Heterologous Biosynthesis of Amidated Polyketides with Novel Cyclization Regioselectivity from Oxytetracycline Polyketide Synthase

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Heterologous expression of the minimal oxytetracycline polyketide synthase and the amidotransferase OxyD in *Streptomyces coelicolor* strain CH999 afforded two novel amidated polyketides, WJ85 (**4**) and WJ85b (**5**). WJ85 is a C-9 unreduced decaketide that is primed by a malonamyl starter unit. WJ85 is cyclized via an unusual C-11 to C-16 intramolecular aldol condensation not observed among known aromatic polyketides. The structures of WJ85 and the previously characterized WJ35 suggest that the presence of an amide starter unit has a profound effect on the cyclization regioselectivity and reactivity of a polyketide backbone. WJ85b is an anthraquinone and is an oxidized product of WJ85.

Aromatic polyketides are biosynthesized by bacterial type II polyketide synthases (PKSs) using malonyl-CoA as building blocks.[1] The minimal PKS that is required to biosynthesize an elongated polyketide chain includes the ketosynthase-chain length factor (KS-CLF, also known as $KS_\alpha-KS_\beta$) pair, an acyl carrier protein (ACP), and the malonyl-CoA:ACP transacylase (MAT) shared between the PKS and endogenous fatty acid synthase (FAS).[2] The starter units for most aromatic polyketides are acetate groups derived from the decarboxylation of malonyl-ACP.[3] Nonacetate starter units such as propionate,[4] pentonoate,[5] isopentanoate,[5] hexanoate,[5] and benzoate[6] are also incorporated via different mechanisms by selected PKSs.[7] The tetracycline family of aromatic polyketides is primed by a polar amide starter unit. The amide starter unit is introduced into the polyketide backbone via a unique malonamyl thioester. We and others have sequenced the complete gene cluster encoding the oxytetracycline (*oxy*) PKS[8−14] and identified an amidotransferase, OxyD, that is essential for biosynthesis of an amidated decaketide.[8] We suggested that OxyD selectively amidates either malonyl-CoA or malonyl-ACP to yield malonamyl-CoA or malonamyl-ACP, respectively, which serves as the starter units in the biosynthesis of oxytetracycline. When OxyD is overexpressed in the presence of the *oxy* minimal PKS and the C-9 specific ketoreductase OxyJ (pWJ35, Table 1) in *Streptomyces coelicolor* strain CH999, a novel amidated polyketide, WJ35 (**1**) (Scheme 1), was synthesized in high yield.[8] In the absence of downstream cyclases, the presence of the amide starter unit affected cyclization regioselectivity of the reduced polyketide backbone. The nucleophilicity of the amide group also resulted in the formation of an alkaloid-like isoquinoline structure previously not observed in the type II PKS literature.

In this report, we studied the cyclization pattern of an amidated polyketide in the absence of C-9 ketoreduction, which is a naturally occurring tailoring step during the biosynthesis of oxytetracycline. We constructed the plasmid pWJ85 (Table 1), which is an *E. coli*−*S. coelicolor* shuttle vector that encodes the minimal *oxy* PKS and *oxyD*. The plasmid pYT319, which encodes only the minimal *oxy* PKS, was constructed as a control. Both plasmids were introduced into CH999 via PEG-mediated protoplast fusion. CH999 is an engineered *S. coelicolor* strain in which the endogenous PKS genes have been deleted[15] and is a useful host for investigating the functions of heterologous PKS enzymes. The metabolites of the transformed strains were extracted and analyzed with HPLC and LC/MS (Figure 1). The control strain CH999/pYT319 afforded only acetate-primed decaketides SEK15[16] (**2**) and SEK15b[17] (**3**), consistent with similar results reported by Fu et al.[18] In addition to **2** and **3**, a major new compound, **4**, was detected in the extract of CH999/pWJ85. LC/MS revealed **4** had a mass of 366 $(M - H)^-$, suggesting that the compound may contain a nitrogen atom. Using negative ion extraction of 366, no trace of **4** could be detected in the extracts of CH999/pYT319, further hinting that **4** was a novel compound produced by the *oxy* minimal PKS in the presence of OxyD.

Table 1. Plasmid Constructions and Resulting Polyketide Products[a]

plasmid	genes	major product(s)
pYT319	*oxyABC*	**2**, **3**
pWJ35	*oxyABCDJ*	**1**
pWJ85	*oxyABCD*	**2**, **3**, **4**, **5**
pWJ98	*oxyABCDK*	**2**, **3**, **4**, **5**
pWJ77	*oxyABCD mtmQ*	**2**, **3**, **4**, **5**

[a] *Streptomyces coelicolor* strain CH999 is used as the host for polyketide biosynthesis. Each plasmid is derived from pRM5. For references to compounds, see text.

