A BioBricks® toolbox for metabolic engineering of the tetracenomycin pathway

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ABSTRACT

Background/Goal/Aim: The tetracenomycins are aromatic anticancer polyketides that inhibit peptide translation via binding to the large ribosomal subunit. Here, we expressed the elloramycin biosynthetic gene cluster in the heterologous host *Streptomyces coelicolor* M1146 to facilitate the downstream production of tetracenomycin analogs.

Main Methods and Major Results: We developed a BioBricks® genetic toolbox of genetic parts for substrate precursor engineering in *S. coelicolor* M1146::cos16F4iE. We cloned a series of integrating vectors based on the VWB, TG1, and SV1 integrase systems to interrogate gene expression in the chromosome. We genetically engineered three separate genetic constructs to modulate tetracenomycin biosynthesis: 1) the *vhb* hemoglobin from obligate aerobe *Vitreoscilla stercoraria* to improve oxygen utilization; (2) the *accA2BE* acetyl-CoA carboxylase to enhance condensation of malonyl-CoA; (3) lastly, the *sco6196*

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acyltransferase, which is a “metabolic regulatory switch” responsible for mobilizing triacylglycerols to β-oxidation machinery for acetyl-CoA. In addition, we engineered the *tcmO* 8-*O*-methyltransferase and newly identified *tcmD* 12-*O*-methyltransferase from *Amycolatopsis sp.* A23 to generate tetracenomycins C and X. We also co-expressed the *tcmO* methyltransferase with oxygenase *urdE* to generate the analog 6-hydroxy-tetracenomycin C.

Conclusions and Implications: Altogether, this system is compatible with the BioBricks® [RFC 10] cloning standard for the co-expression of multiple gene sets for metabolic engineering of *Streptomyces coelicolor* M1146::cos16F4iE. This production platform improves access to potent analogs, such as tetracenomycin X, and sets the stage for the production of new tetracenomycins via combinatorial biosynthesis.

KEYWORDS

Tetracenomycins, polyketides, *Streptomyces*, metabolic engineering, BioBricks

Introduction

The tetracenomycins are a family of aromatic polyketides produced by *Streptomyces glaucescens* GLA.0 and *Streptomyces olivaceus* Tü2353, respectively [1,2]. 8-demethyltetracenomycin C (8-DMTC, **1**), tetracenomycin C (**2**), tetracenomycin X (**3**), 6-hydroxy-tetracenomycin C (**4**), and elloramycin (**5**) are structurally representative compounds from this family (Figure 1). **1** - **5** exhibit antibacterial activity against gram-positive microorganisms and anticancer activity against a variety of mammalian cancer cell lines, though **2** and **3** are the most potent compounds described to date [3,4]. [3]Recently, Osterman and coworkers demonstrated that **3** inhibits peptide translation via binding to the

large ribosomal subunit polypeptide exit channel [5]. Impressively, Osterman et al. demonstrated that **3** binds to the same binding site within the exit channel of the *E. coli* 50S ribosomal subunit and the *H. sapiens* 60S ribosomal subunit, a stunning display of evolutionarily conserved molecular recognition that accounts for the cytotoxic activities of the TCMs [5]. This sets the stage for the development of tetracenomycin analogs with improved potency and anticancer activity.

The gene cluster for *Streptomyces glaucescens* GLA.0 was previously sequenced, revealing the full biosynthetic gene cluster for **1**, spectinomycin, and acarbose [6]. The biosynthesis of **1** has been studied extensively for the past three decades and has served as a model system for understanding type II polyketide biosynthesis (Figure 2) [7–11]. Decker et al. previously isolated the gene cluster for elloramycin biosynthesis and cloned it onto cosmid cos16F4 [12]. Heterologous expression of cosmid cos16F4 resulted in heterologous production of **1** and production of penultimate intermediate tetracenomycin A2 (Figure 2). Cosmid cos16F4 was also discovered to encode the *elmGT* gene, which encodes the glycosyltransferase responsible for the transfer of TDP-L-rhamnose to the 8-position of **1** [13]. ElmGT has been shown to exhibit donor substrate flexibility towards >20 TDP-deoxysugar donors [14–20]. Therefore, ElmGT and the cos16F4 heterologous expression system are significant tools for the generation of a library of tetracenomycin analogs. These tetracenomycin analogs will be instrumental in investigating the anticancer mechanism of action activity for this class of compounds and the role of the carbohydrate moiety in binding to the large mammalian ribosomal subunit.

The heterologous expression of extrachromosomal sequences in *Streptomyces spp*. is subject to genetic instability [21]. Alternatively, cloning of genes via the well-characterized actinophage integrases (e.g. ϕC31, ϕBT1, SV1, TG1, SAM2, VWB) into *attB* sites in the *Streptomyces* spp. chromosome can result in the stable incorporation of heterologously expressed genes [22]. Therefore, we incorporated the elloramycin biosynthetic gene cluster encoded on the C31-integrating cassette cos16F4iE into the genome of the superhost

Streptomyces coelicolor M1146. *Streptomyces coelicolor* M1146 has been genome minimalized for the removal of the actinorhodin, undecylprodigiosin, coelimycin P1, and the calcium-dependent antibiotic gene clusters [23]. Therefore, *S. coelicolor* M1146 exhibits fungible metabolism that can be channeled towards the synthesis of natural products through the heterologous expression of biosynthetic gene clusters (BGCs).

