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Cinnamic acid is a precursor of benzoic acids in cell cultures of *Hypericum androsaemum* L. but not in cell cultures of *Centaurium erythraea* RAFN

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Abstract. Benzoic acids are precursors of xanthone biosynthesis which has been studied in cell cultures of *Hypericum androsaemum* (Hypericaceae) and *Centaurium erythraea* (Gentianaceae). In both cell cultures, methyl jasmonate induces the intracellular accumulation of a new xanthone. Under these inductive conditions, feeding experiments were performed with [U-^{14}C]L-phenylalanine, [7-^{14}C]benzoic acid and [7-^{14}C]3-hydroxybenzoic acid. All three precursors were efficiently incorporated into the elicited xanthone in *H. androsaemum*, whereas 3-hydroxybenzoic acid was the only precursor to be incorporated into xanthones in *C. erythraea*. In addition, an appreciable increase in phenylalanine ammonia-lyase activity occurred only in methyl-jasmonate-treated cell cultures of *H. androsaemum*. Benzoic acids thus appear to be formed by different pathways in the two cell cultures studied. In *H. androsaemum*, benzoic acid is derived from cinnamic acid by side-chain degradation. In *C. erythraea* 3-hydroxybenzoic acid appears to originate directly from the shikimate pathway.

Key words: Benzoic acid biosynthesis – *Centaurium* (cell culture) – *Hypericum* (cell culture) – Methyl jasmonate – Xanthone biosynthesis

Introduction

Benzoic acids serve important functions in plants. They are precursors of a number of natural products, e.g. cocaine contains a benzoyl residue, shikonin arises from 4-hydroxybenzoic acid, and gallic acid is a component of gallotannins and ellagitannins (Dewick 1997). 4-Hydroxybenzoic acid also functions as a precursor of ubiquinones. Salicylic acid acts as an endogenous signal molecule (Klessig and Malamy 1994). It is required for the establishment of systemic acquired resistance, i.e. the development of enhanced resistance to a secondary infection. In addition, salicylic acid was found to be identical to "calorigen" which induces thermogenesis in *Arum* lilies (Raskin et al. 1987). Methyl salicylate may serve as an airborne signal activating defense-related genes in neighbouring plants and in the healthy tissue of the infected plant (Shulaev et al. 1997). 2,3-Dihydroxybenzoic acid and 4-hydroxybenzoic acid were reported to accumulate in elicitor-treated cell cultures (Kauss et al. 1993; Moreno et al. 1994).

Benzoic acids are biogenetically related to aromatic aldehydes and alcohols. For example, vanillin is the main flavour compound in vanilla, and the glucoside of saligenin occurs in willow bark known for its analgesic and antipyretic effects (Dewick 1997). Benzyl and 3-hydroxybenzyl residues are present in some cytokinins, with *meta*-topolin being the most active compound among the aromatic cytokinins (Werbrouck et al. 1996). Simple phenols such as the widespread hydroquinone arise from benzoic acids by oxidative decarboxylation.

Little is known about the biosynthesis of benzoic acids in plants. They can be formed from cinnamic acids by side-chain degradation, for which two mechanisms have been proposed. The oxidative and CoA-dependent pathway appears to operate in cell cultures of *Lithospermum erythrorhizon*, which accumulate shikonin, the biosynthesis of which proceeds via 4-hydroxybenzoic acid (Löscher and Heide 1994). β-Oxidation is also the most likely mechanism underlying the early steps of salicylic acid biosynthesis in tobacco (Ribnicky et al. 1998). The final reaction of this pathway is 2-hydroxylation of benzoic acid catalysed by a soluble cytochrome P450 enzyme (Léon et al. 1995). The non-oxidative and CoA-independent route has been postulated to occur in cell cultures of *Daucus carota*, which incorporate 4-hydroxybenzoic acid into the cell walls upon elicitation (Schnitzler et al. 1992).

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Abbreviations: LS = Linsmaier-Skoog; NMR = nuclear magnetic resonance; PAL = phenylalanine ammonia-lyase

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Potato tubers apparently use the same pathway to form this benzoic acid (French et al. 1976).

