Bioactive Saponins and Glycosides. XIV.[1] Structure Elucidation and Immunological Adjuvant Activity of Novel Protojujubogenin Type Triterpene Bisdesmosides, Protojujubosides A, B, and B_1, from the Seeds of *Zizyphus jujuba* var. *spinosa* (Zizyphi Spinosi Semen)

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Following the elucidation of jujubosides A_1 and C and acetyljujuboside B, novel protojujubogenin type triterpene bisdesmosides, protojujubosides A, B, and B_1, were isolated from Zizyphi Spinosi Semen, the seeds of *Zizyphus jujuba* MILL. var. *spinosa* HU. The structures of protojujubosides A, B, and B_1 were determined on the basis of chemical and physicochemical evidence, which included the conversion of protojujubosides to known jujubosides using enzymatic hydrolysis. Protojujubosides A and jujubosides A, B, and C were found to show potent immunological adjuvant activity.

Key words protojujubosides; Zizyphi Spinosi Semen; *Zizyphus jujuba* var. *spinosa*; protojujubogenin type triterpene bisdesmoside; immunological adjuvant activity

In the course of our studies in search of bioactive saponins and glycosides from natural medicine[1,2] and medicinal foodstuffs,[3] we have isolated three new jujubogenin oligoglycosides [jujubosides A_1 (**5**) and C (**6**) and acetyljujuboside B (**8**)] together with three known jujubogenin oligoglycosides [jujubosides A (**4**), B (**7**), and B_1 (**9**)], three flavonoid glycosides (spinosin, 6″-feruloylspinosin, and vicetin-2), and an alkaloid (magnoflorin) from a Chinese natural medicine, Zizyphi Spinosi Semen, the seeds of *Zizyphus jujuba* MILL. var. *spinosa* HU (Rhamnaceae).[4] As a continuation of this study, we isolated three novel protojujubogenin type triterpene bisdesmosides called protojujubosides A (**1**), B (**2**), and B_1 (**3**) from the seeds of *Zizyphus jujuba* MILL. var. *spinosa* HU. In this paper, we describe the structure elucidation of these protojujubosides (**1**, **2**, **3**). In addition, since this Chinese natural medicine has been known to show a tonic activity in Chinese traditional medicine, we examined the immunological adjuvant activity of protojujubosides and jujubosides.

Structures of 1, 2, and 3 Protojujuboside A (**1**) was isolated as colorless fine crystals of mp 200—202 °C from $CHCl_3$–MeOH. The IR spectrum of **1** showed absorption bands ascribable to carbonyl and olefin functions at 1719 and 1638 cm^{-1} and broad bands at 3419 and 1075 cm^{-1} suggestive of an oligoglycosidic structure. The molecular formula $C_{64}H_{106}O_{32}$ was elucidated by the negative-ion and positive-ion FAB-MS and by high-resolution MS measurement. Namely, a quasimolecular ion peak was observed at *m/z* 1385 $(M-H)^-$ in the negative-ion FAB-MS of **1**, while the positive-ion FAB-MS of **1** showed a quasimolecular ion peak at *m/z* 1409 $(M+Na)^+$. Methanolysis of **1** with 9% hydrogen chloride in dry methanol liberated ebelin lactone (**10**)[5] and 17(*Z*)-ebelin lactone (**11**)[4] in *ca.* 1 : 1 ratio, both of which were obtained by methanolysis of jujubogenin glycosides (**4**—**9**),[4,5] together with the methyl glycosides of arabinose, glucose, rhamnose, and xylose in *ca.* 1 : 3 : 1 : 1 ratio.[6] However, the ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[7] of **1** indicated the presence not of a jujubogenin moiety but a new keto-dammarane type triterpene moiety [δ 0.80, 1.13, 1.63, 1.75, 1.77 (all s, 19, 18, 26, 21, 27-H_3), 1.12 (s, 28, 29-H_3), 2.46, 2.77 (ABq, J=15.9 Hz, 15-H_2), 3.12 (dd-like, 3-H), 5.30 (m, 23-H), 5.62 (d, J=6.9 Hz, 24-H); δ_C 219.3 (16-C)] along with an α-L-arabinopyranosyl moiety [δ 4.92 (d-like, 1′-H)], three β-D-glucopyranosyl moieties [δ 4.90 (d, J=6.7 Hz, 1‴″-H), 4.98 (d, J=7.4 Hz, 1‴-H), 5.03 (d, J=7.6 Hz, 1‴″-H)], a α-L-rhamnopyranosyl moiety [δ 1.67 (d-like, 6″-H_3), 5.95 (br s, 1″-H)], and a β-D-xylopyranosyl moiety [δ 5.38 (d, J=7.8 Hz, 1‴″-H)]. The dammarane-type triterpene structure of **1** having the 16-keto and 23-glucosyl groups was characterized by a heteronuclear multiple bond correlation (HMBC) experiment, which showed long-range correlations between the 15,17-protons and the 16-carbon and between the 1‴″-anomeric proton and the 23-carbon, and by rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiments as shown in Fig. 1. The oligoglycoside structure of the 3-position in **1** was characterized by a HMBC experiment. That is, long-range correlations were observed between the following protons and carbons: 1′-H and 3-C; 1″-H and 2′-C; 1‴-H and 3′-C; 1‴″-H and 2‴-C; 1‴″-H and 6‴-C. The enzymatic hydrolysis of **1** with β-glucosidase furnished a jujubogenin tetraglycoside, jujuboside B (**7**).[4,8] This finding indicates that the keto-type structure of protojujubogenin part (A) obtained by enzyme hydrolysis of **1** is unstable and is immediately transformed to the stable ketal-type (the conformation of jujubogenin part B). Consequently, the structure of protojujuboside A has been elucidated as 23-*O*-β-D-glucopyranosyl-3β,20*S*,23*S*,30-tetrahydroxydammar-24-en-16-on-3-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-{*O*-β-D-glucopyranosyl-(1→6)-*O*-[β-D-xylopyranosyl-(1→2)]-β-D-glucopyranosyl-(1→3)}-α-L-arabinopyranoside (**1**).

