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Isolation and Identification of Steroidal Saponins in Taiwanese Yam Cultivar (*Dioscorea pseudojaponica* Yamamoto)

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A new furostanol pentaoligoside and spirostanol tetraoligoside were isolated for the first time from yam tubers (*Dioscorea pseudojaponica* Yamamoto) from Taiwan, together with four known yam saponins, methyl protodioscin, methyl protogracillin, dioscin, and gracillin. Their structures were characterized as 26-*O*-β-D-glucopyranosyl-22α-methoxyl-(25*R*)-furost-5-en-3β,26-diol, 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-{[α-L-rhamnopyranosyl-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]}-β-D-glucopyranoside, and (25*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-{[α-L-rhamnopyranosyl-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]}-β-D-glucopyranoside. The structural identification was performed using LC-MS and ^{1}H and ^{13}C NMR. The methanol extract of yam tubers was fractionated by XAD-2 column chromatography using a methanol/water gradient elution system to yield furostanol and spirostanol glycoside fractions. Preparative high-performance liquid chromatography, employing a C18 column and a mobile phase of methanol/water (69:31, v/v), was used to separate each furostanol glycoside, whereas a mobile phase of methanol/water (79:21, v/v) was used to resolve the individual spirostanol glycosides. The conversions from steroid saponins to diosgenin after acid hydrolysis were around 68 and 90% for furostanol and spirostanol glycosides, respectively.

KEYWORDS: ***Dioscorea pseudojaponica*; diosgenin; furostanol glycoside; spirostanol glycoside; yam**

INTRODUCTION

The tuber of the yam (*Dioscorea* spp.) is consumed as a food and is also widely used in traditional Chinese medicine (*1*). It is suggested to have a hypoglycemic effect (*2*) and promote the health of elderly women (*3*, *4*). Hu et al. (*5*, *6*) have isolated seven steroidal saponins from *Dioscorea colletti* var. *hypoglauca*. They are four furostanol glycosides, including protoneodioscin, protodioscin, protoneogracillin, and protogracillin, and three spirostanol glycosides, including dioscin**,** prosapogenin A of dioscin, and gracillin. They also deduced that methylation of the hydroxyl group at position C-22 on furostanol glycoside structures, resulting in the formation of methyl esters, including methyl protoneodioscin, methyl protodioscin, methyl protoneogracillin, and methyl protogracillin, would occur during the isolation process using MeOH as solvent. Among different yam species, the closed-ring type of saponin (spirostanol glycosides) has been found more often. Hoyer et al. (*7*) found five spirostanol glycosides, trillin, prosapogenin A, prosapogenin B, dioscin, and 3-*O*-{[α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl]-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyl} diosgenin, and two furostanol glycosides, 26-[β-D-glucopyranosyloxy]-(25*R*)-furost-5-en-3β,22α-diol and 3-β-{-[α-L-rhamnopyranosyl-(1→4)]-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranosyloxy}-26-[β-D-glucopyranosyloxy]-(25*R*)-furost-5-en-3β,22α-ol, from *Dioscorea floribunda*. Two spirostanol saponins, 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl diosgenin and 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl diosgenin, were found in *Dioscorea prazeri* by Wij et al. (*8*). *Dioscorea composita* contained four major spirostanol glycosides: trillin, dioscin, 3-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl] diosgenin, and 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl] diosgenin (*9*). Tang and Jiang (*10*) isolated three spirostanol saponins from *Dioscorea zingiberensis*, named zingiberosides A1, A2, and A3, and Haraguchi et al. (*11*) found diosgenin 3-*O*-β-D-glucopyranoside in *Dioscorea olfersiana.* Yams have become popular as a functional food in recent years. Several native and new cultivar lines have been grown to some extent in Taiwan. However, the saponins present in the yam cultivars grown in Taiwan have not yet been investigated.

Saponins have many biological activities such as hemolytic (*12*, *13*), antithrombotic (*13*, *14*), anti-antineoplastic (*5*, *6*), hypoglycemic (*15*), hypocholesterolemic (*16*, *17*), antiviral (*18*), and anticancer (*19*, *20*). Diosgenin, the aglycon part of the yam steroid saponin, is a principal raw material for the industrial production of steroid drugs, which is obtained after hydrolysis of yam saponins (*21*, *22*).

Yam saponins have traditionally been isolated by silica gel column chromatography (*5*−*7*, *9*, *10*, *23*), which is tedious and time-consuming. In this investigation, a method with a simpler procedure and higher efficiency was developed for the isolation

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and purification of steroidal saponins from a Taiwanese native yam (*Dioscorea pseudojaponica* Yamamoto), and the structures of the purified saponins have been elucidated. The conversion from each steroidal saponin to diosgenin after acidic hydrolysis was also determined.

MATERIALS AND METHODS

Yam Sample. Tubers of yam (*D. pseudojaponica* Yamamoto), a Taiwanese native variety, were purchased from Chidu in Keelung City, Taiwan. The tuber has a common name of Kee-Lung yam and is cylindrical in shape with a white cortex and flesh. After the yam had been peeled and cut into slices, it was dried with FreeZone 18 L Freeze-Dry System (Labconco Co., Kansas City, MO) and ground in an RT08 grinder (Rong-Tsong Co., Taipei, Taiwan) to pass through a 40 mesh screen.

