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Three new pimaren diterpenoids from marine mangrove plant, *Bruguiera gymnorrhiza*

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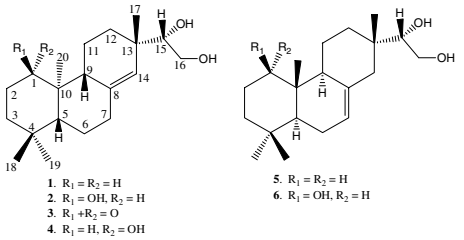
Three new pimaren diterpenoids, *ent*-8(14)-pimarene-15*R*,16-diol (**1**), *ent*-8(14)-pimarene-1α,15*R*,16-triol (**2**), and (5*R*, 9*S*, 10*R*, 13*S*, 15*S*)-*ent*-8(14)-pimarene-1-oxo-15*R*,16-diol (**3**), along with three known diterpenoids (**4**—**6**) have been isolated from the stem of mangrove plant *Bruguiera gymnorrhiza*. The structures of compounds **1**—**3** were determined by extensive spectroscopic (2D NMR, MS, IR, and CD) analysis, and **3** and **5** showed moderate cytotoxic activities against L-929 and K562, respectively.

1. Introduction

Bruguiera gymnorrhiza (L.) (Rhizophoraceae) is an evergreen mangrove tree, widely distributed on tropical and subtropical coastlines. A previous phytochemical study on this plant from India has resulted in the presence of diterpenes, triterpenes and flavonoids (Sarkar et al. 1978; Misra et al. 1984; 1987), steroids, hydrocarbons and wax esters (Subrahmanyan et al. 1999). In our former work, examination of the same plant from the tropical mangrove garden of China resulted in the isolation of thirteen diterpenoids (Han et al. 2004). In the continuation to systematically investigate the secondary metabolites from this plant, additional six pimaren diterpenoids involving three new compounds (**1**–**3**) were isolated. The known diterpenes were identical to *ent*-8(14)-pimarene-1β,15*R*,16-triol (**4**) (Subrahmanyan et al. 1999; Rojas et al. 2001), isopimar-7-ene-15*S*,16-diol (**5**) (Subrahmanyan et al. 1999), and isopimar-7-ene-1β, 15*S*, 16-triol (**6**) (Anjaneyulu and Rao 2002). In this paper, we deal with the structural elucidation of the new compounds.



2. Investigations, results and discussion

The molecular formula of **1** was determined as $C_{20}H_{34}O_2$ on the basis of HRESIMS (m/z 329.2458, calcd 329.2456 for $[M + Na]^+$). The IR absorptions at 1681 and 3255 cm^{-1} suggested the presence of olefinic and hydroxyl groups. The ^1H NMR spectrum exhibited signals for an olefinic proton at δ 5.24 (1 H, s), an oxygenated methine at δ 3.57 (1 H, dd, J = 2.3, 9.2 Hz), an oxygen-bearing methylene at δ 3.67 (1 H, dd, J = 2.3, 10.3 Hz) and 3.48 (1 H, dd, J = 10.3, 9.2 Hz), and four methyl groups at δ 0.75 (3 H, s), 0.81 (3 H, s), 0.85 (3 H, s), and 0.87 (3 H, s). The ^1H and ^{13}C NMR data of **1** (Table 1) were characteristic for a 8(14)-pimarene-type diterpenoid, comparable to those of *ent*-8(14)-pimarene-1β,15*R*,16-triol (**4**). The NMR data of **1** differed from **4** solely at ring A where a hydroxylated methine C-1 of **4** was replaced by a methylene group [δ_H 1.65 (m), 0.99 (m), and δ_C 39.2 (t)]. This evidence was supported by the HMBC correlation from the methyl protons at δ 0.75 (3 H, s, H_3-20) to C-1, C-5 (δ 54.9, d), and C-9 (δ 50.4, d). The gross structure of **1** was further confirmed by detailed elucidation of DQFCOSY, HMQC and HMBC spectra. The stereochemistry of **1** was proposed on the basis of a NOESY spectrum and by comparison of its ^{13}C values with those of **4**, whose stereochemistry was previously established by X-ray diffraction (Subrahmanyan et al. 1999). The NOE correlations between H-5/H-9, H_3-17/ H-14, H-14/ H-7α, H-5/H-18, H_3-20/H-15, in NOESY were in agreement with the β-orientation for H-5, H-9 and H_3-17, and in turn the α-orientation for H_3-20. The comparable values of optical rotation between **1** ($[\alpha]_D^{20}$ −32.0°) and **4** ($[\alpha]_D^{20}$ −35.0°) (Subrahmanyan et al. 1999) indicated that **1** was a *ent*-pimarene-type diterpenoid. The absolute configuration of asymmetric center at C-15 was determined as 15*R* due to the ^{13}C chemical shift of C-15 (δ 78.7) virtually identical to that of **4**, whereas the 15 (*S*)-isomer is at δ 73.0 ppm (Rojas 2001; Subrahmanyam et al. 1999). Accordingly, the structure of **1** was determined as *ent*-8(14)-pimarene-15*R*,16-diol.

