In Vitro Biosynthesis of Unnatural Enterocin and Wailupemycin Polyketides[⊥]

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Nature has evolved finely tuned strategies to synthesize rare and complex natural products such as the enterocin family of polyketides from the marine bacterium *Streptomyces maritimus*. Herein we report the directed ex vivo multienzyme syntheses of 24 unnatural 5-deoxyenterocin and wailupemycin F and G analogues, 18 of which are new. We have generated molecular diversity by priming the enterocin biosynthesis enzymes with unnatural substrates and have illustrated further the uniqueness of this type II polyketide synthase by way of exploiting its unusual starter unit biosynthesis pathways.

Enzymes in organic synthesis are typically employed in stereochemical resolution and functional group alteration reactions due to their regiochemical and stereoselective prowess. In the synthesis of polyketide natural products, for instance, biosynthetic enzymes have been utilized as powerful biocatalysts in numerous transformations that include oxidation, glycosylation, and macrocyclization reactions.[1] In some cases, however, de novo syntheses of complex products from simplified building blocks have been reported that nicely illustrate the power of multienzymatic synthesis.[2]

We recently reconstituted the biosynthesis of the antibiotic polyketide enterocin from benzoic and malonic acids, which involved upward of a dozen recombinant and commercial enzymes.[3] By controlling the composition of the biosynthetic enzymes, we were able to produce a series of natural wailupemycin and enterocin polyketides derived from the marine bacterium *Streptomyces maritimus*.[4] In this study we set out to evaluate a collection of benzoic acid substrate analogues in the enzymatic assembly of a library of enterocin and wailupemycin derivatives in which we could control the input of substrates and enzyme biocatalysts.

The enterocin and wailupemycin polyketides are assembled from benzoic acid and seven molecules of malonyl-CoA by the enc type II polyketide synthase (PKS) complex (Figure 1).[5] Initiation of the biosynthetic reaction is carried out by EncN, which catalyzes the ATP-dependent activation and transfer of benzoate to the acyl carrier protein (ACP), EncC. The migration of the benzoyl unit from EncC to the ketosynthase heterodimer EncA-EncB allows for the subsequent malonation of *holo*-EncC by malonyl-CoA:ACP transacylase (FabD), which sets up the first Claisen condensation reaction between the benzoyl and malonyl units. This process is repeated six additional times to yield an octaketide that is further processed by the ketoreductase EncD to yield a pathway intermediate common to the wailupemycin and enterocin natural products. Without further enzyme processing, this reactive intermediate undergoes nonenzymatic cyclization reactions to give wailupemycins D−G. Alternatively, the key rearrangement catalyst EncM oxidatively converts the linear polyketide intermediate in a Favorskii-like reaction into the enterocin tricyclic scaffold. Further tailoring by the *O*-methyltransferase EncK and the cytochrome P450 hydroxylase EncR completes the pathway to enterocin.

All of the enterocin pathway enzymes have been prepared as recombinant proteins and subsequently reconstituted to recapitulate the biosynthetic pathway in a test tube.[3] This provided us the opportunity to compare the synthesis of unnatural enc-based analogues produced in vitro against a prior collection prepared in vivo. We previously exploited the exogenous *S. maritimus* pathway to benzoyl-CoA[6] to create a small library of enterocin and wailupemycin analogues through mutasynthesis in which the disruption of the phenylalanine ammonia lyase *encP* gene allowed for pathway rescue by chemical complementation with a series of aryl acids.[7] However, although the benzoate:CoA ligase EncN displayed a broad in vitro substrate tolerance, this agility was not mirrored in vivo for the whole pathway reconstitution with unnatural primers. Thus in a very controlled manner by limiting substrates and enzyme biocatalysts, we set out to explore whether we could extend the in vivo library.

Figure 1. In vitro biosynthesis and structures of wailupemycins F and G and desmethyl-5-deoxyenterocin (R = benzyl) by recombinant enterocin enzymes. Replacement of the benzoic acid primer with other mono- and disubstituted benzoates and heteroaromatic carboxylates yielded new structural analogues (see Figure 3).

