EncM, a versatile enterocin biosynthetic enzyme involved in Favorskii oxidative rearrangement, aldol condensation, and heterocycle-forming reactions

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The bacteriostatic natural product enterocin from the marine microbe "*Streptomyces maritimus*" has an unprecedented carbon skeleton that is derived from an aromatic polyketide biosynthetic pathway. Its caged tricyclic, nonaromatic core is derived from a linear poly-β-ketide precursor that formally undergoes a Favorskii-like oxidative rearrangement. *In vivo* characterization of the gene *encM* through mutagenesis and heterologous biosynthesis demonstrated that its protein product not only is solely responsible for the oxidative C—C rearrangement, but also facilitates two aldol condensations plus two heterocycle forming reactions. In total, at least five chiral centers and four rings are generated by this multifaceted flavoprotein. Heterologous expression of the enterocin biosynthesis genes *encABCDLMN* in *Streptomyces lividans* resulted in the formation of the rearranged metabolite desmethyl-5-deoxyenterocin and the shunt products wailupemycins D–G. Addition of the methyltransferase gene *encK*, which was previously proposed through mutagenesis to additionally assist EncM in the Favorskii rearrangement, shifted the production to the *O*-methyl derivative 5-deoxyenterocin. The *O*-methyltransferase EncK seems to be specific for the pyrone ring of enterocin, because bicyclic polyketides bearing pyrone rings are not methylated *in vivo*. Expression of *encM* with different combinations of homologous actinorhodin biosynthesis genes did not result in the production of oxidatively rearranged enterocin–actinorhodin hybrid compounds as anticipated, suggesting that wild-type EncM may be specific for its endogenous type II polyketide synthase or for benzoyl-primed polyketide precursors.

flavoprotein | methyltransferase | polyketide synthase | *Streptomyces*

Multicyclic aromatic polyketides such as the clinically important tetracyclines, anthracyclines, and angucyclines are biosynthesized in actinomycetes on heterodimeric type II polyketide synthases (PKSs) (1, 2). These dissociable complexes of monofunctional enzymes contain a "minimal" set of proteins [the two ketosynthase subunits KS_α and KS_β (also referred to as the chain length factor; ref. 3), acyl carrier protein (ACP), and malonyl-CoA:ACP acyltransferase] that are required for the biosynthesis of the polyketide chain. Additional PKS subunits, including ketoreductases (KR), cyclases, aromatases, oxygenases, etc., are responsible for modification of the nascent poly-β-carbonyl intermediate to form the aromatic product. The context-dependent nature of the type II PKS-encoding gene set thus dictates the metabolic outcome of the pathway, which is dominated by planar, polyaromatic natural products.

The bacteriostatic agent enterocin (**1**), however, stands apart from all other type II PKS-derived polyketides (4). Enterocin does not contain a planar polyaromatic core structure, but rather uniquely has a nonaromatic caged core resulting from a branched intermediate. Although the early stages of enterocin biosynthesis have been shown to proceed by means of a typical aromatic polyketide pathway (5) (Fig. 1), labeling (6) and genetic (7) experiments revealed that its biosynthesis involves an unprecedented oxidative rearrangement of its nascent poly-β-carbonyl intermediate. This carbon skeletal Favorskii-like rearrangement prevents successive cyclizations by means of aldol condensations to characteristic multiaromatic end products. The branched intermediate gives rise not only to the tricyclic caged core of enterocin, but also, after decarboxylation, to the structurally diverse wailupemycins A to C (**2**–**4**) (8).

Biosynthetic carbon skeletal rearrangements involving oxidative 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 (9) and the dinoflagellate polyether okadaic acid (10). The recent cloning and sequencing of the enterocin biosynthetic gene cluster in the marine isolate "*Streptomyces maritimus*" has now allowed us to examine this biosynthetic reaction in greater detail (5). Mutational analysis revealed that the putative FAD-dependent oxygenase EncM and the methyltransferase EncK jointly catalyze the rearrangement because inactivation of either encoding gene terminated enterocin (**1**) production and caused the accumulation of the nonrearranged and nonmethylated polyketides wailupemycins D to G (**5**–**8**) (Fig. 1) (7). This result suggested that the rearrangement could be radical in nature, with EncK providing two biosynthetic functions, first as a radical source and second as a source of the *O*-methyl group. In this study, we set out to further examine this highly unusual biosynthetic rearrangement in a series of heterologous expression experiments.

