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Two new phenol derivatives from the cold seep-derived fungus *Aspergillus insuetus* SD-512

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Two new phenol derivatives, namely insphenol A (**1**) and acetylpeniciphenol (**2**), along with seven known analogues (**3**–**9**), were isolated from the deep-sea cold seep-derived fungus, *Aspergillus insuetus* SD-512. The structures of **1** and **2** were established by extensive interpretation of NMR and mass spectroscopic data. The absolute configuration of **1** was determined by the combination of coupling constant analysis and acid hydrolysis. Among the isolated compounds, insphenol A (**1**) represents the first example of isopentenyl phenol derivative with a unique 1-glycosylation from the species *Aspergillus insuetus*. The isolated new compounds were evaluated for antibacterial activities against six human or aquatic pathogens, while compound **2** exhibited inhibitory effect against *Edwardsiella tarda*, *Vibrio alginolyticus*, and *V. vulnificus*, with MIC values of 4, 8, and 8 μg/mL, respectively.

Keywords: *Aspergillus insuetus*, cold seep-derived fungus, marine natural products, antibacterial activity

Introduction

Deep-sea has been proved to be a great treasure for structural unique and biological active natural products in last two decades.[1-3] Cold seeps are the typical deep-sea chemosynthetically-driven ecosystems, characterized as methane-rich fluid emissions and distinctive sulfur oxidation-reduction reactions, which led to high abundance of specialized cold seep microorganism.[4-5] Microorganisms from deep-sea cold seeps, which could be a new source for biomedically important compounds, are only beginning to be investigated.[6-7] The great potential of the capability for natural product biosynthesis of deep-sea cold seep microbes will undoubtedly accelerated investigation of deep sea microbes for new drugs.

In our continuing research for bioactive compounds from deep sea extreme environment-derived fungi,[8-10] we perform chemical investigations on the extract of the fungus *A. insuetus* SD-512, a strain of deep sea-derived fungus isolated from the sediments in cold seep area in the South China Sea at a depth of 1331m. As a result, two new phenol derivatives, insphenol A (**1**) and acetylpeniciphenol (**2**), together with seven known compounds, penicisochroman E (**3**),[11] asperisocoumarin D (**4**),[12] (−)-brassicadiol (**5**),[13] daldinin C (**6**),[14] penicisochroman I (**7**),[13] TMC-120B (**8**) and C (**9**)[15] (*Figure 1*), were isolated and identified. Among the isolated compounds, insphenol A (**1**) represents the first example of isopentenyl phenol derivative with a unique 1-glycosylation from the species *Aspergillus insuetus*. The assignment of the absolute configuration of **1** was confirmed by the coupling constant of the oxygenated methine proton and acid hydrolysis. This paper describes the isolation, structural elucidation, and biological evaluation of the isolated compounds.

Results and Discussion

Structure Elucidation of Compounds **1** and **2**

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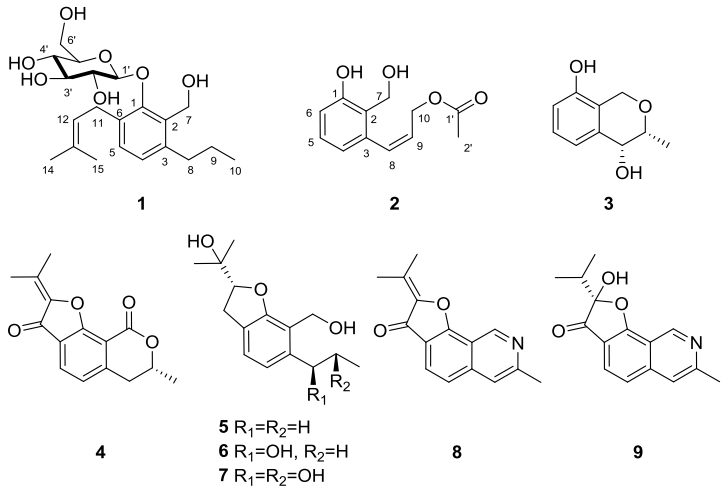


Figure 1. Chemical structures of the identified compounds **1**–**9**.

