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STRUCTURE AND BIOSYNTHESIS OF PROTOTOKORONIN IN TISSUE CULTURES OF *DIOSCOREA TOKORO**

YUTAKA TOMITA and ATSUKO UOMORI

Shionogi Research Laboratory, Shionogi & Co., Ltd., Fukushima-ku, Osaka, 553, Japan

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Key Word Index—*Dioscorea tokoro*; Dioscoreaceae; biosynthesis; prototokoronin; tissue cultures: furostanol glucoside.

Abstract—The furostanol glucoside prototokoronin (**1**) has been isolated from tissue cultures of *Dioscorea tokoro* M., and labelled yonogenin and diosgenin were incorporated into (**1**). Sapogenin and protosaponin are thus seen to be interconvertible in tissue cultures.

INTRODUCTION

STEROIDAL sapogenin components in *Dioscorea tokoro* have previously been investigated and diosgenin, yonogenin, tokorogenin, isodiotigenin, and igagenin isolated; a 3α-hydroxyl group and 5β-configuration have been established as characteristic structural features.[1]

In a previous report we showed that the callus derived from seedling of *D. tokoro* retains the ability to synthesize diosgenin, yonogenin, and tokorogenin; but not isodiotigenin and kogagenin.[2] Furthermore, we proposed the biosynthetic pathway for these sapogenins in the callus on the basis of tracer experiments.[3]

Recently Tschesche *et al.*,[4] Kiyosawa,[5] and Kawasaki *et al.*[6] isolated prototype saponins having a furostanol glucoside structure and confirmed Marker's hypothesis[7] that spirostanol is an artifact produced by acidic treatment. This paper reports the structure of prototokoronin and the biosynthetic relationship between furostanol glucosides and spirostanol sapogenins.

RESULTS AND DISCUSSION

Cells of *D. tokoro* tissue cultures grown in Linsmaier–Skoog medium fortified with 2,4-D and kinetin were extracted with 70% methanol at room temperature. Prototokoronin (**1**) was isolated from the extract by preparative TLC on silica gel (R_f 0·30, in $CHCl_3$–MeOH–H_2O, 30:10:1) and recrystallized from hot water to give colourless needles,

* Part IV in a projected series entitled "Biosynthesis of Isoprenoids". For Part III see (1973). *J. Chem. Soc. Perkin Trans. I* 2656.

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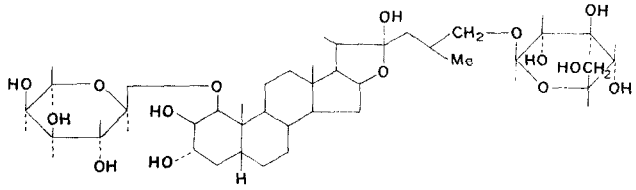
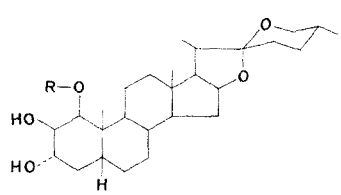
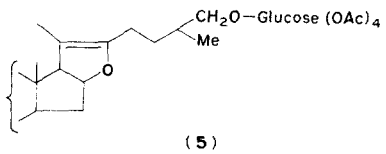


(1)



(2) R = arabinose
(3) R = H



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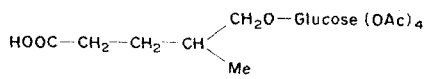
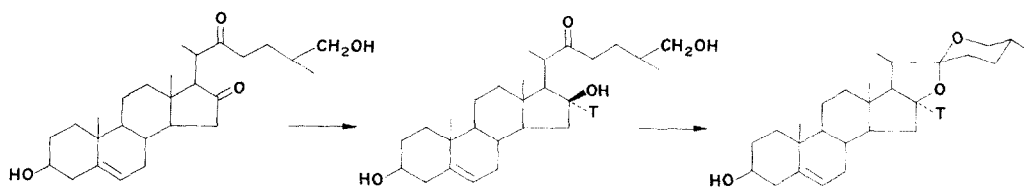


