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Linckosides C–E, three new neuritogenic steroid glycosides from the Okinawan starfish *Linckia laevigata*

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Abstract—Three new steroid glycosides, linckosides C–E, were isolated from the Okinawan starfish *Linckia laevigata*. Their structures and partial stereochemistry were elucidated by spectroscopic methods and chemical derivatization. These metabolites are additional members of the linckosides that were previously discovered as a novel class of neuritogenic compounds. Each of them possesses two monosaccharide units at C-3 of a polyhydroxylated steroidal aglycon and at the side chain (C-28 or C-29). Linckosides C and D are the first steroids that possess a hydroxyisopropyl substituent at C-24 of the side chain. These compounds are not only potent inducers of neurite outgrowth on PC12 cells but also significant enhancers of nerve growth factor (NGF) to induce the neurite outgrowth. The structure–activity relationships within the linckosides revealed that the presence of xylopyranose at the side chain was important rather than arabinofuranose, but that the diversity of the side chain carbon skeleton was not.

1. Introduction

Nerve growth factor (NGF) is the first and best characterized neurotrophic factor, and essential for the neuronal differentiation, growth, survival, function maintenance, and prevention of aging in the central and peripheral systems.[1–5] Although NGF has been considered as a drug candidate for the prevention and treatment of neurodegenerative diseases, its chemical property limits the medical usage of NGF. Therefore, exogenous low-molecular-weight compounds that mimic or enhance the neuritogenic activity of NGF might be developed as promising therapeutic drugs to treat neurodegenerative diseases such as Alzheimer disease.[6] The PC12 cell line derived from the rat pheochromocytoma cells has been used as an in vitro assay system for screening such substances by researchers because it expresses neuronal properties in response to NGF.[7]

In our previous search for such substances from natural sources, we had found six novel cerebrosides, termitomycesphins A–F, from an edible Chinese mushroom as mimics of the NGF activity.[8,9] Subsequently, two steroid glycosides, linckosides A (**1**) and B (**2**), had been isolated from the Okinawan blue starfish *Linckia laevigata* as mimics and synergists of the NGF activity.[10] A further examination of the starfish extract has led us to the purification of three congeners named linckosides C (**3**), D (**4**), and E (**5**) (Fig. 1). In this paper, we wish to report the isolation, structures and biological activities of these new bioactive compounds.

2. Results and discussion

2.1. Isolation

The starfish *L. laevigata* (911 g, wet wt) collected in Okinawa, Japan, was dried and extracted with MeOH. The MeOH extract was defatted and then partitioned between EtOAc and water. The aqueous layer was subjected to ODS column chromatography followed by repeated reversed-phase HPLC to yield linckosides C (**3**, 0.0005%) and D (**4**, 0.0006%). An examination of an HPLC fraction obtained in the previous work using another starfish (285 g, wet wt)[10] afforded an additional congener linckoside E (**5**, 0.0008%).

2.2. Structural elucidation

Linckoside C (**3**) possesses the molecular formula $C_{41}H_{70}O_{14}$ determined by an HR ESI-MS measurement.

