Biosynthesis of (25*S*)- and (25*R*)-Furostanol Glycosides from [1,2-$^{13}C_2$]Acetate in *Dioscorea tokoro* Tissue Cultures†

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The biosynthesis of (25*S*)- and (25*R*)-furostanol glycosides, protoneotokorin (**13**) and prototokoronin (**12**), in cell cultures of *Dioscorea tokoro* fed [1,2-$^{13}C_2$]acetate, was investigated by ^{13}C n.m.r. spectroscopy. The ^{13}C-labelling patterns of neotokorogenin (**19**) and tokorogenin (**16**), obtained from (**13**) and (**12**), indicate that the hydrogen atom at C-25 is introduced on the 25-*si* face of the Δ^{24}-intermediate, followed by oxidation of the *pro-R* (C-26) and the *pro-S* (C-27) methyl group at C-25 leading to (25*S*)- and (25*R*)-furostanol glycosides, respectively. The results were confirmed from the labelling patterns of yamogenin (**21**) and diosgenin (**20**), isolated by hydrolysis of crude furostanol glycosides. The oxidation at the *pro-R* methyl group was accelerated by increasing the concentration of sodium acetate without any effect on the ^{13}C-labelling patterns.

26-Hydroxycholesterol has been shown to be a biosynthetic intermediate in the formation of steroidal sapogenin;[1] the mechanism is likely to involve the reduction of a Δ^{24} double bond in a precursor (**1**) having an unsaturated sterol side chain, followed by oxidation of one of the methyl groups at C-25. The reduction of the double bond leads to two stereochemically different isopropyl intermediates as the hydrogen at C-25 can be added to one or both of the two faces: (i) to the *re*-face in which case the (*E*)-methyl group of the Δ^{24}-intermediate (**1**) becomes the isopropyl *pro-S* methyl group (**3**); and/or (ii) to the *si*-face, in which case the same methyl group becomes the isopropyl *pro-R* methyl group (**2**). Oxidation of one of the methyl groups of each saturated side chain can then take place leading to both (25*S*)- and (25*R*)-26-hydroxycholesterols [(**4**), (**6**) and (**5**), (**7**), respectively].

(25*S*)-Spirostanols are frequently isolated together with their (25*R*)-isomers from acid hydrolysis of their glycosides.[2] Recently, we found that the aerial parts and cell cultures of *Dioscorea tokoro* Makino contain (25*S*)- and (25*R*)-furostanol glycosides, which are hydrolysed to the (25*S*)- and (25*R*)-spirostanols, neotokorogenin (**19**), neoyonogenin (**17**), and yamogenin (**21**), and tokorogenin (**16**), yonogenin (**14**), and diosgenin (**20**),[3] respectively. This paper reports the stereochemistry of the reduction and the hydroxylation reactions of the sterol side chain in the biosynthesis of (25*S*)- and (25*R*)-furostanol glycosides in cultured cells of *D. tokoro*.

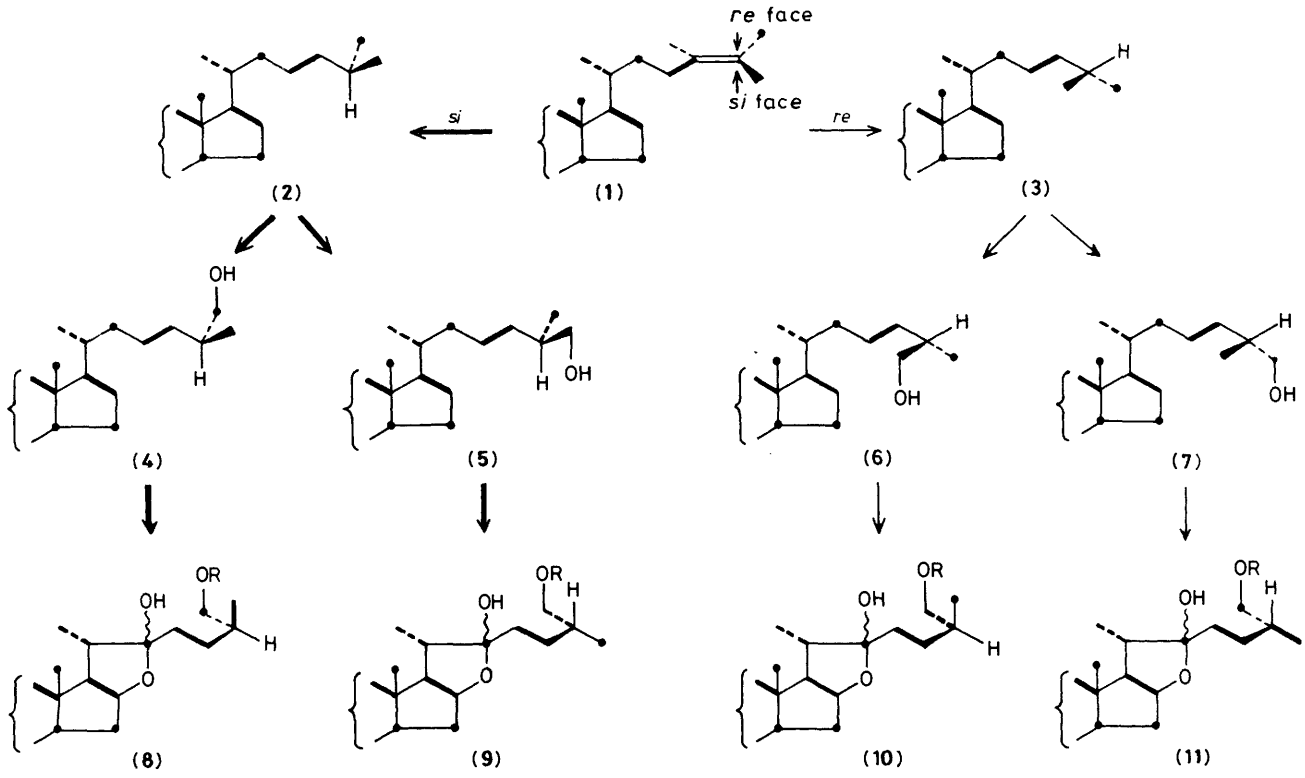
As a fairly high concentration of the ^{13}C-labelled precursor for biosynthetic studies may alter the metabolite, the optimum concentration of sodium acetate (170 mg/l), which did not inhibit cell growth of *D. tokoro* callus was determined (see Experimental section). More than 500 mg/l of acetate inhibited the growth, but the pH value of the broth was unchanged after 3 weeks of incubation. Sodium [1,2-$^{13}C_2$]acetate was administered to the 2-week-old cells grown in Linsmaier–Skoog (LS) liquid medium and the cells were incubated for 2 more weeks. Crude prototokoronin was isolated from the furostanol glycoside fraction by a combination of silica gel and reverse-phase high-performance liquid chromatography.

