Dammarane-Type Saponins from *Panax japonicus* and Their Neurite Outgrowth Activity in SK-N-SH Cells

Kun Zou,[†,‡] Shu Zhu,[†] Meselhy R. Meselhy,[†] Chihiro Tohda,[†] Shaoqing Cai,[§] and Katsuko Komatsu[*,†]

Research Center for Ethnomedicines, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan, Chemistry and Life Science College, Three Gorges University, Yichang, Hubei 443003, People's Republic of China, and School of Pharmaceutical Sciences, Peking University, 38 Xue-yuan Road, Haidian District, Beijing 100083, People's Republic of China

Four new dammarane-type saponins (**1**–**4**), named yesanchinosides G–J, together with nine ginsenosides (**5**–**13**) were isolated from Ye-Sanchi, the underground part of *Panax japonicus* collected in the south of Yunnan Province, China. Their structures were elucidated on the basis of spectroscopic and chemical methods. Ginsenosides Rb₁ and Rb₃ and notoginsenosides R₄ (**6**) and Fa (**7**) showed significant neurite outgrowth enhancing activities in human neuroblastoma SK-N-SH cells.

Ye-Sanchi (local name in Chinese), the underground parts of *Panax japonicus* C. A. Meyer (Araliaceae), has long been used in the south of Yunnan Province, China, as a tonic and to promote blood circulation and eliminate blood stasis. As a part of our systematic study on quality evaluation of *Panax* species, we recently reported the isolation of 17 saponins from the underground part of *P. japonicus* (Ye-Sanchi) collected in the south of Yunnan, and the ocotillol-type saponins were found to be the major compounds.[1] Moreover, our phylogenetic analysis[2] on the basis of 18S ribosomal RNA gene and *mat*K gene sequences of *Panax* species revealed that the title plant had a close relationship with *P. vietnamensis* Ha et Grushv., in which ocotillol-type saponins were also present in abundance. Saponins from *Panax* species have been reported to demonstrate a number of actions on the central nervous system, including CNS stimulation or depression, antifatigue and antistress activity, and learning and memory improving effects.[3] Ginsenosides Rb₁ and Rg₁ have been reported to enhance learning and memory ability by facilitating cholinergic function[4] and increasing synaptophysin levels in the hippocampus.[5] These findings suggest that these compounds may be useful in the treatment of neurodegenerative diseases, which are most often associated with neuronal death and atrophy of neurites. Accordingly, compounds enhancing neurite outgrowth in remaining neurons could possibly initiate a recovery of brain function. In view of the chemotaxonomical and pharmacological interest, we have investigated the saponins from the MeOH extract of Ye-Sanchi and further examined the neurite outgrowth activity of the MeOH extract as well as the isolated compounds in a human neuroblastoma cell line, SK-N-SH. In the present paper we report the structure elucidation of four new compounds, yesanchinosides G–J (**1**–**4**), as well as the neurite outgrowth activity of these and other compounds isolated in sufficient amount.

Results and Discussion

The BuOH-soluble fraction of the MeOH extract of Ye-Sanchi was subjected to column chromatography over Sephadex LH-20, normal-phase and reversed-phase silica gel, followed by HPLC to afford four new saponins,

yesanchinosides G–J (**1**–**4**) and nine known compounds (**5**–**13**). The spectral data of **5**–**13** were identical with those reported for notoginsenoside G (**5**),[6] notoginsenoside R₄ (**6**),[7] notoginsenoside Fa (**7**),[8] ginsenoside Rb₁ (**8**),[9] notoginsenoside Fc (**9**),[8] ginsenoside Rc (**10**),[9] notoginsenoside Fe (**11**),[8] ginsenoside Rb₃ (**12**),[9] and ginsenoside Rd (**13**).[9] The structure determination of the four new compounds is described as follows.

