Bioactive *ent*-Clerodane Diterpenoids from the Aerial Parts of *Baccharis gaudichaudiana*

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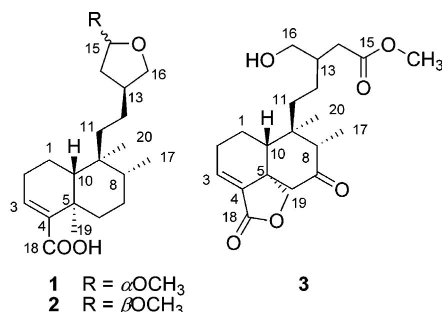
Three new *ent*-clerodane diterpenes (**1**−**3**) were isolated from the aerial parts of *Baccharis gaudichaudiana*, and their structures were elucidated as 15,16-epoxy-15α-methoxy-*ent*-clerod-3-en-18-oic acid (**1**), 13-*epi*-15,16-epoxy-15α-methoxy-*ent*-clerod-3-en-18-oic acid (**2**), and 7-oxo-16-hydroxy-*ent*-clerod-3-en-15-oic acid methyl ester-18,19-olide (**3**), on the basis of spectroscopic data analysis. A known compound, 7-oxo-*ent*-clerod-3-en-15,16:18,19-diolide, was also identified. Compounds **1** and **2** showed enhancing activity of nerve growth factor (NGF)-induced neurite outgrowth in PC 12D cells.

Baccharis gaudichaudiana DC. (Compositae) is used in Paraguay as a folk medicine for the treatment of diabetes and as a tonic, and for the relief of gastrointestinal ailments. Previous phytochemical investigations on this species resulted in the isolation of labdane-type and clerodane-type diterpenes.[1−5] Several labdane diterpene arabinosides with a sweet taste[1] evoked our attention to this plant. In the course of our search for the new natural products with NGF-potentiating activity from medicinal plants, we have studied the chemical constituents of a methanol extract of the aerial parts of *B. gaudichaudiana*, which increased the NGF (2 ng/mL)-induced proportion of neurite-bearing cells by 52% at a concentration of 60 μg/mL. The extract was subjected to silica gel column chromatography, followed by reversed-phase HPLC on an ODS column, to yield three new clerodane diterpenes (**1**−**3**) and a known analogue. In this paper, we report the isolation and structural elucidation of these clerodane diterpenes and their biological activities as enhancers of NGF action. The known compound 7-oxo-*ent*-clerod-3-en-15,16:18,19-diolide was identified by comparing its spectroscopic data with those reported in the literature.[6]



Compound **1** was obtained as a colorless oil. Its molecular formula was determined as $C_{21}H_{34}O_4$ from its HREIMS (*m/z* 349.2376, $[M - H]^+$, calcd for $C_{21}H_{33}O_4$, 349.2379). The IR spectrum of **1** indicated the presence of a hydroxy group (3500−2500 cm^{-1}) and a carbonyl group (1681 cm^{-1}). The ^{1}H NMR data (Table 1) for **1** exhibited typical signals for a diterpene with a clerodane-type skeleton,[3−6] including one secondary methyl group at δ 0.75 (3H, d, $J = 5.5$ Hz, H_3-17), two tertiary methyl groups at δ 0.68 (3H, s, H_3-20) and 1.19 (3H, s, H_3-19), and one α,β-unsaturated proton at δ 6.78 (1H, s, H-3). In addition, one methoxy group signal at δ 3.29 (3H, s, OCH_3-15) was also displayed in the ^{1}H NMR spectrum of **1**. The ^{13}C NMR spectroscopic data showed 21 carbon signals that were classified into three methyls [δ 16.0 (C-17), 20.6 (C-19), and 18.4 (C-20)], eight methylenes [δ 17.5 (C-1), 27.3 (C-2), 35.8 (C-6), 27.2 (C-7), 36.2 (C-11), 27.5 (C-12), 39.4 (C-14), and 72.7 (C-16)], five methines [δ 140.0 (C-3), 37.3 (C-8), 46.6 (C-10), 37.6 (C-13), and 105.3 (C-15)], four quaternary carbons [δ 141.7 (C-4), 37.6 (C-5), 38.6 (C-9), and 172.7 (C-18)], and one methoxyl group [δ 54.6 (OCH_3-15)]. 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[6−8] confirmed that **1** is a diterpene with an *ent*-clerodane-type skeleton (Table 1). By analyzing the HMQC, HMBC, and ^{1}H−^{1}H COSY NMR spectra, all the proton and carbon signals were assigned unambiguously.

