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Exploiting the Reaction Flexibility of a Type III Polyketide Synthase through in Vitro Pathway Manipulation

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Polyketides represent a diverse family of natural products found in plants, fungi, and soil bacteria, which have found extensive use as pharmaceuticals (e.g., antibacterials, antivirals, antineoplastics, among others),[1] food ingredients and nutraceuticals,[2] pigments,[3] and veterinary products.[4] Although naturally occurring polyketides have diverse structures, the majority are produced by three broad classes of polyketide synthases (PKSs) sharing a common mechanism involving sequential decarboxylative condensation reactions.[5] While the vast majority of studies have been focused on the multienzyme type I and iterative type II PKSs, the homodimeric type III PKSs, which give rise to a range of aromatic compounds including the flavonoids and chalcones,[6] represent an untapped source of structural diversity. The type III PKS RppA from *Streptomyces griseus*, the first bacterial type III PKS to be characterized, has been shown to possess significant substrate tolerance.[7] RppA catalyzes the sequential decarboxylative condensation, intramolecular cyclization, and aromatization of five units of malonyl-CoA to give 1,3,6,8-tetrahydroxynaphthalene (THN), which spontaneously oxidizes to flaviolin (**1**) upon exposure to air. THN has been hypothesized to be a key intermediate in the biosynthesis of numerous natural products (e.g., prenylated naphthaquinones, echinochrome, and microbial melanin) that possess antibacterial and tumor-cytotoxic activity against antibiotic-resistant pathogens.[8] The kinetics of RppA have been previously reported,[9] and the crystal structure of the protein has been solved.[10] The structure of the active site region appears to indicate the presence of a unique cavity that may enable acceptance of a wide range of starter units with capability for multiple polyketide extensions.

The breadth of natural products arising from RppA suggests that a still wider array of natural product-like compounds can be generated by incorporating RppA into a synthetic metabolic pathway that consists of non-PKS enzymes that also possess broad substrate specificity. To that end, in the current work we have combined peroxidase catalysis with the RppA from *S. coelicolor*[11] to expand the structural diversity of the products from type III PKS catalysis. The approach developed in this work, therefore, provides a new model to exploit biocatalysis in the synthesis of complex natural product derivatives.

RppA catalyzes the condensation of five units of malonyl-CoA to **1** in ~30% yield (Figure 1). We then examined various commercially available acyl-CoA starter substrates in reactions with malonyl-CoA as the extender unit. A total of 23 polyketide products were obtained (Figure 1), and with the exception of the full-length pentaketide flaviolin, all of the products were pyrones based on tri-, tetra-, and hexaketides. The reactivity of the *S. coelicolor* RppA appears to be broader than that of the *S. griseus* enzyme.[7] For example, lauroyl-CoA and benzoyl-CoA served as starter units to provide **2f** and **3f**, and **2h** and **3h**, respectively; neither starter unit appears to react in any more than a trace yield with the *S. griseus* enzyme.[7] Moreover, conversions to RppA-generated products ranged from 40% for isovaleryl-CoA to 27% for lauroyl-CoA and octanoyl-CoA to 14% for butyryl-CoA, based on the starter unit concentration. These relatively high yields lend further support that the enzyme is inherently flexible toward an array of substrates. In most cases, a single starter unit yielded two products (**2** and **3**), indicating different degrees of condensation. Hence, depending on the starter unit, RppA can catalyze differential chain extension.

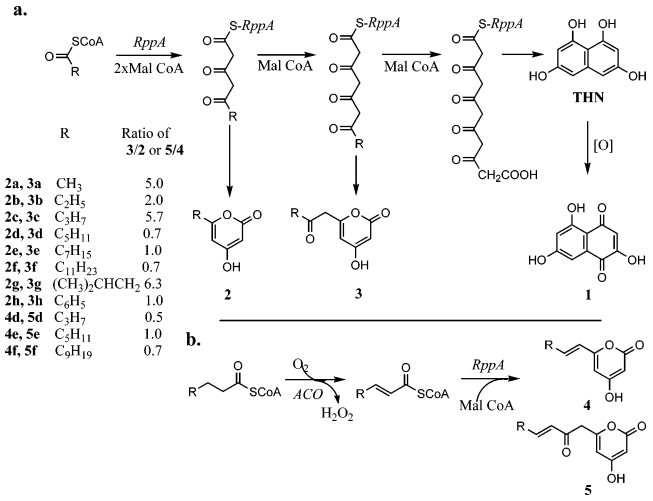

Figure 1. RppA catalysis using various starter CoA substrates. Reactions were performed in 1-mL volumes containing 1 mM malonyl-CoA or 1 mM each of malonyl-CoA and an alternative acyl-CoA starter unit and 0.1 mg of RppA. The reaction mixtures were incubated for 2 h at 37 °C after which 50 μL of 4 N HCl was added to stop the reactions. Products were extracted into 1 mL of ethyl acetate and analyzed by HPLC (see Supporting Information). (a) For alternate CoA starter units, the reaction was primed with the acyl-CoA prior to addition of the malonyl-CoA. (b) For the dehydro-CoA starter units, R = C_5H_{11}, C_7H_{15}, and $C_{11}H_{23}$ were oxidized by ACO prior to incubating with RppA (bottom scheme) to give **4** and **5**. Compounds **2a**, **b**, **f**, and **h**, and **3a**, **b**, **f**, and **h** are novel to RppA.

