Engineered Biosynthesis of a Novel Amidated Polyketide, Using the Malonamyl-Specific Initiation Module from the Oxytetracycline Polyketide Synthase

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Tetracyclines are aromatic polyketides biosynthesized by bacterial type II polyketide synthases (PKSs). Understanding the biochemistry of tetracycline PKSs is an important step toward the rational and combinatorial manipulation of tetracycline biosynthesis. To this end, we have sequenced the gene cluster of oxytetracycline (oxy and otc genes) PKS genes from Streptomyces rimosus. Sequence analysis revealed a total of 21 genes between the otrA and otrB resistance genes. We hypothesized that an amidotransferase, OxyD, synthesizes the malonanate starter unit that is a universal building block for tetracycline compounds. In vivo reconstitution using strain CH999 revealed that the minimal PKS and OxyD are necessary and sufficient for the biosynthesis of amidated polyketides. A novel alkaloid (WJ35, or compound 2) was synthesized as the major product when the oxy-encoded minimal PKS, the C-9 ketoreductase (OxyJ), and OxyD were coexpressed in CH999. WJ35 is an isoquinolone compound derived from an amidated decaketide backbone and cyclized with novel regioselectivity. The expression of OxyD with a heterologous minimal PKS did not afford similarly amidated polyketides, suggesting that the oxy-encoded minimal PKS possesses novel starter unit specificity.

MATERIALS AND METHODS

Bacterial strains and general techniques for DNA manipulation. Streptomyces coelicolor strain CH999 was used as a host for the transformation of shuttle vectors. Protoplast preparation and polyethylene glycol-assisted transformation were performed as described by Hopwood et al. (23). Escherichia coli XL1-Blue (Stratagene) was used for the manipulation of plasmid DNA. Streptomyces rimosus (ATCC 10970) was obtained from ATCC and was cultured for extraction of genomic DNA. Unmethylated DNA was obtained using the methylation-deficient strain GM2163 (New England Biolabs).

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Tetracyclines are among the most important antibiotics known to mankind in the last half century (15). The broad-spectrum antimicrobial activities of tetracyclines have resulted in their widespread clinical use against infectious gram-positive and gram-negative bacteria (21). The emergence of microbial resistance to tetracyclines has severely limited their effectiveness and has prompted the search for analogs that can overcome the known modes of antibiotic resistance (14, 40, 55). The recently published, elegant total synthesis of 6-deoxytetracyclines by Myers’s group has highlighted the importance of being able to access structurally diverse tetracycline derivatives (13).

Considering the structural complexity of natural tetracycline products, engineered biosynthesis is an attractive route of generating pharmaceutically important analogs (27). Tetracyclines are aromatic polyketides synthesized by soilborne actinomycetes using type II polyketide synthases (PKSs) (49). The carbon skeleton of an aromatic polyketide is assembled from malonate-derived building blocks through iterative Claisen-like condensations catalyzed by the minimal PKS, which consists of the ketosynthase (KS), the chain length factor (CLF, or KS₉), and the acyl carrier protein (ACP) (12). Dedicated tailoring enzymes then transform the carbon backbone into fused, richly substituted compounds. Shunt products of the oxytetracycline pathways have been characterized, which enabled deduction of the putative biosynthetic pathway (Fig. 1) (9, 37, 50). The gene clusters responsible for oxytetracycline (compound 1; oxy and otc genes) and chlorotetracycline have been located on the genomes of Streptomyces rimosus (2, 9) and Streptomyces aureofaciens (43), respectively. For the oxy (otc) cluster, gene sequences for the minimal PKS (28), a putative D-ring cyclase (46), and a downstream anhydrotetracycline (ATC)-oxygenase (45) have been deposited in GenBank.

One of the distinguishing structural features of tetracyclines compared to other aromatic polyketides is the universal presence of an amide unit at one terminus of the polyketide backbone (Fig. 1). Substrate feeding studies have suggested that the amide unit stems from an intact malonate unit (59, 60). The enzymes involved in the biosynthesis of the amide starter unit have not been identified to date. The biosynthesis of the polar amide starter unit is especially interesting from a combinatorial perspective, since all other aromatic polyketides are primed by chemically inert aliphatic and aromatic starter units (41). The polar amide unit can serve as a useful reaction handle for orthogonal semisynthetic modifications of polyketides. Elucidating the formation of the amide group will therefore enable the exploration and engineering of tetracycline biosynthesis as well as expand the repertoire of tools that can be used in combinatorial biosynthesis of other polyketides.

