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BRUGNANIN, A NEW SYN-2,3-DIHYDROBENZOFURAN NEOLIGNAN DIOATE FROM THE MANGROVE *Bruguiera gymnorrhiza*

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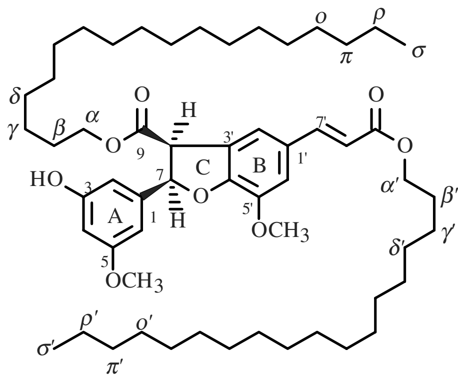
*Brugnanin (**1**), a neolignan dioate, was isolated from a mangrove plant* Bruguiera gymnorrhiza. *Based on spectroscopic interpretation of MS, IR, and NMR data, **1** was elucidated as (7R*,8S*,E)-3-hydroxy-5,5′-dimethoxy-7-*O*-4′,8-3′-neolignan-7′-ene-9,9′-dioic acid dioctadecyl ester. MTT assay showed that **1** had weak inhibitory activity against growth of CNE-1 nasopharyngeal carcinoma cell line.*

Key word: brugnanin, mangrove, *Bruguiera gymnorrhiza*, MTT, CNE-1.

Bruguiera gymnorrhiza is one of the ecosystemically important mangrove plants in the estuaries of Guangxi Zhuang Autonomous Region, China [1]. It has been used as folk medicine for the treatment of diseases such as fever, diarrhea, haemorrhage, inflammation, and malaria [2]. Previous natural products chemistry efforts on this plant species have yielded some structurally interesting natural products such as diterpenes [3, 4], cyclic disulfides [5] and polydisulfides [6], and aromatic compounds [7]. As part of our investigations of bioactive compounds in mangrove plants indigenous to Guangxi Zhuang Autonomous Region, China, we examined the stem bark of *B. gymnorrhiza* collected from Shankou Town, Hepu County. As a result, we isolated and identified a previously unknown neolignan diacid ester, brugnanin (**1**), which possesses a unique syn-2,3-dihydrobenzofuran skeleton.

The air-dried outer layer and inner layer of the stem bark of *Bruguiera gymnorrhiza* were extracted with 95% ethanol and partitioned with petroleum ether. The partition of petroleum ether was then subjected to silica gel column chromatography, and from the eluting fractions of 10% ethyl acetate in petroleum ether, brugnanin (**1**) was obtained.



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Brugnanin (**1**) was obtained as a white amorphous powder. The fast atom bombardment mass spectrum (FAB-MS) of **1** presented a quasi-molecular ion peak $[M+H]^+$ at *m/z* 891. The high resolution matrix-assisted laser desorption ionization mass (HRMALDI-MS) spectrum of **1** gave a quasi-molecular ion peak $[M+Na]^+$ at *m/z* 913.6549. Combined with other spectroscopic data, these mass data allowed us to establish the molecular formula $C_{56}H_{90}O_8$, which has a calculated relative molecular weight of 890.6636.

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TABLE 1. NMR Spectroscopic Data of Brugnanin (**1**) (500 MHz, δ, ppm, TMS =0)

