Reviews

Plant-like Biosynthetic Pathways in Bacteria: From Benzoic Acid to Chalcone[1]

Bradley S. Moore,*[,†,‡] Christian Hertweck,[‡,⊥] Jörn N. Hopke,[†,‡,∥] Miho Izumikawa,[†] John A. Kalaitzis,[†] George Nilsen,[†] Thomas O'Hare,[†,▽] Jörn Piel,[‡,#] Paul R. Shipley,[†,⊗] Longkuan Xiang,[†] Michael B. Austin,[§] and Joseph P. Noel[§]

Division of Medicinal Chemistry, College of Pharmacy, P.O. Box 210207, University of Arizona, Tucson, Arizona 85721, Department of Chemistry, P.O. Box 351700, University of Washington, Seattle, Washington 98195, and the Structural Biology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037

Received May 17, 2002

Although phenylpropanoids and flavonoids are common plant natural products, these major classes of biologically active secondary metabolites are largely absent from bacteria. The ubiquitous plant enzymes phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) are key biosynthetic catalysts in phenylpropanoid and flavonoid assembly, respectively. Until recently, few bacterial counterparts were known, thus reflecting the dearth of these plant natural products in bacteria. This review highlights our progress on the biochemical and genetic characterization of recently identified streptomycete biosynthetic pathways to benzoic acid and type III polyketide synthase (PKS)-derived products. The sediment-derived bacterium "*Streptomyces maritimus*" produces benzoyl-CoA in a plant-like manner from phenylalanine involving a PAL-mediated reaction through cinnamic acid during the biosynthesis of the polyketide antibiotic enterocin. All but one of the genes encoding benzoyl-CoA biosynthesis in "*S. maritimus*" have been cloned, sequenced, and inactivated, providing a model for benzoate biosynthesis not only in this bacterium, but in plants where benzoic acid is an important constituent of many products. The recent discovery that bacteria harbor homodimeric PKSs belonging to the plant CHS superfamily of condensing enzymes has further linked the biosynthetic capabilities of plants and bacteria. A bioinformatics approach led to the prediction that the model actinomycete *Streptomyces coelicolor* A3(2) contains up to three type III PKSs. Biochemical analysis of one of the recombinant type III PKSs from *S. coelicolor* demonstrated activity as a 1,3,6,8-tetrahydroxynaphthalene synthase (THNS). A homology model of THNS based upon the known three-dimensional structure of CHS was constructed to explore the structural and mechanistic details of this new subclass of bacterial PKSs.

Introduction

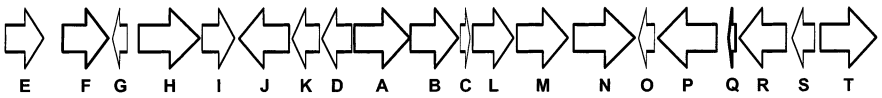
Phenylpropanoids and flavonoids are major classes of natural products commonly associated with plants. These secondary metabolites are structurally diverse, serve important physiological functions in plants, and exhibit numerous pharmacological properties. The ubiquitous plant enzymes phenylalanine ammonia-lyase (PAL, EC 4.3.1.5)[2] and chalcone synthase (CHS)[3] are key biosynthetic catalysts in phenylpropanoid and flavonoid assembly, respectively, and until recently were thought to be largely restricted to plants. The absence of phenylpropanoids and flavonoids in bacteria apparently mirrors the absence of these two key enzymes.

Our laboratory recently characterized biosynthetic pathways closely related to these formerly plant-restricted routes in actinomycetes. We discovered that the sediment-derived bacterium "*Streptomyces maritimus*" produces benzoyl-CoA in a plant-like manner from phenylalanine involving a PAL-mediated reaction through cinnamic acid during the biosynthesis of the polyketide bacteriostatic agent enterocin.[4−7] All but one of the genes encoding benzoyl-CoA biosynthesis in "*S. maritimus*" have been cloned, sequenced, and inactivated, providing a model for benzoate biosynthesis not only in this bacterium, but in plants, where it is an important constituent of numerous natural products.

Although a *bone fide* CHS has not yet been characterized in a bacterium, homologues belonging to the CHS superfamily of plant condensing enzymes called type III polyketide synthases (PKSs) have recently been discovered.[8] Total genome sequencing of several bacteria has identified numerous plant-like PKSs of unknown functions. For instance, the model actinomycete *Streptomyces coelicolor* A3(2) contains up to three copies of a type III PKS gene. Thus far, type III PKSs have been shown to be responsible for the biosynthesis of natural products such as 1,3,6,8-tetrahydroxynaphthalene (THN)[9,10] and the formation of key components of more complex molecules such as the antimicrobial agent vancomycin.[11−13] Progress on the biochemical and structural characterization of THN synthase (THNS) in *S. coelicolor*, as well as its putative role in the

* Address correspondence to this author at the University of Arizona. Tel: 520-626-6931. Fax: 520-626-2466. E-mail: moore@pharmacy.arizona.edu.
† University of Arizona.
‡ University of Washington.
⊥ Present address: Hans-Knoell-Institute for Natural Products Research, Beutenbergstr. 11a, 07745 Jena, Germany.
∥ Present address: Aventis Cambridge Genomics Center, 26 Landsdowne St., Cambridge, MA 02139.
▽ Present address: Vollum Institute, Oregon Health & Sciences, Portland, OR 97201.
Present address: Max Planck Institute of Chemical Ecology, Department of Bioorganics, Winzerlaer Str. 10, D-07745 Jena, Germany.
⊗ Present address: Department of Chemistry, Okanagan University College, 3333 College Way, Kelowna, B.C. V1V 1V7, Canada.
§ The Salk Institute for Biological Studies.

