Bioactive Saponins and Glycosides. XIX.[1] Notoginseng (3): Immunological Adjuvant Activity of Notoginsenosides and Related Saponins: Structures of Notoginsenosides-L, -M, and -N from the Roots of *Panax notoginseng* (BURK.) F. H. CHEN.

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Received June 12, 2001; accepted July 19, 2001

New dammarane-type triterpene saponins, notoginsenosides-L, -M, and -N, were isolated from the glycosidic fraction of the dried roots of *Panax notoginseng* (BURK.) F. H. CHEN. Their structures were elucidated on the basis of chemical and physicochemical evidence. Immunological adjuvant activities of the principal notoginsenosides and related dammarane-type triterpene saponins were examined and notoginsenosides-D, -G, -H, and -K were found to increase the serum IgG level in mice sensitized with ovalbumin.

Key words notoginsenoside; *Panax notoginseng*; immunological adjuvant activity; bioactive saponin; Araliaceae

The Araliaceae plant *Panax notoginseng* (BURK.) F. H. CHEN. is cultivated on a large scale in Yunnan and Guang-Xi provinces, China. The main roots of this plant, notoginseng (三七人参 or 田七人参 in Japanese), is used for treatment of trauma and bleeding due to internal and external injury. As the principal constituents of this plant, various dammarane-type triterpene saponins have been characterized from the roots, leaves, and buds.[2] During the course of our chemical and pharmacological studies on the saponin constituents of natural medicines[1,3] and medicinal foodstuffs,[4] we found that the saponin fraction from notoginseng was found to show potent protective effect on liver injury induced by D-galactosamine and lipopolysaccharide.[5] From the active saponin fraction, we have hitherto characterized ten dammarane-type triterpene saponins termed notoginsenosides-A (**4**), -B, -C (**6**), -D (**7**), -E, -G (**11**), -H (**13**), -I (**8**), -J (**14**), and -K (**5**) and an acetylenic fatty acid glycoside called notoginsenic acid β-sophoroside together with eighteen known saponins.[5]

As a continuing study, we have further isolated a new dammarane-type triterpene tetraglycoside called notoginsenoside-L (**1**) and two new dammarane-type triterpene triglycosides having an α-glucosidic linkage named notoginsenosides-M (**2**) and -N (**3**) together with ginsenoside-Rb_3[6] from the glycosidic fraction, whereas four known acetylenes, panaxynol,[7] panaxydol,[7] panaxytriol,[8] and PQ-1,[9] and a known sesquiterpene, $1\beta,6\alpha$-dihydroxy-4(15)-eudesmene,[10] were isolated from the ethyl acetate-soluble portion. In this paper, we describe the isolation and structure elucidation of three new notoginsenosides (**1**—**3**). Furthermore, since notoginseng as well as ginseng (*Panax ginseng* C. A. MAYER, roots) and American ginseng (*Panax quinquefolium* L., roots) has been known to show tonic activity in Chinese traditional medicine, we examined the immunological adjuvant effect of the principal eleven notoginsenosides (**1**—**8**, **11**, **13**, **14**), two ginsenosides (**10**, **18**),[11] and five quinquenosides (**9**, **12**, **15**—**17**)[12] from notoginseng, ginseng, and American ginseng, respectively.

