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A New Mechanism for Benzopyrone Formation in Aromatic Polyketide Biosynthesis

Wenjun Zhang,[†] Burkhardt I. Wilke,[‡] Jixun Zhan,[†] Kenji Watanabe,[§] Christopher N. Boddy,*[,‡] and Yi Tang*[,†]

Department of Chemical and Biomolecular Engineering, University of California at Los Angeles, Los Angeles, California 90095, Department of Chemistry, Syracuse University, Syracuse, New York 13244, and Department of Pharmaceutical Sciences, School of Pharmacy, University of Southern California, Los Angeles, California 90089

Received May 22, 2007; E-mail: cnboddy@syr.edu; yitang@ucla.edu

Aromatic polyketides, an important class of pharmaceutical agents, are all biosynthesized from highly reactive poly-β-keto acid intermediates. Keys to introducing the vast chemical diversity seen in these natural products are the enzymatic and non-enzymatic tailoring chemistries that occur after biosynthesis of the poly-β-keto backbone. In this work, we expand the scope of non-enzyme-catalyzed modifications and show that primary amides can act in vivo as electrophiles, facilitating the formation of benzopyrones. We demonstrate this mechanism can be rationally introduced into an engineered biosynthetic pathway to produce new compounds. This mechanism is of particular note since it demonstrates the use of a "protecting group" in polyketide biosynthesis.

There are two known fates for a primary amide functional group during aromatic polyketide biosynthesis. The amide can remain unreacted through the biosynthetic pathway, as seen with tetracycline.[1] Alternatively, primary amides can be transformed to pyridones, as is seen in the natural products lysolipin,[2] xantholipin,[3] and fredericamycin A.[4] Our previous work with the oxytetracycline minimal polyketide synthase (PKS) has shown that pyridone formation is non-enzymatic.[5,6] Expression of the minimal PKS in conjunction with amidotransferase OxyD in *Streptomyces coelicolor* CH999 generates the decaketide backbone **2** (Scheme 1). In the absence of tailoring enzymes, the novel isoquinoline compound **6** (WJ85) was produced.[6] Addition of the C-9 specific ketoreductase OxyJ led to production of **10** (WJ35).[5] The alkaloid-like benzopyridone structures observed in **6** and **10** are derived from a spontaneous nucleophilic attack of the amide group on a proximal backbone carbonyl, showing the importance of non-enzyme-catalyzed reactivity of the amide group in aromatic polyketide tailoring.

The unique tailoring chemistry observed from the poly-β-keto amide intermediate **2** prompted us to explore the biosynthesis of other nitrogen-containing polyketides through coexpression of additional tailoring enzymes. Coexpression of the bifunctional cyclase/dehydratase OxyK with the minimal PKS, OxyJ, and OxyD in CH999 afforded a new metabolite **15** (WJ78) in exceptionally high yield (150 mg/L). Surprisingly, high-resolution mass spectrometry indicated a molecular formula of $C_{19}H_{12}O_7$ (m/z = 375.0462 $[M + Na]^+$, Δ = 1.9 mmu), lacking the anticipated nitrogen atom. Extensive one- and two-dimensional NMR characterizations were performed to reveal that **15** is a novel dibenzopyrone as shown in Scheme 1. Confirmation of the ester connectivity in **15** was obtained by comparison of the key phenolic ^{13}C NMR signal (δ_{C-11} = 151 ppm) of **15** to synthetic dibenzopyrone and dibenzopyridone standards (Supporting Information). The structure of the dibenzopyrone portion of **15** is related to the well-known fungal mycotoxin alternariol[7,8] and graphislactones.[9]

