Benzophenone synthase from cultured cells of *Centaurium erythraea*

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Abstract A central step in xanthone biosynthesis is the formation of the C_{13} skeleton, i.e. an intermediate benzophenone. Biosynthesis of 2,3',4,6-tetrahydroxybenzophenone from *m*-hydroxybenzoyl-CoA and malonyl-CoA was shown in cell-free extracts from cultured cells of *Centaurium erythraea*. The enzyme catalyzing this reaction was named benzophenone synthase.

Key words: Benzophenone synthase; *Centaurium erythraea*; Xanthone biosynthesis; *m*-Hydroxybenzoyl-CoA

1. Introduction

Benzophenones are the immediate precursors of xanthones in higher plants, as shown by feeding experiments with differentiated plant organs [1–3]. In addition, 2,3',4,6-tetrahydroxybenzophenone was recently found to be converted regioselectively to isomeric xanthones in cell-suspension cultures of *Centaurium erythraea* and *Hypericum androsaemum* (Peters, Schmidt and Beerhues, unpublished results). The two plant species studied belong to the Gentianaceae and the Hypericaceae from which the majority of xanthones have been isolated [4].

The cell culture of *C. erythraea* accumulates 3,5,6,7,8-pentamethoxy-1-*O*-primeverosylxanthone [5]. A central step in the proposed biosynthetic pathway of this constituent is the formation of the C_{13} skeleton, i.e. an intermediate benzophenone [6].

Feeding experiments with intact plants of *Garcinia mangostana* (Hypericaceae) have shown that *m*-hydroxybenzoic acid is an efficient precursor of xanthone biosynthesis [3]. This appeared to be also true for xanthone formation in cultured cells of *C. erythraea* because these accumulate, when treated with a yeast extract, 1,5-dihydroxy-3-methoxyxanthone [6] the 5-hydroxy group of which corresponds to the 3-hydroxy group of *m*-hydroxybenzoic acid. These findings led us to synthesize the CoA ester of *m*-hydroxybenzoic acid and to incubate it with malonyl-CoA and a protein extract from *C. erythraea*, resulting in the enzymic formation of 2,3',4,6-tetrahydroxybenzophenone.

2. Materials and methods

2.1. Cell culture

The cell-suspension culture of *C. erythraea* was grown as described earlier [5].

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Abbreviations: DTT, dithiothreitol; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

2.2. Chemicals

Benzoyl-CoA and malonyl-CoA were purchased from Sigma (Deisenhofen, Germany). 2,4,6-Trihydroxybenzophenone was obtained from ICN (Meckenheim, Germany). *p*-Hydroxybenzoyl-CoA was a kind gift of Dr. Heide (Tübingen, Germany).

2.3. Analytical procedures

HPLC was carried out on Multospher RP-8 (5 μm, i.d. 0.4×25 cm) at a flow rate of 1 ml min^{-1} using H_2O (A) and MeOH (B) as solvents. After isocratic elution at 30% B for 3 min a gradient from 30 to 50% B in 17 min was employed. Detection was at 306 nm.

TLC was carried out on silica gel 60 F_{254} coated aluminium sheets (Merck, Darmstadt, Germany). The solvents were chloroform/ethanol (9:1 v/v) and toluol/ethyl acetate/acetic acid (25:20:1, v/v). When cellulose plates (Merck) were used the solvent was 15% (v/v) ethanol. Protein concentration was measured according to [7].

2.4. Enzyme extraction

Cells (20 g) were collected by suction filtration, mixed with 2 g Polyclar AT (Serva, Heidelberg, Germany) and homogenized in 20 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 1 mM DTT (buffer A). After centrifugation at 10000×*g* for 15 min the supernatant was subjected to fractionated ammonium sulphate precipitation. The protein precipitating between 50 and 80% saturation was resuspended in buffer A to give a final volume of 2.5 ml. After passage through a PD_{10} column (Pharmacia, Freiburg, Germany) equilibrated with buffer A the protein solution was used for the enzyme assays. The procedure was carried out at 0–4°C.

