# Tigogenin Hexasaccharides from *Camassia cusickii*. Structural Elucidation by Modern NMR Techniques

Yoshihiro MIMAKI, Yutaka SASHIDA,* Tamotsu NIKAIDO,† and Taichi OHMOTO†
Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03
†Faculty of Pharmaceutical Sciences, Toho University,
2-2-1 Miyama, Funabashi, Chiba 274
(Received September 20, 1991)

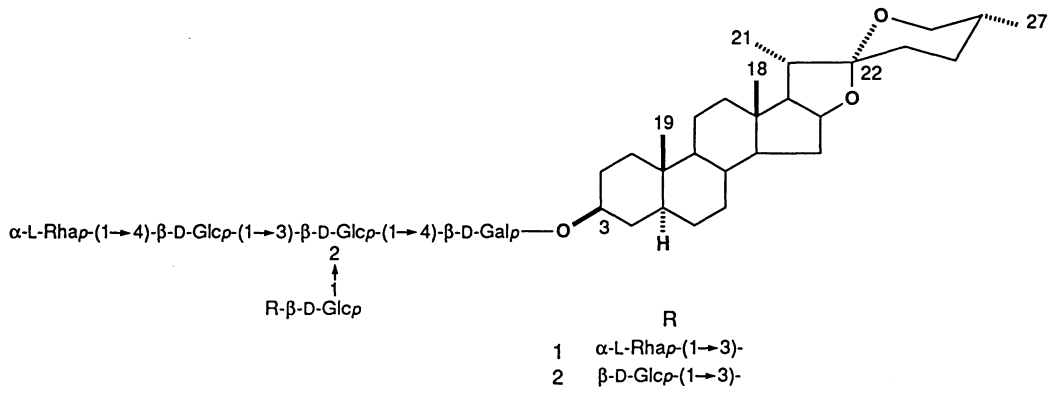
Two steroidal saponins containing six carbohydrate residues have been isolated from the bulbs of *Camassia cusickii*. Their structures were determined by modern NMR techniques, $^1$H–$^1$H COSY, HOHAHA, $^1$H–$^{13}$C COSY, and HMBC spectra to be (25*R*)-5α-spirostan-3β-yl 4-*O*-[2-*O*-[3-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-3-*O*-[4-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-β-D-glucopyranosyl]-β-D-galactopyranoside (**1**) and (25*R*)-5α-spirostan-3β-yl 4-*O*-[2-*O*-[3-*O*-(β-D-glucopyranosyl)-β-D-glucopyranosyl]-3-*O*-[4-*O*-(α-L-rhamnopyranosyl)-β-D-glucopyranosyl]-β-D-glucopyranosyl]-β-D-galactopyranoside (**2**), respectively. Compound **1** showed inhibitory activity on cyclic AMP phosphodiesterase.

Liliaceae plants are known as a rich source of steroidal saponins.[1] We have isolated a number of steroidal saponins from *Lilium*,[2] *Allium*,[3] *Smilax*[4] and *Camassia*[5] plants. Some of the saponins are unique in structure. Previously, we have reported that the bulbs of *Camassia cusickii* abundantly contain steroidal saponins: The main saponins are chlorogenin di- and trisaccharides, which have a bitter taste. The bulbs are not good to eat. Additional research on the most polar fraction of the 1-butanol soluble phase of the methanolic bulb extract of *C. cusickii* has resulted in the isolation of two steroidal saponins (**1** and **2**) with up to six sugars each. While a serious difficulty in assigning the structures of saponins is identifying the oligosaccharide unit, the concerted use of modern two-dimensional (2D) NMR techniques, $^1$H–$^1$H COSY, Homonuclear Hartmann-Hahn (HOHAHA), $^1$H–$^{13}$C COSY, and $^1$H-Detected Multiple-Bond Heteronuclear Multiple Bond Connectivity (HMBC) spectra, readily allowed the elucidation of the structure of the sugar components without such chemical degradation studies as per-methylation followed by hydrolysis and/or partial hydrolysis, which often consume relatively large amounts of material. This paper reports the structural elucidation of the saponins and their inhibitory activity on cyclic adenosine monophosphate (AMP) phosphodiesterase.

