Discovery of novel antibiotics by metabolic engineering of *Streptomyces* soil bacteria

Master's thesis

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Department of Life Technologies
Molecular Biosciences, Biochemistry

June 2023

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TIRKKONEN, HELI: Discovery of novel antibiotics by metabolic engineering of *Streptomyces* soil bacteria

Master's thesis, 100 p.

Molecular Biosciences, Biochemistry

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Antimicrobial resistance is a rapidly developing threat that has been estimated to kill 10 million people yearly by 2050. This underscores the urgent need for novel antibiotics that do not share cross-resistance with existing ones. Recently, tetracenomycins were discovered to bind to the ribosome at a distinct site within the polypeptide exit tunnel, exhibiting no cross-resistance with other classes of antibiotics. However, tetracenomycins also displayed cytotoxic effects on human cell lines, which prevents the use of currently existing tetracenomycins as antibiotics.

The aim of this study was to develop novel antibiotics based on the tetracenomycin scaffold that would display improved specificity against bacterial ribosomes, while harboring low affinity towards human ribosomes to mitigate cytotoxicity issues. To achieve this goal, we cloned 22 different sugar biosynthetic pathways into a modified *Streptomyces* strain that harbored genes for production of the tetracenomycin aglycone moiety, 8-demethyl-tetracenomycin C. The strain also contained a promiscuous glycosyltransferase, ElmGT, which has been shown to be able to transfer various carbohydrates to the aglycone. This approach resulted in the production of ten glycosylated analogs of 8-demethyl-tetracenomycin C, which were detected through HPLC-MS analysis. Furthermore, eight glycosylated analogs were isolated and purified using a combination of chromatographic techniques, and their chemical structures were elucidated through HR-MS and NMR analysis.

The glycosylation of 8-demethyl-tetracenomycin C successfully eliminated the cytotoxicity observed in the compounds when tested against human cancer cell lines. Additionally, it was observed that methylation of either the sugar moiety or the aglycone moiety increased cytotoxicity. The attachment of sugar moieties also significantly lowered the antibacterial activity and influenced the target specificity against bacterial strains. Future studies with a broader range of compounds, strains, or target-drug structures will be required to establish more comprehensive structure-activity relationships of tetracenomycins.

Keywords: antibiotics, *Streptomyces*, glycodiversification, metabolic engineering

The likelihood of the experiment's success is inversely proportional to the level of desire for its success.

-Anonymous researcher

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Abbreviations

1D	one-dimensional
2D	two-dimensional
8-DMTC	8-demethyl-tetracenomycin C
ACP	Acyl carrier protein
AT	Acyl transferase
BGC	Biosynthetic gene cluster
COSY	Correlation spectroscopy
Cryo-EM	Cryo-electron microscopy
dd	doublet of doublets
DH	Dehydratase
ER	Enoyl reductase
ESI	Electrospray ionization
GTP	Guanosine-5'-triphosphate
HMBC	Heteronuclear multiple bond correlation spectroscopy
HPLC	High-performance liquid chromatography
HSQC	Heteronuclear single quantum coherence spectroscopy
KR	Ketoreductase
KS	Ketosynthase
LPS	Lipopolysaccharide
mRNA	Messenger RNA
MS	Mass spectrometer
MW	Molecular weight
NDP	Nucleoside diphosphate
NMP	Nucleoside monophosphate
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser effect spectroscopy
NRP	Nonribosomal peptide
NRPS	Nonribosomal peptide synthetases
NTP	Nucleoside triphosphate
PGN	Peptidoglycan
PKS	Polyketide synthase
RiPP	Ribosomally synthesized and post-translationally modified
RNAP	RNA polymerase
RND	Resistance-nodulation-cell division
rRNA	Ribosomal RNA
TDP	Thymidine diphosphate
TE	Thioesterase
TOCSY	Total correlation spectroscopy
TOF	Time-of-flight
tRNA	Transfer RNA
UDP-MurNac	Uridine-5’-diphosphate-*N*-acetylmuramyl
UV/Vis	Ultraviolet/visible light

1 Introduction

Natural products refer to compounds synthesized by living organisms, including bacteria, fungi, plants, and marine animals. They hold significant importance in medicine, with approximately 75 % of approved drugs being either natural products or derived from them (Newman and Cragg 2020). The vast majority of these natural products are produced by bacteria, especially Actinobacteria (Baltz 2008). Bacterial natural products are classified as secondary metabolites, meaning they are not essential for the organism's survival but instead serve as in the competition for resources and habitats against other organisms (Barka et al. 2015). The majority of natural product-derived drugs function as intended by nature, primarily as antibacterial and antifungal agents. However, their application extends beyond anti-infectives, as numerous drugs have been derived from natural products, notably anticancer agents, as well as treatments for hypercholesterolemia and type 2 diabetes. (Newman and Cragg 2020.)

Bioactive natural products provide their host organisms with a selective advantage, driving the evolutionary pressure to generate chemical diversity (Fischbach et al. 2008). In comparison to synthetic chemistry, natural products often exhibit a higher number of stereogenic centers and intricate architectural designs (Figure 1). Synthetic chemists face challenges in achieving stereospecific hydroxylation, a process commonly observed in natural products. (Clardy and Walsh 2004.) The biosynthesis of natural products relies on the host organism's enzymatic capabilities. These compounds are assembled through coordinated enzyme cascades that combine primary metabolites and employ tailoring enzymes to modify core structures. Enzyme evolution within nature has played a pivotal role in creating diversity, leading to the development of compounds with novel or enhanced bioactivities. (Fewer and Metsä-Ketelä 2020.)

Evolution chooses no favorites, as disease-causing bacteria have in response developed resistance to antibiotics used for treating infections. In 2019 alone, drug-resistant bacteria contributed to 4.95 million deaths (Murray et al. 2022), and projections indicate that drug-resistant diseases could lead to as many as 10 million annual deaths by 2050 (World Health Organization 2019). These alarming statistics emphasize the urgent requirement for new antibiotics with novel targets or mechanisms of action to evade cross-resistance to existing drugs. Hence, this study has two primary objectives. Firstly, it aims to review the progress made thus far in developing antibiotics based on natural products. Secondly,

it seeks to generate novel antibiotics by genetic engineering of *Streptomyces* soil bacteria. By exploring the potential of these organisms, the study aims to contribute to the discovery of innovative antibiotics that can combat drug-resistant bacteria effectively.

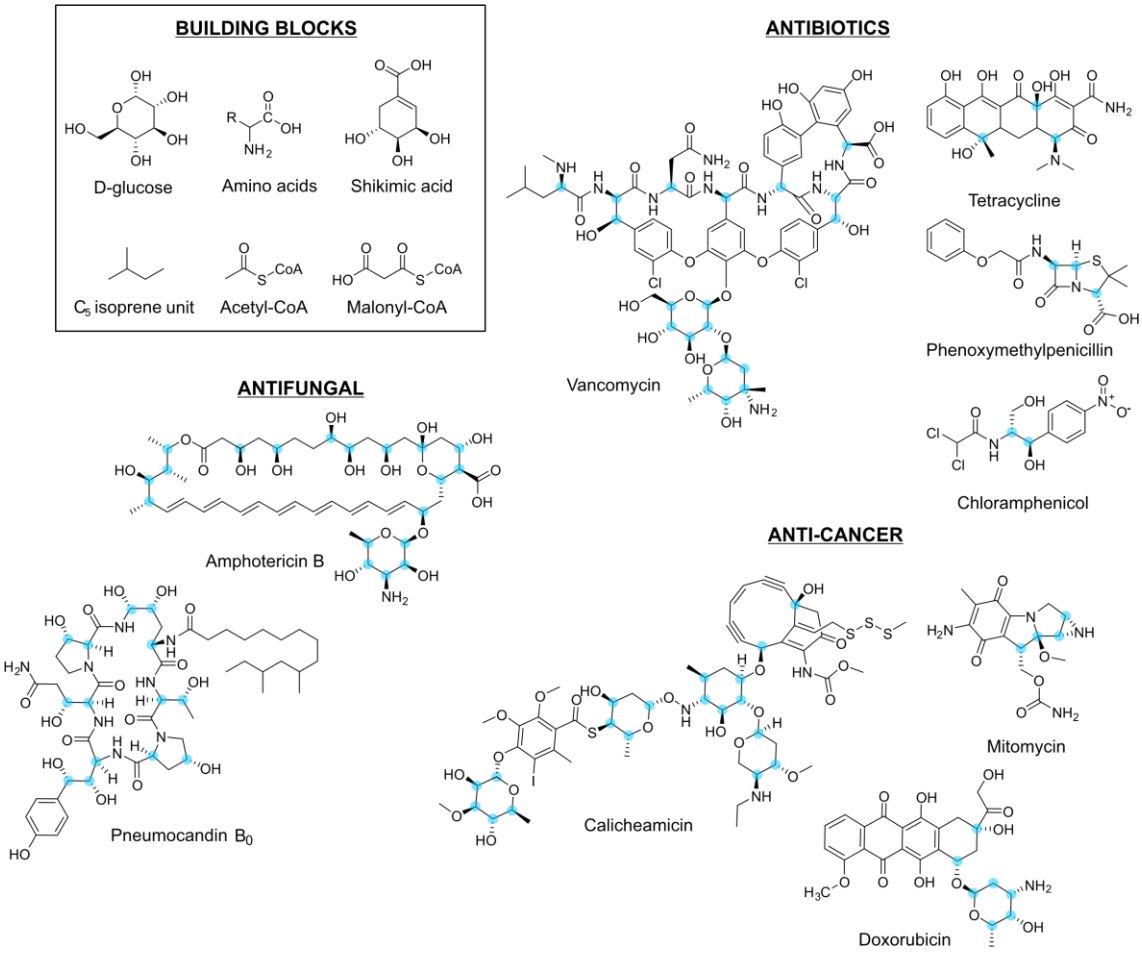

Figure 1. **The diversity of natural products.** Selected examples of natural products are shown along with their simple building blocks. Stereochemical centers are colored blue.

1.1 History of antibiotic discovery

The history of natural products dates back more than 3000 years, with evidence found in an ancient Egyptian papyrus that describes the utilization of moldy bread and medicinal soil for treating open wounds (Haas 1999). However, the significant breakthrough in the field of anti-infectives emerged in the early 1900s with the remarkable discovery of penicillin by Alexander Fleming (Fleming 1929), which became the most widely recognized example. The foundational work in this area was carried out by Selman Waksman, who systematically examined Actinobacteria obtained from soil samples. His research led to the discovery of numerous antibiotics produced by these bacteria,

including streptomycin (Waksman et al. 2010). This marked the beginning of what is often referred to as "the Golden Age of Antibiotics." During this period, the identification of new antibiotics was relatively straightforward, and many of the antibiotic classes that are widely used today were identified (Figure 2) (da Cunha et al. 2019).

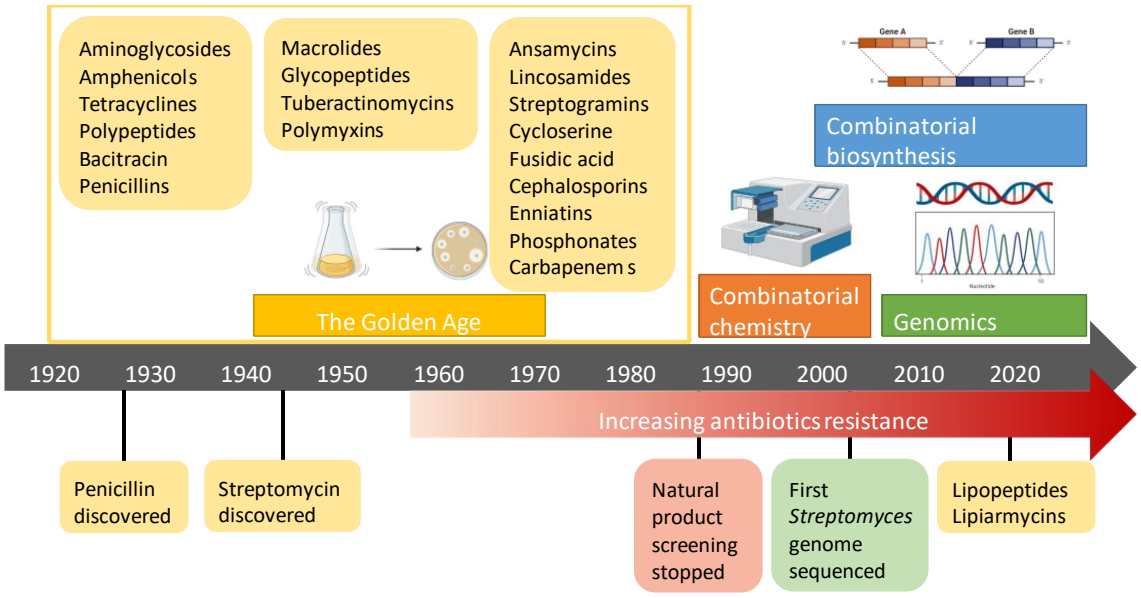

Figure 2. Timeline of antibiotic discovery. The antibiotic classes of microbial origin are listed in yellow boxes.

During the Golden Age of antibiotic discovery (1940-1970), the identification of new anti-infectives primarily involved cultivating various microorganisms in flasks and testing the antibacterial or antifungal activity of their culture extracts. Antibiotic production was assessed through simple phenotype screens, where the compounds were examined for their ability to inhibit bacterial growth on agar plates. In an effort to discover new *Streptomyces* species, a prolific antibiotic-producing genus of Actinobacteria, pharmaceutical companies embarked on acquiring soil samples from around the world. (Katz and Baltz 2016.) While natural products were valuable discoveries, they were often unsuitable for direct use in humans, necessitating chemical modifications through synthesis. The goal was to develop derivatives of natural products with enhanced potency, solubility, stability, pharmacokinetics, or reduced toxicity (Guo 2017). The Golden Age yielded the identification of over 1000 bioactive natural products, many of which are still in use today, either in their original form or as semi-synthetic derivatives (Katz and Baltz 2016).

The pace of natural product discovery began to decline after the 1970s, even though the screening of natural product extracts continued. By this time, the most abundant antibiotics had already been identified, and the remaining antibiotics, which were less abundant, became increasingly challenging to find. It was estimated that for every ten million natural product extracts screened, researchers expected to discover one previously unknown antibiotic. (Baltz 2008.) The screening process itself was time-consuming. Actinobacteria, which are a common source of natural products, have a slow growth rate. Extracts obtained from these bacteria had to undergo careful fractionation to isolate the active component. Furthermore, the active component often existed in small quantities. This posed a challenge as the active component remained unknown during the initial screening, leading to the possibility of rediscovering already known natural products. (Ortholand and Ganesan 2004.) As a result of these challenges, many pharmaceutical companies shifted their focus away from natural product screening in the 1990s and turned towards combinatorial chemistry and high-throughput screening methods (Katz and Baltz 2016).

Combinatorial chemistry is a methodology that involves the covalent linking of different building blocks to create a vast number of diverse compounds. Initially, libraries constructed using this approach focused on peptides, but later expanded to include small molecules. These compounds were often synthesized on solid supports, such as beads, facilitating their screening for binding affinity to specific targets of interest. (Liu et al. 2017.) In parallel, the development of high-throughput screening in the 1990s perfectly complemented the large compound libraries generated through combinatorial chemistry. High-throughput screening involves the use of automated robotics and microtiter plate readers to rapidly test a large number of compounds. (Dias et al. 2012.) Furthermore, advancements in molecular biology played a crucial role in understanding the biological targets responsible for the bioactivities of natural products. This knowledge facilitated the screening of compounds that specifically interacted with target molecules, allowing for more targeted and efficient screening processes. (Katz and Baltz 2016.) In contrast, natural product extracts relied on phenotype screens rather than target-based screening, which made the testing process slower. (Dias et al. 2012)

Despite screening millions of compounds generated through combinatorial chemistry between 1990 and 2010, only one de novo combinatorial new chemical entity was approved by the U.S. Food and Drug Administration. This can be attributed to the fact

that compounds produced by combinatorial chemistry lacked the intricate architectural designs and stereogenic centers found in natural products, which contribute to their specific bioactivities. (Newman 2008.) Combinatorial chemistry found its niche in lead optimization, leveraging natural products as initial templates for further modification (Ortholand and Ganesan 2004). However, as antibiotic resistance continued to be a pressing issue, a new approach was needed. By this time, the most common biosynthetic pathways of natural products had been elucidated through DNA cloning and sequencing techniques, particularly in Actinobacteria. This led to the emergence of combinatorial biosynthesis, which involved the rational design of compounds by modifying the biosynthetic pathways of natural products. (Katz and Baltz 2016.)

Combinatorial biosynthesis offers two main strategies: targeted modifications within a biosynthetic pathway and the combination of genes from different pathways (Floss 2006). Among the targets for combinatorial biosynthesis, polyketide synthases (PKSs) have attracted attention due to their systematic structure. These multienzyme complexes consist of multiple modules, with each module responsible for attaching and modifying a specific building block. Within each module, various domains carry out specific catalytic functions. (Weissman 2016.) Early attempts in the 2000s encountered challenges in combinatorial biosynthesis. Construction of mutant strains was time-consuming, biosynthetic enzymes often rejected unnatural substrates, and yields of new compounds were low (Floss 2006). However, recent advancements in genetic engineering tools and the discovery of more substrate-flexible enzymes have addressed some of these challenges. Despite these improvements, low yields still remain an obstacle. Combinatorial biosynthesis has proven more effective in optimizing existing compounds rather than generating large compound libraries through gene combinations. (Kim et al. 2015.) Nevertheless, there is optimism for the future of combinatorial biosynthesis. Ongoing bacterial genome sequencing efforts continually provide new genes for exploration, expanding the pool of available genetic material. Additionally, understanding protein-protein interactions in PKSs enables more meaningful connections between genes, allowing for the exploration of novel combinations (Baltz 2018).

In 2002, the complete genome sequence of Streptomyces Coelicolor A3(2) was deciphered, revealing that many gene clusters responsible for secondary metabolite production were not expressed under laboratory conditions (Bentley et al. 2002). It was found that only around 10% of secondary metabolites produced by Actinobacteria were

detectable in laboratory settings, while the rest required specific environmental triggers for activation (Katz and Baltz 2016). This discovery reignited interest in natural product exploration, as scientists aimed to uncover the potential products of these cryptic gene clusters (Baltz 2008). The challenge now lies in identifying the most promising gene clusters for the discovery of novel structures, considering the vast amount of genetic information available. Bioinformatics plays a crucial role in this process, as it helps interpret genetic information into compound structures. However, bioinformatics is not the only hurdle to overcome. Another challenge is activating silent gene clusters, as manipulating complex metabolic networks is a daunting task (Kalkreuter et al. 2020.) Nevertheless, microbial genome mining provides a solution to the problem of rediscovering known natural products, as known compound scaffolds share genetic similarities (Baltz 2021).

Despite being temporarily overlooked after the Golden Ages, natural products still hold great potential for the development of novel antibiotics. The World Health Organization has established four criteria for innovative antibiotics, namely the absence of cross-resistance to existing antibiotics, belonging to a new chemical class, targeting a new molecular target, or employing a new mechanism of action (World Health Organization 2019). In recent years, several promising novel microbial natural products, such as teixobactin (Ling et al. 2015) and darobactin (Imai et al. 2019; Kaur et al. 2021), have been discovered that meet these criteria. Hence, while the antibiotic development pipeline has been severely impaired in recent decades, there is renewed optimism that new classes of antibiotics can be found from nature and introduced into the clinic in the future.

1.2 Antibiotics and their mechanisms of action

The term "antibiotic" was coined by Selman Waksman, who originally defined it as compounds produced by microbes that exert harmful effects on the growth of other microbes (Waksman 1973). However, the term has now evolved to include any organic molecule, including synthetic compounds, that can inhibit the growth of microbes (Davies and Davies 2010). Antibiotics can have either bactericidal or bacteriostatic effects on microbes. Bactericidal antibiotics effectively kill bacteria, while bacteriostatic antibiotics merely suppress their growth. Bacterial cell death can be attributed to two main causes: disruption of cell envelopes or irreversible damage to DNA. (Baquero and Levin 2021.) There are various routes through which different antibiotics can achieve these ultimate

causes, leading to their categorization based on their mechanisms of action (Table 1). Currently, most antibiotic classes used in clinical practice target either protein synthesis or cell wall synthesis. This is because there are structural differences in the ribosomes of prokaryotes and eukaryotes, and mammalian cells lack cell walls. In the following sections, I will explore the mechanisms of action outlined in Table 1, providing examples from selected antibiotic classes. While this review primarily focuses on antibiotics derived from microorganisms, it is important to note that there are also synthetic antibiotics that inhibit DNA replication and one carbon folate metabolism, further expanding the repertoire of mechanisms of action (Fernández-Villa et al. 2019; van Eijk et al. 2017).

Table 1. Antibiotic classes in clinical use originating from microbes and their mechanism of action.

Mechanism of action	Antibiotic classes
Inhibition of cell wall synthesis	β-Lactams, polypeptides, cycloserines, phosphonates, phosphoglycolipids
Cell membrane disruption	Lipopeptides, lantibiotics
Inhibition of protein synthesis	Aminoglycosides, tetracyclines, amphenicols, macrolides, lincosamides, streptogramins, pleuromutilins, tuberactinomycins, mupirocin, fusidic acid
Inhibition of RNA synthesis	Ansamycins, lipiarmycins

1.2.1 Inhibition of cell wall synthesis

The bacterial cell wall plays a crucial role in maintaining the structural integrity of bacteria and protecting the protoplast from osmotic lysis. Gram-positive bacteria have a thick cell wall that surrounds their inner membrane, while gram-negative bacteria possess a thinner cell wall along with both inner and outer membranes. (Lima et al. 2020.) The limited availability of antibiotics effective against gram-negative bacteria stems from the challenge of penetrating the outer membrane, which acts as a barrier for drug entry. Diffusion through pores or uptake by transporters in the outer cell membrane are key considerations for antibiotics targeting gram-negative bacteria (Braun et al. 2001). In general, the size and polarity of compounds influence membrane permeability, with smaller molecular weight (less than 600 Da) and high polarity being favorable attributes for passage through the outer membrane porins of gram-negative bacteria (O'Shea and Moser 2008).

Peptidoglycan (PGN) is the primary component of the bacterial cell wall and consists of linear glycan chains cross-linked by peptide bridges. The key precursor molecule for PGN, known as Lipid II, comprises two sugar units, five amino acids, a pyrophosphate moiety, and a C55-undecaprenyl lipid (Figure 3a). (Müller et al. 2017.) The biosynthesis of the cell wall occurs in three stages: cytoplasmic, membrane-associated, and exocytoplasmic. In the cytoplasmic stage, enzymes in the cytoplasm synthesize uridine-5'-diphosphate-*N*-acetylmuramyl (UDP-MurNAc) pentapeptide (Figure 3b). Subsequently, in the membrane-associated stage, UDP-MurNAc pentapeptide is attached to a C55-undecaprenyl phosphate lipid carrier on the membrane, along with *N*-acetylglucosamine (GlcNAc), forming the monomeric precursor Lipid II. (Blumberg and Strominger 1974.) In the final stage, Lipid II is transported outside the cell and incorporated into the polymeric PGN through the transglycosylation of disaccharide units and the transpeptidation of pentapeptide chains (Cochrane and Lohans 2020). During the membrane-associated stage, the polypeptide portion of PGN can be modified in a species-specific manner. For example, *Staphylococcus aureus* attaches a pentaglycine chain to the pentapeptide (Müller et al. 2017). The currently used antibiotics target various stages of cell wall biosynthesis, with the exocytoplasmic stages being more accessible for drug action (Figure 3b).

Inhibition of the cytoplasmic stage of PGN biosynthesis can be achieved by two small molecules: fosfomycin and D-cycloserine (Figure 3c). Fosfomycin functions by covalently binding to the MurA enzyme, thereby obstructing the substrate binding site and impeding the initial step of peptidoglycan synthesis (Figure 3b). As an analogue of the MurA enzyme substrate phosphoenolpyruvate, fosfomycin possesses a reactive epoxide ring that enables it to irreversibly bind to MurA. (Kahan et al. 1974.) On the other hand, D-cycloserine targets both D-Ala-D-Ala ligases and D-Ala racemases, which are crucial for incorporating the final two amino acid residues into the UDP-MurNAc pentapeptide (Figure 3b). Acting as an analogue of D-alanine, D-cycloserine competitively binds to the substrate site of D-Ala-D-Ala ligases (Neuhaus and Lynch 1964) and forms an adduct with D-Ala racemase cofactors (Lambert and Neuhaus 1972). Consequently, the formation of peptidoglycan occurs without the essential D-Ala-D-Ala dipeptide required for transpeptidation in the exocytoplasmic stage (Cochrane and Lohans 2020). Although inhibitors of other enzymes involved in the cytoplasmic stage of PGN synthesis have been identified, their clinical efficacy has been limited thus far (Zhou et al. 2022).

