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Furostanol saponins from the rhizomes of *Dioscorea japonica* and their effects on NGF induction

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ABSTRACT

The rhizome of *Dioscorea japonica* is a food and medicinal source known as 'San Yak' in Korea. Two new furostanol saponins, coreajaponins A (**1**) and B (**2**), together with 10 known compounds (**3**–**12**) were isolated from the rhizomes of *D. japonica*. Their structures were determined by spectroscopic methods, including 1D and 2D NMR techniques, HRMS, and chemical methods. Nerve growth factor (NGF), a crucial factor for neuronal survival and differentiation, can potentially improve neurodegenerative diseases and diabetic polyneuropathy. We evaluated the effects of isolates (**1–12**) on NGF induction in a C6 rat glioma cell line. Coreajaponin B (**2**) upregulated NGF content without significant cell toxicity, as did **6**, **8**, **9**, and **11**.

Nerve growth factor (NGF) was first discovered by Levi-Montalcini in 1966.[1] NGF has neurotrophic actions that protect cholinergic neurons of the basal forebrain against axotomy-induced neurodegeneration and aged-related atrophy.[2,3] Exogenous NGF improves impaired function of the cholinergic neuron system, such as neuronal degeneration,[4] and has therapeutic potential for neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease[5] and diabetic polyneuropathy.[6] However, NGF cannot pass through the blood–brain barrier (BBB) and therefore requires neurosurgical approaches for administration.[7] The development of small molecules that induce NGF secretion or mimic NGF activity is therefore desirable.

In our continuing search for bioactive constituents from Korean plant sources, we found that the rhizomes of *Dioscorea japonica* Thunb. (Dioscoreaceae) induce increases in endogenous NGF levels, and thereby have a protective effect against diabetic neuropathy.[8] The rhizome of *D. japonica*, naturally distributed in East Asia, China, Japan, and Korea, is a food and medicinal source known as 'San Yak' in Korea. The plant has been used to strengthen stomach function, improve anorexia, eliminate diarrhea, dilute sputum, and moisturize skin in traditional Chinese medicine.[9] Previous phytochemical investigations of *D. japonica* revealed active hypoglycemic compounds (dioscorans A–F),[10] sesquiterpene, and acetophenone.[11] The steroidal constituents of this herb, including spirostane, furostane, and cholestane types, are main secondary metabolites of *Dioscorea* species,[12] but have been rarely described and sparsely investigated.

In the present work, to identify the active constituents responsible for induction of NGF secretion, we performed a phytochemical investigation of the rhizomes of *D. japonica*. Two new furostanol saponins (**1–2**), together with 10 known compounds (**3–12**), were isolated from its methanolic extract. Their structures were determined by spectroscopic methods, including 1D and 2D NMR techniques, HRMS, and chemical methods. Herein, we report the isolation and structural elucidation of the new compounds and NGF regulation of isolates (**1–12**).

Dried and pulverized rhizomes of *D. japonica* were extracted with 50% aqueous EtOH. The extract suspended in water was successively extracted with *n*-hexane, $CHCl_3$, EtOAc, *n*-BuOH, and acetone. Purification of the $CHCl_3$-soluble and *n*-BuOH-soluble fractions by multiple chromatographic steps (Supplementary data) led to the isolation of two new furostanol saponins, coreajaponins A (**1**) and B (**2**), and ten known compounds (**3–12**) (Fig. 1). The known compounds were identified as batatasin IV (**3**),[13] raspberry ketone (**4**),[14] 2-methoxy-4′-hydroxyacetophenone (**5**),[15] (3*R*,5*S*)-3,5-dihydroxy-1,7-bis(4-hydroxy-3-methoxyphenyl)heptane (**6**),[16] β-sitosterol (**7**),[17] blumenol A (**8**),[18] dihydropinosylvin (**9**),[19] stilbostemin N (**10**),[20] butyl-β-D-fructofuranoside (**11**),[21] and allantoin (**12**),[22] by comparison of their spectroscopic and physical data with reported values. To the best of our knowledge, four isolates (**5–6**, **8**, and **11**) are here reported for the first time from *Dioscorea* species.

