Sterol and Triterpenoid Constituents of *Verbena littoralis* with NGF-Potentiating Activity

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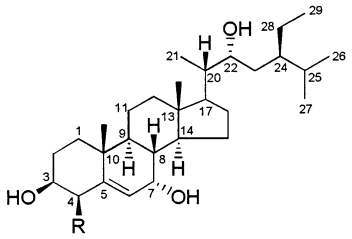
Two new sterols, stigmast-5-ene 3β,4β,7α,22α-tetraol (**1**) and stigmast-5-ene 3β,7α,22α-triol (**2**), were isolated from the aerial parts of a Paraguayan medicinal plant, *Verbena littoralis*, together with the known compounds ursolic acid (**3**) and oleanolic acid (**4**). The structures of **1** and **2** were elucidated by spectroscopic analyses. Compounds **2**−**4** showed an enhancing activity of nerve growth factor (NGF)-mediated neurite outgrowth in PC12D cells.

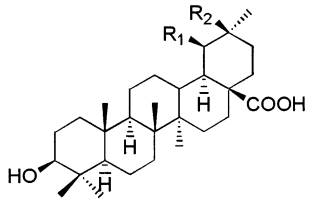
Verbena littoralis H. B. K. (Verbenaceae) has been used widely as a traditional folk medicine for diarrhea, typhoid fever, and tonsillitis in South America.[1] In 1999, a collection of the aerial parts of *V. littoralis* from Paraguay gave an extract that showed an enhancing activity of nerve growth factor (NGF)-mediated neurite outgrowth from PC12D cells. Activity-guided fractionation led to the isolation of gelsemiol, littoralisone, and verbenachalcone as enhancers of the action of nerve growth factor from the methanol extract of *V. littoralis*.[2−4] This extract was further studied by monitoring the potentiation of NGF action to afford two new sterols, stigmast-5-ene 3β,4β,7α,22α-tetraol (**1**) and stigmast-5-ene 3β,7α,22α-triol (**2**), along with the known compounds ursolic acid (**3**) and oleanolic acid (**4**). This paper deals with the isolation and structure elucidation of compounds **1** and **2** and the biological activity of **1**−**4** as enhancers of NGF action.

The ethyl acetate-soluble portion of the methanol extract of the aerial parts of *V. littoralis* was subjected to silica gel column chromatography (EtOAc−MeOH, 100:0 → 0:100) to give fractions I−X, followed by a series of bioassay-directed chromatographic separations, employing passage over Sephadex LH-20 and silica gel, and reversed-phase semipreparative HPLC, culminating in recrystallization. As a result, the two new sterols **1** and **2** were isolated from fraction IX, and the two known compounds **3** and **4** from fraction V, respectively.

Sterol **1** showed a molecular ion peak at *m/z* 462 in the EIMS, and its molecular formula was determined to be $C_{29}H_{50}O_4$ by HREIMS [*m/z* 462.3687, Δ −2.3 mmu]. The IR spectrum of **1** showed an absorption band at 3373 cm^{-1} characteristic of a hydroxyl group. A close scrutiny of the ^{1}H and ^{13}C NMR spectra of **1** (Table 1) by DEPT and HMQC experiments revealed the presence of six methyl groups, including one primary (C-29), three secondary (C-21, C-26, and C-27), and two tertiary (C-18 and C-19) groups. Also observed were eight methylenes (C-1, C-2, C-11, C-12, C-15, C-16, C-23, and C-28), 11 sp^3-hybridized methines (C-3, C-4, C-7, C-8, C-9, C-14, C-17, C-20, C-22, C-24, and C-25), including four hydroxymethines (C-3, C-4, C-7, and C-22), two quaternary sp^3-carbons (C-10 and C-13), and one trisubstituted double bond (C-5 and C-6). The ^{1}H−^{1}H COSY analysis allowed the covalent connectivities of **1** to be established (shown by bold-faced lines in Figure S1, Supporting Information), which were supported by HMQC and HMBC correlations. The connections of these partial structures were determined using the HMBC correlations. On the basis of this evidence, the planar structure of sterol **1** could be elucidated.