Materials and Methods

General Experimental Details. NMR spectra were recorded on Bruker (Billerica, MA) DRX-300 and DRX-600 spectrometers. ^1H and ^{13}C chemical shifts were referenced to the solvent peak (DMSO-d_6) δ 2.49 and 39.5 ppm, respectively. Standard parameters were used for 1D and 2D NMR spectra obtained, which included ^1H, ^{13}C, heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple-bond correlation (HMBC). Optical rotation was measured on a Jasco (Easton, MD) P-1010 polarimeter. A Waters 600 pump equipped with a Waters 2487 dual λ absorbance detector was used for semi-preparative HPLC separations. A Waters 996 photodiode array detector was used for analytical HPLC. High-resolution fast atom bombardment MS (HRFABMS) were recorded on a JEOL HX110A high resolution mass spectrometer at the Mass Spectrometry Facility, Department of Chemistry, University of Arizona.

Bacterial Strains and General Techniques for DNA Manipulation. *Streptomyces lividans* K4-114 was used as a host for transformation of plasmids (11). *Escherichia coli* XL1-Blue was used for the

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Abbreviations: ACP, acyl carrier protein; KR, ketoreductase; PKS, polyketide synthase; HMBC, heteronuclear multiple-bond correlation; HRFABMS, high-resolution fast atom bombardment MS.

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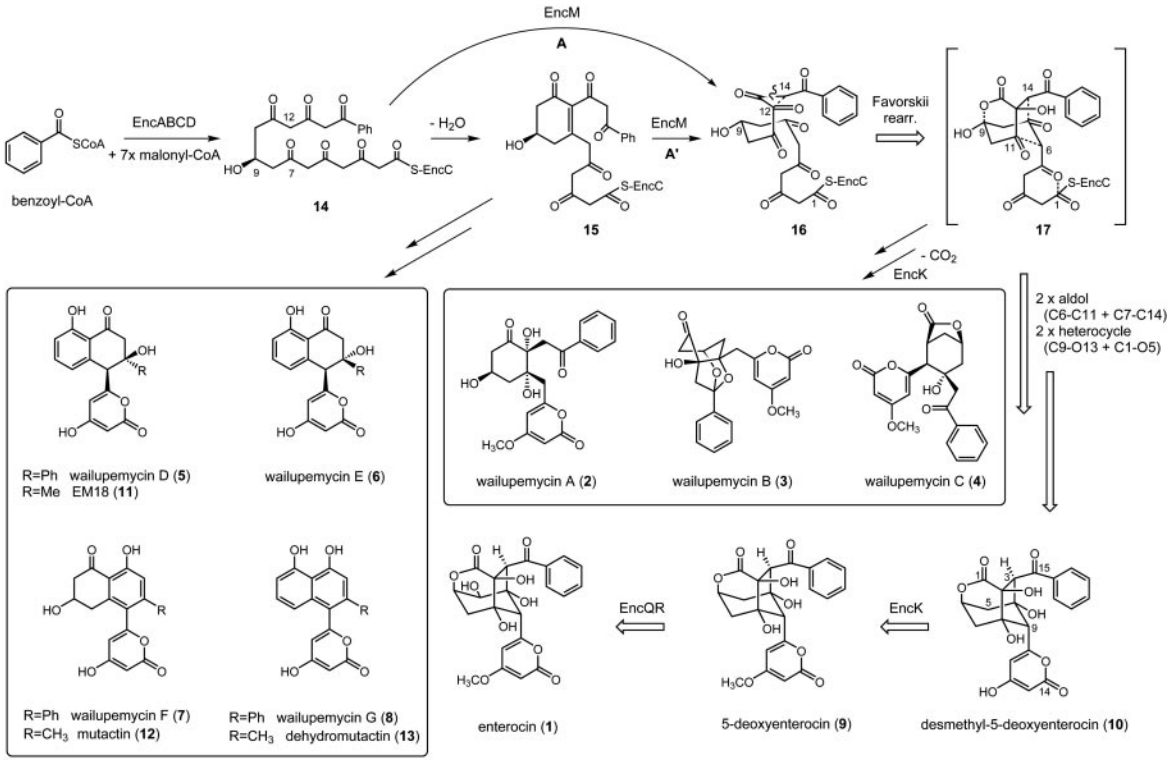

Fig. 1. Proposed biosynthetic pathway to *enc*-based polyketides (**1**–**10**) and structures of analogous *act*-derived polyketides (**11**-**13**). Path A involves monooxidation of **14** to the trione intermediate **16**, whereas path A′ involves a dioxygenase role of EncM in which the C7–C12 olefin of the aldol product **15** is oxidatively cleaved to **16**. The Favorskii-like rearrangement is speculated to occur on a putative ACP (EncC)-bound intermediate. The relative size of the reaction arrows reflects the flux in the pathway to the major product **1** as can be observed chromatographically (see Fig. 2). Dashed lines in structures **16** and **17** represent newly created bonds. In compounds **14**–**17**, the carbon numbering is based on linear **14** in which priority is given to the carboxyl carbon; the numbering in compounds **1**, **9**, and **10** is in contrast based on that defined in ref. 18 for **9**.

