Steroidal Saponins from the Underground Parts of *Hosta longipes* and Their Inhibitory Activity on Tumor Promoter-Induced Phospholipid Metabolism

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Phytochemical study on the underground parts of *Hosta longipes* gave six new steroidal saponins together with a known one. The structures of the new compounds were determined by detailed analysis of their ^{1}H- and ^{13}C-NMR spectra including two-dimensional NMR spectroscopy, acid-catalyzed hydrolysis followed by chemical correlation, and by comparison with spectral data of known compounds. The isolated saponins and their aglycones were examined for inhibitory activity on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated ^{32}P-incorporation into phospholipids of HeLa cells to identify new antitumor-promoter compounds.

Key words *Hosta longipes*; Liliaceae; steroidal saponin; phospholipid metabolism; antitumor-promoter activity

The steroidal saponins are plant glycosides with common properties such as froth formation, haemolytic activity, toxicity to fish and complex formation with cholesterol. Recently, some steroidal saponins have been isolated as active compounds through research to identify new antidiabetics,[1] antitumor-agents in association with modification of the immune system,[2] antitussives for dry cough,[3] and platelet aggregation inhibitors[4] of natural origin, which prompted us to engage in systematic studies of the steroidal saponins of the Liliaceae plants.[5]

The genus *Hosta* with about 20 species belongs to the subfamily Asphodeloideae in Liliaceae and has a distribution in East Asia. The young leaves and buds of the plants are edible and the leaves and rhizomata have been used as a folk medicine in China and Japan.[6] Several steroidal sapogenins were isolated from the neutral fraction of the saponified methanolic extracts of *Hosta* (*H.*) *plantaginea*, *H. sieboldiana*, *H. longipes*, *H. montana* var. *liliflora* and *H. kiyosumiensis* in the 1960's,[7] however, no steroidal saponin appears to have been isolated from the *Hosta* species. Our attention to the steroidal saponins in the underground parts of *H. longipes* has resulted in finding six new steroidal saponins and a known one. This paper reports the structural determination of these new saponins by detailed analysis of their ^{1}H- and ^{13}C-NMR spectra including two-dimensional NMR spectroscopy, acid-catalyzed hydrolysis followed by chemical correlation, and by comparison with spectral data of known compounds. In addition, inhibitory activity on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated ^{32}P-incorporation into phospholipids of HeLa cells by the isolated saponins and their aglycones is also discussed.

Fresh underground parts of *H. longipes* (10 kg) were extracted with hot methanol. A series of chromatographic separations of the 1-butanol-soluble phase of the methanolic extract gave compounds **1**—**7**.

Compound **1** ($C_{50}H_{82}O_{23}$) was a known steroidal saponin and identified by negative-ion FAB-MS, IR, ^{1}H- and ^{13}C-NMR spectra as (25*R*)-5α-spirostane-2α,3β-diol (gitogenin) 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}, *i.e.*, F-gitonin.[8]

Compound **2** ($C_{50}H_{80}O_{23}$) was obtained as a white amorphous powder. The ^{1}H-NMR spectrum showed signals for four anomeric protons at δ 5.57 (d, J=7.4 Hz), 5.23 (d, J=7.7 Hz), 5.18 (d, J=7.9 Hz) and 4.85 (d, J=7.7 Hz), and four characteristic steroid methyls: two appeared as singlets at δ 1.08 and 0.66, indicating the presence of two angular methyl groups and the other two as doublets at δ 1.35 (J=6.9 Hz) and 0.70 (J=5.4 Hz) assignable to secondary methyl groups. The presence of a carbonyl group in **2** was recognized by the IR (1700 cm^{-1}) and ^{13}C-NMR spectra (δ 212.7). When **2** was submitted to acid hydrolysis with 1 N hydrochloric acid (dioxane–H_2O, 1 : 1), **2** was hydrolyzed to yield an aglycone (**2a**), identified as (25*R*)-3β-hydroxy-5α-spirost-5-en-12-one (hecogenin),[9] and D-glucose, D-galactose and D-xylose in a ratio of 2 : 1 : 1. The results of acid hydrolysis and the ^{1}H- and ^{13}C-NMR spectral data provided evidence for the identity of the saccharide sequences between **1** and **2** (Table 1). Thus, the structure of **2** was shown to be hecogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}. The C-25 epimer (25*S*) of **2** has been already isolated from *Chlorophytum malayense*.[10] To the best of our knowledge, **2** has not been reported previously.

Compoud **3** ($C_{51}H_{84}O_{24}$) gave gitogenin (**3a**), and D-glucose and D-galactose in a ratio of 3 : 1 on acid hydrolysis. All the spectral features were similar to those of **1**. On comparison of the ^{13}C-NMR spectrum of **3** with that of **1**, a set of signals, corresponding to a terminal β-D-glucopyranosyl unit appeared instead of the terminal β-D-xylopyranosyl unit; all other signals remained almost unaffected, indicating that *O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-

