 8.9 Hz, 1H), 7.69 (s, 1H), 7.58 (t,  $J$  = 8.0 Hz, 1H), 7.45 (s, 1H), 7.28 (dd,  $J$  = 33.5, 8.0 Hz, 1H), 7.21 – 6.99 (m, 2H), 6.68 (s, 2H), 5.59 (d,  $J$  = 5.1 Hz, 1H), 5.53 (s, 1H), 5.15 (d,  $J$  = 10.0 Hz, 1H), 4.11 – 4.04 (m, 1H), 3.48 (comp, 4H), 2.22 (s, 3H), 1.27 (s, 3H), 1.05 (s, 3H).



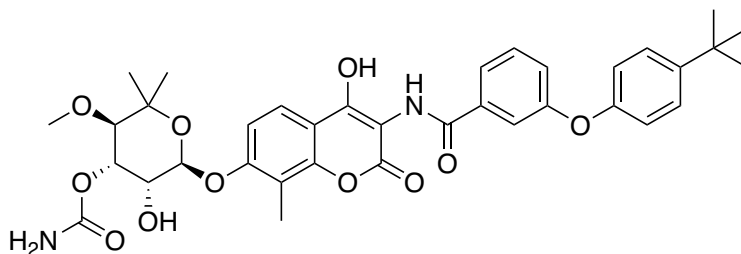
**MDM98**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.88 (s, 1H), 9.50 (s, 1H), 7.79 (d,  $J$  = 7.9 Hz, 1H), 7.73 (d,  $J$  = 8.8 Hz, 1H), 7.64 (d,  $J$  = 8.2 Hz, 1H), 7.56 (d,  $J$  = 2.2 Hz, 1H), 7.52 (d,  $J$  = 7.9 Hz, 1H), 7.42 (t,  $J$  = 7.7 Hz, 1H), 7.27 (t,  $J$  = 7.8 Hz, 1H), 7.17 (q,  $J$  = 9.8, 8.5 Hz, 2H), 6.67 (comp, 2H), 5.58 (d,  $J$  = 5.2 Hz, 1H), 5.55 – 5.50 (m, 1H), 5.15 (dd,  $J$  = 9.9, 3.2 Hz, 1H), 4.11 – 4.05 (m, 1H), 3.46 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



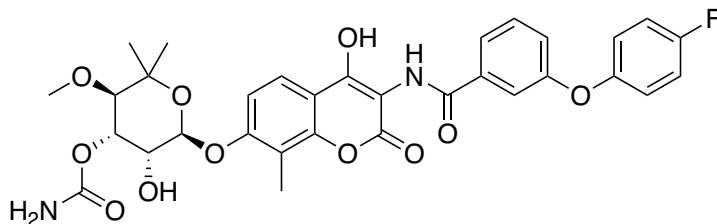
**MDM99**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.97 (s, 1H), 9.11 (s, 1H), 7.78 (d,  $J$  = 7.8 Hz, 1H), 7.67 (d,  $J$  = 8.8 Hz, 1H), 7.58 (s, 1H), 7.49 (t,  $J$  = 7.9 Hz, 1H), 7.29 (t,  $J$  = 7.8 Hz, 1H), 7.19 (d,  $J$  = 8.2 Hz, 1H), 6.98 (comp, 2H), 6.86 (comp, 2H), 6.66 (comp, 2H), 5.54 (d,  $J$  = 3.8 Hz, 1H), 5.48 (s, 1H), 5.15 (dd,  $J$  = 9.5, 2.9 Hz, 1H), 4.10 – 4.03 (m, 1H), 3.47 (comp, 4H), 2.31 (s, 3H), 2.18 (s, 3H), 1.26 (s, 3H), 1.07 (s, 3H).



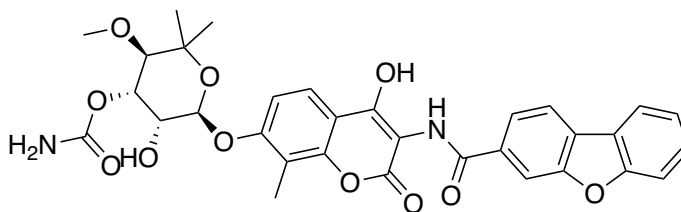
**MDM100**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.90 (s, 1H), 9.35 (s, 1H), 7.77 (d,  $J = 7.8$  Hz, 1H), 7.70 (d,  $J = 8.8$  Hz, 1H), 7.59 (s, 1H), 7.50 (t,  $J = 8.0$  Hz, 1H), 7.46 – 7.40 (m, 2H), 7.21 (d,  $J = 8.2$  Hz, 1H), 7.14 – 7.06 (m, 1H), 6.98 (d,  $J = 8.5$  Hz, 2H), 6.69 (comp, 2H), 5.57 (d,  $J = 5.1$  Hz, 1H), 5.51 (s, 1H), 5.15 (dd,  $J = 9.7, 2.9$  Hz, 1H), 4.10 – 4.03 (m, 1H), 3.47 (comp, 4H), 2.20 (s, 3H), 1.29 (s, 9H), 1.26 (s, 3H), 1.06 (s, 3H).



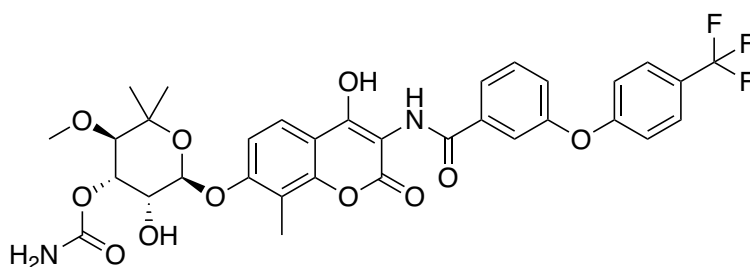
**MDM101**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.87 (s, 1H), 9.50 (s, 1H), 7.76 (comp, 2H), 7.63 – 7.58 (m, 1H), 7.53 (t,  $J = 7.9$  Hz, 1H), 7.25 (comp, 3H), 7.14 (comp, 3H), 6.67 (comp, 2H), 5.59 (d,  $J = 5.2$  Hz, 1H), 5.53 (d,  $J = 2.5$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.10 – 4.05 (m, 1H), 3.48 (comp, 4H), 2.22 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



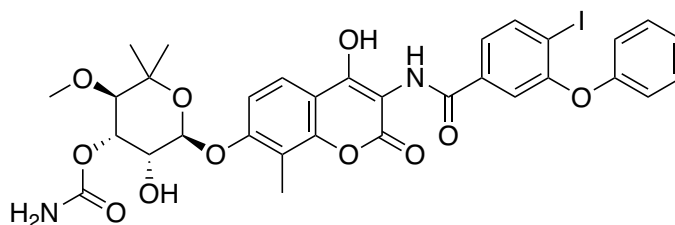
**MDM102**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.97 (s, 1H), 9.66 (s, 1H), 8.30 (comp, 3H), 8.09 (dd,  $J = 8.1$ , 1.4 Hz, 1H), 7.78 (comp, 2H), 7.66 – 7.56 (m, 1H), 7.47 (t,  $J = 7.6$  Hz, 1H), 7.19 (d,  $J = 9.1$  Hz, 1H), 6.69 (comp, 2H), 5.67 – 5.58 (m, 1H), 5.58 – 5.52 (m, 1H), 5.17 (dd,  $J = 10.1$ , 2.7 Hz, 1H), 4.14 – 4.04 (m, 1H), 3.48 (comp, 4H), 2.24 (s, 3H), 1.28 (s, 3H), 1.07 (s, 3H).



