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Linckosides A and B, Two New Neuritogenic Steroid Glycosides from the Okinawan Starfish *Linckia laevigata*

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Abstract—Two new steroid glycosides, named linckosides A and B, were isolated from the Okinawan starfish *Linckia laevigata*, and their stereostructures were elucidated by spectroscopic methods and chemical derivatization. Each of them possesses two monosaccharide units at C-3 and C-29 of a polyhydroxylated steroid aglycon. These steroid glycosides showed not only notable neuritogenic activity against PC12 cells but also significant synergistic effects on the NGF-induced neuronal differentiation of PC12 cells. Linckoside B showed higher activities than those of linckoside A, though the structural difference is only the kind of a sugar.

Introduction

Neurotrophic factors (NTFs), such as NGF and BDNF, are well known to be proteins essential for the growth, development, survival and function maintenance of neurons in the central and peripheral systems.[1–5] NTFs have been considered as good candidates for the prevention and treatment of neurodegenerative diseases. Since such endogenous factors are too large in size to pass through the blood–brain barrier, exogenous low-molecular-weight compounds that mimic the activity of NTFs might be developed as promising therapeutic drugs to treat various neurodegenerative diseases. It is well known that the PC12 cells, rat pheochromocytoma cells, respond to NGF by switching from an immature chromaffin-cell-like phenotype to a sympathetic-neuron-like one with the outgrowth of long, branching neurites.[6] In the present study, it was used as a bioassay system to guide the isolation of novel neuritogenic compounds.

A search for such neuritogenic compounds was recently performed in our laboratory using the above assay system, and resulted in the isolation of novel cerebrosides from an edible Chinese mushroom.[7,8] Furthermore, a study on Okinawan marine samples led to the isolation of two new steroid glycosides, linckosides A (**1**) and B (**2**) (Fig. 1), from the starfish *Linckia laevigata* (LINNE). Here, we describe the isolation, structures and biological activities of these neuritogenic compounds.

Results and Discussion

The EtOH/MeOH (4:1) extract of *L. laevigata* was washed with hexane, then partitioned between EtOAc and water to give an active aqueous fraction. The aqueous fraction was chromatographed on ODS, followed by reversed-phase HPLC to yield linckosides A (**1**, 0.0048% of dry wt) and B (**2**, 0.0026%).

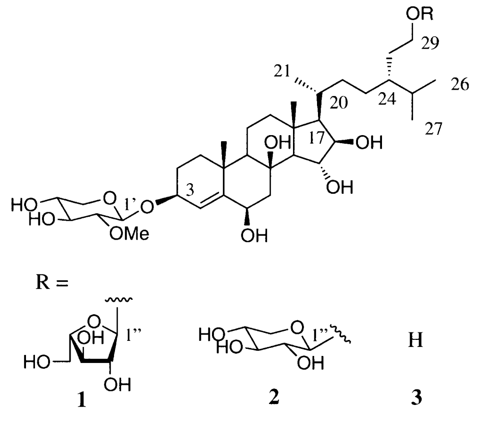

Figure 1. Structures of linckosides A (**1**), B (**2**) and dearabinosyl linckoside A (**3**).

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Linckoside A (**1**) was obtained as a colorless powder and possessed the molecular formula $C_{40}H_{68}O_{14}$ determined by an HRFABMS measurement. The IR spectrum of **1** showed a strong absorption centered at 3420 cm^{-1} due to hydroxyl groups. The ^{1}H NMR, ^{13}C NMR and DEPT spectra of **1** revealed the presence of twelve oxymethines, three oxymethylenes, one oxygenated quaternary carbon, and one methoxyl group (δ_H 2.81–4.85, δ_C 61.1–85.3, 104.6 and 109.5), a trisubstituted double bound (δ_H 5.63, δ_C 126.9 and 148.6) and two quaternary *sp*^{3} carbons (δ_C 37.7 and 45.1) (Table 1). The remaining proton and carbon signals were attributed to five methyls, six methines and eight methylenes. The anomeric resonances of protons and carbons (δ_H/δ_C: 4.41/104.6 and 4.85/109.5) unveiled the presence of two sugar moieties. The analysis of these signals by the DQFCOSY and HMQC experiments led to the partial structures depicted in Figure 2. These partial structures were connected by the long-range C-H correlations observed in an HMBC experiment to give a gross structure of **1** (Fig. 3). The HMBC correlations establishing the gross structure of **1** were as follows: methyl protons (H-19) to C-1, C-5, C-9 and C-10; methyl protons (H-18) to C-12, C-13, C-14 and C-17; H-3 to C-4 and C-5; H-4 to C-6; H-6 to C-8 and C-10; H-7 to C-5 and C-8; H-9 to C-8; H-14 to C-13; H-15 to C-8; H-2′ to C-6′. The correlations from anomeric H-1′ to C-3 and H-1″ to C-29 indicated the location of the sugar moieties.

