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NOTE

Cladosporone A, a new dimeric tetralone from fungus *Cladosporium* sp. KcFL6' derived of mangrove plant *Kandelia candel*

Wen Ai[1,2,4], Xiuping Lin[1,4], Zhen Wang[3], Xin Lu[3], Fredimoses Mangaladoss[1], Xianwen Yang[1], Xuefeng Zhou[1], Zhengchao Tu[3] and Yonghong Liu[1]

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The mangroves habitats in tropical regions represent the terrestrial fringe of the marine habitats. Mangrove forests represent an ecosystem of high biodiversity. Their unique living conditions are thought to predestine mangroves as promising sources for isolation of endophytic fungi. Mangrove plants and their endophytic fungi have been a rich source of bioactive molecules.[1,2] More than 200 species of endophytic fungi were isolated and identified from various species of mangrove plants and have been proven to be a well-established source for structurally diverse and biologically active second metabolites.[3] The genus *Cladosporium* was reported as a kind of main endophytic fungus of mangrove plant, which can produce remarkable diversity of secondary metabolites such as alkaloids,[4] lactones,[5,6] diterpenoids[7] and quinones.[8] Cladosporol (=cladosporol A) was isolated from cultures of *Cladosporium cladosporioides*, which is plant growth regulator and antifungal agent in agriculture.[9] Cladosporol inhibits colon cancer cell proliferation by modulating p21waf1/cip1 expression.[10] PPARγ mediates the antiproliferative action of cladosporol in colorectal cancer cells.[11] *In vivo*, cladosporol also shows antitumor activity in nude mice bearing MGC-803 gastric-cancer xenografts.[12] Alterfungin is a chiral isomers of cladosporol which has strong broad-spectrum antifungal activity.[13] Cladosporols B-E[14] were isolated from *C. tenuissinnum*, a known hyperparasite of several rust fungi.[15] These compounds were active in inhibiting the urediniospore germination of the bean rust agent *Uromyces appendicalatus*.

In our previous research toward the discovery of biologically active metabolites from endophytic fungus of Chinese mangrove plants, we obtained many active compounds.[16–18] In our ongoing research, a new dimeric tetralone bridged through C–C linkage, was isolated, along with cladosporol (**2**), cladosporols C (**3**) and D (**4**). Herein, the isolation, structure elucidation and biological activities of metabolites **1–4** were presented.

Cladosporone A (**1**) was isolated as yellow-brown powder. The molecular formula of **1** was determined to be $C_{20}H_{16}O_6$ (13 degrees of unsaturations) by HRESI-MS analysis (351.0866 $[M\text{-}H]^-$), which was also supported by the ^1H and ^{13}C NMR data (Table 1). ^1H and ^{13}C NMR, DEPT and HSQC data revealed that all protons and carbons and six oxygens in the molecule of **1** were assigned as two methylenes, eight methines, seven quaternary carbons, two carbonyls and three hydoxyls. The connectivities among these units were determined by the analysis of ^1H-^1H COSY and HMBC spectra. Analysis of the COSY data led to the identification of two isolated proton spin systems corresponding to the C-2-C-3-C-4 and C-6-C-7 subunits of structure **1**. HMBC correlations of H-2 with carbonyl C-1, C-3 and C-4, H-3 with C-1, C-2, C-4 and C-10, H-4 with C-2, C-5, C-9 and C-10 led to the assignment of the nonaromatic ring. Correlation of H-6 to C-5, C-7, C-8, C-9 and C-10, H-7 to C-5, C-6, C-9 and C-10 enabled the connection of the C-9 and C-10 of the aromatic ring to C-1 and C-4, resulting in a tetralone moiety. The other tetralone structure was constructed from HMBC. The two structures were connected between C-8 and C-4′ because of the correlations of H-4′ to C-7, C-8 and C-9, thereby completing the planar structure of **1** as shown in Figure 1.

The large $^3J_{2H,3H}$ (8.5 Hz) and the small $^3J_{3H,4H}$ (4.5 Hz) coupling constants indicated that H-2 and H-3 have 2,3-*trans* relative configuration and 3,4-*cis* relative configuration. Then, the position of expoxide was determined at 3,4-position by HMBC corelations. Thereby, the relative configuration of **1** was showed in Figure 1.

The CD pattern of **1** paralleled that reported for the cladosporol,[9] this fact permitting us to assign the absolute configuration of C-4 and C-4′. The absolute configuration of **1** was also assumed by analogy with the co-occurring compounds **2–4**.

