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Identification of a Pentaketide Stilbene Produced by a Type III Polyketide Synthase from *Pinus sylvestris* and Characterisation of Free Coenzyme A Intermediates

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The stilbene synthase (STS) from Scots pine (*Pinus sylvestris*), which is a type III polyketide synthase, produces the well known tetraketide resveratrol from coumaroyl-CoA and three molecules of malonyl-CoA. The same stilbene synthase, however, also generates the previously unknown pentaketide 2-malonylresveratrol from coumaroyl-CoA and four molecules of malonyl-CoA; this indicates that the enzyme does not precisely control the number of condensations leading to diverse products. Tetraketide- and pentaketide-CoA intermediates of the STS were identified by LC-MS/MS; this suggests that CoA-bound polyketide intermediates are involved in the product formation of type III polyketide synthases.

Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene, **4**; Scheme 1) is a phytoalexin produced by a number of plants. It is found in the skin of grapes, in peanuts, blueberries and knotweed, and also in some pines, such as Scots pine (*Pinus sylvestris*) and Eastern white pine (*Pinus strobus*). There has been great interest in the biological properties of resveratrol (**4**) since it was demonstrated that it interferes with all three stages of carcinogenesis—initiation, promotion and progression. Recently it has also been shown to significantly extend the lifespan of the yeast *Saccharomyces cerevisiae*, which adds further to its growing list of intriguing properties.[1]

The enzyme responsible for the formation of resveratrol is stilbene synthase (STS),[2–4] which belongs to the type III family of polyketide synthases. Resveratrol (**4**) is generated by sequential condensation of three acetate units derived from malonyl-CoA with the aromatic starter unit coumaroyl-CoA (**1**) to form the tetraketide intermediate **2**, which is then cyclised and aromatised to form the stilbene nucleus (Scheme 1). STS also accepts a number of other starter units, such as cinnamoyl-CoA and phenylpropanoyl-CoA.[5,6] From the crystal structure of the STS from *P. sylvestris* it has been possible to identify important residues in the active site that control the mode of cyclisation.[7] However, it has proved difficult to investigate the precise mechanism of cyclisation of the tetraketide intermediate because it is assembled on the enzyme and such a reactive compound would be very difficult to access synthetically. A key point in the mechanism is whether decarboxylation occurs before cyclisation has taken place, concomitantly with cyclisation, or after cyclisation (Scheme 1). To investigate this we have carried out detailed analysis of the reaction using LC-MS and NMR spectroscopy with a view to isolating intermediates and minor products that could reveal the true reaction pathway to resveratrol (**4**).

Results and Discussion

A pentaketide product of the *P. sylvestris* stilbene synthase

For this study we chose the well characterised STS from *P. sylvestris*, which accepts either coumaroyl-CoA (**1**) or cinnamoyl-CoA as starter units to produce resveratrol or pinosylvin through the addition of three acetate units.[8] The heterologous recombinant STS was obtained from *E. coli* BL21(DE3), into which the cDNA encoding the STS gene from *P. sylvestris* was introduced by use of the expression vector pET28. The purified protein was homogenous as judged by SDS-PAGE and LC/ESI-MS. Gel filtration analysis suggested that the protein forms a homodimer, as previously found for other members of the chalcone synthase (CHS) superfamily.[3,4]

After incubation of the STS with coumaroyl-CoA as starter unit and malonyl-CoA, the solution was acidified to precipitate the enzyme and subjected to LC-MS analysis. To our surprise, the total ion current and UV chromatogram of the reaction mixture revealed two major peaks at 16.9 and 18.0 min, showing $[M+H]^+$ values of 315 and 271, respectively. The relative abundance of the latter peak increased over time whereas the