Coreajaponin A (**1**) was obtained as a white amorphous powder. The molecular formula was determined to be $C_{50}H_{82}O_{22}$ from the molecular ion peak $[M+H]^+$ at *m/z* 1035.5387 (calcd for

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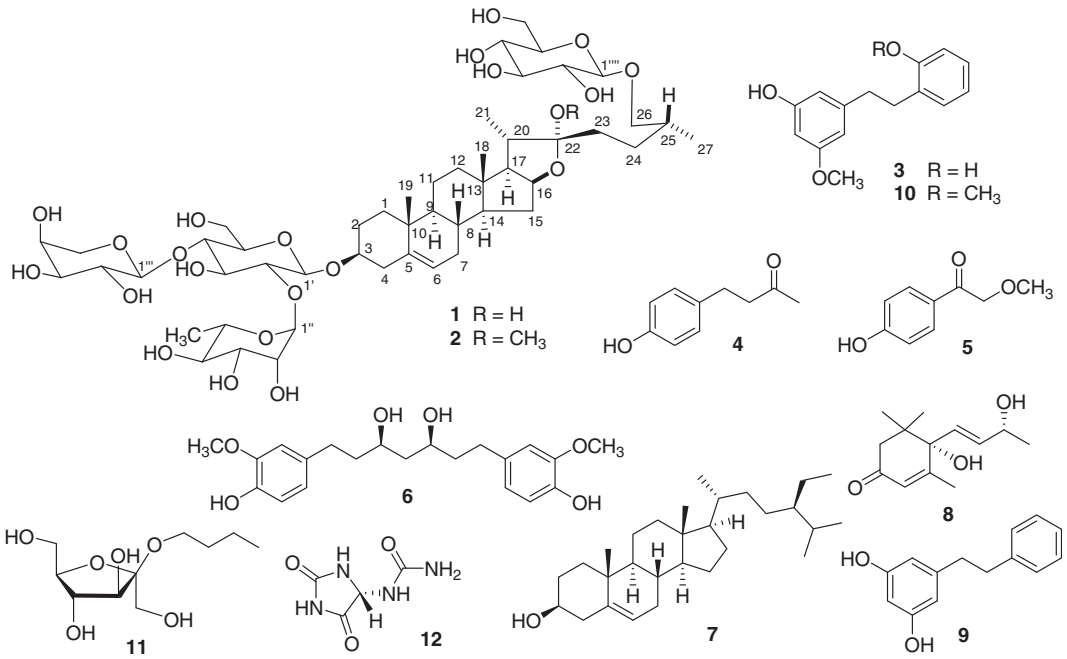

Figure 1. Chemical structures of compounds **1**–**12**.

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of compounds **1**–**2** (δ in ppm, J values in parentheses)[a]

Aglycone	**1**		**2**		Sugar	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C		δ_H	δ_C	δ_H	δ_C
1	1.78 m, 1.05 m	37.1	1.78 m, 1.04 m	37.4	Glc-1′	4.53 d (7.5)	98.9	4.51 d (7.5)	100.1
2ax	1.58 m	29.3	1.59 m	30.1	2′	3.41 m	77.0	3.42 m	77.8
2eq	2.11 m		2.10 m						
3	3.58 m	77.9	3.58 m	78.5	3′	3.42 m	77.1	3.44 m	77.3
4ax	2.30 t (13.0)	39.4	2.29 t (13.0)	39.2	4′	3.35 m	79.5	3.34 m	81.4
4eq	2.45 dd (13.0, 2.5)		2.44 dd (13.0, 2.5)						
5		140.4		140.8	5′	3.28 m	76.1	3.27 m	76.3
6	5.40 br d (4.5)	121.2	5.38 br d (4.0)	121.5	6′a	3.86 dd (12.5, 1.5)	60.4	3.87 dd (12.5, 1.5)	61.6
					6′b	3.81 dd (12.5, 3.5)		3.80 dd (12.5, 3.5)	
7	1.99 m, 1.60 m	31.7	1.97 m, 1.60 m	32.2	Rha-1″	5.23 d (0.5)	100.5	5.22 d (0.5)	102.5
8	1.80 m	31.3	1.82 m	31.6	2″	3.88 m	70.7	3.87 m	72.5
9	0.99 m	50.2	0.99 m	50.3	3″	3.67 dd (9.5, 3.0)	71.1	3.66 dd (9.5, 3.0)	72.8
10		36.6		37.1	4″	3.52 m	72.9	3.52 m	74.2
11	1.66 m, 1.63 m	20.5	1.69 m, 1.63 m	21.1	5″	3.81 dd (9.5, 6.0)	68.5	3.80 dd (9.5, 6.0)	69.4
12	1.78 m, 1.17 m	39.4	1.78 m, 1.18 m	39.7	6″	1.25 d (6.0)	16.5	1.23 d (6.0)	18.9
13		40.4		40.7	Ara-1‴	4.27 d (7.5)	104.0	4.26 d (7.5)	105.9
14	1.15 m	56.3	1.16 m	56.5	2‴	3.68 dd (8.0, 7.0)	70.9	3.66 dd (8.0, 7.0)	72.6
15	2.03 m, 1.57 m	31.3	2.01 m, 1.55 m	32.0	3‴	3.51 m	73.7	3.50 m	74.6
16	4.37 m	81.0	4.37 m	81.3	4‴	3.80 m	68.3	3.80 m	69.6
17	1.73 dd (8.0, 6.5)	63.6	1.73 dd (8.0, 6.5)	64.1	5‴a	3.92 dd (12.0, 1.5)	66.3	3.92 dd (12.0, 1.5)	67.8
					5‴b	3.60 dd (12.0, 3.0)		3.60 dd (12.0, 3.0)	
18	0.84 s	15.3	0.83 s	17.1	Glc-1⁗	4.25 d (8.0)	103.1	4.23 d (7.5)	104.9
19	1.06 s	18.4	1.04 s	19.3	2⁗	3.39 m	74.7	3.38 m	75.1
20	2.15 m	39.7	2.17 m	41.2	3⁗	3.28 m	76.7	3.27 m	78.3
21	1.01 d (7.0)	14.6	0.99 d (7.0)	16.2	4⁗	3.29 m	70.3	3.30 m	71.7
22		112.5		112.6	5⁗	3.40 m	76.4	3.40 m	78.6
23	1.78 m	29.9	1.75 m	30.6	6⁗a	3.85 dd (12.5, 1.5)	61.4	3.84 dd (12.5, 1.5)	62.5
					6⁗b	3.68 dd (12.5, 3.5)		3.68 dd (12.5, 3.5)	
24	1.82 m, 1.75 m	27.5	1.82 m, 1.75 m	28.1					
25	1.79 m	33.5	1.79 m	34.2					
26a	3.72 dd (10.5, 6.0)	74.5	3.72 dd (10.5, 6.0)	75.1					
26b	3.40 m		3.40 m						
27	0.96 d (7.0)	15.8	0.95 d (7.0)	18.6					
OCH_3			3.14 s	47.2					