Benzoic acids are also involved in the biosynthesis of benzophenones and xanthones. These classes of natural products include numerous bioactive compounds. A series of polyprenylated benzophenones, the guttiferones, exhibit pronounced anti-microbial and anti-HIV activities (Hussain et al. 1982; Fuller et al. 1999). Some prenylated xanthones possess strong anti-bacterial activity against methicillin-resistant *Staphylococcus aureus*, and they inhibit efficiently the DNA topoisomerases I and II (Iinuma et al. 1996; Tosa et al. 1997). The furanoxanthone psorospermin is active against drug-resistant human leukemia cell lines and AIDS-related lymphoma, for which the mechanism of action has recently been clarified (Kwok and Hurley 1998). Some polyhydroxyxanthones exhibit pronounced anti-malarial activity by inhibiting heme polymerization in *Plasmodium falciparum* (Ignatushchenko et al. 1997).

In xanthone biosynthesis, either benzoyl-CoA or 3-hydroxybenzoyl-CoA is subjected to sequential chain elongation by three acetate units from malonyl-CoA to yield an intermediate benzophenone (Beerhues 1996; Schmidt and Beerhues 1997). The preceding esterification of the benzoic acids is catalysed by 3-hydroxybenzoate:CoA ligase which has recently been purified and characterised (Barillas and Beerhues 2000). Xanthone biosynthesis has been studied in cell cultures of *Hypericum androsaemum* and *Centaurium erythraea* (Beerhues et al. 1999). Here, we report feeding experiments with radiolabelled cinnamic, benzoic and 3-hydroxybenzoic acids, which indicate that the formation of benzoic acids proceeds by different pathways in the two cell cultures studied.

Materials and methods

Chemicals and enzymes

[U-^{14}C]L-Phenylalanine (11.8 GBq mmol^{-1}) was obtained from Hartmann Analytic (Braunschweig, Germany); [7-^{14}C]benzoic acid (0.6 GBq mmol^{-1}) was purchased from NEN (Köln, Germany); [7-^{14}C]3-hydroxybenzoic acid (0.6 GBq mmol^{-1}) was from Biotrend (Köln, Germany). Phenylalanine ammonia-lyase (PAL) was obtained from Sigma, and methyl jasmonate was from Serva.

Cell cultures

Tissue cultures of *Hypericum androsaemum* L. were established as described previously (Peters et al. 1998). Callus tissue grown on solid LS medium (Linsmaier and Skoog 1965) was transferred to liquid LS medium and cultivated in the dark. The resulting cell suspension cultures were shaken in 300-ml Erlenmeyer flasks at 100 rpm and 25 °C. Cells were inoculated into fresh medium (50 ml) at 7-d intervals. Cell cultures of *C. erythraea* were grown as described earlier (Beerhues and Berger 1994).

Extraction of constituents

Xanthones were extracted as described previously (Beerhues and Berger 1995).

Analytical methods

High-performance liquid chromatography was carried out on an RP-8 column (25 cm long, 0.4 cm i.d.; Nucleosil 100–5; Macherey-Nagel, Düren, Germany) at a flow rate of 1 ml min^{-1}. The solvents were water (A) and methanol (B) both containing 1% (v/v) formic acid. The following gradient was employed: 40% B for 5 min, 40–80% B in 20 min, then isocratic elution at 80% B. Detection was at 254 nm.

Thin layer chromatography was performed on silica gel 60 F_{254}-coated aluminium sheets (Merck). Cyclohexane:dichloromethane:ethyl formate:formic acid (35:30:30:1, by vol.) was used as solvent for the separation of xanthones, and toluene:acetic acid (8:2, v/v) was the solvent for purification of radioactive cinnamic acid.

Treatment with methyl jasmonate

Methyl jasmonate (5 µl) was diluted with ethanol (440 µl). An aliquot of this solution (100 µl) was added to the cell cultures, resulting in a final concentration of 0.1 mmol l^{-1}. Control cells were treated with the same volume of ethanol only.

