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Triazole and Dihydroimidazole Alkaloids from the Marine Sediment-Derived Fungus *Penicillium paneum* SD-44

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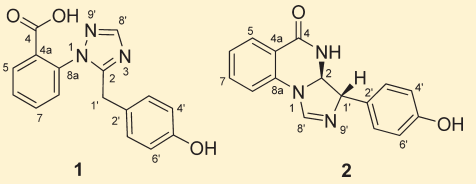
Supporting Information

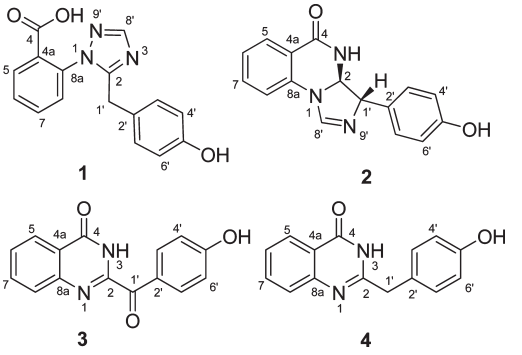
ABSTRACT: A novel triazole carboxylic acid, penipanoid A (**1**), two new quinazolinone alkaloids, penipanoids B (**2**) and C (**3**), and a very recently reported quinazolinone derivative (**4**) were isolated from the marine sediment-derived fungus *Penicillium paneum* SD-44. Their structures were elucidated by spectroscopic analysis, and the structure for **1** was confirmed by X-ray crystallographic analysis. Compound **1** represents the first example of a triazole derivative from marine sediment-derived fungi, and compound **2** is a rare quinazolinone derivative having a dihydroimidazole ring system. The cytotoxicity of compounds **1** and **4** and the antimicrobial activity of **1**−**4** were evaluated.



1 **2**

Marine microorganisms, especially marine fungi, have become one of the richest sources of structurally novel and biologically active metabolites in the marine environment.[1−3] Among them, fungi derived from the deep sea have recently attracted great interest as a promising target for the discovery of pharmaceutically important metabolites due to their extreme environment.[4−8] During our ongoing search for bioactive metabolites from marine-derived fungi,[9−13] the extract of *Penicillium paneum* SD-44, isolated from a deep sea sediment sample that was collected from the South China Sea, displayed cytotoxicity in a preliminary bioassay. Extraction of the fermentation culture and fractionation led to the isolation of three new alkaloids including the novel triazole penipanoid A (**1**) and two new quinazolinone alkaloids, penipanoids B and C (**2** and **3**). In addition, a structurally related quinazolinone derivative (**4**), which was very recently reported from the *Cordyceps*-colonizing fungus *Isaria farinosa*,[14] was also isolated. Compound **1** represents the first triazole derivative isolated from marine sediment-derived fungi, while compound **2** is a rare quinazolinone derivative having a dihydroimidazole ring system. Details of the isolation, structure elucidation, and cytotoxicity of these alkaloids are reported herein.

The fermented substrate in rice medium was exhaustively extracted with EtOAc to yield an organic extract, which was fractionated by a combination of column chromatography (CC) including Si gel, reversed-phase Si gel C18, Sephadex LH-20, and semipreparative HPLC, as well as preparative thin-layer chromatography (pTLC), to yield four alkaloids (**1**−**4**). Known compound **4** was identified by detailed NMR spectroscopic analysis as well as by comparison with recently reported literature data.[14]



1 **2** **3** **4**

Penipanoid A (**1**) was isolated as a single crystal. Its molecular formula was demonstrated as $C_{16}H_{13}N_3O_3$ by HRESIMS, with 12 degrees of unsaturation. The IR absorption bands at 1738 and 3447 cm^{-1} implied the presence of carbonyl and hydroxy groups, respectively. Exhaustive analyses of the 1D NMR data for **1** (Table 1) indicated the presence of one carbonyl, *ortho*- and *para*-disubstituted benzene rings, two additional sp^2 carbons (one quaternary and one methine), and one methylene carbon. Among them, the deshielded sp^2 carbons resonating at δ_C 155.9 (C-5′), 155.4 (C-2), and 150.6 (C-8′) should be connected with oxygen and/or nitrogen atoms. The HMBC correlations from H-5 to C-4a and to the carbonyl carbon C-4 suggested the location of the carbonyl group at C-4 (Figure 1). Additionally,