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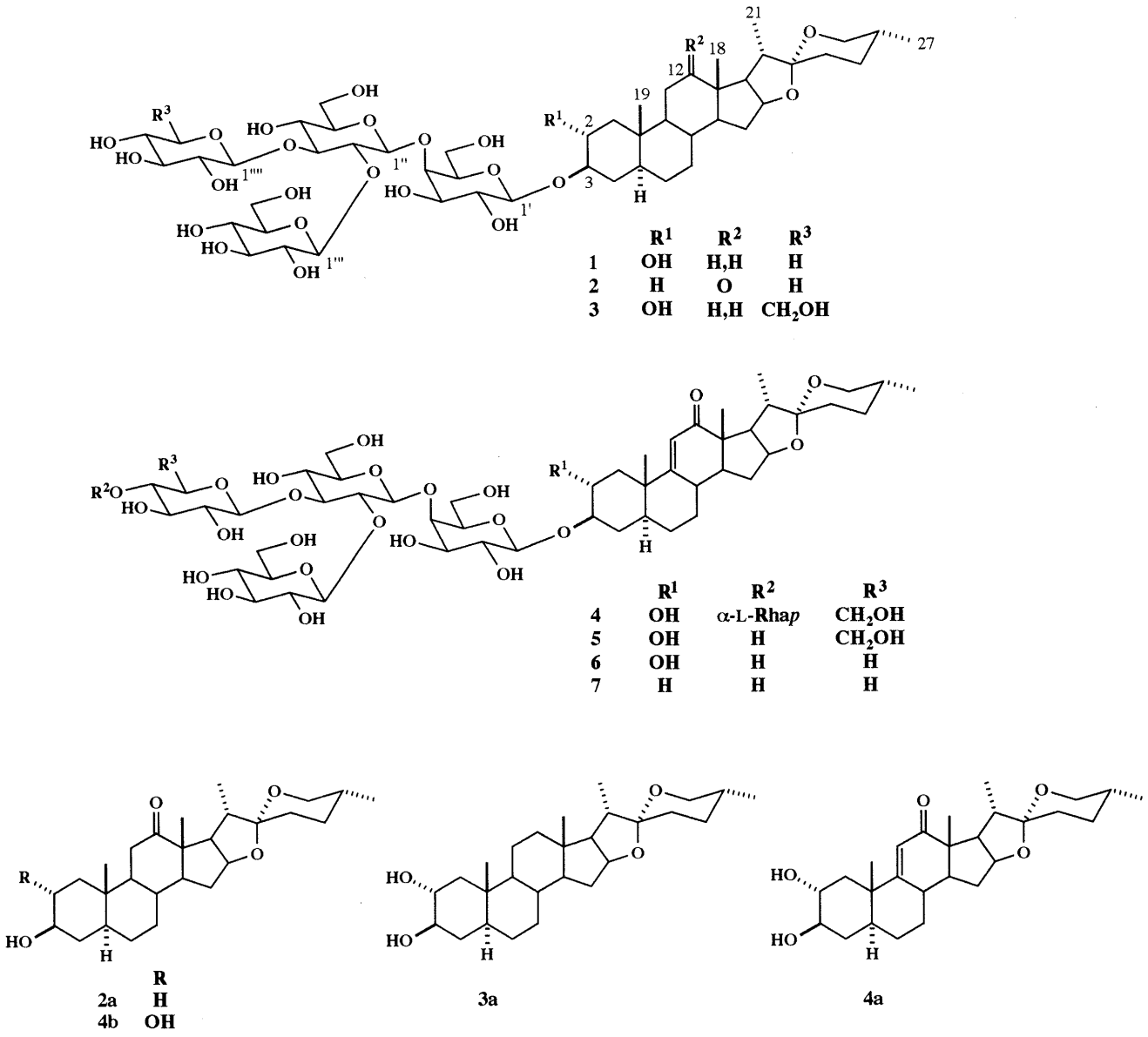

Chart 1

(1→4)-β-D-galactopyranosyl moiety[11] was linked to the C-3 hydroxyl group of gitogenin. The structure of **3** was assigned as gitogenin 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}.

Compound **4** was obtained as a white amorphous powder with the molecular formula $C_{57}H_{90}O_{29}$, which was deduced from the negative-ion FAB-MS (*m/z* 1238 $[M]^-$), ^{13}C-NMR spectrum (57 signals) and elemental analysis. The IR, UV and ^{13}C-NMR spectra were consistent with the presence of a conjugated carbonyl group in **4** [IR: 1655 cm^{-1}; UV λ_{max}: 238 nm (log ε 3.95); ^{13}C-NMR δ: 204.2 (C=O), 170.5 (C) and 120.1 (CH)]. The ^{1}H-NMR spectrum of **4** displayed signals for five anomeric protons at δ 5.73 (br s), 5.59 (d, *J*=7.8 Hz), 5.27 (d, *J*=7.9 Hz), 5.14 (d, *J*=7.9 Hz) and 4.90 (d, *J*=7.7 Hz) and an olefinic proton at δ 5.94 (1H, br s) as well as four steroid methyls at δ 1.39 (d, *J*=6.8 Hz), 0.71 (d, *J*=5.3 Hz), 1.00 (s) and 0.90 (s). Acid hydrolysis of **4** gave an aglycone ($C_{27}H_{40}O_5$) (**4a**), and D-glucose, D-galactose and L-rhamnose in a ratio of 3:1:1. The above data indicated that **4** was a spirostanol pentasaccharide with a conjugated carbonyl group.

The ^{1}H–^{1}H correlation spectroscopy (COSY), homonuclear Hartmann–Hahn (HOHAHA), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) and ^{1}H-detected heteronuclear multiple-bond connectivity (HMBC) spectra, which were measured in a mixed solvent of pyridine-d_5–methanol-d_4 (11:1) to eliminate the exchangeable protons and minimize signal overlap, allowed us to assign all the ^{1}H and ^{13}C signals from the aglycone moiety as shown in Table 2. In the HMBC spectrum, the quaternary carbon signal observed at δ 51.5 showed correlation peaks with the two-bond coupled proton at $\delta_{17\text{-H}}$ 2.57 and three-bond coupled proton at $\delta_{20\text{-H}}$ 1.97 and was assigned to C-13. Another quaternary carbon signal at δ 40.6 was assignable to C-10, which was correlated to both 1β(eq)-H at δ 2.17 and 1α(ax)-H at δ 1.53. The correlations between $\delta_{C\text{-}13}$ 51.5 and δ_{Me} 0.98, and $\delta_{C\text{-}10}$ 40.6 and δ_{Me} 0.92 led to the assignments of the signals at δ 0.98 and 0.92 to 18-Me and 19-Me, respectively. Cross peaks between $\delta_{C=O}$ 204.6 and each $\delta_{18\text{-Me}}$ 0.98 and

Table 1. ^{13}C-NMR Spectral Data for Compounds **1**, **2**, **2a**, **3**, **4**, **4a**, **4b** and **5**—**7**[a]