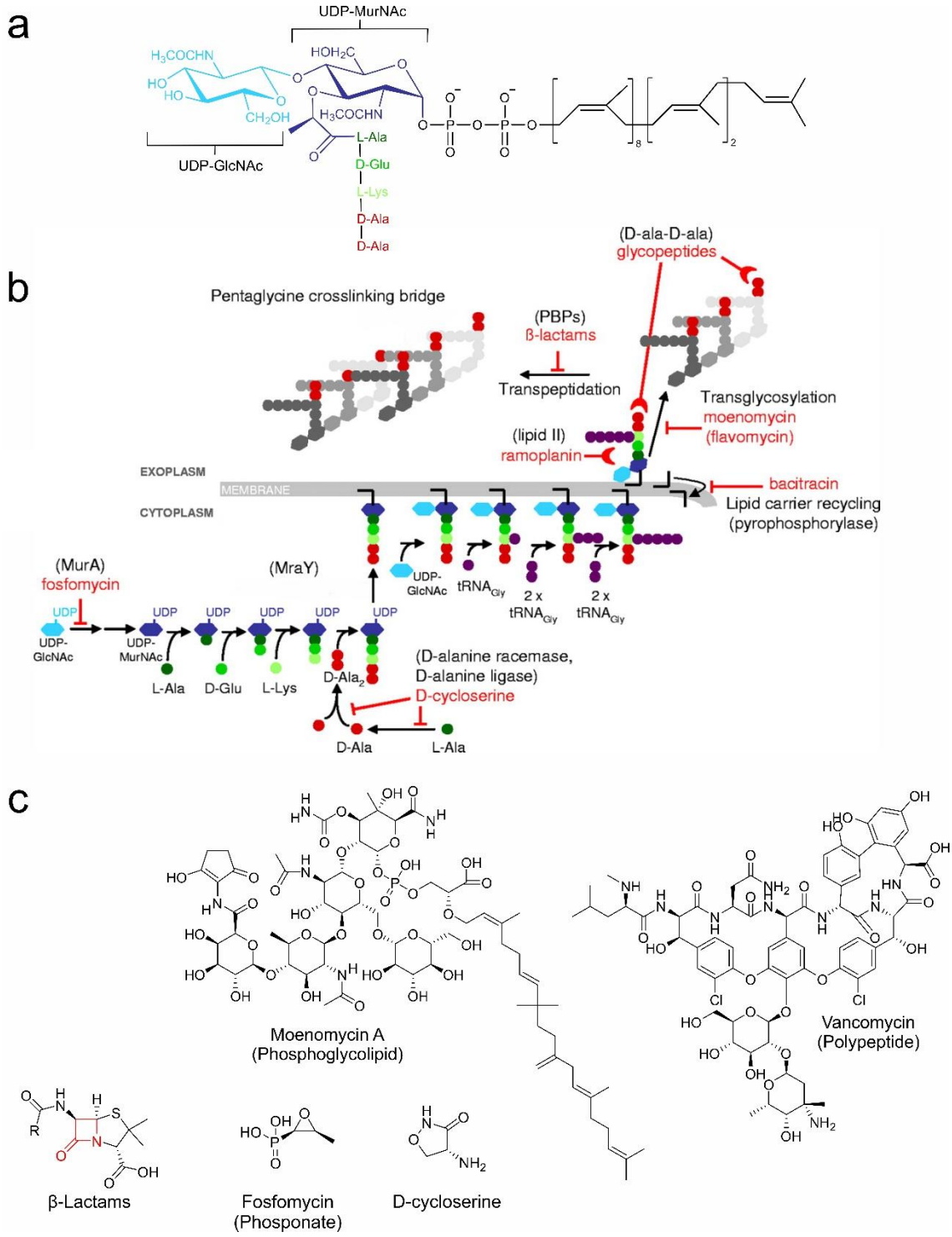

Figure 3. **Cell wall synthesis is an important target for antibiotics. a)** Chemical structure of Lipid II produced by S. aureus. **b)** Biosynthesis of peptidoglycan in S. aureus and antibiotic targets. Enzyme inhibition is represented with a blocked arrow, and antibiotics binding to a peptidoglycan biosynthesis precursor are depicted with half-moon symbols. Figure adapted from McCallum et al. 2011. **c)** Examples of chemical structures from the five classes of antibiotics that target cell wall biosynthesis.

The membrane-associated stage of peptidoglycan (PGN) biosynthesis can be inhibited by the polypeptide antibiotic bacitracin. Bacitracin, a cyclic polypeptide, binds to C55-undecaprenyl pyrophosphate, preventing its recycling for subsequent rounds of PGN synthesis (Siewert and Strominger 1967). It is important to note that although bacitracin is categorized within the membrane-associated stage, its target is located on the outer surface of the inner membrane. This is because bacitracin has a high molecular weight (1.4 kDa), which prevents it from crossing the inner membrane. Due to its limited oral absorption and high systemic toxicity, bacitracin is primarily used as a topical ointment rather than an orally administered antibiotic (Zintel et al. 1949).

The exocytoplasmic stage of peptidoglycan (PGN) biosynthesis is a common target for many antibiotics due to its accessibility outside the inner membrane. Within this stage, there are three primary targets for antibiotics: transpeptidase enzymes, transglycosylase enzymes, and their substrate, Lipid II. Transpeptidases play a crucial role in the cross-linking of peptide chains in PGN (Blumberg and Strominger 1974). They target the D-Ala-D-Ala dipeptide in Lipid II, forming a transient covalent bond with the fourth D-Ala residue, while the fifth D-Ala residue is cleaved off. In the second step of the reaction, an amino acid residue from another peptide chain attacks the enzyme-D-Ala bond, forming a new amide linkage between the peptide chains, and the transpeptidase is released. (Cochrane and Lohans 2020.) Antibiotics belonging to the β-lactam class share structural similarity with the D-Ala-D-Ala dipeptide, allowing them to covalently bind to transpeptidases. This binding inactivates the transpeptidases by preventing the second step of the reaction from occurring due to steric incompatibilities. (Lima et al. 2020.)

Transglycosylases play a crucial role in building the carbohydrate chain of PGN, which consists of repeating MurNAc and GlcNAc sugar units. They can be present as domains attached to transpeptidase enzymes or as separate transglycosylase proteins (Ostash and Walker 2005.) While transglycosylases have the potential to be effective antibiotic targets similar to transpeptidases, only the moenomycin antibiotic family has successfully been able to bind and inhibit transglycosylases. The limited availability of antibiotics targeting transglycosylases is attributed to several factors, including the presence of multiple transglycosylases in bacteria, which would require the inhibition of all of them by antibiotics, as well as the incomplete understanding of the structures and functions of transglycosylases. (Halliday et al. 2006.) Moenomycin A, a phosphoglycolipid antibiotic, consists of a pentasaccharide sugar unit, 3-phosphoglyceric acid, a C_{25} isoprene chain,

and a 2-aminocyclopentane-1,3-dione chromophore (Figure 3c) (Huber et al. 1965). It bears structural similarities to peptidoglycan (Ritzeler et al. 1997) and inhibits transglycosylase activity by binding to the substrate binding site (Lovering et al. 2007). However, moenomycin A has limited oral absorption, rendering it unsuitable for use in humans (Goldman and Gange 2000).

Lipid II serves as the primary building block of PGN, and its conserved structure across bacteria makes it an appealing target for antibiotics. Glycopeptide antibiotics specifically bind to Lipid II, physically obstructing the binding site for transpeptidases. (Müller et al. 2017.) Vancomycin, a glycopeptide antibiotic (Figure 3c), forms hydrogen bonds with the D-Ala-D-Ala dipeptide in Lipid II, which is the same site where transpeptidases bind (Reynolds 1989). To enhance the membrane binding affinity of glycopeptide antibiotics and bring them closer to their substrate, semisynthetic glycopeptide antibiotics have been developed by incorporating lipophilic side chains onto the glycopeptide core. (Müller et al. 2017.) Ramoplanin is another antibiotic that targets Lipid II and inhibits transglycosylation during the exocytoplasmic stage (Helm et al. 2002). It is based on a cyclic polypeptide core and includes a fatty acid unit, two sugar units, and an ester bond replacing the amide bond. Ramoplanin binds to the MurNAc-Ala-γ-D-Glu pyrophosphate portion of Lipid II, which is a distinct binding site from that of glycopeptides (Cudic et al. 2002.)

In summary, the antibiotics discussed above all work by inhibiting the synthesis of PGN, leading to the disruption of the bacterial cell envelope, which becomes vulnerable without a properly formed cell wall. (Baquero and Levin 2021.) It is worth noting that only a limited number of enzymes involved in the entire PGN biosynthesis pathway are targeted by current antibiotics. Fosfomycin and D-cycloserine are the only antibiotics that target intracellular reactions, while the remaining antibiotics predominantly target the machinery outside the inner membrane, which is more easily accessible. However, it is possible that future antibiotics may also target intracellular enzymes. When it comes to gram-negative bacteria, they are primarily targeted by fosfomycin, D-cycloserine, and certain members of the β-lactam family of antibiotics (Lima et al. 2020; Sarkar et al. 2017). Other antibiotics have high molecular weights or other unsuitable attributes that prevent them from effectively crossing the outer membrane, thereby limiting their activity to gram-positive bacteria.

1.2.2 Cell membrane disruption

Both bacteria and humans have cell membranes; therefore, a higher level of selectivity is required for antibiotics targeting the cell wall. Many drug screens generate thousands of antibacterial compounds that disrupt membranes but are unsuitable for human use as they also disturb mammalian membranes (Payne et al. 2007). The outer membrane of gram-negative bacteria is an asymmetrical lipid bilayer: the periplasmic layer consists of phospholipids similar to mammalian membranes, while the surface-exposed layer is composed of lipopolysaccharides (LPS) and Mg^{2+}/Ca^{2+} cations (Figure 4a). (Wesseling and Martin 2022). The Mg^{2+}/Ca^{2+} cations neutralize the negatively charged LPS phosphate heads, enabling tight packing of the hydrophobic acyl chains. Consequently, the surface-exposed side of the outer membrane exhibits low fluidity and prevents the diffusion of hydrophobic compounds through the membrane. (Nikaido 2003.) Despite impeding antibiotic diffusion, LPS is unique to bacteria and serves as a specific target for antibiotics (Raetz and Whitfield 2002). The inner membrane of both gram-positive and gram-negative bacteria comprises phospholipids, but there is one distinction compared to mammalian membranes. Bacterial cell membranes possess exposed anionic lipids on the surface, whereas in mammalian cell membranes, these anionic lipids are concealed within the interior-facing monolayer. (Epand et al. 2016.)

There are currently three different targets in the cell membrane for antibiotics: anionic lipids, LPS, and the Lipid II component of cell wall biosynthesis (Figure 4b). Anionic lipids are targeted by an acidic cyclic lipopeptide, daptomycin (Figure 4c) (Debono et al. 1987). Daptomycin binds to Ca^{2+} ions and acts as a cationic peptide, thus binding to anionic lipid phosphatidylglycerol (Jung et al. 2008). The insertion of daptomycin into the cell membrane causes depolarization through the formation of transient ionophores. However, the exact mechanism of action of daptomycin is still poorly understood, which hinders the design of daptomycin derivatives to combat resistance. (Huang 2020.) Polymyxins, similar to daptomycin, are cyclic lipopeptides (Figure 4c), but they target LPS. Polymyxins have cationic L-α-γ-diaminobutyric acid side chains that bind to the negatively charged phosphates of LPS, displacing bound Mg^{2+}/Ca^{2+} cations. (Nang et al. 2021.) This leads to the formation of hexagonal crystalline structures on the outer membrane, causing the formation of bulges. Eventually, these bulges rupture the outer membrane, allowing the polymyxins to bind to LPS on the inner membrane, where LPS is synthesized before being transported to the outer membrane. (Manioglu et al. 2022.)

Polymyxin binding to the inner membrane LPS also disrupts and permeabilizes the inner membrane, resulting in the leakage of cellular contents (Sabnis et al. 2021). The last target specific to bacterial cell membranes is Lipid II, which is targeted by lantibiotics. Lantibiotics are antibacterial peptides that contain lanthionines, generated through post-translational thioether linkages (Figure 4c). (Draper et al. 2015.) Nisin, the most well-known lantibiotic, functions by binding to the pyrophosphate moiety of Lipid II. This inhibits PGN synthesis and also forms pores on the cell membrane when higher-order oligomeric complexes are formed from nisin-Lipid II complexes. (Hsu et al. 2004.)

All antibiotics targeting the cell membrane permeabilize the membrane to varying degrees, eventually leading to cell death by causing cytoplasmic leakage through direct pore formation or by interfering with lipid organization and membrane-associated processes (Müller et al. 2016). Hydrophobic lipid tails and cationic moieties are crucial chemical features for membrane-binding antibiotics. However, lipopeptides and lantibiotics are effective against gram-positive bacteria only, as their sizes are too large to penetrate the outer membrane of gram-negative bacteria. Polymyxins, on the other hand, specifically target gram-negative bacteria because their target, LPS, is present exclusively in gram-negative bacteria. Polymyxins can also have a synergistic effect by permeabilizing the outer membrane of gram-negative bacteria, thus allowing the entry of other antibiotics that typically cannot penetrate the outer membrane (Wesseling and Martin 2022). This approach enables the utilization of many antibiotics that are active only against gram-positive bacteria to be effective against gram-negative bacteria as well.

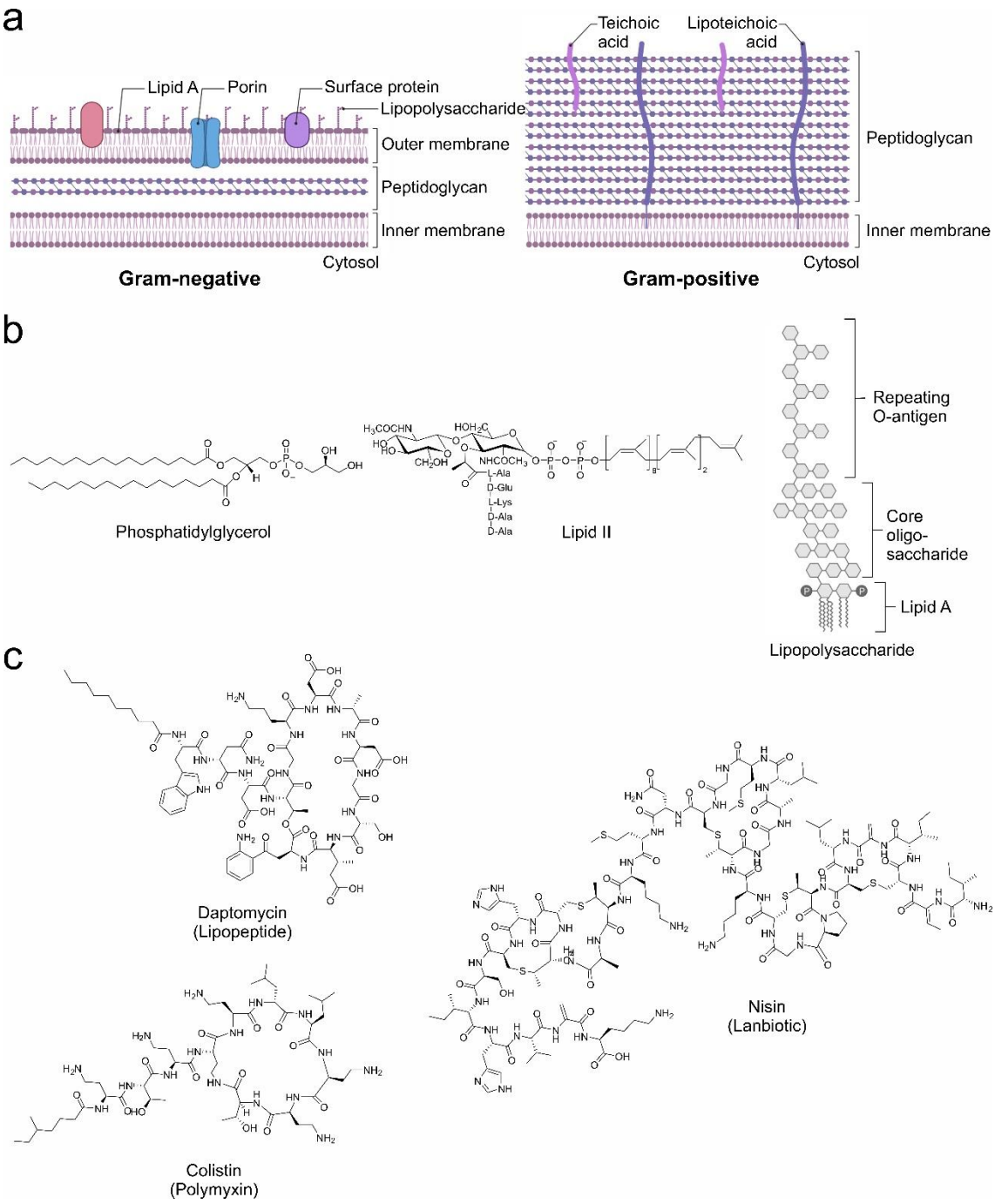

Figure 4. a) Structures of gram-negative (left) and gram-positive (right) bacterial cell membranes. **b)** Chemical structures of antibiotic target compounds on the cell membrane. **c)** Example chemical structures of antibiotics targeting the bacterial cell membrane.

1.2.3 Inhibition of protein synthesis

Proteins are synthesized by ribosomes in both bacteria and eukaryotes. Ribosomes consist of a small subunit responsible for decoding the messenger RNA (mRNA) and a large subunit responsible for forming peptide bonds. Both subunits contain ribosomal RNA (rRNA), which carries out enzymatic activities, and ribosomal proteins that stabilize the

negatively charged rRNA. (Lafontaine and Tollervey 2001.) The process of protein synthesis involves four steps: initiation, elongation, termination, and recycling of ribosomes.

During the initiation step, small and large ribosomal subunits assemble around the mRNA and initiator transfer RNA (tRNA) (Figure 5a). The role of tRNA is to deliver the correct amino acids to the ribosome, and within the ribosome, tRNA occupies three binding sites: aminoacyl (A), peptidyl (P), and exit (E) sites. (Polikanov et al. 2018.) In the elongation step, an aminoacylated tRNA enters the A-site of the ribosome, and a peptide bond is formed between the aminoacyl tRNA and the growing peptidyl-tRNA at the P-site. This is followed by translocation, where the empty tRNA moves from the P site to the E site, and the peptidyl-tRNA moves from the A site to the P site (Figure 5a). Termination occurs when a stop codon enters the ribosome, triggering termination release factors that hydrolyze the peptidyl-tRNA bond. (Wilson 2014.)

While ribosomes share a conserved structural core, there are differences between bacteria and eukaryotes, including specific additional proteins and extensions of conserved proteins and rRNAs. The small subunit, in particular, exhibits extensive differences due to variations in translation initiation between bacteria and eukaryotes. Bacteria bind to a Shine-Dalgarno sequence in mRNA and initiate translation from that point, whereas eukaryotes bind to the 5' cap of mRNA and need to scan the mRNA to locate the start codon further downstream. Another significant structural difference lies in the peptide exit tunnel, which is larger in bacteria, allowing antibiotics to enter. (Melnikov et al. 2012.) Despite the major differences in initiation, most currently used antibiotics target the elongation cycle. The primary mechanisms of action for these antibiotics include preventing tRNA binding, impeding tRNA movement through the ribosome, and blocking the polypeptide exit channel. (Wilson 2014.)

Antibiotics targeting the bacterial small ribosomal subunit (30S) act through two main mechanisms: interfering with tRNA delivery to the A site or blocking mRNA-tRNA complex translocation (Wilson 2014). Tetracycline antibiotics, for instance, bind to the A site of the small subunit in a way that clashes with the anticodon of incoming tRNA (Brodersen et al. 2000). Tetracyclines are composed of four fused rings with multiple hydroxyl and keto functionalities (Figure 5b) and interact with the small subunit through hydrogen bonds with the phosphate backbone of the 16S rRNA and stacking interactions

with nucleobases of the rRNA. These interactions are not sequence-specific, so the specificity of tetracycline antibiotics for bacteria is likely due to uptake differences in eukaryotes. (Nguyen et al. 2014.)

Aminoglycoside antibiotics, on the other hand, can inhibit mRNA-tRNA complex translocation and also promote translational misreading while inhibiting ribosome recycling (Wilson 2014). Aminoglycosides consist of an aminocyclitol core connected to amino sugars, rendering them polycationic through protonated amines (Figure 5b). The polycationic nature of aminoglycosides allows them to bind with high affinity to negatively charged rRNAs. (Kotra et al. 2000.) Translational misreading occurs when aminoglycosides bind to the A site of the small ribosomal subunit, causing a helix of the 16S rRNA to flip out of its position, enabling the accommodation of incorrect tRNA at the A site (Chellat et al. 2016). Aminoglycosides also have a second binding site on another helix of the 16S rRNA, which is involved in the formation of inter-subunit bridges. This prevents the movement of the small ribosomal subunit relative to the large subunit, thereby inhibiting translocation and ribosome recycling. (Borovinskaya et al. 2007.) The specificity of aminoglycosides for bacterial ribosomes is attributed to a distinct conformation of one nucleotide in the 16S rRNA, which prevents aminoglycosides from entering the binding site in eukaryotic ribosomes (Garreau de Loubresse et al. 2014).

Antibiotics targeting the bacterial large ribosomal subunit (50S) act through two main mechanisms: interfering with the correct binding positions of tRNA in the peptidyl transferase center responsible for peptide bond formation, or blocking the polypeptide exit channel (Wilson 2014). Similar to the small ribosomal subunit, antibiotics can compete with tRNA binding to the A site on the large ribosomal subunit. Chloramphenicol, from the amphenicols antibiotic family, achieves this by forming π-stacking interactions with nucleobases of the A-site rRNA (Figure 5b) (J. Lin et al. 2018). Antibiotics can also bind to the P site of the large ribosomal subunit, but their mechanism of action still involves interfering with tRNA binding (Eyal et al. 2016).

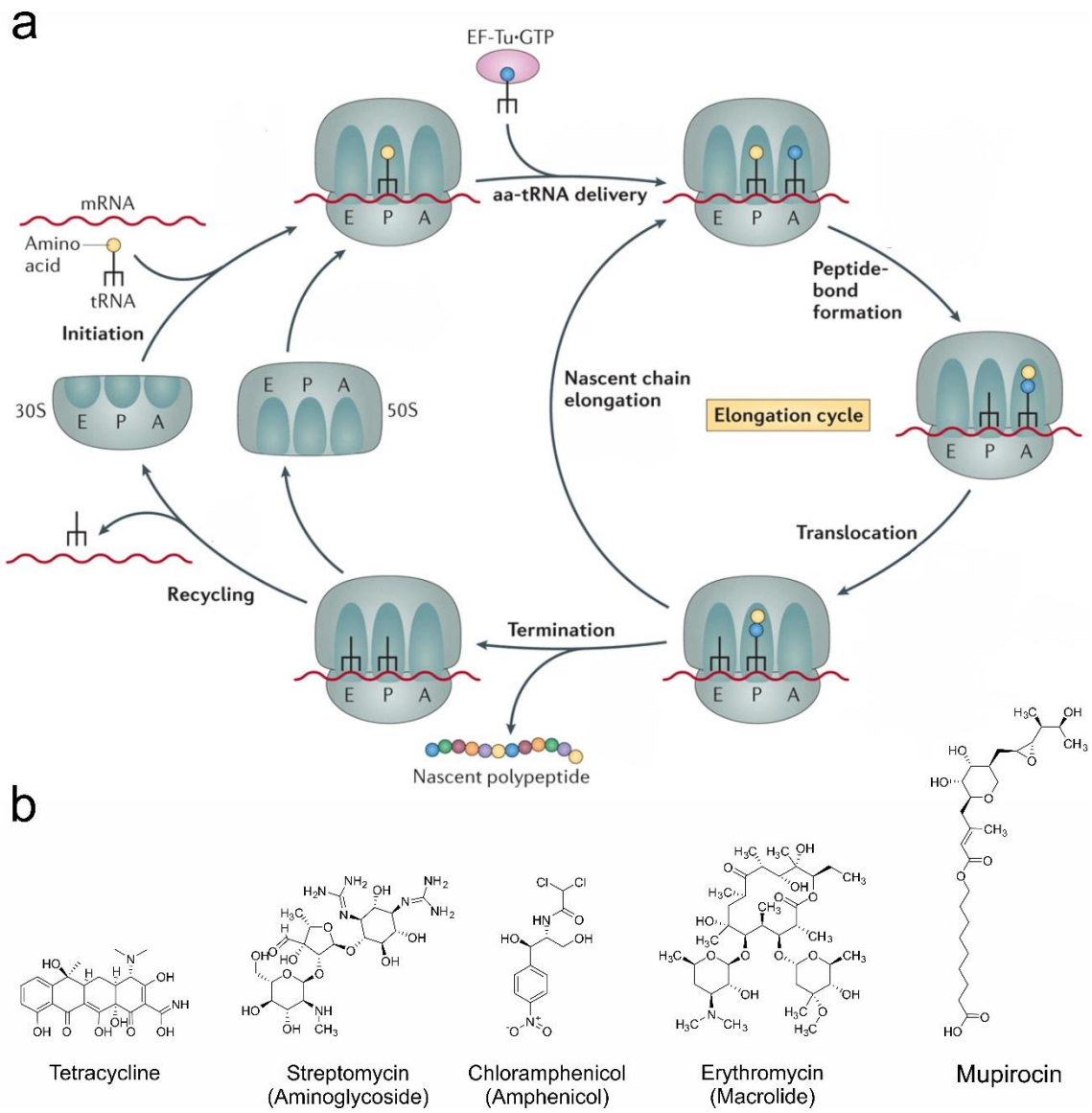

Figure 5. a) Bacterial protein synthesis cycle**.** Figure adapted from Wilson 2013. **b)** Example chemical structures of antibiotics that inhibit protein synthesis.

Macrolides, another antibiotic family, bind to the nascent peptide exit tunnel of the large ribosomal subunit, restricting its size. Macrolides consist of polyketide lactone rings with neutral or amino sugars as substituents (Figure 5b). Binding to the exit channel is achieved through hydrogen bonds between the hydroxyl groups of macrolides and amino acid residues of the exit channel. (Chellat et al. 2016.) Interestingly, macrolides do not completely block the exit channel, but instead interfere with the nascent polypeptide chain, preventing the formation of peptide bonds for specific combinations of amino acids. (Kannan et al. 2014.)

Another target in protein synthesis is bacterial isoleucyl-tRNA synthase, which is inhibited by mupirocin (Sutherland et al. 1985). Mupirocin has a structural similarity to

isoleucine (Figure 5b), enabling it to bind to the substrate binding pocket of isoleucyl-tRNA synthase and impede the synthesis of isoleucyl-tRNA. (Khoshnood et al. 2019). The variations in sequence between human and bacterial tRNA synthases allow mupirocin to selectively bind to bacterial tRNA synthases only. However, mupirocin is rapidly hydrolyzed in the human digestive system, limiting its use to topical applications on the skin. (Thomas et al. 2010.)

Antibiotics that interfere with protein synthesis can exhibit either bacteriostatic or bactericidal effects. The bacteriostatic mechanism is straightforward, as bacteria are unable to grow when their ability to synthesize new proteins is disrupted. The exact mechanism underlying bactericidal protein synthesis antibiotics is still a subject of debate, but it has been suggested that the depletion of proteins involved in the detoxification of harmful chemicals plays a key role (Svetlov et al. 2017). The chemical features crucial for protein synthesis antibiotics are determined by rRNA. Cationic moieties are required to counter the negative charges of rRNA phosphates, while aromatic rings facilitate π-stacking interactions with nucleobases, enabling binding at the active site of the ribosome. Many of the aforementioned antibiotics are active against both gram-negative and gram-positive bacteria due to their small sizes (less than 600 Da). For instance, tetracyclines can traverse the porin channels on the outer membrane of gram-negative bacteria as complexes with positively charged cations (Chopra and Roberts 2001). Only macrolides and mupirocin are effective solely against gram-positive bacteria due to their larger sizes and lower polarity. One drawback of ribosome-targeting antibiotics is their potential impact on mitochondrial ribosomes. Mitochondrial ribosomes are more susceptible to antibiotics compared to other eukaryotic ribosomes because they have a bacterial origin and share structural similarities with bacterial ribosomes (Arenz and Wilson 2016).