Since distinguishing the C-27 signals from the other methyl signals, especially from the C-21 signals, in the ^{13}C n.m.r. spectra was extremely important in our study, we assigned the ^{13}C signals of prototokoronin (**12**) by a selective ^{13}C-{^1H} decoupling experiment.[3] The 21-H and 27-H methyl signals were confirmed in the ^1H n.m.r. spectrum of prototokoronin (**12**) in a mixture with protoneotokoronin (**13**), as follows. In the ^1H n.m.r. spectrum of (**12**) in [$^2H_5$]pyridine at 80 °C, the angular methyl signal appeared at δ_H 0.88 (18-H) and 1.35 (19-H). However, a pair of doublets (*J* 7 Hz) were discernible for 21-H and 27-H at δ_H 1.26 and at 0.99, respectively. In the double resonance experiment the 25-H frequency was irradiated (δ_H 1.95) revealing the 27-H (δ_H 0.99) and 26-H signals (δ_H 3.60 and 4.03). Furthermore, the assignments of C-27 and C-21 of compound (**12**) were supported by the following results using the 25-deuteriated furostanol derivative (**25**). The deuterium atoms at C-20 and C-23 in [20,23,23,25-$^2H_4$]tigogenin (**23**) were removed by heating with hydrochloric acid in methanol[4] followed by acetylation to give [25-^2H]tigogenin acetate (**24**). Hydrogenolysis of compound (**24**) on platinum gave [25-^2H]-3β-acetoxy-26-hydroxyfurostan (**25**) together with the 3,26-*O*-diacetate (**26**). The signal at δ 16.6 (C-27) in (**25**) was shifted by −0.1 p.p.m. corresponding to the β-deuterium isotope shift, while the signal at δ 18.9 (C-21) was not isotopically shifted. The signal at δ 17.4 (C-27) in prototokoronin (**12**) was shifted from that of (**25**) by +0.8 p.p.m., which is in the region of the γ-glucosidation shift,[5] and the Δδ value (−2.8 p.p.m.) for the C-21 signal between compounds (**12**) and (**25**) is due to the γ-hydroxylation shift.[6]

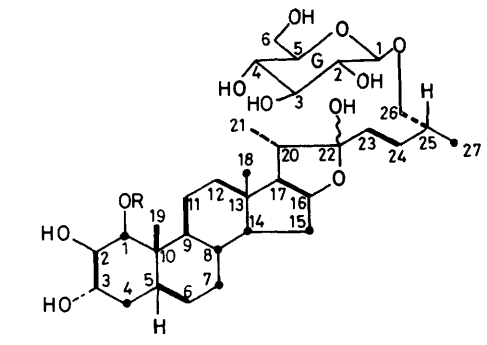
As shown in the Table, the labelling patterns of the (25*R*)-isomer, prototokoronin (**12**), from [1,2-$^{13}C_2$]acetate could be deduced from those of tokoronin (**15**) and those of the (25*S*)-isomer (**13**) were then established from those of neotokorogenin (**19**), obtained by controlled acid hydrolysis of the saponin mixture. The ^1H complete-decoupled ^{13}C n.m.r. spectrum of the ^{13}C-enriched crude prototokoronin (25*R*)-(**12**) was compared with that of the unlabelled compound. The labelled patterns are shown in the Table. Since prototokoronin (**12**) in [$^2H_5$]pyridine is dehydrated to the Δ^{22} compound during the n.m.r. measurements,[3] H_2O (10 μl; 2 mol equiv.) was added in an attempt to avoid this reaction; small signals from the dehydrated derivative were still observed however around the signal due to C-27 in the spectrum of (**12**).

