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Malonyl carba(dethia)- and Malonyl oxa(dethia)-coenzyme A as Tools for Trapping Polyketide Intermediates

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In order to study intermediates in polyketide biosynthesis two nonhydrolyzable malonyl coenzyme A analogues were synthesised by a chemoenzymatic route. In these analogues the sulfur atom of CoA was replaced either by a methylene group (carbadethia analogue) or by an oxygen atom (oxadethia analogue). These malonyl-CoA analogues were found to compete with the natural extender unit malonyl-CoA and to trap intermediates from stilbene synthase, a type III polyketide synthase (PKS). From the reaction of stilbene synthase with its natural phenylpropanoid substrates, diketide, triketide and tetraketide species were successfully off-loaded and characterised by LC-MS. Moreover, the reactivity of the nonhydrolyzable analogues offers insights into the flexibility of substrate alignment in the PKS active site for efficient malonyl decarboxylation and condensation.

Introduction

Among natural products polyketides constitute a unique pool of structurally and functionally diverse compounds endowed with extraordinary medicinal activity. Examples of polyketides in clinical use include erythromycin (antibiotic), epothilone (anticancer), lovastatin (anticholesterol drug) and rapamycin (immunosuppressant).[1]

Polyketides are produced in bacteria, fungi, plants and protists by polyketide synthases (PKSs), and are still usefully classified in three main categories according to their biosynthetic mechanism, although examples of intermediate types of PKS are increasing in number.[2] Type I modular PKSs are giant multifunctional enzymes in which modules of fatty acid synthase-related activities each catalyze a specific step of polyketide chain elongation. These modular synthases are often referred to as "multidomain assembly lines" and their typical products are complex macrolides, which can be further decorated by "post-PKS" modifications, such as methylation and glycosylation.[3] In classic type I iterative systems a single module is used iteratively to synthesize the polyketide chain.[4,5] In type II PKSs several discrete mono- or bifunctional enzymes act in an iterative manner during biosynthesis to produce complex aromatic compounds.[6,7] Type III PKSs are homodimers with a single active site where all the catalytic transformations take place to produce simple aromatic compounds.[8] For type III PKSs the substrates and intermediates are attached to coenzyme A (CoA), whereas for type I and II the intermediates are attached to acyl carrier proteins (ACPs), which are discrete proteins (type II) or are domains in giant multifunctional enzymes (type I).

Despite the differences in enzyme architecture, all polyketide synthases generate a growing polyketide chain in the same way by repetitive condensation steps that attach malonyl (or substituted malonyl units) to a starter unit with concomitant decarboxylation (Scheme 1 A). In contrast to saturated fatty acid biosynthesis,[2,9,10] either no reduction, partial reduction, or full reduction of the carbonyl group can occur in each cycle of chain extension. This and other modifications, such as cyclization and aromatization, account for the large variety of structures produced by PKSs.

A key role in PKSs is played by a flexible phosphopantetheine arm attached to the ACP or part of coenzyme A. This arm is responsible for the delivery of the malonyl units to the ketosynthase (KS) active site and it carries the extended polyketide chain throughout its elaboration. There is intense current interest in understanding the evolution of PKS catalysis and in manipulating the pathways towards the creation of new compounds of increased medicinal value.[11–13] Since the first pioneering experiments with isotopic labelling enormous progress in this field has been made thanks to genetic approaches and latterly to X-ray crystallography,[14,15] but many details remain obscure, such as the timing of chain termination, release and cyclization and the ability of iterative PKSs to differentiate between substrates that vary both in their chain length and state of reduction.[1,8]

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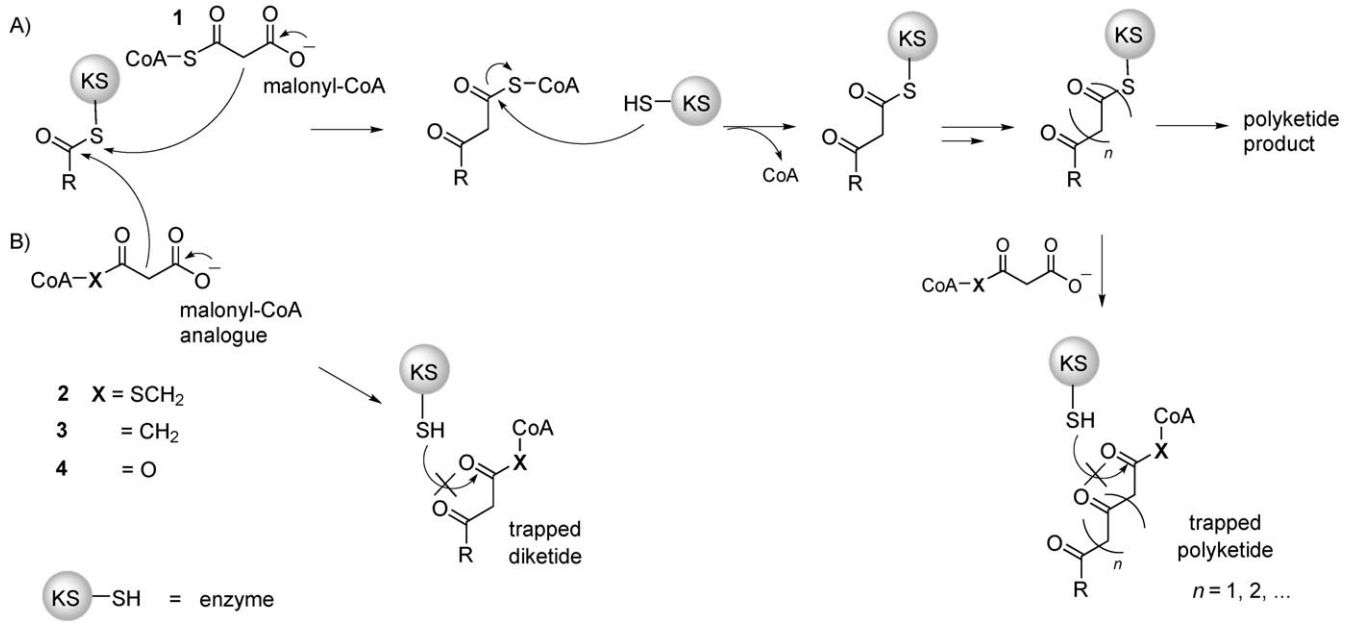


Scheme 1. A) Polyketide formation by decarboxylative malonate condensation (type III PKS); B) Off-loading of polyketide intermediates by using malonyl-CoA analogues;[22] KS: ketosynthase.

