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Bioorganic & Medicinal Chemistry 14 (2006) 4458–4465

Bioorganic & Medicinal Chemistry

Structure–activity relationships of novel neuritogenic steroid glycosides from the Okinawan starfish *Linckia laevigata*

Chunguang Han, Jianhua Qi and Makoto Ojika*

Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan

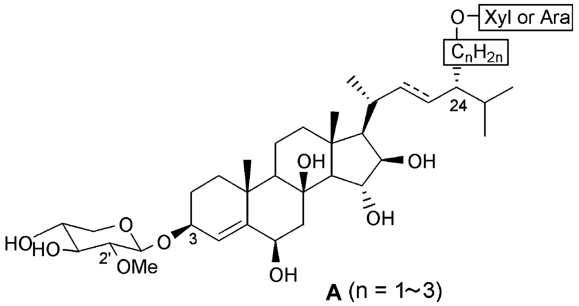
Received 12 December 2005; revised 15 February 2006; accepted 16 February 2006
Available online 9 March 2006

Abstract—Six new steroid glycosides, linckosides F–K, and a related metabolite were isolated from the Okinawan blue starfish *Linckia laevigata* as mimics or enhancers of nerve growth factor (NGF). Their structures and stereochemistry were elucidated by spectroscopic methods and chemical derivatization. Structure–activity relationships suggest that both a carbon branch modified by a pentose at the side chain and 2′-*O*-methylxylopyranose at C-3 of the aglycon are important for neuritogenic activity.

1. Introduction

Nerve growth factor (NGF) is a neurotrophic factor essential for neuronal differentiation, growth, survival, function maintenance, and prevention of aging in the central and peripheral systems.[1–4] Recently, administration of NGF resulted in recovery of learning and memory functions in an Alzheimer mouse model exhibiting NGF-deficiency.[5] Although NGF is considered a drug candidate for the prevention and treatment of neurodegenerative diseases, its chemical properties have limited its usage as a medicine. Therefore, exogenous low-molecular-weight compounds that mimic or enhance the neuritogenic activity of NGF are promising therapeutic drugs for the treatment of neurodegenerative diseases such as Alzheimer's disease.[6] The PC12 cells, derived from rat pheochromocytoma cells, has been used as an in vitro assay system for screening therapeutic substances because the cell system expresses neuronal properties in response to NGF.[7]

In previous studies of the isolation of similar substances from natural sources, we identified five novel steroid glycosides, linckosides A–E, from the Okinawan blue starfish *Linckia laevigata*, which act as mimics and enhancers of NGF activity.[8,9] The common structure of the linckosides is a polyhydroxylated steroid that is glycosylated with 2′-*O*-methyl-β-D-xylose at the position C-3 (structure **A**). The most unusual structural feature of these steroid glycosides is the presence of a carbon branch (C_1–C_3) modified with another pentose (xylose or arabinose) at the position C-24 of the side chain. Such type of steroid diglycosides is quite rare, and only one example, echinasteroside F,[10] was known until the discovery of the linckosides. Since the linckosides are the first members that mimic and enhance NGF among a numerous number of starfish steroid glycosides, such unique structures seem to be responsible for the neuritogenic activity. Our previous studies indeed indicate that the pentose at the side-chain branch should be an important factor for the biological activity: the xylosides (linckosides B–D) are twice as active as arabinosides (linckosides A and E). To confirm this and to reveal more detailed structure–bioactivity relationships, we carried out a large-scale extraction of the same starfish to obtain six additional congeners, linckosides F (**1**), G (**2**), H (**3**), I (**4**), J (**5**), K (**6**), and a known metabolite, echinasteroside C (**7**) (Fig. 1). Here, we report their isolation, structural elucidation, and detail structure–neuritogenic activity relationships.



A (n = 1∼3)

Keywords: Linckosides; Steroid glycosides; Marine natural products; Neuritogenic activity.

* Corresponding author. Tel./fax: +81 52 789 4284; e-mail: ojika@agr.nagoya-u.ac.jp

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doi:10.1016/j.bmc.2006.02.032

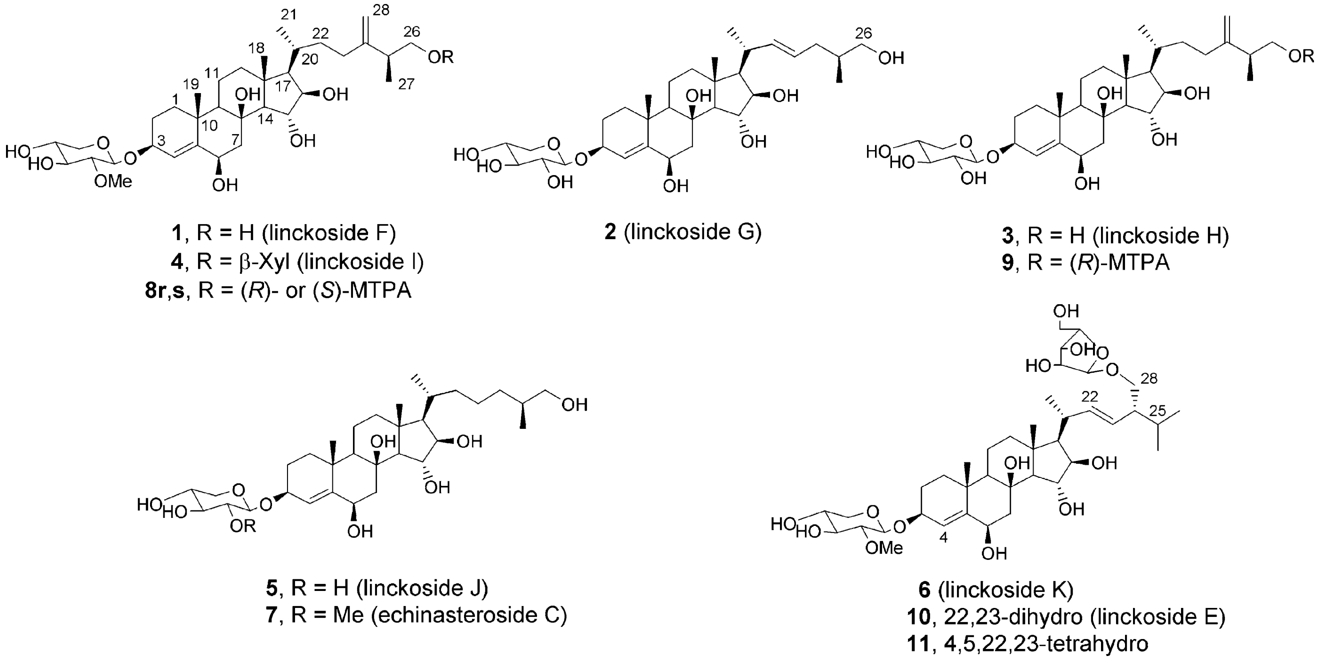

Figure 1. Structures of linckosides F–K (**1**–**6**) and related compounds.

