Enzyme Reaction Mechanisms

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A Method for Trapping Intermediates of Polyketide Biosynthesis with a Nonhydrolyzable Malonyl-Coenzyme A Analogue**

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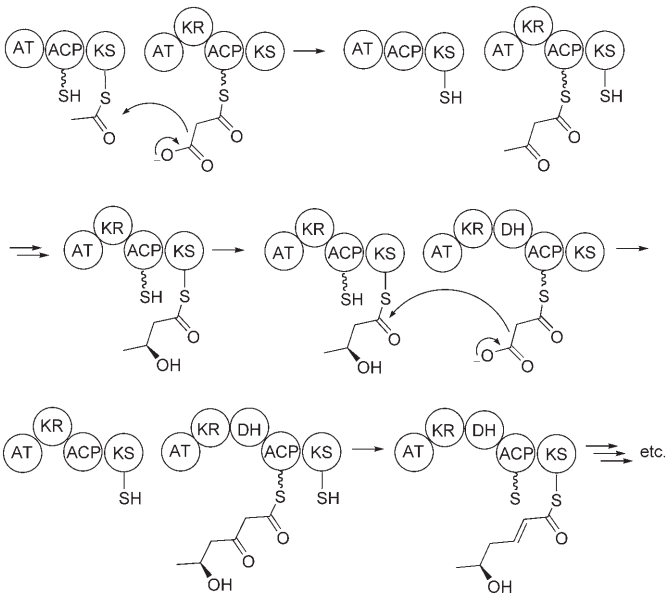
There are currently about 10000 natural products that have been classified as polyketides. Their structures range from simple molecules, such as 6-methylsalicylic acid,[1] to complex natural products, such as brevetoxin.[2] Polyketides are an invaluable source of bioactive compounds with many being clinically important, for example, the antibiotic erythromycin and the immunosupressant rapamycin.[3] Despite their diversity, all polyketides share a common biosynthetic pathway; a starter unit, which is normally acetyl coenzyme A (CoA), undergoes sequential condensation with an extender unit (usually malonyl or (*S*)-methylmalonyl-CoA) in a similar manner to fatty acid biosynthesis. However, unlike fatty acid biosynthesis, the carbonyl group is not always completely reduced after each condensation, which partly accounts for the huge variety of polyketide structures (Scheme 1).[3]

Experiments with labeled precursors, in combination with mechanistic considerations, have been extensively used to investigate the details of polyketide biosynthesis.[1,3] However, it has proven very difficult to isolate intermediates directly as they are covalently attached to the polyketide synthases (PKSs).[3] One approach that has been possible with the cloning and sequencing of genes for many PKSs[4] is to design mutants that halt the biosynthesis at a desired point. For instance, by genetically engineering the fusion of the thioesterase from the third subunit of the deoxyerythronolide synthase onto the first subunit, deoxyerythronolide biosynthesis was stopped after two rounds of (*S*)-methylmalonyl-CoA condensations and the intermediate removed in sufficient quantity to be fully characterized.[5] Whereas this strategy has potential with all type I modular PKSs, it is very limited when applied to type I iterative, type II, and type III PKSs, as the same catalytic activities are usually used repeatedly. For example, if a mutation is made in the ketosynthase, polyketide synthesis could not occur at all. To address this point, we sought to develop a chemical approach that used a non-hydrolyzable malonyl-CoA analogue which can potentially trap polyketide intermediates from these classes of PKSs.



Scheme 1. Schematic diagram of the mechanism of two rounds of polyketide biosynthesis by a polyketide synthase (AT: acyl transferase, KS: ketosynthase, ACP: acyl carrier protein, DH: dehydratase, KR: ketoreductase). In type III polyketide synthases, such as the stilbene synthase from *Pinus sylvestris*, malonyl-CoA is used directly for the elongation instead of the corresponding ACPs.

Analogues of coenzyme A[6–8] have been used previously to investigate a range of enzyme reaction mechanisms, such as the condensation of acetyl-CoA with oxaloacetate catalyzed by citrate synthase[7] and the enzymic carboxylation of acetyl-CoA by acetyl-CoA carboxylase.[9] However, malonyl-CoA analogues have never been used before to investigate the biosynthesis of polyketides. In our strategy, the malonyl-CoA analogue would react with the growing polyketide chain but would not be able to undergo transesterification back onto the PKS resulting in the accumulation of polyketide intermediates attached to the analogue (Scheme 2a).

