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NEW BIOACTIVE SESQUITERPENOID FROM MALAYSIAN SOFT CORAL GENUS *LEMNALIA*

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Abstract – One new sesquiterpenoid, parathyrsoidin K (**1**) along with seven known related secondary metabolites, parathyrsoidin J (**2**), lemnal-1(10)-ene-7β,12ζ-diol (**3**), linardosinene C (**4**), paralemnolin J (**5**) and K (**6**), 1*S**,4*S**,5*S**,10*R**-4,10-guaianediol (**7**), and 4-acetoxy-2,8-neolemnadien-5-one (**8**), were isolated from the organic extracts of soft coral *Lemnalia* sp. Their structures were elucidated based on spectroscopic analyses and these compounds were evaluated to anti-inflammatory activity against lipopolysaccharide-stimulated RAW 264.7 macrophages and antifungal activity against seven fungal strains.

The soft corals of genus *Lemnalia* (Cnidaria, Anthozoa, Octocorallia, Alcyonacea, and Nephtheidae) are widely distributed in the oceans from tropics and subtropics such as Taiwan, South China Sea, off the coast of Australia, and Kenya and consist of more than 30 species.[1] In addition, *Lemnalia* has long been known as a rich source of many structurally diverse and biologically active sesquiterpenes.[2] Many of these secondary metabolites are reported to exhibit potent biological activities such as cytotoxicity, antivirus, neuroprotection, immunosuppression, and anti-inflammatory.[3-7] In our effort to better understand the diversity of marine compounds from the Malaysian soft coral populations, we have discovered a series of bioactive natural products such as sesquiterpenes, diterpenes and others.[8-14] However, there is still a shortage of information on the diversity of bioactive compounds from *Lemnalia* sp. in tropical waters of South China Sea. As part of our ongoing research initiative, the organic extracts of *Lemnalia* sp. from Guhuan Bay, Tun Mustapha Marine Park, North Borneo, Sabah, Malaysia, led to the isolation of one new

sesquiterpenoid (**1**), along with seven known secondary metabolites (**2**-**8**). Here, we report the isolation, structure elucidation, anti-inflammatory and antifungal activity of these compounds.

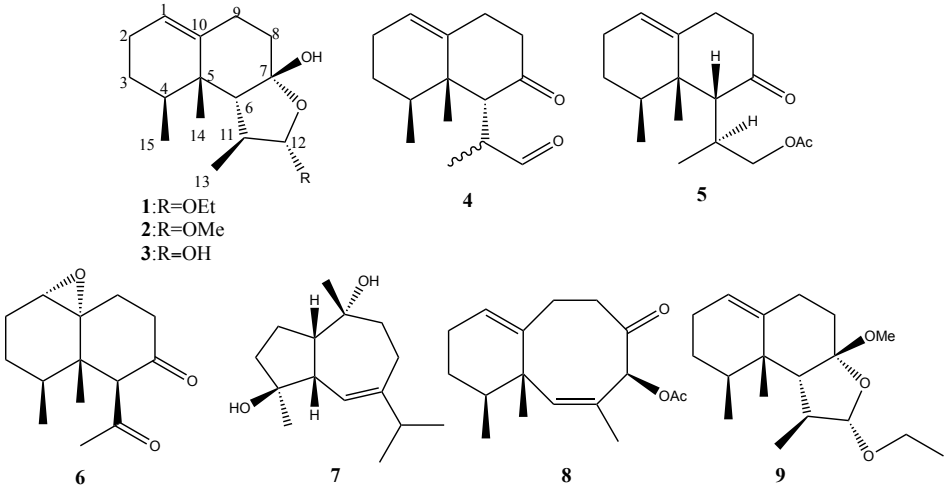
Compound **1** was isolated as a yellow oil with $[\alpha]_D^{25}$ -14 (*c* 0.94, $CHCl_3$). Its molecular formula was established as $C_{17}H_{28}O_3$ based on HRESIMS, the positive ion at *m/z* 263.1995 $[M + H - H_2O]^+$ (calcd 263.2006). Its IR absorption at 3443, 1717, 1458, 1108, and 1035 cm^{-1} indicated the presence of hydroxy and alkoxy groups. The ^{13}C-NMR data (Table 1) revealed the presence of 17 carbon signals, where their multiplicities were confirmed by DEPT and HSQC measurements as four methyls (including one ethoxy), five sp^3 methylenes, four sp^3 methines (include one oxymethine), one sp^2 methine and three quaternary carbons. One trisubstituted double bond was identified from NMR data (Table 1) at δ_C 139.0 and 123.7, δ_H 5.50 (d, J = 5.5 Hz). The above data accounted for one of the four degrees of unsaturation, thus, the tricyclic structure of **1** was revealed.

