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ISOLATION OF 7-DEHYDROCHOLESTEROL FROM CELL CULTURES OF *SOLANUM MALACOXYLON*

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Key Word Index—*Solanum malacoxylon*; *Solanum glaucophyllum*; Solanaceae; *in vitro* cultures; biosynthesis; 7-dehydrocholesterol; cholecalciferol; 25-hydroxycholecalciferol; calcitriol.

Abstract—The presence of cholesterol, 7-dehydrocholesterol, cholecalciferol (vitamin D_3) and its hydroxylated metabolites, 25-hydroxycholecalciferol (25-OH D_3) and 1α,25-dihydroxycholecalciferol (1α,25-diOH D_3), was demonstrated in cell cultures derived from sterile leaves of *Solanum malacoxylon*. When calli were grown in the dark, 7-dehydrocholesterol was found and identified on the basis of its chromatographic and spectroscopic properties. The analysis of calli grown in the light revealed the presence of vitamin D_3 and 25-OH D_3, where 7-dehydrocholesterol was not detected. In suspended cells, maintained in the light, vitamin D_3, 25-OH D_3 and 1α,25-diOH D_3 were found, but no 7-dehydrocholesterol was detected. Our results suggest that 7-dehydrocholesterol may arise as an intermediate in the biosynthesis of vitamin D_3 in *S. malacoxylon*. A photoreaction from 7-dehydrocholesterol may be operative in the biosynthesis of cholecalciferol, thus suggesting that in this plant there is a similar pathway to that in animals. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

It is now well established that the active principle in *Solanum malacoxylon* Sendt., a calcinogenic plant that grows in South America, is calcitriol (1α,25-dihydroxycholecalciferol, 1α,25-diOH D_3). This finding could explain the pathological calcinosis in grazing animals that ingest the leaves, considered the main site of calcinogenic activity in this plant. In fact, Weissenberg *et al.* [1] reported such activity in both the aglycone and glycoside form in the berries, stems and roots. Appreciable 1α,25-diOH D_3 activity, determined by means of a radial immunodiffusion assay, has been found in cell cultures grown in the dark [1]. Furthermore, the capability of *S. malacoxylon* tissue cultures to produce steroids (sitosterol, diosgenin and solasodine) has also been reported [2]. In our previous paper [3] we investigated the nutritional requirements and sterol production of callus cultures from hypocotyl, root and leaf explants of *S. malacoxylon*. This paper describes our results on the production, in different experimental conditions, of metabolites correlated to the vitamin D_3 biosynthetic pathway in cell cultures derived from *S. malacoxylon* leaves.

RESULTS AND DISCUSSION

We made a comparison of the extracts obtained from calli grown on MS-1W in the dark for more than two years, and those cultivated on the same medium in the light for three weeks, after induction in the dark. The calli were extracted with chloroform–methanol–NH_4OH and then with *n*-butanol. Butanolic extracts, which contained glycoside derivatives, were hydrolysed enzymically with mixed glycosidases and then analysed as chloroformic extracts. The analysis on reverse phase HPLC of the chloroform–methanol–NH_4OH extract of the calli grown in the dark revealed the presence of 7-dehydrocholesterol, a compound not previously reported before as a constituent of *S. malacoxylon*. The extract was partially purified by silica gel column chromatography (chloroform–methanol 47:3) and the fractions which contained 7-dehydrocholesterol were collected and further purified by preparative TLC (10 $\mu g\, g^{-1}$ fr. wt calli). The chemical identity of this compound was established by its ^1H NMR and mass spectra by comparison with an authentic sample. No 7-dehydrocholesterol, vitamin D_3 or its hydroxylated metabolites were detected in the butanolic extract obtained from these calli after hydrolysis. When calli were cultured in the light, TLC and HPLC analysis of the chloroform–methanol–NH_4OH extract and co-chromatography with authentic standards revealed the presence of vitamin D_3 and 25-OH D_3, whereas 7-dehydrocholesterol was not detected. The compounds were quantified by HPLC which revealed the presence of 0.44 and 0.88 $\mu g\, g^{-1}$ fr. wt calli, vitamin D_3 and 25-OH D_3, respectively. Apart from the free forms, the

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corresponding glycosidic metabolites were also found. The concentration of free metabolites was much higher than that of the glycosidic forms. From an analysis of the extracts from calli maintained in the dark, which contained cholesterol, as described by us before, and 7-dehydrocholesterol, and those maintained in the light, which contained vitamin D_3 and 25-OH D_3, we are able to hypothesize that in the cells of *S. malacoxylon* there could be a biosynthetic pathway for vitamin D_3 production as in the skin of vertebrates. However, the calli cultivated in the dark could not use light for the conversion of 7-dehydrocholesterol into vitamin D_3; thus, the biosynthesis was blocked at this stage. On the other hand, for the calli grown in the light this pathway was available.

