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Novel NGF-potentiating diterpenoids from a Brazilian medicinal plant, *Ptychopetalum olacoides*

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ABSTRACT

From the MeOH extract of *Ptychopetalum olacoides*, which is used in Brazilian folk medicine for the treatment of chronic degenerative conditions of the nervous system, four novel clerodane-type diterpenoids named 6α,7α-dihydroxyannonene (**1**), 7α,20-dihydroxyannonene (**2**), 7α-hydroxysolidagolactone I (**3**), and ptycho-6α,7α-diol (**4**) were isolated by bioassay-directed fractionation using NGF-differentiated PC12 cells. The structures of **1**–**4** were established by extensive NMR spectroscopic analyses and chemical conversion. Compounds **1** and **2** significantly enhanced NGF-mediated neurite outgrowth in PC12 cells at concentrations ranging from 0.1 to 50.0 μM for **1** and 0.1 to 30.0 μM for **2**, whereas **3** and **4** had no morphological effect on NGF-mediated PC12 cells in the same concentration range. The structure–activity relationship of these compounds is also discussed.

A remarkable pathological symptom of Alzheimer's disease (AD) is the loss of neuronal cells in the brain. Correspondingly, the overall strategy for treatment of AD is to prevent neuronal death or to produce new neuronal cells in the degenerative regions. Nerve growth factor (NGF), a neurotrophin, is recognized as an important regulatory substance in the nervous system.[1] Thus, NGF is expected to have therapeutic efficacy for the treatment of AD. However, it cannot cross brain–blood barrier because of the properties of its high molecular polypeptide and is easily metabolized by peptidases under physiological conditions.[2] To address this issue, considerable efforts have been made to find small molecules that have neurotrophic properties or are capable of enhancing the action of NGF in appropriate cell populations.[3] For example, TMC-95A, a potent proteasome inhibitor,[4] and two iridoids, picrosides I and II,[2] have been demonstrated to show neurite outgrowth-promoting activity in NGF-mediated PC12 cells. Also some synthetic compounds, *N*-benzyloxycarbonyl-leu-leu-leucinal (ZLLLal),[5] AIT-082,[6] SR57746,[7] and Aroclor 1254,[8] have been reported to accelerate the action of NGF in PC12 cells. Recently, we reported that a few natural products such as merrilactone A[9] and merrilactones B and C[10] from *Illicium merrillianum*, 11-*O*-debenzoyltashironin from *I. merrillianum*,[11] and isodunnianol[12] from *Illicium fargesii* exhibit neurotrophic activity in primary cultured rat cortical neurons.

Ptychopetalum olacoides Benth. (Olacaceae), a medicinal plant indigenous to the Amazon in Brazil, is known as 'Marapuama or Muirapuama' and is used for the treatment of chronic degenerative conditions of the nervous systems.[13,14] Previous pharmacological studies have indicated that the EtOH extract of *P. olacoides* produces a series of beneficial effects on the central nervous system in mice.[15–19] As part of our efforts to discover natural products with neurotrophic properties,[12] we investigated the MeOH extract of the barks of *P. olacoides* that exhibited neurite outgrowth-promoting activity in NGF-mediated PC12 cells at 50 μg/mL, resulting in the isolation of four new diterpenoids, designated as 6α,7α-dihydroxyannonene (**1**), 7α,20-dihydroxyannonene (**2**), 7α-hydroxysolidagolactone I (**3**), and ptycho-6α,7α-diol (**4**). In this letter, we report the structures of **1**–**4** and their NGF-potentiating activity on PC12 cells and discuss their structure–activity relationship.

The MeOH extract of the dried barks (2 kg) of *P. olacoides* was fractionated by column chromatography on silica gel with *n*-hexane/ethyl acetate in order of increasing polarity to give ten fractions. Active fractions 6 and 7 were further purified by bioassay-guided fractionation using NGF-mediated PC12 cells to give compounds **1** (7.8 mg) and **2** (9.5 mg), and compounds **3** (1.5 mg) and **4** (2.2 mg), respectively.