Keywords: Steroid glycosides; NMR; Natural products; Structural identification; Linckosides.
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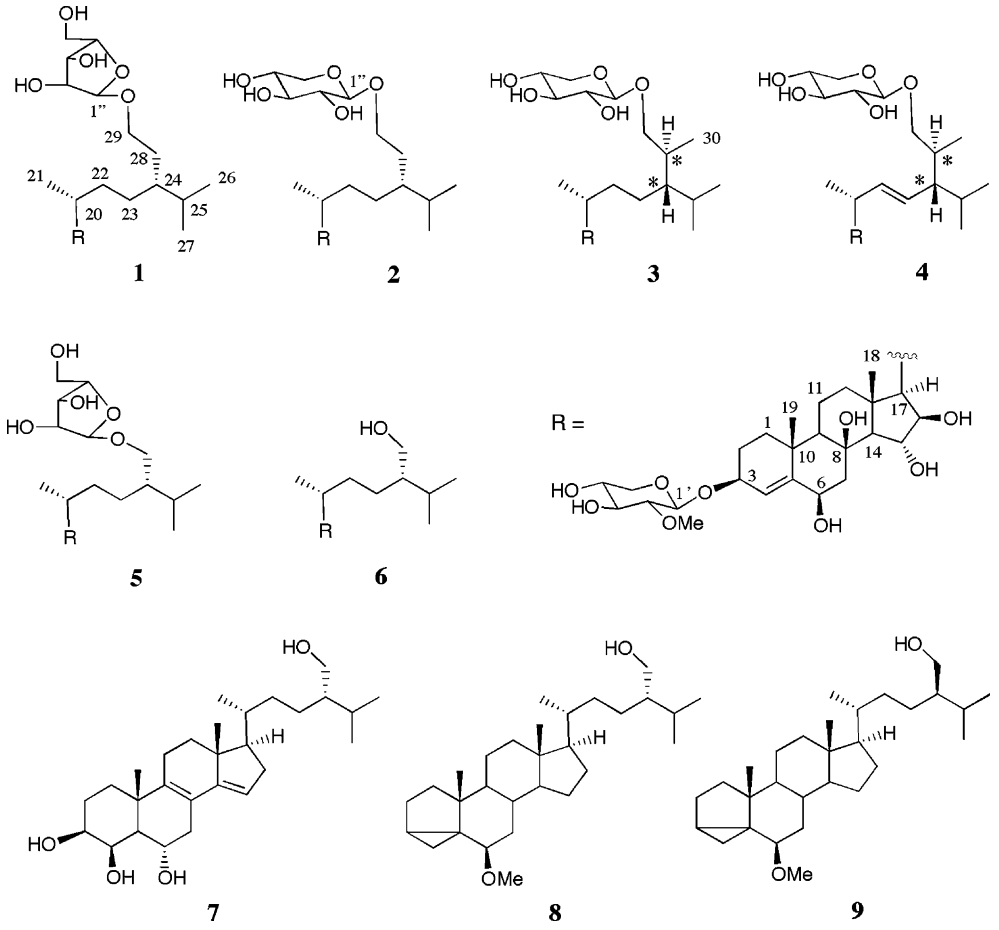

Figure 1. Structures of linckosides A–E (**1**–**5**) and some related steroidal compounds **6**–**9**. The asterisks at C-24 and C-28 of **3** and **4** indicate relative stereochemistry.

The IR absorption band at 3421 cm^{-1} suggested the presence of hydroxyl groups. The ^{1}H and ^{13}C NMR spectra of **3** as well as an HMQC experiment showed the presence of 10 oxymethines, 3 oxymethylenes, 1 oxygenated quaternary carbon, and 1 methoxyl group (δ_H 2.82–4.30, δ_C 61.1–84.9), two anomeric carbons (δ_H 4.41 and 4.17, δ_C 104.6 and 105.6, respectively), 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 six methyls, seven methines, and seven methylenes. The resonances due to anomeric protons and carbons and other many oxygenated functions suggested the presence of two sugar moieties. The analysis of DQFCOSY and HOHAHA spectra led to the determination of the partial structures depicted with the bold bonds in Figure 2. Although the DQFCOSY correlation between H-23 and H-24 was unclear, the HOHAHA spectrum confirmed their connectivity. These partial structures were connected by the long-range H–C correlations obtained by an HMBC experiment to give a gross structure of **3**. Important HMBC correlations are summarized in Figure 2 with arrows. The correlations from anomeric H-1′ to C-3 and anomeric H-1″ to C-29 revealed the location of the sugar moieties. The sugar connected to C-3 was determined as 2-*O*-methylxylopyranose by the NOE correlations of H-1′/H-3′, H-1′/H-5′a, H-2′/H-4′, and H-3′/H-5′a (Fig. 3), and by comparison of the ^{13}C NMR data with the reported data.[11] The β-configuration of this sugar unit was deduced by the coupling constant $J_{1'-2'} = 7.6$ Hz. Another sugar moiety linked to C-29 was determined as β-xylopyranose on the basis of the NOE correlations of H-1″/H-3″, H-1″/H-5″a and H-2″/H-4″ (Fig. 3), the coupling constant $J_{1''-2''} = 7.6$ Hz, and the reported ^{13}C NMR data.[11]

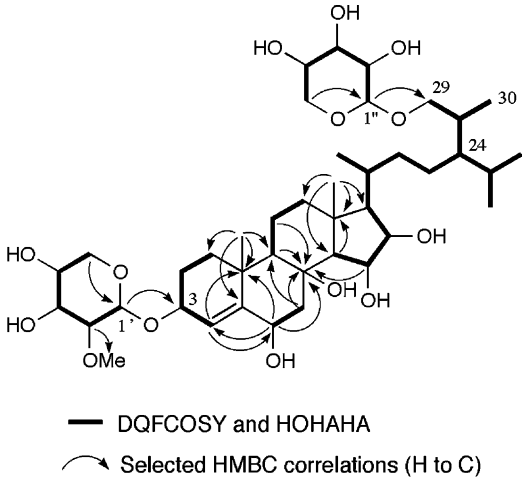

Figure 2. Gross structure of linckoside C (**3**) with 2D NMR correlations.