In this work, we developed a BioBricks® [RFC 10] biosynthetic toolbox for the engineering of central carbon metabolism in elloramycin biosynthesis. First, we engineered the cos16F4iE cluster into *Streptomyces coelicolor* M1146 to generate an improved production host for tetracenomycins, as compared to the original *Streptomyces lividans* TK24 (cos16F4) host. In addition, we generated integrating plasmid cassettes based on plasmids pENSV1, pENTG1, and pOSV808 vectors to site-specifically introduce genes into the SV1, TG1, and VWB actinophage *attB* sites, respectively [24–27]. Using these different integrating vectors, we engineered three different gene cassettes into different genomic loci to determine the optimal arrangement for enhancement of tetracenomycin production and biomass accumulation. First, we engineered the *vhb* hemoglobin gene from *Vitreoscilla stercoraria* to enhance aerobic respiration of *S. coelicolor* M1146::cos16F4iE in shake flasks [28,29]. Secondly, we engineered the acetyl-CoA carboxylase *accA2BE* operon from *S. coelicolor* M145 under the control of the constitutive *ermE*p* promoter to enhance the production of malonyl-CoA [30]. Thirdly, we engineered the *sco6196* acyltransferase, previously identified as a “metabolic switch” during the transition from triacylglycerol synthesis to polyketide biosynthesis in the stationary phase [31]. Overexpression of the *accA2BE* operon and *sco6196* metabolic switches resulted in a 3-fold improvement in **1** production titer, as compared to the original production host. Finally, we utilized the improved production host expressing the *accA2BE* operon to engineer in the *tcmO* 8-*O*-methyltransferase and the *tcmD* 12-*O*-methyltransferase from *Amycolatopsis sp. A23* to biosynthesize tetracenomycin C and tetracenomycin X. To the best of our knowledge, this is the first report to describe the functional characterization of *tcmD* in the biosynthesis of

tetracenomycin X.

Materials and Methods

Bacterial strains and growth conditions

E. coli JM109 and *E. coli* ET12567 were grown at 37°C in LB broth or LB agar as previously described [32]. *E. coli* JM109 was used for plasmid propagation and subcloning, while *E. coli* ET12567/pUZ8002 was used as the conjugation donor host for mobilizing expression vectors into *Streptomyces coelicolor* M1146 as previously described [33]. When appropriate, ampicillin (100 μg mL^{-1}), kanamycin (25 μg mL^{-1}), apramycin (25 μg mL^{-1}), viomycin (30 μg mL^{-1}), hygromycin (50 μg mL^{-1}), and nalidixic acid (35 μg mL^{-1}) were supplemented to media to select for recombinant microorganisms.

Streptomyces coelicolor M1146 and derivative strains were routinely maintained on Soya-Mannitol Flour (SFM) agar supplemented with 10 mM $MgCl_2$ and International Streptomyces Project medium #4 (ISP4) (BD Difco) at 30°C as described previously [34]. For liquid culturing, *Streptomyces coelicolor* M1146::cos16F4iE derivative strains were grown in TSB media for the production of seed culture and modified SG-TES liquid medium (soytone 10 g, glucose 20 g, yeast extract 5 g, TES free acid 5.73 g, $CoCl_2$ 1 mg, per liter) [17]. All media and reagents were purchased from Thermo-Fisher Scientific.

General genetic manipulations

Routine genetic cloning and plasmid manipulation were carried out in *E. coli* JM109 (New England Biolabs). *E. coli* ET12567/pUZ8002 was used as the host for intergeneric conjugation with *Streptomyces coelicolor* as previously described [34]. *E. coli* chemically competent cells were prepared using the Mix and Go! *E. coli* Transformation Kit® (Zymo Research). *E. coli* was transformed with plasmid DNA via chemically competent heat-shock

transformation as described previously [32]. Plasmid DNA was isolated via the Wizard® *Plus* SV Minipreps DNA Purification System by following the manufacturer's protocols (Promega). All molecular biology reagents and enzymes used for plasmid construction were purchased from New England Biolabs. The conjugation donor host *E. coli* ET12567/pUZ8002 was transformed with constructs for mobilization into *Streptomyces coelicolor* M1146::cos16F4iE, as previously described [8]. For each transformation, 9 to 12 independent exconjugants were plated to DNA plates supplemented with antibiotics and grown for 4 to 5 days until the formation of vegetative mycelium.

Ethics Clearance

This study did not require the use of animal or human subjects.

Results

Heterologous expression of cos16F4iE results in production of 1

Initially, we sought to generate an improved host for the production of **1** and **5** analogs for downstream cytotoxicity studies. The initial host *Streptomyces lividans* TK24 (cos16F4) was based on a cosmid expression system that resulted in the production of **1** and tetracenomycin B3 at a yield of approximately 15 – 20 mg/L [12]. *Streptomyces lividans* TK24 (cos16F4) has been used to generate novel glycosylated tetracenomycins via co-expression of "deoxysugar plasmids" that could direct the biosynthesis of TDP-deoxysugars for glycosylation onto the 8-DMTC aglycone via ElmGT [15]. In our hands, *S. lividans* TK24 (cos16F4) expression host demonstrates segregation of cos16F4 or "deoxysugar plasmids" during scaled-up fermentations [17].

We obtained the integrating vector cos16F4iE for introduction in the improved heterologous expression host *S. coelicolor* M1146. The vector cos16F4iE features the ϕC31 integrase and *attP* attachment site for recombination into the *attB* site of the *S. coelicolor* chromosome [39]. The structure of the cos16F4iE construct was reported previously [40]. Integration of this cassette could ensure stable expression of the core 8-DMTC biosynthetic genes and could avoid the instability issues observed previously with *S. lividans* TK24 (cos16F4). The *Streptomyces coelicolor* M1146 expression host has several advantages over *S. lividans* TK24, including deletion of four major biosynthetic gene clusters, resulting in a host with fungible metabolism for heterologous expression of type II polyketide synthase gene clusters [23]. Introduction of cos16F4iE into *S. coelicolor* M1146 via intergeneric conjugation resulted in several apramycin-resistant exconjugants that produced an orange-red pigmented color when plated on SFM agar. 12 independent clones were grown up for 5 days and extracted. The methanolic extracts for all twelve strains indicated significant production of **1** and tetracenomycin B3, as compared to an authentic standard of **1** (t_R = 8.76 min) [12] (See Supplementary Information). The yield of **1** from the clones ranged from 100 – 160 mg/L, which is a 5 to 8-fold improvement over the original production host. One high-producing clone was carried forward for further experiments.

