Evaluating precursor-directed biosynthesis towards novel erythromycins through *in vitro* studies on a bimodular polyketide synthase

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Background: Modular polyketide synthases (PKSs) catalyse the biosynthesis of complex polyketides using a different set of enzymes for each successive cycle of chain extension. Directed biosynthesis starting from synthetic diketides is a potentially valuable route to novel polyketides. We have used a purified bimodular derivative of the erythromycin-producing polyketide synthase (DEBS 1-TE) to study chain extension starting from a variety of diketide analogues and, in some cases, from the alternative acyl-CoA thioester substrates.

Results: Chain initiation *in vitro* by DEBS 1-TE module 2 using a synthetic diketide analogue as a substrate was tolerant of significant structural variation in the starter unit of the synthetic diketide, but other changes completely abolished activity. Interestingly, a racemic β-keto diketide was found to be reduced *in situ* on the PKS and utilised in place of its more complex hydroxy analogue as a substrate for chain extension. The presence of a diketide analogue strongly inhibited chain initiation via the loading module. Significantly higher concentrations of diketide *N*-acetylcysteamine analogues than their corresponding acyl-CoA thioesters are required to achieve comparable yields of triketide lactones.

Conclusions: Although a broad range of variation in the starter residue is acceptable, the substrate specificity of module 2 of a typical modular PKS *in vitro* is relatively intolerant of changes at C-2 and C-3. This will restrict the usefulness of approaches to synthesise novel erythromycins using synthetic diketides *in vivo*. The use of synthetic β-keto diketides *in vivo* deserves to be explored.

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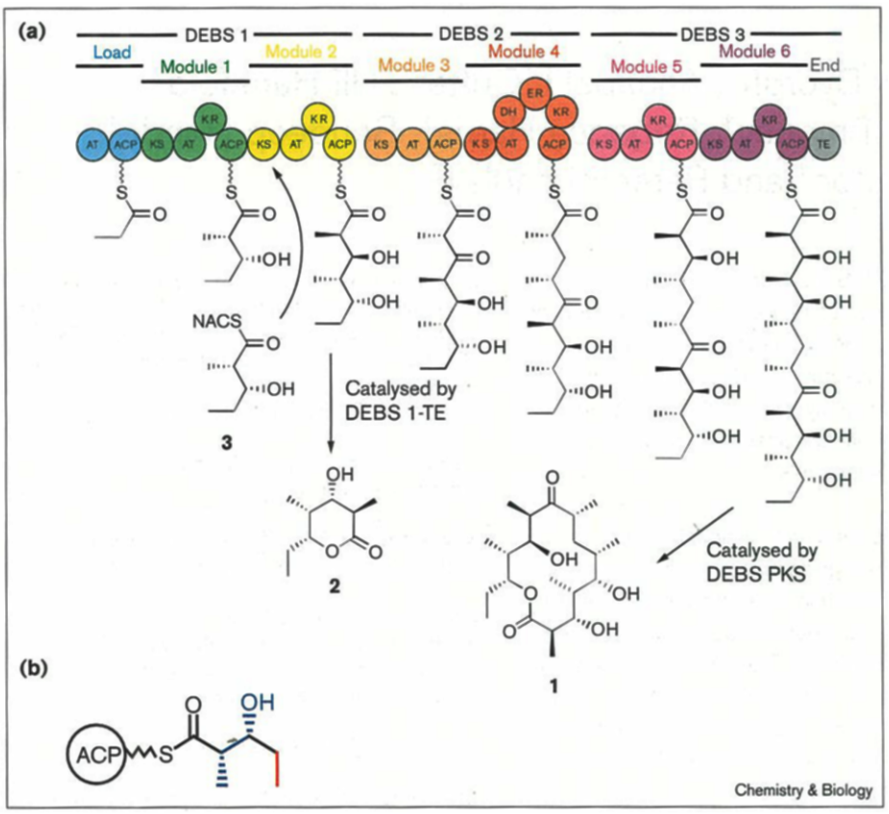
Introduction

Complex or reduced polyketides, such as the antibacterial erythromycin A, constitute a large and structurally diverse class of natural products generated mainly by *Streptomyces* and related filamentous bacteria. They are derived from simple carboxylic acid precursors by stepwise chain assembly on modular polyketide synthases (PKSs). PKSs are giant multienzyme complexes [1–8] that contain a different set or module of enzyme domains to accomplish each successive cycle of chain extension; for example, 6-deoxyerythronolide B synthase (DEBS), responsible for the biosynthesis of the aglycone core of erythromycin A (**1**), contains six such modules housed in three large polypeptides, DEBS 1, DEBS 2 and DEBS 3 (Figure 1a) [9]. Because of the modular nature of these synthases, considerable attention has focused on the possibility of generating novel antibiotics through combinatorial manipulation of the genes for their constituent domains [10–13]. The diversity of such combinatorial libraries can be specifically enhanced by altering the chain initiation process to accept a broad range of alternative starter units [14–16]. A complementary approach to altering the chain initiation step [17] follows from early experiments in which synthetic analogues of putative intermediates, particularly diketides as their *N*-acetylcysteamine (NAC) thioesters, were shown to be successfully incorporated into macrocyclic polyketides [18–22].

There are many factors that together determine the efficiency with which a synthetic substrate analogue can be utilised by a polyketide synthase *in vivo*, including cell permeability, toxicity to the cell, susceptibility to β-oxidation or other types of degradation, a requirement for derivatisation to an active species such as a CoA-thioester and the specificity of the PKS to which it is directed. It is therefore of great interest to obtain a clearer idea of the potential for precursor-directed biosynthesis, using purified enzymes *in vitro*. In earlier *in vitro* experiments to study the loading of diketides at the module 2 ketosynthase domain (KS2) [23,24], Chuck *et al.* [24] relied on their bimodular derivative of DEBS (DEBS 1 + TE) [25] that catalyses the formation of triketide lactone **2** from one equivalent of a starter CoA-thioester (normally propionyl-CoA) and two

Figure 1



(a) Organisation of the 6-deoxyerythronolide B synthase (DEBS). The DEBS system is composed of three bimodular polypeptides, DEBS 1, DEBS 2 and DEBS 3, that co-operate to form the erythromycin aglycone, 6-deoxyerythronolide B **1**. DEBS 1-TE, a truncated form of the polyketide synthase (PKS) created by adding a copy of the terminal thioesterase domain to the end of DEBS 1, produces the triketide lactone, (2*R*,-3*S*, 4*R*, 5*R*)-3, 5-dihydroxy-2, 4-dimethyl-*n*-heptanoic acid δ-lactone **2**. Incorporation of a synthetic diketide intermediate, derivatised as its *N*-acetylcysteamine (NAC) thioester **3**, has been shown to occur at the ketosynthase domain of module 2. **(b)** Structural analysis of the natural diketide intermediate formed by condensation of propionyl-CoA and (2*S*)-methylmalonyl-CoA. The acyl-terminal portion (shown in red) is determined by the choice of starter unit, whereas the structure shown in blue, which includes the functionality at C-2, and the stereochemistry about the C-2, C-3 bond, is established during chain extension. Both portions of the diketide were varied in designing our synthetic analogues. AT, acyltransferase; ACP, acyl carrier protein; KR, β-ketoacyl reductase; KS, β-ketoacyl synthase; ER, enoyl reductase; TE, thioesterase.

equivalents of chain-extending (2*S*)-methylmalonyl-CoA [26] (Figure 1a). More specifically, they used a version of this protein in which the KS1 domain was inactivated to suppress its reported methylmalonyl-CoA decarboxylase activity [27,28].

In the present investigation we have used a similar bimodular PKS, DEBS 1-TE [29], in which we have shown previously that DEBS 1-catalysed decarboxylation does not occur to any detectable extent [30]. Using our system we have comprehensively explored the potential for stereochemical variation in module 1 by evaluating diketide analogues in which the stereochemistry at either or both of the C-2 and C-3 centres has been altered. We have also addressed the feasibility of increasing the steric bulk of the branching sidechain. We have further investigated the potential for structural variation in the starter acid residue, and have carried out complementary studies to compare chain initiation from the equivalent starter CoA derivatives. Finally, we have evaluated the practicality of using synthetically more accessible racemic β-keto diketides in place of their corresponding β-hydroxy analogues.

Results and discussion

Design of diketide analogues

In the normal course of erythromycin biosynthesis, a diketide intermediate is formed by the condensation of a propionyl-CoA starter unit with one equivalent of chain-extending (2*S*)-methylmalonyl-CoA [26]. Diketide structure can, therefore, be considered in two parts: the chain extension structure including the functionality at C-2 and the stereochemistry, if any, about the C-2, C-3 bond (Figure 1b), and the functionality at the acyl terminus (determined by the choice of starter unit).