(7)

$C_{38}H_{64}O_{15}\cdot 3H_2O$, m.p. 177–178°, $[\alpha]_D$ −3·8° (MeOH). Compound (**1**) gave a positive Ehrlich colour test and lacked the characteristic absorption band of a spiroketal linkage in its IR spectrum. Hydrolysis of prototokoronin with β-glucosidase afforded glucose and tokoronin (**2**) which gave tokorogenin (**3**) and arabinose by hydrolysis with 5% HCl. Treatment[4] of the nonaacetate $C_{56}H_{82}O_{24}$ (**4**) of prototokoronin in acetic acid gave an anhydro derivative $C_{56}H_{80}O_{23}$ (**5**) m.p. 138–140°, which was degraded with ozone to give the ester (**6**). Saponification of **6** with sodium carbonate followed by neutralization and acetylation after drying gave the acetyl glucoside (**7**). The MS of the methyl ester of (**7**) showed peaks corresponding to glucose[8] (*m/e* 331, 243, 242, 200, 169, 157, 145, 141, 140, 115, 109, 103 and 98) and methyl γ-methyl-δ-hydroxyvalenate[4] (*m/e* 129 and 97). These results were identical with those of degradation studies on sarsaparilloside described by Tschesche *et al.*[4] Thus, prototokoronin has structure **1**.



(8) (9)

SCHEME 1.

Furthermore, we have investigated the biosynthetic relationship between spirostanol sapogenin and furostanol glucoside in the tissue cultures, since the co-occurrence of sapogenin and saponin in the intact plant has been pointed out by Akahori *et al.*[9] For study of the incorporation of sapogenin into protosaponin in *D. tokoro* tissue cultures, labelled diosgenin and yonogenin were synthesized as follows. Reduction of kryptogenin (**8**) with an equivalent of tritiated $NaBH_4$ in ethanol yielded [16α-$^3H_1$]-3,16,26-trihydroxycholest-5-en-22-one with a small amount of tetrahydroxycholest-5-ene, and the former compound was converted into [16α-$^3H_1$]diosgenin (**9**) by treatment with acetic acid.

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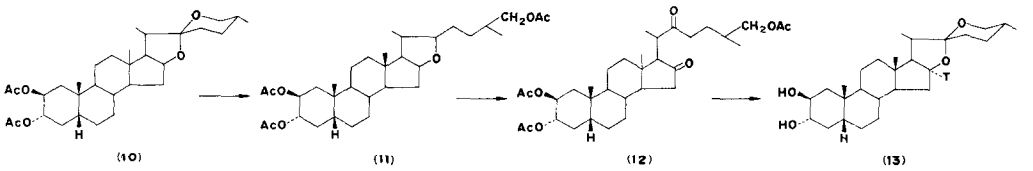


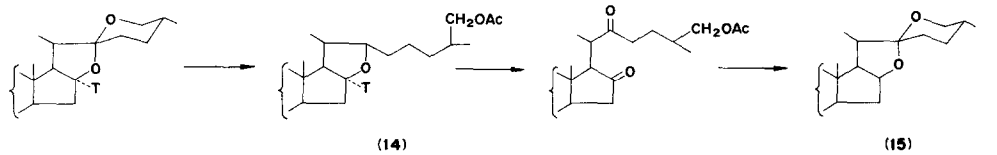
SCHEME 2.

