Precursor-Directed Biosynthesis: Stereospecificity for Branched-Chain Diketides of the β-Ketoacyl-ACP Synthase Domain 2 of 6-Deoxyerythronolide B Synthase

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Dedicated with thanks and admiration to Professor *Duilio Arigoni*, teacher and friend, on the occasion of his 75th birthday

Modular polyketide synthases such as 6-deoxyerythronolide B synthase (DEBS) catalyze the biosynthesis of structurally complex natural products. *Streptomyces coelicolor* CH999/pJRJ2 harbors a plasmid encoding DEBS(KS1^0), a mutant form of 6-deoxyerythronolide B synthase that is blocked in the formation of 6-deoxyerythronolide B (**1**, 6-dEB) due to a mutation in the active site of the ketosynthase (KS1) domain that normally catalyzes the first polyketide chain-elongation step of 6-dEB biosynthesis. Administration of (2*S*,3*R*,4*S*)- and (2*S*,3*R*,4*R*)-3-hydroxy-2,4-dimethylhexanoic acid *N*-acetylcysteamine (SNAC) thioesters (= *S*-[2-(acetylamino)ethyl] (2*S*,3*R*,4*S*)- and (2*S*,3*R*,4*R*)-3-hydroxy-2,4-dimethylhexanethioates) **3** and **4** in separate experiments to cultures of *Streptomyces coelicolor* CH999/pJRJ2 led to production of the corresponding (14*S*)- and (14*R*)-14-methyl analogues of 6-dEB, **10** and **11**, respectively. Unexpectedly, when a 3:2 mixture of **4** and **3** was fed under the same conditions, exclusively branched-chain macrolactone **11** was isolated. In similar experiments, feeding of **3** and **4** to *S. coelicolor* CH999/pCK16, an engineered strain harboring DEBS1 + TE(KS1^0), resulted in formation of the branched-chain triketide lactones **13** and **14**, while feeding of the 3:2 mixture of **4** and **3** gave exclusively **14**. The biochemical basis for this stereochemical discrimination was established by using purified DEBS module 2 + TE to determine the steady-state kinetic parameters for **3** and **4**, with the k_{cat}/K_M for **4** shown to be sevenfold greater than that of **3**.

Introduction. – Modular polyketide synthases (PKSs) are large (M_r 100–1,000 kDa) multifunctional enzyme systems that are responsible for the biosynthesis of structurally complex natural products from simple building blocks such as malonyl-, methylmalonyl-, ethylmalonyl-, and methoxymalonyl-CoA [1]. Polyketide natural products possess a wide variety of pharmacologically important activities, including antibiotic, antifungal, antiparasitic, antitumor, and immunosuppressive properties [2]. Modular polyketide synthases are organized into coordinated groups of active sites, known as modules, in which each module is responsible for one complete cycle of polyketide chain extension and functional-group modification [3]. The individual modules are, in turn, made up of a set of catalytic domains of 100–400 amino acids, each of which is similar in both function and amino acid sequence to the analogous enzymes of fatty acid biosynthesis. The polyketide chain-building step is a decarboxylative condensation reaction catalyzed by a β-ketoacyl-ACP synthase (β-ketosynthase, KS) domain [4]. Almost all PKS modules possess a core set of three domains: a β-ketosynthase (KS), an acyl transferase (AT), and an acyl carrier protein (ACP)

domain. (Interestingly, *Shen* and co-workers have recently reported that the individual modules of the gene cluster encoding leinamycin biosynthesis lack their own AT domains [5].) The degree of functional-group modification taking place during each chain-elongation cycle is determined by the specific combination of additional β-keto reductase (KR), dehydrase (DH), and enoyl reductase (ER) domains found in each module. Further structural diversity arises from the use of different starter and chain-elongation units as well as the generation of new stereogenic centers. Modular PKSs are responsible for the biosynthesis of the polyketide precursors of a wide variety of metabolites including erythromycin [6], methymycin [7], tylosin [8] epothilone [9], rapamycin [10] and rifamycin [11].

The best-understood modular PKS is 6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea*, which catalyzes the formation of 6-deoxyerythronolide B (6-dEB, **1**), the parent macrolide aglycone of the broad-spectrum antibiotic erythromycin A [3] [6] (*Fig. 1,a*). The formation of 6-dEB involves six condensation steps beginning with a propionyl-CoA starter unit and six methylmalonyl-CoA extender units. The six modules of DEBS are organized into three bimodular, homodimeric proteins, DEBS1, DEBS2, and DEBS3, each of subunit $M_r > 330$ kDa [12]. At the N-terminus of module 1 in DEBS1 is a specialized set of AT and ACP domains that, together, are responsible for loading of the propionyl-CoA primer unit. The C-terminus of DEBS3 carries a thioesterase (TE) domain that releases the heptaketide product from ACP6 by cyclization to the 14-membered macrolactone 6-dEB (**1**).

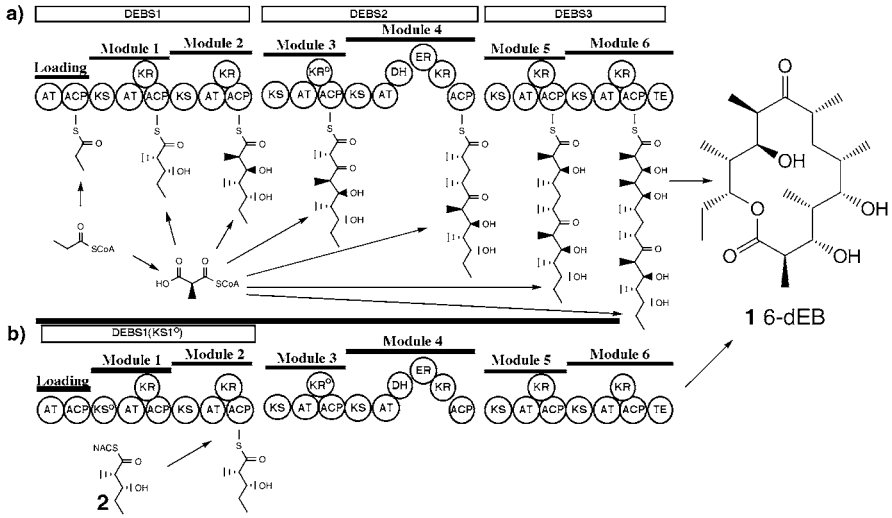


Fig. 1. *Modular organization of DEBS. a*) Each of the six modules contains a ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP), as well as variable numbers of supplementary domains, such as ketoreductase (KR), dehydratase (DH), and enoylreductase (ER). The loading didomain primes module 1 and a thioesterase (TE) domain cyclizes the heptaketide and generates 6-dEB (**1**). *b*) Formation of 6-dEB by DEBS(KS1^0) is blocked but can be restored by addition of the diketide-SNAC **2**.

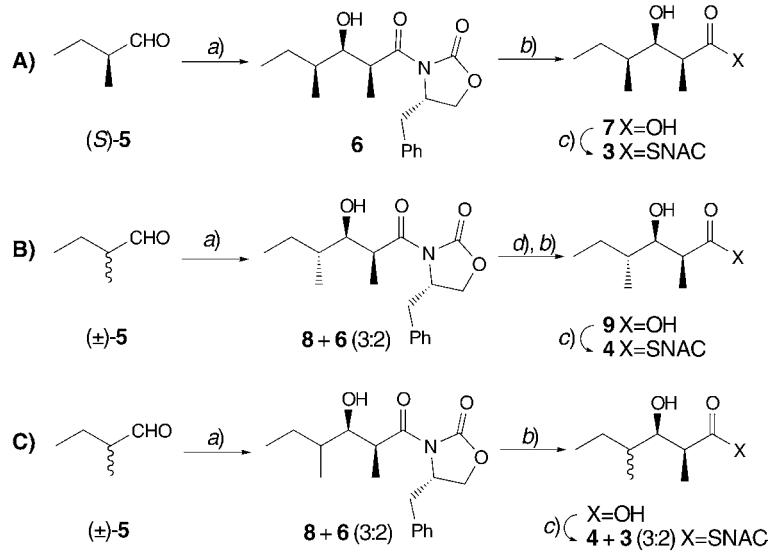
Expression of the entire set of DEBS structural genes in heterologous hosts such as *Streptomyces coelicolor* CH999 or engineered strains of *Escherichia coli*, which carry auxiliary genes for post-translational pantotheinylation of the individual ACP domains of the PKS and for biosynthesis of the necessary propionyl-CoA and methylmalonyl-CoA building blocks, results in accumulation of substantial titers of 6-dEB [13][14]. Inactivation by site-directed mutagenesis of the KS1 domain that normally catalyzes the first condensation step of 6-dEB biosynthesis gives a mutant construct, DEBS(KS1^0), that no longer produces 6-dEB. Formation of 6-dEB (**1**) can be restored, however, when (2*S*,3*R*)-3-hydroxy-2-methylpentanoic acid-SNAC thioester (**2**), an *N*-acetylcysteamine (=2-(acetylamino)ethanethiol, HSNAC) thioester derivative of the natural diketide chain elongation intermediate, is administered to cultures of *S. coelicolor* CH999 harboring plasmid pJRJ2 encoding DEBS(KS1^0) [15a] (*Fig. 1,b*). Moreover, by utilizing surrogate diketide and triketide analogues, it is possible to exploit the apparently broad substrate specificity of the DEBS KS2 domain to generate a wide range of 6-dEB derivatives [15]. We have recently extended the versatility of this precursor-directed biosynthesis technology by the development of an *E. coli* strain harboring a DEBS mutant from which the entire loading didomain and DEBS module 1 have been deleted [15e].

To further explore the utility of this methodology and to better define the substrate specificity of the DEBS KS2 domain, we have now extended these studies to an investigation of the utilization of the diastereoisomeric branched-chain diketide substrates **3** and **4**. Both **3** and **4** share a common substitution pattern and configuration at C(2) and C(3) with the natural diketide **2**. Diketides **3** and **4** can be thought of as diastereoisomeric branched-chain analogues of **2** or, alternatively, as 5-deoxy-6-demethyl analogues of a triketide intermediate. The results of our studies are detailed below.

