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Mutational Analysis of the Enterocin Favorskii Biosynthetic Rearrangement

Longkuan Xiang,[†] John A. Kalaitzis,[†] George Nilsen,[†] Li Chen,[†] and Bradley S. Moore*[,†,‡]

College of Pharmacy and the Department of Chemistry, University of Arizona, Tucson, Arizona 85721

moore@pharmacy.arizona.edu

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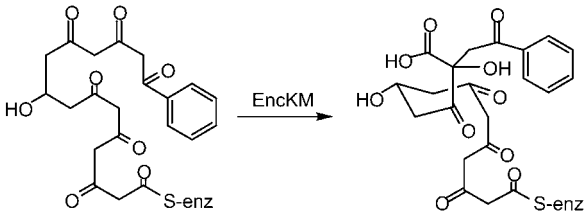
ABSTRACT



A mutational analysis of the enterocin biosynthesis genes revealed that the putative oxygenase and the methyltransferase gene products EncM and EncK, respectively, jointly catalyze a biosynthetic Favorskii-like rearrangement. Inactivation of either gene terminated enterocin production and caused the accumulation of four nonrearranged, nonmethylated polyketides. The structure elucidation of the new wailupemycins E–G is reported.

Enterocin (**1**) and wailupemycins A−C are bacteriostatic polyketides possessing unprecedented carbon skeletons.[1] Biosynthetic studies have shown that these type II polyketide synthase (PKS)-derived products originate from an intermediate assembled from an uncommon benzoyl-coenzyme A (benzoyl-CoA) starter unit[2] and seven malonate molecules that has undergone a rare Favorskii-like rearrangement (Scheme 1).[3] Seto and co-workers demonstrated in 1976 that carbons 1 and 3 of enterocin (**1**) were biosynthetically derived from the same acetate molecule and proposed an intramolecular rearrangement of a linear poly-β-ketoacyl thioester intermediate.[1c] Support that this group of polyketides is derived from a linear intermediate was further provided by the recent identification of wailupemycin D (**3**),[3] a nonrearranged, minor component from the marine actinomycete "*Streptomyces maritimus*". This carbon skeletal rearrangement prevents successive cyclizations via aldol condensations to multiaromatic end products as seen in all other iterative type II PKS systems studied to date.[4] Instead, this derailed type II PKS product gives rise to the enterocin and wailupemycin family of largely chiral, nonaromatic polyketides.

Biosynthetic carbon skeletal rearrangements involving Favorskii-like chemistry have been proposed in only a few secondary metabolic pathways on the basis of feeding experiments with isotopically labeled biosynthetic intermediates. Additional examples include the fungal polyketide aspyrone,[5] the streptomycete antibiotic lankacidin,[6] and the dinoflagellate polyether okadaic acid.[7] In each of these cases,

* To whom correspondence should be addressed at the College of Pharmacy.
[†] College of Pharmacy.
[‡] Department of Chemistry.

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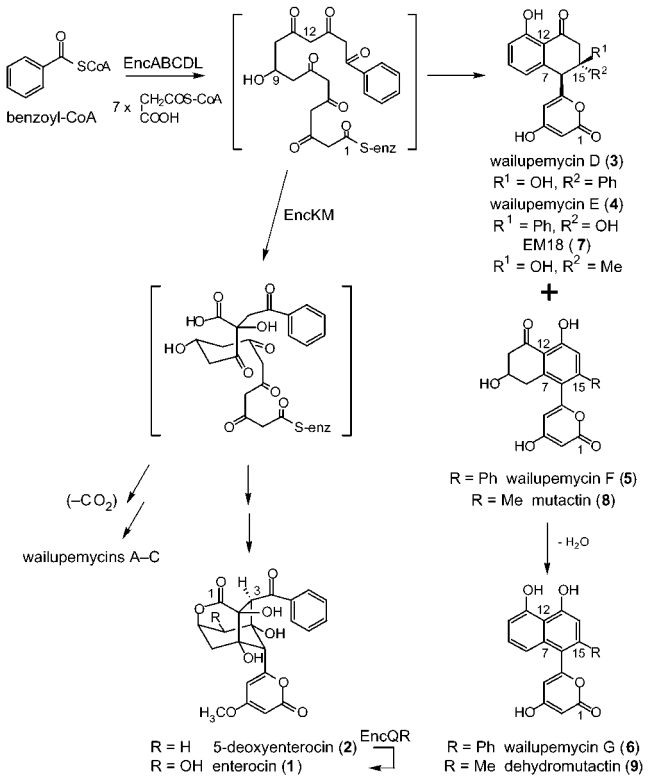
Scheme 1. Structure and Proposed Biosynthesis of Enterocin (**1**), 5-Deoxyenterocin (**2**), and Wailupemycins D−G (**3**−**6**)[a]