Protojujuboside B (**2**) was also isolated as colorless fine crystals of mp 200—204 °C from $CHCl_3$–MeOH and its IR spectrum showed absorption bands at 3419, 1719, 1638, and 1075 cm^{-1} due to hydroxyl, carbonyl, and olefin functions. In the negative-ion FAB-MS of **2**, a quasimolecular ion peak was observed at *m/z* 1223 $(M-H)^-$, while the positive-ion FAB-MS of **2** showed a quasimolecular ion peak at *m/z* 1247 $(M+Na)^+$ and high-resolution MS analysis revealed the mol-

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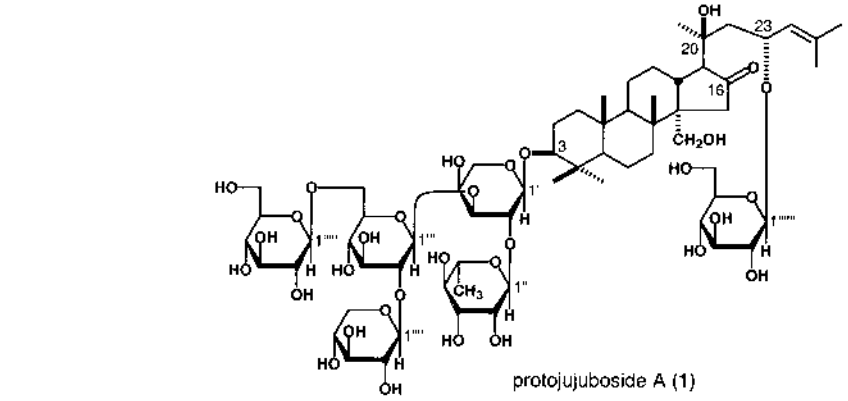
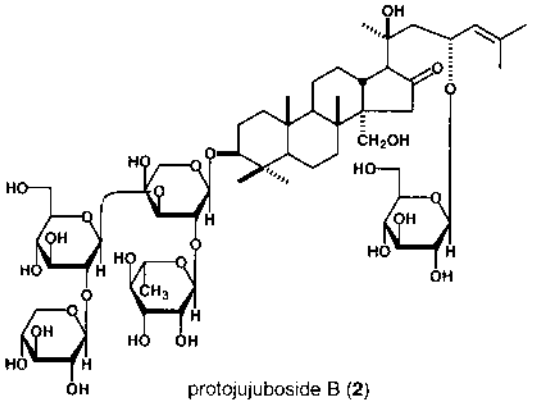




