Structures of Steroidal Saponins from the Tubers of *Brodiaea californica* and Their Inhibitory Activity on Tumor Promoter-Induced Phospholipid Metabolism

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Phytochemical examination of the fresh tubers of *Brodiaea californica* resulted in the isolation of four new steroidal saponins. Their structures were determined, by extensive spectral analysis including two-dimensional (2D) NMR spectroscopy and acid-catalyzed hydrolysis, to be (25*S*)-spirost-5-ene-1*β*,3*β*-diol [(25*S*)-ruscogenin] 1-*O*-{*O*-*β*-D-glucopyranosyl-(1→3)-*O*-*α*-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside} (1), (25*S*)-ruscogenin 1-*O*-{*O*-*β*-D-glucopyranosyl-(1→3)-*O*-*α*-L-rhamnopyranosyl-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranoside} (2), the C-20 and C-22 isomer of 2 (3) and the 6′-*O*-acetyl derivative of 2 (4), respectively. The conformations of the tetrasaccharide moiety of 2 and 4 were inspected through molecular mechanics and molecular dynamics calculation studies, showing that the acetyl group attached to C-6 of the inner glucose was near the C-21 methyl of the aglycon in the calculated preferred conformation of 4, which must cause the downfield shift of 21-Me by 0.07 ppm in comparing the ^{1}H-NMR of 4 with that of 2. The inhibitory activity of the isolated saponins on 12-*O*-tetradecanoylphorbor-13-acetate (TPA)-stimulated ^{32}P-incorporation into phospholipids of HeLa cells was evaluated to identify new antitumor-promoter compounds.

Key words *Brodiaea californica*; Liliaceae; steroidal saponin; conformational analysis; phospholipid metabolism inhibition

Our previous chemical analysis disclosed that the plants belonging to the subfamily Allioideae in Liliaceae, such as *Agapanthus inapertus*,[1)] *Ipheion uniflorum*[2)] and *Triteleia lactea*,[3)] as well as the plants of the genus *Allium*,[4)] contained certain amounts of steroidal saponins. As a continuation, we have investigated the tubers of *Brodiaea californica*,[5)] an Allioideae plant indigenous to north California, resulting in the isolation of four new steroidal saponins, (25*S*)-spirost-5-ene-1*β*,3*β*-diol [(25*S*)-ruscogenin] 1-*O*-{*O*-*β*-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-*β*-D-glucopyranoside} (**1**), (25*S*)-ruscogenin 1-*O*-{*O*-*β*-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*β*-D-xylopyranosyl-(1→3)]-*β*-D-glucopyranoside} (**2**), the C-20 and C-22 isomer of **2** (**3**) and the 6′-*O*-acetyl derivative of **2** (**4**). This paper reports the structural elucidation of **1**—**4** on the basis of spectroscopic analysis and acid-catalyzed hydrolysis, and the conformations of the tetrasaccharide moiety of **2** and **4** are shown by molecular mechanics and molecular dynamics calculation studies. Furthermore, the inhibitory activity of the isolated saponins on 12-*O*-tetradecanoylphorbor-13-acetate (TPA)-stimulated ^{32}P-incorporation into phospholipids of HeLa cells are discussed.

The concentrated 1-butanol-soluble fraction of the methanolic extract of *Brodiaea californica* tubers was repeatedly subjected to column chromatography on silica-gel, Diaion HP-20 and octadecylsilanized (ODS) silica-gel, and to HPLC to yield compounds **1**—**4**.

Compound **1** was obtained as an amorphous powder, $[\alpha]_D$ −54.0° (methanol) and assigned the molecular formula $C_{45}H_{72}O_{18}$ by the ^{13}C-NMR spectrum with 45 signals, negative-ion FAB-MS (*m/z* 899 $[M-H]^-$) and high-resolution positive-ion FAB-MS (*m/z* 923.4616 $[M+Na]^+$, Δ −0.1 mmu). The IR spectrum of **1** was consistent with the presence of hydroxyl groups (3405

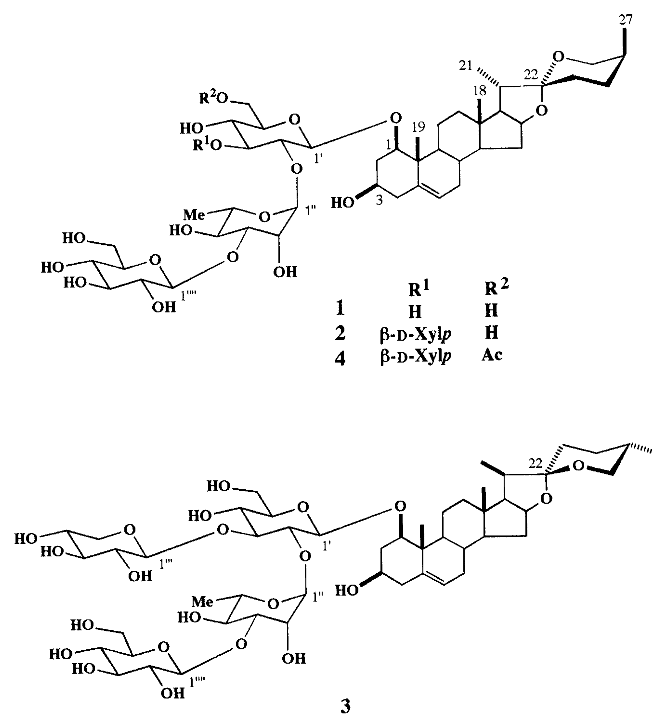

Chart 1

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Table 1. ^{13}C-NMR Spectral Data of Compounds **1**, **1a**, **1c** and **2**—**4**[a)]

