

## ORIGINAL ARTICLE

# C-glycosylation of anhydrotetracycline scaffold with SsfS6 from the SF2575 biosynthetic pathway

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Glycosylation with deoxysugar is a common strategy used by nature to introduce structural diversity and biological activities among natural products. In this study, we biochemically confirmed the activities of SsfS6, a C-glycosyltransferase in the SF2575 biosynthetic pathway, as a regioselective D-olivose transferase that acts on the C-9 position of an anhydrotetracycline aglycon. To perform the glycosyl transfer reaction using *Escherichia coli* as a whole-cell biocatalyst, we reconstituted the biosynthesis of TDP-D-olivose using a heterologous pathway. Under *in vivo* conditions, SsfS6 transferred multiple endogenous sugar substrates, in addition to D-olivose, to the anhydrotetracycline substrate, demonstrating broad substrate tolerance and potential as a tetracycline-diversifying enzyme.

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## INTRODUCTION

Natural products (NPs) and NP derivatives are important resources of therapeutic drugs.<sup>1,2</sup> Deoxysugar moieties are commonly found attached to biologically active NPs, and are critical in the binding of NP to biological targets and the observed therapeutic activities.<sup>3–5</sup> Frontline antibiotics such as erythromycin and vancomycin are examples of glycosylated NPs of which removal of the deoxysugar groups leads to loss of biological activities. As a result, regioselective glycosylation is not only a critical component in the synthesis and biosynthesis of NPs, but also an attractive engineering target in the generation of NP derivatives.

Owing to the structural complexity of many glycosylated NPs, synthetic manipulation of these compounds can be a daunting task. On the other hand, elucidation of numerous biosynthetic pathways of glycosylated NPs has provided enzymatic tools for the generation of glycosylated NPs.<sup>6,7</sup> Typically, dedicated genes are present in NP gene clusters for the transformation of glucose-1-phosphate into specific NDP-deoxysugar moieties, and one or more glycosyltransferases are responsible for the regiospecific transfer of the NDP-deoxysugar to the completed aglycon.<sup>6</sup> The substrate promiscuities of numerous glycosyltransferases have been explored for the production of new NP derivatives *in vitro*, as exemplified in the generation of over 70 glycosylated analogs of calicheamicin and vancomycin variants.<sup>8</sup> Therefore, the continued accumulation of glycosyltransferases that can modify pharmacologically important NP scaffolds is of significant importance toward expanding our bioengineering toolboxes.

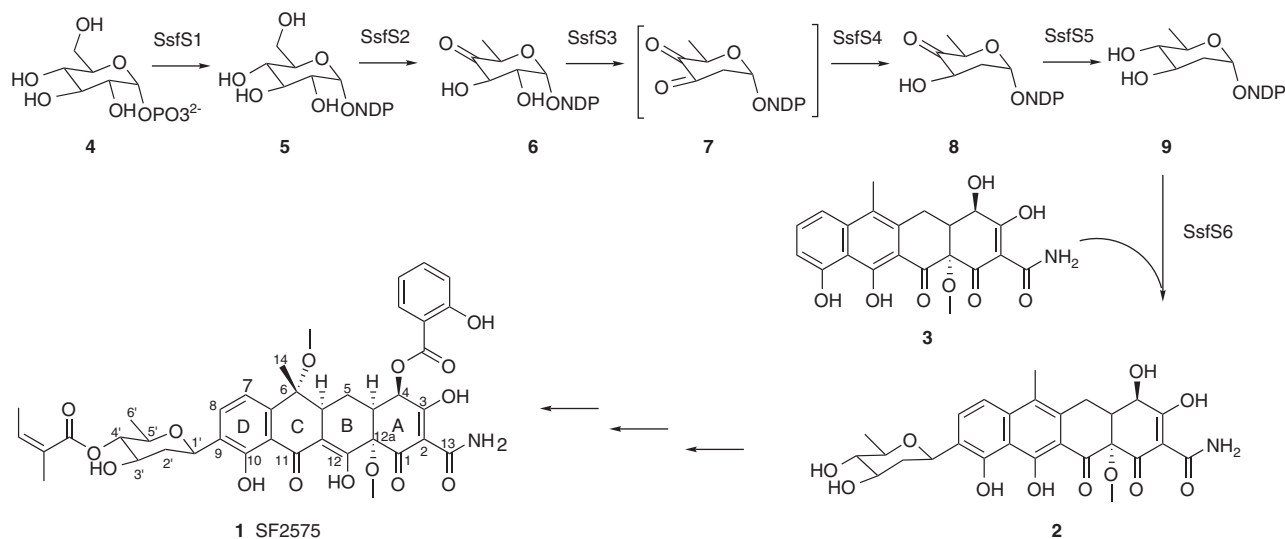
The tetracycline scaffold is considered to be one of the privileged scaffolds for drug discovery. Ever since the first generation of tetracycline antibiotics, which included oxytetracycline **15** and chlorotetracycline **16**, the 2-carboxamide tetracyclic structure has been modified to yield more effective antibiotics, including the second-generation doxycycline<sup>9</sup> and minocycline,<sup>10</sup> and, more recently, the third-generation tigecycline.<sup>11</sup> Therefore, having a glycosyltransferase that can modify the tetracycline or related scaffolds may be a new approach toward further structural diversification. Recently, our group identified the biosynthetic pathway of an unusually decorated tetracycline SF2575 (**1**),<sup>12</sup> which displays highly potent antitumor activities.<sup>13,14</sup> Among the numerous modifications to the classic tetracycline scaffold, one notable addition is a C-glycosylated D-olivose moiety at the C-9 position of **1** (Scheme 1). The C-9 position is a particularly fruitful position for medicinal-chemical modification of tetracyclines,<sup>15</sup> as illustrated in the introduction of *N,N*-dimethylglycylamido group at C-9 of minocycline to yield tigecycline.