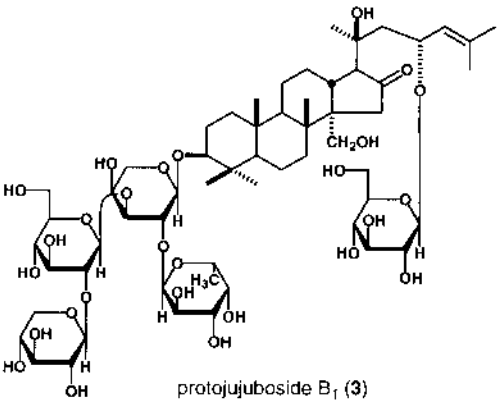
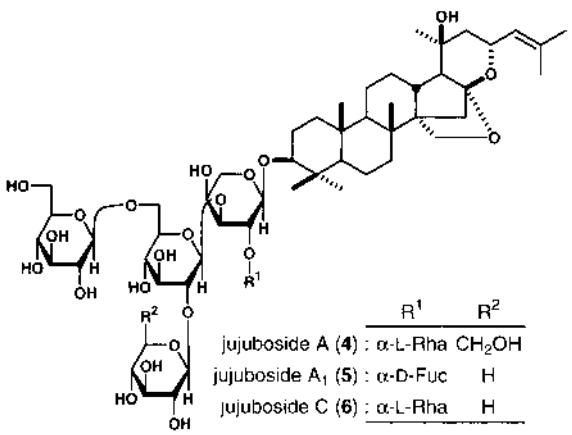
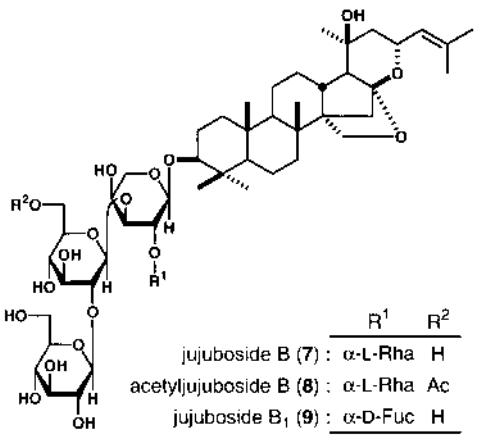


Chart 1

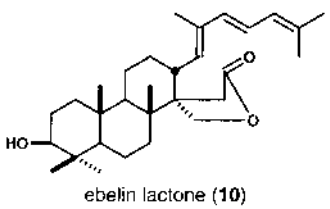


	R^1	R^2
jujuboside A (**4**) :	α-L-Rha	CH_2OH
jujuboside A_1 (**5**) :	α-D-Fuc	H
jujuboside C (**6**) :	α-L-Rha	H



	R^1	R^2
jujuboside B (**7**) :	α-L-Rha	H
acetyljujuboside B (**8**) :	α-L-Rha	Ac
jujuboside B_1 (**9**) :	α-D-Fuc	H