Results. – *Preparation of the Branched-Chain Diketide Analogues* **3** *and* **4**. The branched-chain diketide substrate (2*S*,3*R*,4*S*)-3-hydroxy-2,4-dimethylhexanoic acid *N*-acetylcysteamine (SNAC) thioester **3** was readily prepared from the known (*S*)-2-methylbutanal ((*S*)-**5**) by using the chiral oxazolidinone method of *Evans et al.* [16] to prepare the acid **7** followed by conversion to the corresponding thioester **3** (*Scheme 1*). The diastereoisomeric (2*S*,3*R*,4*R*)-thioester **4** was in turn synthesized from racemic (±)-**5**, by using silica-gel chromatography to separate the resulting mixture of diastereoisomeric acyloxazolidinones **6** and **8**. The (4*R*)-epimer **8** was then converted to the corresponding (2*S*,3*R*,4*R*)-3-hydroxy-2,4-dimethylhexanoic acid SNAC thioester **4**. In like manner, the mixture of **8** and **6** was also used to prepare a 3:2 mixture of (2*S*,3*R*,4*R*)-**4** and (2*S*,3*R*,4*S*)-**3**.

Feeding of the Branched-Chain Diketides to S. coelicolor *CH999/pJRJ2.* According to previously developed protocols [15d], the diastereoisomeric branched-chain diketide substrates **3** and **4** were individually fed to 3-day-old agar cultures of *S. coelicolor* CH999/pJRJ2 harboring DEBS(KS1^0). After an additional 4 d incubation, the combined mycelia and media from 20 plates (25 ml each) were homogenized and extracted with AcOEt, and the resulting polyketide products were purified from the concentrated extract by SiO_2 column chromatography and preparative TLC. The isolated macrolactones were analyzed by a combination of 1D- and 2D-NMR

Scheme 1. *Synthesis of Branched-Chain Diketide-SNAC Esters. A*) Preparation of (4*S*)-**3**; *B*) preparation of (4*R*)-**4**; *C*) preparation of 3:2 mixture of **4** and **3**.

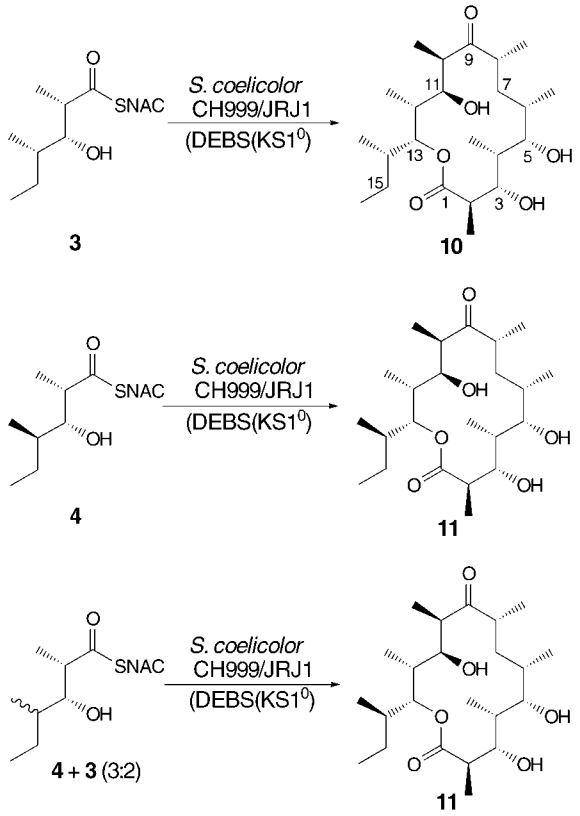


a) Bu_2BOTf, $(i\text{-}Pr)_2NEt$, (4*S*)-4-benzyl-3-propanyl-oxazolidinone. *b*) LiOH, H_2O_2. *c*) *i.* $(PhO)_2PN_3$, Et_3N; *ii.* HSNAC. *d*) SiO_2 Column chromatography.

spectroscopy and HR-FAB-MS, as well as by comparison to the spectra of 6-dEB (**1**). Both branched-chain diketide substrates **3** and **4** were recognized by DEBS module 2 and processed by the downstream modules to give the expected macrolactone products, the (14*S*)-13-(*s*-butyl) 6-dEB analogue **10** (6% yield, based on diketide **3**) and the (14*R*)-13-(*s*-butyl) 6-dEB analogue **11** (25% yield, based on diketide **4**; *Scheme 2*). Unexpectedly, feeding of the 3:2 (4*R*/4*S*) mixture of **4** and **3** under the same conditions gave a single macrolide product (40% yield, based on diketide **4**), identical in all respects to the sample of **11** that had been obtained from the feeding of the (4*R*)-branched-chain diketide thioester **4** alone.

Feeding of the Branched-Chain Diketides to S. coelicolor *CH999/pCK16. S. coelicolor* CH999/pCK16 is an engineered strain harboring DEBS1 + TE($KS1^0$), corresponding to the loading didomain and the first two modules of DEBS, but with an inactive KS1 domain [17]. Feeding of the natural diketide **2** to *S. coelicolor* CH999/pCK16, which can bypass the biochemical block in module 1, has been shown to result in production of the well-known triketide lactone **12**, the product of TE-catalyzed cyclization of the acyclic triketide acyl-ACP intermediate normally generated by DEBS module 2 (*Scheme 3*). Administration of the individual branched-chain diketides **3** and **4** to agar cultures of *S. coelicolor* CH999/pCK16 gave the predicted triketide lactones **13** and **14**, respectively, isolated in 10 and 50% yield based on administered branched-chain diketide diastereomer (*Scheme 3*). Similar to the results obtained with the complete DEBS($KS1^0$) system, feeding of the 3:2 mixture of **4** and **3** to *S. coelicolor* CH999/pCK16 yielded only triketide lactone **14** as a single component (60% yield

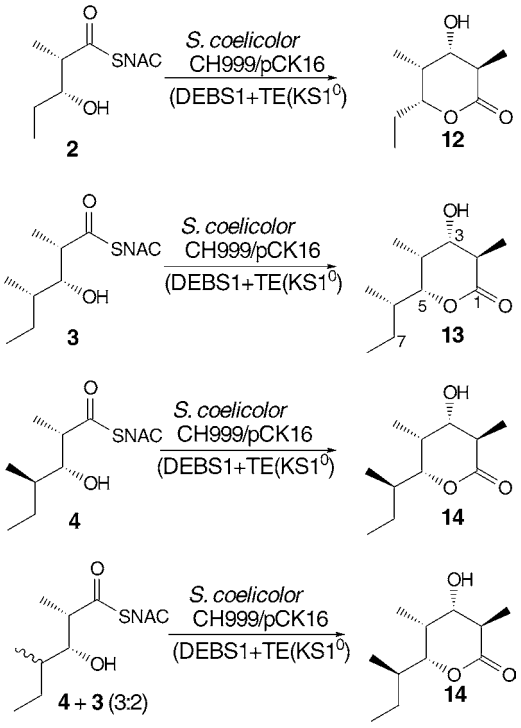
Scheme 2. *Conversion of Branched-Chain Diketide-SNAC Esters to 14-Methyl-6dEb Analogues by* S. coelicolor *CH999/JRJR Harboring DEBS(KS1^0)*



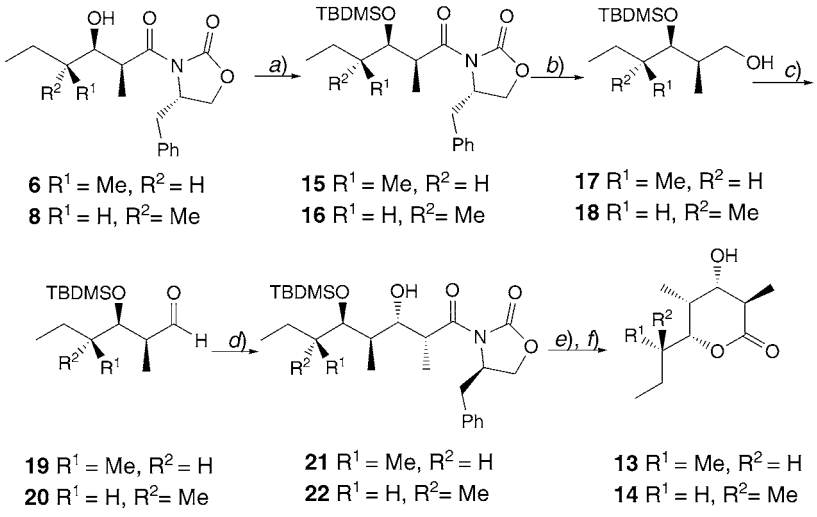
based on **4**), identical to that obtained from the feeding the (4*R*)-branched-chain diketide thioester **4**.

Synthesis of Triketide Lactones. To confirm the structure and the configurational assignments of the triketide lactones **13** and **14** that were produced by precursor-directed biosynthesis from branched-chain diketides with *S. coelicolor* CH999/pCK16, authentic samples of both **13** and **14** were each prepared directly from the individual acyloxazolidinone intermediates **6** and **8**, respectively (*Scheme 4*). The β-OH groups of each diastereoisomer were protected with (*t*-Bu)Me_2SiOTf (TBDMSOTf), affording the TBDMS ethers **15** and **16**, which were converted to the aldehydes **19** and **20**, respectively, by straightforward $LiAlH_4$ reduction followed by PDC oxidation. These aldehydes each served as substrates for a second asymmetric aldol reaction, yielding compounds **21** and **22**, respectively, which were each cleaved from the chiral auxiliary with $LiOH/H_2O_2$. Treatment of the resulting carboxylic acids **23** and **24** with HF effected silyl ether cleavage and subsequent lactonization to form **13** and **14**. The ^{1}H- and ^{13}C-NMR spectra of the individual synthetic triketide lactones **13** and **14** were

Scheme 3. *Conversion of Diketide-SNAC Esters to Triketide Lactones by* S. coelicolor *CH999/pCK16 Harboring DEBS1 + TE(KS1^0)*



Scheme 4

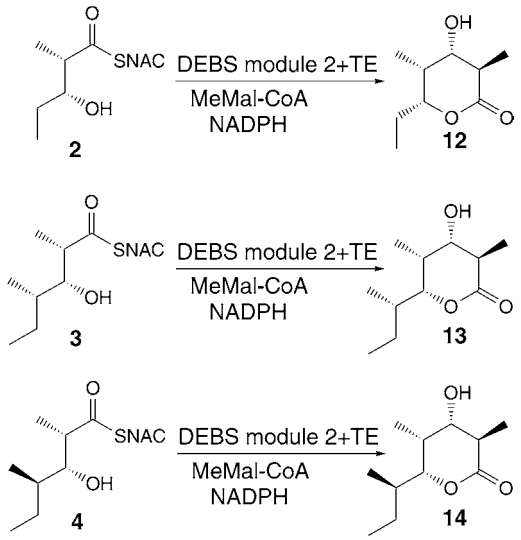


a) TBDMSOTf, (i-Pr)$_2$EtN. *b*) $LiAlH_4$. *c*) PDC. *d*) Bu_2BOTf, (i-Pr)$_2$NEt, (4*R*)-4-benzyl-propanoyloxazolidinone. *e*) LiOH, H_2O_2. *f*) HF.

identical to those of the compounds obtained from the feeding of the branched-chain diketides **3** and **4** to *S. coelicolor* CH999/pCK16.

