

Aminoglycoside-Based Antibiotic Adjuvants: A Platform for Drug Discovery

by

Temilolu O. Idowu, B.Pharm, MSc

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Department of Chemistry

University of Manitoba

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“whether you think you can, or you think you can’t, you are right”

– *Henry Ford*

“if I have seen further than others, it is by standing upon the shoulders of giants”

– *Isaac Newton*

Abstract

Antimicrobial resistance is a global phenomenon that poses a serious threat to public health. Pathogenic bacteria, especially Gram-negative bacilli, express multiple resistance mechanisms that work concertedly to shield them from the actions of antibiotics. Prior works, as reviewed in chapters 1 and 2, have identified several signature pathways by which Gram-negative bacteria become refractory to available treatment options. One of such is the chromosomally-encoded intrinsic mechanism of resistance – defined by reduced outer membrane (OM) permeability and over-expressed efflux pumps. This means that antibiotics (including Gram-positive only agents) may become inactive against Gram-negative bacteria as a result of low bioaccumulation.

Aminoglycoside antibiotics are an interesting class of Gram-negative-active agents because of their pleiotropic mechanism of action. They are polybasic hydrophilic molecules that permeate the OM by displacing stabilizing divalent cations responsible for maintaining OM integrity, in a concentration dependent manner. Next, they are believed to navigate the hydrophobic inner membrane via an energy dependent process. Thirdly, they bind to and inhibit the fidelity of ribosomal protein translation at low concentrations (typically $\leq 4\mu\text{g/mL}$). Lastly, they can also disrupt the integrity of the OM at higher concentrations, typically $\geq 8\mu\text{g/mL}$. Unsurprisingly, the response of bacteria to this class of drugs is efficient, holistic, and co-ordinated.

My doctoral work describes efforts directed at uncoupling the multifunctional properties of aminoglycosides, with a view to abolishing ribosomal effects and amplifying OM permeabilizing properties. It was hypothesized, and my data later supports, that a non-ribosomal aminoglycoside will not trigger a counter response in bacteria, will be non-toxic (possibly less toxic than ribosomal aminoglycosides) versus eukaryotic cells, and will restore full antibacterial potency of otherwise inactive antibiotics against multidrug and extensively drug-resistant Gram-negative bacteria. These types of compounds are known as antibiotic adjuvants, a strategy that is fast gaining momentum in the field of antimicrobial chemotherapy to preserve and/or restore potency of our armamentarium. As a

representative example of the aminoglycoside class, I thoroughly explored the chemistry and non-ribosomal properties of tobramycin as it is one of the most widely used aminoglycoside to treat Gram-negative bacterial infections, especially those caused by *Pseudomonas aeruginosa*. Three different series of tobramycin-based conjugates with different spectrum of activity are described in chapters 4, 5, and 6. Their synthesis, purification, and characterization are reported. A broad-screen campaign aimed at identifying the ‘ideal’ partner antibiotic molecules for these compounds was initiated. Mechanistic studies to understand their mode(s) of action, pattern of synergism, and mechanism of resistance development were conducted. Preliminary *in vivo* efficacies of identified pairs were evaluated using a *Galleria mellonella* larvae infection model. Toxicological liabilities of the newly synthesized molecules, alone and in combination with their partner antibiotics, were also investigated.

In summary, our optimization campaign led to the discovery of a novel broad-spectrum homodimeric tobramycin-based scaffold that is non-toxic and more potent than the current gold standard potentiator molecule, polymyxin B nonapeptide (PMBN) (chapter 6). We also identified a non-toxic narrow-spectrum tobramycin-cyclam conjugate that restores full antibiotic potency of β -lactam antibiotics against recalcitrant *P. aeruginosa* (chapter 5).

This study expands our understanding of the multifunctional properties of aminoglycosides, hints at how we can win a war by losing a battle and provides a template to exploit the resistance pathways of bacteria to mine novel therapeutics. It is hoped that this study, and the concepts therein, will contribute meaningfully towards combating the threats of antimicrobial resistance.

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List of Abbreviations

| | |
|----------|---|
| AMA | aspergillomarasmine A |
| AME | aminoglycoside-modifying enzyme |
| AG | aminoglycoside |
| ATCC | American Type Cell Culture |
| Boc | <i>tert</i> -butyloxycarbonyl |
| CAM | chloramphenicol |
| CAN-ICU | Canadian National Intensive Care Unit |
| CANWARD | Canadian Ward |
| CDC | Centers for Disease Control and Prevention |
| CF | cystic fibrosis |
| CFU | colony forming unit |
| CLSI | the Clinical and Laboratory Standard Institute |
| DOS | deoxystreptamine |
| DOX | doxycycline |
| ESBL | extended-spectrum β -lactamase |
| ESI-MS | electrospray ionization mass spectrometer |
| FDA | the United States Food and Drug Administration |
| FIC | fractional inhibitory concentration |
| HPLC | high-performance liquid chromatography |
| ICU | intensive care unit |
| IDSa | the Infectious Diseases Society of America |
| IM | inner membrane |
| <i>J</i> | NMR coupling constant (in hertz) |
| LB | lysogeny broth |
| LPS | lipopolysaccharide |
| MALDI-MS | matrix-assisted laser desorption ionization mass spectrometry |
| MBL | metallo- β -lactamase |
| MDR | multidrug-resistant |
| MHB | Mueller-Hinton Broth |
| MIC | minimum inhibitory concentration |
| NMR | nuclear magnetic resonance |

| | |
|----------|--|
| OM | outer membrane |
| PBS | phosphate-buffered saline |
| PBP | penicillin binding protein |
| PDR | pandrug-resistant |
| PMF | proton-motive force |
| RNA | ribonucleic acid |
| RND | resistance-nodulation division |
| SAR | structure-activity relationship |
| TBAHS | tetrabutylammonium hydrogen sulphate |
| TBDMS | <i>tert</i> -butyldimethylsilyl ether |
| TFA | trifluoroacetic acid |
| UTI | urinary tract infection |
| WHO | the World Health Organization |
| WT | wild type |
| XDR | extensively drug-resistant |
| δ | NMR chemical shift in part per million |

1 CHAPTER ONE:

Background and Introduction

1.1 Preface

There is consensus in the scientific community, and a growing awareness in general society, that antimicrobial resistance constitutes a serious threat to the expected standard of medical care. In 2016, a one-day high-level meeting convened by the United Nation General Assembly on ‘Antimicrobial Resistance’ had a primary objective of:

*“summoning and maintaining strong national, regional and international political commitment in addressing antimicrobial resistance comprehensively and multi-sectorally, and to increase and improve awareness of antimicrobial resistance”.*¹

This was only the fourth time a health issue was discussed by the UN General Assembly,² demonstrating the urgency of this threat. A ‘One Health’ approach that recognizes that the health of people is connected to the health of animals and the environment, and encourages collaboration across topics and areas for the best health outcome was emphasized.²

In 2017, five months after the UN General Assembly meeting, the WHO published its first ever list of antibiotic-resistant “priority pathogens” – a catalogue of 12 families of bacteria that pose the greatest threat to human health.³ Unsurprisingly, the most critical group of all, i.e. the priority one pathogens, include multidrug resistant Gram-negative bacteria that pose a particular threat in hospitals, nursing homes, and among patients whose care requires devices such as ventilators and blood catheters. These pathogens include carbapenem-resistant strains of *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and various Enterobacteriaceae (including *Klebsiella*, *Escherichia coli*, *Serratia*, and *Proteus*).³ The phenomenon of antimicrobial resistance is more pronounced in Gram-negative bacteria because of the dual nature of their membrane topology that confers intrinsic resistance to most antibiotics that are active against Gram-positive bacteria. Even among Gram-negative bacteria, the susceptibility patterns of *P. aeruginosa* to antimicrobial agents differ in many respects to other pathogens due to differences in the lipid A component of their lipopolysaccharide. *P. aeruginosa* is almost always an exception to the rules governing antimicrobial chemotherapy of Gram-

negative bacteria,⁴ and they are frequently multidrug-resistant. Multidrug resistance (MDR) is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, extensively-drug resistance (XDR) as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories), and pan-drug resistance (PDR) as non-susceptibility to all agents in all antimicrobial categories.⁵ Due to the paucity of new chemical entities in our antibiotic pipelines against MDR/XDR/PDR pathogens, the use of drug combinations to extend the life of antibiotics is a budding strategy to address the immediate need of effective treatment options.⁶

Congruous combinations of antibiotics are based on compounds that individually have cell growth inhibition activity towards the target organism and is the basis for most existing antibiotic combination therapies, e.g., a combination of rifampin-isoniazid-pyrazinamide in the treatment of tuberculosis. By contrast, syncretic combinations include at least one component that does not have overt antibiotic activity, as typified by the classic β -lactam- β -lactamase inhibitor pairs. Compounds with no intrinsic activity in a syncretic combination are termed “antibiotic adjuvants”.⁶ A third type of combination, termed coalism, occurs between compounds that alone have no antibiotic activity but that together are active. Reference 6 provides a comprehensive review on antibiotic drug combinations.

This study focuses on the development of tobramycin-based conjugates as antibiotic adjuvants that can restore meaningful activity and/or enhance efficacy of bona fide antibiotics against recalcitrant Gram-negative bacteria, with special interest in *P. aeruginosa*. To achieve this, a hybrid understanding of two different, but complementary topics is essential: 1) Microbiology and the molecular basis of antibiotic actions, 2) Organic synthesis and chemistry of drug discovery. In this chapter, mini-reviews on *P. aeruginosa* (one of the most feared pathogens in intensive care units of hospitals) and aminoglycoside antibiotics (one of the most potent antipseudomonal agents) are presented. The physicochemical requirements, and the exceptions thereof, of developing antipseudomonal agents, viz-a-viz the chemistry of selection and considerations during lead optimization is also discussed.

1.2 *Pseudomonas aeruginosa*: An Embodiment of Resistance

1.2.1 Introduction

Pseudomonas aeruginosa, an opportunistic Gram-negative bacterium that invades compromised tissues, is the leading cause of nosocomial infections in immunocompromised and debilitated patients, as well as chronic lung infections in cystic fibrosis patients.⁷ It is well known for its capacity to engender resistance,⁸ due to its low outer membrane permeability (~ 8 % of *Escherichia coli*),^{9,10} and secretes an array of virulence factors with which it damages its host cells.^{11,12} *P. aeruginosa* is particularly difficult to eradicate because it adapts to the environment seamlessly, and its large genome provides a great amount of flexibility and metabolic capability needed to thrive in environments that are inhospitable to most other organisms.¹³ The complete genome sequencing of wild-type *P. aeruginosa* (PAO1) at the turn of the century provided a great deal of useful information not just about its pathogenicity but also its potential for resistance development.¹³ Yet, the continued dwindling of therapeutic options available to treat this pathogen has conferred an invincible status on them, resulting into significant morbidity and mortality.

1.2.2 Pathogenesis of Infection

P. aeruginosa is a metabolically versatile Gram-negative bacteria that thrives and mostly inhabits the soil, marshes and surfaces in aqueous environments, and can survive in a wide range of natural and artificial conditions, including surfaces in medical facilities.¹² It occasionally colonize human body sites, with preference for moist areas such as the perineum, axilla, ear, nasal mucosa and throat, and are readily found in water-sinks, mops and respiratory equipment of intensive care units (ICU) of hospitals.¹⁴ It rarely infects healthy tissues, hence its opportunistic nature, and nearly all clinical cases of infection can be associated with compromise of the host defenses such as in neutropenia, severe burns, cystic fibrosis etc.¹⁵ This perhaps explains why most pseudomonal

infections are nosocomial. *P. aeruginosa* is the major cause of respiratory infections in hospitalized patients and is also a significant source of bacteremia in burn victims, urinary-tract infections (UTIs) in catheterized patients and swimmers' ear infection.¹⁵ Community-acquired respiratory infections caused by *P. aeruginosa* are very rare (possible when there is an underlying defect in immunity)¹⁶ while the more prevalent hospital-acquired infections could either be acute or chronic. Acute nosocomial infections results mainly from a direct trauma or assault on epithelial lining such as a breach during intubation (endotracheal tube in airway infections or catheterization in UTIs), smoke inhalation or pre-existing ulcerative conditions; while chronic infections are more severe and are mostly associated with underlying predisposing immune deficiencies such as in cystic fibrosis, neutropenia due to cancer chemotherapy, old age, immunosuppression due to organ transplant etc. The best-known cases of chronic pseudomonal lung infections are those in patients with cystic fibrosis, most of whom develop an acute *Pseudomonas* lung infection by adolescence and can live with such an infection for 20 years or more.¹⁷ The physical presence of this bacterium and its toxins in the airway surface causes a persistent immunological stimulation, whereas the altered cystic fibrosis transmembrane regulator in cystic fibrosis patients impairs the counteractive immune responses.^{17,18} This results in chronic lung inflammation that progressively leads to the deterioration and eventual destruction of lung functions.¹⁸ It is important to note that the ubiquitous nature of *P. aeruginosa* in hospitals and the typical immunocompromised state of ICU patients is the combination needed to trigger a chronic pseudomonal infection.

1.2.3 Defensive and Virulence Factors

P. aeruginosa is well-equipped to circumvent the many mechanisms by which conventional hospital-acquired infections are treated, including host defense clearance. It could be described as an organism endowed with native and acquired arsenal of virulence capabilities. These capabilities are further exacerbated by the nature of their cell wall and the ability to survive in very harsh environment

due to their low nutritional requirements. Pathogenicity is often mediated by a combination of several virulence factors such as the motile surface appendages used for attachment, lipopolysaccharides (LPS), quorum sensing and biofilm formation, type 3 secretion system (T3SS) and several other secretions used to disable the epithelial cells of their host.

1.2.3.1 *Flagella and Pili*

The surface appendages (flagella and pili) of *P. aeruginosa*, which are protein in nature, serve as the major means of motility (chemotaxis) as well as the tethers that facilitate initial binding to the asialylated glycolipid (asialoGM1) receptor on the host's epithelial cells. This is subsequently followed by a LPS-mediated irreversible adhesion to the epithelial cells, an initial critical step in the colonization of respiratory epithelium that allows the organism to destroy the protective glycocalyx and disseminate various secretions.¹⁹ Binding of the bacterium's flagellum to asialoGM1 elicits a strong NF κ B-mediated inflammatory response via TLR5 (toll-like receptor-5) and a caspase-1-mediated response via the Nod-like receptor Ipaf,²⁰ while the pili (type 4 being the most effective) are involved in the formation of aggregates and biofilm micro colonies that offers protection from antibiotics and host immune system.²¹ The cystic fibrosis transmembrane conductance regulator (CFTR) is a membrane protein and chloride ion channel used by epithelial cells as a receptor for internalization of *P. aeruginosa* via endocytosis and subsequent removal from the airway.²² In CF patients where CFTR is dysfunctional, mutated or completely absent, the rapid NF κ B-mediated nuclear translocation of the innate immunity in response to binding of *P. aeruginosa* to the epithelial cells is impeded, thereby leading to increased bacterial loads in the lungs and consequently a chronic infection.^{22,23}

1.2.3.2 Lipopolysaccharides (LPS)

The cell wall of Gram-negative bacteria is made up of a distinct outer and inner membrane that forms a defined periplasmic space, a concentrated gel-like matrix that serves as a barrier before getting to the cytoplasm. The outer membrane (OM) is composed of a complex glycolipid (LPS) that forms the outer leaflet, and phospholipids that makes up the inner leaflet. LPS constitutes a physical barrier that protects the bacterium from host defenses, prevents external molecules from getting into the cell, mediate direct interactions with antibiotics and host cell receptors, and initiate some of the signaling events that leads to host tissue damage.²⁴ The LPS of *P. aeruginosa* is fundamentally made up of three domains – a membrane-anchored lipid A, a core oligosaccharide region, and a highly variable *O*-specific polysaccharide (or *O*-antigen or *O*-polysaccharide) (Figure 1.1). The lipid A component anchors the LPS into the OM such that when the organism binds to host cell receptors, it activates the NFκB signaling pathways (via TLR4) that triggers the production of pro-inflammatory cytokines and chemokines, leading to inflammation and eventually endotoxic shock.²⁴ Modifications to lipid A is known to alter the susceptibility of *P. aeruginosa* to cationic antimicrobial peptides (including polymyxins), and different strains have been identified to have different acylated LPS.^{25,26} The lipid A, a disaccharide backbone bearing several fatty acid chains, is usually attached to a nine or ten-sugar branched oligosaccharide core. A large proportion of the LPS molecules on the surface of many cells have only these two domains, and such molecules are sometimes referred to as lipid A-core. The third LPS domain consists of a repetitive carbohydrate polymer that is covalently attached to the core and can be referred to as *O*-antigen, *O*-polysaccharide or *O*-specific polysaccharide. *P. aeruginosa* cells can simultaneously produce two types of *O*-polysaccharide (A- or B-band) in the same cell, and are the basis for the specificity of each serotypes.²⁴ The *O*-polysaccharides are involved in the interactions between the bacterium and the environment (or host) during infections, with the A-band polysaccharides eliciting a weak antibody response and more common while the B-band elicits a strong antibody response and protects the bacteria from phagocytosis and oxidative stress.²⁴ Many strains of

P. aeruginosa, especially those isolated from chronic infections, have been observed to lose their ability to express the B-band polysaccharide, thereby making the A-band become the dominant antigen over time.²⁴ This perhaps is in response to the selective pressure to evade the host adaptive immune responses.

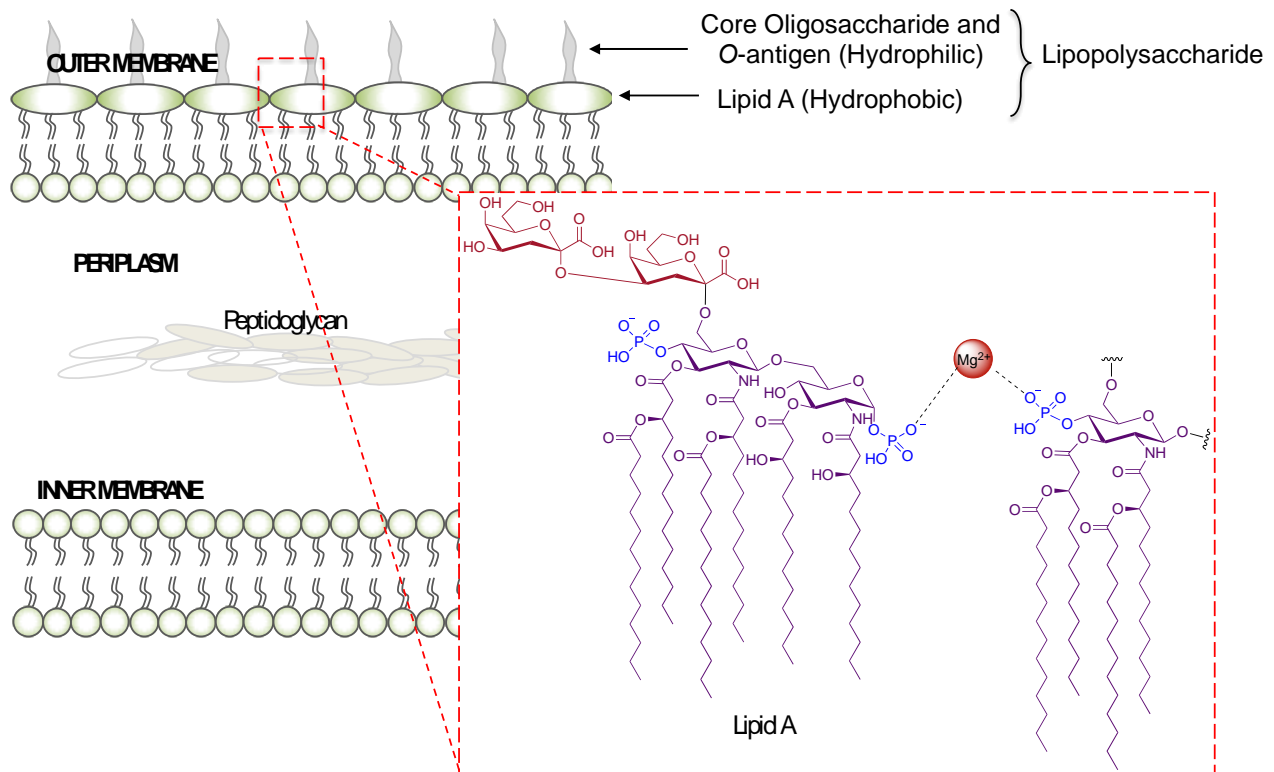


Figure 1.1. Dual (outer and inner) membrane topology of Gram-negative bacteria showing a cross-section of the lipid A component of lipopolysaccharide

1.2.3.3 Quorum Sensing and Biofilm formation

Quorum sensing is a mechanism by which bacteria coordinate gene expressions in response to fluctuations in cell-population density, via the constitutive production and release of membrane-diffusible signaling molecules known as autoinducers.²⁷ This coordination works on the principle of the concentration of autoinducer molecules in the medium being proportional to the concentration of bacteria, such that when the bacterial population reaches a critical mass (i.e. forms a ‘quorum’), the concentration of autoinducers also becomes sufficiently high to downregulate their physiological

activities in a coordinated response across the entire population. The detection of a minimal threshold stimulatory concentration of an autoinducer leads to an alteration in gene expression. In general, Gram-negative bacteria use acylated homoserine lactones as autoinducers, and Gram-positive bacteria use processed oligopeptides to communicate.²⁷ In *P. aeruginosa*, quorum sensing is regulated by a hierarchical LuxI/LuxR circuit comprising of two homologues – LasI/LasR and RhII/RhIR – that control cell survival, biofilm formation and virulence in an intricately delicate fashion.¹⁹ Both LasI and RhII are autoinducer synthase that catalyze the formation of the homoserine autoinducers *N*-(3-oxo-dodecanoyl)-homoserine lactone and *N*-butyryl homoserine lactone, respectively.²⁷ A novel, additional non-homoserine autoinducer, 2-heptyl-3-hydroxy-4-quinolone, has also been demonstrated to be involved in quorum sensing in *P. aeruginosa*.²⁸

Microbial biofilms are structurally and dynamically complex populations of microorganisms concentrated at an interface (usually solid-liquid) and surrounded by an extracellular polymeric substance (EPS) matrix that includes polysaccharides, nucleic acids, lipids and proteins.²⁹ Biofilms are not just passive assemblages of cells stuck to surfaces, but they are cells that have an entirely different lifestyle from their planktonic counterparts due to the relative oxygen and nutrients limitations within the biofilm matrix. The architecture and robustness of the biofilm EPS protects the bacteria from mechanical forces (e.g. flowing water) and decreases the penetration of toxic chemicals (e.g. antibiotics, host defenses). Quorum sensing play a major regulatory role in the transitioning of *P. aeruginosa* from the motile to sessile state within the biofilm, and vice-versa, via loss and acquisition of extracellular appendages.³⁰

1.2.3.4 Secretions

P. aeruginosa injects toxins directly into host cells via a needle-like appendage of the type 3 secretion system (T3SS) that permits the translocation of effector proteins (exoY, exoS, exoT and exoU) through a pore formed on the membrane of the host cell.³¹ ExoU, being the most potent

cytotoxin (about 100 times more than exoS), is capable of causing rapid death of host eukaryotic cell due to loss of plasma membrane integrity consistent with necrosis.³¹ This is believed to fully allow the organism exploit breaches in the epithelial barrier by antagonizing wound healing during colonization and/or to promote cell injury, leading to the symptoms of bacterial pneumonia. T3SSs are essential for pathogenicity of many pathogenic bacteria while a defective T3SS may render a bacterium non-pathogenic. Other secretions such as proteases (that degrade immunoglobulins, fibrins and epithelial tight junctions),¹⁹ exotoxin A (that inhibits protein synthesis thereby resulting into cell death as well as repression of host immune response),³² lipases and phospholipases (that break down surfactant lipids and phospholipids of host cell membranes),¹⁹ and pyocyanin (that causes oxidative stress to the host by disrupting mitochondrial electron transport)³³ are all used by *P. aeruginosa* to weaken their host cells.

In summary, invasion, dissemination and extensive tissue damage are generally more predominant in acute infections, and isolates from these infections differ substantially in phenotypes from those isolated from chronic infections.³⁴ Isolates from acute infections express a wealth of virulence factors whereas those from chronic cystic fibrosis lung infections lack some of the most inflammatory and immunogenic bacterial features such as the T3SS and motile appendages (flagella and pili) in order to avoid clearance by host defences.¹¹ The chronic infection isolates however form biofilm communities much more readily than the acute isolates.¹¹

1.2.4 Mechanism of Resistance to Antibiotics

Several factors are known to contribute to the inability of antibiotics to fully eradicate Gram-negative bacteria or completely sterilize an infection. The form and functions, capabilities and flexibility, properties and constituents of microorganisms make them arguably live the most successful forms of life on earth. Indeed, quorum sensing is believed to bestow some qualities of higher organisms

on bacteria. *P. aeruginosa* is well known for its intrinsic, acquired and secondary adaptive capabilities to evade the actions of antibiotics.

1.2.4.1 *Intrinsic Resistance*

Intrinsic resistance to antibiotics is usually encoded in the chromosomes of microorganisms. *P. aeruginosa* is known for the extremely low permeability of its outer membrane (OM), constitutive overexpression of membrane efflux pumps, and the natural occurrence of an inducible chromosomal AmpC β -lactamase enzyme.^{8,35} The low OM permeability of *P. aeruginosa* (~ 8 % that of *E. coli*)³⁵ makes it act as a selective barrier that prevents the uptake of antibiotics while passage of hydrophilic molecules via the water-filled porin channels are size-dependent and often limited by the selective and narrow nature of the porins.^{9,36} The self-promoted uptake of polycationic antibiotics across the OM is also quickly equilibrated with a corresponding gradient of hydrophilic molecules, and often further opposed by active efflux mediated by multidrug efflux transporters, particularly the resistance-nodulation division (RND) system MexAB-OprM and MexXY-OprM, as well as AmpC β -lactamase enzyme production.^{36–38} A combination of some or all of these, and/or other redundant resistance pathways result in an organism that is very adaptive and fairly difficult to eradicate.

1.2.4.2 *Acquired Resistance*

Asides its chromosomally-encoded intrinsic mechanism of resistance, *P. aeruginosa* can also acquire inheritable traits via horizontal transfer of genetic elements or mutational resistance.³⁵ DNA elements such as plasmids and transposons can be passed between bacteria via conjugation, transformation, or transduction, and can impart resistance to one or more antibiotics in an otherwise susceptible recipient. *P. aeruginosa* acquires resistance to aminoglycoside (aminoglycoside modifying enzymes) and β -lactam (plasmids encoding new β -lactamases and metallo- β -lactamases) antibiotics

mainly via horizontal transfer, although such has been observed for several other classes of antibiotics as well.³⁵ Mutational resistance involves the dysregulation of a preexisting resistance mechanism, and could be significantly aggravated in the presence of DNA-damaging agents or during growth in a biofilm. Some *P. aeruginosa* isolates from cystic fibrosis patients have been found to be strong hypermutators (*mutL* and *mutS*),³⁹ while other mutations make the organism untreatable by antibiotics due to overexpression of efflux pumps, reduced uptake of antibiotics, hyperproduction of β -lactamases and altered drug targets. Interestingly, some of these mutations have been observed to increase the susceptibility of *P. aeruginosa* to some antibiotics. For example, strains with mutations in genes affecting DNA replication, recombination and repair showed an increase in ciprofloxacin susceptibility while mutations in alginate production led to increased susceptibility to the β -lactam imipenem.^{40,41}

1.2.4.3 Adaptive Resistance

Adaptive resistance is an inducible phenomenon that is dependent on the continuing presence of an antibiotic and/or environmental stimulus and is easily reversed when such factor or condition is removed. Sub-inhibitory antibiotic concentrations or growth states such as biofilm formation, swarming, pH, cations etc. can lead to defined changes in the gene expression pattern of *P. aeruginosa* that allow the bacterium to withstand subsequent exposures to lethal concentrations of the inducing agents.¹² These triggering factors can either modulate the expression of genes encoding efflux pumps (e.g. aminoglycosides induce MexXY efflux pumps), cell envelope and enzymes. Under specific inducing conditions (such as limiting Mg^{2+} , exposure to peptides and polymyxins, and epithelial cell interaction), a variety of sensor kinases induce addition of 4-aminoarabinose to lipid A of LPS, thus blocking the self-promoted uptake of polycationic antimicrobial agents by lowering the overall anionic charge of the LPS.⁴² Pre-exposure of chromosomally-encoded β -lactamase-producing *P. aeruginosa* to β -lactam antibiotics have also been shown to cause enzymatic inactivation of some β -lactams (such as ceftazidime and cefotaxime), while some fourth generation β -lactams (such as cephalosporins,

cefepime and ceftazidime) show weaker or no upregulation of AmpC.³⁵ In summary, sub-lethal conditions confer adaptive resistance on *P. aeruginosa* but these effects are usually transient and susceptibility can be restored when the inducing conditions are reversed.

1.2.5 Current Therapeutic Options

The current guidelines from the Infectious Disease Society of America (ISDA) and the American Thoracic Society (ATS) on the management of community and hospital-acquired infection advocate a therapeutic selection based on the severity of the infection, awareness of underlying risk factors and co-morbid diseases, recognition of the epidemiology and resistance phenotypes in individual settings, and knowledge of pharmacokinetic–pharmacodynamics parameters.^{43,44} First, any suspicion of pseudomonal infection should require bacteriological documentation, including the antibiotic susceptibility profile (useful for epidemiological database), followed by an early initiation of therapy (as soon as clinical samples has been collected) using the best available knowledge.⁴⁴ This is important because early therapy has been associated with better outcome,⁴⁵ and the choice of therapy should be informed by patient’s risk factors and local epidemiology. Initial therapy often includes an antipseudomonal β -lactam (penicillin, monobactam, cephalosporin, or carbapenem) associated with either an aminoglycoside (such as inhaled tobramycin) or fluoroquinolone (preferably ciprofloxacin).⁴⁶ The third stage involves treatment de-escalation and/or fine-tuning of therapy once laboratory data of clinical samples are available. This is pertinent in order to limit antibiotic pressure, and by extension limit selection of resistance, to improve chances of therapeutic outcome.^{47,48} Finally, therapeutic drug monitoring and patient’s condition should be constantly re-evaluated so as to make an informed decision on whether antibiotics should be continued or not.⁴⁷ On the contrary, the situation might be much more complex when confronted with multidrug-resistant isolates for which the activity of at least three major antibiotic classes is compromised.⁵ The use of combination therapy (adapted on a case-by-case basis and considering susceptibility testing) is usually preferred, even though the exact

benefit of this approach remains controversial.^{43,49} Early treatment in this scenario often includes inhaled colistin or aminoglycoside (usually tobramycin) and/or oral ciprofloxacin.⁵⁰ Colistin is however usually used as drug of last resort because of its associated dose-limiting toxicities.

1.2.6 Conclusions

Despite the comprehensive and advanced knowledge on *P. aeruginosa*, effective treatment strategy is still far from reach. Fortunately, most human hosts counteract the infectious process quite effectively via the innate immune system. However, given its ubiquitous habitat, metabolic versatility, complex regulatory controls, and the frequency with which the organism is encountered, it is unlikely that *P. aeruginosa* will ever be completely wiped out from hospitals, thereby putting immunocompromised patients at a great disadvantage. Preventive and early interventions are likely to remain the most effective safety valve, at least for the foreseeable future. Also, the development of novel therapeutics that take advantage of the pathogen's Achilles' heel, especially in their mode of growth, adaptation, and restrictive permeability, might be the way to go in the development of future antipseudomonal drugs.

1.3 Aminoglycoside Antibiotics: Physicochemical properties, Activity, and Resistance

1.3.1 Introduction

Aminoglycosides (AGs) are natural product broad-spectrum antibiotics produced as secondary metabolites by the soil-dwelling bacteria species *Streptomyces* (–mycins) and *Micromonospora* (–micins). They are primarily agents of bacterial warfare secreted to kill other bacteria or fungi in the evolutionary struggle to gain advantage over other species competing for the same ecological niche. Interestingly, they rarely achieve the concentration necessary for antibiotic activity within their natural producer organism, and the discovery of their antimicrobial potency against human pathogens is a fortunate coincidence. Since the first AG (streptomycin, Figure 1.2) was isolated from *Streptomyces griseus* in 1944 and used to successfully treat tuberculosis, several milestone analogs (such as kanamycin, neomycin, sisomicin, gentamicin, tobramycin, etc.) have been introduced to treat both Gram-positive and Gram-negative bacterial infections, including the development of some semi-synthetic derivatives such as dibekacin, amikacin, arbekacin, plazomicin (FDA-approved), etc. (Figure 1.3). Most of the semi-synthetic AGs were developed to overcome the setbacks of the naturally occurring analogs.

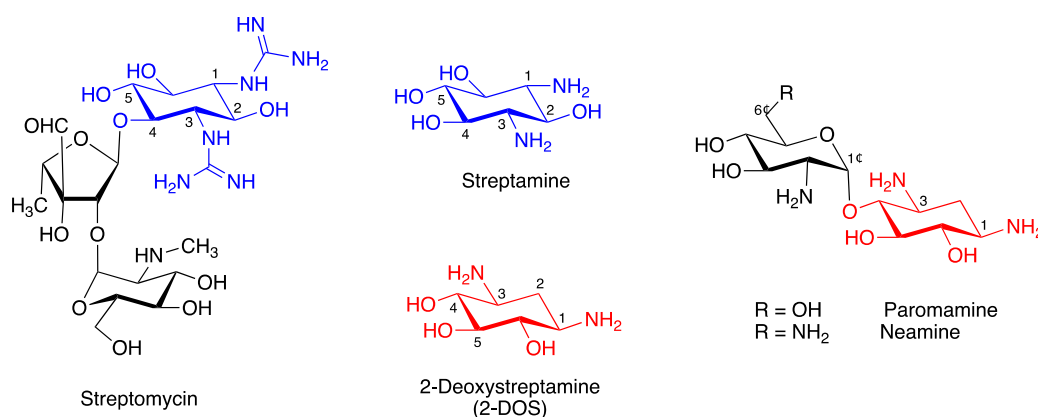


Figure 1.2. Streptomycin and core aminoglycoside structural elements. Coloured structure(s) indicate core scaffold (2-DOS).

AGs are structurally more selective for bacterial (prokaryotic) ribosomes because of the nucleotide substitutions at two key binding residues on human (eukaryotic) ribosomes, i.e. prokaryotic A1408 and G1491 residues are replaced with guanine and adenine respectively.⁵¹ Unfortunately, eukaryotic mitochondrial ribosomes contain similar prokaryotic residues (A1555G and C1494T) that bind AGs, and could likely be the basis for their side effects (ototoxicity) in humans.⁵² Over the years, these toxicological liabilities, notably ototoxicity and nephrotoxicity, and the development of resistance by microorganisms have limited the use of AGs against clinically important pathogens. However, the emergence of multidrug resistant pathogens that are only susceptible to AGs, their broad antimicrobial spectrum, rapid bactericidal action, ability to act synergistically with other drugs, recent development of designer analogs that prevent cochlear hair cell and hearing loss,^{53,54} as well as their optimized dosing regimens have rejuvenated interest in this class of drugs.

1.3.2 Chemistry of Aminoglycosides

AGs are hydrophilic compounds saturated with several functional groups, mainly amines and hydroxyls. Since their amine moieties (having a wide range of pK_a values) are mostly protonated at physiological pH,⁵⁵ they could be considered as polycationic species (for the purpose of understanding their biological interactions). The noticeably high affinity of AGs for nucleic acids, especially their strong binding affinity for negatively charged pockets in structured RNAs, can be attributed to their polycationic nature.⁵⁶ AGs are highly soluble in water but not in lipids, with enhanced antimicrobial activity in alkaline rather than acidic environments. As a result, they are minimally absorbed from the gastrointestinal tract and unable to penetrate the blood-brain barrier, as they do not readily cross cellular membranes.⁵⁷ Intravenous, intramuscular and intrathecal administration is therefore an integral part of the systemic use of AGs, as well as in achieving therapeutic concentrations in the CNS.⁵⁷

The basic chemical structure required for both potency and spectrum of antimicrobial activity is that of a dibasic aminocyclitol nucleus connected to one or several amino sugars via glycosidic

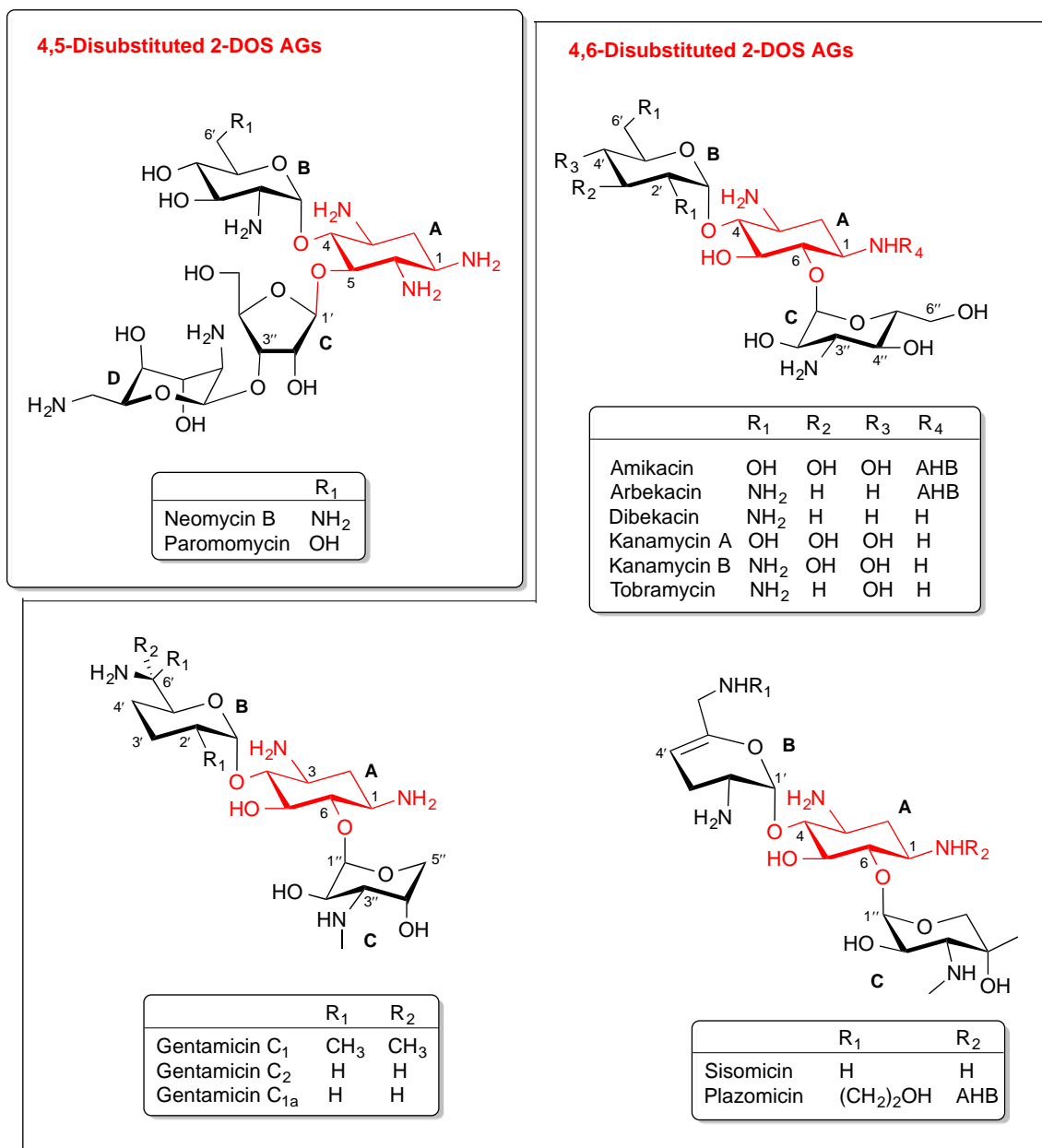


Figure 1.3. Structures of the main classes of 2-deoxystreptamine aminoglycoside. Coloured structure(s) indicate core scaffold (2-DOS).

linkages, hence, the name “aminoglycosides”. Based on these core structural elements, AGs can be categorized into three groups, namely: streptamine, 2-deoxystreptamine (which could be 4,5- or 4,6-disubstituted) and non-deoxystreptamine (unusual) scaffolds (Figure 1.2 to Figure 1.4). For most clinically relevant AGs, the cyclitol is a 2-deoxystreptamine (2-DOS) moiety, except for streptomycin

that has a streptamine (actually a streptidine) aminocyclitol ring. The binding mode of the 2-DOS core to prokaryotic ribosomes is highly conserved across all genera even though different AGs of the 2-DOS scaffold may each bind slightly differently to the 16S RNA.^{58,59} The hydroxyl and amino groups, which can also contain further substituents, are the key binding elements that interact with the RNA of the 30S subunit of the ribosome where they interfere with protein translation (Figure 1.5). It is therefore not surprising that the most prevalent mechanism of resistance to AGs is the enzymatic modification of the drugs at these key groups, in a bid to prevent rRNA recognition and interaction (see chapter 1.3.4.1). Indeed, the chemical structures of different analogs determine their level of susceptibility to various aminoglycoside-modifying enzymes (AMEs), the exact setback that most synthetic derivatives seek to circumvent.

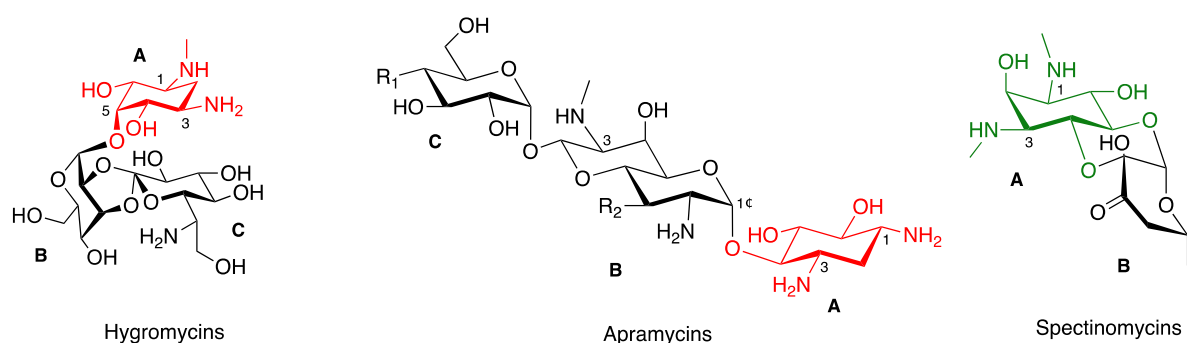


Figure 1.4. Structures of some unusual aminoglycoside scaffolds. Coloured structures indicate core scaffold (2-DOS).

1.3.3 Basis of Antibiotic Action

The bio- and physicochemical properties of AGs play a decisive role in the mechanism of their cellular uptake and mode of action. Being polybasic species, they naturally show binding affinity for negatively charged residues in the outer membrane (OM) of Gram-negative bacteria and in the rRNA. High-resolution x-ray crystallographic structures have also broadened our understanding of the interactions between AGs and their target site nucleotides, and how these translate into cell death and/or resistance.

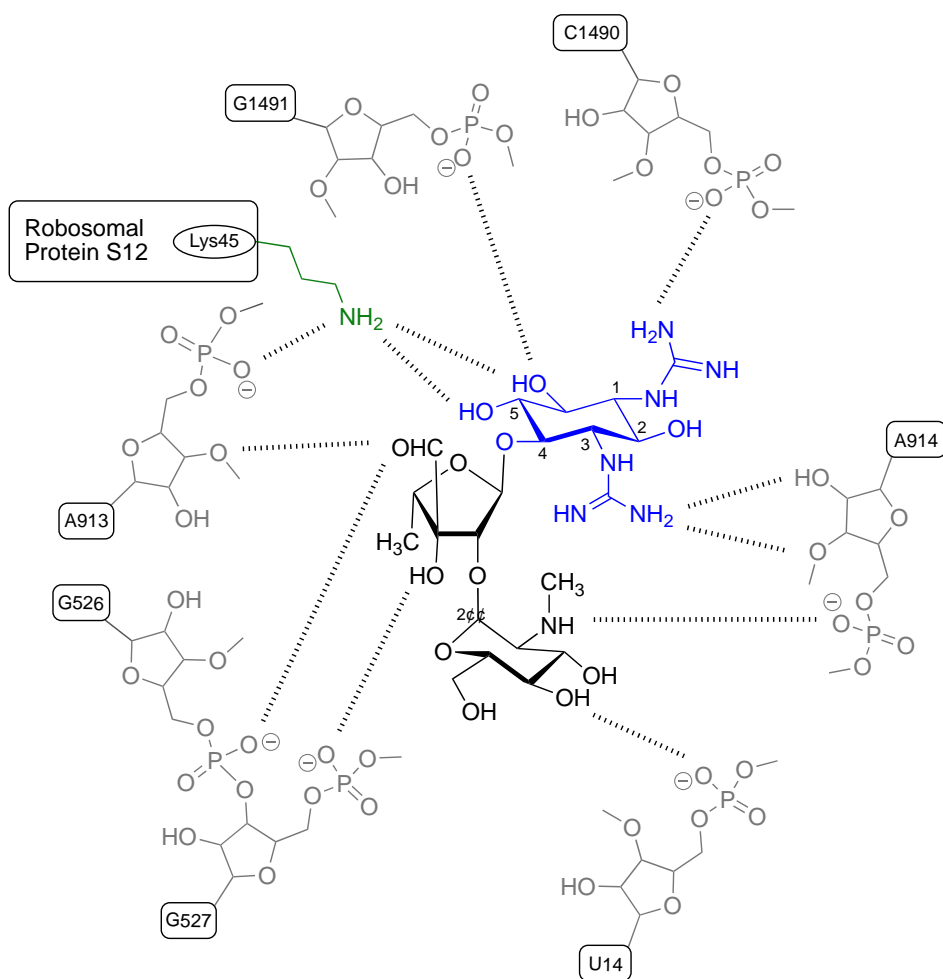


Figure 1.5. Interactions of streptomycin at its binding site.⁶⁰ Coloured structure(s) indicate core scaffold (2-DOS).

1.3.3.1 Cellular Uptake

The exact mechanism by which AGs penetrate bacteria cells is unclear, but a widely acceptable model of cellular uptake in Gram-negative bacteria proposes three different stages.^{61–63} First, there is an electrostatic interaction between the positively charged AGs and the negatively charged phosphate residues of lipopolysaccharides (LPS) on the outer leaflet of bacterial OM. The AG antibiotics then displace the cross-bridging magnesium ion that stabilizes the OM, thereby perturbing and compromising its integrity. This is known as the self-promoted uptake mechanism of AGs and it is a

concentration-dependent process. Another hypothesis suggests that the cationic hydrophilic nature of AGs enables them to penetrate the Gram-negative bacterial cell wall through the water-filled porin channels rather than direct diffusion through the phospholipid bilayer.⁶⁴ Some porin-deficient mutants have however been reported not to show any alteration in AG susceptibility and were still capable of AG-uptake.⁶⁵ In Gram-positive bacteria, this first stage involves the ionic binding of the polycationic drug to anionic phospholipids and lipoteichoic acids on the surface of bacteria cells.⁶²

The second and third stages are energy-dependent uptake processes known as energy-dependent phases I (EDP-I) and II (EDP-II) respectively. EDP-I is characterized by a slow rate energy-dependent uptake of AGs across the cytoplasmic membrane (as a direct consequence of stage I), resulting into an initial ribosomal binding and protein mistranslation.⁶¹ The incorporation of the misread proteins into the cytoplasmic membrane triggers an EDP-II process, a more rapid cytosolic accumulation of AGs that uses energy from electron transport chain and ATP hydrolysis.^{61,66} The EDP-II further compromises the integrity and functions of the cytoplasmic membrane, leading to an autocatalytic cycle of AG uptake and eventual cell death.⁶⁶ This model explains the reason anaerobes are generally ‘immunized’ to AGs, since they lack a membrane potential and the requisite electron transport upkeep.^{61,63} The secondary effects of mistranslated proteins have been hypothesized to be the true mechanism of AG lethality.

1.3.3.2 *Mode of Action*

Upon getting into the cytosol of prokaryotic cells, AGs target the ribosomes and act primarily by interfering with the translational fidelity and translocation step of protein synthesis. Specifically, AGs with 2-DOS scaffold interact with prokaryotic cells by binding to the A-site located on the 16S rRNA of 30S ribosomal subunit, thereby disrupting protein synthesis either by inhibiting the initiation step, causing mistranslation, or truncating the entire process.^{63,67} The 16S rRNA from *Escherichia coli* is a well-studied molecule as far as its interactions with various AGs are concerned. Treatment of

rRNA with an AG protects several nucleic acid bases in rRNA from chemical modification, implying that these molecules possess high affinities for certain sites in the rRNA (Figure 1.5).⁶⁷ When bound to 16S rRNA, crystallographic models have shown that AGs make numerous contacts with tRNA via its amino and hydroxyl functional groups, and induce a closed conformational change that mimics the correct codon-anticodon match of normally active ribosomes.^{68,69} For example, Puglisi and coworkers provided structural evidence on the mode of interactions of paromomycin with a 27-nucleotide RNA template that was designed to mimic the A-site region of the 16S rRNA in *E. coli* (Figure 1.6).⁷⁰ The solution structure of tobramycin-RNA aptamer has also been solved, with its conformation described as being similar to those of the hairpin loops in tRNA and rRNA.⁷¹ These interactions by AGs deceive the bacteria by signaling the continuation of translation process despite the incorrect mRNA-tRNA pairing, resulting into mistranslated proteins. Recent reports have shown that the energetic changes induced by AG binding might actually be more complex than initially understood.⁷²⁻⁷⁴ Some 2-DOS AGs (neomycin B, gentamicin and paromomycin) have been reported to bind not only to 16S RNA of 30S subunit, but also to a secondary site on the ribosome (the major groove of helix 69 of 23S RNA of the 50S subunit).^{74,75} Binding at this allosteric site has been demonstrated to impede the mobility of ribosomal subunit, which consequently interferes with protein translation and ribosome recycling.⁷⁵ Non-DOS (and streptomycin) AGs differ slightly from the 2-DOS AGs in that they do not bind directly to the A-site, but to a different location very close to it.⁶⁰ Nevertheless, they still interfere with the translocation of proteins. For example, streptomycin has a different binding site and interferes with the initial tRNA selection,⁷⁶ while spectinomycin and hygromycin have been shown to cause steric block in translation and inhibit protein biosynthesis respectively.^{77,78} The binding interactions for most AGs may be slightly different, depending on the structural complementarity, but they all lead to the same loss of translational fidelity.⁵⁹ The details of how all of these result into cell death is still an ongoing discussion, but evidences from viable ribosomal mutants with reduced translational accuracy

suggests this to be either due to the insertion of misread proteins or a complete overwhelming and inhibition of ribosomal activity.⁶⁰

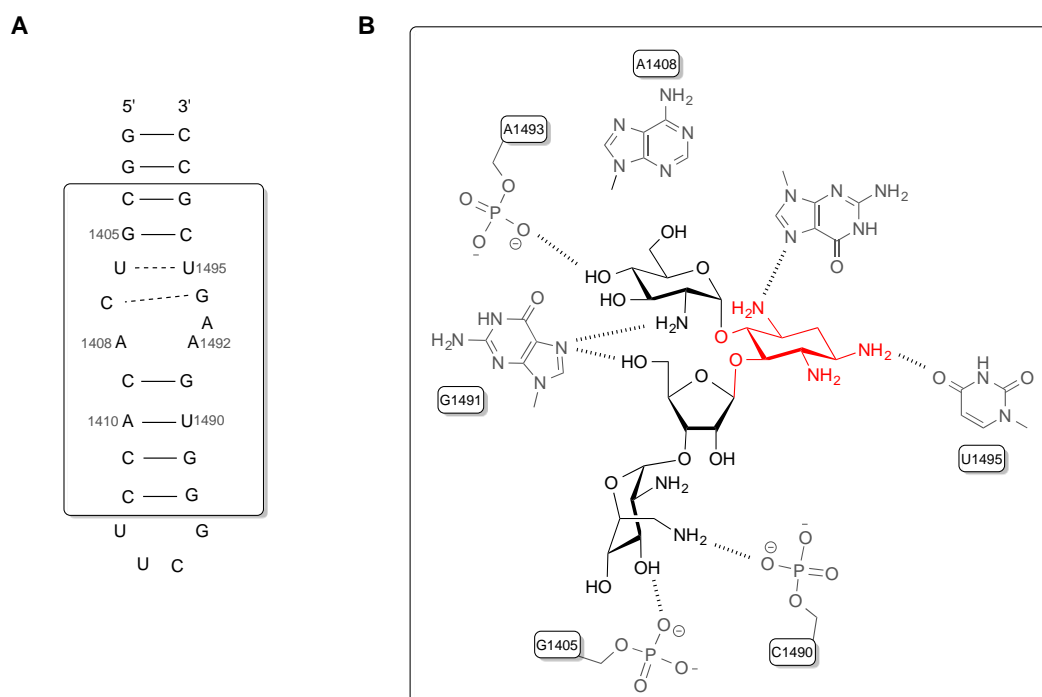


Figure 1.6. (A) Model of the A-site RNA template used to study the interactions of paromomycin. The box represents the portion of the rRNA that is homologous to the A-site.⁷⁹ (B) Structural interactions of paromomycin with the 16S rRNA in the A-site.⁶⁰ Coloured structure indicate core scaffold.

1.3.4 Mechanism of Resistance

Antibiotic resistance can generally be classified into three main categories: intrinsic, adaptive, and acquired resistance.^{35,80} Intrinsic antibiotic resistance is a chromosomally-encoded mechanism that naturally confers resistance capabilities on bacteria e.g. low permeability of the bacteria cell wall limits the uptake of many antibiotics, including AGs, hence resistance. Adaptive resistance is an inducible phenomenon that is dependent on the continuing presence of an external stimuli or sub-inhibitory concentrations of antibiotics and is easily reversed when the precipitating trigger is removed. Acquired resistance involves the acquisition and incorporation of exogenous genetic materials, usually a plasmid carrying multiple resistance genes, or via mutation of existing genes. This is known as horizontal

genetic transfer and could occur between bacteria of similar or different species. Although more pronounced in *Pseudomonas aeruginosa*, the above mechanisms as described in Chapter 1.2.4 hold true for most clinically relevant antibiotics, including AGs.

The resistance mechanisms specific to AG molecules include a combination of any or all of the followings: a) deactivation by AMEs via *N*-acetylation, adenylation or *O*-phosphorylation, b) target modifications via alteration of the 30S ribosomal unit by mutation and/or methylation of AG binding sites, and c) reduction of intracellular AG concentrations by changes in OM permeability, decreased inner membrane transport, active efflux, and drug trapping.^{79,81} Others include, but not limited to, proteolysis of mistranslated proteins by membrane proteases, and biofilms formation in chronic infections that binds cationic molecules such as AGs.⁸¹

1.3.4.1 *Aminoglycoside-modifying enzymes (AMEs)*

The most common mechanism of AG resistance is via the enzymatic activities of AMEs that catalyze covalent modification of specific amino or hydroxyl function, leading to a chemically modified drug that is unable to bind (or binds poorly) to ribosomes, and for which the EDP-II accelerated drug uptake also fails to occur.⁶³ The AMEs, which most likely evolved from enzymes of normal cellular metabolism due to the selective pressure from AGs,⁸² are classified into three categories based on the chemical modifications they impart to their substrates. They include: a) aminoglycoside *N*-acetyltransferases (AACs) that catalyze the acetylation of amino groups, b) aminoglycoside *O*-nucleotidyltransferases (ANTs) that facilitate the transfer of an AMP group from ATP to an hydroxyl group on AG molecule, and c) aminoglycoside *O*-phosphotransferases (APHs) that phosphorylate hydroxyl groups, introduce a negative charge to the molecule and thus decrease their ability to bind to negatively charged RNA due to electrostatic repulsion (Figure 1.7).⁸³ These are usually an acquired form of resistance. The conventional nomenclature for these enzymes consists of a three-letter code that describes their activity (AAC, ANT or APH), followed by a number in

parenthesis that identifies the specific site of AG modification, then a roman numeral that describes a particular resistance profile that is evoked in the host, and the last lower case letter as individual identifier.⁶⁰ For example, AAC(3)-IIa mediates *N*-acetylation at position 3 of its substrate, while some other bifunctional AMEs such AAC(3)-Ib/AAC(6')-Ib' and AAC(6')-30/AAC(6')-Ib from *P. aeruginosa* are capable of multiple types of AG modifications.⁸¹

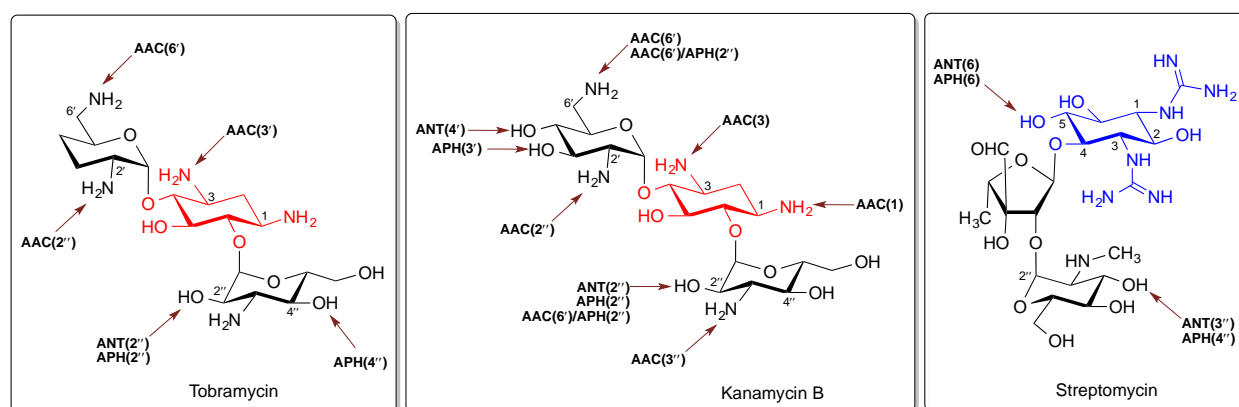


Figure 1.7. Sites of chemical modification by aminoglycoside-modifying enzymes (AMEs) on select aminoglycoside analogs. Coloured structure(s) indicate core scaffold (2-DOS).

The AACs are the largest group of the AME families, with four different classes (1, 3, 2' and 6') and a number of subclasses. AAC(6') enzymes are by far the most prevalent and clinically relevant AMEs, since the 6'-amino group in AG plays a critical role in rRNA and binding of this amino group causes resistance to the majority of clinically useful AGs except gentamicin.⁸⁴ They are also well studied and over fifty variants have been identified in both Gram-negative and Gram-positive species.⁸³ The ANTs, although divided into five classes (6, 9, 4', 2'' and 3''), are the smallest AME family by number, with ANT(4') being the only class with two subclasses I and II. They are nonetheless of significant clinical importance because both tobramycin and amikacin, as well as gentamicin are susceptible to ANT(2'').⁶⁰ The APHs include different classes and subclasses such as APH(4)-I, APH(6)-I, APH(9), APH(3')-I to -VII, APH (2'')-I to -IV, APH(3''), and APH(7'')-I. Of

these, the largest class is the APH(3') enzymes that phosphorylate the 3-hydroxyl in the B-ring of many AGs, while APH(6) and APH(3'') confer resistance to streptomycin.⁸³

In summary, the AMEs are a superfamily of enzymes that modify virtually all known AGs, and many of these AMEs are encoded on plasmids, transposons, integrons, and other transposable gene elements, which makes them highly mobile and facilitate the spreading of resistance via horizontal transfer, often along with other resistance genes (such as β -lactamases).⁶⁰

1.3.4.2 *Target Modification*

Asides the predominant mechanism of AG resistance by AMEs, prevention of binding via target site alteration can also occur by ribosomal mutations or enzymatic modifications. AGs target the A-site of the bacteria ribosome, but direct mutations in this highly conserved region are very rare, as changes to this vital cellular machinery are often lethal to the survival of the bacteria themselves.⁵⁸ Rather, changes that prevent binding of AGs with 2-DOS scaffolds occur mainly in the 16S rRNA codon-decoding A-site (points of contacts with the tRNA) of the ribosomal 30S subunit.⁵⁹ This explains why the binding mode of the 2-DOS core to prokaryotic ribosomes is highly conserved across all genera despite their varying levels of inactivation.

1.3.4.2.1 *Ribosomal Mutations*

A classic example of a successful mutation in the ribosomal 30S subunit-binding site is the mutation of A1408 in the single-stranded region of the 16S rRNA loop of *E. coli* that confers high-level resistance to neomycin and the gentamicins, as well as other members of the 2-DOS class, by interrupting key interactions with AGs.⁸⁵ Mutations of a single base pair in the 16S portion of the ribosome and the S12 protein of *Mycobacterium tuberculosis* have also been shown to confer resistance to the non-DOS AG, streptomycin.⁸⁶

1.3.4.2.2 Ribosomal Modifications by methyltransferases

Ribosomal methyltransferases (MTases) mediate series of enzymatic modifications of the target-binding site that transform nucleotides into its corresponding 7-methyl derivatives. MTases are divided into two families: those that methylate at the *N*7 position of nucleotide G1405 such as ArmA and Rmt(A to H), and those that methylate at the *N*1 position of A1408 (NpmA). The methylation of A1408 in the *N*1 position confers resistance to kanamycin, tobramycin, sisomicin, and apramycin but not to gentamicin, whereas methylation of G1405 in the *N*7 position confers resistance to the 4,6-disubstituted DOS, including gentamicin.⁷⁹ Methylation of these nucleotides presumably abolishes the intermolecular contacts that they make with the drugs. MTases that target the 16S rRNA were originally found in *Actinomycetes*, the natural producers of AGs, as one of the mechanisms to protect themselves from the toxicity of their own metabolites.⁸⁷ However, plasmid-mediated MTases that lead to a very high-level resistance to AGs have been recently reported in a number of pathogens including *P. aeruginosa*, *E. coli*, *Klebsiella pneumonia*, *Serratia marcescens*, *Proteus mirabilis* and *Acinetobacter baumannii*, thereby making further spread to other strains more likely.⁶⁰ Even though the RmtA and RmtB genes were only recently found in Japan where arbekacin has been in extensive use since 1990, and are currently not of serious clinical significance, the MTases pose a major threat because of their ability to confer an almost complete resistance to AGs.^{87,88}

1.3.4.3 Decreased Uptake and Increased Efflux

AGs must traverse a complex multilayered cell wall, especially in Gram-negative bacteria, before reaching its cytosolic target. Modifications to the cell wall in a way that significantly lower their permeability is an innate mechanism of resistance for Gram-negative bacteria and mycobacteria. For example, since the first stage of AG uptake involves an electrostatic attraction between the protonated molecules and the negatively charged LPS, incorporation of a positively charged 4-amino-4-deoxy-L-arabinose sugar into the LPS effectively reduces the net negative charge of LPS, hence,

decreasing affinity for AGs.⁸⁹ This is a common mechanism found in *P. aeruginosa* where the PhoP-PhoQ two-component regulatory system responsible for these changes are usually upregulated by Mg^{2+} starvation or the presence of polyamines.⁸⁹ Furthermore, it has been shown that nitric oxide-mediated repression of respiratory activity was able to block EDPI and EDPII in *Salmonella*, *P. aeruginosa*, *Staphylococcus aureus*, and *Bacillus*, by depleting the energy and proton motive force required for the transport of AG across the cytoplasmic membrane, thereby diminishing the effectiveness of AG therapy.^{90,91}

On the other hand, AGs have been demonstrated to be substrates for very few efflux pumps, perhaps due to their polybasic and polar nature, with the main efflux pump in Gram-negative bacteria (AcrAD) being a multidrug transporter and a member of the resistance-nodulation division (RND) family.^{60,81} The AcrAD-TolC describes a three-component (tripartite) system that spans the cell envelope, i.e. AcrD spans the innermost cellular membrane and functions as a drug-proton antiporter, AcrA is a membrane fusion protein found in the periplasm, and TolC is the OM component of the pump.⁸¹ Mutations in the regulatory genes of this pump or induction of expression in the presence of its substrate (AGs) can lead to overexpression of the originally constitutive pump genes.⁸¹ This contributes to resistance in *Pseudomonas*, *Acinetobacter*, *Brucella*, *Burkholderia*, *Enterobacter*, *Escherichia*, *Helicobacter*, and *Stenotrophomonas* spp.³⁸ AGs have also been demonstrated to induce *mexXY* expression in *P. aeruginosa*, leading to overexpression of MexXY-OprM that has been implicated in the resistance of this pathogen to several antibiotics, including AGs themselves.⁹² In summary, the overall contribution of efflux to AG resistance is modest at best, and seems to play a more important role only in the adaptive AG resistance in *P. aeruginosa*.⁹²

1.3.4.4 Membrane Proteases, Biofilms

Although not conventionally considered as a mechanism of resistance, membrane proteases that are responsible for the proteolysis of mistranslated proteins could protect the bacteria from the

lethal effects of AGs. After translocation of proteins across the membrane, several overlapping cell envelope maintenance and stress response systems are responsible for the quality control of protein biosynthesis. This includes signal sequence cleavage, regulation of protein abundance and degradation of misfolded and mistargeted proteins.⁹³ It is only if and when the expression levels of the misread proteins (caused by AGs) are higher than the ability of the membrane proteases to identify and degrade, will faulty proteins be able to accumulate enough to perturb membrane integrity. Deletion of the membrane protease FtsH regulators in *E. coli*, the *hflK* or *hflC* genes, led to increased susceptibility towards gentamicin compared to the wild-type, whereas treatment with norfloxacin (DNA gyrase inhibitor) showed no significant difference, demonstrating the importance of FtsH for bacteria survival when challenged with an AG.⁹⁴ Proteolysis has also been demonstrated to be part of the intrinsic resistance of *P. aeruginosa* to AGs, with mutations and inactivation of genes relating to FtsH and its regulation leading to significant increase in tobramycin sensitivity.⁹⁵ Multiple mutations led to synergistic effects, increasing the sensitivity against several classes of antibiotics and tobramycin sensitivity up to 500-fold.⁹⁵ Another study revealed an adaptive resistance of *P. aeruginosa* when exposed to tobramycin, where there was an increased expression of *asrA* genes that encodes a Lon-type protease, as well as heat shock genes.⁹⁶ This is common in chronic *Pseudomonas* infections of cystic fibrosis patients.

Furthermore, the adaptive resistance to AGs in chronic *P. aeruginosa* infections is usually associated with the bacteria existing as a biofilm. Biofilm matrix has been hypothesized to decrease the efficacy of cationic molecules (including AGs) by binding tightly to them, as well as upregulate the expression of MexXY efflux pump genes.⁸¹ Meta-analysis revealed that a variety of genes and proteins undergo subtle expression changes in biofilm when compared to non-biofilm cells, yet, they do not provide conclusive evidence on the exact mechanism that protects the biofilm from the activities AGs.⁹⁷

1.3.5 Conclusions and Perspectives

We have come a long way using AGs as antibiotics, and bacteria also have got them selected with harder resistance mechanisms. The origin of the naturally occurring AGs appears to be the main source of these resistance mechanisms, as most of the AG-producing species evolved strategies to avoid the deleterious effects of the antimicrobial metabolites they produce themselves or that are produced by others. These, together with various toxicological liabilities, led to a temporary decline in the use of AGs, especially with the discovery of safer antibiotics such as fluoroquinolones with equal spectrum of activity. The increased occurrence of nosocomial infections caused by multidrug resistant strains, particularly Gram-negative bacteria, has however rekindled enthusiasm about AGs and led to increased research efforts in both academia and industry. Due to the clinical prominence of AMEs in AG resistance, efforts are aimed at elucidating and understanding structural relationships of AMEs, with many useful structural information generated over the last couple of years. A comprehensive list of resolved crystal structures of AMEs is available in a recent review.⁹⁸ The strategy of developing semi-synthetic derivatives that seek to circumvent AMEs, as well as improve activity against many resistant strains, has resulted into analogs such as arbekacin derivatives (in early development in industry, with promising MICs) and Achaogen's plazomicin.^{57,60} Although no case of nephrotoxicity or ototoxicity reported for plazomicin in some clinical trials,^{99,100} this has remained largely inconclusive. Furthermore, the development of designer AG analogs that prevent cochlear hair cell and hearing loss offers a ray of light on how to possibly mitigate AG toxicity in human.^{53,54,101} Plazomicin (ZEMDRI) has now been approved by FDA^a for adults with complicated urinary tract infections (cUTI), including pyelonephritis, caused by *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, or *Enterobacter cloacae*, in patients who have limited or no alternative treatment options.¹⁰² More recently, our group demonstrated that amphiphilic tobramycin possess a host directed

^a Approval was declined for treating bloodstream infections due to lack of effectiveness.¹⁰²

clearance effect that is reminiscent of host defense peptides,¹⁰³ and that tobramycin-ciprofloxacin hybrids destabilize the OM of *P. aeruginosa* and enhance fluoroquinolone efficacy.¹⁰⁴ All of these espouse the renewed interest in AG antibiotics, and demonstrates the enormous traction and momentum it has garnered in the last few years.

The development of resistance to AGs via ribosomal modifications by MTases is also an emerging concern. MTases have the ability to completely inactivate AGs,⁸⁸ with a high mobility and substrate scope. Apramycin, a licensed AG for veterinary use, is the only exception to the activities of these enzymes,⁶⁰ and its unusual structure might offer a glimpse to ways of overcoming this mechanism of resistance. They are also the only known currently-used AG that do not display ototoxicity, as it shows little activity towards eukaryotic ribosomes.¹⁰⁵

Our understanding of the biology, advances in chemistry, and a good grasp of the pharmacology of AGs have placed us in a good position to take advantage of the excellent scaffold provided by nature to generate novel derivatives against resistant bacteria strains. Unfortunately, the exact mechanism of action of AGs, which is believed to be pleiotropic, is yet to be fully understood and our level of imagination might be our ultimate limitation. Going forward, it could be projected that the development of a fully synthetic labeled derivative that would allow an in-depth probe of the mode of action is just a matter of time. In the meantime, to combat multi-faceted bacterial resistance, clinicians will need to be able to execute a breadth of tailored therapies that maximize the benefits of the improved dosage regimens and therapeutic drug monitoring, for safer empirical and directed therapy.

1.4 Development of Ideal Antipseudomonal Agents: The Relevance and Limitations of Metrics

1.4.1 Introduction

Antibiotics are typically not ‘made’, they are often discovered, and the discovery process is a profound scientific challenge that involves lots of screening, prioritization of hits, careful analysis, as well as elements of luck. Our current arsenal is mainly made up of compounds that were derived from natural sources, the very source of their woes. However, the discovery pipeline has witnessed a significant attrition of drug candidates in the past few decades,¹⁰⁶ suggesting we have either exhausted our cheap supplies from nature, harvested all the low-hanging fruits, or simply ran out of steam to develop new scaffolds. Our inability to culture some uncultivable soil bacteria,¹⁰⁷ stringent regulatory policies, and lack of appreciable quantity of lead compounds for initial studies further aggravate the problem of antibacterial drug discovery. In spite of the enormous investment in high throughput screening (HTS) and target-based screening using the knowledge of bacteria genomics and proteomics,¹⁰⁸ antibacterial drug discovery has remained driven by serendipity and luck, as opposed to the much anticipated rational design and/or planned outcomes.

A complementary strategy to make up for this drug attrition is the chemical synthesis of completely new scaffolds, or the derivatization of existing classes of antibiotics to produce their next generation. This approach is expected to generate molecules that have never been encountered by microbes before, thus reducing the likelihood of an early development and spread of resistance.¹⁰⁹ The first crucial decision is whether to randomly synthesize/modify molecules and screen them against a panel of organisms, or to follow a set of rules/predictors for antibacterial drug-likeness. Both options have their merits and demerits, as exemplified in the diverse structural scaffolds that have resulted from approaches. The former approach is driven by luck and fortunate coincidence (how most antibiotics were discovered) while the latter is often guided by a carefully thought-out process

anchored on a multidisciplinary knowledge base (very few drugs have resulted from this approach). Irrespective of the approach, physicochemical properties are known to play significant roles in the biological functions of drugs,¹⁰⁶ except for antibacterial agents where such relationship seems to be ambiguous.¹¹⁰ This is perhaps due to the complex membrane topology of microorganisms, especially in Gram-negative bacteria, a major concern in antibacterial drug discovery.

For instance, *P. aeruginosa*, a soil-dwelling Gram-negative bacteria that has made its way into intensive care units of hospitals, is one of the most difficult pathogens to treat due to its ability to engender resistance to all known classes of antibiotics.³⁵ The low permeability of its outer membrane (OM) and its highly selective porin channels result in a reduced drug uptake, while the constitutively over-expressed efflux pumps and drug-degrading enzymes lead to very low accumulation of drugs within the cytosol.³⁵ These, coupled with the ability to evade the immune system, explain why *P. aeruginosa* is refractory to treatment and capable of living in chronically infected hosts for several years. It therefore implies that the development process of an ideal antipseudomonal agent must consider not just the properties of an ideal antibacterial drug, but also the unique and evolving characteristics of the pathogen as well.

1.4.2 Properties of an Ideal Drug

Drugs are usually not directly discovered but optimized from prototypical lead compounds by amplifying desirable pharmacological properties and repressing unwanted characteristics. This optimization process is guided by pharmacodynamic (PD) and pharmacokinetic (PK) principles. PD of a drug is the observed biochemical and physiological effects that result from the administration of a known drug concentration, its mechanism of action, and its dose-response relationships, while PK describes the drug concentration-time courses in body fluids resulting from the administration of certain drug dose.¹¹¹ In simple terms, PD is the effect of a drug on the body while PK is what the body

does to the drug. Both parameters encapsulate the ideal properties that a chemical entity is expected to have or display before it could be regarded as a potential drug.

1.4.2.1 *Pharmacodynamics*

The ideal PD of a drug stipulates a specificity of action. This means that a drug must have a specific target such as receptors (where it could act as an agonist or antagonist), enzymes (where it could act as a catalyst or inhibitor), DNA (where it could bind reversibly or irreversibly), etc., and must also elicit a dose-dependent therapeutic response. Such a drug would normally have a high therapeutic index (TI) that requires a low dose to elicit its therapeutic effect and a high concentration to reach its toxic threshold. TI is a measure of the comparison of the amount of drug that causes therapeutic effect to the amount that causes toxicity, and is calculated based on plasma exposure level, i.e. $TI = \frac{LD_{50}}{ED_{50}}$ (where LD_{50} and ED_{50} is the lethal and effective doses respectively for 50 % of population). The higher the LD_{50} , relative to ED_{50} , the higher the TI, and the safer the drug. Drugs with low TI could sometimes be used if the condition is life-threatening and no alternative exists. Furthermore, since only a small part of a lead compound, known as the *pharmacophore* of the drug, is usually involved in the PD interactions with drug targets, this part is rarely modified (depending on how the lead was discovered). The extraneous parts, known as *auxophores*, function by maintaining the integrity of the molecule and holding the pharmacophoric group in appropriate positions. They may be systematically modified, but care must be taken not to compromise their essential functions.

1.4.2.2 *Pharmacokinetics*

PK of a drug is described in terms of absorption, distribution, metabolism, and excretion of such drug at a particular concentration during its time-course. It is a robust mathematical representation that is subject to physicochemical properties of drug as well as the physiological state of the patient.

PK is also influenced by drug-drug interactions, route of administration, drug formulations, co-morbidity that could affect clearance from the body, etc., thus making it difficult to predict. It could also vary within an individual patient as condition changes. In general, PK profiles helps in the determination of useful therapeutic information such as half-life of the drug, bioavailability, onset and duration of action, maximum plasma concentration, etc. that helps in dose and dose regimen determination.

1.4.2.3 *Physicochemical Properties*

Physicochemical properties of drugs are responsible for their PD and PK interactions, and Lipinski's landmark study represented the first systematic attempt to correlate these properties with predicted drug absorption, oral bioavailability, target interactions, etc.¹¹² This correlation is famously known as 'Lipinski's rule' or the 'rule of five', and it states that poor absorption or permeation of a drug is more likely when: a) there are more than 5 hydrogen bond donors (expressed as the sum of hydroxyls and amines), b) the molecular weight (MW) is over 500, c) the LogP (lipophilicity as a measure of calculated logarithm of the 1-octanol/water partition coefficient) is over 5, d) there are more than 10 hydrogen bond acceptors (expressed as the sum of oxygen and nitrogen atoms), and e) compound classes that are substrates for biological transporters are exceptions to the rule.¹¹² These descriptors are better appreciated when drug-receptor interactions (such as electrostatic, dipole, hydrogen/halogen bonding, charge-transfer, hydrophobic, and Vander Waals) are considered at the molecular level (Figure 1.8). Although the 'rule of five' do not define a drug-like chemical space *per se*, it is a good predictor for oral bioavailability and the awareness of this has had a major impact on drug discovery.¹¹³ It is now very common to analyze these rules prior to synthesizing, optimizing or developing any novel drug candidate.

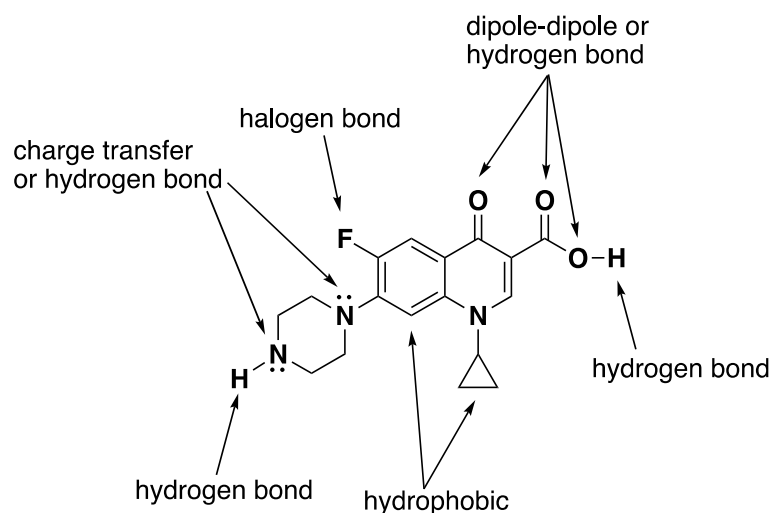


Figure 1.8. Example of potential multiple drug–target interactions of ciprofloxacin.

1.4.3 Ideal Properties of Antibacterial Agents

A limitation of Lipinski’s rule of five is its inability to predict the physicochemical properties of antibacterial agents and correlate bioavailability to their activities.¹¹² Broadly speaking, antibacterial agents often deviate from Lipinski’s metrics towards higher MW and increased polarity, suggesting that specific physicochemical requirements for bacterial action is as well critical for any potential biocidal agent.^{112,114,115} The orthogonal nature of the dual membrane topology of Gram-negative bacteria, i.e. a hydrophilic outer membrane and a hydrophobic inner membrane, further complicates the development of Gram-negative active agents. To put this observation into context, O’Shea and Moser studied 147 active antibacterial compounds and identified that the average MW of Gram-positive only agents was even much higher than those of Gram-negative agents (Table 1.1).¹¹⁶ The major contributors for the high MW of Gram-positive agents are the cell wall-active glycopeptides, macrolides, streptogramins and the lipopeptide daptomycin, while the exceptions to the low MW of Gram-negative only agents are azithromycin (749 Da) and polymyxin B (1203 Da), both of which have special permeability properties.^{117,118} A substantial increase in polarity (calculated as clogp values) was further observed in the Gram-negative group, when compared to Gram-positive agents.¹¹⁶

The disparity between these groups was more striking when the $\text{clogD}_{7.4}$ values that account for the charged state of molecules at neutral pH were compared, an effect that reflects significant increase in relative polar surface area (PSA), hydrogen bond donor, and hydrogen bond acceptor numbers for Gram-negative bacteria compared to Gram-positive (Table 1.1).¹¹⁶ These observations can easily be explained by the differences in the cell wall architecture of bacteria cells, where Gram-positive bacteria that have a thicker peptidoglycan layer will require a more hydrophobic molecule to diffuse across this layer, while Gram-negative bacteria with a polar outer membrane may only allow the passage of hydrophilic molecules via their porins. The exceptions to the less polar Gram-positive agents are membrane-active molecules and cell wall biosynthesis inhibitor that neither require the penetration of a lipid membrane nor directly affect cellular permeability. e.g. daptomycin, gramicidin, vancomycin.¹¹⁶ All these mean that the physicochemical requirements for bacterial actions may not necessarily correlate with Lipinski's metrics for oral bioavailability, yet, these drugs are active and used in humans. The only antibacterial drugs that fit well within the property space of Lipinski's rules are the sulfa drugs and fluoroquinolones (Table 1.2).¹¹⁶ Interestingly, these two classes of drugs are synthetic in origin and they have become the benchmark for the ideal physicochemical properties of antibacterial agents. Fluoroquinolones particularly have good PK properties, a relatively low level of serum protein binding, and an excellent safety profile.¹¹⁹

Table 1.1. Comparison of average (mean) compound property values of three drug sets representing general (CMC, excluding antibiotics) and antibacterial drugs.¹¹⁶ Abbreviations: CMC, comprehensive medicinal chemistry; MW, molecular weight; PSA, polar surface area

| Parameter | CMC data set | Antibacterial Activity | |
|-----------------------|--------------|------------------------|---------------|
| | | Gram-positive | Gram-negative |
| MW | 338 | 813 | 414 |
| clogP | 2.7 | 2.1 | −0.1 |
| clogD _{7.4} | 1.6 | −0.2 | −2.8 |
| PSA (Å ²) | 70 | 243 | 165 |
| rel. PSA (%) | 22 | 30 | 42 |
| H-donor | 1.6 | 7.1 | 5.1 |
| H-acceptor | 4.9 | 16.3 | 9.4 |

Table 1.2. Average (mean) physicochemical parameters for antibacterial classes.¹¹⁶ *n* is the number of representative examples for each class.

| Class | <i>n</i> | MW (Da) | clogP | clogD _{7.4} | PSA (Å ²) | rel. PSA (%) | H-donor | H-acceptor |
|------------------|----------|---------|-------|----------------------|-----------------------|--------------|---------|------------|
| Glycopeptides | 5 | 1740 | 1.3 | −1.8 | 586 | 37 | 22.8 | 37.2 |
| Macrolides | 8 | 790 | 3.5 | 2.6 | 189 | 23 | 3.6 | 15 |
| Penicillins | 14 | 413 | 1.4 | −2.4 | 149 | 39 | 2.8 | 8.5 |
| Cephems | 28 | 452 | 0.1 | −3.0 | 210 | 51 | 4.1 | 10.8 |
| (carba)Penems | 6 | 397 | −3.0 | −5.8 | 159 | 43 | 4.5 | 9 |
| Sulfa drugs | 19 | 273 | 0.6 | −0.1 | 112 | 45 | 3.1 | 6.2 |
| Fluoroquinolones | 24 | 371 | 1.3 | −0.8 | 82 | 25 | 2.1 | 6.5 |
| Tetracyclines | 10 | 481 | −0.7 | −3.6 | 184 | 40 | 7.1 | 10.5 |
| Aminoglycosides | 12 | 526 | −2.9 | −8.1 | 279 | 54 | 14.8 | 15.4 |

1.4.4 Developing an Ideal Antipseudomonal Agent

The synopsis of the foregoing is that Gram-negative only active agents generally have low MW (usually < 600 Da), with the exception of compounds that target the OM or compounds with special permeability properties (such as self-promoted or active transport mechanisms). This criterion is even more critical in *P. aeruginosa* because of its reduced membrane permeability, very selective porin channels, and highly efficient and diverse efflux pumps.^{9,10,13} As expected, the physicochemical property space for antipseudomonal agents is more narrowly defined than the larger set of Gram-negative antibacterial agents, and their lipophilicity requirement is shifted towards even higher polarity.¹¹⁶ The success of antipseudomonal agents depends greatly on their ability to intricately balance hydrophilic-hydrophobic characters in such a way that hydrophilic requirements to cross the outer membrane do not compromise hydrophobic criteria to traverse the inner membrane and/or mediate biological effects. It is therefore not surprising that fluoroquinolones, β -lactams and aminoglycosides are the widely used antipseudomonal agents.⁴³ Aminoglycosides, being highly polar molecules, are used because of their self-promoted uptake mechanism across the outer membrane and energy-dependent uptake mechanism across the inner membrane (Chapter 1.3.3.1).

1.4.4.1 Chemistry

Antipseudomonal drugs typically contain diverse heteroatoms (such as oxygen, nitrogen, sulfur, fluorine, etc.) that contribute to their polarity, hydrogen bonding and PK. H-bonding is essential in the PD interactions of drugs with receptors, enzymes, nucleic acids etc., while fluorine atom has been observed to improve PK, e.g. fluoroquinolones.^{119,120} Also, most antipseudomonal agents are protonated prior to entering the bacterial cells, an important first step for their electrostatic interaction with the negatively-charged LPS of the pathogen. For instance, aminoglycosides and fluoroquinolones exist in their protonated states just prior to getting into bacterial cells, and could be electrostatically

repulsed by *P. aeruginosa* via insertion of positively-charged 4-amino-L-arabinose into its LPS.³⁵ Antipseudomonal drug should be capable of existing in both charged (preferably zwitterionic) and non-charged form, the former to penetrate porins or interact with LPS and the latter to be absorbed in the gut. Also, the *pka* of the amines (or any other basic group) must be modest (not too high) and protonated at physiological pH of ≈ 7.4 , implying a careful positioning of electron-withdrawing substituents (electronic effects) in a rational design approach.¹²¹ Other chemical properties that could influence biological activities include the number of rotatable bonds, polar surface area, aromatic ring count, steric effects (presence of bulky groups), etc.¹²² For porin-mediated uptake, compounds with low MW and relatively small size/dimensions are often desirable, except for molecules that are either facilitated by active transport system, are self-promoting, or can form hydrates, where MW size restriction might not be rigid.¹²³

1.4.4.2 *Mechanism of Action*

A clear knowledge of likely target(s) or mode of action of a proposed drug is an invaluable working tool in lead optimization and rational drug design. Unfortunately, this is usually not the case, as evident in the disappointments of genomic-based antibiotic discovery.¹²⁴ Most rationally-designed drug candidates deviate from their proposed target or anticipated mechanism *in vivo*, have poor PK, or might even lack the relevant pharmacological activity. Nonetheless, the identification of a potential target as either extracellular or intracellular is critical in the overall design and development of an ideal antipseudomonal drug. Extracellularly-acting agents hardly need to comply with size and/or MW restrictions, whereas agents with intracellular targets must be able to navigate the complex membrane topology of *P. aeruginosa*. For example, the high MW polymyxins only retain antipseudomonal activity because of their capability to disrupt the outer and inner membrane, while fluoroquinolones that act intracellularly are able to pass through the porin because of their low MW. Unfortunately, currently available antipseudomonal agents only inhibit a short list of cellular targets, i.e.

fluoroquinolones stabilize the ternary complex of DNA gyrase (and topoisomerase IV),¹²⁰ aminoglycosides inhibit fidelity of ribosomal functions,¹²⁵ and β -lactams shut down cell wall biosynthesis.¹²⁶ Moreover, highly conserved genes and amino acid residues that could serve as targets for antibiotics, as revealed by genomics, have the potential liability of an easy evolution of point-mutation resistance.¹²⁷ Consequently, current efforts are aimed at novel targets such as the extracellular (flagellum, pili, LPS) and intracellular (T3SS, quorum sensing) pathogenic mechanisms of *P. aeruginosa* that encodes different virulence.¹⁹ Our group has also reported the disruption and/or destabilization of the OM as a viable therapeutic strategy to restore potent antimicrobial activities of legacy antibiotics against *P. aeruginosa*.^{104,128,129} In summary, different target sites and mechanisms of penetration require different physicochemical properties that must be built into a drug during design.

1.4.4.3 *Spectrum of Activity*

Antimicrobial chemotherapy could be narrow- or broad-spectrum based. Narrow-spectrum antibiotics could be useful for directed therapies when the causative organism and its susceptibility profile is known, thus reducing the likelihood of early development and rapid dissemination of resistance (due to their specificity). They could also help preserve the normal flora in the body and prevent unnecessary overexposure of the immune system. Unfortunately, causative organism(s) have to be fully characterized and identified before narrow-spectrum antibiotics can be effective, leading to loss of valuable therapeutic time. There is also a high chance of therapy failure if the drug is not carefully selected. Conversely, broad-spectrum antibiotics affect a wider range of bacteria species (Gram-positive and Gram-negative) and/or serotypes. They are often used as prophylaxis after surgical operations or for empirical treatments when causative organism is unknown or in doubt. Although using a broad-spectrum antibiotic is a safe guess, the risks of resistance dissemination across different bacteria species and the possibility of a superinfection (due to the destruction of normal flora) is very high, a major reason why their use should be dictated by necessity. Fortunately, the sequencing of

multiple strains of *P. aeruginosa* revealed that the genome is arranged as an assortment of conserved regions interspersed by ‘regions of genomic plasticity’ that contain genes unique to each strain.¹² The identification of *P. aeruginosa* as having a ‘core’ genome (containing a conserved set of genes common to the species and comprising as much as 90% of the genomic content) and an ‘accessory’ genome (containing genes that are generally found in only a few strains),¹² provides an avenue to develop agents that may be specific for *Pseudomonas*. A broader implication of this for drug discovery is that even within *Pseudomonas* species, antipseudomonal agents could either target the core or accessory genome. It is a considered view that an ideal antipseudomonal drug should have species-based narrow spectrum of activity (specific for *Pseudomonas*), target important cellular machinery and/or multiple sites on the core genome (to prevent point-mutation resistance), and be bactericidal.

1.4.4.4 Toxicity

The therapeutic index (TI) of a drug is an indication of the safety margin of that drug. The higher the TI, the safer the drug, and vice-versa. Even at that, drugs rarely mediate clean pharmacological effects and most antipseudomonal drugs have toxicological profiles that limit their clinical use.^{52,130} Although the genesis of any toxicological outcome is multifactorial and difficult to predict, a probabilistic quantification of likely parameters such as GSK’s 4/400 and Pfizer’s 3/75 rules at the design stage could greatly improve chances of success.^{131,132} GlaxoSmithKline (GSK)’s 4/400 rule assumes higher risks of toxicity, interactions with off-targets or difficulties during development if $\log P > 4$ and $MW > 400$,¹³³ while Pfizer 3/75 rule states that a compound has a 6-fold reduction in preclinical toxicity when $\text{ClogP} < 3$ and a topological polar surface area (tPSA) $> 75 \text{ \AA}^2$ (and 24-fold reduction for basic compounds).¹³¹ These rules are not set in stone, as they have the tendencies to give false negatives, however, antibiotics are usually administered at gram scale per day hence the need for strict toxicological profiles and selectivity for prokaryotic cells.

1.4.5 Outlook

The reality is that ideal drugs do not exist, at least not yet. The pharmacology of first-generation antibiotics is often not ideal, reflecting their origin as specialized microbial metabolites and not medicines. Drug targets and mechanisms of action are also mostly understood (if at all) long after efficacy and toxicity have been established, and PK optimization is continuous throughout the entire life of a drug. As much as Lipinski's rule of five describes molecular properties important for a drug's PK in humans, it does not predict whether a compound will be pharmacologically active or not. The metrics also tend to populate synthetic libraries with antibacterial agents that are optimized for human biology and not the pathogen, making Lipinski's rule a necessary but insufficient condition for drug discovery. Furthermore, natural-product antibiotics are usually complex and polar molecules, with challenging and intractable synthetic routes that make it difficult to prepare derivatives with suitable product profile and/or spectrum of activity. This makes the development of drug candidates a matter of synthetic convenience rather than design.¹³⁴ Lastly, for a new antibiotic to succeed, it has to either; a) satisfy an unmet medical need, b) exhibit superiority over existing treatments in terms of potency, selectivity, safety or act via a new mechanism, or c) provide specific PK advantages such as convenient dosing regimen, improved drug interaction profile, stability/metabolism, etc. The economic risks associated with these led to the abandonment of antibacterial research by big pharmaceutical companies, resulting in a decline in antibiotic discovery.¹³⁵

The solution to this stalemate lies in the development of synthetic libraries that capture the chemical diversity of natural products, and the physicochemical properties of ideal drugs. The practice of best guess medicine and empirical treatment should also be discouraged and avoided when possible. Government incentives for antibacterial research and regulatory policies that reflect the current economic realities could perhaps attract the big players back into the industry. Although the pattern of resistance development by Gram-negative bacteria, especially *P. aeruginosa*, is unpredictable, it is an inevitable phenomenon that we must all be prepared for.

1.5 References

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2 CHAPTER TWO:

Bifunctional Conjugates as Antibiotic Agents and Adjuvants against Gram-negative Bacteria

2.1 Preface

The work presented in this chapter has been published as a review article in:

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Any update since the publication of this article is noted in the footnotes.

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2.1.1 Authors' Contributions

Ronald Domalaon and Temilolu Idowu contributed equally to this work.[#] Ronald Domalaon conducted literature review and wrote the sections on antibiotic hybrids as agents against Gram-negative pathogens. Temilolu Idowu conducted literature review and wrote the sections on antibiotic hybrids as adjuvants against Gram-negative pathogens. George G. Zhanel and Frank Schweizer provided guidance and feedbacks. All authors were responsible for the final form of the manuscript.

2.2 Summary

The global incidence of drug-resistant Gram-negative bacillary infections has been increasing, and there is a dire need to develop novel strategies to overcome this problem. Intrinsic resistance in Gram-negative bacteria, such as their protective outer membrane and constitutively overexpressed efflux pumps, is a major survival weapon that renders them refractory to current antibiotics. Several potential avenues to overcome this problem have been at the heart of antibiotic drug discovery in the past few decades. We review some of these strategies, with emphasis on antibiotic hybrids either as stand-alone antibacterial agents or as adjuvants that potentiate a primary antibiotic in Gram-negative bacteria. Antibiotic hybrid is defined in this review as a synthetic construct of two or more pharmacophores belonging to an established agent known to elicit a desired antimicrobial effect. The concepts, advances, and challenges of antibiotic hybrids are elaborated in this article. Moreover, we discuss several antibiotic hybrids that were or are in clinical evaluation. Mechanistic insights into how tobramycin-based antibiotic hybrids are able to potentiate legacy antibiotics in multidrug-resistant Gram-negative bacilli are also highlighted. Antibiotic hybrids indeed have a promising future as a therapeutic strategy to overcome drug resistance in Gram-negative pathogens and/or expand the usefulness of our current antibiotic arsenal.

2.3 Introduction

The rapid global dissemination of Gram-positive and Gram-negative bacterial pathogens that are resistant to currently available antimicrobial therapies, in both hospital and community settings, marks the onset of a possible severe worldwide health crisis (1–3). Out of all these pathogens, the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) bacteria (4) account for the majority of

nosocomial infections worldwide, with an increasing incidence of drug resistance every year (3, 5). The incidence of clinical isolates belonging to the ESKAPE group that exhibit either multidrug resistance (MDR), extensive drug resistance (XDR), or pandrug resistance (PDR) is quite alarming (6–8). MDR is defined as non-susceptibility to at least one agent in ≥ 3 chemically dissimilar antibiotic classes, XDR is defined as non-susceptibility to at least one agent in all but ≥ 2 chemically dissimilar antibiotic classes, and PDR is defined as non-susceptibility to all agents in all antibiotic classes (9). However, the problem is arguably more serious for Gram-negative organisms, which are more frequently MDR and for which no novel antibacterial drug entities with novel modes of action (only new drug combinations) have been approved for clinical use in 5 decades (3, 10, 11). Indeed, four out of the six ESKAPE pathogens (*K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* spp.) are Gram-negative bacilli.

Various health organizations have been vocal about the urgent need to develop new antibiotics, especially against drug-resistant Gram-negative ESKAPE bacilli. For instance, the World Health Organization (WHO) has raised its utmost concern about the possibility of a post-antibiotic era where common infections and minor injuries may result in significant morbidity and mortality (12). World leaders convened in September 2016 during the 71st United Nations General Assembly (UNGA) to discuss the issue of antimicrobial resistance, an event that resulted in each governing body taking a unified stance toward preventing a post-antibiotic era (13–15). The increasing frequency of bacterial infections caused by MDR pathogens and the lack of effective therapeutic options for treatment are apparent worldwide. In response to the dwindling antibiotic pipeline, the Infectious Diseases Society of America (IDSA) launched the “10 × ‘20 Initiative” in 2010 that challenged stakeholders to advance 10 new U.S. Food and Drug Administration (FDA)-approved systemic agents to treat bacterial infections by 2020 (16). In a follow-up report 3 years later (2013), the IDSA noted definite but slow progress toward achieving the goal of the 10 × ‘20 Initiative, wherein only one systemic agent (ceftaroline fosamil) had materialized as of that time (17). As of November 2017, nine new FDA-

approved systemic new molecular entity (NME) antibiotics have been developed (Table 2.1),^b with a projection that the goal of the IDSA will most likely come to fruition. However, only six (ceftaroline fosamil, ceftolozane-tazobactam, ceftazidime-avibactam, delafloxacin, meropenem-vaborbactam, and secnidazole) out of the nine systemic agents are used for the treatment of drug-resistant Gram-negative bacterial infections. Two agents (fidaxomicin and finafloxacin otic suspension) are approved as non-systemic antibacterial agents.

Table 2.1. FDA-approved New Molecular Entities (NME) antibiotics from 2010 – November 2017

| Year | Name | Class | Able to treat antibiotic-resistant Gram-negative ESKAPE bacterial infection? | Route of drug administration |
|------|------------------------------|--|--|------------------------------|
| 2010 | ceftaroline fosamil | cephalosporin | Yes | Systemic |
| 2011 | fidaxomicin | macrolide | No | Non-systemic |
| 2014 | dalbavancin | lipoglycopeptide | No | Systemic |
| 2014 | oritavancin | lipoglycopeptide | No | Systemic |
| 2014 | tedizolid phosphate | oxazolidinone | No | Systemic |
| 2014 | ceftolozane-tazobactam | cephalosporin + β -lactamase inhibitor | Yes | Systemic |
| 2014 | finafloxacin otic suspension | fluoroquinolone | Yes | Non-systemic |
| 2015 | ceftazidime-avibactam | cephalosporin + β -lactamase inhibitor | Yes | Systemic |
| 2017 | delafloxacin | fluoroquinolone | Yes | Systemic |
| 2017 | meropenem-vaborbactam | carbapenem + β -lactamase inhibitor | Yes | Systemic |
| 2017 | secnidazole | nitroimidazole | Yes | Systemic |

^b As of April 2019, three more systemic agents (eravacycline, omadacycline, and plazomicin) have been approved by FDA for the treatment of drug-resistant Gram-negative bacterial infections ([fda.gov](https://www.fda.gov)).

The limited availability of antibiotics to treat MDR Gram-negative bacterial infections remains a serious problem. It is therefore imperative to develop new agents or new therapeutic strategies that are able to overcome drug resistance in these organisms.

2.3.1 Permeability Is an Important Consideration in Developing Antibiotics for Gram-negative Bacteria

Bacteria are classified as Gram-positive and Gram-negative (with some exceptions, such as mycobacteria) based on their prokaryotic cell membrane structure. Gram-positive bacteria possess a thick cell wall that consists of peptidoglycan and teichoic acid layers anchored to the cytoplasmic membrane. On the other hand, Gram-negative bacteria have a thin peptidoglycan layer that is surrounded by an inner membrane (IM) and an outer membrane (OM), thus forming the periplasmic space (Figure 1.1). The double layer of protection in Gram-negative bacteria, in addition to an abundance of efflux pumps and highly selective porins, makes it more difficult for an intracellularly targeting agent to elicit its antibacterial function (18).

2.3.1.1 *The outer membrane is efficient in restricting molecular passage*

The OM is an asymmetric bilayer (Figure 1.1) with an inner leaflet consisting solely of phospholipids and an outer leaflet that contains an abundance of lipopolysaccharides (LPS). The polymeric LPS is composed of three domains: the hydrophobic lipid A, the hydrophilic core oligosaccharides, and the hydrophilic *O*-antigen. Lipid A is responsible for forming a lipid bilayer with the inner leaflet. Core oligosaccharides and *O*-antigen, which extend outwards to the extracellular environment, are responsible for cellular recognition and virulence (among other functions). The presence of the OM makes Gram-negative bacteria intrinsically resistant to many antibiotics,

especially those with a high molecular weight and hydrophobicity. For instance, the LPS structure renders the bacterial OM more restrictive to hydrophobic antibiotics than the IM (19). It has been argued that the hydrophilic carbohydrate component of LPS creates a hydration sphere that restricts the movement and passage of hydrophobic molecules across the membrane (20). The efficient packing of lipid A, due to its molecular organization and lower unsaturated fatty acid content than that of a normal phospholipid bilayer, results in lower OM fluidity (21–23), thus limiting the membrane permeation of hydrophobic agents. Integral membrane proteins that interact directly with LPS, such as outer membrane protein H (OprH) in *P. aeruginosa* (24) and the Tol-Pal complex in *Escherichia coli* (25), further augment stability and, therefore, the impermeability of the membrane. Experimental evidence shows 50- to 100-fold-lower hydrophobic probe permeation rates in lipid bilayers that contain lipopolysaccharide (reflective of the OM) than in bilayers that consist of phospholipids only (reflective of the IM) (26). Structural variabilities and modifications in the LPS, especially the lipid A portion, result in significant differences in drug permeation rates among Gram-negative organisms (27). It is therefore clear that the OM constitutes a major hurdle for drug uptake in Gram-negative bacteria, especially for *P. aeruginosa*, which has 12- to 100-fold reduced outer membrane permeability relative to that of *E. coli* (28).

2.3.1.2 Membrane permeation is a limitation to most but not all agents with a high molecular weight

The glycopeptide antibiotic vancomycin, with a molecular mass of 1449.3 g/mol, lacks antibacterial activity against most clinically relevant Gram-negative bacteria. Vancomycin inhibits peptidoglycan synthesis by sequestering peptidoglycan precursors that ultimately prevent glycan cross-linking. In Gram-positive organisms, vancomycin exerts its antibacterial activity uninhibited, as its target is located at the cell membrane. However, it must traverse the OM and reach the periplasmic space to elicit its function in Gram-negative organisms, a feat which vancomycin is incapable of

achieving due to the protective membrane barrier. The loss of antibacterial activity due to membrane impermeability is true for almost all clinically used glycopeptide antibiotics (29, 30). Most antibiotics and biomolecules with molecular masses of > 600 g/mol are incapable of traversing the OM (31), with few exceptions, including polybasic amphiphiles, such as polymyxins and antimicrobial peptides, and non-basic energy sources, such as maltohexaoses (32, 33).

Charged (cationic or zwitterionic) or noncharged small hydrophilic molecules are typically able to enter the periplasmic space via nonspecific protein channels called porins. Examples of such antibiotics with porin-dependent uptake include β -lactams, fluoroquinolones, and sulfonamides. These β -barrel-structured integral protein channels allow water-soluble molecules to traverse the restrictive hydrophobic membrane through their water-filled cavity (34–36). However, porins impose molecular sieving properties, as only small molecules, typically ≤ 600 g/mol, are believed to pass through their narrow channels (37–39). Drug permeation through porins may also vary among Gram-negative bacteria. For instance, *P. aeruginosa* possesses lower outer membrane permeability than *E. coli*, as it expresses a more selective outer membrane protein F (OprF) porin (28, 39). The OprF porin constitutes the majority of the porins present in *P. aeruginosa* (40). Porins that are present in relatively smaller amounts in *P. aeruginosa* include OprB, OprC, OprD, OprE, OprF, OprG, OprH, and others (41, 42). The OprF porin has been shown to also allow solute diffusion much more slowly than classical porins as a consequence of its structural conformation (39, 43). For example, the monosaccharide L-arabinose was found to diffuse 50 times slower in the OprF porin in *P. aeruginosa* than in the OmpF porin channel of *E. coli* (44). Low intracellular drug concentrations due to slow porin-mediated influx in *P. aeruginosa* are exacerbated by the abundance of multidrug efflux pumps and resistance-encoding genes (28, 43, 45).

However, several antibiotics with a high molecular mass (> 600 g/mol) are able to pass through the OM in a mode of uptake independent of porins or passive diffusion. These compounds are mostly

cationic and are hydrophilic (such as aminoglycosides [46]) or amphiphilic (such as polymyxins) in nature (46). They are able to transit the OM via a “self-promoted” uptake mechanism, which is characterized by the initial displacement of divalent cations (Ca^{2+} or Mg^{2+}) that results in OM destabilization (47). Electrostatic interactions between the positively charged divalent cations and the negatively charged phosphate groups on lipid A stabilize the LPS structure (48–50). It is perceived that the subsequent localized OM disruption from divalent cation displacement facilitates the penetration of the antibiotic into the periplasmic space (51, 52). It was widely documented in early years that the antibacterial activities of aminoglycosides and polymyxins are antagonized by the exogenous addition of Mg^{2+} and Ca^{2+} cations (53–56). This observation was later attributed as being a hallmark of the self-promoted uptake mechanism, that it entails the displacement of divalent cation LPS bridges and that exogenous supplementation of the divalent cations immediately arrests the process (57). The physicochemical requirements for a molecule to display self-promoted uptake are yet to be fully understood, although the propensities of a molecule to be protonated (to effectively carry one or more positive charges) under physiological conditions and to strongly interact with LPS may be necessary characteristics.

2.3.1.3 The inner membrane as a second restrictive barrier for agents with cytosolic targets

The phospholipid bilayer that comprises the IM greatly limits the diffusion of hydrophilic molecules. Compared to the OM, hydrophobic molecules easily traverse the IM through passive diffusion. However, charged solutes such as sodium cations and hydrophilic nutrients such as glucose enter freely into the cytosol once they traverse the OM. Their uptake is achieved through the use of solute-specific energy-dependent transporter proteins (58). Some weakly charged or neutral amphiphilic compounds may also enter the cytosol by utilizing proton motive force (PMF) (59, 60). Bacterial PMF is governed by the proton gradient, ΔpH , and membrane potential, $\Delta\Psi$. The ΔpH is

believed to facilitate the diffusion of weakly charged molecules through charge neutralization, while the $\Delta\Psi$ is perceived to stimulate electrochemical interactions that lead to molecular uptake (61, 62). For instance, the cytoplasmic uptake of the sulfonamide (63) and tetracycline (64) classes of antibiotics is ΔpH dependent, while the uptake of the aminoglycoside class of antibiotics appears to be $\Delta\Psi$ dependent (65).

2.3.1.4 *Intracellular drug concentrations are greatly affected by efflux*

Once a drug makes its way intracellularly, it could be effluxed out before it mediates its antibacterial action. Efflux pumps are membrane proteins that can expel their substrates from the cytosol into the periplasm or from the periplasm into the external environment. So far, all studied Gram-negative organisms are known to express at least one multidrug efflux pump (66). Bacterial efflux systems have been extensively reviewed in the literature (see references 67–72). However, it should be noted that drug efflux affects the intracellular concentration of a therapeutic agent and that the overexpression of multidrug efflux pumps confers intrinsic antibiotic resistance on the pathogen.