α-D-Fuc : α-D-fucopyranosyl; α-L-Rha : α-L-rhamnopyranosyl



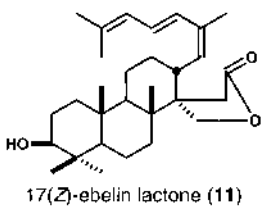


Chart 2

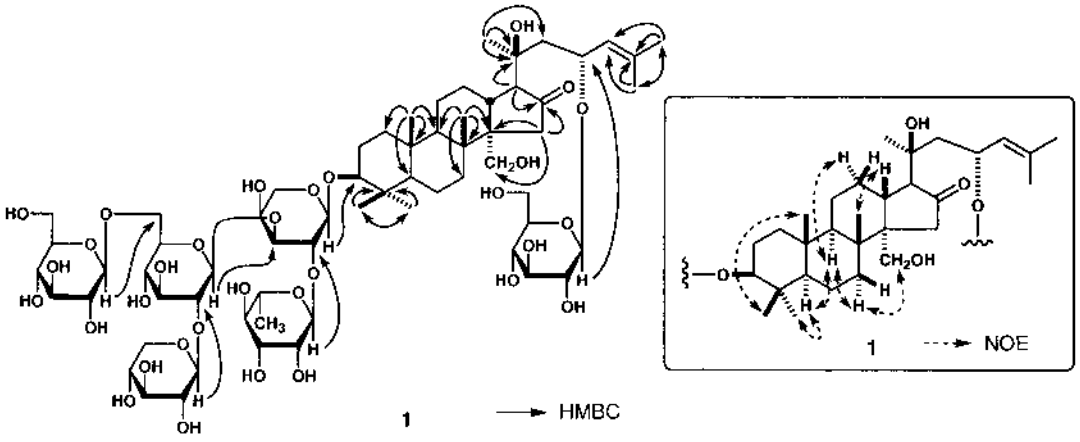


Fig. 1. HMBC and NOE Correlations of **1**

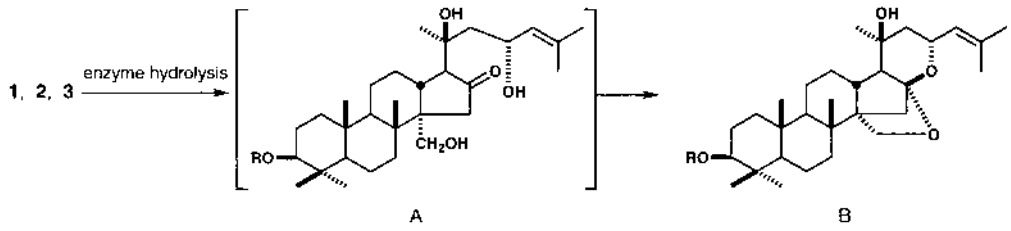


Fig. 2

ecular formula of **2** to be $C_{58}H_{96}O_{27}$. The methanolysis of **2** liberated **10** and **11** (*ca.* 1 : 1 ratio) together with the methyl glycosides of arabinose, glucose, rhamnose, and xylose in *ca.* 1 : 2 : 1 : 1 ratio. The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra of **2**, which were assigned by various NMR analytical methods,[7] indicated the presence of the 16-keto-protojujubogenin type triterpene moiety [δ 0.82, 1.10, 1.63, 1.72, 1.77 (all s, 19, 29, 26, 21, 27-H_3), 1.12 (s, 18, 28-H_3), 2.43, 2.75 (ABq, J=14.6 Hz, 15-H_2), 3.17 (dd-like, 3-H), 5.28 (m, 23-H), 5.65 (d, J=9.2 Hz, 24-H)], an α-L-arabinopyranosyl moiety [δ 4.92 (d-like, 1′-H)], two β-D-glucopyranosyl moieties [δ 4.99 (d, J=8.0 Hz, 1‴″-H), 5.11 (d, J=7.5 Hz, 1‴-H)], a α-L-rhamnopyranosyl moiety [δ 1.65 (d-like, 6″-H_3), 5.95 (br s, 1″-H)], and a β-D-xylopyranosyl moiety [δ 5.35 (d, J=7.5 Hz, 1‴″-H)]. The carbon signals due to the 3-*O*-glycosidic structure in the ^{13}C-NMR data of **2** were superimposable on those of jujuboside B (**7**),[4,8] whereas the signals of the 23-*O*-glucosyl protojujubogenin moieties were very similar to those of **1**. The HMBC experiment on **2** showed long-range correlations between the following protons and carbons: 1′-H and 3-C; 1″-H and 2′-C; 1‴-H and 3′-C; 1‴″-H and 2‴-C; 1‴″-H and 23-C. Furthermore, **7** was obtained on the enzymatic hydrolysis of **2** with β-glucosidase. On the basis of the above evidence, the structure of protojujuboside B was formulated as 23-*O*-β-D-glucopyranosyl-3β,20*S*,23*S*,30-tetrahydroxydammar-24-en-16-on-3-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranoside (**2**).