2. Results and discussion

2.1. Isolation

The starfish *L. laevigata* (7.7 kg, wet wt) collected in Okinawa, Japan, was dried and extracted with MeOH. The MeOH extract was partitioned between EtOAc and water. The aqueous layer was chromatographed on ODS, then on silica gel, followed by repeated reversed-phase HPLC to yield linckosides F (**1**, 0.001% based on wet weight of animals), G (**2**, 0.00001%), H (**3**, 0.0003%), I (**4**, 0.00008%), J (**5**, 0.0005%), K (**6**, 0.00003%), and echinasteroside C (**7**, 0.001%) as well as the previously isolated compounds, linckosides A (0.002%),[8] B (0.001%),[8] and E (**10**, 0.0003%).[9]

2.2. Structural elucidation

Linckoside F (**1**) possessed the molecular formula $C_{34}H_{56}O_{10}$, determined by HR ESI-TOF-MS measurements. The IR spectrum of **1** showed a strong signal centered at 3417 cm^{-1} due to hydroxyl groups. The ^{1}H NMR, ^{13}C NMR, and DEPT spectra of **1** revealed the presence of seven oxymethines, two oxymethylenes, one oxygenated quaternary carbon, and one methoxyl group (CH_nO: δ_H 2.82–4.41, δ_C 61.1–84.9), one acetal methine (δ_H 4.41, δ_C 104.6), a trisubstituted double bond (δ_H 5.64, δ_C 126.9 and 148.5), a 1,1-disubstituted double bond (δ_H 4.74 and 4.81, δ_C 154.0 and 109.2), and two quaternary sp^3 carbons (δ_C 37.7 and 45.1) (Tables 1 and 2). The remaining proton and carbon signals were assigned to four methyls, five methines, and seven methylenes. The resonances due to acetal and several oxygen functionalities suggest the presence of one sugar moiety. DQFCOSY analysis led to the determination of the partial structures depicted in Figure 2. The connections between these partial structures were determined by long-range C–H correlations via an HMBC experiment to give **1**. The HMBC correlations are summarized in Figure 2. The correlation from anomeric H-1′ to C-3 indicates the sugar moiety is connected to C-3 of the steroidal aglycon. The sugar was determined to be 2′-*O*-methylxylopyranose by NOESY correlations of H-1′/H-3′, H-1′/H-5′a, H-2′/H-4′, and H-3′/H-5′a (Fig. 3), by large vicinal *J* values (~10 Hz), and by comparison of ^{13}C NMR spectra with reported data.[8] The β-configuration of this sugar unit was deduced by the coupling constant $J_{1'-2'} = 7.8$ Hz. The stereostructure of the steroidal core was established by NOESY correlations, shown in Figure 3. The 20*R*-configuration was assigned on the basis of NOE correlations of H-18/H-20 and H-18/H-21 and the large coupling constant $J_{17-20} = 10.8$ Hz. Next, an extension of Mosher's method was applied for determining the configuration at C-25 on the side chain.[11] Linckoside F (**1**) was converted to the (*R*)- and (*S*)-MTPA esters **8r** and **8s**. The chemical shift difference between two 26-methylene protons in the ^{1}H NMR spectrum of the (*R*)-MTPA ester **8r** was 0.13 ppm (δ 4.33 – 4.20), while the difference in the ^{1}H NMR spectrum of the (*S*)-MTPA ester **8s** was the larger value of 0.21 ppm (δ 4.37 – 4.16). These results empirically indicated a 25*S*-configuration for **1**.

Linckoside G (**2**) possessed the molecular formula $C_{32}H_{52}O_{10}$, determined by HR ESI-TOF-MS measurements. The IR spectrum of **2** showed a strong absorption centered at 3417 cm^{-1} due to hydroxyl groups. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) for **2** were similar to those of **1** except for a double bond on the side chain and the lack of a 2′-*O*-methyl group in xylose. The double bond at C-22–C-23 was confirmed by DQF-COSY correlations, and the *E*-configuration was determined from the coupling constant $J_{22-23} = 15.3$ Hz.

Table 1. ^{1}H data for linckosides F–K (**1**–**6**) in CD$_3$OD at 600 MHz[a]

