Isolation and characterization of an intermediate steroid metabolite in diosgenin biosynthesis in suspension cultures of *Dioscorea deltoidea* cells

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(1) The aglycon form of the steroidal sapogenin furost-5-ene-3 β ,22,26-triol, 3 β -chacotrioside 26 β -D-glucopyranoside was isolated from cell suspension cultures of *Dioscorea deltoidea* and its molecular structure was determined by mass spectrometry and ^1H and ^{13}C n.m.r. spectroscopy. (2) From kinetic studies and incorporation experiments with [1- ^{14}C]acetate it was concluded that the steroidal compound (in the glycoside form) is an intermediate *in vivo* in diosgenin biosynthesis. It accumulated in growing cells of *D. deltoidea* and was metabolized to diosgenin (in the glycoside form, i.e. dioscin) in non-dividing cells.

Steroidal sapogenins (spirostanols) e.g. diosgenin (Fig. 1) are synthesized from cholesterol in several plants (Bennett & Heftmann, 1965; Bennett *et al.*, 1967; Warma *et al.*, 1969; Stohs *et al.*, 1974; Eichenberger, 1982; Mahato *et al.*, 1982), but the intermediate biosynthetic steps have not yet been completely elucidated. Steroidal saponins in which the side chain is held open by glycoside formation (furostanols) are naturally occurring glycosides in several plant species (Joly *et al.*, 1969a; Eichenberger, 1982; Sharma *et al.*, 1982).

These glycosides are converted *in vitro* to spirostanols by the elimination of the glucose molecule at C-26 and ring closure by the action of glucosidases (see Fig. 1 for the formation of an analogous compound, asparanin D) (Marker & Lopez, 1947; Schreiber & Ripperger, 1966; Ripperger *et al.*, 1967; Tschesche *et al.*, 1967, 1972; Eichenberger, 1982; Sharma *et al.*, 1982). Radioactively labelled furost-5-ene-3 β , 22,26-triol 3 β -chacotrioside 26 β -D-glucopyranoside (furostanol I; Fig. 1) was converted to dioscin (the glycoside form of diosgenin) by

Fig. 1. Intermediate compounds in sapogenin biosynthesis

leaf homogenates of *Dioscorea floribunda* (Joly *et al.*, 1969a). These results suggest that the biosynthesis of diosgenin from cholesterol proceeds via furostanol I, similar to the proposed biosynthetic pathways of other spirostanols from their corresponding furostanols in various plants (Eichenberger, 1982; Sharma *et al.*, 1982).

We present data supporting the hypothesis that furostanol I is an intermediate *in vivo* for diosgenin.

Materials and methods

Growth conditions

Dioscorea deltoidea cells were grown in 100 ml of Murashige & Skoog (1962) medium supplemented with 2,4-dichlorophenoxyacetic acid (0.1 mg/l). Growth of cells in suspension cultures was described previously (Tal & Goldberg, 1982; Tal *et al.*, 1982).

Isolation of diosgenin and the putative steroidal intermediate

The fractionation of steroidal sapogenins from *D. deltoidea* was previously described (Tal & Goldberg, 1981). Diosgenin and the putative intermediate were separated by h.p.l.c. and fractions containing these compounds were collected (Tal & Goldberg, 1981). Each fraction was separated on silica gel 60 plates (Merck) with chloroform/acetone (4:1, v/v) as a solvent. The R_F values of diosgenin and the steroid intermediate were 0.48 and 0.49, respectively. Both were extracted from the t.l.c. plates with tetrahydrofuran and applied to HPTLC RP-18 plates (Merck) and developed in acetonitrile/methanol/chloroform (41:5:4, by vol.). The R_F values of diosgenin and the putative intermediate were 0.18 and 0.47, respectively. The putative intermediate spot on the HPTLC plates was extracted and identified by h.p.l.c.; diosgenin was identified by co-chromatography with standard diosgenin (Sigma).

Characterization of the putative steroidal intermediate

The purified compound was analysed by mass spectrometry in an LKB model 2091 g.l.c.-m.s. at an ionization energy of 70 eV. The ^1H and ^{13}C n.m.r. spectra of the purified steroidal compound and diosgenin were recorded at 300 MHz and 75.46 MHz, respectively, with a Bruker WH-300 spectrometer in the FT mode in C_2HCl_3 . Chemical shifts (δ) are given in p.p.m. with tetramethylsilane as a reference. All the ^{13}C n.m.r. spectra were recorded in the proton noise-decoupling mode.