The molecular formula of **2** was established as $C_{20}H_{34}O_3$ by HRESIMS at m/z 345.2401 (calcd 345.2406 for $[M + Na]^+$), the same as that of **4**. Comparison of the ^1H and ^{13}C NMR spectra between **2** and **4** (Table 1) showed that both of them shared most NMR data. However, the ^{13}C NMR resonance for **2** at C-1 shifted significant up-

Table 1: ^{1}H and ^{13}C NMR data of 1–3

No	**1**[a]		**2**[a]		**3**[a]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	α 1.65 1 H, m[c]	39.2	α 3.67 1 H, brd, 5.5	71.8		217.0
	β 0.99 1 H, m[c]					
2	α 1.50 1 H, m[c]	18.9	α 1.85 1 H, m[c]	25.9	α 2.80 1 H, td, 13.1, 5.1	36.3
	β 1.43 1 H, m[c]		β 1.54 1 H, m[c]		β 2.10 1 H, dt, 13.1, 4.3	
3	α 1.40 1 H, m[c]	42.1	α 1.17 1 H, dt[b]	34.1	α 1.78 1 H, m[c]	43.4
	β 1.15 1 H, m[c]		β 1.67 1 H, td[b]		β 1.68 1 H, m[c]	
4		33.3		33.0		33.5
5	1.03 1 H, dd, 12.4, 2.5	54.9	1.42 1 H, dd, 13.0, 2.2	47.8	1.42 1 H, dd[b]	57.3
6	α 1.30 1 H, m[c]	22.6	α 1.33 1 H, m[c]	22.5	α 1.45 1 H, m[c]	23.2
	β 1.60 1 H, m[c]		β 1.62 1 H, m[c]		β 1.60 1 H, m[c]	
7	α 2.26 1 H, m[c]	36.3	α 2.25 1 H, m[c]	35.9	α 2.25 1 H, m[c]	36.4
	β 2.03 1 H, m[c]		β 2.03 1 H, m[c]		β 1.98 1 H, m[c]	
8		140.1		140.4		138.0
9	1.68 1 H, t[b]	50.4	2.27 1 H, t[b]	43.2	2.30 1 H, t[b]	42.6
10		38.3		42.0		53.0
11	α 1,51 1 H, m[c]	18.9	α 1.56 1 H, m[c]	18.2	α 1.50 1 H, m[c]	20.4
	β 1.53 1 H, m[c]		β 1.65 1 H, m[c]		β 2.00 1 H, m[c]	
12	α 1.62 1 H, m[c]	32.4	α 1.63 1 H, m[c]	32.3	α 1.52 1 H, m[c]	31.3
	β 1.13 1 H, m[c]		β 1.20 1 H, m[c]		β 1.21 1 H, m[c]	
13		37.1		37.1		36.9
14	5.24 1 H, s	126.9	5.29 1 H, s	127.3	5.40 1 H, s	129.2
15	3.57 1 H, dd, 9.2, 2.3	78.7	3.59 1 H, brd, 9.6	78.7	3.52 1 H, dd, 9.2, 2.3	78.8
16	3.48 1 H, dd, 9.2, 10.3	63.3	3.50 1 H, dd, 9.6, 10.0	63.3	3.50 1 H, dd, 10.3, 9.2	63.2
	3.67 1 H, dd, 2.3, 10.3		3.70 1 H, brd, 10.0		3.68 1 H, dd, 10.3, 2.3	
17	0.87 3 H, s	23.6	0.89 3 H, s	23.6	0.89 3 H, s	23.1
18	0.85 3 H, s	33.7	0.91 3 H, s	33.5	0.93 3 H, s	32.4
19	0.81 3 H, s	22.1	0.85 3 H, s	22.3	1.05 3 H, s	22.7
20	0.75 3 H, s	15.0	0.77 3 H, s	15.5	1.12 3 H, s	14.4