Enzymatic preparation of [U-^{14}C]trans-cinnamic acid

[U-^{14}C]L-Phenylalanine was deaminated using PAL. The enzyme assay had a total volume of 500 µl and was composed of 125 µl phenylalanine solution, 80 µl PAL solution and 295 µl 0.1 mol l^{-1} borate buffer pH 8.8. After incubation at 36 °C for 3 h the assay mixture was acidified by addition of 3.5 µl hydrochloric acid (32%) and extracted with ethyl acetate. The organic phase was evaporated to dryness and the radioactive cinnamic acid was purified by preparative TLC. [U-^{14}C]*trans*-Cinnamic acid was dissolved in ethanol (50 µl).

Assay for PAL

The activity of PAL was determined as described above, except that the incubation time was 1 h and product analysis was carried out by HPLC. Detection was at 290 nm.

Protein determination

The method for protein quantification is described elsewhere (Beerhues and Berger 1995).

Feeding experiments

In contrast to the routine cultivation of cell cultures, cells (0.6 g) were inoculated into 5 ml LS medium in 25-ml Erlenmeyer flasks. At day 5 of cell culture growth, methyl jasmonate (10 µl) was added, giving a final concentration of 0.1 mmol l^{-1}. Six hours later, [U-^{14}C]*trans*-cinnamic acid (10 µl), [7-^{14}C]benzoic acid (25 µl) and [7-^{14}C]3-hydroxybenzoic acid (25 µl) were added. Each aliquot contained 92.5 kBq of radioactivity. After a 24-h incubation the cultured cells were harvested by suction filtration and extracted as described above. Aliquots of these extracts were analysed by HPLC coupled with a radioactivity-detector (Berthold, Wildbad, Germany). The radiolabelled xanthones were isolated and their radioactivity was determined in a liquid scintillation counter (1409; Wallac, Turku, Finland). The residual radioactivity in the culture medium following the 24-h period was also measured. All experiments were carried out at least in triplicate and average values were calculated. Incorporation rates of cinnamic acid were not corrected for loss of

two carbon atoms caused by side-chain degradation because the released C_2 unit has been shown to be incorporated to a high extent via the acetate-malonate pathway (Bennett et al. 1990).

Results

Growth and xanthone formation of H. androsaemum *cell cultures in LS medium*

Our previous enzymological studies of xanthone biosynthesis in *H. androsaemum* were performed with cell cultures grown in modified B5 medium (Schmidt and Beerhues 1997). These cell cultures accumulate an array of prenylated xanthone aglycones and their glucosides (Schmidt et al. 2000). When they are treated with methyl jasmonate no appreciable changes in the pattern of their constituents occur. The present observations were therefore made with cell cultures grown in LS medium in the dark. Typical growth curves were obtained (Fig. 1). Relative to the dry weight, the linear growth phase occurred between days 3 and 11. Analysis of cell extracts by HPLC showed that the cell cultures contained one major constituent, which was identified as γ-mangostin (Fig. 2). The UV, mass, and ^1H NMR spectra agreed with published data (Ishiguro et al. 1993). γ-Mangostin was formed in the early linear growth phase (Fig. 1). Its extractable amount decreased in the late linear growth phase to the starting level, which was not due to release of the compound into the culture medium. γ-Mangostin was only present in cell extracts. The maximum xanthone content was approx. 0.2% of dry weight.

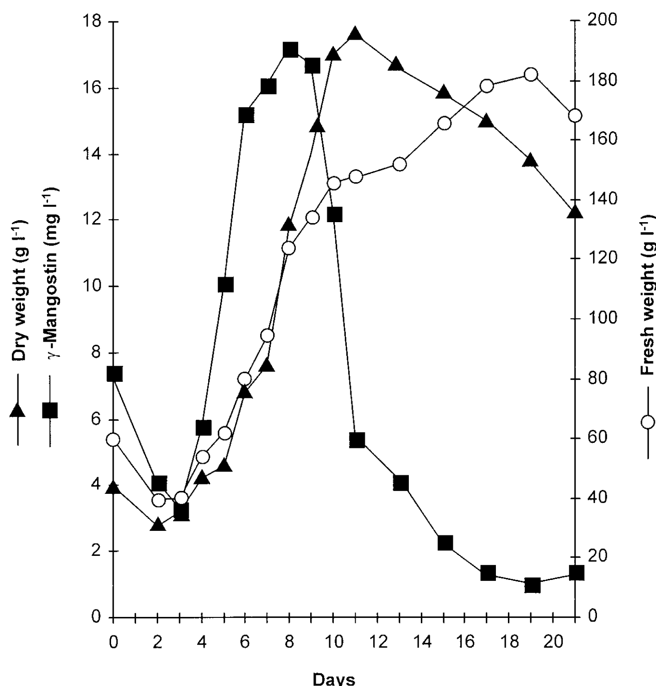

Fig. 1. Growth of a representative *H. androsaemum* cell culture in LS medium in the dark and changes in the γ-mangostin content