C	1	1a	1c	2	3	4
1	84.1	78.1	83.6	85.2	85.3	86.1
2	38.3	44.0	37.7	38.3	38.4	38.5
3	68.1	68.1	68.1	68.1	68.2	68.3
4	43.8	43.6	43.8	43.7	43.8	43.6
5	139.5	140.4	139.5	139.5	139.4	139.3
6	124.8	124.4	124.7	124.9	124.9	124.9
7	32.0	32.3	31.9	31.9	31.9	32.0
8	33.1	33.0	33.1	33.1	32.4	33.2
9	50.6	51.9	50.4	50.5	50.6	50.6
10	42.9	43.6	42.8	42.8	42.8	42.7
11	24.1	24.2	24.2	24.1	23.8	24.2
12	40.5	40.6	40.5	40.4	41.3	40.2
13	40.3	40.3	40.3	40.3	41.6	40.4
14	57.1	57.0	57.0	57.0	57.6	57.3
15	32.4	32.5	32.4	32.3	35.4	32.4
16	81.2	81.2	81.2	81.2	83.3	81.3
17	63.0	63.0	62.9	62.9	60.5	63.0
18	16.8	16.7	16.8	16.9	16.5	17.0
19	15.1	13.9	15.1	15.2	15.2	15.1
20	42.5	42.5	42.5	42.5	46.3	42.5
21	14.8	14.9	14.8	14.8	10.2	15.0
22	109.7	109.7	109.7	109.7	106.9	109.8
23	26.4	26.4	26.4	26.4	34.6	26.4
24	26.2	26.2	26.2	26.2	29.6	26.2
25	27.6	27.6	27.6	27.6	30.3	27.6
26	65.1	65.1	65.0	65.0	67.8	65.1
27	16.3	16.3	16.3	16.3	17.3	16.3
1′	100.4		99.8	100.5	100.6	100.9
2′	79.7		79.9	75.0	75.1	74.5
3′	75.8		76.8	88.8	88.8	88.6
4′	72.8		72.7	70.2	70.2	69.6
5′	77.8		77.8	77.8	77.7	74.2
6′	63.7		63.7	63.2	63.3	64.2
1″	101.1		101.6	101.2	101.2	101.2
2″	72.0		72.6	71.9	71.9	71.9
3″	82.7		72.9	82.6	82.6	82.4
4″	73.2		74.2	73.3	73.3	73.2
5″	69.3		69.4	69.3	69.3	69.3
6″	18.7		19.1	18.9	18.9	18.9
1‴				105.4	105.4	105.4
2‴				74.6	74.6	74.6
3‴				78.3	78.3	78.4
4‴				70.6	70.6	70.6
5‴				67.2	67.3	67.2
1⁗	106.5			106.4	106.4	106.3
2⁗	76.1			76.2	76.2	76.2
3⁗	78.3			78.3	78.3	78.3
4⁗	71.8			71.7	71.7	71.8
5⁗	78.4			78.4	78.5	78.5
6⁗	62.6			62.6	62.6	62.6
Ac						170.5
						21.1

a) Spectra were measured in pyridine-d_5.

cm^{-1}). The ^{1}H-NMR spectrum exhibited signals for four typical steroid methyls at δ 1.46 and 0.90 (each s), 1.09 (d, $J=7.0$ Hz) and 1.06 (d, $J=7.1$ Hz), three anomeric protons at δ 6.49 (br s), 5.63 (d, $J=7.7$ Hz) and 4.81 (d, $J=7.7$ Hz), and an olefinic proton at δ 5.55 (br d, $J=5.3$ Hz). The ^{13}C-NMR spectrum showed six signals in the field lower than 100 ppm. The signals at δ 106.5, 101.1 and 100.4 were anomeric carbons, and the signal at δ 109.7 was assignable to the C-22 carbon of the (25*S*)-spirostan skeleton.[6)] Two olefinic carbons were observed at δ 139.5 (C) and 124.8 (CH), respectively. The above data suggested that **1** was a (25*S*)-spirostenol trisaccharide.

Acid hydrolysis of **1** with 1 N hydrochloric acid in dioxane–H_2O (1 : 1) gave an aglycon, identified as (25*S*)-ruscogenin (**1a**),[7)] and D-glucose and L-rhamnose in relations of 2 : 1. The ^{13}C-NMR assignments of the saccharide moiety of **1** were achieved by referring to those of authentic methyl glycosides, taking into account the known effects of *O*-glycosylation,[6,8)] which indicated the presence of a terminal β-D-glucopyranosyl unit (δ 106.5, 76.1, 78.3, 71.8, 78.4 and 62.6), a 3-substituted α-L-rhamnopyranosyl unit (δ 101.1, 72.0, 82.7, 73.2, 69.3 and 18.7) and a 2-substituted β-D-glucopyranosyl unit (δ 100.4, 79.7, 75.8, 72.8, 77.8 and 63.7). This was further supported by the ^{1}H-NMR data of the acetyl derivative (**1b**) of **1**, in which the 3-H methine proton of rhamnose and 2-H of the inner glucose appeared at δ 4.32 (dd, $J=9.9$, 2.7 Hz) and 3.78 (dd, $J=9.6$, 7.9 Hz), whereas the other hydroxymethine and hydroxymethylene protons appeared downfield by *O*-acylation. The above data led to two possible structures of the oligoside moiety: glucosyl-(1→3)-rhamnosyl-(1→2)-glucose or glucosyl-(1→2)-glucosyl-(1→3)-rhamnose. Mild hydrolysis of **1** with 0.2 N hydrochloric acid at 100 °C for 30 min gave D-glucose and a partial hydrolysate (**1c**), the ^{13}C-NMR spectrum of which showed the presence of a terminal α-L-rhamnopyranosyl unit (δ 101.6, 72.6, 72.9, 74.2, 69.4 and 19.1) and a 2-substituted β-D-glucopyranosyl unit (δ 99.8, 79.9, 76.8, 72.7, 77.8 and 63.7). Thus, it was evident that the structure of the trisaccharide was *O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranose. This was well supported by the fragment-ion peaks at m/z 738 $[M-\text{glucosyl}]^-$ and 591 $[M-\text{glucosyl}-\text{rhamnosyl}]^-$ in the negative-ion FAB-MS of **1**.

