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Enzymatic Formation of an Unnatural C_6–C_5 Aromatic Polyketide by Plant Type III Polyketide Synthases

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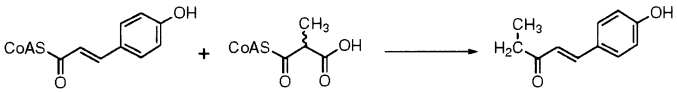
ABSTRACT



Substrate specificities of plant polyketide synthases (PKSs) were investigated using analogues of malonyl-CoA, the extension unit of the polyketide chain elongation reactions. When incubated with methylmalonyl-CoA and 4-coumaroyl-CoA, plant PKSs (chalcone synthase from *Scutellaria baicalensis*, stilbene synthase from *Arachis hypogaea*, and benzalacetone synthase from *Rheum palmatum*) afforded an unnatural C_6–C_5 aromatic polyketide, 1-(4-hydroxyphenyl)pent-1-en-3-one, formed by one-step decarboxylative condensation of the two substrates. In contrast, succinyl-CoA was not accepted as a substrate by the enzymes.

Type III poyketide synthases (PKSs) are pivotal enzymes for construction of the basic skeletons of plant polyketides with diverse structures and biological activities. Chalcone synthase (CHS) (EC 2.3.1.74) and stilbene synthase (STS) (EC 2.3.1.95) catalyze a sequential condensation of the C_6−C_3 unit of 4-coumaroyl-CoA (**1**) as a starter with three C_2 units from malonyl-CoA (**2**) (Scheme 1).[1] After three rounds of the polyketide chain elongation reaction, cyclization and aromatization of the enzyme-bound tetraketide intermediate leads to formation of 4,2′,4′,6′-tetrahydroxychalcone (naringenin chalcone) (**3**) and *trans*-3,4′,5-trihydroxystilbene (resveratrol) (**4**), respectively. Further, in enzyme reactions in vitro, pyrone derivatives 4-coumaroyltriacetic acid lactone (CTAL) (**5**)[2] and bis-noryangonin (BNY) (**6**)[3] are formed as early-released derailment byproducts. On the other hand, recently reported benzalacetone synthase (BAS) (EC 2.3.1.-) catalyzes a one-step decarboxylative condensation of 4-coumaroyl-CoA with malonyl-CoA to produce the C_6−C_4 skeleton of benzalacetone (**7**) (Scheme 1), a precursor of medicinally important phenylbutanoids such as antiinflammatory lindleyin and gingerols.[4]

The plant PKSs are homodimeric enzymes sharing 60−75% amino acid sequence identity with other members of the CHS-superfamily enzymes, including 2-pyrone synthase (2PS)[5] and acridone synthase (ACS).[6] Recent crystallographic and structure-based mutagenesis studies on *Medicago sativa* CHS2[7] and *Gerbera hybrida* 2PS[8] revealed the structural details of the active site govering the enzyme reaction, i.e., starter molecule loading, malonyl-CoA decarboxylation, polyketide chain elongation, and regiospecificities of the