## Results and Discussion

Compounds **1** and **2** were isolated from the most polar fraction of the 1-butanol soluble phase of the methanolic bulb extract of *C. cusickii*.

Compound **1** was obtained as a white amorphous powder, $[\alpha]_D$ −43.3° (pyridine). The molecular formula, $C_{63}H_{104}O_{31}$, was confirmed by the negative-ion fast-atom-bombardment (FAB) mass spectrum (*m/z* 1355 $[M-H]^-$), elemental analysis, and the $^{13}$C NMR spectrum. The (25*R*)-spirostan skeleton of **1** was suggested by the occurrence of a resonance at δ=109.2 (C-22) in the $^{13}$C NMR spectrum,[6] and signals at δ=3.59 (dd, *J*=10.5 and 3.2 Hz, H-26a) and 3.51 (dd, *J*=10.5 and 10.5 Hz, H-26b) in the $^1$H NMR spectrum, and was supported by the characteristic absorption bands at 980, 915, 895, and 860 cm$^{-1}$ with the absorption at 895 cm$^{-1}$ being of greater intensity than that at 915 cm$^{-1}$ in the IR spectrum.[7] The $^1$H NMR spectrum of **1** contained signals for six anomeric protons at δ=6.11 (br s), 5.78 (br s), 5.48 (d, *J*=8.0 Hz), 5.13 (d, *J*=7.7 Hz), 5.10 (d, *J*=8.0 Hz), and 4.86 (d, *J*=7.7 Hz), four methyl protons of the $CH_3$–CH groups at δ=1.69 (d, *J*=6.2 Hz), 1.62 (d, *J*=6.2 Hz), 1.14 (d, *J*=6.9 Hz), and 0.70 (d, *J*=5.5 Hz), and two angular methyl protons at δ=0.83 (s) and 0.68 (s). The signals at δ=1.69 and 1.62 were due to the methyl



groups of 6-deoxyhexoses. Acid hydrolysis of **1** with 1 M (1 M=1 mol dm$^{-3}$) hydrochloric acid (dioxane–$H_2O$, 1 : 1) yielded D-glucose, D-galactose, and L-rhamnose as the carbohydrate compounds, and an aglycon, which was identified as (25*R*)-5$\alpha$-spirostan-3$\beta$-ol, that is, tigogenin. The above data indicated that **1** was a tigogenin hexasaccharide.

The determination of the sequence of the carbohydrate chains and the binding site at the aglycon residue were carried out by a series of NMR experiments. All proton signals of the carbohydrate groups could be assigned using a combination of the $^1$H–$^1$H COSY and 2D-HOHAHA spectra, the latter spectrum provided valuable information for the assignments as only the proton resonances of the same carbohydrate unit are observed clearly and undisturbed, and no signal of the other residues appears. Once the protons of each sugar were assigned, identification of the carbon signals was readily accomplished by tracing out the one-bond $^1$H–$^{13}$C connectivities through the use of the $^1$H–$^{13}$C COSY spectrum. Comparison of the carbon assignments with those of reference methyl glycosides clarified the attachment positions in the sugar chains through the glycosidation-induced shifts and the configurations at the anomeric centers.[6] Thus, **1** was shown to contain two terminal $\alpha$-L-rhamnopyranosyl units ($\delta$=102.7, 72.4, 72.6, 74.2, 69.7, and 18.6; $\delta$=102.5, 72.5, 72.8, 74.0, 70.3, and 18.5), a 3-substituted $\beta$-D-glucopyranosyl unit ($\delta$=104.3, 76.5, 83.6, 69.2, 78.3, and 62.2), a 4-substituted $\beta$-D-glucopyranosyl unit ($\delta$=104.2, 75.8, 76.4, 77.5, 77.3, and 61.1), a 2,3-disubstituted $\beta$-D-glucopyranosyl unit ($\delta$=104.6, 81.0, 88.8, 70.6, 77.4, and 63.0), and a 4-substituted $\beta$-D-galactopyranosyl unit ($\delta$=102.4, 73.1, 75.6, 80.0, 75.3, 60.7). Finally, the $^1$H–$^{13}$C long-range correlation from each anomeric proton across the glycosidic bond to the carbon of another