1.2.4 Inhibition of RNA synthesis

RNA polymerase (RNAP) is responsible for synthesizing RNA from a DNA template. It exists in both bacteria and eukaryotes, but there are distinct differences that contribute to the selectivity toward bacterial RNAPs. Consisting of five conserved subunits, RNAP forms a crab claw-shaped structure (Figure 6a). The active site of RNAP contains a catalytically important Mg^{2+} ion, which can be accessed through three channels that allow entry of DNA-RNA hybrids and nucleoside triphosphates (NTPs), as well as the exit of nascent RNA. (Kirsch et al. 2022.) The disparities between bacterial and eukaryotic

RNAPs are attributed to variations in transcription factors and the presence of up to ten additional protein subunits exclusive to eukaryotic RNAPs. Notably, transcription initiation factors and transcript cleavage factors differ between bacteria and eukaryotes, lacking homology. (Werner and Grohmann 2011.) Antibiotics targeting RNAP focus on two specific regions: the primary channel and the switch region.

The primary channel within RNAP serves as a conduit for the DNA-RNA hybrid and downstream DNA molecules, with its active site positioned on the channel's back wall (Kirsch et al. 2022). Rifamycins, antibiotics belonging to the ansamycin family, target the primary channel. Ansamycins consist of a polyketide chain that connects to an aromatic naphthalene moiety (Figure 6b). The polyketide chain plays a vital role in their activity, as modifications to it have been shown to decrease their biological efficacy. (Adams et al. 2021) Rifamycins bind inside the primary channel, in close proximity to the active site, forming van der Waals interactions with the hydrophobic naphthalene ring and establishing five hydrogen bonds with both the naphthalene ring and the polyketide chain. This binding effectively obstructs the path of the elongating RNA transcript after 2 or 3 nucleotides. Importantly, the rifamycin binding site exhibits no conservation between prokaryotes and eukaryotes, enabling selective inhibition of bacterial RNAP. (Campbell et al. 2001.)

The switch region of RNAP plays a crucial role in orchestrating the conformational changes that occur when the template DNA enters the active center. These changes, in turn, affect the size of the primary channel and govern the interactions with the DNA template and the nascent RNA transcript. (Ma et al. 2016.) Lipiarmycin A3, an antibiotic belonging to the lipiarmycin family (Figure 6b), specifically targets the switch region. Lipiarmycin A3 is composed of an 18-membered macrocyclic lactone ring, which is further modified by two sugar units and an aromatic dichlorohomoorsellinic acid subunit (Coronelli et al. 1975). The binding of lipiarmycin A3 to the switch region occurs through hydrogen bonds and a cation-π interaction involving an arginine residue and the macrolide core (Cao et al. 2022). As a result of this binding, RNAP is trapped in an open conformation, wherein the template DNA can enter the active center, but transcription initiation cannot take place. This is because the RNAP is unable to simultaneously bind the DNA promoter elements -10 and -35 in the open conformation. (Lin et al. 2018.)

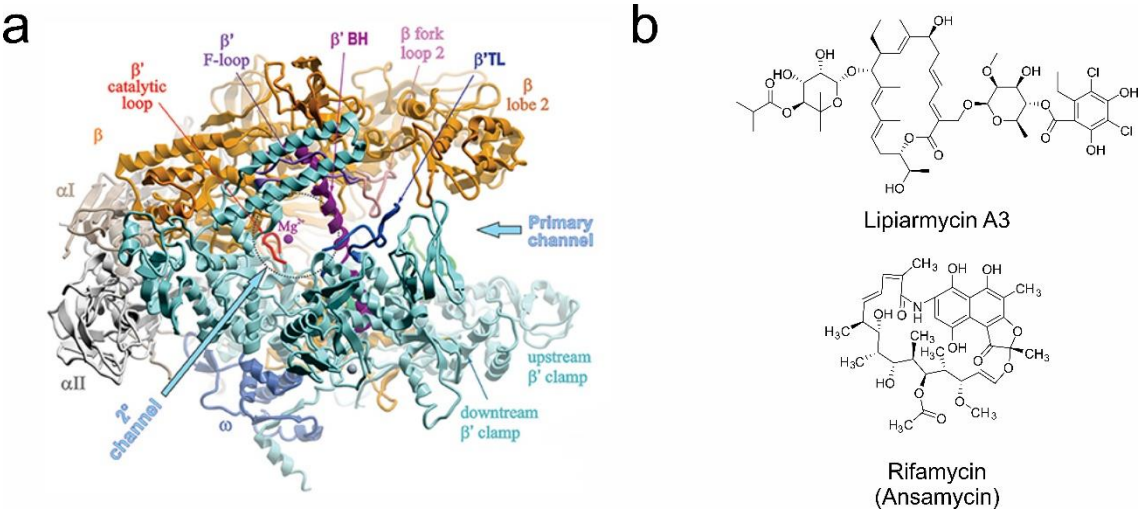

Figure 6. a) The structure of bacterial RNAP. Figure adapted from Lee and Borukhov, 2016. **b)** Chemical structures of antibiotics that inhibit RNA synthesis.

Similar to inhibitors of protein synthesis, RNAP inhibitors ultimately halt the production of new proteins as mRNA is degraded and the synthesis of new mRNA is prevented. Both hydrophobic interactions and hydrogen bonding play crucial roles in binding to the target sites of RNAP. However, lipiarmycin A3 encounters challenges due to its low water solubility, which hampers its systemic absorption (Dorst et al. 2020). Ansamycins are broad-spectrum antibiotics with a bactericidal mechanism. In contrast, lipiarmycins are not effective against gram-negative bacteria due to the absence of a positive amino acid residue in the switch region and concerns regarding outer membrane permeability (Cao et al. 2022). RNAP is a large multisubunit protein with numerous potential drug target sites, yet only two antibiotics have been clinically used thus far. Nevertheless, recent advancements in cryo-electron microscopy (cryo-EM) have made it easier for researchers to obtain structures of RNAP bound to antibiotics, providing a foundation for the rational design of new antibiotics targeting RNAP in the future. (Lin et al. 2018.)

1.3 Resistance mechanisms

When bacteria are exposed to antibiotics, the bacterial strains with an antibiotic resistance mechanism are more likely to survive and become dominant in the population (Lai et al. 2022). Antibiotic resistance can arise through spontaneous gene mutations or through gene transfer from other bacteria. Antibiotic resistance genes have existed in natural antibiotic-producing bacterial strains for billions of years as a self-protection mechanism (D'Costa et al. 2011). These resistance genes can be transferred from harmless bacteria

to pathogenic ones through horizontal gene transfer. The three main mechanisms of horizontal gene transfer are conjugation, transformation, and transduction. Conjugation involves direct transfer between two bacterial cells, transformation is the uptake of naked DNA from the surroundings, and transduction involves phages as DNA delivery agents. (Arnold et al. 2022.) Conjugation is considered the primary pathway for the emergence of resistant bacteria, with plasmids and transposons being the mobile genetic elements that carry the resistance genes (Munita and Arias 2016). While spontaneous mutations are not the major cause for the short-term spread of resistance genes, they play a crucial role in the long-term evolution of these genes (Woodford and Ellington 2007). Antibiotic resistance mutations can incur a fitness cost in the absence of antibiotics, but bacteria can acquire additional mutations that compensate for the initial loss of fitness. Moreover, the selective pressure from antibiotics can favor bacteria with higher mutation rates, making the emergence of resistance mutations easier. (Durão et al. 2018.)

Bacteria can evade the effects of antibiotics even in the absence of resistance genes by residing within a biofilm. A biofilm is a community of aggregated bacterial cells that are surrounded by an extracellular matrix. This matrix acts as a protective barrier, hindering the diffusion of antibiotics and rendering the bacteria inside the biofilm less susceptible to their effects. Furthermore, bacteria within a biofilm tend to exhibit slower growth or enter a dormant state, making them less responsive to bacteriostatic antibiotics. (Ciofu et al. 2022.) Apart from biofilms, a subpopulation of bacteria can display persistence or tolerance to antibiotics without possessing specific resistance mechanisms. This is often attributed to the slower metabolism of persistent or tolerant bacteria. (Balaban et al. 2019.) Antibiotics primarily target specific cellular components, which subsequently trigger stress responses or deplete essential cellular resources, ultimately leading to bacterial death. In slow-growing bacteria, the targeted cellular components are less active, resulting in reduced activation of downstream processes, thereby decreasing the effectiveness of antibiotics. (Yang et al. 2017.)

Bacteria can possess either intrinsic or acquired resistance mechanisms to antibiotics. Intrinsic resistance mechanisms are present regardless of antibiotic exposure and are not dependent on selective pressure. For instance, gram-negative bacteria have an outer membrane that acts as an intrinsic barrier, preventing the entry of many antibiotics. Additionally, membrane efflux pumps are another intrinsic resistance mechanism that actively pumps out toxic substances from the bacterial cell, including antibiotics. (Cox

and Wright 2013.) On the other hand, acquired resistance can be more problematic as bacteria can acquire multiple resistance genes, making them resistant to different classes of antibiotics and leading to multidrug resistance (Murray et al. 2022). There are four main molecular mechanisms involved in acquired antibiotic resistance, namely prevention of intracellular antibiotic accumulation, target modification, antibiotic inactivation, and bypassing the target metabolic pathway (Figure 7). These mechanisms will be briefly discussed in the following sections.

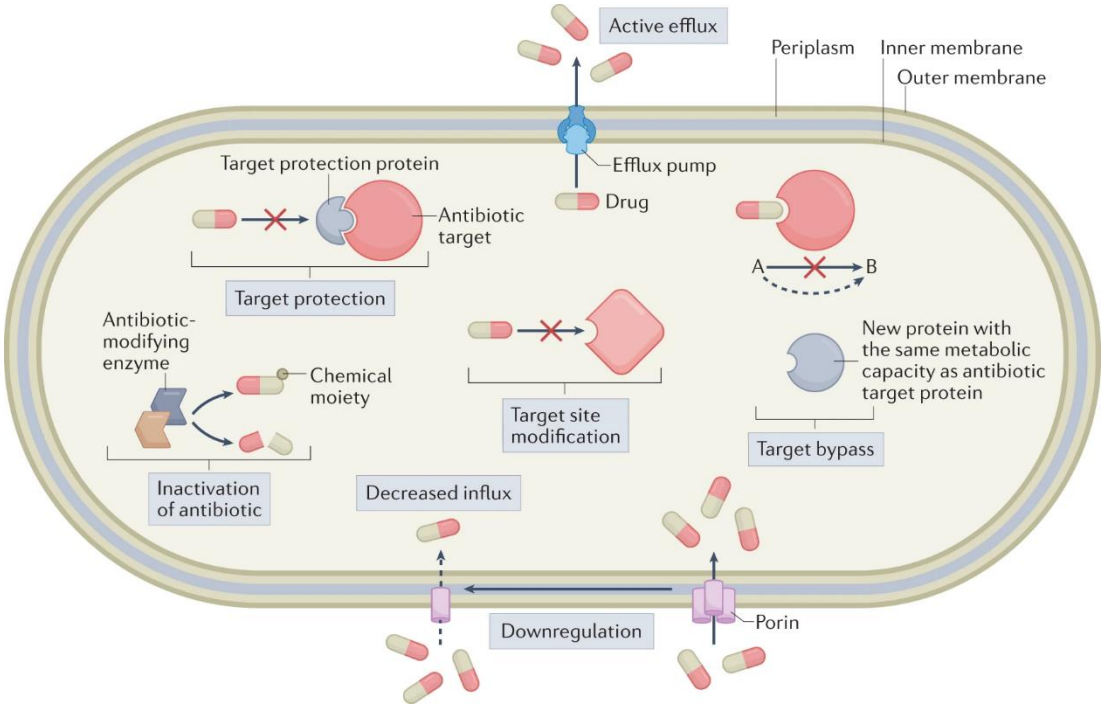

Figure 7. Most common antibiotic resistance mechanisms. Intracellular accumulation of antibiotics can be prevented by increased efflux or decreased influx of the antibiotic. The target of the antibiotic can be directly modified or a separate target protection protein can be involved. Inactivation of antibiotics is the most direct route of resistance and has less fitness cost than reprogramming the whole metabolic pathway to bypass the target of the antibiotic. Figure adapted from Darby et al. 2022.

1.3.1 Prevention of intracellular antibiotic accumulation

Most antibiotics require entry into the bacterial cell to inhibit their target, making the prevention of antibiotic diffusion through the cell membrane and cell wall an effective resistance strategy. The bacterial cell membrane is impermeable to hydrophilic and charged solutes, but bacteria have porins, nonspecific diffusion channel-forming proteins, on their cell membranes. Porins are transmembrane β-barrels that allow the passage of

hydrophilic molecules with a MW of less than 600 Da. (Nikaido 2003.) Porins can be nonspecific or substrate-specific, with antibiotics typically passing through the larger, nonspecific porins. Antibiotic resistance can be mediated through porins by the expression of different porins or by mutations within the porin channel, often combined with reduced expression of the main porins. (Pagès et al. 2008.) Since antibiotics are usually larger than common nutrients, the expression of porins with smaller channels effectively blocks antibiotic entry while allowing essential nutrients to be transported into the cell (Nikaido 2003). Porin channels may contain a constricting loop inside the channel, affecting the diffusion rates of polar molecules. Mutations in this loop can confer antibiotic resistance by selectively blocking the passage of the antibiotic. (Vergalli et al. 2020.) Gram-negative bacteria, in particular, heavily rely on reduced diffusion due to their impenetrable outer membrane for both hydrophilic and hydrophobic molecules. However, gram-positive bacteria have also been found to alter their cell membrane lipid composition to hinder antibiotic entry (Mishra et al. 2012). While the cell wall is generally permeable to antibiotics, *Staphylococcus aureus* can develop vancomycin resistance by producing a thickened cell wall that prevents vancomycin from reaching its target, the cell wall precursors near the cell membrane. (Srinivasan et al. 2002.)

In addition to impeding antibiotic influx, bacteria often increase the efflux of antibiotics by upregulating the expression of efflux pumps on their cell membranes. Efflux pumps are transmembrane protein complexes capable of actively transporting various compounds that are toxic to bacteria. While most efflux pumps are located on the cytoplasmic membrane, gram-negative bacteria possess efflux pumps that span the entire distance from the inner to the outer membrane. (Lai et al. 2022.) Efflux pumps are ubiquitous in bacteria, and any bacterial strain is likely to have efflux pumps from all the different efflux pump families (Henderson et al. 2021.) Gram-negative bacteria, once again, pose significant challenges as they rely on resistance-nodulation-cell division (RND) family efflux pumps, which contribute to the antibiotic resistance of clinically relevant gram-negative pathogens (Darby et al. 2022.) RND efflux pumps form complexes with periplasmic proteins and outer membrane channels, enabling them to expel antibiotics directly from inside the cell to the extracellular medium. Moreover, RND efflux pumps have large binding pockets, resulting in a broad substrate specificity. (Yu et al. 2003.) However, there is a counteractive strategy to overcome this resistance mechanism: efflux pump inhibitors. By combining efflux pump inhibitors with

antibiotics, the resistant bacterial strain can become susceptible to antibiotics once again. (Tambat et al. 2022.)

1.3.2 Target modification or protection

Clinically effective antibiotics exhibit high specificity in binding to their intended targets, minimizing the potential for toxic side effects associated with nonspecific binding to other molecules. However, bacteria have the ability to evade the action of antibiotics by modifying the binding site of the target while preserving its original function. Resistance can occur through two mechanisms: target site modification or the presence of separate target protection molecules. (Darby et al. 2022.) Target site modification has been observed as a resistance mechanism for all previously identified antibiotic targets. This modification can result from genetic mutations that lead to amino acid substitutions in the active site of the target protein or enzymatic modification of the active site itself. These modifications ultimately reduce the binding affinity of the antibiotic to the target site. (Blair et al. 2015.) For instance, antibiotics that target ribosomes can be rendered ineffective through methylation of the 16S or 23S rRNA. Methylation near the antibiotic binding site disrupts the binding affinity by causing steric clashes. (Schaenzer and Wright 2020.) Target modification can also occur in the context of the cell membrane, such as in polymyxin resistance. Polymyxins act on the LPS of the outer membrane in gram-negative bacteria, and bacteria counteract their effects by modifying their LPS molecules. LPS molecules are decorated with positively charged components, which repel the cationic polymyxins away from their intended target. (Olaitan et al. 2014.)

Target protection proteins can employ three distinct mechanisms: direct contact to dislodge the antibiotic, allosteric conformational changes in the target to dislodge the antibiotic, or enabling the target to function despite the presence of the bound antibiotic. (Wilson et al. 2020.) One example of target protection proteins is ribosome protection proteins, which play a key role in conferring resistance to tetracycline antibiotics. These proteins can dislodge tetracyclines from stalled ribosomes by binding to the same site as the antibiotic. (Arenz et al. 2015.) Additionally, it has been proposed that ribosome protection proteins induce conformational changes in nearby active site nucleotides to prevent tetracycline from rebinding to the ribosome (Dönhöfer et al. 2012). Another class of target protection proteins is the antibiotic resistance ATP-binding cassette proteins of the F-subtype. These proteins can rescue stalled ribosomes by inducing allosteric

conformational changes in the peptidyl transferase center of the 23S rRNA, leading to the dissociation of bound antibiotics. (Crowe-McAuliffe et al. 2021.) To overcome target protection mechanisms, the development of new antibiotics with improved binding capabilities to overcome target protection or the discovery of target protection protein inhibitors is necessary (Wilson et al. 2020).

1.3.3 Antibiotic inactivation via destruction or modification

Direct inactivation of the antibiotic itself is a common strategy employed by bacteria to develop resistance. This involves acquiring enzymes capable of using the antibiotic as a substrate, without the need to modify other biochemical processes within the bacterial cell. (Darby et al. 2022.) One of the most prevalent types of enzymes involved in antibiotic destruction is hydrolases, particularly for β-lactam antibiotics. β-lactamases, for instance, can hydrolyze the β-lactam ring through a nucleophilic attack on the carbonyl unit of the ring, followed by the addition of water. (Lima et al. 2020.) Other common antibiotic-modifying enzymes are acetylases, phosphorylases, and nucleotidyltransferases. These enzymes typically target hydroxyl or amino groups in antibiotics because oxygen and nitrogen are more electronegative than carbon or hydrogen. The difference in electronegativity polarizes the covalent bonds, making all participants of the bond more chemically reactive. The attachment of a chemical moiety to the antibiotic usually results in steric hindrance, which is its main effect. (Munita and Arias 2016.). Aminoglycosides serve as a common example of antibiotic modification, as they are prone to modification by all of the previously mentioned enzyme groups. This is due to their high number of exposed oxygens and nitrogens (Blair et al. 2015). To combat this mode of resistance, enzyme inhibitors or new generations of antibiotics are required (Darby et al. 2022).

1.3.4 Changes in the target metabolic pathway

Bacteria can develop resistance to antibiotics by bypassing a single step of the target metabolic pathway or by modifying multiple steps. Methicillin-resistant strains of *S. aureus*, for instance, have acquired an exogenous gene encoding penicillin-binding protein 2a through horizontal gene transfer. This protein exhibits low affinity for β-lactam antibiotics, allowing it to catalyze transpeptidation for cell wall synthesis even in the presence of these antibiotics. (Lim and Strynadka 2002.) Another simple mechanism to

overcome the effects of antibiotics is through the overexpression of their target. This strategy is viable when the antibiotic solely inhibits its target without inducing any harmful reactions upon binding. (Palmer and Kishony 2014.) One of the most complex resistance mechanisms involves the reprogramming of the entire intracellular peptidoglycan synthesis pathway to gain vancomycin resistance (Figure 8). Vancomycin binds the D-Ala-D-Ala dipeptide of Lipid II and inhibits transpeptidation reactions. However, bacteria have discovered a way to alter the D-Ala-D-Ala dipeptide to either D-Ala-D-lac or D-Ala-D-Ser dipeptides, to which have lower affinity for vancomycin. This resistance method relies on five core enzymes that catalyze the synthesis of the alternative dipeptides, accompanied by two enzymes capable of degrading the original D-Ala-D-Ala dipeptide. (Stogios and Savchenko 2020.) It is worth noting that it took approximately 30 years for resistance to develop after the introduction of vancomycin to the clinic, underscoring how bacteria are likely to find ways to develop resistance against all antibiotics eventually (Leclercq et al. 1988).

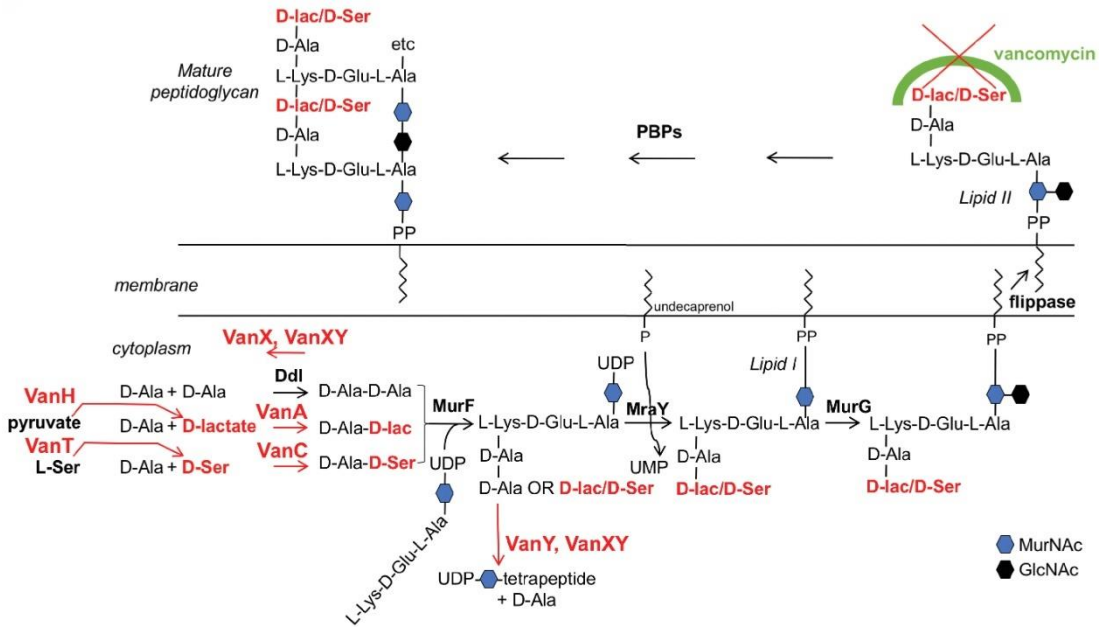

Figure 8. An example of bypassing the target of the antibiotic by modifying the whole metabolic pathway. Vancomycin binds the D-Ala-D-Ala dipeptide of Lipid II but has low affinity for lipid II precursors with a different amino acid residue at the end of the peptide. Differences compared to the normal pathway are marked in red. In total, seven different enzymes are utilized to accomplish this resistance mechanism. Figure adapted from Stogios and Savchenko, 2020.

1.4 Biosynthesis of antibiotics

The biosynthesis of antibiotics relies on several core biosynthetic pathways. Diversity is generated by utilizing steps from these pathways in different combinations and through extensive post-processing reactions catalyzed by specific enzymes. The most common antibiotics are derived from polyketides, peptides, and sugar moieties. Brief reviews of the biosynthesis of each molecule type will be provided below, offering a foundation from which new antibiotics can be rationally designed.

1.4.1 Polyketide antibiotics

Polyketides are one of the largest of all natural product families. Polyketides, one of the largest families of natural products, are synthesized by polyketide synthases (PKSs). PKSs can be categorized into different subtypes based on the functionality of their domains and their organization as standalone proteins (type II) or as components of megaenzymes (type I). (Staunton and Weissman 2001.) The biosynthesis of polyketides involves the repetitive addition of an acetate unit to an activated acyl unit through decarboxylative Claisen condensation, resulting in the extension of the polyketide backbone by two carbon atoms (Figure 9). (Heath and Rock 2002.) Polyketide synthesis requires the involvement of seven different types of enzymes: acyl carrier protein (ACP), ketoacylsynthase (KS), acyl transferase (AT), ketoreductase (KR), dehydratase (DH), enoyl reductase (ER), and thioesters (TE). The core domains involved in each round of polyketide chain elongation are ACP, KS, and AT, which facilitate the addition of the extender unit to the growing polyketide chain. (Staunton and Weissman 2001.) The KS domain catalyzes decarboxylative Claisen condensation through a two-step mechanism. In the first step, the acyl unit is transferred from ACP to a cysteine residue at the active site of KS. In the second step, malonyl-ACP enters the active site of KS, undergoes decarboxylation, and forms a carbanion. This carbanion then attacks the acyl unit bound to KS, leading to the release of the acyl unit from KS and its subsequent covalent bonding with malonyl-ACP. (Mindrebo et al. 2021.) Additionally, the extender unit added to the polyketide chain can undergo reduction by the KR, DH, and ER domains. The TE domain is responsible for releasing the fully formed polyketide chain from the PKS. (Staunton and Weissman 2001.)

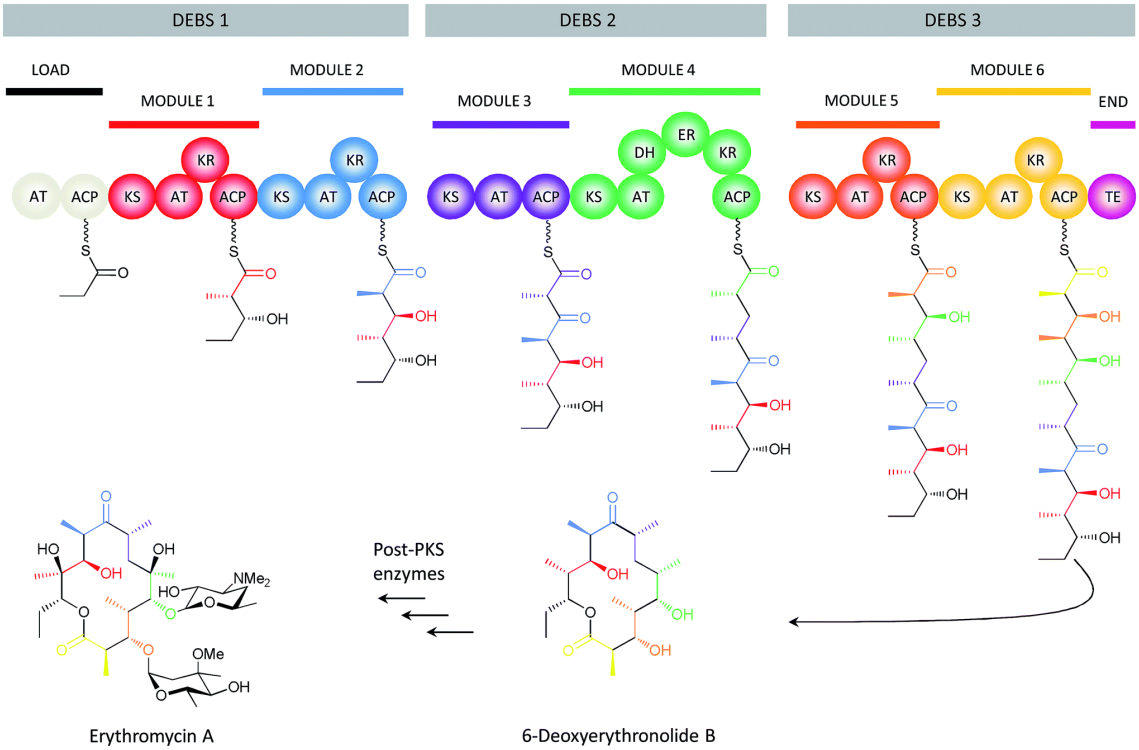

Figure 9. Biosynthetic logic of polyketides. Erythromycin polyketide synthase (type I) is a megaenzyme with six modules, each of which have the core enzymes (AT, ACP, KS) and a different amount of optional reduction enzymes. Each module adds a two carbon β-ketoacyl group to the polyketide chain. The TE domain releases and cyclizes the polyketide chain, after which post-PKS enzymes glycosylate the polyketide. Type II polyketides are synthesized with same enzymes, except they are multiple monofunctional enzymes instead of one megaenzyme. Figure adapted from Weissman 2016.