Protojujuboside B_1 (**3**), obtained as colorless fine crystals of mp 201—203 °C, showed absorption bands at 3418, 1719, 1638, and 1075 cm^{-1} ascribable to hydroxyl, carbonyl, and olefin groups in its IR spectrum. The molecular formula $C_{58}H_{96}O_{27}$ of **3** was determined from the quasimolecular ion peaks [*m/z* 1223 $(M-H)^-$ and 1247 $(M+Na)^+$] in the negative- and positive-ion FAB-MS of **3** and by high-resolution MS measurement. The methanolysis of **3** liberated **10** and **11** (*ca.* 1 : 1 ratio) together with the methyl glycosides of arabinose, fucose, glucose, and xylose in *ca.* 1 : 1 : 2 : 1 ratio. The enzymatic hydrolysis of **3** furnished jujuboside B_1 (**4**).[4,8] The ^{1}H-NMR (pyridine-d_5) and ^{13}C-NMR (Table 1) spectra[7] of **3** showed signals assignable to the protojujubogenin type triterpene part, an α-L-arabinopyranosyl moiety [δ 4.80 (d-like, 1′-H)], a α-D-fucopyranosyl moiety [δ 1.56 (d, J=6.0 Hz, 6″-H_3), 6.15 (br s, 1″-H)], two β-D-glucopyranosyl moieties [δ 5.01 (d, J=7.3 Hz, 1‴″-H), 5.14 (d, J=7.3 Hz, 1‴-H)], and a β-D-xylopyranosyl moiety [δ 5.44 (d, J=6.6 Hz, 1‴″-H)]. The proton and carbon signals in the ^{1}H-NMR and ^{13}C-NMR spectra of **3** significantly resembled those of **2**, except for the signals due to the 2′-*O*-fucopyranosyl moiety. Finally, the oligoglycosidic structure of **3** was characterized from the HMBC experiment, which showed long-range correlations between the following protons and carbons: 1′-H and 3-C; 1″-H and 2′-C; 1‴-H and 3′-C; 1‴″-H and 2‴-C; 1‴″-H and 23-C. Consequently, the structure of protojujuboside B_1 has been determined as 23-*O*-β-D-glucopyranosyl-3β,20*S*,23*S*,30-tetrahydroxydammar-24-en-16-on-3-yl *O*-α-L-fucopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→2)-β-D-glucopyranosyl-(1→3)]-α-L-arabinopyranoside (**3**).

Many jujubogenin oligoglycosides have been isolated from various Rhamnaceae[4,5,8] and Scrophulariaceae plants.[9] Since protojujubogenin type oligoglycosides such as protojujubosides (**1**—**3**) were easily changed to jujubogenin oligoglycosides by partial hydrolysis with β-glucosidase, protojujubosides (**1**—**3**) might be contained in high yields in the fresh seeds and other Rhamnaceae plants.

Table 1. ^{13}C-NMR Data for **1**—**3** (Pyridine-d_5)

	1	2	3		1	2	3
C-1	39.2	39.2	39.2	Ara-1′	104.0	104.1	104.6
C-2	26.6	26.6	26.7	2′	75.1	74.9	74.5
C-3	88.4	88.4	88.0	3′	82.8	81.9	82.6
C-4	39.7	39.7	39.6	4′	67.9	67.8	67.8
C-5	56.4	56.4	56.4	5′	63.4	63.6	64.7
C-6	18.6	18.6	18.6	Rha or Fuc-1″	101.6	101.6	101.8
C-7	36.4	36.4	36.4	2″	72.4	72.3	67.8
C-8	40.7	40.7	40.7	3″	72.5	72.5	72.1
C-9	51.8	51.8	51.8	4″	73.9	74.0	74.2
C-10	37.3	37.4	37.3	5″	70.1	70.1	67.0
C-11	21.9	21.9	21.9	6″	18.6	18.5	17.3
C-12	27.6	27.6	27.6	Glc-1‴	104.0	103.7	103.4
C-13	41.8	41.9	41.9	2‴	83.1	83.4	82.5
C-14	49.4	49.4	49.4	3‴	78.0	78.2	78.5
C-15	45.2	45.2	45.2	4‴	71.4	71.2	71.6
C-16	219.3	219.4	219.3	5‴	76.6	78.5	78.4
C-17	59.8	59.8	59.7	6‴	70.3	62.5	62.4
C-18	16.9	16.9	17.1	Xyl-1⁗	106.4	106.3	105.9
C-19	16.7	16.7	16.7	2⁗	76.3	76.1	75.8
C-20	74.5	74.5	74.5	3⁗	78.0	78.1	78.1
C-21	27.3	27.3	27.3	4⁗	70.8	70.8	70.8
C-22	45.2	45.4	45.3	5⁗	67.9	67.8	67.7
C-23	76.6	76.5	76.5	Glc-1⁗′	105.2	104.5	104.6
C-24	128.8	128.8	128.8	2⁗′	75.4	75.5	75.5
C-25	132.6	132.6	132.6	3⁗′	78.2	78.9	78.9
C-26	25.8	25.8	25.8	4⁗′	71.4	71.7	71.2
C-27	18.3	18.3	18.3	5⁗′	78.4	78.3	78.5
C-28	28.0	28.1	28.0	6⁗′	62.5	62.4	62.5
C-29	17.1	17.1	16.7	Glc-1⁗″	104.6		
C-30	62.7	62.8	62.8	2⁗″	75.5		
				3⁗″	78.9		
				4⁗″	71.6		
				5⁗″	78.4		
				6⁗″	62.5		