In this work, we report the identification of an amidotransferase, OxyD, encoded in the oxy (otc) gene cluster, that is involved in the biosynthesis of the amidated backbone. Using a heterologous host, we show that coexpression of OxyD with the minimal oxy-encoded PKS affords a novel isoquinolone compound derived from an amidated decaketide backbone.
The cluster are cross-referenced in Table 1. To simplify naming and analysis of the cluster for subsequent studies, the genes were renamed and the putative roles of the proteins were assigned using protein-protein BLAST and Pfam analysis. To obtain the sequence information, primer walking techniques was used to obtain the sequence information. Open reading frames (ORFs) were detected and analyzed using Frameplot software. The biosynthetic pathway was established previously by Hunter and coworkers based on an exhaustive analysis of shunt products. The assignments of the tailoring enzymes are based on their functions obtained through protein-protein BLAST analysis. We confirmed in this report the role of OxyD and the minimal PKS in the biosynthesis of an amidated decaketide. We also confirmed the role of OxyJ as the C-9 ketoreductase.

Sequence of oxy (ote) cluster. The complete genomic DNA library of *S. rimosus* was constructed using a pWEB cosmids cloning kit (Epicenter). The cosmids clone pYT264, which harbors the 21.2-kb oxy gene cluster flanked by *ote1* (18) and *oteB*, was identified by PCR screening. A combination of shotgun and primer walking techniques was used to obtain the sequence information. Open reading frames (ORFs) were detected and analyzed using Frameplot software (http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl), and the putative roles of the proteins were assigned using protein-protein BLAST and Pfam analysis. To simplify naming and analysis of the cluster for subsequent studies, the genes were renamed *oxy* to *ote* in a linear fashion. The five previously sequenced genes of the cluster are cross-referenced in Table 1.

Construction of shuttle plasmids for biosynthesis. The following primers were used to amplify the individual genes: for *oxyA*, 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (PaeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI); for *oxyB*, 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (PaeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI); for *oxyC*, 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (PaeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI); for *oxyD*, 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (PaeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI); and for *oxyP*, 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (PaeI/XbaI) and 5'-GGGTAATAAGAGGAGGACC AGACATGGCAATGATCCTAGGGCCTGCGGCTGCTC-3' (SpeI/XbaI).

TABLE 1. Genes in the oxytetracycline biosynthetic cluster and deduced roles based on sequence homology

