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4-HYDROXY-2-PYRONE FORMATION BY CHALCONE AND STILBENE SYNTHASE WITH NONPHYSIOLOGICAL SUBSTRATES

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Key Word Index—*Pinus sylvestris*; Scots pine; *Humulus lupulus*; hop; biosynthesis; chalcone; stilbene; phlorisovalerophenone; phlorisobutyrophenone; 4-hydroxy-2-pyrone.

Abstract—Valerophenone synthase (VPS) is a polyketide synthase that catalyzes the formation of the phloroglucinol derivatives in the synthesis of the bitter acids in hop (*Humulus lupulus*). The reaction uses isovaleryl-CoA or isobutyryl-CoA, but otherwise it is identical to that of the chalcone synthase in flavonoid biosynthesis. Our study showed that chalcone synthase can perform the function of VPS, but not perfectly, because the majority of the reactions terminated after two condensation reactions (products: 4-hydroxy-2-pyrone derivatives). The same experiments with stilbene synthase yielded exclusively the 4-hydroxy-2-pyrone derivatives, not the products expected from three condensation reactions. The results are discussed in the context of the functional diversity and evolution in the family of CHS-related polyketide synthases.

INTRODUCTION

Chalcone synthase (CHS) (EC 2.3.1.74) synthesizes the chalcone backbone that is the precursor for a large number of biologically important products. The reaction is complex, involving a starter CoA-ester from the phenylpropanoid pathway, three extension reactions by condensations with malonyl-CoA and the formation of a new aromatic ring system from a linear tetraketide intermediate (Fig. 1). The reactions of stilbene synthases (STS) are identical up to the tetraketide stage, but a different aromatic ring system is formed and the terminal carboxyl group is removed (Fig. 1). CHS and STS are closely related (>65% protein identity). They are members of a plant-specific polyketide synthase family that also includes the acridone synthase which performs a CHS-type reaction with *N*-methylanthraniloyl-CoA (reviewed in Ref. [1]).

CHS-type reactions are also carried out by enzymes in other pathways of plant secondary metabolism. An interesting example is the valerophenone synthase (VPS, Fig. 2) which uses isovaleryl-CoA and isobutyryl-CoA to synthesize phlorisovalerophenone (**9**) and phlorisobutyrophenone (**10**), the intermediates in the biosynthesis of the bitter acids in hop (*Humulus lupulus*) [2].

The experiments in this work aim at understanding the protein changes involved in the evolution of the functional diversity in the family of CHS-related polyketide synthases. The functional similarities with CHS suggest that VPS is a candidate for being a member of the family of CHS-related proteins. It was therefore an interesting question whether typical CHS would accept the VPS substrates to synthesize the same products. Potentially even more intriguing was the question whether or not a STS would accept the VPS substrates to synthesize the products expected from a STS-type ring-folding.

RESULTS AND DISCUSSION

Figure 2 shows the reaction performed by VPS with isovaleryl-CoA or isobutyryl-CoA to syn-

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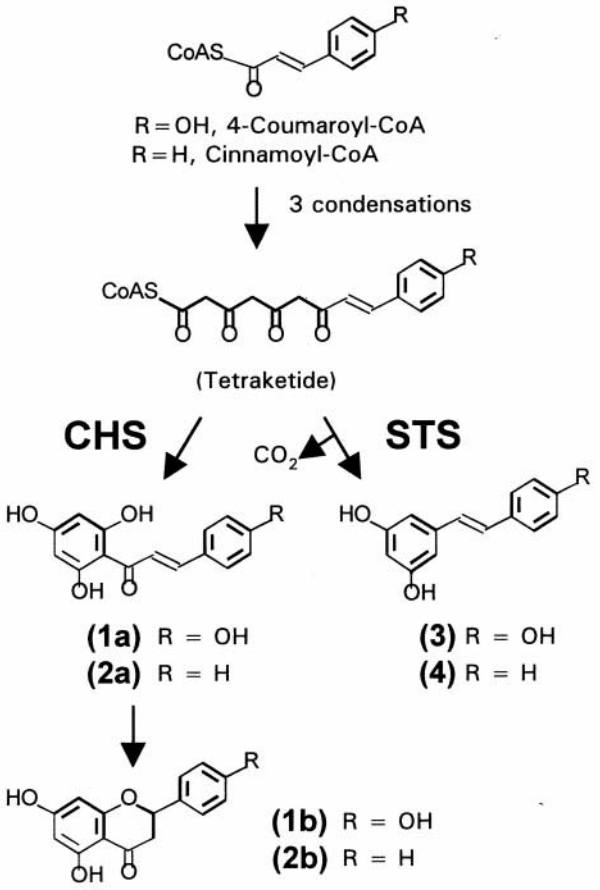