[a] ^{1}H and ^{13}C NMR data of **1** and ^{1}H NMR data of **2** in CD_3OD; ^{13}C NMR data of **2** in C_5D_5N; The assignments were based on DEPT, ^{1}H,^{1}H-COSY, HMQC and HMBC experiments.

$C_{50}H_{83}O_{22}$, 1035.5376) in the positive-ion HRFABMS. The glycosidic nature of **1** was shown by strong IR absorptions at 3400 and 1060 cm^{-1}. The ^{1}H NMR spectrum of **1** (Table 1) showed signals for two tertiary methyl groups at δ_H 1.06 and 0.84 (each 3H, s) and two secondary methyl groups at δ_H 1.01 and 0.96 (each 3H, d, J = 7.0 Hz), typical steroid methyls, as well as a signal for an

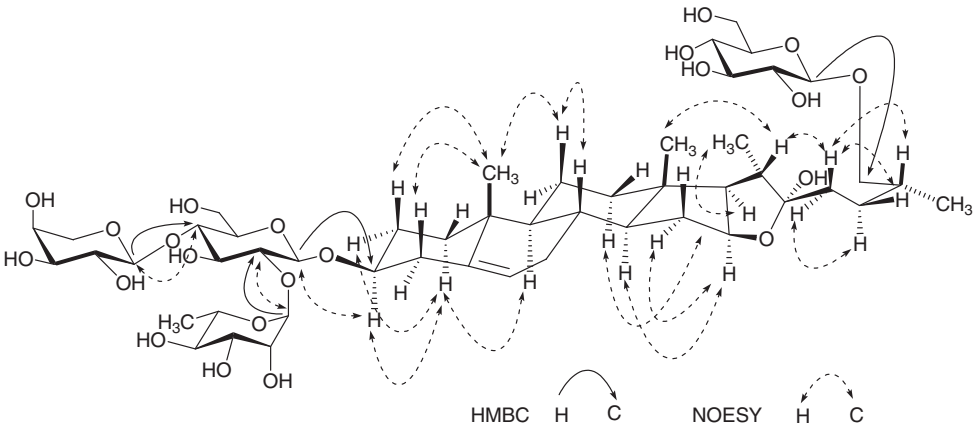

Figure 2. Key HMBC and NOESY correlations of **1**.