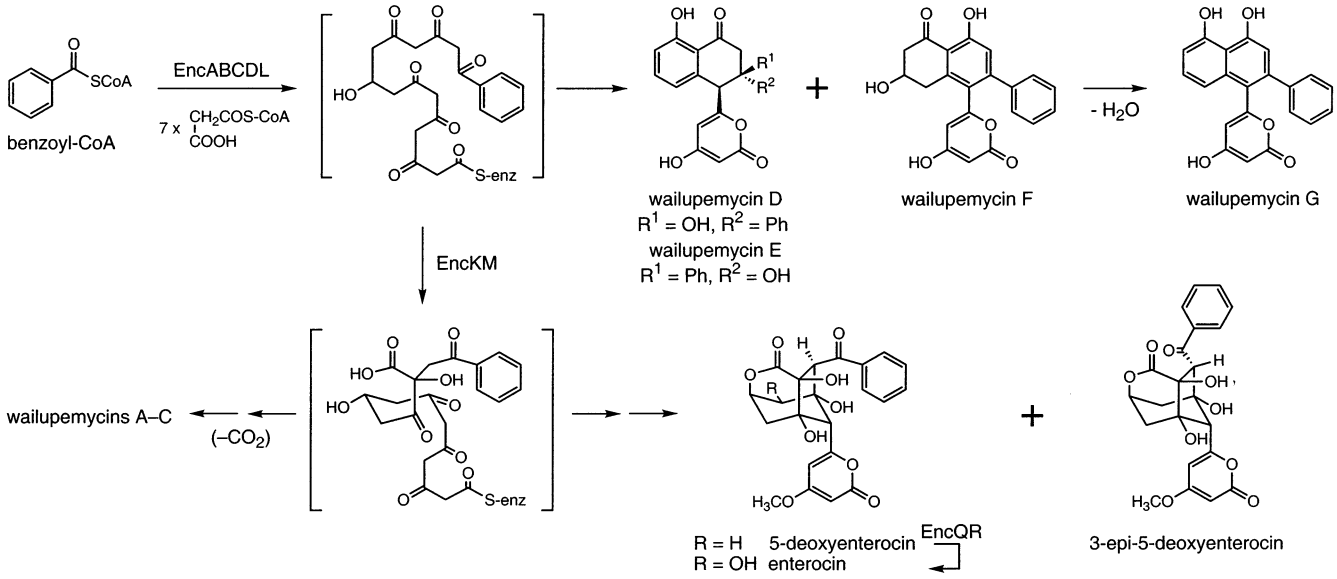
10.1021/np020230m CCC: $22.00
Published on Web 11/12/2002



enc gene product	Proposed function	Sequence similarity
EncA	Ketosynthase-alpha	NcnA, *S. arenae* (AAD20267)
EncB	Ketosynthase-beta	Frn2, *S. roseofulvus* (AAA19617)
EncC	Acyl carrier protein	ActI ORF3, *S. coelicolor* (CAA45045)
EncD	Ketoreductase	AknIII, *S. galilaeus* (BAA03128)
EncE	Regulatory protein	NolA, *B. elkanii* (P50329)
EncF	Transcriptional activator	FrnG, *S. roseofulvus* (AAC18102)
EncG	Unknown	MtmX, *S. argillaceus* (S58172)
EncH	Cinnamoyl-CoA ligase	CaiC, *E. coli* (I41013)
EncI	Cinnamoyl-CoA hydratase	YngF, *B. subtilis* (CAA74218)
EncJ	β-Oxoacyl-CoA thiolase	PaaJ, *E. coli* (CAA66099)
EncK	Unknown	PemT, *A. aceti* (BAA34057)
EncL	Benzoyl-CoA:ACP transferase	DpsD, *S. peucetius* (AAA65209)
EncM	Unknown	MitR, *S. lavendulae* (AAD28454)
EncN	Benzoyl-CoA ligase	BadA, *R. palustris* (AAA92151)
EncO	Unknown	
EncP	Phenylalanine ammonia-lyase	PAL, *T. subterraneum* (P45734)
EncQ	Ferredoxin	RapO, *S. hygroscopicus* (CAA60464)
EncR	Cytochrome P-450 hydroxylase	Orf3, *S. lavendulae* (AAD28449)
EncS	Regulatory protein	BetI, *E. coli* (P17446)
EncT	Efflux protein	CmcT, *S. lactamdurans* (Q04733)

Figure 1. Organization of the enterocin biosynthetic gene cluster (*enc*). The orientation of the arrows indicates the direction of each ORF.

Scheme 1. Proposed Biosynthetic Pathway from Benzoyl-CoA to Enterocin and the Wailupemycins



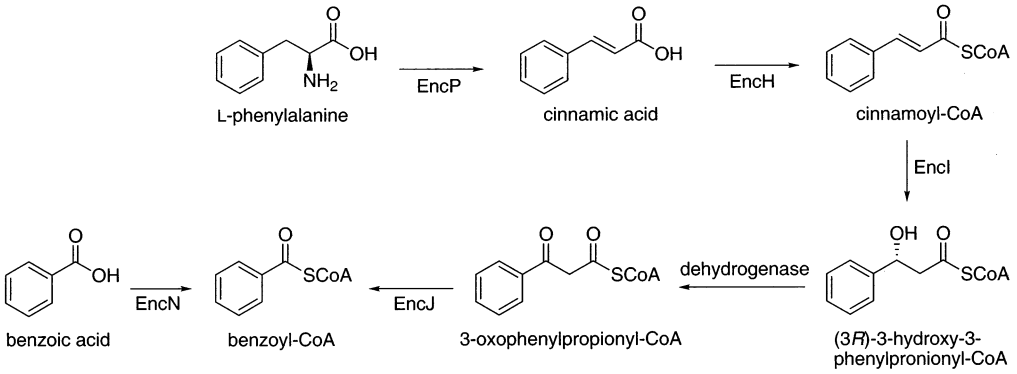
biosynthesis of the prenylated naphthoquinone cytotoxin marinone in a marine sediment-derived actinomycete,[14] is reported.

In this article, we highlight our progress on the biochemical and genetic characterization of these recently identified bacterial enzymes and pathways with ties to the plant world.

