ORIGINAL ARTICLE

Cytochrome *P*450-mediated hydroxylation is required for polyketide macrolactonization in stambomycin biosynthesis

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Many polyketide antibiotics contain macrolactones that arise from polyketide synthase chain release via thioesterase (TE) domain-catalyzed macrolactonization. The hydroxyl groups utilized in such macrolactonization reactions typically derive from reduction of β -keto thioester intermediates in polyketide chain assembly. The stambomycins are a group of novel macrolide antibiotics with promising anticancer activity that we recently discovered via rational activation of a silent polyketide biosynthetic gene cluster in *Streptomyces ambofaciens*. Here we report that the hydroxyl group utilized for formation of the macrolactone in the stambomycins is derived from cytochrome *P*450-catalyzed hydroxylation of the polyketide chain rather than keto reduction during chain assembly. This is a novel mechanism for macrolactone formation in polyketide antibiotic biosynthesis.

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INTRODUCTION

Several polyketide natural products with important applications in medicine and agriculture contain a macrolactone. Examples include the antibacterial erythromycin A,¹ the antifungals amphotericin B and nystatin,^{2,3} the insecticide spinosyn A⁴ and the avermectins⁵ that are used as both insecticides and anthelmintics. Such macrolactones are typically biosynthesized by thioesterase (TE) domains located at the C-terminus of type I modular polyketide synthase (PKS) assembly lines.^{6–10} The fully assembled polyketide chain is transferred from the acyl carrier protein domain within the last module of the PKS to a conserved Ser residue in the active site of the TE domain.¹¹ The resulting ester undergoes general base-assisted intramolecular nucleophilic attack by a hydroxyl group, generated by reduction of a β -keto thioester intermediate in polyketide chain assembly.¹¹ This leads to formation of a tetrahedral intermediate that collapses to release the product of the PKS as a macrolactone (Figure 1).¹¹

We recently reported the discovery of the stambomycins **1–4**, a remarkable family of 51-membered macrolide antibiotics with promising activity against human cancer cell lines, via rational activation of a silent gene cluster in *Streptomyces ambofaciens* ATCC23877 that encodes a cryptic modular PKS assembly line (Figure 2).¹² Although the stambomycin PKS contains a C-terminal TE domain that is

proposed to catalyze macrolactone formation,¹² the C-50 hydroxyl group utilized for this process does not appear to derive from PKS-mediated keto reduction during polyketide chain assembly. Instead, we hypothesized that this hydroxyl group and the C-28 hydroxyl group are derived from molecular oxygen via cytochrome *P*450 (CYP)-catalyzed hydroxylation reactions (Figure 2).¹² Here we report the results of experiments aimed at investigating this hypothesis.

MATERIALS AND METHODS

HR MS, MS/MS and UHPLC-MS were carried out using a Bruker MaXis electrospray ionization time-of-flight (ESI-TOF) MS connected, where appropriate, to a Dionex 3000 RS UHPLC fitted with an Agilent Zorbax Eclipse Plus (Agilent, Wokingham, UK) C18 column (100 × 2.1 mm, 1.8 μ m). NMR spectra were recorded at 25 °C in CD₃OD on a Bruker Avance II 700 MHz spectrometer (Bruker, Coventry, UK) equipped with a TCI cryoprobe. Stambomycin-related metabolites accumulated in *S. ambofaciens* mutants were purified by semi-preparative HPLC on an Agilent 1100 instrument fitted with a C-18 column (100 × 21 mm, 5 μ m, Agilent). The strains and plasmids used in this study are listed in Table 1.

Deletion of *samR0478* and *samR0479* in *S. ambofaciens*

The *samR0478* and *samR0479* genes in *S. ambofaciens* ATCC/OE484¹² were replaced with a spectinomycin resistance cassette using the REDIRECT system,

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Figure 1 Mechanism for polyketide chain release from type I modular polyketide synthases (PKSs) via macrolactonization. The hydroxyl group required for macrolactonization is typically generated by reduction of a β -ketothioester intermediate in polyketide chain assembly; in this example, the ketoreductase (KR) domain within module 1 of 6-deoxyerythronolide B synthase catalyzes this reaction. The fully assembled polyketide chain is transferred from the phosphopantetheine thiol of the acyl carrier protein (ACP) domain within the last module of the PKS to the active site serine residue of the thioesterase (TE) domain at the C-terminus of the last module. The TE domain then utilizes an active site His residue (B) as a general base to form the macrolactone (in this case 6-deoxyerythronolide B) via an addition–elimination mechanism.

