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Identifying the Minimal Enzymes Required for Anhydrotetracycline Biosynthesis

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The tetracycline family of broad-spectrum antibiotics are synthesized by soil-borne streptomycetes using type II polyketide synthases (PKSs).¹ The heavily decorated cyclohexenone ring A of tetracycline possesses unique structural features not observed among other aromatic polyketides. These functional groups, which are essential for the antibiotic properties of tetracyclines, include the C2 primary amide, the C4 dimethylamine, and the C12a tertiary alcohol. Although the biosynthesis of tetracyclines has been well-studied using blocked mutants of tetracycline producers,² the enzymology of these distinctive structural features has remained unresolved. Here we report the identification and reconstitution of the minimum set of enzymes required for the biosynthesis of anhydrotetracycline (ATC, **5**), the first intermediate in the tetracycline biosynthetic pathway that contains the fully functionalized ring A.

Enzymatic conversion of the aromatic intermediate 6-methylpretetramid **1** into **5** requires a cascade of tailoring reactions catalyzed by unidentified enzymes. The key reaction is the double hydroxylation of ring A, in which two oxygen atoms are introduced into **1** to yield the proposed intermediate 4-keto-ATC **2**. In addition to the well-studied ATC-oxygenase *oxyS* (*otcC*),³ the oxytetracycline (**10**) gene cluster encodes four uncharacterized oxygenase genes (*oxyL*, *oxyE*, *oxyG*, and *oxyR*). We hypothesized that one or a combination of these *oxy* oxygenases may be responsible for forming the cyclohexendione ring A via insertion of oxygen at the C12a and the C4 positions. The resulting C4 ketone moiety is then converted to an amine group through reductive amination. The *oxy* gene cluster contains two nitrogen-inserting enzymes (OxyD and OxyQ), of which OxyD is an amidotransferase that installs the C2 amide group.⁴ We putatively assigned the PLP-dependent aminotransferase OxyQ to catalyze the conversion of **2** to 4-amino ATC **3**. Last, a *N,N*-dimethylation reaction, presumably catalyzed by the (*S*)-adenosylmethionine (SAM)-dependent methyltransferase homologue OxyT, yields **5**.

We have recently identified the enzymes required for the biosynthesis of **1**. Using the heterologous host/vector pair *Streptomyces coelicolor* CH999/pWJ119, expression of the minimal *oxy* PKS (OxyABC), OxyD, C-9 ketoreductase (OxyJ), cyclases (OxyKN), and C-methyltransferase (OxyF) led to the biosynthesis of **1** in high yield, which was completely oxidized to **7** (Figure 1).⁵ To examine the roles of the aforementioned set of enzymes (OxyLEGRQT) in transforming **1** into **5**, the six genes were assembled into a single operon and inserted into pWJ119 to yield pWJATC1. CH999 transformed with pWJATC1 developed a dark brown color, and the organic extract contained a single predominant

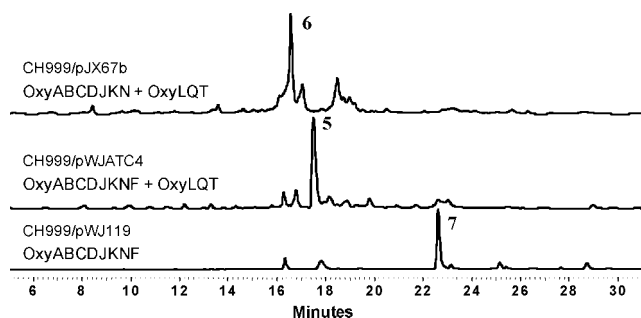


Figure 1. HPLC analysis (260 nm) of organic extracts from CH999 strains transformed with different combinations of *oxy* genes.

product with a yield of ~10 mg/L. The HPLC retention time, UV spectrum, mass (m/z 427 [$M + H$]⁺), and NMR data all matched precisely to those of the commercial **5** standard, confirming the successful heterologous biosynthesis of **5**.

To probe the necessity of individual enzymes toward formation of **5**, we systematically removed each of the newly added genes from pWJATC1 and compared the product profiles of the resulting transformed strains. Deletion of either OxyQ or OxyT completely abolished **5** biosynthesis from CH999, demonstrating that both enzymes are indispensable. Interestingly, removal of OxyL from the operon led to complete loss of **5** and recovery of **7**, while removal of OxyG, OxyE, or OxyR had no effect on either the product profile or the yield of **5**. These results suggest that OxyL may be the only oxygenase required during the biosynthesis of **5**. Indeed, coexpression of OxyL, OxyQ, and OxyT in CH999/pWJATC4 afforded **5** as the predominant polyketide product (Figure 1, CH999/pWJATC4). These three enzymes therefore constitute the minimum set of enzymes required to convert **1** into **5**.