[a] The carbons in **3**−**9** are labeled according to their number in the polyketide backbone. Proposed functions of the *enc* gene products are shown for each catalytic reaction. The structures of EM18 (**7**), mutactin (**8**), and dehydromutactin (**9**) are provided for comparison with **3**−**6**. The stereochemistry of **3** and **4** is relative and is unknown in **7**.

the nature of the rearrangement is oxidative, thereby suggesting a catalytic role of an oxygenase.

The recent cloning, sequencing, and heterologous expression of the 21.3 kb enterocin biosynthetic gene cluster from "*S. maritimus*" provides the genetic information to allow the analysis of a biosynthetic Favorskii-like rearrangement.[3,8] The enterocin minimal PKS genes *encABC* are centrally located in the 20 open reading frame (ORF) gene set and are flanked by a number of genes encoding putative polyketide tailoring enzymes (Figure 1). Nucleotide sequence analysis revealed at least four ORFs whose predicted gene products are involved in polyketide tailoring reactions. These include the oxygenases EncM and EncR, the ketoreductase EncD, and the hypothetical methyltransferase EncK. We previously demonstrated that EncR is a cytochrome P450 hydroxylase that converts 5-deoxyenterocin to enterocin and that the ActIII-homologous EncD catalyzes the ketoreduction at C-9 of the nascent polyketide chain (Scheme 1).[8] By default, we proposed that EncM and EncK were responsible for the Favorskii-like rearrangement and the O-methylation of the

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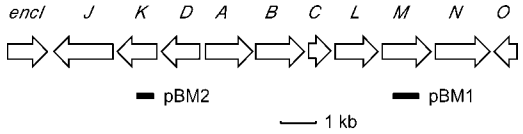

Figure 1. Partial map of the enterocin biosynthetic gene cluster (*enc*) in "*S. maritimus*". Each arrow represents the direction of transcription of an ORF. Plasmids pBM1 and pBM2 contain internal fragments of *encM* and *encK*, respectively, and were used for gene inactivation by single-crossover recombination. The proposed functions of the depicted *enc* genes: *encA*−*encB* (ketosynthase $\alpha\beta$ subunits); *encC* (acyl carrier protein); *encD* (ketoreductase); *encI* (cinnamoyl-CoA hydratase); *encJ* (3-oxo-phenylpropionyl-CoA thiolase); *encK* (methyltransferase); *encL* (acyltransferase); *encM* (oxygenase); *encN* (benzoyl-CoA ligase); *encO* (unknown).

pyrone ring, respectively. Comparison of the deduced amino acid sequence of *encM* with sequences available in the databases revealed significant similarity to a broad group of flavin-adenine dinucleotide (FAD)-dependent oxygenases, suggesting its role as a "favorskiiase". The gene product of *encK*, on the other hand, was weakly homologous to a phosphatidylethanolamine *N*-methyltransferase from *Acetobacter aceti* (GenBank accession number BAA34057) and contained a probable *S*-adenosyl methionine (AdoMet)-binding motif, thereby suggesting its role as a methyltransferase with possible broad substrate specificity.