The *ssf* gene cluster encoded enzymes responsible for the synthesis of TDP-D-olivose (SsfS1–S5) and a putative glycosyltransferase SsfS6 (Scheme 1). Our group recently reconstituted the biosynthesis of **1** in *Streptomyces lividans*, which led to the genetic confirmation of SsfS6 as a C-glycosyltransferase that is required for the addition of D-olivose.<sup>16</sup> SsfS6 therefore represents the first glycosyltransferase that can act on a tetracycline or a tetracycline-related intermediate, such as anhydrotetracycline **12**. SsfS6 is also the first enzyme that can regioselectively modify the C-9 position of tetracyclines. Recently,

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Scheme 1 Biosynthetic pathway of SF2575.

Table 1 Plasmids used in this study

Plasmid	Properties	Source or reference
pKW423	T7 promoter, <i>bla</i> , expression vector,	21
pLN2	Plasmid contains genes involved in the biosynthesis of TDP-L-oleandrose	19
pHJK2014	<i>tal</i> gene cloned in pET-28b	20
pLL16	<i>tal</i> gene ligated with pKW423 in <i>Nde</i> I and <i>Hind</i> III sites	This study
pLL19	<i>oleV</i> ligated with pKW423 in <i>Nde</i> I and <i>Eco</i> RI sites	This study
pLL20	<i>oleW</i> ligated with pKW423 in <i>Nde</i> I and <i>Eco</i> RI sites	This study
pLL21	<i>ssfS1</i> ligated with pKW423 in <i>Nde</i> I and <i>Eco</i> RI sites	This study
pLL24	<i>ssfS2</i> ligated with pKW423 in <i>Nde</i> I and <i>Eco</i> RI sites	This study
pLL25	<i>ssfS6</i> cloned in pKW423	This study
pLL39	<i>ssfS1</i> , <i>ssfS2</i> , <i>oleV</i> , <i>oleW</i> , <i>tal</i> and <i>ssfS6</i> cloned in pKW423	This study
pLL42	<i>ssfS1</i> , <i>ssfS2</i> , <i>oleV</i> , <i>oleW</i> and <i>tal</i> cloned in pKW423	This study

Table 2 Primers used in this study

Primers	Sequence (5'–3')
Ssf1NDF	<i>ACATATGAAAGCGCTAGTTCTGTC</i>
Ssf1ERIR	<i>AGAATTCTCATGCCGAGATCTGCGCTT</i>
ssfS2NDF	<i>ACATATGACCACCGCATCTCTGGT</i>
ssfS2ERIR	<i>AGAATTCTCAGAGGGCGGCCCGCTGCT</i>
ssfS6NDF	<i>ACATATGAGAATCCTGGTCATCGC</i>
ssfS6ERIR	<i>AGAATTCTCACGCTGCTCGATCAGCC</i>
OleVnDf	<i>AACATATGATATGGGAATCCCGCGAT</i>
OleVHDIIIr	<i>AAAGCTTTCAGCTCAGGGCCTGGATGC</i>
OleWnDf	<i>AACATATGCCCTCCCCCGTCTGCGCTT</i>
OleWEIR	<i>AAGAATTCTCAGCACCAGCGCACCCGCG</i>
TalNDF	<i>ACATATGACCCTCCGCACACGGCA</i>
TalHDIIIr	<i>AAAGCTTTTATGCGACCCGCGCGGT</i>

