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Enzymatic formation of an unnatural methylated triketide by plant type III polyketide synthases

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Abstract—Octaketide synthase, a novel plant-specific type III polyketide synthase from *Aloe arborescens*, efficiently accepted (2*RS*)-methylmalonyl-CoA as a sole substrate to produce 6-ethyl-4-hydroxy-3,5-dimethyl-2-pyrone. On the other hand, a tetraketide-producing chalcone synthase from *Scutellaria baicalensis* and a diketide-producing benzalacetone synthase from *Rheum palmatum* also yielded the unnatural methylated C_9 triketide pyrone as a single product by sequential decarboxylative condensations of three molecules of (2*RS*)-methylmalonyl-CoA.

The broad substrate tolerance and catalytic potential of the chalcone synthase (CHS) (EC 2.3.1.74) superfamily of type III polyketide synthases (PKSs) are remarkable.[1,2] Octaketide synthase (OKS) is a recently reported novel plant-specific type III PKS from *Aloe arborescens*, catalyzing sequential condensation of eight molecules of malonyl-CoA to produce aromatic C_{16} octaketides SEK4 and SEK4b (Scheme 1), the longest polyketides known to be synthesized by the structurally simple homodimeric type III PKS.[3] Like other type III PKSs,[4,5] *A. arborescens* OKS exhibits unusually broad, promiscuous substrate specificities; the enzyme accepts a variety of non-physiological substrates, including aromatic and aliphatic CoA thioesters, to generate an array of chemically and structurally distinct unnatural polyketides.[3] Here we now report enzyme reactions using (2*RS*)-methylmalonyl-CoA, instead of malonyl-CoA, as a sole substrate. It was interesting to test whether the octaketides-producing *A. arborescens* OKS accepts (2*RS*)-methylmalonyl-CoA, with an additional bulky methyl group, as a ***starter*** substrate and extends an intermediate to produce novel octaketides.

In previous studies, we have demonstrated that a tetraketide-producing *Scutellaria baicalensis* CHS[4a] and a diketide-producing *Rheum palmatum* benzalacetone synthase (BAS)[6] accepted (2*RS*)-methylmalonyl-CoA as an ***extender*** substrate to produce unnatural polyketides.[4d,e] Further, it has been reported that *Pinus strobus* CHS2 carried out a one-step condensation of a diketide *N*-acetylcysteaminethioester and (2*RS*)-methylmalonyl-CoA to produce a methylated triketide styrylpyrone.[7]

Recombinant *A. arborescens* OKS was expressed in *E. coli*, and purified by Ni-chelate affinity chromatography as described before.[3,8] When incubated with (2*RS*)-methylmalonyl-CoA as a sole substrate, *A. arborescens* OKS efficiently afforded a single product (UV λ_{max} 292 nm) (Fig. 1A).[9] The LC–ESIMS spectrum gave a parent ion peak $[M+H]^+$ at *m*/*z* 169, suggesting that the reaction had terminated after two condensations of (2*RS*)-methylmalonyl-CoA, and in MS/MS (precursor ion at *m*/*z* 169), the fragment at *m*/*z* 125 corresponded to $[M+H-CO_2]^+$, suggesting the presence of an α-pyrone ring. Further, the ^1H NMR spectrum of the product obtained from a large scale enzyme reaction (80% yield from 4 mg of (2*RS*)-methylmalonyl-CoA) revealed signals at δ 2.54 (q, 2H, $J = 7.5$ Hz), 1.21 (t, 3H, $J = 7.5$ Hz), 1.98 (s, 3H), and 1.96 (s, 3H), indicating the presence of one ethyl