Structural variation in the chain extension unit: C-2, C-3 stereochemistry

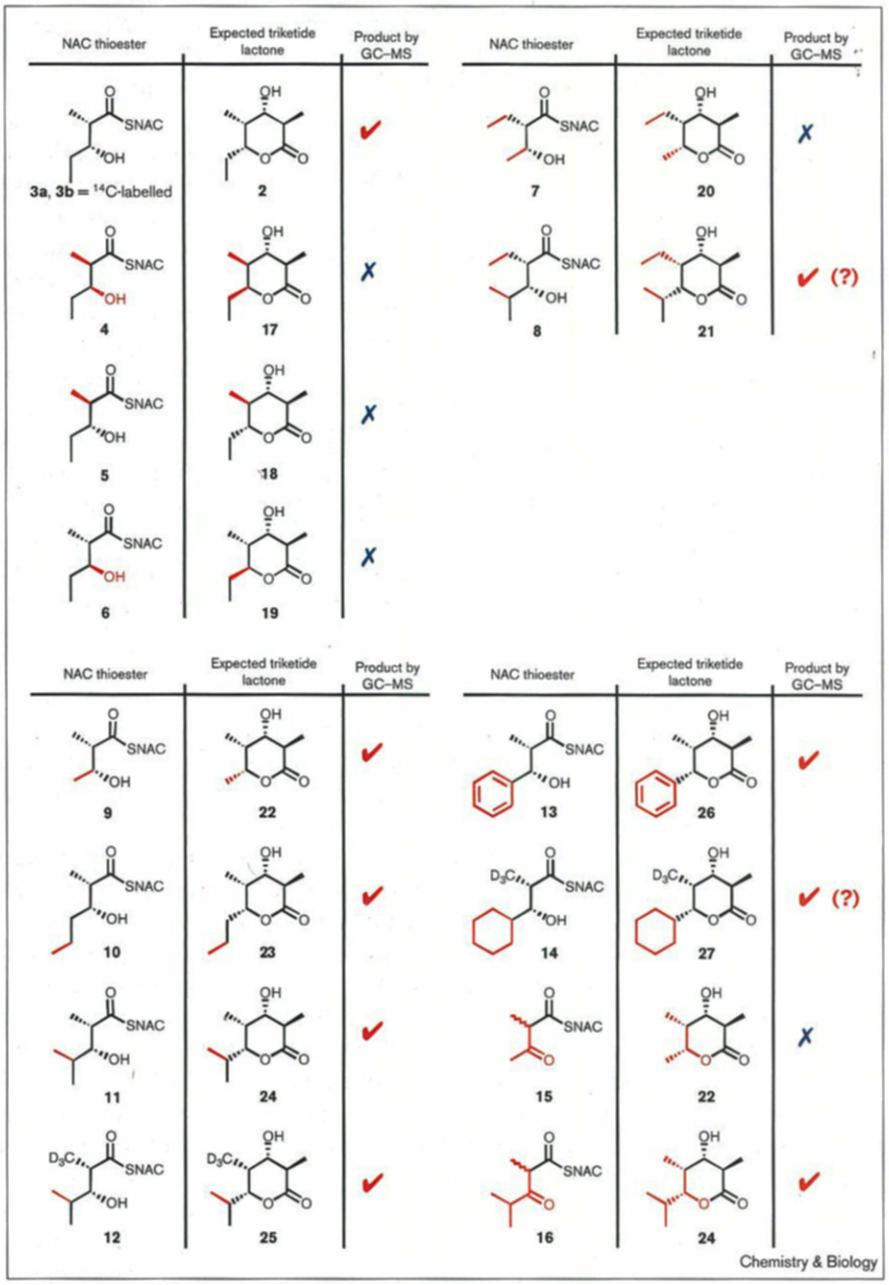
The feasibility of altering the stereochemistry of chain extension in module 1 was assessed by feeding analogues **4**–**6** (Figure 2), which exhibit, respectively, all three non-natural C-2, C-3 configurations (in previous work, compound **5** failed to become incorporated into triketide lactone [24]). Syntheses of diketide analogues, with the exception of **3b** and **14**–**16** given here (see the Materials and methods section), have been published previously [31].

Structural variation in the chain extension unit: C-2 alkyl substituent

In a previous study, Chuck *et al.* [24] reported the incorporation of (3*R*)-3-hydroxypentanoic acid NAC, a diketide analogue lacking a methyl group at the C-2 position; such an intermediate would arise by chain extension from malonyl-CoA. In this study, the sterically more demanding C-2 ethyl group was instead introduced into

Figure 2

Diketide **3** is the *N*-acetylcysteamine thioester of the natural intermediate in the biosynthesis of the triketide lactone **2** by DEBS 1-TE and 6-deoxyerythronolide B **1** by the complete DEBS. Diketides **4–16** are synthetic analogues of **3**, which incorporate variations in functionality, stereochemistry, and level of β-keto reduction. Compounds **17–27** are the lactone products expected upon successful processing of these 'unnatural' diketides by DEBS 1-TE. A check mark indicates that the triketide lactone product was observed by GC-MS and/or radiochemical TLC.



analogues **7** and **8** to model chain extension by ethylmalonyl-CoA.

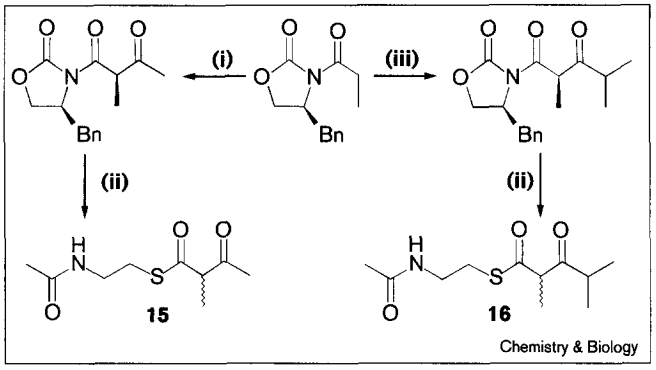
Structural variation in the starter unit

To evaluate the potential for using synthetic diketide analogues as a means of altering the starter unit residue of polyketides, we prepared the three compounds **9–11** (and **12**; Figure 2) that correspond to the incorporation of acetyl-CoA, *n*-butyryl-CoA and *iso*-butyryl-CoA, respectively, but maintain the natural C-2, C-3 stereochemistry of the diketide intermediate. These three non-natural starter units have been shown previously to incorporate into triketide lactones [32–34]. Analogues **13** and **14**, corresponding to processing of benzoyl-CoA and cyclohexyl-CoA, respectively, were also investigated.

Incorporation of β-keto diketides

We also examined the processing of the racemic β-keto analogues **15** and **16** (for synthesis, see Figure 3 and the Materials and methods section) to triketides; these compounds also contained the unnatural starter units acetyl-CoA and *iso*-butyryl-CoA, respectively. Two alternative outcomes were envisaged for these substrates: the keto groups at C-3 would be stereoselectively reduced by the

Figure 3



Synthesis of racemic β-keto diketide NAC analogues **15** and **16**. For experimental details, see the Materials and methods section. Reagents and conditions: **(i)** 1.2 equivalents LDA, THF, −78°C, 2 h; 1.2 equivalents $MgBr_2 \cdot OEt_2$ in Et_2O, −78°C→0°C, 2 min, then 0°C→−78°C, 2 min; 1.3 equivalents acetyl chloride, −78°C, 30 min; **(ii)** 2 equivalents $LiN(SiMe_3)_2$, 1.2 equivalents NAC, THF, −78°C, 15 min, then 0°C, 45 min. **(iii)** 1.2 equivalents LDA, THF, −78°C, 2 h; 1.2 equivalents $MgBr_2 \cdot OEt_2$ in Et_2O, −78°C→0°C, 2 min, then 0°C→−78°C, 2 min; 1.3 equivalents *iso*-butyryl chloride, −78°C, 30 min.

ketoreductase domains of module 1 (KR1) and module 2 (KR2). Reduction of the natural β-keto diketide analogue as its NAC thioester has been shown to occur without loading onto the PKS (I.E. Holzbaur, personal communication). The resulting β-hydroxy diketides could then be accepted by KS2 for chain extension to the triketide, or the unreduced racemic analogues would load directly onto KS2. Processing in module 2 would produce triketides lacking the terminal hydroxyl group necessary for lactonisation, and so presumably the chains would be released from the thioesterase as free acids [35]. If the former outcome were observed, ketoreductase-catalysed reductions could be exploited as a general means of establishing the stereochemistry of synthetic diketides, greatly simplifying the preparation of these analogues.

Choice of analytical methods

In earlier work with diketides, analysis by radiochemical thin layer chromatography (TLC) and comparison to synthetic standards proved adequate for most substrates [24]. The resolution provided by TLC, however, proved inadequate for the much wider range of structural and stereochemical variation used in the present work. Gas chromatography–mass spectrometry (GC–MS) analysis was therefore routinely used for structure determination and assignments were confirmed by comparison with synthetic lactone standards (for all standards but lactone **26**; full details of the syntheses to be given elsewhere). As quantification using GC–MS proved difficult, however, once a product structure was identified and the absence of stereoisomers confirmed, radiochemical TLC analysis was used to determine lactone yields.

Choice of assay conditions

In initial assays, the concentration of the natural diketide (2*S*, 3*R*)-2-methyl-3-hydroxy pentanoic acid NAC thioester **3a** was varied, whereas (2*RS*)-methylmalonyl-CoA was maintained at 600 μM (previously found to be a saturating concentration for this substrate [27,28]) and NADPH was used at 1 mM (no further increase in production of triketide lactone was seen using NADPH at higher concentrations). In order to discriminate between biosynthesis of lactones from any propionyl-CoA contaminant in the methylmalonyl-CoA [30] and from the diketide substrate, radioactivity was added in the form of [^{14}C]-(2*S*, 3*R*)-2-methyl-3-hydroxy pentanoic acid NAC thioester **3b**. Use of this reagent ensured that any radiolabelled product with the same R_f as authentic lactone standard was derived from diketide only. At the highest level of diketide NAC thioester used (6 mM), the yield of triketide lactone under these assay conditions was comparable to that seen previously using the normal propionyl-CoA substrate to provide starter units. In all subsequent experiments, diketide NAC substrates were used at a standard concentration of 6 mM.

In other assays, propionyl-CoA or alternative acyl-CoA esters were added at 300 μM, a concentration that, in preliminary experiments, was found to give the optimum yields of triketide lactone from both acetyl-CoA and *n*-butyryl-CoA, under the conditions used (data not shown).

Results of incorporation experiments

Structural variation in the chain extension unit: C-2, C-3 stereochemistry

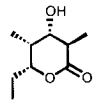
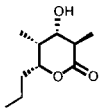
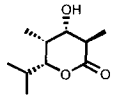
The natural diketide intermediate has a (2*S*, 3*R*)-2-methyl-3-hydroxyl configuration, and its incorporation *in vitro* can be detected using GC–MS. In order to discriminate between biosynthesis from contaminant propionyl-CoA in the methylmalonyl-CoA, and from the diketide substrate, specifically deuterated CD_3-methylmalonyl-CoA was also used. The detection in these experiments of triketide lactone molecules containing a single CD_3 group, by GC–MS analysis, confirmed the incorporation of the natural diketide analogue (data not shown). Chuck *et al.* [24] showed previously that the diastereomeric analogue **5** failed to incorporate. We have re-evaluated this compound as well as the other two stereochemical variants, analogues **4** and **6**. Although identical in functionality to the natural substrate, all three failed to incorporate to yield lactones **17–19**, respectively, above GC–MS detection limits.

Structural variation in the chain extension unit: C-2 alkyl substituent

We looked initially at the feasibility of extending the functionality at C-2 from a methyl group to the sterically more demanding ethyl group, as well as removing the methyl group as had been done previously [24]. Two C-2 ethyl diketide analogues were evaluated, one of which, **7**, has a smaller size starter unit, and the other, **8**, has a larger

Table 1

Comparison of triketide lactone yields from CoA thioesters (300 μM) and their corresponding diketide analogues (6 mM).