Table 1. $^1$H and $^{13}$C NMR Chemical Shifts for Oligosaccharide Units of **1** and **2**[a]

| | **1** ($^1$H NMR) | **1** ($^{13}$C NMR) | | **2** ($^1$H NMR) | **2** ($^{13}$C NMR) |
|---|---|---|---|---|---|
| Gal 1′ | 4.86 d (7.7) | 102.4 | Gal 1′ | 4.86 d (7.7) | 102.5 |
| 2′ | 4.43 | 73.1 | 2′ | 4.37 | 73.1 |
| 3′ | 4.09 | 75.6 | 3′ | 4.08 | 75.6 |
| 4′ | 4.56 br s | 80.0 | 4′ | 4.56 br s | 79.9 |
| 5′ | 3.98 | 75.3 | 5′ | 3.98 | 75.3 |
| 6′ | 4.66 dd (9.9, 9.9) | 60.7 | 6′ | 4.65 dd (9.9, 9.9) | 60.7 |
| | 4.20 | | | 4.20 | |
| Glc 1″ | 5.10 d (8.0) | 104.6 | Glc 1″ | 5.11 d (7.8) | 104.7 |
| 2″ | 4.21 | 81.0 | 2″ | 4.28 | 80.8 |
| 3″ | 4.06 | 88.8 | 3″ | 4.09 | 88.2 |
| 4″ | 3.71 dd (9.1, 9.1) | 70.6 | 4″ | 3.72 dd (9.4, 9.4) | 70.7 |
| 5″ | 3.76 | 77.4 | 5″ | 3.78 | 77.4 |
| 6″ | 4.42 | 63.0 | 6″ | 4.40 | 63.0 |
| | 3.97 | | | 3.96 | |
| Glc 1‴ | 5.48 d (8.0) | 104.3 | Glc 1‴ | 5.52 d (6.7) | 104.0 |
| 2‴ | 3.95 | 76.5 | 2‴ | 4.06 | 74.7 |
| 3‴ | 4.22 | 83.6 | 3‴ | 4.02 | 88.0 |
| 4‴ | 4.12 dd (9.5, 9.5) | 69.2 | 4‴ | 4.06 | 69.4 |
| 5‴ | 3.68 | 78.3 | 5‴ | 3.74 | 78.1 |
| 6‴ | 4.43 | 62.2 | 6‴ | 4.41 | 62.1 |
| | 4.32 | | | 4.24 | |
| Glc 1⁗ | 5.13 d (7.7) | 104.2 | Glc 1⁗ | 5.17 d (7.8) | 104.2 |
| 2⁗ | 3.95 | 75.8 | 2⁗ | 3.93 | 75.6 |
| 3⁗ | 3.99 | 76.4 | 3⁗ | 4.05 | 76.5 |
| 4⁗ | 4.34 | 77.5 | 4⁗ | 4.31 | 78.0 |
| 5⁗ | 3.77 | 77.3 | 5⁗ | 3.77 | 77.2 |
| 6⁗ | 4.22 | 61.1 | 6⁗ | 4.20 | 61.2 |
| | 4.07 | | | 4.04 | |
| Rha 1′′′′′ | 6.11 br s | 102.7 | Glc 1′′′′′ | 5.08 d (7.8) | 105.6 |
| 2′′′′′ | 4.68 br d (3.7) | 72.4 | 2′′′′′ | 3.97 | 75.6 |
| 3′′′′′ | 4.47 (9.8, 3.7) | 72.6 | 3′′′′′ | 4.12 | 78.0 |
| 4′′′′′ | 4.27 | 74.2 | 4′′′′′ | 4.10 | 71.6 |
| 5′′′′′ | 4.89 dq (9.4, 6.2) | 69.7 | 5′′′′′ | 3.86 | 78.5 |
| 6′′′′′ | 1.62 d (6.2) | 18.6 | 6′′′′′ | 4.45 | 62.5 |
| | | | | 4.23 | |
| Rha 1′′′′′′ | 5.78 br s | 102.5 | Rha 1′′′′′′ | 5.74 br s | 102.7 |
| 2′′′′′′ | 4.61 br d (3.7) | 72.5 | 2′′′′′′ | 4.60 br d (2.9) | 72.5 |
| 3′′′′′′ | 4.51 (9.8, 3.7) | 72.8 | 3′′′′′′ | 4.50 dd (9.3, 2.9) | 72.7 |
| 4′′′′′′ | 4.31 | 74.0 | 4′′′′′′ | 4.29 | 73.9 |
| 5′′′′′′ | 4.94 dq (9.4, 6.2) | 70.3 | 5′′′′′′ | 4.89 | 70.4 |
| 6′′′′′′ | 1.69 (6.2) | 18.5 | 6′′′′′′ | 1.67 d (6.1) | 18.5 |