Chemicals. Solvents used for the extraction and separation of steroidal saponins, *n*-butanol (*n*-BuOH), chloroform ($CHCl_3$), 95% ethanol (EtOH), methanol (MeOH), hydrochloric acid (HCl), and sulfuric acid, were purchased from Tedia Co. (Fairfield, OH). NMR solvent, pyridine-d_5, was purchased from Merck Co. (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water purification system (Millipore Co., Bedford, MA) and was degassed under vacuum followed by filtering through a 0.22 μm membrane filter prior to use. Diosgenin standard and *p*-(dimethylamino)benzaldehyde (for preparation of the Ehrlich reagent) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium hydroxide was purchased from Showa Chemical Co. (Tokyo, Japan).

Isolation and Purification of Steroidal Saponins 1—6. *XAD-2 Column Liquid Chromatography.* The method was based on that reported by DeMarino et al. **(***24*) and Chludil et al. (*25*). Freeze-dried yam powder (600 g) was extracted at room temperature with 6 L of MeOH for 24 h, followed by filtration and concentration in the rotary evaporator (Büchi Co., Flawil, Switzerland), and 5 g of MeOH extract was obtained. The extract was then suspended in 50 mL of water and partitioned against 50 mL of *n*-BuOH three times to yield the saponin extract. The extract was washed with 50 mL of distilled water three times and then concentrated to dryness by removing the solvent in a rotary evaporator. The total yield of *n*-BuOH extract was ~3 g. The saponin extract was dissolved in 10 mL of 60% MeOH after vortexing**.** The resultant solution was mixed with 10 mL of Amberlite XAD-2 hydrophobic aromatic resin (Sigma Chemical Co.) and put on top of the XAD-2 column for chromatographing. The column was 50 cm × 2.5 cm i.d., and the XAD-2 resin was suspended in 60% MeOH and filled to 35 cm. After the saponin extract had been loaded, the column was eluted with 300 mL of 60% MeOH to wash off the impurities. The column was then eluted with a MeOH/H_2O gradient system, from 900 mL of 60% MeOH to 900 mL of 100% MeOH, at a flow rate of 0.8 mL/min. Each 150 mL of the column eluate was collected by a fraction collector, and a total volume of 1800 mL of eluate was collected.

Thin-Layer Chromatography (TLC). Each fraction was concentrated to dryness and then dissolved in 1 mL of MeOH. A TLC method similar to that developed by Konishi et al. (*26*) and Pierre et al. (*27*) was used to determine the presence of steroidal saponins in each fraction. A 20 cm × 20 cm silica gel 60 F_{254} TLC plate (Merck Co.) was developed in $CHCl_3$/MeOH/H_2O, 8:4:1 (v/v/v), after spotting each fraction. Steroid saponins were detected by spraying two kinds of visualizing agents separately. To detect furostanol glycosides, the TLC plate was sprayed with Ehrlich's reagent [3.2 g of *p*-(dimethylamino)benzaldehyde in 60 mL of 95% EtOH and 60 mL of 12 N HCl] and heated at 110 °C for 5 min to develop bright red spots, whereas spirostanol glycosides did not show any color change. Both furostanol and spirostanol glycosides became yellow-green when sprayed with 10% sulfuric acid in MeOH, followed by heating at 110 °C for 5 min.

Purification with Reversed-Phase Preparative High-Performance Liquid Chromatography (RP-HPLC). The fractions containing furostanol or spirostanol glycosides from the XAD-2 column were collected and purified via preparative HPLC. A Hitachi L-7100 HPLC pump (Hitachi Instruments Inc., Tokyo, Japan) with a Hitachi L-7420 UV−vis detector was employed. Reversed-phase chromatographic separation was carried out on a preparative 250 mm × 20 mm i.d., 5 μm, Cosmogel C18 column (Nacalai Tesque Inc., Kyoto, Japan), the Colbox column oven (Hipoint Scientific Co., Kaohsiung, Taiwan) was set at 45 °C, and isocratic separation was performed using a mixture of MeOH/H_2O (69:31, v/v) to produce compounds **1** (29 mg), **2** (30 mg), and **3** (28 mg) or using MeOH/H_2O (79:21, v/v) to yield compounds **4** (11 mg), **5** (16 mg), and **6** (12 mg) at a flow rate of 5 mL/min with 203 nm detection. A CHEM-WIN computer software system (Shuen-Hua Co., Taipei, Taiwan) was used for data processing.

Identification of Steroid Saponins 1—6. Purified steroidal saponins (compounds **1**−**6**) were determined by MS and NMR. The mass spectra were performed on a VG Platform II LC-MS (Micromass Co., Cheshire, U.K.) operated in the ESI positive ion mode. The cone voltage was set at 40 eV and the source temperature at 200 °C. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR, including two-dimensional NMR [correlated spectroscopy (COSY), nuclear Overhauser spectroscopy (NOESY), heteronuclear multiple bond coherence (HMBC), and heteronuclear multiple-quantum coherence (HMQC)], spectra were taken on a Bruker DMX-500 MHz FT-NMR (Bruker Co., Karlsruhe, Germany). Melting points were measured on a Yanaco MP-S3 micromelting point apparatus (Yanaco Analytical Instruments Co., Kyoto, Japan). Optical rotations were obtained on a Jasco DIP-181 polarimeter (Jasco International Co. Ltd., Tokyo, Japan).

