Notes

Clerodane Diterpenoids with NGF-Potentiating Activity from *Ptychopetalum olacoides*

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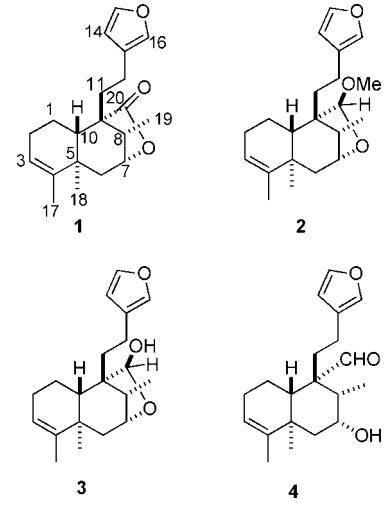
Received July 2, 2008

Four new clerodane-type diterpenoids, ptychonolide (**1**), 20-*O*-methylptychonal acetal (**2**), and an equilibrium mixture of ptychonal hemiacetal (**3**) and ptychonal (**4**), were isolated from the MeOH extract of the bark of a Brazilian plant, *Ptychopetalum olacoides*. The structure of **1** was elucidated as a clerodane-type diterpenoid on the basis of spectroscopic data, whereas **2** was assigned to an acetal derivative of **1**. Compounds **3** and **4** existed as an equilibrium mixture. A mixture of compounds **3** and **4** was found to exhibit neurite outgrowth-promoting activities on NGF-mediated PC12 cells at concentrations ranging from 0.1 to 10.0 μM.

Ptychopetalum olacoides Benth. (Olacaceae), a medium-sized tree, 4−5 m in height, is a medicinal plant native to the Amazon in Brazil.[1] *P. olacoides* roots and bark are known as "Marapuama or Muirapuama" in Brazil and have been used in folk medicine for the treatment of chronic degenerative conditions of the nervous system by Amazonian people.[2] Traditional remedies prepared with *P. olacoides* are known as a "nerve tonic" for use as an aphrodisiac, appetite moderator, and an antitremor agent in Amazonian communities.[3] Previous pharmacological studies indicated that a *P. olacoides* EtOH extract potentiated yohimbine-induced lethality, reversed reserving-induced ptosis, and prevented apomorphine-induced stereotypy in mice.[4] Additionally, the EtOH extract was found to have an anxiogenic effect,[5] antioxidant activity,[6] memory enhancement,[7] anticholinesterase activity,[8] and a neuroprotective effect[9] in mice. Judging from the folk medicinal uses and these pharmacological data of *P. olacoides* formulations, the title plant would be expected to produce compounds that may have positive effects on some brain functions; however, little is known about the chemistry and active ingredients of this plant.[10,11] As part of our search for neurotrophic natural products,[12] we investigated the MeOH extract of the bark of *P. olacoides*, which exhibited neurite outgrowth-promoting activity on NGF-mediated PC12 cells at 50 μg/mL. As a result, four new clerodane-type diterpenoids, namely, ptychonolide (**1**), 20-*O*-methylptychonal acetal (**2**), and an equilibrium mixture of ptychonal hemiacetal (**3**) and ptychonal (**4**), were isolated. In this paper, we elucidate the structures of **1**−**4** and their NGF-potentiating activity in PC12 cells.

Compound **1** was isolated as white crystals and had the molecular formula $C_{20}H_{26}O_3$, as deduced by using high-resolution CIMS, indicating 8 degrees of unsaturation. The IR spectrum revealed the presence of five-membered lactone (1767 cm^{-1}) and furan (1501, 1449 cm^{-1}) rings. The ^{13}C NMR spectrum showed 20 carbon signals, the low-field signals of which, on the basis of DEPT and HMQC spectra, were assigned to an ester carbonyl carbon at δ_C 179.4 (C-20), an oxygen-bearing methine at δ_C 82.4 (C-7), two olefinic carbons at δ_C 119.4 (C-3) and 145.2 (C-4), and a furan ring with signals at δ_C 124.2 (C-13), 110.6 (C-14), 143.0 (C-15), and 138.5 (C-16). The remaining high-field resonances were ascribed to three methyl, five methylene, two methine, and two quaternary carbons resonating at δ_C 38.1 (C-5) and 50.2 (C-9). The ^{1}H NMR spectrum of **1** showed signals



