Littorachalcone, a New Enhancer of NGF-Mediated Neurite Outgrowth, from *Verbena littoralis*

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A new dihydrochalcone dimer, 2′,4′,3″,2‴,4‴-pentahydroxy-4-*O*-4″-tetrahydrobichalcone, given the name littorachalcone, was isolated from the aerial parts of *Verbena littoralis* H. B. K. along with two known flavonoids 4′-hydroxywogonin and 8,3′-dimethoxy-5,7,4′-trihydroxyflavone. Littorachalcone caused a significant enhancement of nerve growth factor-mediated neurite outgrowth from PC12D cells.

Key words *Verbena littoralis*; dihydrochalcone dimer; littorachalcone; nerve growth factor-action; PC12D cell

In the course of our investigations of neuritogenically active substances from medicinal plants we have devoted our attention to the occurrence of natural products having new skeletons. We isolated a new dimeric dihydrochalcone, littorachalcone (**1**), which showed a considerable activity at enhancing nerve growth factor-mediated neurite outgrowth from PC12D cells, from *Verbena littoralis* H. B. K. (Verbenaceae) by a series of bioassay-directed chromatographic separations. Compounds that possess properties which enhance the ability of a nerve growth factor to stimulate neurite outgrowths 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).[1—7]

The EtOAc soluble materials of MeOH extracts of the aerial parts of *V. littoralis* collected in Paraguay were repeatedly subjected to silica gel columns (EtOAc/MeOH) and Sephadex LH-20 column (MeOH) followed by reversed-phase semi-preparative HPLC on a YMC-pack ODS-AM324 column (78% MeCN in H_2O) to yield littorachalcone (**1**), together with two known flavonoids, 4′-hydroxywogonin (**2**) and 8,3′-dimethoxy-5,7,4′-trihydroxyflavone (**3**).

Littorachalcone (**1**) showed a molecular ion peak at *m/z* 514 in the electron impact (EI)-MS spectrum, and its molecular formula was determined to be $C_{30}H_{26}O_8$ by high resolution (HR)-EI-MS (*m/z* 514.1650, $[M]^+$, Δ +2.2 mmu). The IR spectrum of **1** indicated the presence of hydroxyl (3600—3300 cm^{-1}), hydrogen-bonded carbonyl (1635 cm^{-1}), and aromatic ring (1565, 1504 cm^{-1}) moieties. The structural characterization of **1** was performed by analyzing its EI-MS and one dimensional (1D) and 2D NMR spectroscopic data (see Table 1, Figs. 1—3). The ^{1}H-NMR spectrum disclosed the presence of 13 signals for aromatic hydrogen atoms, eight signals for methylene hydrogens, and two hydroxyl groups. Analysis of the ^{13}C-NMR, distortionless enhancement by polarization transfer (DEPT), and ^{1}H-detected heteronuclear multiple quantum coherence (HMQC) spectra of **1** indicated the presence of 24 aromatic carbons, four sp^3 methylene carbons, and two carbonyls. These features of the ^{1}H- and ^{13}C-NMR spectra of **1** were similar to those of verbenachalcone.[5]

In the ^{1}H-NMR spectrum, two pairs of A_2B_2-type signals were observed, one pair at δ 3.21 (2H, t, J=7.3 Hz, H-α) and 3.05 (2H, t, J=7.3 Hz, H-β), and another at δ 3.11 (2H, t, J=7.3 Hz, H-α') and 2.89 (2H, t, J=7.3 Hz, H-β'). From a detailed analysis of the ^{1}H–^{1}H correlation spectroscopy (COSY) and heteronuclear multiple bond connectivity (HMBC) NMR spectral data of **1**, it was concluded that the first pair belonged to the α (δ_C 39.56) and β (δ_C 30.33) positions of one dihydrochalcone moiety and the second to the α' (δ_C 39.86) and β' (δ_C 30.02) positions of another dihydrochalcone moiety. Three sets of ABX patterned signals

Table 1. ^{1}H- and ^{13}C NMR Data for Littorachalcone (**1**) ($CDCl_3$, ^{1}H-NMR 600 MHz, ^{13}C-NMR 150 MHz)