<table>
<thead>
<tr>
<th>Gene</th>
<th>Start position</th>
<th>Stop position</th>
<th>Predicted size (kDa)</th>
<th>Cts homolog (reference)</th>
<th>Homologs</th>
<th>Protein (reference)</th>
<th>Deduced role</th>
<th>% Identity</th>
</tr>
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<tr>
<td>oxyTA (ote2)</td>
<td>134</td>
<td>625</td>
<td>18</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>oxyA (oteY1-1)</td>
<td>770</td>
<td>2047</td>
<td>45</td>
<td>TcsD (43)</td>
<td></td>
<td>CAD15553 (52)</td>
<td>Transcription regulation</td>
<td>40</td>
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<tr>
<td>oxyB (oteY2-2)</td>
<td>2044</td>
<td>3312</td>
<td>44</td>
<td>TcsE (43)</td>
<td></td>
<td>ChAb (61)</td>
<td>Ketosynthase</td>
<td>70</td>
</tr>
<tr>
<td>oxyC (oteY1-3)</td>
<td>3388</td>
<td>3675</td>
<td>10</td>
<td>TcsF (43)</td>
<td></td>
<td>Sim4 (20)</td>
<td>Acyl carrier protein</td>
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<td>oxyD (oteY1-4)</td>
<td>3686</td>
<td>5524</td>
<td>69</td>
<td>TcsG (43)</td>
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<td>AsnB (8)</td>
<td>Asparagine synthetase</td>
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<td>oxyE (oteY1-5)</td>
<td>5521</td>
<td>6777</td>
<td>46</td>
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<td></td>
<td>MtmOII (32)</td>
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<td>51</td>
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<tr>
<td>oxyF (oteY2-5)</td>
<td>7846</td>
<td>8089</td>
<td>37</td>
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<td></td>
<td>MtmO1 (32)</td>
<td>Oxygenase</td>
<td>51</td>
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<tr>
<td>oxyG (oteY1-2-4)</td>
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<td>7899</td>
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<td>MtmL (32)</td>
<td>Acyl-CoA ligase</td>
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<td>oxyH (oteY1-2-3)</td>
<td>9833</td>
<td>8241</td>
<td>59</td>
<td>Yes</td>
<td></td>
<td>MtmX (32)</td>
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<td>oxyI (oteY2-1)</td>
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<td>9872</td>
<td>17</td>
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<td></td>
<td>Sim5 (20)</td>
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<td>oxyJ (oteY1-2)</td>
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<td>11384</td>
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<td>Yes</td>
<td></td>
<td>ChaF (61)</td>
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<td>14059</td>
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<td>oxyM (oteD3)</td>
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<td>14835</td>
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<td></td>
<td>MtmM (32)</td>
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<td>oxyN (oteD4)</td>
<td>14855</td>
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<td>28</td>
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<td></td>
<td>ZP00569712</td>
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<td>oxyO (oteD5)</td>
<td>15734</td>
<td>16795</td>
<td>38</td>
<td>No</td>
<td></td>
<td>AfnK (16)</td>
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<tr>
<td>oxyP (oteX3)</td>
<td>17835</td>
<td>18613</td>
<td>35</td>
<td>No</td>
<td></td>
<td>AspB2 (44)</td>
<td>Aminotransferase</td>
<td>56</td>
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<tr>
<td>oxyQ (oteX2)</td>
<td>18911</td>
<td>19782</td>
<td>38</td>
<td>No</td>
<td></td>
<td>ActVA ORF2 (11)</td>
<td>PNP-oxidase</td>
<td>50</td>
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<tr>
<td>oxyR (oteX1)</td>
<td>19336</td>
<td>18908</td>
<td>16</td>
<td>Yes</td>
<td></td>
<td>MtmOIV (32)</td>
<td>Oxygenase</td>
<td>46</td>
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<tr>
<td>oxyS (oteC)</td>
<td>19492</td>
<td>21003</td>
<td>54</td>
<td>Yes</td>
<td></td>
<td>MtmMI (32)</td>
<td>N-Methyltransferase</td>
<td>45</td>
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<td>oxyT (oteZ)</td>
<td>21064</td>
<td>22098</td>
<td>37</td>
<td>Yes</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

Notes:
- a We compared the partially annotated chlorotetracycline PKS cluster (43, 51) from *Streptomyces aureofaciens* with the oxy c-encoded PKS cluster.
- b Gene names in parentheses are loci of the oxy (ote) PKS previously identified through mutant complementation studies. Sequence information for these genes is not available publicly, except for that shown in bold. The cluster was renamed in this study to facilitate sequence analysis. References for previously sequenced genes are listed in the text.
- c No stop codon was detected. The putative protein size was based on alignment with closely related acyl-CoA ligases.
- d A 60-bp deletion at the 3′ end was found in the deposited *oteD1* sequence and has been corrected here.
To study the biosynthetic pathway of a novel amidated polyketide, we sequenced the oxytetracycline (oxy and otc) gene cluster using a combination of shotgun sequencing and cosmid walking techniques. The gene cluster was previously mapped to be between the two resistance genes *otrA* (18) and *otrB* (2, 10, 38). A total of 21 ORFs were clustered between *otrA* and *otrB* (Fig. 2 and Table 1), including the previously sequenced minimal PKS (*otcY1-1, otcY1-2, and otcY1-3*) (28), cyclase (*otcD1*) (46), and ATC oxidase (*otcC*) (2, 45) genes. Sequencing information for the remainder of the cluster, which has been studied through mutant complementation experiments (24), was not available from GenBank prior to this study. To simplify designation of the gene cluster, we named the oxy genes *oxyA* to *oxyT* from end to end, as graphically represented in Fig. 2. The functions of the proteins were assigned based on sequence similarities to known aromatic PKS enzymes and are listed in Table 1.

