Matrix Metalloproteinase-1 Inhibitor from the Stem Bark of *Styrax japonica* S. *et* Z.

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A bioassay-guided fractionation of the ethyl acetate soluble fraction from the stem bark of *Styrax japonica* S. *et* Z. (Styracaceae) yielded two new lignan compounds, styraxjaponoside A (1) and styraxjaponoside B (2), along with three known compounds, matairesinoside (3), egonol glucoside (4), and dihydrodehydrodiconiferyl alcohol 9′-*O*-glucoside (5). The structures of compounds 1—5 were determined by spectroscopic method, as well as 1D- and 2D-NMR (HSQC, ^{1}H–^{1}H COSY, and HMBC) spectroscopy. Among them, compound 2 exhibited potent inhibitory activity against matrix metalloproteinase (MMP)-1, and prevented the UV-induced changes in the MMP-1 expression. In addition, compounds 3 and 5 were isolated from this plant for the first time.

Key words *Styrax japonica*; Styracaceae; lignan glycoside; styraxjaponoside B; matrix metalloproteinase (MMP)-1 inhibitor

Styrax japonica S. *et* Z. is a member of the Styracaceae family, which is a shrub found in Central America and Mexico, including South Anatolia.[1)] The resin from this species has been used in traditional medicine to treat inflammatory diseases.[2)] An earlier investigation on the phytochemical constituents of the *Styrax* species revealed them to be a rich source of benzofurans,[3)] benzofuran glycosides,[4)] and saponins.[5)] Recently, we reported the isolation of pentacyclic triterpenoids from this plant, in addition to their cytotoxic activity.[6)] The matrix metalloproteinases (MMPs) are essential for tissue remodeling, as well as the healing cascade under normal physiological conditions. It has been suggested that alterations in collagen, which is the major structural component of the skin, are the cause of changes observed in naturally aged and photoaged skin such as skin wrinkling and a loss of elasticity.[7)] In an ongoing investigation into MMP-1 inhibitory compounds from *S. japonica*, two new lignan glycosides (**1**, **2**) and three known compounds (**3**—**5**) were isolated from the ethyl acetate soluble fraction. This paper reports the isolation and structure determination of the constituents from the stem bark of *S. japonica* as well as the effect on the type I procollagen and MMP-1 expression of compound **2**.

Results and Discussion

Activity-guided fractionation and repeated column chromatography of the ethyl acetate soluble fraction from the stem bark of *S. japonica* S. *et* Z. yielded two new lignan glycosides and three known compounds (Fig. 1).

Styraxjaponoside A (**1**) was obtained as colorless crystals. The molecular formula of **1** was found to be $C_{26}H_{30}O_{13}$ by HR-FAB-MS spectrometry (*m/z* 551 $[M+H]^+$). The UV spectrum exhibited absorption maxima at 234 and 281 nm, which are characteristic absorption bands of a butyrolactone-type lignan. In the IR spectrum, the signals for a hydroxyl (3410 cm^{-1}), γ-lactone carbonyl (1751 cm^{-1}), aromatic ring (1600, 1502 cm^{-1}), and methylenedioxyl (925 cm^{-1}) groups were observed. The ^{1}H- and ^{13}C-NMR spectra showed a typical pattern of methylenedioxygenated dibenzylbutyrolactone-type lignan, and the structure of **1** was similar to that of bursehernin,[8)] except for the 7,7′-dihydroxy group and glucosyl moiety. Moreover, the anomeric proton at δ 4.86 (1H, d, J=7.5 Hz) suggested the presence of a glucosyl moiety. Based on the coupling constant value of the anomeric proton, the configuration of the glucosidic linkage was determined to be β.[9)] On acid hydrolysis,[10)] **1** gave D-(+)-glucose, which was identified by co-TLC with standard D-(+)-glucose. Compared with the chemical shifts of H_2-7 and 7′ (δ 2.51, 4H, br s), and H-8 and 8′ (δ 3.00—2.80, 2H, m) of bursehernin,[8)] downfield shifts of H-7 (δ 5.31, 1H, d, J=4.0 Hz), H-7′ (δ 5.36, 1H, d, J=4.0 Hz), H-8 (δ 3.53, 1H, d, J=4.0 Hz), and H-8′ (δ 3.27, 1H, m) were observed in **1**, and these chemical shifts strongly suggested that the hydroxy group was located at C-7 and 7′. In the HMBC spectrum of

