Chemistry & Biology, Vol. 9, 131–142, January, 2002, ©2002 Elsevier Science Ltd. All rights reserved. PII S1074-5521(02)00089-3

Precursor-Directed Biosynthesis: Biochemical Basis of the Remarkable Selectivity of the Erythromycin Polyketide Synthase toward Unsaturated Triketides

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Summary

The structural basis for the striking stereochemical discrimination among triketide analogs has been investigated by incubating a series of N-acetyl cysteamine (-SNAC) esters of unsaturated triketides with DEBS module 2+TE. The triketide analogs were first screened under a standard set of short-term incubation conditions in the presence of the extender substrate methylmalonyl-CoA and NADPH. For those triketide analogs that served as substrates for module 2+TE, the relative specificity, represented by the k_{cat}/K_M values, was quantitated. Triketide diastereomers that were converted in precursor-directed biosynthesis experiments to unsaturated 16-membered ring macrolides by DEBS(KS1°) were good to excellent substrates for DEBS module 2+TE, whereas analogs that were converted to the 14-membered ring analogs of 10,11-dehydro-6-deoxyerythronolide B by DEBS(KS1°) were not turned over at all by module 2+TE.

Introduction

The most extensively characterized modular polyketide synthase is the 6-deoxyerythronolide B synthase (DEBS) of *Saccharopolyspora erythraea*, the producer of the broad-spectrum antibiotic erythromycin A [1–4]. DEBS catalyzes the formation of the erythromycin macrolide aglycone, 6-deoxyerythronolide B (6-dEB, **1**), from a propionyl-CoA primer and six methylmalonyl-CoA extender units (Figure 1). Each of the six rounds of polyketide chain condensation and functional group modification is mediated by a dedicated group of active sites organized into a functional unit known as a module. Each polyfunctional protein module is in turn made up of a set of catalytic domains of 100–400 amino acids each that are similar in both function and sequence to individual enzymes of fatty-acid biosynthesis. Within each module, a keto synthase (β-keto acyl—acyl carrier protein synthase, KS) domain is responsible for the key chain-building step, involving a decarboxylative condensation of methylmalonyl-ACP with the growing polyketide acyl chain. The methylmalonyl moiety is itself loaded onto the acyl carrier protein (ACP) domain by a dedicated acyl transferase (AT) domain. The extent of modification of the resultant β-keto acylthioester is controlled by the specific combination of keto reductase (KR), dehydrase (DH), and enoyl reductase (ER) domains within each module. At the N terminus of module 1 are specialized AT and ACP loading domains that are responsible for priming the keto synthase domain of module 1 with propionyl-CoA [5], while a unique thioesterase (TE) domain at the C terminus of module 6 is responsible for the release of the mature heptaketide product and formation of the 14-membered lactone of 6-dEB. In DEBS, pairs of modules are covalently linked to form three large polypeptides, designated DEBS1, DEBS2, and DEBS3, each of which has a subunit M_r >330,000 Da and is active only as the corresponding homodimer [6–8].

Precursor-directed biosynthesis is a promising approach to generating novel analogs of erythromycin and other polyketides [9–11]. This technique is based on the use of a modular polyketide synthase mutant in which one or more of the early-stage enzyme activities have been eliminated by inactivation or deletion of the corresponding PKS domain. The resultant mutant is thus blocked in the biosynthesis of the natural polyketide product. Formation of this natural polyketide can be restored, however, by introduction of a synthetic derivative of a natural biosynthetic intermediate, usually in the form of the N-acetyl cysteamine (SNAC) thioester. Furthermore, by using analogs of natural intermediates, it has been possible to utilize mutant polyketide synthases to generate a range of structural variants of the natural product itself. The three main requirements for successful precursor-directed biosynthesis are (1) the analog to be introduced, whether by feeding to intact cells or by incubation with the mutant polyketide synthase, must be chemically stable under the conditions used; (2) the analog must be taken up and processed by a PKS domain downstream of the site of the mutation; and (3) the intermediates generated by the processing of the substrate analog must themselves be accepted and processed by the remaining downstream PKS domains.

DEBS(KS1°) is a mutant of DEBS that has been inactivated by site-directed mutagenesis of the β-ketoacyl-ACP synthase domain of module 1 (KS1). Because the KS1 domain would normally catalyze the first condensation step of 6-deoxyerythronolide B biosynthesis, the DEBS(KS1^0) mutant is incapable of carrying out the first round of polyketide chain elongation and is thus unable to catalyze the formation of the macrolide 6-dEB (**1**). We have described precursor-directed biosynthesis experiments in which a series of unsaturated triketides were tested as substrates for DEBS(KS1°) [9, 12, 13]. The unsaturated triketides were designed as stable analogs of the natural triketide product of DEBS module 2, the covalently bound 2,4-dimethyl-3,5-dihydroxy triketide acyl-ACP intermediate, which normally serves as the substrate for the ketosynthase domain of DEBS module 3. The enzyme-free N-acetyl cysteamine thioester of this acyclic substrate undergoes rapid, non-enzyme-

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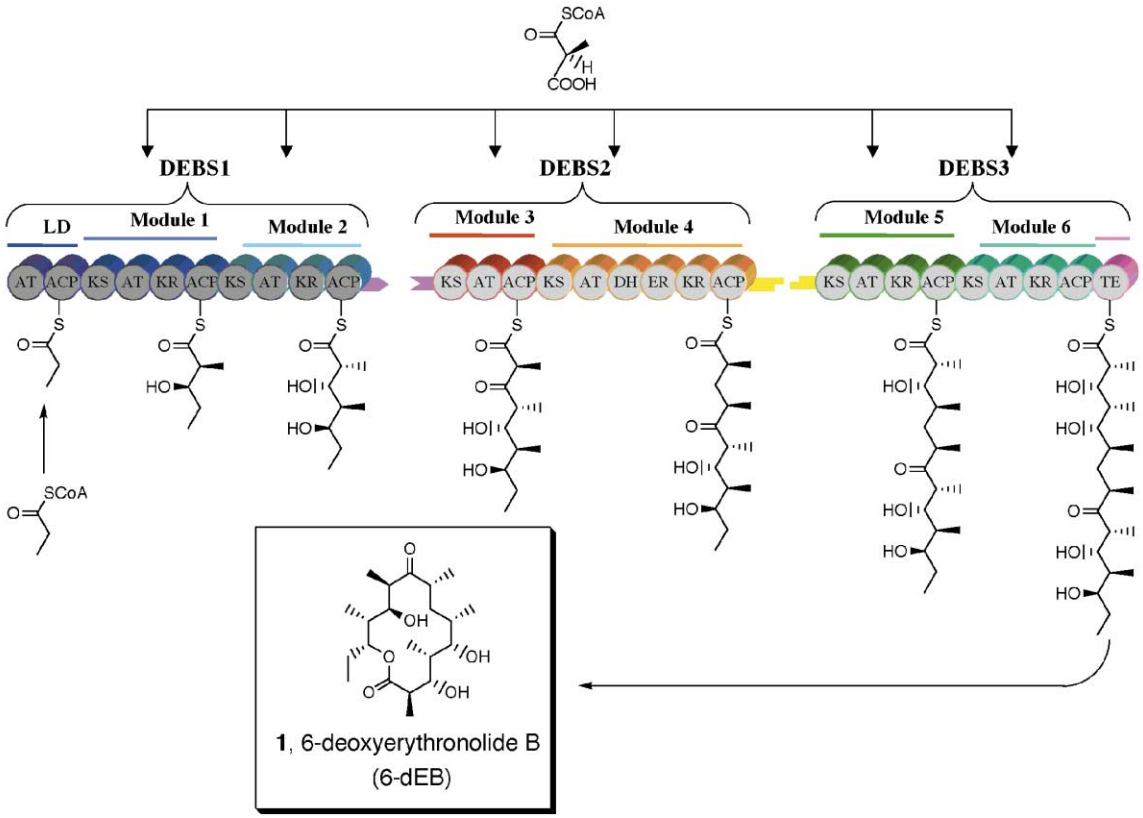

Figure 1. Organization of the Modular PKS, 6-Deoxyerythronolide B Synthase (DEBS)

Each DEBS protein consists of two modules, each of which is made up of a core set of β-ketoacyl-ACP synthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains, plus additional β-ketoreductase (KR), dehdyratase (DH) and enoylreductase (ER) domains, as required for each round of polyketide chain extension by methyl malonyl-CoA- and NADPH-dependent functional-group modification. At the N terminus of DEBS1 is a loading didomain responsible for priming module 1 with the starter propionyl-CoA and at the C terminus of DEBS3 is a thioesterase (TE) domain that releases the mature heptaketide and catalyzes the formation of the 6-deoxyerythronolide B. Short, complementary peptide sequences at the C and N termini, respectively, of the module 2/module 3 and module 4/module 5 pairs mediate specific protein-protein interactions and facilitate the intermodular transfer of polyketide chain elongation intermediates to the correct downstream module [14, 15, 27].

catalyzed lactonization even at neutral pH, precluding its direct use as a protein-free substrate. When the synthetic triketide **2**, which has a stringent geometric impediment to cyclization, was administered to cultures of *Streptomyces coelicolor* CH999/pJRJ2, the strain harboring DEBS(KS1°), the predicted unsaturated 14-membered lactone, 10,11-anhydro-6-dEB (**3**), was formed, as a result of uptake and processing of **2** by DEBS module 3 ([12]; Figure 2). Unexpectedly, however, feeding of the corresponding (4*S*)-4-methyl triketide diastereomer **4a** to the same engineered strain of *S. coelicolor* gave none of the corresponding C-12 epimer of **3**, but instead led to production of the derived 16-membered unsaturated macrolactone **5a** [9] (Figure 2). Similarly, administration of the chain-shortened (4*S*)-4-methyl triketide **4b**, as well as the 4-desmethyl unsaturated triketides **4c** and **4d**, to *S. coelicolor* CH999/pJRJ2 resulted in the formation of the corresponding 16-membered unsaturated macrolactones **5b–5d** [13] (Figure 2). The latter results were startling and indicated that the unsaturated triketides **4a–4d** had all been selectively recognized and processed by DEBS module 2 rather than DEBS module 3, as if each were a *diketide* analog.