Compound **1**[20] was isolated as needles crystals, and had a molecular formula $C_{20}H_{30}O_3$ as deduced by HR-EI-MS at *m/z* 318 $[M]^+$. Its IR spectrum revealed the presence of hydroxy (3357 cm^{-1}) group and a furan (1502, 1447 cm^{-1}) ring. The ^1H NMR spectrum (Table 1) showed signals due to a secondary methyl group at δ_H

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Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of **1–4** in $CDCl_3$[a]

Position	1		2		3		4	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	17.4	1.66 m (α) 1.68 m (β)	19.0	1.66 m (α) 1.70 m (β)	17.9	1.54–1.55 m	17.4	1.58 m (α) 1.56 m (β)
2	26.5	2.09 m (α) 2.01 m (β)	27.0	2.02–2.08 m	26.7	2.09 m (α) 1.98 m (β)	26.4	2.12 m (α) 2.09 m (β)
3	121.4	5.20 br s	119.5	5.14 br s	119.6	5.16 br s	121.4	5.19 br s
4	144.5		145.5		144.9		144.3	
5	43.5		37.0		37.5		43.5	3.45 d (3.6)
6	75.7	3.47 d (3.6)	43.3	2.12 dd (14.4, 2.9, α) 1.43 dd (14.4, 3.6, β)	42.9	2.13 dd (14.0, 2.7, α) 1.42 dd (14.0, 4.0, β)	75.6	3.45 d (3.6)
7	77.5	3.94 dd (3.6, 3.6)	72.6	4.01 ddd (3.6, 3.1, 2.9)	73.4	4.05 ddd (4.0, 3.3, 2.7)	77.4	3.93 dd (3.6, 3.6)
8	38.4	1.70 qd (7.1, 3.6)	39.1	1.77 qd (7.4, 3.1)	39.2	1.53 qd (7.1, 3.3)	38.4	1.63 qd (7.1, 3.6)
9	37.9		43.6		38.2		37.9	
10	45.4	1.44 dd (9.4, 4.7)	46.5	1.63 m	46.7	1.40 dd (12.1, 1.8)	45.4	1.38 dd (12.0, 2.0)
11	39.7	1.56 m 1.60 m	33.7	1.68 m 1.80 m	36.4	1.57 m 1.66 m	36.8	1.52 m 1.54 m
12	18.1	2.21 m 2.22 m	18.2	2.27–2.30 m	22.4	2.23 m	18.9	2.02 m 2.10 m
13	125.4		125.3		170.8		139.1	
14	110.7	6.25 dd (3.2, 0.8)	111.0	6.27 dd (1.8, 0.8)	115.1	5.85 br t (1.9)	141.2	6.76 d (2.7)
15	142.7	7.35 dd (3.2, 1.6)	142.8	7.35 dd (1.8, 1.8)	174.0		102.5	5.73 d (2.7)
16	138.4	7.26 dd (1.6, 0.8)	138.5	7.22 dd (1.8, 0.8)	73.0	4.74 d (1.9, 2H)	171.3	
17	22.2	1.87 br s	18.6	1.64 br s	18.0	1.63 br s	22.1	1.86 br s
18	16.4	1.24 s	21.6	1.36 s	21.7	1.31 s	16.4	1.24 s
19	12.3	1.09 d (7.1)	13.2	1.12 d (7.4)	12.5	1.04 d (7.1)	12.3	1.08 d (7.1)
20	19.6	1.00 s	65.4	3.72 d (11.6) 3.78 d (11.6)	19.8	1.06 s	19.5	1.01 s
OMe							57.1	3.57 s

[a] All assignments were made by extensive analyses of 1D and 2D NMR (COSY, DEPT, HMQC, and HMBC).