Development of orthogonal BioBricks® vectors for integration in the Streptomyces coelicolor chromosome

Next, we set out to develop a set of orthogonal BioBrick® [RFC-10] vectors for integration of gene cassettes into the chromosome of *Streptomyces coelicolor* M1146::cos16F4iE. We designed new BioBricks® vectors based on the SV1 and TG1 actinophage integrases that could be used for the expression of gene circuits from orthogonal promoters (Supplementary Figure 1). The SV1 and TG1 actinophage integrases were chemically synthesized (Genscript). pENSV1 incorporates the SV1 actinophage

integrase, the *attP* site, *oriT* for mobilization from *E. coli* ET12567/pUZ8002 via conjugation, and the *aadA* spectinomycin resistance gene for site-specific recombination into the *sco4383* in the middle of the chromosome [27]. Simultaneously, pENTG1 incorporates the TG1 actinophage integrase, *oriT, attP* site, and the *vph* viomycin resistance gene for single-copy chromosomal engineering (Supplementary Figure 1). The TG1 integrase recombines into the *sco3658* aminotransferase gene in the middle of the chromosome [25]. In addition, we obtained the BioBrick-compatible vector pOSV808, which includes the VWB actinophage integrase, *attP* site, *oriT*, and the *amilCP* gene for screening of recombinant clones [37].

pENSV1, pENTG1, and pOSV808 were successfully transformed into *S. coelicolor* M1146 and *S. coelicolor* M1146::cos16F4iE via intergeneric conjugation. This demonstrated that these vectors could potentially be useful for shuttling gene cassettes into *S. coelicolor* for pathway engineering. We next used these vectors to clone in different operons for substrate precursor engineering of **1**.

Engineering precursor metabolite pools increases production titer of 1

Next, we decided to use the pENSV1, pENTG1, and pOSV808 expression vectors to engineer precursor substrate pools within *S. coelicolor* M1146::cos16F4iE to produce higher levels of **1**. Substrate precursor engineering has been used to increase the production of a variety of aromatic polyketides, including mithramycin, tetracenomycin C, actinorhodin, nogalamycin, and steffimycin [41,42]. The proposed biosynthesis of **1** requires condensation of 1 molecule of acetyl-CoA and 9 molecules of malonyl-CoA via the ElmKLM minimal PKS (Figure 2). Cyclases ElmNI and ElmJ generate the tricyclic tetracenomycin F2, which is cyclized by ElmI to form tetracenomycin F1, oxidatively modified by ElmH to form tetracenomycin D3, and undergoes consecutive *O*-methylations at the 3-*O*-position by ElmNII and at the 9-*O*-position by ElmP [40]. ElmG carries out a triple hydroxylation of the penultimate intermediate to form **1** [10]**.** We engineered three different gene cassettes to

enhance substrate precursor pools for **1.** First, we engineered the *Streptomyces coelicolor* M145 acetyl-CoA carboxylase complex (i.e. *accA2BE*) under the control of the constitutive *ermE*p* promoter to enhance condensation of acetyl-CoA to malonyl-CoA (Figure 2). This strategy has been successfully used to enhance the production of actinorhodin by 6-fold [30]. Second, we engineered the acyltransferase *sco6196* under the control of the constitutive *ermE*p* promoter to increase carbon flux from triacylglycerols to beta-oxidation, which increases acetyl-CoA precursor [31]supply to polyketide biosynthesis when it is most active [31]. *Sco6196* is a highly active acyltransferase that plays a major role as a "metabolic switch" during stationary phase, which mobilizes triacylglycerols to the beta-oxidation machinery to produce acetyl-CoA, which is then diverted towards polyketide biosynthesis [31]. Lastly, we decided to engineer the *vhb* hemoglobin gene from the obligate aerobe *Vitreoscilla stercoraria* under the control of its oxygen-sensitive promoter. Expression of *vhb* in *S. coelicolor* M1146::cos16F4iE is expected to enhance biomass formation and availability of oxygen for the electron transport chain.

We also hypothesized that the expression of different gene cassettes from unique loci in the *S. coelicolor* chromosome might lead to the identification of "chromosomal position effects", due to some regions of the chromosome being transcribed more frequently than other regions, which could lead to improved product formation. This strategy was exploited by Bilyk et al. to array production of aranciamycin over an 8-fold range dependent on the *attB* site of recombination [43]. We cloned *vhb*, *ermE*p-accA2BE*, and *ermE*p-sco6196* onto pENSV1, pENTG1, and pOSV808 to splice the gene constructs into the SV1, TG1, and VWB *attB* sites of *S. coelicolor* (See Supplementary Information). We observed that the engineering the integrating cassettes into the different *attB* sites resulted in decreasing rank order of **1** production titer as follows: SV1 > VWB > TG1. The recombinant strains were grown in SG liquid media in shake flasks for 5 days. After five days, biomass measurements were conducted, and the cultures were extracted to determine **1** production titers via HPLC-MS analysis (Figure 3). Each experiment used 4 – 6 biological replicates, which were