Figure 2 Chemical structures of stambomycins A–D 1–4. The two oxygen atoms proposed to derive from cytochrome P450-catalyzed hydroxylation of the polyketide chain rather than keto reduction during polyketide chain assembly are boxed.

as described previously.¹³ The spectinomycin resistance cassette, derived from the plasmid pIJ778,¹³ was used as the template for PCR reactions with the primers 5'-TGCAGACGGGCTGCTCGACGCGGGGAGTGACGGCATGTG TAGGCTGGAGCTGCTTC-3' (forward)/5'-GTCCCGGGCTCGGGCCGCGCC TTTGCCGCTGCCGACTCATTCGGGGATCCGTCGACC-3' (reverse) and 5'-GAGCGCCGGGAGGCGGGCGCGCGCAGTCGGCGTTCGTAGGC TGGAGCTGCTTC-3' (forward)/5'-CGCCCCGACGACACGGCAACCACCG ACGTAGAGGGATGATTCGGGGATCCGTCGACC-3' (reverse) to create disruption cassettes (~1400 bp) for *samR0478* and *samR0479*, respectively.

Escherichia coli BW25113/pKD20 was transformed with the bacterial artificial chromosome (BAC) BBC and then separately with each of the PCR products to replace either *samR0478* or *samR0479* in the BAC by homologous recombination. *E. coli* ET12567/pUZ8002 was separately transformed with each of the mutated BACs (BBC/ Δ 478::oriT-*aadA* and BBC/ Δ 479::oriT-*aadA*) that were subsequently transferred independently to *S. ambofaciens* ATCC/OE484 by conjugation. Spectinomycin-resistant colonies were picked and grown in Hickey-Tresner (HT) liquid medium.¹⁴ After several rounds of growth, cultures were spread on HT agar medium, and individual colonies were picked and analyzed by PCR and Southern hybridization to identify those in which the desired gene replacements had occurred via double crossover homologous recombination. The resulting mutants were named ATCC/OE484/ Δ 478 and ATCC/OE484/ Δ 479.

Incorporation of $^{18}\text{O}_2$ into stambomycins A–D 1–4

The ATCC/OE484 strain was cultured on MP5 agar medium overlaid with a sterile cellophane membrane as described previously¹² in a glove bag filled with $^{18}\text{O}_2$ gas. After 4 days of incubation at 30 °C, the cellophane membrane was lifted off the plate and the mycelia were scraped into a 50 ml plastic tube. Then, 10 ml of methanol was added and the mixture was sonicated for 10 min. The mycelia were pelleted by centrifugation for 10 min at 4 °C and the supernatant was analyzed by HR LC-MS using a flow rate of 0.2 ml min⁻¹ and water containing 0.1% formic acid (solvent A)/acetonitrile containing 0.1% formic acid (solvent B) as eluents. The following elution profile was used: 80% solvent A/20% solvent B for 5 min; gradient to 100% solvent B in 15 min; 100% solvent B for 5 min; gradient back to 80% solvent A/20% solvent B in 3 min. The MS was calibrated at the beginning of each run via loop injection of 20 μ l of 10 mM sodium formate and was operated in positive ion mode with settings as follows. Full MS scan range: 50–2000 *m/z*; End plate offset: –500 V;

Table 1 Strains, plasmids and bacterial artificial chromosomes (BACs) used in this work

Strain, BAC or plasmid	Principal characteristics ^a	Reference
Strains:		
<i>Streptomyces ambofaciens</i>		
ATCC23877	Wild type	16
ATCC/OE484	Second copy of <i>samR0484</i> under the control of the <i>ermE</i> * promoter	12
ATCC/OE484/Δ478	Second copy of <i>samR0484</i> under the control of the <i>ermE</i> * promoter and <i>samR0478</i> replaced by a spectinomycin resistance cassette	This work
ATCC/OE484/Δ479	Second copy of <i>samR0484</i> under the control of the <i>ermE</i> * promoter and <i>samR0479</i> replaced by a spectinomycin resistance cassette	This work
<i>Escherichia coli</i>		
DH5α	General cloning strain	17
ET12567/pUZ8002	Nonmethylating strain used for conjugal transfer of mutated BACs to <i>S. ambofaciens</i>	18
BW25113/pKD20	Strain used for PCR-targeting mutagenesis (<i>gam</i> , <i>bet</i> , <i>exo</i> , <i>bla</i>)	19
BAC or plasmid:		
BBC	BAC from the genomic library of <i>S. ambofaciens</i> (<i>cat</i>)	20
BBC/Δ478::oriT- <i>aadA</i>	<i>samR0478</i> replaced by the spectinomycin resistance cassette in BBC (<i>cat</i> , <i>aadA</i>)	This work
BBC/Δ479::oriT- <i>aadA</i>	<i>samR0479</i> replaced by the spectinomycin resistance cassette in BBC (<i>cat</i> , <i>aadA</i>)	This work
pIJ778	Contains the <i>oriT-aadA</i> spectinomycin resistance cassette	13