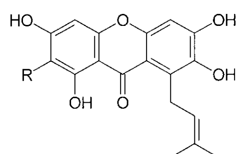

Fig. 2. 1,3,6,7-Tetrahydroxy-8-prenylxanthone (R = H) and γ-mangostin (R = 3,3-dimethylallyl)

Growth and xanthone formation of *C. erythraea* cell cultures have been reported previously (Beerhues and Berger 1994). The major constituent of these cell cultures is 3,5,6,7,8-pentamethoxy-1-O-primeverosylxanthone, the accumulation of which parallels cell culture growth.

Methyl-jasmonate-induced xanthone accumulation

Cell cultures of *C. erythraea* respond to addition of methyl jasmonate with the formation of 1-hydroxy-3,5,6,7-tetramethoxyxanthone (Beerhues and Berger 1995). The intracellular accumulation of this induced compound starts around 6 h after methyl jasmonate addition.

Cell cultures of *H. androsaemum* grown in LS medium also accumulate a new constituent inside the cells when treated with methyl jasmonate. This compound was isolated and identified by UV, mass, and ^1H NMR spectroscopy as 1,3,6,7-tetrahydroxy-8-prenylxanthone (Fig. 2). Its spectroscopic properties were in accord with literature data (Nielsen and Arends 1979). This xanthone has been found recently as the major constituent in cell cultures grown in modified B5 and MS media (Dias et al. 2000; Schmidt et al. 2000), its formation paralleling cell culture growth.

The accumulation of the induced xanthone in cell cultures grown in LS medium started around 3 h after addition of methyl jasmonate and reached a plateau after 12 h (Fig. 3). Control cells, to which only ethanol was added, contained low amounts of 1,3,6,7-tetrahydroxy-8-prenylxanthone.

Feeding experiments with radioactive cinnamic, benzoic and 3-hydroxybenzoic acids

The methyl-jasmonate-induced xanthone accumulation in the two cell cultures was used to perform tracer studies. Six hours after addition of methyl jasmonate the cell cultures were fed with [U-^{14}C]*trans*-cinnamic acid, [7-^{14}C]benzoic acid and [7-^{14}C]3-hydroxybenzoic acid. Cinnamic acid had been derived enzymatically from [U-^{14}C]phenylalanine. After 24 h of incubation around 80% of the added radioactivity was taken up from the culture medium. Acetone extracts of the cultured cells were prepared and analysed by HPLC coupled with a radioactivity-detector.

In cell cultures of *H. androsaemum*, cinnamic acid and the two benzoic acids were incorporated to a high extent into the elicited 1,3,6,7-tetrahydroxy-8-prenylxanthone. This labelled compound was isolated from the extracts and its radioactivity was determined. Incorpo-

