Verbenachalcone, a Novel Dimeric Dihydrochalcone with Potentiating Activity on Nerve Growth Factor-Action from *Verbena littoralis*

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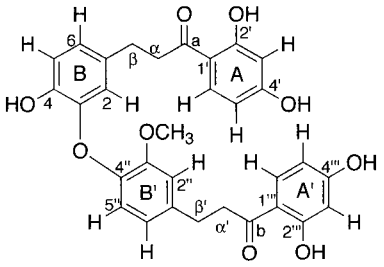
Received December 15, 2000

A novel dimeric dihydrochalcone, verbenachalcone (**1**), was isolated from the aerial parts of *Verbena littoralis*. Its structure was elucidated, on the basis of spectral data interpretation, as 4,2′,4′,2‴,4‴-pentahydroxy-3″-methoxy-3-*O*-4″-tetrahydrobichalcone. This compound caused a significant enhancement of nerve growth factor-mediated neurite outgrowth from PC12D cells.

Verbena littoralis H. B. K. (Verbenaceae) has been used widely as a traditional folk medicine for diarrhea, typhoid fever, and tonsillitis in Paraguay. In the course of our investigations of biologically active substances from medicinal plants,[1−4] we have devoted our attention to the discovery of natural products having neuritogenic activity since these compounds may be useful for the medical treatment of dementia.[5] Recently, we found that a crude extract of *V. littoralis* caused a greater number of PC12D cells to generate neurites in the presence of NGF than treatment of the cells with NGF alone. This extract was fractionated by monitoring the potentiating activity of NGF-action to give a novel compound having a dimeric dihydrochalcone skeleton, for which we have given the name verbenachalcone (**1**).



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The ethyl acetate-soluble portion of the methanolic extract of *V. littoralis* collected in Paraguay was chromatographed over Si gel to give eight fractions, of which the activity was evaluated. Of these, fraction 5 was the most active and was subjected to passage over Sephadex LH-20 to give eight subfractions. Active subfraction 5 was purified by reversed-phase semipreparative HPLC, to give verbenachalcone (**1**).

HREIMS determined the molecular formula for verbenachalcone (**1**) to be $C_{31}H_{28}O_9$. The structural characterization of **1** was performed by analyzing its EIMS and 1D and 2D NMR spectroscopic data (see Table 1 and Figure 1). The ^{1}H NMR spectrum displayed 12 signals for aromatic hydrogen atoms, eight signals for methylene hydrogens, and five hydroxyl groups (two chelated and three nonchelated). The analysis of the ^{13}C NMR, DEPT, and HMQC spectra of **1** indicated the presence of 24 aromatic carbons, four sp^3 methylene carbons, two carbonyls, and one methoxy carbon.

Table 1. ^{1}H and ^{13}C NMR Data for Verbenachalcone (**1**) ($CDCl_3$, ^{1}H NMR 500 MHz, ^{13}C NMR 125 MHz)[a]

position	^{13}C	^{1}H
1	138.02 s	
2	113.18 d	6.80, 1H, d, *J* = 2.0 Hz
3	144.94 s	
4	143.48 s	
5	115.92 d	6.90, 1H, d, *J* = 8.1 Hz
6	120.91 d	6.73, 1H, dd, *J* = 8.1, 2.0 Hz
1′	113.80 s[b]	
2′	165.32 s[c]	
3′	103.61 d	6.34, 1H, d, *J* = 2.5 Hz
4′	162.82 s[c]	
5′	107.83 d[d]	6.32, 1H, d, *J* = 8.7, 2.5 Hz
6′	132.39 d	7.56, 1H, d, *J* = 8.7 Hz[e]
α	39.38 t	3.20, 2H, t, *J* = 7.1 Hz
β	30.79 t	3.03, 1H, t, *J* = 7.1 Hz
a	203.88 s	
1″	132.65 s	
2″	117.46 d	6.56, 1H, d, *J* = 2.0 Hz
3″	150.81 s	
4″	145.47 s	
5″	120.91 d	6.88, 1H, d, *J* = 8.1 Hz
6″	123.67 d	6.81, 1H, dd, *J* = 8.1, 2.0 Hz
1‴	114.54 s[b]	
2‴	165.32 s[c]	
3‴	103.61 d	6.35, 1H, d, *J* = 2.5 Hz
4‴	165.39 s[c]	
5‴	107.73 d[d]	6.32, 1H, d, *J* = 8.7, 2.5 Hz
6‴	132.39 d	7.54, 1H, d, *J* = 8.7 Hz[e]
α′	39.78 t	3.09, 1H, t, *J* = 7.1 Hz
β′	30.04 t	2.87, 2H, t, *J* = 7.1 Hz
b	204.11 s	
OH-4		5.98, 1H, s
OH-2′		12.6, 1H, s[f]
OH-4′		6.32, 1H, br s[g]
OH-2‴		12.7, 1H, s[f]
OH-4‴		5.96, 1H, br s[g]
CH_3O-3″	56.00 q	3.78, 1H, s

[a] Spectra determined in $CDCl_3$; data reported in ppm. All protons and carbons were assigned by ^{1}H−^{1}H COSY, DEPT, HMQC, and HMBC spectra. [b−g] Signals may be interchangeable.