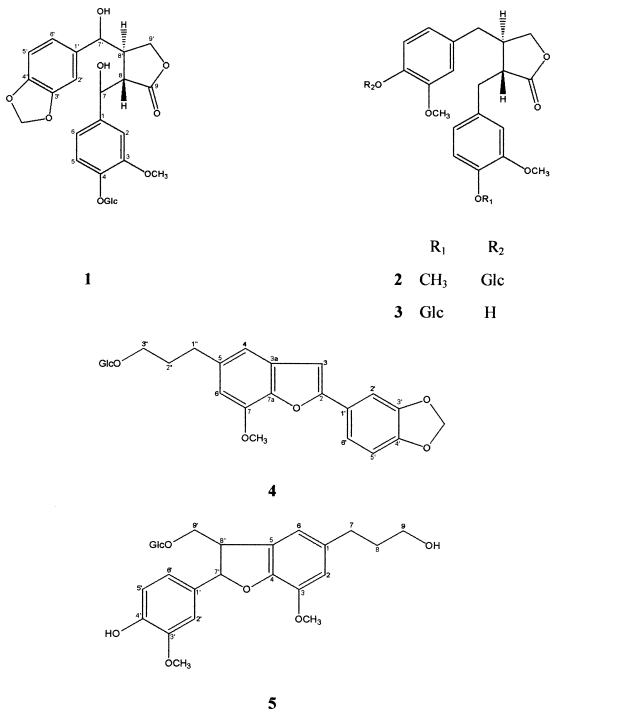

Fig. 1. Structures of Compounds **1**—**5** Isolated from *S. japonica*

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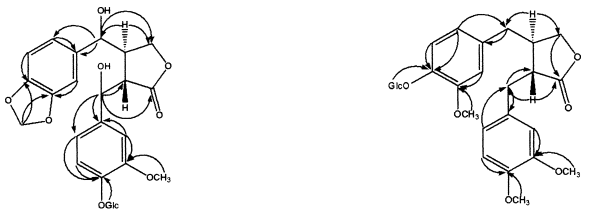

1 2

Fig. 2. HMBC Correlation for Compounds **1** and **2**

1, the carbon signals at δ 149.04 (C-3′) and 148.74 (C-4′) showed a correlation with the proton signal at δ 5.98 (OCH_2O), and the carbon signals at δ 135.93 (C-1) and 133.54 (C-1′) correlated with the proton signals at δ 5.31 (H-7) and 5.36 (H-7′), respectively. Furthermore, the carbon signals at δ 53.86 (C-8) and 50.32 (C-8′) correlated with the proton signals at δ 4.34 and 4.05 (methylene protons, H_2-9′). These results indicate that the skeleton of **1** was a methylenedioxygenated dibenzylbutyrolactone-type lignan. In addition, the ^1H–^{13}C long-range correlation between the anomeric proton (δ 4.86, 1H, d, J=7.5 Hz) and the C-4 carbon (δ 146.63) suggest that the sugar was located at C-4. Based on these observations, styraxjaponoside A (**1**) was determined to be 7,7′-dihydroxybursehernin 4-β-D-glucoside.

Styraxjaponoside B (**2**) was obtained as colorless crystals. The molecular formula of **2** was found to be $C_{27}H_{34}O_{11}$ by HR-FAB-MS spectrometry (*m/z* 557 $[M+Na]^+$). The UV spectrum exhibited absorption maxima at 232 and 279 nm, which are characteristic absorption bands of a butyrolactone-type lignan. In the IR spectrum, signals for γ-lactone carbonyl (1760 cm^{-1}) and aromatic ring (1590, 1510 cm^{-1}) were observed. The ^1H- and ^{13}C-NMR spectra of **2** were similar to matairesinol,[11] butyrolactone-type lignan, except for the glucosyl moiety. Li *et al.*[12] proposed the reference to the chemical shifts of the *cis*- and *trans*-dibenzylbutyrolactones. The ^1H-NMR spectrum of **2** exhibited the characteristic signals of a *trans*-8,8′-dibenzylbutyrolactone lignan, so its relative configuration was determined to be *trans*. The position of the glucosyl group was confirmed by the HMBC spectrum. A ^1H–^{13}C long-range correlation between the C-4′ carbon (δ 146.81) and the anomeric proton (δ 4.86, 1H, d, J=7.5 Hz) was observed. Based on the coupling constant value of the anomeric proton, the configuration of the glucosidic linkage was determined to be β.[9] On acid hydrolysis,[10] **2** gave D-(+)-glucose, which was identified by co-TLC with standard D-(+)-glucose. Therefore, the structure of styraxjaponoside B (**2**) was determined to be 4-methoxymatairesinol 4′-β-D-glucoside.