1.09 (d, J = 7.1 Hz, H_3-19); three tertiary methyl groups at δ_H 1.87 (br s, H_3-17), 1.24 (s, H_3-18), and 1.00 (s, H_3-20); and a β-furan ring at δ_H 6.25 (dd, J = 3.2, 0.8 Hz, H-14), 7.35 (dd, J = 3.2, 1.6 Hz, H-15), and 7.26 (dd, J = 1.6, 0.8 Hz, H-16). The ^{13}C NMR data (Table 1) for **1** showed 20 carbon signals, the low-field signals of which, on the basis of DEPT and HMQC spectra, were assigned to two oxygen-bearing methines at δ_C 75.7 (C-6) and 77.5 (C-7), two olefinic carbons at δ_C 121.4 (C-3) and 144.5 (C-4), and a β-furan ring resonating at δ_C 125.4 (C-13), 110.7 (C-14), 142.7 (C-15), and 138.4 (C-16), and the remaining high-field data were ascribed to four methyl, four methylene, two methine, and two quaternary carbons resonating at 43.5 (C-5) and 37.9 (C-9). These spectroscopic data with the aid of routine analyses of 2D NMR spectra (COSY, HMQC, and HMBC) culminated in the plane structure of annonene (**6**),[21,22] except for the two hydroxy groups at C-6 and C-7. The presence of the neighboring 1,2-diol was substantiated by converting **1** to acetonide **1a** with 2,2-dimethoxypropane and Amberlyst® (H^+) as an acid-catalyst. This means that **1** corresponds to 6α,7β-dihydroxyannonene (**5**), which was isolated previously from *Croton sonderianus*.[23] However, the NMR data of **1** were not consistent with those of **5**, indicating that **1** is a stereoisomer of **5** with regard to two hydroxy groups at the C-6 and C-7 positions. The observed smaller vicinal couplings ($J_{6,7}$ = 3.6 Hz and $J_{7,8}$ = 3.6 Hz) indicated a cis-configuration for an equatorial H-6 and an axial H-7 in **1** in comparison with those ($J_{6,7}$ = 10.4 Hz and $J_{7,8}$ = 12.2 Hz) in 6α,7β-dihydroxyannonene (**5**), which has a trans diaxial H-6/H-7 relationship. Additionally, the relative configuration of **1** was supported by the results of a NOESY experiment (Fig. 2). The NOESY correlations of H-10 to H-1β, H-6, H-8, and H-11 suggested that H-10, H-6, H-8, and the C11–C12 side chain bearing the furan ring have the same spatial β-orientation, indicating an α-orientation for the methyl groups at C-20 and C-19 opposite to H-8.

Additional NOESY correlations of H_3-18 with H-1α and H_3-20 suggested that the C-18 methyl group has an α-axial configuration that is anti to H-10. Thus, compound **1** was established to be 6α,7α-dihydroxyannonene.[24]

Compound **2**[25] was assigned the same molecular formula ($C_{20}H_{30}O_3$) as **1** by HR-EI-MS. The NMR data (Table 1) of **2** were similar to those of **1** except for the following differences: **2** lacks the hydroxy group at C-6 and H_3-20 methyl group that exist in **1,** and has a methylene group [δ_C 43.3; δ_H 1.43 (dd, J = 14.4, 3.6 Hz, H-6β) and 2.12 (dd, J = 14.4, 2.9 Hz, H-6α)] and an isolated oxymethylene group [(δ_C 65.4; δ_H 3.72 (d, J = 11.6 Hz, H-20) and 3.78 (d, J = 11.6 Hz, H-20)]. These spectroscopic data implied that **2** is 7α,20-dihydroxyannonene. This postulated plane structure was confirmed by the HMBC correlations of H_2-20 (δ_H 3.72 and 3.78) with C-8 (δ_C 39.1), C-9 (δ_C 43.6), C-10 (δ_C 46.5), and C-11 (δ_C 33.7) as well as of H-7 (δ_H 4.01) with C-5 (δ_C 37.0), C-6 (δ_C 43.3), and C-8. The NOESY correlations of H_2-20 with H-1α and H_3-18 showed that the H_2-20 oxymethylene has an α-axial orientation, whereas the H-7 oxymethine has a β-equatorial orientation on the base of its small vicinal coupling constants (3.6, 3.1, and 2.9 Hz) and the NOESY interactions between H-7 and H-6β and H-8. Thus, **2** was defined as 7α,20-dihydroxyannonene.

Compound **3**[26] had the same molecular formula ($C_{20}H_{30}O_3$) as **1** and **2** as deduced by HR-EI-MS. Its IR spectrum revealed the presence of a hydroxy (3498 cm^{-1}) group and an α,β-unsaturated γ-lactone (1743 cm^{-1}) ring but the absence of the furan ring that is present in **1** and **2**. In the case of **3**, the β-furan ring has presumably been transformed to the corresponding α,β-unsaturated γ-lactone moiety, the presence of which was supported by the HMBC correlations of H-12 (δ_H 2.23) to C-13 (δ_C 170.8), C-14 (δ_C 115.1), and C-16 (δ_C 73.0) and H-16 (δ_H 4.74) to C-15 (δ_C 174.0). In comparison of the NMR data of **3** with those of **1**, the furan ring and the C-6 hydroxy group occurring in **1** were replaced with an α,β-unsaturated γ-lactone ring [δ_C 170.8 (C-13), 115.1 (C-14), 174.0 (C-15), 73.0 (C-16); δ_H 5.85 (br t, J = 1.9 Hz, H-14), 4.74 (d, J = 1.9 Hz, H-16)] and a methylene group resonating at δ_H 1.42 (dd, J = 14.0, 4.0 Hz, H-6β) and 2.13 (dd, J = 14.0, 2.7 Hz, H-6α), respectively. This structure proposed for **3** is closely related to solidagolactone IV (**7**), which was iso-