A putative oxytetracycline biosynthetic pathway was previously established, largely aided by the identification of metabolic shunt products (Fig. 1) (9, 24). The minimal PKS (OxyA, OxyB, and OxyC) accepts a malonamyl starter unit and condenses eight equivalents of malonate to afford an amidated decaketide backbone. Two distinct nitrogen-inserting enzymes (OxyD and OxyO) were found to be encoded in the oxy gene cluster. OxyD is an amidotransferase and participates in the biosynthesis of the amide starter unit (see below). OxyO is homologous to aspartate/tyrosine/aromatic aminotransferases involved in amino acid metabolism (22) and is likely the enzyme that transaminates C-4 of 4-keto-ATC, using pyridoxal 5'-phosphate as a cofactor. OxyR, the putative pyridoxamine 5-phosphate oxidase, is translationally coupled to OxyO with overlapping stop/start codons. A small set of proteins homologous to OxyR are found in the literature, with the most notable being ActVA-ORF2 (11), an enzyme of unknown function involved in actinorhodin biosynthesis.

Two ORFs (*oxyJ* and *oxyM*) encoding NADP-dependent ketoreductases (KR) are present in the gene cluster. OxyJ catalyzes regiospecific C-9 reduction of the oxytetracycline backbone (see below). The specific role of OxyM in the oxy cluster is unresolved (24) and perhaps reflects redundancy in function. Only one KR gene is present in the recently sequenced chlorotetraycline gene cluster (51). The bifunctional cycloase/dehydratase OxyK (OtcD1) was previously identified by Petkovic and coworkers and was assigned to catalyze formation of the D ring (46). OxyN shows strong sequence similarity to second-ring cyclases (such as DpsY from the daunorubicin cluster [33]) and presumably catalyzes the aldol reaction between the C-5 carbonyl and the acidic C-14 meth-
TABLE 2. Plasmid constructs and resulting polyketide products*  

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Genes</th>
<th>Major product</th>
<th>Approx. yield (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYT319</td>
<td>oxyABC</td>
<td>SEK15</td>
<td>30</td>
</tr>
<tr>
<td>pYT318</td>
<td>oxyABCJ</td>
<td>RM20b</td>
<td>30</td>
</tr>
<tr>
<td>pWJ35</td>
<td>oxyABCDJ</td>
<td>Compound 2</td>
<td>20</td>
</tr>
<tr>
<td>pWJ35a</td>
<td>oxyABCDJ</td>
<td>Compound 2</td>
<td>20</td>
</tr>
<tr>
<td>pWJ40</td>
<td>actI ORFs I-II oxyCDJ</td>
<td>Mutacin</td>
<td>20</td>
</tr>
<tr>
<td>pWJ48</td>
<td>tcmKL oxyCDJ</td>
<td>RM20b</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* Streptomyces coelicolor strain CH999 was used as the host for polyketide biosynthesis. Each plasmid is derived from pRM5.

ylene to form the C ring. Subsequent formation of the B ring should be spontaneous, as seen for other aromatic polyketides such as aklanonic acid (33). Formation of the A ring is putatively assigned to methylate C-6 of pretetramid that primes the free amine present in 4-amino-ATC to yield ATC. OxyF is putatively assigned to methylate C-6 of pretetramid that yields 6-methyl-pretetramid. Four oxygenase genes are present in the oxy gene cluster, including that for the previously identified ATC oxygenase OxyS (OtcC) (2, 45). Interestingly, all four oxygenases are homologous to enzymes encoded in the mithramycin gene cluster in Streptomyces argillaceous (32). OxyE, OxyL, OxyG, and OxyS (OtcC) show strong sequence similarities to MtmOII, MtmOIII, MtmOIV, and MtmOIV, respectively, indicating that the oxy and mtm gene clusters are evolutionarily closely related. Prado and coworkers showed that MtmOII is involved in an early hydroxylation step to yield the mithramycin precursor 4-demethylpremithramycinone (47). Thus, OxyL is assigned to catalyze an analogous reaction in the biosynthesis of compound 1, using 6-methyl-pretetramid as a substrate. OxyG is a small (11-kDa) quinone-forming oxygenase and is also homologous to ElmH from the tetranomycin gene cluster (48). OxyG is therefore possibly involved in the quinone formation of ring A in 4-keto-ATC. OxyE is a flavin adenine dinucleotide-dependent monooxygenase and is putatively assigned to catalyze the C-5 oxidation of 5a,11α-dehydrotetracycline to yield 5a,11α-dehydroxytetracycline.

The last step in the enzymatic cascade is the reduction of 5α,11α-dehydroxytetracycline to compound 1. The gene encoding the reductase (TchA) responsible for the same step in chlorotetracycline biosynthesis was mapped outside the chlorotetracycline gene cluster (42). We have used degenerate primers and isolated a homologous ORF in the S. rimosus genome (result not shown).