To establish the functions of EncM and EncK in "*S. maritimus*", the corresponding genes were inactivated by single-crossover homologous recombination (Figure 1).[9] Conjugal transfer of the pKC1139[10]-based temperature-sensitive plasmids pBM1 and pBM2 from *Escherichia coli* S17-1 to "*S. maritimus*" and growth of the resulting transconjugants under selective conditions resulted in the mutant strains KM and KK, respectively.[11] Southern blot hybridization of genomic DNA from the wild type and the mutants with biotinylated DNA probes carrying *encM* or

(9) The genes *encM* and *encK* are present on different transcripts. Their respective downstream genes *encN* and *encJ* are also inactivated as a result of polar effects in each mutant. Inactivation of these starter unit biosynthesis genes resulted in a 0% and 25% reduction in **1** biosynthesis, respectively, suggesting that EncN is extraneous and that other cellular thiolases can complement the loss of the dedicated thiolase EncJ (Xiang, L.; Moore, B. S., unpublished results).

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(11) The *encK* disruption vector pBM2 was constructed as follows. A 479 bp internal fragment of *encK* was PCR amplified from the cosmid clone pJP15F11[8] with the primers 5′-GTGTGTTTCACGCCCTGGGAC-3′ and 5′-CTTCGAGGGCCAGTCCTAC-3′ and ligated into the vector pCR2.1-TOPO (Invitrogen). The *Eco*RI fragment from the resulting plasmid was cloned into pKC1139 to create pBM2, which was conjugated into "*S. maritimus*" type strain BD26T (GenBank AF233338) as described by Bierman et al.[10] The *encK* single-crossover mutant *S. maritimus* KK was selected after propagating transconjugants on SGGP plates at 37 °C, and apramycin-resistant colonies were confirmed by Southern hybridization with biotinylated *encK*. Biotin-labeling and detection of chemiluminescent positives were performed with the DNADetector HPR Southern Blotting Kit (KPL, Inc.) according to the manufacturer. In a similar fashion, the *encM* disruption vector pBM1 was constructed from an 879 bp internal fragment of *encM* amplified with the primers 5′-CGGACACAGCATG-GCCGGGC-3′ and 5′-GTCGTCGGGCACGCGCGCC-3′ and conjugated into "*S. maritimus*" to yield the *encM* single-crossover mutant "*S. maritimus*" KM.

encK verified the gene disruptions. Predicted band shifts were detected in *Nco*I digests of the total DNA (data not shown). The two mutant strains did not exhibit any different phenotypes in comparison to the wild-type strain.

HPLC-MS analysis of an organic extract from the *encMN*-inactivated strain "*S. maritimus*" KM demonstrated that although enterocin was no longer synthesized, four naturally occurring polyketides, including wailupemycin D (**3**), were produced in elevated levels (Figure 2).[12] To our surprise,

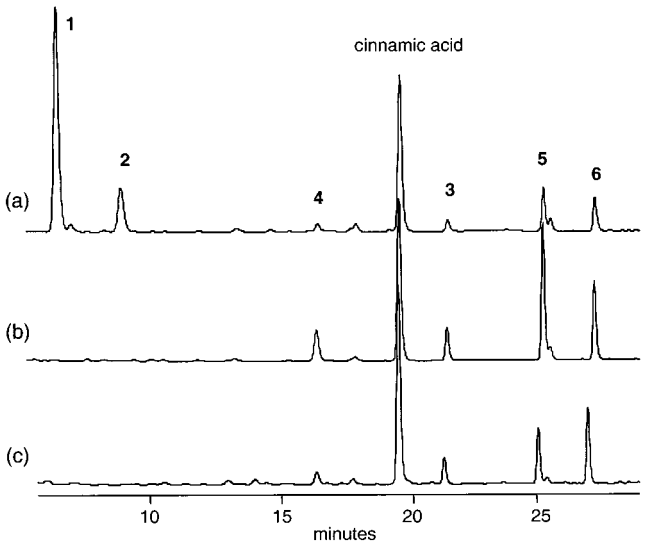

Figure 2. HPLC analysis at 254 nm of crude extracts of (a) wild-type "*S. maritimus*", (b) "*S. maritimus*" KM, and (c) "*S. maritimus*" KK. In addition to polyketides **1**−**6**, cinnamic acid, which is a biosynthetic intermediate in the formation of the benzoyl-CoA starter unit, is observed.