manipulation of plasmid DNA. *Streptomyces coelicolor* A3(2) was used to extract genomic DNA for cloning *act* genes, and the cosmid clone pJP15F11 (5), which harbors the entire *enc* biosynthesis gene cluster, was used as the source of DNA for cloning *enc* genes. The *E. coli–Streptomyces* shuttle pSEK4 and pRM5-based vectors were used for all expression experiments in *Streptomyces* (3). Recombinant DNA procedures were performed by standard techniques (12, 13). Restriction enzyme-digested DNA fragments were recovered from agarose gels with the QIAquick DNA Purification Kit (Qiagen, Valencia, CA). Oligonucleotides were obtained from Sigma Genosys. PCR was carried out on a PTC-2000 Peltier thermal cycler (MJ Research, Cambridge, MA) with PfuTurbo (Stratagene) DNA polymerase. DNA sequencing by BigDye terminator cycle sequencing reaction using an ABI 377 sequencer (Applied Biosystems) was performed at the Laboratory of Molecular Systematics and Evolution at the University of Arizona.

Construction of Expression Plasmids. pBM43 is a derivative of pMP6 (14) in which the *encD* gene downfield of the *actIII* promoter was replaced by a 1.6-kb fragment harboring *encDK*. The *encDK* cassette was PCR amplified from the cosmid clone pJP15F11 (5) with the primers 5′-GGTTAATTAACCGGCCGCCCCGACGAAGG-3′ (forward, *Pac*I site underlined) and 5′-GGTGGCCCCGGACGTCATGC-3′ (reverse), and cloned into pCR-Blunt (Invitrogen) to create pBM41. The sequence-verified plasmid was sequentially digested, first with *Pac*I, blunt-ended with shrimp alkaline phosphatase, and secondly with *Xba*I. The resulting 1.6-kb fragment was ligated into the *Xba*I and blunted *Nde*I sites of pHGF7505 (15) to create pBM42. A 3.2-kb *Pac*I–*Hin*dIII fragment containing *encDK* and the divergent *act* promoters was cloned into analogous sites of pMP6 to create pBM43.

pBM47 is a pSEK4 derivative in which a 6.1-kb *Pac*I/*Eco*RI fragment harboring *actI-ORFsI,II,III* and *encMN* was cloned into the analogous sites. A 3.0-kb fragment containing *actI-ORFsI,II,III* was PCR amplified from *S. coelicolor* A3 (2) genomic DNA by using primers 5′-GCATTAATTAAGCGGCGGTCGAAGGGAGATG-3′ (forward, *Pac*I underlined) and 5′-GCCTCTAGACCACCCGGTGTTCTCCCGG-3′ (reverse, *Xba*I underlined) and cloned into pCR-Blunt to create pBM44. The 3.0-kb *Xba*I-*Hin*dIII fragment harboring *act*I-*ORFsI,II,III* from pBM44 was ligated into the analogous sites of pBM45 to create pBM46. pBM45 is a pCR-Blunt-derived plasmid in which an *Xba*I/*Nhe*I 3.1-kb fragment containing *encMN* was cloned into the analogous sites. *encMN* was PCR amplified from pJP15F11 by using primers 5′-GACTCTAGAGCGCCCGTCGTGGCGCCG-3′ (forward, *Xba*I underlined) and 5′-GCAGCTAGCTGGTCACACGGCGTGGGC-3′ (reverse, *Nhe*I underlined).

*Pac*I-*Hin*dIII fragments from pMP6 (harboring *encD*), pBM43 (harboring *encDK*), and pRM5 (harboring *actIII*) were separately cloned into the analogous sites of pBM47 to create pBM48, pBM49, and pBM50, respectively.

pBM56 and pBM57 are derivatives of pBM55 in which *Pac*I/*Hin*dIII fragments of pMP6 and pBM43 harboring *encD* and *encDK*, respectively, were cloned into the analogous sites. pBM55 is a pSEK4-derived plasmid in which the *act* genes contained within the *Pac*I/*Eco*RI site were replaced by a *Pac*I/*Eco*RI cassette harboring *actI-ORFsI,II,III-encMNK* from pBM54. The *actI-ORFsI,II,III-encMNK* cassette was constructed from a *Nhe*I-*EcoR*I fragment harboring *encK* from pBM51,

which was ligated into the pCR-Blunt-derived pBM45 (harboring *encMN*) to create pBM53. An *Hin*dIII-*Xba*I fragment from pBM52 harboring *actI-ORFsI,II,III* was then cloned into the analogous sites of pBM53 to create pBM54. pBM51 is a pCR-Blunt-derived plasmid harboring *encK* that was PCR amplified from pJP15F11 by using primers 5′-GTCGCTAGCAGTTACTGACGCTTCGAGTG-3′ (forward, *Nhe*I underlined) and 5′-GGTGGCCCCGGACGTCATGC-3′ (reverse).