The introduced restriction enzymes sites are shown in italics and underlined.

the crystal structure of SsfS6 in complex with TDP was reported, and interactions between SsfS6 and tetracyclic substrates were predicted based on docking studies.<sup>17</sup>

In this work, we biochemically investigated the functions of SsfS6 by confirming its natural substrate as 4-hydroxy-12a-methoxy-anhydrotetracycline **3**. We also reconstituted the TDP-D-olivose biosynthetic pathway in *Escherichia coli* using heterologous components and established a whole-cell-based SsfS6 transformation of **3**, demonstrating the potential of this enzyme for use as a biocatalyst.

## MATERIALS AND METHODS

### Bacterial strains, cultures, plasmids and DNA manipulation

The SF2575 producer, *Streptomyces* sp. SF2575, was obtained from Meiji Co. (Tokyo, Japan). The plasmids used in this study are summarized in Table 1. Cultivation of *Streptomyces* strains was performed in tryptic soy broth liquid media or R5 solid agar as described by Kieser *et al.*<sup>18</sup> All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). Amplification of target genes was performed using PCR with *pfx* PCR polymerase from Invitrogen (Grand Island, NY, USA). To amplify *ssfS1–S6*, fosmid 11A12 was used as PCR template; for *oleV–W* and *tal*, PCR templates were pLN2 and pHJK-2014, respectively.<sup>19,20</sup> PCR products were cloned into

pCR-Blunt vector (Invitrogen) and sequenced (Laragen, Culver City, CA, USA). PCR primers used in amplification of different genes are listed in Table 2. To reconstitute the biosynthetic pathway of TDP-D-olivose, associated genes flanked by *Xba*I and *Spe*I were ligated sequentially into pKW423.<sup>21</sup>

### Expression and purification of recombinant proteins

Individual genes, including *ssfS1*, *ssfS2*, *ssfS3*, *ssfS4*, *ssfS5*, *ssfS6*, *oleV*, *oleW* and *tal*, were cloned into pKW423 vector and expressed in *E. coli* BL21(DE3). The transformants were grown in LB media containing 100 mg l<sup>-1</sup> ampicillin. The overnight cultures were inoculated into 500 ml LB media with antibiotic selection in 2-l flasks to an OD<sub>600</sub> of 0.4 at 37 °C. The cultures were transferred to an 18 °C shaker and induced by 0.3 mM isopropyl-β-D-thiogalactopyranoside overnight. Purification of His-tagged SsfS6 was carried out with Ni-NTA resin (Qiagen) at 4 °C. Cells were collected by centrifugation and resuspended in Buffer A (50 mM Tris-HCl, pH 8.0 and 50 mM NaCl), then lysed by sonication. After centrifugation at 15 000 g for 30 min, the supernatant was incubated in Ni-NTA resin for 2 h with gentle rotation. The mixture was transferred to a column and eluted with Buffer A plus imidazole at different concentrations. Buffer exchange into Buffer B (50 mM Tris-HCl, pH 8.0, 2 mM EDTA, 2 mM DTT) and concentration of SsfS6 were carried out with a 30-kDa MWCO Amicon filtration column (Millipore). The purified proteins were aliquoted and stored at –80 °C with 20% glycerol. The concentration of proteins was measured by Bradford assay using a ready-to-use Protein Assay reagent (Bio-Rad).

### In vitro assay of SsfS6

To test the activity of SsfS6 *in vitro*, the reaction was carried out in 100  $\mu$ l Buffer E (50 mM Tris-HCl, pH 7.4, 2 mM MgCl<sub>2</sub>) containing 2.5  $\mu$ M purified SsfS6, 1  $\mu$ M TDP-D-olivose (from Prof. Jürgen Rohr) and 1  $\mu$ M **3**. The solution was incubated at 30 °C for 3 h, and then extracted twice with 200  $\mu$ l ethyl acetate. The organic phase was evaporated *in vacuo* and then resuspended in 20  $\mu$ l methanol. The samples were analyzed by HPLC or LC-MS. HPLC was carried out on a Beckman Gold system with reverse-phase C18 column (Phenomenex Luna 5  $\mu$ m, 4.6  $\times$  250 mm) and a linear gradient of 5–95% CH<sub>3</sub>CN in water (0.1% trifluoroacetic acid) in 30 min at the flow rate of 1 ml min<sup>-1</sup>. LC-MS was performed on a Shimadzu 2010EV system with RP-C18 column (Phenomenex Luna 3.5  $\mu$ m, 2.0  $\times$  100 mm) in positive ionization mode, and the mobile phase is a linear gradient of 5–95% CH<sub>3</sub>CN in water (0.1% formic acid) in 30 min at the flow rate of 0.1 ml min<sup>-1</sup>.