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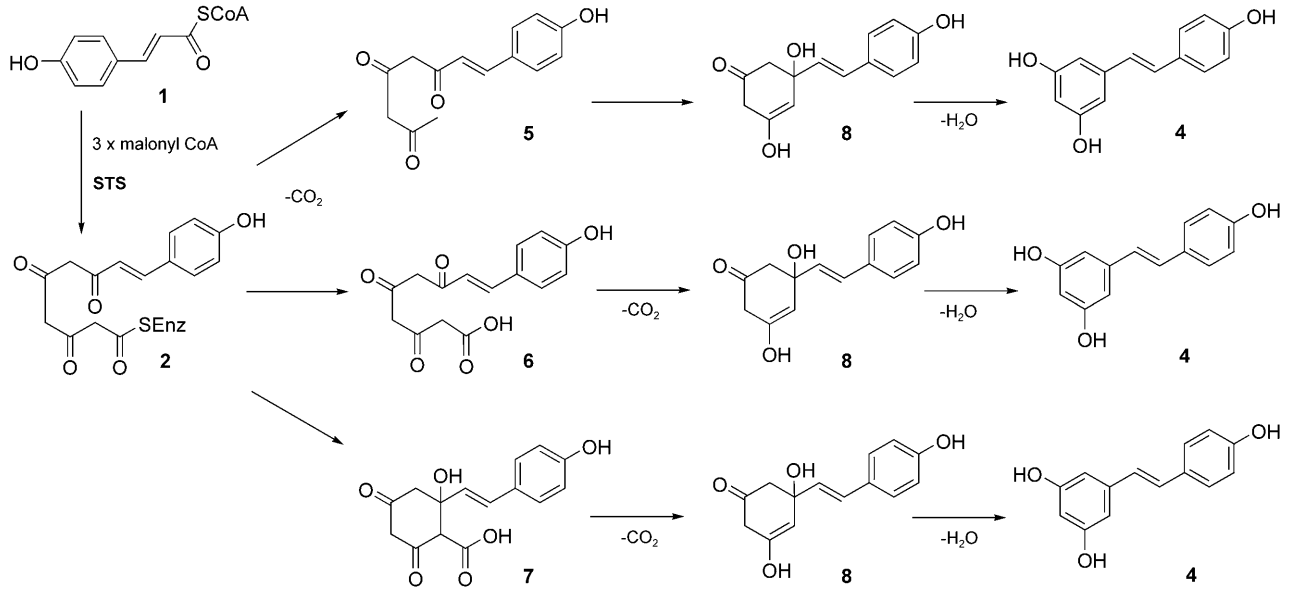
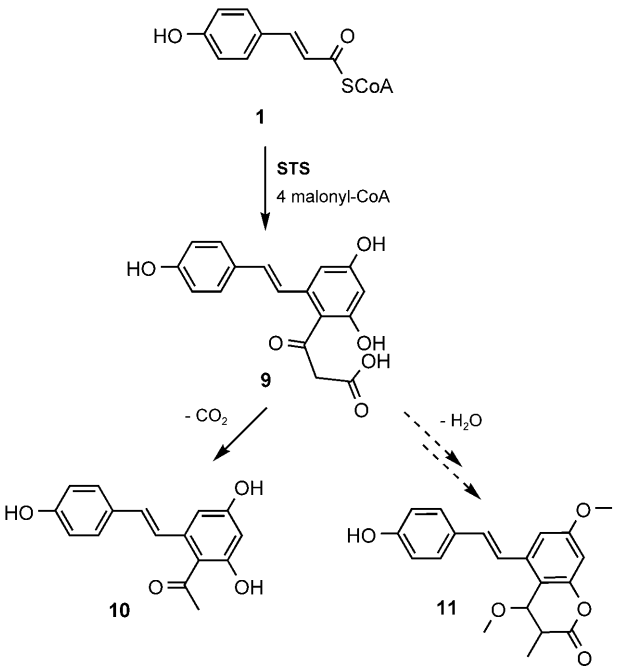


Scheme 1. Possible mechanistic scenarios for resveratrol (**4**) formation by the stilbene synthase from *P. sylvestris*.[2–4]



Scheme 2. Pentaketide stilbene products of the STS from *P. sylvestris* with 4-hydroxycinnamoyl-CoA (**1**) as starter unit. The closely related pentaketide stilbene (**11**) has been isolated from the root bark of *Ekebergia benguelensis*.[9]

former peak decreased. The difference of 44 amu suggested that one is the decarboxylation product of the other. LC-MS/MS analysis demonstrated that both undergo similar MS fragmentation. The elemental compositions were determined by high-resolution mass spectrometry as $C_{17}H_{14}O_6$ and $C_{16}H_{14}O_4$, respectively, and the structure **10** was confirmed for the decarboxylated product by NMR spectroscopy (Scheme 2, Figure 1 and the Supporting Information).

