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SYNTHETIC BIOLOGY APPROACH FOR POLYKETIDE ENGINEERING AND ENZYME FUNCTION ELUCIDATION

Magdalena Joanna Niemczura

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
SARJA – SER. AI OSA – TOM. 744 | ASTRONOMICA – CHEMICA – PHYSICA – MATHEMATICA | TURKU 2025

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Life imitates art

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ABSTRACT

For decades, actinomycetes have been a major source of antibacterial and antiproliferative compounds, providing medicine with drugs invaluable for the treatment of a variety of diseases and types of cancer. The discovery of antibiotics allowed humanity to combat infections that would have been lethal otherwise, which puts them among the most transformative medical innovations in human history. Nowadays, widespread antibiotic use in agriculture and farming has led to the rise in bacterial antibiotic resistance, which, together with a rising number of cancer cases worldwide, creates a critical need for the discovery and synthesis of new bioactive compounds. Synthetic biology offers a comprehensive toolbox for the development of new, biologically active natural products, presenting a compelling alternative to conventional drug discovery methods.

The research presented in this thesis demonstrates how a synthetic biology approach, particularly the BioBrick technique, can facilitate the discovery and production of secondary metabolites in *Streptomyces* strains. First, we developed a BioBrick-based synthetic biology toolbox for the *de novo* construction of biosynthetic pathways, which proves to be especially effective in stepwise pathway building. We assembled eight deoxysugar pathways and expressed them in a heterologous *Streptomyces* host carrying 8-demethyl-tetracenomycin C aglycone, which resulted in the production of eight compounds, including four new glycosylated tetracenomycins.

Secondly, we characterised the functions of key enzymes in the chartreusin pathway using a BioBrick-based synthetic biology platform. We discovered a three-enzyme cascade responsible for converting an intermediate aglycone, auramycinone, into resomycin C through a double dehydration reaction. We proved that the 9,10-dehydration of auramycinone is catalysed by an enzyme pair ChaX/ChaU, while 7,8-dehydration is catalysed by the ChaJ enzyme.

Together, these findings establish synthetic biology as a valuable tool in both pathway assembly and enzyme function elucidation. They shed a new light on the elloramycin glycodiversification as well as the process of double dehydration of auramycinone in chartreusin biosynthesis. The synthetic biology approach used here can help expand our ability to engineer and manipulate biosynthetic pathways for the future production of valuable compounds.

TURUN YLIOPISTO
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TIIVISTELMÄ

Aktinobakteerit ovat olleet vuosikymmenien ajan merkittävä antibakteeristen ja antiproliferatiivisiten yhdisteiden lähde, ja ne ovat tarjonneet lääketieteelle korvaamattomia lääkkeitä erilaisten sairauksien ja syöpien hoitoon. Antibioottien löytyminen mahdollisti muuten kohtalokkaiden infektioiden torjumisen, mikä tekee niistä yhden merkittävimmistä lääketieteen innovaatioista ihmishistoriassa. Kuitenkin nykyään antibioottien laaja käyttö maataloudessa ja karjanhoidossa on johtanut bakteerien antibioottiresistenssin kasvuun. Antibioottiresistenssi sekä maailmanlaajuisesti kasvava syöpätapausten määrän luovat kiireellisen tarpeen uusien bioaktiivisten yhdisteiden löytämiselle ja syntetisoimiselle. Synteettinen biologia tarjoaa kattavan työkalusarjan uusien ja biologisesti aktiivisten luonnonyhdisteiden kehittämiseen ja toimii vaihtoehtona perinteisille lääkkeiden kehittämismenetelmille.

Tämä väitöskirjatutkimus osoittaa, kuinka synteettisen biologian lähestymistapa, erityisesti BioBrick-tekniikka, voi helpottaa luonnonyhdisteiden löytämistä ja tuottamista Streptomyces-bakteerikannoissa. Ensiksi kehitimme BioBrick-pohjaisen synteettisen biologian työkalusarjan biosynteettisten reittien de novo -rakentamiseen, mikä osoittautui erityisen tehokkaaksi vaiheittaisessa biosynteesireitin rakentamisessa. Kokosimme kahdeksan deoksisokerireittiä ja ilmensimme ne heterologisessa Streptomyces-isäntäkannassa, joka tuotti 8-demetyyli-tetrasenomysiini C -aglykonia. Tämä johti kahdeksan yhdisteen tuotantoon, joista neljä oli uusia glykosyloituja tetrasenomysiinejä.

Seuraavaksi karakterisoimme chartreusiinireitin keskeisten entsyymien toimintoja käyttämällä BioBrick-pohjaista synteettisen biologian työkalusarjaa. Löysimme kolmen entsyymin kaskadin, joka vastaa välituoteaglykonin, auramysiinin, muuntamisesta resomysiini C:ksi kaksoisdehydraatioreaktion kautta. Auramysiinin 9,10-dehydraatio katalysoituu entsyymiparilla ChaX/ChaU, kun taas 7,8-dehydraatio katalysoituu ChaJ-entsyymillä.

Yhteenvetona väitöskirjatutkimuksen löydöt vahvistavat synteettisen biologian toimivuutta biosynteesireittien rakentamisessa, että entsyymien toiminnan selvittämisessä. Tutkimus toi myös uutta tietoa elloramysiinin glykodiversifikaatiosta ja auramysiinin kaksoisdehydraatioprosessista chartreusiinin biosynteesissä. Tutkimuksessa käytetty synteettisen biologian lähestymistapa voi auttaa laajentamaan kykyämme suunnitella ja manipuloida biosynteettisiä reittejä uusien luonnonyhdisteiden tuottamiseksi tulevaisuudessa.

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MAGDALENA JOANNA NIEMCZURA: Zastosowanie metod biologii syntetycznej w projektowaniu poliketydów oraz badaniu mechanizmów działania enzymów.
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STRESZCZENIE

Przez dziesięciolecia promieniowce były głównym źródłem związków antybakteryjnych i przeciwrakowych, dostarczając medycynie leków nieocenionych w leczeniu różnorodnych infekcji i nowotworów. Odkrycie antybiotyków pozwoliło ludzkości zwalczać choroby, które w przeciwnym razie byłyby śmiertelne, co stawia je wśród najbardziej przełomowych innowacji medycznych w historii. Obecnie powszechne stosowanie antybiotyków w rolnictwie i hodowli zwierząt doprowadziło do wzrostu oporności bakterii na antybiotyki, co wraz z rosnącą liczbą przypadków nowotworów na całym świecie stwarza potrzebę odkrywania bądź syntezy nowych związków bioaktywnych. Biologia syntetyczna oferuje kompleksowy zestaw narzędzi do opracowywania nowych, biologicznie aktywnych metabolitów wtórnych, stanowiąc przekonującą alternatywę dla konwencjonalnych metod odkrywania związków chemicznych.

Badania przedstawione w tej pracy doktorskiej pokazują, jak podejście z zakresu biologii syntetycznej, szczególnie technika BioBricks, może ułatwić odkrywanie i produkcję metabolitów wtórnych w szczepach bakterii z rodziny Streptomyces. Po pierwsze, opracowaliśmy oparty na technice BioBricks zestaw narzędzi pozwalających na konstrukcję szlaków biosyntetycznych de novo. Zbudowalismy osiem szlaków deoksycukrów, które potem ekspresjonowaliśmy heterologicznie w biosyntetycznym szczepie Streptomyces niosącym aglikon 8-demetylo-tetracenomycyny C, co zaowocowało produkcją ośmiu związków, w tym czterech nowych glikozylowanych tetracenomycyn.

Po drugie, scharakteryzowaliśmy funkcje kluczowych enzymów w szlaku chartreusyny, używając narzędzi biologii syntetycznej opartych na technice BioBricks. Odkryliśmy kaskadę trzech enzymów odpowiedzialnych za konwersję pośredniego aglikonu, auramycynonu, w resomycynę C poprzez jego podwójną dehydratację. Udowodniliśmy, że 9,10-dehydratacja auramycynonu jest katalizowana przez parę enzymów ChaX/ChaU, podczas gdy 7,8-dehydratacja jest katalizowana przez enzym ChaJ.

Łącznie te odkrycia ugruntowują biologię syntetyczną jako cenne narzędzie zarówno w budowie szlaków metabolicznych, jak i w opisywaniu funkcji enzymów. Rzucają one nowe światło na glikodywersyfikację elloramycyny, a także na proces podwójnej dehydratacji auramycynonu w biosyntezie chartreusyny. Strategia oparta na biologii syntetycznej zastosowana w poniższej pracy może pomóc w rozszerzeniu naszej zdolności do inżynierii i manipulacji szlakami biosyntetycznymi w celu przyszłej produkcji cennych związków.

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Abbreviations

ACP	Acyl carrier protein
ALL	Lymphoblastic leukemia
AML	Acute myeloid leukemia
BGC	Biosynthetic gene cluster
CFU	Colony forming unit
CLF	Chain Length Factor
CoA	Coenzyme-A
DAD	Diode array detector
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
GFP	Green fluorescence protein
HPLC	High pressure liquid chromatography
iGEM	International Genetically Engineered Machine
KS	Ketosynthase
LA	Lysogeny agar
LB	Lysogeny broth
LC-MS	Liquid chromatography-Mass spectrometry
MH	Muller Hinton media
MIT	Massachusetts Institute of Technology
MQ	Milli-Q
MRSA	Methicillin-resistant Staphylococcus aureus
MS	Mannitol-soy flour
MS/MS	Mass spectrometry/mass spectrometry
MSSA	Methicillin-susceptible Staphylococcus aureus
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide
NHL	Non-Hodgkin lymphoma
NMR	Nuclear magnetic resonance
NOMAD	Nucleic acid ordered module assembly with directionality

NRP	Non-ribosomal peptides
ORF	Open reading frame
PCR	Polymerase chain reaction
pDALO	TDP-D-allose
pDBOV	TDP-D-boivinose
pDDIG	TDP-D -digitoxose
pDFUCO	TDP-D-fucofuranose
pDMYC	TDP-D-mycarose
pDOLV	TDP-D-olivose
PEG	Polyethylene glycol
PKS	Polyketide synthase
RBS	Ribosome binding site
RFP	Red fluorescence protein
RiPP	Ribosomally synthesized and post-translationally modified peptides
RNA	Ribonucleic acid
SARP	Streptomyces antibiotic regulatory protein
SG	Soytone-glucose
TDP	Thymidyne diposphate
TES	Triethylsilane
TFA	Trifluoroacetic acid
TSB	Tryptone soy broth
UHPLC	Ultra high-pressure liquid chromatography
UTR	Untranslated region
UV	Ultraviolet
WHO	World Health Organisation

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I **Engineering BioBricks for Deoxysugar Biosynthesis and Generation of New Tetracenomycins** Tirkkonen H, Brown K, Niemczura M, Faudemer, Z, Brown C., Ponomareva L, Helmy Y., Thorson J., Nybo S.E., Metsä-Ketelä M., Shaaban K. *ACS Omega (2023) 8(23) 21237-21253*

II **Three-enzyme cascade catalyzes conversion of auramycinone to resomycin in chartreusin biosynthesis** Niemczura M., Nuutila A., Wang R., Rauhanen K., Nybo S.E. and Metsä-Ketelä M., (Accepted, ACS Chemical Biology, 2025)

1 Introduction

1.1 Actinomycetes and their natural products

Antibiotics are one of the most important groups of compounds known to man, as they revolutionized our capacity to fight diseases that plagued humankind for millennia. Already in ancient times people noticed that using a certain plant reduces symptoms of an infection [1]. Traces of tetracyclines were discovered in bones of people living in ancient Sudan, dating back to 350-550 CE, which suggests presence of tetracycline-containing foods in their diet [2]. Traditional Chinese medicine has known methods of treating diseases with plants that later were found to contain active natural agents [3]. After Alexander Fleming noticed that his bacteria do not grow around certain types of mould [4], the focus shifted towards microbes as producers of antibiotics. During the "Golden era" of antibiotics between 1950s and 1970s, the research on the natural producers was thriving, which resulted in the discovery of over 20 new classes of antibiotics [5].

Cancer is a leading and continuously rising concern for modern medicine. Growing pollution and other environmental factors as well as an aging population significantly contribute to an increase in the number of cases. According to WHO's estimates, in 2022 alone it was responsible for the deaths of nearly 10 million people, which places it as the second most common cause of death in the world [6]. Given the circumstances, antiproliferative drugs are now sought after more than ever and the demand for anticancer therapies is at an all-time high [7]. In the antibiotic "Golden era" many compounds were discovered which, while showing minimal antibiotic activity, exhibited potent antiproliferative properties. One of them was an anthracycline compound daunorubicin which was isolated from *Streptomyces peucetius* in the 1960s and quickly became the preferred chemotherapeutic for certain types of cancer due to its robust antiproliferative activity. More than half a century after their discovery, *Streptomyces*-derived natural products are still prominent anticancer agents and are included in the WHO list of drugs essential for cancer treatment [6]. They are used alone or in combination with other chemotherapeutics in treatment of lymphomas, leukemias as well as solid tumours in stomach, ovaries, breast or brain [8].

The phylum that has contributed the most to the antibiotic and anticancer repository of modern medicine is Actinobacteria. To illustrate their prevalence, in just one gram of soil, there are about 10^9 colony-forming units (CFU), with an estimated 10^7 of these being Actinomycetes [9]. One of the greatest producers of secondary metabolites are soil dwelling bacteria from the *Streptomycetaceae* family. They diverged from *Kitasatospora* in the Devonian period, around 382 million years ago, which was most likely caused by the appearance of photosynthesizing plants whose dead tissues the bacteria could feed on [10]. The family *Streptomycetaceae*, the largest within the phylum Actinomycetota, comprises of a variety of aerobic, Gram-positive, non-pathogenic bacteria species characterized by high genomic GC content ranging from 69% to 78% [11].

The genus *Streptomyces*, which includes over 800 bacterial species, was established in 1943 and since then has been the most important subject of antibiotic research. Their preferred environment is soil rich in organic matter, but they also inhabit a variety of terrestrial and marine environments such as deserts [12], deep sea [13][14] or even the inner surface of marine sponges [15]. Evolutionarily, *Streptomyces* specialized in producing and secreting antibacterial natural products, initially meant as a competitive strategy against other microorganisms [16]. Bacterial secondary metabolites are also valuable to plants with which *Streptomyces* become symbiotic and protect the plant from pathogens [16]. The first *Streptomyces*-derived antibiotics were introduced into clinical use in the 1940s. They were first classified as antibiotics thanks to their antibacterial properties, but another very important feature is their anticancer properties [17][18][10]. As of 2018, 17% of all biologically active natural products came from *Streptomycetes* [9]. Thanks to them we have such widely used antibiotics as kanamycin [19], tetracycline [20], daptomycin [21], erythromycin [22] or rifampicin [23], to name just a few. The discovery of this group of compounds contributed immensely to the overall well-being of humanity, treating diseases ranging from typhoid and leprosy to acne.

1.1.1 The need for new natural products in drug development

Antibiotics gave humanity a powerful tool to combat pathogenic bacteria, which led to a rapid and significant decrease in deaths caused by bacterial diseases worldwide. However, microbes quickly developed numerous mechanisms to gain antibiotic resistance [24], and recently the pace has picked up at an alarming rate, which poses a major threat to global health. The ratio of the number of bacterial strains becoming insensitive to antibiotics to the number of new antibiotics being developed is very unfavourable for the latter, with only a few new active compounds in clinical trials [25]. Over the course of recent years, inconsiderate use of antibiotics in medicine,

agriculture and animal breeding has led to a significant increase in antibiotic resistance in bacteria [1]. As of 2023, there was not even one antibiotic in clinical use to which there were no resistant strains [25]. The United States alone was responsible for 46% of global antibiotic use as of 2013 [26] and the trend has continued to escalate, with worldwide antibiotic consumption increasing by nearly 50% between 2000 and 2018 [26].

Contemporary cancer treatment also remains far from ideal with a lot of currently used anticancer compounds exhibiting unfavourable side effects. Doxorubicin, one of the most popular anticancer drugs in treatment of sarcomas in children, acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) or breast cancer has dose-dependent cardiotoxic side effects with an elevated risk of developing a heart condition later in life [27]. Another popular chemotherapeutic bleomycin, used for treatment of Hodgkin lymphoma, non-Hodgkin lymphoma (NHL) or various types of squamous cell carcinoma [28] was shown to have a connection with vascular malformations [29]. Dactinomycin, a chemotherapeutical used in treatment of Ewing sarcoma in adults and children, gestational trophoblastic neoplasia, rhabdomyosarcoma, and solid tumours, [30] and mitomycin used against gastric and pancreatic adenocarcinoma are both hepatotoxic [31]. The main goal of current research on new anticancer compounds is to focus on improving the selectivity, efficiency and safety of the drug while maintaining its bioavailability and anticancer activity [11]

1.1.2 Genomic organization, diversity and expression control of *Streptomyces*-derived natural products

Actinomycetes produce a wide variety of natural products such as polyketides, peptides (non-ribosomal peptides (NRPs), [32] and ribosomally synthesized or post-translationally modified peptides (RiPPs) [33]), aminoglycosides [34], and many more. Genes responsible for natural product biosynthesis are typically clustered within the genome into biosynthetic gene clusters (BGCs). A recent genome-wide study of various Actinomycete species revealed that a single bacterial genome can encode from 25 to 70 diverse BGCs [35]. The size of a single biosynthetic gene cluster differs between strains and the type of molecule it encodes. Different genera of bacteria within the Actinomycetes class also have different secondary metabolite production patterns. For example, NRP biosynthetic gene clusters are more prevalent within the genus *Rhodococcus* than in other genera, while in *Streptomyces* polyketide BGCs are the majority [36].

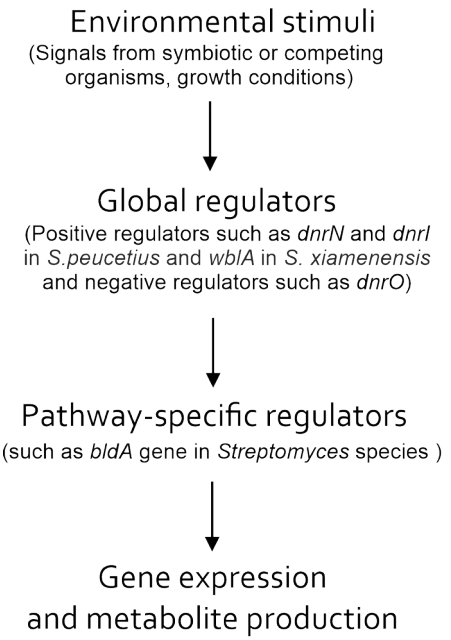

Figure 1: Different types of gene expression regulators in *Streptomyces*.

Early research on Actinomycetes focused on naturally expressed compounds, however after genome sequencing became more accessible and routinely used, it became obvious that the number of potential BGCs present in the bacterial genome is significantly higher than the number of compounds produced by the strain. This is because the majority of biosynthetic gene clusters are not expressed under laboratory conditions [37]. The non-expressed BGCs were called cryptic, sleeping or silent clusters. In fact, only about 1% of environmental bacteria can be cultivated under laboratory conditions and only a small fraction of existing biosynthetic pathways have ever been experimentally tested [38][39]. The control of the activation of these clusters is tied to the original preferred environmental conditions of the strain, where external stimuli such as interactions with other microorganisms, nutrient scarcity and morphological factors [40] trigger the production of the antibiotic secondary metabolites [41]. Actinomycetes can also maintain symbiotic interactions with plants, saprophytes and insects such as parasitic wasps, as well as marine organisms such as sea cucumbers or sponges [42]. In these relationships the secondary metabolites are being produced as a response to very specific communication with the mutualistic partner [42].

The expression of genes within biosynthetic gene clusters (BGCs) is also controlled through multiple internal regulatory mechanisms (Figure 1). The environmental signal is typically detected by two-component regulatory system, which is composed of a transmembrane sensor kinase that phosphorylates an intracellular regulatory protein upon binding the external molecule triggering the environmental response [43][44]. The regulatory genes that mediate the environmental signal further include both global regulators such as the *bldA* gene and pathway-specific controllers, such as members of the SARP (*Streptomyces* antibiotic regulatory protein) family [40]. The *bldA* gene, is responsible for triggering the antibiotic production and plays a crucial role in the regulation of transcriptional expression [45]. Global regulatory systems can have both positive and negative control over secondary metabolite biosynthesis. Positive regulators include *dnrN* and *dnrI* in *S. peucetius* and *wblA* in *S. xiamenensis,* while negative

regulators can be represented by *dnrO* [46]. SARPs are responsible for the transcriptional control of the expression of genes within a biosynthetic gene cluster [47], such as ActII-ORF4 in actinorhodin pathway, CdaR in the calcium-dependent antibiotic or RedD in undecylprodigiosin, the best studied regulatory cascade of the antibiotic producing clusters in *Streptomyces* [46]. In doxorubicin pathway we have a three-protein regulatory cascade responsible for the activation of the BGC, and jadomycin BGC encodes five, including the pathway specific activator JadR1 [46].

1.1.3 Discovery of novel microbial natural products

Despite the large number of microbial natural products currently in clinal use, there is a constant demand for improved drugs. Every year researchers discover new natural, biosynthetic or semi-biosynthetic active compounds produced by *Streptomyces*, but the demand is at an all-time high. A classic way of sourcing new strains of Streptomyces would be to venture out into the world and collect a soil sample, which would later be analyzed for the presence of *Actinomycetes*, and all the metabolites would be characterized [12]. Between 2015 and 2020, research on Actinomycetes has resulted in the discovery of an exceptionally high number of new *Streptomyces* strains, Actinomycete-derived antibiotics and antiproliferative agents [11]. The new natural products include compounds such as branimycins B and C discovered in 2017 [48], ghanamycins A and B [49] isolated from *S. ghanaensis*, a strain known before for the production of moenomycins [50], or benz[a]anthracene polyketides - donghaecyclinones [51]. Given the number of Streptomyces-derived natural products that are currently being used in clinical settings, Actinobacteria as a phylum to this day are and will still be incredibly significant for antibiotic development [52]. The main issue with the traditional methods is that it is affected by diminishing yields due to the decades of screening of soil samples. Estimates suggest that the rediscovery rate of known compounds is close to 99 % [38].

Another strategy for obtaining improved active compounds is their repurposing. Many microbial natural products have been shown to harbor multiple biological activities, and several antibiotics have recently been repurposed as anticancer agents. Daptomycin for example, a compound used for treating difficult infections, was shown to act in a suppressive manner towards breast cancer cells [53]. Another example of successful antibiotic repurposed as anticancer drug is doxycycline, which is a broad-spectrum antibiotic effective against gram-positive and gram-negative, aerobic and anaerobic bacteria. Its spectrum is so broad that it found use in treatment of rosacea but also anthrax. As an anticancer agent, doxocycline shows activity against breast cancer [54] and various cancer cell lines including lymphoma, prostate cancer or leukemia [55]. Another low-side-effect drug, erythromycin, exhibited suppressive antitumour activity in mice and other rodents [56]. Aureolic acids were

also first studied as antibacterial compounds, only to later be used as treatment of several types of cancer [57]. Overall, it is a promising route to repurpose antibacterial compounds as anticancer drugs.

One of the more exciting contemporary opportunities for discovery of natural products is synthetic biology, which offers both a powerful new approach to the production of unknown secondary metabolites and a way to refine the already known pathways. The constantly expanding genome sequence databases have revealed the presence of numerous novel BGCs both within genomes of isolated microbes and environmental DNA sequencing samples [35]. A variety of advanced bioinformatic tools have been developed to efficiently analyze, annotate and categorize BGCs within a *Streptomyces* genome [58]. Deep learning methods such as DeepBGC [59] and e-DeepBGC [60], assembly graph analysis by biosyntheticSPAdes [61] and BGC analysis software antiSMASH [62], that utilizes large-scale comparative databases such as MIBIG [63] or BiG-FAM [64] are crucial to the discovery and identification of new BGCs for natural product discovery. The cluster discovery methods are constantly improved for even more accurate and precise BGC annotation.

On top of that, advances in genetic engineering and constantly expanding genetic toolbox for actinomycetes allowed for greater precision in pathway manipulation [65]. Traditionally, silent gene clusters within the *Streptomyces* genome were activated by removing repressors or introducing activators [66]. Today, advanced tools such as various synthetic biology techniques allow for immensely more precise and tuned engineering biosynthetic pathways for optimal natural product expression [52][66][67]. Combinatorial metabolic engineering revolutionized the production of antibiotics and opened a new door for yield improvement and novel compound discovery in bacteria. Techniques such as gene cluster manipulation, where specific genes within a cluster are duplicated, deleted or multiplicated through multi-copy integration, are incredibly valuable for increasing titers [68][69]. An alternative approach to combinatorial biosynthesis is regulatory gene engineering, which relies on increasing the expression of resistance genes and deletion of repressors which could potentially affect the yields [70][71]. A common practice is also to deactivate native pathways in the strain that is meant to serve as a heterologous host, to redirect the intracellular precursors towards the desired pathway [72]. Another key technique in combinatorial biosynthesis is glycodiversification of compounds via a promiscuous glycosyltransferase. The enzyme will attach a variety of carbohydrate units to an aglycone, resulting in the creation of new secondary metabolites [73]. This can be achieved also through enzyme swapping, where a protein from the original pathway will be replaced by an oftentimes better functioning homolog [74]. A very important technique in pathway engineering is the CRISPR-Cas system. In *Streptomyces* this technique found application in activating silent BGCs [75],

identifying potential BGC regions, and enabling targeted modifications within the bacterial genome such as deletion of entire clusters [76]. CRISPR-Cas systems are constantly evolving and expanding to include more *Streptomyce*s species and editing targets [76]. Synthetic biology utilizes the tools of engineering to recontextualize the current view on developing new derivatives of natural products and discovering unknown clusters within the Actinomycetes class. Currently most of the synthetic biology research focuses on already known groups of natural products that have been studied for decades in attempts to develop a new synthetic or semi-synthetic version of the compound in hopes that the derivative will lack the adverse side effects of its predecessor [77].

1.2 Type II polyketides

Polyketides are a highly diverse group of natural products, among which we can name type I, II and III polyketides. Type I polyketide synthases are large, modular multi-domain enzymes found in bacteria and fungi, where each module performs one specific step in building a polyketide chain. They are non-iterative, which means that each module is used only once during the process. Among type I polyketides we can find groups of compounds such as macrolides or polyenes. Type III polyketide synthases are smaller and simpler homodimeric enzymes acting iteratively to build a polyketide compound. Type III polyketides are small, monocyclic or bicyclic compounds such as flavolin [78].

The research described in this work focuses on type II polyketides, which include a variety of secondary metabolites possessing valuable, clinically significant activities. They are abundantly present in Actinomycetes, especially within the *Streptomyces* genus [79] and despite their diversity share a common biosynthetic logic and core structural features. They all start as a long polyketide chain which can then be cyclized in an array of different ways to yield different polyaromatic structures. Aromatic polyketides can be divided into groups based on their carbon scaffold. Within type II polyketides we can distinguish anthracyclines, angucyclines, tetracenomycins, tetracyclines, aureolic acids, pradimicin-type pentangular polyphenols, and benzoisochromanequinones [80].