^a*bla*, ampicillin resistance gene; *oriT*, origin of transfer; *aadA*, spectinomycin/streptomycin resistance gene; *gam*, inhibitor of the host exonuclease V; *bet*, single-stranded DNA binding protein; *exo*, exonuclease promoting recombination along with *bet*; *cat*, chloramphenicol resistance gene.

capillary: −4000 V; nebulizer gas (N₂): 1.6 bar; dry gas (N₂): 8 l min^{−1}; dry temperature: 180 °C.

Analysis of the production of stambomycins and related metabolites in *S. ambofaciens* ATCC/OE484/Δ478 and ATCC/OE484/Δ479

S. ambofaciens ATCC/OE484/Δ478 was grown and analyzed as described above for the incorporation of ¹⁸O₂ into the stambomycins. *S. ambofaciens* ATCC/OE484/Δ479 was grown in liquid MP5 medium, as described previously.¹² The culture supernatant was separated from the biomass by centrifugation and passed through a 0.4 μm filter. The filtrate was analyzed by LC-MS, as described above for the incorporation of ¹⁸O₂ into the stambomycins.

Isolation of 28-deoxystambomycins A–D 5–8 from *S. ambofaciens* ATCC/OE484/Δ478

The 28-deoxystambomycins A–D 5–8 were isolated from cultures of *S. ambofaciens* ATCC/OE484/Δ478 grown on solid MP5 medium, as described previously for the isolation of stambomycins A–D 1–4 from *S. ambofaciens* ATCC/OE484.¹²

Isolation of shunt metabolites 9–12 from *S. ambofaciens* ATCC/OE484/Δ479

Twenty 50 ml cultures of *S. ambofaciens* ATCC/OE484/Δ479 were grown in MP5 liquid medium, as described previously.¹² The cultures were combined and centrifuged for 20 min at 4000 r.p.m. (3220 g). The supernatant was freeze-dried and the residue was re-suspended in 25 ml of 1:4 MeOH/H₂O. The resulting solution was passed through a 0.4 μm syringe filter and separated by HPLC, monitoring absorbance at 240 nm. The column was eluted with water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) using the following elution profile: 80% solvent A/20% solvent B for 10 min; gradient to 100% solvent B in 10 min; 100% solvent B for 15 min. Fractions containing 9–12 were identified using ESI-MS, combined and evaporated under reduced pressure. The residue was resuspended in 5 ml of 50% aqueous methanol and further purified by HPLC using the following elution conditions: 0 min, 50% A/50% B; 15 min, 5% A/95% B; 20 min, 100% B; 25 min, 100% B. Fractions containing an inseparable mixture of 9 and 10 were collected, combined and lyophilized, as were fractions containing an inseparable mixture of 11 and 12, yielding 4.3 and 3.2 mg, respectively.

Figure 3 Mass spectra from LC-MS analysis of the incorporation of ¹⁸O₂ into stambomycins A/B 1/2. The top panel shows the mass spectrum of a mixture of singly and doubly ¹⁸O-labeled stambomycins A/B; none of the unlabeled metabolites are observed. The middle panel shows the simulated mass spectrum for a species with the molecular formula C₇₃H₁₃₄N₁₆O₂₁¹⁸ONa²⁺, corresponding to the doubly charged ion of singly labeled stambomycins A/B. The bottom panel shows the simulated mass spectrum for a species with the molecular formula C₇₃H₁₃₄N₁₆O₂₀¹⁸O₂Na²⁺, corresponding to the doubly charged ion of doubly labeled stambomycins A/B.