Table 1. ^{1}H-(600 MHz) and ^{13}C-NMR (150 MHz) spectroscopic data of **1**, **2** and **9** in $CDCl_3$, δ in ppm and J in Hz

	1		**2**		**9** †	
Position	**δ_H (Mult. *J*)**	**δ_C**	**δ_H (Mult. *J*)**	**δ_C**	**δ_H (Mult. *J*)**	**δ_C**
1	5.50 (d, 5.5)	123.7	5.52 (d, 5.5)	123.7	5.46 (brd, 4.9)	123.0
2	1.96-2.02 (m) 1.88-1.94 (m)	25.7	1.94-2.02 (m) 1.88-1.94 (m)	25.7	1.91 (m) 1.86 (m)	25.6
3	1.34-1.40 (m) 1.34-1.40 (m)	26.8	1.36-1.42 (m) 1.36-1.42 (m)	26.8	1.40 (m) 1.40 (m)	26.9
4	1.69-1.75 (m)	35.2	1.68-1.72 (m)	35.2	1.66 (m)	35.2
5	-	40.4	-	40.4	-	40.3
6	1.74 (d, 11.0)	59.7	1.75 (d, 10.3)	59.9	1.95 (d, 10.2)	54.1
7	-	106.6	-	106.7	-	109.0
8	1.99-2.05 (m) 1.78-1.84 (m)	36.3	1.99-2.03 (m) 1.80-1.86 (m)	36.3	1.91 (m) 1.89 (m)	33.2
9	2.40-2.48 (m) 2.00-2.04 (m)	28.9	2.41-2.49 (m) 1.99-2.03 (m)	28.7	2.35 (m) 2.05 (m)	28.1
10	-	139.0	-	138.9	-	139.4
11	1.88-1.92 (m)	42.5	1.88-1.94 (m)	42.5	1.85 (m)	42.2
12	4.63 (d, 4.1)	108.4	4.54 (d, 4.1)	109.8	4.59 (d, 4.9)	108.7
13	1.21 (d, 6.9)	20.0	1.22 (d, 6.9)	20.1	1.16 (d, 6.8)	19.5
14	1.12 (s)	20.7	1.13 (s)	20.7	1.03 (s)	20.3
15	0.83 (d, 6.9)	16.5	0.84 (d, 6.9)	16.5	0.81 (d, 6.3)	16.5
16	3.75 (dq, 9.6, 6.9) 3.40 (dq, 9.6, 6.9)	64.0	-	-	-	-
17	1.18 (s)	15.3	-	-	3.76 (dq, 14.1, 6.8) 3.43 (dq, 14.1, 6.8)	64.3
12-OMe	-	-	3.35 (s)	55.9	1.18 (t, *6.8*)	15.3