Yesanchinoside G (**1**) was obtained as a white amorphous powder. The API (atmospheric pressure ionization) mass spectra of **1** showed quasimolecular ion peaks (positive and negative modes) at m/z 1115 [M + Na]⁺ and 1091 [M − H]⁻, which suggested the molecular formula $C_{53}H_{88}O_{23}$ for **1**. This was confirmed by elemental analysis. Acid hydrolysis of **1** with 10% HCl yielded D-glucose and D-xylose as the sugar components, and their configurations were confirmed by GC analysis. The IR spectrum of **1** showed absorption bands assignable to hydroxyl (3394 cm⁻¹) and olefinic (1647 and 1078 cm⁻¹) groups. In the ¹H NMR spectrum of **1**, diagnostic signals were found for a sapogenin moiety (Table 1), namely, signals for eight methyls (δ_H 1.64, 1.64, 1.63, 1.46, 1.39, 1.24, 1.12, and 1.12), two olefinic protons [δ_H 5.80 (br s, H-6) and 5.04 (t like, J = 8.5 Hz, H-24)], and three oxymethines [δ_H 3.24 (dd, J = 12.0, 4.6 Hz, H-3), 4.68 (d, J = 2.4 Hz, H-7), and 4.10 (m, H-12)]. The ¹³C chemical shift values (Table 2) due to the sapogenin moiety were similar to those reported for notoginsenoside G (**5**)[6] and quinquenoside IV (**14**).[10] This was further evident by the presence of daughter ion peaks at m/z 473 [sapogenin − H]⁻ and 455 [sapogenin − H₂O − H]⁻ in the API-MS/MS (negative mode) spectrum of a parent ion peak at m/z 1091 [M − H]⁻. The ¹H and ¹³C NMR spectra of **1** analyzed by the aid of ¹H–¹H COSY and HMQC spectra displayed features for a triterpene glycoside with four sugar units, which were assignable to a β-sophorosyl moiety and a disaccharide moiety composed of D-glucose and D-xylose. A fragment ion peak at m/z 959 [M − xyl − H]⁻ in API-MS suggested that xylose was a terminal unit. Location of the β-sophorosyl moiety was confirmed to be at 3-OH of the sapogenin by HMBC correlation between carbon signals at δ_C 88.0 (C-3), 104.8 (C-1′), 83.5 (C-2′) and proton signals at δ_H 4.85 (H-1′), 3.24 (H-3), 5.32 (H-1″), respectively. Similarly, long-range correlations between the carbon signal at δ_C 83.6 (C-20) and the proton signal at δ_H 5.12 (H-1‴) and between δ_C 70.0 (C-6‴) and δ_H 4.96 (H-1⁗) ascertained that the β-D-
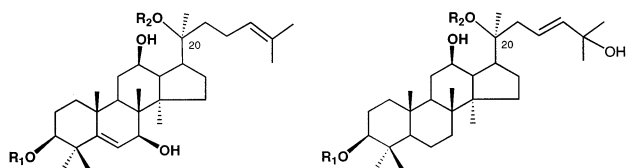
	R_1	R_2
1	Glc^2—Glc	Glc^6—Xyl
5	Glc^2—Glc	Glc
14	Glc^2—Glc	Glc^6—Glc

	R_1	R_2
2	Glc^2—Glc	Glc^6—Xyl
15	Glc^2—Glc	Glc^6—Glc
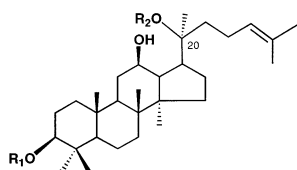
	R_1	R_2
4	$Glc^2{\overset{6}{\diagdown}}Glc$ — Ac	Glc^6—Glc^6—Xyl
6	Glc^2—Glc	Glc^6—Glc^6—Xyl
7	Glc^2—Glc^2—Xyl	Glc^6—Glc
8	Glc^2—Glc	Glc^6—Glc
9	Glc^2—Glc^2—Xyl	Glc^6—Glc
10	Glc^2—Glc	Glc^6—Arap
11	Glc	Glc^6—Araf
12	Glc^2—Glc	Glc^6—Xyl
13	Glc^2—Glc	Glc
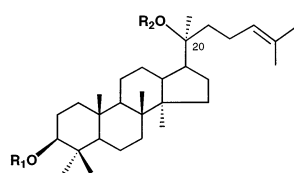
	R_1	R_2
3	Glc^2—Glc	Glc^6—Glc^6—Xyl
16	Glc^2—Glc	Glc^6—Glc

Glc: β-D-glucopyranosyl
Ac: acetyl
Xyl: β-D-xylopyranosyl
Arap: α-L-arabinopyranosyl
Araf: α-L-arabinofuranosyl

xylopyranosyl-(1→6)-β-D-glucopyranose chain is linked to 20-OH of the sapogenin. The 20*S* configuration of **1** was clearly revealed by comparison of ^{13}C NMR data of the aglycone moiety of **1** (Table 2) with **5**[6] and **14**,[10] especially the chemical shifts of C-17 (δ_C 51.1), C-21 (δ_C 22.4), and C-22 (δ_C 36.5), which have been used as the diagnostic signals for determination of the stereochemistry of C-20 [for aglycones with the 20*R* configuration, the upfield shifts at C-17 (−4.1 ppm) and C-21 (−4.2 ppm) and downfield shift at C-22 (+7.4 ppm) have been observed, comparable to the 20*S* configuration].[11,12] NOESY correlations between H₃-21 (δ_H 1.64) and H-17 (δ_H 2.30) confirmed this result.[12,13] Accordingly, the structure of yesanchinoside G (**1**) was established to be 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20-*O*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl-3β,7β,12β,20(*S*)-tetrahydroxydammar-5,24-diene.

The API-MS (positive mode) of yesanchinoside H (**2**) showed a quasimolecular ion peak at *m/z* 1117 [M + Na]⁺ (*m/z* at 1093 [M − H]⁻ in the negative mode), suggesting a molecular formula of $C_{53}H_{90}O_{23}$, which was confirmed by elemental analysis. D-Glucose and D-xylose were identified in the acid hydrolysate of **2**, and GC analysis confirmed their absolute configuration. Absorption bands for hydroxyl and olefinic groups were observed in its IR spectrum. The ^{1}H NMR spectrum of **2** displayed signals for eight methyl [δ_H 1.64, 1.62, 1.58, 1.24, 1.22, 1.14, 1.08, and 0.84], two

