Engineered Biosynthesis

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Heterologous Expression and Manipulation of Three Tetracycline Biosynthetic Pathways**

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The engineered biosynthesis of natural products is a powerful approach towards the generation of structural diversity.[1,2] To fruitfully manipulate a biosynthetic pathway, it is desirable to have a robust heterologous host[3] and a set of enzymatic tools that can modify the target compound. Analogues of numerous important natural products, including erythromycin,[4] novobiocin,[5] and daptomycin,[6] have been obtained through the shuffling of biosynthetic genes in genetically amendable heterologous hosts. The tetracycline family of natural products has been highly important to human health, as evident in the continual development of semisynthetic tetracyclines as new antibiotics in the last 40 years.[7,8] The 2-carboxamide tetracyclic structure universal to all tetracyclines has been recognized as a privileged scaffold for drug discovery.[9] We report herein the heterologous expression and genetic manipulation of three structurally diverse natural tetracyclines: the classic antibiotic oxytetracycline (**1**),[10] the antitumor agent SF2575 (**2**),[11] and dactylocycline (**3**), which is active against tetracycline-resistant bacterial strains.[12] Numerous new tetracycline compounds were obtained from the engineered host/pathway pairs, which led to the discovery of a new set of tailoring enzymes that can modify the tetracycline scaffold at different positions.

Our first objective was to establish a heterologous host for reconstitution of the *oxy* cluster and biosynthesis of **1** to circumvent genetic manipulation in its native *Streptomyces rimosus*.[13] Whereas the systematic buildup of the *oxy* genes in *Streptomyces coelicolor* CH999 has provided key insight into the earlier steps of the pathway,[14,15] the biosynthesis of **1** by this approach has been hampered by incomplete understanding of the last few steps of the pathway, namely, from anhydrotetracycline (**5**) to **1** (Scheme 1). Despite previous reports of the production of **1** from *Streptomyces lividans*[16] and *Myxococcus Xanthus*,[17] we did not detect the biosynthesis of **1** in the model heterologous host *S. lividans* K4-114[18] upon insertion of the *oxy* cluster (*oxyA-T,* 25 kb) into its chromosome through the use of the integrative vector pOTC. To enhance the transcription of the *oxy* pathway, a *Streptomyces* antibiotic regulatory protein (SARP), encoded by *ctc11* from the chlorotetracycline gene cluster,[19] was placed under the constitutive *ermE** promoter[20,21] in pCTC11 and cotransformed into K4-114 with pOTC to give the strain K4/pOTC-Ctc11 (see Table S1 in the Supporting Information). We chose *ctc11* because no corresponding SARP is found in the vicinity of the *oxy* cluster. HPLC–MS analysis showed the production of **1** as the predominant product at a concentration of approximately 20 mg L^{-1} (Figure 1C). With pOTC as the template, we used the λ-Red-mediated recombination method[22,23] to delete *oxyS* encoding

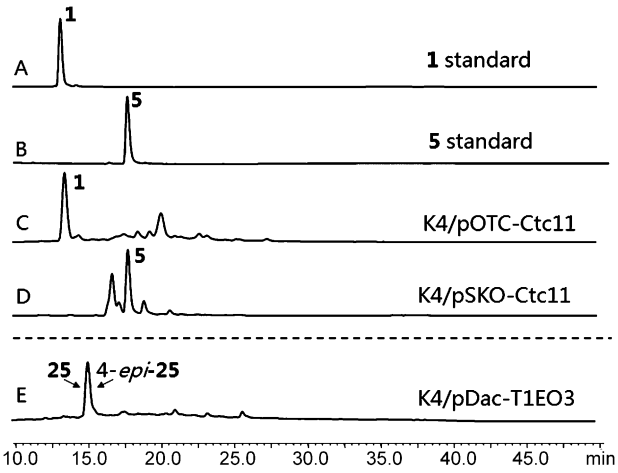

Figure 1. HPLC profiles of extracts from cultures of different K4 strains. A, C, E) λ = 358 nm; B, D) λ = 430 nm.

the anhydrotetracycline oxygenase (Scheme 1A). Cotransformation of the resulting plasmid with pCTC11 into K4-114 led to the accumulation of **5** and disappearance of **1** in K4/pSKO-Ctc11(Figure 1D). Although OxyS has been linked to the C6 hydroxylation of **5** to yield 5a,11a-dehydrotetracycline (**4**),[24,25] previous knockout studies in *S. rimosus* did not lead to the isolation of **5**, presumably as a result of off-pathway modifications by endogenous enzymes.[26] Therefore, the availability of the "clean" heterologous host should enable complete understanding of the enzymology between **5** and **1**, a critical requirement for the generation of tetracycline-like analogues.