Relative yields from feeding experiments can provide at best only a qualitative, and even sometimes misleading, estimate of the relative efficiency with which different PKS substrates are processed. To understand rigorously the biochemical basis for the apparent differential selectivity of the DEBS polyketide synthase for unsaturated triketide analogs, it is essential to carry out experiments at the cell-free level in order to obtain a quantitative measure of the relative k_{cat}/K_M values for each substrate analog. Fortunately, it is not necessary to work with the entire DEBS multienzyme system in such studies. Instead, it is now possible to investigate the specificity of individual DEBS modules. We have recently expressed and purified from *Escherichia coli* DEBS modules 2, 3, 5, and 6, each with the natural DEBS thioesterase (TE) domain fused downstream of the respective ACP domain so as to facilitate release of polyketide products produced by each module [14]. Incubation of (2*S*, 3*R*)-2-methyl-3-hydroxypentanoyl-SNAC diketide **6** with modules 2, 5, or 6+TE in the presence of methylmalonyl-CoA and NADPH resulted in formation of the triketide lactone **7**, whereas incubation of **6** with module 3+TE gave rise to the triketide ketolactone **8** (Fig-

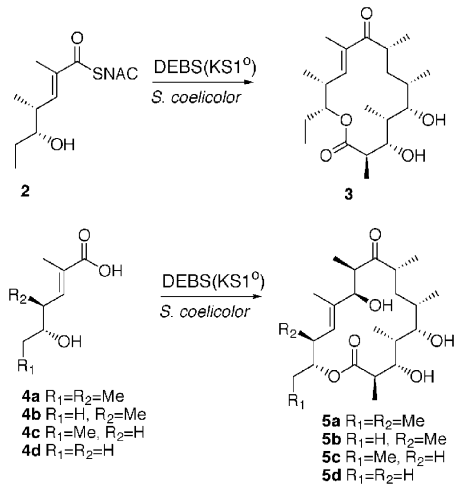

Figure 2. Precursor-Directed Biosynthesis of 14-Membered and 16-Membered Macrolides

Unsaturated triketide-SNAC (N-acetyl cysteaminine) thioesters were fed to a culture of *S. coelicolor* CH999 harboring a plasmid encoding DEBS(KS1°), a 6-deoxyerythronolide B synthase mutant that has been inactivated by site-directed mutagenesis of the KS1 domain. The 16-membered macrolides **5a–5d** are isolated as the corresponding 5,9-hemiketals [13].

ure 3). We have also compared the intrinsic substrate specificity of each these recombinant modules for the natural (2*S*, 3*R*)-2-methyl-3-hydroxypentanoyl-SNAC diketide **6** and its three diastereomers [15, 16]. In all cases, the syn-diketide **6** was preferred over its enantiomer by a ratio of from 15:1 to 100:1, depending on the specific module, as measured by relative k_{cat}/K_M values. Notably, neither of the anti-diketide-SNAC derivatives, for example **9**, was converted to detectable quantities of the corresponding triketide lactone **10**. More recently we have found that the corresponding diketide-ACP thioesters are substantially better substrates for each module,

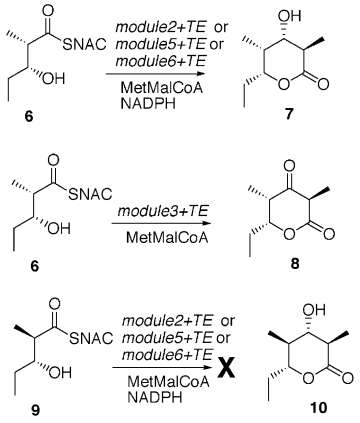

Figure 3. Incubation of NAC Thioesters of the Diketides **6** and **9** with Individual Recombinant DEBS Modules Expressed and Purified from *E. coli*

Each module mediates a single round of chain extension, and the appended TE domain catalyzes the formation of the derived triketide lactone **6** or triketide ketolactone **8**. The anti-diketide-SNAC diastereomer **9** is not processed by any of the DEBS modules.

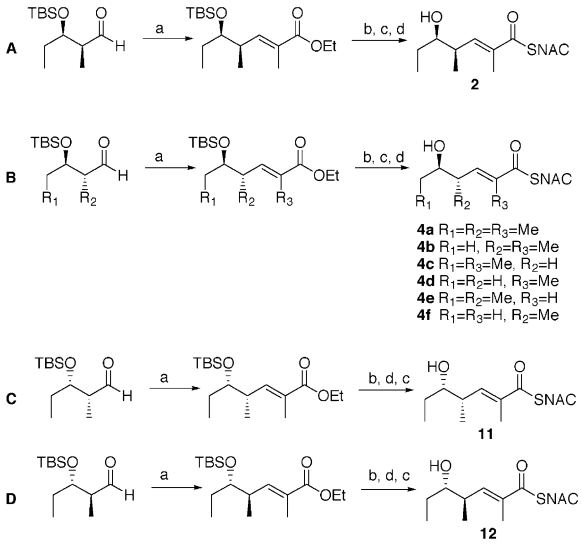

Figure 4. Synthesis of Unsaturated Triketide Analogs

For experimental details see reference [13]. Conditions were as follows: (A) (carboxyethylidene)triphenylphosphorane or (carboxymethylene)triphenyl-phosphorane, THF, reflux, 12–24 hr. (B) K_2CO_3, 3:1 MeOH/H_2O, reflux, 3-6 hr. (C) (1) $(PhO)_2PON_3$, Et_3N, DMF, 0°C, 2 hr; (2) N-acetylcysteamine. (D) HF (48% aqueous solution), 5:1 acetonitrile/H_2O, room temperature, 2 hr.

largely but not exclusively because of significant decreases in the K_M for each substrate, emphasizing the critical role played by protein-protein interactions in delivering polyketide chain elongation intermediates to the cognate elongation modules [17]. Interestingly, even the anti-diketide-ACP thioesters were turned over by each recombinant DEBS module, albeit at substantially reduced rates. We now report the results of investigating the substrate specificity of module 2+TE toward a series of unsaturated triketide-SNAC analogs and their 5-deoxy derivatives. Several of these triketides are not processed into full-length macrolactones by DEBS(KS1°).

Results

Preparation of Unsaturated Triketide Analogs and Tetraketide Products

The unsaturated triketides **2** and **4a–4f** were each prepared as previously described [9, 12, 13], with the key step being the reaction of the corresponding protected aldehyde with the appropriate Wittig reagent, (carboxyethylidene)triphenylphosphorane or (carboxymethylene)-triphenyl-phosphorane (Figures 4A and 4B). Triketides **11** and **12**, which are the enantiomers of the known triketides **2** and **4a**, were prepared from the appropriate β-hydroxyaldehyde enantiomers by a minor variation of the methodology previously described [13] (Figures 4C and 4D). The diastereomeric purity of **2**, **4a**, **11**, and **12** was verified by chiral HPLC analysis. Each of the unsaturated 5-deoxy esters **13** and **14a–14c** was synthesized from the corresponding 2-methyl or straight chain aliphatic aldehyde and (carboxyethylidene)triphenylphosphorane (Figure 5).

For confirmation of the structures of enzymatic-reac-

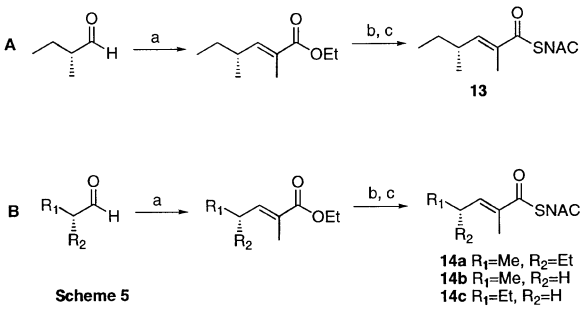

Figure 5. Synthesis of 5-Deoxy-Unsaturated Triketide Analogs

For experimental details see the Experimental Procedures section. Conditions were as follows: (A) (carboxyethylidene)triphenylphosphorane, THF, reflux, 12 hr. (B) K_2CO_3, 3:1 MeOH/H_2O, reflux, 3–6 hr. (C) (1) $(PhO)_2PON_3$, Et_3N, DMF, 0°C, 2 hr; (2) N-acetylcysteamine.

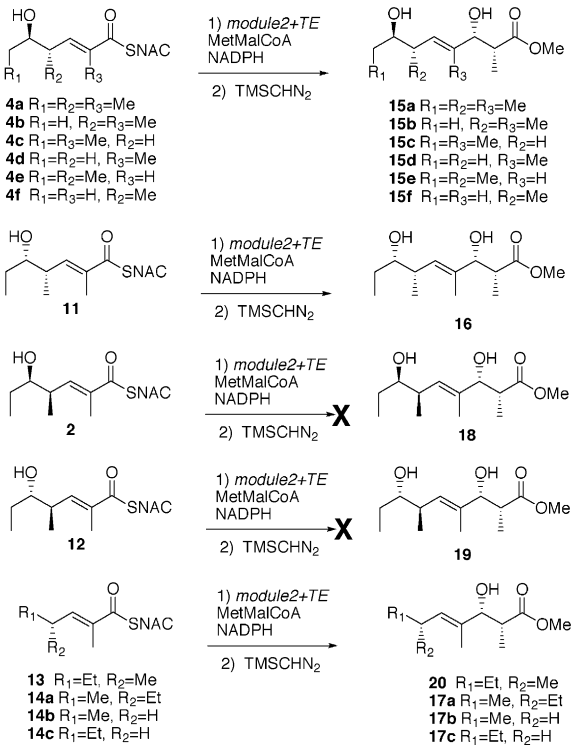

Figure 7. Conversion of Unsaturated Triketides to (2*R*,3*S*)-2-Methyl-3-Hydroxy-Tetraketide Methyl Esters by Incubation with Module 2+TE, Methylmalonyl-CoA, and NADPH

For experimental details see the Experimental Procedures section. Neither **2** nor **12** are turned over by module 2+TE, even upon prolonged incubation.

tion products, we also required authentic samples of the methyl esters of each of the prospective products of incubation of the individual triketide substrates with module 2+TE. Each of the requisite reference products was prepared from the appropriate unsaturated carboxylic acid that had already been prepared for the synthesis of the corresponding -SNAC esters (Figure 6). In each case, reduction with diisobutylaluminum hydride followed by pyridinium dichromate oxidation gave the unsaturated aldehyde, which was subjected to chiral aldol reaction with the Evans reagent, (*4R*)-*N*-propionyl-4-benzyloxazolidinone [18]. Hydrolysis [19], deprotection as needed, and treatment with TMS-diazomethane gave the desired (2*R*,3*S*)-2-methyl-3-hydroxy methyl esters **15–17**.