lated from *Solidago altissima*,[27] except it has a hydroxy group at the C-6 position, disclosing that **3** is a C-7 hydroxy regioisomer of solidagolactone IV. The relative stereochemistry for this hydroxy group at C-7 was determined to be an α and axial configuration by its small *J* values for the H-7 resonating at δ_H 4.05 (ddd, *J* = 4.0, 3.3, 2.7 Hz) and the NOESY correlations of H-7 with H-6β and H-8. Accordingly, the structure of **3** was defined as 7α-hydroxysolidagolactone I as depicted in Figure 1.

Compound **4**[28] had a molecular formula $C_{21}H_{32}O_5$ as deduced by HR-EI-MS. Its IR spectrum showed the presence of a hydroxy (3501 cm^{-1}) group and a γ-lactone (1765 cm^{-1}) ring. The NMR data of **4** (Table 1) were similar to those of **1** except for the lack of a furan ring and the presence of an α,β-unsaturated γ-lactone ring [δ_C 139.1 (C-13), 141.2 (C-14), 102.5 (C-15), 171.3 (C-16); δ_H 6.76 (d, *J* = 2.7 Hz, H-14) and 5.73 (d, *J* = 2.7 Hz, H-15)] and a methoxy group (δ_C 57.1; δ_H 3.57, s). The attachment of the methoxy group at C-15 was confirmed by a HMBC correlation of the OCH_3 signal to C-15. The C-16 position of the lactone carbonyl opposite to that of **3** was supported by a HMBC correlation of H-12 with the lactone carbonyl as well as by a downfield chemical shift (δ_H 6.76) for H-14. According to the small *J* value ($J_{6,7}$ = 3.6 Hz) and the same NOESY correlations as **1**, the relative configuration of **4** was determined to be identical to that of **1** except for the configuration of the methoxy group at C-15. Thus, the structure of ptycho-6α,7α-diol was represented as **4**.

Compounds **1**–**4** were isolated from fractions that promoted neurite outgrowth from NGF (20 ng/mL)-mediated PC12 cells. They were evaluated for PC12 cells neurite outgrowth according to a previously reported experiment procedure.[4,29–31] None of the compounds showed any morphological effects on PC12 cells in the absence of NGF, whereas in the presence of NGF (20 ng/mL) 6α,7α-dihydroxyannonene (**1**) and 7α,20-dihydroxyannonene (**2**) significantly promoted neurite outgrowth from PC12 cells in a dose-dependent manner at concentrations ranging from 0.1 to 50.0 μM and from 0.1 to 30.0 μM, respectively, but **2** showed toxicity at concentrations higher than 50.0 μM (Figs. 3 and 4). The degree of the effects on neurite outgrowth in PC12 cells was demonstrated by morphological observations (Fig. 3) and quantitative analysis of the neurite length extending from the cell bodies (Fig. 4). The average neurite length (205.3 μm at 50.0 μM, 201.5 μm at 30.0 μM, 173.1 μm at 10.0 μM, 115.8 μm at 1.0 μM, and 104.0 μm at 0.1 μM) of 20 ng/mL NGF-mediated PC12 cells treated with **1** increased dose-dependently longer than that (85.7 μm) of the control (Fig. 4). In the case of **2**, according to the measurement of the extended cell neurites (Fig. 3), it was shown that the control average neurite length (78.6 μm) of 20 ng/mL NGF-mediated PC12 cells was extended to 236.3 μm (30.0 μM), 106.5 μm (10.0 μM), 101.7 μm (1.0 μM), and 95.3 μm (0.1 μM), respectively (Fig. 4). 7α-Hydroxysolidagolactone I (**3**), ptycho-6α,7α-diol (**4**) and acetonide **1a** showed no activity at concentrations ranging from 0.1 to 50.0 μM in the presence of 20 ng/mL NGF.