compared to a standard curve of authentic **1** (Figure 4). The recombinant strains harboring pENSV1-*vhb*, pENSV1-*ermE*p-accA2BE*, and pENSV1-*ermE*p-sco6196* exhibited the highest increases in **1** product titer (Figure 3). The cos16F4iE line produced a mean of 166 mg/L **1**, whereas the cos16F4iE::pENSV1-*vhb* line exhibited 32% increased production of **1** (e.g. 220.3 ± 15.3 mg/L, p = 0.0168), the cos16F4iE::pENSV1-*sco6196* line exhibited 2.2-fold increased production of **1** (e.g. 366.6 ± 67.8 mg/L, p=0.0465), and cos16F4iE::pENSV1-*accA2BE* line exhibited the greatest increase in production titer of **1** of 2.4-fold (e.g. 403 ± 83.6 mg/L, p=0.0304) (Figure 3, Supplementary Table 2). HPLC-MS analysis of the different lines revealed an increase in production of **1**, and significant production of the penultimate intermediate tetracenomycin B3 (Figure 4). In addition, the transformation of pOSV808-based constructs resulted in statistically significant increases in titer of **1.** The cos16F4iE::pOSV808-*vhb* strain produced 224.5 ± 18.68 mg/L of **1** (p = 0.0415), cos16F4iE::pOSV808-*sco6196* produced 249.2 ± 11.88 mg/L of **1** (p = 0.0007), and cos16F4iE::pOSV808-*accA2BE* produced 327.3 ± 40.2 mg/L of 1 (p = 0.0131) (Supplementary Table 2). Surprisingly, engineering of pENTG1-based constructs resulted in statistically significant decreases in **1** production for all combinations attempted, which requires further investigation. [44]In addition, no statistically significant differences were detected in biomass between the control line and the experimental lines, except for a decrease in biomass for the cos16F4iE::pENTG1-*vhb* line (p = 0.0039). This result demonstrates that the engineering of specific genes into different *attB* sites may have unanticipated effects on the growth of the strain, therefore, interrogation of several different *attB* sites might be required to identify the optimal chromosomal locus for expression of a given gene cassette.

Combinatorial biosynthesis of tetracenomycin pathway enzymes facilitates production of biosynthetic analogs

We decided to employ our improved production strains for the generation of tetracenomycin analogs **2 - 4** [3,7,45]. The heterologous production of **2** and **3** which feature multiple *O*-methylations, and **4**, which features an additional hydroxyl group, could be useful for downstream structure-activity relationships studies (Figure 1). The biosynthesis for **2** diverges from **1** at tetracenomycin B3: TcmO catalyzes *O*-methylation at the 8-position to form tetracenomycin E, which is *O*-methylated at the 9-position by ElmP (i.e., TcmP homolog from *S. olivaceus* Tü 2353) to afford tetracenomycin A2, which is hydroxylated at 4, 4a, and 12a positions by ElmG (i.e., TcmG homolog from *S. olivaceus* Tü 2353) (Figure 2). **1** biosynthesis does not undergo 8-*O*-methylation (since the 8-position is glycosylated by ElmGT), therefore, tetracenomycin B3 is *O*-methylated at 9-position by ElmP then hydroxylated by ElmG to afford **1.** In summation, **1** is a shunt product with respect to the 8-*O*-methyltransferase TcmO, and the capacity of the elloramycin pathway enzymes to modify late-stage noncanonical tetracenomycin substrates is unknown. In specific, the capability for ElmP to *O*-methylate tetracenomycin E or ElmG to hydroxylate tetracenomycin A2 to **2** is uncertain.

To test this hypothesis, we synthesized a codon-optimized version of the *tcmO* gene from the *S. glaucescens* GLA.0 pathway and expressed it under the control of the *sf14p* promoter in a pENSV1 vector in *S. coelicolor* M1146::cos16F4iE and *S. coelicolor* M1146::cos16F4iE::pOSV808-*accA2BE*. Both strains accumulated minor quantities of **2** as determined via comparison to an authentic standard of **2** (t_R = 9.39 min) (Figure 5). We also sought to characterize the recently sequenced *tcmO* homolog from the **3** producer *Amycolatopsis spp.* A23. We synthesized the codon-optimized version of the *tcmO* homolog from *Amycolatopsis spp.* A23 and similarly expressed it under the control of the *sf14p* promoter in a pENSV1 vector in *S. coelicolor* M1146::cos16F4iE and *S. coelicolor* M1146::cos16F4iE ::pOSV808-*accA2BE*. Again, both strains accumulated minor quantities of **2** as expected. This result shows that the *tcmO* homolog from *Amycolatopsis spp*. A23 encodes a tetracenomycin B3 8-*O*-methyltransferase. In addition, this result demonstrates

that ElmP is capable of *O*-methylating tetracenomycin E and ElmG is flexible enough to convert tetracenomycin A2 to **2.** It was difficult to determine the production titer for these metabolites since the amount of **2** produced by each strain was <1% of the total amount of TCMs detected in the HPLC chromatogram. We were able to quantify relative production based on filtering the data in single-ion monitoring (SIM) in ESI negative ionization mode by searching for the [M-H] = 471 *m/z* ion.

Secondly, we decided to build on this previous result by incorporating the *urdE* oxygenase from the urdamycin pathway to hydroxylate **2** to **4** (Figure 2). UrdE was previously shown to accept **2** as an alternative substrate to its preferred angucyclinone substrate and can carry out hydroxylation at the 6-position of **2** [45]. We synthesized a codon-optimized version of *urdE* and cloned it under the *p* promoter into our pENSV1-*sf14p-tcmO* vector and expressed it in *S. coelicolor* M1146::cos16F4iE. Analysis of methanolic extracts from this strain resulted in the detection of **4** in SIM ESI positive ion mode using the [M-H] = 489 *m/z* ion as compared to an authentic standard of **4** (t_R = 9.64 min) (Figure 5)**.** The yield of this compound was very low, <1% of total TCMs, most likely owing to the relatively high level of metabolic flux towards **1** production. All attempts to transform the pENSV1-*sf14p-tcmO-sf14p-urdE* construct into *S. coelicolor* M1146::cos16F4iE::pOSV808 resulted in transformants that failed to grow on agar plates. One possible explanation for this observation could be that the ElmE elloramycin permease does not actively transport **4** outside of the cell, which could lead to toxicity due to the intracellular accumulation of **4.**