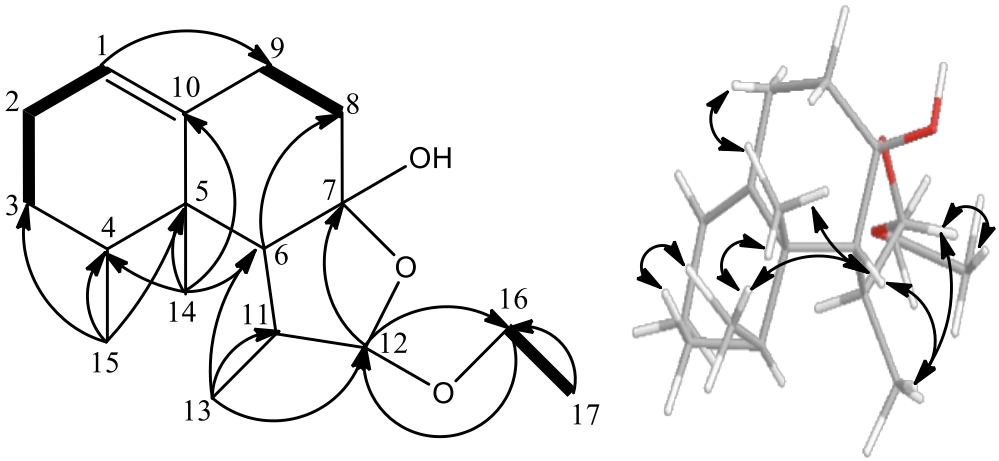
† **Note:** [15] Kapojos *et al., Chem. Pharm. Bull.*, 2008, **56**, 332.

Three ^{1}H-^{1}H COSY connectivities were present; H-1/H_2-2/H_2-3, H_2-8/H_2-9, and H_2-16/H_3-17. Key HMBC of H_3-17 to C-16: H_2-16 to C-12; H_3-15 to C-3, C-4, and C-5; H_3-14 to C-4, C-5, C-6, and C-10; H_3-13 to C-6, C-11, and C-12; H-12 to C-7, and C-16; H-6 to C-8; H-1 to C-9 permitted the establishment of the nardosinane-type skeleton of **1** (Scheme 1). In addition, careful examination of NMR data (Table 1) revealed structures of **1** and **2** were identical except for the replacement of an ethoxy in **1** by a methoxy in **2**.



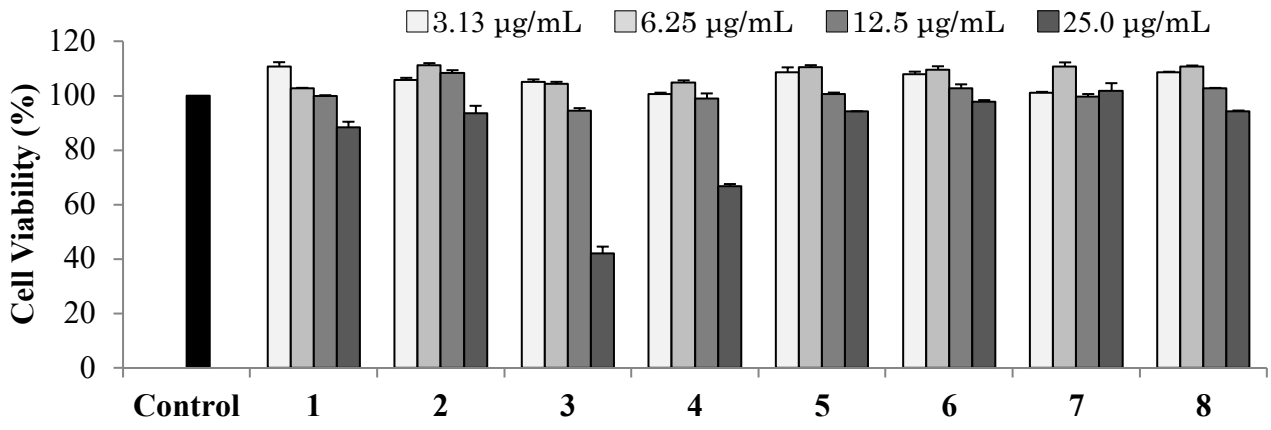
Scheme 1. Structures of the isolated compounds **1**-**8** from the *Lemnalia* sp. and related compound **9**