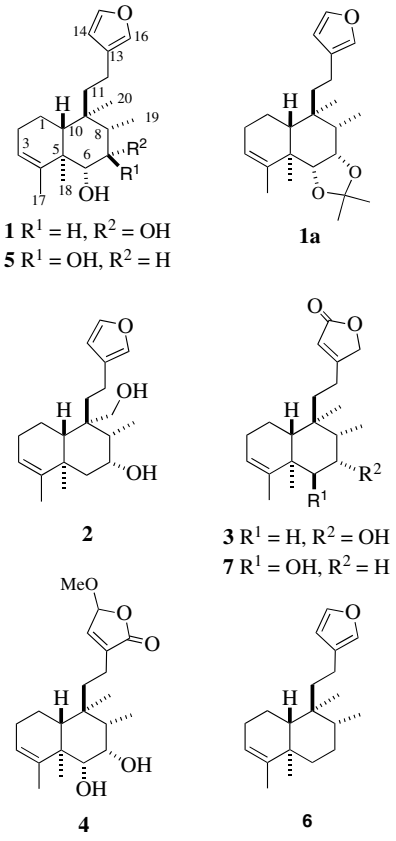

Figure 1. Structures of compounds **1**–**7**.

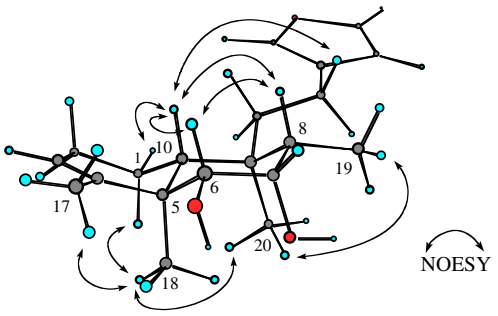

Figure 2. Representative NOESY correlations of **1**.

The above-mentioned results suggest that both compounds **1** and **2** possess strong NGF-potentiating activity on PC12 cells. In a comparison of the average neurite lengths between **1** and **2** (Fig. 5), **1** seemed to be a more potent NGF-potentiator than **2**. However, the activity of **2** became more potent degree than **1** at 30.0 μM and was greatly reduced due to toxicity at 50.0 μM as shown in Figure 5. The two adjacent hydroxy groups in **1** presumably contribute to increase its activity, because the acetonide **1a** has no activity at the same concentrations. When the two hydroxy groups are placed far away from each other as in **2**, the activity tends to decrease. Compounds **3** and **4** did not show activity at the same concentrations. This result suggests that the furan ring present in **1** and **2** plays an important role in the emergence of NGF-potentiating activity.

In conclusion, four novel diterpenoids 6α,7α-dihydroxyannonene (**1**), 7α,20-dihydroxyannonene (**2**), 7α-hydroxysolidagolactone I (**3**), and ptycho-6α,7α-diol (**4**) were isolated from *P. olacoides*. Compounds **1** and **2** showed NGF-potentiating activity on PC12 cells in a dose-dependent manner, and thereby were identified to be responsible for the neurite outgrowth-promotion of NGF-mediated PC12 cells observed by the MeOH extract of *P. olacoides*. It should be noted that the present studies have provided chemical evidence to support the folk medicinal use of *P. olacoides* for the treatment of chronic degenerative conditions of the nervous systems. Furthermore, compounds **1** and **2** have the ability to enhance the activity of NGF, which is capable of stimulating neurite outgrowth in PC12 cells, suggesting that they may be useful candidates for developing drugs for the treatment of neurodegenerative diseases such as AD.