2.3.1.5 *There is an urgent need for guidelines to develop agents that are able to penetrate both outer and inner membranes*

It is evident that drug permeability in Gram-negative bacteria is more challenging for antibiotics with cytosolic targets, as they must transit two protective lipid bilayers (73). One approach to overcome this involves appending or tweaking different functional groups on a lead structure, in the hope of generating more-amenable derivatives with enhanced biological activity and cellular permeation. “Rule-of-thumb” knowledge in structural modification is used by medicinal chemists to make rational decisions for drug optimization. Lipinski’s rule of five for favorable drug oral bioavailability (74) has become a popular *in silico* guideline for the development of therapeutic agents

that are able to cross intestinal epithelial cells. To possess good pharmacokinetics in the human body, Lipinski's rule proposes that an agent may not have (i) more than five hydrogen bond donors, (ii) more than 10 hydrogen bond acceptors, (iv) a molecular mass of > 500 g/mol, and (v) a lipophilicity factor ($\log P$) of > 5 , as measured by the octanol-water partition coefficient (74, 75). Unfortunately, Lipinski's metrics do not hold true for antibacterial agents that require bacterial membrane penetration. Molecular passage through the OM appears to be governed by a different set of physicochemical rules that are orthogonal to the IM (18). Compounds that are optimized solely to traverse the OM most likely would not be able to cross the IM and vice versa. A widely acceptable set of membrane permeation rules for antibacterial agents appears to be nonexistent (76). An attempt was previously made to formulate a guideline by binning all antibiotics in the pipeline and in clinical evaluation to correlate discernible physicochemical parameters with antibacterial activity (77). Compounds were binned into three categories, namely, compounds that have activity against only Gram-positive organisms, compounds that have activity against Gram-negative organisms, and compounds that are antipseudomonal. A high polarity (for porin uptake) and a reasonable level of lipophilicity (to ensure lipid membrane penetration) were observed to be ideal for agents against Gram-negative organisms (77). By exploiting the ideal physicochemical properties revealed from binning antibiotics, an effort to optimize the activity of oxazolidinones against Gram-negative bacteria was recently described (78). Members of the oxazolidinone class of antibiotics, such as linezolid, do not have potent activity against most Gram-negative organisms, presumably due to permeation impediments across the OM and/or efflux (79). The most active prepared oxazolidinone analog demonstrated only a modest enhancement of activity against *E. coli*, and those authors noted that a fully realized set of permeation guidelines is direly needed for optimizing lead compounds (78). A recent article emphasized the inherent hurdles in developing agents that are able to permeate Gram-negative membranes and suggested that the binning process should be further refined to include the route(s) of cellular entry for each antibacterial agent (18). However, the development of reliable methods that allow data mining for cellular entry

and the accumulation of antibiotics is necessary to realize such a suggestion. At this point, several experimental protocols may hold the key to tackling this proposition. For instance, the elucidation of bacterial uptake mechanisms and the subsequent quantification of cytoplasmic accumulation that utilize techniques such as tandem liquid chromatography-mass spectrometry (LC-MS) (80, 81), Raman spectroscopy (82), and microspectroscopy (83) have been reported. A proof-of-concept study that utilized LC-MS for quantification and several correlation programs for analysis of 10 sulfonyladenosine-containing agents in terms of their membrane permeation in *E. coli*, *Bacillus subtilis*, and *Mycobacterium smegmatis* was reported (84). This systematic approach successfully delineated the relationship between the physicochemical properties and the cytoplasmic accumulation of sulfonyladenosines. For instance, the cytoplasmic accumulation of the 10 sulfonyladenosine-containing compounds in *E. coli* was positively correlated with hydrophobicity but negatively correlated with polarity (84). This platform is envisioned by those authors (84) to be applicable to a larger diverse panel of chemical agents and other bacterial organisms and may therefore be utilized for formulating a set of antibacterial permeation rules in the future.

The impermeability of Gram-negative bacterial membranes greatly limits our ability to develop new antibiotics, and there is an apparent void in the fundamental understanding of physicochemical properties necessary for an agent to overcome the double barrier of protection in Gram-negative bacteria. However, recent advances have shed some light on this hurdle (84, 85). It was recently shown that for small molecules to accumulate in the Gram-negative bacterium *E. coli*, they must contain an amine group (a primary amine is preferred over a secondary or tertiary amine), be amphiphilic, be rigid, and have low globularity (defined as the spatial parameter of the molecule) (85). By applying these rules, the natural product deoxynybomycin, which targets DNA gyrase, and which is active against Gram-positive bacteria only, was converted into an antibiotic with activity against a diverse panel of multidrug-resistant Gram-negative pathogens, excluding *P. aeruginosa* (85).

It may also be argued that instead of spending great efforts and resources on the development of intracellularly targeting antibacterials, one may focus on exploring membrane targets that are easily accessible (86). For instance, the membranolytic function of antimicrobial peptides and amphiphilic agents (87–90) or the inhibition of essential outer membrane proteins (91, 92) may be exploited. Looking forward, we foresee the materialization of the essential paradigm to predict bacterial membrane penetration. But for how long? Only time will tell.

2.3.2 Therapeutic Approaches to Overcome Antimicrobial Resistance

Clinicians have been saddled with the onerous task of refining medical practices and procedures to combat the spread of antibiotic resistance, but only so much can be achieved if new agents are not developed to supplement our current antibiotic arsenal. Pathogens have shown their resilience in withstanding antibiotic monotherapy due to their rapid doubling times and high mutation rates. Some pathogens, such as those of the *Mycobacterium* genus or those that form persister cells, display antibiotic tolerance due to their slow growth or dormancy (93). However, the notion of antibiotic tolerance has been observed only *in vitro*, and a recent *in vivo* experiment suggests that non-replicating bacteria might not necessarily confer resistance (94). Acquired resistance is due mostly to the selective pressure that an agent exerts on a bacterial population. A mutation(s) that confers overall fitness under such antibiotic stress (causing the bacteria to survive) is propagated in surviving cells and therefore gives rise to a drug-resistant population. Moreover, it has been documented that some pathogens under antibiotic monotherapy may induce resistance mechanisms that confer cross-resistance to other chemically unrelated antibiotic classes (95). Conversely, resistance mechanisms that confer hypersusceptibility to other antibiotics, known as collateral susceptibility, have also been reported (96). Drug-resistant bacteria may overexpress genes that encode molecular defense mechanisms such as efflux pumps or drug-inactivating enzymes. These resistance genes can

disseminate to a different organism via horizontal gene transfer of mobile genetic elements such as plasmids, transposons, and integrons (97). One way to solve this problem is to continually develop new antibiotics and/or new drug classes that delay the evolution of drug resistance. Further understanding of the molecular interplay that governs pathogenic responses during antibiotic therapy is, however, essential to guide the developmental process of overcoming drug resistance. Fundamental progress in basic science is as vital as it is in clinical science. A close rapport between clinicians and scientists is indeed critical to address the problem of antibiotic resistance development and dissemination. Here, we discuss some therapeutic approaches that may be able to delay the development of antibiotic resistance and briefly elucidate the hypotheses behind them.

2.3.2.1 Antivirulence therapy

The development of agents that are not bactericidal but that indirectly inhibit the molecular pathway responsible for bacterial communication is a viable strategy to address the problem of antibiotic resistance (98–100). This therapeutic approach is based on the purported delayed development of bacterial resistance, as it is perceived that such agents exert reduced evolutionary selective pressure (101). On the other hand, agents that challenge bacterial survival by directly inhibiting a molecular target may result in higher rates of resistance development. For example, blocking bacterial quorum sensing may be a feasible approach. Quorum sensing is characterized by the production, release, and group-wide detection of autoinducer molecules by bacteria as a mode of communication of bacteria with their neighbors (102). This network of communication is triggered by environmental factors within the microbial community, such as differences in bacterial density or the presence of environmental challenges (either physical or chemical) (103, 104). Once these signaling molecules are detected, cascades of physiological and metabolic changes occur by orchestrated alterations in bacterial gene expression, resulting in the secretion of biomolecules needed for biofilm formation and virulence (105). Therefore, hindering quorum sensing may result in the pathogen not

being able to cause harm to the host. Extensive discussions of bacterial quorum sensing and the development of agents that are able to quench this bacterial process have been reported (see references 103 and 105–108). Several agents that block quorum sensing are in preclinical development. For example, the synthetic agent *meta*-bromothiolactone (*m*BTL) has been reported to curb the production of the virulence factor pyocyanin and biofilm formation in *P. aeruginosa* by affecting the regulation of the Las and Rhl quorum-sensing systems (109). Moreover, *in vitro* protection of human lung epithelial cells and *in vivo* protection of *Caenorhabditis elegans* against *P. aeruginosa* by *m*BTL were described (109). A follow-up report detailed the optimization of *m*BTL for enhanced stability, as the thiolactone ring is susceptible to chemical and enzymatic hydrolysis (110). Other anti-quorum-sensing agents (111– 114) have also been reported to exhibit similar promising *in vitro* and *in vivo* results. However, this paradigm was recently challenged (115, 116), and several clinical isolates have been reported to be resistant to established anti-quorum-sensing agents (117). Anti-quorum-sensing agents are yet to reach clinical trials.

2.3.2.2 *Combination therapy*

Combination therapy has been well received by the scientific and medical communities and has existed for more than 3 decades (118). Clinicians often prescribe two or more antibiotics concomitantly during empirical treatment to ensure the coverage of all possible bacterial pathogens and resistance profiles. It was later realized that the use of multiple antibiotic agents in a therapeutic cocktail may limit the development of resistance *in vitro* in comparison to drug monotherapy. The overall expected clinical outcome for this strategy is lower patient mortality rates. However, combination therapy is not limited to antibiotic agents but includes therapeutic interventions that may use bioactive helper molecules, also known as adjuvants, to enhance the efficacy of a primary antibiotic. In fact, it has been argued that the adjuvant-antibiotic combination approach offers a more attractive option for the treatment of drug-resistant bacterial infections than the use of multiple

antibiotics (119). Here, we discuss combination therapy as (i) an antibiotic-adjuvant approach and (ii) an antibiotic-antibiotic approach.

2.3.2.2.1 *Antibiotic-adjuvant combination approach*

Arguably the most successful therapeutic strategy of the 21st century, the antibiotic-adjuvant approach has resulted in several drug entities on the market. The paradigm entails the use of bioactive adjuvants that augment the antibiotic efficacy of a primary antibiotic against drug-resistant pathogens. The adjuvant may possess weak to no antibacterial activity on its own but is able to either impede antibiotic resistance mechanisms or potentiate antibiotic action. An adjuvant may be an efflux pump inhibitor (EPI) (to prevent the extrusion of drugs), a membrane permeabilizer (to increase the number of molecules that penetrate the membrane), or an enzyme inhibitor (to prevent the degradation of drugs before they reach their targets).

(a) *β -Lactam– β -lactamase inhibitor combination.* Augmentin is a clinically used broad-spectrum antibiotic combination of amoxicillin and clavulanic acid (120). Clavulanic acid is a β -lactamase inhibitor that acts in synchrony with the β -lactam amoxicillin to prevent bacterial growth. β -Lactamase inhibitors, such as clavulanic acid, block the function of β -lactamases or β -lactam-hydrolyzing enzymes by forming an irreversible bond with the enzyme's functional/reactive site. Clavulanic acid by itself possesses poor intrinsic activity against pathogens, but it efficiently inhibits widespread β -lactamases such as many types of the extended-spectrum β -lactamase (ESBL) family (121). Inhibition of ESBLs is especially important, as members of this group of β -lactamases^c are promiscuous and are able to hydrolyze penicillins, cephalosporins (first, second, and third generations), and monobactams

^c Carbapenemases which belong to class A, B, and D represent the most versatile family of β -lactamases and they have the ability to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems. (Queenan, A. M.; Bush, K. Carbapenemases: The Versatile β -Lactamases. *Clin. Microbiol. Rev.* **2007**, 20 (3), 440–458).

(such as aztreonam) (122, 123). Augmentin was first introduced in 1981 by GlaxoSmithKline and is useful in the clinic even today (124, 125). It is not surprising for a β -lactam to be a cornerstone antibiotic in an antibiotic-adjuvant approach, as β -lactams are considered to be ideal drugs in terms of their efficacy and tolerability. Unfortunately, their “idealness” has been significantly threatened by the global spread of bacterial β -lactamase-encoding genes. The pursuit of adjuvants that inhibit β -lactamases is therefore crucial for retaining the clinical effectiveness of the β -lactam class of antibiotics. The recent approvals of ceftolozane-tazobactam in 2014, ceftazidime-avibactam in 2015, and meropenem-vaborbactam in 2017 by the FDA (Table 2.1) for the treatment of drug-resistant Gram-positive and Gram-negative bacterial infections are indicative of the continued interest in the development of combination therapies that include a β -lactam and a β -lactamase inhibitor. At least four more β -lactam-based antibiotic-adjuvant combinations are currently in clinical trials (126, 127). The popularity of the antibiotic-adjuvant strategy is apparent in the number of drug combinations under preclinical evaluation. We briefly highlight three examples, although extensive reviews were reported previously (see references 119 and 128–130).

(b) Imipenem-cilastatin-relebactam triple combination. In 1985, the combination of the carbapenem imipenem and the adjuvant cilastatin was approved for use in the United States under the trade name Primaxin (131). Imipenem is a broad-spectrum antibiotic that is rapidly degraded by the human renal enzyme dehydropeptidase 1, and the resulting metabolite poses the potential for nephrotoxicity (132). Thus, the addition of the dehydropeptidase 1 inhibitor cilastatin to imipenem prevents imipenem’s degradation and nephrotoxicity. Cilastatin also blocks megalin-mediated proximal tubule uptake of cationic antibiotics (133), further lowering the risk of kidney damage. However, the recent increase in the prevalence of bacterial infections caused by carbapenemase-producing organisms that inactivate imipenem calls for an improvement in this therapy. The combination of imipenem-cilastatin with the addition of the diazabicyclooctane β -lactamase inhibitor relebactam (also known as MK-7655) is

currently in a phase 3 clinical trial for the treatment of Gram-negative bacterial infections (134). The adjuvant relebactam is able to inhibit the activity of ESBLs and class A (e.g., KPC) and class C (e.g., AmpC) β -lactamases against imipenem by irreversibly blocking their functional/reactive site (135). The triple combination was found to be generally well tolerated in patients, with commonly reported adverse effects being nausea, vomiting, and diarrhea (136). Recently, a phase 3, randomized, double-blind, noninferiority study of imipenem-cilastatin-relebactam in comparison to imipenem-cilastatin-colistimethate sodium for the treatment of hospital-acquired bacterial pneumonia (HABP), ventilator-associated bacterial pneumonia (VABP), complicated intra-abdominal infection (cIAI), and complicated urinary tract infection (cUTI) (<https://clinicaltrials.gov/ct2/show/study/NCT02452047>) was completed. Results are yet to be disclosed.^d Another phase 3, randomized, double-blind, noninferiority study of imipenem-cilastatin-relebactam versus piperacillin-tazobactam for the treatment of HABP or VABP is currently recruiting volunteers (<https://clinicaltrials.gov/ct2/show/NCT02493764>).^e Moreover, a phase 3, nonrandomized, open-label study of the efficacy and safety of imipenem-cilastatin-relebactam for the treatment of cIAI and cUTI is currently ongoing in Japan (<https://clinicaltrials.gov/ct2/show/NCT03293485>). The activity of the triple combination, unfortunately, is very limited against organisms that harbor metallo- β -lactamases such as New Delhi metallo- β -lactamase 1 (NDM-1), imipenemase (IMP), and Verona integron-encoded metallo- β -lactamase (VIM) (134, 135).^f

(c) *Aspergillomarasmine A*. The adjuvant aspergillomarasmine A (AMA) was recently discovered to resuscitate the biocidal activity of the carbapenem drug meropenem against metallo- β -lactamase-

^d Results have now been disclosed at <https://clinicaltrials.gov/ct2/show/results/NCT02452047>

^e As of April 2, 2019, study is currently active and no longer recruiting
<https://clinicaltrials.gov/ct2/show/study/NCT02493764>

^f Entasis Therapeutics recently announced positive topline results from a Phase 2 clinical trial of ETX2514SUL, a quadruple combination therapy involving two β -lactamase inhibitors (ETX2514 and sulbactam) in combination with imipenem/cilastatin for the treatment of complicated urinary tract infections
<https://clinicaltrials.gov/ct2/show/study/NCT03445195>.

producing organisms (137). The fungal metabolite AMA was first isolated in the 1960s (138) and was later evaluated for its antihypertensive properties (139, 140). In an antibiotic era where enzymes that are capable of degrading even the most powerful β -lactam (e.g., carbapenems) are abundant, it is promising to find that AMA is able to inhibit metallo- β -lactamases such as the NDM-1 enzyme. AMA was found to sequester zinc cations (137), which are essential for the hydrolytic activity of metallo- β -lactamases (141, 142). In a mouse model of NDM-1-positive *K. pneumoniae* infection, a single dose of the combination of meropenem (10 mg/kg of body weight) and AMA (30 mg/kg) led to > 95% survival after 5 days post infection (137). The use of meropenem alone (10 mg/kg) or AMA alone (30 mg/kg) resulted in 0% survival (137). These promising results stimulate the need for an optimized dosing regimen of AMA in combination with carbapenems for the treatment of metallo- β -lactamase-producing pathogens. Currently, medicinal chemists are looking into optimizing the chemical structure of this adjuvant. The total synthesis (143), structure-activity relationship (144), and structural reassignment (145) of AMA have all been recently reported.

(d) *SPR741*. A polymyxin-based antimicrobial peptide, SPR741 (formerly NAB741), is currently being developed by Evotec AG and Spero Therapeutics as an adjuvant that potentiates antibiotics against Gram-negative pathogens (146). The recently completed, randomized, quadruple-blind, phase 1 clinical study of the safety and tolerability of this adjuvant in healthy volunteers (<https://clinicaltrials.gov/ct2/show/NCT03022175>) yielded favorable results. SPR741 was well tolerated by healthy adult volunteers in a single dose of up to 800 mg and at doses of up to 600 mg every 8 h for 14 days (Spero Therapeutics). In contrast to polymyxins, SPR741 has poor activity against Gram-negative pathogens on its own but can permeabilize the outer membrane to facilitate the entry of other antibiotics into the bacterial cell (147). For instance, SPR741 was reported to sensitize *Enterobacteriaceae* and *A. baumannii* but not *P. aeruginosa* to an extensive panel of antibiotics, including clarithromycin, fusidic acid, and rifampin (148–150). These three antibiotics are not classical drugs used to treat Gram-negative bacillary infections due to intrinsic resistance, notably OM

impermeability. At 2 µg/ml of SPR741, the MIC₅₀ and MIC₉₀ of rifampin against a panel of MDR *E. coli* isolates were 0.016 and 0.06 µg/ml, respectively (149). Rifampin alone has MIC₅₀ and MIC₉₀ values of 16 and > 128 µg/ml, respectively, against the same panel of *E. coli* strains (149). At similar concentrations of SPR741, strong rifampin potentiation against a panel of MDR *A. baumannii* isolates was also described (149). The *in vivo* efficacy of the SPR741-rifampin combination was shown in murine thigh and lung infection models (151, 152). Interestingly, the characteristic nephrotoxicity concerns usually associated with polymyxins (153, 154) were not observed with SPR741 at a dose of 60 mg/kg/day in cynomolgus monkeys after 7 days of a 1 h infusion three times daily (155).

2.3.2.2.2 Antibiotic-antibiotic combination approach

The use of two or more antibiotic agents that have different targets, which may or may not be for a single biochemical process, presents another attractive strategy to overcome drug resistance. The hypotheses of the antibiotic-antibiotic combination approach are (i) to achieve drug synergism between each drug component in a way that enhances treatment efficacy and (ii) to simultaneously impact multiple targets in pathogens, resulting in the suppression of antibiotic resistance development and the complete eradication of bacterial strains with intermediate susceptibility or resistance to one of two antibiotics. The assumption is that the bacterial cell will have difficulty surviving multiple “hits” at the same time. Clinicians sometimes employ this strategy during empirical treatment of infection, and such an approach might indeed prolong the clinical utility of antibiotics. For instance, the combination of trimethoprim-sulfamethoxazole has been in use since 1968 for the treatment of bacterial infections caused by the *Enterobacteriaceae* family and nonfermentative opportunistic pathogens (156, 157). Both antibiotics work together to inhibit sequential steps in bacterial folic acid synthesis, which is detrimental, as most bacteria are obligate folate synthesizers while humans acquire folate through diet. The sulfonamide sulfamethoxazole inhibits dihydropteroate synthase that converts *para*-aminobenzoic acid to dihydrofolate, and trimethoprim inhibits dihydrofolate reductase that converts

dihydrofolate to tetrahydrofolate (folic acid's bioactive form) (157). Trimethoprim-sulfamethoxazole is an efficacious antibiotic used to treat urinary tract and select gastrointestinal bacterial infections (158, 159). Sulfamethoxazole may be replaced with the sulfonamide sulfametrole in some European Union countries, although both agents, when combined with trimethoprim, exhibit the same clinical efficacy (160). However, the success of the trimethoprim-sulfamethoxazole combination has been affected by the dissemination of resistance mechanisms that prevent both antibiotics from eliciting their biological functions. The overexpression of multidrug efflux pumps that are able to expel both trimethoprim and sulfamethoxazole out of the cell and membrane modifications that limit their intracellular permeation are problematic (156). Many other antibiotic-antibiotic combinations are used in the clinic, including those of tigecycline-gentamicin, tigecycline-colistin, and carbapenem-colistin (161), to name a few.

2.3.2.3 *Challenges of combination therapy*

Considering the “success” of several antibiotic-antibiotic combinations in the past few decades, the strategy remains fallible, as several important pharmacological questions remain unanswered. For instance, other than for tuberculosis, there is no clinical evidence to support the notion that antibiotic resistance is suppressed by antibiotic-antibiotic combinations (162). This is a tough concept to prove, as clinical studies are usually designed not to measure the emergence of antibiotic resistance but to prevent or treat it. These data are often extrapolated from *in vitro* studies and in animal models and may be different from what is obtainable in human hosts. The clinical translatability of drug synergy *in vitro* and in animal models to humans is also debatable (162). The limited available clinical evidence suggests a statistically insignificant difference between antibiotic-antibiotic combination therapy and monotherapy for the treatment of Gram-negative bacterial infections in terms of mortality rates, which is the postulated clinical outcome for synergistic drug combinations (163–165). For instance, a

systematic study reported no appreciable improvement for the combination of a β -lactam–aminoglycoside over β -lactam monotherapy for the treatment of endocarditis caused by a Gram-positive bacterium (e.g., *Staphylococcus aureus*), even though the combination shows synergism *in vitro* (166). Another study showed that the combination of a β -lactam and either an aminoglycoside or a fluoroquinolone in comparison to β -lactam monotherapy imposes no benefit for patient mortality for the treatment of infections caused by the Gram-negative organism *P. aeruginosa* (164). However, it should be noted that the supporting evidences for these studies were based on meta-analyses of a small amount of available clinical data that included those during early years when drug resistance was not as prevalent. Caution should therefore be taken in the extrapolation of data to fit the current landscape, where incidences of MDR and XDR bacterial infections are much higher. Currently, there seems to be an apparent consensus that combination therapy is preferred for the treatment of MDR pathogen infections of severely ill patients and for empirical therapy (167). A lack of pharmacokinetic complementarity between different drugs might indeed contribute to the discrepancies between *in vitro* data and clinical observations (168). Each drug component may be absorbed or distributed in the human body to different degrees. Pharmacokinetic variances, but also the patient's overall condition, would certainly impose a challenge in fine-tuning the dosages of administered drugs to replicate their observed *in vitro* synergy, as both drugs are required to be located at the site of infection at their optimal concentrations simultaneously. Noncomplementary absorption and distribution rates may therefore be circumvented by fusing both pharmacophoric molecules together to make a single hybrid antibacterial agent. Here, the fundamental concept behind antibiotic hybrids is discussed.

2.4 Antibiotic Hybrids against Antibiotic-resistant Bacteria

The antibiotic hybrid strategy was precipitated from countless attempts to discover new synthetic scaffolds that may yield antibiotics capable of overcoming drug resistance. Scientific ingenuity led to the development of molecular hybrids (Figure 2.1) by fusing different biologically active agents into one heteromeric entity with the hope of retaining the biological actions of the constituent fragments. A molecular linker/tether is often used to append the participating agents together via a covalent bond, although molecules could also be fused together directly. The mode of covalent attachment could also be designed to be either cleavable or non-cleavable (Figure 2.1). A cleavable linker is expected to be enzymatically bio-transformed once the hybrid reaches its site of action (the bacteria), while a non-cleavable linker remains unchanged throughout its time course in the body. The former constitutes a hybrid prodrug approach, while the latter constitutes a hybrid drug approach. The hypothesis of antibiotic hybrids integrates the working concept of suppressing drug resistance evolution in combination therapy into monotherapy, thus presenting a molecular agent (instead of two) with a single pharmacokinetic profile. It also eliminates the problem of noncomplementary pharmacodynamics. Hybrid drugs are also postulated to eradicate bacterial strains with intermediate susceptibility or resistance to one of the covalently linked drug fragments. Although unpredictable, retaining antibacterial potency against pathogens that possess intermediate susceptibility or resistance to both drug components is possible, as the process of hybridization may also impart additional physicochemical properties that could alter the hybrid's pharmacological spectrum. For instance, the hybridization of two therapeutic agents may enhance the efficacy or even impart a new mechanism of antibacterial action to the resulting hybrid agent.

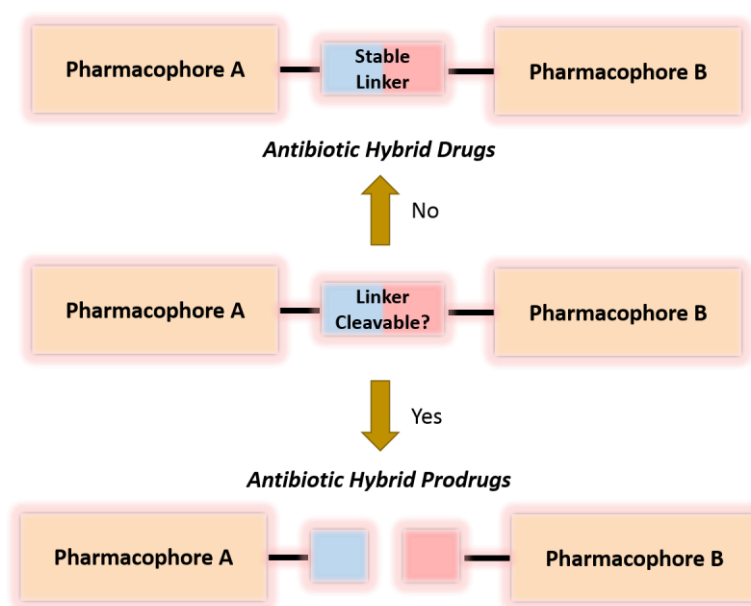


Figure 2.1. Two different pharmacophoric domains attached covalently by a linker domain. The lability of the linker determines the type of antibiotic hybrid generated. A linker that can be enzymatically degraded (preferably by only bacterium-specific enzymes) gives rise to two functional pharmacophoric entities that are thus used in the antibiotic hybrid prodrug strategy. A linker that is inert to enzymatic degradation is used to hold the two pharmacophoric domains together in the antibiotic hybrid drug strategy.

2.4.1 Definition of an Antibiotic Hybrid

What defines a hybrid agent? The literature offers various subjective definitions of hybrid agents, depending on the context in which they are being used. In this account, however, we define a hybrid antibiotic as a synthetic construct of two or more pharmacophores belonging to an established agent known to elicit a desired antimicrobial effect. This encompasses agents described as being either dual-action antibiotic hybrids (169, 170), chimeric antibiotics (171, 172), multivalent/divalent antibiotics (173– 175), or antibiotic conjugates (176). Moreover, the antibiotic hybrid approach is not confined to the covalent fusion of antibacterial agents and may also include beneficial adjuvants such

as resistance enzyme inhibitors, membrane permeabilizers, siderophores, and efflux pump inhibitors. The notion of bimodality (coined “dual action” in 1994 [169]) in prospective antibiotic hybrids suggests the need for the covalently appended agents to retain their known biological actions. However, our experience reveals that it might indeed not be necessary to retain both known activities, as an unexpected third mode of action may arise from the fusion of two therapeutic agents (see below). Moreover, some antibiotic hybrids are able to “resuscitate” the antibacterial potency of legacy antibiotics against drug-resistant pathogens. The term legacy antibiotics pertains to widely used antibacterial agents that have been clinically used for decades and whose clinical efficacy is currently being challenged by the rise of antibiotic resistance mechanisms. In this review, we highlight promising antibiotic hybrids with emphasis on those that are successful in inhibiting or eradicating Gram-negative pathogens. We also discuss our recent discovery that we believe could expand the utility of the antibiotic hybrid approach.

2.4.2 Conceptual Challenges in Designing Antibiotic Hybrids

Several inherent problems need to be addressed before the development of an antibiotic hybrid, especially if the agent is directed at Gram-negative pathogens. Limited cellular penetration across the dual layer of protection of Gram-negative bacteria is the first major concern for hybrid agents that have a molecular mass of more than 600 g/mol. As mentioned above, there are currently no infallible permeation guidelines to assist medicinal chemists in designing therapeutic agents that are capable of traversing bacterial lipid bilayers. Antibacterial agents with a high molecular mass will not pass through nonselective porin channels, restricting cellular uptake to receptor-mediated endocytosis or passive diffusion. One way to overcome this permeability problem is to design an antibiotic hybrid that retains the porin-independent uptake mechanism of one or more of the parent antibiotic components. Periplasmic entry through a self-promoted uptake mechanism may be leveraged, as has

been widely reported for amphiphilic molecules regardless of their molecular masses (28, 45, 46). Subsequent passage through the inner membrane, which may be dependent on PMF or may occur just through passive diffusion, is then required for entry into the cytosol. For instance, antibiotics of the aminoglycoside class are known to enter the OM of Gram-negative bacteria by a self-promoted uptake mechanism followed by two-step energy-dependent IM uptake to reach the cytosol and elicit their antibacterial function (177). Thus, an aminoglycoside-containing hybrid can be prepared in the hopes of retaining the aminoglycoside's inherent mode of uptake. One may also develop a hybrid that has multiple non-intracellular targets to circumvent the permeability issue altogether. Indeed, permeability impediments due to high molecular mass is a major reason why most antibiotic hybrids have limited activity against Gram-negative bacteria (178).

Another problem lies within the fundamental idea of covalently linking two pharmacological agents together. The point of attachment and the physicochemical properties of the chosen linker are crucial for the overall activity of the hybrid. Ideally, the two molecules should be attached at a non-pharmacophoric region to retain the integrity of the functional domains. According to the International Union of Pure and Applied Chemistry (IUPAC), a pharmacophore is defined as “the collective steric and electronic properties of a molecule that are essential for interaction with the biological target and to elicit response” (179). Any obstruction of the pharmacophoric region of the appended agents might be detrimental to the antibacterial potency of the resulting hybrid. Moreover, steric overlap between the two therapeutic agents, due to proximity, may also result in compromised activity. For instance, an enzyme-targeting antibiotic domain requires that its pharmacophoric region be devoid of obstruction to dock properly on the target enzymatic pocket. The adjacent antibiotic domain may impose unwanted steric bulk that could hinder the docking sequences, thus preventing the desired enzymatic inhibition. It is therefore necessary to probe the optimal spatial length of the linker during the early stages of development by utilizing *in silico* modeling programs and/or synthesizing several analogs that differ in linker length to evaluate the optimal spatial separation. The properties of the

linker/tether could also be manipulated to expand the overall chemical space of the hybrid molecule. For instance, the hydrophobicity of the tether segment may be tinkered with by substituting a polycyclic aromatic-based or a polyethylene glycol-based linker instead of an aliphatic hydrocarbon. While conceptualizing an ideal blueprint seems trivial, the molecular manipulation involved in the synthesis of antibiotic hybrids is quite demanding, and the narrow window of chemical reactions may drive a certain bias during the developmental process (180). This usually makes the development of drug candidates a matter of synthetic convenience rather than design (181).

2.4.3 Antibiotic Hybrid Prodrugs against Antibiotic-Resistant Gram-negative Bacteria

2.4.3.1 *Concept and hypothesis*

A mutual prodrug, as defined by the IUPAC, is the covalent attachment of two drugs to form a unique molecule that undergoes biotransformation (such as bond cleavage) to exhibit its pharmacological effects (179). To achieve this for antibiotic hybrids, a prodrug strategy necessitates the use of a cleavable linker/tether with bacterium-specific lability. The linker/tether must deliver both constituent molecules to their site of action (bacterial compartment) before being degraded. The resulting antibiotic hybrid prodrug may or may not possess biological activity by itself.

Hybrid prodrugs in the literature are comprised mostly of β -lactams linked to other therapeutic agents. The foundation of antibiotic hybrid prodrugs is premised on extensive mechanistic studies of the β -lactam core structure. In 1965, it was discovered that some molecular substituents adjacent to carbon-3 of the cephalosporin (a type of an unsaturated β -lactam/penem) backbone was displaced following β -lactam ring hydrolysis (182). Mechanistically (Figure 2.2), it was perceived that the

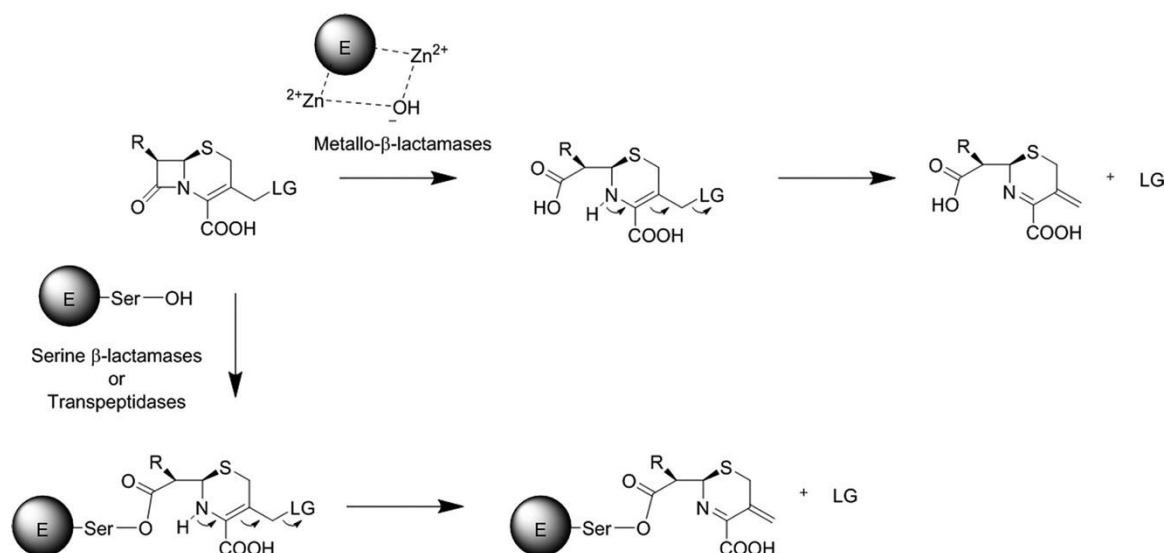


Figure 2.2. β -Lactam hydrolysis of cephalosporin followed by non-enzymatic release of a leaving group (LG). Enzymatic hydrolysis may be due to β -lactamases, such as serine- or metallo-based β -lactamases, or β -lactam target enzyme transpeptidases. Most hybrid prodrug utilize this mechanism to release another antibiotic as a leaving group. R, any molecular substituent; E, enzyme.

liberated secondary amine (from β -lactam ring hydrolysis) would delocalize its electrons on carbon-2 to form an imine and consequently displace the unsaturated bond (carbon-carbon double bond) along carbon positions 2 and 3. Electron delocalization would ultimately release the molecular substituent or leaving group (LG) adjacent to carbon-3. This knowledge was then used to construct cephalosporin-based hybrid prodrugs where an antibiotic was designed to be the leaving group adjacent to carbon 3. Notably, most investigational antibiotic hybrid prodrugs are cephalosporin based (169, 183). Approximately 3 decades later, it was discovered that the installation of an *S*-aminosulfenimine moiety at carbon-6 of penicillin (a type of saturated β -lactam/penam) resulted in the rapid intramolecular displacement of the *S*-amino substituent following β -lactam ring hydrolysis (Figure 2.3) (184). Nuclear magnetic resonance (NMR) studies of this phenomenon (185) led to the proposition that the release of the substituent at penicillin position 6 is due to the formation of a disulfide bond-characterized

intermediate that further decomposes to yield several by-products (Figure 2.3A). Interestingly, a vinyl ester linkage (186) was reported to be a viable alternative to the *S*-aminosulfenimine linkage, as it was also found to be displaced following β -lactam hydrolysis (Figure 2.3B). However, we have yet to see any prospective β -lactam-based hybrid prodrug with either a cleavable *S*-aminosulfenimine or vinyl ester linkage in the literature.

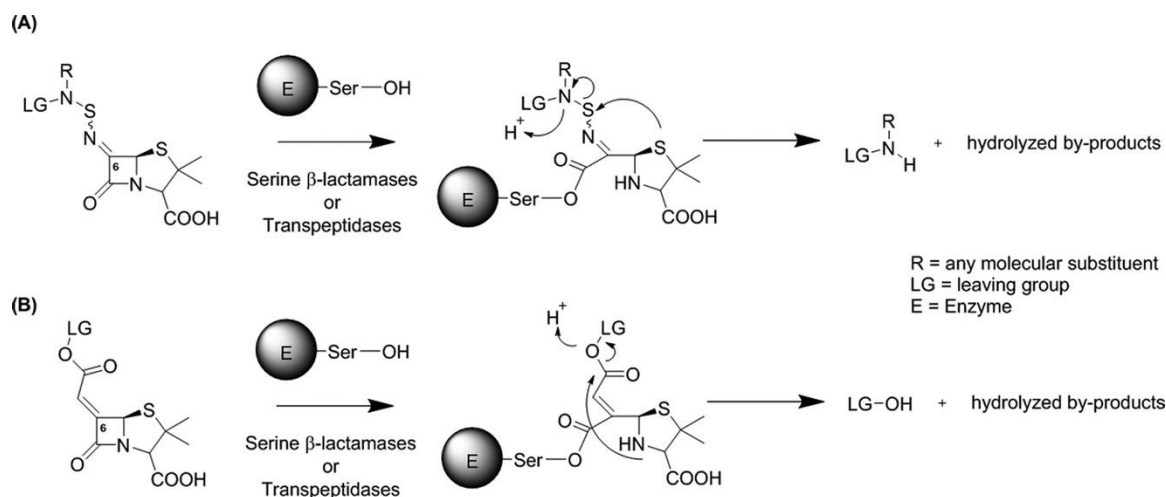


Figure 2.3. β -Lactam hydrolysis of penicillin that contains an installed leaving group at position 6 via an *S*-aminosulfenimine (A) or a vinyl ester (B) linkage. The mechanisms for the nonenzymatically driven release of the leaving group for panels A and B were elucidated thorough NMR experiments (185, 186). Enzymatic hydrolysis may be due to β -lactamases, such as serine or metallo-based β -lactamases (not depicted above), or the β -lactam target enzyme transpeptidase.

β -Lactam-based prodrugs function via a two-step process: (i) the β -lactam antibiotic elicits its activity by inhibiting the enzyme transpeptidase via acylation (ester bond formation) of the active-site serine residue at the hydroxyl side chain, which consequently results in β -lactam ring amide bond hydrolysis, followed by (ii) the release of another functional antibiotic to elicit its own antibacterial activity. In the presence of serine-based β -lactamases that confer enzymatic drug resistance to β -lactam

antibiotics, the β -lactam-containing prodrug could serve as a sacrificial adjuvant to inhibit the resistance enzyme. The drug appended to the hydrolyzed sacrificial adjuvant can then be released to elicit its biological action. Inhibition of serine-based β -lactamases occurs via acylation of the active site of serine at the hydroxyl side chain. Unfortunately, β -lactam-based prodrugs do not have any benefit against metallo- β -lactamases, as this resistance enzyme utilizes zinc ions that activate water molecules to hydrolyze the β -lactam ring and therefore would not be inhibited by acylation. It would be interesting to see the future development of antibiotic hybrid prodrugs that contain zinc-sequestering agents, such as the recently reported agent aspergillomarasmine A (137), to combat metallo- β -lactamase-producing Gram-negative bacteria, as this enzyme is credited as being the main cause of pandrug resistance worldwide (187).

2.4.3.2 *Advantages and disadvantages*

Several advantages and disadvantages are discussed above. In addition, achieving synergistic antibacterial activity through the hybrid prodrug approach is a possibility. As the prodrug reaches its target bacterial compartment, the hybrid entity is expected to be cleaved into two functioning drugs that may have synergistic interactions. Through the prodrug strategy, prediction of *in vivo* drug synergism from *in vitro* combination studies may be easier, as the problem of noncomplementary pharmacokinetics, a concern in combination therapy, as mentioned above, is bypassed. However, this may hold true only for drug combinations with a 1:1 optimal concentration ratio. Nonetheless, it may be ideal to consider any advantageous synergistic relationship of the antibiotics sought to be fused together.

The major challenge with this approach is the difficulty in designing a bacterium-specific cleavable linker, as the human body is a complex biological system comprising specific and nonspecific enzymes capable of degrading various covalent bonds. If the cleavable linker of the

prodrug is degraded before it reaches the bacteria, then the design would no longer be different from that of combination therapy. Therefore, it is crucial to select a linker that is bacterium selective and capable of withstanding human metabolic enzymes. The stability of the cleavable linker in the human body and the drug permeability impediments in Gram-negative bacteria are the major limitations of the hybrid prodrug approach. However, like the mechanistic findings for β -lactam hydrolysis, more and more data on the bacterium-specific molecular interplay become unraveled as we progress into the future, and there is great optimism that human ingenuity will pave the way for the next generations of hybrid prodrugs.

2.4.3.3 *Examples*

The majority of hybrid prodrugs in the literature consist of a non- β -lactam antibiotic fused to the adjacent carbon-3 of the cephalosporin core structure (second antibiotic) (Figure 2.4). The earliest hybrid prodrug, reported in 1976, was a cefamandole (cephalosporin) derivative linked to omadine at carbon-3 (hybrid prodrug **1**) (188). Omadine, also known as 2-mercaptopyridine-*N*-oxide or pyrithione, inhibits bacterial ATP synthesis and is known as a metal chelator (189). Hybrid prodrug **1** was shown to be active against a panel of Gram-negative pathogens with a geometric mean MIC of 0.5 to 16 $\mu\text{g/ml}$ (188). The mode of action of hybrid prodrug **1** was hinted to be mainly that of a cephalosporin, with some contributions from its omadine pharmacophore, as its MIC was reduced by 4- to 32-fold in bacterial strains that express β -lactamases. As noted by those authors, that study validated the concept of a hybrid prodrug but was of no significant therapeutic relevance, as omadine displays undesirable systemic toxicity. Ten years later, a desacetylcephalothin (cephalosporin) linked to a chloroalanyl dipeptide inhibitor of alanine racemase (hybrid **2**) was reported to be active against *E. coli* (MIC of 7.05 to 14.1 $\mu\text{g/ml}$) (190, 191). The cephalosporin pharmacophore serves as an essential structural component for most hybrid prodrugs, and some of the resulting hybrid entities advanced to preclinical/clinical trials. For instance, NB2001 (hybrid **3**) by NewBiotics Inc., which

consists of a desacetylcephalothin fused with a chlorine-containing phenolic triclosan, was reported to possess potent broad-spectrum bacteriostatic activity against Gram-positive and Gram-negative bacteria (192). However, NB2001 (hybrid **3**) was inactive (MIC of $> 32 \mu\text{g/ml}$) against *P. aeruginosa* (192), which was not surprising due to the organism's restrictive OM and abundance of multidrug efflux pumps. No data were reported for *A. baumannii*. The antibacterial activity of NB2001 was attributed to the release of its triclosan component, as the intact hybrid exhibited significantly reduced *in vitro* binding to transpeptidases relative to that of cephalothin, suggesting a diminished mode of action of cephalosporin (193). Moreover, the intact hybrid was unable to inhibit the enzyme enoyl-acyl carrier protein reductase FabI (target enzyme for triclosan) (193). NB2001 (hybrid **3**) entered preclinical evaluation for bacterial and nosocomial infections but was discontinued in 2005 for undisclosed reasons. Another worthy example of a β -lactam-based hybrid prodrug is Ro 23-9424, a story of high hopes but dashed dreams.

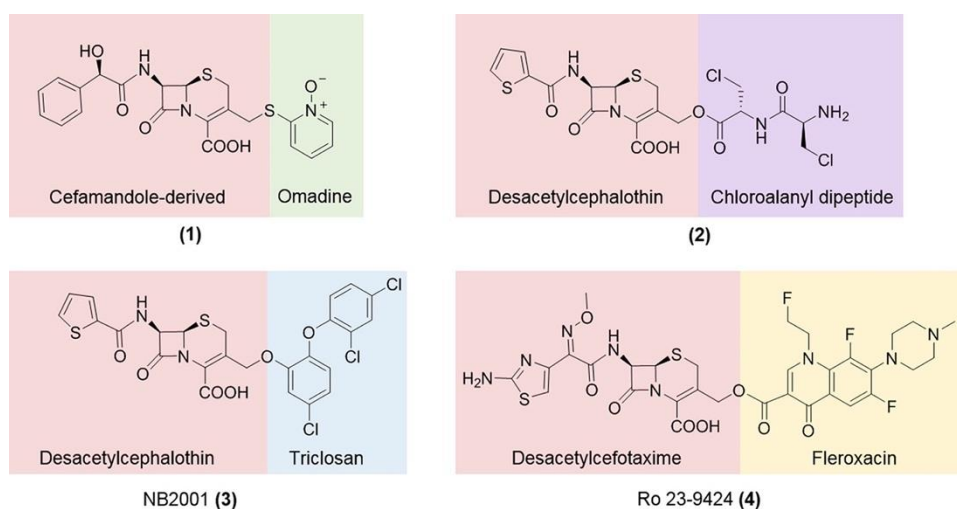


Figure 2.4. Examples of antibiotic hybrid prodrugs: a cefamandole derivative linked to omadine (hybrid **1**), desacetyl-cephalothin linked to the alanine racemase inhibitor chloroalanyl dipeptide (hybrid **2**), desacetylcephalothin linked to triclosan-NB2001 (hybrid **3**), and desacetylcefotaxime linked to fleroxacin-Ro 23-9424 (hybrid **4**). The majority of antibiotic hybrid prodrugs consist of a cephalosporin.

2.4.3.4 *Ro 23-9424: struggle, triumph, and failure — a story from the past*

In 1989, Roche pharmaceuticals announced the development of a cephalosporin-fluoroquinolone ester hybrid prodrug called Ro 23-9424 (hybrid **4**) (Figure 2.4) with potent broad-spectrum bactericidal activity against Gram-positive and Gram-negative organisms (194). The hybrid prodrug contains a desacetylcefotaxime (a cephalosporin) covalently linked to a fleroxacin (a fluoroquinolone) via a cleavable ester linkage adjacent to carbon-3, which is consequently cleaved following enzymatic hydrolysis of the β -lactam ring structure (Figure 2.5). Its mode of uptake was proposed to be porin mediated (195, 196) (even though it has a modestly high molecular mass of 764.7 g/mol). Ro 23-9424 (hybrid **4**) displayed only limited activity against *P. aeruginosa* (194), which may be attributed to reduced OM permeability (due to the selective OprF porin that is majorly expressed) and/or an abundance of multidrug efflux pumps of the organism. Cephalosporins inhibit peptidoglycan synthesis by acetylating the active site of transpeptidases, while fluoroquinolones inhibit DNA synthesis via the inhibition of DNA gyrase and topoisomerase IV. Ro 23-9424 (hybrid **4**) acts initially as a cephalosporin, where the hydrolysis of the β -lactam ring results in a fluoroquinolone secondary mode of action (197). The intact hybrid prodrug exhibits only minimal DNA synthesis inhibition (195). As conceptualized, the antibacterial activity was retained in *E. coli* strains that are resistant to either β -lactams, fluoroquinolones, or both agents (196). The *in vitro* half-life of the hybrid prodrug in human serum was reported to be 6.3 h (198), suggesting an adequate stability of the cleavable ester linkage toward nonspecific enzymatic degradation. pH-dependent stability in an aqueous phosphate buffer solution was also described, where half-lives of 6.9 and 3.0 h were observed at pH 6.5 and 7.4, respectively (198). Ro 23-9424 (hybrid prodrug **4**) was also shown to enhance the induction of LPS-stimulated tumor necrosis factor alpha (TNF- α), yet it reduced the production of the proinflammatory cytokine interleukin-1 β (IL-1 β) in human monocytes (199), suggesting a potential immunomodulatory benefit in reducing the possibility of LPS-induced septic shock. Promising preclinical *in vivo*

pharmacokinetic parameters and tolerability were described for mouse, rat, dog, and baboon models with single- or multiple-dose intravenous administration (200). Excellent *in vivo* efficacy was reported for systemic mouse infection models of Gram-positive and Gram-negative bacterial infections, including strains that are resistant to cefotaxime and fleroxacin (198, 201). For instance, subcutaneously administered Ro 23-9424 (hybrid **4**) was more active (50% effective dose [ED₅₀] of 17 mg/kg) than cefotaxime (ED₅₀ of 50 mg/kg) and fleroxacin (ED₅₀ of > 100 mg/kg) in a murine meningitis model of infection by the Gram-positive organism *S. pneumoniae* (201). The hybrid prodrug resulted in an efficacy (ED₅₀ of 13 mg/kg) that was similar to that of fleroxacin (ED₅₀ of 9 mg/kg) but better than that of cefotaxime (ED₅₀ of > 100 mg/kg) in a murine meningitis model of infection by the Gram-negative organism *K. pneumoniae* (201). These promising preclinical data advanced the hybrid prodrug to phase 1 clinical trial for bacterial infections. Unfortunately, the trial was discontinued around the mid-1990s for undisclosed reasons. Several explanations were speculated (202), such as the fact that the *in vivo* drug stability in humans did not replicate the initial observations *in vitro* and in animal models, i.e., that the ester linkage connecting the cephalosporin and fluoroquinolone fragments was degraded by nonspecific enzymes present in humans. Moreover, in contrast to the expected multimodal suppression of drug resistance evolution, development of resistance (16- to 128-fold increase) to Ro 23-9424 (hybrid prodrug **4**) was described for several bacterial strains after 2 weeks of serial passage at subinhibitory concentrations (203). Resistance in *E. coli* was attributed to two factors: (i) decreased Ro 23-9424 outer membrane permeation due to altered porin uptake and (ii) impeded fluoroquinolone activity as demonstrated by a replicative DNA biosynthesis assay in toluene-permeabilized cells (204). However, caution in interpreting the reported mechanism of resistance against Ro 23-9424 (hybrid **4**) is advised, as efflux-mediated resistance was not fully recognized at the time of publication of that study. Fluoroquinolone resistance via efflux pumps was first reported in 1994 (205) and has since been known to be a major mechanism of resistance against this class. Sadly, Ro 23-9424 (hybrid **4**) presented the antithesis to the idea of

delayed drug resistance generation with antibiotic hybrids. This story exemplified the ingenuity of bacteria in coping with chemical assault by restricting their cellular entry. It may be beneficial to design future antibiotic hybrids as agents that can enter bacterial cells via mechanisms independent of porins, as these protein channels can be easily modified genetically by the pathogen to confer drug resistance.

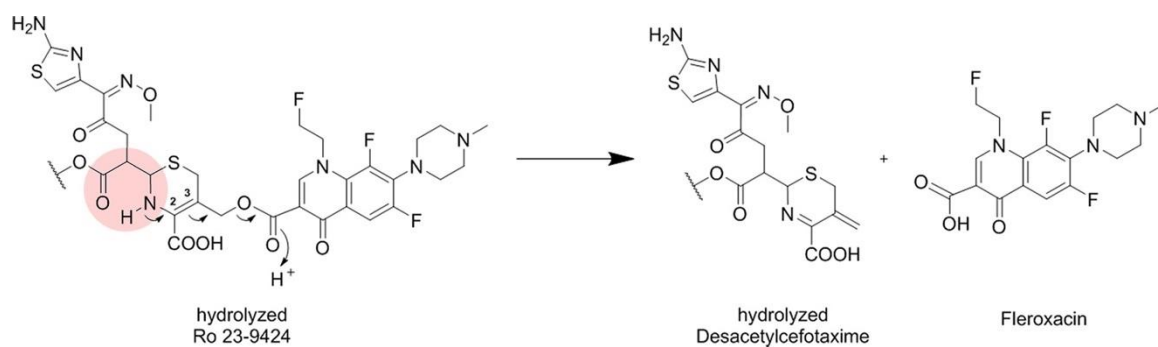


Figure 2.5. Electron transfer of hydrolyzed Ro 23-9424 results in the formation of hydrolyzed desacetylcefotaxime and fleroxacin by-products. Ro 23-9424 (hybrid **4**) initially acts as a cephalosporin, followed by the release of a functional fluoroquinolone as a product of β -lactam hydrolysis. The highlighted pink circle is the hydrolyzed β -lactam ring.

2.4.4 Antibiotic Hybrid Drugs against Antibiotic-Resistant Gram-negative Bacteria

2.4.4.1 Concept and hypothesis

Classical antibiotic hybrid drugs can be distinguished from prodrugs through the stability of their linker/tether. In the typical hybrid drug approach, the participating therapeutic agents are covalently linked by a robust non-cleavable molecular linker that can withstand enzymatic and non-enzymatic assaults throughout its time course in the body. Upon entering the pathogen, a prototypical hybrid drug is expected to elicit its antibacterial action by utilizing either of its pharmacophoric

domains or both domains simultaneously. However, it should be noted that the development of a hybrid drug that is able to simultaneously inhibit both drug targets by utilizing only a singular molecular entity at the same time is a difficult feat to achieve. Ideally, the molecular targets of the conjoined pharmacophores should be in close proximity, while the linker/tether should be of an appropriate spatial length to efficiently anchor them. This imposes a conceptual challenge that is difficult to be satisfied. For example, a single hybrid molecule that has two different pharmacophoric components — one that inhibits DNA synthesis and another that inhibits protein synthesis — will not be able to inhibit both targets at the same time due to the spatial separation of the target compartments. For now, antibiotic hybrid drugs in the literature are believed to interact with only one of the possible targets at a given time (170). However, this does not nullify the concept of multimodal mechanisms for hybrid antibiotics as long as they retain the interactions of both individual antibacterial pharmacophores.

The antibiotic hybrid drug approach is considerably more popular in the literature than the prodrug approach. This is attributed mainly to the limited number of accessible bacterium-specific cleavable linkers for an effective prodrug delivery approach. Either way, it should be noted that both hybrid strategies require tremendous synthetic efforts, as therapeutic agents typically possess dissimilar molecular stabilities and reactivities under different preparative conditions. Designing a specific linker that is expected to be bio-transformed by a bacterium-specific enzyme under specific physiological conditions therefore makes hybrid prodrugs relatively more difficult to prepare than classical hybrid drugs. Nevertheless, the characteristic high molecular weight (> 600 g/mol) of hybrid drugs makes it challenging to generate agents that are able to permeate the dual membrane of Gram-negative bacteria. Emerging reports, however, project a good prognosis for this strategy, as several hybrid drugs (discussed below) that are capable of eradicating MDR Gram-negative bacteria and presumably able to delay the onset of drug resistance are already in preclinical/clinical evaluation (<http://www.pewtrusts.org/en/multimedia/data-visualizations/2014/antibiotics-currently-in-clinical-development>).

2.4.4.2 *Advantages and disadvantages*

Similar to the advantageous pharmacokinetic properties of hybrid prodrugs, a hybrid drug is expected to remain a unimolecular entity as it travels to the site of infection and traverses the bacterial membrane into inner compartments (periplasmic and/or cytosolic space). However, differences in the two therapeutic approaches lie in how they elicit their biological function. A hybrid prodrug is subjected to enzymatic biotransformation, as it enters the bacterial cell, to yield two functional therapeutic entities, while a hybrid drug would remain a single entity throughout its time course in the body. Therefore, hybrid drugs may be advantageous in terms of their kinetics (drug metabolism and elimination), as they are expected to be cleared from the host as a single molecule. A hybrid prodrug, on the other hand, is designed to be cleaved into two separate functional molecules that may possess different pharmacokinetics after intracellular biotransformation. Drug metabolism and excretion are important factors that influence dosing regimens, as bioaccumulation above a certain threshold may result in toxicity. This, along with the above-mentioned advantages and disadvantages, should be taken into consideration when designing an antibiotic hybrid.

2.4.4.3 *Examples*

A substantial number of hybrid drugs that are in development or have entered clinical trials possess limited antibacterial activity against Gram-negative pathogens (178, 206, 207). This is attributed mainly to impeded bacterial cellular uptake due to a high molecular mass of > 600 g/mol and physicochemical properties that render the hybrid drug unable to penetrate both outer and inner membranes. Drug uptake through nonselective porin channels in bacteria is restricted to low-molecular mass (typically < 600 g/mol) and highly polar compounds. With this in mind, we performed a literature search for hybrid drugs that are potent against Gram-negative bacteria. Here, we highlight select antibiotic hybrids from recent literature (2010 to early 2017).

2.4.4.3.1 Most hybrid drugs contain a fluoroquinolone pharmacophore

Interestingly, most of the recently reported antibiotic hybrid drugs that are active against Gram-negative bacteria possess a fluoroquinolone pharmacophore (mostly ciprofloxacin) (Figure 2.6). The choice of incorporating a fluoroquinolone as a parent drug may be attributed to its robust chemical properties that are stable under many reaction conditions. Synthetically, it may be easier to append fluoroquinolones to other therapeutic agents than, for example, β -lactams with narrow windows of chemical stability. Moreover, the well-elucidated structure-activity relationship of fluoroquinolone antibiotics and their broad-spectrum of activity make them an attractive class of antibiotics (208–210).

In 2010, a patent (211) describing a series of benzyl pyrimidines linked to fluoroquinolones was filed by MerLion Pharmaceuticals Pte. Ltd. Trimethoprim was fused to ciprofloxacin and other fluoroquinolones, yielding hybrids that displayed activity against Gram-positive (*Staphylococcus aureus* and *Staphylococcus epidermidis*) and Gram-negative (*E. coli*) pathogens. The antibacterial profile of the whole series was better than that of the parent drug, trimethoprim, while some entries showed activity comparable to that of ciprofloxacin. BP-4Q-002 (hybrid **5**), consisting of a trimethoprim attached to the piperazine ring of ciprofloxacin, is an example that displayed potent activity against *S. aureus* (MIC of 0.5 $\mu\text{g/ml}$) and *E. coli* (MIC of 1 $\mu\text{g/ml}$) (211). This hybrid also has an MIC value of 1 $\mu\text{g/ml}$ against ciprofloxacin-resistant *S. aureus* strain NRS19 (ciprofloxacin MIC of 32 $\mu\text{g/ml}$, trimethoprim MIC of 4 $\mu\text{g/ml}$, and MIC of the equimolar mixture of 8 $\mu\text{g/ml}$) (211). The observed activity of BP-4Q-002 (hybrid **5**) against this drug-resistant *S. aureus* strain validates a fundamental concept in the antibiotic hybrid strategy, that hybrid agents may eradicate strains that are intermediately susceptible or resistant to one of the parent drugs. Furthermore, the 4-fold and 8-fold reductions of the MIC of BP-4Q-002 (compound **5**) against *S. aureus* NRS19 compared to those of the parent drug trimethoprim and the equimolar mixture of the parent drugs, respectively, hint that the hybridization process imparted additional benefits to the hybrid's biological activity. However, there

were no mode-of-action studies disclosed, and therefore, inferences of whether the hybridization of trimethoprim to ciprofloxacin influenced the resulting hybrid's mechanism of action cannot be made. A report in 2012 attempted to further fine-tune the trimethoprim- ciprofloxacin pairing by rationally synthesizing chimeric composites of both parent drug with several truncations, with the hope of improving antibacterial activity (171). Rationally guided truncations were performed on the non-pharmacophoric portions of both parent drugs. Unfortunately, none of the newly synthesized hybrids showed improved antibacterial activity relative to that of BP-4Q-002 (171).

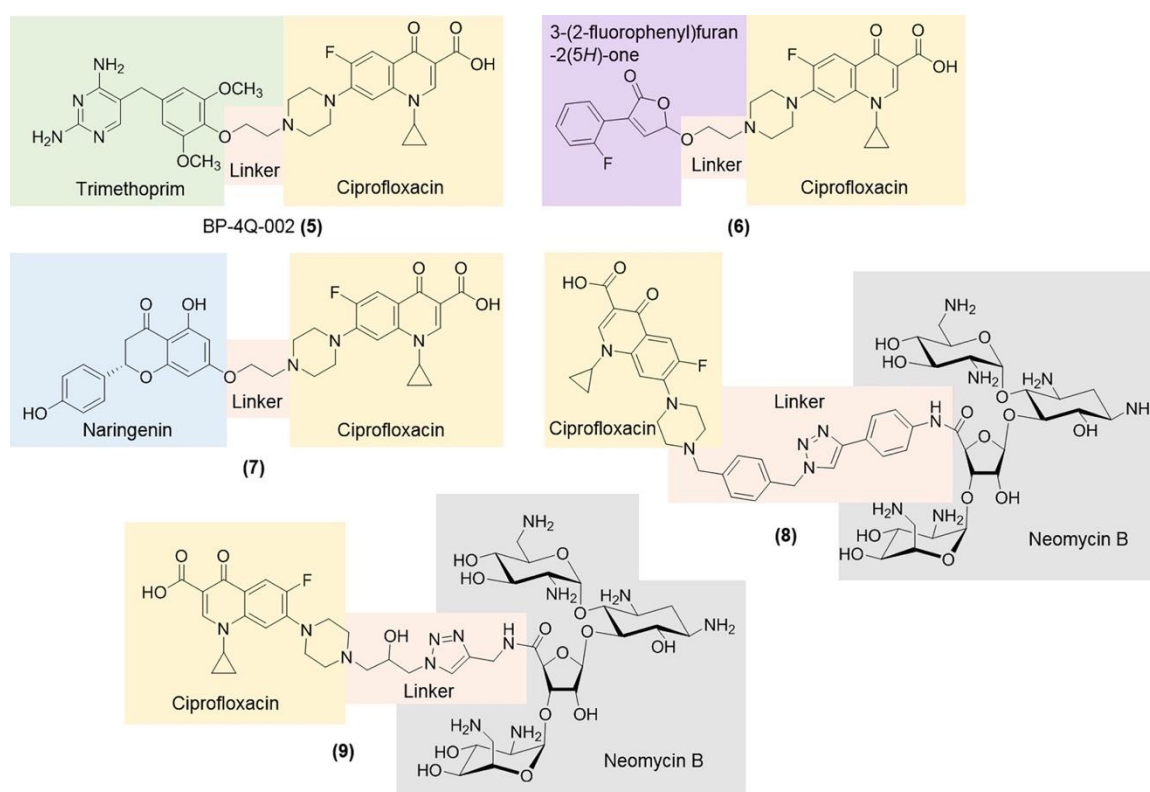


Figure 2.6. Examples of antibiotic hybrid drugs that are active against Gram-negative pathogens: trimethoprim linked to ciprofloxacin–BP- 4Q-002 (hybrid **5**), the TyrRS inhibitor 3-(2-fluorophenyl)furan-2(5H)-one linked to ciprofloxacin (hybrid **6**), the flavonoid naringenin linked to ciprofloxacin (hybrid **7**), neomycin B linked to ciprofloxacin via an aromatic triazole linker (hybrid **8**), and neomycin B linked to ciprofloxacin with a hydroxyl group-containing aliphatic triazole linker (hybrid **9**). Most antibiotic hybrid drugs consist of a fluoroquinolone.

A library of the tyrosyl-tRNA synthetase (TyrRS) inhibitor 3-arylfuran-2(5*H*)-one covalently linked to fluoroquinolones that possess broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria was described in 2014 (212). The most active hybrid drug comprised 3-(2-fluorophenyl)furan-2(5*H*)-one, a recently developed TyrRS inhibitor that compromises bacterial protein synthesis (213). This pharmacophore was linked to ciprofloxacin, and the resulting hybrid (hybrid **6**) displayed a potent MIC₅₀ of 0.11 µg/ml against MDR *E. coli* (ciprofloxacin MIC₅₀ of 5.65 µg/ml) (212). It was found to inhibit DNA gyrase *in vitro* better than ciprofloxacin itself (50% inhibitory concentration [IC₅₀] of 1.15 µM, versus 5.23 µM for ciprofloxacin) and has *in vitro* TyrRS-inhibitory activity comparable to those of established 3-arylfuran-2(5*H*)-one- based TyrRS inhibitors (212). Indeed, this hybrid possesses a bimodal antibacterial mode of action *in vitro*.

Another set of hybrids that contain phenolic flavonoids linked to fluoroquinolone has been described (214). The most promising entry was a fused naringenin-ciprofloxacin hybrid (hybrid **7**) that possesses potent antibiotic activity against Gram-positive bacteria (MIC₅₀ of 0.29 µg/ml against methicillin-resistant *S. aureus* [MRSA]), Gram-negative bacteria (MIC₅₀ of 0.71 µg/ml against MDR *E. coli*), and fungi (MIC₅₀ of 0.14 µg/ml against amphotericin B-resistant *Candida albicans*). Naturally occurring flavonoids have been reported to possess antibacterial activity, but their mode of action has been only loosely elucidated, with broadly suggested mechanisms such as membrane permeabilization and nucleic acid synthesis inhibition (215, 216). The flavonoid naringenin, however, was previously reported to have no antibacterial activity (217) against *E. coli* but is capable of selectively inhibiting drug efflux mechanisms in cancer cells (218). The naringenin-ciprofloxacin hybrid (hybrid **7**) displayed 23-fold- higher inhibitory activity against DNA gyrase than that of ciprofloxacin alone. Moreover, the hybrid was found to accumulate intracellularly in MRSA at levels approximately 5-fold higher than those of the parent drug ciprofloxacin (efflux rate of 9.8% for hybrid **7**, in comparison to 46.7% for ciprofloxacin) (214). The excellent antibacterial activity and high intracellular accumulation

of this hybrid molecule were attributed to the possibility of the flavonoid pharmacophore serving as an efflux pump inhibitor that prevents the agent from being expelled out of the cell. The bactericidal activity of hybrid **7** was credited to the fluoroquinolone pharmacophore and the covalent attachment of naringenin directly to ciprofloxacin, which imparted an enhanced physicochemical property for a stronger DNA gyrase interaction (214).

2.4.4.3.2 *Neomycin B-ciprofloxacin hybrid drugs delayed development of drug resistance*

A series of aminoglycoside-fluoroquinolone hybrid drugs that exhibit good activity against Gram-positive and Gram-negative bacteria has been reported (219). The hybrids consist of neomycin B (aminoglycoside) and ciprofloxacin with different linkers/tethers, with the aim of probing the optimal spatial length and physicochemical property needed for optimal activity. Hybrid **8**, with an aromatic triazole linker, and hybrid **9**, with a hydroxyl group-containing aliphatic triazole linker, are highlighted for several reasons. The neomycin B-ciprofloxacin hybrids exhibited activity against the Gram-positive organisms *Bacillus subtilis* (MICs of 1.5 µg/ml and 3 µg/ml for hybrids **8** and **9**, respectively) and MRSA (MICs of 3 µg/ml and 12 µg/ml for hybrids **8** and **9**, respectively). Both compounds also showed potent activity against Gram-negative drug-resistant *E. coli* strains (MIC ranges of 0.75 to 3 µg/ml and 0.75 to 12 µg/ml for hybrids **8** and **9**, respectively) that harbor several aminoglycoside-modifying enzymes (219). All the reported neomycin B-ciprofloxacin hybrids displayed significantly better antibacterial activity than neomycin B but not ciprofloxacin. Hybrid **8** inhibited protein translation *in vitro* similarly to the parent drug neomycin B. Interestingly, hybrid **8** displayed 15-fold-higher DNA gyrase and 20-fold-higher topoisomerase IV *in vitro* inhibitory activities than those of the parent drug ciprofloxacin (no data were reported for hybrid **9**). It is apparent that the degree of inhibition of *in vitro* DNA synthesis for hybrid **8** is not correlated with its whole-cell activity (MIC values) in that stronger DNA gyrase and topoisomerase IV inhibition should yield a more potent activity for hybrid **8** than for ciprofloxacin. This observation strongly suggests that the

neomycin B-ciprofloxacin hybrids suffer from membrane permeability issues. This may be due to the relatively high molecular masses of the neomycin B-ciprofloxacin hybrids (1,204 g/mol for hybrid **8** and 1,095 g/mol for hybrid **9**). The above-described hybrid drugs BP-4Q-002 (hybrid **5**), 3-(2-fluorophenyl)furan-2(5*H*)-one-ciprofloxacin (hybrid **6**), and naringenin-ciprofloxacin (hybrid **7**) have molecular masses of 633 g/mol, 551 g/mol, and 629 g/mol, respectively. The high molecular masses of antibiotic hybrids, as mentioned above, may become a liability, as large molecules are typically perceived to be OM impermeable, are not able to pass through nonselective porin channels, and therefore are unable to reach their intracellular targets. However, this appears to be untrue for the neomycin B-ciprofloxacin hybrids, as they still retain antibacterial activity, although it is not as potent as that of the parent drug ciprofloxacin. It could then be suggested that the hybrids somehow were able to enter the bacterial cell to reach their targets, although their level of intracellular accumulation was probably not as high as that of ciprofloxacin (which traverses the OM via porins and passively diffuses in the IM).

The reported neomycin B-ciprofloxacin hybrids **8** and **9** were further assessed for their therapeutic potential. No drug resistance was observed for the Gram-positive organism *B. subtilis* and the Gram-negative organism *E. coli* following 15 serial passages of hybrid **8** at subinhibitory (1/2 MIC) concentrations (220). Under similar experimental conditions, MIC increases of 37.5-fold, 8-fold, and 7.6-fold against *B. subtilis* were observed for ciprofloxacin, neomycin B, and an equimolar mixture of both agents, respectively (220). Similarly, MIC increases of 75-fold, 4-fold, and 20-fold against *E. coli* were found for ciprofloxacin, neomycin B, and an equimolar mixture of both agents, respectively (220). The fact that an equimolar combination of ciprofloxacin and neomycin B was not able to suppress drug resistance whereas the hybrid, which also contains equimolar components, was able to suppress resistance stimulated significant interest. This observation corroborates the hypothesized benefit of hybridizing two therapeutic agents into a unified entity, that it may impart additional properties that are otherwise absent in individual molecules (e.g., in combination therapy). The delayed

resistance evolution observed with hybrid **8** supports this notion. Further molecular analysis of the mechanistic interplay between *E. coli* and hybrid **9**, in a follow-up report (221), revealed that the bulk of the antibacterial activity of the neomycin B-ciprofloxacin hybrids is mediated mainly by the ciprofloxacin pharmacophore. On the other hand, the neomycin B pharmacophore was found to be mainly responsible for delaying the emergence of drug resistance that may arise from genetic mutations such as a mutation of the multiple-antibiotic-resistance (*marR*) repressor gene that leads to efflux-mediated resistance (as observed for ciprofloxacin) (221).

2.4.4.3.3 *Other hybrid drugs that are active against Gram-negative pathogens*

Other recent hybrid drugs with activity against Gram-negative pathogens include a berberine pharmacophore fused with either metronidazole (222) or benzimidazole (223), both of which displayed low MICs against *E. coli* and *P. aeruginosa*. Fusions of neomycin B with various phenolic antimicrobial agents such as chloroxylenol, triclosan, and clofoctol were also reported (224). An article (225) describes the *in vitro* and *in vivo* antibacterial evaluation of hybrids composed of the antimicrobial peptide tridecaptin linked to either rifampin, vancomycin, or erythromycin. Tridecaptins are naturally occurring lipopeptides that have potent activity against Gram-negative, but not Gram-positive, bacteria and low toxicity toward mammalian cells (226, 227). In that article, the tridecaptin utilized was either unacylated or acylated with octanoic acid at the *N* terminus. The reported hybrids exhibit low *in vitro* activities against *E. coli*, *K. pneumoniae*, and *A. baumannii* relative to an equimolar combination of tridecaptin and an antibiotic. Against *K. pneumoniae* ATCC 13883, the unacylated tridecaptin-erythromycin hybrid displayed an MIC of 50 μ M, while an equimolar combination of both components showed an MIC of 0.4 μ M (225). However, the unacylated tridecaptin-erythromycin hybrid displayed significantly better *in vivo* efficacy in a moribund mouse model of *K. pneumoniae* pulmonary infection than erythromycin alone or an equimolar combination of unacylated tridecaptin and erythromycin (225). The survival rates were 80%, 40%, and 40%, respectively, after 7 days (225).

This report suggests that a potent *in vitro* activity of an antibiotic-antibiotic combination is not always translatable *in vivo* and that the covalent linking of two pharmacophores rather than a concoction may enhance/retain *in vivo* efficacy.

2.4.4.3.4 *Cefiderocol (S-649266): a Trojan horse strategy*^g

The β -lactam–siderophore subclass of antibiotic hybrids is an emerging type of hybrid drug for Gram-negative pathogens. The idea of covalently attaching a siderophore (Greek for iron carrier) pharmacophore to a biocidal pharmacophore exploits a “Trojan horse” strategy that deceives bacteria to actively transport the antibiotic into the cell. Iron, in the form of ferric ion, is essential to bacteria, especially in an iron-deficient environment such as that of the mammalian host. Bacteria scavenge iron from their environment through the production of small-molecule siderophores that efficiently form complexes with iron. These siderophore-iron complexes are then taken up via active transport systems, such as TonB-dependent transporters (228–231). Through the attachment of the iron-chelating siderophore adjuvant, significantly higher intracellular drug concentrations can be achieved by hijacking the bacterial iron transport system. Several of these β -lactam–siderophore hybrid drugs have entered preclinical/clinical trials (232–236), notably the cephalosporin-catechol hybrid cefiderocol, which has advanced to and is currently in phase 3 clinical trials.

Cefiderocol (hybrid **10**), also known as S-649266 (Figure 2.7), is currently being developed by Shionogi & Co. Ltd. for complicated urinary tract infections and carbapenem-resistant Gram-negative bacterial infection. This hybrid is composed of the catechol 2-chloro-3,4-dihydroxybenzoic acid covalently appended via a non-cleavable linker/tether at carbon-3 of the cephalosporin ceftazidime.

^g We recently published a comprehensive review on this drug, Cefiderocol: A Siderophore Cephalosporin with Activity Against Carbapenem-Resistant and Multidrug-Resistant Gram-negative Bacilli. G.G. Zhanel et al, [Drugs 2019, 79, 3, 271-289](#).

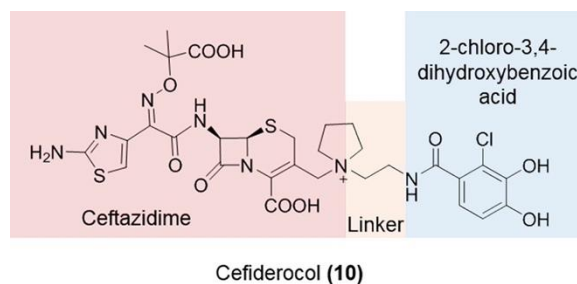


Figure 2.7. Structure of cefiderocol (hybrid **10**), previously known as S-649266, derived by linking ceftazidime to the siderophore catechol 2-chloro-3,4-dihydroxybenzoic acid. This β -lactam–siderophore hybrid possesses potent antibacterial activity against metallo- β -lactamase-producing Gram-negative bacilli.

Cefiderocol (hybrid **10**) possesses potent activity against Gram-negative ESKAPE pathogens. It exhibited MIC₉₀ values of 1 μ g/ml and 0.125 μ g/ml against randomly collected clinical isolates of the *Enterobacteriaceae* *E. coli* ($n = 106$) and *K. pneumoniae* ($n = 105$), respectively (237, 238). Furthermore, MIC₉₀ values of 1 μ g/ml and 2 μ g/ml were found for *P. aeruginosa* ($n = 104$) and *A. baumannii* ($n = 104$) isolates, respectively, among which were carbapenem-resistant strains (237, 238). In a recent study, cefiderocol (hybrid **10**) displayed MIC₉₀ values of 4, 1, and 8 μ g/ml against carbapenem-nonsusceptible *Enterobacteriaceae* ($n = 1022$), MDR *P. aeruginosa* ($n = 262$), and MDR *A. baumannii* ($n = 368$) isolates, respectively (239). These clinical isolates were randomly collected from 52 countries from 2014 to 2016 (239). In fact, cefiderocol (hybrid **10**) has been described to possess higher potency (237, 238) and better β -lactamase stability (240) than cefepime, ceftazidime, and meropenem against organisms that produce carbapenemases belonging to the class A, B (metallo- β -lactamases), and D β -lactam-hydrolyzing enzymes. The superior antibacterial activity of this enhanced cephalosporin-based hybrid relative to clinically relevant β -lactams was attributed to the covalently appended catechol pharmacophore. Cefiderocol (hybrid **10**) has been shown to chelate ferric irons that consequently facilitate its active transport into *P. aeruginosa* via iron transporters,

besides entering through nonselective porin channels, resulting in enhanced intracellular entry (241). The hybrid cefiderocol was also described to be 10 to 100 times more stable *in vitro* against several carbapenemases than its parent antibiotic ceftazidime (240). This enhanced stability might be credited to the steric hindrances imparted by the linker and catechol domains on the resistance enzymes.

The observed *in vitro* potency of cefiderocol (hybrid **10**) against Gram-negative bacilli was well translated *in vivo*. Cefiderocol was found to display good efficacy and significantly reduced the numbers of viable bacterial cells in murine models of pulmonary infection caused by MDR carbapenem-resistant *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *A. baumannii* (239, 242). This hybrid drug accelerated into phase 1 clinical trials in 2012 to evaluate its safety, tolerability, and pharmacokinetics in single or multiple intravenous administrations. An intravenous dosage of 2,000 mg every 8 h was well tolerated for a duration of 10 days in healthy individuals, and the drug is eliminated mainly through renal excretion (243, 244). Currently, cefiderocol (hybrid **10**) is being assessed for its efficacy in a phase 3, randomized, open-label study for the treatment of serious infections caused by carbapenem-resistant Gram-negative pathogens (<https://clinicaltrials.gov/ct2/show/NCT02714595>). Another phase 3, randomized, triple-blind clinical study of cefiderocol (hybrid **10**) in comparison to meropenem for the treatment of nosocomial pneumonia caused by Gram-negative pathogens has recently commenced (<https://clinicaltrials.gov/ct2/show/NCT03032380>). The urgent need to develop new antibiotics to treat MDR bacterial infections, especially infections by carbapenem-resistant Gram-negative bacilli, is apparent. With a phase 3 clinical trial under way, cefiderocol may very well be the first FDA-approved

NME systemic drug to be developed in the 21st century that is able to treat infections by MDR ESKAPE pathogens.^h

2.4.5 Antibiotic Hybrids can adopt new mechanistic actions that differ from those of their constituent Pharmacophores

Although antibiotic hybrids are designed to retain the dual mechanistic functions of their constituent pharmacophores, it is clearly an unpredictable science. Most antibiotic hybrids reported in the literature either retain the activity of only one of the domains or lose the activities of both domains (206, 207, 245, 246), and their antibacterial properties often resemble those of the dominant parent drug. Rational design is particularly challenging for Gram-negative bacteria because of the requisite physicochemical properties needed to navigate the orthogonal sieving nature of the outer and inner membranes (as discussed in Chapter 2.3.1). Moreover, hybridization of drug scaffolds to produce a unimolecular hybrid entity often leads to a high molecular weight that negates uptake through size-exclusive OM porin channels. However, it is possible for an antibiotic hybrid, being an entirely new chemical entity, to pharmacodynamically behave differently from its parent drugs. If hybridization results in a new or additional mode of biological action, it can also, in principle, interact differently with other classes of antibiotics (as an adjuvant). An antibiotic hybrid may interfere with nonbiocidal processes that directly or indirectly aid in, potentiate, or prevent the inactivation of a primary antibiotic-adjuvant property. Antibiotic hybrids are usually designed as stand-alone antibacterial agents, but the idea of investigating them as adjuvants for currently used antibiotics, especially those with high incidence rates of resistance development, is a novel approach that has opened a new

^h As of April 2, 2019, Shionogi Inc. (Japan) is currently recruiting participants for a study to determine the degree of penetration of cefiderocol into infected lung tissue in hospitalized adults with bacterial pneumonia who are being mechanically ventilated <https://clinicaltrials.gov/ct2/show/study/NCT03862040>.

paradigm in drug discovery. Several tobramycin-containing hybrids (Figure 2.8 Figure 2.9) have been reported to possess intrinsic physicochemical properties capable of “resuscitating” the efficacy of currently used antibiotics against multidrug-resistant Gram-negative bacteria, especially *P. aeruginosa* (247–252). These effects were determined and described as a measure of the fractional inhibitory concentration index (FICI), a numerical quantification of the interactions between antibiotics. FICIs of ≤ 0.5 , 0.5 to 4, and > 4 indicate synergism, no (or an additive) interaction, and antagonism, respectively (253). It was clear that the tobramycin-hybrid scaffold, depending on the second participating domain, exhibited a different spectrum of biological activities (247–252). These data, to the best of our knowledge, represent the only known data to date on the adjuvant properties of any hybrid antibiotics against Gram-negative bacteria.

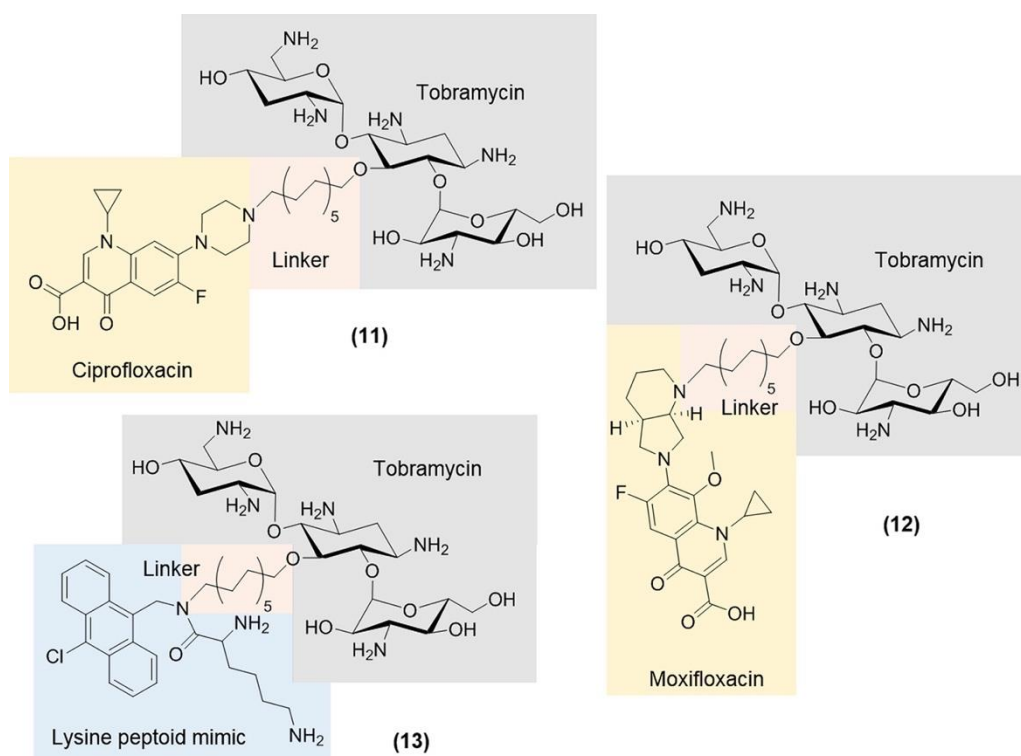


Figure 2.8. Examples of tobramycin-based hybrids: tobramycin linked to ciprofloxacin (hybrid **11**), tobramycin linked to moxifloxacin (hybrid **12**), and tobramycin linked to a lysine peptoid mimic (hybrid **13**). All three tobramycin-based hybrids contain a 12-carbon-long aliphatic (C₁₂) hydrocarbon linker.

2.4.5.1 Tobramycin-based hybrids as adjuvants that potentiate legacy antibiotics against *Pseudomonas aeruginosa*

The opportunistic pathogen *P. aeruginosa* is the leading cause of nosocomial infections in immunocompromised patients and is abundantly found on many medical devices in the hospital. *P. aeruginosa*, alongside other ESKAPE pathogens, is a major threat to public health for which effective therapy is rapidly becoming elusive (3, 254, 255). In 2017, the WHO ranked carbapenem-resistant *P. aeruginosa* as critical (priority 1) in its list of the world's most dangerous superbugs that pose a serious threat to human health (<http://www.who.int/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/>). Since the major impediment that antibiotics face against *P. aeruginosa* is low penetration/uptake across its impermeable outer membrane; the first tobramycin-based hybrid analog, hybrid **11**, was initially conceptualized to take advantage of the self-promoted uptake mechanism of aminoglycosides to deliver a second antibiotic into the periplasm of the cell. Moreover, aminoglycosides possess a pleiotropic mechanism of antibacterial action. They interact with rRNA to inhibit protein translation at lower concentrations ($\leq 4 \mu\text{g/ml}$), while they are known to disrupt the bacterial membrane at higher concentrations ($\geq 8 \mu\text{g/ml}$) (256). Tobramycin is a particularly attractive aminoglycoside, as it is currently one of the most active and effective agents for the treatment of *P. aeruginosa* infections (257).

It was shown previously that tobramycin-based hybrid drugs that lose innate *in vitro* antibacterial activity (Table 2.2) are able to enhance the potency of other antibiotic classes (except aminoglycosides and carbapenems) against *P. aeruginosa* (Table 2.2 and Figure 2.10) (247).ⁱ These synergistic effects are exclusive to the hybrids, as their composing antibiotic fragments do not exhibit

ⁱ Further investigations showed that specific scaffolds of molecules conjoined to tobramycin can generate adjuvants that restore full carbapenem (and other β -lactam antibiotics) activity against carbapenem-resistant *P. aeruginosa* clinical isolates. See Chapters 5 and 6.

this property. For example, a tobramycin-ciprofloxacin hybrid (hybrid **11**) that displayed weak antibacterial activity as a stand-alone agent (MIC of $\geq 16 \mu\text{g/ml}$) was found to restore the efficacies of ciprofloxacin and moxifloxacin (FICI of 0.03 to 0.38) against ciprofloxacin-resistant MDR or XDR *P. aeruginosa* clinical isolates (247).

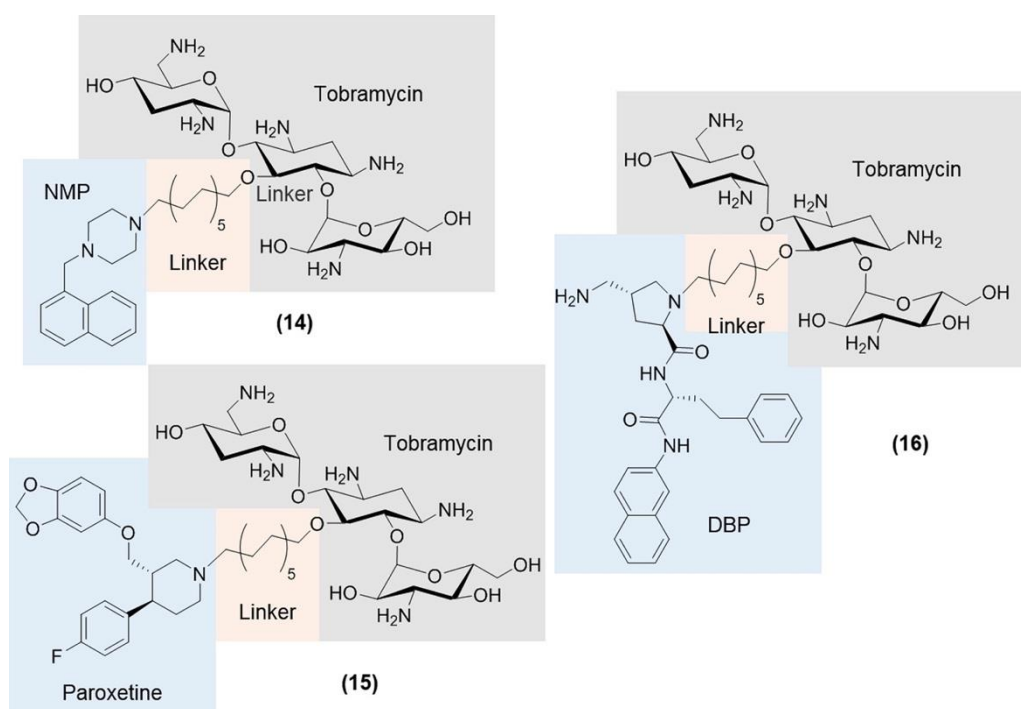


Figure 2.9. Examples of tobramycin-efflux pump hybrids: tobramycin linked to 1-(1-naphthylmethyl)-piperazine (NMP) (hybrid **14**), tobramycin linked to paroxetine (hybrid **15**), and tobramycin linked to the dibasic peptide (DBP) analog D-Ala–D- hPhe–aminoquinoline (MC-04,124) (hybrid **16**). All three tobramycin-based hybrids contain a 12-carbon-long aliphatic (C_{12}) hydrocarbon linker.

It is noteworthy that some of these isolates were also resistant to colistin and carbapenems, our last line of defense. Importantly, ciprofloxacin-susceptible (MIC of $\leq 1 \mu\text{g/ml}$) or -intermediate (MIC of $2 \mu\text{g/ml}$) CLSI breakpoints were reached for most of the fluoroquinolone-resistant clinical isolates in the presence of $\leq 8 \mu\text{g/ml}$ ($6 \mu\text{M}$) of hybrid **11** (247). Measurable *in vivo* potency of this hybrid was demonstrated by using the *Galleria mellonella* larvae infection model. In spite of the inherent limitations of this model, including a lack of adaptive immune responses in insects and the simple one-

compartment distribution, the remarkable similarities between the innate immune response of *G. mellonella* and those of vertebrates make this model desirable for studying the virulence of bacteria and the efficacy of antimicrobial agents (258). Upon injection of the larvae with a lethal load of XDR *P. aeruginosa* strain 101856, a 1:1 concoction of hybrid **11** and moxifloxacin (37.5 mg/kg each) administered as a single dosage at 2 h post infection resulted in 86% survival of the larvae after 24 h. In comparison, single-dose monotherapy with either hybrid **11** or moxifloxacin alone (50 mg/kg each) was ineffective and resulted in 100% killing within the same time frame. *In vitro* biochemical assays revealed that the DNA gyrase A- and topoisomerase IV-inhibitory activities of the ciprofloxacin domain of hybrid **11** were retained (3- to 5-fold higher than those of ciprofloxacin), whereas the protein translation-inhibitory properties of the tobramycin domain were lost (156- to 1,290-fold lower than those of tobramycin) (247). These *in vitro* biochemical observations are consistent with the activities of other aminoglycoside-fluoroquinolone hybrids, e.g., neomycin B-ciprofloxacin hybrids **8** and **9**, that were previously reported by another group (219). Mechanistic studies correlated the observed adjuvant effect of tobramycin-ciprofloxacin hybrid **11** to its ability to perturb the OM of *P. aeruginosa* in a dose-dependent manner, thus facilitating the influx (and bioaccumulation) of antibiotics that are typically unable to cross the OM, such as rifampin, novobiocin, vancomycin, and erythromycin (247). Since one of the main mechanisms of resistance to fluoroquinolones in *P. aeruginosa* is the overexpression of multidrug efflux pumps composed of a tripartite protein assembly spanning the inner and outer membranes (28), it is possible that the perturbation of the membrane's lipid composition, particularly the lipids localized around the transmembrane protein, could compromise the integrity of efflux pumps and restore the potency of fluoroquinolones. This hypothesis was tested by assessing the interaction(s) between fluoroquinolones and known permeabilizers in *P. aeruginosa*. Colistin, cetrimonium bromide, benzethonium chloride, and C₁₆-(Dab)₄-NH₂ (259) were unable to potentiate

Table 2.2. MICs of tobramycin-based hybrids and select antibiotics against wild-type and clinical isolates of Gram-positive and Gram-negative bacteria^a

| Organisms | MIC (µg/mL) | | | | | | | | | | | | |
|--|--------------------------|---------|---------|-------------|-------|-------|-----|-----|-----|-----|------------|-----|-----|
| | Tobramycin-based hybrids | | | Antibiotics | | | | | | | | | |
| | TOB-CIP | TOB-LYS | TOB-NMP | TOB | MOX | CIP | MIN | RMP | CAZ | CAM | ERY | TMP | COL |
| <i>Gram-positive bacteria</i> | | | | | | | | | | | | | |
| <i>S. aureus</i> ATCC 29213 | 64 | 8 | 32 | ≤0.25 | ≤0.25 | ≤0.25 | | | | | | | |
| MRSA ATCC 33592 | 32 | 8 | 64 | ≤0.25 | ≤0.25 | ≤0.25 | | | | | | | |
| MSSE 81388 ^{b,d} | 16 | 2 | 16 | ≤0.25 | ≤0.25 | ≤0.25 | | | | | | | |
| MRSE 61589 ^{b,e} | 32 | 4 | 8 | 1 | 64 | 128 | | | | | Not tested | | |
| <i>E. faecalis</i> ATCC 29212 | 128 | 16 | 128 | 8 | ≤0.25 | 1 | | | | | | | |
| <i>E. faecium</i> ATCC 27270 | 64 | 8 | 64 | 8 | 2 | 8 | | | | | | | |
| <i>S. pneumoniae</i> ATCC 49619 | 64 | 32 | 64 | 2 | ≤0.25 | 1 | | | | | | | |
| <i>Gram-negative bacteria</i> | | | | | | | | | | | | | |
| <i>E. coli</i> ATCC 25922 | 2 | 32 | 512 | 0.5 | ≤0.25 | ≤0.25 | | | | | | | |
| <i>E. coli</i> 61714 ^{b,e,f} | 64 | 32 | 512 | 8 | 0.5 | ≤0.25 | | | | | | | |
| <i>E. coli</i> 63074 ^{b,e,h} | 64 | 16 | 512 | 8 | 1 | ≤0.25 | | | | | | | |
| <i>E. coli</i> 97615 ^{b,d} | 512 | 32 | 512 | 128 | 32 | 256 | | | | | Not tested | | |
| <i>S. maltophilia</i> 62584 ^{b,e} | >512 | >128 | >512 | >512 | 4 | 32 | | | | | | | |
| <i>A. baumannii</i> 63169 ^{b,e} | 128 | >128 | 512 | 32 | 1 | 2 | | | | | | | |
| <i>K. pneumoniae</i> ATCC 13883 | 64 | >128 | >512 | ≤0.25 | ≤0.25 | ≤0.25 | | | | | | | |

| | | | | | | | | | | | | | |
|---|-----|----|-------|------|-----|-----|-----|------|-----|------|------|-------|-------|
| <i>P. aeruginosa</i> ATCC 27853 | 4 | 32 | 256 | 0.5 | 4 | 1 | | | | | | | |
| <i>P. aeruginosa</i> 62308 ^{b,ef} | 32 | 16 | 64 | 16 | 16 | 2 | | | | | | | |
| <i>P. aeruginosa</i> 96846 ^{b,d,f,g} | 64 | 32 | 256 | 256 | 16 | 4 | | | | | | | |
| <i>P. aeruginosa</i> PAO1 | 32 | 32 | 64 | 0.25 | 1 | ND | 8 | 16 | 2 | 64 | 256 | 256 | 1 |
| <i>P. aeruginosa</i> 100036 ^b | 128 | 32 | 256 | 64 | 128 | 64 | 32 | 16 | 8 | >512 | 256 | 256 | 2 |
| <i>P. aeruginosa</i> 101885 ^b | 256 | 16 | 256 | 0.25 | 64 | 32 | 32 | 16 | 8 | 512 | 256 | >512 | 0.5 |
| <i>P. aeruginosa</i> P259-96918 ^b | 128 | 64 | >1024 | 128 | 512 | 128 | 32 | 16 | 512 | 512 | 256 | 512 | 0.5 |
| <i>P. aeruginosa</i> P262-101856 ^b | 64 | 32 | 64 | 512 | 128 | 32 | 256 | 1024 | 16 | 1024 | 1024 | >1024 | 2 |
| <i>P. aeruginosa</i> P264-104354 ^b | 32 | 32 | 128 | 128 | 128 | 32 | 64 | 16 | 64 | 1024 | 256 | 256 | 4 |
| <i>P. aeruginosa</i> 91433 ^{b,c} | 16 | 8 | 32 | 8 | 8 | 2 | 64 | 16 | 256 | 16 | 512 | 512 | 32 |
| <i>P. aeruginosa</i> 101243 ^{b,c} | 16 | 16 | 64 | 256 | 4 | 2 | 4 | 8 | 64 | 4 | 1024 | 1024 | >1024 |

^aSee references (230, 249, 261). TOB, tobramycin; MOX, moxifloxacin; CIP, ciprofloxacin; MIN, minocycline; RMP, rifampin; CAZ, ceftazidime; CAM, chloramphenicol; ERY, erythromycin; TMP, trimethoprim; COL, colistin; TOB-CIP, tobramycin-ciprofloxacin hybrid **11**; TOB-LYS, tobramycin-lysine peptoid **13**; TOB-NMP, tobramycin-NMP hybrid **14**; ND, not determined. MRSA, methicillin-resistant *S. aureus*; MSSE, methicillin-susceptible *S. epidermidis*; MRSE, methicillin-resistant *S. epidermidis*. ^bClinical isolate. ^cColistin-resistant strain. ^dCANWARD (Canadian Ward surveillance). ^eCAN-ICU (Canadian National Intensive Care Unit surveillance). ^fGentamicin-resistant; ^gTobramycin-resistant; ^hThe MIC of amikacin is 32 µg/ml.

moxifloxacin in XDR *P. aeruginosa* 96918. This suggests that tobramycin-ciprofloxacin hybrid **11** either possesses another biological mechanism aside from membrane permeabilization or exhibits an augmented membrane interaction relative to those of common permeabilizers. Indeed, fluoroquinolones are known to be rarely potentiated by classical adjuvants against MDR Gram-negative bacteria, especially *P. aeruginosa* (260). Tobramycin-ciprofloxacin hybrid **11** was relatively non-toxic to human epithelial breast (JIMT1) and prostate (DU145) cancer cell lines (the effect was comparable to those of ciprofloxacin and moxifloxacin), as determined by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (261), and was non-hemolytic to human erythrocytes (< 10% at 1,000 µg/ml) (247). Ciprofloxacin and moxifloxacin are known to exhibit modest cytotoxicity toward these cell lines (262) and were thus used to assess the cytotoxicity of hybrid **11**.

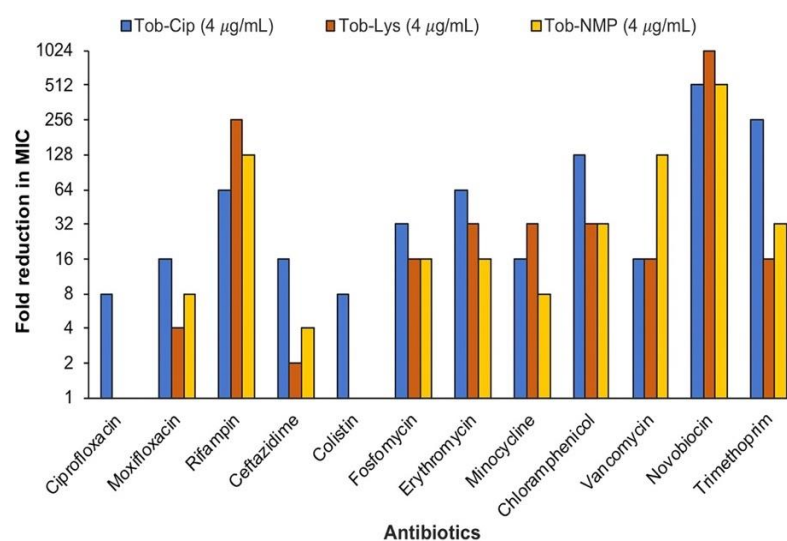


Figure 2.10. Potentiation of legacy antibiotics against *P. aeruginosa* PAO1 by tobramycin-based hybrids: tobramycin-ciprofloxacin hybrid **11** (Tob-Cip), tobramycin-lysine peptoid hybrid **13** (Tob-Lys), and tobramycin-NMP hybrid **14** (Tob-NMP). Tobramycin-lysine peptoid and tobramycin-NMP could not potentiate colistin and were not tested with ciprofloxacin.

Interestingly, amphiphilic aminoglycosides composed of aliphatic hydrocarbons attached to tobramycin (e.g., tobramycin-C₁₄ tether fragment of the hybrids) were found to possess beneficial immunomodulatory functions that may be exploited therapeutically. These amphiphilic tobramycin analogs selectively induced the chemokine IL-8 in macrophages but not growth-related oncogene (Gro- α), proinflammatory cytokines (TNF- α and IL-1 β), or the IL-1 antagonist IL-1RA (263). IL-8 is a potent neutrophil chemotactic factor responsible for the migration and activation of monocytes, lymphocytes, basophils, and eosinophils necessary for the resolution of infections (264). Moreover, the production of the LPS-induced proinflammatory cytokine TNF- α was abrogated by amphiphilic tobramycin via a mechanism that is independent of LPS interaction/neutralization, perhaps through an alteration of intracellular signaling downstream of pattern recognition in macrophages (263). This finding was of importance because the significant production of proinflammatory cytokines induced by endotoxins such as LPS has been implicated in septic shock (265, 266). Tobramycin by itself is not known to possess these immunomodulatory properties, but a tobramycin-copper complex has been reported to possess anti-inflammatory properties (267). Host defense peptides (HDPs) such as cathelicidin LL-37 and indolicidin are also known to be potent inducers of IL-8 (268–270), but they can also induce the production of other chemokines (such as monocyte chemoattractant protein 1 [MCP-1] and Gro- α) (268, 271), suggesting a selective chemoattractant ability of amphiphilic tobramycins. Relative to tobramycin, the tested amphiphilic tobramycin analogs exhibited negligible to <10% cytotoxicity against human monocytic THP-1 cells (ATCC TIB-202) at working concentrations (263).

2.4.5.2 *Structure optimization strategy for the tobramycin-based hybrid scaffold*

Enthused by the unexpected biological properties of the tobramycin-ciprofloxacin hybrid, the core (and least possible) structural fragment necessary for the adjuvant effect of this scaffold was investigated. It was reasoned (and data later confirmed) that the tobramycin fragment might be critical to the hybrid's scaffold, due to its well-known pleiotropic mechanism of action and/or perhaps its self-promoted uptake mechanism. A nonclassical structure-activity relationship study was therefore instituted to replace the ciprofloxacin domain of tobramycin-ciprofloxacin hybrid **11** with other pharmacophoric fragments (Figure 2.8 and Figure 2.9).

2.4.5.2.1 *Tobramycin-moxifloxacin hybrid retains a new mechanism of action*

The effect of replacing ciprofloxacin in the hybrid scaffold with another fluoroquinolone, while keeping the tobramycin fragment and the C₁₂ linker/tether, was investigated. Moxifloxacin (Figure 2.8) was selected because it is clinically relevant in the treatment of *P. aeruginosa* infections, is less affected by bacterial efflux systems due to its bulky C-7 substituent and is robust enough to withstand chemical manipulations (209, 210, 272). Tobramycin-moxifloxacin hybrid **12** retained the adjuvant properties of hybrid **11** and potentiated a range of legacy antibiotics against *P. aeruginosa* in a similar fashion (248). Mechanistic studies also showed that hybrid **12** perturbed the OM of *P. aeruginosa* PAO1 and induced a dose-dependent depolarization of the cytoplasmic membrane in a manner comparable to that of membrane-acting colistin. The ability of this compound to strongly reduce the flagellum-dependent swimming motility of *P. aeruginosa* PAO1 at sub-MIC values in a concentration-dependent manner, a function that requires intact PMF (273), supports the notion that it dissipates the cytoplasmic membrane PMF. This perhaps explains the strong synergistic effects observed with efflux-susceptible antibiotics (Figure 2.10) at sublethal concentrations (248), as PMF energizes RND-

based efflux pumps in *P. aeruginosa* (274, 275). Moreover, the observed dissipation of PMF by hybrid **12** is consistent with its antagonistic or weakly additive effects with aminoglycosides (248), as they require the electrical component ($\Delta\psi$) of an intact PMF for cytosolic uptake (65). Other nonbiocidal agents that dissipate bacterial PMF have also been shown to synergize the activities of some pH-dependent antibiotics (e.g., minocycline) against Gram-negative bacteria, at one-half the MIC for each strain (276). Thus, the replacement of ciprofloxacin in hybrid **11** with moxifloxacin in hybrid **12** did not alter the characteristic adjuvant properties of this scaffold, suggesting the indispensability of amphiphilic tobramycin as one of the primary domains required for their observed adjuvant properties.

2.4.5.2.2 *Tobramycin-lysine peptoid conjugates re-sensitize MDR P. aeruginosa to minocycline and rifampin*

Tobramycin-based hybrids that contain an appended fluoroquinolone pharmacophore seem to possess intrinsic properties that make them permeabilize the OM and dissipate the PMF across the inner membrane. These compounding membrane effects make membrane-impermeable or efflux-susceptible legacy antibiotics able to reach their corresponding cytosolic targets in *P. aeruginosa*. From previous studies, the tobramycin domain linked to the C₁₂ tether (also considered an amphiphilic tobramycin) appears to be responsible for the observed membrane effects, while that of the fluoroquinolone domain is unclear. The necessity of having a fluoroquinolone pharmacophore and to what extent it affects the observed biological activity (adjuvant property) of the tobramycin-based hybrids were then probed. Tobramycin- ciprofloxacin hybrid **11** potentiated other classes of antibiotics but displayed poor activity on its own (Figure 2.10), even though its ability to inhibit DNA synthesis, a characteristic function of fluoroquinolones, was retained. This suggests that the contribution of the fluoroquinolone to the overall chemical nature is perhaps just a physicochemical modulation. In this light, the fluoroquinolone fragment was replaced with a lysine-based peptoid mimic (Figure 2.8) that is capable of membrane permeabilization and depolarization (277). Several compounds of this peptoid

scaffold were reported to exhibit wide-spectrum potency against Gram-positive and Gram-negative bacteria (277). Tobramycin was conjugated at the terminal end of the aliphatic C₁₂ chain in the lysine-based peptoid, such that the amphiphilic tobramycin and the lysine peptoid domains shared a common alkyl chain. Tobramycin-lysine hybrid **13** displayed good activity (MICs of 2 to 8 µg/ml) against staphylococci but only weak activity (MIC of ≥ 16 µg/ml) against the tested Gram-negative bacilli (Table 2.2). Hybrid **13** displayed strong synergistic interactions with membrane-impermeable and efflux-susceptible antibiotics, consistent with other tobramycin-based hybrids (Figure 2.10). For instance, 4 µg/ml of hybrid **13** lowered the MICs of minocycline and rifampin against wild-type *P. aeruginosa* PAO1 by 32- and 256-fold, respectively (Table 2.3) (250). Synergy between hybrid **13** and other antibiotics was also observed for MDR/XDR *P. aeruginosa*, *A. baumannii*, *Enterobacter cloacae*, and *K. pneumoniae* clinical isolates. Extrapolating the CLSI breakpoints of minocycline against *Acinetobacter* spp. (MIC of ≤ 4 µg/ml) and of rifampin against *Enterococcus* spp. (≤ 1 µg/ml) as interpretive MIC standards (278), susceptible breakpoints for these antibiotics were reached in most MDR and XDR Gram-negative bacillus isolates by using just 4 µg/ml (3 µM) of tobramycin-lysine peptoid conjugate **13**. However, it could not potentiate β -lactams, colistin, and aminoglycosides.^j It is noteworthy that β -lactamase inhibitors like tazobactam or avibactam require concentrations of about 12 to 15 µM to potentiate β -lactam antibiotics in *in vitro* studies (121).

Mechanistic studies revealed that tobramycin-lysine hybrid **13** is rapidly bactericidal after 2 h at an MIC value of 32 µg/ml (250). The combination of hybrid **13** with minocycline (both at 1/8× MIC) or rifampin (both at 1/4× MIC) rendered *P. aeruginosa* PAO1 cells completely nonviable after 2 h and 4 h, respectively. Tobramycin-lysine hybrid **13** was found to permeabilize the outer membrane and depolarize the inner membrane, which is reflective of the biological action of the lysine-based

^j Most of the newly developed tobramycin-based conjugates described in this thesis (Chapters 5 and 6) potentiate β -lactam antibiotics. None of the conjugates synthesized to date potentiate any aminoglycoside.

peptoid fragment. Indeed, these membrane effects are also inherent to amphiphilic tobramycins, suggesting that the strong membrane effects of hybrid **13** stemmed from both fragments of the hybrid structure.

Table 2.3. MIC₅₀ and MIC₉₀ values of ciprofloxacin, minocycline, or rifampin alone and in combination with either tobramycin-ciprofloxacin hybrid **11**, tobramycin-lysine peptoid hybrid **13**, or tobramycin-NMP hybrid **14** against MDR/XDR *P. aeruginosa* isolates (*n* = 10)^a

| Antimicrobial(s) | MIC ₅₀ (µg/ml) | MIC ₉₀ (µg/ml) | MIC range (µg/ml) |
|------------------------------------|---------------------------|---------------------------|-------------------|
| CIP | > 32 ⊥ | > 64 ⊥ | 2 – 256 |
| MIN | 64 ⊥ | 256 ⊥ | 4 – 256 |
| RMP | 16 ⊥ | > 16 ⊥ | 8 – 1,024 |
| TOB-CIP | > 64 ⊥ | > 128 ⊥ | 4 – 256 |
| TOB-NMP | > 128 ⊥ | > 256 ⊥ | 2 – 1,024 |
| TOB-LYS ^b | 32 ⊥ | 64 ⊥ | 8 – 64 |
| CIP + 8 µg/ml TOB-CIP | 1 † | 4 ⊥ | < 1 – 4 |
| CIP + 4 µg/ml TOB-NMP | 6 ⊥ | 16 ⊥ | 0.1 – 16 |
| CIP + 4 µg/ml TOB-LYS ^b | ≤ 8 ⊥ | 32 ⊥ | 0.125 – 32 |
| MIN + 4 µg/ml TOB-CIP | 0.25 † | 4 † | 0.25 – 4 |
| MIN + 4 µg/ml TOB-NMP | 1 † | 4 † | 0.5 – 4 |
| MIN + 4 µg/ml TOB-LYS ^b | 0.5 † | 2 † | 0.5 – 2 |
| RMP + 1 µg/ml TOB-CIP | 1 † | 2 ‡ | 0.25 – 16 |
| RMP + 4 µg/ml TOB-NMP | 0.25 † | 8 ⊥ | 0.125 – 8 |
| RMP + 4 µg/ml TOB-LYS ^b | 0.25 † | 16 ⊥ | 0.0625 – 16 |

^aSee references 247–251. CIP, ciprofloxacin; MIN, minocycline; RMP, rifampin; TOB-CIP, tobramycin- ciprofloxacin hybrid **11**; TOB-LYS, tobramycin-lysine peptoid hybrid **13**; TOB-NMP, tobramycin-NMP hybrid **14**. †, susceptible; ‡, intermediately resistant; ⊥, resistant. ^b*n* = 5.