The relative stereochemistry was deduced from the NOESY NMR spectrum of **1**. 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-positions with an axial orientation, and H_3-17 was in a α-position with an equatorial orientation, respectively. These assignments were consistent with the configurations of H-10, H_3-17, H_3-19, and H_3-20 of *ent*-clerodane-type diterpenes reported in the literature.[6−8] Furthermore, the evident NOEs of H-13/H-14α, H-14β/H-15, and H-13/H-16α, but not H-13/H-14β, implied that H-13 and H-15 are oriented on different sides of a furan ring in an α configuration and in a β configuration, respectively. By comparing the furan ring of compound **1** with those of similar compounds reported in the literature,[6] the relative stereochemistry of this ring was established. However, the absolute configuration of the furan ring was not able to be determined. Thus, compound **1** was elucidated as the new *ent*-clerodane diterpene 15,16-epoxy-15α-methoxy-*ent*-clerod-3-en-18-oic acid.

Compound **2** was obtained as a colorless oil. The molecular formula of **2** was determined to be $C_{21}H_{34}O_4$ by HREIMS (*m/z* 349.2363, $[M - H]^+$). The IR spectrum of **2** indicated the same functionalities as in **1**. As a result of comparing the ^{1}H and ^{13}C NMR spectroscopic data of **2** with those of **1** (Table 1), **2** was found to be an *ent*-clerodane diterpene related structurally to **1**. The chemical shifts of all the carbon signals except for C-13 (δ 39.1) in **2** were comparable with those of **1**, which occurred at δ 37.6 for C-13 in **1**. On the basis of an analysis of the HMQC, HMBC, and ^{1}H−^{1}H COSY spectra, the planar structure of **2** was deduced and was identical to **1**. The NOESY spectrum of **2** allowed the stereochemical features of **2** to be assigned. The NOESY correlations of H-10/H-6β, H-10/H-8, H-6β/H-8, and H_3-19/H_3-

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1**−**3** ($CDCl_3$, ^{1}H NMR 600 MHz, ^{13}C NMR 150 MHz)[a]

