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STEROIDAL SAPOGENINS AND STEROLS IN TISSUE CULTURES OF *DIOSCOREA TOKORO**

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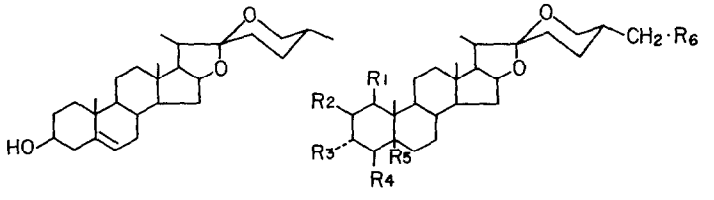
Abstract—The tissue cultures derived from the seedlings of *Dioscorea tokoro* Makino retain the ability to synthesize tokorogenin (0·1%), diosgenin, yonogenin, β-sitosterol, stigmasterol, campesterol and cholesterol even after being subcultured for 1 yr. The ratio of the sapogenin content is similar to the ratio of that in dormant seeds, except for kogagenin.

INTRODUCTION

THE FORMATION of alkaloids, sterols, triterpenes, sesquiterpenes and coumarins by tissue cultures has been studied. Several plants containing steroidal sapogenins have been found to be adequate as tissue cultures[1–3] but only diosgenin was reported as a sapogenin component. Heble *et al.*[4] isolated diosgenin and β-sitosterol from the tissue cultures of *Solanum xanthocarpum*. Recently Kaul and Staba[5] also isolated diosgenin (1%) from the undifferentiated tissue cultures of *Dioscorea deltoides*; however, its differentiated tissue cultures produced diosgenin (I) only in trace amounts. Steroidal sapogenins in *D. tokoro* Makino have previously been investigated, and diosgenin, yonogenin (II), yamogenin, tokorogenin (III), neotokorogenin, isodiotigenin (IV), igagenin (VI) and kogagenin (V) were isolated. The characteristic structures, having an α-hydroxy group at C-3 and A/B *cis* ring junction were established.



Diosgenin (I) R1, R4, R5, R6 = H; R2, R3 = OH Yonogenin (II)

R4, R5, R6 = H; R1, R2, R3 = OH Tokorogenin (III)

R1, R5, R6 = H; R2, R3, R4 = OH Isodiotigenin (IV)

R4, R6 = H; R1, R2, R3, R5 = OH Kogagenin (V)

R1, R4, R5 = H; R2, R3, R6 = OH Igagenin (VI)

* Part II in the series "Chemical Studies on Plant Tissue Cultures"; for Part I, see *Phytochem.* **8**, 2249 (1969).

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[3] L. G. NICKELL, in *Advan. in Appl. Microbiol.*, Vol. 4, p. 213. Academic Press, New York (1962).
[4] M. R. HEBLE, S. NARAYANASWAMI and M. S. CHADHA, *Science* **161**, 1145 (1968).
[5] B. KAUL and E. J. STABA, *Lyloydia* **31**, 171 (1968).

We now report on isolation and identification of diosgenin, yonogenin and tokorogenin in the tissue cultures derived from the seedlings of *D. tokoro* Makino. Moreover, β-sitosterol, stigmasterol, campesterol and cholesterol were detected by gas chromatography.

RESULTS AND DISCUSSION

The callus derived from seedlings of *Dioscorea tokoro* was subcultured for 1 yr on Linsmeier–Skoog agar medium fortified with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. After 1 yr the callus was established as a suspension tissue culture. The actively proliferating tissue cultures derived from the seedlings were extracted with 70% ethanol. The extracts were hydrolysed with 5% HCl in ethanol and the sapogenins were extracted with ethyl acetate. TLC of the extract was carried out on silica-gel plates using acetone and benzene (1:1). Tokorogenin, diosgenin and yonogenin were detected by spraying the plates with a solution of cinnamic aldehyde in methanol and dilute H_2SO_4. These sapogenins gave a characteristic yellow colour reaction and R_fs were identical with those of authentic samples. The i.r. spectra of tokorogenin was also identical with that of an authentic sample. However, the other sapogenins could not be detected by TLC and gas chromatography was used; β-sitosterol, campesterol, stigmasterol and cholesterol were detected in the relative ratios shown in Table 1.