	C	1	2	2a	3	4	4a	4b	5	6	7
	1	45.6	36.7	37.0	45.6	43.5	44.4	45.9	43.5	43.4	35.0
	2	70.4	29.6	32.2	70.5	70.3	72.9	72.8	70.3	70.4	29.6
	3	84.3	77.1	70.3	83.2	83.4	76.1	76.5	83.4	83.5	76.7
	4	34.1	34.7	39.1	34.1	33.8	36.8	37.0	33.8	33.8	34.6
	5	44.6	44.5	45.0	44.6	42.5	43.1[b]	45.0	42.5	42.5	42.5
	6	28.1	28.6	28.7	28.1	27.2	27.4	28.0	27.2	27.1	27.9
	7	32.1	31.5[b]	31.5[b]	32.1	32.5	32.7	31.5[b]	32.5	32.5	32.6
	8	34.6	34.4	34.4	34.6	36.2	36.3	33.8	36.2	36.2	36.9
	9	54.4	55.5	55.7	54.4	170.5	170.9	55.6	170.5	170.5	171.3
	10	36.9	36.3	36.4	36.9	40.6	40.5	37.9	40.6	40.5	39.5
	11	21.4	38.0	38.1	21.4	120.1	120.2	38.2	120.1	120.1	120.0
	12	40.1	212.7	212.8	40.1	204.2	204.3	212.6	204.2	204.2	204.3
	13	40.8	55.4	55.4	40.8	51.4	51.4	55.4	51.4	51.4	51.3
	14	56.3	55.9	56.1	56.3	52.7	52.8	55.9	52.7	52.7	52.7
	15	32.2	31.8[b]	31.8[b]	32.2	31.8	31.9[c]	31.8[b]	31.8	31.8	31.8
	16	81.3	79.8	79.7	81.1	80.2	80.3	79.7	80.2	80.2	80.2
	17	63.0	54.3	54.3	63.0	54.6	54.6	54.3	54.6	54.5	54.5
	18	16.6	16.1	16.1	16.6	15.2	15.3	16.1	15.2	15.2	15.2
	19	13.4	11.7	11.9	13.4	19.4	19.6	13.2	19.4	19.4	18.3
	20	42.0	42.6	42.7	42.0	43.0	43.0[b]	42.7	43.0	43.0	43.0
	21	15.0	13.9	13.9	15.0	13.7	13.8	13.9	13.7	13.7	13.7
	22	109.2	109.3	109.3	109.2	109.5	109.5	109.3	109.5	109.5	109.5
	23	31.8	31.7[b]	31.8[b]	31.8	31.8	31.8[c]	31.7[b]	31.8	31.8	31.8
	24	29.3	29.2	29.2	29.3	29.2	29.2	29.2	29.2	29.2	29.2
	25	30.6	30.6	30.6	30.6	30.6	30.6	30.6	30.6	30.5	30.5
	26	66.9	67.0	67.0	66.9	67.0	67.0	67.0	67.0	67.0	67.0
	27	17.3	17.3	17.3	17.3	17.3	17.3	17.3	17.3	17.3	17.3
Gal	1′	103.3	102.4		103.3	103.3			103.2	103.2	102.5
	2′	72.5	73.2		72.6	72.5			72.5	72.5	73.1
	3′	75.5	75.6		75.5	75.4			75.5	75.5	75.6
	4′	79.4	79.9		79.7	79.8			79.8	79.5	79.9
	5′	75.7	75.4		75.7	75.7			75.7	75.8	75.4
	6′	60.6	60.6		60.5	60.0			60.6	60.6	60.6
Glc	1″	104.8[b]	105.0[c]		104.8[b]	104.8			104.8[b]	104.7[b]	104.9[b]
	2″	81.1	81.3		81.3	81.3			81.3	81.2	81.3
	3″	87.0	86.9		88.7	88.4			88.7	87.1	86.8
	4″	70.4	70.5		70.7	70.5			70.8	70.3	70.5
	5″	77.6	77.6		77.5	77.5			77.6	77.6	77.6
	6″	62.7	62.5		62.6	61.2			62.7	62.7	62.5
Glc	1‴	104.7[b]	104.8[c]		104.7[b]	104.7			104.7[b]	104.7[b]	104.8[b]
	2‴	76.1	76.2		76.0	76.0			76.0	76.0	76.2
	3‴	78.1	78.7		78.2	78.3			78.3	78.1	78.6[c]
	4‴	71.4	71.1		71.3	71.4			71.3	71.4	71.1
	5‴	78.5	77.8		78.6[c]	78.5			78.6[c]	78.5	77.8
	6‴	63.0	63.0		63.0	62.7			63.0	62.9	63.0
Xyl	1⁗	105.0	105.1		104.5	104.3			104.6	104.9	105.1
(Glc)	2⁗	75.1	75.1		75.3	75.5			75.4	75.1	75.1
	3⁗	78.7	78.7		78.7[c]	76.8			78.7[c]	78.7	78.7[c]
	4⁗	70.8	70.7		71.6	78.6			71.7	70.8	70.7
	5⁗	67.3	67.3		78.4	77.2			78.4	67.3	67.3
	6⁗				62.3	62.9			62.4		
Rha	1⁗′					102.8					
	2⁗′					72.5					
	3⁗′					72.7					
	4⁗′					73.9					
	5⁗′					70.6					
	6⁗′					18.5					

a) Spectra were measured in pyridine-d_5. b, c) Assignments may be interchanged.

$\delta_{17\text{-H}}$ 2.57, and between δ_C 170.8 and $\delta_{19\text{-Me}}$ 0.92, accounted for the 9-en-12-one structure (Fig. 1). The nuclear Overhauser effect (NOE) correlations, 19-Me/4β(ax)-H, 19-Me/8β(ax)-H, 18-Me/20-H, 17-H/14-H, 16-H and 21-Me, and 20-H/23(eq)-H in the phase-sensitive nuclear Overhauser effect spectroscopy (PHNOESY) spectrum of **4** provided evidences for the usual A/B *trans*, C/D *trans* and D/E *cis* ring junctions, and 20*S* and 22*R* configurations. Other NOE networks, 2-H/19-Me, and 3-H/1α(ax)-H and 5α(ax)-H confirmed 2β(ax)-H and 3α(ax)-H configurations (Fig. 2). The stereochemistry of C-25 was determined to be *R*, the ^{1}H-NMR parameters of the 26-H_2 protons were δ 3.56 (dd, J=10.5, 3.3 Hz) and 3.46 (dd, J=10.5, 10.5 Hz) and there was an NOE correlation

Table 2. ^{1}H- and ^{13}C-NMR Chemical Shifts for Aglycone Moiety of **4**[a)]