[a] In $CDCl_3$; [b] Multiplicity deduced from HMQC spectra; [c] Overlapping signals

field to δ 71.8 (d) ppm instead of δ 79.1 (d, C-1) ppm of **4**, indicating that **2** is an epimeric isomer of **4** at C-1, which was further supported by NOE correlation between H-1 and H-20 as well as the J value of H-1 (δ 3.67 brd, J = 5.5 Hz) for equatorial orientation. The NOESY spectrum indicated that the tricyclic nucleus of **2** possesses the same stereochemistry as **1** and **4**, and the optical rotation suggested that **2** was also an *ent*-pimarene type. The structure of **2** was thus determined as *ent*-8(14)-pimarene-1α,15*R*,16-triol.

The molecular weight of **3** was 2 mass units smaller than that of **2**, suggesting the loss of two protons, which was confirmed by HRESIMS at m/z 343.2262 (calcd for $C_{20}H_{32}O_3Na$, 343.2249). The IR absorptions at 3383, 1702, 1683 cm^{-1} suggested the presence of an additional keto group by comparison with those data of **2**. The ^{1}H and ^{13}C NMR data of **3** (Table 1) were very similar to those of **2**, except for the ring A where **3** showed a keto carbon (δ 217.0, s) instead of the hydroxylated methine at C-1 of **2**. The HMBC correlation between methyl protons Me-20 (δ 1.12, s) and δ 217.0 (s) confirmed the location of the keto group. The NOESY correlations of **3** (Table 2) indicated that its stereochemistry was in agreement with that of **1** and **2**. The CD spectrum of **3** showed a negative Cotton effect at 277 nm ($n \rightarrow \pi^*$ transition) (Fig. 2). Analysis of the geometrical arrangement of the molecule in the eight octants formed by the symmetry planes of the orbital of the keto group (Klyne et al. 1967) indicated the absolute configuration as 5*R*, 9*S*, 10*R*, and 13*S*. Therefore, the structure of **3** was determined as (5*R*,9*S*,10*R*,13*S*)-*ent*-8(14)-pimarene-1-oxo-15*R*,16-diol.

Compounds **1** to **6** were tested against the tumor cell lines of L-929 (mouse fibroblasts), K562 (human chronic myeloid leukemia) and Hela (human cervix carcinoma), and **3** and **5** showed moderate activity against L-929 (IC_{50} 9.8 μg/ml) and K562 (IC_{50} 7.0 μg/ml), respectively, while the other compounds had no activity.