Culture Conditions and Purification of Polyketides. Plasmids were introduced into *S. lividans* K4-114, and transformants were selected and grown on solid R2YE with 20 μg/ml thiostrepton as described (14). Spores from single colonies were used as inoculum for liquid fermentations in R2YE containing 20 μg/ml thiostrepton. Cultures were grown at 30°C with shaking (250 rpm) for 4–5 days. Where appropriate, benzoic acid (0.8 mM) was added to the culture after 24 h. Liquid cultures were acidified to pH 5 with 1 M HCl and then extracted with EtOAc. Extracts were dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. Polyketide production was monitored by analytical HPLC as described (14).

The *S. lividans* K4-114/pBM43 crude extract (294 mg) from a 1-liter fermentation was subjected to stepwise normal-phase silica gel (Merck, 230–400 mesh) flash column chromatography with 1:1 hexane/EtOAc, EtOAc, 9:1 EtOAc/MeOH, 1:1 EtOAc/MeOH, and MeOH. Each fraction was analyzed by reversed-phase C_{18} analytical HPLC as described (14). The fraction eluting with 1:1 EtOAc/MeOH was subjected to HPLC purification. Compounds were purified by using a YMC-Pack ODS-A HPLC column (YMC, Kyoto) (250 × 20 mm, 10 μm), employing a gradient from 20% to 70% MeOH in 0.15% trifluoroacetic acid (TFA) over 75 min at a flow rate of 9.5 ml/min with UV detection at 254 nm. Desmethyl-5-deoxyenterocin (**10**, 9.3 mg) eluted between 12.5 and 14 min.

S. lividans K4-114/pBM57 was similarly grown and extracted, yielding 250 mg of crude extract, which was subjected to flash column chromatography as described above. The EtOAc/MeOH fraction was separated by using the same column as above, employing a gradient from 40% MeOH in 0.15% trifluoroacetic acid to 100% MeOH over 50 min. B26 (**20**, 14 mg) and dehydroSEK4b (**21**, 1.5 mg) eluted at 20 and 21.5 min, respectively.

Desmethyl-5-Deoxyenterocin (10). α_D −21.4° (c 0.042, MeOH); ^{1}H NMR (DMSO-d_6) δ (multiplicity, assignment, coupling constants, HMBCs): 1.63 (brd, H7$_{eq}$, J = 13.2 Hz, C5, C6, C8, C9), 2.06 (brd, H5$_{eq}$, J = 14.4 Hz, C3, C4, C6, C7, C9), 2.26 (dd, H7$_{ax}$, J = 14.1, 3.0 Hz, C2, C8, C9), 2.65 (dd, H5$_{ax}$, J = 15.0, 4.2 Hz, C3, C4, C9, C15), 4.01 (s, H3, C1, C2, C4, C5, C8, C9, C10, C15), 4.58 (s, H9, C3, C4, C5, C7, C8, C10, C11), 4.83 (brs, H6, C1, C4, C8), 5.29 (d, H13, J = 1.8 Hz, C11, C12, C14), 6.22 (d, H11, J = 1.8 Hz, C9, C10, C12, C13), 7.50 (t, H18/18′, J = 7.8 Hz, C16, C18/18′), 7.60 (t, H19, J = 7.8 Hz, C17/17′), 7.79 (d, H17/17′, J = 7.8 Hz, C15, C17/17′, C19), 11.7 (s, 12-OH, C10, C11, C12, C13); ^{13}C NMR (DMSO-d_6) δ: 36.6 (C7), 39.3 (C5), 54.5 (C9), 60.9 (C3), 72.6 (C6), 75.9 (C4), 77.0 (C8), 79.7 (C2), 89.0 (C13), 104.9 (C11), 127.9 (C17/17′), 128.4 (C18/18′), 132.4 (C19), 139.4 (C16), 162.9 (C10), 163.7 (C14), 170.1 (C12), 174.0 (C1), 195.1 (C15); HRFABMS m/z = 415.1025 ($C_{21}H_{19}O_9$ [M+H]$^+$, 415.1029 calculated).

