Bufadienolide and Spirostanol Glycosides from the Rhizomes of *Helleborus orientalis*

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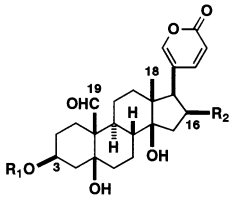
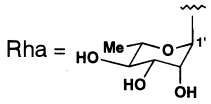
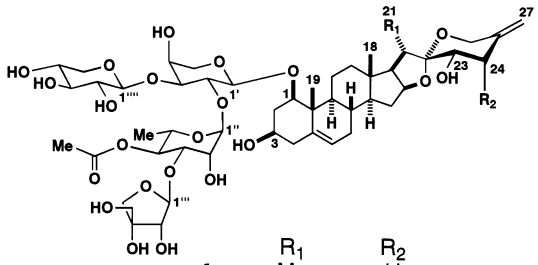
The rhizomes of *Helleborus orientalis* have been analyzed for the bufadienolide glycoside and spirostanol saponin constituents, resulting in the isolation of a new bufadienolide rhamnoside (**1**), along with two known bufadienolide glycosides (**2** and **3**) and five new spirostanol saponins (**4**−**8**). The structures of the new compounds were determined on the basis of extensive spectroscopic analysis, including 2D NMR, and the results of hydrolytic cleavage. The isolated compounds were evaluated for their cytotoxic activities against cultured tumor and normal cells.

Helleborus orientalis Lam. is a perennial plant belonging to the family Ranunculaceae and is indigenous to Greece and Turkey.[1] Its rhizomes have been used as a folk medicine in Europe for the treatment of cardiac insufficiency and constipation. A literature survey concerning the secondary metabolites of *H. orientalis* showed that it has been suggested to contain bufadienolide glycosides and steroidal saponins,[2] but no systematic phytochemical examination has been carried out on this plant. The present investigation on the bufadienolide glycoside and steroidal saponin constituents of the rhizomes of *H. orientalis* has resulted in the isolation of a new bufadienolide rhamnoside (**1**), along with two known bufadienolide glycosides (**2** and **3**) and five new spirostanol saponins (**4**−**8**). This paper reports the structural determination of the new compounds on the basis of extensive spectroscopic analysis, including 2D NMR, and the results of hydrolytic cleavage. The cytotoxic activities of the isolated compounds against cultured cells are also described.

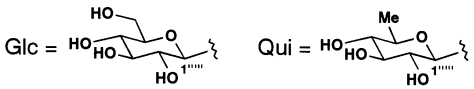


	R_1	R_2
1	Rha	OH
2	Rha	H
3	Glc	H





	R_1	R_2
4	Me	H
5	Me	OH
6	CH_2OAc	OH
7	CH_2OAc	O-Glc
8	CH_2OAc	O-Qui



Results and Discussion

The fresh rhizomes of *H. orientalis* (2.7 kg) were extracted with hot MeOH, and the MeOH extract was passed through a porous-polymer resin (Diaion HP-20) column. The 80% MeOH eluate fraction, with enriched steroidal glycosides, was subjected to column chromatography over silica gel and octadecylsilanized (ODS) silica gel, as well as preparative HPLC, giving compounds **1** (18.9 mg), **2** (193 mg), **3** (26.2 mg), **4** (30.2 mg), **5** (31.8 mg), **6** (34.3 mg), **7** (14.0 mg), and **8** (98.8 mg). Compounds **2** and **3** were identified as 5β,14β-dihydroxy-19-oxo-3β-[(α-L-rhamnopyranosyl)oxy)]bufa-20,22-dienolide[3] and 5β,14β-dihydroxy-19-oxo-3β-[(β-D-glucopyranosyl)oxy)]bufa-20,22-dienolide,[4] respectively.

Compound **1** was obtained as an amorphous solid, and its ^{1}H and ^{13}C NMR spectral data were very similar to those of **2**, suggesting that **1** is a bufadienolide rhamnoside structurally related to **2**. However, the molecular formula of **1**, $C_{30}H_{42}O_{11}$, which was derived from a combination of the positive-ion FABMS (*m*/*z* 601 $[M + Na]^+$), ^{13}C NMR spectral (30 carbon signals), and elemental analysis data, is one oxygen atom in excess of **2**. The C-16 methylene carbon signal observed at δ 29.7 in the ^{13}C NMR spectrum of **2** was displaced by an oxymethine signal at δ 72.4 in that of **1**. Treatment of **1** with Ac_2O in C_5H_5N gave a tetraacetate (**1a**). On comparison of the ^{1}H NMR spectrum of **1a** with that of **1**, a downfield shift from δ 4.85 (t-like, J = 7.8 Hz) to δ 5.73 (t-like, J = 9.0 Hz) was observed for the signal assignable to the H-16 proton, confirming the presence of a hydroxyl group at C-16. A key NOE correlation between the proton signals of H-16 and H-12α (δ 1.36) in the phase-sensitive NOESY spectrum of **1** showed the

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β-configuration of the C-16 hydroxyl group. Thus, the structure of **1** was assigned as 5β,14β,16β-trihydroxy-19-oxo-3β-[(α-L-rhamnopyranosyl)oxy)]bufa-20,22-dienolide.

