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Differential accumulation of xanthones in methyl-jasmonate- and yeast-extract-treated cell cultures of *Centaurium erythraea* and *Centaurium littorale*

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Abstract. Cell-suspension cultures of *Centaurium erythraea* and *Centaurium littorale* (Gentianaceae) respond to methyl jasmonate and yeast extract with a differential accumulation of xanthones. Methyl jasmonate induced the formation of 1-hydroxy-3,5,6,7-tetramethoxyxanthone, the amount of which increased in both cell cultures around 10 h after addition. A substantial increase in the activity of phenylalanine ammonia-lyase (PAL) was not observed. When challenged with yeast extract the cell cultures accumulated 1,5-dihydroxy-3-methoxyxanthone. This appeared rapidly after addition of yeast extract in *C. erythraea* but its amount in *C. littorale* increased only after a lag phase of 25 h. While PAL activity in *C. erythraea* was strongly suppressed a fourfold increase in its activity was found in *C. littorale*. Both elicited xanthones accumulated intracellularly. A scheme for xanthone biosynthesis in the two cell cultures is proposed.

Key words: *Centaurium* (cell cultures) – Methyl jasmonate – Xanthone (differential accumulation) – Yeast extract

Introduction

Xanthones represent a class of natural products that exhibit interesting pharmacological properties. A number of compounds are strong and selective inhibitors of the monoamine oxidase isoform A and, thus, potential antidepressive drugs (Hostettmann and Hostettmann 1989, and literature cited therein; Sparenberg et al. 1993; Rocha et al. 1994). Other xanthones show significant cytotoxicity and antitumour activity against leukemia and mammary and colon tumour systems (Abou-Shoer et al. 1988). Furthermore, some compounds possess anti-inflammatory, antimutagenic, antimicrobial, antioxidative, antiplatelet, tuberculostatic and protein-kinase inhibitory properties (Bennett and Lee 1989, and literature cited therein; Jinsart et al. 1992; Lin et al. 1993; Minami et al. 1993; Schimmer and Mauthner 1994).

The majority of xanthones have been found in only two plant families, the Gentianaceae and the Hypericaceae. Glycosylated xanthones are common in the Gentianaceae, whereas prenylated compounds are widely distributed in the Hypericaceae. A small number of xanthones have been isolated from a few other families of higher plants and ferns (Bennett and Lee 1989; Hostettmann and Hostettmann 1989).

Xanthones arise biosynthetically from the shikimate and the acetate-malonate pathways, as shown by feeding experiments (Gupta and Lewis 1971; Fujita and Inoue 1977, 1981; Bennett and Lee 1988). In *Garcinia mangostana* (Hypericaceae), cinnamic acid, benzoic acid, *m*-hydroxybenzoic acid and malonic acid were found to be efficient precursors of mangostin, indicating that a C_6–C_1 intermediate and three acetate units form the C_{13}-skeleton (Bennett and Lee 1988). In contrast, biosynthesis of mangiferin in *Anemarrhena asphodeloides* (Liliaceae) was postulated to occur via intact incorporation of a C_6–C_3 precursor and addition of two acetate units (Fujita and Inoue 1977, 1981). All tracer experiments demonstrated that first an intermediate benzophenone is formed which is subsequently, most likely by phenol oxidative coupling, converted to the corresponding xanthone (Bennett and Lee 1989).

It is our goal to isolate and characterize the enzymes of xanthone biosynthesis. Cell-suspension cultures of *Centaurium erythraea* Rafn. and *Centaurium littorale* (Tuner) Gilmour (Gentianaceae) which contain 3,5,6,7,8-pentamethoxy-1-0-primeverosylxanthone (*13* in Fig. 4) and the corresponding aglycone (*12* in Fig. 4) as the main constituents (Beerhues and Berger 1994) were treated with biotic and abiotic elicitors in order to stimulate xanthone biosynthesis, and changes in the constituent patterns and in the activity of phenylalanine ammonia-lyase (PAL) were analyzed. It was found that the spectrum of compounds detected depended on the type of elicitor employed.