	1		**2**		**3**	
position	^{13}C	^{1}H (J = Hz)	^{13}C	^{1}H (J = Hz)	^{13}C	^{1}H (J = Hz)
1	17.5 t	α 1.41, 1H, m	17.5 t	α 1.39, 1H, m	20.3 t	α 1.12,1H, m
		β 1.57, 1H, m		β 1.58, 1H, m		β 1.78, 1H, br. s
2	27.3 t	α 2.28, 1H, m	27.3 t	α 2.25, 1H, m	27.5 t	α 2.44, 1H, m
		β 2.10, 1H, m		β 2.17, 1H, m		β 2.19, 1H, m
3	140.0 d	6.78, 1H, s	140.1 d	6.79, 1H, s	137.6 d	6.80, 1H, d, J = 6.2
4	141.7 s		141.7 s		136.2 s	
5	37.6 s		37.6 s		43.9 s	
6	35.8 t	α 2.36, 1H, d, J = 11.0	35.8 t	α 2.36, 1H, d, J = 12.4	50.6 t	α 2.58, 1H, m
		β 1.08, 1H, m		β 1.08, 1H, m		β 2.30, 1H, m
7	27.2 t	α 1.42, 1H, m	27.5 t	α 1.43, 1H, m	210.1 s	
		β 1.34, 1H, m		β 1.33, 1H, m		
8	37.3 d	1.15, 1H, m	37.5 d	1.15, 1H, m	51.5 d	2.54, 1H, m
9	38.6 s		38.6 s		48.2 s	
10	46.6 d	1.22, 1H, m	46.5 d	1.26, 1H, m	47.7 d	2.33, 1H, m
11	36.2 t	a 1.41, 1H, m	36.2 t	a 1.42, 1H, m	35.1 t	a 1.54, 1H, m
		b 1.07, 1H, m		b 1.07, 1H, m		b 1.35, 1H, m
12	27.5 t	a 1.06, 1H, m	26.5 t	a 1.14, 1H, m	23.9 t	a 1.37, 1H, m
		b 1.23, 1H, m		b 1.31, 1H, m		b 1.25, 1H, m
13	37.6 d	2.25, 1H, m	39.1 d	2.02, 1H, m	37.9 d	1.95, 1H, br s
14	39.4 t	α 2.02, 1H, m	39.2 t	α 1.45, 1H, m	36.3 t	a 2.44, 1H, m
		β 1.48, 1H, m		β 2.24, 1H, m		b 2.28, 1H, m
15	105.3 d	4.96, 1H, d, J = 4.8	105.7 d	4.98, 1H, d, J = 3.4	173.7 s	
16	72.7 t	α 4.02, 1H, t, J = 8.2	72.2 t	α 3.41, 1H, t, J = 8.3	64.8 t	3.57, 2H, m
		β 3.42, 1H, t, J = 7.6		β 3.96, 1H, t, J = 7.6		
17	16.0 q	0.75, 3H, d, J = 5.5	16.0 q	0.75, 3H, d, J = 4.8	7.7 q	0.92, 3H, d, J = 6.2
18	172.7 s		172.7 s		168.3 s	
19	20.6 q	1.19, 3H, s	20.8 q	1.19, 3H, s	71.3 t	a 3.94, 1H, d, J = 7.6
						b 3.86, 1H, d, J = 7.6
20	18.4 q	0.68, 3H, s	18.4 q	0.68, 3H, s	19.2 q	0.55, 3H, s
OMe-15	54.6 q	3.29, 3H, s	55.0 q	3.31, 3H, s	51.8 q	3.64, 3H, s

[a] All assignments are based on the measurement of 1D and 2D NMR spectra.

20, but not H-10/H_3-20 (H_3-17, H_3-19), revealed that the configurations of the two fused six-membered rings were the same as those of **1**. However, a different configuration of the furan ring in **2** was indicated by the NOE interactions observed between H-13/H-14β, H-14β/H-15, and H-13/H-16β, but not H-13/H-14α and H-13/H-16α, suggesting that H-13 and H-15 are oriented on the same side of the furan ring in a β configuration.[6,7] Furthermore, in the NOE difference spectrum of **2**, the irradiation of H-13 resulted in the enhancement of H-14β and H-16β, but not of H-14α, which confirmed the configuration of the furan ring. Thus, compound **2** was identified as 13-*epi*-15,16-epoxy-15α-methoxy-*ent*-clerod-3-en-18-oic acid.