### Whole-cell glycosyltransferase assay in *E. coli*

To reconstitute the biosynthetic pathway of TDP-D-olivose and Ssf6 activity in *E. coli*, BL21(DE3) cells transformed with pLL25, pLL39, pLL41 and pLL42 were used as a host. A 5-ml overnight culture, grown in LB media with ampicillin (100  $\mu$ g ml<sup>-1</sup>), was used to inoculate a 500-ml culture and grown until an OD<sub>600</sub> of 0.4 was reached. Isopropyl- $\beta$ -D-thiogalactopyranoside was added to a final concentration of 0.3 mM, and the culture was transferred to 25 °C for incubation overnight. When the cell growth plateaued, 20 mg of **3** (dissolved in 200  $\mu$ l DMSO) was added into the culture. After 48 h, the culture was extracted with 1 l ethyl acetate twice. The organic phase was combined and evaporated *in vacuo*, and resuspended in methanol for analytical HPLC and LC-MS analysis as described before.

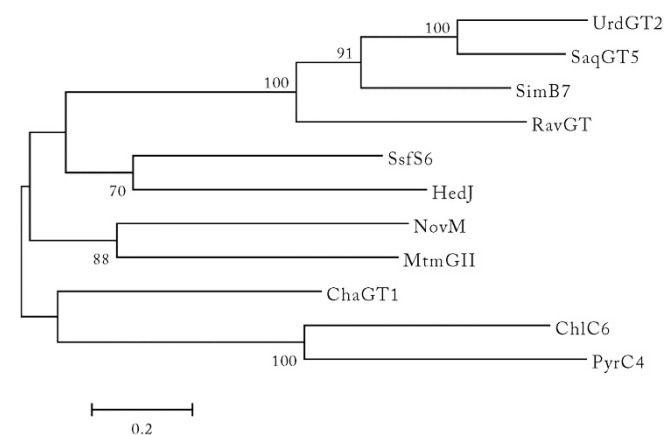
## RESULTS AND DISCUSSION

### Activities of recombinant SsfS6 *in vitro*

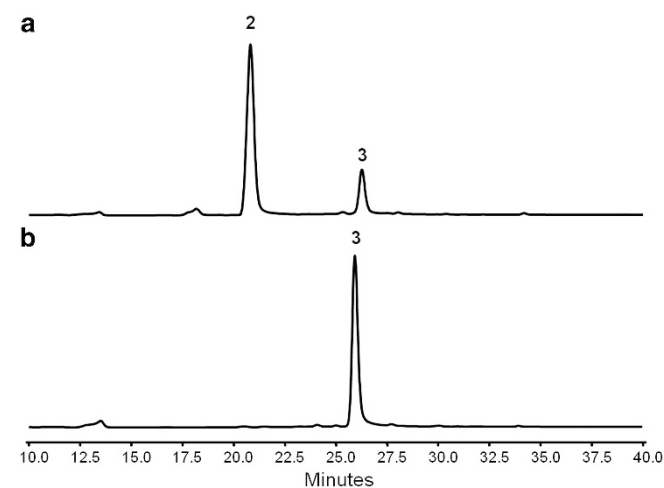
SsfS6, the only glycosyltransferase encoded in the *ssf* pathway, shows high sequence homology to HedJ (40% identity) involved in hedamycin biosynthesis<sup>22</sup> and other characterized C-glycosyltransferases. The aglycon of hedamycin contains a planar 4H-anthra[1,2-b]pyran tetracyclic scaffold, and presents the same phenolic D ring as found in **3**. Protein-sequence-based phylogenetic analysis of selected glycosyltransferases (listed in Table 3) gives the distance between C-glycosyltransferase and O-glycosyltransferase by using maximum likelihood analysis based on the JTT-matrix-based mode<sup>23</sup> (Figure 1). The glycosyl acceptor substrates of the C-glycosyltransferase with the nearest distances to SsfS6 in the phylogenetic tree are all found in the gene clusters of aromatic polyketides, indicating the likely distribution of these glycosyltransferase via horizontal gene transfers with whole antibiotic biosynthetic gene clusters.

