Biochemical and Biophysical Research Communications **279,** 190–195 (2000)
doi:10.1006/bbrc.2000.3920, available online at http://www.idealibrary.com on IDEAL®

Enzymatic Formation of Unnatural Aromatic Polyketides by Chalcone Synthase

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Received November 6, 2000

Substrate specificity of recombinant chalcone synthase (CHS) from *Scutellaria baicalensis* (Labiatae) was investigated using chemically synthesized aromatic and aliphatic CoA esters. It was demonstrated for the first time that CHS converted benzoyl-CoA to phlorobenzophenone (2,4,6-trihydroxybenzophenone) along with pyrone by-products. On the other hand, phenylacetyl-CoA was enzymatically converted to an unnatural aromatic polyketide, phlorobenzylketone (2,4,6-trihydroxyphenylbenzylketone), whose structure was finally confirmed by chemical synthesis. Furthermore, in agreement with earlier reports, *S. baicalensis* CHS also accepted aliphatic CoA esters, isovaleryl-CoA and isobutyryl-CoA, to produce phloroacylphenones. In contrast, hexanoyl-CoA only afforded pyrone derivatives without formation of a new aromatic ring. It was noteworthy that both aromatic and aliphatic CoA esters were accepted in the active site of the enzyme as a starter substrate for the complex condensation reaction. The low substrate specificity of CHS thus provided further insight into the structure and function of the enzyme.

Key Words: **chalcone synthase; polyketide synthase; substrate specificity; phenyl propanoid; benzophenone; phlorophenone; aromatic polyketide.**

Chalcone synthase (CHS) (EC 2.3.1.74) is a plant specific polyketide synthase that catalyzes a condensation of the C_6–C_3 unit of *p*-coumaroyl-CoA (**1a**) with three C_2 units from malonyl-CoA to produce a new aromatic ring system (Scheme 1) (1). The reaction is initiated by binding of *p*-coumaroyl-CoA followed by formation of a thioester at the active site cystein residue of the enzyme. After three rounds of sequential decarboxylative Claisen condensation, cyclization and aromatization of the enzyme-bound tetraketide intermediate lead to formation of naringenin chalcone (**2a**), the key intermediate in the biosynthesis of flavonoids. In addition to chalcone, two pyrone derivatives; bisnoryangonin (BNY) (**3a**) (2) and *p*-coumaroyltriacetic acid lactone (CTAL) (**4a**) (3) are formed as early released derailment by-products when the enzyme reaction is carried out *in vitro*.

CHS functions as a homodimer of a 41- to 44-kDa polypeptide and shares 65–75% amino acid sequence identity with other members of the plant CHS-superfamily enzymes including stilbene synthase (1) and 2-pyrone synthase (4). Recently reported crystal structure of CHS from *Medicago sativa* (Leguminosae) revealed that the dimer contains two functionally independent active sites; the coumaroyl-binding pocket and the cyclization pocket, defined by four residues (Cys164, Phe215, His303, and Asn336) conserved in all the known CHS-superfamily enzymes (5). 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 (6). To control the stereospecificity of the enzyme reaction, precise molecular interactions are required for the enzyme-substrate complexes. The structure-function relationship of the CHS-superfamily enzymes have thus elicited intense chemical and biological interest.

In the previous paper, we investigated substrate specificity of recombinant CHS from *Scutellaria baicalensis* (Labiatae) using chemically synthesized *p*-coumaroyl-CoA analogs (7). When 4-fluorocinnamoyl-CoA was incubated, a fluorinated chalcone along with pyrone by-products were obtained, while other 4-substituted analogs (4-Cl, Br, or OCH_3) afforded only pyrone derivatives. On the other hand, analogs in which the coumaroyl aromatic ring was replaced by furan or thiophene were efficiently converted to novel unnatural polyketides containing the heteroaromatic ring. It was thus demonstrated that the steric and/or electronic perturbations by the substituents may alter the stability of the enzyme-bound tetraketide interme-

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0006-291X/00 $35.00

diate or the optimally folded conformation in the cyclization pocket of the active site of the enzyme.