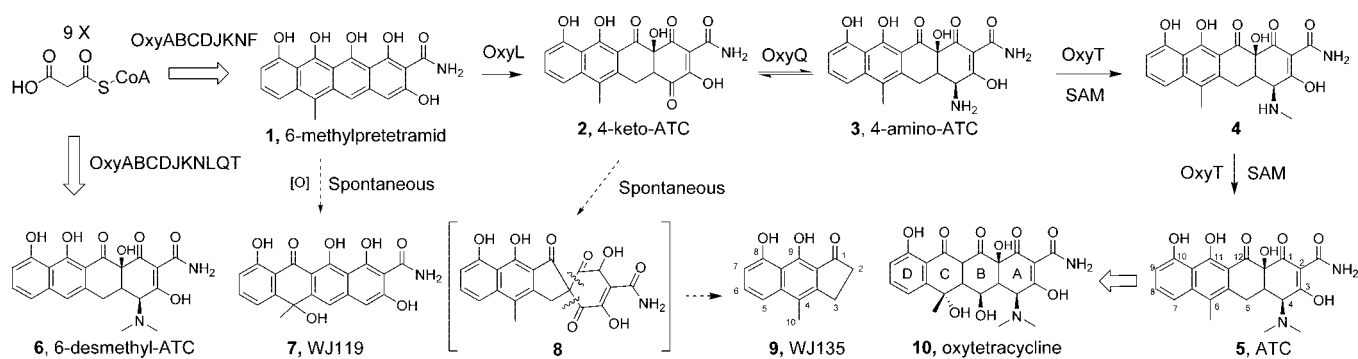
We then characterized the functional roles of three individual enzymes. OxyL is a FAD-binding protein that displays moderate sequence homology to TcmG (27% identity), which catalyzes the triple hydroxylation of Tcm A1 in a monooxygenase–dioxygenase mechanism to yield the highly hydroxylated Tcm C.⁶ Coexpression of OxyL alone with OxyABCDJKNF in CH999/pWJ135 led to the disappearance of **7** and the emergence of WJ135 **9** (m/z 227 [$M - H$]⁻) as the predominant product (Figure S2). NMR characterization revealed **9** is a tricyclic ketone (Table S3) and is a truncated compound that retained the D and C rings of **1**. We were not able to detect **2** in the extract using HPLC/MS. We reason that **2** may be unstable and can undergo spontaneous intramolecular rearrangement of the B ring to afford **8**, which can then undergo two tandem retro-Claisen cleavages to yield the observed degradation product **9** (Scheme 1 and Figure S6). Indeed, the facile *in vitro* conversion of **2** to **9** was reported by Scott et al.⁷ To further confirm the dioxygenation activity of OxyL in converting **1** to **2**,

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Scheme 1



soluble, holo-OxyL was overexpressed from *Escherichia coli* and purified (50 mg/L). The substrate **1** was prepared as described previously.⁸ Due to the instability of both the substrate and product, the reaction containing OxyL (20 μ M), **1** (~0.2 mM), and NADPH (2 mM) was incubated at 25 °C for 1 h, after which the supernatant was immediately injected into HPLC/MS. As expected, **1** was converted to a polar compound with mass (m/z 396 [M - H]⁻) consistent with that of **2** (Figure S8). The combination of in vivo and in vitro results strongly indicates the role of OxyL as a NADPH-dependent dioxygenase that hydroxylates **1** at both C12a and C4 positions, possibly via a monooxygenase–monooxygenase mechanism (Figure S5).

Coexpression of OxyQ and OxyL in CH999/pWJ209 yielded a new compound with a nearly identical UV spectrum as **5** (Figures S2 and S11). The compound was more stable than **2** and was verified to be **3**⁹ using high-resolution mass spectrometry (m/z = 421.1006 [M + Na]⁺, C₂₀H₁₈N₂O₇Na, calcd: 421.1012). Biosynthesis of **3** confirmed OxyQ is a reductive transaminase, which is the first of its kind found among bacterial type II PKSs.

Purification of **3** from the above strain enabled us to verify the role of the *N,N*-dimethyltransferase OxyT in vitro. To date, the only other enzymes found in PKS pathways capable of *N,N*-dimethylation are all associated with the biosynthesis of amino deoxysugars such as desosamine.¹⁰ OxyT was overexpressed from *E. coli* and purified to homogeneity (50 mg/L). OxyT (200 nM) was then incubated with purified **3** (0.2 mM) and SAM (2 mM) at 25 °C. HPLC/MS analysis of the reaction mixture after short reaction times revealed the presence of an intermediate (t_R = 11.0 min), which was verified by LC/MS to be the monomethylated species **4**.¹¹ Prolonged incubation led to the conversion of both **3** and **4** into the expected **5** (Figure 2). Removal of SAM abolished the synthesis of **4** and **5**, establishing SAM as the essential methyl donor in the reaction. Fitting the conversion data to the rate laws for an irreversible unimolecular consecutive reaction (**3** → **4** → **5**) led to the apparent kinetic constants of 0.009 and 0.08 min⁻¹ for the mono- and dimethylation steps, respectively.⁹ These results

confirmed the role of OxyT in the *N,N*-dimethylation of **3**. OxyT is therefore the first *N,N*-dimethyltransferase to use an aromatic polyketide aglycon as a substrate.

