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PALMARIOLS A AND B, TWO NEW CHLORINATED DIBENZO-α-PYRONES FROM DISCOMYCETE *LACHNUM PALMAE*

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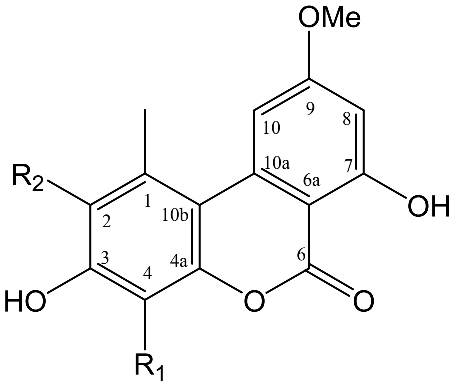
Abstract – Two new chlorinated dibenzo-α-pyrones, palmariols A (**1**) and B (**2**), together with alternariol 9-methyl ether (**3**), were isolated from the mycelial extract of discomycete *Lachnum palmae* (NRBC-106495), and the structures of **1** and **2** were elucidated using spectroscopic data. Palmariols A (**1**) and B (**2**) are rare dizenzo-α-pyrones containing a chlorine. Compounds **1**~**3** exhibited weak antimicrobial activity against *Mucor racemosus* and *Bacillus subtilis*.

INTRODUCTION

For the purpose of screening of new bioactive metabolites, fungi of the order Leotiales, Discomycetes, the member of which have been underutilized for microbial screenings, were collected and isolated.[1] Therefore, we noticed discomycete fungi as reseach source for new bioactive substances. In our screening for antimicrobial activity of various strains of discomycete fungi in the National Museum of Nature and Science, *Lachnum palmae* was selected. A series of the genus *Lachnum* is one of the most popular and remarkable inoperculate discomycetes. The genus of *Lachnum* is known to embrace some 150 species and yet more members have been being added to science.[2] Antimicrobial and nematicidal pentaketide compounds have been isolated from *Lachnum papyraceum*[3-5] and weak antibacterial compounds have been isolated from *Lachnum* sp.[6] However, bioactive compounds from *L. palmae* have not been reported. In this paper, we reported the isolation and structure elucidation of chlorinated dizenzo-α-pyrone compounds from mycelial extract of *L. palmae* and their antimicrobial activities against *Mucor racemosus* and *Bacillus subtilis.*

RESULTS AND DISCUSSION

The cultured mycelia of *L. palmae* were extracted with $CHCl_3$/MeOH (2:1). The extracts were partitioned between EtOAc and H_2O. The EtOAc-soluble portion was furthermore partitioned between *n*-hexane and 90% MeOH. The 90% MeOH-soluble portion was subjected to C_{18} Sep-Pak cartridge and reversed-phase HPLC to yield two new dibenzo-α-pyrones, palmariols A (**1**) and B (**2**) together with alternariol 9-methyl ether (**3**).



1: R_1 = Cl, R_2 = H

2: R_1 = H, R_2 = Cl

3: R_1 = R_2 = H

Figure 1. Chemical structures of **1**~**3**

Table 1. ^1H and ^{13}C NMR Data of Palmariols A (**1**) and B (**2**)

	Palmariol A (**1**)			Palmariol B (**2**)		
	$\delta_C{}^a$	$\delta_H{}^a$ (*J* in Hz)	HMBC^c	$\delta_C{}^b$	$\delta_H{}^a$ (*J* in Hz)	HMBC^c
1	136.3, C			139.2, C		
2	116.6, CH	6.89, s	3, 4, 10b, Me-1	125.1, C		
3	152.3, C			153.4, C		
4	106.5, C			106.3, CH	6.95, s	2, 3, 10b
4a	147.9, C			152.1, C		
6	164.4, C			166.9, C		
6a	98.9, C			100.8, C		
7	165.2, C			167.7, C		
8	99.4, CH	6.58, d (2.1)	6a, 7, 10	100.6, CH	6.58, d (2.1)	6a, 7
9	166.6, C			168.8, C		
10	105.0, CH	7.26, d (2.1)	8, 9, 10b	105.8, CH	7.18, d (2.1)	8, 9, 10b
10a	137.5, C			136.8, C		
10b	111.9, C			111.9, C		
Me-1	25.6, CH_3	2.79, s	1, 2, 10b	24.5, CH_3	2.78, s	1, 2, 10b
MeO-9	55.8, CH_3	3.92, s	9	57.1, CH_3	3.92, s	9
OH-7		11.78, s			11.80,s	

^{a} in $CDCl_3$, ^{b} in CD_3OD, ^{c}HMBC correlations are from proton(s) stated to the indicated carbon.

