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Carbonarones A and B, New Bioactive γ-Pyrone and α-Pyridone Derivatives from the Marine-derived Fungus *Aspergillus carbonarius*

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Abstract Two new secondary metabolites, carbonarones A (**1**) and B (**2**), were obtained from the culture of the marine-derived fungus *Aspergillus carbonarius* isolated from the marine sediment collected at Weizhou island of China. Based on ESIMS, 1D and 2D NMR data, and the X-ray crystallographic analysis, their structures were elucidated as 6-benzyl-4-oxo-4*H*-pyran-3-carboxamide and 6-benzyl-4-hydroxy-2-oxo-1, 2-dihydropyridine-3-carbaldehyde, respectively. **1** and **2** showed moderate cytotoxicity against K562 cells with IC_{50} values of 56.0 and 27.8 μg/ml, respectively.

Keywords *Aspergillus carbonarius*, carbonarones, marine-derived microorganisms, cytotoxicity

Introduction

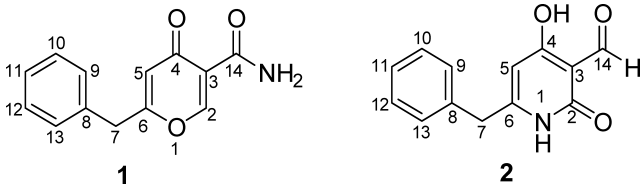
Marine microorganism is a rich source of secondary metabolites with diverse structures and various biological activities, such as cytotoxic [1], antitumor [2], antimicrobial [3], and antimycobacterial [4] activities. In our continuing research on antitumor secondary metabolites from marine-derived microorganisms, a culture extract of an *Aspergillus carbonarius* strain isolated from the marine sediment collected at Weizhou Island, China, showed significant cytotoxic activity against K562 cell line at 10.0 μg/ml. It was reported that the secondary metabolites of *A. carbonarius* include ochratoxin A [5], citric acid, fonsecin [6] and the carbonarins A~H [7]. The latter were effectively used to control Coleopteran and Lepidopteran insects. Further chemical investigation on metabolites of this fungal species was carried out, which led to the isolation and structural elucidation of two new compounds, carbonarones A (**1**) and B (**2**), (Fig. 1) *via* bioassay-guided isolation procedure.

The bioactive secondary metabolites of the *A. carbonarius* were obtained from the EtOAc extract of the fermentation broth and mycelia. Isolation of the compounds was achieved by repeated chromatographic steps, including column chromatography on silica gel, Sephadex LH-20 and reversed-phase semipreparative HPLC.

Materials and Methods

Strain

The fungus strain *Aspergillus carbonarius* WZ-4-11 was isolated from the marine sediments collected in Nov. 2003 at Weizhou Island, Guangxi Province, China and identified according to its morphological characteristic by Prof. Li Tian (First Institute of Oceanography, State Oceanic



1 **2**

Fig. 1 Structures of carbonarones A (**1**) and B (**2**).

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Administration, Qingdao, China). Working stocks were prepared on Potato Dextrose agar slants stored at 4°C.

Fermentation

The strain *A. carbonarius* WZ-4-11 was grown under static conditions at 24°C for 30 days in one hundred and fifty 1000-ml conical flasks containing the liquid medium (300 ml/flask) composed of (g/liter): glucose (20.0), peptone (5.0), malt extract (3.0) and yeast extract (3.0) and sea-water (pH adjusted to 7.0).

Extraction and Isolation

The culture broth (45 liters) was filtered through cheese cloth to separate the mycelia. The filtrate (40 liters) was concentrated under reduced pressure to about a quarter of the original volume and then extracted with ethyl acetate (3×13 liters). The wet mycelium was extracted with acetone (3×3 liters) which was later removed under reduced pressure. The resulting aqueous solution was extracted with ethyl acetate (3×3 liters). All ethyl acetate solutions were combined and evaporated to dryness under reduced pressure to give a crude extract (124.1 g).

The crude extract (124.1 g) was separated into 15 fractions by column chromatography on silica gel (200~300 mesh, column size 90×400 mm, content of silica 230.0 g) using a step gradient elution (chloroform/methanol). The active fraction 2 (403 mg) was crystallized from methanol yielding 20 mg of **2** as colorless needles. The mother liquor was subjected to column chromatography on Sephadex LH-20 with $CHCl_3$ - MeOH (50 : 50) as eluting agent and was separated into 3 subfractions. Subfraction 2~3 (52 mg) was further separated by semipreparative HPLC using an ODS column (Capcell Park C_{18}, column size 10×250 mm, particle size 5 μm, flow rate 4 mL/minute) with 65% MeOH - H_2O. Fractions containing **1** were combined according to the UV characteristics and the retention time and then evaporated to dryness to obtain the pure compound **1** (6.0 mg).