Characterisation of intermediates of polyketide biosynthesis has proved difficult, as they remain covalently attached to the PKS throughout their elaboration. The cloning and sequencing of genes for many modular PKSs have enabled the design of mutants that alter or halt biosynthesis at a desired point,[16,17] which leads to the accumulation of polyketide precursors.[5] However, this approach is quite laborious and of limited use for iterative PKSs. Advances in mass spectrometry techniques have allowed the direct observation of some enzyme-bound species. Using tandem-proteolysis mass spectrometry, Schnarr and co-workers have detected KS-bound diketide intermediates from DEBS3 fed with *N*-acetylcysteamine-derived synthetic substrates.[18]

Kelleher and co-workers have applied FT mass spectrometry to monitor KS-AT and ACP/PCP-bound diketide/dipeptide intermediates upon limited proteolysis[19,20] and, very recently, ACP-bound late-stage precursors of the aflatoxin biosynthesis in vitro.[21]

In our laboratory we have been pursuing an alternative strategy towards the isolation and characterisation of polyketide intermediates—their chemical off-loading from a PKS by using nonhydrolyzable malonyl coenzyme A analogues.[22] As malonyl-CoA (**1**) is responsible for polyketide chain extension (Scheme 1 A), we had envisaged that a nonhydrolyzable analogue might intercept the growing polyketide chain, but would then not be susceptible to its subsequent transesterification onto the cysteine group of the KS. As a result, polyketide intermediates should accumulate, attached to the analogue (Scheme 1 B). The thioether analogue **2** was originally prepared in our laboratory and when used in the reaction of type III PKS stilbene synthase (STS) with 4-hydroxyphenylacetyl-CoA as starter unit, a trapped diketide and triketide were identified.[22] However, no intermediates were detected for STS with its true substrate coumaroyl-CoA; this suggests that the extra methylene group between the thiol and the malonate moiety could have been detrimental to the correct positioning of the analogue in the active site.

Here, we present a significant improvement of this off-loading strategy using new malonyl-CoA analogues (**3** and **4**) that more closely resemble malonyl-CoA (**1**).

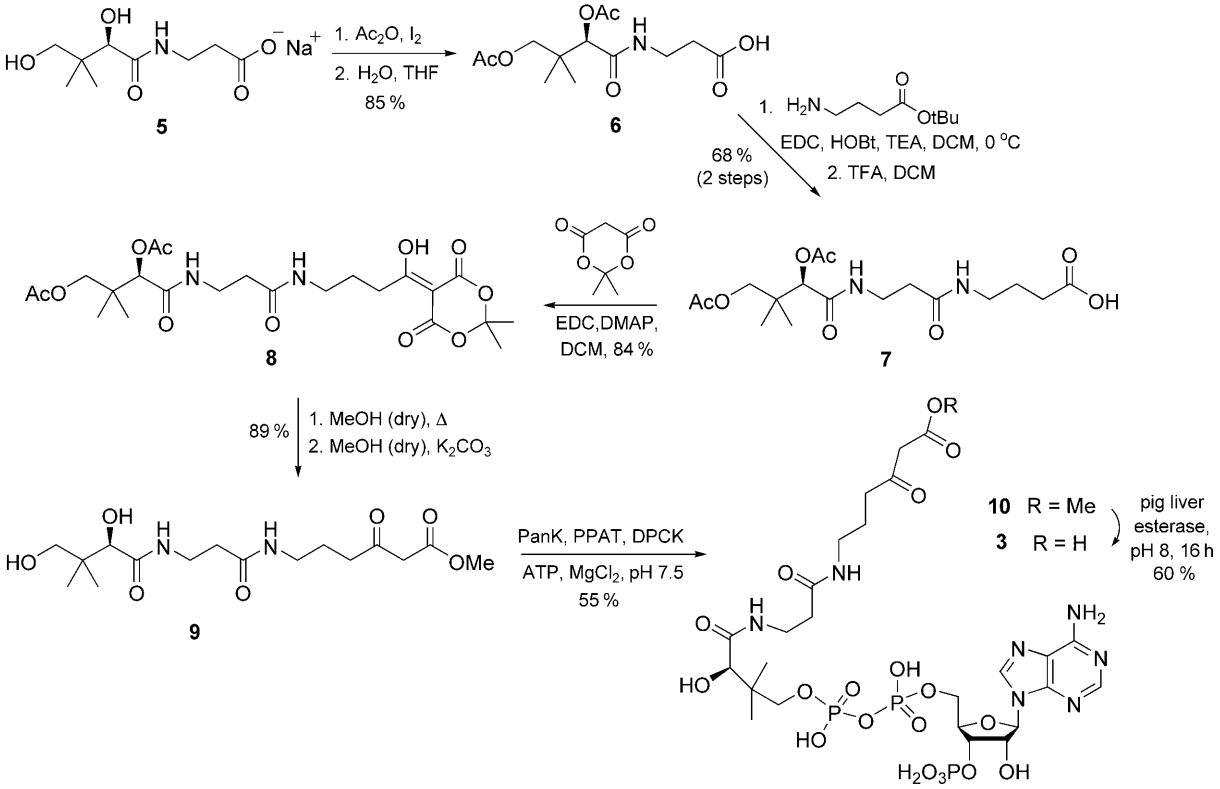
Results and Discussion

Preparation of malonyl carba(dethia)- and malonyloxa-(dethia)-coenzyme A

The use of carba(dethia)- and oxa(dethia)-CoA derivatives for mechanistic investigations is well documented.[23–25] To the best of our knowledge malonyl carba(dethia)-CoA (**3**) has been previously obtained only in situ by enzymatic reaction of acetyl carba(dethia)-CoA with acetyl-CoA carboxylase.[26]

Our approach to the preparation of the malonyl-CoA analogue **3** consisted of the chemical synthesis of a protected malonyl carba(dethia)pantetheine (**9**) and its conversion to the corresponding CoA derivative **10** using the *E. coli* enzymes for the biosynthesis of coenzyme A (Scheme 2).[27,28]

Briefly, sodium pantothenate (**5**) was acetylated in acetic anhydride with catalytic iodine to afford a protected mixed anhydride,[29] which was promptly hydrolyzed to the corresponding acid **6** (85% over two steps). EDC/HOBt coupling of **6** to γ-aminobutyric acid *tert*-butyl ester and subsequent treatment with TFA gave the acid **7** in 68% yield. This substrate was further treated with Meldrum's acid and EDC/DMAP to yield the cyclic adduct **8** (85%). After reflux in dry methanol[30] and deacetylation by potassium carbonate in methanol, the deprotected β-ketoester **9** was obtained in 89% yield. The enzymes pan-



Scheme 2. Preparation of malonylcarba(dethia)-CoA (**3**).

tothenate kinase (PanK), phosphopantotheine adenyltransferase (PPAT) and dephosphocoenzyme A kinase (DPCK) from *E. coli*[27] were over-expressed as His-tagged proteins (see the Supporting Information).

