Alternative pathways of xanthone biosynthesis in cell cultures of *Hypericum androsaemum* L.

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Abstract The biosynthesis of xanthones was studied in cell cultures of *Hypericum androsaemum* L. We have detected a new benzophenone synthase, for which the preferred substrate is benzoyl-CoA, itself supplied by 3-hydroxybenzoate:coenzyme A ligase. The stepwise condensation of benzoyl-CoA with three molecules of malonyl-CoA, catalyzed by benzophenone synthase, yields 2,4,6-trihydroxybenzophenone. This intermediate is subsequently converted by benzophenone 3′-hydroxylase, a cytochrome P450 monooxygenase. These biosynthetic steps, leading to the formation of 2,3′,4,6-tetrahydroxybenzophenone, represent an alternative pathway to that recently proposed for cell cultures of *Centaurium erythraea* [Peters et al., Planta (1997) in press].

Key words: *Hypericum androsaemum* cell culture; Xanthone biosynthesis; 3-Hydroxybenzoate:CoA ligase; Benzophenone synthase; Benzophenone 3′-hydroxylase; Cytochrome P450 monooxygenase

1. Introduction

Extracts from *Hypericum* species (Hypericaceae) have been shown to exert marked effects on the central nervous system [2,3]. Best known is the extract from *H. perforatum* (St. John's wort) which is widely used as an antidepressant drug [2]. The question of the active principle(s) has not yet been clarified. Here we report studies on the biosynthesis in cell suspension cultures of the xanthones known to be constituents present in Hypericaceae [4].

Since cultured cells of *H. perforatum* failed to accumulate xanthones (Schmidt and Beerhues, unpublished results), we used *H. androsaemum*. These cells contain a number of prenylated and/or *C*-glucosylated derivatives of 1,3,6,7-tetrahydroxyxanthone such as γ-mangostin (Fig. 1) when grown in a modified B-5 medium (Schmidt, Wolfender, Hostettmann and Beerhues, unpublished results).

Our second model system consists of cultures of *Centaurium erythraea* (Gentianaceae) in which enzymes for the biosynthesis of xanthones have been detected for the first time [1,5,6]. Very recently, we showed that the immediate precursor of xanthones, 2,3′,4,6-tetrahydroxybenzophenone (Fig. 1), is cyclized regioselectively to 1,3,5- and 1,3,7-trihydroxyxanthones by the xanthone synthases from *C. erythraea* and *H. androsaemum*, respectively [1].

In this paper we report the detection and partial characterization of enzymes leading to the formation of 2,3′,4,6-tetrahydroxybenzophenone in cultured *H. androsaemum* cells. Our findings are suggestive of alternative xanthone biosynthetic pathways in this species.

2. Materials and methods

2.1. Cell cultures

Cell suspension cultures of *H. androsaemum* were grown as described previously [1].

2.2. Chemicals

The coenzyme A esters of 2-, 3- and 4-hydroxybenzoic acids were synthesized as described earlier [6]; benzoyl-CoA, malonyl-CoA, acetophenone, benzaldehyde, salicylic acid, plumbagine and cytochrome *c* were obtained from Sigma (Deisenhofen, Germany); benzophenone, 4-hydroxybenzophenone, 2,4-dihydroxybenzophenone and 3- and 4-hydroxybenzoic acids were purchased from Fluka (Buchs, Switzerland); benzoic acid, cinnamic acid, 2-hydroxybenzophenone and menadione were from Merck (Darmstadt, Germany); 2,4,6-trihydroxybenzophenone was obtained from ICN (Meckenheim, Germany) and 2,3′,4,6-tetrahydroxybenzophenone was synthesized as described earlier [1].

2.3. Enzyme extraction and preparation of microsomes

These procedures were carried out according to the methods reported in the literature [1,5,6].

2.4. Enzyme assays

The activity of 3-hydroxybenzoate:CoA ligase was measured according to a published method [5]. The activity of benzophenone synthase was determined as described previously [6], except that the final dithiothreitol concentration used was 7.5 μM. The assay of benzophenone 3′-hydroxylase was similar to that for xanthone synthase [1], with the exception that 2,4,6-trihydroxybenzophenone replaced 2,3′,4,6-tetrahydroxybenzophenone. The determination of the substrate specificities of the enzymes was performed in triplicate. All substrate concentrations were saturating; therefore the values given are relative V_{max} values. An enzymatic oxygen-scavenging system was used as described earlier [1].