1.2.1.1 Anthracyclines

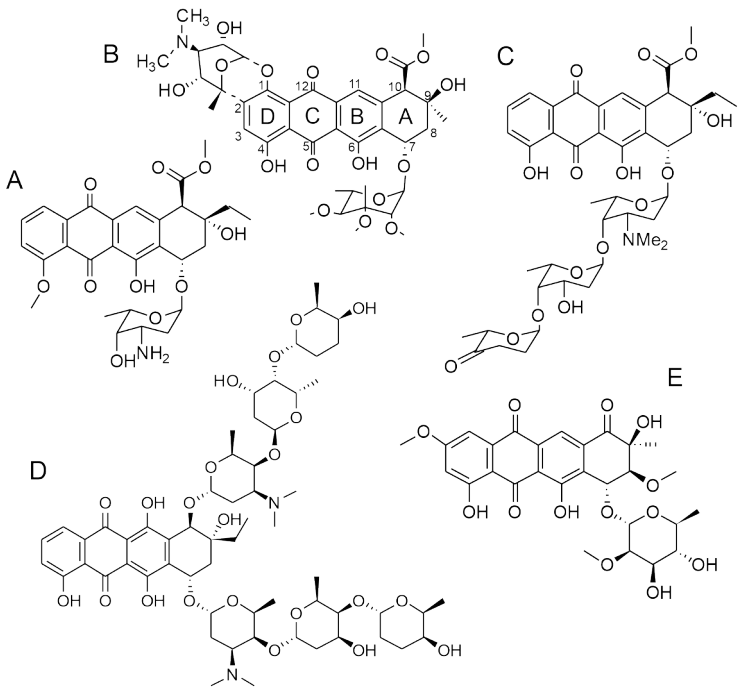

Figure 2: Examples of anthracyclines: (A) daunorubicin. (B) nogalamycin. (C) aclacinomycin A. (D) cosmomycin D. (E) steffimycin.

Anthracyclines are the largest subgroup within type II polyketides [46]. Structurally, anthracyclines consist of a tetracyclic 7,8,9,10-tetrahydro-5,12-naphtaceno-quinone aglycone and a saccharide moiety [81] (Figure 2). Their mechanism of action involves intercalating to DNA and poisoning topoisomerase II [82].

The anthracycline aglycone comprises a planar, linear anthraquinone chromophore composed of four rings: D, C, B, and A. The fourth ring serves as a primary binding site for various side chains and is the most common location for the attachment of saccharide moieties [6]. The anthraquinone skeleton acts as a chromophore and is responsible for the red colour characteristic for this group of compounds. Anthracyclines are usually glycosylated at the C7 position of ring A, with a few exceptions such as nogalamycin, glycosylated both at the C7 and at C1 positions. [83].

Anthracycline aglycones exhibit structural diversity through various substitutions at different carbons. For example, substitutions at the C10 can differ from none at all in dauno- and doxorubicin, through a keto group in steffimycin [84] and hydroxyl group in cosmomycin D to a branched carboxymethyl group in nogalamycin and aclacinomycin (Figure 2) [85]. Another popular site for structural substitution is the C9 carbon, where a whole array of different alkyl side chains can be attached. In nogalamycin we see a methyl group at C9, while in aclacinomycin, steffimycin and cosmomycin D an ethyl group is found at that position [46]. Doxorubicin and daunorubicin differ at the C9 position by one OH group, the side

chain of daunorubicin bearing a primary alcohol and the side chain of doxorubicin a methyl group. This position is also important for the determination of the stereochemistry of the molecule [81].

1.2.1.2 Tetracyclines

One of the most widely used groups of antibiotics known to science are tetracyclines, secondary metabolites produced by various *Streptomyces* species. This group contains antibiotics such as doxycycline, chlortetracycline or oxytetracycline (Figure 3), which are effective against both Gram-positive and Gram-negative bacteria and even some protozoan parasites [86]. Their mechanism of action involves inhibition of protein synthesis through blocking the bacterial ribosome and therefore inhibiting the binding of aminoacyl-tRNA [86].

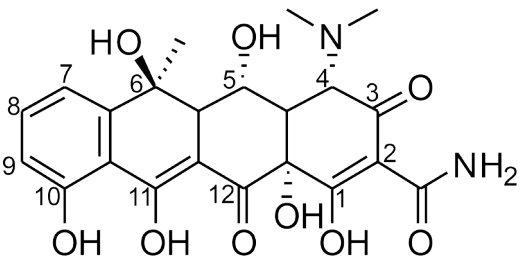

Figure 3: Chemical structure of oxytetracycline.

Their broad spectrum of action established tetracyclines as the second most used group of antibiotics in the world as of 2020 [87]. Unfortunately, their widespread use in agriculture and animal breeding made them the main contributor for the rising antibiotic resistance in nature [88]. The basis of the tetracycline antibiotic is a four-ringed linear structure with an array of different functional groups determining the properties of the compound [89]. Development of new tetracycline antibiotics has been achieved by shuffling these functional groups around to come up with a new molecule. The key positions for attachment of functional groups to obtain new derivatives are C9 and C7 [86] with 6-deoxy-6-demethyltetracycline being the minimal tetracycline pharmacophore, the simplest tetracycline derivative to still possess pharmacological properties. Development of new tetracyclines constantly progresses and nowadays we have three generations of biosynthetic, synthetic and semi-synthetic tetracycline antibiotics [86].

1.2.1.3 Tetracenomycins

Tetracenomycins are a small group of type II polyketides produced by the *Streptomycetaceae* and *Nocardiaceae* families. The first known tetracenomycin was tetracenomycin C (Figure 4) discovered in 1979 [90]. Despite being type II polyketides, which makes them structurally related to tetracyclines and anthracyclines, tetracenomycins have remarkably few close relatives. Some tetracenomycins retain more aromaticity during their biosynthesis than others, whose aromatic ring gets oxidated into a diol and therefore partially saturated [91]. Similarly to other type II polyketide natural products, they exhibit anticancer and antibiotic properties [80]. Their mechanism of action manifests in inhibition of the process of translation by binding to the larger ribosomal subunit [92]. Most studied tetracenomycins include tetracenomycin C produced by *S. glaucescens* and elloramycins A-F produced by *S. olivaceus* Tu2353, as well as tetracenomycin D3 or tetracenomycin B3 which were found to be intermediates of both elloramycin and tetracenomycin C [91].

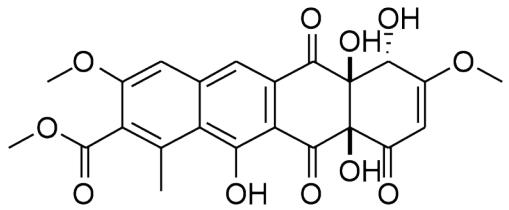

Figure 4: Chemical structure of tetracenomycin C.

1.2.1.4 Angucyclines

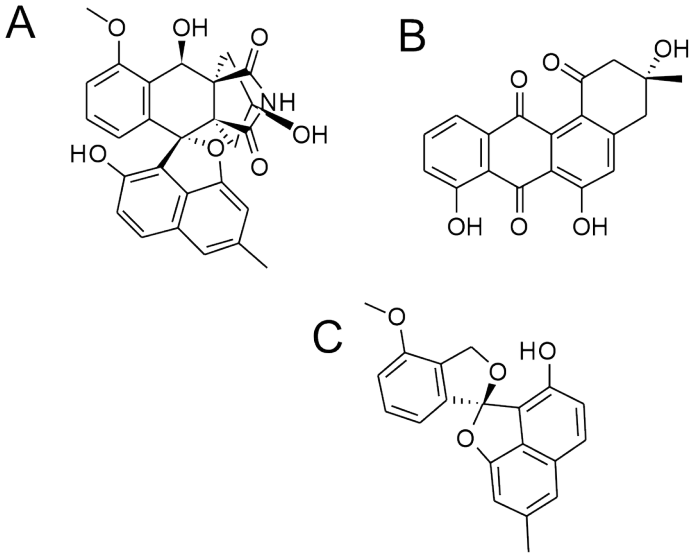

Figure 5: Examples of angucyclines(A) lugdunomycin, (B) rabelomycin, (C) elmonin.

Another large group of type II polyketides synthesized by various species of Actinobacteria are angucyclines, glycosylated decaketides possessing antibacterial, antifungal and antitumour properties. The first angucycline was characterized in 1965 and as of 2024 there are over 300 angucyclines known to science, including compounds such as landomycin, griseomycin, lugdunomycin (Figure 5),

aquayamycin or chlorocyclinones [93][94]. The high structural variation of angucyclines is reflected in their biological activities and diverse mechanisms of action. They can be further divided into three groups: classic angucyclines, rearranged angucyclines and those with C-ring cleavage such as elmonin. C-ring cleavage occurs when the C ring of the quinone part of the molecule is oxidated, which results in opening of the lactone ring and subsequent structure rearrangement. Most angucyclines, such as rabelomycin (Figure 5) have benz(a)anthraquinone as their tetracyclic backbone [93]. Unlike tetracenomycins or anthracyclines, the decaketide chain of angucyclines undergoes angular cyclization, hence their name [94]. Despite the large diversity in this family of natural products and their anticancer and antibacterial therapeutic potential, none of them reached the final stages of clinical trials due to their high toxicity and solubility issues [93]. Currently most of the research on angucyclines focuses on the discovery and synthesis of new, less toxic derivatives that might have a greater clinical potential [93] [94].

1.2.1.5 Pradimicin-type pentangular polyphenols

Pradimicin-type pentangular polyphenols such as pluramycins, pradimicin A and benzo(a)naphthacenes are a group of Actinomycete-derived type II polyketides closely related to angucyclines, also featuring an angular ring system. They exhibit a broad-spectrum of antiviral and antifungal properties and their mechanism of action relies on disrupting the fungal cell wall by binding to its terminal D-mannoside [79][95]. Pradimicin A (Figure 6) is an antifungal agent consisting of a benzo(a)naphthacene aglycone decorated with two chiral alcohols at positions C6 and C5 of the D ring and two saccharide moieties, D-xylopyranose and 4-methylamino-4-deoxy-D-fucopyranose, attached to one of them through an oxygen atom [96]. Pradimicin is one of the few type II polyketide compounds that feature an amino acid moiety, D-alanine, attached to the E ring at the C2 position.

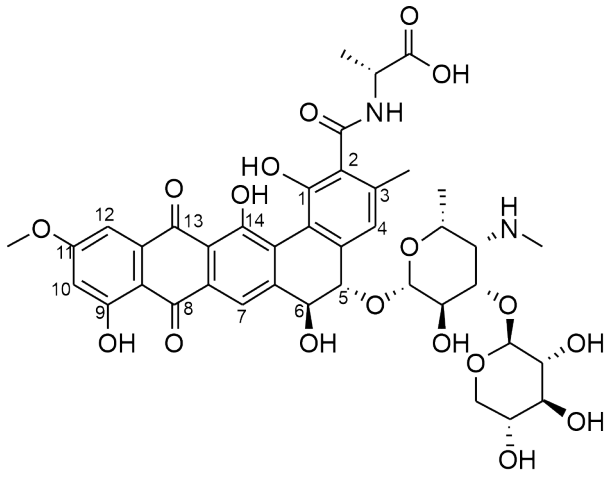

Figure 6: Chemical structure of pradimicin A.

1.2.1.6 Aureolic acids

Aureolic acids are a family of type II tricyclic polyketides produced by various *Streptomyces* species. The first member of this family, aureolic acid, also known as mithramycin (Figure 7), was described in 1953 [57]. Later other members of the family followed, such as chromocyclomycin, olivomycin and chromomycin A3. Aureolic acids are fluorescent, intensely yellow compounds characterized by a tricyclic ring system with a long dihydroxy-methoxy-oxo-pentyl aliphatic chain attached to it at C2 position. There are certain structural differences between the representatives of the aureolic acids family, such as differences in the number of rings in the aglycone, the presence of a small methyl or isobutyl residue attached to C7 carbon, the length and composition of the C2 aliphatic chain or the type of the oligosaccharide residues at C1 and C6 [97].

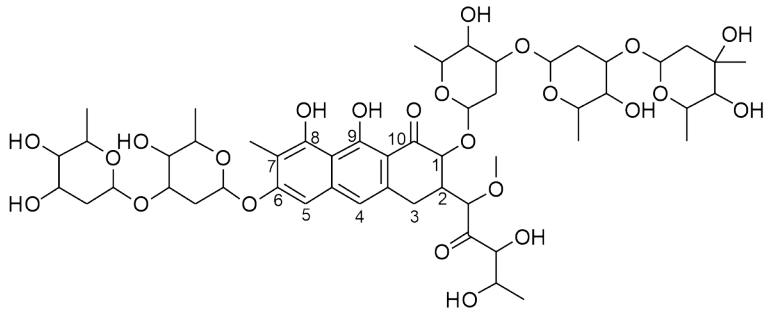

Figure 7: Chemical structure of mithramycin A.

Mechanism of action of aureolic acids relies on their nonintercalative interaction with the minor groove of the DNA double helix in GC rich regions. They inhibit transcription, which significantly affects the downstream protein production [57] [97]. Aureolic acids were first studied as antibacterial compounds, however due to the lack of any activity against gram-negative bacteria, the focus of the research was shifted to testing their antiviral and anticancer properties. Nowadays they are being used in the treatment of malignant tumours, testicular cancer and acute myeloid leukemia. They also proved to be effective against HIV proteins [57]. Unfortunately, the clinical applications of these compounds are limited by their toxicity [97].

1.2.1.7 Benzoisochromanequinones

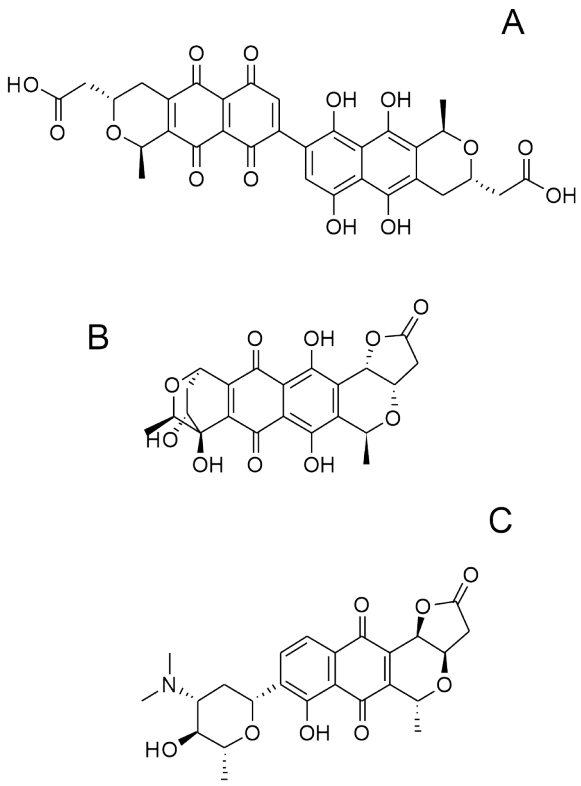

Figure 8: Chemical structures of (A) Actinorhodin. (B) Granaticin. (C) Medermycin.

Benzoisochromanequinones are a group of aromatic antibacterial and anticancer type II polyketides produced by Actinomycetes containing a quinone isochromane derivative in their chromophore. The most researched benzoisochromanequinone is actinorhodin from *S. coelicolor*, one of the best studied type II polyketides to date. Other molecules include medermycin and granaticin from *S. olivaceus* (Figure 8) [79]. Benzoisochromanequinones can be glycosylated, like medermycin, non-glycosylated, like actinorhodin or glycosylated in an atypical way like granaticin. The saccharide moiety involved in the biosynthesis of medermycin is an aminosugar, dTDP-D-angolosamine coming from the deoxyhexose pathway [98]. Medermycin has a strong activity against Gram-positive bacteria, as well an anti-platelet-aggregation activity. Due to their diversity, the mechanism of action of benzoisochromanequinones differs between compounds [99].

1.2.2 Typical type II polyketide biosynthetic gene cluster (BGC)

Extensive research conducted on the biosynthesis of various polyketides showed that a typical type II polyketide gene cluster contains around 30 genes encoding a plethora of enzymes such as cyclases, ketoreductases or transferases [98]. Although type II PK BGCs are the most abundant among Actinobacteria, there is evidence of II PK BGCs found in non-Actinobacterial species such as *Ktenobacter* or *Streptococcus* [100]. The following sections discuss the logic of the type II polyketide PKS biosynthesis using primarily anthracyclines as examples.

1.2.3 Biosynthetic pathways of type II polyketides and their core enzymes

1.2.3.1 Aglycone biosynthesis

A typical type II polyketide biosynthetic pathway starts with a minimal polyketide synthase (minPKS), comprised of three components: a ketosynthase (KS), a chain length factor (CLF) and an acyl carrier protein (ACP) [101]. This protein complex is responsible for assembling a polyketide chain through iterative Claisen condensation of malonyl-CoA extender units to a variety of different starter units [78]. In the KS/CLF heterodimer, the KS enzyme contains amino acids responsible for catalysis, while the chain length factor (CLF) part determines the length of the synthesized polyketide chain [102]. The growing polyketide chain is attached to an acyl carrier protein, to protect the highly reactive polyketide intermediates from premature cyclization [103]. After the elongation is finished, the polyketide chain detaches from ACP, however the exact moment or the mechanism of the detachment is unknown. This process results in the creation of an initial polyketide structure [104] [46].

In biosynthetic pathways of type II polyketides, we can observe different types of starting units. In anthracyclines such as aclacinomycin or daunorubicin we have a propionyl-CoA starting unit (Figure 9 B), which requires two additional genes for chain initiation, *dpsC* and *dpsD*. In oxytetracycline we have a malonamyl-CoA starting unit and in anthraquinones such as R1128 A-D the polyketide chain is initiated from a short chain carboxylic acid such as butyrate and isobutyrate [105]. An interesting group of polyketides are compounds produced by the marine *Streptomyces maritimus*, such as enterocin, a synthesis of which is initiated from a cyclic starting unit, benzoyl-CoA (Figure 9 D) [106]. The extender unit for type II polyketides is always malonyl-CoA [107].

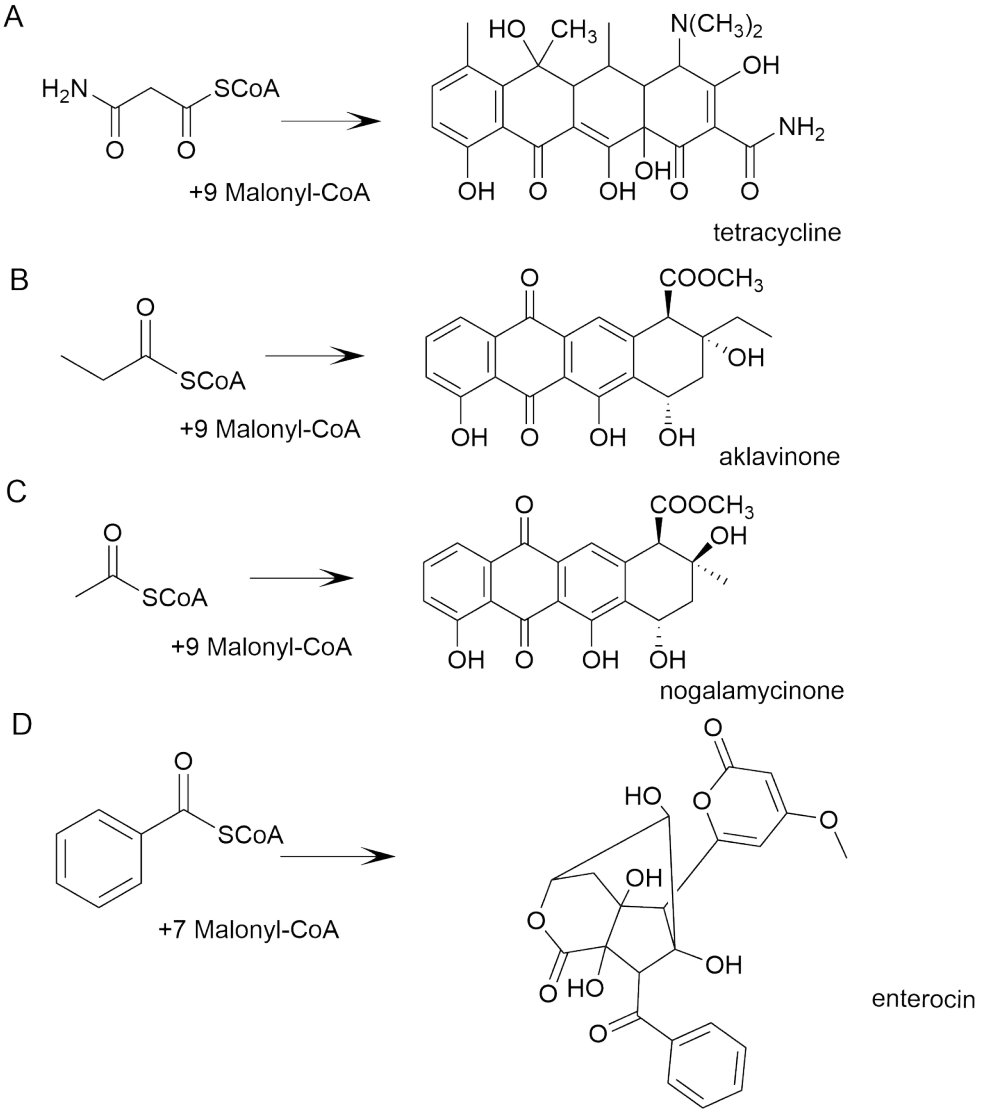

Figure 9: The diversity of starting units among type II polyketides: (A) malonamyl-CoA starting unit in tetracycline biosynthesis. (B) propionyl-CoA in aclacinomycin biosynthesis. (C) acetyl-CoA in nogalamycin biosynthesis. (D) benzoyl-CoA in enterocin biosynthesis.

After the polyketide chain is formed, cyclases determine the orientation, the cyclization pattern and therefore the final configuration of the structure of the future compound [108]. The polyketide undergoes a series of enzyme-catalyzed ketoreduction and aromatization reactions to form a planar aglycone structure. As an example, in anthracycline biosynthesis (Figure 10) the initial polyketide chain undergoes 9-ketoreduction on its ninth carbon (C-2 of the final molecule), then the

D ring is closed by an aromatase, followed by closure of the other two rings of the anthracene aglycone, B and C. The final ring A is closed in a reaction called aldol condensation, performed by a cyclase, which determines the stereochemistry of the final compound. The final step of the synthesis of the aglycone is 7-ketoreduction, which allows for the potential attachment of a sugar moiety to the aglycone [109].

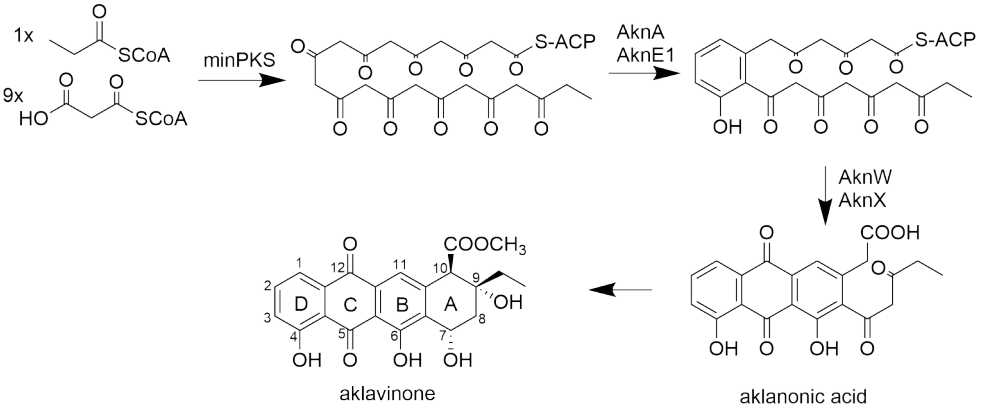

Figure 10: Type II polyketide aglycone biosynthesis on the example of aklavinone. The biosynthesis starts with a polyketide chain assembled by the minimal PKS. The polyketide chain is later modified through a variety of enzymatic reactions to yield a final product.

1.2.3.2 Glycosylation

Glycosylation patterns significantly contribute to the bioactivity of the compounds. There are both glycosylated and non-glycosylated type II polyketides. Prior to attachment to the polyketide aglycones by glycosyltransferases, the carbohydrates are synthesized and modified as TDP (thymidine diphosphate) sugars. TDP sugar biosynthetic pathway starts with D-glucose phosphate which then undergoes multiple enzymatic modifications that allow for the synthesis of a variety of sugars. To yield TDP-L-rhamnose D-glucose phosphate is converted into TDP-D-glucose, which then undergoes 6-dehydrogenation and becomes TDP-4-keto-6-deoxy-D-glucose. An epimerase then converts it to TDP-4-keto-L-rhamnose, which is a substrate for a reductase that completes the synthesis by yielding TDP-L-rhamnose (Figure 11) [73]. Examples of compounds decorated with TDP-deoxysugars are novobiocin glycosylated [110] with TDP-noviose, or an angucycline antibiotic urdamycin glycosylated with TDP-D-olivose and two TDP-D-rhodinoses [111].

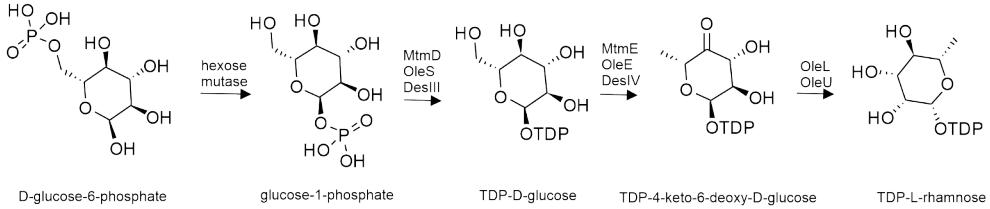

Figure 11: Biosynthesis of a TDP carbohydrate on the example of TDP-L-rhamnose. TDP-L-rhamnose starts as a D-glucose-6-phosphate, which is then modified by various enzymes to achieve the final product.

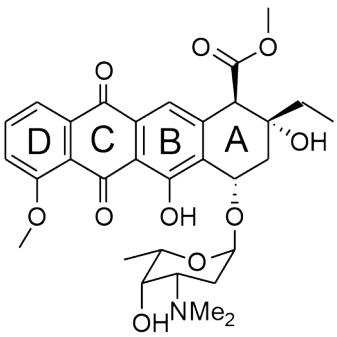

Figure 12: Chemical structure of daunorubicin.

Type II polyketides can be glycosylated in various ways. There are O-glycosylations, which are the most common, less typical C-glycosylations, occurring in medermycin and angucyclines, and even rare glycosylations through two bonds, such as the fused ring system like granaticin (Figure 8) [80] and nogalamycin (Figure 13). The number of saccharide moieties attached to an aglycone can vary from just one to five or more per molecule, like in a tetracycline hainancycline, whose BGC includes six glycosyltransferase genes responsible for attaching five carbohydrate moieties [112].

Another differentiating factor in type II polyketides is the positioning of the glycoside moiety. As an example, in anthracyclines the most common position for the sugar attachment is the C7 position of the A ring (Figure 12), however there is a plethora of differently glycosylated type II PKs. One of the examples is nogalamycin, which has a neutral sugar attached to the A ring at the C7 position and an additional aminosugar attached to the D ring (Figure 13) [85].

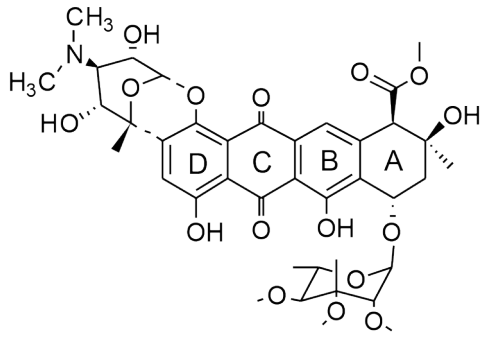

Figure 13: Chemical structure of nogalamycin.