trans-olefinic protons (δ_H 5.81 and 6.10), and two oxymethines (δ_H 3.24 and 4.23). The ^{13}C NMR data (Tables 2 and 3) of **2** were very similar to those reported for **15**,[14] except for some data due to the sugar chain linked to the hydroxyl at the C-20 position of the sapogenin moiety, suggesting that **2** possesses the same sapogenin as that of notoginsenoside A.[14] This was further concluded by API-MS/MS (negative mode), namely, a daughter ion peak at *m/z* 475 [M − 3glc − xyl − H]⁻ of a parent ion peak at *m/z* 1093 [M − H]⁻ was observed. HMBC correlation between ^{13}C signals at δ_C 88.9 (C-3), δ_C 83.7 (C-2′) and ^{1}H signals at δ_H 4.89 (H-1′), δ_H 5.34 (H-1″), respectively, indicated a β-sophorosyl moiety at 3-OH of the sapogenin. However, the β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl group (similar to that in **1**) was linked at 20-OH of the sapogenin, as further confirmed by long-range correlations between δ_C 83.4 (C-20) and δ_H 5.23 (H-1‴) and between δ_C 70.6 (C-6‴) and δ_H 4.97 (H-1‴′) in the HMBC spectrum. The stereochemistry at C-20 was concluded to be *S* from comparison of the ^{13}C NMR spectral data of **2** (Table 2) with those of **15**[14] and the NOESY spectrum, which showed NOE correlations between H₃-21 (δ_H 1.58) and H-17 (δ_H 2.53).[12,13] Thus, **2** was elucidated as 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20-*O*-[β-D-xylopyranosy-(1→6)-β-D-glucopyranosyl-3β,12β,20(*S*),25-tetrahydroxydammar-23-ene.

The molecular formula of yesanchinoside I (**3**) was established as $C_{59}H_{100}O_{26}$ on the basis of API-MS ([M + Na]⁺ at *m/z* 1247 in the positive mode and [M − H]⁻ at *m/z* 1223 in the negative mode) and elemental analysis results. TLC and GC analysis identified D-glucose and D-xylose as the sugar components in the acid hydrolysate of **3**. The ^{13}C chemical shift values (Tables 2 and 3) due to the sapogenin moiety of **3** were superimposable on those of **16**,[6] whereas the carbon signals of the sugar moieties closely resembled those of **6**.[7] The ^{1}H NMR spectrum of **3** showed signals for five sugar units, namely, two anomeric protons of a β-sophorosyl moiety (δ_H 4.92 and 5.35), two anomeric protons of a β-gentiobiosyl moiety (δ_H 5.10 and 5.07), and an anomeric proton of a β-D-xylopyranosyl unit (δ_H 4.94). A fragment ion peak at *m/z* 1091 [M − xyl − H]⁻ observed in the negative mode API-MS of **3** suggested that D-xylose was a terminal unit. Long-range correlations in the HMBC spectrum between the carbon signals at δ_C 105.0 (C-1′) and 89.1 (C-3) and proton signals at δ_H 3.23 (H-3) and 4.92 (H-1′), respectively, indicated that the β-sophorosyl moiety was at 3-OH of a dammarenediol II aglycone. Similarly, a β-D-xylopyranosyl-(1→6)-β-gentiobiosyl moiety was found to attach to 20-OH of the sapogenin on the basis of the HMBC spectrum. The stereostructure of **3** was confirmed to be *S* from the ^{13}C NMR data and the NOESY spectrum. Therefore, **3** was identified as 3-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-20-*O*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-3β,20(*S*)-dihydroxydammar-24-ene.

The IR spectrum of yesanchinoside J (**4**) showed strong absorption bands at 3398 (OH) and 1739 (C=O) cm⁻¹. The quasimolecular ion peaks (positive and negative modes) at *m/z* 1305 [M + Na]⁺, 1283 [M + H]⁺, and 1281 [M − H]⁻ in the API-MS spectra indicated a molecular formula of $C_{61}H_{102}O_{28}$, which was supported by elemental analysis. The ^{1}H and ^{13}C NMR spectra (Tables 1 and 3) of **4** analyzed by the aid of [1]H−^{1}H COSY and HMQC experiments showed features typical of a protopanaxadiol-type saponin similar to those of **6−13**. Signals for five anomeric protons at δ_H 4.84, 5.36, 5.10, 4.91, and 4.93 indicated the presence of five sugar moieties. A singlet methyl group at δ_H 2.49/δ_C 20.8 and a carbonyl signal at δ_C 170.7 were assignable to

Table 1. Selected ^{1}H NMR Data of Compounds **1**–**4**^{a} (in C_5D_5N)