Proton	**1**	**2**	**3**	**4**	**5**	**6**
1	1.28 m, 1.80 m	1.27 m, 1.80 m	1.28 m, 1.79 m	1.28 m, 1.80 m	1.27 m, 1.78 m	1.28 m, 1.78 m
2	1.76 m, 1.99 m	1.77 m, 1.96 m	1.77 m, 1.97 m	1.74 m, 1.97 m	1.74 m, 1.96 m	1.74 m, 1.96 m
3	4.18 m	4.19 m	4.19 m	4.18 m	4.19 m	4.18 m
4	5.64 s	5.67 s	5.67 s	5.64 s	5.67 s	5.64 s
6	4.30 br s	4.30 br s	4.30 br s	4.30 br s	4.30 br s	4.31 br s
7a	1.49 dd (14.4, 3.0)	1.49 dd (14.4, 3.0)	1.49 dd (14.7, 3.0)	1.50 dd (14.4, 3.0)	1.49 dd (14.7, 3.0)	1.49 dd (14.4, 3.0)
7b	2.57 dd (14.4, 2.4)	2.56 dd (14.4, 2.4)	2.57 dd (14.7, 2.4)	2.57 dd (14.4, 2.4)	2.57 dd (14.7, 2.4)	2.58 dd (14.4, 2.4)
9	1.03 m	1.03 m	1.02 m	1.03 m	1.01 m	1.04 m
11	1.48 m, 1.89 m	1.50 m, 1.88 m	1.48 m, 1.88 m	1.49 m, 1.87 m	1.47 m, 1.84 m	1.50 m, 1.87 br q (13.2)
12	1.18 m, 1.97 m	1.21 m, 1.96 m	1.17 m, 1.97 m	1.19 m, 1.97 m	1.18 m, 1.97 m	1.21 m, 1.93 br d (12.6)
14	1.01 d (10.5)	1.01 m	1.02 d (10.5)	1.02 d (10.8)	1.00 d (10.8)	1.03 m
15	4.15 dd (10.5, 2.4)	4.15 dd (10.5, 2.4)	4.15 dd (10.5, 2.4)	4.15 dd (10.5, 2.4)	4.15 dd (10.8, 2.4)	4.16 dd (11.1, 2.4)
16	3.98 dd (7.8, 2.4)	3.88 dd (7.5, 2.4)	3.98 dd (7.5, 2.4)	3.98 dd (7.5, 2.4)	3.96 dd (7.5, 2.4)	3.93 dd (7.2, 2.4)
17	1.22 dd (10.8, 7.8)	1.24 dd (10.8, 7.2)	1.22 dd (10.8, 7.5)	1.22 dd (11.1, 7.5)	1.20 m	1.26 dd (10.8, 7.2)
18	1.13 s	1.15 s	1.13 s	1.13 s	1.12 s	1.16 s
19	1.36 s	1.36 s	1.35 s	1.36 s	1.35 s	1.37 s
20	1.88 m	2.51 m	1.87 m	1.87 m	1.86 m	2.53 m
21	0.95 d (6.6)	1.02 d (7.2)	0.95 d (6.6)	0.95 d (6.6)	0.92 d (6.6)	1.04 d (6.6)
22	1.21 m, 1.73 m	5.50 dd (15.3, 6.6)	1.21 m, 1.73 m	1.19 m, 1.70 m	1.01 m, 1.56 m	5.47 dd (15.0, 9.0)
23	1.99 m, 2.12 m	5.48 dd (15.3, 7.2)	1.99 m, 2.12 m	1.98 m, 2.14 m	1.21 m, 1.47 m	5.34 dd (15.0, 9.6)
24	—	1.84 m, 2.10 m	—	—	1.03 m, 1.41 m	2.05 m
25	2.29 m	1.62 m	2.28 m	2.54 m	1.56 m	1.64 m
26a	3.35 dd (10.8, 7.2)	3.34 dd (10.8, 6.0)	3.35 dd (10.2, 7.2)	3.32 m[b]	3.31 m[b]	0.85 d (6.6)
26b	3.56 dd (10.8, 6.0)	3.40 dd (10.5, 6.0)	3.56 dd (10.5, 6.0)	3.84 dd (9.6, 5.4)	3.41 dd (10.8, 6.0)	
27	1.05 d (7.2)	0.88 d (6.6)	1.05 d (6.6)	1.09 d (6.6)	0.90 d (6.6)	0.90 d (6.6)
28	4.74 s, 4.81 s	—	4.74 s, 4.81 s	4.76 s, 4.80 s	—	3.32 m[b], 3.80 m
1′	4.41 d (7.8)	4.36 d (7.8)	4.36 d (7.8)	4.41 d (7.2)	4.36 d (7.2)	4.41 d (7.2)
2′	2.82 dd (9.0, 7.8)	3.14 dd (9.0, 7.8)	3.14 dd (8.7, 7.8)	2.82 dd (9.0, 7.8)	3.14 dd (9.0, 7.8)	2.82 dd (9.0, 7.8)
3′	3.29 m[b]	3.29 m[b]	3.29 m[b]	3.30 m[b]	3.30 m[b]	3.31 m[b]
4′	3.47 ddd (10.2, 9.0, 5.4)	3.47 ddd (10.2, 9.0, 5.4)	3.47 ddd (10.5, 9.0, 5.4)	3.47 ddd (10.2, 9.0, 5.4)	3.47 ddd (10.2, 9.0 5.4)	3.47 ddd (10.2, 9.0, 5.4)
5′a	3.15 dd (11.4, 10.2)	3.19 dd (11.4, 10.2)	3.19 dd (11.4, 10.5)	3.16 m	3.19 dd (11.4, 10.2)	3.16 dd (11.4, 10.2)
5′b	3.80 dd (11.4, 5.4)	3.83 dd (11.4, 5.4)	3.83 dd (11.4, 5.4)	3.80 dd (11.4, 5.4)	3.83 dd (11.4, 5.4)	3.80 dd (11.4, 5.4)
OMe	3.57 s			3.57 s		3.57 s
1″				4.19 d (7.8)		4.81 m[b]
2″				3.15 m		4.01 dd (4.2, 2.4)
3″				3.30 m[b]		3.82 m
4″				3.47 ddd (10.2, 9.0, 5.4)		3.91 m
5″a				3.17 m		3.61 dd (11.7, 5.4)
5″b				3.84 dd (11.4, 5.4)		3.74 dd (11.7, 3.0)

[a] Coupling constants (*J* in hertz) are in parentheses.

[b] Signals overlap the solvent signal.