Enzymatic Conversion of Branched-Chain Diketides to Triketide Lactones. To probe further the biochemical basis for the apparent stereoselectivity for the (4*R*)-methyl branched-chain diketide **4** compared to its diastereoisomer, (4*S*)-**3**, we carried out individual incubations of both **3** and **4** with isolated DEBS module 2 + TE [18] in the presence of [2-^{14}C]methylmalonyl-CoA and NADPH to give the expected triketide lactones **13** and **14**, as determined by radio-TLC/phosphoimaging (*Scheme 5*). We then

Scheme 5. *Enzymatic Conversion of Diketide-SNAC Esters to Triketide Lactones Catalyzed by DEBS Module 2 + TE*



carried out a series of incubations with varying concentrations of each substrate (*Fig. 2*), and fit the resulting data to the *Michaelis–Menten* equation to calculate the characteristic k_{cat}, K_M, and k_{cat}/K_M values for each branched-chain diketide (*Table 1*). For comparison, we also measured the steady-state kinetic parameters for the natural diketide **2** by using the same enzyme preparations of DEBS module 2 + TE. Notably, at concentrations above 1 mM (*ca.* 2 times K_M), (4*R*)-methyl diketide **4** showed pronounced substrate inhibition (*Fig. 2*,*c*). The relevant steady-state parameters for **4** were, therefore, calculated from the data obtained at concentrations of **4** $\leq$ 1 mM. The specificity constant k_{cat}/K_M for each substrate could also be calculated directly from the slope of the *V vs.* [S] curve at low substrate concentrations (0.1 – 1 mM). Comparison of the relative k_{cat}/K_M values revealed that the specificity for (4*S*)-methyl **3** was similar to that of the natural diketide substrate **2**, while the (4*R*)-methyl diastereoisomer **4** showed a sevenfold increase in k_{cat}/K_M relative to **3** (5.5-fold relative to **2**).

Competition experiments were also carried out with DEBS module 2 + TE in which variable concentrations of natural diketide **2** were incubated with [2-^{14}C]methylmal-

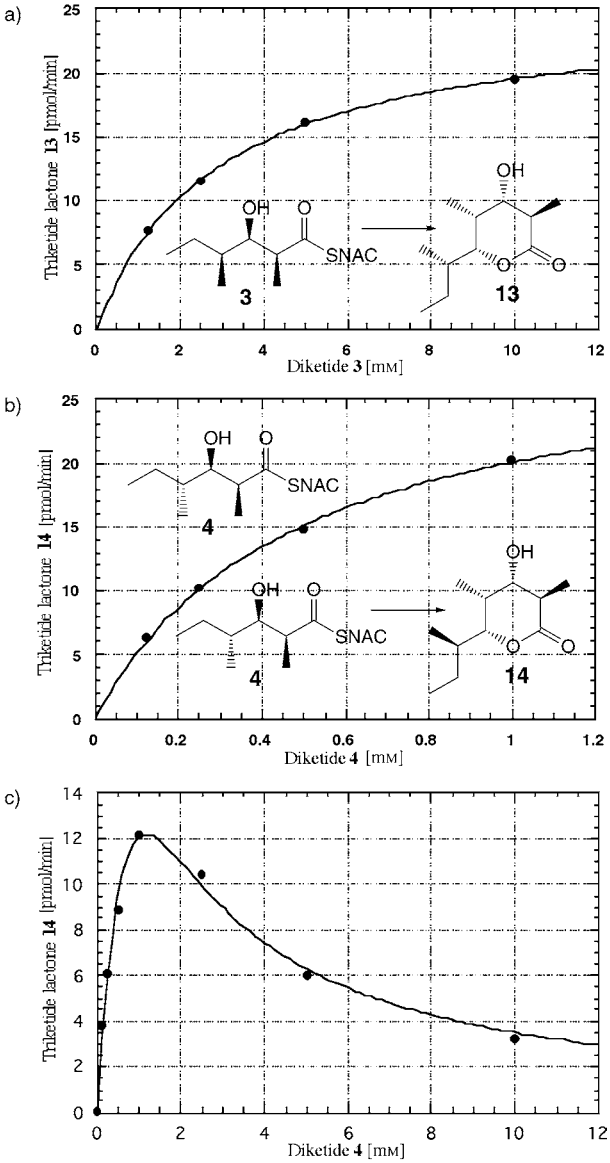


Fig. 2. *Plot of* V vs. *[S] for incubation of branched-chain diketide-SNAC substrates with DEBS module 2 + TE. a*) (4*S*)-Methyl diketide **3**; *b*) (4*R*)-methyl diketide **4**, 0.15 – 1.0 mM; *c*) (4*R*)-methyl diketide **4**, 0.15 – 10.0 mM, showing substrate inhibition

onyl-CoA and NADPH in the presence of 0, 0.2, and 0.5 mM **4**, with the formation of triketide lactone **12** assayed by TLC/phosphoimaging. As is evident from the double reciprocal *Lineweaver–Burk* plot for these reactions (*Fig. 3*), **4** behaved as a typical noncompetitive inhibitor, based on the observed decrease in V_{max} but absence of

Table 1. *Steady-State Kinetic Parameters for Incubation of Branched-Chain Diketides with DEBS Module 2 + TE*

Substrate	K_M [mM]	k_{cat} [min^{-1}]	k_{cat}/K_M [$mmol^{-1}min^{-1}$]	k_{cat}/K_M [rel]
2[a])	4.37 ± 0.61	1.56 ± 0.12	0.36	100
(4*R*)-**3**	2.90 ± 0.13	0.82 ± 0.02	0.28	78
(4*S*)-**4**	0.49 ± 0.04	0.96 ± 0.04	1.98	550

[a]) The steady-state parameters previously determined for **2** for DEBS module 2 + TE were $K_M > 3.2$ mM, $k_{cat} > 4.6$ min^{-1}, k_{cat}/K_M 0.75 $mmol^{-1}$ min^{-1} [18].

change in the apparent V_{max}/K_M for **2**. Direct fitting of these limited data to the appropriate version of the *Michaelis–Menten* equation gave a calculated K_i of *ca.* 0.3 mM for **6** *vs.* natural diketide **2**, close to the independently measured K_M for **4** of 0.5 mM.

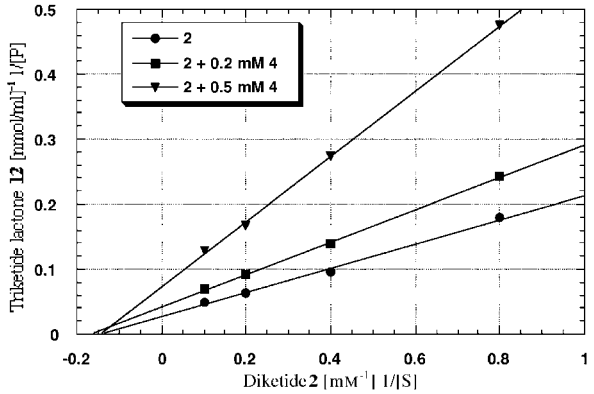


Fig. 3. Lineweaver–Burk *plot showing noncompetitive inhibition by branched-chain diketide* **4** *for processing of natural diketide* **2** *by DEBS module 2 + TE*

Discussion. – The use of modular polyketide synthases blocked in specific reactions in combination with surrogate substrates has provided a powerful tool for the engineered biosynthesis of novel analogues of medicinally important natural products [15]. A major requirement for the successful implementation of this promising tool is a better understanding of the structural and configurational features that influence the processing of substrate analogues by individual polyketide synthase modules. DEBS(KS1^0) is a mutant of the parent DEBS PKS in which the β-ketoacyl-ACP synthase domain of module 1 (KS1) has been inactivated by replacement of the active-site cysteine by alanine [15a]. A variety of analogues of the natural (2*S*,3*R*)-3-hydroxy-2-methylpentanoyl-SACP diketide, the normal product of module 1, have been fed as the corresponding SNAC thioesters to cultures of *S. coelicolor* harboring plasmid pJRJ2 that encodes DEBS(KS1^0), resulting in the formation of the corresponding structural variants of 6-deoxyerythronolide B (**1**) [15].

Although feeding analogues of natural biosynthetic intermediates to strategically blocked PKS mutants harbored within intact microbial hosts has substantial utility for

the controlled generation of novel bioactive polyketides, only qualitative information about relative substrate specificity can be gleaned from comparison of the relative product yields, due to lack of information about numerous experimental variables, including differences in precursor stability, uptake, competing metabolism, and end-product stability, as well as variations in *in vivo* enzyme expression and activity. Fortunately, the availability of substantial quantities of recombinant PKS modules, expressed and purified from *E. coli*, has made possible quantitative evaluation of substrate specificity under well-defined experimental conditions. In comparing the behaviors of different substrate analogues with a single module, the most-informative kinetic parameter is the specificity constant, k_{cat}/K_M. Since k_{cat}/K_M for any enzyme is a function of only individual rate constants up to and including the first irreversible reaction and since the KS-catalyzed decarboxylative condensation is effectively irreversible, it is evident that the observed k_{cat}/K_M values for diketides **2**, **3**, and **4** reflect exclusively the properties of the KS domain of DEBS module 2.

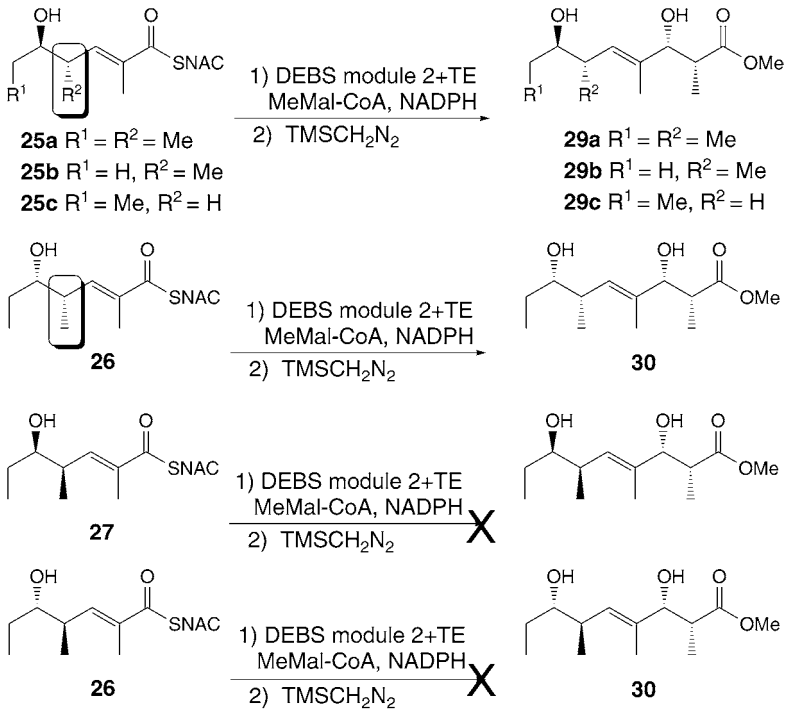
Significantly, both diastereoisomeric branched-chain diketides, **3** and **4**, when fed individually to *S. coelicolor* CH999 harboring either DEBS(KS1^0) or DEBS1+TE(KS1^0), were converted efficiently to the corresponding branched-chain analogues of 6-dEB, **10** and **11**, or to the triketide lactones **13** and **14**, respectively (*Schemes 2* and *3*). Surprisingly, however, when 3:2 mixtures of **4** and **3** were fed to the same strains of *S. coelicolor*, the only isolated lactone products were the single diastereomers **11** and **14**, respectively, corresponding effectively to exclusive mobilization of the (4*R*)-4-methyl diketide **4**.