The highly modified compound **2** produced from *Streptomyces* sp. 2575 contains unique modifications around its tetracycline aglycone, including the 4-O salicylate, the D-olivose at C9, the 18-O angelate, and the methoxy groups at C12a and C6. Unveiling of the biochemistry behind these reactions could therefore lead to a new group of tetracycline-specific transferases. However, owing to genetic difficulties in

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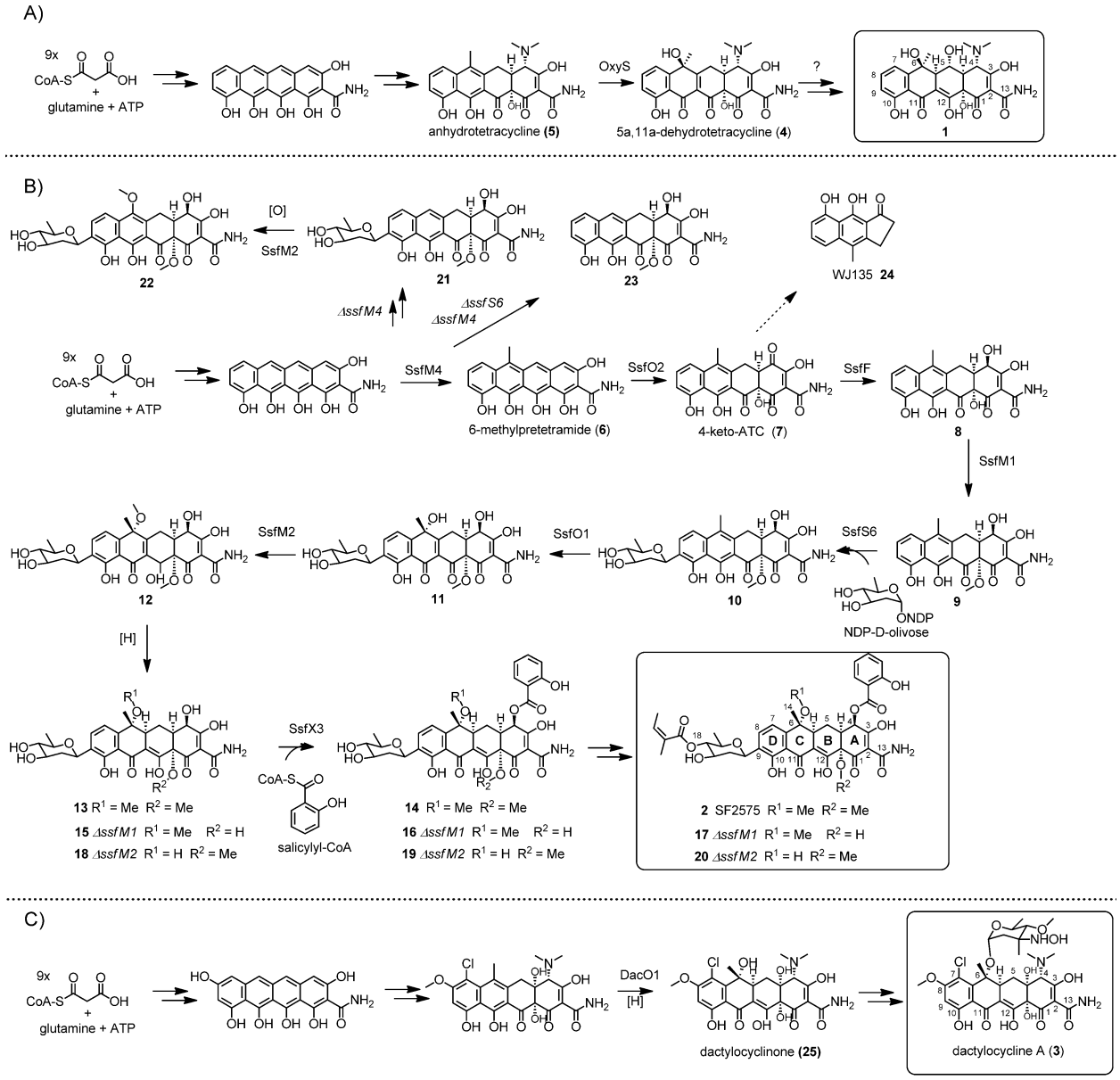