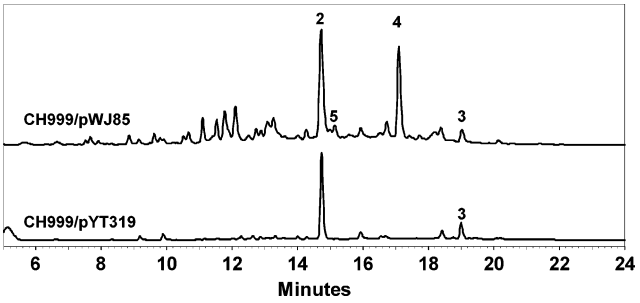

Figure 1. HPLC (280 nm) analysis of extracts from CH999/pYT319 (bottom) and CH999/pWJ85 (top).

Compound **4** was extracted from 2 L of fermentation broth of CH999/pWJ85 and purified by silica gel chromatography and C18 reversed-phase HPLC (approximate yield 9 mg/L). HRESIMS of **4** revealed the molecular formula $C_{19}H_{13}NO_7$ (m/z = 390.0588 $[M + Na]^+$, Δ = 2 mmu). This formula is consistent with an amidated decaketide (10 C=O units) backbone that has undergone four dehydration/cyclization steps. The ^1H, ^{13}C, and $^1H-^{13}C$ HMBC data are summarized in Table 2.

Nineteen ^{13}C signals were observed, consistent with the molecular structure suggested by HRESIMS. No methyl signals were present

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Scheme 1. Compounds Discussed in the Text

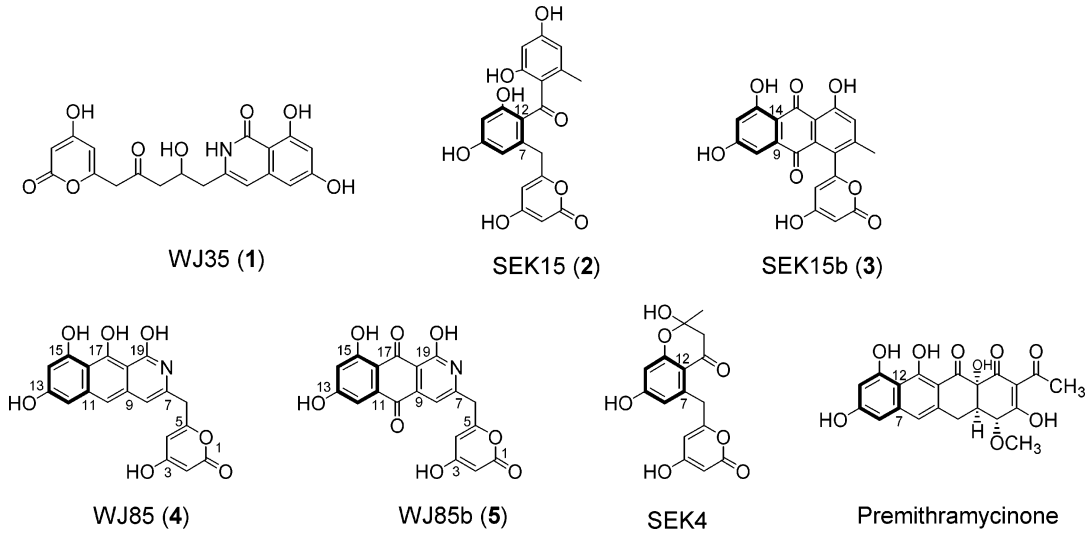


Table 2. ^{1}H and ^{13}C NMR Data for WJ85 (**4**) and WJ85b (**5**)[a]