Fig. 1. Reactions of CHS and STS. Both perform with CoA-esters from the phenylpropanoid pathway three condensation reactions, followed by the formation of new aromatic rings. The difference is in the type of ring-folding and in the decarboxylation performed by STS.

thesize phlorisovalerophenone (**9**) and phlorisobutyrophenone (**10**). Because it was not clear whether CHS would perform the complete reaction with the non-physiological substrates, we also considered the products expected from reactions that prematurely terminated after only one (**5**, 1-isobutylethanone; **6**, 1-isopropylethanone) or two condensation reactions (**7**, 6-isobutyl-4-hydroxy-2-pyrone; **8**, 6-isopropyl-4-hydroxy-2-pyrone). The structures correspond to the compounds described as major byproducts of CHS reactions with phenylpropanoid starter substrates under non-optimal assay conditions [3–6]. The experiments were performed with CHS from plants that are not known to contain secondary products derived from these substrates. We used the enzymes after heterologous expression in *E. coli* and this excluded possible effects by other plant components or by the presence of isoenzymes that are often present in plant preparations.

Figure 3 shows TLC radioscans of the products obtained with purified *Pinus sylvestris* CHS. A single product (**1b**, naringenin under the assay conditions, see Section 3) was identified from the physiological substrate 4-coumaroyl-CoA and no significant amounts of byproducts from a premature termination of the reaction were present. The scan for the products from isovaleryl-CoA is representative for all experiments with the unphysiological substrates, including those with crude extracts. The scan revealed three peaks of radioactivity. The material remaining at the origin of the chromatogram could not be identified. The amount varied in independent experiments, and it may represent a mixture of polymerized products. The substance in the second peak (R_f 0.64–0.69) comigrated with authentic phlorisovalerophenone (**9**), the product expected from a VPS reaction. It also comigrated with **9** in the HPLC analysis (Fig. 4) and the identity was confirmed by comparing the UV-spectra and the M_r obtained with LC/MS. The data are summarized in Table 1. The radioactive substance in the third peak (R_f 0.8–0.85) represented a new product. It was also detected in the HPLC analysis [Fig. 4(A)]. Its UV spectrum and M_r (Table 1) indicated that it represented **7**, the product expected from two condensation reactions (Fig. 2). The CHS also accepted isobutyryl-CoA as starter substrate. The TLC analysis of radioactive products obtained with labelled malonyl-CoA also revealed two peaks (not shown), and these were also detected by HPLC

