PERGAMON

Phytochemistry 53 (2000) 427–431

PHYTOCHEMISTRY

www.elsevier.com/locate/phytochem

Xanthone 6-hydroxylase from cell cultures of *Centaurium erythraea* RAFN and *Hypericum androsaemum* L.

Werner Schmidt [1], Stefan Peters [2], Ludger Beerhues*

Institut für Pharmazeutische Biologie, Universität Bonn, Nußallee 6, D-53115 Bonn, Germany

Received 10 September 1999; received in revised form 19 October 1999; accepted 20 October 1999

Abstract

Xanthone 6-hydroxylase activity was detected in the microsomal fractions from two plant cell cultures. The enzyme from cultured cells of *Centaurium erythraea* (Gentianaceae) exhibited absolute specificity for 1,3,5-trihydroxyxanthone as substrate, whereas xanthone 6-hydroxylase from cell cultures of *Hypericum androsaemum* (Hypericaceae) preferred the isomeric 1,3,7-trihydroxyxanthone but used 1,3,5-trihydroxyxanthone also to a small extent. Both xanthones were regioselectively hydroxylated in position 6. The xanthone 6-hydroxylases are cytochrome P450 monooxygenases, as shown by their dependence on NADPH and molecular oxygen and their inhibition by carbon monoxide and typical P450 inhibitors. In both cell cultures, xanthone accumulation was preceded by an increase in xanthone 6-hydroxylase activity.

Keywords: *Centaurium erythraea*; Gentianaceae; *Hypericum androsaemum*; Hypericaceae; Cell cultures; Xanthone biosynthesis; Xanthone 6-hydroxylase; Cytochrome P450 monooxygenase

1. Introduction

Many pharmacologically active plant xanthones possess a 6-hydroxy group. For example, rubraxanthone exhibits efficient antibiotic activity against methicillin-resistant strains of *Staphylococcus aureus* (Iinuma et al., 1996), subelliptenone F is a strong inhibitor of the DNA topoisomerases I and II (Tosa et al., 1997), and some polyhydroxyxanthones possess pronounced antimalarial activity against multiresistant strains of *Plasmodium falciparum* (Ignatushchenko, Winter, Bächinger, Hinrichs & Riscoe, 1997).

In order to study xanthone biosynthesis in plants we use cell cultures of *Centaurium erythraea* RAFN (Gentianaceae) and *Hypericum androsaemum L.* (Hypericaceae). A number of xanthones have been isolated from these two cell cultures. The major constituents in cultured cells of *H. androsaemum* are derivatives of 1,3,6,7-tetrahydroxyxanthone (Schmidt, Abd El-Mawla, Wolfender, Hostettmann & Beerhues, 1999), whereas xanthone formation in cell cultures of *C. erythraea* was found to proceed via the isomeric 1,3,5,6-tetrahydroxyxanthone (Fig. 1) (Beerhues & Berger, 1994). The immediate precursors of these two isomers are 1,3,5- and 1,3,7-trihydroxyxanthones both of which lack the hydroxy group in position 6. They have been shown recently to arise by enzyme-catalyzed oxidative phenol couplings from the same intermediate 2,3′,4,6-tetrahydroxybenzophenone (Peters, Schmidt and Beerhues, 1998).

In this study we report the detection and characterization of the xanthone 6-hydroxylases from cell cultures of *C. erythraea* and *H. androsaemum*.

* Corresponding author. Tel.: +49-288-732678; fax: +49-228-733250.

E-mail address: beerhues@uni-bonn.de (L. Beerhues).

[1] Present address: Institute of Plant Sciences, Swiss Federal Institute of Technology, ETH-Zentrum, Universitätsstraße 2, CH-8092 Zürich, Switzerland.