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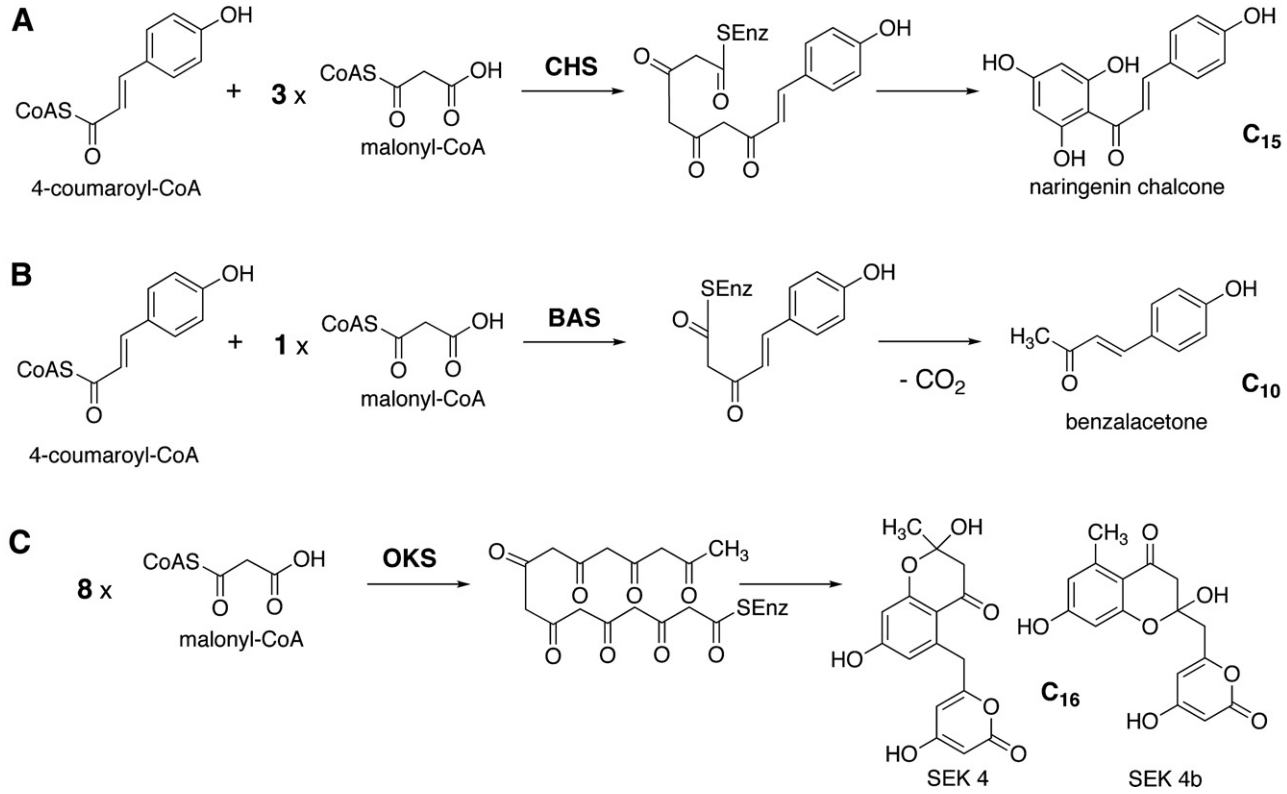


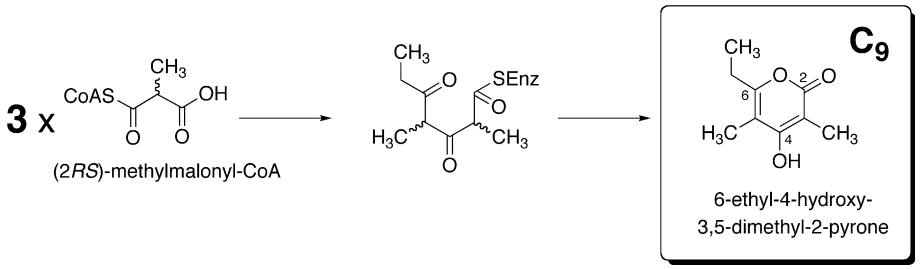
Scheme 1. Proposed mechanism for the formation of (A) naringenin chalcone from 4-coumaroyl-CoA and three molecules of malonyl-CoA by CHS, (B) benzalacetone from 4-coumaroyl-CoA and one molecule of malonyl-CoA by BAS, and (C) SEK4 and SEK4b from eight molecules of malonyl-CoA by OKS.