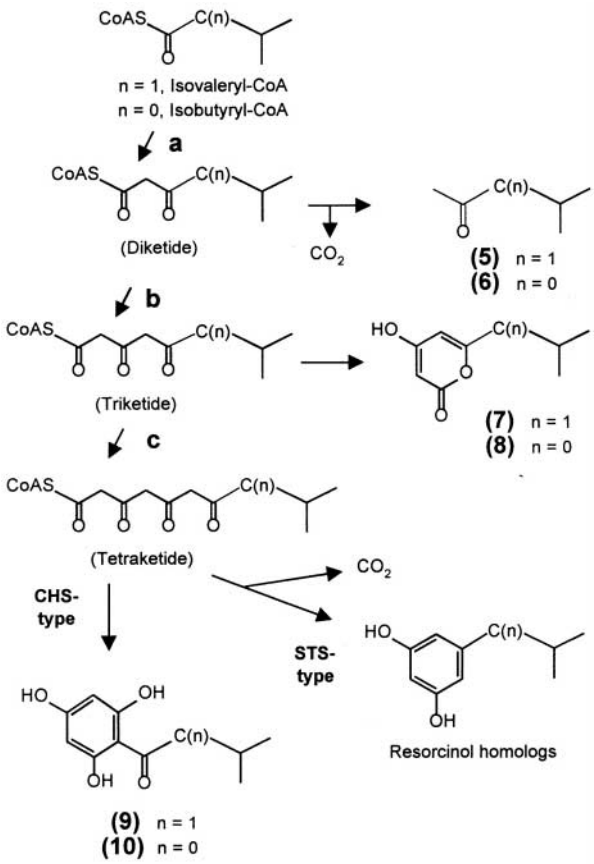

Fig. 2. Reactions of valerophenone synthase (VPS) with isovaleryl-CoA and isobutyryl-CoA. Also shown are the products expected from an STS reaction and the products expected from the termination of the reactions after one or two condensation reactions.

analysis [Fig. 4(B)]. The UV spectra and M_r values (Table 1) indicated that the two substances were **10** and **8**, i.e. the products expected from three and two condensations, respectively (Fig. 2).

The quantification of the data in Fig. 3 suggested a **9**/**7** ratio of *ca.* 0.6, but in terms of nmol synthesized product it was lower because three radioactive malonyl-CoA were incorporated into **9**, while only two were used in the formation of the pyrone derivative **7**. With isobutyryl-CoA as substrate, the ratio of the corresponding products, **10** and **8**, was even lower (see also Fig. 4).

The use of the non-physiological substrates was not a property unique to the CHS from *P. sylvestris*, because they were also accepted by the enzymes from *Pinus strobus* and *Sinapis alba*, and the same products were synthesized. In all cases the efficiency was lower than with the physiological substrates and isovaleryl-CoA was preferred against isobutyryl-CoA (Table 2).

A further characterization was carried out with the enzyme from *P. sylvestris*. The pH dependence for both the non-physiological substrates revealed the highest activity at pH 6 (25% increase against pH 7, 35% increase against pH 5.5) but the distribution between the two products was not significantly different. Therefore the subsequent incubations, in particular for the product identification, were performed at pH 6. The substrate dependence with isovaleryl-CoA (range from 2 to 20 μM) revealed in three independent experiments apparent K_m values of 4 ± 1 and 6 ± 3 μM for the formation of **9** and **7**, respectively, and the ratio between the two products did not vary significantly. These values are in the range determined for 4-coumaroyl-CoA with CHS from several other sources [7].

Previous experiments with the CHS from parsley (*Petroselinum crispum*) had already shown that the enzyme was active with aliphatic substrates (e.g. butyryl-CoA, hexanoyl-CoA) [6]. The phloroacylphenones were among the products, but other major products had not been identified. The biological relevance of our work is that isovaleryl-CoA and isobutyryl-CoA are the physiological substrates for a CHS-type reaction in hop. Our data provide evidence that CHS enzymes with the physiological role in flavonoid biosynthesis can function with aliphatic substrates as VPS and as pyrone synthase. With respect to the possible evolution of VPS, the

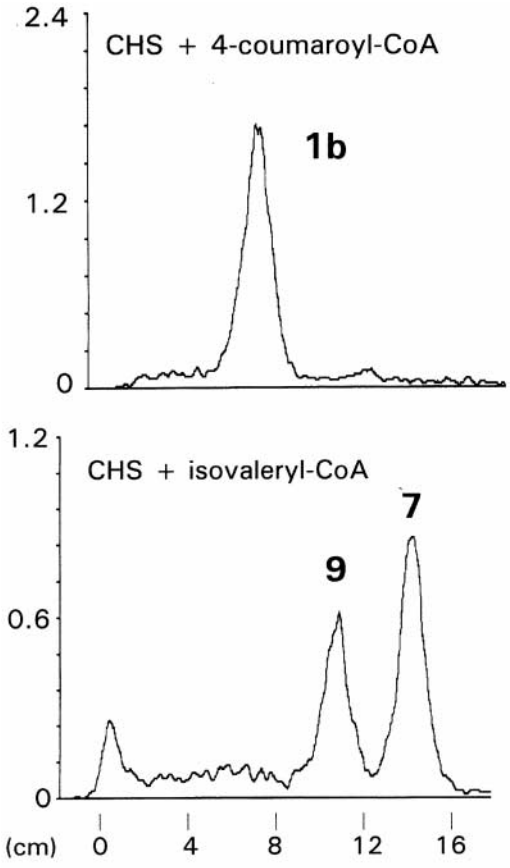

Fig. 3. TLC radioscan of the products of purified CHS from *P. sylvestris* with the physiological substrate 4-coumaroyl-CoA (top) and with isovaleryl-CoA (bottom). The numbers at the ordinate indicate dpm $\times 10^{-3}$. The incubations were performed at pH 7, the optimal pH for the physiological substrate.