The suspension cell cultures, derived from leaves' calli grown in the dark, were maintained in MS-1W or B5 medium in the light. Suspended cell cultures were harvested after 12 and 72 hr in MS-1W and 12, 24, 36, 48 and 72 hr in B5. The results of the analysis by TLC and HPLC of the chloroform extracts of the cell cultures maintained in MS-1W and harvested after 12 hr revealed the presence of 7-dehydrocholesterol (5 $\mu g\, g^{-1}$ cells fr. wt), but no vitamin D_3 or its hydroxylated metabolites were found. With a suspension cell culture cultivated in B5 in the light and harvested after 12 hr, the extracts of the cell cultures contained 7-dehydrocholesterol (58 $\mu g\, g^{-1}$ of cells fr wt) and vitamin D_3 (2.2 $\mu g\, g^{-1}$ of cells fr. wt). The HPLC analysis of the extracts harvested after 24 hr revealed the presence of vitamin D_3 (16.6 $\mu g\, g^{-1}$ cells fr. wt), but no other compounds were detected. Also, after 36 hr the extracts contained only vitamin D_3 (38.6 $\mu g\, g^{-1}$ cells fr. wt). On the other hand, the cell cultures harvested after 48 hr contained, besides vitamin D_3 (42.1 $\mu g\, g^{-1}$ cells fr. wt), two of its hydroxylated metabolites 25-OH D_3 (1 $\mu g\, g^{-1}$ cells fr. wt) and 1α,25-diOH D_3 (0.1 $\mu g\, g^{-1}$ cells fr. wt). Vitamin D_3 and 25-OH D_3 were separated by flash chromatography (petrol–ethyl acetate, 7:3) and then purified by preparative TLC (chloroform–methanol, 19:1). The identity of vitamin D_3 was confirmed by its ^1H NMR and mass spectra, while 25-OH D_3 was identified by its mass spectrum. An indication of the presence of 1α,25-diOH D_3 in the fractions collected after flash chromatography (petrol–ethyl acetate, 3:7) was obtained by HPLC analysis. The UV spectrum of the peak with R_f identical to that of the authentic sample had λ_{max} = 264 nm, characteristic of vitamin D_3 compounds. After 72 hr the extracts contained only vitamin D_3 (5.2 $\mu g\, g^{-1}$ cells fr. wt). On the other hand, the HPLC analysis of the butanolic extracts revealed the presence of small quantities of the corresponding glycosides. The results obtained from the analysis of extracts from suspension cell cultures contained in MS-1W and B5 medium for different incubation times are reported in Table 1. In the suspension cell cultures cultivated in the light in B5 medium, 7-dehydrocholesterol was present only after 12 hr. On the other hand, the quantity of vitamin D_3 gradually increased up to 48 hr and then decreased. From the results in Table 1 it can be seen also that the B5 medium stimulates the production of the compounds involved in the biosynthetic pathway of vitamin D_3 and confirms our previous results on cholesterol production. It is interesting to note that 7-dehydrocholesterol, produced from cell cultures cultivated for 12 hr in B5, was found mainly in the culture medium, as well as vitamin D_3 after 24 hr. Moreover, at the other times (36, 48 and 72 hr) the latter was distributed between cells and culture media. This suggests that the cell cultures of *S. malacoxylon* are not only able to synthesize these compounds, but can also release them into the medium. Earlier workers in this field have suggested that vitamin D_3 and its metabolites may be present in *S. malacoxylon* as glycosides [14]. In our experiments these substances were present in the free form, but only traces of glycosylated metabolites were found.

These results suggest that vitamin D_3 may be synthesized in *Solanum malacoxylon* by the photolytic activation of a precursor, possibly 7-dehydrocholesterol, just as in the skin of vertebrates. Vitamin D_3 is further transformed into the hydroxylated metabolites 25-OH D_3 and 1α,25-diOH D_3. Cholecalciferol and its hydroxylated metabolites are of high pharmacological and commercial interest. Thus, future work concerning biotransformation techniques will be aimed at increasing the calcitriol yield.