Scheme 1. Biosynthetic pathways of A) oxytetracycline; B) SF2575; C) dactylocycline. ATC = anhydrotetracycline, NDP = nucleotide diphosphate.

the manipulation of the *ssf* pathway in *S.* sp. 2575, only limited in vitro experiments have been performed on the *ssf* tailoring enzymes, such as the salicylate transferase SsfX3.[11] To construct a K4-114 host for the production of **2**, we integrated pSF2575 encoding the entire *ssf* gene cluster (40 genes, 47 kb)[27] into the chromosome and overexpressed the SARP SsfT1 under the *ermE** promoter to yield K4/pSF2575-T1 (see Table S1). HPLC–MS analysis showed the production of **2** and two intermediates **13** and **14** with a combined titer of approximately 127 mg L^{-1} (Figure 2A and Scheme 1B).

Starting with K4/pSF2575-T1, we first probed the roles of four *ssf S*-adenosylmethionine-dependent methyltransferases through the individual gene inactivation of *ssfM1*, *ssfM2*, *ssfM3*, and *ssfM4* by λ-Red recombination as described for *oxyS* (see Table S1). HPLC–MS analysis showed that Δ*ssfM1* produced the three major compounds **15**, **16**, and **17**, the masses of which corresponded to the structures of the desmethyl derivatives of **13**, **14**, and **2**, respectively (Figure 2B). The isolation and full characterization of **17** by NMR spectroscopy revealed the compound to be the previously isolated TAN-1518A (see Table S3 and Figure S5 in the Supporting Information),[28] which differs from **2** by the presence of a hydroxy group at C12a. Similarly, the inactivation of *ssfM2* led to the isolation of three different desmethyl compounds, **18**, **19**, and **20** (Figure 2C). Compound **20** was characterized by NMR spectroscopy to be the 6-hydroxy derivative of **2** (see Table S4 and Figure S6). The complementation of Δ*ssfM1* and Δ*ssfM2* strains with plasmid-borne copies of *ssfM1* and *ssfM2*, respectively, each restored the production of **2** in the host (see Figure S4). Therefore, we

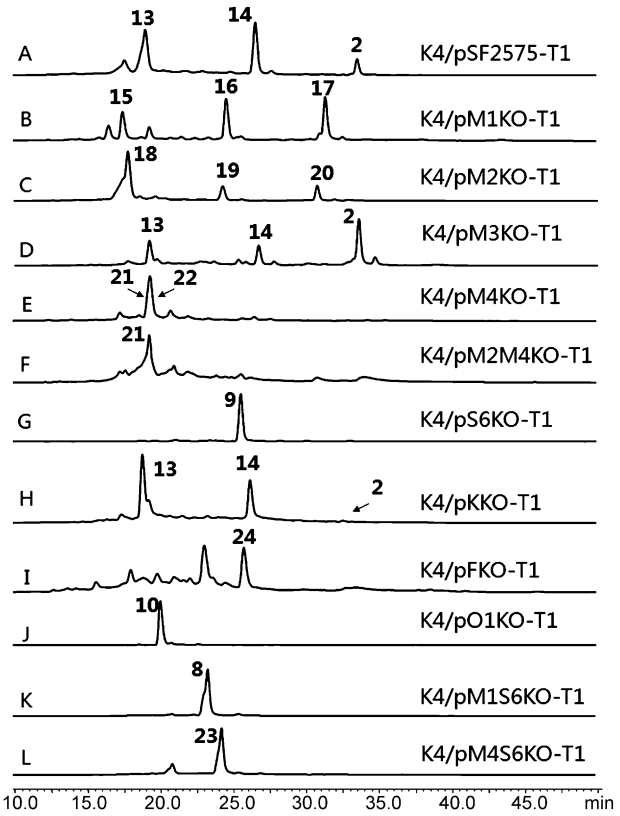

Figure 2. HPLC profiles of extracts from cultures of K4-114 integrated with different knockouts of the *ssf* pathways. A–D, H) $\lambda = 358$ nm; E–G, J–L) $\lambda = 430$ nm; I) $\lambda = 400$ nm.

assigned SsfM1 and SsfM2 as the 12a-O- and 6-O-methyltransferases, respectively; both activities are new to the tetracycline family.