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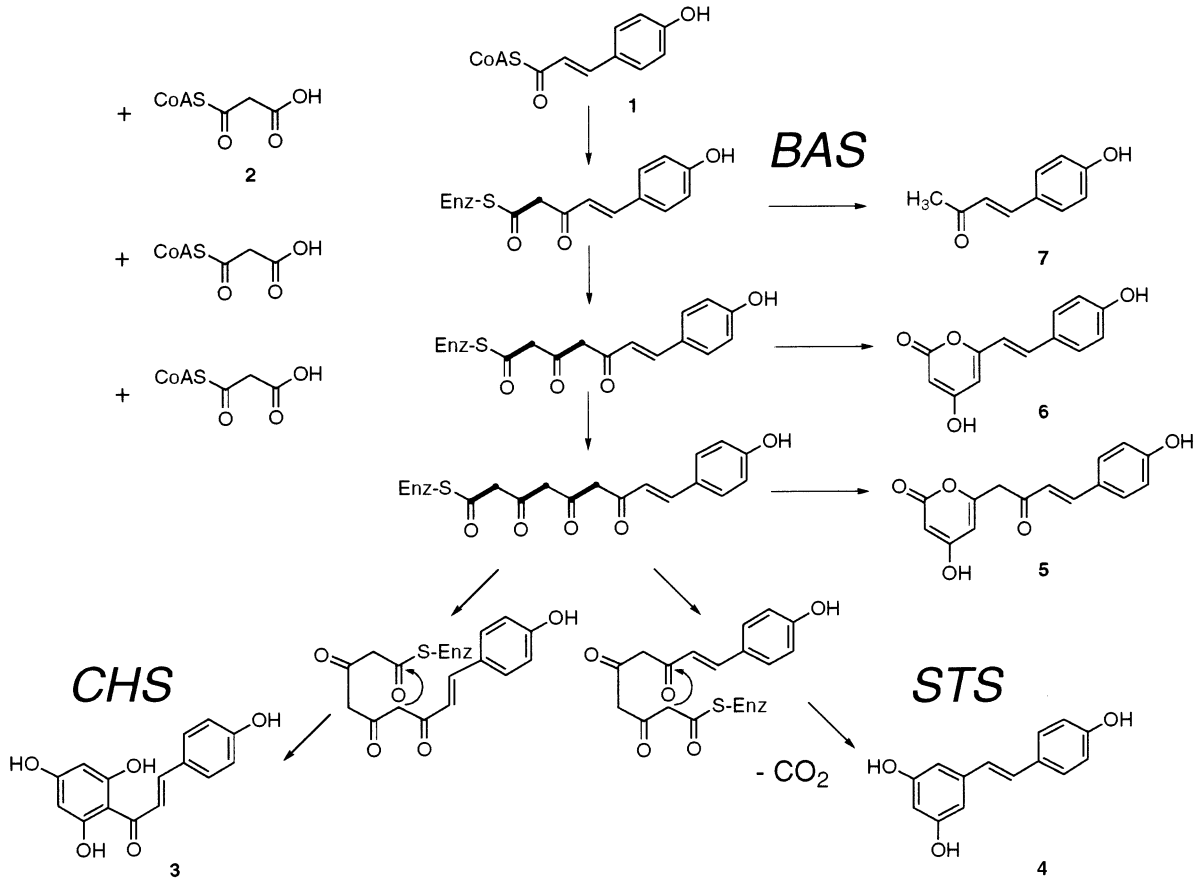
Scheme 1. Proposed Mechanism for the conversion of 4-Coumaroyl-CoA (**1**) and Malonyl-CoA (**2**) to Naringenin Chalcone (**3**) by CHS, to Resveratrol (**4**) by STS, and Benzalacetone (**5**) by BAS.[a]



[a] Pyrone derivatives, BNY (**6**), and CTAL (**7**) are common byproducts of the CHS and STS enzyme reactions in vitro.

cyclization reactions. The catalytic center of the *M. sativa* CHS2 enzyme is composed of four amino acid residues (Cys164, Phe215, His303, and Asn336) well conserved in all the known type III PKSs. The only exception is the BAS that lacks the active site Phe215 and has a Leu at this position.[4] Phe215 has been proposed to facilitate malonyl-CoA decarboxylation and help orient substrates and intermediates during the sequential condensation reactions in CHS.[7b,e] These hypotheses may explain why the polyketide chain elongation reaction is terminated at the diketide stage in BAS.

In previous studies, we have demonstrated that CHSs from *Scutellaria baicalensis*[9] and STS from *Arachis hypogaea*[10] have remarkably broad substrate specificity toward the starter molecule of the enzyme reaction. Instead of 4-coumaroyl-CoA, the enzymes accepted disparate starter molecules, both aromatic and aliphatic CoA esters of different chain length, and efficiently performed sequential condensation and cyclization reactions to produce a series of chemically and structurally different unnatural polyketides. Manipulation of the enzyme reaction through the use of artificial substrates thus led to development of a chemical library of unnatural novel polyketides.