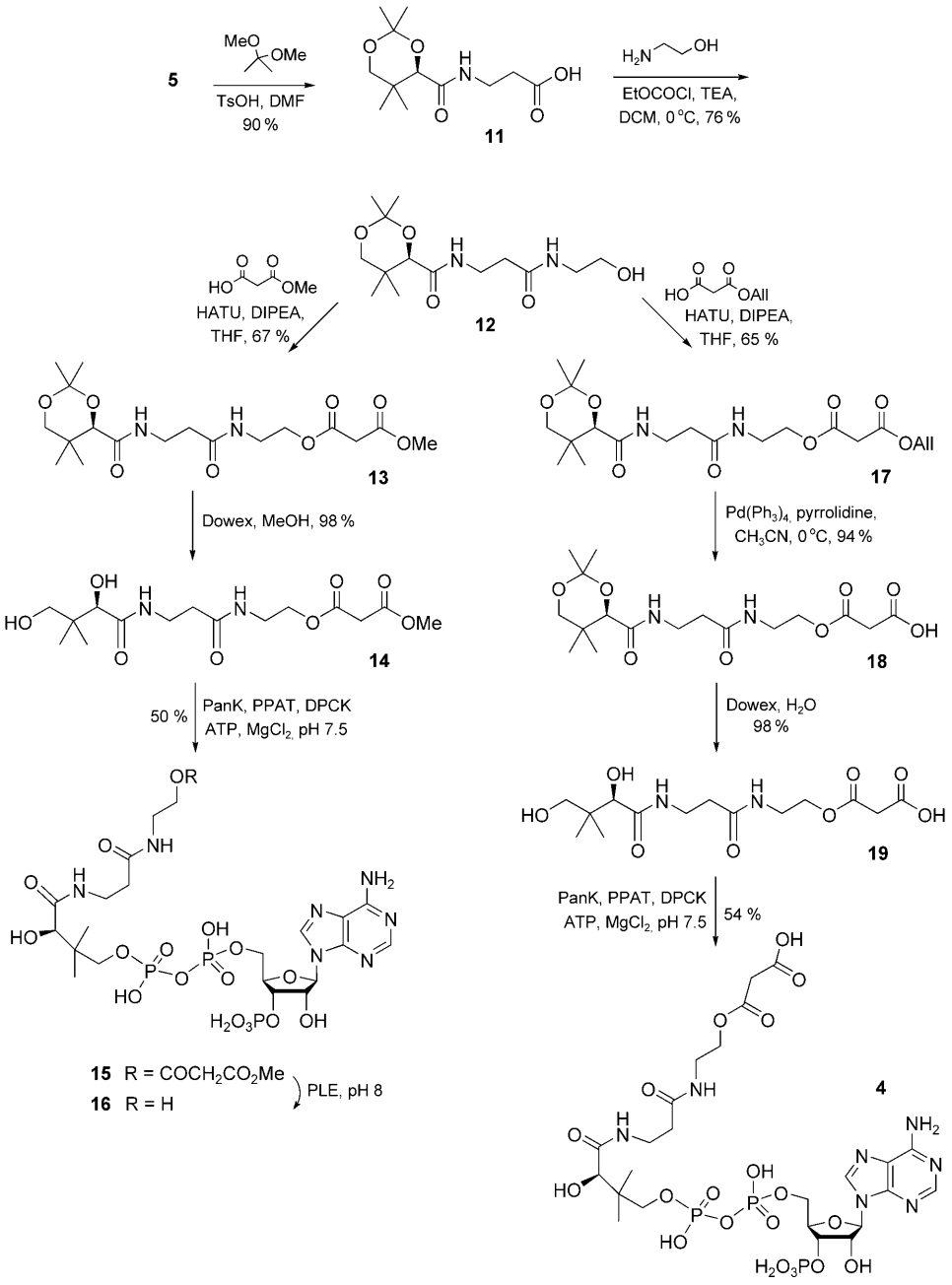
The enzymes quantitatively converted malonyl carba(dethia)pantetheine methyl ester (**9**) into the corresponding malonyl carba(dethia)-CoA analogue (**10**) in the presence of ATP at pH 7.5. After protein precipitation, **10** was purified by RP-HPLC and then subjected to selective hydrolysis with pig liver esterase (PLE)[31] to generate **3** in situ. This last step required a basic pH, which led to partial decarboxylation of **3** to acetyl carba(dethia)-CoA (see the Supporting Information). Nonetheless this enzymatic deprotection is selective and mild compared to standard chemical methods, and it can be directly carried out in the presence of the PKS, which makes the generated **3** immediately available for the reaction with the natural biosynthetic intermediates (Scheme 1 B).

Preparation of the malonyloxa(dethia)-CoA (**4**) was initially pursued by following a similar strategy with modification of the diol protection for sodium pantothenate (**5**; Scheme 3).

Isopropylidene pantothenic acid (**11**) was isolated in high yield from the treatment of **5** with 2,2-dimethoxypropane and *p*-toluenesulfonic acid in dry DMF;[32] **11** was then converted, in situ, to a mixed anhydride by treatment with ethylchloroformate, and nucleophilic attack of ethanolamine under basic conditions resulted in the formation of the alcohol **12** (76%). Compound **12** was initially coupled to malonic acid monomethylester (obtained by methanolysis of Meldrum's acid)[33] by using HATU in the presence of *N,N'*-diisopropylethylamine (67%). Isopropylidene deprotection of **13** resulted in the malonyl oxa(dethia)pantetheine methyl ester (**14**). Compound **14** was efficiently converted to the corresponding CoA derivative **15** and subjected to PLE hydrolysis. Unfortunately, this last step was not regioselective and oxa(dethia)-CoA (**16**)[34] was obtained as the major product. Therefore, it was necessary to modify our protection strategy for the malonyl moiety. Compound **12** was successfully coupled to malonic acid allyl monoester by using HATU and *N,N'*-diisopropylethylamine (65%). The allyl ester of **17** was selectively removed with the aid of $Pd(Ph_3)_4$ in acetonitrile to generate **18**. Treatment of **18** with Dowex 50-X8-400 gave the malonyl oxa(dethia)pantetheine (**19**; 98%), which was enzymatically converted to **4**.