The nature of the sugar that connected to C-3 was determined as 2-*O*-methyl-xylopyranose by the DQFCOSY, NOESY correlations (H-1′/H-3′ and H-5′a, H-2′/H-4′, H-3′≥5/H-5′a; Fig. 4), and the comparison of the ^{13}C NMR data with the reported one.[9] The β configuration of this sugar unit was deduced by the coupling constant $J_{1'-2'} = 7.7$ Hz. Another sugar moiety linked at C-29 was determined as α-arabinofuranose on the basis of the DQFCOSY, NOESY correlations (H-5″/H-1″ and H-3″, H-4″/H-29) (Fig. 4), the coupling constant $J_{1''-2''} = 1.8$ Hz, and the ^{13}C NMR data for the reported one.[9]

The 24*R* configuration was assigned on the basis of the difference of ^{13}C chemical shifts between the C-26 and C-27 resonances, δ 19.0 and 20.0, respectively (Δδ 1.0), which was compared with those reported for 24*R* (δ 18.6 and 19.7; Δδ 1.1) and 24*S* (δ 19.2 and 19.3; Δδ 0.1) synthetic steroids.[10] The degradation of **1** was then carried out to determine the whole stereochemistry of both the aglycon and the sugar moieties. Mild hydrolysis of **1** with aqueous acetic acid gave dearabinosyl linckoside A (**3**, Fig. 1) and sugar containing fractions. The former **3** was identical with desulfated echinasteroside B, because ^{1}H NMR data of **3** showed the excellent agreement with the reported data (Table 2, assigned by DQFCOSY and HOHAHA measurements).[11,12] Thus, the stereostructure of 20*R*, 24*R*, Δ^4, 3β, 6β, 8β, 15α 16β, 29-hexaol of the aglycon of **1** was established and further confirmed by the NOESY correlations of H-19/H-1b and H-11, H-3/H-1a, H7a/H-6 and H-9, H-7b/H-15, H-16/H-14 and H-17, H-18/H-15 and H-20 (Fig. 4). The sugar-containing fractions obtained on the above mild hydrolysis of **1** was combined with **3** and other byproducts, and subjected to methanolysis (HCl, MeOH) followed by benzoylation (*p*-Br-C_6H_4COCl and 4-dimethylaminopyridine) to give di- and tribenzoyl sugar derivatives. The D-configuration of 2-*O*-methylxylose and the L-configuration of arabinose were established by CD measurements of the products: methyl 3,4-di-*O*-(*p*-bromobenzoyl)-2-*O*-methyl-α-D-xylopyranoside (λmax/min 239/256 nm, Δε +21/−41) and methyl 2,3,4-tri-*O*-(*p*-bromobenzoyl)-β-L-arabinopyranoside (λmax/min 240/256 nm, Δε−36/+84).[13]

Table 1. ^{1}H and ^{13}C NMR data for linckosides A (**1**) and B (**2**) in CD_3OD