Structures of Notoginsenosides-L (1), -M (2), and -N (3) Notoginsenoside-L (**1**) was obtained as colorless fine crystals of mp 195—197 °C from aqueous methanol. The IR spectrum of **1** showed strong absorption bands at 3407, 1077, and 1047 cm^{-1} suggestive of the oligoglycosidic structure and a weak band at 1655 cm^{-1} due to an olefin function. The molecular formula $C_{53}H_{90}O_{22}$ was determined from the quasimolecular ion peaks observed in the positive-ion and negative-ion fast atom bombardment (FAB)-MS and high-resolution MS measurement. Namely, a quasimolecular ion peak was observed at m/z 1101 $(M+Na)^+$ in the positive-ion FAB-MS of **1**, while its negative-ion FAB-MS showed the quasimolecular ion peak at m/z 1077 $(M-H)^-$ in addition to fragment ion peaks at m/z 945 $(M-C_5H_9O_4)^-$ and m/z 915 $(M-C_6H_{11}O_5)^-$, which were derived by cleavage of the glycoside linkages of the terminal pentose and hexose, respectively. Acid hydrolysis of **1** with 5% aqueous sulfuric acid–dioxane (1 : 1, v/v) liberated D-glucose and D-xylose, which were identified by gas–liquid chromatography (GLC) analysis[13] of their trimethylsilyl (TMS) thiazolidine derivatives. The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[14] of **1** showed signals assignable to three β-D-glucopyranosyl moieties [δ 4.90 (d, J=7.6 Hz, 1′-H), 5.05 (d, J=7.6 Hz, 1″″-H), 5.10 (d, J=7.9 Hz, 1‴-H)] and a β-D-xylopyranosyl moiety [δ 5.23 (d, J=7.4 Hz, 1″-H)] together with eight tertiary methyls [δ 0.85, 0.97, 0.98, 1.10, 1.30, 1.62, 1.65, 1.67 (all s, 19, 18, 30, 29, 28, 26, 21, 27-H_3)], two methine protons bearing an oxygen function [δ 3.28 (dd, J=4.2, 11.2 Hz, 3-H), 4.15 (m, 12-H)], and a trisubstituted olefin proton [δ 5.31 (t-like, 24-H)]. The carbon signals of the sapogenol part in the ^{13}C-NMR (Table 1) of **1** were found to be superimposable with those of 20(S)-protopanaxadiol glycosides,[5,11,12,15] while the carbon signals of the disaccharide moiety were similar to those of ginsenoside-Rb_1 (**10**)[11,15] except for the terminal 2′-O-β-D-xylopyranosyl moiety. The disaccharide structures bonding to the 3 and 20-hydroxyl groups in the 20(S)-protopanaxadiol part were determined by a heteronuclear multiple bond correlation (HMBC) experiment (J_{CH}=8 Hz) on **1**. Namely, long-range correlations were observed between the 1″-proton and the 2′-carbon, between the 1′-proton and the 3-carbon, between the 1″″-proton and the 6‴-carbon, and between the 1‴-proton and the 20-carbon. On the basis of this

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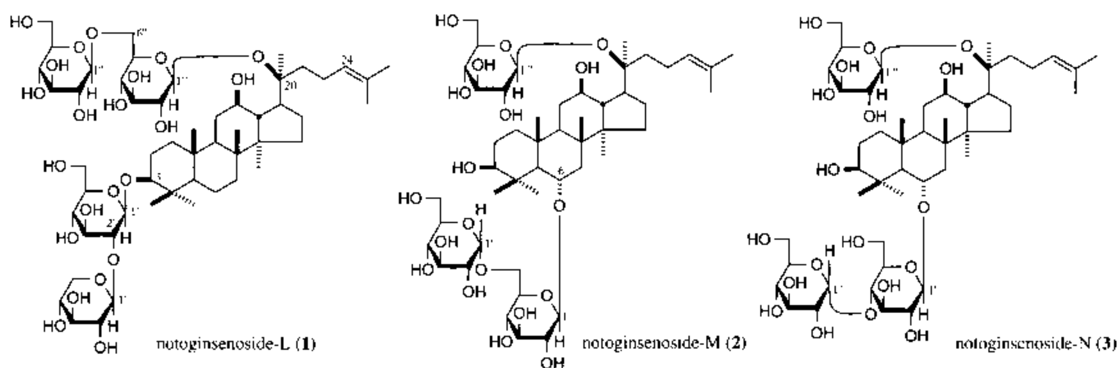

Chart 1

notoginsenoside-A (4) : R=OH
notoginsenoside-K (5) : R=OOH

notoginsenoside-C (6)