In addition to the starter substrate specificity, it is also interesting to test if the enzymes can accept analogues of the extension unit of the sequential condensation reactions. We now describe enzymatic reactions utilizing methylmalonyl-CoA (**8**) and succinyl-CoA (**9**), both having one more carbon atom than malonyl-CoA (**2**), by plant PKS enzymes. We also reasoned that the nucleophilicity of the α-carbon atom of succinyl-CoA (**9**) is significantly lower compared with that of malonyl-CoA having a β-carbonyl group, and succinyl-CoA may be accepted by the enzymes and activated for the decarboxylative Claisen condensation reactions. Evidence favoring selection of methylmalonyl-CoA as a starter has been obtained for CHS2 from *Pinus strobes*.[11] This enzyme was reported to be completely inactive with malonyl-CoA as a substrate; however, it catalyzed a one-step condensation of methylmalonyl-CoA (**8**) with a diketide derivative (*N*-acetylcysteaminethioester of 3-oxo-5-phenyl-

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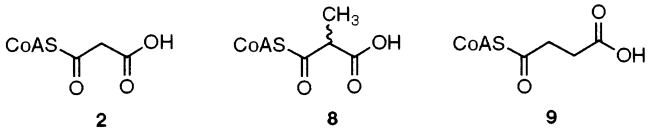
pent-4-enoic acid) to produce a methylated triketide styrylpyrone. Here in this letter, we now report on the detailed enzymatic conversion studies of the extension unit analogues by (i) CHS from *S. baicalensis*, (ii) STS from *A. hypogaea*, and (iii) BAS from *Rheum palmatum*.[12]



When incubated with methylmalonyl-CoA (racemic) (**8**) and 4-coumaroyl-CoA (**1**) as substrates, both *S. baicalensis* CHS and *A. hypogaea* STS afforded 1-(4-hydroxyphenyl)-pent-1-en-3-one (**11**) as a major product along with the BNY-type (**12**) and the CTAL-type (**13**) pyrone byproducts, while *R. palmatum* BAS yielded the C_6-C_5 aromatic polyketide (**11**) as a single product (Figure 1).[13] Here 4-coumaric acid (**10**) was also obtained as a result of hydrolysis of **1**. It should be noted that formation of the products were detected only after 12 h of incubation, instead of 20 min of incubation for the standard assay with malonyl-CoA. The formation of the phenylpentenone was thus apparently at least 100 times slower than that of regular products of the enzymes (CHS, STS, and BAS).[13]

Confirmation of the structure of the major product **11** was obtained as follows.[14] First, the LC-ESIMS spectrum of the product gave a parent ion peak $[M + H]^+$ at *m*/*z* 177, and its UV spectrum with a λ_{max} at 322 nm was similar to that of benzalacetone (**7**). Further, the ^{1}H NMR spectrum of the product obtained from a large-scale enzyme reaction (69% yield from 7.5 mg of 4-coumaroyl-CoA) showed A_2B_2-type (δ 7.40, 2H, d, J = 8.4 Hz, and 6.71, 2H, d, J = 8.4 Hz) aromatic signals together with a pair of trans-coupled α,β-unsaturated olefinic protons (δ 7.48 and 6.56, each 1H, d, J = 16.4 Hz) and α-ethyl protons (2.61, 2H, q, J = 7.2 Hz, and 1.02, 3H, t, J = 7.2 Hz). The structure was finally verified by HMQC and HMBC experiments. On the other hand, the structures of the two byproducts were respectively determined to be the BNY-type (**12**)[15] and the CTAL-type pyrone (**13**)[16] on the basis of the LC-ESIMS and UV spectra. The LC-ESIMS spectrum of **12** gave a parent ion peak $[M + H]^+$ at *m*/*z* 259, and in MS/MS (precursor ion at *m*/*z* 259) the fragment at *m*/*z* 215 corresponded to $[M + H - CO_2]^+$, while that of **13** gave a parent ion peak $[M + H]^+$ at *m*/*z* 315, and in MS/MS (precursor ion at *m*/*z* 315) the fragment