We hypothesized that the unexpected pyrone formation in **15** occurred via non-enzyme-catalyzed nucleophilic attack of the C-11 phenol in **14** on the amide carbonyl (Scheme 1). Intermediate **14** was formed from **7** via OxyK-catalyzed C-7/C-12 cyclization, dehydration of the first ring, and spontaneous C-13/C-18 cyclization. The intramolecular nucleophilic attack of the C-11 phenol on the amide, forming the six-membered aromatic lactone, is enthalpically favorable and is likely to proceed under in vivo conditions. Intermediate **12** is a key branch point in this pathway and can be processed via two pathways to produce either dibenzopyrone **15** or the benzopyridone analogue. Rapid OxyK-catalyzed dehydration of the first ring converts the electrophilic C-11 ketone in **12** into a nucleophilic phenol, enabling dibenzopyrone formation. If the dehydration activity of OxyK is slowed, spontaneous nucleophilic attack of the amide on the C-11 keto group of **12** can occur, generating the corresponding benzopyridone. This alternate route is analogous to the mechanism for formation of **6** and **10**.

The lack of nitrogen in **15** suggests that the amidotransferase OxyD may not be required for formation of **15**. To test this hypothesis, the minimal PKS, OxyJ, and OxyK were expressed in *S. coelicolor* CH999. This strain did not produce any detectable levels of **15**, indicating the essential role of the amidotransferase in the biosynthesis of **15**. The role of the amide group in the biosynthesis of **15** is akin to a protecting group as used in synthetic organic chemistry. By masking the terminal carboxylate group of the poly-β-keto chain as an amide, spontaneous decarboxylation of C-19 during polyketide processing is prevented. The amide is then removed once decarboxylation is no longer problematic.

To demonstrate that benzopyrone formation is spontaneous under physiological conditions, a simplified model of compound **15** was synthesized (**22**, Scheme 2). Construction of the biphenyl core occurred via Suzuki coupling of the commercially available phenyl boronic acid **16** and the known arylbromide **17**.[8] Pinnick oxidation of the aldehyde gave carboxylic acid **19**. Attempts to directly convert the acid into amide **20** via formation of an activated ester were unsuccessful, leading to exclusive formation of lactone **21**. The unanticipated benzyl deprotection is likely due to activation of the aryl benzyl ether oxygen followed by nucleophilic cleavage of the benzyl group.[10] Amide **20** was generated through a two-step process, avoiding activation of the carbonyl. Esterification of **19** followed by treatment with BuLi and anhydrous NH_3 gave the key amide **20**. To model the aqueous environment in vivo, the benzyl group in **20** was removed with Raney-Ni in aqueous conditions (pH 7.4) to generate the free phenol, which quantitatively displaced the amide, leading to production of benzopyrone **21**. Deprotection of the methoxy groups with BBr_3 afforded the final product **22**.

[†] University of California at Los Angeles.
[‡] Syracuse University.
[§] University of Southern California.