2.5. Analytical procedures

The products formed in the incubations of 3-hydroxybenzoate:CoA ligase and benzophenone synthase were analyzed by HPLC according to methods described in [5] and [6], respectively. The HPLC of the products formed in the benzophenone 3′-hydroxylase assay was carried out on an RP-8 column with water:acetonitrile (74:26, v/v) as solvent and a flow rate of 1 ml/min. TLC was performed on silica gel 60 F_{254} coated aluminum sheets and preparative layer chromatography was carried out on silica gel 60 F_{254} coated glass plates with a concentrating zone (Merck, Darmstadt, Germany). The solvent used contained cyclohexane:dichloromethane:ethyl formate:formic acid (35:30:30:1, by vol.). 2,4,6-Trihydroxybenzophenone and 2,3′,4,6-tetrahydroxybenzophenone had R_f values of 0.35 and 0.21, respectively.

3. Results

3.1. Detection of 3-hydroxybenzoate:coenzyme A ligase

Incubation of 3-hydroxybenzoic acid, coenzyme A and ATP with a desalted cell-free extract from cell cultures of *H. androsaemum* resulted in the formation of 3-hydroxyben-

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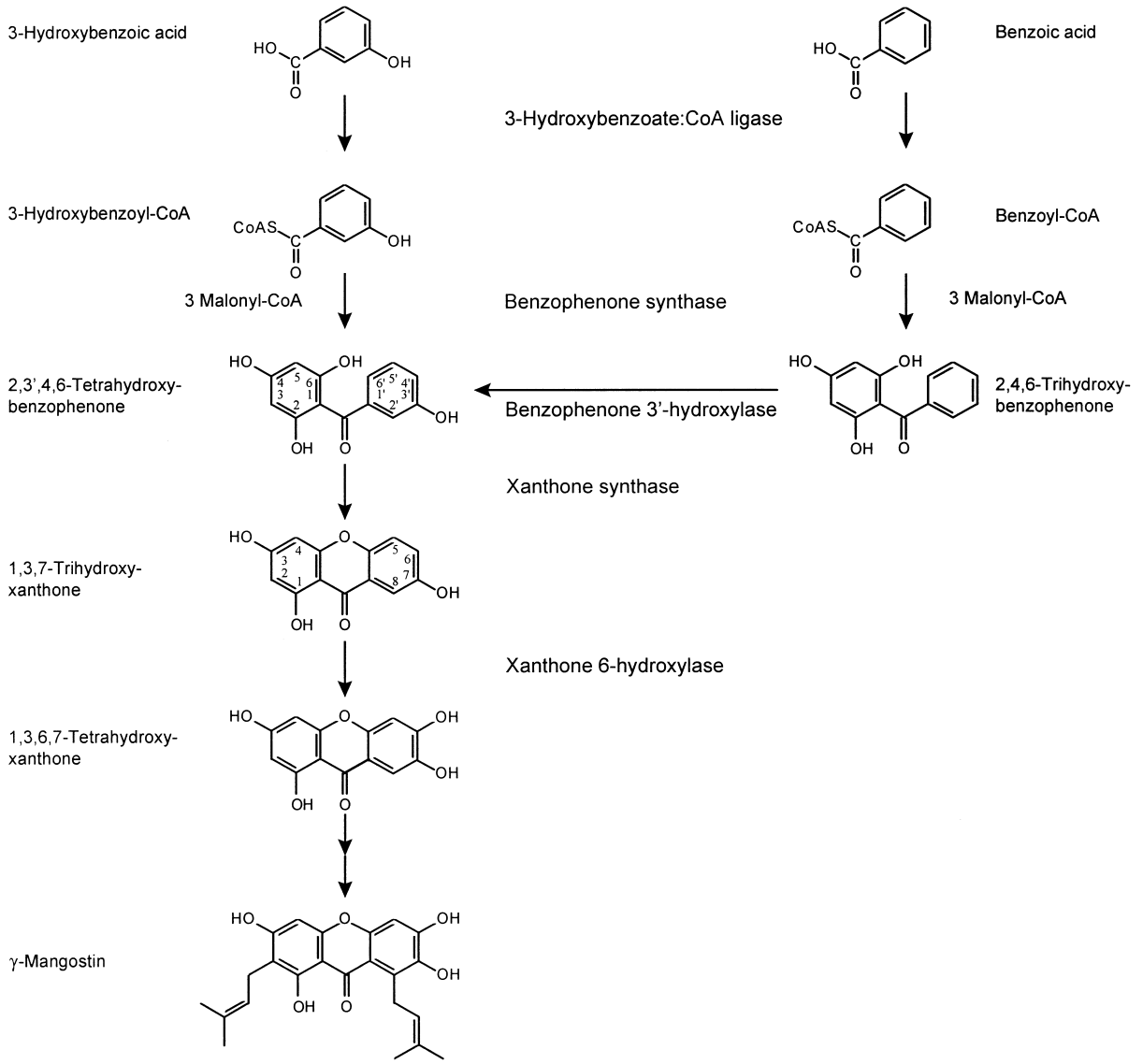