	R^1	R^2	R^3
notoginsenoside-D (7) :	-Glc $\frac{2\text{-}1}{}$ Glc $\frac{2\text{-}1}{}$ Xyl	-OH	-Glc $\frac{6\text{-}1}{}$ Glc $\frac{6\text{-}1}{}$ Xyl
notoginsenoside-I (8) :	(S1)	-H	(S2)
quinquenoside V (9) :	(S1)	-OH	-Glc $\frac{6\text{-}1}{}$ Glc $\frac{4\text{-}1}{}$ Glc*
ginsenoside Rb_1 (10) :	(S1)	-OH	(S2)

notoginsenoside-G (11): R=Glc
quinquenoside IV (12): R=(S2)

notoginsenoside-H (13)

notoginsenoside-J (14)

ginsenoside Rg_1 (18)

	R^1	R^2	R^3
quinquenoside I (15) :	-Glc	-H	(2-butenoyl, CH_3)
quinquenoside II (16) :	(S2)	-H	(2-octenoyl, CH_3)
quinquenoside III (17) :	-Glc	-Ac	-H

Ac: acetyl
Glc: β-D-glucopyranosyl
Glc*: α-D-glucopyranosyl

Chart 2

Table 1. ^{13}C-NMR Data of Notoginsenosides-L (**1**), -M (**2**), and -N (**3**)

	1	2	3		1	2	3
C-1	39.5	39.7	39.7	1′	105.0	106.1	105.7
2	26.9	27.8	27.9	2′	84.1	75.4	74.9
3	89.1	78.7	78.6	3′	78.5	79.3	78.9
4	39.8	40.2	40.4	4′	71.8	71.6	81.3
5	56.6	61.4	61.4	5′	78.1	76.2	76.5
6	18.5	79.6	80.3	6′	63.0	69.1	62.2
7	35.3	45.7	44.9	1″	107.0	101.3	103.0
8	40.1	41.3	41.1	2″	76.5	73.9	74.4
9	50.3	50.0	50.0	3″	78.2	75.2	75.3
10	37.0	39.5	39.4	4″	71.2	72.2	72.0
11	30.9	30.8	30.9	5″	67.5	74.0	75.5
12	70.2	70.3	70.3	6″		62.7	62.9
13	49.6	49.1	49.1	1‴	98.1	98.2	98.3
14	51.5	51.7	51.4	2‴	74.9	75.1	75.2
15	30.8	30.8	30.7	3‴	79.2	79.2	79.2
16	26.7	26.7	26.6	4‴	71.7	71.5	71.6
17	51.8	51.4	51.6	5‴	77.0	78.2	78.2
18	10.5	17.7	17.5	6‴	70.3	62.7	62.9
19	16.3	17.5	17.5	1⁗	105.3		
20	83.5	83.4	83.3	2⁗	75.3		
21	22.5	22.3	22.4	3⁗	78.4		
22	36.3	35.8	36.1	4⁗	71.9		
23	23.3	23.1	23.3	5⁗	78.3		
24	126.0	126.0	126.0	6⁗	62.9		
25	131.1	130.9	131.0				
26	25.8	25.7	25.8				
27	18.0	17.8	18.0				
28	27.9	31.6	31.7				
29	16.3	16.6	16.3				
30	16.1	17.3	17.2				

125 MHz, pyridine-d_5.

evidence, the structure of notoginsenoside-L was characterized as 3-*O*-β-D-xylopyranosyl(1→2)-β-D-glucopyranosyl-20(*S*)-protopanaxadiol 20-*O*-β-D-glucopyranosyl(1→6)-β-D-glucopyranoside (**1**).