The biochemical basis for this apparent kinetic resolution of the diastereoisomeric mixture of branched-chain diketides is evident by comparison of the relative k_{cat}/K_M values for processing of the individual substrates by isolated DEBS module 2+TE, with **4** being favored over **3** by a ratio of $>7:1$, reflecting an intrinsic preference of DEBS KS2 for the (4*R*)-methyl substrate **4**. At a diastereoisomeric ratio of 3:2, this difference in relative k_{cat}/K_M should translate into a competitive kinetic advantage for **4** of $>10:1$. That the preferred branched-chain diketide substrate **4** can act as a *noncompetitive* inhibitor of the diketide **2** at concentrations <1 mM requires that **4** bind to both free protein as well the enzyme-substrate complex of DEBS module 2+TE and **2** with equal affinity. Although the detailed molecular mechanism for this non-competitive inhibition is not known, it is conceivable that binding of branched-chain diketide **4** to one KS2 domain of the DEBS module 2+TE homodimer could influence turnover of a diketide **2** by the paired KS2 domain. Such cross-talk between the paired KS2 domains could also account for the marked substrate self-inhibition displayed by **4** (*Fig. 2*,*c*). Alternatively, the observed noncompetitive inhibition of chain elongation of **2** by **4** could also be accounted for by a relatively rapid rate of acylation but slower rate of release of **4** by the TE domain as compared to **2**. The DEBS TE domain is known to recognize and hydrolyze substituted acyl thioesters, and shows a markedly higher specificity for longer chain substrates such as **4** relative to shorter-chain substrates such as **2** [19]. Since the TE must recognize the distal end of the substrate chain, it is, therefore, possible that the branched-chain diketide might remain more tightly bound to the TE than would diketide **2**. Whatever the mechanism of non-competitive inhibition of turnover of natural diketide **2** by branched-chain diketide **4**, it is likely that **4** might also act as a noncompetitive inhibitor of the turnover of branched-chain

diketide diastereoisomer **3**, whose steady-state kinetic parameters are similar to those of natural diketide **2**. (Direct measurement of such inhibition using TLC/phosphoimaging was complicated by the very similar TLC R_f values of branched-chain triketide lactones **13** and **14**.) Such noncompetitive inhibition would further enhance the intrinsic kinetic advantage for **4** over **3** in processing of the 3:2 mixture by DEBS(KS1^0) or DEBS1 + TE(KS1^0). In any event, formation of minor amounts (< 5% relative yield) of the diastereoisomeric lactones **10** and **13** might well have gone undetected by the isolation and analytical procedures used in the precursor-directed biosynthesis experiments described.

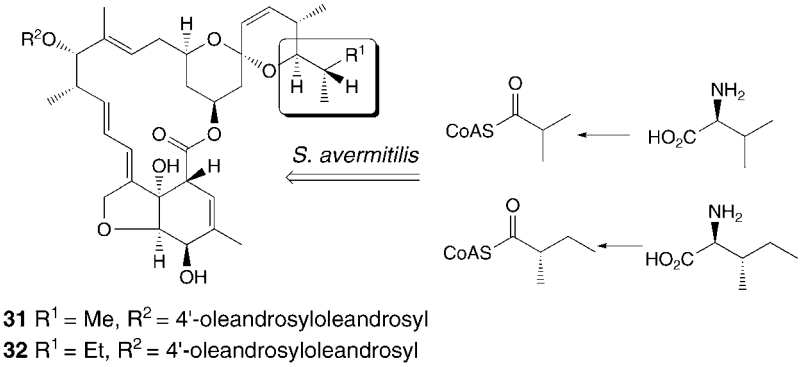
It is interesting to compare the sevenfold relative preference in k_{cat}/K_M for (4*R*)-4-methyl-**4** over (4*S*)-4-methyl-**3** exhibited by DEBS KS2 with the striking diastereoisomeric differentiation shown by DEBS KS2 towards the unsaturated triketides **25**–**28** (*Scheme 6*) [20]. We have previously reported that both **25a** and **26** are efficiently processed to the corresponding tetraketides **29a** and **30** by DEBS module 2 + TE, with relative k_{cat}/K_M values compared to natural diketide **2** ($k_{cat}/K_M = 100$) of 120 and 23, respectively. Significantly, both **25a** and **26** have the same D-methyl configuration at C(4) as the preferred branched-chain diketide **4**. Similarly, the 6-demethyl analogue **25b**, which has the same D-methyl configuration at C(4), is also a good substrate for DEBS module 2 + TE, with a k_{cat}/K_M(rel) of 71, while the 4-demethyl analogue **25c** has a k_{cat}/K_M(rel) of 83. By contrast, neither unsaturated triketide **27** nor **28**, with L-methyl substituents at C(4), is a substrate for DEBS module 2 + TE.

Scheme 6. *Processing of Unsaturated Triketide Analogues by DEBS Module 2 + TE*



Marsden et al. have recently used a chimeric form of DEBS1 + TE ('DEBS1-TE' in their terminology) in which the natural DEBS loading didomain, AT_L-ACP_L, has been replaced by the corresponding loading didomain of the avermectin PKS (AVR_lm) in order to generate polyketides derived from branched-chain starters [21]. The avermectins are tridecaketide macrolides produced by *S. avermitilis*, and are themselves derived from either a (2*S*)-2-methylbutyrate (avermectin B1a (**31**)) or an isobutyrate (avermectin B1b (**32**)) starter unit, the (2*S*)-2-methylbutyryl-CoA originating from (2*S*,3*S*)-isoleucine (*Scheme 7*) [22]. In fact, the avermectin loading domain is known to tolerate a broad range of carboxylic acid starter units, and this natural promiscuity had previously been exploited to generate a variety of avermectin analogues derived from novel starter units [23]. Consistent with this permissiveness toward starter-unit structure, strains of *S. coelicolor* CH999 harboring the hybrid AVR_lm + DEBS1 + TE produced a 8 : 2 : 2 : 5 mixture of four triketide lactones **12**, **33**, **34**, and **35**, in which **35** carried an *s*-Bu substituent of unspecified configuration (*Scheme 8*) [21]. The latter branched-chain triketide lactone was reported to be identical to a synthetically prepared lactone, of known structure but unassigned configuration at C(6) [21][24]. In fact, comparison of the ^{1}H-NMR spectrum reported by *Marsden et al.* [21] for **35** with the spectra of each of the authentic triketide lactones **13** and **14** has now established the identity of **35** and **14**, thereby allowing unambiguous assignment of the (6*R*)-configuration to the *s*-Bu side chain of **35** (*Table 2*).

Scheme 7. *Biosynthesis of Avermectins B1b* (**31**) *and B1a* (**32**) *by* S. avermitilis *from Isobutyryl-CoA and (2*S*)-Methylbutyryl-CoA Derived from Valine and Isoleucine, Respectively*



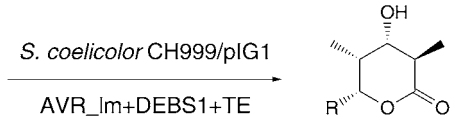
31 R^1 = Me, R^2 = 4'-oleandrosyloleandrosyl
32 R^1 = Et, R^2 = 4'-oleandrosyloleandrosyl

Table 2. *Comparison of ^{1}H-NMR Data for Branched-Chain Triketide Lactones*

H-Atom	δ		
	13	**35**[a])	**14**
H–C(5)	3.85	3.83	3.86
H–C(6)	1.73	1.74	1.75
H_a–C(7)	1.92	1.48	1.51
H_b–C(7)	1.24	1.09	1.05
Me–C(6)	0.89	1.08	1.10

[a]) Supplemental Material: [21].

Scheme 8. *Formation of Triketide Lactones by AVR_lm + DEBS1 + TE*



12 R = Et
33 R = Me
34 R = i-Pr
35 R = *s*-Bu

Interestingly, **35** (=**14**) can only be derived from a (2*R*)-2-methylbutyryl-CoA starter, rather than the (2*S*)-2-methylbutyryl-CoA originating from isoleucine. Thus, although the chimeric AVR_lm + DEBS1 + TE produces a mixture of four triketide lactones, derived more or less indiscriminately from acetate, propionate, isobutyrate, and 2-methylbutyrate starters, it is only the (2*R*)-3-methylbutyrate enantiomer that is utilized by the chimeric AVR_lm + DEBS PKS. It has already been shown that the avermectin loading domain can utilize either enantiomer of the 2-methylbutyrate starter [23b]. The observed chiral discrimination leading to exclusive formation of (6*R*)-methyl triketide lactone **35** (=**14**) by the hybrid AVR_lm + DEBS1 + TE cannot, therefore, be due to AVR_lm, nor can it be attributed to DEBS KS2, since DEBS1 would be already committed irreversibly to synthesizing polyketide by the time that the (4*S*)-4-methyl branched-chain diketide intermediate is generated and presented to DEBS KS2. The apparently strict discrimination against the (2*S*)-2-methylbutyrate starter must, therefore, be due to the intrinsic stereoselectivity of the downstream DEBS KS1 domain.

Tolerance of unnatural intermediates has been widely observed in genetically engineered PKSs. [25]. The results with branched-chain diketides demonstrate the utility of precursor-directed biosynthesis as a method for the controlled generation of novel polyketides having a stereogenic chiral center at C(14) of erythromycin A.