*Compound **1**:* amorphous solid; $[\alpha]_D^{16}$ −86.4° (MeOH; *c* 0.05); mp 189−190 °C (dec); ESI$^+$-MS, *m*/*z* 1231 $[M + Na]^+$, 1215 $[M - CH_3OH + K]^+$, 1177 $[(M + H) - CH_3OH]^+$, 1031 $[(M + H) - CH_3OH - Rha]^+$, 885 $[(M + H) - CH_3OH - 2 \times Rha]^+$, 869 $[(M + H) - CH_3OH - Glc - Rha]^+$, 739 $[(M + H) - CH_3OH - 3 \times Rha]^+$, 723 $[(M + H) - CH_3OH - Glc - 2 \times Rha]^+$, 577 $[(M + H) - CH_3OH - Glc\text{-}3 \times Rha]^+$, 415 $[(M - H) - CH_3OH - 2 \times Glc\text{-}3 \times Rha]^+$; ^{1}H NMR (500 MHz, pyridine-d_5) for the aglycon moiety δ 0.83 (3H, s, CH_3-18), 1.01 (3H, d, J = 6.6 Hz, CH_3-27), 1.06 (3H, s, CH_3-19), 1.20 (3H, d, J = 6.8 Hz, CH_3-21), 3.29 (3H, s, OCH_3 at C-22), 3.89 (1H, m, H-3), 5.34 (1H, br d, J = 5.3 Hz, H-6); and for the sugar moiety δ 1.60 [3H, d, J = 5.0, Hz, CH_3-6‴ of the inner rhamnose attached to the C-4′ of the inner glucose], 1.61 [3H, d, J = 5.5 Hz, CH_3-6⁗ of the terminal rhamnose attached to the C-4‴ of the inner rhamnose], 1.78 [3H, d, J = 6.1 Hz, CH_3-6″ of the terminal rhamnose attached to the C-2′ of the inner glucose], 4.86 (1H, d, J = 7.7 Hz, H-1⁗ of the glucose attached to C-26), 4.96 (1H, d, J = 7.2 Hz, H-1′ of the inner glucose attached to C-3), 5.85 [1H, br s, H-1‴ of the inner rhamnose attached to the C-4′ of the inner glucose], 6.31 [1H, br s, H-1⁗ of the terminal rhamnose attached to the C-4‴ of the inner rhamnose], 6.42 [1H, br s, H-1″ of the terminal rhamnose attached to the C-2′ of the inner glucose]. ^{13}C NMR data (125 MHz, pyridine-d_5) are shown in **Table 1**.

*Compound **2***, 26-*O*-*β*-D-glucopyranosyl-22α-methoxyl-(25*R*)-furost-5-en-3*β*,26-diol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside (methyl protodioscin): amorphous solid; ESI$^+$-MS and ^{1}H and ^{13}C NMR data are consistent with the literature (*6*).

*Compound **3***, 26-*O*-*β*-D-glucopyranosyl-22α-methoxyl-(25*R*)-furost-5-en-3*β*,26-diol 3-*O*-α-L-rhamnopyranosy-(1→2)-*O*-[*β*-D-glucopyranosyl (1→3)]-*β*-D-glucopyranoside (methyl protogracillin): amorphous solid; ESI$^+$-MS and ^{1}H and ^{13}C NMR data are consistent with the literature (*6*).

*Compound **4***: amorphous solid; $[\alpha]_D^{16}$ −104.7° (MeOH; *c* 0.05); mp 243−245 °C (dec); ESI$^+$-MS, *m*/*z* 1053 $[M + K]^+$, 1037 $[M + Na]^+$, 1015 $[M + H]^+$, 869 $[(M + H) - Rha]^+$, 723 $[(M + H) - 2 \times Rha]^+$, 577 $[(M + H) - 3 \times Rha]^+$, 415 $[(M + H) - Glc - 3 \times Rha]^+$; ^{1}H (500 MHz, pyridine-d_5) for the aglycon moiety δ 0.71 (3H, d, J = 5.3 Hz, CH_3-27), 0.85 (3H, s, CH_3-18), 1.06 (3H, s, CH_3-19), 1.15 (3H, d, J = 6.9 Hz, CH_3-21), 3.89 (1H, m, H-3), 5.34 (1H, br d, J = 5.3 Hz, H-6); and for the sugar moiety δ 1.60 [3H, d, J = 5.0 Hz, CH_3-6‴ of the inner rhamnose attached to the C-4′ of the inner glucose], 1.61 [3H, d, J = 5.5 Hz, CH_3-6⁗ of the terminal rhamnose attached to the C-4‴ of the inner rhamnose], 1.79 [3H, d, J = 6.1 Hz, CH_3-6″ of the terminal rhamnose attached to the C-2′ of the inner glucose], 4.96 (1H, d, J = 7.3 Hz, H-1′ of the inner glucose attached to C-3), 5.85 [1H, br s, H-1‴ of the inner rhamnose attached to the C-4′ of the inner glucose], 6.31 [1H, br s, H-1⁗ of the terminal rhamnose attached

