The Journal of Antibiotics (2014) 67, 401–403

www.nature.com/ja

ORIGINAL ARTICLE

Antibacterial α-pyrone derivatives from a mangrove-derived fungus *Stemphylium* sp. 33231 from the South China Sea

Xue-Ming Zhou[1], Cai-Juan Zheng[1], Xiao-Ping Song, Chang-Ri Han, Wen-Hao Chen and Guang-Ying Chen

Two new α-pyrone derivatives, infectopyrones A (1) and B (2), were obtained from the EtOAc extract of the endophytic fungus *Stemphylium* sp. 33231 isolated from the mangrove *Brguiera sexangula var. rhynchopetala* collected in the South China Sea. Their structures were elucidated by the detailed analysis of comprehensive spectroscopic data. Compounds 1 and 2 were evaluated for their antibacterial activities, and they had a broad spectrum of antibacterial activity against five terrestrial pathogenic bacteria.
The Journal of Antibiotics (2014) **67,** 401–403; doi:10.1038/ja.2014.6; published online 19 February 2014

Keywords: α-pyrone derivatives; antibacterial activities; mangrove endophytic fungus; *Stemphylium* sp.

INTRODUCTION

There are various ecosystems in the vast marine environment including hitherto unexploited and geographically and taxonomically undescribed organisms. Marine microorganisms, especially marine fungi, are well known to be rich sources of structurally interesting and biologically active compounds.[1,2] Chemical investigations of mangrove-derived endophytic fungi, especially those from the subtropical island of Hainan, China, have shown a sharp increase in recent years.[3–5] As a result of adaptation to some special environments, mangrove endophytic fungi have formed unique genetic backgrounds and metabolic pathways.[6–8] It is encouraging that bioactive compounds have been obtained from mangrove-derived fungi.[9–12] Endophytic fungi have also proven to be promising sources of new natural products with promising biological and pharmacological activities.[13–15]

In our search for new antibacterial natural products from marine fungi in the South China Sea, a fungus *Stemphylium* sp. 33231 obtained from the leaves of *Brguiera sexangula var. rhynchopetala* attracted our attention. The EtOAc extract of a fermentation broth of the fungus exhibited antimicrobial activities against tested bacterial strains. Bioassay-guided fractionation of the bioactive extract led to the isolation of two new α-pyrone derivatives, infectopyrone A (**1**) and infectopyrone B (**2**) (Figure 1). Compounds **1** and **2** were evaluated for their antibacterial activities. Herein, we report the isolation, structure elucidation and biological activities of these compounds.

RESULTS AND DISCUSSION

Isolation and identification of compounds

The EtOAc extract of the fungal culture was treated with a combination of chromatographic materials, silica gel, octadecyl silane (ODS) column chromatography and Sephadex LH-20 column chromatography. The structures were elucidated by NMR and HRESI-MS spectroscopic data.

Compound **1** was isolated as a light yellow powder and had the molecular formula $C_{14}H_{16}O_6$ (7 degrees of unsaturation) based on the prominent signal at *m/z* 303.0840 $[M+Na]^+$ in the HRESI-MS, and combined with ^1H and ^{13}C NMR spectroscopic data (Table 1). The ^1H NMR spectrum of **1** showed the presence of three olefinic protons in the downfield at δ_H 6.88 (1H, br s), 6.70 (1H, br s) and 5.79 (1H, br s). In the upfield, one oxygenated methylene group at δ_H 4.22 (2H, s), one methoxyl group at δ_H 3.98 (3H, s), two olefinic methyl groups at δ_H 2.09 (3H, s) and 2.25 (3H, s) were also observed. In addition, 14 carbon signals were detected in the ^{13}C NMR spectrum (Table 1) including signals of two methyl groups, one methoxy group, one oxygenated methylene group, eight olefinic carbons, one ester carbonyl group and one carboxy1 group. These ^1H and ^{13}C NMR spectroscopic features suggested that **1** was very similar to infectopyrone. Infectopyrone is an α-pyrone derivative, which has been isolated from the fungus *Alternaria infectoria*,[16] and was not isolated in this study (Figure 1), except for the disappearance of a methyl signal at δ_C 8.8 and δ_H 1.93 (3H, s) for 3, s) in infectopyrone[16] and the presence of an oxygenated methylene signal at δ_C 52.1 and δ_H 4.22

Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, College of Chemistry and Chemical Engineering, Hainan Normal University, Haikou, China
[1]Xue-ming Zhou and Cai-juan Zheng are co-first authors.
Correspondence: Professor G-Y Chen, Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, College of Chemistry and Chemical Engineering, Hainan Normal University, Haikou, Hainan 571158, China.
E-mail: chgying123@163.com
Received 20 September 2013; revised 12 November 2013; accepted 2 December 2013; published online 19 February 2014

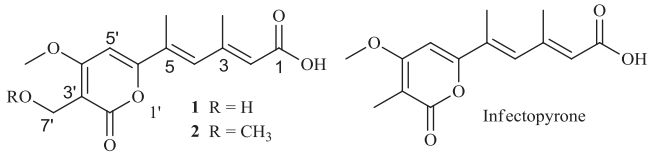

Figure 1 The structure of compounds **1**, **2** and infectopyrone.

Table 1 NMR Data for compounds 1 and 2

	1 *(in DMSO-d_6)*		***2*** *(in $CDCl_3$)*	
Position	δ_H	δ_C	δ_H	δ_C
1	—	167.1, C	—	169.8, C
2	5.79, br s	121.5, CH	5.87, br s	119.6, CH
3	—	150.4, C	—	154.6, C
4	6.88, br s	133.9, CH	7.13, br s	135.6, CH
5	—	129.6, C	—	128.8, C
2′	—	162.7, C	—	163.8, C
3′	—	105.4, C	—	103.3, C
4′	—	167.4, C	—	168.5, C
5′	6.70, br s	95.4, CH	6.32, br s	94.0, CH
6′	—	160.2, C	—	161.5, C
7′	4.22, s	52.1, CH_2	4.41, s	63.1, CH_2
3-Me	2.25, br s	18.6, CH_3	2.36, br s	19.4, CH_3
5-Me	2.09, br s	14.0, CH_3	2.13, br s	14.2, CH_3
4′-OMe	3.98, s	57.0, CH_3	4.01, s	56.7, CH_3
7′-OMe	—	—	3.43, s	58.4, CH_3

(2H, s) for 3, CH_2OH in **1**. These characteristics implied that the methyl group in infectopyrone[16] was replaced by an oxygenated methylene in **1**. These were further supported by the HMBC correlations of the H-7-xygenated methylene, T Figure 2). Detailed analysis of 2D NMR (HSQC, ^1H–^1H COSY, HMBC and NOESY) spectra confirmed that the other parts of the molecule were the same as those of infectopyrone.[16] Thus, the structure of compound **1** was identified as the Figure 1. We name compound **1** as infectopyrone A.

Compound **2** was isolated as a fine light yellow powder and had the molecular formula $C_{15}H_{18}O_6$ (7 degrees of unsaturation) based on the prominent signal at *m/z* 317.0992 $[M+Na]^+$ in the HRESI-MS. The ^1H and ^{13}C NMR data of **2** (Table 1) resembled with those of **1**, except for the existence of one additional methyl group at δ_H 3.43 and δ_C 58.4 for 7′-OMe, which was in accordance with the increase in MW of **2** by 14 a.m.u. compared with **1**. Further confirmation was achieved by the observed HMBC correlations of H-7′ to 7′-OMe, 2′, 3′ and 4′ (Figure 2). Detailed analysis of 2D NMR (HSQC, ^1H–^1H COSY, HMBC and NOESY) spectra confirmed that the other parts of the molecule were the same as those of **1**. Thus, the structure of compound **2** was identified as infectopyrone B (Figure 1).

Biological properties of infectopyrones A and B

Preliminary antibacterial assay results showed that at a concentration of 20 μg ml^{-1}, **1** and **2** exhibited a broad spectrum of antibacterial activity against five terrestrial pathogenic bacteria. The MIC values of **1** and **2** were further tested by the microplate assay method. The results in (Table 2) showed that **1** had moderate antibacterial activity against *Bacillus subtilis* (ATCC 6633), *Micrococcus tetragenus* (ATCC 13623) and *Micrococcus luteus* (ATCC 9341) with MIC values of 10.0 μg ml^{-1} for each. While compound **1** showed significant activity against *S. albus* (ATCC 8799) and *Escherichia coli* (ATCC 25922) with MIC values of 5.0 μg ml^{-1} and 2.5 μg ml^{-1}, respectively. Compounds

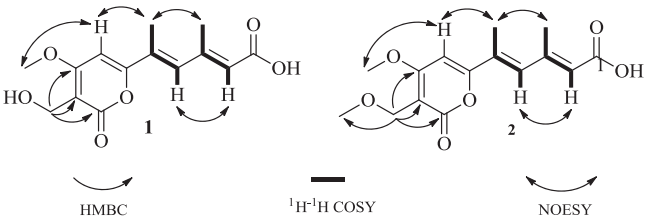

Figure 2 Key partial structure of Compounds **1** and **2** from HMBC data, ^1H–^1H COSY and NOESY correlations.