"*S. maritimus*" KK furnished a nearly identical set of polyketides, suggesting a linked role of EncK with EncM in catalyzing the rearrangement and possibly the pyrone methylation as well. Although both strains were administered d_5-labeled benzoic acid, only polyketides from mutant KK were deuterium labeled at 35%. The benzoyl-CoA ligase gene *encN*, which is located immediately downstream of *encM* and whose product EncN catalyzes the activation of exogenously added benzoic acid, is also inactivated in mutant KM. As a consequence, administered d_5-benzoic acid was not incorporated within the polyketides of mutant KM.

Fermentation of "*S. maritimus*" KM provided sufficient quantities for structural elucidation of the three new polyketides wailupemycins E−G (**4**−**6**), two of which were isomers of the previously reported wailupemycin D (**3**).[13] High-resolution FAB MS analyses confirmed that **4** and **5** were isomers of **3** and that **6** was a dehydration product of **3**−**5**. Furthermore, ^{1}H and ^{13}C NMR spectra, along with COSY and gradient-enhanced HSQC and HMBC spectroscopy, supported the presence of common 4-hydroxy-2-pyrone and monosubstituted phenyl rings in **4**−**6** as previously reported for **3** (Table 1).

Table 1. ^{13}C NMR Data of **3**−**6** Recorded in DMSO-d_6

no.	**3**	**4**	**5**	**6**
1	164.7	162.8	163.4	163.8
2	89.9	88.6	89.3	89.3
3	171.6	169.9	169.7	169.9
4	105.3	102.6	105.4	105.8
5	164.1	162.9	159.7	161.0
6	55.4	55.3	123.0	119.5
7	143.3	141.5	142.8	135.0
8	120.4	120.5	35.7	116.1
9	137.4	136.9	64.3	128.8
10	117.3	116.0	46.2	109.3
11	162.3	161.4	204.6	154.4
12	117.2	116.3	115.5	113.4
13	204.5	204.2	162.0	155.8
14	51.5	45.8	116.1	109.7
15	76.2	74.6	149.4	140.2
16	145.9	144.3	139.1	141.1
17/17′	126.3	125.1	128.0	128.5
18/18′	128.9	128.0	128.4	128.3
19	128.2	127.6	128.2	128.4

Wailupemycin E (**4**) was found to be the diastereomer of **3**.[14] Comparison of NMR data with those obtained previously for wailupemycin D[3] was consistent throughout with only minor differences observed. The relative stereochemistry of **4** was established using ROESY data, providing a *syn* relationship between the pyrone and phenyl rings. These

(12) "*S. maritimus*" KK and KM were grown on A1 agar plates containing apramycin (100 μg/mL) and d_5-benzoic acid (0.5 mg/plate) at 37 °C for approximately 24−30 h until sporulation. The cultured agar was chopped and extracted with 95:5 EtOAc/MeOH (50 mL) at room temperature for 1−2 h. The organic solution was dried ($MgSO_4$), filtered, solvent-evaporated, dissolved in MeOH (0.5 mL), and analyzed (5 μL). A Hewlett-Packard 1050 series HPLC system was linked to a Finnigan MAT TSQ 7000 mass spectrometer, using atmospheric pressure chemical ionization and operating in the positive ion mode. A Vydac 218TP52 4.6 mm × 200 mm C_{18} column was used at a flow rate of 0.2 mL/min with a linear solvent gradient of 0.15% TFA in H_2O to 0.085% TFA in MeCN over a period of 40 min.

(13) The crude extract (300 mg) from "*S. maritimus*" KM grown on 40 A1 plates (1 L) was subjected to silica gel flash column chromatography (~10 g, Merck, grade 9385, 230−400 mesh). Four 100 mL fractions consisting of hexane, hexane/EtOAc, EtOAc, and MeOH were sequentially eluted. EtOAc and MeOH fractions were subjected to semipreparative reversed-phase C_{18} (Waters, 20 mm × 250 mm, 10 μm) HPLC employing gradient elution (40% MeOH/0.15% TFA to 100% MeOH over 50 min) at flow rate of 9.5 mL/min. The resulting fractions were pooled on the basis of retention time in the UV chromatogram yielding wailupemycin E (**4**, 5.9 mg), wailupemycin D (**3**, 3.4 mg), wailupemycin F (**5**, 5.4 mg), and wailupemycin G (**6**, 9.4 mg), which eluted at 17, 22, 27, and 28.5 min, respectively.