EXPERIMENTAL

Cell cultures. Solanum malacoxylon plantlets were obtained in axenic conditions from sterilized seeds as

Table 1. 7-Dehydrocholesterol, vitamin D_3, 25-OH D_3 and 1α,25-diOH D_3 extract content at different times ($\mu g\, g^{-1}$ cells fr. wt)

	12 hr		24 hr	36 hr	48 hr	72 hr	
	MS-1W	B5	(B5)	(B5)	(B5)	MS-1W	B5
7-Dehydrocholesterol	5	58	ND	ND	ND	ND	ND
Vitamin D_3	ND	2.2	16.6	38.6	42.1	ND	5.2
25-OH D_3	ND	ND	ND	ND	1.0	ND	ND
1α,25-DiOH D_3	ND	ND	ND	ND	0.1	ND	ND

ND = not detectable.

described by Suardi *et al.* [3]. The plantlets were multiplied by stem microcuttings in agar-solidified medium containing Murashige–Skoog salts [5] 1/2 strength and 1% sucrose in a growth chamber at 25°, with a day period of 16 hr (light source: Osram L 40W/20 Sa with a fluence rate 34 W m^{-2}). Expanded leaves were produced in 2–3 weeks and used for callus induction. The leaves were cut into thin strips by a scalpel and plated in Petri dishes containing cell proliferation medium. The two media used contained 0.8 agar, Murashige–Skoog salts and vitamins, and the following additions: sucrose 30 g l^{-1}, kinetin 0.3 mg l^{-1}, 2,4-D (2,4-dichlorophenoxyacetic acid) 1 mg l^{-1} (medium MS-1W) or sucrose 20 g l^{-1}, glucose 0.25 g l^{-1}, thiamin 0.8 mg l^{-1}, kinetin 1.2 mg l^{-1}, 2,4-D 2 mg l^{-1} (medium MS-2D). The calli obtained were kept in the dark at 25° and subcultured at 3–4 week intervals. When callus induction was initiated on MS-2D, friable, white calli were induced rapidly (12 days), compared with the 4 weeks needed for induction of callus on MS-1W. However, best results were obtained when callus induction was started on MS-2D and calli were transferred and maintained on MS-1W.

Suspension cultures were initiated by dispersing the friable calli in liquid MS-2D medium, placed in the light at 25° on a rotatory shaker a 120 rev min^{-1}. The suspended cells were subcultured every 2 weeks in a fresh medium (dilution 1:5). The viability of the cells was evaluated by the FDA (fluorescein diacetate) method [6]. Cell growth was estimated as PCV (packed cell volume). The suspended cells of *S. malacoxylon* have their logarithmic growth phase between 3 and 7 days; with an inoculum of 3 g cells in 20 ml culture medium a 6-fold increase in PCV was observed within 2 weeks. For biosynthesis studies, cells in the mid-exponential phase were transferred into liquid MS-1W or Gamborg's B5 [7] (salts and vitamins) supplemented with sucrose (30 g l^{-1}), kinetin (0.3 mg l^{-1}), 2,4-D (1 mg l^{-1}) and vitamins' MS.

Extraction and analysis. Calli grown in the dark (200 g fr. wt) and those grown in the light (200 g fr. wt) in MS-1W were ground in the presence of liquid N_2 and then extracted overnight with $CHCl_3$–MeOH–NH_4OH (3:2:0.3) (600 ml) by percolation. The $CHCl_3$–MeOH–NH_4OH extract was counter-extracted 3× with $CHCl_3$ (150 ml), dried with Na_2SO_4 and filtered. The residue was then percolated overnight in *n*-BuOH (600 ml). The solvents were removed under red. pres. The BuOH extracts were hydrolysed enzymically. The hydrolysis was accomplished with a mixed glycosidase prepn derived from *Turbo cornutus* (ICN Biomedicals) (10 mg for 50 mg BuOH extract) in pH 4 buffer at 37° for 72 hr, then extracted with $CHCl_3$ (5 ml × 3). The organic phase was dried with Na_2SO_4, filtered and the solvent removed under red. pres. $CHCl_3$ extracts were analysed by TLC (Merck silica gel 60 F254); eluents: $CHCl_3$–MeOH (19:1), $CHCl_3$–EtOH–H_2O (183:16:1). The spots were visualized by exposure to UV radiation (254 nm) and by spraying with: (*a*) 10% H_2SO_4 in MeOH and heating at 100° for 40–60 sec, (*b*) anisaldehyde in H_2SO_4 (2%, w/v) mixed with glacial HOAc (1:10, v/v) and warming at 50° for 60–90 sec, (*c*) phosphomolybdic acid in EtOH (10%, v/v) or (*d*) $SbCl_3$ in $CHCl_3$ (20%, w/v). The R_f of the numerous spots obtained from the runs of the extracts were compared with those of authentic samples. TLC and HPLC analyses were confirmed by co-chromatography with authentic standards. The $CHCl_3$ extract obtained from calli grown in the dark was partially purified by silica gel CC (Merck 70–230 mesh), using $CHCl_3$–MeOH (47:3) as eluent. The frs containing spots corresponding to 7-dehydrocholesterol were collected (4.5 mg) and submitted to additional purification by prep. TLC (Merck silica gel 60; 0.25 mm layer, petrol–EtOAc, 7:3; recovered with $CHCl_3$); 2 mg pure 7-dehydrocholesterol were recovered. The chemical identity of this compound was confirmed by its ^1H NMR and mass spectra. Analysis by HPLC of $CHCl_3$ extracts obtained after hydrolysis from calli grown in the dark did not show the presence of 7-dehydrocholesterol, vitamin D_3 or any of its hydroxylated metabolites, but HPLC analysis of $CHCl_3$ extracts obtained after hydrolysis from calli grown in the light revealed the presence of traces of vitamin D_3 and 25-OH D_3.