The analogue of malonyl-CoA **3** used in this study contains an additional methylene unit between the thiol of the coenzyme A and the malonate moiety. This methylene unit could possibly be detrimental to the correct positioning of the analogue at the active site of a PKS, as the malonyl moiety is displaced by one carbon atom. However, we felt that this potential disadvantage was out-weighed by the straightforward preparation of **3** in two steps. Commercially available ethyl 4-chloro-3-oxobutanoate (**1**) was treated with coenzyme A in aqueous 0.11M Li_2CO_3.[7,8] After purification with

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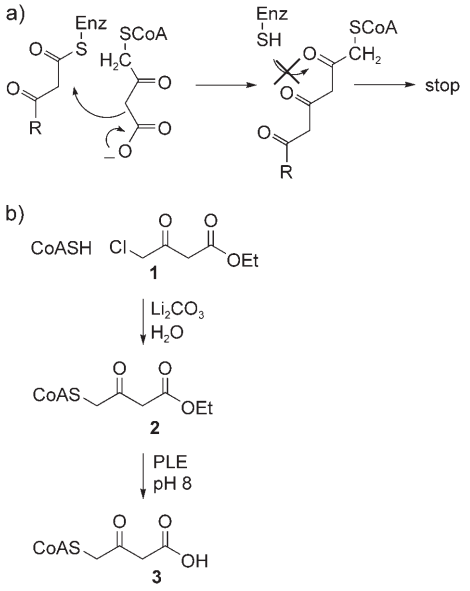
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.



Scheme 2. a) Trapping intermediates of polyketide biosynthesis with a malonyl-CoA analogue **3**; b) synthesis of **3**. Enz = enzyme.

RP-18 HPLC, the ethyl ester **2** was hydrolyzed to **3** with pig liver esterase (PLE; Scheme 2b).[10] As anticipated, **3** was found to undergo decarboxylation more readily than malonyl-CoA (**4**) during purification, therefore it was used directly after precipitation of the PLE.

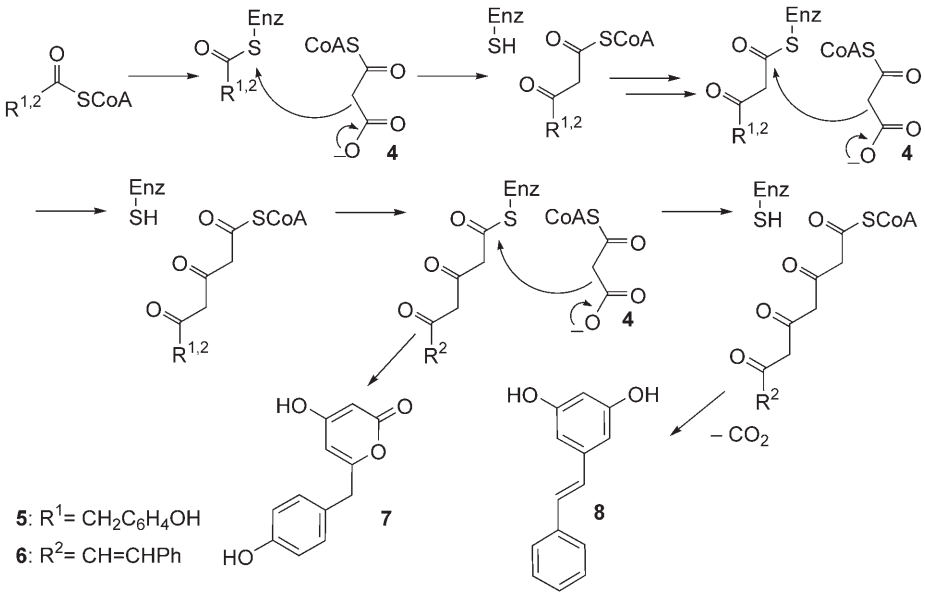
The potential of **3** to trap intermediates of polyketide biosynthesis was investigated with the type III PKS stilbene synthase (STS) from *Pinus sylvestris*[11,12] as a model system. This class of PKS is the simplest to investigate this approach with as it uses **4** directly, in contrast to type I and II PKSs that carry out condensations with malonyl acid carrier proteins (ACPs).[3] STS has a broad substrate specificity with regard to the starter unit, thus producing a number of stilbene and pyrone products.[13] If cinnamoyl-CoA (**6**) is used as the starter unit, the enzyme catalyzes its sequential condensation with three molecules of **4** to form the stilbene pinosylvin (**8**),[12] and with 4-hydroxyphenylacetyl-CoA (**5**), only two condensations with **4** occur to give the pyrone **7** (see Scheme 3).