Fig. 1. Proposed scheme of xanthone biosynthesis in cell cultures of *Hypericum androsaemum*.

zoyl-CoA (Fig. 1), as shown by HPLC analysis. The enzymic product co-chromatographed with a sample of an authentic reference compound. Furthermore its UV spectrum agreed with published data [6]. The esterification was catalyzed by 3-hydroxybenzoate:CoA ligase which activated 3-hydroxybenzoic acid preferentially (Table 1). In addition benzoic acid was a relatively efficient substrate. 3-Hydroxybenzoate:CoA ligase had a pH optimum at 8.5 and a temperature optimum at 25°C.

3.2. Detection of benzophenone synthase

When benzoyl-CoA and malonyl-CoA were incubated with a desalted extract from cell cultures of *H. androsaemum* the enzymatic formation of 2,4,6-trihydroxybenzophenone was observed (Fig. 1). This product was identified by co-chromatography with a sample of an authentic reference compound. Furthermore its UV and mass spectra agreed with those of the reference substance. The preferred substrate of benzophenone synthase was benzoyl-CoA (Table 2). Half-maximal activity

Table 1
Substrate specificity of 3-hydroxybenzoate:CoA ligase from cell cultures of *H. androsaemum*

Substrate	Enzyme activity (% relative to 3-hydroxybenzoic acid)
3-Hydroxybenzoic acid	100[a]
Benzoic acid	69
4-Hydroxybenzoic acid	28
2-Hydroxybenzoic acid	0

[a]Specific enzyme activity: 9.2 μkat/kg protein.

Table 2
Substrate specificity of benzophenone synthase from cell cultures of *H. androsaemum*

Substrate	Enzyme activity (% relative to benzoyl-CoA)
Benzoyl-CoA	100[a]
3-Hydroxybenzoyl-CoA	49
4-Hydroxybenzoyl-CoA	5
2-Hydroxybenzoyl-CoA	0

[a]Specific enzyme activity: 3.6 μkat/kg protein.

Table 3
Substrate specificity of benzophenone 3′-hydroxylase from cell cultures of *H. androsaemum*

Substrate	Enzyme activity (% relative to 2,4,6-trihydroxybenzophenone)
2,4,6-Trihydroxybenzophenone	100[a]
2,4-Dihydroxybenzophenone	49
4-Hydroxybenzophenone	32
2-Hydroxybenzophenone	17
Benzophenone	0
Acetophenone	0
Benzaldehyde	0
Benzoic acid	0
Cinnamic acid	0[b]

[a]Specific enzyme activity: 0.74 μkat/kg protein.
[b]4-Hydroxylation only.

occurred with 3-hydroxybenzoyl-CoA as substrate. The enzyme exhibited optimal product formation at pH 7.0 and 35°C. Its activity was stimulated by dithiothreitol concentrations up to 7.5 μM. Higher concentrations led to the formation of a side-product which was not identified. A similar phenomenon has previously been observed with chalcone synthase [7].

3.3. Detection of benzophenone 3′-hydroxylase

Incubation of 2,4,6-trihydroxybenzophenone with microsomes from cultured cells of *H. androsaemum* in the presence of NADPH resulted in the formation of 2,3′,4,6-tetrahydroxybenzophenone (Fig. 1). The identity of this enzymic product was shown by the UV, mass and ^{1}H NMR spectra, all of which agreed with published data [1]. The enzyme catalyzing the hydroxylation was named benzophenone 3′-hydroxylase. It acted only on hydroxylated benzophenones, with 2,4,6-trihydroxybenzophenone being the preferred substrate (Table 3). This was converted to a single product, indicating that the enzyme hydroxylates specifically in the 3′ position. The enzyme exhibited a pH optimum at 7.5 and a temperature optimum at 30°C.