Moreover, hybrid **13** was found to dissipate PMF and inhibit bacterial swimming motility at 4 µg/ml. It also demonstrated significantly lower rates of ovine erythrocyte hemolysis (< 20%) than the lysine-based peptoid (85%) at a high concentration of 512 µg/ml and was not cytotoxic to JIMT1 and DU145 epithelial cells at 20 µg/ml (250). *In vivo* efficacy studies using XDR *P. aeruginosa* P262-challenged *G. mellonella* larvae demonstrated the ability of hybrid **13** to offer protection when used in combination with minocycline or rifampin, resulting in a 77% survival rate (for both combinations) after 24 h (250).

2.4.5.2.3 *Tobramycin-efflux pump inhibitor conjugates perturb RND efflux pumps*

Since the above-mentioned tobramycin-based hybrids (Figure 2.8) comprise antibacterial agents (ciprofloxacin, moxifloxacin, and lysine peptoid) that are substrates for *P. aeruginosa* RND pumps, the effects and implication of their conjugation to tobramycin on RND efflux pumps have also been examined (279). Tobramycin is known to be a poor substrate for most RND efflux pumps except for MexXY pumps (280, 281). The overall effect of attaching an efflux substrate antibiotic (such as moxifloxacin) to another non-substrate agent (such as tobramycin) on the ability of the resulting molecule to be effluxed was investigated. Although the substrate fragment may still be recognized by the active site of the efflux protein, its extrusion by the pump might be impeded by the hybrid's sheer steric bulk. Previous studies have shown that increasing the size or molecular weight of compounds reduces the likelihood of efflux (282). In concept, this may also alter the protein environment and incapacitate the efflux pump. The effect of efflux on tobramycin-based hybrids was explored by using a MexAB-OprM deletion strain (PAO200) and an efflux-sensitive strain (PAO750) that lacks five different clinically relevant RND pumps (MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK, and MexXY) and the OM protein OpmH (283, 284). Whereas the antibacterial activity of moxifloxacin was greatly affected by the absence of RND pumps (128-fold reduction of the MIC for PAO750) (248), the MICs of tobramycin-based hybrids **11**, **12**, and **13** were quite similar across wild-type *P.*

aeruginosa strains and efflux deletion strains, and only a marginal 2- to 4-fold reduction in the MIC was observed for PAO750 (247, 248, 250). This clearly shows that the hybridization of an efflux substrate antibiotic to a non-substrate antibiotic resulted in an entity that could resist efflux, perhaps due to steric bulk. In terms of their adjuvant properties, a significant potentiation of several classes of antibiotics was retained across these efflux-deficient strains (247, 248, 250), suggesting that the synergistic interactions between these hybrids and antibiotics are independent of clinically relevant RND efflux pumps. However, dissipation of the PMF, which is induced by the tobramycin-based hybrids, may also be implicated in the inactivity of PMF-dependent efflux systems (285).

Overexpressed multidrug efflux pumps that effectively reduce intracellular antibiotic concentrations remain a major problem (23, 28). Efflux pump inhibitors (EPIs) that block active efflux have been demonstrated to potentiate efflux-susceptible antibiotics in Gram-positive and some Gram-negative pathogens but in only a few *P. aeruginosa* isolates (286–289). EPIs work by competing with the antibiotic binding site and/or by perturbing the integrity of the transmembrane protein channel or the RND tripartite protein complex assembly (275). The inability of most EPIs to synergize other antibiotics in *P. aeruginosa* is perhaps attributable to their penetration impediments across the bacterial membrane. However, some EPIs may also be subject to expulsion, as the efflux pump architecture varies among different efflux systems. The possibility of inducing vector-assisted intracellular uptake of EPIs in *P. aeruginosa*, following a hybrid drug approach, by utilizing the self-promoted uptake of aminoglycosides and amphiphilic aminoglycosides was thus investigated (290). Aminoglycosides are desired since they are poor substrates of most efflux pumps in *P. aeruginosa*, especially those of the RND family (291). It was posited that the physicochemical property of the tobramycin-hybrid scaffold required to elicit a biological response (such as PMF dissipation) and the native efflux pump-inhibitory effects of EPIs would be preserved during hybridization. Thus, three EPIs [1-(1-naphthylmethyl)-piperazine (NMP), paroxetine (PAR), and dibasic peptide (DBP)] were appended to tobramycin using aliphatic hydrocarbon tethers, to give hybrids **14**, **15**, and **16**, respectively (Figure 2.9), and screened

against a panel of clinically relevant pathogens (249). It should be noted that DBP is an analog of the dibasic dipeptide D-Ala–D-homophenylalanine (hPhe)–aminoquinoline (MC-04,124) (292), a former drug candidate that was able to potentiate fluoroquinolones via efflux pump inhibition and membrane-destabilizing effects (284, 292, 293). None of the resulting conjugates, hybrids **14**, **15**, and **16**, displayed potent antibacterial activity against both Gram-positive and Gram-negative bacteria (Table 2.2) (249). However, the tobramycin-EPI conjugates retained adjuvant properties similar to those of other tobramycin-based hybrids, as they enhanced the antibacterial activity of minocycline in wild-type, MDR, and XDR *P. aeruginosa* isolates (Table 2.3). On the other hand, neither NMP nor PAR potentiated minocycline. Minocycline is a known substrate of *P. aeruginosa* RND efflux pumps (291). At 8 µg/ml (6.1 to 7.2 µM), all the tobramycin-EPI hybrids (hybrids **14**, **15**, and **16**) reduced the MIC of minocycline below its CLSI interpretive susceptibility breakpoint (≤ 4 µg/ml) in all tested *P. aeruginosa* clinical isolates. Strong synergism was similarly observed with other tetracyclines, such as doxycycline and tigecycline, and potentiation was observed for other Gram-negative pathogens as well (249).

Mechanistic validation for hybrid **14** showed modes of action similar to those of other tobramycin-based hybrids. It permeabilizes the OM in a dose-dependent manner, induces a dose-dependent depolarization of the cytoplasmic membrane, and inhibits the PMF-driven flagellum-dependent motility of *P. aeruginosa* PAO1 at sub-MIC values (249). The combination of hybrid **14**, but also other tobramycin-EPI combinations, with minocycline at a 1:1 mass ratio delayed the emergence of resistance in PAO1 after 25 serial passages. However, minocycline or tobramycin alone resulted in a 16-fold or 256-fold increase in the MIC, respectively. Tobramycin-NMP conjugate **14** displayed *in vivo* potency in XDR *P. aeruginosa* 101856-challenged *G. mellonella* larvae. A single dose of the minocycline-hybrid **14** combination (75 mg/kg each) resulted in 77% survival after 24 h (249). On the other hand, minocycline (75 mg/kg) or hybrid **14** alone (75 mg/kg) resulted in 0% survival after 24 h. Tobramycin-NMP hybrid **14** was shown to be nonhemolytic ($\leq 5\%$ hemolysis of

ovine erythrocytes at 1,000 µg/ml) and displayed low cytotoxicity to human epithelial cancer cell lines (50% cytotoxic concentration [CC₅₀] of >30 µM). CC₅₀ is the concentration of a drug required to reduce the viability of a cell population by 50% relative to untreated controls (294, 295). This rules out the suspicion of a nonspecific mode of action (296), as the hybrid molecule could discriminate prokaryotic from eukaryotic cells. The synergism of minocycline and tobramycin-EPI conjugates was more pronounced in *P. aeruginosa* strains that express RND efflux pumps but was less pronounced in efflux deletion strains (249). This subtle observation may suggest that, in addition to dissipating the PMF, the tobramycin-EPI conjugates may also disrupt active efflux via RND pumps, particularly that of the minocycline-relevant MexAB-OprM RND pump. The ability of tobramycin-EPI conjugates to enhance the uptake of tetracycline in *P. aeruginosa* PAO1 was reversed in the presence of the oxidative phosphorylation uncoupler CCCP (carbonyl cyanide *m*-chlorophenyl hydrazone), an agent that disrupts the proton gradient of Gram-negative bacteria (297). This observation is consistent with the ability of the hybrids to interfere with bacterial PMF.

More recently, a polymyxin B₃-tobramycin hybrid was also reported to potentiate rifampin, minocycline, and vancomycin against MDR and XDR *P. aeruginosa* isolates, in a fashion similar to those of other tobramycin-based hybrids (252).

In summary, tobramycin-based hybrids appear to have an intrinsic physicochemical property that enables the potentiation of legacy antibiotics (Figure 2.10). The pairing of a pharmacophore to tobramycin may impart an additional biological action to the scaffold, and/or hybridization may result in a new (third) mechanism of antibacterial action independent of the composing parent drugs. Table 2.2 summarizes the MICs of tobramycin-based hybrids and some select antibiotics against a panel of Gram-positive and Gram-negative bacteria. Tobramycin-based hybrids, in combination with ciprofloxacin, minocycline, or rifampin, re-sensitized a panel of MDR/XDR *P. aeruginosa* isolates

and decreased their respective MICs from resistant to susceptible or intermediately resistant values (Table 2.3).

2.4.5.3 Re-sensitization of resistant pathogens to antibiotics may be induced by targeting the membrane

A recent review aptly described the role of proton-dependent processes in the propagation of bacterial infections (298). Membrane-active agents are known to disrupt vital bacterial processes by perturbing the well-ordered membrane constituents, resulting in the loss of transmembrane protein integrity and function but also the loss of transmembrane potential (ion/proton balance) (86, 299). The mechanistic action of membrane disruption may stem from membrane perforation via the formation of transient pores (300), lipid disintegration leading to lysis, and/or the segregation of phospholipids (301). These concentration-dependent effects are usually driven by the amphiphilic nature of the compounds and could range from mild to fatal (302–307). Since the bacterial membrane is central to a host of its vital, survival, and adaptive mechanisms such as energy production via the respiratory chain, quorum sensing, and efflux pumps, etc., agents that alter the transmembrane protein environment (such as membrane charge, fluidity, and thickness) and/or steric hindrance of membrane-embedded proteins can theoretically lead to cell death via the inhibition of signaling cascades. For instance, swimming motility is a flagellum-dependent bacterial movement that is governed by the respiratory chain on the cytoplasmic membrane (273). When cytoplasmic membrane potential or PMF is disrupted, electron transfer across the respiratory chain is inhibited, resulting in a reduction of ATP synthesis, which is essential for flagellar function (308). These flagella (and pili) are surface appendages that serve as the major means of motility (chemotaxis) as well as the anchors that facilitate initial binding to the asialylated glycolipid (asialoGM1) receptor on the host's epithelial cells prior to the destruction the protective glycocalyx (309).

Membrane depolarization can therefore result in the re-sensitization of MDR pathogens to antibiotics, as the inhibition of signal transduction coordinated by ATP machinery of the respiratory chain could inflict a crippling fitness cost on the bacteria. Some HDPs are endowed with excellent membrane-disruptive capabilities for selectively interacting with bacterial membranes (310) but have severe limitations of protease cleavage and systemic cytotoxicity, etc. (311). Other polycationic agents such as the pentabasic polymyxin analogs that increase OM permeability in Gram-negative bacteria are also relatively toxic (312). Reducing the number of basic groups in polymyxins may represent a path forward to reduce potential nephro- and/or ototoxicity, as evident in preliminary studies with tribasic SPR741 (150). The reduction in basic groups could, however, compromise the ability of the adjuvant to sensitize certain pathogens, such as the inability of SPR741 to synergize antibiotics in *P. aeruginosa* (149).

2.4.5.4 *Proposed mechanisms of action of tobramycin-based hybrids*

The mechanism of action of tobramycin-based hybrids is quite consistent with that of membrane-active agents that target cellular energetics of prokaryotes. The cellular uptake and mode of action of this scaffold, particularly in but not limited to *P. aeruginosa*, involve the following (Figure 2.11).

Competitive displacement of divalent cation cross-bridges that stabilize the LPS structure on the outer leaflet of the OM, in a fashion analogous to that of the self-promoted uptake mechanism of aminoglycosides and amphiphilic aminoglycosides (90, 224, 312–322), results in OM destabilization. This destabilization facilitates the permeation of other antibiotics into the periplasm and may explain the observed sensitization of MDR Gram-negative bacteria to membrane-impermeable antibiotics such as rifampin, novobiocin, and vancomycin, etc.

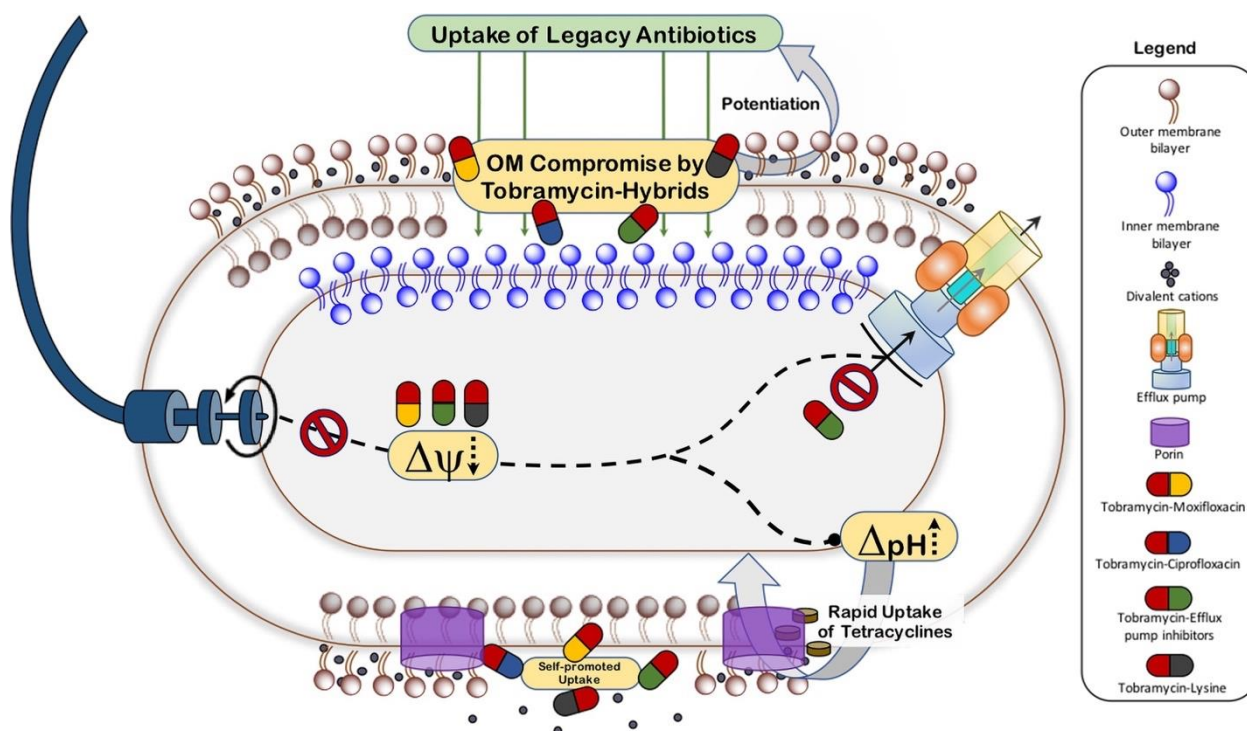


Figure 2.11. Proposed mechanism(s) of action of tobramycin-based antibiotic hybrids.

Insertion into the OM, presumably promoted by the hydrophobic aliphatic hydrocarbon linker, allows tobramycin-based hybrids to reach the periplasm. This may further augment the entry of other membrane-impermeable antibiotics, as reflected by their synergistic relationship with the hybrids.

Perturbation of transmembrane efflux protein domains prevents the relay of signaling cascades required to elicit conformational changes necessary to extrude substrate molecules (such as antibiotics). The loss of protein integrity may be induced by the hybrid directly, via hydrogen bonding with exposed residues, or indirectly, by altering the lipid composition surrounding the protein. This may in part explain the observed enhancement of the intracellular concentration (synergy) of efflux-susceptible antibiotics such as fluoroquinolones.

Dissipation of the membrane potential ($\Delta\psi$) component of PMF leads to compensatory transmembrane pH gradient (ΔpH) adjustments to maintain a constant PMF. PMF is composed of an

electrical component ($\Delta\psi$) and a proton component (ΔpH), which are complementary to one another. If one component is altered, the bacteria compensate for and ensure a stable PMF by adjusting the other complementary component (59). This phenomenon was previously described as being the basis for the potentiating effects of the antidiarrheal drug loperamide (276). Tobramycin hybrids dissipate $\Delta\psi$ (at sub-MIC values), thereby prompting the pathogen to raise its transmembrane ΔpH . This view is supported by the following findings: (i) the swimming motility (a $\Delta\psi$ -controlled process) of *P. aeruginosa* was severely constrained in the presence of the hybrids; (ii) the hybrids could not potentiate the aminoglycoside class of antibiotics, as they require an optimal $\Delta\psi$ component for cytoplasmic uptake; and (iii) the strong synergy between tobramycin-based hybrids and minocycline (and other tetracyclines) is indicative of an increased ΔpH component. Tetracyclines are known to penetrate bacterial cells in a ΔpH -dependent fashion (64). Moreover, the inability of tobramycin-based hybrids to potentiate meropenem, which is exclusively taken up by active transport, may indirectly suggest that their effects on PMF not only affect efflux but also may reduce active energy-dependent transport.

A combination of any or all of the above-described mechanisms, simultaneously or sequentially, is most likely responsible for the adjuvant effects of tobramycin-based hybrids. Although these hybrids target bacterial membrane energetics, some FDA-approved nonantibiotics have also been shown to target and disrupt the physical integrity of this membrane (59, 274, 276). This suggests that these membrane-active compounds may similarly be tolerated in humans, as evident in pilot studies with *G. mellonella* larvae and human epithelial cancer cells.

Indeed, the characteristic mechanism of action of this tobramycin hybrid scaffold is what sets it apart from those of other hybrid antibiotics discussed above, and we encourage everyone involved in antimicrobial drug discovery to partake in this exciting area of investigation.

2.5 Perspectives

Our viewpoint of the current landscape of antibiotic development is simple: there is an urgent need to develop new therapeutics that are able to treat antibiotic-resistant infections, especially those caused by MDR Gram-negative pathogens. However, coming up with a magic bullet to address this problem has proven to be elusive over the years. We highlight several strategies to potentially develop new antibiotics, yet these are only a few of the possible solutions to this ever-worsening global issue. Imagination is perhaps the greatest limitation to drug discovery.

The concept of antibiotic hybrids is attractive considering their apparent advantages, but they are not without an Achilles' heel. Molecular complexity, intractable chemical synthesis, and the rigorous work needed to establish the mode of action and benefit of hybrids over conventional drugs make the hybrid approach daunting. Nonetheless, we argue that drug discovery should be driven not only by synthetic convenience but also by scientific exploration to solve an important problem. Despite its associated challenges, the antibiotic hybrid strategy remains a viable approach to expand the chemotherapeutic space of our current antimicrobial arsenal. Our experience in this research field has broadened our views to rethink the principles of antibacterial drug discovery, that (i) an inactive agent, in terms of its antibacterial activity (MIC), is not necessarily biologically irrelevant, and (ii) molecular fusion/hybridization of pharmacophores could in fact present a new scaffold with a completely different biological activity and pharmacological profile. Our exploration with tobramycin-based hybrids yielded a core scaffold that appears to have intrinsic adjuvant properties and is amenable to modifications.

2.6 Future Outlook: Antibiotic Hybrids may be the Next Generation of Antibiotic Agents and Adjuvants

With the globally escalating incidences of MDR, XDR, and PDR infections in both inpatients and outpatients, especially those caused by the carbapenem-resistant ESKAPE superbugs, there is an obvious need to restock our antibiotic arsenal and stay ahead of these pathogens. Hybridization of legacy antibiotics to generate new drug scaffolds might be the future of antibacterial drug discovery. There are at least two hybrid drug candidates (Figure 2.12) currently in consideration for the treatment of Gram- positive bacterial infections, while only one (Figure 2.7) is under consideration for Gram-negative bacterial infections.

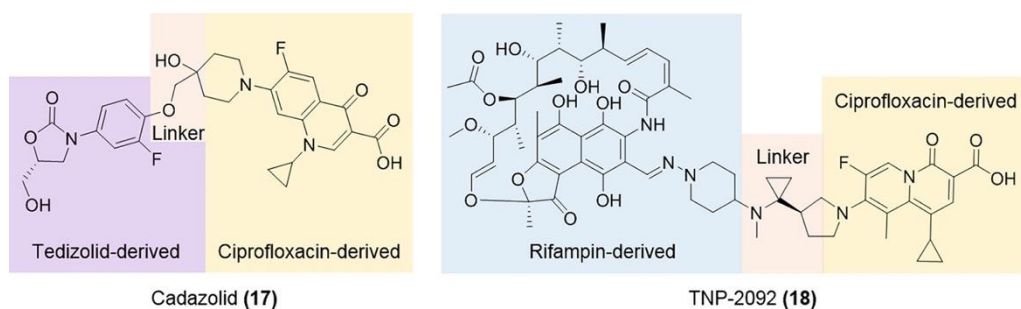


Figure 2.12. Examples of advanced hybrid antibiotics undergoing clinical trials: cadazolid (hybrid **17**) and TNP-2092, also known as CBR-2092 (hybrid **18**). Cadazolid was derived by fusing ciprofloxacin and tedizolid (with overlapping pharmacophores), while TNP-2092 comprises ciprofloxacin and rifampin-derived pharmacophores. These hybrids display limited antibacterial activity against Gram-negative bacilli.

Cadazolid (hybrid **17**) (323–327), a non-cleavable heterodimer consisting of fused pharmacophoric portions derived from ciprofloxacin and tedizolid (Figure 2.12), has completed phase 3, randomized, double-blind clinical trials for the treatment of *Clostridium difficile*-associated diarrhea

in comparison to vancomycin (<https://clinicaltrials.gov/show/NCT01987895>). According to Actelion Pharmaceuticals (now acquired by Johnson & Johnson), the study yielded mixed results: cadazolid met its primary endpoint of clinical cure for its pivotal IMPACT 1 study but failed to do so for its follow-up IMPACT 2 study, raising concerns about its efficacy. CBR-2092 (hybrid **18**) (328, 329), a rifamycin-quinolone hybrid (Figure 2.12), is also in clinical development for acute bacterial skin and skin structure infections but also for hospital-acquired pneumonia, ventilator-associated bacterial pneumonia, and bacteremia. Hybrid **18** was renamed TNP-2092 after Cumbre Pharmaceuticals was acquired by TenNor Therapeutics in 2009 and has successfully completed phase 1 clinical trials. Other hybrid drug candidates in clinical trials for the treatment of Gram-positive bacterial infections include the glycopeptide-cephalosporin hybrid cefilavancin (completed phase 2 trials [<https://clinicaltrials.gov/ct2/show/NCT00442832>]) and the oxazolidinone-quinolone hybrid MCB3837 (currently in phase 1) (<http://www.pewtrusts.org/en/multimedia/data-visualizations/2014/antibiotics-currently-in-clinical-development>).

As elaborated above, the cephalosporin-siderophore hybrid cefiderocol (hybrid **10**) (241, 243, 244) is currently in phase 3 clinical trials for the treatment of severe infections caused by carbapenem-resistant Gram-negative bacteria (<https://clinicaltrials.gov/ct2/show/NCT02714595>) as well as nosocomial pneumonia caused by Gram-negative pathogens (<https://clinicaltrials.gov/ct2/show/NCT03032380>). The fact that cefiderocol is in late stages of clinical trials is very encouraging, as it is one of the very few antibiotic hybrids that retain potency against recalcitrant carbapenem-resistant Gram-negative bacilli.

We envisage an increase in the number of antibiotic hybrids that progress into clinical studies in the near future, either as stand-alone antibacterial agents or as adjuvants. The use of adjuvants to rescue the efficacy of currently used antibiotics is increasingly gaining attention, especially for use against Gram-negative pathogens (11, 119, 127, 146, 330), and antibiotic hybrids certainly have a role to play

in this going forward. With the recent examples of experimental hybrid agents in the literature (245), we are optimistic that a few of these drugs will make it all the way to the clinic for patient treatment. It is our hope that this article and others (169, 207, 245, 246) will stimulate curiosity and interest in the antibiotic-hybrid strategy as a viable way to develop agents that are capable of combating drug-resistant pathogens.

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3 CHAPTER THREE:

Thesis Objectives and Methods

3.1 Preface

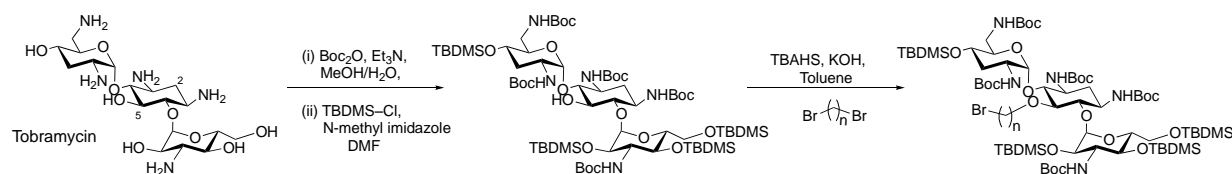
The objective of this thesis is to study the non-classical structure-activity relationships of tobramycin-based bifunctional molecules, with a view to developing antibiotic adjuvants that can potentiate existing classes of antibiotics when used in syncretic combinations. This requires the actualization of two major goals:

- a) To design and synthesize different series of non-ribosomal tobramycin-based conjugates (Chapters 4 – 6). Previous studies with tobramycin-hybrids revealed that an amphiphilic tobramycin fragment is critical to outer membrane destabilization and that the attached secondary domain may modulate/fine-tune this property towards bacteria-specific and/or antibiotic-specific potentiation.¹ Consequently, different derivatives of amphiphilic tobramycin-based conjugates were synthesized to impart different physicochemical properties.
- b) To carry out extensive biological evaluation of the newly prepared compounds, investigate their breadth/spectrum of activity, as well as gain some mechanistic insights into their mode of actions. The best molecule in each series/generation of the synthesized compounds were selected for this study.

3.1.1 Chemistry

The preparation of the new series of tobramycin-based conjugates, i.e. rifampicin-tobramycin conjugates, tobramycin-cyclam conjugates, and tobramycin homodimers, were achieved following a modified protocol of a previously published procedure.² These molecules were all ligated at C-5 of the 4,6-disubstituted 2-deoxystreptamine ring of tobramycin (rationale for each compound are provided in their respective chapters), a position that was selectively assessed, due to sterics, by using bulky protecting groups to protect other functional groups (Boc for amines and TBDMS for hydroxyls). A phase transfer catalyst (TBAHS) was subsequently used to achieve alkylation of the unprotected 5-

OH of the deoxystreptamine ring with different alkyl halides (Scheme 3.1). Detailed synthetic procedures are provided in each respective chapter. Compounds were purified by flash chromatography and characterized using NMR (1D and 2D) and mass spectrometry. Purity of final compounds were evaluated using HPLC analysis or elemental analysis.



Scheme 3.1. General procedure to access and alkylate the 5-OH of 4,6-disubstituted deoxystreptamine of tobramycin.

3.1.2 Biology

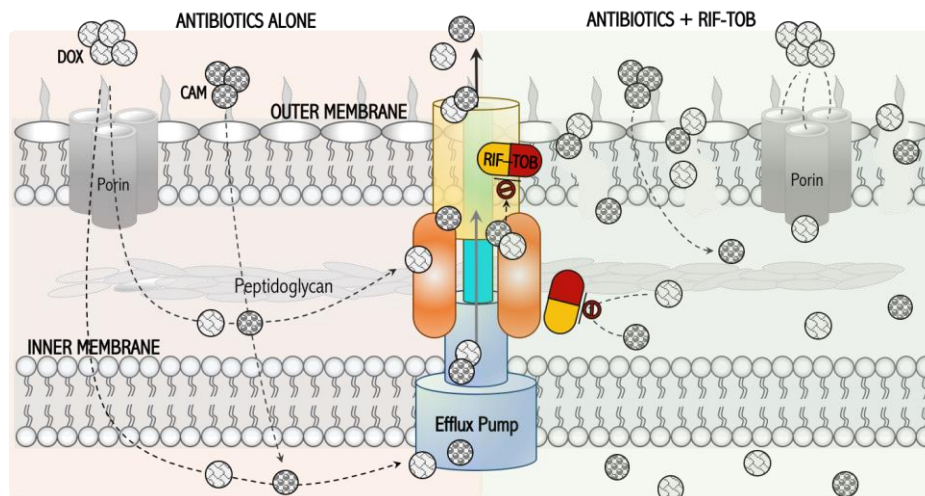
For biological studies, antimicrobial susceptibility and checkerboard assay were determined in accordance with the Clinical Laboratory Standard Institute (CLSI) broth dilution guidelines.³ Synergistic relationships observed in checkerboard assay were further investigated using time-kill assays, to determine the kinetics of killing and log reduction in bacterial burden. Emergence of resistance to mono- and combination therapies were investigated by serial passaging, following an established protocol,⁴ and preliminary *in vivo* efficacy studies were carried out using *Galleria mellonella* (wax moths) larvae infection model. Toxicological evaluations against eukaryotic cells were investigated using i) porcine erythrocytes and ii) human epithelial cancer cells such as human embryonic kidney (HEK293) and liver carcinoma (HepG2) cells, following standard protocols.^{5,6}

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4 CHAPTER FOUR:

Heterodimeric Rifampicin–Tobramycin Conjugates break Intrinsic Resistance of *Pseudomonas aeruginosa*



4.1 Preface

The work presented in this chapter has been published as a research article in:

Temilolu Idowu, Gilbert Arthur, George G. Zhanel, and Frank Schweizer. (2019). Heterodimeric Rifampicin–Tobramycin conjugates break intrinsic resistance of *Pseudomonas aeruginosa* to doxycycline and chloramphenicol *in vitro* and in a *Galleria mellonella in vivo* model.

Eur J Med Chem 174: 16-32. <https://doi.org/10.1016/j.ejmech.2019.04.034>

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4.1.1 Authors' Contributions

Temilolu Idowu performed all chemical synthesis, all biological studies, and all *in vivo* experiments. Gilbert Arthur conducted cytotoxicity testing. George G. Zhanel provided helpful insights on the project. Frank Schweizer supervised the project. Temilolu Idowu analyzed the data and wrote the manuscript. All authors were responsible for the final form of the manuscript.

4.2 Abstract

Intrinsic resistance in *Pseudomonas aeruginosa*, defined by chromosomally encoded low outer membrane permeability and constitutively over-expressed efflux pumps, is a major reason why the pathogen is refractory to many antibiotics. Herein, we report that heterodimeric rifampicin-tobramycin conjugates break this intrinsic resistance and sensitize multidrug and extensively drug-resistant *P. aeruginosa* to doxycycline and chloramphenicol *in vitro* and *in vivo*. Tetracyclines and chloramphenicol are model compounds for bacteriostatic effects, but when combined with rifampicin-tobramycin adjuvants, their effects became bactericidal at sub MIC levels. Potentiation of tetracyclines correlates with the SAR of this class of drugs and is consistent with outer membrane permeabilization and efflux pump inhibition. Overall, this strategy finds new uses for old drugs and presents an avenue to expand the therapeutic utility of legacy antibiotics to recalcitrant pathogens such as *P. aeruginosa*.

4.3 Introduction

The problem of antimicrobial resistance is a global phenomenon that is threatening to reverse the gains and advances in modern medicine.[1–4] Microorganisms are developing resistance to our antibiotic pipeline at a rate that is practically impossible to keep up with and there are some phenotypes that are completely resistant to all available treatment options.[5] Regrettably, no new class of antibiotics with a novel mode of action has been successfully developed in the last few decades.[6,7] It is not surprising that for the first time ever, the WHO in 2017 published a list of pathogens for which effective treatment options are becoming elusive.[8] With a projection that antimicrobial resistance will be a leading cause of death worldwide by 2050,[9] it is expedient to, in the meantime, develop strategies that can expand and/or preserve the therapeutic usefulness of our current armamentarium.

Gram-negative bacteria are more difficult to inhibit or kill because of their outer membrane (OM) that serves as an additional layer of protection.[10] The OM is an asymmetric bilayer that limits or prevents the uptake of potentially noxious compounds into the bacterial cell, especially molecules

that are hydrophobic and/or have high molecular weight.[11] Thus, most antibiotics that are active against Gram-positive bacteria are inactive against Gram-negative bacteria because of their inability to traverse the OM. Furthermore, the physicochemical requirements for a molecule to traverse the OM and inner membrane of Gram-negative bacteria are orthogonal to each other.[12] To cross the OM, a molecule needs to be small and polar (for porin-mediated uptake) or polybasic (for a self-promoted uptake), whereas it needs to be hydrophobic to traverse the inner membrane.[11] Medicinal chemists often struggle with developing agents that can tightrope this dual-membrane topology, accumulate in the cell, and still be able to bind to a distinct target in the cytosol.[12,13] The frustrations expressed by the WHO is therefore apparent and it is clear that the priority one pathogens, which are all carbapenem-resistant (CR) Gram-negative ESKAPE[14] pathogens (i.e. CR *Acinetobacter baumannii*, CR *Pseudomonas aeruginosa*, and various CR *Enterobacteriaceae*), are major threats to public health. Of these pathogens, *P. aeruginosa* is even more difficult to inhibit or kill because of its sophisticated and high level of intrinsic resistance, such as its extremely low OM permeability and constitutively over-expressed efflux pumps of broad substrate specificities that actively extrude drug molecules from its periplasm/cytoplasm.[15] These, coupled with acquired and adaptive resistance mechanisms,[15] make *P. aeruginosa* resistant to many antibiotics that are active against other Gram-negative bacteria. As an opportunistic pathogen, *P. aeruginosa* is the leading cause of nosocomial infections such as respiratory infections in debilitated patients and patients with cystic fibrosis, and it is well known for its ability to evade antibiotic actions.[11,15]

Current thinking in the development of clinically relevant antipseudomonal agents include, but not limited to, developing agents that could potentially weaken or destabilize the OM, such that molecules that are otherwise unable to traverse this barrier can now gain access into the periplasm and/or cytoplasm.[16–19] Even for drugs with porin-mediated uptake mechanisms, OM destabilization ensures that the rate of drug influx overwhelms the rate of efflux thereby enhancing antibiotic accumulation. For instance, SPR741, a polymyxin-based adjuvant is being developed by

Spero Therapeutics as an OM permeabilizer that can facilitate the entry of OM-impermeable antibiotics into Gram-negative bacteria such as *A. baumannii* and *Enterobacteriaceae*. [20] Unfortunately, SPR741 is not effective against *P. aeruginosa*. [20] On the other hand, tobramycin-based heterodimeric scaffolds have been reported to potentiate several legacy antibiotics against *P. aeruginosa* than in other Gram-negative bacteria. [11,21] These two scenarios present intriguing dynamics about the physicochemical requirements for activity and antibiotic potentiation in *P. aeruginosa*. Hybridization of a second bioactive molecule to tobramycin via a tether resulted in conjugates that: i) preserve the original mode of action of the bioactive molecule, ii) abolish the ribosomal effect of tobramycin by itself, and iii) confer adjuvant properties on the resulting conjugate. [11,21] Tobramycin, an aminoglycoside antibiotic that interferes with the fidelity of ribosomal protein translation, propagates its own uptake into *P. aeruginosa* by displacing the stabilizing divalent cations that cross-bridge adjacent lipopolysaccharides on the outer leaflet of the OM. [22] This process is known as the ‘self-promoted uptake mechanism’ and the cationic nature of aminoglycosides is critical to their ability to induce this process.

To put all of these into perspective, we sought to expand the therapeutic usefulness of some OM-impermeable antibiotics by leveraging the self-promoted uptake mechanism of tobramycin to deliver them into Gram-negative bacteria, in a “Trojan-horse” fashion. For example, rifampicin, a large ($M_w = 822.9$ g/mol) hydrophobic molecule that binds to RNA polymerase and inhibit RNA synthesis, is active against Gram-positive bacteria and mycobacteria but inactive against most Gram-negative bacteria because of its inability to cross the OM. Unlike most classes of antibiotics, rifamycins are unique in that they are active against pathogens in slow growing, stationary, and non-replicating metabolic states. [23] Unfortunately, the greatest limitations of this class of drug is the rapid development of a single mutation in the β -subunit of bacterial RNA polymerase (*rpoB*), [24] and the lack of activity against Gram-negative pathogens. Surprisingly, some tobramycin-based conjugates that strongly potentiate the activity of rifampicin against *P. aeruginosa* were also shown to prevent the

development of resistance after 25 serial passages at sub MIC levels.[25] Hence, by directly conjugating rifampicin to a tobramycin scaffold using various tether lengths, we wanted to investigate the possibility of: 1) using tobramycin as a vector to shuttle rifampicin into *P. aeruginosa*, 2) preserving the original mode of action of rifampicin, i.e. RNA polymerase inhibition, in *P. aeruginosa*, 3) modulating the overall physicochemical property of the resulting conjugate such that it preserves or amplifies the known adjuvant properties of tobramycin-derived conjugates. The different tether lengths between the rifampicin and tobramycin domains were meant to investigate the optimal spatial separation between these two drug moieties. Herein, we report the synthesis and evaluation of a series of covalently-attached rifampicin–tobramycin molecules **1 – 3** (Figure 4.1). When hybridized as a single non-cleavable entity, the potency of rifampicin was preserved in some Gram-positive bacteria but was attenuated in *P. aeruginosa* (Table 4.1). However, the resulting conjugates **1 – 3** break the chromosomally-encoded intrinsic resistance of *P. aeruginosa* to doxycycline and chloramphenicol and significantly potentiate their activities against wild-type, multidrug- (MDR) and extensively drug-resistant (XDR) phenotypes *in vitro* and *in vivo*. An isolate is MDR if it is non-susceptible to at least one agent in at least three antimicrobial categories, while an XDR isolate is non-susceptible to at least one agent in all but two or fewer antimicrobial categories.[26] Typically, *P. aeruginosa* is clinically-resistant to doxycycline and chloramphenicol, but in the presence of < 10 µM of compounds **1 – 3**, the Clinical Laboratory Standard Institute (CLSI) susceptibility breakpoints (extrapolated: doxycycline ≤ 4 µg/ml for *Acinetobacter spp*, chloramphenicol ≤ 8 µg/ml for *Enterobacteriaceae*) were reached in 9 out of 10 phenotypes for doxycycline, and 7 out of 10 for chloramphenicol. This strategy expands the chemotherapeutic utility of doxycycline and chloramphenicol to *P. aeruginosa* and makes the pathogen susceptible to, and treatable by, these agents.

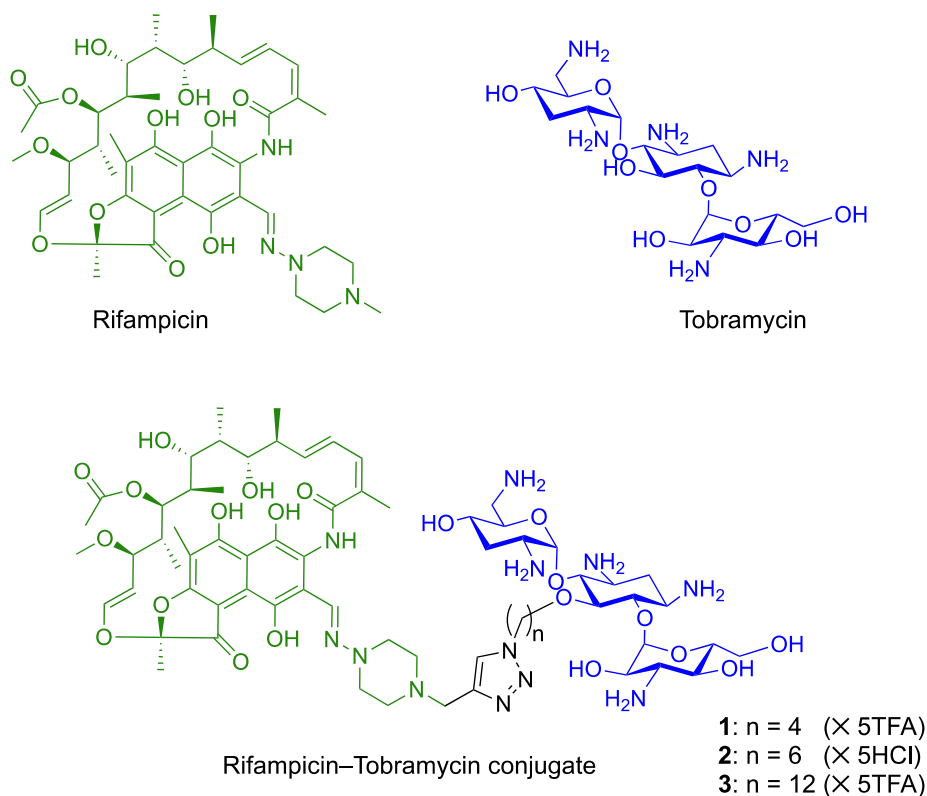


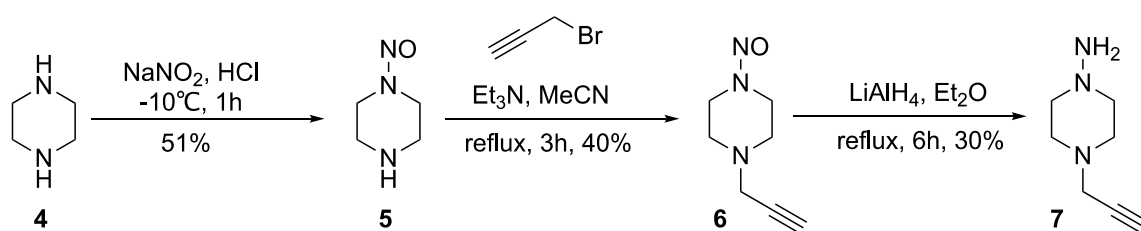
Figure 4.1. Structures of Rifampicin, Tobramycin, and newly synthesized Rifampicin-Tobramycin conjugates **1** – **3**. Conjugates differ in the length of carbon chains.

4.4 Results

4.4.1 Design and Synthesis

To design a covalently hybridized conjugate of two bioactive molecules, the point of attachment on both molecules must be carefully identified in order not to interfere with their biological activities. Tobramycin was conceptualized as a vector to deliver rifampicin into the periplasm, due to its self-promoted uptake mechanism, hence, its cationicity must be preserved. Rifampicin was intended to mediate its antibiotic activity in the cell; hence, the point of attachment must not be directly involved with its target. Therefore, the design of rifampicin-tobramycin conjugates was established from the known structure-activity relationships of the parent molecules. Amphiphilic tobramycins with lipophilic groups at the *C*-5 position have been shown to retain the self-promoted uptake

mechanism.[27,28] Solved crystal structure of RNA polymerase complexed with rifampicin revealed that the terminal piperazine ring of rifampicin is not involved in the binding of the drug to its target.[29] The drugs were therefore linked at these identified positions under a chemically benign reaction condition using aliphatic hydrocarbon of different lengths, i.e. C-4, C-6, and C-12. Rifampicin is hydrolyzed in both acidic and basic conditions, due to its imine and ester groups, respectively, thus, a copper(1)-catalyzed azide-alkyne cycloaddition reaction (“Click Chemistry”)[30] was employed to join both compounds together under neutral conditions. This afforded regioselective 1,4-disubstituted 1,2,3-triazole products **1** – **3** (Figure 4.1). To achieve this, an azide moiety was incorporated on the tether length of an amphiphilic tobramycin, while an alkyne group was installed on the piperazine ring of rifampicin. The tether length was meant to investigate the optimal spatial separation between the two domains. The synthetic strategy for preparing compounds **1** – **3** is outlined in Scheme 4.1, Scheme 4.2, and Scheme 4.3.



Scheme 4.1. Synthesis of 1-amino-4-propargylpiperazine.

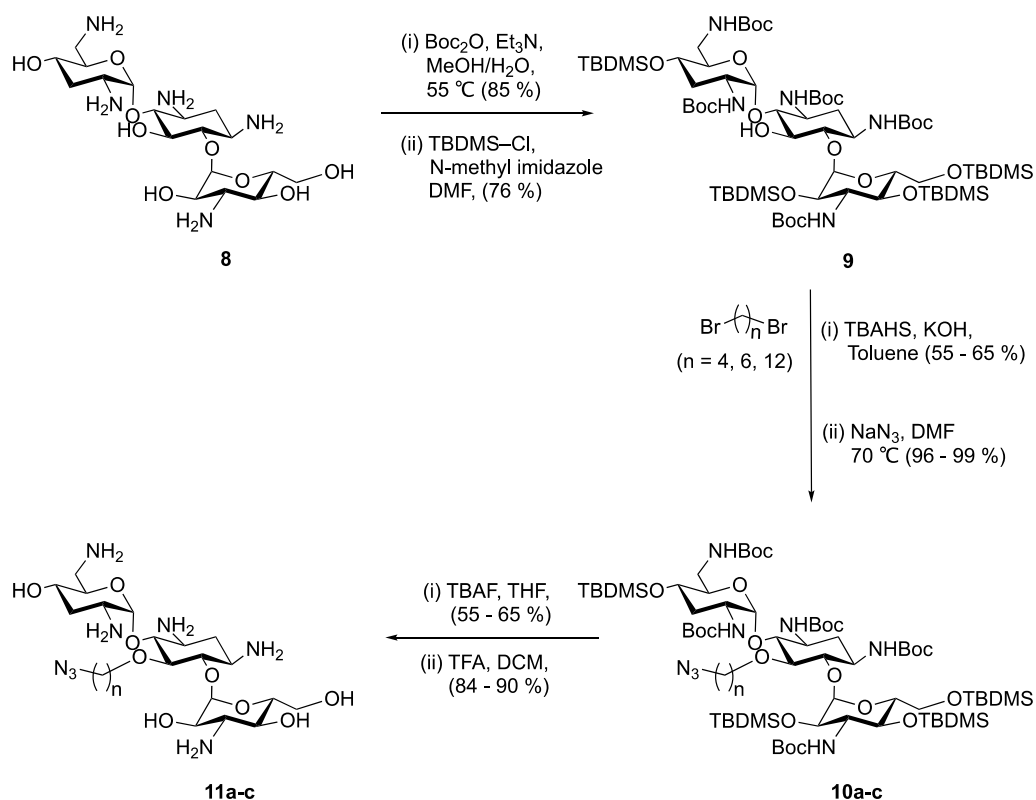
4.4.2 Chemical Synthesis of Rifampicin–Tobramycin Conjugates **1** – **3**

To install a propargyl group on rifampicin, a 1-amino-4-propargylpiperazine **7** precursor was synthesized (Scheme 4.1) and coupled to commercially available 3-formyl rifamycin SV (Scheme 4.3) following established procedure.[31] Briefly, **7** was prepared by converting one of the secondary amines in piperazine **4** to a nitroso- group under acidic conditions to give compound **5**, followed by a nucleophilic conjugation of the second secondary amine to propargyl bromide under basic conditions

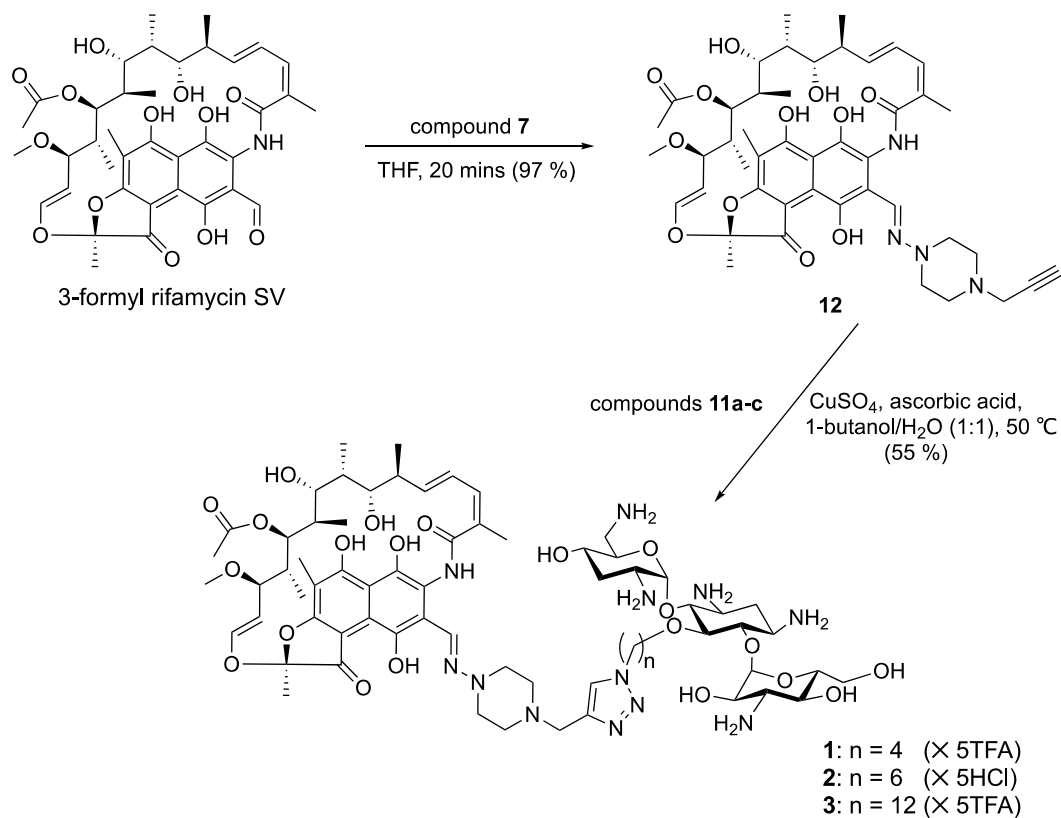
to give 1-nitroso-4-propargylpiperazine **6**. The nitroso group was then reduced to a primary amine in the presence of a strong reducing agent, LiAlH₄, to afford precursor **7** (Scheme 4.1). The amphiphilic tobramycin domain was prepared following previously reported protocol.[27] Tobramycin **8** was purchased from a commercial source and the amino groups were first protected using di-*tert*-butyl dicarbonate (Boc anhydride), followed by silylation of the *N*-Boc-tobramycin intermediate with excess TBDMS-Cl to afford a partially protected derivative **9** with free OH at the C-5 position of the cyclitol ring. Alkylation of **9** in toluene with 1,*n*-dibromoalkane (*n* = 4, 6, 12) in the presence of a phase-transfer catalyst (TBAHS) afforded bromoalkylated TBDMS-Boc-protected tobramycin intermediates. The terminal bromo-groups of these intermediates were then displaced by an azido nucleophile under anhydrous condition to give compounds **10a-c**. The protecting groups were finally removed in a stepwise manner, first by removing TBDMS groups using TBAF, and then *Boc*-protecting groups using TFA, to afford compounds **11a-c** (Scheme 4.2). Rifampicin-tobramycin conjugates **1 – 3** were ligated by coupling **7** to 3-formyl rifamycin SV to give **12**, followed by a “click chemistry” conjugation to **11a-c** under neutral conditions to afford the final compounds (Scheme 4.3). The final compounds were retained on C18 reverse-phase silica in a column and washed with copious amount of deionized water to remove residual copper ions.

4.4.3 Antimicrobial Susceptibility Screening

The antibacterial activities of the newly synthesized conjugates **1 – 3** and reference compounds, rifampicin and tobramycin, were assessed against a panel of Gram-positive and Gram-negative bacteria following the CLSI guidelines. These results are presented as the minimum inhibitory concentrations (MICs) in table 1. As expected, rifampicin by itself was potent against several Gram-positive bacteria (MIC = <0.25 – 1 µg/ml) but not against Gram-negative bacteria (MIC ≥ 8 µg/ml) while tobramycin exhibits potency against most Gram-positive and Gram-negative bacteria. By design, the rifampicin-tobramycin conjugates **1 – 3** are expected to lose the protein translation



Scheme 4.2. Synthesis of n-Azido Amphiphilic Tobramycins **11a-c**.



Scheme 4.3. Synthesis of Rifampicin-Tobramycin conjugates **1 - 3**.

inhibitory effect of tobramycin but retain the RNA polymerase inhibitory properties of rifampicin, as previously reported.[23] Tobramycin conjugated in this fashion are generally non-ribosomal,[21,25,28] hence, it was designed to only shuttle rifampicin into Gram-negative bacteria. Table 4.1 shows that compounds **1** – **3** exhibit slightly potent activity against some Gram-positive bacteria (MIC of 1 – 8 µg/ml) but were generally not potent against Gram-negative bacteria (MIC > 32 µg/ml). This suggests that whereas the conjugates might have retained the RNA polymerase effect of rifampicin against Gram-positive bacteria, they are unable to mediate the same effect in Gram-negative bacteria. The antipseudomonal activities of these conjugates were further assessed against a panel of MDR/XDR phenotypes and the results were consistent with the poor activity against Gram-negative bacteria (Table S1). Against Gram-positive bacteria, compound **1** with the shortest tether length (C-4 aliphatic hydrocarbon chain) appears to be more potent than compound **3** with the longest tether length (C12 chain).

4.4.4 Combination Studies of Rifampicin–Tobramycin conjugates with different classes of antibiotics against wild-type *P. aeruginosa*

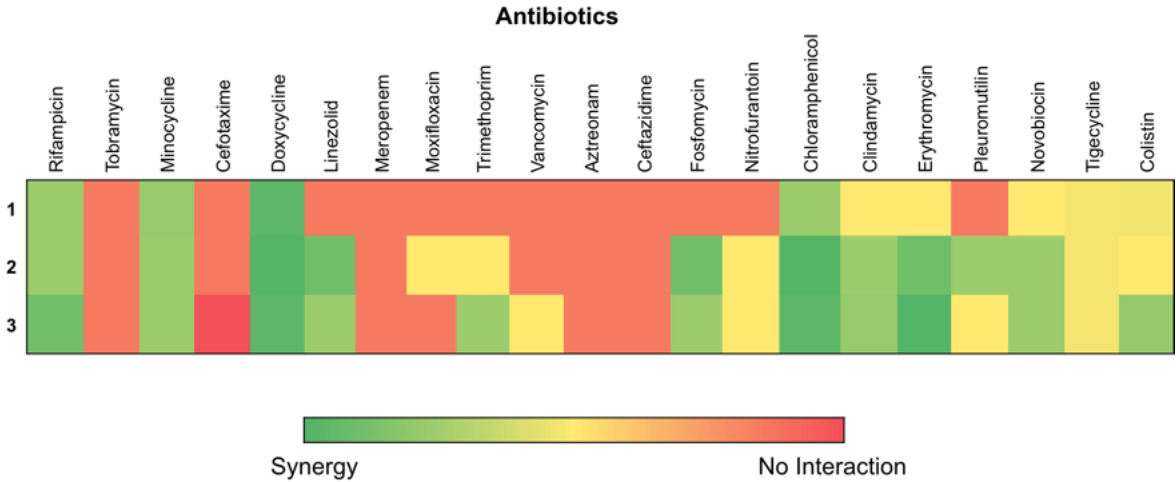
To investigate the impact of hybridizing a highly hydrophobic rifampicin moiety to a richly hydrophilic and polybasic tobramycin domain, the adjuvant properties of the resulting compounds were evaluated against *P. aeruginosa*. Physicochemical modulation of tobramycin-based conjugates is believed to be crucial to the nature, type, and degree of potentiation of different antibiotics.[11] Thus, we assessed the interactions between compounds **1** – **3** and twenty-one different antibiotics (representing all major classes) against wild-type *P. aeruginosa* PAO1 using checkerboard assay (Table S2). Data from this study were interpreted as a function of the fractional inhibitory concentration index (FICI), a numerical quantification of the interactions between antimicrobial agents. FICI of ≤ 0.5 , $> 0.5 - 4$, and > 4 indicate synergy, additive or no interaction, and antagonism, respectively.[11,21] Surprisingly, rifampicin-tobramycin conjugates **1** – **3** were able to potentiate the

Table 4.1. Minimum inhibitory concentrations (MICs, µg/ml) of Rifampicin, Tobramycin, and compounds **1** – **3** against a panel of Gram-positive and Gram-negative bacteria. [#]CANWARD; *CAN-ICU

| Test organism | Rifampicin | Tobramycin | Rifampicin–Tobramycin conjugates | | |
|---|------------|------------|----------------------------------|----------|----------|
| | | | 1 | 2 | 3 |
| <i>S. aureus</i> ATCC 29213 | ≤0.25 | 0.5 | 4 | 16 | 16 |
| MRSA ATCC 33592 | >128 | 0.5 | >32 | >32 | >32 |
| MSSE 81388 [#] | ≤0.25 | ≤0.25 | 2 | 4 | 4 |
| MRSE 61589 (CAZ >32)* | ≤0.25 | 2 | 2 | 4 | 8 |
| <i>E. faecalis</i> ATCC 29212 | 1 | 8 | >32 | >32 | >32 |
| <i>E. faecium</i> ATCC 27270 | ≤0.25 | 16 | 32 | >32 | >32 |
| <i>S. pneumoniae</i> ATCC 49619 | ≤0.25 | 2 | 1 | 2 | 8 |
| <i>E. coli</i> ATCC 25922 | 8 | 0.5 | >32 | >32 | 16 |
| <i>E. coli</i> 61714 (GEN-R)* | 8 | 8 | >32 | >32 | >32 |
| <i>E. coli</i> 63074 (AMK 32)* | 16 | 8 | >32 | >32 | 32 |
| <i>E. coli</i> 97615 (GEN-R, TOB-R, CIP-R) [#] | 16 | 128 | >32 | >32 | 32 |
| <i>P. aeruginosa</i> ATCC 27853 | 32 | 1 | >32 | >32 | >32 |
| <i>P. aeruginosa</i> 62308 (GEN-R)* | 32 | 16 | >32 | >32 | >32 |
| <i>P. aeruginosa</i> 96846 (GEN-R, TOB-R) [#] | 32 | 256 | >32 | >32 | >32 |
| <i>S. maltophilia</i> 62584* | 32 | >512 | >32 | >32 | >32 |
| <i>A. baumannii</i> 63169* | 8 | 32 | >32 | >32 | >32 |
| <i>K. pneumoniae</i> ATCC 13883 | 16 | ≤0.25 | >32 | >32 | >32 |

effects of several antibiotics against WT PAO1, including rifampicin, but not tobramycin or β -lactams (Figure 4.2a, Table S2). Unlike in susceptibility screening against Gram-positive bacteria, conjugates **2** and **3** with longer tether lengths were better potentiators than compound **1** with four carbon chain length (Figure 4.2b). Compounds **2** and **3**, at $\leq 10 \mu\text{M}$, potentiated rifampicin ($\text{FICI} = 0.15 - 0.28$), tetracyclines ($\text{FICI} = 0.06 - 0.50$), chloramphenicol ($\text{FICI} = 0.06 - 0.09$), fosfomycin ($\text{FICI} = 0.15 - 0.28$), linezolid ($\text{FICI} = 0.15 - 0.28$), novobiocin ($\text{FICI} = 0.28$), erythromycin ($\text{FICI} = 0.06 - 0.15$), etc. but not tobramycin ($\text{FICI} = 1.03$), β -lactams ($\text{FICI} = 1.03$), moxifloxacin ($\text{FICI} = 0.53 - 1.03$), and nitrofurantoin ($\text{FICI} = 0.53$) (Figure 4.2a, Table S2). Doxycycline ($\text{FICI} = 0.06 - 0.09$) and chloramphenicol ($\text{FICI} = 0.06 - 0.09$) were exceptionally potentiated by compounds **2** and **3** against PAO1, hence, these combinations were further investigated against highly resistant phenotypes of *P. aeruginosa*. It should be noted that doxycycline and chloramphenicol are typically not used to treat *P. aeruginosa* infections because of poor susceptibility due to intrinsic resistance.

a)



b)

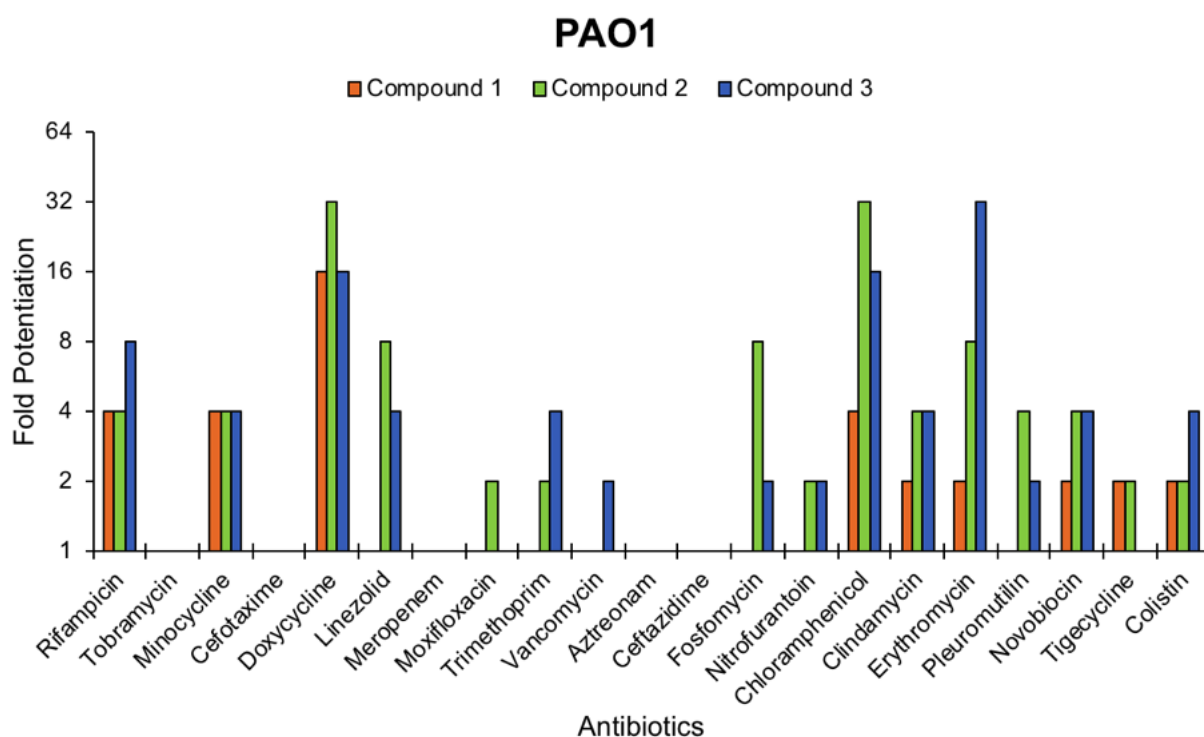


Figure 4.2. (a) Interactions of compounds **1** – **3** (at $\leq 10 \mu\text{M}$, i.e. 2 -16 $\mu\text{g/ml}$) with different antibiotics against WT *P. aeruginosa* PAO1. FIC < 0.4 = Green; FIC ≥ 0.5 but < 0.6 = Yellow; FIC > 0.6 but < 3 = Red, (b) Fold potentiation of several classes of antibiotics by rifampicin-tobramycin conjugates **1** – **3** (at $\leq 10 \mu\text{M}$) against WT *P. aeruginosa* PAO1

4.4.5 Rifampicin–Tobramycin conjugates strongly potentiate doxycycline and chloramphenicol against multidrug and extensively drug resistant *P. aeruginosa*

A combination of doxycycline or chloramphenicol with compounds **2** or **3** were further assessed against a panel of highly drug-resistant *P. aeruginosa* phenotypes. Most of these isolates are neither susceptible to doxycycline nor chloramphenicol, and are mostly resistant to carbapenems, the drugs of last resort (Table S3). Remarkably, compounds **2** and **3** significantly potentiated the effects

of doxycycline and chloramphenicol against these clinical isolates (Table 4.2). For instance, in the presence of $\leq 10 \mu\text{M}$ of compounds **2** and **3**, susceptibility of *P. aeruginosa* clinical isolates to doxycycline was increased by 4- to 512-fold while susceptibility to chloramphenicol was increased by 4- to 256-fold (Figure S1). These effects were generally dose-dependent against WT and clinical isolates (Figure 4.3), suggesting an involvement of a common mechanism in both phenotypes. On the contrary, the degree of potentiation of doxycycline and chloramphenicol were not as pronounced (nil to 8-fold) in other Gram-negative bacteria (Figure S2), suggesting a strong antipseudomonal potentiating effect for **2** and **3**.

To put these findings into context, extrapolated CLSI susceptibility breakpoints were used as interpretive standards for doxycycline and chloramphenicol since they are not conventional antipseudomonal agents. Breakpoints are discriminatory antimicrobial concentrations used in the interpretation of susceptibility testing to define isolates as susceptible, intermediately-resistant, or resistant. If the MIC of an antibiotic against an organism is less than or equal to its susceptibility breakpoint, the bacterial strain is considered to be susceptible to the antibiotic. The CLSI clinical breakpoint of doxycycline for *Acinetobacter* spp. is $4 \mu\text{g/ml}$ while that of chloramphenicol for *Enterobacteriaceae* is $8 \mu\text{g/ml}$. [32] Susceptibility equal or below these breakpoints were reached for doxycycline in wild-type and eight out of nine clinical isolates, and for chloramphenicol in wild-type and six out of nine clinical isolates (Table 4.2). CLSI clinical breakpoints could not be reached for both antibiotics in other resistant Gram-negative pathogens such as *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Enterobacter cloacae* in the presence of $10 \mu\text{M}$ of compounds **2** and **3** (Table S4). It is noteworthy that tobramycin alone, rifampicin alone, or a combination of both do not potentiate doxycycline or chloramphenicol against any of the *P. aeruginosa* phenotype used in this study (Table S5).

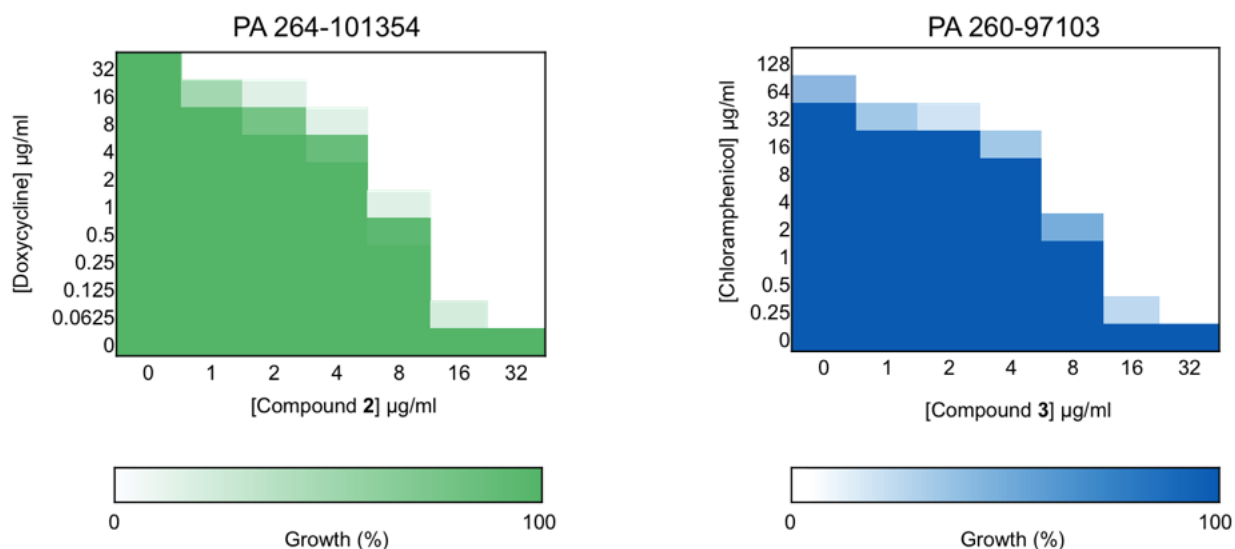


Figure 4.3. Representative checkerboard broth microdilution assays showing dose-dependent potentiation of doxycycline and chloramphenicol in two MDR/XDR *P. aeruginosa* clinical isolates by compounds **2** and **3**. Dark colours represent higher cell density (OD measured at 590 nm); 16 µg/ml of compounds **2** and **3** = 9.8 µM and 7.6 µM, respectively.

4.4.6 Potentiation of doxycycline and chloramphenicol in *P. aeruginosa* is efflux pump-dependent

A major contributor to the intrinsic resistance of *P. aeruginosa* to doxycycline and chloramphenicol is their highly efficient efflux mechanisms, as does the low permeability of the OM. To accumulate in cells, compounds must traverse the OM (or porins) faster than they are pumped out. The MexAB/MexXY efflux systems in *P. aeruginosa* contribute significantly to the extrusion of tetracyclines out of the cell while resistance to chloramphenicol is in part due to the MexAB-OprM efflux system.[33] To investigate the role of these pumps in the ability of compound **2** to increase the susceptibility of *P. aeruginosa* to doxycycline and chloramphenicol in such magnitude, we assessed the synergistic relationships of these combinations in two mutant strains, PAO200 and PAO750. PAO200 is a *mexA-mexB-oprM* deletion strain while PAO750 lacks five clinically important RND

Table 4.2. Synergistic effects of 16 µg/ml each of compounds **2** (9.8 µM) and **3** (7.6 µM) with doxycycline (DOX) and chloramphenicol (CAM) against MDR/XDR *P. aeruginosa* clinical isolates. MICs are reported in µg/ml. In the presence of < 10 µM of compounds **2** and **3**, CLSI breakpoints (DOX = ≤ 4 µM; CAM = ≤ 8 µM) were reached in eight out of nine clinical isolates for doxycycline and six out of nine for chloramphenicol.

| Strain | Antibiotic (MIC) | Conjugate (MIC) | MIC _{Antibiotic} in the presence of < 10 µM of conjugate | FICI |
|-----------|------------------|-----------------|---|--------|
| PA 100036 | DOX (64) | 2 (256) | 1 | 0.078 |
| | | 3 (256) | 0.5 | 0.070 |
| | CAM (1024) | 2 (256) | 4 | 0.066 |
| | | 3 (256) | 4 | 0.067 |
| PA 264 | DOX (64) | 2 (256) | 0.125 | 0.064 |
| | | 3 (256) | 1 | 0.078 |
| | CAM (4096) | 2 (256) | 128 | 0.063 |
| | | 3 (256) | 128 | 0.063 |
| PA 101243 | DOX (4) | 2 (256) | 0.25 | 0.125 |
| | | 3 (256) | 0.125 | 0.094 |
| | CAM (1) | 2 (256) | 0.125 | 0.187 |
| | | 3 (256) | 0.0625 | 0.125 |
| PA 262 | DOX (1024) | 2 (>256) | 8 | <0.070 |
| | | 3 (>256) | 8 | <0.070 |
| | CAM (2048) | 2 (>256) | 128 | <0.125 |
| | | 3 (>256) | 128 | <0.125 |
| PA 101885 | DOX (64) | 2 (256) | 1 | 0.078 |
| | | 3 (256) | 2 | 0.094 |
| | CAM (512) | 2 (256) | 8 | 0.078 |
| | | 3 (256) | 4 | 0.070 |
| PA 259 | DOX (32) | 2 (>128) | 1 | <0.156 |
| | | 3 (>128) | 0.25 | <0.133 |
| | CAM (1024) | 2 (>128) | 256 | <0.375 |
| | | 3 (>128) | 512 | <0.625 |
| PA 260 | DOX (16) | 2 (>128) | 0.125 | <0.133 |
| | | 3 (>128) | 0.125 | <0.133 |

| | | | | |
|-----------|-----------|-----------------|------|--------|
| | CAM (128) | 2 (>128) | 2 | <0.141 |
| | | 3 (>128) | 0.5 | <0.129 |
| PA 91433 | DOX (32) | 2 (128) | 4 | 0.250 |
| | | 3 (128) | 0.25 | 0.133 |
| | CAM (8) | 2 (128) | 2 | 0.375 |
| | | 3 (128) | 1 | 0.250 |
| PA 114228 | DOX (32) | 2 (>128) | 1 | <0.156 |
| | | 3 (>128) | 2 | <0.187 |
| | CAM (64) | 2 (>128) | 16 | <0.281 |
| | | 3 (>128) | 8 | <0.156 |

pumps (MexAB–OprM, MexCD–OprJ, MexEF–OprN, Mex JK, and MexXY) and the OM protein OpmH.[34,35] As expected, the efflux-mutant strains were hypersusceptible to both doxycycline and chloramphenicol (Table 4.3), consistent with known contributions of RND efflux pumps to the intrinsic resistance to these agents.[36] However, at a fixed concentration of compound **2**, its ability to potentiate doxycycline and chloramphenicol was diminished in efflux-deficient mutants compared to the wild-type strain (Table 4.3). For instance, compound **2** (at 9.8 μ M) increased the susceptibility of WT PAO1 to doxycycline by 32-fold while an increase in susceptibility of 8- and 4-fold was observed in PAO200 and PAO750, respectively. The same phenomenon was observed for the potentiation of chloramphenicol (Table 4.3 and Figure S3). Even between the two mutants, the test antibiotics were more potentiated in PAO200 which is deficient in only one of its pumps, compared to PAO750 which lacks five different pumps. This indicates that interaction with and/or inhibition of RND efflux pumps play an important role in the degree of potentiation seen in PAO1 but, expectedly, not in efflux-deficient mutants. However, inhibition of efflux pumps does not explain the partial increase in susceptibilities of the efflux mutants, suggesting that other mechanisms such as OM permeabilization might concomitantly be at play. OM permeabilization is indeed consistent with the ability of these conjugates to potentiate some OM-impermeable antibiotics such as rifampicin, erythromycin, etc. Overall, interaction with RND efflux pumps seems to play a role in the ability of compound **2** to

potentiate doxycycline and chloramphenicol, and strains with higher levels of expression will likely be more susceptible to these combinations.

Table 4.3. Potentiation of doxycycline and chloramphenicol in *P. aeruginosa* by compound **2** (≤ 9.8 μ M) is dependent on RND efflux pumps. PAO1 = wild-type, PAO200 and PAO750 are efflux-deficient mutants. MICs are reported in μ g/ml.

| Antibiotics | MIC of antibiotic alone in | | | MIC of antibiotic (fold change) + Compound 2 in | | |
|-----------------|----------------------------|--------|--------|---|------------|-----------|
| | PAO1 | PAO200 | PAO750 | PAO1 | PAO200 | PAO750 |
| Doxycycline | 16 | 0.5 | 0.125 | 0.5 (32) | 0.063 (8) | 0.031 (4) |
| Chloramphenicol | 32 | 1 | 0.5 | 1 (32) | 0.063 (16) | 0.125 (4) |

4.4.7 Rifampicin–Tobramycin conjugate potentiates rifampicin and other members of tetracycline against MDR/XDR *P. aeruginosa*

To ascertain whether the ability of rifampicin-tobramycin conjugates to potentiate doxycycline is conserved across the entire class of tetracyclines, synergistic relationships between compound **2** and minocycline or tigecycline were evaluated against wild-type and MDR/XDR *P. aeruginosa*. Results of this study show that minocycline and tigecycline were similarly potentiated as doxycycline but to a lesser degree (Figure 4.4, Table S6). The degree of potentiation of tetracyclines is: doxycycline > minocycline > tigecycline (Figure 4.4), an observation that seems to correlate with the structure-activity relationships of tetracyclines. This variation is perhaps due to the fact that tigecycline evades acquired efflux and target-mediated resistance to classical tetracyclines[37] but remains vulnerable to the chromosomally-encoded multidrug efflux pumps of *P. aeruginosa*. [38] Thus, inhibition of acquired efflux pumps by compound **2** will have lesser consequential effects on tigecycline than it will on doxycycline. Rifampicin, an OM-impermeable antibiotic, was also potentiated against wild-type

and MDR/XDR *P. aeruginosa* isolates (Figure 4.4, Table S6), suggesting a role for OM permeabilization as part of the mechanism of potentiation by compound **2**. Rifampicin is not a substrate for RND efflux pumps in *P. aeruginosa* (Table S1).

4.4.8 Time-kill Assay

Time-kill curves can be used to monitor bacterial growth and death over a wide range of antimicrobial concentrations to evaluate the effect of antimicrobials over time. An antibiotic could either be bactericidal or bacteriostatic under specific growth conditions. Bactericidal activity is defined as a ≥ 3 -log reduction in the total CFU/mL from the original inoculum over 24 hours while bacteriostatic activity is defined as maintenance of < 3 -log reduction in the total CFU/mL from the original inoculum.[32] Thus, the time-kill kinetics of wild-type *P. aeruginosa* PAO1 when incubated

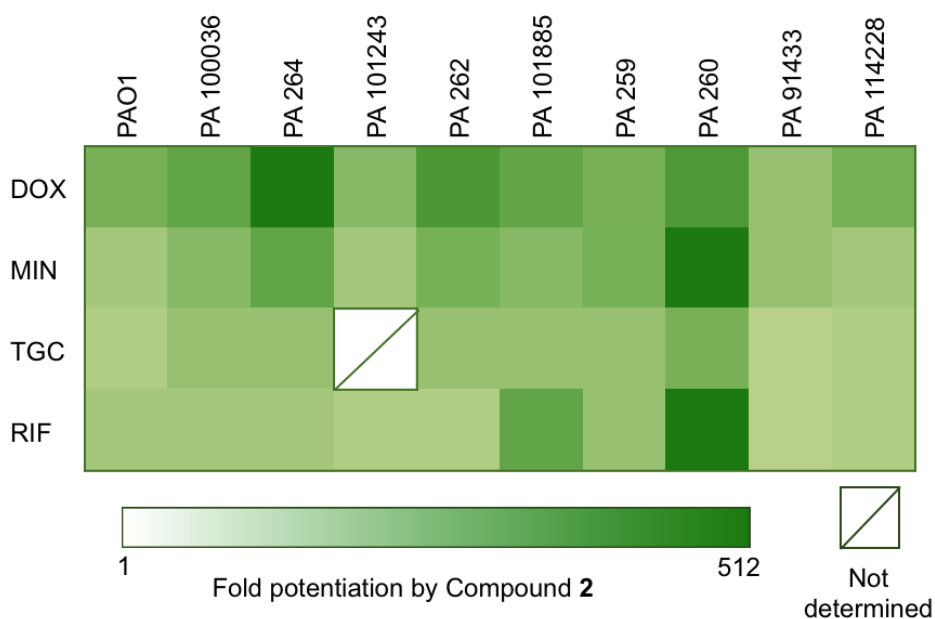


Figure 4.4. Fold change in susceptibilities of wild-type and MDR/XDR *P. aeruginosa* to doxycycline (DOX), minocycline (MIN), tigecycline (TGC), and rifampicin (RIF) in the presence of $\leq 9.8 \mu\text{M}$ of compound **2**.

with doxycycline or chloramphenicol alone, or in combination with compound **2**, was assessed. Preliminary growth curve was performed to ensure that PAO1 could be grown from a starting inoculum of about 10^6 CFU/ml under our assay conditions, and that pre-incubation in antimicrobial-free LB medium will reach a stable early- to mid-log phase after 4 h. The time-kill curves of doxycycline (at 32 μ g/ml, i.e. $2\times$ MIC) and chloramphenicol (at 32 μ g/ml, i.e. $1\times$ MIC) looked similar, with almost no killing of bacteria within the assay period (Figure 4.5). Bacterial growth was completely inhibited by both drugs at these concentrations, an effect that is consistent with known bacteriostatic properties of tetracyclines (doxycycline) and chloramphenicol.[39] When PAO1 was incubated with sub MICs of 4 μ g/ml doxycycline alone or 8 μ g/ml chloramphenicol alone, i.e. their respective extrapolated CLSI breakpoints, there was bacterial growth as early as 3 h, similar to the control without antibiotics (Figure 4.5). However, in the presence of 4.9 μ M or 9.8 μ M of compound **2**, doxycycline became strongly bactericidal and chloramphenicol became weakly bactericidal at their respective CLSI breakpoints. Indeed, a combination of 4 μ g/ml of doxycycline and 9.8 μ M of compound **2** reduced the viability of *P. aeruginosa* PAO1 below the limits of detection (< 10 CFU/mL) after 9 h of incubation (Figure 5). Synergistic effect is defined as ≥ 2 -log decrease in the number of CFU/mL between the combination and the most active component of the combination after 24 h (at least one of the drugs must be present at a concentration that does not affect the growth curve of the test organism).[40] It is clear that compound **2** exhibited synergistic relationships with both doxycycline and chloramphenicol against *P. aeruginosa* PAO1 in a dose-dependent manner and there was no re-growth after 24 h of incubation.

4.4.9 *In Vivo* Efficacy Studies using *Galleria mellonella* Infection Model

In vitro synergy of many antibiotic/antibiotic or antibiotic/adjuvant combinations against Gram-negative bacteria have been shown to be often incongruent with *in vivo* synergy.[41,42] To investigate whether the *in vitro* synergy observed with compound **2** and doxycycline or chloramphenicol is translated *in vivo*, we examined the ability of different combination concentrations

to offer therapeutic protection against MDR *P. aeruginosa*-infected *Galleria mellonella* wax moths. The capability of this infection model to determine virulence of *P. aeruginosa* strains as well as

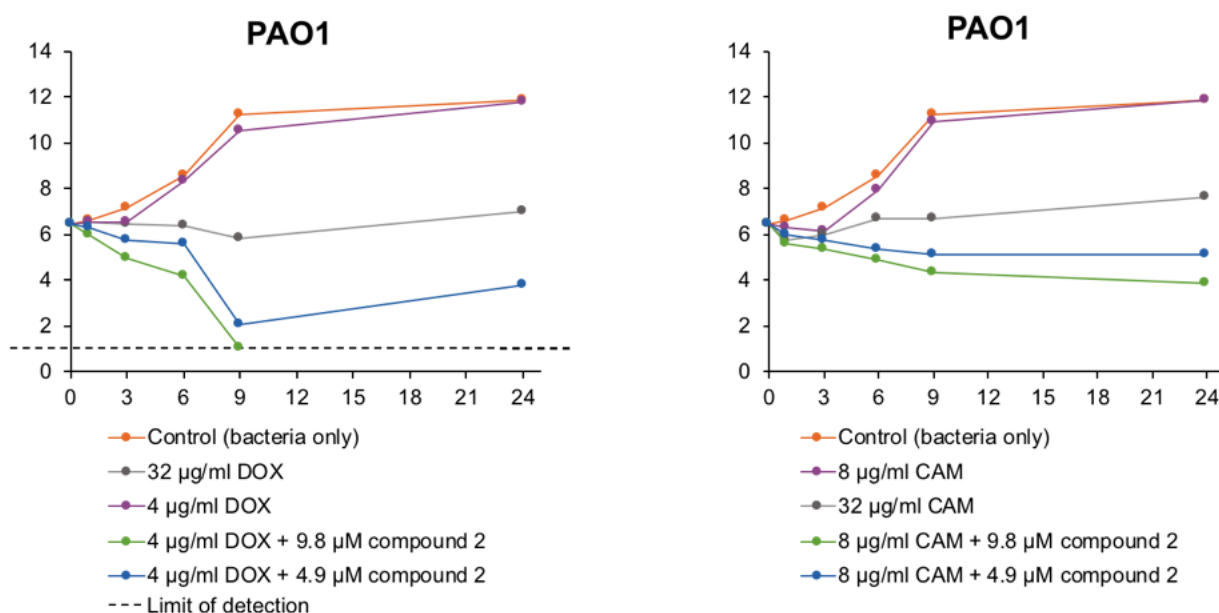
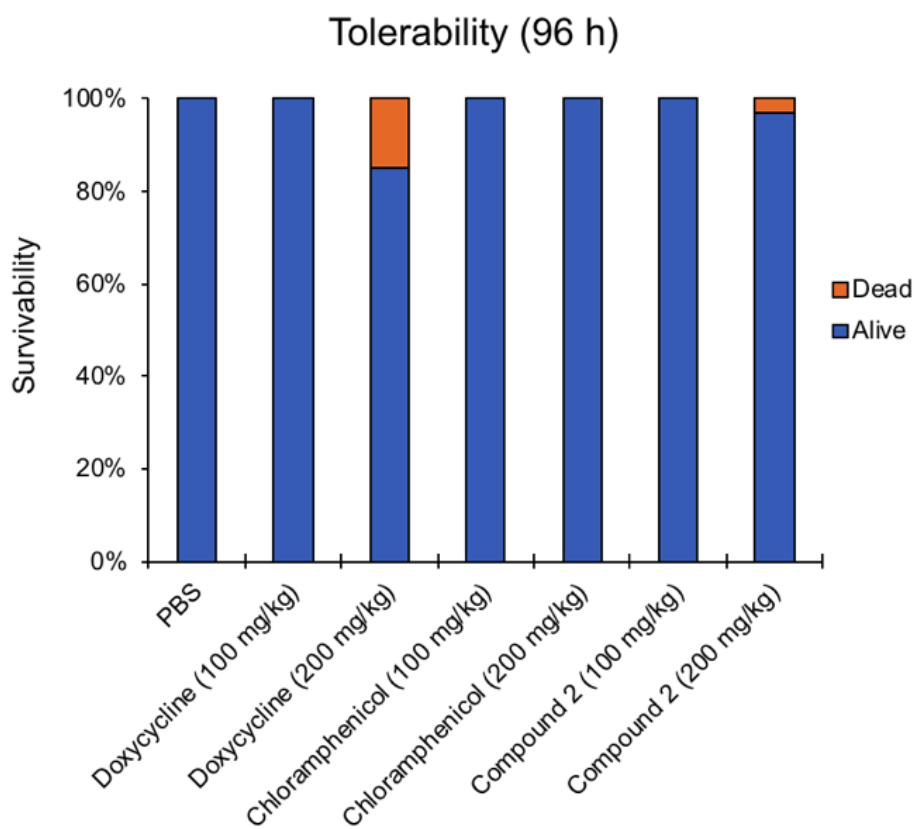


Figure 4.5. Time-kill kinetics of doxycycline (DOX) and chloramphenicol (CAM), alone and in combination with different concentrations of compound **2**, on the viability of wild type *P. aeruginosa* PAO1 grown in LB media. MICs of DOX and CAM are 16 µg/ml and 32 µg/ml, respectively. CLSI susceptibility breakpoint of DOX (against *Acinetobacter spp*) and CAM (against *Enterobacteriaceae*) are 4 µg/ml and 8 µg/ml, respectively. Colony-forming units (CFU) of PAO1 were reduced below the limits of detection when incubated with 4 µg/ml DOX and 9.8 4 µM of compound **2**. Each data point is an average of three independent determinations.

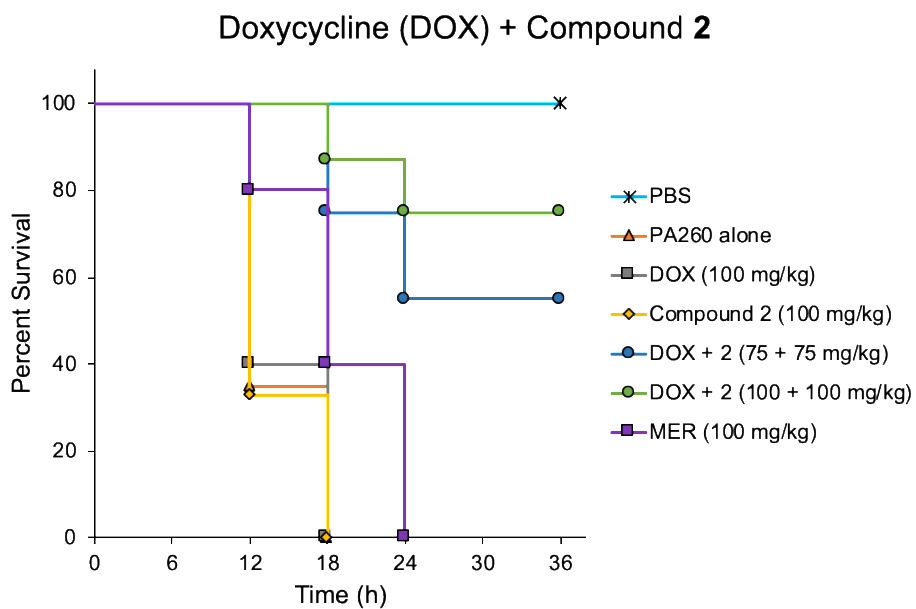
to offer therapeutic protection against MDR *P. aeruginosa*-infected *Galleria mellonella* wax moths. The capability of this infection model to determine virulence of *P. aeruginosa* strains as well as efficacy and pharmacokinetics of antipseudomonal agents have been widely demonstrated.[11,21,25,42,43] The maximum tolerable dose was first determined by injecting high concentrations of doxycycline, chloramphenicol, and compound **2** (100 mg/kg and 200 mg/kg each)

into the larvae and survivability was scored for 96 h (4 days). 100 % survival was recorded in groups injected with 100 mg/kg doxycycline, 100 and 200 mg/kg chloramphenicol, and 100 mg/kg of compound **2**, while groups injected with 200 mg/kg each of doxycycline and compound **2** recorded 85 % and 97 % survival, respectively, after 4 days (Figure 4.6a). This indicates that the compounds were relatively non-toxic to the larvae by themselves, an effect that is consistent with other *in vitro* toxicity studies (*vide infra*). Also, an inoculum size of ~5 CFU of PA260, an extensively drug-resistant *P. aeruginosa* clinical isolate, achieved 100% lethality in the larvae after 18 h. Next, the ability of the drugs, alone and in combination, to protect the larvae from XDR PA260 3 h post infection was determined using single doses of 100 mg/kg each as monotherapy, or 75 + 75 mg/kg or 100 + 100 mg/kg as combination therapy. PA260 is expected to have reached a stable early- to mid-log phase in the larvae after 3 h of infection. Like the untreated control, 100 % mortality was observed in the monotherapy treatments with all drugs (Figure 4.6b). However, a single dose combination of doxycycline and compound **2** using 75 + 75 mg/kg and 100 + 100 mg/kg resulted in 75 % and 87 % survival, respectively after 18 h, and 55 % and 75 % survival, respectively after 24 h (Figure 4.6b). Similarly, a single dose combination of chloramphenicol and compound **2** using 75 + 75 mg/kg and 100 + 100 mg/kg resulted in 55 % and 72 % survival, respectively after 18 h, and 10 % and 45 %, respectively after 24 h (Figure 4.6b). This demonstrates the ability of compound **2** to synergize with doxycycline and chloramphenicol *in vivo* and offer therapeutic protection to PA260-infected larvae in a dose-dependent manner at tolerable concentrations. The higher level of survival seen with doxycycline + compound **2** relative to chloramphenicol + compound **2** is perhaps attributable to the susceptibility of PA260 to these agents. The absolute MIC (in the presence of compound **2**) of doxycycline against PA260 is 0.125 µg/ml while that of chloramphenicol is 2 µg/ml (Table 4.2). Colistin, which is the only antibiotic that PA260 is susceptible to besides amikacin (Table S3), served as the positive control for this experiment while treatment with PBS only served as negative control.

a)



b)



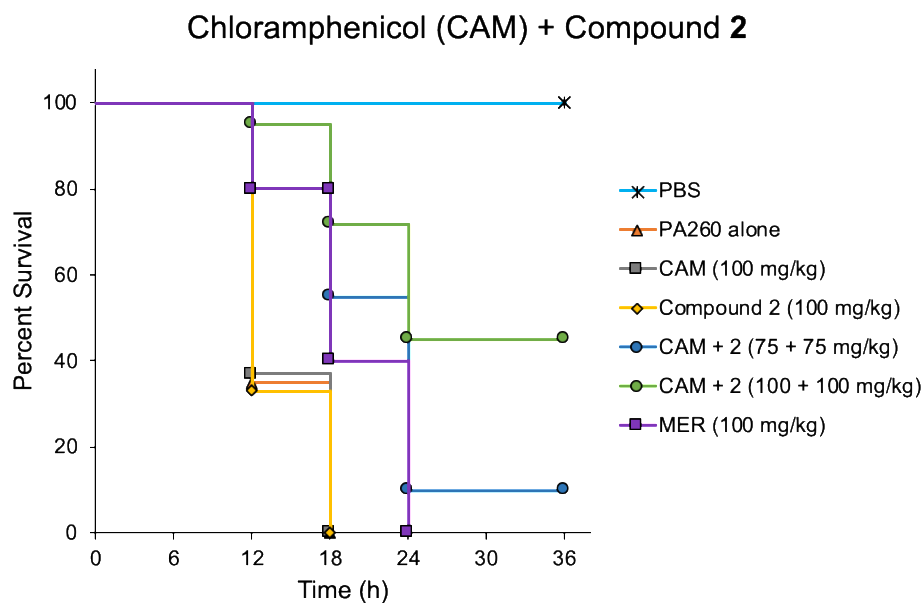
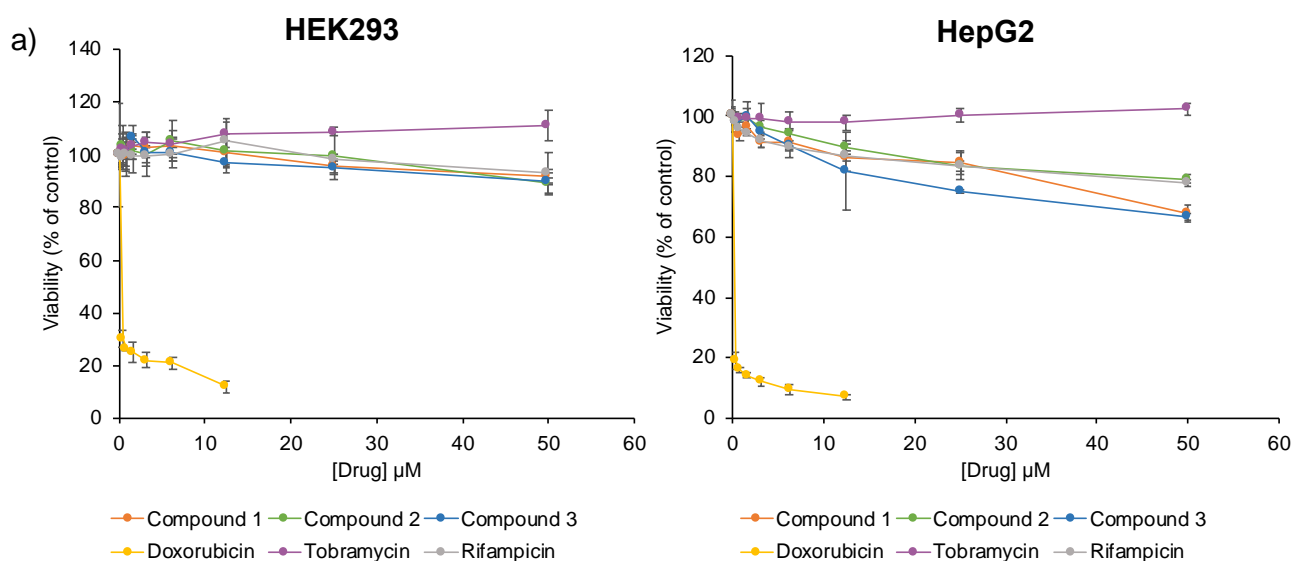


Figure 4.6. *In vivo* dose-dependent efficacy of a combination therapy of compound **2** and doxycycline (DOX) or chloramphenicol (CAM) demonstrated in *Galleria mellonella* infection model. a) Tolerable doses were determined by injecting 100 and 200 mg/kg of test compounds alone into the larvae and scored for survivability for 96 h (4 days). b) Efficacy studies using a single dose administration of different concentrations of mono- and combination therapies to treat PA260-challenged larvae 3 h post infection. Survivability of the larvae was scored every 6 h for 36 h.

4.4.10 Toxicity Studies

To evaluate *in vitro* toxicity and margin of therapeutic safety of compounds **1** – **3** against eukaryotic cells, they were screened against human liver (HepG2) and kidney (HEK293) cells, as well as porcine erythrocytes. The results of these studies were compared to the approved parent antibiotics, rifampicin and tobramycin. For cytotoxicity against human cells, doxorubicin, a very potent anticancer drug, was used as a positive control for this experiment. The results of the study showed that none of the rifampicin-tobramycin conjugates **1** – **3**, rifampicin, or tobramycin was toxic to HepG2 and HEK293 (Figure 4.7a). At the effective adjuvant concentration of $< 10 \mu\text{M}$ ($< 16 \mu\text{g/mL}$) used for this

study, cell viability was greater than 90 % for both cells, and greater than 70 % at the highest concentration tested (50 μ M, i.e. > 100 μ g/mL). Expectedly, doxorubicin reduced the viability of HepG2 and HEK293 cells to less than 10% at about 12 μ M (6.5 μ g/mL), consistent with its cytotoxic properties (Figure 4.7a). We also investigated the toxicity of the combination therapies against HepG2 and HEK293 cells (i.e. compound **2** + doxycycline and compound **2** + chloramphenicol) and found that the combination regimen did not elevate toxicological profiles of the primary antibiotics (Figure 4.7b). For toxicity against freshly collected porcine erythrocytes, 0.1% Triton X-100 served as the positive control and was used to calculate percent hemolysis. All the newly synthesized conjugates **1** – **3** exhibited insignificant hemolytic effects (< 2%) at very high concentrations of 1024 μ g/mL (Figure 4.7c), a 64-fold higher dose than the maximum synergistic concentration used in the study.



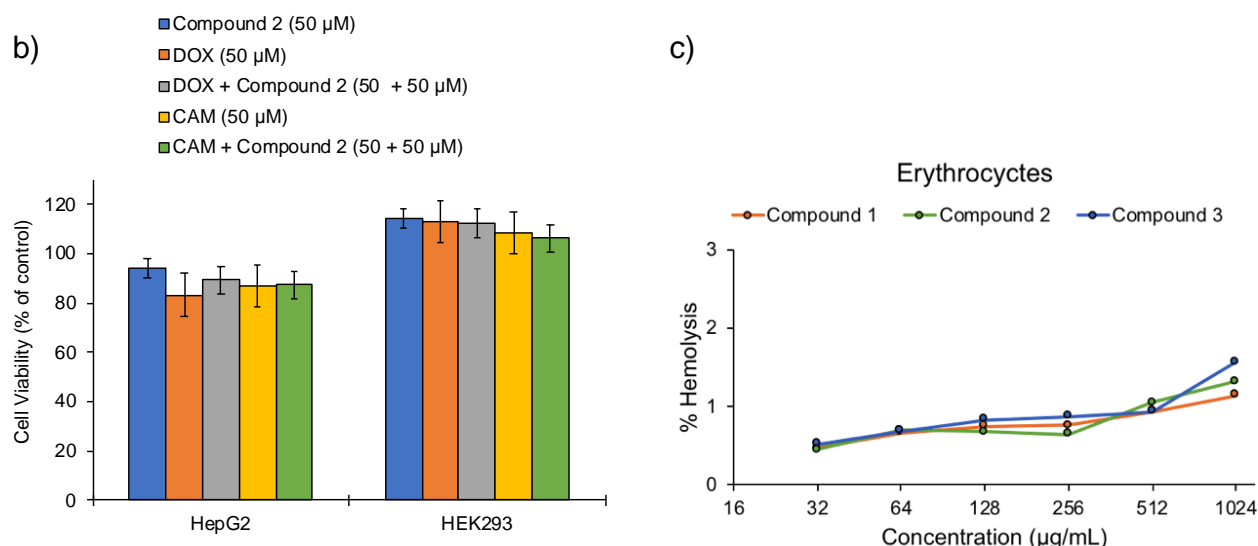


Figure 4.7. a) Cytotoxicity of compounds **1** – **3**, doxorubicin, tobramycin, and rifampicin against human liver carcinoma (HepG2) cells and human embryo kidney (HEK293) cells using PrestoBlue cell viability assay. Doxorubicin was used as positive control. Error bars denote standard deviation of at least four replicates. b) Cytotoxic evaluation of compound **2** + (doxycycline, DOX or chloramphenicol, CAM) combinations (at 50 μ M) against HepG2 and HEK293 cell lines. c) Hemolytic activity of compounds **1** – **3** evaluated against porcine erythrocytes at different concentrations. 0.1% Triton X-100 (100 % hemolysis) was used as positive control to calculate percent hemolysis. The result represents the mean of three independent determinations.

4.5 Discussion

The sustained and emerging resistance of recalcitrant pathogens to our antibiotic armamentarium constitutes an acute threat to public health and a cause for alarm. The growing gap between clinical needs and drug innovation is a direct consequence of the difficulty in identifying and bringing new drugs into market, especially against Gram-negative pathogenic bacteria. Intrinsic resistance in Gram-negative bacteria, such as their protective outer membrane and constitutively overexpressed efflux pumps, is a major survival weapon that renders them untreatable by most

antibiotics.[11] To fill the current void in antibacterial drug discovery against Gram-negative bacteria, a plausible strategy that has gained wide attention is the development of adjuvants that could either rescue/preserve the efficacy of available treatment options,[17] or expand the therapeutic usefulness of antibiotics that are not clinically indicated for such infections.[11,20,25] For example, tazobactam, avibactam, and varborbactam are adjuvants that have all been approved by FDA to rescue/preserve the efficacies of ceftolozane, ceftazidime, and meropenem, respectively, while membrane permeabilizing agents (e.g. SPR741) have been shown to be capable of expanding the therapeutic usefulness of OM-impermeable drugs (e.g. rifampicin) to Gram-negative bacteria.[11,25,44]

To cross the OM, a molecule must either pass through the porin (usually very small and polar, e.g. fluoroquinolones, β -lactams),[45] have an active transport mechanism, or induce a self-promoted uptake mechanism (could be big, e.g. colistin, aminoglycosides). Cationicity is critical for a self-promoted uptake mechanism across the OM and hydrophobicity facilitates penetration of membrane-active compounds across bacterial membrane. Consequently, amphiphilicity have often been employed to achieve the dual physicochemical requirement of electrostatic interactions and transmembrane navigation in cells.[13,46,47] Rifampicin–Tobramycin conjugates **1** – **3** were designed such that rifampicin could be shuttled into the periplasm of Gram-negative bacteria, especially *P. aeruginosa*, using tobramycin as a vector due its self-promoted uptake mechanism. The hydrophobic rifampicin domain may also facilitate uptake across the inner membrane. Based on earlier studies, tobramycin was expected to lose its ability to inhibit protein translation,[21,47] but rifampicin was expected to retain its activity.[29] A different group had previously used similar ‘Trojan-horse’ approach to deliver OM-impermeable erythromycin as an effective treatment of *Klebsiella pneumonia* *in vivo*. [31] The resulting conjugates **1** – **3** retained activity as standalone agents against some Gram-positive bacteria but not against Gram-negatives (Table 4.1). This observation is similar to that of a dual-acting rifampicin-ciprofloxacin hybrid, TNP-2092, a former drug candidate that was only active against Gram-positive bacteria.[23] Since conjugated tobramycins modified at the C-5 position are

completely inactive against Gram-positive and Gram-negative bacteria,[25] the activity of compounds **1** – **3** against some Gram-positive pathogens is most likely driven by the rifampicin domain, suggesting that ligation to its piperazine moiety do not significantly affect its biological activities. Previous SAR study of tobramycin-based compounds have shown that the second domain of this scaffold is amenable, and that the spectrum of activity, degree of potentiation, and type of antibiotics it potentiates can be altered by changing the second domain.[11] Thus, we investigated the physicochemical impact of hybridizing a hydrophobic rifampicin with a cationic hydrophilic tobramycin domain, as it relates to the ability to permeate bacterial membrane and potentiate legacy antibiotics. The conjugates (Figure 4.1) were found to potentiate different classes of Gram-positive-only antibiotics against *P. aeruginosa*, most especially doxycycline and chloramphenicol (Figure 4.2), while the individual parent molecules alone or in combination could not (Table S5). Compound **1** with the shortest tether was more active than **2** and **3** as standalone antibacterial agents, while compounds **2** and **3** were more potent than **1** as adjuvants. Indeed, compounds **1** – **3** potentiated antibiotics that are clinically not indicated for the treatment of *Pseudomonas* infections but did not potentiate typical antipseudomonal agents (Figure 4.2). This suggests a mechanism that involves the reversal of intrinsic resistance of *P. aeruginosa* to these agents, especially resistance to doxycycline and chloramphenicol.

P. aeruginosa is clinically resistant to tetracyclines and chloramphenicol due to its intrinsic and adaptive resistance mechanisms. Tetracyclines enter Gram-negative bacteria via a porin-mediated process and they inhibit the elongation phase of protein synthesis by blocking the association of aminoacyl tRNA with the bacterial ribosome.[48] On the other hand, chloramphenicol, which also prevents protein chain elongation by inhibiting the peptidyl transferase activity of bacteria ribosome,[49] is believed to be taken up into the cell via an energy-dependent process.[50] A major mechanism of resistance to these agents by *P. aeruginosa* is by actively extruding them out of the cell using its abundantly expressed efflux pumps.[48] The ability of compounds **2** and **3** to potentiate the effects of doxycycline and chloramphenicol were found to be dependent on efflux pumps (Table 4.3),

suggesting that mitigating the effects of these pumps, in addition to outer membrane permeabilization, might be responsible for the sensitization of *P. aeruginosa* to these agents. Outer membrane permeabilization is a known property of all tobramycin conjugates synthesized to date.[11,21,25] Agents that alter transmembrane protein environment (such as membrane charge, fluidity, and thickness) and/or steric hindrance of membrane-embedded proteins can prevent the relay of signaling cascades required to elicit conformational changes necessary to extrude substrate molecules by efflux pumps.[11] The perturbation of transmembrane efflux protein domains by compounds **2** and **3** via alteration of lipid composition surrounding the protein may, therefore, contribute to their ability to potentiate doxycycline and chloramphenicol in *P. aeruginosa*. Indeed, rapid influx and reduced efflux are simultaneous complementary processes necessary for enhanced drug bioaccumulation in bacteria cell.

Tetracyclines and chloramphenicol often act as model compounds for bacteriostatic effects,[50] and the association of tetracycline with the ribosome is reversible,[48] providing an explanation for the bacteriostatic nature of this class of drugs. The concentration-independent bacteriostatic effects of doxycycline and chloramphenicol against wild-type PAO1 were confirmed by growth curves that do not indicate any decrease in the number of CFU/mL from the initial inoculum at MIC and supra MIC levels (Figure 4.5). However, in the presence of compound **2**, *P. aeruginosa* was sensitized to doxycycline and chloramphenicol (Table 4.2) and their effects became bactericidal (Figure 4.5). The ability to switch the bacteriostatic effects of these drugs to bactericidal effects (in combination with compound **2**) in the same organism, under the same growth conditions, is quite remarkable. Given the lack of antimicrobial activity of compounds **2** and **3** against Gram-negative bacteria (Table 4.1 and S1), the mechanism of synergistic relationships with doxycycline and chloramphenicol is not immediately obvious. It is possible that compounds **2** and **3** disrupt the OM of *P. aeruginosa*, thus, enhancing bioaccumulation of doxycycline and chloramphenicol in the cytosol. However, this does not fully explain the bactericidal effects of these combinations, given that very high concentrations of

tetracyclines and chloramphenicol are often bacteriostatic against *P. aeruginosa*. [25,50] It is therefore conceivable that the bactericidal effect of these combinations emanates from the antimicrobial/biochemical potentiation of both participating molecules. For example, ribosome-targeting antibiotics (tetracyclines, chloramphenicol, aminoglycosides) are known to preferentially inhibit the biosynthesis of envelop proteins, [48–50] thus, impacting the OM and facilitating the entry of some chemical entities. [51] We speculate that interference with the fidelity of ribosomal proteins by doxycycline and chloramphenicol enhances the uptake of compounds **2** and **3** across a structurally-weakened OM, thereby promoting the antibiotic efficacy of the rifampicin domain. Rifampicin is bactericidal against WT *P. aeruginosa*. [25] This scenario is consistent with the activity of compounds **2** and **3** against some Gram-positive bacteria that lack an OM (Table 4.1), an effect that is believed to be mediated by the rifampicin domain. The membrane-weakening effects of doxycycline and chloramphenicol possibly augment the OM-permeabilizing properties of the amphiphilic tobramycin domain in compounds **2** and **3**, thus facilitating their uptake across the OM, while the rifampicin domain possibly anchors the conjugate into the periplasm to mediate its antibacterial effect. The lack of meaningful synergistic relationship between a double and triple combination of doxycycline or chloramphenicol and tobramycin or rifampicin (Table S5) underscores the importance of linking the two domains in compounds **2** and **3**. Aminoglycosides (tobramycin) are taken up across the cytoplasmic membrane via an energy-dependent process [22] while rifampicin lacks the requisite physicochemical properties to navigate the OM.