Thirdly, we decided to investigate the biosynthesis of **3**, which is previously uncharacterized. We hypothesized that an *S*-adenosyl-L-methionine-dependent 12-*O*-methyltransferase (i.e. SAM-dependent *O*-MT) methylates **2** to **3** (Figure 2). Further investigation in SIM ESI negative ion mode of the extracts from *S. coelicolor* M1146::cos16F4iE::pENSV1-*tcmO* revealed the presence of another methylated tetracenomycin with a mass of 486 *amu* and a later elution profile than **2** (t_R = 10.15 min) (Figure 5). We identified the peak as **3** as compared to an authentic standard. This result

demonstrates that the elloramycin 12-*O*-methyltransferase, ElmD, is capable of methylating **2** to form **3.** Knowing that the **3** gene cluster from *Amycolatopsis* spp. A23 would likely include an ElmD paralogue, we conducted a translated nucleotide basic local alignment search tool (BLASTX®) search of the *Amycolatopsis* spp. A23 genome with the ElmD nucleotide sequence as a search query. The search resulted in the identification of a 296 amino acid SAM-dependent *O*-MT (Accession Number WP_155542896.1) with significant sequence homology (e.g. 54% identical/67% similar) to ElmD. We decided to call this enzyme TcmD, and we proceeded to synthesize a codon-optimized version of *tcmD* for co-expression in our pENSV1-*sf14p-tcmO* construct. We cloned *tcmD* under a copy of an additional *sf14p* promoter and spliced it at the 3'-end of *tcmO.* We expressed the resulting pENSV1-*sf14p-tcmO-sf14p-tcmD* construct in both *S. coelicolor* M1146::cos16F4iE and *S. coelicolor* M1146::cos16F4iE::pOSV808-*accA2BE* (Figure 5). While quantification of the production titer of **3** was not possible due to the low level of production, <1% of all TCMs, we were able to determine relative production amounts of **2** and **3** via intensity counts from the mass spectrometer. Expression of *tcmO* itself lead to approximately equimolar production of **2** and **3** in extracts from *S. coelicolor* M1146::cos16F4iE ::pOSV808-*accA2BE*::pENSV1-*sf14p-tcm*O. Co-expression of *tcmD* in *S. coelicolor* M1146::cos16F4iE:: pENSV1-*sf14p-tcmO+tcmD* and *S. coelicolor* M1146::cos16F4iE::pOSV808-accA2BE-pENSV1-*sf14p-tcmO+tcmD* resulted in a ten-fold increase in **3** production titer, as well as a significant increase in **2** production as compared to the strains only expressing *tcmO*. The highest yields resulted from co-expression of *tcmO* and *tcmD* in the line harboring the acetyl-CoA carboxylase complex, which provided a modest benefit in production of **2** and **3** compared to *S. coelicolor*::M1146iE. In summation, to the best of our knowledge, this is the first report in which *tcmD* has been characterized via heterologous expression as the 12-*O*-MT responsible for the biosynthesis of **3.**

Discussion

In this report, we developed a series of orthogonal integrating vectors based on the SV1, TG1, and VWB actinophage integrases and used these vectors to engineer in *vhb* hemoglobin, *accA2BE* acetyl-CoA carboxylase, and *sco6196* acyltransferase gene cassettes. This multiplexed metabolic engineering strategy resulted in improved production strains of *S. coelicolor* M1146::cos16F4iE, especially those lines expressing *accA2BE* or *sco6196*, which resulted in the highest production titer of 486 mg/L. Previously, Li et al. engineered the **2** biosynthetic pathway in a knockout mutant of the industrial monensin producer, *Streptomyces cinnomonaeus* [46]. The highest reported production of **3** in this strain was 440 mg/L, which indicates that our production methodology compares favorably with this industrial host. Furthermore, industrial hosts often result from iterative cycles of random mutagenesis and screening for mutants with desired production characteristics. Our methodology provides a rational approach for improving type II polyketide production titers based on several complementary approaches, including overexpression of the acetyl-CoA carboxylase complex to enhance malonyl-CoA concentration, overexpression of *sco6196* to enhance acetyl-CoA levels, and overexpression of the *vhb* hemoglobin to enhance oxygen concentrations in submerged liquid fermentation.

We used this enhanced production platform as a showcase for combinatorial biosynthesis of tetracenomycin analogs **2 – 4**. These analogs are thought to be more valuable anticancer compounds than **1**, owing to the *O*-methyl groups at 8- and 12-positions, which enhance binding to the large ribosomal polypeptide exit channel [3,5]. [3,5]Engineering of *tcmO* orthologs from two different actinomycetes, *S. glaucescens* GLA.0 and *Amycolatopsis* sp. A23 resulted in the production of **2** and **3.** The heterologous expression of *urdE* also resulted in the production of **4**, as previously described [45]. Most importantly, the heterologous expression of the newly characterized *tcmD* gene resulted in a ten-fold increase in production of **3**, which provides good evidence for its role as a tetracenomycin C 12-*O*-methyltransferase. The utility of this production method is diminished, however, by the significant metabolic flux away from **2 – 4** to production of **1** at the tetracenomycin B3 step.

Tetracenomycin B3 likely represents a branch point for the glycosylated elloramycins and methylated tetracenomycins. Future studies should focus on engineering combinations of *tcmD* and *urdE* in the *Streptomyces glaucescens* GLA.0 tetracenomycin C wildtype producer. In this strain, we expect that higher production titers of **3** and **4** could be realized, in addition to the potential for producing new tetracenomycin analogs.

