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Biosynthesis of Heartwood Tropolones I. Incorporation of Mevalonate and Acetate into β-Thujaplicin (Hinokitiol) of *Cupressus lusitanica* Cell Cultures

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In order to obtain fundamental knowledge on the biosynthetic route of β-thujaplicin, tracer experiments were conducted on cell cultures of *Cupressus lusitanica* using ^{14}C-labeled possible precursors. Radioactivity of [U-^{14}C]glucose and [2-^{14}C]mevalonate was incorporated into β-thujaplicin produced in the callus cultures whereas almost no [2-^{14}C]malonate was incorporated into β-thujaplicin. In the suspension culture of *C. lusitanica* cells, the radiolabel of [1-^{14}C]acetate was successively incorporated into mevalonate, geranyl pyrophosphate, and β-thujaplicin. In addition, the production of β-thujaplicin was significantly suppressed by the addition of an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, compactin, to the elicited cultures, in agreement with the involvement of mevalonate in the β-thujaplicin synthesis in *C. lusitanica* cells. All these results strongly indicate for the first time that β-thujaplicin is synthesized via the mevalonate pathway.

Keywords: *Cupressus lusitanica*, biosynthesis, cell culture, β-thujaplicin, mevalonate pathway.

INTRODUCTION

Certain wood tropolones, α-, β- and γ-thujaplicins that have C_7 rings and isopropyl side chains occur in heartwood of most species in the family Cupressaceae. They are responsible for the durability and resistance to insect attack and decay (Gardner, 1962, Dev, 1989),which are characteristics of the heartwood of these species. Okabe *et al.* (1994) noted the antibacterial activity of β-thujaplicin (hinokitiol in Japaneses) against hospital-acquired infections. Its efficient extraction from the hiba wood with supercritical carbon dioxide was reported recently (Ohira *et al.*, 1996). Oster *et al.* (1996) recently found that a β-thujaplicin treatment induced a nearly complete inhibition of protochlorophyllide synthesis in dark-grown cress seedlings.

The biosynthesis of thujaplicins is of interest because of their strong antibiotic activities. A plausible route for their formation via 3-carene was proposed in 1952 by Erdtman (Charlwood and Banthorpe, 1978, Croteau and Johnson, 1985). An alternative route is through the polyacetate pathway whereby 3 molecules of malonyl-CoA condense with 3,3-dimethylacryl-CoA to form a branched chain C_{11} polyketide that can cyclize,

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decarboxylate and be functionalized (Charlwood and Banthorpe, 1978). On the other hand recent developments in the plant isoprenoid biochemistry suggest that biosynthesis of many isoprenoids does not proceed via the classical acetate/mevalonate pathway, but via a recently found glyceraldehyde/pyruvate route (Rohmer *et al.*, 1993, Schwender *et al.*, 1996, Eisenreich *et al.*, 1996). However, biochemistry of wood tropolones has not been explored in any detail and nothing is known of their routes of biosynthesis. No tracer studies have been carried out for biosynthesis of wood tropolones, probably because they are naturally formed in the interior of mature wood (Hillis, 1987) where biochemical experiments are quite difficult to conduct.

In vitro cell culture systems of woody plants can be useful for studying the biosynthetic routes of their heartwood constituents if the culture can produce them. We have established a fast growing callus culture line derived from a young seedling of *Cupressus lusitanica* Miller and have maintained it for more than 7 years (Sakai *et al.*, 1994). The callus grows fast on the Gamborg B5 medium (Gamborg *et al.*, 1968) supplemented with growth regulators. When elicited with yeast extract the callus started *de novo* synthesis of β-thujaplicin (Inada *et al.*, 1993, Sakai *et al.*, 1994). We have been able to conduct tracer experiments with this callus culture system to obtain fundamental knowledge on the biosynthetic route of β-thujaplicin since the elicitation phenomenon is highly reproducible. In the present paper we deal with feeding experiments with ^{14}C-labeled possible precursors on the cell cultures of *C. lusitanica*.