Table 1. ^{13}C NMR (500 MHz) Data (δ) for Yam Saponins (in Pyridine-d_5)

		compd			compd	
carbon	diosgenin	**1**	**4**	carbon	**1**	**4**
1	38.3	38.0	38.0	3-O-Glc (inner)		
2	33.0	30.5	30.5	1′	100.8	100.8
3	71.7	78.6	78.6	2′	78.2	78.2
4	43.9	39.4	39.4	3′	78.5	78.4
5	142.4	141.3	141.3	4′	79.1	79.2
6	121.1	122.3	122.3	5′	77.5	77.5
7	32.8	32.8	32.8	6′	61.7	61.7
8	32.2	32.1	32.1	Rha (1 → 2)		
9	50.9	50.8	50.8	1″	102.7	102.7
10	37.5	37.6	37.6	2″	73.0	72.8
11	21.7	21.5	21.6	3″	73.3	73.3
12	40.4	40.2	40.3	4″	74.6	74.6
13	40.9	41.3	40.9	5″	70.1	70.1
14	57.2	57.1	57.1	6″	18.9	18.9
15	32.7	32.6	32.7	Rha (1 → 4)		
16	81.6	81.8	81.6	1‴	102.7	102.7
17	63.4	64.6	63.3	2‴	73.3	73.3
18	16.9	16.7	16.8	3‴	73.3	73.3
19	20.1	19.9	19.9	4‴	80.9	80.8
20	42.4	41.0	42.4	5‴	70.9	70.9
21	15.5	16.7	15.5	6‴	19.1	19.1
22	109.7	113.2	109.8	Rha (1 → 4)		
23	32.3	31.3	32.3	1⁗	103.8	103.8
24	29.7	28.6	29.7	2⁗	73.0	72.9
25	31.1	34.7	31.1	3⁗	73.3	73.3
26	67.3	75.7	67.3	4⁗	74.5	74.3
27	17.8	17.6	17.8	5⁗	70.1	70.1
C_{22}-OCH_3		47.8		6⁗	19.3	19.3
				26-O-Glc		
				1⁗′	105.4	
				2⁗′	75.7	
				3⁗′	78.6	
				4⁗′	72.2	
				5⁗′	79.0	
				6⁗′	63.3	

to the C-4‴ of the inner rhamnose], 6.41 [1H, br s, H-1″ of the terminal rhamnose attached to the C-2′ of the inner glucose]. ^{13}C NMR data (125 MHz, pyridine-d_5) are shown in **Table 1.**

Compound ***5***, (25*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside (dioscin): amorphous solid; ESI^+-MS and ^1H and ^{13}C NMR data are consistent with the literature (*5*, *37*).

Compound ***6***, (25*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-β-D-glucopyranoside (gracillin): amorphous solid; ESI^+-MS and ^1H and ^{13}C NMR data are consistent with the literature (*5*, *37*).

Acid Hydrolysis. The method was based on that reported by Taylor et al. (*28*). Each compound (1 mg) was added to 2 mL of 2 N HCl in MeOH and heated in a sealed tube at 80 °C for 2 h. After hydrolysis, neutralization with 4 N aqueous NaOH, and evaporation of the alcohol at 35 °C in a rotary evaporator, the residues were extracted three times with 2 mL of hexane for 5 min each time. The aqueous fraction was diluted with water and was used for sugar composition analysis. The sugar composition was determined by high-performance anion-exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). The system was a Bioscan 817 Metrohm IC system (Metrohm, Herisau, Switzerland) including an IC pump 709, an injection valve unit 812 with a 20 μL loop, and an electrochemical detector (gold working electrode: $E_1 = 0.05$ V, 0.44 s; $E_2 = 0.80$ V, 0.18 s; $E_3 = -0.30$ V, 0.36 s). A 250 mm × 4 mm i.d. CarboPac PA1 column (Dionex, Sunnyvale, CA) was used, and isocratic elution was performed at 1 mL/min with 10 mM NaOH containing 2 mM $Ba(OAc)_2$ (*29*). The retention times (t_R) of rhamnose and glucose were 5.30 and 6.75 min, respectively. The hexane extracts were combined and concentrated to dryness by evaporating the solvent in the rotary evaporator and then dissolved in 1 mL of methanol. The analysis of diosgenin was performed with a Hitachi L-7100 HPLC pump (Hitachi Instruments Inc., Tokyo, Japan) with a 250 mm × 4.6 mm i.d., 5 μm, Luna C18 column (Phenomenex, Torrance, CA); the mobile phase was an isocratic elution using a binary solvent system consisting of acetonitrile and deionized water (95:5, v/v) at a flow rate of 1 mL/min. This method is based on that reported by Oncina et al. (*30*). An ELSD 2000 evaporative light-scattering detector (Alltech Associates Inc., Deerfield, IL) at a tube temperature of 80 °C and air flow rate of 2.5 L/min was used to detect diosgenin ($t_R = 12.05$ min). After acid hydrolysis, the conversions from steroidal saponins to diosgenin were subjected to analysis of variance and Duncan's multiple-range test procedures for statistical analysis. All analyses were carried out in triplicate, and the mean values were determined.