Lactone		Yield from diketide NAC (nmol)	Yield from CoA thioester (nmol)
2		13*	16
22		3.9	6.0
23		17	14
24		20 from **11**/ 3.2 from **16**	4.7

*Assuming complete suppression of biosynthesis from contaminant propionyl-CoA.

one. Using GC–MS analysis, we found no evidence for incorporation of the acetate-terminal diketide **7**. Although there was a peak corresponding to incorporation of **8** with the correct mass and fragmentation pattern for the expected lactone **21**, its identity could not be confirmed due to lack of the appropriate synthetic standard. As the levels of incorporation were small, we did not go on to synthesise this standard. From this preliminary evidence, it appears that increased bulk at C-2 is only poorly accommodated. This result is in agreement with a recent report that replacement of the module 2 acyl transferase (AT) of DEBS with the ethylmalonate-specific AT from the niddamycin PKS resulted in only minor amounts of the expected ethyl-substituted erythromycin analogue [36].

Structural variation in the starter unit

DEBS 1-TE has been shown to accept the unnatural starter acids acetyl-CoA, *n*-butyryl-CoA and *iso*-butyryl-CoA *in vitro* [32–34]. Consequently, diketides including these functionalities (**9–12**) would be expected to incorporate into triketide lactones. The conversion of analogues **9** and **10** to lactones **22** and **23** has been reported previously [24,33,34], and was reproduced under these conditions. Likewise, analogues **11** and **12** were shown to incorporate into lactones **24** and **25**, respectively, using GC–MS. In addition, as observed previously, the extent of conversion, as measured using radiochemical TLC, improved with increased chain length of the starter unit [24] (Table 1). Although incorporation of the corresponding starter units has not been reported, we also investigated the benzoyl diketide **13** and the CD_3-labelled cyclohexyl diketide **14** under the same conditions. By GC–MS analysis and comparison with synthetic standard **26** (for synthesis, see Figure 4 and the Materials and methods section), both the benzoyl diketide **13** and the starter unit benzoyl-CoA were shown to incorporate. Although a significant peak with the anticipated mass and fragmentation pattern of lactone **27** was detected, incorporation of analogue **14** could not be conclusively established without the appropriate synthetic standard (cyclohexyl-CoA is not commercially available).

Figure 4

Synthesis of triketide lactone standard **26**. For experimental details, see the Materials and methods section. Reagents and conditions: **(i)** 1.2 equivalents Bu_2BOTf, 1.4 equivalents Et_3N, CH_2Cl_2, 0°C, 20 min; 1.1 equivalents benzaldehyde, −78°C, 20 min, then 0°C, 2 h; **(ii)** 1.1 equivalents TBDMS triflate, 1.8 equivalents *i*-Pr_2EtN, CH_2Cl_2, −78°C, 2 h; **(iii)** 1.1 equivalents $LiBH_4$, 1.1 equivalents water, Et_2O, 0°C→room temperature, then room temperature, 1 h; **(iv)** 1.1 equivalents Dess-Martin reagent, CH_2Cl_2, room temperature, 40 min; **(v)** (a) 1.2 equivalents Bu_2BOTf and 1.3 equivalents Et_3N added to 1 equivalent (4*R*)-3-propionyl-4-phenylmethyl-2-oxazolidinone **29**, CH_2Cl_2, −78°C, 20 min; (b) 1.1 equivalents **28**, −78°C, 20 min, then 0°C, 2 h; **(vi)** (a) 4.6 equivalents LiOOH, THF/water (3:1), 0°C, 20 min; (b) 1 M HCl, THF, 40°C, 4 h.

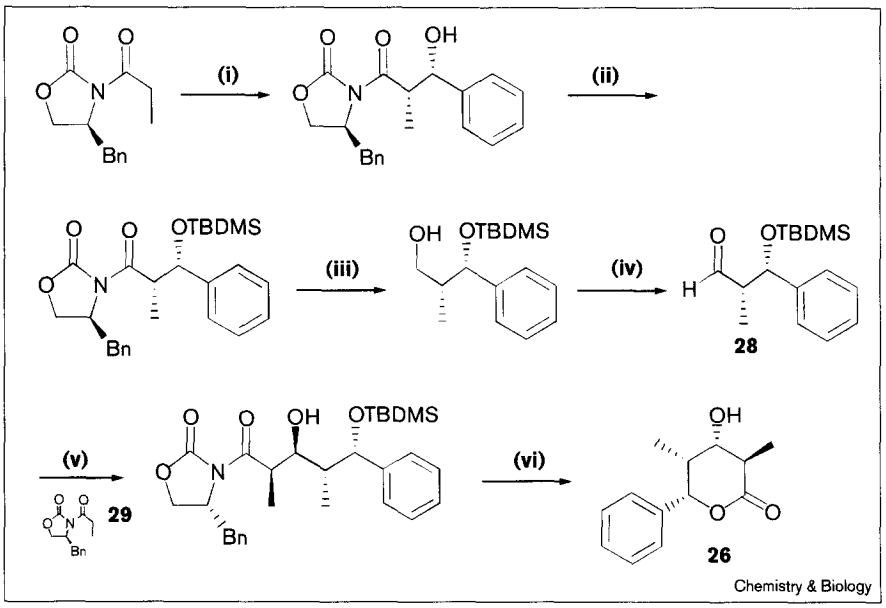
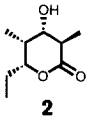
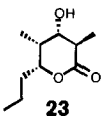
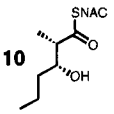
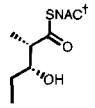


Table 2

Suppression of lactone biosynthesis from CoA thioesters (300 μM) by diketide NAC thioesters (6 mM).

Starter unit		Diketide	Yield  (nmol) **2**	Yield  (nmol) **23**
Propionyl-CoA		–	4.4	–
Propionyl-CoA	+	**10** 	0.18	1.6
Propionyl-CoA	+	Free NACSH	1.2	–
n-Butyryl-CoA†		–	6.6*	82
n-Butyryl-CoA	+	**3a** 	31	5.0

*Lactone **2** produced from propionyl-CoA contaminant in the added methylmalonyl-CoA. †Assay(s) conducted with a different batch of proteins, hence disparity in yields.

Comparison of biosynthesis through the loading domain/module 1 and through diketide incorporation at module 2

The data presented here demonstrate that under the chosen conditions *in vitro*, the same range of starter unit structure can be incorporated through either unnatural starter acids or their corresponding diketides, even though the erythromycin loading domain has been thought to have a restricted specificity [32]. As mentioned earlier, however, there are many factors that determine the efficiency with which substrate analogues can be incorporated *in vivo*. A quantitative comparison of efficiency of chain initiation by starter CoA-thioesters via the loading domain/KS1 [37] or through diketide analogues at module 2 will require, at the least, the future development of more sensitive assay methods and the elimination of contaminant propionyl-CoA to allow initial rates to be measured with acceptable accuracy. Meanwhile, it is pertinent to point out that, in qualitative terms, to achieve comparable yields of triketide lactones from diketide NAC analogues required significantly higher concentrations of theses substrates than of the corresponding acyl-CoA thioesters (Table 1).

Suppression of lactone biosynthesis from acyl CoA thioesters by diketide NAC thioesters

In monitoring triketide lactone synthesis using radiochemical TLC, it appeared that diketide NAC thioesters suppress chain initiation from CoA-thioesters. To study this effect, assays were conducted in the presence of 300 μM starter (either propionyl-CoA or *n*-butyryl-CoA), 6 mM diketide NAC (*n*-butyryl diketide NAC **10** or propionyl diketide NAC **3a**, respectively), as well as 600 μM methylmalonyl-CoA, 6 μM [^{14}C]-(2*RS*)-methylmalonyl-CoA and 1 mM NADPH, and the yields of lactones were compared to those from the starter only. An assay was also conducted in the presence of propionyl-CoA starter and free NACSH (6 mM), because, as shown using GC–MS analysis, NACSH is present in the assays with added diketides. The data are shown in Table 2. From the results it can be concluded that the presence of a diketide NAC substrate strongly inhibits biosynthesis from a CoA thioester, even when the starter unit is present at saturating concentrations. The nature of this inhibitory effect is unclear, but if it operates *in vivo* it should tend to favour incorporation of exogenous diketides into wild-type PKSs.

Incorporation of β-keto diketides

The utility of directed biosynthesis would increase if synthetic diketide analogues could be supplied to the enzyme not as β-hydroxy esters but as racemic β-keto esters. To assess the viability of this approach, the β-keto analogues **15** and **16** were incubated with DEBS 1-TE and the reaction mixture was analysed for the presence of lactones **22** and **24** with the natural stereochemistry, incorporating either an acetate or *iso*-butyrate starter residue, respectively (although KR2 might have reduced the keto group to give the opposite C-2 and C-3 configuration, we had

already demonstrated that this stereochemistry is not accepted by the PKS).

Compound **15** failed to be processed by DEBS 1-TE to any significant extent, but GC–MS analysis of methylated extracts demonstrated that diketide **16** had been reduced stereoselectively and incorporated into lactone **24** (there was no evidence for the methyl ester of the triketide corresponding to processing of the unreduced analogue by module 2). It appears, therefore, that KR1 will reduce a diketide keto ester stereospecifically, as has been observed previously (I.E. Holzbaur, personal communication), and that the resultant β-hydroxy compound is then recognised by KS2 for chain extension to the triketide. Depending on the substrate tolerance of KR1, feeding of racemic β-keto esters could greatly simplify the synthetic work that is required for diketide incorporation studies.

Significance

Antibiotic-producing modular polyketide synthases (PKSs) are giant multienzymes in which a different set of enzyme activities is used to catalyse each successive round of chain extension. Previous work has shown that analogues of putative enzyme-bound intermediates, particularly synthetic diketides as their *N*-acetylcysteamine thioesters, can be incorporated intact *in vivo* into full-length products. We have explored the versatility of this strategy through *in vitro* experiments with a fully purified bimodular derivative (DEBS 1-TE) of the 6-deoxyerythronolide B synthase. We show here that there is considerable scope for variation in the starter unit. Increased bulk at the alkyl branch (ethyl for methyl), however, is not tolerated to an efficient extent, nor is alteration of stereochemistry at C-2 and C-3. The constituent enzymes of module 2, in this PKS at least, are apparently specific enough that the usefulness of diketide analogue feeding as a method of generating additional diversity in polyketide products will be confined to variations in starter unit structure, a type of alteration already conveniently achieved by engineering broader specificity into the loading module of a PKS. More encouragingly, racemic keto analogues of diketides, which are synthetically more accessible, are alternatives to hydroxy intermediates in mutasynthesis.