Suspended cells (about 10 g fr. wt in 100 ml nutritive medium for each time), cultivated in MS-1W for 12 and 72 hr and in B5 for 12, 24, 36, 48 and 72 hr, were extracted under the same conditions. Culture medium and cells were sepd by centrifugation (2400 rev min^{-1}, 20 min). Cells were ground in the presence of liquid N_2 and macerated with 10 ml 10% NH_4OH for 20 min, then extracted 3× with 50 ml $CHCl_3$–MeOH (2:1) and 3× with 50 ml *n*-BuOH. The $CHCl_3$–MeOH–NH_4OH extract was counter-extracted 3× with $CHCl_3$ and then treated as for calli. The culture medium was extracted 3× with $CHCl_3$, then with *n*-BuOH. The BuOH extracts were hydrolysed enzymically as mentioned before. $CHCl_3$ extracts were analysed by TLC/HPLC and quantified by HPLC. Analysis of the $CHCl_3$ extracts obtained from cell cultures maintained in B5 for 48 hr revealed the presence of vitamin D_3, 25-OH D_3 and 1,25-diOH D_3. Ten replicates (each about 10 g cells in 100 ml medium) under these conditions (B5 for 48 h) were made. The combined $CHCl_3$ extracts were submitted to flash chromatography (Merck Kieselgel 230–400 mesh) using petrol–EtOAc (7:3) and then petrol–EtOAc (3:7). The frs containing vitamin D_3 (10.5 mg) were collected and further purified, after concn *in vacuo* by prep. TLC ($CHCl_3$–MeOH, 19:1). The portion corresponding to vitamin D_3 was removed and extracted 3× with $CHCl_3$, $CHCl_3$–MeOH (2:1) and MeOH. The combined organic solvents were evapd under red. pres.; 4 mg pure vitamin D_3 were recovered. Its chemical identity was confirmed by its ^1H NMR and mass spectra. The frs containing 25-OH D_3 (2.9 mg) were purified by prep. TLC ($CHCl_3$–MeOH, 19:1); the portion corresponding to the authentic sample of 25-OH D_3 was removed and extracted 3× with $CHCl_3$, $CHCl_3$–MeOH (1:1) and MeOH. The combined or-

ganic solvents were evapd under a stream of N_2; 0.1 mg of pure 25-OH D_3 was recovered. The MS of this compound was identical to that of the standard sample of 25-OH D_3 and in accordance with published data [4]. The frs which revealed the presence of 1α,25-diOH D_3 on TLC analysis were further analysed by HPLC. The R_fs in HPLC and UV ($\lambda = 264$ nm) were identical to those of an authentic sample. HPLC analysis of $CHCl_3$ extracts obtained after enzymic hydrolysis from suspended cells indicated that only traces of vitamin D_3 and 25-OH D_3 were present.

HPLC analysis. HPLC analysis was carried out on a system consisting of a Waters pump 600MS, a Waters model U6K sample injector and a Waters model 991 photodiode array assembly. $CHCl_3$ extracts were dissolved in MeOH and analysed by a reverse phase column Nova-Pak C18 (150 × 3.9 mm). Gradient elution was performed [15 min isocratic conditions MeOH–H_2O (3:1) and in 15 min to 100% MeOH] at 1 ml min^{-1}, with UV monitoring at 264 nm, comparing R_fs to those of standards: 7-dehydrocholesterol (29.1 min), vitamin D_3 (26.5 min), 25-OH D_3 (21.2 min) and 1α,25-diOH D_3 (10.3 min). Quantification in HPLC was by comparison of peak areas with those of standards.

Spectrometric methods. ^{1}H NMR spectra were recorded on a Varian XL200 and MS on a VG 7070 EQ instrument.

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