2.5. Enzyme assay

The incubation assay contained in a total volume of 1 ml 830 μl of 0.1 M potassium phosphate buffer, pH 7.5, 100 μl protein solution (containing up to 100 μg protein), 30 μl of 0.23 mM *m*-hydroxybenzoyl-CoA solution and 40 μl of 0.47 mM malonyl-CoA solution to give final substrate concentrations of 6.8 and 18.7 μM, respectively. After incubation at 40°C for 30 min the reaction mixture was extracted twice with 1 ml ethyl acetate. The organic phases were combined and evaporated to dryness. The residue was taken up in 50 μl methanol and analyzed by HPLC. For large-scale assays the incubation volume was increased 50-fold.

2.6. Synthesis of hydroxybenzoic acid CoA esters

Synthesis of *m*-hydroxybenzoyl-CoA was performed according to [8]. The intermediate *N*-hydroxysuccinimide ester was chromatographed twice on a silica gel column (2.8×50 cm) using chloroform/methanol (20 : 1, v/v) and ethyl acetate as solvents. Fractions containing the intermediate ester were pooled, combined and evaporated to dryness. UV λ_{max} (MeOH) nm: 224, 243, 307. EI-MS *m/z* (rel. int.): 235 $[M]^+$ (5), 121 (100; *m*-hydroxybenzoyl), 93 (41; hydroxyphenyl), 65 (20).

After transesterification which also followed the method of [8] the CoA ester was purified as described in [9]. A column of DEAE-Sephacel (Sigma, Deisenhofen, Germany; 2.6×11.5 cm) equilibrated with 0.1 M formic acid was used. Elution was performed with a gradient of 250 ml of 0.1 M formic acid and 250 ml of 2 M formic acid/NaOH, pH 3.5. Fractions of 160 drops were collected and the fractions containing the CoA ester (28–32) combined and passed through a column of Dowex 50 Wx8 (3×5 cm). The eluate was lyophilized and the CoA ester identified by UV, mass and ^{1}H-NMR spectroscopy. The UV spectrum was in accord with data reported by [10]. FAB-MS *m/z* 886 $[M-H]^-$. ^{1}H-NMR (500 MHz, D_2O) and COSY assignments: 0.83 (3 H, s), 0.96 (3 H, s), 2.47 (2 H, t, J=6.3 Hz), 3.17 (2 H, m, J=6.3), 3.46 (4 H, m, J=6.3/6.3), 3.65 (1 H, dd, J=9.9/4.0), 3.90 (1 H, dd, J=9.9/4.0), 4.04 (1 H, s), 4.31 (2 H, br s),

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Table 1
Effect of the DTT concentration on benzophenone synthase activity

DTT concentration (μM)	Relative enzyme activity (%)
0.0	0
6.3	49
12.5	66
18.8	83
25.0	100
50.0	98
100.0	98

4.01 (1 H, br s), 4.86 (1 H, m), 4.90 (1 H, m), 6.14 (1 H, d, J = 5.8), 7.04 (1 H, dd, J = 7.9/1.9, H-4), 7.19 (1 H, t, J = 1.9, H-2), 7.29 (1 H, t, J = 7.9, H-5), 7.35 (1 H, d, J = 7.9, H-6), 8.32 (1 H, s), 8.61 (1 H, s). The spectrum agreed with the spectroscopic properties reported previously [11].

p-Hydroxybenzoyl-CoA was synthesized in the same way. Its ^{1}H-NMR spectrum was in accord with that of *m*-hydroxybenzoyl-CoA, except the signals of the protons of the benzoyl moiety in the range of 6.9–7.9 ppm: 6.98 (1 H, dt, J = 7.9/0.8, H-5), 7.00 (1 H, dd, J = 7.9/0.8, H-3), 7.54 (1 H, dt, J = 7.9/1.5, H-4), 7.84 (1 H, dd, J = 7.9/1.5, H-6). It closely resembled the spectra of *N*-methylanthraniloyl-CoA except the N-CH_3 signal and of 2-aminobenzoyl-CoA [9,12].