1.2.3.3 Tailoring

Tailoring reactions in the type II polyketide biosynthesis, are steps where the molecules undergo a series of modifications to maximize their chemical diversity (Figure 14). Each molecule has a pathway-specific set of tailoring enzymes, such as 3-ketoreductase in actinorhodin biosynthesis [113] or cytochrome P-450

monooxygenase in doxorubicin pathway responsible for converting daunorubicin to doxorubicin [114] (Figure 14 C). Tetracenomycins introduce tailoring modifications

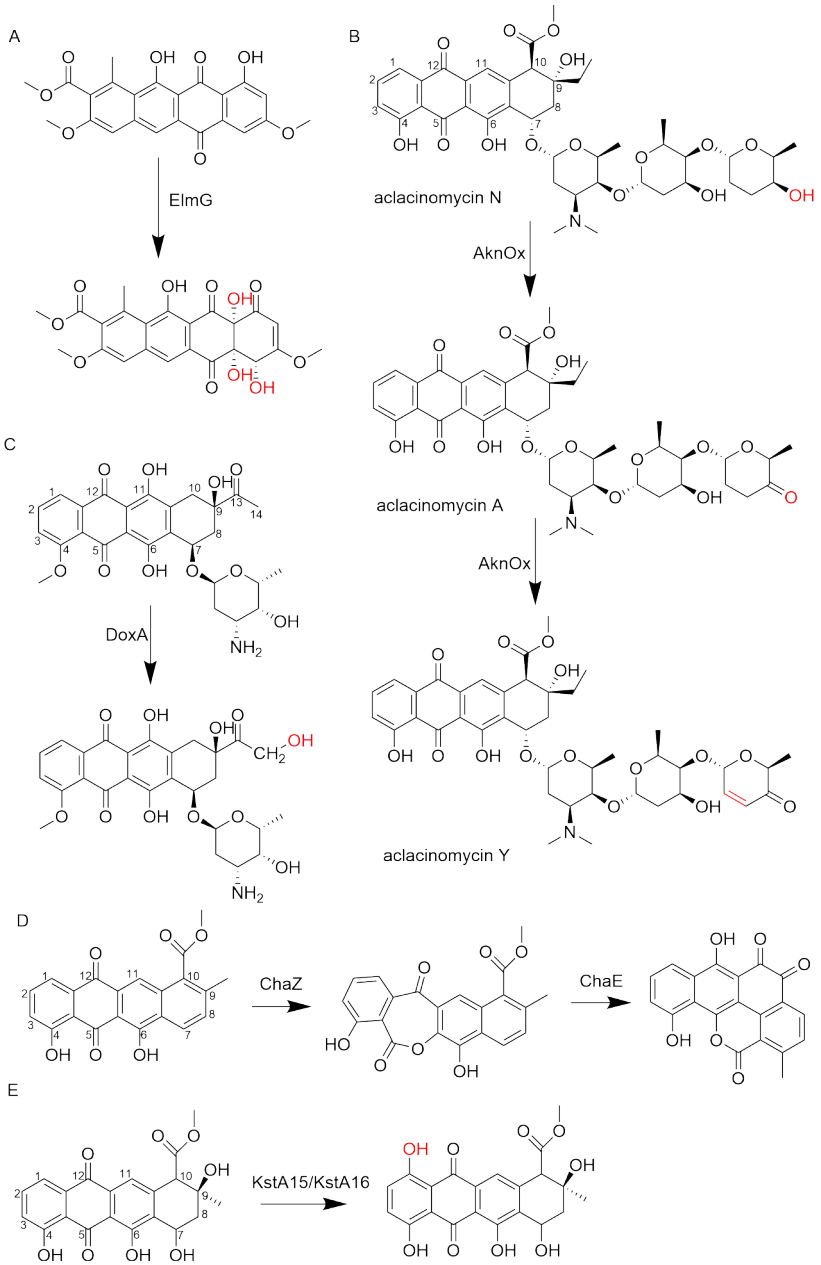

Figure 14: The diversity of tailoring strategies for type II polyketides: (A) Conversion of tetracenomycin A2 to Tetracenomycin C by ElmG. (B) Double oxidation of aclacinomycin N to aclacinomycin A to aclacinomycin Y. (C) 14-hydroxylation of daunorubicin to doxorubicin (D) Carbon ring rearrangement in chartreusin biosynthesis. (E) 1-hydroxylation in kosinostatin pathway.

to partially saturate an aromatic ring through oxidation, resulting in a diol structure [91]. Also, in tetracenomycins, an oxygenase ElmG present in elloramycin pathway catalyzes a triple hydroxylation of tetracenomycin A2 to tetracenomycin C [115] (Figure 14 A). Tetracyclines are tailored in a way that the tailoring enzymes desaturate the tetracyclic structure of the molecule and add various functional groups [80]. In aclacinomycin biosynthetic pathway an interesting oxidoreductase, the enzyme AknOx, oxidates the outermost L-rhodosamine sugar of aclacinomycin N turning it into aclacinomycin A and then oxidates it again to turn it into aclacinomycin Y [116] (Figure 14 B). Final steps of nogalamycin biosynthesis involve 1-hydroxylation by a two-component hydroxylase system, which leads to creation of an atypical ring system. For chartreusin, the tailoring steps involve a rearrangement of the carbon structure of the aglycone and addition of two lactone rings (Figure 14 D). In kosinostatin pathway we can observe a two-enzyme 1-hydroxylation mechanism, where enzymes KstA15 and KstA16 together catalyze a reaction yielding a 1,4-dihydroxy-anthracycline [117] (Figure 14 E).

1.3 Synthetic biology approach to antibiotic discovery

Synthetic biology is an interdisciplinary field that extends beyond traditional genetic engineering by using standardized biological parts, computational design, and engineering principles to construct or redesign biological systems. This includes assembling genetic circuits, creating synthetic pathways, and even engineering microbial communities, with the goal of making biology more modular, and engineerable [118]. The functional biological entity assembled from the parts can be one of two things: it can either reproduce an already existing biological entity, or it can comprise a gain of function [119]. However, no matter how well-designed and well-thought-through synthetic biology systems are, one needs to remember they are still involving a living organism, which can sometimes be unpredictable and needs always to be taken into consideration [118]. Synthetic biology flourished as computational design methods became more reliable and gene synthesis from single nucleotides became more affordable, which eliminated the tedious process of amplification of single parts via PCR [120]. As the number of standard biological parts grew, a repository holding all the information on the biological parts called iGEM was created [121]. All parts in this database were analyzed and characterized either experimentally or via software tools.

1.3.1 Expression systems in *Streptomyces* for efficient engineering of biosynthetic pathways

There is a comprehensive genetic toolbox of specific biological parts, that allow for the heterologous expression of the recombinant or synthetic systems in a *Streptomyces* host [65], the design of which depends very much on the desired outcome. Various expression systems serve different applications, such as activating cryptic biosynthetic gene clusters through promoter region modification, introducing activators, deleting negative regulators, or even a complete pathway reconstruction.

Pathway refactoring is an efficient synthetic biology strategy, where the whole biosynthetic pathway is assembled *de novo* from artificially produced DNA. The goal of pathway refactoring is to improve yields of natural products of known pathways or to activate silent BGCs by introducing native or synthetic regulators [122]. Using advanced cloning techniques like BioBricks, Gibson Assembly or Golden Gate every single module of the pathway is separately cloned in, including genes, promoters, RBSs, terminators and reporters [123]. To successfully express a gene, one needs to think of all the elements necessary, starting from promoters and RBSs, through plasmids and inducers, and finishing with fully engineered strains, resistance mechanisms, and reporter genes.

1.3.1.1 Vectors and integration sites

When creating an expression system, selecting an appropriate vector for the pathway assembly is crucial. Various cloning vectors are available which, thanks to their small size, provide a great scaffolding for pathway assembly [124], however classic *E. coli* cloning vectors such as pET and pUC vectors also are suitable for that task. The typical workflow involves initially assembling the pathway in a strictly *E. coli* amplifiable vector before transferring it into a shuttle vector. Shuttle vectors fuse themselves with the *Streptomyces* host genome through a phage-derived integrating site. Vector selection depends heavily on the desired integration location within the bacterial genome, as different vectors have different integration sites (Table 1)[125]. Incorporation of the heterologous DNA into the bacterial chromosome via phage integrase sites significantly increases stability of the construct [126].

Table 1: Genetic parts for the construction of expression systems in *Streptomyces*. [123] (Modified)

GENETIC PART	*FEATURE*	*REF.*
Constitutive Promoters		
ermE promoter	From promoter region of erythromycin resistance gene of *S. erythraeus*	[127]
kasOP promoter	Promoter of SARP family regulator from coelimycin cluster in *S. coelicolor* A3	[128]
gapdh promoter	Derived from the promoter region of glyceraldehyde-3-phosphate dehydrogenase in *S. griseus*	[129]
SP44	The strongest constitutive promoter engineered from the promoter kasOp, with twice the activity of the latter	[130]
rpsL promoter	Taken from the promoter region of *rpsL* gene in *S. griseus*	[129]
195 native or synthetic promoters	High-throughput screening in *S. venezuelae*	[131]
32 native promoters	Transcriptome -based promoter collection in *S. albus*	[132]
166 native promoters	Transcriptome-based promoter collection in *S. coelicolor*	[133]
2 native promoters	Multi-omics -based promoter collection in *S. coelicolor*	[134]
Inducible Promoters		
tipA promoter	Thiostrepton-induced promoter	[131]
nitA promoter	e-caprolactam-induced promoter	[135]
xylA promoter	Xylose-induced promoter	[136]
tcp830	Tetracycline-induced promoter	[137]
Terminators		
fd	Terminator derived from *E. coli* phage fd	[138]
TD1	Terminator derived from *Bacillus subtilis* phage $29	[139]
ttsbiB	Highly efficient terminator in *S. lividans*, used also in *Mycobacteria*	[140]
U term	Terminator from WebGeSTer, a database of intrinsic transcription terminators	[140]
V term	Terminator from WebGeSTer, a database of intrinsic transcription terminators	[140]
RBS		
AAAAAGGAAG	Native RBS sequence of *S. coelicolor*	[141]
192 native or synthetic RBSs	High-throughput screening in *S. venezuelae*	[131]
4 native RBSs	Multi-omics data-based selection in *S. coelicolor*	[134]
Reporter genes		
luxAB cassette	n-Decanal as substrate; absorbance at 490 nm wavelength	[142]
amy gene	An assay of reducing sugars released from starch with 3,5-dinitrosalycilic acid (DNS) as substrate; absorbance at 540 nm wavelength	[131]

xyle gene	Catechol as substrate; absorbance at 375 nm wavelength	[143]
gusA gene	p-Nitrophenyl-β-D-glucuronide as substrate; absorbance at 415 nm wavelength	[144]
eGFP	Green fluorescent protein; excitation wavelength 470 - 490 nm and emission wavelength 515 nm	[145]
fGFP	Green fluorescent protein; excitation wavelength 488 nm and emission wavelength 500 - 550 nm	[131]
mRFP	Red fluorescent protein; excitation wavelength 584 nm and emission wavelength 607 nm	[146]
mCherry	Red fluorescent protein; excitation wavelength 587 nm and emission wavelength 610 nm	[147]
Phage integration sites		
ΦC31	Sourced from *Streptomyces* phage ϕC31	[148] [149]
ΦBT1	Sourced from *Streptomyces* phage ϕBT1	[150]
VWB	Sourced from bacteriophage VWB	[151]
RP3	Sourced from *Streptomyces rimosus* phage RP3	[151]
R4	Sourced from *S. parvulus* phage R4	[152] [153]
TG1	Sourced from *Streptomyces cattleya* phage TG1	[152] [154]
Bxb1	Sourced from Mycobacteriophage Bxb1	[155]
SV1	Sourced from *S. venezuelae* phage SV1	[156]
ΦJoe	*Streptomyces* phage ϕJoe, introduced to SCO2603, an ancestral phage fragment, in *S. coelicolor*	[157]
Φ1/6	Sourced from prophage Φμ1/6, introduced to chromosome of tetracycline producing strains, *S. aureofaciens*	[158]

1.3.1.2 Promoters

Another essential factor for the successful gene expression in *Streptomyces* is an appropriate promoter. Selection of a promoter of the right strength is crucial for the correct gene expression and therefore the optimal production of the compound of interest [123]. There are both constitutive and inducible promoters of varied strength available for *Streptomyces* expression systems (Table 1). Constitutive promoters provide continuous gene expression, while inducible promoters require an inducer, a specific compound, which can be a small organic molecule like resorcinol or cumate, sugar, for example xylose, or antibiotic, such as tetracycline or thiostrepton [137]. Constitutive promoters are most commonly used in pathway building, unless the gene of interest encodes a product that is highly toxic for the cell and therefore its expression needs to be tightly controlled. One of the most popular *Streptomyces* promoters is the ermEp, a promoter region taken from in front of the *ermE* gene, which gives *Saccharopolyspora erythraea* resistance to erythromycin and

lincomycin [159]. Another popular choice is kasOp promoter from a cluster responsible for the production of coelimycin in *S. coelicolor* [128]. Its sequence, just like the one of ermEp resembles the sequence of the consensus prokaryotic promoter [130]. Another promoter worth mentioning is derived from the promoter region of actinobacterial glyceraldehyde-3-phosphate dehydrogenase of *S. griseus*, gapdh. It was noticed that the *gapdh* and *rpsL* genes were expressed at a significantly higher levels than others and upon investigation, it turned out that it was thanks to the gapdhP promoter, which then, due to its strength, became a staple in the synthetic biology toolkit [130].

1.3.1.3 Ribosome binding sites

The next step after selecting a promoter, is to choose an appropriate ribosome binding site (Table 1). A ribosome binding site is a DNA sequence that lies within a region called 5'-UTR located upstream of the start codon. This sequence, called a Shine-Dalgarno sequence is usually rich in adenines and guanines and is relatively conserved amongst bacterial species. The efficiency of translation depends heavily on the choice of RBS and the 5'-UTR as well as on their pairing with an appropriate promoter [160]. While native RBSs may be suitable in some cases, a synthetic RBS is typically preferred due to its standardized nature [161].

1.3.1.4 Terminators

Since *Streptomyces* genomic structure is strictly operon-based, it is important to ensure transcriptional termination at the end of each synthetic operon. Earlier, the number of characterized terminators which are suitable for *Streptomyces* was limited, however recently that number has significantly increased [123] (Table 1). The most commonly used is the fd terminator, originating from the lambda coliphage [162], which can be used both in *E. coli* and *Streptomyces* strains.

1.3.1.5 Reporter genes

In synthetic biology, fluorescent reporters are commonly used to measure expression levels of individual genetic components like promoters and ribosome binding sites, as well as sophisticated genetic circuits. Fluorescent proteins are used for example as biosensors, genetic pattern reporters in plants [163], and as protein localisation indicators [164]. The intensity of fluorescence is proportional to the protein abundance, which is therefore a great indicator of the expression rate. Fluorescence is measured at a wavelength specific to the protein. Interestingly, a reporter gene does not always have to be a fluorescent protein. There are several colorimetric

methods based on the enzymatic reaction of the expressed protein with added substrate, for example the *luxAB* luciferase cassette from *Vibrio harveyi* [165], *gusA* encoding a β-glucuronidase [166] or *xylE*, a product of which converts catechol into a yellow oxidation product [167]. Each of the colorimetric assays works by the principle of the colourful product absorbing light at a certain wavelength which can later be measured, and gene expression levels can be quantified. Neither of these methods are without limitations, because the product compound in the colorimetric method can interfere with cell metabolism and the fluorometric method can be misleading due to *Streptomyces* having significant natural fluorescence [161].

1.3.2 *Streptomyces* strains as versatile hosts for antibiotic production

A common practice in synthetic biology is to heterologously express a BGC from one *Streptomyces* strain in a model host strain, which is more amenable to genetic engineering. It also often improves the yield of the desired molecule, since the heterologous host strains have been specifically designed for overexpression of secondary metabolites [168]. An ideal *Streptomyces* host should be relatively free of secondary-metabolite-encoding clusters to limit the interactions between intermediates from various pathways, should provide a high level of precursors to facilitate product formation, have a clean production profile and increased yields of the desired compound compared to the native strain [169]. There are several *Streptomyces* strains developed specifically for the heterologous production of secondary metabolites, such as *S. coelicolor* 145 derivatives, *S. lividans* TK24, *S. avermitilis* and *S. albus* J1074 [169].

1.3.2.1 Streptomyces coelicolor

One of the most popular strains for secondary metabolite production is *S. coelicolor* M145 and its derivatives [170]. M145 was engineered from the actinorhodin producer *Streptomyces coelicolor A3* [171][172], which is a stable, easy to manipulate strain with a genome of a little over 8,7Mb. M145 lacks two plasmids, SCP1 and SCP2, that are present in the parental strain. Further modifications of *S. coelicolor* M145 included deletion of four antibiotic-producing gene clusters, actonorhodin, undecylprodigiosin, a calcium-dependent peptide antibiotic cluster and coelimycin via homologous recombination, which yielded a *S. coelicolor* M1146 strain [168]. M1146 was further modified to enhance the production of secondary metabolites, which resulted in development of *S. coelicolor* M1154 strain which, aside from the quadruple deletion of the metabolic clusters, carried a mutation in *rpoB*, a gene encoding the β subunit of bacterial RNA polymerase, and in *rpsL*,

encoding ribosomal protein S12 [168]. Point mutations C1298T in *rpoB* and A262G in rpsL resulted in a strain with improved antibiotic production without growth impairments. Another engineered *S. coelicolor* derivative strain was designed specifically for the expression of type III polyketide biosynthetic clusters. Strain M1317 was built on the basis of *S. coelicolor* M1152 which already carried multiple mutations enhancing the yields of natural products. In addition to the *Δact Δred Δcpk Δcda* deletions and *rpoB* (C1298T) mutation, M1317 carries three additional deletions in genes typical for type III polyketides, *gcs, srs* and *rppA*, resulting in a strain tailored for the expression of type III polyketides [173].

1.3.2.2 Streptomyces lividans

A close relative of *S. coelicolor* is *S. lividans*, an easily manipulated strain with low endogenous protease activity [169]. The most popular *S. lividans* derivative is TK24 [170], which carries the mutation (K88E) in *rpsL* gene encoding ribosomal protein S12 [174], which increases the yields of metabolite production. Other attempts to increase the yields of natural products were made by deleting the whole silent actinorhodin producing cluster, which yielded strains *S. lividans* K4-114 and K4-155. Recent advances on strain improvement yielded the strain *RedStrep*, which, through multiple deletions of biosynthetic clusters such as actinorhodin, undecylprodigiosin, calcium dependent antibiotic or melanin (RedStrep *Δact Δred Δcda Δmel*), shows significantly increased yields of mithramycin [175].

1.3.2.3 Streptomyces albus

Another widely used *Streptomyces* strain for heterologous gene expression is *S. albus* J1074, with disabled endonuclease *salI* gene that facilitates phage transfection of the strain. The engineered strain lacks the restriction activity of SalI, which would otherwise recognize and cleave specific sequences within the phage Pal6 genome [176]. Among the advantages of this strain are rapid growth rate (complete life cycle in 4 days on solid media) and a very small chromosome consisting of only a little over 6Mb, encoding 5,832 genes [177]. Despite carrying over two dozen biosynthetic gene clusters, it is still one of the most successful strains for heterologous expression of secondary metabolites. Compounds such as stefimycin, iso-migrastatin [178], greocycline [179] or landomycin [180] were successfully expressed in this strain, giving better yields than their native hosts. In 2017, a derivative *S. albus* strain was created with a translocated *attB* integration site of phage φC31 to eliminate the chromosomal position effect. The chromosomal position effect occurs when a heterologous biosynthetic gene is integrated into a bacterial chromosome within a silent cluster or a region subjected to repression,

which results in lowered expression levels and therefore lower yields of a secondary metabolite [181]. This shows how important positioning of the integration site is for the future yields of the desired compound [181]. The availability of more than one *attB* site also significantly influences the yields, which was also demonstrated in *S. albus* J1074 [169]. *S. albus J1074* has been further developed into the derivative strain Del14, where 15 of the biosynthetic gene clusters present in original J1074 were deleted, yielding a chassis strain with an extremely simplified production profile [169].

1.3.2.4 Streptomyces venezuelae

An emerging candidate for becoming a heterologous host for secondary metabolite production is *S. venezuelae*. Its rapid growth rate, easy genetic manipulation, high conjugation efficiency [182] and relatively homogenous mycelium proved to be very valuable for research [183]. The native chloramphenicol producer is described as a strain most suitable for industrial antibiotic production due to relatively dispersed growth [184]. Both short pathways as well as those encoding entire biosynthetic gene clusters were successfully heterologously expressed in *S. venezuelae* [185]. Several mutant strains emerged over the years, such as *S. venezuelae YJ003* or DHS2001 derived from *S. venezuelae* ATTC15439. DHS2001 was created through the replacement of the *pikA* I-IV genes from pikromycin biosynthetic gene cluster with a hygromycin resistance gene [186]. Another mutant strain, YJ003 was developed through deletion of the *desI-desVIII* and *desR* genes from deosamine biosynthetic cluster, native to the parental strain [187]. Later, as proof of principle, the entire kanamycin A biosynthetic gene cluster from *S. kanamyceticus* was expressed in that strain, which led to the discovery of previously unknown kanamycin intermediates and production of both kanamycin A and B [188]. Finally, a double mutant strain was created, where both pikromycin and deosamine biosynthetic gene clusters were deleted from the bacterial chromosome of the *S. venezuelae* parental strain resulting in a strain named YJ028 [189].

1.3.2.5 Streptomyces avermitilis

Finally, there is also *S. avermitilis*, a strain naturally producing the widely used anthelmintic agent avermectin. This strain is not as widely used in genetic engineering as the *S. lividans* or *S. coelicolor*, however it is more stable than them in terms of genome instability and terminal inverted repeats (TIR). In 2010, a deletion mutant of *S. avermitilis* was created, where a deletion of over 1,4Mb was removed, containing mostly non-essential genes. The strains called SUKA2-17 carried various deletions and were not able to produce the secondary metabolites

native to the parent strain [190]. Development of the SUKA strains encouraged the heterologous expression endeavors using *S. avermitilis* as host [191].

1.3.3 Pathway assembly methods

1.3.3.1 BioBricks

One main goal of synthetic biology is to simplify and standardize the workflow of genetic engineering to make the process more straightforward and efficient. The key step in standardization of a biological system is to come up with a definition of a standard biological part. In the late 1990s, there were attempts at developing a standardized biological engineering platform called NOMAD, however the system was not well received by the scientific community and therefore was reluctantly used beyond its lab of origin [124]. The NOMAD system was proposed in 1996 and had certain downsides, such as no clear distinction between front and back inserts, and the restriction enzymes used in the system were not user friendly [192]. An innovation came in 2003 when Knight et.al. at a MIT Computer Science and Artificial Intelligence lab proposed the concept of a BioBrick. The first approaches to the creation of a BioBrick standard part were not without flaws. The original BioBrick scar consisted of 8 nucleotides, which would later disrupt the open reading frame and subsequently compromise the downstream protein production [193]. This was later corrected and the whole idea was pitched to the public at the Synthetic Biology 1.0 conference in 2004. The inventors compared BioBricks to a set of screw threads that all fit together thanks to their standardized nature. Knight was among the early advocates of the concept of "biological simplicity" and compared the parts to Lego building blocks, which were even depicted on the cover of his first BioBrick manual handbook [194]. The goal of this technique was to eliminate the need to design a whole new expression system from the experiment planning, which significantly speeds up the progress of research.

1.3.3.1.1 BioBricks Registry of Standard Biological Parts

BioBricks are a collection of interchangeable biological parts allowing for the rapid and smooth cloning of synthetic DNA fragments into a biosynthetic pathway. In the BioBrick system, a biological part is a nucleic acid sequence encoding a biological function. One BioBrick part can be combined with any other BioBrick part, creating a seamless joint. This allows for the creation of a global collection of standard biological parts, from which an engineer can take whatever they need and implement it into their research [195]. The standard nature of the BioBrick design also allows for smoother collaboration between labs in different parts of the world, because it

ensures that whatever fragments of pathways they design, will always be joinable [124].

The global database of BioBrick parts is called the iGEM (International Genetically Engineered Machines) parts registry. It was launched in 2003 as an internal MIT collection of biological parts stored in an -80°C freezer in a basement, meant for a course for students taught therein. Later, as the database expanded, it was offered to the public as Registry of Standard Biological Parts [193]. It is said that the repository is a constant work in progress due to its communal and collaborative nature. By 2010 iGEM contained 7000 physical samples of biological parts and continues to grow to this day, shipping a great number of parts across the globe every year [193]. As of 2024 it contains tens of thousands of entries, each of them including all the necessary information about a certain biological part that a biologist or engineer might need to include in their design.

1.3.3.1.2 Principles of the modular BioBrick design

The principle of the BioBrick system is very simple and relies on recycling of restriction sites. By definition, any BioBrick part must be able to be connected with

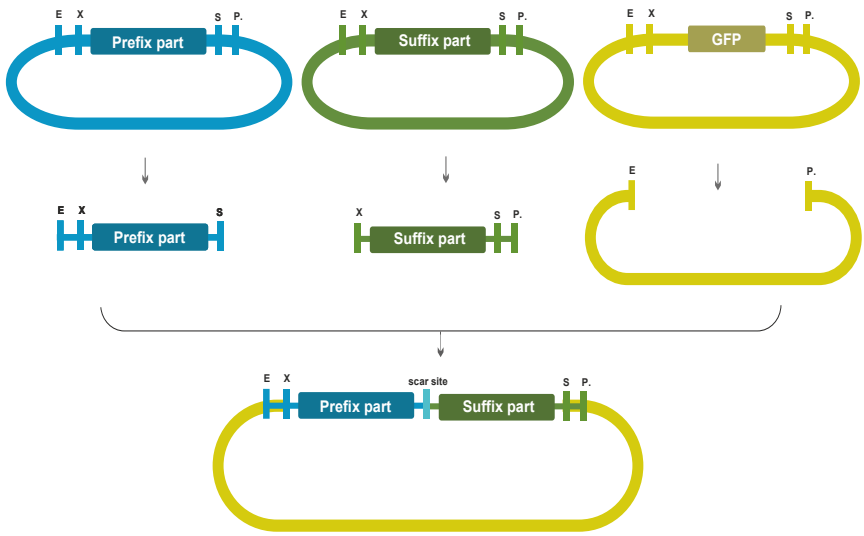

Figure 15: A standard assembly of BioBrick parts. The prefix fragment is digested with EcoRI and SpeI, the suffix fragment is digested with XbaI and PstI. The acceptor vector is digested with EcoRI and PstI. The parts are ligated so that the EcoRI sites from the prefix fragment and PsiT site from the suffix part fuse with the corresponding sites in the acceptor vector. SpeI site from the prefix fragment and XbaI site from the suffix part create a 'scar site' which cannot be re-cut by neither of the enzymes.

another BioBrick part, which means that a standard BioBrick part must be flanked by four specific restriction sites: EcoRI and XbaI upstream and SpeI and PstI downstream of the coding sequence. By cutting the fragment with appropriate enzymes one can create a prefix part, which can be cloned in front of an existing BioBrick sequence, or a suffix part, which can be cloned after an existing sequence [194]. Additionally, SpeI and XbaI share a very useful feature, which allows for the recycling of restriction sites. After digestion, the sticky ends created by these enzymes (TCTAGA and ACTAGT) can be sealed together by a ligase into an ACTAGA scar site, which then cannot be cut again by either of the two enzymes [194]. This means that once ligated, two BioBrick parts cannot be re-cut, but that also means that after ligation of two parts, SpeI and XbaI can be safely used again without the risk of them cutting the already assembled fragments [194].

BioBrick parts can be either very small or relatively big. A whole cloning vector can be a single BioBrick part, but it can also be built out of BioBrick parts itself. There are parts as small as plasmid replication origins, terminators, RBSs or antibiotic resistance markers, which allow for the construction of cloning vectors perfectly tailored to individual research needs [124].

