Steroidal Glycosides from the Rhizomes of *Dioscorea spongiosa*

Jun Yin,[†] Kyoji Kouda,[†] Yasuhiro Tezuka,[†] Quan Le Tran,[†] Tatsuro Miyahara,[§] Yingjie Chen,[‡] and Shigetoshi Kadota*[,†]

Institute of Natural Medicine and Faculty of Pharmaceutical Science, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan, and Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110015, People's Republic of China

Received December 20, 2002

A water extract of the rhizomes of *Dioscorea spongiosa*, which showed antiosteoporotic activity, was examined, and four new pregnane glycosides, named spongipregnolosides A−D (**1**−**4**), and two new cholestane glycosides, named spongiosides A (**5**) and B (**6**), were isolated together with 15 known glycosides. Their structures were determined on the basis of spectroscopic analysis and chemical methods. Among the isolated compounds, spongioside A (**5**), hypoglaucin G (**7**), methylprotodioscin (**8**), and (*R*)-oct-1-en-3-yl *O*-α-L-arabinopyranosyl-(1→6)-α-D-glucopyranoside (**9**) showed potent inhibition against bone resorption induced by parathyroid hormone in a bone organ culture system.

Osteoporosis, which has been defined as a "state of low bone mass", is one of the major problems in our aging society.[1] In Chinese traditional medicine, many natural crude drugs have been used to treat bone diseases, but only a small amount of laboratory work on their active constituents has been reported.[2−4]

In a search for natural crude drugs having antiosteoporotic activity, we screened 30 plants used in Chinese traditional medicine for tonifying the kidneys and strengthening the bone, both for stimulatory activity on the proliferation of osteoblasts and for inhibitory activity on the formation of osteoclasts.[5] Among the crude drugs investigated, the water extract of rhizomes of *Dioscorea spongiosa* J. Q. Xi, M. Mizuno et W. L. Zhao (Dioscoreaceae) showed both activities. This plant is distributed in the southern part of People's Republic of China and is used for the treatment of rheumatism and for urethral and renal infections in Chinese traditional medicine.[6] Earlier chemical studies on this plant have resulted in the isolation of steroidal sapogenins (tokorogenin, kogagenin, yamogenin) and steroidal saponins (dioscin, gracillin, yononin, tokoronin, furost-5-en-3,22,26-triol 3,26-di-*O*-glycoside).[7,8] Moreover, its antiatherosclerosis activity was also reported.[9] In the present work, bioactivity-guided fractionation of the water extract of *D. spongiosa* led to the isolation of six new steroidal glycosides, named spongipregnolosides A−D (**1**−**4**) and spongiosides A (**5**) and B (**6**), together with 15 known compounds. Herein we report the isolation and characterization of the new compounds and the inhibitory activity of all isolated compounds on bone resorption induced by parathyroid hormone (PTH) in a bone organ culture model.

Results and Discussion

The water extract of rhizomes of *D. spongiosa* showed potent stimulatory activity (67%) on the proliferation of an osteoblast-like UMR106 cell line at a concentration of 400 μg/mL[5] and the complete inhibition of the formation of osteoclast-like multinuclear cells at a concentration of 200 μg/mL. Accordingly, the water extract was chromatographed over a Diaion HP-20 column, using a H_2O−EtOH solvent system, to give four (H_2O, 30%, 60%, and 90% EtOH−H_2O) fractions. They showed 11.2, 14.7, 86.7, and 89.5% stimulation of the proliferation of the osteoblast-like UMR106 cell line at a concentration of 200 μg/mL, and all showed 100% inhibition of the formation of osteoclast-like multinuclear cells at the same concentration. On the other hand, only the 90% EtOH−H_2O fraction inhibited the bone resorption induced by PTH in the bone organ culture system at a concentration of 440 μg/mL (82.8% inhibition). Thus, this fraction was further separated by a combination of normal- and reversed-phase column chromatography and preparative TLC, to afford four new pregnane glycosides, named spongipregnolosides A−D (**1**−**4**), and two cholestane glycosides, named spongiosides A (**5**) and B (**6**), together with 15 known glycosides.

