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Two New Bisorbicillinoids Isolated from a Deep-sea Fungus, *Phialocephala* sp. FL30r

Dehai Li, Fengping Wang, Shengxin Cai, Xiang Zeng, Xiang Xiao, Qianqun Gu, Weiming Zhu

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Abstract Two new bisorbicillinoids, named oxosorbiquinol (**1**) and dihydrooxosorbiquinol (**2**), were isolated from a deep-sea fungus, *Phialocephala* sp., and their structures established using spectroscopic methods. The absolute configurations of **1** and **2** were determined by their biosynthesis route and analysis of the CD spectrum. Their cytotoxic effects on P388, A-549, HL60, BEL7402 and K562 cell lines were examined by the MTT method.

Keywords bisorbicillinoids, deep-sea fungus, *Phialocephala* sp., cytotoxicity

Introduction

Sorbicillinoids, the related dimeric bisorbicillinoids, and other natural products have been found in a wide variety of fungal sources. Some of the bisorbicillinoids may be biosynthesized *via* an unusual type of Diels-Alder reaction and also show promising biological properties, such as antifungal, antitumor and antioxidant activities [1, 2].

During a program to discover and isolate novel bioactive natural products, over 300 strains of microorganisms derived from deep-sea samples have been screened against a K562 cell line. Among them, the fungal strain FL30r, identified as *Phialocephala* sp., showed cytotoxicity *in vitro*. The strain was isolated from a deep-sea sediment sample (depth 5059 m) collected from ES304 (W145°23′03″, N8°19′50″) in 2003. Studies of the active constituents of this fungus led to the isolation of two new bisorbicillinoids, named oxosorbiquinol (**1**) and dihydrooxosorbiquinol (**2**) (Fig. 1). In this paper we report their isolation, structure elucidation and cytotoxic activities.

Materials and Methods

General

Optical rotations were obtained on a JASCO P-1020 digital polarimeter. IR spectra were taken on a NICOLET NEXUS

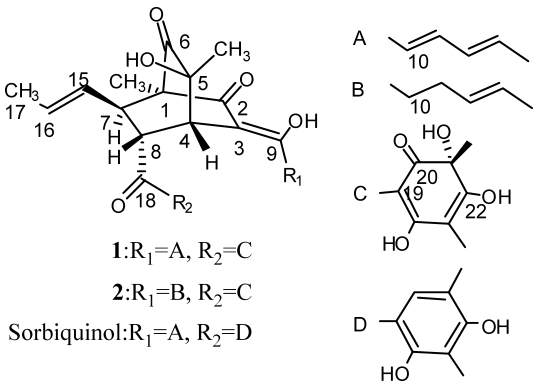

Fig. 1 Structures of oxosorbiquinol (**1**) and dihydrooxosorbiquinol (**2**).

Q. Gu, W. Zhu (Corresponding author), **D. Li, S. Cai:** Key Laboratory of Marine Drugs, Chinese Ministry of Education; Institute of Marine Drugs and Food, Ocean University of China, Qingdao 266003, PR China,
E-mail: weimingzhu@ouc.edu.cn, guqianq@ouc.edu.cn

F. Wang, X. Zeng, X. Xiao: Key Laboratory of Marine Biogenetic Resources, Third Institute of Oceanography, The State Oceanic Administration, Xiamen, PR China

470 spectrophotometer in KBr discs. UV spectra were recorded on Beckman DU® 640 spectrophotometer. ESI-MS was measured on a Q-Tof Ultima GLOBAL mass spectrometer. ^{1}H-, ^{13}C-NMR and DEPT spectra and 2D-NMR were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard. Semi-preparative HPLC was performed using an ODS column (YMC-Pack ODS-A, 10×250 mm, 5 μm).