RESULTS

If our hypothesis for the origin of the C-28 and C-50 oxygen atoms is correct, we would expect two oxygen atoms of the stambomycins to be derived from molecular oxygen. Thus, we investigated the incorporation of ¹⁸O₂ into stambomycins A–D 1–4. The previously reported stambomycin producer *S. ambofaciens* ATCC/OE484¹² was grown on a solid medium under an ¹⁸O₂ atmosphere and methanol extracts of the mycelia were analyzed by HR LC-MS. An approximately 3:1 mixture of doubly and singly ¹⁸O-labeled stambomycins A and B 1 and 2 was observed (Figure 3). Analogous

$^{18}\text{O}_2$ incorporation patterns were observed for stambomycins C and D **3** and **4**.

The *samR0478* and *samR0479* genes within the stambomycin biosynthetic gene cluster encode proteins with sequence similarity to CYPs (Figure 4a).¹² Thus, we hypothesized that SamR0478 and SamR0479 catalyze the hydroxylation of C-28 and C-50 (or vice versa) of the stambomycin polyketide chain. To investigate the role played by these enzymes in stambomycin biosynthesis, we independently replaced *samR0478* and *samR0479* on the chromosome of *S. ambifaciens* ATCC/OE484 with a spectinomycin resistance cassette.

HR LC-MS analysis of methanol extracts of the *samR0478* mutant showed that the production of stambomycins A–D **1–4** was abolished and identified new metabolites with molecular formulae corresponding to stambomycin derivatives lacking an oxygen atom (calculated for $\text{C}_{73}\text{H}_{134}\text{NO}_{21}\text{Na}^{2+}$: 691.9668, found: 691.9682; calculated for $\text{C}_{72}\text{H}_{132}\text{NO}_{21}\text{Na}^{2+}$: 684.9589, found: 684.9601) (Supplementary Information). The new metabolites were purified from extracts of

large-scale cultures of the *samR0478* mutant by semipreparative HPLC. ^1H and ^{13}C NMR data for the purified metabolites were very similar to those for stambomycins A–D **1–4** (Supplementary Information). Only the signals due to C-28 differed significantly ($\delta_{\text{H}} = 3.47$ p.p.m. and $\delta_{\text{C}} = 74.2$ p.p.m. for stambomycins A/B; $\delta_{\text{H}} = 1.30$, 1.58 p.p.m. and $\delta_{\text{C}} = 35.8$ p.p.m. for the corresponding novel metabolites), leading us to conclude that the novel metabolites are 28-deoxystambomycins A–D **5–8** (Figure 4b and Supplementary Information).

The production of stambomycins A–D **1–4** was also found to be abolished in the *samR0479* mutant, which accumulated new metabolites with the molecular formulae $\text{C}_{65}\text{H}_{120}\text{O}_{19}$ (calculated for $[\text{M} + \text{H}]^+$: 1205.8497, found: 1205.8488) and $\text{C}_{64}\text{H}_{118}\text{O}_{19}$ (calculated for $[\text{M} + \text{H}]^+$: 1191.8340, found: 1191.8332) (Supplementary Information). A neutral loss of 44 Da, corresponding to CO_2 , was observed in negative ion mode MS/MS analyses of the purified metabolites (Supplementary Information). This indicated that they are the shunt metabolites **9–12**, resulting from hydrolytic release and

Figure 4 The stambomycin biosynthetic gene cluster and the structures of stambomycin-related metabolites accumulated in the *samR0478* and *samR0479* mutants of *Streptomyces ambifaciens*. **(a)** Organization of the stambomycin biosynthetic gene cluster. The *samR0478* and *samR0479* genes, which were independently deleted in this study, are black. The polyketide synthase (PKS) genes are dark gray and other genes are light gray. The *samR0487* to *samR0478* and *samR0473*–*samR0468* regions are magnified 5 and 10 times, respectively, compared with the regions containing the PKS genes. **(b)** Structures of 28-deoxystambomycins A–D **5–8** accumulated in the *samR0478* mutant. **(c)** Structures of the shunt metabolites **9–12** that accumulate in the *samR0479* mutant showing observed COSY correlations (black lines) and key heteronuclear multiple-bond correlations (HMBC; arrows).