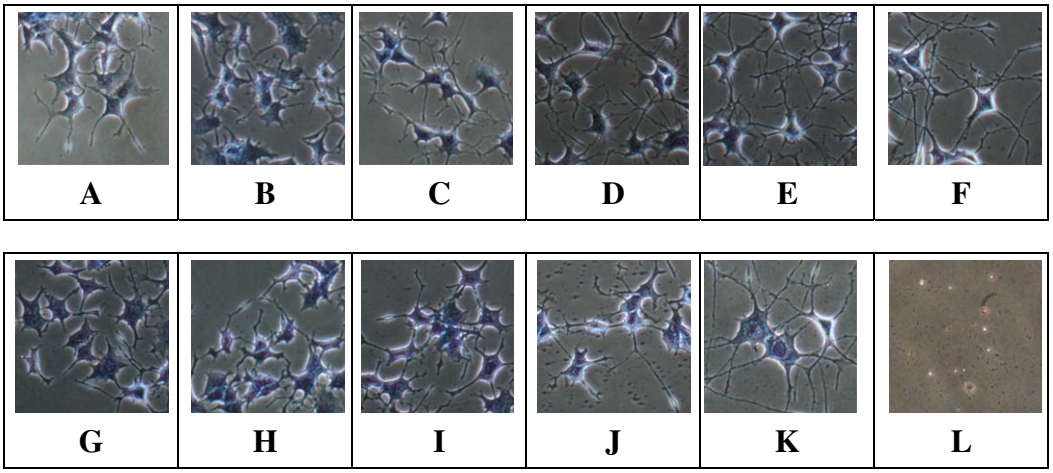

Figure 3. Morphological changes of PC12 cells after treatment with (A) NGF 20 ng/mL, (B) **1** (0.1 μM) + NGF 20 ng/mL, (C) **1** (1.0 μM) + NGF 20 ng/mL, (D) **1** (10.0 μM) + NGF 20 ng/mL, (E) **1** (30.0 μM) + NGF 20 ng/mL, (F) **1** (50.0 μM) + NGF 20 ng/mL, (G) NGF 20 ng/mL, (H) **2** (0.1 μM) + NGF 20 ng/mL, (I) **2** (1.0 μM) + NGF 20 ng/mL, (J) **2** (10.0 μM) + NGF 20 ng/mL, (K) **2** (30.0 μM) + NGF 20 ng/mL, (L) **2** (50.0 μM) + NGF 20 ng/mL.

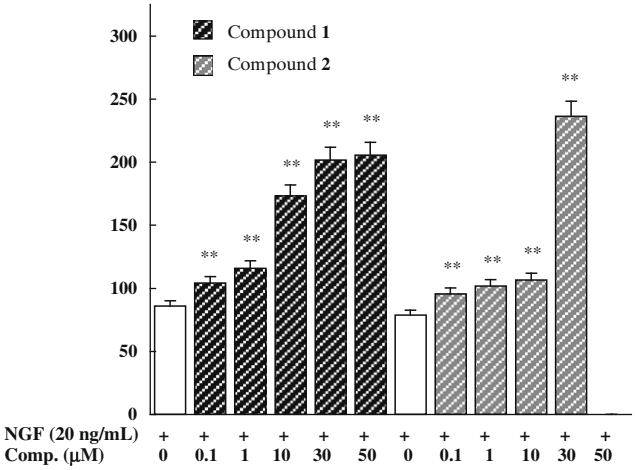

Figure 4. Quantitative analysis of the neurite outgrowth promoted by **1** or **2**. PC12 cells were cultured in two 24-well plates in DMEM + 10% HS and 5% FBS for 24 h at a density of 1×10^3 cells/cm^2, and then medium was changed to DMEM + 2% HS and 1% FBS with NGF (20 ng/mL) containing **1** and **2**, respectively. After 4 days, the neurite lengths of PC12 cells were quantified. Data are expressed as the mean ± SE ($n = 75$). $^{**}P < 0.01$ vs control; Dunnett's *t*-test.

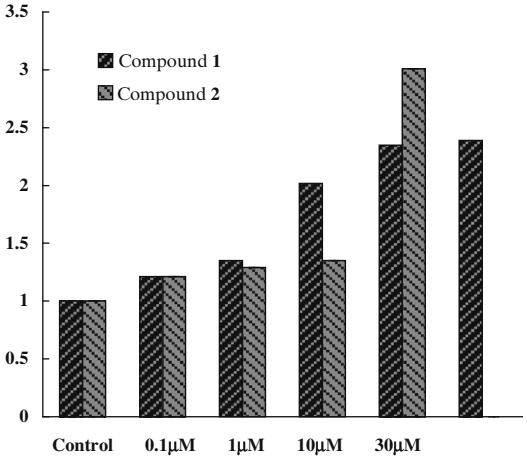

Figure 5. A comparison of the relative average neurite length of PC12 cells and control promoted by **1** and **2**.