Fungus and Culture

A small loop of spores growing on a PDA slant was inoculated into a 250-mL Erlenmeyer flask containing 75 mL sea-water-based culture medium (glucose 2.0%, potato extract 20%, yeast extract 0.2%, peptone 0.3%, NaCl 1%, $MgCl_2 \cdot 6H_2O$ 0.08%, KCl 0.1%) and cultured at 28°C for 2 days on a rotary shaker at 120 rpm. Then, 10 mL of the resultant seed culture was inoculated into a 500-mL Erlenmeyer flask containing 150 mL of the above culture medium and incubated (500 flasks) for 10 days under the same conditions.

Isolation

Seventy liters of whole broth was filtered through cheesecloth to separate the supernatant and mycelia. The former was extracted with ethyl acetate, while the latter was extracted with acetone. The acetone extraction was evaporated under reduced pressure to afford an aqueous solution and then extracted with ethyl acetate. The two ethyl acetate extracts were combined and concentrated *in vacuo* to give a crude extract (50.0 g). This was subjected to silica gel column chromatography eluted in gradient ratios with petroleum ether - chloroform and chloroform - methanol, respectively. And the active fragment (petroleum ether : chloroform 1 : 5) was further chromatographed on Sephadex LH-20 using chloroform : methanol 1 : 1 as elution, repeatedly. Further purification was carried about using HPLC on a ODS semi-preparative column (gradient eluted with 80~90% methanol/water containing TFA 3%) to obtain **1** (6.5 mg) and **2** (1.6 mg).

Physico-chemical Properties

1: brown syrup; $[\alpha]_D^{20}$ +255° (*c* 0.10, MeOH); HRESI-MS *m/z* 511.1950 $[M-H]^-$ (calcd for $C_{28}H_{31}O_9$, 511.1968); UV λ_{max}^{MeOH} nm (log ε) 232 (2.8), 341 (4.0); CD (MeOH) λ_{max} nm ($\Delta\varepsilon$) 366 (+34.8), 310 (−44.7), 249 (+14.1), 229 (+0.4), 205 (+6.2); IR ν_{max} (KBr) cm^{-1} 3421, 2929, 1730, 1670, 1631, 1605, 1560, 1454, 1382, 1247, 997.

2: brown syrup; $[\alpha]_D^{20}$ +94° (*c* 0.08, MeOH); HRESI-MS *m/z* 537.2110 $[M+Na]^+$ (calcd for $C_{28}H_{34}O_9Na$, 537.2101); UV λ_{max}^{MeOH} nm (log ε) 232 (2.5), 279 (3.7); CD (MeOH) λ_{max} nm ($\Delta\varepsilon$) 328 (+59.5), 288 (−74.3), 249 (+15.1), 239 (+8.1), 221 (+23.5); IR ν_{max} (KBr) cm^{-1} 3419, 2936, 1728, 1639, 1631, 1601, 1537, 1449, 1247, 968.

Biological Assay

Cytotoxic activity was evaluated by the MTT [2, 3] method. The IC_{50} values were obtained using the Bliss method.

Results and Discussion

Structure Determination

1: Its molecular formula, $C_{28}H_{32}O_9$, was established by HRESI-MS, which was in agreement with its ^{1}H- and ^{13}C-NMR data. The IR absorptions at 3421, 1670, 1631, 1605 and 1560 cm^{-1} were suggestive of the presence of hydroxy, α,β-unsaturated carbonyl and two enolized diketones [4], which were proved by the very low field chemical shifts of two hydroxys (δ: 18.50 and 14.00) in the ^{1}H-NMR spectrum. The ^{1}H-NMR spectrum displayed the presence of a sorbyl group [4, 5] [5.81 (1H, d, 14.8 Hz), 7.22 (1H, dd, 14.8, 10.3 Hz), 6.08 (1H, m), 6.14 (1H, m) and 1.85 (3H, d, 6.4 Hz)] and a substituted dihydrosorbyl group [1, 6] [3.19 (1H, dd, 10.3, 5.8 Hz), 4.37 (1H, d, 3.8 Hz), 5.04 (1H, dd, 14.8, 11.6 Hz), 5.41 (1H, m) and 1.60 (3H, d, 4.5 Hz)], which were also confirmed by the ^{1}H-^{1}H COSY and HMBC spectra. The *E* configuration of the two double bonds in the sorbyl residue and the double bond in the dihydrosorbyl chain was determined *via* the large coupling constants observed, and further confirmed by the observed correlation between H-10 and H-12, H-11 with H-13, and H-17 and H-15 in the NOESY spectrum, respectively.