Palmariol A (**1**) showed pseudomolecular ion peaks at *m/z* 307 and 309 (3:1) $(M+H)^+$ in the ESIMS. The molecular formula of **1** was deduced as $C_{15}H_{12}ClO_5$ from HRESIMS [*m/z* 307.03685 $(M+H)^+$, Δ-0.48 mmu]. The IR spectrum indicated the presence of hydroxyl (3417 cm^{-1}) and unsaturated carbonyl (1672 and 1623 cm^{-1}) groups, whereas the UV absorptions at 224 and 252 nm implied that **1** possesses pyrone ring. The gross structure of **1** was dedued by detailed analyses of the ^1H and ^{13}C NMR data (Table 1) aided by 2D NMR experiments (HMQC and HMBC) (Figure 2). ^1H NMR spectrum at δ_H 2.79 (3H, s) and 3.92 (3H, s) implied the presence of one methyl group and one methoxy group, respectivly. ^1H NMR

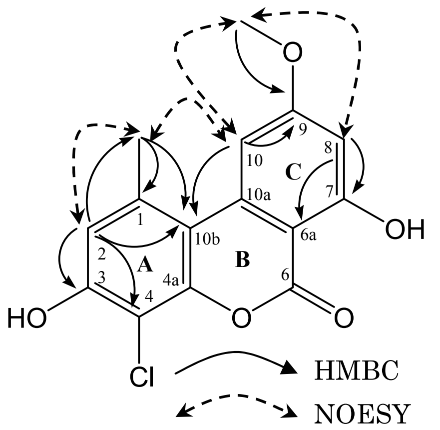


Figure 2. 2D NMR correlations of **1**.

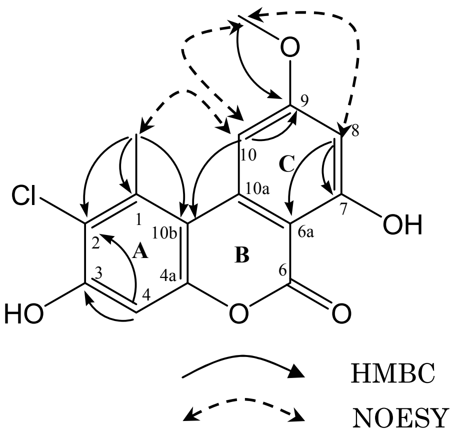


Figure 3. 2D NMR correlations of **2**.

spectrum at δ_H 6.58 (d, J = 2.1 Hz), 7.26 (d, J = 2.1 Hz), and 6.89 (s) indicated the existence of a 1,2,4,6-tetrasubstituted and 1,3,4,5,6-pentasubstituted benzene rings. One doublet with J value of 2.1 Hz between δ_H 6.58 and 7.26 confirmed the presence of *meta* position aromatic protons. A proton signal at δ_H 11.78 (s) indicated the presence of a hydrogen bond between a hydroxy group and a carbonyl group. The ^{13}C NMR data indicated **1** possessed one unsaturated carbonyl carbon, twelve aromatic carbons, one methoxy carbon, and one aromatic methyl carbon (Table 1). The UV and ^{1}H NMR spectra of **1** were similar to those of alternariol 9-methyl ether (**3**).[7,8] The HMBC correlation between MeO-9 and C-9 (δ_C 166.6) revealed the linkage of methoxy group at C-9. The HMBC correlations of H-8 to C-6a (δ_C 98.9) and C-10 (δ_C 105.0) and H-10 to C-8 (δ_C 99.4) and C-9 revealed the structure of ring C. The HMBC correlations of a methyl proton at δ_H 2.79 to C-1 (δ_C 136.3), C-2 (δ_C 116.6), and C-10b (δ_C 111.9) revealed that a methyl group is attached at C-1. The HMBC correlations of H-2 to C-3 (δ_C 152.3), C-4 (δ_C 106.5), Me-1 (δ_C 25.6), and C-10b revealed the structure of ring A. Especially, the HMBC correlations of H-2 to C-4 and Me-1, a NOESY correlation (Figure 2) between H-2 and Me-1, and the chemical shift (δ_C 106.5) of the quaternary carbon (C-4) revealed the presence of a chlorine at C-4. The HMBC correlations of H-2, H-10, and Me-1 to C-10b revealed connectivity to rings A, B, and C. The carbon signals of C-10a, C-4a, and C-6 were assigned by comparison of the ^{13}C NMR data of **1** with those of **3**.[8] The NOESY correlations of MeO-9 to H-8 and H-10 and Me-1 to H-2 and H-10 were also supported the connectivity to rings A, B, and C. Therefore, palmariol A (**1**) was assigned 4-chloro-3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo[*b,d*]pyran-6-one.