The linkage position between the trisaccharose and the aglycon was established by the following spectral data. In the ^{13}C-NMR spectrum of **1**, the signal due to C-1 shifted to a lower field by 6.0 ppm, whereas the signal due to C-2 moved to an upper field by 5.7 ppm, as compared with those of **1a**. All other signals remained almost unaffected. Accordingly, the structure of **1** was determined to be (25*S*)-ruscogenin 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside}.

Compound **2**, $[\alpha]_D$ −62.0°, was a more polar constituent than **1**. The molecular formula $C_{50}H_{80}O_{22}$ was deduced by the ^{13}C-NMR spectrum with 50 signals, negative-ion FAB-MS (m/z 1031 $[M-H]^-$) and high-resolution positive-ion FAB-MS (m/z 1055.5028 $[M+Na]^+$, Δ −1.2 mmu). The ^{1}H-NMR spectrum exhibited four anomeric proton signals at δ 6.50 (br s), 5.67 (d, $J=7.8$ Hz), 4.88 (d, $J=7.6$ Hz) and 4.72 (d, $J=7.8$ Hz). Complete hydrolysis of **2** with 1 N hydrochloric acid gave (25*S*)-ruscogenin and D-glucose, D-xylose and L-rhamnose in relations of 2 : 1 : 1. On comparison of the whole ^{13}C-NMR spectrum of **2** with that of **1**, a set of five additional signals, corresponding to a terminal β-D-xylopyranosyl unit, appeared at δ 105.4 (CH), 74.6 (CH), 78.3 (CH), 70.6 (CH) and 67.2 (CH_2), and the signals due to the inner glucose moiety varied, while all other signals remained almost unaffected. It was observed that the signal of C-3 of the inner glucose was markedly displaced downfield at δ 88.8 compared with that of **1**, indicating

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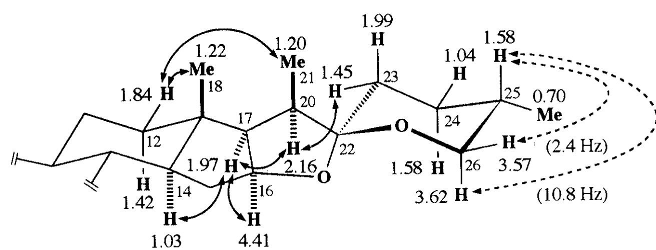


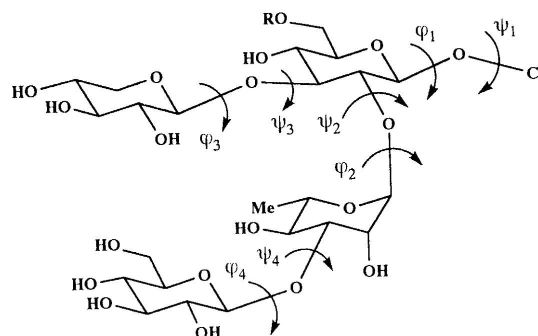
Fig. 1. ^{1}H-NMR Chemical Shifts (ppm) and NOE Correlations of **3** in Pyridine-d_5–Methanol-d_4 (10:1)

that the C-3 hydroxy position of the glucose was the glycosylated position to which the additional D-xylose was linked. Mild acid hydrolysis of **2** with 0.2 N hydrochloric acid gave **1** as a partial hydrolysate. Thus, the structure of **2** was formulated as (25*S*)-ruscogenin 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranoside}.

Compound **3** had the same molecular formula as **2**, $C_{50}H_{80}O_{22}$, and the ^{1}H- and ^{13}C-NMR spectra immediately indicated the identity of the saccharide part between **2** and **3**. Compound **3** also gave (25*S*)-ruscogenin, and D-glucose, D-xylose and L-rhamnose in a ratio of 2:1:1 on acid hydrolysis; however, in the ^{13}C-NMR spectrum of **3**, signals due to the E- and F-ring carbons did not coincide with those of **2**. Significant differences were also observed in the shift values of the 18-Me, 21-Me, and 27-Me protons between the ^{1}H-NMR spectra of **2** and **3**. These data suggested that the aglycon of **3** might be different in stereostructure from that of **2** with respect to the E- and F-ring parts. The phase-sensitive NOE correlation spectroscopy (PHNOESY) spectrum provided certain information for the stereostructure assignment. The ^{1}H signals were assigned by ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum combined with homonuclear Hartmann–Hahn (HOHAHA) spectrum prior to inspection of the NOESY spectrum, and they were recorded in a mixed solvent of pyridine-d_5–methanol-d_4 (10:1) to eliminate the exchangeable protons and minimize signal overlap. The 17-H showed clear NOE correlations with 14-H, 16-H, and 20-H, indicating a D/E *cis* ring junction, and C-20*R* configuration, which were both supported by an intense NOE between 12β(eq)-H and 21-Me. The 20-H, in turn, showed an NOE with 23(eq)-H, giving evidence for C-22*S* (Fig. 1). The C-25*S* configuration was corroborated by the fact that **3** was transformed into (25*S*)-ruscogenin on acid treatment. The chair-form conformation of the F-ring was confirmed by the ^{1}H-NMR parameters of the 26-H_2 protons ($^3J_{26(ax)\text{-H}(\delta\,3.62)-25\text{-H}}=10.8$ Hz and $^3J_{26(eq)\text{-H}(\delta\,3.57)-25\text{-H}}=2.4$ Hz). Thus, the structure of the aglycon moiety of **3** was presumed to be (20*R*,22*S*,25*S*)-spirost-5-ene-1β,3β-diol and the full structure was concluded to be (20*R*,22*S*,25*S*)-spirost-5-ene-1β,3β-diol 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-β-D-glucopyranoside}.