Table 1. ^{1}H and ^{13}C NMR data for linckosides C (**3**), D (**4**), and E (**5**) in CD_3OD[a]

Carbon	3		4		5	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1a	1.28 m	39.7	1.28 m	39.7	1.26 m	39.7
1b	1.79 m		1.79 m		1.78 m	
2a	1.75 m	27.9	1.75 m	27.9	1.75 m	27.9
2b	1.98 m		1.96 m		1.96 m	
3	4.18 m	77.5	4.18 m	77.5	4.16 m	77.5
4	5.63 s	126.9	5.64 s	126.9	5.63 s	126.9
5	—	148.6	—	148.6	—	148.6
6	4.30 dd (2.8, 2.6)	76.4	4.30 dd (3.6, 3.0)	76.4	4.30 dd (3.2, 2.9)	76.4
7a	1.51 dd (14.7, 2.6)	44.5	1.50 dd (14.2, 3.6)	44.4	1.47 dd (14.0, 3.2)	44.4
7b	2.58 dd (14.7, 2.8)		2.56 dd (14.2, 3.0)		2.56 dd (14.0, 2.9)	
8	—	76.2	—	76.2	—	76.2
9	1.04 m	57.9	1.04 m	57.9	1.02 m	57.9
10	—	37.7	—	37.8	—	37.7
11a	1.46 m	19.5	1.48 m	19.5	1.48 m	19.5
11b	1.88 m		1.87 m		1.86 m	
12a	1.18 m	43.0	1.21 m	43.0	1.17 m	43.0
12b	1.97 m		1.95 m		1.95 m	
13	—	45.1	—	45.1	—	45.1
14	1.01 d (10.6)	63.7	1.02 d (10.5)	63.8	1.03 d (10.8)	63.7
15	4.14 dd (10.6, 2.4)	81.1	4.16 dd (10.5, 2.5)	80.4	4.15 dd (10.8, 2.0)	81.0
16	3.99 dd (7.8, 2.4)	82.9	3.93 dd (7.0, 2.5)	83.5	3.97 dd (7.9, 2.0)	82.9
17	1.21 dd (10.0, 7.8)	60.4	1.26 dd (12.0, 7.0)	61.0	1.21 m	60.4
18	1.12 s	16.8	1.17 s	17.0	1.12 s	16.8
19	1.37 s	22.7	1.37 s	22.7	1.36 s	22.7
20	1.84 m	31.4	2.55 m	34.9	1.81 m	31.1
21	0.93 d (6.7)	18.6	1.03 d (6.7)	20.8	0.93 d (6.8)	18.6
22a	1.07 m	37.5	5.41 dd (15.4, 8.1)	140.7	1.04 m	34.8
22b	1.58 m				1.60 m	
23a	1.09 m	25.4	5.26 dd (15.4, 9.9)	129.0	1.18 m	25.9
23b	1.41 m				1.46 m	
24	1.09 m	48.1	1.59 ddd (9.9, 7.5, 5.2)	54.1	1.35 m	46.0
25	1.80 m	29.7	1.82 m	29.3	1.80 m	29.6
26	0.85 d (6.8)	19.2	0.79 d (6.8)	18.4	0.89 d (6.8)	20.0
27	0.91 d (6.8)	22.1	0.88 d (6.7)	22.3	0.90 d (6.8)	20.1
28a	1.86 m	37.3	1.84 m	36.2	3.32 m	70.1
28b	—		—		3.72 dd (9.1, 5.0)	
29a	3.28 m	75.1	3.23 dd (9.0, 7.0)	74.6	—	—
29b	3.89 dd (8.2, 3.2)		3.87 dd (9.0, 3.8)		—	—
30	0.98 d (6.7)	16.6	0.98 d (6.7)	16.9	—	—
1′	4.41 d (7.6)	104.6	4.41 d (7.6)	104.6	4.41 d (7.6)	104.6
2′	2.82 dd (8.9, 7.6)	84.9	2.82 dd (9.0, 7.6)	84.9	2.82 dd (8.8, 7.6)	84.9
3′	3.29 m	77.5	3.31 m	77.5	3.31 m	77.5
4′	3.47 m	71.3	3.46 m	71.3	3.46 m	71.3
5′a	3.14 dd (11.2, 10.5)	66.8	3.14 m	66.8	3.15 dd (11.2, 10.4)	66.8
5′b	3.80 dd (11.2, 5.0)		3.81 m		3.80 dd (11.2, 4.5)	
2′-OMe	3.57 s	61.1	3.57 s	61.1	3.57 s	61.1
1″	4.17 d (7.6)	105.6	4.15 d (7.5)	105.6	4.83 d (1.8)	109.7
2″	3.14 m	75.0	3.17 m	75.0	3.94 dd (3.9, 1.8)	83.7
3″	3.29 m	77.9	3.29 m	77.9	3.82 m	78.8
4″	3.47 m	71.3	3.46 m	71.3	3.91 m	85.2
5″a	3.18 m	66.9	3.18 m	67.0	3.62 dd (11.7, 5.2)	63.1
5″b	3.84 m		3.84 m		3.73 dd (11.7, 2.9)	

