A New *ent*-Clerodane Diterpene from the Aerial Parts of *Baccharis gaudichaudiana*

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A new *ent*-clerodane diterpene, named baccharіol (1) was isolated from the aerial parts of *Baccharis gaudichaudiana* DC. (Compositae), together with known *ent*-clerodane diterpenes (2, 3), eight known flavonoids (4—11) and 3,5-dicaffeoylquinic acid (12). Their structures were determined by spectroscopic analyses. Flavonoids (7, 8, 11) and 12 showed moderate scavenging activities toward 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals.

Key words *ent*-clerodane diterpene; *Baccharis gaudichaudiana*; Paraguayan folk medicine; baccharіol; antioxidant

Baccharis gaudichaudiana DC. (Compositae) is used as a folk medicine for antidiabetic and tonic, and for the treatment of gastrointestinal disease in Paraguay, where it is known by the local name "chilca melosa". A previous phytochemical investigation on this species resulted in the isolation of labdane-type diterpene glycosides,[1,2] apigenin, hispidulin, spathulenol and ursolic acid.[3] In the course of screening for antioxidants in medicinal plants,[4] the extract of the aerial parts of *B. gaudichaudiana* showed scavenging activity towards 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical in the TLC autographic assay.

This paper deals with the structural elucidation of a newly isolated *ent*-clerodane diterpene (**1**) and antioxidant activity of isolated compounds towards DPPH radical.

Results and Discussion

The 75% ethanol extract of the aerial parts of *B. gaudichaudiana* (620 g) was separated into *n*-hexane, EtOAc, *n*-BuOH and H_2O-soluble fractions. According to the activity of the EtOAc-soluble fraction in the TLC autographic assay,[5] repeated silica gel (*n*-hexane/EtOAc, $CHCl_3$/MeOH, $CHCl_3$/acetone), Sephadex LH-20 (H_2O/MeOH) and Cosmosil 75C_{18}-OPN (H_2O/MeOH) column chromatographies (CC) were performed to give a new compound, named baccharіol (**1**, bitter taste, 0.0288%/g dried material), and known flavonoids jaceosidin (**4**, 0.0007%),[6] desmethoxycentaureidin (**5**, 0.0006%),[7] hispidulin (**6**, 0.0035%),[8,9] quercetin-3-*O*-L-α-rhamnoside (**7**, 0.0463%),[10,11] eupafolin (**8**, 0.0050%),[8,12] eupatilin (**9**, 0.0025%)[8,13] and 3,5-dicaffeoylquinic acid (**12**, 0.0050%).[14] Isoschaftoside (**10**, 0.0542%)[15] and rutin (**11**, 0.0129%)[16] were isolated from the *n*-BuOH-soluble fraction by Toyopearl HW-40 (H_2O/MeOH), Sephadex LH-20 (H_2O/MeOH) and MCI-gel CHP20 (H_2O/MeOH) CC. An attempt to separate Dragendorff's reagent positive compounds in the EtOH extract resulted in isolation of two known *ent*-clerodane diterpenes (**2**, **3**, bitter taste, 0.0972, 0.0112%, respectively),[17,18] as described in experimental section. **2** and **3** were identified by comparing their spectroscopic data with values in the literatures (Table 1).

Baccharіol (**1**) was obtained as a white powder and the molecular formula was determined to be $C_{20}H_{24}O_6$ from high resolution (HR)-FAB mass spectrometry. IR spectrum of **1** showed an absorption at 3448 cm^{-1} for a hydroxyl group, absorptions at 1773, 1742, 1717 and 1180 cm^{-1} for two lactones and one ketone in **1**. The ^{1}H- and ^{13}C-NMR spectra of **1** were similar to those of the known *ent*-clerodane diterpene **2**[17,18] (Table 1). The ^{13}C-NMR spectrum of **1** showed 20 signals which revealed the presence of two sets of α,β-unsaturated carbonyl systems, one ketone carbon, seven methylene carbons, two methyl carbons, three quaternary carbons and one methine carbon. In the ^{1}H-NMR spectrum of **1**, signals of two methyl groups [δ 0.61 (s) and 1.32 (s)] were observed, and one [δ 1.32 (s)] of them was shifted to the downfield and observed as a singlet peak compared to the signal [δ 0.95 (d, J=6.7 Hz)] of the methyl group in **2**. The signal of a hydroxyl group [δ 2.63 (s)] was observed in the spectrum of **1**, and the group was thought to be attached to the deshielded carbon at 8 position (δ 82.6) by consideration of heteronuclear multiple bond connectivity (HMBC) correlations between the hydroxyl proton and carbons at 8 and 9 position (Fig. 2). From these results, **1** was deduced to be the 8-hydroxyl derivative of **2**. The planar structure was also supported by other HMBC correlations (Fig. 2). To confirm the

