Substrate Specificity of Chalcone Synthase: Enzymatic Formation of Unnatural Polyketides from Synthetic Cinnamoyl-CoA Analogues

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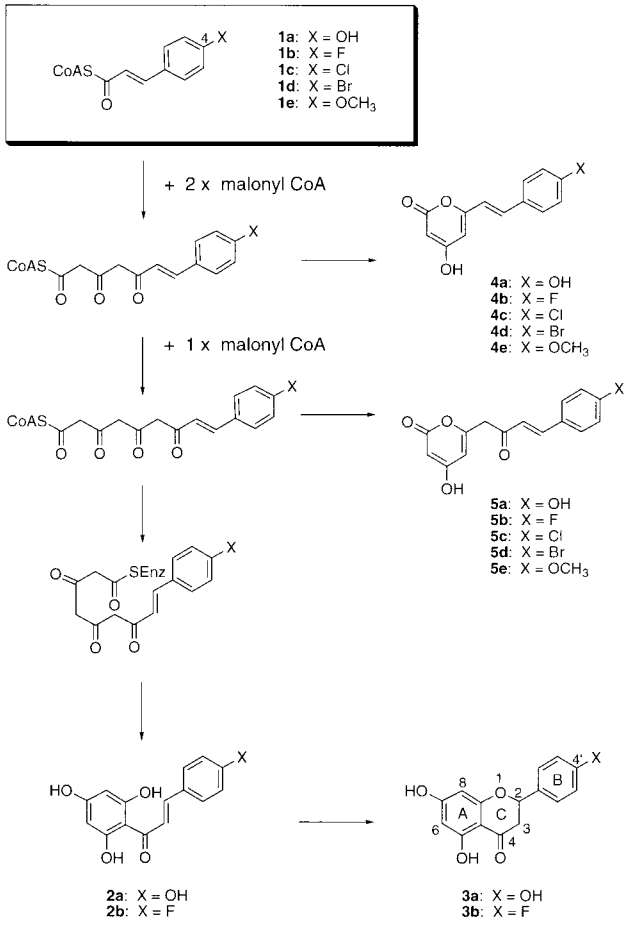
Chalcone synthase (CHS) (EC 2.3.1.74) is a plant specific polyketide synthase that plays a pivotal role in the biosynthesis of flavonoids.[1] CHS catalyzes a sequential condensation of the phenylpropanoid unit of *p*-coumaroyl-CoA (**1a**) as a starter with three two-carbon units from malonyl-CoA (Scheme 1). The reaction is thought to be initiated by binding of *p*-coumaroyl-CoA followed by formation of a coumaroyl thioester at Cys164 at the active site of the enzyme. After three rounds of stepwise condensation of acetate units from malonyl-CoA, cyclization and aromatization of the enzyme-bound tetraketide intermediate lead to formation of naringenin chalcone (**2a**). Naringenin chalcone is then converted to (−)-(2*S*)-5,7,4′-trihydroxyflavanone (naringenin) by chalcone isomerase; however, in the absence of the enzyme, chalcone spontaneously forms racemic (2*RS*)-naringenin (**3a**) through a nonstereospecific ring-C closure (Scheme 1).[2]

CHSs, along with other plant CHS-like enzymes including stilbene synthase, have been cloned from more than 40 plant species, and many of them have been functionally expressed in *E. coli*.[1] CHS functions as a homodimer of a 42 kDa polypeptide. The recently reported three-dimensional crystal structure of CHS from alfalfa (*Medicago sativa*) revealed that the dimer contains two functionally independent active sites: the coumaroyl-binding pocket and the cyclization pocket, defined by four residues conserved in all the known CHS-related enzymes (Cys164, Phe215, His303, and Asn336).[3] The coumaroyl-binding pocket has been proposed to lock the moiety in the position, while the cyclization pocket accommodates the elongating polyketide and this is where the cyclization and aromatization of the new ring takes place.[4]

In this paper, we report substrate specificity of CHS from *Scutellaria baicalensis*,[5,6] using chemically synthesized substrate analogues, and describe enzymatic formation of novel unnatural polyketides. First, we prepared *p*-coumaroyl-CoA analogues in which the 4-hydroxyl group was substituted by halogen or a methoxy group (**1b**−**e**) (Scheme 1) to test the effect of the substitution on the enzyme reaction. We then tested analogues in which the coumaroyl aromatic ring was replaced by heteroaromatic moieties, furan (**1f**) or thiophene (**1g**) (Scheme 2). The two-step synthesis of the cinnamoyl-CoA analogues (**1b**−**e**) involved generation of the *N*-hydroxysuccinimide esters of the corresponding 4-substituted cinnamic acid followed by a thioester exchange with CoA as originally described by Stöckigt and Zenk (see Supporting Information).[7] Analogue compounds **1f** and **1g** were prepared in the same way from commercially available *trans*-3-furanacrylic acid and *trans*-3-(3-thienyl)acrylic acid, respectively.