Negative-ion HRFABMS of spongipregnoloside A (**1**) displayed a quasimolecular ion at *m*/*z* 621.3304, indicating a molecular formula of $C_{33}H_{50}O_{11}$. The ^{1}H NMR spectrum of **1** showed signals ascribable to three tertiary methyls (δ_H 0.93, 1.04, 2.22), two olefinic protons (δ_H 5.37, 6.59), a characteristic doublet of a 6-deoxyhexopyranosyl group (δ_H 1.71, d, J = 6.4 Hz), and two anomeric protons (δ_H 4.89, 6.31). Moreover its ^{13}C NMR spectrum showed 33 carbon signals including four primary, eight secondary, 16 tertiary, and five quatenary carbons (Table 1), suggesting **1** to be a steroidal diglycoside. Analysis of the COSY and HMQC spectra, together with the molecular formula, suggested **1** to be a pregnane glycoside, whose aglycon was the same as that of pregnadienolone 3-*O*-*β*-gracillimatriose (**10**) and pregnadienolone 3-*O*-*β*-chacotrioside (**11**).[10] The sugar part of **1** was determined to be a combination of D-glucose and L-rhamnose on the basis of the ^{13}C NMR data and GC analysis of a chiral derivative of the sugars in an acidic hydrolysate.[11] The location of the D-glucose and L-rhamnose was determined to be at C-3 of the aglycon and at C-2 of the D-glucopyranosyl unit, on the basis of a glycosylation shift of C-3 (δ_C 78.1) of the aglycon and C-2 (δ_C 77.8) of the glucose, respectively. These were confirmed by the HMBC correlations between the anomeric proton of the glucopyranosyl unit (δ_H 4.89) and C-3 of the aglycon, and between the anomeric proton of the rhamnopyranosyl unit (δ_H 6.31) and C-2 of the glucopyranosyl unit. The anomeric configuration of D-glucose and L-rhamnose was determined to be *β* and α, respectively, on the basis of the coupling constant

* To whom correspondence should be addressed. Tel: +81-76-434-7625. Fax: +81-76-434-5059. E-mail: kadota@ms.toyama-mpu.ac.jp.
[†] Institute of Natural Medicine, Toyama Medical and Pharmaceutical University.
[§] Faculty of Pharmaceutical Science, Toyama Medical and Pharmaceutical University.
[‡] Shenyang Pharmaceutical University.