Table 1. ^{13}C-NMR Data for Compounds **1**—**3** (125 MHz, $CDCl_3$)

C	1	2	3
1	20.6	20.2	20.4
2	27.4	27.3	27.4
3	137.3	137.0	137.0
4	136.1	136.0	136.2
5	47.7	47.8[a]	43.6
6	45.4[a]	50.5	50.5
7	208.6	208.6	209.0
8	82.6	51.3	51.4
9	45.6[a]	43.4	48.0[a]
10	42.3	47.7[a]	47.8[a]
11	34.3	34.8	35.7[b]
12	25.0	22.3	26.9
13	170.9	168.8	35.9[b]
14	114.7	115.6	34.5
15	174.0	173.4	176.3
16	73.2	72.9	73.0
17	18.1	7.8	7.8
18	168.0	167.7	167.8
19	71.0	71.0	71.0
20	19.7	18.8	19.0

δ in ppm from TMS. *a*), *b*) Assignments may be interchanged in each column.

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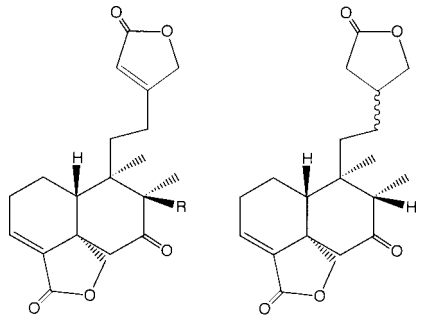

Fig. 1. Structures of *ent*-Clerodane Diterpenes **1**—**3**

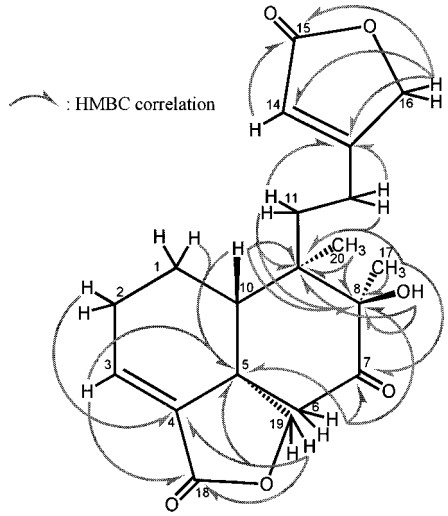

Fig. 2. Structure and Important HMBC Correlations for **1**

relative structure of **1**, the differential nuclear Overhauser effect (NOE) experiment was adapted. When the methyl protons (H-20) were irradiated, differential NOEs were observed at methyl proton signal (H-17, 3.3%) and 19α proton signal [δ 3.88 (dd, J=8.2, 2.1 Hz)] (3.7%), not at 10β proton signal [δ 2.99 (d, J=12.2 Hz)]. The differential NOE was observed at 6β proton signal [δ 3.14 (dd, J=12.1, 2.1 Hz)] (6.0%), not at methylene proton signal (H-19), when irradiated at 10β proton. Thus, the relative structure of **1** was the same as that of **2**. Furthermore, circular dichroism (CD) spectra of **1** ([θ]$_{245}$ −57900, [θ]$_{278}$ −5200, [θ]$_{309}$ −12300) was similar to that of **2**,[17,18] indicating that the absolute structure of **1** was the same as that of **2**. From these results, the structure of **1** was finally concluded to be (5*S*,8*R*,9*R*,10*R*)-8-hydroxy-7-oxo-*ent*-clerodan-3,13-dien-18,19:15,16-diolide (Fig. 1).