In continuation of the study, we now report enzymatic conversion of benzoyl-CoA (**1b**) and phenylacetyl-CoA (**1c**) to unnatural aromatic polyketides by recombinant *S. baicalensis* CHS. In an early report, benzoyl-CoA has been shown to be accepted as a substrate by CHS from cell suspension cultures of *Petroselium crispum* (formally called *Petroselium hortense*) (Umbelliferae), however, the enzyme reaction products had not been identified (8). Further, in addition to the aromatic CoA esters, we also reexamined substrate specificity of the enzyme toward aliphatic CoA esters for which enzymatic conversion to phloroacylphenones have been reported (8, 9).

MATERIALS AND METHODS

Chemicals

Benzoyl-CoA (**1b**) was chemically synthesized according to the reported method (10). Thus, the two-step synthesis involved generation of the *N*-hydroxysuccinimide esters followed by a thioester exchange with Coenzyme A. Phenylacetyl-CoA (**1c**), hexanoyl-CoA (**1d**), isovaleryl-CoA (**1e**), isobutyryl-CoA (**1f**), and malonyl-CoA were purchased from Sigma.

Phlorobenzophenone (2,4,6-trihydroxybenzophenone) (**2b**) and phlorobenzylketone (2,4,6-trihydroxyphenylbenzylketone) (**2c**) were respectively synthesized by Friedel-Crafts acylation of phloroglucinol (1.1 eq) with phenylacetyl chloride (1.0 eq) or benzoylchloride (1.0 eq) in the presence of $AlCl_3$ (1.0 eq).

Recombinant Enzyme

The CHS used in this study was cloned from young leaves of *S. baicalensis* (11). The recombinant enzyme with an additional hexahistidine tag at the C-terminal was subcloned into pET-22b(+) (Novagen), expressed in *E. coli*, and purified by Ni-chelate affinity chromatography as described before. Thus, *E. coli* BL21(DE3)pLysS harboring the plasmid was cultured to an A_{600} of 0.6 in LB medium containing 100 μg/mL of ampicillin at 30°C. Then, 0.4 mM IPTG (final concentration) was added to induce protein expression, and the culture was incubated further at 30°C for 16 h. Cells were collected by centrifugation and resuspended in 50 mM K-phosphate buffer, pH 8.0, containing 1 M NaCl. Cell lysis was carried out by the freeze-thaw method, and centrifuged at 25,000*g* for 20 min. The supernatant was passed through a column of Pro-Bond resin (Invitrogen) in which Ni^{2+} was retained as an affinity ligand. After washing with 50 mM K-phosphate buffer, pH 6.0, containing 1 M NaCl and 30 mM imidazole, the recombinant CHS was eluted with 15 mM K-phosphate buffer, pH 6.5, containing 10% glycerol and 500 mM imidazole. Finally, the enzyme preparation was desalted by Bio-Gel P6DG Desalting gel. The purified enzyme showed the K_M = 36.1 μM and k_{cat} = 1.26 min^{-1} for *p*-coumaroyl-CoA.

Enzyme Reaction

The standard reaction mixture contained 27 nmol of substrate analog (**1b**–**1f**), 54 nmol of malonyl-CoA, and 105 pmol of the purified recombinant CHS in a final volume of 500 μL of 100 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA. Incubations were carried out at 30°C for 1 h, and stopped by adding 50 μL of 20% HCl. The products were then extracted with 800 μL of ethyl acetate, and concentrated by N_2 flow. The residue was dissolved in aliquot of MeOH containing 0.1% TFA, and separated by HPLC as described below.

For large-scale enzyme reactions, benzoyl-CoA (**1b**) (11.2 mg, 11.9 μmol), or phenylacetyl-CoA (**1c**) (10.0 mg, 11.3 μmol), were respectively incubated with 15 mg of purified recombinant CHS in 200 mL of 100 mM phosphate buffer, pH 7.5, containing malonyl-CoA (20.0 mg, 23.1 μmol) and 1 mM EDTA at 30°C for 3 h. The reactions were quenched by addition of 20% HCl (15 mL), and extracted with ethyl acetate (200 mL × 2). Extracts were dried over sodium sulfate and evaporated to dryness. After HPLC separation, phlorobenzophenenone **2b** (0.14 mg, yield 5.2%) or phlorobenzylketone **2c** (0.12 mg, 4.4%) were obtained, respectively.