The ultimate success of this enzymatic total synthesis approach with unnatural substrates initially hinges upon the ability of EncN to transfer unnatural substrates to the ACP EncC. We monitored this transfer reaction by FTMS analysis[8] of the *holo*-EncC protein (12 145.11 Da; 12 144.97 Da theor, Δ 0.14 amu). Upon incubation with EncN, benzoate was transferred in typical nonribosomal peptide synthetase fashion, resulting in a +104 Da mass shift to 12 249.55 Da (12 249.00 Da theor, Δ 0.55 amu) (Figure 2A/B). While benzoic acid is the preferred physiological substrate of EncN,[7] as demonstrated with competition binding experiments with salicylic and 2,3-dihydroxybenzoic acids (Figure 2C), in its absence, EncN preferentially activates and loads salicylate (12 265.56 Da;

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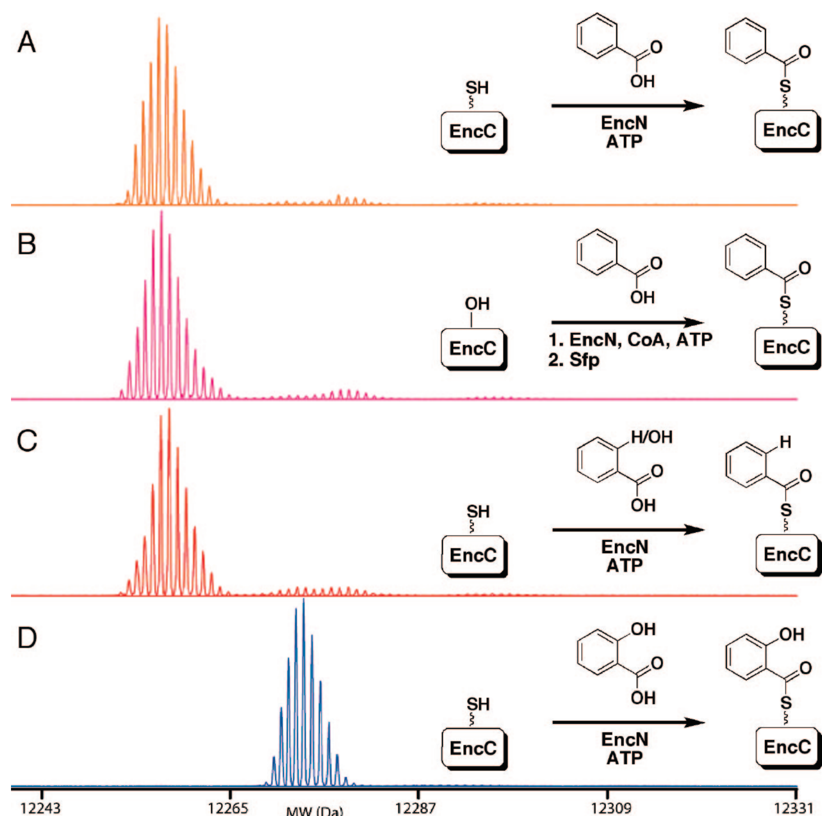

Figure 2. FTMS monitoring of the loading of benzoate and salicylate on the ACP EncC. (A) ATP (10 mM)-dependent transfer of benzoic acid (10 mM) by EncN (2 μM) on *holo*-EncC (20 μM). (B) Sfp-catalyzed phosphopantheteinylation of *apo*-EncC (20 μM) by benzoyl-CoA[7] (~0.25 mM) generated in situ. (C) Same reaction conditions as panel A except that equimolar amounts of benzoic, salicylic, and 2,3-dihydroxybenzoic acids were added. (D) Same reaction conditions as panel A except that benzoic acid was omitted.