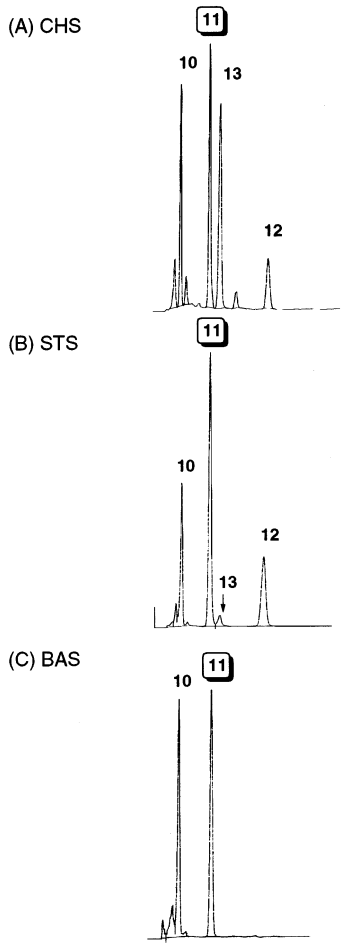


Figure 1. HPLC profile of the enzyme reaction products from 4-coumaroyl-CoA (**1**) and methylmalonyl-CoA (racemic) (**8**) by (A) *S. baicalensis* CHS, (B) *A. hypogaea* STS, and (C) *R. palmatum* BAS. 4-Coumaric acid (**10**) (R_t = 3.7 min), 1-(4-hydroxyphenyl)-pent-1-en-3-one (**11**) (R_t = 9.8 min), the BNY-type pyrone (**12**) (R_t = 20.0 min), and the CTAL-type pyrone (**13**) (R_t = 11.6 min). Here 4-coumaric acid (**10**) was obtained as a result of hydrolysis of **1**.

(12) Recombinant *S. baicalensis* CHS,[9] *A. hypogaea* STS,[10] and *R. palmatum* BAS[4] with an additional hexahistidine tag at the C-terminal were expressed in *E. coli* and purified by Ni-chelate affinity chromatography as described before. The purified enzymes showed the following K_M and k_{cat} values for 4-coumaroyl-CoA: CHS (36.1 μM and 1.26 min^{-1}), STS (11.2 μM and 1.20 min^{-1}), and BAS (10.0 μM and 1.79 min^{-1}).