Incubation with DEBS Module 2+TE—Relative Productivity

As the first step in evaluating the effectiveness of each unsaturated -SNAC derivative as a substrate for DEBS module 2+TE, we determined the relative productivity for conversion of each substrate by carrying out a series of incubations under a standard set of conditions, including the use of 2 mM -SNAC ester, 0.2 mM DL-[2-methyl-^{14}C]methylmalonyl-CoA, 1 mM NADPH, and 1 μM purified module 2+TE for 2 hr at 30°C (Figure 7). In order to avoid errors due to substrate depletion, we adjusted the reaction conditions so that no more than 1% of any triketide substrate would be converted to tetraketide during the 2 hr incubation period. To facilitate

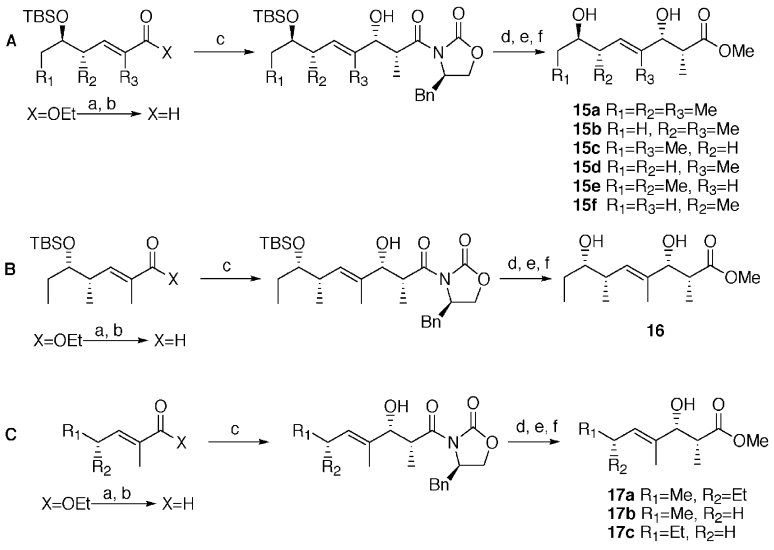

Figure 6. Synthesis of (2*R*,3*S*)-2-Methyl-3-Hydroxy-Tetraketide Methyl Esters

For experimental details see the Experimental Procedures section. Conditions were as follows: (A) DIBAL, CH_2Cl_2, 0°C, 2 hr. (B) PDC, CH_2Cl_2, room temperature, overnight. (C) (1) (*4R*)-3-Propionyl-4-Benzyl-2-Oxazolidinone, Bu_2BOTf, iPr_2EtN, CH_2Cl_2, 0°C, 0.5 hr, then −78°C, 0.5 hr; (2) add aldehyde, −78°C, 0.5 hr. (D) H_2O_2, LiOH, 4:1 THF:H_2O, room temperature, 12 hr. (E) 6 M HCl, 0°C. (F) $TMSCHN_2$, 1:1 benzene:MeOH, room temperature, 0.5 hr.

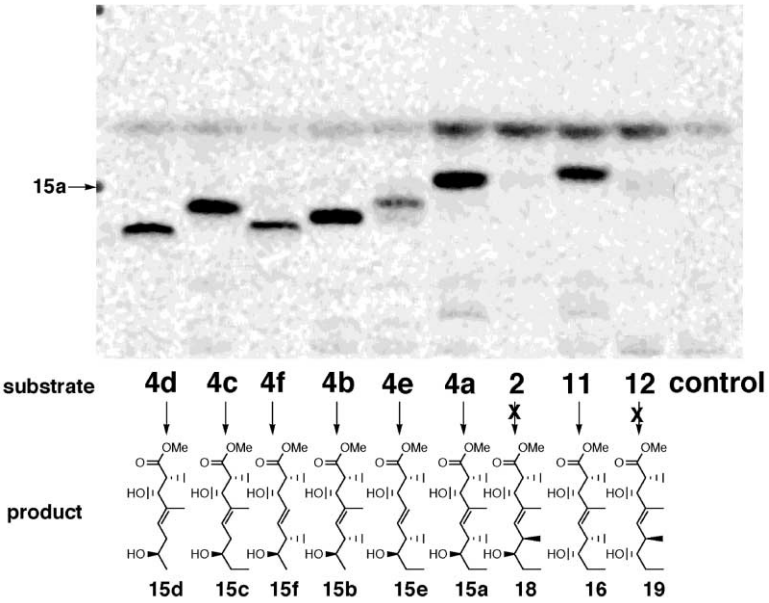

Figure 8. Phosphoimaging of Radioactive Products of the Incubation of Unsaturated Triketide Analogs with DEBS Module 2+TE

Each substrate (2 mM) was incubated with module 2+TE plus DL-[2-*methyl*-^{14}C]methylmalonyl-CoA and NADPH as described in the Experimental Procedures. The ethyl acetate-soluble products were methylated with TMS-diazomethane, and the entire mixture was subjected to silica gel TLC developed with 4:1 EtOAc/hexane. The products were analyzed by phosphoimaging. In the control incubation the triketide substrate was omitted. The less-polar band visible in each lane is dimethyl methylmalonate. Lanes **4a** –**12** were run with a separate batch of enzyme, and the results in Figure 9 have been normalized to adjust for differences in the absolute yield of **4a**.

separation and analysis of the enzymatic incubation products, we converted the resulting 2-methyl-3-hydroxy carboxylic acids to the corresponding methyl esters by treatment of the crude extract with trimethylsilyldiazomethane. The identities of the individual tetraketide products were verified by comigration on SiO_2 TLC with samples of the synthetic methyl esters, and the yields of each were quantitated by phosphoimaging (Figure 8). The structure of the tetraketide **15a** produced by the action of module 2+TE on triketide **4a** was further confirmed by feeding **4a** to a culture of *S. coelicolor* CH999 harboring DEBS1(KS1°)+TE, a mutant of the DEBS1+TE ion in which KS1 has been inactivated [20], and methylating the resulting product. (Data not shown.) The isolated tetraketide methyl ester was identical to synthetic **15a** by direct NMR comparison. To calibrate the activity of the protein preparations, we also incubated module 2+TE with 2 mM (2*S*,3*R*)-2-methyl-3-hydroxy diketide **6** under conditions identical to those used with each triketide analog.

As illustrated in Figures 7 and 9, the anti-(4*S*,5*R*)-triketide **4a** was converted by module 2+TE to the expected tetraketide **15a** with a productivity comparable to that for the processing of **6**, the -SNAC analog of the natural diketide substrate. By contrast, the syn-(4*R*,5*R*)-triketide **2** was not a substrate for module 2+TE and produced no detectable tetraketide product **18** even after prolonged incubation. Interestingly, the diastereomeric syn-(4*S*,5*S*)-triketide **11** was turned over with a productivity approximately 60% that of **4a**, whereas the corresponding anti-(4*R*,5*S*)-triketide **12** was not a substrate for the module 2+TE-catalyzed reaction and yielded none of the tetraketide **19**. As expected, the 6-desmethyl, 4-desmethyl, and 4,6-bis(desmethyl) analogs **4b–4d** of the anti-(4*S*,5*R*)-triketide **4a**, each of which had each been converted by cultures of *S. coelicolor* harboring DEBS(KS1°) to the corresponding 16-membered lactones [13], were themselves converted to the predicted tetraketides **15b**, **15c**, and **15d**, respectively, by module 2+TE, with relative productivities ranging from 33% to 70% that exhibited by **4a**. The two 2-desmethyl triketides **4e** and **4f,** which were turned over with approximately one-sixth the efficiency of triketide **4a**, were also substrates for module 2+TE. Finally, the un-

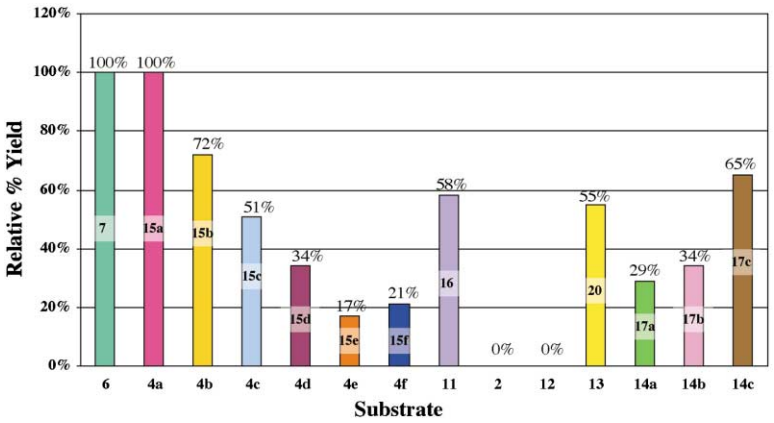

Figure 9. Relative Yields of 2-Methyl-3-Hydroxy Tetraketides

Relative yields of 2-methyl-3-hydroxy tetraketides resulting from the incubation of unsaturated triketide analogs with DEBS module 2+TE plus DL-[2-*methyl*-^{14}C]methylmalonyl-CoA and NADPH, as described in the Experimental Procedures.

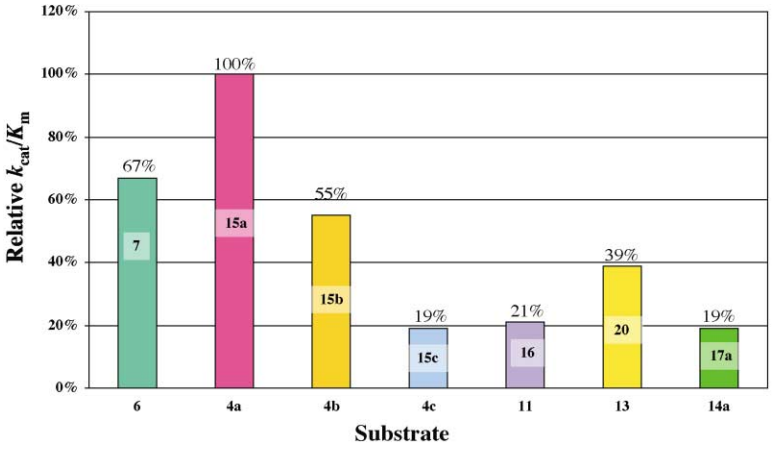

Figure 10. Relative k_{cat}/K_M Values of Substrate Pairs

Relative k_{cat}/K_M values determined by competitive incubation of pairs of substrates with DEBS module 2+TE plus DL-[2-*methyl*-^{14}C]methylmalonyl-CoA and NADPH, and measurement of the relative rates of product formation, as described in the Experimental Procedures. **4a–4c** and **11** were each coincubated with the deoxy analog **13** while **13** and **14a** were each incubated in competition with the diketide **6**. The relative k_{cat}/K_M values were all normalized, with that of **4a** set as 100%.

saturated -SNAC esters, **13** and **14a–14c**, each of which can be thought of as a 5-deoxy triketide analog, were all processed with efficiencies 25%–67% that of the standard **4a**.

