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PROTOYONOGENIN AND PROTONEOYONOGENIN FROM THE AERIAL PARTS AND TISSUE CULTURES OF *DIOSCOREA TOKORO*

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Key Word Index—*Dioscorea tokoro*, Dioscoreaceae, tissue cultures, furostanol glycosides, steroidal sapogenins, protoyonogenin, protoneoyonogenin

Abstract—The aerial parts and cell cultures of *Dioscorea tokoro* were found to contain two new furostanol glucosides, 26-*O*-β-D-glucopyranosyl-(25*R*)- and (25*S*)-5β-furostan-2β,3α,22ξ,26-tetraol (protoyonogenin and protoneoyonogenin respectively) The structures of these compounds were elucidated chemically and confirmed by ^{13}C NMR spectroscopy Their biosynthetic relationship to the 1-arabinosides of this plant is indicated and spectral evidence is given for the production of a Δ^{22}-furostene on dehydration of the protosaponin in pyridine

INTRODUCTION

Steroidal sapogenins in the acid hydrolysate from cell cultures of *Dioscorea tokoro* have previously been investigated and (25*R*)-steroidal sapogenins such as diosgenin, yonogenin (**2**), and tokorogenin (**4**) have been isolated and identified [1] Moreover, tokorogenin (**4**) exists for the most part in the form of the furostanol glucoside, prototokoronin (**6a**), in the cells [2] The biosynthetic pathway leading to these saponins and sapogenins has already been proposed on the basis of tracer experiments [3]

In this paper, we report the isolation and structural determination of two minor furostanol glucosides, protoyonogenin (**1a**) and protoneoyonogenin (**1b**), from both cell cultures and intact plants of *Dioscorea tokoro*

RESULTS AND DISCUSSION

Cells of *D tokoro* tissue cultures were extracted with methanol at room temperature and the extract was separated into *n*-hexane-soluble and *n*-butanol-soluble fractions The latter, containing the crude saponin mixture, was then further separated into an acetone-soluble and a methanol-soluble fraction The acetone-soluble fraction was subjected to prep TLC on Si gel (chloroform–methanol–water, 30 10 1) and the band (R_f 0 43) giving a positive Ehrlich colour test was recovered and recrystallized from hot water–acetone yielding colourless powder (**1**), $C_{33}H_{56}O_{10}$ $\frac{5}{2}H_2O$ mp 128–131°, $[\alpha]_D^{26}$ −17 8° (methanol) This compound was also obtained from aerial parts of intact *D tokoro* plants The IR spectrum of **1** lacked the absorption band characteristic of a spirostanol linkage [4] On hydrolysis with β-glucosidase or with 5% hydrochloric acid in methanol followed by acetylation, **1** afforded glucose peracetate, yonogenin diacetate (25*R*), and a small mount of neoyonogenin diacetate (25*S*) (see Experimental) Therefore, **1** was assumed to be an epimeric mixture of the furostanol glucosides of **2** and **3**, protoyonogenin (**1a**) and protoneoyonogenin (**1b**), respectively

Recently, structural determination of natural plant glucosides containing an aglycone which is unstable under the acid hydrolysis conditions has successfully been achieved by using ^{13}C NMR spectroscopy [5], and all the

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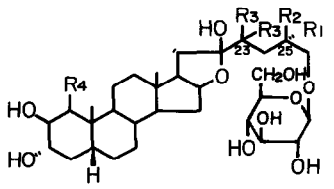
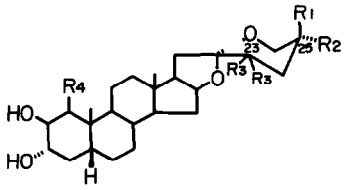