Compound **4** was shown to have the molecular formula $C_{50}H_{76}O_{22}$ on the basis of the negative-ion FABMS (*m/z* 1027 $[M - H]^-$), ^{13}C NMR spectral (50 carbon signals), and elemental analysis data. The ^1H NMR spectrum contained signals for two angular methyl groups at δ 1.33 and 1.04 (each s), and the ^{13}C NMR spectrum showed an acetal carbon signal at δ 111.7,[5] suggesting **4** to have a spirostan skeleton. Furthermore, the ^1H NMR spectrum of **4** displayed four anomeric proton signals due to monosaccharide units at δ 6.46 (br s), 5.94 (d, $J = 3.0$ Hz), 4.92 (d, $J = 7.3$ Hz), and 4.62 (d, $J = 7.7$ Hz), as well as a three-proton doublet signal at δ 1.39 ($J = 6.1$ Hz), which was associated with the methyl carbon signal at δ 18.3, indicating that one of the four sugars is a 6-deoxyhexose. The presence of an acetyl group in **4** was shown by the signals at δ_H 2.23 (3H, s) and δ_C 170.7 (C═O) and 21.1 (Me). Acid hydrolysis of **4** with 0.2 M HCl in dioxane−H_2O (1:1) gave D-apiose, L-arabinose, L-rhamnose, and D-xylose as the carbohydrate moieties, while the labile aglycon was decomposed under acid conditions. The monosaccharides, including their absolute configurations, were identified by direct HPLC analysis of the hydrolysate, which was performed on an aminopropyl-bonded silica gel column using MeCN−H_2O (17:3) as solvent system, with detection being carried out using a combination of RI and optical rotation (OR) detectors. Comparison of the ^1H and ^{13}C NMR signal assignments of the aglycon moiety of **4**, which were established by analysis of the $^1H-^1H$ COSY, 2D TOCSY, HMQC, and HMBC spectra, with those of spirosta-5,25(27)-diene-1β,3β-diol (neoruscogenin) 1-*O*-glycosides, abundantly present in *Ruscus aculeatus*,[6] revealed that the structure of the A−E-ring parts (C-1−C-21) of **4** was identical to that of the reference compounds, including the orientation of the C-1 and C-3 oxygen atoms (1β-equatorial, 3β-equatorial), ring junctions (B/C *trans*, C/D *trans*, D/E *cis*), and C-20α and C-22α configurations, but with significant differences in the signals from the F-ring portion. The $^1H-^1H$ COSY spectrum showed that the structural fragments of the F-ring were an oxymethine adjacent to a methylene group appearing as an ABX-like spin system (AB part: δ 2.90, 2.79; X part: δ 3.91), as well as an exomethylene [δ 4.82 and 4.80 (each br s)] and an oxymethylene [δ 4.40 and 3.98 (ABq, $J = 12.4$ Hz)] group, attributable to H_2-27 and H_2-26, respectively. The oxymethine proton was coupled with the methylene protons with *J* values of 10.2 and 5.3 Hz and showed NOE correlations with both the H-20 (δ 3.00) and Me-21 [δ 1.09 (d, $J = 7.0$ Hz)] protons. On acetylation (**4a**), the oxymethine proton was shifted downfield by 1.25 ppm and was observed at δ 5.16 (dd, $J = 11.7$, 5.3 Hz). When the ^{13}C NMR spectrum of **4** was compared with that of neoruscogenin 1-*O*-glycosides, the resonance assignable to C-20 was shifted upfield by about 6 ppm, which was presumed to be due to the γ-gauche effect of a C-23*S* hydroxyl group. Thus, the presence of the (23*S*)-hydroxyl group was disclosed, and the structure of the aglycon was assigned as (23*S*)-spirosta-5,25(27)-diene-1β,3β,23-triol. The $^1H-^1H$ COSY and 2D TOCSY experiments allowed the sequential assignments from H-1 to CH_2-5 and Me-6 of three monosaccharides. Their signal multiplet patterns and coupling constants enabled the identification of an α-L-arabinopyranosyl ($^4C_1$) unit, an α-L-rhamnopyranosyl ($^1C_4$) unit, and a β-D-xylopyranosyl ($^4C_1$) unit (Table 1). In addition, the ^1H NMR signals at δ 5.94 and 4.65 ($J = 3.0$ Hz), 4.56 and 4.23 ($J = 9.3$ Hz), and 4.07 and 4.04 ($J =$ 11.5 Hz), along with the results of acid hydrolysis, were indicative of an apiofuranosyl unit. The relatively large *J* values of the anomeric protons of the arabinosyl (7.7 Hz) and xylosyl (7.3 Hz) moieties indicated an α anomeric orientation for the arabinosyl and β for the xylosyl. For the rhamnosyl moiety, the large $^1J_{C,H}$ value (174.6 Hz) confirmed that the anomeric proton was equatorial, thus possessing an α-pyranoid anomeric form.[7] The ^{13}C NMR shifts of the anomeric carbon of the apiosyl at δ 112.0 indicated a β-orientation of the anomeric center.[8] All the proton signals for the sugar moiety thus assigned were associated with the one-bond coupled carbon signals using the HMQC spectrum. The apiosyl and xylosyl residues were considered to be the terminal units, as shown by the absence of any glycosylation shift for their carbon resonances, while C-2 and C-3 of the arabinosyl unit and C-3 and C-4 of the rhamnosyl unit were suggested to be substituted by comparison with those of authentic methyl glycosides.[9] In the HMBC spectrum, the anomeric proton of the apiosyl at δ 5.94 showed a $^3J_{C,H}$ correlation with C-3 of the rhamnosyl at δ 77.6, whose anomeric proton at δ 6.46, in turn, showed a long-range correlation with C-2 of the arabinosyl at δ 72.4. The anomeric proton of the xylosyl at δ 4.92 was correlated to C-3 of the arabinosyl at δ 85.2. The arabinosyl moiety was thus shown to be glycosylated at C-2 and C-3, and its anomeric proton at δ 4.62 exhibited an HMBC correlation with C-1 of the aglycon at δ 84.2. A long-range correlation between the acetyl carbonyl carbon signal at δ 170.7 and the H-4 signal of the rhamnosyl at δ 5.86 (t-like, $J = 9.8$ Hz) indicated that the C-4 hydroxyl group of the rhamnosyl residue is acetylated. Accordingly, the structure of **4** was determined to be (23*S*)-3β,23-dihydroxyspirosta-5,25(27)-dien-1β-yl *O*-β-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside.

Table 1. ^1H NMR Data for the Sugar Moiety of Compound **4**[a]

proton	^1H	multiplicity	*J* (Hz)
1′	4.62	d	7.7
2′	4.56	dd	9.3, 7.7
3′	4.01	dd	9.3, 4.3
4′	4.39	br s	
5′a	4.22	dd	11.2, 1.6
b	3.66	br d	11.2
1″	6.46	br s	
2″	4.92	br d	3.0
3″	4.73	dd	9.8, 3.0
4″	5.86	t-like	9.8
5″	4.91	dq	9.8, 6.1
6″	1.39	d	6.1
1‴	5.94	d	3.0
2‴	4.65	d	3.0
3‴	–		
4‴a	4.56	d	9.3
b	4.23	d	9.3
5‴a	4.07	d	11.5
b	4.04	d	11.5
1⁗	4.92	d	7.3
2⁗	3.88	dd	8.5, 7.3
3⁗	4.14	t-like	8.5
4⁗	4.09	ddd	11.2, 8.5, 5.1
5⁗a	4.25	dd	11.2, 5.1
b	3.67	t-like	11.2
Ac	2.23	s	

[a] Spectrum was measured in C_5D_5N.

