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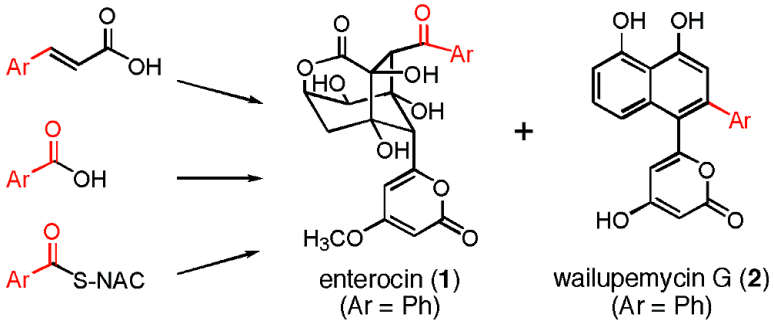

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Mutasynthesis of Enterocin and Wailupemycin Analogues

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Polyketide synthases (PKSs) utilize a wide assortment of starter units, such as short-chain (branched) fatty acids, various alicyclic and aromatic acids, and amino acids, in the assembly of their products.[1] In many cases, the nature of the primer unit provides important structural and biological features to the molecule. Blocked mutants associated with modular type I PKSs that are deficient in starter unit biosynthesis often tolerate unnatural primer units, and in the case of the erythromycin and avermectin systems, the loading acyltransferase can be rather flexible.[2] While modular PKSs can often be reprogrammed to load unnatural primer units to the megasynthase, iterative type II PKSs have not been exploited by mutasynthesis to generate structural diversity as most employ malonyl-coenzyme A (-CoA) as both starter and extender units. We report here the generation of a series of type II PKS products generated through mutasynthesis with assorted aryl acid primer units by manipulating the benzoyl-CoA primed enterocin type II PKS.

Enterocin and the wailupemycins are structurally diverse bacteriostatic polyketides produced by the marine bacterium *Streptomyces maritimus*.[3] This family of iterative type II PKS-derived natural products[4] originates from a common biosynthetic intermediate assembled from a phenylalanine-derived benzoyl-CoA starter unit[5] and seven malonate molecules, and in the case of enterocin and wailupemycins A−C, has undergone a rare Favorskii-like rearrangement.[6] The starter unit is biosynthesized from L-phenylalanine, which is deaminated to *trans*-cinnamic acid by the novel bacterial phenylalanine ammonia lyase (PAL) EncP, followed by a single round of β-oxidation (Figure 1). Feeding experiments with fluorinated benzoic acids in the enterocin-producing bacterium *S. hygroscopicus* No. A-5294 resulted in modest yields of mono- and difluorinated enterocins,[7] hence suggesting that the enterocin PKS can accept unnatural starter units. However, other substituted benzoates were not incorporated into new analogues in *S. maritimus*. To eliminate the natural background production of the benzoyl-CoA starter unit in *S. maritimus*, we inactivated the PAL encoding gene *encP* by double crossover homologous recombination, resulting in a 441-bp in-frame deletion (Figure 2).[8] Although the Δ*encP* mutant *S. maritimus* XP did not produce the benzoate-primed polyketides enterocin and wailupemycins D−G, wild-type levels were completely restored by the addition of cinnamic and benzoic acids.

A series of aryl acids, including monosubstituted benzoates, heteroaromatic carboxylates, and cyclohex-1-enecarboxylate, were administered to the Δ*encP* mutant, and the resulting enterocin and wailupemycin derivatives were characterized by HPLC−MS (Table 1). *p*-Fluorobenzoic acid was the only substituted benzoate that alternated as a starter unit, resulting in 20-fluoroenterocin, 5-deoxy-20-fluoroenterocin, and 19-fluorowailupemycins D−G. 2-Thiophenecarboxylate and 3-thiophenecarboxylate were similarly incorporated into enterocin- and wailupemycin-based metabolites in a 1:2 ratio, whereas other heterocyclic compounds such as furoic acid and nicotinic acid failed to assimilate. Administration of cyclohex-1-enecarboxylic acid, on the other hand, led to the formation of enterocin analogues only. In all cases other than with nicotinic acid, the unreacted aryl acids were reisolated, indicating that the precursor pools were not depleted by competing biochemical reactions. Taken together, these results suggest strict starter unit specificity at the stage of either carboxyl activation by the benzoate:CoA ligase EncN, transfer of the aryl-CoA to either the acyl carrier protein EncC or the ketosynthase α-subunit (KS_α) EncA, or during polyketide assembly itself.