As the critical signal at δ 75.2 (C-26) of (**12**) is unfortunately very close to other signals, we decided to use spirostanol glycoside instead of furostanol to analyse the ^{13}C-labelling patterns of C-26 and C-27. The configuration of C-25 of sapogenin has been reported to be epimerized by acid treatment.[7] To avoid this we hydrolysed the enriched crude prototokoronin (**12**) with β-glucosidase at pH 4.5 to give tokoronin (**15**) with a small amount of neotokoronin (**18**). The

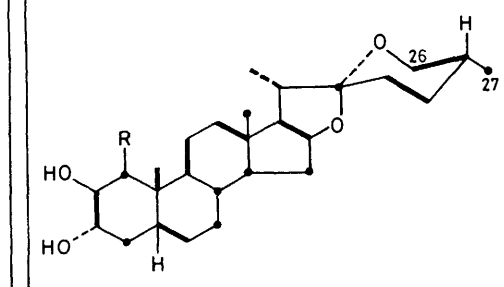
† For preliminary report see S. Seo, K. Tori, A. Uomori, and T. Yoshimura, *J. Chem. Soc., Chem. Commun.*, 1981, 895.
‡ Deceased.



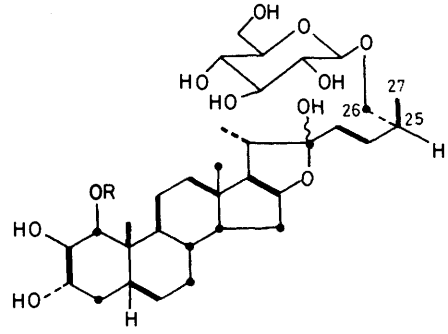
R = β-D-glucopyranosyl



(12) R = α-L-Ara



(14) R = H
(15) R = O-α-L-Ara
(16) R = OH



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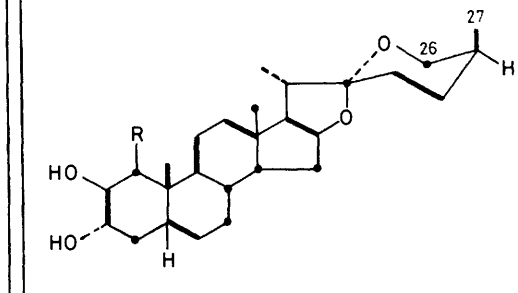
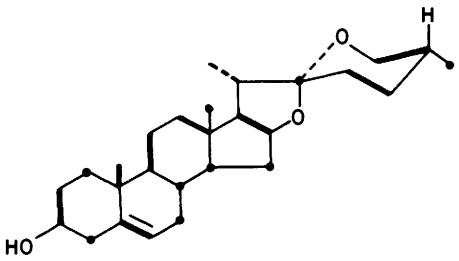
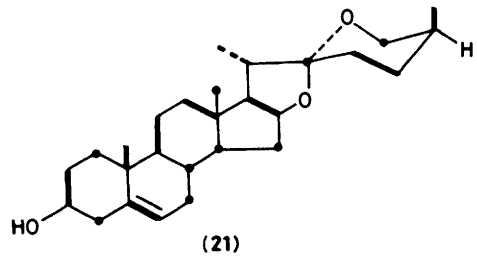


(17) R = H
(18) R = O-α-L-Ara
(19) R = OH



(20)



(21)

α-L-Ara :

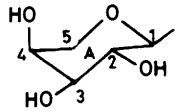


Table. ^{13}C N.m.r. spectra data† of saponins and sapogenins from [1,2-$^{13}C_2$]acetate (δ_C/p.p.m. and J/Hz)