Compound **5** was deduced as $C_{50}H_{76}O_{23}$ from the positive-ion FABMS (*m/z* 1067 $[M + Na]^+$), ^{13}C NMR spectral (50 carbon signals), and elemental analysis data. The ^1H and ^{13}C NMR spectra implied that **5** was closely related to **4** and that one more hydroxyl group was present at the F-ring portion of **5**. The oxymethine proton signal at δ 3.93

showed an HMBC correlation with C-22 and NOE correlations with H-20 and Me-21 and was assigned to H-23. The H-23 proton had a spin-coupling correlation with an adjacent oxymethine proton at δ 4.70 with a J value of 3.8 Hz. A clear NOE correlation was observed between the two oxymethine proton signals. On acetylation (**5a**), the δ 3.93 and 4.70 resonances were moved downfield by 1.38 and 1.45 ppm to δ 5.31 and 6.15, respectively. Thus, the presence of a (24*S*)-hydroxyl group in addition to a (23*S*)-hydroxyl group was evident. The structure of **5** was defined as (23*S*,24*S*)-3β,23,24-trihydroxyspirosta-5,25(27)-dien-1β-yl *O*-β-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside.

Compound **6** was analyzed for $C_{52}H_{78}O_{25}$ by the combined positive-ion FABMS (*m*/*z* 1125 $[M + Na]^+$), ^{13}C NMR spectral (52 carbon signals), and elemental analysis data. Comparison of the ^1H and ^{13}C NMR spectra of **6** with those of **5** showed their considerable structural similarity. However, the Me-21 signal, which was observed at δ 1.10 (d, J = 6.9 Hz) in **5**, was displaced by the oxymethylene signals at δ 4.41 (dd, J = 10.7, 6.9 Hz) and 4.37 (dd, J = 10.7, 6.8 Hz) in **6**. Furthermore, the presence of one more acetyl group in addition to that attached at C-4 of the rhamnosyl group in **6** was indicated by the ^1H [δ 1.93 (3H, s)] and ^{13}C NMR [δ 170.8 (C=O) and 20.9 (Me)] spectra. The ester linkage at the aglycon C-21 in **6** was formed from acetic acid, as was evident from HMBC correlations from δ_H 4.41 and 4.37 to δ_C 170.8. The structure of **6** was elucidated as (23*S*,24*S*)-21-acetoxy-3β,23,24-trihydroxyspirosta-5,25(27)-dien-1β-yl *O*-β-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside.

Compound **7**, obtained as an amorphous solid, exhibited a molecular formula of $C_{58}H_{88}O_{30}$ on the basis of its positive-ion FABMS (*m*/*z* 1287 $[M + Na]^+$), negative-ion FABMS (*m*/*z* 1263 $[M - H]^-$), ^{13}C NMR spectral (58 carbon signals), and elemental analysis data. The deduced molecular formula was higher by $C_6H_{10}O_5$ than that of **6**, and the ^1H and ^{13}C NMR spectra showed five anomeric proton and carbon signals at δ_H 6.44 (d, J = 1.3 Hz), 5.93 (d, J = 3.0 Hz), 5.38 (d, J = 7.9 Hz), 4.91 (d, J = 7.4 Hz), and 4.64 (d, J = 7.5 Hz); δ_C 112.2, 106.7, 106.2, 100.8, and 100.6 Acid hydrolysis of **7** with 0.2 M HCl in dioxane−H_2O (1:1) gave D-apiose, L-arabinose, D-glucose, L-rhamnose, and D-xylose as the carbohydrate moieties. On comparison of the whole ^{13}C NMR spectrum of **7** with that of **6**, a set of six additional signals corresponding to a terminal β-D-glucopyranosyl moiety appeared at δ 106.2 (CH), 75.9 (CH), 78.6 (CH), 71.5 (CH), 78.6 (CH), and 62.5 (CH_2), and the carbon signals due to the F-ring part varied, while all other signals remained almost unaffected. In the HMBC spectrum, the oxymethine carbon signal at δ 82.2 showed long-range correlations with the H-27a olefinic proton at δ 5.20 (br s) and the C-26 equatorial proton at δ 3.96 (d, J = 12.0 Hz) and was assigned to C-24. An HMBC correlation from the anomeric proton signal of the glucosyl moiety at δ 5.38 to the C-24 carbon gave ample evidence for the glucosyl group linkage to the aglycon C-24 hydroxyl group. The structure of **7** was shown to be (23*S*,24*S*)-21-acetoxy-24-[(*O*-β-D-glucopyranosyl)oxy]-3β,23-dihydroxyspirosta-5,25(27)-dien-1β-yl *O*-β-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside.

Compound **8** was shown to have the molecular formula $C_{58}H_{88}O_{29}$ on the basis of the positive-ion FABMS (*m*/*z* 1271 $[M + Na]^+$), ^{13}C NMR spectral (58 carbon signals), and elemental analysis data. Analysis of the ^1H and ^{13}C NMR spectra of **8** and comparison with those of **7** implied that the aglycon and the tetraglycoside attached at C-1 of the aglycon were identical with those of **7**, but differed from **7** in terms of the monosaccharide linked to C-24 of the aglycon. Instead of the signals for a glucosyl moiety, signals assignable to a β-D-quinovopyranosyl residue were observed at δ_{H-1} 5.18 (d, J = 7.7 Hz) and δ_{Me-6} 1.53 (d, J = 6.0 Hz) and δ_C 105.7 (CH), 75.9 (CH), 78.1 (CH), 76.7 (CH), 73.1 (CH), and 18.6 (Me) in the ^1H and ^{13}C NMR spectra of **8**. Acid hydrolysis of **8** with 0.2 M HCl in dioxane−H_2O (1:1) gave D-apiose, L-arabinose, D-quinovose, L-rhamnose, and D-xylose. The glycosidic linkage of the quinovosyl moiety to C-24 of the aglycon was ascertained by an HMBC correlation from H-1 of the quinovosyl to C-24 at δ 82.2. Thus, the structure of **8** was formulated as (23*S*,24*S*)-21-acetoxy-3β,23-dihydroxy-24-[(*O*-β-D-quinovopyranosyl)-oxy]spirosta-5,25(27)-dien-1β-yl *O*-β-D-apiofuranosyl-(1→3)-*O*-(4-*O*-acetyl-α-L-rhamnopyranosyl)-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-α-L-arabinopyranoside.