Table 2. ^{13}C NMR data for linckosides F-K (**1-6**) in CD_3OD at 150 MHz

Carbon	**1**	**2**	**3**	**4**	**5**	**6**
1	39.7	39.7	39.7	39.7	39.7	39.7
2	27.9	27.7	27.7	27.9	27.7	27.9
3	77.5	77.1	77.1	77.5	77.1	77.5
4	126.9	126.9	127.0	126.9	127.0	127.0
5	148.5	148.3	148.5	148.6	148.3	148.6
6	76.4	76.3	76.3	76.4	76.4	76.4
7	44.4	44.4	44.4	44.4	44.4	44.4
8	76.1	76.2	76.2	76.2	76.2	76.1
9	57.8	57.8	57.8	57.8	57.8	57.9
10	37.7	37.7	37.7	37.7	37.7	37.8
11	19.5	19.5	19.5	19.5	19.5	19.5
12	43.0	43.0	43.0	43.0	43.0	43.0
13	45.1	45.1	45.1	45.1	45.1	44.9
14	63.7	63.6	63.7	63.7	63.7	63.0
15	80.9	80.3	80.9	81.0	80.9	79.8
16	82.8	83.3	82.8	82.8	82.9	83.2
17	60.5	61.0	60.5	60.5	60.7	60.8
18	16.8	16.9	16.8	16.8	16.8	17.0
19	22.7	22.6	22.6	22.7	22.6	22.7
20	30.6	34.5	30.6	30.6	30.7	35.7
21	18.4	20.5	18.4	18.4	18.5	21.1
22	35.5	139.5	35.5	35.5	37.2	141.0
23	32.9	127.7	32.9	32.8	24.8	130.3
24	154.0	37.9	154.0	154.1	34.9	51.0
25	43.4	37.3	43.4	41.1	37.0	30.1
26	67.5	68.1	67.5	75.4	68.4	19.8
27	17.2	17.2	17.2	17.7	17.3	21.3
28	109.2		109.2	109.3		71.5
1′	104.6	104.4	104.3	104.6	104.4	104.6
2′	84.9	75.1	75.0	84.9	75.1	84.9
3′	77.5	78.0	78.0	77.5	78.0	77.5
4′	71.2	71.2	71.2	71.2	71.2	71.3
5′	66.8	66.9	67.0	66.9	67.0	66.8
OMe	61.1			61.2		61.1
1″				105.5		109.8
2″				74.9		83.2
3″				77.8		78.3
4″				71.2		84.9
5″				66.8		63.0

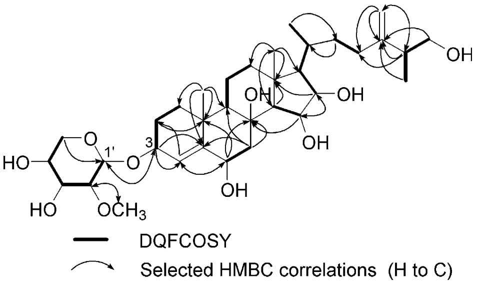

Figure 2. Gross structure of linckoside F (**1**) with 2D NMR correlations.

The β-configuration of xylopyranose was determined from the coupling constant $J_{1'-2'} = 7.8$ Hz. The stereochemistry of the steroidal core and C-20 was assigned on the basis of ^{1}H NMR data and NOE experiments (data not shown). The 25*S*-configuration was suggested from the chemical shift difference $\Delta\delta_{26a-26b} = 0.06$; the differences were 0.05–0.07 ppm for 25*S* and 0.09 ppm for 25*R*.[12]

Linckoside H (**3**) possessed a molecular formula of $C_{33}H_{54}O_{10}$, determined by HR ESI-TOF-MS measurements. The ^{1}H and ^{13}C NMR data (Table 1 and 2) for **3** were almost superimposable on those of **1** except for the lack of 2′-*O*-methyl signals in the β-xylopyranose moiety. The location of the xylose was determined by HMBC correlations from H-1′ to C-3. Sugar type and β-configuration were determined on the basis of NOE correlations (H-1′/H-5′a, H-2′/H-4′, H-3′/H-5′a, and H-4′/H-5′b) and the coupling constant $J_{1'-2'} = 7.8$ Hz. The 20*R*-configuration was determined on the basis of NMR data and NOE experiments. The *S*-configuration at C-25 was confirmed by analogy with **1**. Thus, the (*R*)-MTPA ester **9** was prepared from **3**. The chemical shift difference between the two protons of the 26-methylene group was 0.13 ppm (δ: 4.33 – 4.20), indicating a 25*S*-configuration.[11]

Linckoside I (**4**) possessed the molecular formula of $C_{39}H_{64}O_{14}$, as determined by HR ESI-TOF-MS measurements. The ^{1}H and ^{13}C NMR data of **4** were almost superimposable on those of **1** (Tables 1 and 2), except for the signals due to an additional sugar. The location of this sugar moiety was determined by HMBC correlations from the anomeric H-1″ to C-26 and from H-26 to C-1″.

The sugar moiety linked to C-26 was determined to be β-xylopyranose on the basis of NOE correlations (H-1″/H-5″a, H-2″/H-4″, and H-3″/H-5″a) and the coupling constant $J_{1''-2''} = 7.8$ Hz. The *S*-configuration at C-25 was determined by the conversion to **1** by enzymatic hydrolysis with *exo*-1,4-β-D-xylosidase.[13]

Linckoside J (**5**) produced NMR spectra almost superimposable on those of a known starfish metabolite, echinasteroside C (**7**),[10] expect for the lack of a methyl ether group at the 2′-*O*-position in **7**. The molecular formula obtained by HR MS analysis supported this 2′-*O*-demethyl structure. Therefore, **5** was determined to be 2′-*O*-demethyl echinasteroside C. The related metabolite **7** was identified as echinasteroside C based on reported spectral data.[10]

Linckoside K (**6**) possessed the molecular formula $C_{39}H_{62}O_{14}$ (HR ESI-TOF-MS). The ^{1}H and ^{13}C NMR data (Tables 1 and 2) for **6** were similar to those of linckoside E (**10**)[9] except for the presence of an additional double bond on the side chain. The *E* double bond at C-22–C-23 was confirmed by the DQFCOSY correlations (H-21–H-22–H-23–H-24) and coupling constant $J_{22-23} = 15.3$ Hz. 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 sugar 2′-*O*-methyl-β-D-xylopyranose at C-3 was determined based on the NMR signals that were superimposable on those of the 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″ and H-3″/H-5″a, the coupling constant $J_{1''-2''} = 2.4$ Hz, and the ^{13}C NMR data from the reported data.[9] The stereochemistry of the steroidal moiety including C-20 and C-24 was assigned on the basis of

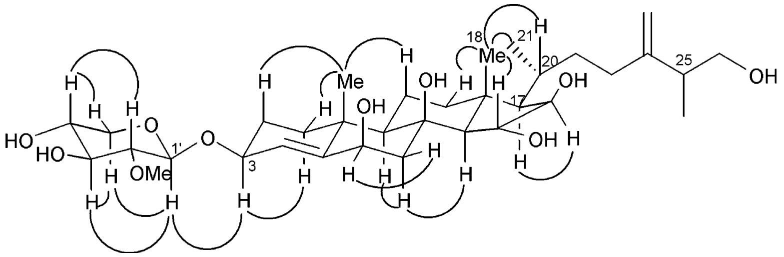

Figure 3. Important NOE correlations determined by NOESY experiments of linckoside F (**1**).