It is gratifying to note that, consistent with *in vitro* potentiation, synergy was also observed *in vivo* between doxycycline or chloramphenicol and compound **2** in *G. mellonella in vivo* infection model (Figure 4.6). A 100 mg/kg monotherapy each of doxycycline, chloramphenicol, or compound **2** resulted in 100 % mortality of PA260-challenged *G. mellonella* larvae after 18 h, while a single dose administration of 100 + 100 mg/kg combination therapy each of doxycycline + compound **2** or chloramphenicol + compound **2** resulted in 87 % and 72 % survival, respectively, after 18 h in a dose-

dependent manner (Figure 4.6). This indicates the therapeutic potential of rifampicin-tobramycin conjugates in combination with doxycycline or chloramphenicol to treat infections caused by MDR/XDR *P. aeruginosa*. A 100 mg/kg of meropenem alone resulted in 100% mortality of the larvae after 24 h while colistin, the only antibiotic (besides amikacin) that the strain is susceptible to, require up to 50 mg/kg to confer $\geq 50\%$ survivability on the larvae after 24 h. Despite the obvious limitation of this model such as the simple body plan and lack of many defined organs/tissues in the larvae, the similarities between the innate immune responses in *G. mellonella* larvae and humans, the demonstrable virulence of pathogenic bacteria and efficacy of therapies in both *G. mellonella* larvae and humans, and the logistical and ethical relief of using *G. mellonella* make it a suitable preliminary model to determine efficacy of therapeutic agents.[11,21,25,42,43] Moreover, unlike other invertebrate models such as *Caenorhabditis elegans* and *Drosophila melanogaster*, *G. mellonella* larvae can survive at 37 °C and therefore allow the investigation of temperature-dependent microbial virulence and antibiotic action.[52] However, further studies of this combination is needed in mice infection model.

The newly synthesized compounds **1 – 3**, alone and in combination with antibiotics, were found to be non-cytotoxic to human kidney (HEK293) and liver (HepG2) cells *in vitro*, non-hemolytic to porcine erythrocytes *in vitro* (Figure 4.7), and non-toxic to *G. mellonella* larvae *in vivo* (Figure 4.6a) at relatively high concentrations. As a result, it rules out the suspicion of a non-specific mode of action and demonstrates the relative safety of these compounds against eukaryotic cells. Amphiphilic aminoglycosides have previously been shown to target bacterial membranes more selectively than eukaryotic membranes.[53]

4.6 Conclusions

The lack of a robust pipeline of new agents, particularly against resistant Gram-negative bacteria, emphasizes the importance of optimizing our current antimicrobials. Rifampicin–

Tobramycin conjugates have been shown to break the intrinsic resistance of *P. aeruginosa* to doxycycline and chloramphenicol *in vitro* and *in vivo*, thus expanding the therapeutic usefulness of these agents. In the presence of <10 μM of compounds **2** or **3**, extrapolated CLSI susceptibility breakpoints were reached in nine out of ten resistant *P. aeruginosa* phenotypes for doxycycline (≤ 4 $\mu\text{g/ml}$), and seven out of ten for chloramphenicol (≤ 8 $\mu\text{g/ml}$). A single dose combination of compound **2** with doxycycline or chloramphenicol also confer survivability on MDR *P. aeruginosa*-challenged *G. mellonella* larvae while a monotherapy of compound **2**, doxycycline, or chloramphenicol does not. Whereas doxycycline alone exhibits bacteriostatic effects against *P. aeruginosa* at supra MIC levels, when combined with compound **2**, its effect became bactericidal at sub MIC levels. In developed countries, usage of chloramphenicol as broad-spectrum antibiotics has diminished over the years due to increasing resistance and its well-described adverse effects, but the ability to achieve potent bactericidal effects at lower concentrations could rejuvenate interest in this drug, especially in this era of unabating resistance development. The current study has also demonstrated that chemical entities with no intrinsic activity as standalone agents against Gram-negative bacteria does not necessarily mean they are biologically irrelevant. Indeed, the attainment of specific physicochemical threshold in a molecule is central to permeating the outer membrane of *P. aeruginosa*. Further studies with tobramycin-based conjugates in general are warranted to carefully elucidate and situate their role in clinical practice.

4.7 Experimental Section

4.7.1 Chemistry

All chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) except 3-formyl rifamycin SV and tobramycin that were purchased from AK Scientific Inc. (CA, USA). The chemicals were all used without further purification. Air and moisture-sensitive reactions

were performed with dry solvents under nitrogen atmosphere. Thin-layer chromatography (TLC) was carried out on aluminum-backed silica gel 60 F₂₅₄ GF plates (0.25 mm) and/or aluminum-backed reverse phase silica gel 60 RP-18 F₂₅₄S plates (Merck KGaA, Germany) with the indicated solvents, and visualized under ultraviolet light and/or by staining within ninhydrin solution in *n*-butanol. Compounds were purified by flash chromatography on silica gel 60 (230-400 ASTM mesh) and/or reverse-phase C18 silica gel (Silicycle, USA). Yields refer to chromatography-purified homogenous materials, except otherwise stated. ¹H and ¹³C NMR spectra were recorded on Bruker AMX-300 and AMX-500 spectrometers (Germany) as solutions and reported in the order of chemical shifts (δ) in ppm relative to the indicated solvent, multiplicity (s, singlet; d, doublet; t, triplet and m, multiplet), number of protons, and coupling constants (*J*) in hertz (Hz). ¹H and ¹³C of compounds were assigned using 1D and 2D NMR experiments such as Proton, COSY, Carbon-13, DEPT-135, HSQC, and HMBC. ESI-MS and MALDI-TOF MS analyses were performed on Varian 500-MS ion trap mass spectrometer (USA) and Bruker Daltonics Ultraflexextreme MALDI TOF/TOF mass spectrometer (Germany), respectively. Analytical HPLC was performed on Breeze HPLC Waters equipped with W2998 PDA detector (1.2 nm resolution) coupled to Phenomenex Synergi Polar (50 \times 4.6 mm) 4 μ m reverse-phase column with phenyl ether-linked stationary phase and a detection wavelength of 332 nm. The purity of final compounds as determined by HPLC analysis was > 95 %.

4.7.1.1 *General Procedure A: Copper(I)-catalyzed azide-alkyne cycloaddition reaction (“Click Chemistry”) for the Preparation of compounds 1 – 3.*

Compounds **11a-c** (2 equiv.) and **12** (1 equiv.) were dissolved in a 1:1 mixture of H₂O/1-butanol (2.0 mL). A 100 mM solution of CuSO₄·5H₂O (1 equiv.) and freshly prepared 500 mM solution of ascorbic acid (4 equiv.) were added, and the mixtures were stirred at 50 °C for 4 h. The reaction mixtures were concentrated and purified by reverse-phase column chromatography (100% water to water/acetonitrile, 85/15, v/v, spiked with 0.1% v/v formic acid) to give the final compounds **1 – 3** (45

– 55%) isolated as reddish-brown solids. Excess **11a–c**, CuSO₄, and ascorbic acid were eluted on RP-18 column using a copious amount of 100% water while the desired compounds **1 – 3** were retained in the column until 15% v/v acetonitrile in water. The potential retention of some residual copper ions did not impact cytotoxicity of the final compounds *in vitro* and *in vivo*, consistent with an earlier report.[31] The purification process is laborious and the final compounds are stable at neutral conditions but are labile in acidic or basic pH. CD₃CN was used as co-solvent for acquisition of NMR spectra for compounds **2** and **3**.

4.7.1.2 General Procedure B: 5-O-alkylation of Boc and TBDMS protected tobramycin for the Preparation of compounds **10a–c**

A solution of **9** (1 equiv.) in toluene was treated with KOH (3 equiv.), 1,*n*-dibromoalkane (3 equiv.), and a catalytic amount of tetrabutylammonium hydrogen sulphate, TBAHS (0.1 equiv.). The reaction mixture was stirred at RT overnight, dispersed in water and extracted with an equal volume of ethyl acetate (×3). The organic layers were combined, washed with brine (×1), dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The crude products were then purified by column chromatography (hexanes/ethyl acetate, 100/0 to 100/10, v/v). The resulting compounds were dried and subsequently dissolved in anhydrous DMF, treated with NaN₃ (20 equiv.) and stirred at 70 °C for 3 h under nitrogen atmosphere. The crude mixtures were concentrated under *vacuo* and re-dissolved in ethyl acetate. The organic layers were then washed with water (×2) and brine (×1), dried over anhydrous Na₂SO₄, and concentrated under *vacuo* to afford compounds **10a–c** as white solids.

4.7.1.3 General Procedure C: Deprotection of hydroxyls and amines (Removal of TBDMS and Boc protecting groups) for Preparation of compounds **11a–c**

A solution of TBDMS- and Boc-protected compounds **10a–c** in anhydrous THF (5.0 mL) were treated with tetrabutylammonium fluoride (TBAF, 6 equiv.) and stirred under nitrogen atmosphere for

2 h. The reaction mixture was concentrated under *vacuo*, dissolved in water and extracted with DCM (×3). The organic layers were combined, dried over anhydrous Na₂SO₄, concentrated *in vacuo*, and purified by column chromatography (dichloromethane/methanol, 40/1, v/v). A solution of the resulting compounds in DCM (2.0 mL) were further treated with trifluoroacetic acid (2.0 mL), stirred at RT for 1 h and concentrated under low *vacuo*. 2% methanol in ether (2.0 mL) was then added, stirred gently for 1 min and the solvent carefully decanted to give off-white solid compounds. The crude products were subsequently purified by reverse-phase flash chromatography (eluted with 100% deionized water) to afford analytically pure compounds **11a–c** as off-white TFA salt solid compounds. Compound **11b** was converted to HCl salt before use by treating with stoichiometric amount of aqueous HCl solution.

4.7.1.3.1 Rifampicin-*C*₄-Tobramycin conjugate.5TFA (**1**).

Compounds **11a** (0.100 g, 0.088 mmol) and **12** (0.037 g, 0.044 mmol) were conjoined via click chemistry as described in general procedure A to afford a final compound **1** (0.027 g, 45%) as a reddish brown solid. ¹H NMR (500 MHz, D₂O) δ 8.27 (s, 1H, triazole), 7.66 (s, 1H, imine), 6.69 – 6.42 (br. m, 2H), 6.25 – 6.04 (br. m, 2H), 5.40 (s, 1H), 5.30 – 5.04 (m, 3H), 4.52 – 4.45 (m, 3H), 4.32 – 4.21 (m, 3H), 3.94 – 3.54 (m, 30H), 3.41 – 3.30 (m, 6H), 3.20 – 3.14 (m, 3H), 3.10 – 3.05 (m, 2H), 2.58 – 2.49 (m, 2H), 2.28 – 2.22 (m, 5H), 2.11 – 2.03 (m, 5H), 1.97 – 1.91 (m, 3H), 1.84 – 1.77 (m, 3H), 1.66 – 1.57 (m, 5H), 1.52 – 1.48 (m, 2H), 1.40 – 1.27 (m, 10H), 0.97 (br. s, 3H), 0.80 (br. s, 3H), 0.58 (br. s, 3H), -0.04 (br. s, 3H). ¹³C NMR (126 MHz, D₂O) δ 199.3, 176.2, 173.6, 147.9, 147.5, 144.9, 143.7, 142.9, 141.4, 139.8, 138.2, 137.2, 136.3, 134.8, 130.5, 127.5, 125.0, 108.3, 106.2, 101.3, 92.5, 82.1, 81.8, 77.3, 76.4, 75.6, 73.5, 73.2, 73.0, 72.1, 71.7, 69.7, 68.6, 64.8, 63.7, 63.6, 62.6, 60.4, 59.3, 56.7, 54.7, 50.6, 49.8, 48.5, 47.4, 39.2, 38.6, 37.4, 35.6, 32.6, 29.9, 29.3, 28.3, 27.6, 25.8, 25.7, 25.1, 24.6, 20.5, 20.3, 17.4, 9.9, 9.7, 9.6, 9.0. MALDI TOF-MS *m/e* calcd for C₆₇H₁₀₃N₁₂O₂₁, 1411.7282; measured *m/e*, 1411.7285 [M + H]⁺.

4.7.1.3.2 Rifampicin-*C*₆-Tobramycin conjugate.5HCl (**2**).

Compounds **11b** (0.100 g, 0.086 mmol) and **12** (0.036 g, 0.043 mmol) were conjoined via click chemistry as described in general procedure A to afford a final compound **2** (0.032 g, 52%) as a reddish brown solid. ¹H NMR (500 MHz, D₂O + CD₃CN) δ 8.03 (s, 1H, triazole), 7.66 (s, 1H, imine), 6.63 (br., 1H), 6.49 (d, *J* = 10.7 Hz, 1H), 6.26 (d, *J* = 12.6 Hz, 1H), 6.12 (br., 1H), 5.44 – 5.39 (m, 1H), 5.28 (d, *J* = 10.0 Hz, 1H), 5.25 – 5.22 (m, 1H), 5.18 – 5.13 (m, 1H), 4.32 – 4.28 (m, 2H), 4.23 (t, *J* = 9.6 Hz, 2H), 4.02 – 3.84 (m, 15H), 3.80 – 3.72 (m, 11H), 3.69 – 3.66 (m, 2H), 3.62 – 3.43 (m, 12H), 3.37 – 3.34 (m, 3H), 3.25 – 3.21 (m, 2H), 3.20 – 3.17 (m, 1H), 3.13 (s, 2H), 3.10 – 3.08 (m, 1H), 2.83 – 2.73 (m, 3H), 2.59 – 2.50 (m, 2H), 2.35 – 2.33 (m, 1H), 2.31 – 2.25 (m, 5H), 2.02 – 1.95 (m, 4H), 1.83 (s, 3H), 1.71 – 1.63 (m, 6H), 1.58 – 1.54 (m, 2H), 1.52 – 1.50 (m, 1H), 1.43 – 1.34 (m, 14H), 1.05 (d, *J* = 7.0 Hz, 3H), 0.99 (t, *J* = 6.7 Hz, 3H), 0.88 (d, *J* = 6.9 Hz, 3H), 0.65 (d, *J* = 6.8 Hz, 3H), 0.15 (br. s, 3H). ¹³C NMR (126 MHz, D₂O + CD₃CN) δ 196.6, 185.3, 178.1, 175.5, 171.1, 169.2, 163.3, 151.1, 144.8, 137.6, 133.3, 132.8, 131.2, 130.1, 128.7, 127.7, 118.8, 115.2, 112.9, 110.6, 104.1, 95.0, 85.0, 84.7, 79.2, 79.0, 78.2, 76.7, 76.3, 76.2, 75.2, 75.0, 72.5, 71.4, 67.6, 66.6, 65.4, 62.3, 59.3, 57.5, 53.2, 52.6, 51.3, 51.2, 50.2, 41.8, 41.5, 40.3, 38.4, 35.5, 34.3, 32.5, 32.4, 32.2, 32.1, 32.0, 31.9, 31.3, 31.0, 30.3, 28.7, 28.2, 27.9, 25.1, 23.4, 23.1, 22.5, 20.2, 16.3, 12.7, 9.9. MALDI TOF-MS *m/e* calcd for C₇₅H₁₁₉N₁₂O₂₁, 1523.8534; measured *m/e* 1523.8541 [M + H]⁺. MALDI TOF-MS *m/e* calcd for C₆₉H₁₀₆N₁₂O₂₁Na, 1461.7493; measured *m/e*, 1461.7498 [M + Na]⁺.

4.7.1.3.3 Rifampicin-*C*₁₂-Tobramycin conjugate.5TFA (**3**).

Compounds **11c** (0.100 g, 0.081 mmol) and **12** (0.036 g, 0.041 mmol) were conjoined via click chemistry as described in general procedure A to afford a final compound **3** (0.034 g, 55%) as a reddish brown solid. ¹H NMR (500 MHz, D₂O + CD₃CN) δ 8.02 (s, 1H, triazole), 7.69 (s, 1H, imine), 6.58 (br. d, *J* = 10.5 Hz, 1H), 6.42 (d, *J* = 11.0 Hz, 1H), 6.18 (d, *J* = 12.5 Hz, 1H), 6.06 (br. d, *J* = 10.5, 1H), 5.34 (d, *J* = 2.7 Hz, 1H), 5.20 (d, *J* = 10.1 Hz, 1H), 5.17 (d, *J* = 3.5 Hz, 1H), 5.09 (dd, *J* = 12.5, 6.1 Hz, 1H), 4.24 – 4.16 (m, 2H), 4.12 (br. s, 1H), 3.95 (t, *J* = 9.6 Hz, 1H), 3.91 (dd, *J* = 11.0, 3.4 Hz, 2H),

3.87 – 3.75 (m, 9H), 3.74 – 3.32 (m, 25H), 3.27 (d, $J = 6.6$ Hz, 3H), 3.17 – 3.13 (m, 1H), 3.10 (dd, $J = 10.3, 2.3$ Hz, 1H), 3.06 (s, 3H), 2.99 – 2.91 (m, 3H), 2.53 – 2.49 (m, 1H), 2.29 (s, 3H), 2.25 – 2.15 (m, 7H), 1.92 – 1.87 (m, 3H), 1.76 (s, 3H), 1.74 – 1.71 (m, 1H), 1.66 – 1.55 (m, 8H), 1.50 – 1.43 (m, 3H), 1.38 – 1.22 (m, 32H), 0.97 (d, $J = 6.9$ Hz, 3H), 0.91 (t, $J = 6.7$ Hz, 3H), 0.77 (d, $J = 6.9$ Hz, 3H), 0.57 (d, $J = 6.8$ Hz, 3H), 0.06 (br. s, 3H). ^{13}C NMR (126 MHz, $\text{D}_2\text{O} + \text{CD}_3\text{CN}$) δ 196.6, 185.3, 178.1, 175.5, 171.1, 169.2, 163.3, 151.1, 144.8, 137.6, 133.3, 132.8, 131.2, 130.1, 128.7, 127.7, 118.8, 115.2, 112.9, 110.6, 104.1, 95.0, 85.0, 84.7, 79.2, 79.0, 78.2, 76.7, 76.3, 76.2, 75.2, 75.0, 72.5, 71.4, 67.6, 66.6, 65.4, 62.3, 59.3, 57.5, 53.2, 52.6, 51.3, 51.2, 50.2, 41.8, 41.5, 40.3, 38.4, 35.5, 34.3, 32.5, 32.4, 32.2, 32.1, 32.0, 31.9, 31.3, 31.0, 30.3, 28.7, 28.2, 27.9, 25.1, 23.4, 23.1, 22.5, 20.2, 16.3, 12.7, 9.9. MALDI TOF-MS m/e calcd for $\text{C}_{75}\text{H}_{119}\text{N}_{12}\text{O}_{21}$, 1523.8534; measured m/e 1523.8541 $[\text{M} + \text{H}]^+$.

4.7.1.3.4 1-Nitrosopiperazine (7).

Piperazine **4** (5.16 g, 60.0 mmol) was dissolved in 6 M HCl (36.0 mL) and cooled to -10°C in ice bath. A solution of NaNO_2 (4.14 g, 60.0 mmol) in water (72.0 mL) was slowly added (using a separatory funnel) to the reaction mixture in ice bath over 1 h. The reaction mixture was adjusted to pH 10 with 3 M NaOH and extracted with CHCl_3 (100 mL, $\times 3$). The combined organic extracts were dried over anhydrous Na_2SO_4 , concentrated *in vacuo* and purified by flash column chromatography (hexanes/ ethyl acetate, 4:1 to 2:1, v/v) to give **5** as a yellow oil (3.56 g, 51%). ^1H NMR (300 MHz, CDCl_3) δ 3.72 (m, 2H), 3.29 (m, 2H), 2.57 (m, 2H), 2.32 (m, 2H). ^{13}C NMR (75 MHz, CDCl_3) δ 50.8, 46.2, 44.6, 40.7. ESI-MS: m/z calcd for $\text{C}_4\text{H}_{10}\text{N}_3\text{O}$, 116.08; found 116.2 $[\text{M} + \text{H}]^+$.

4.7.1.3.5 1-Nitroso-4-propargylpiperazine (6).

A solution of 1-nitrosopiperazine **5** (2.61 g, 22.48 mmol) in anhydrous acetonitrile (20.0 mL) was treated with propargyl bromide (3.37 g of 80% solution in toluene, 22.48 mmol) and Et_3N (6.3 mL, 44.96 mmol). The reaction mixture was refluxed for 3 h and concentrated *in vacuo*. The crude product was subsequently dissolved in 10 % NaOH (50.0 mL) and extracted with CH_2Cl_2 (50.0 mL,

×3). The combined organic phase was dried over anhydrous Na₂SO₄, concentrated *in vacuo* and purified by column chromatography (hexanes/ ethyl acetate, 4:1, v/v) to yield **6** as an orange oil (1.39 g, 40%). ¹H NMR (300 MHz, CDCl₃) δ 3.98 – 3.92 (m, 2H), 3.50 (dd, *J* = 6.0, 4.9 Hz, 2H), 3.09 (d, *J* = 2.6 Hz, 2H, CH₂CCH), 2.48 – 2.42 (m, 2H), 2.23–2.17 (m, 3H). ¹³C NMR (75 MHz, CDCl₃) δ 77.3, 74.2, 51.6, 50.1, 49.1, 46.3, 39.0. ESI-MS: *m/z* calcd for C₇H₁₂N₃O, 154.09; found 154.1 [M + H]⁺.

4.7.1.3.6 1-Amino-4-propargylpiperazine (**7**).

LiAlH₄ (0.59 g, 15.58 mmol) was added to a flame-dried RBF, suspended in anhydrous Et₂O (15.0 mL) and stirred vigorously for 5 mins. A solution of **6** (1.20 g, 7.79 mmol) in anhydrous Et₂O (5.0 mL) was slowly added in ice bath, stirred for 2 mins at RT, and then refluxed for 6 h. The reaction mixture was then cooled in ice bath, quenched with 2 M HCl and filtered through celite. The celite was subsequently washed with 2 M HCl (100 mL) and the filtrate adjusted to pH 10 with 10% NaOH. The aqueous phase was extracted with CH₂Cl₂ (100 mL, ×3), dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The crude product was purified by column chromatography (dichloromethane/ methanol, 20:1 to 10:1, v/v) to yield compound **7** (0.33 g, 30%). ¹H NMR (300 MHz, CDCl₃) δ 3.28 (br. s, 2H), 3.24 (d, *J* = 1.5 Hz, 2H, CH₂CCH), 2.80 – 2.74 (m, 2H), 2.61 – 2.43 (br. m, 4H), 2.42 – 2.36 (m, 2H), 2.16 (t, *J* = 2.5, 1H, CH₂CCH). ¹³C NMR (75 MHz, CDCl₃) δ 73.4, 73.3, 58.9, 52.5, 51.4, 46.2, 45.46. ESI-MS: *m/z* calcd for C₇H₁₄N₃, 140.11; found 140.2 [M + H]⁺.

4.7.1.3.7 1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-Tobramycin (**9**).

Commercial tobramycin (4.0 g, 8.56 mmol) was dissolved in a 2:1 mixture of methanol and water (90.0 mL) and treated with Boc₂O (18.7 g, 85.56 mmol) in the presence of Et₃N (5.0 mL). The reaction mixture was stirred under reflux (at 55 °C) overnight, concentrated under vacuo and thoroughly dried under high vacuum for 24 h to afford a white powdery solid (7.04 g, 85%). The dried crude penta-*N*-boc-protected tobramycin (7.0 g, 7.27 mmol) was dissolved in anhydrous DMF (6.0 mL) and treated with *tert*-butyldimethylsilyl chloride, TBDMSCl (11.0 g, 72.98 mmol) and *N*-

methylimidazole (4.0 mL). The reaction was stirred at RT for 5 days under nitrogen gas atmosphere, and the resulting mixture was poured into water (100.0 mL) and extracted with DCM (×3). The organic layer was dried over anhydrous Na₂SO₄, concentrated in *vacuo*, and purified by flash chromatography using gradient elution (hexanes/ ethyl acetate, 15/1 to 8/1, v/v) to afford **9** (7.87 g, 76%) as a white solid. NMR data are consistent with an earlier report.[21]

4.7.1.3.8 *5-O-(n-Azidoalkyl)-1,3,2',6',3''-penta-N-Boc-4',2'',4'',6''-tetra-O-TBDMS-Tobramycin (10a–c).*

Compounds **10a**, **10b**, and **10c** were prepared by treating **9** with 1,4-dibromobutane, 1,6-dibromohexane, and 1,12-dibromododecane, respectively, according to general procedure B.

5-O-(4-Azidobutyl)-1,3,2',6',3''-penta-N-Boc-4',2'',4'',6''-tetra-O-TBDMS-Tobramycin

(10a). Yield (55%). ¹H NMR (500 MHz, CDCl₃) δ 5.27 – 4.98 (m, 2H, anomeric), 4.32 – 4.00 (m, 2H), 3.87 – 3.06 (m, 17H), 2.43 (d, *J* = 7.5 Hz, 1H), 2.06 – 1.95 (m, 2H), 1.91 – 1.72 (m, 2H), 1.57 – 1.35 (m, 49), 0.98 – 0.79 (m, 36H), 0.19 – 0.04 (m, 24H, Si(CH₃)₂). ¹³C NMR (126 MHz, CDCl₃) δ 156.9, 156.8, 156.5, 156.2, 156.0, 97.9 (anomeric), 96.6 (anomeric), 85.7, 79.7, 79.0, 75.3, 73.3, 72.9, 71.5, 68.0, 66.8, 63.1, 57.3, 50.5, 49.0, 48.5, 41.6, 35.9, 35.7, 34.1, 32.8, 30.6, 29.4, 28.4, 26.1, 26.0, 22.0, 18.0, 17.9, 17.8, 17.6, -4.7, -5.1, 5.5, -5.9, -6.3, -6.4. ESI-MS: *m/z* calcd for C₇₁H₁₄₀N₈O₁₉Si₄Na, 1543.9; found 1544.2 [M + Na]⁺.

5-O-(6-Azidohexyl)-1,3,2',6',3''-penta-N-Boc-4',2'',4'',6''-tetra-O-TBDMS-Tobramycin

(10b). Yield (59%). ¹H NMR (500 MHz, CDCl₃) δ 5.28 – 4.99 (m, 2H, anomeric), 4.31 – 3.97 (m, 2H), 3.87 – 3.05 (m, 17H), 2.42 (d, *J* = 7.5 Hz, 1H), 2.06 – 1.96 (m, 2H), 1.92 – 1.72 (m, 2H), 1.57 – 1.35 (m, 49), 0.98 – 0.80 (m, 40H), 0.19 – 0.04 (m, 24H, Si(CH₃)₂). ¹³C NMR (126 MHz, CDCl₃) δ 157.0, 156.7, 156.5, 156.1, 156.0, 97.8 (anomeric), 96.6 (anomeric), 85.7, 79.8, 78.9, 75.4, 73.3, 72.9, 71.5, 68.1, 66.7, 63.1, 57.3, 50.3, 49.1, 48.5, 41.5, 35.9, 35.5, 34.1, 32.8, 30.6, 29.4, 28.4, 26.1, 26.0,

22.0, 18.0, 17.9, 17.8, 17.6, -4.7, -5.1, 5.5, -5.9, -6.3, -6.4. ESI-MS: m/z calcd for $C_{73}H_{144}N_8O_{19}Si_4Na$, 1571.95; found 1571.8 $[M + Na]^+$.

5-*O*-(12-Azidododecyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-

Tobramycin (10c). Yield (66%). 1H NMR (500 MHz, $CDCl_3$) δ 5.26 – 4.97 (m, 2H, anomeric), 4.32 – 4.00 (m, 2H), 3.87 – 3.06 (m, 17H), 2.43 (d, $J = 7.5$ Hz, 1H), 2.06 – 1.95 (m, 2H), 1.91 – 1.72 (m, 2H), 1.57 – 1.35 (m, 49), 0.98 – 0.79 (m, 42H), 0.19 – 0.04 (m, 24H, $Si(CH_3)_2$). ^{13}C NMR (126 MHz, $CDCl_3$) δ 156.9, 156.8, 156.5, 156.2, 156.0, 97.9 (anomeric), 96.6 (anomeric), 85.7, 79.7, 79.0, 75.3, 73.3, 72.9, 71.5, 68.0, 66.8, 63.1, 57.3, 50.5, 49.0, 48.5, 41.6, 35.9, 35.7, 34.1, 32.8, 30.6, 29.4, 28.4, 26.1, 26.0, 22.0, 18.0, 17.9, 17.8, 17.6, -4.7, -5.1, 5.5, -5.9, -6.3, -6.4. ESI-MS: m/z calcd for $C_{79}H_{156}N_8O_{19}Si_4Na$, 1656.04; found 1656.2 $[M + Na]^+$.

4.7.1.3.9 5-*O*-(*n*-Azidoalkyl)-Tobramycin.5TFA (11a–c).

Compounds **11a**, **11b**, and **11c** were prepared by treating compounds **10a–c** with tetrabutylammonium fluoride and trifluoroacetic acid successively, according to general procedure C.

5-*O*-(4-Azidobutyl)-Tobramycin.5TFA (11a). 1H NMR (300 MHz, D_2O) δ 5.39 (d, $J = 2.4$ Hz, 1H, anomeric), 5.20 (d, $J = 3.4$ Hz, 1H, anomeric), 4.34 – 4.17 (m, 2H), 4.00 – 3.71 (m, 11H), 3.63 – 3.52 (m, 3H), 3.49 – 3.27 (m, 4H), 2.61 – 2.48 (m, 1H), 2.36 – 2.19 (2H), 2.11 – 1.93 (m, 1H), 1.81 – 1.56 (m, 4H). ^{13}C NMR (75 MHz, D_2O) δ 101.1 (anomeric), 92.6 (anomeric), 81.9, 81.7, 76.6, 75.7, 73.1, 72.7, 68.5, 64.8, 63.2, 59.3, 54.8, 51.0, 49.6, 48.4, 47.3, 38.5, 28.0, 27.7, 26.7, 24.6. MALDI TOF-MS m/e calcd for $C_{22}H_{44}N_8O_9$, 565.3231; measured m/e 565.35541 $[M + H]^+$.

5-*O*-(6-Azidohexyl)-Tobramycin.5HCl (11b). 1H NMR (500 MHz, D_2O) δ 5.31 (d, $J = 2.6$ Hz, 1H, anomeric), 5.10 (d, $J = 3.6$ Hz, 1H, anomeric), 4.23 – 4.17 (m, 1H), 4.14 (dd, $J = 11.1, 8.5$ Hz, 1H), 3.88 – 3.61 (m, 11H), 3.59 – 3.47 (m, 3H), 3.37 – 3.30 (m, 1H), 3.27 – 3.21 (m, 3H), 2.49 – 2.42 (m, 1H), 2.23 – 2.10 (m, 2H), 1.99 – 1.89 (m, 1H), 1.63 – 1.49 (m, 4H), 1.36 – 1.19 (m, 5H). ^{13}C NMR (126 MHz, D_2O) δ 101.3 (anomeric), 92.7 (anomeric), 81.9, 81.8, 76.6, 75.6, 73.5, 73.2, 68.6, 64.9,

63.3, 59.3, 54.8, 51.2, 49.8, 48.5, 47.4, 38.6, 29.4, 28.2, 28.0, 27.8, 26.2, 25.3, 24.8, 19.4. MALDI TOF-MS m/e calcd for $C_{24}H_{48}N_8O_9Na$, 615.3442; measured m/e 615.3555 $[M + Na]^+$.

5-*O*-(12-Azidododecyl)-Tobramycin.5TFA (11c). 1H NMR (300 MHz, D_2O) δ 5.38 (d, J = 2.4 Hz, 1H, anomeric), 5.15 (d, J = 3.4 Hz, 1H, anomeric), 4.28 (dd, J = 8.8, 4.1 Hz, 1H), 4.20 – 4.07 (m, 1H), 3.99 – 3.48 (m, 16H), 3.45 – 3.24 (m, 4H), 2.61 – 2.47 (m, 1H), 2.30 – 2.20 (m, 2H), 2.02 – 1.85 (m, 1H), 1.73 – 1.52 (m, 4H), 1.46 – 1.20 (m, 18H). ^{13}C NMR (75 MHz, D_2O) δ 101.4 (anomeric), 92.7 (anomeric), 81.9, 81.8, 76.9, 76.0, 73.8, 73.2, 68.5, 64.8, 63.1, 59.3, 54.8, 51.3, 49.7, 48.3, 47.2, 38.3, 29.4, 28.9, 28.8, 28.7, 28.6, 28.4, 28.2, 28.1, 28.0, 27.7, 26.0, 25.3. MALDI TOF-MS m/e calcd for $C_{30}H_{60}N_8O_9$, 676.4483; measured m/e 676.4492.

4.7.1.3.10 *N*-propargyl-Rifampicin (12).

A solution of Rifaldehyde (3-formyl rifamycin SV) (0.62 g, 0.854 mmol) suspended in dry THF (5.0 mL) was treated with 1-amino-4-propargylpiperazine (**7**) (0.12 g, 0.854 mmol) and stirred at RT for 20 mins. The reaction mixture was diluted with DCM (10.0 mL) and washed with a solution of ascorbic acid (2.0 g) in 3:1 H_2O /brine (40.0 mL). The aqueous layer was then extracted with DCM (30.0 mL, $\times 3$), and the combined organic layers were dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude product was purified by reverse-phase (C18) column chromatography using a gradient elution of acetonitrile /water, 1:1 to 4:1, v/v, to yield rifampicin alkyne (**12**) as a reddish orange solid (0.70 g, 97%). 1H NMR (300 MHz, CD_3OD) δ 8.00 (s, 1H, imine), 6.64 – 6.51 (m, 1H), 6.45 (dd, J = 11.3, 1.6 Hz, 1H), 6.18 (dd, J = 12.8, 0.9 Hz, 1H), 5.91 (dd, J = 15.3, 4.7 Hz, 1H), 5.05 – 4.88 (m, 2H), 3.72 (dd, J = 9.2, 1.7 Hz, 1H), 3.43 – 3.33 (m, 2H), 3.32 – 3.26 (m, 1H), 3.22 – 3.03 (m, 4H, piperazine), 2.99 (dd, J = 10.3, 2.4 Hz, 1H), 2.91 (s, 3H), 2.86 – 2.64 (m, 4H, piperazine), 2.33 – 2.20 (m, 1H), 2.08 (s, 3H), 2.04 (s, 3H), 1.96 (s, 1H, alkyne), 1.95 (s, 3H), 1.77 (s, 3H), 1.65 – 1.53 (m, 1H), 1.33 – 1.16 (m, 2H), 1.02 – 0.80 (m, 6H), 0.46 (d, J = 6.8 Hz, 3H), -0.55 (d, J = 6.7 Hz, 3H). ^{13}C NMR (75 MHz, CD_3OD) δ 194.6, 173.8, 171.0, 169.5, 147.2, 143.3, 141.8, 138.4, 134.4, 133.7

(imine), 130.0, 123.5, 119.4, 117.4, 112.3, 111.0, 109.1, 105.4, 77.1, 76.6, 74.3, 74.0, 71.4, 55.5, 50.2, 49.6, 45.7, 40.1, 38.6, 38.1, 33.0, 29.3, 20.9, 19.4, 17.3, 9.6, 8.0, 7.8, 6.7, -0.6. MALDI TOF-MS *m/e* calcd for C₄₅H₅₈N₄O₁₂, 846.4051; measured *m/e* 846.4068.

4.7.2 Microbiology.

Bacterial isolates were either obtained from the American Type Culture Collection (ATCC), the Canadian National Intensive Care Unit (CAN-ICU) surveillance study[54], or the Canadian Ward (CANWARD) surveillance study[55,56]. Clinical isolates obtained as part of the CAN-ICU and CANWARD studies from participating medical centers across Canada were cultured from body fluids and tissues of patients suffering from presumed “clinically significant” infectious diseases. Antimicrobial susceptibilities of clinical isolates were evaluated (using ATCC strains as quality control strains) and categorized, where appropriate, as either multidrug resistant (MDR), extensively drug-resistant (XDR), or pan drug-resistant (PDR). MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, XDR as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories), and PDR as non-susceptibility to all agents in all antimicrobial categories.[26]

4.7.2.1 Antimicrobial Susceptibility Assay

The *in vitro* antimicrobial activity of all compounds/antibiotics against a panel of bacteria was evaluated by microbroth dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines. Overnight grown bacterial cultures were diluted in saline to achieve a 0.5 McFarland turbidity, followed by 1:50 dilution in Mueller-Hinton broth (MHB) for inoculation to a final concentration of approximately 5×10^5 CFU/mL. The antimicrobial agents were 2-fold serially diluted in MHB in a 96-well plate and incubated at 37 °C with equal volumes of inoculum for 18 h. The lowest concentration that prevented the visible growth of bacteria was defined as the MIC for each

antimicrobial agent. The broth with or without bacterial cells was used as positive or negative control, respectively.

4.7.2.2 Checkerboard Assay

Combination studies with different antibiotics were performed in 96-well plates as previously described.[25] Briefly, the antibiotic of interest was serially diluted in MHB along the abscissa while the adjuvant (newly synthesized conjugates) was serially diluted in MHB along the ordinate. This creates a 10×7 matrix wherein each well consists of a combination of different antibiotic and adjuvant concentrations. Overnight grown bacterial cultures were diluted in saline to achieve a 0.5 McFarland turbidity, followed by 1:50 dilution in Mueller-Hinton broth (MHB) for inoculation to a final concentration of approximately 5×10^5 CFU/mL. Equal volume of this bacterial culture was then added to each well and incubated at 37 °C for 18 h. After incubation, the plates were read on EMax[®] Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 590 nm. MIC was recorded as wells with the lowest concentration of drugs with no bacterial growth. The fractional inhibitory concentration (FIC) for each antibiotic was calculated by dividing the MIC of the antibiotic in the presence of adjuvant by the MIC of the antibiotic alone. Similarly, the FIC of adjuvant was calculated by dividing the MIC of the adjuvant in the presence of antibiotic by the MIC of the adjuvant alone. FIC index is the sum of both FICs. FIC indices of < 0.5 were deemed synergistic; $0.5 - 4$, no interaction; and > 4 , antagonistic.

4.7.2.3 Time-kill assay

Time-kill curve analyses were performed by culturing *P. aeruginosa* in LB medium, in the presence of antibiotics alone and in combination with test adjuvants. MICs of antibiotics and adjuvants were determined before the experiment following CLSI microbroth dilution guidelines. Growth curves were initially performed to confirm that all strains will reach a stable early- to mid-log phase after 4 h of pre-incubation in antimicrobial-free LB medium. A 0.5 McFarland inoculum of each strain was

prepared in sterile 0.85 % saline solution from an overnight grown culture. For each strain, 30 µl of the prepared inoculum was diluted to 3 ml of LB broth (containing different combinations of antimicrobial agents and adjuvants) and incubated at 37 °C shaking at 250 rpm. At specific time intervals (0, 1, 3, 6, 9, and 24 h), 100 µl was taken from each sample, serially diluted in sterile PBS, plated on LB agar plates, and incubated at 37 °C in a humid 5 % CO₂-enriched atmosphere. Bacterial colonies were counted after 20 h of incubation.

4.7.2.4 *In vivo* larvae–infection model

In vivo synergistic effects were determined using *Galleria mellonella* infection model, as previously described.[21] Briefly, wax worms were purchased from The Worm Lady Live Feeder (ON, Canada), stored in their natural habitat at 16 °C, and used within 10 days of delivery. The larvae (average weight of 250 mg) were used for tolerability and efficacy studies. Tolerability study was performed by injecting 10 µL of antimicrobial agents only at concentrations equivalent to 100 mg/kg or 200 mg/kg. The larvae (ten in each group) were incubated at 37 °C and monitored for 96 h. For efficacy studies, the virulence and bacterial load required to kill 100 % of the larvae within 12 – 18 h was first determined, which is approximately 5 CFU. Overnight grown culture of extensively-resistant *P. aeruginosa* PA260 was standardized to 0.5 McFarland standard and diluted in PBS to a final concentration of 5×10^2 CFU/mL. 10 µL of this solution (5 CFU) was injected into each larva and incubated for 3 h at 37 °C. After the 3 h challenge, larvae in monotherapy experimental groups (fifteen per group) were treated with 10 µL injection of doxycycline (100 mg/kg), chloramphenicol (100 mg/kg), compound **2** (100 mg/kg), or PBS alone. The larvae in combination therapy groups were treated with doxycycline + compound **2** (75 + 75 mg/kg or 100 + 100 mg/kg) or chloramphenicol + compound **2** (75 + 75 mg/kg or 100 + 100 mg/kg). Larvae treated with 10 µL PBS or colistin (50 mg/kg, 75 mg/kg or 100 mg/kg) served as negative and positive control, respectively. The larvae were incubated in Petri dishes lined with filter paper at 37 °C and scored for survivability every 6 h for 36

h. This experiment was repeated to give a total of thirty larvae ($n = 30$) in each case. Survival data curves were plotted using Kaplan-Meier survival analysis. Larvae were considered dead if they do not respond to touch.

4.7.2.5 Cytotoxicity Assay

Human embryonic kidney cells (HEK293) and HepG2 cells were grown in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum in a humidified 5% atmospheric incubator at 37 °C. Equal number of cells (100 µl of media containing ~8000 cells) were dispersed into 96-well plates and wells with medium but no cells were used as blanks. After incubating for 24 h, 100 µl of varying concentrations of test compounds (at twice the desired concentrations) were added to each well, including the blanks. The treated cells were then incubated further for 48 h, after which PrestoBlue reagent was added to each well. The plates were then incubated for an additional hour on a nutator mixer in a 5% CO₂ incubator. The fluorescence was read at 490 nm on a SpectraMax M2 plate reader (Molecular Devices, USA). Cell viability were interpreted as previously described.[46,57] The values of blank were subtracted from each value, and the viability values of the treated samples relative to the controls with vehicle were calculated. The values for the plots are the means \pm standard deviation.

4.7.2.6 Hemolytic Assay

The hemolytic activities of the newly synthesized compounds were determined and quantified as the amount of hemoglobin released by lysing ovine erythrocytes. Fresh blood drawn from the antecubital vein of a pig (Animal Care and Use Program, University of Manitoba) was centrifuged at 1000 g at 4 °C for 10 mins, washed with PBS thrice and resuspended in the same buffer. The final cell concentration used was 3×10^8 cells/mL. Compounds were serially diluted with PBS and added to wells in a 96-well plate at twice the desired concentrations. Equal volumes of erythrocyte solution were then added to each well and incubated at 37 °C for 1 h. Intact erythrocytes were subsequently

pelleted by centrifuging at 1000 g at 4 °C for 10 mins, and the supernatants were transferred to a new 96-well plate. Hemoglobin release was determined by measuring the absorbance on EMax[®] Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. Blood cells in PBS (0% hemolysis) and 0.1 % Triton X-100 (100% hemolysis) were used as negative and positive controls, respectively. Percent hemolysis was calculated as $[\% \text{ hemolysis} = (X - 0\%) / (100\% - 0\%)]$, where X is the optical density values of the compounds at different concentrations.

4.7.3 Supporting Information

Supplementary data A to this article is provided at the end of this chapter and can also be found online at <https://doi.org/10.1016/j.ejmech.2019.04.034>.

Appendix A: NMR spectra, HPLC purity analysis.

4.8 References

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4.9 Supplementary Data A

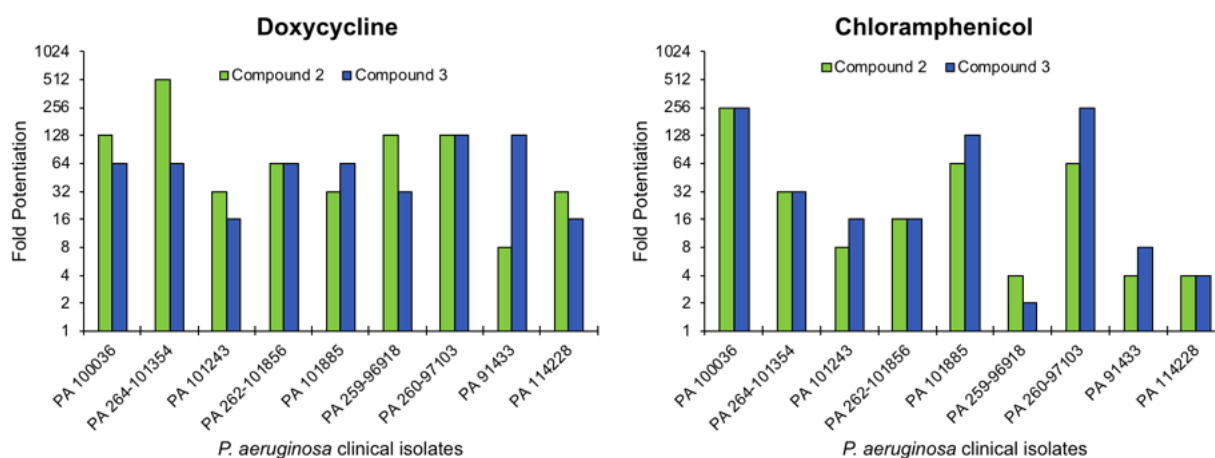


Figure S1. Fold change in susceptibility of *P. aeruginosa* (PA) clinical isolates to doxycycline and chloramphenicol in the presence of 16 $\mu\text{g/ml}$ each of compounds **2** (9.8 μM) and **3** (7.6 μM).

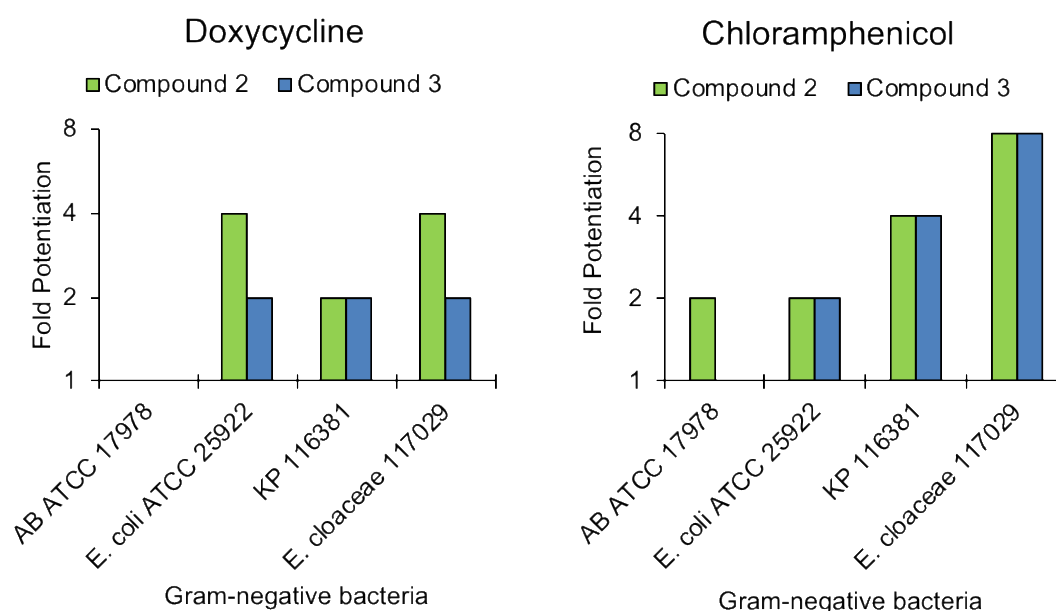


Figure S2. Fold change in susceptibility of other Gram-negative bacteria to doxycycline and chloramphenicol in the presence of 16 $\mu\text{g/ml}$ each of compounds **2** (9.8 μM) and **3** (7.6 μM). AB = *Acinetobacter baumannii*; KP = *Klebsiella pneumoniae*.

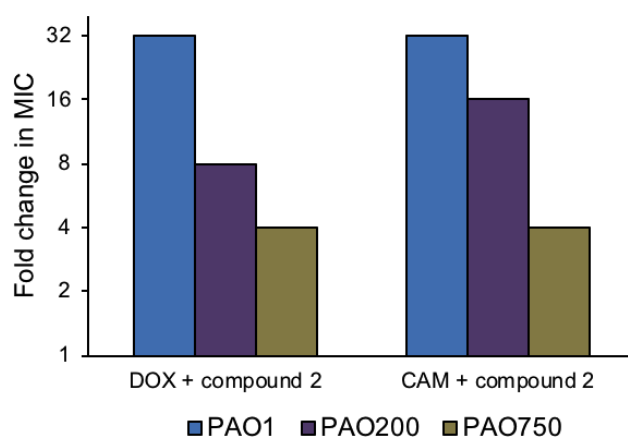


Figure S3. Fold change in susceptibilities of wild-type (PAO1) and efflux-deficient mutants (PAO200 and PAO750) of *P. aeruginosa* to doxycycline (DOX) and chloramphenicol (CAM) in the presence of 9.8 μ M of compound **2**.

Table S1. Minimum inhibitory concentrations (MICs, μ g/ml) of Rifampicin, Tobramycin, and compounds **1** – **3** against a panel of wild-type*, MDR and XDR *P. aeruginosa*. **Efflux mutant.

| <i>P. aeruginosa</i> strains | Rifampicin | Tobramycin | Rifampicin–Tobramycin conjugates | | |
|------------------------------|------------|------------|----------------------------------|----------|----------|
| | | | 1 | 2 | 3 |
| PAO1* | 16 | 1 | 512 | 512 | 512 |
| 100036 | 32 | 128 | >256 | 256 | 256 |
| 101885 | 32 | 1 | 256 | 256 | 256 |
| 259 – 96918 | 16 | 256 | >128 | >128 | >128 |
| 260 – 97103 | 16 | 32 | >128 | >128 | >128 |
| 262 – 101856 | 512 | 1024 | >256 | >256 | >256 |
| 264 – 101354 | 16 | 128 | 256 | 256 | 256 |
| 91433 | 16 | 16 | >128 | >128 | >128 |
| 101243 | 4 | 128 | 256 | 256 | 256 |
| 114228 | 16 | 8 | >128 | >128 | >128 |
| 200** | 16 | 0.5 | 256 | 128 | 128 |
| 750** | 32 | 0.5 | >256 | 256 | 128 |

Table S2. Combination studies of compounds **1** – **3** with different antibiotics against WT *P. aeruginosa* PAO1. MICs are reported in µg/ml. FICI = Fractional inhibitory concentration index. FICI is the sum of the FICs of antibiotic and adjuvant. FICI of ≤ 0.5 , $> 0.5 - 4$, and > 4 indicate synergy, additive or no interaction, and antagonism, respectively. Synergistic combinations are highlighted in green.

| Antibiotics (MIC alone) | MIC of Antibiotics (FICI) in the presence of ≤ 10 µM of | | |
|-------------------------|--|------------|-------------|
| | 1 | 2 | 3 |
| Rifampicin (16) | 4 (0.28) | 4 (0.28) | 2 (0.15) |
| Tobramycin (1) | 1 (1.03) | 1 (1.03) | 1 (1.03) |
| Minocycline (8) | 2 (0.26) | 2 (0.28) | 2 (0.28) |
| Cefotaxime (16) | 16 (1.03) | 16 (1.03) | 16 (1.03) |
| Doxycycline (16) | 1 (0.09) | 0.5 (0.06) | 1 (0.09) |
| Tigecycline (4) | 2 (0.51) | 2 (0.51) | 2 (0.51) |
| Linezolid (1024) | 1024 (1.03) | 128 (0.15) | 256 (0.28) |
| Meropenem (1) | 1 (1.03) | 1 (1.03) | 1 (1.03) |
| Moxifloxacin (1) | 1 (1.03) | 0.5 (0.53) | 1 (1.03) |
| Trimethoprim (64) | 64 (1.03) | 32 (0.53) | 16 (0.28) |
| Vancomycin (256) | 256 (1.03) | 256 (1.03) | 128 (0.53) |
| Aztreonam (4) | 4 (1.03) | 4 (1.03) | 4 (1.03) |
| Ceftazidime (2) | 2 (1.03) | 2 (1.03) | 2 (1.03) |
| Fosfomycin (16) | 16 (1.03) | 2 (0.15) | 4 (0.28) |
| Nitrofurantoin (512) | 512 (1.00) | 256 (0.53) | 256 (0.53) |
| Chloramphenicol (32) | 8 (0.28) | 1 (0.06) | 2 (0.09) |
| Clindamycin (1024) | 512 (0.53) | 256 (0.28) | 256 (0.26) |
| Erythromycin (256) | 128 (0.53) | 32 (0.15) | 8 (0.06) |
| Pleuromutilin (512) | 512 (1.03) | 128 (0.28) | 256 (0.53) |
| Novobiocin (512) | 256 (0.53) | 128 (0.28) | 128 (0.28) |
| Colistin (1) | 0.5 (0.53) | 0.5 (0.53) | 0.25 (0.26) |

Table S3. Susceptibility profiles of MDR/XDR *P. aeruginosa* isolates used in this study

| Stock # | PTZ | A/C | AZT | FOX | CFZ | CTR | CPM | CAZ | IMI | MER | DOR | ETP | CIP | MOX | TOB | GEN | AMK | TGC | MIN | DOX | CST | CAM | RIF |
|-------------|-----|-----|-----|-----|------|-----|-----|------|-----|------|-------|-----|-----|-----|------|------|-----|-----|-----|------|-----|------|-----|
| 100036 | 8 | >32 | 16 | >32 | >128 | 32 | 4 | 8 | 8 | 4 | 16 | >32 | >16 | >16 | 128 | >32 | 32 | 32 | 16 | 64 | 2 | 1024 | 16 |
| 101885 | 16 | >32 | 16 | >32 | >128 | 32 | 8 | 8 | 1 | 4 | 4 | 32 | >16 | >16 | 1 | ≤0.5 | ≤1 | 8 | 8 | 64 | 1 | 512 | 32 |
| P259-96918 | 64 | >32 | 32 | >32 | >128 | >64 | >64 | 512 | 32 | 1024 | >1024 | >32 | >16 | >16 | 256 | >32 | >64 | 32 | 32 | 32 | 1 | 1024 | 16 |
| P260-97103 | 128 | >32 | 64 | >32 | >128 | >64 | 16 | 32 | 32 | 16 | 16 | >32 | 16 | >16 | 32 | >32 | 4 | 16 | 16 | 16 | 1 | 128 | 16 |
| P262-101856 | 64 | >32 | 32 | >32 | >128 | 64 | 32 | 16 | 32 | 32 | 16 | >32 | >16 | >16 | 1024 | >32 | >64 | 32 | 64 | 1024 | 1 | 2048 | 512 |
| P264-104354 | 256 | >32 | 64 | >32 | >128 | >64 | 32 | 128 | 32 | 64 | 16 | >32 | >16 | >16 | 128 | >32 | 8 | 32 | 32 | 64 | 1 | 4096 | 16 |
| 91433 | 64 | >32 | 512 | >32 | >128 | >64 | 16 | 1024 | 32 | 16 | 16 | >32 | 2 | 16 | 16 | 32 | >32 | 32 | 16 | 32 | 4 | 8 | 16 |
| 101243 | 128 | >32 | 32 | >32 | >128 | >64 | 64 | 64 | 16 | 16 | 16 | >32 | 1 | 8 | 128 | >32 | >64 | ND | 2 | 4 | >16 | 1 | 4 |

PTZ: piperacillin-tazobactam; A/C: amoxicillin-clavulanic acid; AZT: aztreonam; FOX: ceftiofur; CFZ: cefazolin; CTR: ceftriaxone; CPM: cefepime; CAZ: ceftazidime; IMI: imipenem; MER: meropenem; DOR: doripenem; ETP: ertapenem; CIP: ciprofloxacin; MOX: moxifloxacin; TOB: tobramycin; GEN: gentamicin; AMK: amikacin; TGC: tigecycline; MIN: minocycline; DOX: doxycycline; CST: colistin; CAM: chloramphenicol; RIF: rifampicin; ND: not determined.

Table S4. Synergistic effects of compounds **2** and **3** with doxycycline (DOX) and chloramphenicol (CAM) against other Gram-negative bacteria. MICs are reported in µg/ml. AB is *Acinetobacter baumannii*; KP is *Klebsiella pneumoniae*. Synergistic combinations are highlighted in green.

| Strain | Antibiotic (MIC) | Conjugate (MIC) | MIC _{Antibiotic} in the presence of conjugate | FICI |
|---------------------------|------------------|-----------------|--|--------|
| AB ATCC 17978 | DOX (0.25) | 2 (64) | 0.25 | 1.250 |
| | | 3 (64) | 0.25 | 1.250 |
| | CAM (64) | 2 (64) | 32 | 0.750 |
| | | 3 (64) | 64 | 0.750 |
| <i>E. coli</i> ATCC 25922 | DOX (0.5) | 2 (32) | 0.125 | 0.500 |
| | | 3 (16) | 0.25 | 0.625 |
| | CAM (2) | 2 (32) | 1 | 0.750 |
| | | 3 (16) | 1 | 0.625 |
| KP 16381 | DOX (128) | 2 (>128) | 64 | <0.516 |
| | | 3 (>128) | 64 | <0.516 |
| | CAM (1024) | 2 (>128) | 256 | <0.375 |
| | | 3 (>128) | 256 | <0.281 |
| <i>E. cloacae</i> 117029 | DOX (64) | 2 (64) | 16 | 0.500 |
| | | 3 (64) | 32 | 0.375 |
| | CAM (8) | 2 (16) | 1 | 0.375 |
| | | 3 (16) | 1 | 0.375 |

Table S5.

A) Combination of tobramycin (TOB) alone or rifampicin (RIF) alone with doxycycline (DOX) or chloramphenicol (CAM) against MDR/XDR *P. aeruginosa* (PA) isolates used in this study.

| PA strain | MIC _{DOX} | MIC _{Combination} | FIC _{DOX} | MIC _{TOB} | MIC _{Combination} | FIC _{TOB} | FICI | Interpretation |
|-----------|--------------------|----------------------------|--------------------|--------------------|----------------------------|--------------------|-------|----------------|
| 100036 | 64 | 64 | 1 | 128 | 64 | 0.5 | 1.5 | No Interaction |
| 264 | 64 | 64 | 1 | 128 | 64 | 0.5 | 1.5 | No Interaction |
| 101243 | 4 | 4 | 1 | 128 | 64 | 0.5 | 1.5 | No Interaction |
| 262 | 1024 | 1024 | 1 | >256 | 256 | ≤0.5 | ≤1.5 | No Interaction |
| 259 | 32 | 32 | 1 | 256 | 32 | 0.125 | 1.125 | No Interaction |
| 260 | 16 | 16 | 1 | 32 | 16 | 0.5 | 1.5 | No Interaction |
| 91433 | 32 | 32 | 1 | 16 | 8 | 0.5 | 1.5 | No Interaction |

| PA strain | MIC _{DOX} | MIC _{Combination} | FIC _{DOX} | MIC _{RIF} | MIC _{Combination} | FIC _{RIF} | FICI | Interpretation |
|-----------|--------------------|----------------------------|--------------------|--------------------|----------------------------|--------------------|------|----------------|
| 100036 | 64 | 64 | 1 | 16 | 4 | 0.25 | 1.25 | No Interaction |
| 264 | 64 | 64 | 1 | 8 | 2 | 0.25 | 1.25 | No Interaction |
| 262 | 1024 | 1024 | 1 | 512 | 256 | 0.5 | 1.5 | No Interaction |
| 101243 | 4 | 4 | 1 | 4 | 2 | 0.5 | 1.5 | No Interaction |

| PA strain | MIC _{CAM} | MIC _{Combination} | FIC _{CAM} | MIC _{TOB} | MIC _{Combination} | FIC _{TOB} | FICI | Interpretation |
|-----------|--------------------|----------------------------|--------------------|--------------------|----------------------------|--------------------|------|----------------|
| 100036 | 1024 | 1024 | 1 | 128 | 64 | 0.5 | 1.5 | No Interaction |
| 264 | 4096 | 2048 | 0.5 | 128 | 64 | 0.5 | 1.0 | No Interaction |
| 101243 | 1 | 1 | 1 | 128 | 64 | 0.5 | 1.5 | No Interaction |
| 262 | 2048 | 1024 | 0.5 | >256 | 256 | ≤0.5 | ≤1.0 | No Interaction |
| 259 | 1024 | 1024 | 1 | 256 | 64 | 0.25 | 1.25 | No Interaction |
| 260 | 128 | 64 | 0.5 | 32 | 16 | 0.5 | 1.0 | No Interaction |
| 91433 | 8 | 8 | 1 | 16 | 8 | 0.5 | 1.5 | No Interaction |

| PA strain | MIC _{CAM} | MIC _{Combination} | FIC _{CAM} | MIC _{RIF} | MIC _{Combination} | FIC _{RIF} | FICI | Interpretation |
|-----------|--------------------|----------------------------|--------------------|--------------------|----------------------------|--------------------|------|----------------|
| 100036 | 1024 | 1024 | 1 | 16 | 8 | 0.5 | 1.5 | No Interaction |
| 264 | 4096 | 4096 | 1 | 8 | 4 | 0.5 | 1.5 | No Interaction |
| 262 | 1024 | 1024 | 1 | 512 | 256 | 0.5 | 1.5 | No Interaction |
| 101243 | 1 | 1 | 1 | 4 | 2 | 0.5 | 1.5 | No Interaction |

B) Combination studies of doxycycline + tobramycin + rifampicin, and chloramphenicol + tobramycin + rifampicin. *Wild-type (concentration of tobramycin used = 0.25 µg/mL).

| PA strain | MIC of Doxycycline | | | | Interpretation |
|-----------|--------------------|---------------|-----------------|-------------------------------|----------------|
| | Alone | + 8 µg/mL TOB | + (x µg/mL) RIF | + 8 µg/mL TOB + (x µg/mL) RIF | |
| 100036 | 64 | 64 | 64 (4) | 32 (4) | No Interaction |
| 259 | 32 | 32 | 32 (2) | 16 (2) | No Interaction |
| 260 | 16 | 16 | 16 (2) | 8 (2) | No Interaction |
| 262 | 1024 | 1024 | 1024 (16) | >256 (16) | No Interaction |
| 264 | 64 | 64 | 32 (4) | 32 (4) | No Interaction |
| PAO1* | 16 | 8 | 16 (8) | 8 (8) | No Interaction |

| PA strain | MIC of Chloramphenicol | | | | Interpretation |
|-----------|------------------------|---------------|-----------------|-------------------------------|----------------|
| | Alone | + 8 µg/mL TOB | + (x µg/mL) RIF | + 8 µg/mL TOB + (x µg/mL) RIF | |
| 100036 | 1024 | 1024 | 1024 (4) | 1024 (4) | No Interaction |
| 259 | 1024 | 1024 | 1024 (2) | 1024 (2) | No Interaction |
| 260 | 128 | 128 | 128 (2) | 64 (2) | No Interaction |
| 262 | 2048 | 2048 | 2048 (16) | >512 (16) | No Interaction |
| 264 | 4096 | 4096 | 4096 (4) | >512 (4) | No Interaction |
| PAO1* | 32 | 32 | 32 (8) | 16 (8) | No Interaction |

Table S6. Synergistic effects of compound **2** with tigecycline (TGC), minocycline (MIN), and rifampicin (RIF) against MDR/XDR *P. aeruginosa* (PA) clinical isolates. MICs are reported in µg/ml. FICI of ≤ 0.5 , $> 0.5 - 4$, and > 4 indicate synergy, additive or no interaction, and antagonism, respectively. ND = not determined. Synergistic combinations are highlighted in green.

| PA strain | Antibiotic | MIC _{Antibiotic} | MIC _{Combination} | FIC _{Antibiotic} | MIC ₂ | MIC _{Combination} | FIC ₂ | FICI |
|-----------|------------|---------------------------|----------------------------|---------------------------|------------------|----------------------------|------------------|-------|
| 100036 | TGC | 32 | 4 | 0.125 | 256 | 1 | 0.004 | 0.13 |
| | MIN | 16 | 1 | 0.063 | 256 | 16 | 0.063 | 0.13 |
| | RIF | 16 | 4 | 0.250 | 256 | 16 | 0.063 | 0.31 |
| 101885 | TGC | 8 | 1 | 0.125 | 256 | 16 | 0.063 | 0.19 |
| | MIN | 8 | 0.5 | 0.063 | 256 | 16 | 0.063 | 0.13 |
| | RIF | 32 | 0.5 | 0.016 | 256 | 16 | 0.063 | 0.08 |
| 259 | TGC | 32 | 4 | 0.125 | >128 | 16 | <0.125 | <0.25 |
| | MIN | 32 | 1 | 0.031 | >128 | 16 | <0.125 | <0.16 |
| | RIF | 16 | 2 | 0.125 | >128 | 16 | <0.125 | <0.25 |
| 260 | TGC | 16 | 0.5 | 0.031 | >128 | 16 | <0.125 | <0.16 |
| | MIN | 16 | 0.03 | 0.002 | >128 | 16 | <0.125 | <0.13 |
| | RIF | 16 | 0.5 | 0.031 | >128 | 16 | <0.125 | <0.16 |
| 262 | TGC | 32 | 4 | 0.125 | >256 | 16 | <0.063 | <0.19 |
| | MIN | 64 | 2 | 0.031 | >256 | 16 | <0.063 | <0.09 |
| | RIF | 512 | 256 | 0.500 | >256 | 2 | <0.008 | <0.51 |
| 264 | TGC | 32 | 4 | 0.125 | 256 | 16 | 0.063 | 0.19 |
| | MIN | 32 | 0.5 | 0.016 | 256 | 16 | 0.063 | 0.08 |
| | RIF | 16 | 4 | 0.250 | 256 | 4 | 0.016 | 0.27 |
| 91433 | TGC | 32 | 32 | 1.000 | >128 | 16 | <0.125 | 1.13 |
| | MIN | 16 | 2 | 0.125 | >128 | 16 | <0.125 | <0.25 |
| | RIF | 16 | 16 | 1.000 | >128 | 16 | <0.125 | 1.13 |
| 101243 | TGC | ND | ND | ND | >256 | ND | ND | ND |
| | MIN | 2 | 0.5 | 0.250 | >256 | 16 | <0.063 | <0.31 |
| | RIF | 4 | 2 | 0.500 | 256 | 16 | 0.063 | 0.56 |
| 114228 | TGC | 16 | 8 | 0.500 | >128 | 4 | <0.031 | <0.53 |
| | MIN | 16 | 4 | 0.250 | >128 | 4 | <0.031 | <0.28 |
| | RIF | 8 | 4 | 0.500 | >128 | 4 | <0.031 | <0.53 |

HPLC Analysis

Method

Mobile Phase: A = 0.05% FA in water. B = 0.05% FA in acetonitrile.

Column = SynergiTM 4 μ m Polar-RP 80 Å LC column (50 \times 4.6 mm, Phenomenex).

Flow rate = 0.7 mL/min

UV detection wavelength = 332 nm

Gradient

| Time (min) | % Buffer A | % Buffer B |
|------------|------------|------------|
| 0 | 90 | 10 |
| 4 | 90 | 10 |
| 4.5 | 85 | 14 |
| 5.5 | 80 | 20 |
| 7 | 70 | 30 |
| 8 | 65 | 35 |
| 9.5 | 65 | 35 |
| 11 | 50 | 50 |
| 13 | 50 | 50 |
| 16 | 70 | 30 |
| 19 | 85 | 15 |

5 CHAPTER FIVE:

Tobramycin–Cyclam Conjugates Overcome Resistance to β -Lactam Antibiotics

5.1 Preface

The work presented in this chapter has been published as a research article in:

Temilolu Idowu, Derek Ammeter, Gilbert Arthur, George G. Zhanel, and Frank Schweizer (2019). Potentiation of β -Lactam Antibiotics and β -Lactam/ β -Lactamase Inhibitor Combinations against Multidrug and Extensively Drug-resistant *Pseudomonas aeruginosa* using Non-ribosomal Tobramycin–Cyclam Conjugates. *J Antimicrob Chemother.* <https://doi.org/10.1093/jac/dkz228>

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5.1.1 Authors' Contributions

Temilolu Idowu and Derek Ammeter carried out chemical synthesis and compound characterization. Temilolu Idowu performed all biological studies and *in vivo* experiments. Gilbert Arthur conducted cytotoxicity testing. George G. Zhanel provided helpful insights on the project. Frank Schweizer supervised the project. Temilolu Idowu analyzed the data and wrote the manuscript. All authors were responsible for the final form of the manuscript.

5.2 Abstract

Objectives: Our aim was to develop a multifunctional adjuvant molecule that can rescue β -lactam antibiotics and β -lactam/ β -lactamase inhibitor combinations from resistance in carbapenem-resistant *Pseudomonas aeruginosa* clinical isolates

Methods: Preparation of adjuvant was guided by structure-activity relationships, following standard protocols. Susceptibility and checkerboard studies were assessed using serial 2-fold dilution assays. Toxicity was evaluated against porcine erythrocytes, human embryonic kidney (HEK293) and liver carcinoma (HepG2) cells via MTS assay. Preliminary *in vivo* efficacy was evaluated using *Galleria mellonella* infection model.

Results: Conjugation of tobramycin and cyclam abrogates the ribosomal effects of tobramycin but confers a potent adjuvant property that restores full antibiotic activity of meropenem and aztreonam against carbapenem-resistant *P. aeruginosa*. Therapeutic levels of susceptibility, as determined by CLSI susceptibility breakpoints, were attained in several multidrug-resistant clinical isolates and time-kill assays revealed a synergistic dose-dependent pharmacodynamic relationship. A triple combination of the adjuvant with ceftazidime/avibactam (approved), aztreonam/avibactam (Phase III), and meropenem/avibactam enhances the efficacies of β -lactam/ β -lactamase inhibitors against recalcitrant strains, suggesting rapid access of the combination to their periplasmic targets. The newly developed adjuvants, and their combination thereof, were non-haemolytic and non-cytotoxic, and preliminary *in vivo* evaluation in *G. mellonella* suggests therapeutic potentials for the double and triple combinations.

Conclusions: Non-ribosomal tobramycin-cyclam conjugate mitigates the effect of OprD/OprF porin loss in *P. aeruginosa* and potentiates β -lactam/ β -lactamase inhibitors against carbapenem-resistant clinical isolates, highlighting the complexity of resistance to β -lactam antibiotics. Our strategy presents an avenue to further preserve the therapeutic utility of β -lactam antibiotics.

5.3 Introduction

The emergence of carbapenem-resistant Gram-negative bacteria is considered a public health crisis and a major global threat to all the clinical procedures that rely on effective antibiotic therapy.¹ Carbapenem resistance in Gram-negative pathogens is almost always associated with resistance to several other classes of antibiotics because carbapenemase-encoding genes are located on mobile genetic elements that frequently carry genes responsible for resistance to other antibiotics.^{1,2} β -Lactam antibiotics (penicillins, cephalosporins, carbapenems and monobactams) are cornerstone agents in the treatment of Gram-negative bacterial infections, especially against *Pseudomonas aeruginosa* with low outer membrane (OM) permeability and limited treatment options.^{3–5} They are the most widely-used group of antibiotics and they exhibit their bactericidal effects by covalently binding to the penicillin-binding proteins (PBPs) involved in cell wall synthesis.⁶ Unfortunately, primary mechanisms of resistance such as changes in the active site of PBPs, decreased porin expression, augmented antibiotic efflux and the dissemination of β -lactamase-encoding gene have threatened the continuous use of this highly important class of antibiotics from an already shrinking antibiotic arsenal.⁷ To preserve the therapeutic utility of β -lactams, efforts have been geared towards developing newer generations that can withstand enzymatic degradation and/or develop partner molecules that could shield the β -lactam core from enzymatic inactivation.⁷ However, bacteria often develop multiple resistance mechanisms that work concertedly to stop antibiotic activity,⁵ including lipid A modification that prevents permeation of polybasic molecules.^{8,9} Importantly, complete loss or diminished expression of the OprD and OprF OM proteins, especially in *P. aeruginosa* that can survive by utilizing other protein channels^{10–12} can bestow microbiological resistance to both β -lactam antibiotics and β -lactamase inhibitors.^{13,14} OprD protein is a common channel for uptake of basic amino acids and small peptides that share structural similarities with carbapenems; thus, amino acids can induce up-regulation of OprD protein expression, while trace metals such as zinc and copper can decrease OprD expression in *P. aeruginosa* via two-component regulatory systems, CzcR–CzcS and copR–copS, respectively.^{15–17}

Therefore, OM permeabilization and/or induction of porin expression may restore potent activity of β -lactam antibiotics against MDR pathogens.

Aminoglycosides (AGs) are a unique class of antipseudomonal agents in that they promote their own uptake via a porin-independent mechanism.¹⁸ AGs interfere with the fidelity of ribosomal protein translation at low concentrations (≤ 4 mg/L) and disrupt the OM at higher concentrations (≥ 8 mg/L).¹⁹ The consequence of the ribosomal effects of AGs, as do other antibiotics, is the selection of resistant genotypes and phenotypes.²⁰ To exploit the OM properties of AGs without generating resistant mutants, specific modifications and systematic conjugation to other moieties have been employed to decouple OM effects from ribosomal functions.^{21,22} These compounds were found to potentiate several OM-impermeable antibiotics against Gram-negative bacteria but, surprisingly, do not potentiate carbapenems.^{23,24}

Putting these findings into perspective, we envisaged the development of a multifunctional adjuvant molecule that combines the OM-permeabilizing properties of non-ribosomal amphiphilic tobramycins with a metal-chelating property of a secondary moiety. By sequestering divalent metals, a chelating agent may not only augment the OM-disrupting properties of amphiphilic tobramycin, but it may also prevent downregulation of OprD proteins by inactivating the CzcR–CzcS/copR–copS two-component regulatory systems.^{15–17} EDTA is presumably toxic at clinically-relevant concentrations,²⁵ but chelating agents such as aspergillomarasmine A²⁶ and cyclam^{27,28} are non-toxic at therapeutic concentrations. Cyclam (1,4,8,11-tetraazacyclotetradecane; Figure 5.1) was therefore investigated as a potential companion molecule to an amphiphilic tobramycin. Herein, we report the development of a non-toxic antibiotic adjuvant that overcomes resistance to β -lactam antibiotics and restores full antibiotic activity against carbapenem-resistant clinical isolates.

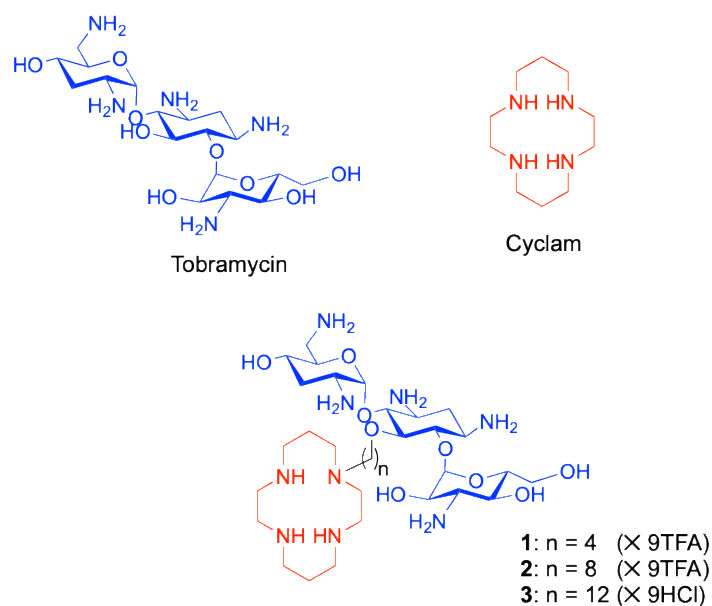


Figure 5.1. Structures of Tobramycin, Cyclam, and newly synthesized Tobramycin-Cyclam conjugates **1** – **3**. Conjugates differ in the length of carbon chains.

5.4 Materials and Methods

5.4.1 Design and Synthesis

The design of adjuvants **1–3** (Figure 5.1) was established from previous structure–activity relationship studies.²³ Amphiphilic tobramycins conjugated at the 5-OH of deoxystreptamine lose ribosomal activities but retain membrane effects.^{22,23} A lipophilic neamine grafted to cyclam has also been reported to permeabilize the OM of *E. aerogenes*.²⁹ Thus, tobramycin and cyclam were conjoined at the C-5 position of tobramycin, via reductive amination, using aliphatic hydrocarbons of different tether lengths (Scheme S1, available as Supplementary 5.8). The prepared compounds were characterized by NMR and mass spectrometry. The purity (>95%) of the compounds was determined by elemental analysis (Table S1). These data, together with the method of preparation, are presented in the supplementary data (5.8) available at JAC online (<http://jacoxfordjournals.org/>).

5.4.2 Bacterial strains

Bacterial isolates were either obtained from either the American Type Culture Collection (ATCC), CAN-ICU³⁰ or CANWARD^{31,32} surveillance studies. Clinical isolates were cultured from body fluids and tissues of patients suffering from presumed “clinically-significant” infectious diseases. Antimicrobial susceptibilities of clinical isolates were categorized, where appropriate, as either MDR, XDR, or pan-drug resistant (PDR).³³

5.4.3 Susceptibility and Checkerboard Assays.

Antimicrobial susceptibility of bacteria were assessed by the microbroth dilution method, in accordance with CLSI guidelines,³⁴ and synergistic interactions were assessed by checkerboard assays, as previously described (Chapters 4.7.2.14.7.2.2).²⁴ Fractional inhibitory concentration indices (FICI) of ≤ 0.5 were deemed synergistic; 0.5–4, as no interaction; and >4 , as antagonistic.³⁵

5.4.4 Time-kill assay

Time-kill curve analyses were performed by culturing *P. aeruginosa* in LB medium in the presence of antibiotics, alone and in combination with compound **2**. A 30 μ L aliquot of a 0.5 McFarland inoculum of each strain was diluted to 3 mL of LB broth (containing different combinations of antimicrobial agents and adjuvants) and incubated at 37°C with shaking at 250 rpm. At specific time intervals, 100 μ L was taken from each sample, serially diluted in sterile PBS, plated on LB agar plates and incubated at 37°C in a humid 5% CO₂-enriched atmosphere. Bacteria colonies were counted after 20 h of incubation.

5.4.5 *In vivo* larvae infection model

Preclinical *in vivo* synergistic effects were determined using the *Galleria mellonella* infection model, as previously described (Chapter 4.7.2.4).²³ The virulence and bacterial load required to kill 100% of the larvae within 12–18 h (no treatment) was determined to be ~5 cfu. A 10 µL aliquot of the standardized culture solution was injected into each larva, incubated at 37°C for 3 h and then treated with different concentrations of mono-, double and triple combination therapy. Larvae treated with 10 µL of PBS or high concentrations of test antibiotics served as a negative and positive control, respectively. Upon treatment, the larvae were further incubated at 37°C and scored for survivability every 6 h for 48 h. The experiment was repeated to give a total of 30 worms in each case. Larvae were considered dead if they do not respond to touch.

5.4.6 Cytotoxicity Assay

Human embryonic kidney (HEK293) cells and liver carcinoma (HepG2) cells were grown in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a humidified 5% atmospheric CO₂ incubator at 37°C. Cytotoxicity of compounds was evaluated as previously described (Chapter 4.7.2.5),^{36,37} using doxorubicin as a positive control.

5.5 Results

5.5.1 Susceptibility and Toxicity Screening

The antimicrobial activities of the resulting compounds **1–3** were assessed against a panel of Gram-positive and Gram-negative bacteria and compared to the parent molecule, tobramycin (Table S2). Tobramycin exhibited low MIC values (≤ 2 mg/L) against susceptible strains, indicating potency, while the MICs of adjuvants **1–3** were significantly higher against all strains (≥ 16 mg/L), indicating

loss of activity. This is consistent with our hypothesis that a C-5 conjugation will abolish the ribosomal effects of tobramycin. To establish toxicity profiles, we investigated and found that compounds **1–3** were: i) non-hemolytic (<1% hemolysis at 1024 mg/L), ii) non-cytotoxic against HEK293 and HepG2 cells at 50 μ M (>80 % viability at ~128 mg/L), and iii) non-toxic *in vivo* in *Galleria mellonella* wax moths at 200 mg/kg (Figure S1). Conversely, a 100 mg/kg colistin resulted in 70% mortality of the larvae after 48 h (Figure S1).

5.5.2 Evaluation of Compound Synergies with different Antibiotics

The lack of intrinsic activity and non-toxic properties of compounds **1–3** encouraged us to screen their adjuvant properties. An ideal adjuvant is a bioactive helper molecule that is inactive by itself but can potentiate the activity of a primary antibiotic.³⁸ To investigate this, checkerboard assay was used to assess interactions between compounds **1–3** and twenty-three different antibiotics against wild-type *P. aeruginosa* PAO1. *P. aeruginosa* was selected because OM permeability is a major mechanism by which it evades antibiotic activity.⁵ Theoretically, synergy is defined by the ability of compound A at $\frac{1}{4}$ MIC to reduce the MIC of compound B by at least 4-fold.³⁹ In practice, a low concentration of the adjuvant is often desired. The MICs of compounds **1–3** against PAO1 is ≥ 512 mg/L, giving a theoretical synergistic concentration of ≥ 128 mg/L. However, a working concentration of ≤ 16 mg/L (≤ 10 μ M) was used in this study, a concentration that may be readily achieved in human plasma based on the achievable plasma concentrations of aminoglycosides (20–200 μ M, i.e. 10–113 mg/L).^{40,41} Compounds **1–3** strongly potentiate the effects of different classes of antibiotics against PAO1, but not tobramycin, nitrofurantoin and colistin (Figure 5.2A, Table S3). None of the combinations exhibited an antagonistic relationship, while tobramycin by itself did not potentiate any antibiotic against PAO1 (FICI ≥ 0.75) (Table S4). Potentiation of β -lactam antibiotics was quite remarkable as they are not typically potentiated by traditional OM-permeabilizers (including polymyxin B nonapeptide) and other amphiphilic tobramycins at such concentrations.^{22–24} Compound

2 is the most potent and least toxic of the three analogs (Figure 5.2A and Figure S1), hence, it was selected for further studies with β -lactam antibiotics.

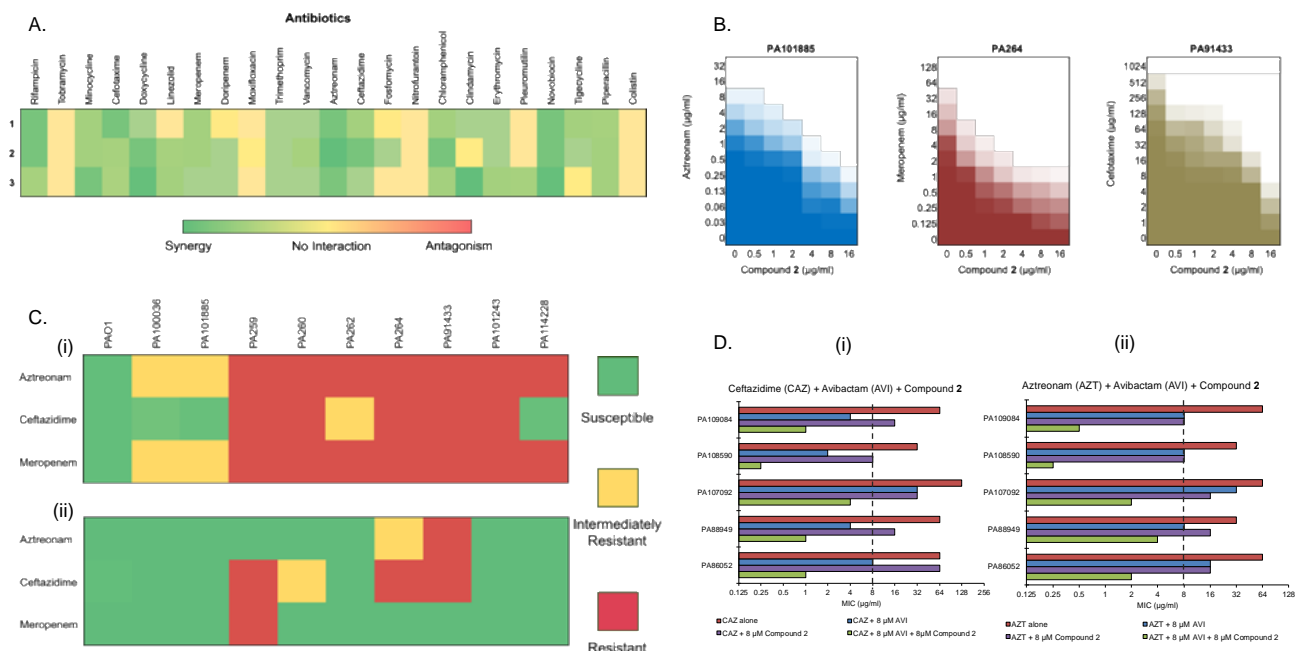


Figure 5.2. A) Interactions of compounds **1** – **3** ($\leq 10 \mu\text{M}$) with different antibiotics against *P. aeruginosa* PAO1. $\text{FIC} \leq 0.5$ = Green; $0.5 < \text{FIC} < 4$ = Yellow. B) Dose-dependent potentiation of β -lactam antibiotics against MDR *P. aeruginosa*. C) CLSI susceptibility profiles of WT and MDR *P. aeruginosa* when treated with select β -lactam antibiotics i) alone, ii) in combination with $8.7 \mu\text{M}$ of compound **2**. D) Triple combination therapy versus double combination versus monotherapy against β -lactamase-harboring MDR *P. aeruginosa* isolates. $8 \mu\text{M}$ avibactam $\sim 2 \text{ mg/L}$; $8 \mu\text{M}$ compound **2** $\sim 16 \text{ mg/L}$.

5.5.3 Restoration of β -lactam Activities against Carbapenem-resistant *P.*

aeruginosa Clinical Isolates

To investigate whether potentiation is conserved beyond wild-type PAO1, synergistic relationships between compound **2** and six β -lactam antibiotics were assessed against nine MDR/XDR *P. aeruginosa* isolates exhibiting multiple resistance patterns (Table S5). Compound **2** retained the ability to potentiate all six antibiotics against all isolates in a dose-dependent fashion (Figure 5.2B), and increased susceptibilities by 4- to 128-fold (Table 5.1, Figure S2). Notably, susceptibilities equal to or below CLSI breakpoints were attained for aztreonam (≤ 8 mg/L) in seven out of nine isolates, ceftazidime (≤ 8 mg/L) in three out of six and meropenem (≤ 2 mg/L) in eight out of nine carbapenem-resistant isolates (Figure 5.2C). In contrast, none of the combinations attained the susceptibility breakpoint in other Gram-negative bacteria, except for ceftazidime/compound **2** combination against *Acinetobacter baumannii* 110193 (Table S6). Aztreonam and meropenem were more potentiated in *P. aeruginosa* than in other Gram-negative bacteria while piperacillin and ceftazidime were more potentiated in MDR *A. baumannii* than in *P. aeruginosa* (Table 5.1 and S6). Overall, nine out of nine MDR/XDR *Pseudomonas* phenotypes demonstrate the attainment of therapeutic levels of susceptibilities to at least one of the six β -lactam/compound **2** combinations, with aztreonam and meropenem showing the best therapeutic potential in seven out of nine and eight out of nine, respectively (Figure 5.2C). Tobramycin alone, cyclam alone or a combination of both did not potentiate aztreonam or meropenem against any isolate (FICI >0.56) (Table S7). To establish whether synergy was specific to Gram-negative bacteria, we assessed the combination against Gram-positive bacteria and found no synergistic relationship (Table S8), suggesting OM permeabilization in Gram-negative but not Gram-positive bacteria as the primary mechanism of action.

Table 5.1. **Compound 2 (8.7 μ M) potentiates β -lactam antibiotics against MDR/XDR *P. aeruginosa* clinical isolates.** MICs are reported in mg/L. FICI of ≤ 0.5 , $> 0.5 - 4$, and > 4 indicate synergy, additive or no interaction, and antagonism, respectively. AZT = aztreonam; CAZ = ceftazidime; PIP = piperacillin; MER = meropenem; DOR = doripenem; CEF = cefotaxime; ND = not determined.

| Strain | Antibiotic | MIC (mg/L) of Antibiotic | | Fold Potentiation | FICI |
|-----------|------------|--------------------------|--------------|-------------------|-------|
| | | Alone | + Compound 2 | | |
| PA 100036 | AZT | 16 | 2 | 8 | <0.16 |
| | CAZ | 8 | 2 | 4 | <0.28 |
| | PIP | 64 | 64 | 1 | <1.06 |
| | MER | 4 | 2 | 2 | <0.51 |
| | DOR | 16 | 8 | 2 | <0.56 |
| | CEF | 32 | 8 | 4 | <0.31 |
| PA 101885 | AZT | 16 | 0.5 | 32 | <0.09 |
| | CAZ | 8 | 1 | 8 | <0.16 |
| | PIP | 16 | 4 | 4 | <0.31 |
| | MER | 4 | 0.5 | 8 | <0.16 |
| | DOR | 4 | 1 | 4 | <0.27 |
| | CEF | 128 | 8 | 16 | <0.13 |
| PA 259 | AZT | 32 | 4 | 8 | <0.19 |
| | CAZ | 512 | 64 | 8 | <0.19 |
| | PIP | 512 | 256 | 2 | <0.50 |
| | MER | 1024 | 256 | 4 | <0.31 |
| | DOR | >1024 | ND | ND | ND |
| | CEF | 2048 | 512 | 4 | <0.31 |
| PA 260 | AZT | 64 | 8 | 8 | <0.19 |
| | CAZ | 32 | 16 | 2 | <0.52 |
| | PIP | 512 | 512 | 1 | <1.06 |
| | MER | 8 | 1 | 8 | <0.16 |
| | DOR | 16 | 8 | 2 | <0.53 |
| | CEF | 1024 | 512 | 2 | <0.53 |
| PA 262 | AZT | 32 | 8 | 4 | <0.28 |
| | CAZ | 16 | 4 | 4 | <0.31 |

| | | | | | |
|-----------|-----|------|------|-----|-------|
| | PIP | 1024 | 512 | 2 | <0.52 |
| | MER | 32 | 2 | 8 | <0.13 |
| | DOR | 16 | 4 | 4 | <0.31 |
| | CEF | 128 | 32 | 4 | <0.31 |
| PA 264 | AZT | 64 | 16 | 4 | <0.28 |
| | CAZ | 128 | 32 | 4 | <0.28 |
| | PIP | 2048 | 1024 | 2 | <0.52 |
| | MER | 64 | 2 | 32 | <0.05 |
| | DOR | 16 | 8 | 2 | <0.51 |
| | CEF | 2048 | 512 | 4 | <0.31 |
| PA 91433 | AZT | 512 | 512 | 1 | <1.06 |
| | CAZ | 1024 | 512 | 2 | <0.50 |
| | PIP | ND | ND | ND | ND |
| | MER | 16 | 0.25 | 64 | <0.03 |
| | DOR | 16 | 1 | 16 | <0.09 |
| | CEF | 1024 | 8 | 128 | <0.07 |
| PA 101243 | AZT | 32 | 8 | 4 | <0.26 |
| | CAZ | 64 | 8 | 8 | <0.13 |
| | PIP | 128 | 128 | 1 | <1.06 |
| | MER | 8 | 2 | 16 | <0.25 |
| | DOR | 16 | 8 | 2 | <0.50 |
| | CEF | 256 | 128 | 2 | <0.50 |
| PA 114228 | AZT | 32 | 4 | 8 | <0.14 |
| | CAZ | 8 | 1 | 8 | <0.19 |
| | PIP | 16 | 8 | 2 | <0.50 |
| | MER | 8 | 2 | 4 | <0.28 |
| | DOR | 8 | 1 | 8 | <0.14 |
| | CEF | 128 | 16 | 8 | <0.14 |

5.5.4 Potentiation of β -lactams is Independent of Resistance–Nodulation–

Division (RND) Efflux Pumps

A previous study has shown that potentiation of aminoglycoside by meropenem is dependent on MexXY efflux pumps.⁴² To investigate whether non-ribosomal tobramycin-cyclam conjugate **2** also interferes with efflux mechanisms in increasing susceptibility of *P. aeruginosa* to β -lactams, two mutant strains, PAO200 and PAO750, lacking different clinically-relevant efflux pumps were assessed. Compound **2** retained its ability to potentiate aztreonam, ceftazidime, meropenem and doripenem in these strains (Table 5.2), an indication that potentiation in *P. aeruginosa* is not dependent on the presence of, or interactions with, these pumps.

Table 5.2. Potentiation of β -lactam antibiotics in *P. aeruginosa* by compound **2** ($\leq 8.7 \mu\text{M}$) is independent of RND efflux pumps. PAO1 = wild-type, PAO200 ($\Delta\text{mexA-mexB-oprM}$) and PAO750 ($\Delta\text{mexAB-oprM}$, $\Delta\text{mexCD-oprJ}$, $\Delta\text{mexEF-oprN}$, $\Delta\text{mex JK}$, ΔmexXY , and ΔopmH) are efflux-deficient mutants. MICs are reported in mg/L.

| Antibiotics | MIC of antibiotic alone in | | | MIC of antibiotic (fold change) + Compound 2 in | | |
|--------------|----------------------------|--------|--------|---|------------|------------|
| | PAO1 | PAO200 | PAO750 | PAO1 | PAO200 | PAO750 |
| Aztreonam | 4 | 0.25 | 0.5 | 0.25 (16) | 0.016 (16) | 0.031 (16) |
| Ceftazidime | 4 | 2 | 1 | 0.5 (8) | 0.125 (16) | 0.125 (8) |
| Piperacillin | 8 | 1 | 2 | 2 (4) | 1 (1) | 0.5 (4) |
| Meropenem | 1 | 0.25 | 1 | 0.25 (4) | 0.063 (4) | 0.25 (4) |
| Doripenem | 1 | 1 | 1 | 0.5 (2) | 0.25 (4) | 0.25 (4) |
| Cefotaxime | 16 | 8 | 32 | 4 (4) | 4 (2) | 2 (16) |

5.5.5 A Triple Combination Enhances β -Lactam/ β -Lactamase Inhibitors

(BLIs) against β -Lactamase-harboring *P. aeruginosa*

We investigated whether a triple combination of β -lactam/BLI/compound **2** will be more efficacious than a double combination of β -lactam/BLI or β -lactam/compound **2**. We evaluated susceptibilities of five β -lactamase-harboring MDR isolates (Table S9) to β -lactam antibiotics (ceftazidime, aztreonam and meropenem) in combination with fixed equimolar concentrations (8 μ M) of avibactam and compound **2**. Avibactam is a BLI that is able to inhibit a wide range of serine β -lactamases (class A, C and D) but not metallo- β -lactamases (class B).⁴³ Our results show that addition of compound **2** to conventional β -lactam/BLI enhances the potency of these combinations *in vitro* (Table S10). Compound **2** potentiated ceftazidime/avibactam by 4- to 16-fold, aztreonam/avibactam by 4- to 32-fold and meropenem/avibactam by 2- to 16-fold (Figure 5.2D and S3). In contrast, a triple combination of compound **2**/ceftolozane/tazobactam was not significantly better than a double combination of compound **2**/ceftolozane (Figure S3).

5.5.6 Time-kill Assay

To gain insights into the kinetics of bacterial growth and death, and provide more complementary data for the checkerboard results, time-kill curves of wild-type PAO1, MDR PA101885 and MDR PA108590 using monotherapy, and double and triple combination therapy were determined. For PAO1, aztreonam (4 mg/L) and meropenem (1 mg/L) exhibited time-dependent bactericidal effects within 6 h and regrowth after 24 h (Figure S4). However, combinations of aztreonam (4 mg/L) and compound **2** (≤ 8.7 μ M) were synergistic and prevented bacterial regrowth, while combinations of meropenem (1 mg/L) and compound **2** resulted in growth curves identical to those of meropenem alone. For carbapenem-resistant PA101885, 8 mg/L of aztreonam resulted in regrowth as early as 6 h post-inoculation, while a combination of aztreonam (8 mg/L) + compound **2**

($\leq 8.7 \mu\text{M}$) resulted in a bactericidal and synergistic relationship with no regrowth (Figure 5.3Ai). A combination of meropenem (2 mg/L) + compound **2** ($\leq 8.7 \mu\text{M}$) resulted in a synergistic relationship against MDR 101885 after 6 h and re-growth after 24 h (Figure S4).

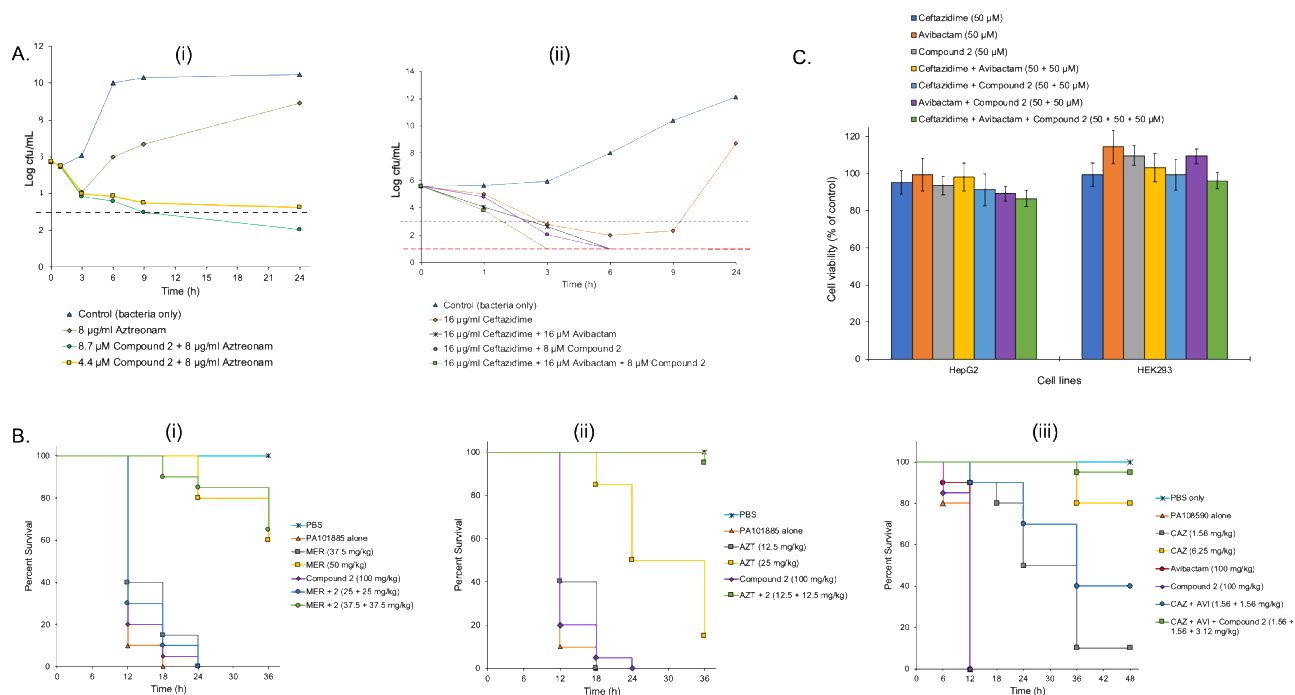


Figure 5.3. A) Time-kill kinetics of i) aztreonam alone and in combination with different concentrations of compound **2** on the viability of MDR *P. aeruginosa* 101885; ii) ceftazidime + avibactam + compound **2** on the viability of MDR *P. aeruginosa* 108590. Dashed black line represents bactericidal level while dashed red line represents the limit of detection. Each data point is an average of three independent determinations. B) Efficacy studies in *G. mellonella* using a single dose administration of i) meropenem (MER), ii) aztreonam (AZT), iii) ceftazidime (CAZ) + avibactam (AVI), alone or in combination with compound **2**. C) Toxicity studies of double and triple combination against HEK293 and HepG2 cell lines. Each data point is an average of four independent determinations.

Similarly, a triple combination of ceftazidime, avibactam and compound **2** was assessed against a β -lactamase-harboring MDR strain, PA108590. Ceftazidime and avibactam were studied at a clinically used ratio of 4 parts to 1 part, and compound **2** was studied at an equimolar concentration to avibactam. At 16 mg/L, ceftazidime alone was bactericidal after 3h while a combination of ceftazidime (16 mg/L) and avibactam (4 mg/L) resulted in an ~ 2 log reduction in bacterial burden within 1 h (Figure 5.3Aii). After 3h of incubation, the bacterial loads of ceftazidime alone and ceftazidime/avibactam were similar, suggesting saturation of β -lactamase enzymes. Conversely, the bacterial load of ceftazidime/avibactam/compound **2** was identical to that of ceftazidime/avibactam after 1h but >2 log lower after 3h. This implies that whereas avibactam exhibited a rapid synergistic effect with ceftazidime after 1h, compound **2** further potentiated the effect of this combination within 3 h. Indeed, the bacterial culture was completely sterilized within 3 h of treatment with a triple combination of ceftazidime, avibactam and compound **2** (~ 6 log reduction). Overall, the ability of compound **2** to potentiate β -lactams is both time and concentration dependent, and reflects, to a large extent, the fold potentiation in checkerboard assay.

5.5.7 In vivo Efficacy in *Galleria mellonella* wax moths

Preliminary *in vivo* investigation on the potency of the combinations was assessed using a *Galleria mellonella* larvae infection model. Despite its limitations, the use of *G. mellonella* larvae to study host-pathogen interactions, virulence, toxicity and efficacy of novel compounds has been demonstrated using clinically-approved agents.^{44–46} The larvae tolerated 200 mg/kg each of β -lactams and compound **2**, while 100 mg/kg of colistin resulted in 70% mortality after 48 h (Figure S1). Untreated larvae challenged with MDR PA101885 and PA108590 died within 18 h of infection. A single dose monotherapy of aztreonam (12.5 mg/kg), meropenem (37.5 mg/kg) or compound **2** (100 mg/kg) also resulted in 0% survival after 24 h. However, a single dose combination therapy of aztreonam/compound **2** (12.5/12.5 mg/kg) and meropenem/compound **2** (37.5/37.5 mg/kg) resulted in

100% and 85% survival, respectively, after 24 h (Figure 5.3B). Furthermore, ceftazidime alone (1.56 mg/kg) and ceftazidime/avibactam (1.56/1.56 mg/kg) resulted in 50% and 70% survival of PA108590-challenged larvae after 24 h while ceftazidime/avibactam/compound **2** (1.56/1.56/3.12 mg/kg) resulted in 100% survival after 24 h.

To ascertain that the combination therapies do not elevate toxicity against eukaryotic cell, we assessed toxicities of different combinations against HEK293 and HepG2 cell lines and found that both double and triple combination therapies were not cytotoxic (Figure 5.3C), ruling out a non-specific mode of action.

5.6 Discussion

The dearth of novel antibacterial drugs in the pipeline means that we must conserve the efficacy of existing antibacterial drugs as much as practically possible. β -Lactam antibiotics are important agents for preventing and treating life-threatening nosocomial infections which are often associated with debilitated patients and/or techniques developed in modern medicine.⁴⁷ Sadly, the global dissemination of β -lactamases and adaptive bacterial responses such as porin loss and overexpressed efflux pumps are threats to the continued utility of β -lactam antibiotics.⁷ With no reprieve in sight, the pursuit of adjuvants that can inhibit the actions of β -lactamases and/or reverse intrinsic/adaptive resistance is critical for the survival of β -lactam antibiotics.

We developed non-toxic non-ribosomal tobramycin-cyclam adjuvants that combine the OM-permeabilizing properties of an amphiphilic aminoglycoside with the chelating properties of a cyclam domain, with a view to destabilizing the OM of *P. aeruginosa* and sequestering divalent metal ions used to downregulate porin expression.^{15–17} The adjuvants potentiate the activities of meropenem, aztreonam and ceftazidime against MDR/XDR *P. aeruginosa*, consistent with an augmented OM permeabilization mechanism, whereas tobramycin alone or other amphiphilic-like tobramycin-based adjuvants do not.^{23,24} A previous report has shown the reversal of carbapenem resistance using

aspergillomarasmine A, a chelating agent that binds the divalent zinc metal used by metallo- β -lactamases (MBLs) to hydrolyse carbapenems.²⁶ However, tobramycin-cyclam adjuvants most probably act via MBL-independent mechanisms due to their ability to potentiate meropenem against both MBL-expressing and MBL-non-expressing strains. Moreover, monobactams are resistant to MBL actions⁴⁸ hence, the ability to strongly potentiate aztreonam supports this hypothesis. The addition of excess MgCl_2 (100 μM) to the media reduced the ability of compound **2** (8 μM) to potentiate β -lactams while ZnSO_4 (100 μM) had no effect. This is consistent with OM permeabilization (Mg^{2+} displacement) but not MBL inhibition.

There is a known history of β -lactam/AG synergism in *P. aeruginosa*,^{42,49–51} with the underlying mechanism believed to be the destruction of cell wall peptidoglycan polymers by β -lactams, thus, facilitating the uptake/entry of AGs that subsequently exert their bactericidal effects. The lack of potent activity by compounds **1–3** indicates abrogation of the ribosomal properties of AGs; hence, synergistic interaction between compound **2** and β -lactams is not due to the intrinsic activity of tobramycin. Moreover, tobramycin alone was found to display an additive relationship with β -lactams ($\text{FICI} > 0.56$; Table S7), consistent with a previous report that identifies tobramycin as an exception to β -lactam/AG synergism.⁴² The involvement of RND efflux pumps in the synergistic interactions of compound **2** and β -lactams appear unlikely because of the ability of compound **2** to potentiate this class of antibiotics in both wild-type and efflux-deficient mutants (Table 5.2). However, agents that alter the transmembrane protein environment (such as membrane charge, fluidity and thickness) and/or steric hindrance of membrane-embedded proteins may prevent the relay of signaling cascades and/or conformational changes necessary to extrude substrate molecules by efflux pumps.^{22,52,53}

P. aeruginosa is often regarded as a highly challenging model organism for new antibiotics but our study revealed that potentiation of aztreonam and meropenem was more pronounced in *P. aeruginosa* than in other Gram-negative bacilli (Table 5.1 and S6). Although differences in exo-/lipopolysaccharides can be hypothesized as a possible reason for this, the best potentiation of

meropenem (64-fold) was achieved in a colistin-resistant isolate PA91433 while susceptibility breakpoints were reached for aztreonam and meropenem in PA101243 (MIC colistin = 1024 mg/L). Resistance to colistin often involves modifications to lipid A of LPS,⁹ suggesting that the action of compound **2** is independent of lipid A modifications.

With the exception of combination treatment for tuberculosis, β -lactam/BLIs is the most successful combination therapy used in clinics,⁷ as evident in the successes of amoxicillin/clavulanic acid, ampicillin/sulbactam, piperacillin/tazobactam and, more recently, ceftazidime/avibactam, meropenem/vaborbactam and ceftolozane/tazobactam. At least four more β -lactam-based antibiotic-adjuvant combinations are currently in clinical trials.^{54,55} Unfortunately, resistance to these combinations is rapidly emerging.⁷ β -Lactamases are typically found in the periplasmic space of Gram-negative bacteria, and the rapidity with which inhibitors can access their targets is critical for successful inhibition.⁷ We show that the addition of compound **2**, a second non-antibiotic component, can further potentiate the effects of β -lactam/BLIs against recalcitrant pathogens (Figure 5.2D). Time-kill kinetics further revealed that whereas avibactam alone, at high concentrations, reverses resistance to ceftazidime in MDR PA108590, the addition of compound **2** (8 μ M) resulted in complete sterilization of the culture within 3 h (Figure 5.3Aii). Compound **2** did not improve the therapeutic efficacy of ceftolozane/tazobactam combination (Figure S3) against *P. aeruginosa*, perhaps due to their intrinsic susceptibility to the combination. Ceftolozane is the most potent cephalosporin against *P. aeruginosa* and is unaffected by the multitude of resistance mechanisms employed by this pathogen.^{56,57} Preliminary evaluation using the *G. mellonella* infection model did not reveal any toxic interaction between the combination but rather suggests a therapeutic potential *in vivo*.

In summary, we report that a tobramycin-cyclam conjugate can mitigate the effect of OprD/OprF porin loss in microbiologically-resistant *P. aeruginosa* and facilitate the delivery of β -lactam/BLIs to the periplasmic space quantitatively. β -Lactam/BLIs are arguably the most successful combination therapy against Gram-negative bacteria,⁷ and it is evident that preserving their therapeutic

utility requires a second, or even a third resistance breaker. Studies are ongoing in Phase 3 clinical trials to validate a triple or four-regimen combination therapy for β -lactams,^{58,59} and we propose that non-ribosomal tobramycin-derived adjuvants will be a useful addition to addressing the dwindling fortunes of this highly important class of drugs.

5.6.1 Supporting Information

Supplementary data B to this article is provided at the end of this chapter and can also be found online at *JAC* Online (<http://jac.oxfordjournals.org/>).

Appendix B: NMR spectra of compounds **1** – **3**.