Substrate Specificity of DEBS Module 2+TE—Competition Experiments

In order to obtain a more accurate measure of the relative substrate specificity of module 2+TE toward different triketide analogs, we carried out competition experiments in which pairs of analogs were coincubated with module 2+TE plus [^{14}C]methylmalonyl-CoA and NADPH. By monitoring the relative rates of formation of the derived pairs of tetraketide products by TLC-phosphoimaging, we could calculate the relative k_{cat}/K_M for each pair. Substrate pairs were chosen such that the two resulting products were well resolved on TLC. Thus each of the four unsaturated triketides **4a–4c** and **11** were coincubated with the deoxy analog **13**, while the two deoxy analogs **13** and its enantiomer **14a** were each incubated in competition with the diketide **6**. The normalized results are illustrated in Figure 10.

Interestingly, in these experiments the anti-(4*S*,5*R*)-triketide **4a** actually displayed a relative k_{cat}/K_M 1.5 times that of the natural syn-diketide substrate **6**. The two desmethyl triketides **4b** and **4c** exhibited relative k_{cat}/K_M values that were 55% and 19%, respectively, that of **4a** (82% and 29% that of the natural diketide **6**). The syn-triketide diastereomer **11** showed a comparable k_{cat}/K_M relative to **4a** of 21%. The enantiomeric 5-deoxy unsaturated triketides **13** and **14a** were also efficient substrates, in spite of the absence of any hydroxyl moiety, with relative k_{cat}/K_M values of 39% and 19%, respectively, compared to **4a** (58% and 29% compared to **6**).

Steady-State Kinetic Parameters

As a complement to the competition experiments, we also directly determined the intrinsic k_{cat}/K_M values for module 2+TE of the six unsaturated triketides **4a–4c**, **11**, **13**, and **14a**, as well as the natural diketide **6**, by carrying out steady-state kinetic measurements on each substrate. (Table 1) The values obtained essentially mirrored the relative k_{cat}/K_M specificities determined in the competition experiments, although the 4-desmethyl triketide **4c** appeared to be a somewhat better substrate, whereas the 5-deoxy triketides **13** and **14a** were somewhat poorer. The triketides **4a–4c** and **11** showed relatively minor differences in k_{cat}, all about 30%–40% that of the diketide **6**, with most of the observed variations in k_{cat}/K_M apparently due to changes in K_M.

Discussion

Screening of the relative productivity of module 2+TE for the four diastereomers of the unsaturated triketide **4a** as well as a series of desmethyl analogs of **4a** confirmed and extended the results of the previous in vivo incorporation experiments that had been carried out with *S. coelicolor* CH999/pJRJ2 harboring the DEBS(KS1°) mutant [9, 12, 13]. Thus, the anti-(4*S*,5*R*)-triketide **4a** was converted by module 2+TE to the expected (2*R*,3*S*)-2-methyl-3-hydroxy unsaturated tetraketide **15a** in a relative yield comparable under the assay conditions to that of triketide **7** from the natural diketide substrate **6**. The relative k_{cat}/K_M for triketide **4a** was actually 25%–50% higher than that of natural diketide **6**. By contrast, the unsaturated syn-(4*R*,5*R*)-triketide **2** was not a substrate at all for module 2, consistent with the exclusive formation of the 10,11-dehydro-6-dEB (**3**) from the syn-triketide **2** [12], evidently because of exclusive turnover by the downstream DEBS module 3, which ordinarily processes (saturated) diketide substrates generated by module 2. Unexpectedly, module 2+TE also processed the unsaturated syn-triketide **11**, the enantiomer of **2**, although the corresponding anti-triketide **12**, the enantiomer of **4a**, was not a substrate for module 2. These results suggest that the configuration of the 4*S*-methyl substituent is more important in the recognition and processing of the unsaturated triketide by module 2 than is the configuration of the 5-hydroxyl moiety. Interestingly, DEBS module 2 normally processes the (2*S*,3*R*)-2-methyl-3-hydroxy diketide; it prefers this syn-diastereomer of the -SNAC ester by a factor of 100:1 over the enantiomeric syn-diketide and completely excludes both of the anti-diketides.

Also consistent with the previously reported precursor-directed biosynthesis experiments with *S. coelicolor* [13] was the processing by module 2+TE of the 6-desmethyl analog **4b**, the 4-desmethyl analog **4c**, and the 4,6-bis-desmethyl analog **4d**. The turnover of **4c** and **4d**

Table 1. Steady-State Kinetic Parameters for Incubation of Unsaturated Triketides with Module 2+TE

Substrate	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M ($mmol^{-1}$ min^{-1})	k_{cat}/K_M (rel)
6	5.0[a]	1.2[a]	0.24[a]	83
4a	1.7	0.50	0.29	100
4b	2.6	0.43	0.17	59
4c	1.6	0.36	0.20	69
11	7.2	0.41	0.056	19
13	5.5	0.46	0.073	25
14a	3.4	0.19	0.056	19

[a] The steady-state parameters previously determined for **6** for module 2+TE expressed in *E. coli* BL21(DE3) were $K_M > 3.2$ mM, $k_{cat} > 4.6$ min^{-1}, k_{cat}/K_M 0.75 $mmol^{-1}$ min^{-1} [15].

further confirms that the methyl group at C-4 is not essential for recognition by module 2. Indeed, the relative productivities and k_{cat}/K_M values of **4b–4d** are all less than the k_{cat}/K_M value of **4a** within a factor of 5. Interestingly, the corresponding 2-desmethyl analogs **4e** and **4f** were also reasonably good substrates for module 2+TE, with relative productivities and k_{cat}/K_M values all within a factor of 5–6 the k_{cat}/K_M value of **4a**. In previous whole-cell feeding experiments of **4e** to *S. coelicolor* CH999/pJRJ2, by contrast, we had not detected any of the corresponding unsaturated 16-membered lactone or any other recognizable macrolactone. It is not known whether the absence of such products was simply a function of the reduced efficiency of turnover of **4e** by module 2, discrimination against the derived 4-desmethyl tetraketide product by downstream DEBS modules, or simply an artifact of the in vivo feeding and fermentation conditions. The latter results emphasize the fact that whereas incorporation of a substrate analog in precursor-directed biosynthesis experiments can be taken as positive evidence that the analog and its derived intermediates can be processed by the relevant PKS modules, the failure to observe the expected products in such an experiment cannot be used to draw conclusions about the relative substrate specificity or tolerance of the PKS.

Although the anti-unsaturated triketide **4a** is normally never encountered by DEBS module 2 (or any other DEBS module), it is in fact a natural intermediate of biosynthesis of the 16-membered lactone tylactone (**21**), normally generated by module 2 of the TylG gene product, which includes the requisite DH domain ([21]; Genbank accession number U78289) (Figure 11). Similarly, the 2-desmethyl anti-triketide **4e** is a presumed precursor of **22**, the aglycone of the mycinamicins, based on the isolation of the corresponding unsaturated acid (**4g**) from a wild-type strain of *Micromonospora griseorubida* [22]. Intriguingly, all known 16-membered macrolides that have a methyl at C-14 have the same 14*S*-(D) stereochemistry and either a C-12,13 double bond or a derived 12,13-epoxide [23]. By contrast, 10,11-unsaturated 12- and 14-membered macrolides with a methyl group at C-12 or C-14, such as 10-deoxymethynolide (**24**) and narbonolide (**25**), the precursors of methymycin and picromycin, respectively, all have the epimeric L-methyl configuration, identical to that of the corresponding methyl group in the prototype macrolide aglycone, 6-dEB (**1**), and are derived from precursors such as **23** with the syn-(4*S*,5*R*)-4-methyl-5-hydroxy stereochemistry [24]. The reasons for this divergence in substrate stereochemistry among macrolides of different ring size is not known, but it is noteworthy that the relevant PKS modules have evolved with different intrinsic substrate specificities and levels of tolerance for structural variation.

The -SNAC thioesters **13** and **14a–14c** can be considered as 5-deoxy-2,3-unsaturated triketides. The fact that all four analogs are turned over by module 2+TE indicates that the 5-hydroxyl moiety of **4a–4f** is not essential for recognition and processing of unsaturated triketide substrates by this module. In a comparison of **13** with its structural analog **4b**, the absence of the 5-hydroxyl

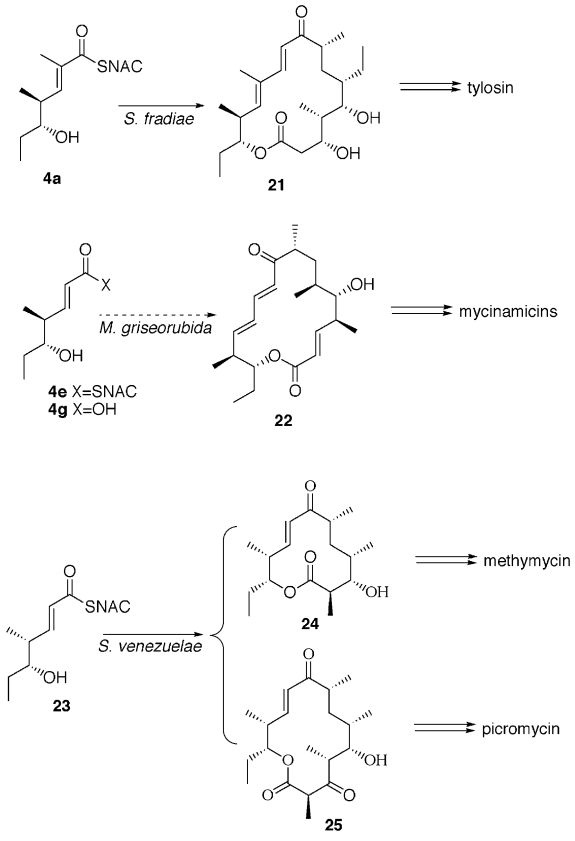

Figure 11. Unsaturated Triketides as Natural Intermediates in the Biosynthesis of Macrolides

Both **4a** and **23** have been incorporated into the corresponding macrolide aglycones tylactone (**21**) and 10-deoxymethynolide (**25**), whereas the unsaturated acid **4g** has been isolated from the mycinamicin producer *M. griseorubida*.

group results in no more than a 1.5- to 2.5-fold reduction in relative k_{cat}/K_M values. Notably, module 2+TE shows only a relatively modest 2-fold preference in k_{cat}/K_M for **13** over its enantiomer **14a**. Interestingly, in this case, a 2.5 advantage for **13** in k_{cat} is offset by a 40% reduction in K_M for **14a**. It is possible that the two enantiomers are bound by module 2+TE (presumably by the KS2 domain, see below) in different conformations, in which the ethyl group of **14a** occupies the binding site that interacts with the corresponding methyl group of **13**.