The DPPH radical scavenging activity was evaluated as IC$_{50}$ and Trolox equivalent value[19] for the isolated compounds. Quercetin-3-*O*-L-α-rhamnoside (**7**), eupafolin (**8**), rutin (**11**) and 3,5-dicaffeoylquinic acid (**12**) showed moderate activity (TEAC value 1.2, 1.1, 1.5, 1.2 mM, respectively) comparable to Trolox (1.0 mM) or quercetin (1.9 mM) as a positive control. Other isolated flavonoids, however, showed no activity probably due to the absence of a catechol function in the molecule. A new *ent*-clerodane diterpene (**1**) and two *ent*-clerodane diterpenes (**2**, **3**) did not show any antioxidant activity.

Some diterpenes are known to have antihyperglycemic activity.[20,21] Further study on a biological activity of those isolated *ent*-clerodane diterpenes is under consideration. Abundance of flavonoids in this plant may account for its folkloric use.

Experimental

General Melting points were determined on a Yanako melting point apparatus and are uncorrected. UV spectra were recorded with a Hitachi U-3200 spectrophotometer. IR spectra were recorded with a JASCO FT/IR-230 spectrophotometer. Optical rotations were measured with a JASCO DIP-140 digital polarimeter. CD curves were recorded on a JASCO J-720WI. NMR spectra were recorded on a JEOL JNM-α 400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C) or a JEOL JNM-α 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C). Chemical shifts are shown as δ values, using tetramethylsilane (TMS) as an internal reference. Correlation spectroscopy (COSY), ^{1}H-detected heteronuclear multiple quantum coherence (HMQC), HMBC and differential NOE experiments were obtained with the usual pulse sequence, and data processing was performed with the standard JEOL software. The J value in HMBC experiments was 8 Hz. FAB-MS were taken on a JEOL JMS-HX110A mass spectrometer in *m*-nitrobenzylalcohol (NBA) matrix in the positive mode. Column chromatography was carried out on Kieselgel 60 (70—230 mesh, 230—400 mesh)(Merck), Cosmosil 75C$_{18}$-OPN (Nacalai Tesque), Sephadex LH-20 (Amersham Biosciences), MCI-gel CHP20 (Mitsubishi Chemical Co.) and Toyopearl HW-40 (Tosoh Co.). TLC was performed on precoated silica gel 60 F$_{254}$ (0.25 mm) (Merck) or RP-18 F$_{254}$ (0.25 mm) (Merck), and spots were detected by UV (254 nm) or by 50% H_2SO_4 spraying reagent followed by heating.

Plant Material The aerial parts of *Baccharis gaudichaudiana* DC. (Compositae) were collected at Asunción suburb, Paraguay in May 2000, and identified by Dr. S. Seo. A voucher specimen (JH054002) is deposited in the Herbarium of Graduate School of Pharmaceutical Sciences, Chiba University, Japan.

Extraction and Isolation The dried aerial parts of *B. gaudichaudiana* (620 g) were extracted with 75% EtOH at room temperature. The solvent was evaporated under reduced pressure to give the extract (167 g). A portion (13.0 g) of the EtOH extract was acidified with 5% HCl after suspension with H_2O, and then extracted with $CHCl_3$, because some spots on TLC were detected with Dragendorff's reagent which suggested the presence of basic compounds in the EtOH extract. A portion (5.0 g) of the resulting $CHCl_3$ fraction (6.0 g) was subjected to silica gel CC ($CHCl_3$/MeOH) to give 8 fractions. Fraction 2 was subjected to silica gel CC to provide *ent*-clerodane diterpene (**2**, 39.1 mg, 0.0972%/g dried material) and was further separated by repeated silica gel (*n*-hexane/EtOAc, $CHCl_3$/EtOAc, MeOH) and ODS (H_2O/acetone) CC to provide *ent*-clerodane diterpene (**3**, 4.5 mg, 0.0112%). *ent*-Clerodane diterpenes (**2**, **3**) showed false positive for Dragendorff's reagent.