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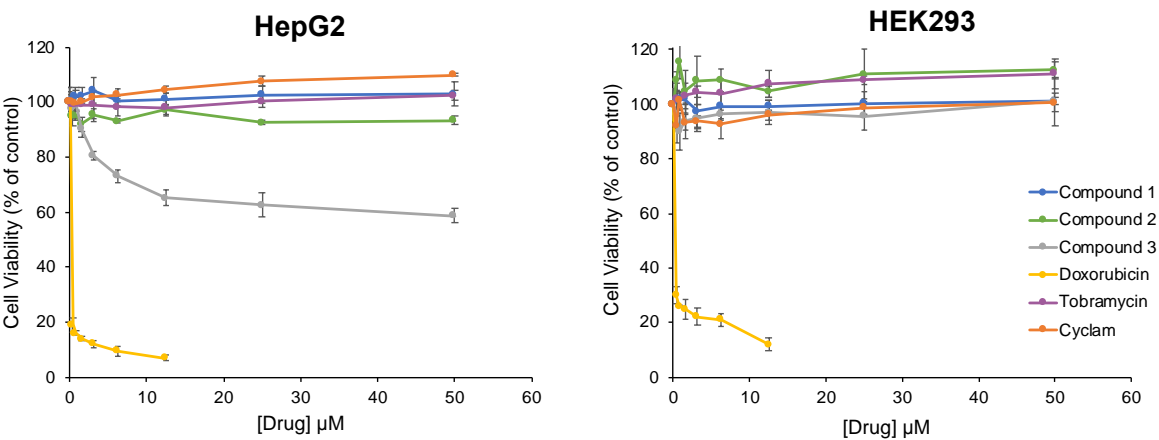
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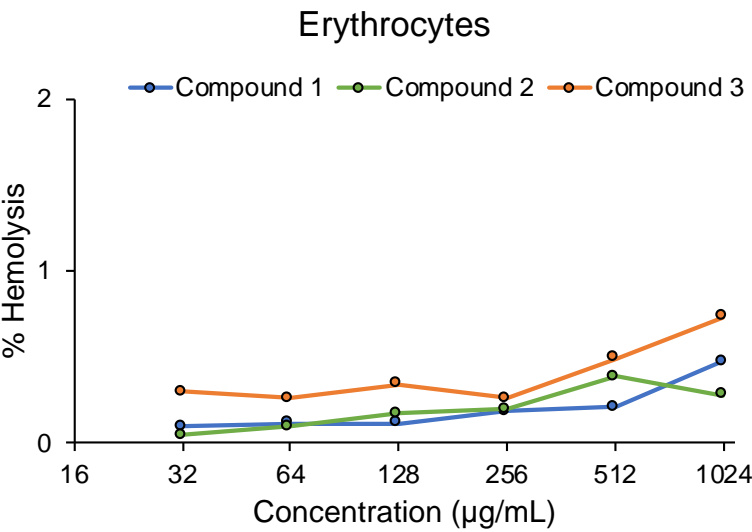
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5.8 Supplementary Data B

a)



b)



c)

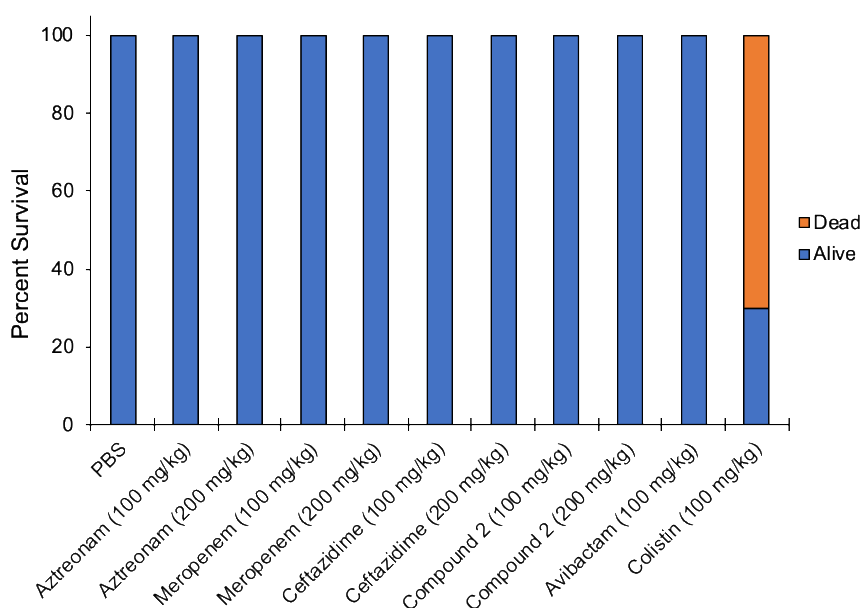


Figure S1. Cytotoxicity of compounds **1** – **3**, doxorubicin, tobramycin, and cyclam against human liver carcinoma (HepG2) cells and human embryo kidney (HEK293) cells using PrestoBlue cell viability assay. Doxorubicin was used as positive control. Error bars denote standard deviation of at least four replicates. b) Hemolytic activity of compounds **1** – **3** evaluated against porcine erythrocytes at different concentrations. 0.1% Triton X-100 (100 % hemolysis) was used as positive control to calculate percent hemolysis. The result represents the mean of three independent determinations. c) Tolerability/Toxicity studies in *Galleria mellonella* larvae. Tolerable doses were determined by injecting 100 and 200 mg/kg of test compounds, alone and in combination, into the larvae and survivability was scored for 48 h (2 days). Compound **2** is non-toxic to *G. mellonella* larvae.

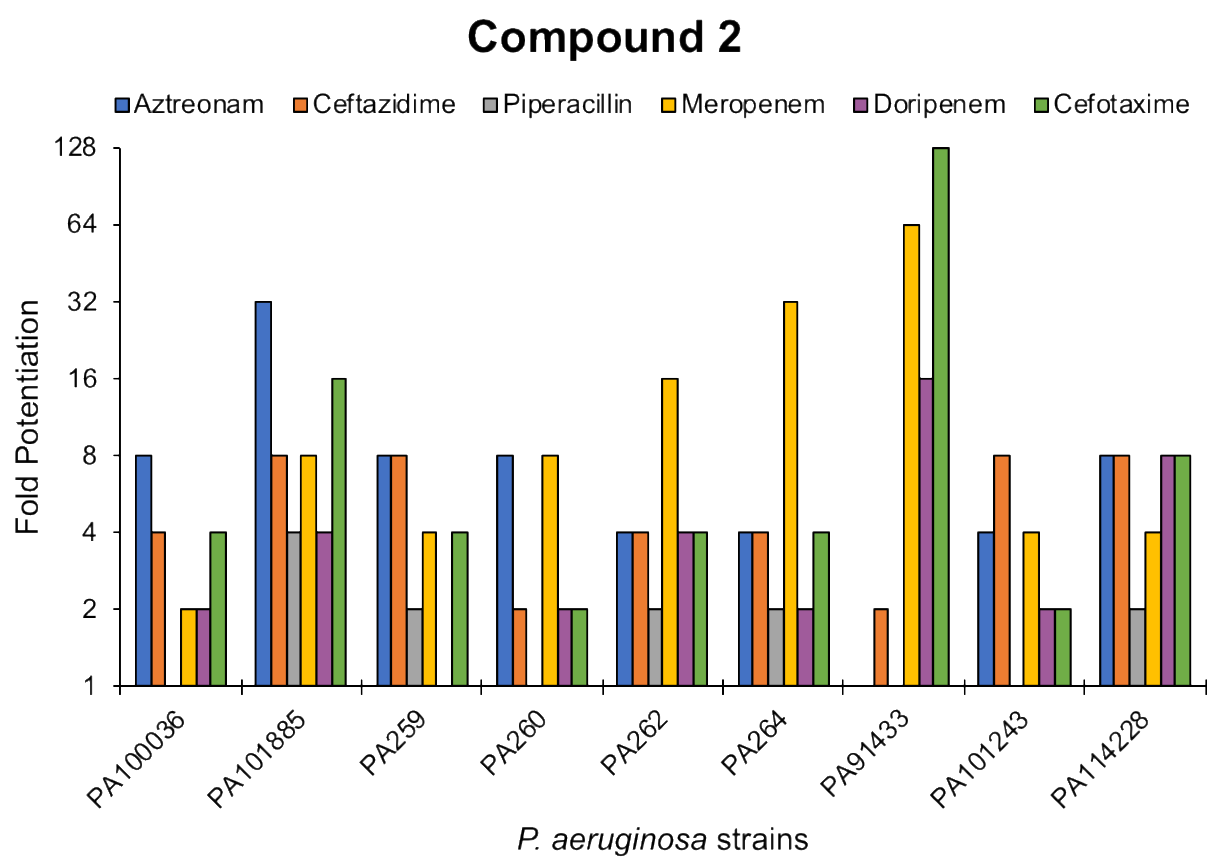


Figure S2. Fold change in susceptibility of resistant clinical isolates of *P. aeruginosa* (PA) to select β -lactam antibiotics in the presence of 8.7 μ M of compound **2**.

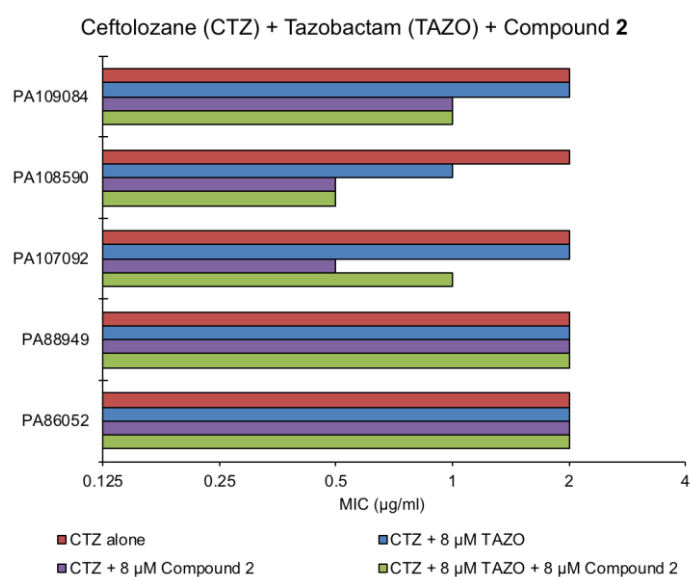
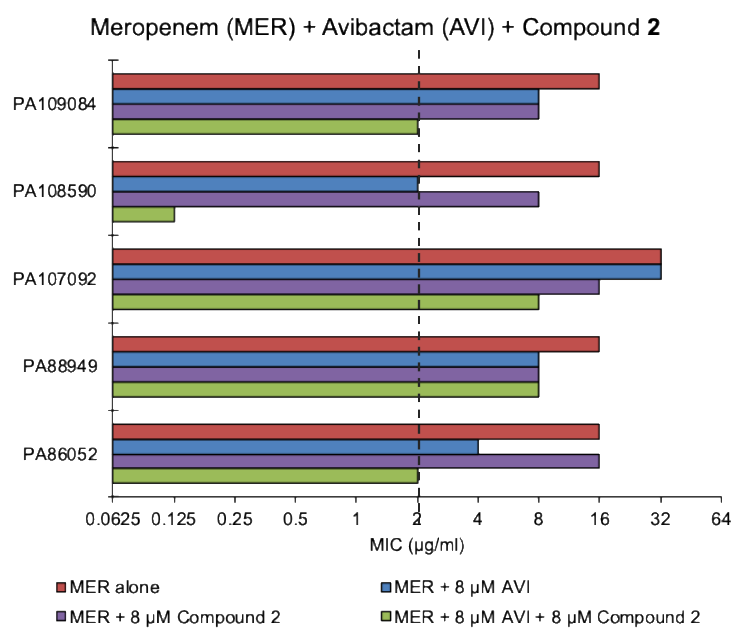
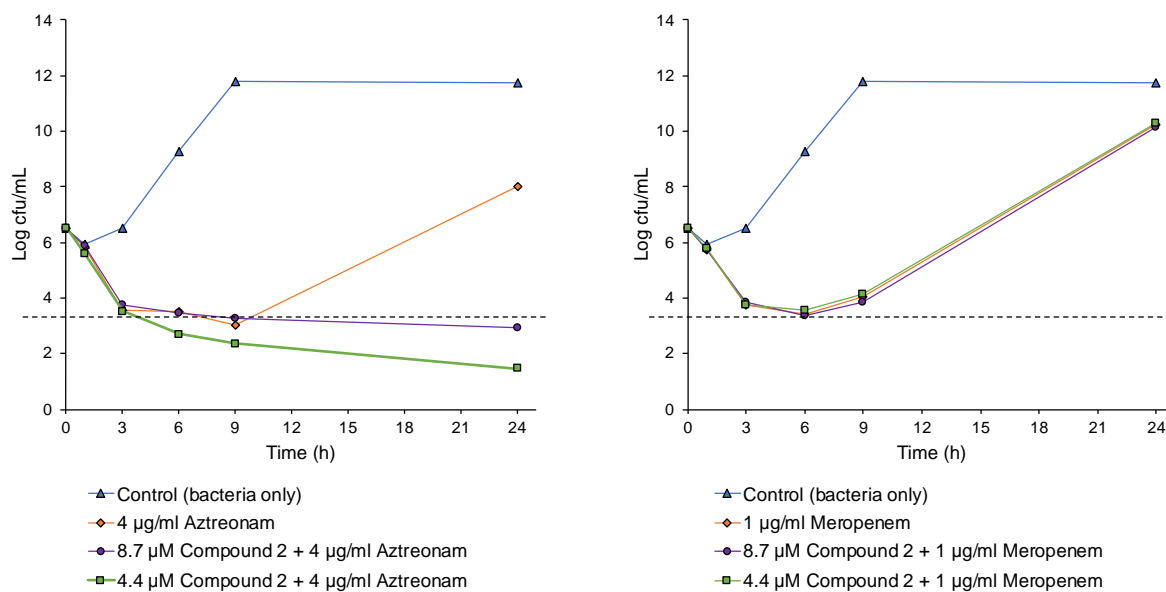


Figure S3. Triple combination therapy versus double combination versus monotherapy against β -lactamase-harboring MDR *P. aeruginosa* isolates. MER = meropenem; AVI = avibactam; CTZ = ceftolozane, TAZO = tazobactam. 8 μ M Avibactam \sim 2 mg/L; 8 μ M Tazobactam \sim 2.4 mg/L; 8 μ M compound 2 \sim 16 mg/L.

A)



B)

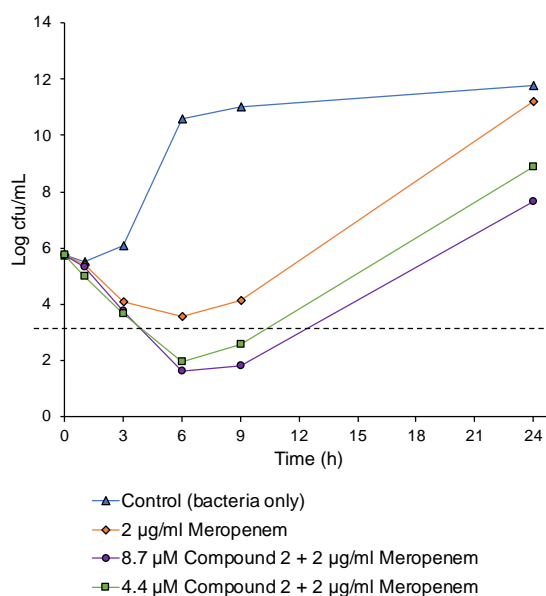


Figure S4. Time-kill kinetics of A) aztreonam or meropenem, alone and in combination with different concentrations of compound **2** on the viability of wild-type *P. aeruginosa* PAO1; B) meropenem, alone and in combination with different concentrations of compound **2** against MDR *P. aeruginosa* 101885. Each data point is an average of three independent determinations.

Table S1. Elemental analysis of compounds **1** – **3**. C = calculated; F = found.

| Sample ID | Carbon (%) | | Hydrogen (%) | | Nitrogen (%) | |
|-------------------|------------|--------|--------------|-------|--------------|--------|
| | C | F | C | F | C | F |
| 1 (× 9TFA) | 34.35 | 32.988 | 4.38 | 4.422 | 7.21 | 6.976 |
| 2 (× 9TFA) | 35.95 | 35.763 | 4.69 | 4.704 | 6.99 | 6.897 |
| 3 (× 9HCl) | 41.34 | 40.951 | 7.98 | 7.924 | 10.85 | 10.773 |

Table S2. Minimum inhibitory concentrations (MICs, mg/L) of Tobramycin and compounds **1** – **3** against a panel of Gram-positive and Gram-negative bacteria. [#]CANWARD; * CAN-ICU

| Test organism | Tobramycin | Tobramycin–cyclam conjugates | | |
|--|------------|------------------------------|----------|----------|
| | | 1 | 2 | 3 |
| <i>S. aureus</i> ATCC 29213 | 0.5 | 64 | 128 | >128 |
| MRSA ATCC 33592 | 0.5 | 64 | 64 | >128 |
| MSSE 81388 [#] | ≤0.25 | 16 | 16 | 16 |
| MRSE 61589 (CAZ >32)* | 2 | 64 | 128 | 32 |
| <i>E. faecalis</i> ATCC 29212 | 8 | >128 | >128 | >128 |
| <i>E. faecium</i> ATCC 27270 | 16 | >128 | >128 | >128 |
| <i>S. pneumoniae</i> ATCC 49619 | 2 | >128 | >128 | >128 |
| <i>E. coli</i> ATCC 25922 | 0.5 | 64 | 64 | 64 |
| <i>E. coli</i> 61714 (GEN-R)* | 8 | 128 | 128 | >128 |
| <i>E. coli</i> 63074 (AMK 32)* | 8 | >128 | >128 | >128 |
| <i>E. coli</i> 97615 (GEN-R, TOB-R, CIP-R)* | 128 | >128 | >128 | >128 |
| <i>P. aeruginosa</i> ATCC 27853 | 1 | >128 | >128 | >128 |
| <i>P. aeruginosa</i> 62308 (GEN-R)* | 16 | >128 | >128 | >128 |
| <i>P. aeruginosa</i> 96846 (GEN-R, TOB-R) [#] | 256 | >128 | >128 | >128 |
| <i>S. maltophilia</i> 62584* | >512 | >128 | >128 | >128 |
| <i>A. baumannii</i> 63169* | 32 | >128 | >128 | >128 |
| <i>K. pneumoniae</i> ATCC 13883 | ≤0.25 | 32 | 128 | 64 |

Table S3. Combination studies of compounds **1** – **3** with different antibiotics against WT *P. aeruginosa* PAO1. MICs are reported in mg/L. FICI = Fractional inhibitory concentration index. FICI of ≤ 0.5 , $> 0.5 - 4$, and > 4 indicate synergy, additive or no interaction, and antagonism, respectively. Synergistic combinations are highlighted in green.

| Antibiotics (MIC alone) | MIC of Antibiotics (FICI) in the presence of $\leq 10 \mu\text{M}$ of | | |
|-------------------------|---|-------------|-------------|
| | 1 | 2 | 3 |
| Rifampicin (16) | 2 (0.13) | 2 (0.13) | 4 (0.25) |
| Tobramycin (1) | 1 (1.01) | 1 (1.00) | 1 (1.01) |
| Minocycline (8) | 2 (0.25) | 2 (0.25) | 1 (0.17) |
| Cefotaxime (16) | 2 (0.13) | 4 (0.26) | 4 (0.27) |
| Doxycycline (8) | 4 (0.50) | 1 (0.13) | 0.5 (0.08) |
| Linezolid (1024) | 1024 (1.00) | 256 (0.26) | 256 (0.25) |
| Meropenem (1) | 0.25 (0.25) | 0.25 (0.26) | 0.5 (0.50) |
| Doripenem (1) | 0.5 (0.51) | 0.5 (0.50) | 0.5 (0.50) |
| Moxifloxacin (1) | 1 (1.00) | 0.5 (0.51) | 1 (1.01) |
| Trimethoprim (64) | 32 (0.50) | 32 (0.50) | 32 (0.50) |
| Vancomycin (256) | 128 (0.50) | 64 (0.26) | 128 (0.50) |
| Aztreonam (4) | 0.5 (0.13) | 0.25 (0.07) | 0.5 (0.14) |
| Ceftazidime (2) | 0.5 (0.25) | 0.25 (0.13) | 0.5 (0.25) |
| Fosfomycin (16) | 8 (0.51) | 8 (0.50) | 16 (1.02) |
| Nitrofurantoin (1024) | 1024 (1.00) | 1024 (1.00) | 1024 (1.02) |
| Chloramphenicol (32) | 8 (0.25) | 4 (0.13) | 8 (0.25) |
| Clindamycin (1024) | 512 (0.50) | 512 (0.51) | 64 (0.07) |
| Erythromycin (512) | 256 (0.50) | 256 (0.50) | 128 (0.25) |
| Pleuromutilin (512) | 512 (1.00) | 512 (1.00) | 128 (0.25) |
| Novobiocin (512) | 64 (0.13) | 64 (0.13) | 32 (0.08) |
| Colistin (1) | 2 (2.00) | 2 (2.00) | 1 (1.02) |
| Tigecycline (4) | 2 (0.25) | 2 (0.50) | 2 (0.52) |
| Piperacillin (8) | 2 (0.26) | 2 (0.25) | 2 (0.25) |

Table S4. **Tobramycin alone does not potentiate antibiotics against *P. aeruginosa* PAO1.** MIC of tobramycin against PAO1 = 1 µg/mL. FICI > 0.5 but ≤ 4 indicates no interaction.

| Antibiotics | MIC (µg/ml) of Antibiotic | | FICI |
|-----------------|---------------------------|------------------------|------|
| | Alone | + 0.5 µg/mL Tobramycin | |
| Rifampicin | 16 | 8 | 1.00 |
| Minocycline | 8 | 8 | 1.50 |
| Cefotaxime | 16 | 8 | 1.00 |
| Doxycycline | 8 | 8 | 1.50 |
| Linezolid | 1024 | 1024 | 1.50 |
| Meropenem | 1 | 0.5 | 1.00 |
| Moxifloxacin | 1 | 0.5 | 1.00 |
| Ciprofloxacin | 0.125 | 0.125 | 1.50 |
| Levofloxacin | 0.5 | 0.5 | 1.50 |
| Vancomycin | 256 | 128 | 1.00 |
| Aztreonam | 4 | 1 | 0.75 |
| Fosfomycin | 16 | 8 | 1.00 |
| Chloramphenicol | 32 | 16 | 1.00 |
| Clindamycin | 1024 | 1024 | 1.50 |
| Novobiocin | 512 | 128 | 0.75 |

Table S5. Susceptibility profiles of MDR/XDR *P. aeruginosa* isolates used in this study

| Stock # | PTZ | A/C | AZT | FOX | CFZ | CTR | CPM | CAZ | IMI | MER | DOR | ETP | CIP | MOX | TOB | GEN | AMK | TGC | MIN | DOX | CST | CAM | RIF |
|-------------|-----|-----|-----|-----|------|-----|-----|------|-----|------|-------|-----|-----|-----|------|------|-----|-----|-----|------|------|------|-----|
| 100036 | 8 | >32 | 16 | >32 | >128 | 32 | 4 | 8 | 8 | 4 | 16 | >32 | >16 | >16 | 128 | >32 | 32 | 32 | 16 | 64 | 2 | 1024 | 16 |
| 101885 | 16 | >32 | 16 | >32 | >128 | 32 | 8 | 8 | 1 | 4 | 4 | 32 | >16 | >16 | 1 | ≤0.5 | ≤1 | 8 | 8 | 64 | 1 | 512 | 32 |
| P259-96918 | 64 | >32 | 32 | >32 | >128 | >64 | >64 | 512 | 32 | 1024 | >1024 | >32 | >16 | >16 | 256 | >32 | >64 | 32 | 32 | 32 | 1 | 1024 | 16 |
| P260-97103 | 128 | >32 | 64 | >32 | >128 | >64 | 16 | 32 | 32 | 16 | 16 | >32 | 16 | >16 | 32 | >32 | 4 | 16 | 16 | 16 | 1 | 128 | 16 |
| P262-101856 | 64 | >32 | 32 | >32 | >128 | 64 | 32 | 16 | 32 | 32 | 16 | >32 | >16 | >16 | 1024 | >32 | >64 | 32 | 64 | 1024 | 1 | 2048 | 512 |
| P264-104354 | 256 | >32 | 64 | >32 | >128 | >64 | 32 | 128 | 32 | 64 | 16 | >32 | >16 | >16 | 128 | >32 | 8 | 32 | 32 | 64 | 1 | 4096 | 16 |
| 91433 | 64 | >32 | 512 | >32 | >128 | >64 | 16 | 1024 | 32 | 16 | 16 | >32 | 2 | 16 | 16 | 32 | >32 | 32 | 16 | 32 | 4 | 8 | 16 |
| 101243 | 128 | >32 | 32 | >32 | >128 | >64 | 64 | 64 | 16 | 16 | 16 | >32 | 1 | 8 | 128 | >32 | >64 | ND | 2 | 4 | 1024 | 1 | 4 |

PTZ: piperacillin-tazobactam; A/C: amoxicillin-clavulanic acid; AZT: aztreonam; FOX: cefoxitin; CFZ: cefazolin; CTR: ceftriaxone; CPM: cefepime; CAZ: ceftazidime; IMI: imipenem; MER: meropenem; DOR: doripenem; ETP: ertapenem; CIP: ciprofloxacin; MOX: moxifloxacin; TOB: tobramycin; GEN: gentamicin; AMK: amikacin; TGC: tigecycline; MIN: minocycline; DOX: doxycycline; CST: colistin; CAM: chloramphenicol; RIF: rifampicin; ND: not determined.

Table S6. Interactions of compound **2** (8.7 μ M) with aztreonam (AZT), ceftazidime (CAZ), piperacillin (PIP), meropenem (MER), doripenem (DOR), and cefotaxime (CEF) against other Gram-negative bacteria. MICs are reported in mg/L. FICI of ≤ 0.5 , $> 0.5 - 4$, and > 4 indicate synergy, additive or no interaction, and antagonism, respectively. AB = *Acinetobacter baumannii*; KP = *Klebsiella pneumoniae*; ND = not determined. Synergistic combinations are highlighted in green.

| Strain | Antibiotic | MIC (mg/L) of Antibiotic | | Fold Potentiation | FICI |
|---------------------------|------------|--------------------------|---------------------|-------------------|-------|
| | | Alone | + Compound 2 | | |
| AB ATCC 17978 | AZT | 64 | 16 | 4 | <0.28 |
| | CAZ | 32 | 16 | 2 | <0.56 |
| | PIP | 256 | 256 | 1 | <1.06 |
| | MER | 0.5 | 0.25 | 2 | <0.53 |
| | DOR | 2 | 1 | 2 | <0.53 |
| | CEF | 64 | 32 | 2 | <0.51 |
| AB 110193 | AZT | 256 | 64 | 4 | <0.31 |
| | CAZ | 512 | 8 | 64 | <0.08 |
| | PIP | 512 | 32 | 16 | <0.13 |
| | MER | 0.5 | 0.25 | 2 | <0.51 |
| | DOR | 2 | 1 | 2 | <0.56 |
| | CEF | 256 | 64 | 4 | <0.25 |
| <i>E. coli</i> ATCC 25922 | AZT | 0.25 | 0.016 | 16 | <0.13 |
| | CAZ | 0.5 | 0.25 | 2 | <0.53 |
| | PIP | 4 | 1 | 4 | <0.28 |
| | MER | 0.016 | 0.016 | 1 | <1.06 |
| | DOR | 0.063 | 0.063 | 1 | <1.06 |
| | CEF | ND | ND | ND | ND |
| <i>E. coli</i> 107115 | AZT | 512 | 512 | 1 | <1.06 |
| | CAZ | 512 | 128 | 4 | <0.27 |
| | PIP | >1024 | ND | ND | ND |
| | MER | 64 | 16 | 4 | <0.26 |
| | DOR | 128 | 32 | 4 | <0.27 |
| | CEF | 1024 | 512 | 2 | <0.53 |
| KP 116381 | AZT | 128 | 64 | 2 | <0.53 |

| | | | | | |
|-----------------------------|-----|-------|-------|-----|-------|
| | CAZ | 32 | 16 | 2 | <0.56 |
| | PIP | 1024 | 1024 | 1 | <1.06 |
| | MER | 0.125 | 0.063 | 2 | <0.50 |
| | DOR | 1 | 0.5 | 2 | <0.50 |
| | CEF | 1024 | 1024 | 1 | <1.06 |
| <i>E. cloacae</i> 117029 | AZT | 0.5 | 0.063 | 8 | <0.19 |
| | CAZ | 1 | 0.125 | 8 | <0.19 |
| | PIP | 4 | 1 | 4 | <0.28 |
| | MER | 0.016 | 0.031 | 0.5 | <2.00 |
| | DOR | 0.063 | 0.125 | 0.5 | <2.00 |
| | CEF | 1 | 0.5 | 2 | <0.50 |

Table S7. Tobramycin alone and/or cyclam alone do not potentiate β -lactam antibiotics. against wild-type* and MDR/XDR *P. aeruginosa* (PA) isolates. TOB = tobramycin; CYC = cyclam; AZT = aztreonam; CAZ = ceftazidime; PIP = piperacillin; MER = meropenem, DOR = doripenem; CEF = cefotaxime.

| PA strain | MIC _{AZT} | MIC _{Combination} | FIC _{AZT} | MIC _{TOB} | MIC _{Combination} | FIC _{TOB} | FICI | Interpretation |
|-----------|--------------------|----------------------------|--------------------|--------------------|----------------------------|--------------------|-------|----------------|
| PAO1* | 4 | 1 | 0.25 | 1 | 0.5 | 0.5 | 0.75 | No Interaction |
| 10036 | 16 | 4 | 0.25 | 128 | 64 | 0.5 | 0.75 | No Interaction |
| 260 | 64 | 32 | 0.5 | 32 | 8 | 0.25 | 0.75 | No Interaction |
| 101885 | 16 | 8 | 0.5 | 1 | 0.25 | 0.25 | 0.75 | No Interaction |
| 259 | 32 | 8 | 0.25 | 256 | 128 | 0.5 | 0.75 | No Interaction |
| 262 | 32 | 2 | 0.0625 | >256 | 128 | <0.5 | 0.56 | No Interaction |
| 114228 | 32 | 4 | 0.125 | 2 | 1 | 0.5 | 0.625 | No Interaction |

| PA strain | MIC _{MER} | MIC _{Combination} | FIC _{MER} | MIC _{TOB} | MIC _{Combination} | FIC _{TOB} | FICI | Interpretation |
|-----------|--------------------|----------------------------|--------------------|--------------------|----------------------------|--------------------|-------|----------------|
| PAO1* | 1 | 0.5 | 0.5 | 1 | 0.5 | 0.5 | 1.00 | No Interaction |
| 260 | 8 | 4 | 0.5 | 32 | 8 | 0.25 | 0.75 | No Interaction |
| 264 | 64 | 32 | 0.5 | 128 | 32 | 0.25 | 0.75 | No Interaction |
| 101885 | 4 | 2 | 0.5 | 1 | 0.25 | 0.25 | 0.75 | No Interaction |
| 262 | 32 | 16 | 0.5 | >256 | 128 | <0.5 | 1.00 | No Interaction |
| 91433 | 16 | 2 | 0.125 | 16 | 8 | 0.5 | 0.625 | No Interaction |
| 114228 | 8 | 2 | 0.25 | 2 | 1 | 0.5 | 0.75 | No Interaction |

| Strain | Antibiotic | MIC _{Antibiotic} | MIC _{Combination} | FIC _{Antibiotic} | MIC _{CYC} | MIC _{Combination} | FIC _{CYC} | FICI | Interpretation |
|--------|------------|---------------------------|----------------------------|---------------------------|--------------------|----------------------------|--------------------|------|----------------|
| PAO1* | AZT | 4 | 4 | 1 | >64 | >64 | 1 | 2 | No Interaction |
| | CAZ | 2 | 2 | 1 | >64 | >64 | 1 | 2 | No Interaction |
| | PIP | 4 | 4 | 1 | >64 | >64 | 1 | 2 | No Interaction |
| | MER | 1 | 1 | 1 | >64 | >64 | 1 | 2 | No Interaction |
| | DOR | 1 | 1 | 1 | >64 | >64 | 1 | 2 | No Interaction |
| | CEF | 16 | 16 | 1 | >64 | >64 | 1 | 2 | No Interaction |

Table S8: Checkerboard studies of compound **2** with aztreonam (AZT), ceftazidime (CAZ), or meropenem (MER) against Gram-positive bacteria. MICs are reported in µg/ml. FICI of > 0.5 but < 4 indicate no interaction. MRSA = methicillin-resistant *Staphylococcus aureus*; MRSE = methicillin-resistant *Staphylococcus epidermis*.

| Strain | Antibiotic | MIC _{Antibiotic} | MIC _{Combination} | FIC _{Antibiotic} | MIC ₂ | MIC _{Combination} | FIC ₂ | FICI |
|--------------------|------------|---------------------------|----------------------------|---------------------------|------------------|----------------------------|------------------|-------|
| MRSA | AZT | >256 | >256 | 1 | 64 | 16 | 0.25 | 1.25 |
| ATCC | CAZ | 128 | 128 | 1 | 64 | 16 | 0.25 | 1.25 |
| 33592 | MER | 16 | 16 | 1 | 64 | 16 | 0.25 | 1.25 |
| MRSE 61589 | AZT | >256 | >256 | 1 | 128 | 16 | 0.125 | 1.125 |
| | CAZ | 256 | 256 | 1 | 128 | 16 | 0.125 | 1.125 |
| | MER | 64 | 64 | 1 | 128 | 16 | 0.125 | 1.125 |
| <i>E. faecalis</i> | AZT | >256 | >256 | 1 | >128 | 16 | <0.125 | <1.13 |
| ATCC | CAZ | 32 | 32 | 1 | >128 | 16 | <0.125 | <1.13 |
| 29212 | MER | 2 | 2 | 1 | >128 | 16 | <0.125 | <1.13 |
| <i>E. faecium</i> | AZT | >256 | >256 | 1 | >128 | 16 | <0.125 | <1.13 |
| ATCC | CAZ | >512 | >512 | 1 | >128 | 16 | <0.125 | <1.13 |
| 27270 | MER | 4 | 2 | 0.5 | >128 | 16 | <0.125 | <1.13 |

Table S9. Susceptibility profiles of β -lactamase-harboring MDR *P. aeruginosa* isolates used for triple combination study.

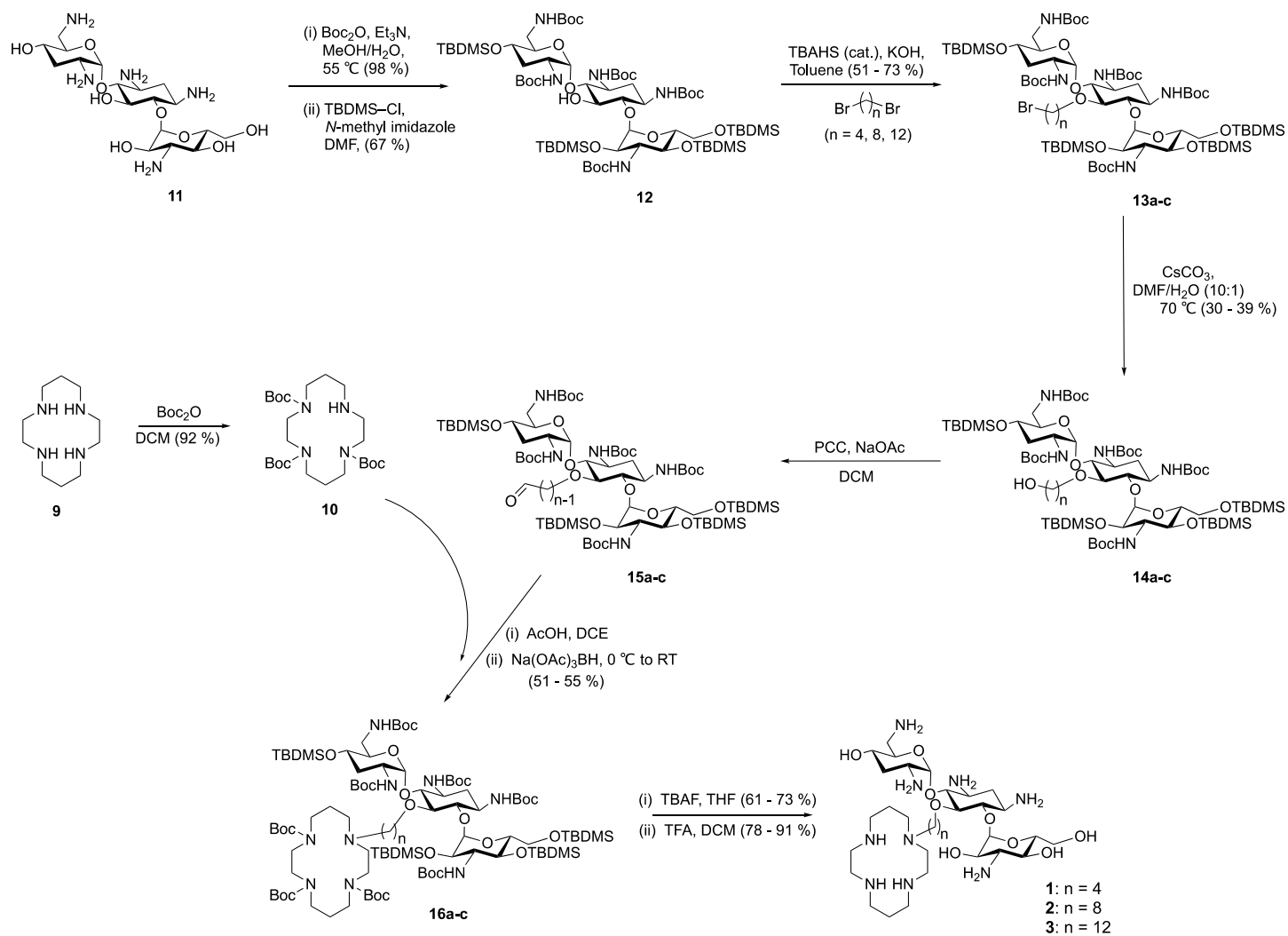
| Stock # | PTZ | A/C | AZT | FOX | CFZ | CTR | CPM | CTP | C/T | CAZ | CAZ-AVI | IMI | MER | ETP | CIP | MOX | TOB | GEN | AMK | DOX | CST | SXT | NFN |
|-----------|-----|-----|-----|-----|------|-----|-----|-----|-----|-----|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|
| PA 86052 | 256 | >32 | 64 | >32 | >128 | >64 | 32 | 16 | 2 | 64 | 4 | 32 | 16 | >32 | >16 | >16 | 1 | 4 | 16 | >32 | 1 | >8 | >512 |
| PA 88949 | 256 | >32 | 32 | >32 | >128 | >64 | 32 | 32 | 2 | 64 | 4 | >32 | 16 | 32 | 4 | 8 | 4 | 16 | 32 | >32 | 2 | >8 | >512 |
| PA 107092 | 512 | >32 | 64 | >32 | >128 | >64 | 64 | 32 | 2 | 128 | 16 | 32 | 32 | >32 | >16 | >16 | >64 | >32 | 8 | >32 | 1 | >8 | >512 |
| PA 108590 | 256 | >32 | 32 | >32 | >128 | >64 | 32 | 16 | 2 | 32 | 2 | 32 | 16 | >32 | 4 | 16 | 4 | 16 | 32 | >32 | 1 | >8 | >512 |
| PA 109084 | 512 | >32 | 64 | >32 | >128 | >64 | 32 | 16 | 2 | 64 | 4 | 32 | 16 | >32 | 4 | 16 | 4 | 16 | 32 | 16 | 1 | 4 | >512 |

PTZ: piperacillin-tazobactam; A/C: amoxicillin-clavulanic acid; AZT: aztreonam; FOX: cefoxitin; CFZ: cefazolin; CTR: ceftriaxone; CPM: cefepime; CTP: ceftobiprole; C/T: ceftolozane-tazobactam; CAZ: ceftazidime; CAZ-AVI: ceftazidime-avibactam; IMI: imipenem; MER: meropenem; ETP: ertapenem; CIP: ciprofloxacin; MOX: moxifloxacin; TOB: tobramycin; GEN: gentamicin; AMK: amikacin; DOX: doxycycline; CST: colistin; SXT: trimethoprim-sulfamethoxazole; NFN: nitrofurantoin. [Avibactam] = 4 mg/L.

Table S10. Synergistic effects of compound **2** or avibactam (AVI) with aztreonam (AZT), ceftazidime (CAZ), meropenem (MER), or ceftolozane (CTZ) against multidrug resistant *P. aeruginosa* clinical isolates. MICs are reported in µg/mL. FICI of ≤ 0.5 , $> 0.5 - 4$, and > 4 indicate synergy, additive or no interaction, and antagonism, respectively. Synergistic combinations are highlighted in green.

| Strain | Antibiotic | MIC _{Antibiotic} | MIC _{Combination} | FIC _{Antibiotic} | MIC ₂ | MIC _{Combination} | FIC ₂ | FICI |
|--------------|------------|---------------------------|----------------------------|---------------------------|------------------|----------------------------|------------------|-------|
| PA 86052 | AZT | 64 | 16 | 0.25 | >256 | 16 | <0.0625 | <0.31 |
| | CAZ | 64 | 64 | 1 | >256 | 16 | <0.0625 | <1.06 |
| | MER | 16 | 8 | 0.5 | >256 | 8 | <0.03125 | <0.53 |
| | CTZ | 2 | 2 | 1 | >256 | 16 | <0.0625 | <1.06 |
| PA 88949 | AZT | 32 | 16 | 0.5 | >256 | 8 | <0.03125 | <0.53 |
| | CAZ | 128 | 32 | 0.25 | >256 | 16 | <0.0625 | <0.31 |
| | MER | 16 | 8 | 0.5 | >256 | 8 | <0.03125 | <0.53 |
| | CTZ | 2 | 2 | 1 | >256 | 16 | <0.0625 | <1.06 |
| PA 107092 | AZT | 64 | 16 | 0.25 | >256 | 16 | <0.0625 | <0.31 |
| | CAZ | 128 | 32 | 0.25 | >256 | 8 | <0.03125 | <0.28 |
| | MER | 32 | 16 | 0.5 | >256 | 8 | <0.03125 | <0.53 |
| | CTZ | 2 | 0.5 | 0.25 | >256 | 16 | <0.0625 | <0.31 |
| PA 108590 | AZT | 32 | 8 | 0.25 | >256 | 8 | <0.03125 | <0.31 |
| | CAZ | 32 | 8 | 0.25 | >256 | 4 | <0.01563 | <0.27 |
| | MER | 16 | 8 | 0.5 | >256 | 8 | <0.03125 | <0.53 |
| | CTZ | 2 | 0.5 | 0.25 | >256 | 16 | <0.0625 | <0.31 |
| PA 109084 | AZT | 64 | 8 | 0.125 | >256 | 16 | <0.0625 | <0.19 |
| | CAZ | 64 | 16 | 0.25 | >256 | 8 | <0.03125 | <0.28 |
| | MER | 16 | 8 | 0.5 | >256 | 8 | <0.03125 | <0.53 |
| | CTZ | 2 | 0.5 | 0.25 | >256 | 4 | <0.01563 | <0.27 |

| Strain | Antibiotic | MIC _{Antibiotic} | MIC _{Combination} | FIC _{Antibiotic} | MIC _{AVI} | MIC _{Combination} | FIC _{AVI} | FICI |
|--------------|------------|---------------------------|----------------------------|---------------------------|--------------------|----------------------------|--------------------|-------|
| PA 86052 | AZT | 32 | 2 | 0.0625 | >128 | 4 | <0.03125 | <0.09 |
| | CAZ | 64 | 2 | 0.03125 | >128 | 16 | <0.125 | <0.16 |
| | MER | 16 | 1 | 0.0625 | >128 | 8 | <0.0625 | <0.13 |
| PA 88949 | AZT | 32 | 4 | 0.125 | >128 | 8 | <0.0625 | <0.19 |
| | CAZ | 64 | 4 | 0.0625 | >128 | 4 | <0.03125 | <0.09 |
| | MER | 16 | 4 | 0.25 | >128 | 8 | <0.0625 | <0.31 |
| PA 107092 | AZT | 64 | 16 | 0.25 | >128 | 16 | <0.125 | <0.38 |
| | CAZ | 128 | 8 | 0.0625 | >128 | 8 | <0.0625 | <0.13 |
| | MER | 32 | 16 | 0.5 | >128 | 8 | <0.0625 | <0.56 |
| PA 108590 | AZT | 32 | 8 | 0.25 | >128 | 2 | <0.01563 | <0.27 |
| | CAZ | 32 | 1 | 0.03125 | >128 | 8 | <0.0625 | <0.09 |
| | MER | 16 | 1 | 0.0625 | >128 | 4 | <0.03125 | <0.09 |
| PA 109084 | AZT | 64 | 8 | 0.125 | >128 | 2 | <0.01563 | <0.14 |
| | CAZ | 64 | 4 | 0.0625 | >128 | 2 | <0.01563 | <0.08 |
| | MER | 16 | 8 | 0.5 | >128 | 2 | <0.01563 | <0.52 |



Scheme S1. Synthesis of Tobramycin–Cyclam conjugates **1** – **3**.

Chemistry. All chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) except tobramycin that was purchased from AK Scientific Inc. (CA, USA). The chemicals were all used without further purification. Air and moisture-sensitive reactions were performed with dry solvents under nitrogen atmosphere. Thin-layer chromatography (TLC) was carried out on aluminum-backed silica gel 60 F₂₅₄ GF plates (0.25 mm) and/or aluminum-backed reverse phase silica gel 60 RP-18 F₂₅₄S plates (Merck KGaA, Germany) with the indicated solvents, and visualized by staining within ninhydrin solution in *n*-butanol. Intermediate compounds were purified by flash chromatography on silica gel 60 (230-400 ASTM mesh) and final compounds were purified on reverse-phase C18 silica gel (Silicycle, USA). Yields refer to chromatography-purified homogenous materials, except otherwise stated. ¹H and ¹³C NMR spectra were recorded on Bruker AMX-300 and AMX-500 spectrometers (Germany) as solutions and reported in the order of chemical shifts (δ) in ppm relative to the indicated solvent, multiplicity (s, singlet; d, doublet; t, triplet and m, multiplet), number of protons, and coupling constants (*J*) in hertz (Hz). ¹H and ¹³C of compounds were assigned using 1D and 2D NMR experiments such as Proton, COSY, Carbon-13, DEPT-135, HSQC, and HMBC. ESI-MS and MALDI-TOF MS analyses were performed on Varian 500-MS ion trap mass spectrometer (USA) and Bruker Daltonics Ultraflexxtreme MALDI TOF/TOF mass spectrometer (Germany), respectively. The purity of final compounds as determined by elemental analysis was > 95 %.

General Procedure A: 5-*O*-alkylation of *Boc* and TBDMS protected Tobramycin for the Preparation of Compounds 13a–c. A solution of **12** (1 equiv.) in toluene was treated with KOH (3 equiv.), 1,*n*-dibromoalkane (3 equiv.), and a catalytic amount of tetrabutylammonium hydrogen sulphate, TBAHS (0.1 equiv.). The reaction mixture was stirred at RT overnight, dispersed in water and extracted with an equal volume of ethyl acetate (×3). The organic layers were combined, washed with brine (×1), dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The crude products were

then purified by column chromatography (hexanes/ethyl acetate, 12:1 to 10:1, v/v) to afford compounds **13a-c** as white solids.

General Procedure B: Hydroxylation of 5-*O*-(*n*-Bromoakyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin for the Preparation of Compounds 14a-c. A solution of compounds **13a-c** (1 equiv.) in a mixture of DMF (20.0 mL) and water (2.0 mL) was treated with Cs₂CO₃ (2 equiv.) and stirred overnight at 70 °C. The crude mixture was then dispersed in water and extracted with ethyl acetate (50 mL ×3). The combined organic layer was washed with saturated brine (×1), dried over anhydrous Na₂SO₄, concentrated in *vacuo*, and purified by flash chromatography (hexanes/ethyl acetate, 10:1 to 8:1, v/v) to afford compounds **14a-c** as white solids.

General Procedure C: Oxidation of 5-*O*-(*n*-Hydroxyldodecyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin to Aldehydes for the Preparation of Compounds 15a-c. Compounds **14a-c** (1 equiv.) dissolved in dry DCM were treated with PCC (pyridinium chlorochromate, 3 equiv.) in the presence of NaOAc, and stirred under nitrogen gas for 3 h at RT. The resulting crude was filtered through a pad of silica and concentrated under reduced pressure to give compounds **15a-c**. These compounds were used immediately without further purification.

General Procedure D: Reductive Amination of Aldehydes with (Boc)₃-protected Cyclam for the Preparation of Compounds 16a-c. A solution of compounds **15a-c** (1 equiv.) in dry DCE was treated with (Boc)₃-protected cyclam **10** (1.5 equiv.) and two drops of acetic acid. The reaction was stirred for 7 h at RT under nitrogen gas. The reaction mixture was cooled in ice, treated with Na(OAc)₃BH (3 equiv.), and stirred overnight under nitrogen atmosphere from 0 °C to RT. The resulting mixture was quenched with saturated Na₂CO₃, extracted with DCM (×3), concentrated in *vacuo*, and purified by flash chromatography (hexanes/ethyl acetate, 10:1 to 4:1, v/v) to yield compounds **16a-c**.

General Procedure E: Deprotection of Hydroxyls and Amines (Removal of TBDMS and *Boc* Protecting Groups) for Preparation of Compounds 1 – 3. A solution of TBDMS- and *Boc*-protected compounds **16a–c** in anhydrous THF (5.0 mL) were treated with tetrabutylammonium fluoride (TBAF, 6 equiv.) and stirred under nitrogen atmosphere for 2 h. The reaction mixture was concentrated under *vacuo*, dissolved in water and extracted with DCM (×3). The organic layers were combined, dried over anhydrous Na₂SO₄, concentrated *in vacuo*, and purified by column chromatography (hexanes/ethyl acetate, 1:1, v/v, then dichloromethane/methanol, 25:1 to 20:1, v/v). A solution of the resulting compounds in DCM (2.0 mL) were further treated with trifluoroacetic acid (2.0 mL), stirred at RT for 1 h and concentrated under low *vacuo*. 2% methanol in diethylether (2.0 mL) was then added, stirred gently for 1 min and the solvent carefully decanted to give off-white solid compounds. The crude products were subsequently purified by reverse-phase flash chromatography (eluted with 100% deionized water) to afford analytically pure compounds **1 – 3** as off-white TFA salt solid compounds.

5-*O*-(Butylcyclam)–tobramycin.9TFA (1). Compound **16a** was deblocked according to general procedure E. ¹H NMR (500 MHz, D₂O) δ 5.36 (d, *J* = 2.5 Hz, 1H, anomeric), 5.15 (d, *J* = 3.4 Hz, 1H, anomeric), 4.25 (dd, *J* = 9.4, 3.6 Hz, 1H), 4.11 (t, *J* = 9.7 Hz, 1H), 3.83 – 3.52 (m, 12H), 3.51 – 3.20 (m, 19H), 3.19 – 3.10 (m, 3H), 2.38 (dd, *J* = 12.7, 4.4 Hz, 1H), 2.15 – 2.07 (m, 2H), 2.07 – 1.98 (m, 4H), 1.79 (m 1H), 1.71 – 1.60 (m, 2H), 1.57 – 1.49 (m, 2H). ¹³C NMR (126 MHz, D₂O) δ 101.0 (anomeric), 92.8 (anomeric), 82.3, 81.6, 77.1, 76.1, 73.2, 72.1, 68.6, 64.9, 63.1, 59.4, 55.8, 54.9, 49.5, 48.3, 47.7, 47.3, 44.7, 41.5, 40.9, 40.8, 38.4, 37.4, 36.6, 28.0, 27.7, 26.4, 20.5, 18.4, 17.6. MALDI TOF-MS *m/e* calcd for C₃₂H₆₈N₉O₉, 722.514; measured *m/e*, 722.519 [M + H]⁺.

5-*O*-(Octylcyclam)–tobramycin.9TFA (2). Compound **16b** (0.068 g, 0.04 mmol) was deblocked according to general procedure E to give **2** as a white solid (0.06 g, 78%). ¹H NMR (500 MHz, D₂O)

δ 5.26 (d, $J = 2.6$ Hz, 1H, anomeric), 5.03 (d, $J = 3.5$ Hz, 1H, anomeric), 4.15 (dd, $J = 8.6, 3.8$ Hz, 1H), 4.01 (m, 1H), 3.85 – 3.54 (m, 11H), 3.54 – 3.33 (m, 13H), 3.32 – 3.23 (m, 7H), 3.21 – 3.14 (m, 3H), 2.42 (dd, $J = 12.7, 4.4$ Hz, 1H), 2.16 – 2.09 (m, 2H), 2.11 – 2.01 (m, 4H), 1.82 (m, 1H), 1.68 – 1.59 (m, 2H), 1.56 – 1.44 (m, 2H), 1.28 – 1.09 (m, 8H). ^{13}C NMR (126 MHz, D_2O) δ 101.3 (anomeric), 92.7 (anomeric), 81.9, 81.9, 76.8, 75.8, 73.7, 73.1, 68.5, 64.7, 63.1, 59.2, 56.1, 54.8, 49.7, 48.3, 47.5, 47.3, 44.2, 41.3, 40.7, 40.7, 38.4, 37.2, 37.1, 36.3, 29.4, 28.8, 28.3, 28.0, 27.7, 25.6, 25.1, 24.0, 18.2, 17.4. MALDI TOF-MS m/e calcd for $\text{C}_{36}\text{H}_{76}\text{N}_9\text{O}_9$, 778.577; measured m/e , 778.579 $[\text{M} + \text{H}]^+$.

5-*O*-(Dodecylcyclam)–tobramycin.9HCl (3). Compound **16c** (0.044 g, 0.03 mmol) was deblocked according to general procedure E and the resulting compound was treated with stoichiometric amount of aqueous HCl solution to give **3** as a white solid HCl salt (0.046 g, 91%). ^1H NMR (500 MHz, D_2O) δ 5.22 (d, $J = 2.6$ Hz, 1H, anomeric), 4.99 (d, $J = 3.5$ Hz, 1H, anomeric), 4.11 (dd, $J = 9.3, 3.8$ Hz, 1H), 3.97 (t, $J = 9.8$ Hz, 1H), 3.82 – 3.50 (m, 11H), 3.48 – 2.98 (m, 23H), 2.37 (dd, $J = 12.6, 4.4$ Hz, 1H), 2.12 – 2.05 (m, 2H), 2.03 – 1.90 (m, 4H), 1.77 (m, 1H), 1.60 – 1.52 (m, 2H), 1.51 – 1.39 (m, 2H), 1.24 – 1.06 (m, 16H). ^{13}C NMR (126 MHz, D_2O) δ 101.4 (anomeric), 92.8, 82.0, 81.9, 76.9, 76.0, 73.9, 73.2, 68.6, 64.8, 63.1, 59.2, 55.4, 54.8, 49.7, 48.4, 48.0, 47.3, 44.9, 41.8, 41.4, 41.1, 38.4, 37.84, 37.81, 37.1, 29.5, 29.0, 29.0, 28.9, 28.8, 28.7, 28.3, 28.1, 27.7, 25.7, 25.4, 23.8, 18.9, 18.0. MALDI TOF-MS m/e calcd for $\text{C}_{40}\text{H}_{84}\text{N}_9\text{O}_9$, 834.639; measured m/e , 834.640 $[\text{M} + \text{H}]^+$.

(Boc)₃-cyclam (10). Synthesis was accomplished following previously reported procedure.⁷⁶ 1,4,8,11-tetraazacyclotetradecane (cyclam **9**) (0.35 g, 1.75 mmol) was dissolved in DCM (5.0 mL). Di-*tert*-butoxy-dicarbonate (Boc_2O) (1.0 mL, 4.35 mmol) was dissolved separately in DCM (5.0 mL) and added dropwise over 15 mins and allowed to stir overnight. The reaction mixture was concentrated, re-dispersed in hexanes (45.0 mL) and the insoluble were filtered. The solvent was removed in *vacuo* and purified by flash chromatography (ethyl acetate/hexanes, 4:1, v/v) to afford compound **10** (0.67 g,

92%) as white foam. ^1H NMR (300 MHz, CDCl_3) δ 3.48 – 3.19 (m, 12H), 2.78 (t, J = 5.3 Hz, 2H), 2.61 (t, J = 5.6 Hz, 2H), 2.01 – 1.81 (m, 2H), 1.76 – 1.63 (m, 2H), 1.45 (m, 27H, *Boc*). ^{13}C NMR (75 MHz, CDCl_3) δ 156.31, 155.48, 79.46, 60.35, 50.56, 50.02, 47.68, 46.73, 45.87, 44.07, 28.51, 28.49, 21.02, 14.19. ESI-MS: m/z calcd for $\text{C}_{25}\text{H}_{49}\text{N}_4\text{O}_6\text{Na}^+$, 501.7; found 502.0 $[\text{M} + \text{Na}]^+$.

1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (12). Commercial tobramycin (0.50 g, 1.01 mmol) was dissolved in a 2:1 mixture of methanol and water (75.0 mL) and treated with Boc_2O (1.63 g, 7.49 mmol) in the presence of Et_3N (1.0 mL). The reaction mixture was stirred under reflux (at 55 °C) overnight, concentrated under vacuo and thoroughly dried under high vacuum for 24 h to afford a white powdery solid (1.04 g, 100 %). The dried crude penta-*N*-*boc*-protected tobramycin (1.04 g, 1.07 mmol) was dissolved in anhydrous DMF (6.0 mL) and treated with *tert*-butyldimethylsilyl chloride, TBDMSCl (1.13 g, 7.49 mmol) and *N*-methylimidazole (0.6 mL). The reaction was stirred at RT for 4 days under nitrogen gas atmosphere, and the resulting mixture was poured into water (50.0 mL) and extracted with DCM ($\times 3$). The organic layer was dried over anhydrous Na_2SO_4 , concentrated in *vacuo*, and purified by flash chromatography using gradient elution (hexanes/ ethyl acetate, 15:1 to 8:1, v/v) to afford **16** (1.05 g, 67%) as a white solid. NMR data are consistent with an earlier report.²⁰

5-*O*-(*n*-Bromoalkyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (13a-c).

Compounds **13a**, **13b**, and **13c** were prepared by treating **12** with 1,4-dibromobutane, 1,8-dibromooctane, and 1,12-dibromododecane, respectively, according to general procedure A.

5-*O*-(4-Bromobutyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (13a).

Yield (47%). ^1H NMR (300 MHz, CDCl_3) δ 5.24 – 5.12 (m, 2H, anomeric), 4.28 – 4.09 (m, 3H), 3.93 – 3.14 (m, 17H), 2.61 – 2.37 (m, 1H), 2.14 – 1.84 (m, 5H), 1.72 – 1.56 (m, 3H), 1.61 – 1.35 (m, 45H, *Boc*), 1.11 – 0.72 (m, 36H, TBDMS, *tert*-butyl), 0.24 – -0.09 (m, 24H, TBDMS $-\text{CH}_3$). ESI-MS: m/z calcd for $\text{C}_{71}\text{H}_{140}\text{BrN}_5\text{O}_{19}\text{Si}_4\text{Na}^+$, 1583.2; found 1583.2 $[\text{M} + \text{Na}]^+$.

5-*O*-(8-Bromooctyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (13b).

Yield (51%). ¹H NMR (300 MHz, CDCl₃) δ 5.26 – 5.12 (m, 2H, anomeric), 4.39 – 3.97 (m, 3H), 3.89 – 3.07 (m, 16H), 2.47 (d, *J* = 12.8 Hz, 1H), 2.08 – 1.94 (m, 2H), 1.93 – 1.77 (m, 2H), 1.65 (m, 1H), 1.56 – 1.39 (m, 45H, Boc), 1.39 – 1.14 (m, 8H), 1.13 – 1.02 (m, 1H), 1.05 – 0.75 (m, 36H, TBDMS *tert*-butyl), 0.34 – -0.15 (m, 24H, TBDMS –CH₃). ESI-MS: *m/z* calcd for C₇₅H₁₄₈BrN₅O₁₉Si₄Na⁺, 1613.90; found 1613.94 [M + Na]⁺.

5-*O*-(12-Bromododecyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (13b).

Yield (73%). ¹H NMR (300 MHz, CDCl₃) δ 5.31 – 5.15 (m, 2H, anomeric), 4.36 – 4.01 (m, 3H), 3.93 – 3.07 (m, 17H), 2.58 – 2.35 (m, 1H), 2.13 – 1.98 (m, 1H), 1.98 – 1.72 (m, 3H), 1.53 – 1.38 (m, 45H, Boc), 1.38 – 1.23 (m, 19H), 1.05 – 0.80 (m, 36H, TBDMS *tert*-butyl), 0.27 – -0.03 (m, 24H, TBDMS –CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 85.77, 79.42, 79.24, 57.27, 50.52, 34.06, 32.88, 32.83, 31.60, 30.67, 30.05, 29.67, 29.58, 29.47, 29.44, 28.82, 28.66, 28.52, 28.42, 28.22, 28.18, 26.16, 26.04, 26.01, 25.80, 18.52, 18.36, 18.12, 17.93, -3.38, -3.77, -4.17, -4.93, -5.06, -5.21. ESI-MS: *m/z* calcd for C₇₉H₁₅₆BrN₅O₁₉Si₄Na⁺, 1692.955; found 1692.972 [M + Na]⁺.

5-*O*-(*n*-Hydroxylalkyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (14a–c).

Compounds **14a–c** were prepared by treating compounds **13a–c** with Cs₂CO₃ in aqueous conditions, according to general procedure B.

5-*O*-(4-Hydroxylbutyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (14a).

Yield (27%). ¹H NMR (300 MHz, CDCl₃) δ 5.31 – 5.16 (m, 2H, anomeric), 4.74 – 4.51 (m, 1H), 4.27 – 3.93 (m, 2H), 3.92 – 3.07 (m, 17H), 2.55 – 2.32 (m, 1H), 2.28 – 2.12 (m, 1H), 2.09 – 1.93 (m, 1H), 1.74 – 1.53 (m, 4H), 1.49 – 1.30 (m, 45H, Boc), 1.00 – 0.80 (m, 36H, TBDMS), 0.21 – -0.03 (m, 24H, TBDMS). ESI-MS: *m/z* calcd for C₇₁H₁₄₁N₅O₂₀Si₄Na⁺, 1518.9; found 1519.2 [M + Na]⁺.

5-*O*-(8-Hydroxyoctyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (14b).

Yield (30%). ¹H NMR (300 MHz, CDCl₃) δ 5.26 – 5.12 (m, 2H), 4.30 – 4.02 (m, 3H), 3.87 – 3.06 (m,

16H), 2.44 (d, $J = 12.5$ Hz, 1H), 2.22 (s, 1H), 2.06 – 1.92 (m, 2H), 1.59 (m, 1H), 1.52 – 1.33 (m, 45H, *Boc*), 1.30 – 1.18 (m, 8H), 1.12 – 0.99 (m, 1H), 1.00 – 0.72 (m, 36H, TBDMS *tert*-butyl), 0.21 – -0.20 (m, 24H, TBDMS –CH₃). C₇₅H₁₄₉N₅O₂₀Si₄Na⁺, 1575.0; found 1575.4 [M + Na]⁺.

5-*O*-(12-Hydroxyldodecyl)-1,3,2',6',3''-penta-*N*-*Boc*-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin

(14c). Yield (39%). ¹H NMR (300 MHz, CDCl₃) δ 5.31 – 5.15 (m, 2H, anomeric), 4.36 – 4.01 (m, 3H), 3.93 – 3.07 (m, 17H), 2.58 – 2.35 (m, 1H), 2.13 – 1.98 (m, 1H), 1.98 – 1.72 (m, 3H), 1.53 – 1.38 (m, 45H, *Boc*), 1.38 – 1.23 (m, 19H), 1.05 – 0.80 (m, 36H, TBDMS *tert*-butyl), 0.27 – -0.03 (m, 24H, TBDMS –CH₃). ESI-MS: m/z calcd for C₇₉H₁₅₇N₅O₂₀Si₄Na⁺, 1631.0; found 1631.5 [M + Na]⁺.

5-*O*-(*n*-Alkanal)-1,3,2',6',3''-penta-*N*-*Boc*-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (15a–c).

Compounds **15a–c** were prepared by treating compounds **14a–c** with pyridinium chlorochromate, according to general procedure C.

5-*O*-(12-Dodecanal)-1,3,2',6',3''-penta-*N*-*Boc*-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin (15c).

Yield (85%). ¹H NMR (300 MHz, CDCl₃) δ 9.71 (s, 1H, aldehyde), 5.23 – 4.58 (m, 2H), 4.21 – 3.95 (m, 4H), 3.91 – 2.95 (m, 14H), 2.45 – 2.28 (m, 2H), 2.03 – 1.96 (m, 4H), 1.64 – 1.35 (m, 45H, *Boc*), 1.25 – 0.99 (m, 20H), 1.04 – 0.79 (m, 36H, TBDMS *tert*-butyl), 0.31 – -0.13 (m, 24H, TBDMS –CH₃).

5-*O*-[alkyl-(*Boc*)₃-cyclam]-1,3,2',6',3''-penta-*N*-*Boc*-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin

(16a–c). Compounds **16a–c** were prepared by treating compounds **15a–c** each with (Boc)₃-cyclam **10** via reductive amination, according to general procedure D.

5-*O*-[Butyl-(*Boc*)₃-cyclam]-1,3,2',6',3''-penta-*N*-*Boc*-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin

(16a). ¹H NMR (300 MHz, CDCl₃) δ 5.25 – 5.08 (m, 2H, anomeric), 5.05 – 4.93 (m, 1H), 4.89 – 4.68 (m, 1H), 4.26 – 4.06 (m, 2H), 3.87 – 3.11 (m, 26H), 2.63 – 2.28 (m, 7H), 2.03 – 1.77 (m, 3H), 1.68 – 1.21 (m, 80H), 0.97 – 0.79 (m, 36H, TBDMS *tert*-butyl), 0.25 – -0.14 (m, 24H, TBDMS –CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 155.63, 154.67, 79.50, 79.32, 57.15, 50.70, 48.26, 46.93, 35.77, 28.64,

28.54, 28.50, 28.44, 26.13, 26.01, 25.79, 18.48, 18.30, 18.08, 17.91, -3.31, -3.72, -4.17, -4.86, -4.91, -4.99, -5.14. ESI-MS: m/z calcd for $C_{96}H_{188}N_9O_{25}Si_4^+$, 1980.3; found 1981.3 $[M + H]^+$.

5-*O*-[Octyl-(Boc)₃-cyclam]-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin

(16b). 1H NMR (300 MHz, $CDCl_3$) δ 5.21 (m, 1H, anomeric), 5.17 – 5.10 (m, 1H, anomeric), 5.12 – 4.94 (m, 1H), 4.77 (m, 1H), 4.34 – 3.98 (m, 3H), 3.91 – 3.04 (m, 26H), 2.63 – 2.27 (m, 6H), 2.11 (m, 1H), 2.07 – 1.95 (m, 3H), 1.95 – 1.81 (m, 2H), 1.71 – 1.60 (m, 2H), 1.57 – 1.30 (m, 76H, Boc), 1.28 – 1.18 (m, 9H), 1.12 – 1.00 (m, 1H), 0.99 – 0.79 (m, 36H, TBDMS *tert*-butyl), 0.30 – -0.11 (m, 24H, TBDMS $-CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.67, 154.72, 79.31, 79.24, 60.34, 48.38, 35.68, 30.29, 29.78, 28.64, 28.53, 28.50, 28.41, 27.93, 26.14, 26.03, 26.00, 25.78, 21.01, 18.48, 18.33, 18.10, 17.91, 14.18, -3.78, -4.19, -4.88, -5.16. ESI-MS: m/z calcd for $C_{100}H_{195}N_9O_{25}Si_4Na^+$, 2058.84; found 2058.88 $[M + Na]^+$

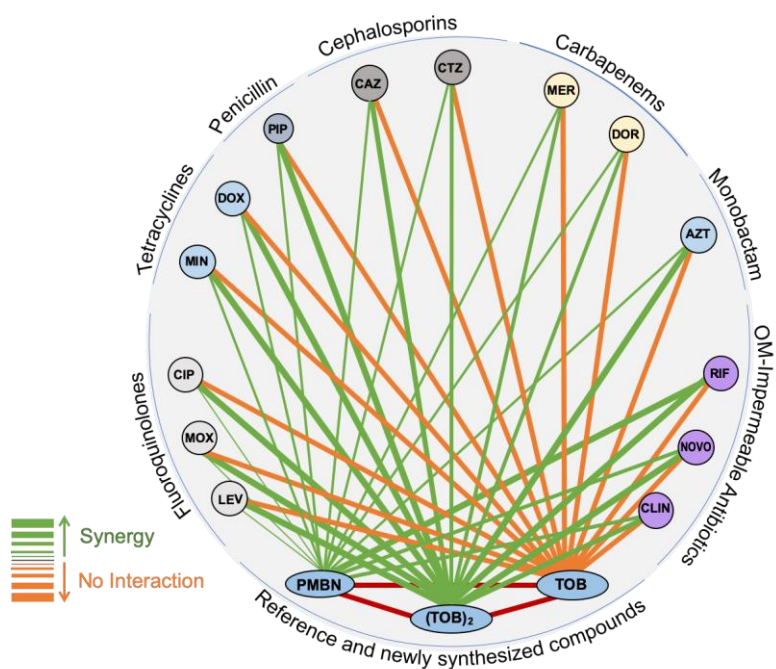
5-*O*-[Dodecyl-(Boc)₃-cyclam]-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin

(16c). 1H NMR (300 MHz, $CDCl_3$) δ 5.28 – 5.10 (m, 2H, anomeric), 3.85 – 3.04 (m, 25H), 2.59 – 2.25 (m, 6H), 1.99 – 1.78 (m, 3H), 1.73 – 1.55 (m, 4H), 1.45 – 1.34 (m, 55H), 1.28 – 1.12 (m, 20H), 0.93 – 0.71 (m, 36H, TBDMS *tert*-butyl), 0.18 – -0.09 (m, 24H, TBDMS $-CH_3$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 155.68, 155.53, 154.81, 154.56, 96.46, 91.67, 85.72, 79.89, 79.53, 79.35, 79.19, 75.25, 73.33, 72.68, 71.53, 68.05, 66.83, 63.09, 60.32, 57.26, 55.48, 50.53, 48.36, 46.97, 45.67, 36.66, 35.61, 30.64, 30.06, 29.77, 29.71, 29.68, 28.62, 28.47, 28.38, 27.69, 26.22, 26.11, 26.00, 25.97, 25.76, 24.69, 23.54, 20.97, 18.46, 18.30, 18.08, 17.89, 14.15, 7.93, -3.46, -3.81, -4.22, -4.91, -4.96, -5.09, -5.19, -5.26. MALDI TOF-MS m/e calcd for $C_{104}H_{203}N_9O_9Si_4Na^+$, 2113.386; measured m/e , 2113.381 $[M + Na]^+$.

6 CHAPTER SIX:

Broad-Spectrum Homodimeric Tobramycin Adjuvants

Repurposes Novobiocin against Gram-negative Bacteria



6.1 Preface

The work presented in this chapter has been submitted as a research article to the Journal of Medicinal Chemistry (American Chemical Society).

Temilolu Idowu, Derek Ammeter, Heather Rossong, George G. Zhanel, and Frank Schweizer (2019). Homodimeric Tobramycin Adjuvant Repurposes Novobiocin as an Effective Antibacterial Agent against Gram-negative Bacteria. *J Med Chem* (Revision Submitted).

6.1.1 Authors' Contributions

Temilolu Idowu, Derek Ammeter and Heather Rossong carried out chemical synthesis and compound characterization. Temilolu Idowu performed all biological studies and *in vivo* experiments. George G. Zhanel provided helpful insights on the project. Frank Schweizer supervised the project. Temilolu Idowu analyzed the data and wrote the manuscript. All authors were responsible for the final form of the manuscript.

6.2 Abstract

Low permeability across the outer membrane is a major reason why most antibiotics are ineffective against Gram-negative bacteria. Agents that permeabilize the outer membrane are typically toxic at their effective concentrations. Here, we report the development of a broad-spectrum homodimeric tobramycin adjuvant that is non-toxic and more potent than the gold standard permeabilizing agent, polymyxin B nonapeptide. In pilot studies, the adjuvant confers potent bactericidal activity on novobiocin against Gram-negative bacteria, including carbapenem-resistant and colistin-resistant strains bearing plasmid-borne *mcr-1* genes. Resistance development to the combination was significantly reduced, relative to novobiocin alone, and there was no induction of cross-resistance to other antibiotics, including the gyrase-acting fluoroquinolones. Tobramycin homodimer may allow the use of lower doses of novobiocin, overcoming its twin-problem of efficacy and toxicity.

6.3 Introduction

The problem of antibacterial drug resistance is complex and multifaceted, and antibiotic drug development is being massively outpaced by emerging resistance against available antibiotics.¹ Gram-negative bacteria are more difficult to eradicate because of their dual membrane topology and over expressed efflux pumps of broad substrate specificities.^{2,3} The outer membrane (OM) of Gram-negative bacteria, which serves as a barrier to the permeation of potentially noxious molecules, including antibiotics, is composed of lipopolysaccharide (LPS) on the outer leaflet and phospholipid on the inner leaflet. The mechanism by which bacteria assemble this well-organized protective barrier has been well elucidated.⁴ To breach the OM, agents that interact directly with LPS stability (e.g. polybasic molecules such as polymyxins, aminoglycosides (AGs), and chelating agents such as EDTA)⁵ or indirectly with mechanisms that synthesize, assemble, and/or transport the LPS (e.g. LpxC

inhibitors, novobiocin, etc.)^{6,7} have been investigated as potential resistance breakers for Gram-negative bacteria.

Polybasic compounds destabilize the OM by displacing divalent metals that cross-bridge adjacent phosphate groups attached to LPS core sugar, thereby altering the well-ordered polyelectrolyte barrier. Truncation and/or modification of the polymyxin class of drugs has resulted into adjuvants such as polymyxin B nonapeptide (PMBN)⁸ and SPR741⁹, where intrinsic antimicrobial activity was decoupled from their OM-destabilizing properties. Whereas PMBN seems to potentiate several OM-impermeable antibiotics against various colistin-susceptible Gram-negative bacteria,⁸ the activity of SPR741 excludes antipseudomonal effects.⁹ PMBN was shown to be generally less toxic than polymyxin B, but it causes similar proximal renal tubular injury in male rats.¹⁰ Similarly, conjugation and site-specific modification of AGs has resulted into scaffolds that lose the primary ribosomal properties of AGs but adopts an enhanced membrane effect.^{11–13} Among membrane-acting AGs, tobramycin-based adjuvants seem to be more effective against *Pseudomonas aeruginosa* than other Gram-negative bacteria.¹⁴ A major problem associated with the use of AGs is their propensity to cause irreversible hearing loss, an effect linked to the lack of precise selectivity for prokaryotic ribosomes.^{15–17} Synthetic AG analogs with lower bacterial and human mitochondrial ribosome specificities have been shown to exhibit reduced ototoxic potentials in cochlear explants, in culture and in guinea pig.¹⁸ Hence, non-ribosomal AGs may exhibit lower idiosyncratic toxicities and drug-induced hearing loss. Consequently, we sought to expand the spectrum of activity of tobramycin-derived adjuvants beyond *P. aeruginosa* by dimerizing the core scaffold of an amphiphilic tobramycin, such that the hydrophobic domain is sandwiched between two identical polar heads (Figure 6.1). This was hypothesized to overcome the hemolytic problem of classic cationic amphiphiles.^{19–22}

As a proof of concept, we investigated the possibility of repurposing novobiocin against clinically-relevant Gram-negative bacteria using the newly synthesized adjuvant. Novobiocin (Figure 6.1) is an orally active dihydroxy-glycosylated aminocoumarin antibiotic that inhibits DNA gyrase by

binding the ATP-binding site in the ATPase subunit.⁷ In 2011, the oral form of novobiocin (novobiocin sodium capsule, 250 mg) was withdrawn from US market for “reasons of safety or effectiveness”.²³ However, recent pharmacokinetic trials in non-infected subjects (Phase I and II studies) have demonstrated novobiocin plasma concentration of 150 μ M (~ 90 – 100 mg/L) for 24 h after a 5.5 g dose, with no serious toxicities.^{24,25} Novobiocin displays limited activity against Gram-negative bacteria (MICs far higher than clinically achievable serum concentrations), even though their GyrB is sensitive to the antibiotic, due to the LPS-containing OM that act as a permeability barrier. PMBN had previously been investigated in combination with novobiocin to increase penetrance,^{26,27} but effective concentrations and dose-limiting toxicities are of serious concern. Herein, we report the development of a non-toxic broad-spectrum antibiotic adjuvant that is more potent than PMBN and restores potent GyrB-dependent activity of novobiocin against multidrug (MDR) and extensively drug-resistant (XDR) Gram-negative bacteria. Concentrations as low as 0.25 μ g/ml (0.1 μ M) of the adjuvant were enough to cause a measurable effect and the addition of ≤ 7.1 μ M resulted in the attainment of MICs levels below clinically achievable plasma concentration of novobiocin in all 28 isolates studied. We also provide insights into the mechanism of action and resistance development to this combination.

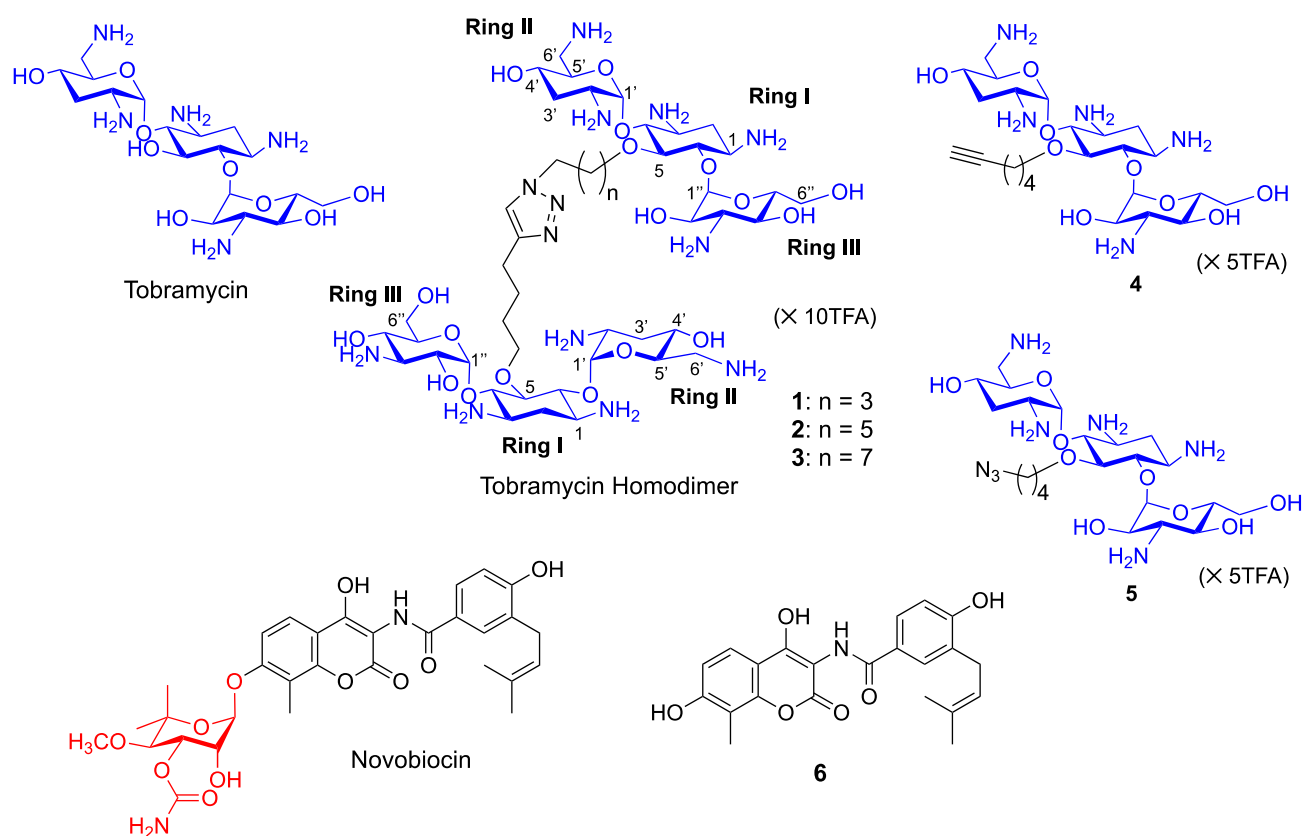


Figure 6.1. Structures of newly synthesized and reference compounds. Compounds **1–3** are tobramycin homodimers conjugated at the C-5 position of tobramycin with different tether lengths, compounds **4** and **5** are fragments of lead structure **1**, and compound **6** is an aglycone derivative of novobiocin.

6.4 Results and Discussion

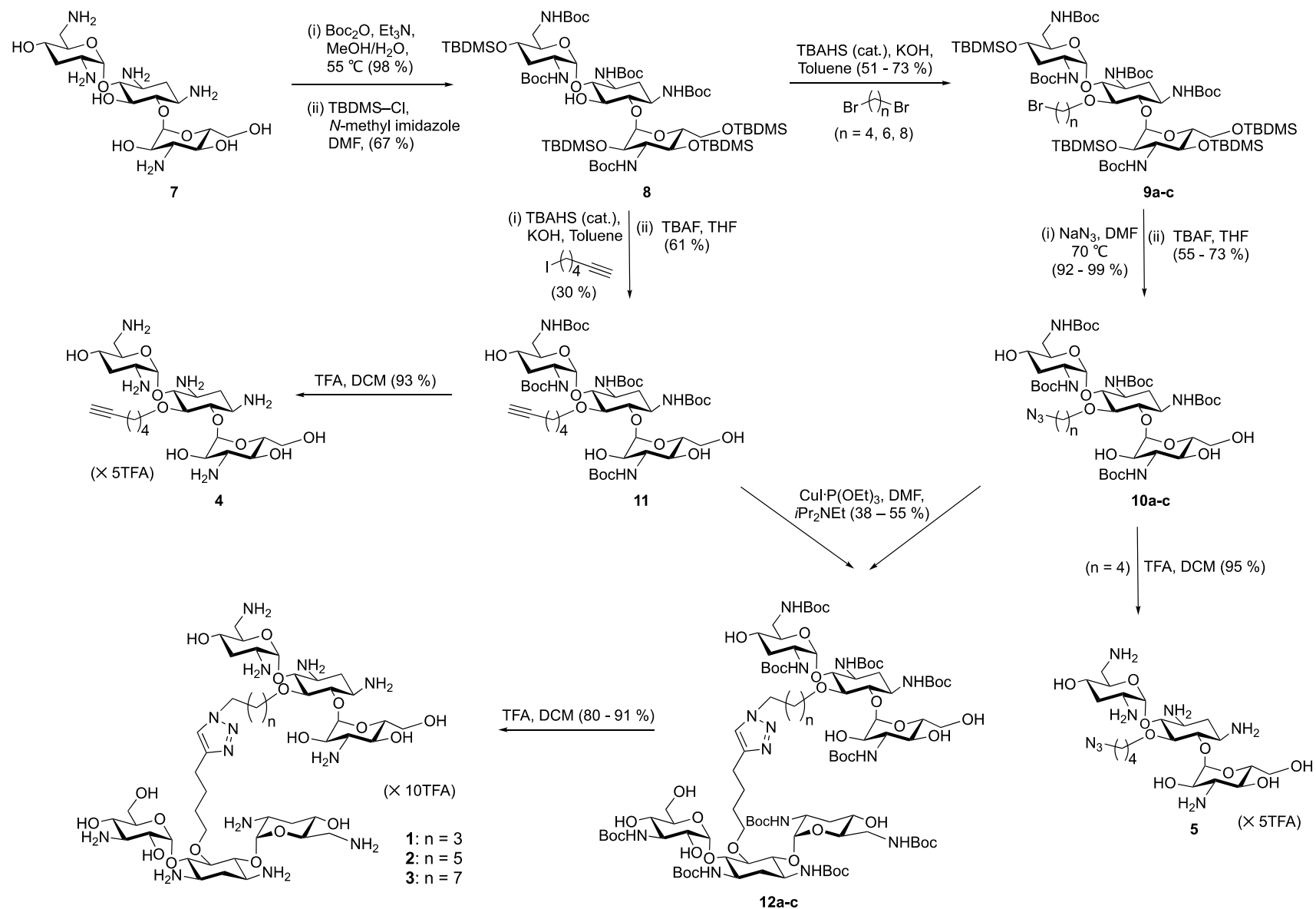
6.4.1 Design and Synthesis

The design of tobramycin homodimers **1–3** was guided by previous SAR.¹³ Amphiphilic tobramycins with lipophilic groups at the 5-OH of deoxystreptamine (ring I; Figure 6.1) have been shown to lose ribosomal activities but retain the ability to permeabilize the OM.^{13,14} Dimerization of ribosome-targeting antibiotics has also been shown to result in poor inhibitors of *in vitro* protein translation.²⁸ Hence, to prepare non-ribosomal amphiphilic-like tobramycin homodimers with potentially broad-spectrum OM permeabilizing properties, we dimerized two fragments of short-chain

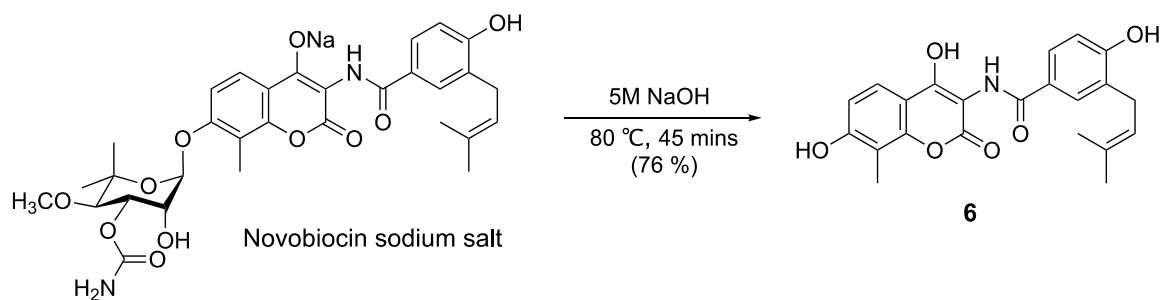
amphiphilic tobramycins ligated at the 4,6-disubstituted 2-deoxystreptamine via a copper(I)-catalyzed azide-alkyne cycloaddition reaction (Scheme 6.1). This afforded regioselective 1,4-disubstituted 1,2,3-triazole products **1–3**. Analogs with different tether length were synthesized to investigate the optimal spatial separation between the two domains while compounds **4** and **5** were prepared to study the SAR of the lead compound **1**. The full synthetic strategy for preparing compounds **1–5** is outlined in Scheme 6.1. Compound **6**, an aglycone derivative of novobiocin, was prepared to investigate the role of the L-noviose sugar on the gyrase activity of novobiocin (Scheme 6.2).

6.4.2 Chemical Synthesis of Tobramycin Homodimers (**1–3**), Fragments **4–5** and Novobiocin Aglycone (**6**)

The two amphiphilic tobramycin domains **4** and **5** were prepared following previously reported protocol.¹³ Tobramycin **7** was purchased from a commercial source and the amino groups were first protected using di-*tert*-butyl dicarbonate (Boc anhydride), followed by silylation of the *N*-Boc-tobramycin intermediate with excess TBDMSCl to afford a partially protected derivative **8** with free OH at the *C*-5 position of the deoxystreptamine ring. In the presence of a phase-transfer catalyst (TBAHS), **8** was alkylated with 1,*n*-dibromoalkane ($n = 4, 6, 8$) in toluene to afford alkylated TBDMS-Boc-protected tobramycin intermediates **9a–c**. Similarly, alkylation of **8** with iodoheptyne under the same conditions followed by TBDMS deprotection afforded **11**. The terminal bromine of **9a–c** was then displaced by an azido nucleophile under anhydrous condition and the TBDMS protecting groups were deblocked – using TBAF – to give compounds **10a–c**, followed by Boc deprotection (using TFA) to give compound **5**. Similarly, compound **4** was prepared by deblocking compound **11** with TFA. Dimerization of tobramycin was achieved by conjoining compounds **10a–c** and **11** via “click chemistry” to afford **12a–c**. Global deprotection of Boc-protecting groups using TFA afforded the final compounds **1–3** (Scheme 6.1). Compound **6** was prepared by exposing novobiocin sodium salt to a highly basic condition (5M NaOH) at 80 °C (Scheme 6.2).



Scheme 6.1. Synthesis of Tobramycin homodimers **1–3** and fragments **4–5**. Conjugates differ in the length of tether.



Scheme 6.2. Synthesis of novobiocin aglycone **6**

6.4.3 Susceptibility and Toxicity Screening

Susceptibilities of different Gram-positive and Gram-negative bacteria to the newly synthesized molecules **1–3** were determined and compared to the progenitor molecule tobramycin. The lack of activity of compounds **1–3** ($\text{MIC} \geq 16 \mu\text{g/ml}$, Table S1) against a panel of organisms, relative to tobramycin, is consistent with loss of ribosomal binding. To investigate toxicity, we tested and found that compounds **1–3** were: i) non-hemolytic against porcine erythrocytes at $1024 \mu\text{g/ml}$, ii) non-cytotoxic to human embryonic kidney (HEK293) and human liver carcinoma (HepG2) cells at $50 \mu\text{M}$ ($>128 \mu\text{g/ml}$), and iii) non-toxic *in vivo* against *Galleria mellonella* wax moths at 200 mg/kg (Figure S1). On the contrary, a single dose administration of 100 mg/kg colistin was toxic to *G. mellonella* and killed 90 % of the larvae after 96 h (Figure S1c).

6.4.4 Checkerboard Assay with different Classes of Antibiotics

The lack of antibacterial activity and non-toxic properties of **1–3** further encouraged us to screen their adjuvant properties. An ideal adjuvant is a bioactive helper molecule that is inactive by itself but can potentiate the activity of a primary antibiotic and/or delay resistance development when used in combination. These types of molecules are less likely to select for resistance.²⁹ To investigate

this, checkerboard assay was used to assess the interactions between compounds **1–3** and nineteen different antibiotics (representing all major classes) against wild-type *P. aeruginosa* PAO1. *P. aeruginosa* was selected for this initial screen because OM permeability is a major mechanism of intrinsic resistance to antibiotics,³⁰ and it is often regarded as a highly challenging model organism for new antibiotics.³¹ Compounds **1–3**, investigated at $\leq 7.1 \mu\text{M}$ based on achievable plasma concentrations (20 – 200 μM) of aminoglycosides,^{32,33} exhibit concentration-dependent synergistic relationships with all antibiotics tested against PAO1, except tobramycin, vancomycin, and colistin (Figure 6.2, Table S2). OM-impermeable antibiotics (such as rifampicin, linezolid, clindamycin and novobiocin), efflux-prone antibiotics (such as tetracyclines, fluoroquinolones, chloramphenicol, etc.), β -lactam antibiotics (such as monobactams, carbapenems, cephalosporins, and penicillins), and fosfomycin were all potentiated by 4- to 128-fold (Table S2). Tobramycin by itself is not synergistic with these antibiotics (Table S3). The antagonistic relationship between compounds **1–3** and tobramycin or colistin (FICI > 4) is consistent with observed antagonism between tobramycin and colistin at high concentrations (Figure S2). This is perhaps due to competition for LPS binding by both polybasic molecules. The lack of potentiation of vancomycin is consistent with other OM permeabilizing agents such as PMBN and pentamidine,^{26,34} where synergy is generally more pronounced with large hydrophobic molecules (e.g. rifampicin) than with large hydrophilic molecules (e.g. vancomycin). Compound **1** is the most potent (Table S2) and least toxic of the three (Figure S1), hence, it was used for further studies.

To investigate the spectrum of activity of the newly synthesized adjuvants, we examined a combination of compound **1** (at $\leq 7.1 \mu\text{M}$) and novobiocin against MDR/XDR *P. aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* exhibiting multiple resistance patterns (Table S4). Novobiocin, an ATPase inhibitor of bacterial DNA gyrase and topoisomerase IV, was selected for this study because of its unique mechanism of action but lack of meaningful activity against Gram-negative bacteria even though their GyrB is sensitive to

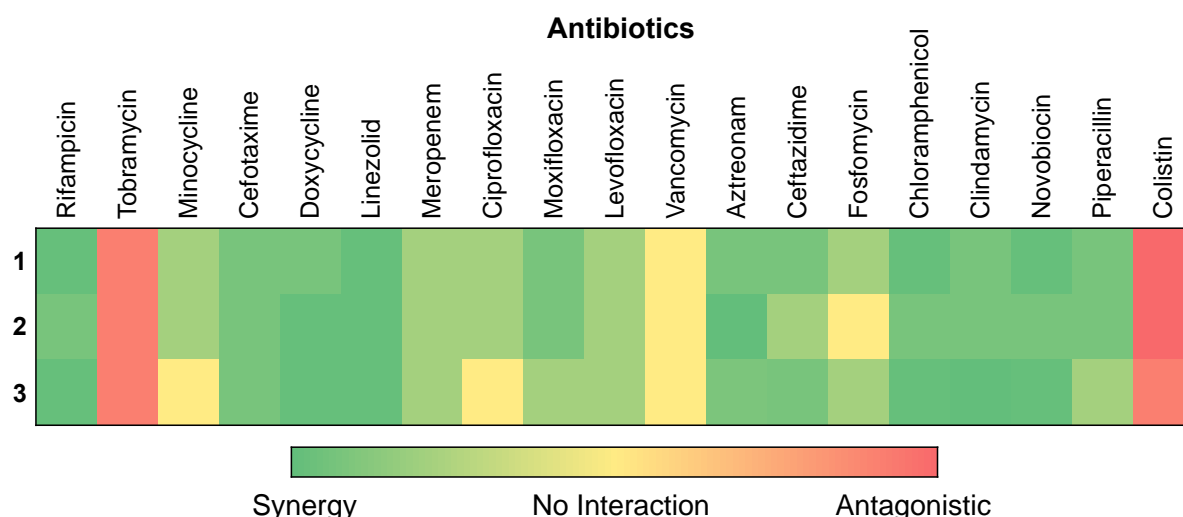


Figure 6.2. Interactions of compounds **1–3** (at $\leq 7.1 \mu\text{M}$) with different antibiotics against *P. aeruginosa* PAO1. FICI ≤ 0.5 = Green (synergistic); FICI > 0.5 but < 1 = Yellow (no interaction); FICI > 4 = Red (antagonistic)

the antibiotic.^{26,27} Synergy was retained in 100% of all isolates tested (8- to 256-fold potentiation; Table 6.1) – including carbapenem- and colistin-resistant clinical isolates (Table S4), isolates bearing GyrA mutations (Table S5), and strains carrying the mobilized colistin resistance (*mcr-1*) gene that confer plasmid-mediated resistance to colistin.³⁵ We conclude that resistance to novobiocin in Gram-negative bacteria is primarily due to OM permeability, and that resistance to fluoroquinolones (via GyrA mutations) does not necessarily confer resistance to novobiocin. To contextualize the potency of compound **1**, we compared its ability to potentiate novobiocin to the ‘gold standard’ potentiator molecule, PMBN, and observed that at equimolar molar concentrations of $7.1 \mu\text{M}$, compound **1** reproducibly displayed superior activity to PMBN against colistin-resistant Gram-negative bacteria (Table 6.2, Figure S3a).

Table 6.1. **Tobramycin homodimer potentiates novobiocin against Gram-negative bacteria.**

Synergistic effects of novobiocin and compound **1** (at $\leq 7.1 \mu\text{M}$, i.e. $\leq 16 \mu\text{g/ml}$) against wild-type and clinical isolates of Gram-negative bacteria. MIC of compound **1** is $> 128 \mu\text{g/ml}$ against all strains.

[†]Wildtype; [‡]Clinical isolate; [§]Carbapenem-resistant; *Colistin resistant; [#]*mcr-1* gene positive.

| Bacteria | Strain | MIC ($\mu\text{g/ml}$) of Novobiocin | | Fold Potentiation |
|----------------------|---------------------------|--|---------------------|-------------------|
| | | Alone | + Compound 1 | |
| <i>P. aeruginosa</i> | PAO1 [†] | 512 | 4 | 128 |
| | 259-96918 ^{‡,§} | 512 | 8 | 64 |
| | 260-97103 ^{‡,§} | 256 | 1 | 256 |
| | 262-101856 ^{‡,§} | 512 | 8 | 64 |
| | 264-104354 ^{‡,§} | 512 | 8 | 64 |
| | 91433 ^{‡,§,*} | 16 | 1 | 16 |
| | 100036 ^{‡,§} | 1024 | 8 | 128 |
| | 101243 ^{‡,§,*} | 128 | 1 | 128 |
| | 101885 ^{‡,§} | 1024 | 8 | 128 |
| | 114228 ^{‡,§,*} | 512 | 16 | 32 |
| <i>A. baumannii</i> | ATCC 17978 [†] | 16 | 0.5 | 32 |
| | 027 [‡] | 8 | 0.5 | 16 |
| | 031 [‡] | 8 | 0.5 | 16 |
| | 92247 ^{‡,*} | 4 | 0.25 | 16 |
| | 110193 [‡] | 256 | 1 | 256 |
| | LAC-4 [‡] | 8 | 0.5 | 16 |
| <i>E. coli</i> | ATCC 25922 [†] | 64 | 2 | 32 |
| | 94393 ^{‡,*#} | 128 | 2 | 64 |
| | 94474 ^{‡,*#} | 256 | 16 | 16 |
| | 107115 ^{‡,§} | 128 | 8 | 16 |
| <i>K. pneumoniae</i> | 113250 ^{‡,*} | 512 | 16 | 32 |
| | 113254 ^{‡,*} | 256 | 16 | 16 |
| | 116381 [‡] | 1024 | 8 | 128 |
| <i>E. cloacae</i> | 117029 [‡] | 1024 | 8 | 128 |
| | 118568 ^{‡,*} | 1024 | 32 | 32 |
| | 121187 ^{‡,*} | 64 | 8 | 8 |

Next, we investigated whether tobramycin by itself would potentiate novobiocin against Gram-negative bacteria. We tested a combination of tobramycin and novobiocin against tobramycin-susceptible and tobramycin-resistant Gram-negative bacteria strains and observed no synergy ($FICI \geq 0.75$) (Table S6). Other antibiotics such as minocycline, ceftazidime, rifampicin, etc. also did not show any synergy with novobiocin, consistent with a previous chemical screen of 30,000 small molecules that identified just a handful of compounds (four hits) capable of synergizing with novobiocin.³⁶ To establish whether the synergistic relationship of compound **1** with novobiocin was specific to Gram-negative bacteria, we assessed the combination against Gram-positive bacteria. Whereas novobiocin displayed potent activity against MRSA ATCC 33592 (MIC, 0.125 $\mu\text{g/ml}$), MRSE 61589 (MIC, 0.0625 $\mu\text{g/ml}$), and *E. faecium* ATCC 27270 (MIC, 2 $\mu\text{g/ml}$), consistent with prior literature,³⁷ addition of compound **1** did not improve the activity of novobiocin further (Table S7). This suggests that OM-permeabilization in Gram-negative bacteria, but not in Gram-positive, is the predominant mechanism by which compound **1** (and PMBN) enhances the activity of novobiocin. Notably, a high level of susceptibility to novobiocin ($\text{MIC} \leq 1 \mu\text{g/ml}$) was attained in wild-type and MDR *A. baumannii* clinical isolates in the presence of $\leq 7.1 \mu\text{M}$ of compound **1** (Table 6.1).

To investigate SAR of tobramycin homodimers, we prepared the constituent fragments (alkyne **4** and azide **5**) of compound **1**, the most potent of the synthesized derivatives (Scheme 1). We also examined the adjuvant properties of a Boc-protected derivative **12a** to investigate the role of free amines as it relates to OM destabilization. Compounds **4** and **5** did not potentiate novobiocin against *P. aeruginosa* PAO1 (Table S8), suggesting that covalent linkage of both domains is critical for the function of the adjuvant. This observation is consistent with optimal hydrophobic-charge threshold that must be maintained by cationic amphiphiles in order to destabilize bacterial membranes.^{19–22} Similarly, compound **12a** did not potentiate novobiocin against *P. aeruginosa* PAO1 (Table S8), indicating that electrostatic interactions between the positively charged amines in compound **1** and the

negatively charged phosphate residues on the OM of Gram-negative bacteria is central to the potency of the adjuvant.

Table 6.2. Compound **1** potentiates Novobiocin better than PMBN against Gram-negative bacteria.

PA = *P. aeruginosa*; AB = *A. baumannii*; KP = *K. pneumoniae*; EC = *E. cloacae*. [†]Wild type; [‡]Clinical isolate.

| Organism | MIC (μg/mL) of Novobiocin | | |
|-----------------------------------|---------------------------|-----------------|---------------------|
| | Alone | + PMBN (7.1 μM) | + 1 (7.1 μM) |
| PAO1 [†] | 512 | 4 | 4 |
| PA 91433 [‡] | 16 | 4 | 1 |
| PA 114228 [‡] | 512 | 128 | 16 |
| PA 101243 [‡] | 128 | 8 | 1 |
| <i>E. coli</i> 94393 [‡] | 128 | 4 | 2 |
| <i>E. coli</i> 94474 [‡] | 256 | 32 | 16 |
| AB 92247 [‡] | 4 | 1 | 0.25 |
| KP 113254 [‡] | 256 | 64 | 16 |
| EC 118568 [‡] | 1024 | 512 | 32 |

6.4.5 Potentiation of Novobiocin is Independent of RND Efflux Pumps

To investigate whether efflux pumps play a role in the restoration of potent antibacterial activity of novobiocin by tobramycin homodimers, we assessed and compared the presence of synergy in efflux-deficient *P. aeruginosa* strains to wild type PAO1. Efflux mutants PAO200 and PAO750, lacking different clinically-relevant efflux pumps that extrude different classes of antimicrobial agents, exhibited higher level of susceptibilities to novobiocin (MIC, 32 μg/ml) than wild type PAO1 (MIC, 512 μg/ml), suggesting that novobiocin is a substrate of the RND efflux pumps. The MIC of compound **1** was unaffected by these pumps (Table 6.3). However, compound **1** (at 7.1 μM) further increased the

susceptibilities of the mutants to novobiocin and lowered its MICs in both strains from 32 µg/ml to 0.125 µg/ml (256-fold potentiation) (Table 6.3). This suggests that whereas novobiocin is a substrate of the RND pumps, its potentiation by compound **1** is independent of these pumps.

Table 6.3. Potentiation of novobiocin in *P. aeruginosa* by compound **1 is independent of RND efflux pumps.** PAO1 = wild-type, PAO200 (Δ mexAB-oprM) and PAO750 (Δ mexAB-oprM, Δ mexCD-oprJ, Δ mexEF-oprN, Δ mexJK, Δ mexXY, and Δ opmH outer membrane) are efflux-deficient strains.^{38,39} MICs are reported in µg/ml.

| Strain | MIC of Compound 1 | MIC of Novobiocin | | Fold Potentiation |
|--------|-----------------------------|-------------------|-------------------------------|-------------------|
| | | Alone | + 7.1 µM Compound 1 | |
| PAO1 | >128 | 512 | 4 | 128 |
| PAO200 | >128 | 32 | 0.125 | 256 |
| PAO750 | >128 | 32 | 0.125 | 256 |

6.4.6 Time-Kill Assay

To scrutinize the data generated by checkerboard assay and provide more complementary evidence for the observed synergy, time-kill assays were performed on four different Gram-negative bacteria. Novobiocin is predominantly bacteriostatic versus most bacteria but could also be bactericidal against some pathogens. We investigated the kinetics of killing in LB media at fixed concentrations of 32 µg/ml (50.4 µM) for novobiocin and 16 µg/ml (7.1 µM) for compound **1** because these concentrations (in combination) inhibit visible growth of all isolates studied in MHB media (Table 6.1). For PAO1 and *K. pneumoniae* 116381, 32 µg/ml of novobiocin alone resulted in bacterial growth identical to their respective controls (without drug) while a combination of novobiocin (32 µg/ml) and compound **1** (7.1 µM) inhibited the growth of both pathogens in LB media (Figure 3). The combination resulted

in bactericidal (PAO1) and synergistic relationships against both strains after 24 h. For *A. baumannii* ATCC 17978, 32 µg/ml novobiocin alone was bacteriostatic after 24 h, while a combination of novobiocin (32 µg/ml) and compound **1** (7.1 µM) was bactericidal (> 3-Log reduction) and synergistic after 9 h of incubation (Figure 3). After 24 h, the bacterial culture containing novobiocin and compound **1** was completely sterilized, representing > 5-Log reduction from the starting inoculum. For *E. coli* ATCC 25299, 32 µg/ml novobiocin alone was bactericidal after 24 h, while a combination of novobiocin (32 µg/ml) and compound **1** (7.1 µM) exhibited bactericidal effects after 6 h of incubation (Figure 6.3). Novobiocin and compound **1** became synergistic against *E. coli* ATCC 25922 after 9 h of incubation and the culture was completely sterilized within this period. Overall, the species-dependent degree of bacterial load reduction reflects, to a large extent, the fold potentiation of novobiocin by compound **1** in checkerboard assay.

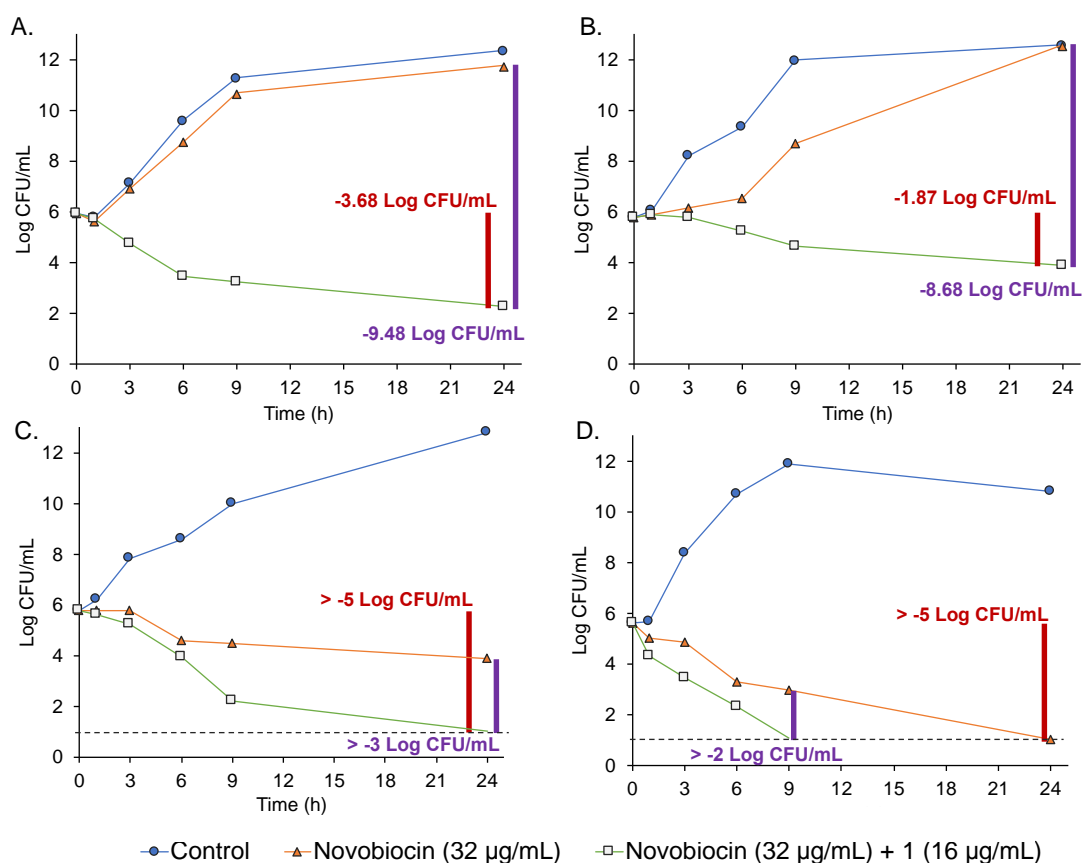


Figure 6.3. Time-kill synergy graphs. The activities of novobiocin (32 µg/ml) in combination with compound 1 (16 µg/ml, i.e. 7.1 µM) against (A) *P. aeruginosa* PAO1, (B) *K. pneumoniae* 116381, (C) *A. baumannii* ATCC 17978, (D) *E. coli* ATCC 25922. Red bars and numbers indicate differences in bacterial concentrations between the starting inoculum and drug combination at 24 h. Purple bars and numbers indicate differences in bacterial concentrations between the combination and the most active single agent at 24 h (9 h for *E. coli*). The dashed lines represent the lower limit of detection. Each data point represents an average of three independent determinations.