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20. Data for **1** [6α,7α-dihydroxy-cleroda-3,13(16),14-triene-15,16-oxide]: needles, mp 140–141°; $[\alpha]_D^{16} = -1.9$ (*c* 0.8, EtOH); CD (EtOH) $\Delta\varepsilon$ (295) −16.4, $\Delta\varepsilon$ (243) +4.9; IR ν_{max} 3357 (OH), 2947, 1502, 1447 cm^{-1}; HR-EI-MS *m/z* 318.2186 (M$^+$, calcd for $C_{20}H_{30}O_3$, 318.2195); ^{1}H and ^{13}C NMR, see Table 1.
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24. For clarifying the absolute configuration for **1**, attempts to prepare dibenzoate and MTPA ester derivatives were failed presumably due to the axial orientation of the hydroxy group at C-7 and the hydroxy group at C-6 hindered by two axial C-5 methyl and C-7 hydroxy groups in spite of its equatorial disposition.
25. Data for **2** [7α,20-dihydroxy-cleroda-3,13(16),14-triene-15,16-oxide]: pale yellow oil; $[\alpha]_D^{22} = -7.4$ (*c* 0.9, $CHCl_3$); IR ν_{max} 3258 (OH), 2936, 1559, 1501, 1456 cm^{-1}; HR-EI-MS *m/z* 318.2186 (M^+, calcd for $C_{20}H_{30}O_3$, 318.2195); ^1H and ^{13}C NMR, see Table 1.
26. Data for **3** (7α-hydroxy-cleroda-3,13-dien-15,16-olide): colorless oil; $[\alpha]_D^{21} = -18$ (*c* 0.4, acetone); CD ($CHCl_3$) $\Delta\varepsilon$ (301) −4.7, $\Delta\varepsilon$ (244) +6.8; IR ν_{max} 3498 (OH), 2939, 1743, 1448 cm^{-1}; HR-EI-MS *m/z* 318.2179 (M^+, calcd for $C_{20}H_{30}O_3$, 318.2195); ^1H and ^{13}C NMR, see Table 1.
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28. Data for **4** (6α,7α-dihydroxy-15-methoxy-cleroda-3,13-dien-16,15-olide): colorless oil; $[\alpha]_D^{23} = -32$ (*c* 1.2, $CHCl_3$); CD ($CHCl_3$) $\Delta\varepsilon$ (334) −7.2, $\Delta\varepsilon$ (294) +7.8, $\Delta\varepsilon$ (254) −80.5; IR ($CHCl_3$) ν_{max} 3501 (OH), 2956, 1765, 1447, 1366 cm^{-1}; HR-EI-MS *m/z* 364.2253 (M^+, calcd for $C_{21}H_{32}O_5$, 364.2250). ^1H and ^{13}C NMR, see Table 1.
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31. PC12 (pheochromocytoma) cells were cultured in a 24-well plate at a density of 1×10^3 cells/cm^2 in DMEM containing 10% horse serum (HS), 5% fetal bovine serum (FBS), and 100 IU/mL penicillin and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 for 24 h. The culture medium was then changed to DMEM, 2% HS, 1% FBS, and 100 IU/mL penicillin and 100 μg/mL streptomycin. At the same time, different concentrations of test samples with or without NGF were added. One concentration experiment was repeated in three wells. After incubation with samples for 4 days, the cultures were fixed with 4% paraformaldehyde/PBS, and stained with methylene blue. Cell morphology was observed under a phase-contrast microscope and neurite length was quantified. Five images were selected randomly under microscope for each well and five cells bearing well-developed neurite in each photo were selected and the average length of the longest neurite on selected cells was measured. At least 75 cells were calculated for each concentration. Statistical analyses were performed using Dunnett's *t*-test. Compound **1** showed significant neurite outgrowth-promoting activity on 20 ng/mL NGF-mediated PC12 cells at the concentrations ranging from 0.1 to 50.0 μM (Figs. 3 and 4). Compound **2** also promoted the neurite length of 20 ng/mL NGF-mediated PC12 cells at the concentrations ranging from 0.1 to 30.0 μM (Figs. 3 and 4) and showed cell toxicity at 50.0 μM. Compounds **3** and **4**, and derivative **1a** showed no activity on 20 ng/mL NGF-potentiating PC12 cells at the concentrations ranging from 0.1 to 50.0 μM, respectively.