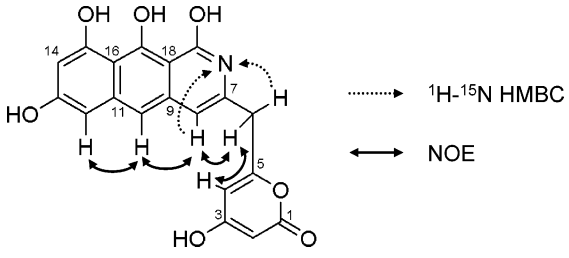
	WJ85 (**4**)			WJ85b (**5**)	
C no.[b]	δ_C	δ_H (m, J_{HH} (Hz), area)	$^{1}H-^{13}C$ HMBC	δ_C	δ_H (m, J_{HH} (Hz), area)
1	163.54			163.79	
2	88.9	5.265 (d, 2.2, 1H)	C1, C3, C4	89.18	5.242 (d, 2.2, 1H)
3 (OH)	170.24	11.739 (s, 1H)	C2, C3, C4	170.94	11.746
4	101.12	6.045 (d, 2.2, 1H)	C2, C5, C6	100.81	5.92 (d, 2.2, 1H)
5	162			161.6	
6	36.2	3.709 (s, 2H)	C4, C5, C7, C8	40.2	4.398 (s, 2H)
7	134.38			151.06	
8	107.08	6.454 (s, 1H)	C6, C7, C10, C18	111.1	6.973 (s, 1H)
9	140			142.03	
10	110.52	7.099 (s, 1H)	C8, C9, C12, C16, C18	181.7	
11	133			135.11	
12	100.33	6.553 (d, 2.2, 1H)	C10, C13, C14, C16	107.78	7.090 (d, 2.2, 1H)
13 (OH)	159.86	10.076 (s, 1H)	C12, C13, C14	164.96	11.24 (s, 1H)
14	100.79	6.307 (d, 2.2, 1H)	C13, C15, C16	109.23	6.622 (d, 2.2, 1H)
15 (OH)	157.86	9.765 (s, 1H)	C14, C15, C16	165.2	13.116 (s, 1H)
16	106.28			111.14	
17 (OH)	161.97	16.996 (s, 1H)		184.23	
18	101.46			118.31	
19 (OH)	168.19	11.507 (s, 1H)	C18	162.6	12.949 (s, 1H)

[a] Spectra were obtained at 500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR and were recorded in DMSO-d_6. [b]Carbons are labeled as shown in Scheme 1.

in the ^{1}H and ^{13}C NMR spectra, in accordance with the lack of an acetate starter unit. ^{1}H ($\delta_{H-2} = 5.27$, $\delta_{H-4} = 6.02$) and ^{13}C NMR ($\delta_{C-2} = 88.9$, $\delta_{C-4} = 101.1$) data interpretations readily established the presence of a 4-hydroxy-2-pyrone moiety commonly observed among aromatic polyketides as a result of O-1/C-5 cyclization.[19] The C-6 methylene ($\delta_{H-6} = 3.71$) was identified from HMBC experiments and is analogous to the same proton present in both SEK4 and SEK15 (Scheme 1).[16] The first aromatic ring formed as a result of an unexpected C-11 to C-16 intramolecular aldol condensation was established from $^{1}H-^{13}C$ HMBC data. The H-12 and H-14 protons were both sharp doublets with an expected *meta*-coupling constant of $J_{HH} = 2.2$ Hz.

Two remaining aromatic proton signals ($\delta_{H-8} = 6.45$ and $\delta_{H-10} = 7.10$) were both uncoupled and indicated the presence of an extended aromatic framework. Additional 2D NMR data revealed the tricyclic, fused-ring structure shown in **4** (Scheme 2). The H-10 proton displayed strong NOESY correlations to both H-12 and H-8 and strong HMBC correlations to C-12 and C-8. Furthermore, the NOESY correlation of H-8 to H-6 and HMBC correlations of H-8 to C-6 established the connectivity between the pyrone and the tricyclic rings. The structure of **4** was unequivocally confirmed by a $^{1}H-^{15}N$ HMBC experiment, in which both H-8 and H-6 displayed correlation to the unique nitrogen atom (Scheme 2).

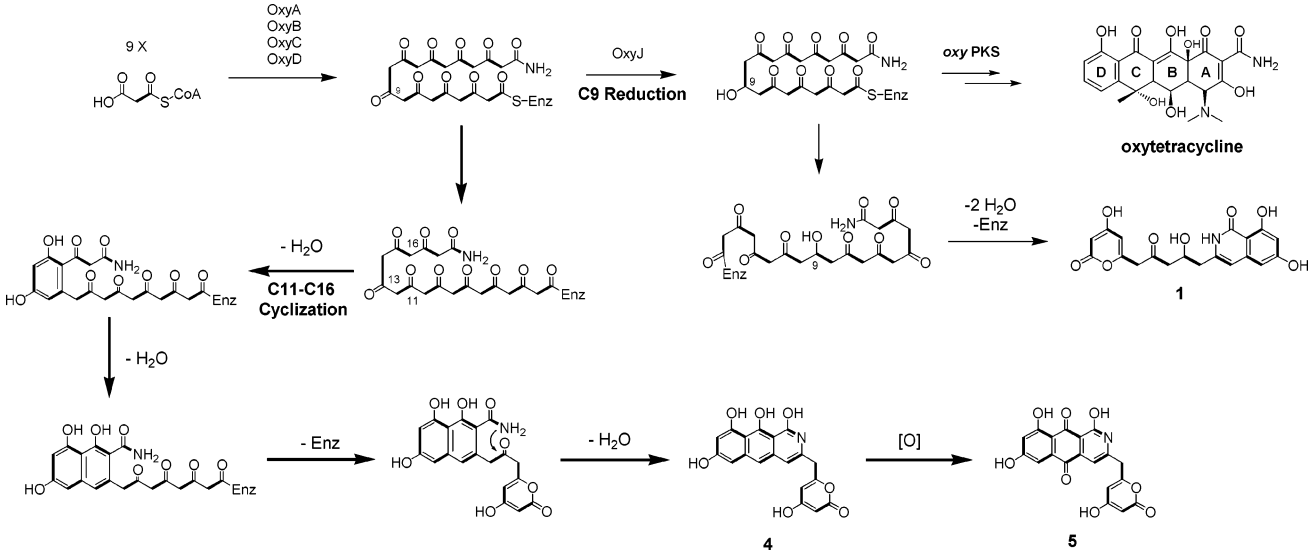
Scheme 2. Observed NOESY and $^{1}H-^{15}N$ HMBC Signals for **4**[a]