	^{1}H-NMR	^{13}C-NMR
1eq	2.18 dd (12.5, 4.8)	43.4
1ax	1.53	
2	3.97	70.3
3	3.80	83.3
4eq	1.92 ddd (11.6, 4.8, 4.8)	33.7
4ax	1.50 ddd (11.6, 11.6, 11.6)	
5	1.17 dddd (11.6, 11.6, 4.8, 4.8)	42.6
6a	1.27	27.2
6b	1.20	
7eq	1.82	32.6
7ax	0.95	
8	2.42	36.3
9	—	170.8
10	—	40.6
11	5.87 s	120.1
12	—	204.6
13	—	51.5
14	1.75	52.8
15α	2.19	31.8
15β	1.61	
16	4.49	80.3
17	2.57 dd (7.4, 7.4)	54.6
18	0.98 s	15.3
19	0.92 s	19.4
20	1.97 dq (7.4, 6.9)	43.0
21	1.34 d (6.9)	13.7
22	—	109.6
23eq	1.72	31.8
23ax	1.60	
24 (2H)	1.55	29.3
25	1.56	30.6
26eq	3.57 dd (10.5, 3.3)	67.1
26ax	3.46 dd (10.5, 10.5)	
27	0.71 d (5.8)	17.3

a) Spectra were measured in pyridine-d_5–methanol-d_4 (11 : 1). J values in parentheses are expressed in Hz.

between 26(ax)-H and 27-Me. Thus, the structure of the aglycone moiety of **4** was confirmed to be (25*R*)-2α,3β-dihydroxy-5α-spirost-9-en-12-one. This was further supported by the following chemical transformations of the aglycone (**4a**). Catalytic hydrogenation of **4a** over platinum (IV) oxide in ether–acetic acid (3 : 1) gave a dihydro derivative (**4b**). The ^{13}C shifts of the B—F ring carbons were in good agreement between **4b** and **2a**.

The structure of the pentasaccharide moiety and its linkage position to the aglycone were determined based on the following data. On comparison of the ^{13}C signals from the saccharide moiety of **4** with those of **3**, a set of six additional signals, corresponding to a terminal α-L-rhamnopyranosyl unit appeared. The assignments of the ^{1}H and ^{13}C signals due to the saccharide moiety was as shown in Table 3 through interpretation of the ^{1}H–^{1}H COSY spectrum combined with the HOHAHA and HMQC spectra. The $^3J_{C,H}$ correlation from each anomeric proton, across the glycosidic bond to the carbon of another substituted monosaccharide or the aglycone, revealed the sugar sequences. In the HMBC spectrum, the anomeric proton signals at δ 5.61 (terminal rhamnose), 5.51 (terminal glucose), 5.20 (4-substituted glucose), 5.06 (2,3-disubstituted glucose) and 4.84 (4-substituted galactose) exhibited correlations with the ^{13}C signals at δ 78.5 (C-4 of 4-substituted glucose), 81.3 (C-2 of 2,3-disubstituted glucose), 88.2 (C-3 of 2,3-disubstituted glucose), 79.8 (C-4 of 4-substituted galactose) and 83.3 (C-3 of aglycone), respectively (Fig. 3). The above data unambiguously identified the pentasaccharide structure as *O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside and indicated its linkage to the aglycone C-3 hydroxyl group. The FAB-MS fragments were assigned as shown in Fig. 3. According-

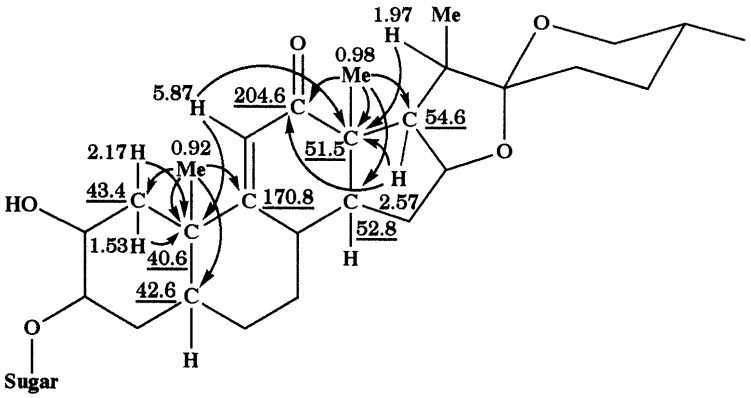

Fig. 1. ^{1}H–^{13}C Long-Range Correlations of the Aglycone Moiety of **4** in Pyridine-d_5–Methanol-d_4 (11 : 1)

$^nJ_{C,H}$ was optimized for 8 Hz. Underlined figures indicate ^{13}C-NMR chemical shifts.

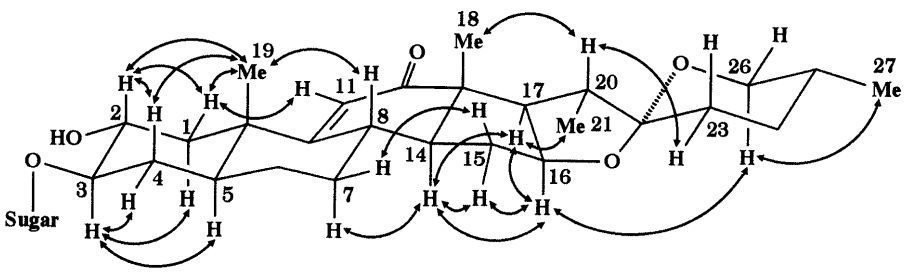

Fig. 2. NOE Correlations of Aglycone Moiety of **4** in Pyridine-d_5–Methanol-d_4 (11 : 1)

ly, the structure of **4** was formulated as (25*R*)-2α,3β-dihydroxy-5α-spirost-9-en-12-one 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}.

The structures of **5** ($C_{51}H_{80}O_{25}$) and **6** ($C_{50}H_{78}O_{24}$) were readily determined by comparison of the ^{1}H- and ^{13}C-NMR spectra with those of **1**—**4**. The aglycone moiety of **5** and **6** agreed with that of **4**. The structure of the saccharide moiety of **5** corresponded to that of **3**, and **6** to that of **2**. Thus, the structures of **5** and **6** were assigned as 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-glucopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} and 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside} of (25*R*)-2α,3β-dihydroxy-5α-spirost-9-en-12-one, respectively.