Enzymes putatively involved in malonamyl starter unit biosynthesis. We previously showed that nonacetate-primed aromatic PKSs contain discrete initiation modules that typically consist of (i) a second ACP (ACP₂) that primes the minimal PKS with the nonacetate unit (34), (ii) a ketosynthase III that synthesizes the ACP₂-bound primer unit (39), and (iii) a potent acetyl-ACP thiolase that hydrolyzes the competing acetyl-ACP species that may otherwise initiate polyketide assembly with acetate (56). An ORF (oxyP) 15 kb downstream of the minimal PKS genes encodes an enzyme of high sequence homology to acetyl-ACP thiolases, such as ZhuC from the R1128 PKS (34) (49% identity) and FrnK from the frenolicin PKS (1) (43% identity). Based on sequence homology, OxyP may play an analogous role in the biosynthesis of compound 1 to ensure correct chain initiation by the malonamyl starter unit.

Surprisingly, no homologs of ketosynthase III and ACP₂ are present in the oxy cluster, suggesting that the oxy-encoded PKS employs a distinct set of enzymes to initiate polyketide biosynthesis. OxyD, encoded immediately downstream of the minimal PKS genes (oxyABC), is a 613-amino-acid protein that shows high sequence identity (60%) to the type II (also known as the Ntn family) asparagine synthases (31). Asparagine synthase converts aspartic acid to asparagus in an ATP-dependent two-step reaction, using glutamine or ammonia as the amine donor (3–5). OxyD contains the conserved N-terminal nucleophilic cysteine (Cys2) which is involved in the hydrolysis of glutamine (64) as well as the conserved adenyl domain that activates the acid moiety of the amine acceptor (6). We hypothesize that OxyD may therefore amidate either malonyl-coenzyme A (malonyl-CoA) or malonyl-ACP to yield malonamyl-CoA or malonyl-ACP (see Fig. 4), which then primes the oxy-encoded KS-CLF for chain elongation.

Biosynthesis of an amidated polyketide by extended minimal oxy-encoded PKS. To examine the roles of OxyD in the biosynthesis of an amidated polyketide in vivo, a series of Streptomyces coelicolor shuttle vectors derived from pRM5 were constructed (35). The plasmids were transformed into S. coelicolor strain CH999 (35) and were analyzed for polyketide biosynthesis (Table 2).

The minimal oxy-encoded PKS (oxyABC), in pYT319 produced ample amounts of SEK15 (see Fig. 4) (53) (30 mg/liter), while the addition of OxyJ (pYT318) yielded reduced decaketide RM20b as the major product (30 mg/liter), consistent with the data reported by Fu et al. (19). OxyJ is therefore the regiospecific C-9 ketoreductase in the oxy-encoded PKS. Therefore, to reconstitute the steps of oxytetracycline biosynthesis, OxyJ was coexpressed in all subsequent studies.

Significantly, a new polyketide product was identified when OxyD was coexpressed with the minimal oxy-encoded PKS and OxyJ. The oxyD gene encoding the amidotransferase was cloned into pYT318 to yield pWJ35. CH999 transformed with pWJ35 produced an intense yellow pigmentation on solid R5 medium that was not observed before. HPLC analysis of the CH999/pWJ35 extract revealed that a new metabolite (WJ35, or compound 2) was synthesized as the major product, with an excellent yield (>20 mg/liter) (Fig. 3). The acetate-primed RM20b product was also synthesized by this strain, although in much lower quantities (<5 mg).

The mass ([M + H]+) of compound 2 was detected to be 388. LC-MS analysis of the CH999/pYT318 extract with positive ion extraction for m/z 388 showed that no trace of compound 2 was produced in the absence of OxyD, confirming the essential role of OxyD in the biosynthesis of compound 2. A sufficient amount of compound 2 was extracted from 2 liters of CH999/pWJ35 fermentation and was purified to homogeneity.
using reverse-phase preparative HPLC and normal-phase thin-layer chromatography. HRMS indicated a molecular formula of C_{19}H_{17}NO_{8} (m/z = 388.1027 [M + H]⁺; difference of 0.0016), consistent with the molecular composition of an amidated decaketide that has been subjected to one ketoreduction at C-9 and two cyclization/dehydration events. When OxyJ was replaced by the heterologous ActIII KR (pWJ35a; Table 2), no change in yield and selectivity of compound 2 was observed.