TABLE 1. RATIO OF PHYTOSTEROL CONTENT IN *D. tokoro* TISSUE CULTURES

Compound	Ret. time	R.R.T.†	Relative amounts
Cholestane*	7·68′	1·00	—
Cholesterol-TMS	15·43′	2·01	0·3
Campesterol-TMS	19·68′	2·56	1·0
Stigmasterol-TMS	20·81′	2·73	2·6
β-sitosterol-TMS	24·27′	3·16	2·4

* Standard.
† Retention times relative to cholestane.

It has been noted that the ratio of the secondary metabolites in tissue cultures usually differs from the ratio in the original plants. The difference between the two ratios is important when considering the utilization of tissue cultures in the manufacture of these compounds. Therefore, quantitative analyses of the sapogenins in the tissue cultures and seedlings were carried out by preparative TLC and gas chromatography (Tables 2 and 3).

TABLE 2. RETENTION TIMES OF SAPOGENINS AND THEIR TRIMETHYLSILYL ETHERS FOUND IN THE *D. tokoro* TISSUE CULTURES

			TMS derivative	
Compound	Ret. time	R.R.T.†	Ret. time	R.R.T.
Cholestane*	2·30′	1·00	—	1·00
Diosgenin	5·25′	2·28	6·15′	2·67
Yonogenin	7·65′	3·33	7·70′	3·35
Tokorogenin	11·45′	4·98	10·20′	4·43

* Standard.
† Retention times relative to cholestane.

TABLE 3. RELATIVE RATIO OF SAPOGENIN CONTENT IN THE TISSUE CULTURES, SEEDLINGS AND SEEDS OF *D. tokoro*

Compound	Tissue cultures	Seedling	Seed[7]
Diosgenin	2·5	1	1
Yonogenin	1	1	2·5
Tokorogenin	15	2·5	30
Isodiatigenin	—	11	—
Kogagenin	—	+	+

+ = trace amount.

As shown in Table 3, the main component found in the seedling is isodiotigenin (IV) and its presence is 4·4 times higher than the amount of tokorogenin (III). Kogagenin (V) was present in a trace amount and was detected by its colour reaction on TLC plates with cinnamic aldehyde and H_2SO_4. On the other hand, the tissue cultures produce tokorogenin as the major sapogenin. Akahori *et al.*[6] have reported on a marked change in sapogenin content with age in the whole plant, but according to them, tokorogenin is never the main component. They also reported on the sapogenin content in dormant seeds of *D. tokoro*,[7] and the relative ratio of the sapogenin content found in the actively proliferating tissue cultures of *D. tokoro* is similar to that in dormant seeds, except for kogagenin. Kogagenin and isodiotigenin could not be detected in *D. tokoro* tissue cultures, a result which suggests that enzymic hydroxylations at C-4 and C-5 occurring in the intact plant do not take place in the tissue cultures.

EXPERIMENTAL

Source of Plant Material

The seeds of *Dioscorea tokoro* Makino that were used were collected at the Aburahi Research Laboratory, Shionogi and Co., Ltd., Shiga Pref., Japan.

Tissue Culture and Preparative Media

The seeds of *D. tokoro* were sterilized in 70% alcohol followed by immersion in 0·2% $HgCl_2$ solution for 20 min, and were then washed 3 times with sterile water. The sterilized seeds were germinated at 25° on agar medium without growth regulators. One week after the germination, the seedlings were placed under sterile conditions on Linsmeier–Skoog medium containing 0·8% agar, 0–2·0 ppm kinetin and 0–10^{-5} M 2,4-D. After 3–4 weeks, a yellowish-white callus was formed from the seedlings inoculated on to the agar medium containing 10^{-5}–10^{-6} M 2,4-D, regardless of the kinetin concentration. This callus was subcultured on to Linsmeier–Skoog agar medium supplemented with 10^{-6} M 2,4-D, every 4 weeks.

Extraction of Sapogenins from the D. tokoro *Tissue Cultures*

The callus (dry wt. 3 g) derived from the seedlings of *D. tokoro* was refluxed with 5% (w/v) HCl in 70% ethanol for 5 hr and the solution, after adding water, was extracted 3 times with ethyl acetate. The extracts were washed with water, dried and the solvent removed. The residue (300 mg) was analysed by TLC and gas chromatography.