Table 3. ^{1}H- and ^{13}C-NMR Chemical Shifts for Oligosaccharide Units of **4**[a]

	^{1}H-NMR	^{13}C-NMR
Gal 1′	4.84 d (7.8)	103.1
2′	4.42 dd (9.8, 7.8)	72.4
3′	4.05	75.4
4′	4.50	79.8
5′	3.98	75.7
6′	4.49	60.6
	4.13	
Glc 1″	5.06 d (7.9)	104.7
2″	4.22 dd (8.4, 7.9)	81.3
3″	4.11 dd (8.4, 8.4)	88.2
4″	3.67 dd (8.4, 8.4)	70.5
5″	3.73	77.4
6″	4.34	62.8
	3.94	
Glc 1‴	5.51 d (8.0)	104.6
2‴	3.93 dd (8.9, 8.0)	75.9
3‴	4.08 dd (9.4, 8.9)	78.1
4‴	4.04 dd (9.4, 9.4)	71.2
5‴	3.82	78.4
6‴	4.44	62.5
	4.35	
Glc 1⁗	5.20 d (7.9)	104.2
2⁗	3.86 dd (8.6, 7.9)	75.4
3⁗	4.00 dd (9.3, 8.6)	76.6
4⁗	4.21 dd (9.3, 9.3)	78.5
5⁗	3.73	77.2
6⁗	4.15	61.1
	3.98	
Rha 1⁗′	5.61 br s	102.8
2⁗′	4.49	72.4
3⁗′	4.38 dd (3.3, 9.3)	72.5
4⁗′	4.20 dd (9.3, 9.5)	73.7
5⁗′	4.75 dq (9.5, 6.2)	70.5
6⁗′	1.63 d (6.2)	18.4

a) Spectra were measured in pyridine-d_5–methanol-d_4 (11 : 1). *J* values in parentheses are expressed in Hz.

All spectral data of **7** ($C_{50}H_{78}O_{23}$) showed a close similarity to those of **6**. On comparison of the ^{13}C-NMR spectrum of **7** with that of **6**, the signal due to the C-2 carbon, which was observed at δ 70.4 in **6**, appeared at δ 29.6 as a methylene carbon signal, accompanied by upfield shifts of the signals due to C-1 and C-3 by 8.4 and 6.8 ppm, respectively, indicating the missing hydroxyl group at C-2 in **7**. The structure of **7** was shown to be (25*R*)-3β-hydroxy-5α-spirost-9-en-12-one 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside}.

Compounds **3**—**7** are new compounds as well as **2**.

The isolated saponins and their aglycones were evaluated for TPA-stimulated ^{32}P-incorporation into phospholipids of HeLa cells. This is known as an excellent pri-

Table 4. Inhibitory Effects of the Isolated Saponins and Their Aglycones on TPA-Enhanced ^{32}P-Incorporation into Phospholipids of HeLa Cells[a]

Compounds	Inhibition (%) 50 μg/ml	Inhibition (%) 5 μg/ml
1	23.1	—
2	b)	24.2
2a	78.3	—
3	b)	8.9
3a	3.9	—
4	10.6	—
4a	58.1	—
5	b)	0
6	b)	14.5
7	b)	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.

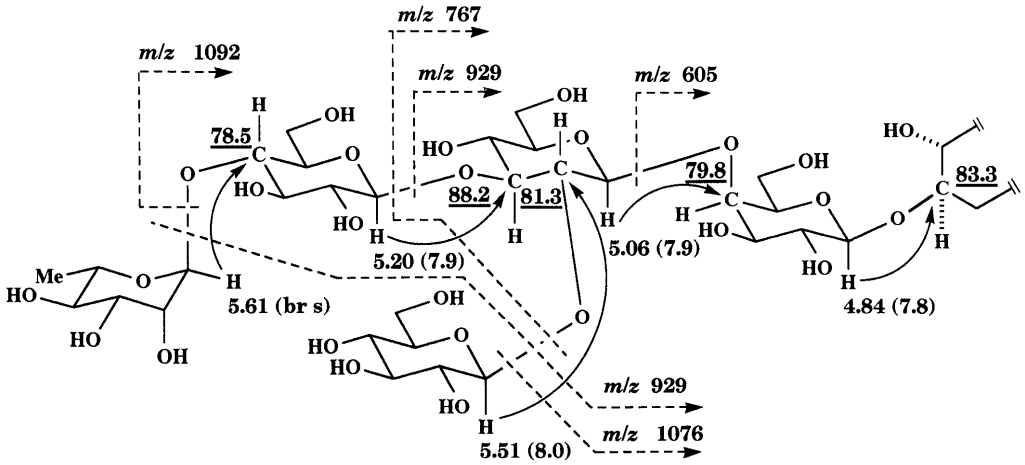

Fig. 3. ^{1}H–^{13}C Long-Range Correlations and FAB-MS Fragments of the Oligosaccharide Moiety of **4** in Pyridine-d_5–Methanol-d_4 (11 : 1)
J values (Hz) in the ^{1}H-NMR spectrum are given in parentheses. Underlined figures indicate ^{13}C-NMR chemical shifts.

mary screening test to identify new antitumor-promoter compounds.[12] The results are shown in Table 4. Compounds **2**, **3**, **5**, **6** and **7** were cytotoxic towards HeLa cells at a sample concentration of 50 μg/ml, and at a lower concentration (5 μg/ml), **2** exhibited 24.2% inhibition while **3**, **5**, **6** and **7** were less potent (**3**, 8.9%; **5**, 0%; **6**, 14.5%; **7**, 0%). The aglycone, **2a** and **4a** showed relatively high inhibition at 50 μg/ml (**2a**, 78.3%; **4a**, 57.8%) without any cytotoxicity towards HeLa cells while **3a** was far less active (3.9%), suggesting that the C-12 carbonyl group is important for activity. Compounds **2a** and **4a** are considered promising enough to be subjected to the two-stage carcinogenesis inhibition test *in vivo*.