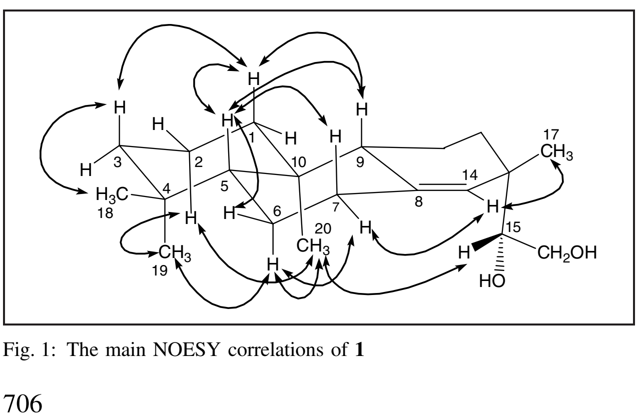


Fig. 1: The main NOESY correlations of **1**

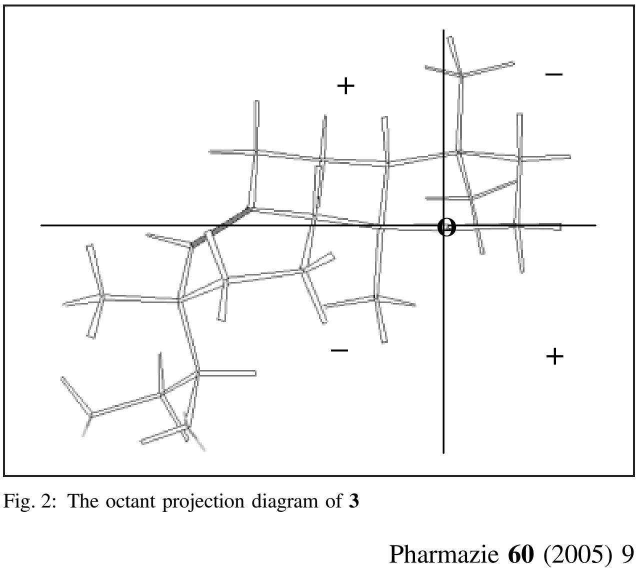


Fig. 2: The octant projection diagram of **3**

Table 2: Main HMBC and NOESY correlations of 1–to 3[a]

atom	**1**	
	HMBC	NOESY
H-1	C-3, C-5, C-9	
H-2		
H-5		H-1β, H-7β, H-9
H-7	C-5, C-9, C-14	
H-9	C-5, C-7, C-14, C-20	H-1β, H-7β, H-5
H-14	C-7, C-9, C-12, C-17	H-7α, H-16a, H-17
H-15	C-12, C-14, C-17	H-14, H-16, H-20
H-16	C-13, C-15	H-14, H-17
H-17	C-12, C-14, C-15	H-14
H-18	C-3, C-4, C-5, C-19	H-5, H-6β, H-19
H-19	C-3, C-4, C-5, C-18	H-2α, H-6α, H-18, H-20
H-20	C-1, C-5, C-9, C-10	H-6α, H-11α, H-15, H-19
atom	**2**	
	HMBC	NOESY
H-1	C-3, C-5, C-20	H-5, H-9
H-2		
H-5		H-1β, H-7β, H-9, H-18
H-7	C-5, C-9, C-14	
H-9	C-5, C-7, C-12, C-14, C-20	H-1β, H-7β, H-5
H-14	C-7, C-9, C-12, C-15, C-17	H-7α, H-16a, H-17
H-15	C-12, C-14, C-16, C-17	H-14, H-16, H-20
H-16	C-13, C-15	H-14, H-17
H-17	C-12, C-13, C-14, C-15	H-14
H-18	C-3, C-4, C-5, C-19	H-5, H-6β, H-19
H-19	C-3, C-4, C-5, C-18	H-2α, H-6α, H-18, H-20
H-20	C-1, C-5, C-9, C-10	H-1α, H-6α, H-11α, H-15, H-19
atom	**3**	
	HMBC	NOESY
H-1		
H-2	C-1, C-4, C-10	
H-5		H-1β, H-7β, H-9, H-18
H-7	C-5, C-9, C-14	
H-9	C-1, C-5, C-7, C-14, C-20	H-5, H-7β, H-12β
H-14	C-7, C-9, C-12, C-15, C-17	H-7α, H-15, H-17
H-15	C-12, C-14, C-16, C-17	H-14, H-16, H-20
H-16	C-13, C-15	H-12α, H-14
H-17	C-12, C-13, C-14, C-15	H-12β, H-14
H-18	C-3, C-4, C-5, C-19	H-3α, H-3β, H-5, H-6β, H-19
H-19	C-3, C-4, C-5, C-18	H-2α, H-6α, H-18, H-20
H-20	C-1, C-5, C-9, C-10	H-2α, H-6α, H-11α, H-19

[a] HMBC (H → C)

3. Experimental

3.1. General

Optical rotations were detected on a Propol digital automatic polarimeter, and IR spectra were recorded by a spectra IFS55 spectrometer (Bruker, Germany). CD spectrum was detected on J-810-150s spectropolarimeter (Jasco, Germany). ^{1}H and ^{13}C NMR spectra were measured on DPX-300, and DPX-500 (Bruker, Germany). ESIMS including HRMS were obtained by a triple quadrupole mass spectrometer Quattro (VG Biotech, England). Column chromatography was performed on silica gel 60 M (230–400 mesh, Macherey-Nagel, Germany). Sephadex LH-20 (Parmacia Biotech AB, Sweden). TLC was used with silica gel plates (Sil G/UV$_{254}$, 0.20 mm, Macherey-Nagel, Germany), and spots were detected under a UV spraying with anisaldehyde reagent.