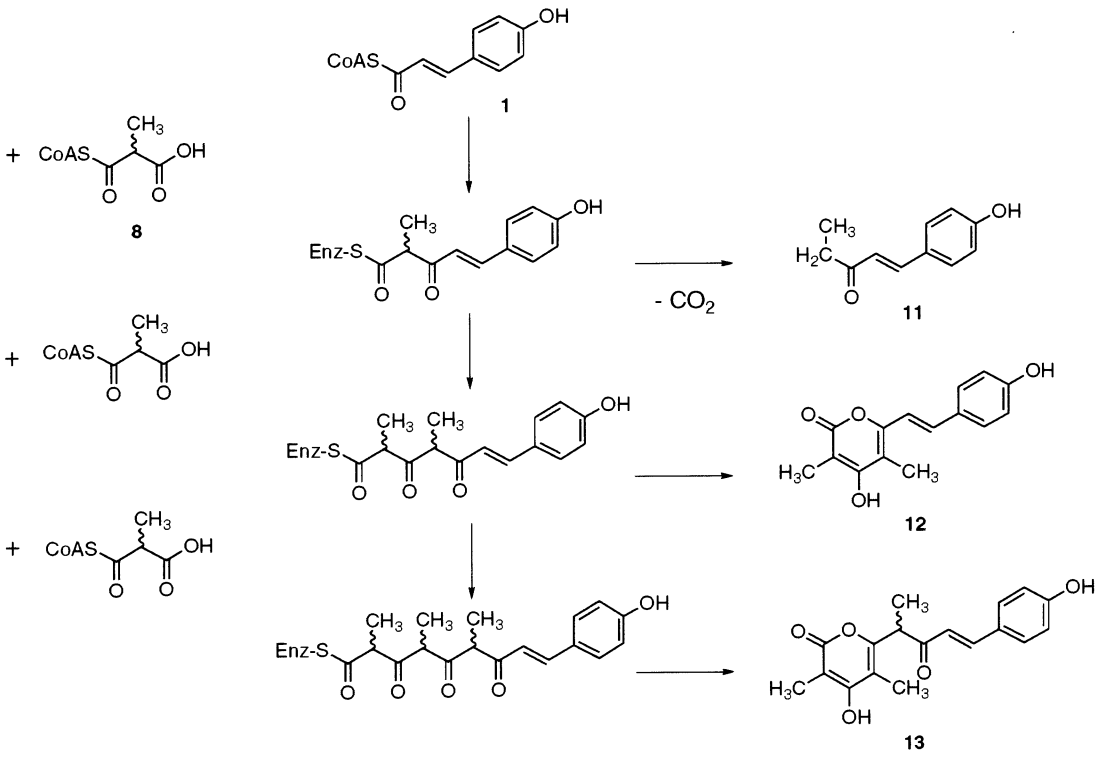
(13) The reaction mixture contained 54 μM 4-coumaroyl-CoA, 108 μM methylmalonyl-CoA (racemic), and 20 μg of the purified recombinant enzyme in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 8.0, containing 1 mM EDTA. Incubations were carried out at 30 °C for 18 h and stopped by addition of 50 μL of 20% HCl. The products were then extracted with 600 μL of ethyl acetate and separated by reverse-phase HPLC (column, TSK-gel ODS-80Ts, 4.6 × 150 mm, Tosoh Co., Ltd., Japan; eluent, 50% aqueous MeOH containing 0.05% TFA; flow rate, 0.8 mL/min). For large-scale enzyme reactions, 4-coumaroyl-CoA (7.5 mg, 8.2 μmol) and methylmalonyl-CoA (racemic, 15.0 mg, 17.3 μmol) were incubated with 50 mg of purified recombinant CHS in 750 mL of 100 mM phosphate buffer, pH 8.0, containing 1 mM EDTA at 30 °C for 18 h. The apparent K_M and k_{cat} values for methylmalonyl-CoA for the formation of the unnatural C_6-C_5 aromatic polyketide (**11**) are as follows: CHS (129 μM and 0.0103 min^{-1}), STS (79.3 μM and 0.0194 min^{-1}), and BAS (50.4 μM and 0.0360 min^{-1}).

(14) HPLC: R_t = 9.8 min. LC-ESIMS: *m*/*z* 177 $[M + H]^+$. UV: λ_{max} 322 nm. ^{1}H NMR (400 MHz, CD_3OD): δ 7.48 (H-1, d, J = 16.4 Hz), 7.40 (H-2′ and H-6′, d, J = 8.4 Hz), 6.71 (H-3′ and H-5′, d, J = 8.4 Hz), 6.56 (H-2, d, J = 16.4 Hz), 2.61 (CH_2, q, J = 7.2 Hz), 1.02 (CH_3, t, J = 7.2 Hz). ^{13}C NMR (100 MHz, CD_3OD): δ 203.9 (C-3), 161.6 (C-1), 144.8 (C-4′), 131.4 (C-2′ and C-6′), 127.1 (C-2), 123.6 (C-1′), 116.9 (C-3′ and C-5′), 34.3 (C-4), 8.8 (C-5). HRMS (FAB): found for $[C_{11}H_{13}O_2]^+$ 177.0919, calcd 177.0916.