The ^{13}C-NMR spectrum of **1** showed 28 carbons. Careful analysis and comparison of the ^{13}C-NMR data and DEPT spectrum with sorbiquinol [6] revealed that **1** should have the same skeleton as that of sorbiquinol, except for the C-20 carbonyl (195.9) instead of an sp^2 methine (129.0) and C-21 as sp^3 carbon (75.8) instead of sp^2 carbon (115.0), which was verified by the HMBC correlatons between CH_3-21 and C-20, C-21, and C-22 (Table 1).

The H-4 and CH_3-1 were located on the equatorial bond of the bicyclo [2, 2, 2] moiety which in the boat form [7]. In the NOESY spectrum (Fig. 2), the cross peaks observed between H-7 with CH_3-1 indicated that they were in *cis* arrangement. The coupling constant between H-7 and H-8 ($J_{7,8}$=5.8 Hz) implied that H-8 was *trans* to H-7 (the coupling constant over 10 Hz in the *cis* form) [7]. The cross peaks of H-8 with H-15 established that the 1-propenyl group was *cis* to H-8.

The absolute stereochemistry of **1** was determined from the biosythesis route and analysis of the CD spectrum. Recent research has shown that known sobicillin dimeric

Table 1 ^{1}H and ^{13}C NMR data for oxosorbiquinol (**1**) and dihydrooxosorbiquinol (**2**) ($CDCl_3$, 600 and 150 MHz, TMS, δ ppm)

NO	**1**			**2**		
	δ_C	δ_H (*J* in Hz)	HMBC (H→C)	δ_C	δ_H (*J* in Hz)	HMBC (H→C)
1	63.3			62.7		
2	198.1			197.0		
3	107.1			106.9		
4	45.3	3.48 (1H, br.s)	2, 3, 5, 6, 7	45.8	3.43 (1H, br.s)	2, 3, 5, 6, 7, 9
5	75.5			75.5		
6	210.6			214.1		
7	47.2	3.19 (1H, dd, 10.3 5.8)	6, 8, 15	47.4	3.15 (1H, dd, 10.8 5.9)	6
8	48.0	4.37 (1H, br.d, 3.8)	7, 18	47.8	4.32 (1H, d, 3.2)	3
9	168.6			180.0		
10	117.2	5.81 (1H, d, 14.8)	12	31.8	2.02 (1H, m); 2.24 (1H, m)	9
11	142.4	7.22 (1H, dd, 14.8 10.3)	9, 13	29.0	2.15 (2H, m)	9
12	130.7	6.08 (1H, m)	11	126.6	5.26 (1H, m)	14
13	140.0	6.14 (1H, m)	14	128.8	5.35 (1H, m)	14
14	18.8	1.85 (3H, d, 6.4)	12, 13	17.8	1.58 (3H, d, 6.6)	12, 13
15	128.6	5.04 (1H, dd, 14.8 11.6)		128.4	5.02 (1H, dd, 14.8, 10.8)	16
16	130.1	5.41 (1H, m)	17	130.1	5.26 (1H, m)	17
17	17.8	1.60 (3H, d, 4.5)	16	17.7	1.59 (3H, d, 6.0)	15, 16
18	200.5			200.5		
19	103.8			99.9		
20	195.9			196.0		
21	75.8			76.3		
22	170.1			170.3		
23	104.4			104.3		
24	190.0			190.8		
CH_3-1	10.1	1.15 (3H, s)	1, 2, 6, 7	10.0	1.14 (3H, s)	1, 2, 6, 7
CH_3-5	24.4	1.26 (3H, s)	4, 5, 6	24.6	1.28 (3H, s)	4, 5, 6
CH_3-21	30.5	1.60 (3H, s)	20, 21, 22	30.7	1.58 (3H, s)	20, 21, 22
CH_3-23	6.9	1.82 (3H, s)	22, 23, 24	6.9	1.86 (3H, s)	22, 23, 24
OH-24		18.5 (1H, s)	18, 19, 23, 24		18.8 (1H, s)	
OH-9		14.0 (1H, s)	3, 9, 10		14.5 (1H, s)	3, 9, 10