3.2. Plant material

The stems of *B. gymnorrhiza* were collected in Xiamen, People's Republic of China, in June, 2002, and the species was identified by Prof. Peng Lin (Xia Men University). The Voucher sample (M004) is deposited in State Key Laboratory of Natural and Biomimetic Drugs, Peking University.

3.3. Extraction and isolation

The pulverized plant material (6.1 kg) was percolated in methanol (25 L) at room temperature for 2 weeks. The methanol extracts were concentrated under vacuum to yield a crude extract (282.6 g). The crude extract was partitioned between H_2O-EtOAc to obtain 23.6 g of EtOAc extract. The EtOAc extract was subjected to silica gel column chromatography (gradient $CHCl_3$/MeOH = 50 : 1/1 : 1). The eluted fractions were monitored by TLC to afford 25 fractions. Fraction 12 (513 mg) (10 : 1) was subjected to Sephadex LH-20 column chromatography by eluting with $CHCl_3$ to obtain **1** (12.6 mg) and **5** (16.0 mg). Fraction 16 (469 mg) (5 : 1) was separated by repeated silica gel chromatography (cyclohexane-EtOAc = 2 : 1) to get **3** (14.4 mg). Fraction 17 (398 mg) (4 : 1) was subjected to Sephadex LH-20 column chromatography by eluting with MeOH to afford **4** (20.0 mg). Fraction 24 (529 mg) (1 : 1) was separated on silica gel column chromatography with cyclohexane/EtOAc (3 : 2) as eluent to yield **2** (7.6 mg) and **6** (6.0 mg).

3.3.1. Ent-8(14)-pimarene-15R,16-diol (**1**)

White amorphous solid; $[\alpha]_D^{20}$ −32.0° (c 0.48, $CHCl_3$); IR (film) ν_{max} cm^{-1} 3255, 2921, 2360, 2341, 1681, 1472, 1364, 1049; ^{1}H and ^{13}C NMR data, see Table 1; (+)ESIMS m/z 329 $[M + Na]^+$, 324 $[M + NH_4]^+$, 307 $[M + H]^+$; HRESIMS m/z 329.2458 (calcd for $C_{20}H_{34}NaO_2$, 329.2456).

3.3.2 Ent-8(14)-pimarene-1α,15R,16-triol (**2**)

White amorphous solid; $[\alpha]_D^{20}$ −18.2° (c 0.12, $CHCl_3$); IR (film) ν_{max} cm^{-1} 3384, 2937, 2360, 2341, 1682, 1507, 1217, 1046; ^{1}H and ^{13}C NMR, see Table 1; (+)ESIMS m/z 345 $[M + Na]^+$, 340 $[M + NH_4]^+$, 323 $[M + H]^+$; HRESIMS m/z 345.2401 (calcd for $C_{20}H_{34}NaO_3$, 345.2406).

3.3.3. (5R,9S,10R,13S)-Ent-8(14)-pimarene-1-oxo-15R,16-diol (**3**)

White amorphous solid; $[\alpha]_D^{20}$ −36.5° (c 0.30, $CHCl_3$); CD (c 6.29 × 10^{-5} mol/L, MeOH); −867 (277 nm); IR (film) ν_{max} cm^{-1} 3383, 2936, 2360, 2341, 1702, 1683, 1457, 1367, 1070, 1019; ^{1}H and ^{13}C NMR data, see Table 1; (+)ESIMS m/z 321 $[M + H]^+$, 338 $[M + NH_4]^+$, 343 $[M + Na]^+$; HRESIMS m/z 343.2262 (calcd for $C_{20}H_{32}NaO_3$, 343.2249).

3.4. Biological testing

Compounds **1**–**6** were tested against L-929 (DSM ACC 2), K562 (DSM ACC 10) and Hela (DSM ACC 57) for their cytotoxic activities according to Dahse et al. (2001).

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