3. Results

3.1. Detection of benzophenone synthase

m-Hydroxybenzoyl-CoA was synthesized via the *N*-hydroxysuccinimide ester [8] and its identity confirmed by UV, mass and ^{1}H-NMR spectroscopy. The CoA ester was incubated with malonyl-CoA and a desalted crude protein extract from 5-d-old cultured cells of *C. erythraea* that were expected from the growth curves [5] to contain high enzyme activity. The formation of an enzymic product was shown by HPLC analysis of the ethyl acetate soluble compounds extracted from the enzyme assay. When *m*-hydroxybenzoyl-CoA or malonyl-CoA were omitted from the incubation and when heat-denatured enzyme was used no product formation occurred. To increase the product yield the enzyme was enriched by fractionated ammonium sulphate precipitation. The protein fraction obtained between 50 and 80% saturation contained high enzyme activity and was used for further incubations.

The enzymic product was isolated from large-scale assays by HPLC and TLC and identified as 2,3′,4,6-tetrahydroxybenzophenone by UV, mass and ^{1}H-NMR spectroscopy. The spectra agreed with those of an authentic reference substance that had been chemically synthesized (Peters and Beerhues, unpublished results). Furthermore, this synthetic compound and the enzymic product exhibited the same R_f and R_t values (TLC in three solvent systems and HPLC).

3.2. Partial enzyme characterization

Benzophenone synthase activity was strictly dependent on dithiothreitol, a final concentration of 25 μM leading to maximum activity (Table 1). The most efficient substrate of benzophenone synthase was *m*-hydroxybenzoyl-CoA (Table 2). Benzoyl-CoA also served as a substrate and was converted to 2,4,6-trihydroxybenzophenone the identity of which was ascertained by UV spectroscopy and co-chromatography with an authentic reference compound (TLC in three solvent systems and HPLC). The pH optimum of the enzyme was at 7.5 and the temperature optimum at 40–45°C (Fig. 1). The high temperature optimum might be related to the occurrence of *C. erythraea* in dry grounds.

Table 2
Substrate specificity of benzophenone synthase

Substrate	Relative amount of benzophenone (%)
m-Hydroxybenzoyl-CoA	100
Benzoyl-CoA	44
o-Hydroxybenzoyl-CoA	0
p-Hydroxybenzoyl-CoA	0

Benzophenone formation was linear with time for nearly 15 min and with the protein concentration up to 100 μg in the standard assay. The specific enzyme activity was 3.0 μkat kg^{-1} protein in the crude extract and 15.0 μkat kg^{-1} protein in the 50–80% ammonium sulphate precipitated protein fraction. Using this for incubation the amount of product formed in the standard assay was about 0.5 μg.

Treatment of cultured *C. erythraea* cells with methyl jasmonate did not lead to an increase in benzophenone synthase activity.

4. Discussion

The formation of 2,3′,4,6-tetrahydroxybenzophenone was shown in cell-free extracts from cultured cells of *C. erythraea*. The enzyme catalyzing this reaction was detected for the first time and will be termed benzophenone synthase. The reaction mechanism is most likely, in analogy to chalcone, stilbene, bibenzyl and acridone biosynthesis [9,13–15], a sequential condensation of three molecules of malonyl-CoA with one molecule of *m*-hydroxybenzoyl-CoA (Fig. 2). The strict dependency of the enzyme activity on DTT is suggestive of an essential cysteine that binds the substrate and has already been shown in chalcone, stilbene and acridone synthases [16,17].