The negative-ion FAB-MS of **4** exhibited an $[M-H]^-$ ion at *m/z* 1073, which exceeded that of **2** by 42 mass units. The presence of an acetyl group in **4** was shown by the IR (ν_{max} 1740 cm^{-1}), ^{1}H-NMR [δ 2.10 (3H, s)] and

Table 2. Torsion Angles (°) of the Saccharide Part of **2** and **4**



2: R=H
4: R=Ac

φ	2	4	ψ	2	4
1	26.51	45.46	1	32.88	16.79
2	51.50	49.29	2	25.30	20.85
3	−29.43	−27.13	3	−21.26	−22.82
4	56.79	55.47	4	−20.78	−19.25

^{13}C-NMR [δ 170.5 (C=O) and 21.1 (Me)]. On the treatment of **4** with 3% sodium methoxide in methanol, **4** was hydrolyzed to yield **2**. Therefore, **4** must be a monoacetate of **2**. In the ^{13}C-NMR spectrum of **4**, the siganl due to the C-6 carbon of the inner glucose was shifted to a lower field by 1.0 ppm, whereas the signal due to C-5 shifted to an upper field by 3.6 ppm. Thus, the acetyl group was proven to be linked to the C-6 hydroxyl group of the inner glucose, and the structure of **4** was determined to be (25*S*)-ruscogenin 1-*O*-{*O*-β-D-glucopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-6-*O*-acetyl-β-D-glucopyranoside}.

Compounds **2**—**4**, named brodiosides A—C, have an oligoside unit composed of four monosaccharides with new sequences attached to the C-1 hydroxyl group of the aglycon. The conformations of the tetrasaccharide moiety of **2** and **4** were inspected through molecular mechanics and molecular dynamics calculation studies. The starting geometries were generated by the Metropolis Monte Carlo search method [9] and submitted to energy minimization using the Discover-cff91 force field program.[10] The local minima thus found were taken as starting structures for molecular dynamics calculations *in vacuo* at 300 K. The calculation results are shown in Table 2, in which the glycosidic torsion angles are expressed as φ (H1–C1–O1–C_x) and ψ (C1–O1–C_x–H_x). The preferred conformations calculated of **2** and **4** were essentially identical to each other except for the torsion of φ_1 and ψ_1. In **2**, the 5-H of the rhamnose was revealed to be in close proximity to 19-Me of the aglycon at a distance of 2.55 Å, which was consistent with an NOE observed between them (Fig. 2). On comparison of the ^{1}H-NMR of **4** with that of **2**, 21-Me of **4** was shifted downfield by 0.07 ppm (Fig. 3). This must be caused by the anisotropic effect of the carbonyl group of the acetyl moiety attached to the inner glucose in **4**. In the calculated preferred conformation of **4**, the carbonyl group was near the C-21 carbon at a distance of 4.36 Å (Fig. 2).

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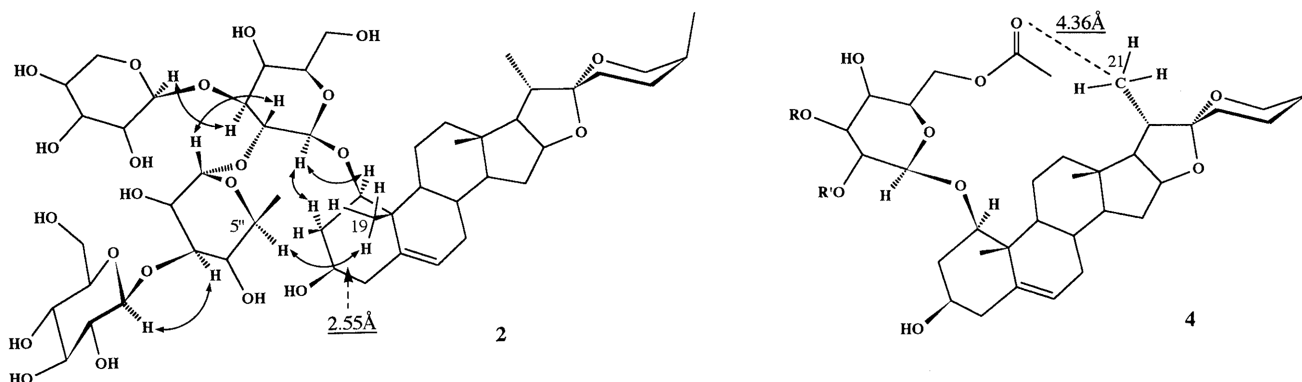

Fig. 2. Calculated Preferred Conformations of **2** and **4**
Arrows indicate the NOE correlatons.

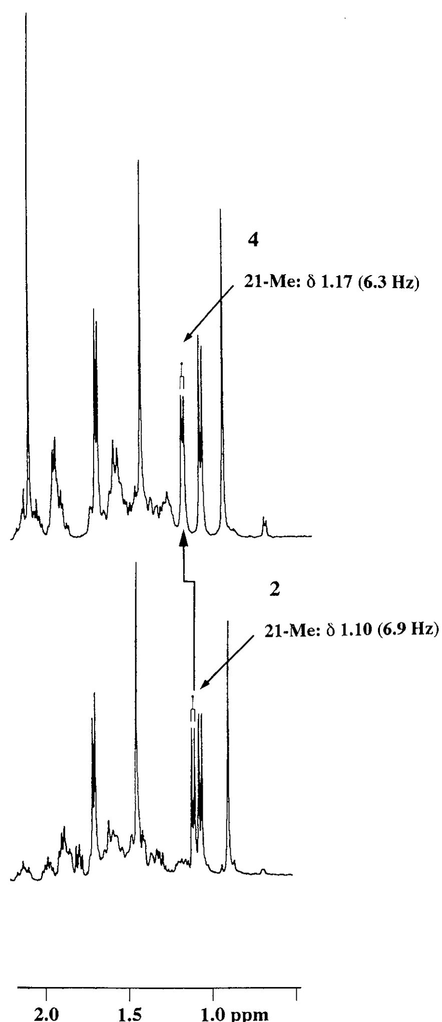

Fig. 3. ^{1}H-NMR Spectra of **2** and **4** in Pyridine-d_5

Table 3. Inhibitory Effect of the Isolated Saponins on TPA-Enhanced ^{32}P-Incorporation into Phospholipids of HeLa Cells[a]

Compounds	Inhibition (%) 50 μg/ml	Inhibition (%) 5 μg/ml
1	12.8	—
2	[b]	1.1
3	54.7	—
4	[b]	0.0

a) Data expressed as percentage of inhibition on TPA-enhanced ^{32}P incorporation, the deviations of which are within 5%. *b*) The samples exhibited cytotoxicity towards HeLa cells. —, not measured.