Experimental

Optical rotations were measured with a JASCO DIP-360 automatic digital polarimeter. IR spectra were recorded on a Hitachi 260-30 instrument and MS on a VG AutoSpec E machine. Elemental analysis was performed on a Perkin–Elmer 240B elemental analyzer. NMR spectra were recorded with a Bruker AM-400 spectrometer for one dimensional (1D) NMR, and a Bruker AM-500 for 2D NMR employing the standard Bruker software. 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 octadecylsilanized (ODS) silica-gel (Nacalai Tesque) were used for column chromatographies. TLC was carried out on precoated Kieselgel 60 F_{254} (0.25 or 0.5 mm thick, Merck) and RP-18 F_{254} S (0.25 mm thick, Merck) plates, and spots were visualized by spraying with 10% H_2SO_4 followed by heating. HPLC was performed using a Tosoh HPLC system (Tosoh: pump, CCPM; controller, CCP controller PX-8010; detector, UV-8000) equipped with a TSK-gel ODS-Prep column (Tosoh, 4.6 mm i.d. × 250 mm, ODS, 5 μm) or a TSK-gel Silica-60 column (Tosoh, 4.6 mm i.d. × 250 mm, silica-gel, 5 μm). TPA was obtained from Pharmacia PL Biochemicals. Radioactive inorganic phosphate (^{32}P, carrier-free) was purchased from the Japan Radioisotope Associations. All other chemicals were of biochemical-reagent grade.

Plant Material Fresh underground parts of *H. longipes* (10 kg) were collected in Gunma prefecture, Japan, and a voucher specimen is on file in our laboratory.

Extraction and Isolation The underground parts were extracted with hot MeOH. The MeOH extract, after removal of the solvent under reduced pressure, was partitioned between *n*-BuOH and H_2O. Silica-gel column chromatography of the *n*-BuOH phase was carried out and elution was performed with CH_2Cl_2–MeOH, increasing the proportion of MeOH (9:1; 4:1; 2:1; 1:1) and then finally with MeOH. Fractions with the same TLC profile were combined to recover three fractions (I—III). After removal of saccharides from fraction III by column chromatography on Diaion HP-20, eluting with an increasing amount of MeOH in H_2O, it was chromatographed on silica-gel eluting with $CHCl_3$–Et_2O–MeOH–H_2O (7:7:8:2) and then divided into four fractions (IIIa—IIId). Fraction IIIc was subjected to a silica-gel column eluting with $CHCl_3$–Et_2O–MeOH–H_2O (7:7:8:2; 5:5:4:1) and an ODS silica-gel column with MeOH–H_2O (4:1) to give compounds **1** (753 mg), **2** (151 mg), **5** (688 mg), **6** (960 mg) and **7** (100 mg). Chromatography of fraction IIId on silica-gel eluting with $CHCl_3$–Et_2O–MeOH–H_2O (5:5:4:1) and ODS silica-gel with MeOH–H_2O (3:2; 1:1) gave compound **3** (527 mg) in a pure form and **4** with a few impurities. Final purification of **4** was performed by preparative TLC developing with a mixed solvent composed of $CHCl_3$–Et_2O–MeOH–H_2O (7:7:8:2) to furnish **4** (120 mg) as a pure compound.

Compound 1 An amorphous solid, $[\alpha]_D^{25}$ −42.0° (c=0.10, $CHCl_3$–MeOH (1:1)). Negative-ion FAB-MS m/z: 1050 $[M]^-$. IR ν_{max}^{KBr} cm^{-1}: 3410 (OH), 2930 (CH). ^{1}H-NMR (pyridine-d_5) δ: 5.59 (1H, d, J=7.8 Hz, 1‴-H), 5.26 (1H, d, J=7.8 Hz, 1⁗-H), 5.22 (1H, d, J=7.9 Hz, 1″-H), 4.92 (1H, d, J=7.7 Hz, 1′-H), 3.59 (1H, dd, J=10.6, 3.0 Hz, 26a-H), 3.51 (1H, dd, J=10.6, 10.6 Hz, 26b-H), 1.13 (3H, d, J=6.7 Hz, 21-Me), 0.81 (3H, s, 19-Me), 0.70 (3H, s, 18-Me), 0.69 (3H, d, J=5.8 Hz, 27-Me).

Compound 2 An amorphous solid, $[\alpha]_D^{25}$ −29.0° (c=0.10, $CHCl_3$–MeOH (1:1)). Negative-ion FAB-MS m/z: 1047 $[M-H]^-$, 915 $[M-xylosyl]^-$, 885 $[M-glucosyl]^-$, 753 $[M-xylosyl-glucosyl]^-$, 591 $[M-xylosyl-glucosyl\times2]^-$. IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2930 (CH), 1700 (C=O), 1450, 1420, 1365, 1155, 1060, 1035, 975, 915, 890, 860. ^{1}H-NMR (pyridine-d_5) δ: 5.57 (1H, d, J=7.4 Hz, 1‴-H), 5.23 (1H, d, J=7.7 Hz, 1⁗-H), 5.18 (1H, d, J=7.9 Hz, 1″-H), 4.85 (1H, d, J=7.7 Hz, 1′-H), 3.59 (1H, dd, J=10.5, 2.3 Hz, 26a-H), 3.49 (1H, dd, J=10.5, 10.5 Hz, 26b-H), 1.35 (3H, d, J=6.9 Hz, 21-Me), 1.08 (3H, s, 18-Me), 0.70 (3H, d, J=5.4 Hz, 27-Me), 0.66 (3H, s, 19-Me).

Compound 3 An amorphous solid, $[\alpha]_D^{25}$ −52.0° (c=0.10, $CHCl_3$–MeOH (1:1)). Negative-ion FAB-MS m/z: 1079 $[M-H]^-$, 917 $[M-glucosyl]^-$, 756 $[M-glucosyl\times2]^-$, 594 $[M-glucosyl\times3]^-$. IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 2935 (CH), 1445, 1375, 1235, 1150, 1050, 915, 890, 860, 745. ^{1}H-NMR (pyridine-d_5) δ: 5.58 (1H, d, J=7.8 Hz, 1‴-H), 5.29 (1H, d, J=7.8 Hz, 1⁗-H), 5.16 (1H, d, J=7.9 Hz, 1″-H), 4.90 (1H, d, J=7.8 Hz, 1′-H), 3.58 (1H, dd, J=10.5, 3.1 Hz, 26a-H), 3.50 (1H, dd, J=10.5, 10.5 Hz, 26b-H), 1.13 (3H, d, J=6.9 Hz, 21-Me), 0.81 (3H, s, 18-Me), 0.71 (3H, s, 19-Me), 0.70 (3H, d, J=5.3 Hz, 27-Me).