[a] 600 MHz for ^{1}H and 150 MHz for ^{13}C, coupling constants (J in Hz) are in parentheses.

To determine the absolute stereochemistry of the sugar moieties of **3**, methanolysis (HCl, MeOH) followed by benzoylation (*p*-Br-C_6H_4COCl and 4-dimethylaminopyridine) was performed to give di- and tribenzoyl sugar derivatives. The D-configuration of both sugar moieties were established by CD measurements of the products: methyl 2,3,4-tri-*O*-(*p*-bromobenzoyl)-α-D-xylopyranoside (CD: $\lambda_{max/min}$ 241/251 nm, $\Delta\varepsilon + 8.2/-1.5$) and methyl 3,4-di-*O*-(*p*-bromobenzoyl)-2-*O*-methyl-α-D-xylopyranoside (CD: $\lambda_{max/min}$ 241/256 nm, $\Delta\varepsilon + 13.4/-35.0$).[12] The *R*-configuration at C-20 was determined on the basis of the NOE correlations of H-18/H-20 and H-18/H-21 (Fig. 3), and the large coupling constant $J_{17-20} = 10.0$ Hz that suggested the anti-relationship between H-17 and H-20. Since linckoside C (**3**) is a dihydro derivative of linckoside D (**4**) isolated from

the same animal, the relative stereochemistry at C-24 and C-28 is supposed to be the same as that of **4** (see below).

Linckoside D (**4**) possesses the molecular formula $C_{41}H_{68}O_{14}$ determined by an HR ESI-MS measurement. The ^{1}H and ^{13}C NMR data for **4** were almost superimposable on those of **3** (Table 1) except for the signals around the positions C-22 and C-23 of the side chain, which was substituted by a double bond in **4** (Fig. 1). The double bond at C-22–C-23 was confirmed by the DQFCOSY correlations, and the *trans*-geometry was determined from the coupling constant $J_{22-23} = 15.4$ Hz. The β-configuration of both xylopyranoses was determined from the coupling constants $J_{1'-2'} = 7.6$ Hz and $J_{1''-2''} = 7.5$ Hz. Moreover, the configuration of the sugar moieties was determined by derivatization of **4** and CD measurements in a similar manner to the case of **3**. Thus, methanolysis of **4** followed by benzoylation gave the two sugar derivatives same as those obtained from **3**. The 20*R*-configuration was assigned on the basis of the NOE correlations of H-18/H-20 and H-18/H-21 (Fig. 4), and the coupling constant $J_{17-20} = 12.0$ Hz. Furthermore, the relative stereochemistry of 24*R**, 28*S** was demonstrated by the NOE correlations and coupling constants shown in Figure 4.