HPLC and HPLC–ESIMS

The enzyme reaction products were separated by reverse-phase HPLC (JASCO 880, JASCO) on a TSK-gel ODS-80Ts column (4.6 × 150 mm, TOSOH) with a flow rate of 0.8 ml/min. Gradient elution was performed with H_2O and MeOH, both containing 0.1% TFA. The gradient profiles were as follows: 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. Elutions were monitored by a multichannel UV detector (MULTI 340, JASCO) at 290, 330, and 360 nm; UV spectra (198–400 nm) were recorded every 0.4 s.

On-line HPLC–ESIMS spectra were measured with a Hewlett–Packard HPLC 1100 series (Wilmington, DE) coupled to a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA) fitted with an ESI source. HPLC separations were carried out under the same conditions as described above. The ESI capillary temperature and capillary voltage were 275°C and 3.0 V, respectively. The tube lens offset was set at 20.0 V. All spectra were obtained in the negative and positive mode; over a mass range of *m/z* 120–350, at a range of one scan every 2 s. The collision gas was helium, and the relative collision energy scale was set at 30.0% (1.5 eV).

Spectroscopic Data for the Enzyme Reaction Products

*Products of benzoyl-CoA (**1b**).* Phlorobenzophenone (**2b**): HPLC: R_t = 19.2 min. LC–ESIMS: MS, *m/z* 231 $[M + H]^+$, 229 $[M - H]^-$, MS/MS (precursor ion at *m/z* 231), *m/z* 185 (29), 171 (18), 153 (100), 105 (13). UV: λ_{max} 313 nm. ^1H NMR (400 MHz, $(CD_3)_2CO$): δ 7.58 (2H, m), 7.48 (1H, m), 7.38 (2H, m), 6.00 (2H, s). Compound **3b:** HPLC: R_t = 21.0 min. LC–ESIMS: MS, *m/z* 189 $[M + H]^+$, 187 $[M - H]^-$, MS/MS (precursor ion at *m/z* 187), *m/z* 143 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 325 nm. Compound **4b:** HPLC: R_t = 17.0 min. LC–ESIMS: MS, *m/z* 231 $[M + H]^+$, 229 $[M - H]^-$, MS/MS (precursor ion at *m/z* 229), *m/z* 185 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 288 nm.

*Products of phenylacetyl-CoA (**1c**).* Phlorobenzylketone (**2c**): HPLC: R_t = 25.9 min. LC–ESIMS: MS, *m/z* 245 $[M + H]^+$, 243 $[M - H]^-$, MS/MS (precursor ion at *m/z* 245), *m/z* 227 (100), 203 (50), 161 (34), 153 (2), 91 (19). UV: λ_{max} 294 nm. ^1H NMR (400 MHz, $(CD_3)_2CO$): δ 7.28 (4H, m), 7.21 (1H, m), 5.94 (2H, s), 4.42 (2H, s). Compound **3c:** HPLC: R_t = 20.9 min. LC–ESIMS: MS, *m/z* 203 $[M + H]^+$, 201 $[M - H]^-$, MS/MS (precursor ion at *m/z* 201), *m/z* 157 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 294 nm. Compound **4c:** HPLC: R_t = 17.5 min. LC–ESIMS: MS, *m/z* 245 $[M + H]^+$, 243 $[M - H]^-$, MS/MS (precursor ion at *m/z* 243), *m/z* 199 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 289 nm.

*Products of hexanoyl-CoA (**1d**).* Compound **3d:** HPLC: R_t = 26.1 min. LC–ESIMS: MS, *m/z* 183 $[M + H]^+$, 181 $[M - H]^-$, MS/MS (precursor ion at *m/z* 181), *m/z* 137 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 294 nm. Compound **4d:** HPLC: R_t = 21.2 min. LC–ESIMS: MS, *m/z* 225 $[M + H]^+$, 223 $[M - H]^-$, MS/MS (precursor ion at *m/z* 223), *m/z* 179 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 291 nm.