Several cardenolides and bufadienolides have been reported to show cytotoxic activity against cultured tumor cells.[10] The bufadienolide glycosides (**1**−**3**) isolated in this study also exhibited potent cytotoxicities, with HSC-2 human squamous cell carcinoma cells and A375 human melanoma cells showing particular sensitivity, but HepG2 human hepatoma cells being relatively resistant to them. Although the bufadienolide glucoside **3** was cytotoxic to both the tumor cells and normal human pulp cells (HPC), the bufadienolide rhamnosides **1** and **2** had higher tumor specificity and therefore showed only weak cytotoxicity against HPC. These results suggested that the monosaccharides attached at the bufadienolide aglycon contribute to the mediation of the tumor specificity among these bufadienolides. The spirostanol saponins (**4**−**8**) did not show significant cytotoxicities when evaluated against the same cell lines.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a JASCO DIP-360 (Tokyo, Japan) automatic digital polarimeter. IR spectra were recorded on a JASCO FT-IR 620 spectrophotometer. NMR spectra were recorded on a Bruker DRX-500 spectrometer (500 MHz for ^1H NMR, Karlsruhe, Germany) using standard Bruker pulse programs. Chemical shifts are given as δ values with reference to tetramethylsilane (TMS) as internal standard. MS were recorded on a Finnigan MAT TSQ-700 (San Jose, CA) mass spectrometer, using a dithiothreitol and dithioerythritol (3:1) matrix. Elemental analysis was carried out using an Elemental Vario EL (Hanau, Germany) elemental analyzer. Silica gel (Fuji-Silysia Chemical, Aichi, Japan), ODS Si gel (Nacalai Tesque, Kyoto, Japan), and Diaion HP-20 (Mitsubishi-Chemical, Tokyo, Japan) were used for column chromatography. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck, Darmstadt, Germany) and RP-18 $F_{254}S$ (0.25 mm thick, Merck) plates, and spots were visualized by spraying the plates with 10% H_2SO_4 solution, followed by heating. HPLC was performed using a system comprised of a Tosoh CCPM pump (Tokyo, Japan), a Tosoh CCP PX-8010 controller, a Tosoh RI-8010 detector, a Shodex OR-2 detector (Showa-Denko, Tokyo, Japan), and a Rheodyne injection port. A Capcell Pak C_{18} UG120 column (10 mm i.d. × 250 mm, 5 μm, Shiseido, Tokyo, Japan) was employed for preparative HPLC. The following reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (Gibco, Grand Island, NY); fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS); penicillin and streptomycin sulfate (Meiji-Seica, Tokyo, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bro-

mide (MTT) and α-minimum essential medium (α-MEM) (Sigma, St. Louis, MO). All other chemicals used were of biochemical reagent grade.

Plant Material. *Helleborus orientalis* was purchased from a nursery in Heiwaen, Japan, in November 1999 and was identified by one of the authors (Y.S.). A voucher specimen has been deposited in our laboratory (voucher No. 99-11-7-HO, Laboratory of Medicinal Plant Science).

Extraction and Isolation. The plant material (fresh weight, 2.7 kg) was extracted with hot MeOH twice. The MeOH extract was concentrated under reduced pressure, and the viscous concentrate (300 g) was passed through a Diaion HP-20 column (30% MeOH, 80% MeOH, MeOH, EtOH, and EtOAc). The 80% MeOH eluate portion (90 g) was chromatographed on silica gel eluting with $CHCl_3$−MeOH gradients (9:1, 4:1, 2:1) and finally MeOH to give subfractions I−VIII. Fraction III was dissolved in MeOH, and the deposited precipitate was filtered off to give **2** (139 mg). Fraction V was subjected to silica gel column chromatography eluting with $CHCl_3$−MeOH−H_2O (50:10:1), ODS silica gel with MeCN−H_2O (5:8; 1:2; 4:9), and finally preparative HPLC using MeCN−H_2O (3:10) to furnish **1** (18.9 mg), **3** (26.2 mg), **4** (30.2 mg), **5** (31.8 mg), and **6** (34.3 mg). Fraction VI was further divided by subjecting it to an ODS silica gel column eluting with MeCN−H_2O (1:4) into four fractions (VIa−VId). Fraction VIb was chromatographed on silica gel eluting with $CHCl_3$−MeOH−H_2O (20:10:1) to give **8** with a few impurities. Final purification of **8** (98.8 mg) was carried out by preparative HPLC using MeCN−H_2O (4:11). Compound **7** (14.0 mg) was isolated from fraction VIc by subjecting it to column chromatography on silica gel by elution with $CHCl_3$−MeOH−H_2O (20:10:1) and by passage through an ODS silica gel column eluting with MeOH−H_2O (8:5).

Compound 1: amorphous solid; $[\alpha]^{27}_D$ −36.0° (*c* 0.10, $CHCl_3$−MeOH, 1:1); UV (MeOH) (log ϵ) λ_{max} 296 (3.64) nm; IR (film) ν_{max} 3288 (OH), 2948 and 2888 (CH), 1714 and 1703 (C=O), 1545, 1446, 1417, 1375, 1312, 1145, 1093, 1051, 1028, 981, 831 cm^{-1}; ^1H NMR (C_5D_5N) δ 10.40 (1H, s, H-19), 8.55 (1H, dd, J = 9.7, 2.5 Hz, H-22), 7.52 (1H, d, J = 2.5 Hz, H-21), 6.32 (1H, d, J = 9.7 Hz, H-23), 5.49 (1H, d, J = 1.5 Hz, H-1′), 4.85 (1H, t-like, J = 7.8 Hz, H-16), 4.51 (1H, dd, J = 3.3, 1.5 Hz, H-2′), 4.44 (1H, dd, J = 9.1, 3.3 Hz, H-3′), 4.32 (1H, br s, H-3), 4.30 (1H, dd, J = 9.4, 9.1 Hz, H-4′), 4.23 (1H, dq, J = 9.4, 6.1 Hz, H-5′), 2.85 (1H, d, J = 7.8 Hz, H-17), 2.63 (1H, dd, J = 14.5, 7.8 Hz, H-15α), 2.21 (1H, br d, J = 14.5 Hz, H-15β), 1.67 (3H, d, J = 6.1 Hz, Me-6′), 1.02 (3H, s, Me-18); ^{13}C NMR (C_5D_5N) δ 18.7 (C-1), 25.6 (C-2), 73.1 (C-3), 35.4 (C-4), 74.0 (C-5), 37.0 (C-6), 25.0 (C-7), 42.3 (C-8), 39.6 (C-9), 55.3 (C-10), 22.8 (C-11), 41.0 (C-12), 49.4 (C-13), 84.3 (C-14), 42.3 (C-15), 72.4 (C-16), 58.9 (C-17), 17.1 (C-18), 208.4 (C-19), 119.2 (C-20), 150.7 (C-21), 151.3 (C-22), 112.6 (C-23), 162.2 (C-24), 100.6 (C-1′), 72.5 (C-2′), 72.9 (C-3′), 70.8 (C-4′), 73.6 (C-5′), 18.6 (C-6′); FABMS (positive mode) m/z 601 $[M + Na]^+$; *anal.* C 59.45%, H 7.82% (calcd for $C_{30}H_{42}O_{11}\cdot 3/2H_2O$, C 59.49%, H 7.49%).