B26 (20). ^{1}H NMR (DMSO-d_6) δ (multiplicity, assignment, coupling constants, HMBCs): 2.28 (s, H16, C14, C15), 4.35 (s, H6, C4, C5, C7, C8, C12), 5.17 (d, H2, J = 1.8 Hz, C1, C3, C4), 5.56 (d, H4, J = 1.1 Hz, C2, C3, C5, C6), 6.01 (s, H14, C12, C16), 6.72 (d, H8, J = 2.2 Hz, C6, C9, C10, C12), 6.77 (d, H10, J = 2.6 Hz, C8, C9, C11, C12), 10.8 (s, 9-OH, C7, C8, C9, C10), 11.5 (s, 3-OH, C1, C2, C3, C4, C5); ^{13}C NMR (DMSO-d_6) δ: 19.4 (C16), 37.3 (C6), 88.2 (C2), 99.5 (C4), 102.0 (C10), 110.6 (C14), 113.7 (C12), 117.4 (C8), 137.4 (C7), 159.2 (C11), 161.2 (C9), 163.9 (C1), 164.4 (C15), 165.7 (C5), 170.4 (C3), 177.6 (C13); HRFABMS m/z = 301.0712 ($C_{16}H_{13}O_6$ [M+H]$^+$, 301.0712 calculated).

Table 1. Plasmid constructions and resulting polyketide products in *S. lividans* K4–114

Plasmid	Genes	Major products
pMP6	*encABCLMN/encD*	**5–8, 10***
pBM43	*encABCLMN/encDK*	**5–10***
pBM47	*actI*-ORFsI–III-*encMN*	**18, 19**†
pBM48	*actI*-ORFsI–III-*encMN/encD*	**11–13**‡
pBM49	*actI*-ORFsI–III-*encMN/encDK*	**11–13**‡
pBM50	*actI*-ORFsI–III-*encMN/actIII*	**11–13**‡
pBM55	*actI*-ORFsI–III-*encKMN*	**18, 19**†
pBM56	*actI*-ORFsI–III-*encKMN/encD*	**11–13**‡
pBM57	*actI*-ORFsI–III-*encKMN/encDK*	**18–21**

*In the presence of 0.8 mM benzoic acid.
†**20** and **21** produced in trace amounts.
‡**18** and **19** produced in trace amounts.

DehydroSEK4b (21). ^{1}H NMR (DMSO-d_6) δ (multiplicity, assignment, HMBCs): 2.64 (s, H16, C10, C14, C15), 3.87 (s, H6, C4, C7, C8), 5.28 (s, H2, C1, C3, C4), 6.09 (s, H8, C6, C7, C9, C10), 6.16 (s, H4, C2, C3, C5, C6), 6.60 (s, H12, C10, C11, C13, C14), 6.62 (s, H14, C10, C12, C13, C16), 10.6 (s, 13-OH, C12, C13, C14), 11.8 (s, 3-OH, C2, C3, C4); ^{13}C NMR (DMSO-d_6) δ: 22.4 (C16), 37.0 (C6), 89.2 (C2), 100.5 (C12), 101.9 (C4), 112.1 (C8), 114.3 (C10), 116.8 (C14), 141.7 (C15), 159.5 (C11), 160.8 (C5), 161.1 (C7), 161.6 (C13), 163.9 (C1), 170.2 (C3), 178.0 (C9); HRFABMS m/z = 301.0714 ($C_{16}H_{13}O_6$ [M+H]$^+$, 301.0712 calculated).

Bioconversion of Desmethyl-5-Deoxyenterocin to 5-Deoxyenterocin. Desmethyl-5-deoxyenterocin (1 mg) dissolved in DMSO (0.1 ml) was added to a 1-day-old 30-ml culture of *S. lividans* K4-114/pBM43. After an additional 24 h, the culture was extracted with EtOAc and analyzed by analytical HPLC as described above, showing an ≈30–40% conversion to 5-deoxyenterocin.

Results

Identification of *encM* as the Enterocin Favorskiiase-Encoding Gene. On the basis of our earlier report on the identification of the genes *encM* (FAD-dependent oxygenase) and *encK* (methyltransferase) as playing a joint role in the enterocin favorskii rearrangement in *S. maritimus* (7), we set out to heterologously express these genes with those encoding the enterocin minimal PKS to further elucidate this biosynthetic event. We earlier prepared a series of pRM5-based expression plasmids for expression in the engineered host strain *S. lividans* K4-114 and demonstrated that the minimal enterocin PKS requires its endogenous KR EncD for activity (14). With this in mind, we constructed the plasmid pBM43 by introducing the *encDK* gene cassette into pMP6 (14) downstream of the *act*III promoter (Table 1). This plasmid, which additionally carries the *encABCLMN* gene cassette downstream of the divergent *act*I promoter, contains the machinery for the synthesis of the starter unit benzoyl-CoA from exogenous benzoic acid by the benzoate:CoA ligase EncN (16, 17), its loading onto the enterocin ACP EncC, the heterodimeric $KS_{\alpha,\beta}$ EncA-B, and the acyltransferase EncL. This expression plasmid was introduced by means of transformation into the *S. lividans* K4-114 (11) and grown in a benzoate-enriched medium. As projected, the *S. lividans* K4-114/pBM43 transformant produced 5-deoxyenterocin (**9**) as the major product together with the nonrearranged polyketides wailupemycins D to G (**5-8**) (Fig. 2*A*), thereby establishing that no other gene