a) Spectra were measured in pyridine-$d_5$. *J* values in parentheses are expressed in Hz.

substituted monosaccharide or the aglycon confirmed the sugar sequence. In the HMBC spectrum, the anomeric proton signals at $\delta$=6.11 (rhamnose), 5.78 (rhamnose), 5.48 (3-substituted glucose), 5.13 (4-substituted glucose), 5.10 (2,3-disubstituted glucose), and 4.86 (4-substituted galactose) showed correlations with the carbon signals at $\delta$=83.6, 77.5, 81.0, 88.8, 80.0, and 77.5, respectively (Fig. 1). Accordingly, the structure of the saccharide moiety was resolved, which was supported by the fragmentation patterns observed in the negative-ion FAB mass spectrum. The structure of **1** was elucidated as (25*R*)-5$\alpha$-spirostan-3$\beta$-yl 4-*O*-[2-*O*-[3-*O*-($\alpha$-L-rhamnopyranosyl)-$\beta$-D-glucopyranosyl]-3-*O*-[4-*O*-($\alpha$-L-rhamnopyranosyl)-$\beta$-D-glucopyranosyl]-$\beta$-D-glucopyranosyl]-$\beta$-D-galactopyranoside.

Compound **2**, $C_{63}H_{104}O_{32}$, $[\alpha]_D$ −53.3° (pyridine), was also a tigogenin hexasaccharide. The analysis of the $^1$H–$^1$H COSY, 2D-HOHAHA, and $^1$H–$^{13}$C COSY spectra revealed that the monosaccharides constituting **2** were a terminal $\alpha$-L-rhamnopyranoside ($\delta$=102.7, 72.5, 72.7, 73.9, 70.4, and 18.5), a terminal $\beta$-D-glucopyranoside ($\delta$=105.6, 75.6, 78.0, 71.6, 78.5, and 62.5), a 3-substituted $\beta$-D-glucopyranoside ($\delta$=104.0, 74.7, 88.0, 69.4, 78.1, and 62.1), a 4-substituted $\beta$-D-glucopyranoside ($\delta$=104.2, 75.6, 76.5, 78.0, 77.2, and 61.2), a 2,3-disubstituted $\beta$-D-glucopyranoside ($\delta$=104.7, 80.8, 88.2, 70.7, 77.4, and 63.0), and a 4-substituted $\beta$-D-galactopyranose ($\delta$=102.5, 73.1, 75.6, 79.9, 75.3, and 60.7). The anomeric proton signals at $\delta$=5.74 (rhamnose), 5.08 (glucose), 5.52 (3-substituted glucose), 5.17 (4-substituted glucose), 5.11 (2,3-disubstituted glucose), and 4.86 (4-substituted galactose) showed long-range correlations with the carbon signals at $\delta$=78.0, 88.0, 80.8, 88.2, 79.9, and 77.5, respectively, in the HMBC spectrum (Fig. 2). The structure of **2** was characterized as (25*R*)-5$\alpha$-spirostan-3$\beta$-yl 4-*O*-[2-*O*-[3-*O*-($\beta$-D-glucopyranosyl)-$\beta$-D-glucopyranosyl]-3-*O*-[4-*O*-($\alpha$-L-rhamnopyranosyl)-$\beta$-D-glucopyranosyl]-$\beta$-D-glucopyranosyl]-$\beta$-D-galactopyranoside.