The structure of compound 2 was elucidated using a combination of 1D and 2D NMR experiments (Table 3) and is shown in Fig. 4. Data obtained from $^{13}$C, distortionless enhancement by polarization transfer (90, 135), and HMQC experiments identified 19 carbon signals, supporting the number of carbon atoms predicted by HRMS. No methyl carbon atom is present, consistent with the lack of an acetate starter unit. The presence of the α-pyrone moiety as a result of O-1/C-5 cyclization is clearly evident from both $^1$H (δ_{H} = 5.3 and δ_{H} = 6.1) and $^{13}$C (δ_{C} = 63) data. The C-6 methylene was assigned using $^{1}$H-$^{13}$C HMBC (Table 3). The linear connectivities among C-6 to C-9 were readily established using correlation spectroscopy and $^1$H-$^1$H HMBC. The $^1$H multiplet at δ_{H} = 4.5 is assigned to H-9 and is nearly identical to the H-9 protons present in mutactin (66) and YT46 (57), with each containing a 9-OH as a result of C-9 ketoreduction (Fig. 4). The assignments of the C-8/C-10 methylenes were readily achieved using correlation spectroscopy, HMBC, and HMQC experiments. The key assignment of the C-7 aliphatic ketone enabled us to eliminate the common C-7/C-12 intramolecular cyclization observed among aromatic polyketides (36). The remaining carbon backbone cyclizes and dehydrates through a C-13/C-18 intramolecular aldol condensation, which is confirmed by the coupling between H-14 and H-16 (J_{HH} = 2.1 Hz). The nucleophilic amide group is thus favorably positioned to attack the electrophilic C-11 to yield, after dehydration, an isoquinolone heterocycle. The sharp proton singlet at δ_{H} = 10.2, which shows long-range coupling to C-16, C-17, and C-18, is assigned to OH-17. The broad singlet at δ_{H} = 10.2 is assigned to the NH proton. These assignments are consistent with the isoquinolone protons observed in the natural product fredericamycin (7). The presence of a lactam ring was unambiguously supported by the $^1$H-$^{15}$N HMBC experiment, in which both H-10 and H-12 showed long-range coupling to the nitrogen (Table 3). Therefore, WJ35 is a shunt product of the oxy-encoded PKS in the absence of the first-ring cyclase.

**OxyD does not interact with heterologous minimal PKSs.** We assayed whether heterologous minimal PKSs that are normally primed by acetate can interact productively with OxyD and OxyC to yield amidate polyketides. The act-encoded KS-CLF (65) and the tcm-encoded KS-CLF (54) were each coexpressed in the presence of Act KR, OxyD, and OxyC in CH999. Heterologous KS-CLFs have been shown to function with the OxyC ACP to produce acetate-primed compounds (26). To our surprise, no amidated polyketides were recovered from these strains (Table 2). We were able to detect only acetate-primed muactin and RM20b from CH999/pWJ40 and CH999/pWJ48, respectively. This is in sharp contrast to the broad compatibility between the R1128 initiation module and heterologous minimal PKSs (57). Hence, fundamental differences exist between oxy-encoded and other minimal PKSs that allow the former to accept both amide and acetate starter units.

**DISCUSSION**

The tetracyclines are universally primed with a unique malonamate starter unit not found in any other polyketides. Past work has cloned five oxy (otc) genes and shed light on the minimal PKS. However, the initiation mechanism that leads to the production of the unique malonamate starter unit is not well understood. The lack of this information has hampered efforts to engineer the initiation of tetracycline biosynthesis. Understanding the biosynthesis and incorporation of the novel amide functionality is therefore a top priority in studying tetracycline biosynthesis.

In this work, we sequenced the previously mapped oxy (otc) gene cluster from *S. rimosus*, which allowed us to propose that OxyD is involved in the formation of the malonamate starter unit. We reconstituted the minimal oxy-encoded PKS in CH999,

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**TABLE 3. Proton and carbon NMR data for WJ35 (compound 2)**