Linckoside E (**5**) possesses the molecular formula $C_{39}H_{66}O_{14}$ determined by an HR ESI-MS measurement. The ^{1}H and ^{13}C NMR data (Table 1) for **5** were quite similar to those for linckoside A (**1**) except for the lack of methylene signals due to C-28 of **1**. The location of two sugar moieties was determined by the HMBC correlations from H-1″ to C-3 and from H-1″ to C-28. The nature of the sugar connected to C-3 was determined as 2-*O*-methyl-β-D-xylopyranose based on the NMR signals due to this sugar and the neighborhood of the steroidal core, which was superimposable on those for other linckosides. Another sugar moiety linked to C-28 was determined as α-arabinofuranose on the basis of the DQFCOSY, the NOE correlations of H-1″/H-3″, H-1″/H-5″a, and H-3″/H-5″a, the coupling constant $J_{1''-2''} = 1.8$ Hz, and the ^{13}C NMR data for the reported data.[11] To determine the absolute configuration at C-24, **5** was converted to dearabinosyl linckoside E (**6**) by selective hydrolysis. The 24*S*-configuration of **5** was thus demonstrated by comparison of the selected ^{1}H NMR data for **6** with the corresponding data for some related steroidal compounds, **7**[13], **8**,[14] and **9**[14] (Table 2, Fig. 1). The absolute configuration of arabinofuranose of **5** should be the same as that of linckoside A (**1**) isolated from the same starfish, though not confirmed by the chemical means due to small amount of the sample.

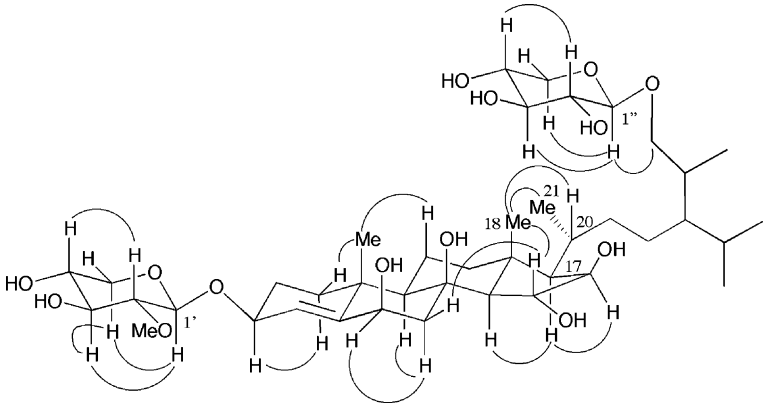

Figure 3. Selected NOE correlations determined by an NOESY experiment of linckoside C (**3**).

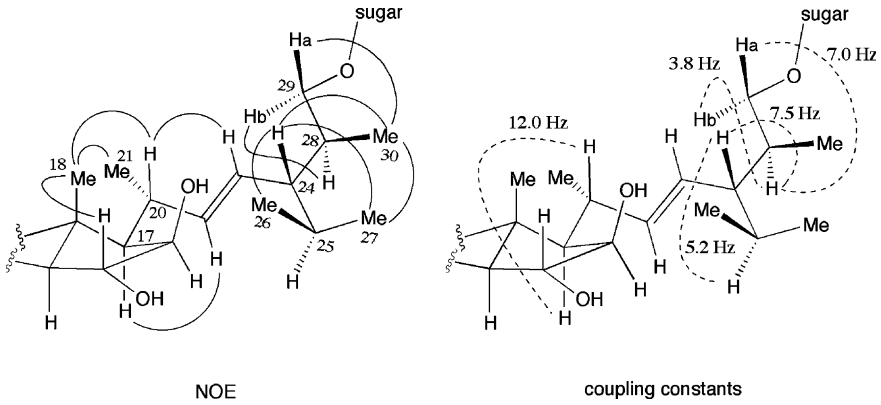

Figure 4. Partial structures of linckoside D (**4**) with selected NOE correlations and coupling constants.

Table 2. Selected ^{1}H NMR data for **6**, **7**, **8**, and **9**[a]

Position	**6**[b]	**7**[13] (24*S*)	**8**[14] (24*S*)	**9**[14] (24*R*)
H-26	0.93 d (6.6)	0.92 d (7.0)	0.92 d (6.5)	0.91 d (6.5)
H-27	0.93 d (6.6)	0.93 d (7.0)	0.93 d (6.5)	0.94 d (6.5)
H-28a	3.50 dd (11.0, 5.0)	3.46 dd (10.0, 6.5)	3.47 dd (10.0, 6.5)	3.52 br d (5.0)
H-28b	3.57 dd (11.0, 5.0)	3.56 dd (10.0, 6.5)	3.56 dd (10.0, 6.5)	3.52 br d (5.0)

[a] Measured in CD_3OD at 600 MHz. Coupling constants (J in Hz) are in parentheses.
[b] The chemical shifts were exceptionally referenced to the solvent peak at δ_H 3.34 to compare with the data in Refs. 13 and 14.