[2] Present address: Thiemann Arzneimittel GmbH, Im Wirrigen 25, D-45731 Waltrop, Germany

0031-9422/00/$ - see front matter
PII: S0031-9422(99)00566-X

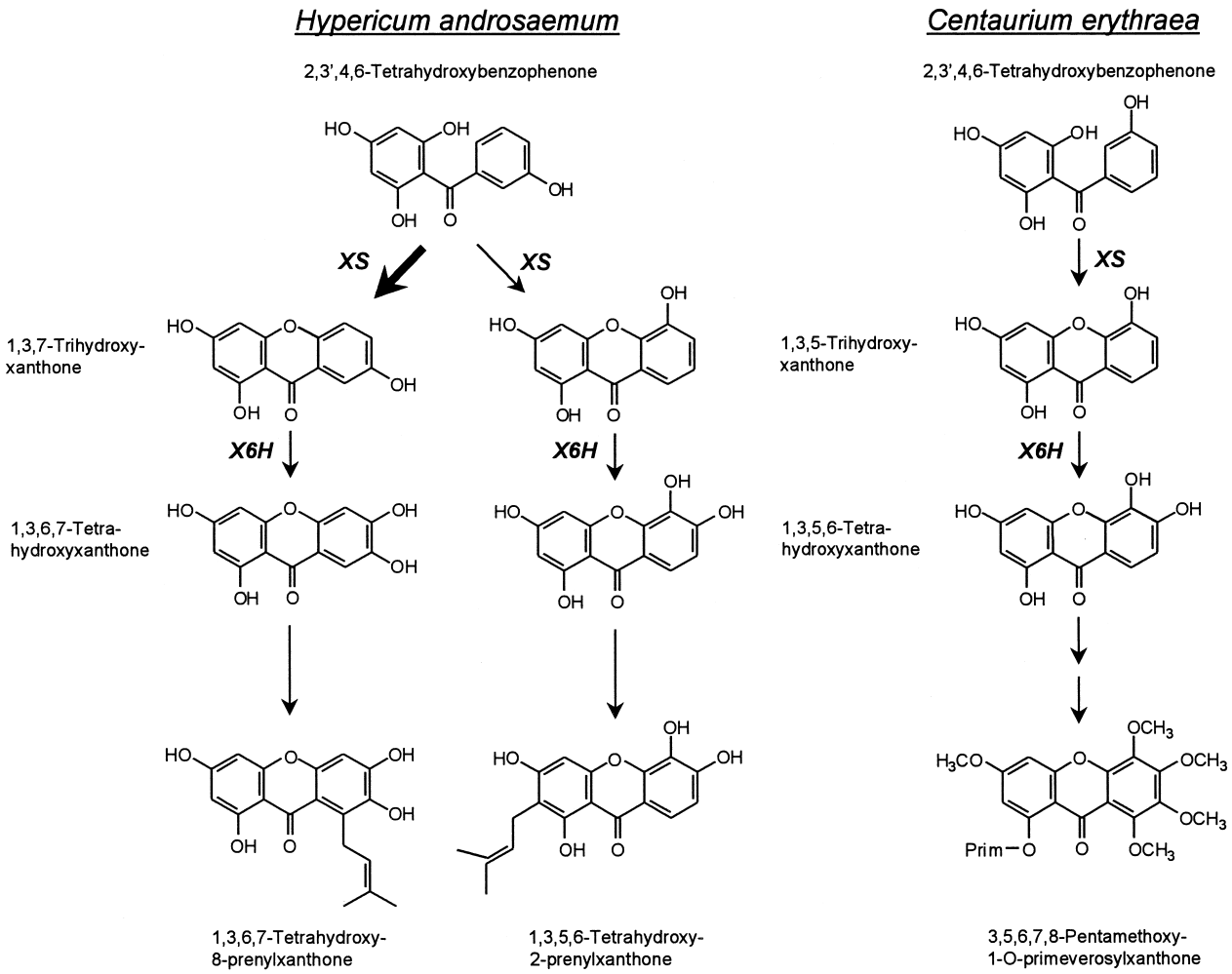

Fig. 1. Metabolism of 2,3′,4,6-tetrahydroxybenzophenone in cell cultures of *H. androsaemum* and *C. erythraea*. XS = xanthone synthase. X6H = xanthone 6-hydroxylase.