Chemical diversity in polyketides arises from four main factors: 1) selection of starter and extender units, 2) reduction and stereochemical control of the newly attached β-ketoacyl group, 3) polyketide backbone cyclization upon release from PKS, and 4) post-PKS modifications. In bacterial PKSs, extender units such as malonate, methylmalonate, and heterosubstituted malonates are commonly used, while starter units can vary, including acetate, propionate, longer chains, branched chains, or cyclic moieties. (Hertweck 2009.) Therefore, the majority of chemical diversity in polyketides originates from factors other than substrate selection. The stereochemistry of polyketides is mainly controlled by KR domains, which can generate L- or D-β-hydroxyl groups through reduction of β-ketoacyl and also epimerize the possible α-substituent of the β-ketoacyl. Additionally, the DH domain's reduction of the hydroxyl group can result in either a *cis*- or *trans*-double bond. (Keatinge-Clay 2016.) These enzymatic steps contribute to the presence of hydrophilic

keto- or hydroxyl groups, hydrophobic partially reduced double bonds, or fully reduced carbon chains with varying stereochemistries.

The final step performed by PKS, releasing the polyketide chain covalently bound to the PKS enzyme, significantly impacts the chemical structure of the polyketide. This release often occurs through nucleophilic attack on the thioester, leading to various cyclization, reduction, or fusion reactions with other moieties. Cyclization reactions, in particular, contribute to the three-dimensional diversity of the compound's structure. (Little and Hertweck 2022). Post-PKS modifications play a crucial role in introducing a wide range of chemical functionalities to the polyketide molecule. These modifications can include oxidation, reduction, methylation, glycosylation, amination, and even halogenation (Figure 9). Enzymatic cyclization of the polyketide is another essential type of post-PKS modification, which can transform linear polyketide chains into polycyclic aromatic molecules. (Olano et al. 2010.) In summary, PKS biosynthesis gives rise to molecules of varying sizes and hydrophobicities, which can be further tailored through post-PKS modifications to fine-tune their properties.

1.4.2 Peptide antibiotics

There are two primary biosynthetic pathways for peptide antibiotics: nonribosomal peptides (NRPs) and ribosomally synthesized and post-translationally modified peptides (RiPPs). The key distinction between NRPs and RiPPs lies in their building blocks during the initial peptide chain formation. RiPPs are typically limited to the 22 proteinogenic amino acids, whereas NRPs often incorporate unusual, non-proteinogenic amino acids. (Winn et al. 2016.) The biosynthesis of NRPs involves three main steps: 1) generation of non-proteinogenic amino acids, 2) synthesis of the polypeptide chain, and 3) modification of the peptide through tailoring enzymes. Non-proteinogenic amino acids are typically produced by standalone enzymes that modify substrates from primary metabolism. These non-proteinogenic amino acids often possess additional hydroxyl or methyl groups, N-based side chains, phenyl groups, halogen atoms, and undergo cyclization. (Süssmuth and Mainz 2017.) NRPs are synthesized by nonribosomal peptide synthetases (NRPSs), which follow a similar biosynthetic logic as PKSs. NRPSs are organized into modules, with each module incorporating one amino acid into the growing peptide chain. The NRPS modules consist of three core domains: adenylation, thiolation, and condensation. Similar to the AT and ACP domains in PKSs, the adenylation domain selects the substrate

and covalently attaches it to the thiolation domain. The condensation domain, although resembling the KS domain in PKSs, does not have a bound peptide chain but instead catalyzes the formation of peptide bonds between two amino acids attached to adjacent thiolation domains from different modules. (Finking and Marahiel 2004.) The newly incorporated amino acid residue can undergo further modifications by optional enzymes, which can carry out processes such as methylation, epimerization, cyclization, reduction, formylation, and oxidation. After the completion of the polypeptide chain, a thioesterase domain facilitates chain release and potential macrocyclization. (Süssmuth and Mainz 2017.)

Amino acids exhibit a range of chemical properties, including variations in size, charge, and hydrophobicity. However, much of the chemical diversity in peptide antibiotics arises after the formation of the peptide chain, as NRPs and RiPPs undergo modifications by tailoring enzymes. These modifications encompass processes such as halogenation, acylation, glycosylation, and sulfation. Post-NRPS enzymes often acylate the N-terminus of NRPs, impacting the molecule's affinity for cell membranes (Süssmuth and Mainz 2017). Glycosylation not only influences the biological activity of the peptides, but also enhances their solubility in aqueous solutions, making them more promising as drug candidates (Winn et al. 2016). Sulfation has been demonstrated to impede the induction of glycopeptide resistance without affecting the bioactivity of the antibiotic itself (Kalan et al. 2013). Halogenation generally increases the hydrophobicity of the peptide, thereby influencing its self-assembly and bioactivity (Molchanova et al. 2020). Cyclization is another crucial modification mechanism for both NRPs and RiPPs. RiPPs, in particular, exhibit numerous post-translational cyclizations that generate unusual heterocyclic features or even lassopeptides, where the C-terminal tail is threaded through the macrocyclic peptide ring. (Hudson and Mitchell 2018.) Cyclization renders peptide antibiotics generally resistant to proteases found in both prokaryotes and eukaryotes (Lucana et al. 2021). Peptide antibiotics can range in size from small peptides to larger ones, introducing structural diversity, but the size can also hinder their entry into cells, particularly in gram-negative bacteria.

1.4.3 Sugar containing antibiotics

There exist antibiotics that are purely sugar-based, such as aminoglycosides, as well as antibiotics that have sugar units attached to an aglycone part of the molecule. Sugars play

a significant role in natural products, with more than one-fifth of all bacterial natural products containing at least one sugar moiety (Elshahawi et al. 2015). There are no common enzyme scaffolds for sugar units as there are for PKs or NRPs. Instead, they are attached and modified by multiple stand-alone enzymes in a sequential manner. In many sugar-containing antibiotics, the sugar moieties are derived from D-glucose-6-phosphate or D-fructose-6-phosphate (Figure 10), which are common intermediates in primary metabolism. (Thibodeaux et al. 2007.) Sugars are often activated through the attachment of a nucleoside monophosphate (NMP) to a sugar-1-phosphate, resulting in the formation of a nucleoside diphosphate (NDP) derivative. The NDP moiety serves as a good leaving group in substitution reactions, facilitating the attachment of the sugar to another molecule.

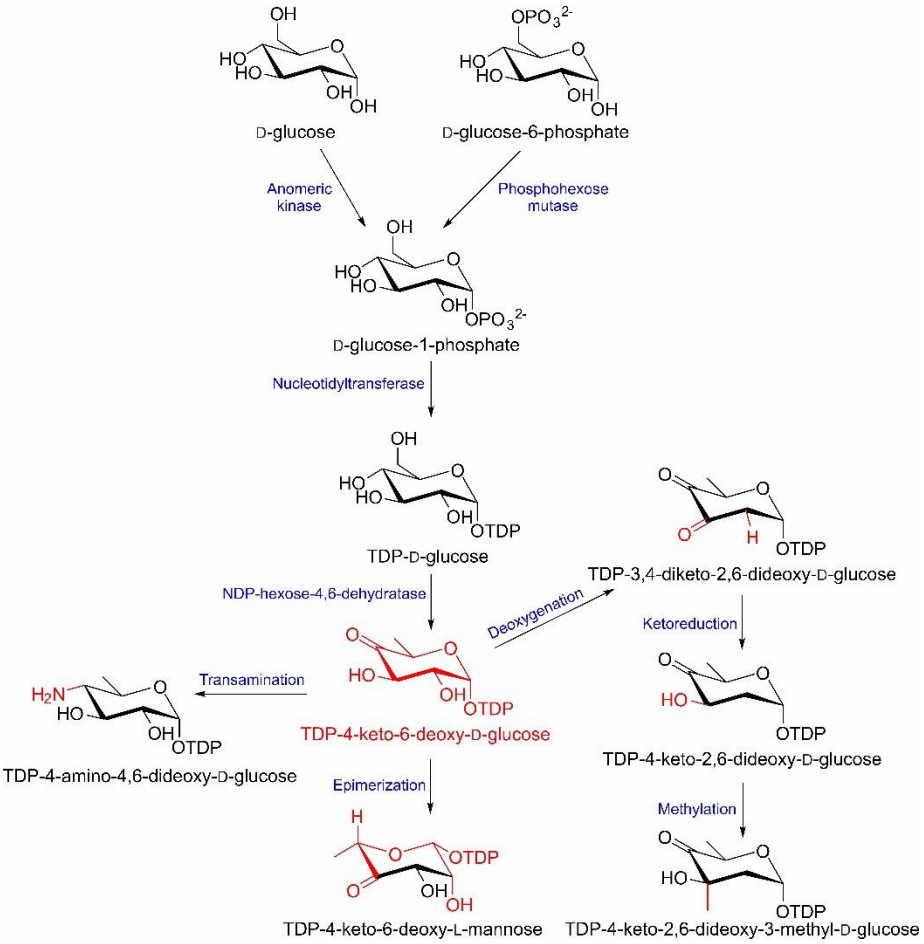

Figure 10. Biosynthetic reactions of natural product sugar moieties. D-glucose is modified to produce a common intermediate, TDP-4-keto-6-deoxy-D-glucose. TDP-4-keto-6-deoxy-D-glucose can be modified by pathway-dependent reactions to produce unique sugar moieties.

Sugar-1-phosphates can be generated from sugar-6-phosphates through the action of mutases or by the phosphorylation of D-glucose by anomeric kinases. Thymidine

diphosphate (TDP)-D-glucose is the most common type of activated sugar. In the biosynthesis of bacterial deoxysugars, TDP-D-glucose is typically dehydrated into TDP-4-keto-6-deoxy-D-glucose (Figure 10). The presence of the 4-keto group lowers the pK_a of adjacent protons, making them more chemically reactive. (Thibodeaux et al. 2008.) From this point, biosynthetic pathways diverge as TDP-4-keto-6-deoxy-D-glucose undergoes various modifications, including deoxygenation, transamination, ketoreduction, epimerization, or *C*-, *N*-, or *O*-methylation. These sugar modifications primarily occur prior to attachment to the acceptor molecule, although post-glycosylation modifications are also possible, particularly *O*-methylation (Salas and Méndez 2007.)

Glycosyltransferases play a crucial role in catalyzing glycosylation reactions, where the anomeric substituent of the sugar unit is replaced by a nucleophilic group from the acceptor molecule (Thibodeaux et al. 2008). The sugar unit can be attached to various atoms of the acceptor molecule, including O-, N-, C-, or S-atoms, although O-glycosylation is the most common (Liang et al. 2015). Through the characterization of sugar biosynthetic pathways, it has been discovered that many glycosyltransferases involved in secondary metabolism exhibit broad substrate specificities. These enzymes can display promiscuity towards sugar donors, sugar acceptors, or both. (Salas and Méndez 2007.) The substrate promiscuity of enzymes is facilitated by dynamic changes in enzyme conformations. Flexible loops and side chains can adopt different conformations, allowing the active site to accommodate diverse substrates. (Tokuriki and Tawfik 2009). Factors such as larger active sites and non-specific interactions, like hydrophobic interactions, also contribute to substrate promiscuity (Copley 2017). This promiscuity of glycosyltransferases can be harnessed in combinatorial biosynthesis approaches to attach non-native sugar units to various aglycone acceptors, expanding the chemical diversity of the resulting compounds.

1.5 Future of antibiotic development

The development of novel antibiotics can generally be categorized into two main approaches: discovering antibiotics from natural sources and creating unnatural antibiotics through synthetic chemistry or by utilizing existing biosynthetic pathways. Antibiotics obtained from natural sources serve as valuable starting points for the creation of unnatural antibiotics because they often possess the necessary pharmacophore for binding to the target of interest. Thus, discovering novel antibiotics from natural sources

not only provides potential new drugs but also introduces new pharmacophores, expanding the chemical diversity of antibiotics. The search for novel antibiotics from natural sources can involve exploring less studied targets that have been challenging to obtain or cultivate. Additionally, computational approaches can be employed to predict potential antibiotics from metagenomic data.

Unnatural antibiotics can be generated through various methods, such as combinatorial chemistry. However, it is important to note that the vast number of possible small molecules with drug-like characteristics exceeds an astronomically large number (more than 10^{60}) (Wetzel et al. 2011). Moreover, since proteins and their binding sites are evolutionarily conserved, the number of distinct binding sites for small molecules is limited. Therefore, rational design based on existing antibiotic core structures becomes advantageous when creating novel antibiotics. Combinatorial biosynthesis is an approach that leverages the building blocks already present in nature and the promiscuity of enzymes involved in secondary metabolism. This technique enables the combination of building blocks in non-natural ways, leading to the creation of complex molecules with defined stereocenters. Defined stereocenters are especially difficult to create through synthetic chemistry. To conclude this review, we will discuss novel sources for antibiotics, followed by an exploration of considerations for designing unnatural antibiotics. Lastly, we will present glycodiversification as an example of combinatorial biosynthesis, which offers a potential solution for creating novel antibiotics.

1.5.1 Discovery of novel antibiotics from nature

Antibiotics have traditionally been discovered primarily from soil-derived Actinobacteria and plants. However, due to the lack of new discoveries in these sources, researchers have expanded their search to less studied targets, such as microorganisms from extreme environments (Li and Vederas 2009). Marine-derived counterparts of Actinobacteria have been found to produce a wide range of potent bioactive compounds that are not typically found in terrestrial environments. Seawater contains approximately 10^6 bacterial cells per milliliter, and the ocean sediment has a similarly diverse microbial population. Therefore, marine organisms produce antibacterial compounds as chemical warfare against sea-dwelling bacteria, and these compounds often have high potencies due to the diluting effect of seawater environment. (Hughes and Fenical 2010.) Nevertheless, the study of antibiotic-producing organisms in marine environments poses challenges due to

difficulties in obtaining and cultivating them in laboratory conditions. Bacteria naturally inhabit complex environments alongside numerous other organisms, making it challenging to replicate these conditions in pure cultures. (Stewart 2012.) This can be alleviated by experimenting with the culture conditions that simulate the natural environment or by co-culturing the bacteria of interest with “helper” bacteria. The “helper” bacteria often provide an essential process for the growth of the bacteria that have lost this ability. (Lewis et al. 2010).

The vast array of secondary metabolites encoded in bacterial genomes provides an additional avenue for the discovery of new metabolites. However, these metabolites are often not expressed under standard laboratory conditions. Therefore, their discovery requires either activating silent biosynthetic gene clusters (BGCs) within the original host or transferring the BGCs to a heterologous host where they can be expressed. The global regulation of metabolite expression in bacteria can be modulated by altering growth conditions. This can be as straightforward as varying the composition of the culture media. However, in some cases, more sophisticated strategies are necessary. For instance, microbial interactions in co-cultures can stimulate metabolite production as a response. (Rutledge and Challis 2015.) Additional methods to induce changes in metabolite expression include the addition of elicitor molecules to the culture, introducing global mutations to the genome, manipulating transcriptional regulators, or engineering the transcription or translation machinery. In cases where the original host organism is not amenable to genetic manipulation, the entire biosynthetic pathway of the metabolite can be transferred to a heterologous host. Successful implementation of these approaches relies on a deep understanding of the specific biosynthetic pathway involved. (Mao et al. 2018.)

1.5.2 Design of novel antibiotics

The rational design of unnatural antibiotics requires a comprehensive understanding of the necessary chemical properties for successful human use. There are three primary factors that may render an antibiotic unsuitable for drug development: poor solubility or permeability, toxicity, or insufficient efficacy (Kola and Landis 2004). Traditionally, the solubility and permeability properties of effective drug lead compounds have been evaluated using the "rule of 5." According to this rule, if more than two of the following conditions are met, absorption or permeation is likely to be poor: more than 5 hydrogen

bond donors, a molecular weight over 500, a log*P* (octanol-water partition coefficient) value over 5, or more than 10 hydrogen bond acceptors. However, it has been observed that natural products often deviate from the "rule of 5". (Lipinski et al. 2001.) Although Lipinski's "rule of 5" may not directly apply to natural products, successful natural product drugs still tend to adhere to the principles of logP and hydrogen bond donors. An increased logP value typically leads to low solubility in aqueous solutions, while a higher number of hydrogen bond donors incurs a greater desolvation cost for cell membrane permeation and target binding. However, natural products can overcome the desolvation cost by forming intramolecular hydrogen bonds, allowing for more flexibility in adhering to the hydrogen bond rule. (Ganesan 2008.)

O'Shea et al. conducted a study to determine the favorable chemical properties of antibiotics. It was found that antibiotics generally exhibit higher polarity and lower lipophilicity compared to other drugs. This trend was particularly prominent in antibiotics targeting gram-negative bacteria, which can be attributed to the limitations imposed by outer membrane porins during cellular entry. Furthermore, the study revealed that antibiotics targeting gram-negative bacteria had a stricter molecular weight restriction of 600 Da, whereas antibiotics with activity against gram-positive bacteria had higher molecular weights, especially in the case of cell-wall active compounds. (O'Shea and Moser 2008.) Another study identified key properties necessary for the accumulation of compounds within gram-negative *Escherichia coli*. It was found that an effective compound should possess both hydrophilic and hydrophobic moieties, as well as a positive charge, preferably in the form of a non-sterically encumbered primary amine. Rigidity and low globularity of the compound were also observed to be favorable for accumulation in *E. coli*. (Richter et al. 2017.) It is worth noting that there is a delicate balance between the polarity and lipophilicity of an antibiotic. While polarity is essential for solubility, a certain level of lipophilicity is necessary for the compound to permeate cellular lipid membranes.

1.5.3 Combinatorial biosynthesis: glycodiversification

Bacteria utilize a wide range of sugar moieties to generate chemical diversity, with at least 344 distinct sugar moieties identified in bacterial natural products (Elshahawi et al. 2015). The incorporation of sugar units into antibiotics enhances their polarity, resulting in increased water solubility and potentially higher intracellular or extracellular

concentrations. Moreover, sugar units can contribute to the chemical stability of the antibiotic's aglycone moiety and enhance target binding affinity and selectivity. (Liang et al. 2015.) One notable example is the ability of sugar units to bind to DNA in both the minor and major grooves through hydrogen bonding and van der Waals contacts. Deoxysugars, which are moderately hydrophobic, are often involved in DNA binding. The specific pattern of hydrogen bonding and hydrophobic interactions between the sugar unit and DNA can influence substrate selectivity. (Křen and Řezanka 2008.) Glycosylation plays a crucial role in the mechanism of ribosome inhibition by macrolide antibiotics. The sugar unit attached to the macrolactone core determines the length of the oligopeptide synthesized before the macrolide antibiotic halts further elongation (Hansen et al. 2002). Hydroxyl, amino, and methyl groups are important chemical features of sugar units. Hydroxyl and amino groups are capable of forming hydrogen bonds, while methylation of hydroxyl groups or deoxygenation increases hydrophobicity. Furthermore, amino groups are often positively charged under biological conditions, allowing sugar units to engage in ionic interactions.

Glycodiversification involves the creation of compound analogues by attaching different sugar moieties to a core molecule, thereby generating chemical diversity. The three main components of glycodiversification are the sugar, the aglycone, and the glycosyltransferase responsible for connecting them. (Thibodeaux et al. 2008.) The key to linking the aglycone and non-natural sugar moieties lies in the promiscuity of the glycosyltransferase, which determines the possible combinations of sugars that can be attached to the aglycone. The sugar and aglycone moieties can be synthesized chemically or produced by either the native or a heterologous host. In terms of cost efficiency, *in vivo* production is preferable for both the glycosyltransferase and its substrates. (Gantt et al. 2011.) When biosynthesizing the substrates in a heterologous host, several factors come into play, including understanding the relevant biosynthetic pathways, the host's genetic manipulability, and the compatibility between the host and the expressed gene(s). Ideally, the sugar, aglycone, and glycosyltransferase genes would all be expressed in a genetically tractable heterologous host that does not produce other natural products. (Salas and Méndez 2007.)

A notable example of glycodiversification involves the utilization of the sugar promiscuous glycosyltransferase ElmGT from the elloramycin A (**1**) pathway. ElmGT has been employed to create non-native glycosylated versions of the pathway

intermediate 8-demethyl-tetracenomycin C (**2**) (Figure 11a) (Blanco et al. 2001). The natural substrate for ElmGT is permethylated L-rhamnose, which is attached to C8 position of **2** through an *O*-glycosidic bond. Remarkably, ElmGT exhibits broad substrate specificity, enabling the transfer of various sugar moieties to the C8 position of **2**. This includes L- and D-configured 6-deoxysugars, 2,6-dideoxysugars, 2,3,6-trideoxysugars, branched chain sugars, ketosugar, and even a disaccharide (Figure 11b) (Heinrich Decker et al. 1995; Fischer et al. 2002; Lombó et al. 2004; Nybo et al. 2012; Pérez et al. 2005; Rodriguez et al. 2000; Rodríguez et al. 2002; Wohlert et al. 1998). Furthermore, the biosynthetic genes responsible for the production of **2** have been elucidated, allowing glycodiversification be performed in a heterologous host (H. Decker et al. 1995). The success of ElmGT highlights the potential of glycodiversification, although only a fraction of possible combinations has been explored.

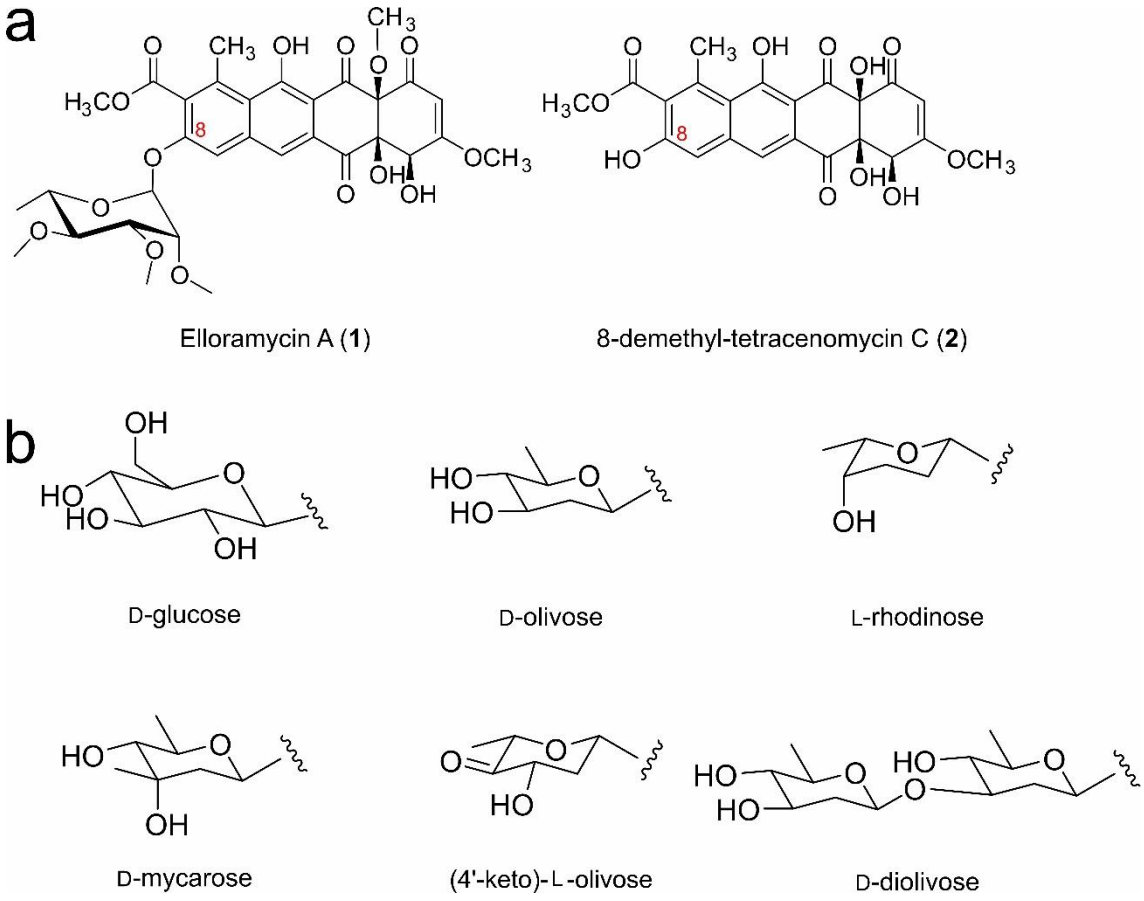

Figure 11. a) Chemical structures of elloramycin A (**1**) and 8-demethyl-tetracenomycin C (**2**). **b)** Examples of the chemically diverse sugar moieties attached to C8 of **2** by ElmGT.

2 Aims of the Study

This study focuses on the development of novel antibiotics through glycodiversification of tetracenomycins. Tetracenomycins have not been extensively used as antibiotics and are unlikely to exhibit cross-resistance to existing antibiotics due to a unique binding site recently discovered in the ribosome.

The specific aims of this study are as follows:

1) Glycodiversification of tetracenomycins to enhance their physicochemical properties and increase their specificity towards prokaryotic ribosomes

2) Elucidation of the structure-activity relationships based on the glycosylation patterns of tetracenomycins

3 Materials and Methods

3.1 Reagents

All reagents were bought from SigmaAldrich unless stated otherwise.