Compounds **3**—**5** were identified as matairesinoside,[13] egonol glucoside,[3] and dihydrodehydrodiconiferyl alcohol 9′-*O*-glucoside,[13] respectively, by comparing the NMR spectral data with those reported in the literature. Compounds **3** and **5** have not been previously isolated from *S. japonica*.

The *in vitro* cell cytotoxicity was evaluated according to reference,[14] and the compounds **1**—**5** showed no cytotoxicity against the human dermal fibroblasts in the test dose (0.1—10 μM, $p<0.001$ compared to the control). This study examined the effects of compounds **1**—**5** on the expression of type I procollagen, and the MMP-1 proteins in cultured human dermal fibroblasts. The human dermal fibroblasts were treated with 10 μM for 48, 72, and 96 h, and the type I procollagen and MMP-1 expression levels were then determined in the culture media by Western blot analysis. Epigallocatechin-3-gallate (EGCG) was used as a positive control throughout the experiments (Tables 1, 2). Antioxidants, such as EGCG, Vitamin C, and E inhibit the MMP-1 expression level in human dermal fibroblasts.[14] EGCG is a major constituent polyphenol in green tea and commonly used for preventing UV-induced adverse skin reaction such as sunburn and photoaging.[15,16] Compound **2** increased the type I procollagen protein expression level by 518.9±18.0% ($p<0.05$, n=5) at 10 μM (Table 1), and decreased the MMP-1 protein expression level significantly in a dose-dependent manner by an average of 62.1±8.3% ($p<0.05$, n=5) at 10 μM, compared with the vehicle-treated control cells (Table 1). In order to determine if **2** has any inhibitory effects on the UV-induced changes in the MMP-1 protein expression level, human dermal fibroblasts were irradiated with 100 mJ/cm^2 of UV light with or without treatment of **2** (0.1, 1, 10 μM). The UV-induced MMP-1 protein expression level was significantly inhibited by 63.5±17.6% ($p<0.05$, n=5) at 10 μM by a pretreatment with **2** in the cultured human dermal fibroblasts in a dose-dependent manner (Table 2). Throughout the experiment, **2** exhibited almost equivalent effects on type I procollagen and MMP-1 expression to that of EGCG, which is used as a positive control.

In conclusion, styraxjaponoside B (**2**) can be used for the treatment and prevention of the skin aging processes, based on the following results: 1) Topical application of **2** in the human dermal fibroblasts increases the type I procollagen expression level and decreases the MMP-1 expression level. 2) Styraxjaponoside B (**2**) prevents the UV-induced changes in the MMP-1 expression level.

Table 1. The Effects of **2** on the Type I Procollagen and MMP-1 Expressions

Conc. (μM)	Compd.	Type I procollagen		MMP-1	
		EGCG[a]	**2**	EGCG	**2**
0		100	100	100	100
0.1		156.4±6.44	126.1±12[b]	76.4±9.48	76.4±9.48[b]
1		296.9±2.36	205.3±16[b]	58.2±6.64	68.5±6.64[b]
10		515.6±13.47	518.9±18[b]	43.2±8.30	62.1±8.30[b]

a) EGCG (epigallocatechin-3-gallate) was used as a positive control. *b*) $p<0.05$ compared with the control. Each value represents a mean±S.E.M. (n=5).

Table 2. The Effect of **2** on the UV-Induced in the MMP-1 Expression in the Human Dermal Fibroblasts[a]

Conc. (μM)	Compound	
	EGCG[c]	**2**
0[b]	100	100
0	100	100
0.1	61.2±11.6	83.5±10.5[d]
1	62.2±9.07	58.1±14.2[d]
10	22.9±5.31	63.5±17.6[d]

a) The cells were pretreated with compound prior to UV irradiation (100 mJ/cm^2) and harvested 72 h later. *b*) None UV. *c*) Used as a positive control. *d*) $p<0.05$ compared with the control. Each value represents a mean±S.E.M. (n=5).

