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Neuroprotective Bibenzyl Glycosides of *Stemona tuberosa* Roots

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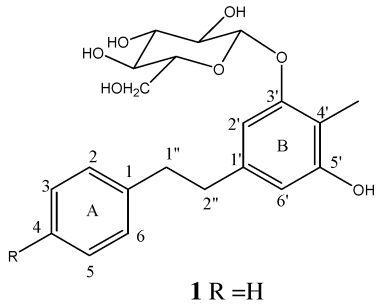
Three new bibenzyl glycosides characterized as stilbostemin B 3′-*β*-D-glucopyranoside (**1**), stilbostemin H 3′-*β*-D-glucopyranoside (**2**), and stilbostemin I 2″-*β*-D-glucopyranoside (**3**) were isolated from the roots of *Stemona tuberosa*. All three bibenzyl glycosides significantly protected human neuroblastoma SH-SY5Y cells from 6-hydroxydopamine-induced neurotoxicity.

Parkinson's disease (PD) is a neurodegenerative disorder with clinical symptoms of tremor, rigidity, and slowness in movement.[1] Although its prevalence has been on the increase for decades, the current therapeutics for PD improve only the motor symptoms in the early stages but do not prevent the progression.[2] Therefore, there has been an urgent need for the development of more effective therapeutics for PD. We have searched for neuroprotective compounds from natural products employing 6-hydroxydopamine (6-OHDA)-injured SH-SY5Y cells as an in vitro assay system. The human neuroblastoma SH-SY5Y cells possess many characteristics of dopaminergic neurons such as dopamine transporter.[3] 6-OHDA is a selective neurotoxin that causes dopaminergic neuronal cell death in vivo and in vitro.[4]

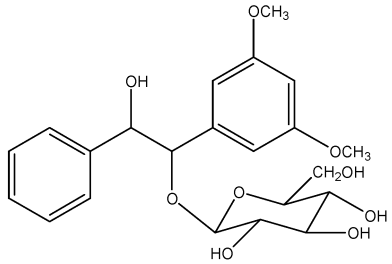
In our screening system, the *n*-BuOH fraction of *Stemona tuberosa* roots exhibited significant neuroprotective activity against 6-OHDA-induced neurotoxicity in SH-SY5Y cells. *Stemonae radix*, known as "Bai-Bu" in Traditional Chinese Medicine, is derived from the root of *S. tuberosa* Lour. The extract of the roots was found to have antibacterial, antifungal, antiviral, and insecticidal activities.[5] However, to our knowledge no studies related to neuroprotective activity have been linked to this root. In the present study, we report the isolation and structural elucidation of three new bibenzyl glycosides (**1**−**3**) from *S. tuberosa*, as well as their neuroprotective activities.

Compound **1** was isolated as a colorless powder. The molecular formula was determined to be $C_{21}H_{26}O_7$ from the HRFABMS at *m*/*z* 413.1588 $[M + Na]^+$ (calcd for $C_{21}H_{26}O_7Na$, *m*/*z* 413.1576). The fragment ion peak at *m*/*z* 228 ($[M - 162]^+$) indicated the loss of one glucosyl unit. The ^1H and ^{13}C NMR (Table 1) spectra indicated that **1** has a stilbostemin B aglycone moiety from the observed signals, consistent with published data.[6−8] The ^1H NMR spectrum showed the presence of a monosubstituted aromatic moiety (ring A) with signals at *δ* 7.15 (3H, m, H-3,4,5) and 7.24 (2H, m, H-2,6) and signals for a 1,3,4,5-tetrasubstituted aromatic moiety (ring B) at *δ* 6.32 (1H, s, H-2′) and 6.46 (1H, s, H-6′) and two methylene protons at *δ* 2.78 (2H, m, H-2″) and 2.89 (2H, m, H-1″). The correlations between C2 (6)/H-1″ and C-6″/H-2″ in the HMBC spectrum revealed the C1″/C1 and C2″/C1′ linkages, respectively. In addition, the HMBC correlation between the anomeric proton at *δ* 4.75 (1H, d, *J* = 7.4 Hz) and C-3′ (*δ* 158.70) indicated the presence of a *β*-glucopyranosyl moiety, attached to C-3′ of the stilbostemin B moiety through the anomeric carbon. Furthermore, the D configuration of the glucose unit was determined by acid hydrolysis of **1** and conversion of the resultant sugar to a trimethylsilyl derivative followed by comparison to an authentic sample using GC.[9] All carbon resonances of **1** were fully assigned by ^{13}C NMR, $^1H-^1H$ COSY, and HMQC spectra. Therefore, **1** was assigned as the new dihydrostilbene stilbostemin B 3′-*β*-D-glucopyranoside.