due to a secondary methyl group at δ_H 1.06 (d, J = 6.9 Hz, H_3-19), two tertiary methyl groups at δ_H 1.65 (brs, H_3-17) and 1.21 (s, H_3-18), and a β-furan ring at δ_H 6.29 (dd, J = 1.6, 0.8 Hz, H-14), 7.37 (dd, J = 1.6, 1.6 Hz, H-15), and 7.26 (dd, J = 1.6, 0.8 Hz, H-16). Four connections of the protonated carbons (C10−C1−C2−C3; C6−C7; C8−C19; C11−C12) were obtained from the COSY and HMQC spectra. The H-7 oxymethine vicinal to H-8 and H-6β appeared as a doublet with J = 4.7 Hz. As the dihedral angles between H-7 and H-8, H-6β are close to 90°, the coupling constants between H-7 and H-8, H-6β would be zero. Thus two structural units (C6−C7 and C8−C19) could be joined to give the new fragment C6−C7−C8−C19, the presence of which was supported by the HMBC correlation of H_3-19 to C-7. An HMBC experiment was applied to connect these structural fragments to the nonprotonated carbon atoms (C-4, C-5, C-9). Namely, HMBC correlations (C-4/H_3-17 and H_3-18; C-5/H_3-17 and H_3-18) allowed the connection of the C-17 and C-18 methyl groups to C-4 and C-5, which bonded to each other. The H-10 resonance at δ_H 2.03 showed HMBC correlations to C-5, supporting the bond formation between C-5 and C-10. The H_3-19 secondary methyl and H-10 signals correlated to C-9 at δ_C 50.2, which furthermore had correlations with H-11. In addition, the H_3-18 methyl signal showed a HMBC correlation with C-6. These observations allowed C-9 and C-5 to connect

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10.1021/np8004002 CCC: $40.75
Published on Web 09/27/2008

Table 1. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR Data of **1** and **2** in $CDCl_3$[a]

	1		2	
position	δ_C	δ_H	δ_C	δ_H
1α	18.4	1.69 m	18.6	1.68 m
1β		1.77 m		1.79 m
2α	25.2	2.12 m	25.6	2.13 m
2β		2.15 m		2.15 m
3	119.4	5.09 brd	118.7	5.03 brd
4	145.2		146.3	
5	38.1		38.5	
6α	38.2	2.16 dd (14.0, 4.7)	40.6	1.91 dd (13.4, 4.7)
6β		1.73 d (14.0)		1.48 d (13.4)
7	82.4	4.47 d (4.7)	84.1	4.25 d (4.7)
8	47.9	2.09 q (6.9)	46.6	1.75 q (7.1)
9	50.2		52.3	
10	47.5	2.03 dd (13.2, 2.8)	47.1	1.85 dd (13.4, 2.6)
11	27.0	1.63 m	26.8	1.64 m
		2.10 m		1.94 m
12	18.6	2.23 m	19.2	2.25 m
		2.43 m		2.41 m
13	124.2		125.3	
14	110.6	6.29 dd (1.6, 0.8)	110.9	6.30 dd (1.6, 0.8)
15	143.0	7.37 dd (1.6, 1.6)	142.7	7.35 dd (1.6, 1.6)
16	138.5	7.26 dd (1.6, 0.8)	138.5	7.24 dd (1.6, 0.8)
17	18.0	1.65 brs	18.0	1.60 brs
18	23.9	1.21 s	23.5	1.27 s
19	14.4	1.06 d (6.9)	15.1	1.13 d (7.1)
20	179.4		106.0	4.64 s
−OMe			55.4	3.32 s

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

to C-10, C-11, and C-8, and C-6, respectively. A β-furan ring was proved to be at the C-12 position by the HMBC correlations of H-12 with C-13, C-14, and C-16. These spectroscopic data implied that **1** was a clerodane-type diterpenoid,[13] but one C-20 tertiary methyl group typical in normal clerodanes was missing. The HMBC correlation of H-10 to the C-20 carbonyl suggested that the C-20 methyl was replaced with the ester carbonyl, which formed a γ-lactone ring with the C-7 oxygen, according to the key HMBC correlation from H-7 resonating at δ_H 4.47 (d, J = 4.7 Hz) to C-20. Thus, compound **1** was a clerodane-type diterpenoid.