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds 1−3 (DMSO-d_6, δ ppm)

	penipanoid A (**1**)[a]		penipanoid B (**2**)[b]		penipanoid C (**3**)[a]	
position	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
2	155.4, C		73.9, CH	5.25, d (4.1)	150.4, C	
3		13.10, br s		8.44, br s		
4	165.8, C		162.8, C		161.3, C	
4a	129.4, C		119.5, C		122.6, C	
5	130.8, CH	7.97, dd (7.3, 1.5)	127.6, CH	7.79, dd (7.7, 1.4)	125.9, CH	8.19, d (7.8)
6	130.0, CH	7.66, ddd (7.6, 7.3, 1.5)	122.9, CH	7.12, td (7.7, 0.8)	127.9, CH	7.62, t (7.8)
7	132.6, CH	7.70, ddd (7.6, 7.3, 1.5)	133.6, CH	7.54, td (7.7, 1.4)	134.5, CH	7.88, t (7.8)
8	128.5, CH	7.34, dd (7.6, 1.5)	120.1, CH	7.39, d (7.7)	127.9, CH	7.76, d (7.8)
8a	135.9, C		143.0, C		147.3, C	
1′	30.8, CH_2	3.83, s	59.6, CH	4.32, dd (4.1, 1.5)	185.1, C	
2′	126.0, C		127.0, C		125.0, C	
3′	129.5, CH	6.83, d (8.5)	128.9, CH	7.04, d (8.5)	133.6, CH	8.05, d (8.7)
4′	115.0, CH	6.58, d (8.5)	115.8, CH	6.76, d (8.5)	115.6, CH	6.90, d (8.7)
5′	155.9, C		157.1, C		163.9, C	
6′	115.0, CH	6.58, d (8.5)	115.8, CH	6.76, d (8.5)	115.6, CH	6.90, d (8.7)
7′	129.5, CH	6.83, d (8.5)	128.9, CH	7.04, d (8.5)	133.6, CH	8.05, d (8.7)
8′	150.6, C	7.98, s	148.0, CH	7.25, d (1.5)		
5′-OH		9.24, br s		9.57, br s		

[a] Measured at 500 and 125 MHz for ^{1}H and ^{13}C, respectively. [b] Measured at 600 and 150 MHz for ^{1}H and ^{13}C, respectively.

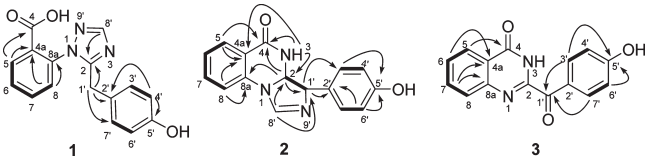

Figure 1. Key HMBC (arrows) and ^{1}H−^{1}H COSY (bold lines) correlations of compounds **1**−**3**.

the observed HMBC correlations from the methylene (H_2-1′) to C-2′ and C-3′/C-7′ and from H-4′/H-6′ to C-2′ and C-5′ revealed the presence of a 4-hydroxybenzyl moiety. Besides the above two deduced benzene rings, the assignments of the remaining elements ($C_2H_2N_3$) were difficult because only the correlations of H_2-1′ and H-8′ to C-2 were observed. Fortunately, compound **1** yielded a crystal suitable for X-ray analysis, and a crystallographic study was conducted. A 1,2,4-triazole ring system and connections of the azole ring with a benzoic acid at N-1 and the benzyl group at C-2 were established (Figure 2). The structure of **1** was therefore determined as 2-(5-(4-hydroxybenzyl)-1*H*-1,2,4-triazol-1-yl)benzoic acid, which was named penipanoid A. The X-ray crystallographic data indicated that penipanoid A (**1**) presents an asymmetric unit containing two independent molecules, which are in different rotational conformations, with one having the COOH and 5′-OH groups on the same side of the triazole plane and the other having them on either side of the plane. Both conformationally distinct molecules of **1** are H-bonded to a neighboring molecule, which results in the different rotational conformations of **1** in the solid state (Figure 2).