Palmariol B (**2**) showed pseudomolecular ion peaks at *m/z* 305 and 307 (3:1) $(M-H)^-$ in the ESIMS. The molecular formula of **2** was deduced as $C_{15}H_{12}ClO_5$ from HRESIMS [*m/z* 305.02116 $(M-H)^-$, Δ -0.52 mmu], indicating **2** was an isomer of **1**. The IR spectrum indicated the presence of hydroxyl (3435 cm^{-1}) and unsaturated carbonyl (1655 and 1630 cm^{-1}) groups, whereas the UV absorptions at 218 and 257 nm implied that **2** possesses pyrone ring. The gross structure of **2** was dedued by detailed analyses of the ^{1}H and ^{13}C NMR data (Table 1) aided by 2D NMR experiments (HMQC and HMBC) (Figure 3). ^{1}H NMR spectrum at δ_H 2.78 (3H, s) and 3.92 (3H, s) the implied the presence of one methyl group and one methoxy group, respectively. One doublet with *J* value of 2.1 Hz between δ_H 6.58 and δ_H 7.18 confirmed the presence of *meta* position aromatic protons. Comparison of the UV, IR, ESIMS, and ^{1}H NMR spectra of **1** and **2** indicated **2** was a regioisomer of **1** with a chlorine. An HMBC cross peak of H-4 to C-2 (δ_C 125.1) was observed, while a NOESY correlation between H-2 and Me-1 (Figure 3) in palmariol A (**1**) was not observed, indicating the presence of a chlorine at C-2 (Figure 3) in **2**. Therefore, palmariol B (**2**) was assigned 2-chloro-3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo[*b,d*]pyran-6-one.

Palmariols A (**1**) and B (**2**) are dibenzo-α-pyrones with a chlorine at C-4 and C-2, respectively. Since only one natural chlorinated dibenzo-α-pyrone has been reported,[9] compounds **1** and **2** are second example. Alternariol 9-methyl ether (**3**) and other dibenzo-α-pyrones were reported to show antimicrobial activity.[10,11] Therefore, compounds **1 - 3** were tested antimicrobial activities against various microorganisms of four gram positive-bacteria, five gram-negative bacteria, two fungi, and two yeasts by plate diffusion assay. Compounds **1 - 3** showed weak antifungal activity against *M. racemosus*, while compound **1** showed weak antibacterial activity against *B. subtilis* (Table 2).

Table 2. Antimicrobial activities of **1**~**3** in the plate diffusion assay.

Organism	Diameter of inhibition zone (mm)		
	1	2	3
Mucor racemsus KF223	8	8	8
Bacillus subtilis KB27	9	-*	-

Concentrations tested: 20µM/paper disc (i. d. 6 mm)
*-: no inhibition zone

EXPERIMENTAL

General Procedures.

UV spectra were recorded on a HITACHI U-2000A spectrometer. IR spectra were recorded on a JASCO FT/IR-300 spectrometer. ^{1}H and ^{13}C NMR spectra were measured and recorded on a Bruker Avance 500 MHz spectrometer in $CDCl_3$ and CD_3OD. The resonances of $CDCl_3$ at δ_H 7.26 and δ_C 77.0 and CD_3OD at δ_H 3.35 and δ_C 49.8 were used as internal references for the ^{1}H and ^{13}C NMR spectra, respectively. ESIMS were recorded on a JEOL JMS-T100LC mass spectrometer.

Fungal Material.

The isolate NBRC 106495, deposited in the National Institute of Technology and Evaluation, Biological

Resource Center (NBRC) was obtained by a single ascospore isolation using a Skerman's micromanipulator[12] from an apothecium of *Lachnum palmae* (Kanouse) Spooner produced on the decaying leaves of *Livistona*, collected in Suzaki, Shimoda, Shizuoka Prefecture in July, 2004. The specimen was preserved as TNS-F-11197 in the National Museum of Nature and Science. The isolate was grown and kept on potato dextrose agar (PDA, Nissui).

Fermentation.

Fermentation of 100 mL Erlenmeyer flasks (× 5) each containing 30 mL of the seed medium (PYG: 20 g of glucose, 10g of polypeptone, 5 g of yeast extract, and 1 L of deionized water; pH was adjusted to 7.5 before autoclaving) were inoculated with some pieces of cut from agar slants. After incubation on a rotary shaker at 180 rpm, 23 ºC for 13 days, each seed culture was transferred into 500 mL Erlenmeyer shaking flasks (× 20) each containing 100 mL of PYG medium and incubated on rotary shaker at 210 rpm, 23 ºC for 1 week. A series of fermentation repeated twenty times and finally 20 L of culture broth were obtained. The culture broth was filtered to give a wet mycelial mat.

Extraction and Isolation.