	R
1	OH
2	H



	R_1	R_2
3	CH_3	H
4	H	CH_3

The stereochemistry of **1** was established by a combination of observed coupling constants and NOESY data (Table 1 and Figure S2, Supporting Information). The NOEs from H_3-19 to H-2β and from H-3 to H-1α, H-2α, and H-4 and the coupling constants between H-3 and H-2β (J = 11.8 Hz) and H-4 (J = 2.8 Hz) suggested that the A-ring exists in a chair conformation. The 3- and 4-hydroxy groups were oriented on the same side of A-ring in a β-position and therefore in an equatorial and an axial arrangement, respectively. The 10-methyl group was in a β-position and in an axial arrangement. The observed NOEs from H-7 to

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Table 1. ^{1}H NMR and ^{13}C NMR Data of **1**[a] (CD_3OD, ^{1}H NMR 600 MHz, ^{13}C NMR 150 MHz)

position	^{13}C	^{1}H
1	36.9 t	α 1.13, 1H, m
		β 1.84, 1H, m
2	24.4 t	α 1.56, 1H, m
		β 1.95, 1H, m
3	72.2 d	3.50, 1H, dt, J = 11.8, 2.8 Hz
4	77.1 d	4.09, 1H, d, J = 2.8 Hz
5	146.6 s	
6	128.6 d	5.75, 1H, d, J = 5.3 Hz
7	64.6 d	3.83, 1H, t, J = 4.4 Hz
8	37.8 d	1.56, 1H, dd, J = 11.8, 4.4 Hz
9	42.5 d	1.27, 1H, m
10	36.8 s	
11	23.9 t	α 1.15, 1H, m
		β 1.81, 1H, m
12	39.2 t	α 1.18, 1H, m
		β 2.03, 1H, dt, J = 12.4, 3.5 Hz
13	42.2 s	
14	49.0 d	1.46, 1H, m
15	19.9 t	1.50, 2H, m
16	27.5 t	α 1.82, 1H, m
		β 1.41, 1H, m
17	53.0 d	1.15, 1H, m
18	10.7 q	0.74, 3H, s
19	18.6 q	1.18, 3H, s
20	42.9 d	1.71, 1H, m
21	11.6 q	0.94, 3H, d, J = 6.9 Hz
22	70.5 d	3.70, 1H, dd, J = 10.7, 2.8 Hz
23	29.4 t	a 1.03, 1H, dd, J = 13.2, 10.7 Hz
		b 1.29, 1H, m
24	41.1 d	1.35, 1H, m
25	28.8 d	1.78, 1H, m
26	16.9 q	0.81, 3H, d, J = 6.9 Hz
27	19.4 q	0.91, 3H, d, J = 6.9 Hz
28	22.8 t	a 1.25, 1H, m
		b 1.38, 1H, m
29	10.8 q	0.88, 3H, d, J = 6.9 Hz

[a] Spectra were determined in CD_3OD; data are reported in ppm. All protons and carbons were assigned by ^{1}H−^{1}H COSY, long-range ^{1}H−^{1}H COSY, DEPT, NOESY, HMQC, and HMBC spectra.

H-8, from H-8 to H-11β, H_3-19, and H_3-18, from H-9 to H-12α and H-14, from H_3-18 to H-11β and H-15β, and from H-14 to H-16α, as well as a coupling constant between H-7 and H-8 (J = 4.4 Hz), implied that the B- and C-rings exist in a twist-chair and chair conformation, respectively. The H-7 proton was in a β-position and arranged *cis*-equatorially to H-8, and OH-7 *cis*-axially to H-9 and H-14, with the 13-methyl group arranged axially. The C/D-rings were fused with a *trans*-orientation. The presence of a cross-peak between H-14 and H-17 provided evidence that the C-17 side chain was in a β-position.

The C-20*S* and C-22*R* configuration of **1** was suggested by the NOEs from H-21 to H-12β, H-17, and H-18, from H-20 to H-16β, H-18, and H-21, and from H-22 to H-16α, H-20, and H-24. The utility of the ^{13}C NMR chemical-shift difference of C-20 has been reported to discriminate (22*R*)- and (22*S*)-hydroxycholesterols, where the observed chemical shifts of C-20 were δ_C 42.6 and 40.3, respectively.[5] The application of this chemical shift rule for **1** (δ_C 42.89 for C-20) supported the configuration of C-22 as *R*, as described above. The assignment of the configuration at C-24 was suggested as *R* by comparing the ^{13}C NMR data of C-20 to C-25 of **1** (Table 1) with those of schleicherastatin 1 [δ_C 42.5 (C-20), 12.3 (C-21), 71.3 (C-22), 29.9 (C-23), 41.4 (C-24), and 28.7 (C-25)], whose stereochemistry at the C-24 position had been established by X-ray crystal structure determination.[6] According to this evidence, the configuration of C-24 was deduced as *R*. Thus, the new sterol **1** was determined to be stigmast-5-ene 3β,4β,7α,22α-tetraol.