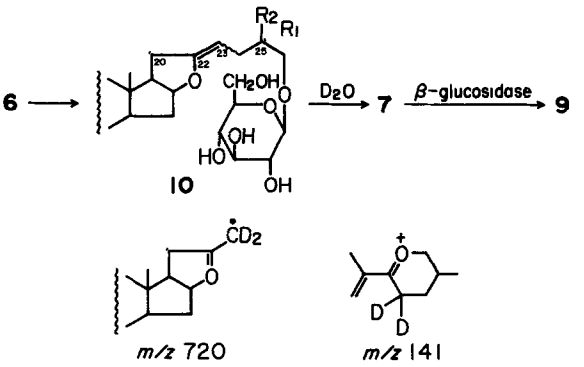
1 a R1,R3,R4=H, R2=Me (25*R*)
b R1=Me, R2,R3,R4=H(25*S*)
6 a R1,R3=H, R2=Me, R4=O-α-L-Ara (25*R*)
b R1=Me, R2,R3=H, R4=O-α-L-Ara (25*S*)
7 a R1=H, R2=Me, R3=D, R4=O-α-L-Ara (25*R*)
b R1=Me, R2=H, R3=D, R4=O-α-L-Ara (25*S*)



2 R1,R3,R4=H, R2=Me
3 R1=Me, R2,R3,R4=H
4 R1,R3=H, R2=Me, R4=OH
5 R1=Me, R2,R3=H, R4=OH
8 a R1,R3=H, R2=Me, R4=O-α-L-Ara (25*R*)
b R1=Me, R2,R3=H, R4=O-α-L-Ara (25*S*)
9 a R1=H, R2=Me, R3=D, R4=O-α-L-Ara (25*R*)
b R1=Me, R2=H, R3=D, R4=O-α-L-Ara (25*S*)

^{13}C NMR signals of (25*R*)- and (25*S*)-steroidal sapogenins were assigned unambiguously [6] Thus, the ^{13}C NMR spectrum of **6** was first investigated in [$^2H_5$]-pyridine at 80° to help elucidate the structure of **1**

^{13}C NMR signals of **6a** were assigned by comparing the chemical shifts with those of **4** [6], tokoronin (**8a**), and methyl glucoside and arabinoside, using known chemical-shift rules [7] The C-23 signal was assigned by the deuterium labelling method 23,23-Dideuteriopototo-koronin mixture (**7**) was prepared in good yield by heating **6** with dried molecular sieves in dry pyridine at 80°, followed by hydration with D_2O and repetition of these two procedures The position and the extent of deuterium incorporation were examined by mass spectrometry of the 23,23-dideuteriotokoronin peracetate mixture, which had been obtained by hydrolysis of **7** with β-glucosidase followed by acetylation Mass spectral peaks at *m/z* 792 $[M]^+$, 720, 517, 457, 415, 397 and 141 indicated that almost all of the hydrogen atoms at C-23 of **6** had been displaced by deuterium atoms [4] A ^{13}C NMR signal at δ 36 9 (C-23) in **6a** disappeared in the spectrum of **7a** Moreover, the signals at 110 8 (C-22) and 28 3 (C-24) in the spectrum of **6a** were shifted upfield by 0 1 and 0 2 ppm, respectively, by the deuterium isotope effect [6] However, the intensity of the C-20 signal (δ 40 6) in the spectrum of **6a** was not affected by deuteration These



facts strongly suggest the presence of a $\Delta^{22(23)}$ intermediate (**10**) in the course of this reaction It is noteworthy that the dehydration of furostanol in pyridine gave a $\Delta^{22(23)}$ derivative (**10**), while dehydration in acetic anhydride was reported to yield a $\Delta^{20(22)}$ derivative [8] The C-21 and C-27 signals were discriminated by a ^1H selective decoupling experiment because the H-21 and H-27 methyl signals could be assigned in the ^1H NMR spectrum of **6**, as described below Table 1 shows the ^{13}C NMR data for **4** [6], **6a**, and **8a**

Table 1 ^{13}C NMR chemical shifts of yonogenin (**2**), **1a**, tokorogenin (**4**), tokoronin (**8a**) and prototokoronin (**6a**) (δ, in C_5D_5N)*