The NMR analysis also revealed the presence of the *cis* isomer of 2-acetylresveratrol (**10**). It is well documented that stilbenes can undergo rapid *cis*/*trans* isomerisation when exposed to acid or light.[10] When the workup of the enzyme assay was carried out by avoiding acidic conditions (extraction with ethyl acetate) and with exclusion of light, only the *trans* isomer was found, which shows that the enzyme forms exclusively the *trans* stilbene. The biosynthesis of **9** and **10** by the STS was further investigated by using [$^{13}C_3$]-malonyl-CoA.[11] The LC-MS analysis revealed a gain of eight mass units for **9** and seven mass units for **10**, as would be expected for the incorporation of four [$^{13}C_2$]-acetyl units, which confirms that **9** and **10** are indeed pentaketide derivatives.[3] The other known type III polyketide synthases (PKSs) that produce pentaketide products are the bacterial enzyme RppA,[12] the plant enzyme PCS,[13] and the fungal enzyme ORAS.[14] None of them, however, has been reported to produce both aldol-type tetraketide and pentaketide products.

Interestingly, Chávez et al. recently reported the isolation of the pentaketide stilbene **11** (Scheme 2) from the root bark of *Ekebergia benguelensis*.[9] Such a new stilbene-like compound might be a pentaketide product of an STS similar to that from *P. sylvestris*.

Coenzyme A intermediates of the stilbene synthase

Besides the major products, four minor peaks were observed in the LC-MS. One of these peaks (*m/z* 231 at 17.8 min) had the correct mass to be a triketide pyrone formed from coumaroyl-CoA (**1**) and two malonyl units. Such derailment products are commonly observed with type III PKSs.[5,15] A peak with *m/z* 229 corresponding to resveratrol (**4**) nearly co-eluted with 2-acetylresveratrol (**10**).[16] Interestingly, the detection of resveratrol by LC-MS is at least one order of magnitude less sensitive than that for the tetra- and pentaketides **9** and **10**.