Benzophenone 3′-hydroxylase possessed an absolute requirement for NADPH with a strict dependence on molecular oxygen, as shown by preincubation with an enzymatic oxygen-scavenging system [8]. The enzyme was strongly inhibited by known P450 inhibitors, of which plumbagine was the most potent. The IC_{50} values calculated from the measured dose-response curves are given in Table 4. The enzyme was also strongly inhibited by a $CO:O_2$ (9:1) gas mixture in the dark. Under these conditions it exhibited only 17% of its maximal activity in the standard assay. Upon illumination with white light the inhibitory effect was partly reversed and enzyme activity increased to 31%. Potassium cyanide stimulated the enzyme activity by about 15%. Taken together, these features indicate that benzophenone 3′-hydroxylase is a cytochrome P450 monooxygenase.

Table 4
Concentrations of cytochrome P450 inhibitors which led to 50% inhibition of benzophenone 3′-hydroxylase (IC_{50} values)

P450 inhibitor	IC_{50} (μM)
Plumbagine	4
Cytochrome *c*	30
Menadione	30
Tetcyclacis	33
BAS 111	> 2500

4. Discussion

The products in cell cultures of *H. androsaemum* arise biosynthetically from 1,3,7-trihydroxyxanthone which is, in turn, formed by regioselective cyclization of 2,3′,4,6-tetrahydroxybenzophenone (Fig. 1) [1]. In cultured *C. erythraea*, this benzophenone has been shown recently to originate from the sequential condensation of one molecule of 3-hydroxybenzoyl-CoA with three molecules of malonyl-CoA; the reaction is catalyzed by benzophenone synthase [6]. In contrast to the enzyme from *C. erythraea*, however, benzophenone synthase from *H. androsaemum* acts most efficiently on benzoyl-CoA. 3-Hydroxybenzoyl-CoA is also converted but to a lesser extent. Both benzophenone synthases lack appreciable affinity for the CoA esters of salicylic acid and 4-hydroxybenzoic acid. Among the plant polyketide synthases, different substrate specificities have previously been observed with two stilbene synthases [9].

The stepwise condensation of benzoyl-CoA with three molecules of malonyl-CoA yields 2,4,6-trihydroxybenzophenone (Fig. 1). This raises the question whether this intermediate is 3′-hydroxylated subsequently. It has been found recently that 2,3′,4,6-tetrahydroxybenzophenone is intramolecularly coupled to 1,3,7-trihydroxyxanthone in cell cultures of *H. androsaemum* [1]. Indeed we detected a microsomal cytochrome P450 monooxygenase acting preferentially on 2,4,6-trihydroxybenzophenone and hydroxylating the 3′ position of this substrate specifically. The enzyme was named benzophenone 3′-hydroxylase (Fig. 1). It does not hydroxylate the 3 position of benzoic acid or cinnamic acid and is thus not involved in the early steps of xanthone biosynthesis. Hydroxylation of cinnamic acid in the 4 position occurred readily and was probably due to the activity of the well known cinnamic acid 4-hydroxylase [10].

Since benzophenone synthase from *H. androsaemum* was most active with benzoyl-CoA as substrate, we expected the CoA ligase supplying this substrate to activate benzoic acid most efficiently. The preferred substrate of this enzyme, however, turned out to be 3-hydroxybenzoic acid. Benzoic acid was also a relatively efficient substrate but its conversion rate was somewhat lower. By comparison, in cell cultures of *C. erythraea*, benzoic acid is a poor substrate for 3-hydroxybenzoate:CoA ligase, indicating that in this species 3-hydroxybenzoic acid functions as the sole physiological substrate [5].

In conclusion, the substrate specificities of 3-hydroxybenzoate:CoA ligase and benzophenone synthase, as well as the occurrence of benzophenone 3′-hydroxylase, strongly suggest that alternative pathways lead to the formation of 2,3′,4,6-tetrahydroxybenzophenone in cell cultures of *H. androsaemum* (Fig. 1). The 3′-hydroxy group, which is essential for the subsequent oxidative phenol coupling reaction catalyzed by xanthone synthase [1], is introduced either at the benzophenone level or at an earlier, as yet unknown stage in the xanthone biosynthetic route.

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