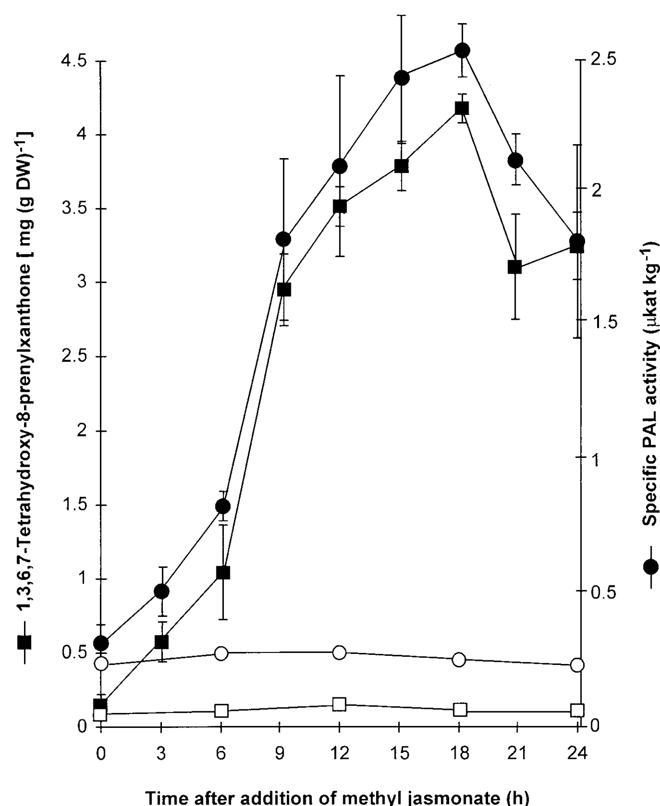

Fig. 3. Accumulation of 1,3,6,7-tetrahydroxy-8-prenylxanthone (■) and time course of PAL activity (●) in *H. androsaemum* cell cultures treated with methyl-jasmonate. *Open symbols* indicate xanthone content and PAL activity in control cells

ration rates of 5.5% for cinnamic acid, 12.7% for benzoic acid and 6.0% for 3-hydroxybenzoic acid were found (Table 1). No label was present in γ-mangostin.

In cell cultures of *C. erythraea*, 3-hydroxybenzoic acid was the only precursor to be incorporated into the induced 1-hydroxy-3,5,6,7-tetramethoxyxanthone (Table 2). The incorporation rate was 6.1%. The constitutively formed 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone was also labelled. When cinnamic acid and benzoic acid were fed no incorporation of label into these xanthones occurred.

Changes in PAL activity

A 10-fold increase in PAL activity was observed in *H. androsaemum* cell cultures treated with methyl jasmonate. Enzyme activity started to change around 3 h after addition of methyl jasmonate and increased dramatically in the 6- to 9-h period (Fig. 3). No changes in enzyme activity were observed in control cells to which only ethanol was added. When *C. erythraea* cell cultures were treated with methyl jasmonate, no substantial increase in PAL activity occurred, as reported previously (Beerhues and Berger 1995).

Discussion

Benzoic acids are precursors of xanthone biosynthesis. Their CoA esters are stepwise condensed with three molecules of malonyl-CoA to give an intermediate benzophenone, which undergoes cyclization to a xanthone (Beerhues 1996; Schmidt and Beerhues 1997; Peters et al. 1998). 3-Hydroxybenzoyl-CoA is the starter substrate in cell cultures of *C. erythraea*, whereas both benzoyl-CoA and 3-hydroxybenzoyl-CoA are used in cell cultures of *H. androsaemum*. How these benzoic acids are formed is so far unclear. Here we present evidence that different pathways of benzoic acid bio-

Table 1. Incorporation of radiolabelled potential precursors into methyl-jasmonate-induced 1,3,6,7-tetrahydroxy-8-prenylxanthone in cell cultures of *H. androsaemum*

Precursor	Concentration of tracer in culture medium[a] (μmol l^{-1})	Uptake of radioactivity within 24 h[b] (%)	Radioactivity found in induced xanthone[c] (kBq)	Incorporation rate (%)
[U-^{14}C]*trans*-cinnamic acid	1.6	88	4.5 ± 0.47	5.5
[7-^{14}C]benzoic acid	30.8	73	8.6 ± 0.37	12.7
[7-^{14}C]3-hydroxybenzoic acid	30.8	78	4.3 ± 0.26	6.0

[a] 92.5 kBq each was added
[b] Radioactivity added to culture medium = 100%. Radioactivity uptake was determined by its loss from culture medium. The labelled xanthone was not released into the culture medium
[c] Mean values of three replicates ± SD

Table 2. Incorporation of radiolabelled potential precursors into methyl-jasmonate-induced 1-hydroxy-3,5,6,7-tetramethoxyxanthone in cell cultures of *C. erythraea*

Precursor	Concentration of tracer in culture medium[a] (μmol l^{-1})	Uptake of radioactivity within 24 h[b] (%)	Radioactivity found in induced xanthone[c] (kBq)	Incorporation rate (%)
[U-^{14}C]*trans*-cinnamic acid	1.6‘	90	0	0
[7-^{14}C]benzoic acid	30.8	79	0	0
[7-^{14}C]3-hydroxybenzoic acid	30.8	71	4.0 ± 0.45	6.1

[a] 92.5 kBq each was added
[b] Radioactivity added to culture medium = 100%. Radioactivity uptake was determined by its loss from culture medium. The labelled xanthone was not released into the culture medium
[c] Mean values of three replicates ± SD

synthesis appear to operate in the two cell cultures studied.