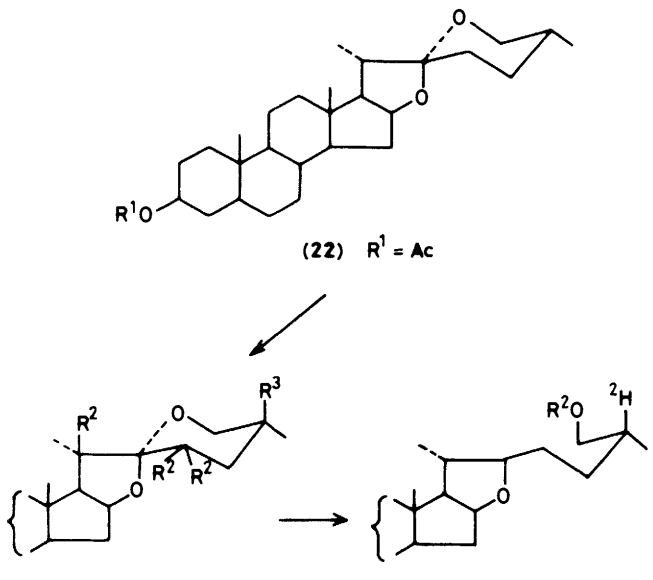
Carbon atom	(12)[a] δ_C	J	(15)[a] δ_C	J	(16)[a] δ_C	J	(20)[b] δ_C	J	(18)[a] δ_C	J	(19)[a] δ_C	J	(21)[a] δ_C	J
C-1	88.9	s	88.9	s	76.6	s	37.3	s	88.9	s	76.6	s	37.3	s
					(75.6	s)	(37.0	s)			(75.6	s)	(37.0	s)
C-2	74.9	39[c]	74.8	39	74.3	39	31.6	36	75.0	39	74.3	39	31.6	36
					(70.8	f)	(27.8	37)			(70.8	f)	(27.8	37[c])
C-3	71.6	39	71.6	39	71.3	39	71.7	36	71.6	39	71.3	39	71.7	36
					(70.8	f)	(73.9	37)			(70.8	f)	(73.9	37)
C-4	34.9	s	35.0	s	35.2	s	42.3	s	35.0	s	35.2	s	42.3	s
					(31.0	s)	(38.1	s)			(31.0	s)	(38.2	s)
C-5	36.5	n.d.	36.5	36[c]	36.0	33[c]	140.9	72	36.5	36[c]	36.0	33[c]	140.9	72
					(35.8	36[c])	(139.8	72)			(35.8	35[c])	(139.8	72)
C-6	26.2	n.d.	26.2	36[c]	26.6	33	121.4	72	26.2	36[c]	26.6	33	121.4	72
					(25.5	36[c])	(122.4	72)			(25.5	35[c])	(122.4	72)
C-7	26.5	s	26.5	s	26.6	s	32.1	s	26.5	s	26.6	s	32.1	s
					(26.0	s)	(32.1	s)			(26.0	s)	(32.1	s)
C-8	35.7	s	35.8	s	35.8	s	31.5	s	35.8	s	35.8	s	31.5	s
					(35.4	s)	(31.5	s)			(35.4	s)	(31.5	s)
C-9	42.4	34[c]	42.4	35	42.3	35[c]	50.1	34	42.4	35	42.3	35	50.1	34[c]
					(42.1	34)	(50.0	34)			(42.1	34)	(50.1	35)
C-10	41.7[e]	n.d.	41.7	36	41.3	n.d.	36.7	35	41.7	36	41.3	n.d.	36.7	35[c]
					(40.6	37[c])	(36.8	35)			(40.6	36[c])	(36.8	n.d.)
C-11	21.1	32[c]	21.2	35	21.4	35	20.9	34	21.2	35	21.4	35	20.9	34[c]
					(20.9	34)	(20.9	34)			(20.9	34)	(20.9	35[c])
C-12	40.3	n.d.	40.2	n.d.[d]	40.3	n.d.[d]	39.8	n.d.[d]	40.2	n.d.[d]	40.3	n.d.[d]	39.8	n.d.
					(39.9	n.d.[d])	(39.8	32)			(39.9	n.d.[d])	(39.8	n.d.[d])
C-13	41.1	n.d.	40.7	n.d.[d]	40.7	n.d.[d]	40.3	n.d.[d]	40.7	n.d.[d]	40.7	n.d.	40.3	n.d.
					(40.4	n.d.[d])	(40.3	32)			(40.4	n.d.[d])	(40.3	n.d.[d])
C-14	56.3	s	56.4	s	56.5	s	56.6	s	56.4	s	56.5	s	56.6	s
					(56.0	s)	(56.5	s)			(56.0	s)	(56.5	s)
C-15	32.3	s	32.2	s	32.2	s	31.9	s	32.1	s	32.2	s	31.9	s
					(31.8	s)	(31.9	s)			(31.7	s)	(31.9	s)
C-16	81.1	32	81.1	32	81.3	32	80.9	32	81.1	34	81.3	32	80.9	32
					(80.7	34)	(80.8	35)			(80.8	32)	(80.9	33)
C-17	63.9	32[c]	63.3	32	63.3	32	62.2	32	63.1	34	63.2	32	62.0	32
					(62.4	34)	(62.2	34)			(62.1	32)	(62.1	33)
C-18	16.6	s	16.5	s	16.6	s	16.3	s	16.5	s	16.6	s	16.3	s
					(16.4	s)	(16.3	s)			(16.4	s)	(16.3	s)
C-19	19.1	37	19.1	36	18.9	37	19.4	35	19.1	36	18.9	37	19.4	35
					(17.9	37[c])	(19.3	35)			(17.9	36)	(19.4	35)
C-20	40.7	n.d.	42.1	37	42.1	36[c]	41.7	37	42.7	n.d.	42.7	36	42.2	37[c]
					(41.7	36)	(41.7	36)			(42.2	37)	(42.2	38)
C-21	16.1	37[c]	14.9	37	14.9	36	14.5	37	14.7	38	14.7	36	14.3	37
					(14.5	36)	(14.5	36)			(14.3	37)	(14.4	38)
C-22	110.8	s	109.2	s	109.2	s	109.3	s	109.7	s	109.7	s	109.8	s
					(109.3	s)	(109.3	s)			(109.8	s)	(109.8	s)
C-23	36.9	n.d.	32.0	35[c]	32.0	32[c]	31.5	32	26.6	n.d.[d]	26.6	n.d.[d]	26.0	n.d.[d]
					(31.5	34[c])	(31.5	33)			(26.0	n.d.[d])	(26.1	n.d.[d])

Table. (*cont.*)

Carbon atom	(12)[a] δ_C	(12)[a] J	(15)[a] δ_C	(15)[a] J	(16)[a] δ_C (δ_C)	(16)[a] J (J)	(20)[b] δ_C (δ_C)	(20)[b] J (J)	(18)[a] δ_C	(18)[a] J	(19)[a] δ_C (δ_C)	(19)[a] J (J)	(21)[a] δ_C (δ_C)	(21)[a] J (J)
C-24	28.3	34	29.3	35[c]	29.4 (28.9	32 34)	28.8 (28.9	32 33)	26.3	n.d.[d]	26.3 (25.8	n.d.[d] n.d.[d])	25.8 (25.9	n.d.[d] n.d.[d])
C-25	34.2	40[c]	30.6	36	30.7 (30.3	36 36[c])	30.3 (30.3	35 35)	27.6	36	27.6 (27.1	35[c] 35)	27.1 (27.2	35 36[c])
C-26	75.2	38[c]	67.0	36	67.0 (66.9	36[c] 36)	66.9 (66.9	35 35)	65.3	s	65.3 (65.2	s s)	65.2 (65.2	s s)
C-27	17.4	s	17.2	s	17.2 (17.1	s s)	17.1 (17.1	s s)	16.3	n.d.	16.3 (16.1	35 36)	16.1 (16.1	35 36)
A-1	107.6		107.7						107.7					
A-2	73.8		73.9						73.9					
A-3	75.0		75.0						75.0					
A-4	69.6		69.6						69.6					
A-5	67.3		67.3						67.3					
G-1	104.6													
G-2	75.0													
G-3	78.4													
G-4	72.0													
G-5	77.9													
G-6	63.0													

† Data for peracetates were measured in [^{2}H]chloroform and are shown in parentheses. s: Single label. n.d.: Not detected. f: Singlet because δ_C signals of the two carbons are co-incidentally equivalent.