2.3. Biological activity

The neuritogenic activity of linckosides C–D (**3**–**5**) was evaluated using PC12 cells in comparison with NGF and the previously isolated congeners **1** and **2**. Figure 5 shows the time-dependent increase of the percentage of the cells with obvious neurite outgrowth induced on PC12 cells. The concentrations were set to 25 or 12.5 μM that were the maximum concentrations without cytotoxicity. The linckosides showed a gradually increasing activity, while the activity of the positive control NGF (10 ng/mL) rapidly increased within 3 days and then reached the plateau. The solvent control did not induce any neurite outgrowth. Linckosides B (**2**), C (**3**), and D (**4**) that possess a xylose at the side chain showed significant neuritogenic activities of 62%, 56%, and 73%, respectively, on day 6 after the treatment. These activities were comparable with that of NGF. On the other hand, linckosides A (**1**) and E (**5**) that have an arabinose at the side chain exhibited lower activities (33% and 29%, respectively) than the others. This result indicates that the nature of the sugar moiety at the side chain plays an important role for the neuritogenic activity. However, other structural factors, for example, the distance of the sugar from the branching point C-24, the presence or absence of the methyl branch at C-28 and the double bond at C-22, seemed not to be important.

All the linckosides significantly enhanced NGF to induce neurite outgrowth of PC12 cells (Fig. 6). On day 3 after the treatment, each linckoside alone induced only

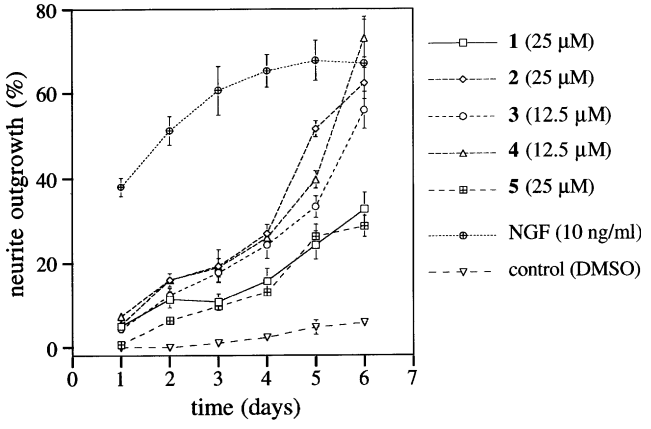

Figure 5. Time course of neuritogenic activity of linckosides A–E (**1**–**5**) in comparison with NGF as a positive control. The activity was represented by the percentages of the PC12 cells with a longer neurite outgrowth than the cell diameter. All linckosides showed cytotoxicity at a higher concentration than the indicated ones.

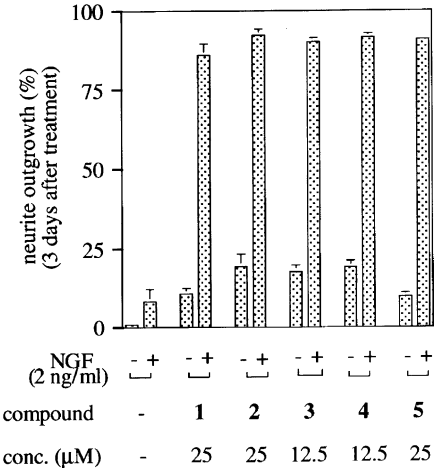

Figure 6. Neuritogenic activities of NGF at a low concentration, linckosides A–E (**1**–**5**) at the indicated concentrations, and the mixtures of them. Synergistic effects were observed in the coexistence of the NGF and a linckoside.

10–20% neurite outgrowth at the concentration of 25 or 12.5 μM. NGF at a low concentration of 2 ng/mL induced only 8% neurite outgrowth. However, this low activity of NGF was enhanced up to 86–92% in the coexistence of linckosides A–E (**1**–**5**). Thus, the structural difference within the linckosides had little effects on such synergistic phenomenon between NGF and linckosides. The NGF-like neuritogenic activity of the linckosides depends on the structural diversity of these steroid glycosides, whereas the enhancement of the neuritogenic activity of NGF seems to be a common characteristic of the linckosides.