We previously deleted *ssfS6* in the SF2575 pathway and recovered an analog of **12**, 4-hydroxy-12a-methoxy-anhydrotetracycline **3**, from

the fermentation culture.<sup>16</sup> This indicated that the substrate of SsfS6 contains a naphthacenedione present in anhydrotetracyclines, instead of the matured tetracycline scaffold that contains the oxidized and dearomatized C-ring. Docking studies of **3** in the SsfS6 catalytic site showed four putative binding poses, in which Glu316 or Asp58 was speculated to be the catalytic residue that initiates the Friedel–Crafts glycosyltransfer at C-9 through deprotonation of the C-10 hydroxyl group.<sup>17</sup> To verify **3** is indeed the substrate of SsfS6, recombinant SsfS6 was expressed and purified as a hexahistidine-tagged fusion protein from *E. coli* BL21(DE3) with a yield of  $\sim$ 10 mg l<sup>-1</sup>. When combined with **3** and TDP-D-olivose in Tris buffer containing Mg<sup>2+</sup>, we were able to detect the formation of a new product **2** in the organic extract of the reaction (Figure 2). Compound **2** showed the same retention time and *m/z* ( $[M+H]^+ = 544$ ) as a standard of 4-hydroxy-9-D-olivosyl-12a-methoxy-anhydrotetracycline isolated from the fermentation broth of a mutant blocked in the next step of the *ssf* biosynthetic pathway, thereby confirming the substrate and C-9 regioselectivity of SsfS6.<sup>16</sup> Under the experimental condition, about 90% of **3** was transformed into **2** following incubation at 30 °C



**Figure 1** Phylogenetic analysis of SsfS6 and selected glycosyltransferases in Table 1. Numbers at the nodes represent bootstrap percentages obtained from 1000 replicates, and the values <70 were eliminated.



**Figure 2** HPLC analysis of SsfS6-catalyzed conversion of **3** to **2** *in vitro*. (a) Reaction conditions: 2.5  $\mu$ M SsfS6, 1  $\mu$ M TDP-D-olivose and 1  $\mu$ M **3** in 100  $\mu$ l Buffer E (50 mM Tris-HCl, pH 7.4, 2 mM MgCl<sub>2</sub>); reaction quenched and extracted after 3 h. (b) Negative control using boiled SsfS6.

**Table 3** Selected glycosyltransferases involved in natural product biosynthesis

Name	Strain	Accession number
SsfS6	<i>Streptomyces</i> sp. SF2575	ADE34512.1
HedJ	<i>Streptomyces griseoruber</i>	AAP85354.1
UrdGT2	<i>Streptomyces fradiae</i>	AAF00209.1
ChaGT1	<i>Streptomyces chartreusis</i>	CAH10164.1
NovM	<i>Streptomyces caeruleus</i>	AAF67506.1
SaqGT5	<i>Micromonospora</i> sp. Tu 6368	ACP19370.1
MtmGII	<i>Streptomyces argillaceus</i>	CAK50792.1
SimB7	<i>Streptomyces antibioticus</i>	AEU17886.1
RavGT	<i>Streptomyces ravidus</i>	CBH32797.1
ChlC6	<i>Streptomyces antibioticus</i>	AAZ77691.1
PyrC4	<i>Streptomyces rugosporus</i>	AFV71301.1

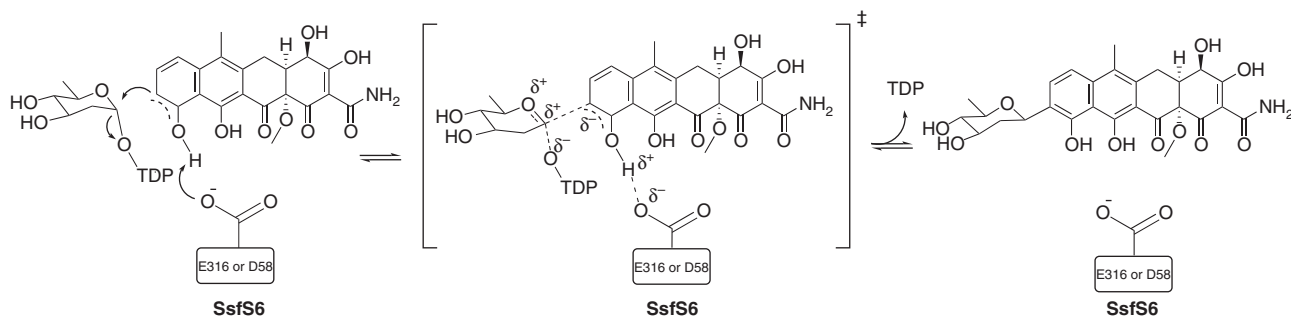
for 3 h. The proposed mechanism of SsfS6 is shown in Scheme 2, and is expected to be consistent with those proposed for other C-glycosyltransferases in which the C-9 carboanion nucleophile is formed through deprotonation of the adjacent C-10 hydroxyl in **3**, followed by nucleophilic displacement of the TDP group on TDP-D-olivose to yield **2**.<sup>24</sup>

