Clerodane Diterpenoids and Flavonoids with NGF-Potentiating Activity from the Aerial Parts of *Baccharis gaudichaudiana*

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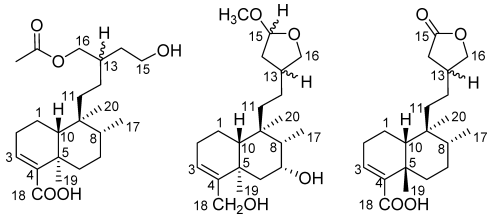
A new clerodane diterpene, 15-hydroxy-16-acetoxy-*ent*-clerod-3-en-18-oic acid (1), together with three known clerodane diterpenes (2—4) and three known flavones (5—7), were isolated from the aerial parts of *Baccharis gaudichaudiana*. Their structures were elucidated on the basis of spectroscopic analysis. Compounds 2, 3, and 5 showed enhancing activity of nerve growth factor (NGF)-induced neurite outgrowth in PC 12D cells.

Key words *Baccharis gaudichaudiana*; clerodane diterpene; flavone; nerve growth factor (NGF)-potentiating activity

Baccharis gaudichaudiana DC. (Compositae) is used in Paraguay as a folk medicine against diabetes, as a tonic, and for the treatment of gastrointestinal disease. Previous phytochemical investigations on the species resulted in the isolation of labdane-type diterpene glycosides,[1,2] clerodane diterpenes.[3,4] In the course of our search for new natural products with nerve growth factor (NGF)-potentiating activity from medicinal plants, we studied the chemical constituents of methanol extract of the aerial parts of *B. gaudichaudiana*, which showed moderate NGF-potentiating activity.[5] The extract was repeatedly subjected to silica gel column chromatography followed by reversed-phase HPLC on an ODS column to yield a new clerodane diterpene, 15-hydroxy-16-acetoxy-*ent*-clerod-3-en-18-oic acid (**1**), three known clerodane diterpenes, 15,16-epoxy-7α,18-dihydroxy-15-methoxy-*ent*-clerod-3-ene (**2**),[6] 13,14-dihydro-marrubiagenine (**3**),[7] gaudichanolide A (**4**),[4] and three known flavones, 5,7,4′-trihydroxyflavone (**5**),[8] 5,7,3′,4′-tetrahydroxyflavone (**6**),[8] 5-methoxy-7,4′-dihydroxyflavone (**7**).[8] The known compounds were identified by comparing their spectroscopic data with those reported in the literature. Herein, we report the isolation and structural elucidation of the new clerodane diterpene and the biological activities of the isolated compounds as enhancers of NGF action.

Compound **1** was obtained as a colorless oil. Its molecular formula was determined as $C_{22}H_{36}O_5$ from its HR-FAB-MS (*m*/*z* 403.2468, $[M+Na]^+$, Calcd for $C_{22}H_{36}O_5Na$, 403.2460). The IR spectrum of **1** indicated the presence of hydroxyl (3500—2500 cm^{-1}) and carbonyl (1716, 1681 cm^{-1}) groups. The ^{1}H-NMR data (Table 1) for **1** exhibited typical signals for a diterpene with a clerodane-type skeleton, including one secondary methyl group at δ 0.76 (3H, d, J=5.4 Hz, H_3-17), two tertiary methyl groups at δ 0.71 (3H, s, H_3-20) and 1.22 (3H, s, H_3-19), and one α,β-unsaturated proton at δ 6.81 (1H, br s, H-3). In addition, one methyl proton signal of acetoxy at δ 2.04 (3H, s, $OCOCH_3$-16) was displayed in the ^{1}H-NMR spectrum of **1**. The ^{13}C-NMR spectroscopic data showed 22 carbon signals that were discriminated into three



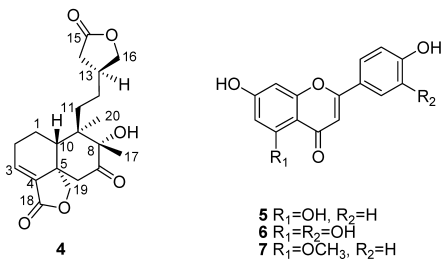


Table 1. ^{1}H- and ^{13}C-NMR Data of Compound **1** ($CDCl_3$, ^{1}H-NMR 600 MHz, ^{13}C-NMR 150 MHz)[a]