Immunological Adjuvant Activity of Protojujubosides and Jujubosides Recently, a number of studies were reported on the adjuvant effect of saponins, especially saponin fraction Quil A and purified QS-21 from *Quillaja saponaria* Molina.[10] However, there are few studies about other saponins except for those of *Q. saponaria* and some of these adjuvant-active saponins had potent haemolytic effects on red blood cells in an *in vitro* assay.[10]

The seeds of *Zizyphus jujuba* Mill. var. *spinosa* Hu have been used as a tonic and for treatment of insomnia in Chinese traditional medicine. Several triterpene oligoglycosides from medicinal herbs or foodstuffs with tonic and nutritive effects were found to show immunological adjuvant activity.[11] In this study, we examined the immunological adjuvant effect of the dammarane-type triterpene oligoglycosides, jujubosides and protojujubosides, to characterize the traditional pharmacological effect of this Chinese medicinal herb.

As shown in Table 2, **1** and **2** and **4**, **7**, **9**, and **6** potently increased the value of passive haemagglutination (PHA) titer. Particularly, the values of **1**, **4**, and **9** were almost equivalent to that of QS-21 and **6** was found to show an exceptionally potent effect. Furthermore, **6** significantly increased serum IgG levels, and **1**, **2**, **4**, and **7** also tended to increase these levels. These active constituents except for **9** have an α-L-rhamnopyranosyl moiety at the 2′-position, whereas **3** and **5** having a β-D-fucopyranosyl moiety at the 2′-position lacked the activity. These results indicated that the structure of the 3-glycoside moiety is important to show the activity.

Table 2. Effects of Saponin Constituents from Chinese Zizyphi Spinosi Semen on Serum Anti-OVA Antibody Levels in OVA-immunized Mice

Compound	PHA titer	ELISA value (Abs. 492 nm)
Protojujuboside A (**1**)	194	1.240±0.455
Protojujuboside B (**2**)	97	1.289±0.379
Protojujuboside B_1 (**3**)	64	0.572±0.153
Jujuboside A (**4**)	169	1.140±0.348
Jujuboside A_1 (**5**)	39	0.382±0.087
Jujuboside B (**7**)	97	1.329±0.430
Jujuboside B_1 (**9**)	194	1.351±0.193
Jujuboside C (**6**)	1092	2.113±0.234*
QS-21	239	1.805±0.588**
Control (non-adjuvant)	37	0.270±0.024

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 antibody levels were determined using PHA test and ELISA system. ELISA values represent the mean±S.E. Asterisks denote significant differences from the control at $*p<0.01$, $**p<0.05$.

Experimental

The following instruments were used to obtain physical data: Melting points, Yanagimoto micro-melting point apparatus MP-500D (values are uncorrected); specific rotations, Horiba SEPA-300 digital polarimeter (l=5 cm); GLC, Shimadzu GC-14A; IR spectra, Shimadzu FTIR-8100 spectrometer; FAB-MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; ^{1}H-NMR spectra, JEOL EX-270 (270 MHz) and JNM LA-500 (500 MHz) spectrometer; ^{13}C-NMR spectra, JEOL EX-270 (68 MHz) and JNM LA-500 (125 MHz) spectrometer with tetramethylsilane as an internal standard.

The following experimental conditions were used for chromatography: Ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., 150—350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh); TLC, pre-coated TLC plates with Silica gel 60F_{254} (Merck, 0.25 mm) (ordinary phase) and Silica gel RP-18 60F_{254} (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, pre-coated TLC plates with Silica gel RP-18 60WF_{254S} (Merck, 0.25 mm); detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 and heating.

Isolation of Protojujubosides A (1), B (2), and B_1 (3) from Chinese Zizyphi Spinosi Semen Fraction 3 (2.5 g), which was obtained from the seeds of *Zizyphus jujuba* Mill. var. *spinosa* Hu (8.0 kg, purchased from Tochimoto Tenkaido Co., Ltd., Lot. No. 406-C211, 1995) as reported previously,[4] was purified by reversed-phase silica gel column chromatography [Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd.), MeOH–H_2O] and HPLC [YMC-Pack R & D ODS-5 (YMC Co., Ltd.), 250×20 mm, i.d., MeOH–H_2O) to give protojujubosides A (**1**, 0.0004%), B (**2**, 0.0018%), and B_1 (**3**, 0.0005%).