(15) HPLC: R_t = 20.0 min. LC-ESIMS: *m*/*z* 259 $[M + H]^+$, MS/MS (precursor ion at *m*/*z* 259), *m*/*z* 229 (100) $[M + H - 2Me]^+$, 215 (51) $[M + H - CO_2]^+$, 140 (23), 119 (27). UV: λ_{max} 370 nm. HRMS (FAB): found for $[C_{15}H_{15}O_4]^+$ 259.0981, calcd 259.0970.

(16) HPLC: R_t = 11.6 min. LC-ESIMS: *m*/*z* 315 $[M + H]^+$, MS/MS (precursor ion at *m*/*z* 315), *m*/*z* 271 (13) $[M + H - CO_2]^+$, 195 (11), 147 (100), 119 (37). UV: λ_{max} 332 nm. HRMS (FAB): found for $[C_{18}H_{19}O_5]^+$ 315.1220, calcd 315.1232.

Scheme 2. Enzymatic Conversion of 4-Coumaroyl-CoA (**1**) and Methylmalonyl-CoA (**8**) to 1-(4-Hydroxyphenyl)pent-1-en-3-one (**11**), the BNY-Type Pyrone (**12**), and the CTAL-Type Pyrone (**13**).



at *m*/*z* 271 corresponded to $[M + H - CO_2]^+$, confirming the presence of α-pyrone ring. Furhermore, UV spectrum of **12** (λ_{max} at 370 nm) and **13** (λ_{max} at 332 nm) respectively showed good similarity to that of BNY (**6**) and CTAL (**5**), supporting the structures.

These experiments were the first to demonstrate that plant PKSs can catalyze the formation of the unnatural C_6-C_5 aromatic polyketide (**11**) from methylmalonyl-CoA and 4-coumaroyl-CoA (Scheme 2). The C_6-C_5 skeleton was produced by one-step decarboxylative condensation of the C_6-C_3 unit of 4-coumaroyl-CoA with the C_2 unit of methylmalonyl-CoA, as in the case of the formation of the C_6-C_4 benzalacetone (**7**) from malonyl-CoA and 4-coumaroyl-CoA. Thus, most of the polyketide chain elongation reactions were terminated at the diketide stage presumably due to the presence of the additional bulky methyl group of the extension unit analogue. Moreover, it is noteworthy that since diketide carboxylic acid was not detected in the reaction mixture, decarboxylation of the enzyme-bound diketide intermediate should be tightly controlled by the enzyme.

In contrast, when incubated with succinyl-CoA (**9**) and 4-coumaroyl-CoA (**1**) as substrates, none of the enzymes (CHS, STS, and BAS) afforded any reaction products, indicating succinyl-CoA was not accepted as a substrate for the polyketide chain elongation reaction. In addition to the steric factor that succinyl-CoA has one more carbon atom than malonyl-CoA, the nucleophilicity of the α-carbon of succinyl-CoA may not be high enough to perform the decarboxylative Claisen condensation reaction.

In summary, this is the first demonstration of the enzymatic formation of the unnatural C_6-C_5 aromatic polyketide by plant PKSs. The enzymes accepted the nonphysiological extension unit analogue in addition to a wide variety of starter molecule analogues. Manipulation of the plant PKS enzyme reactions by substrate analogues along with rationally engineered mutant enzymes would lead to further production of chemically and structurally disparate unnatural novel polyketides, which is now in progress in our laboratories.

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Supporting Information Available: Comparative NMR data for 1-(4-hydroxyphenyl)pent-1-en-3-one (**11**) and benzalacetone (**7**), NMR spectra of **11** (^1H and ^{13}C NMR, HMQC, and HMBC), and LC/MS/MS data of the pyrone byproducts **12** and **13**. This material is available free of charge via the Internet at http://pubs.acs.org.

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