In *C. erythraea*, 2,3′,4,6-tetrahydroxybenzophenone is converted to 1,3,5-trihydroxyxanthone by xanthone synthase, the next enzyme in the biosynthetic pathway, however, in *Hypericum androsaemum*, it is cyclized to the isomeric 1,3,7-trihydroxyxanthone (Peters, Schmidt and Beerhues, unpublished results). These two isomers are the precursors of the majority

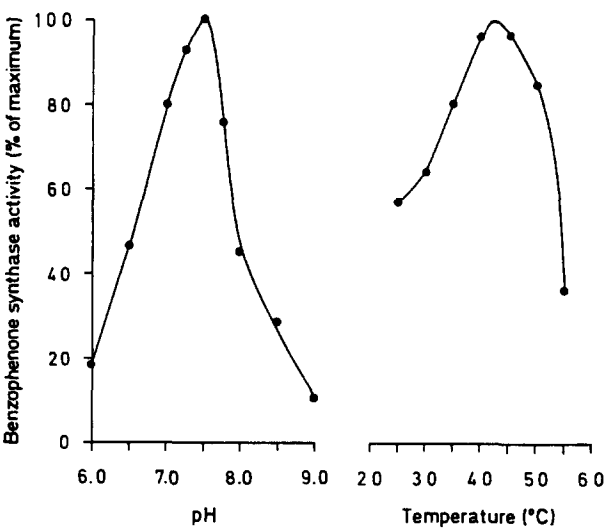

Fig. 1. pH and temperature optima of benzophenone synthase.

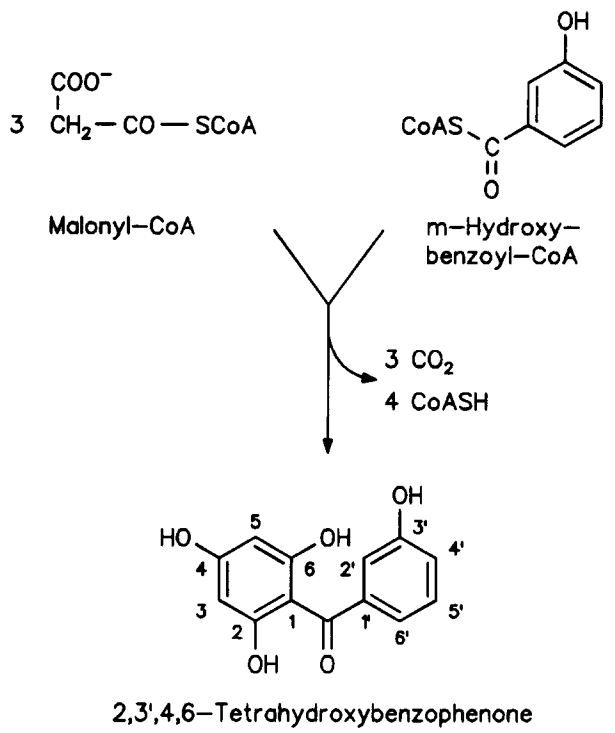

Fig. 2. Reaction catalyzed by benzophenone synthase.

of higher plant xanthones [4], thus, 2,3′,4,6-tetrahydroxybenzophenone represents a central intermediate in xanthone biosynthesis.

The reaction mechanism underlying the conversion of the benzophenone to the isomeric xanthones is most likely an oxidative phenol coupling. This is dependent on the 3′-hydroxy group and, thus, supported by the substrate specificity of benzophenone synthase that converts most efficiently *m*-hydroxybenzoyl-CoA, yielding 2,3′,4,6-tetrahydroxybenzophenone. When benzoyl-CoA acts as a substrate 3′-hydroxylation might occur at the benzophenone level. Benzoyl-CoA has previously been found to be converted by parsley chalcone synthase, however, the product formed was not identified [18].

m-Hydroxybenzoyl-CoA is formed in the cell culture of *C. erythraea* by *m*-hydroxybenzoate:CoA ligase that was separated from *p*-coumarate:CoA ligase also occurring in the cell culture (Barillas and Beerhues, unpublished results).

When cultured *C. erythraea* cells were treated with methyl jasmonate 1-hydroxy-3,5,6,7-tetramethoxyxanthone accumulated but the activity of phenylalanine ammonia lyase did not appreciably increase [6]. Similarly, benzophenone synthase activity is also not stimulated by addition of methyl jasmonate, indicating that this potential signal molecule in the elicitation process [19] affects only peripheral reactions of the constitutive xanthone biosynthetic pathway in *C. erythraea*, as discussed earlier [6].

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