Protojujuboside A (**1**): Colorless fine crystals from $CHCl_3$–MeOH, mp 200–202 °C, $[\alpha]_D^{29}$ −37.6° (c=0.25, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{64}H_{106}O_{32}Na$ $(M+Na)^+$: 1409.6565. Found: 1409.6581. IR (KBr): 3419, 1719, 1638, 1075 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.80, 1.13, 1.63, 1.75, 1.77 (3H each, all s, 19, 18, 26, 21, 27-H_3), 1.12 (6H, s, 28, 29-H_3), 1.67 (3H, d-like, 6″-H_3), 2.46, 2.77 (2H, ABq, J=15.9 Hz, 15-H_2), 2.71 (1H, m, 13-H), 2.95 (1H, m, 17-H), 3.12 (1H, dd-like, 3-H), 4.90 (1H, d, J=6.7 Hz, 1⁗′-H), 4.92 (1H, d-like, 1′-H), 4.98 (1H, d, J=7.4 Hz, 1‴-H), 5.03 (1H, d, J=7.6 Hz, 1⁗″-H), 5.30 (1H, m, 23-H), 5.38 (1H, d, J=7.8 Hz, 1⁗-H), 5.62 (1H, d, J=6.9 Hz, 24-H), 5.95 (1H, br s, 1″-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1. Negative-ion FAB-MS: m/z 1385 $(M-H)^-$. Positive-ion FAB-MS: m/z 1409 $(M+Na)^+$.

Protojujuboside B (**2**): Colorless fine crystals from $CHCl_3$–MeOH, mp 200—204 °C, $[\alpha]_D^{29}$ −25.8° (c=0.25, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{58}H_{96}O_{27}Na$ $(M+Na)^+$: 1247.6036. Found: 1247.6053. IR (KBr): 3419, 1719, 1638, 1075 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.82, 1.10, 1.63, 1.72, 1.77 (3H each, all s, 19, 29, 26, 21, 27-H_3), 1.12 (6H, s, 18, 28-H_3), 1.65 (3H, d-like, 6″-H_3), 2.43, 2.75 (2H, ABq, J=14.6 Hz, 15-H_2), 2.85 (1H, m, 13-H), 2.95 (1H, m, 17-H), 3.17 (1H, dd-like, 3-H), 4.92 (1H, d-like, 1′-H), 4.99 (1H, d, J=8.0 Hz, 1⁗′-H), 5.11 (1H, d, J=7.5 Hz, 1‴-H), 5.28 (1H, m, 23-H), 5.35 (1H, d, J=7.5 Hz, 1⁗-H), 5.65

(1H, d, J=9.2 Hz, 24-H), 5.95 (1H, br s, 1″-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1. Negative-ion FAB-MS: m/z 1223 $(M-H)^-$. Positive-ion FAB-MS: m/z 1247 $(M+Na)^+$.

Protojujuboside B_1 (**3**): Colorless fine crystals from $CHCl_3$–MeOH, mp 201—203 °C, $[\alpha]_D^{29}$ −24.4° (c=0.25, MeOH). High-resolution positive-ion FAB-MS: Calcd for $C_{58}H_{96}O_{27}Na$ $(M+Na)^+$: 1247.6037. Found: 1247.6049. IR (KBr): 3418, 1719, 1638, 1075 cm^{-1}. ^{1}H-NMR (500 MHz, pyridine-d_5) δ: 0.82, 0.98, 1.11, 1.13, 1.63, 1.74, 1.77 (3H each, all s, 19, 29, 28, 18, 26, 21, 27-H_3), 1.56 (3H, d, J=6.0 Hz, 6″-H_3), 2.54, 2.76 (2H, ABq, J=15.4 Hz, 15-H_2), 2.85 (1H, m, 13-H), 2.94 (1H, m, 17-H), 3.25 (1H, dd-like, 3-H), 4.80 (1H, d-like, 1′-H), 5.01 (1H, d, J=7.3 Hz, 1‴″-H), 5.14 (1H, d, J=7.3 Hz, 1‴-H), 5.27 (1H, m, 23-H), 5.44 (1H, d, J=6.6 Hz, 1‴-H), 5.62 (1H, d-like, 24-H), 6.15 (1H, br s, 1″-H). ^{13}C-NMR (125 MHz, pyridine-d_5) δ_C: given in Table 1. Negative-ion FAB-MS: m/z 1223 $(M-H)^-$. Positive-ion FAB-MS: m/z 1247 $(M+Na)^+$.