Dedicated to Professor R. Wiermann on the occasion of his 60th birthday

Abbreviations: TLC = thin-layer chromatography; NMR = nuclear magnetic resonance; PAL = phenylalanine ammonia-lyase

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Materials and methods

Suspension cultures. Cultured cells were maintained as described previously (Beerhues and Berger 1994).

Preparation of fungal elicitors. Potentially phytopathogenic fungi (see *Results*) were obtained from the Institut für Pflanzenkrankheiten (Universität Bonn, Germany) and grown in malt extract/peptone medium (10 $g \cdot l^{-1}$ malt extract, 3 $g \cdot l^{-1}$ soya peptone, pH 5.6). Their mycelia were collected by suction filtration and processed essentially according to the method described by Ayers et al. (1976). Yeast extract (10 g; GIBCO BRL, Paisley, UK) was taken up in 50 ml methanol (80%). After centrifugation the supernatant was evaporated to dryness and the residue dissolved in 12.5 ml water. After extraction with 25 ml ethyl acetate and centrifugation the aqueous phase was sterile-filtered. Preliminary studies had shown that this fraction contained the elicitor activity.

Elicitor treatments. To test the effect of elicitors, 1.5 g *Centaurium* cells were transferred to 25 ml Linsmaier and Skoog (1965) medium containing 5% sucrose in 100-ml Erlenmeyer flasks. The elicitors were applied after 6–9 d of growth at the following final concentrations: 50–100 $\mu g \cdot ml^{-1}$ fungal elicitor preparations, 0.5 and 1.0 $mmol \cdot l^{-1}$ $AgNO_3$, $CuSO_4$, and $Pb(NO_3)_2$, 1 and 3 $mmol \cdot l^{-1}$ glutathione, 8 $mg \cdot ml^{-1}$ yeast extract, 100 $\mu mol \cdot l^{-1}$ methyl jasmonate. Control cultures were treated with an identical amount of water (fungal elicitors, heavy metal ions, and glutathione) or ethanol instead of methyl jasmonate. After 3 d, cells and culture media were separated by vacuum filtration and analyzed. To monitor changes in the level of individual xanthones and in PAL activity after addition of methyl jasmonate and yeast extract, the cell suspensions of about 20 flasks were combined immediately before treatment to get a homogeneous suspension, aliquots of which were then redistributed to the flasks. At various time points after the onset of treatment the cells were harvested by suction filtration and frozen. Culture media were also stored at −20 °C. Two independent experiments were performed and, after analysis, average values determined.

Extraction and analysis of constituents. Cells (1 g) were ground in 7 ml acetone and the homogenate was filtered. The residue was extracted twice with 5 ml acetone. The acetone phases were combined and evaporated to dryness. The residue was redissolved in 1 ml methanol and subjected to HPLC analysis. Culture media were extracted twice with 15 ml ethyl acetate. The organic phases were combined and evaporated to dryness. The residue was taken up in 0.5 ml methanol and also analyzed by HPLC. This was performed on an RP-8 column (Nucleosil 100–5; 25 cm long, 0.4 cm i.d.; Macherey-Nagel, Düren, Germany) using water (A) and methanol (B) as the solvents. The following gradient was employed: 35% B for 2 min, 35–70% B within 20 min, then isocratic elution at 70% B. The flow rate was 1 $ml \cdot min^{-1}$ and detection at 254 nm. Thin-layer chromatography was carried out on silica gel 60 F_{254} coated aluminium sheets (Merck, Darmstadt, Germany). Solvents were as follows: toluene:petroleum ether (40–60 °C):ethyl formate:formic acid = 42:42:14:2 (by vol.); toluene:ethyl acetate = 3:1 (v/v).