10.1021/np0205957 CCC: $25.00
Published on Web 05/03/2003

Chart 1

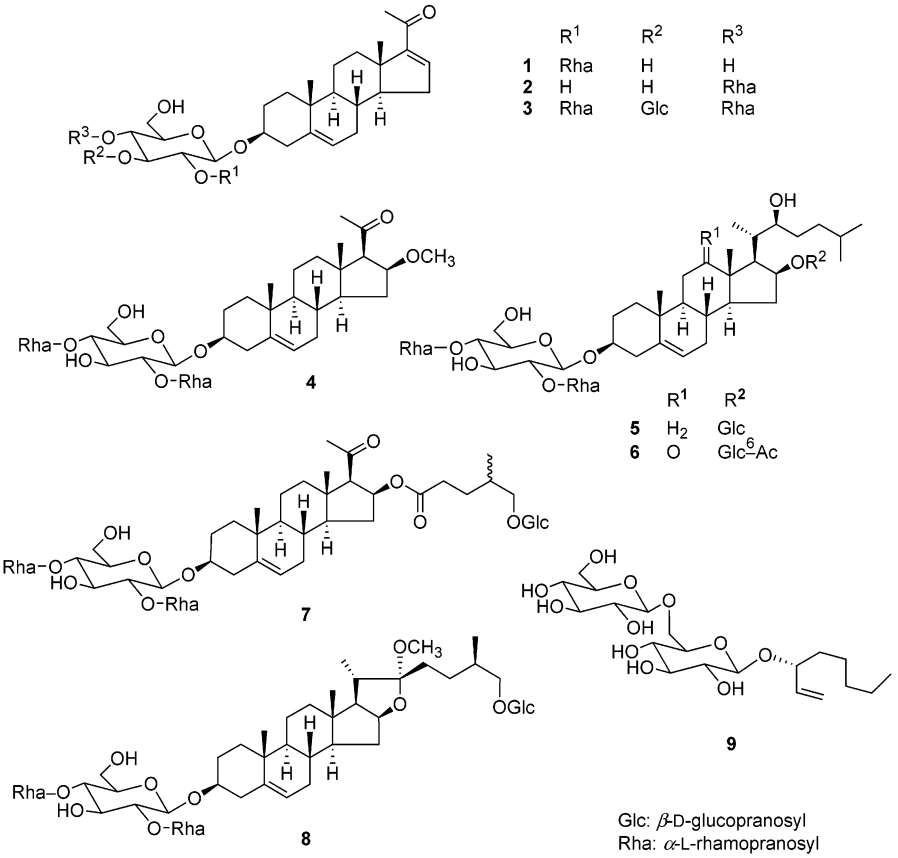

Table 1. ^{13}C NMR Data (δ) of Spongiosides A−F (**1**−**6**) in Pyridine-d_5

position	1	2	3	4	5	6	position		1	2	3	4	5	6
1	37.3	37.4	37.3	37.4	39.8	38.0	glc	1′	100.3	100.4	100.3	100.3	100.3	100.2
2	30.1	30.1	30.1	30.1	29.9	30.1		2′	77.8	75.5	77.7	77.6	77.7	77.5
3	78.1	78.1	78.3	78.0	78.2	77.8		3′	79.5	76.7	89.5	77.7	89.5	77.9
4	38.9	39.0	39.1	39.1	39.1	38.8		4′	71.7	78.1	78.7	78.8	78.7	78.8
5	141.3	141.3	141.2	140.8	141.8	140.6		5′	78.1	77.1	76.9	76.9	76.9	76.8
6	121.4	121.5	121.5	121.6	122.6	121.8		6′	62.6	61.5	61.3	61.4	61.3	61.3
7	31.8	31.8	31.8	31.6	31.8	31.8								
8	30.3	30.4	30.3	31.5	31.2	32.1	rha	1″	101.9		102.0	102.0	102.0	101.9
9	50.7	50.8	50.7	50.1	50.1	54.7		2″	72.4		72.4	72.7	72.5	72.5
10	37.1	37.2	37.1	37.0	37.0	38.1		3″	72.7		72.8	74.1	72.8	72.7
11	20.8	20.9	20.9	20.9	21.1	37.2		4″	74.0		74.1	74.0	74.1	74.1
12	35.0	35.1	35.1	38.7	37.1	213.9		5″	69.3		69.5	69.5	69.5	69.3
13	46.2	46.3	46.3	39.0	42.4	57.2		6″	18.5		18.6	18.6	18.6	18.6
14	56.4	56.5	56.4	44.4	55.2	57.3								
15	32.3	32.3	32.3	32.2	32.1	37.3	glc	1‴			104.5		107.0	106.9
16	144.3	144.6	144.7	81.8	82.7	82.1		2‴			75.0		75.6	71.6
17	155.2	155.2	155.2	54.6	57.6	49.3		3‴			77.8		78.7	77.9
18	15.9	15.9	15.9	14.5	13.5	13.3		4‴			69.6		71.7	75.1
19	19.2	19.3	19.2	19.4	19.4	19.0		5‴			78.0		78.1	75.3
20	196.2	196.2	196.2	207.5	35.9	35.3		6‴			62.5		62.9	64.6
21	27.0	27.1	27.1	31.6	12.6	13.3		6‴-Ac						20.9
22					73.1	72.9								172.1
23					33.9	33.9	rha	1⁗		102.7	102.9	102.7	102.9	102.9
24					36.7	36.9		2⁗		72.6	72.5	72.8	72.5	72.5
25					28.9	28.9		3⁗		72.8	72.7	73.9	72.7	72.6
26					23.0	23.1		4⁗		74.0	73.9	74.0	73.9	73.8
27					23.1	23.1		5⁗		70.4	70.4	70.4	70.4	70.4
OMe				57.0				6⁗		18.5	18.5	18.5	18.5	18.5

of the anomeric proton in glucose (7.5 Hz) and the chemical shift for the anomeric proton in rhamnose (δ_H 6.31).[12] From these data, spongipregnoloside A was determined to be 3β-[(*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl)oxy]-pregna-5,16-dien-20-one (**1**).

The negative-ion HRFABMS data of spongipregnoloside B (**2**) indicated the same molecular formula, $C_{33}H_{50}O_{11}$, as that of **1**. The ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1**, indicating **2** also to be a pregnane diglycoside. The ^{13}C NMR data and GC analysis of the acid hydrolysate of **2** showed that **2** had the same sugar composition as **1**. The α-L-rhamnose unit (anomeric proton: δ_H 5.89) was located at C-4 of the β-D-glucose unit (anomeric proton: J = 7.5 Hz), instead of at C-2 of that in

1, which was confirmed by the HMBC spectrum. Thus, spongipregnoloside B was determined to be 3*β*-[(*O*-α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl)oxy]pregna-5,-16-dien-20-one (**2**).

The molecular formula of spongipregnoloside C (**3**) was determined by negative-ion HRFABMS to be $C_{45}H_{70}O_{20}$. The ^{1}H and ^{13}C NMR spectra of **3** indicated it also to be a pregnane glycoside. The NMR data for the sugar moiety of **3** and GC analysis of chiral derivatives of the sugars in the acidic hydrolysate of **3** revealed the presence of two *β*-D-glucose units and two α-L-rhamnose units. The connectivity of the sugars was determined on the basis of the HMBC correlations of the anomeric proton of the inner glucose (δ_H 4.96) with C-3 of the aglycon (δ_C 78.3), of the anomeric proton of the outer glucose (δ_H 5.12) with C-3 of the inner glucose (δ_C 89.5), and of the anomeric protons of two rhamnoses with C-2 and C-4 of the inner glucose. Thus, the structure of spongipregnoloside C was concluded to be 3*β*-[(*O*-*β*-D-glucopyranosyl-(1→3)-*O*-[α-L-rhamnopyranosyl-(1→2)]-*O*-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranosyl)oxy]pregna-5,16-dien-20-one (**3**).