Although relative productivity measurements, based on the relative yield of products under standard incubation conditions, are useful for determining whether or not a given analog is actually a substrate for a given module, in the absence of information about either k_{cat} or K_M values for each substrate, such experiments allow only qualitative comparisons of candidate substrates. More quantitative evaluations of the relative specificity for each substrate requires determination of the relative or absolute k_{cat}/K_M values, either by pairwise competition assays or by direct steady-state kinetic measurements, respectively. In fact, the results of both sets of experiments for analogs **4a–4f**, **13**, and **14a** are essentially in agreement, with the exception of minor differences in the relative k_{cat}/K_M values obtained for the 4-desmethyl analog **4c** and the deoxy analog **13**. These analogs all have relative k_{cat}/K_M values that are less than the k_{cat}/K_M value of the parent analog **4a** within a factor of 5, with most of the variation being due to increases in K_M. Interestingly, the slight (20%–50%) preference for the unsaturated triketide **4a** over the natural diketide **6** apparently results from the 3.2-fold K_M reduction that compensates for the 0.4-fold reduction in k_{cat}.

The relative k_{cat}/K_M values for the processing of each substrate are a function of only those kinetic events up to and including the first irreversible enzymatic step. The observed differences in k_{cat}/K_M values for module 2+TE of the various unsaturated triketides compared to the natural diketide therefore necessarily reflect the intrinsic substrate specificity of *only* the ketosynthase domain, KS2, and are insensitive to the intrinsic specificities of any of the domains (KR2, TE) catalyzing downstream reactions. We have previously reported that processing of the diketide-SNAC **6** by DEBS module 2 involves covalent acylation of the active site cysteine of KS2 [25], and we have demonstrated that this acylated protein is catalytically competent [2]. In addition to the rates of binding and dissociation of each substrate analog to the KS2 domain, the k_{cat}/K_M is a function of the rate constants for acylation and deacylation of the active site cysteine, as well as the *irreversible* decarboxylative condensation with methymalonyl-ACP2. Of course, should the original acylation also be irreversible, then only the acylation step would influence k_{cat}/K_M. We are currently examining the relative contribution of KS-catalyzed acylation and condensation to substrate discrimination. In spite of the remarkably broad substrate tolerance of module 2, this module strictly excludes both unsaturated triketides **2** and **12**. The fact that, nonetheless, DEBS(KS1°) will convert **2** to 10,11-dehydro-6dEb (**3**) indicates that the latter triketide diastereomer must be a viable substrate for module 3. Indeed, we have now found that recombinant module 3+TE can in fact process triketide **2** and the anti-triketide **4a** to give the corresponding tetraketides with equal catalytic efficiency, but at relative rates that are 10% of those for the processing of triketide **4a** by module 2+TE.

Significance

Precursor-directed biosynthesis has already proven to be a powerful tool for the rational synthesis of structural analogs of erythromycin and related macrolides. Successful exploitation of this technique, as well as of related approaches involving combinatorial biosynthesis methodology, requires a detailed understanding of the structural and stereochemical factors that control substrate specificity and tolerance by target PKS modules. The demonstration that the differential processing of syn- and anti-diastereomers of unsaturated triketides by blocked mutants of DEBS is due to the intrinsic kinetic selectivity of DEBS module 2 for substrate analogs of different structure establishes the biochemical basis for the observed product distribution resulting from the in vivo feeding experiments with cells harboring the DEBS(KS1°) mutant. By combining this information with knowledge of specific protein-protein interactions within pairs of ACP donor domains and downstream KS acceptor domains, one should be able to optimize the rational production of polyketides of designed structure by chimeric PKS constructs based on a judicious choice of the proper combination of modules, linkers, and substrate analogs. In particular, the generation of a library of hybrid 14- to 16-membered macrolide antibiotics may be possible in the near future.

Experimental Procedures

Materials

DL-[2-*methyl*-^{14}C]Methylmalonyl-CoA (56.4 mCi/mmol) was obtained from ARC. DL-MethylmalonylCoA and NADPH were obtained from Sigma. (2*S*,3*R*)-2-Methyl-3-hydroxy pentanoyl N-acetylcysteamine (**6**) was synthesized as previously described [26]. All other reagents and solvents were commercially available products.

Product Analysis and Characterization

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-300 NMR spectrometer. Chemical shifts are reported in ppm relative to tetramethylsilane, and *J* values are in Hz. A Kratos MS80RFA Mass Spectrometer was used to obtain high-resolution mass spectra (reported as *m/z*) by fast atom bombardment (FAB). High-pressure liquid chromatography (HPLC) was performed on a Rainin Dynamax HPLC system equipped with a Chiralcel OC column (Diacel, 25 cm × 4.6 mm inner diameter; guard column, 5 cm × 4.6 mm inner diameter); a mobile phase of 10% of ethanol in hexane at a flow rate of 1 ml/min was used. The elution was monitored by UV at 232 nm. Enzymatically generated products were separated on silica gel TLC plates, and the radioactivity of individual components was quantitated by phosphoimaging with a Bio-Rad GS-363 Molecular Imager and Molecular Analyst software; standards of known radioactivity were used.

Isolation and Purification of DEBS Module 2+TE

The DEBS module 2+TE was expressed and purified by a variation of the previously published method [14, 15]. *E. coli* BL21-CodonPlus(DE3)-RP (Stratagene) harbors plasmid pRSG64, which encodes module 2+TE, and a plasmid containing the *sfp* phosphopantetheinyl transferase gene from *Bacillus subtilis* [14]. The Sfp protein is essential for the activity of module 2+TE because it catalyzes post-

translational phosphopantetheinylation of the apo-ACP2 domain. A 500-ml culture was grown at 37°C in LB medium supplemented with ampicillin, kanamycin, and chloamphenicol until $OD_{600} = 0.6$. After cooling to 26°C–28°C, the cells were induced with 1 mM IPTG and incubated at this temperature for 10 hr (final $OD_{600} = 2.5$). The harvested cells were disrupted with a French Press (1000 psi) in the previously described disruption buffer [14, 15], and the resulting supernatant was treated with DNase (20 μg/ml, 10 mM $MgCl_2$) for 20 min, followed by precipitation with 0.15% polyethylenimine (PEI) for 20 min. After ultracentrifugation, the cell-free extract was diluted twice by buffer A (0.1 M sodium phosphate buffer [pH 7.2], 20% glycerol) and then directly loaded on Q Sepharose (Amersham Pharmacia Biotech AB) gel. The module 2+TE protein was eluted with a linear gradient of 0.2 M (100 ml) to 0.5 M (100 ml) NaCl, emerging at approximately 0.25 M NaCl. The combined fractions containing module 2+TE protein were loaded on a 1 ml Ni^{2+} affinity column (Amersham Pharmacia Biotech AB) and washed with buffer A + 20 mM imidazole. Purified module 2 eluted with buffer A + 500 mM imidazole and was concentrated by Centricon 30 with buffer B (0.1 M sodium phosphate buffer [pH 7.2], 20% glycerol, 2 mM EDTA, 2.5 and mM DTT). The pure enzyme could be stored at −80°C without significant loss of activity over several months.

Assays

Productivity assays were carried out with 1 μM module 2+TE protein, 2 mM substrate, 1 mM NADPH, 200 μM DL-[2-*methyl*-^{14}C]methylmalonyl-CoA (1 mCi/mmol), 100 mM Na-phosphate buffer (pH 7.2), 20% glycerol, 10% DMSO (to solubilize the substrate), 2.5 mM DTT, and 2 mM EDTA in a total volume of 100 μl at 30°C for 1 or 2 hr. The incubation mixture was saturated with solid NaCl and extracted with ethyl acetate. The residue after evaporation of the solvent was treated with 10 μl of 2 M TMS-diazomethane in 40 μl of 50% CH_3OH/benzene at 37°C for 1 hr to generate the methyl ester of the product. The entire methylation reaction mixture was then applied to a TLC plate, which was developed with either 1:1 or 4:1 EtOAc/hexane and analyzed by phosphoimaging (Figures 8 and 9).

For competition assays, pairs of substrates (2 mM each; total DMSO concentration, 10%) were coincubated with the standard assay mixture for 1 hr and 2 hr, followed by extraction and methylation as above. Over the 2 hr time period, less than 5% of each substrate was consumed. Substrate pairs were chosen so that the derived tetraketide products were readily resolved by TLC, and the yields at each time point were quantitated by phosphoimaging. Each incubation was carried out in triplicate, and the results were averaged to calculate the relative k_{cat}/K_M values illustrated in Figure 10.

For steady-state kinetic assays, incubations were carried out for 1 hr with a range of triketide concentrations from 0.5 to 4 mM. The natural diketide substrate **6** was also assayed for comparison purposes (Table 1).

Substrate Synthesis

Triketide-SNAC esters **2** and **4a–4f** were synthesized as previously described [9, 12, 13], whereas **11** (the enantiomer of **2**) and **12** (the enantiomer of **4a**) were each prepared by similar methods from the corresponding enantiomeric protected 3-hydroxyaldehydes, with deprotection of the 5-hydroxy group preceding formation of the -SNAC ester. The enantiomeric purity of **2**, **4a**, **11**, and **12** was verified by HPLC with a Chiralcel OC column under the conditions described above. Retention time: **2**, 40.55 min; **4a**, 34.01 min; **11**, 37.28 min; **12**, 38.91 min.