*Products of isovaleryl-CoA (**1e**).* Phlorisovalerophenone (**2e**): HPLC: R_t = 27.0 min. LC–ESIMS: MS, *m/z* 211 $[M + H]^+$, 209 $[M - H]^-$, MS/MS (precursor ion at *m/z* 211), *m/z* 193 (37), 165 (4), 155 (100), 123 (1). UV: λ_{max} 288 nm. Compound **3e:** HPLC: R_t = 20.2 min.

LC–ESIMS: MS, *m/z* 169 $[M + H]^+$, 167 $[M - H]^-$, MS/MS (precursor ion at *m/z* 167), *m/z* 123 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 295 nm. Compound **4e:** HPLC: R_t = 16.9 min. LC–ESIMS: MS, *m/z* 211 $[M + H]^+$, 209 $[M - H]^-$, MS/MS (precursor ion at *m/z* 209), *m/z* 165 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 288 nm.

*Products of isobutyryl-CoA (**1f**).* Phlorisobutyrophenone (**2f**): HPLC: R_t = 22.5 min. LC–ESIMS: MS, *m/z* 197 $[M + H]^+$, 195 $[M - H]^-$, MS/MS (precursor ion at *m/z* 197), *m/z* 179 (100), 151 (50), 109 (1). UV: λ_{max} 294 nm.. Compound **3f:** HPLC: R_t = 16.4 min. LC–ESIMS: MS, *m/z* 155 $[M + H]^+$, 153 $[M - H]^-$, MS/MS (precursor ion at *m/z* 153), *m/z* 109 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 295 nm.. Compound **4f:** HPLC: R_t = 12.0 min. LC–ESIMS: MS, *m/z* 197 $[M + H]^+$, 195 $[M - H]^-$, MS/MS (precursor ion at *m/z* 195), *m/z* 151 $[M - H\text{-}CO_2]^-$. UV: λ_{max} 289 nm.

RESULTS AND DISCUSSION

When benzoyl-CoA (**1b**) and malonyl-CoA were incubated with the recombinant *S. baicalensis* CHS overexpressed in *E. coli,* three products were isolated by HPLC (Fig. 1B). The first product showed a UV spectrum (λ_{max} 313 nm) similar to that of benzophenone, suggesting the structure of phlorobenzophenone (2,4,6-trihydroxybenzophenone) (**2b**) (Scheme 1). The LC–ESIMS spectrum gave a parent ion peak $[M - H]^-$ at *m/z* 229, and the ^{1}H NMR spectrum of **2b** obtained from large scale incubation (5.2% yield from 11 mg of **1b**) showed seven aromatic protons (δ 7.3–7.6, 5H, and δ 6.00, 2H, s), supported the structure. It was finally confirmed by direct comparison (MS, ^{1}H NMR) with synthetic compound which was obtained by Friedel-Crafts acylation of phloroglucinol with benzoylchloride.

The other two products, **3b** and **4b,** respectively showed a UV spectrum similar to that of bisnoryangonin (BNY) (**3a**) and *p*-coumaroyltriacetic acid lactone (CTAL) (**4a**), the early released derailment by-products of CHS enzyme reactions *in vitro* when the reaction mixtures are acidified before extraction. The LC–ESIMS spectrum of the BNY-type compound **3b** gave a parent ion peak $[M - H]^-$ at *m/z* 187, indicating the reaction had terminated after two condensation reactions of malonyl-CoA, and in MS/MS (precursor ion at *m/z* 187), the fragment at *m/z* 143 corresponded to $[M - H\text{-}CO_2]^-$, consistent with the presence of a α-pyrone ring. Enzymatic formation of **3b** from benzoyl-CoA and two units of malonyl-CoA has been reported for 2-pyrone synthase from *Gerbera hybrida* (Asteraceae) (4). On the other hand, the CTAL-type compound **4b** gave a parent ion peak $[M - H]^-$ at *m/z* 229, supporting the successive condensation of three units of malonyl-CoA, and in MS/MS (precursor ion at *m/z* 229), the fragment at *m/z* 185 corresponded to $[M - H\text{-}CO_2]^-$, confirming the presence of α-pyrone ring. The enzyme reaction was therefore terminated without aromatic ring formation.