For our initial experiments, **5** was used as a starter unit and the ratio of **3** to **4** was varied. Formation of the products was followed by LC-MS, and across a range of conditions (a ratio of 3:4 of **3**/**4** was found to give the highest yield) we observed ions at *m*/*z* 958 and 1000 that correspond to one and two condensation steps, in addition to the ion at *m*/*z* 219 for the normal pyrone product **7** (Figure 1).

Interestingly, we also observed the ions at *m*/*z* 958 and 1000 at very low levels in the control samples without the addition of the enzyme. Although not totally unexpected, to our knowledge, the non-enzymic formation of polyketides from malonyl-CoA has not been previously reported.

Several closely eluting peaks, all exhibiting *m*/*z* 1000, were observed in some LC-MS traces of **10** instead of a single one, thus suggesting the presence of isomers of **10** probably formed by enolization and/or cyclization.

The MS/MS spectrum of both intermediates that exhibit ($[M+H]^+$) peaks at *m*/*z* 958 and 1000 showed characteristic fragments for the presence of coenzyme A derivatives (see Figure 2 and the Supporting Information). In the case of the triketide intermediate **10**, the fragments at *m*/*z* 591 and 428 are characteristic for the cleavage of the phosphodiester bond of the coenzyme A unit of the molecule (Figure 2). Furthermore, a fragment at *m*/*z* 493 was also observed that corresponds to loss of the 3′-phospho adenosine diphosphate moiety from the alkyl chain. The observed fragmentation pattern for the intermediates of *m*/*z* 958 and 1000 is in agreement with a study of the MS/MS fragmentation of other coenzyme A derivatives by Burns et al.[14]



Scheme 3. Formation of the pyrone-type and stilbene-type products **7** and **8**, respectively, by stilbene synthase from *Pinus sylvestris* using either **5** or **6** as the starter unit and **4** for chain elongation.

To provide further evidence for the isolation of these intermediates, assays were carried out with [$^{13}C_3$]malonyl-CoA.[15] The incorporation of the labeled substrate should shift the mass of the $[M+H]^+$ ion to 1002 amu in the case of **10**, as one molecule of [$^{13}C_3$]malonyl-CoA is utilized in its formation. Figure 2 clearly shows that the MS/MS spectra complement and confirm the MS/MS spectra of the unlabeled assay. All signals at *m*/*z* 1002, 984, 593, 575, 495, and 477, which contain the polyketide chain in their fragments, are shifted by 2 amu, whereas the signals at *m*/*z* 428 and 410, which correspond to the 3′-phospho adenosine diphosphate moiety, remained unchanged.

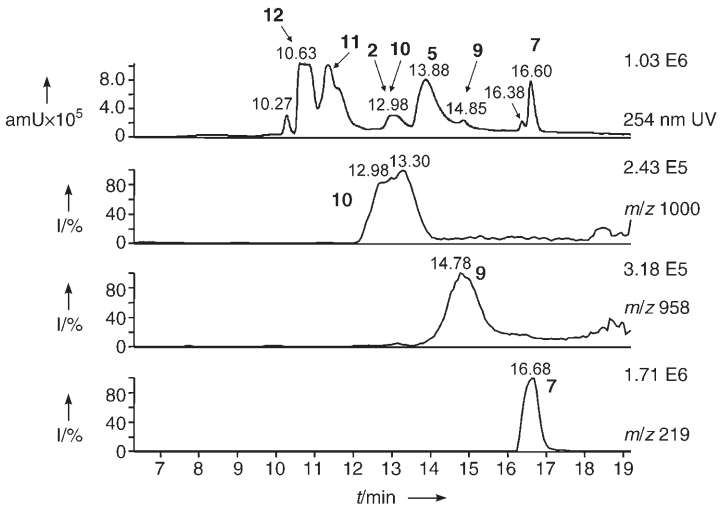


Figure 1. UV chromatogram and ion traces *m/z* 219, 958, and 1000 observed after the reaction of the stilbene synthase with **5** and **4** in presence of **3.** Peaks: Coenzyme A (**12**); decarboxylated malonyl-CoA analogue **11**; ethylmalonyl-CoA analogue **2**; STS intermediate formed from two condensations condensations **10**; 4-hydroxyphenylacetyl-CoA **5**; STS intermediate formed from one condensation **9**; 4-hydroxyphenylacetylpyrone (**7**).