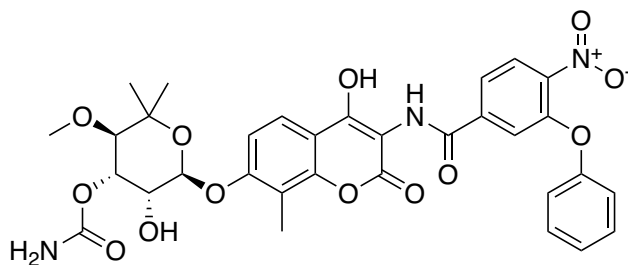
**MDM103**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.88 (s, 1H), 9.53 (s, 1H), 7.89 (d,  $J = 7.8$  Hz, 1H), 7.76 (comp, 4H), 7.62 (d,  $J = 8.5$  Hz, 1H), 7.39 (d,  $J = 8.0$  Hz, 1H), 7.18 (comp, 3H), 6.64 (comp, 2H), 5.59 (d,  $J = 4.1$  Hz, 1H), 5.56 – 5.50 (m, 1H), 5.15 (dd,  $J = 9.7$ , 3.0 Hz, 1H), 4.11 – 4.04 (m, 1H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



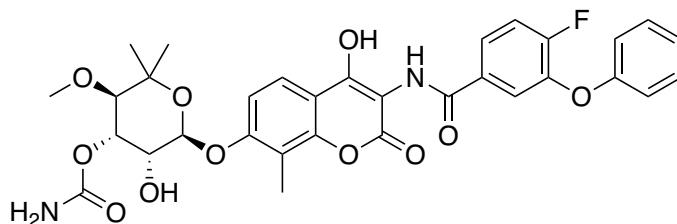
**MDM104**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.92 (d,  $J = 17.8$  Hz, 1H), 9.49 (s, 1H), 8.07 (d,  $J = 8.2$  Hz, 1H), 7.75 – 7.54 (m, 3H), 7.42 (t,  $J = 7.7$  Hz, 1H), 7.08 (comp, 4H), 6.65 (comp, 2H), 5.57 (d,  $J = 5.2$  Hz, 1H), 5.51 (s, 1H), 5.15 (dd,  $J = 9.8$ , 3.1 Hz, 1H), 4.33 (d,  $J = 4.2$  Hz, 1H), 4.09 – 4.03 (m, 1H), 3.46 (comp, 4H), 2.19 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



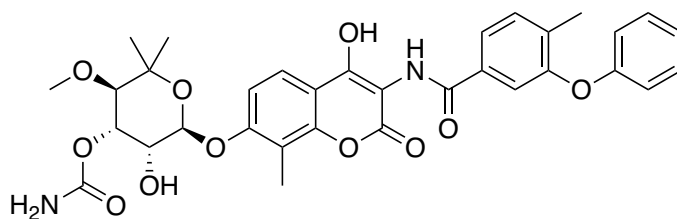
**MDM105**

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.94 (s, 1H), 9.68 (s, 1H), 8.18 (d,  $J$  = 8.5 Hz, 1H), 7.90 (d,  $J$  = 8.5 Hz, 1H), 7.72 – 7.63 (m, 2H), 7.44 (t,  $J$  = 7.8 Hz, 2H), 7.25 – 7.18 (m, 1H), 7.11 (d,  $J$  = 8.0 Hz, 3H), 6.57 (comp, 2H), 5.54 (d,  $J$  = 5.2 Hz, 1H), 5.51 – 5.46 (m, 1H), 5.11 (dd,  $J$  = 9.8, 3.1 Hz, 1H), 4.07 – 4.00 (m, 1H), 3.44 (comp, 4H), 2.16 (s, 3H), 1.22 (s, 3H), 1.01 (s, 3H).



**MDM106**

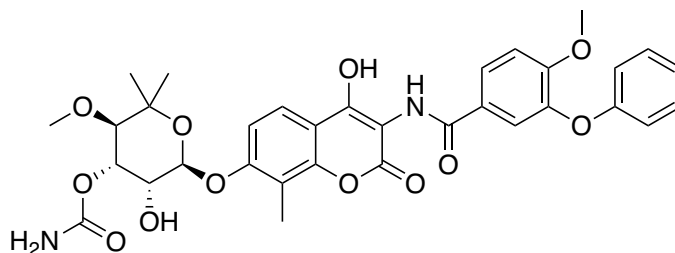
$^1\text{H}$  NMR (400 MHz DMSO- $d_6$ )  $\delta$  11.87 (s, 1H), 9.54 (s, 1H), 7.89 (d,  $J$  = 8.5 Hz, 1H), 7.80 (d,  $J$  = 8.0 Hz, 1H), 7.73 (d,  $J$  = 8.9 Hz, 1H), 7.60 – 7.52 (m, 1H), 7.42 (t,  $J$  = 7.7 Hz, 2H), 7.16 (comp, 2H), 7.05 (comp, 2H), 6.61 (comp, 2H), 5.59 (s, 1H), 5.53 (s, 1H), 5.19 – 5.10 (m, 1H), 4.10 – 4.05 (m, 1H), 3.47 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H).



**MDM107**

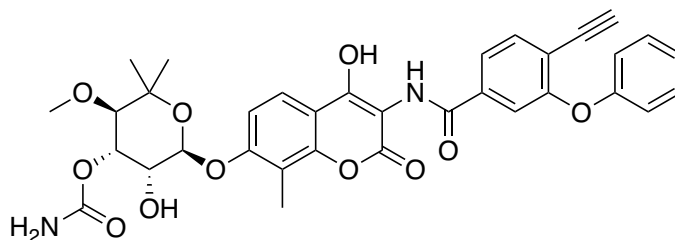


$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.84 (s, 1H), 9.45 (s, 1H), 7.75 (comp, 2H), 7.56 (s, 1H), 7.48 (d,  $J = 7.9$  Hz, 1H), 7.39 (comp, 2H), 7.14 (comp, 2H), 6.95 (comp, 2H), 6.72 (comp, 2H), 5.59 (s, 1H), 5.53 (d,  $J = 2.8$  Hz, 1H), 5.15 (d,  $J = 9.4$  Hz, 1H), 4.10 – 4.04 (m, 1H), 3.46 (comp, 4H), 2.26 (s, 3H), 2.21 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H).



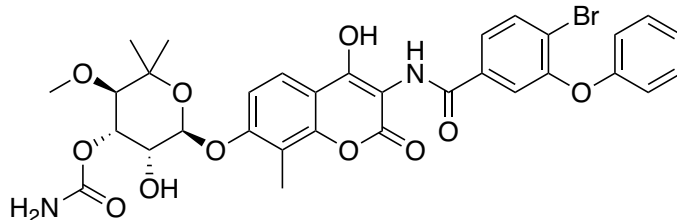
**MDM108**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.84 (s, 1H), 9.40 (s, 1H), 7.92 (dd,  $J = 8.6, 2.2$  Hz, 1H), 7.73 (comp, 2H), 7.33 (comp, 3H), 7.16 (d,  $J = 9.1$  Hz, 1H), 7.07 (d,  $J = 7.4$  Hz, 1H), 6.89 (comp, 2H), 6.66 (comp, 2H), 5.59 (d,  $J = 5.2$  Hz, 1H), 5.53 (d,  $J = 2.4$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.11 – 4.05 (m, 1H), 3.84 (s, 3H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