Cloning, Sequencing, and Heterologous Expression of the Enterocin Biosynthesis Gene Cluster. The bacteriostatic agents enterocin and the wailupemycins are structurally diverse polyketides produced by "*S. maritimus*" (Scheme 1).[15] Classical feeding experiments by Seto and co-workers demonstrated that enterocin is derived from an uncommon benzoic acid starter unit and seven acetate extensions.[16] The biosynthetic pathway includes a rare biological example of a Favorskii-like rearrangement. The α-pyrone unit is reminiscent of a large number of mutant and recombinant aromatic polyketides generated from iterative type II PKSs[17] and indicated that this family of metabolites is probably synthesized via an aberrant type II rather than a modular type I polyketide pathway.[18,19] Southern blot analyses with type II PKS gene probes from the actinorhodin biosynthesis gene set revealed three distinct gene clusters in the "*S. maritimus*" genome.[5] Spot sequencing of cosmid clone pJP15F11 identified a minimal PKS clustered with genes putatively involved in the formation of the benzoate starter unit. The enterocin biosynthesis gene cluster was unambiguously confirmed on this cosmid by heterologous expression using the *Escherichia coli*−*Streptomyces* shuttle cosmid pJP15F11 in the engineered host strain *Streptomyces lividans* K4-114.[4] LCMS analysis of organic extracts from the resulting transformant verified enterocin as the major polyketide metabolite and revealed a series of uncharacterized analogues. We isolated and characterized wailupemycin D as a new member of this structural family. Unlike other members of this series, wailupemycin D is derived from a linear polyketide intermediate. Identification of this metabolite provided further support that the enterocin family of polyketides originates from a carbon rearrangement of a type II PKS-generated linear poly-*β*-ketide.

We next cloned and sequenced the 21.3 kb *en*terocin (*enc*) biosynthesis gene cluster (Figure 1).[5] Central to the 20 open reading frame (ORF) cluster are *encABCD*, which encode the minimal *enc* PKS (consisting of the ketosynthase subunits KS_α and KS_β and the acyl carrier protein) and

Scheme 2. Proposed Biosynthesis of the Enterocin PKS Starter Unit Benzoyl-CoA in "*S. maritimus*"



the ketoreductase. Noticeably absent from the octaketide-homologous type II PKS gene cluster are cyclase or aromatase genes that typify aromatic PKS clusters. This significant architectural difference between the *enc* cluster and all other known type II PKS gene sets provided insight into the observed product diversity of the pathway. Cyclases not only function to direct regiospecific aldol condensations from a myriad of chemically feasible reactions on highly reactive intermediates, but their unnatural removal from their coupled minimal PKS can result in spontaneous chemistry.[17]

Biosynthesis of Benzoyl-CoA, the Enterocin PKS Starter Unit. One of the unusual features of the *enc* pathway involves the benzoate-derived starter unit.[20] Benzoyl-CoA has been implicated as a PKS starter unit in only one other bacterium, the myxobacterium *Sorangium cellulosum*, which produces the macrolide soraphen A.[21] Conversely, benzoic acid is a common metabolite in eukaryotic systems and is a component of many important natural products, including salicylic acid, cocaine, and taxol in plants and the zaragozic acids in fungi. Despite its common occurrence in plant products, benzoic acid biosynthesis is not well defined. Two pathways have been proposed in plants that similarly involve conversion of phenylalanine to cinnamic acid by PAL.[22,23] The routes diverge at cinnamate, one involving a β-oxidation pathway and the second a retro-aldol path through benzaldehyde. Until recently, the only known bacterial benzoate pathway was anaerobic and involved transamination of phenylalanine to phenylpyruvate followed by two successive α-oxidative decarboxylations.[24]

Sequence analysis of the *enc* gene cluster suggested that the benzoate starter unit is biosynthesized in a plant-like manner.[5] Genes putatively encoding benzoic acid biosynthesis were arranged on either side of the minimal *enc* PKS on four transcripts. The initial biosynthetic step was proposed to involve a unique bacterial PAL catalyzed conversion by the *encP* gene product (Scheme 2). Activation of cinnamic acid to its coenzyme A (CoA) thioester by the acyl-CoA ligase EncH followed by a series of β-oxidation reactions would directly provide the benzoyl-CoA primer unit. Two of the three β-oxidation enzymes were identified on the sequenced fragment, a hypothetical enoyl-CoA hydratase (EncI) and a β-ketothiolase (EncJ). A dedicated β-hydroxyacyl-CoA dehydrogenase, however, is missing and implied that this protein is encoded on an unsequenced region of the cluster or chromosome. The aryltransferase EncL presumably loads the benzoate unit onto the *enc* ACP to initiate polyketide assembly.

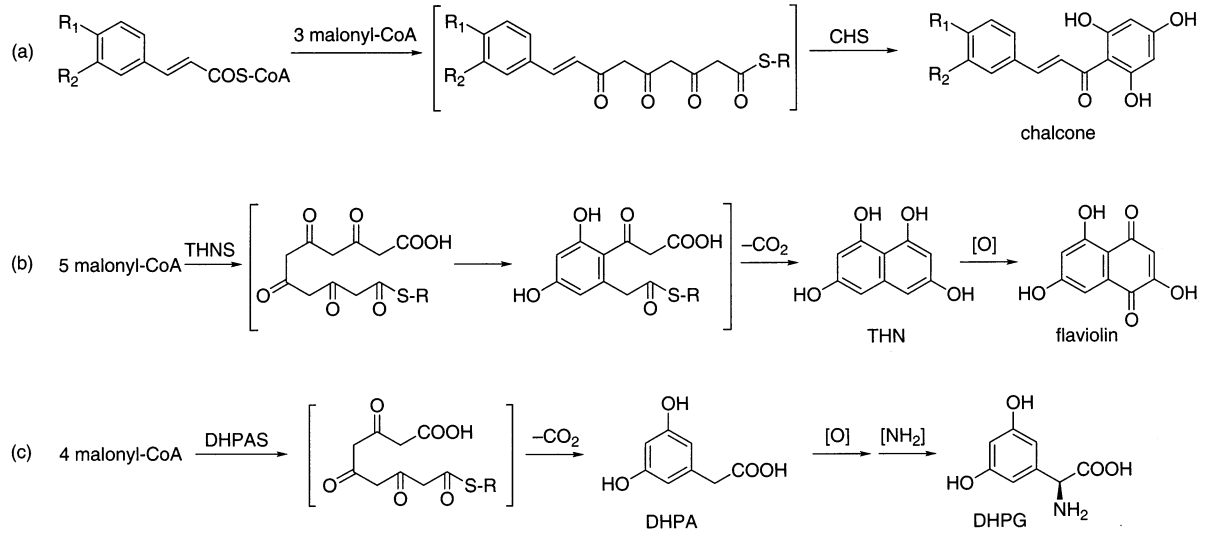
Feeding experiments with a series of doubly labeled [ring-$^2H_5$,1,2-$^{13}C_2$]phenylpropanoid biosynthetic intermediates were conducted to differentiate between β-oxidative and retro-aldol routes and to establish whether β-ketophenylpropionyl-CoA, a proposed intermediate, may serve as an alternative primer unit, thus skipping the first malonate extension.[6] MS analysis indicated that all the intermediates were incorporated into enterocin, yet only after the loss of the side chain $^{13}C_2$-label. This result indicated that the side chain is cleaved before incorporation of the phenyl unit, confirming benzoyl-CoA and not β-ketophenylpropionyl-CoA as the starter unit. Furthermore, the labeling experiments supported the involvement of the β-oxidative pathway as predicted by genetic analysis of the *enc* gene cluster.