Notoginsenoside-M (**2**) was also isolated as colorless fine crystals of mp 187—189 °C and its IR spectrum showed absorption bands at 3410, 1647, and 1076 cm^{-1} assignable to hydroxyl and olefin functions. In the positive-ion and negative-ion FAB-MS of **2**, quasimolecular ion peaks were observed at *m/z* 985 $(M+Na)^+$ and *m/z* 961 $(M-H)^-$ and high-resolution MS analysis revealed the molecular formula of **2** to be $C_{48}H_{82}O_{19}$. Furthermore, fragment ion peaks at *m/z* 799 $(M-C_6H_{11}O_5)^-$ and *m/z* 637 $(M-C_{12}H_{21}O_{10})^-$ were observed in the negative-ion FAB-MS. The acid hydrolysis of **2** liberated D-glucose, which was identified by GLC analysis of the TMS thiazolidine derivative.[13] The ^{1}H-NMR (pyridine-d_5) spectrum[14] of **2** showed signals assignable to two β-D-glucopyranosyl moieties [δ 5.03 (d, J=7.6 Hz, 1′-H), 5.16 (d, J=7.6 Hz, 1‴-H)], an α-D-glucopyranosyl moiety [δ 5.50 (d, J=3.7 Hz, 1″-H)], and a 20(*S*)-protopanaxatriol moiety [δ 0.91, 0.98, 1.21, 1.53, 1.59, 2.07 (all s, 30, 19, 18, 29, 27, 28-H_3), 1.56 (s, 21, 26-H_3), 3.49 (dd, J=4.6, 11.3 Hz, 3-H), 4.14 (m, 12-H), 4.35 (m, 6-H), 5.22 (t-like, 24-H)]. The carbon signals in the ^{13}C-NMR (Table 1) spectrum[14] of **2** closely resembled those of ginsenoside-Rg_1 (**18**),[11,15] except for the signals due to the terminal α-D-glucopyranosyl moiety in **2**. Furthermore, comparison of the ^{13}C-NMR data for **2** with those for **18** revealed a glycosilation shift around the 6′-position of the ginsenoside-Rg_1 moiety of **2**. The bisdesmoside structure of **2** was characterized from the HMBC experiment of **2**, which showed long-range correlations between the following protons and carbons: 1″-H and 6′-C; 1′-H and 6-C; 1‴-H and 20-C. Comparison of the NMR data for **2** with those for several 20(*S*)-protopanaxatriol glycosides having an α-D-glucopyranosyl moiety[13,15] led us to elucidate the structure of notoginsenoside-M as 6-*O*-α-D-glucopyranosyl(1→6)-β-D-glucopyranosyl 20(*S*)-protopanaxatriol 20-*O*-β-D-glucopyranoside (**2**).

Notoginsenoside-N (**3**), isolated as colorless fine crystals of mp 186—188 °C, exhibited absorption bands due to hydroxyl and olefin groups (3432, 1638, 1075 cm^{-1}) in the IR spectrum. The molecular formula $C_{48}H_{82}O_{19}$, which was the same as that of notoginsenoside-M (**2**), was determined from the quasimolecular ion peaks [*m/z* 985 $(M+Na)^+$ and *m/z* 961 $(M-H)^-$] in the positive-ion and negative-ion FAB-MS and by high-resolution MS measurement. By the acid hydrolysis of **3**, D-glucose was detected by GLC analysis of the TMS thiazolidine derivative.[13] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[14] of **3** showed signals due to two β-D-glucopyranosyl moieties [δ 4.89 (d, J=7.6 Hz, 1′-H), 5.17 (d, J=7.6 Hz, 1‴-H)] and an α-D-glucopyranosyl moiety [δ 5.88 (d, J=3.7 Hz, 1″-H)] together with a protopanaxatriol part. The carbon signals in the ^{13}C-NMR spectrum of **3** were very similar to those for **2**, except for the signals of the 4′, 6′, and 1″-positions. In the HMBC experiment of **3**, long-range correlations were observed between the 1″-proton and the 4′-carbon, between the 1′-proton and the 6-carbon, and between the 1‴-proton and the 20-carbon. Consequently, the structure of notoginsenoside-N was determined as 6-*O*-α-D-glucopyranosyl(1→4)-β-D-glucopyranosyl-20(*S*)-protopanaxatriol 20-*O*-β-D-glucopyranoside (**3**).