[a] Spectra were measured in [$^2H_5$]pyridine at 80 °C. [b] Spectra were measured in [^{2}H]chloroform at 30 °C. [c] J Value accurate to ±2 Hz because one of the doublet signals overlapped other signals [d] AB quartet of which the small peaks could not be detected. [e] The assignment for C-10 (δ_C 41.0) in our previous report, ref. 3, should read δ_C 41.7 p.p.m.



^{13}C n.m.r. spectrum of the enriched crude tokoronin was analysed on the basis of recent signal assignments of spirostanol derivatives.[3,4] The signal at δ 67.0 due to C-26 of tokoronin (**15**) appeared as a doublet ($^1J_{CC}$ 36 Hz) coupled to C-25 (δ 30.6), while C-27 (δ 17.2) appeared as a singlet. These results indicate that prototokoronin (25*R*)-(**12**) is labelled as in structure (**9**) from [1,2-$^{13}C_2$]acetate. The signals due to neotokoronin (25*S*)-(**18**) were not strong enough to allow identification of the labelling patterns.

To observe the labelling patterns more precisely, (25*S*)-furostanol glycoside was transformed into (25*S*)-spirostanol sapogenin by acid hydrolysis. We confirmed previously that the configuration at C-25 of spirostanol is almost completely retained under acid hydrolysis in methanol.[4]

The rest of the enriched furostanol glycoside mixture was hydrolysed with 1.8% HCl in methanol. From the sapogenin mixture obtained, enriched tokorogenin (**16**), neotokorogenin (**19**), diosgenin (**20**), and yamogenin (**21**) were isolated and purified as their acetates. The ^{13}C n.m.r. data and the labelling patterns are shown in the Table. The (25*S*)-spirostanols, neotokorogenin (**19**) and yamogenin (**21**), each had a singlet for C-26 (δ 65.3 and 65.2, respectively) and a doublet for C-27 (δ 16.3 and 16.1, respectively). The ^{13}C-labelling patterns for the (25*R*)-spirostanols, tokorogenin (**16**) and diosgenin (**20**), confirmed the results obtained for tokoronin (**15**). These results indicate that C-26 of (25*S*)-furostanols and C-27 of the (25*R*)-isomers are derived stereospecifically from C-6 of mevalonic acid.

Finally, we examined the effect of sodium acetate on the biosynthesis of furostanol glycoside in tissue cultures of *D. tokoro*. The cells were incubated in the LS medium containing sodium acetate (200 mg/l) for 6 weeks with transfer into fresh medium every 2 weeks. A mixture of neotokoronin (**18**) and tokoronin (**15**) was obtained by hydrolysis with β-glucosidase. The ratio of (**18**) to (**15**) based on the ^{13}C signal intensities increased (1:8 to 1:1) with the amount of acetate (0—300 mg/l).

These results raised the question of whether the ^{13}C-labelling patterns might be affected by a high concentration of sodium acetate. The cells in the LS medium with [1,2-$^{13}C_2$]acetate (220 mg/l), after incubation for 1 week with the addition of the same amount of labelled sodium acetate, were harvested after a further 10 days of incubation (total amount of acetate, 440 mg/l). The tokorogenin (**16**) and neotokorogenin (**19**) obtained had the same ^{13}C-labelling patterns as those in the case of the acetate (170 mg/l) administration mentioned above, except in the product ratio [(**16**) to (**19**), 1:1].

These results indicate that in the biosynthesis of (25*S*)- and (25*R*)-furostanol glycoside, reduction of the 24(25) double bond of the intermediate (**1**) occurs from the 25-*si* face of the double bond in such a way that the (*E*)- and (*Z*)-methyl group,

respectively, assumed the *pro-R* and the *pro-S* position [(**1**) ⟶ (**2**)] at C-25. This prochirality of the methyl groups at C-25 in the intermediate (**2**) has the same biosynthetic origin as that for cholesterol in mammals,[8] isofucosterol in *Pinus pinea*,[9] and sitosterol and stigmasterol in the cultured cells of some higher plants.[10] Solasodin[11] and tomatidin[12] also seem to have an intermediate with the same prochirality at C-25. The subsequent oxidation of the *pro-R* methyl (derived from C-2 of MVA) gives (25*S*)-furostanol glycoside (**8**) and of the *pro-S* methyl (derived from C-6 of MVA) at C-25 gives (25*R*)-furostanol glycoside (**9**). The results for (25*R*)-furostanol are in good agreement with the former conclusion for tigogenin[13] and diosgenin.[1] Acceleration of the oxidation of the *pro-R* methyl group at C-25 on increasing the concentration of sodium acetate suggests either that a hydroxylating enzyme for the *pro-R* methyl group is activated relative to one for the *pro-S* methyl group or that the enzyme loses its stereospecificity.