Position	^{13}C	^{1}H
1	136.60 s	
2	129.93 d	7.16, 1H, d, J=8.6 Hz
3	118.44 d	6.87, 1H, d, J=8.6 Hz
4	155.10 s	
5	118.44 d	6.87, 1H, d, J=8.6 Hz
6	129.93 d	7.16, 1H, d, J=8.6 Hz
1′	113.90 s *b)*	
2′	165.31 s *c)*	
3′	103.64 d	6.36, 1H, d, J=2.3 Hz
4′	162.89 s	
5′	107.90 d *d)*	6.34, 1H, dd, J=8.6, 2.3 Hz
6′	132.52 d	7.55, 1H, d, J=8.6 Hz
α	39.56 t	3.21, 2H, t, J=7.3 Hz
β	30.33 t	3.05, 2H, t, J=7.3 Hz
a	203.90 s	
1″	133.80 s	
2″	118.19 d	6.61, 1H, d, J=2.0 Hz
3″	143.58 s	
4″	144.94 s	
5″	116.11 d	6.95, 1H, d, J=8.6 Hz
6″	124.16 d	6.87, 1H, dd, J=8.6, 2.0 Hz
1‴	114.55 s *b)*	
2‴	165.32 s *c)*	
3‴	103.73 d	6.37, 1H, d, J=2.3 Hz
4‴	162.83 s	
5‴	107.75 d *d)*	6.34, 1H, dd, J=8.6, 2.3 Hz
6‴	132.48 d	7.58, 1H, d, J=8.6 Hz
α'	39.86 t	3.11, 2H, t, J=7.3 Hz
β'	30.02 t	2.89, 2H, t, J=7.3 Hz
b	203.90 s	
OH-2′		12.61, 1H, s *e)*
OH-2‴		12.76, 1H, s *e)*

a) Spectra determined in $CDCl_3$; data reported in ppm. All protons and carbons were assigned by ^{1}H-^{1}H COSY, long range ^{1}H-^{1}H COSY, DEPT, HMQC and HMBC spectra. *b—e*) Signals may be interchangeable.

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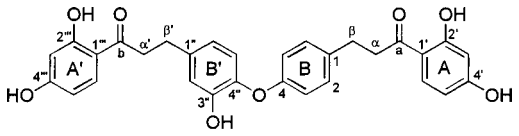
were found at δ 7.55 (1H, d, J=8.6 Hz, H-6′), 6.34 (1H, dd, J=8.6, 2.3 Hz, H-5′) and 6.36 (1H, d, J=2.3 Hz, H-3′) in ring A, at δ 7.58 (1H, d, J=8.6 Hz, H-6‴), 6.34 (1H, dd, J=8.6, 2.3 Hz, H-5‴), and 6.37 (1H, d, J=2.3 Hz, H-3‴) in ring A′, and at δ 6.95 (1H, d, J=8.6 Hz, H-5″), 6.87 (1H, dd, J=8.6, 2.0 Hz, H-6″), and 6.61 (1H, d, J=2.0 Hz, H-2″) in ring B′, and these proton signals were assigned to the corresponding positions by analyzing the data of the ^{1}H–^{1}H COSY, HMQC, and HMBC NMR spectra. The ^{1}H-NMR spectrum also showed a pair of two-proton doublets (J=8.6 Hz) at δ 7.16 (H-2, H-6) and 6.87 (H-3, H-5). This substructure of ring B was further confirmed by the detailed analysis of correlations H-2, 6/H-3, 5 in the ^{1}H–^{1}H COSY spectrum, and H-2/C-6, H-2/C-3, H-3/C-5, H-3/C-2, H-5/C-6 and H-6/C-3 in the HMBC spectrum.

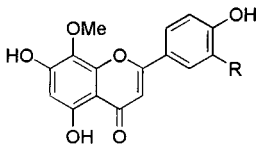
Two D_2O-exchangeable signals at 12.61 (OH-2′) and 12.76 (OH-2‴) were attributed to hydrogen-bonded hydroxyl groups at the C-2′ (δ_C 165.31) and C-2‴ (δ_C 165.32) positions, respectively. The remaining three hydroxyl groups were connected to C-4′ (δ_C 162.89), C-3″ (δ_C 143.58), and C-4‴ (δ_C 162.83) separately by analysis of the ^{1}H- and ^{13}C-NMR spectral data. The connections of the four aromatic rings were unambiguously determined by analyzing the correlations of the HMBC and nuclear Overhauser effect spectroscopy (NOESY) spectra. The first 4,2′,4′-trisubstituted dihydrochalcone monomer was confirmed by the correlations of C-a/H-α, C-a/H-β, C-a/H-6′, H-α/C-1, H-β/C-1, H-β/C-2, and H-β/C-6 in the HMBC spectrum, and H-6′/H-α, H-2/H-α and H-6/H-β in the NOESY spectrum. The second 3″,4″,2‴,4‴-tetrasubstituted dihydrochalcone monomer was established by the correlations of C-b/H-α', C-b/H-β', C-b/H-6‴, H-α'/C-1″, H-β'/C-1″, H-β'/C-2″, and H-β'/C-6″ in the HMBC spectrum, and H-2″/H-α', H-6″/H-β' and H-6‴/H-α' in the NOESY spectrum.