The molecular formula of spongipregnoloside D (**4**) was determined by negative-ion HRFABMS to be $C_{40}H_{64}O_{16}$. Its ^{1}H and ^{13}C NMR spectra were similar to those of **11**, but they showed the absence of the olefinic group at C-16 and the presence of a methine (δ_H 2.66, δ_C 54.6), an oxymethine (δ_H 4.53, δ_C 81.8), and a methoxyl group (δ_H 3.23, δ_C 57.0). These data suggested that **4** should have a methoxyl group at C-16, which was confirmed by the HMBC correlations. The α-orientation of H-16 was established by the ROESY correlations between H-16 and H-17, between the methoxyl protons and H_3-21, and between the methoxyl protons and H_3-18. Thus, the structure of spongipregnoloside D was determined to be 16*β*-methoxy-3*β*-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranosyl)oxy]pregna-5,16-dien-20-one (**4**).

Negative-ion HRFABMS of spongioside A (**5**) showed a quasimolecular ion at *m*/*z* 1033.5577, corresponding to the molecular formula $C_{51}H_{86}O_{21}$. The ^{1}H NMR spectrum of **5** displayed signals ascribable to two tertiary methyls (δ_H 0.99, 1.05), three secondary methyls (δ_H 0.94, 0.94, 1.20), an olefinic proton (δ_H 5.22), and four anomeric protons (δ_H 4.74, 4.93, 5.83, 6.37), while the ^{13}C NMR spectrum of **5** showed 51 signals (Table 1). Analysis of the COSY and HMQC spectra, together with the molecular formula, suggested **5** to be a cholestane glycoside with oxygen functionalities at C-3 (δ_C 78.2), C-16 (δ_C 82.7), and C-22 (δ_C 73.1). The sugar part of **5** was determined to be composed of two D-glucose units and two L-rhamnose units from the NMR data and GC analysis of chiral derivatives of the sugars in an acidic hydrolysate. In the HMBC spectrum, the anomeric protons of the two glucoses at δ_H 4.93 and 4.74 showed correlations with C-3 and C-16 of the aglycon, respectively, indicating the two glucose units to be located at C-3 and C-16, respectively. The location of the two rhamnose units was indicated to be at C-2 and C-4 of the glucose unit attached at C-3 of the aglycon by the HMBC correlations of the anomeric protons of two rhamnoses at δ_H 6.37 and 5.83 with C-2 and C-4 of the inner glucose, respectively. The α-orientation of H-16 (δ_H 4.49) was established by the ROESY correlation with H-17 (δ_H 1.98) and by the low-field shift of H_3-18 (δ_H 0.99) compared to that of cholesterol (δ_H 0.70).[13] The *S*-configuration of C-20 was also determined by the ROESY correlation between H_3-18 (δ_H 0.99) and H-20 (δ_H 2.55), as reported for 9*β*,19-cyclosterol[14] and (22*S*)-3*β*,11α,22-trihydroxycholesta-5,24-dien-16*β*-yl *O*-α-L-rhamnopyranoside,[15] while the *S*-configuration at C-22 was inferred from an agreement of its ^{1}H and ^{13}C NMR data with those of (22*S*)-16*β*-[(α-L-rhamnopyranosyl(1→3)-*β*-D-galactopyranosyl)oxy]-22-hydroxycholesta-5-en-3*β*-yl *O*-*β*-D-glucopyranoside.[13] Thus, spongioside A was concluded to be (22*S*)-16*β*-[(*β*-D-glucopyranosyl)oxy]-22-hydroxycholest-5-en-3*β*-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside (**5**).

Table 2. Inhibitory Activities of the Glycosides **5** and **7**−**9**[a]

compound	^{45}Ca release (%)	compound[b]	^{45}Ca release (%) 200 μM	^{45}Ca release (%) 20 μM
control	15.4 ± 1.3	**5**	17.1 ± 4.5*	30.9 ± 4.5
PTH	25.3 ± 2.6#	**7**	19.2 ± 2.1*	22.8 ± 7.9
elcitonin	18.4 ± 0.7**	**8**	17.4 ± 1.6**	24.9 ± 1.2
		9	20.7 ± 1.1*	23.8 ± 1.6

[a] Control: bones were cultured without PTH and compound. PTH: bones were cultured with PTH (2×10^{-9} M). Elcitonin: bones were cultured with PTH and elcitonin (2 U/mL). Sample: bones were cultured with PTH (2×10^{-9} M) and test compound. Each datum represents mean ± SE. # $p < 0.01$, significantly different from control. Significant decrease of ^{45}Ca release compared to PTH, * $p < 0.05$, ** $p < 0.01$. [b] Only active compounds are included.