Experimental

^{13}C Fourier transform (FT) n.m.r. spectra were recorded on a Varian XL-100-12A or a JEOL FX-200 spectrometer operating at 25.16 or 50.18 MHz in [^2H]chloroform at 30 °C or [$^2H_5$]pyridine at 80 °C using a 5-mm spinning tube. FT n.m.r. measurement conditions were as follows: spectral width, 6 016 or 10 000 Hz; pulse flipping angle, 45°; acquisition time, 1 s; number of data points, 12 284 or 16 384. ^1H N.m.r. spectra were recorded on a Varian EM-390 spectrometer operating at 90 MHz. Chemical shifts are given in δ (in p.p.m.) from internal tetramethylsilane. The accuracies of δ_C, δ_H, and J_{CC} values are *ca.* ±0.05 p.p.m., ±0.01 p.p.m., and ±1 Hz, respectively. Sodium [1,2-$^{13}C_2$]acetate was purchased from M.S.D. (Canada) and β-glucosidase was purchased from Sigma. The cells of *D. tokoro* were incubated at 25 °C in the dark on a rotary shaker (120 r.p.m.). Mass spectra were recorded on an RMV-8GN or M-68 spectrometer and optical rotations were determined on a Hitachi Perkin-Elmer 141 instrument.

[20,23,23,25-$^2H_4$]*Tigogenin* (**23**).—A mixture of tigogenin [(25*R*)-5α-spirostan-3β-ol] (**22**) (180 mg) and conc. deuteriated hydrochloric acid (99 atom % ^2H, 2.6 ml) in ethan[^2H]ol (99 atom % ^2H, 6.7 ml) was refluxed for 72 h. After addition of deuteriated water (20 ml) and extraction with ethyl acetate–$NaHCO_3$, the residue was subjected to chromatography on a Lobar-B column with n-hexane–chloroform–ethyl acetate (3:1:1) and afforded the crude product (**23**) (131 mg), which showed β-[^2H]isotope shifted ^{13}C-signals at C-17, -21, -22, -24, and -27 ($\Delta\delta_C$ −0.1, −0.1, −0.1, −0.2, and −0.1, respectively) due to ^2H on C-20, -23, -23, and -25: δ_C ($CDCl_3$) 37.0 (C-1), 31.6 (C-2), 71.3 (C-3), 38.3 (C-4), 44.9 (C-5), 28.7 (C-6), 32.3 (C-7), 35.2 (C-8), 54.4 (C-9), 35.6 (C-10), 21.1 (C-11), 40.1 (C-12), 40.6 (C-13), 56.4 (C-14), 31.8 (C-15), 80.9 (C-16), 62.3 (C-17), 16.5 (C-18), 12.4 (C-19), 41.7 (C-20 with reduced intensity), 14.5 (C-21), 109.2 (C-22), 31.4 (C-23 with reduced intensity), 28.9 (C-24), 30.3 (C-25 with reduced intensity and with γ isotope shifted signal), 66.9 (C-26 br), and 17.1 p.p.m. (C-27); δ_H ($CDCl_3$) 3.58 (3-H, m), 4.38 (16-H, m), 0.75 (18-H, s), 0.82 (19-H, s), 0.95 (21-H, d, *J* 6 Hz), 0.94 [21-H (20-^2H), s, ratio of s to d, 7:3], 3.25—3.50 (26-H, m), and 0.76 (27-H, s); *m/z* 420 (M^+, $^2H_4$), 419 ($^2H_3$), 418 ($^2H_2$), 417 ($^2H_1$), and 416 ($^2H_0$), (1:1:0.5:0.4:0.5, respectively).

[25-^2H]*Tigogenin Acetate* (**24**).—Compound (**23**) (170 mg) was heated in methanol (63 ml) containing conc. hydrochloric acid (13 ml) for 72 h. The solution was mixed with water (200 ml), extracted with ethyl acetate, and washed with 5% sodium hydrogen carbonate solution and water. Purification of the extracts, as described above, by column chromatography gave crude [25-^2H]tigogenin (135 mg), m.p. 199—200 °C, which showed isotopically highfield shifted (−0.1 p.p.m.) ^{13}C-signals at C-24, C-26, and C-27 due to the presence of the ^2H on C-25: δ_C ($CDCl_3$) 37.0 (C-1), 31.5 (C-2), 71.3 (C-3), 38.3 (C-4), 44.9 (C-5), 28.7 (C-6), 32.3 (C-7), 35.2 (C-8), 54.4 (C-9), 35.6 (C-10), 21.1 (C-11), 40.1 (C-12), 40.6 (C-13), 56.4 (C-14), 31.8 (C-15), 80.9 (C-16), 62.3 (C-17), 16.5 (C-18), 12.4 (C-19), 41.7 (C-20), 14.5 (C-21), 109.3 (C-22), 31.4 (C-23), 28.8 (C-24), 30.3 (C-25), 66.9 (C-26), and 17.1 p.p.m. (C-27); δ_H($CDCl_3$) 3.58 (3-H, m), 4.38 (16-H, m), 0.75 (18-H, s), 0.82 (19-H, s), and 0.95 (21-H, d, *J* 7 Hz); *m/z* 417 (M^+, $^2H_1$) and 416 ($^2H_0$). This compound was acetylated and purified by chromatography on a Lobar-B column with n-hexane–chloroform–ethyl acetate (15:1:1), giving after crystallization [25-^2H]tigogenin acetate (**24**) (105 mg), m.p. 205—208 °C (from MeOH).