A BioBrick part can either be synthesized via *de novo* DNA synthesis offered commercially or amplified using PCR from native DNA template. Literature offers pre-designed PCR primers for optimal amplification of a BioBrick part from a native template [196]. The primers are designed to add 8nt at the 5' terminus, which enables more efficient cleavage of the prefix restriction sites and promote the addition of a 3' overhang by Taq polymerase which facilitates downstream cloning. While designing a part, certain criteria need to be met, and rules need to be followed. Each standard BioBrick part, with an exception for protein expression parts, must be prefixed with a 5'-GAATTCGCGGCCGCTTCTAGAG-3' sequence. In protein encoding parts the prefix sequence is shorter by 2nt at the 3' terminus, which ensures the optimal spacing between the Shine-Dalgarno sequence and the start codon [197]. The suffix sequence for the standard BioBrick part is always 5'-TACTAGTAGCGGCCGCTGCAG-3' (Figure 16). Standard BioBrick parts must not contain the four BioBrick restriction sites within their coding sequence to prevent unintended cleavage [196].

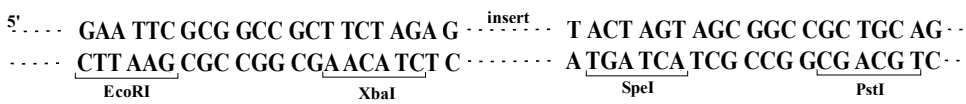

Figure 16: A standard BioBrick part flanked with EcoRI, XbaI, SpeI and PstI restriction sites.

The most popular way of joining multiple BioBrick parts together is a method called a 3-way assembly (Figure 15), where the destination vector is cut with EcoRI and PstI to create a standard BioBrick opening. Then two fragments of choice are cut, one with EcoRI and SpeI to create a prefix part and the other with XbaI and PstI to create a suffix part. These two parts, when joined together, create an SpeI/XbaI scar site ACTAGA upon ligation. Then the EcoRI and PstI sites of the vector are ligated to the corresponding sites on the prefix and suffix parts, respectively. The 3-way assembly is a fast and efficient way to join several BioBrick parts at once. The method can be performed without having to purify the fragments from a gel as long as the three BioBrick parts to be joined come from vectors with different resistance markers [196].

1.3.3.2 Gibson Assembly

Classical cloning techniques usually take multiple steps to assemble more than two DNA fragments into a pathway. However, in the past decade several new cloning methods were developed to facilitate the process, such as Gibson assembly or Golden Gate cloning. When it comes to pathway building, time and efficiency are of the highest value, therefore any technique that facilitates the assembly of the biosynthetic pathway is invaluable.

The Gibson assembly is a molecular cloning method that has been perfected and improved over the years. The general principle of Gibson assembly is ligation of digested DNA fragments in vitro, using a thermocycler. There are two versions of Gibson assembly, the first involves joining up to five DNA fragments in a thermocycler in two steps, and the other, thanks to the addition of exonuclease III and antibody-bound Taq DNA polymerase, can be performed in just one step. The method uses a process called overlap assembly instead of restriction enzymes [198] and can be used for both simple cloning of small genes and joining multiple fragments of large sizes [199].

The main difference between using the BioBrick 3-way assembly and the Gibson assembly is that for the latter the DNA fragments need to have at least 40bp homologous overlapping sequences at the 5’ of one and 3’ end of the other. A BioBrick can become a part for the Gibson assembly if it undergoes a round of PCR where PCR primers are used to create those overlapping sequences using a BioBrick part as a template. They add approximately 20 nucleotides at the end of one BioBrick that are identical with the standard 5’ BioBrick prefix sequence of the other [200].

The process of Gibson assembly starts with at least two PCR fragments, amplified so that they share a homologous fragment of at least 40bp at their 5’ and 3’ termini (Figure 17). These homologous fragments are later cut with T5

endonuclease to generate sticky ends, which anneal to each other at 50°C. Then the DNA polymerase fills in the gaps in the strands between the double stranded structure and the annealed fragments. Finally, the ligase seals the strands amplified by the polymerase with the rest of the double stranded fragment [198]. All three enzymes do not interfere with each other, so they can all be present in the same reaction tube at the same time. The reaction master mix contains all the necessary components for the enzymes to perform their tasks: NAD as a cofactor for the ligase, PEG-8000 as a crowding agent necessary to create a cell-like environment for the reaction to occur, $MgCl_2$ as a cofactor for the polymerase and DTT as a reducing agent [198][199].

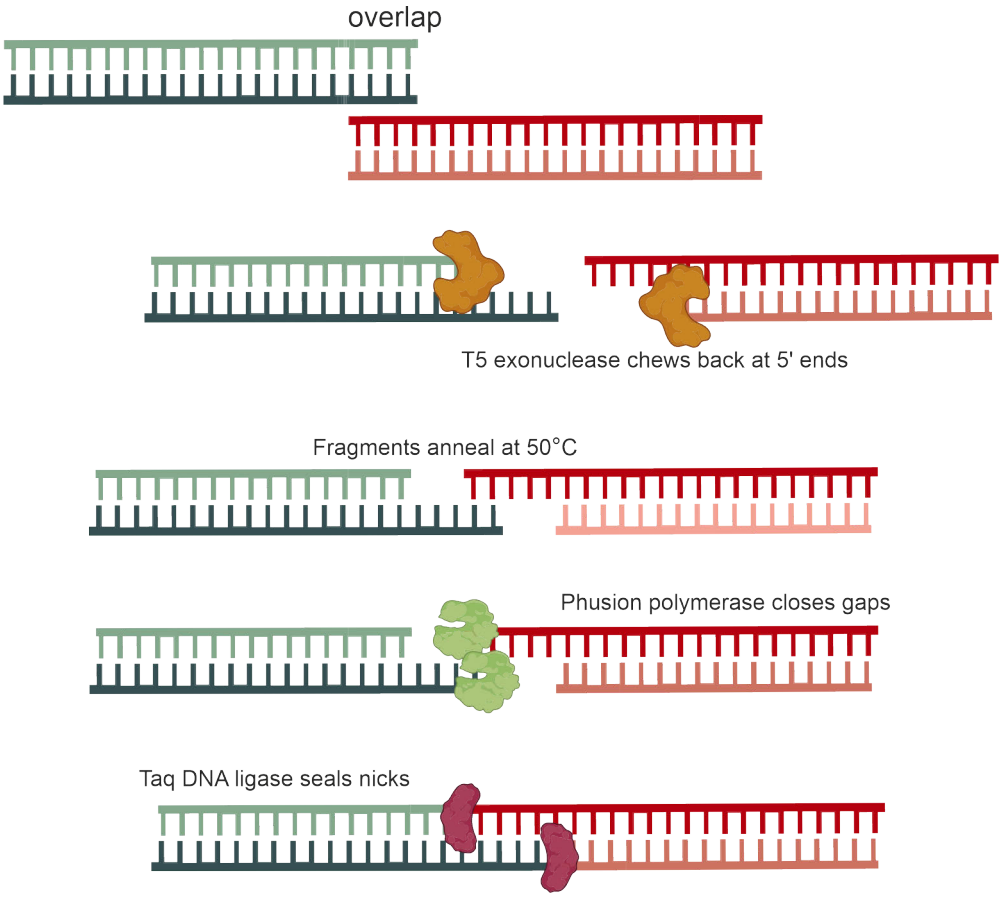

Figure 17: The principle of Gibson Assembly. The process begins with two PCR fragments with 40 bp homologous regions at their termini. T5 exonuclease generates sticky ends by digesting the 5' ends, which anneal to each other at 50°C. DNA polymerase then fills gaps in the strands, and DNA ligase seals nicks, completing the assembly of the recombinant DNA fragment.

1.3.3.3 Golden Gate cloning

Golden Gate cloning is another one-step cloning method developed to facilitate the speed of assembling DNA fragments into a functional pathway. It allows for the assembly of up to 52 DNA fragments in one step (Figure 18) [201].

The main principle of Golden Gate cloning is joining DNA fragments cut by Type IIS restriction enzymes. The key feature of Type IIS restriction enzymes is that their recognition sequences are distinct from their cleavage sites. They identify specific DNA sequences called recognition sites and then cleave the DNA strand at a fixed distance from these recognition sites. Another important feature of type IIS endonucleases is that the cleavage site is not sequence specific. In traditional cloning type I restriction enzymes are used to cut within their recognition site. For type IIS restriction enzymes, the recognition site can even be outside of the DNA fragment of interest, which allows for the donor and recipient DNA fragments to be cut and then ligated seamlessly [202]. The joint is considered seamless because the ligated fragment does not contain any scar site or restriction enzyme restriction site [203].

Golden Gate assembly starts with a reaction mix containing the donor and recipient vectors, T4 ligase and type IIS restriction enzymes. The donor DNA fragments, and the recipient vector can be provided as circular DNA [204]. First the donor and recipient fragments bearing the restriction enzyme recognition sites are cut by the restriction enzyme at a restriction site [203]. Sticky ends created by these enzymes are then ligated by T4 ligase. Whatever fragments fail to ligate in a seamless manner and still contain the original recognition sites, will be re-cut by the restriction enzymes always present in the reaction mix, which also means that the concentration of correctly ligated fragments increases proportionally to the incubation time [202]. The reaction is conducted in a thermal cycler at 37°C for 60-120min.

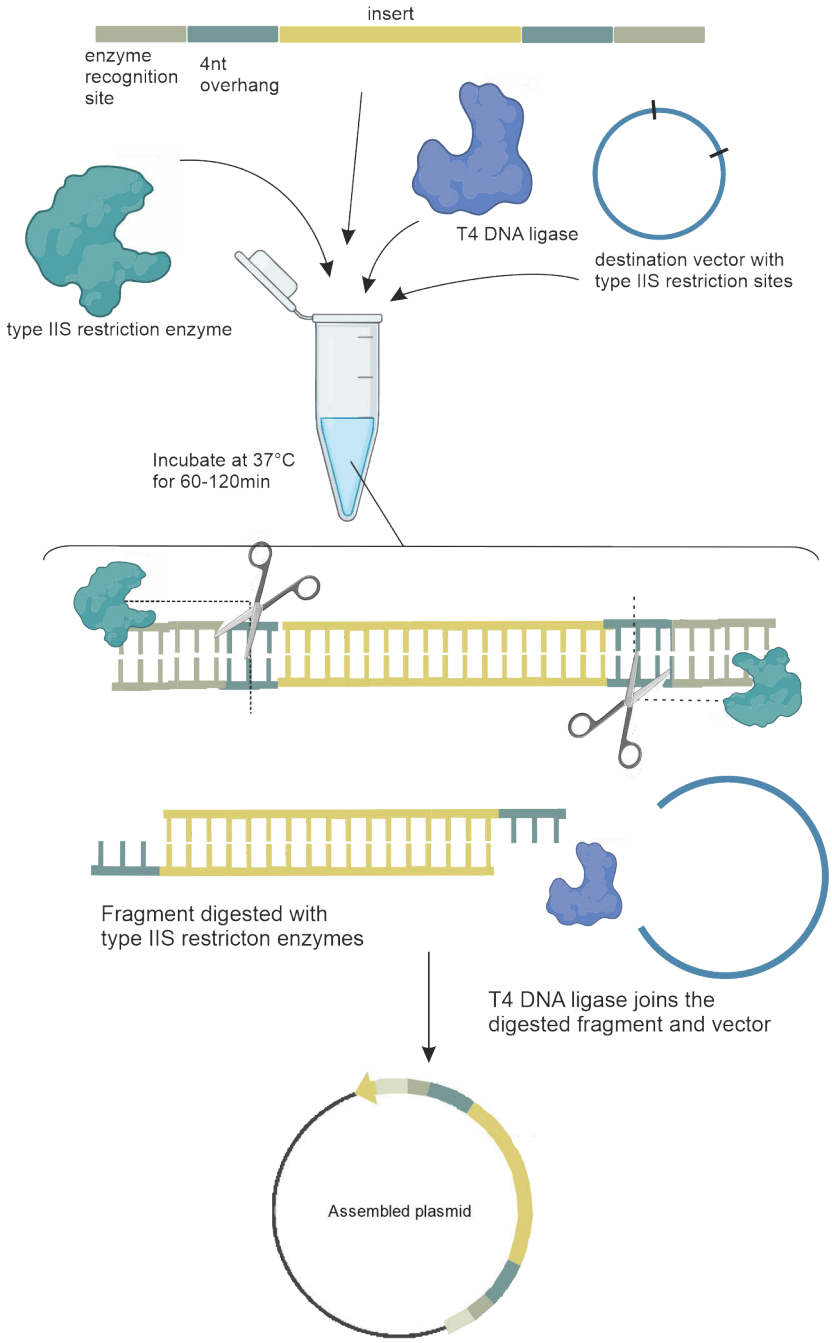

Figure 18: The principle of Golden Gate cloning. First, the enzymes cleave the donor and recipient fragments at their recognition sites, generating compatible sticky ends. T4 ligase then joins these ends, while any un-ligated products are re-cut by the restriction enzymes and subsequently re-ligated. This iterative process results in a growing number of correctly assembled fragments as the time of the reaction progresses.

1.3.4 Advantages and limitations of pathway assembly methods

BioBricks, Golden Gate cloning, and Gibson Assembly each offer unique advantages in terms of efficiency, scalability, and precision, yet come with their own sets of limitations. Understanding the comparative strengths and weaknesses of these techniques is essential for selecting the most appropriate approach for a specific experiment.

BioBricks have found applications in a variety of biological research projects. Researchers started building on the foundation set by Knight et.al., tailoring the technique to their own needs, making it one of the most prominent tools in modern synthetic biology. Many new, specific BioBrick-based systems were developed, building genetic toolboxes for certain organisms such as *Bacillus subtilis* [205], *E. coli* [206], the *Streptomycetaceae* family [125][65] or even for less studied organisms such as *Rhodococcus* [207] , *Acinetobacter* [208] or mycobacteria [209]. The main advantage of BioBricks lies in their modularity and the ability of mixing and matching the parts to achieve the most optimal expression, which is very useful when designing branched pathways or optimizing yields. The standardized nature of BioBricks is also a great asset, as it facilitates efficient cloning. BioBricks are not only used in genetic modifications of bacteria, but also eucaryotic organisms such as yeast [210] or plants [211]. Synthetically engineered standardized biological components find application in a vast variety of fields, such as drug development, biofilm research, protein production and amino acid synthesis, to just name a few.

While BioBricks provide versatility across organisms and applications, other assembly methods offer distinct advantages for specific cloning challenges. For example, Gibson Assembly is an optimal method for cloning large DNA fragments. One of its many advantages is the ability to join fragments as large as 580kb. Another is the fact that the whole reaction can be performed in a single tube, all the reagents are commercially available and the whole reaction takes about 15 minutes to complete [199]. Cloning high GC-content DNA using Gibson Assembly has classically been challenging, but a recently developed improved protocol allows for cloning fragments high in GC content, such as *Streptomyces* genes, which gives a new perspective for the *Streptomyces* pathway refactoring [212].

Gibson assembly can be used by synthetic biologists to create entire biosynthetic pathways for producing biofuels, pharmaceuticals, and to create DNA libraries. It can also be used as a tool for introducing mutations in genes for a phenotype change [213] or construction of infectious viral clones [214], which makes it very popular in plant synthetic biology, especially in research on plant-infecting viruses [214]. There are multiple examples of assembling an entire viral genome via Gibson assembly [215]. Sometimes the technique is paired with Crispr-Cas9 for seamless insertion of a fragment into a linearized vector. In *Streptomyces* Gibson Assembly

can be used for gene cluster engineering. It was used to introduce a duplicate of the whole pristinamycin II biosynthetic gene cluster into a *S. pristinaespiralis* HCCB10218 strain to increase yields of the molecule. Gibson Assembly is also used for expression of entire gene clusters in a heterologous *Streptomyces* host [216].

While Gibson Assembly excels with large fragments and high GC content, Golden Gate cloning offers its own set of advantages in terms of assembly efficiency and precision. One of many advantages of Golden Gate cloning is the speed of assembly, with the reaction being just a one-step reaction. Moreover, Golden Gate cloning joins DNA fragments in a seamless manner, which allows for the engineering of fusion in-frame constructs, for example proteins with linkers and tags [203]. Joining more than two fragments requires very careful design of the recognition and restriction sites. By using a single type IIS restriction enzyme one can create a multitude of different fragment-specific sticky ends [202]. In 2011 Weber et.al. developed another cloning strategy based on the principle of Golden Gate cloning, called Molecular Cloning, or MoClo [204]. Golden Gate cloning can be joined with Crispr-Cas system to achieve targeted modification of the Streptomyces chromosome, which allows for genome editing in any given locus [217]. There are also methods combining Golden Gate cloning with BioBricks, where the DNA fragment of interest is flanked not only with BioBrick restriction sites but also with type IIS restriction sites [218] [219].

2 Aims

The main aim of this research was to develop a BioBrick-based synthetic biology platform for the engineering, assembly and functional characterisation of biosynthetic gene clusters within microbial hosts.

More specifically the aims were:

I. To develop a BioBrick-based synthetic biology toolbox enabling efficient pathway engineering to facilitate the discovery of novel antiproliferative and antibiotic compounds.
II. To demonstrate the versatility and applicability of the BioBrick toolbox through glycodiversification of type II polyketides.
III. To leverage the BioBrick toolbox for functional characterization of previously unidentified steps within the chartreusin biosynthetic pathway.

3 Materials and Methods

3.1 Gene synthesis and cloning

The genetic components were synthesized (Genewiz from Azenta Life Science, South Plainfield, NJ (Article II) and GenScript, Piscataway, NJ (Article I)) as standard BioBrick parts following the [RFC10] guidelines, each having the appropriate BioBrick prefix (5′-GAATTCGCGGCCGCTTCTAGAG-3′) and suffix (5′-TACTAGTAGCGGCCGCTGCAG-3′). The biosynthetic genes were codon-optimized for efficient expression in *S. coelicolor* (Article I). A robust ribosome binding site (BBa_B0034) was added at the front of each fragment within the BioBrick prefix (5′-GAATTCGCGGCCGCTTCTAGAGAAAGAGGAGAAATACTAG, underlined). The BioBrick parts were cloned using standard BioBrick EcoRI, XbaI, SpeI, and PstI restriction enzymes. For cloning and gene amplification, high copy number vectors pUC57 pSBIC3-J04450 (Article II) and pSB1C3-J04450, pSB1K3-J04450, and pSB1A3-J04450 (Article I) were utilized. The restriction enzymes and T4 DNA ligase for cloning were sourced from Fisher Scientific (Waltham, MA) (Article II) and NEB (New England Biolabs, Ipswich, MA) (Article I).

3.2 Bacterial strains and growth conditions

E. coli TOP10 (Fisher Scientific, Waltham, MA) (Article II) and *E. coli* JM109 (Promega) (Article I) strains were used for routine cloning of the BioBrick parts, amplification and maintenance of the plasmids. Chemically competent cells were prepared following standard protocols [220]. The *E. coli* strain transformed with an appropriate vector was grown on LA (lysogeny agar) agar plates and in LB (lysogeny broth) liquid medium supplemented with antibiotics at the following concentrations: 100μg/mL ampicillin and 50μg/mL apramycin (Article II) and 25 μg/mL apramycin, 25 μg/mL kanamycin, 100 μg/mL ampicillin, and 35 μg/mL chloramphenicol (Article II). *E. coli* ET12567 / pUZ8002 was used for intergeneric conjugation.

For heterologous expression in Streptomyces, *S. coelicolor* M1152ΔmatAB (Article II) and *S. coelicolor* M1146::cos16F4iE were used. The strains and their derivatives were routinely cultured on R5 and MS (mannitol-soy flour) solid agar

plates. Spore conjugation of the shuttle vector pENTG3 (Article II) and pUWL201PWBB-based plasmids (Article I) carrying the constructs into *Streptomyces* was performed according to the standard protocol [221]. *E. coli* ET12567 / pUZ8002 carrying the desired constructs were washed and mixed with spores of a target *Streptomyces* strain. The bacterial suspension was streaked on solid MS agar plates supplemented with 10mM MgCl2 and incubated at +30°C. After 24 hours, the plates were overlaid with 500µL of sterile MQ water containing appropriate antibiotics and 25µg/ml nalidixic acid and incubated for 72 hours at +30°C. After the appearance of exconjugants, six of them (Article II) or ten to twelve (Article I) were streaked onto a secondary MS (Article II) or R5 (Article I) agar plate with the same antibiotic combination and incubated for an additional three days at +30°C.

For the production of metabolites, vegetative mycelia were collected from the MS plates and suspended in liquid TSB (tryptone soy broth) medium. Seed cultures were grown with appropriate antibiotic selection for three days at +30°C and 300rpm. A 100µl inoculum from the seed culture was added to 250ml flasks containing 50ml SG media (10g soytone, 20g glucose, 5g yeast extract, 5,73g TES buffer, 1mg cobalt chloride per one liter) and cultured for 7 days (30°C, 300rpm). The preculture (100µl) was then transferred to 250ml flasks, each containing 50ml of SG media, and incubated for 7 days (30°C, 300rpm). Cultivations were performed in triplicate.

For large scale production of compounds for NMR, compounds **2, 8, 10** (Article I) were produced by culturing S. coelicolor M1146::cos16F4iE/pDOLV, S. coelicolor M1146::cos16F4iE/pDMYC, S. coelicolor M1146::cos16F4iE/ pDFUCO2, and S. coelicolor M1146::cos16F4iE/pDALO in 4 L of SG-TES liquid media (100 mL × 40 shake flasks, 250 mL Erlenmeyer baffled flask) at 220rpm for 5-6 days. Compounds **7, 9, 11,** and **14** (Article I) were produced by growing *S. coelicolor* M1146/cos16F4iE strains on MS agar plates (containing 20 g/L each of mannitol, soya flour, and agar) with thiostrepton and apramycin for 5 days at 30°C. Spores from these transformants were used to seed 50 mL E1 media (containing 20 g/L glucose, 20 g/L starch, 5 g/L cottonseed flour, 2.5 g/L yeast extract, 1.3 g/L K2HPO4·3H2O, 1 g/L MgSO4·7H2O, 3 g/L NaCl, and 3 g/L CaCO3) in 250 mL flasks, supplemented with the same antibiotics and incubated for 3 days (30°C, 300 rpm). The preculture was then used to inoculate (4% v/v) main cultures containing 250 mL E1 media with antibiotics in 2 L flasks, which were grown for 7 days (30°C, 250 rpm).

Compound **9** (Article II) was produced by conducting a large-volume enzymatic reaction combining auramycinone, KstA16, ChaU, and NADPH.

Compounds **4, 6** and **7** (Article II) were produced by culturing appropriate bacterial strains were first grown in 50 mL SG media for 5 days (30°C, 300rpm).

The preculture (100µl) was then transferred to ten 250ml flasks, each containing 50ml of SG-TES media and 1ml of LXA1180 resin slurry, and incubated for 7 days (30°C, 300rpm).

3.3 Extraction and purification of the compounds

In Article I the extraction of compounds was performed by adding 25mL of ethyl acetate +0,1% formic acid to 25mL of culture. The organic top phase was collected, and the solvent was evaporated in a rotary evaporator (Buchi, Flavil, Switzerland). The dried compound was resuspended in 4mL of methanol for further analysis.

In Article II the compounds were extracted from the LXA1180 resin with acidic chloroform (1% of 37% HCl in chloroform), dried using a vacuum concentrator (Fisher Scientific, Waltham, MA), and resuspended in 100µl of DMSO for UPLC analysis.

For large scale purification for structure elucidation the cells were centrifuged at 3000rpm for 10 minutes and the pellet was extracted with 1L of methanol. The supernatant was extracted 3 times with 4L of ethyl acetate +0,1% formic acid. Then the solvents were combined and dried in a rotary evaporator. The dried extract was dissolved in 9:1 chloroform/methanol, dry-loaded on a 25 g silica cartridge, and fractionated on a silica cartridge (24 g silica RediSep Rf Gold) on a Teledyne Combiflash 100 instrument using a gradient of chloroform to 9:1 chloroform/methanol at a flow rate of 30 mL/min over 15 min. The resulting fractions were further purified using preparative HPLC. The obtained fraction F1 was purified by preparative HPLC using method A afforded 8-demethyl-8-*O*-β-D-mycarosyl-tetracenomycin C and 8- demethyl-tetracenomycin C as yellow solids. Similarly, fraction F2 was purified using Sephadex LH-20 (MeOH; 2.0 × 30 cm2), followed by prep-HPLC to afford 8-demethyl-8-β-D-olivosyl-tetracenomycin C in pure form as a yellow solid.

In Article II compound **9** was isolated through serial chloroform extractions, followed by methanol dissolution and subsequent purification using preparative HPLC (Agilent Technologies, Santa Clara, CA) with a Kinetex 5 µm Phenyl-Hexyl 250×21.2mm column (Phenomenex, Torrance, CA, USA). The isolated compounds were dried by rotary evaporation, weighed, and dissolved in 700µl deuterated chloroform before transfer to NMR tubes.

Compounds **4, 6** and **7** (Article II) were extracted through acidified chloroform extraction (1% HCl), followed by concentration using a rotary evaporator (Buchi, Flavil, Switzerland) and DMSO resuspension. Compound purification was performed via preparative HPLC (Agilent Technologies, Santa Clara, CA) using a Kinetex 5µm EVO C18 250x21.1mm column (Phenomenex, Torrance, CA, USA).

The isolated compounds were dried by rotary evaporation, weighed, and dissolved in 700µl deuterated chloroform before transfer to NMR tubes.

3.4 Analysis of the compounds

In Article I the compounds were analyzed on an Agilent 1260 Infinity II LC/MSD iQ single quadrupole HPLC-MS (Agilent Technologies, Santa Clara, CA) using Poroshell 120 Phenyl-Hexyl column (ID 2.7µm, 4.6 mm × 100 mm). The diode array detector (DAD) was set to monitor UV−vis absorbance at 290 and 410 nm. 10µL of the sample was injected into the system and analyzed with a 15-minute-long gradient method (0 min, 95% solvent A and 5% solvent B; 0−10 min, 95% solvent A and 5% solvent B to 5% solvent A and 95% solvent B; 10−13 min, held at 5% solvent A and 95% solvent B; 13.1 min, re-equilibrate to 95% solvent A and 5% solvent B; and 13.1−15.1 min, 95% solvent A and 5% solvent B). Solvent A was 0,1% formic acid in MQ water, solvent B was 0,1% formic acid in acetonitrile.

In Article II the samples were analyzed using an UHPLC system (Shimadzu) with a DAD detector using a Kinetex C18 1.7µm, 100Å, 100x2.1mm column as stationary phase. The samples were run for 20 minutes in a gradient of buffer A (acetonitrile + 0.1% TFA), and buffer B (MQ water + 0.1% TFA) as mobile phase. The method used was a gradient from 10% to 90% (1-15 min), 100% (15-17 min) and 10% (17-20 min).

LCMS analysis was done using an Agilent 1290 Infinity LC and Agilent 6460 Triple Quadrupole Jetstream MS/MS (Agilent Technologies, Santa Clara, CA). Samples were analyzed on Kinetex C18 1,7 µm 100Å 100×2,1 mm (Phenomenex, Torrance, CA) column using a 20 min gradient method from 10% to 90% (1-15 min), 100% (15-17 min) and 10% (17-20 min). Mobile phase: buffer A was 100% acetonitrile; buffer B was MQ water. Samples were analyzed in both positive and negative ionization modes, with DAD focusing on UV absorbance at 430nm and 450nm. Mestrelab Research Analytical Chemistry Software (Mestrelab, Santiago de Compostela, Spain) was used for data analysis.