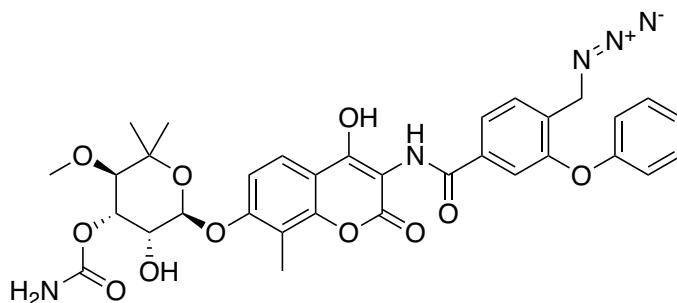
**MDM109**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.87 (s, 1H), 9.58 (s, 1H), 7.82 (d,  $J = 8.7$  Hz, 1H), 7.73 (d,  $J = 8.4$  Hz, 2H), 7.58 (s, 1H), 7.43 (t,  $J = 7.9$  Hz, 2H), 7.17 (t,  $J = 9.1$  Hz, 2H), 7.04 (d,  $J = 8.0$  Hz, 2H), 6.59 (comp, 2H), 5.53 (comp, 2H), 5.15 (d,  $J = 9.7$  Hz, 1H), 4.48 (s, 1H), 4.08 (s, 1H), 3.48 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



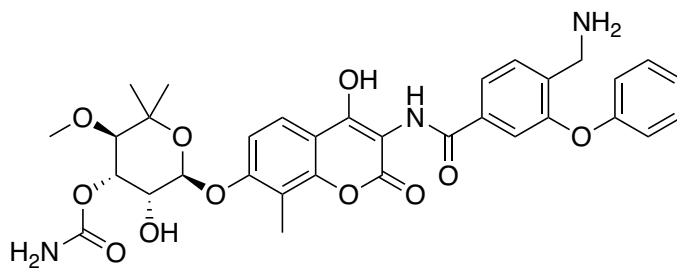
### MDM110

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  9.59 (s, 1H), 8.60 (s, 1H), 7.94 – 7.81 (m, 1H), 7.80 – 7.65 (m, 3H), 7.44 (d,  $J$  = 8.0 Hz, 2H), 7.17 (comp, 2H), 7.02 (d,  $J$  = 8.1 Hz, 2H), 6.61 (comp, 2H), 5.59 (s, 1H), 5.53 (s, 1H), 5.19 – 5.10 (m, 1H), 4.10 – 4.06 (m, 1H), 3.48 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



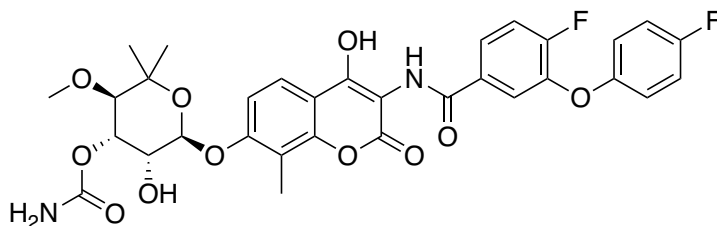
### MDM111

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.86 (s, 1H), 9.53 (s, 1H), 7.85 (d,  $J$  = 8.1 Hz, 1H), 7.73 (d,  $J$  = 9.0 Hz, 1H), 7.65 (d,  $J$  = 8.0 Hz, 1H), 7.52 (s, 1H), 7.45 (comp, 2H), 7.18 (comp, 2H), 7.06 (comp, 2H), 6.63 (comp, 2H), 5.59 (s, 1H), 5.53 (s, 1H), 5.15 (d,  $J$  = 9.8 Hz, 1H), 4.58 (s, 2H), 4.11 – 4.05 (m, 1H), 3.47 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



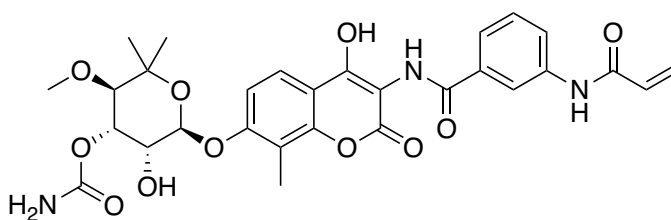
### MDM112

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.66 (s, 1H), 7.83 (d,  $J = 8.0$  Hz, 1H), 7.59 (comp, 2H), 7.43 (comp, 3H), 7.18 (t,  $J = 7.4$  Hz, 1H), 7.07 (comp, 2H), 6.86 (comp, 2H), 6.60 (comp, 2H), 5.49 (d,  $J = 5.1$  Hz, 1H), 5.40 (d,  $J = 2.6$  Hz, 1H), 5.15 (dd,  $J = 9.7, 3.2$  Hz, 1H), 4.03 (s, 3H), 3.44 (comp, 4H), 2.14 (s, 3H), 1.25 (s, 3H), 1.08 (s, 3H). (missing two signals, likely those of the primary amine  $\text{NH}_2$ ).



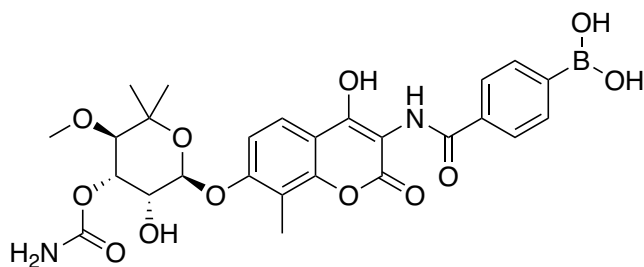
**MDM113**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.87 (s, 1H), 9.53 (s, 1H), 7.74 (comp, 2H), 7.55 (comp, 2H), 7.19 (comp, 4H), 7.09 – 7.01 (m, 1H), 6.65 (comp, 2H), 5.59 (br, 1H), 5.53 (d,  $J = 2.4$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.08 (s, 1H), 3.46 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



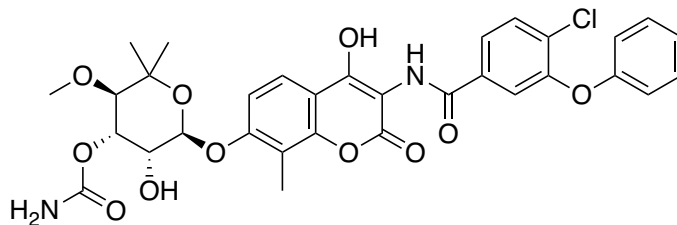
**MDM114**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.93 (s, 1H), 10.33 (d,  $J = 2.6$  Hz, 1H), 9.44 (s, 1H), 8.16 (s, 1H), 7.94 (dd,  $J = 24.3, 8.4$  Hz, 1H), 7.75 (comp, 2H), 7.47 (t,  $J = 8.0$  Hz, 1H), 7.16 (d,  $J = 8.7$  Hz, 1H), 6.65 (comp, 2H), 6.51 – 6.36 (m, 1H), 6.29 (d,  $J = 17.1$  Hz, 1H), 5.78 (d,  $J = 10.0$  Hz, 1H), 5.62 – 5.56 (m, 1H), 5.54 (s, 1H), 5.16 (d,  $J = 9.9$  Hz, 1H), 4.11 – 4.04 (m, 1H), 3.47 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



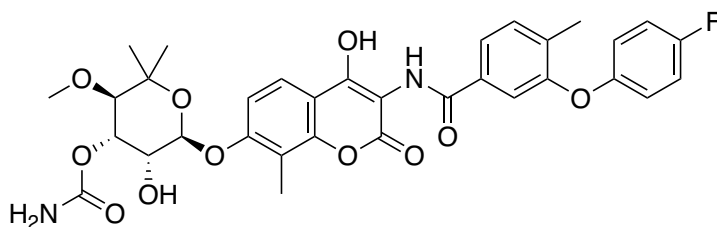
### MDM115

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.91 (s, 1H), 9.46 (s, 1H), 8.23 (d,  $J$  = 5.3 Hz, 2H), 7.97 (d,  $J$  = 7.8 Hz, 2H), 7.90 (comp, 2H), 7.75 (d,  $J$  = 8.9 Hz, 1H), 7.17 (d,  $J$  = 9.0 Hz, 1H), 6.60 (comp, 2H), 5.59 (d,  $J$  = 5.2 Hz, 1H), 5.56 – 5.52 (m, 1H), 5.16 (dd,  $J$  = 9.8, 3.1 Hz, 1H), 4.10 – 4.05 (m, 1H), 3.47 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (d,  $J$  = 1.6 Hz, 3H).