Acid Hydrolysis of 2 and 3 A solution of **2** (20 mg) in 1 N HCl (dioxane–H_2O (1:1)) was heated in a boiling-water bath for 2 h under an Ar atmosphere. After cooling, the reaction mixture was neutralized by passing it through an Amberlite IRA-93ZU (Organo) column. A Sep-Pak C_{18} cartridge (Waters) was used to fractionate the reaction mixture into a sugar fraction eluting with H_2O (20 ml) and into a sapogenin fraction eluting with MeOH (20 ml). The sapogenin fraction was chromatographed on silica-gel using $CHCl_3$–Me_2CO (4:1) to yield an aglycone (**2a**) (4.9 mg), identified as hecogenin. The sugar fraction (2 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 using H_2O–MeCN (4:1, 10 ml) and then MeCN (10 ml). The MeCN eluate was further passed through a TOYOPAK IC-SP M cartridge (Tosoh) using 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.[13] Derivatives of D-glucose, D-galactose and D-xylose were detected in a ratio of 2:1:1. Following this procedure, **3** (20 mg) was subjected to acid hydrolysis to yield an aglycone (**3a**) (7.3 mg), identified as gitogenin, and D-glucose and D-galactose in a ratio of 3:1.

Compound 4 An amorphous solid, $[\alpha]_D^{25}$ −40.0° (c=0.10, $CHCl_3$–MeOH (1:1)). *Anal.* Calcd for $C_{57}H_{90}O_{29}\cdot H_2O$: C, 54.45; H, 7.38. Found: C, 54.67; H, 7.37. Negative-ion FAB-MS m/z: 1238 $[M]^-$, 1092 $[M-rhamnosyl]^-$, 1076 $[M-glucosyl]^-$, 929 $[M-rhamnosyl-glucosyl]^-$, 767 $[M-rhamnosyl-glucosyl\times2]^-$, 605 $[M-rhamnosyl-glucosyl\times3]^-$. UV λ_{max}^{MeOH} nm (log ε): 238 (3.95). IR ν_{max}^{KBr} cm^{-1}: 3425 (OH), 2930 (CH), 1655 (C=O), 1595 (C=C), 1450, 1370, 1175, 1155, 1065, 1035, 975, 915, 895. ^{1}H-NMR (pyridine-d_5) δ: 5.94 (1H, br s, 11-H), 5.73 (1H, br s, 1⁗-H), 5.59 (1H, d, J=7.8 Hz, 1‴-H), 5.27 (1H, d, J=7.9 Hz, 1⁗-H), 5.14 (1H, d, J=7.9 Hz, 1″-H), 4.90 (1H, d, J=7.7 Hz, 1′-H), 3.59 (1H, dd, J=10.6, 2.8 Hz, 26a-H), 3.49 (1H, dd, J=10.6, 10.6 Hz, 26b-H), 1.69 (3H, d, J=6.1 Hz, 6⁗-Me), 1.39 (3H, d, J=6.8 Hz, 21-Me), 1.00 (3H, s, 18-Me), 0.90 (3H, s, 19-Me), 0.71 (3H, d, J=5.3 Hz, 27-Me).

Acid Hydrolysis of 4 Compound **4** (100 mg) was hydrolyzed with 1 N HCl and the crude reaction mixture was partitioned by passing it through a Sep-Pak C_{18} cartridge eluting with H_2O (20 ml) and into a sapogenin fraction eluting with MeOH (20 ml). The MeOH eluate was chromatographed on silica-gel using $CHCl_3$–Me_2CO (2:1) to yield an aglycone (**4a**) (34.7 mg). The monosaccharides contained in the H_2O eluate were converted to the corresponding 1-[(*S*)-*N*-acetyl-α-methylbenzylamino]-1-deoxyalditol acetate derivatives, which were then analyzed by HPLC to reveal derivatives of D-glucose, D-galactose and D-xylose in a ratio of 3:1:1. Compound **4a**: an amorphous solid, $[\alpha]_D^{25}$ −8.0° (c=0.05, $CHCl_3$–MeOH (1:1)). EI-MS m/z: 444.2873 $[M]^+$ (Calcd for $C_{27}H_{40}O_5$: 444.2876). UV λ_{max}^{MeOH} nm (log ε): 239 (4.00). IR ν_{max}^{KBr} cm^{-1}: 3370 (OH), 2955, 2925 and 2855 (CH), 1665 (C=O), 1595 (C=C), 1450, 1375, 1340, 1260, 1240, 1180, 1155, 1130, 1095, 1070, 1055, 1025, 1005, 980, 920, 895, 860, 800. ^{1}H-NMR (pyridine-d_5) δ: 6.00 (1H, d, J=1.6 Hz, 11-H), 4.53 (1H, q-like, J=8.4 Hz, 16-H), 4.10 (1H, ddd, J=12.5, 10.3, 4.5 Hz, 2-H), 3.80 (1H, ddd, J=10.3, 10.3, 5.0 Hz, 3-H), 3.60 (1H, dd, J=10.5, 3.6 Hz, 26a-H), 3.50 (1H, dd, J=10.5, 10.5 Hz, 26b-H), 1.41 (3H, d, J=6.9 Hz, 21-Me), 1.06 (3H, s, 18-Me), 1.03 (3H, s, 19-Me), 0.70 (3H, d, J=5.7 Hz, 27-Me).