Radioactivity experiments

Pulse experiment. On various days (Table 2), $10\ \mu\text{Ci}$ of $[1-^{14}\text{C}]$ acetate (2 mCi/mmol; New Eng-

land Nuclear, Boston, MA, U.S.A.) were added to 500 ml flasks containing 100 ml of cell culture. The cells were filtered after 6 h and the remaining radioactivity was determined in the culture broth. The radioactivity was measured in purified diosgenin and steroidal compound; a constant specific radioactivity of diosgenin was obtained after separation by the first silica gel t.l.c. system while a constant specific radioactivity of the steroidal compound was obtained after separation by the second reversed-phase t.l.c. system.

Pulse-chase experiment. Cells were grown for 3 days and then $10\ \mu\text{Ci}$ of $[1-^{14}\text{C}]$ acetate was added to cell suspensions for 6 h. Cells were filtered, washed with medium containing 25 mM-acetate and resuspended in 3-day-old medium obtained from another culture into which labelled acetate was not added. The cells were harvested at different times and diosgenin and steroidal compound were prepared and counted for radioactivity as described above.

Results

Kinetics of growth and production of steroidal metabolites by *D. deltoidea* cells

The pattern of diosgenin accumulation in relation to growth of *D. deltoidea* cells in a batch culture is shown in Fig. 2. A maximal amount of biomass (6.5 g of cell dry wt./l; cellular yield 0.43 g of cell dry wt./g of sucrose utilized) was obtained 10 days after inoculation followed by a pronounced reduction in cell dry weight. Diosgenin synthesis started 5 days after inoculation and reached a level of 184 mg/l (7.25% of dry weight) after 29 days.

Fig. 2. Kinetics of growth and product formation by *D. deltoidea* cells in suspension cultures

The relative amount of the steroidal compound was calculated from the peak area of this compound in the h.p.l.c. chromatogram (see Fig. 3).

Fig. 3. H.p.l.c. chromatogram of *D. deltoidea* extract (a) and diosgenin (D) and steroidal compound (X) content in extracts of *D. deltoidea* cells grown for 3, 8 and 20 days (b)

During the course of analysis of cell extracts, an unidentified steroidal compound (see below) was detected in the h.p.l.c. chromatograms (Fig. 3a). The peak area of this compound was greater in extracts obtained from 8-day-old cultures than in 3-day-old cultures, and decreased to a negligible level in 20-day-old suspension cultures (Fig. 3b). Detailed kinetics showed (Fig. 2) that the accumulation of the steroidal compound (putative intermediate) proceeded in parallel with cell growth and, after the amount of biomass reached a maximum, the level of this compound decreased rapidly (5% of its maximal level). The results suggested that a precursor-product relationship exists between the steroidal compound and diosgenin.

Isolation and characterization of the steroidal compound

The steroidal compound had a retention time of 1.25 min when applied on a reversed-phase h.p.l.c. column. Diosgenin had a retention time of 6 min (Fig. 3a). This suggested that the steroidal compound is more polar than diosgenin.

The mass spectrum of the purified compound contained signals of m/z 414 (M^+), 398, 355, 342, 327, 300, 282, 271 and 126. For diosgenin signals were observed at m/z 414 (M^+), 398, 355, 342, 327, 300, 282, 271 and 126. The signals are identical for the two compounds in spite of the pronounced difference in their polarity in h.p.l.c. A possible explanation is that during the mass spectra

determinations one molecule of water was eliminated from the steroidal compound. Thus, the molecular weight of this compound may be 432.

^1H and ^{13}C n.m.r. spectroscopies were used to verify the molecular structure of the purified steroid. The assignments of the signals of this compound in the spectra were obtained by comparison with the corresponding signals of diosgenin. The following identical resonances were observed in the ^1H n.m.r. spectra of the steroidal compound and diosgenin: δ 0.79 (s, C-18 methyl), 0.79 (d, $J = 6.2\text{ Hz}$; C-27 methyl), 0.79 ($J = 7.1\text{ Hz}$; C-21 methyl), 1.03 (s, C-19 methyl), 3.38 (t, $J = 10.6\text{ Hz}$; C-26 α -H), 3.47 (d, dd, $J = 10.5\text{ Hz}$ and J approx. 4 Hz; C-26 β -H), 3.49 (broad, C-3 α -H), 4.41 (q, $J = 7.1\text{ Hz}$; C-16H), 5.35 (broad d, $J = 5.3\text{ Hz}$; C-6H) (see for comparison Blanden *et al.*, 1981). The methylenic envelopes in both spectra were very similar and they differed only by a single peak at δ 2.18 having an intensity corresponding to two protons in the spectrum of the steroidal compound. This peak is probably best rationalized as two hydroxyl groups at C-22 and C-26.