(4R)-2,4-Dimethyl-2-Hexenoic Acid N-Acetylcysteamine Thioester (13)

(*R*)-(+)-4-benzyl-2-oxazolidinone (5.1 g, 28.8 mmol) was stirred in the THF (50 ml) at −78°C for 10 min. To the mixture, n-butyllithium (12 ml of 2.5 M solution in hexane, 30 mmol) was added via syringe. The mixture was stirred for 10 min, and butyryl chloride (4.56 g, 42.8 mmol) was added. After 10 min the cooling bath was removed, and the reaction was allowed to warm to 0°C over 30 min. The reaction was quenched with 20 ml of saturated NH_4Cl solution at 0°C. The THF was removed by rotary evaporation, and the mixture was extracted with 3 × 50 ml of CH_2Cl_2. The combined organic extract was washed with 20 ml of 1 N NaOH and subsequently with 20 ml of brine. The organic phase was dried with Na_2SO_4 and concentrated. Chromatography (150 g silica, 40% EtOAc/hexane) afforded 7.8 g (100%) of (4*R*)-4-benzyl-3-butyryl-2-oxazolidinone as a colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.04 (t, $J = 7.37$, 3H, 4′-H), 1.75 (m, 2H, 3′-H), 2.78 (dd, $J = 9.64$ and 13.4, 1H, 6b-H), 2.95 (m, 2H, 2′-H), 3.32 (dd, $J = 3.29$ and 13.4, 1H, 6a-H), 4.20 (m, 2H, 5-H), 4.69 (m, 1H, 4-H), 7.22–7.38 (m, 5H, Ph). To 7.8 g, (31.1 mmol) of this imide in THF (50 ml) at −78°C was added NaHMDS (35 ml of 1 M solution in THF, 34.3 mmol) via syringe. The mixture was stirred for 30 min, and methyl iodide (11 g, 77.8 mmol) was added. After 2 hr, the reaction was quenched with 30 ml of saturated NaCl solution. The THF was evaporated, and the mixture was extracted with 3 × 50 ml of CH_2Cl_2. Drying (Na_2SO_4), concentration, and silica gel chromatography (200 g silica gel, 20% ether/pentane) afforded 5.5 g (67%) of (4*R*,2′*R*)-4-benzyl-3-(2′-methylbutyryl)-2-oxazolidinone as a colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.95 (t, $J = 7.42$, 3H, 4′-H), 1.24 (d, $J = 6.78$, 3H, 2′-CH_3), 1.5 and 1.8 (m, 2H, 3′-H), 2.79 (dd, $J = 9.57$ and 13.4, 1H, 6b-H), 3.29 (dd, $J = 3.28$ and 13.4, 1H, 6a-H), 3.66 (m, 1H, 2′-H), 4.20 (m, 2H, 5-H), 4.70 (m, 1H, 4-H), 7.22–7.38 (m, 5H, Ph). The oxazolidinone (3.6 g, 13.9 mmol) in Et_2O (50 ml) was reduced with $LiAlH_4$ (42 ml of 1 M solution in THF, 42 mmol) for 1 hr at −78°C, after which the reaction was quenched with successive portions of 1.6 ml water, 1.6 ml 15% NaOH solution, and 4.8 ml water. After filtration and washing with ether, the organic phase was dried (Na_2SO_4) and concentrated. Chromatography (50 g silica, 20% EtOAc/hexane) afforded 1.2 g (95%) of (2*R*)-2-methyl-1-butanol as a colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.94 (d, $J = 6.65$, 3H, 2-CH_3), 0.95 (t, $J = 7.39$, 3H, 4-H), 1.2 and 1.5 (m, 3H, 2-H, and 3-H), 3.48 (m, 2H, 1-H). (2*R*)-2-methyl-1-butanol (300 mg, 3.4 mmol) was stirred in CH_2Cl_2 (10 ml) at room temperature. PDC (3.9 g, 10.2 mmol) was added, and the mixture was stirred overnight. The solution was then applied to a silica gel (20 g) column that was eluted with CH_2Cl_2. After rotary evaporation (bath temperature, 0°C), the desired (2*R*)-2-methylbutyraldehyde was obtained; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.97 (t, $J = 7.43$, 3H, 4-H), 1.11 (d, $J = 6.99$, 3H, 2-CH_3), 1.45 and 1.75 (m, 2H, 3-H), 2.31 (dt, $J = 1.94$ and 6.86, 1H, 2-H), and 9.65 (d, $J = 2.0$, 1H, 1-H). The aldehyde was dissolved in 30 ml of THF and treated with 1.7 g (4.5 mmol) of (carbethoxyethylidene)triphenylphosphorane at reflux for 12 hr. The THF was evaporated, and residue was subjected to silica filtration with 10% EtOAc/hexane to remove triphenylphosphine oxide. Concentration gave ethyl (2*E*, 4*R*)-2,4-dimethyl-2-hexenoate (130 mg, 22% from alcohol); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.86 (t, $J = 7.42$, 3H, 6-H), 0.99 (d, $J = 6.68$, 3H, 4-CH_3), 1.3 (t, $J = 7.14$, 3H, -CH_2CH_3), 1.3–1.5 (m, 2H, 5-H), 1.83 (d, $J = 1.49$, 3H, 2-CH_3), 2.41 (m, 1H, 4-H), 4.19 (q, $J = 7.15$, 2H, -CH_2CH_3), 6.52 (dq, $J = 1.4\sim1.5$, and 10.1, 1H, 3-H). The ethyl ester (130 mg, 0.76 mmol) was dissolved in 30 ml of methanol and 10 ml of H_2O containing 1.6 g (12 mmol) of K_2CO_3, and the mixture was stirred at reflux for 6 hr. The methanol was evaporated, and the remaining aqueous layer was acidified to pH 2 with concentrated HCl, saturated with NaCl, and extracted with EtOAc. The combined organic extracts were dried over Na_2SO_4 and concentrated. (2*E*, 4*R*)-2,4-dimethyl-2-hexenoic acid (100 mg, 90%) was obtained as a colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.86 (t, $J = 7.39$, 3H, 6-H), 1.02 (d, $J = 6.68$, 3H, 4-CH_3), 1.25–1.53 (m, 2H, 5-H), 1.86 (d, $J = 1.25$, 3H, 2-CH_3), 2.45 (m, 1H, 4-H), 6.71 (dd, $J = 1.21$ and 10.1, 1H, 3-H). Under N_2 atmosphere, 100 mg (0.7 mmol) of this acid was dissolved in 5 ml of DMF and treated with diphenylphosphoryl azide (170 μl, 0.77 mmol) and 136 μl (0.84 mmol) of Et_3N at 0°C for 2 hr, after which N-acetylcysteamine (84 mg, 0.7 mmol) was added. After the mixture was stirred at room temperature for 5 hr, 10 ml of H_2O was added, and the mixture was extracted with EtOAc. Drying over Na_2SO_4, concentration, and flash chromatography (silica gel 20 g, 50% EtOAc/hexane) yielded 85 mg (50%) of **13** as a colorless oil; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.89 (t, $J = 7.47$, 3H, 6-H), 1.04 (d, $J = 6.67$, 3H, 4-CH_3), 1.25–1.53 (m, 2H, 5-H), 1.91 (d, $J = 1.35$, 3H, 2-CH_3), 1.99 (s, 3H, C(O)CH_3), 2.48 (m, 1H, 4-H), 3.08 (t, $J = 6.0$, 2H, S-CH_2), 3.48 (q, $J = 6.0$, 2H, N-CH_2), 5.9 (broad, 1H, NH), 6.56 (dq, $J = 1.3\sim1.4$ and 9.88, 1H, 3-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.3, 13.0, 19.9, 23.6, 28.8, 30.0, 35.5, 40.2, 135.0, 147.9, 170.7, 194.7; $[\alpha]_D$ −35.5° (c = 1.0, $CHCl_3$); HRMS (FAB^+, NBA/NaI), calculated for $(C_{12}H_{21}NO_2S)Na^+$ 266.1191, found 266.1200. The enantiomeric purity of **13** was determined by conversion of a portion of the (4*R*)-2,4-dimethyl-2-hexenoic acid to the corresponding (4*S*)-4-benzyl-2-oxazolidinone imide by treatment with oxalyl chloride and reaction

of the resulting acid chloride with (4*S*)-4-benzyl-2-oxazolidinone. Measurement of the relative ^{1}H NMR signal intensity for the (4′*R*)-4′-CH_3 group (δ 1.01 (d, *J* = 6.67 Hz) and that of the (4′*S*)- 4′-CH_3 group (δ 1.02 (d, *J* = 6.67 Hz) of the minor 4′*S* –diastereomer established the 4′*R*/4′*S* ratio to be 94:6.

(4S)-2,4-Dimethyl-2-Hexenoic Acid N-Acetylcysteamine Thioester (14a)

14a was prepared by the procedure described for **13** from (2*S*)-(-)-2-methyl-1-butanol (Aldrich). The ^{1}H and ^{13}C NMR spectra were identical to those of **13**. **14a**: $[\alpha]_D$ +34.4° (c = 0.5, $CHCl_3$); HRMS (FAB^+, NBA/NaI), calculated for $(C_{12}H_{21}NO_2S)Na^+$ 266.1191, found 266.1198. ^{1}H NMR analysis of the corresponding (4*S*,2′*E*,4′*R*)-4-benzyl-3-(2′,4′-dimethyl-2′-hexenoyl)-2-oxazolidinone gave a 4′*S*/4′*R* ratio of 84:16.

2-Methyl-2-Pentenoic Acid N-Acetylcysteamine Thioester (14b)

14b was prepared by a procedure analogous to that used for **13**. **14b**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.09 (t, *J* = 7.57, 3H, 5-H), 1.87 (d, *J* = 1.01, 3H, 2-CH_3), 2.00 (s, 3H, C(O)CH_3), 2.24 (m, 2H, 4-H), 3.08 (t, *J* = 6.1, 2H, S-CH_2), 3.47 (q, *J* = 6.0, 2H, N-CH_2), 6.05 (broad, 1H, NH), 6.76 (tq, *J* = 1.39 and 7.24, 1H, 3-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.7, 13.3, 22.5, 23.6, 28.8, 40.2, 135.7, 143.9, 170.7, 194.4; HRMS (FAB^+, NBA/NaI), calculated for $(C_{10}H_{17}NO_2NS)Na^+$ 238.0878, found 238.0879.