Carbon No	2†,‡	1a‡	4†,‡	8a§	6a§	Carbon No	2†,‡	1a‡	4a†,‡	8a§	6a§
C-1	44 7	44 8	76 6	88 9	88 8	C-21	15 0	16 4	15 0	14 9	16 1
							(14 9)		(14 9)	(14 7)	
C-2	71 3	71 3	74 2	75 0	74 9‖	C-22	109 2	110 7	109 2	109 2	110 8
C-3	77 0	77 0	71 2	71 6	71 6		(109 7)		(109 7)	(109 7)	
C-4	35 5	35 6	35 3	35 0	34 9	C-23	31 9	37 2	31 9	32 0	36 9
							(26 4)	(37 1)	(26 4)	(26 4)	
C-5	42 3	42 4	35 9	36 5	36 4	C-24	29 3	28 3	29 3	29 3	28 3
C-6	26 9	26 9	26 5	26 2	26 1		(26 2)		(26 2)	(26 2)	
C-7	26 9	26 9	26 5	26 5	26 4	C-25	30 6	34 2	30 6	30 6	34 2
							(27 5)	(34 3)	(27 5)	(27 6)	(34 3)
C-8	35 7	35 7	35 6	35 8	35 7	C-26	66 9	75 3	66 9	67 0	75 2
C-9	42 3	42 4	42 0	42 4	42 3		(65 1)		(65 1)	(65 3)	
C-10	37 2	37 2	41 2	41 7	41 0	C-27	17 3	17 4	17 3	17 2	17 3
							(16 3)		(16 3)	(16 4)	(17 2)
C-11	21 1	21 1	21 2	21 2	21 1	G-1′	—	104 8	—	—	104 5
C-12	40 2	40 2	40 1	40 2	40 2			(105 0)			(104 7)
C-13	40 8	41 2	40 6	40 7	41 0	G-2′	—	75 1	—	—	74 9‖
C-14	56 3	56 2	56 3	56 4	56 2	G-3′	—	78 5‖	—	—	78 3
C-15	32 1	32 3	32 1	32 2	32 3	G-4′	—	71 7	—	—	71 9
C-16	81 2	81 2	81 1	81 1	81 1	G-5′	—	78 4‖	—	—	77 8
C-17	63 1	63 9	63 1	63 3	63 8	G-6′	—	62 8	—	—	62 9
	(62 9)		(62 9)	(63 1)							
C-18	16 6	16 7	16 6	16 5	16 6	A-1	—	—	—	107 7	107 5
C-19	23 6	23 6	19 1	19 1	19 0	A-2	—	—	—	73 9	73 8
C-20	42 0	40 6	42 0	42 1	40 6	A-3	—	—	—	75 0	75 0‖
	(42 5)		(42 5)	(42 7)		A-4	—	—	—	69 6	69 5
						A-5	—	—	—	67 3	67 3

*Data in parentheses are those for the (25*S*)-epimers Recently, the C-23, C-24 and C-25 signals of the (25*S*)-steroidal sapogenins were reassigned since they had been wrongly assigned [14, 15]

†Data taken from ref [6]

‡Measured at 30°

§Measured at 80°

‖Assignments can be interchanged in each vertical column

In the ^{13}C NMR spectrum of **6** a few small signals accompanied those due to carbons neighbouring C-25 (Table 1) The ^1H NMR spectrum of **6** was thus determined in [$^2H_5$]pyridine at 80° The angular methyl signals appeared at δ 0 88 (H-18) and 1 35 (H-19) However, pairs of doublets ($J \simeq 7$ Hz) were discernible for each H-21 and H-27 signal at 1 26 and 1 25, and at 0 99 and 1 02, respectively Furthermore, a pair of doublets ($J \simeq 7$ Hz) was observed at 0 90 and 0 87 for the H-27 signal in the ^1H NMR spectrum of the peracetate of **6** determined in $CDCl_3$ at 80° The H-18, H-19 and H-21 signals appeared at 0 66, 1 13 and 0 89, respectively These signal pairs seemed to correspond to those due to the (25*R*)- and (25*S*)-epimers, respectively

Acid hydrolysis of **6** followed by acetylation afforded tokorogenin triacetate together with a trace amount of neotokorogenin triacetate Therefore, **6** was concluded to be 1β-*O*-α-L-arabinopyranosyl-26-*O*-β-D-glucopyranosyl-(25*R*)-5β-furostan-1β,2β, 3α, 22ξ, 26-pentaol [prototokoronin (**6a**)] containing some of its (25*S*)-epimer, 1β-*O*-α-L-arbinopyranosyl-26-*O*-β-D-glucopyranosyl-(25*S*)-5-β-furostan-1β, 2β, 3α, 22ξ, 26-pentaol [protoneotokoronin (**6b**)]