Hydrogenation of yonogenin diacetate (**10**) with PtO_2 in acetic acid containing a small amount of perchloric acid gave furostanol derivative $C_{33}H_{46}O_8$ (**10**), which was oxidized with chromic acid to give the diketone (**12**). Reduction of the diketone (**12**) with an equivalent of tritiated sodium borohydride followed by saponification and acidic treatment gave [16α-$^3H_1$]yonogenin (**13**). These sapogenins were purified by preparative TLC on silica gel. Solutions of [16α-$^3H_1$]diosgenin and [16α-$^3H_1$]yonogenin were separately added to callus grown on agar medium under sterile conditions. After 1 week, cells were harvested, and prototokoronin was isolated as described above and recrystallized to constant radiospecific activity after addition of pure carrier prototokoronin. As shown in Table 1, prototokoronins obtained in both experiments were radioactive; they were degraded to determine the location of tritium in the following ways (Scheme 3). Radioactive prototokoronin derived from [16α-$^3H_1$]yonogenin was hydrolyzed with HCl to give radioactive tokorogenin, which was acetylated with acetic anhydride and pyridine. Hydrogenation of the acetate with PtO_2 in acetic acid-perchloric acid gave furostanol derivative (**14**),

TABLE 1. INCORPORATION OF LABELLED SAPOGENINS INTO PROTOTOKORONIN IN TISSUE CULTURES OF *Dioscorea tokoro*

Compounds added	Prototokoronin (dpm)	Incorporation (%)
(16α-T_1)diosgenin 50 μCi	$2{\cdot}11 \times 10^4$	0·019
(16α-T_1)yonogenin 50 μCi	$3{\cdot}36 \times 10^4$	0·031

which was oxidized with chromic acid to give the diketone (**15**). The diketone (**15**) was converted into tokorogenin by reduction with $NaBH_4$ followed by saponification and acid treatment. This tokorogenin now possessed no radioactivity. Radioactive prototokoronin derived from [16α-$^3H_1$]diosgenin was also converted into tokorogenin via diketone derivative (**15**), and the tokorogenin obtained had no radioactivity. Thus the tritium atoms in both radioactive prototokoronin were located at C-16 in the tokorogenin skeleton, and labelled diosgenin and yonogenin were incorporated into prototokoronin without scrambling. These results show that spirostanol sapogenin can be converted into furostanol glucoside with cleavage of the spiroketal linkage. Sapogenin and protosaponin are, there-



SCHEME 3.

fore, seen to be interconvertible, since β-glucosidase is usually present in plants. That only the proto-form of saponin exists in the tissue culture would indicate that the tokoronin-prototokoronin equilibrium lies in favour of the proto-form, owing to the high glucose concentration in the medium. However, it is not yet known that whether biosynthesis of steroidal saponin proceeds in the form of a furostanol glucoside.

EXPERIMENTAL

General procedure. M.ps were determined on a hot-stage apparatus and are uncorrected. Tritiated $NaBH_4$ (142 mCi/mmol) was purchased from Daiichi Pure Chemical Co., Ltd. (Tokyo). Radioactive measurements were made on Beckman DPM 100 Scintillation counter. Gas chromatography was run on a Shimazu Gas Chromatograph GC-4A (RF) instrument fitted with a H_2 FID.

Tissue cultures of *Dioscorea tokoro* Makino derived from seedling were cultured on Linsmaier-Skoog agar medium fortified with 2,4-D (10^{-6} mmol) and kinetin (0·2 ppm).

Isolation of prototokoronin (**1**). Callus derived from *D. tokoro* were cultured on Linsmaier–Skoog agar medium containing 2,4-D (10^{-6} mmol) and kinetin (0·2 ppm) for 4 weeks, and cells (wet wt 506 g) were harvested and extracted 3 × with 70% MeOH for 48 hr at room temp. The extracts were combined and the solvent was removed. The residue was applied on silica gel plates (0·3 mm) and developed with $CHCl_3$–MeOH–H_2O (30:10:1) and the prototokoronin fraction (R_f 0·3) was eluted with MeOH and rechromatographed on the same system for further purification. The crude prototokoronin was dissolved in hot water (2 ml), refluxed for 2 hr, and then concentrated to one-third of its original vol. After cooling, the amorphous precipitate was recrystallized 3 × from hot water to give colourless needles, m.p. 177–180°, $[\alpha]_D$ −3·8°(MeOH) (Found: C, 55·90; H, 8·26. $C_{38}H_{64}O_{15}\cdot 3H_2O$ requires: C, 56·00; H, 8·66%).