the superimposable ^{1}H NMR data for tetrahydro derivative **11** on those for linckoside E (**10**).[9]

2.3. Biological activity and structure–activity relationships

The discovery of a variety of steroid glycosides in this study made it possible to reveal more detailed structure–activity relationships as follows. The neuritogenic activity of linckosides F–K (**1**–**6**) and echinasteroside C (**7**) was evaluated using PC12 cells in comparison with NGF. Figure 4 shows the NGF-mimic activity represented by the percentage of the cells with obvious neurite outgrowth induced on PC12 cells six days after treatment at a concentration of 40 μM, which is approximately the maximum concentration that does not produce cytotoxicity. Linckoside K (**6**) showed the highest neuritogenic activity (50%) among the seven compounds **1**–**7**. The activity was comparable to that of NGF at 10 ng/mL. A photomicrograph indicates the induction of multipolar processes on PC12 cells by **6** (Fig. 5c) like NGF (Fig. 5b), while the control experiment did not express any neurite outgrowth (Fig. 5a). One of the most interesting structure–activity relationships is the importance of the carbon branch modified by a pentose at the side chain. Linckoside K (**6**) is an additional member of diglycosides of this type like the previously isolated linckosides A–E. This type of structures might be the best for the NGF-mimic activity. The importance of such a rare side-chain structure was supported on the basis of a little weaker activity (40%) of linckoside I (**4**) possessing a pentose migrated from the carbon branch to the isopropyl terminus, a weaker activity (30%) of linckoside F (**1**) lacking the pentose at the carbon branch, and a much weaker activity (18%) of echinasteroside C (**7**) lacking both the pentose and carbon branch (Fig. 6). Even a pentose at the side chain terminus seems to play a role for the activity in comparison between **4** and **1** like the relationship between **6** and **1**. Another interesting structure–activity relationship is the role of 2′-*O*-methyl ether group of xylose at C-3. Linckoside F (**1**) and echinasteroside C (**7**) possessing a 2′-*O*-methyl ether group are approximately three times as active as the corresponding non-methylated derivatives, linckosides H (**3**) and J (**5**), respectively (Fig. 6).

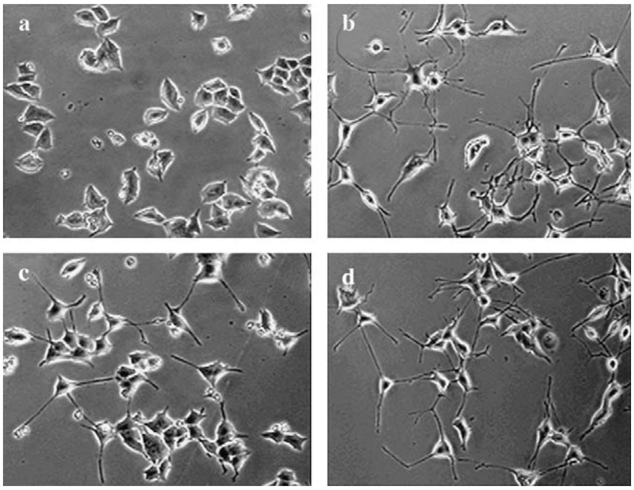

Figure 5. Phase-contrast photomicrographs of PC12 cell six days after treatment. (a) control (no sample), (b) NGF (10 ng/mL), (c) linckoside K (**6**, 40 μM), and (d) linckoside K (**6**, 40 μM) + NGF (1.5 ng/mL).

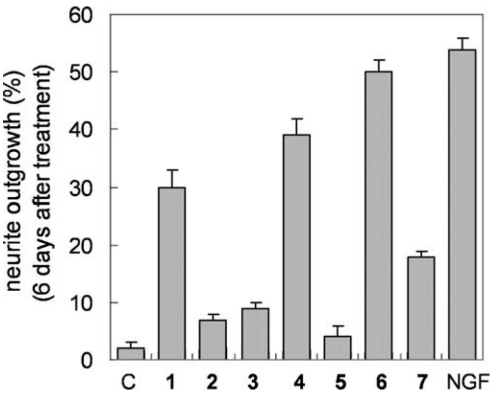

Figure 4. NGF-mimic activity of linckosides F–K (**1**–**6**) and echinasteroside C (**7**) at 40 μM six days after treatment. Activity is represented by the percentages of the PC12 cells with a neurite outgrowth longer than the cell diameter. C: control (1% DMSO). NGF (10 ng/mL): positive control.

All the linckosides significantly enhanced the neuritogenic activity of NGF against PC12 cells (Fig. 7). Three days after treatment, linckosides at a concentration of 40 μM induced only 0–15% neurite outgrowth; NGF at a concentration of 1.5 ng/mL induced only 6% neurite outgrowth. However, the low activity of NGF was increased to 40–98% by addition of a linckoside. This enhancement of NGF activity (ratio of NGF activity at 1.5 ng/mL with sample to that without sample) was approximately 15-, 7-, 8-, 16-, 8-, 17-, and 11-fold for **1**–**7**, respectively. Figure 5d indicates an obvious enhancement of the neuritogenic activity of a trace amount of NGF by linckoside K (**6**). The enhancement

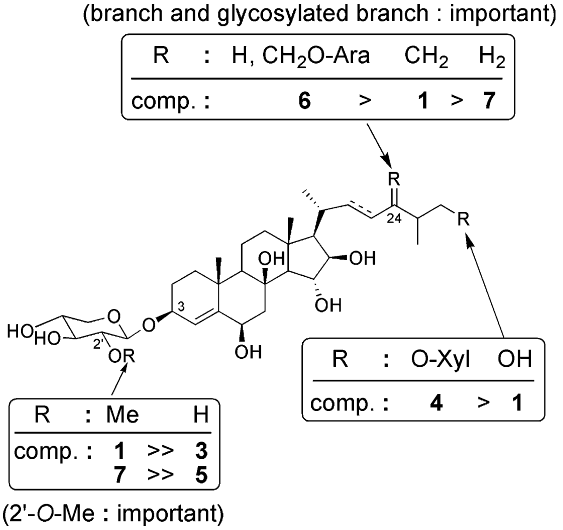

Figure 6. Summary of the structure–neuritogenic activity relationships of the linckosides.