Mycelia of *L. palmae* (1.95 kg, wet weight) was extracted with $CHCl_3$/MeOH (2:1) at room temperature and evaporated to dryness *in vacuo* 35 ºC. The extract (65.8 g) was partitioned between EtOAc (400 ml×3) and H_2O (400 ml). The EtOAc–soluble portion (5.33 g) was furthermore partitioned between *n*-hexane (200 ml×3) and 90% MeOH (200 ml). The 90% MeOH-soluble portion (990 mg) was divided into 11 fractions using C_{18} Sep-Pak cartridge (Waters; MeOH/H_2O, 20:80 →100:0) and the fraction (32.0 mg) containing alternariol analogs judging from the ^{1}H NMR spectrum was further separated by reversed-phase HPLC [TSK-gel ODS-80Ts, TOSOH, ϕ7.8×25 cm, flow rate 2.0 ml/min; MeOH/H_2O, 70:30 → 100:0 (0 to 50 min)] to give alternariol 9-methyl ether (**3**) (2.6 mg, t_R 22 min), palmariols A (**1**) (4.2 mg, t_R 24 min) and B (**2**) (2.3 mg, t_R 28 min).

Palmariol A (1): Colorless powder; IR (KBr) ν_{max} 3417, 2925, 1672, 1623, and 1572 cm^{-1} ; UV (MeOH) λ_{max} (logε) 224 (4.6), 252 (4.6), 284 (sh, 4.3), 297 (4.2), and 340 (4.2) nm; ^{1}H and ^{13}C NMR (Table 1); ESIMS (positive ion) *m/z* 307 and 309 (3:1) $(M+H)^+$; HRESIMS (positive ion) *m/z* 307.03685 $(M+H)^+$, (calcd for $C_{15}H_{12}{}^{35}ClO_5$, 307.03733).

Palmariol B (2): Colorless powder; IR (KBr) ν_{max} 3435, 2924, 1655, 1630, and 1385cm^{-1}; UV (MeOH) λ_{max} (logε) 208 (4.2), 218 (4.5), 257 (4.5), 282 (sh, 4.1), 302 (4.0), and 344 (4.0) nm; ^{1}H and ^{13}C NMR

(Table 1); ESIMS (negative ion) *m/z* 305 and 307 (3:1) $(M-H)^-$; HRESIMS (negative ion) *m/z* 305.02116 $(M-H)^-$, (calcd for $C_{15}H_{10}{}^{35}ClO_5$, 305.02168).

Alternariol 9-mehyl ether (3): Colorless powder; IR (KBr) ν_{max} 3437, 2925, 1655, 1618, and 1234 cm^{-1}; UV (MeOH) λ_{max} (logε), 218 (4.9), 248 (5.0), 256 (5.0), 288 (4.6), 289 (4.6), and 335 (4.6) nm; ^{1}H NMR (500 MHz, CD_3OD): 7.32 (1H, d, *J* = 2.1 Hz, H-10), 6.74 (1H, d, *J* = 2.4 Hz, H-2), 6.65 (1H, d, *J* = 2.4 Hz, H-4), 6.59 (1H, d, *J* = 2.1 Hz, H-8), 3.97 (3H, s, MeO-9), and 2.81 (3H, s, Me-1); ^{13}C NMR (125 MHz, CD_3OD): 169.0 (C-9), 167.6 (C-7), 167.1 (C-6), 160.9 (C-3), 155.3 (C-4a), 140.6 (C-10a), 140.5 (C-1), 119.5 (C-2), 111.8 (C-10b), 105.7 (C-10), 103.7 (C-4), 100.8 (C-6a), 100.8 (C-8), 57.1 (MeO-9), and 25.6 (Me-1); HMBC correlations (CD_3OD, H/C): 2/4, 2/Me-1, 4/2, 4/3, 4/4a, 4/10b, 8/6a, 8/7, 8/9, 8/10, 10/8, 10/9, 10/10b, Me-1/1, Me-1/2, Me-1/10b, and MeO-9/9.

Antimicrobial test.

Antimicrobial activity against four gram positive-bacteria (*Bacillus subtilis* KB27, *Staphylococcus aureus* KB210, *Micrococcus luteus* KB212, and *Mycobacterium smegmatis* KB42), five gram-negative bacteria (*Esherichia coli* KB213, *Esherichia coli* KB176, *Pseudomonas aeruginosa* KB105, *Xanthomonas campestris* pv. *Oryzae* KB88, and *Bacteroides fragilis* KB169), two fungi (*Aspergillus niger* KF103 and *Mucor racemosus* KF223), and two yeasts (*Candida albicans* KF11 and *Saccharomyces cerevisiae* KF26) were tested by plate diffusion assay using 6 mm paper disk. Palmariols A (**1**) and B (**2**) and **3** solutions (1 mM) were prepared by dissolving each compound in acetone. Each adjusted solution were added in paper disk (20 μl) and paper disk were drying. The paper disks were set on the agar plate suspended tested microorganisms. After cultivating microorganisms certain times, the strength of antimicrobial activity was estimated by measuring the diameter length of inhibition zone (mm).

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