Compound **1** was obtained as colorless oil. The elemental composition was established to be $C_{21}H_{32}O_7$ supported by ion peak at *m/z* 419.2038 $[M + Na]^+$ (calculated for $C_{21}H_{32}O_7Na$), indicating 6 degrees of unsaturation. In the ^1H NMR spectrum, the signals for a pair of typical ortho-coupled aromatic protons were present at δ_H 6.93 (d, J = 7.8 Hz, H-C(4))/δ_H 6.99 (d, J = 7.8 Hz, H-C(5)), suggesting the presence of a 1,2,3,4-*tetra*-substituted benzene ring system. Meanwhile, a tri-substituted double bond (δ_H 5.26, t, J = 7.4 Hz, H-C(12)) were also observed. The ^{13}C NMR spectrum revealed signals attributable to three methyls, five sp^3-hybridized methylenes (two oxygenated), three sp^2- and five sp^3-hybridized methines, and five quaternary carbons. Further detailed analysis of 1D and 2D NMR data indicated that a hydroxymethyl group, a *n*-propyl group, an isoprenyl group, and a glucopyranose moiety were attached to the benzene ring bonded to C(2), C(3), C(6), and C(1), respectively (*Figure S3, S4,* and *S5*). Thus, the planar structure of **1** was determined and it was named as insphenol A.

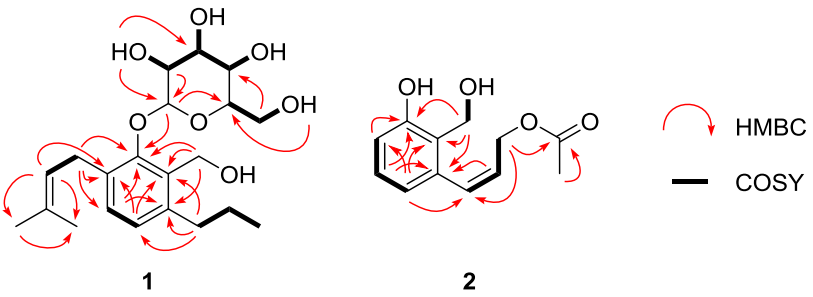
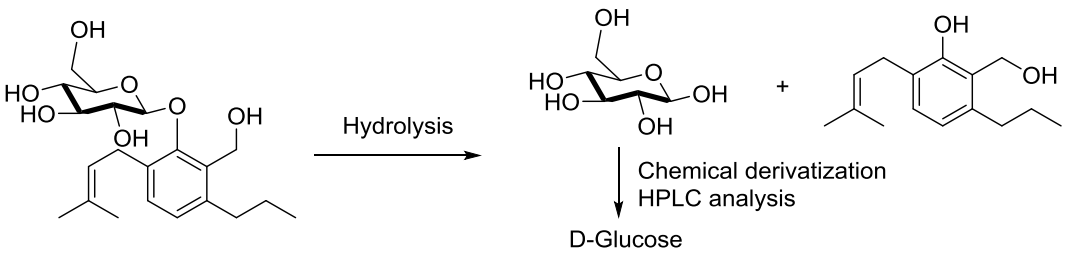


Figure 2. Key COSY (bold lines in black) and HMBC (arrows in red) correlations for compounds **1** and **2**.

The coupling constant of the anomeric proton at H-C(1') (δ_H 4.52, d, J = 7.8 Hz) revealed the β-configuration of the glycosidic linkage in **1**.[16] To confirm the identity of the glucose and to establish its absolute configuration, hydrolysis of **1** was performed with 3N HCl to give the sugar residue, which, together with the authentic D- and L-glucose, was further derivatized with L-cysteine methyl ester hydrochloride and σ-tolylisothiocyanate (*Scheme 1*).[16] HPLC analysis showed that the retention time of the derivative of sugar residue of **1** was identical with that of D-glucose (*Figure S8*). Thus, the sugar in **1** was determined as β-D-glucose.



Scheme 1. Determination of absolute configurations of **1** by chemical derivatization.

Table 1. ^{1}H- and ^{13}C-NMR data of compounds **1** and **2**.