3. Experimental

3.1. General procedures

Preparative HPLC was performed on an HPLC system equipped with JASCO PU-980 intelligent HPLC pumps and a JASCO UV-970 intelligent UV/VIS detector. Thin layer chromatography (TLC) was conducted with silica gel 60 F_{254} plates (Merck). Optical rotations were measured on a JASCO DIP-370 digital polarimeter. IR spectra were recorded on a JASCO FT/IR-7000S. HR ESI-TOF-MS were recorded on an Applied Biosystems Mariner Biospectrometry Workstation using polypropylene glycol and PEG 600 as calibration standards in the negative and positive mode, respectively. NMR spectra

were recorded on a Bruker AMX2-600 spectrometer. NMR chemical shifts in δ (ppm) were referenced to the solvent peaks of δ_C 49.0 and δ_H 3.30 for CD_3OD.

3.2. Extraction and isolation

The starfish *L. laevigata* (911 g, wet wt) was collected off Akajima Island in Okinawa, Japan. The freeze-dried sample (400 g) was powdered and soaked in MeOH (3000 mL) for 5 days at room temperature. The supernatant was separated by filtration and concentrated to give 25.9 g of a crude extract, which was dissolved in 90% aqueous MeOH (300 mL) and then washed twice with hexane (150 mL). The concentrated aqueous methanolic fraction was dissolved in H_2O (300 mL) and then washed three times with EtOAc (150 mL). The aqueous layer was freeze-dried to give 20.6 g of a powder, which was chromatographed on ODS (Cosmosil 75 C18-OPN, 100 g, Nacalai Tesque) eluted with MeOH–H_2O (5:5, 6:4, 7:3, 8:2, and 10:0) to afford six fractions. The third fraction (40.8 mg) eluted with MeOH–H_2O (8:2) was subjected to HPLC [TSKgel ODS-120T (ϕ 20×250 mm), flow rate: 8 mL/min, 77% aq MeOH] to give two fractions containing steroid glycosides (3.0 and 3.5 mg, t_R = 53.5–57.5 and 59.0–65.5 min, respectively). These fractions were individually purified by HPLC [Develosil ODS-HG-5 (ϕ 10×250 mm), Nomura chemical, flow rate: 2 mL/min, 43% aq MeCN and 41% aq MeCN, respectively] to yield linckosides C (**3**) (2.0 mg, t_R = 29.0 min) and D (**4**) (2.2 mg, t_R = 48.4 min), respectively.

On the other hand, a fraction (14.2 mg, t_R = 81.0–108.5 min) eluted prior to **1** and **2** in a preparative HPLC separation, which was reported in the previous paper using another *L. laevigata* (285 g, wet wt),[10] was further subjected to HPLC [TSKgel ODS-120T (ϕ 20×250 mm), 60–90% aq MeOH in 90 min linear gradient] to yield linckoside E (**5**) (1.0 mg, t_R = 65.0 min).

3.2.1. Linckoside C (3). Colorless powder, $[\alpha]_D^{25}$ −30 (*c* 0.10, MeOH), IR (KBr) 3421, 2950, 1650, 1056, 880 cm^{-1}; HR ESI-TOF-MS *m/z* 831.4737 $(M+COOH)^-$, calcd for $C_{42}H_{71}O_{16}$ 831.4742; for ^1H and ^{13}C NMR see Table 1.

3.2.2. Linckoside D (4). Colorless powder, $[\alpha]_D^{25}$ −43 (*c* 0.12, MeOH), IR (KBr) 3422, 2930, 1647, 1058, 982, 881 cm^{-1}; HR ESI-TOF-MS *m/z* 829.4580 $(M+COOH)^-$, calcd for $C_{42}H_{69}O_{16}$ 829.4586; for ^1H and ^{13}C NMR see Table 1.

3.2.3. Linckoside E (5). Colorless powder, $[\alpha]_D^{25}$ −34 (*c* 0.06, MeOH), IR (KBr) 3417, 2948, 1650, 1057, 882 cm^{-1}; HR ESI-TOF-MS *m/z* 803.4424 $(M+COOH)^-$, calcd for $C_{40}H_{67}O_{16}$ 803.4429; for ^1H and ^{13}C NMR see Table 1.