2-Methyl-2-Hexenoic Acid N-Acetylcysteamine Thioester (14c)

14c was prepared by a procedure analogous to that used for **13**. **14c**: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.96 (t, *J* = 7.39, 3H, 6-H), 1.50 (tq, *J* = 7.44, 2H, 5-H), 1.88 (d, *J* = 1.09, 3H, 2-CH_3), 2.00 (s, 3H, C(O)CH_3), 2.21 (m, 2H, 4-H), 3.08 (t, *J* = 6.1, 2H, S-CH_2), 3.47 (q, *J* = 6.0, 2H, N-CH_2), 6.02 (broad, 1H, NH), 6.79 (tq, *J* = 1.4 and 7.35, 1H, 3-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.8, 14.3, 22.1, 23.6, 28.8, 31.2, 40.3, 136.3, 142.5, 170.8, 194.4; HRMS (FAB^+, NBA/NaI), calculated for $(C_{11}H_{19}NO_2S)Na^+$ 252.1035, found 252.1027.

Product Synthesis

Methyl (2R, 3S, 6S, 7S)-3, 7-Dihydroxy-2,4,6-Trimethyl-4-Nonenoate (16)

Ethyl (2*E*,4*S*,5*S*)-5-(*tert*-butyldimethylsiloxy)-2,4-dimethyl-2-heptenoate (180 mg, 0.57 mmol) was dissolved in CH_2Cl_2, and treated with DIBAL (1.2 ml of 1 M hexane, 1.2 mmol) at 0°C. After 2 hr, the reaction was quenched with 2 ml of EtOAc followed by 5 ml of 1 M tartaric acid. The mixture was extracted with CH_2Cl_2, and the organic extract was dried with $MgSO_4$ and concentrated to yield (2*E*,4*S*,5*S*)-5-(*tert*-butyldimethylsiloxy)-2,4-dimethyl-2-heptenol: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.04 (s, 6H, $Si(CH_3)_2$), 0.85 (t, *J* = 7.5, 3H, 7-H), 0.90 (s, 9H, $SiC(CH_3)_3$), 0.94 (d, *J* = 6.7, 3H, 4-CH_3), 1.45 (m, 2H, 6-H), 1.68 (d, *J* = 1.4, 3H, 2-CH_3), 2.51 (m, 1H, 4-H), 3.44 (m, 1H, 5-H), 4.00 (s, 2H, 1-H), 5.28 (dd, *J* = 1.3 and 10.1, 1H, 3-H). This product was reacted with PDC (430 mg, 1.14 mmol) in 10 ml CH_2Cl_2 at room temperature overnight. The resulting mixture was loaded on a silica gel (20 g) column that was eluted with CH_2Cl_2 to give (2*E*,4*S*,5*S*)-5-(*tert*-butyldimethylsiloxy)-2,4-dimethyl-2-heptenal (91 mg, two steps 59%); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.04 (s, 6H, $Si(CH_3)_2$), 0.88 (t, *J* = 7.5, 3H, 7-H), 0.90 (s, 9H, $SiC(CH_3)_3$), 1.05 (d, *J* = 6.7, 3H,4-CH_3), 1.35–1.65 (m, 2H, 6-H), 1.76 (d, *J* = 1.2, 3H, 2-CH_3), 2.82 (m, 1H, 4-H), 3.56 (m, 1H, 5-H), 6.42 (dd, *J* = 1.2 and 9.7, 1H, 3-H), 9.39 (s, 1H, H-1). (4*R*)-3-propionyl-4-benzyl-2-oxazolidinone (78 mg, 0.33 mmol) was dissolved in 10 ml of dry CH_2Cl_2 at 0°C and treated with Bu_2BOTf (366 μl of 1 M in CH_2Cl_2, 0.37 mmol) and iPr_2EtN (70 μl, 0.4 mmol) for 30 min at 0°C followed by 30 min at −78°C. The unsaturated aldehyde (91 mg, 0.33 mmol) was added in one portion. The reaction mixture was stirred at −78°C for 30 min and then allowed to warm to room temperature over 2 hr. After cooling to 0°C, the reaction was quenched by addition of 5 ml of phosphate buffer (pH 7.4), poured into a 250-ml flask containing 8 ml of methanol, cooled to 0°C, and treated with a solution of 10 ml of precooled 30% aqueous H_2O_2 for 1 hr. The organic solvents were removed by rotary evaporation, aqueous $NaHCO_3$ (20 ml) was added, and the resultant solution was extracted with CH_2Cl_2. The organic layer was treated with 20 ml of 2 M Na_2SO_3 for 30 min and was again extracted with CH_2Cl_2. The combined extracts were dried over $MgSO_4$ and concentrated. Flash chromatography (15 g silica, 20% EtOAc/hexane) afforded 50 mg (56%) of recovered aldehyde and 70 mg (43%) of (4*R*,2′*R*,3′*S*,6′*S*,7′*S*)-4-benzyl-3-[7′-(*tert*-butyldimethylsiloxy)-3′-hydroxy-2′,4′,6′-trimethyl-4′-nonenoyl]-2-oxazolidinone as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.04 and 0.06 (s, 6H, $Si(CH_3)_2$), 0.83 (t, *J* = 7.46, 3H, 9′-H), 0.90 (s, 9H, $SiC(CH_3)_3$), 0.94 (d, *J* = 6.72, 3H, 6′-CH_3), 1.19 (d, *J* = 7.00, 3H, 2′-CH_3), 1.35-1.55 (m, 2H, 8′-H), 1.63 (d, *J* = 1.01, 3H, 4′-CH_3), 2.51 (m, 1H, 6′-H), 2.79 (dd, *J* = 9.46 and 13.4, 1H, 6b-H), 3.27 (dd, *J* = 3.19 and 13.4, 1H, 6a-H), 3.47 (m, 1H, 7′-H), 3.98 (m, 1H, 2′-H), 4.20 (m, 2H, 5-H), 4.36 (broad, 1H, 3′-H), 4.68 (m, 1H, 4-H), 5.42 (d, *J* = 9.91, 1H, 5′-H), 7.2-7.4 (m, 5H, Ph). To a solution of oxazolidinone (30 mg, 0.06 mmol) in THF/H_2O (5 ml, 4:1) at 0°C, 30% aqueous hydrogen peroxide (100 μl), followed by LiOH•H_2O (100 mg), was added slowly. The mixture was stirred at room temperature for 12 hr. The reaction was quenched with a solution of sodium sulfite (3 ml of 20% aqueous solution) and stirred until peroxides were no longer detectable (30 min). The THF was evaporated and resulting solution was extracted with CH_2Cl_2. After acidification at 0°C with 6 M HCl, the water layer was extracted with ethyl acetate. The combined ethyl acetate layer were concentrated, and the residue was dissolved in 5% $NaHCO_3$. After extraction with CH_2Cl_2 and acidification (as above), the water layer was extracted with ethyl acetate. During the extraction, the TBS group was deprotected spontaneously. The combined ethyl acetate layers were dried over Na_2SO_4 and concentrated to yield (2*R*,3*S*,6*S*,7*S*)-3,7-dihydroxy-2,4,6-trimethyl-4-nonenoic acid; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.94 (t, *J* = 7.45, 3H, 9-H), 0.99 (d, *J* = 6.76, 3H, 6-CH_3), 1.19 (d, *J* = 6.99, 3H, 2-CH_3), 1.3–1.6 (m, 2H, 8-H), 1.65 (d, *J* = 0.83, 3H, 4-CH_3), 2.53 (m, 1H, 6-H), 2.72 (m, 1H, 2-H), 3.39 (m, 1H, 7-H), 4.28 (d, *J* = 6.08, 1H, 3-H), 5.38 (d, *J* = 9.86, 1H, 5-H). The carboxylic acid was dissolved in 1 ml of 50% benzene/methanol and treated with 50 μl of trimethylsilyldiazomethane (2 M in hexane) for 30 min at room temperature. After concentration, chromatography (20% EtOAc/hexane) afforded 5.2 mg (two steps, 30%) of **16**; ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.95 (t, *J* = 7.4, 3H, 9-H), 0.99 (d, *J* = 6.7, 3H, 6-CH_3), 1.16 (d, *J* = 7.0, 3H, 2-CH_3), 1.2–1.6 (m, 2H, 8-H), 1.63 (d, *J* = 1.3, 3H, 4-CH_3), 2.49 (m, 1H, 6-H), 2.70 (m, 1H, 2-H), 3.31 (m, 1H, 7-H), 3.68 (s, 3H, -OCH_3), 4.25 (d, *J* = 5.5, 1H, 3-H), 5.34 (dt, *J* = 1.2 and 9.9, 1H, 5-H). ^{1}H NMR (300 MHz, CD_3OD): δ 0.92 (t, *J* = 7.3, 3H, 9-H), 0.99 (d, *J* = 6.7, 3H, 6-CH_3), 1.18 (d, *J* = 6.9, 3H, 2-CH_3), 1.2–1.6 (m, 2H, 8-H), 1.62 (d, *J* = 1.3, 3H, 4-CH_3), 2.37 (m, 1H, 6-H), 2.68 (m, 1H, 2-H), 3.31 (m, 1H, 7-H), 3.62 (s, 3H, -OCH_3), 4.06 (d, *J* = 7.9, 1H, 3-H), 5.22 (dt, *J* = 1.0 and 9.9, 1H, 5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.7 (C9), 12.0 (2-CH_3), 13.1 (4-CH_3), 16.5 (6-CH_3), 27.5 (C8), 38.4 (C6), 43.4 (C2), 52.2 (OCH_3), 77.5 (C7), 77.6 (C3), 130.4 (C5), 134.8 (C4), 176.6 (C1). ^{13}C NMR (75.5 MHz, CD_3OD): δ 9.67 (C9), 11.2 (2-CH_3), 12.3 (4-CH_3), 16.0 (6-CH_3), 27.8 (C8), 38.7 (C6), 44.3 (C2), 51.1 (OCH_3), 77.2 (C7), 78.6 (C3), 131.0 (C5), 135.0 (C4), 175.9 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{13}H_{24}O_4)Na^+$ 267.1573, found 267.1570.

Triketide-SNAC thioesters **15a–15f** and **17a–17c** were prepared by procedures analogous to those used to synthesize **16**.