3.2 Bacterial strains and growth conditions

S. coelicolor M1146::cos16F4iE transformants were maintained on mannitol soya flour (MS) plates (Mannitol 20 g/L, soya flour 20 g/L, agar 20 g/L) supplemented with thiostrepton and apramycin at +30 ℃ for five days. The resulting *S. coelicolor* M1146/cos16F4iE transformant spores were used to inoculate 250 mL Erlenmeyer flasks containing 50 mL of E1 media. E1 media consisted of glucose 20 g/L, starch 20 g/L, cottonseed flour 5 g/L, yeast extract 2.5 g/L, K_2HPO_4 x 3 H_2O 1.3 g/L, $MgSO_4$ x 7 H_2O 1 g/L, NaCl 3 g/L, and $CaCO_3$ 3 g/L, and it was supplemented with thiostrepton and apramycin, and incubated for three days (+30 ℃, 300 rpm). This seed culture was used to inoculate (4 % v/v) 2 L Erlenmeyer flasks containing 250 mL of E1 media supplemented with thiostrepton and apramycin, which were incubated for seven days (+30 ℃, 250 rpm).

3.3 Isolation and purification of compounds

3.3.1 Isolation of compounds

From 50 mL cultures, the compounds were extracted by adding an equal volume of ethyl acetate/0.1 % formic acid. Ethyl acetate phase was collected, dried in rotary evaporator, and resuspended in methanol. From 250 mL cultures, compounds were extracted by adding 20 g/L LXA1180 resin (SUNRESIN) and incubating overnight (180 rpm, +30 ℃). LXA-1180 resin was separated from the culture and the compounds were eluted from the resin with methanol. Methanol was evaporated using a rotary evaporator and compounds were resuspended in minimal volume of methanol.

3.3.2 Detection of compounds

Compounds were detected by HPLC-UV/Vis and HPLC-MS analyses from the ethyl acetate extracts. HPLC-UV/Vis analyses were carried out using a Shimadzu Nexera X3 system equipped with a photodiode array detector and a Kinetex C18 column (1.7 µm, 100 Å, 100 x 2.1 mm, Phenomenex) [Method: solvent A: H2O/0.1 % TFA; solvent B: CH3CN; flow rate: 0.3 mL/min; 0-2 min, 0 % B; 2-22 min, 0-100 % B; 22-24 min, 100 % B; 24-29 min, 0 % B]. HPLC-MS analyses were carried out using an Agilent 6120 Quadrupole LCMS system linked to an Agilent Technologies 1260 infinity HPLC system using identical columns, gradients, and buffers as for HPLC-UV analyses.

3.3.3 Purification of compounds 2, 6, 8, 11, 12, and 13.

Methanol extracts from cultures were purified by silica chromatography. Silica chromatography was performed using high-purity grade silica (pore size 60 Å, 230-400 mesh particle size) and a gradient elution from 99:0:1 $CHCl_3$/MeOH/HCOOH to 0:99:1 $CHCl_3$/MeOH/HCOOH. Fractions from silica chromatography containing compounds of interest were evaporated using a rotary evaporator and suspended in minimal volume of methanol for semi-preparative HPLC. Semi-preparative HPLC were carried out using a LC-20AP/CBM-20A system with a diode array detector (Shimadzu) and EVO C18, 5 µm, 100 Å, 250 x 21.2 mm Kinetex column (Phenomenex) [Method A: solvent A: H_2O/0.1 % TFA; solvent B: CH_3CN; flow rate: 20 mL/min; 0-2 min, 0 % B; 2-22 min, 0-100 % B; 22-24 min, 100 % B; 24-29 min, 0 % B; Method B: solvent A: 50 % 60 mM ammonium acetate – acetic acid pH 3.6, 15 % CH_3CN, 35 % H_2O; solvent B: CH_3CN; flowrate: 20 mL/min; 0-2 min, 0 % B; 2-20 min, 0-60 % B; 20-24 min, 100 % B; 24-29 min, 0 % B]. Semi-preparative HPLC method A was utilized for **12** and method B for **2**, **6**, **8**, **11**, and **13**. Fractions containing pure compounds were extracted with ethyl acetate, dried using rotary evaporator and desiccator, and resuspended in deuterated solvents for NMR measurements.

3.3.4 Purification of compound 4

Methanol extracts from cultures were purified by size exclusion chromatography using Sephadex LH-20 with methanol as eluent. Fractions containing compounds of interest were evaporated using a rotary evaporator and suspended in minimal volume of methanol for semi-preparative HPLC. Semi-preparative HPLC were carried out using a LC-20AP/CBM-20A system with a diode array detector (Shimadzu) and EVO C18, 5 µm,

100 Å, 250 x 21.2 mm Kinetex column (Phenomenex) [Method: solvent A: 50 % 60 mM ammonium acetate – acetic acid pH 3.6, 15 % CH_3CN, 35 % H_2O; solvent B: CH_3CN; flowrate: 20 mL/min; 0-2 min, 0 % B; 2-20 min, 0-60 % B; 20-24 min, 100 % B; 24-29 min, 0 % B]. Fractions containing pure compounds were extracted with ethyl acetate, dried using rotary evaporator and desiccator, and resuspended in deuterated solvents for NMR measurements.

3.4 HR-MS and NMR analysis of compounds

NMR spectra were recorded with 600 Mhz Bruker AVANCE-III system with liquid nitrogen cooled Prodigy TCI cryoprobe or 500 Mhz Bruker AVANCE-III system with liquid nitrogen cooled Prodigy BBO cryoprobe. All NMR spectra were processed in Bruker TopSpin 4.1.3 version and the signals were internally referenced to the solvent signals or tetramethylsilane. High resolution electrospray ionization mass spectra were recorded on Bruker Daltonics micrOTOF system.

4 Results

Tetracenomycins are polyketide antibiotics with a naphthacenequinone chromophore that resembles anthracyclines and tetracyclines. However, they are distinguished by a unique cyclohexanone moiety that is highly hydroxylated (Figure 12a) (Rohr and Zeeck 1990). Previous studies have shown that tetracenomycins exhibit moderate antibacterial activity against gram-positive bacteria (Weber et al. 1979). Recently, the mechanism of action for tetracenomycins has been elucidated. Tetracenomycins bind to a distinct site in the polypeptide exit tunnel of the large ribosomal subunit, which prevents elongation of the nascent RNA (Figure 12b). Notably, this unique binding site sets tetracenomycins apart from other antibiotics, such as macrolides, and results in a lack of cross-resistance. (Osterman et al. 2020.) Consequently, tetracenomycins meet the World Health Organization's criteria for innovative antibiotics due to their absence of cross-resistance (World Health Organization 2019). However, it should be noted that tetracenomycins exhibit cytotoxicity towards human cell lines as well (Osterman et al. 2020; Rohr and Zeeck 1990). These findings prompted us to develop novel antibiotics based on the tetracenomycins scaffold, with the goal of achieving specificity for prokaryotic ribosomes while maintaining a lack of cross-resistance with other antibiotics.

Glycosylation plays a significant role in the ribosome inhibition mechanism of macrolide antibiotics (Hansen et al. 2002). Similarly, glycosylation could also influence the ribosome binding specificity of tetracenomycins, which share the same mechanism of action as macrolides. Tetracenomycins adhere to the two essential rules of Lipinski's rule of 5. For instance, 8-demethyl-tetracenomycin C (**2**) possesses five hydrogen bond donors and a calculated log*P* of -1.41 (Figure 12a). However, the polarity of tetracenomycins remains below the average for antibiotics with activity against gram-negative bacteria (O'Shea and Moser 2008). It has been observed that effective antibiotics targeting gram-negative bacteria should contain both hydrophilic and hydrophobic moieties (Richter et al. 2017). The polycyclic aromatic core of tetracenomycins is hydrophobic, and an attached sugar unit could function as the desired hydrophilic moiety for accumulation inside gram-negative bacteria. The molecular weight of **2** is 458 Da, thus it is possible to add one sugar moiety to **2** while still maintaining a low enough molecular weight (<600 Da), which is required for penetrating the outer membrane of gram-negative *E. coli* (O'Shea and Moser 2008). Therefore, novel glycosylated analogues of **2** could exhibit increased specificity towards prokaryotic ribosomes over human ribosomes, antibacterial

activity against both gram-positive and gram-negative bacteria, and improved physicochemical properties.

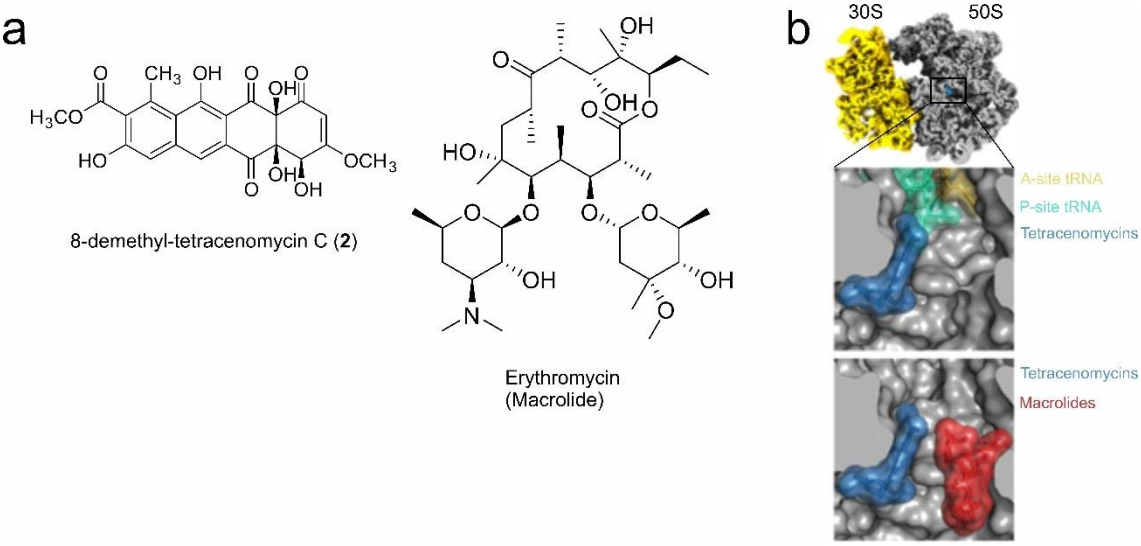

Figure 12. a) Chemical structure of 8-demethyl-tetracenomycin C (**2**) and an example structure of a macrolide, erythromycin. **b)** Both tetracenomycins and macrolides bind to the polypeptide exit tunnel of ribosomes, but at opposite sites from each other.

4.1 Design of bacterial strains to increase the glycodiversity of tetracenomycins

The genes necessary for biosynthesis of **2** have previously been identified and isolated on the cosmid cos16F4 (Blanco et al. 2001). Additionally, cos16F4 contains the genes encoding a sugar promiscuous glycosyltransferase, ElmGT, which attaches a TDP-L-rhamnose to the C8-position of **1** as its native function. To enhance the genetic stability of the production of **2**, Nguyen et al. developed an integrating vector cos16F4iE, which was used to insert cos16F4 into the genome of *S. coelicolor* M1146 (Nguyen et al. 2022). The endogenous antibiotic biosynthetic gene clusters have been removed from the genome of *S. coelicolor* M1146, which increases the precursor availability for heterologously expressed compounds and makes compound detection and purification easier (Gomez-Escribano and Bibb 2011). Therefore, *S. coelicolor* M1146::cos16F4iE was chosen as the heterologous host for glycodiversification of tetracenomycins in this study. *S. coelicolor* M1146::cos16F4iE is capable of producing **2** and the glycosyltransferase ElmGT, thus only the biosynthetic genes required for modifying the primary metabolism metabolite glucose-1-phosphate need to be added to the host (Figure 13a).

BioBricks is an assembly standard that facilitates the sharing of genetic parts among researchers worldwide. This standardization is achieved through the use of circular carrier plasmids containing standardized restriction enzyme sites that flank the DNA sequence, known as “BioBricks parts”. The restriction enzyme sites serve as connection points, allowing any two BioBricks parts to be joined together. Importantly, the restriction enzyme sites remain unchanged after the parts are ligated together (Figure 13b). (Knight 2003.) This feature simplifies the process of assembling multiple BioBricks parts without the need for additional design and manipulation. Consequently, BioBricks was chosen for this study, as the constructed BioBricks parts can be reused to generate different sugars in future experiments.

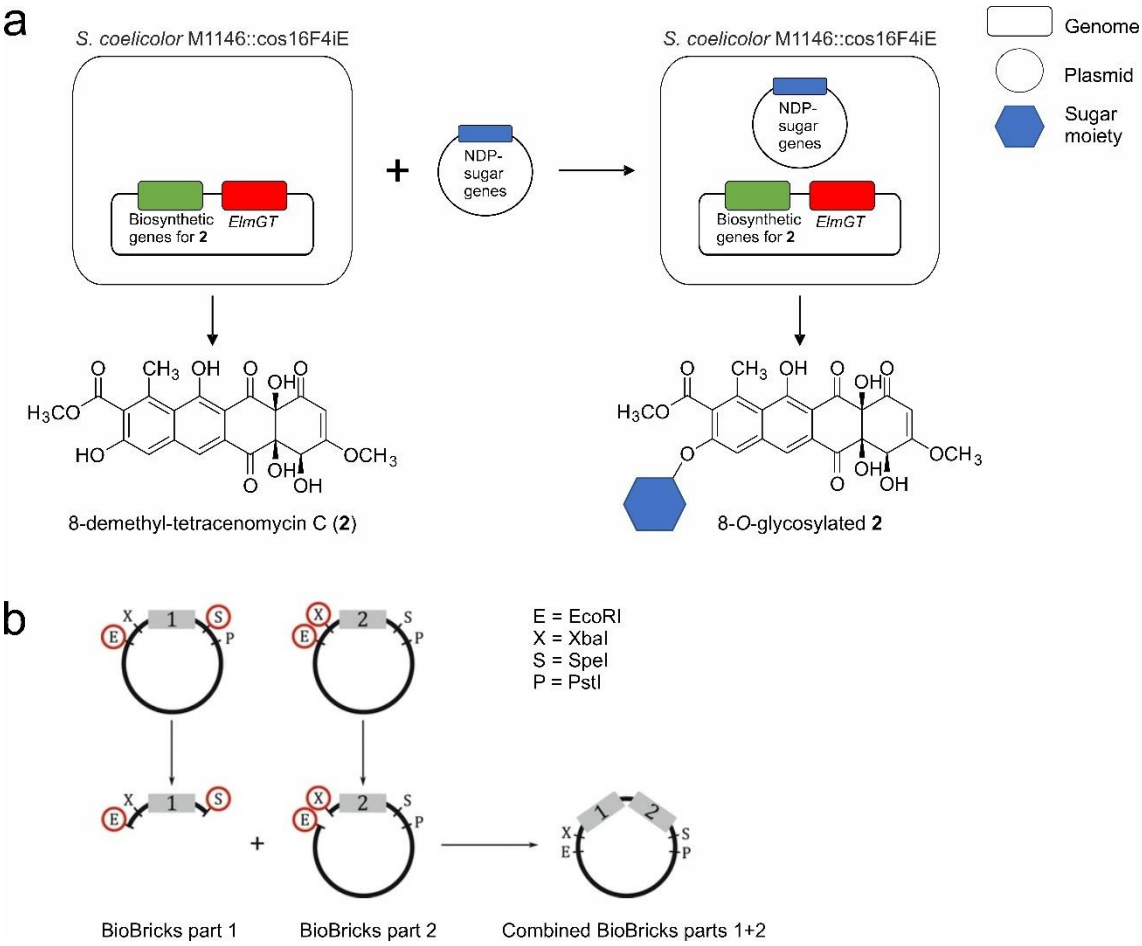

Figure 13. a) Heterologous expression strategy for glycodiversification of tetracenomycins. *S. coelicolor* M1146::cos16F4iE has the genes for production of **2** and ElmGT integrated into the genome, therefore by different plasmids containing sugar biosynthetic genes will result in the production of differently glycosylated versions of **2**. **b)** BioBricks assembly standard RFC[10]. Four restriction enzyme sites flank the DNA

fragments 1 & 2, which can be cut and ligated together while preserving the restriction enzyme sites. Figure adapted from Røkke et al. 2014.

The selection of sugar moieties for the glycodiversification of tetracenomycins was based on several factors, including the availability of characterized sugar biosynthetic pathways, knowledge of ElmGT's promiscuity, and the goal of achieving chemical diversity. A total of 22 unique sugar moieties were chosen for this study (Figure 14). Among these, ten sugar moieties had previously been successfully attached to compound **2** by ElmGT, while the remaining twelve had not been tested with ElmGT before. The selected sugars are chemically diverse ranging from hydrophilic to moderately hydrophobic. They include various structural groups of sugars in L- and/or D-configuration, such as 6-deoxysugars in both pyranose and furanose forms, 2,6-dideoxysugars, 2,3,6-trideoxysugars, 3-amino-2,3,6-trideoxysugars, 6-deoxy-3-methyl sugar, 6-deoxy-4-methyl sugar, and 2,6-dideoxy-3-methyl sugar (Figure 14). ElmGT is an inverting glycosyltransferase, meaning that the anomeric configuration of sugar moieties is inverted during their attachment to **2** (Lombó et al. 2004). The addition of an amine moiety, particularly a non-sterically encumbered primary amine, has been shown to enhance the accumulation of the antibiotics in gram-negative *E. coli* (Richter et al. 2017). While aminosugars have not been tested as substrates for ElmGT, their inclusion in the glycodiversification process could be beneficial for generating a broad-spectrum antibiotic.

In addition to the glycodiversification of tetracenomycins, the construction of sugar plasmids provided an opportunity to investigate the functions of uncharacterized enzymes from sugar biosynthetic pathways. One such example is the rare deoxysugar D-fucofuranose, which is found in the gilvocarcin pathway. The biosynthetic pathway for D-fucofuranose not yet been fully elucidated. The 4-ketoreductase GilU has been identified as responsible for reducing the intermediate TDP-4-keto-6-deoxy-D-glucose to TDP-D-fucopyranose (T. Liu et al. 2009). However, the enzyme involved in the ring contraction of 6-membered TDP-D-fucopyranose to 5-membered TDP-D-fucofuranose has not to be discovered (Figure 14). Among the genes in the gilvocarcin gene cluster, the functions of almost all have been determined or rendered unnecessary, with the exception of the gene *GilV*, which is a potential candidate for producing the enzyme required for the ring contraction reaction (Fischer et al. 2003). Therefore, we aimed to

test the role of *GilV* by constructing a plasmid containing the biosynthetic genes for producing TDP-D-fucopyranose, with *GilV* included in the operon.

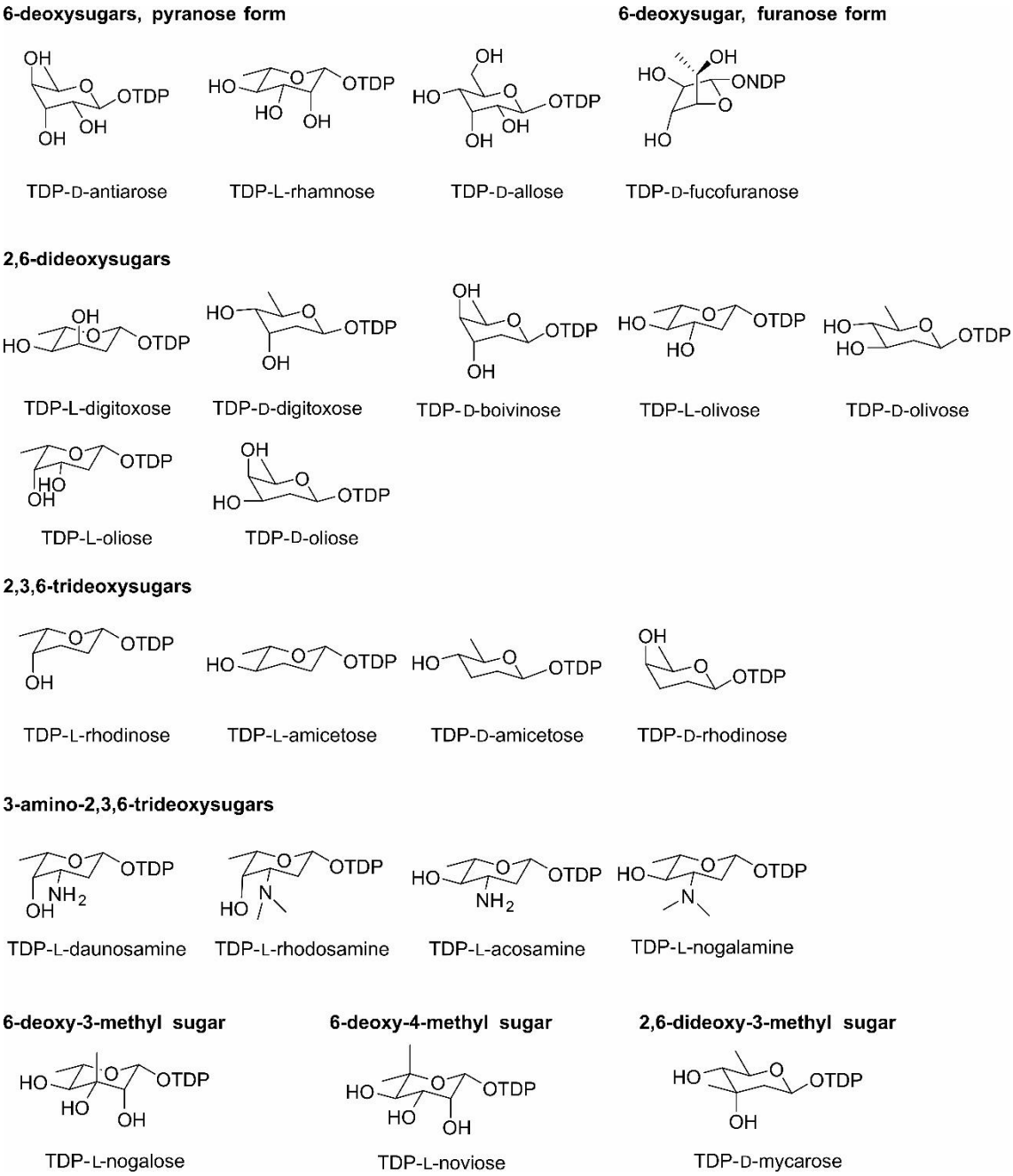

Figure 14. Chemical structures of sugar moieties used for glycodiversification of **2** in this study.

In this study, a total of 22 plasmids containing biosynthetic genes for different sugar moieties were generated by Prof. Eric Nybo's group according to the BioBricks RFC[10] standard (Table 2) (Knight 2003). The biosynthetic genes for the sugars were cloned as a single operon under a strong *rCFp* promoter into a *Streptomyces-E. coli* multi-copy shuttle vector pUWL201PWBB (Shao et al. 2013) (Kieser et al. 1982). The generated

plasmids were conjugated from *E. coli* to *S. coelicolor* M1146::cos16F4iE by M. Sc. Magdalena Niemczura (Table 3). All the plasmids shared two common genes, namely TDP-D-glucose synthase (desIII) and TDP-D-glucose-4,6-dehydratase (desIV), which are necessary for the biosynthesis of the common intermediate TDP-4-keto-6-deoxy-D-glucose. The specific biosynthetic genes for each sugar moiety varied depending on the respective biosynthetic pathway.

Table 2. Plasmids constructed and/or used for this study. The sugar moieties that have not been previously tested with ElmGT are in bold. The sugar biosynthetic genes are in a single operon under control of *rCFp* promoter in pUWL201PWBB plasmid.

Plasmid	Sugar biosynthetic genes	Expected product
pDALO	***desIII+desIV+gerF+gerK1***	**TDP-D-allose**
pDAMI	*desIII+desIV+oleVW+urdQ+urdR*	TDP-D-amicetose
pDARO	***desIII+desIV+rubS3***	**TDP-D-antiarose**
pDBOV	*desIII+desIV+oleV+eryBII+oleU*	TDP-D-boivinose
pDDIG	*desIII+desIV+oleV+eryBII+urdR*	TDP-D-digitoxose
pDFUCO	***desIII+desIV+gilU+gilV***	**TDP-D-fucofuranose**
pDMYC	*desIII+desIV+oleVW+cmmC+mtmTIII*	TDP-D-mycarose
pDOLI	***desIII+desIV+oleVWU***	**TDP-D-oliose**
pDOLV	*desIII+desIV+oleVW+urdR*	TDP-D-olivose
pDRHO	***desIII+desIV+oleVW+urdQZ3***	**TDP-D-rhodinose**
pLACO	***desIII+desIV+dnmTJU+aveBIV+snogAX***	**TDP-L-acosamine**
pLAMI	*desIII+desIV+oleVWL+urdQ+eryBIV*	TDP-L-amicetose
pLDAU	***desIII+desIV+dnmTJUV***	**TDP-L-daunosamine**
pLDIG	*desIII+desIV+oleV+eryBII+oleL+eryBIV*	TDP-L-digitoxose
pLNGA	***desIII+desIV+dnmTJU+avrE+snogAX***	**TDP-L-nogalamine**
pLNGO	***desIII+desIV+SnogG2FC***	**TDP-L-nogalose**
pLNOV	***desIII+desIV+novWUS***	**TDP-L-noviose**
pLOLI	***desIII+desIV+oleVWL+urdR***	**TDP-L-oliose**
pLOLV	*desIII+desIV+oleVWLU*	TDP-L-olivose
pLRHA	*desIII+desIV+oleLU*	TDP-L-rhamnose
pLRHO	*desIII+desIV+oleVWL+urdQZ3*	TDP-L-rhodinose
pLRAM	***desIII+desIV+dnmTJUV+snogAX***	**TDP-L-rhodosamine**

Table 3. Bacterial strains used/generated for this study.