Position	^{13}C	^{1}H (J=Hz)
1	17.5 t	α 1.43, 1H, m
		β 1.61, 1H, m
2	27.3 t	α 2.27, 1H, m
		β 2.12, 1H, m
3	140.0 d	6.81, 1H, br s
4	141.7 s	
5	37.6 s	
6	35.9 t	α 2.38, 1H, d, J=12.4
		β 1.12, 1H, m
7	27.6 t	α 1.45, 1H, m
		β 1.37, 1H, m
8	36.2 d	1.43, 1H, m
9	38.7 s	
10	46.6 d	1.29, 1H, m
11	35.0 t	a 1.41, 1H, m
		b 1.26, 1H, m
12	24.1 t	a 1.09, 1H, m
		b 1.28, 1H, m
13	34.9 d	1.74, 1H, m
14	34.5 t	1.59, 2H, m
15	60.8 t	3.66—3.70, 2H, m
16	67.0 t	4.00, 2H, m
17	15.9 q	0.76, 3H, d, J=5.4
18	172.2 s	
19	20.6 q	1.22, 3H, s
20	18.5 q	0.71, 3H, s
16-$COCH_3$	171.4 s	
	21.0 q	2.04, 3H, s

a) All assignments based on the extensive 1D and 2D NMR spectra.

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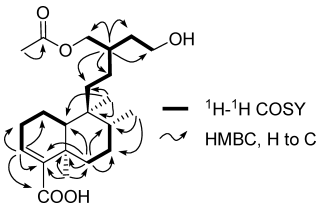

Fig. 1. Selected ^{1}H–^{1}H COSY and HMBC Correlations of Compound **1**

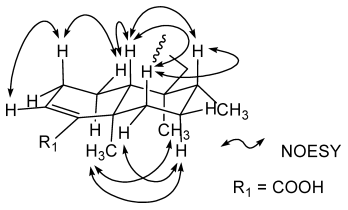

Fig. 2. Selected NOESY Correlations of Compound **1**

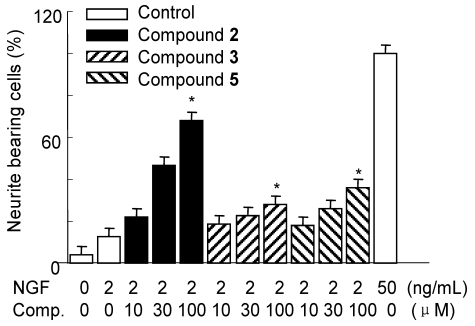

Fig. 3. Effects of Compounds **2**, **3** and **5** on the Proportion of Neurite-Bearing PC12D Cells in the Presence or Absence of NGF

The proportion of neurite-bearing cells is expressed as a percentage against the maximum response to NGF (50 ng/ml, 100%) in the absence of compounds. Values are mean±S.E. from four experiments. A statistically siginificant difference ($*p<0.01$) from the control (2 ng/ml NGF) in the absence of compounds was apparent.

methyls [δ 15.9 (C-17), 20.6 (C-19), and 18.5 (C-20)], nine methylenes [δ 17.5 (C-1), 27.3 (C-2), 35.9 (C-6), 27.6 (C-7), 35.0 (C-11), 24.1 (C-12), 34.5 (C-14), 60.8 (C-15), and 67.0 (C-16)], four methines [δ 140.0 (C-3), 36.2 (C-8), 46.6 (C-10), and 34.9 (C-13)], four quaternary carbons [δ 141.7 (C-4), 37.6 (C-5), 38.7 (C-9), and 172.2 (C-18)], and one acetoxy [δ 171.4, 21.0 (OCOCH$_3$-6)]. Close similarities of the chemical shifts from C-1 to C-10 and C-17 to C-20 for **1** with reported values for similar compounds[7,9,10] revealed that **1** is a diterpene with a clerodane-type skeleton. Comparison of the ^{13}C-NMR spectroscopic data of **1** (Table 1) with those of ballodiolic acid, a very similar clerodane,[10] indicated the presence of one acetoxy group [δ 171.4, 21.0 (OCOCH$_3$-16)] in compound **1**. By further analysis of HMQC, HMBC, and ^{1}H–^{1}H COSY spectra, all the proton and carbon signals were assigned unambiguously.

The relative stereochemistry of **1** was deduced from NOESY NMR spectrum. NOESY correlations observed for H-10/H-6β, H-10/H-8, and H-6β/H-8, but not for H-10/H$_3$-20 (H$_3$-17, H$_3$-19), suggested that two six-membered rings were fused with a *trans*-orientation and existed in a twist-chair and chair conformation. The H-10 proton was in a β-position with an axial orientation, H$_3$-19 and H$_3$-20 were in α-position with an axial orientation, and H$_3$-17 was in α-position with an equatorial orientation. These assignments were consistent with the configurations of H-10, H$_3$-17, H$_3$-19, and H$_3$-20 of clerodane-type diterpenes reported in the literature.[3,6,9] However, the stereochemistry of C-13 could not be deduced spectroscopically. Thus compound **1** was elucidated as the new clerodane diterpene, 15-hydroxy-16-acetoxy-*ent*-cleroda-3-en-18-oic acid.