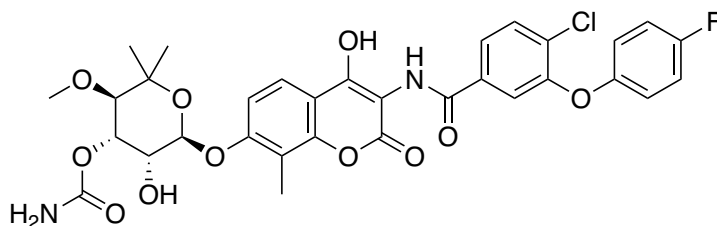
### MDM116

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.89 (s, 1H), 9.56 (s, 1H), 7.77 (comp, 4H), 7.41 (comp, 2H), 7.16 (comp, 2H), 7.03 (comp, 2H), 6.62 (comp, 2H), 5.62 – 5.55 (m, 1H), 5.53 (s, 1H), 5.15 (dd,  $J$  = 9.5, 2.8 Hz, 1H), 4.10 – 4.03 (m, 1H), 3.47 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H).



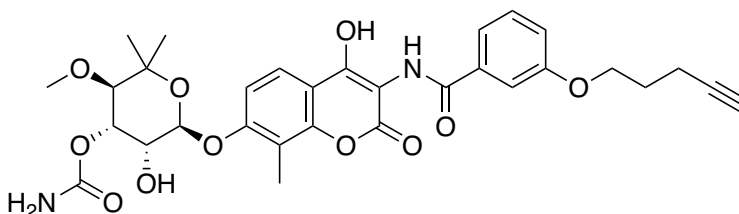
### MDM117

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.85 (s, 1H), 9.42 (s, 1H), 8.58 (s, 1H), 7.75 (dd,  $J = 18.6, 8.7$  Hz, 2H), 7.49 (d,  $J = 11.8$  Hz, 2H), 7.41 – 7.36 (m, 1H), 7.27 – 7.22 (m, 1H), 7.15 (comp, 1H), 7.01 (d,  $J = 6.5$  Hz, 1H), 6.67 (comp, 2H), 5.58 (d,  $J = 5.2$  Hz, 1H), 5.56 – 5.47 (m, 1H), 5.15 (dd,  $J = 10.8, 2.0$  Hz, 1H), 4.11 – 4.03 (m, 1H), 3.47 (comp, 4H), 2.28 (s, 3H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



**MDM118**

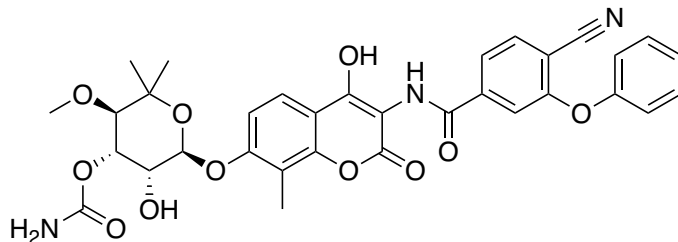
$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.90 (s, 1H), 9.54 (s, 1H), 8.58 (d,  $J = 4.8$  Hz, 1H), 7.87 – 7.78 (m, 1H), 7.76 – 7.70 (m, 1H), 7.65 (s, 1H), 7.39 (dd,  $J = 7.6, 5.0$  Hz, 1H), 7.27 (comp, 2H), 7.14 (d,  $J = 9.0$  Hz, 1H), 7.11 – 7.07 (m, 1H), 6.66 (comp, 2H), 5.58 (d,  $J = 5.1$  Hz, 1H), 5.54 – 5.49 (m, 1H), 5.15 (dd,  $J = 10.0, 3.1$  Hz, 1H), 4.10 – 4.05 (m, 1H), 3.48 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



**MDM119**

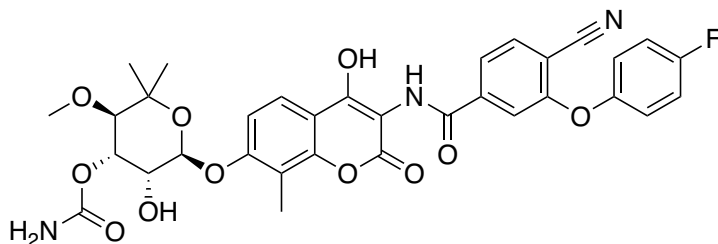
$^1\text{H}$  NMR (500 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.88 (s, 1H), 9.46 (s, 1H), 7.75 (d,  $J = 8.8$  Hz, 1H), 7.58 (comp, 2H), 7.42 (t,  $J = 8.1$  Hz, 1H), 7.16 (comp, 2H), 6.54 (comp, 2H), 5.60 (d,  $J = 5.2$  Hz, 1H), 5.54 (d,  $J = 2.5$  Hz, 1H), 5.16 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.11 (comp, 3H), 3.48 (comp, 4H), 2.83 (t,  $J = 2.7$

Hz, 1H), 2.36 (dd,  $J = 7.0, 2.7$  Hz, 2H), 2.22 (s, 3H), 1.93 (q,  $J = 6.5$  Hz, 2H), 1.27 (s, 3H), 1.06 (s, 3H).



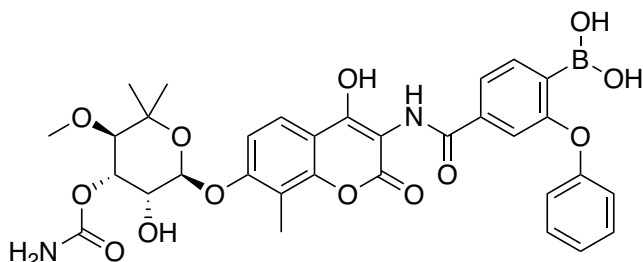
**MDM120**

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.95 (s, 1H), 9.69 (s, 1H), 8.06 (dd,  $J = 16.3, 8.1$  Hz, 1H), 7.87 (dd,  $J = 8.1, 1.5$  Hz, 1H), 7.79 – 7.70 (m, 1H), 7.56 – 7.49 (m, 3H), 7.33 – 7.20 (m, 4H), 7.14 (d,  $J = 9.0$  Hz, 1H), 6.66 (comp, 2H), 5.58 (d,  $J = 5.2$  Hz, 1H), 5.52 (d,  $J = 2.4$  Hz, 1H), 5.14 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.11 – 4.03 (m, 1H), 3.46 (comp, 4H), 2.20 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H).



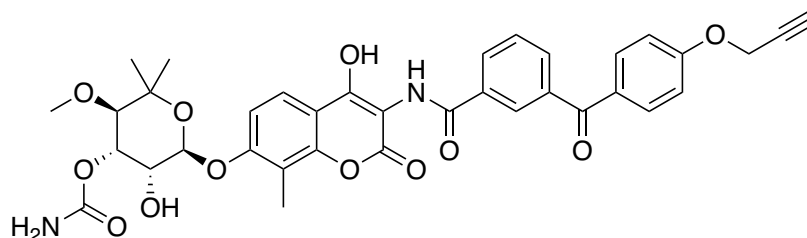
**MDM121**

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.96 (s, 1H), 9.69 (s, 1H), 8.08 (d,  $J = 8.1$  Hz, 1H), 7.86 (dd,  $J = 8.2, 1.5$  Hz, 1H), 7.72 (d,  $J = 8.9$  Hz, 1H), 7.45 (d,  $J = 1.4$  Hz, 1H), 7.34 (comp, 4H), 7.15 (d,  $J = 9.0$  Hz, 1H), 6.66 (comp, 2H), 5.58 (d,  $J = 5.2$  Hz, 1H), 5.52 (d,  $J = 2.5$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.10 – 4.01 (m, 1H), 3.47 (comp, 4H), 2.20 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H).