NMR analysis of the samples was conducted on a Bruker Avance Neo 500 with an Oxford 500MHz magnet and BB/1H Smartprobe. The NMR spectra were analyzed using TopSpin 4.1.3 (Bruker BioSpin Corporation, Billerica, MA) and *J*-coupling constants were simulated and extracted using ChemAdder (Spin Discoveries Ltd.).

3.5 Antibacterial activity testing

Antibacterial properties of the compounds were evaluated (Article I) against various pathogens using a microdilution well assay according to established protocols. The

test organisms included *E. coli* O157:H7 (ATCC 35150), *S. enterica ser.* Typhimurium (ATCC 14028), *L. monocytogenes* (ATCC 15313), methicillin-susceptible *S. aureus* (MSSA; ATCC 12600), methicillin-resistant *S. aureus* (MRSA; ATCC 43300), which were cultured in LB media at 37°C for 12 hours. *C. jejuni* (ATCC 33560) was grown in MH media at 42°C for 48 hours, while *S. prasinus* NRRL B-12101 (ATCC 13879) and *S. violaceusniger* NRRL B-1476 (ATCC 27477) were cultured in ISP broth. Each bacterial suspension (100 µL at 0.05 OD_{600}, approximately 1 × 107 CFU/mL) was exposed to test compounds (1 µL, 100 µM final concentration) in appropriate media using 96-well plates. Controls included DMSO, chloramphenicol (1 µL, 20 µg/mL), and sterile culture media.

3.6 Cancer cell line viability assay

The effects of the compounds on cell viability were tested (Article I) in triplicate on multiple human cancer cell lines: A549 (non-small cell lung), PC3 (prostate), HCT116 (colorectal), and Merkel cell lines MKL1 and MCC26, following established methods80-83. Tests included negative controls using DMSO and positive controls using actinomycin D and H_2O_2 (for A549, PC3, MKL1, and MCC26) at concentrations of 20 µM and 1 mM respectively.

4 Results

This thesis investigates the use of synthetic biology for both applied and basic research on the secondary metabolism of *Streptomyces*. I utilized BioBricks for the heterologous expression of biosynthetic genes in *Streptomyces* bacteria, with a focus on the production of novel antiproliferative and anticancer compounds and to investigate the biosynthesis of secondary metabolites. The results presented in this chapter are derived from two studies: Article I, which examined the production of novel secondary metabolites through glycodiversification, and Article II, which explored the unknown functions of enzymes involved in the chartreusin biosynthetic pathway. Together, these studies provide complementary insights into the importance of a well-tuned synthetic biology platform for the progress of research on secondary metabolites.

4.1 Glycodiversification of elloramycin using BioBricks

Article I demonstrates the use of BioBrick gene cassettes to modify and diversify the sugar configurations of tetracenomycins, aiming to engineer new variants of tetracenomycin antibiotics with different carbohydrates, including the creation of four new tetracenomycin compounds with unique sugars.

Tetracenomycins and elloramycins are type II polyketides produced by *Streptomyces* bacteria. Elloramycin is a natural product of *S. olivaceus*, while tetracenomycin C is produced by *S. glaucescens*. Both tetracenomycins and elloramycins exhibit antibacterial and anticancer properties through binding to the polypeptide exit channel of the large ribosomal subunit and therefore inhibiting translation. Naturally occurring tetracenomycins have a binding affinity for both eucaryotic and prokaryotic ribosomes, although evidence suggests that there is a potential for selective binding of glycosylated tetracenomycins to bacterial but not to eucaryotic ribosome [92]. The binding site itself is located in a unique position specific for tetracenomycins, in the polypeptide exit tunnel within the large ribosomal subunit [222]. The main objective of the study described in Article I was to develop elloramycin derivatives which would exhibit enhanced binding selectivity towards bacterial ribosomes.

Elloramycins and tetracenomycins differ by one O-methylation present at the C12a position of elloramycin and absent at the C12a position of tetracenomycin C and 8-demethyl tetracenomycin C (Figure 19). Additionally, tetracenomycin C has a methyl group at C8 while 8-demethyltetracenomycin does not. Elloramycin as a glycosylated compound, has a saccharide moiety, 2′,3′,4′-tri-*O*-methyl-α-L-rhamnose, attached at the C8 position [184].

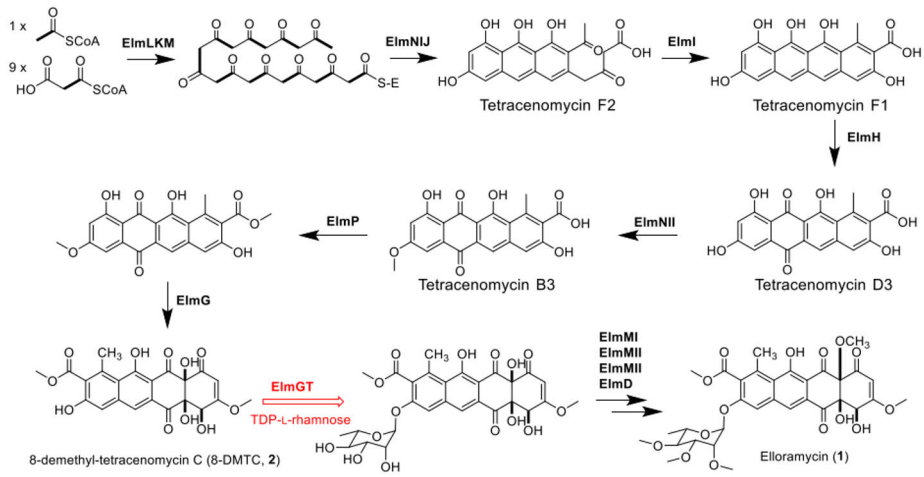

Figure 19: Bosynthetic pathway of elloramycin. Elloramycin starts as a decaketide chain assembled by a type II polyketide synthase. Then it undergoes a series of enzymatic modifications including methylations, cyclizations and aromatisations to yield a tetracyclic tetracenomycin molecule.

All three compounds start as a linear decaketide chain composed of nine malonyl-CoA moieties and one acetyl-CoA moiety which are assembled by a type II polyketide synthase consisting of the *elmKLM* genes. Later aromatase ElmNI and third ring cyclase ElmJ form an early tetracenomycin intermediate, tetracenomycin F2 [185]. Then the fourth ring cyclase ElmI closes ring A to form another intermediate, tetracenomycin F1, later converted to tetracenomycin D3 by monooxygenase ElmH, which then is O-methylated into tetracenomycin B3 by ElmNII. Tetracenomycin B3 then undergoes another O-methylation catalyzed by ElmP into tetracenomycin A2. Finally, ElmG enzyme is responsible for triple hydroxylation of the tetracenomycin A2 into 8-demethyl tetracenomycin C [185].

4.1.1 Deoxysugar biosynthesis

Deoxysugar biosynthesis begins with D-glucose-1-phosphate, which undergoes a series of enzymatic transformations involving phosphorylation, thymidine monophosphate transfer, dehydration, epimerization, and reduction. Through these

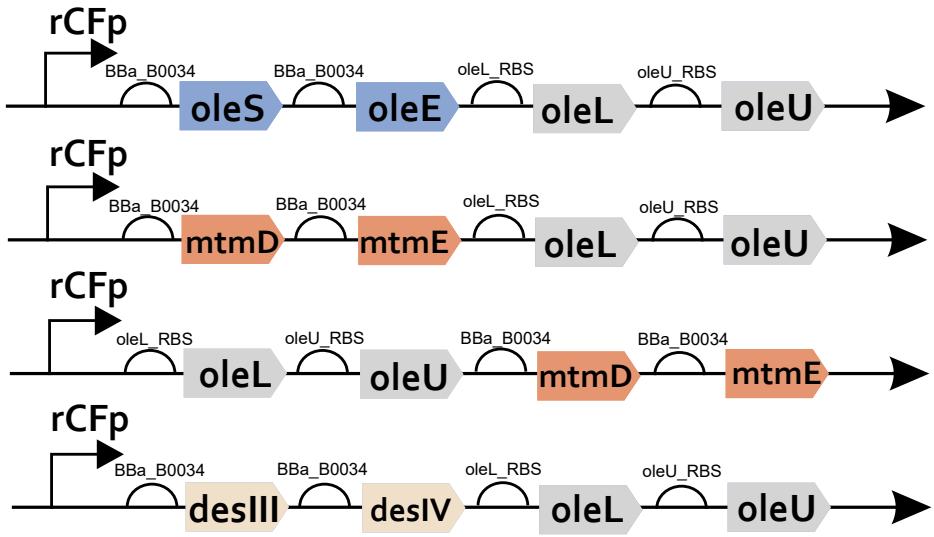

Figure 20: SBOL vector diagrams for TDP-L-rhamnose sugar plasmids tested in Article I in order to assess the best performing producing construct for further pathway bulding.[73]

steps, key enzymes like TDP-D-glucose synthase (OleS, DesIII, MtmD), TDP-D-glucose-4,6-dehydratase (MtmE, OleE, DesVI), epimerase OleL, and ketoreductase OleU progressively modify the glucose molecule. The final product of this biochemical pathway is TDP-L-rhamnose, the native sugar attached to 8-demethyl tetracenomycin C to form elloramycin. We tested four different BioBrick constructs for the most efficient synthesis of TDP-D-rhamnose when expressed in the 8-demethyl tetracenomycin C producing strain M1146::cos16F4iE (Figure 20). The best performing construct proved to be pRHAM3, which featured genes from the mithramycin biosynthetic pathway and oleandomycin pathway. The strain produced elloramycin as expected, but through chromatographic analysis we detected a second glycosylated compound, which was proved to be 8-demethyl-8-*O*-D-glucosyl-tetracenomycin C. This compound is a presumed result of the relaxed substrate preference of ElmGT. Both compounds were purified and analyzed using various chromatography techniques and their structures were confirmed with 1D and 2D NMR spectroscopy.

4.1.2 Construction of carbohydrate cassettes

The glycodiversification of elloramycin was first performed by using a promiscuous glycosyltransferase ElmGT to glycosylate 8-demethyl tetracenomycin C with different neutral sugars to yield new compounds (Figure 21). The sugar cassettes were designed and cloned into a pUWL201PWBB plasmid at the laboratory of a

collaborator at Ferris State University, College of Pharmacy. My role in the progress of this project was conjugating the sugar plasmids into *S. coelicolor M1146ΔmatAB::cos16F4iE*, culturing them and analyzing the production profile. As a result, eight carbohydrate cassettes were assembled and expressed in a heterologous *Streptomyces* host.

Figure 21: Deoxysugar nucleotide pathways engineered in this work. (A) TDP-D-allose, (B) TDP-D-fucofuranose, (C) TDP-D-olivose, (D) TDP-D-mycarose, (E) TDP-D-digitoxose, (F) TDP-D-boivinose, (G) TDP-D-antiarose, (H) TDP-D-quinovose.

4.1.2.1 2,6-dideoxysugars

Initially, we engineered expression cassettes for the biosynthesis of thymidine diphosphate (TDP)-activated deoxysugars, specifically TDP-D-olivose, TDP-D-digitoxose, TDP-D-boivinose, and TDP-D-mycarose (Figure 22). In 2,6-deoxysugars, the biosynthetic pathway starts with TDP-4-keto-6-deoxy-D-glucose and then is modified by a variety of enzymes such as OleV, OleW, UrdR, and EryBII to yield a range of TDP-deoxysugars. By exchanging pathway-specific genes for a gene from different BGCs, different sugar variants could be created.

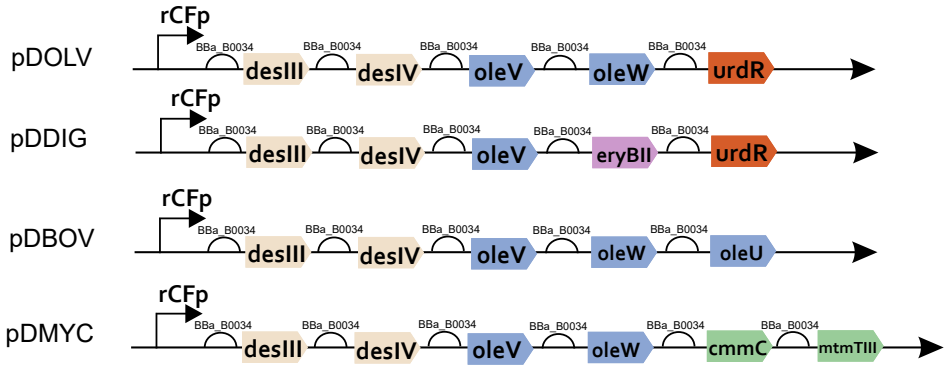

Figure 22: SBOL diagrams of TDP-2,6-dideoxysugar gene cassettes. pDOLV – TDP-D-olivose, pDDIG – TDP-D-digitoxose, pDBOV – TDP-D-boivinose, pDMYC – TDP-D-mycarose used in this work. The sugar cassettes were then introduced to a *Streptomyces* host for the production of new 8-demethyl-tetraceomycin C derivatives. [73]

For the production of TDP-D-olivose, the biosynthetic pathway has to feature genes such as *oleV*, *oleW* and then *urdR*, which performs a 4-ketoreduction to yield TDP-D-olivose. Upon expression, the TDP-D-olivose biosynthetic cassette was able to convert approximately 84% of the 8-demethyl-tetracenomycin C to 8-demethyl-8-*O*-β-D-olivosyl-tetracenomycin C.

To yield TDP-D-digitoxose, the intermediate compound created by OleW needs to undergo 3-ketoreduction catalyzed by EryBII from the erythromycin pathway and then 4-ketoreduction performed by UrdR. For the TDP-D-digitoxose cassette the conversion rate to the glycosylated product, 8-demethyl-8-*O*-β-D-digitoxosyl-tetracenomycin C was at 60-65%.

Upon exchange of UrdR enzyme from TDP-D-olivose pathway to OleU to yield TDP-D-boivinose, we observed the expression of a new glycosylated compound, 8-demethyl-8-*O*-(4′-keto)-β-D-digitoxosyl-tetracenomycin C. This suggested that OleU might not be functioning correctly due to a sequential discrepancy, so the gene

was resynthesized and expressed in M1146ΔmatAB::cos16F4iE, which resulted in production of the expected compound, 8-demethyl-8-*O*-β-D-boivinosyl-tetracenomycin C.

TDP-D-olivose and TDP-D-mycarose are, together with TDP-D-oliose the native sugars attached to the mithramycin aglycone. We decided to design the full TDP-D-mycarose biosynthetic pathway to demonstrate the ability of ElmGT to attach branched sugar to a tetracenomycin aglycone. We assembled the cassette adding a *cmmC* gene from chromomycin A3 pathway, hypothesizing that it would act as a methyltransferase, without the ketoreductase activity characteristic to MtmC, an enzyme native to the mithramycin biosynthetic pathway. As expected, the strain carrying *mtmC* gene produced 8-demethyl-8-*O*-β-D-mycarosyl-tetracenomycin C with 80% efficiency.

4.1.2.2 6-deoxysugar cassettes

The next step of the project was to focus on engineering 6-deoxysugar cassettes. Given that TDP-D-rhodinose, a carbohydrate native to the elloramcyin pathway, is a 6-deoxysugar, we hypothesized that ElmGT will be able to attach other 6-deoxysugars and therefore focused on TDP-D-allose from the dihydrochalcomycin pathway.

Figure 23: SBOL diagrams of TDP6-dideoxysugar gene cassettes pDALO – TDP-D-allose, pDFUCO – TDP-D-fucofuranose. pDALO and pDFUCO were 6-deoxysugars attached to the 8-demethyl-tetracenomycin C aglycone for production of novel secondary metabolites. [73]

TDP-D-allose is synthesized from TDP-4-keto-6-deoxy-D-glucose through the activity of epimerase GerF and 4-ketoreductase GerK1 (Figure 23). We expressed the TDP-D-allose cassette in *S. coelicolor* M1146::cos16F4iE. The resulting strains produced two novel tetracenomycin compounds, which suggested successful attachment of two different 6-deoxysugars to the 8-demethyl-tetracenomycin C aglycone. Upon LCMS and NMR analysis the glycosylated compounds were characterized as 8-demethyl-8-*O*-β-D-quinovosyl-tetracenomycin C and 8-demethyl-8-*O*-β-D-allosyl-tetracenomycin C. The generation of D-quinovose was

unexpected, yet not unprecedented, as D-quinovosyl-narbonolide production had previously been described in extracts from *S. venezuelae* ATCC15439 strains with a deleted *desI* aminotransferase gene [223]. This result could be attributed to either GerK1 activity on TDP-4-keto-6-deoxy-D-glucose or potentially to an alternate 4-ketoreductase encoded within the host *S. coelicolor* genome with a moonlighting activity.

Next, we decided to assemble the TDP-D-fucofuranose cassette. TDP-D-fucofuranose is a carbohydrate native to the gilvocarin V biosynthetic pathway [224]. Extensive research has been conducted on gilvocarin pathway in order to characterize the functions of its most crucial enzymes, however the enzyme responsible for the TDP-D-fucopyranose to TDP-D-fucofuranose conversion was never described. We transformed *S. coelicolor* M1146::cos16-F4iE with a construct containing *desIII, desIV, gilU* and *gilV*, which resulted in the production of two glycosylated compounds, one of which was 8-demethyl-8-*O*-β-D-quinovosyl-tetracenomycin C, and the other was a new compound, 8-demethyl-8-*O*-D-fucosyl-tetracenomycin C.

Finally, we focused on assembling a TDP-D-antiarose biosynthetic pathway from the rubterolone A pathway [225]. The rare deoxysugar TDP-D-antiarose, characterized by axial 3′-OH and 4′-OH groups, is synthesized from TDP-4-keto-6-deoxy-D-glucose through the catalytic activity of an unusual enzyme possessing both reductase and epimerase activity, RubS3. We created a construct encoding the TDP-D-antiarose biosynthetic pathway (containing *desIII, desIV* and *rubS3*) however, expression of this construct in *S. coelicolor* M1146::cos16F4iE did not yield any new glycosylated tetracenomycins. We hypothesized that this lack of new tetracenomycin formation was due to ElmGT's inability to use the TDP-D-antiarose sugar as substrate.

4.1.3 Bioactivity testing

All of the compounds generated in this study were tested for antiproliferative (Figure 22) and antibacterial (Figure 23) properties. The assays were conducted at the University of Kentucky, Pharmaceutical Sciences Dept. by a collaborator.

4.1.3.1 Cancer cell cytotoxicity assay

The compounds were assayed individually with cancer cell lines [A549 (nonsmall cell lung), PC3 (prostate), and HCT116 (colorectal) human cancer cell lines and Merkel cells (MKL1 and MCC26] and the only compounds that exhibited moderate cytotoxic activities were elloramycin, tetracenomycin C and 6-hydroxy-tetracenomycin C (Figure 24), highlighting the significance of a carbohydrate or methyl group at the C8 and C12 positions for antiproliferative activity. Likewise, 8-

demethyl-8-*O*-(2′-methoxy)-α-L-rhamnosyl-tetracenomcyin C showed minimal activity against the MKL1 cancer cell line and demonstrated lower cytotoxicity compared to elloramycin. This result suggests that the permethylated L-rhamnose in elloramycin pathway plays a crucial role in both binding the 50S ribosome subunit and cytotoxicity. Among the compounds developed in this study, 8-demethyl-8-*O*-β-D-mycarosyl-tetraceomycin C containing the branched D-mycarose sugar demonstrated the most significant activity in the single-dose assay on MKL1 cell line. The glycosylated analogues with neutral sugars showed low cytotoxicity across the tested cancer cell lines.

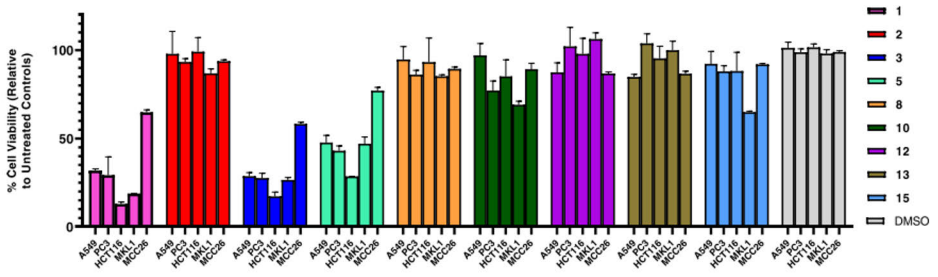

Figure 24: % Viability of A549 (nonsmall lung), PC3 (prostate), and HCT116 (colorectal) human cancer cell lines and Merkel cells (MKL1 and MCC26) (after 72 h) at 80 μM concentration of representative compounds **1** (elloramycin A), **2** (8-demethyltetracenomycin C), **3** (tetracenomycin C), **5** (6-hydroxy-tetracenomycin C), **8** (8-demethyl-8-*O*-β-D-olivosyl-tetracenomycin C), **10** (8-demethyl-8-*O*-β-D-mycarosyl-tetracenomycin C), **12** (8-demethyl-8-*O*-β-D-quinovosyl-tetracenomycin C), **13** (8-demethyl-8-*O*-β-D-allosyl-tetracenomycin C), and **15** (8-demethyl-8-*O*-(2′-methoxy)-α-L-rhamnosyl-tetracenomycin C). [73].

These results suggest that hydrophobic sugars or methyl substitutions at the C8 position may enhance cytotoxicity. Further research strategies for creating new elloramycin glycoside derivatives could focus on creating tetracenomycins with alkyl chains or other nonpolar substitutions at the 8-position to develop more effective ribosome inhibitors with improved antibiotic activity.

4.1.3.2 Antibacterial assay

Elloramycin exhibited the highest activity against methicillin-susceptible Staphylococcus aureus (MSSA; ATCC 12600) and methicillin-resistant S. aureus (MRSA; ATCC 43300) (90−95% inhibition, at 100 μM concentration) (Figure 24). This suggests that L-rhamnose is crucial for binding the 50S ribosomal polypeptide exit channel. Another finding in the antibacterial study was that tetracenomycin C and 6-hydroxy-tetracenomycin C inhibited the growth of *Streptomyces violaceusniger* NRRL B-1476 (ATCC 27477) (80−90% inhibition, at 100 μM

concentration). The glycosylated compounds developed in this study presented moderate antibacterial properties with a narrow spectrum of activity. The compounds were active against the *Streptomyces* strains, with 40-70% inhibition of bacterial growth at 100µM concentration on *S.prasinus*, which was previously shown to be resistant to tetracenomycins. No significant antibiotic activity was detected against MSSA and MRSA (Figure 25)

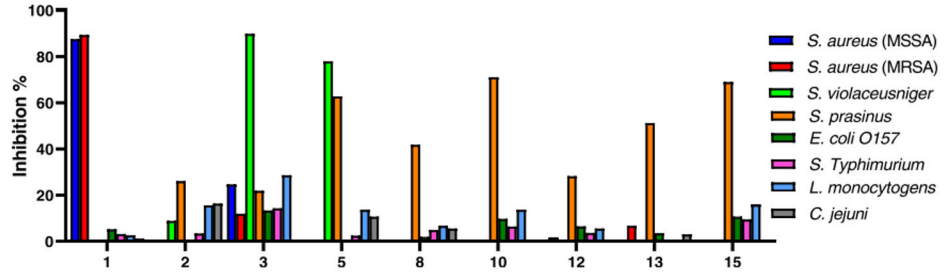

Figure 25: Antibacterial activities ofthe representative compounds 1 (elloramycin A), 2 (8-demethyltetracenomycin C) ,3 (tetracenomycin C), 5 (6-hydroxy-tetracenomycin C), 8 (8-demethyl-8-*O*-β-D-olivosyl-tetracenomycin C), 10 (8-demethyl-8-*O*-β-D-mycarosyl-tetracenomycin C), 12 (8-demethyl-8-*O*-β-D-quinovosyl-tetracenomycin C), 13 (8-demethyl-8-*O*-β-D-allosyl-tetracenomycin C), and 15 (8-demethyl-8-*O*-(2′-methoxy)-α-L-rhamnosyl-tetracenomycin C) (% inhibition). [73]

4.2 Elucidating the chartreusin biosynthetic pathway using BioBricks

Chartreusin is a type II polyketide natural product which was first described in 1953 as a product of *Streptomyces chartreusis* isolated from African soil. The name owas derived from the greenish yellow colour of the dried purified compound. Initial bioactivity tests showed antibacterial activity against Gram-positive bacteria and Mycobacteria [226]. The chemical structure of chartreusin was established in 1964 and revealed that the compound is composed of an angular pentacyclic bislactone aglycone called chartarin decorated with two saccharide moieties, D-fucose and D-digitalose attached to the aglycone at C7 position through an O-glycosydic bond. Similar compounds, elsamicin A and B, sharing the same chartarin aglycone, but differing in the saccharide moieties attached to it, were isolated from another Actinomycete strain in 1986. Another interesting chartreusin derivative is the synthetic prodrug IST-622, which showed anticancer and antibacterial activity similar to chartreusin and elsamicin A (Figure 26) [227].

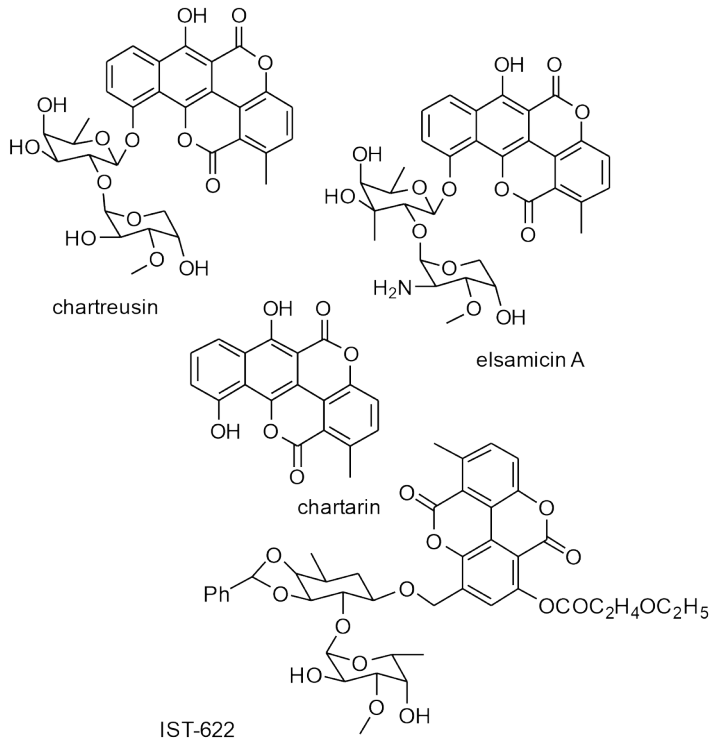

Figure 26: Chemical structures of chartreusin, chartarin, elsamicin A and IST-622.

Chartreusin itself exhibited moderate anticancer activity against human liver cancer cell lines Hep3B2.1-7 and H1299 [228] as well as murine leukemia cancer cell lines P388 and L1210 and melanoma cell line B16. The mechanism of action of chartreusin and related compounds manifests in their ability to poison the

topoisomerase II, bind to DNA and generate radical-mediated single-strand breaks [229].

At first, thanks to its unusual structure and high antiproliferative activity *in vitro*, chartreusin was anticipated to proceed to clinical trials, however due to its low solubility and therefore bioavailability, it was not selected. Elsamicin A showed better bioavailability and reached phase II of clinical trials, just like the synthetic chartreusin prodrug, IST-622. IST-622 was tested in clinical setting in Japan as an orally administered therapeutic for breast cancer. The compound exhibited a series of dose-limiting hematologic side effects, although they were mild enough to let the drug move on to phase II of clinical trials [230]. Recent research has focused on developing synthetic and semisynthetic chartreusin derivatives with improved bioavailability and higher solubility than the precursor compound but retaining their anticancer and antibacterial properties.