1 R =H

2 R=OCH_3



3

Compound **2** was isolated as a colorless powder. The molecular formula was determined to be $C_{22}H_{28}O_8$ from the HRFABMS at *m*/*z* 421.1851 $[M + H]^+$ (calcd for $C_{22}H_{29}O_8$, *m*/*z* 421.1862). The fragment ion peak at *m*/*z* 258 ($[M - 162]^+$) indicated the loss of one glucosyl unit. The ^1H and ^{13}C NMR (Table 1) spectra were very similar to those of **1**. However, the ^1H NMR spectrum showed the presence of one methoxy group at *δ* 3.74 (3H, s) and a 1,4-disubstituted aromatic moiety (ring A) with signals at *δ* 6.80 (2H, d, *J* = 8.6 Hz, H-3,5) and 7.05 (2H, d, *J* = 8.6 Hz, H-2,6). In addition, the HMBC correlation between the methoxy protons at *δ* 3.74 (3H, s) and C-4 (*δ* 160.09) indicated the position of this methoxy group to be C-4. Therefore, the aglycone of **2** was assigned as 4-methoxy-3′,5′-dihydroxy-4′-methylbibenzyl, named as stilbostemin H. In addition, the HMBC correlation between the anomeric proton at *δ* 4.73 (1H, d, *J* = 7.5 Hz) and C-3′ (*δ* 158.65) indicated the presence of a *β*-glucopyranosyl moiety, attached to C-3′ of the stilbostemin H moiety through the anomeric carbon. Furthermore, the D configuration of the glucose unit was determined by acid hydrolysis of **2** and conversion of the resultant sugar to a trimethylsilyl derivative followed by comparison to authentic sample using GC.[9] Therefore, **2** was assigned as stilbostemin H 3′-*β*-D-glucopyranoside.

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Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1**−**3** in CD_3OD (*J* values in parentheses)

	1		**2**		**3**	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		144.04		136.01		140.26
2	7.24 m	130.07	7.05 d (8.6)	131.35	7.21 m	129.14
3	7.15 m	130.43	6.80 d (8.6)	115.42	7.14 m	129.37
4	7.15 m	127.62		160.09	7.21 m	129.07
5	7.15 m	130.43	6.80 d (8.6)	115.42	7.14 m	129.37
6	7.24 m	130.07	7.05 d (8.6)	131.35	7.21 m	129.14
1′		142.14		142.21		142.48
2′	6.32 s	111.25	6.30 s	111.25	6.30 s	108.72
3′		158.70		158.65		162.31
4′		113.66		113.54	6.30 s	102.2
5′		157.68		157.64		162.31
6′	6.46 s	109.08	6.42 s	109.09	6.30 s	108.72
1″	2.89 m	39.70	2.83 m	38.83	5.06 s	78.64
2″	2.78 m	39.89	2.75 m	40.13	5.06 s	84.06
4′-Me	2.07 s	9.45	2.06 s	9.45		
OMe			3.74 s	56.40	3.62 s	56.42
					3.62 s	56.42
1‴	4.75 d (7.4)	103.54	4.73 d (7.5)	103.53	4.16 d (7.4)	101.56
2‴	3.40 m	72.28	3.37 m	72.24	3.27 m	72.55
3‴	3.45 m	75.88	3.44 m	75.83	3.33 m	76.07
4‴	3.37 m	78.83	3.31 m	78.81	3.26 m	78.53
5‴	3.43 m	79.08	3.37 m	79.07	3.09 m	78.78
6‴	3.87 dd (1.6, 13.0)	63.40	3.86 dd (2.0, 12.1)	63.37	3.87 dd (2.1, 11.9)	63.53
	3.69 dd (5.1, 12.1)		3.69 dd (5.2, 12.0)		3.67 dd (6.0, 11.9)	