Experimental

General Procedures The melting points were determined using a Fisher Scientific 307N0043 melting point apparatus and are uncorrected. The optical rotations were measured using an Autopol-IV polarimeter (Rudolph Research Flangers). UV spectra were obtained on a Shimadzu UV/Visible spectrophotometer (UV-1601PC). The IR spectra were measured in KBr pellets using an IMS 85 (Bruker). The NMR spectra were recorded on a Varian Unity Inova 500 spectrometer. The HR-FAB-MS was recorded on a JEOL JMS 700 mass spectrometer. TLC and preparative TLC were carried out on precoated silica gel 60 F_{254} (Merck, art. 5715) and RP-18 F_{254}s (Merck, art. 15389) plates. TLC of **1**—**5** was performed on precoated silica gel 60 F_{254} and RP-18 F_{254}s plates, which were developed with $CHCl_3$–MeOH–Me_2CO–H_2O (17 : 3 : 3 : 0.3, solvent A) and MeOH–H_2O (45 : 55, solvent B). A 10% H_2SO_4 reagent (in EtOH) was sprayed for detection and then heated. Column chromatography was performed on silica gel 60 (Merck, 40—63 and 63—200 μm), TSK gel TOYOPEARL HW-40© (TOSOH, cat. no. 07449), and Sephadex LH-20 (Sigma, 25—100 μm). Silver carbonate (Ag_2CO_3, Aldrich Co.) and D-(+)-glucose ($C_6H_{12}O_6$, Sigma) were used as a neutralization substance and standard sugar on acidic hydrolysis, respectively. Epigallocatechin-3-gallate (EGCG, m.w. 458.4) was used as a positive control and obtained from Pharmafood (Kyoto, Japan).

Plant Material The stem bark of *S. japonica* was collected from Jogyesan, Suncheon, Chonnam, Korea, in September 2002. A voucher specimen is deposited in the Herbarium of the College of Pharmacy, Chosun University, Korea (CSU-964-17).

Extraction and Isolation The air-dried stem bark of *S. japonica* (654 g) was cut and extracted with MeOH (3 l×3) at 80 °C for 4 h (×3). The MeOH extract (120 g) was suspended in water (1.3 l) and then partitioned sequentially with equal volumes of dichloromethane, ethyl acetate, and *n*-butanol. Each fraction was evaporated *in vacuo* to yield the residues of CH_2Cl_2 (13 g), EtOAc (12 g), *n*-BuOH (75 g), and water (2.9 g) extract. Among these fractions, the ethyl acetate extract had a strong MMP-1 expression inhibition effect; the MMP-1 expression decreased to 68% at 10 μg/ml and to 41% at 100 μg/ml compared with the control. The ethyl acetate extract (4 g) was subjected to column chromatography over silica gel (250 g, 4.5×68 cm) by eluting with an EtOAc–*i*-PrOH–H_2O (20 : 1 : 0.5). The fractions were pooled into 7 fractions based on their TLC profiles. The subfraction, E4 (430.4 mg) containing **1**, **3**, and **4** was purified by column chromatography on silica gel (135 g, 2.3×70 cm, $CHCl_3$–MeOH–Me_2CO–H_2O, 50 : 4 : 2 : 0.3→30 : 4 : 2 : 0.3) and was followed by gel filtration column chromatography (Sephadex LH-20, 48 g, 2.0×69 cm, MeOH–H_2O, 50 : 50→65 : 35), preparative TLC (RP-18 F_{254}s, 0.5 mm, MeOH–H_2O, 50 : 50), and finally with TOYOPEARL HW-40© (55 g, 2.8×70 cm, MeOH–H_2O, 50 : 50) to give **1** (50 mg, *Rf* A=0.22, B=0.14), **3** (28 mg, *Rf* A=0.15, B=0.29), and **4** (5 mg, *Rf* A=0.22, B=0.01), respectively. The subfraction, E5 (829.4 mg) was chromatographed on a column (3.3×55 cm) packed with 170 g of silica gel and eluted with $CHCl_3$–MeOH–Me_2CO–H_2O (50 : 4 : 2 : 0.3). The fractions were pooled into 7 fractions according to their TLC profiles. Gel filtration column chromatography (Sephadex LH-20, 51 g, 2.0×69 cm, MeOH–H_2O, 50 : 50) of fraction E5.6 (144 mg) yielded colorless crystals of **5** (20 mg, *Rf* A=0.12, B=0.26). The subfraction, E6 (1.1 g) on a column (4.0×69 cm) packed with 175 g of the silica gel was eluted with a $CHCl_3$–MeOH–Me_2CO–H_2O (30 : 3 : 2 : 0.3). Gel filtration column chromatography (Sephadex LH-20, 53 g, 2.1×69 cm) of fraction E6.2 (386 mg) using MeOH–H_2O (50 : 50) yielded colorless crystals of **2** (150 mg, *Rf* A=0.22, B=0.24).