Immunological Adjuvant Activity of Notoginsenosides and the Related Dammarane-Type Triterpene Saponins Recently, the saponin fraction Quil A and purified QS-21 from *Quillaja saponaria* MOLINA were reported to show an immunological adjuvant effect (an immunostimulating effect).[16] In our previous study, several dammarane-type saponins from the seeds of *Zizyphus jujuva* var. *spinosa*, which has been used for tonic in Chinese traditional medicines, showed immunological adjuvant activity.[17] In the present study, we examined the immunological adjuvant activity of the dammarane-type triterpene glycosides, notoginsenosides, ginsenosides, and quiquenosides, to characterize the traditional pharmacological effect of this Chinese medicinal herb.

As shown in Fig. 1, notoginsenosides-D (**7**), -G (**11**), -H (**13**), and -K (**5**) were found to show an increase in the serum IgG levels in chiken ovalbumin (OVA)-immunized mice. Notoginsenosides-A (**4**), -C (**6**), -I (**8**), -L (**1**), and -N (**3**) and quiquenosides III (**17**), IV (**12**), and V (**9**) tended to show this activity.

Experimental

The instruments used for obtaining physical data and experimental conditions for chromatography were the same as described in our previous paper.[4]

Isolation of Acetylenic Compounds and Sesquiterpene from the Ethyl Acetate-Soluble Portion The AcOEt-soluble portion (44 g, 0.75%), obtained from the roots of *Panax notoginseng* as reported previously,[4] was subjected to ordinary-phase silica gel column chromatography [BW-200 (Fuji Silysia Chemical, Ltd.), *n*-hexane–AcOEt (10 : 1→2 : 1, v/v)→$CHCl_3$–MeOH (5 : 1→2 : 1, v/v)] to furnish five fractions [fr. 1 (10.1 g), fr. 2 (4.2 g), fr. 3 (4.4 g), fr. 4 (2.3 g), fr. 5 (15.6 g)]. Fraction 2 (2.1 g) was purified by reversed-phase silica gel column chromatography [MeOH–H_2O

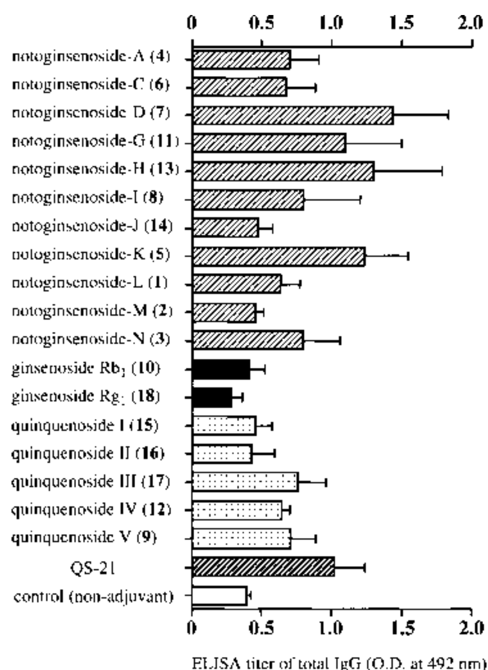

Fig. 1. Effects of Notoginsenosides (**1**—**8**, **11**, **13**, **14**), Ginsenosides (**10**, **18**), and Quinquenosides (**9**, **12**, **15**—**17**) on Serum IgG in Mice Sensitized with OVA

Female ddY mice were immunized with OVA (10 μg/0.1 ml PBS/mouse, *i.m.*) containing 20 μg of test compound. After 4 weeks, blood samples were collected, and then anti-OVA IgG levels were determined using ELISA system. Values represent the means with S.E.

(80 : 20→90 : 10, v/v)] followed by HPLC [YMC-Pack ODS, MeOH–H_2O (80 : 20, v/v)] to give panaxynol (450 mg, 0.0015%) and panaxydol (54 mg, 0.0018%). Fraction 3 (2.2 g) was also separated with reversed-phase silica gel column chromatography and HPLC [MeOH–H_2O (65 : 35→95 : 5, v/v)] to provide 1β,6α-dihydroxy-4(15)-eudesmene (9.3 mg, 0.00031%) and PQ-1 (63 mg, 0.0021%). Fraction 4 (2.3 g) was purified by reversed-phase silica gel column chromatography and HPLC [MeOH–H_2O (50 : 50→70 : 30, v/v)] to give panaxytriol (102 mg, 0.0017%). These known compounds were identified by comparison of their physical data ([α]$_D$, IR, ^{1}H, ^{13}C-NMR) with reported values.[7—9)]