Preparative Thin-layer Chromatography of Sapogenins

The crude residue was applied to silica-gel thin-layer plates (0·3 mm) and developed with benzene–acetone (1:1). To visualize the sapogenins, the plates were sprayed with water. Tokorogenin (R_f 0·43), yonogenin (R_f 0·57) and diosgenin (R_f 0·71) were extracted 3 times with $CHCl_3$–MeOH (1:1). The crude tokorogenin

[6] A. AKAHORI, F. YASUDA, I. OKUNO, M. TOGAMI, T. OKANISHI and T. IWAO, *Phytochem.* **8**, 45 (1969).
[7] A. AKAHORI, I. OKUNO, T. OKANISHI and T. IWAO, *Chem. Pharm. Bull.* **16**, 1994 (1968).

(4·7 mg) was recrystallized from MeOH, m.p. 256–258°, yield 3 mg (0·1%). The crude yonogenin was purified by repeated TLC, using the same solvents and 0·2 mg of yonogenin were obtained in pure form, m.p. 237–238°. The diosgenin fraction was purified by preparative TLC (×2) on silica gel using *n*-hexane–$CHCl_3$–EtOAc (20:5:5) and 0·5 mg of diosgenin were obtained in pure form, m.p. 198–200°.

Gas Chromatography of Sapogenins

The crude residue was applied to silica-gel plates and developed with benzene–acetone (1:1). The fractions on the TLC, corresponding to diosgenin (R_f 0·5–0·8), yonogenin (R_f 0·39–0·40), tokorogenin and isodiotigenin (R_f 0·21–0·32) and kogagenin (R_f 0·07–0·14) were extracted with a mixture of $CHCl_3$ and acetone. Each extract and its trimethylsilyl ether, prepared in the usual manner, were analysed by gas chromatography. All gas chromatographic analyses were run on a Shimadzu Gas-Chromatograph GC-4A (PF) instrument fitted with a hydrogen flame ionization detector. A glass U-column (1·5 mm × 4 m i.d.) packed with 1% OV-1 on Gas-Chrom Q (80–100 mesh) was operated under the following conditions. The temperatures of the injection port, detector block and column oven were 257°, 282° and 280°, respectively. The carrier gas was nitrogen, with a flow rate of 45 ml/min.

Extraction of Phytosterols

The fresh callus (10 g) was extracted 3 times with boiling methanol for 4 hr each time. The extracts were evaporated to dryness under reduced pressure and the residue was saponified with 5% KOH in ethanol. After the addition of water, the solutions were extracted 3 times with petroleum ether and the extracts were washed with water, dried, and evaporated to dryness.

Thin-layer and Gas Chromatography of Phytosterols

The phytosterol extract was applied to thin-layer plates (0·3 mm) and developed with *n*-Hexane–$CHCl_3$–EtOAc (20:5:5). A mixture of phytosterols was detected as a white zone on the plates by spraying with water, and this zone was extracted twice with acetone. The extracts were combined and the solvent was evaporated to dryness. The residue and its trimethylsilyl ether, prepared in the usual manner, were quantitatively analysed for cholesterol, campesterol, stigmasterol and β-sitosterol by gas chromatography with a hydrogen flame ionization detector. A glass U-column (4 mm × 3 m i.d.) packed with 1% OV-101 on Gas-Chrom Q (100–120 mesh) was operated at 265°. The carrier gas was nitrogen, with a flow rate of 60 ml/min.

Extraction of Sapogenins from the Seedlings of D. tokoro

After 2 months following germination, the seedlings (dry wt. 6 g) of *D. tokoro* were harvested and were extracted twice with boiling methanol. The sapogenin components were isolated as described for the tissue cultures. Diosgenin, yonogenin, tokorogenin and isodiotigenin were isolated by TLC using benzene–acetone (1:1). These sapogenins were recrystallized from MeOH. Isodiotigenin, (22 mg), tokorogenin (5 mg), yonogenin (2 mg) and diosgenin (2 mg) were obtained in pure form.

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