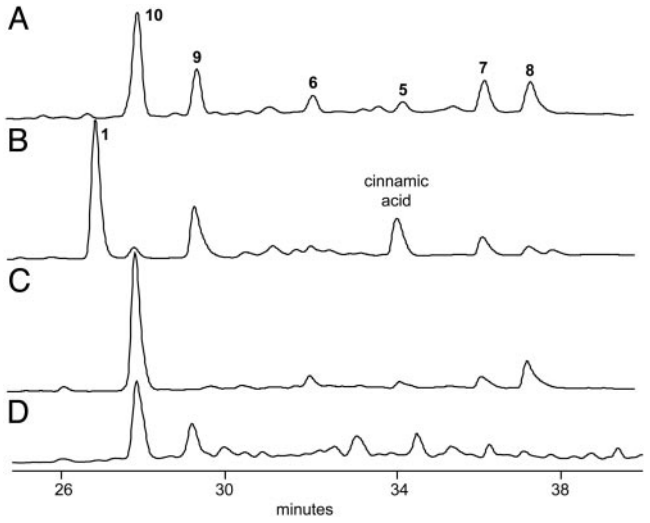

Fig. 2. HPLC analysis at 254 nm of crude extracts from *S. lividans* K4-114/pBM43 plus benzoic acid (trace A), wild-type *S. maritimus* (trace B), *S. lividans* K4-114/pMP6 plus benzoic acid (trace C), and the biotransformation of **10** to **9** in *S. lividans* K4-114/pBM43 (trace D). Cinnamic acid is an intermediate in the biosynthesis of the benzoyl-CoA starter unit from L-phenylalanine (17) as seen in trace A.

product is required for the rearrangement and subsequent cyclization reactions. In addition to these compounds, a new compound (**10**) was produced when the transformant was fermented in liquid R2YE media rather than on solid agar plates in ≈100-fold excess.

Reversed-phase HPLC purification of compound **10** from a 1-liter fermentation provided 9.3 mg for structure characterization. Comparison of the ^{1}H and ^{13}C NMR data with those for 5-deoxyenterocin (18) suggested that **10** was the desmethyl derivative. The absence of the methyl singlet at δ 3.80 in the ^{1}H NMR spectrum supported the loss of the pyrone methyl group. Furthermore, a new, exchangeable proton signal at δ 11.7 showing HMBC correlations into the pyrone confirmed that the pyrone was not methylated, and thus the structure could be assigned as desmethyl-5-deoxyenterocin (**10**). This assignment was further established by HRFABMS with a molecular formula of $C_{21}H_{19}O_9$ (m/z = 415.1029 $[M+H]^+$, Δ −0.4 millimass units). HPLC analysis of the wild-type *S. maritimus* strain showed that **10** is a very minor natural constituent that was never characterized (Fig. 2*B*) (8).

Assignment of EncK as an *O*-Methyltransferase. In contrast to our earlier *in vivo* mutagenesis experiments (7), the heterologous expression experiment implies that the oxygenase EncM is solely responsible for catalyzing the Favorskii rearrangement and that EncK may act only as a methyltransferase with rearranged polyketide substrates (Fig. 1). To test this new hypothesis, we recultured the Δ*encK* mutant *S. maritimus* KK in liquid media, because this mutant when previously grown on solid media produced only wailupemycins D to G and not desmethyl-5-deoxyenterocin (7). HPLC analysis confirmed our new suspicion, because **10** was produced by liquid cultures of the Δ*encK* mutant (data not shown). We next turned our attention to the transformant *S. lividans* K4-114/pMP6 (*encABCLMN*/*encD*), which, when grown on solid media, produced only wailupemycins D to G (14). Once again, when fermented in liquid media, **10** was additionally produced (Fig. 2*C*). These two experiments clearly showed that the influence of fermentation conditions led initially to the incorrect functional assignment of the *encK* gene product (7).

The role of EncK as an *O*-methyltransferase was further confirmed through a biotransformation experiment in which **10** was added to a culture of *S. lividans* K4-114/pBM43 (*encABCLMN*/*encDK*). In the absence of supplemental benzoate, this transformant does not produce *enc*-based polyketides. Thus, when pure **10** was added to the fermentation, we observed by HPLC the production of 5-deoxyenterocin (Fig. 2*D*), thereby confirming that methylation is the penultimate step in enterocin biosynthesis. EncK is most homologous to AurI, another Streptomycete polyketide pyrone *O*-methyltransferase (19), with a similarity/identity score of 54/36 and has been shown to complement an *aurI* genetic mutation in aureothin biosynthesis (J. He, C. Hertweck, L.X., and B.S.M., unpublished observation).