Table 2 Antibacterial activity for compounds 1 and 2

	MIC (μg ml^{-1})				
Compound	S. albus	E. coli	B. subtilis	M. tetragenus	M. luteus
1	5.0	2.5	10.0	10.0	10.0
2	10.0	2.5	>20.0	10.0	10.0
Ciprofloxacin[a]	0.6	0.3	0.6	0.3	0.3

[a]Ciprofloxacin was used as a positive control.

1 and **2** had similar antibacterial activity, but **2** showed weaker activity against *S. albus* (ATCC 8799) with an MIC value of 10.0 μg ml^{-1}. These results indicated that the 7′-OH group was important for antibacterial activity. Both compounds were tested for the microwell cytotoxicity assay using *Artemia salina* (brine shrimp).[17] No significant cytotoxic activities against *A. salina* were observed at 50 μg ml^{-1}.

METHODS

Fungal materials

The fungal strain *Stemphylium* sp. 33231 was isolated from the mangrove *Brguiera sexangula var. rhynchopetala* collected in the South China Sea in August, 2012. The strain was deposited in the Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, College of Chemistry and Chemical Engineering, Hainan Normal University of China, Hainan. The fungal strain was cultivated in 30 l potato glucose liquid medium (15 g of glucose and 30 g of sea salt in 1 l of potato infusion, in 1-l Erlenmeyer flasks each containing 300 ml of culture broth) at 25 °C without shaking for 4 weeks.

Identification of the fungus

The Fungus was identified according to its morphological characteristics and a molecular biological protocol by 18S rRNA amplification and sequencing of the ITS region. The sequence data have been submitted to GenBank, with an accession number KF479349, and the fungal strain was identified as *Stemphylium* sp.

General experimental procedures

Silica gel (Qing Dao Hai Yang Chemical Group Co., Qingdao, China; 200–300 mesh), octadecylsilyl silica gel (YMC, Kyoto, Japan; 12 nm–50 μm) and Sephadex LH-20 (GE Healthcare, Shanghai, China) were used for column chromatography (CC). Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co., Yantai, China; G60, F-254) were used for TLC. ^1H and ^{13}C NMR spectra were recorded on a Bruker AV spectrometer (Bruker, Zurich, Switzerland) at 400 MHz in $CDCl_3$ or DMSO-d_6. Chemical shifts δ are reported in p.p.m., using tetramethylsilane (TMS) as an internal standard, and coupling constants (*J*) are in Hz. ESI-MS and HRESI-MS spectra were measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer. IR spectra were recorded on a Nicolet 6700 spectrophotometer (Thermo Fisher Scientific Co., Shanghai, China).

Extraction and isolation

The fungal cultures were filtered through cheesecloth and the filtrate was extracted with EtOAc (3 × 30 l, 10 h each). The EtOAc extracts were

concentrated *in vacuo* to yield an oily residue (25.2 g). that was subjected to silica gel CC (petroleum ether/EtOAc v/v, 100:0–0:100) to generate five fractions (Fr. 1–Fr. 5). Fr. 4 was isolated by CC on silica gel eluted with petroleum ether—EtOAc (1:1), and then subjected to Sephadex LH-20 CC eluting with mixtures of petroleum $CHCl_3$—MeOH (2:3) and further purified by using octadecylsilyl silica gel eluted with 50% MeOH/H_2O to obtain compound **2** (6.2 mg). Fr. 5 was isolated by CC on silica gel eluted with petroleum ether—EtOAc (1:2), and then subjected to Sephadex LH-20 CC eluting with mixtures of petroleum $CHCl_3$—MeOH (2:3) and further purified by using octadecylsilyl silica gel eluted with 40% MeOH/H_2O to obtain compound **1** (11.2 mg).

Physical properties of compounds 1 and 2

Infectopyrone A (**1**): light yellow powder; UV (MeOH) λ_{max} (log ε) 334 (1.12), 213 (2.29) nm; IR (KBr) ν_{max} 3429, 1572, 1408 cm^{-1}; ^1H and ^{13}C NMR: see Table 1; HRESI-MS *m/z* 303.0840 $[M+Na]^+$ (calcd. for $C_{14}H_{16}O_6Na$, 303.0839).