position	1	2	3	4
3	3.24 (dd, 12.0, 4.6)	3.24 (dd, 12.0, 4.9)	3.23 (dd, 12.0, 4.4)	3.23 (dd, 11.7, 4.4)
5		0.71 (m)	0.69 (m)	0.70 (m)
6	5.80 (br s)	1.61 (m), 1.30 (m)	1.55 (m), 1.29 (m)	1.61 (m), 1.28 (m)
9	1.57 (m)	1.40 (m)	1.33 (m)	1.42 (m)
12	4.10 (m)	4.23 (m)	1.69 (m), 1.32 (m)	3.89 (dd, 9.5, 4.6)
13	1.98 (m)	1.91 (m)	0.72 (m)	1.91 (dd, 10.2, 9.5)
17	2.30 (m)	2.53 (m)	2.58 (m)	2.30 (dd, 10.2, 8.4)
18	1.24 (s)	1.14 (s)	0.83 (s)	0.95 (s)
19	1.12 (s)	1.08 (s)	0.70 (s)	0.83 (s)
21	1.64 (s)	1.58 (s)	1.44 (s)	1.59 (s)
23		6.10 (m)		
24	5.04 (t, 8.5)	5.81(d, 15.4)	5.28 (t, 8.4)	5.29 (t, 8.4)
26	1.64 (s)	1.62 (s)	1.42 (s)	1.63 (s)
27	1.63 (s)	1.64 (s)	1.44 (s)	1.63 (s)
28	1.46 (s)	1.24 (s)	1.23 (s)	1.26 (s)
29	1.39 (s)	1.22 (s)	1.04 (s)	1.08 (s)
30	1.12 (s)	0.84 (s)	0.99 (s)	0.95 (s)
CH_3CO				2.48 (s)
1′	4.85 (d, 7.3)	4.89 (d, 7.3)	4.92 (d, 7.6)	4.84 (d, 7.4)
1″	5.32 (d, 7.6)	5.34 (d, 7.3)	5.35 (d, 7.8)	5.36 (d, 7.8)
1‴	5.12 (d, 8.3)	5.23 (d, 6.4)	5.10 (d, 7.8)	5.10 (d, 7.6)
1⁗	4.96 (d, 7.3)	4.97 (d, 7.4)	5.07 (d, 7.2)	4.91 (d, 6.6)
1‴″			4.94 (d, 7.6)	4.93 (d, 7.3)

^{a} Chemical shifts in ppm (δ) and coupling constants (in parentheses) in Hz.

Table 2. ^{13}C NMR Data (δ) of Sapogenin Moieties in Compounds **1**–**4** (in C_5D_5N)

position	1	2	3	4
1	39.5	39.2	39.4	39.2
2	27.1	26.8	26.8	26.6
3	88.0	88.9	89.1	89.4
4	42.7	39.5	39.7	39.7
5	147.1	56.3	56.4	56.4
6	127.4	18.4	18.4	18.4
7	71.2	35.1	35.6	35.1
8	42.3	40.0	40.8	40.1
9	47.4	50.0	51.0	50.3
10	38.0	36.9	36.9	36.2
11	33.1	30.9	21.9	30.7
12	69.7	70.0	25.8	70.1
13	50.4	49.4	42.6	49.5
14	51.0	51.5	50.6	51.4
15	34.4	30.9	31.5	30.9
16	27.1	26.4	28.0	26.8
17	51.1	52.1	48.4	51.6
18	10.7	16.3	15.7	16.2
19	20.3	16.4	16.4	16.0
20	83.6	83.4	82.4	83.4
21	22.4	23.1	21.4	22.3
22	36.5	39.5	40.6	36.9
23	23.2	122.9	23.2	23.1
24	126.0	142.3	126.2	126.0
25	131.0	70.1	130.6	131.0
26	25.7	30.5	25.8	25.8
27	17.9	30.7	18.0	17.9
28	28.3	28.0	28.0	28.1
29	23.8	16.7	16.6	16.6
30	18.2	17.4	16.8	17.4

Table 3. ^{13}C NMR Data of Sugar Moieties in Compounds **1**–**4** (in C_5D_5N)

position	1	2	3	4
6-*O*-sugar	Glc	Glc	Glc	Glc
1′	104.8	105.0	105.0	105.0
2′	83.5	83.7	83.1	83.1
3′	78.2	78.2	78.2	78.0
4′	71.6	71.6	71.7	70.4
5′	77.9	78.1	78.0	75.1
6′	62.7	62.8	62.8	64.4
CO				170.7
CH_3				20.8
	Glc(1→2)	Glc(1→2)	Glc(1→2)	Glc(1→2)
1″	106.0	106.0	105.8	105.9
2″	77.1	77.1	77.0	76.9
3″	77.9	77.9	77.9	79.3
4″	71.6	71.6	71.6	71.7
5″	78.2	78.2	78.3	78.2
6″	62.7	62.7	62.7	62.7
20-*O*-sugar	Glc	Glc	Glc	Glc
1‴	98.2	98.1	98.6	98.0
2‴	74.8	74.8	75.5	74.6
3‴	79.2	79.2	78.8	78.0
4‴	71.0	71.0	71.6	71.6
5‴	76.9	76.9	76.6	76.9
6‴	70.0	70.6	70.5	71.4
	Xyl(1→6)	Xyl(1→6)	Glc(1→6)	Glc(1→6)
1⁗	105.7	106.5	105.4	105.4
2⁗	74.9	74.9	75.0	74.8
3⁗	79.3	79.3	78.2	78.1
4⁗	71.1	71.1	71.5	71.7
5⁗	67.6	67.6	76.8	77.0
6⁗			69.8	69.9
			Xyl(1→6)	Xyl(1→6)
1‴″			105.8	106.0
2‴″			74.8	74.8
3‴″			78.0	78.1
4‴″			71.0	71.1
5‴″			67.0	67.0

an acetyl group. Upon alkaline hydrolysis of **4** with 10% NH_4OH, notoginsenoside R_4 (**6**) was obtained. The NMR data of **4** were mostly superimposed on those of **6** and suggested that the acetyl group should be on one of the sugar moieties. Comparison of the ^{13}C chemical shifts of the sugar part in **4** with those of **6** revealed an acylation shift at C-6′ of ca. 1.8 ppm (**4**, δ_C 64.4; **6**, δ_C 62.6). Long-range correlations were observed between the methylene protons at δ_H 4.30 (H$_2$-6′) and the carbonyl carbon signal at δ_C 170.7 (C=O) and between the carbon signal at δ_C 89.4 (C-3) and H-1′ at δ_H 4.84 in the HMBC spectrum. Therefore, the acetyl group was placed at 6′-OH of the inner glucose unit of the sugar chain linked to 3-OH of the

sapogenin. This was further ascertained by observing a fragment ion peak at *m/z* 629 [M − xyl − 3glc − 2H$_2$O + H]$^+$ observed in the positive-ion API-MS of **4**. The stereo-chemistry of **4** was *S* at C-20 following from the close similarity of the ^{13}C chemical shift values with those of **6**−**13** as well as from the spatial correlation between