Combinatorial Biosynthesis of Hybrid Enterocin–Actinorhodin Constructs. The antibiotic actinorhodin, whose type II PKS has served as a prototype system in bacteria, is an all malonyl-CoA-derived octaketide (1, 2). Although actinorhodin and enterocin are structurally dissimilar upon initial inspection, their biosynthetic pathways share many early features. The *act* PKS shunt products EM18 (**11**), mutactin (**12**), and dehydromutactin (**13**) are identical to wailupemycins D (**5**), F (**7**), and G (**8**), respectively, except for the nature of the starter unit, acetate versus benzoate (Fig. 1) (7). Different sets of polyketide tailoring reactions are responsible for taking these compounds down such diverse biosynthetic paths. We thus set out to probe whether the favorskiiase EncM could be used to derail other type II PKSs, such as the *act* PKS, from producing aromatic polyketides to rather synthesize acetate-primed enterocin-like compounds. A series of hybrid *act–enc* gene combinations were thus constructed in pRM5 and pSEK4-based *act* PKS expression vectors (3) and expressed heterologously in *S. lividans* K4-114 (Table 1).

The nonreduced shunt octaketides SEK4 (**18**) and SEK4b (**19**) are products of the minimal *act* PKS ActI-ORFsI-III when expressed in the absence of the KR ActIII (1, 2). We reengineered the plasmid pSEK4 (3) by replacing the *actVII* (cyclase) and *actIV* (aromatase) genes with *encMN* and *encKMN* to give pBM47 and pBM55, respectively. In both cases, *S. lividans* K4-114 transformants produced only the known *act* compounds SEK4 and SEK4b (Fig. 3), suggesting that the enterocin favorskiiase EncM and the *O*-methyltransferase EncK were not able to use the *act* minimal PKS products as substrates. To evaluate whether a C9 ketoreduced *act* substrate is preferred, much like in *enc* biosynthesis, we next expressed the *act* minimal PKS with either its endogenous KR ActIII or with the homologous enterocin KR EncD, which has been successfully used before in conjunction with the *act* PKS (5). Several plasmids containing the *act* minimal PKS and a KR with different combinations of the genes *encKMN* (Table 1) all yielded roughly the same polyketide profile when expressed in *S. lividans* K4-114 (data not shown). In each case, in addition to the KR-reduced *act* polyketides mutactin, dehydromutactin, and EM18, the nonreduced SEK4 and SEK4b compounds were measured at various levels along with two new compounds. These molecules were isolated by reversed-phase HPLC and characterized by MS and NMR. Proton and carbon NMR data, along with gradient-enhanced heteronuclear multiple quantum correlation (HMQC) and HMBC spectroscopy, quickly revealed that **20** and **21** were dehydrated analogs of SEK4 and SEK4b, respectively, which was supported in each case by HRFABMS. Dehydro SEK4 (aka B26, **20**) was previously characterized from the heterologous expression of the naphthocyclinone PKS, although its structure was never assigned by NMR (20). Key HMBC correlations supporting the structure **20** were observed from the H14 olefin at δ 6.01 to the aromatic carbon at δ 113.7 (C12) and the methyl carbon at δ 19.4 (C16). Similarly, the placement of the olefin in dehydro SEK4b (**21**) was established through HMBC correlations from the H-8 olefin at δ 6.09 into the benzene ring and the C-6 methylene bridge to the

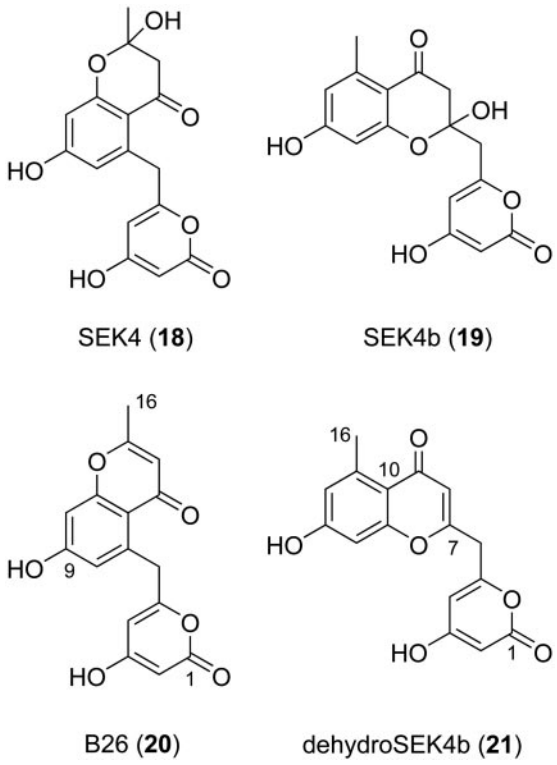

Fig. 3. Structures of *act*-derived polyketides **18**–**21** from the expression of *S. lividans* K4-114/pBM57.