[25-^2H]-3β-*Acetoxy*-26-*hydroxy*-5α-*furostan* (**25**) *and* [25-^2H]-3β,26-*Diacetoxy*-5α-*furostan* (**26**).—(*a*) Tigogenin acetate (**22**) (200 mg) was shaken with platinum dioxide (105 mg) in glacial acetic acid (5 ml) activated with 60% perchloric acid (1 drop) under hydrogen gas for 10 min. After filtration and extraction with ethyl acetate–$NaHCO_3$, the residue (205 mg) was purified by chromatography on a Lobar-B column with n-hexane–chloroform–ethyl acetate (1.5:1:1) and gave the amorphous *product* (**25**) (84 mg) (Found: C, 75.3; H, 10.2. $C_{29}H_{48}O_4$ requires C, 75.60; H, 10.50%); $[\alpha]_D$ −10.5°, $[\alpha]_{365}$ −29.1° (1.0% in $CHCl_3$); δ_C($CDCl_3$) 36.8 (C-1), 27.5 (C-2), 73.7 (C-3), 34.1 (C-4), 44.7 (C-5), 28.5 (C-6), 32.2 (C-7), 35.3 (C-8), 54.3 (C-9), 35.6 (C-10), 20.9 (C-11), 39.7 (C-12), 41.1 (C-13), 56.7 (C-14), 32.2 (C-15), 83.3 (C-16), 65.3 (C-17), 16.6 (C-18), 12.3 (C-19), 38.0 (C-20), 18.9 (C-21), 90.3 (C-22), 30.5 (C-23), 30.2 (C-24), 35.8 (C-25), 68.1 (C-26), 16.6 (C-27), 21.4 (3-OCO*C*H_3), and 170.7 p.p.m. (3-O*C*OCH$_3$); δ_H($CDCl_3$) 4.67 (3-H, m), 4.28 (16-H, m), 0.77 (18-H, s), 0.82 (19-H, s), 0.98 (21-H, d, *J* 7 Hz), 3.31 (22-H, m), 3.47 (26-H, d, *J* 6 Hz), 0.90 (27-H, d, *J* 7 Hz), and 1.99 (3-OCOC*H*$_3$, s); and 3β,26-*diacetoxy*-5α-*furostan* (**26**) (104 mg), m.p. 116—117 °C (Found: C, 74.1; H, 9.95. $C_{31}H_{50}O_5$ requires C, 74.06; H, 10.03%); $[\alpha]_D$ −11.2°, $[\alpha]_{365}$ −34.1° (1.0% in $CHCl_3$); δ_C($CDCl_3$) 36.8 (C-1), 27.5 (C-2), 73.7 (C-3), 34.0 (C-4), 44.7 (C-5), 28.5 (C-6), 32.2 (C-7), 35.3 (C-8), 54.3 (C-9), 35.6 (C-10), 20.9 (C-11), 39.7 (C-12), 41.0 (C-13), 56.7 (C-14), 32.2 (C-15), 83.3 (C-16), 65.3 (C-17), 16.6 (C-18), 12.3 (C-19), 38.0 (C-20), 18.9 (C-21), 90.2 (C-22), 30.8 (C-23), 30.5 (C-24), 32.8 (C-25), 69.4 (C-26), 16.8 (C-27), 21.4 (3-OCO*C*H_3), 170.7 (3-O*C*OCH$_3$), 21.0 (26-OCO*C*H$_3$), and 171.3 p.p.m. (26-O*C*OCH$_3$); δ_H($CDCl_3$) 4.67 (3-H, m), 4.28 (16-H, m), 0.76 (18-H, s), 0.82 (19-H, s), 0.98 (21-H, d, *J* 7 Hz), 3.30 (22-H, m), 3.96 and 3.86 (26-H, AB part of ABX, J_{AB} 11 Hz, J_{AX} 6 Hz, and J_{BX} 7 Hz), 0.91 (27-H, d, *J* 7 Hz), 1.99 (3-OCOCH_3, s), and 2.03 (26-OCOCH_3, s).

(*b*) Compound (**24**) (100 mg) was shaken under hydrogen gas for 1.5 h with platinum dioxide (50 mg) in glacial acetic acid (4 ml) activated with 68% perchloric acid (1 drop). After filtration and extraction with ethyl acetate–$NaHCO_3$, the residue was purified as above and gave the product (**25**) (72 mg); δ_C($CDCl_3$) 30.2 (C-24), 30.0 [C-24(25-^2H)], 67.9 (C-26), 67.8 [C-26(25-^2H)], 16.6 (C-27), and 16.5 p.p.m. [C-27(25-^2H)]; δ_H($CDCl_3$) 4.67 (3-H, m), 4.27 (16-H, m), 0.77 (18-H, s), 0.82 (19-H, s), 0.97 (21-H, d, *J* 7 Hz), 3.31 (22-H, m), 3.37—3.53 (26-H, m), 0.90 (27-H, d, *J* 7 Hz), 0.88 [27-H(25-^2H), s], and 1.99 (3-OCOCH$_3$, s); and compound (**26**) (16 mg): δ_C($CDCl_3$) 30.4 (C-24), 30.3 [C-24(25-^2H)], 32.8 (C-25), 69.4 (C-26), 69.3$_5$[C-26(25-^2H)], 16.8 (C-27), and 16.6 p.p.m. [C-27(25-^2H)]; δ_H($CDCl_3$) 4.68 (3-H, m), 4.28 (16-H, m), 0.76 (18-H, s), 0.82 (19-H, s), 0.98 (21-H, d, *J* 7 Hz), 0.92 (27-H, d, *J* 7 Hz), 0.91 [27-H(25-^2H), s], 1.99 (3-OCOCH$_3$, s), and 2.03 (26-OCOCH$_3$, s).