We next determined the absolute configuration of the intermediate 3-hydroxy-3-phenylpropionyl-CoA by synthesizing and feeding both enantiomers of the corresponding [ring-d_5]-labeled free acid.[7] The wild-type strain preferentially incorporated the (3*R*)-enantiomer 5:1 over the (3*S*)-isomer. The absolute configuration of this intermediate was identical to that observed in the benzoyl-CoA biosynthesis pathway in the terrestrial plants *Nicotiana attenuata*[7] and *Erythroxylum coca*[25] and in fatty acid β-oxidation. Furthermore, there is considerable amino acid sequence homology between the "*S. maritimus*" cinnamoyl-CoA hydratase EncI and fatty acid enoyl-CoA hydratases. In particular, the EncI amino acid residues Gly-116, Glu-119, and Glu-139, which are essential for catalytic activity in homologous fatty acid-type hydratases,[26] suggest that the two enzyme systems operate by a common mechanism.

Mutational Analysis of the *enc* Benzoyl-CoA Encoding Genes. To gain insight into the roles of *enc* genes involved in benzoyl-CoA biosynthesis, we developed a genetic system in "*S. maritimus*" and disrupted several candidate genes via single-crossover homologous recombination.[27,28] We employed an *E. coli* to "*S. maritimus*" conjugal transfer of pKC1139-based temperature-sensitive plasmids carrying internal regions of target *enc* genes. The benzoyl-CoA biosynthesis genes *encH*, *-I*, *-J*, *-N*, and *-P* were individually disrupted, and growth of the resulting transconjugants under selective conditions resulted in the mutant strains KH, KI, KJ, KN, and KP, respectively.

Inactivation of the unique PAL-encoding gene *encP* completely abolished the biosynthesis of cinnamic acid and the benzoate-primed enterocin family of polyketides in the *encP* mutant strain KP.[27] Enterocin biosynthesis could be restored in this mutant by supplementing with cinnamic or benzoic acid as well as complementing with wild-type *encP*. Heterologous expression of the *encP* gene in *S. coelicolor* yielded cinnamic acid, confirming that the gene product EncP is a novel bacterial PAL. The bacterial PAL EncP is a considerably smaller protein than plant PALs (522 versus 680−720 amino acids). EncP more closely resembles bacterial histidine ammonia-lyase (HAL, EC

Scheme 3. Biosynthesis of the Type III PKS Products: (a) Chalcone in Higher Plants, (b) THN in *S. griseus* and *S. coelicolor*, and (c) DHPA in *A. mediterranei* and *A. orientalis*



4.3.1.3) in size. One key difference between EncP and HAL occurs at amino acid position 83. HALs harbor a conserved histidine residue at this position which coordinates its imidazole group through a hydrogen bond to that of the bound histidine substrate.[29] Plant PALs, on the other hand, carry residues such as valine and isoleucine at this position, which is analogous to Val83 of EncP, to provide a hydrophobic environment for the benzene ring of the substrate phenylalanine.

The fatty acid β-oxidation related genes *encH*, *-I*, and *-J* were similarly shown by target-directed mutagenesis to be involved in, but not absolutely required for, the biosynthesis of benzoyl-CoA.[28] The yield of benzoyl-CoA-primed enterocin dropped significantly below wild-type levels in each mutant strain. We attributed the reduced benzoyl-CoA formation in these specific mutants to functional substitution and cross-talk between the products of genes *encH*, *-I*, and *-J* and their corresponding fatty acid β-oxidation homologues.

Missing from the sequenced *enc* cluster is a homologue of β-hydroxyacyl-CoA dehydrogenase, the β-oxidation enzyme needed to catalyze the dehydrogenation of (3*R*)-3-hydroxy-3-phenylpropionoyl-CoA and complete the oxidative cycle. The search extended 2 kb upstream and downstream of the 21.3 kb *enc* cluster.[5] As heterologous expression of the *enc*-containing *E. coli*-streptomycete shuttle cosmid pJP15F11 was sufficient to produce enterocin, either a dedicated dehydrogenase gene is located elsewhere on the cosmid or a dehydrogenase isoenzyme is provided by the streptomycete host. This second scenario is plausible, as inactivation of the enterocin ketothiolase *encJ* gene in mutant KJ resulted in only a 25% loss in enterocin production, suggesting strong complementation by the corresponding ketothiolase associated with fatty acid β-oxidation. The benzoyl-CoA pathway dehydrogenase may be entirely supplied through primary metabolism.