Materials and methods

Materials

Coenzyme A thioesters were purchased from Sigma, DL-2-[methyl-^{14}C]-methylmalonyl-CoA (56.4 mCi/mmol) from DuPont NEN and HPLC grade ethyl acetate from Fisher. THF and Et_2O were distilled from lithium aluminium hydride, hexane from calcium hydride, and CH_2Cl_2 from sodium hydride immediately before use. *N*, *N*′-dimethylpropyleneurea was refluxed over calcium hydride, distilled at reduced pressure, and stored over 4 Å molecular sieves. *N*, *N*-Diisopropylethylamine, *N*, *N*-diisopropylamine and triethylamine were refluxed over calcium hydride, distilled, and stored over calcium hydride or 4 Å molecular sieves. Flash column chromatography was performed using Merck Kieselgel 60 (40–60 μm), and the indicated solvent systems. All reactions were performed under argon.

Protein isolation

Purification of DEBS 1-TE was performed essentially as described for DEBS multienzymes from *S. erythraea* [38], and for over-expressed DEBS 3 in *Escherichia coli* [39].

Diketide incorporation assays

Assays (45 h, 30°C) were conducted in which the concentrations of diketide NAC and NADPH were varied relative to a constant level of (2*RS*)-methylmalonyl-CoA (600 μM) as follows: [**3a**] = 600 μM and [NADPH] = 600 μM, 1.2 mM, 3 mM; [**3a**] = 1.2 mM and [NADPH] = 1.2 mM, 2.4 mM, 5.8 mM; [**3a**] = 3.0 mM and [NADPH] = 3.4 mM, 6.4 mM and 14.6 mM; and [**3a**] = 6.0 mM and [NADPH] = 1.0 mM, 6.3 mM, 12.4 mM and 27.6 mM. Radioactivity was added in the form of [^{14}C]-(2*S*, 3*R*)-2-methyl-3-hydroxypentanoic acid NAC **3b** (250 μCi/mmol, 2% of total diketide concentration). The assays were carried out in 400 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA and 20% glycerol in a total volume of 500 μl. Protein concentration (250 ± 10 μg/ml) was determined by the method of Bradford [40]. The reaction mixtures were quenched by extraction with two equal volumes of HPLC-grade ethyl acetate. The ^{14}C-labeled products were separated on TLC plates using diethyl ether as the mobile phase, and were counted on a Phosphorimager (Molecular Dynamics) relative to a lactone standard of known radioactivity.

Further incubations (21–45 h, 30°C) were carried out in the presence of various diketide NAC thioesters (6 mM), (2*RS*)-methylmalonyl-CoA (600 μM), NADPH (1.0–6.0 mM), and DEBS 1-TE. When used, radioactivity was added in the form of [^{14}C]-(2*RS*)-methylmalonyl-CoA (6.0 μM, 54.6 μCi/mmol). The assays were carried out in 400 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA in a final volume of 200–500 μl. Protein concentrations (100–250 ± 10 μg/ml) were determined by the method of Bradford [40]. The reaction mixtures were in most cases acidified to pH 2–3, quenched by extraction with 2–3 equal volumes of HPLC-grade ethyl acetate, and the products analysed by GC–MS or by radiochemical TLC. Conditions for the competition experiments were as above, but a starter-CoA thioester was also present at 300 μM and [^{14}C]-methylmalonyl-CoA at 6.0 μM (54.6 μCi/mmol).

Acyl-Coenzyme A thioester assays

Incubations (23 h, 30°C) were carried out in the presence of various starter acyl-CoA thioesters (100, 300 or 600 μM), (2*RS*)-methylmalonyl-CoA (600 μM), [^{14}C]-(2*RS*)-methylmalonyl-CoA (6.0 μM, 54.6 μCi/mmol), NADPH (1.0 mM), and DEBS 1-TE. The assays were carried out in 400 mM potassium phosphate buffer (pH 7.5), containing 1 mM EDTA in a total volume of 200–250 μl. Protein concentrations (100–250 ± 10 μg/ml) were determined by the method of Bradford [40]. The reaction mixtures were acidified to pH 2–3, quenched by extraction with 2 equal volumes of HPLC-grade ethyl acetate, and the products analysed by radiochemical TLC.

Product analysis and characterisation

GC–MS analysis was carried out with chemical ionisation (methane or ammonia as ionisation gas) on a Finnigan/MAT GCQ instrument using an Anachem SGE BPX5 5% phenyl polysilphenylene-siloxane column (inner diameter 0.22 mm, film width 0.25 mm, length 25 m). The following temperature program was used to analyse the extraction mixtures: 2 min 70°C, then 20 min, 10 deg/min to 250°C. Melting points were determined on a Gallenkamp hot stage melting point apparatus, and are uncorrected. Specific optical rotations were recorded on a Perkin-Elmer 241 polarimeter (concentration, c, g/100 ml solvent). Infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR spectrometer as solutions between sodium chloride plates. ^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded on Bruker WM250, AC250, AM400 or AC400 NMR spectrometers. Chemical shifts are reported in parts per million relative to tetramethylsilane. Mass spectra were recorded on an AEI MS 30 double beam spectrometer (EI) or on a Bruker CM547e Fourier Transform Ion Cyclotron Resonance (FT-ICR) spectrometer.

(4S)-3-Acetyl-4-phenylmethyl-2-oxazolidinone [41,42]
n-Butyl lithium (10.7 ml of a 1.6 M solution in hexane, 17.1 mmol, 1.0 eq) was added dropwise to a stirred solution of (4*S*)-4-phenylmethyl-2-oxazolidinone (3.0 g, 17 mmol, 1.0 eq) in tetrahydrofuran (THF) (150 ml) at 0°C. The solution was stirred at 0°C for 5 min, cooled to −78°C, and acetyl chloride (2.2 ml, 2.6 g, 34 mmol, 2.0 eq) was added dropwise. The resulting solution was stirred at −78°C for 1.5 h, allowed to warm to 0°C over 30 min, and then a saturated sodium bicarbonate solution (60 ml) was added. The mixture was extracted with diethyl ether (3 × 100 ml), the extracts washed with brine (50 ml), then dried (Na_2SO_4) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 20:80 ethyl acetate/hexane) to give 3.4 g (15 mmol, 91%) of (4*S*)-3-acetyl-4-phenylmethyl-2-oxazolidinone. $[\alpha]_D = +71.5°$ (c 1.1 CH_2Cl_2). ^1H NMR ($CDCl_3$ 400 MHz) δ 7.34–7.16 (m, 5H, Ph), 4.65 (m, 1H, C(4)-H), 4.10 (m, 2H, C(5)-H_2), 3.28 (dd, J = 13.4, 3.3 Hz, 1H, one of Ph-CH_2), 2.76 (dd, J = 13.4, 9.5 Hz, 1H, one of Ph-CH_2), 2.53 (s, 3H, C(2′)-H_3). ^{13}C NMR ($CDCl_3$ 100 MHz) δ 170.3, 153.7, 135.3, 129.4, 129.0, 127.4, 66.1, 55.0, 37.8, 23.8. IR (thin film, cm^{-1}): 1782, 1697. M^+ calc'd for $C_{12}H_{13}NO_3$: 219.0895; found: 219.0903.

(4S)-3-[3′-^{14}C]-Propionyl-4-phenylmethyl-2-oxazolidinone [43]
Sodium bis(trimethylsilylamide) (1.0 M in THF, 2.5 ml, 2.5 mmol, 1.1 eq) was cooled to −78°C with stirring and a solution of (4*S*)-3-acetyl-4-phenylmethyl-2-oxazolidinone (0.50 g, 2.3 mmol, 1.0 eq) in dry THF (15 ml) and *N*, *N*′-dimethylpropyleneurea (DMPU) (2.8 ml, 23 mmol, 10 eq) added dropwise via cannula. The resulting solution was stirred at −78°C for 1 h, a solution of [^{14}C]-methyl iodide (1 mCi) and methyl iodide (0.43 ml, 6.8 mmol, 3.0 eq) in dry THF (5 ml) was added and then the solution stirred at −78°C overnight. The reaction was quenched by the addition of saturated ammonium chloride (10 ml) and the volatiles removed *in vacuo*. The slurry was extracted with ether (4 × 40 ml), the extracts washed with brine (40 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 20% ethyl acetate in hexane) to give 0.18 g (0.74 mmol, 32%) of (4*S*)-3-[3′-^{14}C]-propionyl-4-phenylmethyl-2-oxazolidinone. Radiochemical yield 600 μCi, 60%. Specific activity 820 μCi/mmol.

(4S)-3-((2′S, 3′R)-3′-Hydroxy-2′-[^{14}C]-methylpentanoyl-4-phenylmethyl-2-oxazolidinone [41]
Di-*n*-butylboron triflate (0.20 ml, 0.80 mmol, 1.1 eq) and *N*, *N*-diisopropylethylamine (0.15 ml, 0.88 mmol, 1.2 eq) were added dropwise to a stirred solution of (4*S*)-3-[3′-^{14}C]-propionyl-4-phenylmethyl-2-oxazolidinone (0.17 g, 0.73 mmol, 1.0 eq) in dry dichloromethane (5 ml) at 0°C. The resulting solution was stirred at −78°C for 30 min. Propionaldehyde (2.0 M in dichloromethane (CH_2Cl_2), 0.44 ml, 0.88 mmol, 1.2 eq) was added dropwise and then the solution stirred at −78°C for 30 min and at 0°C for 2 h. The reaction was quenched by the addition of pH 7 phosphate buffer (2 ml) and methanol (6 ml). A 2:1 mixture of methanol/hydrogen peroxide (27 wt% in H_2O) (6 ml) was added dropwise at 0°C and the resulting solution stirred at 0°C for 1 h. The volatiles were removed *in vacuo* and the slurry extracted with ether (4 × 30 ml). The extracts were washed with 5% sodium hydrogen carbonate (20 ml) and brine (20 ml), dried (Na_2SO_4) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 5% ethyl acetate in hexane) to afford 0.14 g (0.48 mmol, 65%) of (4*S*)-3-((2′*S*, 3′*R*)-3′-hydroxy-2′-[^{14}C]-methylpentanoyl-4-phenylmethyl-2-oxazolidinone. Specific activity 810 μCi/mmol.