Isolation of Notoginsenosides-L (1), -M (2), and -N (3) and Ginsenoside-Rb$_3$ from the Glycosidic Fraction Earlier saponin fractions, fr. 3-3 (0.2 g) and fr. 4-1 (0.5 g), obtained from the roots of *Panax notoginseng* (cultivated in Yunnan Province, China and purchased through Teikoku Seiyaku Co., Ltd., Japan) as reported previously,[4)] were subjected to reversed-phase silica gel column chromatography [Chromatorex ODS DM 1020T (Fuji Silysia Chemical Ltd., MeOH–H_2O (60 : 40, v/v)] followed by HPLC [YMC-Pack ODS (250×20 mm i.d., YMC Co., Ltd.), MeOH–H_2O (55 : 45, v/v)] to give notoginsenoside-L (**1**, 24.2 mg, 0.0014%) and ginsenoside-Rb$_3$ (22.3 mg, 0.0011%) from fr. 3-3 and notoginsenosides-M (**2**, 57.0 mg, 0.0035%) and -N (**3**, 23.9 mg, 0.0014%).

Notoginsenoside-L (**1**): Colorless fine crystals from aqueous MeOH, mp 195—197 °C, $[\alpha]_D^{28}$ +20.4° (c=0.1, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{53}H_{90}O_{22}Na$ $(M+Na)^+$: 1101.5821. Found: 1101.5798. IR (KBr): 3407, 1655, 1077, 1047 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.85, 0.97, 0.98, 1.10, 1.30, 1.62, 1.65, 1.67 (3H each, all s, 19, 18, 30, 29, 28, 26, 21, 27-H_3), 3.28 (1H, dd, J=4.2, 11.2 Hz, 3-H), 4.15 (1H, m, 12-H), 4.90 (1H, d, J=7.6 Hz, Glc-1′-H), 5.05 (1H, d, J=7.6 Hz, Glc-1⁗-H), 5.10 (1H, d, J=7.9 Hz, Glc-1‴-H), 5.23 (1H, d, J=7.4 Hz, Xyl-1″-H), 5.31 (1H, t-like, 24-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_c: given in Table 1. Positive-ion FAB-MS: m/z 1101 $(M+Na)^+$. Negative-ion FAB-MS: m/z 1077 $(M-H)^-$, 945 $(M-C_5H_9O_4)^-$, 915 $(M-C_6H_{11}O_5)^-$.

Notoginsenoside-M (**2**): Colorless fine crystals from aqueous MeOH, mp 187—189 °C, $[\alpha]_D^{24}$ +24.7° (c=0.3, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{48}H_{82}O_{19}Na$ $(M+Na)^+$: 985.5348. Found: 985.5347. IR (KBr): 3410, 1647, 1076 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.91, 0.98, 1.21, 1.53, 1.59, 2.07 (3H each, all s, 30, 19, 18, 29, 27, 28-H_3), 1.56 (6H, s, 21, 26-H_3), 3.49 (1H, dd, J=4.6, 11.3 Hz, 3-H), 4.14 (1H, m, 12-H), 4.35 (1H, m, 6-H), 5.03 (1H, d, J=7.6 Hz, Glc-1′-H), 5.16 (1H, d, J=7.6 Hz, Glc-1‴-H), 5.22 (1H, t-like, 24-H), 5.50 (1H, d, J=3.7 Hz, Glc-1″-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_c: given in Table 1. Positive-ion FAB-MS: m/z 985 $(M+Na)^+$. Negative-ion FAB-MS: m/z 961 $(M-H)^-$, 799 $(M-C_6H_{11}O_5)^-$, 637 $(M-C_{12}H_{21}O_{10})^-$.