2. Results

2.1. Detection of xanthone 6-hydroxylase activity in cell cultures of C. erythraea and H. androsaemum

Incubation of the microsomal fraction from cell cultures of *C. erythraea* with 1,3,5-trihydroxyxanthone and NADPH resulted in the formation of 1,3,5,6-tetrahydroxyxanthone, as shown by HPLC analysis. The identity of the enzymatic product was confirmed by UV and mass spectroscopy. The spectra obtained agreed with the properties reported in the literature for 1,3,5,6-tetrahydroxyxanthone (Abou-Shoer, Suwanborirux, Habib, Chang & Cassady, 1993; Sia, Bennett, Harrison & Sim, 1995). No product formation was observed when NADPH was omitted, the microsomes were heat-inactivated and 1,3,7- instead of 1,3,5-trihydroxyxanthone was used as substrate (Table 1).

In contrast, the microsomal fraction derived from *H. androsaemum* cell cultures catalyzed, in the presence of NADPH, the hydroxylation of both 1,3,7- and 1,3,5-trihydroxyxanthones. The products formed enzymatically were isolated using TLC and HPLC and subjected to UV and mass spectroscopy. Their properties were in accordance with the data published for 1,3,5,6- and 1,3,6,7-tetrahydroxyxanthones (Chen, Lin &

Table 1
Substrate specificities of the xanthone 6-hydroxylases from cell cultures of *C. erythraea* and *H. androsaemum*

Substrate	Relative xanthone 6-hydroxylase activity (%)	
	C. erythraea	*H. androsaemum*
1,3,5-Trihydroxyxanthone	100[a]	65
1,3,7-Trihydroxyxanthone	0	100[b]

[a] 100% corresponds to 384 nkat/mg protein.
[b] 100% corresponds to 512 nkat/mg protein.

Hung, 1975). The enzyme from *H. androsaemum* cell cultures preferred 1,3,7-trihydroxyxanthone (Table 1). Its relative activity with 1,3,5-trihydroxyxanthone was 65%. Both hydroxylation reactions did not proceed using heat-inactivated microsomes.

2.2. Characterization of the xanthone 6-hydroxylases

The enzyme from cell cultures of *H. androsaemum* exhibited maximal activity at pH 7.0 (half-maximal activity at pH 6.3 and 7.7) and an incubation temperature of 30°C. The hydroxylation reactions required strictly molecular oxygen, as demonstrated by preincubation with an oxygen-consuming system. Established cytochrome P450 inhibitors blocked the enzyme activity significantly. The IC_{50} values determined were 1 μM for tetcyclacis and plumbagin and 11 μM for cytochrome *c*, respectively. Xanthone 6-hydroxylase was also inhibited by treatment with a $CO:O_2$ (9:1) gas mixture in the dark. Under these conditions, enzyme activity was 52% of that in the standard incubation. The inhibitory effect was restored partly by illuminating the incubation assay with white light, resulting in a relative enzyme activity of 73%. Xanthone 6-hydroxylase was not inhibited by potassium cyanide at 100 μM, a potent inhibitor of peroxidases (Fujita, Ôba & Uritani, 1982). On the contrary, enzyme activity was stimulated by 20%.

Similar properties were observed for the enzyme from cell cultures of *C. erythraea*. Our experimental findings clearly demonstrate that the xanthone 6-hydroxylases from the two cell cultures studied are membrane-bound cytochrome P450 monooxygenases.

2.3. Changes in xanthone 6-hydroxylase activity during the growth of the two cell cultures

Maximum xanthone 6-hydroxylase activity in *H. androsaemum* cell cultures occurred around day 3 when the xanthone content started to increase (Fig. 2). At day 7, xanthone formation reached a plateau and the enzyme activity decreased rapidly. In cell cultures of *C. erythraea* xanthone 6-hydroxylase exhibited highest activity between day 4 and 6, after which the increase in the xanthone content started. At day 10 xanthone 6-hydroxylase activity was low.