(2S, 3R)-3-Hydroxy-2-[^{14}C]-methylpentanoic acid [44]
(4*S*)-3-((2′*S*, 3′*R*)-3′-Hydroxy-2′-[^{14}C]-methylpentanoyl-4-phenylmethyl-2-oxazolidinone (0.14 g, 0.47 mmol, 1.0 eq) was dissolved in 3:1 THF/water (30 ml) at 0°C. Hydrogen peroxide (27 wt% in H_2O, 0.72 ml, 5.7 mmol, 12 eq) was added dropwise followed by lithium hydroxide (0.060 g, 1.4 mmol, 3.0 eq). The resulting solution was stirred at room temperature for 75 min before cooling to 0°C and quenching with sodium sulphite (1.5 M, 3.2 ml, 4.7 mmol, 10 eq). The volatiles were removed *in vacuo* and the remaining aqueous solution extracted with dichloromethane (4 × 20 ml). The aqueous layer was cooled to 0°C and acidified to pH 1 with 3 M hydrochloric acid. This layer was then extracted with saturated sodium chloride, extracted with ethyl acetate (4 × 20 ml), dried (Na_2SO_4) and concentrated *in vacuo* to give 0.063 g (0.47 mmol, 100%) of (2*S*, 3*R*)-3-hydroxy-2-[^{14}C]-methylpentanoic acid. Specific activity 800 μCi/mmol.

*2′-(Acetylamino)ethyl (2S, 3R)-3-hydroxy-2-[^{14}C]-methylpentanethioate **3b** [45,46]*
(2*S*, 3*R*)-3-Hydroxy-2-[^{14}C]-methylpentanoic acid (0.063 g, 0.47 mmol, 1.0 eq) was dissolved in dry dichloromethane (2.0 ml) at 0°C and 4-dimethylaminopyridine (12.0 mg, 0.095 mmol, 0.20 eq) added. *N*-acetylcysteamine (1.0 M in CH_2Cl_2, 0.71 ml, 0.71 mmol, 1.5 eq) and 1, 3-dicyclohexylcarbodiimide (DCC) (1.0 M in CH_2Cl_2, 0.52 ml, 0.52 mmol, 1.1 eq) were added dropwise and the resulting solution stirred at 0°C for 10 min and then at room temperature overnight. The precipitate was removed by filtration through Celite and the filtrate concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel with 1 cm plug of $CuSO_4$-impregnated silica, 5% methanol in ethyl acetate) to give 0.082 g (0.35 mmol, 74%) of 2′-(acetylamino)ethyl (2*S*,3*R*)-3-hydroxy-2-[^{14}C]-methylpentanethioate **3b**. Specific activity 780 μCi/mmol.

(4S)-3-Acetyl-4-isopropyl-2-oxazolidinone [41,42]
n-Butyl lithium (18.5 ml of a 1.6 M solution in hexane, 29.6 mmol, 1.1 eq) was added dropwise to a stirred solution of (4*S*)-4-isopropyl-2-oxazolidinone (3.56 g, 27.6 mmol, 1.0 eq) in tetrahydrofuran (THF) (80 ml) at 0°C. The solution was stirred at 0°C for 20 min, cooled to −78°C, and acetyl chloride (2.2 ml, 2.4 g, 31 mmol, 1.0 eq) was added dropwise. The reaction was allowed to warm to 0°C over 1 h and a saturated sodium bicarbonate solution (50 ml) was added. The mixture was extracted with diethyl ether (3 × 50 ml), and then the extracts combined, dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 30:70 ethyl acetate/hexane) to give 4.29 g (26.9 mmol, 91%) of (4*S*)-3-acetyl-4-isopropyl-2-oxazolidinone. $[\alpha]_D = +105°$ (c 1.4 CH_2Cl_2). ^1H NMR ($CDCl_3$ 400 MHz) δ 4.34 (ddd, 1H, J = 8.2, 3.3, 3.3 Hz, C(4)-H), 4.19 (dd, J = 9.1, 8.2 Hz, 1H, one of C(5)-H_2), 4.13 (dd, 1H, J = 9.1, 3.3 Hz, one of C(5)-H_2), 2.43 (s, 3H, C(2′)-H_3), 2.33–2.25 (m, 1H, C(6)-H), 0.83 (d, J = 7.1 Hz, 3H, one of C(6)-CH_3), 0.78 (d, J = 7.0 Hz, 3H, one of C(6)-CH_3). ^{13}C NMR ($CDCl_3$ 100 MHz) δ 170.0, 154.1, 63.0, 58.1, 28.3, 23.5, 17.7, 14.4. IR (thin film, cm^{-1}): 1782, 1704. M^+ calc'd for $C_8H_{13}NO_3$: 171.0895; found: 171.0890.

(4S)-4-Isopropyl-3-([3′-$^2H_3$]-propionyl)-2-oxazolidinone [43]
A −78°C solution of (4*S*)-3-acetyl-4-isopropyl-2-oxazolidinone (1.31 g, 7.66 mmol, 1.0 eq) and DMPU (9.2 ml, 9.8 g, 77 mmol, 10 eq) in THF (15 ml) was added dropwise via cannula to a stirred solution of sodium hexamethyldisilylamide (8.5 ml of a 1 M solution in THF, 8.5 mmol, 1.1 eq) at −78°C. The mixture was stirred for 1 h, and then a −50°C solution of [$^2H_3$]-methyl iodide (2.2 ml, 5.0 g, 34 mmol, 4.5 eq) in dry THF (3 ml) was added via cannula. The reaction was allowed to warm to room temperature over 16 h and then quenched by the addition of saturated ammonium chloride (30 ml). The resulting mixture was extracted with ether (3 × 50 ml) and then the combined extracts dried (Na_2SO_4) and evaporated *in vacuo*. The crude product was purified by flash chromatography (silica gel, 30:70 ethyl acetate/hexane) to give 1.01 g (5.36 mmol, 70%) of (4*S*)-4-isopropyl-3-([3′-$^2H_3$]-propionyl)-2-oxazolidinone. $[\alpha]_D = +91.8°$ (c 1.2 CH_2Cl_2). ^1H NMR ($CDCl_3$ 250 MHz) δ 4.39 (ddd, 1H, J = 9.0, 3.1, 3.1 Hz, C(4)-H), 4.22 (dd, J = 9.0, 9.0 Hz, 1H, one of C(5)-H_2), 4.18 (dd, 1H, J = 9.0, 3.3 Hz, one of C(5)-H_2), 2.92 (d, J = 17.7 Hz, 1H, one of C(2′)-H_2), 2.84 (d, J = 17.7 Hz, 1H, one of C(2′)-H_2), 2.34 (dqq, J = 7.0, 7.0, 3.1 Hz, 1H, C(6)-H), 0.88 (d, J = 7.0 Hz, 3H, one of C(6)-CH_3), 0.84 (d, J = 7.0 Hz, 3H, one of C(6)-CH_3). ^{13}C NMR ($CDCl_3$ 100 MHz) δ 174.0, 154.1, 63.2, 58.3, 28.8, 28.3, 17.9, 14.5, 8.4 (septet, J_{C-D} 19.4 Hz, CD_3). IR (thin film, cm^{-1}): 1781, 1702. M^+ calc'd for $C_9H_{12}D_3NO_3$: 188.1240; found: 188.1245.

(4S)-3-((2′R,3′S)-[2″-$^2H_3$]-3′-Cyclohexyl-3′-hydroxyl-2′-methylpropionyl)-4-isopropyl-2-oxazolidinone [41]

N, N-Diisopropylethylamine (0.40 ml, 0.30 g, 2.3 mmol, 1.1 eq) and di-*n*-butyl boron triflate (0.60 ml, 0.66 g, 2.4 mmol, 1.2 eq) were added sequentially to a stirred solution of (4*S*)-4-isopropyl-3-([3′-$^2H_3$]-propionyl)-2-oxazolidinone (0.36 g, 2.0 mmol, 1.0 eq) in dry dichloromethane (4 ml) at 0°C. The resulting solution was stirred at 0°C for 45 min and then cooled to −78°C. Cyclohexanecarboxaldehyde (0.30 ml, 0.28 g, 2.4 mmol, 1.2 eq) was added dropwise and the solution stirred at −78°C for 30 min, warmed to 0°C and stirred for a further 2 h. The reaction was quenched by the addition of pH 7 phosphate buffer (3 ml), the organic layer separated and the aqueous layer extracted with ether (3 × 10 ml). All organic extracts were combined, the solvent removed *in vacuo*, and the crude product was re-dissolved in a 1:1 mixture of pH 7 phosphate buffer/methanol (6 ml). This solution was cooled to 0°C, hydrogen peroxide (0.83 ml of a 30% solution in water, 7.3 mmol, 3.6 eq) added carefully and the resulting mixture stirred at 0°C for 1 h. Methanol was removed *in vacuo* and the aqueous layer extracted with dichloromethane (3 × 25 ml). The extracts were combined, dried (Na_2SO_4) and the solvent removed *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 25:75–35:65 ethyl acetate/hexane) to give 0.50 g (1.7 mmol, 83%) of (4*S*)-3-((2′*S*, 3′*R*)-[2″-$^2H_3$]-3′-cyclohexyl-3′-hydroxyl-2′-methylpropionyl)-4-isopropyl-2-oxazolidinone. m.p. 82–84°C. $[\alpha]_D = +68.8°$ (c 1.05 $CHCl_3$). ^1H NMR ($CDCl_3$ 400 MHz) δ 4.45 (ddd, 1H, J = 9.0, 3.1, 3.0 Hz, C(4)-H), 4.28 (dd, J = 9.0, 9.0 Hz, 1H, one of C(5)-H_2), 4.21 (dd, 1H, J = 9.0, 3.1 Hz, one of C(5)-H_2), 3.93 (d, J = 2.3 Hz, 1H, C(2′)-H), 3.57 (ddd, J = 8.7, 2.9, 2.3 Hz, 1H, C(3′)-H), 3.00 (d, J = 2.9 Hz, 1H, OH), 2.38–2.30 (m, 1H, C(6)-H), 2.11–2.08 (m, 1H), 1.75–1.71 (m, 2H), 1.66–1.62 (m, 2H), 1.41–1.35 (m, 1H, C(4′)-H), 1.25–1.09 (m, 3H), 1.00–0.95 (m, 2H), 0.91 (d, J = 7.0 Hz, 3H, one of C(6)-CH_3), 0.87 (d, J = 6.8 Hz, 3H, one of C(6)-CH_3). ^{13}C NMR ($CDCl_3$ 100 MHz) δ 178.2, 153.4, 75.1, 63.3, 58.2, 40.0, 38.9, 29.6, 28.8, 28.3, 26.3, 26.0, 25.9, 17.9, 14.7, 9.5 (septet, J_{C-D} 19.4 Hz, CD_3). IR (thin film, cm^{-1}): 3600–3450, 1779, 1682. M^+ calc'd for $C_{16}H_{24}D_3NO_4$: 301.2206; found: 301.2214.