Strain	Genotype/comments
Streptomyces coelicolor M1146::cos16F4iE	SCP1- SCP2- Δact Δred Δcpk Δcda , cos16F4iE integrated into ϕC31 attB chromosomal locus
Streptomyces coelicolor M1146::cos16F4iE/pDALO	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pDALO
Streptomyces coelicolor M1146::cos16F4iE/pDAMI	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pDAMI
Streptomyces coelicolor M1146::cos16F4iE/pDARO	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pDARO
Streptomyces coelicolor M1146::cos16F4iE/pDBOV	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid PDBOV
Streptomyces coelicolor M1146::cos16F4iE/pDDIG	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pDDIG
Streptomyces coelicolor M1146::cos16F4iE/pDFUCO	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pDFUCO
Streptomyces coelicolor M1146::cos16F4iE/pDMYC	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pDMYC
Streptomyces coelicolor M1146::cos16F4iE/pDOLI	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pDOLI
Streptomyces coelicolor M1146::cos16F4iE/pDOLV	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pDOLV
Streptomyces coelicolor M1146::cos16F4iE/pDRHO	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pDRHO
Streptomyces coelicolor M1146::cos16F4iE/pLACO	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLACO
Streptomyces coelicolor M1146::cos16F4iE/pLAMI	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLAMI
Streptomyces coelicolor M1146::cos16F4iE/pLDAU	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLDAU
Streptomyces coelicolor M1146::cos16F4iE/pLDIG	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLDIG
Streptomyces coelicolor M1146::cos16F4iE/pLNGA	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLNGA
Streptomyces coelicolor M1146::cos16F4iE/pLNGO	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLNGO
Streptomyces coelicolor M1146::cos16F4iE/pLNOV	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLNOV
Streptomyces coelicolor M1146::cos16F4iE/pLOLI	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLOLI
Streptomyces coelicolor M1146::cos16F4iE/pLOLV	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLOLV
Streptomyces coelicolor M1146::cos16F4iE/pLRHA	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLRHA
Streptomyces coelicolor M1146::cos16F4iE/pLRHO	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLHRO
Streptomyces coelicolor M1146::cos16F4iE/pLRAM	*Streptomyces coelicolor* M1146::cos16F4iE transformed with plasmid pLRAM

4.2 Analysis of the compounds produced by the generated bacterial strains

The compounds produced by the generated bacterial strains were extracted from the culture using ethyl acetate, which is a non-miscible solvent with water. Ethyl acetate was chosen for its moderately polarity, volatility, and ability to effectively extract compound **2** from an aqueous solution. This extraction process enables subsequent drying of the extract, which concentrates the compounds and facilitates detection of small quantities. The produced compounds were initially detected from the ethyl acetate extracts using reverse phase high-performance liquid chromatography (HPLC) coupled with ultraviolet/visible light (UV/Vis) detector. Tetracenomycins have a distinct UV/Vis spectrum with maxima at 285, 395, and 412 nm (Figure 15a). As a result, compound **2** can be identified in the HPLC chromatogram trace at 412 nm (Figure 15a). The attachment of the sugar moiety to **2** does not significantly alter its chromophore. Therefore, the UV/Vis spectrum remains highly similar between **2** and its glycosylated analogues.

Reverse phase HPLC with a C18 column separates compounds based on their affinity to the nonpolar C18 stationary phase. Tetracenomycins consist of a nonpolar naphthacenequinone core, which is further decorated with polar hydroxyl groups, nonpolar methyl groups, and moderately polar ether and methyl ester groups. The selected sugar moieties for this study contain a 5- or 6-membered heterocyclic ring with one oxygen atom, which are further decorated with polar hydroxyl or amine groups, or nonpolar methyl groups to varying extents. Overall, the sugar moieties increase the polarity of **2**. As a result, the glycosylated analogues of **2** are expected to elute earlier than non-glycosylated **2** in the HPLC analysis. To detect production of glycosylated analogues of **2**, the strain *S. coelicolor* M1146::cos16F4iE, which lacks the plasmids containing the sugar biosynthetic genes, was used as a negative control (Figure 15b,c). This negative control strain produces only the non-glycosylated **2**. Hence, if successful glycosylation occurs in the generated strains, a new peak should appear in the HPLC chromatogram trace at 412 nm, with a retention time earlier than that of **2**, while exhibiting a similar UV/Vis spectrum.

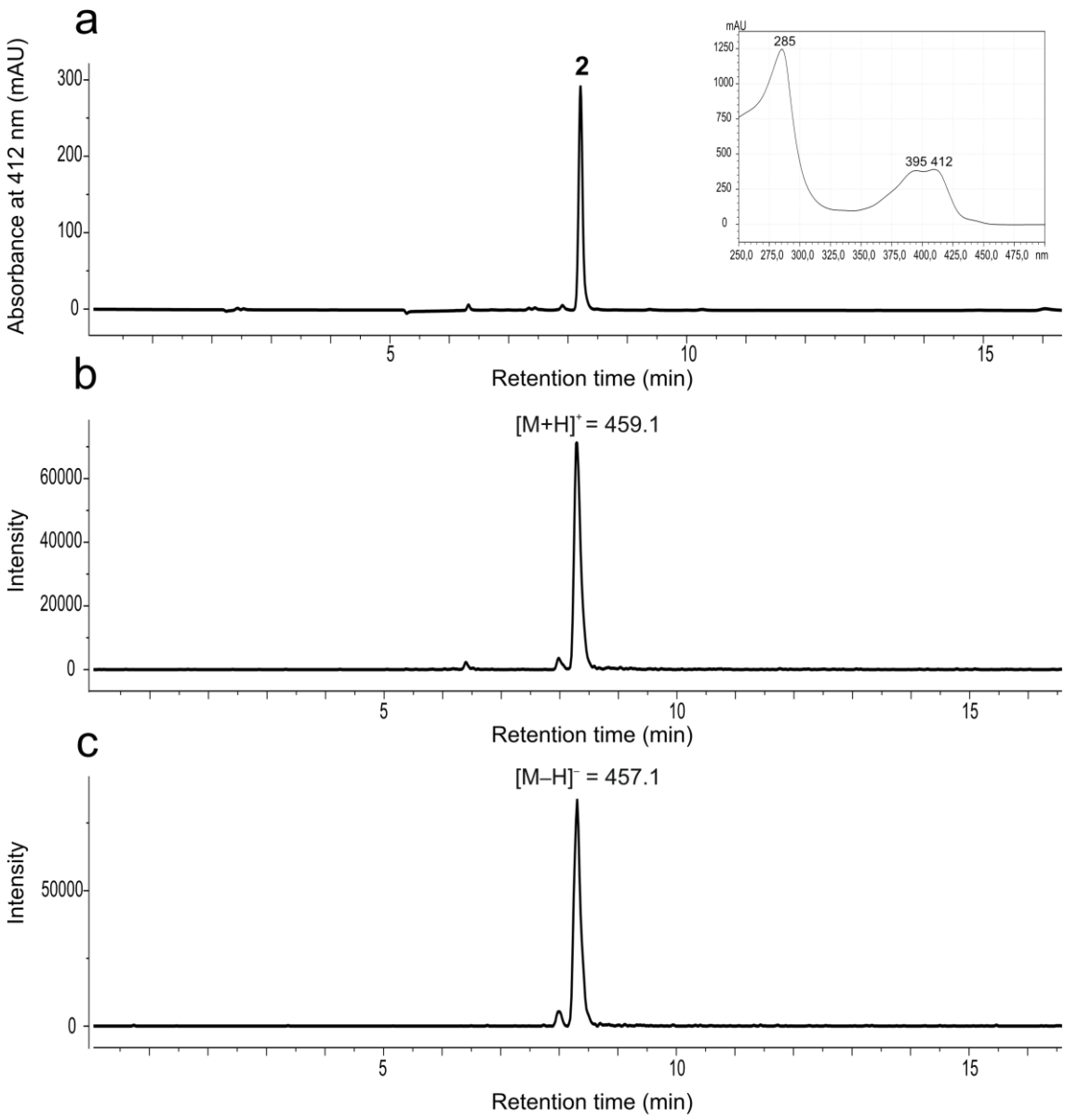

Figure 15. The negative control strain *S. coelicolor* M1146::cos16F4iE was analyzed by HPLC-UV/Vis-MS. **a)** The compound **2** can be detected in the HPLC chromatogram trace at 412 nm and by the typical tetracenomycin UV/Vis spectrum. **b)** Extracted ion chromatogram from positive ionization mode using the m/z value $[M+H]^+ = 459.1$ with 0.05 Da tolerance to detect **2**. **c)** Extracted ion chromatogram from negative ionization mode using the m/z value $[M–H]^– = 457.1$ with 0.05 Da tolerance to detect **2**.

Out of the 22 generated bacterial strains, 16 exhibited new peaks in the HPLC chromatogram trace at 412 nm compared to the negative control strain that produced only **2**. The compound analysis and purification were a collaborative effort with Asst. Prof. Khaled Shaaban's group, therefore, three of the generated strains with new peaks (*S. coelicolor* M1146::cos16F4iE/pDALO, *S. coelicolor* M1146::cos16F4iE/pDMYC, and *S. coelicolor* M1146::cos16F4iE/pLRHA) were analyzed by Asst. Prof. Khaled

Shaaban's group. These three strains produced the desired glycosylated analogues of **2**. The main peaks at 412 nm from the remaining 13 strains assigned to our group were subjected to HPLC-mass spectrometry (MS) analysis (Figure 16). HPLC-MS analysis enables the detection of the mass-to-charge ratio (m/z) values associated with the peaks observed in the 412 nm chromatogram trace. By identifying the correct molecular ion, such as $[M+H]^+$ in positive ionization mode or $[M–H]^-$ in the negative ionization mode, along with the characteristic UV/Vis spectrum of tetracenomycins, it is possible to detect successful glycosylation of **2**. Moreover, the electrospray ionization (ESI) voltage causes a small amount of fragmentation of the sugar moiety from **2**, therefore the molecular ion for **2** is also detectable at the glycosylated peaks. This further confirms that the assigned peaks are derivatives of **2**.

Out of the 13 strains analyzed, eight successfully produced the intended glycosylated analogues of **2**, although with varying conversion efficiencies ranging from 2 % to 40 %. The detection of the corresponding molecular ion in HPLC-MS analysis confirmed the presence of glycosylated analogues (**Figure 17**). Among the strains containing biosynthetic genes for 2,6-dideoxysugars, five strains produced the correct glycosylated analogues of **2**. The detection of the correct molecular ions ($[M+H]^+$ = 589.2, $[M–H]^-$ = 587.1) supported the identification of these analogues, which share the same molecular weight as the differences in these sugars are in the stereochemical configuration only. The glycosylated analogues identified were 8-demethyl-8-*O*-D-boivinosyl-tetracenomycin C (**3**), 8-demethyl-8-*O*-D-digitoxosyl-tetracenomycin C (**4**), 8-demethyl-8-*O*-D-oliosyl-tetracenomycin C (**5**), 8-demethyl-8-*O*-D-olivosyl-tetracenomycin C (**6**), and 8-demethyl-8-*O*-L-olivosyl-tetracenomycin C (**7**) (Figure 16a-e). Out of these, **5** is a novel compound; however, its production yield was too low to confirm its structure through nuclear magnetic resonance (NMR) spectroscopy.

Unfortunately, none of the intended aminosugars were successfully attached to **2**, as their molecular ions were not detected in the HPLC-MS analysis (Figure 16f-i). Instead, the molecular ion corresponding to 8-demethyl-8-*O*-D-glucosyl-tetracenomycin C (**8**, $[M+H]^+$ = 621.1, $[M–H]^-$ = 619.1) was detected (Figure 16). TDP-D-glucose is a common intermediate generated from D-glucose-1-phosphate by TDP-D-glucose synthase (desIII) included in all the plasmids, which has been previously attached to **2** by ElmGT (Fischer et al. 2002). Among the strains containing biosynthetic genes for 2,3,6-trideoxysugars, two out of the four strains produced the correct glycosylated analogues of **2** based on the

presence of correct molecular ions ($[M+H]^+$ = 573.1, $[M–H]^–$ = 571.2) (Figure 16j-l). These were 8-demethyl-8-*O*-D-amicetosyl-tetracenomycin C (**9**) and 8-demethyl-8-*O*-D-rhodinosyl-tetracenomycin C (**10**). The strain containing the biosynthetic genes for the formation of 6-deoxysugar, TDP-D-fucofuranose, showed two peaks on the HPLC chromatogram trace at 412 nm with correct molecular ions ($[M+H]^+$ = 605.1, $[M–H]^–$ = 603.1), indicating the successful attachment of either TDP-D-fucose or TDP-D-fucofuranose to **2** (Figure 16m). However, it is not possible to distinguish between TDP-D-fucose and TDP-D-fucofuranose based on mass spectroscopy alone, as they have the same chemical formula and molecular mass.

Among the strains, some produced glycosylated analogues of **2** with an attached ketosugar, as indicated by the presence two molecular ions with mass difference of 18 Da. Ketosugars are interconverted to the hydrate form by the addition of water (18 Da) to the keto group under aqueous conditions. While ketosugars are typically not substrates for glycosyltransferases as pathway intermediates, ElmGT has been previously shown to accept ketosugars (Nybo et al. 2012). Surprisingly, seven of the strains started to produce a peak with molecular mass of 444.1 Da ($[M+H]^+$ = 445.1, $[M–H]^–$ = 443.1). This indicates the presence of a molecule with a mass difference of 14 Da to **2** (M = 458.1 Da), which is typically caused by the absence of a methyl group. Therefore, the molecular mass of 444 Da most likely corresponds to didemethyl-tetracenomycin C (Figure 16). The biosynthetic pathway of **2** includes two methyltransferases (ElmNII/ElmP), and the detection of the didemethyl-tetracenomycin C peak suggests that one of these methyltransferases did not function properly in these strains. Furthermore, some of the glycosylated analogues did not have **2** as their aglycone, but instead, a methylated version of **2** ($[M+H]^+$ = 473.2, $[M–H]^–$ = 471.1). This is most likely 8-demethyl-12a-*O*-methyl-tetracenomycin C, as the 12a-*O*-methyltransferase ElmD is included in cos16F4iE. (Ramos et al. 2008).

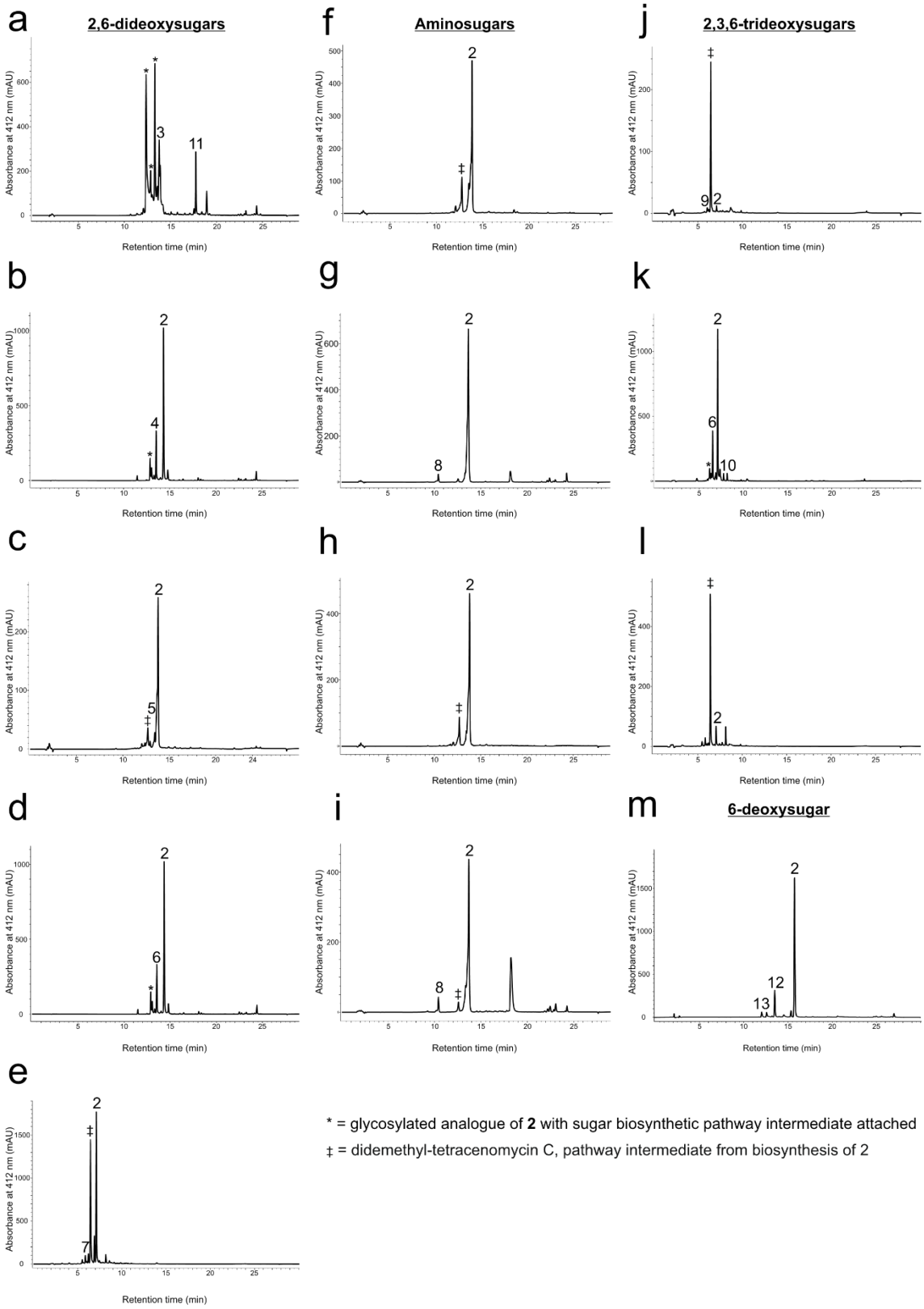

Figure 16. HPLC-UV/Vis chromatograms at 412 nm showing different production profiles obtained from the cell extracts of *Streptomyces coelicolor* M1146::cos16F4iE transformed with different plasmids: **a)** pDBOV, **b)** pDDIG, **c)** pDOLI, **d)** pDOLV, **e)**

pLOLV, **f)** pLACO, **g)** pLDAU, **h)** pLNGA, **i)** pLRAM, **j)** pDAMI, **k)** pDRHO, **l)** pLRHO, and **m)** pDFUCO.

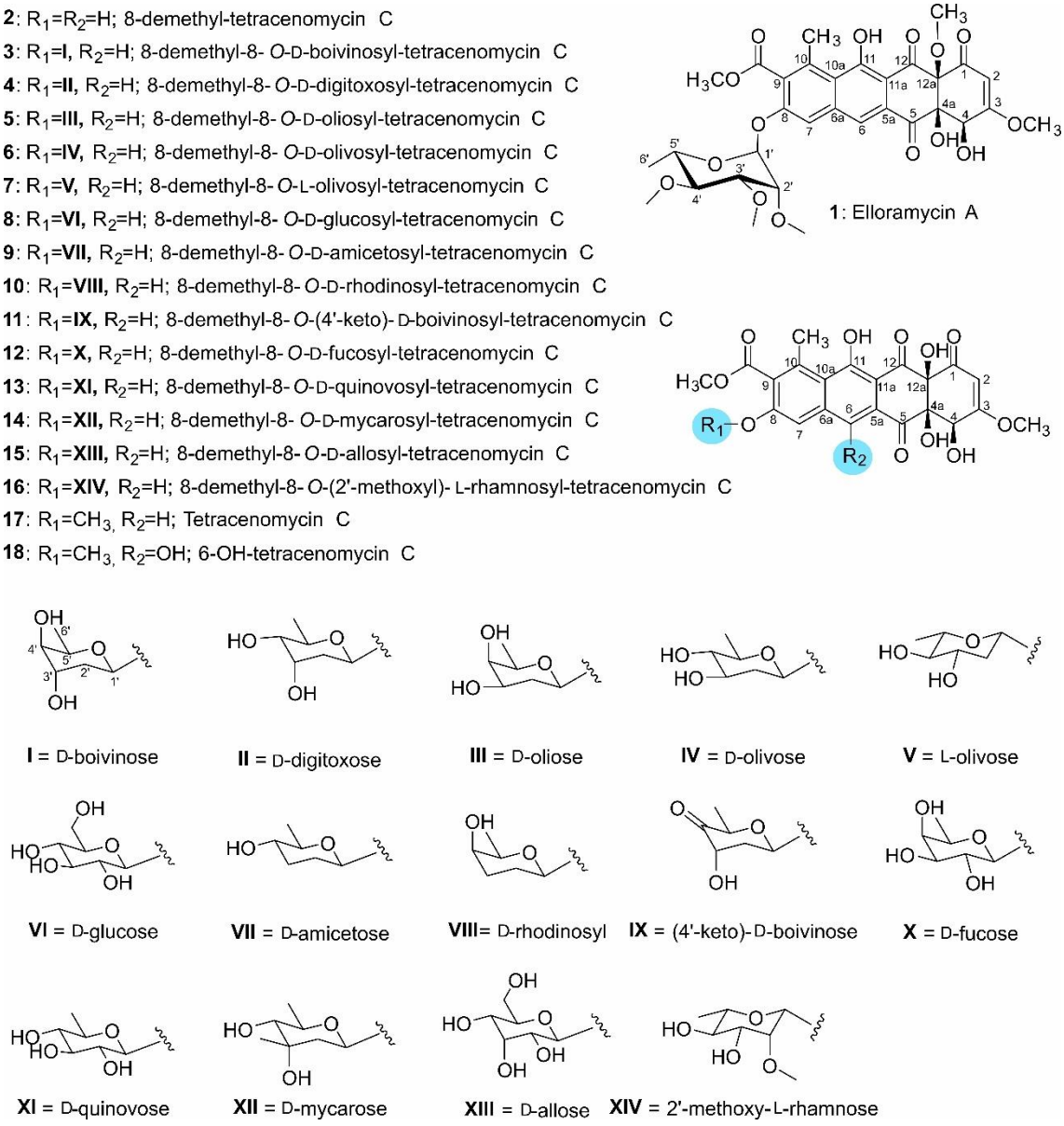

Figure 17. Chemical structures of compounds successfully produced and purified in this study.

4.3 Up-scaled production and purification of compounds

Up-scaled production of the compounds was necessary for two reasons: 1) bioactivity measurements require highly pure compounds, and 2) novel compounds need to undergo full NMR spectroscopy characterization to confirm their structures. NMR spectroscopy typically requires approximately 1-5 mg of purified compound, which could not be obtained from the 50 mL cultures. Therefore, the strains containing the following

plasmids were selected for up-scaled production and purification: pDBOV, pDDIG, pDOLV, pDRHO, and pDFUCO. The remaining strains either did not produce any glycosylated analogues of **2** or exhibited very low production levels, making it impractical to isolate the compounds (less than 3% of all tetracenomycins produced). The compounds were produced under conditions as identical as possible to the small-scale cultures, including media composition and temperature, but in larger volumes (250 mL) and culture flasks (2 L). However, the slight changes in culture conditions led to alterations in the production profile of *Streptomyces coelicolor* M1146::cos16F4iE/pDRHO, from which no glycosylated analogues of **2** could be produced in up-scaled conditions. Despite numerous attempts, it was not feasible to obtain sufficient quantities of **10** for purification. Glycosylated analogues of **2** from the other strains selected for up-scaled production had good yields.

For the up-scaled cultures, the compounds were extracted from the cultures with LXA1180 resin instead of ethyl acetate. LXA1180 is a polymeric adsorbent that binds compounds through hydrophobic interactions. Since the naphthacenequinone core of tetracenomycins is hydrophobic, LXA1180 efficiently absorbs both **2** and its glycosylated analogues from the cultures. Methanol is then used to elute the compounds from LXA1180, requiring smaller volumes compared to ethyl acetate, which simplifies the compound drying process. Subsequently, the compound extracts were purified using silica chromatography, a rapid method capable of separating tetracenomycins from other compound groups. Silica serves as a polar stationary phase in chromatography; compounds are separated based on their ability to engage in polar interactions. The sugar moieties attached to **2** slightly increase their retention time compared to the retention time of **2**. As a result, some of the glycosylated analogues of **2** were effectively separated from the non-glycosylated **2** during silica chromatography.

In order to achieve better separation of the majority of glycosylated analogues of **2**, semi-preparative HPLC with a C18 column was employed. Semi-preparative HPLC offers improved control of the gradient and increased flow rates, resulting in enhanced compound separation compared to silica chromatography. However, it has a smaller maximum sample loading capacity. Through semi-preparative HPLC purification, purities exceeding 95% were obtained for all compounds except compound **4**. In the case of compound **4**, the retention time difference between the glycosylated form and the non-glycosylated **2** was too small to achieve efficient separation using semi-preparative

HPLC. Therefore, an alternative separation strategy was employed, namely size exclusion chromatography using Sephadex LH-20. Sephadex LH-20 separates compounds based on their size, and there was a sufficient size difference (148 Da) between **4** and **2**. This allowed for the successful purification of **4** to a purity of over 95 % using this separation method.

A total of seven different compounds were successfully purified. Compounds **2**, **4**, and **6** were purified from their respective strains. The strain *Streptomyces coelicolor* M1146::cos16F4iE/pDFUCO yielded two compounds with the correct molecular mass for the attachment of either TDP-D-fucose or TDP-D-fucofuranose to **2**. Compound 8, corresponding to the attachment of TDP-D-glucose, was isolated from multiple strains since TDP-D-glucose is a common sugar pathway intermediate in all strains included in this study. Compound **3** was not successfully isolated from the strain *Streptomyces coelicolor* M1146::cos16F4iE/pDBOI due to the abundance of different sugar pathway intermediates attached to **2** in the culture extract. These intermediates posed a challenge during the purification process, as several different compounds eluted with nearly identical retention times (Figure 16a). Nonetheless, a ketosugar intermediate was isolated from this strain, as the compound had a distinct later elution time and could therefore be separated from the other intermediates.

4.4 Structural elucidation of the compounds via HR-MS and NMR analysis

The structural elucidation of the compounds involves the use of both high-resolution mass spectrometry (HR-MS) and NMR spectroscopy. HR-MS offers several advantages over low-resolution MS techniques, such as higher resolution and improved mass accuracy. Low-resolution MS instruments, quadrupole mass spectrometers, can only measure the m/z ratio of an ion to the nearest integral value, which can be informative if the chemical formula of the compound is known. On the other hand, HR-MS instruments, such as time-of-flight (TOF) mass spectrometers, provide a higher level of mass accuracy (typically within 1-2 ppm). This enables the determination of the elemental composition of the compound. The elemental composition acquired from HR-MS measurements should match the numbers of carbons and protons assigned to the compound in NMR spectroscopy.

NMR spectroscopy is typically utilized to detect hydrogen-1 (referred to as protons) and carbon-13 nuclei of organic compounds, as well as their connectivity to one another. NMR experiments require a strong magnetic field to align the magnetic nuclear spins of the compound. During the NMR measurement, the alignment of the nuclear spins is perturbed by a radio-frequency pulse. Each nucleus of the compound absorbs at a specific resonance frequency determined by its isotope and chemical environment. The chemical environment refers to the electrons surrounding the nucleus, which generate local magnetic fields that shield the nucleus and cause a shift in its resonance frequency. Subsequently, the nuclei relax back to equilibrium and emit radio-frequency radiation at their specific frequency, which is measured and mathematically transformed into chemical shifts. The chemical environment of a nucleus can therefore be deduced from the chemical shift. For instance, electronegative oxygens attract nearby electrons from the nuclei connected to oxygen, resulting in a downward shift in the resonance frequency of those nuclei. NMR can only measure nuclei with a non-zero magnetic spin. The most common hydrogen isotope with non-zero magnetic spin is ^{1}H, which has a natural abundance of almost 100 %. On the other hand, the most common carbon isotope with non-zero magnetic spin is ^{13}C, which has a natural abundance of 1 %. As a result, ^{1}H NMR is more sensitive and requires a smaller amount of compound for detection. However, elucidating a novel structure almost always requires ^{13}C measurements to be accurate.