Penipanoid B (**2**) was obtained as a white powder. Its molecular formula, $C_{16}H_{13}N_3O_2$, was determined by negative HR-ESIMS, indicating 12 degrees of unsaturation. Comparison of the NMR data of **2** with those of **4** indicated that the structures of these two compounds are very similar. However, the methylene

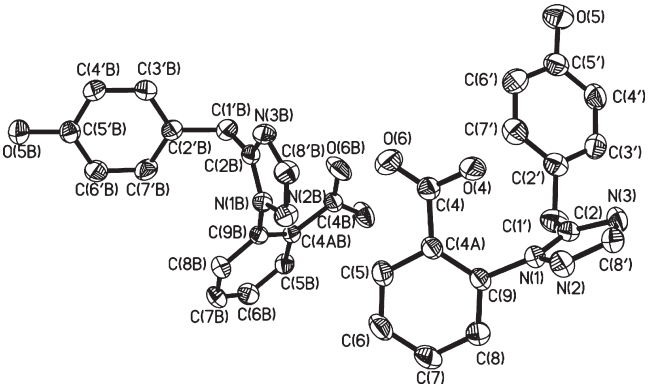

Figure 2. X-ray structure of compound **1**. (Note: A different numbering system is used for the structural data in the Supporting Information and those deposited with the CCDC).

carbon signal at δ_C 39.5 (C-1′) and the quaternary carbon resonating at δ_C 156.5 (C-2) in **4**[14] were absent in the ^{13}C NMR spectrum of **2**. Instead, two sp^3- and one nitrogen-substituted sp^2-hybridized methines at δ_C 59.6 (C-1′) and 73.9 (C-2) and at δ_C 148.0 (C-8′), respectively, were observed in the ^{13}C NMR spectrum of **2**. The ^{1}H−^{1}H COSY spectrum obviously indicated that the two sp^3 methines (CH-1′ and CH-2) were connected to each other. In the HMBC spectrum, the observed cross-peaks from H-1′ to C-2, C-2′, and C-3′/C-7′ and from H-2 to C-1′, C-4, and C-8a placed the two sp^3 methines as C-1′ and C-2. Additionally, only two obvious HMBC correlations were observed from the nitrogen-connected sp^2 methine H-8′ to C-1′ and C-2. These observations could establish two possible subunits (A or B) for **2**, as shown in Figure 3. The observed HMBC correlations of the exchangeable proton (H-3) to C-2, C-4, and C-4a implied that moiety A was the more reasonable subunit for **2**. The relative

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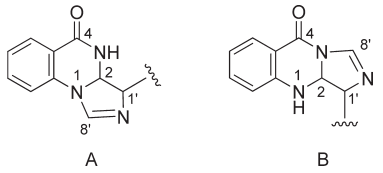

A B

Figure 3. Two possible subunits for compound **2**.

configuration of compound **2** was established through analysis of its NOESY spectrum. No NOE correlations between H-1′ and H-2 could be detected, which implies the opposite orientation for the proton pair, and this was verified by the obvious NOE correlation observed between H-1′ and H-3.

Penipanoid C (**3**) was obtained as a yellowish solid. The molecular formula $C_{15}H_{10}N_2O_3$ (12 degrees of unsaturation) was determined by HRESIMS. The ^{1}H and ^{13}C NMR data of **3** and **4** matched well, except for the carbonyl carbon resonating at δ_C 185.1 (C-1′) in **3** replaced the sp^3 methylene at δ_C 39.5 (C-1′) in **4**.[14] Accordingly, the methylene protons resonating at δ_H 3.81 (H-1′) in **4** were not present in the ^{1}H NMR spectrum of **3**. These observations indicated that the methylene in **4** was replaced by a carbonyl group in **3**, and this deduction was verified by the HMBC correlations from H-3′/H-7′ to the carbonyl carbon C-1′. The structure of compound **3** was thus established to be 2-(4-hydroxybenzoyl)-4(3*H*)-quinazolinone.