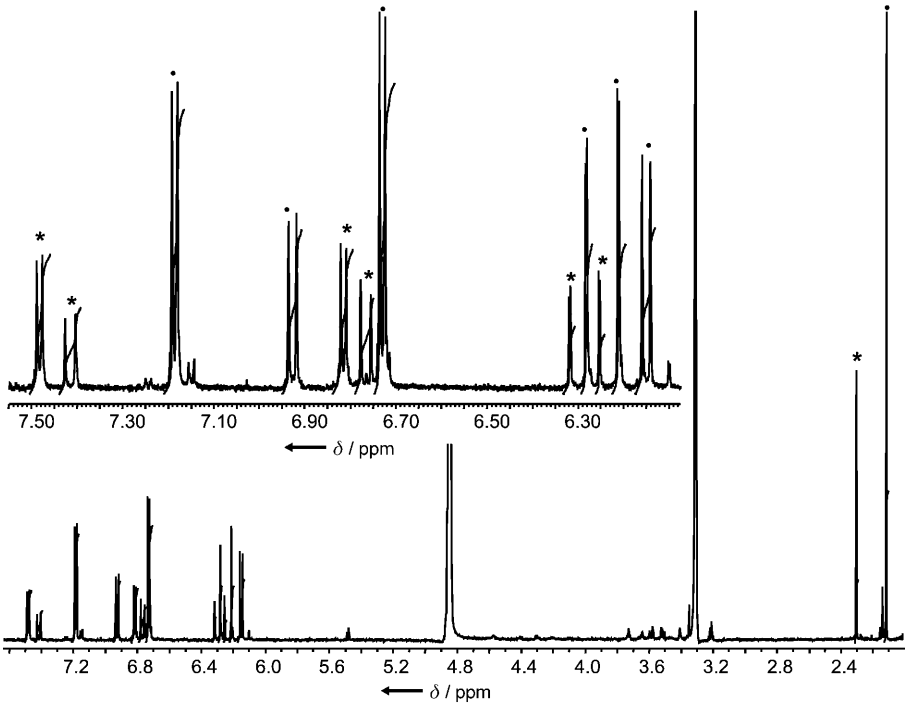


Figure 1. ^{1}H NMR spectra of 2-acetylresveratrol (**10**) formed by the STS from *P. sylvestris*. In CD_3OD two datasets corresponding to *cis*-2-acetylresveratrol (**10**, ●) and *trans*-2-acetylresveratrol (**10**, *) were observed.

Because resveratrol virtually coelutes with the 2-acetylresveratrol, an estimation of the product ratio by mass spectrometry or UV spectroscopy was unreliable. We, therefore, estimated the product ratio by NMR spectroscopy, by comparing the ^{1}H integrals of the crude assay mixture after ethyl acetate extraction. Although there was some variation, the ratio of 2-acetylresveratrol to resveratrol typically fell in the range of 1–2 to 4.

Two other peaks at 12.8 and 14.1 min and with much higher masses (*m/z* 1040 and 1082) also attracted our attention. The high masses of these two compounds suggest that they might contain CoA (Figure 2).

In assays incubated with [$^{13}C_3$]-malonyl-CoA the masses of the two compounds had in-