Acetylation of 1. Compound **1** (2.3 mg) was acetylated with Ac_2O (1 mL) in C_5H_5N (1 mL) at room temperature for 20 h. The crude acetate was chromatographed on silica gel eluting with $CHCl_3$−MeOH (22:1) to give tetraacetate **1a** (2.1 mg).

Compound 1a: amorphous solid; $[\alpha]^{26}_D$ −20.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3363 (OH), 2920 and 2850 (CH), 1744 and 1716 (C=O), 1538, 1453, 1372, 1247, 1226, 1133, 1042, 981, 949, 836 cm^{-1}; ^1H NMR (C_5D_5N) δ 10.37 (1H, s, H-19), 8.62 (1H, br d, J = 9.8 Hz, H-22), 7.74 (1H, br s, H-21), 6.37 (1H, d, J = 9.8 Hz, H-23), 5.73 (1H, t-like, J = 9.0 Hz, H-16), 5.72 (1H, dd, J = 9.8, 3.2 Hz, H-3′), 5.58 (1H, br d, J = 3.2 Hz, H-2′), 5.56 (1H, t-like, J = 9.8 Hz, H-4′), 5.40 (1H, br s, H-1′), 4.31 (1H, dq, J = 9.8, 6.2 Hz, H-5′), 4.29 (1H, br s, H-3), 3.07 (1H, d, J = 9.0 Hz, H-17), 2.79 (1H, dd, J = 15.2, 9.0 Hz, H-15α), 2.12 (1H, br d, J = 15.2 Hz, H-15β), 2.08, 1.98, 1.97, and 1.80 (each 3H, s, Ac × 4), 1.37 (3H, d, J = 6.2 Hz, Me-6′), 0.97 (3H, s, Me-18).

Compound 4: amorphous solid; $[\alpha]^{26}_D$ −64.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3388 (OH), 2927 and 2852 (CH), 1731 (C=O), 1453, 1373, 1251, 1043, 987, 837 cm^{-1}; ^1H NMR (C_5D_5N) δ 6.46 (1H, br s, H-1″), 5.94 (1H, d, J = 3.0 Hz, H-1‴), 5.59 (1H, br d, J = 5.6 Hz, H-6), 4.92 (1H, d, J = 7.3 Hz, H-1⁗), 4.82 (1H, br s, H-27a), 4.80 (1H, br s, H-27b), 4.62 (1H, d, J = 7.7 Hz, H-1′), 4.60 (1H, m, H-16), 4.40 (1H, d, J = 12.4 Hz, H-26ax), 3.98 (1H, d, J = 12.4 Hz, H-26eq), 3.91 (1H, dd, J = 10.2, 5.3 Hz, H-23), 3.88 (1H, br m, $W_{1/2}$ = 16.7 Hz, H-3), 3.77 (1H, dd, J = 12.0, 4.0 Hz, H-1), 3.00 (1H, m, H-20), 2.90 (1H, dd, J = 12.5, 10.2 Hz, H-24ax), 2.79 (1H, dd, J = 12.5, 5.3 Hz, H-24eq), 2.23 (3H, s, Ac), 1.39 (3H, d, J = 6.1 Hz, Me-6″), 1.33 (3H, s, Me-19), 1.09 (3H, d, J = 7.0 Hz, Me-21), 1.04 (3H, s, Me-18); ^{13}C NMR, see Table 2; FABMS (negative mode) m/z 1027 $[M - H]^-$, 985 $[M -$ acetyl$]^-$, 967 $[M - H -$ AcOH$]^-$, 895 $[M -$ (apiosyl or xylosyl)$]^-$; *anal.* C 54.00%, H 7.79% (calcd for $C_{50}H_{76}O_{22}\cdot 9/2H_2O$, C 54.09%, H 7.72%).

Table 2. ^{13}C NMR Data for Compounds **4**−**8**[a]