Methanolysis of 1—3 1) A solution of protojujubosides (10 mg each of **1**, **2**, and **3**) in 9% HCl–dry MeOH (1.0 ml) was heated under reflux for 1 h. After cooling, the reaction solution was poured into ice-water and the whole mixture was extracted with $CHCl_3$. The $CHCl_3$ extract was washed with aqueous saturated $NaHCO_3$ and brine and dried over $MgSO_4$. After removal of the solvent from the $CHCl_3$ extract under reduced pressure, the residue was subjected to ordinary-phase silica gel column chromatography [1.0 g, n-hexane–AcOEt (5 : 1)] and HPLC [MeOH–H_2O (85 : 15)] to give ebelin lactone (**10**, 1.0 mg from **1**; 1.2 mg from **2**; 0.9 mg from **3**) and 17(*Z*)-ebelin lactone (**11**, 1.0 mg from **1**; 0.9 mg from **2** and **3**), which were identified by comparison of their physical data ($[\alpha]_D$, IR, ^{1}H- and ^{13}C-NMR, MS) with authentic samples.

2) A solution of **1**, **2**, and **3** (1 mg each) in 9% HCl–dry MeOH (0.5 ml) was heated under reflux for 2 h. After cooling, the reaction mixture was neutralized with Ag_2CO_3 and the insoluble portion was removed by filtration. After removal of the solvent from the filtrate under reduced pressure, the residue was dissolved in pyridine (0.01 ml) and the solution was treated with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (0.02 ml) for 1 h. The reaction solution was subjected to GLC analysis to identify the trimethylsilyl (TMS) derivatives of methyl glycosides [methyl arabinoside (i), methyl glucoside (ii), methyl rhamnoside (iii), methyl xyloside (iv), and methyl fucoside (v): i, ii, iii, iv from **1** and **2**; i, ii, iii, v from **3**]. GLC conditions : column CBR1-M25-025 [Shimadzu Co., 0.25 mm (i.d.)×25 m], injector temperature : 140 °C, detector temperature: 280 °C, column temperature: 140—240 °C, 5 °C/min, initial time: 5 min, He flow rate: 15 ml/min, t_R (min): i: 13.2, 13.3, 13.6, 14.4; ii: 21.3, 21.7; iii: 14.1, 14.5; iv: 16.0, 16.5; v: 14.8, 15.4.

Partial Hydrolysis of 1—3 with β-Glucosidase A solution of protojujubosides (**1**, **2**, **3**, 10 mg each) in 0.1 M acetate buffer (pH 4.4, 5 ml) was treated with β-glucosidase from almond (50 mg, Oriental Yeast Co., Ltd.) and the reaction mixture was stirred at 40 °C for 98 h. After treatment of the reaction solution with EtOH, the entire mixture was concentrated under reduced pressure to give a residue. The residue was purified by reversed-phase silica gel column chromatography [3 g, MeOH–H_2O (30 : 70)→MeOH] and HPLC [MeOH–H_2O (65 : 35)] to give jujubosides B (**7**, 1.6 mg from **1**, 1.1 mg from **2**) and B_1 (**9**, 1.5 mg from **3**). The jujubosides B and B_1 were identical with authentic samples by TLC, HPLC, $[\alpha]_D$, and ^{1}H-NMR spectra comparisons.

Bioassay Methods Female ddY mice (6 weeks old) were immunized with chicken ovalbumin (OVA, Grade VI, Sigma) [10 μg/0.1 ml phosphase 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 a PHA test[12] and enzyme-linked immunosorbent assay (ELISA).

PHA Test: The PHA test was performed using round bottomed 96 well plates. Serial 2-fold dilutions of sera in gelatin veronal buffer (GVB) were prepared at the volume of 100 μl/well on the plates. Twenty-five μl of 1% sheep red blood cells (SRBC) conjugated with OVA in GVB were added to the wells, and the plates were incubated overnight at room temperature. PHA titer was defined as the highest reciprocal dilution showing complete agglutination of SRBC. Values are expressed as geometrical mean in each group (n=10).

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). The anti-mouse IgG was diluted in Tween–PBS to 1 : 4000, and 100 μl/well was added to the wells. 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 2 N H_2SO_4/well and read using a microplate reader at 492 nm. Each value represents the mean±S.E. of 5 mice. Statistical significance of differences was estimated by analysis of variance (ANOVA) followed by Dunnett's test.

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