Compound **3** was obtained as a colorless oil. Its molecular formula, $C_{21}H_{30}O_6$, was determined by HREIMS (m/z 378.2037, $[M]^+$). The IR spectrum of **3** showed three absorption bands at 3550−3400 cm^{-1} (hydroxyl group) and 1771 and 1704 cm^{-1} (carbonyl groups). Signals for methyl signals at δ 0.55 (3H, s, H_3-20) and 0.92 (3H, d, J = 6.2 Hz, H_3-17), a methoxy at δ 3.64 (3H, s, OCH_3-15), and a α,β-unsaturated proton at δ 6.80 (1H, d, J = 6.2 Hz, H-3) were displayed in the ^{1}H NMR spectrum. The ^{13}C NMR spectroscopic data indicated 21 carbon signals: two methyls [δ 7.7 (C-17) and 19.2 (C-20)], eight methylenes [δ 20.3 (C-1), 27.5 (C-2), 50.6 (C-6), 35.1 (C-11), 23.9 (C-12), 36.3 (C-14), 64.8 (C-16), and 71.3 (C-19)], four methines [δ 137.6 (C-3), 51.5 (C-8), 47.7 (C-10), and 37.9 (C-13)], six quaternary carbons [δ 136.2 (C-4), 43.9 (C-5), 210.1 (C-7), 48.2 (C-9), 173.7 (C-15), and 168.3 (C-18)], and one methoxy group [δ 51.8 (OCH_3-15)]. These data suggested that compound **3** possesses an *ent*-clerodane-type skeleton.[6−8] On comparing the ^{13}C NMR spectroscopic data of **3** with those of 7-oxo-16,19-dihydroxy-*ent*-clerod-3-en-15,20-diacid-dilactone[9] and gaudichanolide B,[5] the chemical shift value at δ 173.7 (C-15) in **3** was shifted upfield to about 2.9 ppm, corresponding to the carbon signal (δ 176.6, C-15) of the known compound, which implied the cleavage of the furan ring situated at C-12. All the proton and carbon signals of **3** were assigned unambiguously by analyzing its HMQC, HMBC, and ^{1}H−^{1}H COSY NMR spectra. Furthermore, the NOESY spectrum of **3** showed correlations of H-10/H-6β, H-10/H-8, H-6β/H-8, and H_3-20/H_2-19, but not of H-10/H_3-20 (H_3-17, H_2-19), and 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 determined as being in a β-position and arranged *cis*-axially to H-6β and H-8, with H_3-20 in an α-position with an axial orientation and H_3-17 in a α-position with equatorial orientation, respectively.[6−9] Therefore, the structure of compound **3** was elucidated as 7-oxo-16-hydroxy-*ent*-clerod-3-en-15-oic acid methyl ester-18,19-olide.

The effect of four *ent*-clerodane diterpenes on neurite outgrowth from PC12D cells was assessed as previously reported.[10] In control experiments, the percentages of neurite-bearing cells were 12% and 100% following incubation with 2 and 50 ng/mL NGF after 48 h, respectively. Compounds **1** and **2** (10, 30, and 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 49% and 53%, respectively. However, compound **3** and the known *ent*-clerodane diterpene 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).[11,12]

Experimental Section

General Experimental Procedures. Melting points were measured on a Yanaco micro-melting point apparatus without correction. Optical rotations were measured with a JASCO DIP-370 polarimeter. The 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 system, equipped with a UV-7000 detector at 205 nm, and a J'Sphere ODS-M80 (250 × 20 mm) column. EIMS and HREIMS were recorded on JMS DX-303 and JMS AX-700, respectively. 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 Seiwa Pharmaceuticals Co.

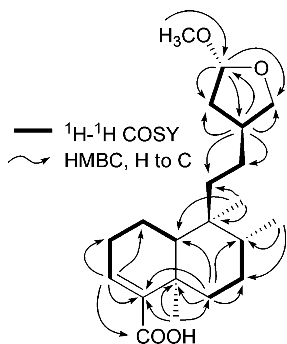

Figure 1. Selected $^1H-^1H$ COSY and HMBC correlations of compound **1**.

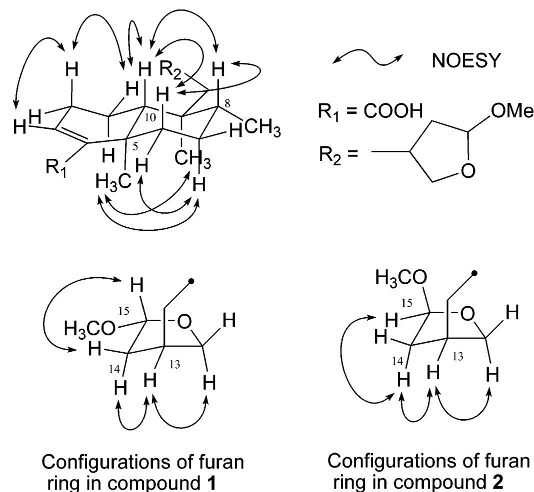

Figure 2. Selected NOESY correlations of compounds **1** and **2**.