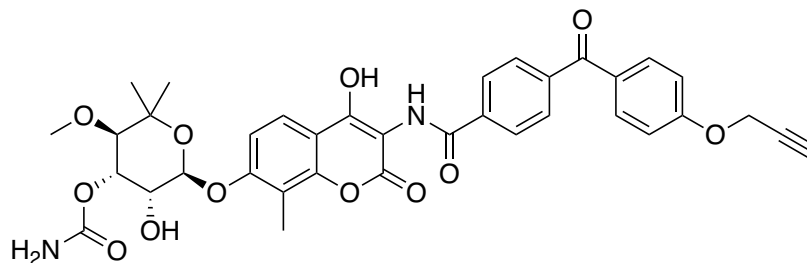
### MDM122

NMR data unavailable



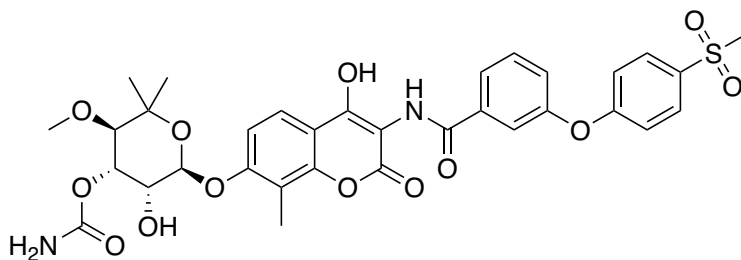
### MDM123

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.93 (s, 1H), 9.66 (s, 1H), 8.34 (s, 1H), 8.25 (d,  $J$  = 7.8 Hz, 1H), 7.89 (d,  $J$  = 7.7 Hz, 1H), 7.81 (comp, 2H), 7.72 (comp, 2H), 7.18 (comp, 3H), 6.61 (comp, 2H), 5.59 (d,  $J$  = 5.2 Hz, 1H), 5.56 – 5.51 (m, 1H), 5.16 (dd,  $J$  = 9.9, 3.1 Hz, 1H), 4.95 (comp, 2H), 4.12 – 4.04 (m, 1H), 3.65 (t,  $J$  = 2.4 Hz, 1H), 3.48 (comp, 4H), 2.22 (s, 3H), 1.27 (s, 3H), 1.05 (s, 3H).



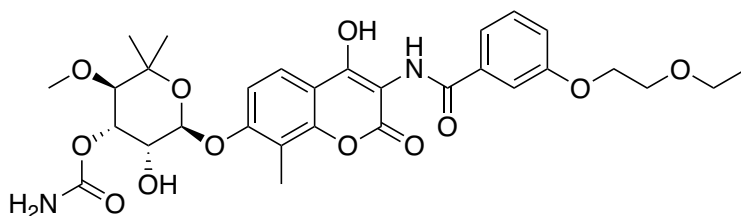
### MDM124

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.97 (s, 1H), 9.66 (s, 1H), 8.16 (comp,  $J$  = 8.1 Hz, 2H), 7.79 (comp, 5H), 7.17 (comp, 3H), 6.65 (comp, 2H), 5.60 (d,  $J$  = 5.2 Hz, 1H), 5.55 (d,  $J$  = 2.4 Hz, 1H), 5.16 (dd,  $J$  = 9.9, 3.1 Hz, 1H), 4.95 (comp, 2H), 4.14 – 4.03 (m, 1H), 3.66 (t,  $J$  = 2.4 Hz, 1H), 3.48 (comp, 4H), 2.23 (s, 3H), 1.27 (s, 3H), 1.06 (s, 3H).



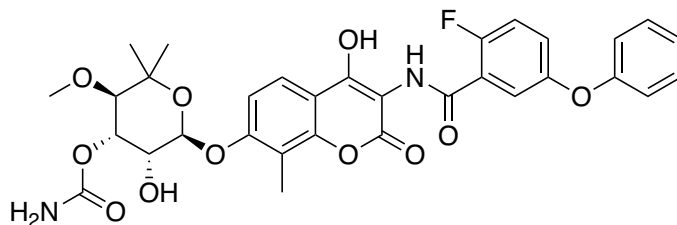
**MDM125**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.88 (s, 1H), 9.56 (s, 1H), 7.94 (comp, 3H), 7.76 (comp, 2H), 7.63 (t,  $J = 8.0$  Hz, 1H), 7.41 (d,  $J = 8.3$  Hz, 1H), 7.22 (comp, 2H), 7.17 (d,  $J = 9.0$  Hz, 1H), 6.62 (comp, 2H), 5.59 (d,  $J = 5.0$  Hz, 1H), 5.54 (d,  $J = 2.5$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.08 (s, 1H), 3.48 (comp, 4H), 3.21 (s, 3H), 2.22 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



**MDM126**

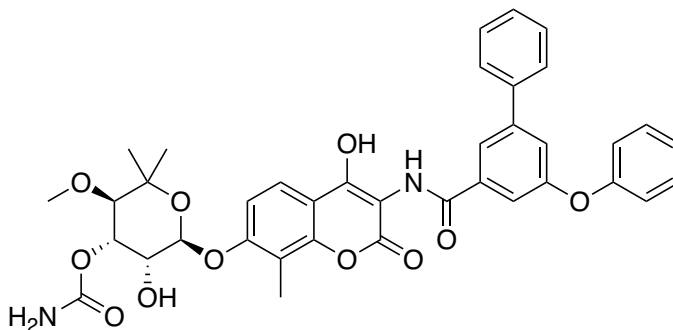
$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.87 (s, 1H), 9.45 (s, 1H), 7.75 (d,  $J = 8.9$  Hz, 1H), 7.58 (comp, 2H), 7.42 (t,  $J = 7.8$  Hz, 1H), 7.17 (comp, 2H), 6.66 (comp, 2H), 5.59 (d,  $J = 5.2$  Hz, 1H), 5.54 (d,  $J = 2.4$  Hz, 1H), 5.16 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.17 (comp, 2H), 4.11 – 4.05 (m, 1H), 3.73 (comp, 2H), 3.48 (comp, 6H), 2.23 (s, 3H), 1.27 (s, 3H), 1.14 (comp, 3H), 1.06 (s, 3H).





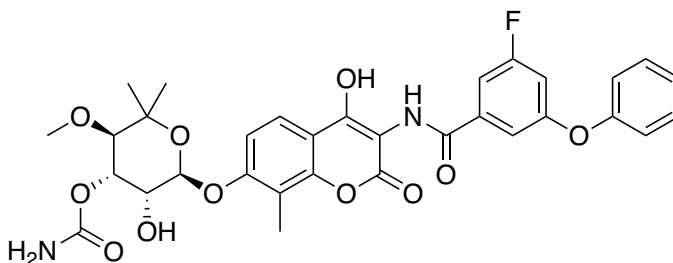
### MDM127

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.96 (s, 1H), 9.29 (s, 1H), 7.74 (d,  $J = 8.9$  Hz, 1H), 7.56 – 7.47 (m, 1H), 7.40 (comp, 3H), 7.30 – 7.23 (m, 1H), 7.16 (comp, 2H), 7.06 (comp, 2H), 6.65 (comp, 2H), 5.58 (d,  $J = 5.1$  Hz, 1H), 5.56 – 5.50 (m, 1H), 5.15 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.16 – 4.00 (m, 1H), 3.46 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



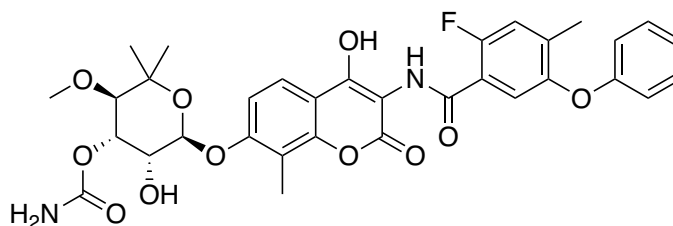
### MDM128

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.87 (s, 1H), 9.67 (s, 1H), 8.15 (s, 1H), 7.77 (comp, 3H), 7.61 – 7.49 (m, 4H), 7.44 (comp, 5H), 7.20 (comp, 2H), 7.14 (comp, 2H), 5.63 – 5.56 (m, 1H), 5.55 – 5.51 (m, 1H), 5.16 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.14 – 4.02 (m, 1H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.27 (s, 3H), 1.05 (s, 3H).