The effects of compounds **1**—**7** on neurite outgrowth from PC12D cells were assessed as previously reported.[11] In control experiments, the percentages of neurite-bearing cells were 13% and 100% following 48-h incubation with NGF 2 and 50 ng/ml, respectively. Compounds **2**, **3**, and **5** (10, 30, 100 μmol) had no effect on neurite outgrowth from PC12D cells in the absence of NGF, but at 100 μmol markedly increased the NGF (2 ng/ml)-induced proportion of neurite-bearing cells by 68%, 28%, and 36%, respectively. However, compounds **1**, **4**, **6**, and **7** showed no activity on the proportion of neurite-bearing cells in either the absence or presence of NGF (2 ng/ml). Compounds that possess the property to enhance the action of NGF to stimulate neurite outgrowth from PC12D cells may be useful in the treatment of neurological disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and human immunodeficiency virus-associated dementia (HAD).[12,13]

Experimental

General Experimental Procedures Melting points were measured on a Yanaco micro-melting point apparatus without correction. Optical rotations were measured by JASCO DIP-370 polarimeter. Ultraviolet spectra were recorded on a Shimadzu UV-260 spectrophotometer, and the infrared spectra were obtained on a Shimadzu IR-408 spectrometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ and CD_3OD on an ECP-600 instrument. Preparative HPLC separations were performed on a Shimadzu LC-6A, equipped with UV-7000 detector at 205 nm, and a J'Sphere ODS-M80 (250×20 mm) column. FAB-MS and HR-FAB-MS were recorded on JMS-700 spectrometer. Chemicals for the biological studies were purchased from Wako Pure Chemical (Tokyo, Japan).

Plant Material The aerial parts of *B. gaudichaudiana*, which were imported from Paraguay, were provided by Serwa Pharmaceuticals Co., Ltd. (Ibaragi, Japan) in April 2000. The botanical identification was made by Mr. Tetsuo Nakasumi and a voucher specimen (No. 68536) was deposited in the Graduate School of Pharmaceutical Sciences, Tohoku University (Sendai, Japan).

Extraction and Isolation The aerial parts of *B. gaudichaudiana* (800 g) were extracted with MeOH (3×6 l), and the MeOH extracted (80 g) was subjected to silica gel (600 g; 70—230 mesh) column chromatography, using a gradient of EtOAC in *n*-hexane to give nine fractions (1—9). Then, fraction 4 was chromatographed by HPLC (YMC-Pack J'Sphere ODS-M80, 250×20 mm, 72% MeOH in H_2O, UV detector, 205 nm) to afford compound **1** (6.2 mg). Compounds **2** (4.7 mg), **3** (5.7 mg), and **4** (6.0 mg) were isolated from fraction 3 by HPLC (YMC-Pack J'Sphere ODS-M80, 250×20 mm, 76% MeOH in H_2O, UV detector, 205 nm). Purification of fraction 8 by HPLC (YMC-Pack J'Sphere ODS-M80, 250×20 mm, 68% MeOH in H_2O, UV detector, 205 nm) resulted in the isolation of compounds **5** (7.0 mg), **6** (7.2 mg), and **7** (6.3 mg).

Compound **1**: Colorless oil; $[\alpha]_D^{26}$ −94.1° (c=0.4, MeOH). UV λ_{max} (MeOH) nm: 214; IR (film) ν_{max} cm^{-1}: 2954,1716, 1681, 1384, 1240. ^{1}H- and ^{13}C-NMR see Table 1. Positive-ion FAB-MS *m/z* 403 $[M+Na]^+$, positive-ion HR-FAB-MS 403.2468 $[M+Na]^+$ (Calcd for $C_{22}H_{36}O_5Na$, 403.2460).

Bioassay Procedure The enhancing activity of NGF-mediated neurite outgrowth in PC12D cells was examined for the isolated compounds by the method previously reported.[12] PC12D cells were dissociated by incubation with 1 mmol of ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) in phosphate-buffered saline (PBS) for 30 min then seeded in 24-well culture plates (2×10^4 cells/well) coated with poly-1-lysine. After 24 h, the medium was changed to test medium containing various

concentrations of NGF (50 ng/ml for positive control; 2 ng/ml for test samples and significant difference control), 1% fetal calf serum, 2% horse serum, and various concentrations of test compounds (10, 30, 100 μmol). After 48 h the cells were fixed with 1% glutaraldehyde at 37 °C. The neurite outgrowth was assessed under a phase-contrast microscope. Neurite processes with a length equal to or greater than the diameter of the neuron cell body were scored as a neurite-bearing cell. The ratio of neurite-bearing cells to total cells (with $\geqslant$100 cells examined/viewing area; three viewing areas/well; six wells/sample) was determined and expressed as a percentage. Statistical comparisons were made using the Student *t*-test.

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