The inactivation of *ssfM3* did not abolish the production of **2** (Figure 2D). Sequence analysis of SsfM3 showed significant homology (39% identity) to MtmMII, which is a C9 C-methyltransferase in the mithramycin biosynthetic pathway.[29] Considering that the same position in **2** is decorated with D-olivose, it is likely that SsfM3 is rendered inactive in this pathway. Indeed, the conserved histidine residue that serves as a general base in the catalytic pocket is substituted with Phe in SsfM3 (see Figure S17).[30]

SsfM4 was previously identified to catalyze C6-methylation by heterologous reconstitution in K4-114.[27] To examine the effect of removing this enzyme on the *ssf* pathway, we generated the Δ*ssfM4* mutant K4/pM^{4}KO-T1. HPLC–MS analysis revealed the biosynthesis of **21** and **22** (Figure 2E and Scheme 1B), both of which exhibited identical UV spectra to that of **5** (see Figures S7 and S8). Following full NMR spectroscopic analysis (see Tables S5 and S6 and Figures S7 and S8), **21** was confirmed to be an analogue of **5** with a D-olivose substituent at C9, a methoxy group at C12a, and a hydroxy group at C4. Hence, inactivation of the C6 methyl transfer, which takes place early in the pathway to generate 6-methylpretetramide (**6**), does not affect the functions of the glycosyltransferase and tailoring reactions on rings B and A. The unexpected isolation of **22** suggests that **21** can undergo oxidation in ring C, and the resulting phenol can be methylated by SsfM2. This hypothesis was confirmed by the further inactivation of *ssfM2* in the Δ*ssfM4* mutant, which produced only **21** (Figure 2F).

The attachment of D-olivose in **21** also suggests that the glycosylation of C9 takes place on the naphthacene intermediate, instead of on the mature tetracycline scaffold. To confirm this timing, we inactivated *ssfS6*, which encodes the only glycosyltransferase in the *ssf* cluster. A single metabolite **9** was found in the extract of the mutant (Figure 2G), which was characterized as the 4-hydroxy, 12a-methoxy derivative of **5** (see Table S7 and Figure S9). The Friedel–Crafts C-glycosylation of **9** with D-olivose is analogous to that catalyzed by the glycosyltranferase UrdGT2 in the urdamycin pathway.[31] Hence, SsfS6 is the first enzyme identified that can modify anhydrotetracycline-like molecules regioselectively at C9, which is a position that has been fruitfully modified to yield newer generations of tetracyclines.[8]

The elucidated timing of SsfS6 suggests that the hydroxylation of the C6 position by an OxyS homologue in the *ssf* pathway probably involves a glycosylated substrate. Indeed, the inactivation of *ssfO1*, which encodes a flavin-dependent monooxygenase that shows 54% sequence similarity to OxyS, led to the disappearance of **2** and the emergence of a single compound **10** (Figure 2J). The complementation of Δ*ssfO1* restored the biosynthesis of **2** in the K4 host (see Figure S4). Structural characterization revealed **10** to be the glycosylated derivative of **9** (see Table S8 and Figure S10), which indicates that SsfO1 functions immediately following SsfS6. The methoxy-substituted C6 center in **2** was previously determined to have the *R* configuration by X-ray crystal-structure analysis,[32] as opposed to the *S* configuration of this center in **1**. Therefore, we expect the binding of **5** and **10** in OxyS and SsfO1, respectively, to be significantly different to facilitate the hydroxylation from opposite faces of the naphthacenedione ring.

An interesting structural feature shared by **9** and **10** is the C4 hydroxy group, which is the site of salicylyl transfer catalyzed by SsfX3 much later in the pathway.[11] In the *oxy* pathway, the C4 ketone group in **7** is reductively aminated and N,N-dimethylated to yield **5**.[15] The presence of the hydroxy group at C4 (*R*) in **9** indicates that ketoreduction at C4 by a ketoreductase should take place early in the pathway. This process was further verified in the Δ*ssfS6*/Δ*ssfM1* double knockout, which produced a single product **8** (Figure 2K); this compound was verified by NMR spectroscopy to be 4-hydroxyanhydrotetracycline (see Table S9 and Figure S11). To identify which of the two unassigned ketoreductases (SsfK and SsfF) is involved in the reduction of **7** to **8**, we inactivated both genes independently and then carried out metabolite analysis. Since **7** can spontaneously undergo a series of rapid retro-Claisen cleavage processes to yield the shunt product WJ135 (**24**),[15] inactivation of the C4 ketoreductase should yield **24** as a major product. Whereas Δ*ssfK* continued to produce the compounds **13, 14**, and **2** (Figure 2H), Δ*ssfF* led to the complete abolishment of **2** along with the emergence of **24** (Figure 2I). The complementation of *ssfF* in K4/pFKO-T1 restored the production of **2** (see Figure S4) and thus confirmed the unique role of SsfF in catalyzing ketoreduction

at *R*-configured C4: the diverging point between the *oxy* and *ssf* pathways.