Table 4. Neurite Outgrowth Activities of 15 Saponins Isolated from Ye-Sanchi[a]

compound	cells with neurites (% of control)
protopanaxadiol-type	
ginsenoside Rb$_1$ (**8**)	178.0 ± 21.30[b]
ginsenoside Rb$_3$ (**12**)	228.0 ± 20.00[b]
notoginsenoside Fa (**7**)	147.0 ± 3.27[b]
notoginsenoside R$_4$ (**6**)	150.9 ± 19.84[b]
yesanchinoside J (**4**)	87.9 ± 17.18
protopanaxatriol-type	
ginsenoside Rg$_1$[1]	107.3 ± 24.57
ginsenoside Re[1]	83.5 ± 12.81
notoginsenoside R$_1$[1]	84.6 ± 6.20
20-*O*-Glc-ginsenoside R$_f$[1]	96.6 ± 4.80
ocotillol-type	
majonoside R$_2$[1]	109.6 ± 8.86
(24*S*)-pseudoginsenoside F$_{11}$[1]	103.0 ± 11.40
(24*S*)-pseudoginsenoside RT$_4$[1]	91.4 ± 12.90
vina-ginsenoside R$_1$[1]	82.2 ± 12.61
vina-ginsenoside R$_2$[1]	97.0 ± 7.19
vina-ginsenoside R$_6$[1]	104.1 ± 7.93

[a] The cells were treated with each compound at a concentration of 100 μM. Yesanchinoside J and (24*S*)-pseudoginsenoside F$_{11}$ were used at 10 μM, since they were toxic at 100 μM. Each value represents the mean ± sem of eight sights. [b] $p < 0.05$ compared with control.

H$_3$-21 and H-17 observed in the NOESY spectrum.[12,13] Thus, **4** was identified as 3-*O*-[β-D-glucopyranosyl-(1→2)-6-*O*-acetyl-β-D-glucopyranosyl]-20-*O*-[β-D-xylopyranosyl-(1→6)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-3β,-12β,20(*S*)-trihydroxydammar-24-ene.

It should be noted that the constituent spectrum of Ye-Sanchi was very similar to that of Vietnamese ginseng, the underground part of *P. vietnamensis* collected from central Vietnam.[12,15,16] However, most of these compounds have not been reported, so far, from *P. japonicus* specimens collected from Japan (Chikusetsu-Ninjin in Japanese), the latter containing oleanolic acid saponins in abundance.[17,18] This finding suggested that *P. japonicus* drugs Chikusetsu-Ninjin collected from Japan and Ye-Sanchi from Yunnan Province of China are at least chemotaxonomically different.

The MeOH extract of Ye-Sanchi (50 μg/mL) significantly increased neurite outgrowth in human neuroblastoma SK-N-SH cells. Among 30 saponins isolated from Ye-Sanchi (including 17 other saponins reported in our previous paper[1]), 15 compounds in sufficient amount representative of three types of *Panax* saponin were selected for further investigation of their neurite outgrowth activities (Table 4). At a concentration of 100 μM, ginsenosides Rb$_1$ (**8**) and Rb$_3$ (**12**) and notoginsenosides R$_4$ (**6**) and Fa (**7**) possessing the common protopanaxadiol-type core significantly increased the percentage of cells with neurites in SK-N-SH cells, whereas the other compounds had no effect. Moreover, **6**–**8** and **12** significantly increased the total length of neurites and the number of varicosities, the site of synaptic connection (Table 5). Especially, cells treated with **8** and **12** increased the total length of neurites by more than 3 times compared with control cells (Table 5). When examined under the same experimental conditions, oleanolic acid saponins isolated from other *Panax* species were found inactive (data not shown). This observation clarified that protopanaxadiol-type saponins increased the neurite outgrowth in SK-N-SH cells, but not protopanaxatriol-type, ocotillol-type, and oleanolic acid saponins. These results suggest that the difference in constituent spectra of *Panax* drugs should also be taken into consideration in their utilities and also in evaluating their efficacy.