6.4.7 Outer Membrane Permeabilization Assay

To investigate whether compound **1** enhances the uptake of novobiocin by permeabilizing the OM, we measured the intensity of fluorescence of the nonpolar probe 1-*N*-phenylnaphthylamine (NPN) using NPN uptake assay.⁴⁰ An intact OM will ordinarily prevent the uptake of NPN dye which fluoresces strongly in phospholipid environments but only weakly in an aqueous environment. We observed that tobramycin homodimer **1** permeabilizes the OM of *P. aeruginosa* PAO1 in a dose-dependent manner, reminiscent of PMBN, while tobramycin alone showed weak fluorescence (Figure 6.4). PMBN is a known OM permeabilizer,²⁶ and our results showed that compound **1** (at 16 $\mu\text{g/ml}$) caused an increased fluorescence of NPN relative to PMBN (8 $\mu\text{g/ml}$) at similar micromolar concentrations (Figure 6.4). This observation is consistent with data from checkerboard assay (Table 6.2).

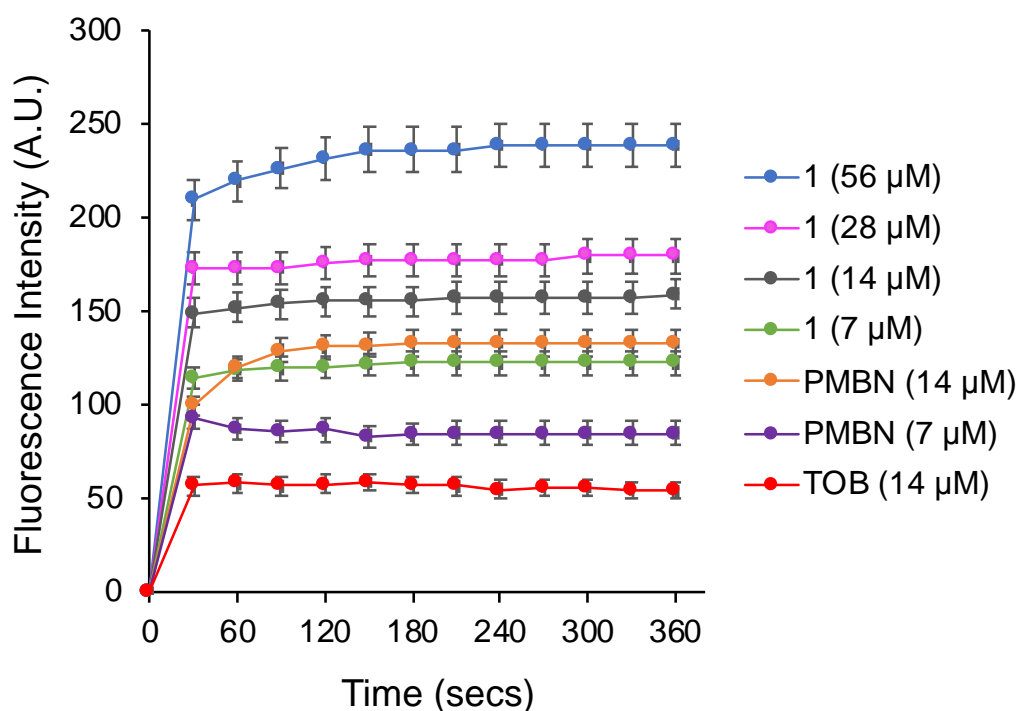


Figure 6.4. Outer membrane permeabilization by compound **1**, polymyxin B nonapeptide (PMBN) and tobramycin (TOB) was determined by measuring the accumulation of 1-*N*-phenylnaphthylamine

(NPN) in *P. aeruginosa* PAO1 cells. Each data point is an average of four independent determinations \pm SD.

6.4.8 Mechanism of Resistance Study

To investigate the possible mechanisms of resistance development to the combination, we generated some *A. baumannii* mutants by exposing wild-type ATCC 17978 to sub-MICs of novobiocin, alone and in combination with compound **1**. *A. baumannii* was used for this study because all strains exhibited a high level of susceptibility to novobiocin + compound **1** combination (Table 6.1). Tobramycin-resistant and colistin-resistant mutants were also generated to investigate the mechanism and pattern of resistance development. Emergence of resistance to novobiocin alone was fast and high level, consistent with GyrB mutation,⁴¹ with a 64-fold change in MIC (from 8 μ g/ml to 512 μ g/ml) after just 3 days and a 256-fold change in MIC (from 8 μ g/ml to 2048 μ g/ml) after 5 days (Figure 6.5). On the other hand, a combination of novobiocin and compound **1** (7.1 μ M) resulted in a slow and steady 2-fold increase in MIC every generation, with a 4-fold increase in MIC (from 0.5 μ g/ml to 2 μ g/ml) after 3 days and a 16-fold change in MIC (from 0.5 to 8.0 μ g/ml) after 5 days (Figure 6.5). Tobramycin alone resulted in a 512-fold increase in MIC (from 1 to 512 μ g/ml) after 7 days while colistin resulted in a 1024-fold change in MIC after 7 days (Figure 6.5).

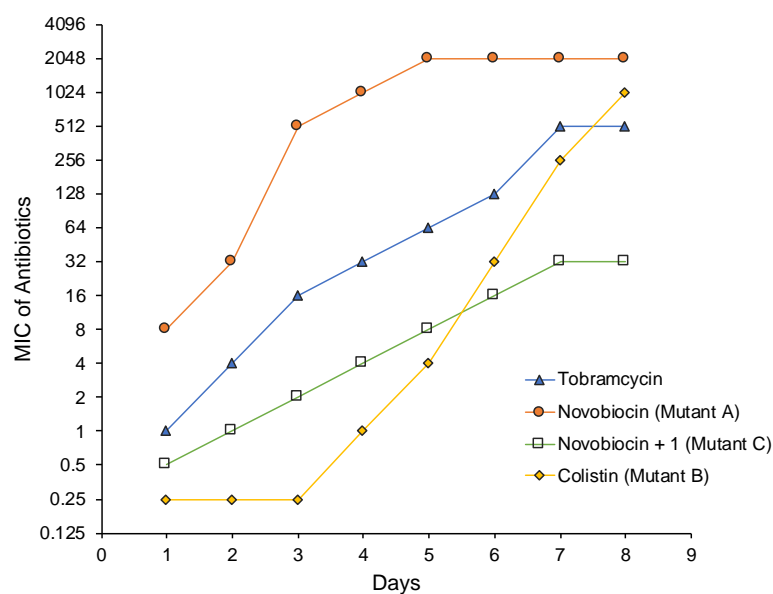


Figure 6.5. Emergence of resistance study. Resistance acquisition during serial passaging of *A. baumannii* ATCC 17978 in the presence of sub-MIC levels of antibiotics. For combination study, compound **1** was kept constant at a concentration of 7.1 μ M throughout the experiment.

To gain insights into the emerging mechanism(s) of resistance to novobiocin/compound **1** combination, we assessed the susceptibilities of all generated mutants to different antibiotics (Table 4) and performed checkerboard assay on the novobiocin-resistant and colistin-resistant mutants A and B, respectively. Exposure of *A. baumannii* ATCC 17978 to sub-MIC levels of tobramycin homodimer **1** for seven serial passages did not generate tobramycin-resistant phenotype, whereas tobramycin alone did, suggesting that tobramycin homodimers do not bind to any critical target in the bacteria. Novobiocin-resistant mutant A (Table 6.4) was found to be resistant to ciprofloxacin (MIC, 16 μ g/ml) and colistin (MIC, 8 μ g/ml), suggesting gyrase mutation(s) as a primary mechanism of resistance but also LPS modification and efflux. Compound **1** was able to further potentiate novobiocin against this highly novobiocin-resistant mutant by 16-fold (Figure S3b). Surprisingly, the generated colistin-resistant mutant B was hyper-susceptible to all antibiotics tested, consistent with a complete loss of

LPS production that resulted in collateral sensitivity to antibiotics,⁴² or/and mutations in the PmrAB two-component system.⁴³ The lipid A component of LPS is critical for the activity of colistin,⁴² and the development of such high level resistance to colistin under clinical conditions might constitute a huge fitness cost to the pathogen. PMBN and compound **1** did not potentiate novobiocin further against this strain (Figure S3c), suggesting that LPS interaction is critical for OM destabilization by both compounds. Resistance to novobiocin/compound **1** combination did not confer resistance to tobramycin, ciprofloxacin, and colistin, but conferred low level resistance to novobiocin alone (Mutant C; Table 6.4), suggesting that the combination did not trigger the production of aminoglycoside modifying enzymes (AMEs), overexpression of efflux pumps, and/or LPS modifications. By comparing the MICs of novobiocin versus mutants A and C, it is evident that compound **1** suppressed the development of resistance to novobiocin. Overall, the mechanism of resistance development to novobiocin/compound **1** combination is consistent with gyrase mutation, not LPS modification and AMEs production, and compound **1** significantly delayed this process.

Table 6.4. Susceptibility profiles (MIC in $\mu\text{g/ml}$) of wild-type *A. baumannii* ATCC 17978 versus resistant mutants generated from seven serial passages (Day 8) of exposure to sub-MICs novobiocin (Mutant A), colistin (Mutant B) and novobiocin + 7.1 μM compound **1** (Mutant C).

| Antibiotics | Wild-type | Mutant A | Mutant B | Mutant C |
|-----------------------|-----------|----------|----------|----------|
| Novobiocin | 16 | 2048 | <0.031 | 128 |
| Tobramycin | 1 | 1 | 0.25 | 1 |
| Ciprofloxacin | 1 | 16 | 0.0625 | 1 |
| Minocycline | 0.125 | 0.5 | <0.031 | 0.125 |
| Rifampicin | 2 | 2 | <0.031 | 2 |
| Ceftazidime | 16 | 32 | 1 | 16 |
| Chloramphenicol | 64 | 32 | 8 | 64 |
| Colistin | 0.063 | 8 | 1024 | 0.031 |
| Novobiocin + 1 | 0.5 | 128 | <0.031 | 16 |

6.4.9 Potentiation of Novobiocin by Compound **1** is dependent on Gyrase B Activity

A study has shown that synergistic relationship between novobiocin and polymyxin B is independent of gyrase activity of novobiocin.⁴⁴ To ascertain whether potentiation of novobiocin by compound **1** is indeed based on the binding of novobiocin to the ATP-binding site in the ATPase subunit of GyrB, we prepared novobiocin aglycone **6** (Scheme 6.2) lacking the L-noviose sugar of novobiocin, which is known to make important contacts with GyrB.⁴⁵ Consistent with a loss of gyrase activity, potent antibacterial activity was completely lost in compound **6**, even against Gram-positive organisms that are susceptible to novobiocin (Table S9). Novobiocin aglycone **6** has been reported to lose *in vitro* supercoiling activity against *A. baumannii* gyrase by at least 260-fold, relative to novobiocin.⁴⁴ We thereafter evaluated whether any synergistic relationship exists between compounds **1** and **6** against *P. aeruginosa* PAO1, *A. baumannii* ATCC 17978, and *E. coli* ATCC 25922.

Tobramycin homodimer **1** potentiates novobiocin against all of these strains (Table 6.1) but there was no potentiation of compound **6** against any of these pathogens (FICI > 1) (Table S10a). On the contrary, compound **6** was found to potentiate colistin against *A. baumannii* ATCC 17978 and *E. coli* ATCC 25922 (Table S10b), similar to a previous report.⁴⁴ This suggests that whereas the synergistic relationship between colistin and novobiocin (or compound **6**) might not be gyrase-dependent, the synergistic relationship between tobramycin homodimers and novobiocin is DNA-gyrase dependent.

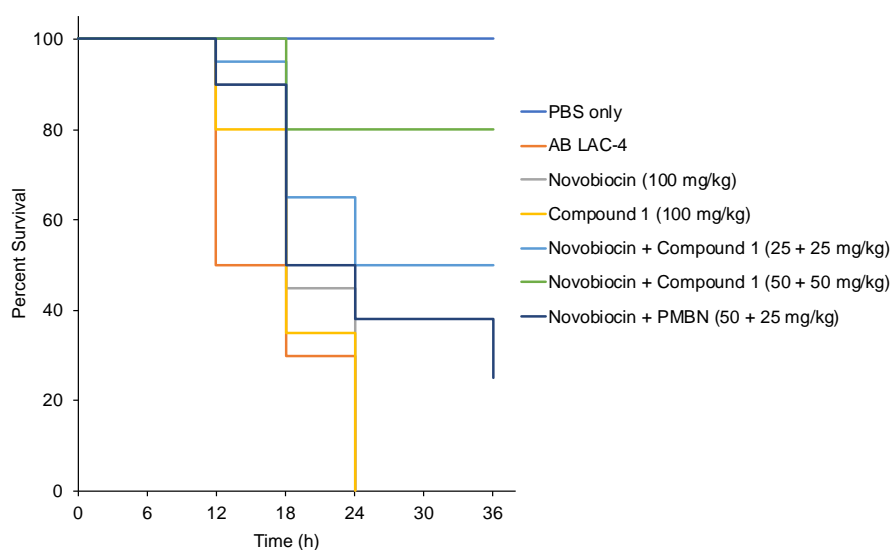
6.4.10 *In Vivo* Efficacy in *Galleria mellonella* Larvae

To expand the robustness of the data generated using *in vitro* assays, we performed preliminary *in vivo* investigation on the ability of novobiocin and compound **1** to protect *G. mellonella* moths from two different MDR *A. baumannii* infections. Despite its well-described limitations, the use of *G. mellonella* larvae as preliminary *in vivo* model system to study host-pathogen interactions, virulence, toxicity, and efficacy of novel compounds has been demonstrated with clinically used therapeutic agents.^{46–49} We found that the larvae tolerated 200 mg/kg each of compound **1** and novobiocin alone, and a 100 + 100 mg/kg combination of both, for more than 4 days, whereas exposure to 100 mg/kg of colistin (single dose administration) resulted in 90 % mortality after 4 days (Figure S1c). A multiple, but not single, dose administration of 25 mg/kg novobiocin + 50 mg/kg PMBN had previously been reported to protect mice challenged with MDR *P. aeruginosa* or *K. pneumoniae*.²⁶ In pilot studies, we investigated the *in vivo* therapeutic potential of novobiocin + compound **1** (25 + 25 and 50 + 50) mg/kg in the infection model. Untreated larvae challenged with MDR *A. baumannii* died within 24 h of infection. A single dose administration of 100 mg/kg each of novobiocin alone or compound **1** alone also resulted in 100 % mortality of infected larvae after 24 h. However, a single dose administration of novobiocin + compound **1** (50 + 50 mg/kg) protected ~80 % of the larvae after 24 h for more than

96 h (Figure 6.6). An approximate equimolar concentration of novobiocin + PMBN (50 + 25 mg/kg) only protected ~35 % of the larvae from the same infection (Figure 6.6).

To ascertain that the combination therapy does not elevate toxicity against eukaryotic cells, we assessed the toxicity of a combination of compound **1** and novobiocin against HEK293 and HepG2 cell lines and found the combination to be non-cytotoxic (Figure S1d). These preliminary data provide a baseline for future studies in mice.

A)



B)

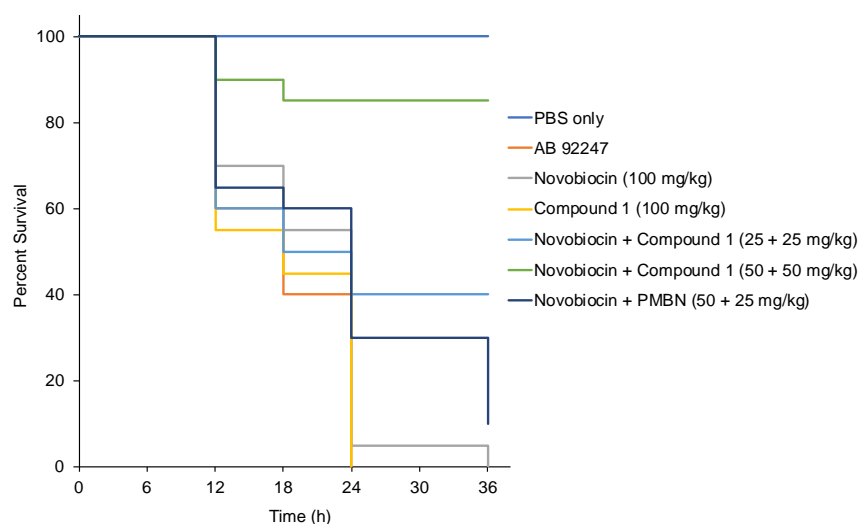


Figure 6.6. Preliminary efficacy studies of mono- and combination therapies in MDR *Acinetobacter baumannii* (AB)-challenged *Galleria mellonella* wax worms. A) AB Lac-4, a multidrug-resistant

hypervirulent strain isolated from a hospital outbreak in LA county, California. B) AB 92247, a colistin-resistant MDR clinical isolate. Survivability of the larvae was scored every 6 h for 96 h.

6.5 Conclusion

The problem of antimicrobial resistance is forcing a re-think and re-evaluation of our old antibiotics, with a view to repurposing them to combat emerging threats. Antibiotic adjuvants are becoming increasingly attractive as a strategy to address the immediate needs of effective treatment options against MDR pathogens. We report the development of a broad-spectrum homodimeric tobramycin adjuvant that is more potent than the gold standard potentiator molecule, PMBN, and confers potent bactericidal activity on novobiocin against MDR/XDR Gram-negative bacteria. *A. baumannii* isolates exhibit a high level of susceptibility to this combination, allowing the use of lower doses of novobiocin that could consequently improve tolerability and safety. Our data and others²⁶ suggest that intrinsic, but not acquired, resistance is the predominant mechanism of resistance to novobiocin in Gram-negative bacteria. Emergence of resistance to novobiocin alone by *A. baumannii* was spontaneous and conferred resistance to ciprofloxacin (consistent with target mutation)³⁷ whereas a combination of novobiocin and compound **1** suppressed this process and did not induce cross-resistance to other antibiotics. Preliminary *in vivo* evaluation in *G. mellonella* wax moths did not reveal any toxic interaction between the combination (colistin alone was toxic), but rather suggests a therapeutic potential for this combination. Future work will involve efficacy studies in animals and optimization of dosing regimens for each constituent molecule.

6.6 Experimental Section

6.6.1 Chemistry

All chemicals and reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada) except tobramycin that was purchased from AK Scientific Inc. (CA, USA). The chemicals were all used without further purification. Air and moisture-sensitive reactions were performed with dry solvents under nitrogen atmosphere. Thin-layer chromatography (TLC) plates were visualized by staining within ninhydrin solution in n-butanol. Yields refer to chromatography-purified homogenous materials, except otherwise stated. ^1H and ^{13}C NMR spectra were recorded on Bruker AMX-300 and AMX-500 spectrometers (Germany) as solutions and reported in the order of chemical shifts (δ) in ppm relative to the indicated solvent, multiplicity (s, singlet; d, doublet; t, triplet and m, multiplet), number of protons, and coupling constants (J) in hertz (Hz). ESI-MS and MALDI-TOF MS analyses were performed on Varian 500-MS ion trap mass spectrometer (USA) and Bruker Daltonics Ultraflextreme MALDI TOF/TOF mass spectrometer (Germany), respectively. Purity of final compounds, as determined by elemental analysis, was >95 % (Table S11).

6.6.1.1 *General Procedure A: Global Amine Deprotection (Removal of Boc Protecting Groups) for Preparation of Compounds 1–3.*

Solution of Boc-protected compounds **12a–c** in DCM (2.0 mL) were treated with trifluoroacetic acid (2.0 mL), stirred at RT for 1 h and concentrated under low *vacuo*. 2% methanol in diethylether (2.0 mL) was then added, stirred gently for 1 min and the solvent carefully decanted to give off-white solid compounds. The crude products were subsequently purified by reverse-phase flash chromatography (eluted with 100% deionized water) to afford analytically pure compounds **1–3** (80 – 91 %) as off-white TFA salt solid compounds.

6.6.1.1.1 Tobramycin Homodimers 1–3.

Final compounds **1–3** were prepared by global amine deprotection of compounds **12a–c**, according to general procedure A.

Tobramycin Homodimer 1. Yield (80 %). ^1H NMR (500 MHz, D_2O) δ 7.75 (s, 1H, CH of triazole), 5.19 (d, $J = 2.5$ Hz, 1H, anomeric H-1'), 5.17 (d, $J = 2.5$ Hz, 1H, anomeric H-1'), 4.97 (d, $J = 2.7$ Hz, 2H, anomeric H-1''), 4.30 (t, $J = 7.0$ Hz, 2H, N-CH₂ of linker), 4.13 – 4.06 (m, 2H, H-5'), 3.96 (t, $J = 9.7$ Hz, 2H, H-5''), 3.78 – 3.49 (m, 22H, H-5, H-6, H-4, H-2', H-4', H-2'', H-4'', H-6'', O-CH₂ of linker $\times 2$), 3.44 – 3.31 (m, 6H, H-1, H-3, H-3''), 3.25 – 3.17 (m, 2H, H-6'), 3.14 – 3.08 (m, 2H, H-6'), 2.61 (m, 2H, CH₂ of linker), 2.36 (dt, $J = 12.9, 4.3$ Hz, 2H, H-2), 2.09 – 2.00 (m, 4H, H-3'), 1.84 – 1.71 (m, 4H, H-2, CH₂ of linker), 1.57 – 1.41 (m, 6H, CH₂ of linker $\times 3$), 1.19 – 1.16 (m, 2H). ^{13}C NMR (125 MHz, D_2O) δ 163.28 (TFA), 163.00 (TFA), 162.72 (TFA), 162.44 (TFA), 124.16 (CH of triazole), 117.92 (TFA) 115.54 (TFA), 101.27 (C-1''), 101.17 (C-1''), 92.76 (C-1'), 82.09 (C-4''), 81.99 (C-4''), 81.80 (C-5), 81.77 (C-5), 77.07 (C-5''), 76.96 (C-5''), 76.14 (C-5'), 75.99 (C-5'), 73.21 (C-4), 73.18 (C-4), 73.06 (O-CH₂ of linker), 72.51 (O-CH₂ of linker), 68.55 (C-2''), 64.86 (C-6), 64.80 (C-6), 63.10 (C-4'), 59.35 (C-6''), 59.24 (C-6''), 54.82 (C-3''), 54.44, 50.86 (CH₂ of linker), 49.67 (C-1), 49.61 (C-1), 48.36 (C-3), 47.27 (C-2'), 47.24 (C-2'), 38.36 (C-6'), 38.32 (C-6'), 28.89 (CH₂ of linker), 27.98 (C-3'), 27.70 (C-2), 26.39 (CH₂ of linker), 25.99 (CH₂ of linker), 24.97 (CH₂ of linker), 24.12 (CH₂ of linker), 17.79, 16.32. MALDI: m/e calcd for $\text{C}_{46}\text{H}_{89}\text{N}_{13}\text{O}_{18}\text{H}^+$, 1112.653; found 1112.650 $[\text{M} + \text{H}]^+$

Tobramycin Homodimer 2. Yield (85 %). ^1H NMR (500 MHz, D_2O) δ 7.68 (s, 1H, CH of triazole), 5.21 (d, $J = 2.6$ Hz, 1H, anomeric H-1'), 5.20 (d, $J = 2.6$ Hz, 1H, anomeric H-1'), 4.99 (d, $J = 3.5$ Hz, 2H, anomeric H-1''), 4.24 (t, $J = 7.1$ Hz, 2H, N-CH₂ of linker), 4.14 – 4.07 (m, 2H, H-5'), 3.97 (td, $J = 9.8, 4.8$ Hz, 2H, H-5''), 3.79 – 3.50 (m, 22H, H-5, H-6, H-4, H-2', H-4', H-2'', H-4'', H-6'', O-CH₂ of linker $\times 2$), 3.46 – 3.34 (m, 6H, H-1, H-3, H-3''), 3.27 – 3.19 (m, 2H, H-6'), 3.18 – 3.08 (m, 2H, H-6'), 2.65 – 2.54 (m, 2H, CH₂ of linker), 2.37 (dt, $J = 12.8, 4.4$ Hz, 2H, H-2), 2.14 – 1.97 (m,

4H, H-3'), 1.82 – 1.69 (m, 4H, H-2, CH₂ of linker), 1.60 – 1.41 (m, 6H, CH₂ of linker ×3), 1.21 – 1.11 (m, 4H, CH₂ of linker ×2). ¹³C NMR (125 MHz, D₂O) δ 163.37 (TFA), 163.09 (TFA), 162.81 (TFA), 162.53 (TFA), 123.62 (CH of triazole), 119.96 (TFA), 117.67 (TFA), 115.35 (TFA), 113.04 (TFA), 101.32 (C-1''), 101.30 (C-1''), 92.76 (C-1'), 81.96 (C-4''), 81.84 (C-5), 76.93 (C-5''), 76.88 (C-5''), 75.99 (C-5'), 75.89 (C-5'), 73.49 (O-CH₂ of linker), 73.18 (C-4), 73.13 (O-CH₂ of linker), 68.55 (C-2''), 64.79 (C-6), 64.78 (C-6), 63.14 (C-4'), 63.10 (C-4'), 59.23 (C-6''), 54.82 (C-3''), 50.59 (CH₂ of linker), 49.69 (C-1), 48.37 (C-3), 47.29 (C-2'), 47.25 (C-2'), 38.41 (C-6'), 38.34 (C-6'), 29.41 (CH₂ of linker), 29.28 (CH₂ of linker), 28.85 (CH₂ of linker), 28.05 (C-3'), 27.98 (C-3'), 27.71 (C-2), 25.83 (CH₂ of linker), 25.19 (CH₂ of linker), 24.65 (CH₂ of linker), 24.31 (CH₂ of linker). MALDI: *m/e* calcd for C₄₈H₉₃N₁₃O₁₈H⁺, 1140.684; found 1140.695 [M + H]⁺

Tobramycin Homodimer 3. Yield (91 %). ¹H NMR (500 MHz, D₂O) δ 7.73 (s, 1H, CH of triazole), 5.21 (d, *J* = 2.6 Hz, 1H, anomeric H-1'), 5.20 (d, *J* = 2.6 Hz, 1H, anomeric H-1'), 4.98 (d, *J* = 3.4 Hz, 2H, anomeric H-1''), 4.25 (t, *J* = 7.0 Hz, 2H, N-CH₂ of linker), 4.10 (dt, *J* = 8.4, 3.8 Hz, 2H, H-5'), 3.97 (td, *J* = 9.8, 5.1 Hz, 2H, H-5''), 3.80 – 3.50 (m, 22H, H-5, H-6, H-4, H-2', H-4', H-2'', H-4'', H-6'', O-CH₂ of linker ×2), 3.46 – 3.33 (m, 6H, H-1, H-3, H-3''), 3.27 – 3.20 (m, 2H, H-6'), 3.16 – 3.09 (m, 2H, H-6'), 2.66 – 2.56 (m, 2H, CH₂ of linker), 2.37 (dt, *J* = 12.5, 4.3 Hz, 2H, H-2), 2.09 – 2.01 (m, 4H, C-3'), 1.84 – 1.68 (m, 4H, H-2, CH₂ of linker), 1.61 – 1.41 (m, 6H, CH₂ of linker ×3), 1.19 – 1.06 (m, 8H, CH₂ of linker ×4). ¹³C NMR (125 MHz, D₂O) δ 163.35 (TFA), 163.07 (TFA), 162.79 (TFA), 162.50 (TFA), 123.92 (CH of triazole), 120.02 (TFA), 117.70 (TFA), 115.37 (TFA), 113.05 (TFA), 101.38 (C-1''), 101.27 (C-1''), 92.75 (C-1'), 81.97 (C-4''), 81.91 (C-5), 81.81 (C-4''), 76.90 (C-5''), 76.87 (C-5''), 75.96 (C-5'), 75.91 (C-5'), 73.75 (O-CH₂ of linker), 73.18 (C-4), 73.06 (O-CH₂ of linker), 68.56 (C-2''), 64.81 (C-6), 64.77 (C-6), 63.15 (C-4'), 63.12 (C-4'), 59.23 (C-6''), 59.21 (C-6''), 54.82 (C-3''), 50.98 (CH₂ of linker), 49.72 (C-1), 49.68 (C-1), 48.38 (C-3), 47.30 (C-2'), 47.25 (C-2'), 38.41 (C-6'), 38.35 (C-6'), 29.42 (CH₂ of linker), 29.30 (CH₂ of linker), 28.82 (CH₂ of linker), 28.78 (CH₂ of linker), 28.18 (CH₂ of linker), 28.06 (C-3'), 27.99 (C-3'), 27.70

(C-2), 25.57 (CH₂ of linker), 25.19 (CH₂ of linker), 25.09 (CH₂ of linker), 24.12 (CH₂ of linker).

MALDI: m/e calcd for C₅₀H₉₇N₁₃O₁₈H⁺, 1168.715; found = 1168.719 [M + H]⁺

6.6.1.2 5-*O*-(Hexyne)-Tobramycin (**4**).

Compound **4** was prepared by exposing compound **11** to TFA according to general procedure A. Yield (93 %). ¹H NMR (500 MHz, D₂O) δ 5.40 (d, J = 2.4 Hz, 1H, anomeric), 5.18 (d, J = 3.4 Hz, 1H, anomeric), 4.26 – 4.22 (m, 1H), 4.07 (t, J = 9.7 Hz, 1H), 3.97 – 3.71 (m, 11H), 3.61 – 3.51 (m, 2H), 3.48 – 3.44 (m, 1H), 3.44 – 3.29 (m, 3H), 2.49 – 2.45 (m, 1H), 2.45 – 2.40 (m, 1H), 2.33 – 2.25 (m, 3H), 2.25 – 2.16 (m, 1H, alkyne), 1.91 (m, 1H), 1.82 – 1.72 (m, 2H), 1.64 – 1.50 (m, 2H). ¹³C NMR (126 MHz, D₂O) δ 101.3 (anomeric), 92.7 (anomeric), 81.9, 81.8, 76.6, 75.6, 73.5, 73.2, 68.6, 64.9, 63.3, 59.3, 54.8, 51.2, 49.8, 48.5, 47.4, 38.6, 29.4, 28.2, 28.0, 27.8, 26.2, 25.3, 24.8, 19.4. MALDI TOF-MS m/e calcd for C₂₄H₄₅N₅O₉, 547.3217; measured m/e 548.3219 [M + H]⁺

6.6.1.3 5-*O*-(4-Azidobutyl)-Tobramycin (**5**).

Compound **5** was prepared by deprotecting compound **10a** according to general procedure A. Yield (95 %). ¹H NMR (300 MHz, D₂O) δ 5.39 (d, J = 2.4 Hz, 1H, anomeric), 5.20 (d, J = 3.4 Hz, 1H, anomeric), 4.34 – 4.17 (m, 2H), 4.00 – 3.71 (m, 11H), 3.63 – 3.52 (m, 3H), 3.49 – 3.27 (m, 4H), 2.61 – 2.48 (m, 1H), 2.36 – 2.19 (2H), 2.11 – 1.93 (m, 1H), 1.81 – 1.56 (m, 4H). ¹³C NMR (75 MHz, D₂O) δ 101.1 (anomeric), 92.6 (anomeric), 81.9, 81.7, 76.6, 75.7, 73.1, 72.7, 68.5, 64.8, 63.2, 59.3, 54.8, 51.0, 49.6, 48.4, 47.3, 38.5, 28.0, 27.7, 26.7, 24.6. MALDI TOF-MS m/e calcd for C₂₂H₄₄N₈O₉, 565.3231; measured m/e 565.35541 [M + H]⁺.

6.6.1.4 Novobiocin Aglycone (**6**)

Compound **6** was prepared as previously reported and NMR data were consistent with literature.⁴⁴

6.6.1.5 *1,3,2',6',3''-penta-N-Boc-4',2'',4'',6''-tetra-O-TBDMS-tobramycin (8).*

Commercial tobramycin **7** (4.00 g, 8.56 mmol) was dissolved in a 2:1 mixture of methanol and water (150 mL) and treated with Boc₂O (14.25 g, 65.29 mmol) in the presence of Et₃N (8.0 mL, 57.4 mmol). The reaction mixture was stirred under reflux (at 55 °C) overnight (~ 20 h), concentrated under vacuo and thoroughly dried under high vacuum for 24 h to afford a white powdery solid (7.48 g, 90 %). The dried crude penta-*N*-boc-protected tobramycin (1.04 g, 1.07 mmol) was dissolved in anhydrous DMF (6.0 mL) and treated with *tert*-butyldimethylsilyl chloride, TBDMSCl (1.13 g, 7.49 mmol) and *N*-methylimidazole (0.6 mL, 7.49 mmol). The reaction was stirred at RT for 4 days under nitrogen gas atmosphere, and the resulting mixture was poured into water (50.0 mL) and extracted with DCM (50 mL, ×3). The organic layer was dried over anhydrous Na₂SO₄, concentrated in *vacuo*, and purified by flash chromatography using gradient elution (hexanes/ ethyl acetate, 15:1 to 8:1, v/v) to afford **8** (1.05 g, 67%) as a white solid. NMR data are consistent with an earlier report.¹³

6.6.1.6 *General Procedure B: 5-O-Alkylation of Boc and TBDMS protected Tobramycin for the Preparation of Compounds 9a–c.*

A solution of **8** (1 equiv.) in toluene was treated with KOH (3 equiv.), 1,*n*-dibromoalkane (3 equiv.), and a catalytic amount of tetrabutylammonium hydrogen sulphate, TBAHS (0.1 equiv.). The reaction mixture was stirred at RT overnight, dispersed in water and extracted with an equal volume of ethyl acetate (×3). The organic layers were combined, washed with brine (×1), dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The crude products were then purified by column chromatography (hexanes/ethyl acetate, 12:1 to 10:1, v/v). to afford compounds **9a–c** as white solids.

6.6.1.6.1 5-*O*-(*n*-Bromoalkyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin

(**9a–c**).

Compounds **9a**, **9b**, and **9c** were prepared by treating **8** with 1,4-dibromobutane, 1,6-dibromohexane, and 1,8-dibromooctane, respectively, according to general procedure B.

5-*O*-(4-Bromobutyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin

(**9a**). Yield (51 %). ¹H NMR (300 MHz, CDCl₃) δ 5.24 – 5.12 (m, 2H, anomeric), 4.28 – 4.09 (m, 3H), 3.93 – 3.14 (m, 17H), 2.61 – 2.37 (m, 1H), 2.14 – 1.84 (m, 5H), 1.72 – 1.56 (m, 3H), 1.61 – 1.35 (m, 45H, *Boc*), 1.11 – 0.72 (m, 36H, TBDMS, *tert*-butyl), 0.24 – -0.09 (m, 24H, TBDMS –CH₃). ESI-MS: *m/z* calcd for C₇₁H₁₄₀BrN₅O₁₉Si₄Na⁺, 1583.2; found 1583.2 [M + Na]⁺.

5-*O*-(6-Bromohexyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin

(**9b**). Yield (73 %). ¹H NMR (300 MHz, Chloroform-*d*) δ 5.24 – 5.07 (m, 3H), 5.05 – 4.95 (m, 2H), 4.17 – 4.00 (m, 3H), 3.79 – 3.60 (m, 5H), 3.60 – 3.26 (m, 11H), 3.24 – 3.15 (m, 2H), 2.47 – 2.32 (m, 1H), 1.98 – 1.88 (m, 1H), 1.82 – 1.69 (m, 3H), 1.54 – 1.24 (m, 52H), 0.96 – 0.73 (m, 36H), 0.15 – -0.10 (m, 24H). ¹³C NMR (75 MHz, CDCl₃) δ 96.34, 85.70, 79.81, 79.29, 79.16, 79.11, 75.20, 73.04, 71.49, 68.03, 66.96, 63.09, 57.18, 50.50, 48.85, 48.27, 36.66, 35.67, 33.54, 33.32, 32.73, 30.32, 28.57, 28.43, 28.35, 27.19, 26.06, 25.93, 25.73, 25.23, 24.67, 18.40, 18.23, 18.02, 17.84, -3.54, -3.85, -4.26, -4.94, -4.98, -5.10, -5.21, -5.26. MALDI: Exact mass calcd for C₇₃H₁₄₄BrN₅O₁₉Si₄Na⁺, 1608.861; found 1608.886[M + Na]⁺

5-*O*-(8-Bromooctyl)-1,3,2',6',3''-penta-*N*-Boc-4',2'',4'',6''-tetra-*O*-TBDMS-tobramycin

(**9c**). Yield (60 %). ¹H NMR (300 MHz, CDCl₃) δ 5.26 – 5.12 (m, 2H, anomeric), 4.39 – 3.97 (m, 3H), 3.89 – 3.07 (m, 16H), 2.47 (d, *J* = 12.8 Hz, 1H), 2.08 – 1.94 (m, 2H), 1.93 – 1.77 (m, 2H), 1.65 (m, 1H), 1.56 – 1.39 (m, 45H, *Boc*), 1.38 – 1.14 (m, 8H), 1.13 (m, 1H), 1.05 – 0.75 (m, 36H, TBDMS *tert*-butyl), 0.34 – -0.15 (m, 24H, TBDMS –CH₃). ¹³C NMR (75 MHz, CDCl₃) δ 85.77, 79.42, 79.24, 57.27, 50.52, 34.06, 32.88, 32.83, 31.60, 30.67, 30.05, 29.67, 29.58, 29.47, 29.44, 28.82, 28.66, 28.52, 28.42,

28.22, 28.18, 26.16, 26.04, 26.01, 25.80, 18.52, 18.36, 18.12, 17.93, -3.38, -3.77, -4.17, -4.93, -5.06, -5.21. ESI-MS: m/z calcd for $C_{75}H_{148}BrN_5O_{19}Si_4Na^+$, 1636.89; found 1636.80 $[M + Na]^+$.

6.6.1.7 General Procedure C: Conversion of Bromoalkyl to Azide.

A solution of bromoalkylated compounds **9a-c** (1 equiv.) in anhydrous DMF was treated with sodium azide (10 equiv.) and stirred at 70 °C for 3 h. The resulting mixture was concentrated in *vacuo*, re-dispersed in water and extracted with ethyl acetate. The combined organic layers were subsequently washed with brine ($\times 1$), dried over anhydrous Na_2SO_4 and concentrated in *vacuo* to give yellow solids.

6.6.1.8 General Procedure D: Deprotection of Hydroxyl Groups

(Removal of TBDMS Protecting Groups).

A solution of TBDMS- and Boc-protected compounds in anhydrous THF (5.0 mL) were treated with tetrabutylammonium fluoride (TBAF, 6 equiv.) and stirred under nitrogen atmosphere for 2 h. The reaction mixture was concentrated under *vacuo*, dissolved in water and extracted with DCM ($\times 3$). The organic layers were combined, dried over anhydrous Na_2SO_4 , concentrated *in vacuo*, and purified by column chromatography (hexanes/ethyl acetate, 1:1, v/v, then dichloromethane/methanol, 25:1 to 20:1, v/v) to afford off-white solids.

6.6.1.8.1 5-O-(*n*-Azidoalkyl)-1,3,2',6',3''-penta-*N*-Boc-tobramycin (**10a-c**).

Compounds **10a-c** were prepared by converting **9a-c** to azido compounds, according to general procedure C, and subsequently removing the TBDMS protecting groups according to general procedure D.

5-O-(4-Azidobutyl)-1,3,2',6',3''-penta-*N*-Boc-tobramycin (10a). Overall yield (45 %). 1H NMR (300 MHz, $CDCl_3$) δ 5.35 – 5.27 (m, 1H), 5.19 – 5.09 (m, 1H), 3.98 – 3.59 (m, 12H), 3.55 – 3.35 (m, 4H), 3.35 – 3.19 (m, 3H), 2.24 – 2.05 (m, 2H), 1.73 – 1.58 (m, 5H), 1.55 – 1.33 (m, 45H), 1.31 – 1.12 (m, 4H). ESI-MS: m/z calcd for $C_{47}H_{84}N_8O_{19}Na^+$, 1087.58; found 1087.61 $[M + Na]^+$

5-*O*-(6-Azidohexyl)-1,3,2',6',3''-penta-*N*-Boc-tobramycin (10b). Overall yield (65 %). ¹H NMR (300 MHz, CDCl₃) δ 5.33 – 5.12 (m, 3H), 4.31 – 4.09 (m, 1H), 3.96 – 3.53 (m, 12H), 3.48 – 3.33 (m, 3H), 3.31 – 3.05 (m, 4H), 2.22 – 2.01 (m, 2H), 1.76 – 1.46 (m, 6H), 1.46 – 1.08 (m, 52H). ¹³C NMR (75 MHz, CDCl₃) δ 80.59, 80.01, 79.40, 78.06, 73.25, 72.42, 70.23, 64.99, 62.08, 56.52, 52.33, 51.31, 49.10, 33.12, 29.66, 28.67, 28.47, 28.40, 28.33, 26.68, 25.21, 20.07, 13.53. ESI-MS: *m/z* calcd for C₄₉H₈₈N₈O₁₉Na⁺, 1116.3; found 1116.9 [M + Na]⁺

5-*O*-(8-Azidooctyl)-1,3,2',6',3''-penta-*N*-Boc-tobramycin (10c). Overall yield (57 %). ¹H NMR (300 MHz, CDCl₃) δ 5.49 – 5.35 (m, 1H), 5.25 – 5.11 (m, 1H), 3.88 – 3.30 (m, 16H), 3.23 – 3.02 (m, 4H), 2.14 – 1.90 (m, 2H), 1.73 – 1.04 (m, 62H). ¹³C NMR (75 MHz, CDCl₃) δ 158.77, 157.88, 155.70, 155.30, 155.09, 96.60, 80.36, 79.83, 79.50, 79.20, 73.21, 70.31, 64.94, 61.95, 56.33, 53.42, 51.30, 48.99, 32.98, 29.76, 29.53, 29.38, 28.94, 28.68, 28.44, 28.34, 28.27, 28.03, 26.49, 25.53. MALDI: *m/e* calcd for C₅₁H₉₂N₈O₁₉Na⁺, 1143.638; found 1143.659 [M + Na]⁺

6.6.1.9 5-*O*-Hexyne-1,3,2',6',3''-penta-*N*-Boc-tobramycin (**11**).

Compound **11** was prepared by reacting **8** with 6-iodohexyne, according to general procedure B, followed by TBDMS deprotection following general procedure D. Yield (30 %). ¹H NMR (500 MHz, CDCl₃) δ 5.31 – 5.23 (m, 2H), 5.15 – 4.97 (m, 2H), 3.95 – 3.50 (m, 14H), 3.48 – 3.34 (m, 4H), 3.29 – 3.20 (m, 1H), 3.18 – 3.06 (m, 1H), 2.24 – 2.06 (m, 4H), 1.97 – 1.93 (m, 1H, alkyne), 1.73 – 1.62 (m, 3H), 1.60 – 1.31 (m, 49H), 1.27 – 1.20 (m, 2H). MALDI: *m/e* calcd for C₄₉H₈₅N₅O₁₉Na⁺, 1070.574; found 1070.596 [M + Na]⁺

6.6.1.10 General Procedure E: Copper(1)-catalyzed azide-alkyne cycloaddition reaction (“Click Chemistry”) for the Preparation of compounds 12a-c.

Compounds **10a-c** (2 equiv.) and **11** (1 equiv.) were dissolved in an anhydrous DMF (4.0 mL) and treated with CuI·P(OEt)₃ (3 equiv.) and *i*Pr₂NEt (3 equiv.) The reaction was stirred under nitrogen gas for 2 h. The reaction mixture was concentrated under *vacuo*, dissolved in water and extracted with DCM (×3). The organic layers were combined, dried over anhydrous Na₂SO₄, concentrated *in vacuo*, and purified by column chromatography (dichloromethane/methanol, 40:1 to 20:1, v/v) to afford compounds **12a-c** (38 – 55 %) as white solids.

6.6.1.10.1 5-O-Alkylated-1,3,2',6',3''-penta-N-Boc-tobramycin homodimers (12a-c).

Compounds **12a-c** were prepared via a copper(1)-catalyzed azide-alkyne cycloaddition reaction between **10a-c** and **11**, according to general procedure E.

Compound 12a. Yield (38 %). ¹H NMR (300 MHz, CDCl₃) δ 5.39 – 4.99 (m, 4H), 4.32 (s, 2H), 3.88 – 3.33 (m, 27H), 3.15 (m, 2H), 2.87 – 2.52 (m, 2H), 2.45 – 2.02 (m, 5H), 2.03 – 1.81 (m, 1H), 1.66 – 1.05 (m, 103H). MALDI: *m/e* calcd for C₉₆H₁₆₉N₁₃O₃₈Na⁺, 2135.159; found 2135.169 [M + Na]⁺

Compound 12b. Yield (55 %). ¹H NMR (300 MHz, CDCl₃) δ 5.38 – 4.98 (m, 4H), 4.42 – 4.21 (m, 1H), 4.02 – 3.24 (m, 25H), 3.23 – 2.88 (m, 2H), 2.78 – 2.56 (m, 1H), 2.32 – 2.07 (m, 2H), 1.98 – 1.82 (m, 2H), 1.69 – 0.88 (m, 106H). MALDI: *m/e* calcd for C₉₈H₁₇₃N₁₃O₃₈Na⁺, 2164.514; found 2164.519 [M + Na]⁺

Compound 12c. Yield (42 %). ¹H NMR (500 MHz, CDCl₃) δ 7.37 (s, 1H), 5.28 – 5.11 (m, 5H), 4.28 (t, *J* = 6.5 Hz, 2H), 4.13 (s, 2H), 3.93 – 3.25 (m, 34H), 3.20 – 3.00 (m, 2H), 2.80 – 2.63 (m, 2H), 2.48 (s, 3H), 2.29 – 1.98 (m, 4H), 1.93 – 1.77 (m, 2H), 1.71 – 1.04 (m, 108H). ¹³C NMR (125 MHz, CDCl₃) δ 158.83, 157.95, 155.99, 155.24, 155.17, 121.36, 96.64, 80.49, 80.04, 79.42, 79.38,

73.16, 70.88, 70.58, 65.21, 56.47, 53.39, 50.59, 50.09, 49.26, 49.15, 40.88, 33.20, 31.89, 29.66, 29.19, 28.47, 28.44, 28.42, 28.39, 28.34, 25.16, 24.97, 22.65, 14.08. MALDI: m/e calcd for $C_{100}H_{177}N_{13}O_{38}Na^+$, 2191.222; found 2191.233 $[M + Na]^+$

6.6.2 Microbiology.

Bacteria isolates were either obtained from the American Type Culture Collection (ATCC), the Canadian National Intensive Care Unit (CAN-ICU) surveillance study⁴³, or the Canadian Ward (CANWARD) surveillance study^{44,45}. Clinical isolates obtained as part of the CAN-ICU and CANWARD studies from participating medical centers across Canada were cultured from body fluids and tissues of patients suffering from presumed “clinically significant” infectious diseases. Antimicrobial susceptibilities of clinical isolates were evaluated (using ATCC strains as quality control strains) and categorized, where appropriate, as either multidrug resistant (MDR), extensively drug-resistant (XDR), or pan drug-resistant (PDR). MDR is defined as acquired non-susceptibility to at least one agent in three or more antimicrobial categories, XDR as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories), and PDR as non-susceptibility to all agents in all antimicrobial categories.⁵³

6.6.2.1 Antimicrobial Susceptibility Assay.

The *in vitro* antimicrobial activity of all compounds/antibiotics against a panel of bacteria was evaluated by microbroth dilution method in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (using ATCC strains as quality control strains).⁴⁹ Overnight grown bacterial cultures were diluted in saline to achieve a 0.5 McFarland turbidity, followed by 1:50 dilution in Mueller-Hinton broth (MHB) for inoculation to a final concentration of approximately 5×10^5 CFU/mL. The antimicrobial agents were 2-fold serially diluted in MHB in a 96-well plate and

incubated at 37 °C with equal volumes of inoculum for 18 h. The lowest concentration that prevented the visible growth of bacteria was defined as the MIC for each antimicrobial agent. The broth with or without bacterial cells was used as positive or negative control, respectively.

6.6.2.2 *Checkerboard Assay.*

Combination studies with different antibiotics were performed in 96-well plates as previously described.¹⁹ Briefly, the antibiotic of interest was serially diluted in MHB along the abscissa while the adjuvant (tobramycin homodimer) was serially diluted in MHB along the ordinate. This creates a 10 × 7 matrices wherein each well consists of a combination of different antibiotic and adjuvant concentrations. Overnight grown bacterial cultures were diluted in saline to achieve a 0.5 McFarland turbidity, followed by 1:50 dilution in Mueller-Hinton broth (MHB) for inoculation to a final concentration of approximately 5×10^5 CFU/mL. Equal volume of this bacterial culture was then added to each well and incubated at 37 °C for 18 h. After incubation, the plates were read on EMax[®] Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 590 nm. MIC was recorded as wells with the lowest concentration of drugs with no bacterial growth. The fractional inhibitory concentration (FIC) for each antibiotic was calculated by dividing the MIC of the antibiotic in the presence of adjuvant by the MIC of the antibiotic alone. Similarly, the FIC of adjuvant was calculated by dividing the MIC of the adjuvant in the presence of antibiotic by the MIC of the adjuvant alone. FIC index is the sum of both FICs. FIC indices of ≤ 0.5 were deemed synergistic; $> 0.5 - 4$, no interaction; and > 4 , antagonistic.

6.6.2.3 *Cytotoxicity Assay.*

Human embryonic kidney cells (HEK293) and HepG2 cells were grown in Dulbecco's modified eagle's medium supplemented with 10% fetal bovine serum in a humidified 5% atmospheric incubator

at 37 °C. Equal number of cells (100 µl of media containing ~8000 cells) were dispersed into 96-well plates and wells with medium but no cells were used as blanks. After incubating for 24 h, 100 µl of varying concentrations of test compounds (at twice the desired concentrations) were added to each well, including the blanks. The treated cells were then incubated further for 48 h, after which PrestoBlue reagent was added to each well. The plates were then incubated for an additional hour on a nutator mixer in a 5% CO₂ incubator. The fluorescence was read at 490 nm on a SpectraMax M2 plate reader (Molecular Devices, USA). Cell viability were interpreted as previously described.^{21,47} The values of blank were subtracted from each value, and the viability values of the treated samples relative to the controls with vehicle were calculated. The values for the plots are the means ± standard deviation.

6.6.2.4 *Hemolytic Assay*

The hemolytic activities of the newly synthesized compounds were determined and quantified as the amount of hemoglobin released by lysing porcine erythrocytes. Fresh blood drawn from the antecubital vein of a pig (Animal Care and Use Program, University of Manitoba) was centrifuged at 1000 g at 4 °C for 10 mins, washed with PBS thrice and resuspended in the same buffer. The final cell concentration used was 3×10^8 cells/mL. Compounds were serially diluted with PBS and added to wells in a 96-well plate at twice the desired concentrations. Equal volumes of erythrocyte solution were then added to each well and incubated at 37 °C for 1 h. Intact erythrocytes were subsequently pelleted by centrifuging at 1000 g at 4 °C for 10 mins, and the supernatants were transferred to a new 96-well plate. Hemoglobin release was determined by measuring the absorbance on EMax[®] Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm. Blood cells in PBS (0% hemolysis) and 0.1 % Triton X-100 (100% hemolysis) were used as negative and positive controls,

respectively. Percent hemolysis was calculated as $[\% \text{ hemolysis} = (X - 0\%) / (100\% - 0\%)]$, where X is the optical density values of the compounds at different concentrations.⁴⁸

6.6.2.5 *Time-kill Assay.*

Time-kill curve analyses were performed by diluting a 30 μ l aliquot of 0.5 McFarland standard of overnight culture to 3 ml of LB broth (containing novobiocin alone, and in combination with compound 1) and incubated at 37 °C with shaking at 250 rpm. At specific time intervals (0, 1, 3, 6, 9, and 24 h), 100 μ l was taken from each sample, serially diluted in sterile PBS, plated on LB agar plates, and incubated at 37 °C in a humid 5 % CO₂-enriched atmosphere. Bacterial colonies were counted after 20 h of incubation.

6.6.2.6 *Outer Membrane Permeabilization Assay.*

The ability of the newly synthesized and reference compounds (compound 1, PMBN and tobramycin) to permeabilize the outer membrane of *P. aeruginosa* PAO1 was assessed using the nonpolar membrane-impermeable fluorescent probe 1-*N*-phenyl naphthylamine (NPN).⁴⁰ Briefly, an overnight grown *P. aeruginosa* PAO1 culture was subcultured (1 in 100) in fresh LB broth and grown to a mid-logarithmic phase (approximately 2 h, OD₆₀₀ = 0.5 – 0.6). The cells were harvested by centrifuging for 10 min at 1000 *g* and room temperature, washed twice in PBS, and resuspended in half volume of PBS. This suspension was used in standard microtiter plate assay. Care was taken not to cool the suspension at any stage, since cooling the bacterial suspension below room temperature (i.e. during refrigerated centrifugation) could cause considerable increase in initial NPN uptake levels.⁴⁰ To a black 96-well plate containing the cell culture was added NPN (10 μ M final concentration), alone and in combination with various concentrations of test compounds. The resulting change in NPN fluorescence was measured immediately and continuously (every 30 secs) for 10 mins, with

intermittent shaking, on a FlexStation 3 (Molecular Devices, Sunnyvale, USA) microplate reader at an excitation wavelength of 350 nm and emission wavelength of 420 nm. PMBN, a known outer membrane permeabilizer, served as a positive control while cells + NPN (without test compounds) served as a negative control. Four independent replicates were conducted, and the data were corrected for any background fluorescence.

6.6.2.7 *Development of Resistance Study.*

The ability of novobiocin + compound **1** to suppress resistance development was determined by serial passaging, as previously described.^{19,50} Briefly, wild-type *A. baumannii* ATCC 17978 cells were grown in 1 mL MHB media containing novobiocin (at ¼ MIC, ½ MIC, 1× MIC, 2× MIC, and 4× MIC), alone and in combination with compound **1**. Tobramycin and colistin were included as controls. At 24-hour intervals, the cultures were assessed for growth. Cultures from the second highest concentrations that allowed visible growth were harvested and diluted to 0.5 McFarland in sterile PBS, followed by 1:50 dilution into fresh MHB media containing ¼ MIC, ½ MIC, 1× MIC, 2× MIC, and 4× MIC of each antibiotic. This serial passaging was repeated for 8 days. For novobiocin/compound **1** combination, the concentration of compound **1** was kept constant at 7.1 µM throughout the experiment. For cultures that grew at higher than the MIC levels, cultures in highest concentrations that permit growth were passaged on drug-free LB plates and the MICs were determined by microbroth dilution in MHB.

6.6.2.8 *Galleria mellonella In vivo Larvae–Infection Model.*

In vivo synergistic effects were determined using *Galleria mellonella* infection model, as previously described.⁴⁸ Briefly, larvae were purchased from The Worm Lady® Live Feeder (ON, Canada), stored in their natural habitat at 16 °C, and used within 10 days of delivery. The larvae (average weight of

250 mg) were used for tolerability and efficacy studies. Tolerability study was performed by injecting a 10 μ L aliquot of antimicrobial agents only at concentrations equivalent to 100 mg/kg or 200 mg/kg. The larvae (ten in each group) were incubated at 37 °C and monitored for 96 h. For efficacy studies, the virulence and bacterial load required to kill 100 % of the larvae within 24 h (with no treatment) was first determined, which is approximately 10 CFU. Overnight grown culture of respective MDR *A. baumannii* isolate was standardized to 0.5 McFarland standard and diluted in PBS to a final concentration of 10³ CFU/mL. A 10 μ L aliquot of this solution (~10 CFU) was injected into each larva and incubated for 3 h at 37 °C. After the 3 h challenge, larvae in monotherapy experimental groups (fifteen per group) were treated with a 10 μ L aliquot solution (containing different concentrations) of novobiocin, PMBN, compound **1**, or PBS alone. The larvae in combination therapy groups were treated with novobiocin + compound **1** (25 + 25 mg/kg or 50 + 50 mg/kg) and novobiocin + PMBN (50 + 25 mg/kg). Larvae treated with 10 μ L of PBS or high concentrations of test antibiotics served as negative and positive control, respectively. The larvae were incubated at 37 °C in Petri dishes lined with filter paper and scored for survivability every 6 h for up to 36 h. This experiment was repeated to give a total of 30 larvae in each case. Survival data curves were plotted using Kaplan-Meier survival analysis. Larvae were considered dead if they do not respond to touch.

6.7 References

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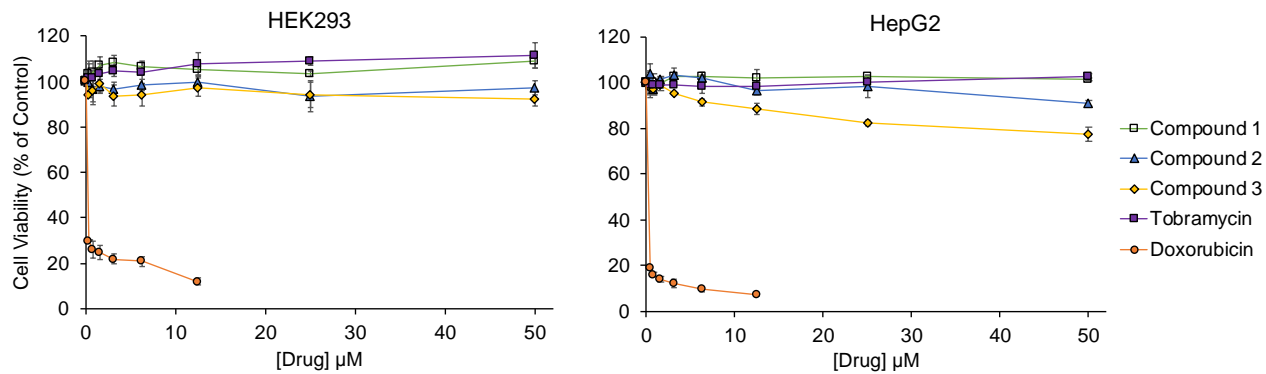
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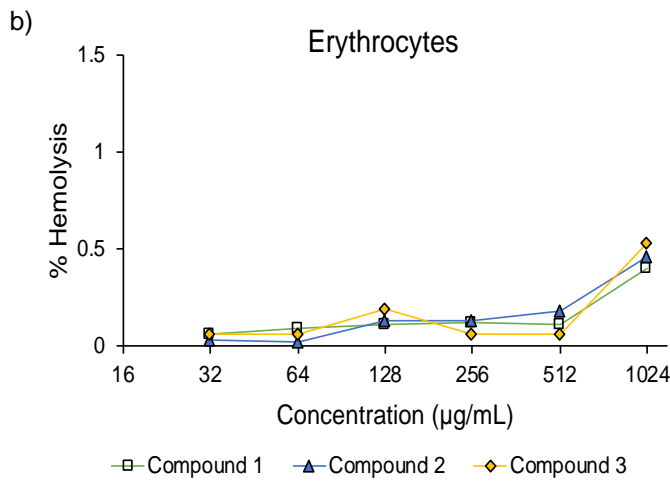
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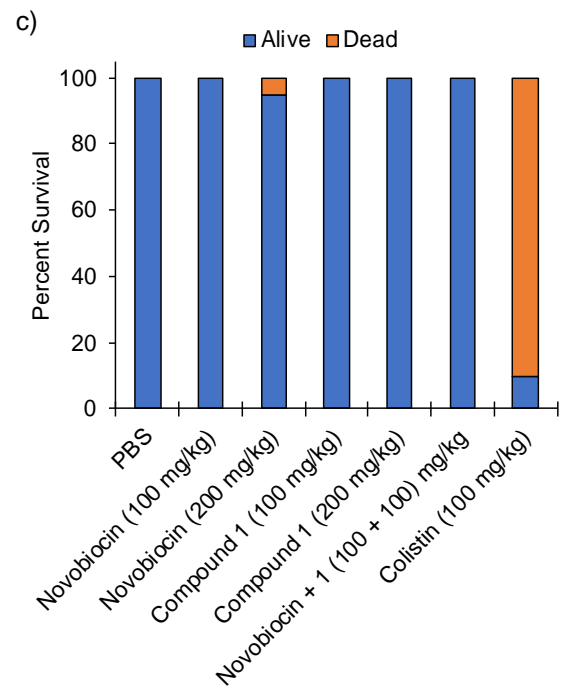
6.8 Supplementary Data C



a.



b)



c)

d.

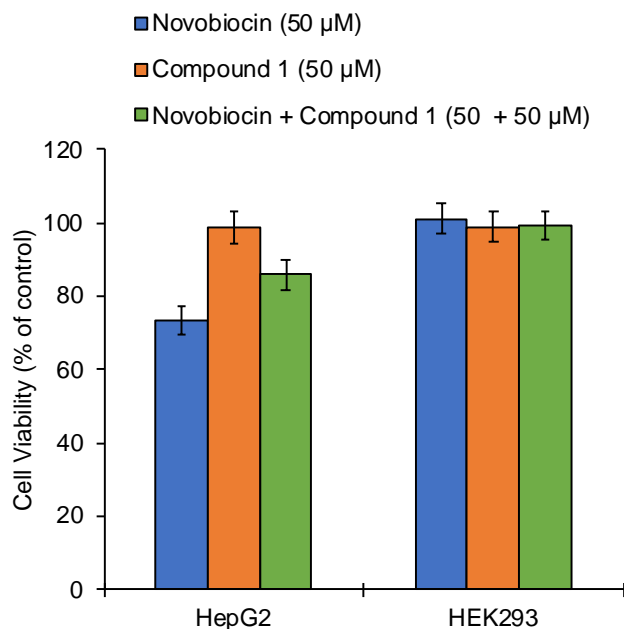


Figure S1. **Tobramycin homodimers are non-cytotoxic and non-hemolytic.** a) Cytotoxicity of compounds **1–3**, tobramycin, and doxorubicin against human embryonic kidney (HEK293) cells and human liver carcinoma (HepG2) cells using PrestoBlue cell viability assay. Doxorubicin was used as positive control. Error bars denote standard deviation of at least four replicates. b) Hemolytic activity of compounds **1–3** evaluated against porcine erythrocytes at different concentrations. 0.1% Triton X-100 (100 % hemolysis) was used as positive control to calculate percent hemolysis. The result represents the mean of three independent determinations. c) Tolerability/Toxicity studies in *Galleria mellonella* larvae. Tolerable doses were determined by injecting 100 and 200 mg/kg of test compounds, alone and in combination, into the larvae and survivability was scored for 96 h (4 days). Compound **1**, alone and in combination with novobiocin, is non-toxic to *G. mellonella* larvae. d) A combination therapy of 50 μM each of compound **1** (~ 128 μg/ml) and novobiocin (32 μg/ml) did not elevate toxicity against HEK293 and HepG2 epithelial cells.

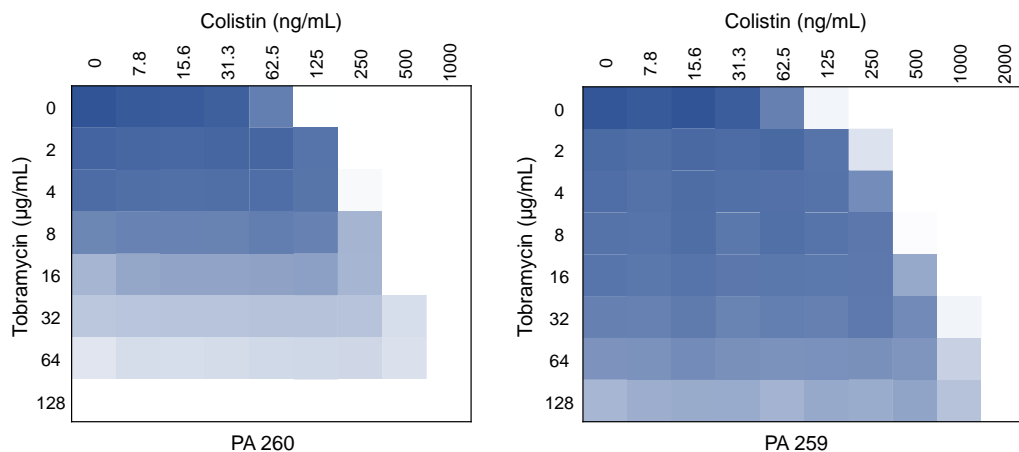
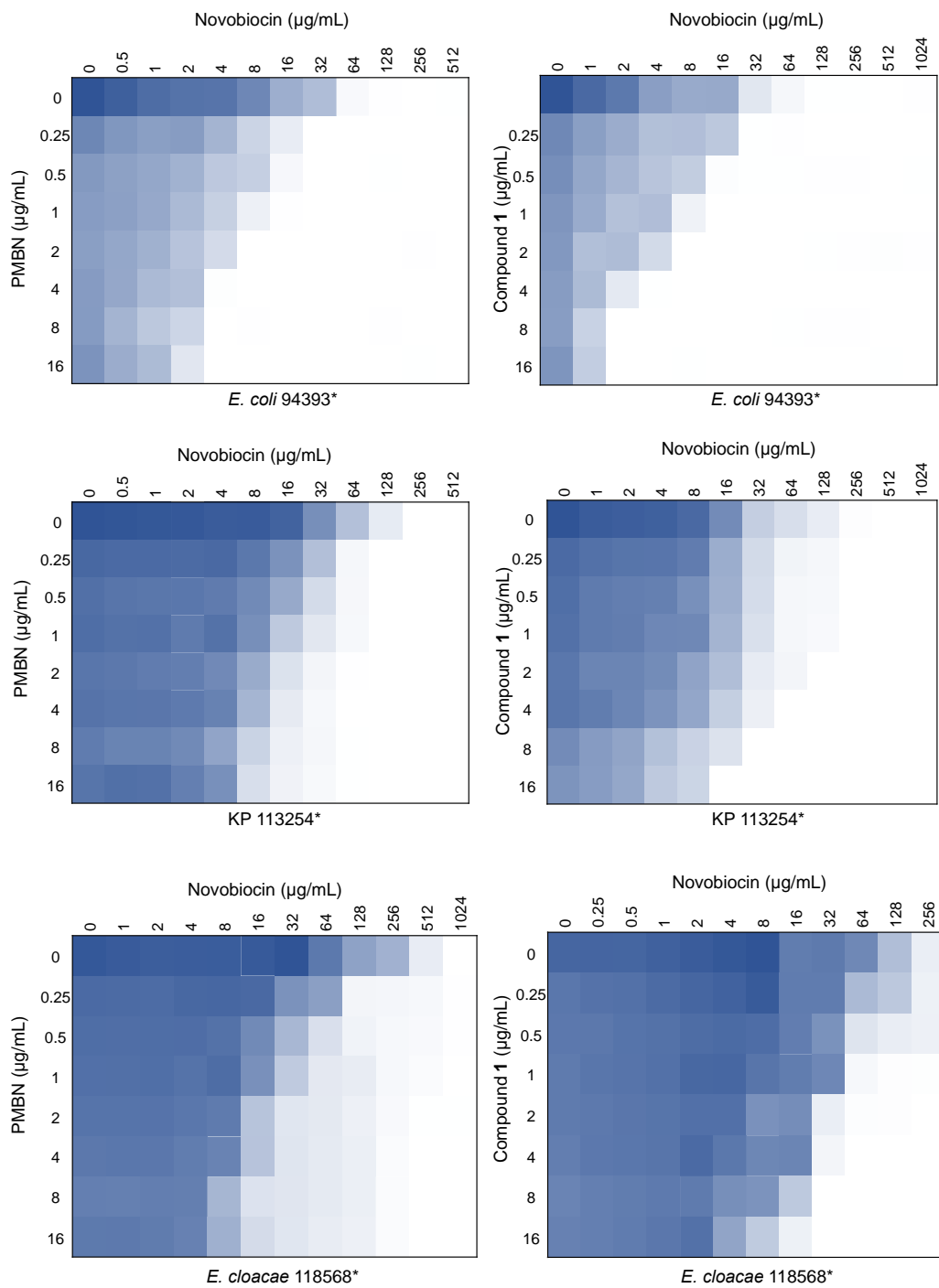
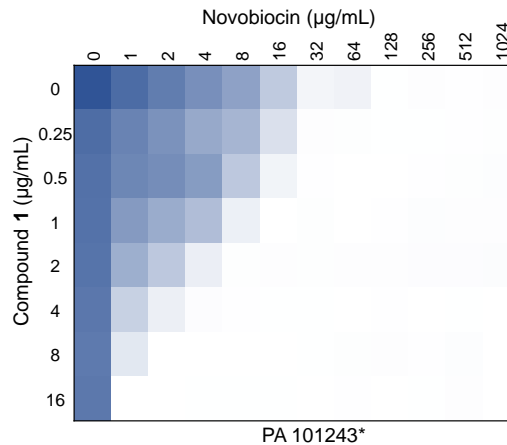
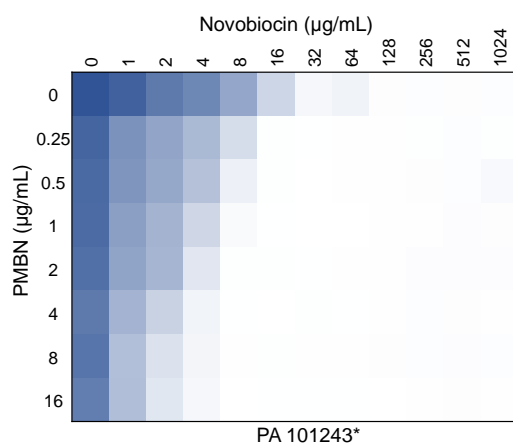
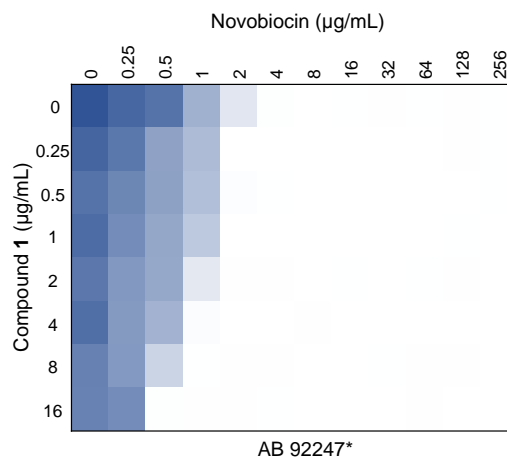
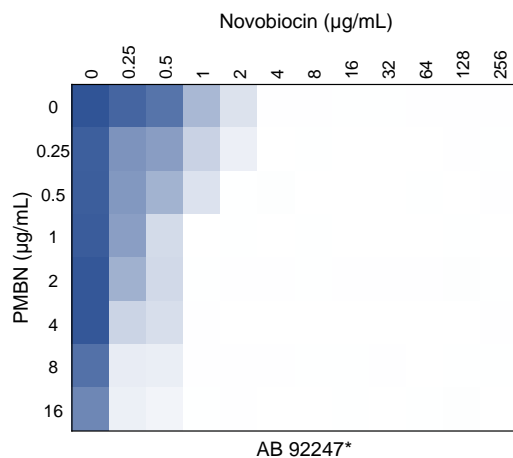


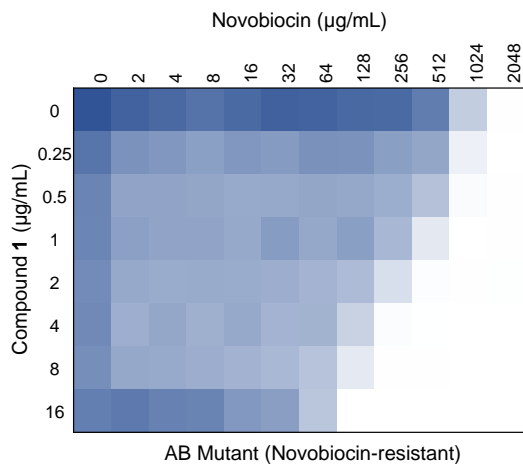
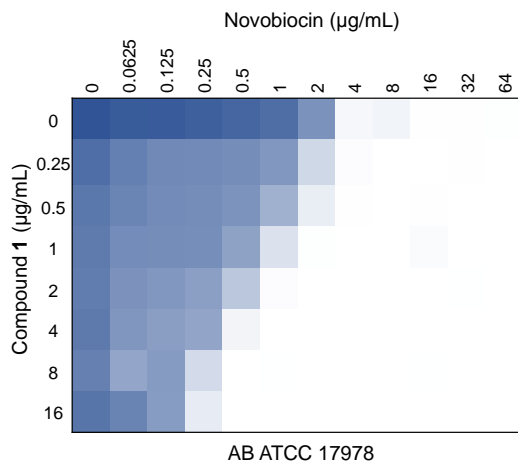
Figure S2. Tobramycin (at high concentrations) exhibits antagonistic relationship ($FICI > 4$) with colistin against tobramycin-resistant *P. aeruginosa* isolates. Dark colours represent higher cell density (OD measured at 590 nm).

A.





B.



C.

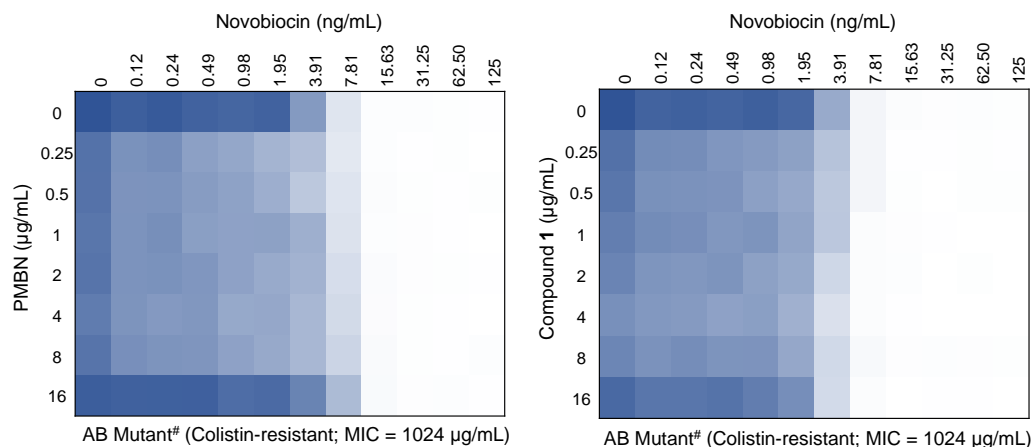


Figure S3. **Potential of novobiocin by compound 1 is dependent on LPS interactions.**

Checkerboard broth microdilution assay between novobiocin and compound **1** (or PMBN) against different Gram-negative bacteria strains. A) LPS modifications in colistin-resistant clinical isolates reduces novobiocin potentiation by PMBN, but not compound **1**; B) Compound **1** potentiates novobiocin in wild-type and novobiocin resistant *A. baumannii*; C) PMBN and compound **1** do not potentiate novobiocin in an *A. baumannii* mutant strain, consistent with a complete loss of LPS production as previously reported (collateral sensitivity to all antibiotics).¹ Dark colours represent higher cell density (OD measured at 590 nm). 16 µg/mL of compound **1** = 7.1 µM; 8 µg/mL PMBN ~ 7.1 µM. PMBN = Polymyxin B nonapeptide; AB = *Acinetobacter baumannii*; PA = *Pseudomonas aeruginosa*; KP = *Klebsiella pneumoniae*. *Colistin-resistant clinical isolate. #Colistin-resistant mutant generated in the study.

Table S1. **Dimerization abrogates intrinsic activity of tobramycin.** Minimum inhibitory concentrations (MICs, µg/ml) of Tobramycin and compounds **1–3** against a panel of Gram-positive and Gram-negative bacteria. ^a(GEN-R, TOB-R, CIP-R) aac(3')iia; ^b(GEN-R, TOB-R).

| Test Organism | Tobramycin homodimers | | | |
|--|-----------------------|----------|----------|----------|
| | Tobramycin | 1 | 2 | 3 |
| <i>S. aureus</i> ATCC 29213 | 0.5 | 32 | >128 | >128 |
| MRSA ATCC 33592 | 0.5 | 32 | 128 | 128 |
| MSSE CANWARD-2008 81388 | ≤0.25 | 16 | 16 | 16 |
| MRSE CAN-ICU 61589 (CAZ >32) | 2 | 64 | 128 | 128 |
| <i>E. faecalis</i> ATCC 29212 | 8 | >128 | >128 | >128 |
| <i>E. faecium</i> ATCC 27270 | 16 | >128 | >128 | >128 |
| <i>S. pneumoniae</i> ATCC 49619 | 2 | >128 | >128 | >128 |
| <i>E. coli</i> ATCC 25922 | 0.5 | >128 | >128 | >128 |
| <i>E. coli</i> CAN-ICU 61714 (GEN-R) | 8 | >128 | >128 | >128 |
| <i>E. coli</i> CAN-ICU 63074 (AMK 32) | 8 | >128 | >128 | >128 |
| <i>E. coli</i> CANWARD-2011 97615 ^a | 128 | >128 | >128 | >128 |
| <i>P. aeruginosa</i> ATCC 27853 | 1 | >128 | >128 | >128 |
| <i>P. aeruginosa</i> CAN-ICU 62308 (GEN-R) | 16 | >128 | >128 | >128 |
| <i>P. aeruginosa</i> CANWARD-2011 96846 ^b | 256 | >128 | >128 | >128 |
| <i>S. maltophilia</i> CAN-ICU 62584 | >512 | >128 | >128 | >128 |
| <i>A. baumannii</i> CAN-ICU 63169 | 32 | >128 | >128 | >128 |
| <i>K. pneumoniae</i> ATCC 13883 | ≤0.25 | 128 | >128 | >128 |

Table S2. **Tobramycin homodimer 1–3 potentiates several antibiotics.** Combination studies of compounds **1–3** with different antibiotics against WT *P. aeruginosa* PAO1. MICs are reported in µg/ml. FICI = Fractional inhibitory concentration index. FICI of ≤ 0.5 , $> 0.5 - 4$, and > 4 indicate synergy, additive or no interaction, and antagonism, respectively. Synergistic combinations are highlighted in green.

| Antibiotics (MIC alone) | MIC of Antibiotics (FICI) in the presence of ≤ 7.1 µM of | | |
|-------------------------|---|--------------|--------------|
| | 1 | 2 | 3 |
| Rifampicin (16) | 0.5 (0.09) | 1 (0.13) | 1 (0.09) |
| Tobramycin (1) | 4 (4.02) | 4 (4.02) | 4 (4.00) |
| Minocycline (8) | 2 (0.31) | 2 (0.31) | 4 (0.53) |
| Cefotaxime (16) | 2 (0.19) | 2 (0.19) | 2 (0.16) |
| Doxycycline (8) | 0.5 (0.13) | 0.5 (0.08) | 1 (0.14) |
| Linezolid (1024) | 32 (0.09) | 64 (0.09) | 32 (0.06) |
| Meropenem (1) | 0.25 (0.31) | 0.25 (0.31) | 0.25 (0.28) |
| Moxifloxacin (1) | 0.125 (0.19) | 0.125 (0.19) | 0.25 (0.28) |
| Ciprofloxacin (0.125) | 0.031 (0.31) | 0.031 (0.31) | 0.063 (0.52) |
| Levofloxacin (0.5) | 0.125 (0.28) | 0.125 (0.28) | 0.125 (0.28) |
| Vancomycin (256) | 128 (0.53) | 128 (0.52) | 128 (0.52) |
| Aztreonam (4) | 0.25 (0.13) | 0.5 (0.19) | 0.5 (0.16) |
| Ceftazidime (2) | 0.25 (0.19) | 0.5 (0.28) | 0.25 (0.16) |
| Fosfomycin (16) | 4 (0.26) | 8 (0.52) | 4 (0.28) |
| Chloramphenicol (32) | 1 (0.09) | 2 (0.09) | 2 (0.08) |
| Clindamycin (1024) | 128 (0.19) | 64 (0.13) | 128 (0.14) |
| Novobiocin (512) | 4 (0.07) | 8 (0.08) | 8 (0.05) |
| Colistin (1) | 4 (4.02) | 4 (4.02) | 4 (4.01) |
| Piperacillin (16) | 1 (0.13) | 1 (0.13) | 2 (0.16) |

Table S3. **Tobramycin alone does not potentiate antibiotics against *P. aeruginosa* PAO1.** MIC of tobramycin against PAO1 = 1 µg/ml. FICI > 0.5 but ≤ 4 indicates no interaction.

| Antibiotics | MIC (µg/ml) of Antibiotic | | FICI |
|-----------------|---------------------------|------------------------|------|
| | Alone | + 0.5 µg/ml Tobramycin | |
| Rifampicin | 16 | 8 | 1.00 |
| Minocycline | 8 | 8 | 1.50 |
| Cefotaxime | 16 | 8 | 1.00 |
| Doxycycline | 8 | 8 | 1.50 |
| Linezolid | 1024 | 1024 | 1.50 |
| Meropenem | 1 | 0.5 | 1.00 |
| Moxifloxacin | 1 | 0.5 | 1.00 |
| Ciprofloxacin | 0.125 | 0.125 | 1.50 |
| Levofloxacin | 0.5 | 0.5 | 1.50 |
| Vancomycin | 256 | 128 | 1.00 |
| Aztreonam | 4 | 1 | 0.75 |
| Fosfomycin | 16 | 8 | 1.00 |
| Chloramphenicol | 32 | 16 | 1.00 |
| Clindamycin | 1024 | 1024 | 1.50 |
| Novobiocin | 512 | 128 | 0.75 |

Table S4. Susceptibility profiles of MDR/XDR Gram-negative clinical isolates used in the study

a) *Pseudomonas aeruginosa* isolates

| Stock # | PTZ | A/C | AZT | FOX | CFZ | CTR | CPM | CAZ | C/T | IMI | MER | DOR | ETP | CIP | MOX | TOB | GEN | AMK | TGC | MIN | DOX | CST | CAM | RIF |
|-------------|-----|-----|-----|-----|------|-----|-----|------|-----|-----|------|-------|-----|-----|-----|------|------|-----|-----|-----|------|------|------|-----|
| 100036 | 8 | >32 | 16 | >32 | >128 | 32 | 4 | 8 | 0.5 | 8 | 4 | 16 | >32 | >16 | >16 | 128 | >32 | 32 | 32 | 16 | 64 | 2 | 1024 | 16 |
| 101885 | 16 | >32 | 16 | >32 | >128 | 32 | 8 | 8 | 0.5 | 4 | 4 | 4 | 32 | >16 | >16 | 1 | ≤0.5 | ≤1 | 8 | 8 | 64 | 1 | 512 | 32 |
| P259-96918 | 64 | >32 | 32 | >32 | >128 | >64 | >64 | 512 | >16 | 32 | 1024 | >1024 | >32 | >16 | >16 | 256 | >32 | >64 | 32 | 32 | 32 | 1 | 1024 | 16 |
| P260-97103 | 128 | >32 | 64 | >32 | >128 | >64 | 16 | 32 | 1 | 32 | 16 | 16 | >32 | 16 | >16 | 128 | >32 | 4 | 16 | 16 | 16 | 1 | 128 | 16 |
| P262-101856 | 64 | >32 | 32 | >32 | >128 | 64 | 32 | 16 | 1 | 32 | 32 | 16 | >32 | >16 | >16 | 1024 | >32 | >64 | 32 | 64 | 1024 | 1 | 2048 | 512 |
| P264-104354 | 256 | >32 | 64 | >32 | >128 | >64 | 32 | 128 | 2 | 32 | 64 | 16 | >32 | >16 | >16 | 128 | >32 | 8 | 32 | 32 | 64 | 1 | 4096 | 16 |
| 91433 | 64 | >32 | 512 | >32 | >128 | >64 | 16 | 1024 | 0.5 | 32 | 16 | 16 | >32 | 2 | 16 | 16 | 32 | >32 | 32 | 16 | 32 | 4 | 8 | 16 |
| 101243 | 128 | >32 | 32 | >32 | >128 | >64 | 64 | 64 | 1 | 16 | 16 | 16 | >32 | 1 | 8 | 128 | >32 | >64 | ND | 2 | 4 | 1024 | 1 | 4 |

b) *Escherichia coli* isolates

| Stock # | PTZ | A/C | AZT | FOX | CFZ | CTR | CPM | CAZ | C/T | IMI | MER | ETP | CIP | MOX | TOB | GEN | AMK | TGC | DOX | CST | SXT | NTR |
|----------------------------|------|-----|-------|-----|------|-------|-------|-------|------|------|-------|-------|-------|-------|------|------|-----|------|-----|-----|-------|-----|
| ATCC 25922 | 2 | 8 | ≤0.12 | 4 | 1 | ≤0.25 | ≤0.25 | ≤0.25 | ND | 0.12 | ≤0.03 | ≤0.03 | ≤0.06 | ≤0.06 | 2 | ≤0.5 | ≤1 | 0.12 | 1 | 0.5 | ≤0.12 | 8 |
| 94393 (<i>mcr</i> -1 +ve) | ≤1 | 4 | ≤0.12 | 4 | 1 | ≤0.25 | ≤0.25 | ≤0.25 | 0.25 | 0.25 | ≤0.03 | ≤0.03 | 0.5 | 1 | ≤0.5 | ≤0.5 | 2 | 0.25 | 4 | 4 | ≤0.12 | 16 |
| 94474 (<i>mcr</i> -1 +ve) | 16 | >32 | ≤0.12 | 16 | 4 | ≤0.25 | ≤0.25 | 0.5 | 0.5 | 0.25 | ≤0.03 | ≤0.03 | >16 | 16 | 32 | 16 | 2 | 1 | >32 | 16 | >8 | 64 |
| 107115 | >512 | >32 | >64 | >32 | >128 | >64 | >64 | >32 | >16 | 8 | 32 | >32 | >16 | 16 | 8 | >32 | 2 | 0.25 | >32 | 0.5 | >8 | 16 |

mcr = mobilized colistin resistance gene.

c) Other Gram-negative bacteria

| Stock # | Organism | PTZ | A/C | AZT | FOX | CFZ | CPM | CAZ | CAZ-AVI | C/T | CTX | IMI | MER | ETP | CIP | MXF | TOB | GEN | AMK | TGC | DOX | CST | SXT | RIF |
|---------|----------------------|-----|-----|-------|-----|------|-------|------|---------|------|-------|------|-------|-------|-------|-------|------|------|-----|------|-----|------|------|------|
| 113250 | <i>K. pneumoniae</i> | 4 | 4 | ≤0.12 | 1 | 1 | 1 | 0.5 | ND | 2 | ND | 0.25 | ≤0.03 | ≤0.03 | ≤0.06 | ≤0.06 | ≤0.5 | ≤0.5 | ≤1 | ND | 2 | >16 | ≤0.1 | ND |
| 113254 | <i>K. pneumoniae</i> | ≤1 | 2 | ≤0.12 | 1 | 1 | 1 | ≤0.2 | ND | 0.5 | ND | 0.12 | ≤0.03 | ≤0.03 | ≤0.06 | ≤0.06 | ≤0.5 | ≤0.5 | ≤1 | ND | 2 | >16 | ≤0.1 | ND |
| 116381 | <i>K. pneumoniae</i> | 8 | 16 | 16 | 16 | >128 | 16 | 8 | 0.5 | 1 | >64 | 0.5 | ≤0.03 | 0.12 | >16 | >16 | 4 | ≤0.5 | ≤1 | 1 | >32 | 0.5 | >8 | >128 |
| 117029 | <i>E. cloacae</i> | 2 | 16 | ≤0.12 | >32 | >128 | ≤0.25 | 0.5 | 0.25 | 0.25 | ≤0.25 | 0.25 | ≤0.03 | ≤0.03 | ≤0.06 | ≤0.06 | 2 | ≤0.5 | 2 | 0.5 | >32 | 0.25 | >8 | 8 |
| 118564 | <i>E. cloacae</i> | 2 | >32 | ≤0.12 | >32 | >128 | 0.25 | 0.5 | ND | ND | ND | ND | 0.12 | ND | 0.06 | 0.12 | 1 | 1 | 2 | ND | 4 | >16 | ND | ND |
| 121187 | <i>E. cloacae</i> | 1 | 8 | ≤0.12 | >32 | 32 | 0.25 | 0.5 | ND | ND | ND | ND | 0.06 | ND | 0.25 | 1 | 32 | >32 | 1 | ND | >32 | >16 | ND | ND |
| AB027 | <i>A. baumannii</i> | 512 | ND | ND | ND | >128 | ND | ND | ND | >16 | ND | 32 | 16 | ND | >16 | 8 | ND | 32 | >64 | 4 | ND | >256 | >8 | ND |
| AB031 | <i>A. baumannii</i> | 4 | ND | ND | ND | >128 | ND | ND | ND | >16 | ND | 0.25 | 1 | ND | 0.25 | 0.12 | ND | <0.5 | 2 | 8 | ND | 16 | 4 | ND |
| 92247 | <i>A. baumannii</i> | <1 | ND | ND | 32 | 128 | 4 | ND | ND | 2 | ND | ND | 4 | ND | ≤0.06 | ND | ND | ND | <1 | 0.25 | ND | 4 | ≤0.1 | ND |
| LAC-4 | <i>A. baumannii</i> | ND | ND | ND | ND | ND | ND | >16 | ND | 8 | 1 | <1 | <1 | ND | >4 | ND | >4 | >4 | 4 | <4 | <4 | ND | ND | ND |

PTZ: piperacillin-tazobactam; A/C: amoxicillin-clavulanic acid; AZT: aztreonam; FOX: cefoxitin; CFZ: cefazolin; CTR: ceftriaxone; CPM: cefepime; CAZ: ceftazidime; IMI: imipenem; MER: meropenem; DOR: doripenem; ETP: ertapenem; CIP: ciprofloxacin; MOX: moxifloxacin; TOB: tobramycin; GEN: gentamicin; AMK: amikacin; TGC: tigecycline; MIN: minocycline; DOX: doxycycline; CST: colistin; CAM: chloramphenicol; RIF: rifampicin; ND: not determined

Table S5. **Potentialiation of novobiocin by tobramycin homodimer 1 is independent of *gyrA* mutations.**

Analysis of *gyrA* mutations (red) in *P. aeruginosa* strains used in the study. Codon: PAO1, 91433, 101243

= ACC; Others = ATC. Concentration of compound **1** = 7.1 μ M. *Colistin-resistant.

| Strain | <i>gyrA</i> mutations | MIC (μ g/mL) | | | |
|------------|-----------------------|-------------------|--------------|------------|-----------------------|
| | | Ciprofloxacin | Moxifloxacin | Novobiocin | Novobiocin + 1 |
| PAO1 | 81 GDTAVYDTIV 90 | 0.125 | 1 | 512 | 4 |
| PA 259 | 81 GDIAVYDTIV 90 | 256 | 512 | 512 | 8 |
| PA 260 | 81 GDIAVYDTIV 90 | 32 | 64 | 256 | 1 |
| PA 262 | 81 GDIAVYDTIV 90 | 32 | 128 | 512 | 8 |
| PA 264 | 81 GDIAVYDTIV 90 | 16 | 32 | 512 | 8 |
| PA 91433* | 81 GDTAVYDTIV 90 | 4 | 8 | 16 | 1 |
| PA 100036 | 81 GDIAVYDTIV 90 | 64 | 128 | 1024 | 8 |
| PA 101243* | 81 GDTAVYDTIV 90 | 2 | 8 | 128 | 1 |
| PA 101885 | 81 GDIAVYDTIV 90 | 32 | 128 | 1024 | 8 |

Table S6. A) **Tobramycin alone does not potentiate novobiocin against Gram-negative bacteria.**

Interactions of tobramycin (TOB) in combination with novobiocin (NOVO) against Gram-negative bacteria. PA = *P. aeruginosa*; AB = *A. baumannii*; KP = *K. pneumoniae*. MICs are reported in µg/ml. FICI > 0.5 but ≤ 4 indicates no interaction.

| Organism | MIC _{NOVO} | MIC _{Combination} | FIC _{NOVO} | MIC _{TOB} | MIC _{Combination} | FIC _{TOB} | FICI |
|---------------------------|---------------------|----------------------------|---------------------|--------------------|----------------------------|--------------------|-------|
| PAO1 | 512 | 128 | 0.25 | 1 | 0.5 | 0.50 | 0.75 |
| PA 260 | 256 | 128 | 0.50 | 128 | 32 | 0.25 | 0.75 |
| AB ATCC 17978 | 16 | 8 | 0.50 | 1 | 0.5 | 0.50 | 1.00 |
| <i>E. coli</i> ATCC 25922 | 64 | 32 | 0.50 | >2 | 2 | <1.00 | <1.50 |
| KP 116381 | 512 | 512 | 1.00 | 4 | 2 | 0.50 | 1.50 |
| <i>E. cloacae</i> 117029 | 256 | 64 | 0.25 | 2 | 1 | 0.50 | 0.75 |

B) Other antibiotics do not potentiate novobiocin against Gram-negative bacteria

| Organism | MIC _{NOVO} | MIC _{Combination} | FIC _{NOVO} | Antibiotic (MIC) | MIC _{Combination} | FIC _{Antib.} | FICI |
|---------------------------|---------------------|----------------------------|---------------------|---------------------|----------------------------|-----------------------|------|
| PAO1 | 512 | 512 | 1.00 | Rifampicin (16) | 16 | 1.00 | 2.00 |
| PA 262 | 512 | 512 | 1.00 | Rifampicin (512) | 256 | 0.50 | 1.50 |
| PA 264 | 512 | 512 | 1.00 | Rifampicin (16) | 16 | 1.00 | 2.00 |
| <i>E. coli</i> ATCC 25922 | 64 | 32 | 0.50 | Rifampicin (4) | 2 | 0.50 | 1.00 |
| AB ATCC 17978 | 16 | 8 | 0.50 | Minocycline (0.125) | 0.0625 | 0.50 | 1.00 |
| AB ATCC 17978 | 16 | 16 | 1.00 | Ceftazidime (16) | 8 | 0.50 | 1.50 |

Table S7. Tobramycin homodimer 1 does not potentiate novobiocin against Gram-positive bacteria.

Interactions of compound **1** with novobiocin (NOVO) against Gram-positive bacteria. MICs are reported in µg/ml. FICI > 0.5 but ≤ 4 indicates no interaction.

| Organism | MIC _{NOVO} | MIC _{Combination} | FIC _{NOVO} | MIC ₁ | MIC _{Combination} | FIC ₁ | FICI |
|-------------------------------|---------------------|----------------------------|---------------------|------------------|----------------------------|------------------|-------|
| MRSA ATCC 33592 | 0.125 | 0.125 | 1.00 | 32 | 16 | 0.50 | 1.50 |
| MRSE 61589 | 0.0625 | 0.0625 | 1.00 | 64 | 16 | 0.25 | 1.25 |
| <i>E. faecium</i> ATCC 27270 | 2 | 2 | 1.00 | >128 | 16 | <0.0625 | >1.00 |
| <i>E. faecalis</i> ATCC 29212 | 8 | 8 | 1.00 | >128 | 16 | <0.0625 | >1.00 |

Table S8. **Dimeration and cationity are critical for the potency of Tobramycin homodimers.** Fragments **4–5** and Boc-protected Tobramycin Homodimer **12a** do not potentiate novobiocin against *P. aeruginosa* PAO1.

| Compound | MIC Novobiocin (µg/ml) | |
|------------|------------------------|-----------------------|
| | Alone | + Compound (16 µg/ml) |
| 4 | 512 | 512 |
| 5 | 512 | 512 |
| 12a | 512 | 512 |

Table S9. MICs of novobiocin versus novobiocin aglycone **4** against Gram-positive and Gram-negative bacteria. PA = *P. aeruginosa*. *Efflux-deficient strains

| Bacteria Strains | MIC (µg/ml) | |
|--------------------------------|-------------|-------------------|
| | Novobiocin | Compound 6 |
| MRSA ATCC 33592 | 0.125 | 512 |
| MRSE 61589 | 0.0625 | 512 |
| <i>E. faecium</i> ATCC 27270 | 2 | 512 |
| <i>E. faecalis</i> ATCC 29212 | 8 | 512 |
| PAO1 | 512 | 512 |
| PAO200* | 32 | 256 |
| PAO750* | 32 | 256 |
| <i>A. baumannii</i> ATCC 17978 | 16 | 512 |
| <i>E. coli</i> ATCC 25922 | 32 | 512 |

Table S10. Checkerboard studies. MICs are reported in $\mu\text{g/ml}$.

a) Compound **1** does not synergize with compound **6** against Gram-negative bacteria. PA = *P. aeruginosa*; AB = *A. baumannii*. FICI > 0.5 but ≤ 4 indicates no interaction.

| Organism | MIC ₆ | MIC _{Combination} | FIC ₆ | MIC ₁ | MIC _{Combination} | FIC ₁ | FICI |
|---------------------------|------------------|----------------------------|------------------|------------------|----------------------------|------------------|-------|
| PAO1 | 512 | 512 | 1.00 | 256 | 16 | 0.0625 | 1.06 |
| AB ATCC 17978 | 512 | 512 | 1.00 | >128 | 16 | <0.0625 | >1.00 |
| <i>E. coli</i> ATCC 25922 | 512 | 512 | 1.00 | >128 | 16 | <0.0625 | >1.00 |

b) Compound **6** potentiates colistin against wild type *A. baumannii* (AB) and *E. coli*.

| Organism | MIC _{Colistin} | MIC _{Comb} | FIC _{Colistin} | MIC ₆ | MIC _{Comb} | FIC ₆ | FICI | Fold Potentiation |
|---------------------------|-------------------------|---------------------|-------------------------|------------------|---------------------|------------------|-------|-------------------|
| PAO1 | 1 | 0.5 | 0.5 | 512 | 512 | 1.00 | 1.50 | 1 |
| AB ATCC 17978 | 0.25 | 0.031 | 0.125 | 512 | 128 | 0.25 | 0.375 | 8 |
| <i>E. coli</i> ATCC 25922 | 0.5 | 0.016 | 0.031 | 512 | 64 | 0.125 | 0.156 | 32 |

Table S11. Elemental analysis of compounds **1** – **3**. C = calculated; F = found.

| Compound (× 10TFA) | Carbon (%) | | Hydrogen (%) | | Nitrogen (%) | |
|------------------------------|-------------------|----------|---------------------|----------|---------------------|----------|
| | C | F | C | F | C | F |
| 1 | 35.19 | 35.13 | 4.43 | 4.41 | 8.08 | 8.01 |
| 2 | 35.81 | 35.11 | 4.55 | 4.54 | 7.98 | 7.72 |
| 3 | 36.42 | 35.21 | 4.67 | 4.51 | 7.89 | 7.49 |

Sequencing of *parC*, *parE*, *gyrA*, and *gyrB*

Fluoroquinolone target genes in *P. aeruginosa* were amplified, as previously reported,² using the following primers sets, *parC*: *parCFwd_P_aeruginosa* (ATG AGC GAA CTG GGG CTG GAT) and *parCRev_P_aeruginosa* (ATG GCG GCG AAG GAC TTG GGA); *parE*: *parEFwd_P_aeruginosa* (CGG CGT TCG TCT CGG GCG TGG TGA AGG A) and *parERev_P_aeruginosa* (CGG CGT TCG TCT CGG GCG TGG TGA AGG A); *gyrA*: *gyrAFwd_P_aeruginosa* (TTA TGC CAT GAG CGA GCT GGG CAA CGA CT) and *gyrARev_P_aeruginosa* (AAC CGT TGA CCA GCA GGT TGG GAA TCT T); and *gyrB*: *gyrBFwd_P_aeruginosa* (AGC TCG CAG ACC AAG GAC AAG) and *gyrBRev_P_aeruginosa* (GGG CTG GGC GAT GTA GAT GTA). PCR products were gel purified and sequenced at the core facility of Genome Quebec, Montreal Canada.

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7 CHAPTER SEVEN

Conclusions and Future Work

7.1 Concluding Remarks

Bacterial resistance to antibiotics has been a recognized reality almost since the dawn of the antibiotic era and the dearth of novel antibacterial drugs in the pipeline means that we must conserve the efficacy of existing antibacterial drugs as much as practically possible. Standing on the shoulders of leading experts and their work, I reviewed the relevant challenges of antibacterial discovery and potential solutions to this problem in chapters 1 and 2. Indeed, no new class of antimicrobial agent with novel mode of action has been approved for Gram-negative bacterial infections in the last few decades.^{1,2} With no reprieve in sight, the pursuit of adjuvants that can rejuvenate and/or preserve the utility of current (actually old) antibiotics is an attractive strategy to extend the life of our antibiotic arsenal.³

During my doctoral research, I identified a core tobramycin-based antibiotic scaffold that is amenable to modifications and that can be fine-tuned to suit different purposes. For example, chapter 4 describes the development of rifampicin-tobramycin conjugates that were found to break the intrinsic resistance of *Pseudomonas aeruginosa* to tetracyclines and chloramphenicol *in vitro* and in a *Galleria mellonella* larvae infection model. A few things are worthy of note: 1) rifampicin, tetracyclines, and chloramphenicol are not used to treat *P. aeruginosa* infection due to intrinsic resistance; 2) tobramycin was designed to lose its ribosomal functions (inactive), hence, it is no longer an antipseudomonal agent. However, when used in combination, rifampicin-tobramycin conjugates sensitized extensively drug-resistant *P. aeruginosa* clinical isolates to tetracyclines and chloramphenicol, but not typical antipseudomonal agents. These suggests (based on data) that the conjugates primarily reverse intrinsic resistance in *P. aeruginosa*, but not in other Gram-negative pathogens. This strategy finds new uses for old drugs versus hard-to-treat bacterial infections, reminiscent of teaching an old dog a new trick.

In chapter 5, we report the discovery of a novel tobramycin-cyclam conjugate that reverse resistance to β -lactam antibiotics (and β -lactamase inhibitors) and restore full potency against colistin-

and carbapenem-resistant *P. aeruginosa*. This was particularly exciting for us as the adjuvant was the first tobramycin-based conjugate that restored the potency of carbapenem against carbapenem-resistant *P. aeruginosa*. β -Lactam antibiotics (penicillins, cephalosporins, carbapenems and monobactams) are cornerstone agents in the treatment of Gram-negative bacterial infections, and they are the most widely prescribed.⁴ Traditionally observed synergistic relationship between aminoglycosides and β -lactam antibiotics is due to the intrinsic activity of both drugs, however, the prepared tobramycin-cyclam conjugates were inactive by themselves, hence, synergistic interaction with β -lactam antibiotics is not due to the ribosomal effect of the aminoglycoside domain. We conclude (based on data) that non-ribosomal tobramycin-cyclam conjugate mitigates the effect of OprD/OprF porin loss in *P. aeruginosa* and potentiates β -lactam / β -lactamase inhibitors against carbapenem-resistant clinical isolates, highlighting the complexity of resistance to β -lactam antibiotics. Given the clinical difficulties presented by drug-resistant organisms expressing multiple β -lactamases and reduced permeability, a triple combination strategy comprising two different adjuvants (OM permeabilizers and β -lactamase inhibitors) and one β -lactam antibiotics, as described in chapter 5, should be given serious considerations. The microbiological advantages of various triple combination over conventional β -lactam/ β -lactamase inhibitor combinations were demonstrated *in vitro* and *in vivo* in a *G. mellonella* larvae infection model. I am particularly curious and optimistic about a triple combination of tobramycin-cyclam conjugate + aztreonam + avibactam versus β -lactamase-producing *P. aeruginosa* clinical isolates in higher animals.

In chapter 6, we initiated an optimization strategy to, based on previous knowledge, develop a broad-spectrum tobramycin-based molecule that would rival the potency of the gold standard potentiator molecule, polymyxin B nonapeptide (PMBN), but obviate its associated toxicities. This led to the discovery of tobramycin homodimers that are non-toxic and more potent than PMBN. In pilot studies using novobiocin, a Gram-positive-only antibiotic, as a companion molecule, we show that tobramycin

homodimers (at $\leq 7 \mu\text{M}$) confer potent activity on novobiocin versus 100 % of all clinically-relevant Gram-negative bacteria tested. These pathogens include *Escherichia coli*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*. Importantly, the adjuvant significantly suppressed the emergence of resistance to novobiocin in *A. baumannii* and did not induce cross-resistance to other classes of antibiotics, even the closely acting fluoroquinolones. This molecule is quite exciting and further studies in animal models should be aggressively pursued. So far, toxicity has not been picked up for this molecule *in vitro* and in preliminary *in vivo* testing in *G. mellonella* larvae.

All things being equal, non-ribosomal aminoglycosides should be less toxic and more tolerated than ribosomal aminoglycosides as most eukaryotic toxicities (mainly ototoxicity) have been linked to lack of precise selectivity for prokaryotic ribosomes.^{5–7} If safety is demonstrated in higher animals, non-ribosomal aminoglycosides may become a drug discovery platform to mine novel antibiotic adjuvants that can expand the chemotherapeutic space of our current armamentarium.

7.2 Future Work

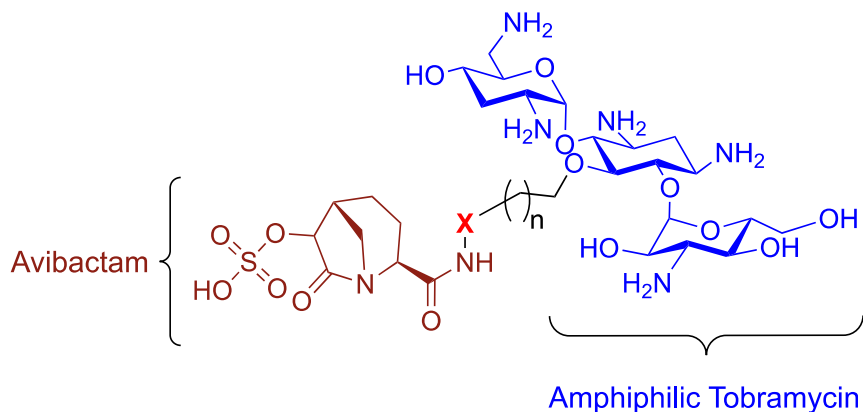
Evidently, there is a need to expand and build on the studies presented in this thesis especially as it relates to β -lactam antibiotics. β -Lactam antibiotics are the most important class of antibiotics in our antibiotic arsenal and all hands must be on deck to preserve their therapeutic utility(ies). We have demonstrated an approach to enhance cellular bioaccumulation of this class of drug in resistant Gram-negative bacterial phenotypes and genotypes, using tobramycin-based adjuvants, and we envisage that the concept therein will be useful towards developing a holistic strategy to address the problem of antimicrobial resistance. Some plausible pathways towards expanding this project may include, but not limited to:

7.2.1 Chemistry

7.2.1.1 *Synthesis of Tobramycin–Avibactam Conjugates*

Avibactam is a clinically used non- β -lactam β -lactamase inhibitor that inhibits a wider range of β -lactamases, including class A, C and some class D (Ambler classification) enzymes.⁶ However, bacteria often express multiple resistance pathways that work in tandem to prevent the actions of antibiotics (or adjuvants). In chapter 5, we demonstrated that β -lactamase inhibitors (using avibactam as an example) may also suffer from outer membrane permeability impediments, wherein an outer membrane permeabilizer may enhance their efficacy in a triple combination regimen. To reduce the number of drug combinations and possible pharmacokinetic interactions that may be associated therein, it is conceivable that an outer membrane permeabilizer and a β -lactamase inhibitor can possibly be linked as a single entity and may still retain the individual activities. Our experience with amphiphilic tobramycin analogs (chapters 4 to 6) and the various derivatives of avibactam currently in clinical trials⁷ support this

hypothesis. These compounds should be ligated (using a stable linker) at the amenable site of avibactam (shown below),⁷ with the hope that the amphiphilic-tobramycin domain will anchor avibactam across porin-deficient Gram-negative bacteria (especially *P. aeruginosa*) into the periplasm where it will inhibit β -lactamase enzymes. A concise synthetic strategy for this labile and highly constrained scaffold has been reported, and a different study has also reported the synthesis of orally absorbed avibactam derivatives.⁸

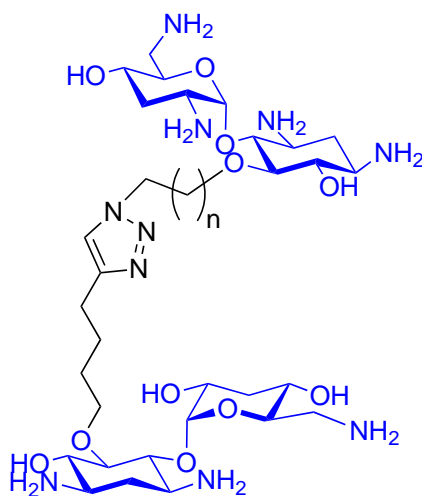


X = different linkers; n = tether length

7.2.1.2 Synthesis of Nebramine Homodimers

Nebramine is a tobramycin derivative in which one α -D-glucose of tobramycin is cleaved. We have reported some nebramine-based derivatives of previously reported tobramycin conjugates that retained the ability to permeabilize the outer membrane of Gram-negative bacteria. Nebramine derivatives have two main benefits: a) the reduced molecular weight of the final compounds makes them more drug-like, b) the reduced number of overall cationic charges may be relevant in avoiding some off-target interactions downstream. This compound can easily be synthesized by subjecting tobramycin homodimers to strong acidic conditions (40% methanolic HCl) at high temperature.