Acetylation of prototokoronin (**1**). Prototokoronin (30 mg) was acetylated with Ac_2O and pyridine. The product (ex MeOH × 3) was obtained as colourless needles, m.p. 129–131·5°, $[\alpha]_D$ +5·4°(pyridine) (Found: C, 58·70; H, 7·19. $C_{56}H_{82}O_{24}$ requires: C, 59·04; H, 7·25%).

Hydrolysis of prototokoronin (**1**). A buffer soln (AcOH–AcONa, pH 4·5) of prototokoronin (15 mg) was treated with emulsin (50 mg) for 48 hr at 30°. The soln, after addition of H_2O, was extracted 2 × with *n*-BuOH and the extracts were combined, washed, dried and evaporated. The residue was recrystallized from MeOH to give colourless needles (7·3 mg), m.p. 278–280°, $[\alpha]_D$ −12·2° (pyridine), IR 860–850, 890, 900, 920 cm^{-1} (corresponding to the spiroketal linkage). Penta-acetate, m.p. 235–237° (from MeOH), $[\alpha]_D$ −3·1° ($CHCl_3$) (Found: C, 64·04; H, 7·76. $C_{42}H_{62}O_{14}$ requires: C, 63·78; H, 7·90%). The acetate was identical with tokoronin acetate in its IR and m.m.p. The aq. soln was evaporated to dryness under reduced pressure and the residue was acetylated with pyridine and Ac_2O. The product was identical with penta-acetyl D-glucose in its TLC (R_f 0·13, *n*-hexane–$CHCl_3$–MeOH, 4:1:1) and GLC (1% QF-1 on Gas Chrom Q (100/120), glass U column, 1·5 m × 4 mm o.d., column temp. 170°, N_2 45 ml/min).

Preparation of anhydroprototokoronin acetate (**5**). Prototokoronin nonaacetate (23 mg) was dissolved in HOAc (2 cm^3) and refluxed for 2 hr, the soln was extracted with CH_2Cl_2 after addition of H_2O. The extract was washed and then dried. After removal of the solvent the residue (21·4 mg) was applied to silica gel plates and developed with *n*-hexane–EtOAc (2:3). Anhydroprototokoronin acetate (R_f 0·31) was eluted and recrystallized from MeOH to give colourless needles (11 mg), m.p. 138–140° [M^+, 1121].

Ozone degradation of anhydroprototokoronin acetate (**5**). A soln of anhydroprototokoronin acetate (44 mg) in $CHCl_3$ was treated with O_3 at −70° for 20 min. After removal of the solvent, H_2O was added dropwise to the ozonide with stirring. After continuous stirring for 2 hr at room temp., EtOH (10 ml) and Na_2CO_3 (1 g) was added to the soln and stirred for 1 hr at 50°. The soln was neutralized with 1 NHCl and evaporated to dryness under reduced pressure, and the residue was acetylated with Ac_2O and pyridine. To a soln of the acidic product (1·7 mg) in acetone (1 ml) added a soln of CH_2N_2 (0·5 ml) in ether. After 1 hr the solvent was evaporated and the residue was applied to silica gel plates and developed with C_6H_6–Et_2O (1:1). The methyl ester (R_f 0·26) was eluted and rechromatographed by the same system for further purification. Yield 1·6 mg. MS of (**6**) shows the peaks corresponding to acetyl glucose (*m/e* 331, 243, 242, 200, 169, 157, 145, 141, 140, 115, 109, 103 and 98) and methyl γ-methyl δ-hydroxyvalenate (*m/e* 129 and 97).