To examine the aglycon specificity of SsfS6, we repeated the same assay using a variety of anhydrotetracycline (**10–14**) and tetracycline (**15, 16**) compounds available in our laboratory (Scheme 3). In all cases, we were not able to observe any glycosylated products from the reactions, indicating that SsfS6 has a high substrate specificity toward the aglycon. In the case of anhydrotetracycline analogs, addition of a methoxy group at C-8 is not tolerated. The enzyme is also highly sensitive to remote substitutions, as replacing the (*R*)-C-4 hydroxyl with (*S*)-C-4 dimethyl amine in **12** also led to no activity. We can exclude the C12a methoxy group as a requirement, however, as the C12a hydroxy version of **3** (that is, 4-hydroxyanhydrotetracycline) can be efficiently converted to the glycosylated form and was recovered from mutants blocked in the C12a *O*-methylation step.<sup>16</sup> In contrast to the high selectivity of SsfS6 toward the natural aglycon, the C-glycosyltransferase UrdGT2 from the urdamycin pathway was

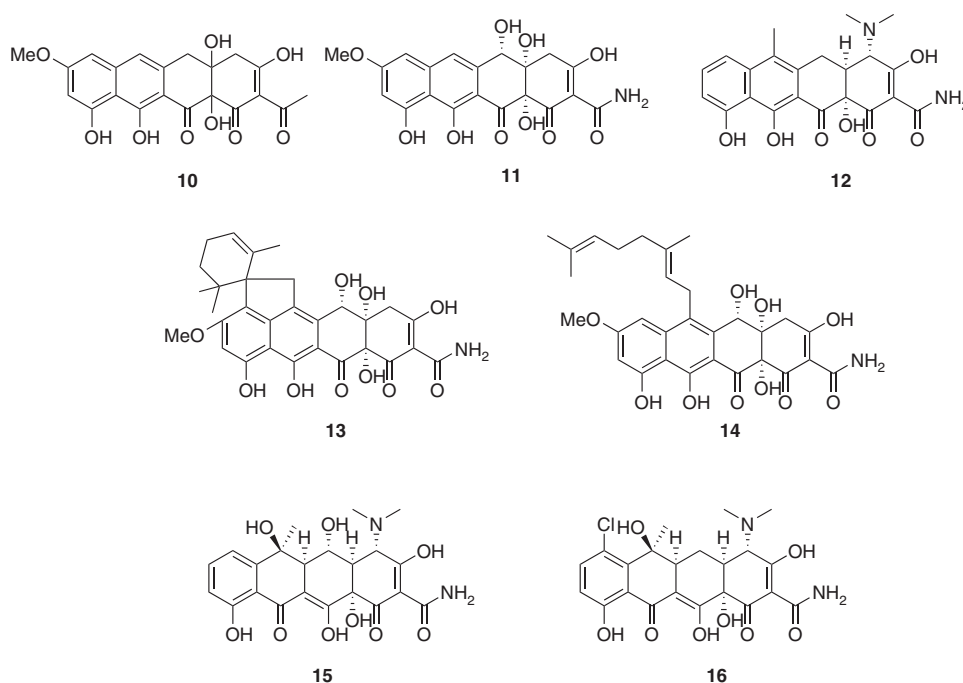
shown to be capable of glycosylating different aromatic aglycons in addition to the natural benz[ $\alpha$ ]anthraquinone-derived substrates.<sup>25,26</sup> The substrate specificity of SsfS6 can be gleaned from the X-ray crystal structure; the turnover of **3** to **2** requires a precise arrangement between the aglycone and TDP-D-olivose, during which the C-9 of **3** is placed at the attacking distance from the activated anomeric carbon of TDP-D-olivose. Alterations to the aglycon, such as replacing the C-4 dimethylamine or adding the C-8 methoxy, might therefore lead to complete loss of binding or alternative orientation of the aglycon in the active site and thus they are unable to undergo the reaction. With the structure in hand, protein-engineering approaches can be used to expand the specificity of SsfS6 toward substrates that have substituted and unnatural aglycons.