The *cha* biosynthetic gene cluster was first described in *Streptomyces chartreusis* strain HKI-249. The 37kb long cluster is flanked by housekeeping genes involved in primary metabolism and consists of 35 open reading frames [231]. The biosynthesis of chartreusin has been extensively investigated, however not all chartreusin biosynthetic genes have been assigned with an experimentally proven function. The functions of a few gene products have remained unknown to this day and the functional assignment of others is just assessed based on homology to known proteins.

4.2.1 Chartreusin biosynthetic pathway

The *cha* biosynthetic gene cluster was first described in *Streptomyces chartreusis* strain HKI-249. The 37kb long cluster is flanked by housekeeping genes involved in primary metabolism and consists of 35 open reading frames [231]. Chartreusin begins as a decaketide chain, which is later cyclized, aromatized and reduced into a tetracyclic anthracycline intermediate, resomycin C, which has been experimentally confirmed to be a substrate for a flavin-dependent monooxygenase ChaZ (Figure 27). To achieve the pentacyclic bislactone aglycone characteristic for chartreusin, the linear structure of resomycin C needs to undergo two reactions. The first reaction is catalyzed by enzymes ChaZ and ChaE. ChaZ is a flavin-dependent monooxygenase responsible for the Baeyer-Villiger oxidation and lactonization of the tetracyclic linear intermediate resomycin C [232]. After that, a NADPH-dependent ketoreductase ChaE rearranges the ring structure, which yields a pentacyclic angular aglycone intermediate. The next step is a cleavage of C-C bond followed by another ring lactonization catalyzed by ChaP. Activity of these three tailoring enzymes yields a pentacyclic bislactone carbon skeleton of the chartreusin atypical aglycone, chartarin. Chartarin is later double-glycosylated by two

glycosyltransferases, ChaGT1 attaching D-fucose to C7 position of chartarin and ChaGT2 responsible for the attachment of D-digitalose to D-fucose through an O-glycosidic bond [233].

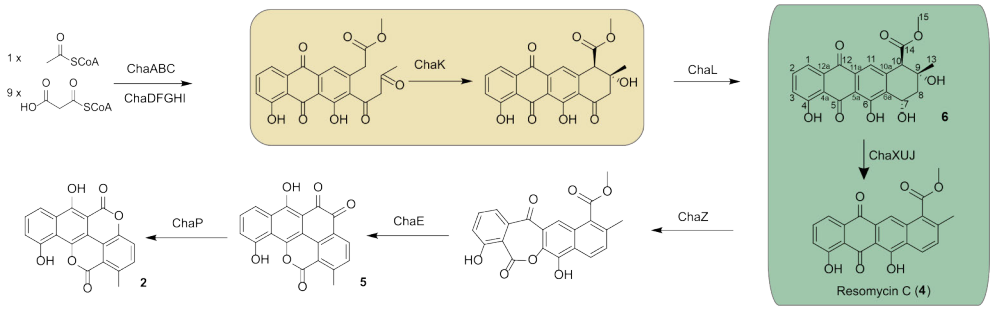

Figure 27: Chartarin biosynthesis. Chartarin begins as a polyketide chain assembled by the type II polyketide synthase. The polyketide chain is then cyclized, aromatized and reduced into a pentacyclic bislactone compound.

The biosynthesis of chartreusin has been extensively investigated, but the steps from auramycinone to resomycin C are still unknown. Correspondingly, not all chartreusin biosynthetic genes have been assigned with an experimentally proven function. The functions of *chaU*, *chaX*, *chaJ* or *chaK* products have remained unknown to this day and the functional assignment of others was assessed based only on their homology to known proteins [231].

4.2.2 Phylogenetic analysis

We performed phylogenetic analysis on cyclase-like proteins from the chartreusin biosynthetic gene cluster (BGC), comparing them with known fourth ring cyclases and 1-hydroxylases (Figure 28). After aligning representative proteins using MUSCLE [234], we built a phylogenetic tree using PhyML+LG model in SeaView 5 [235]. Our analysis revealed two distinct clusters of cyclase-like proteins: one group containing fourth ring cyclases (SnoaL, AknH, DnrD) along with two chartreusin BGC proteins (ChaK and ChaJ), and another group containing C1-hydroxylation enzymes (SnoaL2, KstA15) and the chartreusin pathway protein ChaU.

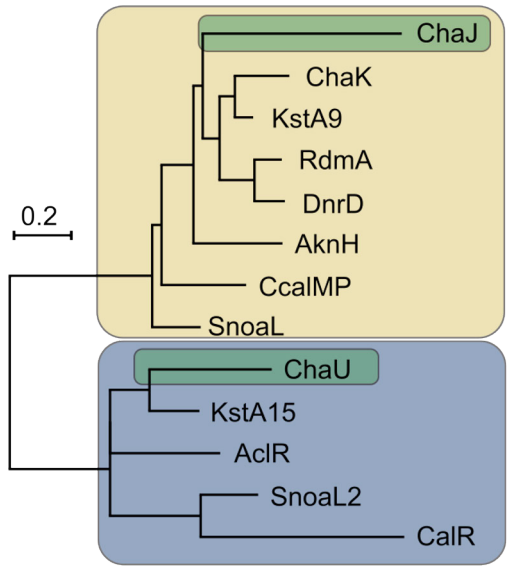

Figure 28: Phylogenetic tree showing homology between 4th ring cyclase enzymes, 1-hydroxylases and cyclase-like proteins.

4.2.3 Enzymatic assays

A co-author overexpressed ChaJ, ChaX and ChaU in *E. coli* as recombinant proteins carrying a N-terminal 6xHis tag. Upon failing to produce ChaX as a soluble protein, we conducted the assays involving ChaX using a cell-free bacterial lysate. Based on the phylogenetic analysis we speculated that the function of KstA16 is homologous to the function of ChaX, which was confirmed by the assays, where both ChaX and KstA16 performed a 1-hydroxylation reaction on the auramycinone molecule in the presence of NADH, yielding 1-hydroxy-auramycinone. The incubation of ChaX with ChaU in the presence of NADH and using auramycinone as substrate, yielded a new product, 9,10-dehydroauramycinone. These results confirmed that the functions of ChaX and KstA16 are identical and KstA16 can therefore be used as a substitute for ChaX in enzymatic assays.

To fully determine the functions of all three enzymes, ChaX, ChaJ and ChaU, we assayed them in various combinations using auramycinone as a substrate, in the presence of NADH and KstA15 (Figure 14). None of the enzymes showed any activity on the substrate when tested alone. The combination of ChaJ and KstA16 did not exhibit any activity either, but upon addition of ChaU, all three enzymes together converted auramycinone into a new product. We confirmed it to be

resomycin C based on LC-MS and NMR analysis. To further investigate the reaction mechanism, we incubated the intermediate, 9,10-dehydroauramycinone with each of the enzymes alone in the presence of NADH. Assay with KstA16 or ChaU showed no activity, however ChaJ was able to perform a 7,8-dehydration of 9,10-dehydroauramycinone which resulted in production of resomycin C. What is more, ChaJ was able to perform the reaction without any cofactors.

4.2.4 Assembly of the Biosynthetic Pathway

I assembled the biosynthetic pathway leading to production of resomycin C in *S. coelicolor* M1152Δ*matAB* using synthetic BioBrick DNA fragments (Figure 29). For the production of auramycinone we improved our previously cloned construct,

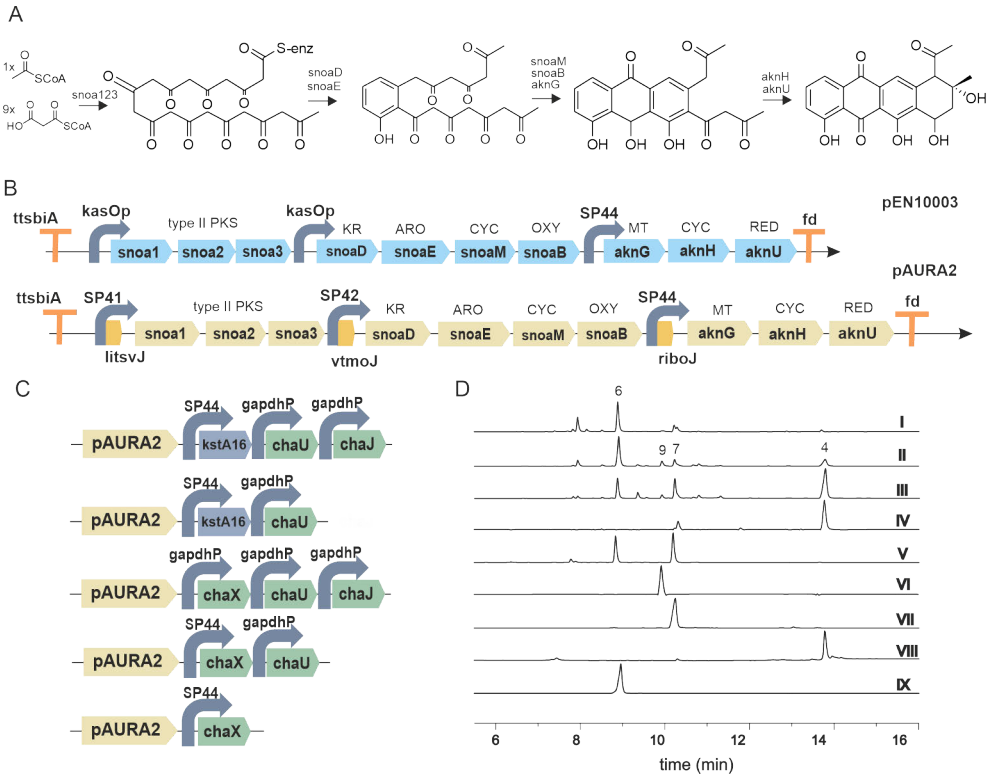

Figure 29: (A) Assembly of the resomycin C biosynthetic pathway in Streptomyces coelicolor M1152ΔmatAB. (A) Production of auramycinone was increased via the use of genetic insulators LitsvJ, VtmoJ and RiboJ (B) SBOL diagrams for the BioBrick expression constructs used to study the functions of kstA16, chaX, chaU and chaJ. (C) UPLC chromatogram traces recorded at 450nm of culture extracts. Legend: I, Streptomyces coelicolor M1152ΔmatAB_pAURA2; II, pAURA2_kstA16 chaU; III, pAURA2_k16 chaU chaJ; IV, pAURA2 chaU chaX chaJ; V, pAURA2_chaX; VI, purified 9,10-dehydroauramycinone; VII, purified 7-deoxyauramycinone; VIII, purified resomycin C; IX, purified auramycinone. KR – ketoreductase, ARO – aromatase, CYC – cyclase, MT – methyltransferase, RED – reductase.

pEN10003 by adding ribozyme insulators and terminators to enhance stability of mRNA, which increased the yields to 77,78mg/L. Then we cloned *chaU*, *chaX/kstA16* and *chaJ* into a pENTG3 integrating vector under the strong SP44, or natural gapdhP promoter. At first, we detected only trace amounts of resomycin C, which was caused by using an inappropriate solvent in the extraction process. Resomycin C is very poorly soluble in organic solvents and after switching to DMSO as the main solvent for the compound, we observed a significant increase in the yield.

Expressing *chaU* together with *kstA16* yielded small amounts of resomycin C, 9,10-dehydroauramycinone and 7-deoxyauramycinone. Addition of *chaJ* to *kstA16* and *chaU* resulted in an incomplete conversion of auramycinone to resomycin C. Upon switching *kstA16* for *chaX*, native to the chartreusin pathway, we observed an almost complete conversion of auramycinone to resomycin C. When *chaX* was expressed alone, the strain yielded a shunt product, 7-deoxyauramycinone, which has been characterized in the past [236].

4.2.5 The three-enzyme cascade reaction mechanism

The results let us describe ChaX as a NAD(P)H dependent reductase, ChaU as a cyclase-like protein and ChaJ as a cofactor-independent cyclase-like protein. We propose that the conversion of auramycinone to 9,10-dehydroauramycinone is catalyzed by a pair of enzymes, ChaU and ChaX, whereas the 7,8-dehydration of 9,10-dehydroauramycinone is performed by ChaJ.

Based on the findings from both in vitro reaction and in vivo studies we proposed a mechanism of reaction for the three-enzyme cascade. The double dehydration takes place with first ChaX donating a hydride from NAD(P)H to C5 of the substrate, similarly to what happens in reactions involving SnoaW and KstA16 [236] [117]. The negative charge of the formed carbanion delocalizes over the anthraquinone ring structure and therefore can contribute to resonance stabilisation. We propose that ChaU, by participating in the stabilization of the molecule, facilitates the detachment of the hydroxyl group from C9 and formation of the double bond between C9 and C10.

The second dehydration reaction catalyzed by ChaJ. The hydroxyl group at C7 is protonated and subsequently detached, which leads to the formation of a double bond between C7 and C8, aromatization of the A ring and therefore formation of resomycin C.

5 Discussion and conclusions

Actinomycetes are a rich source of antiproliferative and antibacterial compounds, the discovery of which opened a completely new chapter in the history of medicine and allowed for advancement never seen before. Nowadays, the overuse of antibiotics in farming and agriculture contributed to a rise in antibiotic resistance amongst bacterial strains, which has generated an urgent demand for new antibacterial compounds. Similarly, the growing number of cancer cases worldwide, coupled with an aging population, influences the demand for innovative cancer treatments and further emphasizes the necessity for innovative therapeutic strategies. Traditional approaches to developing antibacterial and anticancer compounds face serious challenges and constraints, which encourages researchers to turn towards other, new methods, such as synthetic biology. Synthetic biology allows for de novo construction of biosynthetic pathways by selecting and combining specific genes to achieve a functional biosynthetic pathway with the best yield. This has great potential for accelerating the future development of novel secondary metabolites with therapeutic antibacterial and antiproliferative properties.

In our research, we demonstrate the scope of applications of synthetic biology technique, more specifically the BioBrick cloning technique. Our findings indicate that the BioBricks approach is particularly effective for developing hierarchical deoxysugar pathways. By adding or removing dehydratases, ketoreductases, or methyltransferases, we can produce sugar cassettes with predictable stereochemistry and structural modifications. This approach allowed us to successfully reconstruct known pathways with good conversion efficiency, including TDP-D-glucose, TDP-D-olivose, TDP-D-digitoxose, TDP-D-mycarose, and TDP-L-rhamnose. We also expanded tetracenomycin chemical diversity by characterizing a new ketosugar (TDP-4′-keto-D-digitoxose) and new D-series 6-deoxysugars (TDP-D-allose, TDP-D-quinovose, and TDP-D-fucose). Although the novel tetracenomycin derivates did not appear to gain desired specificity towards bacterial ribosomes, the work provided important structure-activity relationship information on this class of microbial natural products. Overall, the BioBricks cloning system in *S. coelicolor* M1146::cos16F4iE proves to be an excellent method for assembling sugar

nucleotide pathways in vivo and serves as a foundation for deeper understanding of structure-activity relationships in this important polyketide class.

We also used the synthetic biology approach for characterization of the functions of key enzymes in the chartreusin biosynthetic pathway. Through stepwise pathway assembly we discovered a three-enzyme cascade catalyzing conversion of auramycinone, an anthracycline aglycone intermediate, into resomycin C through a double dehydration reaction. The first dehydration reaction is catalyzed by ChaX/KstA16 and ChaU, through a carbanion intermediate, while the second dehydration is catalyzed by ChaJ and proceeds involving a carbocation intermediate. ChaX and ChaU catalyze a 9,10-dehydration of auramycinone, yielding an intermediate molecule, 9,10-dehydroauramycinone. 9,10-dehydroauramycinone is further dehydrated by ChaJ leading to full aromatization of the A ring and formation of resomycin C. This discovery contributed to better understanding of the chartreusin BGC as well as the biosynthesis of microbial natural products in general, which forms the basis for all applied aspects such as pathway engineering.

The results described in this thesis demonstrate the efficacy of synthetic biology platforms in facilitating the identification of novel antibiotic and antiproliferative compounds, as well as characterization of previously unidentified genes within biosynthetic gene clusters. The BioBricks technique provides researchers with a comprehensive toolkit for the design and optimization of synthetic biological systems, thereby enhancing efficiency and effectiveness in drug discovery and metabolic pathway elucidation. By employing BioBrick, we successfully assembled deoxysugar pathways, expanding the structural diversity of tetracenomycins and revealed key enzymatic mechanisms in chartreusin biosynthesis. The modular pathway design strategy using standardized genetic parts allowed us to bypass the unpredictability of traditional methods, significantly shortening development timelines, which facilitates discovery of new compounds. Moreover, merging BioBricks with other molecular biology techniques such as Golden Gate (GoldBricks) could overcome speed limitations of classic BioBricks. Moving forward, combining synthetic biology with more advanced computational tools and deep learning models may even more accelerate the process of drug development. This paves the way for a new chapter where synthetic biology not only supplements traditional drug discovery but becomes its driving engine.

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Turku, August 2025
Magdalena Niemczura

List of References

[1] D. De Simeis and S. Serra, "Actinomycetes: A never-ending source of bioactive compounds—an overview on antibiotics production," *Antibiotics*, vol. 10, no. 5, 2021, doi: 10.3390/antibiotics10050483.

[2] M. L. Nelson, A. Dinardo, J. Hochberg, and G. J. Armelagos, "Brief communication: Mass spectroscopic characterization of tetracycline in the skeletal remains of an ancient population from Sudanese Nubia 350-550 CE," *Am J Phys Anthropol*, vol. 143, no. 1, pp. 151–154, 2010, doi: 10.1002/ajpa.21340.

[3] X. Zhang *et al.*, "Endophytic Streptomyces sp Y3111 from traditional Chinese medicine produced antitubercular pluramycins," *Appl Microbiol Biotechnol*, vol. 98, no. 3, pp. 1077–1085, 2014, doi: 10.1007/s00253-013-5335-6.

[4] I. Oa. F. Joseph *et al.*, "Proliferation of antibiotic-producing bacteria and concomitant antibiotic production as the basis for the antibiotic activity of Jordan's red soils," *Appl Environ Microbiol*, vol. 75, no. 9, pp. 2735–2741, 2009, doi: 10.1128/AEM.00104-09.

[5] A. R. Coates, G. Halls, and Y. Hu, "Novel classes of antibiotics or more of the same?," *Br J Pharmacol*, vol. 163, no. 1, pp. 184–194, 2011, doi: 10.1111/j.1476-5381.2011.01250.x.

[6] M. B. Martins-Teixeira and I. Carvalho, "Antitumour Anthracyclines: Progress and Perspectives," *ChemMedChem*, vol. 15, no. 11, pp. 933–948, 2020, doi: 10.1002/cmdc.202000131.

[7] M. Volkova and R. Russell, "Anthracycline Cardiotoxicity: Prevalence, Pathogenesis and Treatment," *Curr Cardiol Rev*, vol. 7, no. 4, pp. 214–220, 2012, doi: 10.2174/157340311799960645.

[8] J. Huang, R. Wu, L. Chen, Z. Yang, D. Yan, and M. Li, "Understanding Anthracycline Cardiotoxicity From Mitochondrial Aspect," *Front Pharmacol*, vol. 13, no. February, pp. 1–15, 2022, doi: 10.3389/fphar.2022.811406.

[9] M. Harir, H. Bendif, M. Bellahcene, and Z. Fortas and Rebecca Pogni, "Streptomyces Secondary Metabolites," *Basic Biology and Applications of Actinobacteria*, 2018, doi: 10.5772/intechopen.79890.

[10] K. F. Chater, "Streptomyces inside-out: A new perspective on the bacteria that provide us with antibiotics," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 361, no. 1469, pp. 761–768, 2006, doi: 10.1098/rstb.2005.1758.

[11] L. Donald, A. Pipite, R. Subramani, J. Owen, R. A. Keyzers, and T. Taufa, "Streptomyces: Still the Biggest Producer of New Natural Secondary Metabolites, a Current Perspective," *Microbiol Res (Pavia)*, vol. 13, no. 3, pp. 418–465, 2022, doi: 10.3390/microbiolres13030031.

[12] K. K. Sivakala *et al.*, "Desert environments facilitate unique evolution of biosynthetic potential in Streptomyces," *Molecules*, vol. 26, no. 3, pp. 1–15, 2021, doi: 10.3390/molecules26030588.

[13] X. Li, S. Dobretsov, Y. Xu, X. Xiao, O. Hung, and P. Y. Qian, "Antifouling diketopiperazines produced by a deep-sea bacterium, Streptomyces fungicidicus," *Biofouling*, vol. 22, no. 3, pp. 187–194, 2006, doi: 10.1080/08927010600780771.

[14] R. Subramani and D. Sipkema, *Marine rare actinomycetes: A promising source of structurally diverse and unique novel natural products*, vol. 17, no. 5. 2019. doi: 10.3390/md17050249.

[15] C. Viegelmann *et al.*, "Metabolomic profiling and genomic study of a marine sponge-associated Streptomyces sp," *Mar Drugs*, vol. 12, no. 6, pp. 3323–3351, 2014, doi: 10.3390/md12063323.
[16] R. E. de Lima Procópio, I. R. da Silva, M. K. Martins, J. L. de Azevedo, and J. M. de Araújo, "Antibiotics produced by Streptomyces," *Brazilian Journal of Infectious Diseases*, vol. 16, no. 5, pp. 466–471, 2012, doi: 10.1016/j.bjid.2012.08.014.
[17] K. Alam *et al.*, "Streptomyces: The biofactory of secondary metabolites," *Front Microbiol*, vol. 13, no. September, pp. 1–21, 2022, doi: 10.3389/fmicb.2022.968053.
[18] B. Abdul and R. Hassan, *Advances in Precision Medicine Oncology*. 2021. doi: 10.5772/intechopen.91507.
[19] H. Umezawa *et al.*, "Production and Isolation of a New Antibiotic, Kanamycin," *J Antibiot (Tokyo)*, vol. 181–188, 1957.
[20] M. L. Nelson and S. B. Levy, "The history of the tetracyclines," *Ann N Y Acad Sci*, vol. 1241, no. 1, pp. 17–32, 2011, doi: 10.1111/j.1749-6632.2011.06354.x.
[21] M. Heidary, A. D. Khosravi, S. Khoshnood, M. J. Nasiri, S. Soleimani, and M. Goudarzi, "Daptomycin," *Journal of Antimicrobial Chemotherapy*, vol. 73, no. 1, pp. 1–11, 2018, doi: 10.1093/jac/dkx349.
[22] R. S. Griffith and H. R. Black, "Erythromycin.," *Med Clin North Am*, vol. 54, no. 5, pp. 1199–1215, 1970, doi: 10.1016/s0025-7125(16)32587-1.
[23] P. Sensi, "History of the Development of Rifampin," *Reviews on Infectious Diseases*, vol. 5, pp. 4–8, 1983, doi: 10.1201/b10256-3.
[24] N. A. Church and J. L. McKillip, "Antibiotic resistance crisis: challenges and imperatives," *Biologia (Bratisl)*, vol. 76, no. 5, pp. 1535–1550, 2021, doi: 10.1007/s11756-021-00697-x.
[25] E. M. Darby *et al.*, "Molecular mechanisms of antibiotic resistance revisited," *Nat Rev Microbiol*, vol. 21, no. 5, pp. 280–295, 2023, doi: 10.1038/s41579-022-00820-y.
[26] A. J. Browne *et al.*, "Global antibiotic consumption and usage in humans, 2000–18: a spatial modelling study," *Lancet Planet Health*, vol. 5, no. 12, pp. e893–e904, 2021, doi: 10.1016/S2542-5196(21)00280-1.
[27] P. Vejpongsa and E. T. H. Yeh, "Prevention of anthracycline-induced cardiotoxicity: Challenges and opportunities," *J Am Coll Cardiol*, vol. 64, no. 9, pp. 938–945, 2014, doi: 10.1016/j.jacc.2014.06.1167.
[28] "National Cancer Institute - Bleomycin sulfate," cancer.gov. [Online]. Available: https://www.cancer.gov/about-cancer/treatment/drugs/bleomycin
[29] J. M. Mack, G. T. Richter, D. Becton, O. Salem, S. E. M. Hill, and S. E. Crary, "Short-term side effects and patient-reported outcomes of bleomycin sclerotherapy in vascular malformations," *Pediatr Blood Cancer*, vol. 65, no. 6, pp. 1–4, 2018, doi: 10.1002/pbc.27008.
[30] "National Cancer Institute - Dactinomycin," cancer.gov. [Online]. Available: https://www.cancer.gov/about-cancer/treatment/drugs/dactinomycin
[31] W. M. C. van den Boogaard, D. S. J. Komninos, and W. P. Vermeij, "Chemotherapy Side-Effects: Not All DNA Damage Is Equal," *Cancers (Basel)*, vol. 14, no. 3, pp. 1–27, 2022, doi: 10.3390/cancers14030627.
[32] L. Du and L. Lou, "PKS and NRPS release mechanisms," *Nat Prod Rep*, vol. 27, no. 2, pp. 255–278, 2010, doi: 10.1039/b912037h.
[33] G. A. Hudson and D. A. Mitchell, "RiPP antibiotics: biosynthesis and engineering potential," *Curr Opin Microbiol*, vol. 45, pp. 61–69, 2018, doi: 10.1016/j.mib.2018.02.010.
[34] K. M. Krause, A. W. Serio, T. R. Kane, and L. E. Connolly, "Aminoglycosides: An overview," *Cold Spring Harb Perspect Med*, vol. 6, no. 6, pp. 1–18, 2016, doi: 10.1101/cshperspect.a027029.
[35] K. C. Belknap, C. J. Park, B. M. Barth, and C. P. Andam, "Genome mining of biosynthetic and chemotherapeutic gene clusters in Streptomyces bacteria," *Sci Rep*, vol. 10, no. 1, pp. 1–9, 2020, doi: 10.1038/s41598-020-58904-9.

[36] J. R. Doroghazi and W. W. Metcalf, “Comparative genomics of actinomycetes with a focus on natural product biosynthetic genes,” *BMC Genomics*, vol. 14, no. 611, 2013, doi: 10.1186/1471-2164-14-611.

[37] M. Nett, H. Ikeda, and B. S. Moore, “Genomic basis for natural product biosynthetic diversity in the actinomycetes,” *Nat Prod Rep*, vol. 26, no. 1, pp. 1362–1384, 2009, doi: 10.1039/b817069j.

[38] K. Lewis, S. Epstein, A. D’Onofrio, and L. L. Ling, “Uncultured microorganisms as a source of secondary metabolites,” *Journal of Antibiotics*, vol. 63, no. 8, pp. 468–476, 2010, doi: 10.1038/ja.2010.87.

[39] Y. Zha, H. Chong, P. Yang, and K. Ning, “Microbial Dark Matter: from Discovery to Applications,” *Genomics Proteomics Bioinformatics*, vol. 20, no. 5, pp. 867–881, 2022, doi: 10.1016/j.gpb.2022.02.007.

[40] E. A. Barka *et al.*, “Taxonomy, Physiology and Natural Products of Actinobacteria,” *Microbiology and Molecular Biology Reviews*, vol. 80, no. 1, pp. 1–43, 2016, doi: 10.1128/MMBR.00019-15.Address.

[41] K. Scherlach and C. Hertweck, “Mining and unearthing hidden biosynthetic potential,” *Nat Commun*, vol. 12, no. 1, pp. 1–12, 2021, doi: 10.1038/s41467-021-24133-5.

[42] A. van der Meij, S. F. Worsley, M. I. Hutchings, and G. P. van Wezel, “Chemical ecology of antibiotic production by actinomycetes,” *FEMS Microbiol Rev*, vol. 41, no. 3, pp. 392–416, 2017, doi: 10.1093/femsre/fux005.