7,7′-Dihydroxybursehernin 4-β-D-Glucoside (**1**, Styraxjaponoside A): Colorless crystals. mp 156.8—156.9 °C (decom.). $[\alpha]_D^{25}$ +0.25° (*c*=0.5, MeOH). UV λ_{max} (MeOH) nm (log ε): 234 (1.93), 281 (1.15). IR (KBr) ν_{max} (cm^{-1}): 3410 (OH), 2923, 2880, 1751 (γ-lactone CO), 1600, 1502 (aromatic ring), 925 (methylenedioxy). HR-FAB-MS *m/z* 551.1765 (Calcd for $C_{26}H_{31}O_{13}$ $[M+H]^+$, 551.1764). ^{1}H-NMR (CD_3OD, 500 MHz): δ 7.13 (1H, d, *J*=8.5 Hz, H-5), 6.99 (1H, d, *J*=2.0 Hz, H-2), 6.94 (1H, br d, *J*=8.5 Hz, H-6), 6.81 (2H, d, *J*=2.0 Hz, H-5′, 6′), 6.80 (1H, s, H-2′), 5.98 (2H, s, OCH_2O), 5.36 (1H, d, *J*=4.0 Hz, H-7′), 5.31 (1H, d, *J*=4.0 Hz, H-7), 4.86 (1H, d, *J*=7.5 Hz, Glc-1), 4.34 (1H, dd, *J*=9.7, 4.8 Hz, H-9′a), 4.05 (1H, dd, *J*=9.5, 4.7 Hz, H-9′b), 3.90 (3H, s, OCH_3-3), 3.88 (1H, dd, *J*=12.2, 2.5 Hz, Glc-6a), 3.75 (1H, dd, *J*=12.0, 6.0 Hz, Glc-6b), 3.58 (1H, m, Glc-2), 3.53 (1H, br d, *J*=2.0 Hz, H-8), 3.52—3.50 (1H, m, Glc-3), 3.47 (1H, m, Glc-4), 3.42 (1H, m, Glc-5), 3.27 (1H, m, H-8′). ^{13}C-NMR (CD_3OD, 125 MHz): δ 178.30 (C-9), 150.13 (C-3), 149.04 (C-3′), 148.74 (C-4′), 146.63 (C-4), 135.93 (C-1), 133.54 (C-1′), 119.88 (C-6′), 118.62 (C-6), 117.62 (C-5), 110.24 (C-2), 109.03 (C-5′), 106.41 (C-2′), 102.35 (Glc-1), 102.11 (OCH_2O), 85.75 (C-7), 83.80 (C-7′), 77.15 (Glc-5), 76.78 (Glc-3), 73.94 (Glc-2), 73.40 (C-9′), 70.56 (Glc-4), 62.11 (Glc-6), 56.63 (OCH_3-3), 53.86 (C-8), 50.32 (C-8′).