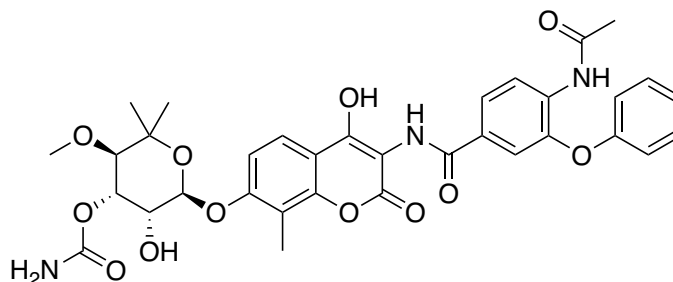
### MDM129

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.90 (s, 1H), 9.59 (s, 1H), 7.74 (d,  $J = 8.9$  Hz, 1H), 7.60 (d,  $J = 9.2$  Hz, 1H), 7.49 – 7.41 (m, 3H), 7.24 (t,  $J = 7.2$  Hz, 1H), 7.15 (comp, 4H), 6.60 (comp, 2H), 5.64 – 5.55 (m, 1H), 5.53 (d,  $J = 2.4$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.3$  Hz, 1H), 4.10 – 4.05 (m, 1H), 3.48 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



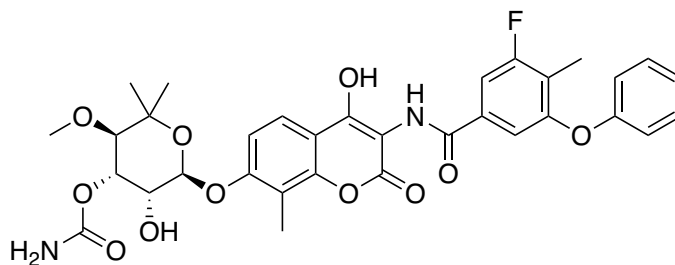
**MDM130**

NMR data unavailable



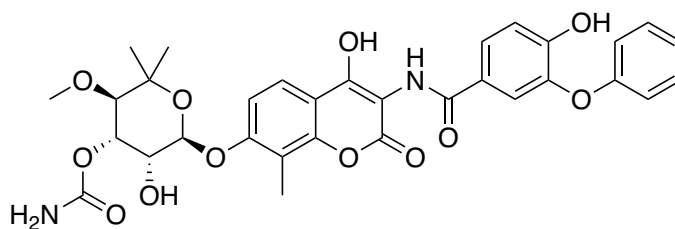
**MDM131**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.82 (s, 1H), 9.72 (s, 1H), 9.40 (s, 1H), 8.25 (t,  $J = 7.7$  Hz, 1H), 7.80 (dd,  $J = 8.8, 2.0$  Hz, 1H), 7.72 (d,  $J = 8.9$  Hz, 1H), 7.52 (d,  $J = 2.0$  Hz, 1H), 7.46 – 7.34 (m, 2H), 7.21 – 7.11 (m, 2H), 7.06 (d,  $J = 8.0$  Hz, 2H), 6.59 (d,  $J = 35.5$  Hz, 2H), 5.58 (d,  $J = 5.2$  Hz, 1H), 5.52 (d,  $J = 2.5$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.09 – 4.04 (m, 1H), 3.46 (comp, 4H), 2.20 (s, 3H), 2.10 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H).



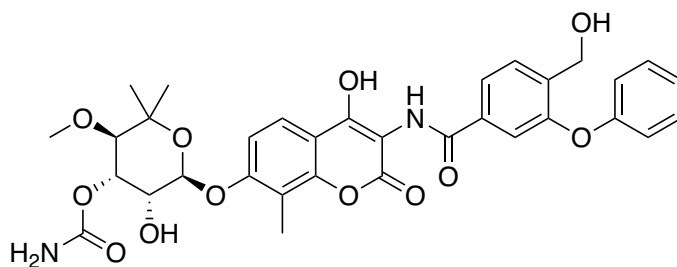
**MDM132**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.90 (s, 1H), 9.47 (s, 1H), 7.68 (comp, 2H), 7.42 (comp, 2H), 7.37 (s, 1H), 7.16 (comp, 2H), 7.06 – 6.97 (m, 2H), 6.66 (s, 2H), 5.58 (d,  $J$  = 5.2 Hz, 1H), 5.52 (d,  $J$  = 2.5 Hz, 1H), 5.15 (dd,  $J$  = 9.8, 3.2 Hz, 1H), 4.07 (dt,  $J$  = 5.4, 2.8 Hz, 1H), 3.46 (comp, 4H), 2.20 (comp, 6H), 1.26 (s, 3H), 1.04 (s, 3H).



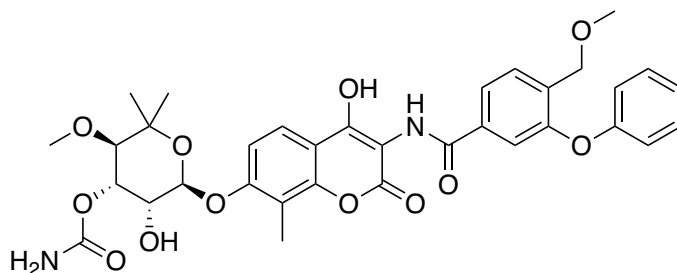
**MDM133**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.83 (s, 1H), 10.26 (s, 1H), 9.29 (s, 1H), 7.73 (comp, 3H), 7.34 (comp, 2H), 7.15 (d,  $J$  = 9.1 Hz, 1H), 7.05 (comp, 2H), 6.90 (comp, 2H), 6.63 (comp, 2H), 5.58 (d,  $J$  = 5.2 Hz, 1H), 5.53 (s, 1H), 5.15 (dd,  $J$  = 9.8, 3.1 Hz, 1H), 4.07 (s, 1H), 3.47 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



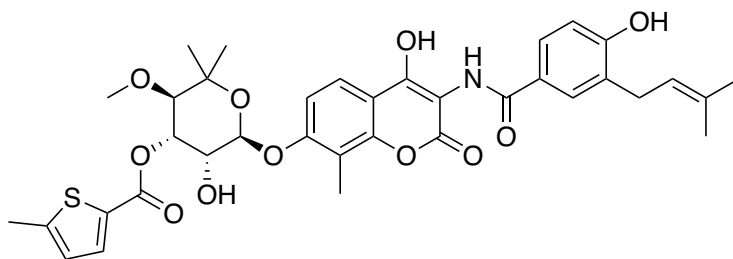
**MDM134**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.84 (s, 1H), 9.45 (s, 1H), 8.57 (d,  $J = 4.7$  Hz, 0H), 7.87 (d,  $J = 8.1$  Hz, 1H), 7.70 (dd,  $J = 15.9, 8.5$  Hz, 2H), 7.51 (d,  $J = 1.7$  Hz, 1H), 7.43 – 7.37 (m, 3H), 7.16 – 7.08 (m, 2H), 6.98 (d,  $J = 8.2$  Hz, 2H), 6.64 (comp, 2H), 5.58 (d,  $J = 5.2$  Hz, 1H), 5.53 (d,  $J = 2.5$  Hz, 1H), 5.33 (t,  $J = 5.5$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.73 (s, 1H), 4.60 (comp, 2H), 4.11 – 4.04 (m, 1H), 3.47 (comp, 4H), 2.21 (s, 3H), 2.08 (s, 1H), 1.26 (s, 3H), 1.05 (s, 3H). (spectrum is messy in aromatic region)