Determination of PAL activity. Frozen cells (1 g) were mixed with 0.1 g Polyclar AT (Serva, Heidelberg, Germany) and homogenized at 0–4 °C in 2.0 ml 0.1 $mol \cdot l^{-1}$ potassium phosphate buffer (pH 7.5), containing 1 $mmol \cdot l^{-1}$ dithiothreitol. After centrifugation, aliquots of the supernatant were used for spectrophotometric determination of PAL activity. The enzyme assay is described elsewhere (Seitz et al. 1985).

Protein determination. Protein was measured according to the method of Bradford (1976) using bovine serum albumin as a standard.

Results

Effect of potential elicitors on secondary product formation. The cell cultures of *C. erythraea* and *C. littorale* were treated with cell wall preparations of various phytopathogenic fungi (*Alternaria alternata, Botrytis cinerea, Cladosporium cucumerinum, Colletotrichum gloeosporioides, Fusarium oxysporum, Phoma betae, Phytophthora megasperma* f.sp. *glycinea* [Pmg], *Pythium ultimum, Rhizopus oligosporus, Septoria nodorum, Verticillium rexianum*). Significant changes in the patterns of cell culture constituents were not observed. The same was true for the addition of heavy metal ions (Ag, Cu, Pb) and glutathione which, in some cell cultures, elicit secondary product formation (Threlfall and Whitehead 1989; Edwards et al. 1991). However, challenge with methyl jasmonate and yeast extract resulted in the accumulation of new constituents.

Metabolic changes after addition of methyl jasmonate. After application of methyl jasmonate at a final concentration of 100 $\mu mol \cdot l^{-1}$ the cells of *C. erythraea* and *C. littorale* turned yellow. The HPLC analysis of extracts from these cells showed that, apart from an increase in the level of 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone (*12*), a new constituent (*8*) accumulated (Fig. 1a, b and d, e). This was isolated by HPLC and TLC and identified as 1-hydroxy-3,5,6,7-tetramethoxyxanthone (*8*) by comparison of its UV, mass, and ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra with published data (Quillinan and Scheinmann 1973). The level of this metabolite increased in *C. erythraea* and *C. littorale* 8 and 12 h after methyl jasmonate addition, with maxima at about 30 and 25 h, respectively, and reached an approximately fivefold higher value in *C. erythraea* (Fig. 2a, c). Addition of methyl jasmonate at final concentrations of 50 and 200 $\mu mol \cdot l^{-1}$ led to similar concentrations. In both cell cultures, 1-hydroxy-3,5,6,7-tetramethoxyxanthone (*8*) accumulated intracellularly, only 1–2% of the total amounts of this metabolite was found in the culture media. While the compound was not detectable in the control cells of *C. littorale*, a slight increase in its concentration was observed in the control cells of *C. erythraea*. This might have been due to the addition of the small volume of ethanol that served as a solvent for methyl jasmonate and was also added to the control cells. It might also be attributable to the handling of the cell cultures immediately prior to treatment (see *Materials and methods*).

Application of methyl jasmonate did not appreciably affect the PAL activity of either cell culture. Around 10 h after the onset of treatment a 1.5-fold increase in enzyme activity was observed (Fig. 2b, d).

Metabolic changes after addition of yeast extract. After treatment with yeast extract the cultured cells of *C. erythraea* and *C. littorale* became brownish. The HPLC analysis showed that the cells again contained an additional constituent (*7*) which, however, was not identical to the methyl-jasmonate-induced compound (*8*) (Fig. 1c, f). The new metabolite was isolated by HPLC and TLC and subjected to UV, mass, and ^{1}H-NMR spectroscopy. Its properties were identical to those published for 1,5-dihydroxy-3-methoxyxanthone (*7*) (Delle Monache et al. 1983). Furthermore, its R_t and R_f values (HPLC and TLC) were in accord with those of authentic reference compound. In both cell cultures, a final concentration of 8 mg yeast