Littorachalcone has eight oxygen atoms, seven of which are now accounted for by five hydroxyl and two carbonyl groups. The remaining oxygen must, therefore, be involved in the biphenyl ether linkage, and the two dihydrochalcone units are linked by an ether bridge between C-4 (δ_C 155.10) of ring B and C-4″ (δ_C 144.94) of ring B′. The HMBC spectrum further confirmed the involvement of C-4 and C-4″ in the C–O–C linkage because H-3 and H-5 showed correlations with C-4″ and H-5″ showed correlation with C-4, respectively (Fig. 2). This was suggested by a cross peak between H-5 and H-2″ in the NOESY spectrum. Full analysis of the ^{1}H–^{1}H COSY, long range ^{1}H–^{1}H COSY, NOESY, DEPT, HMQC and HMBC data enabled the complete assignment of all protons and carbons. Thus, the structure of littorachalcone (**1**) was determined to be 2′,4′,3″,2‴,4‴-pentahydroxy-4-*O*-4″-tetrahydrobichalcone. The rationalization of the structure of **1** was supported by analyzing the EI-MS spectral data (Fig. 3). In the EI-MS spectrum there were fragmental ion peaks at *m/z* 404 $(M-C_6H_5O_2)^+$, 363 $(M-C_8H_7O_3)^+$, 225 $(C_{15}H_{13}O_2)^+$, 165 $(C_9H_9O_3)^+$, 137 $(C_7H_5O_3)^+$, and 110 $(C_6H_5O_2)^+$.

Although there are some reports on dimeric dihydrochalcones,[8,9] this is the second isolation of a dimeric dihydrochalcone with a biphenyl ether linkage through oxygen. The flavonoid compounds 4′-hydroxywogonin (**2**)[10] and 8,3′-dimethoxy-5,7,4′-trihydroxyflavone (**3**)[11,12] were isolated from *V. littoralis* for the first time and their structures



1



R

2 H

3 OMe

Fig. 1. Structures of Compounds **1**—**3**

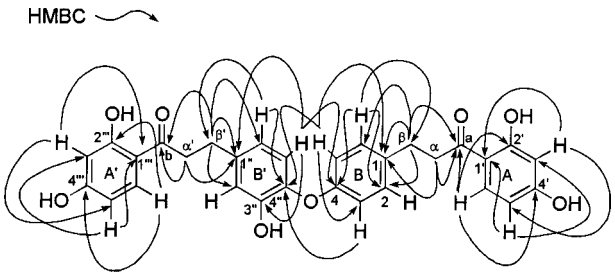


Fig. 2. Selected HMBC Correlations of Littorachalcone

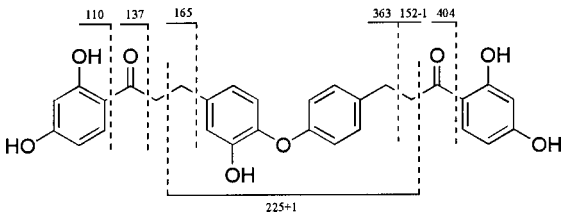


Fig. 3. Selected EI-MS Fragmental Ion Peaks of Littorachalcone

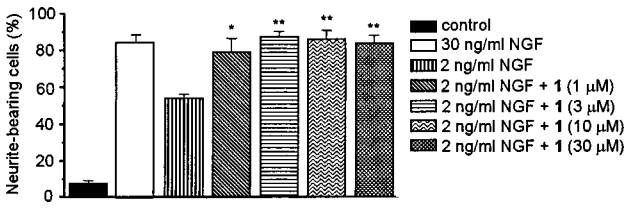


Fig. 4. Enhancement of Effect of NGF on Stimulating Neurite Outgrowths in PC12D Cells with Littorachalcone (**1**)