(14) Data for **4**: α_D −19.9° (0.36 *c*, MeOH). UV (MeOH) λ_{max} (log ϵ) 215 (3.56), 255 (3.25), 332.7 (1.86). ^{1}H NMR (DMSO-d_6) δ (multiplicity, assignment, coupling constants in Hz, *COSY*s, HMBCs, **ROESY**s): 2.93 (d, H14$_S$, J = 17 Hz, *H14$_R$*, C15, **H14$_R$**, **C15 hydroxyl**), 3.95 (d, H14$_R$, J = 17 Hz, *H14$_S$*, C15, **H14$_S$**, **H17/17′**), 4.34 (s, H6, C4, C5, C7, C8, C12, C15, **H4**, **C15 hydroxyl**, **H8**, **H17/17′**), 4.98 (s, H2, C1, C4), 5.67 (s, H4, C2, C5, **H6**, **H17/17′**), 6.10 (s, C15 hydroxyl, **H14$_S$**, **H6**, **H17/17′**), 6.88 (d, H8, J = 7.5 Hz, *H9*, C12, **H6**, **H9**), 6.90 (d, H10, J = 8 Hz, *H9*, C8, C12, **H9**), 7.26 (t, H19, J = 8 Hz, *H18/18′*, C17/C17′), 7.33 (dd, H18/18′, J = 8, 7.5 Hz, *H17/17′*, *H19*, C16, C18/18′), 7.41 (d, H17/H17′, J = 7.5 Hz, *H18/18′*, C15, C17/C17′, C19, **H6**, **H14$_R$**), 7.52 (dd, H9, J = 8, 7.5 Hz, *H8*, *H10*, C7, C11, **H8**, **H10**), 11.6 (s, C3 hydroxyl), 12.5 (s, C11 hydroxyl, C11, C12). See Table 1 for ^{13}C NMR data. HRFABMS m/z = 365.1029 ($C_{21}H_{17}O_6$, Δ + 0.4 mmu).

groups are related *anti* in **3**. Key ROESY correlations included H4 of the pyrone to the ortho hydrogens H17/17′ on the benzene ring and from H6 to the hydroxyl on carbon 15.

The second isomer wailupemycin F (**5**) shared the same carbon skeleton as **3** and **4** but differed in the oxidation pattern of the bicyclic ring system. Detailed NMR spectral analysis showed that **5** is structurally identical to the actinorhodin (*act*) minimal PKS product mutactin (**8**),[15] differing only in the substitution at C15 where **5** and **8** contain phenyl and methyl groups, respectively.[16] This structural difference is reflected in the different choice of starter units, benzoyl-CoA versus acetyl-CoA. All attempts to determine the absolute stereochemistry of **5** at C9 with optically active auxiliary agents were unsuccessful, as previously reported for the related **8**.[15]

The dehydrated analogue wailupemycin G (**6**) was isolated as the major polyketide. The absence of sp^2-hybridized carbons and presence of two phenolic carbon resonances at 154.4 and 155.8 ppm supported the structural assignment of **6** as the dehydrated product of **3**−**5**.[17] HMBCs from protons at δ 6.85 (H10) and 7.40 (H9) to 154.4 ppm (C11) and from δ 6.78 (H14) to 155.8 ppm (C13) confirmed the assignments unambiguously. Furthermore, this structural feature is also present in another *act* PKS product, dehydromutactin (**9**),[18] and shares similar NMR values with it.