Positions	δ_C	δ_H (J, Hz)	COSY	NOESY	HMBC
1	131.6, C				
2	119.5, CH	6.93 m			C-1, 4, 6, 7
3	144.4, C				
4	114.5, C	6.93 m			C-2, 5, 6
5	146.7, C				
6	108.8, CH	6.93 m			
7	87.5, CH	6.13 d (8.2)	H-8	H-8	C-1, 2, 4, 5, 7
8	56.0, CH	4.34 d (8.2)	H-7	H-7	C-2, 6, 9, 3′, 4′
9	170.3, C				C-9, 2′, 3′, 4′
1′	128.7, C				
2′	117.9, CH	7.22 s			C-8, 4′, 6′, 7′
3′	125.9, C				
4′	150.0, C				
5′	146.1, C				
6′	112.2, CH	7.05 d (0.7)			C- 2′, 4′, 7′
7′	144.7, CH	7.65 d (18.8)	H-8′		C-2′, 6′, 9′
8′	116.1, CH	6.33 d (18.8)	H-7′		C-1′, 9′
9′	167.3, C				
α	66.0, CH_2	4.23 m	H-*β*		C-9
β	28.8, CH_2	1.72 m		3-OH	
γ	26.0, CH_2	1.25-1.45 m			
δ-o	29.7-29.2, CH_2	1.25-1.45 m			
π	31.9, CH_2	1.25-1.45 m			
ρ	22.7, CH_2	1.25-1.45 m			
σ	14.1, CH_3	0.90 t			
α′	64.6, CH_2	4.23 m	H-*β*		C-9′
β′	28.6, CH_2	1.72 m	H-*α′*		
γ′	25.9, CH_2	1.25-1.45 m			
δ′-o′	29.7-29.2, CH_2	1.25-1.45 m			
π′	31.9, CH_2	1.25-1.45 m			
ρ′	22.7, CH_2	1.25-1.45 m			
σ′	14.1, CH_3	0.90 t			
3-OH		5.68 s, br		H-*β*	
5-OCH_3	55.7				C-5
5′-OCH_3	56.2				C-5′

Through comprehensive spectral analysis of NMR data (Table 1), **1** was found to have a 2,3-dihydrobenzofuran neolignan skeleton. In the ^{1}H NMR, chemical shift signals at δ_H 7.22 s (H-2′), 7.05 d (J = 0.7 Hz, H-6′), and 6.93 m (H-2, H-4, H-6) demonstrated the existence of benzene rings. Fourteen aromatic or olefinic carbon signals (δ_C 100-150) suggested that **1** has, with high probability, two benzene rings. The aromatic characteristic of **1** could be confirmed also by the maximum IR absorption series at 1607, 1520, 1499, and 1469 cm^{-1}. It was uniquely significant to the structural elucidation that **1** had two saturated protons that resonated at δ_H 6.13d (J = 8.2 Hz, H-7) and 4.35 d (J = 8.2 Hz, H-8), which shifted greatly to downfield where olefinic protons normally appear. HMQC, however, showed they correlated to two saturated carbons at δ_C 87.48 (C-7) and 56.0 (C-8), respectively. In this coupling system, there was a large shift difference, i.e., Du = 1.78 ppm, which defined an AX coupling system. HMBC correlations from oxymethine proton H-7 to aromatic C-3′ and C-4′, from carbonylmethine proton H-8 to aromatic C-2′, C-3′, and C-4′, and from aromatic proton H-2′ to C-8 constituted evidence of the existence of a 2,3-dihydrofuran ring (C ring) fused with a benzene ring (B ring), whereas those from H-7 to C-2 and C-6, from H-2 to C-1, C-4, C-6, and C-7, from H-4 to C-2 and C-6, and from H-6 to C-1, C-2, and C-4 demonstrated that there was a 3,5-disubstituted benzene ring (A ring) attached to C-7 or the 2 position in the dihydrobenzofuran backbone. These outstanding NMR spectral characteristics, altogether, corresponded to the picture of 2,3-dihyrobenzofuranoid-type neolignans.

Functional groups were determined by analysis of other spectral information. Downfield of the ^{1}HNMR, a pair of olenic protons remarkably resonated at δ_H 7.65 d (H-7′) and 6.33 d (H-8′) with a coupling constant J = 18.8 Hz. Also, IR showed maximum absorption at n 1637 cm^{-1}, an absorption that shifted to a relatively low frequency band compared with normal double bonds. It was easy to show that this double bond was a trans-double bond conjugated with an aromatic system. Significant HMBC correlations from the olenic proton H-7′ to aromatic carbon C-2′ and C-6′ and from the olenic proton H-8′ to aromatic carbon C-1′ proved that this trans-double bond was attached to the B-ring benzene of the benzofuran skeleton. Here, it is noteworthy to observe the weakness of the long-range coupling $^{4}J_{HH}$ between meta protons H-2′ and H-6′, the later of which showed a doublet peak in the ^{1}H NMR with a very small coupling constant of 0.7 Hz (making the signal δ_H 7.05 appear almost in the singlet peak), whereas the former of which appeared to be a singlet peak only. The meta relationship between C-2′ and C-6′ can be determined by the observed HMBC correlations from H-6′ to C-2′, and from H-2′ to C-6′.