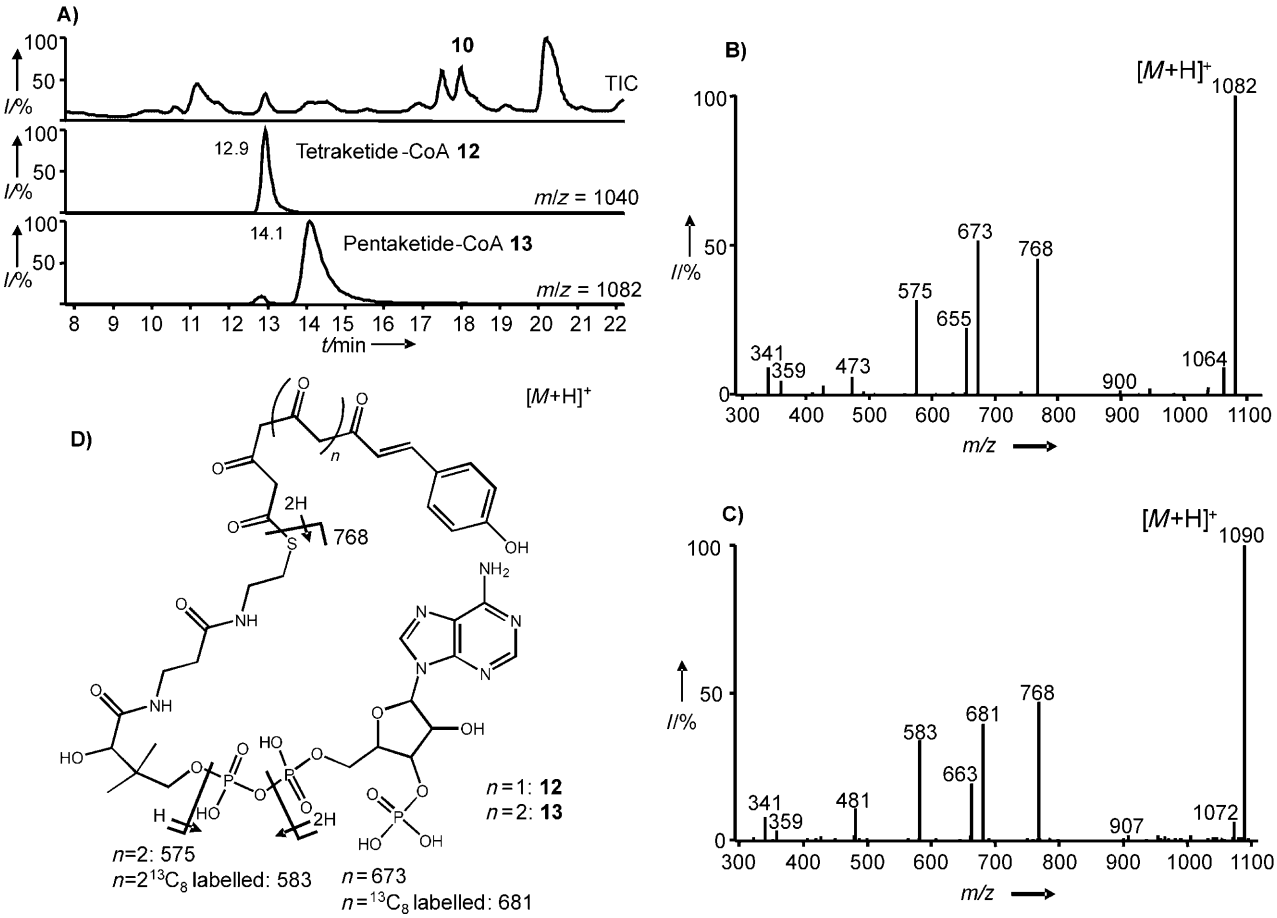


Figure 2. A) LC-MS of tetraketide-CoA **12** and pentaketide-CoA **13**. B) ESI-MS/MS spectra of the pentaketide-CoA intermediate **13** and C) ESI-MS/MS spectra of its corresponding $^{13}C_8$-labelled pentaketide-CoA **13b** formed by the STS from *P. sylvestris*. D) Structures of **12** and **13**.

creased to *m/z* 1046 and 1090, respectively; this suggests their identities to be the tetraketide-CoA (derivative **12**) and the pentaketide-CoA (derivative **13**), respectively. Further confirmation of their structures was obtained by MS/MS analysis, which showed fragmentation[17,18] clearly indicative of cleavage reactions of the CoA parts of the molecules (Figure 2 and the Supporting Information). Owing to the small quantities of material isolated it was not possible to determine the precise structures of these two compounds by NMR analysis.

The parent tetraketide and pentaketide structures **12** and **13** (Figure 2) have the correct masses, but are likely to exist as mixtures of enolic forms and could possibly be already cyclised (but not dehydrated). Although there is circumstantial evidence for the involvement of free CoA intermediates interacting with other enzymes during the biosynthesis of several polyketides produced by plant type III PKSs,[19] and although we recently managed to trap stilbene synthase intermediates using a malonyl-CoA analogue,[18] this is the first time that such compounds have been directly observed. It raises the possibility that events such as cyclisation might occur while the polyketone chain is bound to CoA rather than to the active site cysteine.

Previous studies had established that the STS, like many other type III PKSs, can take a range of starter units.[8] We therefore investigated whether the use of different starter units would also result in the formation of pentaketide products. Assays carried out with either cinnamoyl-CoA or phenylpropionyl-CoA as starter units did result in the formation of the corresponding pentaketide stilbenes, but they were produced to much smaller extents than when coumaroyl-CoA (**1**) served as the starter unit.

Cinnamoyl-CoA and phenylpropionyl-CoA both promoted the formation of substantial amounts of triketide pyrones, relative to the amounts of these compounds formed with coumaroyl-CoA (**1**; 30–40% of the products formed). This implies that precise binding of the starter unit within the active-site cavity of the enzyme is an important factor for determining chain elongation and the mode of cyclisation. The isolation of 2-malonyl-resveratrol (**9**) also demonstrates that the enzyme can carry out the cyclisation without requiring decarboxylation. This could suggest that decarboxylation also does not accompany ring closure for the formation of resveratrol (**4**). Stilbene carboxylate has been isolated from several plants; this suggests a similar mode of cyclisation.[3] But we did not detect any stilbene carboxylate; this indicates that it is probably not an intermediate on the pathway to resveratrol, and might suggest that decarboxylation occurs immediately after cyclisation but before the ring becomes aromatic. However, since we have so far only managed to observe the linear tetraketide-CoA **12** and pentaketide-CoA **13**, further experiments are needed to investigate whether it is the thioester **12** or its free acid derivative that is cyclised before or after decarboxylation to yield resveratrol.