Carbon No.	**1**		**2**	
	^{1}H[a]	^{13}C[b]	^{1}H[a]	^{13}C[b]
1a	1.28 m	39.7	1.29 m	39.7
1b	1.79 m		1.79 m	
2a	1.75 m	27.9	1.75 m	27.9
2b	1.98 m		1.98 m	
3	4.18 m	77.5	4.18 m	77.5
4	5.63 s	126.9	5.63 s	126.9
5	—	148.6	—	148.6
6	4.30 dd (2.9, 2.7)	76.4	4.30 dd (3.5, 3.0)	76.4
7a	1.49 dd (14.8, 2.7)	44.4	1.49 dd (14.1, 3.5)	44.4
7b	2.57 dd (14.8, 2.7)		2.57 dd (14.1, 3.0)	
8	—	76.2	—	76.2
9	1.04 m[c]	57.8	1.04 m[c]	57.8
10	—	37.7	—	37.7
11a	1.48 m	19.5	1.48 m	19.5
11b	1.86 m		1.87 m	
12a	1.18 m	43.0	1.18 m	43.0
12b	1.97 m		1.97 m	
13	—	45.1	—	45.1
14	1.01 d (10.7)	63.7	1.01 d (10.7)	63.7
15	4.15 dd (10.7, 2.5)	81.0	4.15 dd (10.7, 2.5)	81.0
16	3.98 dd (7.8, 2.5)	82.9	3.97 dd (7.4, 2.5)	82.9
17	1.21 m[c]	60.5	1.21 m[c]	60.5
18	1.12 s	16.8	1.12 s	16.8
19	1.36 s	22.7	1.36 s	22.7
20	1.83 m	31.1	1.82 m	31.2
21	0.93 d (6.5)	18.6	0.92 d (6.7)	18.6
22	1.04 m[c]	34.8	1.04 m[c]	34.8
23a	1.16 m	28.6	1.14 m	28.6
23b	1.41 m		1.41 m	
24	1.21 m[c]	42.5	1.21 m[c]	42.3
25	1.74 m	30.6	1.73 m	30.7
26	0.84 d (6.8)	19.0	0.84 d (6.8)	19.0
27	0.86 d (6.8)	20.0	0.86 d (6.8)	20.0
28a	1.43 m	31.6	1.44 m	31.6
28b	1.64 m		1.67 m	
29a	3.42 m	68.1	3.19 m	70.1
29b	3.75 m		3.81 m	
1′	4.41 d (7.7)	104.6	4.41 d (7.7)	104.6
2′	2.81 dd (8.9, 7.7)	84.9	2.81 dd (8.9, 7.7)	84.9
3′	3.31 m	77.5	3.31 m[c]	77.5
4′	3.46 m	71.3	3.47 m[c]	71.3
5′a	3.15 dd (11.3, 10.5)	66.8	3.15 m[c]	66.8
5′b	3.81 dd (11.3, 5.2)		3.81 m[c]	
2′-OMe	3.57 s	61.1	3.57 s	61.1
1″	4.85 d (1.8)	109.5	4.17 d (7.5)	105.2
2″	3.94 dd (3.9, 1.8)	83.6	3.15 m[c]	75.0
3″	3.82 m	78.7	3.31 m[c]	77.9
4″	3.91 m	85.3	3.47 m[c]	71.3
5″a	3.62 dd (12.1, 5.0)	63.0	3.15 m[c]	66.9
5″b	3.73 dd (12.1, 3.0)		3.81 m[c]	

[a] 600 MHz, coupling constants (*J* in Hz) are in parentheses.
[b] 150 MHz.
[c] Overlapping signals.

Linckoside B (**2**) was obtained as a colorless powder and possessed the molecular formula $C_{40}H_{68}O_{14}$ determined by an HRFABMS measurement. The ^{1}H and ^{13}C NMR of **2** were superimposable on those of **1** (Table 1) except for the signals of a sugar and around the position C-29, which linked with the sugar. The location of this sugar moiety was determined by the correlations from the anomeric H-1″ to C-29 and from H-29 to the anomeric C-1″ in an HMBC experiment. The sugar was determined as xylopyranose by the NOESY correlations (H-1″/H-3″ and H-5″a, H-2″/H-4″) and the comparison of the ^{13}C NMR data with the reported one.[9] The β configuration of xylopyranose was determined by the coupling constant $J_{1''-2''} = 7.5$ Hz. Furthermore, the configuration of the sugar moieties was determined by the derivatization of **2** and CD measurements. The methanolysis (HCl, MeOH) of **2** followed by benzoylation using the same conditions as those for **1** gave methyl 2,3,4-tri-*O*-(*p*-bromobenzoyl)-α-D-xylopyranoside (CD: λmax/min 240/253 nm, Δε +6.2/−0.7) and methyl 3,4-di-*O*-(*p*-bromobenzoyl)-2-*O*-methyl-α-D-xylopyranoside (CD: λmax/min 239/256 nm, Δε +15.3/−32.0).[13]