The structural determination of the saponins with up to six sugars using a combination of 2D NMR techniques needed only 10—15 mg of material. The NOE experiments have been used for the determination of the sequence of the oligosaccharide chains and the connection to the aglycon,[8] however, it seemed to be difficult to find NOE between each anomeric proton signal and

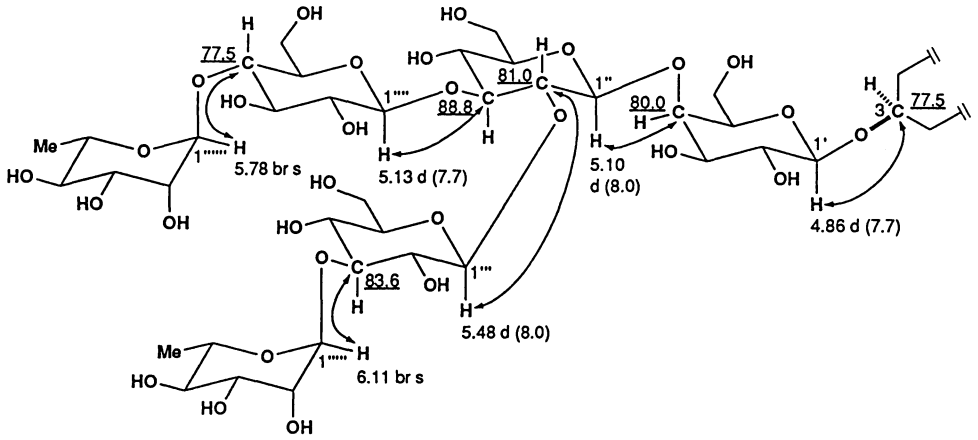



Fig. 1. $^1$H–$^{13}$C long-range correlations of **1** in pyridine-$d_5$. *J* values (Hz) in the $^1$H NMR spectrum are given in parentheses. Underlined figures indicate $^{13}$C NMR chemical shifts.

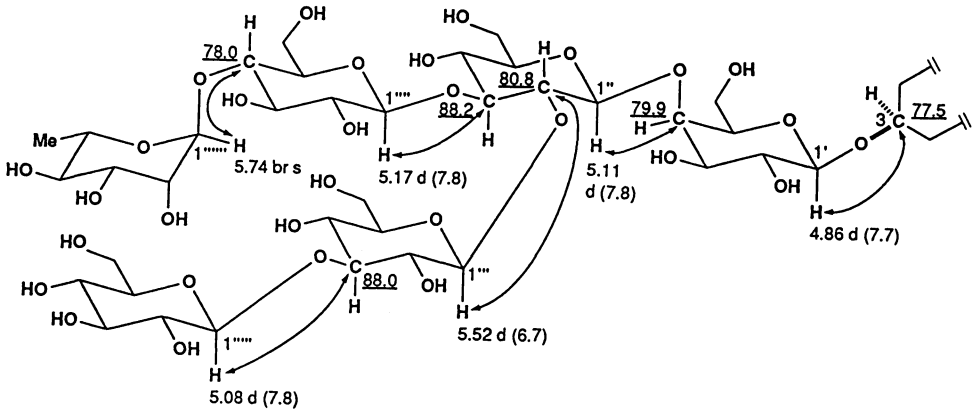



Fig. 2. $^1$H–$^{13}$C long-range correlations of **2** in pyridine-$d_5$.

the crucial methine or methylene proton signal of another substituted saccharide or the aglycon which must be severely overlapped with other sugar proton signals. This study gives a good illustration of the assignment of a complex sugar sequence by $^{1}H$–$^{13}C$ long-range coupling traversing the glycosidic linkage.

Compound **1** is a new steroidal saponin. The proposed structure of **2** is identical with that of yuccaloiside C isolated from the leaves of *Yucca aloifolia*.[9] The structure of yuccaloiside C was only presumed by comparison of the $^{13}C$ NMR data with those of yuccaloiside B and no confirmative evidence was presented.[9] In this study, we determined the structure of **2** by reliable NMR methods.