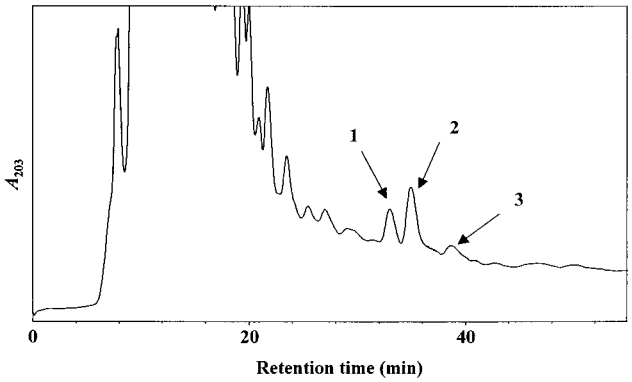

Figure 1. Separation of furostanol glycosides by RP-HPLC. The sample was the fraction obtained from XAD-2 column chromatographic separation of the methanol extract of *D. pseudojaponica* Yamamoto.

RESULTS AND DISCUSSION

Fractionation and Purification of Steroidal Saponins in Yam. The steroidal saponins in yams are usually extracted with MeOH or EtOH and isolated by repeated silica gel column chromatography (*7*, *9*, *10*) or solvent fractionation followed by silica gel column chromatography and preparative HPLC (*5*, *6*, *23*). Silica gel column chromatography for steroidal saponin separation needs a complicated solvent system to achieve good resolution. Recently, XAD-2, a hydrophobic aromatic resin, has been used as a column-packing material for the fractionation of steroidal saponins from starfishes (*24*, *25*, *31*, *32*).

In our study of Taiwanese yam, the steroidal saponins were extracted with MeOH and fractionated using XAD-2 column chromatography. The impurities in the saponin extract were first removed by eluting the XAD-2 column with 60% MeOH after the extract had been loaded on the column. A simple gradient elution solvent system using a MeOH/H_2O mixture was developed in this study. Each fraction was monitored by TLC to determine the presence of furostanol glycosides and spirostanol glycosides. The furostanol glycoside fraction, eluted by 60−77% MeOH, showed a bright red color on the TLC plate after spraying with Ehrlich reagent and a yellow-green color after spraying with 10% sulfuric acid in MeOH. The R_f values were ∼0.44. The spirostanol glycosides fraction, eluted by 83−97% MeOH, showed a yellow-green color after spraying with 10% sulfuric acid in MeOH and had R_f values of ∼0.67.

The fractions containing furostanol glycosides or spirostanol glycosides were further separated and purified by RP-HPLC. As shown in **Figure 1**, the furostanol glycoside fraction could be resolved into three peaks by isocratic elution with 69% aqueous MeOH. Three spirostanol glycoside peaks were found in the mixture of spirostanol glycoside fraction when separated by a mobile phase composed of 79% aqueous MeOH (**Figure 2**). The presence of furostanol or spirostanol glycoside in each fraction was determined by TLC. The neighboring peaks of

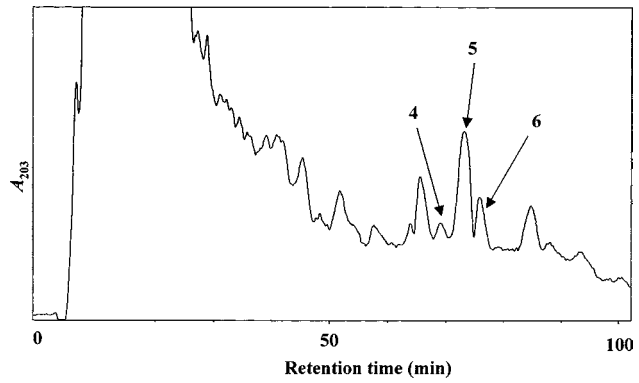

Figure 2. Separation of spirostanol glycosides by RP-HPLC. The sample was the fraction obtained from XAD-2 column chromatographic separation of the methanol extract of *D. pseudojaponica* Yamamoto.

steroidal glycosides on preparative chromatograms were also collected for the TLC test. None of them produced a positive reaction.

Identification of the Purified Furostanol Glycosides and Spirostanol Glycosides. The purified furostanol and spirostanol glycosides were identified by ESI$^+$-MS and ^{1}H and ^{13}C NMR including 2D-NMR (COSY, NOESY, HMBC, and HMQC); moreover, sugar and diosgenin analysis after acid hydrolysis was conducted to help identify the structure.