The isolated saponins were evaluated for inhibitory activity on TPA-stimulated ^{32}P-incorporation into phospholipids of HeLa cells to find new antitumor-promoter agents.[11] Compounds **2** and **4** were cytotoxic towards HeLa cells at the sample concentration of 50 μg/ml, and at the lower concentration (5 μg/ml), they showed no activity. Compound **3**, the C-20 and C-22 epimer of **2**, exhibited 54.7% inhibition at 50 μg/ml, without any cytotoxicity towards HeLa cells. This effect is almost as potent as that of laxogenin isolated from *Allium bakeri* as an antitumor-promoter principle.[12]

Experimental

Optical rotations were measured using a Jasco DIP-360 automatic digital polarimeter. IR spectra were recorded on a Hitachi 260-30 spectrophotometer and MS on a VG AutoSpec E or a JMX-102 instrument. 1D NMR spectra were recorded on a Bruker AM-400 spectrometer (400 MHz for ^{1}H-NMR) and 2D NMR on a Bruker AM-500 instrument (500 MHz for ^{1}H-NMR) using the usual Bruker pulse program. Chemical shifts are given as δ-values with reference to tetramethylsilane (TMS), the internal standard. Silica-gel (Fuji-Silysia Chemical), Diaion HP-20 (Mitsubishi-Kasei) and ODS silica-gel (Nacalai Tesque) were used for column chromatographies. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck) 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 Tosoh HPLC system (Tosoh: pump, CCPM; controller, CCP controller PX-8010; detector, RI-8010 or UV-8000) equipped with a Kapcell Pak C_{18} column (Shiseido, 10 mm i.d. × 250 mm, ODS, 5 μm) for preparative HPLC, and a TSK-gel ODS-Prep column (Tosoh, 4.6 mm i.d. × 250 mm, ODS, 5 μm) and a TSK-gel Silica-60 column (Tosoh, 4.6 mm i.d. × 250 mm, silica-gel, 5 μm) for analytical HPLC. TPA was obtained from Pharmacia PL Biochemicals. Radioactive inorganic phosphate (^{32}P, carrier-free) was purchased from the Japan Radioisotope Association. All other chemicals were of biochemical-reagent grade.

Extraction and Isolation Fresh bulbs of *B. californica* (3.0 kg) purchased from Heiwaen, Japan, were cut into pieces and extracted with MeOH under reflux. The MeOH extract was concentrated almost to dryness under reduced pressure, and the residue, after dilution with H_2O, was extracted with *n*-BuOH. The *n*-BuOH-soluble phase was fractionated on a silica-gel column, eluted with a gradient mixture of CH_2Cl_2–MeOH (6 : 1; 4 : 1; 2 : 1), and finally with MeOH. Fractions with the same TLC profile were combined. Three fractions (I—III) were recovered. Fractions II and III mainly contained steroidal saponins and a considerable amount of saccharides. Further fractionation of fraction II was carried out by passage through a Diaion HP-20 column using H_2O and an increasing amount of MeOH. The MeOH eluate fraction was chromatographed on

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ODS silica-gel eluted with MeOH–H_2O (4 : 1) to give **1** (75.6 mg) and **4** (50.7 mg). Fraction III was subjected to Diaion HP-20 column chromatography with a gradient mixture of H_2O–MeOH and silica-gel column chromatography with $CHCl_3$–MeOH–H_2O (20 : 10 : 1) to give a mixture of **2** and **3**, which were separated by HPLC using a mobile phase consisting of MeOH–H_2O (7 : 3) at the column temperature of 5 °C to furnish **2** (118 mg) and **3** (39.3 mg) as pure compounds.

Compound 1 Amorphous powder, $[\alpha]_D^{26}$ −54.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 899 $[M-H]^-$, 738 $[M-glucosyl]^-$, 591 $[M-glucosyl-rhamnosyl]^-$; High-resolution positive-ion FAB-MS m/z: 923.4616 $[M+Na]^+$ (Calcd for $C_{45}H_{72}O_{18}\cdot Na$: 923.4617). IR ν_{max}^{KBr} cm^{-1}: 3405 (OH), 2935 (CH), 1450, 1370, 1220, 1155, 1125, 1060, 985, 915, 890, 845, 835, 805 (intensity 915>890, (25S)-spiroacetal). ^{1}H-NMR (pyridine-d_5) δ: 6.49 (1H, br s, 1″-H), 5.63 (1H, d, J=7.7 Hz, 1⁗-H), 5.55 (1H, br d, J=5.3 Hz, 6-H), 4.94 (1H, br d, J=2.7 Hz, 2″-H), 4.86 (1H, dd, J=9.5, 2.7 Hz, 3″-H), 4.81 (1H, d, J=7.7 Hz, 1′-H), 4.04 (1H, dd, J=10.9, 2.2 Hz, 26a-H), 3.35 (1H, br d, J=10.9 Hz, 26b-H), 1.72 (3H, d, J=6.1 Hz, 6″-Me), 1.46 (3H, s, 19-Me), 1.09 (3H, d, J=7.0 Hz, 21-Me), 1.06 (3H, d, J=7.1 Hz, 27-Me), 0.90 (3H, s, 18-Me).