The neuritogenic activity of **1** and **2** was evaluated with PC12 cells. Figure 5 shows the time course of the neuronal differentiation (neurite outgrowth) induced in PC12 cells by various concentrations of **1** and **2** in comparison with an active control, NGF (10 ng/mL). Each of them showed a gradually increasing curve, while the activity of NGF rapidly increased within 2 days and then no more change was observed. Linckoside B (**2**) showed the maximum activity of 76% at 40 μM 6 days after the treatment, which was higher than the activity (45%) of NGF at a concentration of 10 ng/mL, whereas **1** showed a lower activity (25%) than that

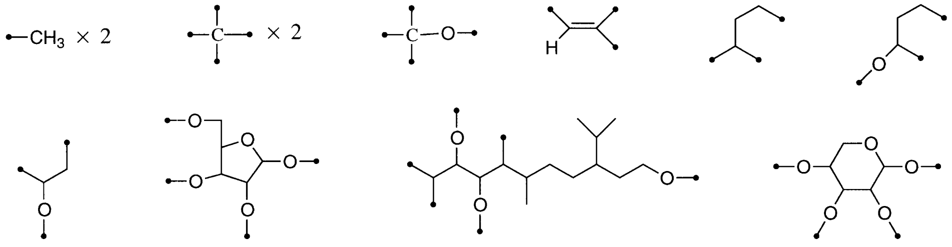

Figure 2. Partial structures for linckoside A (**1**).

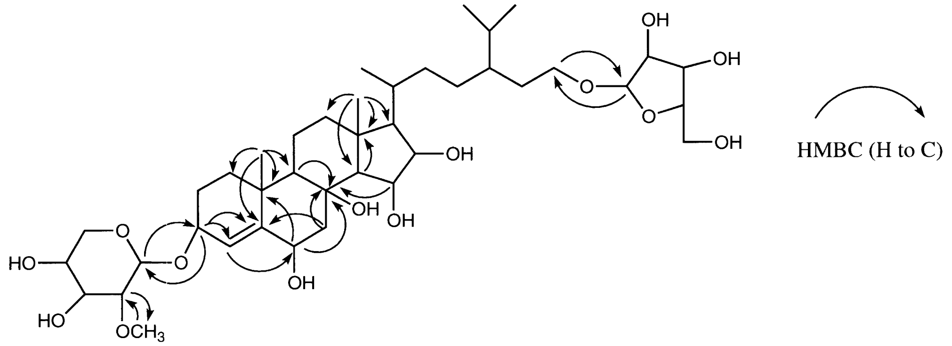

Figure 3. Selected long-range correlations observed in an HMBC spectrum of linckoside A (**1**).

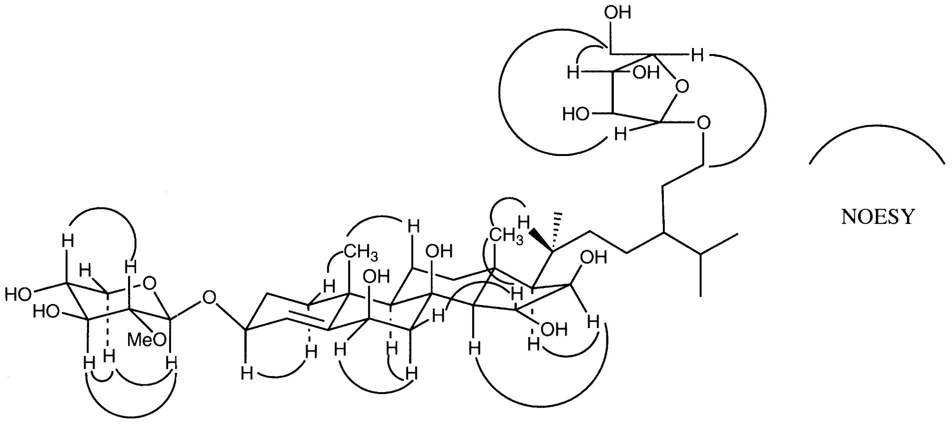

Figure 4. Selected NOESY correlations observed in an NOESY spectrum of linckoside A (**1**).