Negative-ion HRFABMS of spongioside B (**6**) showed a quasimolecular ion at *m*/*z* 1089.5433, corresponding to the molecular formula $C_{53}H_{86}O_{23}$. The ^{1}H and ^{13}C NMR spectra of **6** were similar to those of **5**, except for the presence of a ketone carbon (δ_C 213.9) and an acetyl group (δ_H 2.05; δ_C 20.9, 172.1). The ketone carbon was assigned to C-12 by the HMBC correlation with H_3-18 (δ_H 1.31), while the acetyl group was located at C-6 of the glucopyranosyl unit at C-16 of the aglycon, on the basis of the acylation shift of H_2-6 of the glucose (**6**, δ_H 4.86, 2H; **5**, δ_H 4.38, 4.39) and the HMBC correlation with H_2-6 of the glucose. The stereochemistry of **6** was shown to be the same as that of **5** by analysis of the ROESY data. Thus, spongioside B was determined to be (22*S*)-16*β*-[(6-*O*-acetyl-*β*-D-glucopyranosyl)oxy]-22-hydroxy-3*β*-[(*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranosyl)oxy]cholest-5-en-12-one (**6**).

The known compounds were identified by spectroscopic analysis and comparison with published data as hypoglaucin G (**7**),[16] methylprotodioscin (**8**),[17,18] (*R*)-oct-1-en-3-yl *O*-α-L-arabinopyranosyl-(1→6)-*β*-D-glucopyranoside (**9**),[19] pregnadienolone-3-*O*-*β*-gracillimatriose (**10**),[10] pregnadienolone 3-*O*-*β*-chacotrioside (**11**),[10] daucosterol (**12**), dioscin (**13**),[17,18] prosapogenin A (**14**),[20] isonarthogenin-3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-rhamnopyranosyl-(1→4)]-*β*-D-glucopyranoside (**15**),[21] gracillin (**16**),[22] trigofoenoside D-1 (**17**),[23] glycoside D (**18**),[17,18] protogracillin (**19**),[22] dumoside (**20**),[24] and zizyvoside I (**21**).[25] Among them, compounds **9**, **15**, **18**, **20**, and **21** were isolated for the first time from the genus *Dioscorea*.

All compounds isolated were examined for their inhibitory activity on PTH-stimulated bone resorption at concentrations of 200 and 20 μM. At a concentration of 200 μM, compounds **5**, **7**, **8**, and **9** showed inhibitions of 63.6%, 66.7%, 89.9%, and 61.6%, respectively (Table 2).

Experimental Section

General Experimental Procedures. Optical rotations were determined on a JASCO DIP-140 digital polarimeter at 25 °C. NMR spectra were measured on a JEOL JNM-GX400 spectrometer with tetramethylsilane (TMS) as internal standard. HRFABMS were measured on a JEOL JMS-700T mass spectrometer, and glycerol was used as matrix. Diaion HP-20 was purchased from Mitsubishi Chemical Corporation (Tokyo,

Japan). Silica gel column chromatography was performed with Wakogel C-200 (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and ODS column chromatography utilized Cosmosil 75C_{18}-OPN (Nakalai Tesque, Kyoto, Japan). Analytical and preparative TLC were conducted on precoated Merck Kieselgel 60 F_{254} (0.25 and 0.50 mm) or RP-18 F_{254} (0.25 mm) plates.

Plant Material. Rhizomes of *D. spongiosa* were purchased in March 2000 at a market in Shenyang, People's Republic of China, and identified by Professor Qishi Sun (Division of Pharmacognosy, Shenyang Pharmaceutical University). A voucher specimen (SPU 1129) is deposited at the herbarium of Shenyang Pharmaceutical University.

Extraction and Isolation. The rhizomes of *D. spongiosa* (10 kg) were extracted with refluxing water (10 L, 2 h, 3 times), and the extract was concentrated under reduced pressure to give an aqueous extract (3.6 kg). A part of the aqueous extract (3.0 kg) was subjected to chromatographic separation over Diaion HP-20 (10 kg) with H_2O and 30%, 60%, and 90% EtOH−H_2O to give four fractions. The 90% EtOH−H_2O fraction (80 g) was further subjected to column chromatography on Si gel (500 g) with a $CHCl_3$−MeOH (99:1→1:99) gradient system to give seven additional fractions. Fraction 3 (1.2 g) was then chromatographed on Si gel to give three subfractions. Subfraction 2 (426 mg) was separated by a combination of Si gel ($CHCl_3$−MeOH−H_2O, 65:20:1) and ODS (MeOH−H_2O, 2:1) column chromatography to give **1** (8 mg), **10** (6 mg), **11** (110 mg), and **12** (10 mg). Fraction 4 (2.7 g) was subjected to Si gel column chromatography, followed by reversed-phase preparative TLC (MeOH−H_2O, 5:2), to give **2** (5 mg), **3** (4 mg), **5** (4 mg), **13** (160 mg), and **14** (16 mg). Fraction 5 (1.6 g) was also chromatographed over Si gel with $CHCl_3$−MeOH−H_2O (35:20:1), followed by reversed-phase preparative TLC with MeOH−H_2O (2:1), to give **4** (4 mg), **6** (7 mg), **7** (20 mg), **8** (13 mg), **15** (13 mg), and **16** (34 mg). Fraction 6 (17.5 g) was in turn separated by Si gel column chromatography ($CHCl_3$−MeOH−H_2O, 35:20:1) to give five subfractions, with subfractions 2 and 3 purified by crystallization to yield **8** (3.0 g) and **17** (1.7 g), respectively. Fraction 7 (3.5 g) was separated by a combination of Si gel ($CHCl_3$−MeOH−H_2O, 35:20:1) and ODS (MeOH−H_2O, 2:1) column chromatography and then by normal- and reversed-phase preparative TLC to give **9** (143 mg), **18** (19 mg), **19** (6 mg), **20** (11 mg), and **21** (17 mg).