RESULTS AND DISCUSSION

Incorporation of ^{14}C-labeled glucose and mevalonate into β-thujaplicin in callus cells

Callus of *C. lusitanica* maintained on the Gamborg B5 medium at 25 °C accumulates β-thujaplicin if a yeast extract solution is dropped onto the callus, though almost no thujaplicins were produced while the callus is rapidly growing without the elicitor treatment (Sakai *et al.*, 1994). In order to obtain knowledge of the biosynthetic route of β-thujaplicin, the callus was fed with ^{14}C-labeled compounds by the shot-gun method and simultaneously treated with the elicitor solution. After 10 days of incubation ethyl acetate extract of the callus was analyzed by HPLC, detected both with absorbance at 325 nm (λ_{max} of thujaplicinato-Cu(II)-complex) and radioactivity. [2-^{14}C]Mevalonate was incorporated into β-thujaplicin of the *C. lusitanica* callus as seen from a significant peak with a retention time 17.0 min in the HPLC chromatogram (Fig. 1). A large peak of radioactive mevalonic acid was also observed near 4 min in the same chromatogram. A slight increase in radioactivity at 25–28 min was not identified.

The incorporation of mevalonate strongly suggests that β-thujaplicin is *de novo* synthesized via the mevalonate pathway in the callus. However, specific radioactivity of β-thujaplicin from [U-^{14}C]glucose-fed callus was about 2.5 times larger than that from a [2-^{14}C]mevalonate-feeding experiment as shown in Table 1. The conversion of [U-^{14}C]glucose into β-thujaplicin was 3.7 times that of ^{14}C-labeled mevalonate.

Incorporation of ^{14}C-labeled malonate into β-thujaplicin in callus cells

If the larger incorporation of glucose than mevalonate in Table 1 implies that glucose

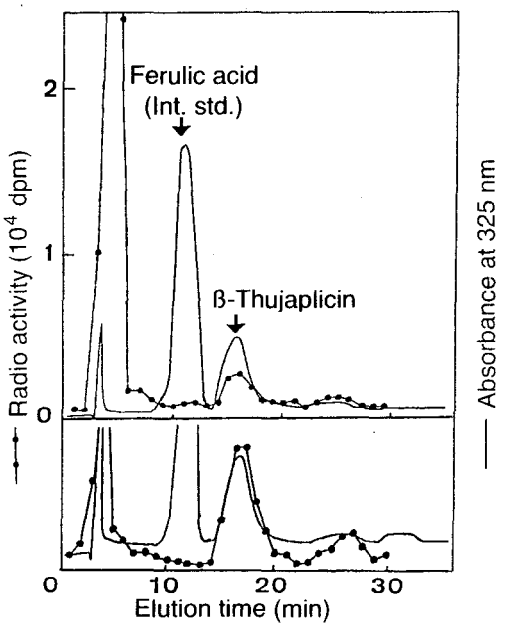

Fig. 1. HPLC of ethyl acetate extract of *C. lusitanica* callus cultures fed with ^{14}C-labeled mevalonate (upper figure) and ^{14}C-labeled glucose (lower figure).

Table 1. Incorporation of radioactivity of [U-^{14}C]glucose and [2-^{14}C]mevalonate into β-thujaplicin produced by *C. lusitanica* callus

Administered		β-thujaplicin	Spec. act	Incorporaition
Compound	Radioactivity (kBq)	(mg/g fr. callus)	(kBq mmol^{-1})	(%)
[U-^{14}C] Glucose	433	0.124±0.001	684±82	0.166±0.010
[2-^{14}C] Mevalonate	431	0.163±0.007	283±34	0.045±0.003

is a closer precursor, β-thujaplicin could be formed through a polyketide pathway in which 3 molecules of malonyl-CoA condensed with 3,3-dimethylacryl-CoA to form a branched chain C_{11} polyketide (Charlwood and Banthorpe, 1978). A shunt pathway of mevalonate metabolism, through which 3,3-dimethylacrylate can be produced from mevalonate (Landau and Brunengraber, 1985), has been known in animal tissues.