Table 2. Selected ^1H NMR data[a] for dearabinosyl linckoside A (**3**) in comparison with desulfated echinasteroside B[11,12]

Position	Dearabinosyl linckoside A (**3**)[b]	Desulfated echinasteroside B
3	4.18 m	4.23 m
4	5.63 br s	5.67 br s
6	4.30 t (3)	4.34 t (3)
7b	2.57 dd (15, 2.7)	2.60 dd (15, 3)
7a	1.49 dd (14.8, 3)	1.52 dd (15, 3)
15	4.15 dd (10.8, 2.4)	4.18 dd (11, 2.5)
16	3.97 dd (7.8, 2.4)	4.00 dd (7.5, 2.5)
18	1.13 s	1.16 s
19	1.36 s	1.40 s
21	0.93 d (6.8)	0.96 d (6.8)
26	0.84 d (6.8)	0.87 d (6.8)
27	0.87 d (6.9)	0.90 d (6.8)
29	3.61 m	3.65 ABq

[a] All the chemical shifts of **3** are a little smaller than those of desulfated echinasteroside B by the fixed range of 0.03–0.05 ppm, indicating the difference is simply due to the chemical shift calibration.

[b] 600 MHz, coupling constants (*J*, Hz) are in parentheses.

of **2** at the same concentration and time. Both compounds induced the neuronal differentiation of PC12 cells at the minimum concentration of 1.6 μM. The control (1% DMSO) did not induce any neuronal differentiation.

Interestingly, **1** and **2** exhibited significant synergistic effects on the NGF-induced neuronal differentiation of PC12 cells. In the presence of linckosides, the activity of NGF was notably increased (Fig. 6). For example, a 5% neuronal differentiation induced by NGF (2.5 ng/mL) was enhanced to 62% and 87% in the presence of **1** and **2** (40 μM), respectively, which alone induced only 15 and 27% activity, respectively, 3 days after the treatment. The synergistic effect of **1** on NGF ranged from 8 to 40 μM, while **2** showed a wider range (1.6–40 μM) than that of **1**. It is fascinating that **1** and **2** showed quite different activities despite their structural similarity, suggesting that the sugar moiety at C-29 of the aglycon plays an important role for the activity of these steroid glycosides.

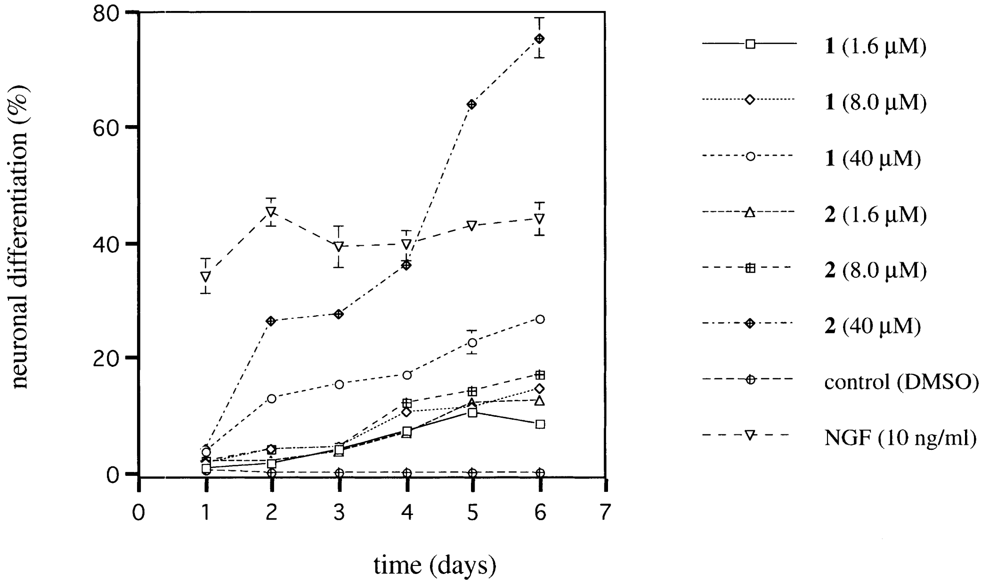

Figure 5. Time course of neuronal differentiation induced in PC12 cells by various concentrations of linckosides A (**1**) and B (**2**) in comparison with NGF as a positive control. The percentages of the cells with longer processes than the diameter of the cell body were plotted as an index of the neuronal differentiation.