3. Discussion

Xanthone 6-hydroxylase activities have been detected in cultured cells of *C. erythraea* and *H. androsaemum*. In both cell cultures, 6-hydroxylation is the first modifying step at the xanthone level. The substrates of the xanthone 6-hydroxylases are supplied by xanthone synthases catalyzing the oxidative phenol couplings of 2,3′,4,6-tetrahydroxybenzophenone (Peters et al., 1998). In *C. erythraea*, the product of this regioselective intramolecular cyclization is 1,3,5-trihydroxyxanthone. This finding is in good agreement with the substrate specificity of xanthone 6-hydroxylase. The enzyme from *C. ery-*

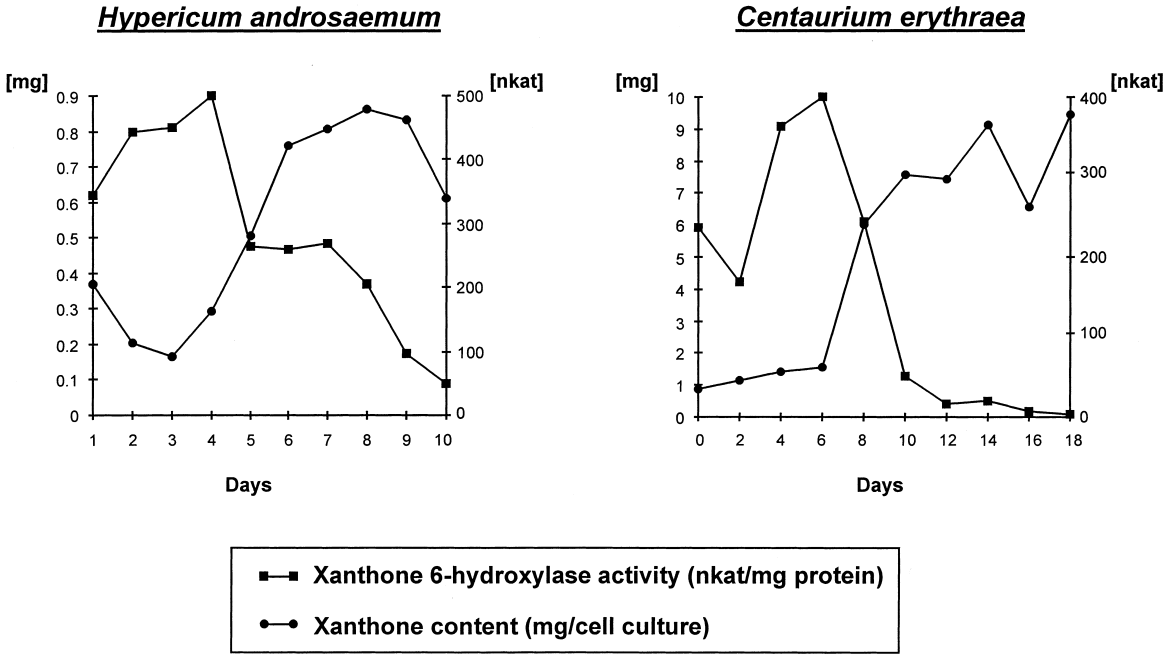

Fig. 2. Changes in xanthone 6-hydroxylase activity and xanthone content during the growth of *H. androsaemum* and *C. erythraea* cell cultures.

thraea exhibits absolute specificity for 1,3,5-trihydroxyxanthone which is regioselectively hydroxylated in position 6. The isomeric 1,3,7-trihydroxyxanthone does not serve as substrate.

In cultured cells of *H. androsaemum* both isomers are converted by xanthone 6-hydroxylase, with 1,3,7-trihydroxyxanthone, however, being the preferred substrate. Since these cell cultures contain primarily derivatives of 1,3,6,7-tetrahydroxyxanthone, metabolic channelling is mainly due to the regioselectivity of the xanthone synthase reaction (Fig. 1). 2,3′,4,6-Tetrahydroxybenzophenone is cyclised preferentially to 1,3,7-trihydroxyxanthone (Peters et al., 1998), the isomeric 1,3,5-trihydroxyxanthone is a minor product. It is yet unclear whether *H. androsaemum* cell cultures contain a single xanthone synthase exhibiting less pronounced regioselectivity or two enzymes differing in their regioselectivities.

In both cell cultures the increase in xanthone 6-hydroxylase activity preceded the increase of xanthone content. When maximum enzyme activity occurred, xanthones started to accumulate. Similar kinetics were found for the enzymes catalyzing the preceding reactions in xanthone biosynthesis (W. Schmidt, S. Peters and L. Beerhues, unpublished results) and have also been observed with enzymes involved in other biosynthetic pathways (Kutchan, Dittrich, Bracher & Zenk, 1991; Chappell & Hahlbrock, 1984).