Biological Assay

Inhibition of the human leukemia cell lines K562 proliferation and cytotoxicity were measured by the sulphorhodamine B (SRB) assay [8]. Cells were plated in 96-well plates and allowed to attach and grow for 24 hours. The compounds or vehicle (MeOH) was added and incubated with the cells for 48 hours. Following drug exposure, the cells were fixed with 10% trichloroacetic acid at 4°C for 1 hour, and then the cell layer was stained with an SRB solution (0.4%) for 30 minutes. Excess stain was washed off with 1% AcOH, and the SRB was solubilized with 10 mM Tris base for 1 hour on an orbital shaker. The absorbance of the SRB solution was measured at 520 nm. Dose-response curves were generated, and the IC_{50} values calculated from the linear portion of the log dose-response curves.

Results and Discussion

Physico-chemical Properties

Carbonarone A (**1**): yellow amorphous powder; HRESIMS *m/z* 230.0819 $[M+H]^+$ (calcd for $C_{13}H_{12}NO_3$, 230.0817). UV (MeOH) λ_{max} (log ε) nm 279 (4.20), 317 (3.66), 401 (3.60); IR ν_{max} cm^{-1} (KBr) 3350, 2952, 2355, 1759, 1679, 1610, 1567, 1414, 1243, 1162, 1070, 937, 718, 697. ^1H ($CDCl_3$, 600 MHz) and ^{13}C ($CDCl_3$, 150 MHz) see Table 1.

Carbonarone B (**2**): colorless needle; mp 209~209.5°C; HRESIMS *m/z* 230.0824 $[M+H]^+$ (calcd for $C_{13}H_{12}NO_3$, 230.0817). UV (MeOH) nm λ_{max} (log ε) 340 (3.87); IR ν_{max} cm^{-1} (KBr) 3032, 2912, 2826, 2754, 1673, 1613, 1560, 1295, 1253, 824, 755, 692, 519. ^1H ($CDCl_3$, 600 MHz) and ^{13}C ($CDCl_3$, 150 MHz) see Table 1.

Structure Determination

Carbonarone A (**1**) was obtained as a yellow amorphous powder. Its molecular formula was determined as $C_{13}H_{11}NO_3$ on the basis of HRESIMS at *m/z* 230.0819 $[M+H]^+$ (calcd. 230.0817 for $C_{13}H_{12}NO_3$) and ^1H and ^{13}C NMR spectra data (Table 1). The IR spectrum of **1** showed strong $-CONH_2$ bands at 3350 and 1680 cm^{-1}, carbonyl band at 1759 cm^{-1}, and phenyl bands at 1567, 718 and 697 cm^{-1}. ^1H NMR data of **1** showed two amide protons at δ 9.07 (br s, 1H) and 5.94 (br s, 1H), five phenyl protons at δ 7.24 (d, J=7.3 Hz, 2H), 7.37 (t, J=7.3 Hz, 2H) and 7.32 (t, J=7.3 Hz, 1H), two aromatic protons at δ 8.73 (s, 1H) and 6.25 (s, 1H), and two methylene protons at δ 3.89 (s, 2H). ^{13}C NMR and DEPT spectra data of **1** revealed two carbonyl carbons and two quaternary carbons at low field (δ 162~178), seven sp^2 methine carbons, and a sp^3 methylene carbon (δ 39.6, t).

Three structural moieties of **1**, including benzyl group (A), carbamino group (B) and 3,6-disubstituted γ-pyrone (C), were deduced by comprehensive interpretation of its ^1H, ^{13}C, DEPT NMR, and ^1H-^1H COSY, HMQC and HMBC spectra (Fig. 2). The key long rang connections between H-2 (δ 8.73) with C-14 (δ 164.0, s), H-5 (δ 6.25) with C-7 (δ 39.6, t), and H-7 (δ 3.89) with C-5 (δ 116.0, d) and C-6 (δ 168.8, s) indicated that benzyl and carbamino- were connected to C-6 and C-3 of γ-pyrone nucleus, respectively. Thus, structure of **1** was elucidated as 6-benzyl-4-oxo-4*H*-pyran-3-carboxamide (Fig. 1).