Scheme 1. Proposed Mechanism for the Conversion of *p*-Coumaroyl-CoA (**1a**) and Its Analogs (**1b**−**e**) to Chalcones (**2a**,**b**), Flavanones (**3a**,**b**), BNY-type (**4a**−**e**), and CTAL-type Compounds (**5a**−**e**)



When 4-fluorocinnamoyl-CoA (**1b**) was incubated with the recombinant CHS,[8] three products were isolated by HPLC. The first product showed a UV spectrum (λ_{max} 296 nm) similar to that of naringenin (**3a**), suggesting the structure of (2*RS*)-4′-fluoro-5,7-dihydroxyflavanone (**3b**) (Scheme 1). The LC-ESIMS spectrum gave a parent ion peak $[M - H]^-$ at *m*/*z* 273, and in MS/MS (precursor ion at *m*/*z* 273), the fragment at *m*/*z* 151 corresponded to $[M - H - C_6H_4F - C_2H_3]^-$. Furthermore, the ^{1}H NMR spectrum of **3b** obtained from a large scale incubation (20% yield from 10 mg of **1b**) showed two aromatic protons of

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(6) The deduced amino acid sequences of *S. baicalensis* CHS showed 77.4% (302/389) identity with those of the alfalfa (*M. sativa*) CHS. The recombinant enzyme with an additional hexahistidine tag at the C-terminal was expressed in *E. coli*, and purified by Ni-chelate chromatography as described before.[5] The enzyme showed $K_M = 36.1$ μM and $k_{cat} = 1.26$ min^{-1} for *p*-coumaroyl-CoA.

(7) Stöckigt, J.; Zenk, M. H. *Z. Naturforsch.* **1975**, *30c*, 352−358.

(8) The standard reaction mixture contained 27 nmol of cinnamoyl-CoA analogue, 54 nmol of malonyl-CoA, 105 pmol of purified recombinant CHS, and 1 mM EDTA in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 7.5. Incubations were carried out at 30 °C for 1 h, and stopped by adding 50 μL of 20% HCl. After extraction with ethyl acetate, reaction products were analyzed by reverse-phase HPLC and LC-ESIMS. For large-scale reactions, 4-fluorocinnamoyl-CoA (**1b**) (10.0 mg, 10.9 μmol), *trans*-3-furanacryloyl-CoA (**1f**) (10.0 mg, 11.3 μmol), or *trans*-3-(3-thienyl)acryloyl-CoA (**1g**) (10.0 mg, 11.1 μmol) were respectively incubated with 56 mg of purified recombinant CHS in 400 mL of 100 mM phosphate buffer containing malonyl-CoA (20.0 mg, 23.1 μmol) and 1 mM EDTA at 30 °C for 3 h.

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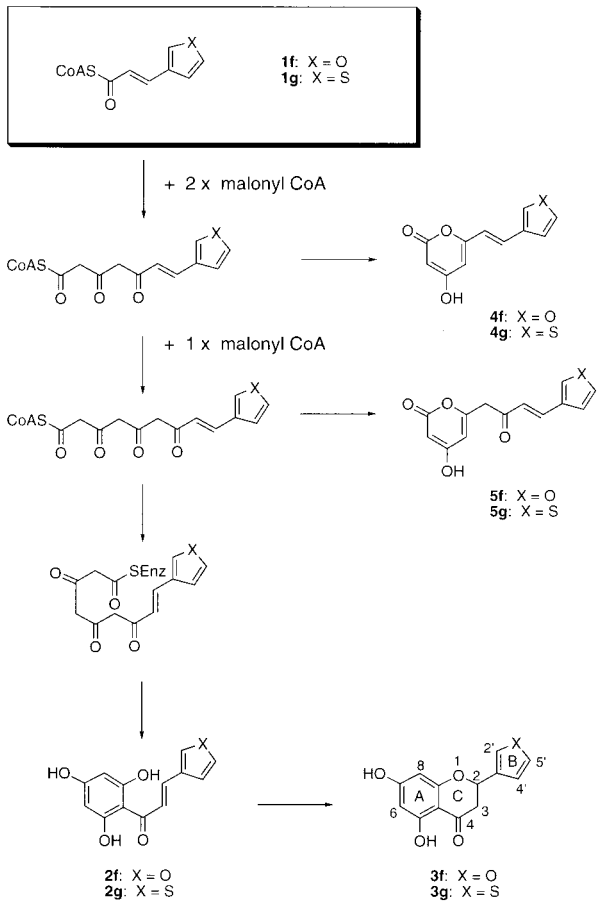
Scheme 2. Proposed Mechanism for the Conversion of *p*-Coumaroyl-CoA Analogs **1f** and **1g**



ring-A (δ 6.01 and 5.99) and two α-carbonyl H-3 protons (δ 3.17 and 2.81) coupled to H-2 proton (δ 5.58), confirming the structure.