Assuming that the polyketide pathway is the case, malonate should be incorporated into β-thujaplicin more favorably than is mevalonate or glucose. To check the liability of this assumption the *C. lusitanica* callus was treated with the elicitor solution and simultaneously fed with [2-^{14}C]malonate, [2-^{14}C]mevalonate, or [U-^{14}C]glucose. After 10 days of incubation, almost no [2-^{14}C]malonate was incorporated, while mevalonate was certainly incorporated into β-thujaplicin that was *de novo* synthesized in *C. lusitanica*

callus cultures (Table 2). This result clearly indicates that mevalonate was not metabolized to 3,3-dimethylacrylate through the shunt pathway (Landau and Brunengraber, 1985) in the biosynthesis route of β-thujaplicin in *C. lusitanica* callus. In other words β-thujaplicin should be biosynthesized though the mevalonate pathway.

The conversion of [2-^{14}C]mevalonate into β-thujaplicin was again smaller than that of [U-^{14}C]-glucose. The smaller incorporation of exogenous mevalonate is presumably due to the subcellular compartmentation of enzyme systems for the β-thujaplicin biosynthesis as suggested for other terpenoid compounds by Croteau *et al.* (1995). The present result probably implies that ^{14}C-labeled mevalonate is hard to incorporate compared with [U-^{14}C]-glucose into the site where β-thujaplicin is synthesized.

Table 2. Incorporation of radioactivity of [U-^{14}C]glucose, [2-^{14}C]mevalonate, and [2-^{14}C]malonate into β-thujaplicin. Administered radioactivity of the ^{14}C-labeled compounds was 74kBq

Administered Compound	β-Thujaplicin	
	Spec. act. (kBq mmol^{-1})	Incorporaition(%)
[U-^{14}C] Glucose	54.0±2.7	0.032
[2-^{14}C] Mevalonate	21.4±2.0	0.020
[2-^{14}C] Melonate	1.4±1.1	0.001

Time-course of incorporation of [1-^{14}C]acetate into β-thujaplicin in suspension cell cultures

To obtain better information on the pathway of β-thujaplicin biosynthesis, [1-^{14}C]-acetate was administered to the suspension cultures of *C. lusitanica*. Its incorporation into mevalonate, geranyl pyrophosphate (GPP) and β-thujaplicin generated in the culture was evaluated at different time intervals as shown in Fig. 2. When the elicited cells were incubated with [1-^{14}C]acetate, radiolabel maxima were observed for mevalonate and GPP on day 1 and day 3, respectively. The accumulation of radiolabel β-thujaplicin was observed until day 5. This successive incorporation of [1-^{14}C]acetate into mevalonate, GPP and then β-thujaplicin strongly supports the involvement of the mevalonate pathway in the β-thujaplicin biosynthesis. Involvement of the recently found glyceraldehyde/pyruvate route (Rohmer *et al.*, 1993, Eisenreich *et al.*, 1996) should be almost rejected due to the above-mentioned incorporation of acetate into β-thujaplicin, since acetate is not a precursor in this non-mevalonate pathway.

Significant differences in the acetate incorporation were observed between control (non elicitation) and elicited cells. In the control (non-elicited) cells, accumulation of labeled GPP was very low and very weak radioactivities were incorporated into β-thujaplicin as compared with elicited cells. This result indicates the pronounced effect of elicitation on the onset of the secondary metabolism that leads to the β-thujaplicin synthesis.