Carbonarone B (**2**), obtained as a colorless needles, mp

Table 1 NMR spectral data of carbonarones A (**1**) and B (**2**) (ppm in $CDCl_3$)[a]

Position	**1**			**2**		
	δ_H (*J* in Hz)	δ_C (m)	HMBC[b] (H→C)	δ_H (*J* in Hz)	δ_C (m)	HMBC[b] (H→C)
1				11.86 br s		
2	8.73 s	162.0 d	3, 4, 6, 14		165.5 s	
3		119.3 s			106.0 s	
4		178.1 s			175.9 s	
5	6.25 s	116.0 d	3, 4, 6, 7	5.80 s	99.8 d	3, 4, 6, 7
6		168.8 s			157.4 s	
7	3.89 s (2H)	39.6 t	5, 6, 8, 9, 13	3.88 s (2H)	40.0 t	5, 6, 8, 9, 13
8		133.7 s			134.5 s	
9	7.24 d (7.3)	129.1 d	7, 10, 11, 13	7.28 d (8.0)	129.2 d	7, 11, 13
10	7.37 t (7.3)	129.1 d	8, 9, 12	7.35 t (7.0, 8.0)	129.1 d	8, 9, 12
11	7.32 t (7.3)	127.8 d	9, 10, 13	7.31 t (7.0)	127.8 d	9, 13
12	7.37 t (7.3)	129.1 d	8, 10, 13	7.35 t (7.0, 8.0)	129.1 d	8, 10, 13
13	7.24 d (7.3)	129.1 d	7, 9, 11, 12	7.28 d (8.0)	129.2 d	7, 9, 11
14		164.0 s		10.07 s	194.0 d	4
14-NH_2	9.07 br s, 5.94 br s					
4-OH				13.67 br s		3, 4, 5

[a] ^1H, ^{13}C NMR and HMBC, ^1H-^1H COSY spectra were obtained at 600 MHz, 150 MHz and 600 MHz, and recorded in $CDCl_3$ at room temperature, respectively. Unless otherwise indicated, all proton signals integrated to 1H.

[b] Carbon atoms coupled with proton. Multiplicity was determined by DEPT data.

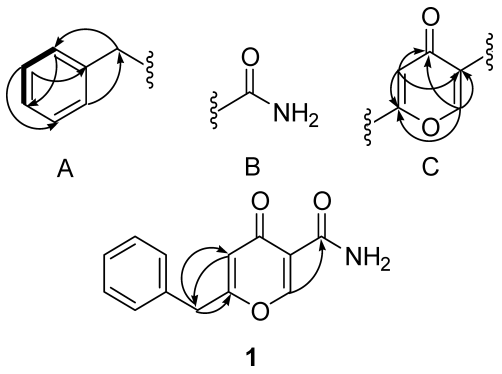

Fig. 2 Key COSY and HMBC correlations for **1**.

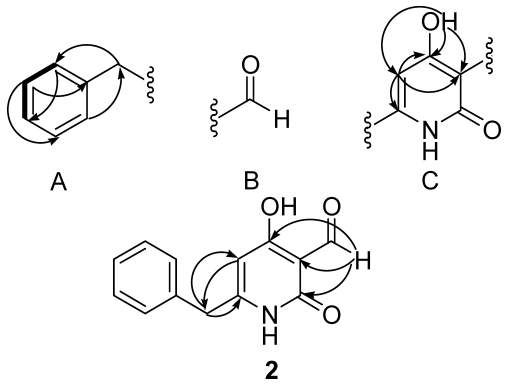

Fig. 3 Key COSY and HMBC correlations for **2**.

209∼209.5°C. The molecular formula of **2** was also determined as $C_{13}H_{11}NO_3$ according to its ^1H and ^{13}C NMR spectra data (Table 1) and HRESIMS at *m/z* 230.0824 $[M+H]^+$ (calcd. 230.0817 for $C_{13}H_{12}NO_3$). The IR spectrum showed –CHO bands at 2826, 2754 cm^{-1}, carbonyl band at 1673 cm^{-1}, and phenyl bands at 1560, 755 and 692 cm^{-1}. The ^1H NMR data of **2** showed one hydroxyl proton at δ 13.67 (br s, 1H), one amide proton at δ 11.86 (br s, 1H), one aldehyde proton at δ 10.07 (s, 1H), five phenyl protons at δ 7.28 (d, J=8.0 Hz, 2H), 7.35 (dd, "t" like, J=7.0, 8.0 Hz, 2H) and 7.31 (t, J=7.0 Hz, 1H), one vinyl proton at δ 5.80 (s, 1H), and two methylene protons at δ 3.88 (s, 2H). ^{13}C NMR and DEPT spectra data of **2** revealed one aldehyde carbon (δ 194.0, d), one carbonyl carbon (δ 165.5, s), six sp^2 methine carbons and four sp^2 quaternary carbons, and one sp^3 methylene carbon (δ 40.0, t) in the high field.