Synthesis of [16α-$^3H_1$]*diosgenin* (**9**). To a soln of kryptogenin (**8**, 12 mg) in EtOH (2 ml) added NaB^3H_4 (1 mg, 3·08 mCi) with stirring at room temp. After 5 hr, the soln was diluted with H_2O and acidified with HOAc. Further stirring for 1 hr at 60° the soln was extracted with Et_2O and the extract was washed and dried. The solvent was removed and the residue was applied to silica gel plates and developed with *n*-hexane–AcOEt–$CHCl_3$ (4:1:1). The bands corresponding diosgenin (R_f 0·2) was eluted, rechromatographed by the same system for further purification, and recrystallized from methanol to give colourless needles, m.p. 198–200°, $4·8 \times 10^8$ dpm/3 mg.

Synthesis of [16α-$^3H_1$]*yonogenin*. A soln of yonogenin acetate (430 mg) in HOAc containing traces of $HClO_4$ was hydrogenated over PtO_2 (100 mg) (uptake 47 ml of H_2 in 6 hr). The catalyst was filtered off and washed with HOAc (1 ml × 2). After the filtrate was diluted with H_2O, the soln was extracted with EtOAc. The extract was combined, washed and dried. After the solvent was evaporated and the residue was acetylated with Ac_2O and

pyridine. A soln of CrO_3 (300 mg) in HOAc (4 ml) and H_2O (1 ml) was added dropwise to a soln of the acetate (**11**, 160 mg) in HOAc (13 ml) during 30 min. After further stirring for 3 hr at 25°, the soln was diluted with H_2O and extracted with Et_2O. The extract was washed and dried. The solvent was removed and the residue was applied to silica gel plates and developed with C_6H_6–Et_2O (1:1). The band corresponding to the diketon (**12**, R_f 0·52) was eluted and rechromatographed by the same system for further purification. Yield 121 mg (Found: C, 69·40; H, 8·40. $C_{33}H_{46}O_8$ requires: C, 69·45; H, 8·12%). To a soln of **12** (12 mg) in EtOH (2 ml) added an equivalent of NaB^3H_4 (1 mg, 3·08 mCi) with stirring at room temp. Further stirring for 3 hr, the soln was diluted with H_2O (3 ml) and extracted with Et_2O. The extract was washed and dried. After removal of the solvent the residue was dissolved in EtOH (5 ml) containing 5% Na_2CO_3 and the soln was refluxed for 1 hr. The soln was acidified with 1 NHCl, stirred for 1 hr at 60°, and extracted with Et_2O. The extract was washed and dried. The solvent was evaporated, and the residue was applied to silica gel plates and developed with C_6H_6–Me_2CO (1:1). The band corresponding yonogenin (R_f 0·57) was eluted with $CHCl_3$–MeOH (3:1) and rechromatographed by the same system. Colour test (cinnamic aldehyde–H_2SO_4), and R_f value (C_6H_6–Me_2CO, 1:1) were identical with those of an authentic sample. Total radioactivity $4·75 \times 10^8$ dpm/3·1 mg.

Incubation of [16α-$^3H_1$]*yonogenin with tissue cultures of* Dioscorea tokoro *Makino.* [16α-$^3H_1$]yonogenin (50 μCi) was mixed with sterile H_2O (5 ml) containing 0·3% of Tween 80 and the mixture was added dropwise to cells of *D. tokoro* tissue cultures. After 7 days cells (wet wt 52 g) were harvested and extracted with 70% MeOH with stirring at room temp. The solvent was removed under reduced pressure below 60° and prototokoronin was isolated by preparative TLC as described above and it was recrystallized from hot H_2O to constant specific radioactivity after addition of carrier prototokoronin (20 mg). Total radioactivity: $3·36 \times 10^4$ dpm.

Incubation of [16α-$^3H_1$]*diosgenin with tissue cultures of* D. tokoro *Makino.* [16α-$^3H_1$]Diosgenin (50 μCi) was mixed with sterile H_2O (5 ml) containing 0·3% of Tween 80 and the mixture was added dropwise to cells of *D. tokoro* tissue cultures. After 7 days cell (wet wt 42 g) were harvested and extracted with 70% MeOH at room temp. Prototokoronin was isolated from the extract by the same manner described above, and it was recrystallized from hot water to constant specific radioactivity after addition of pure carrier compound (20 mg). Total radioactivity, $2·11 \times 10^4$ dpm.