Experimental Part

General. Q Sepharose fast-flow resin was purchased from *Pharmacia*. TLC was carried out on *Whatman* silica-gel plates (*AL SIL G/UV*). [2-^{14}C]Methylmalonyl-coenzyme A (54 mCi/mmol) was purchased from *American Radiolabeled Chemicals, Inc*. All other commerical reagents, buffer components, and enzymes were purchased from *Sigma-Aldrich Chemical Co.* and were of the highest purity available. Culture medium components were purchased from *Difco*. NMR Spectra were recorded on a *Bruker Avance 300* spectrometer. High-resolution mass spectra HR-MS were measured on a *Kratos MS80RFA* mass spectrometer. Quantification of radio-TLC components for kinetic assays utilized a *BioRad GS-363 Molecular Imager* and *Molecular Analyst* software calibrated with standards of known radioactivity. Protein concentration was determined by the method of *Bradford* [26] with the kit from *BioRad*. Kinetic data were analyzed by direct fitting to the relevant version of the *Michaelis–Menten* equation with the *KaleidaGraph* data analysis software (*Synergy Software*).

*(4*S*)-4-Benzyl-3-[(2*S*,3*R*,4*RS*)-3-hydroxy-2,4-dimethylhexanoyl]oxazolidin-2-one* (3:2 mixture **8**/**6**). To a soln. of 1.0 g (4.26 mmol, 1.0 equiv.) of (4*S*)-4-benzyl-3-propanoyloxazolidin-2-one in 20 ml of CH_2Cl_2 at 0° under N_2 was added 4.7 ml of Bu_2BOTf (1.0M in CH_2Cl_2, 1.1 equiv.), followed by the addition of 0.9 ml EtN(i-Pr)$_2$ (5.12 mmol, 1.2 equiv.). The soln. was stirred at 0° for 30 min, then cooled to −78° (dry ice/acetone), stirred for 30 min, and 690 µl of freshly distilled (±)-2-methylbutanal (6.39 mmol, 1.5 equiv.) was added rapidly *via* syringe. The resulting soln. was stirred for 2 h at −78°, then for 1.5 h at 0° before quenching with 20 ml of MeOH

and 50 mM phosphate buffer (pH 7.2). After 5 min, 10 ml of 30% aq. H_2O_2 was added dropwise. After being stirred an additional 20 min at 0°, the mixture was extracted with 3×20 ml CH_2Cl_2, dried (Na_2SO_4), and concentrated. Purification by flash column chromatography (CC; SiO_2; 20% AcOEt/hexanes) afforded 950 mg of a 3:2 mixture **8**/**6** as a colorless oil (70% yield). *Rf* (20% AcOEt/hexanes) 0.28. $[\alpha]_D = +31.2$ ($c = 1.0$, $CHCl_3$); The IR, and ^{1}H- and ^{13}C-NMR data for the mixture corresponded to the sum of the spectra of the individual components **8** and **6**, whose spectroscopic data are given below. HR-FAB-MS (NBA/NaI): 342.1693 ($[M + Na]^+$, $(C_{18}H_{25}NO_4)Na+$; calc. 342.1681).

*(2*S*,3*R*,4*RS*)-3-Hydroxy-2,4-dimethylhexanoic Acid* (3:2 mixture **9**/**7**). To a stirred soln. of 350 mg (1.10 mmol, 1.0 equiv.) of the 3:2 mixture **8**/**6** in 12 ml of THF and 4 ml of H_2O at 0° was added under N_2 1.06 ml (8.8 mmol, 8 equiv.) of 30% aq. H_2O_2, followed by 92 mg (2.20 mmol, 2 equiv.) of $LiOH \cdot H_2O$ in one portion. The resulting mixture was warmed to r.t. overnight before quenching with 1.25 g of Na_2SO_3 in 5 ml of H_2O. THF was evaporated, and the aq. soln. was extracted with CH_2Cl_2 (2×20 ml). The aq. layer was adjusted to pH 1.0 with 1N HCl and extracted with AcOEt (3×20 ml). The org. soln. was dried (Na_2SO_4) and concentrated *in vacuo* to yield 135 mg of a mixture **9**/**7** as a yellow oil, which was used immediately in the next step [16b].

S-*[2-(Acetylamino)ethyl] (2*S*,3*R*,4*RS*)-3-Hydroxy-2,4-hexanethioate* (3:2 mixture **4**/**3**). Crude (2*S*,3*R*,4*RS*)-3-hydroxy-2,4-dimethylhexanoic acid (135 mg, 0.84 mmol, 1.0 equiv.) in 5 ml of DMF under N_2 was treated with diphenyl phosphorylazide (0.28 ml, 1.26 mmol, 1.5 equiv.) and Et_3N (0.24 ml, 1.68 mmol, 2 equiv.) at 0°. After 2 h, 210 mg (1.68 mmol, 2 equiv.) of *N*-acetylcysteamine (=2-(acetylamino)ethanethiol) was added. The mixture was allowed to stir at r.t. for 2 h, followed by addition of 5 ml of H_2O and extraction with AcOEt (3×25 ml). The org. extract was dried (Na_2SO_4) and concentrated *in vacuo*. Purification by CC (SiO_2; 60% AcOEt/hexanes) afforded 178 mg (80%) of a 3:2 mixture of (4*R*)-**4**/(4*S*)-**3** as a colorless oil. R_f (AcOEt) 0.40. The IR and ^{1}H- and ^{13}C-NMR data for the mixture corresponded to the sum of the spectra of the individual components **4** and **3**, whose spectroscopic data are given below. HR-FAB-MS (NBA/NaI): 284.1291 ($[M + Na]^+$, $(C_{12}H_{23}NO_3S)Na^+$; calc. 284.1296).

*(4*S*)-4-Benzyl-3-[(2*S*,3*R*,4*S*)-3-hydroxy-2,4-dimethylhexanoyl]oxazolidin-2-one* (**6**). To a suspension of 3.8 g (10.0 mmol) of pyridinium dichromate in CH_2Cl_2 was added dropwise 440 mg (5.0 mmol) of (2*S*)-2-methylbutan-1-ol at r.t. After 2 h, an additional 3.8 g (10.0 mmol) of pyridinium dichromate was added to the mixture. The mixture was allowed to stir at r.t. overnight and then passed through a short column of *Florisil* (30 mesh, washed with CH_2Cl_2). The filtrate was concentrated under reduced pressure on ice water to afford *(2*S*)-2-methylbutanal* (**5**), which was used immediately in the preparation of **6** by the method described above for the diastereoisomeric mixture **8**/**6**.

Data of **6**: R_f (10% Et_2O/CH_2Cl_2) 0.59. $[\alpha]_D = +41.0$ ($c = 1.0$, $CHCl_3$). IR (film): 3534, 2966, 1778, 1692. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.91 (*t*, *J* = 7.3, Me(6′)); 0.99 (*d*, *J* = 6.6, Me−C(4′)), 1.00–1.20 (*m*, H_b−C(5′)); 1.27 (*d*, *J* = 7.0, Me−C(2′)); 1.40–1.58 (*m*, H−C(4′), H_a−C(5′)); 2.79 (*dd*, *J* = 3.4, 9.5, 1 H, PhCH_2); 3.26 (*dd*, *J* = 13.5, 3.3, 1 H, PhCH_2); 3.69 (*dd*, *J* = 7.1, 3.7, H−C(3′)); 3.97 (*m*, H−C(2′)); 4.21 (*m*, CH_2(5)); 4.71 (*m*, H−C(4)); 7.20–7.38 (*m*, arom. H); ^{13}C-NMR (75.5 MHz, $CDCl_3$): 177.6; 153.0; 135.2; 129.5; 129.0; 127.5; 75.1; 66.2; 55.2; 40.0; 37.8; 37.3; 25.7; 14.6; 11.4. HR-FAB-MS (NBA/NaI): 342.1692. ($[M + Na]^+$ $(C_{18}H_{35}NO_4)Na^+$; calc. 342.1681).

S-*[2-(Acetylamino)ethyl] (2*S*,3*R*,4*S*)-3-Hydroxy-2,4-dimethylhexanethioate* (**3**). Compound **3** was prepared from **6** by the same procedure used to prepare the diastereoisomeric mixture **4**/**3**. R_f (AcOEt). 0.40 $[\alpha]_D = +11.7$ ($c = 1.0$, $CHCl_3$). IR (film): 3293, 3079, 2965, 2933, 2876, 1657, 1554. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.92 (*t*, *J* = 7.4, 3 H); 0.96 (*d*, *J* = 6.8, Me−C(4)); 1.10–1.25 (*m*, H_b−C(5)); 1.27 (*d*, *J* = 7.1, Me−C(2)); 1.35–1.55 (*m*, H_a−C(5), H−C(4)); 1.98 (*s*, COMe); 2.90 (*m*, H−C(2)); 3.05 (*m*, CH_2S); 3.46 (*m*, CH_2N); 3.73 (*t*, *J* = 5.7, H−C(3)); 5.84 (br., NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 204.0; 170.5; 75.4; 51.2; 39.5; 37.1; 28.4; 26.0; 23.2; 13.9; 12.4; 10.9. HR-FAB-MS (NBA/NaI): 284.1290 ($[M + Na]^+$, $(C_{12}H_{23}NO_3S)Na^+$; calc. 284.1296).

*(4*S*)-4-Benzyl-3-[(2*S*,3*R*,4*R*)-3-hydroxy-2,4-dimethylhexanoyl)oxazolidin-2-one* (**8**). Compound **8** was separated by CC (SiO_2; CH_2Cl_2) from the 3:2 mixture **8**/**6**. R_f (10% Et_2O/CH_2Cl_2) $[\alpha]_D = +42.5$ ($c = 1.0$, $CHCl_3$). IR (film): 3525, 2967, 2878, 1781, 1697. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.87 (*d*, *J* = 6.8, Me−C(4′)); 0.93 (*t*, *J* = 7.5, Me(6′)); 1.10–1.25 (*m*, H_b−C(5′)); 1.26 (*d*, *J* = 7.0, Me−C(2′)); 1.45–1.60 (*m*, H_a−C(5′)); 1.75–1.90 (*m*, H−C(4′)); 2.81 (*dd*, *J* = 13.4, 9.5, 1 H, PhCH_2); 3.28 (*dd*, *J* = 13.5, 3.2, 1 H, PhCH_2); 3.64 (*dd*, *J* = 9.1, 2.2 H−C(3′)); 3.97 (*m*, H−C(2′)); 4.22 (*m*, CH_2(5)); 4.73 (*m*, H−C(4)); 7.20–7.38 (*m*, 5 arom. H). ^{13}C-NMR (75.5 MHz, $CDCl_3$): 177.9; 152.8; 135.0; 129.3; 128.8; 127.3; 74.7; 66.0; 55.0; 38.4; 37.6; 36.9; 25.0; 14.6; 10.8. HR-FAB-MS (NBA/NaI): 342.1692. ($[M + Na]^+$), $(C_{18}H_{35}NO_4)Na^+$; calc. 342.1681.