Compound **1** was active as a cyclic AMP phosphodiesterase inhibitor ($IC_{50}$ $13.2\times10^{-5}$ M),[10] while **2** was inactive ($IC_{50}$ $500\times10^{-5}$ M<). The activity seemed to depend on the structures of the terminal monosaccharides in the steroidal saponins and further studies to clarify the structure-activity relationship are now under way.

## Experimental

IR spectra were recorded on a Hitachi 260-30 spectrometer. 1D NMR spectra (ppm, *J* Hz) were taken with a Bruker AM-400 spectrometer, and 2D NMR spectra with a Bruker AM-500 spectrometer employing the standard Bruker software. HPLC was performed on a Tosoh HPLC system (Tosoh Co., Ltd.: pump, Tosoh CCPM; controller, CCP controller PX-8010; detector, Tosoh UV-8000).

**Extraction and Isolation.** The *n*-BuOH soluble phase of the methanolic bulb extract of *C. cusickii* (4.4 kg) was divided into six fractions.[5] Fraction 6 was chromatographed on silica gel with $CHCl_3$–MeOH–$H_2O$ (20 : 10 : 1) to yield a 1 : 1 mixture of **1** and **2**, as was shown by the $^{1}H$ NMR spectrum. To a pyridine solution of the mixture with 4-(dimethylamino)pyridine (200 mg) was added $Ac_2O$ and the solution was left standing overnight. The reaction mixture was then poured into ice-water, and extracted with $Et_2O$. The extract was washed successively with 0.4 M HCl and $H_2O$, and dried over anhydrous $Na_2SO_4$. The acetate was subjected to silica-gel column chromatography with 1-hexane–$Me_2CO$ (2 : 1) to give **1** peracetate and **2** peracetate. Each acetate was hydrolyzed with 4% KOH in EtOH at room temperature for 1 h and neutralized by passing through an Amberlite IR-120B column to give **1** (168 mg) and **2** (167 mg) as pure compounds.

**Compound 1.** A white amorphous powder, $[\alpha]_D^{28}$ −43.3° (*c* 0.30, pyridine). Found: C, 53.44; H, 7.74%. Calcd for $C_{63}H_{104}O_{31}\cdot7/2H_2O$: C, 53.27; H, 7.88%. Negative-ion FABMS *m/z* 1355 $[M-H]^-$, 1210 [M−deoxyhexose]$^-$, 1047 [M−deoxyhexose−hexose]$^-$, 901 [M−2×deoxyhexose−hexose]$^-$, 739 [M−2×deoxyhexose−2×hexose]$^-$, 577 [M−2×deoxyhexose−3×hexose]$^-$, 305, 199, 153, and 122; IR (KBr) 3420 (OH), 2935 (CH), 1450, 1375, 1240, 1065, 980, 915, 895, 860, 835, 810, and 700 $cm^{-1}$ ((25*R*)-spiroacetal, intensity 915<895); $^{1}H$ NMR (pyridine-$d_5$) δ=6.11 (1H, br s, H-1‴″) 5.78 (1H, br s, H-1‴″), 5.48 (1H, d, *J*=8.0 Hz, H-1″), 5.13 (1H, d, *J*=7.7 Hz, H-1‴), 5.10 (1H, d, *J*=8.0 Hz, H-1″), 4.86 (1H, d, *J*= 7.7 Hz, H-1′), 3.91 (1H, m, H-3), 3.59 (1H, dd, *J*=10.5 and 3.2 Hz, H-26a), 3.51 (1H, dd, *J*=10.5 and 10.5 Hz, H-26b), 1.69 (3H, d, *J*=6.2 Hz, H-6‴″), 1.62 (3H, d, *J*=6.2 Hz, H-6‴″), 1.14 (3H, d, *J*=6.9 Hz, H-21), 0.83 (3H, s, H-18), 0.70 (3H, d, *J*=5.5 Hz, H-27), and 0.68 (1H, s, H-19); $^{13}C$ NMR of the aglycon moiety (pyridine-$d_5$) δ=37.2, 29.9, 77.5, 34.9, 44.7, 29.0, 32.4, 35.3, 54.5, 35.9, 21.3, 40.2, 40.8, 56.5, 32.2, 81.2, 63.1, 16.6, 12.4, 42.0, 15.0, 109.2, 31.9, 29.3, 30.6, 66.9, and 17.3 (C-1—C-27).