The structurally related alkaloids **1**−**4** might be derived from the same precursor, e.g., tyrosine. A proposed biogenetic pathway is presented in the Supporting Information (Scheme S1).

The cytotoxity of compounds **1** and **4** and the antimicrobial activity of compounds **1**−**4** were evaluated. In the cytotoxic assays, compound **1** exhibited activity against the SMMC-7721 cell line with an IC_{50} value of 54.2 μM, while compound **4** displayed significant cytotoxic activity against the A-549 and BEL-7402 cell lines with IC_{50} values of 17.5 and 19.8 μM, respectively. The positive control, fluorouracil, showed obvious activities against the above three cell lines with IC_{50} values of 13.0, 13.7, and 21.8 μM, respectively. In the antimicrobial screening, no obvious activity could be observed for the tested compounds **1**−**4** against two bacteria (*Staphylococcus aureus* and *Escherichia coli*) and five plant-pathogenic fungi (*Alternaria brassicae*, *Fusarium oxysporium* f. sp. *vasinfectum*, *Coniella diplodiella*, *Physalospora piricola*, and *Aspergillus niger*).

EXPERIMENTAL SECTION

General Experimental Procedures. The optical rotation was measured on an Optical Activity AA-55 polarimeter. UV spectroscopic data were obtained on a Lengguang Gold S54. IR data were collected on a JASCO FT/IR-4100 Fourier transform infrared spectrometer with KBr pellets. NMR spectra were recorded on Bruker Advance 500 and Bruker Advance 600 spectrometers in DMSO-d_6. Mass spectra were measured on a VG Autospec 3000 mass spectrometer. Column chromatography (CC) was performed with silica gel (200−300 mesh, Qingdao Marine Chemical Factory), Lobar LiChroprep RP-18 (40−63 μm; Merck), and Sephadex LH-20 (18−110 μm, Merck).

Fungal Material. The fungus *Penicillium paneum* SD-44 was isolated from a marine sediment sample collected in the South China Sea at a depth of 201 m, in August 2008. The fungus was identified using a molecular biological protocol by DNA amplification and sequencing of the ITS region, as described in our previous report.[15] The sequenced data derived from the fungal strain have been deposited at Genbank (accession no. HQ703579). The BLAST result showed that the sequence was the most similar (99%) to the sequence of *P. paneum* (compared with AB479336.1). The strain is preserved at the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences.

Fermentation, Extraction, and Isolation. The fermentation was carried out statically in a rice medium (100 mL seawater, 100 g rice, 0.6 g peptone) in 1 L Erlenmeyer flasks for 30 days at room temperature. The fermented rice substrate (30 flasks) was extracted repeatedly with EtOAc (3 × 400 mL for each flask), and the solvent was combined and evaporated to dryness under vacuum to afford an extract (18.0 g), which was fractionated by Si gel vacuum liquid chromatography (VLC) using $CHCl_3$−MeOH gradient elution. The fraction (1.70 g) eluted with $CHCl_3$−MeOH (20:1) was further purified by column chromatography on silica gel eluting with a $CHCl_3$−MeOH gradient (from 40:1 to 5:1) to produce five parts (P.1−P.5). P.2 was further separated by Sephadex LH-20 CC (MeOH) to afford **4** (38.2 mg). P.3 was purified by semi-preparative HPLC (Elite ODS-BP column, 10 μm; 10.0 × 300 mm; 50% MeOH−H_2O, 3 mL/min) to afford **2** (3.4 mg, t_R 16.5 min) and **3** (3.1 mg, t_R 22.8 min). The fraction (2.18 g) eluted with $CHCl_3$−MeOH (10:1) was further separated by CC on reversed-phase silica gel C_{18} eluted with a MeOH−H_2O gradient (20% to 100%) and then purified by CC on Sephadex LH-20 (MeOH) to get compound **1** (26.7 mg) as single crystals.