### Reconstitution of the biosynthetic pathway of TDP-D-olivose in *E. coli*

We next aimed to reconstitute the activities of SsfS6 in *E. coli*, which has been widely used as a platform for whole-cell biocatalysis. Our goal is to install a *de novo* pathway for TDP-D-olivose biosynthesis in *E. coli*, followed by conversion of **3** into **2**. This approach will address



**Scheme 2** Mechanism of SsfS6.



**Scheme 3** Tetracycline substrates examined as substrates for SsfS6.

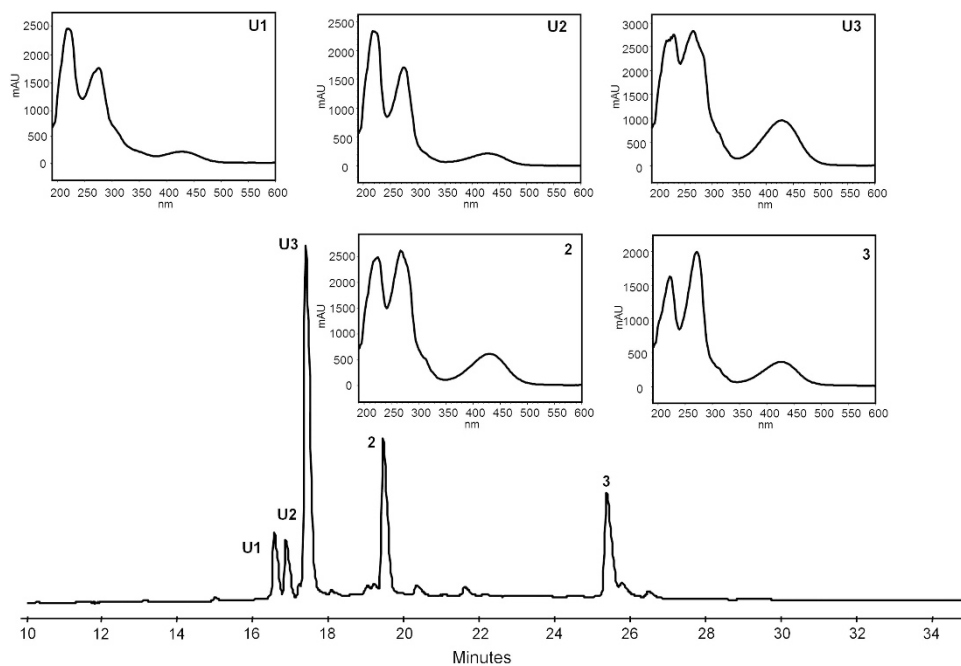
the membrane impermeability of synthetic TDP-D-olivose, as well as circumvent the elaborate synthetic steps required for its preparation.

Five enzymes are proposed to convert glucose-1-phosphate into TDP-D-olivose as shown in Scheme 1.<sup>27</sup> The corresponding enzymes in the *ssf* gene cluster include NDP-glucose synthase (SsfS1), 4',6'-dehydratase (SsfS2), 2',3'-dehydratase (SsfS3), C-3' ketoreductase (SsfS4) and C-4' ketoreductase (SsfS5). Pérez *et al.*<sup>28</sup> successfully reconstituted the biosynthetic pathway of TDP-D-olivose in *S. lividans*; however, reconstitution in *E. coli* has not been demonstrated. Upon cloning each of the enzymes into *E. coli* followed by expression analysis, only SsfS1 and SsfS2 were solubly expressed, while all the rest formed inclusion bodies. We then conducted a search for soluble substitutes of the other three enzymes to assemble a heterologous pathway in *E. coli*. Upon a homology search and *E. coli* expression, we identified OleV (2',3'-dehydratase, 70% similarity to SsfS3) and OleW (C-3' ketoreductase, 58% similarity to SsfS4) in the biosynthetic pathway of oleandomycin (which contains L-oleandrose) in *S. antibioticus* to be suitable replacements of SsfS3 and SsfS4, respectively.<sup>29</sup> We also hypothesized that Tal (C-4' ketoreductase, 56% similarity to SsfS5) in the biosynthetic pathway of TDP-6'-deoxy-L-talose in *Kitasatospora kifunensis* may catalyze the final C-4' reduction to yield TDP-D-olivose.<sup>20</sup> After confirming OleV, OleW and Tal can be solubly expressed in *E. coli*, we constructed the plasmid pLL39, which harbors the six-gene cassette of *ssfS1*, *ssfS2*, *oleV*, *oleW*, *tal* and *ssfS6* and transformed into BL21(DE3). As a control, pLL42 with *ssfS1*, *ssfS2*, *oleV*, *oleW* and *tal* was also constructed.