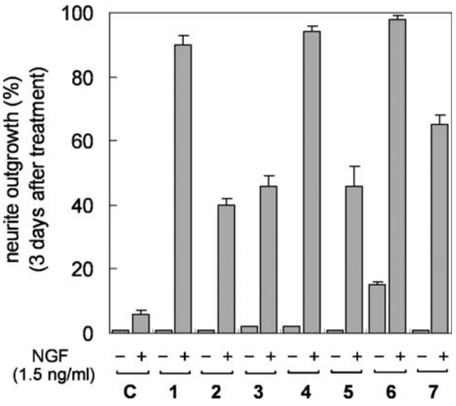

Figure 7. Enhancement of neuritogenic activity of NGF (1.5 ng/mL) by linckosides F–K (**1**–**6**) and echinasteroside C (**7**) at 40 μM.

effect of linckosides F (**1**), I (**4**), and K (**6**) is approximately twice higher than that of the other linckosides, suggesting that the 2′-*O*-methyl ether group of xylose at C-3 plays a role for the significant NGF-enhancing activity. The NGF-mimic activity of the linckosides appears to be sensitive to the structural diversity of the linckosides, whereas the NGF-enhancing activity 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-1586 and JASCO PU-2086 pumps, a JASCO UV-1570 UV/VIS, and a JASCO RI-2301 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 spectra were recorded on an Applied Biosystems Mariner Biospectrometry Workstation using PEG 600 as a calibration standard in the positive mode. NMR spectra were recorded on a Bruker AMX2-600 spectrometer (600 MHz for ^{1}H), and the chemical shifts in δ (parts per million) 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* (7.7 kg, wet wt) was collected in the waters of Akajima Island in Okinawa, Japan. A freeze-dried sample (2.9 kg) was chipped and immersed in MeOH (30 L) for five days with occasional shaking at room temperature. The supernatant was separated by filtration and concentrated to give 340.5 g of a crude extract, which was dissolved in water (1 L), and then adjusted to pH 9.0 with 1 N NaOH (400 mL) and washed twice with EtOAc (2 L). The aqueous layer, after neutralization with 1 N HCl (200 mL), was concentrated to 500 mL and then diluted with EtOH (1 L). The resulting precipitate was isolated and supernatant was concentrated. The product was suspended in 20% aq MeOH (1 L) and chromatographed on ODS (Cosmosil 140C_{18}-OPN, 550 g, Nacalai Tesque) eluted with MeOH–H_2O (5:5, 6:4, 7:3, 8:2, 9:1, and 10:0) to afford five fractions. The third fraction (3.2 g) eluted with MeOH–H_2O (7:3, 8:2, and 9:1) was re-chromatographed on silica gel (Wakogel® C-300, 100 g, Wako Pure Chemical) eluted with $CHCl_3$–MeOH (9:1, 8:1, 7:1, 6:1, 5:1, 4:1, and MeOH, 600 mL each) to afford six fractions. The active fourth fraction (833.4 mg) eluted with $CHCl_3$–MeOH (7:1 and 6:1) was separated by HPLC [TSK gel ODS-120T (∅ 20 × 250 mm), TOSOH, flow rate 8 mL/min, 70% aq MeOH, eight injections] to yield echinasteroside C (**7**, 108.3 mg, t_R = 50.4–54.8 min), linckoside F (**1**, 97.4 mg, t_R = 54.8–59.5 min), and an impure fraction (36.4 mg, t_R = 111.6–121.7 min). This impure fraction was purified by HPLC [Develosil ODS-5 (∅ 20 × 250 mm), Nomura Chemical, flow rate 8 mL/min, 42–52% aq MeCN, 40 min linear gradient] to yield linckoside K (**6**, 2.3 mg, t_R = 20.5–23.5 min). The active fifth fraction (680.7 mg) eluted with $CHCl_3$–MeOH (5:1 and 4:1) was separated by HPLC under the same conditions to give linckoside J (**5**, 45.1 mg, t_R = 38.1–41.6 min), linckoside H (**3**, 22.5 mg, t_R = 41.6–44.0 min), linckoside E (26.5 mg, t_R = 56.5–60.0 min), linckoside A (157.9 mg, t_R = 70.5–91.7 min), linckoside B (113.1 mg, t_R = 95.5–114.0 min), and two fractions containing linckosides [7.1 mg (t_R = 36.0–38.1 min) and 10.9 mg (t_R = 44.0–46.2 min)]. The two impure fractions were purified individually by HPLC [Develosil ODS-UG-5 (∅ 10 × 250 mm), flow rate 2 mL/min, 35% aq MeCN] to yield linckoside G (**2**) (1.0 mg, t_R = 30.9–36.1 min) and linckoside I (**4**) (5.8 mg, t_R = 29.5–40.0 min). Linckoside F (**1**): colorless powder, $[\alpha]_D^{24}$ −13 (*c* 0.045, MeOH), IR (KBr) 3417, 1641, 1059, 881, 839 cm^{-1}; HR ESI-TOF-MS *m*/*z* 647.3809 $(M+Na)^+$, calcd for $C_{34}H_{56}O_{10}Na$: 647.3766. Linckoside G (**2**): colorless powder, $[\alpha]_D^{24}$ −25 (*c* 0.091, MeOH), IR (KBr) 3417, 1632, 1045, 979, 880 cm^{-1}; HR ESI-TOF-MS *m*/*z* 619.3422 $(M+Na)^+$, calcd for $C_{32}H_{52}O_{10}Na$: 619.3452. Linckoside H (**3**): colorless powder, $[\alpha]_D^{24}$ −13 (*c* 0.11, MeOH), IR (KBr) 3418, 1640, 1044, 881 cm^{-1}; HR ESI-TOF-MS *m/z* 633.3632 (M+Na)$^+$, calcd for $C_{33}H_{54}O_{10}Na$: 633.3609. Linckoside I (**4**): colorless powder, $[\alpha]_D^{24}$ −21 (*c* 0.17, MeOH), IR (KBr) 3425, 1641, 1054, 882 cm^{-1}; HR ESI-TOF-MS *m/z* 779.4189 (M+Na)$^+$, calcd for $C_{39}H_{64}O_{14}Na$: 779.4188. Linckoside J (**5**): colorless powder, $[\alpha]_D^{24}$ −21 (*c* 0.32, MeOH), IR (KBr) 3417, 1640, 1044, 880 cm^{-1}; HR ESI-TOF-MS *m/z* 621.3637 (M+Na)$^+$, calcd for $C_{32}H_{54}O_{10}Na$: 621.3609. Linckoside K (**6**): colorless powder, $[\alpha]_D^{24}$ −33 (*c* 0.12, MeOH), IR (KBr) 3418, 1647, 1386, 1059, 882, 842 cm^{-1}; HR ESI-TOF-MS *m/z* 779.4196 (M+Na)$^+$, calcd for $C_{39}H_{64}O_{14}Na$: 779.4188. Echinasteroside C (**7**): $[\alpha]_D^{24}$ −12 (*c* 1.0, MeOH) (reported data: −15);[10] ESI-TOF-MS *m/z* 635 (M+Na)$^+$.