The ^{13}C NMR spectrum of **1** was then examined in [$^2H_5$]pyridine at 80° As shown in Table 1, the resonance positions for the ring carbons of **1a** were essentially the same as those of **2** or **3**, and the resonance positions for the side chain carbons of **1a** were in good agreement with those of **6a** (Table 1) As observed for **6**, small accompanying signals were also discernible in a ratio of 5 2 (Table 1) The ^1H NMR spectrum of **1** in [$^2H_5$]pyridine at 80° showed two singlets at δ 0 87 (H-18) and 0 90 (H-19) and two pairs of doublets ($J \simeq 7$ Hz) at 1 26 and 1 25 (H-21) and 0 99 and 1 02 (H-27) in a ratio of *ca* 5 2 A pair of doublets ($J \simeq 7$ Hz) was also observed at 0 90 and 0 88 for the H-27 signal in the ^1H NMR spectrum of the peracetate of **1** in $CDCl_3$ at 80° The H-18, H-19 and H-21 signals appeared at 0 66, 0 99 and 0 89, respectively Therefore, by taking the acid hydrolysis products from **1** into consideration together with the results obtained above, **1** was assumed to be protoyonogenin (**1a**) containing a small amount of its (25*S*)-epimer (**1b**) Unfortunately, separation of the (25*R*)-furostanol glucosides from their (25*S*)-epimers is difficult and has not been achieved yet

It is already known that, in the biosynthesis of tigogenin (25*R*), C-2 of MVA is incorporated predominantly into the methyl carbon at C-25 [9, 10], and that 27-hydroxycholesterol derived from kryptogenin is hardly incorporated into (25*S*)-spirostanols [11] From the biosynthetic viewpoint, it is suggested that two routes coexist in the cell cultures, oxidation of C-27 (originating from C-6 of MVA) of cholesterol leading to (25*R*)-spirostanols and oxidation of C-26 (from C-2 of MVA) resulting in (25*S*)-epimers Because tokorogenin (**4**) is believed to be synthesized via yonogenin (**2**) [3], compounds **1a** and **1b** obtained in this work are assumed to be the biosynthetic precursors of **6a** and **6b**, respectively, in the cell cultures of *D tokoro*

EXPERIMENTAL

General All mps are uncorr MS were determined at 70 eV Chromatography was achieved using an FMI Lab Pump fitted with a Lobar-B column at a flow rate of 3–4 ml/min Known steroidal sapogenins were identified after acetylation by comparison of spectral data (MS, NMR, IR) with those of authentic samples, by mp and by TLC

^{13}C FT NMR spectra were recorded at 25 160 MHz using *ca* 200 mg/ml of sample solns in $CDCl_3$ or [$^2H_5$]pyridine containing TMS as int reference in 5-mm spinning tubes at 30° and/or 80° FT NMR measurement parameters were spectral width, 4342 Hz, acquisition time, 0 989 sec, pulse width, 13 μsec (flipping angle, 32°), number of data points, 8192, number of transients, 100 000–200 000 ^1H FT NMR spectra were measured at 200 06 MHz using *ca* 2–8 mg/ml of sample solns containing TMS as int reference in $CDCl_3$ and/or [$^2H_5$]pyridine in 5-mm spinning tubes at 23° or 80° FT NMR measurement parameters were, spectral width, 2399 8 Hz, acquisition time, 3 334 sec, pulse width, 3 μsec (flipping angle, 42°), number of data points, 16 000, number of transients, 1000–2000

Extraction of saponins from the cells Initiation of callus from the seedling of *D tokoro* has been described earlier [1] The cell suspension cultures (12 l) of *D tokoro* were grown in a Linsmaier–Skoog medium supplemented with 10^{-6} M 2, 4-D under agitation (140 rpm) at 25° for 4 weeks Cells (fr wt 3 9 kg) were harvested, suspended in MeOH (7 l), and extracted by being subjected to vibration for 5 hr The suspension was filtered and the solid was re-extracted (2 × 4 l) The extracts were combined and solvent was removed The residue (55 6 g) was suspended in 100 ml H_2O and washed with *n*-hexane (3 × 250 ml) The aq soln was extracted with *n*-BuOH (2 × 300 ml) and the combined extracts were concd to yield the crude saponins (12 g) After being suspended in a few ml MeOH, the crude saponin was further fractionated by trituration with Me_2CO (300 ml) to give 623 mg Me_2CO-soluble material The residue (soluble in MeOH) was chromatographed on a Si gel open column with 2 4 1 $CHCl_3$–MeOH–H_2O (65 25 4) and *ca* 400 ml crude prototokoronin fraction (**6**) (dry wt 3 0 g) was obtained after the initial 360 ml fraction was eluted