Trapping polyketide intermediates of the STS reaction

Type III PKSs have recently received much attention due to the discovery of new biological activities of their products (as antioxidants, platelet aggregation inhibitors, anti-inflammatory and anticancer agents).[8] STS is one of the few type III PKSs the structure of which has been elucidated by X-ray crystallography.[35,36] STS produces stilbenes, in particular resveratrol (**26**), and pyrones by condensation of malonyl-CoA (**1**) with phenylpropanoid-CoA esters.[37–39] Resveratrol was originally identified as a phytoalexin, and is currently believed to be the antioxidant responsible for the so-called "French paradox".[40] During the biosynthesis of stilbenes, a C2→C7 intramolecular aldol condensation is thought to occur on a tetraketide intermediate, possibly enzyme bound. Thioester chain hydrolysis, decar-

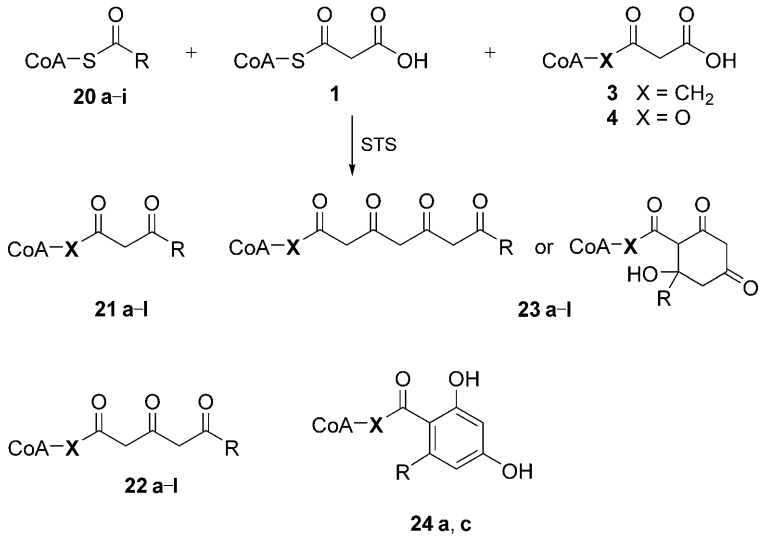


Scheme 3. Preparation of malonyloxa(dethia)-CoA (**4**).

boxylation, dehydration and cyclization are thought to occur to afford the final polyketide, but the order of these events still remains unclear.[8,35] Additionally, we recently found that besides the tetraketide resveratrol, STS produces the pentaketide 2-malonylresveratrol; this indicates that the enzyme is unable to precisely control the number of condensations.[41] Therefore, methodologies to reveal details of the polyketide chain growth within the active site of PKS enzymes are highly desirable; a more detailed understanding of the factors controlling PKS biosynthesis could, for example, furnish valuable information for optimising combinatorial biochemistry approaches. We analyzed the reaction of STS from *Pinus silvestris*[42] with different starter units and malonyl-CoA in the presence of the nonhydrolyzable analogues **3** or **4** (Scheme 4) in an attempt to trap polyketide intermediates following the strategy of Scheme 1 B. Multiple assays were carried out at various temperatures, incubation times and enzyme and reactant concentrations in HEPES buffer (50 mM, pH 7.5–8.5). Assays carried out in the absence of either STS or one of the starter units were used as controls. Malonylcarba(dethia)-CoA (**3**) was either added to standard STS assays after PLE hydrolysis of **10** or it was generated in situ by PLE hydrolysis of **10** in the presence of STS and the substrates. After enzyme precipitation or filtration through a cut-off membrane, the reaction mixtures were analyzed by LC-ESI-MS/MS.

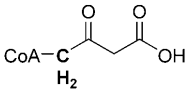
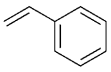
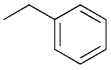
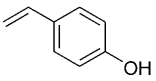
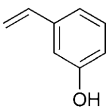
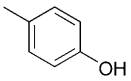
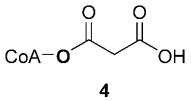
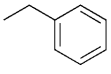
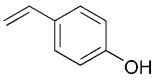
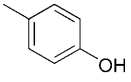
A summary of the outcome of the experiments with malonyl-CoA analogues **3** and **4** as trapping agents is presented in Table 1.

In contrast to the experiments previously carried out with the thioether analogue **2**,[22] trapped polyketide species were found in the reaction mixtures of STS with phenylpropanoid starter units (Table 1, entries a–c and j–k); this indicates the improved properties of **3** and **4**. From the reaction of STS with cinnamoyl-CoA (**20 a**) in the presence of **3**, a diketide (**21 a**), a triketide (**22 a**), a tetraketide (**23 a**) and a dehydrated, possibly cyclized, tetraketide species (**24 a**) were identified (Table 1, entry a, and Figure 1 A–D). These species were absent in control samples and their MS/MS fragmentation pattern was consistent with that of coenzyme A derivatives.[43] While the assignment of *m/z* 922 (Figure 1 A) and 964 (Figure 1 B) to the diketide **21 a** and the triketide **22 a**, respectively, was clear-cut, two distinct peaks were found for *m/z* 1006 at 15.9 and 19.9 min (Figure 1 C and E). Their different MS/MS fragmentation patterns allowed their assignment to the nonhydrolyzable tetraketide **23 a** (Figure 1 C) and to the dehydrated, possibly cyclized, natural tetraketide **25**, respectively (Figure 1 E), with the latter showing the *m/z* 768 fragment diagnostic for a coenzyme A thioester.[41] In addition, a significant peak with *m/z* 988 was found, which corre-



Scheme 4. Trapping intermediates of the STS by using **3** or **4**.

Table 1. Trapped intermediates for type III PKS stilbene synthase.

	Trapping agent	Substrate	R	Trapped intermediates (m/z)[a]	X
a	 **3**	**20 a**[b]		**21 a** (922) **22 a** (964) **23 a** (1006) **24 a** (988)	CH_2
b	**3**	**20 b**		**21 b** (924) **22 b** (966) **23 b** (1008)	CH_2
c	**3**	**20 c**[b]		**21 c** (938)[a,c] **22 c** (980)[c] **23 c** (1022)[c] **24 c** (1004)[c]	CH_2
d	**3**	**20 d**[b]		n.d.	
e	**3**	**20 e**		**21 e** (926) **22 e** (968)	CH_2
f	**3**	**20 f**		n.d.	
g	**3**	**20 g**		**21 g** (914)	CH_2
h	**3**	**20 h**		n.d.	
i	**3**	**20 i**		n.d.	
j	 **4**	**20 b**		**21 j** (926)	O
k	**4**	**20 c**[b]		**21 k** (940)	O
l	**4**	**20 e**		**21 l** (928)	O