Figure 5 Proposed roles of the putative cytochromes P450 (CYPs) encoded by *samR0478* and *samR0479* in stambomycin biosynthesis, and mechanisms for the formation of 28-deoxystambomycins A–D **5–8** and shunt metabolites **9–12** (R = $\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_2\text{CH}_3$, $\text{CH}_2\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_3$, $\text{CH}_2\text{CH}(\text{CH}_3)\text{CH}_3$, or $\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$) in the *samR0478* and *samR0479* mutants, respectively, of *Streptomyces ambifaciens*. SamR0478 and SamR0479 are hypothesized to catalyze hydroxylation of C-28 and C-50, respectively, of the fully assembled polyketide chains attached to the acyl carrier protein (ACP) domain within the last polyketide synthase (PKS) module. Alternatively, these enzymes may catalyze hydroxylation of earlier intermediates in polyketide chain assembly. In the *samR0478* mutant, the ACP-bound intermediates in which X = H and Y = OH are transferred to the active site Ser residue of the thioesterase (TE) domain that catalyzes chain release via macrolactonization utilizing the C-50 hydroxyl group introduced by SamR0479. The resulting 28-deoxystambomycin A–D alkycones **13–16** undergo SamR0481-mediated mycaminosylation, yielding 28-deoxystambomycins A–D **5–8**. In contrast, the ACP-bound intermediates in which X = OH and Y = H accumulate in the *samR0479* mutant. These intermediates are transferred to the active site Ser residue of the TE domain, but cannot be offloaded via macrolactonization because the C-50 hydroxyl group is absent. Instead, the TE domain catalyzes hydrolysis of the enzyme-bound intermediates, yielding shunt metabolites **9–12**.

C-28 hydroxylation of the fully assembled polyketide chains from the stambomycin PKS. ^1H , ^{13}C , COSY, TOCSY, heteronuclear single quantum correlation and heteronuclear multiple-bond correlation NMR spectroscopic analyses confirmed this hypothesis and showed that these metabolites possess the C-28 hydroxyl group, but lack the C-50 hydroxyl group required for macrolide formation (Figure 4c and Supplementary Information). Coupling constants of 15.0 and 15.2 Hz for H-12/H-13 and H-48/H-49, respectively, confirmed the *E* configurations of the C-12/C-13 and C-48/C-49 double bonds in **9** and **10**. Similarly, correlations between the C-10 methyl group and H-12, and the C-24 methyl group and H-26 in the NOESY spectrum of **9** and **10**, were consistent with *E* configurations for the C-10/C-11 and C-24/C-25 double bonds (Supplementary Information).

DISCUSSION

The incorporation of two atoms of oxygen from $^{18}\text{O}_2$ into stambomycins A–D **1–4** is consistent with our hypothesis that the C-28 and C-50 oxygen atoms of these metabolites derive from hydroxylation of the polyketide chain. The accumulation of 28-deoxystambomycins A–D **5–8** in the *samR0478* mutant of *S. ambofaciens* implies that the putative CYP encoded by *samR0478* is responsible for introducing the C-28 hydroxyl group into the stambomycins. Whether this hydroxylation reaction occurs during or after polyketide chain assembly is unclear. The observation that the shunt metabolites accumulated in the *samR0479* mutant have a hydroxyl group at C-28 tempts us to speculate that C-28 hydroxylation occurs during polyketide chain assembly (Figure 5). Several examples of analogous CYP-catalyzed in *trans* oxidations have been reported for intermediates bound to the carrier proteins of nonribosomal peptide synthetases.¹⁵

The accumulation of stambomycin shunt metabolites **9–12**, lacking the C-50 oxygen atom of the macrolide and the mycaminoside residue, in the *samR0479* mutant indicates that the putative CYP encoded by *samR0479* catalyzes hydroxylation of C-50 during (or possibly even before) polyketide chain assembly (Figure 5). It appears that the TE domain of the PKS is unable to catalyze chain release via macrolactonization if the C-50 hydroxyl group is absent from the polyketide chains, despite the availability of several alternative hydroxyl groups resulting from keto reduction during chain assembly. Thus, the fully assembled polyketide chains appear to be offloaded from the PKS in the *samR0479* mutant via hydrolysis (Figure 5). In contrast to the 28-deoxystambomycin A–D aglycones **13–16**, **9–12** are evidently not substrates for the mycaminosyl transferase encoded by *samR0481*¹² (Figure 5).

In conclusion, we have shown that putative CYPs encoded by the *samR0478* and *samR0479* genes are involved in the introduction of the C-28 hydroxyl group and the C-50 oxygen atom, respectively, into the stambomycins. Hydroxylation at C-50 is required for offloading of fully assembled polyketide chains from the stambomycin PKS via macrolactonization. Further insight into the roles played by SamR0478 and SamR0479 in stambomycin biosynthesis could be obtained by investigating the catalytic properties of the purified

recombinant proteins. However, this may prove challenging, given the structural complexity of likely substrates for these enzymes.

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