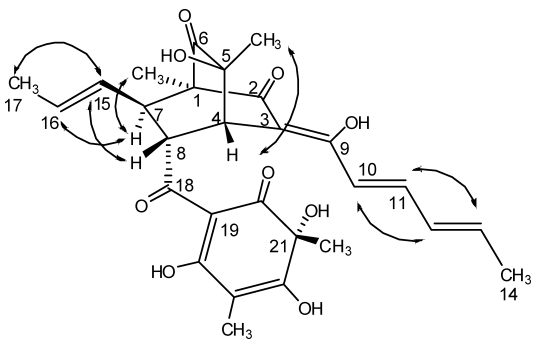

Fig. 2 NOESY correlations for **1**.

compounds, such as bisvertinolone and bisorbibutenolide that were isolated from this strain, were biosynthesized from two molecules of sorbicillinol or oxosorbicillinol by [4+2] cycloadditions or Michael addition ketalization sequences. The configuration at C-6 of sorbicillinol or oxosorbicillinol did not change in the two reactions, so it was also suggested that **1** was likely biosynthesized from sorbicillinol and oxosorbicillinol by [4+2] cycloadditions that are similar to what was proposed for sorbiquinol [6]. The CD spectrum of **1** also showed strong splitting Cotton effect at 366 nm ($\Delta\varepsilon$ +34.8) and 310 nm (−44.7). Therefore, the configurations at C-5 and C-21 of **1** should be respectively similar to sorbicillinol and oxosorbicillinol [8] and may have the (*S*) and (*R*) configurations, from the point of biogenesis as shown in Fig. 1.

Table 2 The activities of compounds **1** and **2** on cancer cell lines

Compound	IC_{50} (μM)				
	P388	A549	HL60	BEL7402	K562
1	29.9	103.5	8.9	12.7	56.3
2	40.3	97.6	10.5	31.8	68.2

2: Its molecular formula was determined to be $C_{28}H_{34}O_9$ by HRESI-MS, which was well consistent with its ^{1}H- and ^{13}C-NMR data. The IR spectrum of **2** showed absorptions at 3419, 1728, 1639, 1631, 1601, 1537, which was very similar to **1**. Detailed analysis and comparison of the ^{1}H- and ^{13}C-NMR data with those of **1** revealed that **2** possesses the same planar structure as **1**, except that the sorbyl group in **1** corresponds to a dihydrosorbyl group in **2**. The conclusion was validated by the HMBC experiments (Table 1). The CD cotton effect [$\Delta\varepsilon$ 328 (+59.5), 288 (−74.3), 249 (+15.1), 239 (+8.1), 221 (+23.5)] revealed the absolute stereochemistry of **2** is the same as that of **1**.

Cytotoxic Activities

The cytotoxic effects of **1** and **2** were preliminarily evaluated in P388, A-549, HL60, BEL7402 and K562 cell lines, as shown in Table 2.

1 and **2** showed weak cytotoxic activities against the five cell lines, except for the A549 cell line.

Most of the bisorbicillinoids were Diels-Alder adductions which occurred between the hexacyclic ring of two sorbicill derivers [1, 2, 4∼7]. The adduction between the hexacyclic ring and sorbyl side chain was unusual in nature, and only one bisorbicillinoid, sorbiquinol [6], has hitherto been found. **1** and **2**, as new members of this kind of bisorbicillinoids, therefore hold promise as potential anticancer agents. Detailed studies on their antitumor activities and related mechanisms of action are being undertaken.

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