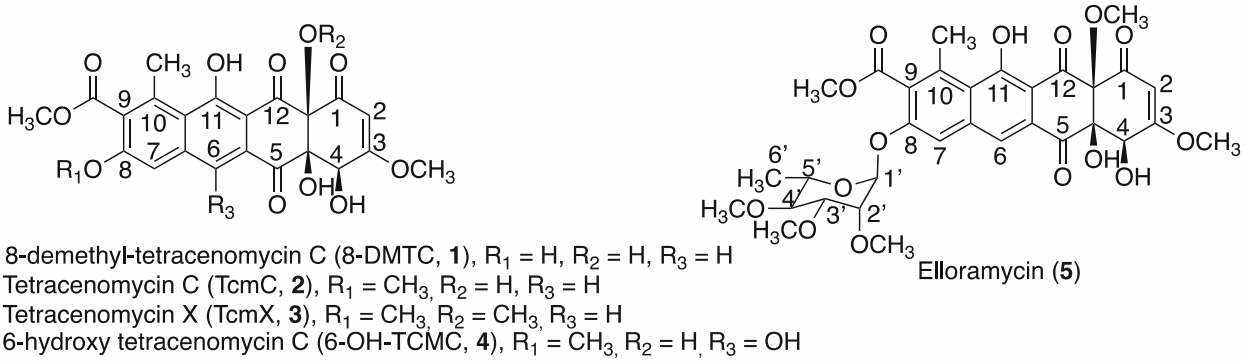

Figure 1. The tetracenomycins are a chemically diverse class of polyketides that feature *O*-methylations and glycosylations.

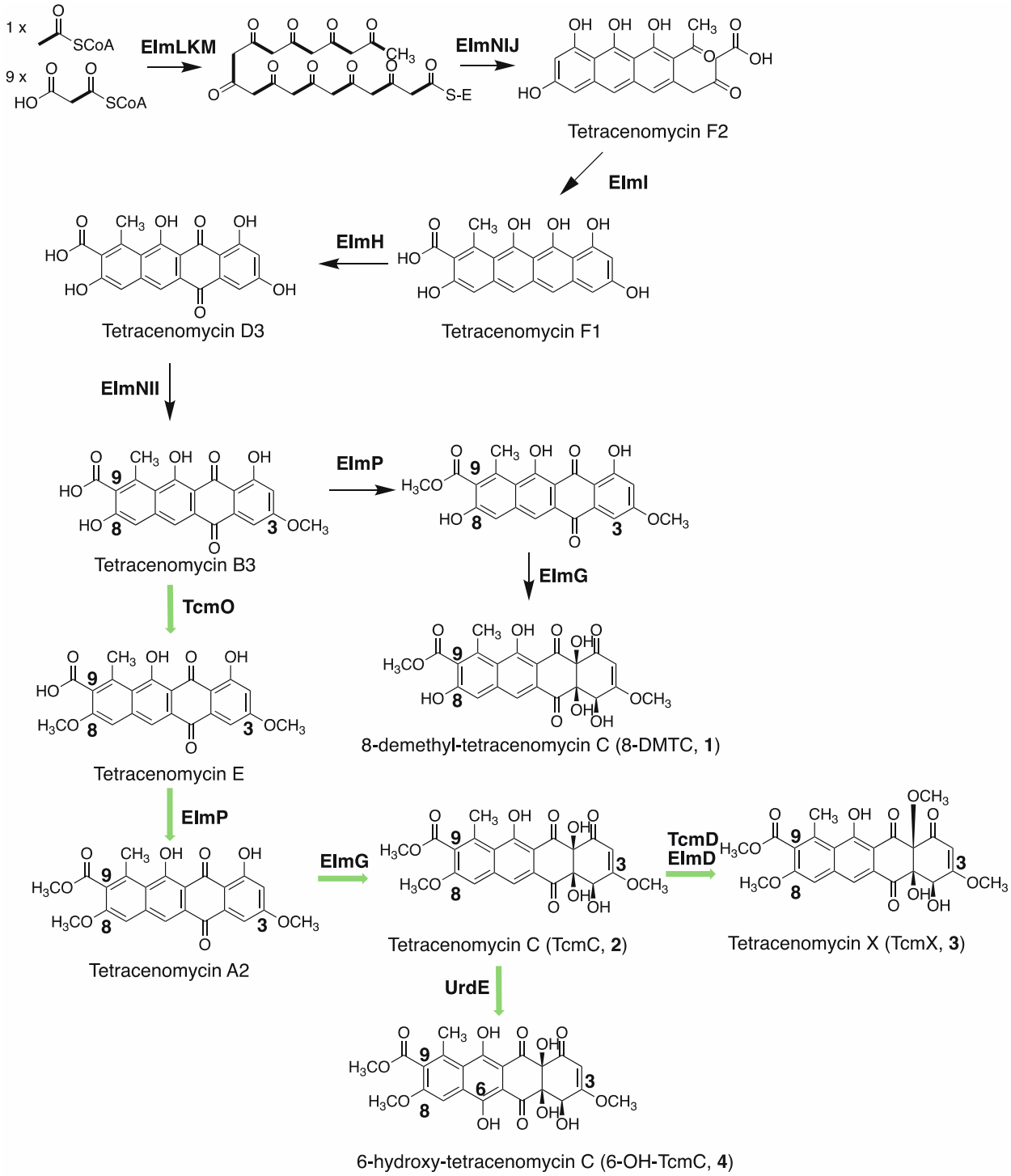

Figure 2. Proposed biosynthetic steps for the biosynthesis of the tetracenomycins.

The native **1** biosynthetic pathway is indicated in black arrows, whereas the engineered tetracenomycin bypass pathway is indicated with bold green arrows.