[43] R. Sánchez de la Nieta, R. I. Santamaría, and M. Díaz, “Two-Component Systems of Streptomyces coelicolor: An Intricate Network to Be Unraveled,” *Int J Mol Sci*, vol. 23, no. 23, 2022, doi: 10.3390/ijms232315085.

[44] R. Cruz-Bautista, B. Ruíz-Villafán, A. Romero-Rodríguez, R. Rodríguez-Sanoja, and S. Sánchez, “Trends in the two-component system’s role in the synthesis of antibiotics by Streptomyces,” *Appl Microbiol Biotechnol*, vol. 107, no. 15, pp. 4727–4743, 2023, doi: 10.1007/s00253-023-12623-z.

[45] S. Hackl and A. Bechthold, “The Gene bldA, a Regulator of Morphological Differentiation and Antibiotic Production in Streptomyces,” *Arch Pharm (Weinheim)*, vol. 348, no. 7, pp. 455–462, 2015, doi: 10.1002/ardp.201500073.

[46] M. B. Hulst, T. Grocholski, J. J. C. Neefjes, G. P. van Wezel, and M. Metsä-Ketelä, “Anthracyclines: biosynthesis, engineering and clinical applications,” *Nat Prod Rep*, vol. 39, no. 4, pp. 814–841, 2021, doi: 10.1039/d1np00059d.

[47] A. Wietzorrek and M. Bibb, “A novel family of proteins that regulates antibiotic production in streptomycetes appears to contain an OmpR-like DNA-binding fold,” *Molecular Microbiology (1997)*, vol. 25, no. 6, pp. 1177–1184, 1997.

[48] A. F. Braña *et al.*, “Branimycins B and C, Antibiotics Produced by the Abyssal Actinobacterium Pseudonocardia carboxydivorans M-227,” *J Nat Prod*, vol. 80, no. 2, pp. 569–573, 2017, doi: 10.1021/acs.jnatprod.6b01107.

[49] J. H. Xu, K. B. Gu, D. J. Zhang, Y. G. Li, and L. Tian, “Ghanamycins A and B, two novel γ-butyrolactones from marine-derived streptomyces ghanaensis TXC6-16,” *Journal of Antibiotics*, vol. 70, no. 6, pp. 733–736, 2017, doi: 10.1038/ja.2017.37.

[50] G. Huber, “Moenomycin and Related Phosphorus-Containing Antibiotics,” *Mechanism of Action of Antibacterial Agents*, no. 1979, pp. 135–153, 1979, doi: 10.1007/978-3-642-46403-4_9.

[51] M. Bae *et al.*, “Donghaecyclinones A-C: New cytotoxic rearranged angucyclinones from a volcanic island-derived marine Streptomyces sp.,” *Mar Drugs*, vol. 18, no. 121, 2020, doi: 10.3390/md18020121.

[52] J. Parra, A. Beaton, R. F. Seipke, B. Wilkinson, M. I. Hutchings, and K. R. Duncan, “Antibiotics from rare actinomycetes, beyond the genus Streptomyces,” *Curr Opin Microbiol*, vol. 76, p. 102385, 2023, doi: 10.1016/j.mib.2023.102385.

[53] S. M. Cho, H. J. Lee, P. Karuso, and H. J. Kwon, “Daptomycin suppresses tumor migration and angiogenesis via binding to ribosomal protein S19 in humans,” *Journal of Antibiotics*, vol. 74, no. 10, pp. 726–733, 2021, doi: 10.1038/s41429-021-00446-x.

[54] L. Zhang, L. Xu, F. Zhang, and E. Vlashi, “Doxycycline inhibits the cancer stem cell phenotype and epithelial-to-mesenchymal transition in breast cancer,” *Cell Cycle*, vol. 16, no. 8, pp. 737–745, 2017, doi: 10.1080/15384101.2016.1241929.

[55] I. Ali, K. O. Alfarouk, S. J. Reshkin, and M. E. Ibrahim, “Doxycycline as Potential Anti-cancer Agent,” *Anticancer Agents Med Chem*, vol. 17, no. 12, pp. 1617–1623, 2017, doi: 10.2174/1871520617666170213111951.

[56] T. Hamoya *et al.*, “Chemopreventive effects of a low-side-effect antibiotic drug, erythromycin, on mouse intestinal tumors,” *J Clin Biochem Nutr*, vol. 60, no. 3, pp. 199–207, 2017, doi: 10.3164/jcbn.16.

[57] F. Lombó, N. Menéndez, J. A. Salas, and C. Méndez, “The aureolic acid family of antitumor compounds: Structure, mode of action, biosynthesis, and novel derivatives,” *Appl Microbiol Biotechnol*, vol. 73, no. 1, pp. 1–14, 2006, doi: 10.1007/s00253-006-0511-6.

[58] M. H. Medema, R. Breitling, and E. Takano, *Synthetic Biology in Streptomyces Bacteria*, 1st ed., vol. 497. Elsevier Inc., 2011. doi: 10.1016/b978-0-12-385075-1.00021-4.

[59] G. D. Hannigan *et al.*, “A deep learning genome-mining strategy for biosynthetic gene cluster prediction,” *Nucleic Acids Res*, vol. 47, no. 18, p. E110, 2019, doi: 10.1093/NAR/GKZ654.

[60] M. Liu, Y. Li, and H. Li, “Deep Learning to Predict the Biosynthetic Gene Clusters in Bacterial Genomes,” *J Mol Biol*, vol. 434, no. 15, p. 167597, 2022, doi: 10.1016/j.jmb.2022.167597.

[61] D. Meleshko *et al.*, “BiosyntheticSPAdes: Reconstructing biosynthetic gene clusters from assembly graphs,” *Genome Res*, vol. 29, no. 8, pp. 1352–1362, 2019, doi: 10.1101/gr.243477.118.

[62] M. H. Medema *et al.*, “AntiSMASH: Rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences,” *Nucleic Acids Res*, vol. 39, no. SUPPL. 2, pp. 339–346, 2011, doi: 10.1093/nar/gkr466.

[63] S. A. Kautsar *et al.*, “MIBiG 2.0: A repository for biosynthetic gene clusters of known function,” *Nucleic Acids Res*, vol. 48, no. D1, pp. D454–D458, 2020, doi: 10.1093/nar/gkz882.

[64] S. A. Kautsar, K. Blin, S. Shaw, T. Weber, and M. H. Medema, “BiG-FAM: The biosynthetic gene cluster families database,” *Nucleic Acids Res*, vol. 49, no. D1, pp. D490–D497, 2021, doi: 10.1093/nar/gkaa812.

[65] R. Wang *et al.*, “A BioBricks Metabolic Engineering Platform for the Biosynthesis of Anthracyclinones in Streptomyces coelicolor,” *ACS Synth. Biol.*, vol. 11, 2022, doi: 10.1021/acssynbio.2c00498.

[66] A. Gavriilidou *et al.*, “Compendium of specialized metabolite biosynthetic diversity encoded in bacterial genomes,” *Nat Microbiol*, vol. 7, no. 5, pp. 726–735, 2022, doi: 10.1038/s41564-022-01110-2.

[67] M. H. Medema, R. Breitling, R. Bovenberg, and E. Takano, “Exploiting plug-and-play synthetic biology for drug discovery and production in microorganisms,” *Nat Rev Microbiol*, vol. 9, no. 2, pp. 131–137, 2011, doi: 10.1038/nrmicro2478.

[68] Z. Zhuang *et al.*, “Combinatorial metabolic engineering of Streptomyces sp. CB03234-S for the enhanced production of anthraquinone-fused enediyne tiancimycins,” *Microb Cell Fact*, vol. 23, no. 1, pp. 1–13, 2024, doi: 10.1186/s12934-024-02399-w.

[69] Z. Li *et al.*, “Stepwise increase of thaxtomins production in Streptomyces albidoflavus J1074 through combinatorial metabolic engineering,” *Metab Eng*, vol. 68, no. July, pp. 187–198, 2021, doi: 10.1016/j.ymben.2021.10.008.

[70] L. Li *et al.*, “A stepwise increase in pristinamycin II biosynthesis by Streptomyces pristinaespiralis through combinatorial metabolic engineering,” *Metab Eng*, vol. 29, pp. 12–25, 2015, doi: 10.1016/j.ymben.2015.02.001.

[71] J. Meng *et al.*, "Improvement of pristinamycin I (PI) production in Streptomyces pristinaespiralis by metabolic engineering approaches," *Synth Syst Biotechnol*, vol. 2, no. 2, pp. 130–136, 2017, doi: 10.1016/j.synbio.2017.06.001.

[72] C. Wang *et al.*, "Generation of Streptomyces hygroscopicus cell factories with enhanced ascomycin production by combined elicitation and pathway-engineering strategies," *Biotechnol Bioeng*, vol. 116, no. 12, pp. 3382–3395, 2019, doi: 10.1002/bit.27158.

[73] H. Tirkkonen *et al.*, "Engineering BioBricks for Deoxysugar Biosynthesis and Generation of New Tetracenomycins," *ACS Omega*, vol. 8, no. 23, pp. 21237–21253, 2023, doi: 10.1021/acsomega.3c02460.

[74] R. Wang *et al.*, "A BioBricks Metabolic Engineering Platform for the Biosynthesis of Anthracyclinones in Streptomyces coelicolor," *ACS Synth Biol*, vol. 11, no. 12, pp. 4193–4209, 2022, doi: 10.1021/acssynbio.2c00498.

[75] A. Ameruoso, M. C. V. Kcam, K. P. Cohen, and J. Chappell, "Activating natural product synthesis using CRISPR interference and activation systems in Streptomyces," *Nucleic Acids Res*, vol. 50, no. 13, pp. 7751–7760, 2022, doi: 10.1093/nar/gkac556.

[76] Y. Lee, S. Hwang, W. Kim, J. H. Kim, B. O. Palsson, and B. K. Cho, "CRISPR-aided genome engineering for secondary metabolite biosynthesis in Streptomyces," *J Ind Microbiol Biotechnol*, vol. 51, no. March, 2024, doi: 10.1093/jimb/kuae009.

[77] O. Genilloud, "Actinomycetes: Still a source of novel antibiotics," *Nat Prod Rep*, vol. 34, no. 10, pp. 1203–1232, 2017, doi: 10.1039/c7np00026j.

[78] B. Shen, "Polyketide biosynthesis beyond the type I , II and III polyketide synthase paradigms," *Curr Opin Chem Biol*, pp. 285–295, 2003, doi: 10.1016/S1367-5931(03)00020-6.

[79] C. Hertweck, A. Luzhetskyy, Y. Rebets, and A. Bechthold, "Type II polyketide synthases: Gaining a deeper insight into enzymatic teamwork," *Nat Prod Rep*, vol. 24, no. 1, pp. 162–190, 2007, doi: 10.1039/b507395m.

[80] M. A. J. Rivers and A. N. Lowell, "Expanding the Biosynthetic Toolbox: The Potential and Challenges of In Vitro Type II Polyketide Synthase Research," *SynBio*, vol. 2, no. 1, pp. 85–111, 2024, doi: 10.3390/synbio2010006.

[81] M. Metsä-Ketelä, J. Niemi, P. Mäntsälä, and G. Schneider, "Anthracycline biosynthesis: Genes, enzymes and mechanisms," *Top Curr Chem*, vol. 282, no. January, pp. 101–140, 2008.

[82] D. M. Rayner and S. M. Cutts, "Anthracyclines," *Side Effects of Drugs Annual*, vol. 36, pp. 683–694, 2014, doi: 10.1016/B978-0-444-63407-8.00045-9.

[83] L. Zhu, X. Cao, W. Chen, G. Zhang, D. Sun, and P. G. Wang, "Syntheses and biological activities of daunorubicin analogs with uncommon sugars," *Bioorg Med Chem*, vol. 13, no. 23, pp. 6381–6387, 2005, doi: 10.1016/j.bmc.2005.06.053.

[84] S. Gullón *et al.*, "Isolation, characterization, and heterologous expression of the biosynthesis gene cluster for the antitumor anthracycline steffimycin," *Appl Environ Microbiol*, vol. 72, no. 6, pp. 4172–4183, 2006, doi: 10.1128/AEM.00734-06.

[85] S. Torkkell, T. Kunnari, K. Palmu, J. Hakala, and K. Ylihonko, "The entire nogalamycin biosynthetic gene cluster of Streptomyces nogalater: characterization of a 20-kb DNA region and generation of hybrid structures," *Mol Genet Genomics*, vol. 266, pp. 276–288, 2001, doi: 10.1007/s004380100554.

[86] A. Rusu and E. L. Buta, "The development of third-generation tetracycline antibiotics and new perspectives," *Pharmaceutics*, vol. 13, no. 12, 2021, doi: 10.3390/pharmaceutics13122085.

[87] J. Leichtweis, Y. Vieira, N. Welter, S. Silvestri, G. L. Dotto, and E. Carissimi, "A review of the occurrence, disposal, determination, toxicity and remediation technologies of the tetracycline antibiotic," *Process Safety and Environmental Protection*, vol. 160, pp. 25–40, 2022, doi: 10.1016/j.psep.2022.01.085.

[88] R. Daghrir and P. Drogui, "Tetracycline antibiotics in the environment: A review," *Environ Chem Lett*, vol. 11, no. 3, pp. 209–227, 2013, doi: 10.1007/s10311-013-0404-8.

[89] I. Chopra and M. Roberts, “Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance,” *Microbiology and Molecular Biology Reviews*, vol. 65, no. 2, pp. 232–260, 2001, doi: 10.1128/mmbr.65.2.232-260.2001.

[90] J. Rohr and A. Zeeck, “Structure-activity relationships of elloramycin and tetracenomycin C,” *J Antibiot (Tokyo)*, vol. 43, no. 9, pp. 1169–1178, 1990, doi: 10.7164/antibiotics.43.1169.

[91] C. R. Hutchinson, “Biosynthetic studies of daunorubicin and tetracenomycin C,” *Chem Rev*, vol. 97, no. 7, pp. 2525–2535, 1997, doi: 10.1021/cr960022x.

[92] I. A. Osterman *et al.*, “Tetracenomycin X inhibits translation by binding within the ribosomal exit tunnel,” *Nat Chem Biol*, vol. 16, no. 10, pp. 1071–1077, 2020, doi: 10.1038/s41589-020-0578-x.

[93] D. Vysloužilová and O. Kováč, “The Chemistry of Angucyclines,” *Chempluschem*, vol. 89, 2024, doi: 10.1002/cplu.202400307.

[94] M. K. Kharel *et al.*, “Angucyclines: Biosynthesis, mode-of-action, new natural products, and synthesis,” *Nat Prod Rep*, vol. 29, no. 2, pp. 264–325, 2012, doi: 10.1039/c1np00068c.

[95] T. J. Walsh and N. Giri, “Pradimicins: A novel class of broad-spectrum antifungal compounds,” *European Journal of Clinical Microbiology and Infectious Diseases*, vol. 16, no. 1, pp. 93–97, 1997, doi: 10.1007/BF01575126.

[96] J. Zhan, K. Qiao, and Y. Tang, “Investigation of tailoring modifications in pradimicin biosynthesis,” *ChemBioChem*, vol. 10, no. 9, pp. 1447–1452, 2009, doi: 10.1002/cbic.200900082.

[97] A. K. Isagulieva, A. N. Tevyashova, and A. A. Shtil, “Aureolic Acid-Derived Antibiotics: Prospects for a Biologically Active Class,” *Russ J Bioorg Chem*, vol. 48, no. 2, pp. 244–255, 2022, doi: 10.1134/S1068162022020121.

[98] C. Risdian, T. Mozef, and J. Wink, “Biosynthesis of polyketides in Streptomyces,” *Microorganisms*, vol. 7, no. 5, pp. 1–18, 2019, doi: 10.3390/microorganisms7050124.

[99] T. Nishiyama, N. Enomoto, R. Nagayasu, and K. Ueda, “Organocatalytic activity of granaticin and its involvement in bactericidal function,” *Sci Rep*, vol. 12, no. 1, pp. 1–11, 2022, doi: 10.1038/s41598-022-10877-7.

[100] C. M. McBride, E. L. Miller, and L. K. Charkoudian, “An updated catalogue of diverse type II polyketide synthase biosynthetic gene clusters captured from large-scale nucleotide databases,” *Microb Genom*, vol. 9, no. 3, pp. 1–9, 2023.

[101] A. Sulpizio, C. E. W. Crawford, R. S. Koweek, and L. K. Charkoudian, “Probing the structure and function of acyl carrier proteins to unlock the strategic redesign of type II polyketide biosynthetic pathways,” *Journal of Biological Chemistry*, vol. 296, p. 100328, 2021, doi: 10.1016/J.JBC.2021.100328.

[102] Y. Tang, S. C. Tsai, and C. Khosla, “Polyketide Chain Length Control by Chain Length Factor,” *J Am Chem Soc*, vol. 125, no. 42, pp. 12708–12709, 2003, doi: 10.1021/ja0378759.

[103] K. V. Brown, B. Nji Wandi, M. Metsä-Ketelä, and S. Eric Nybo, “Pathway engineering of anthracyclines: Blazing trails in natural product glycodiversification,” *Journal of Organic Chemistry*, vol. 19, no. 1, pp. 100–106, 2020, doi: 10.1177/0022146515594631.Marriage.

[104] Y. Ogasawara, B. J. Yackley, J. A. Greenberg, S. Rogelj, and C. E. Melançon, “Expanding our understanding of sequence-function relationships of type II polyketide biosynthetic gene clusters: Bioinformatics-guided identification of frankiamicin A from Frankia sp. EAN1pec,” *PLoS One*, vol. 10, no. 6, pp. 2–3, 2015, doi: 10.1371/journal.pone.0129408.

[105] B. S. Moore and C. Hertweck, “Biosynthesis and attachment of novel bacterial polyketide synthase starter units,” *Nat Prod Rep*, vol. 19, no. 1, pp. 70–99, 2002, doi: 10.1039/b003939j.

[106] J. A. Kalaitzis *et al.*, “Policing starter unit selection of the enterocin type II polyketide synthase by the type II thioesterase EncL,” *Bioorg Med Chem*, vol. 19, no. 22, pp. 6633–6638, 2011, doi: 10.1016/j.bmc.2011.04.024.

[107] C. Hertweck, “The biosynthetic logic of polyketide diversity,” *Angewandte Chemie - International Edition*, vol. 48, no. 26, pp. 4688–4716, 2009, doi: 10.1002/anie.200806121.

[108] S. Xie and L. Zhang, “Type II Polyketide Synthases: A Bioinformatics-Driven Approach,” *ChemBioChem*, vol. 24, no. 9, 2023, doi: 10.1002/cbic.202200775.
[109] J. Kantola, T. Kunnari, A. Hautala, J. Hakala, K. Ylihonko, and P. Mäntsälä, “Elucidation of anthracyclinone biosynthesis by stepwise cloning of genes for anthracyclines from three different Streptomyces spp.,” *Microbiology (N Y)*, vol. 146, no. 1, pp. 155–163, 2000, doi: 10.1099/00221287-146-1-155.
[110] T. J. Oh, S. J. Mo, Y. J. Yoon, and J. K. Sohng, “Discovery and molecular engineering of sugar-containing natural product biosynthetic pathways in actinomycetes,” 2007.
[111] J. Rohr, “Urdamycins, new angucycline antibiotics from Streptomyces fradiae. VI. Structure elucidation and biosynthetic investigations on urdamycin H,” *J Antibiot (Tokyo)*, vol. 42, no. 10, pp. 1482–1488, 1989, doi: 10.7164/antibiotics.42.1482.
[112] L. Y. Li *et al.*, “Resistance and phylogeny guided discovery reveals structural novelty of tetracycline antibiotics,” *Chem Sci*, vol. 24, no. 48, pp. 12892–12898, 2022, doi: 10.1039/d2sc03965f.
[113] S. Okamoto, T. Taguchi, K. Ochi, and K. Ichinose, “Biosynthesis of Actinorhodin and Related Antibiotics: Discovery of Alternative Routes for Quinone Formation Encoded in the act Gene Cluster,” *Chem Biol*, vol. 16, no. 2, pp. 226–236, 2009, doi: 10.1016/j.chembiol.2009.01.015.
[114] R. J. Walczak, M. L. Dickens, N. D. Priestley, and W. R. Strohl, “Purification, properties, and characterization of recombinant Streptomyces sp. strain C5 DoxA, a cytochrome P-450 catalyzing multiple steps in doxorubicin biosynthesis,” *J Bacteriol*, vol. 181, no. 1, pp. 298–304, 1999, doi: 10.1128/jb.181.1.298-304.1999.
[115] E. R. Rafanan, C. R. Hutchinson, and B. Shen, “Triple hydroxylation of tetracenomycin A2 to tetracenomycin C involving two molecules of o2 and one molecule of H2o,” *Org Lett*, vol. 2, no. 20, pp. 3225–3227, 2000, doi: 10.1021/ol0002267.
[116] I. Alexeev, A. Sultana, P. Mäntsälä, J. Niemi, and G. Schneider, “Aclacinomycin oxidoreductase (AknOx) from the biosynthetic pathway of the antibiotic aclacinomycin is an unusual flavoenzyme with a dual active site,” *Proc Natl Acad Sci U S A*, vol. 104, no. 15, pp. 6170–6175, 2007, doi: 10.1073/pnas.0700579104.
[117] Z. Zhang *et al.*, “Hydroxyl regioisomerization of anthracycline catalyzed by a four-enzyme cascade,” *Proc Natl Acad Sci U S A*, vol. 114, no. 7, pp. 1554–1559, 2017, doi: 10.1073/pnas.1610097114.
[118] L. Serrano, “Synthetic biology: Promises and challenges,” *Mol Syst Biol*, vol. 3, no. 158, p. 158, 2007, doi: 10.1038/msb4100202.
[119] S. A. Benner and A. M. Sismour, “Synthetic biology,” *Nat Rev Genet*, vol. 6, no. 7, pp. 533–543, 2005, doi: 10.1038/nrg1637.
[120] F. Meng and T. Ellis, “The second decade of synthetic biology: 2010–2020,” *Nat Commun*, vol. 11, no. 1, pp. 1–4, 2020, doi: 10.1038/s41467-020-19092-2.
[121] “iGEM.” [Online]. Available: https://igem.org/
[122] G. Y. Tan and T. Liu, “Rational synthetic pathway refactoring of natural products biosynthesis in actinobacteria,” *Metab Eng*, vol. 39, no. December 2016, pp. 228–236, 2017, doi: 10.1016/j.ymben.2016.12.006.
[123] N. Lee, S. Hwang, Y. Lee, S. Cho, B. Palsson, and B. K. Cho, “Synthetic biology tools for novel secondary metabolite discovery in streptomyces,” *J Microbiol Biotechnol*, vol. 29, no. 5, pp. 667–686, 2019, doi: 10.4014/jmb.1904.04015.
[124] R. P. Shetty, D. Endy, and T. F. Knight, “Engineering BioBrick vectors from BioBrick parts,” *J Biol Eng*, vol. 2, pp. 1–12, 2008, doi: 10.1186/1754-1611-2-5.
[125] J. T. Nguyen, K. K. Riebschleger, K. V. Brown, N. M. Gorgijevska, and S. E. Nybo, “A BioBricks toolbox for metabolic engineering of the tetracenomycin pathway,” *Biotechnol J*, vol. 17, no. 3, pp. 1–11, 2022, doi: 10.1002/biot.202100371.

[126] J. Kormanec *et al.*, "Recent achievements in the generation of stable genome alterations/mutations in species of the genus Streptomyces," *Appl Microbiol Biotechnol*, vol. 103, no. 14, pp. 5463–5482, 2019, doi: 10.1007/s00253-019-09901-0.

[127] M. J. Bibb, G. R. Janssen, and J. M. Ward, "Cloning and analysis of the promoter region of the erythromycin resistance gene (ermE) of Streptomyces erythraeus (Recombinant DNA; Sl mapping; exonuclease VII; in vitro transcription; Streptomyces lividuns; promoter-probe plasmids; MLS; RNA polymerase)," vol. 38, pp. 215–226, 1985.

[128] W. Wang, X. Li, J. Wang, S. Xiang, X. Feng, and K. Yang, "An engineered strong promoter for streptomycetes," *Appl Environ Microbiol*, vol. 79, no. 14, pp. 4484–4492, 2013, doi: 10.1128/AEM.00985-13.

[129] Z. Shao, G. Rao, C. Li, Z. Abil, Y. Luo, and H. Zhao, "Refactoring the silent spectinabilin gene cluster using a plug-and-play scaffold," *ACS Synth Biol*, vol. 2, no. 11, pp. 662–669, 2013, doi: 10.1021/sb400058n.

[130] M. Myronovskyi and A. Luzhetskyy, "Native and engineered promoters in natural product discovery," *Nat Prod Rep*, vol. 33, no. 8, pp. 1006–1019, 2016, doi: 10.1039/c6np00002a.

[131] C. Bai *et al.*, "Exploiting a precise design of universal synthetic modular regulatory elements to unlock the microbial natural products in Streptomyces," *Proc Natl Acad Sci U S A*, vol. 112, no. 39, pp. 12181–12186, 2015, doi: 10.1073/pnas.1511027112.

[132] Y. Luo, L. Zhang, K. W. Barton, and H. Zhao, "Systematic Identification of a Panel of Strong Constitutive Promoters from Streptomyces albus," *ACS Synth Biol*, vol. 4, no. 9, pp. 1001–1010, 2015, doi: 10.1021/acssynbio.5b00016.

[133] S. Li, J. Wang, X. Li, S. Yin, W. Wang, and K. Yang, "Genome-wide identification and evaluation of constitutive promoters in streptomycetes," *Microb Cell Fact*, vol. 14, no. 1, pp. 1–11, 2015, doi: 10.1186/s12934-015-0351-0.

[134] J. S. Yi *et al.*, "A Novel Approach for Gene Expression Optimization through Native Promoter and 5′ UTR Combinations Based on RNA-seq, Ribo-seq, and TSS-seq of Streptomyces coelicolor," *ACS Synth Biol*, vol. 6, no. 3, pp. 555–565, 2017, doi: 10.1021/acssynbio.6b00263.

[135] S. Herai *et al.*, "Hyper-inducible expression system for streptomycetes," *Proc Natl Acad Sci U S A*, vol. 101, no. 39, pp. 14031–14035, 2004, doi: 10.1073/pnas.0406058101.

[136] Y. Noguchi *et al.*, "Development of a strictly regulated xylose-induced expression system in Streptomyces," *Microb Cell Fact*, vol. 17, no. 1, pp. 1–11, 2018, doi: 10.1186/s12934-018-0991-y.

[137] A. Rodríguez-García, P. Combes, R. Pérez-Redondo, M. C. A. Smith, and M. C. M. Smith, "Natural and synthetic tetracycline-inducible promoters for use in the antibiotic-producing bacteria Streptomyces," *Nucleic Acids Res*, vol. 33, no. 9, pp. 1–8, 2005, doi: 10.1093/nar/gni086.

[138] J. M. Ward, G. R. Janssen, T. Kieser, M. J. Bibb, M. J. Buttner, and M. J. Bibb, "Construction and characterisation of a series of multi-copy promoter-probe plasmid vectors for Streptomyces using the aminoglycoside phosphotransferase gene from Tn 5 as indicator," *MGG Molecular & General Genetics*, vol. 203, no. 3, pp. 468–478, 1986, doi: 10.1007/BF00422072.

[139] D. Pulido, A. Jiménez, M. Salas, and R. P. Mellado, "A Bacillus subtilis phage φ29 transcription terminator is efficiently recognized in Streptomyces lvidans," *Gene*, vol. 56, no. 2–3, pp. 277–282, 1987, doi: 10.1016/0378-1119(87)90144-2.