Compound **1** was isolated as an amorphous solid. The molecular formula $C_{58}O_{26}H_{96}$ was deduced by the ESI$^+$-MS spectrum: $[M + Na]^+$ and $[(M + H) - CH_3OH]^+$ peaks at *m*/*z* 1231 and 1177, suggesting that the molecular weight of compound **1** could be 1208. Other significant peaks at 1031 $[(M + H) - CH_3OH - 146]^+$, 885 $[(M + H) - CH_3OH - 2 \times 146]^+$, 869 $[(M + H) - CH_3OH - 146 - 162]^+$, 739 $[(M + H) - CH_3OH - 3 \times 146]^+$, 723 $[(M + H) - CH_3OH - 2 \times 146 - 162]^+$, 577 $[(M + H) - CH_3OH - 3 \times 146 - 162]^+$, and 415 $[(M + H) - CH_3OH - 3 \times 146 - 2 \times 162]^+$ correspond to the losses of a methoxy group, one to three deoxyhexoses, and one to two hexoses. ^{1}H NMR data of compound **1** revealed the existence of two tertiary methyl groups [δ 0.83, 1.06 (s)] and two secondary methyl groups [δ 1.01 (d, $J = 6.6$ Hz), 1.20 (d, $J = 6.8$ Hz)] corresponding to the angular methyl groups of a steroidal sapogenin. The signal corresponding to an olefinic proton at δ 5.34 (br d, $J = 5.3$ Hz) could be assigned to 5,6-unsaturation. A signal at δ 3.28 (s) was attributed to a methoxyl group. As for the sugar moiety, there existed five anomeric hydrogen signals [δ 4.86 (d, $J = 7.7$ Hz), 4.96 (d, $J = 7.2$ Hz), 5.85 (br s), 6.31 (br s), 6.42 (br s)], which revealed the presence of five momosaccharides. The signals of the three secondary methyl groups [δ 1.60 (d, $J = 5.0$ Hz), 1.61 (d, $J = 5.5$ Hz), 1.78 (d, $J = 6.1$ Hz)] might arise from the three deoxyhexoses. These results were supported by the ^{13}C NMR data of compound **1** (**Table 1**): the signals of the angular methyl groups (δ 16.7, 16.7, 17.6, 19.9) corresponding to the structure of steroidal sapogenin, the signals at δ 47.8 indicating the existence of a methoxyl group, and the olefinic carbons on sapogenin were proven by the signals at δ 122.3 and 141.3. ^{13}C NMR data also support the existence of anomeric carbons (δ 100.8, 102.7, 102.7, 103.8, 105.4), three methyl groups (δ 18.9, 19.1, 19.3) from the three deoxyhexoses on the sugar moiety, and an acetalic quaternary carbon (δ 113.2), which is characteristic for a furostane skeleton possessing a methoxyl group at C-22 in the 26-*O*-glycosidic form (*6*). From the results of MS and ^{1}H and ^{13}C NMR, it is suggested that compound **1** is a methyl furostene pentaoligoside.

From a comparison of the ^{1}H NMR and ^{13}C NMR data (**Table 1**) of the aglycon moiety of compound **1** with those described in the literature (*6*, *33*), it is clear that the aglycon of compound **1** is 22α-methoxyl-(25*R*)-furost-5-en-3β,26-diol. A comparison of the ^{13}C NMR signals and the anomeric protons on ^{1}H NMR of the sugar moiety of compound **1** with those described in the literature (*7*, *34*, *35*) suggested the presence of a terminal β-D-glucose, a 2,4-substituted β-D-glucose, a 4-substituted α-L-rhamnose, and two terminal α-L-rhamnoses. The α anomeric configuration for rhamnose was judged by its C_5 data (δ 70.1, 70.1, 70.9). Evidence supporting the β anomeric configuration for glucose was provided by the large J_{H1-H2} value (7.2 Hz, 7.7 Hz) (>7.0 Hz).

In the HMBC spectrum of compound **1**, the anomeric proton signals at δ 4.96 (H-1′ of the 2, 4-substituted glucose), 6.42 (H-1″ of the terminal rhamnose), 5.85 (H-1‴ of the 4-substituted rhamnose), 6.31 (H-1⁗ of the terminal rhamnose), and 4.86 (H-1⁗′ of the terminal glucose) showed cross-peaks with the carbon signals at δ 78.6 (C-3 of the aglycon), 78.2 (C-2′ of the 2,4-substituted glucose), 79.1 (C-4′ of the 2,4-substituted glucose), 80.9 (C-4‴ of the 4-substituted rhamnose), and 75.7 (C-26 of the aglycon), respectively (**Figure 3**). Accordingly, the structure of compound **1** was identified as 26-*O*-β-D-glucopyranosyl-22α-methoxyl-(25*R*)-furost-5-en-3β,26-diol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-{[α-L-rhamnopyranosyl-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]}-β-D-glucopyranoside. Compound **1** has not been found in yam species yet. Its existence in *Trachycarpus fortunei* (Hook.) H. Wendl. was reported by Hirai et al. (*36*), however.