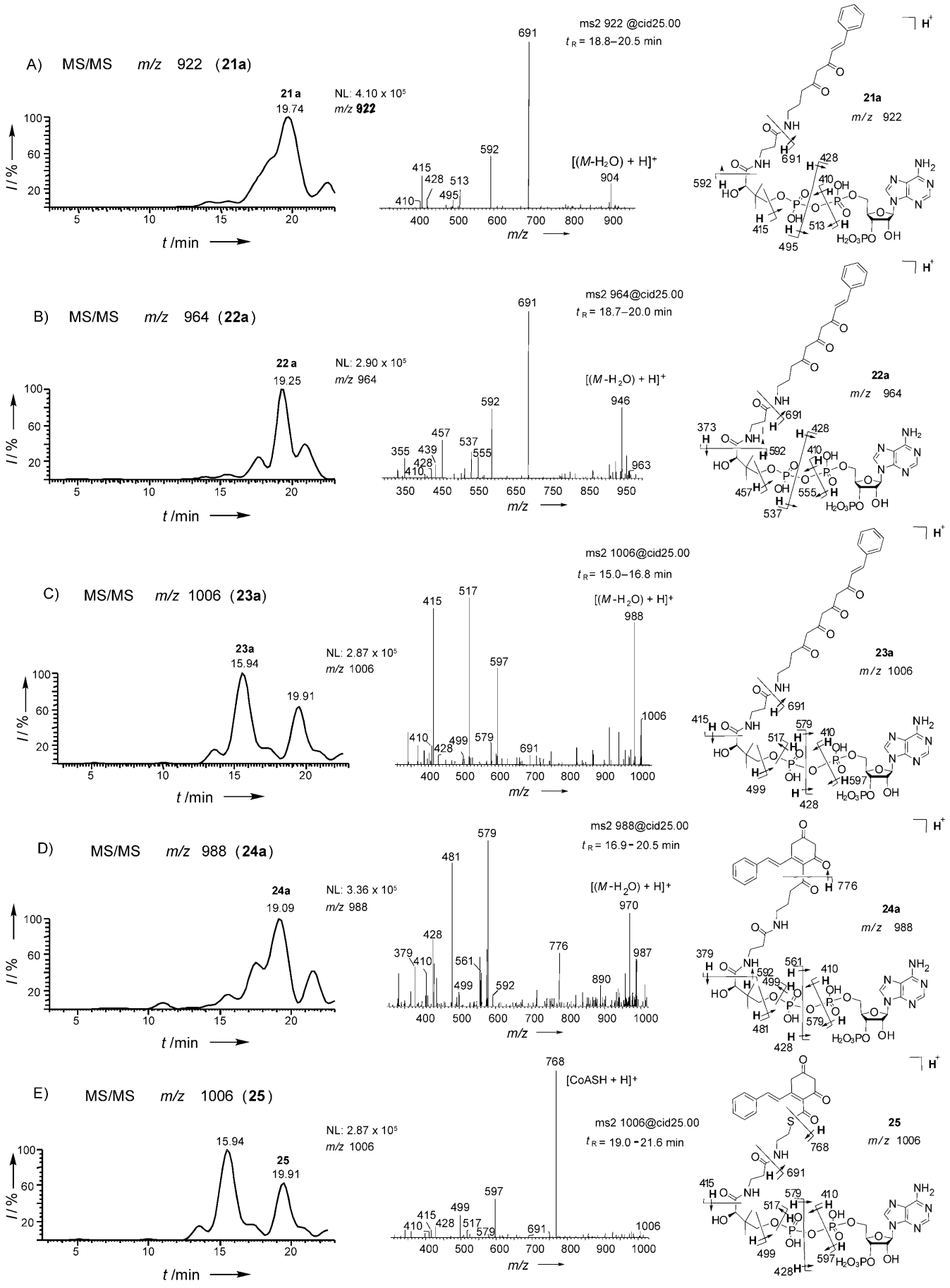
[a] $[M+H]^+$ detected by LCQ (unless stated otherwise); [b] *trans* isomer; [c] $[M+H]^+$ detected by LTQ Orbitrap.

sponds to a dehydrated, likely cyclized, trapped tetraketide **24 a** (Figure 1 D). Its m/z 776 fragment is diagnostic for **23 a**, which originates from the C2→C7 intramolecular aldol condensation and subsequent dehydration.

From the reaction of STS with phenylpropionyl-CoA (**20 b**) in the presence of **3**, three trapped biosynthetic intermediates, **21**–**23 b**, were identified (Table 1, entry b). The amount of the tetraketide **23 b** was considerably lower compared to the other species (see the Supporting Information), but its MS/MS pattern was consistent with that of **23 a**. To provide extra evidence for the trapping of tetraketides, some samples of the STS reaction with coumaroyl-CoA (**20 c**) in the presence of **3** were analyzed by micro-LC-ESI-HR-MS.[44] After incubation for 4 h at 37 °C, the tetraketide **23 c** and its dehydration product **24 c** were identified (Table 1, entry c, Figure 2 and the Supporting Information).

The potential of **3** to trap intermediates of STS was further tested by incubating the enzyme with unnatural substrates. Interestingly, 3-hydroxycinnamoyl-CoA (**20 d**) as starter unit did not yield any intermediates (Table 1, entry d). Incubation with 4-hydroxyphenylacetyl-CoA (**20 e**) yielded the diketide **21 e** and the triketide **22 e** as expected for two rounds of condensation (Table 1, entry e). However, when smaller and shorter starter units, such as phenylacetyl-CoA, 4-fluorobenzoyl-CoA, benzoyl-CoA and cyclohexanoyl-CoA were employed, either only low levels (entry g) or no intermediates (entries f, h–i) were detected. Nonetheless, in all the experiments carried out in the presence of **3** we observed partial inhibition of polyketide production. This is consistent with the mechanism of action proposed for **3** (Scheme 1 B).

When malonyl oxa(dethia)-CoA (**4**) was employed as a trapping agent for STS biosynthetic intermediates, only the oxa(dethia)monoketide species **21 j**–**l** were found (Table 1, entries j–l, Figure 3 and the Supporting Information). The higher efficiency of malonylcarba-