Spongipregnoloside A (1): colorless amorphous solid; $[\alpha]_D$ −18.2° (*c* 0.15, CH_3OH); ^1H NMR (C_5D_5N) δ 6.59 (1H, br s, H-16), 6.31 (1H, br s, H-1″), 5.37 (1H, d, J = 4.1 Hz, H-6), 4.89 (1H, d, J = 7.5 Hz, H-1′), 3.88 (1H, dddd, J = 11.4, 11.4, 4.3, 4.3 Hz, H-3), 2.22 (3H, s, H_3-21), 1.53 (1H, m, H-8), 1.36 (1H, m, H-14), 1.04 (3H, s, H_3-19), 0.99 (1H, m, H-9), 0.93 (3H, s, H_3-18); ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 621.3304 (calcd for $C_{33}H_{49}O_{11}$ 621.3275 [M − H]$^-$).

Spongipregnoloside B (2): colorless amorphous solid; $[\alpha]_D$ −64.6° (*c* 0.05, CH_3OH); ^1H NMR (C_5D_5N) δ 6.58 (1H, br s, H-16), 5.89 (1H, br s, H-1″), 5.33 (1H, br s, H-6), 4.94 (1H, d, J = 7.5 Hz, H-1′), 3.86 (1H, dddd, J = 11.4, 11.4, 4.3, 4.3 Hz, H-3), 2.23 (3H, s, H_3-21), 1.57 (1H, m, H-8), 1.25 (1H, m, H-14), 0.92 (3H, s, H_3-19), 0.85 (1H, m, H-9), 0.91 (3H, s, H_3-18); ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 621.3304 (calcd for $C_{33}H_{49}O_{11}$ [M − H]$^-$ 621.3275).

Spongipregnoloside C (3): colorless amorphous solid; $[\alpha]_D$ −68.7° (*c* 0.10, CH_3OH); ^1H NMR (C_5D_5N) δ 6.62 (1H, br s, H-16), 6.40 (1H, br s, H-1″), 5.85 (1H, br s, H-1‴), 5.35 (1H, d, J = 4.6 Hz, H-6), 5.12 (1H, d, J = 7.6 Hz, H-1‴), 4.96 (1H, d, J = 7.6 Hz, H-1′), 3.92 (1H, m, H-3), 2.25 (3H, s, H_3-21), 1.57 (1H, m, H-8), 1.36 (1H, m, H-14), 1.08 (3H, s, H_3-19), 0.96 (1H, m, H-9), 0.94 (3H, s, H_3-18); ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 929.4423 (calcd for $C_{45}H_{69}O_{20}$ [M − H]$^-$ 929.4383).

Spongipregnoloside D (4): colorless amorphous solid; $[\alpha]_D$ −54.2° (*c* 0.20, CH_3OH); ^1H NMR (C_5D_5N) δ 6.39 (1H, br s, H-1″), 5.82 (1H, br s H-1‴), 5.29 (1H, d, J = 4.0 Hz, H-6), 4.89 (1H, d, J = 7.4 Hz, H-1′), 4.53 (1H, br s, H-16), 3.86 (1H, dddd, J = 11.3, 11.3, 4.6, 4.6 Hz, H-3), 3.23 (3H, s, OCH_3), 2.66 (1H, d, J = 6.4 Hz, H-17), 2.17 (3H, s, H_3-21), 1.55 (1H, m, H-8), 1.30 (1H, m, H-14), 0.98 (1H, m, H-9), 0.94 (3H, s, H_3-19), 0.91 (3H, s, H_3-18); ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 799.4160 (calcd for $C_{40}H_{63}O_{16}$ [M − H]$^-$ 799.4116).