[140] L. Horbal, T. Siegl, and A. Luzhetskyy, "A set of synthetic versatile genetic control elements for the efficient expression of genes in Actinobacteria," *Sci Rep*, vol. 8, no. 1, pp. 1–13, 2018, doi: 10.1038/s41598-017-18846-1.

[141] R. Bunet *et al.*, "Characterization and manipulation of the pathway-specific late regulator AlpW reveals Streptomyces ambofaciens as a new producer of kinamycins," *J Bacteriol*, vol. 193, no. 5, pp. 1142–1153, 2011, doi: 10.1128/JB.01269-10.

[142] C. D. Sohaskey, H. Im, A. D. Nelson, and A. T. Schauer, "Tn4556 and luciferase: synergistic tools for visualizing transcription in Streptomyces," vol. 115, pp. 67–71, 1992.

[143] C. Ingram, M. Brawner, P. Youngman, and J. Westpheling, “xylE functions as an efficient reporter gene in Streptomyces spp.: Use for the study of galP1, a catabolite-controlled promoter,” *J Bacteriol*, vol. 171, no. 12, pp. 6617–6624, 1989, doi: 10.1128/jb.171.12.6617-6624.1989.

[144] M. Myronovskyi, E. Welle, V. Fedorenko, and A. Luzhetskyy, “β-Glucuronidase as a sensitive and versatile reporter in Actinomycetes,” *Appl Environ Microbiol*, vol. 77, no. 15, pp. 5370–5383, 2011, doi: 10.1128/AEM.00434-11.

[145] J. Willemse and G. P. van Wezel, “Imaging of streptomyces coelicolor A3(2) with reduced autofluorescence reveals a novel stage of FtsZ localization,” *PLoS One*, vol. 4, no. 1, pp. 1–5, 2009, doi: 10.1371/journal.pone.0004242.

[146] K. D. Nguyen, S. H. Au-Young, and J. R. Nodwell, “Monomeric red fluorescent protein as a reporter for macromolecular localization in Streptomyces coelicolor,” *Plasmid*, vol. 58, no. 2, pp. 167–173, 2007, doi: 10.1016/j.plasmid.2007.03.005.

[147] R. M. Phelan *et al.*, “Development of next generation synthetic biology tools for use in streptomyces venezuelae,” *ACS Synth Biol*, vol. 6, no. 1, pp. 159–166, 2017, doi: 10.1021/acssynbio.6b00202.

[148] T. Khaleel, E. Younger, A. R. Mcewan, A. S. Varghese, and M. C. M. Smith, “A phage protein that binds φC31 integrase to switch its directionality,” *Mol Microbiol*, vol. 80, no. 6, pp. 1450–1463, 2011, doi: 10.1111/j.1365-2958.2011.07696.x.

[149] N. D. Lomovskaya, N. M. Mkrtumian, N. L. Gostimskaya, and V. N. Danilenko, “ Characterization of Temperate Actinophage φC31 Isolated from Streptomyces coelicolor A3(2) ,” *J Virol*, vol. 9, no. 2, pp. 258–262, 1972, doi: 10.1128/jvi.9.2.258-262.1972.

[150] L. Zhang, B. Zhu, R. Dai, G. Zhao, and X. Ding, “Control of directionality in Streptomyces phage φBT1 integrase-mediated site-specific recombination,” *PLoS One*, vol. 8, no. 11, pp. 1–8, 2013, doi: 10.1371/journal.pone.0080434.

[151] L. Van Mellaert, L. Mei, E. Lammertyn, S. Schacht, and J. Anne, “Site-specific integration of bacteriophage VWB genome into Streptomyces venezuelae and construction of a VWB-based integrative vector,” *Microbiology (N Y)*, vol. 144, no. 12, pp. 3351–3358, 1998, doi: 10.1099/00221287-144-12-3351.

[152] F. Foor *et al.*, “Isolation and characterization of the Streptomyces cattleya temperate phage TG1,” *Gene*, vol. 39, no. 1, pp. 11–16, 1985, doi: 10.1016/0378-1119(85)90101-5.

[153] K. F. Chater and A. T. Carter, “A New, Wide Host-range, Temperate Bacteriophage (R4) of,” *J Gen Microbiol*, vol. 115, pp. 431–442, 1979.

[154] K. Morita *et al.*, “In vitro characterization of the site-specific recombination system based on actinophage TG1 integrase,” *Molecular Genetics and Genomics*, vol. 282, no. 6, pp. 607–616, 2009, doi: 10.1007/s00438-009-0490-2.

[155] P. Ghosh, L. R. Wasil, and G. F. Hatfull, “Control of phage Bxb1 excision by a novel recombination directionality factor,” *PLoS Biol*, vol. 4, no. 6, pp. 0964–0974, 2006, doi: 10.1371/journal.pbio.0040186.

[156] B. Fayed, E. Younger, G. Taylor, and M. C. M. Smith, “A novel Streptomyces spp. integration vector derived from the S. venezuelae phage, SV1,” *BMC Biotechnol*, vol. 14, pp. 1–7, 2014, doi: 10.1186/1472-6750-14-51.

[157] N. S. Bacteriophage, P. C. M. Fogg, J. A. Haley, W. M. Stark, and M. C. M. Smith, “Genome Integration and Excision by a New Streptomyces Bacteriophage, Joe,” vol. 83, no. 5, pp. 1–16, 2017.

[158] J. Farkašovská and A. Godány, “Analysis of the site-specific integration system of the streptomyces aureofaciens phage μ1/6,” *Curr Microbiol*, vol. 64, no. 3, pp. 226–233, 2012, doi: 10.1007/s00284-011-0054-7.

[159] W. Guo *et al.*, “Identification and characterization of a strong constitutive promoter stnYp for activating biosynthetic genes and producing natural products in streptomyces,” *Microb Cell Fact*, vol. 22, no. 1, pp. 1–14, 2023, doi: 10.1186/s12934-023-02136-9.

[160] R. M. Phelan *et al.*, "Development of next generation synthetic biology tools for use in streptomyces venezuelae," *ACS Synth Biol*, vol. 6, no. 1, pp. 159–166, 2017, doi: 10.1021/acssynbio.6b00202.
[161] S. J. Moore *et al.*, "A Streptomyces venezuelae Cell-Free Toolkit for Synthetic Biology," *ACS Synth Biol*, vol. 10, no. 2, pp. 402–411, 2021, doi: 10.1021/acssynbio.0c00581.
[162] J. Otsuka and T. Kunisawa, "Characteristic base sequence patterns of promoter and terminator sites in φX174 and fd phage DNAs," *J Theor Biol*, vol. 97, no. 3, pp. 415–436, 1982, doi: 10.1016/0022-5193(82)90374-5.
[163] N. C. A. De Ruijter, J. Verhees, W. Van Leeuwen, and A. R. Van Der Krol, "Evaluation and comparison of the GUS, LUC and GFP reporter system for gene expression studies in plants," *Plant Biol*, vol. 5, no. 2, pp. 103–115, 2003, doi: 10.1055/s-2003-40722.
[164] J. M. Fernández-Ábalos, H. Fox, C. Pitt, B. Wells, and J. H. Doonan, "Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein localization and mitosis in the filamentous fungus, Aspergillus nidulans," *Mol Microbiol*, vol. 27, no. 1, pp. 121–130, 1998, doi: 10.1046/j.1365-2958.1998.00664.x.
[165] C. D. Sohaskey, H. Im, A. D. Nelson, and A. T. Schauer, "and luciferase: s y n e r g i s t i c tools for visualizing transcription in Streptomyces* (Gene fusion; transposition; sporulation mutants; sapA; Tnlux) Charles D. Sohaskey, Hana Im, Andrea D. Nelson and Alan T. Schauer," vol. 115, pp. 67–71, 1992.
[166] M. Myronovskyi, E. Welle, V. Fedorenko, and A. Luzhetskyy, "β-Glucuronidase as a sensitive and versatile reporter in Actinomycetes," *Appl Environ Microbiol*, vol. 77, no. 15, pp. 5370–5383, 2011, doi: 10.1128/AEM.00434-11.
[167] C. Ingram, M. Brawner, P. Youngman, and J. Westpheling, "xylE functions as an efficient reporter gene in Streptomyces spp.: Use for the study of galP1, a catabolite-controlled promoter," *J Bacteriol*, vol. 171, no. 12, pp. 6617–6624, 1989, doi: 10.1128/jb.171.12.6617-6624.1989.
[168] J. P. Gomez-Escribano and M. J. Bibb, "Engineering Streptomyces coelicolor for heterologous expression of secondary metabolite gene clusters," *Microb Biotechnol*, vol. 4, no. 2, pp. 207–215, 2011, doi: 10.1111/j.1751-7915.2010.00219.x.
[169] M. Myronovskyi and A. Luzhetskyy, "Heterologous production of small molecules in the optimized: Streptomyces hosts," *Nat Prod Rep*, vol. 36, no. 9, pp. 1281–1294, 2019, doi: 10.1039/c9np00023b.
[170] Kieser T, Bibb MJ, Buttner MJ, and Chater KF, *Practical Streptomyces Genetics. 2nd ed.* 2000.
[171] M. Bibb, "The regulation of antibiotic production in Streptomyces coelicolor A3(2)," *Microbiology (N Y)*, vol. 142, no. 6, pp. 1335–1344, 1996, doi: 10.1099/13500872-142-6-1335.
[172] B. A. M. Rudd and D. A. Hopwood, "Genetics of Actinorhodin Biosynthesis by," *J Gen Microbiol*, vol. 3, no. 114, pp. 35–43, 1979.
[173] A. Thanapipatsiri, J. Claesen, J. P. Gomez-Escribano, M. Bibb, and A. Thamchaipenet, "A Streptomyces coelicolor host for the heterologous expression of Type III polyketide synthase genes," *Microb Cell Fact*, vol. 14, no. 1, pp. 1–11, 2015, doi: 10.1186/s12934-015-0335-0.
[174] J. Shima, A. Hesketh, S. Okamoto, S. Kawamoto, and K. Ochi, "Induction of actinorhodin production by rpsL (encoding ribosomal protein S12) mutations that confer streptomycin resistance in Streptomyces lividans and Streptomyces coelicolor A3(2)," *J Bacteriol*, vol. 178, no. 24, pp. 7276–7284, 1996, doi: 10.1128/jb.178.24.7276-7284.1996.
[175] R. Novakova *et al.*, "Increased heterologous production of the antitumoral polyketide mithramycin A by engineered Streptomyces lividans TK24 strains," *Appl Microbiol Biotechnol*, vol. 102, no. 2, pp. 857–869, 2018, doi: 10.1007/s00253-017-8642-5.
[176] K. F. Chater and L. C. Wilde, "Restriction of a bacteriophage of Streptomyces albus G involving endonuclease salI," *J Bacteriol*, vol. 128, no. 2, pp. 644–650, 1976, doi: 10.1128/jb.128.2.644-650.1976.

[177] N. Zaburannyi, M. Rabyk, B. Ostash, V. Fedorenko, and A. Luzhetskyy, "Insights into naturally minimised Streptomyces albus J1074 genome," *BMC Genomics*, vol. 15, no. 1, 2014, doi: 10.1186/1471-2164-15-97.

[178] Z. Feng, L. Wang, S. R. Rajski, Z. Xu, M. F. Coeffet-LeGal, and B. Shen, "Engineered production of iso-migrastatin in heterologous Streptomyces hosts," *Bioorg Med Chem*, vol. 17, no. 6, pp. 2147–2153, 2009, doi: 10.1016/j.bmc.2008.10.074.

[179] O. Bilyk, O. N. Sekurova, S. B. Zotchev, and A. Luzhetskyy, "Cloning and heterologous expression of the grecocycline biosynthetic gene cluster," *PLoS One*, vol. 11, no. 7, pp. 1–17, 2016, doi: 10.1371/journal.pone.0158682.

[180] M. Myronovskyi *et al.*, "Generation of new compounds through unbalanced transcription of landomycin A cluster," *Appl Microbiol Biotechnol*, vol. 100, no. 21, pp. 9175–9186, 2016, doi: 10.1007/s00253-016-7721-3.

[181] B. Bilyk, L. Horbal, and A. Luzhetskyy, "Chromosomal position effect influences the heterologous expression of genes and biosynthetic gene clusters in Streptomyces albus J1074," *Microb Cell Fact*, vol. 16, no. 1, pp. 1–8, 2017, doi: 10.1186/s12934-016-0619-z.

[182] M. Lopatniuk, B. Ostash, A. Luzhetskyy, S. Walker, and V. Fedorenko, "Generation and study of the strains of streptomycetes-heterologous hosts for the production of moenomycin," *Russ J Genet*, vol. 50, no. 4, pp. 360–365, 2014, doi: 10.1134/S1022795414040085.

[183] J. E. Kim, J. S. Choi, and J. H. Roe, "Growth and differentiation properties of pikromycin-producing Streptomyces venezuelae ATCC15439," *Journal of Microbiology*, vol. 57, no. 5, pp. 388–395, 2019, doi: 10.1007/s12275-019-8539-3.

[184] G. P. Van Wezel *et al.*, "Unlocking Streptomyces spp. for use as sustainable industrial production platforms by morphological engineering," *Appl Environ Microbiol*, vol. 72, no. 8, pp. 5283–5288, 2006, doi: 10.1128/AEM.00808-06.

[185] L. P. Thapa *et al.*, "Heterologous expression of the kanamycin biosynthetic gene cluster (pSKC2) in Streptomyces venezuelae YJ003," *Appl Microbiol Biotechnol*, vol. 76, no. 6, pp. 1357–1364, 2007, doi: 10.1007/s00253-007-1096-4.

[186] W. S. Jung *et al.*, "Heterologous expression of tylosin polyketide synthase and production of a hybrid bioactive macrolide in Streptomyces venezuelae," *Appl Microbiol Biotechnol*, vol. 72, no. 4, pp. 763–769, 2006, doi: 10.1007/s00253-006-0318-5.

[187] J. S. J. Hong, S. H. Park, C. Y. Choi, J. K. Sohng, and Y. J. Yoon, "New olivosyl derivatives of methymycin/pikromycin from an engineered strain of Streptomyces venezuelae," *FEMS Microbiol Lett*, vol. 238, no. 2, pp. 391–399, 2004, doi: 10.1016/j.femsle.2004.08.002.

[188] J. W. Park *et al.*, "Discovery of parallel pathways of kanamycin biosynthesis allows antibiotic manipulation," *Nat Chem Biol*, vol. 7, no. 11, pp. 843–852, 2011, doi: 10.1038/nchembio.671.

[189] W. S. Jung *et al.*, "Bioconversion of 12-, 14-, and 16-membered ring aglycones to glycosylated macrolides in an engineered strain of Streptomyces venezuelae," *Appl Microbiol Biotechnol*, vol. 76, no. 6, pp. 1373–1381, 2007, doi: 10.1007/s00253-007-1101-y.

[190] M. Komatsu *et al.*, "Engineered Streptomyces avermitilis host for heterologous expression of biosynthetic gene cluster for secondary metabolites," *ACS Synth Biol*, vol. 2, no. 7, pp. 384–396, 2013, doi: 10.1021/sb3001003.

[191] M. Komatsu, T. Uchiyama, S. Omura, D. E. Cane, and H. Ikeda, "Genome-minimized Streptomyces host for the heterologous expression of secondary metabolism," *Proc Natl Acad Sci U S A*, vol. 107, no. 6, pp. 2646–2651, 2010, doi: 10.1073/pnas.0914833107.

[192] D. Rebatchouk, N. Daraselia, and J. O. Narita, "NOMAD: A versatile strategy for in vitro DNA manipulation applied to promoter analysis and vector design," *Proc Natl Acad Sci U S A*, vol. 93, no. 20, pp. 10891–10896, 1996, doi: 10.1073/pnas.93.20.10891.

[193] L. Campos, "The BioBrick™ road," *Biosocieties*, vol. 7, no. 2, pp. 115–139, 2012, doi: 10.1057/biosoc.2012.6.

[194] T. Knight, "Idempotent Vector Design for Standard Assembly of Biobricks," *MIT Libraries*, pp. 1–11, 2003, [Online]. Available: http://dspace.mit.edu/handle/1721.1/45138

[195] C. D. Smolke, "Building outside of the box: iGEM and the BioBricks Foundation," *Nat Biotechnol*, vol. 27, no. 12, pp. 1099–1102, 2009, doi: 10.1038/nbt1209-1099.
[196] R. Shetty, M. Lizarazo, R. Rettberg, and T. F. Knight, *Assembly of BioBrick standard biological parts using three antibiotic assembly*, 1st ed., vol. 498. Elsevier Inc., 2011. doi: 10.1016/B978-0-12-385120-8.00013-9.
[197] I. Phillips and P. Silver, "A new biobrick assembly strategy designed for facile protein engineering," *DSpace@MIT*, pp. 1–6, 2006, [Online]. Available: http://dspace.mit.edu/handle/1721.1/32535
[198] L. Avilan, *DNA Manipulation and Analysis*, vol. 2633. 2023. [Online]. Available: https://link.springer.com/10.1007/978-1-0716-3004-4
[199] D. G. Gibson, L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison, and H. O. Smith, "Enzymatic assembly of DNA molecules up to several hundred kilobases," *Nat Methods*, vol. 6, no. 5, pp. 343–345, 2009, doi: 10.1038/nmeth.1318.
[200] G. Røkke, E. Korvald, J. Pahr, O. Øyås, and R. Lale, "BioBrick assembly standards and techniques and associated software tools," *Methods in Molecular Biology*, vol. 1116, pp. 1–24, 2014, doi: 10.1007/978-1-62703-764-8_1.
[201] A. P. Sikkema, S. K. Tabatabaei, Y. J. Lee, S. Lund, and G. J. S. Lohman, "High-Complexity One-Pot Golden Gate Assembly," *Curr Protoc*, vol. 3, no. 9, pp. 1–47, 2023, doi: 10.1002/cpz1.882.
[202] J. E. Bird, J. Marles-Wright, and A. Giachino, "A User's Guide to Golden Gate Cloning Methods and Standards," *ACS Synth Biol*, vol. 11, no. 11, pp. 3551–3563, 2022, doi: 10.1021/acssynbio.2c00355.
[203] C. Engler and S. Marillonnet, "Golden Gate cloning," *Methods in Molecular Biology*, vol. 1116, pp. 119–131, 2014, doi: 10.1007/978-1-62703-764-8_9.
[204] E. Weber, C. Engler, R. Gruetzner, S. Werner, and S. Marillonnet, "A modular cloning system for standardized assembly of multigene constructs," *PLoS One*, vol. 6, no. 2, 2011, doi: 10.1371/journal.pone.0016765.
[205] P. F. Popp, M. Dotzler, J. Radeck, J. Bartels, and T. Mascher, "The Bacillus BioBrick Box 2.0: Expanding the genetic toolbox for the standardized work with Bacillus subtilis," *Sci Rep*, vol. 7, no. 1, pp. 1–13, 2017, doi: 10.1038/s41598-017-15107-z.
[206] S. Zucca, L. Pasotti, N. Politi, M. G. Cusella De Angelis, and P. Magni, "A standard vector for the chromosomal integration and characterization of BioBrick™ parts in Escherichia coli," *J Biol Eng*, vol. 7, no. 1, pp. 1–13, 2013, doi: 10.1186/1754-1611-7-12.
[207] J. Ellinger and C. Schmidt-Dannert, "Construction of a BioBrick™ compatible vector system for Rhodococcus," *Plasmid*, vol. 90, pp. 1–4, 2017, doi: 10.1016/j.plasmid.2017.01.004.
[208] S. Santala and V. Santala, "Acinetobacter baylyi ADP1-naturally competent for synthetic biology," *Essays Biochem*, vol. 65, no. 2, pp. 309–318, 2021, doi: 10.1042/EBC20200136.
[209] T. L. Oliveira *et al.*, "A standardized BioBrick toolbox for the assembly of sequences in mycobacteria," *Tuberculosis*, vol. 119, no. April, p. 101851, 2019, doi: 10.1016/j.tube.2019.07.002.
[210] A. G. Venkatesh, A. Sun, H. Brickner, D. Looney, D. A. Hall, and E. Aronoff-Spencer, "Yeast dual-affinity biobricks: Progress towards renewable whole-cell biosensors," *Biosens Bioelectron*, vol. 70, pp. 462–468, 2015, doi: 10.1016/j.bios.2015.03.044.
[211] P. M. Boyle *et al.*, "A BioBrick compatible strategy for genetic modification of plants," *J Biol Eng*, vol. 6, 2012, doi: 10.1186/1754-1611-6-8.
[212] L. Li, W. Jiang, and Y. Lu, "A modified gibson assembly method for cloning large DNA fragments with high GC contents," *Methods in Molecular Biology*, vol. 1671, pp. 203–209, 2018, doi: 10.1007/978-1-4939-7295-1_13.
[213] O. Rudenko and A. C. Barnes, "Gibson Assembly facilitates bacterial allelic exchange mutagenesis," *J Microbiol Methods*, vol. 144, no. October 2017, pp. 157–163, 2018, doi: 10.1016/j.mimet.2017.11.023.

[214] M. M. M. Ferro *et al.*, "New approach for the construction of infectious clones of a circular DNA plant virus using Gibson Assembly," *J Virol Methods*, vol. 263, no. October 2018, pp. 20–23, 2019, doi: 10.1016/j.jviromet.2018.10.017.
[215] V. Wetzel, V. Brault, and M. Varrelmann, "Production of a beet chlorosis virus full-length cDNA clone by means of Gibson assembly and analysis of biological properties," *Journal of General Virology*, vol. 99, no. 11, pp. 1522–1527, 2018, doi: 10.1099/jgv.0.001146.
[216] Y. Zhou, A. C. Murphy, M. Samborskyy, P. Prediger, L. C. Dias, and P. F. Leadlay, "Iterative Mechanism of Macrodiolide Formation in the Anticancer Compound Conglobatin," *Chem Biol*, vol. 22, no. 6, pp. 745–754, 2015, doi: 10.1016/j.chembiol.2015.05.010.
[217] R. E. Cobb, Y. Wang, and H. Zhao, "High-Efficiency Multiplex Genome Editing of Streptomyces Species Using an Engineered CRISPR/Cas System," *ACS Synth Biol*, vol. 4, no. 6, pp. 723–728, 2015, doi: 10.1021/sb500351f.
[218] V. R. Chaudhari and M. R. Hanson, "GoldBricks: An improved cloning strategy that combines features of Golden Gate and BioBricks for better efficiency and usability," *Synth Biol*, vol. 6, no. 1, pp. 1–9, 2021, doi: 10.1093/synbio/ysab032.
[219] S. Kirchmaier, K. Lust, and J. Wittbrodt, "Golden GATEway cloning--a combinatorial approach to generate fusion and recombination constructs.," *PLoS One*, vol. 8, no. 10, pp. 1–9, 2013, doi: 10.1371/journal.pone.0076117.
[220] M. R. Green and J. Sambrook, *Molecular Cloning: A Laboratory Manual*, 4th ed., vol. 4, no. 1. Cold Spring Harbor Laboratory Press, 2012. doi: 10.3724/sp.j.1141.2012.01075.
[221] Hopwood D.A., T. Kieser, M. Bibb, M. Buttner, and K. Chater, *Practical Streptomyces Genetics*. John Innes Foundation, 2000.
[222] V. A. Alferova *et al.*, "Characterization of a novel natural tetracenomycin reveals crucial role of 4-hydroxy group in ribosome binding," *Biochimie*, vol. 206, pp. 150–153, 2023, doi: 10.1016/j.biochi.2022.10.016.
[223] S. A. Borisova, L. Zhao, D. H. Sherman, and H. W. Liu, "Biosynthesis of desosamine: Construction of a new macrolide carrying a genetically designed sugar moiety," *Org Lett*, vol. 1, no. 1, pp. 133–136, 1999, doi: 10.1021/ol9906007.
[224] C. Fischer, F. Lipata, and J. Rohr, "The Complete Gene Cluster of the Antitumor Agent Gilvocarcin V and Its Implication for the Biosynthesis of the Gilvocarcins," *Physiol Behav*, vol. 176, no. 1, pp. 100–106, 2017, doi: 10.1177/0022146515594631.Marriage.
[225] Y. Yan *et al.*, "Biosynthetic access to the rare antiarose sugar: Via an unusual reductase-epimerase," *Chem Sci*, vol. 11, no. 15, pp. 3959–3964, 2020, doi: 10.1039/c9sc05766h.
[226] B. E. Leach, K. M. Calhoun, L. E. Johnson, C. M. Teeters, and W. G. Jackson, "Chartreusin, a New Antibiotic Produced by Streptomyces chartreusis, a New Species," *J Am Chem Soc*, vol. 75, no. 16, pp. 4011–4012, 1953, doi: 10.1021/ja01112a040.
[227] J. Portugal, "Chartreusin, elsamicin A and related anti-cancer antibiotics," *Curr Med Chem Anticancer Agents*, vol. 3, no. 6, pp. 411–420, 2003, doi: 10.2174/1568011033482215.
[228] H. Zhao *et al.*, "New peptidendrocins and anticancer chartreusin from an endophytic bacterium of Dendrobium officinale," *Ann Transl Med*, vol. 8, no. 7, pp. 455–455, 2020, doi: 10.21037/atm.2020.03.227.
[229] X. Salas and J. Portugal, "Map of chartreusin and elsamicin binding sites on DNA," *FEBS Lett*, vol. 292, no. 1–2, pp. 223–228, 1991, doi: 10.1016/0014-5793(91)80872-Z.
[230] G. Asai *et al.*, "Pharmacokinetic and pharmacodynamic study of IST-622, a novel synthetic derivative of chartreusin, by oral administration in a phase II study of patients with breast cancer," *Cancer Chemother Pharmacol*, vol. 49, no. 6, pp. 468–472, 2002, doi: 10.1007/s00280-002-0455-1.
[231] Z. Xu, K. Jakobi, K. Welzel, and C. Hertweck, "Biosynthesis of the antitumor agent chartreusin involves the oxidative rearrangement of an anthracyclic polyketide," *Chem Biol*, vol. 12, no. 5, pp. 579–588, 2005, doi: 10.1016/j.chembiol.2005.04.017.

[232] F. W. Jiao *et al.*, "An NADPH-Dependent Ketoreductase Catalyses the Tetracyclic to Pentacyclic Skeletal Rearrangement in Chartreusin Biosynthesis," *Angewandte Chemie - International Edition*, vol. 60, no. 50, pp. 26378–26384, 2021, doi: 10.1002/anie.202112047.
[233] K. Weissman, "A new origin for chartreusin," *Chem Biol*, vol. 12, no. 5, pp. 512–514, 2005, doi: 10.1016/j.chembiol.2005.05.004.
[234] R. C. Edgar, "MUSCLE: Multiple sequence alignment with high accuracy and high throughput," *Nucleic Acids Res*, vol. 32, no. 5, pp. 1792–1797, 2004, doi: 10.1093/nar/gkh340.
[235] M. Gouy, E. Tannier, N. Comte, and D. Parsons, "Seaview Version 5: A Multiplatform Software for Multiple Sequence Alignment, Molecular Phylogenetic Analyses, and Tree Reconciliation," *Methods Mol Biol*, vol. 2231, pp. 241–260, 2021, doi: 10.1007/978-1-0716-1036-7_15.
[236] B. N. Wandi, P. Dinis, V. Siitonen, G. Schneider, R. Schnell, and M. Metsä-Ketelä, "Mechanism of Two-Component Mono-Oxygenases Involved in Anthracycline 1-Hydroxylation," *ACS Catal*, vol. 14, no. 16, pp. 12359–12371, 2024, doi: 10.1021/acscatal.4c02623.

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