<table>
<thead>
<tr>
<th>Position</th>
<th>$^{13}$C δ (ppm)</th>
<th>$^1$H δ (ppm)</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>165.9</td>
<td>5.35 (d, 1H, 2.1)</td>
<td>C1, C3, C4</td>
</tr>
<tr>
<td>2</td>
<td>90.9</td>
<td>11.68 (s, 1H), OH</td>
<td>C4, C5, C7</td>
</tr>
<tr>
<td>3</td>
<td>172.7</td>
<td>5.09 (d, 1H, 2.0)</td>
<td>C2, C5, C7</td>
</tr>
<tr>
<td>4</td>
<td>105.0</td>
<td>6.09 (d, 1H, 2.0)</td>
<td>C2, C5</td>
</tr>
<tr>
<td>5</td>
<td>160.9</td>
<td>4.92 (3.73, 2H)</td>
<td>C4, C5, C7</td>
</tr>
<tr>
<td>6</td>
<td>204.7</td>
<td>5.07 (2.82, 2H, 5.3)</td>
<td>C7, C9, C10</td>
</tr>
<tr>
<td>7</td>
<td>68.2</td>
<td>4.50 (m, 1H)</td>
<td>C8, C9, C11, C12, N</td>
</tr>
<tr>
<td>8</td>
<td>41.5</td>
<td>2.69 (dd, 1H, 8.0, 14.5)</td>
<td>C8, C9, C11, C12, N</td>
</tr>
<tr>
<td>9</td>
<td>140.7</td>
<td>6.28 (s, 1H)</td>
<td>C10, C11, C13, C14, C18, N</td>
</tr>
<tr>
<td>10</td>
<td>142.8</td>
<td>4.60 (d, 1H, 2.1)</td>
<td>C12, C15, C16, C18</td>
</tr>
<tr>
<td>11</td>
<td>164.9</td>
<td>11.16 (s, 1H), OH</td>
<td>C12, C15, C16, C18</td>
</tr>
<tr>
<td>12</td>
<td>161.7</td>
<td>6.25 (d, 1H, 2.1)</td>
<td>C14, C15, C17, C18</td>
</tr>
<tr>
<td>13</td>
<td>165.2</td>
<td>12.91 (s, 1H), OH</td>
<td>C16, C17, C18</td>
</tr>
<tr>
<td>14</td>
<td>106.2</td>
<td>6.28 (s, 1H)</td>
<td>C16, C17, C18</td>
</tr>
<tr>
<td>15</td>
<td>167.9</td>
<td>10.18 (s, 1H)</td>
<td>NH</td>
</tr>
</tbody>
</table>

* Spectra were obtained at 500 MHz for protons and 125 MHz for carbon and were recorded in acetone-$d_6$ for compound 2.

* Numbering of the carbon backbone is shown in Fig. 4.

* Observed $^1$H-$^{13}$C and $^1$H-$^{15}$N HMBC signals.
as well as an extended minimal PKS including OxyD. Signifi-
cantly, the coexpression of minimal PKS with the C-9 KR and
OxyD produced a unique, amidated polyketide 2 at a high yield
(Table 2). Establishing the origin of the amide unit and con-
structing the complete backbone required for tetracycline bio-
synthesis are significant steps towards rational bioengineering
of this family of compounds. Isolation of this novel isoquin-
olone 2 revealed several important biochemical properties of
the oxy-encoded PKS, as follows.

(i) OxyD is the only enzyme required to biosynthesize and
insert an amide starter unit into the polyketide backbone in the
heterologous host. CH999/pWJ35 produced predominantly the
amidated polyketide, indicating preferential incorporation of a
malonyl unit over acetyl-OxyC by the oxy-encoded minimal
PKS, even in the absence of an acetyl-ACP editing enzyme. A
homologous enzyme (TcsG) from the chlorotetracycline PKS
(43) likely performs the same catalytic function as OxyD dur-
ing chlorotetracycline biosynthesis.

(ii) The amidated compound 2 is derived from an intact
decaketide (10-carbonyl) backbone, indicating that in the het-
erologous host CH999, the oxy-encoded minimal PKS is able to
maintain correct chain length control. We did not find any
truncated polyketide products in the extract of CH999/pWJ35,
presumably because these truncated products are present at
much lower levels in this strain and may have escaped our
purification and detection protocols. We did detect a truncated
polyketide in a pWJ35-derived construct coexpressing OxyK
(OtcD1) (unpublished data), consistent with the conclusion by
Petkovic and coworkers that the oxy-encoded PKS also synthe-
sizes truncated polyketides (46).