Acetylation of 1 Compound **1** (25 mg) was acetylated with Ac_2O in pyridine and the crude acetate was purified by silica-gel column chromatography eluted with hexane–Me_2CO (3 : 1) to give the corresponding decaacetate (**1b**) (29.3 mg) as an amorphous powder. Compound **1b**: IR ν_{max}^{KBr} cm^{-1}: 3460 (OH), 2950 (CH), 1750 (C=O), 1445, 1435, 1365, 1230, 1165, 1125, 1035, 980, 915, 900, 845, 835, 800 (intensity 915>900, (25S)-spiroacetal). ^{1}H-NMR (benzene-d_6) δ: 5.78 (1H, dd, J=9.9, 9.9 Hz, 4″-H), 5.52 (1H, br d, J=5.7 Hz, 6-H), 5.45 (1H, dd, J=9.6, 9.6 Hz, 3′-H), 5.31 (1H, dd, J=9.6, 9.6 Hz, 3⁗-H), 5.30 (1H, br s, 1″-H), 5.29 (1H, br d, J=2.7 Hz, 2″-H), 5.24 (1H, dd, J=9.6, 9.6 Hz, 4⁗-H), 5.20 (1H, dd, J=9.6, 7.8 Hz, 2⁗-H), 5.13 (1H, dd, J=9.6, 9.6 Hz, 4′-H), 4.80 (1H, m, 3-H), 4.59 (1H, q-like, J=7.1 Hz, 16-H), 4.58 (1H, d, J=7.8 Hz, 1⁗-H), 4.50 (1H, dq, J=9.9, 6.2 Hz, 5″-H), 4.36 (1H, dd, J=12.4, 3.6 Hz, 6⁗a-H), 4.32 (1H, dd, J=9.9, 2.7 Hz, 3″-H), 4.29 (1H, dd, J=12.4, 3.7 Hz, 6′a-H), 4.25 (1H, d, J=7.9 Hz, 1′-H), 4.19 (1H, dd, J=12.4, 2.3 Hz, 6⁗b-H), 4.10 (1H, dd, J=11.0, 2.8 Hz, 26a-H), 3.94 (1H, dd, J=12.4, 1.8 Hz, 6′b-H), 3.78 (1H, dd, J=9.6, 7.9 Hz, 2′-H), 3.45 (1H, dd, J=11.8, 4.0 Hz, 1-H), 3.33 (1H, br d, J=11.0 Hz, 26b-H), 3.21 (1H, ddd, J=9.6, 3.6, 2.3 Hz, 5⁗-H), 3.00 (1H, ddd, J=9.6, 3.7, 1.8 Hz, 5′-H), 2.05, 1.97, 1.95, 1.92, 1.88, 1.87, 1.71, 1.65, 1.62×2 (each 3H, s, Ac), 1.42 (3H, d, J=6.2 Hz, 6″-Me), 1.18 (3H, d, J=6.1 Hz, 21-Me), 1.17 (3H, s, 19-Me), 1.08 (3H, d, J=7.0 Hz, 27-Me), 0.94 (3H, s, 18-Me).

Compound 2 Amorphous powder, $[\alpha]_D^{28}$ −62.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 1031 $[M-H]^-$, 899 $[M-xylosyl]^-$, 871 $[M-glucosyl]^-$, 738 $[M-glucosyl-xylosyl]^-$, 723 $[M-glucosyl-rhamnosyl]^-$; High-resolution positive-ion FAB-MS m/z: 1055.5028 $[M+Na]^+$ (Calcd for $C_{50}H_{80}O_{22}\cdot Na$: 1055.5040). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2940 (CH), 1445, 1375, 1220, 1150, 1040, 985, 915, 895, 840, 835, 800 (intensity 915>895, (25S)-spiroacetal). ^{1}H-NMR (pyridine-d_5) δ: 6.50 (1H, br s, 1″-H), 5.67 (1H, d, J=7.8 Hz, 1⁗-H), 5.56 (1H, br d, J=5.7 Hz, 6-H), 4.88 (1H, d, J=7.6 Hz, 1‴-H), 4.72 (1H, d, J=7.8 Hz, 1′-H), 4.03 (1H, dd, J=10.9, 2.4 Hz, 26a-H), 3.35 (1H, br d, J=10.9 Hz, 26b-H), 1.70 (3H, d, J=6.1 Hz, 6″-Me), 1.45 (3H, s, 19-Me), 1.10 (3H, d, J=6.9 Hz, 21-Me), 1.06 (3H, d, J=7.1 Hz, 27-Me), 0.90 (3H, s, 18-Me).

Compound 3 Amorphous powder, $[\alpha]_D^{28}$ −36.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 1031 $[M-H]^-$, 900 $[M-xylosyl]^-$, 870 $[M-glucosyl]^-$, 739 $[M-glucosyl-xylosyl]^-$, 724 $[M-glucosyl-rhamnosyl]^-$. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2925 (CH), 1445, 1375, 1360, 1240, 1150, 1070, 1035, 980, 915, 900, 865, 835; ^{1}H-NMR (pyridine-d_5) δ: 6.51 (1H, br s, 1″-H), 5.68 (1H, d, J=7.6 Hz, 1⁗-H), 5.57 (1H, br d, J=6.0 Hz, 6-H), 4.89 (1H, d, J=7.7 Hz, 1‴-H), 4.73 (1H, d, J=7.8 Hz, 1′-H), 1.69 (3H, d, J=6.1 Hz, 6″-Me), 1.46 (3H, s, 19-Me), 1.24 (3H, s, 18-Me), 1.21 (3H, d, J=7.4 Hz, 21-Me), 0.68 (3H, d, J=5.1 Hz, 27-Me).