Detailed analysis of 1D and 2D NMR spectra data of **2** resulted in the elucidation of three structural moieties, *i.e.* benzyl group (A), aldehyde group (B) and 3,6-disubstituted-4-hydroxy-α-pyridone (C) (Fig. 3). The key HMBC correlations from H-5 (δ 5.80) to C-7 (δ 40.0, t),

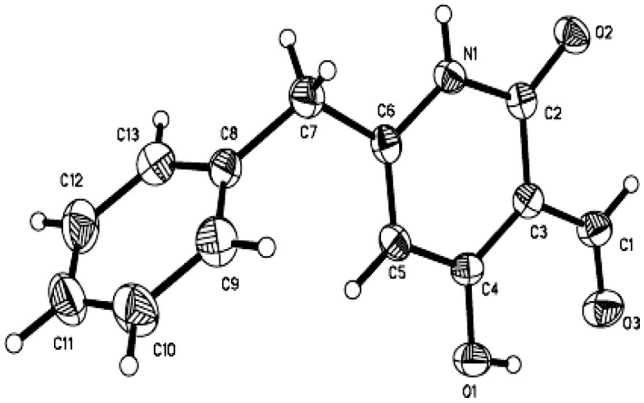

Fig. 4 X-Ray molecular structure of carbonarone B (**2**).

H-7 (δ 3.88) to both C-5 (δ 99.8, d) and C-6 (δ 157.4, s), and H-14 (δ 10.07) to both C-4 (δ 175.9, s) and C-3 (δ 106.0, s) clearly indicated that benzyl and aldehyde group was attached to C-6 and C-3 of α-pyridone nucleus, respectively (Fig. 3), which was further confirmed by X-ray diffraction experiment (Fig. 4).* Thus, structure of **2** was unambiguously elucidated as 6-benzyl-4-hydroxy-2-oxo-1,2-dihydropyridine-3-carbaldehyde.

To the best of our knowledge, compounds with benzyl group substituted α-pyridone are extremely rare in nature, and compounds with benzyl group substituted γ-pyrone from natural resource have not been reported before.

Cytotoxic Activities

The cytotoxic effects of **1** and **2** were preliminarily evaluated in K562, P388, A-549, BEL-7402 and HL60 cell lines. Both compounds **1** and **2** exhibited moderate antiproliferative activity against K562 cell lines with IC_{50} value of 56.0 and 27.8 μg/ml, respectively, while they were inactive against the other tested cell lines ($IC_{50}>100\ \mu$g/ml).

Experimental

Melting points were measured using a Yanaco MP-500D micro-melting point apparatus and were uncorrected. Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on Beckman DU® 640 spectrophotometer. IR spectra were taken on a NICOLET NEXUS 470 spectrophotometer in KBr discs. ^{1}H, ^{13}C NMR and DEPT spectra and 2D-NMR were recorded on a JEOL Eclips-600 spectrometer using TMS as internal standard and chemical shifts were recorded as δ values. ESI-MS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semipreparative HPLC was performed on a SHIMADZU LC-6AD Liquid Chromatograph with SPD-M10A *vp* Diode Array Detector.

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* X-ray crystal structure analysis of compound **2**: colorless needle crystal of $C_{13}H_{11}NO_3$. Space group P-1, a=4.774 (5) Å, b=9.248 (10) Å, c=13.332 (14) Å, α=95.889 (14)°, β=98.411 (15)°, γ=102.340 (15)°, V=563.3 (10) Å^3, Z=2, crystal size 0.70×0.43×0.27 mm^3. A total of 1933 unique reflections ($2\theta<50°$) were collected using graphite monochromated Mo Kα (λ=0.71073 Å) on a CCD area detector diffractometer. The structure was solved by direct methods (SHELXS-97) and expanded using Fourier techniques (SHELXS-97). The final cycle of full-matrix least squares refinement was based on 1933 unique reflections ($2\theta<50°$) and 154 variable parameters and converged with unweighted and weighted agreement factors of R=0.0992, R_w=0.1670 and R_1=0.0622 for $I>2.0\sigma$ (I) data. Crystallographic data (excluding structure factors) for structure **2** in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 615239. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 (0) 1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].

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