X-ray Analysis of 1 (ref 16). A colorless crystal was obtained from a solution of MeOH. All crystallographic data were collected on a Srigaku Mercury CCD/AFCR diffractometer equipped with graphite-monochromatic Mo Kα radiation (λ = 0.71073 Å) at 293(2) K. The data were corrected for absorption by using the program SADABS.[17] The structure was solved by direct methods and subsequent difference Fourier synthesis and refined by full-matrix least-squares techniques with the SHELXTL software package.[18] All non-hydrogen atoms were refined anisotropically. The H atoms belonging to C atoms were calculated theoretically, and those to O atoms were determined by difference Fourier maps.[18]

Crystal data of ***1****:* $C_{16}H_{13}N_3O_3$; F.W. 295.29; monoclinic space group $P2_1/n$; unit cell dimensions a = 15.595(3) Å, b = 11.656(2) Å, c = 17.393(4) Å, V = 2867.4(10) Å^3; α = 90°, β = 114.91(3)°, γ = 90°, Z = 8, d_{calc} = 1.368 Mg/m^3, crystal dimensions 0.20 × 0.20 × 0.20 mm, μ = 0.097 mm^{-1}, F(000) = 1232. The 24 977 measurements yielded 6535 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave R_1 = 0.0481 and wR_2 = 0.1222 [$I > 2\sigma(I)$].

Cytotoxicity Assays. The cytotoxic activities against the SMMC-7721, A-549, and BEL-7402 cell lines were determined according to previously reported methods.[19] Fluorouracil was used as a positive control.

Antimicrobial Assays. The antimicrobial activities against two bacteria (*S. aureus* and *E. coli*) and five plant-pathogenic fungi (*A. brassicae*, *F. oxysporium*, *C. diplodiella*, *P. piricola*, and *A. niger*) were determined using the disk diffusion method.[20] Chloramphenicol and amphotericin B were used as antibacterial and antifungal positive controls, respectively.

Penipanoid A (1): colorless crystals (MeOH); mp 212−214 °C; UV (MeOH) λ_{max} (log ε) 222 (4.16), 275 (3.29) nm; IR (KBr) ν_{max} 3447, 2970, 1738, 1636, 1365, 1229, 1217 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS m/z 294 [M − H]$^-$; HRESIMS m/z 294.0892 (calcd for $C_{16}H_{12}N_3O_3$, 294.0879).

Penipanoid B (2): white powder; $[\alpha]^{25}_D$ +30 (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 224 (3.97) nm; IR (KBr) ν_{max} 3441, 2916, 1688, 1620, 1365, 1445, 1167 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS m/z 278 [M − H]$^-$; HRESIMS m/z 278.0966 (calcd for $C_{16}H_{12}N_3O_2$, 278.0930).

Penipanoid C (3): yellowish solid; UV (MeOH) λ_{max} (log ε) 224 (4.32), 273 (3.11) nm; IR (KBr) ν_{max} 3440, 2919, 1738, 1637, 1365, 1229, 1217, 1162 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESIMS m/z

265 [M − H]$^-$; HRESIMS m/z 265.0645 (calcd for $C_{15}H_9N_2O_3$, 265.0613).

■ ASSOCIATED CONTENT

Supporting Information. Selected 1D and 2D NMR spectra of compounds **1**−**3**, possible biosynthetic pathway of compounds **1**−**4** (Scheme S1), and X-ray crystallographic data of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) Crystallographic data of compound **1** have been deposited with the Cambridge Crystallographic Data Centre (deposition no. CCDC 806074). The data can be obtained free of charge via www.ccdc.cam.ac.uk/data_request/cif (or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, U.K.; fax: (+44) 1223-336-033; e-mail:deposit@ccdc.cam.ac.uk).
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