results suggest that a change in substrate preference and an optimization towards the performance of three condensation reactions would be sufficient to convert a CHS into a VPS. It has been proposed that the present-day STS developed from CHS several times independently by relatively small modifications of the protein [8] and this involved an important functional change to obtain a new type of ring-folding and a decarboxylation (Fig. 1). Given the capacities of CHS with the aliphatic substrates, it could be argued that the conversion of a CHS into VPS would require less dramatic changes and peptide sequences obtained from purified VPS indeed suggest a close relationship to typical CHS (Paniego *et al.*, Leiden University, unpublished).

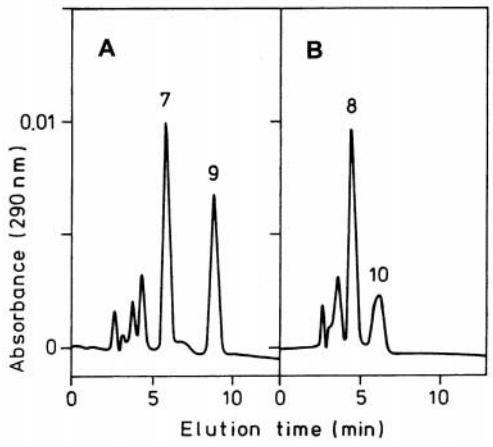

Fig. 4. HPLC elution profiles obtained for the products from CHS with non-physiological substrates. (A) Isovaleryl-CoA and (B) isobutyryl-CoA. See Experimental Section for assay and HPLC conditions.

Table 1. Properties of the products obtained with CHS and STS. The structures are shown in Figs 1 and 2. The M_r and the UV maxima (nm) were determined by LC/MS and HPLC-PDA, respectively

Enzyme	Substrate	Product	M_r	UV max.
CHS	4-coumaroyl-CoA	**1b***	272	290
	isovaleryl-CoA (a)	**7**	168	282
	isovaleryl-CoA (b)	**9***	210	288
	isobutyryl-CoA (a)	**8**	154	280
	isobutyryl-CoA (b)	**10***	196	282
STS	cinnamoyl-CoA	**4**	212	304
	isovaleryl-CoA	**7**	168	282
	isobutyryl-CoA	**8**	154	280

*Confirmed with reference compound.

In view of the findings with CHS it was an interesting question whether STS would accept the aliphatic substrates to form the products expected from a STS-type ring-folding (Fig. 2). The products from reactions terminating after only one or two condensations would be the same as with CHS, because the difference between the two enzymes is after the third condensation reaction (Fig. 1). The experiments were performed with the STS from *P. sylvestris* cloned for heterologous expression in *E. coli*.

Figure 5 shows TLC radioscans of the products obtained with purified STS. The product with the

Table 2. Activities of three cloned CHS with different starter CoA-esters. The assays were performed with lysates from *E. coli* cells expressing the enzymes. The incubations were performed at pH 7, the optimal pH for the physiological substrates. Starter substrates 10 μM, radioactive malonyl-CoA 16 μM. The incubations were stopped after 20 min at 37°C

Substrate	Relative activities (%) with the CHS from		
	P. sylvestris	*P. strobus*	*S. alba*
4-Coumaroyl-CoA	100	90	100
Cinnamoyl-CoA	80	100	70
Isovaleryl-CoA*	30	37	51
Isobutyryl-CoA*	18	22	27

*Sum of 4-hydroxy-2-pyrone and phloroacylphenone derivatives.