Separation of **1** The crude Me_2CO-soluble fraction (623 mg) obtained above was subjected to chromatography on Lobar-B with $CHCl_3$–MeOH–H_2O (30 10 1) and then further purified by prep TLC in the same solvent system The fraction (R_f 0 43) giving a positive Ehrlich colour test was extracted with MeOH and the solvent was evaporated The crude material (24 9 mg) obtained was chromatographed repeatedly on the same system for further purification, and then dissolved in hot H_2O–Me_2CO (2 ml), refluxed for 2 hr, concd to a small vol, cooled and left to stand to give a colourless powder of **1** (5 mg), mp 128–131° Half of this powder was acetylated with Ac_2O and pyridine affording the peracetate (2 mg), mp 78–84°, MS *m/z* 846 [M − 18]$^+$, 787, 515, 498, 457, 395, 373, 331 (glu–OAc), 271, 253, 211, 169, 109, R_f 0 53 in *n*-hexane–EtOAc (2 3) The remaining half of the powder of **1** was treated with excess β-glucosidase (from sweet almonds) in a buffer soln (0 1 M HOAc–NaOAc, pH 4 5) for 24 hr at 30° H_2O was added to the soln, which was then extracted × 3 with EtOAc, washed, dried and evaporated The residue was acetylated with Ac_2O and pyridine and gave a product (0 7 mg) with R_f 0 46 in *n*-hexane–$CHCl_3$–EtOAc (4 1 1), MS *m/z* 516 [M]$^+$, 444, 402, 387, 373, 342, 327, 313, 282, 267, 253, 139, ^1H NMR $CDCl_3$ δ 0 75 (H-18), 0 99 (H-19), 0 96 (H-21), 0 78 (H-27) [12], which was identical to an authentic sample of yonogenin diacetate Another product (0 3 mg) with R_f 0 42 in *n*-hexane–$CHCl_3$–EtOAc (4 1 1) was identified as neoyonogenin diacetate on the basis of the following data MS *m/z* 516 [M]$^+$, 444 [M − 72]$^+$, 402, 387, 373, 342, 327, 313, 282, 267, 253, 139, ^1H NMR $CDCl_3$ δ 0 75 (H-18), 0 99 (H-19), 0 99 (H-21), 1 08 (H-27) [13] The aq soln was neutralized with Na_2CO_3, evaporated to dryness under red pres and the residue was acetylated with Ac_2O and pyridine The product was identical to penta-

acetyl glucose by TLC (R_f 0 13 in *n*-hexane–$CHCl_3$–EtOAc, 4 1 1) and MS comparisons

Isolation of **1** *from intact* D tokoro *plants* The aerial part of intact *D tokoro* plant (fr wt 1 kg) was extracted with MeOH (3 × 7 l) at room temp The extracts were combined and the solvent was removed The residue (52 g) was purified as described above and gave **1** (30 mg) as a colorless powder by crystallization from hot Me_2CO–H_2O, mp 128–131° $[\alpha]_D$ −17 8° (MeOH, *c* 0 21) (Found C, 60 30, H, 9 04 $C_{33}H_{56}O_{10}\cdot\frac{5}{2}H_2O$ requires C, 60 27, H, 9 28%) Compound **1** was further analysed by ^{13}C NMR by comparison with **6** Compound **1** (2 mg) was hydrolysed with β-glucosidase in a buffer soln followed by acetylation to afford yonogenin diacetate (0 7 mg), neoyonogenin diacetate (0 3 mg) and glucose peracetate, which were identical with authentic samples according to TLC and MS