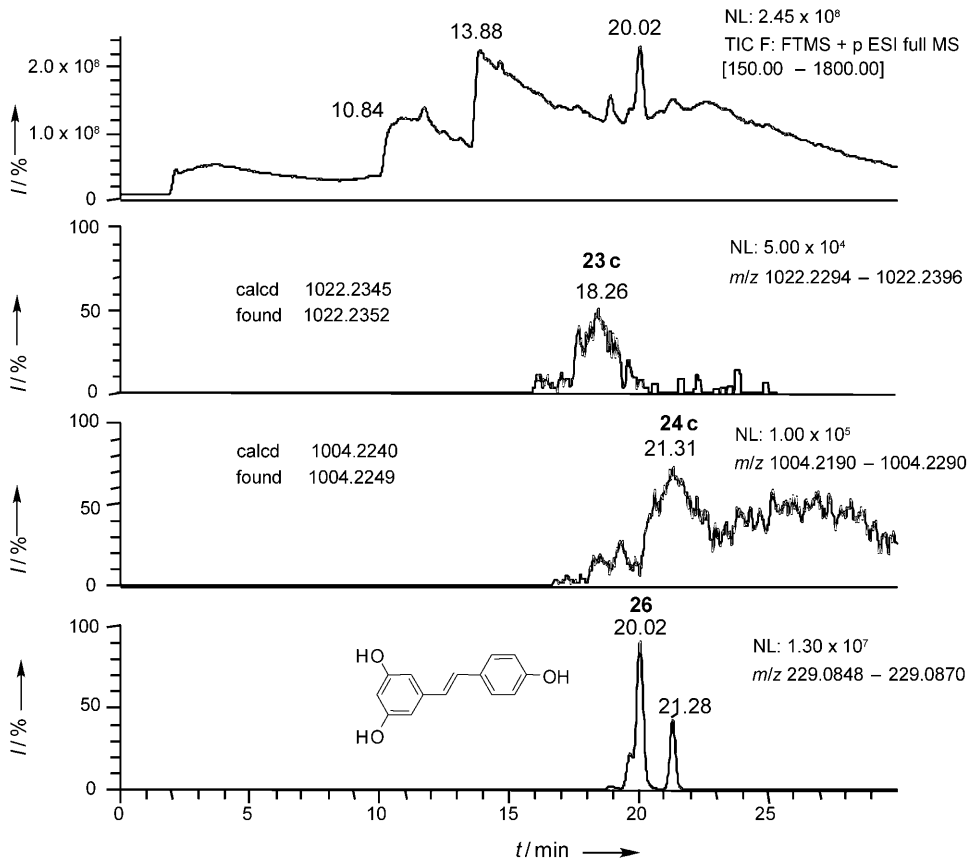


Figure 2. The $[M+H]^+$ ion traces of trapped tetraketides **23 c** and **24 c** from the reaction of STS, malonyl-CoA (**1**), coumaroyl-CoA (**20 c**) and **3**, which was added to the standard assay after 20 min incubation at 37 °C in a 3:4 ratio relative to **1**. After 4 h incubation at 37 °C the mixture was analysed by using a PepMap 100 microcolumn (15 mm × 1 mm, 3 μm). The presence of two resveratrol peaks (**26**) arises from acid- and/or light-catalyzed *cis–trans* isomerization.[41]

In the past, derailed triketide and tetraketide compounds have been isolated from STS-catalyzed reactions as heterocyclic lactones[8,45] and, together with feeding studies,[46] have supported a biosynthetic pathway leading to stilbenes through a tetraketide intermediate.

Herein, we have shown that with **3** and **4**, intermediate CoA-linked polyketides can be off-loaded from a PKS; this provides direct evidence for the formation of these intermediates and of their ability to diffuse in and out of the enzyme active site.[8] In particular, among all the malonyl-CoA analogues prepared so far, the carba(dethia) compound **3** is the most efficient trapping agent because of the polyketide nature of the isolated species and its closer structural resemblance to malonyl-CoA (**1**). Besides the close steric match and the nonhydrolyzability, the behaviour of **3** possibly reflects the importance of decarboxylation of the extender unit for polyketide/fatty acid synthase as a requirement for efficient condensation and chain extension.[47]

(dethia)-CoA (**3**) compared to malonyloxa(dethia)-CoA (**4**) may be explained by its nonhydrolyzability (of the trapped species), and its relative ease of decarboxylation. Indeed **3** decarboxylates at a faster rate than malonyl-CoA (**1**) itself, as expected for a β-keto acid. Compound **4** did not decarboxylate spontaneously to any significant extent, but the detection of one condensation species **21 j–l** in the presence of STS indicates that the enzyme can catalyze this process, although to a low extent and not at the competitive rate required to trap longer chain intermediates.

Because **4** is more resistant to hydrolysis and less reactive than malonyl-CoA (**1**), we speculated whether it would be accepted by STS as an alternative extender unit and still yield a polyketide product or some of its direct precursors. However, even after prolonged incubation of **4** in the absence of **1** with coumaroyl-CoA (**20 c**) and STS neither resveratrol (**26**) nor any intermediates other than the oxa(dethia)monoketide (**21 k**) were found.

Taken together, the results from this study and our earlier work with the thioether **2**[22] offer some insights into the flexibility of substrate alignment in the PKS active site. The simple model in Figure 4 A shows the STS natural starter unit coumaroyl (enzyme bound) and the extender unit malonyl-CoA (and analogues **3** and **4**) positioned in their respective binding pockets[8,35] and optimally arranged for decarboxylation and chain extension at the pockets' interface. Because of the extra methylene unit, it was envisaged that the thioether **2** would be misaligned for reaction with coumaroyl-CoA (**20 c**; Figure 4 B) and indeed no trapped intermediates were found.[22] With 4-hydroxyphenylacetyl-CoA (**20 e**) as starter unit, malonyl-CoA (**1**) and **2** (Figure 4 C, left) however, trapped intermediates were observed.[22] This suggests that substrate realignment probably occurs by "stretching" of the starter unit within its binding pocket (Figure 4 C, right).

Figure 1. (Previous page) LC-ESI-MS analysis of the STS reaction with *trans*-cinnamoyl-CoA (**20 a**; 1 mM), malonyl-CoA (**1**; 3 mM), **10** (3 mM), STS and PLE. The reaction mixture was analyzed after 6 h incubation at room temperature. The panels show ESI-MS $[M+H]^+$ ion traces and the MS/MS fragmentation of trapped: A) diketide **21 a**, B) triketide **22 a**, C) tetraketide **23 a**, D) the dehydrated (possibly cyclized) trapped tetraketide **24 a**, and E) the natural dehydrated (possibly cyclized) tetraketide **25**. For simplicity all the species are represented in their polyketone form only (the degree of enolization/tautomerism is unknown).