The EtOH extract (94 g) was partitioned with H_2O–*n*-hexane and the remaining H_2O layer was successively extracted with EtOAc and *n*-BuOH. A portion (10 g) of the EtOAc-soluble fraction (32.2 g) was subjected to silica gel CC with $CHCl_3$–MeOH of increasing polarity to give 10 fractions. Fraction 5 was applied to silica gel CC (*n*-hexane/EtOAc, MeOH) and further separated by repeated Sephadex LH-20 CC (H_2O/MeOH) to give jaceosidin (**4**, 0.8 mg, 0.0007%) and desmethoxycentaureidin (**5**, 0.6 mg, 0.0006%), respectively. Fraction 6 was applied to silica gel CC (*n*-hexane/EtOAc, MeOH) to provide hispidulin (**6**, 3.8 mg, 0.0035%). Fraction 10 was applied to silica gel ($CHCl_3$/MeOH), ODS (H_2O/MeOH) and Sephadex LH-20 (H_2O/MeOH) CC to provide quercetin-3-*O*-L-α-rhamnoside (**7**, 13.0 mg, 0.0463%), eupafolin (**8**, 1.4 mg, 0.0050%) and 3,5-dicaffeoylquinic acid (**12**, 1.4 mg, 0.0050%). A portion (5.1 g) of the EtOAc-soluble fraction was again separated by silica gel CC with $CHCl_3$/MeOH of increasing polarity to give 10 fractions. Fraction 2 was subjected to silica gel ($CHCl_3$/acetone) and ODS (H_2O/MeOH) CC to provide eupatilin (**9**, 1.4 mg, 0.0025%). Fraction 3 was also repeatedly subjected to silica gel CC (*n*-hexane/EtOAc, $CHCl_3$/acetone) to provide a new compound baccharіol (**1**, 15.9 mg, 0.0288%). Subsequently, a portion (0.62 g) of the *n*-BuOH-soluble fraction (18.6 g) was separated by Toyopearl HW-40 CC (H_2O/MeOH) to give 7 fractions. Fraction 2 was subjected to Sephadex LH-20 CC (H_2O/MeOH) to provide isoschaftoside (**10**, 6.3 mg, 0.0542%). Fraction 6 was applied to Sephadex LH-20 (H_2O/MeOH) and MCI-gel CHP20 (H_2O/MeOH) CC to provide rutin (**11**, 1.5 mg, 0.0129%).

Baccharіol (1) White powder, mp 179—182 °C. [α]$_D^{25}$: −117° (c=0.7, $CHCl_3$). UV λ_{max} (EtOH) nm (log ε): 208 (4.29). IR (KBr) cm^{-1}: 3448, 1773, 1742, 1717, 1261, 1180, 752. Positive HR-FAB-MS *m*/*z*: 361.1632

$[M+H]^+$ (Calcd for $C_{20}H_{25}O_6$: 361.1651). CD (EtOH, c=0.02) [θ] (nm): −57900 (245), −5200 (278), −12300 (309). ^{1}H-NMR (500 MHz, $CDCl_3$): δ: 0.61 (3H, s, H-20), 1.21 (1H, m, H-1), 1.32 (3H, s, H-17), 1.61 (1H, m, H-1), 1.73 (1H, m, H-11), 1.95 (1H, m, H-11), 2.34 (2H, m, H-2, H-12), 2.49 (1H, m, H-2), 2.49 (1H, d, J=12.1 Hz, H-6), 2.63 (1H, s, C-8-OH), 2.99 (1H, d, J=12.2 Hz, H-10), 3.10 (1H, m, H-12), 3.14 (1H, dd, J=12.1, 2.1 Hz, H-6), 3.88 (1H, dd, J=8.2, 2.1 Hz, H-19), 3.99 (1H, d, J=8.2 Hz, H-19), 4.75 (2H, d, J=1.5 Hz, H-16), 5.83 (1H, t, J=1.5 Hz, H-14), 6.84 (1H, dd, J=7.3, 2.1[Hz, H-3). ^{13}C-NMR (125 MHz, $CDCl_3$): See Table 1.