Compound **3** was isolated as a colorless powder. The molecular formula was determined to be $C_{22}H_{28}O_9$ from the HRFABMS at *m*/*z* 459.1615 $[M + Na]^+$ (calcd for $C_{22}H_{28}O_9Na$, *m*/*z* 459.1631). The fragment ion peak at *m*/*z* 257 ($[M - OH - 162]^+$) indicated the loss of one glucosyl unit. The ^{1}H NMR spectrum showed the presence of a monosubstituted aromatic moiety with signals at δ 7.14 (3H, m, H-3,4,5) and 7.21 (2H, m, H-2,6), signals for a 1,3,5-trisubstituted aromatic moiety at δ 6.30 (3H, s, H-2′,4′,6′), signals for two *O*-methyl groups at δ 3.62 (6H, s), and signals for two hydroxymethine protons at δ 5.06 (2H, s, H-1″,2″). Therefore, the aglycone of **3** was assigned as 3′,5′-dimethoxy-1″,2″-dihydroxybibenzyl and named as stilbostemin I. In addition, the HMBC correlation between the anomeric proton at δ 4.16 (1H, d, $J = 7.4$ Hz) and C-1″ (δ 78.74) indicated the presence of a β-glucopyranosyl moiety, attached to C-1″ of the stilbostemin I moiety through the anomeric carbon. Furthermore, the D configuration of the glucose unit was determined by acid hydrolysis of **3** and conversion of the resultant sugar to a trimethylsilyl derivative followed by comparison to an authentic sample using GC.[9] Therefore, **3** was assigned as stilbostemin I 2″-β-D-glucopyranoside.

All three compounds showed significant neuroprotective activity against 6-OHDA-induced neurotoxicity in human neuroblastoma SH-SY5Y cells as assessed by MTT assay (Table 2).

Table 2. Neuroprotective Activity of Compounds **1**−**3** Isolated from *S. tuberosa* against 6-OHDA-Induced Neurotoxicity in SH-SY5Y Cells[a]

	relative protection (%)[b]	
compound	0.1 μM	1 μM
control	100.0 ± 1.8	
6-OHDA-injured	0.0 ± 1.9	
1	50.9 ± 6.4**	72.4 ± 5.6***
2	31.0 ± 9.1	65.6 ± 7.6***
3	29.5 ± 7.9	97.3 ± 8.9**
EGCG[c]	20.1 ± 3.2	42.2 ± 2.4**

[a] Control is the value of SH-SY5Y cells that were not treated with 6-OHDA. 6-OHDA is the value of SH-SY5Y cells that were treated with 25 μM 6-OHDA for 24 h. Optical density (OD) of control and 6-OHDA-treated cultures was 1.02 ± 0.02 and 0.69 ± 0.03, respectively. Compounds were treated 1 h before the 6-OHDA injury. [b] Relative protection (%) was calculated as 100 × (OD of 6-OHDA + sample-treated − OD of 6-OHDA-treated)/(OD of control − OD of 6-OHDA-treated). Mean value is significantly different (**$p < 0.01$, ***$p < 0.001$) from the value of the 6-OHDA-treated. [c] EGCG (epigallocatechin-3-gallate) was used as a positive control.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, HMQC, and HMBC were measured on a Bruker AMX 400 spectrometer in CD_3OD with TMS or solvent signals as internal standards. FAB-MS were recorded on a VG 70-VSEQ mass spectrometer with direct inlet system using PEG600/glycerol as a matrix. High-resolution MS analyses were done on a JEOL JMS AX 505 WA spectrometer. Optical rotation was measured with a Jasco DIP-1000 digital polarimeter. UV spectra were recorded on a Shimadzu UV-2100 spectrophotometer. HPLC was performed with an L-6200 pump (Hitachi, Japan), an L-4000 UV detector (Hitachi, Japan), and a YMC-Pack Pro C18 column (YMC Co., Ltd., Japen).

Plant Material. The roots of *S. tuberosa* (15 kg) were purchased from Kyungdong Oriental Herbal Market, Seoul, Korea, and a voucher specimen (SNUPH-0822) has been deposited in the Herbarium of the Medicinal Herb Garden, College of Pharmacy, Seoul National University.