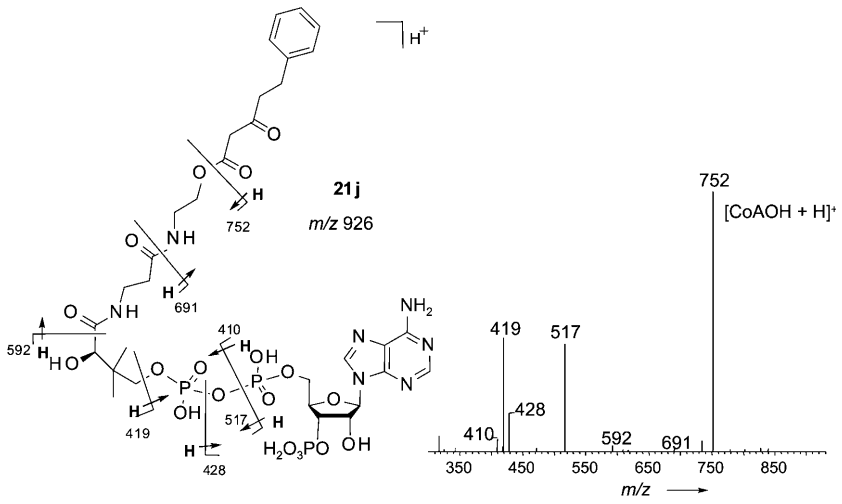


Figure 3. ESI-MS/MS of the $[M+H]^+$ of the trapped oxa(dethia)monoketide **21j** from the reaction of STS and phenylpropionyl-CoA (**20b**) in the presence of **4**.

previously postulated.[35] However, only recently the direct involvement of CoA-bound species has been considered[41] and further work in this direction is in progress. Finally, the isolation of nonhydrolyzable species offers clear advantages for intermediate characterisation; whereas for thioester species, such as **25** (Figure 1E; very rarely detectable), the major mass fragment *m/z* 768 is merely diagnostic of a CoA derivative, the *m/z* 776 fragment of its counterpart **24a** strongly supports its C2→C7 cyclized structure.

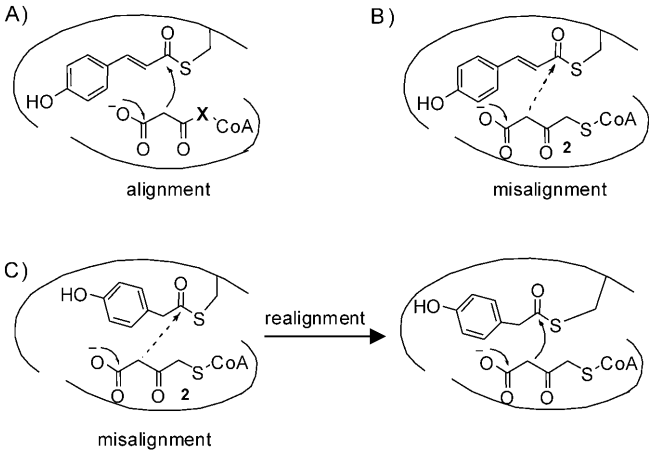


Figure 4. A) Malonyl-CoA (**1**; X = S) and the analogues **3** and **4** (X = CH_2 and O) are optimally aligned with the enzyme-bound coumaroyl group for malonyl decarboxylation and condensation. B) The malonyl analogue **2** is misaligned for condensation with the coumaroyl moiety due to its extra methylene group. C) Compound **2** is initially misaligned with the enzyme-bound 4-hydroxyphenylacetyl group (left), however, "stretching" of the latter within the active site allows substrate realignment and decarboxylative condensation.

Further, the yield of accumulated diketides, triketides and tetraketides seems to depend on the starter unit (Table 1). This could be related to different stereoelectronic effects within the active site, to the local concentration of trapping agent in solution at a particular time and also to possible differences in the kinetics of two/three condensation steps.[8]

Malonyl-CoA analogues effectively act as "reactivity probes" that are able to reveal details of the modality and the timing of polyketide formation. For instance, the presence of the possibly cyclized trapped intermediates **24a** (Figure 1D) and **24c** (Figure 2) in the biosynthesis of stilbenes, besides the natural intermediate **25**, would indicate that cyclization can take place for a CoA-bound tetraketide prior to thioester hydrolysis and decarboxylation. Cyclization followed by decarboxylation to give a readily hydrolyzed tetraketide intermediate has been

Conclusions

In summary, we have chemoenzymatically prepared two novel malonyl-CoA analogues (**3** and **4**) and demonstrated their utility in trapping intermediates of type III polyketide biosynthesis. In particular, the carba(dethia)-CoA analogue **3** has proved to be highly efficient for the direct off-loading of polyketide precursors thanks to its nonhydrolyzability, its close steric match to malonyl-CoA (**1**) and competitive decarboxylation potential.

By using **3**, diketide-, triketide- and tetraketide intermediates were off-loaded from the stilbene synthase, and the likely tetraketide cyclization offers new perspectives on the mechanism and the timing of stilbene biosynthesis. Moreover, comparison of the results from the trapping experiments with the different malonyl-CoA analogues (**2**, **3**, **4**) provides insights into the flexibility/limitation of substrate binding/alignment within the enzyme active site of the STS.

In combination with LC-MS/MS our methodology to trap PKS intermediates using malonyl-CoA analogues allows the straightforward characterisation of biosynthetic intermediates and avoids enzyme reengineering, enzyme feeding with substrate surrogates and laborious work-up procedures. The methodology for polyketide-chain termination also prevents the intramolecular formation of pyrone products,[48] and it can be further extended to other PKS. Analogues of pantetheines have recently been used to tag carrier proteins to trap transient protein–protein interactions[49] or to manipulate the carrier geometry.[50] We anticipate that upon loading nonhydrolyzable malonyl carba(dethia)pantetheine (from **2**) onto the acyl carrier protein (ACP) using phosphopantetheinyl transferase Sfp,[51] our approach will prove to be a powerful tool for studying such complex PKS systems.

Experimental Section

Syntheses and characterisation of compounds **6**–**9**, **11**–**12** and **17**–**19** and related intermediates are described in the Supporting Information.