As products of ketothiolases are acyl-CoAs, we predicted that the homologous *enc*-encoded thiolase EncJ catalyzes the conversion of β-oxophenylpropionyl-CoA directly to benzoyl-CoA. This conversion, however, bypasses the need to directly activate benzoic acid by a dedicated benzoyl-CoA ligase such as EncN, whose encoding gene is present in the *enc* gene cluster. Inactivation of the *encN* gene did not disturb the production of benzoate-primed enterocin, thereby confirming that *encN* is extraneous and that benzoic acid is not a pathway intermediate.[28] Supplemental benzoic acid can efficiently enter the enterocin biosynthetic pathway in the wild-type strain, but not in the corresponding *encN* mutant KN. Hence, EncN rather serves as a substitute pathway for utilizing exogenous benzoic acid. This observation is consistent with our recent analysis of the *encMN* mutant KM, in which the oxygenase *encM* as well as the coupled *encN* were disrupted.[30] Although benzoate-primed polyketides were synthesized in this mutant strain, they were not enriched with administered d_5-benzoic acid.

Bacterial Type III PKSs are Members of the Plant CHS Superfamily of PKSs. A new polyketide biosynthetic pathway for the assembly of small aromatic metabolites has recently been characterized in bacteria.[8] These bacterial PKSs are termed type III PKSs and are members of the CHS superfamily of condensing enzymes previously found only in plants.[31] These enzymes are structurally and mechanistically quite different from the type I and type II PKSs and use free CoA thioesters as substrates without the involvement of 4′-phosphopantetheine residues on acyl carrier proteins. Members of the CHS superfamily of condensing enzymes are relatively modest-sized proteins (40−47 kDa) that function as homodimers and typically select a cinnamoyl-CoA starter unit and carry out three successive extensions with malonyl-CoA. In the case of CHS, release of the tetraketide followed by cyclization yields chalcone (Scheme 3). CHSs appear to be ubiquitous in higher plants and catalyze the first enzymatic reaction to flavonoids, which exhibit a wide range of biochemical, physiological, and ecological roles. The crystal structure of CHS2 from *Medicago sativa* (alfalfa) was recently determined and provides important structural information on the reaction mechanism of a plant PKS.[3,32] Several new additions to the CHS superfamily have emerged from plants and deviate from the chalcone biosynthetic model by utilizing non-phenylpropanoid starter units, varying the number of condensation reactions, and having different cyclization patterns (e.g., 2-pyrone and acridone synthases).[33,34] Thus, plant enzymes in the CHS superfamily are growing in number and function and are not limited to plant-specific cinnamoyl-CoA starter units.

Over the past several years, 12 CHS-homologous bacterial proteins have been reported, suggesting that this new polyketide biosynthetic pathway may be widespread in

bacteria.[8] Most of the type III PKS sequences deposited in genetic databases were identified through total genome sequencing efforts. The results suggest that this third type of bacterial PKS is common, especially among the metabolically rich Actinobacteria. Genome sequence analysis of the model Gram-positive bacteria *S. coelicolor* and *Bacillus subtilis*, the human pathogen *Mycobacterium tuberculosis*, and *Bacillus halodurans* and *Deinococcus radiodurans* revealed a number of CHS homologues of unknown function.[8] Although none of the total genome-based type III PKSs have been biochemically characterized to date, three type III PKSs associated with secondary metabolic biosynthesis gene clusters have been described. The type III PKSs THNS from *S. griseus*[9,10] and 3,5-dihydroxyphenylacetic acid synthase (DHPAS) from several vancomycin group *Amycolatopsis* strains[11−13] have been characterized biochemically. Those enzymes display an absolute requirement for several molecules of malonyl-CoA as substrate (Scheme 3). The biosynthesis of the broad-spectrum antimicrobial agent 2,4-diacetylphloroglucinol in plant-associated fluorescent pseudomonads involves the CHS homologous gene *phlD*.[35,36] Expression of the four-gene cassette *phlACBD* in *E. coli* led to the formation of 2,4-diacetylphloroglucinol.

1,3,6,8-Tetrahydroxynaphthalene Synthase from *S. coelicolor*. *Streptomyces coelicolor* A3(2) is a model actinomycete that has served as a key genetic tool in *Streptomyces* biology. Recently, the sequencing of the 8.7 Mbp *S. coelicolor* genome was completed. Sequence analysis of the deposited genome[37] revealed three putative type III PKS genes (accession numbers CAC01488, CAB94644, and CAC17488), which in relation to *S. griseus* RppA (THNS) had identity/similarity scores of 70/83, 27/39, and 29/43, respectively. The putative *S. coelicolor* THNS encoding gene (CAC01488) was PCR amplified with appropriate restriction sites, cloned into the pHIS8 expression plasmid, and overexpressed in *E. coli* (Izumikawa, M.; Shipley, P. R.; O'Hare, T.; Hopke, J. N.; Moore, B. S., unpublished observations). Upon induction with isopropyl 1-thio-β-D-galactopyranoside, the *E. coli* transformant produced a dark red pigment, which after treatment with acid was extracted into ethyl acetate. Analytical HPLC analysis of the crude organic extract verified the presence of flaviolin, the autoxidation product of THN. MS and NMR analysis of purified flaviolin further confirmed its structure.

Expression of the octahistidyl-tagged protein resulted in large quantities of soluble protein of expected size. SDS-PAGE analysis indicated that the fusion protein was purified to greater than 80% following nickel-affinity chromatography. Incubation with malonyl-CoA resulted in the increase in absorbance at 340 nm, which is indicative of THN. We additionally followed this reaction by HPLC, which showed the complete consumption of malonyl-CoA and the maximum production of THN within 1 h (Figure 2). Over time the amount of THN decreased with the concomitant increase in its autoxidized product flaviolin. The identity of CoA, malonyl-CoA, acetyl-CoA, and flaviolin were confirmed by co-injection with authentic standards, while THN was verified by HPLCMS.