The identification of the minor, wild-type components **3**−**6** as the major polyketides in the mutant strains KM and KK further supports that the unprecedented α-oxidation and Favorskii-like rearrangement take place on the linear, C9 reduced octa-β-ketide (Scheme 1). The oxygenase EncM and the putative methyltransferase EncK were identified as two gene products that participate in the novel oxidative rearrangement and perhaps the pyrone O-methylation as well. As linear poly-β-ketides are extremely reactive and spontaneously cyclize,[19] there must be tight coordination between the enterocin PKS complex with the proteins EncM and EncK to ensure efficient transfer of the reactive biosynthetic intermediate. Their removal, as observed in either mutant strain, resulted in the formation of aborted products that likely derive from spontaneous aldol condensations. To a minor extent, this is mirrored naturally as the wild-type strain "*S. maritimus*" also produces these nonrearranged, nonmethylated polyketides, which are structurally related to the *act* minimal PKS products EM18[20] (**7**), mutactin[15] (**8**), and dehydromutactin[18] (**9**). These *act* minimal PKS-derived shunt products are also thought to result from spontaneous cyclizations that occur in the absence of the stabilizing *act* cyclase and aromatase PKS subunits.

The mechanism of these possibly linked biosynthetic events (oxidative rearrangement and methylation) is presently being investigated at the in vivo and in vitro levels. While the role of EncM may involve the C12 oxidation of the linear octa-β-ketide to a trione intermediate, the functional role of EncK remains unclear. On the basis of sequence analysis, EncK appears to bind the biological methylating agent AdoMet. Although used as a cofactor in methyltransferases, AdoMet is also a source of 5′-deoxyadenosyl radicals, suggesting that the rearrangement could be radical in nature. It is tempting to speculate that EncK could provide two biosynthetic functions, first as a radical source and second as a source of a methyl group.

Acknowledgment. We thank the NIH (AI47818) for generous support and Dr. Paul Shipley for assistance with MS and NMR experiments.

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(16) Data for **5**: α_D 3.5° (*c* 0.36, MeOH). UV (MeOH) λ_{max} (log ϵ) 239 (3.79), 281 (3.53), 338 (3.33). ^{1}H NMR (DMSO-d_6) δ (multiplicity, assignment, coupling constants in Hz, *COSYs*, HMBCs): 2.72 (dd, H10a, J = 17, 7 Hz, *H10b*, *H9*), 2.81 (dd, H8a, J = 16.5, 6 Hz, *H8b*, *H9*, C6, C7, C12), 2.99 (dd, H10b, J = 16.5, 3 Hz, *H10a*, *H9*), 3.07 (dd, H8b, J = 17, 2.5 Hz, *H8a*, *H9*, C6, C7), 4.27 (m, H9, *H8a*, *H8b*, *H10a*, *H10b*), 5.25 (s, H2), 5.82 (s, H4, C5), 6.85 (s, H14, C6, C12, C16), 7.25 (d, H17/17′, J = 7.5 Hz, *H18/18′*, C15, C18/18′, C19) 7.36 (m, H18/18′, *H17/17′*, C16, C17, C18/18′), 7.36 (m, H19), 11.76 (s, C3 hydroxyl), 12.73 (s, C13 hydroxyl, C13, C14). see Table 1 for ^{13}C NMR data. HRFABMS m/z = 365.1024 ($C_{21}H_{17}O_6$, Δ −0.1 mmu).

(17) Data for **6**: UV (MeOH) λ_{max} (log ϵ) 215.2 (3.59), 250 (3.53), 339.9 (3.18). ^{1}H NMR (DMSO-d_6) δ (multiplicity, assignment, coupling constants in Hz, HMBCs): 5.30 (s, H2, C1, C4), 5.85 (s, H4, C2, C5, C6), 6.78 (s, H14, C6, C12, C13, C15), 6.85 (d, H10, J = 8 Hz, C8, C11, C12), 7.08 (d, H8, J = 8.5 Hz, C6, C7, C10, C12), 7.29 (m, H18/18′, C16, C17/17′, C18/18′), 7.33 (m, H19, C17/17′), 7.39 (m, H17/17′, C15, C19), 7.40 (m, H9, C7, C11) 11.8 (s, C3 hydroxyl), 12.7 (s). See Table 1 for ^{13}C NMR data. HRFABMS m/z = 347.0925 ($C_{21}H_{15}O_5$, Δ + 0.6 mmu).

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