The structure of sterol **2** was deduced from the structure of **1** combined with 1D NMR, 2D NMR, and mass spectral interpretations on **2**. Sterol **2** was found to have the molecular formula $C_{29}H_{50}O_3$ by HREIMS [*m*/*z* 446.3737, Δ −2.3 mmu]. The IR spectrum of **2** showed an absorption band at 3370 cm^{-1} (hydroxyl group). The ^{1}H and ^{13}C NMR spectra of sterol **2** revealed that the hydroxyl group at the C-4 position in **1** was replaced by hydrogen in sterol **2**. The chemical shifts of all the carbon signals except for C-2 (δ_C 31.33), C-3 (δ_C 71.32), C-4 (δ_C 41.96), and C-6 (δ_C 123.79) and the coupling constants of H-3 (tt, $J_{2\beta,3} = J_{3,4\beta}$ = 11.4 Hz and $J_{2\alpha,3} = J_{3,4\alpha}$ = 4.8 Hz) and H-22 (br d, $J_{22,23a}$ = 10.4 Hz) in **2** were comparable with those of **1**. The ROESY spectrum of **2** allowed the stereochemical features of **2** to be assigned. The configurations of all the asymmetric centers in sterol **2** were the same as those of the asymmetric centers in sterol **1** apart from C-4, which was a methylene carbon. Thus, the structure of **2** was established as stigmast-5-ene 3β,7α,22α-triol. The 3α isomer of **2**, stigmast-5-ene 3α,7α,22α-triol, was earlier reported from *Corylus avellena*.[7]

The structures of the two known triterpenoid compounds **3** and **4** were determined as ursolic acid and oleanolic acid, respectively, on the basis of spectral data comparison with literature values.[8,9]

The propensity of **1**−**4** to enhance the effects of NGF to stimulate neurite outgrowths was assessed using methodology previously reported.[10] In control experiments, the percentage of neurite-bearing cells was 11.8% and 42.3%, following incubation with 2 and 30 ng/mL NGF, respectively. The NGF (2 ng/mL)-induced increase in the proportion of neurite-bearing cells in PC12D cells (Figure S3, Supporting Information) was enhanced by **2** (3−10 μM), **3** (1−3 μM), and **4** (3−10 μM), but not by **1** (3−10 μM). PC12D cells were not affected morphologically by each compound up to 30 μM in the absence of NGF.

Experimental Section

General Experimental Procedures. Melting points were determined on a Yanaco micro-melting point apparatus and were uncorrected. Optical rotation values were measured with a Horiba SEPA-300 polarimeter. The ultraviolet spectra were recorded on a Hitachi U-2000 spectrophotometer, and the infrared spectra were obtained on a Shimadzu FTIR-8300 spectrophotometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ or CD_3OD on a JEOL GX-500 and a JEOL ECP-600 instrument. Chemical shifts were measured using residual $CHCl_3$ (δ_H 7.24 and δ_C 77.03) and MeOH-d_4 multiplet (δ_H 3.30 and δ_C 49.0) as internal standard. LREIMS and HREIMS were recorded on a JMS DX-303 and a JMS AX-500 spectrometer.

Plant Material. The aerial parts of *V. littoralis* were provided by Seiwa Yakuhin Co., Ltd. (Ibaragi, Japan), on April 20, 2000. The botanical identification was made by Mr. Tetsuo Nakasumi (Instituto de Pesquisas de Plantas Medicinais do Brasil, Sao Paulo, Brazil). A voucher specimen (No. 68531) is deposited in the Graduate School of Pharmaceutical Sciences, Tohoku University (Sendai, Japan).

Extraction and Isolation. The aerial parts of *V. littoralis* (5 kg) were ground and extracted with MeOH (15 L) three times, and the MeOH extract (208 g) was partitioned between EtOAc and H_2O (1:1). Portions (20 g) of the ethyl acetate-soluble fraction (120 g) were subjected to silica gel column chromatography (EtOAc−MeOH) to give fractions I−X. The most active fraction, IX (1.5 g), was separated by a series of bioassay-directed chromatographic separations employing Sephadex LH-20 and silica gel column chromatographic steps and culminating in a reversed-phase semipreparative HPLC separation on a YMC-AM 324 column (ODS, 30 × 1 cm i.d. stainless column, 78% MeOH in H_2O, 1 mL/min) to give the sterols **1** (15.6 mg) and **2** (8.0 mg). The active fraction V (2.0

g) was further separated by a similar procedure and finally purified with a semipreparative HPLC column (YMC-AM 324, ODS, 30 × 1 cm i.d. stainless column, 88% MeOH in H_2O, 1 mL/min) to afford compounds **3** (13.5 mg) and **4** (8.4 mg), respectively.