While type III PKSs, including THNS, are architecturally simple, they arguably represent the most sophisticated PKSs mechanistically. Embodied within their homodimeric architecture is the catalytic machinery necessary for starter molecule recognition and loading, malonyl-CoA decarboxylation and polyketide extension, and ultimately, multiple pathways for termination. We have constructed a homology model of THNS based upon the known three-dimensional

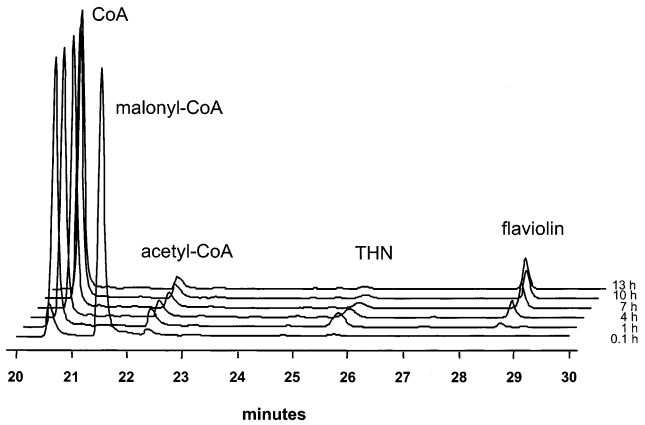


Figure 2. Reaction profile of the conversion of malonyl-CoA to THN and CoA by *S. coelicolor* THNS as monitored by reversed-phase HPLC. Although the enzymatic reaction is complete by 1 h, as shown by the total consumption of malonyl-CoA, THN is oxidized to flaviolin over time. This nonenzymatic conversion can be inhibited when the reaction is carried out under inert conditions.

structure of CHS to explore structural and mechanistic details of this new subclass of bacterial PKSs (Austin, M. B.; Noel, J. P.; Moore, B. S., unpublished observations). Five notable structural features not shared with CHS are associated with this tentative THNS homology model and labeled in Figure 3. An abbreviated N-terminus appears to be a common feature among the bacterial type III PKSs, which often appears at CHS residue 15. The C-terminal extension is variable in other bacterial type III PKSs. For instance, while *S. griseus* THNS is two amino acids shorter than *S. coelicolor* THNS, other bacterial type III PKSs are shorter or longer than CHS. To facilitate the alignment of this region, we used an absolutely conserved structural feature associated with all proteins possessing the CHS fold, namely, the conserved GFGPGxTxE sequence starting at CHS Gly 372 (Figure 3). Assuming the first nine THNS residues following this conserved sequence in the C-terminal sequence completes the beta strand as in CHS, an additional 25 residue THNS C-terminal extension remains, which is likely to form a structural motif distinct from those found in the plant enzymes.

The gap labeled (4) in Figure 3 is the only buried change and therefore the most suspect. This ambiguity is reflected in the sequence alignment. Although the current model reflects the best primary sequence alignment, the conserved helical sequence between gaps (4) and (5) should probably be shifted toward the active site, eliminating gap (4) and increasing the size of gap (5). In other words, contrary to what the sequence alignment predicts, area (4) probably extends as a helix very much like CHS after passing the active site. Soon after passing gap (5) the conserved GFGPG loop begins, whereupon confidence in the alignment picks up again.

Starting with CHS residue 16 and ending with CHS residue 380, *S. coelicolor* THNS covers 340 residues of CHS with gaps extending the alignment. Of the 340 residues aligned, 88 are identical, resulting in 26% (88/340) identity over the aligned portion. A number of active-site features implied by the THNS homology model are currently being explored by site-directed mutagenesis. It is notable that a significant degree of ambiguity remains in this model. The inherent lack of "atomic resolution" obtainable in any homology modeling approach further supports the utility of elucidating the three-dimensional structure of this model bacterial type III PKS. To this end, we have removed the affinity tag and purified the THNS protein to homogeneity.

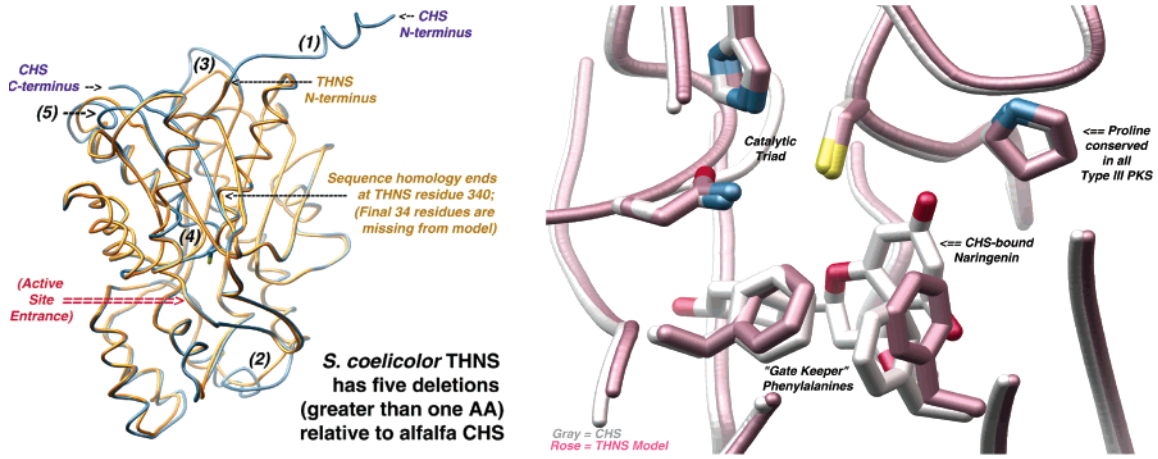
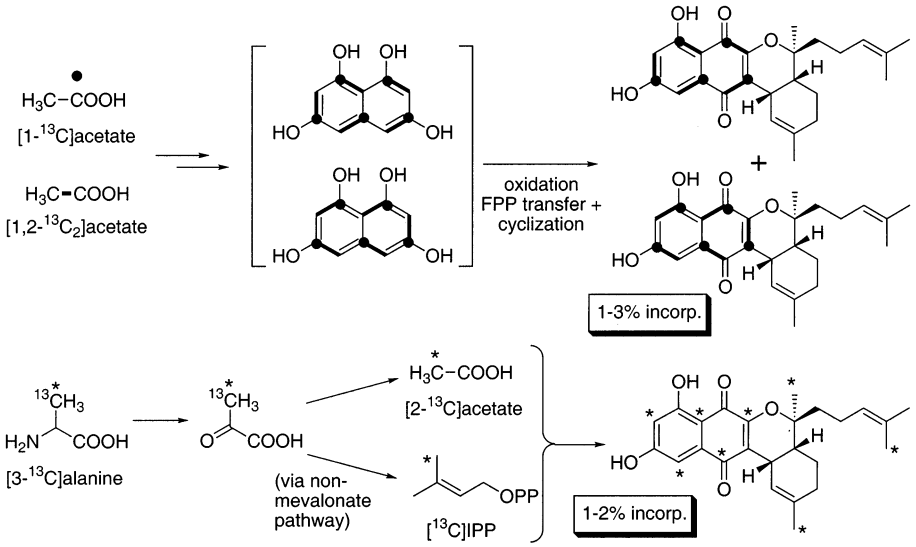