Catalytic Hydrogenation of 4a Prior to catalytic hydrogenation, the 2α,3β-hydroxyl groups of **4a** were protected with acetyl groups by treatment of **4a** (20 mg) with Ac_2O in pyridine. A mixture of the acetate and PtO_2 (5 mg) in Et_2O–AcOH (3:1, 2 ml) was stirred under an H_2

atmosphere at ambient temperature for 24 h. The reaction mixture, after removal of PtO_2 by filtration, was treated with 3% NaOMe in MeOH (3 ml) for 15 min. The reaction mixture was neutralized by passage through an Amberlite IR-120B (Organo) column and chromatographed on silica-gel eluting with ($CHCl_3$–Me_2CO (2:1)) to yield the dihydro derivative (**4b**) (6.8 mg). Compound **4b**: an amorphous solid, $[\alpha]_D^{25}$ −2.0° (c=0.05, $CHCl_3$–MeOH (1:1)). EI-MS m/z: 446.3017 $[M]^+$ (Calcd for $C_{27}H_{47}O_8$: 446.3032). IR ν_{max}^{KBr} cm^{-1}: 3400 (OH), 2920 and 2850 (CH), 1700 (C=O), 1450, 1420, 1370, 1335, 1255, 1235, 1170, 1150, 1090, 1070, 1050, 1020, 1000, 980, 915, 895, 860. ^{1}H-NMR (pyridine-d_5) δ: 4.50 (1H, q-like, J=7.9 Hz, 16-H), 4.00 (1H, ddd, J=12.0, 11.0, 4.4 Hz, 2-H), 3.81 (1H, ddd, J=11.0, 11.0, 5.0 Hz, 3-H), 3.59 (1H, dd, J=10.5, 3.5 Hz, 26a-H), 3.49 (1H, dd, J=10.5, 10.5 Hz, 26b-H), 2.48 (1H, dd, J=14.1, 14.1 Hz, 11a-H), 2.40 (1H, dd, J=14.1, 5.3 Hz, 11b-H), 1.35 (3H, d, J=6.8 Hz, 21-Me), 1.10 (3H, s, 18-Me), 0.89 (3H, s, 19-Me), 0.70 (3H, d, J=5.7 Hz, 27-Me).

Compound 5 An amorphous solid, $[\alpha]_D^{25}$ −32.0° (c=0.10, $CHCl_3$–MeOH (1:1)). Negative-ion FAB-MS m/z: 1091 $[M-H]^-$, 929 $[M-glucosyl]^-$, 768 $[M-glucosyl\times2]^-$, 605 $[M-glucosyl\times3]^-$. UV λ_{max}^{MeOH} nm (log ε): 238 (4.08). IR ν_{max}^{KBr} cm^{-1}: 3380 (OH), 2940 and 2875 (CH), 1660 (C=O), 1595 (C=C), 1455, 1375, 1300, 1240, 1155, 1065, 980, 915, 895. ^{1}H-NMR (pyridine-d_5) δ: 5.94 (1H, d, J=1.1 Hz, 11-H), 5.58 (1H, d, J=7.8 Hz, 1‴-H), 5.28 (1H, d, J=7.8 Hz, 1⁗-H), 5.15 (1H, d, J=7.8 Hz, 1″-H), 4.89 (1H, d, J=7.8 Hz, 1′-H), 3.59 (1H, dd, J=10.6, 3.2 Hz, 26a-H), 3.49 (1H, dd, J=10.6, 10.6 Hz, 26b-H), 1.39 (3H, d, J=6.9 Hz, 21-Me), 1.00 (3H, s, 18-Me), 0.90 (3H, s, 19-Me), 0.71 (3H, d, J=5.6 Hz, 27-Me).

Compound 6 An amorphous solid, $[\alpha]_D^{25}$ −20.0° (c=0.10, $CHCl_3$–MeOH (1:1)). Negative-ion FAB-MS m/z: 1062 $[M]^-$, 930 $[M-xylosyl]^-$, 899 $[M-glucosyl]^-$, 767 $[M-xylosyl-glucosyl]^-$, 767 $[M-xylosyl-glucosyl\times2]^-$. UV λ_{max}^{MeOH} nm (log ε): 237 (4.01). IR ν_{max}^{KBr} cm^{-1}: 3390 (OH), 2920 and 2860 (CH), 1665 (C=O), 1590 (C=C), 1445, 1365, 1295, 1150, 1045, 975, 915, 890. ^{1}H-NMR (pyridine-d_5) δ: 5.94 (1H, d, J=1.3 Hz, 11-H), 5.58 (1H, d, J=7.8 Hz, 1‴-H), 5.24 (1H, d, J=7.8 Hz, 1⁗-H), 5.20 (1H, d, J=7.9 Hz, 1″-H), 4.90 (1H, d, J=7.7 Hz, 1′-H), 3.59 (1H, dd, J=10.6, 2.8 Hz, 26a-H), 3.49 (1H, dd, J=10.6, 10.6 Hz, 26b-H), 1.39 (3H, d, J=6.9 Hz, 21-Me), 1.00 (3H, s, 18-Me), 0.90 (3H, s, 19-Me), 0.71 (3H, d, J=5.5 Hz, 27-Me).

Compound 7 An amorphous solid, $[\alpha]_D^{25}$ −34.0° (c=0.10, $CHCl_3$–MeOH (1:1)). Negative-ion FAB-MS m/z: 1045 $[M-H]^-$, 913 $[M-xylosyl]^-$, 883 $[M-glucosyl]^-$, 751 $[M-xylosyl-glucosyl]^-$, 589 $[M-xylosyl-glucosyl\times2]^-$. UV λ_{max}^{MeOH} nm (log ε): 240 (4.26). IR ν_{max}^{KBr} cm^{-1}: 3420 (OH), 2925 and 2865 (CH), 1665 (C=O), 1595 (C=C), 1445, 1420, 1365, 1300, 1155, 1065, 1040, 975, 915, 895. ^{1}H-NMR (pyridine-d_5) δ: 5.78 (1H, br s, 11-H), 5.58 (1H, d, J=7.4 Hz, 1‴-H), 5.24 (1H, d, J=7.8 Hz, 1⁗-H), 5.19 (1H, d, J=7.9 Hz, 1″-H), 4.86 (1H, d, J=7.6 Hz, 1′-H), 3.59 (1H, dd, J=10.6, 3.1 Hz, 26a-H), 3.50 (1H, dd, J=10.6, 10.6 Hz, 26b-H), 1.41 (3H, d, J=6.9 Hz, 21-Me), 1.01 (3H, s, 18-Me), 0.82 (3H, s, 19-Me), 0.70 (3H, d, J=5.6 Hz, 27-Me).

Cell Culture and Assay of ^{32}P-Incorporation into Phospholipids of Cultured Cells HeLa cells were cultured as monolayers in Eagle's minimum essential medium supplemented with 10% calf serum in 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 and then the radioactivity incorporated into the phospholipid fraction was measured.[12d]

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