3.3. (*R*)-MTPA ester (8r) and (*S*)-MTPA ester (8s) from 1

Linckoside F (**1**, 1.5 mg, 2.4 μmol) was treated with (+)-(*S*)-methoxytrifluoromethylphenylacetyl (MTPA) chloride (3 μL, 16 μmol) in dry pyridine (0.2 mL) for 1 h at room temperature. Three drops of distilled water were then added to stop the reaction. The reaction mixture was evaporated and chromatographed on ODS (Cosmosil 75C$_{18}$-OPN, 0.6 g) eluted with MeOH–H_2O (8.5:1.5) to afford **1** (1.0 mg, 67%) and **8r** (0.6 mg, 40%): ^{1}H NMR (CD_3OD) δ 5.63 (1H, s, H-4), 4.74 and 4.81 (each 1H, s, H-28), 4.41 (1H, d, *J* = 7.6 Hz, H-1′), 4.20 and 4.33 (each 1H, dd, *J* = 10.8, 6.4 Hz, H-26), 4.30 (1H, br s, H-6), 4.18 (1H, m, H-3), 4.15 (1H, m, H-15), 3.96 (1H, dd, *J* = 8.0, 2.8 Hz, H-16), 3.80 (1H, dd, *J* = 11.6, 5.6 Hz, H-5′eq), 3.57 (3H, s, OMe), 3.46 (1H, m, H-4′), 3.30 (1H, m, H-3′), 3.16 (1H, dd, *J* = 10.8, 10.4 Hz, H-5′ax), 2.82 (1H, dd, *J* = 9.2, 7.6 Hz, H-2′), 2.57 (1H, dd, *J* = 11.4, 3.2 Hz, H-7eq), 2.55 (1H, m, H-25), 1.96 and 1.99 (each 1H, m, H-23), 1.77 and 1.99 (each 1H, m, H-2), 1.96 (1H, m, H-12eq), 1.88 (1H, m, H-11ax), 1.88 (1H, m, H-20), 1.28 and 1.81 (each 1H, m, H-1), 1.74 (1H, m, H-22), 1.49 (2H, m, H-7ax and H-11eq), 1.36 (3H, s, H-19), 1.12 (1H, m, H-17), 1.18 (2H, m, H-22 and H-12ax), 1.13 (3H, s, H-18), 1.06 (3H, d, *J* = 6.8 Hz, H-27), 1.03 (1H, m, H-9), 1.00 (1H, m, H-14), 0.92 (3H, d, *J* = 6.8 Hz, H-21). (*S*)-MTPA ester (**8s**) was prepared in 45% yield using (−)-(*R*)-MTPA chloride: ^{1}H NMR (CD_3OD, only signals different from those of **8r**) δ 2.57 (1H, m, H-25), 4.16 and 4.37 (each 1H, dd, *J* = 10.6, 6.4 Hz, H-26), 1.05 (3H, d, *J* = 7.2 Hz, H-27).

3.4. (*R*)-MTPA ester 9 from 3

Linckoside H (**3**, 1.5 mg, 2.45 μmol) was treated with (+)-(*S*)-MTPA chloride (3 μL, 16 μmol) in dry pyridine (0.2 mL) for 1 h at room temperature and followed by addition of three drops of distilled water to stop the reaction. The reaction mixture was evaporated and purified by HPLC [Develosil ODS-UG-5 (∅ 10 × 250 mm), flow rate 2 mL/min, 80–90% aq MeOH, 80 min, linear gradient] to give **3** (1.2 mg, 80%) and (*R*)-MTPA ester **9** (0.2 mg, 13% yield, t_R = 49.0–51.8 min): ^{1}H NMR (CD_3OD) δ 5.64 (1H, s, H-4), 4.77 (1H, s, H-28), 4.36 (1H, d, *J* = 7.8 Hz, H-1′), 4.20 and 4.33 (each 1H, dd, *J* = 10.8, 6.6 Hz, H-26), 4.30 (1H, br s, H-6), 4.18 (1H, m, H-3), 4.14 (1H, dd, *J* = 10.8, 2.4 Hz, H-15), 3.96 (1H, dd, *J* = 7.2, 2.4 Hz, H-16), 3.83 (1H, dd, *J* = 11.4, 5.4 Hz, H-5′eq), 3.57 (3H, s, OMe), 3.47 (1H, m, H-4′), 3.30 (1H, m, H-3′), 3.19 (1H, dd, *J* = 10.8, 10.2 Hz, H-5′ax), 3.14 (1H, dd, *J* = 8.4, 7.8 Hz, H-2′), 2.57 (1H, dd, *J* = 11.4, 3.2 Hz, H-7eq), 2.55 (1H, m, H-25), 1.98 and 2.11 (each 1H, m, H-23), 1.77 and 1.98 (each 1H, m, H-2), 1.96 (1H, m, H-12eq), 1.48 and 1.87 (each 1H, m, H-11), 1.86 (1H, m, H-20), 1.28 and 1.80 (each 1H, m, H-1), 1.73 (1H, m, H-22), 1.49 (1H, dd, *J* = 14.7, 3.0 Hz, H-7ax), 1.35 (3H, s, H-19), 1.20 (1H, dd, *J* = 10.8, 7.8 Hz, H-17), 1.19 (2H, m, H-22 and H-12ax), 1.13 (3H, s, H-18), 1.06 (3H, d, *J* = 6.6 Hz, H-27), 1.02 (1H, m, H-9), 1.01 (1H, d, *J* = 10.2 Hz, H-14), 0.92 (3H, d, *J* = 6.6 Hz, H-21).