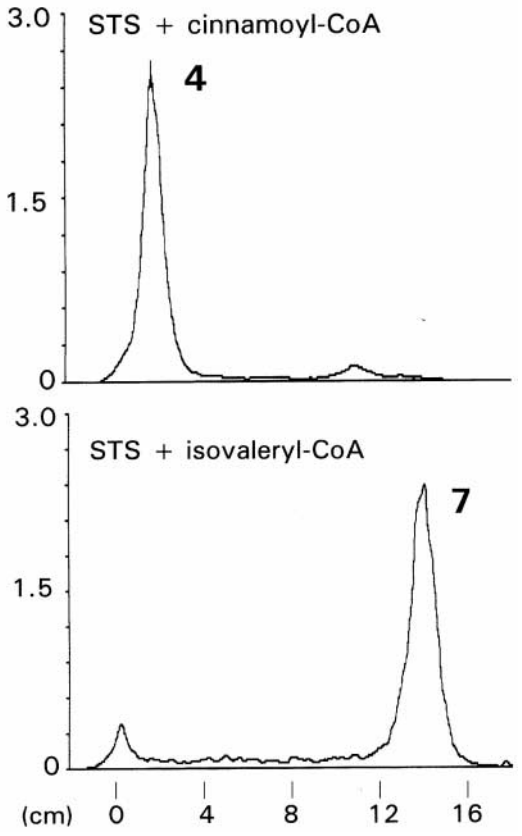

Fig. 5. TLC radioscan of the products of purified STS from *P. sylvestris* with the physiological substrate cinnamoyl-CoA (top) and with isovaleryl-CoA (bottom). The numbers at the ordinate indicate dpm $\times 10^{-3}$. The incubations were performed at pH 7, the optimal pH for the physiological substrate.

physiological substrate cinnamoyl-CoA [9] was pinosylvin (**4**, Fig. 1), and only small amounts of possible byproducts were detectable. The incubations with isovaleryl-CoA led to two radioactive peaks. As with CHS, the material remaining at the origin of the chromatogram could not be identified. The dominant product migrated to the same position as the 4-hydroxypyrone derivative obtained from the CHS reaction and the HPLC-PDA and LC/MS analysis (Table 1) confirmed that the product of the STS reaction was **7**. The experiments with isobutyryl-CoA also revealed a single dominant peak of radioactivity (not shown), and the product analysis indicated that it was the compound **8** expected from two condensation reactions. A comparison of the efficiency of the different starter CoA-esters indicated a ratio of 1:0.7:0.3 between cinnamoyl-CoA, isovaleryl-CoA and isobutyryl-CoA, respectively. The value for isovaleryl-CoA is comparable to that of 4-coumaroyl-CoA which is a phenylpropanoid derivative, but not a physiological substrate for the STS from *P. sylvestris*. The pH dependence for the reaction with isovaleryl-CoA revealed no large differences between pH 6.0 and 7.0, with decreasing activities at pH values higher than 7. These experiments also showed that the pyrone derivatives were poorly extracted at pH values higher than 7 and extraction at low pH was necessary to obtain the products. The material remaining at the origin of the chromatograms increased simultaneously, suggesting that the high pH during the incubation led to the opening of the pyrone ring and polymerization of the resulting triketide. It seems likely that this also explains the presence of this material in the experiments with CHS.

The substrate dependence with isovaleryl-CoA (concentration range from 2 to 20 μM) indicated in three independent experiments an apparent K_m of 3 ± 1.5 μM. The product was in all cases the pyrone, and no significant amount of other radioactive substances was detected. The K_m was comparable to the values determined with cinnamoyl-CoA for the *P. sylvestris* STS (0.2 to 2 μM [9]), for the closely related pinosylvin synthase from *P. strobus* (0.5 to 2.0 μM) [10] and for the resveratrol-forming STS from other plants that prefer 4-coumaroyl-CoA against cinnamoyl-CoA [7, 8, 11].

The exclusive formation of the pyrones by STS with the aliphatic substrates was unexpected and we questioned whether the assay conditions were non-optimal with respect to the substrates. The standard assays, as in most of the literature on CHS and STS [7], used a ratio of malonyl-CoA to starter CoA-ester of 1.6, i.e. lower than the value of three which is the actual use of the substrates in an STS-type reaction. Therefore we tested with the purified enzyme whether increased malonyl-CoA concentrations or other variations in the ratio of chain extender and starter substrate led to additional products. None were detected but the results unexpectedly showed that increasing the isovaleryl-CoA concentration (e.g. to a ratio of 0.8) even improved the yield of the pyrone product. The same effect was observed with 4-coumaroyl-CoA as substrate and the formation of resveratrol (**3**) which requires three condensation reactions. The reasons are not understood but the results argue against the possibility that the pyrone formation was due to a non-optimal ratio between chain extender and starter substrate.