carbon	4	5	6	7	8
1	84.2	84.2	84.3	84.3	84.2
2	37.8	37.9	38.0	38.0	37.9
3	68.0	68.0	68.0	68.0	68.0
4	43.7	43.8	43.8	43.8	43.7
5	139.3	139.3	139.3	139.5	139.4
6	124.9	124.9	124.9	124.8	124.8
7	32.0	32.0	31.9	31.8	31.7
8	32.9	33.0	33.1	33.1	33.0
9	50.3	50.4	50.4	50.3	50.2
10	42.8	42.9	42.8	42.8	42.7
11	23.9	23.9	24.0	24.0	23.9
12	40.5	40.5	40.1	40.0	40.0
13	40.7	40.6	40.9	41.0	40.9
14	56.7	56.8	57.0	56.9	56.8
15	32.3	32.3	32.3	32.5	32.4
16	81.9	83.2	83.8	83.7	83.6
17	62.4	61.3	58.5	58.8	58.7
18	16.8	16.8	16.9	16.8	16.7
19	14.9	14.9	14.9	15.0	15.0
20	35.7	37.0	42.4	42.7	42.6
21	14.5	14.6	65.0	65.1	65.0
22	111.7	112.6	111.8	111.0	110.9
23	68.4	68.0	70.9	71.5	71.3
24	38.8	74.1	73.8	82.2	82.2
25	144.3	146.4	146.0	143.3	143.4
26	64.2	60.8	60.8	61.5	61.4
27	109.3	112.3	112.7	114.5	114.2
Ac			170.8	170.8	170.8
			20.9	20.9	20.9
1′	100.7	100.8	100.8	100.8	100.7
2′	72.4	72.6	72.6	72.7	72.5
3′	85.2	85.2	85.2	85.2	85.1
4′	69.6	69.8	69.7	69.7	69.5
5′	67.2	67.2	67.1	67.1	67.0
1″	100.6	100.7	100.6	100.6	100.5
2″	71.4	71.5	71.6	71.6	71.4
3″	77.6	77.7	77.7	77.8	77.6
4″	74.5	74.6	74.6	74.6	74.5
5″	66.6	66.7	66.6	66.7	66.6
6″	18.3	18.4	18.3	18.4	18.3
1‴	112.0	112.2	112.2	112.2	112.0
2‴	77.8	77.9	77.9	77.9	77.8
3‴	80.0	80.0	80.0	80.0	80.0
4‴	74.9	74.9	74.9	74.9	74.9
5‴	65.2	65.3	65.3	65.3	65.2
1⁗	106.6	106.7	106.7	106.7	106.5
2⁗	74.5	74.5	74.5	74.5	74.5
3⁗	78.4	78.5	78.5	78.5	78.4
4⁗	70.9	70.9	70.9	70.9	70.9
5⁗	67.0	67.1	67.1	67.1	67.0
1⁗′				106.2	105.7
2⁗′				75.9	75.9
3⁗′				78.6	78.1
4⁗′				71.5	76.7
5⁗′				78.6	73.1
6⁗′				62.6	18.6
Ac	170.7	170.6	170.6	170.6	170.7
	21.1	21.1	21.1	21.1	21.1

[a] Spectra were measured in C_5D_5N.

Table 3. Cytotoxic Activities of Compounds **1**−**8** against HSC-2, A-375, and HepG2 Tumor Cells and HPC[a]

	IC_{50} (μg/mL)			
compound	HSC-2	A-375	HepG2	HPC
1	0.0085	0.055	0.74	9.5
2	0.0028	0.0063	0.25	6.7
3	0.0029	0.0086	0.23	0.082
4	27	–[b]	–	–
5	86	–	–	–
6	184	–	–	–
7	>200	–	–	–
8	>200	–	–	–
doxorubicin	2.5	1.8	>40	>40

[a] Key to cell lines: HSC-2 (human squamous cell carcinoma); A-375 (human melanoma); HepG2 (human hepatoma); HPC (normal human pulp cells). [b] Not measured.

Acid Hydrolysis of 4. A solution of **4** (4.8 mg) in 0.2 M HCl (dioxane−H_2O, 1:1, 3 mL) was heated at 95 °C for 30 min under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU (Organo, Tokyo, Japan) column and then chromatographed on Diaion HP-20 eluting with H_2O−MeOH (3:2), followed by Me_2CO−EtOH (1:1), to give a sugar fraction (1.1 mg) and an aglycon fraction (1.3 mg). TLC analysis of the aglycon fraction indicated that it contained several unidentified compounds produced under acid conditions. The sugar fraction was dissolved in H_2O−MeOH (3:2) and passed through a Sep-Pak C_{18} cartridge (Waters, Milford, MA) and a Toyopak IC-SP M cartridge (Tosoh, Tokyo, Japan), which was then analyzed by HPLC under the following conditions: column, Capcell Pak NH_2 UG80 (4.6 mm i.d. × 250 mm, 5 μm, Shiseido, Tokyo, Japan); solvent, MeCN−H_2O (17:3); flow rate, 0.9 mL/min; detection, RI and OR. Identification of D-apiose, L-arabinose, L-rhamnose, and D-xylose present in the sugar fraction was carried out by comparison of their retention times and optical rotations with those of authentic samples: t_R (min) 7.10 (D-apiose, positive optical rotation), 7.39 (L-rhamnose, negative optical rotation), 8.56 (L-arabinose, positive optical rotation), 9.20 (D-xylose, positive optical rotation).

Acetylation of 4. Compound **4** (7.7 mg) was acetylated with a mixture of Ac_2O (1 mL) and pyridine (1 mL) in the presence of 4-(dimethylamino)pyridine (3.5 mg) as catalyst. The crude acetate was chromatographed on silica gel eluting with hexane−Me_2CO (2:1) to afford decaacetate **4a** (6.0 mg) of **4**.

Compound 4a: amorphous solid; $[\alpha]^{26}_D$ −40.0° (*c* 0.10, MeOH); IR (film) ν_{max} 2955, 2924 and 2851 (CH), 1745 (C=O), 1436, 1370, 1227, 1046, 980, 876 cm^{-1}; ^1H NMR (C_5D_5N) δ 5.72 (1H, br s, H-1″), 5.64 (1H, br d, *J* = 5.6 Hz, H-6), 5.47 (1H, d, *J* = 2.9 Hz, H-1‴), 5.16 (1H, dd, *J* = 11.7, 5.3 Hz, H-23), 5.15 (1H, d, *J* = 7.0 Hz, H-1⁗), 4.87 (1H, br s, H-27a), 4.85 (1H, br m, $W_{1/2}$ = 19.2 Hz, H-3), 4.82 (1H, br s, H-27b), 4.56 (1H, m, H-16), 4.49 (1H, d, *J* = 7.5 Hz, H-1′), 4.38 (1H, d, *J* = 12.3 Hz, H-26ax), 3.97 (1H, d, *J* = 12.3 Hz, H-26eq), 3.66 (1H, dd, *J* = 12.0, 4.3 Hz, H-1), 2.85 (1H, dd, *J* = 12.1, 11.7, H-24ax), 2.78 (1H, dd, *J* = 12.1, 5.3 Hz, H-24eq), 2.37, 2.36, 2.21, 2.19, 2.15, 2.11, 2.07, 2.05, 2.01, 1.99, and 1.94 (each 3H, s, Ac × 11), 1.31 (3H, s, Me-19), 1.07 (3H, d, *J* = 7.0 Hz, Me-21), 1.05 (3H, s, Me-18).