The relative configurations at C-4, C-5, C-6, C-7, C-11, and C-12 in **1** were determined as identical to its known analogues and 2-deoxy-12α-ethoxy-7-*O*-methyllemnacarnol (**9**) based on NOE cross peaks (Scheme 2), comparison of chemical shifts and *J*-based configuration. Structure of **1** closely resembled **9**, respectively except the replacement of methoxy group at C-7 by hydroxy group in **1**. The methyls at C-13, C-14, and C-15 were determined as *β* relative configuration based on comparison of ^{13}C-NMR data (Table 1) in **1** (δ_C 20.0, 20.7, and 16.5) with **9** (δ_C 19.5, 20.3, and 16.5).[15] Subsequently, the methine at C-6 and hydroxy group at C-7 were determined as *β* relative configuration due to the nature of decalin system found to be *cis*-fused with a tetrahydrofuran unit.[2,15-17] In addition, this finding was further supported by ^{1}H-^{1}H vicinal coupling constant (Table 1) between methines H-6 and H-11 in **1** ($^{3}J_{6\text{-}11}$ = 11.0 Hz) were closely similar to those of **9** ($^{3}J_{6\text{-}11}$ = 10.2 Hz).[15] The *α* relative configuration was assigned at C-12 based on ^{1}H-^{1}H vicinal coupling constant (Table 1) between methines H-11 and H-12 in **1** ($^{3}J_{11\text{-}12}$ = 4.1 Hz) were closely similar to those of **9** ($^{3}J_{11\text{-}12}$ = 4.9 Hz).[15] Some of the key NOE correlations (Scheme 2) between H_3-15/H_3-14, H_3-14/H-6, H_3-13/H-12, and H_3-13/H-6 in **1** were consistent to those of **9**.[15] The structures of seven known compounds were identified as parathyrsoidin J (**2**),[18] lemnal-1(10)-ene-7β,12ζ-diol (**3**),[16] linardosinene C (**4**),[19] paralemnolin J (**5**) and K (**6**),[20] 1*S**,4*S**,5*S**,10*R**-4,10-guaianediol (**7**),[21] and 4-acetoxy-2,8-neolemnadien-5-one (**8**),[22] by comparing their spectroscopic data and their optical rotation angles with those reported in the literatures (Scheme 1).

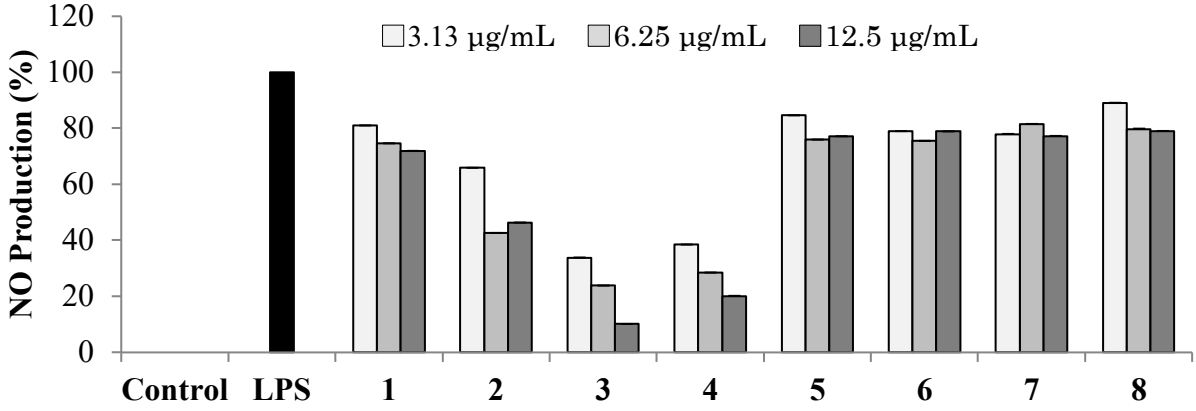


Scheme 2. The key ^{1}H-^{1}H COSY (bold lines) and selective HMBC (single-headed arrows) and NOE (double-headed arrows) correlations of compound **1**