S-*[2-(Acetylamino)ethyl] (2*S*,3*R*,4*R*)-3-Hydroxy-2,4-dimethylhexanethioate* (**4**). Compound **4** was prepared according to the same procedure used to prepare **3**. R_f (AcOEt) 0.40: $[\alpha]_D = +16.3$ ($c = 1.0$, $CHCl_3$). IR (film): 3288, 3081, 2965, 2934, 2877, 1659, 1551. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.86 (*d*, *J* = 6.9, Me−C(4)); 0.93 (*t*,

$J = 7.4$, 3 H); 1.10–1.25 (*m*, H_b–C(5)); 1.21 (*d*, $J = 7.0$, Me–C(2)); 1.35–1.55 (*m*, H_b–C(5)); 1.70–1.85 (*m*, H–C(4)); 1.98 (*s*, COMe); 2.89 (*m*, H–C(2)); 3.05 (*m*, CH_2S); 3.46 (*m*, CH_2N); 3.69 (*dd*, $J = 8.5, 3.2$, H–C(3)); 5.83 (br., NH). ^{13}C-NMR (100 MHz, $CDCl_3$): 204.1; 170.6; 75.6; 50.6; 39.1; 37.1; 28.3; 24.5; 22.8; 15.0; 10.8; 10.1. HR-FAB-MS (NBA/NaI): 284.1292 ($[M + Na]^+$, $(C_{12}H_{23}NO_3S)Na^+$; calc. 284.1296).

*(4*S*)-4-Benzyl-3-[(2*S*,3*R*,4*R*)-3-[(*tert*-butyl)dimethylsiloxy]-2,4-dimethylhexanoyl}oxazolidin-2-one* (**16**). Compound **8** (230 mg, 0.72 mmol, 1.0 equiv.) was dissolved in 5 ml of CH_2Cl_2 and cooled to 0°. EtN(i-Pr)$_2$ (216 µl, 1.18 mmol, 1.7 equiv.) and TBDMSOTf (208 µl, 1.10 mmol, 1.6 equiv.) were added, and the mixture was allowed to warm slowly to r.t., then stirred for an additional 2 h. The reaction was quenched with 1 ml of brine, the mixture was extracted with 4×5 ml CH_2Cl_2, dried (Na_2SO_4), and concentrated. Purification by CC (SiO_2; 5% AcOEt/hexanes) afforded 290 mg (93%) of **16**. Colorless oil. **16**: R_f (10% AcOEt/hexanes) 0.55. $[\alpha]_D = +52.8$ ($c = 1.0$, $CHCl_3$). IR (film): 2959, 2858, 1783, 1699, 1460, 1382, 1210, 1117, 1103, 1016, 969, 868, 837, 774, 700 cm. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.06 (*s*, MeSi); 0.08 (*s*, MeSi); 0.82–0.99 (*m*, Me_3C, Me(6′), Me–C(4′)); 1.13–1.32 (*m*, H_b–C(5′)); 1.16 (*d*, $J = 6.4$, Me–C(2′); 1.38–1.59 (*m*, H–C(4′), H_a–C(5′)); 2.78 (*dd*, $J = 13.3, 9.6$, 1 H, PhCH_2); 3.28 (*dd*, $J = 13.3, 3.1$ 1 H, PhCH_2); 3.88–4.04 (*m*, H–C(2′), H–C(3′)); 4.18 (*m*, CH_2(5)); 4.65 (*m*, H–C(4)); 7.15–7.38 (*m*, 5 arom. H). ^{13}C-NMR (75.5 MHz, $CDCl_3$): 176.0; 152.7; 135.2; 129.3; 129.0; 127.2; 76.1; 65.8; 55.6; 40.8; 40.7; 26.0; 24.8; 15.2; 13.8; 12.3; −2.9; −3.4. HR-FAB-MS (NBA/NaI): 456.2539 ($[M + Na]^+$, $(C_{24}H_{39}NO_4Si)Na^+$; calc. 456.2546.

*(2*S*,3*R*,4*R*)-3-[(*tert*-Butyl)dimethylsiloxy]-2,4-dimethylhexanal* (**20**). Compound **16** (290 mg, 0.67 mmol, 1.0 equiv.) was dissolved in 10 ml of freshly distilled Et_2O and cooled to −78°. $LiAlH_4$ (1.0 ml of a 1.0M soln. in hexanes, 1.0 mmol, 1.5 equiv.) was added by syringe, and the mixture was stirred for an additional 30 min and then warmed to 0°. The reaction was quenched successively with 50 µl of H_2O, 60 µl of 15% NaOH, and 160 µl of H_2O at 0°. After 10 min, the mixture was filtered through a silica-gel layer with Et_2O. The filtrate was dried (Na_2SO_4), and concentrated *in vacuo*. The residue, which consisted of a mixture of **18** and **20**, was used immediately for the next reaction (**18**: R_f (10% AcOEt/hexanes) 0.31. To a stirred suspension of 1.0 g (2.64 mmol) of pyridinium dichromate in 10 ml of CH_2Cl_2 was added dropwise a mixture **18**/**20** in 5 ml of CH_2Cl_2. After 16 h, the mixture was passed through a short column of *Florisil* (30 mesh, 20 g) with 5% AcOEt/hexanes, and the filtrate was concentrated *in vacuo* to afford 36 mg (58% based on **16**) of **20**. R_f (10% AcOEt/hexanes) 0.72. IR (film): 2960, 2860, 1709. ^{1}H-NMR (300 MHz, $CDCl_3$): −0.06 (*s*, MeSi); 0.00 (*s*, MeSi); 0.79–0.87 (*m*, Me_3C, Me(6), Me–C(4)); 1.04 (*d*, $J = 6.9$, Me–C(2)); 0.97–1.12 (*m*, H_b–C(5)); 1.40–1.65 (*m*, H–C(4), H_a–C(5)); 2.32–2.45 (*m*, H–C(2)); 3.96–4.01 (*m*, H–C(3)); 9.75 (br., CHO). ^{13}C-NMR (75.5 MHz, $CDCl_3$): 205.3; 74.9; 50.0; 39.7; 25.9; 25.2; 14.1; 8.6; −4.2.

*(4*R*)-4-Benzyl-3-[(2*R*,3*S*,4*R*,5*R*,6*S*)-5-[(*tert*-butyl)dimethylsiloxy]-3-hydroxy-2,4,6-trimethyloctanoyl}oxazolidin-2-one* (**22**). Bu_2BOTf (110 µl of a 1.0M soln. in CH_2Cl_2) was added by syringe at 0° to 23 mg of (4*R*)-4-benzyl-3-propanoyloxazolidin-2-one (0.1 mmol, 1.0 equiv.) in 0.2 ml of dry CH_2Cl_2, followed by dropwise addition of 21 µl (0.12 mmol, 1.2 equiv.) of EtN(i-Pr)$_2$. The mixture was cooled to −78°, and 20 mg of **20** was added. The mixture was stirred at −78° for 30 min, then warmed to r.t., and stirred for an additional 16 h. A mixture of 0.2 ml of phosphate buffer (1.0M Na_3PO_4, pH 7.4) and 0.4 ml of MeOH was added, followed by dropwise addition of 0.2 ml of 30% H_2O_2. After 30 min, the mixture was extracted with 2×10 ml of CH_2Cl_2. The org. layers were combined, dried (Na_2SO_4), and concentrated *in vacuo*. FC (5 g of SiO_2; 0–10% AcOEt/hexanes) afforded 10 mg (29%) of **22**: R_f (10% AcOEt/hexanes) 0.25. $[\alpha]_D = -22.1$ ($c = 1.0$, $CHCl_3$). IR (film): 2957, 1785, 1681. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.00 (*s*, MeSi); 0.02 (*s*, MeSi); 0.75–0.85 (*m*, Me_3C, Me(8′), Me–C(4′), Me–C(6′)), 0.85–1.02 (*m*, H_b–C(7′)); 0.97–1.12 (*m*, H_b–C(7′)); 1.13 (*d*, $J = 7.0$, Me–C(2′); 1.43–1.58 (*m*, H–C(6′), H_a–C(7′)); 1.27–145 (*m*, H_a–C(7′)); 1.48–1.65 (*m*, H–C(6′)); 1.70–1.88 (*m*, H–C(4′)); 2.69 (*dd*, $J = 9.6, 13.3$, 1 H, PhCH_2); 3.22 (*dd*, $J = 3.2, 13.3$, 1 H, PhCH_2); 3.74–3.90 (*m*, H–C(2′), H–C(3′), H–C(5′)); 4.05–4.20 (*m*, CH_2(5)); 4.60–4.68 (*m*, H–C(4)); 7.10–7.30 (*m*, 5 arom. H). ^{13}C-NMR (75.5 MHz, $CDCl_3$): 177.5; 152.9; 135.2; 129.4; 128.9; 127.4; 72.2; 66.2; 55.4; 39.8; 39.1; 38.5; 37.8; 31.6; 26.0; 22.6; 18.3; 16.2; 14.1; 11.9; 11.2; 8.8; −4.1; −4.3. HR-FAB-MS (NBA/NaI): 514.2985 ($[M + Na]^+$, $(C_{27}H_{45}NO_5Si)Na^+$; calc. 514.2965.

*(2*R*,3*S*,4*R*,5*R*,6*R*)-3-Hydroxy-2,4,6-trimethyloctano-5-lactone* (**14**). Imide **22** (10 mg, 0.02 mmol) was dissolved in 2 ml of THF. H_2O (0.5 ml) was added, and the soln. was cooled to 0° with vigorous stirring, while 20 µl of 30% H_2O_2 was added, followed by 2 mg (0.05 mmol) of LiOH. The mixture was allowed to warm slowly to r.t. After 24 h, the reaction was quenched with 35 mg of Na_2SO_3. After stirring for 10 min, the mixture was concentrated *in vacuo* to remove THF and then acidified to pH 1 by dropwise addition of conc. HCl. The resulting cloudy mixture was extracted with 3×10 ml of CH_2Cl_2. The combined org. layers were dried (Na_2SO_4) and concentrated *in vacuo* to give *(2*R*,3*S*,4*R*,5*R*,6*S*)-5-[(*tert*-butyl)dimethylsiloxy]-3-hydroxy-2,4,6-trimethyloctanoic acid* (**24**). R_f (40% AcOEt/hexanes) 0.38; ^{1}H-NMR (300 MHz, CDCl3): 0.01 (*s*, MeSi); 0.03 (*s*, MeSi);

0.75 (*d*, *J* = 7.1, Me–C(6)); 0.78–0.92 (*m*, Me_3C, Me(8), Me–C(4)); 0.93–1.08 (*m*, H_b–C(7)); 1.12 (*d*, *J* = 7.2, Me–C(2)); 1.45–1.65 (*m*, H–C(6), H_a–C(7)); 1.66–1.80 (*m*, H–C(4)); 2.52–2.62 (*m*, H–C(3)); 3.72–3.75 (*m*, H–C(2)); 3.97–4.03 (*m*, H–C(5)). The sample of **24** was dissolved in 2 ml of MeCN and 0.5 ml of H_2O. To the cloudy mixture, HF (20 µl of a 48% aq. soln.) was added dropwise. The mixture gradually cleared to a colorless soln. and was stirred at r.t. for 4 h before being quenched with sat. aq. $NaHCO_3$ to a pH of 7.5. The resulting mixture was extracted with 3 × 10 ml of CH_2Cl_2. The combined org. extract was dried (Na_2SO_4) and concentrated *in vacuo*. The residue was purified by chromatography (1 g of silica gel, 20% AcOEt/hexanes) to afford **14** (3 mg, 69%) whose physical and spectroscopic properties were identical to those of the biosynthetically-generated lactone **14** described below.