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 extract (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). Of these, fraction 5 was chromatographed by HPLC (YMC-Pack J'Sphere ODS-M80, 250 × 20 mm, 78% MeOH in H_2O, UV detector, 205 nm) to afford compounds **1** (4.2 mg; t_R 46.0 min) and **2** (4.7 mg; t_R 50.0 min). Compound **3** (5.3 mg, t_R 37.0 min) was isolated from fraction 9 by HPLC (YMC-Pack J'Sphere ODS-M80, 250 × 20 mm, 70% MeOH in H_2O, UV detector, 205 nm). Purification of fraction 7 by HPLC (YMC-Pack J'Sphere ODS-M80, 250 × 20 mm, 73% MeOH in H_2O, UV detector, 205 nm) resulted in the isolation of a known clerodane diterpene, 7-oxo-*ent*-clerod-3-en-15,16:18,19-diolide (5.0 mg).

15,16-Epoxy-15α-methoxy-*ent*-clerod-3-en-18-oic acid (1): colorless oil; $[\alpha]^{26}_D$ −111.8 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 215 (4.53) nm; IR (film) ν_{max} 2956, 1681, 1629, 1384, 1262 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 349 $[M - H]^+$ (1), 300 (100), 285 (53), 221 (56), 202 (52), 137 (29), 125 (85); HREIMS *m/z* 349.2376 $[M - H]^+$ (calcd for $C_{21}H_{33}O_4$, 349.2379).

13-Epi-15,16-epoxy-15α-methoxy-*ent*-clerod-3-en-18-oic acid (2): colorless oil; $[\alpha]^{26}_D$ −13.4 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 215 (4.53); IR (film) ν_{max} 2953, 1681, 1629, 1383, 1262 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 349 $[M - H]^+$ (1), 300 (100), 285 (53), 221 (56), 202 (52), 137 (29), 125 (85) (1); HREIMS *m/z* 349.2363 $[M - H]^+$ (calcd for $C_{21}H_{33}O_4$, 349.2379).

7-Oxo-16-hydroxy-*ent*-clerod-3-en-15-oic acid methyl ester-18,-19-olide (3): colorless oil; $[\alpha]^{26}_D$ −92.5 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (4.48) nm; IR (film) ν_{max} 2935, 1771, 1704, 1274, 1173 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; EIMS *m/z* 378 $[M]^+$ (1), 346 (34), 316 (29), 233 (47), 203 (57), 175 (35), 136 (100), 120 (40), 92 (21); HREIMS *m/z* 378.2037 (calcd for $C_{21}H_{30}O_6$, 378.2042).

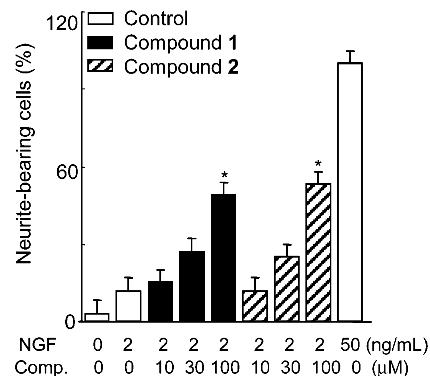

Figure 3. Effects of compounds **1** and **2** on the proportion of neurite-bearing PC12D cells in the presence or absence of NGF. The proportion of neurite-bearing cells is expressed as a percentage against the maximum response to NGF (50 ng/mL, 100%) in the absence of compounds. Values are means ± SE from four experiments. A statistically significant difference from the control (2 ng/mL NGF) in the absence of compounds is indicated in the figure: $*p < 0.01$.

Bioassay Procedure. The enhancing activity of NGF-mediated neurite outgrowth in PC12D cells was examined for the initial methanol extract of *B. gaudichaudiana* and the isolated compounds by a method previously reported.[10] 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 and then seeded in 24-well culture plates (2 × 10^4 cells/well) coated with poly-L-lysine. After 24 h, the medium was changed to test medium containing various concentrations of NGF (50 ng/mL for the positive control, 2 ng/mL for run-compounds), 1% fetal calf serum, 2% horse serum, and various concentrations of test materials (10, 30, and 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 the neurite-bearing cells to total cells (with at least 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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