[a] For $^{1}H-^{13}C$ HMBC signals, see Table 2.

When primed with an acetate starter unit, the *oxy* PKS produced **2** and **3**, in which the first cyclization steps proceed via C-7 to C-12 and C-9 to C-14 aldol condensations, respectively. The unprecedented C-11 to C-16 cyclization observed in **4** (Scheme 3) is unexpected and suggests that amidation of the remote starter unit might direct novel cyclization regioselectivity of the polyketides backbone. Alternatively, the *oxy* KS-CLF ($KS_\alpha-KS_\beta$) may uniquely influence the spontaneous cyclization patterns of nascent polyketide backbones and result in a partially cyclized polyketide before its release from the active site. Cyclization of the second ring via the C-9 to C-18 aldol condensation is presumably spontaneous and

Scheme 3. Proposed Biosynthetic Scheme of Amidated Polyketides **4** and **5** by the *oxy* Minimal PKS and OxyD



favorably positions the nucleophilic C-19 amide to attack the C-7 carbonyl, yielding a six-membered heterocycle similarly observed in **1** (Scheme 3). We attempted to fix the cyclization regioselectivity of the first intramolecular aldol condensation to the commonly observed C-7 to C-12 by coexpressing decaketide cyclases with the minimal *oxy* PKS and OxyD. Coexpression of the bifunctional aromatase/cyclase OxyK[14] from the *oxy* PKS (pWJ98) did not afford C-7 to C-12 cyclized products, suggesting the cyclase requires a C-9 reduced backbone (upon coexpression of OxyJ, OxyK directed C-7 to C-12 cyclization of the reduced polyketide, unpublished data). MtmQ is a monofunctional cyclase from the mithramycin PKS,[20] which catalyzes the C-7 to C-12 cyclization of an acetate-primed, C-9 unreduced decaketide to form the first ring of premithramycinone (Scheme 1). Coexpression of MtmQ (pWJ77) did not result in formation of C-7 to C-12 cyclized products either, indicating that the presence of an amide starter unit may alter the substrate specificity of MtmQ.

During analysis of the CH999/pWJ85 extract, we observed an additional new metabolite (**5**) with UV absorptions characteristic of an anthraquinone. The compound eluted in close proximity to **2** during HPLC and was purified as a yellow solid (2 mg/L). HRESIMS suggested a formula of $C_{19}H_{11}NO_8$, which contains an additional oxygen atom compared to **4**. Further, the ^{1}H and ^{13}C NMR data of **5** revealed the loss of one aromatic proton and one downfield hydroxyl proton and the gain of two downfield carbon signals ($\delta_{C-10} = 181.7$, $\delta_{C-17} = 184.23$) when compared to **4**. This was suggestive of the presence of a quinone moiety in **5**. Analysis of the remaining ^{1}H, ^{13}C, and ^{1}H−^{13}C HMBC signals (Table 2) confirmed that **5** was an anthraquinone and is thus an oxidized derivative of **4** (Scheme 3). The compound was relatively unstable in DMSO and degraded after 1−2 days in solution.