3.3. Sugar derivatives from 3

Linckoside C (**3**, 0.7 mg) in an anhydrous methanolic HCl solution (1.4 M, 0.4 mL) was heated at 80 °C in a sealed tube for 12 h. After being cooled down, 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 3 times with ether–hexane (1:1) (3 mL). The aqueous layer was freeze-dried, dissolved in dry pyridine (1 mL), and then treated with *p*-bromobenzoyl chloride (32 mg) and 4-dimethylaminopyridine (1 mg). The mixture was stirred at 50 °C for 12 h under N_2, and then the reaction was quenched by adding H_2O (0.5 mL). After being stirred for 5 min, the solution was evaporated and the residue was suspended in water and extracted 3 times with $CHCl_3$ (1.5 mL). The combined $CHCl_3$ extracts were washed successively with saturated aqueous $NaHCO_3$ and H_2O. After evaporation of the solvent, the benzoate mixture was purified by preparative TLC [silica gel, Et_2O–hexane (3:7)] to give methyl 2,3,4-tri-*O*-(*p*-bromobenzoyl)-α-D-xylopyranoside (0.2 mg, R_f = 0.38) and a mixture (R_f = 0.12). The latter was further purified by TLC [silica gel, Et_2O–hexane (4:6), developed 3 times] to give methyl 3,4-di-*O*-(*p*-bromobenzoyl)-2-*O*-methyl-α-D-xylopyranoside (0.3 mg, R_f = 0.44).

Methyl 2,3,4-tri-*O*-(*p*-bromobenzoyl)-α-D-xylopyranoside:[10] CD ($CHCl_3$) $\lambda_{max/min}$ 241/251 nm ($\Delta\varepsilon$ + 8.2/ − 1.5).

Methyl 3,4-di-*O*-(*p*-bromobenzoyl)-2-*O*-methyl-α-D-xylopyranoside:[10] CD ($CHCl_3$) $\lambda_{max/min}$ 241/256 nm ($\Delta\varepsilon$ + 13.4/ − 35.0).

3.4. Sugar derivatives from 4

The linckoside D (**4**, 0.7 mg) was subjected to acidic methanolysis (1.4 M HCl in MeOH) followed by benzoylation under the same conditions as already mentioned. The benzoate mixture was purified by TLC [silica gel, Et_2O–hexane (3:7), developed 3 times] to give the following two sugar derivatives.

Methyl 2,3,4-tri-*O*-(*p*-bromobenzoyl)-α-D-xylopyranoside: R_f = 0.63, CD ($CHCl_3$) $\lambda_{max/min}$ 240/252 nm ($\Delta\varepsilon$ + 4.9/ − 1.6).

Methyl 3,4-di-*O*-(*p*-bromobenzoyl)-2-*O*-methyl-α-D-xylopyranoside: R_f = 0.26, CD ($CHCl_3$) $\lambda_{max/min}$ 240/256 nm ($\Delta\varepsilon$ + 15.9/ − 37.0).

3.5. Partial hydrolysis of linckoside E (5) to give 6

A solution of **5** (0.5 mg) in 50% aqueous acetic acid (1 mL) was stirred at 60 °C for 24 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 eluted with 100% MeOH (3 mL). The MeOH fraction dissolved in MeOH (1 mL)

was treated with potassium carbonate (5 mg) overnight at room temperature. The reaction mixture was neutralized with acetic acid (2 μL) and dried up. The residue was separated by TLC (silica gel, $CHCl_3$/MeOH = 5:1, developed twice, $R_f = 0.27$) to give **6** (0.3 mg) as a colorless powder: HR ESI-TOF-MS m/z 649.3912 $(M+Na)^+$, calcd for $C_{34}H_{58}O_{10}Na$ 649.3922; ^1H NMR (CD_3OD, 600 MHz) δ 3.53 (1H, dd, $J = 11.0$, 5.0 Hz, H-28b), 3.46 (1H, dd, $J = 11.0$, 5.0 Hz, H-28a), 1.83 (1H, m, H-25), 1.82 (1H, m, H-20), 1.63 (1H, m, H-22b), 1.45 (1H, m, H-23b), 1.22 (1H, m, H-24), 1.20 (1H, m, H-23a), 1.04 (1H, m, H-22a), 0.94 (3H, d, $J = 7.2$ Hz, H-21), 0.89 (6H, d, $J = 6.6$ Hz, H-26, 27), the other data were identical with those for **5** (see Table 1).

3.6. Bioassay methods

Biological activity was evaluated according to the methods described in our previous paper.[8,10] Briefly, twenty-thousand of PC12 cells in MEME medium (1 mL) 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. In the case for evaluation of the NGF-enhancement effects, the medium was replaced by 1 mL of serum-free MEME medium containing 2.0 ng of NGF (Recombinant Human β-NGF, R&D Systems) in addition to a test sample. The morphological changes of the cells were monitored under a phase-contrast microscope at every 24 h through 6 days. About one hundred cells were counted from a randomly chosen area and this operation was repeated 3 times.

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