Table 5. Neurite outgrowth Activities of Compounds **6**–**8** and **12**[a]

	total length of neurites (μm)	number of varicosity per cell
control	45.3 ± 3.97	0.10 ± 0.071
8	149.3 ± 18.59[b]	0.93 ± 0.210[b]
12	131.4 ± 9.95[b]	0.80 ± 0.082[b]
6	116.1 ± 20.96[b]	0.68 ± 0.278[b]
7	112.5 ± 10.68[b]	0.53 ± 0.131[b]

[a] The cells were treated with each compound at a concentration of 100 μM. Each value represents the mean ± sem of 40 cells. [b] $p < 0.05$ compared with control.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Jasco DIP-360 digital polarimeter. IR spectra were measured in KBr using a Jasco FT/IR-230 infrared spectrometer. ^{1}H and ^{13}C NMR spectra were obtained using a JEOL JNA-LA 400WB-FT spectrometer at 400 MHz for proton and 100 MHz for carbon in C$_5$D$_5$N with TMS as an internal standard. The API-MS (positive and negative modes) and API-MS/MS were recorded using a Perkin-Elmer Sciex API-*III* biomolecular mass analyzer. HPLC was performed using a Jasco PU-1587 intelligent preparative pump, a Jasco UV-1575 intelligent UV/vis detector, and a Jasco 807-IT integrator; column, YMC-Pack ODS-AQ (S-5 μm, 12 nm, 250 × 20 mm i.d.); flow rate, 8.0 mL/min; detection, UV at 203 nm. GC analysis was carried out with a GC-17A gas chromatograph (Shimadzu, Japan) fitted with a DB-1 column (0.25 mm i.d. × 30 m) (J&W Scientific); column temp, 50−230 °C, 15 °C/min; carrier gas, He at a flow rate of 50 mL/min.

Plant Material. The underground part of *P. japonicus* (Ye-Sanchi) was collected from Jinping county of Yunnan Province, China, in August 1999. A voucher specimen (TMPW No. 19759) has been deposited in the Museum of Materia Medica, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama.

Extraction and Isolation. The air-dried, powdered underground part of *P. japonicus* (500 g) was extracted with MeOH (4 × 2.5 L) under reflux. After removal of solvent in vacuo and freeze-drying, the MeOH extract (150 g) was obtained. This extract was suspended in H$_2$O (1.5 L) and successively extracted with ethyl acetate (4 × 300 mL) and *n*-BuOH saturated with H$_2$O (4 × 300 mL). The *n*-BuOH layer was evaporated in vacuo and freeze-dried to give 72 g of dry powder. This powder was subjected to CC over Sephadex LH-20 eluted with MeOH to yield 56 g of crude saponin mixture. This mixture was separated by CC (silica gel 40−63 μm), eluted with a gradient solvent system (CHCl$_3$−CH$_3$OH−H$_2$O, 10:1:0 → 6:4:1), to afford 27 fractions. Fraction 23 (3.5 g) was further separated by CC (reversed-phase silica gel RP-18 63−200 μm) eluted with a gradient solvent system of MeOH−H$_2$O (4:6 → 9:1) to give 17 fractions. Similar fractions were pooled and further purified using HPLC eluted with CH$_3$CN−H$_2$O (28:72 → 38:62). Compounds **1** (17.5 mg), **2** (6.8 mg), and **5** (4.0 mg) were obtained from subfraction 23-11. Compounds **3** (8.6 mg), **6** (71.5 mg), **7** (55.0 mg), and **4** (40.6 mg) were from subfraction 23-13, while **8** (125.6 mg), **9** (23.8 mg), **10** (5.5 mg), and **11** (4.1 mg) were from subfraction 23-15, and **12** (124.3 mg) and **13** (9.2 mg) were from subfraction 23-17.

Yesanchinoside G (1): white amorphous powder, [α]$_D^{20}$ +28.6° (*c* 0.1, 40% CH$_3$CN); IR (KBr) cm^{-1} 3394, 2925, 1647, 1479, 1385, 1171, 1078, 1041; ^{1}H NMR and ^{13}C NMR (see Tables 1, 2, and 3); API-MS (positive mode, NH$_4$OAc) *m/z* 1115 [M + Na]$^+$, 803 [M − glc − xyl − H$_2$O + Na]$^+$, 457 [M − 3glc − xyl − H$_2$O + H]$^+$, 421 [M − 3glc − xyl − 3H$_2$O + H]$^+$; API-MS/MS (positive, parent ion *m/z* 1115) 1115 [M + Na]$^+$, 803 [M − glc − xyl − H$_2$O + Na]$^+$, 621 [M − 2glc − xyl − H$_2$O + H]$^+$, 335 [glc + xyl − H$_2$O + Na]$^+$; (negative, NH$_4$OAc) *m/z* 1091 [M − H]$^−$, 959 [M − xyl − H]$^−$, 797 [M − xyl − glc − H]$^−$, 653 [M − xyl − 2glc + H$_2$O − H]$^−$; API-MS/MS (negative, parent ion *m/z* 1091) 1091 [M − H]$^−$, 929 [M − glc − H]$^−$, 797

$[M - glc - xyl - H]^-$, 635 $[M - 2glc - xyl - H]^-$, 473 $[M - 3glc - xyl - H]^-$; *anal.* C 57.30%, H 8.10%, calcd for $C_{53}H_{88}O_{23}\cdot H_2O$, C 57.20%, H 8.12%.