Compound **4** was isolated as an amorphous solid. The molecular formula $C_{51}O_{20}H_{82}$ was elucidated from the ESI$^+$-MS spectrum: *m*/*z* at 1037 and 1015 correspond to $[M + Na]^+$ and $[M + H]^+$ peaks, suggesting that the molecular weight of compound **4** might be 1014. Other significant peaks at 869 $[(M + H) - 146]^+$, 723 $[(M + H) - 2 \times 146]^+$, 577 $[(M + H) - 3 \times 146]^+$, and 415 $[(M + H) - 3 \times 146 - 162]^+$ correspond to the losses of one to three deoxyhexoses and one hexose. ^{1}H NMR data of compound **4** showed diagnostic signals of two tertiary methyl groups [δ 0.85, 1.06 (s)] and two secondary methyl groups [δ 0.71 (d, $J = 5.3$ Hz), 1.15 (d, $J = 6.8$ Hz)] corresponding to the angular methyl groups of a steroidal sapogenin. The signal corresponding to an olefinic proton at δ 5.34 (br d, $J = 5.3$ Hz) could be attributed to 5,6-unsaturation. As for the sugar moiety, the four anomeric hydrogen signals [δ 4.96 (d, $J = 7.3$ Hz), 5.85 (br s), 6.31 (br s), 6.41 (br s)] suggested the existence of four monosaccharides. The signals of the three secondary methyl groups [δ 1.60 (d, $J = 5.0$ Hz), 1.61 (d, $J = 5.5$ Hz), 1.78 (d, $J = 6.1$ Hz)] were assigned to the three deoxyhexoses. This evidence was further supported by the ^{13}C NMR data of compound **4** (**Table 1**): the signals of the angular methyl groups (δ 16.7, 16.7, 17.6, 19.9) corresponding to a steroidal sapogenin; the signals of olefinic carbons (δ 122.3, 141.3) also supported the structure of the aglycon. ^{13}C NMR data gave further evidence of the structure of the sugar moiety: anomeric carbons (δ 100.8, 102.7, 102.7, 103.8) and three methyl groups (δ 18.9, 19.1, 19.3) from the three deoxyhexoses. The ^{13}C NMR signal at δ 109.8 corresponded to a spiroketal carbon. All of the above spectral data suggested compound **4** to be a spirostene tetraoligoside.

From a comparison of the ^{1}H NMR and ^{13}C NMR data of the aglycon moiety of compound **4** (**Table 1**) with diosgenin and those described in the literature (*5*, *9*, *37*), the structure of the aglycon was confirmed to be (25*R*)-spirost-5-en-3β-ol (diosgenin). The numbers, varieties, and bonding forms of the

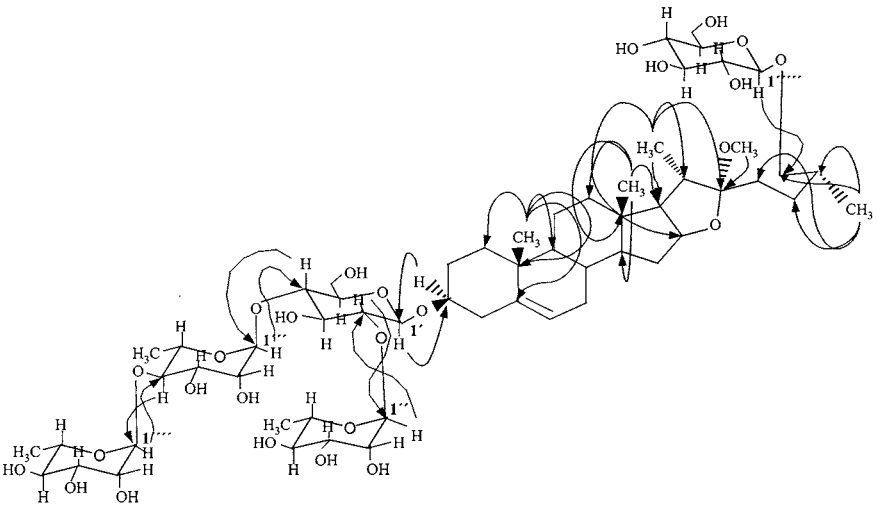

Figure 3. HMBC analysis for compound **1**.