Isolation of [^{13}C]*Prototokoronin* (**12**).—The stock-cultured

cells of *D. tokoro* were transferred into Linsmaier–Skoog (LS) liquid medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 10^{-6}M) and after 4 weeks incubation, were transferred into fresh medium (6 l) containing sodium acetate (90 atom % ^{13}C enriched, 500 mg and unlabelled, 500 mg) distributed in 20 500-ml conical flasks. After 2 further weeks of incubation, the cultured cells (fresh wt. 2 151 g) were collected by filtration and extracted with hot 70% methanol (2 l × 4). The n-butyl alcohol soluble part of the methanol extract (45.5 g) was fractionated by column chromatography [SiO_2 eluted with chloroform, chloroform–methanol (3:1), and chloroform–methanol–water (65:25:4)]. From the last fraction (4.0 g), crude prototokoronin (557 mg) was isolated by h.p.l.c. (LiChrosorb RP-18 25—40 μm, 20 × 500 mm; eluted with H_2O–MeOH, 1:1), followed by recrystallization from water. Based on the ^{13}C n.m.r. spectrum, the ratio of protoneotokoronin (**13**) to prototokoronin (**12**) was *ca.* 1:8.

Tokoronin (**15**) *from Prototokoronin* (**12**).—Crude protokokoronin (300 mg) and β-glucosidase (150 mg) were suspended in M/10 acetate buffer (pH 4.5, 20 ml) and left at room temperature (25—30 °C) for 48 h. The reaction mixture was diluted with water (50 ml) and extracted with ethyl acetate (30 ml × 3), washed with water, dried (Na_2SO_4), and evaporated to dryness. The residue was crystallized from chloroform–methanol as crude tokoronin (145.6 mg, m.p. 278—282 °C).

Effects of Sodium Acetate on the Growth of D. tokoro.—Precultured cells of *D. tokoro* were cultured in LS liquid medium supplemented with 2,4-D (10^{-6}M) containing different concentrations of sodium acetate (0, 50, 150, 200, 300, 500, 1 000 mg/l). After 3 weeks incubation, the cells were collected by filtration and dried. The results are shown in the Figure.

Effects of Higher Concentration of Sodium Acetate on ^{13}C-*Labelling Patterns.*—Precultured cells of *D. tokoro* were cultured in LS liquid medium (1.8 l) containing sodium [1,2-$^{13}C_2$]acetate (200 mg) and unlabelled acetate (200 mg) distributed in 6 500-ml conical flasks. After incubation for 1 week, the same amount of diluted sodium [1,2-$^{13}C_2$]acetate was added under sterile conditions, and the incubation was then continued for 10 more days. The collected cells (fresh wt. 880 g, dry wt. 31 g) were extracted with hot methanol (1 l × 3). The methanol extract (16.8 g) was partitioned between n-butyl alcohol and water. The n-butyl alcohol-soluble fraction (2.1 g) was fractionated on a silica-gel column eluted with chloroform, chloroform–methanol (9:1), and chloroform–methanol–water (65:25:0.5). The fractions containing prototokoronin checked by t.l.c. (chloroform–methanol–water, 85:25:5) were combined and purified by h.p.l.c. (LiChroprep RP-18, 25—40 μm, 20 × 500 mm, eluted with methanol–water 1:1). The crude prototokoronin (284 mg) was refluxed with 1.7% hydrochloric acid in methanol (30 ml for 5 h). The reaction mixture was diluted with water (50 ml) and extracted with ethyl acetate (30 ml × 3) and the organic layer was washed with water, dried (Na_2SO_4), and evaporated to dryness, to give crude tokorogenin (150 mg). This was purified by column chromatography (silica gel Lobar-B, Merck, eluted with benzene–acetone, 6:4) and afforded crystalline sapogenin (64 mg). The ratio of neotokorogenin (**19**) to tokorogenin (**16**) was *ca.* 1:1 according to the ^{13}C n.m.r. spectrum.

The crude tokorogenin (64 mg) in acetic anhydride (2 ml) and pyridine (2 ml) was refluxed for 4 h. Water (30 ml) was added to the reaction mixture under ice-cooling and extracted with ethyl acetate (30 ml × 3). The organic layer was washed with water and dried (Na_2SO_4) and the solvent was evaporated off. The residue (68 mg) was separated by column chromatography (silica gel Lobar-A eluted with n-hexane–ethyl acetate–chloroform, 6:1:1) into tokorogenin acetate (19.9 mg) and neotokorogenin acetate (20 mg).

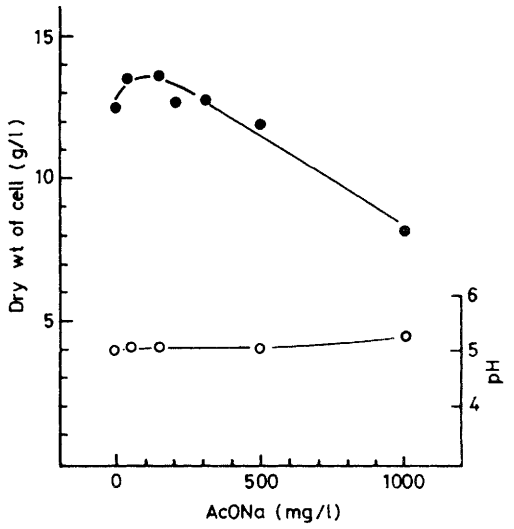


Figure. Effect of sodium acetate on cell growth (●) and pH (○) of broth in tissue cultures of *D. tokoro*

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