Extraction and Isolation. The dried roots (15 kg) of *S. tuberosa* were extracted with 80% MeOH in an ultrasonic apparatus. Upon removal of solvent in vacuo, the MeOH extract yielded 9 kg. This extract was then suspended in H_2O and partitioned successively with *n*-hexane, EtOAc, and *n*-BuOH. The *n*-BuOH fraction (830 g) was chromatographed over HP resin and eluted with a gradient of aqueous MeOH (MeOH/H_2O = 0:1, 4:1, 3:2, 2:3, 4:1, 1:0, 8 L of each solvent). The fraction eluted with MeOH/H_2O (3:2) was further subjected to Sephadex LH-20 (MeOH) and reversed-phase HPLC chromatographic separation (acetonitrile/H_2O = 25:75, flow rate 2.0 mL/min). Three compounds, **1** (t_R = 34.3 min, 11.2 mg), **2** (t_R = 33.3 min, 3.8 mg), and **3** (t_R = 23.0 min, 12.8 mg), were obtained.

Stilbostemin B 3′-β-D-glucopyranoside (1): colorless powder; $[\alpha]^{20}_D$ −12.42 (*c* 0.2, MeOH); UV λ_{max} nm (log ϵ) 217 (2.40), 220 (2.40), 269 (1.65), 277 (1.63); ^{1}H NMR and ^{13}C NMR, see Table 1; FABMS (positive) *m*/*z* 413 $[M + Na]^+$, 228; HRFABMS (positive) *m*/*z* 413.1588 $[M + Na]^+$ (calcd for $C_{21}H_{26}O_7Na$, *m*/*z* 413.1576).

Stilbostemin H 3′-β-D-glucopyranoside (2): colorless powder; $[\alpha]^{20}_D$ −15.51 (*c* 0.2, MeOH); UV λ_{max} nm (log ϵ) 225 (2.45), 276 (1.97); ^{1}H NMR and ^{13}C NMR, see Table 1; FABMS (positive) *m*/*z* 421 $[M + H]^+$, 258; HRFABMS at *m*/*z* 421.1851 $[M + H]^+$ (calcd for $C_{22}H_{29}O_8$, *m*/*z* 421.1862).

Stilbostemin I 2″-β-D-glucopyranoside (3): colorless powder; $[\alpha]^{20}_D$ −21.41 (*c* 0.2, MeOH); UV λ_{max} nm (log ϵ) 209 (2.38), 277 (1.91), 281 (1.91); ^{1}H NMR and ^{13}C NMR, see Table 1; FABMS (positive)

m/*z* 459 $[M + Na]^+$, 257; HRFABMS at *m*/*z* 459.1615 $[M + Na]^+$ (calcd for $C_{22}H_{28}O_9Na$, *m*/*z* 459.1631).

Acid Hydrolysis of Compounds 1−3. The absolute configuration of sugar units was assigned by GC after total acid hydrolysis of each compound. The sugars were compared with those of the authentic samples prepared in the modified manner.[9] In brief, a solution (0.8 mg each) of bibenzyl glycosides in 1 N HCl (0.25 mL) was stirred at 80 °C for 4 h. After cooling, the solution was concentrated by blowing with N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.1 mL), and the solution was stirred at 60 °C for 5 min. After drying the solution with a stream of N_2, the residue was partitioned between H_2O and CH_2Cl_2 (1 mL, 1:1 v/v). The CH_2Cl_2 layer was analyzed by GC using a l-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected at 14.74 min (D-glucose). Retention times for authentic samples after being treated simultaneously with 1-(trimethylsilyl)imidazole in pyridine were detected at 14.76 min (D-glucose) and 15.82 min (L-glucose).

Cell Culture. The human neuroblastoma SH-SY5Y cell line was obtained from KCLB (Korean Cell Line Bank) and was grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 95% air−5% CO_2.

Assessment of Cell Viability. Test compounds were dissolved in DMSO (final concentration in culture, 0.1%). SH-SY5Y cells were treated with test compounds for 1 h and then exposed to 25 μM 6-OHDA. After further 24 h incubation, the cultures were assessed for viability by MTT (3-[4,5-dimethylthiozol-2-yl]-2,5-diphenyltetrazolium bromide) assay. Data are expressed as the percentage protection relative to vehicle-treated control cultures: 100 × [optical density (OD) of 6-OHDA + sample-treated cultures − OD of 6-OHDA-treated cultures]/[OD of control cultures − OD of 6-OHDA-treated cultures]. The evaluation of statistical significance was determined by the one-way ANOVA test with a value of $p < 0.05$ considered to be statistically significant.

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Note Added after ASAP Publication: The stereochemistry of structures **1** and **2** was incorrect in the version posted on Jan 31, 2006. The correct structures appear in the version posted on Feb 9, 2006.

Supporting Information Available: Spectra of compounds **1**−**3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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