Compound 5: amorphous solid; $[\alpha]^{25}_D$ −104.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3387 (OH), 2974 and 2905 (CH), 1728 (C=O), 1451, 1250, 1133, 1043, 994, 959, 897 cm^{-1}; ^1H NMR (C_5D_5N) δ 6.49 (1H, d, *J* = 1.1 Hz, H-1″), 5.94 (1H, d, *J* = 3.0 Hz, H-1‴), 5.61 (1H, br d, *J* = 5.7 Hz, H-6), 5.09 (1H, d, *J* = 1.0 Hz, H-27a), 4.99 (1H, d, *J* = 1.0 Hz, H-27b), 4.93 (1H, d, *J* = 7.0 Hz, H-1⁗), 4.83 (1H, d, *J* = 12.1 Hz, H-26ax), 4.70 (1H, d, *J* = 3.8 Hz, H-24), 4.65 (1H, d, *J* = 7.7 Hz, H-1′), 4.62 (1H, m, H-16), 4.01 (1H, d, *J* = 12.1 Hz, H-26eq), 3.93 (1H, d, *J* = 3.8 Hz, H-23), 3.89 (1H, br m, $W_{1/2}$ = 19.8 Hz, H-3), 3.79 (1H, dd, *J* = 12.0, 3.8 Hz, H-1), 2.98 (1H, m, H-20), 2.21 (3H, s, Ac), 1.42 (3H, d, *J* = 6.2 Hz, Me-6″), 1.36 (3H, s, Me-19), 1.10 (3H, d, *J* = 6.9 Hz, Me-21), 1.03 (3H, s, Me-18); ^{13}C NMR, see Table 2; FABMS (positive mode) *m*/*z* 1067 $[M + Na]^+$; *anal*. C 53.85%, H 7.64% (calcd for $C_{50}H_{76}O_{23}\cdot 4H_2O$, C 53.76%, H, 7.57%).

Acetylation of 5. Compound **5** (8.0 mg) was acetylated with a mixture of Ac_2O (1 mL) and pyridine (1 mL) in the presence of 4-(dimethylamino)pyridine (3.5 mg) as catalyst. The crude acetate was chromatographed on silica gel eluting with hexane−Me_2CO (2:1) to afford undecaacetate **5a** (5.2 mg) of **5**.

Compound 5a: amorphous solid; $[\alpha]^{23}_D$ −40.0° (*c* 0.10, MeOH); IR (film) ν_{max} 2917 and 2850 (CH), 1744 (C=O), 1452, 1372, 1245, 1046, 936, 875 cm^{-1}; ^1H NMR (C_5D_5N) δ 6.15 (1H, d, *J* = 4.2 Hz, H-24), 5.73 (1H, d, *J* = 1.2 Hz, H-1″), 5.66 (1H, br d, *J* = 5.6 Hz, H-6), 5.47 (1H, d, *J* = 2.9 Hz, H-1‴), 5.31 (1H, d, *J* = 4.2 Hz, H-23), 5.21 (1H, br s, H-27a), 5.14 (1H, br s, H-27b), 5.14 (1H, d, *J* = 2.8 Hz, H-1⁗), 4.88 (1H, br m, $W_{1/2}$ = 17.0 Hz, H-3), 4.81 (1H, d, *J* = 12.4 Hz, H-26ax), 4.70 (1H, d, *J* = 7.5 Hz, H-1′), 4.63 (1H, m, H-16), 4.04 (1H, d, *J* = 12.4 Hz, H-26eq), 3.66 (1H, dd, *J* = 12.0, 4.3 Hz, H-1), 2.38, 2.35, 2.21 × 2, 2.19, 2.11, 2.10, 2.07, 2.06, 2.01, 2.00, and 1.94 (each 3H, s, Ac × 12), 1.36 (3H, d, *J* = 6.2 Hz, Me-6″), 1.32 (3H, s, Me-19), 1.08 (3H, d, *J* = 7.0 Hz, Me-21), 1.02 (3H, s, Me-18).

Compound 6: amorphous solid; $[\alpha]^{26}_D$ −78.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3392 (OH), 2972 and 2907 (CH), 1728 (C=O), 1451, 1251, 1043, 992, 836 cm^{-1}; ^1H NMR (C_5D_5N) δ 6.45 (1H, d, *J* = 1.3 Hz, H-1″), 5.93 (1H, d, *J* = 2.9 Hz, H-1‴), 5.61 (1H, br d, *J* = 5.7 Hz, H-6), 5.10 (1H, d, *J* = 1.1 Hz, H-27a), 5.00 (1H, d, *J* = 1.1 Hz, H-27b), 4.91 (1H, d, *J* = 7.7 Hz, H-1⁗), 4.83 (1H, d, *J* = 12.2 Hz, H-26ax), 4.70 (1H, d, *J* = 3.9 Hz, H-24), 4.70 (1H, m, H-16), 4.64 (1H, d, *J* = 7.5 Hz, H-1′), 4.41 (1H, dd, *J* = 10.7, 6.9 Hz, H-21a), 4.37 (1H, dd, *J* = 10.7, 6.8 Hz, H-21b), 4.12 (1H, d, *J* = 3.9 Hz, H-23), 3.98 (1H, d, *J* = 12.2 Hz, H-26eq), 3.89 (1H, br m, $W_{1/2}$ = 16.0 Hz, H-3), 3.78 (1H, dd, *J* = 12.1, 4.0 Hz, H-1), 3.30 (1H, m, H-20), 2.21 and 1.93 (each 3H, s, Ac × 2), 1.39 (3H, d, *J* = 6.2 Hz, Me-6″), 1.35 (3H, s, Me-19), 1.13 (3H, s, Me-18); ^{13}C NMR, see Table 2; FABMS (positive mode) *m*/*z* 1125 $[M + Na]^+$; *anal*. C 54.40%, H 7.58% (calcd for $C_{52}H_{78}O_{25}\cdot 5/2H_2O$, C 54.40%, H 7.92%).