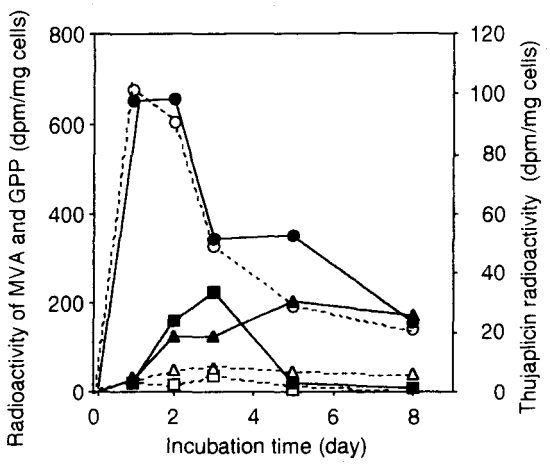

Fig. 2. Time-course of incorporation of [1-^{14}C]acetate into mevalonate (MVA; ● and ○), geranyl pyrophosphate (GPP; ■ and □) and β-thujaplicin (▲ and △) in the suspension culture of *C. lusitanica* with and without elicitor. Solid symbols are for elicited cells and open symbols for control cells.

Effect of hydroxymethylglutaryl CoA reductase inhibitor on the β-thujaplicin production

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is one of the regulatory enzymes of the mevalonate pathway (Goldstein and Brown, 1990). It is therefore expected that inhibitors of HMG-CoA reductase would suppress the β-thujaplicin production if this compound is biologically synthesized through the mevalonate pathway. We added different amounts of compactin, an HMG-CoA reductase inhibitor, to the suspension cell cultures of *C. lusitanica*. The β-thujaplicin content in the compactin-treated cells was determined after incubation for 6 days. As shown in Fig. 3 addition of 87μM compactin to the elicited cell cultures resulted in significant suppression of β-thujaplicin production, in agreement with the involvement of the mevalonate pathway in the β-thujaplicin biosynthesis in *C. lusitanica* cells. This compactin concentration was very high as compared with the case of human fibroblasts where cholesterol synthesis from [^{14}C]acetate was almost completely suppressed by compactin at 0.26μM (Brown *et al.*, 1978). This result also suggests the subcellular compartmentation of the β-thujaplicin biosynthesis site that might not be easily accessible by the exogenous inhibitor.

In conclusion, the data shown in the present paper provide the first experimental results which strongly indicate that β-thujaplicin, one of heartwood tropolones, is of mevalonoid origin but not formed from a polyketide compound through the polyacetate pathway. A revision of the pathway previously proposed (Charlwood and Banthorpe,

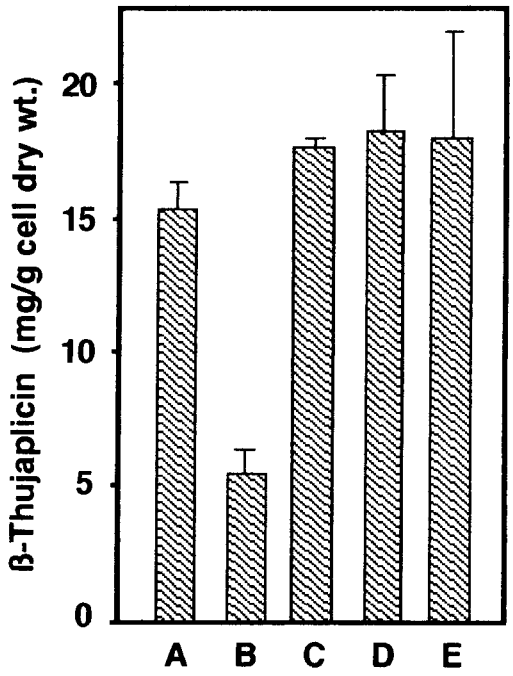

Fig. 3. Effect of HMG-CoA reductase inhibitor, compactin, on the β-thujaplicin production by *C. lusitanica* callus cultures. After elicitation, 87 (B), 46 (C), 8.7 (D), or 0,87 (E) μM of compactin was added to each culture. A is a control containing no compactin.