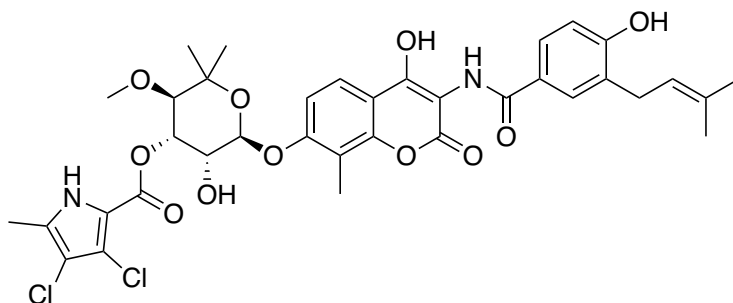
**MDM135**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.85 (s, 1H), 9.48 (s, 1H), 7.85 (d,  $J = 8.0$  Hz, 1H), 7.77 – 7.60 (m, 3H), 7.53 (s, 1H), 7.41 (td,  $J = 8.6, 8.2, 2.5$  Hz, 4H), 7.26 (d,  $J = 1.6$  Hz, 1H), 7.16 (comp, 3H), 7.00 (comp, 3H), 6.66 (comp, 2H), 5.58 (d,  $J = 5.2$  Hz, 1H), 5.52 (s, 1H), 5.35 (t,  $J = 5.7$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.1$  Hz, 1H), 4.61 (d,  $J = 5.0$  Hz, 1H), 4.52 (s, 2H), 4.09 – 4.03 (m, 1H), 3.47 (comp, 4H), 3.34 (s, 3H), 2.21 (s, 3H), 1.26 (s, 3H), 1.04 (s, 3H). (spectrum is messy in aromatic region).



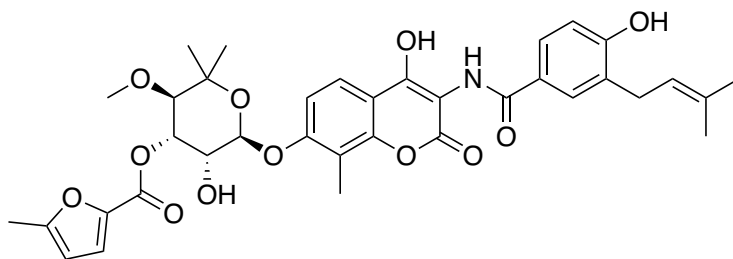
**MDM136**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.00 (s, 1H), 9.99 (s, 1H), 7.72 (comp, 4H), 7.14 (s, 1H), 6.99 (s, 1H), 6.84 (d,  $J = 8.2$  Hz, 1H), 5.86 – 5.77 (m, 1H), 5.64 – 5.53 (m, 1H), 5.46 (d,  $J = 9.9$  Hz, 1H), 5.32 (t,  $J = 7.6$  Hz, 1H), 4.26 – 4.18 (m, 1H), 3.68 (d,  $J = 9.9$  Hz, 1H), 3.51 (comp, 4H), 3.27 (d,  $J = 7.3$  Hz, 2H), 2.54 (s, 3H), 2.24 (s, 3H), 1.70 (comp, 6H), 1.30 (s, 3H), 1.09 (s, 3H).



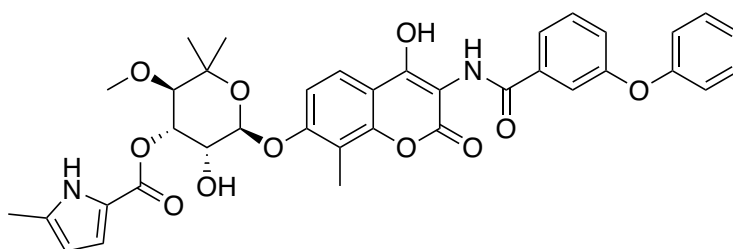
**MDM137**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  12.13 (d,  $J = 2.5$  Hz, 1H), 7.79 (s, 1H), 7.72 (comp, 2H), 7.20 – 7.04 (m, 1H), 6.83 (d,  $J = 2.7$  Hz, 1H), 6.77 (d,  $J = 8.6$  Hz, 1H), 5.73 – 5.67 (m, 1H), 5.61 – 5.56 (m, 1H), 5.48 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.21 – 4.14 (m, 1H), 3.65 (d,  $J = 9.8$  Hz, 1H), 3.47 (s, 3H), 2.79 (d,  $J = 7.1$  Hz, 2H), 2.24 (s, 3H), 2.21 (s, 3H), 1.81 (t,  $J = 6.8$  Hz, 2H), 1.30 (comp, 9H), 1.23 (s, 3H), 1.10 (s, 3H).



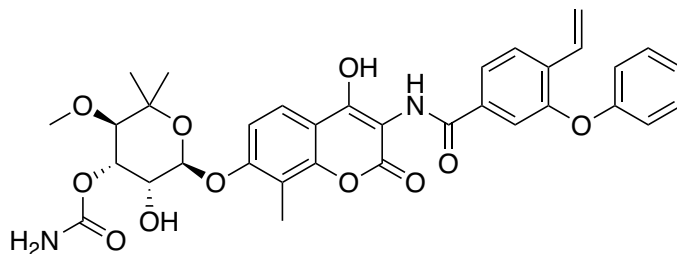
**MDM138**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.99 (s, 1H), 7.71 (comp, 3H), 7.30 (d,  $J$  = 3.3 Hz, 1H), 6.85 (d,  $J$  = 8.4 Hz, 1H), 6.38 (d,  $J$  = 3.5 Hz, 1H), 5.55 (d,  $J$  = 5.4 Hz, 1H), 5.32 (comp, 2H), 4.29 (s, 1H), 3.52 (comp, 4H), 2.39 (s, 3H), 2.18 (s, 3H), 1.70 (s, 6H), 1.37 (s, 3H), 1.24 (s, 6H), 1.12 (s, 3H).



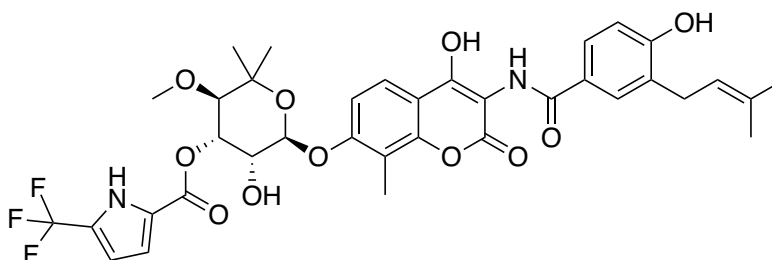
**MDM139**

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO-}d_6$ )  $\delta$  11.92 (s, 1H), 11.66 (s, 1H), 9.49 (s, 1H), 7.80 (d,  $J$  = 7.8 Hz, 1H), 7.74 (d,  $J$  = 8.8 Hz, 1H), 7.65 – 7.61 (m, 1H), 7.54 (t,  $J$  = 7.9 Hz, 1H), 7.43 (comp, 2H), 7.25 (d,  $J$  = 8.5 Hz, 1H), 7.18 (comp, 2H), 7.06 (comp, 2H), 6.81 – 6.75 (m, 1H), 5.93 (t,  $J$  = 2.7 Hz, 1H), 5.69 (d,  $J$  = 5.1 Hz, 1H), 5.61 (s, 1H), 5.48 (dd,  $J$  = 9.8, 3.1 Hz, 1H), 4.20 – 4.14 (m, 1H), 3.65 (d,  $J$  = 9.8 Hz, 1H), 3.47 (s, 3H), 2.25 (comp,  $J$  = 3.2 Hz, 6H), 1.30 (s, 3H), 1.09 (s, 3H).