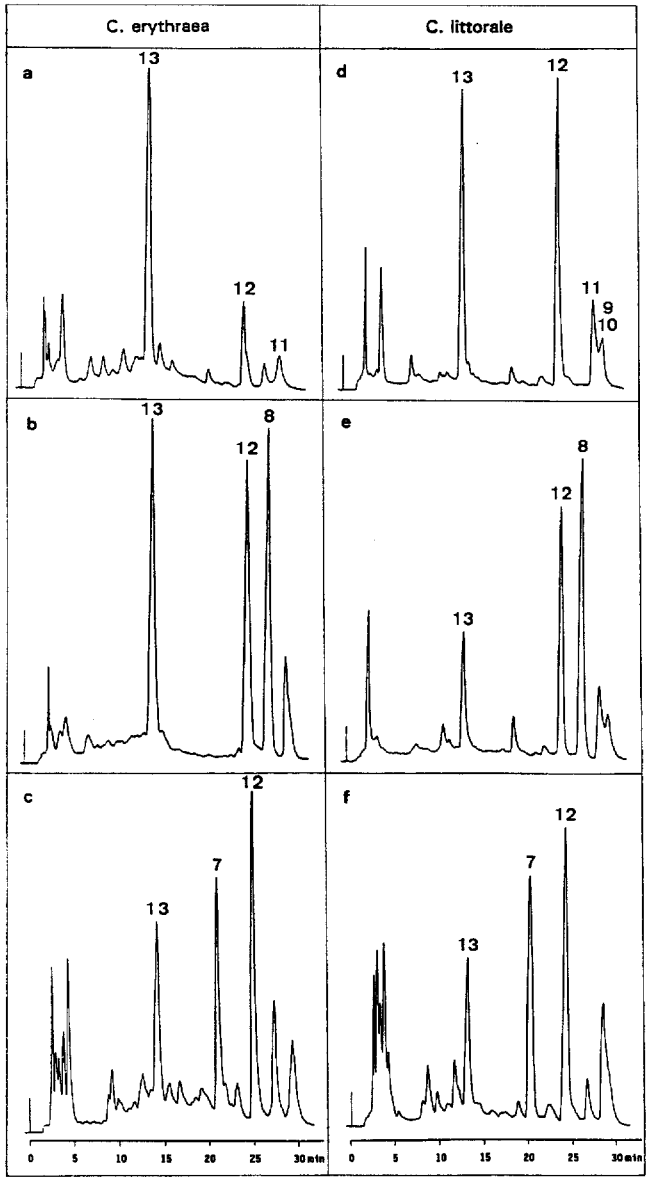

Fig. 1a–f. High-performance liquid chromatographic analysis of extracts from cultured cells of *C. erythraea* (**a–c**) and *C. littorale* (**d–f**). **a,d** Untreated cells, **b,e** methyl-jasmonate-treated cells; **c,f** yeast-extract-treated cells. Numbers refer to the formulae in Fig. 4

extract·ml^{-1} culture medium resulted in maximum accumulation of 1,5-dihydroxy-3-methoxyxanthone (Table 1). While this metabolite accumulated rapidly in yeast-extract-treated cells of *C. erythraea*, its concentration in *C. littorale* increased only after a lag phase of 25 h, maximum levels being observed after 25 and 45 h, respectively (Fig. 3a, c). In both cell cultures the new xanthone was mainly present inside the cells, and the amounts detected in the culture media constituted only 1–2% of the total amounts observed. The control cells to which a comparable volume of water was added contained only low amounts of this xanthone.

Accumulation of 1,5-dihydroxy-3-methoxyxanthone in *C. littorale* was accompanied by an approximately fourfold increase in PAL activity, whereas the enzyme activity in *C. erythraea* was rapidly and strongly suppressed upon addition of yeast extract (Fig. 3b, d).