Compound **2** was identified as cladosporol[9] compared with literatures reported. In CD spectrum, there was a negative cotton

[1]CAS Key Laboratory of Tropical Marine Bio-resources and Ecology/Guangdong Key Laboratory of Marine Materia Medica/Research Center for Marine Microbes, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China; [2]University of Chinese Academy of Sciences, Beijing, China and [3]Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China
[4]These authors contributed equally to this work.
Correspondence: Dr Z Tu and Dr Y Liu, Guangdong Key Laboratory of Marine Materia Medica/Research Center for Marine Microbes, South China Sea Institute of Oceanology, Chinese Academy of Sciences, No 164, West Xinggang Road, Guangzhou, Guangzhou 510301, China.
E-mail: tu_zhengchao@gibh.ac.cn or yonghongliu@scsio.ac.cn
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effect at 230 nm, which certified the stereochemistry of C-4′. The CD pattern of **2** paralleled that reported for the cladosporol.[9]

Compounds **3** and **4** were identified as cladosporols C (**3**) and D (**4**)[15] compared with the literatures reported.

From biosynthetic aspect, compounds **1–4** could be generated from hexaketide or pentaketide to form the key monomer tetralone, and then the tetralone coupled to give the key intermediate **4**. The structure of **4** would be further reacted by different cascades (dehydration, reduction, cyclization and oxidation) as illustrated in Figure 2.

In standard antibacterial disc assays, none of the compounds showed activities against *Acinetobacter baumannii* ATCC 19606, *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Aeromonas hydrophila* ATCC 7966, *Escherichia coli* ATCC 25922 and *Klebsiella pneumonia* ATCC 13883 when concentration of the tested solutions was 50 μg per disc.

In standard antifungal disc assays, none of the compounds showed activities against *Fusarium* sp., *Fusarium oxysporumf.* sp. *niveum*, *F. oxysporumf.* sp. *cucumeris*, *Aspergillus niger* and *Rhizoctonia solani* when concentration of tested solutions was 25 μg per disc.

In anti-COX-2 assay, compounds **1** and **4** showed inhibitory activities (IC_{50} = 49.1 and 60.2 μM, respectively). The cytotoxicities of **1–4** were evaluated against 10 human tumor cell lines. Compounds **1** and **3** exhibited moderate cytotoxicities against human tumor cell lines (Table 2).

Table 1 NMR spectral data for 1 ($CDCl_3$)

No.	^{13}C	^{1}H
1	191.3	
2	63.6	4.91, d, 8.5
3	71.3	4.31, dd, 8.5, 4.5
4	66.1	5.50, d, 4.5
5	155.3	
6	122.1	7.01, d, 8.5
7	132.2	6.89, d, 8.5
8	138.2	
9	124.3	
10	132.1	
1′	205.3	
2′	36.1	2.63, m
3′	30.1	2.13, m, 2.43, m
4′	40.1	5.38, m
5′	119.8	6.35, d, 8.0
6′	136.4	7.30, t, 8.0
7′	116.2	6.82, d, 8.0
8′	163.1	
9′	117.1	
10′	147.4	
–OH		12.57, s

MATERIALS AND METHODS

General experimental procedures

The NMR spectra were recorded on a Bruker AC 500 NMR spectrometer (Bruker, Brenen, Germany) with tetramethylsilane as internal standard. HR-ESI-MS data were measured on a Bruker micro TOF-QII mass spectrometer (Bruker). Optical rotation values were measured with an Anton Paar MCP500 polarimeter (Anton Paar GmbH, Graz, Austria). UV spectra were recorded on a Shimadzu UV-2600 UV–Vis spectrophotometer (Shimadzu, Tokyo, Japan). CD spectra were measured with a chirascan circular dichroism spectrometer (Applied Photophysics, London, UK). The silica-gel GF254 used for TLC were supplied by the Qingdao Marine Chemical Factory, Qindao, China. Sephadex LH-20 gel (GE Healthcare, Uppsala, Sweden) were used. HPLC was carried on Hitachi L-2400 (Hitachi, Tokyo, Japan) with YMC ODS column (YMC America, Allentown, PA, USA). Spots were detected on TLC under UV light or by heating after spraying with 5% H_2SO_4 in EtOH.

Fungal strain

Endophytic fungus strain KcFL6', identified as *Cladosporium* sp., was obtained from the flower of mangrove plant *Kandelia candel*, which was collected at Daya Bay, Shenzhen city, Guangdong province, China, in April 2012.

Fermentation, extraction and isolation

Ascospores were subcultured onto 4 kg of rice in twenty 1 l Erlenmeyer flasks (200 g each) and incubated at 26 °C under 12-h light/12-h dark conditions for 2 months. The fermentation mixture was broken up with a spatula and extracted three times with EtOAc (3 × 4 liters). The combined EtOAc extract was filtered and evaporated to afford 64.3 g of crude extract.