RppA catalysis demonstrated further flexibility by using unsaturated CoA esters generated by acyl-CoA oxidase (ACO). ACO catalyzes the formation of *trans*-2,3-dehydroacyl-CoA esters (Figure 1).[12] This approach provides potential for creating additional structural diversity, particularly through the chemistry of the alkene functionality. To that end, in the presence of ACO hexanoyl-, octanoyl-, and lauroyl-CoA esters were converted to their respective *trans*-2,3-dehydro-CoA esters that subsequently served as starter units for RppA. Conversions to **4f** and **5f**, for example, ranged from 5 to 7%, which indicate that RppA is able to accept alkenoyl-CoA starter units, albeit with low efficiency.

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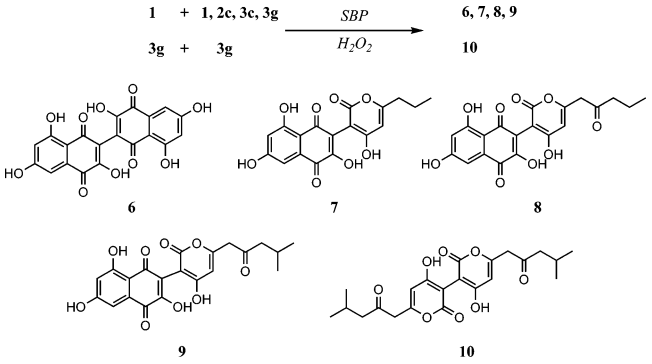

Figure 2. Self- and cross-coupling products catalyzed by SBP from RppA-generated flaviolin and selected pyrones. The bienzymic reaction was performed as described in the text for biflaviolin synthesis, except for reactions with pyrones where a slow feed of H_2O_2 into the reaction mixture (33 μL/h) was performed to minimize biflaviolin formation.

The structural diversity of natural polyketides are in large part due to the various post-PKS tailoring enzymes.[6] To expand beyond the natural polyketides generated solely through RppA catalysis, we sought other enzymes that could accept the wide range of structures generated by the PKS and, in the process, generate a novel in vitro synthetic metabolic pathway. Because flaviolin is a naphthaquinone derivative, we reasoned that it may be a suitable substrate for peroxidases, in general, and the highly active soybean peroxidase (SBP), specifically.[13] To demonstrate the reactivity of flaviolin by SBP, we performed a sequential bienzymic reaction using RppA and SBP.[14] All of the flaviolin generated in the first reaction was converted to the flaviolin dimer, biflaviolin (**6**, Figure 2, conversion of ca. 60% of theoretical maximum). Although biflaviolin has been detected *in vivo*,[15] this is the first report of enzymatic synthesis of biflaviolin *in vitro*, thereby providing evidence that peroxidase-mediated coupling may be involved in the *in vivo* production of biflaviolin.

Encouraged by this result, we examined the self- and cross-coupling of the pyrone products of RppA catalysis, using the pyrone products from butyryl-CoA and isovaleryl-CoA as substrates for SBP. In the presence of the butyryl-CoA products (**2c** and **3c**) cross-coupling of flaviolin (which was present as a coproduct in the RppA reaction) with the two respective pyrones yielded **7** and **8** (Figure 2). The isovaleryl-CoA product (**3g**) underwent both cross-coupling in the presence of the flaviolin coproduct to give **9** and also self-coupling to give the pyrone dimer **10**. The ratio of **9** to **10** was ca. 10:1; hence, cross-coupling was heavily favored. In all cases other than biflaviolin the conversions were 20% of the theoretical maximum. These results demonstrate the ability of PKS products to undergo further transformation to yield higher molecular weight species that have widely different structures.

Another peroxidase with broad specificity is the chloroperoxidase (CPO) from *Caldariomyces fumago*, which catalyzes the chlorination and bromination of a wide range of aromatic and aliphatic compounds, including flavones, in the presence of H_2O_2.[16] We therefore proceeded to perform a sequential bienzymic reaction similar to that described for SBP, except that in the second stage, 20 μg/mL of CPO was added along with 1 mM of H_2O_2 and 40 mM of KCl or KBr. To avoid deactivation of CPO by the relatively high H_2O_2 concentration, we slowly fed the H_2O_2 into the reaction solution. Flaviolin underwent only bromination to give the presumed 3-bromoflaviolin (**11**). Interestingly, the pyrone products from butyryl-CoA and isovaleryl-CoA underwent both chlorination and bromination (Figure 3) to yield several unique products (**12** and **13**).

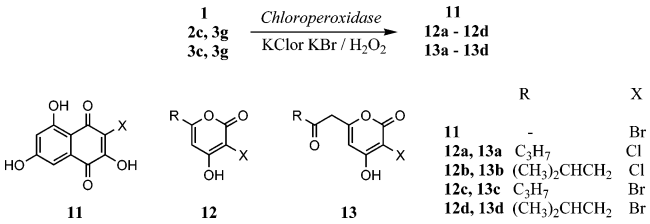

Figure 3. Halogenation of flaviolin, and butyryl- and isovaleryl-based pyrones catalyzed by CPO. The yield of **11** from **1** was ca. 10%.

In summary, we have used the type III PKS RppA *in vitro* to generate flaviolin and 22 structurally different pyrones from malonyl-CoA and other acyl-CoA starter units, including aromatic and unsaturated acyl moieties. The latter was a result of the coupled reaction of acyl-CoA oxidase and RppA. The structural diversity of RppA catalysis was further expanded by coupling RppA with peroxidase catalysis to yield 14 dimeric, chlorinated, or brominated compounds. The coupling of oxidative enzymes with RppA represents an example of the biocatalytic flexibility that extends natural product structural diversity above and beyond native pathway endpoints. Further structural diversity may be achieved by the addition of other enzymes with broad specificity (e.g., hydroxylases and transaminases) in both iterative and combinatorial fashion. This is the subject of our continuing work.

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Supporting Information Available: Cloning and purification of RppA, detailed description of reactions and kinetics, polyketide analysis by HPLC and LC−MS, MS/MS, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

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