olefinic proton at δ_H 5.40 (1H, br d, J = 4.5 Hz). Furthermore, signals for four anomeric protons at δ_H 5.23 (1H, d, J = 0.5 Hz), 4.53 (1H, d, J = 7.5 Hz), 4.27 (1H, d, J = 7.5 Hz), and 4.25 (1H, d, J = 8.0 Hz) showing HMQC correlations with four anomeric carbon signals at δ_C 100.5, 98.9, 104.0, and 103.1, respectively, were assigned, and the signal for the methyl group of a 6-deoxyhexopyranose at δ_H 1.25 (3H, d, J = 6.0 Hz) was also observed in the ^{1}H NMR spectrum. The above NMR data, an acetalic carbon signal at δ_C 112.5 in the ^{13}C NMR spectrum, and a positive red color reaction in Ehrlich's test indicated that **1** is a furostanol saponin with four attached glycosidic units.[23] The chemical shifts of ^{1}H and ^{13}C NMR (including DEPT) and evaluation of 2D NMR, including COSY, HMBC, and NOESY allowed the identification of two β-glucopyranosyl (Glc), one α-arabinopyranosyl (Ara), and one α-rhamnopyranosyl (Rha) units.[24] The relatively large J values (7.5–8.0 Hz) indicated a β-orientation for the anomeric center of Glc and an α-orientation for that of Ara. The J coupling (d, J = 0.5 Hz) of the anomeric proton signal at δ_H 5.23 of Rha indicated an α-orientation. The monosaccharides obtained from the acidic hydrolysis of **1** were identified as L-arabinose, D-glucose, and L-rhamnose by GC analysis of their chiral derivatives.[25,26] The sequence and interglycosidic linkages among the four sugar units and the aglycone were revealed by the HMBC and NOESY experiments (Fig. 2). In the HMBC spectrum, a correlation peak between H-1‴′ of Glc (terminal glucosyl) at δ_H 4.25 and C-26 of the aglycone at δ_C 74.5 implied that one glucose unit is attached to C-26 of the aglycone, which is a structural feature in plant furostanol saponins.[27] That one of the glucose units is attached to C-3 of the aglycone was ascertained by the HMBC correlation between H-1′ of Glc at δ_H 4.53 and C-3 of the aglycone at δ_C 77.9, and by the NOESY correlation between H-1′ of Glc at δ_H 4.53 and H-3 of the aglycone at δ_H 3.58. Furthermore, the anomeric proton of Rha at δ_H 5.23 was correlated with C-2′ of Glc at δ_C 77.0, and that of Ara at δ_H 4.27 was correlated with C-4′ of Glc at δ_C 79.5, which supported the proposed sequence of the oligosaccharidic chain linked at the C-3 of the aglycone.[24] This connection was also deduced by the NOESY correlations between H-1″ of Rha at δ_H 5.23 and H-2′ of Glc at δ_H 3.41, and between H-1‴′ of Ara at δ_H 4.27 and H-4′ of Glc at δ_H 3.35. The identity of the aglycone of **1** was confirmed by the acidic hydrolysis that afforded diosgenin.[28,29] The C-25 configuration of **1** was deduced based on the difference ($\Delta_{ab} = \delta_a - \delta_b$) of the ^{1}H NMR chemical shifts of the H_2-26 geminal protons.[30] The 25*R* configuration of **1** was assigned by the observed difference (Δ_{ab} = 0.32) of the chemical shifts of **1**, which was in agreement with that of 25*R* furostane-type steroidal saponins (Δ_{ab} <0.48 for 25*R*; Δ_{ab} >0.57 for 25*S*).[30] To identify the configuration of C-22, compound **1** was converted to the 22-methoxy form by treatment with hot MeOH,[28,31] which was identical to compound **2** by measurement of ^{1}H NMR data. During the methanolysis, the chiral inversion is possible, but was not detected in same reactions of relative furostanol saponins.[28,31] The C-22 configuration of **1** was confirmed to be α-configuration by analysis of its NOESY experiment showing a correlation between the methoxy proton at δ_H 3.14 and the H-16 proton at δ_H 4.37. Thus, the structure of **1** was established as (25*R*)-26-[(β-D-glucopyranosyl)oxy]-22α-hydroxyfurost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→4)]-β-D-glucopyranoside.