(2S, 3R)-[2′-$^2H_3$]-3-Cyclohexyl-3-hydroxy-2-methylpropionic acid [44]

Hydrogen peroxide (2.6 ml of a 30% solution, 23 mmol, 14 eq) and lithium hydroxide (0.21 g, 5.1 mmol, 3.0 eq) were added to a stirred solution of (4*S*)-3-((2′*S*, 3′*R*)-[2″-$^2H_3$]-3′-cyclohexyl-3′-hydroxyl-2′-methylpropionyl)-4-isopropyl-2-oxazolidinone (0.50 g, 1.7 mmol, 1.0 eq) in a 3:1 mixture of THF/water (32 ml) at 0°C. The reaction mixture was stirred at room temperature for 1 h, cooled to 0°C and quenched with sodium sulphite solution (5.2 ml of a 1.5 M solution in water, 7.8 mmol, 4.6 eq). The THF was removed *in vacuo* and the aqueous solution extracted with dichloromethane (3 × 15 ml). The aqueous layer was acidified to pH 1 by addition of 3 M hydrochloric acid, and then re-extracted with ethyl acetate (3 × 15 ml). The organic extracts were combined, dried (Na_2SO_4) and the solvent removed *in vacuo* to give 0.30 g (1.6 mmol, 95%) of (2*S*, 3*R*)-[2′-$^2H_3$]-3-cyclohexyl-3-hydroxy-2-methylpropionic acid. $[\alpha]_D = +1.3°$ (c 1.2 CH_2Cl_2). ^1H NMR ($CDCl_3$ 400 MHz) δ 7.67 (br s, 1H, OH), 3.71 (dd, J = 8.4, 3.1 Hz, 1H, C(3)-H), 2.69 (d, J = 3.1 Hz, 1H, C(2)-H), 2.08–2.04 (m, 1H), 1.76–1.64 (m, 3H), 1.56–1.51 (m, 1H), 1.41–1.37 (1H, m, C(4)-H), 1.26–1.10 (m, 3H), 1.02–0.93 (m, 2H). ^{13}C NMR ($CDCl_3$ 100 MHz) 181.4, 75.9, 41.0, 39.9, 29.1, 28.9, 26.2, 25.9, 25.9. IR (thin film, cm^{-1}): 3500–3250, 1716. M^+ calc'd for $C_{10}H_{15}D_3O_3$: 190.1552; found: 190.1526.

*S((2S, 3R)-[2′-$^2H_3$]-3-Cyclohexyl-3-hydroxy-2-methylpropionyl)-2-acetamidoethanethiol **14** [45,46]*

A few crystals of 4-dimethylaminopyridine were added to a stirred solution of (2*S*, 3*R*)-[2′-$^2H_3$]-3-cyclohexyl-3-hydroxy-2-methylpropionic acid (0.30 g, 1.6 mmol, 1.0 eq) in dry dichloromethane at 0°C. To this mixture was added *N*-acetylcysteamine (2.4 ml of a 1 M solution in dichloromethane, 2.4 mmol, 1.5 eq) and DCC (2.4 ml of a 1 M solution in dichloromethane, 2.4 mmol, 1.5 eq). The reaction was stirred at °C for 10 min and then at room temperature for 20 h. The mixture was then filtered through Celite, washed with ethyl acetate and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, ethyl acetate) to give 0.32 g (1.1 mmol, 67%) of *S*((2*S*, 3*R*)-[2′-$^2H_3$]-3-cyclohexyl-3-hydroxy-2-methylpropionyl)-2-acetamidoethanethiol **14**. $[\alpha]_D = +23.5°$ (c 1.2 CH_2Cl_2). ^1H NMR ($CDCl_3$ 400 MHz) δ 6.48 (br s, 1H, NH), 3.58 (dd, J = 7.8, 3.6 Hz, 1H, C(3)-H), 3.37–3.35 (m, 2H, CH_2N), 2.94–2.92 (m, 2H, CH_2S), 2.77 (d, J = 3.6 Hz, 1H, C(2)-H), 2.57 (br d, J = 6.6 Hz, OH), 1.88 (s, 3H, CH_3CO), 1.72–1.56 (m, 3H), 1.49–1.46 (m, 2H), 1.30–1.22 (m, 1H), 1.20–1.03 (m, 3H), 0.99–0.89 (m, 2H). ^{13}C NMR ($CDCl_3$ 100 MHz) 204.0, 170.6, 76.1, 50.1, 40.3, 39.1, 29.1, 28.8, 28.3, 26.1, 25.9, 25.7, 22.9. IR (thin film, cm^{-1}): 3302, 1659. M^+ calc'd for $C_{14}H_{22}D_3NO_3S$: 291.1822; found: 291.1804.

(4S)-3-[(2′R)-2′-Methyl-3′-oxobutanoyl]-4-phenylmethyl-2-oxazolidinone [47]

n-Butyl lithium (1.6 ml of a 1.6 M solution in hexane, 2.5 mmol, 1.2 eq) was added to a solution of *N, N*-diisopropylamine (0.35 ml, 2.5 mmol, 1.1 eq) in THF at −78°C. After stirring for 20 min, a solution of (4*S*)-3-propionyl-4-phenylmethyl-2-oxazolidinone (0.50 mg, 2.2 mmol, 1 eq) in THF (5 ml) was added. The resulting mixture was stirred at −78°C for 2 h. A 2.5 M solution of magnesium bromide etherate in ether (1.0 ml, 2.5 mmol, 1.1 eq) was added, and the resulting suspension was allowed to warm towards 0°C for 2 min and then rapidly cooled to −78°C. A solution of acetyl chloride (0.19 ml, 2.7 mmol, 1.2 eq) in THF (3 ml) was added, and the solution left to stir at −78°C for 30 min before quenching with saturated aqueous ammonium chloride (10 ml). The solution was warmed to room temperature, the volatile solvents removed *in vacuo*, and the resulting aqueous slurry was extracted with ether. The organic extracts were washed with saturated aqueous sodium bicarbonate and brine, dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by flash column chromatography (silica gel, 30% ethyl acetate in light petroleum), to yield 0.28 g (1.0 mmol, 48%) of (4*S*)-3-[(2′*R*)-2′-methyl-3′-oxobutanoyl]-4-phenylmethyl-2-oxazolidinone. m.p. 91–93°C. $[\alpha]_D = -38.1°$ (c 1.22 $CHCl_3$). ^1H NMR ($CDCl_3$ 400 MHz) δ 7.35–7.24 (m, 5H, Ph-H), 4.66 (m, 1H, C(4)-H), 4.53 (q, J = 7.3 Hz, 1H, C(2′)-H), 4.17 (m, 2H, C(5)-H_2), 3.43 (dd, J = 13.6, 3.3 Hz, 1H, one of C(4)-CH_2), 2.76 (dd, 1H, J = 13.6, 10.0 Hz, one of C(4)-CH_2), 2.33 (s, 3H, C(4′)-H_3), 1.44 (d, J = 7.3 Hz, 3H, C(2′)-CH_3). ^{13}C NMR ($CDCl_3$ 100 MHz) δ 205.2, 169.8, 153.8, 135.4, 129.5, 129.0, 127.3, 66.4, 55.4, 53.0, 37.5, 28.4, 12.4. IR (thin film, cm^{-1}): 1776, 1718. M^+ calc'd for $C_{15}H_{17}NO_4$: 275.1158; found: 275.1151.

*2′-(Acetylamino)ethyl 2-methyl-3-oxobutanoylthioate **15** [48]*

Lithium bis(trimethylsilylamide) (1.25 ml of a 1 M solution in THF, 1.25 mmol, 2 eq) was added to a solution of *N*-acetylcysteamine (0.19 g, 1.6 mmol, 2.5 eq) in THF (40 ml) at −78°C. The resulting solution was stirred for 15 min at −78°C and then a solution of (4*S*)-3-[(2′*R*)-2′-methyl-3′-oxobutanoyl]-4-phenylmethyl-2-oxazolidinone (0.17 g, 0.64 mmol, 1 eq) in THF (20 ml) was added. The resulting solution was stirred for 15 min at −78°C and at 0°C for 45 min before quenching with saturated aqueous ammonium chloride (20 ml). The volatile solvents were removed and the aqueous slurry was extracted with ether. The organic extracts were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The crude mixture was purified by column chromatography (2 cm copper sulphate-impregnated silica gel, 25:75–35:65 ethyl acetate/hexane), to yield 0.074 g (0.35 mmol, 55%) of 2′-(acetylamino)ethyl 2-methyl-3-oxobutanoylthioate **15**. ^1H NMR ($CDCl_3$ 250 MHz) δ 6.16 (br s, 1H, NH), 3.71 (q, J = 7.1 Hz, 1H, C(2)-H), 3.36 (m, 2H, CH_2N), 3.03 (m, 2H, CH_2S), 2.19 (s, 3H, $NCOCH_3$), 1.92 (s, 3H, C(4)-H_3), 1.33 (d, J = 7.1 Hz, 3H, C(2)-H_3). ^{13}C NMR ($CDCl_3$ 63 MHz) 202.7, 196.9, 170.5, 61.9, 39.2, 28.9, 28.5, 23.1, 13.5. IR (thin film, cm^{-1}): 3294, 3077, 1724, 1666. $[M+H]^+$ calc'd for $C_9H_{16}NO_3S$: 218.0851; found: 218.0852.