In the ^{13}C NMR, quaternary carbons δ_C 170.31 (C-9) and 167.25 (C-9′) typically stood for two ester carbonyls. The HMBC showed both H-7 and H-8 correlated to C-9, whereas both H-7′ and H-8′ correlated to C-9′. Considering that there is a disubstituted phenyl group attached to the 2 position of the 2,3-dihydrofuran ring (C ring), it is obvious that the C-9 carbonyl is attached to the 3 position of the C ring or to C-8. Similarly, since C-7′ is attached to C-1′, C-9′ should be attached to C-8′.

Other substituents in brugnanin (**1**) included two octadecyloxy, two methoxy, and one phenolic hydroxyl group. The chemical shifts δ_H 4.23 m (4H: H-α and H-α'), 1.72 m (4H: H-β and H-β'), 1.25-1.45 m (60H: H-γ-$\gamma'$and H-ρ-ρ'), and 0.90 t (J = 6.8 Hz, 6H: H-$\sigma$and H-σ'), which appeared in pairs, exactly reflected the existence of two long-chain alkyl groups equally in 18-carbon length. The chemical shift value of δ_H 4.23 was typically the signal of the carboxymethylene proton, since the two octadecyloxy groups are attached to C-9 and C-9′ carbonyls, respectively. HMBC correlations from H-α to C-9 and from H-α' to C-9′ further confirm of this proposal for their attachment. FAB-MS fragmentation showed consecutive losses of two $OC_{18}H_{37}$ fragments (m = 269 each) after formation of the quasi-molecular ion $[M-H]^+$ (*m/z* 889). This fact strongly confirmed the equality in carbon numbers of the two long-chain alkyl groups.

In view of the fact that 5-OCH_3 and 5′-OCH_3 are correlated in HMBC respectively to C-5 (δ_C 55.7) and C-5′ (δ_C 56.2), we could easily locate the two methoxy groups. The sole phenolic hydroxyl group was the last group to be located. The fact that in the ^{1}H NMR there appeared a broad single resonance peak at δ_H 5.68, which did not give correlations in both ^{1}H-^{1}H COSY and HMQC, demonstrated that this signal was not a carbon-connected proton. The IR 3412 br cm^{-1} stretch absorption, however, corroborated its existence in the form of a hydroxyl group. Note that the HMBC showed H-2 correlated to C-1, C-4, and C-6, H-4 to C-2 and C-6, and H-6 to C-1 and C-4, this hydroxyl group was phenolic to C-3 of the A ring.

The relative configuration of brugnanin (**1**) was established by analyzing the ^{1}H NMR coupling constant and NOESY correlations and 3D simulation using chemical software. The value of the coupling constant J = 8.2 Hz given by the two ^{1}H-^{1}H COSY-corroborated coupling protons H-7 and H-8 implied the syn relationship between these two protons. The observed nuclear Overhauser effect (NOE) between H-7 and H-8 further illustrated the syn relationship of the single bond between C-7 and C-8. It is noteworthy also that the 3-position phenolic proton in A ring showed a unique NOESY correlation with H-β in the 9-position octadecyloxy chain. Using the software Chem3D Ultra^{TM} 8.0 to perform a 3D structural simulation, we found that **1**, in fact, adopted a conformation where the A ring plane was perpendicular to the 2,3-dihydrobenzofuran plane. This special conformation could generate the NOE from the phenolic proton only to protons whose spatial location and distance were correctly chosen. This special observation demonstrated that the 7-position phenyl group and the 8-position octadecyloxycarbonyl moiety were at the same side, namely, in a syn substitution relationship. Therefore, the relative configuration of **1** was determined as (7*R**, 8*S**)-7-*O*-4′,8-3′-linked lignan.

Taken together, the above spectroscopic interpretations led to elucidation of brugnanin (**1**) as (7*R**, 8*S**, *E*)-3-hydroxy-5, 5′-dimethoxy-7-*O*-4′,8-3′-neolignan-7′-ene-9, 9′-dioic acid dioctadecyl ester, or (2*R**, 3*S**, *E*)-octadecyl-2-(3-hydroxy-5-methoxyphenyl)-7-methoxy-5-(3-(octadecyloxy)-3-oxoprop-1-enyl)-2,3-dihydrobenzofuran-3-carboxylate. In the chemical literature, reported compounds with the 2,3-dihydrobenzofuran skeleton, either from naturally occurring sources or organic synthetic sources, are virtually all in 2,3-*trans* configuration [8–10]. Brugnanin (**1**) presents a novel dihydrofuran neoligan with 2, 3-syn configuration. This is the report of such a neolignan dicarboxylic acid esterified with two particularly unique long-chain fatty alcohols.