(iii) The most surprising structural feature of compound 2 is
perhaps the lack of C-7/C-12 cyclization. The decaketide back-
bone must undergo two unique cyclization steps (C-13/C-18 and
N-19/C-11) to yield the isoquinolone backbone. This is appar-
ently a dominant mode of cyclization, since no alternatively cyclized
amidated polyketides were detected in the fermentation extract.
The cyclization regioselectivity of compound 2 was completely
unanticipated, considering that C-9-reduced, acetate-primed de-
caketides cyclize solely between C-7 and C-12 (as observed in
RM20b/c) (49). From the crystal structures of act-encoded KR
(29) and act-encoded KS-CLF (25), it has been tempting to
propose that the C-7/C-12 connectivity is formed within the
active site of KS, prior to C-9 reduction by the KR. It is evident
from the novel structural features of compound 2 that C-9 keto-
reduction must take place independently of C-7/C-12 cyclization.
OxyJ must therefore recognize an uncyclized polyketide back-
bone to yield compound 2.

(iv) Furthermore, formation of the isoquinolone must take
place immediately after the complete assembly and release of
the polyketide product. Premature C-13/C-18 cyclization will
prevent the correct C-7/C-12 cyclization catalyzed by OxyK
during the tailoring steps of tetracycline biosynthesis (Fig. 1).
Malonate is a very rare metabolite under normal physiolog-
ical conditions and is a toxic compound due to its potent
inhibition of succinate dehydrogenase in the tricarboxylic acid
cycle (30), and hence it is unlikely to serve as a substrate for

![FIG. 4. Biosynthesis of compound 2 by the extended oxy-encoded minimal PKS.](http://aem.asm.org/)](http://aem.asm.org/)
OxyD. Therefore, we hypothesize that biologically plausible malonyl substrates for OxyD are malonyl-CoA and malonyl-OxyC. Malonyl-OxyC may be preferred over malonyl-CoA for the following reasons. (i) Malonyl-CoA is involved in other essential cellular processes, including fatty acid biosynthesis. Amidation of a significant amount of the intracellular malonyl-CoA pool may therefore be detrimental to host strain viability (we did not observe any difference in the growth characteristics of CH999/pWJ35). Malonyl-OxyC, on the other hand, is dedicated to the oxy-encoded PKS and is not involved in the primary metabolism of the host. The robust growth of CH999/pWJ35 supports the above argument that the malonyl-CoA pool is not depleted. (ii) It is known that a cognate ACP-bound acyl substrate has a micromolar Km towards the KS-CLF, while the same acyl-CoA has Km values exceeding 100 μM (56). The lower Km of acyl-ACP is a result of extensive protein–protein interactions between the acyl-ACP and the KS-CLF. Therefore, under in vivo conditions, a significantly lower concentration of malonamyl-ACP than of malonamyl-CoA may be required to prime the KS-CLF. We are currently investigating the substrate specificities of OxyD in vitro.

It is unknown why heterologous pairing of minimal PKSs with OxyD failed to yield amidated polyketides. One likely possibility is that the exclusively acetate-primed KS-CLFs, including the act- and tcm-encoded PKSs, do not tolerate the presence of a polar starter unit in their active sites. Alternatively, OxyD may interact with the KS-CLF heterodimer to shuttles the maloyamyl starter unit to the active site of KS and may require specific residues present on the surfaces of OxyA-OxyB. We have demonstrated that the chain length specificities of KS-CLFs can be drastically altered through rational mutagenesis (58). Therefore, the specificity of oxy-encoded KS-CLF may be similarly engineered to synthesize amidated polyketides of various lengths in coordination with OxyD.

While our in vivo results show that OxyD is a key determinant in the formation of the amide unit in WJ35 and, most logically, oxytetraycline, our results do not completely rule out a late role of OxyD during tetracycline assembly. In this model, the oxy-encoded PKS is primed directly by a malonyl group and proceeds with chain elongation. The acid-primed polyketide can then be amidated by OxyD to yield an amidated polyketide (WJ35) or can undergo spontaneous decarboxylation to yield an acetate-primed compound (RM20b, which will require the oxy-encoded KS to synthesize an 11-carbonyl, C11 backbone). However, it is difficult to hypothesize the exact timing and substrate of the OxyD-catalyzed amidation reaction in this model (e.g., partially elongated, linear, or cyclized). We are currently performing in vitro assays to unequivocally identify the possible malonyl substrates of OxyD, which will provide a definitive mechanism for the formation of the amidated unit.

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REFERENCES