Cells were incubated in the presence of NGF (2 or 30 ng/ml) alone and in the presence of **1** plus NGF (2 ng/ml) for 48 h before being fixed with 2% glutaraldehyde (37 °C, 1 h). The ratio of neurite-bearing cells was determined and expressed as a mean±S.E. (n=12). A statistically significant difference ($*p<0.01$ or $**p<0.001$) from the control (2 ng/ml NGF) in the absence of **1** was apparent.

were identified by comparing their spectral data with those in the literature.

The abilities of **1**, **2** and **3** to enhance the ability of NGF to stimulate neurite outgrowth was assessed utilizing methodology previously reported.[7] In control experiments, the percentages of neurite-bearing cells were 54.1% and 84.2% fol-

lowing incubation with 2 ng/ml and 30 ng/ml NGF, respectively. Littorachalcone (**1**) (1—30 μM) did not affect morphologically PC12D cells in the absence of NGF, but markedly enhanced the NGF (2 ng/ml)-induced increase in the proportion of neurite-bearing cells (Fig. 4). The percentages of neurite-bearing cells presented by **1** (3—30 μM) plus NGF (2 ng/ml) were approximately equal to or were greater than 30 ng/ml NGF alone. The potencies at which **1** (3—30 μM) enhanced the effects of nerve growth factor-mediated neurite outgrowth from PC12D cells were greater than those of verbenachalcone[5] and littoralisone,[6] which were isolated previously from *V. littoralis*. Compounds **2** and **3** had no effect on nerve growth factor action in PC12D cells.

Experimental

General Procedures The melting point was determined on a Yanaco micro-melting point apparatus and was uncorrected. The ultraviolet spectrum was recorded on a Hitachi U-2000 spectrophotometer. The infrared spectrum was recorded on an IR-408 spectrometer. 1D and 2D NMR spectra were recorded in $CDCl_3$ on JEOL GX-500 and JEOL ECP-600 spectrometers. Chemical shifts were measured using residual $CHCl_3$ (δ_H 7.24 and δ_C 77.03) as internal standard. EI-MS and HR-EI-MS were recorded on a JMS AX500 and JMS DX303 spectrometers.

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 extracted with MeOH (15 l) three times, and the MeOH extract (250 g) was partitioned between EtOAc and H_2O (1 : 1). Part (20 g) of the EtOAc–soluble fraction (90 g) was subjected to a silica gel column (8×80 cm, EtOAc/MeOH, 100 : 0→0 : 100) to give 10 fractions, fr. 1 (111 mg), fr. 2 (1106 mg), fr. 3 (457 mg), fr. 4 (6170 mg), fr. 5 (4397 mg), fr. 6 (1592 mg), fr. 7 (100 mg), fr. 8 (5021 mg), fr. 9 (1338 mg), and fr. 10 (423 mg). Further purification of fr. 5 was achieved by a Sephadex LH-20 (5×120 cm) column (MeOH) followed by reversed-phase semi-preparative HPLC (YMC-Pack ODS-AM324, 5 μm, 300×10 mm; eluant, MeCN/H_2O, 78 : 22; flow rate, 1 ml/min; RI detector) to afford littorachalcone (**1**, 1.9 mg), 4′-hydroxywogonin (**2**, 1.8 mg), and 8,3′-dimethoxy-5,7,4′-trihydroxyflavone (**3**, 3.6 mg).

Littorachalcone (1): Yellow powder (CH_3OH), mp 178—180 °C. UV λ_{max} (MeOH) nm (log ε): 215 (2.46), 277 (1.65) and 314 (0.87). IR (neat) cm^{-1}: 3600—3300 (br, OH), 1635, 1565 and 1504. ^1H- and ^{13}C-NMR data, see Table 1. EI-MS *m/z*: 514 (M^+) (5.6), 404 (1.0), 376 (1.5), 363 (3.0), 239 (1.5), 225 (6.0), 211 (4.1), 152 (2.6), 137 (31.7), 110 (21.0). HR-EI-MS *m/z*: 514.1650 (Calcd for $C_{30}H_{26}O_8$: 514.1627).

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