Spongioside A (5): colorless amorphous solid; $[\alpha]_D$ −67.8° (*c* 0.10, CH_3OH); ^1H NMR (C_5D_5N) δ 6.37 (1H, br s, H-1″), 5.83 (1H, br s, H-1‴), 5.22 (1H, br s, H-6), 4.93 (1H, d, J = 6.3 Hz, H-1′), 4.74 (1H, d, J = 6.4 Hz, H-1‴), 4.49 (1H, m, H-16), 4.15 (1H, m, H-22), 3.82 (1H, dddd, J = 11.2, 11.2, 4.7, 4.7 Hz, H-3), 2.55 (1H, m, H-20), 1.98 (1H, m, H-17), 1.38 (1H, m, H-9), 1.23 (1H, m, H-14), 1.20 (3H, d, J = 7.1 Hz, H_3-21), 1.05 (3H, s, H_3-19), 1.08 (1H, m, H-8), 0.99 (3H, s, H_3-18), 0.94 (6H, d, J = 5.6 Hz, H_3-26, H_3-27); ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 1033.5577 (calcd for $C_{51}H_{85}O_{21}$ [M − H]$^-$ 1033.5584).

Spongioside B (6): colorless amorphous solid; $[\alpha]_D$ −46.3° (*c* 0.25, CH_3OH); ^1H NMR (C_5D_5N) δ 6.37 (1H, br s, H-1″), 5.82 (1H, br s, H-1‴), 5.23 (1H, br s, H-6), 4.88 (1H, d, J = 6.8 Hz, H-1′), 4.64 (1H, d, J = 6.8 Hz, H-1‴), 4.46 (1H, m, H-16), 4.25 (1H, m, H-22), 3.78 (1H, m, H-3), 3.02 (1H, dd, J = 12.0, 2.8 Hz, H-17), 2.51 (1H, m, H-20), 1.77 (1H, m, H-8), 1.46 (1H, m, H-9), 1.31 (3H, s, H_3-18), 1.22 (3H, d, J = 7.0 Hz, H_3-21), 1.23 (1H, m, H-14), 1.11 (3H, s, H_3-19), 0.93 (6H, d, J = 5.6 Hz, H_3-26, H_3-27); ^{13}C NMR, see Table 1; HRFABMS *m*/*z* 1089.5433 (calcd for $C_{53}H_{85}O_{23}$ [M − H]$^-$ 1089.5482).

Sugar Analysis of 1−6.[11,26] Each compound (1 mg) was hydrolyzed with 1 M HCl (in H_2O dioxane, 1:1; 0.5 mL) at 80 °C for 3 h. The reaction mixture was neutralized with a small column of Amberlite IRA67 (OH^- form), and the filtrate was concentrated to dryness in vacuo. The residue was dissolved in 0.1 mL of pyridine, to which L-cysteine methyl ester hydrochloride in pyridine (0.1 M, 0.1 mL) was added. The mixture was heated at 60 °C for 2 h. Then, trimethylsilylimidazole (0.1 mL) was added, and the mixture was heated at 60 °C for 1.5 h. The reaction mixture was partitioned between hexane and water (0.1 mL each), and the hexane layer was analyzed with a Shimadzu GC-14AH gas chromatograph; column, DB-5MS 30 m × 0.32 mm (J&W Scientific Inc., Kyoto, Japan); column temperature, 210 °C; detector temperature, 270 °C; injection temperature, 270 °C. Standard D- and L-glucose gave peaks at t_R 15.90 and 16.92 min, respectively, and L-rhamnose at 10.27 min.[27]

Bone Resorbing Activity. An assay method reported by Shigeno et al.[28] was used to evaluate bone resorbing activity, as presented previously.[3,4]

Briefly, 2-day-old mice were injected subcutaneously with $^{45}CaCl_2$ (2 μCi). Two days later, the parietal bones were taken out and cultured in sterile plastic multiwell culture plates (well area, 2 cm^2) on stainless steel grids in the top of glass rings that supported the bones near the gas−liquid interface. Ham's F-12 medium (1 mL/well), which consisted of 10.6 g/L Ham's F-12 medium, 2.2 g/L $NaHCO_3$, 1.0 mM $CaCl_2$, and 5% (v/v) heat-inactivated horse serum, was used. Bones were incubated in an incubator at 37 °C under 5% CO_2 in air. Bones were randomly assigned to control and treated groups, and each group consisted of four bones. After preculturing for 24 h, the medium was removed and fresh medium containing PTH (final concentration, 2×10^{-9} M) and the samples to be tested were added. Then the bones were incubated for 6 days. During the experimental period, after 3 days, the medium was changed to fresh medium. After finishing the culture, bones were removed and put in 0.01 M EDTA-acetate buffer solution (pH 5.5) to extract ^{45}Ca contained in bone. ^{45}Ca released into the culture medium from prelabeled bones at 3 days and 6 days and EDTA solution was counted separately. Bone resorption was obtained as the percentage of total ^{45}Ca that was released into the medium during the culture. ^{45}Ca was measured by liquid scintillation counting.