The 1D NMR signals provide general information about the chemical environment of protons or carbons in a molecule. To determine the connectivity of the chemical bonds, *J*-coupling constants and 2D NMR measurements are necessary. Nuclei possess their own small magnetic fields, which influence the resonance frequency of nearby nuclei through chemical bonds. In ^{1}H NMR, *J*-coupling is observed as splitting of signals into multiplets. This allows determination of the magnitudes of two-bond (^{1}H–C–^{1}H) and three-bond (^{1}H–C–C–^{1}H) *J*-coupling constants for each nearby proton. *J*-coupling is also utilized in homonuclear 2D NMR techniques such as correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY). In these techniques, the coupling between a pair of protons is visualized as a cross-peak in a two-dimensional spectrum. COSY displays cross-peaks for two- and three-bond couplings, while TOCSY shows cross-peaks for all protons belonging to the same spin system. Together, *J*-coupling constants, COSY, and TOCSY provide information about the proton connectivity and bond angles.

Through-bond correlation between ^{1}H and ^{13}C nuclei can be detected using heteronuclear single quantum coherence spectroscopy (HSQC) and heteronuclear multiple bond correlation spectroscopy (HMBC) 2D NMR techniques. HSQC detects correlation between ^{1}H and ^{13}C nuclei separated by one bond, while HMBC detects correlations between ^{1}H and ^{13}C nuclei separated by 2–4 bonds. These methods establish connectivity between ^{1}H and ^{13}C nuclei, and the connectivity between different ^{13}C nuclei can be indirectly deduced. Lastly, nuclear overhauser effect spectroscopy (NOESY) allows the observation of through-space interactions within a molecule. NOESY is based on dipole-dipole interactions of nuclei that are close in space (<5 Å). The relative stereochemistry of a proton can be deduced from the observed NOESY cross-peaks.

The chemical structures of seven different compounds were determined with one-dimensional (1D) NMR measurements (^{1}H, ^{13}C) and two-dimensional (2D) NMR measurements (COSY, TOCSY, HSQC, HMBC, NOESY) (**Table 4** and **Table 5**). The ^{1}H and ^{13}C spectra obtained for compounds **2**, **4**, **6**, and **8** matched previously published data (Fischer et al. 2002; Wohlert et al. 1998). However, the remaining three compounds were new analogues of **2**, and thus required a more detailed investigation for their structural elucidation.

4.4.1 8-demethyl-8-*O*-(4'-keto)-D-boivinosyl-tetracenomycin C (11)

A compound containing a ketosugar intermediate attached to **2** was obtained from Streptomyces *coelicolor* M1146::cos16F4iE/pDBOI, instead of the intended glycosylated analogue. The ^{1}H NMR data for this compound was consistent with **2** for the aglycone moiety of the compound. It exhibited two aromatic protons (δ 8.07 (s, 6-H) and δ 7.77 (s, 7-H)), an olefinic proton (δ 5.63 (s, 2-H)), an oxygenated methine proton (δ 5.07 (s, 4-H)), two methoxy signals (δ 3.95 (s, 9-OCH_3) and δ 3.84 (s, 3-OCH_3)), and an olefinic methyl signal (δ 2.75 (s, 10-CH_3)) (**Figure 18**a). Similarly, the ^{13}C NMR data was consistent with **2** for the aglycone moiety of the compound (**Figure 18**b). Additionally, both ^{1}H and ^{13}C data displayed six additional signals in comparison to **2**, which were attributed to a hexose sugar moiety.

The anomeric 1'-H of the sugar was observed at δ 5.72 as doublet of doublets (dd) with $^{3}J_{H\text{-}H}$ coupling constants of 2.4 Hz and 9.9 Hz. The magnitude of the $^{3}J_{H\text{-}H}$ coupling constants depends on the dihedral torsion angles between the protons, which are located

in either axial or equatorial positions in the chair conformation of cyclohexane. The largest $^{3}J_{H\text{-}H}$ coupling constants are observed between two axial protons (~9–12 Hz), while the $^{3}J_{H\text{-}H}$ coupling constants between axial-equatorial or equatorial-equatorial protons are smaller (~2–5 Hz). The observed large $^{3}J_{H\text{-}H}$ coupling constant of 1'-H (9.9 Hz) indicated that 1'-H is an axial proton, suggesting that the sugar moiety is β-glycosidically bound to the aglycone moiety. Two spin systems were observed in the COSY and TOCSY spectra. The first spin system involved 1'-H (δ 5.72), 2'-H_2 (δ 2.29 and δ 2.13), and 3'-H (δ 4.43) of the sugar moiety. The second spin system involved 5'-H (δ 4.29) and 6'-H_3 (δ 1.36) of the sugar moiety (**Figure 18**c, d). The signal for 4'-H was not observed, indicating the presence of a keto group.

Table 4. ^{13}C NMR spectroscopic data of compounds.

Compound	**2**	**4**	**6**	**8**	**11**	**12**	**13**
Solvent	**DMSO-*d*$_6$**	**Acetone-*d*$_6$**	**Acetone-*d*$_6$**	**CD$_3$OD**	**Acetone-*d*$_6$**	**CD$_3$OD**	**CD$_3$OD**
Transmitter frequency (MHz)	**125**	**125**	**125**	**150**	**125**	**125**	**125**
Position	**δ ppm**	**δ ppm**	**δ ppm**	**δ ppm**	**δ ppm**	**δ ppm**	**δ ppm**
1	191.3	190.8	190.8	192.2	190.7	193.1	193.1
2	100.0	100.0	100.0	99.6	100.1	100.7	100.7
3	174.6	175.4	175.4	174.9	175.4	176.0	176.1
3-OCH3	57.1	57.3	57.3	56.5	57.3	57.6	57.6
4	69.6	70.6	70.6	69.7	70.3	70.8	70.8
4a	85.3	85.2	85.2	84.6	85.1	85.8	85.8
5	194.2	194.1	194.2	193.4	194.2	194.7	194.7
5a	128.5	129.1	129.1	128.6	129.3	129.7	129.7
6	119.1	121.5	121.4	120.9	121.6	121.8	121.9
6a	140.5	141.3	141.2	140.6	141.1	141.6	141.7
7	111.7	112.2	112.1	111.9	112.2	112.5	112.5
8	156.6	156.3	156.1	155.6	156.1	156.8	156.7
9	129.1	130.6	130.5	129.6	130.5	130.8	130.8
9-COOCH3	168.2	168.0	168.0	168.5	167.9	169.6	169.6
9-COOCH3	52.8	52.7	52.7	52.2	52.3	53.3	53.3
10	137.8	138.4	138.4	138.2	138.5	139.3	139.4
10a	119.5	121.9	122.0	121.4	122.1	122.6	122.7
10-CH3	21.1	21.0	21.0	20.1	21.2	21.2	21.2
11	166.7	167.8	167.8	166.7	162.3	167.8	167.8
11a	108.7	110.2	110.3	109.5	110.4	110.5	110.6
12	197.7	198.0	198.0	197.5	198.1	198.4	198.4
12a	83.7	83.6	83.6	83.5	83.6	84.6	84.6
1'		96.8	97.8	100.8	97.3	102.0	101.4
2'		38.1	39.7	73.7	34.2	71.8	75.1
3'		68.1	71.2	77.1	68.7	75.2	77.9
4'		73.4	78.1	70.2	94.1	72.9	76.6
5'		71.3	73.4	77.4	73.8	72.6	73.8
6'		18.6	18.3	61.6	13.0	16.8	18.1

Table 5. ^{1}H NMR spectroscopic data of compounds.

Compound	**2**	**4**	**6**	**8**
Solvent	**DMSO-*d*$_6$**	**Acetone-*d*$_6$**	**Acetone-*d*$_6$**	**CD$_3$OD**
Transmitter frequency (MHz)	**600**	**500**	**600**	**600**
Position	**δ ppm (mult, *J* Hz)**	**δ ppm (mult, *J* Hz)**	**δ ppm (mult, *J* Hz)**	**δ ppm (mult, *J* Hz)**
2	5.55 (s)	5.62 (s)	5.62 (s)	5.63 (s)
3-OCH3	3.73 (s)	3.84 (s)	3.86 (s)	3.83 (s)
4	4.77 (d, 7.8)	5.06 (s)	5.06 (s)	4.90 (s)
6	7.87 (s)	8.06 (s)	8.00 (s)	8.05 (s)
7	7.38 (s)	7.82 (s)	7.80 (s)	7.68 (s)
9-COOCH3	3.87 (s)	3.93 (s)	3.94 (s)	3.98 (s)
10-CH3	2.75 (s)	2.84 (s)	2.84 (s)	2.86 (s)
1'		5.81 (dd, 2.3, 9.2)	5.58 (dd, 2.1, 9.7)	5.20 (d, 7.7)
2'		1.96 (H_{ax}, ddd, 2.2, 9.2, 13.2) 2.22 (H_{eq}, ddd, 2.3, 4.0, 13.2)	1.77 (H_{ax}, ddd, 9.7 12.0 12.0) 2.34 (H_{eq}, ddd, 2.1 4.9 12.0)	3.48 (dd, 7.7, 9.2)
3'		4.18 (dddd, 2.2, 2.2, 2.7, 4.0)	3.73 (m)	3.52 (dd, 9.0. 9.2)
3'-OH		4.03 (d, 2.2)	4.19 (brs)	
4'		3.34 (ddd, 2.7, 5.0, 9.0)	3.06 (t, 9.2)	3.40 (dd, 9.0, 9.8)
4'-OH		3.94 (d, 5.0)	4.24 (brs)	
5'		4.14 (dq, 6.3, 9.0)	3.68 (dq, 6.2, 9.2)	3.63 (ddd, 1.8, 6.3, 9.8)
6'		1.29 (d, 6.3)	1.34 (d, 6.2)	3.71 (H_{ax}, dd, 6.3, 11.8) 3.96 (H_{eq}, dd, 1.8, 11.8)

Compound	**11**	**12**	**13**
Solvent	**Acetone-*d*$_6$**	**CD$_3$OD**	**CD$_3$OD**
Transmitter frequency (MHz)	**600**	**500**	**600**
Position	**δ ppm (mult, *J* Hz)**	**δ ppm (mult, *J* Hz)**	**δ ppm (mult, *J* Hz)**
2	5.63 (s)	5.61 (s)	5.61 (s)
3-OCH3	3.84 (s)	3.81 (s)	3.81 (s)
4	5.07 (s)	4.89 (s)	4.89 (d, 1.4)
6	8.07 (s)	8.01 (s)	8.02 (s)
7	7.77 (s)	7.61 (s)	7.60 (s)
9-COOCH3	3.95 (s)	3.96 (s)	3.96 (s)
10-CH3	2.85 (s)	2.85 (s)	2.86 (s)
1'	5.72 (dd, 2.4, 9.9)	5.16 (d, 7.9)	5.2 (d, 7.7)
2'	2.29 (H_{ax}, ddd, 4.0, 9.9, 13.6) 2.13 (H_{eq}, ddd, 2.4, 2.6, 13.6)	3.76 (dd, 7.9, 9.7)	3.44 (dd, 7.7, 9.5)
3'	4.43 (dd, 2.6, 4.0)	3.61 (dd, 3.6, 9.7)	3.45 (dd, 9.0, 9.5)
3'-OH			
4'		3.70 (d, 3.6)	3.10 (dd, 9.0, 9.4)
4'-OH			
5'	4.29 (q, 6.5)	3.98 (d, 6.6)	3.65 (dq, 6.2, 9.4)
6'	1.36 (d, 6.5)	1.32 (d, 6.6)	1.33 (d, 6.2)

The stereochemistry of the sugar moiety was determined based on the *J*-coupling constants of the sugar protons and NOESY spectra (**Table 5**, **Figure 18**g). The large $^{2}J_{H\text{-}H}$ coupling constant (13.6 Hz) indicated the presence of geminal protons at the C-2' position. Specifically, 2'-H_{ax} (δ 2.29) exhibited a larger $^{3}J_{H\text{-}H}$ coupling constant (9.9 Hz) with 1'-H, while 2'-H_{eq} (δ 2.13) displayed a smaller $^{3}J_{H\text{-}H}$ coupling constant (2.4 Hz) with 1'-H. The small $^{3}J_{H\text{-}H}$ coupling constants of 3'-H (2.6 Hz and 4.0 Hz) indicated its equatorial position. A NOE coupling was observed between 5'-H and the axial 1'-H, confirming that 5'-H is located in the axial position of the cyclohexane ring. The signal of 5'-H appeared as a quartet with $^{3}J_{H\text{-}H}$ coupling constant of 6.5 Hz, indicating its coupling with a methyl group (6'-CH_3).

The HSQC experiment determined the one-bond correlations between the sugar protons and their respective carbons. This allowed the assignment of the observed ^{13}C signals to carbons 1'-C, 2'-C, 3'-C, 5'-C, and 6'-C (**Figure 18**e). The presence of a keto group at 4'-C (δ 94.1) was established based on the HMBC correlations from 6'-CH_3, 5'-H, and 3'-H protons to 4'-C (**Figure 18**e, h). The chemical shift of 4'-C (δ 94.1) suggests that the keto group is primarily present in its hydrate form when measured in acetone-d_6. Furthermore, HMBC correlation from 1'-H to 8-C confirmed that the sugar moiety is attached to the 8-C position of **2**.

The compound isolated from Streptomyces *coelicolor* M1146::cos16F4iE/pDBOI was identified as 8-demethyl-8-*O*-(4'-keto)-D-boivinosyl-tetracenomycin C (**11**) based on the comprehensive 1D and 2D NMR experiments (**Figure 17**). This was further supported by HR-MS measurements as the molecular formula of **11** was determined to be $C_{28}H_{26}O_{14}$ based on (–)-HRESI-MS (obs. $[M–H]^-$ = 585.1250, calc. $[M–H]^-$ = 585.1250) and (+)-HRESI-MS (obs. $[M+H]^+$ = 587.1385, calc. $[M+H]^+$ = 587.1395).

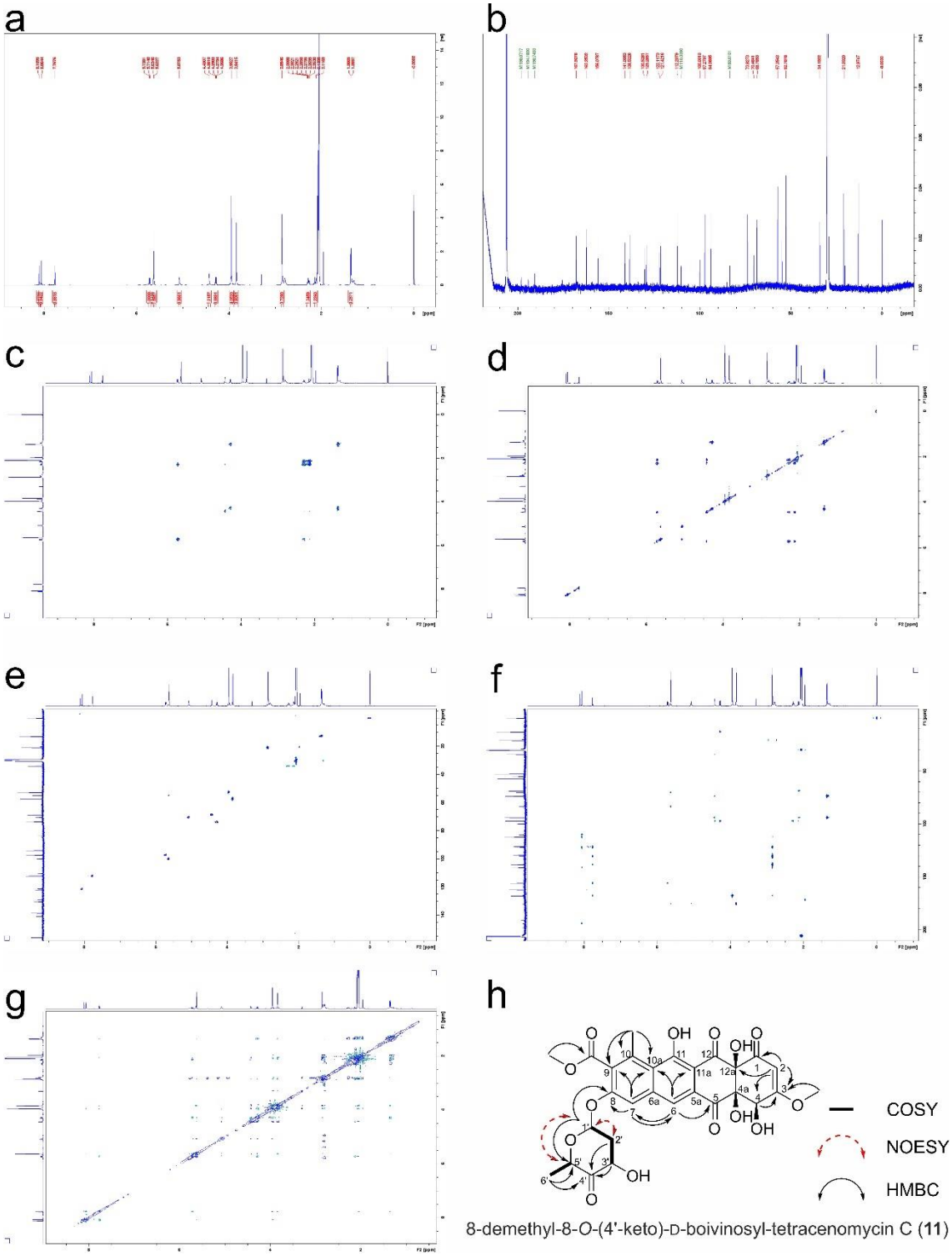

Figure 18. NMR spectra for compound **11**. **a)** ^{1}H, **b)** ^{13}C, **c)** COSY, **d)** TOCSY, **e)** HSQC, **f)** HMBC, **g)** NOESY, and **h)** COSY (—), selected HMBC (→) and selected NOESY ( ) correlations for compound **11**.

4.4.2 8-demethyl-8-*O*-D-fucosyl-tetracenomycin C (12)

Two compounds were isolated from the strain *Streptomyces coelicolor* M1146::cos16F4iE/pDFUCO, both having the appropriate molecular mass for the attachment of either TDP-D-fucose or TDP-D-fucofuranose to **2**. We will first focus on the more abundant glycosylated peak, which exhibits a later retention time compared to the minor glycosylated analogue. The ^{1}H and ^{13}C NMR data for the aglycone portion of this compound were in agreement with the non-glycosylated **2** (**Figure 19**a, b). Moreover, both ^{1}H and ^{13}C data showed the presence of six additional signals, indicating the presence of a hexose sugar moiety.

The anomeric proton 1'-H of the sugar exhibited a doublet signal at δ 5.16 with $^{3}J_{H\text{-}H}$ coupling constant of 7.9 Hz. This observation suggests that the sugar moiety is in an axial position and indicates a β-glycosidic bond between the sugar and the aglycone. In the COSY spectrum, two distinct spin systems were observed. The first spin system involved 1'-H (δ 5.16), 2'-H (δ 3.76), 3'-H (δ 3.61), and 4'-H (δ 3.70) of the sugar moiety. The second spin system was between 5'-H (δ 3.98) and 6'-H_3 (δ 1.32) of the sugar moiety (**Figure 19**c). The absence of a cross-peak between 4'-H and 5'-H in the COSY spectrum can be attributed to weak *J*-coupling between these two protons, which are in equatorial positions.

The stereochemistry of the sugar moiety was determined by analyzing the *J*-coupling constants of the sugar protons and utilizing the NOESY spectra (Table 5, **Figure 19**f). The 2'-H appeared as a dd with two large $^{3}J_{H\text{-}H}$ coupling constant to 1'-H (7.9 Hz) and 3'-H (9.7 Hz), indicating its axial position. Similarly, the 3'-H exhibited a dd signal with a large $^{3}J_{H\text{-}H}$ coupling constant to 2'-H (9.7 Hz) and a smaller $^{3}J_{H\text{-}H}$ coupling constant to 4'-H (3.6 Hz). This established the 3'-H to be in an axial position and the 4'-H to be in an equatorial position. The equatorial position of 4'-H was further supported by the observed NOE correlation with 5'-H (**Figure 19**g). The axial position of 5'-H was confirmed by the NOE correlation between 5'-H and the axial 1'-H. Similar to compound **11**, the presence of the 6'-CH_3 methyl group was detected through the splitting of 5'-H, forming a quartet with $^{3}J_{H\text{-}H}$ coupling constant of 6.6 Hz. The HSQC experiment determined the ^{13}C signals associated to the carbons 1'-C, 2'-C, 3'-C, 4'-C, 5'-C, and 6'-C (**Figure 19**d). The HMBC correlation from 1'-H to 8-C confirmed that the sugar moiety is attached to the 8-C position of **2** (**Figure 19**e, g).

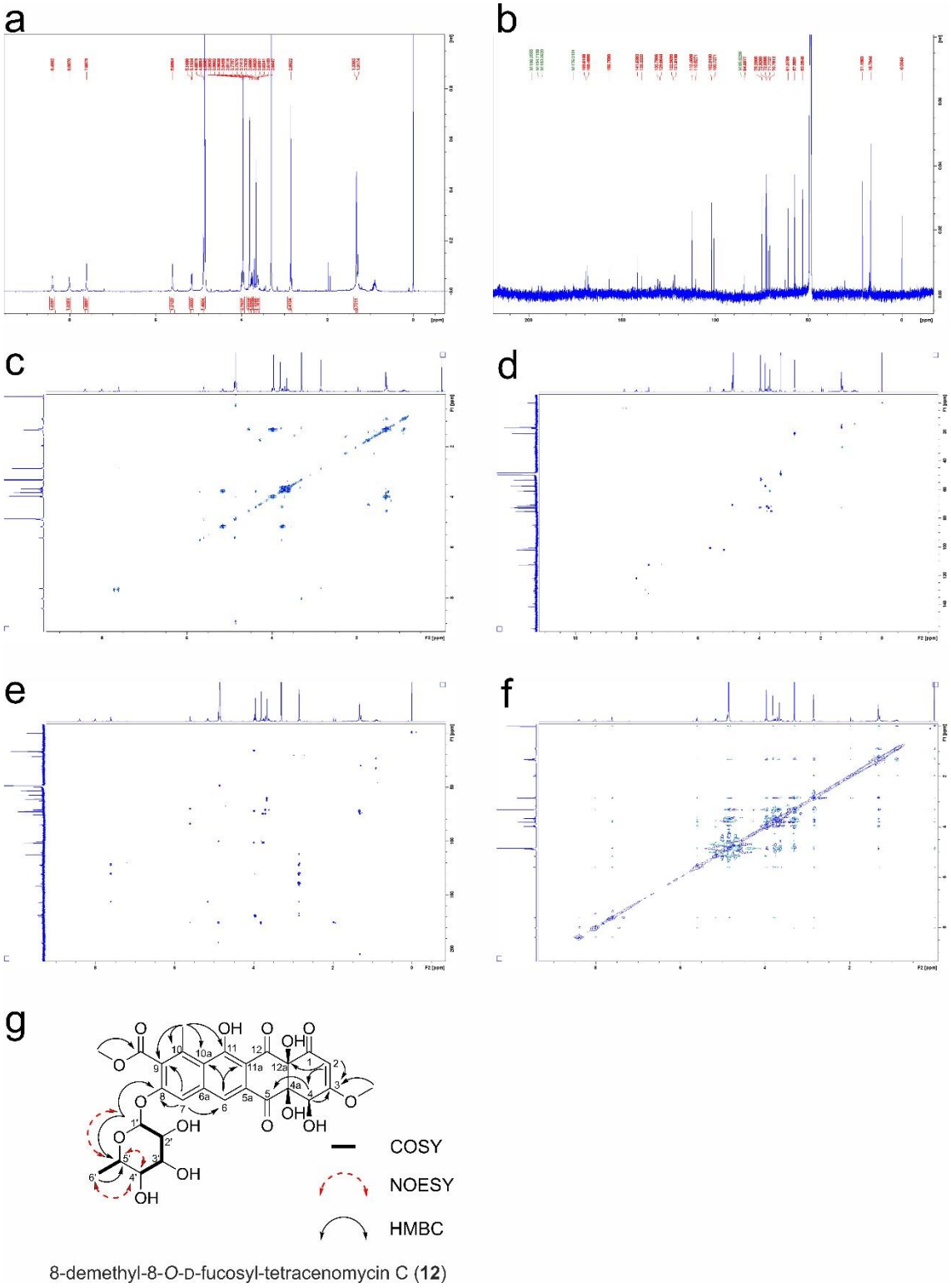

Figure 19. NMR spectra for compound **12**. **a)** ^{1}H, **b)** ^{13}C, **c)** COSY, **d)** HSQC, **e)** HMBC, **f)** NOESY, and **g)** COSY (—), selected HMBC (→) and selected NOESY (↶) correlations for compound **12**.

The compound isolated from Streptomyces *coelicolor* M1146::cos16F4iE/pDBOI was established as 8-demethyl-8-*O*-D-fucosyl-tetracenomycin C (**12**) based on the comprehensive 1D and 2D NMR experiments (**Figure 17**). This was further supported by HR-MS measurements as the molecular formula of **12** was determined to be $C_{28}H_{29}O_{15}$ based on (–)-HRESI-MS (obs. $[M–H]^-$ = 603.1377, calc. $[M–H]^-$ = 603.1355) and (+)-HRESI-MS (obs. $[M+H]^+$ = 605.1508, calc. $[M+H]^+$ = 605.1501).

4.4.3 8-demethyl-8-*O*-D-quinovosyl-tetracenomycin C (13)

A minor glycosylated analogue of **2** was obtained from the strain *Streptomyces coelicolor* M1146::cos16F4iE/pDFUCO, exhibiting the appropriate molecular mass for the attachment of either TDP-D-fucose or TDP-D-fucofuranose to **2**. Similar to the previous compounds, the ^1H and ^{13}C NMR data for the aglycone portion of this compound were consistent with the non-glycosylated **2**, while displaying six additional signals in both the ^1H and ^{13}C spectra, indicative of a hexose sugar moiety (Figure 20a, b). The anomeric proton 1'-H of the sugar was observed at δ 5.20 as doublet with $^3J_{H\text{-}H}$ coupling constant of 7.7 Hz, indicating the axial position of the sugar moiety and a β-glycosidic bond between the sugar and aglycone. In the COSY and TOCSY spectra, a single spin system was observed between 1'-H (δ 5.20), 2'-H (δ 3.44), 3'-H (δ 3.45), 4'-H (δ 3.10), 5'-H (δ 3.65) and 6'-H_3 (δ 1.33) of the sugar moiety (Figure 20c, d).

The stereochemistry of the sugar moiety was determined by analyzing the *J*-coupling constants of the sugar protons and examining the NOESY spectra (Table 5, Figure 20g). This compound exhibited similar *J*-coupling constants and NOE correlations as the previously analyzed compound **12**, except for the 4'-H. In compound **12**, the 4'-H was determined to be in an equatorial position based on a small coupling constant of 3.6 Hz. However, in this compound, the 4'-H displayed two large coupling constants of 9.0 Hz and 9.4 Hz. This established that the 4'-H in this compound is in axial position, indicating that the sugar moiety is D-quinovose. Furthermore, the HSQC experiment facilitated the assignment of the ^{13}C signals corresponding to the carbons ranging from 1'-C to 6'-C in the sugar moiety (Figure 20e). Additionally, the HMBC experiment confirmed the attachment of the sugar moiety to the 8-C position of **2** (Figure 20f, h).