The STS was a pyrone synthase with the aliphatic substrates and the majority of the CHS products also were pyrones. 4-Hydroxy-2-pyrone derivatives are wide-spread but the biosynthetic enzymes have rarely been demonstrated. One example is the 6-styryl-4-hydroxy-2-pyrone synthase (SPS) identified in gametophytes of *Equisetum arvense* [12]. The enzyme uses phenylpropanoid starter CoA-esters but it is not yet known whether the protein is related to CHS. Recent data, however, directly demonstrated that a CHS-related protein from *Gerbera hybrida* [13] is a pyrone synthase (Schröder and collaborators, unpublished). It has *ca.* 70%

identity with typical CHS from the flavonoid pathway but is a 4-hydroxy-2-pyrone synthase specific for small hydrophobic starter CoA-esters. Those substrates are also accepted by the STS from *P. sylvestris* and the same products are formed (Schröder and collaborators, unpublished). Taken together, these findings suggest the possibility that the synthesis of the 4-hydroxy-pyrone backbone in other secondary plant products may be catalyzed by enzymes belonging to the family of CHS-related proteins.

EXPERIMENTAL

Chemicals

[2-^{14}C]Malonyl-CoA (55 mCi/mmol) was obtained from Biotrend (Cologne, Germany). The other CoA-esters were from the laboratory collection or were purchased from Sigma Biochemicals (Germany).

CHS and STS expression plasmids

The proteins were obtained from *E. coli* cells expressing the enzymes. In all cases the protein-coding regions of the cDNAs [8, 10, 14] had been inserted via an introduced *Nco*I-site into the *Nco*I-site of vector pQE-6 [8, 15], thus retaining the optimal promoter-translation-start configuration of the vector. The cDNA for the *P. strobus* CHS will be described elsewhere. The plasmids were maintained in *E. coli* strain RM82 that contained an additional plasmid (pUBS520) with the *argU* (*dnaY*) gene that improves the expression of eucaryotic proteins [16] (a gift from R. Mattes, Stuttgart, FRG).

Enzyme purification

The purification could be followed by visual inspection of the fractions stained with Coomassie-Serva Blue R250 after SDS gel-electrophoretic separation because the proteins constituted *ca.* 10% (CHS) or *ca.* 2% (STS) of the total soluble protein in crude extracts. The harvested *E. coli* cells were resuspended in 0.1 M Tris–HCl (pH 8) containing 1 mM dithiothreitol, broken in a French pressure cell (1018 psi) and centrifuged to remove insoluble material (20 min, 20,000*g* at 4°C).

The purifications used four steps in various combinations: (a) fractionated ammonium sulfate precipitation; with the optimal fractions for CHS (60 to 80% saturation) and STS (50 to 65% saturation), (b) hydrophobic interaction chromatography (Fractogel EMD Butyl 650 S, Merck, Germany), (c) size exclusion chromatography (Fractogel EMD BioSEC S, Merck, Germany) and (d) anion exchange chromatography (Fractogel EMD DEAE 650 S, Merck, Germany). The procedures followed the recommendations supplied by the manufacturer. All buffers contained 1 or 2 mM dithiothreitol. CHS was purified through all four steps to apparent homogeneity; it eluted in step (b) at 0.7 to 0.8 M ammonium sulfate (gradient from 1.5 to 0 M) and in step (d) at 80 mM NaCl (step gradient from 0 to 1 M). STS was purified with step (d), with peak elution at 0.2 M NaCl. The enzyme was *ca.* 90% pure.