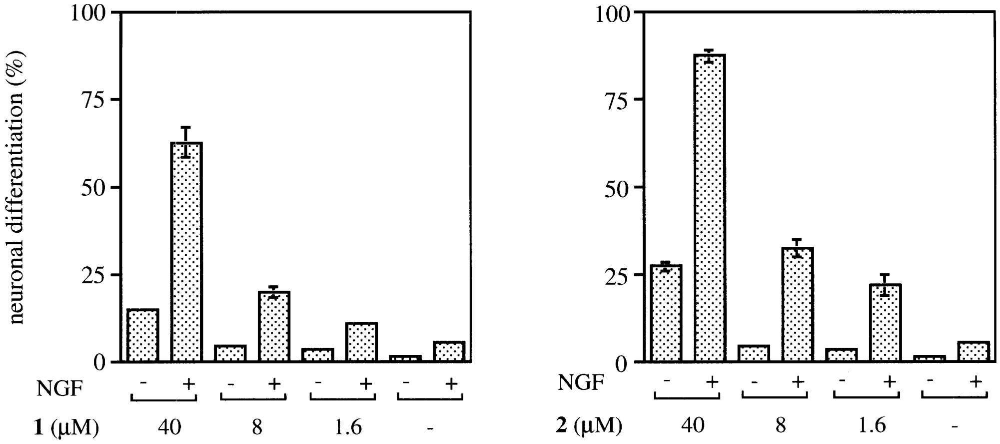

Figure 6. Synergistic effects of linckosides A (**1**) and B (**2**) on the NGF (2.5 ng/mL)-induced neuronal differentiation of PC12 cells, 3 days after the treatment.

Experimental

General procedures

Preparative HPLC was performed using JASCO PU-980 pumps. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. IR spectra were recorded on a JASCO FT/IR-7000S. FABMS (positive) spectra were recorded on a JEOL Mstation JMS-700 mass spectrometer using *m*-nitrobenzyl alcohol as the matrix. High-resolution FABMS spectra were obtained by using a mixture of glycerol/thioglycerol/*m*-nitrobenzyl alcohol (3:1:1) + NaI as the matrix and PEG 1000 as the calibration standard in the positive mode. NMR spectra were recorded on a Bruker AMX-600 or a Bruker ARX-400 spectrometer. NMR chemical shifts in δ (ppm) were referenced to the solvent peak of δ_C 49.0, δ_H 3.30 for CD_3OD and δ_H 7.26 for $CDCl_3$.

Extraction and isolation

The starfish *L. laevigata* (LINNE) were collected in Okinawa in October 1999, Japan. The freeze-dried sample (dry wt: 123 g) was ground and extracted with EtOH/MeOH (4:1; 900 mL). The supernatant was separated by filtration and concentrated to give 3.76 g of an extract, which was dissolved in 90% aqueous MeOH (40 mL) and then washed with hexane (40 mL, 2×20 mL). The concentrated aqueous methanolic fraction was dissolved in H_2O (20 mL) and then extracted with EtOAc (3×20 mL). The aqueous layer was freeze-dried to give 1.95 g of a dried aqueous fraction, which was chromatographed on ODS (Cosmosil 75 C18-OPN, 38 g, Nacalai Tesque) eluted with MeOH/H_2O (1:9, 2:8, 1:1, 8:2, 9:1), MeOH, and then MeOH/$CHCl_3$ (1:1) to afford six fractions. The active fourth fraction (37.0 mg), which was eluted with MeOH/H_2O (8:2), was subjected to HPLC [Develosil ODS-10 (ϕ 20/250 mm), Nomura chemical, flow rate: 8 mL/min, MeOH/H_2O (7:3)] to yield pure linckoside A (**1**) (5.9 mg, $t_R = 113$ min) and linckoside B (**2**) (3.2 mg, $t_R = 140$ min) as active components.

Linckoside A (**1**): a colorless powder, $[\alpha]_D^{25} -35$ ($c = 0.37$, MeOH), IR (KBr) 3420 cm^{-1}. HRFABMS *m*/*z* 795.4469, calcd for $C_{40}H_{68}O_{14}Na$ (M + Na) 795.4507; for ^1H and ^{13}C NMR see Table 1.