Compound 2 Viscous oil. $[\alpha]_D^{25}$: −112° (c=0.15, MeOH). IR (KBr) cm^{-1}: 1784, 1746, 1707, 1636, 1250, 1172, 756. FAB-MS m/z: 345 $[M+H]^+$. CD (MeOH, c=0.025) [θ] (nm): −22700 (245), −3700 (278), −5000 (297). ^{1}H-NMR (500 MHz, $CDCl_3$): δ: 0.62 (3H, s, H-20), 0.95 (3H, d, J=6.7 Hz, H-17), 1.65 (1H, m, H-11), 1.68 (1H, m, H-1), 1.78 (1H, m, H-11), 2.25 (1H, m, H-2), 2.30 (2H, m, H-6, H-12), 2.33 (1H, m, H-10), 2.40 (1H, m, H-12), 2.48 (1H, m, H-2), 2.53 (1H, q, J=6.7 Hz, H-8), 2.65 (1H, d, J=12.5 Hz, H-6), 3.87 (1H, dd, J=8.2, 2.1 Hz, H-19), 3.94 (1H, d, J=8.2 Hz, H-19), 4.73 (2H, s, H-16), 5.84 (1H, t, J=1.8 Hz, H-14), 6.80 (1H, dd, J=7.3, 2.1 Hz, H-3). ^{13}C-NMR (125 MHz, $CDCl_3$): See Table 1.

Compound 3 Viscous oil. $[\alpha]_D^{25}$: −121° (c=0.3, MeOH). IR (KBr) cm^{-1}: 1770, 1705, 1663, 1173, 755. FAB-MS m/z: 347 $[M+H]^+$. CD (MeOH, c=0.025) [θ] (nm): −18800 (245), −3200 (280), −4100 (297). ^{1}H-NMR (500 MHz, $CDCl_3$): δ: 0.61 (3H, s, H-20), 0.97 (3H, d, J=6.7 Hz, H-17), 1.35 (2H, m, H-11, H-12), 1.50 (2H, m, H-11, H-12), 1.70 (2H, m, H-1), 2.19 (1H, dd, J=17.4, 7.6 Hz, H-14), 2.25 (1H, m, H-2), 2.31 (1H, m, H-10), 2.32 (1H, m, H-6), 2.50 (1H, m, H-2), 2.51 (1H, m, H-13), 2.53 (1H, q, J=6.7 Hz, H-8), 2.69 (1H, d, J=17.4, 8.2 Hz, H-14), 2.70 (1H, d, J=12.8 Hz, H-6), 3.89 (1H, dd, J=8.2, 2.1 Hz, H-19), 3.93 (1H, dd, J=9.2, 7.3 Hz, H-16), 3.98 (1H, d, J=8.2 Hz, H-19), 4.46 (1H, dd, J=9.2, 7.3 Hz, H-16), 6.84 (1H, dd, J=7.3, 2.1 Hz, H-3). ^{13}C-NMR (125 MHz, $CDCl_3$): See Table 1.

Preliminary Test of Bitter Taste The bitter taste of *ent*-clerodane diterpenes was determined by a human sensor.

DPPH Radical Scavenging Activity[5,19] DPPH reagent was prepared at 80 μg/ml in MeOH in the TLC autographic assay. For the quantitative test, a test sample (50 μl) was dissolved in EtOH or DMSO and mixed with 100 mM Tris–HCl buffer (pH 7.4, 50 μl), distilled water (50 μl) and 400 μM DPPH ethanolic solution (50 μl). The mixture was shaken well and stood for 20 min in the dark. The absorbance at 515 nm was measured by microtiter plate reader (Biorad, model 550). Vitamin E derivative Trolox and quercetin were used as positive control. The decrease in absorbance/μM of each sample was compared with that of Trolox.

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