In *H. androsaemum* cell cultures, benzoic acid and 3-hydroxybenzoic acid were efficiently incorporated into the methyl-jasmonate-induced 1,3,6,7-tetrahydroxy-8-prenylxanthone. This observation is in accordance with our previous biochemical findings that both benzoic acids are efficient substrates of 3-hydroxybenzoate:CoA ligase and both resulting CoA esters are accepted by benzophenone synthase (Schmidt and Beerhues 1997). This enzyme prefers, however, benzoyl-CoA, which is reflected in the higher incorporation rate of benzoic acid than of 3-hydroxybenzoic acid. Cinnamic acid was also incorporated to a high extent, demonstrating that this precursor is converted to benzoic acid by side-chain shortening. This conclusion is supported by the substantial increase in PAL activity after methyl jasmonate treatment.

Previous feeding experiments with young shoots of *Garcinia mangostana* (Hypericaceae) have also shown that cinnamic, benzoic and 3-hydroxybenzoic acids are efficient precursors of the xanthone mangostin (Bennett and Lee 1988). The labelled mangostin was degraded to yield phloroglucinol. Feeding of radioactive acetate and malonate resulted in a highly labelled phloroglucinol. Phloroglucinol was, however, almost devoid of label when the precursors were benzoic acid, 3-hydroxybenzoic acid or [3-^{14}C]cinnamic acid (Bennett et al. 1990).

By contrast, 3-hydroxybenzoic acid was the only radioactive precursor to be incorporated into xanthones in cell cultures of *C. erythraea*. In this species, benzoic acid is a poor substrate of 3-hydroxybenzoate:CoA ligase (Barillas and Beerhues 1997) and, in addition, benzoyl-CoA is only the second best substrate of benzophenone synthase (Beerhues 1996), explaining why benzoic acid is not incorporated. Furthermore, there is no *meta*-hydroxylation of benzoic acid, as demonstrated by the feeding experiments. The lack of incorporation of cinnamic acid indicates that the formation of 3-hydroxybenzoic acid in *C. erythraea* cell cultures proceeds through a PAL-independent pathway. This finding is in agreement with our previous observation that there is no appreciable increase in PAL activity in methyl-jasmonate-treated cell cultures, although these cultures accumulate high levels of 1-hydroxy-3,5,6,7-tetramethoxyxanthone (Beerhues and Berger 1995). Thus, 3-hydroxybenzoic acid in cultured *C. erythraea* cells appears to be derived directly from the shikimate pathway. This might also explain why tracer studies with rhizome discs of *Gentiana lutea*, likewise belonging to the Gentianaceae, resulted only in a poor incorporation of [U-^{14}C]phenylalanine (0.044%; Gupta and Lewis 1971).

In bacteria, salicylic acid, 2,3-dihydroxybenzoic acid and 4-hydroxybenzoic acid originate directly from intermediates of the shikimate pathway (Marshall and Ratledge 1972; Sakaitani et al. 1990; Siebert et al. 1994). In the plant species *Rhus typhina* (Anacardiaceae) gallic acid is derived from 3-dehydroshikimic acid, as shown recently by quantitative NMR studies with ^{13}C-labelled glucose (Werner et al. 1997). Another metabolite that arises directly from the shikimate pathway is quinate (Herrmann and Weaver 1999). The reversible conversion of 3-dehydroquinate to quinate is catalysed by quinate dehydrogenase. Furthermore, 2,3-dihydroxybenzoic acid accumulating in elicitor-treated tissue cultures of *Catharanthus roseus* has been postulated to originate from chorismate via isochorismate (Moreno et al. 1994).

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