The relative configuration of **1** was deduced by NOESY experiment. First, the disposition of the five-membered lactone ring was automatically defined down because H-7 had a β and equatorial configuration according to its small J value (4.7 Hz). NOESY correlations from H-10 to H-1β, H-6β, H-11, and H-8 suggested that H-10 and the C11−C12 side chain bearing the furan ring were β-oriented, and the C-19 methyl group was α-oriented opposite H-8. Additional NOESY correlations from H_3-18 to H-1α and H-6α suggested that the C-18 methyl group was anti to H-10, indicating that the C-18 methyl group had an α axial disposition. Thus, the relative configuration of ptychonolide was represented as **1**.

Compound **2**, a colorless powder, was assigned the molecular formula $C_{21}H_{30}O_3$ by high-resolution EIMS. The NMR data of **2** were similar to those of **1** (Table 1). The significant difference was that **2** had no carbonyl group, but acetal (δ_C 106.0; δ_H 4.64) and methoxy (δ_C 55.4; δ_H 3.32) groups instead. HMBC correlation of the acetal carbon with the methoxy protons indicated that the lactone carbonyl in **1** was reduced to a methyl acetal in **2**. The OCH_3 group at C-20 was assigned as β from the NOESY correlations of the H-20 acetal signal to H-1α and H_3-18. Thus, **2** was 20-*O*-methylptychonal acetal.

Compounds **3** and **4** were obtained as an equilibrium mixture in a ratio of 3:1. The molecular formula of **3** and **4** was determined to be $C_{20}H_{28}O_3$ by HREIMS. The IR spectrum showed absorption bands ascribable to OH group (3394 cm^{-1}), aldehyde (1710 cm^{-1}), and furan (1455 cm^{-1}) moieties. Comparing the ^{1}H NMR spectrum of the mixture of **3** and **4** with that of **2** showed that the hemiacetal proton (δ_H 5.21) in **3** appeared at lower field than that (δ_H 4.64) in **2**. In the case of **4** an additional aldehyde proton (δ_H10.01) was observed. The results indicated that **4** readily equilibrated with hemiacetal **3** due to the 1,3-diaxial disposition of both the C-20 aldehyde and C-7 hydroxy groups. A mixture of **3** and **4** was left standing in MeOH under acidic conditions to yield **2**. Thus, the structures of ptychonal hemiacetal and ptychonal were assigned as **3** and **4**, respectively, and compound **2** should be regarded as an artifact. It is noted that clerodane diterpenoids bearing an axial oxy-function at the C-7 position are rare, but a few examples such as 7α-acetoxyhardwickiic acid[14] have been documented.

The effects of compounds **1**−**4** on neurite outgrowth from PC12 cells were evaluated according to previously reported procedures.[15,16] Compounds **1** and **2** had no neurite outgrowth effect on PC12 cells in the presence or absence of NGF (20 ng/mL) at concentrations ranging from 0.1 to 10.0 μM. Although the mixture of compounds **3** and **4** showed no morphological effect on PC12 cells in the absence of NGF, in the presence of NGF (20 ng/mL), this mixture significantly promoted neurite outgrowth from NGF-mediated PC12 cells dose-dependently at concentrations ranging from 0.1 to 10.0 μM.[17] This effect was well demonstrated by morphological observations (Figure 1) and ascertained by quantitative analysis of neurite length extending from the cell bodies (Figure 2). Average neurite length (126.4 μm at 10.0 μM, 114.5 μm at 1.0 μM, and 102.5 μm at 0.1 μM) from NGF-mediated PC12 cells treated by a mixture of **3** and **4** increased dose-dependently to longer than the average neurite length (89.9 μm) in a control experiment (Figure 2). These results suggest that compounds **3** and **4** possess the ability to potentiate the activity of NGF to stimulate neurite outgrowth from PC12 cells and that they may be useful candidates to develop drugs for the treatment of neurodegenerative diseases such as Alzheimer's.[16,18]

Experimental Section

General Experimental Procedures. The melting points were measured by a Yanagimoto micro melting point apparatus (not adjusted). Optical rotations were measured using a JASCO P-1030 digital polarimeter. IR and UV spectra were recorded on a JASCO FT-IR 410 infrared and a Shimadzu UV-1650PC spectrophotometer, respectively. Circular dichroism (CD) spectra were recorded on a JASCO-J-725. ^{1}H (600 MHz) and ^{13}C NMR (150 MHz) spectra were measured on a Varian Unity 600 instrument. NMR assignments were made by using DEPT, COSY, NOESY, HMQC, and HMBC experiments. Chemical shifts are given as δ (ppm) with TMS as an internal standard. HRCIMS and HREIMS were recorded on a JEOL JMS-HX 110 instrument. Silica gel column chromatography (CC) was carried out on Kiselgel 60 (70−230 mesh and 230−400 mesh) and Wako C-300. HPLC was performed on a JASCO PU-1580 HPLC equipped with a JASCO UV-1575 detector.