The anti-inflammatory potentials of compounds **1**-**8** were evaluated based on the accumulation of nitric oxide (NO) production in lipopolysaccharide (LPS)-induced RAW 264.7 macrophages. The results showed that **3** and **4** were the most active compounds. The cell viability of RAW 264.7 macrophages against **1**-**8** displayed no significant differences between the compound groups (3.13, 6.25, 12.5, and 25.0 μg/mL) and control in the RAW 264.7 macrophages (Scheme 3). The results indicated that concentrations of up to 12.5 μg/mL did not compromise the cell viability of RAW 264.7 macrophages. Therefore, a concentration of 12.5 μg/mL or less was used for further analysis. The LPS treatment significantly elevated the production of NO (Scheme 4). The effects of **1**-**8** against the accumulation of NO production in LPS-stimulated RAW 264.7 macrophages showed that **3** and **4** were the most active against NO production at concentrations of 12.5 μg/mL as compared to that of negative control (LPS-induced RAW macrophages without the presence of the compound). The cell viability test proved that the inhibitory effect of **1**-**8** on NO production was not due to cytotoxic effects in RAW 264.7 macrophages.



Scheme 3. The effects of **1**-**8** on cell viability in RAW 264.7 macrophages



Scheme 4. The effects of **1**-**8** on NO production in LPS-induced RAW 264.7 macrophages

In addition, the antifungal potential of compounds **1**-**8** was tested against seven fungal strains (*Exophiala* sp. NJM 1551, *Fusarium moniliforme* NJM 8995, *F. oxysporum* NJM 0179, *Haliphthoros milfordensis* IPMB 1603, *H. sabahensis* IPMB 1402, *Lagenidium thermophilum* IPMB 1401, and *Ochroconis humicola* NJM 1503). The results of antifungal screening analysis showed that most active compounds were **3** and **4** with minimum inhibitory concentration (MIC) 25.0 µg/mL against *Exophiala* sp., and **3**, **5**, **6**, and **8** with MIC 25.0 µg/mL against *H. milfordensis* (Table 2).

Table 2. Antifungal activities of compounds **1**-**8**

Strains	MIC (µg/mL)								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**Clo ‡**
Exophiala sp.	50.0	50.0	25.0	25.0	50.0	50.0	50.0	50.0	3.1
F. moniliforme	>100	>100	>100	>100	>100	>100	>100	>100	3.1
F. oxysporum	>100	>100	>100	>100	>100	>100	>100	>100	3.1
H. milfordensis	50.0	50.0	25.0	50.0	25.0	25.0	50.0	25.0	3.1
H. sabahensis	>100	>100	>100	>100	>100	>100	>100	>100	3.1
L. thermophilum	>100	>100	>100	>100	>100	>100	>100	>100	3.1
O. humicola	50.0	>100	50.0	50.0	50.0	>100	50.0	50.0	3.1

‡ Clotrimazole: positive control

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES. The ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectra were recorded on a JEOL ECA 600 NMR spectrometer (Japan) using $CDCl_3$ with TMS as an internal standard. The high-resolution mass spectrum was acquired via Shimadzu LCMS-ESI-IT-TOF (Japan). Rudolph Research Analytical AUTOPOL IV automatic polarimeter (USA) was used to measure the optical rotation. Infrared spectra were recorded on a PerkinElmer Spectrum Two Universal Attenuated Total Reflection Spectrometer (USA). Preparative TLC was performed with Merck Kieselgel 60 F_{254} silica

gel glass plates (USA), and column chromatography (CC) with Merck Kieselgel 60, 70-230 mesh silica gel (USA). RAW 264.7 macrophages were obtained from the Korean Cell Line Bank (Korea). The quantity of formazan was measured at 540 nm by Tecan Co. Ltd. ELISA reader (Australia). Seven fungal strains were obtained from Universiti Malaysia Sabah crab hatchery farms.

BIOLOGICAL MATERIALS. A specimen of *Lemnalia* sp. was collected from Ghuan Bay, North Borneo (7.338711'N, 117.35017'E), in April 2017. The voucher specimen (BORMI0053) was deposited in the BORNEENSIS Collection of Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah.