4-Methoxymatairesinol 4′-β-D-Glucoside (**2**, Styraxjaponoside B): Colorless crystals. mp 84—86 °C (decom.). $[\alpha]_D^{25}$ −5.09° (*c*=0.3, MeOH). UV λ_{max} (MeOH) nm (log ε): 232 (2.35), 279 (1.10). IR (KBr) ν_{max} (cm^{-1}): 3425 (OH), 2925, 2860, 1760 (γ-lactone CO), 1590, 1510 (aromatic ring), 800. HR-FAB-MS *m/z* 557.1999 (Calcd for $C_{27}H_{34}O_{11}Na$ $[M+Na]^+$, 557.1996). ^{1}H-NMR (CD_3OD, 500 MHz): δ 7.05 (1H, d, *J*=8.0 Hz, H-5′), 6.82 (1H, d, *J*=8.5 Hz, H-5), 6.70 (1H, s, H-2), 6.65 (1H, d, *J*=8.5 Hz, H-6), 6.61 (1H, s, H-2′), 6.60 (1H, d, *J*=9.0 Hz, H-6′), 4.86 (1H, d, *J*=7.5 Hz, Glc-1), 4.15 (1H, t, *J*=8.0 Hz, H-9′a), 3.92 (1H, d, *J*=8.0 Hz, H-9′b), 3.87 (1H, br d, *J*=12.0 Hz, Glc-6a), 3.78 (3H, s, OCH_3-4), 3.77 (3H, s, OCH_3-3′), 3.75 (3H, s, OCH_3-3), 3.69 (1H, br dd, *J*=12.0, 5.0 Hz, Glc-6b), 3.49 (2H, m, Glc-2, 3), 3.40 (2H, m, Glc-4, 5), 2.90 (1H, dd, *J*=14.0, 6.8 Hz, H-7a), 2.80 (1H, dd, *J*=14.0, 7.0 Hz, H-7b), 2.66 (1H, q, *J*=6.8 Hz, H-8), 2.55 (1H, m, H-7′a), 2.53 (1H, m, H-7′b), 2.49 (1H, m, H-8′). ^{13}C-NMR (CD_3OD, 125 MHz): δ 181.46 (C-9), 150.90 (C-3′), 150.50 (C-4), 149.42 (C-3), 146.81 (C-4′), 134.95 (C-1′), 132.26 (C-1), 122.95 (C-6), 122.43 (C-6′), 118.08 (C-5′), 114.37 (C-2), 114.24 (C-2′), 113.06 (C-5), 102.99 (Glc-1), 78.26 (Glc-5), 77.93 (Glc-3), 75.01 (Glc-2), 72.95 (C-9′), 71.45 (Glc-4), 62.65 (Glc-6), 56.78 (OCH_3-4), 56.65 (OCH_3-3, 3′), 47.78 (C-8), 42.56 (C-8′), 39.00 (C-7′), 35.49 (C-7).

Matairesinoside (**3**): White powder. mp 93—96 °C. $[\alpha]_D^{25}$ −46° (*c*=0.68, EtOH). UV λ_{max} (EtOH) nm (log ε): 239 (4.21), 281 (3.81). IR (KBr) ν_{max} (cm^{-1}): 3600—3200 (OH), 1755 (γ-lactone CO), 1595, 1510 (aromatic ring). The spectral data were identical with those reported in the literature.[13]

Egonol Glucoside (**4**): Viscous yellowish oil. $[\alpha]_D^{25}$ −15.2° (*c*=0.6, MeOH). UV λ_{max} (MeOH) nm (log ε): 303 (1.05), 318 (1.04). IR (KBr) ν_{max} (cm^{-1}): 3580 (OH), 2940, 1600 (aromatic ring), 1480, 920 (methylenedioxy). The spectral data were identical with those reported in the literature.[3]

Dihydrodehydrodiconiferyl Alcohol 9′-*O*-Glucoside (**5**): Colorless crystals. $[\alpha]_D^{25}$ −15.5° (*c*=2.0, MeOH). UV λ_{max} (MeOH) nm (log ε): 233 (1.66), 282 (1.05). IR (KBr) ν_{max} (cm^{-1}): 3395 (OH), 2900, 1595, 1515 (aromatic ring). The spectral data were identical with those reported in the literature.[13]

Acidic Hydrolysis of 1 and 2 Compounds **1** and **2** (5 mg each) were dissolved in 1 N HCl (1 ml) and MeOH (1 ml) and refluxed at 75 °C for 90 min. The reaction solution was evaporated under reduced pressure, and the hydrolysate was extracted with EtOAc (3 ml×3). The aqueous fraction was neutralized with Ag_2CO_3, filtered, and the filtrate was concentrated under reduced pressure. The residue was compared with standard sugar using TLC ($CHCl_3$–MeOH–H_2O, 6 : 4 : 1),[10] which showed the sugar to be D-(+)-glucose (*Rf*=0.13) in **1** and **2**.

Human Fibroblast Cell Culture The primary cultures of the dermal fibroblasts were established from human adult foreskins in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a 37 °C humidified incubator containing 5% CO_2.

Cell Proliferation Assay and UV Irradiation Cell proliferation was determined by a MTT assay.[14] The UV light source was a F75/85W/UV21 fluorescent sun lamp, which had an emission spectrum between 285—350 nm (peak at 310—315 nm), as described previously.[17]

Western Blot Analysis The soluble protein fraction was extracted from the cultured human fibroblasts with a WCE buffer. The supernatant extracts were centrifuged at 12000×***g*** for 10 min, and the supernatant was used for Western blot analysis.

Statistical Analysis The data was analyzed using a Student's *t*-test. The results are presented as a means±S.E.M. All the *p* values quoted were two-tailed, and a *p* value 0.05 was considered significant.

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