Compound **1** (9 mg) was acetylated with Ac_2O and pyridine and afforded the hexa-acetate of **1** (8 8 mg), mp 78–84°, $[\alpha]_D$ 0 0°, $[\alpha]_{365}^{26}$ + 3 7° (pyridine, *c* 0 27), R_f 0 53 in *n*-hexane–EtOAc (2 3) (Found C, 62 48, H, 7 99 $C_{45}H_{68}O_{16}$ requires C, 62 48, H, 7 92%) MS *m/z* 846 $[M-18]^+$, 787, 515, 498, 457, 395, 373, 331, 271, 253, 211, 169, 109

23, 23-*Dideuterio prototokoronin mixture* (**7**) Crude prototokoronin (**6**) (101 mg) was dissolved in dry pyridine (0 6 ml) and heated in the presence of dried molecular sieves (20 particles) at 80° for 4 hr The soln was mixed with 0 3 ml D_2O (95%) and then evaporated to dryness The residue was dissolved in 0 6 ml D_2O, heated for 2 hr at 100–108° with stirring, and then the D_2O was evaporated These procedures were repeated × 5 The product **7** (90 mg) was dried overnight *in vacuo* and analysed by ^{13}C NMR spectroscopy

23, 23-*Dideuterio tokoronin mixture* (**9**) Compound **7** was suspended in 4 ml 0 1 M acetate buffer (pH 4 5) and treated with β-glucosidase at 32° for 72 hr The soln was mixed with 20 ml H_2O satd with NaCl and extracted with *n*-BuOH (3 × 30 ml) The extracts were combined, washed with H_2O (3 × 30 ml) and evaporated to dryness The residue **9** (40 mg), treated with Ac_2O and pyridine, gave the peracetate which was analysed by mass spectrometry and ^{13}C NMR MS *m/z* 792 $[M]^+$, 720 $[M-72]^+$, 647 $[M-145]^+$, 517 $[M-Ara]^+$, 457 $[517-60]^+$, 415 $[457-42]^+$, 397 $[457-60]^+$, 341, 325, 299, 271, 269, 259, 141, by comparison with data for tokoronin penta-acetate, MS *m/z* 790 $[M]^+$, 718 $[M-72]^+$, 647 $[M-143]^+$, 515, 455, 413, 395, 341, 325, 299, 271, 269, 259, 139

Acid hydrolysis of crude prototokoronin (**6**) Prototokoronin (**6**) (100 mg) was refluxed with 1 7% HCl in MeOH for 5 hr The soln was combined with H_2O and extracted × 3 with EtOAc The extracts were subjected to chromatography on a Lobar-B column with $CHCl_3$–MeOH (9 1) and afforded crude tokorogenin This material was acetylated and purified by chromatography on a Lobar-B column in the solvent system of *n*-hexane–$CHCl_3$–EtOAc (4 1 1), giving tokorogenin triacetate (45 mg) and neotokorogenin triacetate (13 mg) Tokorogenin triacetate (45 mg), mp 266° (lit 255° [16]), IR $\nu_{max}^{CHCl_3}$ cm^{-1} 866, 897, 921, 981 (897 > 921, 25*R*), MS *m/z* 574 $[M]^+$, 502 $[M-72]^+$, 460, 445, 431, 400, 385, 371, 340, 325, 311, 280, 265, 251, 139, ^1H NMR $CDCl_3$ δ 0 77 (H-18), 0 96 (H-19), 0 96 (H-21), 0 78 (H-27) [12] (Found C, 68 84, H, 8 89 $C_{33}H_{50}O_8$ requires C, 68 96, H, 8 77%) Neotokorogenin triacetate (13 mg), mp 203° (lit 185–190° [17]), IR $\nu_{max}^{CHCl_3}$ cm^{-1} 850, 896, 916–921, 986 (896 < 916–921, 25*S*), MS *m/z* 574 $[M]^+$, 502, 460, 445, 431, 400, 385, 371, 340, 325, 311, 280, 265, 251, 139, ^1H NMR $CDCl_3$ δ 0 77 (H-18), 0 96 (H-19), 1 00 (H-21), 1 08 (H-27) [13] (Found C, 68 94, H, 8 84 $C_{33}H_{50}O_8$ requires C, 68 96, H, 8 77%) These were also identified by comparison with their authentic samples by ^{13}C NMR (Table 1) [6]

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