Yesanchinoside H (2): white amorphous powder, $[\alpha]_D^{20}$ +35.5° (*c* 0.1, 40% CH_3CN); IR (KBr) cm^{-1} 3384, 2934, 2855, 1646, 1566, 1459, 1387, 1087, 1044; ^{1}H NMR and ^{13}C NMR (see Tables 1, 2, and 3); API-MS (positive mode, MeOH) *m/z* 1117 $[M + Na]^+$, 971 $[M - glc + K]^+$; API-MS/MS (positive, NH_4OAc, parent ion *m/z* 1117) 1117 $[M + Na]^+$, 805 $[M - glc - xyl - H_2O + Na]^+$, 365 $[2glc + Na]^+$, 335 $[glc + xyl + Na]^+$; (negative, MeOH) *m/z* 1093 $[M - H]^-$; API-MS/MS (negative, parent ion *m/z* 1093) 962 $[M - xyl - H]^-$, 932 $[M - glc - H]^-$, 800 $[M - xyl - glc - H]^-$, 637 $[M - xyl - 2glc - H]^-$, 475 $[M - xyl - 3glc - H]^-$; *anal.* C 54.54%, H 8.40%, calcd for $C_{53}H_{90}O_{23}\cdot 4H_2O$, C 54.49%, H 8.36%.

Yesanchinoside I (3): white amorphous powder, $[\alpha]_D^{20}$ −2.2° (*c* 0.1, 40% CH_3CN); IR (KBr) cm^{-1} 3420, 2928, 1647, 1469, 1375, 1321, 1261, 1181, 1078, 1041; ^{1}H NMR and ^{13}C NMR (see Tables 1, 2, and 3); API-MS (positive mode, MeOH) *m/z* 1263 $[M + K]^+$, 1247 $[M + Na]^+$; API-MS/MS (positive, parent ion *m/z* 1247) 1247 $[M + Na]^+$, 935 $[M - xyl - glc - H_2O + Na]^+$, 773 $[M - xyl - 2glc - H_2O + Na]^+$, 497 $[xyl + 2glc - H_2O + Na]^+$; (negative, MeOH) *m/z* 1223 $[M - H]^-$, 1091 $[M - xyl - H]^-$, 929 $[M - xyl - glc - H]^-$; API-MS/MS (negative, parent ion *m/z* 1223) 1091 $[M - xyl - H]^-$, 1061 $[M - glc - H]^-$, 929 $[M - xyl - glc - H]^-$, 768 $[M - xyl - 2glc - H]^-$, 605 $[M - xyl - 3glc - H]^-$; *anal.* C 57.00%, H 8.12%, calcd for $C_{59}H_{100}O_{26}\cdot H_2O$, C 57.06%, H 8.13%.

Yesanchinoside J (4): white amorphous powder, $[\alpha]_D^{20}$ +0.73° (*c* 0.1, 40% CH_3CN); IR (KBr) cm^{-1} 3398, 2923, 2846, 1739, 1638, 1479, 1375, 1190, 1097, 1041; ^{1}H NMR and ^{13}C NMR (see Table 1, 2, and 3); API-MS (positive mode, NH_4OAc) *m/z* 1305 $[M + Na]^+$, 809 $[M - xyl - 2glc - H_2O + H]^+$; API-MS/MS (positive, parent ion *m/z* 1305) 1305 $[M + Na]^+$, 831 $[M - xyl - 2glc - H_2O + Na]^+$; (negative, NH_4OAc) *m/z* 1281 $[M - H]^-$, 987 $[M - xyl - glc - H]^-$, 640 $[M - xyl - 3glc - Ac + H_2O - H]^-$; API-MS/MS (negative, parent ion *m/z* 1281) 1281 $[M - H]^-$, 1240 $[M - Ac - H]^-$, 1150 $[M - xyl - H]^-$, 1108 $[M - xyl - Ac - H]^-$, 946 $[M - xyl - glc - Ac - H]^-$, 783 $[M - xyl - 2glc - Ac - H]^-$, 621 $[M - xyl - 3glc - Ac - H]^-$; *anal.* C 55.54%, H 8.04%, calcd for $C_{61}H_{102}O_{28}\cdot 2H_2O$, C 55.41%, H 8.00%.

Determination of Sugar Components.[19,20] Compounds **1**−**4** (each 0.8 mg) were separately hydrolyzed with 10% HCl in 40% CH_3CN solution by stirring at 80 °C for 4 h. After neutralization with 2.0 M NaOH, the mixture was extracted with $CHCl_3$. The aqueous layer was filtered through Amberlite MB-3 (desalting) and evaporated to dryness in vacuo. The residue was dissolved in anhydrous pyridine (0.1 mL), and 0.2 mL of 0.1 M L-cysteine methyl ester hydrochloride was added. The reaction mixture was stirred at 60 °C for 1 h. After adding 0.15 mL of HMDS−TMCS (hexamethyldisilazane−trimethyl-chlorosilane−pyridine = 2:1:10), the mixture was stirred at 60 °C for another 30 min. After centrifugation, the supernatant was directly subjected to GC analysis. The sugar derivatives obtained from **1**−**4** showed two peaks at t_R 21.55 and 18.04 min, which were identical with those of authentic D-glucose and D-xylose, respectively.

Alkaline Hydrolysis of Yesanchinoside J (4). A solution of **4** (7.2 mg) in 2.5 mL of 25% NH_4OH was stirred at 35 °C for 16 h. After neutralization with 2.0 M HCl, the solution was desalted with Amberlite MB-3 and the volume of the eluate was concentrated to ca. 1.0 mL in vacuo. This solution was subjected to preparative HPLC (mobile phase, CH_3CN−H_2O, 26:74 v/v; flow rate, 8.0 mL/min) to afford a white amorphous powder (4.9 mg), t_R = 30.7 min. Its t_R, API-MS, IR, and ^{1}H and ^{13}C NMR spectra were identical to those of notoginsenoside R$_4$ (**6**).