The ^{1}H NMR spectrum of **1** showed four sets of ABX system signals at *δ* 6.90 (1H, d, *J* = 8.1 Hz, H-5), 6.73 (1H, dd, *J* = 8.1, 2.0 Hz, H-6), and 6.80 (1H, d, *J* = 2.0 Hz, H-2) in ring B, at *δ* 7.56 (1H, d, *J* = 8.7 Hz, H-6′), 6.32 (1H, dd, *J* = 8.7, 2.5 Hz, H-5′), and 6.34 (1H, d, *J* = 2.5 Hz, H-3′) in ring A, at *δ* 6.88 (1H, d, *J* = 8.1 Hz, H-5″), 6.81 (1H, dd, *J* = 8.1, 2.0 Hz, H-6″), and 6.56 (1H, d, *J* = 2.0 Hz, H-2″)

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10.1021/np000602w CCC: $20.00
Published on Web 05/23/2001

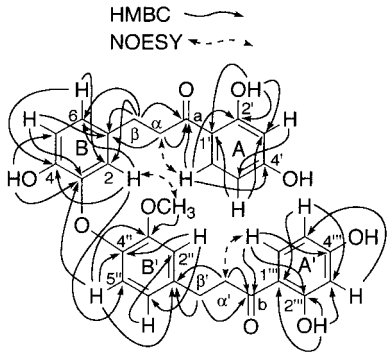

Figure 1. Selected HMBC and NOESY correlations of verbenachalcone (**1**).

in ring B′, and at δ 7.54 (1H, d, J = 8.7 Hz, H-6‴), 6.32 (1H, dd, J = 8.7, 2.5 Hz, H-5‴), and 6.35 (1H, d, J = 2.5 Hz, H-3‴) in ring A′. The ^{1}H NMR spectrum also showed two pairs of triplets of the A_2B_2 type, one pair at δ 3.20 (2H, t, J = 7.1 Hz, H-α) and 3.03 (2H, t, J = 7.1 Hz, H-β), and another at δ 3.09 (2H, t, J = 7.1 Hz, H-α′) and 2.87 (2H, t, J = 7.1 Hz, H-β′). From a detailed analysis of the ^{1}H−^{1}H COSY and HMBC NMR data of **1**, it was concluded that the first pair belonged to the α (δ_C 39.38) and β (δ_C 30.79) positions of one dihydrochalcone moiety and the second to the α′ (δ_C 39.78) and β′ (δ_C 30.04) positions of another dihydrochalcone moiety. The signal at δ 3.78 (3H, s, OCH_3-3″) was assigned to a methoxy group at C-3″ (δ_C 150.81) by the correlation between the −OCH_3 group and C-3″ in the HMBC spectrum.

Two D_2O-exchangeable signals at δ 12.60 (OH-2′) and 12.73 (OH-2‴) were attributed to hydrogen-bonded hydroxyl groups at the C-2′ (δ_C 165.32) and C-2‴ (δ_C 165.32) positions, respectively. Another exchangeable signal at δ 5.98 (OH-4) was assigned to the hydroxyl group at the C-4 (δ_C 143.48) position by the correlations of OH-4/C-3 (δ_C 144.94) and OH-4/C-5 (δ_C 115.92) in the HMBC spectrum. The remaining two hydroxyl groups at δ 6.32 and 5.96 were connected to C-4′ (δ_C 162.82) and C-4‴ (δ_C 165.39), respectively. The chemical shifts of the carbonyl carbons were assigned from the HMBC data of **1**. The carbonyl carbons C-a (δ_C 203.88) and C-b (δ_C 204.11) were correlated with H-α and H-β and with H-α′ and H-β′, respectively.

Verbenachalcone (**1**) has nine oxygen atoms, eight of which were accounted for by five hydroxyl, one methoxyl, and two carbonyl groups. The remaining one oxygen must, therefore, be involved in the inter-dihydrochalcone linkage, and the two dihydrochalcone units were accordingly linked by an ether bridge between C-3 (δ_C 144.94) of ring B and C-4″ (δ_C 145.47) of ring B′. The HMBC spectrum further confirmed the involvement of C-3 and C-4″ in the C−O−C linkage because H-2 and H-5″ showed correlations with C-4″ and with C-3, respectively. This was suggested by a cross-peak between OCH_3-3″ and H-2 in the NOESY spectrum (Figure 1). Analysis of the ^{1}H−^{1}H COSY, NOESY, DEPT, HMQC, and HMBC NMR data enabled the complete assignment of all protons and carbons. Thus, the structure of verbenachalcone (**1**) was determined as 4,2′,4′,2‴,4‴-pentahydroxy-3″-methoxy-3-*O*-4″-tetrahydrobichalcone.