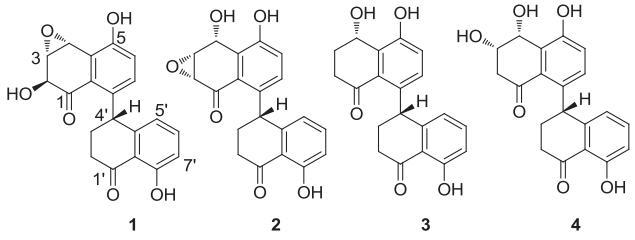

Figure 1 The structures of the compounds **1–4**.

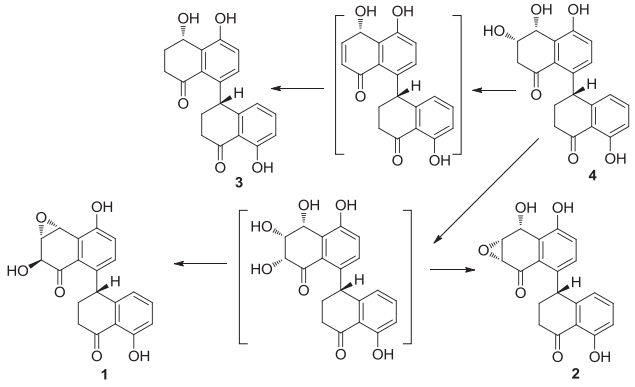

Figure 2 The possible biosynthetic pathways of compounds **1–4**.

Table 2 The cytotoxicities of compounds 1 and 3 (μM)

Entry	*K562*	*A549*	*Huh-7*	*H1975*	*MCF-7*	*U937*	*BGC823*	*HL60*	*Hela*	*MOLT-4*
1	14.3	15.7	29.9	40.6	21.3	10.5	17.0	10.1	53.7	14.6
3	>30	33.9	>30	45.6	>30	>30	>30	72.5	>30	11.4
TSA	0.24	0.05	0.08	0.09	0.78	0.06	0.09	0.09	0.11	0.03

Abbreviation: TSA, Trichostatin A.

The crude EtOAc extract was partitioned between methanol and hexane. The resulting methanol fraction (17.7 g) was subjected to silica-gel column chromatography eluted with petroleum ether/EtOAc in gradient eluant (80:20, 50:50, 30:70) followed by $CHCl_3$/MeOH in gradient eluant (90:10, 80:20, 70:30, 60:40, 0:100) to obtain eight fractions (fractions 1–8) by monitoring with TLC. Fraction 4 was purified by Sephadex LH-20 (GE Healthcare) ($CHCl_3$/MeOH, 1:1) first to give four subfractions (fractions 4.1–4.5). Fraction 4.2 (20.0 mg) was further purified by HPLC (MeOH/H_2O, 75%) to give **1** (2.8 mg). Fraction 5 was purified by Sephadex LH-20 (GE Healthcare) (MeOH) first to give three subfractions (fractions 5.1–5.3). Fraction 5.3 (24.0 mg) was further purified by HPLC (MeOH/H_2O, 70%) to give **2** (2.3 mg) and **3** (3.3 mg). Fraction 7 was purified by Sephadex LH-20 (GE Healthcare) (MeOH) first to give four subfractions (fractions 7.1–7.4). Fraction 7.3 (10.0 mg) was further purified by HPLC (MeOH/H_2O, 75%) to give **4** (2.1 mg).

Cladosporone A (**1**): yellow-brown powder; $[\alpha]^{25}_D = +60.7$ ($c = 0.3$ MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.83), 246 (4.45), 259 (4.51); ^1H and ^{13}C NMR data see Table 1; selected HMBC data, H-3 → C-1; H-4 → C-2, C-3, C-5, C-9, C-10; H-6 → C-5, C-7, C-8, C-10; H-7 → C-5, C-6, C-9; HRESI-MS 351.0866 $[M-H]^-$ (calcd for $C_{20}H_{15}O_6$ 351.0874); ESIMS 351 $[M-H]^-$, 387 $[M+Cl]^-$.