In the course of our profiling, the newly isolated brugnanin (**1**) was tested by MTT assay for cytoxic activity against CNE-1 nasopharyngeal carcinoma cell line, showing that **1** had weak potency ($IC_{50} = 5.72\times10^{-4}$ M) on this tumor cell line.

EXPERIMENTAL

General Experimental Procedures. IR spectra were obtained with a Perkin-Elmer Spectrum One FT-IR spectrometer. ^{1}H, and ^{13}C NMR, COSY, HMQC, NOESY, and HMBC spectra were recorded on a Bruker Avance AV 500 spectrometer. COSY, HMQC, NOESY, and HMBC spectra were obtained using standard Bruker pulse sequences. FAB-MS measurements were recorded on a VG Autospec 3000 mass spectrometer. HRMALDI-MS data were obtained on an IonSpec 4.7 Tesla FTMS.

Biological Material. The stems of *Bruguiera gymnorrhiza* were collected from Shankou town, Hepu county, Guangxi Autonomous Region, People's Republic of China in November 2003. A voucher specimen was deposited at Guangxi Medical University, Department of Chemistry (HSYP-103-3).

Extraction and Isolation. The barks were promptly peeled off the freshly collected stems and sorted into two parts, the outer layer and the inner layer, which weighed 4.5 kg and 5.6 kg after air-drying, respectively. The two parts of air-dried materials were subject to repeated extraction with 95% ethanol at ambient temperature to afford brown gums of 525 g and 218 g, respectively. The combined extract was then partitioned by petroleum ether (60-90°C) to give 100 g brown gum, of which 95 g was subjected to silica gel column chromatography, using petroleum ether (60-90°C) with an increasing proportion of ethyl acetate as eluent to afford 245 fractions (fr. 1-245), 1000 mL/fraction. According to TLC inspection results, repeated crystallization for fr. 107 (10% ethyl acetate) was performed with *n*-hexane to afford brugnan (**1**) (11.3 mg, 0.0001% yield from dry material).

Brugnanin (**1**) : white amorphous powder; mp 114-115°C, TLC R_f 0.46 (petroleum ether-ethyl acetate: 80-20) and 0.60 (benzene-acetone: 90-10); IR (KBr, ν_{max}, cm^{-1}): 3545, 3412, 2955, 2917, 2850, 1727, 1708, 1637, 1607, 1588, 1520, 1499, 1272, 1178, 834, 720; ^{1}H and ^{13}C NMR data, see Table 1; HRMALDI-MS $[M+Na]^+$ at *m/z* 913.6549 (913.6533 calculated for $C_{56}H_{90}O_8Na^+$); FAB-MS *m/z* (%) 891 $[M+H]^+$ (7), 890 M^+ (1), 889 $[M-H]^+$ (1), 862 $[M-CO]^+$ (10), 834 $[M-2CO]^+$ (8), 862 $[M-H-OC_{18}H_{37}]^+$ (12), 592 $[M-H-OC_{18}H_{37}-CO]^+$ (13), 566 $[M-H-OC_{18}H_{37}-CO-C_2H_2]^+$ (2), 351 $(M-H-2OC_{18}H_{37}$, 100), 323 $[M-H-2OC_{18}H_{37}-CO]^+$ (25), 295 $[M-H-2OC_{18}H_{37}-2CO]^+$ (5), 201(6), 83(12), 69(22), 56(67).

Bioactive Assay. Brugnanin (**1**) was tested for its cytotoxic effect on CNE-1 nasopharyngeal carcinoma cell line by MTT assay. CNE-1 nasopharyngeal carcinoma cell culturing and general test procedures were as previously described [11]. In our test, 1 mg of **1** was dissolved in advance with DMSO, which was controlled to 0.5% as the maximum final concentration before it was diluted with culturing solution RPMI 1640. The influence of DMSO was excluded from the calculation of IC_{50} (concentration of compound resulting in 50% inhibition of tumor cell proliferation). Compound **1** was tested in concentration gradients of 200, 100, 50, 25, and 12.5 μg/mL.

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