The isolation and characterization of **1**, **4**, and **5** from engineered strains of CH999/pWJ35[8] and CH999/pWJ85 demonstrate that priming the polyketide backbone with amide starter units can yield novel compounds with unusual cyclization patterns. In both strains, the commonly observed pattern of C-7 to C-12 first-ring cyclization is not observed (see highlighted aromatic rings in Scheme 1). OxyD therefore adds to the repertoire of enzymes in the type II PKS toolbox that can be utilized to afford unnatural aromatic polyketides. Considering nitrogen-containing functional groups are rarely found in type II polyketides,[21] our findings are useful in expanding the chemical functionality and reactivity of the polyketide backbone. The biochemical nature of the unique cyclization regiospecificity is unclear and may be a result of alternative chain conformation in the active site of the *oxy* KS-CLF.

Experimental Section

General Experimental Procedures. *Streptomyces coelicolor* strain CH999 was used as a host for transformation of shuttle vectors. *Escherichia coli* XL-1 Blue (Stratagene) was used for the manipulation of plasmid DNA. *Streptomyces rimosus* (ATCC 10970) and *Streptomyces argillaceus* (ATCC 12956) were obtained from ATCC and were cultured for extraction of genomic DNA. Unmethylated DNA was obtained using the methylase-deficient strain GM2163 (New England Biolabs). HRESIMS was performed at the Vincent Coates Foundation Mass Spectrometry Laboratory at Stanford University using a Micromass Q-Tof hybrid quadrupole-time-of-flight LC-MS. NMR spectra were obtained on Bruker DRX-500 spectrometers at the NMR facility in the Department of Chemistry and Biochemistry at UCLA. ^{15}N NMR experiments were performed on the DRX-600 instrument, and formamide ($\delta = 95.0$, DMSO-d_6) was used as an internal reference.

Molecular Cloning. The *oxyA, oxyB, oxyC, oxyD, oxyJ,* and *oxyK* genes were amplified from *S. rimosus* genomic DNA. The *mtmQ* gene was amplified from *S. argillaceus* genomic DNA. A single *Pac*I/*Xba*I cassette encoding *oxyA* and *oxyB* was cloned into the same sites in pYT128 to yield pYT312. A *Xba*I/*Eco*RI fragment encoding *oxyC* was inserted into pYT312 to yield pYT319. A single *Xba*I/*Eco*RI cassette encoding *oxyC* and *oxyD* was cloned into the same sites in pYT312 to yield pWJ85. The cyclase genes *oxyK* and *mtmQ* were each cloned with flanking *Pme*I and *Eco*RI sites and inserted separately into pWJ85 to yield constructs pWJ98 and pWJ77, respectively. For a list of plasmids discussed in the text, see Table 1.

Extraction and Isolation. Following transformation of the shuttle vectors into CH999, strains bearing the vectors were maintained on R5 agar plates supplemented with 50 mg/L thiostrepton. For LC/MS analysis, a single plate with a confluent lawn of mycelia grown for 7 days at 30 °C was chopped into fine pieces and extracted with 50 mL of EtOAc/MeOH/AcOH (94:5:1). The organic extract was dried with Na_2SO_4 and evaporated to dryness. The residue was redissolved in 500 μL of DMSO, and 10 μL was analyzed by HPLC and LC/MS. The polyketide products were separated by reversed-phase HPLC and detected at 280 and 410 nm using an analytical C18 column (Varian Pursuit, 250 mm × 4.6 mm, 5 μm); linear gradient: 5% CH_3CN in H_2O (0.1% TFA) to 95% CH_3CN in H_2O (0.1% TFA) over 30 min with a flow rate of 1 mL/min.

The large-scale organic extract from a 2 L fermentation of CH999/pWJ85 was loaded onto a normal-phase silica gel column and fractionated by flash chromatography (20% hexane/EtOAc, followed by EtOAc). Fractions containing **4** or **5** (eluted with EtOAc) were detected by analytical TLC and HPLC, combined, dried, and redissolved in small amounts of DMSO. The compounds were further purified by preparative HPLC using a Waters Xterra reversed-phase C18 semi-preprative column (19 mm × 50 mm, 5 μm) with UV detection at 280 and 410 nm. A 30%−80% CH_3CN in H_2O (0.1% TFA) gradient was

used over 45 min with a flow rate of 3 mL/min. Solvent was removed in vacuo from the collected fractions.

WJ85 (4): light yellow solid; UV (CH_3CN) λ_{max} (log ϵ) 262 (sh) (4.05), 293 (4.22), 376 (3.45) nm; ^1H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 390.0588 (calcd for $C_{19}H_{13}NO_7Na$, 390.0568).

WJ85b (5): yellow solid; UV (CH_3CN) λ_{max} (log ϵ) 248 (3.36), 279 (sh) (3.35), 322 (3.46), 431 (2.99); ^1H and ^{13}C NMR, see Table 2; HRESIMS *m*/*z* 380.0409 (calcd for $C_{19}H_{10}NO_8$, 380.0406).

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