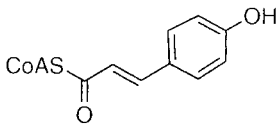
It was for the first time demonstrated that CHS also catalyzed condensation reaction of benzoyl-CoA (**1b**) with three units of malonyl-CoA to form a new aromatic ring system of phlorobenzophenone **2b.** Here it should be noted that benzophenone and its derivatives (e.g., xanthones) have not been isolated from *S. baicalensis.* Further, in addition to *S. baicalensis* (Labiatae) CHS, recombinant CHSs from *Pueraria lobata* (Leguminosae) and *Ipomea purpurea* (Convolvulaceae) also converted benzoyl-CoA to phlorobenzophenone (data not shown). On the other hand, benzophenone synthases from *Centaurium erythraea* (12) and *Hypericum androsaemum* (Hypericaceae) (13), have been shown to catalyze the condensation of 3-hydroxybenzoyl-CoA with three units of malonyl-CoA to produce 2,3′,4,6-tetrahydroxybenzophenone, which is a key intermediate in the biosynthesis of xanthones. Benzophenone synthase is another member of the CHS-superfamily enzymes that does not accept *p*-coumaroyl-CoA as a starter substrate. The structural difference of the active site of the enzyme between CHS and benzophenone synthase is not well understood yet.

Interestingly, *S. baicalensis* CHS also accepted phenylacetyl-CoA (**1c**) to produce a novel unnatural polyketide **2c** (Fig. 1C). The LC–ESIMS spectrum of **2c** gave a parent ion peak $[M - H]^-$ at *m/z* 243, and the ^{1}H NMR spectrum obtained from large scale incubation (4.4% yield from 10 mg of **1c**) showed seven aromatic protons (δ 7.2–7.3, 5H, and δ 5.94, 2H, s) and two benzyl protons (δ 4.42, 2H, s), suggesting the structure of phlorobenzylketone (2,4,6-trihydroxyphenylbenzylketone) (**2c**), which was finally confirmed by direct comparison (MS, ^{1}H NMR) with chemically synthesized compound obtained by Friedel-Crafts acylation of phloroglucinol with phenylacetylchloride. In addition to phlorobenzylketone, phenylacetyl-CoA also afforded pyrone by-products **3c** and **4c** as in the case of other analogs.

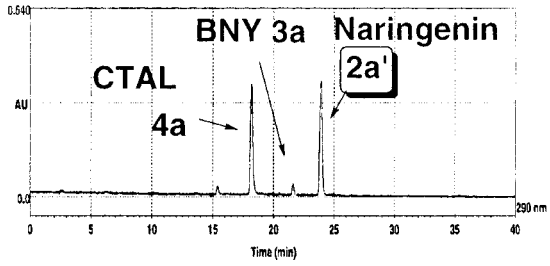
In earlier studies, CHSs have been shown to accept aliphatic CoA esters; hexanoyl-CoA was enzymatically converted to phlorocaprophenones by CHS from cell suspension cultures of *Petroselium crispum* (formally called *Petroselium hortense*) (Umbelliferae) (8), while purified *Pinus sylvestris* (Pinaceae) CHS afforded phlorisovalerophenone and phlorisobutyrophenone respectively from isovaleryl-CoA and isobutyryl-CoA (9). In agreement with these reports, recombinant *S. baicalensis* CHS also converted isovaleryl-CoA (**1e**) and isobutyryl-CoA (**1f**) to phlorisovalerophenone (**2e**) and

FIG. 1. HPLC profile of the enzyme reaction products from malonyl-CoA and (A) *p*-coumaroyl-CoA (**1a**), (B) benzoyl-CoA (**1b**), (C) phenylacetyl-CoA (**1c**), (D) hexanoyl-CoA (**1d**), (E) isovaleryl-CoA (**1e**), and (F) isobutyryl-CoA (**1f**). Note that by acid treatment naringenin chalcone (**2a**) is converted to racemic naringenin (5,7,4′-trihydroxyflavanone) (**2a′**) through a non-stereospecific ring-C closure.