Plant Material. Bark of *Ptychopetalum olacoides* was purchased in Sao Paulo, Brazil, in 2005. Dr. G. Hashimoto (Centro de Pesquisas de Historia Natural) identified the plant, and a voucher specimen (1762BK) has been deposited in the institute of Pharmacognosy, Tokushima Bunri University, Japan.

Extraction and Isolation. The dried bark (2 kg) of *P. olacoides* was powdered and extracted with MeOH (16 L) at room temperature for one month. The MeOH extract was concentrated to 400 mL, 200 mL of which was extracted with hexane (5 × 200 mL) to give a green, oily residue (16.2 g). Water (400 mL) was added to the MeOH layer. This was extracted with EtOAc (4 × 200 mL), dried (Na_2SO_4), and concentrated to give a brown, oily residue (28.2 g). The residue was separated by CC on silica gel with a linear gradient solvent system (100% hexane to 100% EtOAc) to yield 10 fractions (1−10). Fraction 1 was separated by CC on silica gel with hexane−EtOAc (9:1) to give six fractions. The sixth fraction was purified by HPLC on a Cosmosil 5SL-II column (10 × 250 mm) with hexane−EtOAc (9:1, at a flow rate of 2.0 mL/min and UV detector of 254 nm) to yield **1** (10 mg, t_R 19 min). Fraction 6 was subjected to silica gel CC to give eight fractions. The sixth fraction was purified by reversed-phase chromatography on

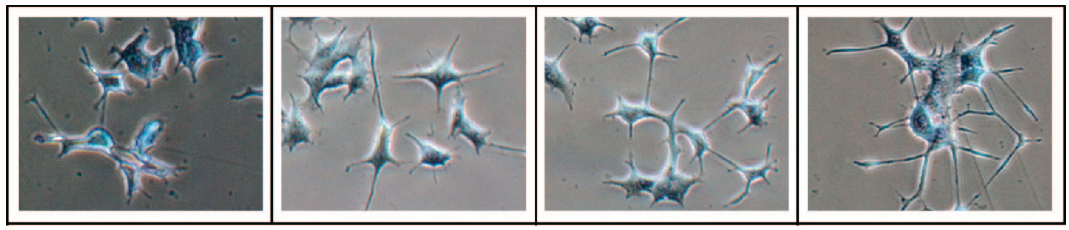

Figure 1. Morphological changes of PC12 cells after treatment with (A) NGF 20 ng/mL, (B) **3** and **4** (0.1 μM) + NGF 20 ng/mL, (C) **3** and **4** (1.0 μM) + NGF 20 ng/mL, and (D) **3** and **4** (10.0 μM) + NGF 20 ng/mL.

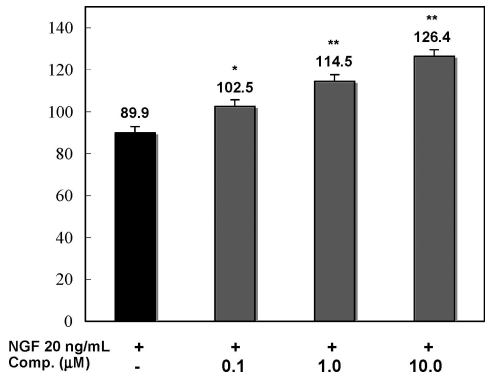

Figure 2. Quantitative analysis of neurite outgrowth promoted by a mixture of **3** and **4**. PC12 cells were cultured in a 24-well plate in DMEM + 10% HS and 5% FBS for 24 h at a density of 8 × 10^3 cells/cm^2, and then medium was changed to DMEM + 2% HS and 1% FBS with NGF (20 ng/mL) containing a mixture of **3** and **4**. After 4 days neurite lengths of PC12 cells were quantified. Data are expressed as the mean ± SE ($n = 75$). *$P < 0.05$, **$P < 0.01$ vs control, respectively; Dunnett's t test.

Cosmosil 75 C_{18}-OPN eluted with MeOH−H_2O (4:1) to give **2** (2.3 mg) and a 3: 1 mixture of **3** and **4** (10.0 mg).