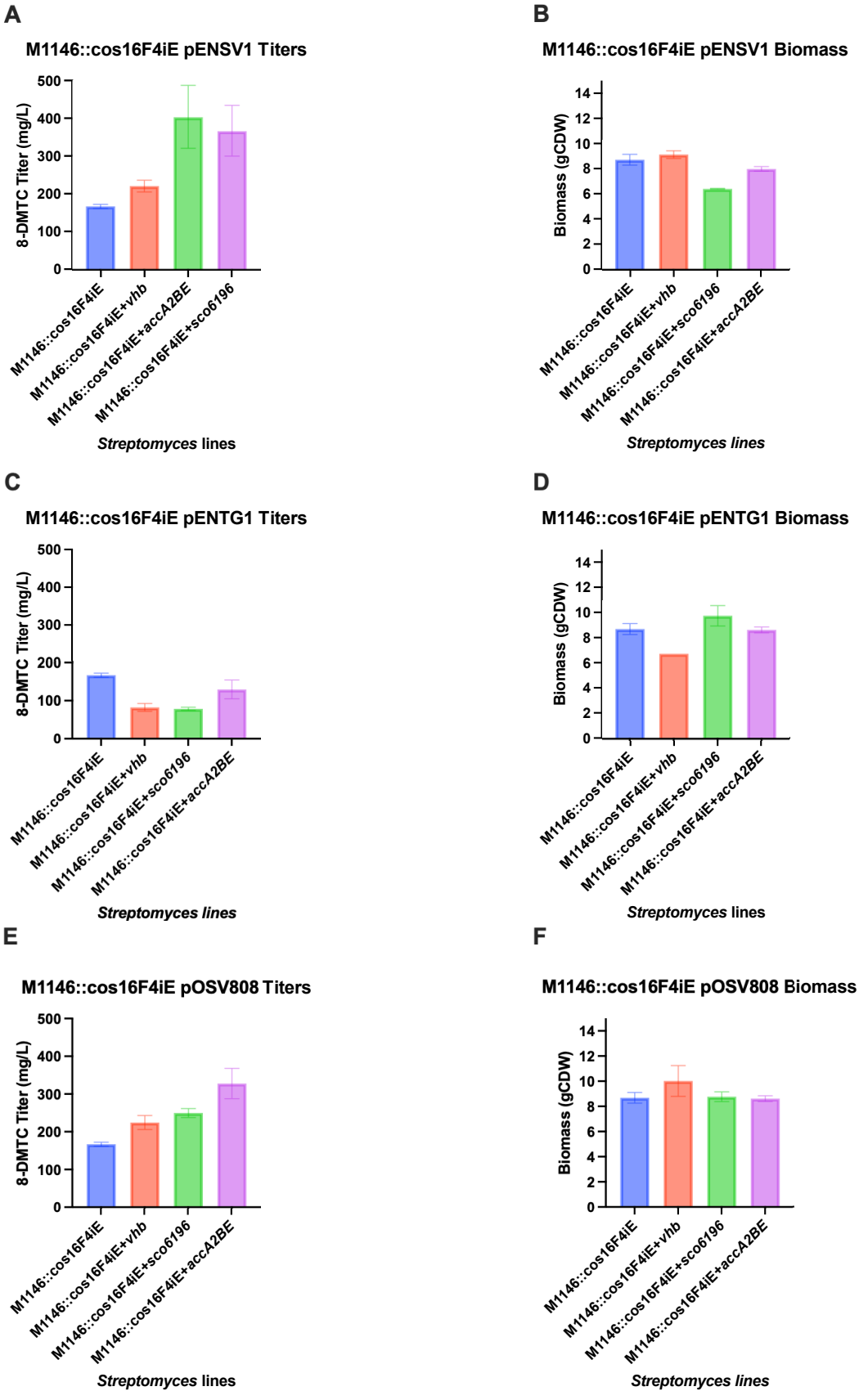

Figure 3 Substrate precursor engineering of *S. coelicolor* M1146::cos16F4iE increases the production of 1. (A) Production titers of 8-DMTC from lines engineered with pENSV1 expression vectors. (B) Biomass measurements of lines engineered with pENSV1 expression vectors. (C) Production titers of **1** from lines engineered with pENTG1 expression vectors. (D) Biomass measurements of lines engineered with pENTG1 expression vectors. (E) Production titers of **1** from lines engineered with pOSV808 expression vectors. (F) Biomass measurements of lines engineered with pOSV808 expression vectors. Experiments were conducted with 4 – 6 biological replicates. Experimental groups were compared using a t-test to determine statistical significance ($p < 0.05$).