A



1a



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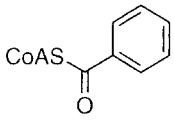

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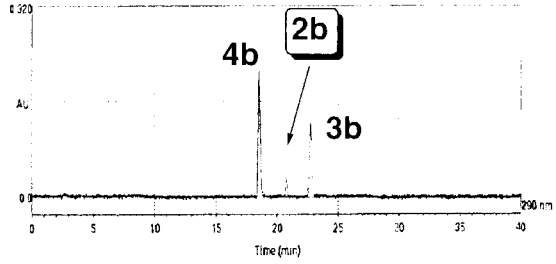

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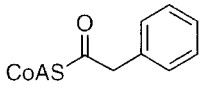

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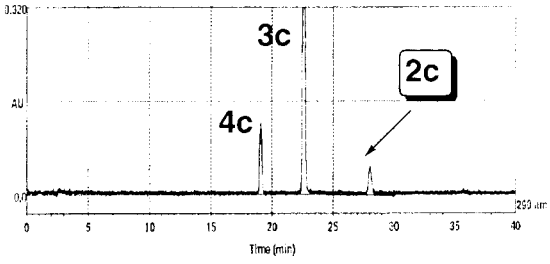

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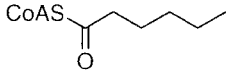
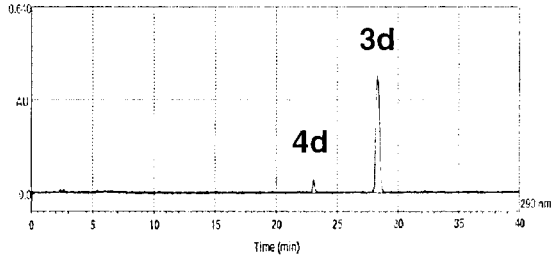


1d



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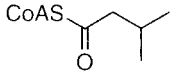

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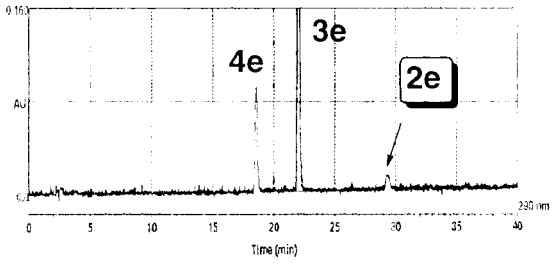

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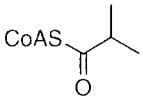

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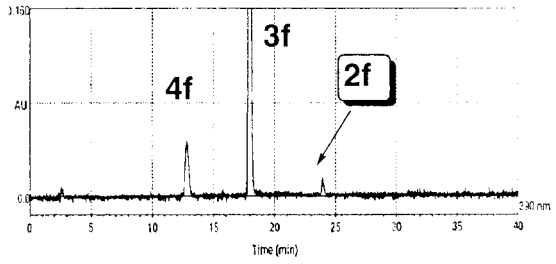