Notoginsenoside-N (**3**): Colorless fine crystals from aqueous MeOH, mp 186—188 °C, $[\alpha]_D^{22}$ +50.0° (c=0.3, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{48}H_{82}O_{19}Na$ $(M+Na)^+$: 985.5348. Found: 985.5366. IR (KBr): 3432, 1638, 1075 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.80, 1.01, 1.15, 1.60, 2.03 (3H each, all s, 30, 19, 18, 29, 28-H_3), 1.61 (9H, s, 21, 26, 27-H_3), 3.51 (1H, dd, J=4.6, 11.6 Hz, 3-H), 4.09 (1H, m, 12-H), 4.32 (1H, m, 6-H), 4.89 (1H, d, J=7.6 Hz, Glc-1′-H), 5.17 (1H, d, J=7.6 Hz, Glc-1‴-H), 5.27 (1H, t-like, 24-H), 5.88 (1H, d, J=3.7 Hz, Glc-1″-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_c: given in Table 1. Positive-ion FAB-MS: m/z 985 $(M+Na)^+$. Negative-ion FAB-MS: m/z 961 $(M-H)^-$, 799 $(M-C_6H_{11}O_5)^-$.

Acid Hydrolysis of Notoginsenosides-L (1), -M (2), and -N (3) A solution of notoginsenosides (**1**, **2**, and **3**, 2 mg each) in 5% aq. H_2SO_4–1,4-dioxane (1 : 1, v/v, 1 ml) was heated under reflux for 2 h. After cooling, the reaction mixture was neutralized with Amberlite IRA-400 (OH^- form) and the resin was filtered. After removal of the solvent from the filtrate *in vacuo*, the residue was passed through a Sep-Pak C18 cartridge with H_2O and MeOH. The H_2O eluate was concentrated and the residue was treated with L-cysteine methyl ester hydrochloride (0.3 mg) in pyridine (0.02 ml) at 60 °C for 1 h. The solution was subsequently treated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (0.01 ml) at 60 °C for 1 h. The supernatant was then subjected to GLC analysis to identify the derivatives of D-glucose (i) from **1**, **2**, and **3** and D-xylose (ii) from **1**. GLC conditions : column: Supelco STB™-1, 30 m×0.25 mm (i.d.) capillary column, injector temperature: 230 °C, detector temperature: 230 °C, column temperature: 230 °C, He flow rate 15 ml/min, t_R: i: 24.2 min, ii: 13.8 min.

Immunological Adjuvant Activity Female ddY mice (6 weeks old) were immunized with OVA (Grade VI, Sigma) [10 μg/0.1 ml phosphate-buffered saline (PBS)/mouse, *i.m.*] containing 20 μg of test compound. After 4 weeks, blood samples were collected, and then anti-OVA antibody levels were determined using enzyme-linked immunosorbent assay (ELISA).

The 96 well microplates were coated with OVA. Briefly, 150 μl of 0.1% OVA in bicarbonate buffer (pH 9.4) was added to each well. Plates were incubated at 37 °C for 2 h, followed by washing three times with PBS containing 0.05% Tween (Tween–PBS). The sera (100-fold diluted) were added to the plate (100 μl/well) and incubated at 37 °C for 1 h. After washing three times with Tween–PBS, the bound antibody was detected using an anti-mouse IgG polyclonal antibody conjugated with horseradish peroxidase (Wako Pure Chemical Industries). The anti-mouse IgG was diluted in Tween–PBS to 1 : 4000, and 100 μl of the diluted IgG was added to each well. After incubation for 0.5 h at 37 °C and three final washes with Tween–PBS, the *o*-phenylenediamine solution [10 mg *o*-phenylenediamine in 25 ml citrate–phosphate buffer (pH 5.1) and 10 μl of 30% H_2O_2] (100 μl/well) was added to the wells and incubated at 37 °C for 30 min. The reaction was stopped by the addition of 50 μl of 1 M H_2SO_4 to the well, and absorbance was read using a microplate reader at 492 nm. Each value represents the mean±S.E. of 5 mice.

References and Notes

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