12 264.99 Da theor, Δ 0.57 amu), which is +16 Da larger than benzoate (Figure 2D). The HPLC levels of unreacted *holo*-EncC are the same in the benzoate species as in the salicylate species, so not only does EncN activate salicylate, it does so with ease. These ACP ligase data support our prior CoA ligase activity observation that EncN has broad substrate tolerance.[7] We tested initially various aryl acids as primers in a minimal enterocin PKS enzyme assay harboring the $KS_{\alpha/\beta}$ heterodimer EncAB, *holo*-EncC, EncD, EncN, and the *Streptomyces glaucescens* FabD (SgFabD) with the appropriate cofactors and the primer unit malonyl-CoA. The products of this enzymatic transformation when supplemented with the natural primer benzoic acid are wailupemycins D−G,[3] which are formed via the spontaneous cyclization of the reduced nascent polyketide. Alternative incubation with *p*-fluorobenzoate and 2- and 3-thiophene carboxylates gave wailupemycin F and G analogues (**1**−**6**) (Figure 3), which were identical upon HPLC-MS comparison with authentic standards generated by in vivo mutasynthesis.[7] With the knowledge that alternate starters could successfully be incorporated in vitro, the same enzymatic assay conditions were employed to probe whether substrates that were activated in vitro by EncN yet failed in vivo[7] could be successfully extended in vitro. To our delight, five new sets of wailupemycin F and G derivatives were generated, including those derived from 3- and 4-hydroxybenzoates, 3,4-dihydroxybenzoate, *p*-chlorobenzoate, and nicotinate (**7**−**16**) (Figure 3). We had thus successfully synthesized in vitro 10 novel unnatural polyketides and a further six unnatural products that we had previously generated in vivo via mutasynthesis.

Upon the addition of the FAD-dependent favorskiiase enzyme EncM to the minimal enterocin synthase system, we generated a series of desmethyl-5-deoxyenterocin analogues. To confirm the syntheses of the predicted polyketides, HPLC-MS was again employed to analyze the resulting organic extracts generated from the enzymatic incubations. Desmethyl-5-deoxyenterocin analogues primed with *p*-fluoro-, *p*-chloro-, and 3- and 4-hydroxybenzoates and 2- and 3-thiophenecarboxylates were detected in the respective organic enzyme assay extracts by LC-MS (**17**−**22**) (Figures 3 and 4). The extracts also contained the wailupemycin F and G analogues as expected. Further inspection of the fluoro and chloro products revealed additional analogues most likely corresponding to the C-3-epimers of **17** and **18** (Figure 3), respectively, which are related to the natural product 3-*epi*-5-deoxyenterocin, a minor metabolite of the wild-type bacterium.[4] Rearranged analogues primed with 3,4-dihydroxybenzoate or nicotinate, on the other hand, were not evident by LC-MS even though the wailupemycin F and G analogues were indeed produced. Further incubation with cyclohex-1-enecarboxylate was unsuccessful, representing the only example where the in vivo mutasynthesis incorporation of a precursor[7] was not mirrored in vitro.

We speculate that the inability to generate dihydroxybenzoyl- or nicotinoyl-derived desmethyl-5-deoxyenterocin analogues may be due to the instability of the enzyme-bound linear poly-β-keto intermediate or its incompatibility with the favorskiiase EncM. We observed that the in vitro production of polyketides across the board tended to favor the wailupemycin shunt products rather than the rearranged enterocin analogues, which was contrary to that observed with the natural primer benzoate, in which the natural enterocins were the major in vitro products.[3] This may be attributed to the in vitro system being more flexible than the in vivo system and the absence of editing enzymes such as the hydrolase EncL (J.A.K., Q.C., B.S.M., unpublished observations).

In conclusion, we successfully directed the one-pot, total enzymatic synthesis of 24 wailupemycin and enterocin analogues, the majority of which are new chemical entities. Although the enterocin benzoate:CoA ligase EncN has broad in vitro substrate specificity toward other aryl acids, corresponding mutasynthesis experiments yielded only a small collection of unnatural analogues.[7]

Figure 3. Analogues of wailupemycins F and G, desmethyl-5-deoxyenterocin, and 3-*epi*-desmethyl-5-deoxyenterocin generated in vitro using purified proteins.