*(4*S*)-4-Benzyl-3-{(2*S*,3*R*,4*S*)-3-[(*tert*-butyl)dimethylsiloxy]-2,4-dimethylhexanoyl}oxazolidin-2-one* (**15**). Compound **6** (40 mg, 0.44 mmol, 1.0 equiv.) was converted to **15** as described above for **16** to afford, after purification, 190 mg (100%) of **15**. Colorless oil. R_f (10% AcOEt/hexanes) 0.55. $[\alpha]_D = +43.3$ (*c* = 1.0, $CHCl_3$). IR (film): 2958, 1783, 1699. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.07 (*s*, MeSi); 0.08 (*s*, MeSi); 0.82–0.99 (*m*, Me_3C, Me(6′), Me–C(4′)); 1.11–1.22 (*m*, H_b–C(5′)); 1.25 (*d*, *J* = 6.8, Me–C(2′)); 1.27–1.43 (*m*, H_a–C(5′)); 1.47–1.59 (*m*, H–C(4′)); 2.76 (*dd*, *J* = 13.3, 9.6, 1 H, PhCH_2); 3.27 (*dd*, *J* = 13.4, 3.1, 1 H, PhCH_2); 3.87–4.02 (*m*, H–C(2′), H–C(3′)); 4.19 (*m*, CH_2(5)); 4.64 (*m*, H–C(4)); 7.19–7.37 (*m*, 5 arom. H). ^{13}C-NMR (75.5 MHz, $CDCl_3$): 176.3; 152.9; 135.3; 129.4; 128.9; 127.3; 76.0; 65.9; 55.5; 41.6; 41.0; 40.6; 37.6; 26.4; 26.1; 25.6; 18.4; 18.0; 14.9; 14.0; 12.3; −3.6; −3.7; −4.0. HR-FAB-MS (NBA/NaI): 456.2545 ($[M + Na]^+$; ($C_{24}H_{39}NO_4Si$)Na^+; calc. 456.2546.

*(2*S*,3*R*,4*S*)-3-[(*tert*-Butyl)dimethylsiloxy]-2,4-dimethylhexan-1-ol* (**17**). Compound **15** (190 mg, 0.44 mmol, 1.0 equiv.) was reduced with $LiAlH_4$ as described above to give 49 mg (43%) of **17** as a colorless oil after purification by FC (5 g of SiO_2; 0–5% AcOEt/hexanes). R_f 0.31 (10% AcOEt/hexanes). IR (film): 3362. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.00 (*s*, MeSi); 0.02 (*s*, MeSi); 0.75–0.85 (*m*, Me_3C, Me(6), Me–C(2), Me–C(4)); 0.96–1.20 (*m*, H_b–C(5)); 1.35–1.50 (*m*, H–C(4), H_a–C(5)); 1.84–1.92 (*m*, H–C(2)); 3.42 (*dd*, *J* = 5.9, 10.5, H_b–C(1)); 3.62–3.70 (*m*, H_a–C(1), H–C(3)). ^{13}C-NMR (75.5 MHz, CDCl3): 66.2; 39.3; 37.7; 27.1; 25.8; 18.1; 15.1; 12.3; −3.8; −3.9.

*(2*S*,3*R*,4*S*)-3-[(*tert*-Butyl)dimethylsiloxy]-2,4-dimethylhexanal* (**19**). Compound **17** was oxidized with pyridinium dichromate to the corresponding aldehyde **19** (36 mg, 73%) as described for **20**. R_f 0.72 (10% AcOEt/hexanes). IR (film): 2959, 1728. ^{1}H-NMR (300 MHz, $CDCl_3$): −0.03 (*s*, MeSi); 0.01 (*s*, MeSi); 0.75–0.95 (*m*, Me_3C, Me(6), Me–C(4)); 1.02 (*d*, *J* = 6.9, Me–C(2)); 1.00–1.18 (*m*, H_b–C(5)); 1.20–1.57 (*m*, H–C(4), H_a–C(5)); 2.40–2.56 (*m*, H–C(2)); 3.90–4.01 (*m*, H–C(3)); 9.75 (br. *s*, CHO); ^{13}C-NMR (75.5 MHz, $CDCl_3$): 205.4; 75.3; 50.8; 39.1; 30.4; 26.5; 25.9; 23.7; 14.1; 9.3; −4.1; −4.3.

*(4*R*)-4-Benzyl-3-{(2*R*,3*S*,5*R*,6*S*)-5-[(*tert*-burtyl)dimethylsiloxy]-3-hydroxy-2-,4-,6-trimethyloctanoyl}oxazolidin-2-one* (**21**). Compound **21** was prepared from **19** as described for **22** to afford, after FC (5 g of SiO_2; 0–10% AcOEt/hexanes) 40 mg (58%) of **21**. R_f 0.25 (10% AcOEt/hexanes). $[\alpha]_D = -13.8$ (*c* = 1.0, $CHCl_3$). IR (film): 2958, 1784, 1682. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.07 (*s*, MeSi); 0.08 (*s*, MeSi); 0.79–1.02 (*m*, Me_3C, Me(8′), Me–C(4′), Me–C(6′)); 1.20 (*d*, *J* = 7.0, Me–C(2′)); 1.08–1.22 (*m*, H_b–C(7′)); 1.27–1.45 (*m*, H_a–C(7′)); 1.48–1.65 (*m*, H–C(6′)); 1.70–1.88 (*m*, H–C(4′)); 2.68 (*dd*, *J* = 9.6, 13.4, 1 H, PhCH_2); 3.25 (*dd*, *J* = 3.3, 13.3, 1 H, PhCH_2); 3.78–4.03 (*m*, H–C(2′), H–C(3′), H–C(5′)); 4.19–4.23 (*m*, CH_2(5)); 4.65–4.72 (*m*, H–C(4)); 7.19–7.37 (*m*, 5 arom. H). ^{13}C-NMR (75.5 MHz, $CDCl_3$): 176.8; 153.1; 135.3; 129.4; 128.9; 127.3; 72.7; 66.2; 55.6; 40.2; 39.4; 37.7; 37.5; 27.7; 26.0; 18.3; 12.0; 8.5; −4.1; −4.3. HR-FAB-MS (NBA/NaI): 514.2975 ($[M + Na]^+$, ($C_{27}H_{45}NO_5Si$)Na^+; calc. 514.2965).

*(2*R*,3*S*,4*S*,5*R*,6*S*)-3-Hydroxy-2,4,6-trimethyloctano-5-lactone* (**13**). Imide **21** (40 mg, 0.08 mmol) was hydrolyzed, lactonized, and deprotected as described for **14** to give, after chromatography (1 g of SiO_2, 20% AcOEt/hexanes), 11 mg (69%) of **13**, whose physical properties were identical to those of the biosynthetically-derived product **13**.

General Precursor Feeding and Extraction Conditions. S. coelicolor CH999/pJRJ2 encoding DEBS($KS1^0$) [15a] and *S. coelicolor* CH999/pCK16 encoding DEBS1 + TE ($KS1^0$) [17] were each cultivated at 30° on 25-ml R2YE agar [27] plates for 3 d. Each plate was then overlaid with 1 ml of diketide soln. in 5% DMSO/H_2O. After an additional 4 d, the combined mycelia and agar (20 plates, 500 ml total) were homogenized and extracted with AcOEt (3 × 500 ml) at *ca.* 40°. The combined org. extracts were dried ($MgSO_4$), concentrated to *ca.* 5 ml *in vacuo*, then washed with sat. $NaHCO_3$ and sat. NaCl, dried (Na_2SO_4), and concentrated *in vacuo*.

Purification and Structure Determination. The crude mixture obtained from each precursor feeding was subjected to initial purification by SiO_2 CC (*ca.* 1 g SiO_2 in a *Pasteur* pipette) with 20% AcOEt/hexanes. The relevant fraction thus obtained was further purified by prep. TLC (6 cm × 10 cm) with 40% AcOEt/hexanes. The product band was excised and eluted with AcOEt, and the resultant product was characterized by ^{1}H-

(300 MHz) and ^{13}C-NMR (75 MHz) spectroscopy, HR-MS, and FT-IR. The ^{1}H- and ^{13}C-NMR spectra of each product was fully assigned by a combination of ^{1}H, ^{1}H-COSY, DEPT and ^{1}H, ^{13}C-HETCOSY spectroscopy.