Diosgenin and the steroidal compound were acetylated in an effort to account for the number of

Table 1. ^{13}C n.m.r. signals and their assignment of diosgenin and the steroidal compound

Carbon atom no.	δ (p.p.m.) for:	
	Diosgenin	Steroidal compound
1	37.17	37.20
2	31.98	32.01
3	71.61	71.70
4	42.24	42.27
5	140.83	140.58
6	121.29	121.35
7	31.42	31.79
8	31.57	31.67
9	50.08	50.14
10	36.58	36.68
11	20.81	20.84
12	39.72	39.79
13	41.56	40.91
14	56.49	56.49
15	31.76	31.79
16	80.73	80.78
17	62.18	62.30
18	16.11	16.11
19	19.26	19.28
20	41.56	41.62
21	14.34	14.27
22	109.16	128.27
23	31.35	31.45
24	28.74	28.80
25	30.20	30.24
26	66.76	66.80
27	16.95	16.95

Table 2. *Pulse labelling of D. deltoidea cells with [1-¹⁴C]acetate*

[1-¹⁴C]Acetate (10 μ Ci) was added on different days to suspension cultures (100 ml) of *D. deltoidea* and after 6 h the cells were harvested. Radioactivity was measured in cells and purified diosgenin and steroidal compound. Because of minor differences in the incorporation of [1-¹⁴C]acetate into cells at various days the specific radioactivities of diosgenin and the steroidal compound were corrected accordingly. Abbreviations used: N.D., not detected.

Pulse given after (days)	Cells		Diosgenin			Steroidal compound	
	Dry wt. 10 ⁻⁶ × Radioactivity (g/l) (d.p.m./100mg)	Total amount (mg/flask)	% of dry wt.	10 ⁻⁴ × Specific radioactivity (d.p.m./mg)	Total amount (peak area/flask)	Specific radioactivity (d.p.m./unit area)	
2	1.8	13.4	0.54	0.3	176	5568	5.08
4	3.6	8.2	3.24	0.9	55	11088	5.46
6	4.9	8.0	5.39	1.1	33	18568	0.71
9	4.3	8.2	7.74	1.8	2	18448	0.27
12	2.3	8.2	9.89	4.3	1	1100	N.D.
16	1.8	6.6	12.42	6.9	0.5	917	N.D.

hydroxyl groups in the molecules. The spectrum of acetylated diosgenin indicated the presence of a single acetoxy group at δ 2.03 due to the acetylation of the C-3 hydroxyl. The spectrum of the acetylated steroid compound consisted of many peaks as a result of the acetylation procedure, indicating that the compound was not stable under these conditions.

The results of the ¹H n.m.r. analysis suggested that the steroidal compound is the aglycon form of furost-5-ene-3 β ,22,26-triol 3 β -chacotrioside 26 β -D-glucopyranoside (furostanol I) and its proposed molecular structure is shown in Fig. 1.

The ¹³C n.m.r. spectra were determined in order to confirm the proposed molecular structure of the steroidal compound in comparison with that of diosgenin. The corresponding chemical shifts and their assignments (Tori *et al.*, 1981; Espejo *et al.*, 1982) are summarized in Table 1. All the corresponding signals of the various carbon atoms of diosgenin were accounted for in the spectrum of the steroidal sapogenin. The chemical shifts were very similar except for that of C-22. This was shifted downfield by about 19 p.p.m. to δ_C = 128.27 in the spectrum of the compound. This shift indicates that ring F is open and C-22 has an additional hydroxyl group as shown in Fig. 1.

The steroidal sapogenin is a possible intermediate in diosgenin biosynthesis

Diosgenin and the steroidal sapogenin were labelled with [1-¹⁴C]acetate in short-time feeding experiments (6 h) and the specific radioactivities were determined (Table 2). The specific radioactivity of diosgenin decreased by about 5-fold from day 2 to day 6 when maximal growth was obtained. After the growth phase the specific radioactivity of diosgenin was further decreased by about 350-fold, 16 days after inoculation. Since the total amount of diosgenin increased 23-fold from

day 2 to day 16 (Table 2), the decrease in the rate of acetate incorporation into diosgenin suggests that most of the diosgenin is not synthesized directly from acetate *in vivo* but rather from an intermediate precursor(s).

The specific radioactivity of the steroidal compound increased slightly from day 2 to day 4 and then decreased. The level of this steroid increased up to day 9 and then decreased to about 5% of its maximal level (Table 2). This would indicate that the rate of synthesis of this compound from acetate was maximal in the early stages of exponential growth. During the later stage of growth (day 6 and day 9) the rate decreased without a parallel decrease in the amount of this compound, suggesting a transient accumulation of the steroidal metabolite before it was further metabolized to diosgenin.