Stigmast-5-ene 3*β*,4*β*,7α,22α-tetraol (1): colorless needle crystals (MeOH); mp 148−150 °C; $[\alpha]^{27}_D$ −32.5° (*c* 0.1, MeOH); UV (MeOH) λ_{max} 205 (log ϵ 4.42) nm; IR (film) ν_{max} 3373, 2931, 1261, 1033, 802 cm^{-1}; ^1H and ^{13}C NMR data (Table 1); LREIMS *m*/*z* 462 $[M]^+$ (7), 444 (73), 408 (59), 397 (48), 316 (100), 298 (83), 273 (36), 229 (22), 174 (44), 163 (50); HREIMS *m*/*z* 462.3687 $[M]^+$ (calcd for $C_{29}H_{50}O_4$, 462.3709).

Stigmast-5-ene 3*β*,7α,22α-triol (2): colorless needle crystals (MeOH); mp 143−145 °C; $[\alpha]^{27}_D$ −23.7° (*c* 0.3, MeOH); UV (MeOH) λ_{max} 205 (log ϵ 4.10) nm; IR (film) ν_{max} 3370, 2932, 1261, 1033, 802 cm^{-1}; ^1H NMR ($CDCl_3$, 500 MHz) δ 0.71 (3H, s, H_3-18), 0.78 (3H, d, J = 7.0 Hz, H_3-26), 0.88 (3H, t, J = 7.0 Hz, H_3-29), 0.89 (3H, d, J = 7.0 Hz, H_3-27), 0.93 (3H, d, J = 6.7 Hz, H_3-21), 0.99 (3H, s, H_3-19), 1.03 (1H, m, H-23a), 1.12 (1H, m, H-1α), 1.15 (1H, m, H-11α), 1.16 (1H, m, H-17), 1.18 (1H, m, H-12α), 1.24 (1H, m, H-23b), 1.25 (2H, m, H-24 and H-28a), 1.26 (1H, m, H-9), 1.38 (2H, m, H-16*β* and H-28b), 1.45 (1H, m, H-8), 1.46 (1H, m, H-14), 1.48 (1H, m, H-15*β*), 1.55 (1H, m, H-2*β*), 1.72 (2H, m, H-15α and H-20), 1.74 (1H, m, H-16α), 1.79 (1H, m, H-25), 1.80 (1H, m, H-11*β*), 1.86 (1H, m, H-1*β*), 1.87 (1H, m, H-2α), 2.01 (1H, dt, J = 12.6, 3.5 Hz, H-12*β*), 2.27 (1H, dd, J = 13.2, 11.4 Hz, H-4*β*), 2.32 (1H, dd, J = 13.2, 4.8 Hz, H-4α), 3.59 (1H, tt, J = 11.4, 4.8 Hz, H-3), 3.72 (1H, br d, J = 10.4 Hz, H-22), 3.83 (1H, br s, H-7), 5.58 (1H, d, J = 4.8 Hz, H-6); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 11.6 (q, C-18), 11.9 (q, C-29), 12.3 (q, C-21), 17.5 (q, C-26), 18.2 (q, C-19), 20.5 (q, C-27), 20.7 (t, C-15), 23.6 (t, C-28), 24.4 (t, C-11), 27.5 (t, C-16), 28.7 (d, C-25), 29.8 (t, C-23), 31.3 (t, C-2), 37.0 (t, C-1), 37.4 (s, C-10), 37.5 (d, C-8), 39.1 (t, C-12), 41.4 (d, C-24), 42.0 (t, C-4), 42.3 (d, C-9 and d, C-13), 42.5 (d, C-20), 49.1 (d, C-14), 52.8 (d, C-17), 65.3 (d, C-7), 71.28 (d, C-22), 71.32 (d, C-3), 123.8 (d, C-6), 146.4 (s, C-5); LREIMS *m*/*z* 446 $[M]^+$ (5), 428 (87), 412 (6), 395 (2), 300 (53), 267 (6), 213 (7), 176 (19), 158 (28); HREIMS *m*/*z* 446.3737 $[M]^+$ (calcd for $C_{29}H_{50}O_3$, 446.3760).

Bioassay Procedure. The neuritogenic activity of **1**−**4** on PC12D cells was examined as previously described.[10]

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Supporting Information Available: Details of the NMR experiments performed on compound **1** are summarized in Figures 1 and 2. The results of the neuritogenic activity examined on compounds **1**−**4** are summarized in Figure 3. Included are 18 pages of actual spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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