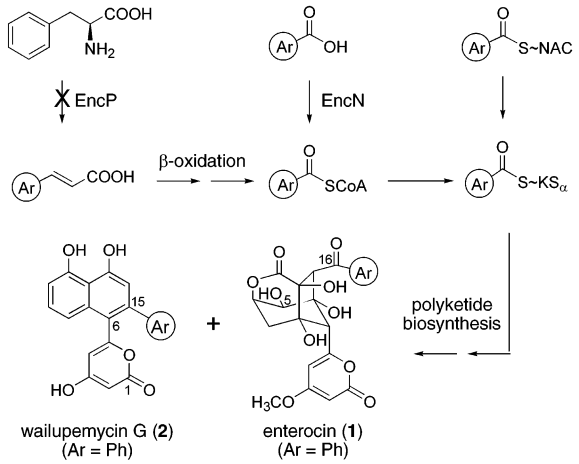

Figure 1. Biosynthesis of enterocin (**1**) and wailupemycin G (**2**) analogues in *S. maritimus* XP from cinnamic acid, benzoic acid, and benzoyl-SNAC derivatives.

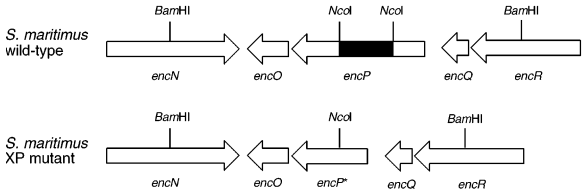

Figure 2. Partial maps of the enterocin biosynthetic gene cluster (*enc*) in the wild-type *S. maritimus* (upper) and the mutant *S. maritimus* XP (lower).

Aryl-CoA thioester mimics based on *N*-acetylcysteamine (NAC) were fed to the Δ*encP* mutant; yet in much the same way as for the free acids, only benzoyl-SNAC and 2-thiophenecarbonyl-SNAC[9] were incorporated into the enterocin and wailupemycin G structures, albeit at lower rates (Table 1). Other derivatives including *p*-toluoyl-SNAC and *p*-chlorobenzoyl-SNAC were not incorporated, suggesting that carbonyl activation may not be the discriminating step in the pathway. This hypothesis was supported by feeding experi-

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Table 1. Retention Times and ESIMS Measurements of Polyketides[a] Generated from Feeding of Cinnamate[b], Benzoate[c] and Benzoyl-SNAC[d] Mimics to *S. maritimus* XP

		1	**2**
Starter Unit Source(s)	Ar	rt (M+H$^+$)	rt (M+H$^+$)
benzoic acid benzoyl-SNAC cinnamic acid		27.8 (444.9)	38.4 (346.8)
p-fluorobenzoic acid *p*-fluorocinnamic acid		29.2 (463.0)	39.1 (365.1)
2-thiophenecarboxylic acid 2-thiophenecarbonyl-SNAC		25.9 (451.0)	38.4 (353.1)
3-thiophenecarboxylic acid		27.1 (451.0)	39.8 (353.1)
cyclohex-1-enecarboxylic acid		26.9 (449.7)	n/d

[a] Retention time and MS data shown in the table is for analogues of enterocin (**1**) and wailupemycin G (**2**) only. Analogues of 5-deoxyenterocin and wailupemycins D−F were also detected for all starter unit sources except for cyclohex-1-enecarboxylate where only enterocin and 5-deoxyenteroicn derivatives were detected. [b] *p*-Methylcinnamate, 3-(2-thienyl)acrylate, and *trans*-3-furanacrylate were not incorporated. [c] *p*-Toluic acid, *p*-anisic acid, 4-chlorobenzoate, 3-bromobenzoate, 3- and 4-hydroxybenzoates, 2-furoic acid, nicotinic acid, phenylacetate, and phthalate were not incorporated. [d] *p*-Toluoyl-SNAC and *p*-chlorobenzoyl-SNAC were not incorporated. rt − LC retention time in minutes. n/d − not detected by LC−MS.