Acid Hydrolysis of 1—3 A solution of **1** (30 mg) in 1 N HCl (dioxane–H_2O, 1 : 1) (4 ml) was heated at 100 °C for 3 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU (Organo) column, and then fractionated by silica-gel CC eluted with $CHCl_3$–MeOH (19 : 1) followed by MeOH to give an aglycon (**1a**) (12.1 mg), identified as (25S)-ruscogenin and the sugar fraction (10.5 mg). (25S)-Ruscogenin: ^{1}H-NMR (pyridine-d_5) δ: 6.22 (1H, br s, 3-OH), 6.02 (1H, br d, J=4.6 Hz, 1-OH), 5.62 (1H, br d, J=5.6 Hz, 6-H), 4.52 (1H, q-like, J=7.1 Hz, 16-H), 4.06 (1H, dd, J=11.1, 2.6 Hz, 26a-H), 3.96 (1H, m, 3-H), 3.82 (1H, ddd, J=12.0, 4.6, 4.6 Hz, 1-H), 3.36 (1H, br d, J=11.1 Hz, 26b-H), 1.35 (3H, s, 19-Me), 1.12 (3H, d, J=6.9 Hz, 21-Me), 1.07 (3H, d, J=7.0 Hz, 27-Me), 0.92 (3H, s, 18-Me). The sugar fraction was shown to contain glucose and rhamnose by preliminary TLC analysis [Glc: Rf 0.38; Rha: Rf 0.66 (n-BuOH–Me_2CO–H_2O, 4 : 5 : 1)]. The sugar fraction (3 mg) was diluted with H_2O (1 ml) and treated with (−)-α-methylbenzylamine (5 mg) and $Na[BH_3CN]$ (8 mg) in EtOH (1 ml) at 40 °C for 4 h, followed by acetylation with Ac_2O (0.3 ml) in pyridine (0.3 ml). The reaction mixture was passed through a Sep-Pak C_{18} cartridge (Waters) with H_2O–MeCN (4 : 1; 1 : 1; 1 : 9, each 10 ml). The H_2O–MeCN (1 : 9) eluate fraction was further passed through a Toyopak IC-SP M cartridge (Tosoh) with EtOH (10 ml) to give a mixture of 1-[(S)-N-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives of the monosaccharides, which was then analyzed by HPLC under the following conditions: column, a TSK-gel ODS-Prep column (Tosoh, 4.6 mm i.d.×250 mm, ODS, 5 μm); solvent, MeCN–H_2O (2 : 3); flow rate, 0.8 ml/min; detection UV 230 nm and a TSK-gel Silica-60 column (Tosoh, 4.6 mm i.d.×250 mm, silica-gel, 5 μm); solvent, hexane–EtOH (19 : 1); flow rate, 0.8 ml/min; detection UV 230 nm.[13] Derivatives of D-glucose and L-rhamnose were detected in a ratio of 2 : 1. Following this procedure, **2** (3 mg) and **3** (11 mg) were subjected to acid hydrolysis. Compound **2** gave (25S)-ruscogenin (1.1 mg), and D-glucose, D-xylose and L-rhamnose (2 : 1 : 1), and **3** also gave (25S)-ruscogenin (3 mg), and D-glucose, D-xylose and L-rhamnose (2 : 1 : 1).

Partial Hydrolysis of 1 A solution of **1** (25 mg) in 0.2 N HCl (dioxane–H_2O, 1 : 1) (4 ml) was heated at 100 °C for 3 h under an Ar atmosphere. The reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU column and was purified by silica-gel column chromatography eluted with $CHCl_3$–MeOH–H_2O (50 : 10 : 1; 30 : 10 : 1) to give a partial hydrolysate, **1c** (4.9 mg) as an amorphous powder. Compound **1c**: $[\alpha]_D^{24}$ −62.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 738 $[M]^-$, 591 $[M-rhamnosyl]^-$. IR ν_{max}^{KBr} cm^{-1}: 3415 (OH), 2930 (CH), 1465, 1450, 1375, 1220, 1125, 1055, 980, 915, 890, 840, 835, 805 (intensity 915>890, (25S)-spiroacetal); ^{1}H-NMR (pyridine-d_5) δ: 6.51 (1H, br s, 1″-H), 5.56 (1H, br d, J=5.4 Hz, 6-H), 4.91 (1H, d, J=7.4 Hz, 1′-H), 4.89 (overlapping with H_2O signal, 5″-H), 4.74 (1H, br d, J=3.6 Hz, 2″-H), 4.64 (1H, dd, J=9.7, 3.6 Hz, 3″-H), 4.52 (1H, br d, J=11.1 Hz, 6′a-H), 4.43 (1H, q-like, J=7.3 Hz, 16-H), 4.35 (1H, dd, J=9.7, 9.7 Hz, 4″-H), 4.30 (1H, dd, J=11.1, 4.3 Hz, 6′b-H), 4.29 (1H, dd, J=8.8, 8.8 Hz, 3′-H), 4.22 (1H, dd, J=8.8, 8.8 Hz, 4′-H), 4.05 (1H, dd, J=10.6, 2.7 Hz, 26a-H), 4.02 (1H, dd, J=8.8, 7.4 Hz, 2′-H), 3.92 (1H, dd, J=11.7, 3.7 Hz, 1-H), 3.87 (1H, br dd, J=8.8, 4.3 Hz, 5′-H), 3.80 (1H, m, 3-H), 3.35 (1H, br d, J=10.6 Hz, 26b-H), 1.80 (3H, d, J=6.1 Hz, 6″-Me), 1.46 (3H, s, 19-Me), 1.11 (3H, d, J=6.9 Hz, 21-Me), 1.07 (3H, d, J=7.1 Hz, 27-Me), 0.92 (3H, s, 18-Me). Following this procedure, **2** (30 mg) were subjected to partial acid hydrolysis to give **1** (3.5 mg) as a partial hydrolysate.