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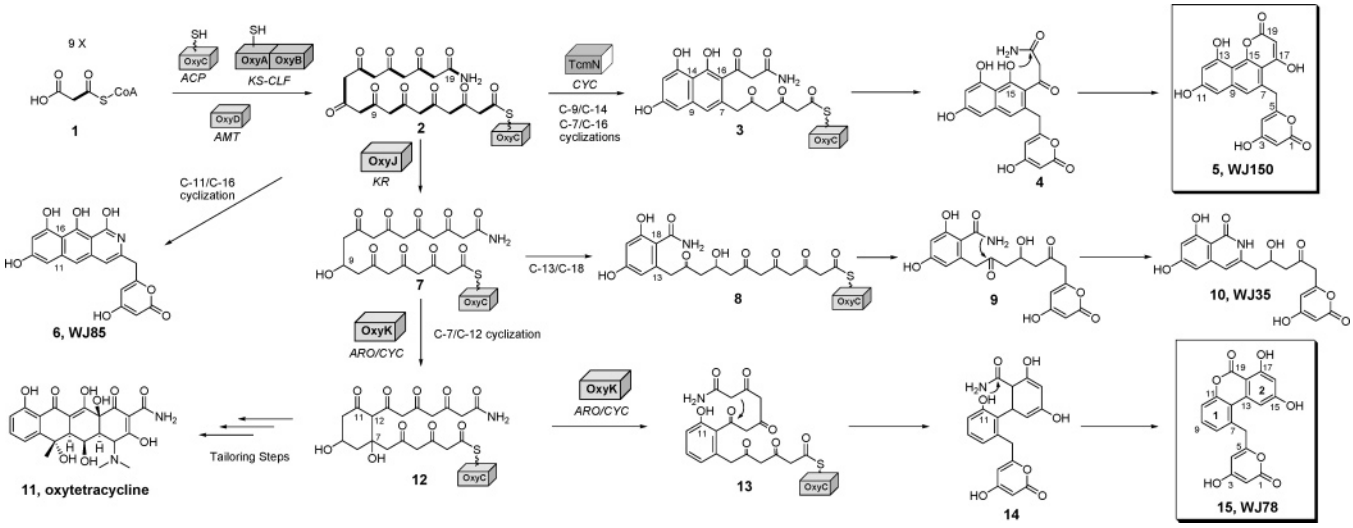
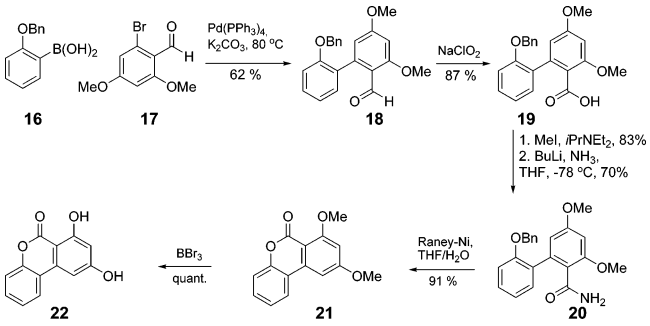
Scheme 1. Biosynthetic Polyketides Derived from Amidated Backbone



Scheme 2. Dibenzopyrone Synthesis via Amide Displacement



This synthetic sequence confirms that the amide group can act as an electrophilic center and be attacked by an intramolecular phenol to generate dibenzopyrone under neutral, low temperature aqueous conditions, further supporting the proposed biosynthetic scheme for **15**.

Having confirmed the feasibility of using the amide group as a precursor for benzopyrone synthesis, we set out to rationally design this reactivity into an aromatic polyketide biosynthetic pathway. We focused on the engineered biosynthesis of benzopyrone **5** (Scheme 1), which is an analogue of the HMG-CoA reductase inhibitor pannorin.[11] We reasoned that the benzopyrone ring (Scheme 1) can be similarly generated from an amidated polyketide intermediate **4**, in which a nucleophilic phenol has been positioned five atoms away from the amide carbonyl at C-19 to facilitate pyrone formation. To satisfy these structural requirements, two intramolecular aldol condensations must take place between C-9/C-14 and C-7/C-16, in contrast to the C-7/C-12 regioselectivity required for the synthesis of **15**. We chose the TcmN cyclase from the tetracenomycin pathway to control cyclization regiochemistry because it has been shown to catalyze the desired cyclizations on an acetate-primed decaketide.[12] When OxyD and TcmN were expressed in *S. coelicolor* with the *oxy* minimal PKS, the expected metabolite WJ150 (**5**) was produced at a titer of 50 mg/L.

In summary, this work demonstrated that, in addition to amide- and pyridone-containing compounds, a primary amide moiety can also serve as an electrophilic center when positioned favorably, readily yielding pyrone-containing polyketides. Benzopyrones are widely present in natural products and are typically formed through esterification of carboxylic acids, intramolecular hydrolysis of enzyme-attached thioesters, or through recently reported oxidative rearrangement.[13] The observed involvement of the amide moiety in pyrone formation may be the mechanism by which pyrones are formed in other aromatic polyketides, including those in the rubromycin family. Interestingly, OxyD-like amidotransferase homologues are present in these gene clusters, while no nitrogen atoms were present in the reported polyketide structures.[4]

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Supporting Information Available: Experimental details, NMR spectroscopy data, and plasmid construction. This material is available free of charge via the Internet at http://pubs.acs.org.

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