To obtain an additional analogue of **5** by using the *ssf* pathway, we constructed a Δ*ssfS6*/Δ*ssfM4* double knockout, which afforded **23**, the 6-desmethyl analogue of **9**, as predicted (Figure 2L and Scheme 1B). To evaluate the antibiotic activities of these new compounds, we measured the minimum inhibitory concentration (MIC) values against both Gram-positive (*Bacillus subtilis*) and Gram-negative (*Salmonella enterica*, *Pseudomonas fluorescens* Pf0-1, and *Escherichia coli* DH10B) bacteria (see Table S10). MIC assays showed that **8**, **9**, and **23** exhibited antimicrobial activities comparable to those of **5**, whereas the C9-glycosylation in **21** and **22** dramatically increased the MIC values for all strains. Compounds **16**, **17**, and **20** showed decreased MIC values for Gram-positive *B. subtilis*. This result reconfirms the importance of hydroxylation at the C6 position for antibiotic activity. Moreover, a further decrease in the MIC values observed for *B. subtilis* upon the attachment of angelic acid at 18-O in **20** implies that the 18-O position is a promising site for the generation of active tetracycline analogues.

To further utilize the K4-114 strain as a heterologous host for tetracycline biosynthesis, we targeted the biosynthesis of **25**, which is the aglycone of **3** (Scheme 1C). Compound **3** is a heavily modified tetracycline isolated from *Dactylosporangium* sp. SC 14051[33] and is active against *Staphylococcus aureus* strains that are resistant towards **1**.[12] However, **3** was shown to be too acid labile to be developed as an antibiotic.[12] Therefore, understanding of the biosynthesis of **3** may lead to the development of better analogues. However, the elucidation of this biosynthetic pathway is particularly challenging in the native host owing to its exceedingly slow growth rate and complete inaccessibility for genetic modification. Reconstitution of the biosynthesis of **25** (and ultimately **3**) in K4-114 may serve as a promising alternative. Towards this end, we sequenced the genome of the producing strain and identified the putative *dac* gene cluster (38 genes, 45 kb; see Table S11 and Figure S1). The *dac* gene cluster contains homologues to numerous *oxy* and *ssf* genes that perform similar functions in the generation of the tetracycline scaffold. Unique tailoring genes are also present, in accordance with the structural features of **3**, including those thought to encode a C7 halogenase DacE, a C4a monooxygenase DacO3, an 8-O methyltransferase DacM3, a 6-O glycosyltransferase DacS8, and a set of enzymes that synthesize the hydroxyamino deoxysugar. A proposed biosynthetic pathway of **3** is shown in Scheme 1C (see also Figure S2).

To transplant the *dac* pathway into K4-114, we constructed two plasmids encoding all the necessary genes proposed for the formation of **25** and overexpression of the SARP DacT1 (see Figure S2) and transformed them into K4-114 to generate K4/pDac-T1O3E. HPLC–MS analysis showed the emergence of two major compounds with the same mass and UV absorption spectra as **25** at a titer of approximately 1 mg L^{-1} (Figure 1E). ^{1}H NMR spectroscopic analysis confirmed one of the compounds to be **25**, whereas the other compound was assigned as the C4 epimer of **25**.[34] The isolation of **25** confirms the role of the *dac* gene cluster in the biosynthesis of **3** and affords a third set of enzymatic tools that can modify the tetracycline scaffold. Furthermore, this study is the first example of the reconstitution of natural products originating from *Dactylosporangium* in *Streptomyces*.

In conclusion, we have reconstituted three tetracycline pathways in the genetically superior host *S. lividans* K4-114. By using the heterologous platforms and newly discovered tailoring enzymes, we will be able to complete our understanding of tetracycline biosynthesis and perform engineered biosynthesis of tetracycline analogues in an efficient manner.

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