References and Notes

(1) McGowan, J. A. *Aging* **1993**, *5*, 81−93.
(2) Li, J. X.; Kadota, S.; Li, H. Y.; Miyahara, T.; Namba, T. *J. Trad. Med.* **1996**, *13*, 50−58.
(3) Li, H.; Miyahara, T.; Tezuka, Y.; Miyahara, T.; Nemoto, N.; Tonami, S.; Seto, H.; Tada, T.; Kadota, S. *Biol. Pharm. Bull.* **1998**, *21*, 1322−1326.
(4) Li, J.; Li, H.; Kadota, S.; Namba, T. *J. Nat. Prod.* **1996**, *59*, 1015−1018.

(5) Yin, J.; Wang, D.; Li, F.; Chen, Y.; Jiang, Z. *J. Shenyang Pharm. Univ.* **2001**, *18*, 279−282.
(6) Zhao, W.; He, J.; Xi, J. *China J. Chin. Mater. Med.* **1994**, *19*, 199−202.
(7) Lou, W.; Chen, Y.-Y. *Acta Bot. Sin.* **1983**, *25*, 353−355.
(8) Kawasaki, T.; Yamauchi, T.; Yamauchi, R. *Chem. Pharm. Bull.* **1962**, *10*, 698−702.
(9) Zhang, K. *Chin. Herbs* **1990**, *21*, 25−27.
(10) Dong, M.; Feng, X.-Z.; Wu, L.-J.; Wang, B.-X.; Ikejima, T. *Planta Med.* **2001**, *67*, 853−857.
(11) Hara, S.; Okabe, H.; Mihashi, K. *Chem. Pharm. Bull.* **1987**, *35*, 501−506.
(12) Kasai, R.; Okihara, M.; Asakawa, J.; Mizutani, K.; Tanaka, O. *Tetrahedron* **1979**, *35*, 1427−1432.
(13) Kawashima, K.; Mimaki, Y.; Sashida, Y. *Chem. Pharm. Bull.* **1991**, *39*, 2761−2763.
(14) Nes, W. D.; Koike, K.; Jia, Z.; Sakamoto, Y.; Satou, T.; Nikaido, T.; Griffin, J. F. *J. Am. Chem. Soc.* **1998**, *120*, 5970−5980.
(15) Kuroda, M.; Mimaki, Y.; Sashida, Y. *Phytochemistry* **1999**, *52*, 445−452.
(16) Hu, K.; Yao, X.-S.; Dong, A.-J.; Kobayashi, H.; Iwasaki, S.; Jing, Y.-K. *J. Nat. Prod.* **1999**, *62*, 299−301.
(17) Shao, Y.; Poobrasert, O.; Kennelly, E.; Chin, C.; Ho, C.; Huang, M.; Garrison, S. A.; Cordell, G. A. *Planta Med.* **1997**, *63*, 258−262.
(18) Watanabe, Y.; Sanada, S.; Ida, Y.; Shoji, J. *Chem. Pharm. Bull.* **1983**, *31*, 3486−3495.
(19) Wang, S.; Ghisalberti, E. L.; Ridsdill-Smith J. *J. Nat. Prod.* **1998**, *61*, 508−510.
(20) Nakano, K.; Murakami, K.; Takaishi, Y.; Tomimatsu, T.; Nohara, T. *Chem. Pharm. Bull.* **1989**, *37*, 116−118.
(21) Abbas, F. A. *Sci. Pharm.* **2001**, *69*, 219−234.
(22) Liu, C.-L.; Chen, Y.-Y. *Acta Bot. Sin.* **1985**, *27*, 635−639.
(23) Mimaki, Y.; Sashida, Y.; Nakamura, O.; Nikaido, T.; Ohmoto, T. *Phytochemistry* **1993**, *33*, 675−682.
(24) Ahmad, V. U.; Khaliq-uz-Zaman, S. M.; Shameel, S.; Perveen, S.; Ali, Z. *Phytochemistry* **1998**, *50*, 481−484.
(25) Okamura, N.; Yagi, A.; Nishioka, I. *Chem. Pharm. Bull.* **1981**, *29*, 3507−3514.
(26) D- and L-Rhamnose are reported to be separated under the conditions. See: (a) Ikeda, T.; Tsumagari, H.; Nohara, T. *Chem. Pharm. Bull.* **2000**, *48*, 362−365. (b) Yokosuka, A.; Mimaki, Y.; Sashida, Y. *J. Nat. Prod.* **2000**, *63*, 1239−1243.
(27) Tran, Q. L.; Adnyana, I K.; Tezuka, Y.; Nagaoka, K.; Tran, Q. K.; Kadota, S. *J. Nat. Prod.* **2001**, *64*, 456−461.
(28) Shigeno, C.; Yamamoto, I.; Dokoh, S.; Hino, M.; Aoki, J.; Yamada, K.; Morita, R.; Kameyama, M.; Torizuka, M. *J. Clin. Endocrin. Metab.* **1985**, *61*, 761−768.

NP0205957