Location of the tritium atom in prototokoronin. (i) Prototokoronin (12·1 mg, $1·93 \times 10^4$ dpm), which was obtained from the tissue cultures incubated with [16α-$^3H_1$]yonogenin, was dissolved in ethanol containing 5% HCl and refluxed for 3 hr. The solution was extracted with EtOAC after addition of H_2O, and the extract was washed and dried. After removal of the solvent, the residue was applied to silica gel plates and developed with C_6H_6–Me_2CO (1:1). Tokorogenin (R_f 0·43) was eluted and rechromatographed by the same system for further purification. Total radioactivity $1·67 \times 10^4$ dpm (6·2 mg). Radioactive tokorogenin obtained was mixed with carrier tokorogenin (14 mg) and acetylated with Ac_2O and pyridine. A soln of the acetate in HOAc–$HClO_4$ was hydrogenated over PtO_2 (20 mg) (uptake *ca.* 5·7 ml of H_2 in 2 hr) and the catalyst was filtered off. After addition of H_2O the filtrate was extracted 2 × with EtOAc, and the extract was combined, washed, and then dried. The solvent was evaporated to dryness and the residue was acetylated with Ac_2O and pyridine. The acetate (**14**) (23 mg) was dissolved in HOAc (2 ml) and a soln of CrO_3 (0·2 ml, see synthesis of [16α-$^3H_1$]yonogenin) was added dropwise to the soln with stirring at room temp. After further stirring for 3 hr, the mixture was extracted with Et_2O after addition of H_2O. The extract was washed and dried, and the solvent was removed to dryness. The diketone (**15**, 9·1 mg, R_f 0·6), isolated from the residue by preparative TLC (C_6H_6–Et_2O, 1:1), was dissolved in EtOH (1 ml) and $NaBH_4$ (1 mg) was added to the soln with stirring. After continuous stirring for 1 hr alcoholic 5% KOH (0·5 ml) was added and stirred for 1 hr at 60°. The soln was extracted with EtOAc after addition of 1 N HCl (2 ml). The extract was washed and dried, and then the solvent evaporated. The residue was applied to silica gel plates and developed with C_6H_6–Me_2CO (1:1). Tokorogenin (R_f 0·43) was eluted and recrystallized from MeOH, yield 3·1 mg. Tokorogenin obtained was not contained radioactivity. (ii) Prototokoronin (11·8 mg, $1·18 \times 10^4$ dpm), which was obtained from the tissue cultures incubated with [16α-$^3H_1$]diosgenin, was dissolved in EtOH containing 5% HCl and refluxed for 3 hr. The soln was extracted with EtOAc after addition of H_2O. The extract was washed and dried. After removal of the solvent, the residue was applied to silica gel plates and developed with C_6H_6–Me_2CO (1:1). Tokorogenin (R_f 0·43) was eluted and rechromatographed by the same system for further purification. Total radioactivity $1·06 \times 10^4$ dpm (6·3 mg). Radioactive tokorogenin (6·3 mg) was mixed with carrier tokorogenin (14 mg) and the mixture was acetylated with Ac_2O and pyridine. Hydrogenation of the acetate (26 mg) with PtO_2 (20 mg) followed by acetylation and oxidation with chromic acid yielded the diketone (**15**). The diketone (7·3 mg) was dissolved in EtOH (2 ml) and $NaBH_4$ (0·7 mg) was added to the soln with stirring. After continuous stirring for 1 hr, alcoholic 5% KOH (0·5 ml) was added and stirred for 1 hr at 60°. The soln, after addition of 1 N HCl (3 ml), was extracted with EtOAc and the extract was washed and dried. The solvent was evaporated and the residue was recrystallized from MeOH to give colourless needles. Yield 2·2 mg. Tokorogenin obtained was not contained radioactivity.