Linckoside B (**2**): a colorless powder, $[\alpha]_D^{25} -27$ ($c = 0.17$, MeOH), IR (KBr) 3419 cm^{-1}. HRFABMS *m*/*z* 795.4504, calcd for $C_{40}H_{68}O_{14}Na$ (M + Na) 795.4507; for ^1H and ^{13}C NMR see Table 1.

Partial hydrolysis of 1 to give 3

A solution of linckoside A (**1**, 2.0 mg) in 50% aqueous acetic acid (1 mL) was stirred at 60 °C for 45 h. The reaction mixture was evaporated and the residue dissolved in H_2O (0.5 mL) was passed through an ODS cartridge (TOYOPAK ODS-M, TOSOH, Japan), which was washed with H_2O (3 mL) and then eluated with 100% MeOH (3 mL). The MeOH fraction was concentrated and separated by TLC (silica gel, MeOH/$CHCl_3$ = 1:9, developed 3 times) to give five fractions. The third fraction ($R_f = 0.46$) containing dearabinosyl linckoside A (**3**) was purified by TLC (silica gel, MeOH/$CHCl_3$ = 15:85, developed twice, $R_f = 0.39$) to give **3** (0.4 mg): FABMS *m*/*z* 663 $(M + Na)^+$, ^1H NMR (CD_3OD, 600 MHz) δ 5.63 (1H, s, H-4), 4.41 (1H, d, *J* = 7.6 Hz, H-1′), 4.30 (1H, t, *J* = 3.0 Hz, H-6), 4.18 (1H, m, H-3), 4.15 (1H, dd, *J* = 10.8, 2.4 Hz, H-15), 3.97 (1H, dd, *J* = 7.8, 2.4 Hz, H-16), 3.81 (1H, m, H-5′b), 3.65 (2H, m, H-29), 3.57 (3H, s, 2′-OMe), 3.46 (1H, m, H-4′), 3.31 (1H, m, H-3′), 3.16 (1H, m, H-5′a), 2.81 (1H, dd, *J* = 8.8, 7.7 Hz, H-2′), 2.57 (1H, dd, *J* = 15, 2.7 Hz, H-7b), 2.12 (1H, m, H-28b), 1.98 (1H, m, H-2b), 1.97–1.04 (5H, m, H-9, H-11a,b, H-12a,b), 1.84 (1H, m, H-20), 1.79 (1H, m, H-1b), 1.76 (1H, m, H-2a), 1.75 (1H, m, H-25), 1.58 (1H, m, H-28a), 1.49 (1H, dd, *J* = 14.8, 3, H-7a), 1.36 (1H, m, H-23b), 1.35 (3H, s, H-19), 1.31 (1H, m, H-24), 1.28 (1H, m, H-1a), 1.21 (1H, m, H-17), 1.19 (1H, m, H-23a), 1.12 (3H, s, H-18), 1.04 (2H, m, H-22), 1.01 (1H, m, H-14), 0.93 (3H, d, *J* = 6.5 Hz, H-21), 0.86 (3H, d, *J* = 6.8 Hz, H-27), 0.84 (3H, d, *J* = 6.8 Hz, H-26).

Sugar derivatives from 1

The sugar-containing fractions described above were combined with **3** and other by-products, and the mixture in anhydrous 1.4 M HCl in MeOH (0.5 mL) was heated at 80 °C for 9 h in a sealed tube. After being cooled, the reaction mixture was neutralized with Ag_2CO_3 and filtrated, and the supernatant was evaporated to dryness under N_2. The residue was dissolved in H_2O (3 mL) and washed with ether/hexane (1:1; 3 mL, 3 times). The dried H_2O layer in dry pyridine (1 mL) was treated with *p*-bromobenzoyl chloride (30 mg) and 4-dimethylaminopyridine (DMAP, 1 mg). The mixture was stirred at 50 °C for 10 h under N_2, and then the reaction was quenched by adding H_2O (0.5 mL). After 10 min, the solution was evaporated and the residue was suspended in water and extracted with $CHCl_3$ (1.5 mL, 3 times). The combined $CHCl_3$ extracts were washed successively with saturated aqueous $NaHCO_3$ and H_2O. After evaporation of the solvent, the residue was separated by TLC (silica gel, Et_2O/hexane = 3:7) to afford three fractions. The second ($R_f = 0.25$) and third ($R_f = 0.12$) fractions were combined and further purified by TLC (silica gel, Et_2O/hexane = 3:7, developed 6 times) to give two major compounds.