1978) will be described in forthcoming papers.

EXPERIMENTAL

Radiochemicals

D-[U-^{14}C]-glucose, DBED salt of (*RS*)-[2-^{14}C]mevalonic acid, [2-^{14}C]malonic acid, and sodium [1-^{14}C]acetate were commercially available from Daiichi Kagaku Co. Ltd., Tokyo.

Callus culture

Calluses of *Cupressus lusitanica* Miller have been subcultured every 3 or 4 weeks for more than 7 years on the Gamborg B5 solid medium (Gamborg *et al.*, 1968) containing 2% sucrose, 10^{-5}M naphthylacetic acid, 10^{-6}M benzylaminopurine and 0.27% Gelrite as previously described (Sakai *et al.*, 1994).

Feeding callus cultures with ^{14}C-labeled glucose, mevalonate and malonate

A solution (1 ml) containing 100 mg of glucose, about 430 kBq (about 26×10^{6}dpm) or

74 kBq (4.44×10^6 dpm) of a ^{14}C-labeled compound and 15 mg of yeast extract was added to about 0.7 g of the callus on the B5 solid medium (10 ml) containing no sugars. The calluses were incubated at 25 °C in the dark for 10 days after the feeding of ^{14}C-labeled compounds and then extracted with ethyl acetate containing 1.2 mg ferulic acid as an internal standard. The ethyl acetate extract was evaporated *in vacuo* and the residue dissolved in a mixture of 0.2% aq. $CuSO_4$ and methanol (57/43) prior to analysis by HPLC equipped with a μBondasphere C_4 column and a UV detector (325 nm) using a 0.2% aq. $CuSO_4$/MeOH (65/35) mixture as an eluent. Fractions eluted were collected every minute and their radioactivities were recorded with a liquid scintillation analyzer using Aquasol-2 as a xylene-based scintillant cocktail.

Conversion of [1-^{14}C]acetate to β-thujaplicin by suspension culture

The callus of *C. lusitanica* (0.5–0.7 g fr. wt.) was pulverized and placed in a 50 ml Erlenmeyer flask which contained 2% glucose in 10 ml of B5 liquid medium. An aqueous solution (1 ml) containing yeast extract (15 mg) and sodium [1-^{14}C]acetate 185 kBq was added into the flask. Water (1 ml) was added in a control experiment instead of the yeast extract. The cultures were incubated at 25 °C in the dark for the periods of time indicated in Fig. 2 and then extracted with ethyl acetate containing 0.08 mg vanillin as an internal standard. Amounts and radioactivity of β-thujaplicin in the extracts were determined as described above.

The extraction residues were filtrated and mevalonic acid in the filtrates was analyzed by HPLC equipped with a HPX-87H column (Biorad) and a refractive index detector. Geranyl pyrophosphate (GPP) was analyzed as geraniol. A part of the filtrate (5 ml) was treated with 10 M NaOH aq. (5 ml) and extracted with diethyl ether. Geraniol liberated in the ether extract was purified using a preparative TLC system, diethyl ether-hexane 1:1 (v/v).

Radioactivity of the fractions collected from HPLC analysis and TLC system was recorded with a liquid scintillation analyzer as described above.

Effect of compactin on the β-thujaplicin production

Compactin in the lactone form (Sigma) 3.9 mg was dissolved in 500 ml ethanol, saponified with 500 ml of 0.2 N NaOH at 50 °C for 1 h (Brown *et al.*, 1978, Endo *et al.*, 1976), and neutralized with HCl at room temperature. Proper amounts of the neutralized compactin (cf. Fig. 3) and yeast extract (15 mg) were added to the suspension cell cultures of *C. lusitanica* in 10 ml aliquots of the liquid medium described before. β-Thujaplicin contents in the cultures were determined after incubation for 6 days.

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