Malonyl carba(dethia)-CoA methylester (10): Purified malonyl carba(dethia)pantetheine methylester (**9**; 4.8 mg, 0.013 mmol) was dissolved in Tris-HCl (50 mM), KCl (20 mM), $MgCl_2$ (10 mM), pH 7.5[27] (1.5 mL). ATP disodium salt (24 mg, 0.046 mmol) was added and the pH was adjusted to 7 by addition of NaOH (4 M). Pantothenate kinase, phosphopantetheine adenyltransferase, and dephosphocoenzyme A kinase (450 μg each) were added to a total volume of 3 mL. After overnight incubation and addition of extra DPCK (50 μg), LC-MS analysis of the mixture showed 100% conversion to the CoA derivative **10**. The enzymes were precipitated by addition of $CHCl_3$ (3 mL); the water layer, which contained the product, was isolated and washed twice with $CHCl_3$ before being freeze dried. This crude material was purified by semipreparative HPLC (t_R 10.5 min gradient elution with water and acetonitrile containing 0.05% TFA) and **10** was obtained as a white powder (6 mg, 55%); ^{1}H NMR (400 MHz, D_2O): $\delta = 8.59$, 8.36 (s, 1H; ArC*H*), 6.14 (d, $^3J = 5.5$ Hz, 1H; C*H*), 4.80 (m, 2H; C*H*, overlapping with HOD), 4.51 (brt, 1H; C*H*), 4.17 (brt, 2H; CH_2), 3.94 (s, 1H; C*H*), 3.77 (dd, $^3J = 4.5$ Hz, $^2J = 10.0$ Hz, 1H; C*H*), 3.63 (s, 3H; OCH_3), 3.51 (dd, 1H; CH_2), 3.38 (t, $^3J = 6.5$ Hz, 2H; CH_2), 3.05 (t, $^3J = 7.0$ Hz, 2H; CH_2), 2.56 (t, $^3J = 7.0$ Hz, 2H; CH_2), 2.36 (t, $^3J = 6.5$ Hz, 2H; CH_2), 1.63 (quint, $^3J = 7.0$ Hz, 2H; CH_2), 0.85, 0.73 (s, 3H; CH_3); ^{13}C NMR (100 MHz, D_2O): $\delta = 207.5$ ($CH_2C{=}OCH_2$), 174.7, 173.8, 170.1 (*C*=O), 149.9 (Ar*C*), 148.5 (Ar*C*), 144.6 (ArCH), 142.5 (ArCH), 118.6 (Ar*C*), 87.4, 83.5, 74.1, 74.0, 73.9 (*C*H), 71.9, 71.8, 65.0 (CH_2), 52.7 (CH_3, OMe), 39.9, 38.4 (CH_2), 38.3 (*C*$(CH_3)_2$), 35.4, 35.3, 22.3 (CH_2), 20.8, 18.2 (C*C*H_3); ^{31}P NMR (162 MHz, D_2O): $\delta = 0.35$, −10.22, −10.68; HR-ESI-MS: $[M+H]^+$ found: 850.1817, calcd: 850.1821.

Malonyl carba(dethia)-CoA (3)

Method A: Pig liver esterase (Sigma, suspension at pH 8) was added (73 units per μmol) to a solution of methylester **10** in HEPES (50 mM), NaCl (25 mM), pH 8.0. The mixture was incubated at room temperature for 16 h, after which LC-MS analysis showed complete consumption of **10**, and **3** was found as the major product (ESI-HR-MS: $[M+H]^+$ found: 836.1658, calcd: 836.1743). Acetyl carba-(dethia)-CoA[52] (ESI-HR-MS: $[M+H]^+$ found: 792.1723, calcd: 792.1766) was also present as a byproduct of decarboxylation of **3** (around 40%, as judged by LC-MS; see the Supporting information). PLE was precipitated by addition of chloroform, and the aqueous layer was used as a source of **3** for the stilbene synthase assays.

Method B: The methylester **10** was directly used in the stilbene synthase assays together with an appropriate amount of PLE.

Malonyl oxa(dethia)-CoA (4): Malonyl oxa(dethia)pantetheine **19** (2.7 mg, 0.008 mmol) was dissolved in Tris-HCl buffer (50 mM, pH 7.5; 0.9 mL). ATP disodium salt (14 mg, 0.025 mmol), PanK, PPAT and DPCK (260 μg each) were then added (final volume adjusted to 2 mL). After overnight incubation and quantitative conversion to **4**, the enzymes were precipitated by addition of $CHCl_3$ (2 mL). The aqueous layer was separated, washed twice with $CHCl_3$ and freeze dried. The crude material was purified by HPLC ($t_R = 9.3$ min gradient elution with water and acetonitrile containing 0.05% TFA) to yield **4** as a white powder (3.5 mg, 54%); ^{1}H NMR: (500 MHz, D_2O): $\delta = 8.72$ (s, 1H; ArC*H*), 8.47 (s, 1H; ArC*H*), 6.26 (d, $^3J = 6.0$ Hz, 1H; C*H*), 4.93–4.88 (m, 2H; C*H*), 4.63 (brs, 1H; C*H*), 4.29 (brs, 2H; CH_2), 4.25 (t, $^3J = 5.0$ Hz, 2H; CH_2), 4.06 (s, 1H; C*H*), 3.89 (brdd, 1H; C*H*), 3.62 (brdd, 1H; C*H*), 3.50 (t, $^3J = 6.3$ Hz, 2H; CH_2), 3.48 (t, $^3J = 4.7$ Hz, 2H; CH_2), 2.50 (t, $^3J = 6.5$ Hz, 2H; CH_2), 0.96, 0.84 (s, 3H; CH_3); ^{13}C NMR (125.7 MHz, D_2O): $\delta = 174.7$, 174.1, 170.8, 169.0 (*C*=O), 150.0, 148.5 (Ar*C*), 144.7, 142.5 (ArCH), 118.6 (Ar*C*), 87.4, 83.6, 74.1, 74.0, 73.9 (*C*H), 71.8, 65.1, 64.1 (CH_2), 38.3 (*C*$(CH_3)_2$), 38.2, 38.0, 35.3, 35.2 (CH_2), 20.8, 18.1 (C*C*H_3); ^{31}P NMR (162 MHz, D_2O): $\delta = 3.05$, −7.46, −8.07; HR-ESI-MS: $[M+H]^+$ found: 838.1460, calcd: 838.1457.

Cloning, expression and purification of PanK, PPAT, DPCK: PanK, PPAT and DPCK were amplified from *E. coli* K12 genomic DNA as previously reported[27] by using modified primers (see the Supporting Information) and cloned into pET29b and pET20b(+) (Novagen). The enzymes were over-expressed in *E. coli* Rosetta BL21(DE3) and Rosetta 2, respectively, and purified according to published procedures.[27]

STS assays and detection of polyketide intermediates: Substrates **20a–i** were prepared according to published procedures.[53] The STS was expressed and purified as previously reported.[22,41] The assays were typically carried out either at room temperature or at 37 °C in HEPES buffer (50 mM, pH 8, 25 mM NaCl) with starter unit CoA (**20a–i**; 1.0 mM), malonyl-CoA (**1**; 3 mM), malonyl-CoA analogues (**3** or **4**; 1–10 mM) and STS (20–200 μg) in a final volume of 100 μL. Control assays lacking either the enzyme or one of the substrates were set up in parallel. After precipitation of the enzyme with HCl (10 μL of 20% solution) or filtration through a 10 kDa cut-off membrane (Microcon YM-10, Millipore), the mixtures were analysed at different reaction times by LC-MS. LC-ESI-MS/MS and HR-ESI-MS spectra of trapped polyketide intermediates are available in the Supporting Information. The best results for intermediate trapping were obtained with a 3:4 ratio of **3** (or **4**):**1**.[22]

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