Having identified and confirmed the roles of OxyL, OxyQ, and OxyT in the biosynthesis of **5**, we set out to rationally biosynthesize the analogue 6-desmethyl-ATC **6** (Scheme 1). The 6-methyl moiety is not necessary for the antibacterial activity of tetracyclines and is absent in semisynthetic tetracyclines such as minocycline. We constructed pJX67b that contained all the enzymes required for the assembly of **5**, except OxyF, which we previously identified as a C6 methyltransferase.⁵ Transformation of CH999 with pJX67b resulted in the biosynthesis of a predominant new product with a titer of 10 mg/L (Figure 1). The identity of the compound was confirmed to be **6** using NMR spectroscopy (Table S4). This result reveals that the three tailoring enzymes studied here have relaxed substrate specificity toward substitutions at C6. The efficient biosynthesis of **6** demonstrates that the heterologous host/vector pair can be a useful platform toward the biosynthesis of tetracycline analogues.

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Supporting Information Available: Experimental procedures, and compound characterizations. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Kim, E. S.; Bibb, M. J.; Butler, M. J.; Hopwood, D. A.; Sherman, D. H. *Gene* **1994**, *141*, 141–142. (b) Binnie, C.; Warren, M.; Butler, M. J. *J. Bacteriol.* **1989**, *171*, 887–895. (c) Butler, M. J.; Binnie, C.; Hunter, I. S.; Sugden, D. A.; Warren, M. *Dev. Ind. Microbiol.* **1990**, *31*, 41–50. (d) Nakano, T.; Miyake, K.; Ikeda, M.; Mizukami, T.; Katsumata, R. *Appl. Environ. Microbiol.* **2000**, *66*, 1400–1404.
- (2) (a) McCormic, J. R. D.; Johnson, S.; Sjolander, N. O. *J. Am. Chem. Soc.* **1963**, *85*, 1692–1694. (b) McCormic, J. R. D.; Joachim, U. H.; Jensen, E. R.; Johnson, S.; Sjolander, N. O. *J. Am. Chem. Soc.* **1965**, *87*, 1793–1794. (c) McCormic, J. R. D.; Jensen, E. R. *J. Am. Chem. Soc.* **1969**, *91*, 206–206.
- (3) (a) Behal, V.; Hostalek, Z.; Vanek, Z. *Biotechnol. Lett.* **1979**, *1*, 177–182. (b) Peric-Concha, N. A.; Borovicka, B.; Long, P. F.; Hrnauei, D.; Waterman, P. G.; Hunter, I. S. *J. Biol. Chem.* **2005**, *280*, 37455–37460.
- (4) Zhang, W.; Ames, B. D.; Tsai, S. C.; Tang, Y. *Appl. Environ. Microbiol.* **2006**, *72*, 2573–2580.
- (5) (a) Zhang, W.; Watanabe, K.; Wang, C. C.; Tang, Y. *J. Biol. Chem.* **2007**, *282*, 25717–25725. (b) Zhang, W.; Wilke, B. I.; Zhan, J.; Watanabe, K.; Boddy, C. N.; Tang, Y. *J. Am. Chem. Soc.* **2007**, *129*, 9304–9305.
- (6) Rafanan, E. R., Jr.; Hutchinson, C. R.; Shen, B. *Org. Lett.* **2000**, *2*, 3225–3227.
- (7) Scott, A. I.; Yamaguchi, E.; Chung, S. K. *Tetrahedron Lett.* **1975**, *16*, 1369–1372.
- (8) Hassall, C. H.; Thomas, G. J. *J. Chem. Soc., Perkin Trans. 1* **1978**, *2*, 145–152.
- (9) Blackwood, R. K.; Stephens, C. R. *J. Am. Chem. Soc.* **1964**, *86*, 2736–2737.
- (10) (a) Chang, C.; Zhao, L.; Yamase, H.; Liu, H. *Angew. Chem., Int. Ed.* **2000**, *39*, 2160–2163. (b) Chen, H.; Yamase, H.; Murakami, K.; Chang, C. W.; Zhao, L.; Zhao, Z.; Liu, H. W. *Biochemistry* **2002**, *41*, 9165–9183.
- (11) Miller, P. A.; Saturnelli, A.; Martin, J. H.; Mitscher, L. A.; Bohonos, N. *Biochem. Biophys. Res. Commun.* **1964**, *16*, 285–291.

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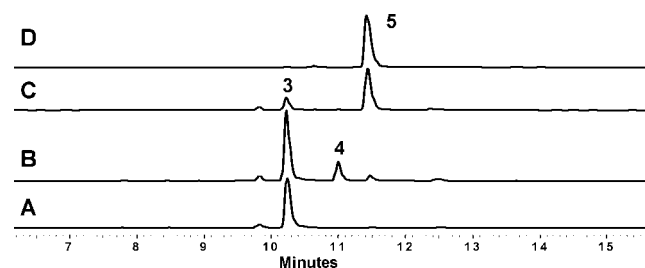


Figure 2. HPLC analysis (270 nm) of the OxyT catalyzed *N,N*-dimethylation of **3** to yield **5**. (A) No OxyT; (B) 10 min after OxyT addition. **4** is the monomethylated compound; (C) 10 h; and (D) **5** standard. Traces not drawn to scale.