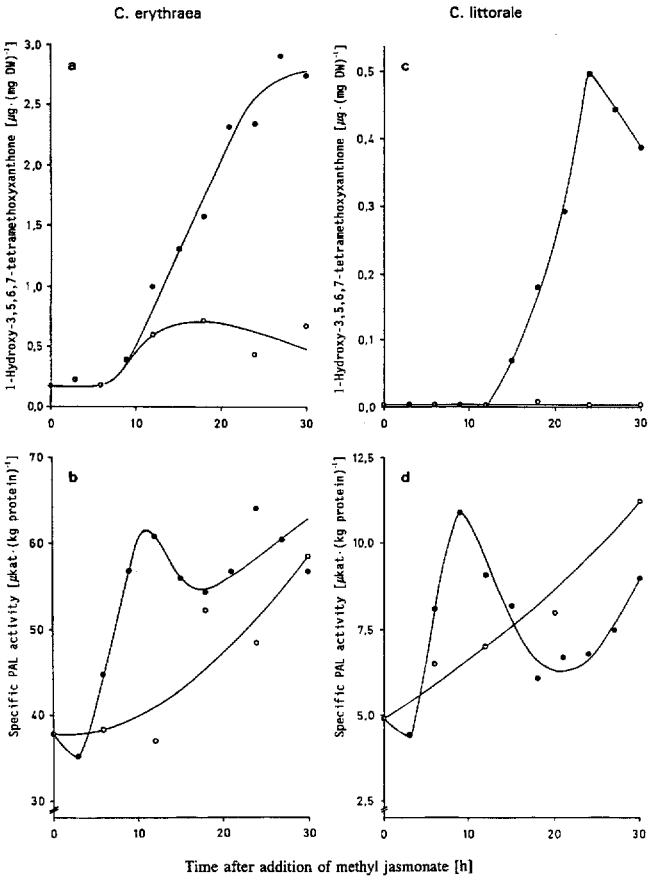

Fig. 2a–d. Accumulation of 1-hydroxy-3,5,6,7-tetramethoxyxanthone (*8*) (**a,c**) and changes in PAL activity (**b,d**) in cell cultures of *C. erythraea* (**a,b**) and *C. littorale* (**c,d**) after addition of methyl jasmonate. ●–●, treated cells; ○–○, control cells

Table 1. Effect of various amounts of yeast extract on accumulation of 1,5-dihydroxy-3-methoxyxanthone (7) in *C. erythraea*

Yeast extract [mg·ml^{-1}]	1,5-Dihydroxy-3-methoxyxanthone [μg·(mg DW)$^{-1}$]
0	0.4
1	0.4
2	0.9
4	2.0
8	3.3
16	3.0

Hypothetical pathway of xanthone biosynthesis in the cell cultures of the two Centaurium species. The feeding experiments primarily of Bennett and Lee (1988) and our own findings are the basis of the xanthone biosynthetic route proposed in Fig. 4. An interesting intermediate in the constitutive pathway concerning the methoxylation sequence is 1,8-dihydroxy-3,5,6-trimethoxyxanthone (*10*). This and 1,8-dihydroxy-3,5,7-trimethoxyxanthone were available as reference compounds (Takagi and Yamaki 1982; Fukamiya et al. 1990). They were not separated by HPLC under the conditions employed; however, the ^{1}H-NMR spectrum and the R_f values in TLC demonstrated unequivocally that the intermediate occurring in the cell cultures is the 6-methoxy isomer.