Coreajaponin B (**2**), obtained as a white amorphous powder, had the molecular formula $C_{51}H_{84}O_{22}$ by the positive-ion HRFABMS (m/z 1071.5330 $[M+Na]^+$). The glycosidic nature of **1** was shown by strong IR absorptions at 3397 and 1061 cm^{-1}. Compound **2** was suggested to be a 22-methoxyfurostanol saponin by Ehrlich's test,[23] and by ^{1}H NMR [δ_H 3.14 (3H, s)] and ^{13}C NMR [δ_C 112.6 (C-22) and 47.2 (OCH_3)] spectrum.[27] Analysis of the ^{1}H and ^{13}C NMR spectrum of **2** and comparison with those of **1** revealed that compound **2** possessed sugar moieties identical to those of **1**, but differed slightly from **1** in the aglycone, with the presence of an additional methoxy group. Acidic hydrolysis of **2** afforded diosgenin, together with L-arabinose, D-glucose, and L-rhamnose, which were confirmed by GC analysis.[25,26] The methoxy group at C-22 was confirmed by HMBC correlation between a methoxy group at δ_H 3.14 and C-22 of the aglycone at δ_C 112.6. An NOESY correlation between the methoxy signal at δ_H 3.14 and the H-16 signal at δ_H 4.37 was consistent with the C-22α configuration. Thus, the structure of **2** was elucidated as (25*R*)-26-[(β-D-glucopyranosyl)oxy]-22α-methoxyfurost-5-en-3β-yl *O*-α-L-rhamnopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→4)]-β-D-glucopyranoside. Compound **2** may be an artifact produced from **1**, since 22-hydroxyfurostane derivatives are readily converted into 22-methoxyfurostane ones in solutions containing MeOH.[31]

Then, we evaluated the effects of isolates (**1**–**12**) on cell viability by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay[32] and on NGF secretion using ELISA development kit (R&D System, Minneapolis, MN, USA) from C6 glial cells to measure NGF release into the medium.[33] As shown in Table 2, compound **2** was the most potent stimulant of NGF release, with 132.9 ± 3.6% stimulation and normal cell viability (98.0 ± 1.5%) at concentration of 20 μM. Interestingly, although the structures of **1** and **2** are quite similar except of the presence of a methoxy group, they differed substantially with respect to their effect on NGF synthesis induction. The obtained data suggest that the presence of the methoxy group at C-22 in furostanol saponins is important for the effect on NGF induction though more saponins need to be tested to confirm this hypothesis. Compounds **6**, **8**, **9** and **11** also increased NGF secretion to 120.7 ± 4.6%, 122.2 ± 4.5%, 117.0 ± 3.4% and 117.5 ± 3.3% of untreated control, without cell toxicity. No other compounds affected NGF release, and no compound was effective at

Table 2
Effects of compounds **1**–**12** on NGF secretion and cell viability in C6 cells[a]

Compounds	NGF secretion	Cell viability
1	107.2 ± 1.2	105.9 ± 4.5
2	132.9 ± 3.6[b]	98.0 ± 1.5
3	106.1 ± 4.3	95.0 ± 4.7
4	106.8 ± 6.3	100.9 ± 2.8
5	105.6 ± 4.1	99.8 ± 1.3
6	120.7 ± 4.6[b]	102.5 ± 1.7
7	106.1 ± 2.8	103.5 ± 2.7
8	122.2 ± 4.5[b]	101.0 ± 2.3
9	117.0 ± 3.4[b]	104.9 ± 1.5
10	104.6 ± 1.1	103.7 ± 4.0
11	117.5 ± 3.3[b]	101.6 ± 1.9
12	102.4 ± 1.4	100.2 ± 2.7

[a] C6 cells were treated with 20 μM of compounds **1**–**12**. After 24 h, the content of NGF secretion in C6-conditioned media was measured by ELISA, and the cell viability was determined by MTT assay. The level of secreted NGF and viable cells are expressed as percentage of the untreated control. The data shown represent the means ± SD of three independent experiments performed in triplicate.
[b] $p < 0.05$ indicates significantly different from control group (Student's *t*-test).

concentrations below 20 μM (data not shown). The above results suggest that the active compounds (**2**, **6**, **8**, **9**, and **11**) promote synthesis of NGF, which might enhance neuron cell survival in neurodegenerative disease models.

In conclusion, the structures of two new furostanol saponins (**1–2**), along with ten known compounds (**3**–**12**) isolated from the rhizomes of *D. japonica* were identified. While steroidal saponins have been described as the primary and characteristic constituents of this species, the presence of furostanol saponins studied here is reported from this plant for the first time. With regard to bioactivity, compounds **2**, **6**, **8**, **9**, and **11** induced NGF secretion in C6 cells at 20 μM. The most potent stimulant of NGF release, coreajaponin B (**2**), may have a potential for neuroprotection via inducing NGF secretion and may deserve further investigation as a candidate for regulation of neurodegenerative diseases and diabetic polyneuropathy. The apparent activity of multiple components from this root suggests the possibility of additive or synergistic effects which merits further investigation.

Acknowledgements

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.02.003.

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