Conclusions

In summary, we have shown that the STS from *P. sylvestris* is capable of producing the previously unknown pentaketide **9** together with established tetraketide stilbenes, such as resveratrol (**4**). The chain length determination system of the STS is thus somewhat flexible and can generate diverse products. The free tetra- and pentaketide-CoA species **12** and **13**, respectively, have been detected for the first time, which offers opportunities for the analysis of more mechanistic details of polyketide formation. Further analysis of intermediates of the polyketide biosynthesis of the STS and those from other systems should provide additional insights into the reaction mechanisms of polyketide synthases.

Experimental Section

General synthetic and analytical methods: Synthetic reactions were performed under N_2. Solvents were dried by using standard methods. ^{1}H NMR and ^{13}C NMR spectra were recorded by using Bruker Avance (700 MHz) or Bruker DRX (500 MHz) spectrometers, each fitted with a cryoprobe. Chemical shifts of ^{1}H NMR and ^{13}C NMR spectra are given in ppm (δ) based on solvent signals: D_2O 4.67 ppm, CD_3OD 3.30 ppm (^{1}H NMR), and CD_3OD 49.0 ppm (^{13}C NMR). LC-ESI-MS/MS spectra were obtained with a ThermoFinnigan LCQ (San Jose, USA) ion trap mass spectrometer hooked to an Agilent HP1100 HPLC system. HPLC separation was performed either with a Phenomenex Prodigy RP18-column (250 mm × 4.6 mm, 5 μm) by gradient elution (standard programme: 95% A to 25% A in 25 min, 25% to 0% A in 5 min, 100% B 8 min; A: H_2O (0.1% TFA), B: MeCN (0.1% TFA); flow rate: 1 mL min^{-1}) or with a Phenomenex Synergy polar RP-column (150 mm × 2 mm, 4 μm) by gradient elution (standard programme: 100% A for 3 min, from 100% A to 0% A in 27 min, 100% B 10 min; A: H_2O (0.1% TFA), B: MeCN (0.1% TFA); flow rate: 0.3 mL min^{-1}). High-resolution mass spectra were recorded with a Micromass Q-TOF (Manchester, UK) instrument fitted with an ESI source by direct injection of the purified sample. Preparative RP-18 HPLC (Phenomenex Luna 250 × 20 mm, 10 μm) was used for purification of the CoA derivatives. The products from large-scale STS assays were purified for NMR spectroscopic characterisation by HPLC (Agilent HP1100) with a fraction collection system fitted with the same analytical column as for the LC-MS analysis. CoA and malonyl-CoA were purchased from Sigma.

Synthesis of CoA-derivatives: The free aromatic acid (0.10 mmol) and triethylamine (0.11 mmol) were stirred at −10 °C under argon in dry THF (1.5 mL), and ethyl chloroformate (0.11 mmol) was added. After 5 min a solution of coenzyme A lithium salt (0.10 mmol) in NaOH (0.5 mL, 0.3 N) was added, and the mixture was stirred at room temperature for 30 min.[20] The progress of the reaction was monitored by LC-MS. THF was removed in vacuo, and the aqueous solution was purified by preparative HPLC on RP-18 (Phenomenex Luna 250 × 20 mm, 10 μm) by using gradient elution (A: H_2O, B: MeOH; 100% A to 100% B in 30 min, flow rate 10 mL min^{-1}); yield: 10–40%. If the ethyl chloroformate reacted with the acidic aromatic hydroxy groups, then deprotection was achieved with pig liver esterase (PLE) while the sample was stirred at room temperature in phosphate buffer (pH 8).