Following induction of protein synthesis in BL21(DE3)/pLL39 and BL21(DE3)/pLL42, **3** was added to the culture at a final concentration of 40 mg l<sup>-1</sup> and biotransformation was allowed to proceed for 2 days at 25 °C. The moderate conversion of **3** to **2** was observed in BL21(DE3)/pLL39, but not in the control strain BL21(DE3)/pLL42 that does not express SsfS6 (Figure 3). This result confirmed that indeed TDP-D-olivose is formed in the *E. coli* strain. Deletion of individual deoxysugar biosynthetic genes abolished the bioconversion, agreeing with the expectation that the entire five-gene cassette is

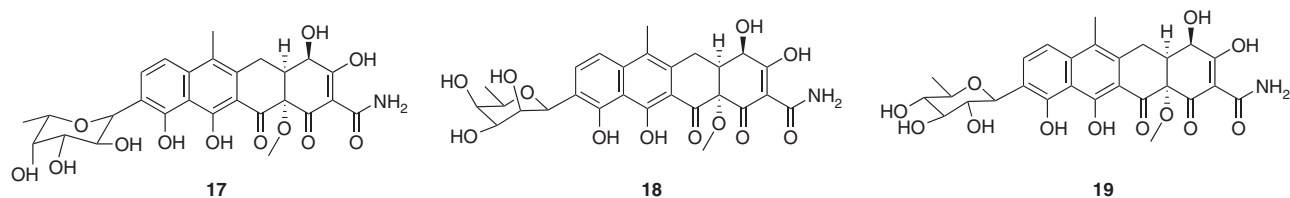
required for formation of TDP-D-olivose. This result also showed that Tal can accept both D- and L-hexose for reduction of the C-4' keto group. A number of 4'-ketoreductases have been observed to display some degree of flexibility. For example, the EryBIV reductase can reduce the 4'-keto group of both 3'-methyl-3'-hydroxyl (axial form) substrate NDP-4-keto-L-mycarose and 3'-demethyl-3'-hydroxyl (equatorial form) substrate NDP-4-keto-L-olivose.<sup>19</sup>

Interestingly, in addition to **2**, three other compounds (U1, U2 and U3) with the same mass ( $m/z$  [M+H]<sup>+</sup> 560) and displaying a similar UV spectrum pattern ( $\lambda_{\max}$  = ~430 nm) to **2** were found from the biotransformation assay (Figure 3), suggesting other sugar moieties may have been added to the anhydrotetracycline substrate **3**. Both the increase in mass (+16 mu) and more polar nature of these compounds hint the sugar groups added to **3** in these compounds are more oxygenated, which points to UDP sugars that originate from the primary metabolism of *E. coli* (Scheme 4). One such putative sugar donor may be GDP-L-fucose, which is abundant in *E. coli*. GDP-L-fucose is synthesized from GDP-4-keto-6-deoxy-D-mannose by the epimerase/reductase Fcl.<sup>30</sup> When we did the bioconversion in the culture tube with BL21(DE3)/pLL25, which only expressed SsfS6, a small peak with similar retention time was found in the HPLC profile, which suggests that endogenous deoxysugars in *E. coli* may be used by SsfS6. Other possible sugar donors may be GDP-6-deoxy-D-talose and TDP-D-quinovose, which may be reduced by Tal using GDP-4'-keto-6'-deoxy-D-mannose and TDP-4'-keto-6'-deoxy-D-glucose as a substrates, respectively.<sup>31</sup> TDP-4'-keto-6'-deoxy-D-glucose, which is the product of SsfS1 and SsfS2, may accumulate inside the cell if the reaction catalyzed by OleV is relatively slower compared with that of Tal. The proposed structures for **U1–U3** are shown as **17**, **18** and **19** in Scheme 4. Unfortunately, due to the small amount of product isolated from the bioconversion, the structures of these compounds were not confirmed by NMR analysis. Nevertheless, the surprise finding that additional glycosylated derivatives of **3** were formed in the biotransformation experiment suggests that although SsfS6 is stringent toward the aglycon substrate, it displays broad and promiscuous substrate



**Figure 3** HPLC profile of the bioconversion of compound **3**. In addition to **2**, three new products, **U1–U3**, were generated from *E. coli*.





**Scheme 4** Possible structures of 17–19.

specificity towards different sugar and deoxysugar substrates. This feature renders SsfS6 a promising enzyme in the bioengineering of glycosylated tetracycline compounds through combinatorial biosynthesis.

#### ACKNOWLEDGEMENTS

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