The xanthone 6-hydroxylases are cytochrome P450 monooxygenases. Their properties are similar to those of other microsomal hydroxylases (Grand, 1984; Song & Brash, 1991; Gabriac, Werck-Reichhart, Teutsch & Durst, 1991; St-Pierre & De Luca, 1995). They are reported here for the first time. Very recently, cell cultures of *H. androsaemum* were shown to contain another membrane-bound monooxygenase, benzophenone 3′-hydroxylase. The detection of this enzyme and the finding that benzophenone synthase from *H. androsaemum* converts most efficiently benzoyl-CoA led us to postulate alternative pathways of xanthone biosynthesis (Schmidt & Beerhues, 1997). The cytochrome P450 enzymes also include the xanthone synthases which, however, are likely to be cytochrome P450 oxidases (Peters et al., 1998).

The products isolated from cell cultures and intact plants of *H. androsaemum* are derivatives of either 1,3,6,7- or 1,3,5,6-tetrahydroxyxanthones (Nielsen & Arends, 1979; Schmidt et al., 1999), demonstrating that further hydroxylations do not occur in this species. In contrast, cell cultures of *C. erythraea* accumulate 3,5,6,7,8-pentamethoxy-1-*O*-primeverosylxanthone (Beerhues & Berger, 1994) the formation of which requires two additional hydroxylation reactions. At present, we are cloning cytochrome P450 enzymes of the two species studied to carry out comparative molecular analyses.

4. Experimental

4.1. Plant cell cultures

Cell suspension cultures of *C. erythraea* and *H. androsaemum* were grown as described previously (Peters et al., 1998).

4.2. Chemicals

1,3,5- and 1,3,7-Trihydroxyxanthones were synthesized and purified as described earlier (Peters et al., 1998); cytochrome *c* and plumbagin were purchased from Sigma (Deisenhofen, Germany); tetcyclacis was a kind gift from Prof. W. Barz (Münster, Germany).

4.3. Enzyme extraction and preparation of microsomes

These procedures were carried out according to published methods (Peters et al., 1998).

4.4. Enzyme assay

The xanthone 6-hydroxylase assay was similar to that for xanthone synthase (Peters et al., 1998), with the exception that 1,3,5- or 1,3,7-trihydroxyxanthone replaced 2,3′,4,6-tetrahydroxybenzophenone. An enzymatic oxygen-scavenging system was used as described earlier (Peters et al., 1998).

4.5. Analytical procedures

HPLC analysis of the ethylacetate-soluble products formed enzymatically was performed on an RP-18 column using water:acetonitrile (66:34) as solvent and a flow rate of 1 ml/min. The formation of 1,3,5,6- and 1,3,6,7-tetrahydroxyxanthones was detected at 326 and 361 nm, respectively. Both products had an R_t value of 9.3 min. Silica gel 60 F_{254} coated aluminium sheets (Merck, Darmstadt, Germany) were used for TLC analysis. Preparative TLC was carried out on silica gel 60 F_{254} coated glass plates with a concentrating zone. The solvent used contained cyclohexane:dichloromethane:ethyl formate:formic acid (35:30:30:1). The R_f value of 1,3,5,6-tetrahydroxyxanthone was 0.12, and that of 1,3,6,7-tetrahydroxyxanthone 0.18.

4.6. Xanthone content

Xanthones were quantified as described by Beerhues & Berger (1994) and Schmidt et al. (1999).

4.7. Protein determination

Protein concentrations were measured by the method of Bradford (1976).

Acknowledgements

The authors thank Dr. G. Eckhardt, Kekulé-Institut für Organische Chemie und Biochemie, Universität Bonn, for his help with mass spectrometry. Prof. W. Barz, Institut für Biochemie und Biotechnologie der Pflanzen, Universität Münster, kindly provided tetcyclacis. This work was financially supported by a grant from the Deutsche Forschungsgemeinschaft, Bonn.