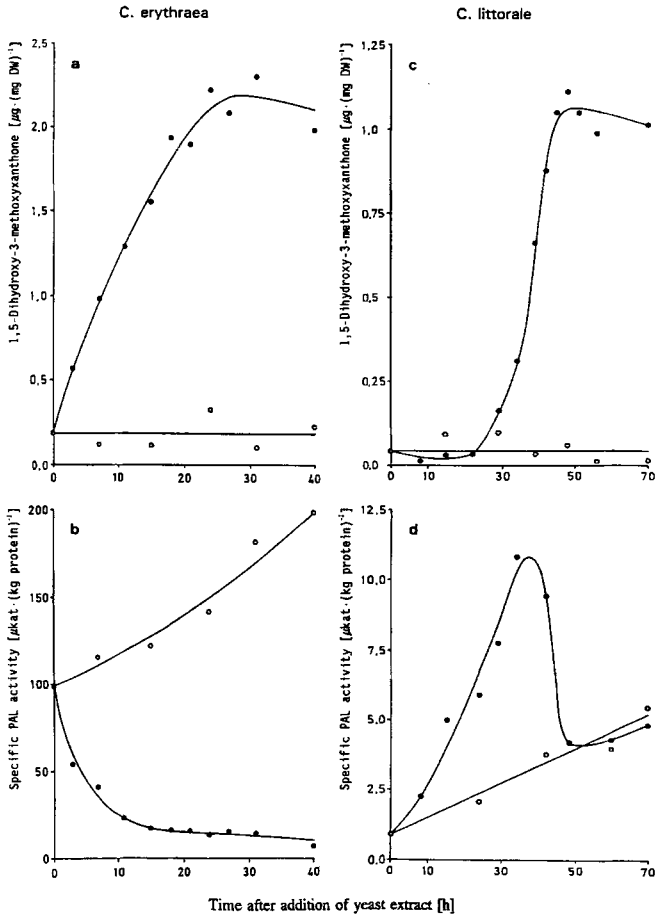

Fig. 3a–d Accumulation of 1,5-dihydroxy-3-methoxyxanthone (*7*) (**a,c**) and changes in PAL activity (**b,d**) in cell cultures of *C. erythraea* (**a,b**) and *C. littorale* (**c,d**) after addition of yeast extract. ●—●, treated cells, ○—○, control cells

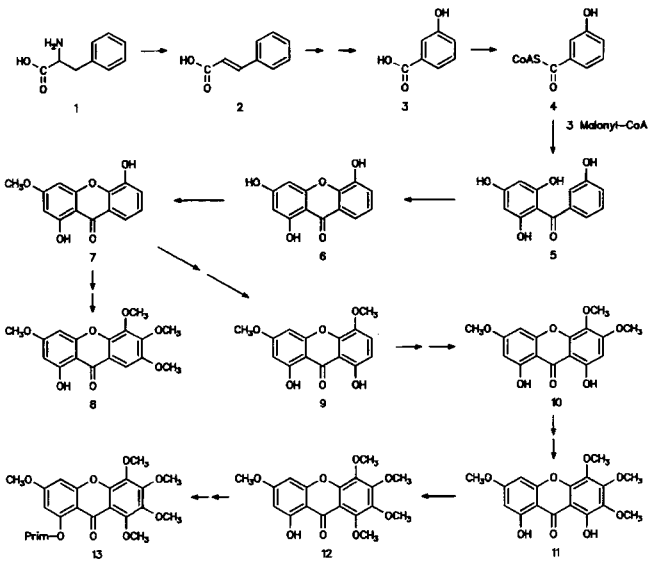

Fig. 4. Proposed pathway of xanthone biosynthesis in cell cultures of *C. erythraea* and *C. littorale* based primarily on the results of Bennett and Lee (1988) and our own data (*1*, phenylalanine; *2*, cinnamic acid; *3*, m-hydroxybenzoic acid; *4*, m-hydroxybenzoyl-CoA; *5*, 2,3′,4,6-tetrahydroxybenzophenone; *6*, 1,3,5-trihydroxyxanthone; *7*, 1,5-dihydroxy-3-methoxyxanthone; *8*, 1-hydroxy-3,5,6,7-tetramethoxyxanthone; *9*, 1,8-dihydroxy-3,5-dimethoxyxanthone; *10*, 1,8-dihydroxy-3,5,6-trimethoxyxanthone; *11*, 1,8-dihydroxy-3,5,6,7-tetramethoxyxanthone; *12*, 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone; *13*, 3,5,6,7,8-pentamethoxy-1-0-primeverosylxanthone).