Figure 3. Overall homology model of the THNS monomer shown on the left panel as a yellow backbone trace. For reference, CHS is shown in blue. Five notable differences relative to CHS are noted. The right panel illustrates the conserved features in the active sites of these diverse type III PKSs. This comparison includes the catalytically essential catalytic triad consisting of Asn, His, and Cys.

Scheme 4. Incorporation of ^{13}C-Labeled Acetates and Alanine into Debromomarinone in the Marine Actinomycete Strain CNH-099



Crystals of THNS have been obtained and a structural analysis is in progress.

Biosynthesis of the Mixed Polyketide-Terpenoid Debromomarinone. The type III PKS product THN or a derivative thereof may be an intermediate in the biosynthesis of a group of prenylated naphthoquinone antibiotic cytotoxins produced by several actinomycete strains. Such members include the monoterpene-substituted naphthoquinones naphterpin and furaquinocin, the sesquiterpene-substituted naphthoquinones marinone and neomarinone, and the dimethylallyl/monoterpene-disubstituted naphthoquinones belonging to the napyradiomycin family.[8] Feeding experiments with singly and doubly ^{13}C-labeled acetate confirmed that the naphthoquinone moiety of debromomarinone is derived from a symmetrical pentaketide intermediate such as THN in the sediment-derived actinomycete strain CNH-099 (Scheme 4) (Nilsen, G.; Kalaitzis, J. A.; Moore, B. S., unpublished observations). The monoterpenoid analogues naphterpin[38] and furaquinocin A[39] similarly derive from a symmetrical acetate-derived naphthalene unit. Although we consistently measured between 1 and 3% incorporation of labeled acetate into the pentaketide residue, the sesquiterpenoid unit of debromomarinone was not enriched. This observation implied that the terpene is not made by the mevalonate pathway, but rather by the recently established non-mevalonate pathway.[40,41] This is in contrast to naphterpin biosynthesis, in which the monoterpene unit is mainly derived from the mevalonate pathway that operates during secondary growth in *Streptomyces aeriouvifer*.[42] Feeding experiments with ^{13}C-labeled glucose and glycerol as general precursors to provide support for the non-mevalonate pathway were unsuccessful, as these sugars suppressed debromomarinone production. We therefore administered [3-^{13}C]alanine to strain CNH-099, which upon metabolism to [3-^{13}C]pyruvate, equally labeled the polyketide (via decarboxylation to [2-^{13}C]acetate) and sesquiterpene (solely via the non-mevalonate pathway) units (Scheme 4).

Concluding Remarks. Two unusual plant-like enzymes have been characterized in streptomycetes, one involved in the biosynthesis of the benzoate-derived moiety of the antibiotic polyketide enterocin and the other in the biosynthesis of small aromatic polyketides. It is tempting to speculate that it may be possible to metabolically engineer simple plant-like phenylpropanoids and flavonoids by heterologously expressing these bacterial enzymes and their derivatives. Work is underway to pathway engineer plant-associated natural products by deploying bacterial systems for the renewable production of small chemical entities.

Acknowledgment. This work was generously supported in part by grants from the National Institutes of Health (AI47818), the National and Washington Sea Grant programs (NA76RG01149, project no. R/B-28), and the donors of the Petroleum Research Foundation administered by the American Chemical Society (34265-G4) to B.S.M. The work in the Noel Laboratory was supported by a grant from the National Science Foundation (MCB9982586) to J.P.N. Fellowships from the Alexander von Humboldt Foundation to C.H. and J.P., the Deutsche Forschungsgesellschaft and the Deutscher Akademischer Austauschdienst to J.H., and the ACS-PRF to T.O. are gratefully acknowledged.