The other two products respectively showed a UV spectrum similar to that of bis-noryangonin (BNY) (**4a**)[9] and *p*-coumaroyltriacetic acid lactone (CTAL) (**5a**),[10] thus suggesting the structures of **4b** and **5b** (Scheme 1). The pyrone derivatives, BNY and CTAL, have been known to be common byproducts of CHS enzyme reactions *in vitro* when the reaction mixtures are acidified before extraction.[10] The LC-ESIMS spectrum of BNY-type compound **4b** gave a parent ion peak $[M - H]^-$ at *m*/*z* 231, indicating the reaction had terminated after two condensation reactions of malonyl-CoA, and in MS/MS (precursor ion at *m*/*z* 231), the fragment at *m*/*z* 187 corresponded to $[M - H - CO_2]^-$, consistent with the presence of a α-pyrone ring. While the LC-ESIMS of CTAL-type compound **5b** gave a parent ion peak $[M - H]^-$ at *m*/*z* 273, indicating the successive condensation of three units of malonyl-CoA, in MS/MS (precursor ion at *m*/*z* 273), the fragment at *m*/*z* 229 corresponded to $[M - H - CO_2]^-$, indicating the presence of a α-pyrone ring. The reaction was therefore terminated without aromatic ring formation.

4-Fluorocinnamoyl-CoA (**1b**) was thus enzymatically converted to fluorinated flavanone **3b** along with the pyrone byproducts **4b** and **5b**. In contrast, incubation of other 4-substituted analogues (**1c**−**e**, X = Cl, Br, and OCH_3) did not yield flavanones, but afforded only the BNY- and CTAL-type products (Scheme 1). It appears that the steric and/or electronic perturbations by the substituents (4-Cl, Br, or OCH_3) larger in size than that of the natural substrate (4-OH) may alter the stability of the enzyme-bound tetraketide intermediate or the optimally folded conformation in the cyclization pocket of the active site of the enzyme.

Interestingly, CHS accepted analogues **1f** and **1g**, in which the coumaroyl ring was replaced by heteroaromatic moieties, furan or thiophene, leading to formation of novel polyketides **3f** and **3g** (Scheme 2). From large-scale incubations, **3f** and **3g** were obtained in 22% and 28% yield, respectively. The LC-ESIMS spectrum of **3f** gave a parent ion peak $[M - H]^-$ at *m*/*z* 245, and in MS/MS (precursor ion at *m*/*z* 245), the fragment at *m*/*z* 151 corresponded to $[M - H - C_4H_3O - C_2H_3]^-$. Further, the ^1H NMR spectrum of **3f** showed two aromatic protons of ring-A (δ 5.86 and 5.84) coupled to each other, and two α-carbonyl H-3 protons (δ 3.00 and 2.72) coupled to the H-2 proton of ring-C (δ 5.43), clearly confirming the structure. In a similar manner, the structure of **3g** was determined. In addition, as in the case of other analogues, incubation of **1f** and **1g** also afforded the pyrone byproducts (Scheme 2).

This is the first demonstration of the enzymatic formation of novel, unnatural polyketides containing the heteroaromatic ring moiety. In particular, it was noteworthy that the enzyme accepted the CoA ester with furan or thiophene smaller in size than the *p*-coumaroyl moiety, and locked in the coumaroyl-binding pocket, the enzyme-bound thioester efficiently initiated the subsequent chain elongation and aromatization reactions. Presumably, the $\pi-\pi$ interaction between the substrate and the active site aromatic residue (e.g. Phe215)[4] would play an important role for guiding the course of the reaction. On the other hand, CHSs have been reported to also accept aliphatic CoA esters (e.g. hexanoyl-CoA and isovaleryl-CoA) as starter molecules to produce phloroacylphenones.[11,12] In contrast, as described, 4-substituted cinnnamoyl-CoA analogues (**1c**−**e**) only afforded pyrone derivatives, suggesting that the steric size of the substrate is crucial for the optimally folded conformation of the polyketide intermediate in the cyclization pocket.

Finally, since as medicinal natural products phenylpropanoids such as chalcone and stilbene exhibit cancer chemopreventive, anti-mitotic, estrogenic, anti-malarial, and anti-asthmatic activities,[3] the observed enzyme reaction products may show a promising profile of important biological activities. Manipulation of the biosynthesis of phenylpropanoids by synthetic analogues would thus lead to development of the chemical library of pharmaceutically important novel polyketides.

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Supporting Information Available: Chemical synthesis of substrate analogues, enzyme reaction conditions, HPLC and LC-ESIMS procedure, and a complete set of spectroscopic data for all the synthetic and the enzyme reaction products (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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