Infectopyrone B (**2**): light yellow powder; UV (MeOH) λ_{max} (log ε) 309 (0.95), 211 (2.20) nm; IR (KBr) ν_{max} 3429, 1570, 1413 cm^{-1}; ^1H and ^{13}C NMR: see Table 1; HRESI-MS *m/z* 317.0992 $[M+Na]^+$ (calcd for $C_{15}H_{18}O_6Na$, 317.0995).

Antibacterial assays

Antibacterial activity was determined against five terrestrial pathogenic bacteria, including *Staphylococcus albus* (ATCC 8799), *E. coli* (ATCC 25922), *B. subtilis* (ATCC 6633), *M. tetragenus* (ATCC 13623) and *M. luteus* (ATCC 9341), by the microplate assay method.[18]

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (21162009, 31360069), 973 Program, Ministry of Science and Technology of China (Grant No. 2011CB512010).

1 Blunt, J. W. *et al*. Marine natural products. *Nat. Prod. Rep.* **30,** 237–323 (2013).
2 Blunt, J. W. *et al*. Marine natural products. *Nat. Prod. Rep.* **29,** 144–222 (2012).
3 Rateb, M. E. & Ebel, R. Secondary metabolites of fungi from marine habitats. *Nat. Prod. Rep.* **28,** 290–344 (2011).
4 Li, L. Y. *et al*. A-seco-oleane-type triterpenes from *Phomopsis* sp. (strain HKI0458) isolated from the mangrove plant *Hibiscus tiliaceus*. *Phytochemistry* **69,** 511–517 (2008).
5 Aly, A. H., Debbab, A., Kjer, J. & Proksch, P. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. *Fungal Divers.* **41,** 1–16 (2010).
6 Debbab, A., Aly, A. H., Lin, W. H. & Proksch, P. J. Bioactive compounds from marine bacteria and fungi. *Microb. Biotechnol.* **3,** 544–563 (2010).
7 Debbab, A., Aly, A. H. & Proksch, P. Endophytes and associated marine derived fungi—ecological and chemical perspectives. *Fungal Divers.* **57,** 45–83 (2012).
8 Strobel, G. *et al*. Taxol from *Pestalotiopsis microspora*, an endophytic fungus of *Taxus wallachiana*. *Microbiology* **142,** 435–440 (1996).
9 Gao, H. *et al*. New steryl esters of fatty acids from the mangrove fungus *Aspergillus awamori*. *Helv. Chim. Acta* **90,** 1165–1178 (2007).
10 Lin, Y. *et al*. Five unique compounds: xyloketals from the mangrove fungus *Xylaria* sp. from the South China Sea coast. *J. Org. Chem.* **66,** 6252–6256 (2001).
11 Lin, Y. C. *et al*. The metabolites of the mangrove fungus *Verruculina enalia* No. 2606 from a salt lake in the Bahamas. *Phytochemistry* **59,** 469–471 (2002).
12 Lin, Z., Zhu, T., Fang, Y. & Gu, Q. ^1H and ^{13}C NMR assignments of two new indolic enamide diastereomers from a mangrove endophytic fungus *Aspergillus* sp. *Magn. Reson. Chem.* **46,** 1212–1216 (2008).
13 Aly, A. H., Debbab, A. & Proksch, P. Fungal endophytes: unique plant inhabitants with great promises. *Appl. Microbiol. Biotechnol.* **90,** 1829–1845 (2011).
14 Aly, A. H., Debbab, A. & Proksch, P. Fungal endophytes from higher plants: a profilic source of phytochemicals and other bioactive natural products. *Fungal Divers.* **41,** 1–16 (2010).
15 Chen, Y. H. *et al*. Pseudoalteromone A: a novel bioactive ubiquinone from a marine bacterium *Pseudoalteromonas* sp. CGH2XX (Pseudoalteromonadaceae). *Tetrahedron Lett.* **53,** 1675–1677 (2012).
16 Larsen, T. O., Perry, N. B. & Andersen, B. Infectopyrone, a potential mycotoxin from *Alternaria infectoria*. *Tetrahedron Lett.* **44,** 4511–4513 (2003).
17 Solis, P. N., Wright, C. W. & Anderson, M. M. A microwell cytotoxicity assay using *Artemia salina* (Brine Shrimp). *Planta Med.* **3,** 250–252 (1993).
18 Pierce, C. G. *et al*. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. *Nat. Protoc.* **3,** 1494–1500 (2008).

Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)