Discussion

Cell cultures of *C. erythraea* and *C. littorale* respond to addition of methyl jasmonate and yeast extract with a differential accumulation of xanthones. Formation to a high level of 1-hydroxy-3,5,6,7-tetramethoxyxanthone (*8*) in methyl-jasmonate-treated cells of *C. erythraea* suggests that the activities of the enzymes involved are remarkably enhanced. However, PAL activity did not substantially increase. Either it is not rate-limiting, at least it was relatively high at the onset of treatment, or accumulation of the induced compound is simply due to an inhibition of the 8-methoxylation. This step leads in the constitutive pathway to formation of 1-hydroxy-3,5,6,7,8-pentamethoxyxanthone (*12*). The concentration of this compound in about 7-d-old cell cultures of *C. littorale* is markedly lower than in those of *C. erythraea* (Beerhues and Berger 1994). This would explain why the methyl-jasmonate-treated cells of *C. littorale* form only a small amount of 1-hydroxy-3,5,6,7-tetramethoxyxanthone (*8*). Accumulation of this xanthone in response to methyl jasmonate might also be due to dehydroxylation of 1,8-dihydroxy-3,5,6,7-tetramethoxyxanthone (*11*) which is an intermediate in the constitutive pathway (Beerhues and Berger 1994). Aromatic dehydroxylations catalyzed by microbial enzymes have been reported by Anderson (1986) and Brackmann and Fuchs (1993).

Jasmonates control diverse processes in plant growth and development, including embryogenesis, seed germination, leaf senescence, and stomatal closure, and are induced in response to various stresses such as desiccation, wounding, and pathogen attack (Sembdner and Parthier 1993). In a number of cell-suspension cultures, exogenously applied jasmonate led to formation of specific low-molecular-weight compounds and, in addition, triggered a significant increase in PAL activity in cultured cells of *Glycine max* and *Lithospermum erythrorhizon* (Gundlach et al. 1992; Mizukami et al. 1993). Furthermore, treatment of cell cultures with a yeast elicitor caused a rapid increase in the level of endogenous jasmonate, strongly suggesting that jasmonate is the signal transducer in the elicitation process leading to formation of high- and low-molecular-weight compounds (Gundlach et al. 1992; Mueller et al. 1993).

Accumulation of 1,5-dihydroxy-3-methoxyxanthone (*7*) in the cell cultures of the two *Centaurium* species after challenge with yeast extract might be due to a suppression of the late steps of the constitutive pathway, which would explain the rapid increase in the concentration of this intermediate in *C. erythraea*. Although PAL activity in this cell culture is also suppressed the residual activity is still higher than the basal level of enzyme activity in *C. littorale*. In the latter species, accumulation of 1,5-dihydroxy-3-methoxyxanthone (*7*) is preceded by an increase in PAL activity, suggesting that the enzyme activities involved are enhanced by addition of yeast

extract. In various cell cultures, yeast extract has previously been used to elicit phytoalexin production and, in addition, was found to greatly increase the activities of enzymes involved in the respective biosynthetic pathways (Schumacher et al. 1987; Sumaryono et al. 1991).

Many cell cultures producing low-molecular-weight compounds upon elicitation excrete the major proportion of these phytoalexins into the culture medium (Hauffe et al. 1986; Keßmann and Barz 1987; Liswidowati et al. 1991). A small number of suspension cultures store the bulk of the induced metabolites inside the cells (Eilert et al. 1985; Mizukami et al. 1993). In the cell cultures of the two *Centaurium* species, both the methyl-jasmonate- and the yeast-extract-elicited xanthones are accumulated intra-cellularly. The proportions released into the medium are negligible.

At present some enzymes involved specifically in xanthone biosynthesis are being studied. Figure 4 depicts a hypothetical scheme of the biosynthetic route in the cell cultures of the two *Centaurium* species. Since these both form xanthones constitutively and respond to application of methyl jasmonate and yeast extract with a differential xanthone accumulation they represent attractive systems to study the biosynthesis of these interesting secondary products.

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