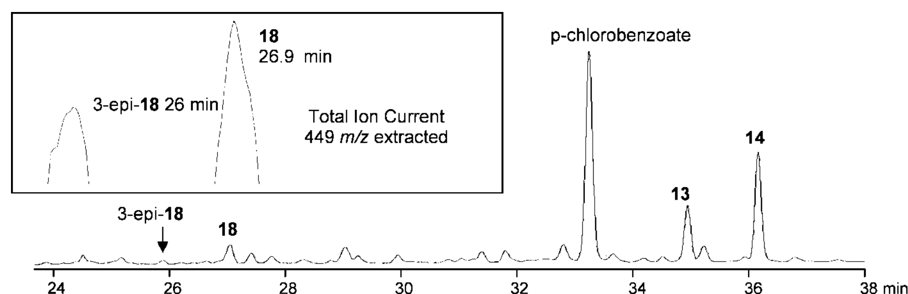

Figure 4. Representative reversed-phase HPLC chromatogram at 340 nm of an organic extract of the EncABCDMN/SgFabD enzymatic incubation of *p*-chlorobenzoate (1 mM) and malonyl-CoA (2.5 mM). Chlorinated products are marked and were analyzed by MS; remaining chromatographic peaks are rather associated with the enzyme/coenzyme mixture.

The in vivo experiment highlighted both the strengths and limitations of precursor-directed biosynthesis with mutant bacteria, as unnatural products were indeed produced, yet the biotransformations were not faithful in the majority of cases. Thus, in vitro total enzyme reconstitution provides an alternative approach to interrogate the dexterity of a biosynthetic pathway without the competing complications of whole cell transformations involving uptake, metabolism, toxicity, and transport. In the suite of experiments described here with alternative priming molecules, we effectively expanded the functional nature of the enterocin starter unit.

Experimental Section

General Experimental Procedures. Protein purification and enzyme assays were performed as described in ref 3. Extracts were analyzed using a Hewlett-Packard 1100 series HPLC system linked to a Hewlett-Packard 1100 MSD mass spectrometer using atmospheric pressure ionization and operating in the positive-ion mode.

In Vitro Synthesis of Wailupemycin Analogues. The in vitro synthesis of wailupemycin analogues followed the published protocol in ref 3 except for the replacement of benzoic acid with 0.5 mM aryl acids. HPLC-MS analysis verified the production of **1** (33.0 min, *m/z* [MH^+] 383.0), **2** (34.5 min, *m/z* [MH^+] 365.0), **3** (31.5 min, *m/z* [MH^+] 371.0), **4** (33.3 min, *m/z* [MH^+] 353.0), **5** (31.3 min, *m/z* [MH^+] 371.0), **6** (33.1 min, *m/z* [MH^+] 353.0), **7** (28.6 min, *m/z* [MH^+] 381.0), **8** (30.8 min, *m/z* [MH^+] 363.0), **9** (27.9 min, *m/z* [MH^+] 381.0), **10** (30.1 min, *m/z* [MH^+] 363.0), **11** (26.4 min, *m/z* [$MH - H_2O^+$] 379.0), **12** (29.4 min, *m/z* [MH^+] 379.0), **13** (35.0 min, *m/z* [MH^+] 399.0/401.1), **14** (36.2 min, *m/z* [MH^+] 381.0/383.0), **15** (21.8 min, *m/z* [MH^+] 366.1), and **16** (23.4 min, *m/z* [MH^+] 348.0).

In Vitro Synthesis of Enterocin and Wailupemycin Analogues. The addition of *holo*-EncM (2 μM) to the reconstituted minimal

enc PKS preceded the addition of the aryl acid primers, per ref 3, to give **17** (24.0 min, *m/z* [MH$^+$] 433.1), 3-epi-**17** (23.0 min, *m/z* [MH$^+$] 433.1), **18** (26.9 min, *m/z* [MH$^+$] 449.0/451.0), 3-epi-**18** (26.0 min, *m/z* [MH$^+$] 449.0/451.0), **19** (20.2 min, *m/z* [MH$^+$] 421.0), **20** (20.0 min, *m/z* [MH$^+$] 421.0), **21** (19.9 min, *m/z* [MH$^+$] 431.0), and **22** (19.2 min, *m/z* [MH$^+$] 431.0).

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