Enzyme assays with radioactive malonyl-CoA and TLC quantification

The preparation of the extracts from *E. coli* cells expressing CHS or STS has been described [17]. Standard incubations (0.1 ml) contained 50 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N*′[2-ethanesulfonic acid]) buffer (pH 7.0), 10 μM starter CoA-ester, 16 μM [2-^{14}C]malonyl-CoA (73,300 dpm per assay), and 2 to 5 μg protein. They were stopped after 20 or 30 min at 37°C. With CHS and 4-coumaroyl-CoA as substrate, the incubations were followed by a treatment at pH 9 to convert the chalcone non-enzymatically into the isomeric flavanone [18]. The products were extracted into EtOAc and subjected to quantitative TLC [18] with 20% HOAc as solvent. The amount of enzyme and the length of incubation were chosen not to exceed the incorporation of 25% of the radioactive substrate. The experiments with purified enzymes were performed with the assay conditions optimized for reactions with the aliphatic starter CoA-esters (pH 6).

Enzyme assays for product detection with HPLC photo diode array (HPLC-PDA) and LC/MS

Volume 0.3 ml; 0.1 M HEPES buffer (pH 6 for CHS, pH 7 for STS), 20 μM isovaleryl-CoA or isobutyryl-CoA, 16 μM unlabelled malonyl-CoA, and 7 to 10 μg purified protein. After incubation at 30°C for 30 min, the reaction was stopped by the addition of 0.4 ml EtOAc. Product extraction was performed by thoroughly mixing the sample, centrifugation for at least 2 min and isolation of the EtOAc layer which contained the products. The extraction was performed twice. The samples were pooled, evaporated to dryness using a vacuum concentrator and the residue was dissolved in 50 μl MeOH (HPLC photodiode array detection) or 25 μl MeOH (LC/MS).

HPLC photodiode array detection (HPLC-PDA)

The system consisted of an LKB Model 2150 HPLC pump, a Waters 600 E system controller, a Waters 712 WISP injector and a Waters 991 photo diode array detector. A reversed phase column [Hypersil 5 μm C18, 250 × 4.6 mm (i.d.)] was used. The injection volume was 40 μl. Detection was performed using a range of 250–390 nm. The mobile phase consisted of MeOH–H_2O–H_3PO_4 (70:34:0.25) (CHS) or MeOH–H_2O–H_3PO_4 (60:40:0.25) (STS). A flow rate of 1 ml/min was used.

LC/MS

The LC-negative ion electrospray (ES) mass spectra were obtained from a Finnigan MAT TSQ-70 (San Jose, CA) triple quadrupole mass spectrometer equipped with a custom made electrospray interface. The stainless steel sampling capillary and ion-source were optimized with respect to sensitivity and kept at 250 and 200°C, respectively. Although the LC/MS experiments were performed in ionspray mode, the optimization of the mass spectrometer was performed in the constant infusion mode, with orcinol and resveratrol as reference compounds, dissolved in a mixture of MeOH–H_2O (80:20) containing 1% HOAc to a concentration of 10 μg/ml and infused with a flow-rate of 2 μl/min by means of a Harvard 2400 syringe pump. For electrical contact a liquid sheath flow of a mixture of MeOH–H_2O (80:20) with 1% HOAc at 2 μl/min was used. This optimization was possible because ion-source parameters such as repeller and tube-lens voltages were independent of ionization mechanisms. Best sensitivity could be obtained in negative ionization mode scanning from m/z 100 to 300 scanning for $[M–H]^-$ and $[M + CH_3COO]^-$ of the compounds of interest.

Both using ionspray and APCI, the isocratic HPLC system consisted of a Model 2150 high pressure pump (LKB, Bromma, Sweden), a Rheodyne 7120 injection valve (Cotati, CA) with a 20 μl loop, a Phenomenex Hypersil 5 C_{18}, 150 × 4.6 mm column and a Waters UV-detector (Waters Associates, Milford, MA) which was installed in-line (254 nm). The mobile phase consisted of a mixture of MeOH–H_2O (60:40) containing 1% HOAc at a flow rate of 1 ml/min.

For ionspray the needle assembly was kept at −3 kV towards the grounded sampling capillary. For best performance, 25 μl of the delivered flow-rate from the HPLC system was directed to the MS by means of a splitting device. For electrical contact 1 μl/min of a mixture of MeOH–H_2O (80:20, v/v) with 1% HOAc was used as a liquid sheath flow (6 l/min). APCI was performed with a coaxial pneumatic nebulizer. The effluent flow of the HPLC system (1 ml/min) was dispersed with helium (6 l/min). The APCI-heater temperature was optimized and kept at 400°C. The corona needle was kept at −3 kV, towards the grounded sampling capillary.

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