(4S)-3-[(2′R)-2′,4′-Dimethyl-3′-oxopentanoyl]-4-phenylmethyl-2-oxazolidinone [47]

n-Butyl lithium (1.6 ml of a 1.6 M solution in hexane, 2.5 mmol, 1.2 eq) was added to a solution of *N, N*-diisopropylamine (0.35 ml, 2.5 mmol,

1.1 eq) in THF at −78°C. After stirring for 20 min, a solution of (4*S*)-3-propionyl-4-phenylmethyl-2-oxazolidinone (0.50 g, 2.2 mmol, 1 eq) in THF (5 ml) was added. The resulting mixture was stirred at −78°C for 2 h. A 2.5 M solution of magnesium bromide etherate in ether (1.0 ml, 2.5 mmol, 1.1 eq) was added, and the resulting suspension was allowed to warm towards 0°C for 2 min and then rapidly cooled to −78°C. A solution of *iso*-butyryl chloride (0.28 ml, 2.7 mmol, 1.2 eq) in THF (3 ml) was added, and the solution left to stir at −78°C for 30 min before quenching with saturated aqueous ammonium chloride (10 ml). The solution was warmed to room temperature, the volatile solvents removed *in vacuo*, and the resulting aqueous slurry was extracted with ether. The organic extracts were washed with saturated aqueous sodium bicarbonate and brine, dried ($MgSO_4$) and concentrated under reduced pressure. The crude product was purified by column chromatography (silica gel, 20% ethyl acetate in light petroleum) to yield 0.37 g (1.2 mmol, 57%) of (4*S*)-3-[(2′*R*)-2′, 4′-dimethyl-3′-oxopentanoyl]-4-phenylmethyl-2-oxazolidinone. m.p. 88–90°C. $[\alpha]_D = -23.5°$ (c 1.02 $CHCl_3$). ^1H NMR ($CDCl_3$ 250 MHz) δ 7.35–7.23 (m, 5H, Ph-H), 4.79 (q, J = 7.0 Hz, 1H, C(2′)-H), 4.65 (m, 1H, C(4)-H), 4.16 (m, 2H, C(5)-H_2), 3.47 (dd, J = 13.5, 3.3 Hz, 1H, one of C(4)-CH_2), 2.86 (m, 1H, C(4′)-H), 2.75 (dd, J = 13.5, 10.2 Hz, 1H, one of C(4)-CH_2), 1.43 (d, J = 7.3 Hz, 3H, C(2′)-CH_3), 1.22 (d, J = 6.9 Hz, 3H, one of C(4′)-CH_3), 1.15 (d, J = 6.8 Hz, 3H, one of C(4′)-CH_3). ^{13}C NMR ($CDCl_3$ 63 MHz) δ 211.6, 170.5, 153.5, 135.5, 129.4, 128.9, 127.2, 66.3, 55.4, 51.2, 39.1, 37.5, 18.8, 18.8, 12.7. IR (thin film, cm^{-1}): 1770, 1714. M^+ calc'd for $C_{17}H_{21}NO_4$: 303.1471; found: 303.1471.

2′-(Acetylamino)ethyl 2,4-dimethyl-3-oxopentanoylthioate ***16*** *[48]*

Lithium bis(trimethylsilylamide) (1.3 ml of a 1 M solution in THF, 1.3 mmol, 2 eq) was added to a solution of *N*-acetylcysteamine (0.20 g, 1.7 mmol, 2.6 eq) in THF (50 ml) at −78°C. The resulting solution was stirred for 15 min at −78°C and then a solution of (4*S*)-3-[(2′*R*)-2′, 4′-dimethyl-3′-oxopentanoyl]-4-phenylmethyl-2-oxazolidinone (0.20 g, 0.64 mmol, 1 eq) in THF (20 ml) was added. The resulting solution was stirred for 15 min at −78°C and at 0°C for 45 min before quenching with saturated aqueous ammonium chloride (20 ml). The volatile solvents were removed and the aqueous slurry was extracted with ether. The organic extracts were washed with brine, dried ($MgSO_4$) and concentrated *in vacuo*. The crude mixture was purified by column chromatography (2 cm copper sulphate-impregnated silica gel, ethyl acetate) to yield 0.11 g (0.46 mmol, 71%) of 2′-(acetylamino)ethyl 2, 4-dimethyl-3-oxopentanoylthioate **16**. ^1H NMR ($CDCl_3$ 250 MHz) δ 6.21 (br s, 1H, NH), 3.93 (q, J = 7.0 Hz, 1H, C(2)-H), 3.34 (m, 2H, CH_2N), 3.01 (m, 2H, CH_2S), 2.78 (sep, J = 7.0 Hz, 1H, C(4)-H), 1.92 (s, 3H, $NCOCH_3$), 1.32 (d, J = 7.0 Hz, 3H, C(2)-CH_3), 1.06 (d, J = 7.0 Hz, 6H, 2 × C(4)-CH_3). ^{13}C NMR ($CDCl_3$ 63 MHz) 208.8, 196.6, 170.5, 59.1, 40.4, 39.2, 28.7, 23.0, 18.5, 18.1, 13.9. IR (thin film, cm^{-1}): 3292, 3078, 1715, 1650, 1556. M^+ calc'd for $C_{11}H_{19}NO_3S$: 245.1086; found: 245.1086.

(4S)-3-[(2′S, 3′S)-3′-Hydroxy-2′-methyl-3′-phenylpropanoyl]-4-phenylmethyl-2-oxazolidinone [41]

Di-*n*-butylboron triflate (6.5 ml, 25 mmol, 1.2 eq) and triethylamine (4.0 ml, 30 mmol, 1.4 eq) were added dropwise to a stirred solution of (4*S*)-3-propionyl-4-phenylmethyl-2-oxazolidinone (5.0 g, 21 mmol, 1.0 eq) in dry dichloromethane (50 ml) at 0°C. The resulting solution was stirred at −78°C for 20 min. Freshly distilled benzaldehyde (2.4 ml, 24 mmol, 1.1 eq) was added dropwise, then the solution stirred at −78°C for 20 min and at 0°C for 2 h. The reaction was quenched by the addition of pH 7 phosphate buffer (24 ml) and methanol (71 ml). A 2:1 mixture of methanol/hydrogen peroxide (27 wt% in H_2O) (71 ml) was added dropwise at 0°C and the resulting solution stirred at 0°C for 1 h. The volatiles were removed *in vacuo* and the slurry extracted with ether (3 × 50 ml). The extracts were washed with 5% sodium sulphite solution (30 ml), and then extracted with diethyl ether (50 ml). The combined extracts were dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 20% ethyl acetate in light petroleum) to afford 5.4 g (16 mmol, 76%) of (4*S*)-3-[(2′*S*, 3′*S*)-3′-hydroxy-2′-methyl-3′-phenylpropanoyl]-4-phenylmethyl-2-oxazolidinone. $[\alpha]_D = +44°$ (c 1.50 $CHCl_3$). ^1H NMR ($CDCl_3$ 400 MHz) δ 7.41–7.17 (m, 10H, 2 × Ph-H), 5.09 (m, 1H, C(3′)-H), 4.60 (m, 1H, C(4)-H), 4.10 (m, 3H, C(5)-H_2 and C(2′)-H), 3.24 (dd, J = 13.4, 3.3 Hz, 1H, one of C(4)-CH_2), 3.09 (d, J = 2.7 Hz, 1H, OH), 2.76 (dd, J = 13.4, 9.5 Hz, 1H, one of C(4)-CH_2), 1.22 (d, J = 6.9 Hz, 3H, C(2′)-CH_3). ^{13}C NMR ($CDCl_3$ 100 MHz) δ 176.8, 152.9, 141.3, 135.0, 129.4, 129.0, 128.3, 127.6, 127.4, 126.1, 73.8, 66.2, 55.2, 44.6, 37.8, 11.0. IR (thin film, cm^{-1}): 1769, 1694. $[M+Na^+]^+$ calc'd for $C_{20}H_{21}NO_4Na$: 362.1363; found: 362.1358.

(4S)-3-[(2′S, 3′S)-3′-[(tert-Butyldimethylsilyl)oxy]-2′-methyl-3′-phenylpropanoyl]-4-phenylmethyl-2-oxazolidinone [49]

N, *N*-Diisopropylethylamine (2.1 ml, 12 mmol, 1.8 eq) and *tert*-butyldimethylsilyl triflate (1.5 ml, 6.9 mmol, 1.1 eq) were added to a solution of (4*S*)-3-[(2′*S*, 3′*S*)-3′-hydroxy-2′-methyl-3′-phenylpropanoyl]-4-phenylmethyl-2-oxazolidinone (2.2 g, 6.5 mmol, 1 eq) in dry dichloromethane (100 ml) at −78°C. The solution was stirred for 2 h at −78°C, and then the volatiles removed *in vacuo*. The crude product was purified by flash column chromatography (30% ethyl acetate in light petroleum) to yield 2.2 g (4.8 mmol, 74%) of (4*S*)-3-[(2′*S*, 3′*S*)-3′-[*tert*-butyldimethylsilyl)oxy]-2′-methyl-3′-phenylpropanoyl]-4-phenylmethyl-2-oxazolidinone. $[\alpha]_D = +59°$ (c 1.86 $CHCl_3$). ^1H NMR ($CDCl_3$ 250 MHz) δ 7.43–7.16 (m, 10H, 2 × Ph-H), 4.84 (d, J 7.0 Hz, 1H, C(3′)-H), 4.26 (m, 1H, C(4)-H), 4.16 (qn, J = 7.0 Hz, 1H, C(2′)-H), 3.98 (dd, J = 8.9, 1.9 Hz, 1H, one of C(5)-H_2), 3.68 (t, J = 8.2 Hz, 1H, one of C(5)-H_2), 3.20 (dd, J = 13.3, 3.2 Hz, 1H, one of C(4)-CH_2), 2.69 (dd, J = 13.3, 9.7 Hz, 1H, one of C(4)-CH_2), 1.30 (d, J = 6.7 Hz, 3H, C(2′)-CH_3), 0.88 (s, 9H, *t*-Bu-H), 0.015 (s, 3H, one of Si-CH_3), −0.23 (s, 3H, one of Si-CH_3). ^{13}C NMR ($CDCl_3$ 63 MHz) δ 174.6, 142.9, 135.4, 129.4, 128.9, 127.9, 127.4, 127.3, 126.7, 76.6, 65.9, 55.7, 46.7, 37.7, 25.7, 18.1, 12.9, −4.7, −5.3. IR (thin film, cm^{-1}): 1783, 1698. $[M+H^+]^+$ calc'd for $C_{26}H_{36}NO_4Si$: 454.2408; found: 454.2399.