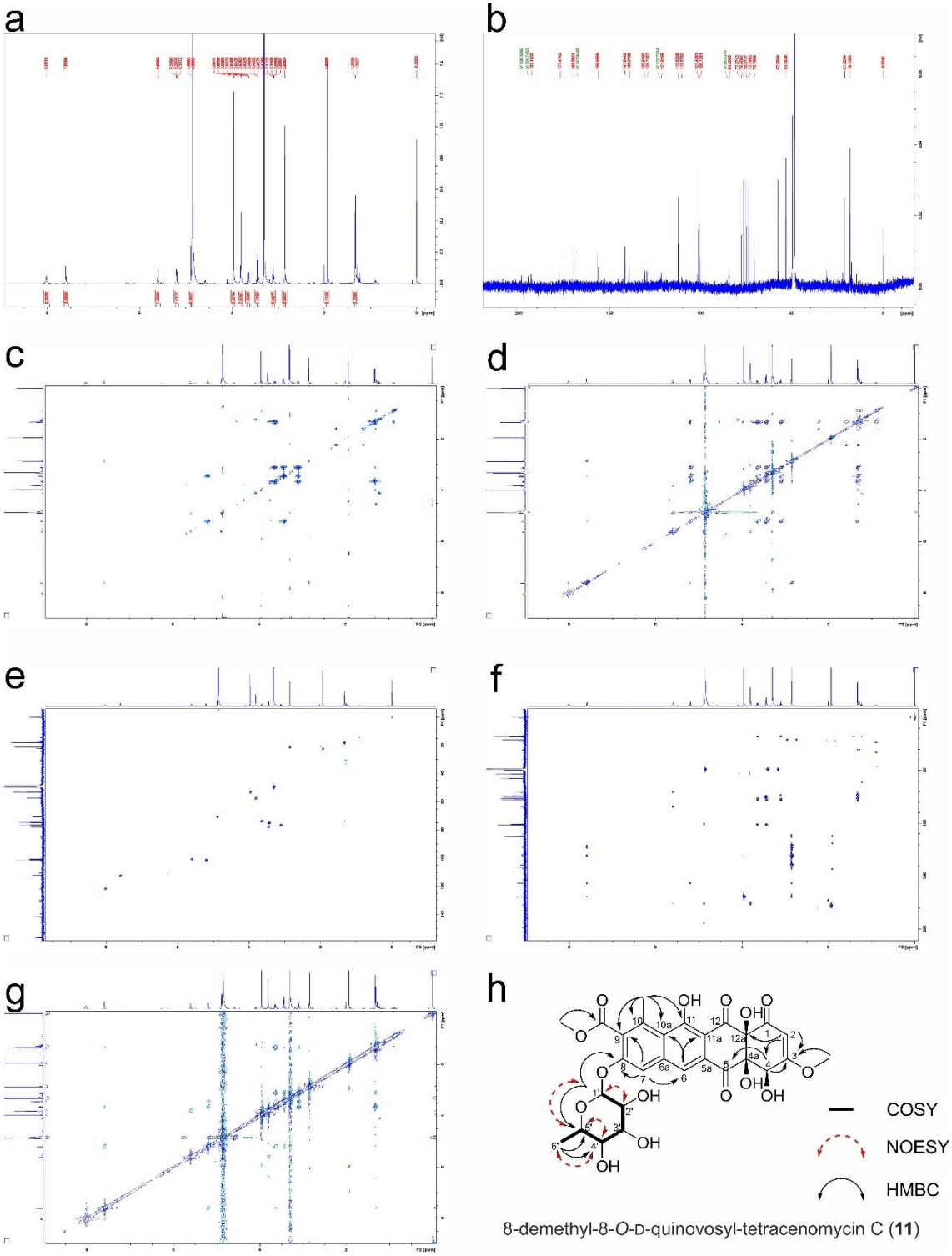

Figure 20. NMR spectra for compound **13**. **a)** ^{1}H, **b)** ^{13}C, **c)** COSY, **d)** TOCSY, **e)** HSQC, **f)** HMBC, **g)** NOESY, and **h)** COSY (—), selected HMBC (→) and selected NOESY ( ) correlations for compound **13**.

The minor glycosylated compound obtained from Streptomyces *coelicolor* M1146::cos16F4iE/pDBOI was established as 8-demethyl-8-*O*-D-quinovosyl-tetracenomycin C (**13**) based on the comprehensive 1D and 2D NMR experiments

(**Figure 17**). This was further supported by HR-MS measurements as the molecular formula of **13** was determined to be $C_{28}H_{29}O_{15}$ based on (+)-HRESI-MS (obs. $[M+H]^+$ = 605.1504, calc. $[M+H]^+$ = 605.1501).

4.5 Antibacterial and cytotoxic activities of the tetracenomycin analogues

The antibacterial activities of the compounds **1**, **2**, **6**, **13**-**18** (**Figure 21**) were assessed at the Center for Pharmaceutical Research and Innovation, University of Kentucky, by Dr. Larissa V. Ponomareva. However, due to limited material availability, the remaining compounds could not be tested. The antibacterial activity of these compounds was evaluated by exposing eight different bacteria to a concentration of 100 μM of each compound. Bacterial growth in the presence of the compounds was compared to bacterial growth in the absence of the compounds. Among the tested bacteria, five were gram-positive, including methicillin-susceptible *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Streptomyces violaceusniger*, *Streptomyces prasinus*, and *Listeria monocytogenes*. The remaining three strains were gram-negative, namely *Escherichia coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *Campylobacter jejuni* 81-176. Methicillin-resistant *S. aureus* and fluoroquinolone-resistant strains of *Salmonellae* and *Campylobacter* are included in the World Health Organization's high priority list for the development of new antibiotics (World Health Organization 2017). *S. aureus* is known to cause skin infections and pneumonia with high mortality rates, ranging from 15 % to 50 % depending on infection type, and it has developed resistance against multiple classes of antibiotics (Turner et al. 2019). *E. coli* O157:H7, *S. enterica*, *L. monocytogenes,* and *C. jejuni* are among the most common bacteria responsible for serious infections associated with food poisoning (Scallan et al. 2011).

Compound **1** exhibited the highest antibacterial activity against both strains of *S. aureus* with a range of 90–95 % inhibition (Figure 21a). Compound **17** also displayed some antibacterial activity against the *S. aureus* strains (10–25 %). However, the remaining compounds did not show any significant activity against the *S. aureus* strains. The main difference between compounds **1** and **17** lies in the presence of a permethylated L-rhamnose sugar moiety attached to the C8-position of **1**. Interestingly, only the non-glycosylated compounds **17** and **18** exhibited antibacterial activity against *S. violaceusniger* (80–90 %). On the other hand, the non-glycosylated compound **2**, which lacks the *O*-methyl group at the C8-position, displayed only slight activity (8 %) against

S. violaceusniger. It appears that the presence of the *O*-methyl group at the C8-position in compounds **17** and **18** is responsible for the enhanced antibacterial activity against *S. violaceusniger* compared to compound **2**.

All compounds in this study, except compound **1**, showed inhibitory effects on the growth of *S. prasinus*, although to varying degrees (25–70 %). The sugar moiety played a role in the activity, as glycosylated compounds **14** and **16** displayed the highest activity against *S. prasinus* (68–70 %), while glycosylated compound **13** exhibited only 28 % inhibition. The difference between these compounds lies in the presence of an additional methyl group in the sugar moiety of compounds **14** and **16** compared to compound **13**. The additional methyl group increases the hydrophobicity of the compounds. However, glycosylated compound **15**, which has a high degree of hydroxylation in the sugar moiety, also showed significant activity against *S. prasinus* (51 %). Interestingly, the non-glycosylated compound **18** displayed high activity against *S. prasinus* (63 %), whereas the non-glycosylated compound **2** exhibited significantly lower activity (25 %). Therefore, it appears that multiple factors contribute to the activity against *S. prasinus*. These findings are consistent with previously published data, as *S. violaceusniger* and *S. prasinus* have been shown to be susceptible to tetracenomycins (Drautz et al. 1985).

The compounds **13**, **14**, **16**, and **17** exhibited slight inhibition of growth against the gram-negative *E. coli* O157:H7 (7–13 %) and *S. enterica* (6–14 %). Notably, the non-glycosylated compound **2** showed no activity against these gram-negative pathogens, indicating that the activity of glycosylated compounds **13**, **14**, and **16** is attributed to the attached sugar moiety. However, the non-glycosylated compound **17** also demonstrated activity against *E. coli* O157:H7 and *S. enterica*, suggesting that glycosylation is not the only factor influencing the activity against these bacteria. Compounds **2**, **14**, **16**, **17**, and **18** exhibited slight antibacterial activity against *L. monocytogenes* with varying degrees of effectiveness (13–16 %). Similarly to the antibacterial activity against *S. prasinus*, there appear to be multiple factors influencing the antibacterial activity against *L. monocytogenes*. Compounds **2** and **18** displayed modest antibacterial activity against *C. jejuni* (10–29 %). The non-glycosylated compounds **2** and **18** are more polar than the non-glycosylated compound **17**, while the glycosylated compounds are larger in size. It has been observed that small and polar molecules penetrate the outer membrane of gram-negative bacteria more efficiently (O'Shea and Moser 2008).

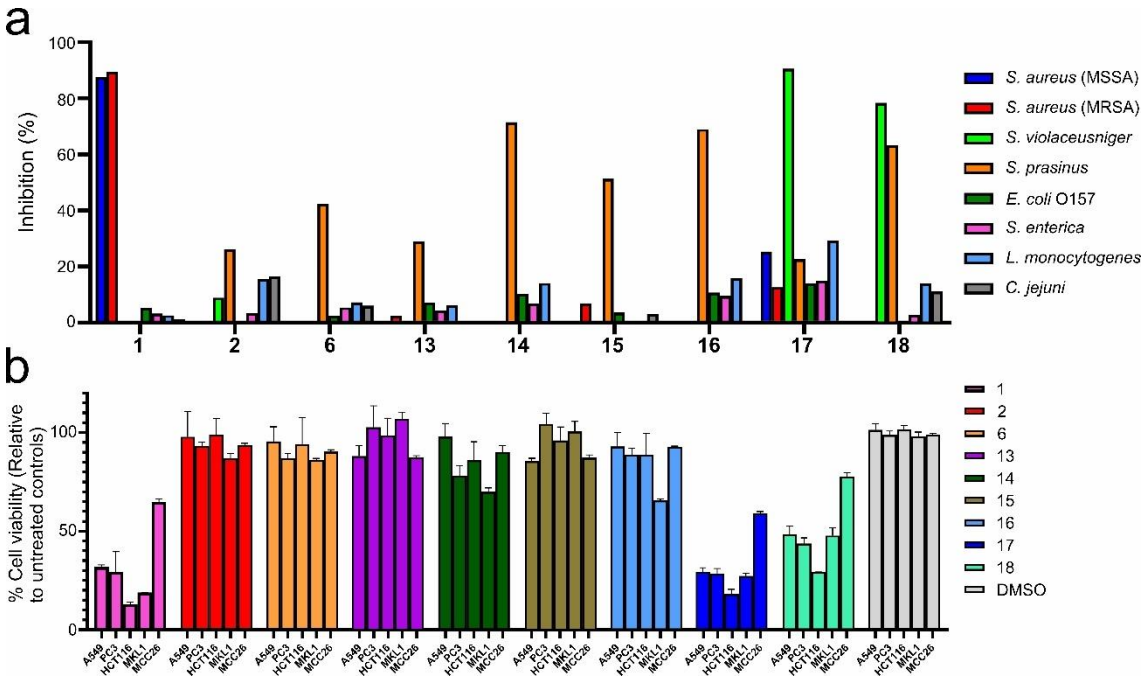

Figure 21. a) Antibacterial activities of compounds **1**, **2**, **6**, **13-18**. Compounds were tested at 100 µM concentration against the selected microbial strains. **b)** Cytotoxic activities of compounds **1**, **2**, **6**, **13-18**. Compounds were tested at 80 µM concentration against the following cancer cell lines: A549 (non-small lung), PC3 (prostate), HCT116 (colorectal), Merkel MKL1 (carcinoma, lymph metastatic site), and Merkel MCC26 (carcinoma, skin).

The cytotoxicity of the compounds was assessed at the Department of Veterinary Science, University of Kentucky, by Dr. Yosra Helmy using a resazurin-based cytotoxicity assay, which measures the reduction of non-fluorescent resazurin to red fluorescent resorufin by metabolically active cells. The decrease in fluorescence compared to untreated cells indicates a loss of cell viability caused by the tested compounds at a concentration of 80 µM. (Shaaban et al. 2013.) Five different human cancer cell lines were selected for the cytotoxicity testing: A549 (non-small lung), PC3 (prostate), HCT116 (colorectal), Merkel MKL1 (carcinoma, lymph metastatic site), and Merkel MCC26 (carcinoma, skin) (Figure 21b).

The compounds **1**, **17**, and **18** exhibited cytotoxic activity against all tested cell lines with varying degrees of effectiveness. Compounds **1** and **17** demonstrated higher cytotoxic compared to compound **18**, which is hydroxylated at the C6-position. In contrast to the other non-glycosylated compounds **17** and **18**, compound **2** did not display cytotoxic activity against the cell lines. Compound **2** lacks the C8-*O*-methyl group present in

compounds **17** and **18**. Interestingly, the glycosylated compounds **6** and **13**–**16** exhibited significantly lower cytotoxicity compared to glycosylated compound **1**. Among the glycosylated compounds, compounds **14** and **16** showed some cytotoxicity against the Merkel MKL1 cell line. Compounds **14** and **16** have an additional methyl group in the sugar moiety compared to compounds **6**, **13**, and **15**. The sugar moiety of **1** is a permethylated L-rhamnose, suggesting that methylation of the sugar moiety may enhance the cytotoxic activity of the compounds.

5 Discussion

Two aims were presented earlier for this study: 1) glycodiversification of tetracenomycins to enhance their physicochemical properties and increase their specificity towards prokaryotic ribosomes, and 2) elucidation of the structure-activity relationships based on the glycosylation patterns of tetracenomycins. The success of these aims will be discussed below.

5.1. Functionality of the generated sugar pathways and glycodiversification

In total, 22 unique sugar moieties with different biosynthetic pathways were inserted into a heterologous host, *S. coelicolor* M1146::cos16F4iE. Among the generated bacterial strains, eight strains showed detectable production of the correct glycosylated analogue of **2**, as confirmed by HPLC-MS measurements. Previous studies have established that six of these sugar moieties are substrates for ElmGT. However, in this study, the remaining four sugar moieties that had been successfully attached to **2** by ElmGT in previous studies were not effectively transferred to the aglycone in the current study. This discrepancy could potentially be attributed to the use of a different heterologous host, as previous studies utilized *Streptomyces lividans* and *Streptomyces albus*, whereas this study employed *S. coelicolor M1146*.

Out of the various sugar moieties attempted, two 6-deoxysugars, five 2,6-dideoxysugars, two 2,3,6-trideoxysugars, and one methylated sugar were successfully attached to **2** by ElmGT. However, none of the aminosugars were effectively attached to **2**. The lack of successful glycosylated analogues of **2** can be attributed to two possible reasons: either the sugar moiety itself was not produced due to non-functional sugar biosynthetic genes, or the glycosyltransferase ElmGT did not recognize the sugar moiety as a substrate. It is worth noting that 21 out of the 22 sugar biosynthetic pathways attempted in this study had been characterized in previous studies, which suggests that ElmGT may not have sufficient promiscuity to accept all the tested sugar moieties. However, it is important to acknowledge that this conclusion is not definitive, as only the glycosylated analogues of **2** were analyzed and detected in this study, while the presence of the sugar moieties in the culture media was not tested.

Numerous ketosugar intermediates were detected from the generated bacterial strains based on HPLC-MS measurements. This observation can be attributed to the utilization of a single operon for expressing the biosynthetic genes. It is possible that the promoter of the operon was not sufficiently strong, leading to lower expression levels of the genes located at the end of the operon compared to those at the beginning. Specifically, the ketoreductase genes responsible for converting the keto group to a hydroxyl group were situated towards the end of the operons and may have been expressed to a lesser extent. An unexpected finding was the production of didemethyl-tetracenomycin C by some of the strains, which is an intermediate in the biosynthesis of **2** and was not observed in the original strain prior to plasmid transformation. This phenomenon could be attributed to genomic mutations in the integrated biosynthetic pathway for **2**. However, it is worth noting that this phenomenon was observed in seven of the generated strains, suggesting that alternative factors may be at play. These factors could include reduced availability of precursors for the biosynthesis of **2** due to the presence of sugar biosynthetic enzymes or heterologous recombination if there were similarities between the transformed plasmids and the biosynthetic genes of **2**.

Only four of the intended glycosylated analogues of **2** were successfully purified for bioactivity measurements. Additionally, four unexpected glycosylated analogues of **2** were also purified. This can be attributed to low production levels of four of the intended analogues (compounds **5**, **7**, **9**, **10**) and challenges encountered during the purification process for compound **3**. Promiscuous enzymes often catalyze non-native reactions at slower rates compared to native reactions (Copley 2017). This can explain the lower yields observed in this study when unnatural sugar moieties were utilized as substrates for ElmGT. The presence of intermediates from the sugar biosynthetic pathway that were also attached to **2** by ElmGT hindered the purification process of a specific glycosylated analogue. Separating highly similar compounds becomes challenging as they possess nearly identical chemical properties, making the separation process based on these properties more difficult.

Four unexpected glycosylated analogues of **2** were successfully purified: compounds **8**, **11**, **12**, and **13**. Compound **8**, which has D-glucose attached, was isolated from multiple strains. Previous studies have shown that D-glucose is a known substrate for ElmGT (Fischer et al. 2002). TDP-D-glucose, a common intermediate of all the sugar biosynthetic pathways in this study, can serve as an alternative substrate for ElmGT when the intended

sugar moiety is not biosynthesized or attached. Compound **11**, which has a 4'-keto-D -boivinose attached, was isolated from the strain intended to produce compound **3** due to purification challenges with compound **3**. Compound **11** represents the final sugar intermediate before the ketoreductase OleU reduces the keto group to a hydroxyl group. Similarly, the strain containing the sugar biosynthetic genes for D-fucofuranose production yielded only the pathway intermediate with D-fucose attached. This suggests that the enzyme GilV may not catalyze the ring contraction from TDP-D-fucopyranose to TDP-D-fucofuranose. Alternatively, it is possible that TDP-D-fucofuranose is not recognized as a substrate by ElmGT. Compound **13**, which has a D-quinovose attached, was also obtained from the strain with the sugar biosynthetic genes for D-fucofuranose production. D-quinovose can be derived from TDP-4-keto-6-deoxy-D-glucose, a common intermediate of all the sugar biosynthetic pathways in this study, through the action of a 4-ketoreductase enzyme. The production of compound **13** may be attributed to the activity of GilU ketoreductase from the D-fucofuranose pathway on TDP-4-keto-6-deoxy-D-glucose or to an endogenous 4-ketoreductase encoded in the *S. coelicolor* genome.

5.2 The impact of the glycosylation pattern on the bioactivities of tetracenomycins

The generation of glycosylation analogues of **2** aimed to achieve three main goals: gaining antibacterial activity against pathogenic bacterial strains, reducing cytotoxicity against human cell lines, and improving the physicochemical properties of the compounds. However, the pharmacokinetic properties of the compounds were not evaluated in this study, as further improvements in the bioactivities of the compounds are still needed. Although the study did not quantify the effect, glycosylation was observed to enhance solubility in polar solvents. This improved solubility could potentially contribute to the observed differences in antibacterial activity and cytotoxicity.

The attachment of a sugar moiety to compound **2** had a significant impact on the target bacterial strain specificity of the tested compounds. For instance, the non-glycosylated compound **17** displayed the highest activity against *S. violaceusniger* among the tested strains in this study. However, the attachment of a sugar moiety in compound **1** shifted its activity towards being active against *S. aureus* instead. Furthermore, compounds **13**, **14**, and **16** exhibited increased antibacterial activity against the gram-negative pathogens *E. coli* O157:H7 and *S. enterica*, whereas the non-glycosylated **2** had no activity against these strains. This suggests that glycosylation can enhance the penetration of the outer

cell wall of gram-negative bacteria. Interestingly, there was an inverse relationship observed in the antibacterial activity against *S. violaceusniger*, where only the non-glycosylated compounds with a C8-*O*-methyl group displayed active against this strain. The effect of the sugar moiety was also evident in the antibacterial activity against *S. prasinus*. However, it is important to note that all tetracenomycins tested in this study had antibacterial activity against *S. prasinus* to varying degrees, making it challenging to establish clear patterns or gains/losses of activity based solely on the chemical structures of the sugars. Nevertheless, compounds with methylated sugar moieties tended to have slightly better activity against *S. prasinus*. The observed differences in antibacterial activity against *S. prasinus* may be attributed to variations in the three-dimensional shapes of the sugar moieties. Overall, the compounds tested in this study demonstrated higher activity against gram-positive bacteria compared to gram-negative bacteria.

The glycosylated compounds generated in this study did not exhibit significant cytotoxic activities against most of the tested human cancer cell lines. This represents a notable improvement compared to the highly cytotoxic glycosylated compound **1**. Methylation of the sugar moiety appears to play a crucial role in the cytotoxicity of glycosylated compounds, as compound **1**, which contains a permethylated L-rhamnose, displayed high cytotoxic activity. Additionally, compounds **14** and **16**, which showed slight cytotoxic activity against the Merkel MKL1 cell line, possess one methyl group in their sugar moieties. The impact of methylation on cytotoxic activity is also evident in non-glycosylated compounds, where compounds **17** and **18**, containing a C8-*O*-methyl group, demonstrated significantly higher cytotoxicity compared to compound **2**, which lacks the C8-*O*-methyl group. The influence of the C8-*O*-methyl group on cytotoxic activity has been previously documented, as 8-*O*-methylelloramycinone exhibited higher cytotoxic activity than elloramycinone (Rohr and Zeeck 1990). Moreover, the presence of a C6-OH group appears to reduce the cytotoxicity of the compounds, as compound **18**, which contains a C6-OH group, displayed lower cytotoxicity compared to compounds **1** and **17**, which lack the C6-OH group. This finding aligns with a previous study, which noted that the C6-OH group reduced the cytotoxicity of tetracenomycin X (Alferova et al. 2022). Therefore, it can be concluded that an increase in hydrophobicity enhances the cytotoxicity of the compounds based on the results obtained in this study.

6 Conclusions and Future Perspectives

The development of novel compounds with antibacterial activities is of great importance due to the increasing rates of antimicrobial resistance. In this study, we successfully generated glycosylated tetracenomycins through metabolic engineering of a heterologous host using the promiscuous glycosyltransferase, ElmGT. The glycodiversification approach resulted in the production of eight glycosylated tetracenomycins, four of which were previously unknown natural products. Moreover, the library of BioBricks parts created for cloning sugar biosynthetic genes in this study holds potential for glycodiversification of other scaffolds beyond tetracenomycins. The reasons behind the unsuccessful attachment of certain sugar moieties to the aglycone moiety remain unknown. To investigate this further, the functionality of the sugar biosynthesis could be tested by extracting sugars from the culture media. Additionally, the promiscuity of ElmGT could be evaluated through in vitro assays using purified ElmGT and the potential substrates. If it is determined that the attachment failure was due to ElmGT's inability to accept certain sugar moieties as substrates, one possible solution could be the generation of more promiscuous glycosyltransferases through directed evolution techniques (Williams et al. 2007).

The glycosylation of the aglycone had a significant impact on the antibacterial and cytotoxic activities of the compounds. Glycosylation generally resulted in the loss of cytotoxicity against most human cancer cell lines. Methylation, whether in the sugar moiety or at the C8-OH group, was observed to increase the cytotoxic activity of the compounds. In terms of antibacterial activities, the attachment of the sugar moiety to the aglycone influenced the bacterial strain specificity of the compounds. For instance, a compound with trimethylated L-rhamnose showed activity against *S. aureus*, while non-glycosylated compounds with a C8-*O*-methyl group were active against *S. violaceusniger*. Furthermore, some of the generated glycosylated tetracenomycins exhibited enhanced penetration of the outer cell wall of gram-negative bacteria compared to the aglycone moiety. However, it should be noted that the antibiotic activity of all tetracenomycin tested was generally low requiring 100 μM concentrations and therefore further development is needed to improve the efficiency of antibacterial activity. These findings highlight the importance of methylation in guiding the future development of glycosylated tetracenomycin analogues.

In future studies, expanding the pool of compounds and bacterial strains to be tested would provide a broader understanding of structure-activity relationships. It would be particularly important to test against all the pathogens listed on the World Health Organization's priority list. To gain more detailed insights into the structure-activity relationships, obtaining the structures of the target with the bound compound through techniques such as crystallography or molecular modeling would be beneficial. Additionally, investigating the pharmacokinetics of the compounds would provide valuable information for the development of antibiotics with desirable properties. In conclusion, this study successfully expanded the chemical diversity of tetracenomycins by introducing a new ketosugar (4'-keto-D-boivinose) and three new 6-deoxysugars (D-fucose, D-quinovose, and D-allose). Glycosylation proved effective in reducing cytotoxicity, but further improvement or testing against different bacterial strains is needed to enhance the antibacterial activities of the compounds.

Acknowledgements

I would like to express my gratitude to the following individuals, who have contributed to the successful completion of this master's thesis work. First and foremost, I am deeply grateful to Prof. Mikko Metsä-Ketelä for welcoming me into his Antibiotic Biosynthesis Engineering Lab (ABE) at the University of Turku, Finland, and for his continuous support, guidance, and encouragement. I would like to extend my appreciation to Assoc. Prof. Eric Nybo's group from Ferris State University, Michigan, USA, for providing the bacterial strains and plasmids that were instrumental in this study. I would also like to acknowledge Asst. Prof. Khaled Shaaban's group from the University of Kentucky, Kentucky, USA, for their assistance with the NMR analysis of the compounds and for providing valuable bioactivity results. I am grateful to MSc. Magdalena Niemczura for her contributions in performing the conjugations of the plasmids to bacterial strains, which were essential for the success of this study. Special thanks go to my students, Zélie Faudemer and Guillaume Mazurier, for their invaluable assistance in conducting the large-scale purification of the compounds. I would like to express my sincere appreciation to all my colleagues at ABE for their support and memorable moments both inside and outside the lab. Lastly, I would like to acknowledge the use of AI tools in improving the grammar of this thesis.

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