References

Abou-Shoer, M., Suwanborirux, K., Habib, A. A. M., Chang, C. J., & Cassady, J. M. (1993). Xanthones and vismiones from *Psorospermum febrifugum*. *Phytochemistry*, *34*, 1413–1420.

Beerhues, L., & Berger, U. (1994). Xanthones in cell suspension cultures of two *Centaurium* species. *Phytochemistry*, *35*, 1227–1231.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*, *72*, 248–254.

Chappell, J., & Hahlbrock, K. (1984). Transcription of plant defence genes in response to UV light or fungal elicitor. *Nature*, *311*, 76–78.

Chen, F. C., Lin, Y. M., & Hung, J. C. (1975). Phenolic compounds from the heartwood of *Garcinia multiflora*. *Phytochemistry*, *14*, 300–303.

Fujita, M., Ôba, K., & Uritani, I. (1982). Properties of a mixed function oxygenase catalyzing ipomeamarone 15-hydroxylation in microsomes from cut-injured and *Ceratocystis fimbriata*-infected sweet potato root tissues. *Plant Physiol*, *70*, 573–578.

Gabriac, B., Werck-Reichhart, D., Teutsch, H., & Durst, F. (1991). Purification and immunocharacterization of a plant cytochrome P450: The cinnamic acid 4-hydroxylase. *Arch. Biochem. Biophys*, *288*, 302–309.

Grand, C. (1984). Ferulic acid 5-hydroxylase: a new cytochrome P-450-dependent enzyme from higher plant microsomes involved in lignin synthesis. *FEBS Lett*, *169*, 7–11.

Ignatushchenko, M. V., Winter, R. W., Bächinger, H. P., Hinrichs, D. J., & Riscoe, M. K. (1997). Xanthones as antimalarial agents; studies of a possible mode of action. *FEBS Lett*, *409*, 67–73.

Iinuma, M., Tosa, H., Tanaka, T., Asai, F., Kobayashi, Y., Shimano, R., & Miyauchi, K. I. (1996). Antibacterial activity of xanthones from guttiferaeous plants against methicillin-resistant *Staphylococcus aureus*. *J. Pharm. Pharmacol*, *48*, 861–865.

Kutchan, T. M., Dittrich, H., Bracher, D., & Zenk, M. H. (1991). Enzymology and molecular biology of alkaloid biosynthesis. *Tetrahedron*, *47*, 5945–5954.

Nielsen, H., & Arends, P. (1979). Xanthone constituents of *Hypericum androsaemum*. *J. Nat. Prod*, *42*, 301–304.

Peters, S., Schmidt, W., & Beerhues, L. (1998). Regioselective oxidative phenol couplings of 2,3′,4,6-tetrahydroxybenzophenone in cell cultures of *Centaurium erythraea* RAFN and *Hypericum androsaemum* L. *Planta*, *204*, 64–69.

Schmidt, W., & Beerhues, L. (1997). Alternative pathways of xanthone biosynthesis in cell cultures of *Hypericum androsaemum* L. *FEBS Lett*, *420*, 143–146.

Schmidt, W., Abd El-Mawla, A.M.A., Wolfender, J.L., Hostettmann, K., & Beerhues, L. (1999). Xanthones in cell cultures of *Hypericum androsaemum*. *Planta Med* (in press).

Sia, G. L., Bennett, G. J., Harrison, L. J., & Sim, K. Y. (1995). Minor xanthones from the bark of *Cratoxylum cochinchinense*. *Phytochemistry*, *38*, 1521–1528.

Song, W. C., & Brash, A. R. (1991). Purification of an allene oxide synthase and identification of the enzyme as a cytochrome P-450. *Science*, *253*, 781–784.

St-Pierre, B., & De Luca, V. (1995). A cytochrome P-450 monooxygenase catalyzes the first step in the conversion of tabersonine to vindoline in *Catharanthus roseus*. *Plant Physiol*, *109*, 131–139.

Tosa, H., Iinuma, M., Tanaka, T., Nozaki, H., Ikeda, S., Tsutsui, K., Tsutsui, K., Yamada, M., & Fujimori, S. (1997). Inhibitory activity of xanthone derivatives isolated from some guttiferaeous plants against DNA topoisomerases I and II. *Chem. Pharm. Bull*, *45*, 418–420.