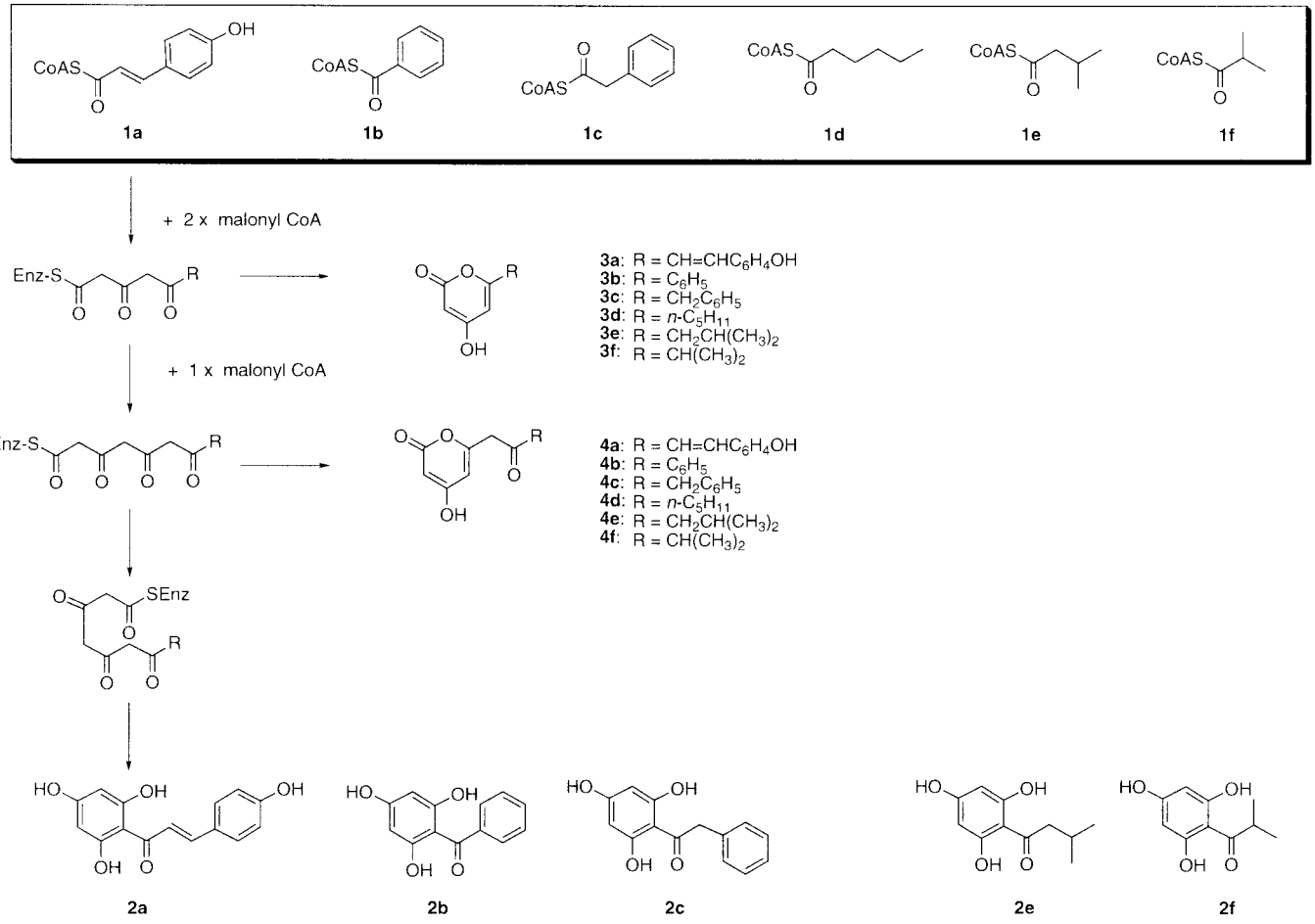

SCHEME 1. Proposed mechanism for the enzymatic conversion of *p*-coumaroyl-CoA (**1a**) and its analogs (**1b**–**1f**) to naringenin chalcone (**2a**), phlorophenones (**2b**–**2f**), BNY-type (**3a**–**3f**), and CTAL-type by-products (**4a**–**4f**).

phlorisobutyrophenone (**2f**), respectively (Figs. 1E and 1F). In contrast, interestingly, hexanoyl-CoA (**1d**) only afforded pyrone derivatives **3d** and **4d;** formation of phlorocaprophenone was not detected in our assay system (Fig. 1D). This may suggest species difference between *S. baicalensis* and *P. crispum* in the active site structure of the enzyme.

The low substrate specificity of CHS for the nonphysiological substrates is remarkable. In particular, it was noteworthy that the enzyme accepted both aromatic and aliphatic CoA esters, smaller in size than the natural substrate, as a starter for the complex condensation reaction. With locked in the coumaroyl-binding pocket of the active site, the enzyme-bound thioester efficiently initiated the subsequent chain elongation and guided the course of the cyclization and aromatization reactions. In contrast, as reported in our previous paper, 4-substituted coumaroyl-CoA analogs (4-Cl, Br, or OCH_3), larger in size than that of *p*-coumaroyl-CoA, only afforded pyrone derivatives without formation of a new aromatic ring. The steric and/or electronic perturbations may thus 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. Further, it should be also noted that even for the smaller starter substrates, the chain elongation always stopped at three rounds of condensation of malonyl-CoA; no evidence was found for enzyme reaction products with more than four units of malonyl-CoA, suggesting the rigid steric requirement for the cyclization pocket in the active site of the enzyme. To obtain further insight into the structure and function of the enzyme, synthesis of other substrate analogs as well as site-directed mutagenesis experiments based on the crystal structure of the enzyme are now in progress.

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