Compound 4 Amorphous powder, $[\alpha]_D^{28}$ −12.0° (c=0.10, MeOH). Negative-ion FAB-MS m/z: 1073 $[M-H]^-$, 1031 $[M-acetyl]^-$, 911 $[M-glucosyl]^-$, 781 $[M-glucosyl-xylosyl]^-$, 765 $[M-glucosyl-rhamnosyl]^-$, 724 $[M-glucosyl-rhamnosyl-acetyl]^-$. IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2935 (CH), 1740 (C=O), 1445, 1370, 1240, 1150, 1035, 985, 915, 895, 845, 835, 805 (intensity 915>895, (25S)-spiroacetal); ^{1}H-NMR (pyridine-d_5) δ: 6.51 (1H, br s, 1″-H), 5.68 (1H, d, J=7.8 Hz, 1⁗-H), 5.60 (1H, br d, J=5.7 Hz, 6-H), 4.87 (1H, d, J=7.6 Hz, 1‴-H), 4.63 (1H, d, J=7.9 Hz, 1′-H), 4.04 (1H, dd, J=11.0, 2.4 Hz, 26a-H), 3.34 (1H, br d, J=11.0 Hz, 26b-H), 2.10 (3H, s, Ac), 1.69 (3H, d, J=6.1 Hz, 6″-Me), 1.43 (3H, s, 19-Me), 1.17 (3H, d, J=6.3 Hz, 21-Me), 1.06 (3H, d, J=7.0 Hz, 27-Me), 0.93 (3H, s, 18-Me).

Alkaline Hydrolysis of 4 Compound **4** (5 mg) was treated with 3% NaOMe in MeOH at room temperature for 30 min. The reaction mixture was neutralized by passing it through an Amberlite IR-120B column (Organo), and the eluate was chromatographed on silica-gel with $CHCl_3$–MeOH–H_2O (20 : 10 : 1) to give **2** (3.4 mg).

Conformational Calculation The starting geometries were generated by the Metropolis Monte Carlo search method[9] using the CONFSEARCH program,[14] giving 10000 conformers at 300 K followed by submission to energy minimization using the Discover-cff91 force field program.[10] The local minima thus found were taken as starting structures for molecular dynamics calculations *in vacuo* at 300 K and at a time step of 1 fs. The equilibration time was 1 ps and the total simulation time was 1000 ps. Trajectory frames were saved every 1 ps. The trajections were then examined with the Analysis module of Insight II.[15] Calculations were performed on an IRIS Indigo Elan computer.

Cell Culture and Assay of ^{32}P-Incorporation into Phospholipids of the

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Cultured Cells HeLa cells were cultured as a monolayer in Eagle's minimum essential medium supplemented with 10% calf serum under a humidified atmosphere of 5% CO_2 in air. HeLa cells were incubated with the test samples (50 μg/ml and/or 5 μg/ml), and after 1 h, ^{32}P (370 kBq/culture) was added with or without TPA (50 nM). Incubation was continued for 4 h, then the radioactivity incorporated into phospholipid fraction was measured.[16]

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References and Notes

1) Nakamura O., Mimaki Y., Sashida Y., Nikaido T., Ohmoto T., *Chem. Pharm. Bull.*, **41**, 1784 (1993).
2) Nakamura O., Mimaki Y., Sashida Y., Nikaido T., Ohmoto T., *Chem. Pharm. Bull.*, **42**, 1116 (1994).
3) Mimaki Y., Nakamura O., Sashida Y., Nikaido T., Ohmoto T., *Phytochemistry*, **38**, 1279 (1995).
4) Sashida Y., Kawashima K., Mimaki Y., *Chem. Pharm. Bull.*, **39**, 698 (1991); Kawashima K., Mimaki Y., Sashida Y., *Phytochemistry*, **30**, 3063 (1991); Kawashima K., Mimaki Y., Sashida Y., *Chem. Pharm. Bull.*, **39**, 2761 (1991); Kawashima K., Mimaki Y., Sashida Y., *Phytochemistry*, **32**, 1267 (1993); Mimaki Y., Kawashima K., Kanmoto T., Sashida Y., *ibid.*, **34**, 799 (1993); Mimaki Y., Nikaido T., Matsumoto K., Sashida Y., Ohmoto T., *Chem. Pharm. Bull.*, **42**, 710 (1994).
5) A part of this paper has been reported in our preliminary communication: Nakamura O., Mimaki Y., Sashida Y., Nikaido T., Ohmoto T., *Chem. Lett.*, **1994**, 805.
6) Agrawal P. K., Jain D. C., Gupta R. K., Thakur R. S., *Phytochemistry*, **24**, 2479 (1985).
7) Watanabe Y., Sanada S., Ida Y., Shoji J., *Chem. Pharm. Bull.*, **31**, 1980 (1983).
8) Agrawal P. K., *Phytochemistry*, **31**, 3307 (1992).
9) Chang G., Guida W. C., Still W. C., *J. Am. Chem. Soc.*, **111**, 4379 (1989).
10) Discover 2.8 Program, Biosym Technol. Inc., San Diego, CA, U.S.A.
11) Nishino H., Iwashima A., Fujiki H., Sugimura T., *Gann*, **75**, 113 (1984); Nishino H., Iwashima A., Nakadate T., Kato R., Fujiki H., Sugimura T., *Carcinogenesis*, **5**, 283 (1984); Nishino H., Nishino A., Takayasu J., Hasegawa T., Iwashima A., Hirabayashi K., Iwata S., Shibata S., *Cancer Res.*, **48**, 5210 (1988).
12) Nishino H., Nishino A., Satomi Y., Takayasu J., Hasegawa T., Tokuda H., Fukuda T., Tanaka H., Shibata S., Fujita K., Okuyama T., *J. Kyoto Pref. Univ. Med.*, **99**, 1159 (1990).
13) Oshima R., Kumanotani J., *Chem. Lett.*, **1981**, 943; Oshima R., Yamauchi Y., Kumanotani J., *Carbohydr. Res.*, **107**, 169 (1982).
14) CONFSEARCH program, Daikin Industries Ltd., Tokyo, Japan.
15) Insight II 2.3.0. Program, Biosym Technol. Inc., San Diego, CA, U.S.A.
16) Nishino H., Fujiki H., Terada M., Sato S., *Carcinogenesis*, **4**, 107 (1983).

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