Conversion of **3** *to (14*S*)-6-Deoxy-14-methylerythronolide B* (**10**) *by DEBS(KS1^{0})*. Feeding of 20 ml of 20 mM **3** to *S. coelicolor* CH999/pJRJ2 gave 10 mg of **10** (6%). White powder. R_f (40% AcOEt/hexanes) 0.49. $[\alpha]_D = -50.2$ ($c = 0.963$, $CHCl_3$). IR (film): 3485, 2972, 2938, 1706. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.88 (*d*, *J* = 6.8 Me–C(12)); 0.91 (*d*, *J* = 6.7, Me–C(14)); 0.94 (*t*, *J* = 7.4, Me(16)); 1.00–1.10 (*m*, Me–C(4), Me–C(6), Me–C(8), Me–C(10)); 1.05–1.20 (*m*, H_b–C(15)); 1.10–1.30 (*m*, H_b–C(7)); 1.30 (*d*, *J* = 6.9, Me–C(2)); 1.40–1.55 (*m*, H_a–C(15)); 1.66 (*m*, H_a–C(7)); 1.75–1.95 (*m*, H–C(4), H–C(12), H–C(14)); 1.95–2.10 (*m*, H–C(6)); 2.50–2.70 (*m*, H–C(8)); 2.70–2.85 (*m*, H–C(10)); 2.81 (*dq*, *J* = 10.4, 6.7, H–C(2)); 3.67 (br. *d*, *J* = 9.2, H–C(11)); 3.92 (*d*, *J* = 10.5, H–C(3)); 4.01 (*dd*, *J* = 5.2, 2.0, H–C(5)); 4.88 (*dd*, *J* = 10.3, 1.3, H–C(13)). ^{13}C-NMR (75 MHz, $CDCl_3$): 6.27 (Me–C(8)); 6.86 (Me–C(4)); 9.14 (Me–C(12)); 10.41 (C(16)); 13.18 (Me–C(10)); 14.69 (Me–C(2)); 15.71 (Me–C(14)); 16.52 (Me–C(6)); 24.76 (C(15)); 35.00 (C(14)); 35.48 (C(6)); 37.32 (C(7)); 37.43 (C(4)); 37.72 (C(12)); 39.19 (C(8)); 43.62 (C(2)); 44.01 (C(10)); 70.80 (C(11)); 76.44 (C(5)); 79.50 (C(13)); 79.5 (C(3)); 178.28 (C(1)); 213.48 (C(9)). HR-FAB-MS (NBA/NaI): 437.2877 ($[M + Na]^+$, $(C_{23}H_{42}O_6)Na^+$; calc. 437.2879.

Conversion of **4** *to (14*R*)-6-Deoxy-14-methylerythronolide B* (**11**) *by DEBS(KS1^{0})*. Feeding of 20 ml of 5 mM **4** to *S. coelicolor* CH999/pJRJ2 gave 10 mg (25%) of **11**. Colorless crystals. M.p. 139–140°. R_f. (40% AcOEt/hexanes) 0.49. $[\alpha]_D = -46.9$ ($c = 0.625$, $CHCl_3$). IR (film): 3487, 2972, 2939, 1704. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.86 (*d*, *J* = 6.9, Me–C(12)); 0.88 (*d*, *J* = 6.7, Me–C(14)); 0.89 (*t*, *J* = 7.4, Me–C(16)); 1.00–1.10 (*m*, Me–C(4), Me–C(6), Me–C(8), Me–C(10)); 1.05–1.20 (*m*, H_b–C(15)); 1.10–1.30 (*m*, H_b–C(7)); 1.29 (*d*, *J* = 6.8); 1.40–1.55 (*m*, H_a–C(15)); 1.66 (*m*, H_a–C(7)); 1.75–1.95 (*m*, H–C(4), H–C(12), H–C(14)); 1.95–2.10 (*m*, H–C(6)); 2.55–2.75 (*m*, H–C(8)), 2.70–2.85 (*m*, H–C(10)); 2.79 (*dq*, *J* = 10.4, 6.8, H–C(2)); 3.67 (br. *d*, *J* = 9.3, H–C(11)); 3.92 (*d*, *J* = 10.5, H–C(3)); 4.01 (*dd*, *J* = 5.2, 2.1, H–C(5)); 4.88 (*dd*, *J* = 10.3, 1.3, H–C(13)). ^{13}C-NMR (75 MHz, $CDCl_3$): 6.27 (Me–C(8)); 6.84 (Me–C(4)); 8.93 (Me–C(12)); 10.39 (C(16)); 13.18 (Me–C(10)); 14.69 (Me–C(2)); 14.72 (Me–C(14)); 16.51 (Me–C(6)); 25.75 (C(15)); 35.13 (C(14)); 35.45 (C(6)); 37.38 (C(7)); 37.58 (C(4)); 37.78 (C(12)); 39.09 (C(8)); 43.62 (C(2)); 44.00 (C(10)); 70.80 (C(11)); 76.41 (C(5)); 78.34 (C(13)); 79.55 (C(3)); 178.29 (C(1)); 213.40 (C(9)). HR-FAB-MS (NBA/NaI): 437.2882 ($[M + Na]^+$, $(C_{23}H_{42}O_6)Na^+$; calc. 437.2879.

Conversion of the 3:2 Mixture **4**/**3** *to* **11** *by DEBS(KS1^{0})*. Feeding of 20 ml of a 5 mM soln. of the 3:2 mixture **4**/**3** to *S. coelicolor* CH999/pJRJ2 gave 10 mg of **11** (40% yield, based on **4**) as colorless crystals, whose ^{1}H- and ^{13}C-NMR spectra were identical with those of **11** obtained from the feeding of **4**.

Conversion of **3** *to* **13** *by DEBS1 + TE(KS1^{0})*. Feeding of 20 ml of 20 mM **3** to *S. coelicolor* CH999/pCK16 (DEBS1 + TE KS1^{0}) gave 4 mg (10%) of **13**. White powder. R_f (40% AcOEt/hexanes) 0.36. $[\alpha]_D = +82.8$ ($c = 1.0$, $CHCl_3$). IR (film): 3440, 1716. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.89 (*d*, *J* = 6.9, Me–C(6)); 0.93 (*t*, *J* = 7.2, Me(8)); 0.97 (*d*, *J* = 7.0, Me–C(4)), 1.24 (*m*, H_b–C(7)); 1.43 (*d*, *J* = 7.1, Me–C(2)); 1.73 (*m*, H–C(6)); 1.92 (*m*, H_a–C(7)); 2.28 (*m*, H–C(4)); 2.48 (*dq*, *J* = 10.4, 7.1, H–C(2)); 3.80 (*dd*, *J* = 10.3, 4.1, H–C(3)); 3.85 (*dd*, *J* = 10.2, 2.1, H–C(5)). ^{13}C-NMR: 173.7; 84.2; 74.2; 39.9; 35.4; 35.3; 24.1; 15.5; 14.4; 10.5; 4.4. HR-FAB-MS (NBA/NaI): 223.1305 ($[M + Na]^+$, $(C_{11}H_{20}O_3)Na^+$; calc. 223.1310.

Conversion of **4** *to* **14** *by DEBS1 + TE(KS1^{0})*. Feeding of 20 ml of 5 mM **4** to *S. coelicolor* CH999/pCK16 (DEBS1 + TE KS1^{0}) gave 5 mg (50%) of **14**. White powder. R_f (40% AcOEt/hexanes) 0.36. $[\alpha]_D = +118$ ($c = 0.35$, $CHCl_3$). IR (film): 3440, 1717. ^{1}H-NMR (300 MHz, $CDCl_3$): 0.94 (*t*, *J* = 7.3, Me(8)); 0.97 (*d*, *J* = 7.0, Me–C(4)); 1.05 (*m*, H_b–C(7)); 1.10 (*d*, *J* = 6.5, Me–C(6)); 1.42 (*d*, *J* = 7.1, Me–C(2)); 1.51 (*m*, H_a–C(7)); 1.75 (*m*, H–C(6)); 2.31 (*m*, H–C(4)); 2.48 (*dq*, *J* = 10.3, 7.1, H–C(2)); 3.80 (*dd*, *J* = 10.4, 4.2, H–C(3)); 3.86 (*dd*, *J* = 10.0, 2.1, H–C(5)). ^{13}C-NMR: 173.7; 83.4; 74.4; 40.0; 35.6; 35.4; 25.3; 14.4; 13.9; 10.6; 4.2. HR-FAB-MS (NBA/NaI): 223.1307 ($[M + Na]^+$, $(C_{11}H_{20}O_3)Na^+$; calc. 223.1310.

Conversion of the 3:2 Mixture **4**/**3** *to* **14** *by DEBS1 + TE(KS1^{0})*. Feeding of 20 ml of a 5 mM soln. of the 3:2 mixture **4**/**3** to *S. coelicolor* CH999/pCK16 (DEBS1 + TE KS1^{0}) gave 4 mg (60% yield based on **4**) of **14** as a white powder, whose ^{1}H- and ^{13}C-NMR spectra were identical with those of **14** obtained from the feeding of **4**.

DEBS Module 2 + TE. DEBS Module 2 + TE was expressed in *E. coli* BL21(DE3) harboring plasmid pRSG64 [18][28] and cotransformed with the sfp plasmid to ensure phosphopantetheinylation of the ACP domain of the ACP domain [29]. The cells were grown in LB medium supplemented with ampicillin, kanamycin, and chloramphenicol as previously described at 37° until $OD_{600} = 0.6$. The cultures were then induced with 1 mM final concentration of IPTG (isopropyl-β-D-thiogalactopyranoside) and grown at r.t. overnight. After harvesting, the cell pellet was washed with buffer *A* (100 mM sodium phosphate (pH 7.2), 2 mM EDTA, 2.5 mM DTT, and 20% glycerol). The washed cells were then resuspended in disruption buffer (200 mM Na_3PO_4 (pH 7.2), 200 mM NaCl, 2.5 mM EDTA, 2.5 mM DTT, 30% glycerol, 1.5 mM benzamidine, 2 mg/l pepstatin, and

2 mg/l leupeptin) and lysed with a *French Press* at 1000 psi. All steps were carried out at 4°. After centrifugation, the supernatant was treated with the final concentration of 50 μg/ml DNase and 10 mM $MgCl_2$ for 20 min at 4°, and then subjected to a 0.15% PEI precipitation for 20 min. The proteins were purified on a 30-ml anion-exchange chromatography column (*Q Sepharose: Fast Flow* resin from *Pharmacia*), which was washed with 100 mM Na_3PO_4 (pH 7.2) containing 20% glycerol and 200 mM NaCl, and then eluted with a gradient from 200 to 500 mM NaCl. The appropriate fractions containing DEBS module 2 (> 70% of protein) eluted at *ca.* 250 mM NaCl were pooled and concentrated on *Centriprep 30* membranes (*Amicon*) to a protein concentration of *ca.* 110 μg/ml. The purity of this protein was determined to be > 60% by SDS-PAGE. The partially purified DEBS module 2 + TE protein soln. was placed in 500-μl *Eppendorf* tubes and stored at − 80° before being used for the kinetic studies.

Kinetic Assays. The kinetic assays were carried out with 0.31 μM DEBS module 2 + TE, with concentrations over a range of 125 μM – 10 mM, plus 200 μM [2-^{14}C]methylmalonyl-CoA (1 mCi/mmol), 1 mM NADPH, and buffer *A* in 100-μl total volume at 30° for 1 h. The reactions were quenched by the addition of 100 μl of AcOEt and subsequent vortexing for 1 min, and then separated by a micro-centrifuge (14000 rpm for 1 min). The AcOEt extracts were loaded onto TLC plates, which were developed with 70% AcOEt/hexanes. Product yields were quantitated by TLC-phosphoimaging.

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