(2S, 3S)-3[(tert-butyldimethylsilyl)oxy]-2-methyl-5-phenylpropanol [50]

Water (0.10 ml, 5.6 mmol, 1.1 eq) and lithium borohydride (2.9 ml of a 2 M solution in hexane, 5.8 mmol, 1.1 eq) were added to a solution of (4*S*)-3-[(2′*S*, 3′*S*)-3′-[*tert*-butyldimethylsilyl)oxy]-2′-methyl-3′-phenylpropanoyl]-4-phenylmethyl-2-oxazolidinone (2.3 g, 5.1 mmol, 1.0 eq) in diethyl ether at 0°C. The solution was warmed to room temperature and stirred for 1 h. The reaction was then quenched with 1 M sodium hydroxide (100 ml), stirred for 10 min and then extracted with diethyl ether (3 × 100 ml). The extracts were washed with brine (100 ml), dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 10% ethyl acetate in light petroleum) to yield 0.72 g (2.6 mmol, 51%) of (2*S*, 3*S*)-3[(*tert*-butyldimethylsilyl)oxy]-2-methyl-5-phenylpentanoyl. $[\alpha]_D = -57°$ (c 2.93 $CHCl_3$). ^1H NMR ($CDCl_3$ 250 MHz) δ 7.40–7.12 (m, 5H, Ph-H), 4.84 (d, J = 4.3 Hz, 1H, OH), 3.57 (m, 1H, one of C(1)-H_2), 3.42 (m, 1H, one of C(1)-H_2), 2.57 (s, 1H, OH), 2.04 (m, 1H, C(2)-H), 0.89 (s, 9H, *t*-Bu-H), 0.74 (d, J = 7.0 Hz, 3H, C(2)-CH_3), 0.040 (s, 3H, one of Si-CH_3), −0.19 (s, 3H, one of Si-CH_3). ^{13}C NMR ($CDCl_3$ 63 MHz) δ 142.4, 127.8, 127.1, 126.8, 65.5, 43.0, 25.8, 18.2, 11.9, −4.65, −5.29. IR (thin film, cm^{-1}): 3387. $[M+H^+]^+$ calc'd for $C_{16}H_{29}O_2Si$: 281.1931; found: 281.1932.

(2S, 3S)-3-[(tert-Butyldimethylsilyl)oxy]-2-methyl-5-phenylpropanal [51–53]

(2*S*, 3*R*)-3[(*tert*-butyldimethylsilyl)oxy]-2-methyl-5-phenylpentanoyl (0.60 g, 2.1 mmol, 1.0 eq) in dichloromethane (50 ml) was added to a suspension of Dess-Martin reagent (1.0 g, 2.4 mmol, 1.1 eq). The reaction was stirred for 40 min at room temperature, and then 1 M sodium hydroxide/ether 5:12 (170 ml) added. The aqueous layer was extracted with diethyl ether (100 ml), and then the organic extracts dried ($MgSO_4$) and the solvent removed *in vacuo*. The crude product was purified by flash column chromatography to yield 0.48 g (1.7 mmol, 71%) of (2*S*, 3*S*)-3-[(*tert*-butyldimethylsilyl)oxy]-2-methyl-5-phenylpropanal. $[\alpha]_D = -32°$ (c 1.31 $CHCl_3$). ^1H NMR ($CDCl_3$ 250 MHz) δ 9.77 (d, J = 1.3 Hz, 1H, C(1)-H), 7.37–7.20 (m, 5H, Ph-H), 5.15 (d, J = 4.3 Hz, C(4)-H), 2.59 (m, 1H, C(2)-H), 1.04 (d, J = 7.0 Hz, 3H, C(2)-CH_3), 0.89 (s, 9H, *t*-Bu-H), 0.035 (s, 3H, one of Si-CH_3), −0.17 (s, 3H, one of Si-CH_3). ^{13}C NMR

($CDCl_3$ 63 MHz) δ 204.2, 142.3, 128.1, 127.4, 126.3, 74.3, 54.8, 25.7, 8.0, −4.6, −5.2. IR (thin film, cm^{-1}): 1782, 1694. $[M+Na^+]^+$ calc'd for $C_{16}H_{26}O_2SiNa$: 301.1594; found: 301.1590.

(4R)-[(2′R, 3′S, 4′R, 5′S)-3′-Hydroxy-2′, 4′-methyl-5′[(tert-butyldimethylsilyl)oxy]-phenylpentanoyl]-4-phenylmethyl-2-oxazolidinone [41]

Di-*n*-butylboron triflate (0.43 ml, 1.7 mmol, 1.2 eq) and triethylamine (0.27 ml, 1.9 mmol, 1.4) were added dropwise to a stirred solution of (4*R*)-3-propionyl-4-phenylmethyl-2-oxazolidinone (0.34 g, 1.4 mmol, 1.0 eq) in dry dichloromethane (20 ml) at 0°C. The resulting solution was stirred at −78°C for 20 min, then (2*S*, 3*S*)-3-[(*tert*-butyldimethylsilyl)oxy]-2-methyl-5-phenylpropanal (0.41 g, 1.5 mmol, 1.1 eq) was added. The solution was stirred at −78°C for 20 min and at 0°C for 2 h. The reaction was quenched by the addition of pH 7 phosphate buffer (1.9 ml) and methanol (71 ml). A 2:1 mixture of methanol/hydrogen peroxide (27 wt% in H_2O) (5.7 ml) was added dropwise at 0°C and the resulting solution stirred at 0°C for 1 h. The volatiles were removed *in vacuo* and the slurry extracted with diethyl ether (3 × 50 ml). The extracts were washed with 5% sodium sulphite solution (30 ml), and then extracted with ether (50 ml). The combined extracts were dried ($MgSO_4$) and concentrated *in vacuo*. The crude product was purified by flash column chromatography (silica gel, 15% ethyl acetate in light petroleum) to afford 0.40 g (0.78 mmol, 56%) of (4*R*)-[(2′*R*, 3′*S*, 4′*R*, 5′*S*)-3′-hydroxy-2′, 4′-methyl-5′[(*tert*-butyldimethylsilyl)oxy]-phenylpentanoyl]-4-phenylmethyl-2-oxazolidinone. $[\alpha]_D = -78°$ (c 2.36 $CHCl_3$). ^1H NMR ($CDCl_3$ 500 MHz) δ 7.36–7.19 (m, 10H, 2 × Ph-H), 5.24 (d, J = 2.0 Hz, 1H, C(5′)-H), 4.73 (m, 1H, C(4)-H), 4.24–4.08 (m, 2H, C(2′)-H and C(3′)-H), 3.88 (m, 2H, C(5)-H_2), 3.28 (dd, J = 13.3, 3.3 Hz, 1H, one of C(4)-CH_2), 2.78 (dd, J = 13.3, 9.6 Hz, 1H, one of C(4)-CH_2), 1.77 (m, 1H, C(4′)-H), 1.23 (d, J = 7.0 Hz, 3H, C(2′)-CH_3), 0.93 (s, 9H, *t*-Bu-H), 0.71 (d, J = 7.0 Hz, C(4′)-CH_3), 0.085 (s, 3H, one of Si-CH_3), −0.16 (s, 3H, one of Si-CH_3). ^{13}C NMR ($CDCl_3$ 100 MHz) δ 177.9, 152.8, 143.6, 135.2, 129.5, 129.0, 127.7, 127.4, 126.7, 126.6, 74.3, 71.8, 66.2, 55.2, 43.5, 39.7, 37.8, 26.0, 18.3, 9.2, −4.6, −5.2. IR (thin film, cm^{-1}): 3528, 1788, 1680. $[M+Na^+]^+$ calc'd for $C_{29}H_{41}O_5NSiNa$: 534.2646; found: 534.2650.

*(2R, 3S, 4R, 5S)-3, 5-Dihydroxy-2, 4-dimethyl-5-phenyl-n-pentanoic acid-δ-lactone **26** [44]*

Hydrogen peroxide (0.35 ml of a 30% solution, 2.8 mmol, 4.6 eq) and lithium hydroxide (0.058 g, 1.4 mmol, 2.3 eq) were added to a stirred solution (4*R*)-[(2′*R*, 3′*S*, 4′*R*, 5′*S*)-3′-hydroxy-2′, 4′-methyl-5′[*tert*-butyldimethylsilyl)oxy]-phenylpentanoyl]-4-phenylmethyl-2-oxazolidinone (0.31 g, 0.61 mmol, 1.0 eq) in a 3:1 mixture of THF/water (20 ml) at 0°C. The reaction mixture was stirred at room temperature for 20 min, cooled to 0°C and quenched with sodium sulphite solution (3.3 ml of a 1.5 M solution in water, 5.0 mmol, 8.2 eq). The THF was removed *in vacuo* and the aqueous solution extracted with dichloromethane (3 × 20 ml). The chiral auxiliary was separated from the acid by flash column chromatography (10% isopropanol in light petroleum), and the resulting acid was stirred at 40°C in a solution of 1 M HCl (10 ml) and THF (2 ml) for 4 h. The solution was extracted with ethyl acetate (3 × 10 ml), the extracts washed with brine, dried ($MgSO_4$) and the solvent removed *in vacuo* to yield 0.060 g (0.25 mmol, 40%) of (2*R*, 3*S*, 4*R*, 5*S*)-3, 5-dihydroxy-2, 4-dimethyl-5-phenyl-n-pentanoic acid-δ-lactone **26**. $[\alpha]_D = +8.7°$ (c 0.470 $CHCl_3$). ^1H NMR ($CDCl_3$ 400 MHz) δ 7.42–7.25 (m, 5H, Ph-H), 5.42 (d, J = 2.5 Hz, 1H, C(5)-H), 4.08 (dd, J = 10.4, 4.3 Hz, 1H, C(3)-H), 2.60 (dq, J = 10.4, 7.1 Hz, 1H, C(2)-H), 2.43 (qdd, J = 7.1, 4.1, 2.6 Hz, 1H, C(4)-H), 1.48 (d, J = 7.0 Hz, 3H, C(2)-CH_3), 0.77 (d, J = 7.1 Hz, 3H, C(4)-CH_3). ^{13}C NMR ($CDCl_3$ 100 MHz) δ 173.2, 137.4, 128.4, 127.8, 125.2, 80.2, 74.0, 40.2, 39.8, 14.3, 4.6. IR (thin film, cm^{-1}): 3480, 1716. $[M+Na^+]^+$ calc'd for $C_{13}H_{16}O_3Na$: 243.0992; found: 243.0985.

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