Coumaroyl-CoA: ^{1}H NMR (500 MHz, D_2O) δ = 0.58 (s, 3H), 0.75 (s, 3H), 2.28 (t, J = 6.45 Hz, 2H), 2.95 (t, J = 6.12 Hz, 2H), 3.16 (s, 1H), 3.19 (s, 1H), 3.24 (t, J = 6.12 Hz, 2H), 3.30 (t, J = 6.45 Hz, 2H), 3.39 (dd, J = 9.60, J = 4.08 Hz, 1H), 3.70 (dd, J = 9.60, J = 4.08 Hz, 1H), 3.87 (s, 1H), 4.08 (s, 1H), 4.39 (s, 2H), 4.60–4.63 (m, 2H) 5.93 (d, J = 5.66 Hz, 1H), 6.45 (d, J = 15.79 Hz, 1H), 6.67–6.73 (m, 2H), 7.24–7.33 (m, 3H), 7.98 (s, 1H), 8.35 ppm (s, 1H); ESI-MS: 936 $[M+Na]^+$ (4),

914 $[M+H]^+$ (100); HR-ESI-MS: *m/z* calcd for $C_{30}H_{42}N_7NaO_{18}P_3S$ $[M+Na]^+$: 936.1418; found: 936.1427; *m/z* calcd for $C_{30}H_{43}N_7O_{18}P_3S$ $[M+H]^+$: 914.1598; found: 914.1490.

Preparation of succinyl-CoA:3-ketoacid-transferase (EC 2.8.3.5):[21] pBR322 containing the gene for succinyl-CoA:3-ketoacid-transferase (EC 2.8.3.5) was a gift from Prof. Dr. Fraser (University of Calgary, Canada). *E. coli* BL21DE3 was transformed with the plasmid. *E. coli* cells were grown at 37 °C in LB medium (1 L) until an OD_{600} of about 0.8 was reached. Protein expression was induced with IPTG (0.5 mM) at 16 °C. After 12 h cells were harvested by centrifugation, resuspended in HEPES buffer (pH 7.8, 50 mM) and ruptured by sonification. $(NH_4)_2SO_4$ was added to the cell-free extract to 45% saturation in order to precipitate proteins. After centrifugation, further $(NH_4)_2SO_4$ was added to the supernatant to 65% saturation. Centrifugation at 10000 *g* for 30 min gave a pellet containing the crude succinyl-CoA-transferase. This crude preparation was used to generate $[^{13}C_3]$-malonyl-CoA.

Preparation of labelled $[^{13}C_3]$-malonyl-CoA:[22] Succinic anhydride (2 mg) was dissolved in acetone (300 μL), an aqueous solution of coenzyme A lithium salt (12 mg) was added quickly, and the mixture was vortexed for several minutes to generate succinyl-CoA. Its formation was monitored by LC-MS. The acetone was removed in an argon stream. $[^{13}C_3]$-Malonate was dissolved in HEPES (pH 7.6, 50 mM, 500 μL), and the pH was readjusted to 7 with the aid of a pH-microelectrode. The $[^{13}C_3]$-malonate was added to the solution containing succinoyl-CoA, and the pH was monitored and adjusted to 7. Finally, a quantity (5–10 mg) from the pellet of the crude succinoyl-CoA:3-ketoacid-transferase was added. After 1 h the reaction was quenched by addition of $CHCl_3$ (300 μL). The enzyme was precipitated by being vortexed for 1 min, the solution was centrifuged, and the aqueous solution was collected. $[^{13}C_3]$-Malonyl-CoA was used for the bioassays. The amount of $[^{13}C_3]$-malonyl-CoA generated by the enzyme was estimated by comparison of the UV-LC trace with a standard solution of malonyl-CoA.