Cladosporol (**2**): white powder; $[\alpha]^{25}_D = +273.3$ ($c = 0.06$ MeOH); ^1H NMR (500 MHz, CD_3OD) δ_H: 4.34 (1H, d, $J = 3.5$ Hz, H-2), 5.21 (1H, d, $J = 3.5$ Hz, H-3), 5.51 (1H, s, H-4), 6.97 (1H, d, $J = 8.5$ Hz, H-6), 6.77 (1H, d, $J = 8.5$ Hz, H-7), 2.66 (2H, m, H-2′), 2.26 (1H, m, H-3′), 2.36 (1H, m, H-3′), 5.51 (1H, s, H-4′), 6.37 (1H, d, $J = 7.5$ Hz, H-5′), 7.32 (1H, t, $J = 8.0$ Hz, H-6′), 6.79 (1H, d, $J = 8.0$ Hz, H-7′); ESIMS 353 $[M+H]^+$, 375 $[M+Na]^+$.

Cladosporol C (**3**): white powder; $[\alpha]^{25}_D = +92.4$ ($c = 0.5$ MeOH); ^1H NMR (500 MHz, CD_3OD) δ_H: 2.55 (1H, dt, $J = 4.5$, 17.0 Hz, H-2), 3.04 (1H, m, H-2), 2.29 (2H, m, H-3), 5.39 (1H, m, H-4), 6.95 (1H, d, $J = 8.5$ Hz, H-6), 6.76 (1H, d, $J = 8.0$ Hz, H-7), 2.59 (2H, t, $J = 6.5$ Hz, H-2′), 2.27 (1H, m, H-3′), 2.32 (1H, m, H-3′), 5.50 (1H, s, H-4′), 6.37 (1H, d, $J = 7.5$ Hz, H-5′), 7.31 (1H, t, $J = 8.0$ Hz, H-6′), 6.81 (1H, d, $J = 8.5$ Hz, H-7′); ESIMS 337 $[M-H]^-$, 373 $[M+Cl]^-$.

Cladosporol D (**4**): white powder; $[\alpha]^{25}_D = +22.5$ ($c = 0.05$ MeOH); ^1H NMR (500 MHz, CD_3OD) δ_H: 2.77 (1H, d, $J = 15.0$ Hz, H-2), 3.05 (1H, dd, $J = 15.0$, 10.5 Hz, H-2), 4.13 (2H, m, H-3), 5.39 (1H, d, $J = 3.0$ Hz, H-4), 6.98 (1H, d, $J = 8.5$ Hz, H-6), 6.77 (1H, d, $J = 8.0$ Hz, H-7), 2.65 (2H, m, H-2′), 2.23 (1H, m, H-3′), 2.29 (1H, m, H-3′), 5.52 (1H, m, H-4′), 6.37 (1H, d, $J = 7.5$ Hz, H-5′), 7.32 (1H, t, $J = 8.0$ Hz, H-6′), 6.85 (1H, d, $J = 8.0$ Hz, H-7′); ESIMS 353 $[M-H]^-$, 389 $[M+Cl]^-$.

Antimicrobial assays

Studies on antibacterial activity. The ready-made nutrient agar medium (38 g) was suspended in distilled water (1000 ml) and heated to boiling until it dissolved completely. The medium and petridishes were autoclaved at a temperature 121 °C for 20 min.

Disc diffusion assay was employed for testing antibacterial activity of the compounds. The medium was poured into sterile petridishes under aseptic conditions in laminar flow chamber. Before the solidification of the medium on the plate, 2% cultured of test organism was inoculated and uniformly spread over the medium. Solutions were prepared by dissolving compound in MeOH and concentration (50 μg μl^{-1}) was made. After incubation, to each disc concentrations of test solutions were added (1 μl). The positive control, Penicillin was maintained with MeOH (50 μg μl^{-1}). The treated materials and the controls were kept in an incubator at 28 °C for 12 h. Inhibition zones were measured and diameter was calculated in mm. Three replicates were maintained for each treatment.

Studies on antifungal activity. The method followed for antifungal bioassay is similar to that followed for antibacterial assay where in the medium is malt extract agar 40 g l^{-1} and the positive control is carbendazim. Concentration (25 μg μl^{-1}) of test solution was tested. Controls were maintained with acetone and carbendazim (25 μg μl^{-1}). The treated and the controls were kept in an incubator at 28 °C for 3 days. Inhibition zones were measured and diameter was calculated in mm. Three replicates were maintained for each treatment.

Cytotoxicity assay

The cytotoxicity activity against K562 (human acute myelocytic leukemia cell line), A549, HL-60 (human promyelocytic leukemia cells), Huh-7, MCF-7 (human breast carcinoma), H1975, U937, BGC823, Hela and MOLT-4. Cell lines were determined according to previously reported method.[19]

COX-2 inhibitory activity assay

The effect of the compounds on human recombinant COX-2 (IC_{50} value, μM) enzymes was determined according to the reported method.[20]

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