It will be interesting to investigate how nebramine homodimers compare with tobramycin homodimers, both in terms of potency and toxicity.



Nebramine Homodimer

n = tether length

7.2.2 Biology

7.2.2.1 Tobramycin-Cyclam and Gallium Interaction

In chapter 5, we describe the activity of tobramycin-cyclam conjugates. Cyclams are very good chelators of many metal ions.⁹ Gallium, which has a nearly identical ionic radius as iron, disrupts iron-dependent processes in bacteria because it cannot be reduced under physiological conditions.¹⁰ Indeed, some bacterial uptake systems are unable to distinguish gallium from iron,^{11,12} and gallium has been shown to be potent against *P. aeruginosa*.¹⁰ This means that under iron-depleted conditions, gallium may be taken up more rapidly in place of iron. Interestingly, gallium nitrate [Ga(NO₃)₃] is already approved by the FDA for a non-infection indication (hypercalcemia of malignancy) and cyclams are excellent chelators of gallium.¹³ Therefore, a viable study will be to investigate the interactions between tobramycin-cyclam conjugates and gallium, and cyclam and gallium under normal and iron-depleted conditions.

7.2.2.2 *Animal (Mice) Studies*

Efficacy studies in animals and pharmacokinetic studies of leading compounds described in this thesis is warranted. Preliminary determinations in *Galleria mellonella* may serve as a baseline for these studies, and optimization of dosing regimen for each constituent molecules is needed to maximize therapeutic potentials. Importantly, pharmacokinetic study of the adjuvant may be useful in matching an appropriate antibiotic to each adjuvant.

7.2.2.3 *Immunomodulatory Properties*

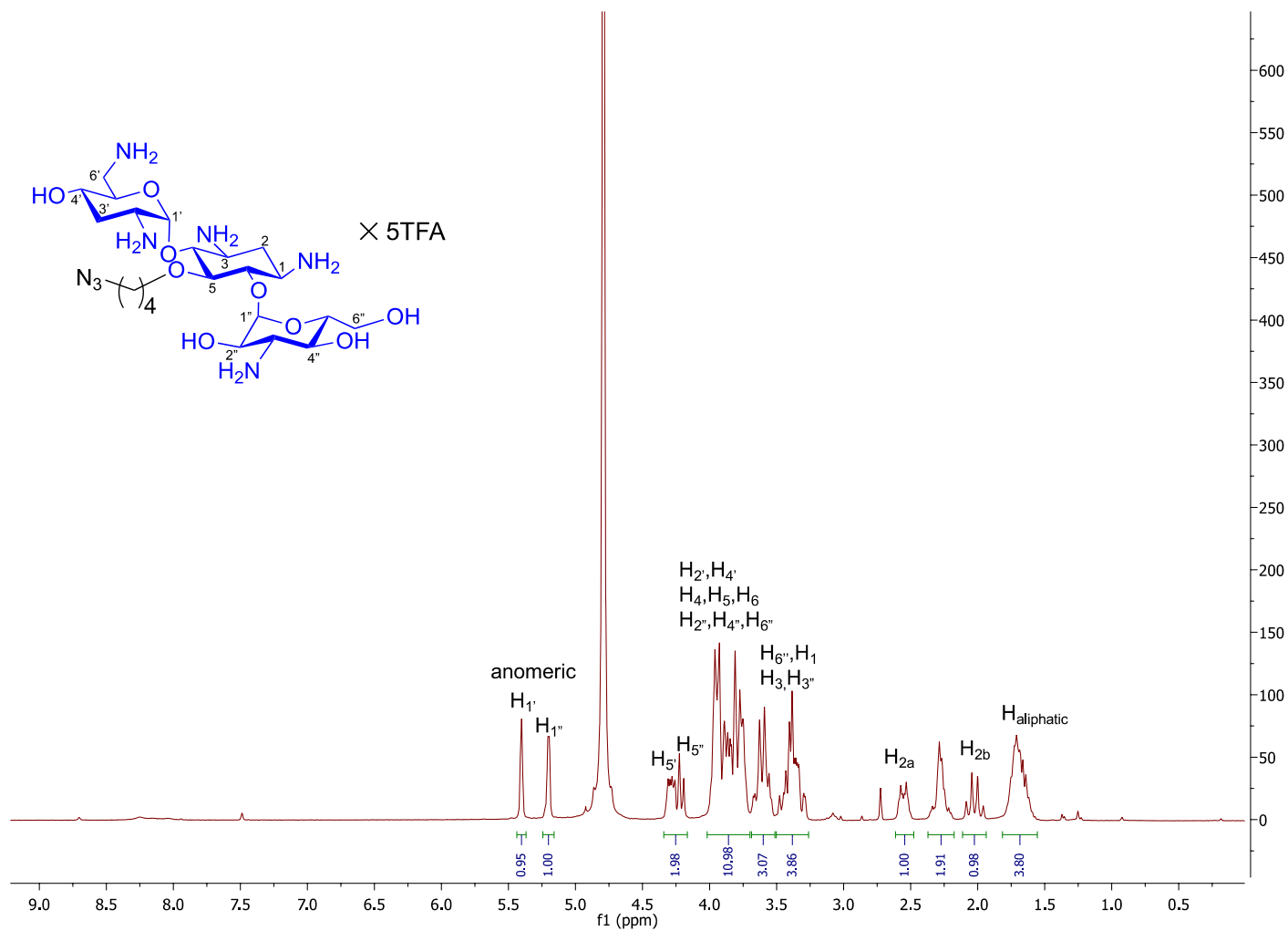
Immunomodulatory properties of the newly synthesized tobramycin-based conjugates should be thoroughly assessed to investigate possible non-antibacterial potentials of this scaffold.

7.3 References

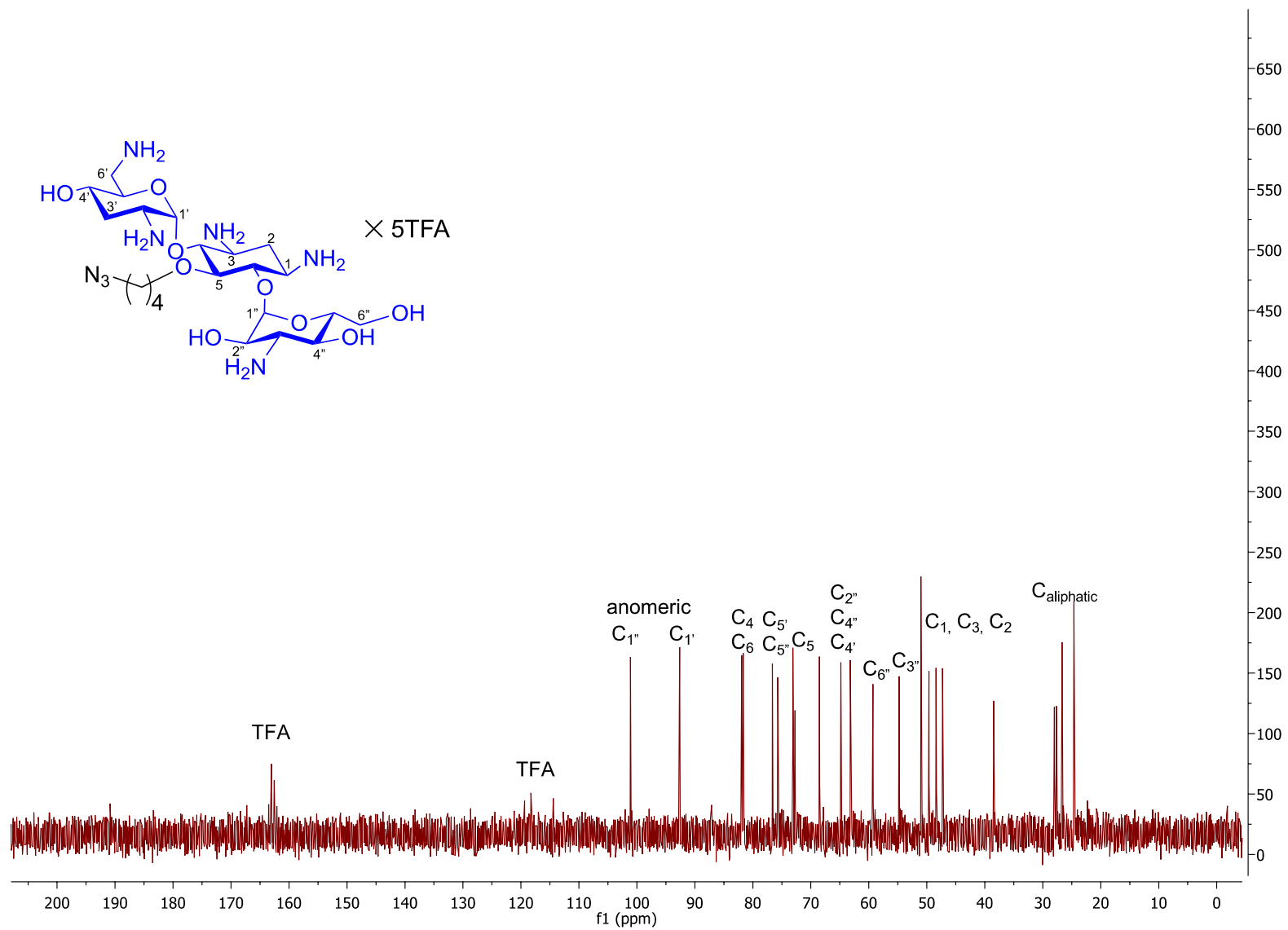
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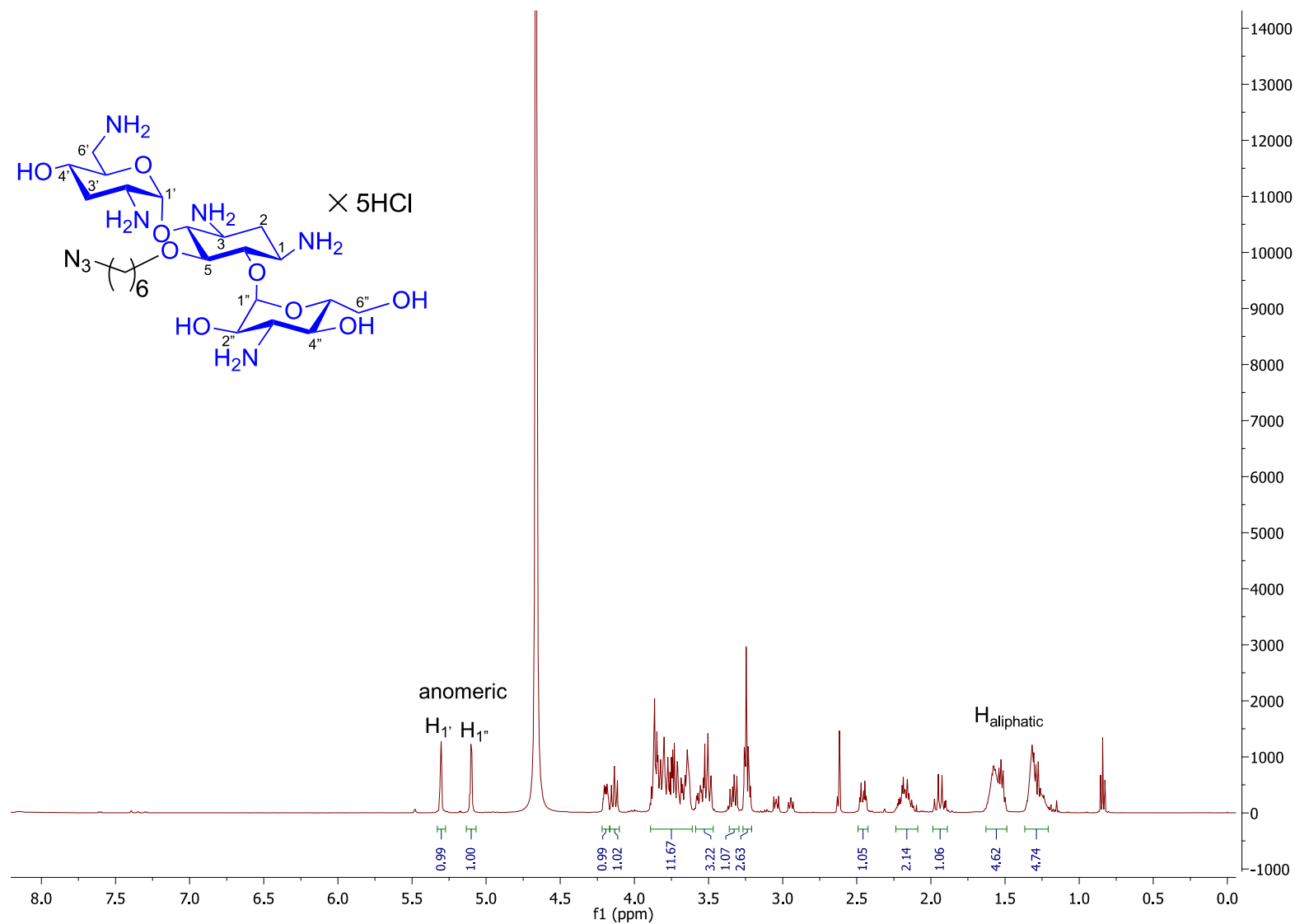
Appendix A



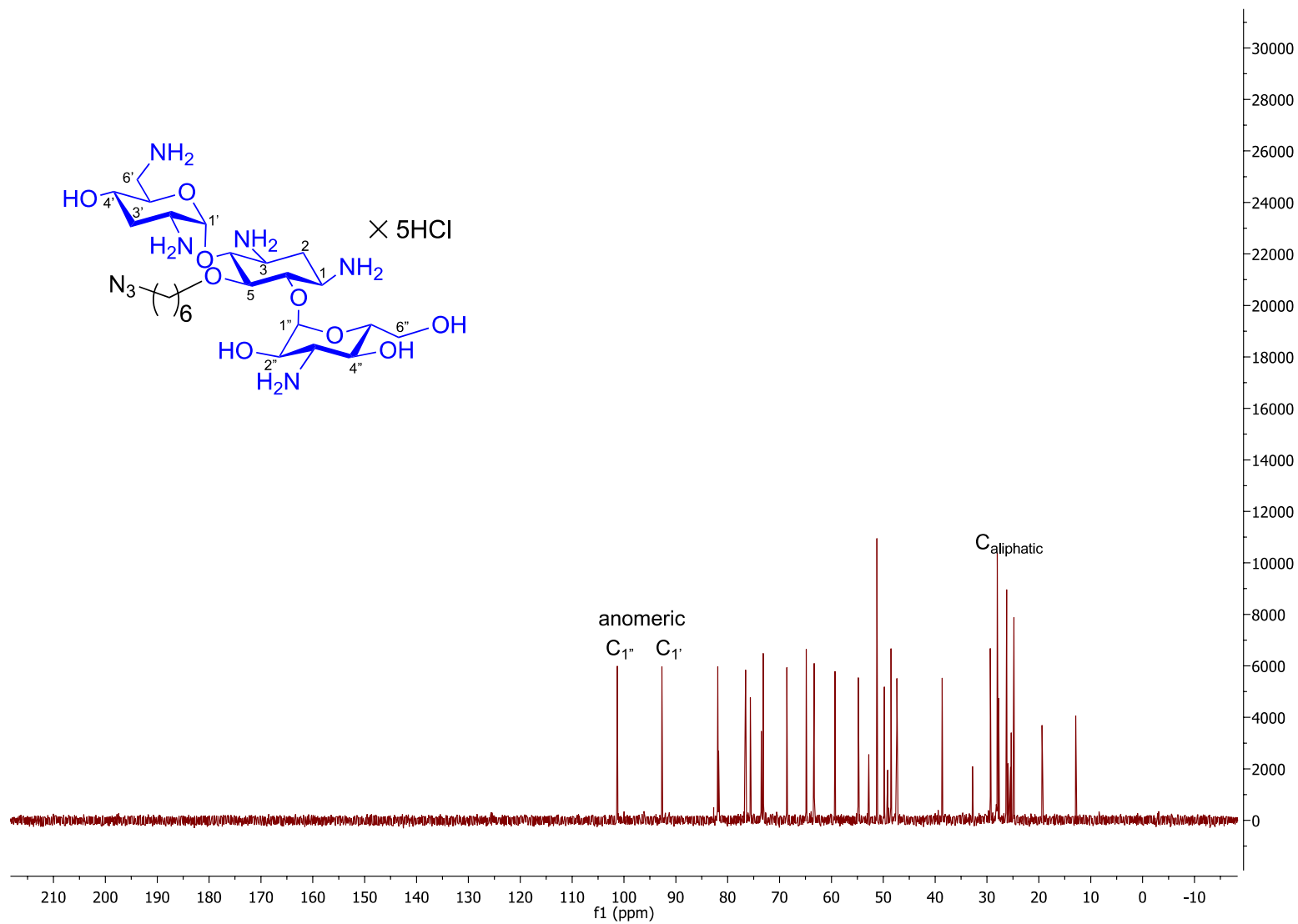
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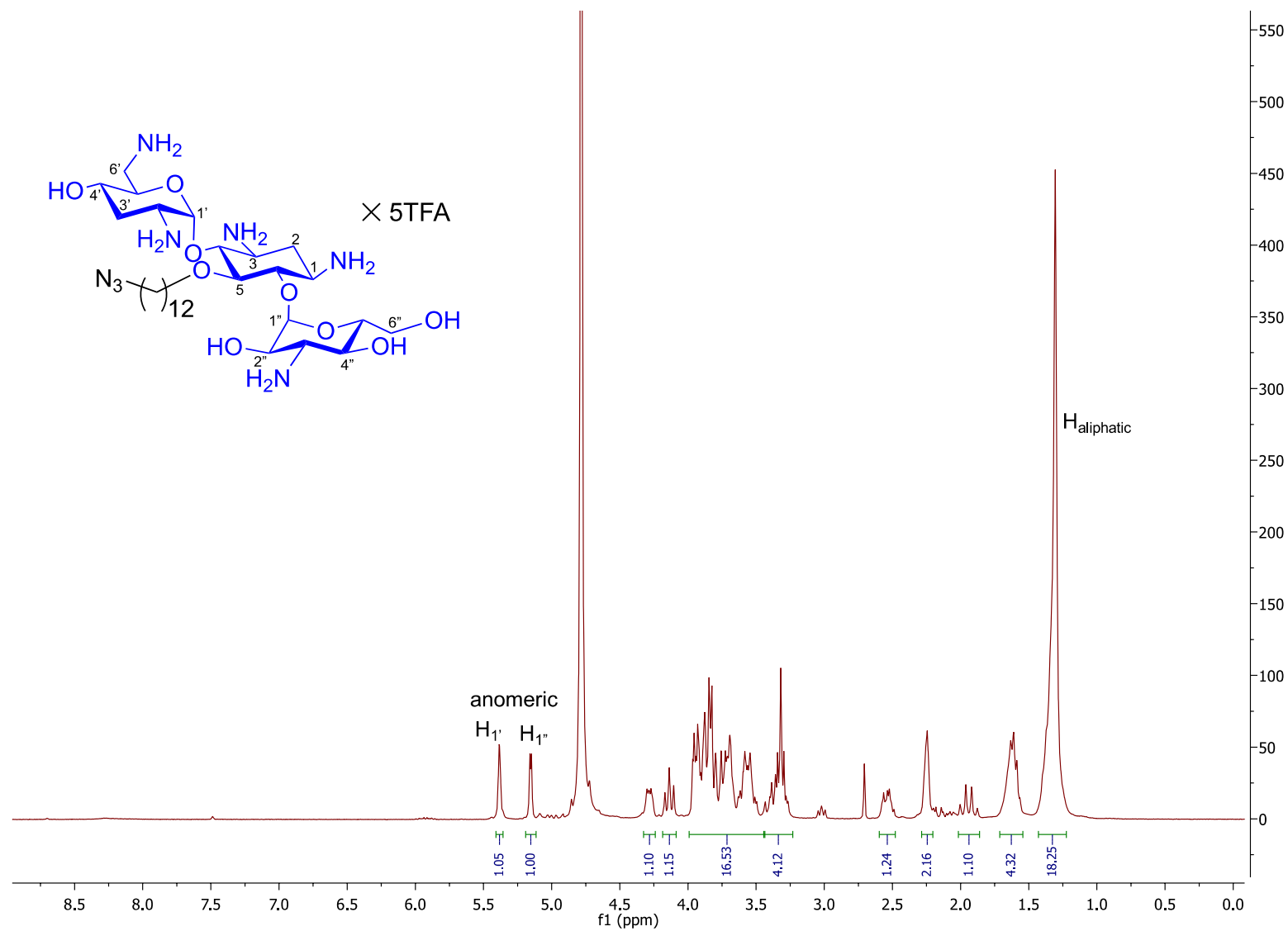
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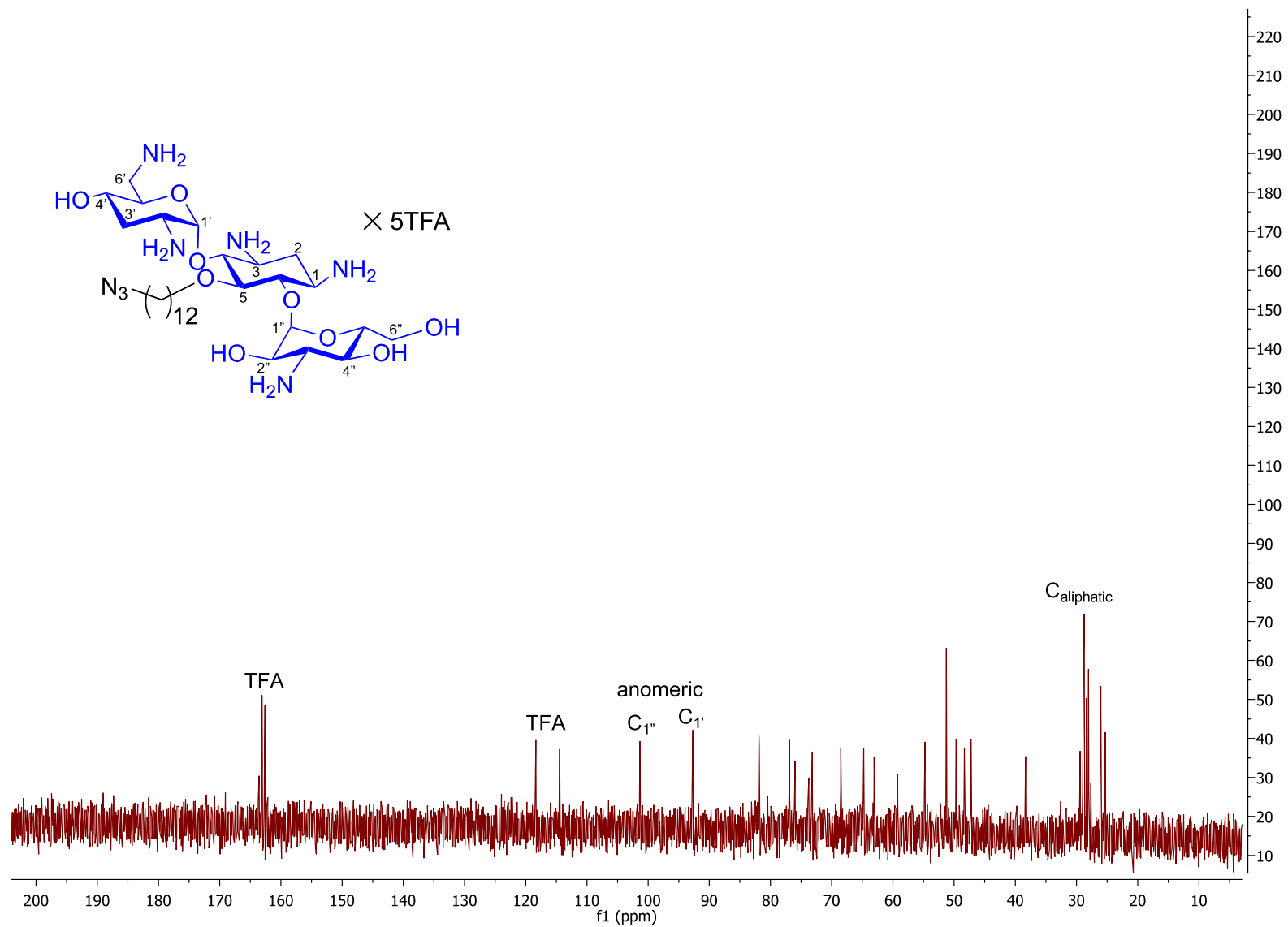
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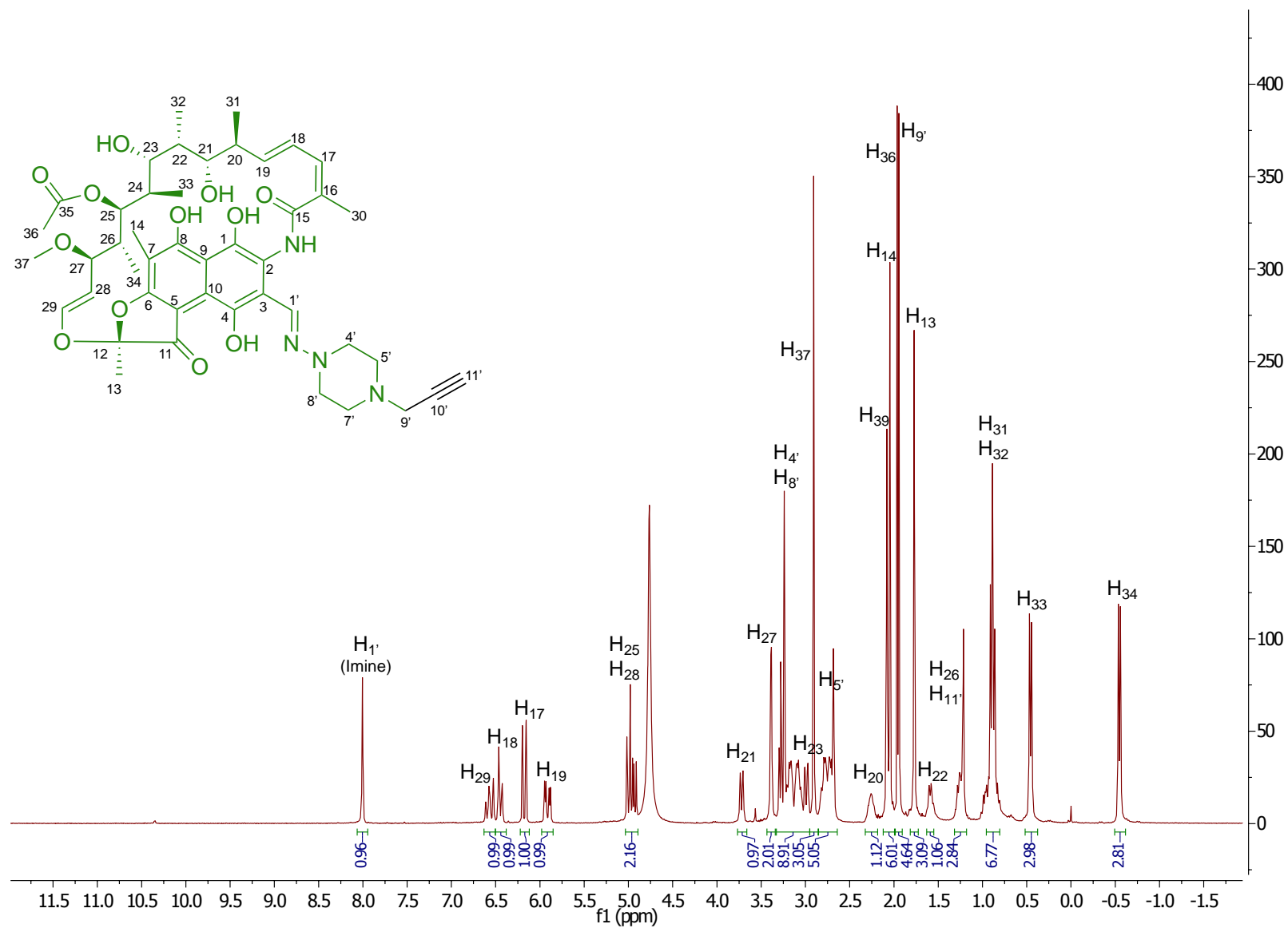
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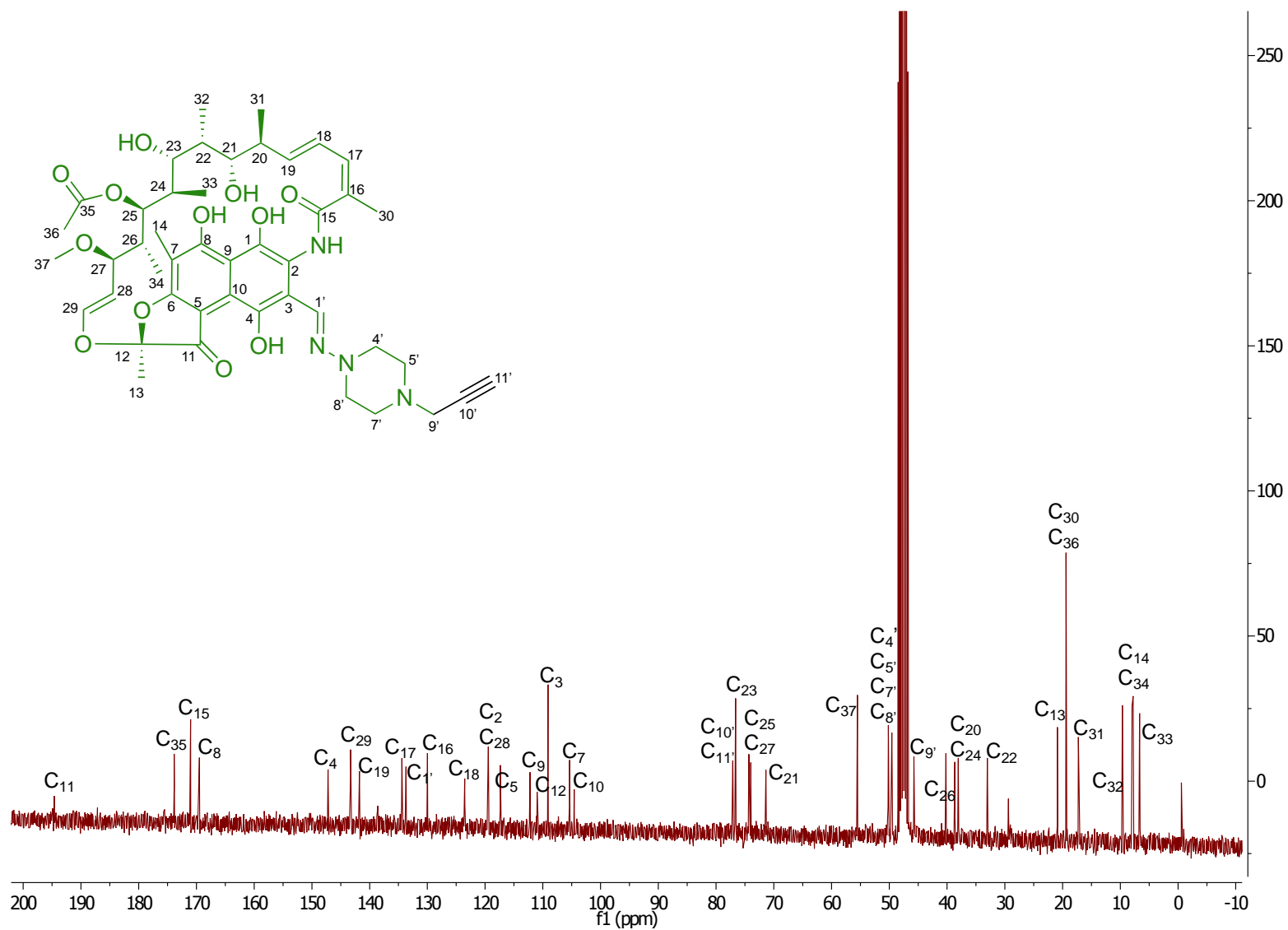
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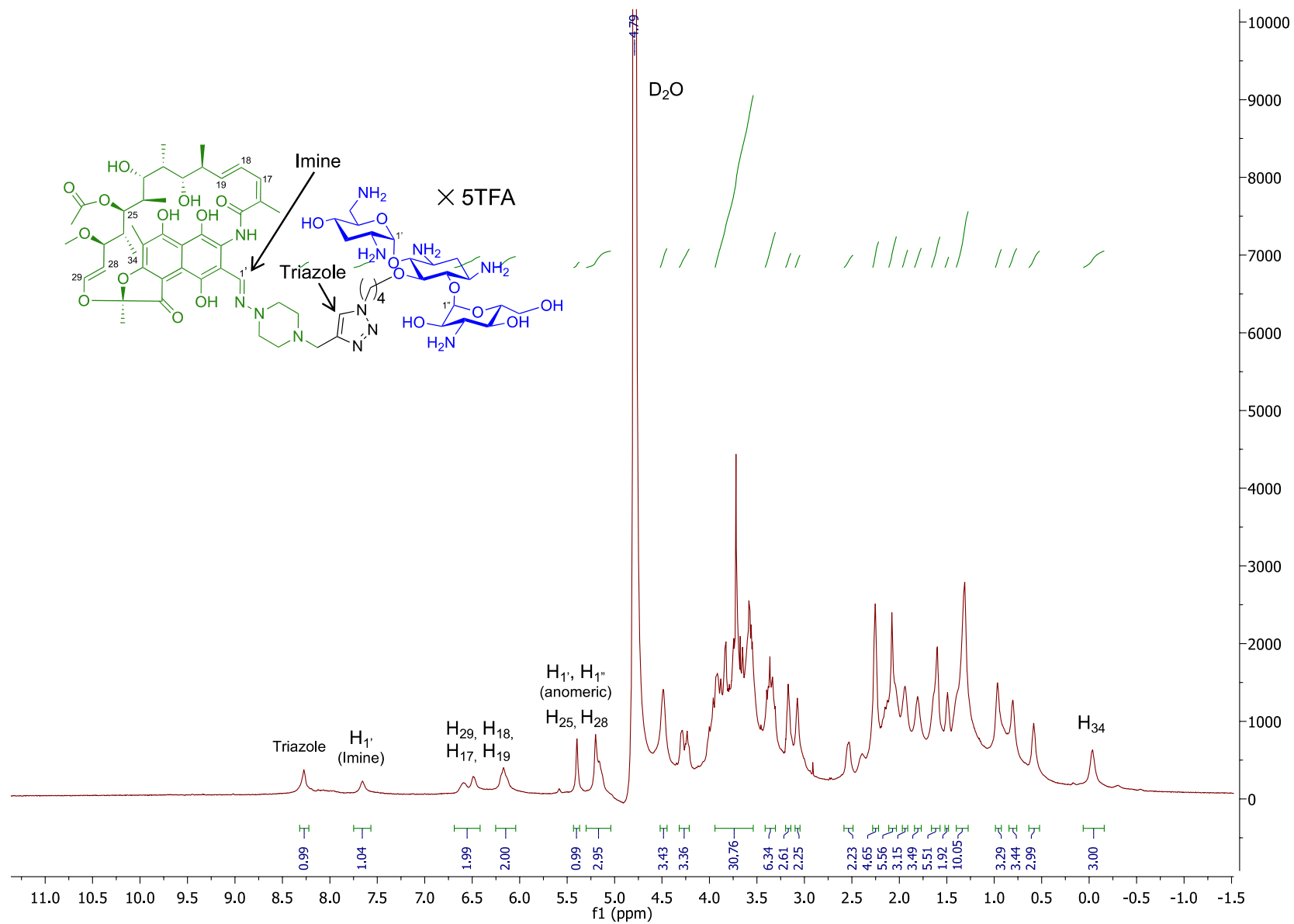
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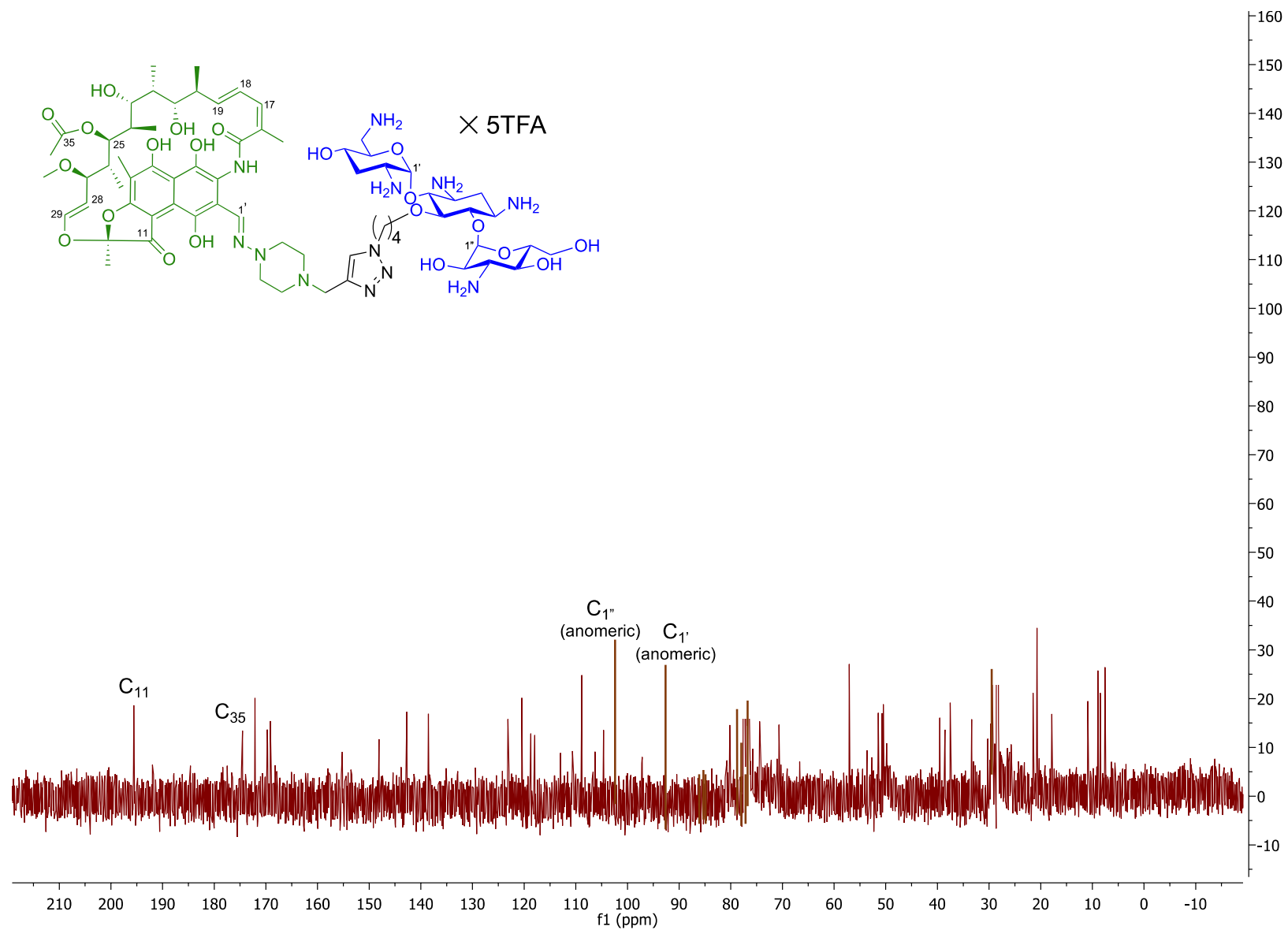
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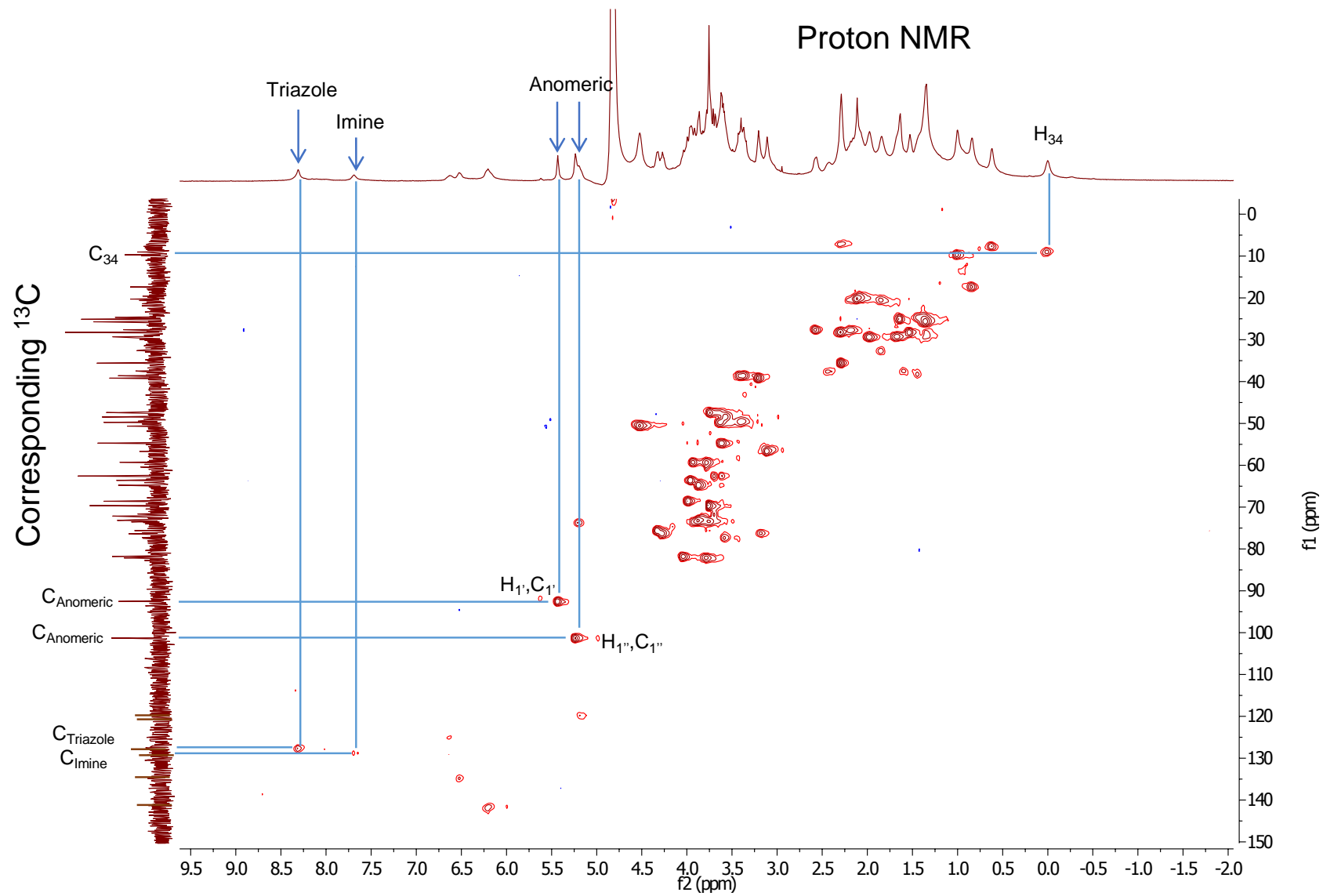
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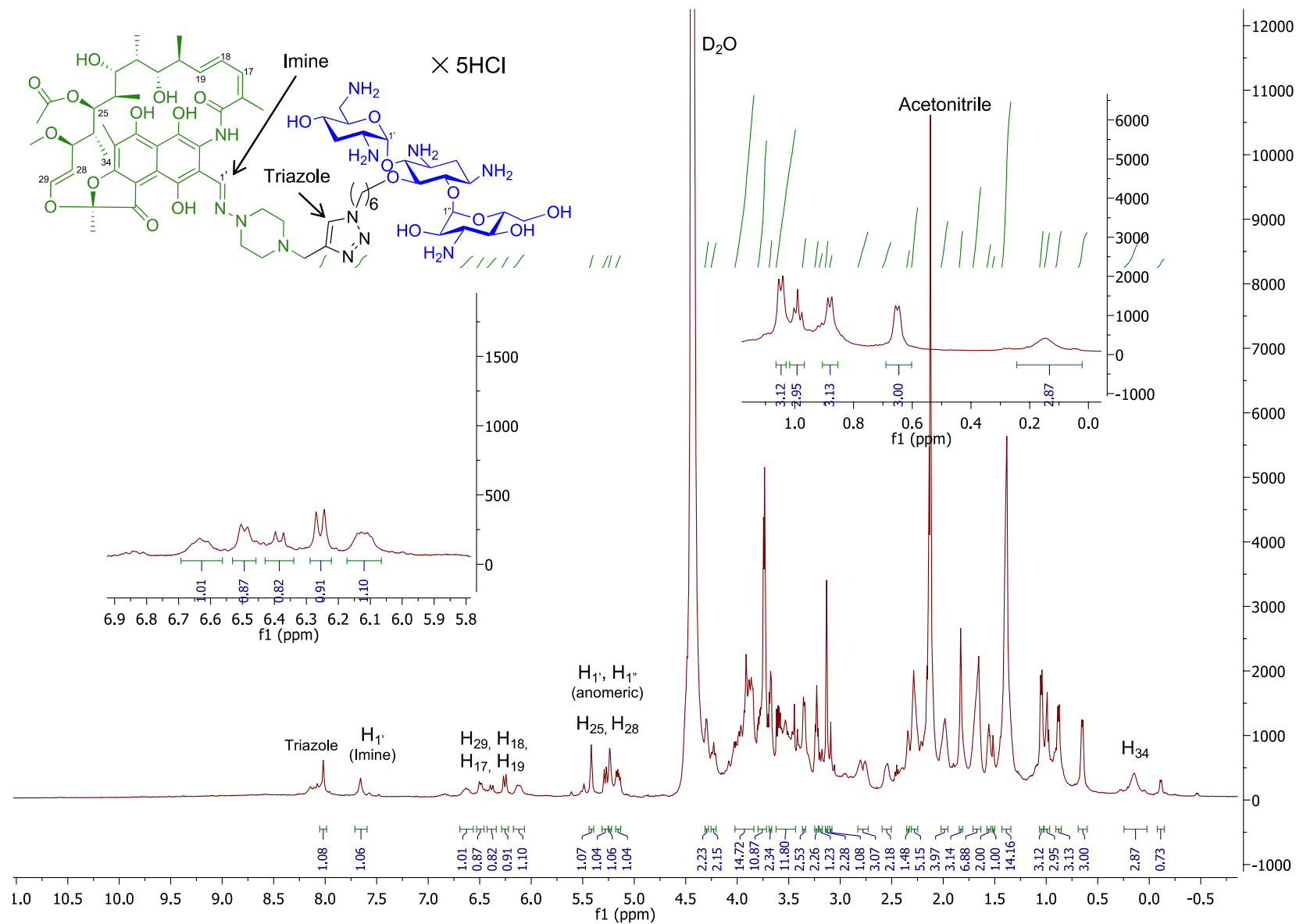
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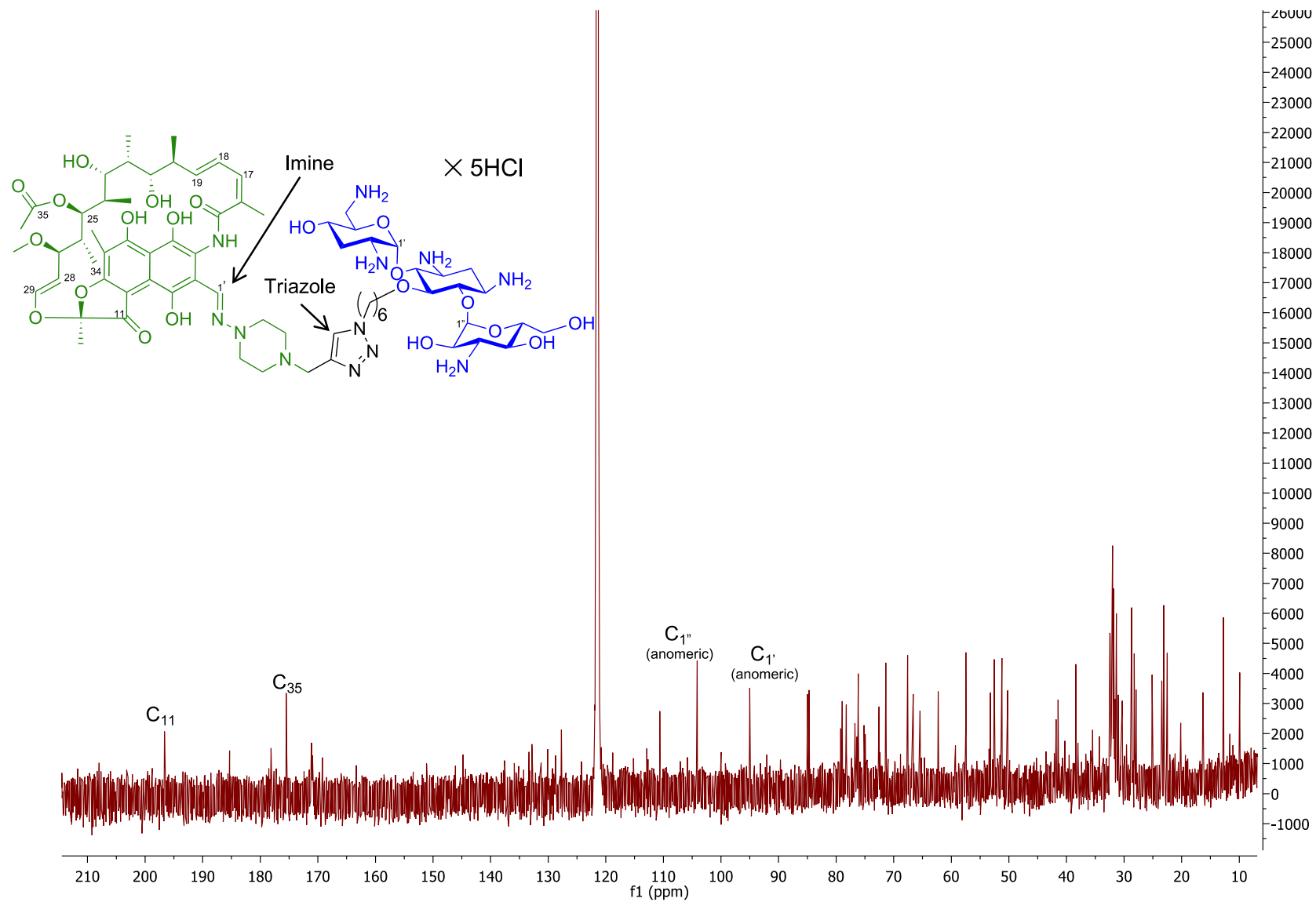
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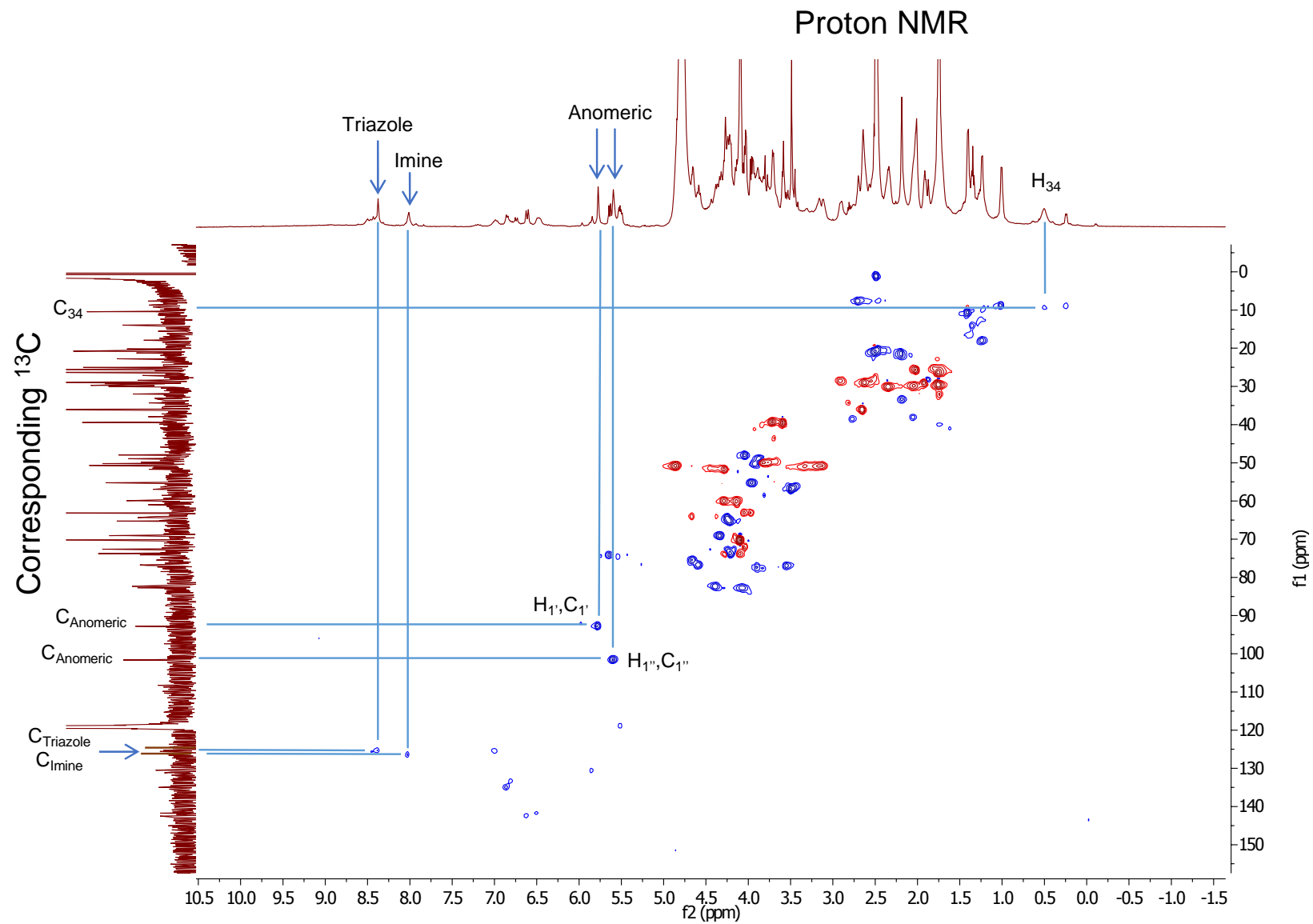
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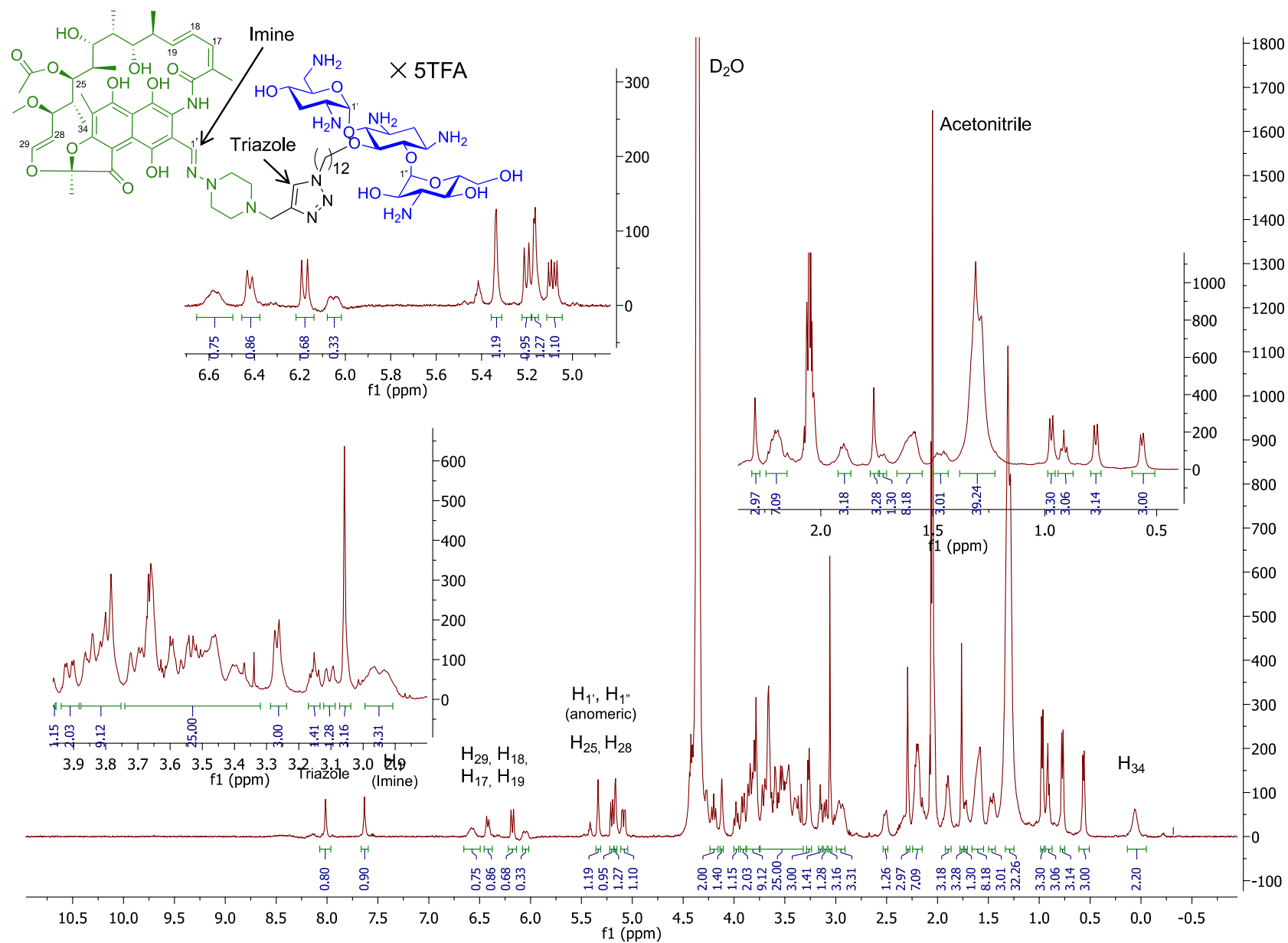
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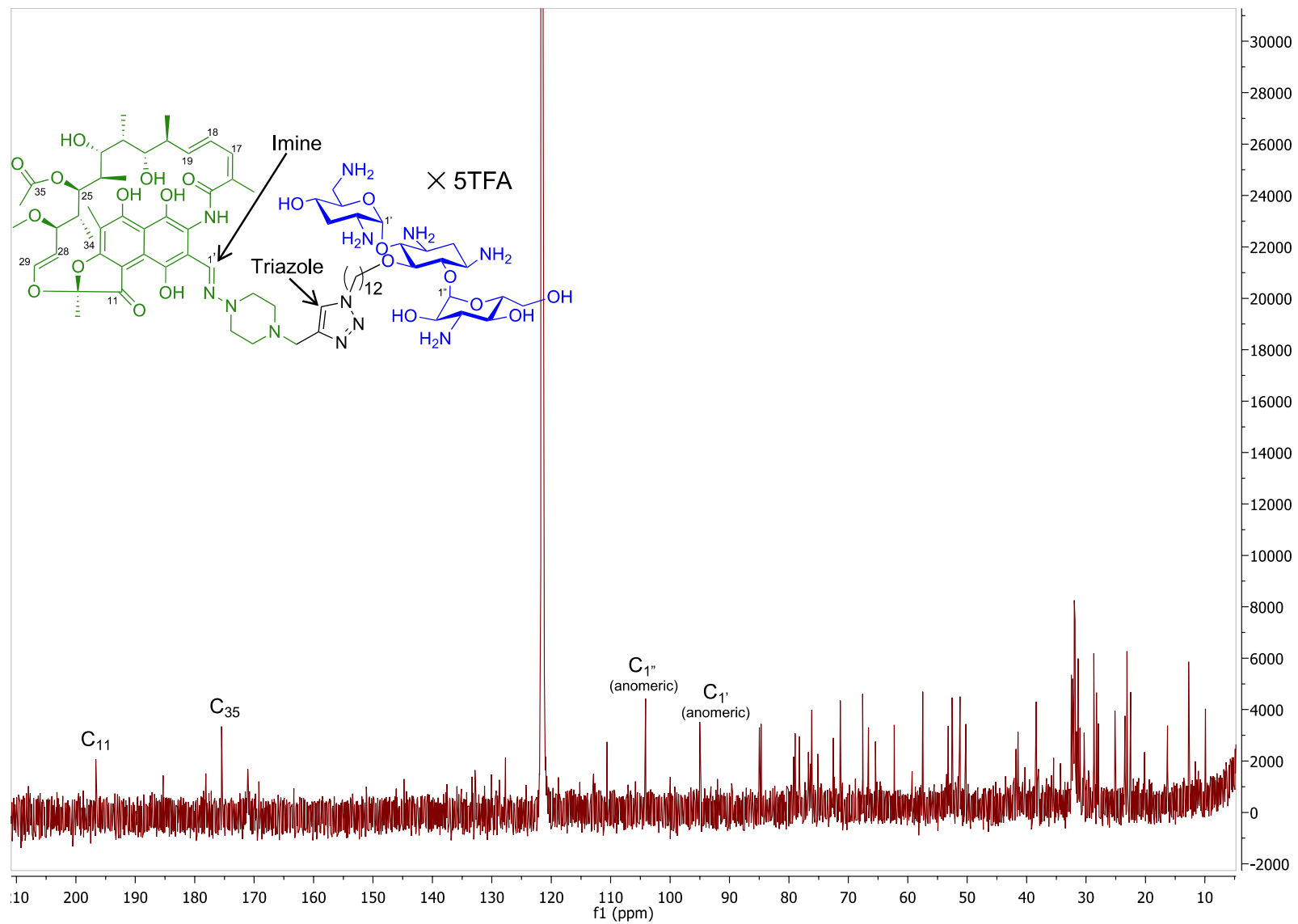
^{13}C NMR of Compound **2** in $\text{D}_2\text{O} + \text{CD}_3\text{CN}$



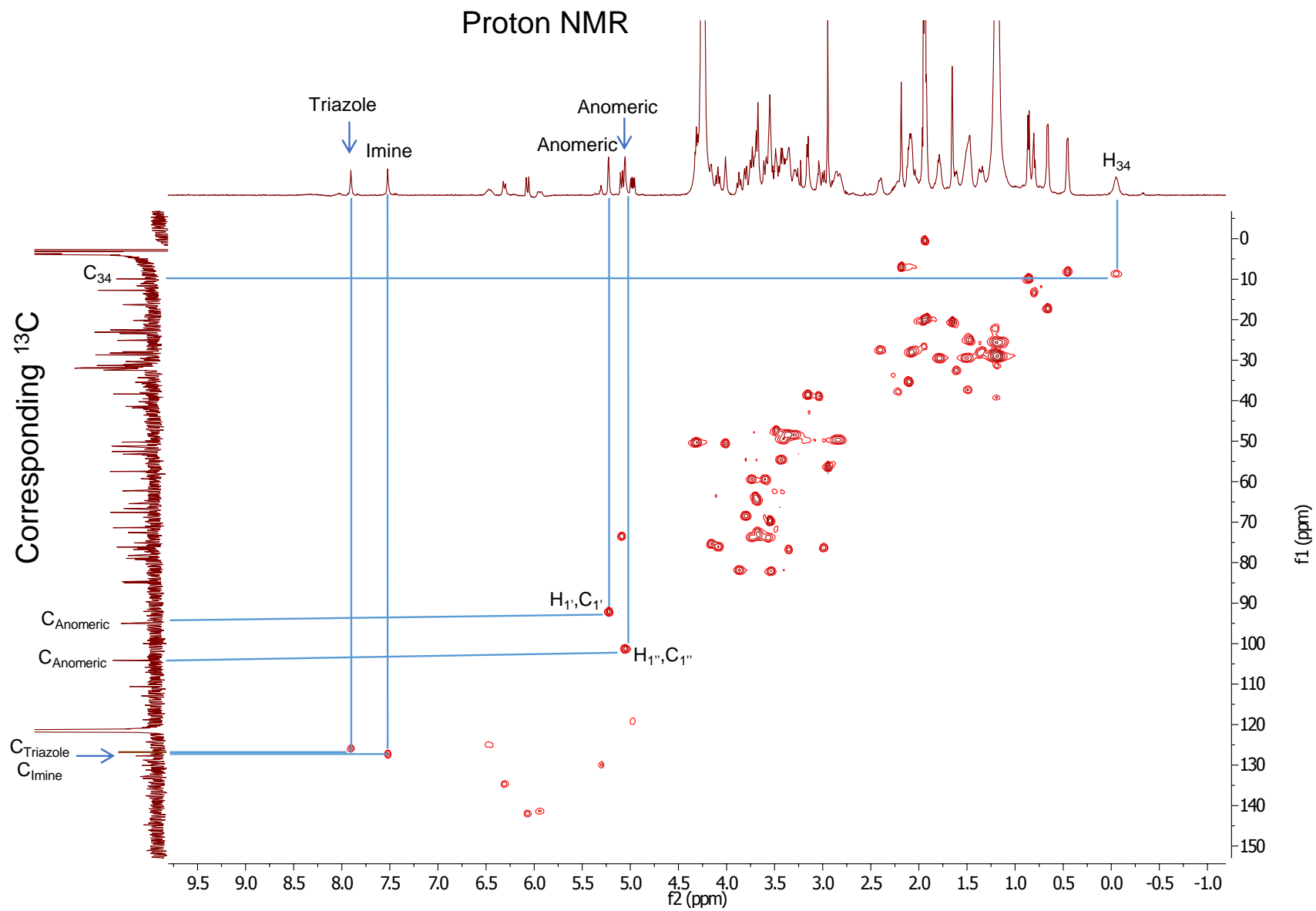
HSQC of Compound **2** in $\text{D}_2\text{O} + \text{CD}_3\text{CN}$



¹H NMR of Compound 3 in D₂O + CD₃CN

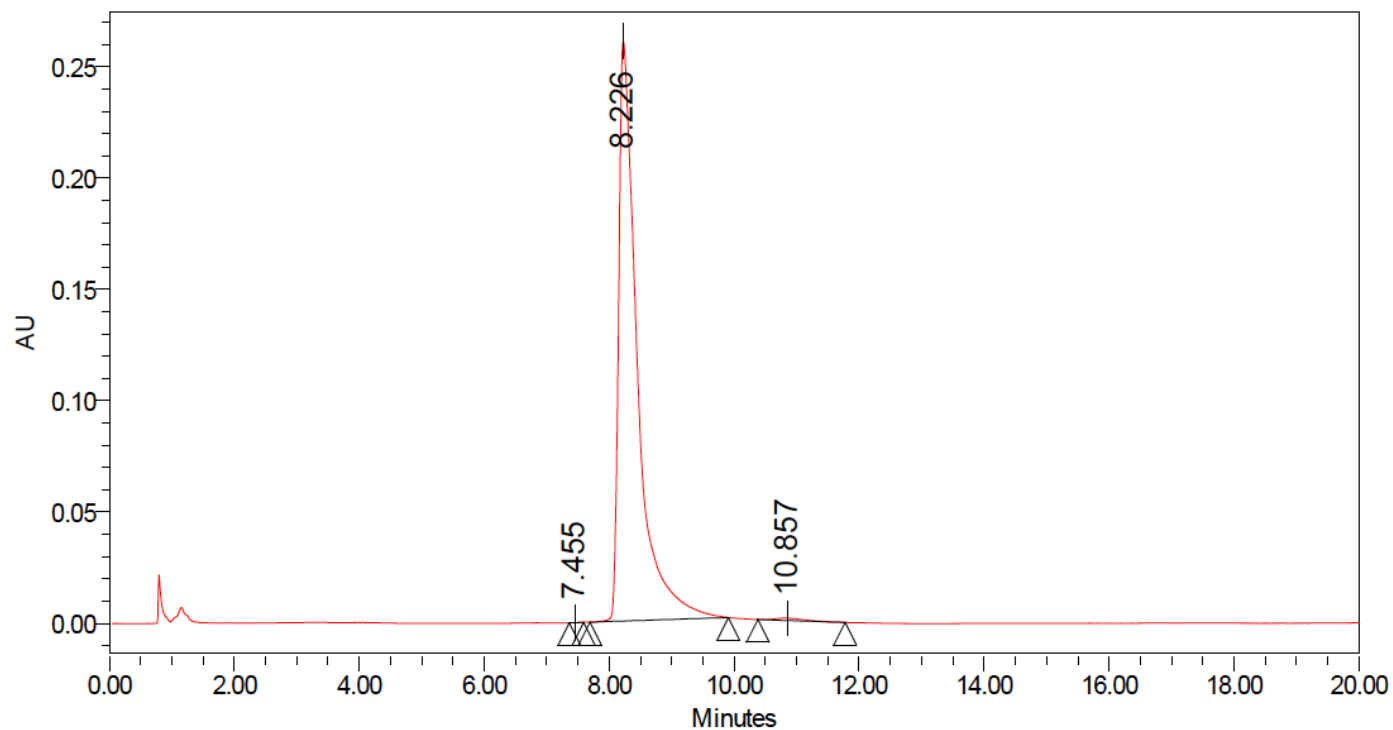


^{13}C NMR of Compound 3 in $\text{D}_2\text{O} + \text{CD}_3\text{CN}$



HSQC of Compound **3** in D₂O + CD₃CN

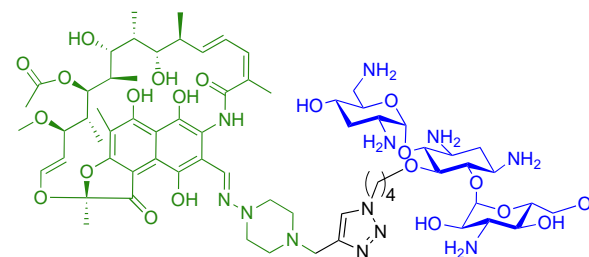
Chromatogram Overlay with Z Axis Offset



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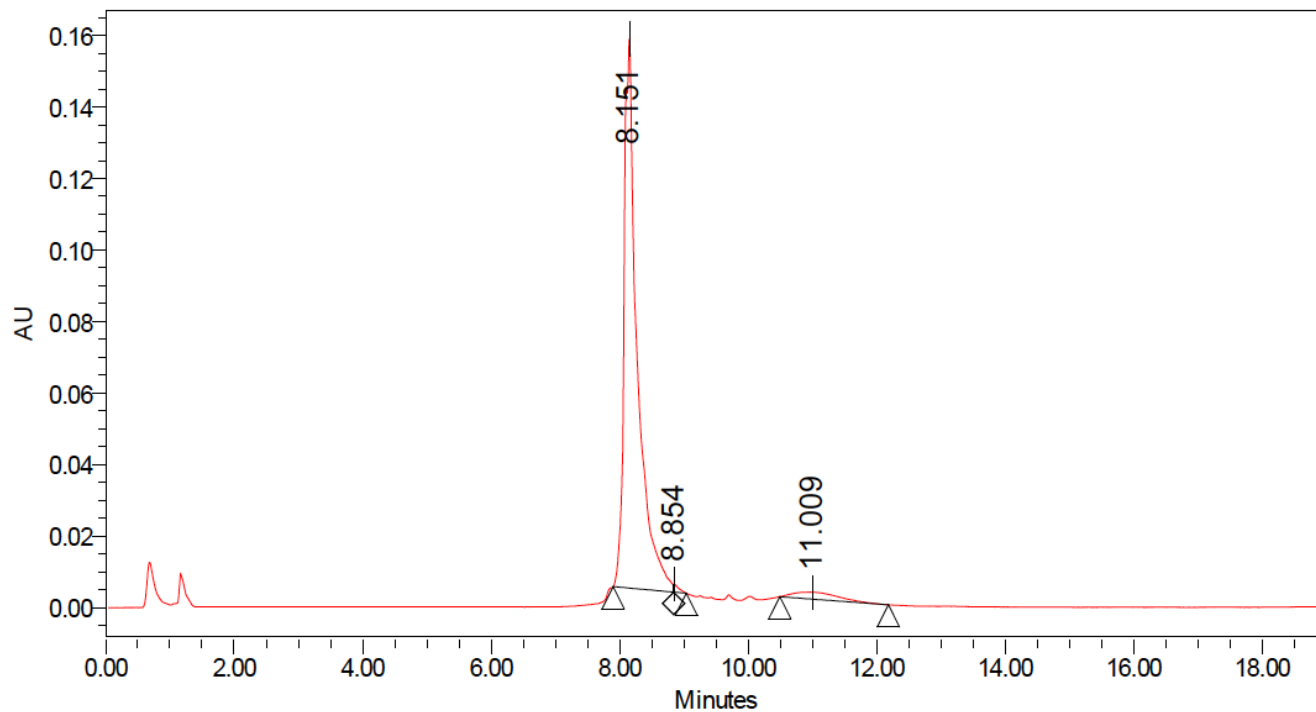
Processed Channel: PDA 332.0 nm

| | Processed Channel | Retention Time (min) | Area | % Area | Height |
|---|-------------------|----------------------|---------|--------|--------|
| 1 | PDA 332.0 nm | 7.455 | 322 | 0.01 | 50 |
| 2 | PDA 332.0 nm | 8.226 | 5403624 | 99.36 | 260517 |
| 3 | PDA 332.0 nm | 10.857 | 34737 | 0.64 | 1129 |



HPLC Purity Analysis of Compound 1

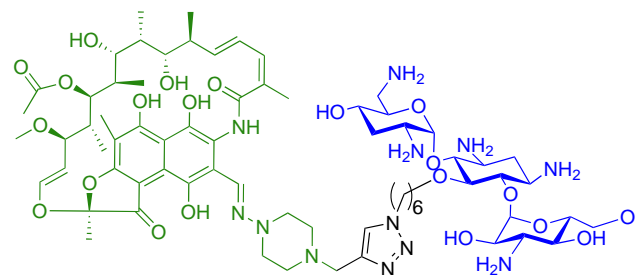
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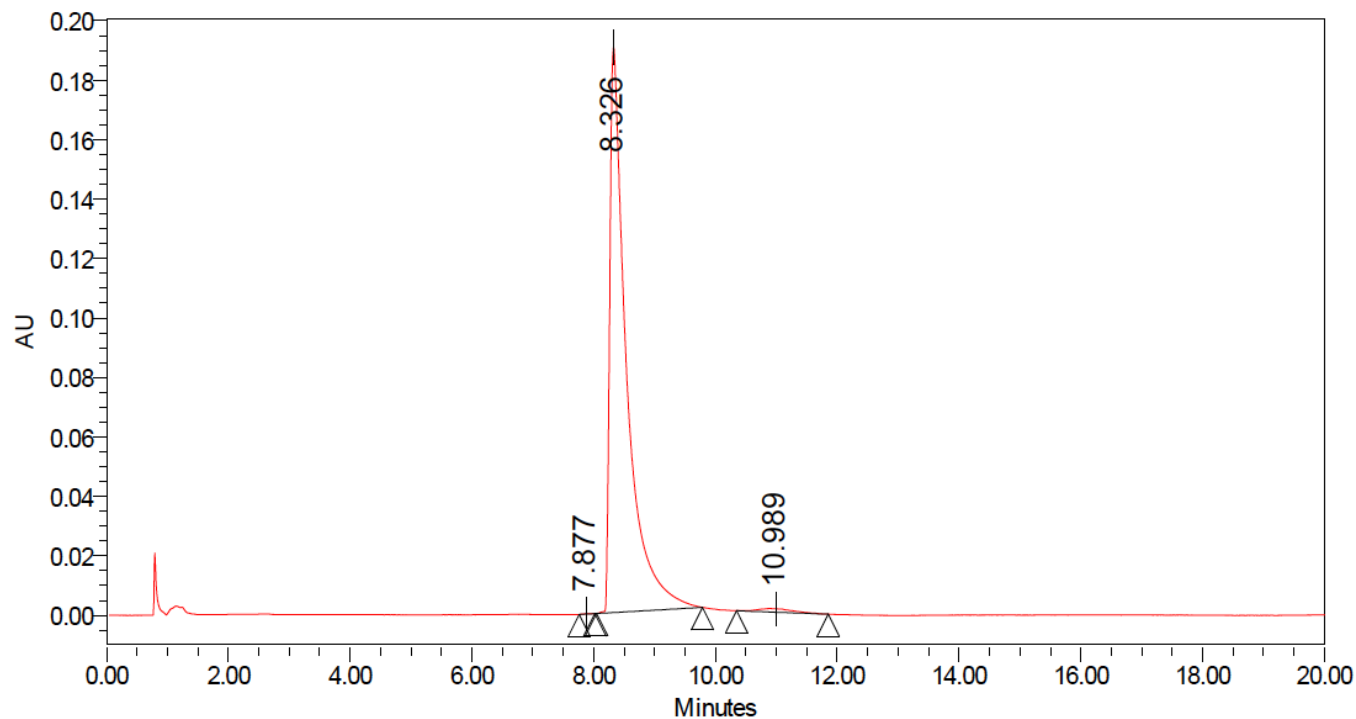
Processed Channel: PDA 332.0 nm

| | Processed Channel | Retention Time (min) | Area | % Area | Height |
|---|-------------------|----------------------|---------|--------|--------|
| 1 | PDA 332.0 nm | 8.151 | 2185518 | 95.29 | 153552 |
| 2 | PDA 332.0 nm | 8.854 | 12467 | 0.54 | 2150 |
| 3 | PDA 332.0 nm | 11.009 | 95626 | 4.17 | 1960 |



HPLC Purity Analysis of Compound 2

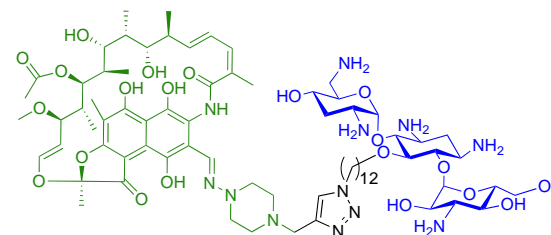
Chromatogram Overlay with Z Axis Offset



— Sample Name: TO-02-063 ; Date Acquired: 24/07/2017 2:33:02 PM CDT

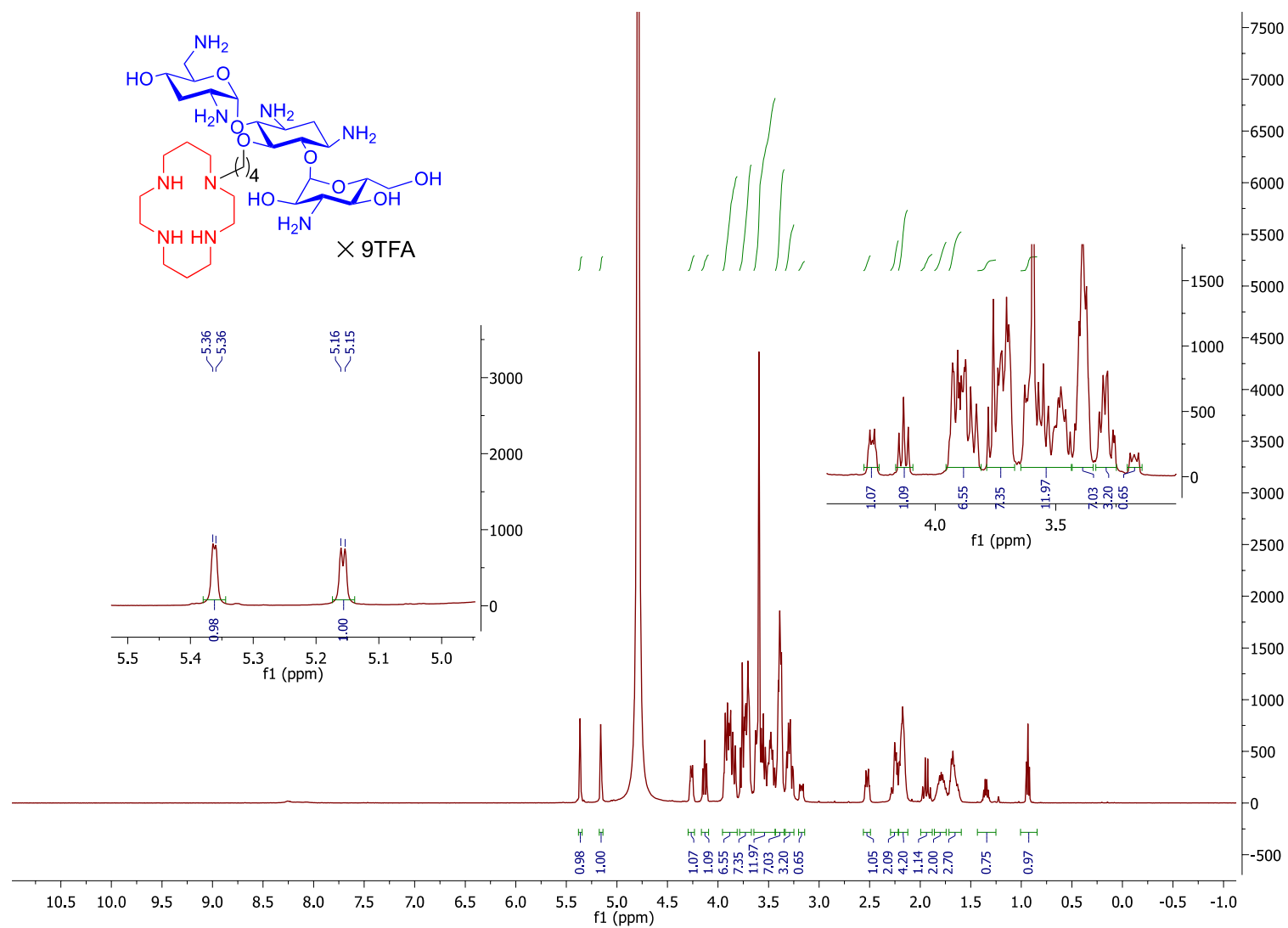
Processed Channel: PDA 332.0 nm

| | Processed Channel | Retention Time (min) | Area | % Area | Height |
|---|-------------------|----------------------|---------|--------|--------|
| 1 | PDA 332.0 nm | 7.877 | 1189 | 0.03 | 140 |
| 2 | PDA 332.0 nm | 8.326 | 3649189 | 98.68 | 190164 |
| 3 | PDA 332.0 nm | 10.989 | 47488 | 1.28 | 1176 |