These results show that the malonyl-CoA analogue **3** can be successfully used to trap the growing polyketide chain. It is also interesting that the tetraketide is not observed. This result indicates that the triketide formed from the starter unit and two malonyl-CoA molecules either rapidly cyclizes to the observed pyrone **7** or is not readily transferred back onto the STS after the final condensation.

To see if longer intermediates could be trapped, we used **6** as starter unit because the favored product in this case is the stilbene **8** formed from the tetraketide intermediate. Therefore, the addition of **3** to the assay could trap three different intermediates during the stilbene formation. However, no intermediates were detected above the background levels under a range of conditions. It is possible that the positioning of the analogue in the active site of the STS is better matched with **5** as a starter unit because it has one less carbon atom than **6** and **3** has one more carbon atom than **4**. This observation indicates that the positioning of the growing polyketide chain bound to the active-site cysteine unit is strongly influenced by the structure of the starter unit. A smaller starter unit may allow the necessary flexibility in the active site for the analogue to be able to be correctly positioned and react.

In summary, we have shown for the first time that intermediates of polyketide biosynthesis can be trapped for detailed analytical studies with a non-hydrolyzable malonyl-CoA analogue. This finding potentially opens the way to the determination of the timing of crucial events, such as cyclization of the polyketone chain. It is highly likely that the analogue can be transferred to ACPs with Sfp, a phosphopantetheinyl transferase from *Bacillus subtilis*, as this enzyme has been shown to have a wide substrate specificity.[16]

This approach should allow type I and II PKSs to be investigated. Experiments to apply this concept to other polyketide synthases and the synthesis of alternative analogues that may be better steric matches for malonyl-CoA are currently under investigation.

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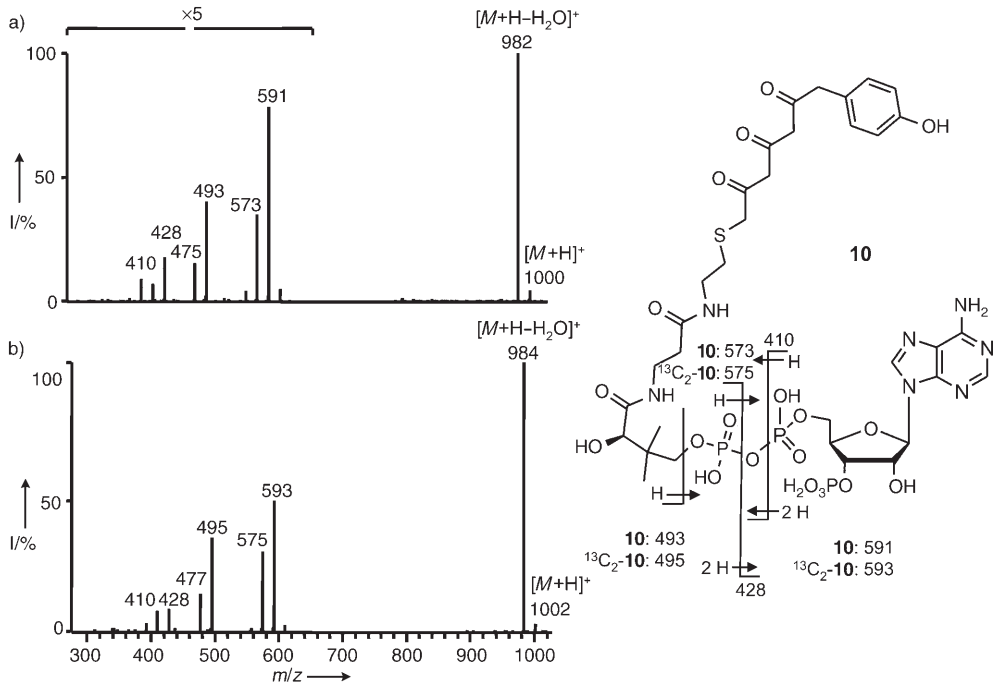

Figure 2. ESI-MS/MS of **10** formed by the stilbene synthase using **5**, **3**, and either a) **4** or b) [$^{13}C_3$]malonyl-CoA.

Keywords: biosynthesis · coenzymes · mass spectrometry · polyketides · synthases

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