**Compound 2.** A white amorphous powder, $[\alpha]_D^{28}$ −53.3° (*c* 0.30, pyridine). Found: C, 52.78; H, 7.71%. Calcd for $C_{63}H_{104}O_{32}\cdot7/2H_2O$: C, 52.67; H, 7.79%. Negative-ion FABMS *m/z* 1371 $[M-H]^-$, 1225 [M−deoxyhexose]$^-$, 1063 [M−deoxyhexose−hexose]$^-$, 901 [M−deoxyhexose−2×hexose]$^-$, 739 [M−deoxyhexose−3×hexose]$^-$, 577 [M−deoxyhexose−4×hexose]$^-$, 306, 199, 168, 153, and 122; IR (KBr) 3400 (OH), 2930 (CH), 1455, 1370, 1240, 1150, 1070, 1035, 980, 915, 895, 805, and 695 $cm^{-1}$ ((25*R*)-spiroacetal, intensity 915<895); $^{1}H$ NMR (pyridine-$d_5$) δ=5.74 (1H, br s, H-1‴″), 5.52 (1H, d, *J*=6.7 Hz, H-1‴), 5.17 (1H, d, *J*=7.8 Hz, H-1‴), 5.11 (1H, d, *J*=7.8 Hz, H-1″), 5.08 (1H, d, *J*=7.8 Hz, H-1‴″), 4.86 (1H, d, *J*=7.7 Hz, H-1′), 3.90 (1H, m, H-3), 3.59 (1H, dd, *J*=10.6 and 3.2 Hz, H-26a), 3.51 (1H, dd, *J*=10.6 and 10.6 Hz, H-26b), 1.67 (3H, d, *J*=6.1 Hz, H-6‴″), 1.14 (3H, d, *J*=7.0 Hz, H-21), 0.83 (3H, s, H-18), 0.70 (3H, d, *J*=5.5 Hz, H-27), and 0.66 (1H, s, H-19). The $^{13}C$ NMR spectral data of the aglycon moiety completely agreed with those of **1**.

**Acid Hydrolysis of 1 and 2.** A solution of each saponin (5 mg) in 1 M HCl (dioxane–$H_2O$, 1 : 1) (2 ml) was heated in a sealed tube at 100 °C for 1 h. After cooling, the reaction mixture was neutralized by passage through an Amberlite IRA-93ZU ($OH^-$ form) column. A Sep-Pak $C_{18}$ cartridge (Waters) was applied to fractionate the reaction mixture into the sugar fraction and the sapogenin fraction using $H_2O$–MeOH (9 : 1→1 : 9) as the eluents. The sapogenin constituting **1** and **2** was identified as tigogenin by direct TLC comparison with an authentic sample ($R_f$ 0.62, $CHCl_3$–MeOH (15 : 1)). Each sugar fraction was treated with (−)-α-methylbenzylamine (7 mg) and $Na[BH_3CN]$ (2 mg) at 40 °C for 3 h, followed by acetylation with $Ac_2O$ in pyridine containing a catalytic amount of 4-(dimethylamino)pyridine. The 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides were analyzed by HPLC.[11] The derivatives of D-glucose, D-galactose, and L-rhamnose were detected in the sugar fractions of **1** and **2**.

**Assay of Phosphodiesterase Activity.** Phosphodiesterase activity was assayed by a modification of the method of Thompson and Brooker as described in the previous paper.[10a]

Our thanks are due to the Laboratory of Organic Chemistry, Taisho Pharmaceutical Co., Ltd., for the measurements of the FAB mass spectra, and to Mrs. C. Sakuma, the Analytical Center of this college, for the measurements of the 2D NMR spectra.

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