Ptychonolide (1): colorless crystal; mp 168−169 °C; $[\alpha]_D^{24}$ −107 (c 1.02, $CHCl_3$); IR ($CHCl_3$) ν_{max} 2944, 1767, 1501, 1449, 1385, 1351, 1252, 1202, 1158, 1132, 1085, 1062, 1025, 991, 949 cm^{-1}; UV (CH_2Cl_2) λ_{max} (log ε) 232 (4.1); CD ($CHCl_3$) $\Delta\varepsilon$ (327) +7.2, $\Delta\varepsilon$ (261) −7.2; ^{1}H and ^{13}C NMR data, see Table 1; HRCIMS m/z 315.1964 $[M + H]^+$ (calcd for $C_{20}H_{26}O_{30}$, 315.1960).

20-*O*-Methylptychonal acetal (2): colorless powder; $[\alpha]_D^{26}$ −10 (c 0.56, MeOH); IR ($CHCl_3$) ν_{max} 2926, 1455, 1087, 1026, 955, 873 cm^{-1}; UV (MeOH) λ_{max} (log ε) 215 (3.5) nm; ^{1}H and ^{13}C NMR data, see Table 1; HREIMS m/z 330.2185 $[M]^+$ (calcd for $C_{21}H_{30}O_3$, 330.2195).

Ptychonal hemiacetal (3) and ptychonal (4): colorless powder; IR ($CHCl_3$) ν_{max} 3394, 2930, 1710, 1455, 1260, 1063, 1026, 990, 873 cm^{-1}; HREIMS m/z 316.2037 $[M]^+$ (calcd for $C_{20}H_{28}O_3$, 316.2038); ^{1}H NMR (300 MHz, $CDCl_3$) of **3** δ 1.19 (3H, d, $J = 7.1$ Hz, H_3-19), 1.25 (3H, s, H_3-18), 1.60 (3H, s, H_3-17), 4.31 (1H, d, $J = 4.8$ Hz, H-7), 5.03 (1H, m, H-3), 5.21 (1H, s, H-20), 6.31 (1H, brd, H-14), 7.26 (1H, brd, H-16), 7.35 (1H, brd, H-15); ^{1}H NMR (300 MHz, $CDCl_3$) of **4** δ 1.19 (3H, d, $J = 7.1$ Hz, H_3-19), 1.25 (3H, s, H_3-18), 1.63 (3H, s, H_3-17), 4.11 (1H, m, H-7), 5.18 (1H, m, H-3), 6.28 (1H, brd, H-14), 7.26 (1H, brd, H-16), 7.35 (1H, brd, H-15), 10.01 (1H, s, H-20).

Conversion of an Equilibrium Mixture of 3 and 4 to 2. A MeOH solution (1 mL) of **3** and **4** (2 mg) was kept in the presence of a piece of Amberlist (H^+) A-21 at room temperature for 24 h. Filtering and removal of MeOH left an oil (2.1 mg), all spectroscopic data of which were identical to those of **2**.

Neurite Outgrowth-Promoting Activity. PC12 (Pheochromocytoma) cells were cultured in a 24-well plate at a density of 8 × 10^3 cells/cm^2 in DMEM + 10% HS, 5% FBS, 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, 100 IU/mL penicillin, and 100 μg/mL streptomycin. At the same time, different concentrations of test samples with or without 20 ng/mL 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 a microscope for each well, and five significantly differentiated cells were selected to measure the longest neurite length extending from a cell body for each picture. At least 75 cells were calculated for each concentration. Statistical analyses were performed using Dunnett's t test. A mixture of compounds **3** and **4** showed neurite outgrowth-promoting activity on NGF-mediated PC12 cells at 0.1, 1.0, and 10.0 μM (Figures 1 and 2).

Acknowledgment. We thank Dr. M. Tanaka and Ms. Y. Okamoto (TBU) for measurements of the 600 MHz NMR and mass spectra. W.T. thanks Dr. K. Hirose, KNC Laboratories Co., Ltd., for outstanding scholarship. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Priority Area, 18032085; 19790027) and the Open Research Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan.

Supporting Information Available: ^{1}H and ^{13}C NMR, COSY, HMQC, HMBC, and NOESY spectra of **1** and **2**, and ^{1}H NMR spectrum of a mixture of **3** and **4**. These materials are available free of charge via the Internet at http://pubs.acs.org.

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(17) A mixture of compounds **3** and **4** showed no morphological effect on NGF-mediated PC12 cells at a concentration of 0.01 μM and was found to be toxic to PC12 cells in the presence of NGF (20 ng/mL) when the concentration was increased to 50.0 μM.

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NP8004002