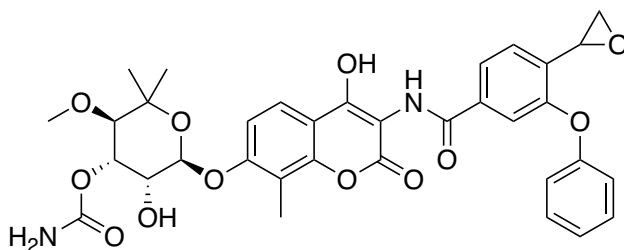
**MDM140**

$^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ )  $\delta$  11.86 (s, 1H), 9.49 (s, 1H), 7.87 (comp, 2H), 7.72 (d,  $J$  = 8.8 Hz, 1H), 7.60 (d,  $J$  = 1.6 Hz, 1H), 7.39 (comp, 2H), 7.13 (comp, 2H), 6.93 (comp, 3H), 6.65 (comp, 2H), 6.05 (d,  $J$  = 17.8 Hz, 1H), 5.58 (d,  $J$  = 5.2 Hz, 1H), 5.52 (d,  $J$  = 2.4 Hz, 1H), 5.44 (d,  $J$  = 11.3 Hz, 1H), 5.15 (dd,  $J$  = 9.9, 3.1 Hz, 1H), 4.09 – 4.05 (m, 1H), 3.47 (comp, 4H), 2.21 (s, 3H), 1.26 (s, 3H), 1.05 (s, 3H).



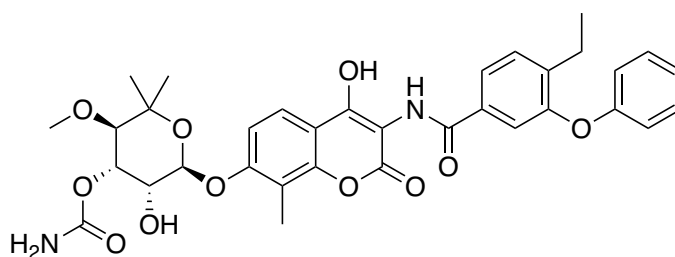
**MDM141**

NMR data unavailable



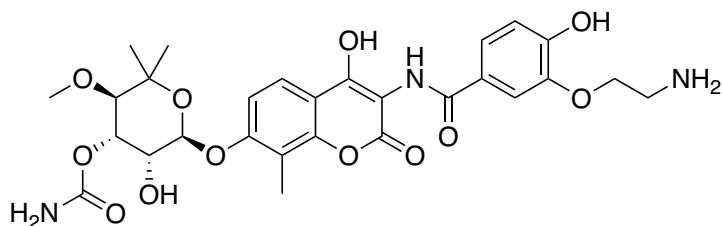
### MDM142

$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.94 (s, 1H), 9.69 – 9.46 (m, 1H), 8.06 (s, 1H), 7.94 (d,  $J = 7.8$  Hz, 1H), 7.74 (comp, 2H), 7.66 (d,  $J = 8.0$  Hz, 1H), 7.56 (t,  $J = 7.8$  Hz, 1H), 7.44 (dt,  $J = 13.8, 7.7$  Hz, 1H), 7.22 (t,  $J = 7.4$  Hz, 1H), 7.18 – 7.12 (m, 1H), 7.06 (dd,  $J = 17.7, 8.0$  Hz, 1H), 6.64 (comp, 2H), 5.65 – 5.59 (m, 1H), 5.56 – 5.50 (m, 1H), 5.16 (dd,  $J = 9.9, 3.3$  Hz, 1H), 4.10 – 4.05 (m, 1H), 3.95 – 3.82 (m, 1H), 3.47 (comp, 4H), 2.22 (s, 3H), 1.99 (s, 2H), 1.26 (s, 3H), 1.05 (s, 3H).



### MDM143

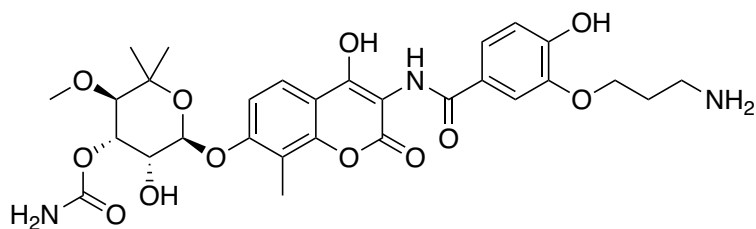
$^1\text{H}$  NMR (600 MHz,  $\text{DMSO}-d_6$ )  $\delta$  11.86 (s, 1H), 9.41 (s, 1H), 7.81 (dd,  $J = 8.0, 1.8$  Hz, 1H), 7.71 (d,  $J = 8.9$  Hz, 1H), 7.56 – 7.51 (m, 1H), 7.49 (d,  $J = 8.0$  Hz, 1H), 7.39 (comp, 2H), 7.12 (comp, 2H), 6.96 (comp, 2H), 6.63 (comp, 2H), 5.60 (d,  $J = 5.2$  Hz, 1H), 5.52 (d,  $J = 2.5$  Hz, 1H), 5.15 (dd,  $J = 9.9, 3.2$  Hz, 1H), 4.09 – 4.05 (m, 1H), 3.47 (comp, 4H), 2.65 (q,  $J = 7.5$  Hz, 2H), 2.20 (s, 3H), 1.26 (s, 3H), 1.18 (t,  $J = 7.5$  Hz, 3H), 1.04 (s, 3H).



### MDM144



$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.33 (comp, 2H), 7.56 (comp, 2H), 6.84 (dd,  $J = 30.1, 8.5$  Hz, 1H), 5.48 – 5.31 (m, 1H), 5.16 (dd,  $J = 9.7, 3.1$  Hz, 1H), 4.16 – 4.08 (m, 1H), 4.06 – 3.99 (m, 1H), 2.15 (s, 3H), 1.26 (s, 3H), 1.09 (s, 3H). (several resonances obscured by solvent peak at 3.38 ppm)



**MDM145**

$^1\text{H}$  NMR (400 MHz,  $\text{DMSO}-d_6$ )  $\delta$  8.35 (s, 1H), 8.24 (s, 1H), 7.61 (d,  $J = 8.6$  Hz, 1H), 7.56 (s, 1H), 7.48 (d,  $J = 8.3$  Hz, 1H), 6.88 (d,  $J = 8.8$  Hz, 1H), 6.82 (d,  $J = 8.2$  Hz, 1H), 6.64 (comp, 2H), 5.55 – 5.45 (m, 1H), 5.41 (d,  $J = 2.6$  Hz, 1H), 5.16 (dd,  $J = 9.8, 3.1$  Hz, 1H), 4.08 (comp, 2H), 4.04 (s, 1H), 3.46 (comp, 4H), 3.01 (t,  $J = 7.1$  Hz, 3H), 2.15 (s, 3H), 2.00 (comp, 2H), 1.26 (s, 3H), 1.09 (s, 3H). ( $\text{NH}_2$  protons obscured).

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