15a: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.9–1.0 (m, 6H, 9-H and 6-CH_3), 1.14 (d, *J* = 7.0, 3H, 2-CH_3), 1.2–1.6 (m, 2H, 8-H), 1.65 (s, 3H, 4-CH_3), 2.50 (m, 1H, 6-H), 2.72 (m, 1H, 2-H), 3.31 (m, 1H, 7-H), 4.27 (d, *J* = 6.4, 1H, 3-H), 5.35 (d, *J* = 10, 1H, 5-H). ^{1}H NMR (300 MHz, CD_2Cl_2): δ 0.9–1.0 (m, 6H, 9-H and 6-CH_3), 1.16 (d, *J* = 7.1, 3H, 2-CH_3), 1.2–1.6 (m, 2H, 8-H), 1.63 (s, 3H, 4-CH_3), 2.43 (m, 1H, 6-H), 2.70 (m, 1H, 2-H), 3.27 (m, 1H, 7-H), 3.66 (s, 3H, -OCH_3), 4.25 (d, *J* = 5.5, 1H, 3-H), 5.33 (5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.3 (C9), 11.9 (2-CH_3), 13.0 (4-CH_3), 17.4 (6-CH_3), 27.6 (C8), 38.5 (C6), 43.5 (C2), 52.3 (OCH_3), 77.2 (C7), 77.6 (C3), 129.2 (C5), 136.3 (C4), 176.3 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{13}H_{24}O_4)Na^+$ 267.1573, found 267.1584.

15b: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.95 (t, *J* = 6.73, 3H, 6-CH_3), 1.15 (d, *J* = 6.21, 3H, 8-H), 1.17 (d, *J* = 7.02, 3H, 2-CH_3), 1.65 (d, *J* = 1.1, 3H, 4-CH_3), 2.37 (m, 1H, 6-H), 2.71 (m, 1H, 2-H), 3.49 (m, 1H, 7-H), 3.68 (s, 3H, -OCH_3), 4.26 (d, *J* = 6.15, 1H, 3-H), 5.31 (d, *J* = 10.0, 1H, 5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.3 (2-CH_3), 13.0 (4-CH_3), 17.3 (6-CH_3), 20.5 (C8), 40.8 (C6), 43.5 (C2), 52.3 (OCH_3), 72.2 (C7), 77.6 (C3), 130.4 (C5), 136.8 (C4), 176.4 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{12}H_{22}O_4)Na^+$ 253.1416, found 253.1414.

15c: ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.96 (t, *J* = 7.41, 3H, 9-H), 1.16 (d, *J* = 7.02, 3H, 2-CH_3), 1.4–1.6 (m, 2H, 8-H), 1.63 (s, 3H, 4-CH_3), 2.23 (m, 1H, 6-H), 2.71 (m, 1H, 2-H), 3.60 (m, 1H, 7-H), 3.68 (s, 3H, -OCH_3), 4.29 (d, *J* = 5.53, 1H, 3-H), 5.53 (t, *J* = 8.0, 1H, 5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.4 (C9), 11.9 (2-CH_3), 13.1 (4-CH_3), 30.1 (C8), 35.6 (C6), 43.5 (C2), 52.2 (OCH_3), 73.3 (C7), 77.3 (C3), 123.5

(C5), 137.6 (C4), 176.5 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{12}H_{22}O_4)Na^+$ 253.1416, found 253.1425.

15d: ^1H NMR (300 MHz, $CDCl_3$): δ 1.16 (d, J = 7.10, 3H, 2-CH_3), 1.21 (d, J = 6.19, 3H, 8-H), 1.64 (s, 3H, 4-CH_3), 2.21 (m, 1H, 6-H), 2.72 (m, 1H, 2-H), 3.68 (s, 3H, -OCH_3), 3.83 (m, 1H, 7-H), 4.29 (d, J = 5.65, 1H, 3-H), 5.52 (t, J = 8.0, 1H, 5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 11.9 (2-CH_3), 13.2 (4-CH_3), 23.2 (C8), 38.0 (C6), 43.4 (C2), 52.3 (OCH_3), 68.1 (C7), 77.6 (C3), 123.3 (C5), 137.7 (C4), 176.5 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{11}H_{20}O_4)Na^+$ 239.1260, found 239.1262.

15e: ^1H NMR (300 MHz, $CDCl_3$): δ 0.96 (t, J = 7.41, 3H, 9-H), 1.04 (d, J = 6.87, 3H, 6-CH_3), 1.19 (d, J = 7.16, 3H, 2-CH_3), 1.3–1.6 (m, 2H, 8-H), 2.22 (m, 1H, 6-H), 2.65 (m, 1H, 2-H), 3.33 (m, 1H, 7-H), 3.71 (s, 3H, -OCH_3), 4.34 (t, J = 5.25, 1H, 3-H), 5.53 (dd, J = 5.84 and 15.6, 1H, 4-H), 5.65 (dd, J = 7.98 and 15.6, 1H, 5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 10.4 (C9), 12.3 (2-CH_3), 17.2 (6-CH_3), 27.5 (C8), 42.8 (C6), 45.5 (C2), 52.3 (OCH_3), 73.6 (C3), 76.6 (C7), 131.3 (C5), 135.1 (C4), 176.1 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{12}H_{22}O_4)Na^+$ 253.1416, found 253.1421.

15f: ^1H NMR (300 MHz, $CDCl_3$): δ 1.02 (d, J = 6.82, 3H, 6-CH_3), 1.16 (d, J = 6.36, 3H, 8-H), 1.19 (d, J = 7.21, 3H, 2-CH_3), 2.13 (m, 1H, 6-H), 2.64 (m, 1H, 2-H), 3.57 (m, 1H, 7-H), 3.70 (s, 3H, -OCH_3), 4.34 (t, J = 5.07, 1H, 3-H), 5.55 (dd, J = 5.88 and 15.6, 1H, 4-H), 5.65 (dd, J = 7.95 and 15.6, 1H, 5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.3 (2-CH3), 16.8 (6-CH_3), 20.6 (C8), 45.1 (C6), 45.5 (C2), 52.3 (OCH_3), 71.4 (C7), 73.6 (C3), 131.6 (C5), 135.3 (C4), 176.1 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{11}H_{20}O_4)Na^+$ 239.1260, found 239.1267.

17a (prepared as a mixture of C-6 diastereomers from racemic **13**): ^1H NMR (300 MHz, $CDCl_3$): δ 0.82 and 0.83 (t × 2, J = 7.40, 3H, 8-H), 0.92 and 0.94 (d × 2, J = 6.66, 3H, 6-CH_3), 1.15 and 1.16 (d × 2, J = 7.04, 3H, 2-CH_3), 1.2-1.4 (m, 2H, 7-H), 1.60 (s, 3H, 4-CH_3), 2.18 and 2.23 (d × 2, J = 3.1, 1H, -OH), 2.26 (m, 1H, 6-H), 2.70 (m, 1H, 2-H), 3.67 and 3.68 (s × 2, 3H, -OCH_3), 4.25 (m, 1H, 3-H), 5.25 (m, 1H, 5-H). ^1H NMR (300 MHz, CD_2Cl_2): 0.83 and 0.86 (t × 2, J = 7.40, 3H, 8-H), 0.92 and 0.94 (d × 2, J = 6.55, 3H, 6-CH_3), 1.13 and 1.14 (d × 2, J = 7.02, 3H, 2-CH_3), 1.15-1.45 (m, 2H, 7-H), 1.61 (d, J = 1.33, 3H, 4-CH_3), 2.18 and 2.23 (d, J = 3.8, 1H, -OH), 2.30 (m, 1H, 6-H), 2.69 (m, 1H, 2-H), 3.65 and 3.66 (s × 2, 3H, -OCH_3), 4.23 (m, 1H, 3-H), 5.22 (d, J = 9.6, 1H, 5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 12.1 (2-CH_3), 12.3 and 12.4 (4-CH_3), 13.2 (C8), 21.0 (6-CH_3), 30.6 (C7), 34.2 (C6), 43.3 and 43.5 (C2), 52.1 (OCH_3), 77.3 and 77.7 (C3), 133.8 (C5), 134.4 (C4), 176.3 and 176.4 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{12}H_{22}O_3)Na^+$ 237.1467, found 237.1461.

17b: ^1H NMR (300 MHz, $CDCl_3$): δ 0.96 (t, J = 7.53, 3H, 7-H), 1.15 (d, J = 7.09, 3H, 2-CH_3), 1.59 (s, 3H, 4-CH_3), 2.04 (m, 1H, 6-H), 2.23 (d, J = 3.60, 1H, -OH), 2.69 (m, 1H, 2-H), 3.68 (s, 3H, -OCH_3), 4.25 (t, J = 4.2, 1H, 3-H), 5.47 (tt, J = 1.30 and 7.19, 1H, 5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 11.7 (2-CH_3), 12.7 (4-CH_3), 14.4 (C7), 21.2 (C6), 43.4 (C2), 52.1 (-OCH_3), 129.3 (C5), 133.6 (C4), 176.4 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{10}H_{18}O_3)Na^+$ 209.1154, found 209.1153.

17c: ^1H NMR (300 MHz, $CDCl_3$): δ 0.90 (t, J = 7.32, 3H, 8-H), 1.15 (d, J = 7.06, 3H, 2-CH_3), 1.38 (tq J = 7.36, 2H, 7-H), 1.60 (d, J = 0.81, 3H, 4-CH_3), 2.01 (q, J = 7.33, 1H, 6-H), 2.24 (broad, 1H, -OH), 2.70 (dq, J = 5.55 and 7.08, 1H, 2-H), 3.68 (s, 3H, -OCH_3), 4.27 (d, J = 5.3, 1H, 3-H), 5.48 (tt, J = 1.2 and 7.24, 1H, 5-H). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ 11.8 (2-CH_3), 12.9 (4-CH_3), 14.2 (C8), 23.0 (C7), 30.0 (C6), 43.4 (C2), 52.1 (OCH_3), 77.4 (C3), 127.6 (C5), 134.3 (C4), 176.4 (C1). HRMS (FAB^+, NBA/NaI) calculated for $(C_{11}H_{20}O_3)Na^+$ 223.1310, found 223.1301.

Acknowledgments

This work was funded by grants from the US National Institutes of Health to D.E.C. (GM22172) and to C. K. (CA66736).

Received: June 22, 2001
Revised: October 1, 2001
Accepted: October 4, 2001

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