Compound 7: amorphous solid; $[\alpha]^{28}_D$ −76.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3415 (OH), 2972 and 2911 (CH), 1728 (C=O), 1647, 1372, 1245, 1042, 899, 836 cm^{-1}; ^1H NMR (C_5D_5N) δ 6.44 (1H, d, *J* = 1.3 Hz, H-1″), 5.93 (1H, d, *J* = 3.0 Hz, H-1‴), 5.66 (1H, br d, *J* = 5.5 Hz, H-6), 5.38 (1H, d, *J* = 7.9 Hz, H-1⁗), 5.20 (1H, br s, H-27a), 5.02 (1H, br s, H-27b), 4.91 (1H, d, *J* = 7.4 Hz, H-1⁗), 4.87 (1H, d, *J* = 12.0 Hz, H-26ax), 4.82 (1H, d, *J* = 4.0 Hz, H-24), 4.67 (1H, m, H-16), 4.64 (1H, d, *J* = 7.5 Hz, H-1′), 4.37 (1H, m, H-21a), 4.34 (1H, m, H-21b), 4.15 (1H, d, *J* = 4.0 Hz, H-23), 3.96 (1H, d, *J* = 12.0 Hz, H-26eq), 3.90 (1H, br m, $W_{1/2}$ = 18.5 Hz, H-3), 3.78 (1H, dd, *J* = 12.0, 4.0 Hz, H-1), 3.26 (1H, m, H-20), 2.21 and 1.92 (each 3H, s, Ac × 2), 1.39 (3H, d, *J* = 6.1 Hz, Me-6″), 1.40 (3H, s, Me-19), 1.08 (3H, s, Me-18); ^{13}C NMR, see Table 2; FABMS (positive mode) *m*/*z* 1287 $[M + Na]^+$; FABMS (negative mode) *m*/*z* 1263 $[M - H]^-$; *anal*. C 51.29%, H 7.45% (calcd for $C_{58}H_{88}O_{30}\cdot 5H_2O$, C 51.40%, H 7.29%).

Acid Hydrolysis of 7. Compound **7** (8.3 mg) was subjected to acid hydrolysis as described for **4** to give a sugar fraction (2.4 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of that of **4** showed the presence of D-apiose, L-arabinose, D-glucose, L-rhamnose, and D-xylose: t_R (min) 7.09 (D-apiose, positive optical rotation), 7.76 (L-rhamnose, negative optical rotation), 9.28 (L-arabinose, positive optical rotation), 9.63 (D-xylose, positive optical rotation), 14.91 (D-glucose, positive optical rotation).

Compound 8: amorphous solid; $[\alpha]^{26}_D$ −72.0° (*c* 0.10, MeOH); IR (film) ν_{max} 3442 (OH), 2975, 2935, and 2908 (CH), 1731 (C=O), 1453, 1372, 1253, 1160, 1041, 897, 879, 837 cm^{-1}; ^1H NMR (C_5D_5N) δ 6.41 (1H, br s, H-1″), 5.93 (1H, d, *J* = 3.1 Hz, H-1‴), 5.62 (1H, br d, *J* = 5.2 Hz, H-6), 5.18 (1H, d, *J* = 7.7 Hz, H-1⁗), 5.23 (1H, br s, H-27a), 5.07 (1H, br s, H-27b), 4.90 (1H, d, *J* = 7.3 Hz, H-1⁗), 4.83 (1H, d, *J* = 11.3 Hz, H-26ax), 4.75 (1H, d, *J* = 3.7 Hz, H-24), 4.63 (1H, m, H-16), 4.59 (1H, d, *J* = 7.2 Hz, H-1′), 4.35 (1H, m, H-21a), 4.31 (1H, m, H-21b), 4.14 (1H, d, *J* = 3.7 Hz, H-23), 3.94 (1H, d, *J* = 11.3 Hz, H-26eq), 3.87 (1H, br m, $W_{1/2}$ = 18.7 Hz, H-3), 3.75

(1H, dd, $J = 11.8$, 4.3 Hz, H-1), 3.23 (1H, m, H-20), 2.23 and 1.91 (each 3H, s, Ac × 2), 1.53 (3H, d, $J = 6.0$ Hz, Me-6‴″), 1.35 (3H, d, $J = 6.3$ Hz, Me-6″), 1.36 (3H, s, Me-19), 1.05 (3H, s, Me-18); ^{13}C NMR, see Table 2; FABMS (positive mode) *m*/*z* 1271 $[M + Na]^+$; *anal*. C 52.59%, H 7.38% (calcd for $C_{58}H_{88}O_{29}\cdot 4H_2O$, C 52.72%, H 7.32%).

Acid Hydrolysis of 8. Compound **8** (11.3 mg) was subjected to acid hydrolysis as described for **4** to give a sugar fraction (3.8 mg). HPLC analysis of the sugar fraction under the same conditions as in the case of that of **4** showed the presence of D-apiose, L-arabinose, D-quinovose, L-rhamnose, and D-xylose: t_R (min) 7.12 (D-apiose, positive optical rotation), 7.41 (L-rhamnose, negative optical rotation), 8.16 (D-quinovose, positive optical rotation), 8.59 (L-arabinose, positive optical rotation), 9.23 (D-xylose, positive optical rotation).

Cell Culture. A375 human melanoma cells were provided through the courtesy of Dr. H. Fukuda, Meikai University School of Dentistry, Saitama, Japan, and HSC-2 human squamous cell carcinoma cells through the courtesy of Prof. M. Nagumo, Showa University, Tokyo, Japan. HepG2 human hepatoma cells were obtained from Dainippon Pharmaceutical (Osaka, Japan). Normal human pulp cells (HPC) were prepared from the explants of pulp of first premolars extracted for orthodontics purposes, after obtaining the Approval by Institutional Review Board, Meikai University School of Dentistry. HPC were used between the fifth and 10th passages. The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate in a humidified 5% CO_2 atmosphere.

Assay for Cytotoxic Activity. Cells were trypsinized and inoculated at 6×10^3 to 1.2×10^4 per each 96-microwell plate (Falcon, flat bottom, treated polystyrene, Becton Dickinson, San Jose, CA) and incubated for 24 h. After washing once with phosphate-buffered saline (PBS, 0.01 M phosphate buffer, 0.15 M NaCl, pH 7.4) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin sulfate, they were treated for 24 h without or with test compounds. They were washed once with PBS and incubated for 4 h with 0.2 mg/mL MTT in DMEM supplemented with 10% FBS. After the medium was removed, the cells were lysed with 0.1 mL of DMSO, and the relative viable cell number was determined by measuring the absorbance at 540 nm of the cell lysate, using Labsystems Multiskan (Biochromatic, Helsinki, Finland) connected to a Star/DOT Matrix printer JL-10.[11,12] The LD_{50} value, which reduces the viable cell number by 50%, was determined from the dose−response curve.

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