Stilbene synthase (STS) enzyme purification: The STS was amplified by PCR from the plasmid pREP4 containing the STS cDNA by using the following primers: P1: 5′-ATC GGC TCA TAT GGG GGG CGT TGA TTT TGA AGG TTT C-3′ (NdeI), P2: 5′-GAC AAG CTT CAT TAC TGG ATC TAT CAA CAG GAG TC-3′ (HindIII). The product was ligated according to the manufacturer's instructions (Novagen) into expression vector pET-28a(+), which provides an N-terminal His_6-tagged fusion protein. DNA sequencing (MWG, Ebersberg, Germany) was carried out for the STS construct and confirmed its 100% identity with the published STS sequence (accession no.: S50350, stilbene synthase, *Pinus sylvestris*). *E. coli* cells were grown at 37 °C in LB medium (1 L) until an A_{600} of about 0.7 was reached. Protein expression was induced with IPTG (0.2 mM) at 16 °C, and after 12 h cells were harvested by centrifugation, resuspended in Tris-HCl buffer (50 mM) containing imidazole-HCl (binding buffer, 10 mM, pH 7.8), and ruptured by sonification. The cell-free extract was applied to a Ni^{2+}-NTA resin column, which was washed successively with binding buffer and wash buffer (30 mM imidazole-HCl) before elution of the target protein with imidazole-HCl (100 mM). The buffer was changed to HEPES buffer (50 mM, pH 7.2) by using Millipore centrifugal filters. The relative molecular mass of the purified STS protein was determined by ESI-MS to be 44760 Da; calcd: 44761 Da. The concentration of the enzyme was measured by using the Bradford assay.

Enzyme assays

Microscale reactions: For the standard assay, starter substrate-CoA (200 nmol) and malonyl-CoA (1 μmol) in HEPES buffer (100–200 μL, 100 mM, pH 7.5, 1 mM EDTA) were added to the STS (ca. 450 pmol). After 1–2 h incubation at room temperature, the sample was either used directly for LC-MS analysis (after precipitation of the STS with diluted HCl) or extracted with ethyl acetate (200 μL) after acidification with dilute HCl. The ethyl acetate was removed in a gentle stream of nitrogen, and the sample was redissolved in methanol (100 μL). For the LC-MS analysis 50 μL were used for injection.

Large-scale reactions: In order to collect enough product for NMR spectroscopy characterisation, the STS (2.5 μmol) was incubated with starter unit-CoA (10 mmol) and malonyl-CoA (50 mmol) in HEPES buffer (10 mL, 100 mM, pH 7.5, 1 mM EDTA) for 10 h at room temperature. The sample was acidified with diluted HCl and extracted with ethyl acetate (3×10 mL). After removal of the ethyl acetate in a nitrogen stream the sample was dissolved in MeOH (0.5 mL). Purification of the products from the STS was achieved by injection of the sample (100 μL) onto a Phenomenex Prodigy HPLC column and 0.5 min fractions were collected with the fraction collector. After being dried, the samples were used for NMR analysis.

1-[2-(4-Hydroxystyryl)-4,6-dihydroxyphenyl]ethanone (10): ^1H NMR (700 MHz, CD_3OD): δ (major isomer) = 2.11 (s, 3H), 6.14 (d, J = 12.5 Hz, 1H), 6.20 (d, J = 2.3 Hz, 1H), 6.27 (d, J = 2.3 Hz, 1H), 6.70–6.74 (m, 2H), 6.92 (d, J = 12.5 Hz, 1H), 7.16–7.20 ppm (m, 2H); δ (minor isomer) = 2.29 (s, 3H), 6.24 (d, J = 2.2 Hz, 1H), 6.31 (d, J = 2.2 Hz, 1H), 6.76 (d, J = 16.0 Hz, 1H), 6.79–6.82 (m, 2H), 7.40 (d, J = 16.0 Hz, 1H), 7.46–7.49 ppm (m, 2H); ESI-MS: 271 $[M+H]^+$ (100), 229 (10), 227 (9); HR-ESI-MS: *m/z* calcd for $C_{16}H_{14}O_4Na$ $[M+Na]^+$: 293.0790; found: 293.0784; *m/z* calcd for $C_{16}H_{15}O_4$ $[M+H]^+$: 271.0970; found: 271.0970.

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