Methyl 2,3,4-tri-*O*-(*p*-bromobenzoyl)-β-L-arabinopyranoside: $R_f = 0.6$; ^1H NMR ($CDCl_3$, 600 MHz) δ 7.94, 7.83, 7.66, 7.63, 7.53, 7.43 (each 2H, d, *J* = 8.5 Hz, Ar-H), 5.88 (1H, dd, *J* = 10.5, 3.5 Hz, 3-H), 5.72 (1H, br s, H-4), 5.64 (1H, dd, *J* = 10.5, 3.5 Hz, 2-H), 5.19 (1H, d, *J* = 3.5 Hz, 1-H), 4.16 and 3.93 (each 1H, br d, *J* = 13.1 Hz, 5-H), 3.47 (3H, s, OCH_3); CD ($CHCl_3$) λmax/min 240/256 nm (Δε−36/ + 84).

Methyl 3,4-di-*O*-(*p*-bromobenzoyl)-2-*O*-methyl-α-D-xylopyranoside: $R_f = 0.42$; ^1H NMR ($CDCl_3$, 600 MHz) δ 7.84–7.51 (8H, m, Ar-H), 5.82 (1H, t, *J* = 10.4 Hz, 3-H), 5.22 (1H, ddd, *J* = 10.4, 10.4, 5.9 Hz, H-4), 4.95 (1H, d, *J* = 3.4 Hz, 1-H), 3.97 (1H, dd, *J* = 10.4, 5.9 Hz, 5-Heq), 3.73 (1H, t, *J* = 10.4 Hz, 5-Hax), 3.55 (1H, dd, *J* = 10.4, 3.4 Hz, H-2), 3.50 and 3.45 (each 3H, s, OCH_3); CD ($CHCl_3$) λmax/min 239/256 nm (Δε +21/−41).

Sugar derivatives from 2

The linckoside B (**2**, 2.0 mg) was subjected to acidic methanolysis (1.4 M HCl in MeOH) followed by benzoylation in the same conditions as those for **1**. The benzoate mixture was purified by TLC (silica gel, Et_2O/hexane = 3:7) to give two major compounds.

Methyl 2,3,4-tri-*O*-(*p*-bromobenzoyl)-α-D-xylopyranoside: $R_f = 0.43$, ^1H NMR ($CDCl_3$, 600 MHz) δ 7.98–7.46 (12H, m, Ar-H), 6.06 (1H, t, $J = 10.5$ Hz, 3-H), 5.38 (1H, ddd, $J = 10.5$, 10.5, 5.9 Hz, 4-H), 5.22 (1H, dd, $J = 10.5$, 3.5 Hz, 2-H), 5.12 (1H, d, $J = 3.5$ Hz, 1-H), 4.06 (1H, dd, $J = 10.5$, 5.9 Hz, 5-Heq), 3.82 (1H, t, $J = 10.5$ Hz, 5-Hax), 3.46 (3H, s, OCH_3); CD ($CHCl_3$) λmax/min 240/253 nm (Δε +6.2/−0.7).

Methyl 3,4-di-*O*-(*p*-bromobenzoyl)-2-*O*-methyl-α-D-xylopyranoside: $R_f = 0.14$, CD ($CHCl_3$) λmax/min 239/256 nm (Δε +15.3/−32.0).

Bioassay methods

Neuronal differentiation activity was evaluated according to the methods described in our previous paper.[7] Briefly, 20,000 of PC12 cells in 1 mL of MEME medium were placed in each well of a 24-well microplate and precultured under a humidified atmosphere of 5% CO_2 at 37 °C. Twenty-four hours later, the medium was replaced by 1 mL of serum-free MEME medium containing 1% DMSO and a test sample at various concentrations. In the case of evaluation of the synergistic effects, the medium was replaced by 1 mL of serum-free MEME medium containing 2.5 ng NGF and a test sample. The morphological changes of the cells were monitored by a phase-contrast microscope at every 24 h through 6 days. About 100 cells were counted from a randomly chosen field and this was repeated 3 times.

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