Neurite Outgrowth Activity.[21] A human neuroblastoma cell line, SK-N-SH (RIKEN, Tsukuba, Japan), was maintained as a monolayer culture in minimum essential medium (GIBCO BRL, Rockville) supplemented with 5% fetal bovine serum at 37 °C in a humidified atmosphere of 95% air/5% CO_2. Cells were plated at a density of 4.7×10^4 cell/mL in 60 mm diameter culture dishes with 2 mm grids (Corning, New York). Extracts (50 μg/mL), isolated compouds (100 μM), or vehicle solution (0.1% DMSO) was added to the culture medium once at the start of culture. Five days after the treatment, the cells (100−300 cells) were examined in four areas (650 × 430 μm). The percentage of cells with neurites longer than 50 μm, the total length of extended neurites, and the number of varicosities were calculated. Statistical comparisons were made by Student's *t*-test; $P < 0.05$ was considered as significant.

References and Notes

(1) Zou, K.; Zhu, S.; Tohda, C.; Cai, S. Q.; Komatsu, K. *J. Nat. Prod.* **2002**, *65*, 346−351.
(2) Zou, K.; Zhu, S.; Cai, S. Q.; Komatsu, K. The 121th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo; Abstracts of Papers; 2001, p 110.
(3) Oura, H.; Ono, T.; Sakanaka, M.; Yoshimura, H. In *Medicinal Ginseng 2000*; Kumagai, A., Ed.; Kyoritsu Press: Tokyo, 2000; pp 23−29, 65−93.
(4) Zhang, J. T.; Qu, Z. W.; Liu, Y.; Deng, H. J. *Chin. Med. J.* **1990**, *103*, 932−938.
(5) Inhee, M. J.; Hong, H. S.; Boo, J. H.; Lee, K. H.; Yun, S. H.; Cheong, M. Y.; Joo, I.; Huh, K.; Jung, M. W. *J. Neurosci. Res.* **2001**, *63*, 509−515.
(6) Yoshikawa, M.; Murakami, T.; Ueno, T.; Hirokawa, N.; Yashiro, K.; Murakami, N.; Yamahara, J.; Matsuda, H.; Saijoh, R.; Tanaka, O. *Chem. Pharm. Bull.* **1997**, *45*, 1056−1062.
(7) Matsuura, H.; Kasai, R.; Tanaka, O.; Saruwatari, Y.; Fuwa, T.; Zhou, J. *Chem. Pharm. Bull.* **1983**, *31*, 2281−2287.
(8) Yang, T. R.; Kasai, R.; Zhou, J.; Tanaka, O. *Phytochemistry* **1983**, *22*, 1473−1478.
(9) Zheng, H. Z.; Yang, C. R., Ed. *Sanqi: Biology and its Application*; Science Press: Beijing, 1994; pp 6−8; 37−39.
(10) Yoshikawa, M.; Murakami, R.; Yashiro, K.; Yamahara, J.; Matsuda, H.; Saijoh, R.; Tanaka, O. *Chem. Pharm. Bull.* **1998**, *46*, 647−654.
(11) Asakawa, J.; Kasai, R.; Yamasaki, K.; Tanaka, O. *Tetrahedron* **1977**, *33*, 1935−1977.
(12) Duc, N. M.; Kasai, R.; Ohtani, K.; Nham, N. T.; Yamasaki, K.; Tanaka, O. *Chem. Pharm. Bull.* **1994**, *42*, 634−640.
(13) Tran, L. Q.; Adnyana, I. K.; Tezuka, Y.; Nagaoka, T.; Tran, Q. K.; Kadota, S. *J. Nat. Prod.* **2001**, *64*, 456−461.
(14) Yoshikawa, M.; Murakami, R.; Ueno, T.; Yashiro, K.; Hirokawa, N.; Murakami, N.; Yamahara, J.; Matsuda, H.; Saijoh, R.; Tanaka, O. *Chem. Pharm. Bull.* **1997**, *45*, 1039−1045.
(15) Duc, N. M.; Nham, N. R.; Kasai, R.; Ito, A.; Yamasaki, K.; Tanaka, O. *Chem. Pharm. Bull.* **1993**, *41*, 2010−2014.
(16) Duc, N. M.; Kasa, R.; Ohtani, K.; Ito, A.; Nham, N. T.; Yamasaki, K.; Tanaka, O. *Chem. Pharm. Bull.* **1994**, *42*, 115−122.
(17) Lin, T. D.; Kondo, N.; Shoji, J. *Chem. Pharm. Bull.* **1976**, *24*, 253−261.
(18) Morita, T.; Kasai, R.; Kohda, H.; Tanaka, O.; Yang, T. R. *Chem. Pharm. Bull.* **1983**, *31*, 3205−3209.
(19) Ma, C. M.; Nakamura, N.; Hattori, M. *Chem. Pharm. Bull.* **1998**, *46*, 982−987.
(20) Hara, S.; Okabe, H.; Mihashi, K. *Chem. Pharm. Bull.* **1987**, *35*, 501−506.
(21) Tohda, C.; Kuboyama, T.; Komatsu, K. *NeuroReport* **2000**, *11*, 1981−1985.