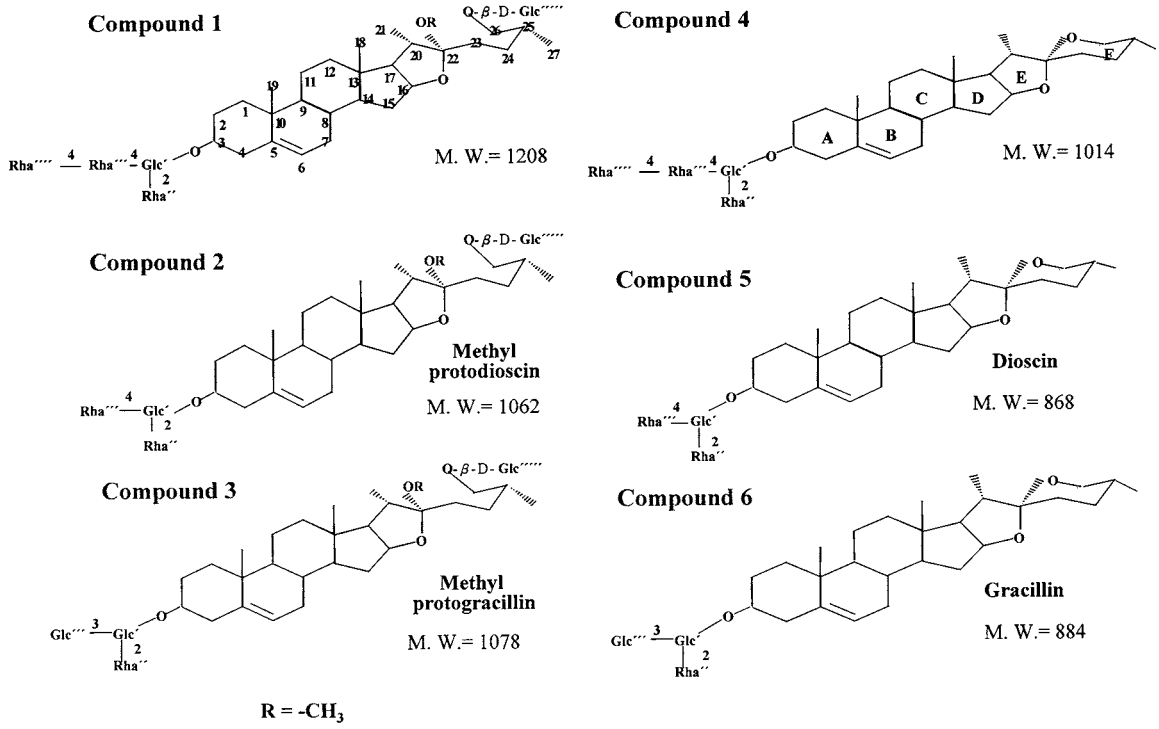

Figure 4. Structures of furostanol and spirostanol glycosides prepared from yam tubers (*D. pseudojaponica* Yamamoto).

carbohydrate groups at C3 on compound **4** were the same as on compound **1** (**Table 1**). Thus, compound **4** was identified as (25*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl -(1→2)-*O*-{[α-L-rhamnopyranosy-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]}-β-D-glucopyranoside. The presence of compound **4** in yam species has also not been reported before. It has been isolated from *Paris polyphylla* Sm. var. *Yunnanensis* (*34*).

The spectroscopic data for compound **2** were consistent with methyl protodiosin, and those for compound **3** were consistent with methyl protogracillin. The spectroscopic data for compounds **5** and **6** were consistent with dioscin and gracillin, respectively. Diosin and gracillin are the two most important yam saponins and have been isolated from various species of yam, such as *D. floribunda* (*7*), *D. composita* (*9*), *Dracaena cambodiana* (*37*), and *D. colletti* var. *hypoglauca* (*5*). Both compounds **2** and **3** have been isolated from *D. colletti* var. *hypoglauca* (*6*). However, both compounds **1** and **4** were isolated and identified for the first time from yams (*Dioscorea* spp.). **Figure 4** shows the structures of the six steroidal saponins isolated from *D. pseudojaponica* Yamamoto.

Acid Hydrolysis. After acid hydrolysis, all of the steroidal glycosides could be converted to diosgenin, glucose, and rhamnose, the ratios of glucose and rhamnose being 2:3, 1:1, 3:1, 1:3, 1:2, and 2:1 for compounds **1**−**6**, respectively, confirming the steroid glycosides structures as identified above. Drapeau et al. (*38*) indicated that *Dioscorea deltoidea* cell cultures contained furostanol and spirostanol saponins, which, upon acid hydrolysis, lose their sugar moieties to become sapogenins, principally diosgenin. Diosgenin is a principal raw material for the industrial production of steroidal drugs. We therefore determined the conversion efficiency from each steroidal glycoside to diosgenin after further acid hydrolysis. **Table 2** shows that after acidic hydrolysis, the conversions from furostanol and spirostanol glycosides to diosgenin were 67.14−68.11 and 89.72−90.44%, respectively. The yield of diosgenin was lower for furostanol glycosides. The conversion of spirostanol

Table 2. Conversion from Each Steroidal Saponin to Diosgenin after Methanolic HCl Hydrolysis

compd	MW	amount of saponin added (mg)	saponins added (μmol)	diosgenin conversion[a] (μmol)	conversion[a,b] (%)
1	1208	1	0.828	0.556 ± 0.017	67.1 ± 2.1a
2	1062	1	0.942	0.641 ± 0.013	68.1 ± 1.4a
3	1078	1	0.928	0.629 ± 0.020	67.8 ± 2.2a
4	1014	1	0.986	0.885 ± 0.012	89.7 ± 2.0b
5	868	1	1.152	1.042 ± 0.009	90.4 ± 1.4b
6	884	1	1.131	1.019 ± 0.013	90.1 ± 1.9b

[a] All values are mean ± SD obtained by three replicate analyses. The molecular weight (MW) of diosgenin is 414. [b] Values bearing different letters are significantly different ($p < 0.05$).

saponins to diosgenin involves only detachment of the sugar moiety at the C-3 position, whereas the conversion of furostanol saponins also involves separation of the sugar moiety at the C-26 position and cyclization of the F-ring (*38*).

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