3.5. Catalytic hydrogenation of 6 to 11

Linckoside K (**6**, 1.0 mg, 1.3 μmol) was dissolved in MeOH (1 mL), and Rh–Al_2O_3 (4.5 mg) was added. The mixture was stirred under a hydrogen atmosphere for 5 h at room temperature. The catalyst was removed through a filter (Minisart RC 4, 0.45 μm, Goettingen, Germany), and the filtrate was evaporated and purified by HPLC [Develosil ODS-UG-5 (∅ 10 × 250 mm), 80% aq MeOH, flow rate 2 mL/min, RI detection] to give **11** (0.4 mg, 40% yield, t_R = 24.0–25.5 min): ^{1}H NMR (CD_3OD) δ 4.83 (1H, m, under HDO signal, H-1″), 4.40 (1H, d, *J* = 7.8 Hz, H-1′), 4.13 (1H, dd, *J* = 10.8, 2.4 Hz, H-15), 3.98 (1H, dd, *J* = 7.8, 2.4 Hz, H-16), 3.95 (1H, dd *J* = 4.2, 1.8 Hz, H-2″), 3.92 (1H, ddd, *J* = 7.2, 5.4, 3.6 Hz, H-4″), 3.86 (1H, br s, H-6), 3.81 (1H, dd, *J* = 7.2, 4.2 Hz, H-3″), 3.79 (1H, dd, *J* = 11.4, 6.0 Hz, H-5′eq), 3.75 (1H, dd, *J* = 12.0, 3.6 Hz, H-5″eq), 3.73 (1H, dd, *J* = 9.6, 6.0 Hz, H-28), 3.68 (1H, m, H-3), 3.63 (1H, dd, *J* = 12.0, 5.4 Hz, H-5″ax), 3.58 (1H, s, OMe), 3.46 (1H, ddd, *J* = 10.2, 9.0, 5.4 Hz, H-4′), 3.32 (1H, m, under solvent signal, H-28), 3.29 (1H, m, under solvent signal, H-3′), 3.14 (1H, dd, *J* = 11.4, 10.8 Hz, H-5′ax), 2.81 (1H, dd, *J* = 9.0, 7.8 Hz, H-2′), 2.42 (1H, dd, *J* = 14.4, 3.0 Hz, H-7eq), 1.95 (1H, br d, *J* = 12.6 Hz, H-12), 1.83 (2H, m, H-11, H-4), 1.82 (1H, m, H-2), 1.80 (2H, m, H-20, H-25), 1.73 (1H, m, H-2), 1.72 (1H, m, H-1), 1.60 (1H, m, H-22), 1.58 (1H, m, H-14), 1.55 (1H, dd, *J* = 14.4, 3.0 Hz, H-7ax), 1.50 (1H, m, H-11), 1.46 (1H, m, H-23), 1.37 (1H, m, H-24), 1.21 (1H, m, H-17), 1.20 (1H, m, H-5), 1.19 (2H, m, H-12, H-23), 1.17 (3H, s, H-19), 1.11 (3H, s, H-18), 1.05 (1H, d, *J* = 10.8 Hz, H-14), 1.04 (1H, m, H-22), 0.96 (1H, m, H-9), 0.94 (1H, m, H-1), 0.93 (3H, d, *J* = 6.6 Hz, H-21), 0.90 (3H, d, *J* = 7.2 Hz, H-27), 0.89 (1H, d, *J* = 6.6 Hz, H-26).

3.6. Enzymatic hydrolysis of 4 with xylosidase[13]

Linckoside I (**4**, 2.0 mg) was dissolved in a mixture of 0.3 mL EtOH, 0.4 mL of 0.1 M acetate buffer (pH 4.0), and 0.2 mL distilled water, followed by the addition of 0.1 mL of *exo*-1,4-β-D-xylosidase (from *Aspergillus niger*, 1.45 U/mL, SIGMA). After incubation for 2 h at 35 °C, 0.55 M Na_2CO_3 solution (0.2 mL) was added to stop the reaction. The reaction mixture was desalinated through ODS (Cosmosil $75C_{18}$-OPN, 3 g) eluted with H_2O and then MeOH–H_2O (8:2 and 10:0). The fraction eluted with MeOH–H_2O (8:2) was evaporated and purified by HPLC [Develosil ODS-UG-5 (∅ 10 × 250 mm), 75% aq. MeOH, flow rate 2 mL/min] to give **4** (1.3 mg, t_R = 27.5–31.0 min) and **1** (0.3 mg, 15%, t_R = 32.8–35.0 min): $[\alpha]_D^{24}$ −11 (*c* 0.027, MeOH).

3.7. Bioassay methods

The neuritogenic (induction of neurite extension) activity was evaluated with PC12 cells according to methods described previously.[8,14] Briefly, 20,000 cells in 1 mL MEME serum 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. For evaluation of the NGF activity enhancement, the medium was replaced by 1 mL of serum-free MEME medium containing 1.5 ng NGF (Recombinant Human NGF, R&D Systems) and a test sample. The morphological changes of the cells were monitored by phase-contrast microscopy every 24 h for six days. Approximately 100 cells were counted from a randomly chosen field; this operation was repeated three times. The activity was represented by the percentages of PC12 cells with a neurite outgrowth longer than the cell diameter.

Acknowledgments

This work was financially supported by KAKENHI (B) (16310151) from JSPS and a Grant-in-Aid for Scientific Research on Priority Areas (17035040) from MEXT. We are grateful to the Akajima Marine Science Laboratory (Establishment of Tropical Marine Ecological Research, Tokyo) for their help with collection of the starfish.

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