group and two methyl groups. The spectroscopic data (LC–ESIMS, UV, MS, and ^{1}H NMR)[10] were identical with those of 6-ethyl-4-hydroxy-3,5-dimethyl-2-pyrone,[11] which was chemically synthesized by condensation of three molecules of propionyl chloride.[11a] The structure of the enzyme reaction product was thus confirmed to be 6-ethyl-4-hydroxy-3,5-dimethyl-2-pyrone, which was consistent with biogenetic reasoning (Scheme 2). Interestingly, the compound has been reported from the fungus *Emericella heterothallica*,[11b] however, it has never been isolated from any plant sources including *A. arborescens*.

The C_{16} octaketides-producing *A. arborescens* OKS, normally catalyzing condensation of eight molecules of malonyl-CoA, thus accepted the bulky (2*RS*)-methylmalonyl-CoA, both as a ***starter*** and an ***extender*** substrate, and carried out sequential decarboxylative condensation to produce the methylated C_9 triketide pyrone (Scheme 2). This is the first demonstration of the enzymatic formation of 6-ethyl-4-hydroxy-3,5-dimethyl-2-pyrone by the structurally simple plant-specific type III PKS.

In addition to *A. arborescens* OKS, a C_{15} tetraketide-producing *S. baicalensis* CHS and a C_{10} diketide-producing *R. palmatum* BAS also accepted (2*RS*)-methylmalonyl-CoA as a ***starter*** substrate and efficiently yielded the unnatural methylated C_9 triketide pyrone as a single product by sequential decarboxylative condensations of three molecules of (2*RS*)-methylmalonyl-CoA (Fig. 1B and C).[8,9] Here, CHS is a pivotal enzyme for flavonoid biosynthesis, catalyzing the formation of naringenin chalcone from 4-coumaroyl-CoA and three molecules of malonyl-CoA (Scheme 1A), whereas BAS carries out a one-step decarboxylative condensation of 4-coumaroyl-CoA with malonyl-CoA to produce a diketide benzalacetone (Scheme 1B). On the other hand, it has been reported that a bacterial modular type I PKS (a cell-free system of recombinant 6-deoxyerythronolide B synthase; DEBS 1 + TE) produced 6-ethyl-4-hydroxy-3,5-dimethyl-2-pyrone from propionyl-CoA, as a ***starter***, and (2*RS*)-methylmalonyl-CoA, as an ***extender***, in the absence of NADPH.[12] In this case, (2*RS*)-methylmalonyl-CoA was not accepted as a ***starter*** substrate by the bacterial modular type I PKS.

It is remarkable that the plant-specific type III PKSs accept (2*RS*)-methylmalonyl-CoA, with an additional bulky methyl group, both as a ***starter*** and an ***extender*** substrate, and catalyze the formation of the non-physiological methylated C_9 triketide pyrone. This demonstrated the further tremendous catalytic potential of the CHS-superfamily type III PKS enzymes. Manipulation of the functionally divergent type III PKSs by utilizing non-physiological substrates as active-site probes would thus lead to further production of unnatural



Scheme 2. Formation of 6-ethyl-4-hydroxy-3,5-dimethyl-2-pyrone by sequential decarboxylative condensations of three molecules of (2*RS*)-methylmalonyl-CoA.