In a pulse-chase experiment (Table 3) the total radioactivity incorporated into diosgenin during

Table 3. *Pulse-chase experiment with [1-¹⁴C]acetate in cell suspension cultures of D. deltoidea*

Cells were grown for 3 days and then 10 μ Ci of [1-¹⁴C]acetate were added to cell suspensions for 6 h. Cells were filtered, washed with medium containing 25 mM-acetate and resuspended in medium (see the Materials and methods section) for the chase period. Radioactivity was determined in purified steroidal compound and diosgenin.

Time of chase (days)	$10^{-3} \times$ Radioactivity (d.p.m.)/ flask in:			
	Steroidal compound		Diosgenin	
	Total	Change	Total	Change
0	190	0	802	0
5	74	-116	931	+129
17	2	-188	1011	+209

the pulse increased during the chase period, while the radioactivity in the steroidal compound decreased. The increase of total radioactivity in diosgenin corresponded to the decrease in the radioactivity incorporated into the steroidal compound, and strongly suggested that the latter sapogenin is a direct intermediate precursor of diosgenin (Table 3).

Discussion

The synthesis of the steroidal sapogenin diosgenin is not associated with active cell growth and occurred mainly in non-dividing cells of *Dioscorea deltoidea* suspension cultures (Stohs *et al.*, 1974; Tal & Goldberg, 1982; Tal *et al.*, 1982). Our working hypothesis was that a precursor is formed *de novo* from acetate during the exponential growth phase, and biotransformed to diosgenin mostly after growth ceased. In order to identify the possible precursor *in vivo* of diosgenin the sterols of *D. deltoidea* cells were analysed. The analysis revealed the presence of a steroidal compound in the h.p.l.c. chromatogram (Fig. 2a). A detailed study of the time course production of diosgenin and the steroidal compound (Fig. 1) indicated that the latter may be an intermediary metabolite in the biosynthetic pathway of diosgenin. On the basis of spectroscopic investigations (mass spectrometry and ^1H and ^{13}C n.m.r.) it was possible to establish the structure of the putative intermediate as the aglycon form of the steroidal sapogenin furost-5-ene-3 β ,22,26-triol 3 β -chacotriose 26 β -D-glucopyranoside (furostanol I; Fig. 1).

Labelled furostanol I was prepared by treatment of *Dioscorea floribunda* plants with [4- ^{14}C]cholesterol (Joly *et al.*, 1969b). It was shown that furostanol I was completely converted to dioscin (the glycoside form of diosgenin) by a leaf homogenate of that species (Fig. 1; see Joly *et al.*, 1969a). These results led to the conclusion that the biosynthesis of dioscin (or diosgenin) from cholesterol proceeds via furostanol I in *D. floribunda* (Joly *et al.*, 1969a). Similar studies (Marker & Lopez, 1947; Schreiber & Ripperger, 1966; Ripperger *et al.*, 1967; Tschesche *et al.*, 1967, 1972; Eichenberger, 1982; Sharma *et al.*, 1982) have demonstrated that different furostanols, isolated from various plants, were converted *in vitro* to the corresponding spirostanols by treatment with β -glucosidases (for example, the conversion of asparoside D to asparanin D shown in Fig. 1). These studies did not show that furostanol I was a precursor *in vivo* of diosgenin nor when it or diosgenin were synthesized.

The results of the pulse-chase incorporation study of [1- ^{14}C]acetate into sterols of *D. deltoidea* cells (Table 3) presented strong evidence for the

biosynthesis of dioscin from a furostanol in which the F-ring is held open by glucosidation at the C-26 hydroxyl (furostanol I). These results also supported the theory (Joly *et al.*, 1969b; Stohs *et al.*, 1969, 1974; Bennett *et al.*, 1970) that in sapogenin biosynthesis oxygenation occurs first at C-26 followed by cyclization of the sterol side-chain. Upon extraction and hydrolysis (see the Materials and methods section) dioscin and furostanol I were obtained as diosgenin and the steroidal compound, respectively.

We have previously argued that the improvement of diosgenin biosynthesis (preferably in a continuous process) is a major factor in the possible utilization of *D. deltoidea* cells for the industrial production of this useful compound (Tal *et al.*, 1983). As the synthesis of diosgenin is not associated with active cell growth, a two-stage continuous process was required with growth in stage I and biotransformation to diosgenin in stage II to obtain appreciable production rates of diosgenin (Tal *et al.*, 1983). The results of the present study, showing that furostanol I is accumulated during growth of *D. deltoidea* cells in suspension cultures, suggest another possible scheme for the efficient production of diosgenin.

Growing cells could be harvested continuously from a chemostat and the steroidal precursor (the aglycon of furostanol I) prepared. The addition of β -glucosidase to purified precursor should transform it to diosgenin. The feasibility and economics of this scheme are being studied.

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