ments with cinnamic acid analogues. Although the feeding of *p*-methylcinnamic acid to the mutant did not yield polyketides, toluic acid was accumulated in the culture broth, thus indicating that it had been β-oxidized directly to toluoyl-CoA, thereby circumventing the ligase EncN (Figure 1). Addition of *p*-fluorocinnamate, on the other hand, efficiently resulted in fluorinated enterocin and wailupemycin molecules.

The substrate specificity of recombinant EncN, which was overexpressed as an NH_2-8xHis tagged protein, was evaluated and remarkably paralleled the in vivo results. EncN has a very high affinity for benzoate (apparent $K_m = 1.1\ \mu M$), which is similar to BadA from the anaerobe *Rhodopseudomonas palustris*.[10] Apparent K_m values of 0.9 μM for ATP and 34.6 μM for CoA were determined and were relatively smaller than those for other benzoate:CoA ligases from *R. palustris*, a denitrifying pseudomonad, and the plant *Clarkia breweri*.[10,11] The substrate specificity of EncN was analyzed by competition assays against benzoate, and product formation was analyzed by LC−MS. EncN converted *p*-fluorobenzoate, cyclohex-1-enecarboxylate, and 2- and 3-thiophenecarboxylates into their corresponding CoA thioesters with the highest relative activities (Table 2), thus mirroring the in vivo results observed with these free acids. EncN showed broad specificity toward most monosubstituted benzoates, heteroaromatic carboxylates, and linear 2-alkenoates, albeit with lower affinity, yet was inactive toward many disubstituted benzoates and cinnamate.

In conclusion, novel enterocin and wailupemycin G analogues were prepared by mutasynthesis in which the natural background of the benzoyl-CoA starter unit was eliminated. In contrast to mutasynthesis experiments with modular type I PKSs which can be rather tolerant toward unnatural starter units,[2] the enterocin iterative type II PKS was more discriminating toward substitute molecules. While the priming mechanism of type II PKSs with nonacetate starter units remains unclear,[1] the specificity of the enterocin PKS system must be downstream of the formation of the substitute aryl-CoA as the CoA ligase EncN has broad substrate specificity and CoA thioesters formed in vivo via β-oxidation from corresponding cinnamates are not incorporated. Mechanistic studies on the transfer of benzoyl-CoA and its analogues to the enterocin type II PKS are presently underway to probe starter unit processing in iterative PKSs.

Table 2. Substrate Specificity of EncN[a]

substrate	rel. act. (%)	substrate	rel. act. (%)
benzoate	100	3,4-dihydroxybenzoate	2
p-fluorobenzoate	17	crotonic acid	2
cyclohex-1-enecarboxylate	16	2,3-dihydroxybenzoate	<1
2-thiophenecarboxylate	16	anthranilate	<1
3-thiophenecarboxylate	14	*m*-aminobenzoate	<1
p-anisic acid	9	*p*-aminobenzoate	<1
p-toluic acid	7	*N*-methylanthranilate	<1
nicotinic acid	5	*o*-anisic acid	<1
4-(chloromethyl)benzoate	3	*m*-anisic acid	<1
tiglic acid	3	4-chlorobenzoate	<1
salicylic acid	3	2-furoic acid	<1
3-hydroxybenzoate	2	pyrrole-2-carboxylate	<1
4-hydroxybenzoate	2	cyclohexanecarboxylate	<1

[a] EncN was inactive toward 3,5-dihydroxybenzoate, 2,5-dihydroxybenzoate, 2,6-dihydroxybenzoate, 3,5-diaminobenzoate, 3-nitrobenzoate, 3,5-dinitrobenzoate, 4-hydroxy-3-methoxybenzoate, phthalate, 3-bromobenzoate, *R*-(−)-thiazolidine-4-carboxylate, thiazolidine-2-carboxylate, cinnamate, phenylacetate, and hippuric acid.

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