EXTRACTION AND ISOLATION. The fresh soft coral (3.7 kg wet wt) was extracted in methanol (MeOH) at room temperature for 3 days, subsequently filtered, concentrated and partitioned between ethyl acetate (EtOAc)/water (H_2O) followed by partitioned with *n*-hexane/90% MeOH from EtOAc fraction. The resulting crude extracts were subjected to CC eluting with a gradient of *n*-hexane/EtOAc (9:1, 8:2, 7:3, 5:5, and 0:10) to yield five fractions 1-5. The *n*-hexane fraction 1 obtained in *n*-hexane/EtOAc (9:1) gave **8** (2.8 mg; 1.8%) after purification by preparative TLC using *n*-hexane. The *n*-hexane fraction 2, obtained in *n*-hexane/EtOAc (8:2), was subjected to preparative TLC with *n*-hexane/EtOAc (9:1) and toluene/EtOAc (9:1) to yield **3** (5.0 mg; 3.1%) and **5** (30 mg; 18.8%). As well as the *n*-hexane fraction 3, obtained in *n*-hexane/EtOAc (7:3), was subjected to preparative TLC with *n*-hexane/EtOAc (7.5:2.5) and toluene/EtOAc (8:2) to yield **1** (16.4 mg; 10.3%). In addition, the *n*-hexane fraction 5 obtained in EtOAc (100%) gave **4** (22.6 mg; 14.1%) after purification by preparative TLC using *n*-hexane/EtOAc (5:5). The MeOH fraction 3, obtained in *n*-hexane/EtOAc (7:3), was subjected to preparative TLC using *n*-hexane/EtOAc (8:2) and toluene/EtOAc (8:2) to yield **2** (9.5 mg; 5.3%) and **6** (28.2 mg; 15.7%). As well as the MeOH fraction 5, obtained in EtOAc (100%), was subjected to preparative TLC with *n*-hexane/EtOAc (5:5) and toluene/EtOAc (5:5) to yield **7** (5.4 mg; 3.0%). Percentages of compounds were the average of the respective compounds in the *n*-hexane and 90% MeOH crudes.

Parathyrsoidin K (1): Yellow oil; $[\alpha]_D^{25}$ -14 (*c* 0.94, $CHCl_3$); IR (KBr): λmax 3443, 1717, 1458, 1108, and 1035 cm^{-1}; HRESIMS $[M + H - H_2O]^+$ *m/z* 263.1995 (calcd for $C_{17}H_{27}O_2$, 263.2006); ^1H-($CDCl_3$, 600 MHz) and ^{13}C-($CDCl_3$, 150 MHz) NMR data, see Tables 1.

ANTI-INFLAMMATORY ASSAY. The cytotoxicity of compounds on RAW264.7 macrophage cells was evaluated using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[12] The cells (1.0×10^5 cells/mL) were seeded in a 96-well plate followed by treatment of compounds at various gradient concentrations (3.13, 6.25, 12.5, and 25.0 μg/mL) respectively in triplicate. The cells were maintained in 5% CO_2 at 37 °C for 24 h. The determination of NO production was performed by pre-cultured RAW 264.7 cells (1.0×10^5 cells/mL) for 24 h, subsequently pre-incubated with compounds for 1 h, followed by treatment with LPS (1 μg/mL) and incubated for 24 h.[12] Griess reagent (1% sulfanilamide

and 0.1% naphthylethylenediamine dihydrochloride in 2.5% phosphoric acid) was used to quantify NO production.

ANTIFUNGAL ASSAY. The MIC against seven fungal strains was performed by incorporating the compound solutions (6.25, 12.5, 25.0, 50.0, and 100 μg/mL) onto peptone yeast glucose sea water agar in petri dish.[13] The fungal strains are as described here; *Exophiala* sp. NJM 1551, *Fusarium moniliforme* NJM 8995, *F. oxysporum* NJM 0179, *Haliphthoros milfordensis* IPMB 1603, *H. sabahensis* IPMB 1402, *Lagenidium thermophilum* IPMB 1401, and *Ochroconis humicola* NJM 1503. The MIC was determined visually as the lowest concentration showing no hyphal growth when they were incubated at 25 °C for 7 days.

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