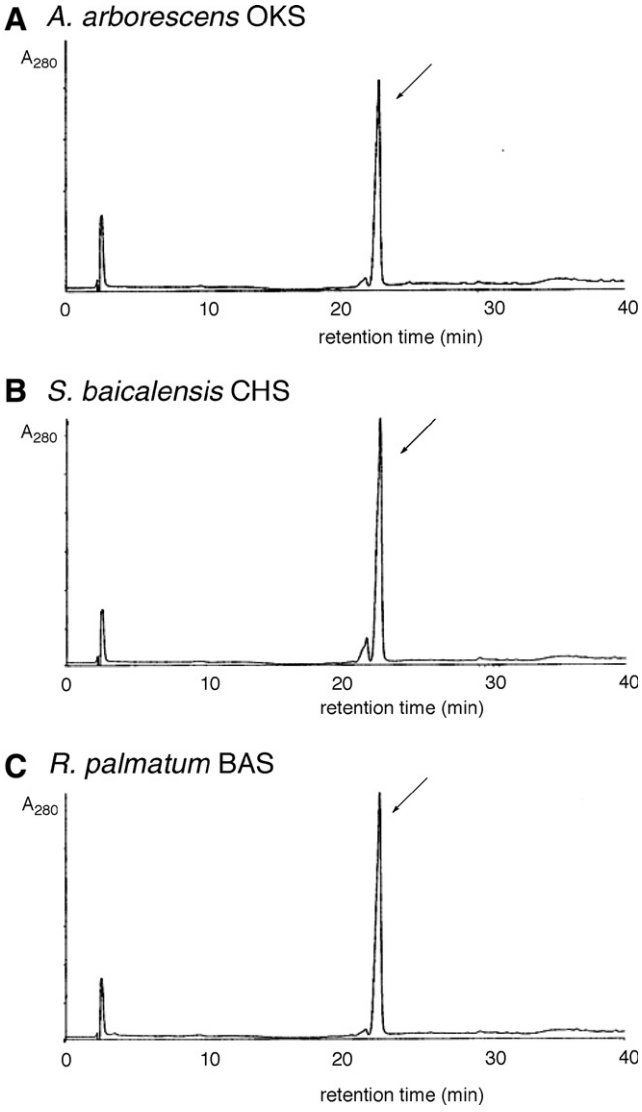


Figure 1. HPLC elution profiles of enzyme reaction products of (A) *A. arborescens* OKS, (B) *S. baicalensis* CHS, and (C) *R. palmatum* BAS.

novel poyketides, which is now in progress in our laboratories.

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8. Recombinant *A. arborescens* OKS,[3] *S. baicalensis* CHS,[4a] and *R. palmatum* BAS[6] 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; OKS (95.0 μM and 0.094 min^{-1} for malonyl-CoA), CHS (36.1 μM and 1.26 min^{-1} for 4-coumaroyl-CoA), and BAS (10.0 μM and 1.79 min^{-1} for 4-coumaroyl-CoA).
9. The reaction mixture contained (2*RS*)-methylmalonyl-CoA (108 μM) and the purified recombinant enzyme (20 μg) in 100 mM potassium phosphate buffer (500 μL, pH 8.0), containing 1 mM EDTA. Incubations were carried out at 30 °C for 12 h, and stopped by addition of 20% HCl (50 μL). The products were then extracted with ethyl acetate (1 mL), and separated by reverse-phase HPLC (column, TSK-gel ODS-80Ts, 4.6 × 150 mm, Tosoh Co. Ltd, Japan; flow rate, 0.8 mL/min). Gradient elution was performed with H_2O and MeOH, both containing 0.1% TFA: 0–5 min, 30% MeOH; 5–17 min, linear gradient from 30% to 60% MeOH; 17–25 min, 60% MeOH; 25–27 min, linear gradient from 60% to 70% MeOH. For large-scale enzyme reactions, (2*RS*)-methylmalonyl-CoA (4 mg) was incubated with purified recombinant enzyme (10 mg) in 100 mM phosphate buffer (40 mL, pH 8.0), containing 1 mM EDTA at 30 °C for 18 h.
10. LC–ESIMS: *m*/*z* 169 $[M+H]^+$. MS/MS (precursor ion at *m*/*z* 169): *m*/*z* 125 $[M+H-CO_2]^+$. UV: λ_{max} 292 nm. ^1H NMR (400 MHz, CD_3OD): δ 2.54 (q, 2H, $J = 7.5$ Hz), 1.98 (s, 3H), 1.96 (s, 3H), 1.21 (t, 3H, $J = 7.5$ Hz). MS (FAB^+): *m*/*z* 168 $[M]^+$, 140, 125 $[M+H-CO_2]^+$, 113. The spectroscopic data were identical with those reported in the literature.[11]
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