References and Notes

(1) Based on an ASP Matt Suffness Award lecture at the 42nd Annual Meeting of the American Society of Pharmacognosy, Oaxaca, Mexico on July 18, 2001.
(2) Langer, B.; Röther, D.; Rétey, J. *Biochemistry* **1997**, *36*, 10867−10871.
(3) Ferrer, J.-L.; Jez, J. M.; Bowman, M. E.; Dixon, R. A.; Noel, J. P. *Nature Struct. Biol.* **1999**, *6*, 775−784.
(4) Piel, J.; Hoang, K.; Moore, B. S. *J. Am. Chem. Soc.* **2000**, *122*, 5415−5416.
(5) Piel, J.; Hertweck, C.; Shipley, P. R.; Hunt, D. M.; Newman, M. S.; Moore, B. S. *Chem. Biol.* **2000**, *7*, 943−955.
(6) Hertweck, C.; Moore, B. S. *Tetrahedron* **2000**, *56*, 9115−9120.
(7) Hertweck, C.; Jarvis, A. P.; Xiang, L.; Moore, B. S.; Oldham, N. J. *ChemBioChem* **2001**, *2*, 784−786.
(8) Moore, B. S.; Hopke, J. N. *ChemBioChem* **2001**, *2*, 35−38.
(9) Funa, N.; Ohnishi, Y.; Fujii, I.; Shibuya, M.; Ebizuka, Y.; Horinouchi, S. *Nature* **1999**, *400*, 897−899.
(10) Funa, N.; Ohnishi, Y.; Ebizuka, Y.; Horinouchi, S. *J. Biol. Chem.* **2002**, *277*, 4628−4635.
(11) Pfeifer, V.; Nicholson, G. J.; Ries, J.; Recktenwald, J.; Schefer, A. B.; Shawky, R. M.; Schroder, J.; Wohlleben, W.; Pelzer, S. *J. Biol. Chem.* **2001**, *276*, 38370−38377.
(12) Li, T.-L.; Choroba, O. W.; Hong, H.; Williams, D. H.; Spencer, J. B. *Chem. Commun.* **2001**, 2156−2157.
(13) Chen, H. W.; Tseng, C. C.; Hubbard, B. K.; Walsh, C. T. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14901−14906.
(14) Pathirana, C.; Jensen, P. R.; Fenical, W. *Tetrahedron Lett.* **1992**, *33*, 7663−7666.
(15) Sitachitta, N.; Gadepalli, M.; Davidson, B. S. *Tetrahedron* **1996**, *52*, 8073−8080.
(16) Seto, H.; Sato, T.; Urano, S.; Uzawa, J.; Yonehara, H. *Tetrahedron Lett.* **1976**, 4367−4370.
(17) Shen, Y.; Yoon, P.; Yu, T.-W.; Floss, H. G.; Hopwood, D. A.; Moore, B. S. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 3622−3627.
(18) Hopwood, D. A. *Chem. Rev.* **1997**, *97*, 2465−2497.
(19) Rawlings, B. J. *Nat. Prod. Rep.* **1999**, *16*, 425−484.
(20) Moore, B. S.; Hertweck, C. *Nat. Prod. Rep.* **2002**, *19*, 70−99.
(21) Wilkinson, C. J.; Frost, E. J.; Staunton, J.; Leadlay, P. F. *Chem. Biol.* **2001**, *8*, 1197−1208.
(22) Ribnicky, D. M.; Shulaev, V.; Raskin, I. *Plant Physiol.* **1998**, *118*, 565−572.
(23) Jarvis, A. P.; Schaaf, O.; Oldham, N. J. *Planta* **2000**, *212*, 119.
(24) Schneider, S.; Mohamed, M. E.; Fuchs, G. *Arch. Microbiol.* **1997**, *168*, 310−320.
(25) Bjorklund, J. A.; Leete, E. *Phytochemistry* **1992**, *31*, 3883−3887.
(26) He, X.-Y.; Yang, S.-Y. *Biochemistry* **1997**, *36*, 11044−11049.
(27) Xiang, L.; Moore, B. S. *J. Biol. Chem.* **2002**, *277*, 32505−32509.
(28) Xiang, L.; Moore, B. S. *J. Bacteriol.*, accepted for publication.
(29) Schwede, T. F.; Rétey, J.; Schulz, G. E. *Biochemistry* **1988**, *27*, 5355−5361.
(30) Xiang, L.; Kalaitzis, J. A.; Nilsen, G.; Chen, L.; Moore, B. S. *Org. Lett.* **2002**, *4*, 957−960.
(31) Schröder, J. In *Comprehensive Natural Products Chemistry*; Sankawa, U., Ed.; Elsevier: Amsterdam, 1999; Vol. 1, pp 749−771.
(32) Jez, J. M.; Bowman, M. E.; Noel, J. P. *Biochemistry* **2001**, *40*, 14829−14838.
(33) Jez, J. M.; Austin, M. B.; Ferrer, J. L.; Bowman, M. E.; Schröder, J.; Noel, J. P. *Chem. Biol.* **2000**, *7*, 919−930.
(34) Lukacin, R.; Schreiner, S.; Matern, U. *FEBS Lett.* **2001**, *508*, 413−417.
(35) Bangera, M. G.; Thomashow, L. S. *J. Bacteriol.* **1999**, *181*, 3155−3163.
(36) Mavrodi, O. V.; Gardener, B. B. M.; Mavrodi, D. V.; Bonsall, R. F.; Weller, D. M.; Thomashow, L. S. *Phytopathology* **2001**, *91*, 35−43.
(37) Bentley, S. D.; Chater, K. F.; Cerdeño-Tárraga, A.-M.; Challis, G. L.; Thomson, N. R.; James, K. D.; Harris, D. E.; Quail, M. A.; Kieser, H.; Harper, D.; Bateman, A.; Brown, S.; Chandra, G.; Chen, C. W.; Collins, M.; Cronin, A.; Fraser, A.; Goble, A.; Hidalgo, J.; Hornsby, T.; Howarth, S.; Huang, C.-H.; Kieser, T.; Larke, L.; Murphy, L.; Oliver, K.; O'Niel, S.; Rabbinowitsch, E.; Rajandream, M.-A.; Rutherford, K.; Rutter, S.; Seeger, K.; Saunders: D.; Sharp, S.; Squares, R.; Squares, S.; Taylor, K.; Warren, T.; Wietzorrek, A.; Woodward, J.; Barrell, B. G.; Parkhill, J.; Hopwood, D. A. *Nature* **2002**, *417*, 141−147.
(38) Shin-ya, K.; Furihata, K.; Hayakawa, Y.; Seto, H. *Tetrahedron Lett.* **1990**, *31*, 6025−6026.
(39) Funayama, S.; Ishibashi, M.; Komiyama, K.; Ōmura, S. *J. Org. Chem.* **1990**, *55*, 1132−1133.
(40) Rohmer, M. *Nat. Prod. Rep.* **1999**, *16*, 565−574.
(41) Rohdich, F.; Kis, K.; Bacher, A.; Eisenreich, W. *Curr. Opin. Chem. Biol.* **2001**, *5*, 535−540.
(42) Seto, H.; Watanabe, H.; Furihata, K. *Tetrahedron Lett.* **1996**, *37*, 7979−7982.

NP020230M