pyrone. Interestingly, the levels of the nonreduced polyketides SEK4 and SEK4b and their dehydrated counterparts increased relative to the KR-reduced compounds as the number of *enc* genes was enlarged. The *enc*-tailoring proteins EncK and EncM may compete with the KR ActIII or EncD in complexing with the minimal *act* PKS, thereby perturbing the ketoreduction process. In no case, unfortunately, did we detect oxidatively rearranged or *O*-methylated *act* products.

Discussion

The *encM* gene encodes a putative 464-aa flavoprotein with remarkable catalytic properties. Sequence analysis suggests that the ''favorskiiase'' EncM has a covalent attachment of FAD at a conserved histidine residue at position 78, which is reminiscent of many oxidases (21, 22), including those involved in the biosynthesis of the natural products mitomycin (23) and griseorhodin A (24). Flavoenzymes are very versatile catalysts and are involved not only in the activation of oxygen for oxidation and hydroxylation reactions, but also in dehydrogenation reactions, in light emission, and in one- and two-electron transfer reactions (25). Flavoprotein-catalyzed oxidative reactions associated with post-PKS modifications include hydroxylations, epoxidations, Baeyer–Villager rearrangements (26), and now Favorskii rearrangements.

The enterocin flavoprotein EncM is implicated in this study not only to catalyze the Favorskii-like oxidative rearrangement, but also must facilitate the two aldol condensation and two heterocycle-forming reactions in the formation of desmethyl-5-deoxyenterocin (**10**). In the course of this series of chemical transformations, at least five new chiral centers and four new rings are generated. The presumed EncM precursor is the linear *enc* PKS C9-reduced octaketide **14** that is oxidized at C12 to form the 11,12,13-trione intermediate **16** (Fig. 1, path A). If EncM functions rather as a dioxygenase, then oxidation of the cyclohexenone intermediate **15** similarly yields **16** (Fig. 1, path A′). Hydration of the C13 carbonyl and scission of the C13–C14 bond with concomitant formation of a new C12–C14 linkage generates the hypothetical Favorskii product **17** bearing a chiral α-hydroxy acid center. This branched intermediate formally undergoes two aldol condensations between C6–C11 and C7–C14 and in the process generates chiral centers at each position. Although cyclases catalyze such aldol condensation reactions during aromatic polyketide assembly (1, 2), the *enc* gene cluster is a notable exception among all other known type II PKS gene sets for its absence of typical cyclase and aromatase encoding genes (5). We hypothesize by virtue of the formation of **10** that EncM likely facilitates these C–C-forming reactions by orienting **17** in a conducive conformation, because linear poly-β-ketides are extremely reactive and spontaneously cyclize (27, 28). The lactone C–O bond between the carboxylate oxygen and C9 is also generated during the course of **10** biosynthesis. On the basis of the presumed 9*R*-configuration that is inferred from the structures of the wailupemycin A and B shunt products (8, 29), the C–O linkage should be generated with inversion of stereochemistry with displacement of the acetate-derived C9 hydroxyl group. Pyrone formation with hydroysis of the thioester linkage to the EncC ACP completes the biosynthetic transformation to **10**. Because the activity of EncM has not yet been reconstituted *in vitro*, there remains the possibility that other host proteins may contribute to the transformation *in vivo*.

We previously proposed on the basis of a gene knockout experiment that the *encK* gene product also participates in the rearrangement reaction (7). Further experimentation in this study, however, conclusively showed this not to be the case with the characterization of **10**. Primary sequence analysis of EncK demonstrates that it belongs to a distinct family of *S*-adenosyl-L-methionine-dependent *O*-methyltransferases involved in the methylation of macrolide antibiotics and plant phenylpropanoids.

Although the *encM* gene product has now been shown to solely catalyze a remarkable series of biosynthetic events during enterocin biosynthesis, its use as a combinatorial biosynthetic reagent to generate ''unnatural'' natural products may unfortunately be limited. This statement is, however, based upon a single observation with the actinorhodin PKS in which the addition of *encM* did not redirect the post-PKS tailoring reactions of the *act* pathway, suggesting that wild-type EncM may be specific for its endogenous type II PKS or for benzoyl-primed polyketide precursors. An in-depth biochemical characterization of EncM will be necessary to probe its mechanism and to evolve it into a suitable reagent for the engineered biosynthesis of new chemical entities.

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