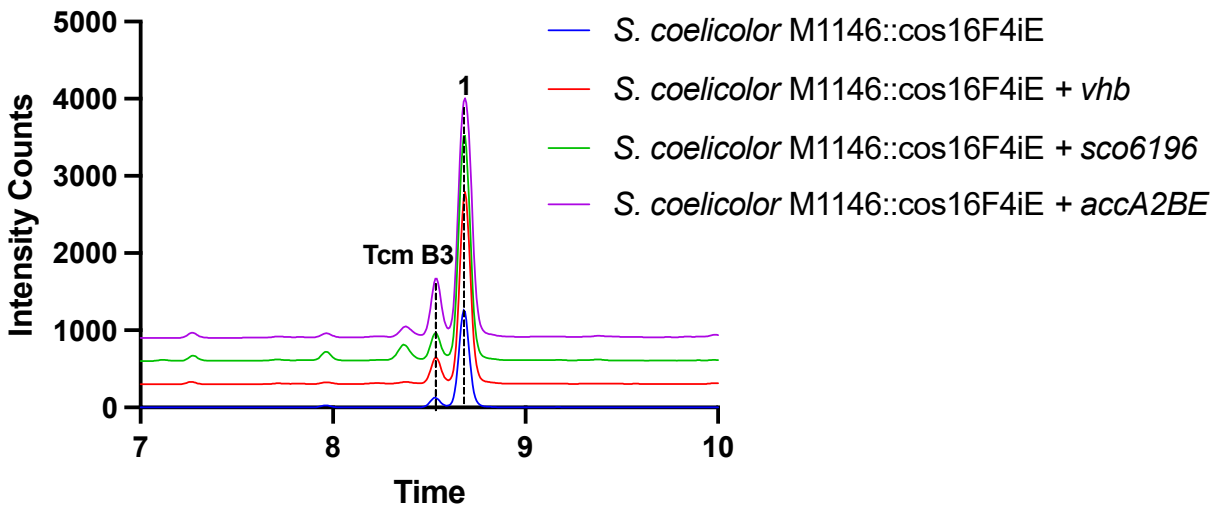

Figure 4 Increased production of 1 via the metabolic engineering of substrate precursor pools is determined via HPLC-MS. Production of **1** from different *Streptomyces coelicolor* M1146::cos16F4iE lines was assessed via HPLC-MS and comparison to authenticated standards. The lines included *Streptomyces coelicolor* M1146::cos16F4iE as a control (blue trace), and lines expressing a chromosomally-integrated copy of the *Vitreoscilla stercoraria* hemoglobin gene (*vhb*, red trace), the *S. coelicolor sco6196* acyltransferase

(*sco6196*, green trace), or the *S. coelicolor* acetyl-CoA carboxylase complex (*accA2BE*, purple trace).

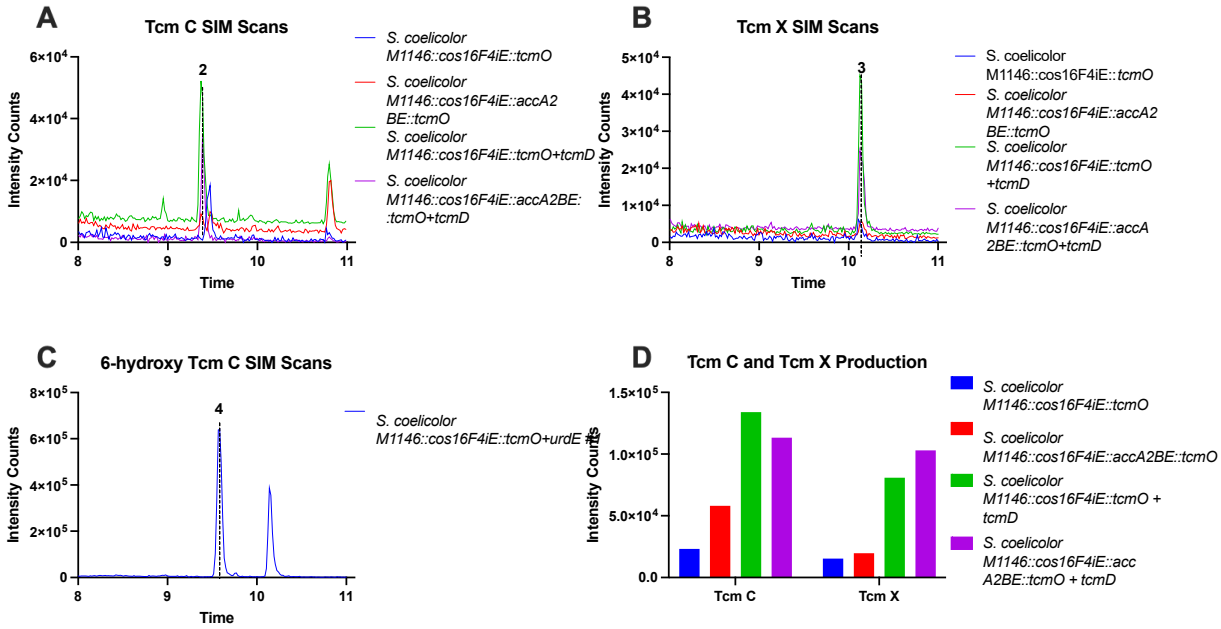

Figure 5 Heterologous expression of *tcmO, tcmD*, and *urdE* in *Streptomyces coelicolor* M1146::cos16F4iE results in production of tetracenomycin analogs. (Panel A) Chromatogram traces of lines producing **2** as analyzed in ESI-MS negative ion SIM mode: [M-H] = 471 *m/z*. (Panel B) Chromatogram traces of lines producing **3** as analyzed in ESI-MS negative ion SIM mode: [M-H] = 485 *m/z*. (Panel C) Chromatogram traces of lines producing **4** as analyzed in ESI-MS positive ion SIM mode: [M+H] = 489 *m/z*. (Panel D) Quantification of relative amounts of **2** and **3** from different production lines based on intensity counts in ESI-MS negative ion SIM mode.

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Data Availability Statement: Data available in article supplementary material**.**

Conflict of Interest

Material published in this report is covered under U.S. Patent Application No. 16/015,821 to Ferris State University.

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[1,2

We developed a BioBricks® toolbox of integrating vectors, promoters, and biosynthetic genes for engineering the tetracenomycin pathway. We improved tetracenomycin polyketide production by overexpressing genes encoding *Vitreoscilla stercoraria* hemoglobin (*vhb*), *Streptomyces coelicolor* acetyl-CoA carboxylase (*accA2BE*), and a *Streptomyces coelicolor* fatty acyl-CoA synthase (*sco6196*) to generate a strain capable of producing 400 mg/L 8-demethyl-tetracenomycin C in shake flask fermentations. Lastly, we diverted the 8-demethyl-tetracenomycin C pathway towards production of the more potent analog, tetracenomycin X, by overexpressing the recently characterized *tcmD* 12-*O*-methyltransferase, thereby setting the stage for generation of new tetracenomycins via combinatorial biosynthesis.