Chalcones have been regarded as key compounds in the biosynthesis of flavonoids. The dihydrochalcones, a variation of the chalcone skeleton, are not abundant in nature and are of sporadic occurrence.[6] The dimeric dihydrochalcones are extremely rare, and only two compounds, brackenin[7] and cinnabarone,[8] which are composed of two dihydrochalcone units linked by a carbon-to-carbon bond, have been reported. To our knowledge, verbenachalcone (**1**) isolated in this study is the first example of a dimeric dihydrochalcone with an interchalcone linkage through oxygen.

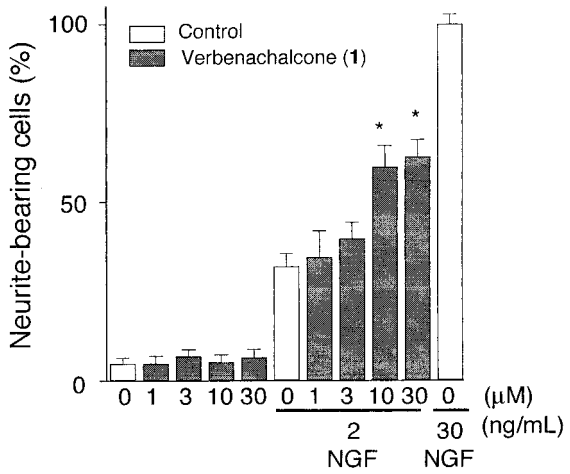

Figure 2. Effects of verbenachalcone (**1**) on the proportion of neurite-bearing PC12D cells in the presence or absence of NGF. The proportion of neurite-bearing cells is expressed as a percentage against the maximum response to NGF (30 ng/mL, 100%) in the absence of verbenachalcone. Values are mean ± SE from four experiments. A statistically significant difference (* P < 0.01) from the control (2 ng/mL NGF) in the absence of verbenachalcone was apparent.

Verbenachalcone (**1**) (1−30 μM) did not induce neurite outgrowth from PC12D cells in the absence of NGF (Figure 2). However, this compound (>10 μM) markedly potentiated the NGF (2 ng/mL)-induced increase in the proportion of neurite-bearing cells (Figure 2).

Experimental Section

General Experimental Procedures. The melting point was determined on a Yanaco micro-melting point apparatus and is uncorrected. The ultraviolet spectrum was recorded on a Hitachi U-2000 spectrophotometer, and the infrared spectrum obtained on a Shimadzu IR-408 spectrometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ on a JEOL GX-500 spectrometer. Chemical shifts were measured using residual $CHCl_3$ (δ_H 7.24 and δ_C 77.03) as internal standard. LREIMS and HREIMS were recorded on a JMS DX-303 and a JMS AX-500 spectrometer, respectively. 7S NGF was obtained from Sigma Chemical Co. (St. Louis, MO). Chemicals for the biological studies were purchased from Wako Pure Chemical (Tokyo, Japan).

Plant Material. The plant material was supplied by Seiwa Pharmaceuticals, Ltd. (Ibaragi, Japan). It was imported from Paraguay and identified as the stems of *Verbena littoralis* H. B. K. by Mr. Tetsuo Nakasumi (Instituto de Pesquisas de Plantas Medicinais do Brazil, 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* (1 kg) were ground and extracted with MeOH (3 L) three times, and the MeOH extract (48 g) was partitioned between EtOAc and H_2O (1:1). The EtOAc-soluble fraction (18 g) was subjected to Si gel (500 g) column chromatography (EtOAc−MeOH, 5:1) to give eight fractions. The most active fraction 5 was passed over a Sephadex LH-20 (500 g) column (EtOAc−MeOH, 1:1) to yield eight subfractions. Final purification of the active subfraction 5 was effected by reversed-phase semipreparative HPLC (YMC-Pack ODS-AM324, 5 μm, 300 × 10 mm; eluent, MeCN−H_2O, 78:22; flow rate, 1 mL/min; UV detector) to afford verbenachalcone (**1**, 4.7 mg).

Verbenachalcone (4,2′,4′,2‴,4‴-Pentahydroxy-3″-methoxy-3-*O*-4″-tetrahydrobichalcone, 1): yellow needle crystals (hexanes−EtOAc); mp 220−224 °C (dec); UV (MeOH) λ_{max}

(log ϵ) 225 (3.36), 278 (3.99), 312 (2.25) nm; IR (film) ν_{max} 3600−3300 (br, OH), 1635 (chelated C═O), 1565 and 1504 (aromatic rings) cm^{-1}; ^1H and ^{13}C NMR, see Table 1; LREIMS *m/z* 544 $[M]^+$ (28), 406 (4), 393 (9), 255 (16), 241 (11), 137 (100); HREIMS *m/z* 544.1725 (calcd for $C_{31}H_{28}O_9$, 544.1734).

Bioassay Procedure. The enhancing activity of NGF-mediated neurite outgrowth in PC12D cells was examined by a method previously described.[1]

Acknowledgment. We thank Dr. Mamoru Sano of Aichi Human Service Center, Aichi, Japan, for kindly providing PC12D cells. We are indebted to Mr. Toyokichi Yoshizawa of Seiwa Pharmaceuticals, Ltd., Ibaragi, Japan, for providing the plant material. This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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NP000602W