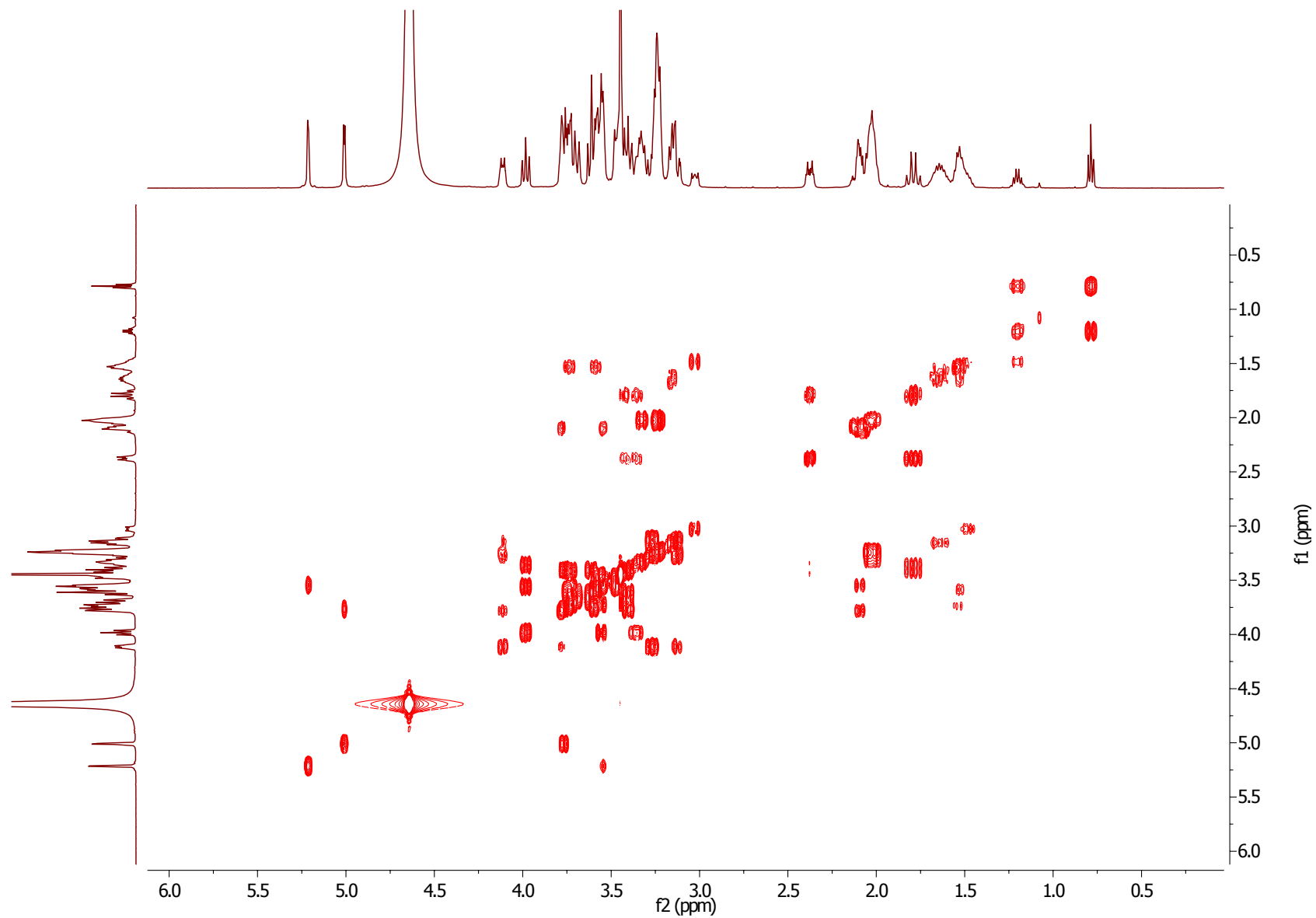


HPLC Purity Analysis of Compound 3

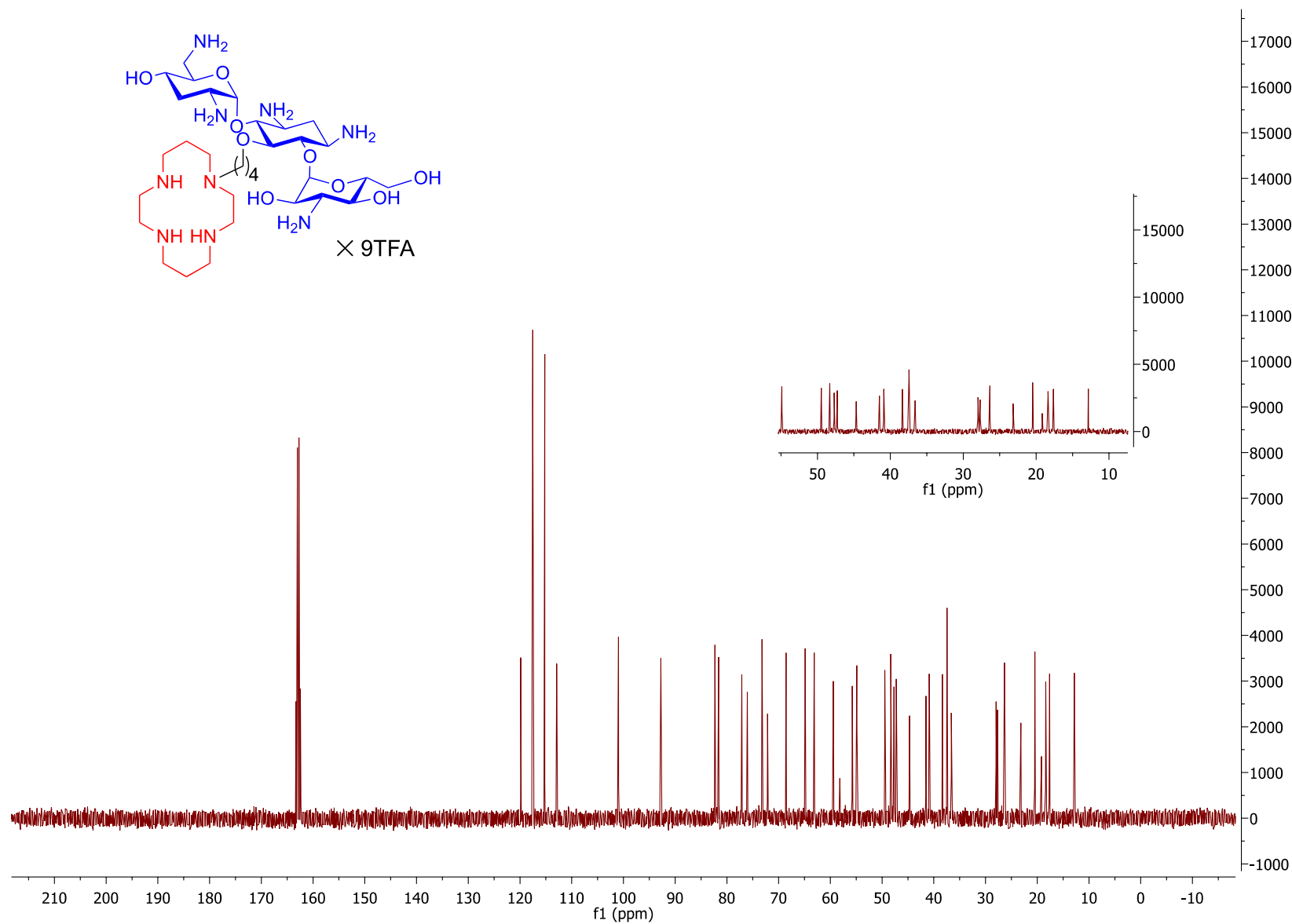
Appendix B



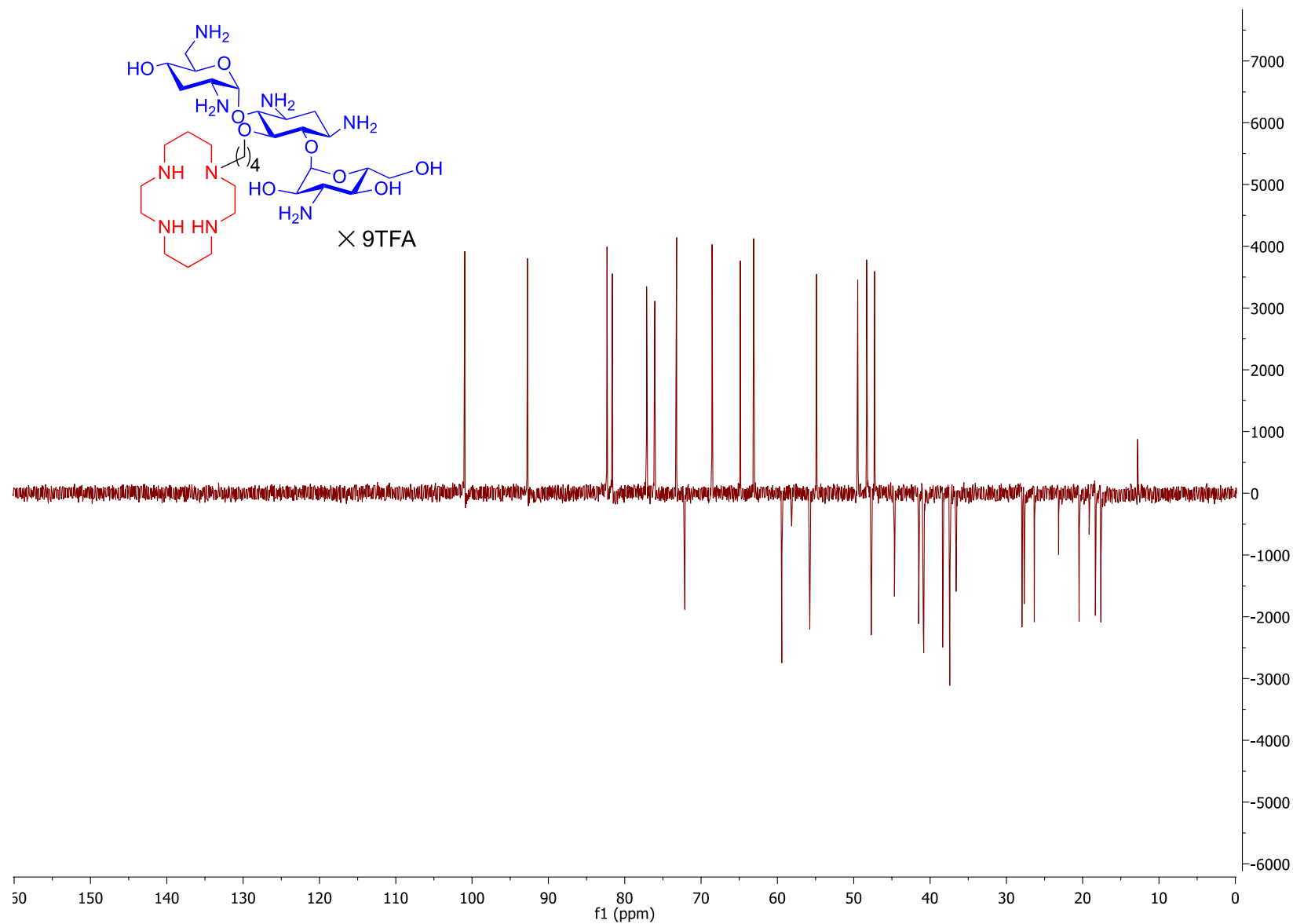
^1H NMR of Compound 1 in D_2O



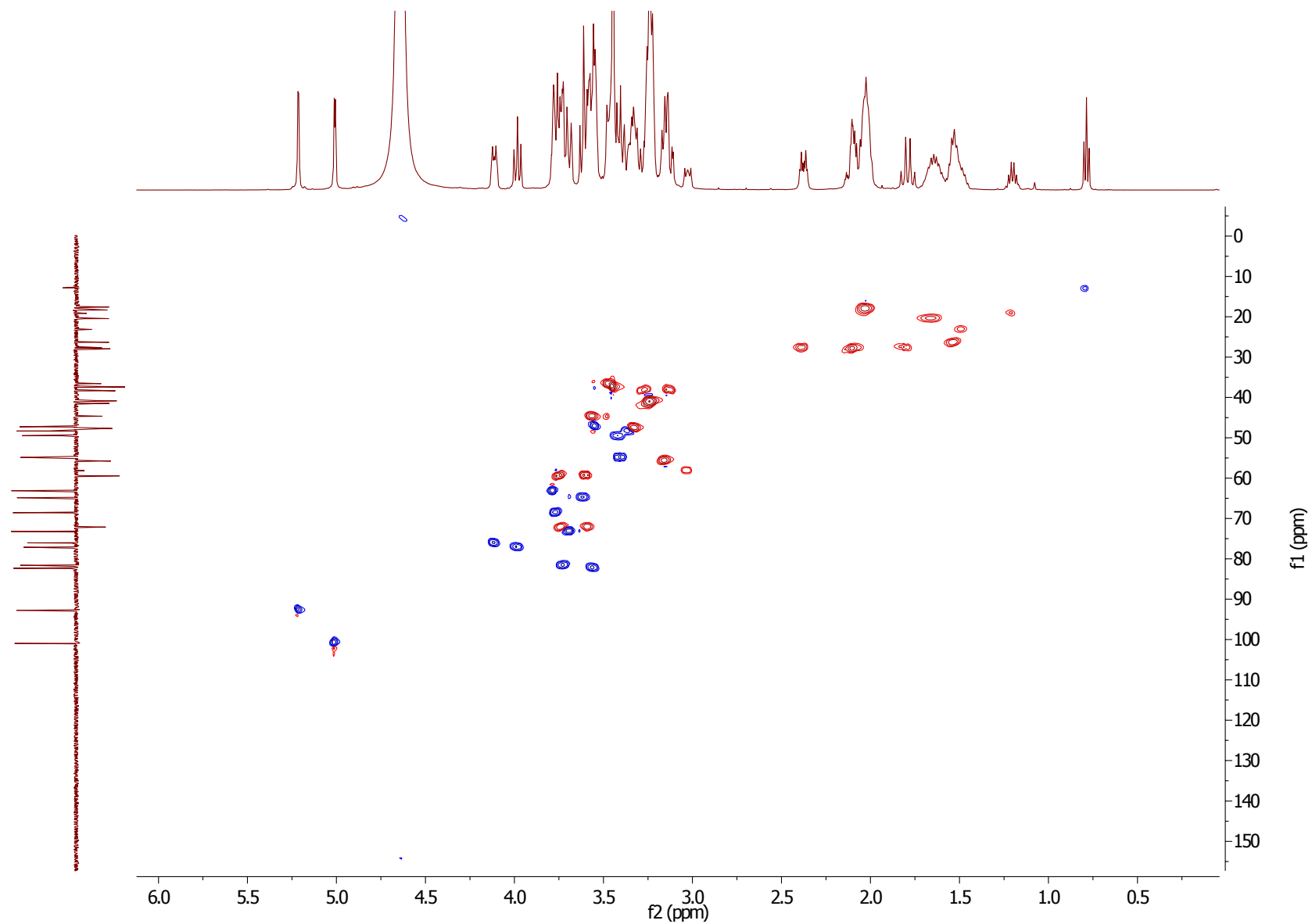
COSY NMR of Compound **1** in D₂O



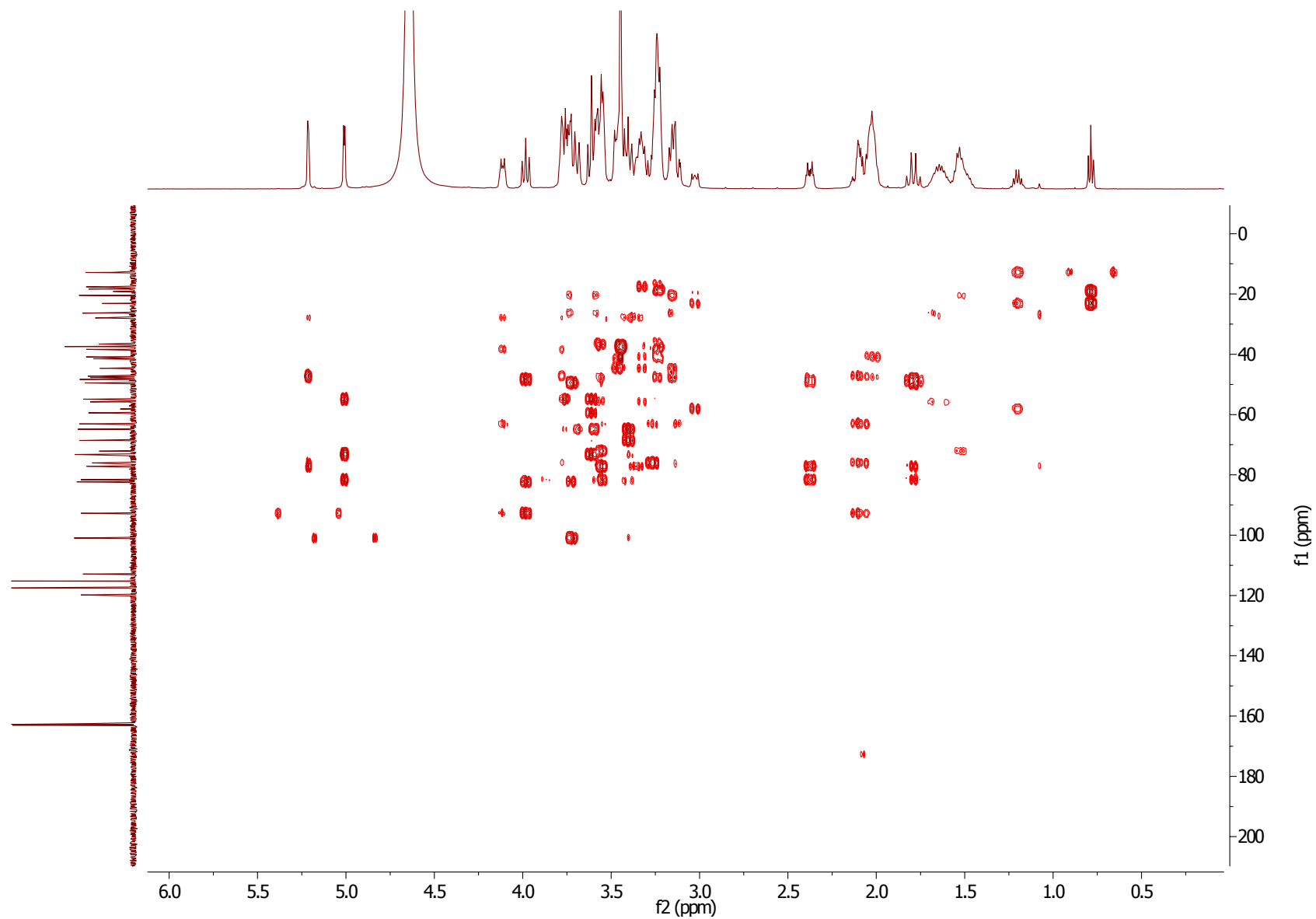
^{13}C NMR of Compound **1** in D_2O



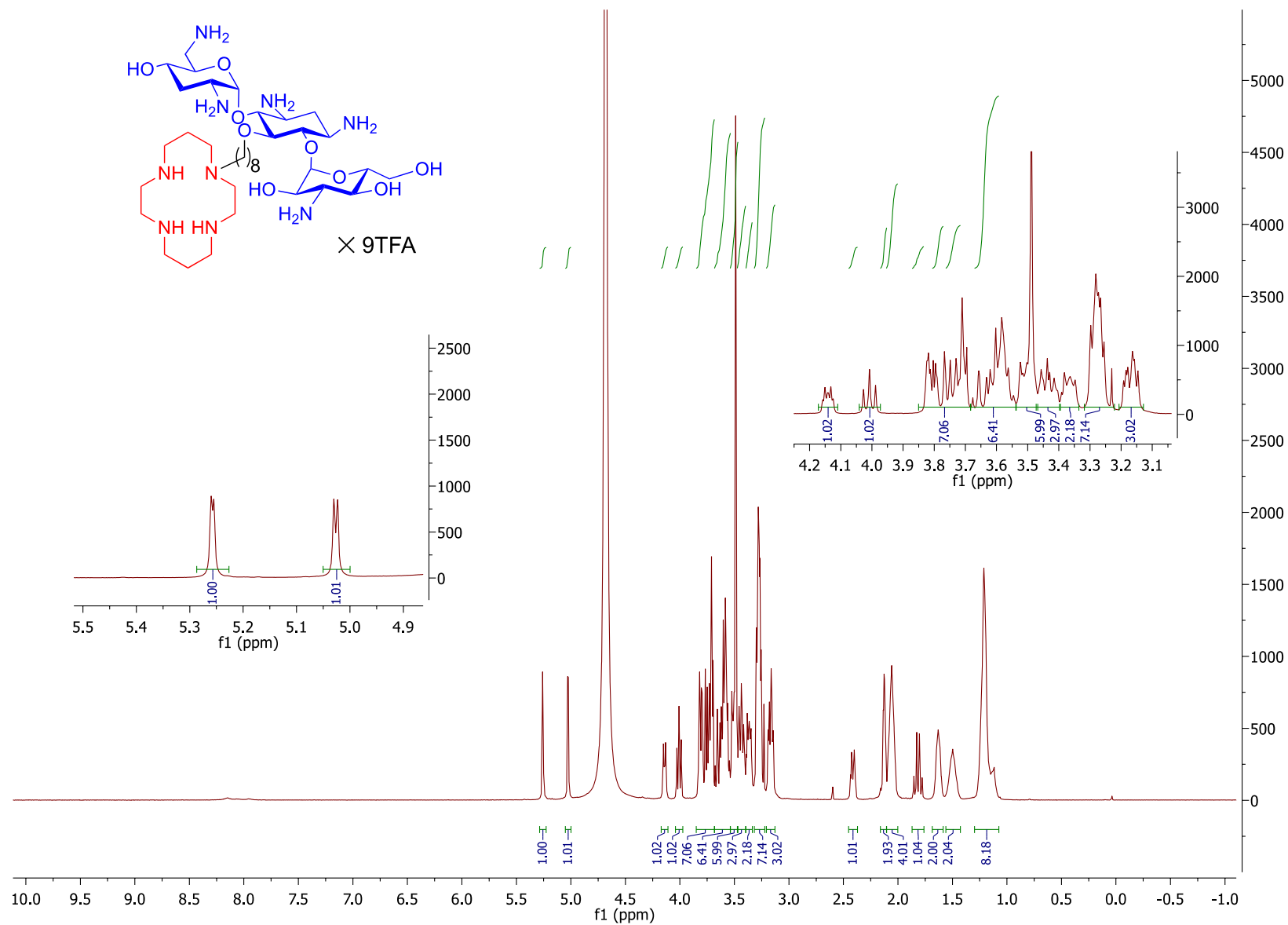
DEPT-135 of Compound **1** in D₂O



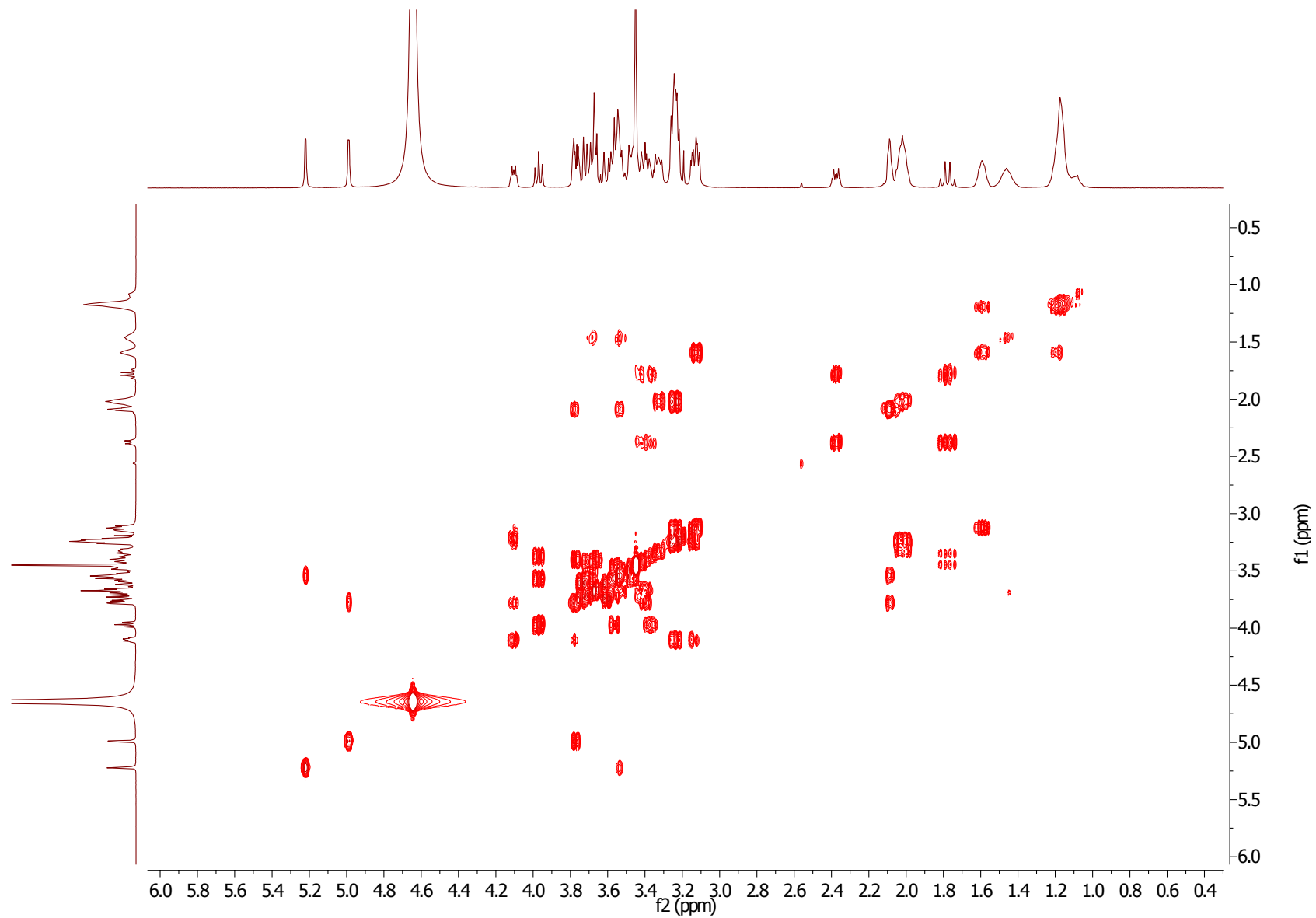
HSQC of Compound **1** in D₂O



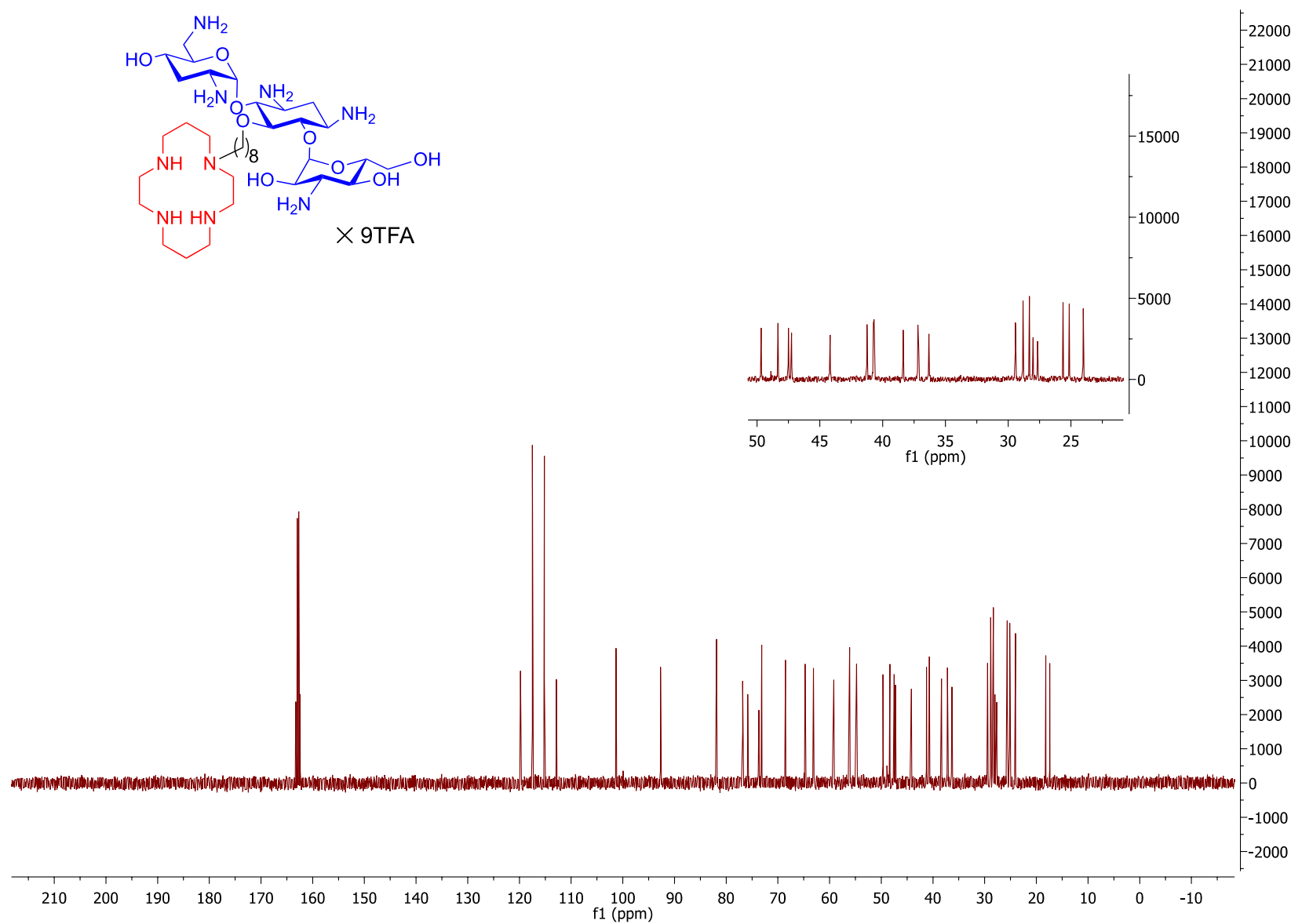
HMBC of Compound **1** in D₂O



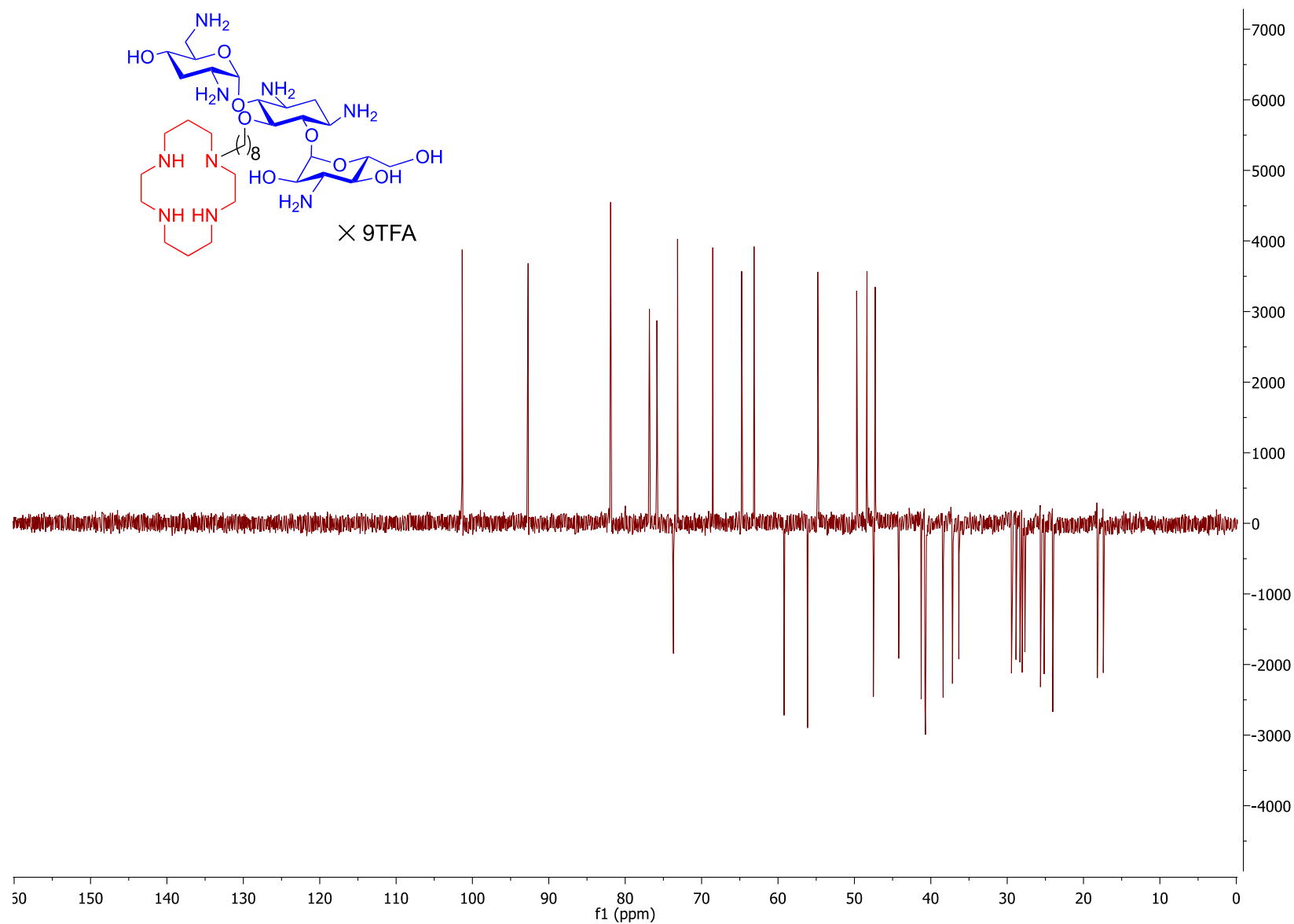
^1H NMR of Compound 2 in D_2O



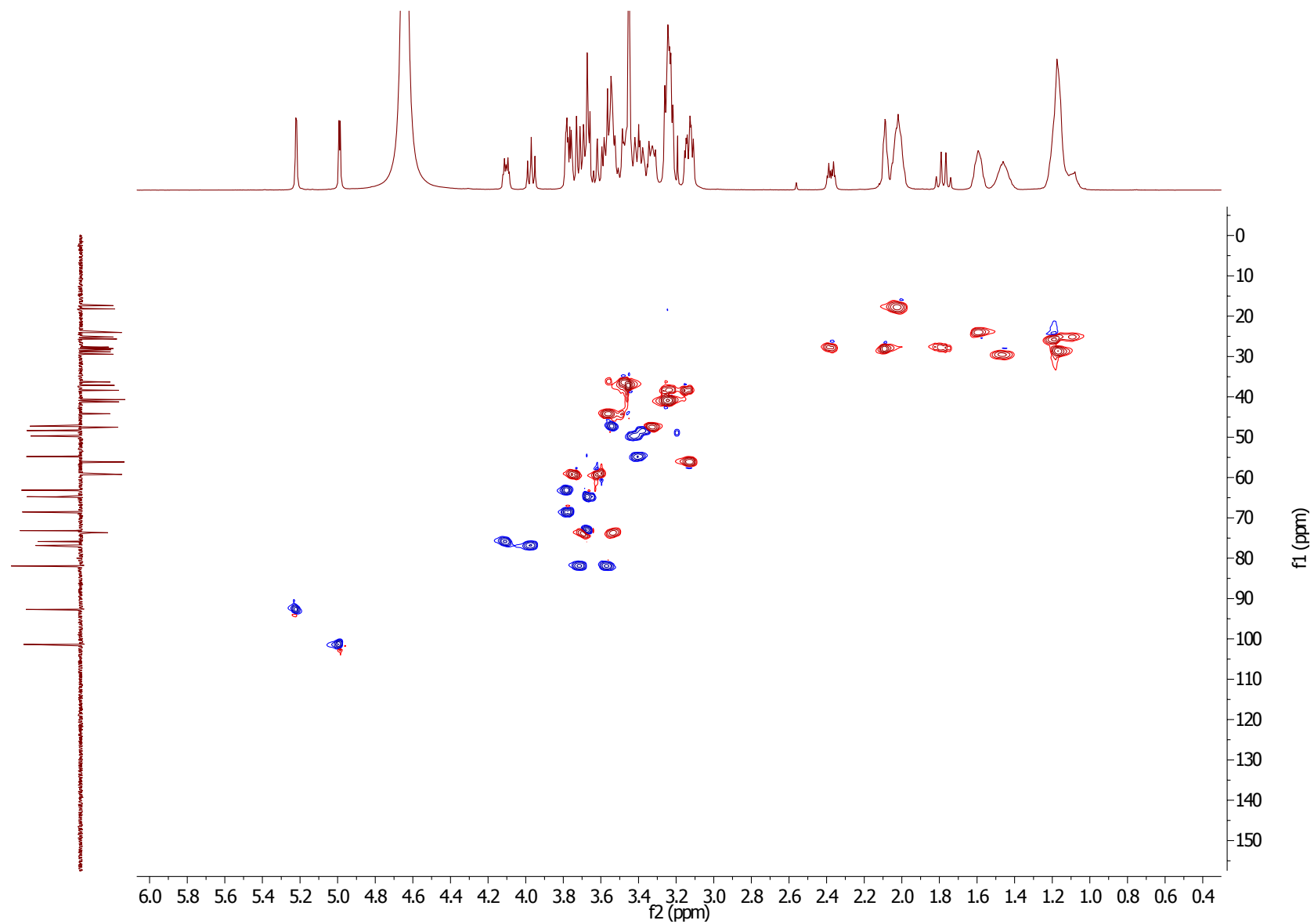
COSY NMR of Compound **2** in D₂O



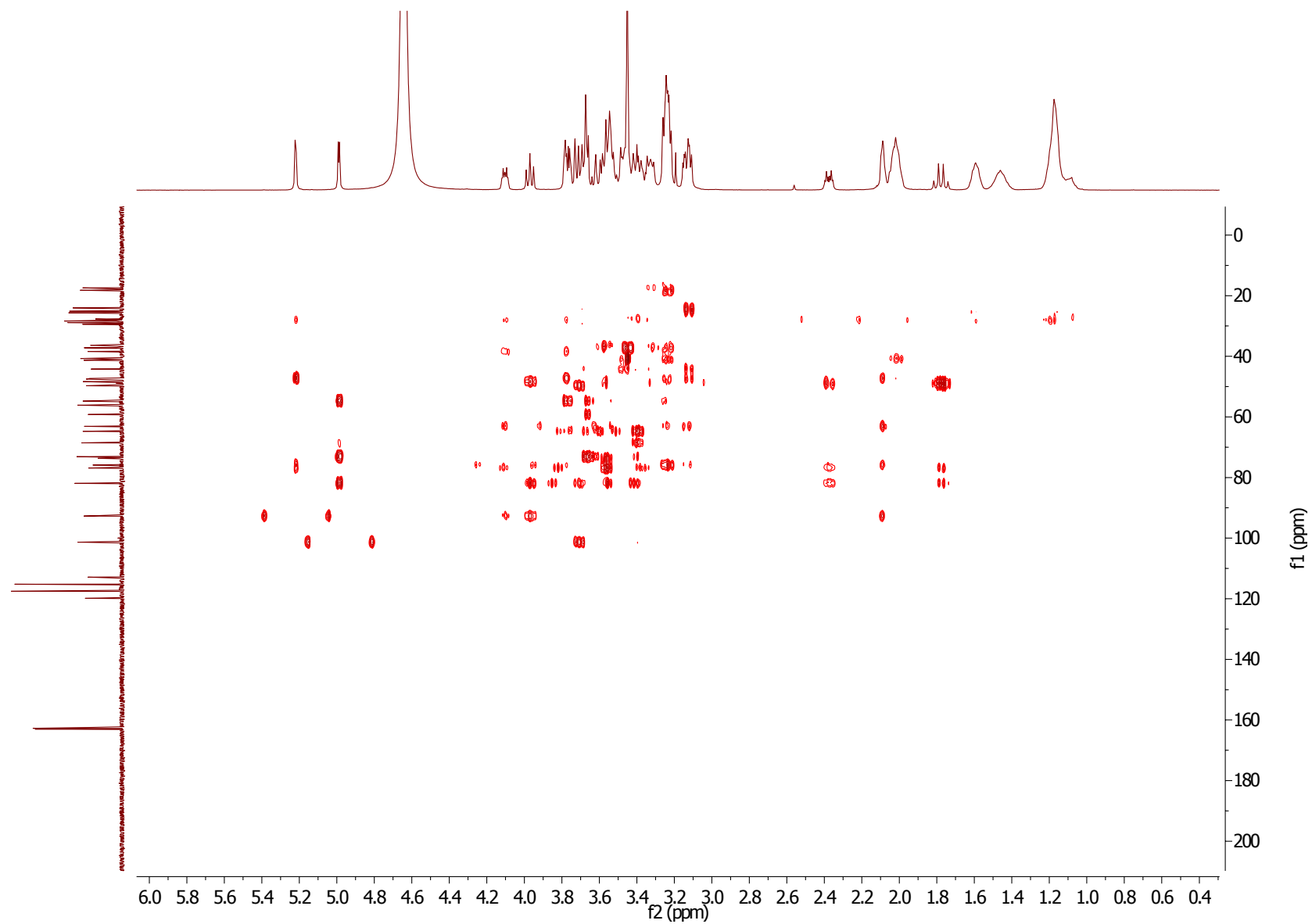
^{13}C NMR of Compound 2 in D_2O



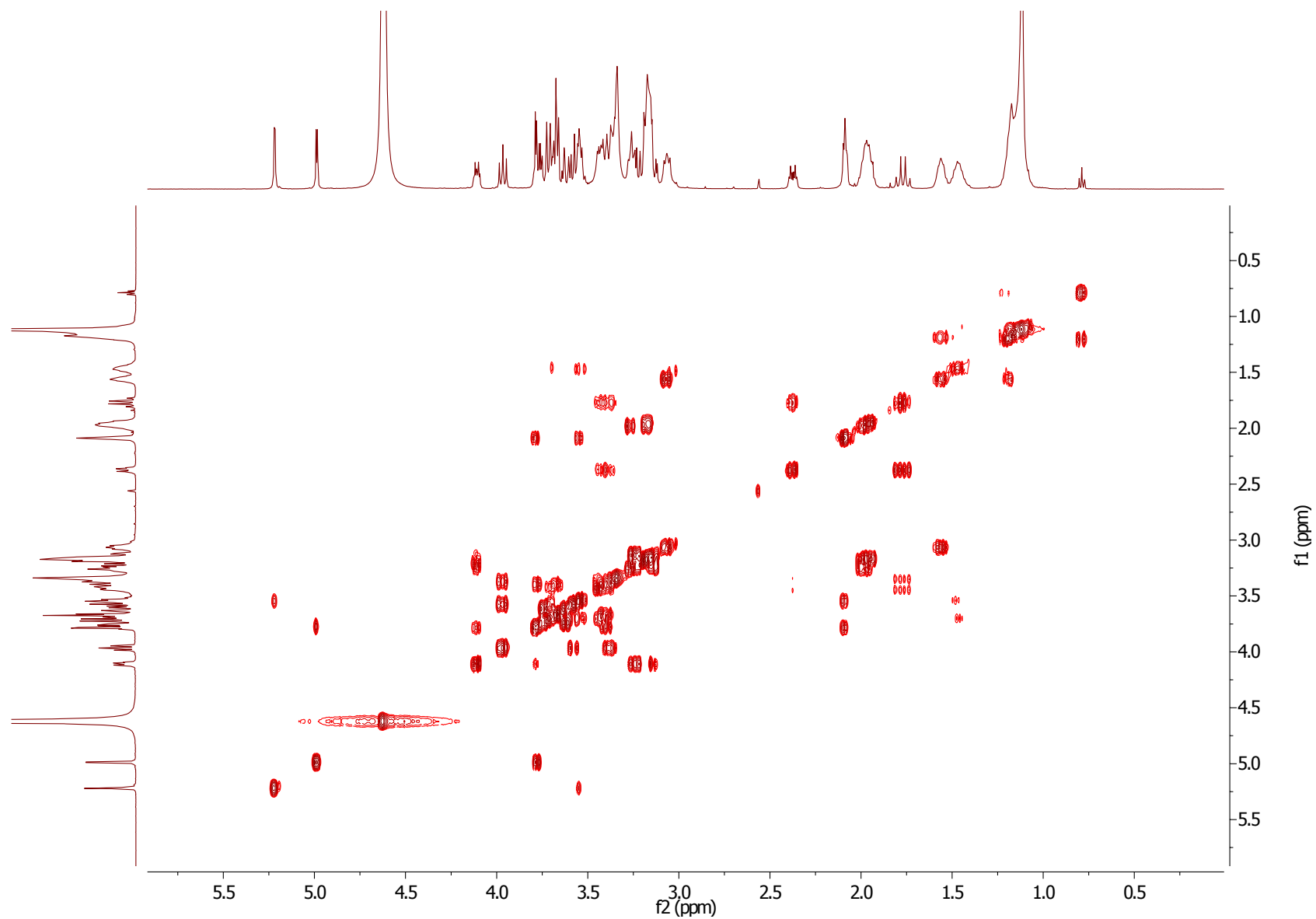
DEPT-135 of Compound **2** in D₂O



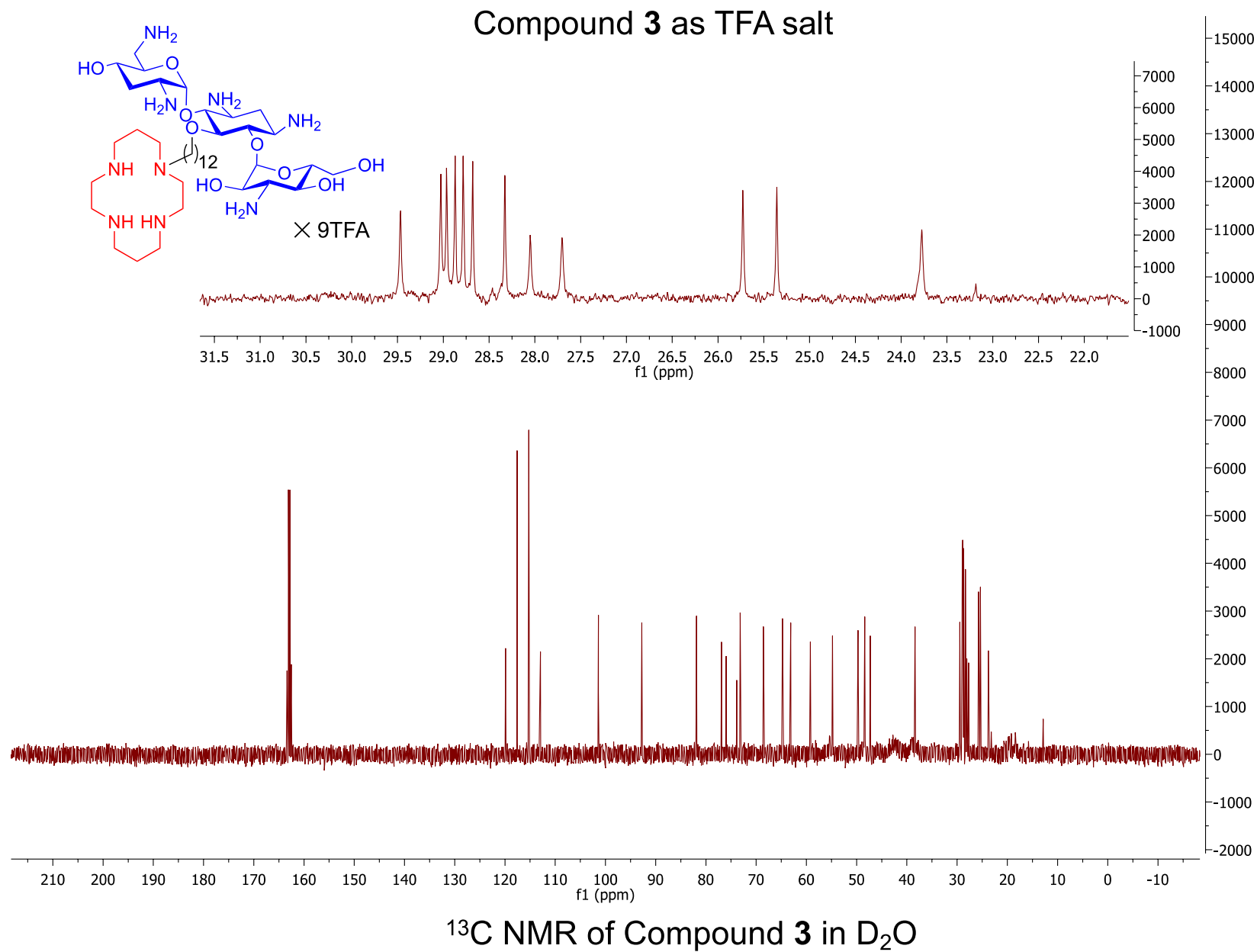
HSQC of Compound **2** in D₂O



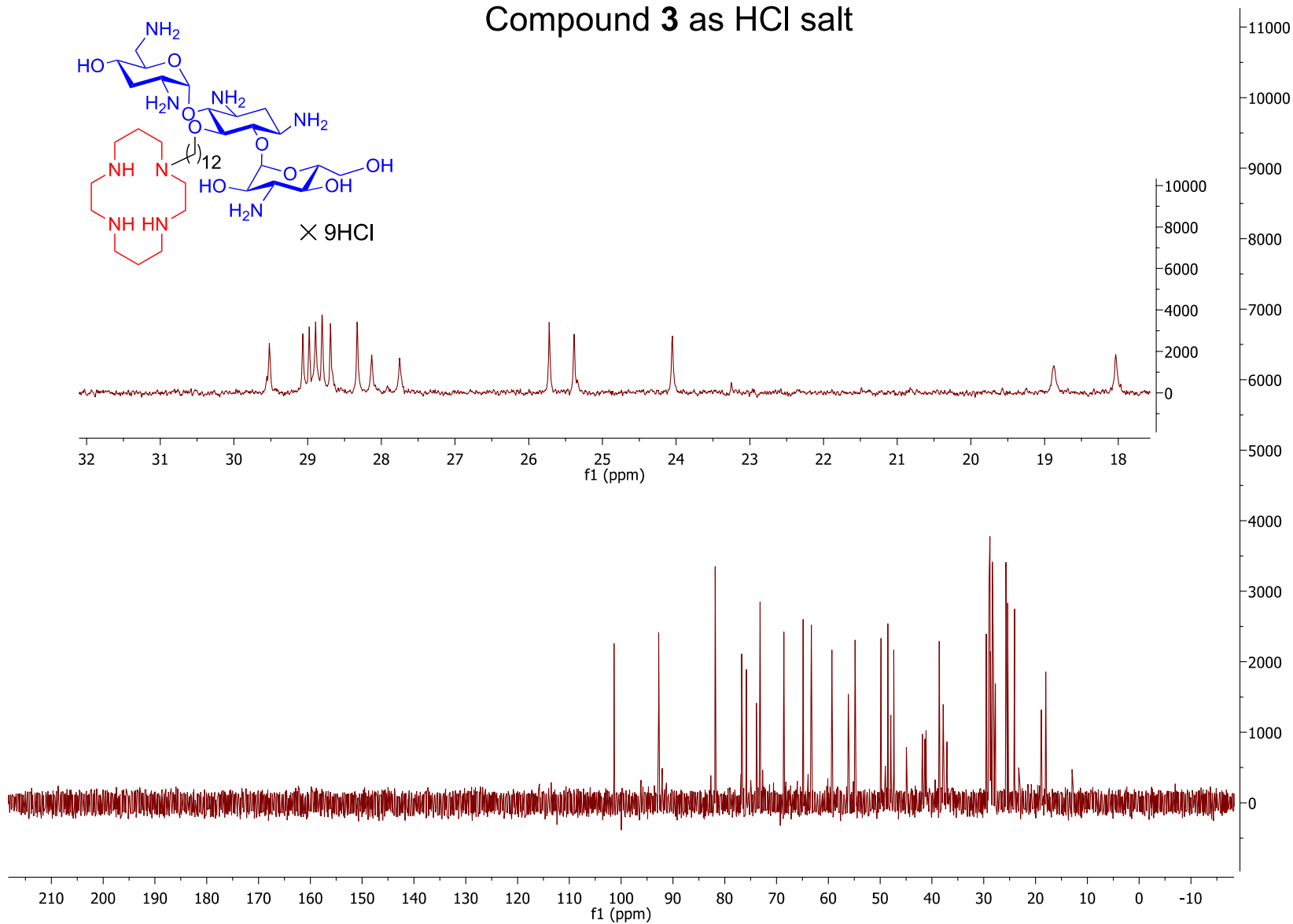
HMBC of Compound **2** in D₂O



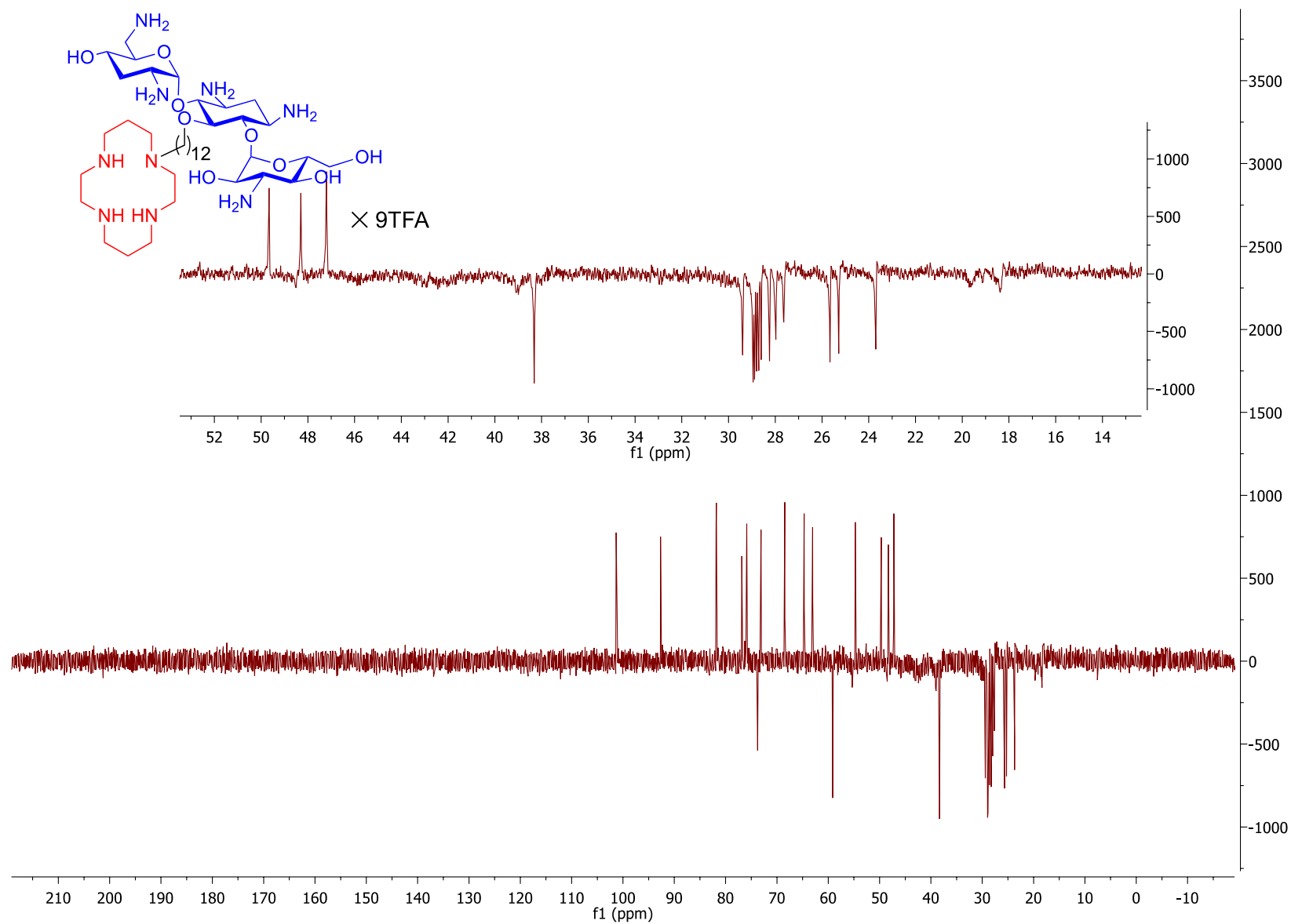
COSY NMR of Compound **3** in D₂O



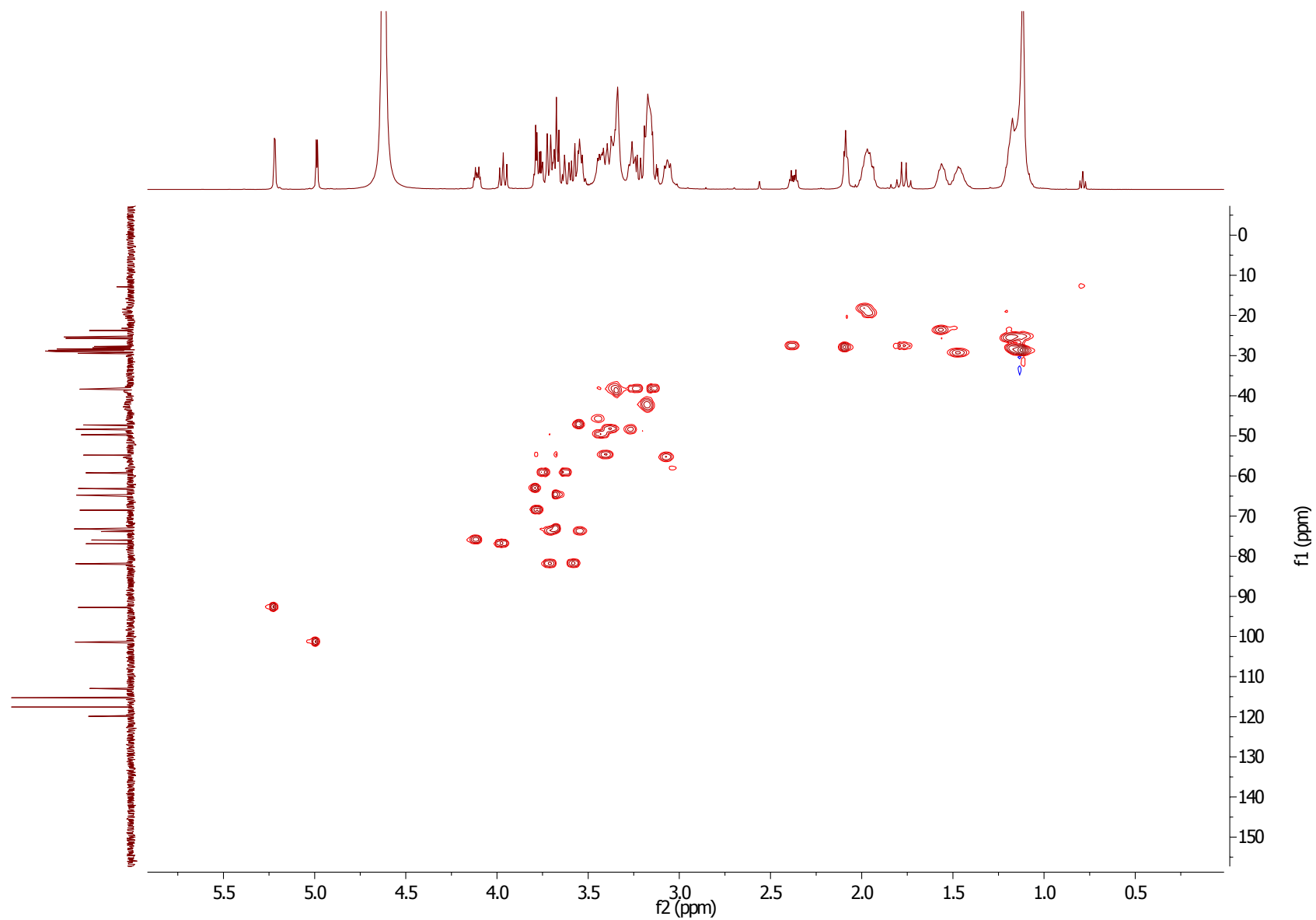
Compound 3 as HCl salt



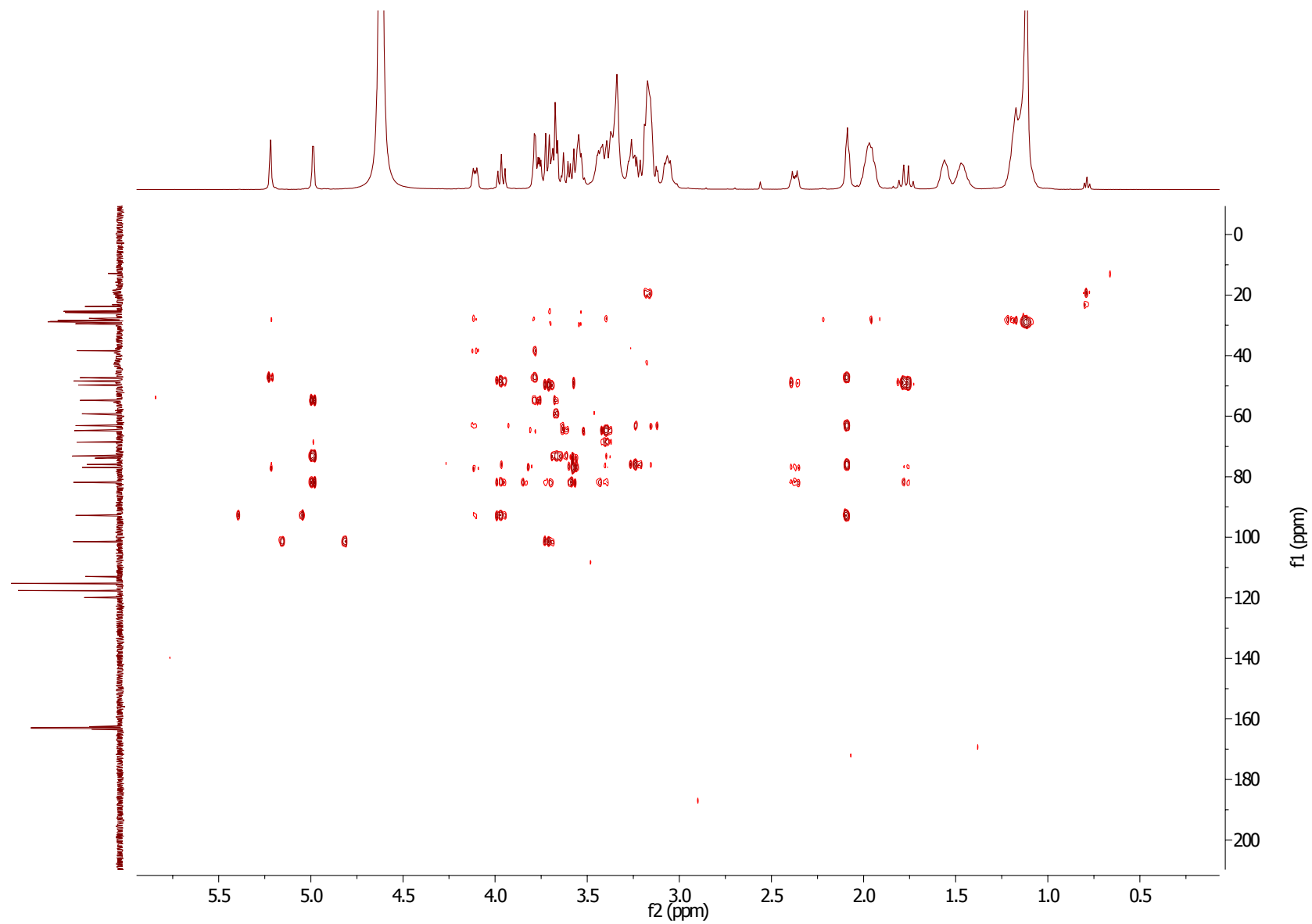
^{13}C NMR of Compound 3 in D_2O



DEPT-135 of Compound **3** in D₂O

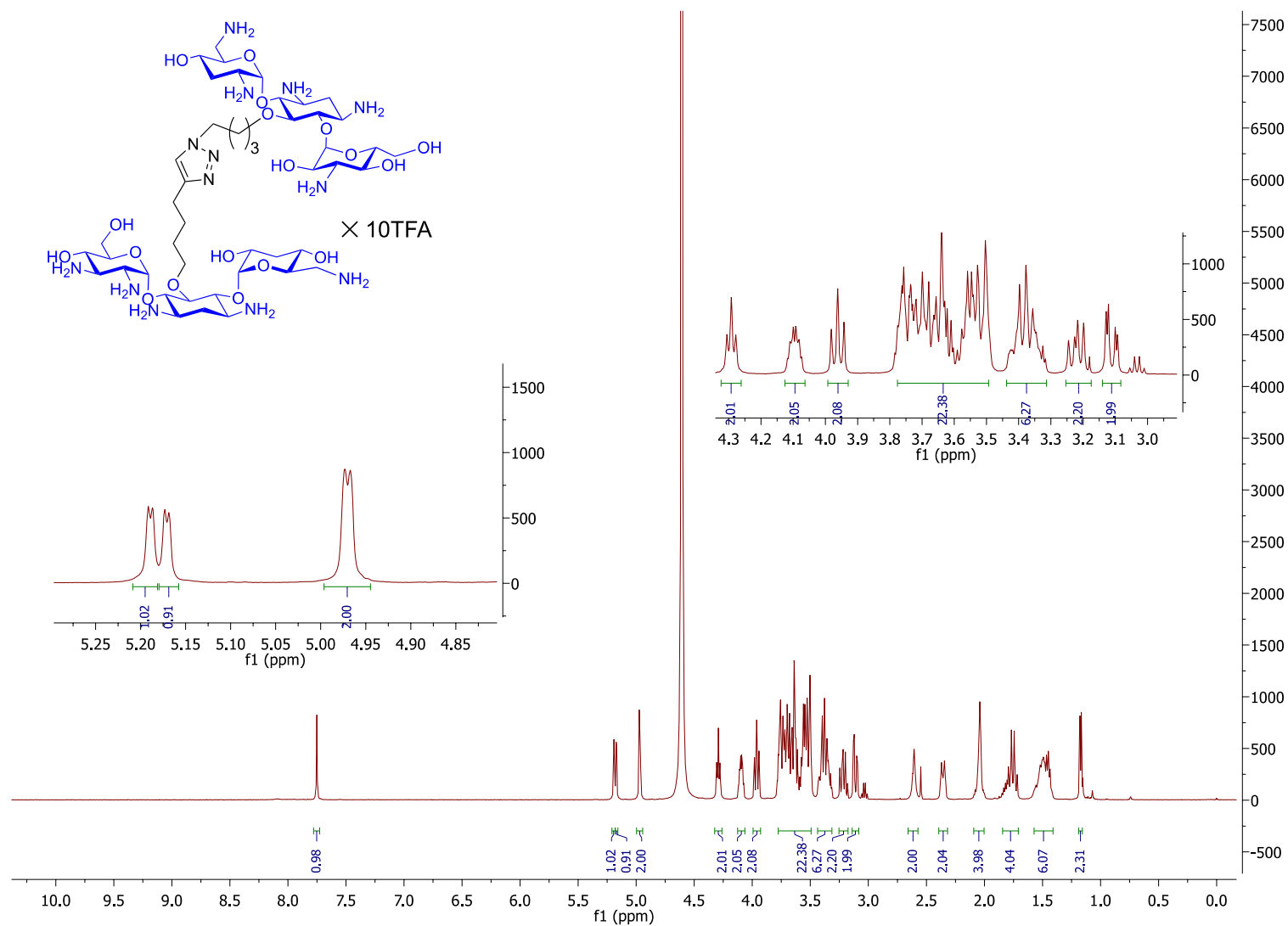


HSQC of Compound **3** in D₂O

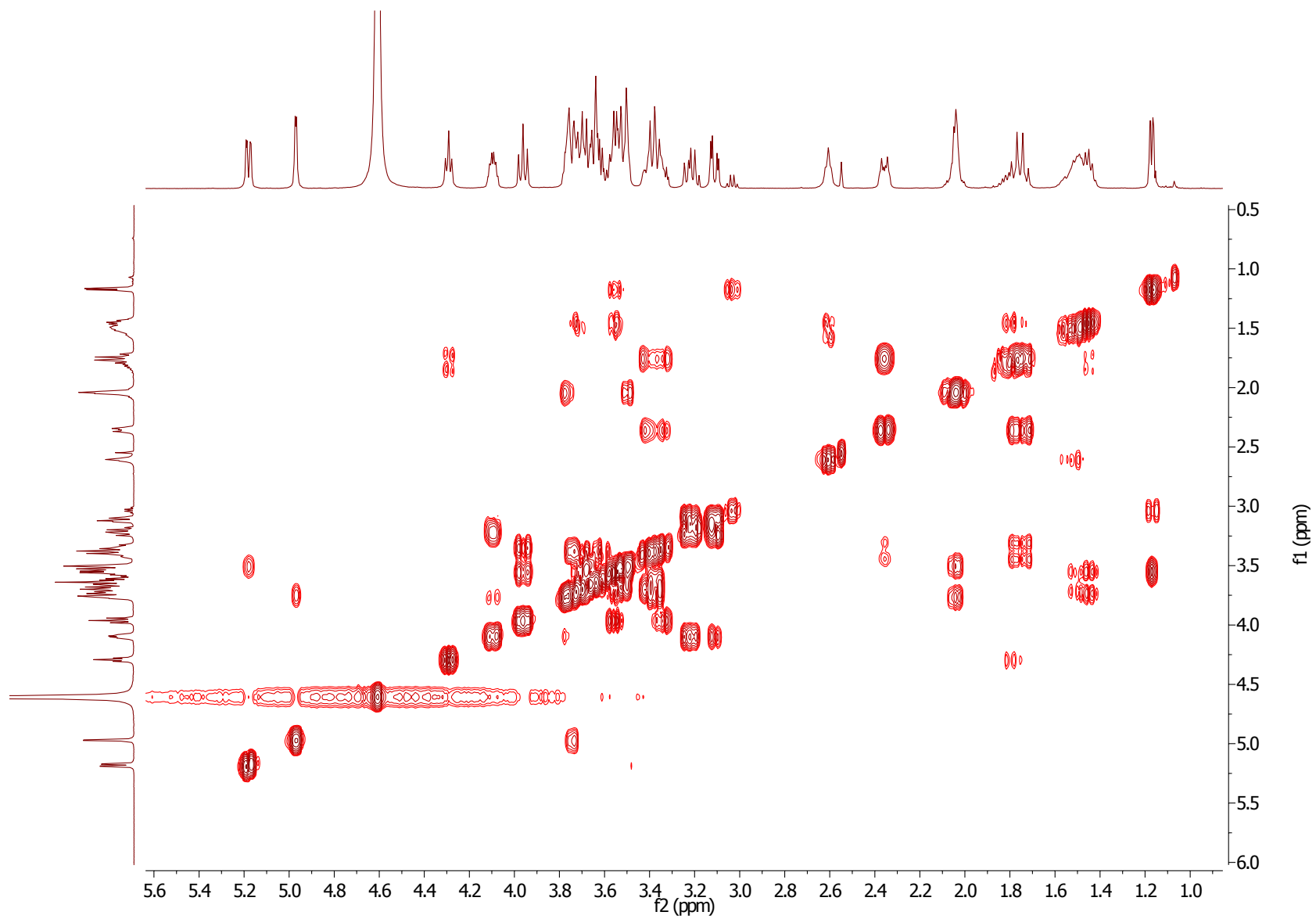


HMBC of Compound **3** in D₂O

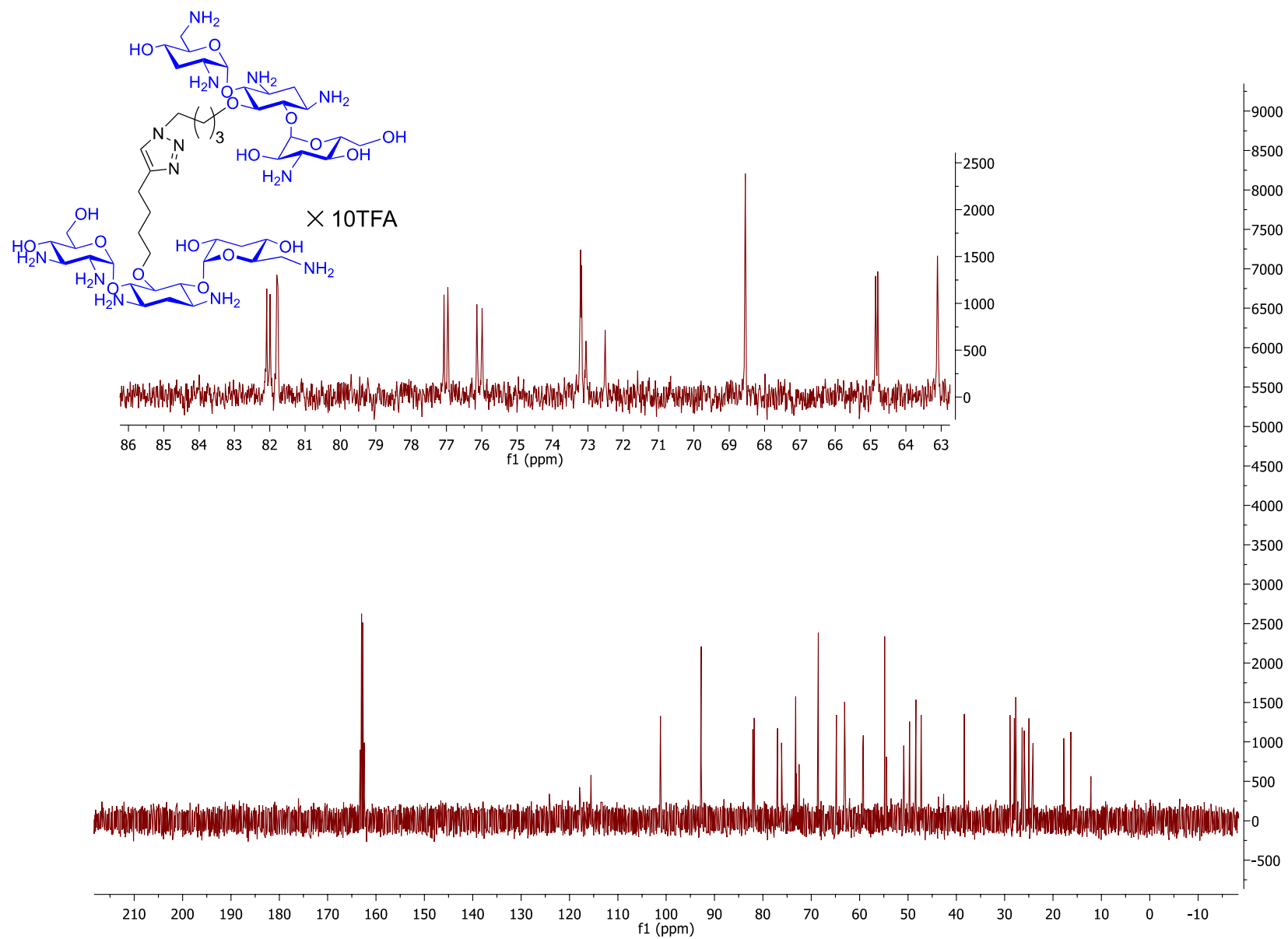
Appendix C



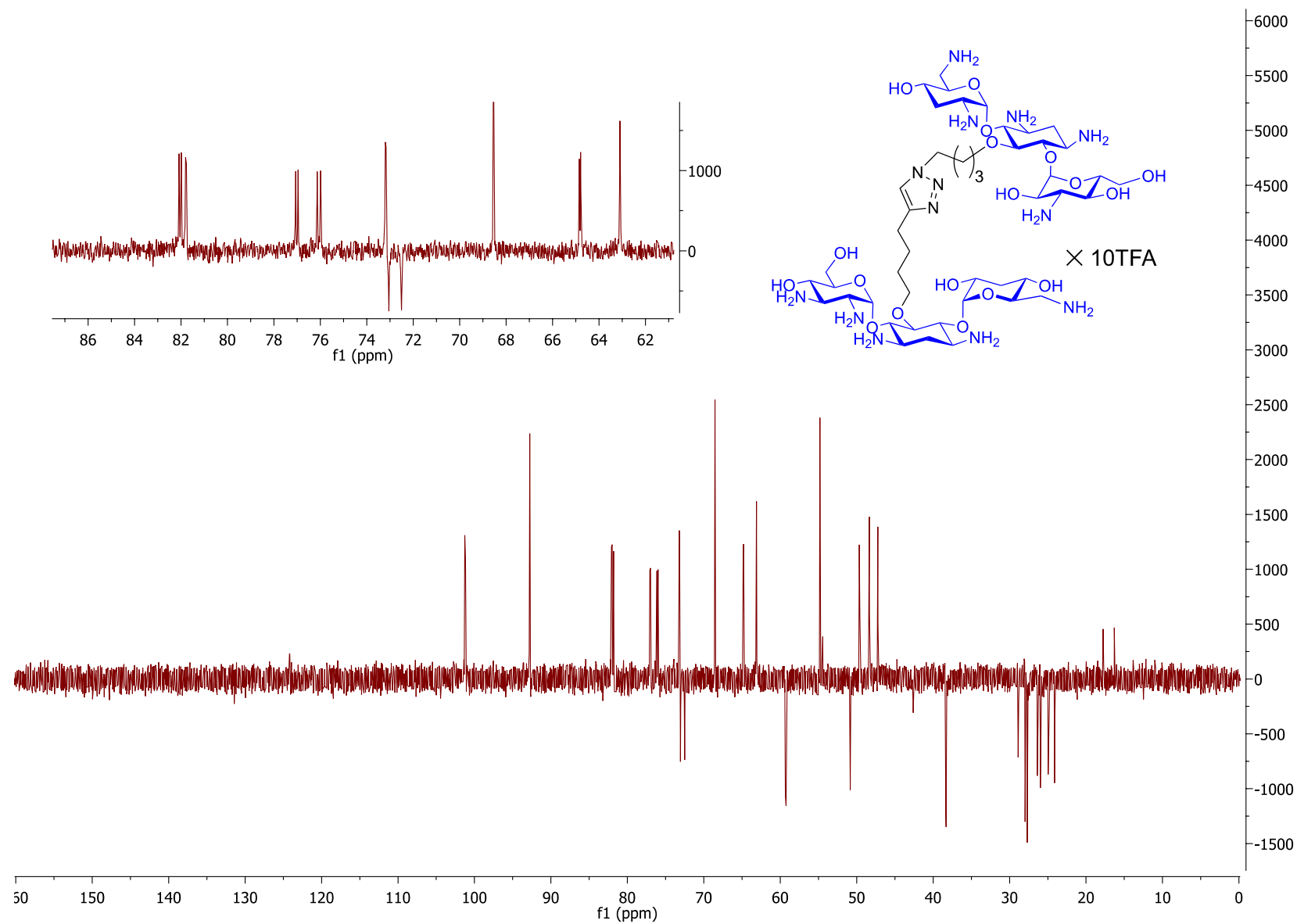
^1H NMR of Compound **1** in D_2O

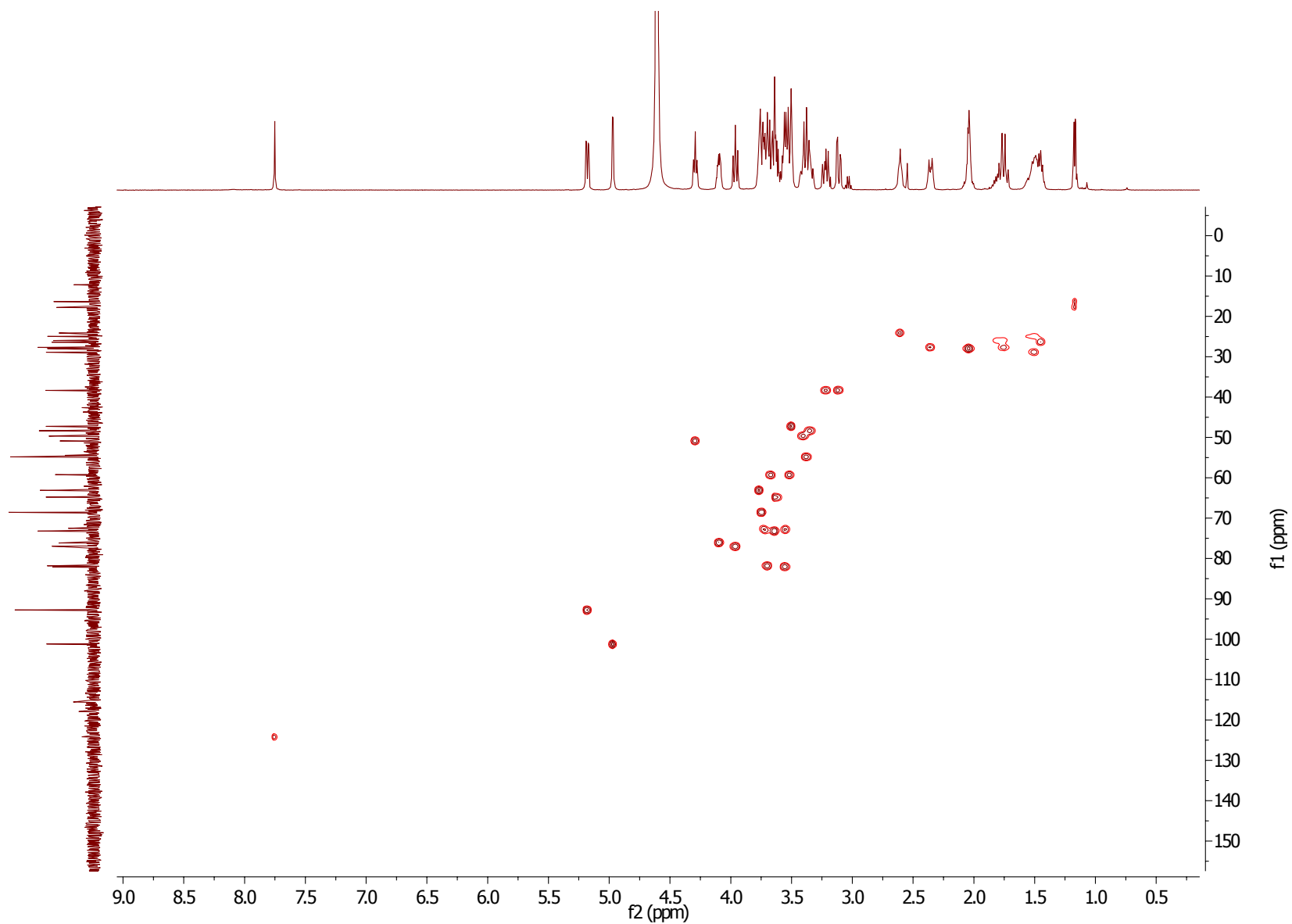


COSY NMR of Compound **1** in D₂O

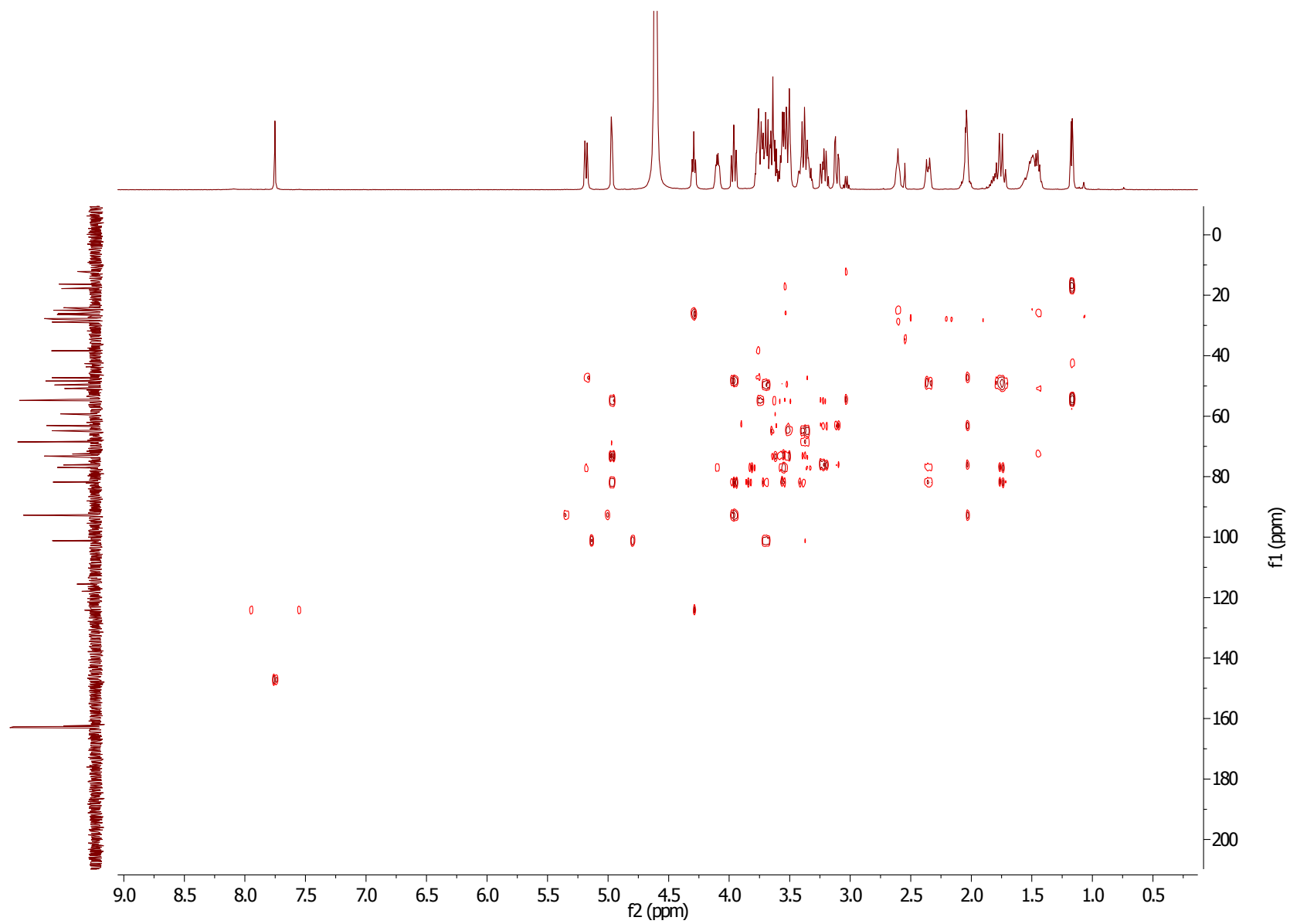


^{13}C NMR of Compound **1** in D_2O

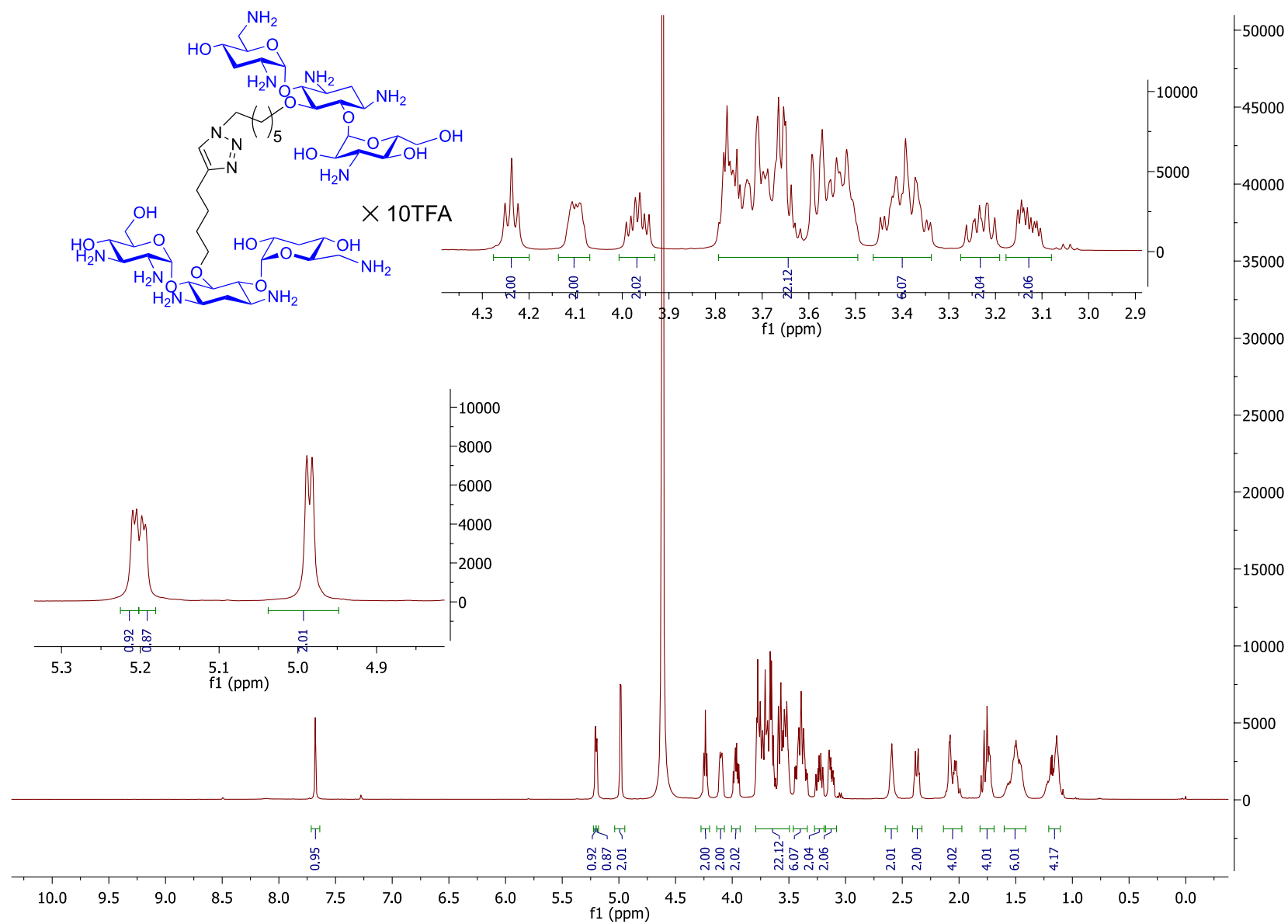




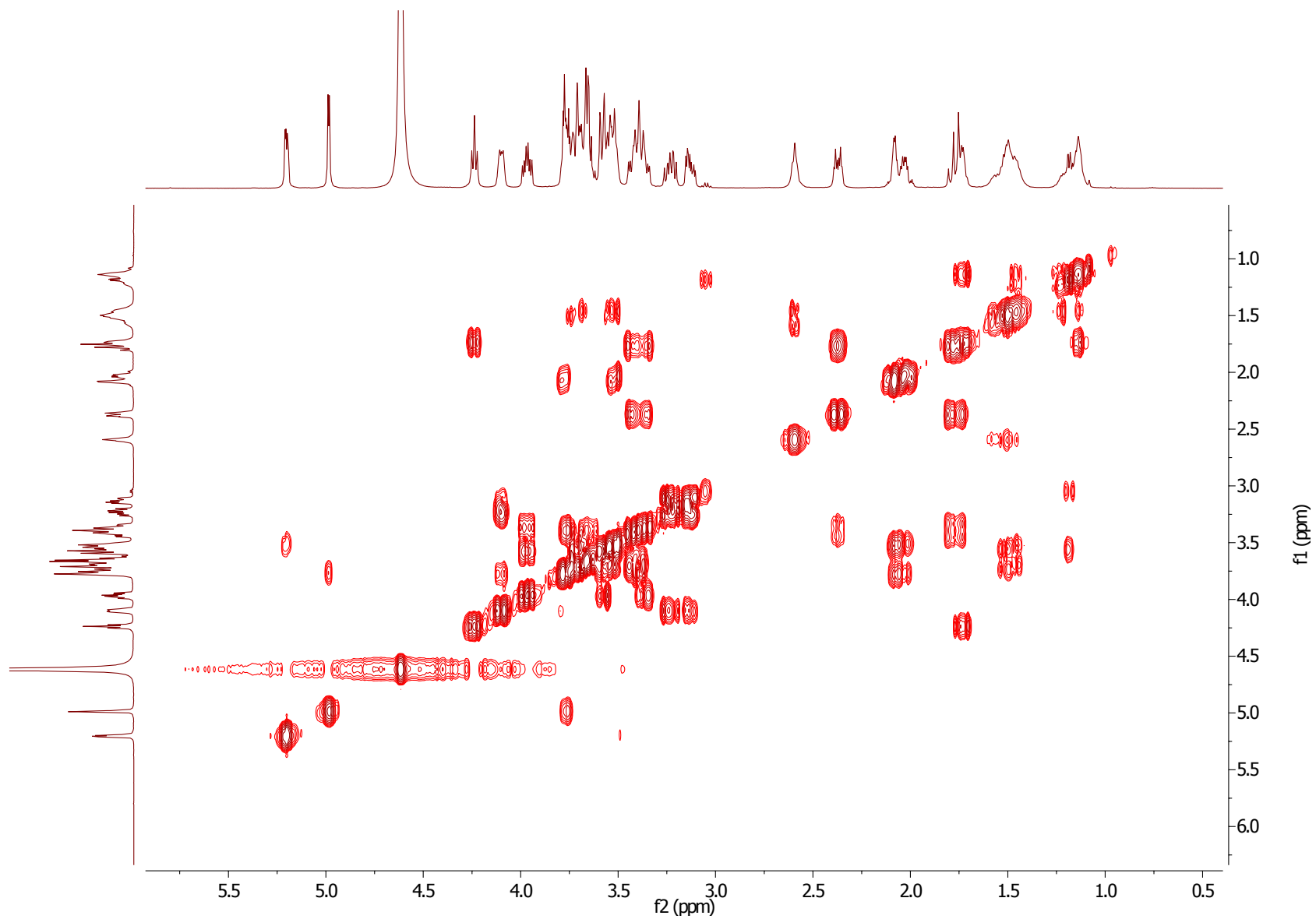
HSQC of Compound **1** in D₂O



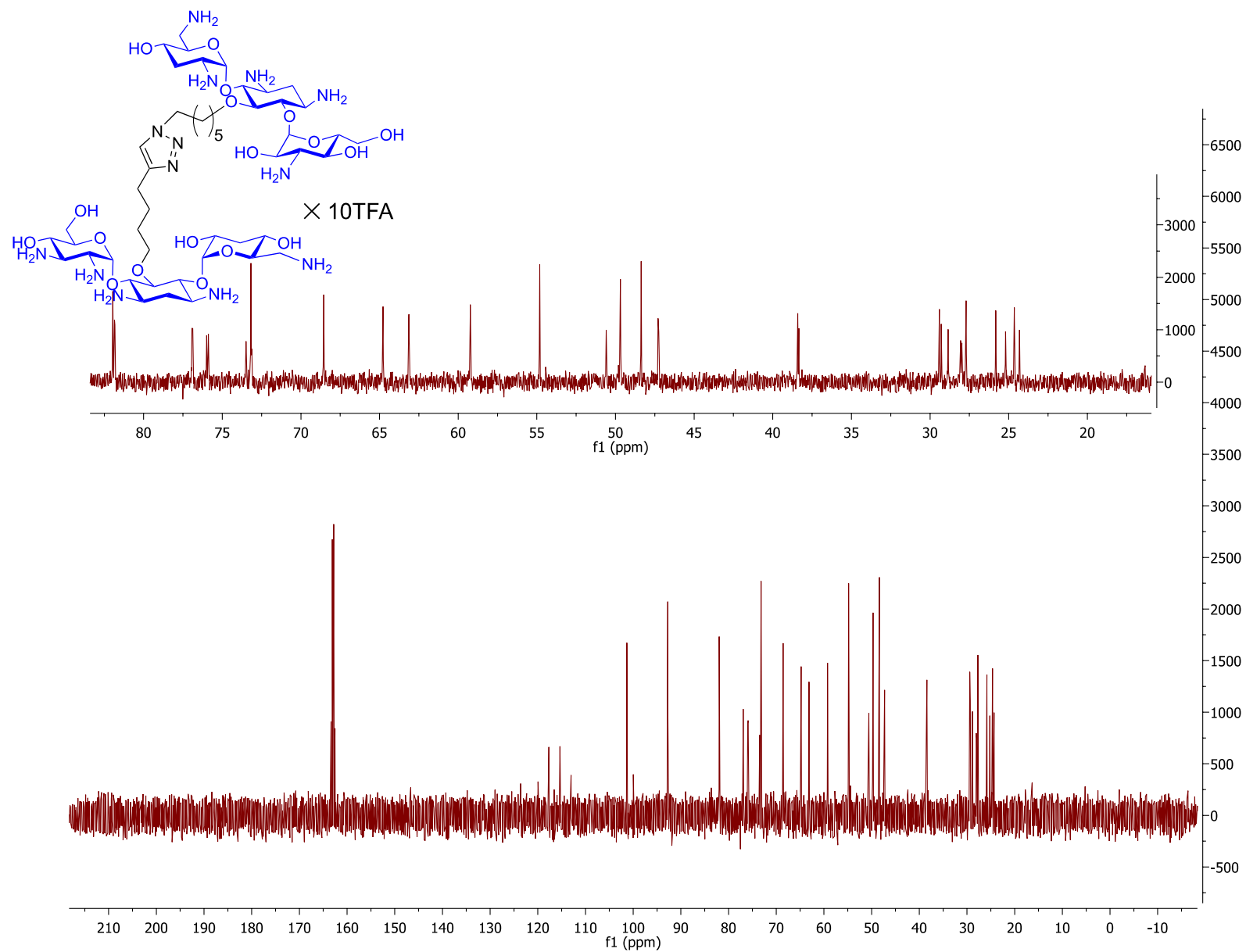
HMBC of Compound **1** in D₂O



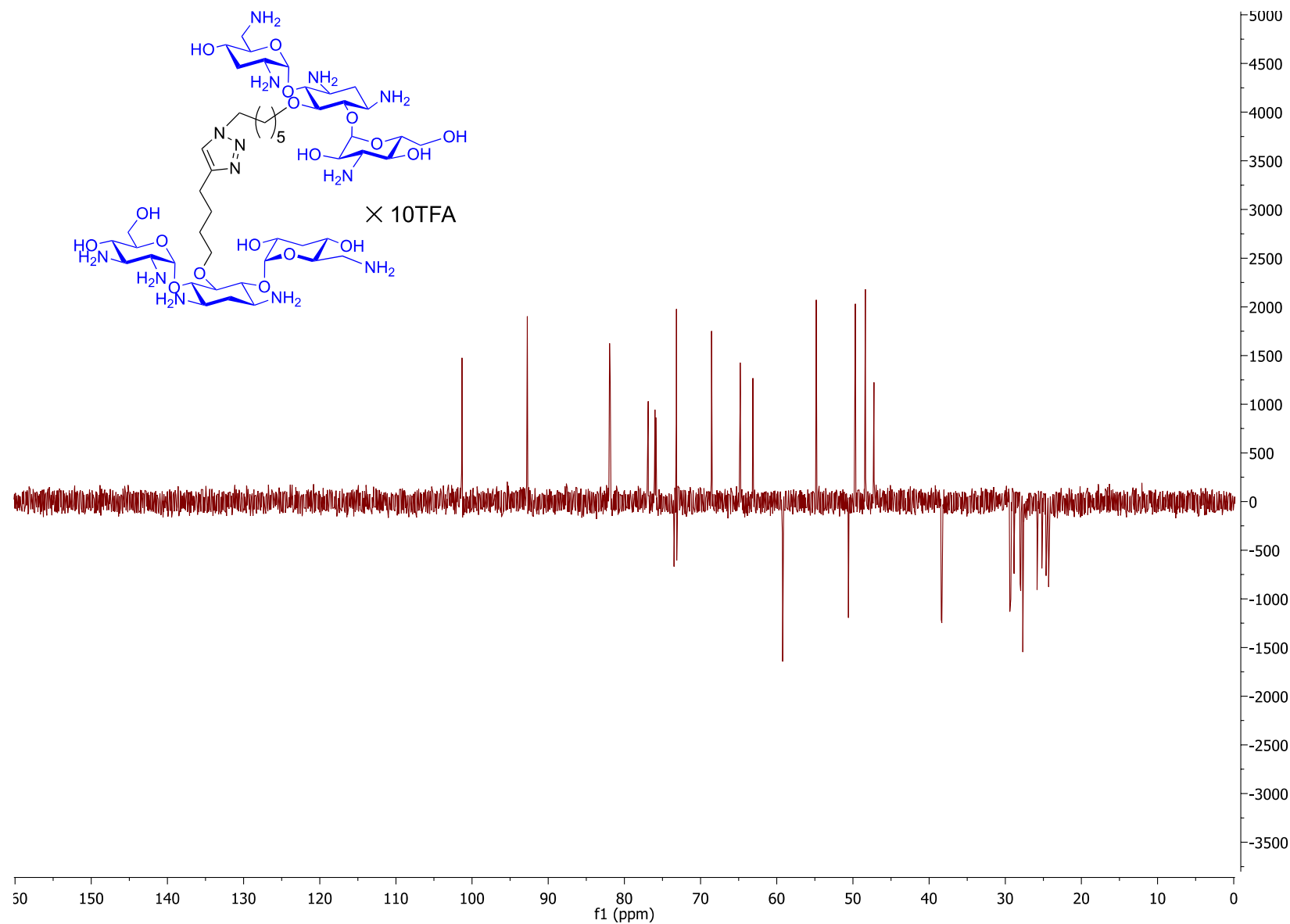
¹H NMR of Compound **2** in D₂O



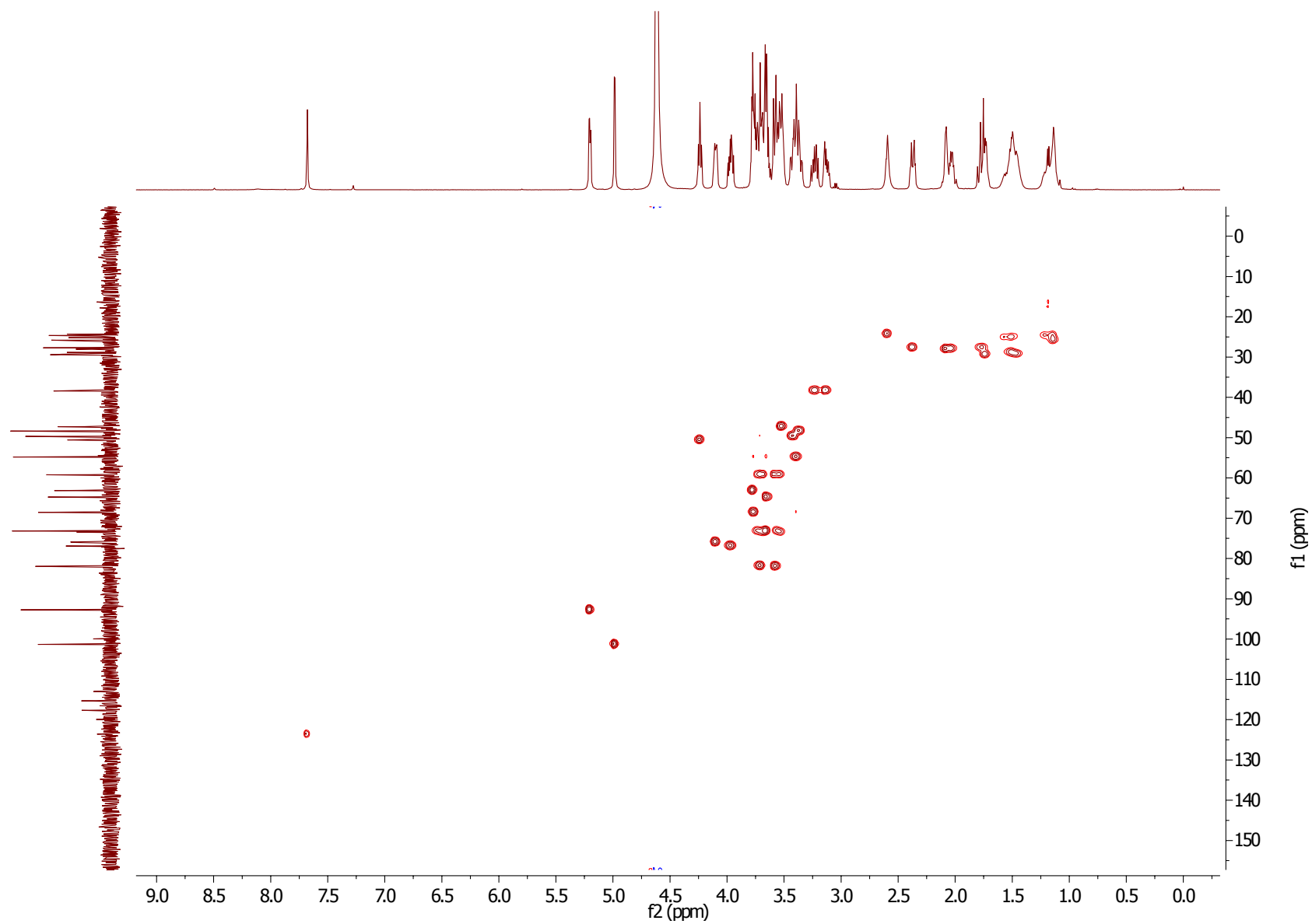
COSY NMR of Compound **2** in D₂O



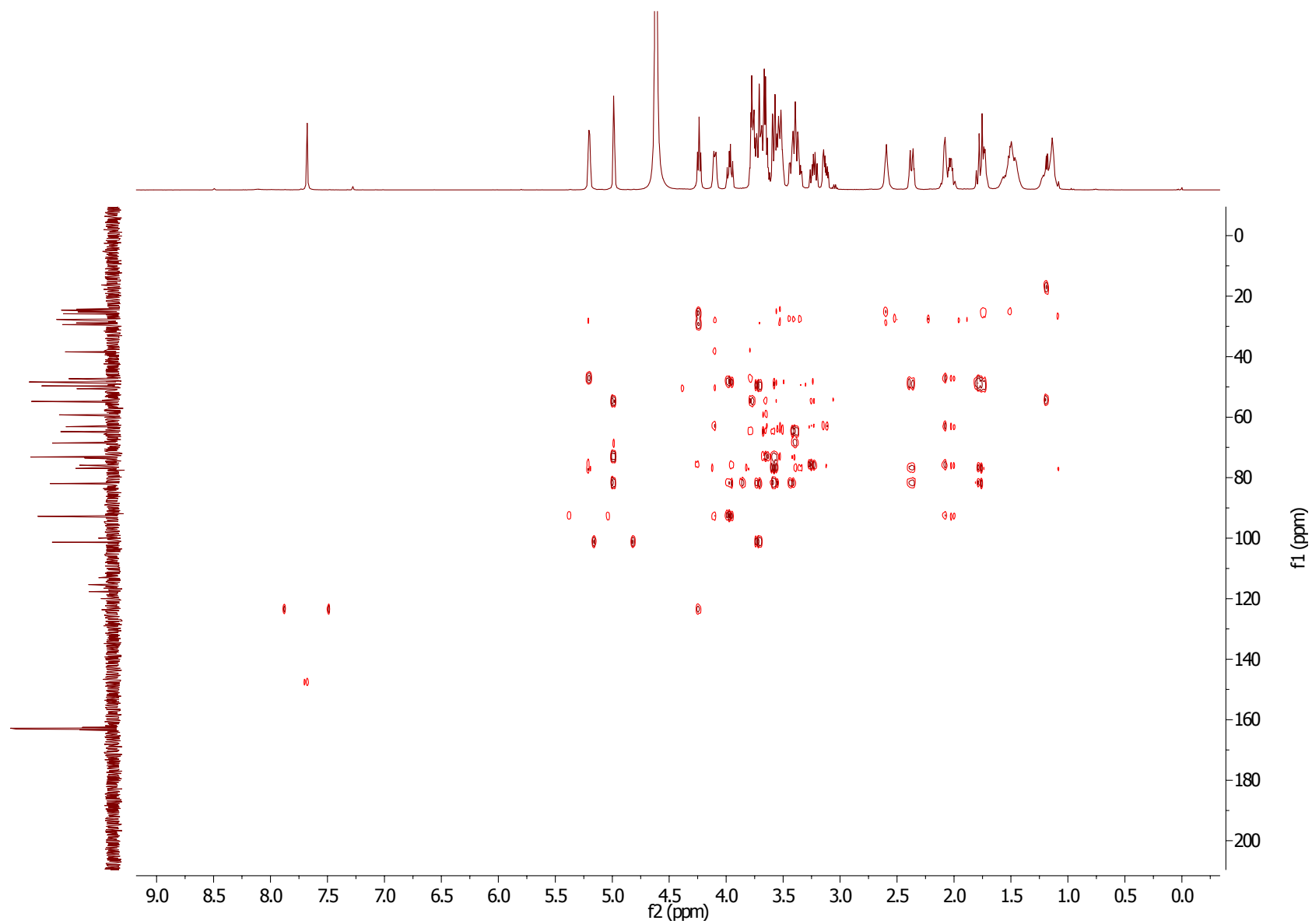
^{13}C NMR of Compound 2 in D_2O



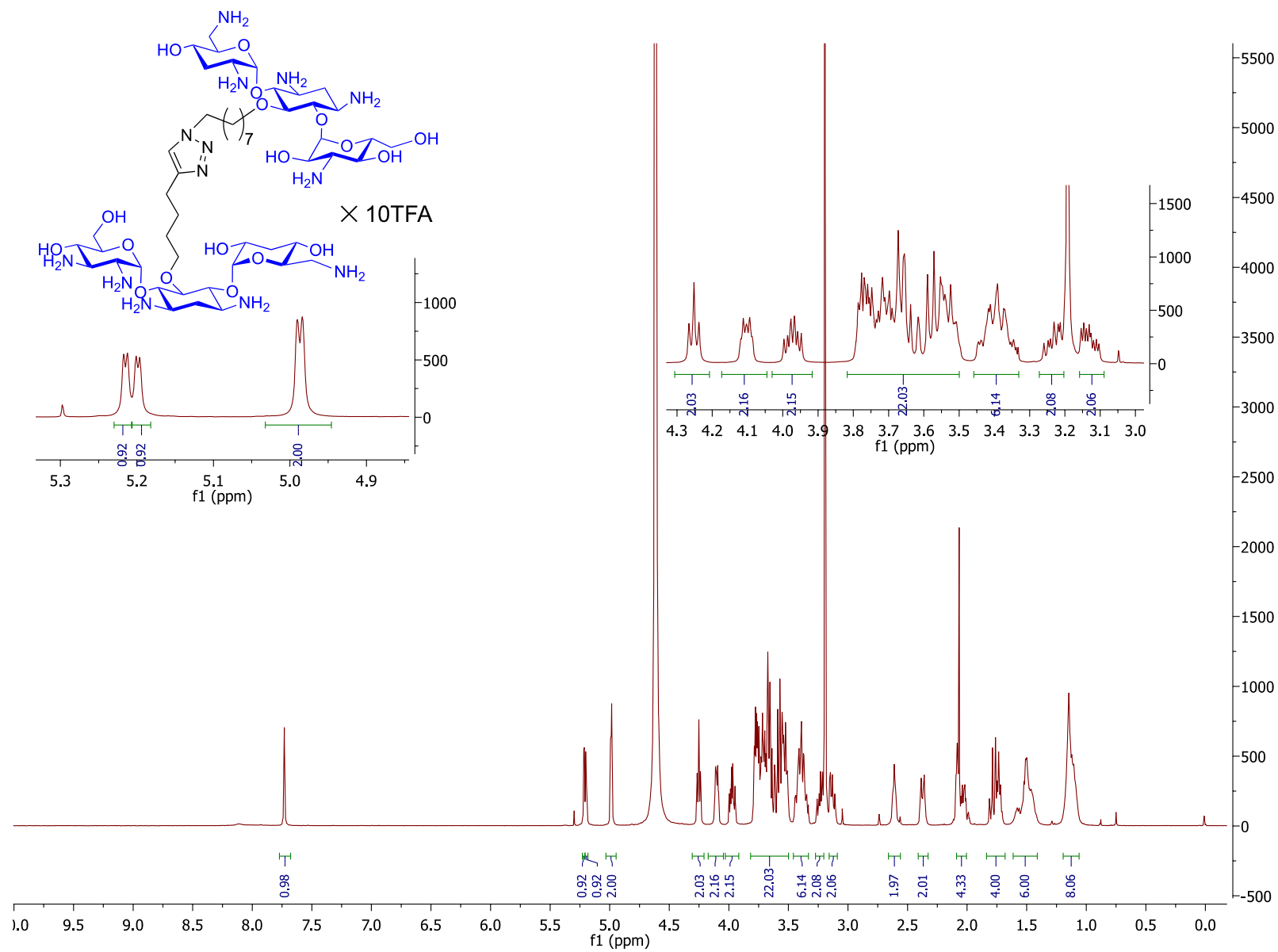
DEPT-135 of Compound **2** in D₂O



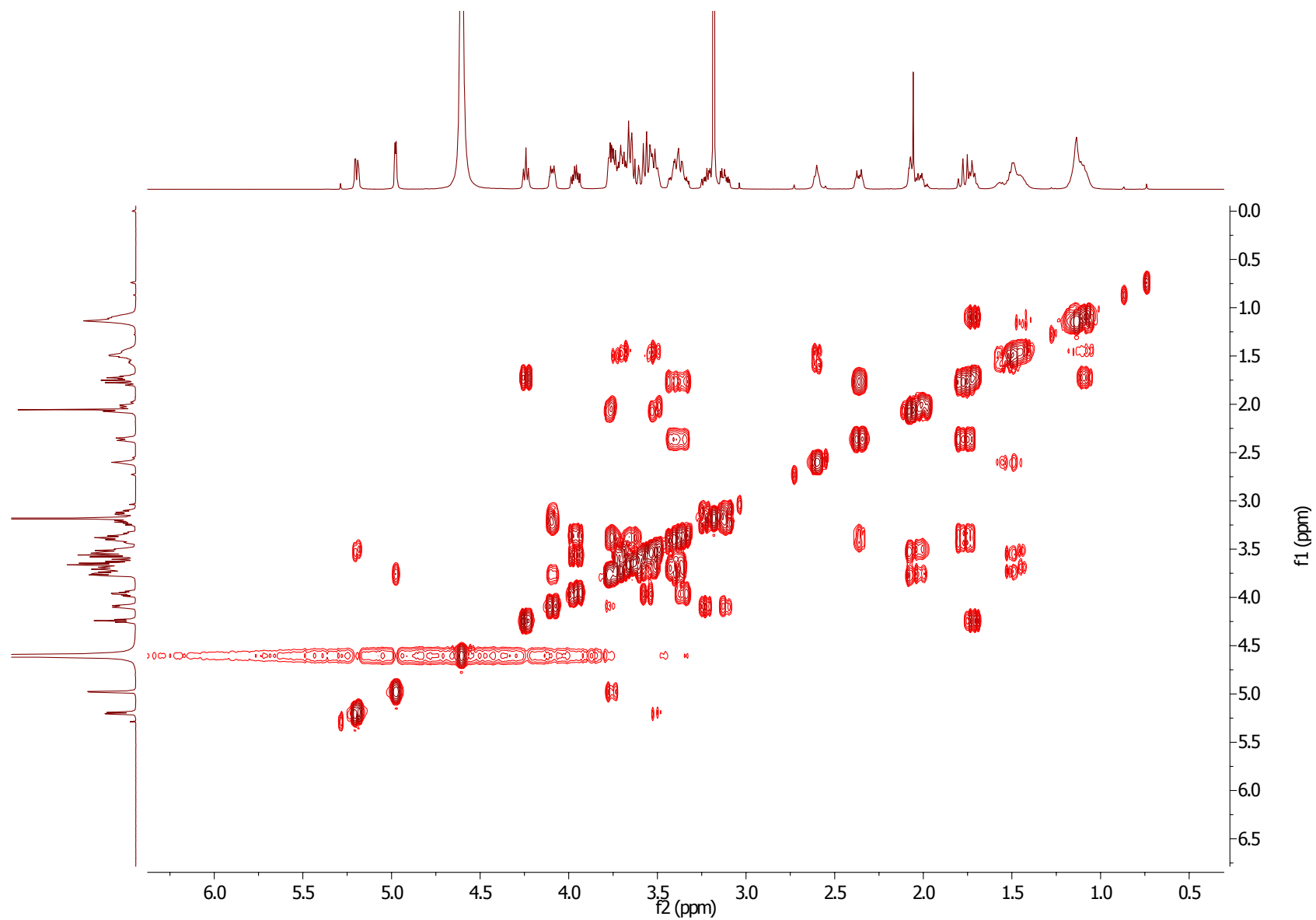
HSQC of Compound **2** in D₂O



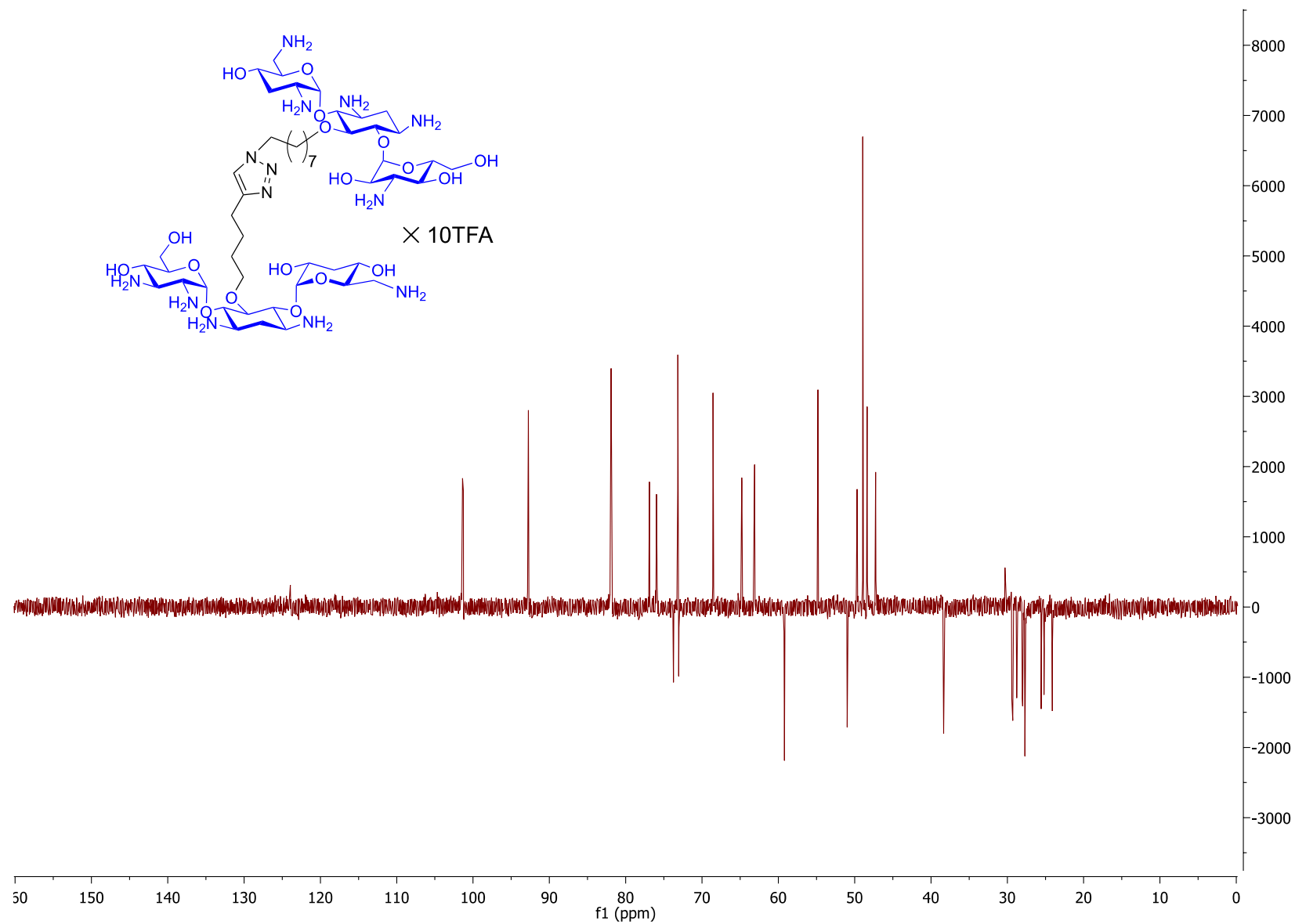
HMBC of Compound **2** in D₂O



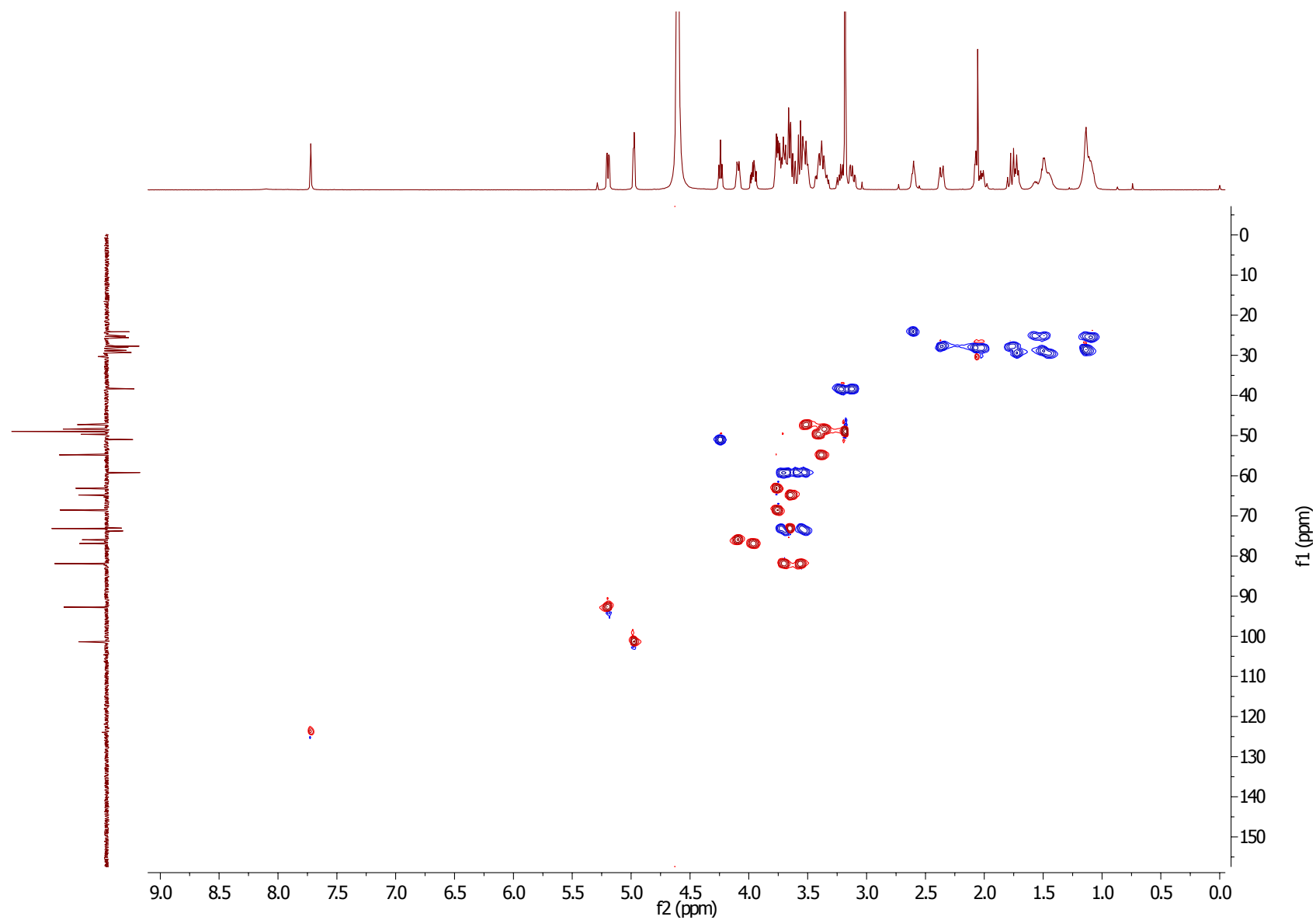
¹H NMR of Compound **3** in D₂O



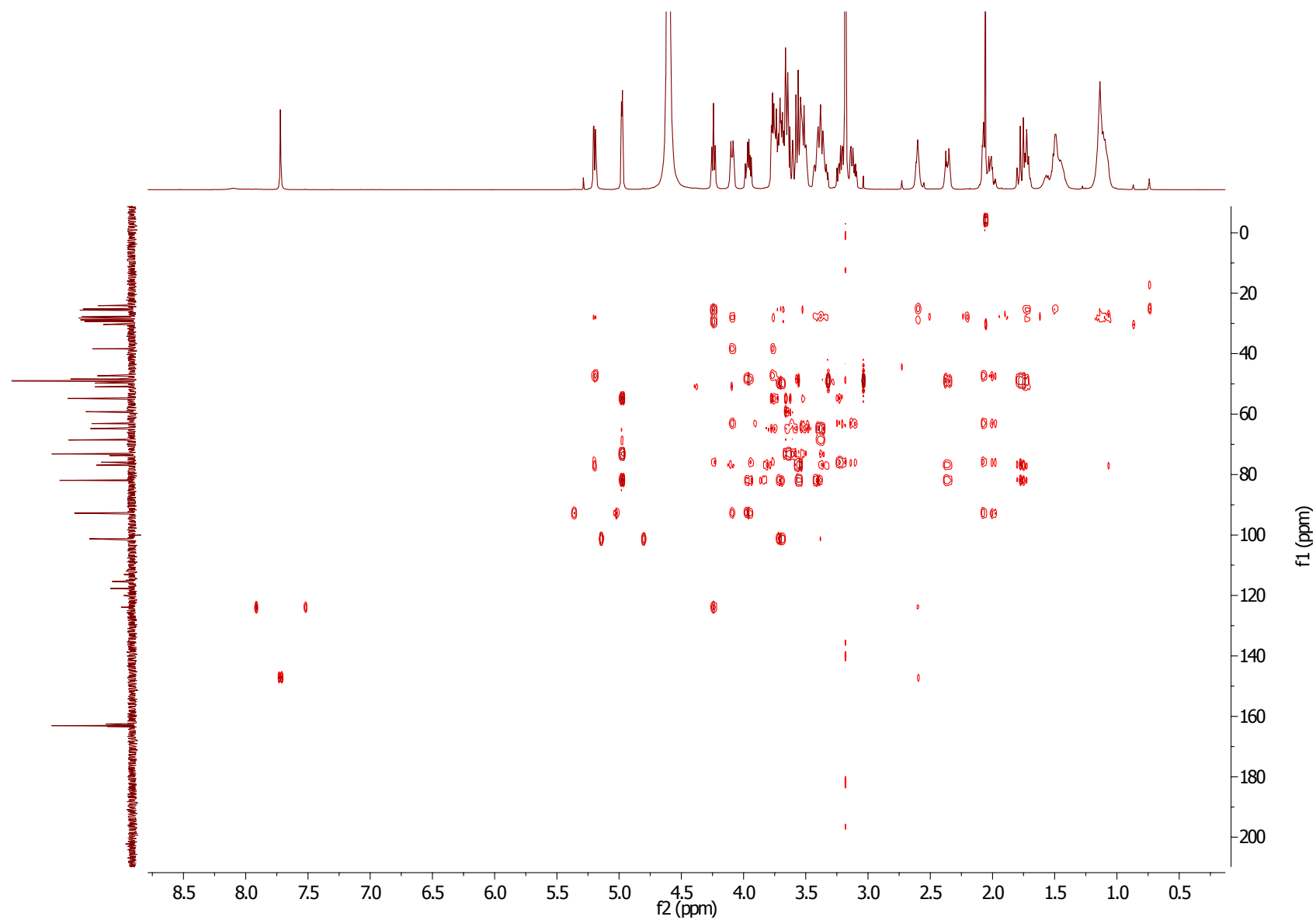
COSY NMR of Compound **3** in D₂O



DEPT-135 of Compound **3** in D₂O



HSQC of Compound **3** in D₂O



HMBC of Compound **3** in D₂O

List of Publications

- (15) **Idowu, T.**; Ammeter, D.; Zhanel, G. G.; Schweizer, F. An Adjunctive Strategy for the Use of Cefiderocol under Normal Physiologic (Iron-Rich) and Infectious (Iron-Depleted) Conditions. [Manuscript in Preparation].
- (14) **Idowu, T.**; Zhanel, G. G.; Schweizer, F. Efficacy of Ceftolozane/Tobramycin Homodimer Combination Versus Ceftolozane/Tazobactam Combination against Multidrug-Resistant *Pseudomonas aeruginosa*. [Manuscript Submitted].
- (13) **Idowu, T.**; Ammeter, D., Rossong, R.; Zhanel, G. G.; Schweizer, F. Homodimeric Tobramycin Adjuvants Repurposes Novobiocin as an Effective Antibacterial Agents against Gram-negative Bacteria. *J. Med. Chem.* **2019** [Revision submitted].
- (12) **Idowu, T.**; Ammeter, D.; Arthur, G.; Zhanel, G. G.; Schweizer, F. Potentiation of β -Lactam Antibiotics and β -Lactam/ β -Lactamase Inhibitor Combinations against Multidrug and Extensively Drug-Resistant *Pseudomonas aeruginosa* using Non-Ribosomal Tobramycin–Cyclam Conjugates. *J. Antimicrob. Chemother.* **2019**. <https://doi.org/10.1093/jac/dkz228>
- (11) **Idowu, T.**; Arthur, G.; Zhanel, G. G.; Schweizer, F. Heterodimeric Rifampicin–Tobramycin Conjugates Break Intrinsic Resistance of *Pseudomonas aeruginosa* to Doxycycline and Chloramphenicol *in Vitro* and in a *Galleria mellonella in Vivo* Model. *Eur. J. Med. Chem.* **2019**, *174*, 16–32. <https://doi.org/10.1016/j.ejmech.2019.04.034>
- (10) Yang, X.; Ammeter, D.; **Idowu, T.**; Domalaon, R.; Brizuela, M.; Okunnu, O.; Bi, L.; Guerrero, Y. A.; Zhanel, G. G.; Kumar, A.; Schweizer, F. Amphiphilic Nebramine-Based Hybrids Rescue Legacy Antibiotics from Intrinsic Resistance in Multidrug-Resistant Gram-Negative Bacilli. *Eur. J. Med. Chem.* **2019**, *175*, 187-200. <https://doi.org/10.1016/j.ejmech.2019.05.003>

- (9) Ammeter, D.; **Idowu, T.**; Zhanel, G. G.; Schweizer, F. Development of a Nebramine–Cyclam Conjugate as an Antibacterial Adjuvant to Potentiate β -Lactam Antibiotics against Multidrug-Resistant *P. aeruginosa*. *Journal of Antibiotics* **2019**.
<https://doi.org/10.1038/s41429-019-0221-9>
- (8) Zhanel, G. G.; Golden, A. R.; Zelenitsky, S.; Wiebe, K.; Lawrence, C. K.; Adam, H. J.; **Idowu, T.**; Domalaon, R.; Schweizer, F.; Zhanel, M. A.; et al. Cefiderocol: A Siderophore Cephalosporin with Activity against Carbapenem-Resistant and Multidrug-Resistant Gram-Negative Bacilli. *Drugs* **2019**, 79 (3), 271–289. <https://doi.org/10.1007/s40265-019-1055-2>
- (7) #Domalaon, R.; #**Idowu, T.**; Zhanel, G. G.; Schweizer, F. Antibiotic Hybrids: The Next Generation of Agents and Adjuvants against Gram-Negative Pathogens? *Clin. Microbiol. Rev.* **2018**, 31 (2), e00077-17. <https://doi.org/10.1128/CMR.00077-17>
#Shared First Authorship
- (6) **Idowu, T.**; Schweizer, F. Ubiquitous Nature of Fluoroquinolones: The Oscillation between Antibacterial and Anticancer Activities. *Antibiotics* **2017**, 6 (4), 26.
<https://doi.org/10.3390/antibiotics6040026>
- (5) Lyu, Y.; Yang, X.; Goswami, S.; Gorityala, B. K.; **Idowu, T.**; Domalaon, R.; Zhanel, G. G.; Shan, A.; Schweizer, F. Amphiphilic Tobramycin–lysine Conjugates Sensitize Multidrug Resistant Gram-Negative Bacteria to Rifampicin and Minocycline. *J. Med. Chem.* **2017**, 60 (9), 3684–3702. <https://doi.org/10.1021/acs.jmedchem.6b01742>
- (4) **Idowu, T.**; Samadder, P.; Arthur, G.; Schweizer, F. Amphiphilic Modulation of Glycosylated Antitumor Ether Lipids Results in a Potent Triamino Scaffold against Epithelial Cancer Cell Lines and BT474 Cancer Stem Cells. *J. Med. Chem.* **2017**, 60, 9724–9738.
<https://doi.org/10.1021/acs.jmedchem.7b01198>
- (3) Ogunsina, M.; Samadder, P.; **Idowu, T.**; Arthur, G.; Schweizer, F. Replacing D-Glucosamine with Its L-Enantiomer in Glycosylated Antitumor Ether Lipids (GAELs) Retains Cytotoxic

Effects against Epithelial Cancer Cells and Cancer Stem Cells. *J. Med. Chem.* **2017**, 60 (5), 2142–2147. <https://doi.org/10.1021/acs.jmedchem.6b01773>

- (2) **Idowu, T.**; Samadder, P.; Arthur, G.; Schweizer, F. Design, Synthesis and Antitumor Properties of Glycosylated Antitumor Ether Lipid (GAEL)-Chlorambucil-Hybrids. *Chem. Phys. Lipids* **2016**, 194, 139–148. <https://doi.org/10.1016/j.chemphyslip.2015.07.003>
- (1) Ogunsina, M.; Samadder, P.; **Idowu, T.**; Arthur, G.; Schweizer, F. Design, Synthesis and Evaluation of Cytotoxic Properties of Bisamino Glucosylated Antitumor Ether Lipids against Cancer Cells and Cancer Stem Cells. *Med. Chem. Commun.* **2016**, 7, 2100–2110. <https://doi.org/10.1039/C6MD00328A>

List of Patents

- (3). Potentiation of Antibiotics using Homodimeric Aminoglycosides. United States. USSN 62/849,264. 2019/05/17.

Inventors: Schweizer, F. and **Idowu, T.**

- (2). Potentiation of β -Lactam Antibiotics and β -Lactam/ β -Lactamase Inhibitor Combinations using Non-Ribosomal Tobramycin-Cyclam Conjugates. United States. USSN 62/770,929. 2018/11/23.

Inventors: Schweizer, F.; **Idowu, T.**; and Ammeter, D.

- (1). Di- and Tri-cationic Glycosylated Antitumor Ether Lipids, L-glucosylated GAELs and Rhamnose-Linked GAELs as Cytotoxic Agents against Epithelial Cancer Cells and Cancer Stem Cells. WO 2015/179983 A1. 2015/12/03.

Inventors: Ogunsina, M.; Samadder, P.; Schweizer, F.; Arthur, G.; and **Idowu, T.**