Position	**1**		**2**	
	δ(H) (Mult, *J* in Hz)[a]	δ(C), Type[b]	δ(H) (Mult, *J* in Hz)[c]	δ(C), Type[d]
1		152.5, C		156.4, C
2		133.4, C		122.2, C
3		141.0, C		134.6, C
4	6.93, d (7.8)	125.4, CH	6.59, d (7.8)	120.5, CH
5	6.99, d (7.8)	128.7, CH	7.15, t (7.8)	126.8, CH
6		131.7, C	6.86, d (7.8)	115.8, CH
7*α*	*α* 4.72, dd (8.2, 6.8)	55.0, CH_2	4.84, s	60.4, CH_2
7*β*	*β* 4.45, dd (8.2, 6.8)			
8	2.59-2.69, m	33.8, CH_2	6.68, d (11.3)	131.3, CH
9	1.51-1.61, m	24.2, CH_2	5.83, dt (11.3, 6.7)	128.2, CH
10	0.93, t (7.3)	14.2, CH_3	4.56, dd (6.7, 1.2)	61.0, CH_2
11	3.46, d (7.4)	27.4, CH_2		
12	5.26, t (7.4)	123.6, CH		
13		131.3, C		
14	1.69, s	25.6, CH_3		
15	1.68, s	17.8, CH_3		
1'	4.52, d (7.8)	104.3, CH		170.6, C
2'	3.31, td (7.8, 2.7)	74.0, CH	2.02, s	20.4, CH_3
3'	3.23, t (7.8)	76.4, CH		
4'	3.05-3.09, m (overlap)	70.5, CH		
5'	3.05-3.09, m (overlap)	76.5, CH		
6'*α*	*α* 3.36-3.40, m	61.4, CH_2		
6'*β*	*β* 3.66, dd (11.7, 3.9)			
1-OH			7.69, s	
7-OH	4.43, d (6.8)		2.87, s	
2'-OH	5.59, d (2.7)			
3'-OH	5.14, s			
4'-OH	5.04, s			
6'-OH	4.70, t (3.9)			

[a] Data collected at 600 MHz in DMSO-d_6. [b] Data collected at 150 MHz in DMSO-d_6. [c] Data collected at 500 MHz in $CDCl_3$. [d] Data collected at 125 MHz in $CDCl_3$.

Compound **2**, acquired as colorless oil, was assigned the molecular formula $C_{12}H_{14}O_4$ by the analysis of HR-ESI-MS data, suggesting the requirement of 6 degrees of unsaturation. The observed signals in ^{1}H NMR spectrum of **2** for three aromatic protons at δ_H 7.15 (t, *J* = 7.8 Hz), δ_H 6.86 (d, *J* = 7.8 Hz) and δ_H 6.59 (d, *J* = 7.8 Hz) were indicative of a 1,2,3-trisubstituted benzene ring system. Meanwhile, protons for a *cis* disubstituted olefin, two oxygenated methylenes, a methyl and two changeable protons were also found in the ^{1}H NMR spectrum. The ^{13}C NMR data of **2** revealed the presence of a methyl group, two oxygenated methylenes, five olefinic/aromatic methines, and four quaternary carbon atoms (including three aromatic carbons and one carbonyl group). Exhaustive comparison of its ^{1}H-NMR data with those of peniciphenol, a known analogue isolated from the sea fan-derived fungus *Penicillium* sp. PSU-F40,[11] revealed the structural similarity between **2** and peniciphenol, except that an additional acetyl group were observed in the NMR spectra of **2**. Compared with peniciphenol, obvious deshielded shift for H-C(10) was also observed. The above observation suggested that compound **2** was a 10-OH acetylated derivative of peniciphenol, which was confirmed by HMBC correlation from H-C(10) to C(1'). Besides, the ^{1}H NMR data (δ_H 6.68, d, *J* = 11.3 Hz; δ_H 5.83, dt, *J* = 11.3, 6.7 Hz) indicated the *cis*-disubstituted double bond in **2**. Thus, the planar structure of **2** was established, and the trivial name acetylpeniciphenol was assigned to compound **2**.

In addition, seven known compounds, including penicisochroman E (**3**),[11] asperisocoumarin D (**4**),[12] (−)-brassicadiol (**5**),[13] daldinin C (**6**),[14] penicisochroman I (**7**),[13] TMC-120B (**8**) and C (**9**),[15] were also isolated and identified. Their structures were elucidated by comparing ^{1}H- and ^{13}C-NMR data with those reported in literature.

Antibacterial Activities

In bioassay, the isolated compounds were assayed for their antimicrobial activities against zoonotic aquatic pathogens in 96-well plates (*Table 2*). Compound **2** showed activity against *Edwardsiella tarda, Vibrio alginolyticus*, and *V. vulnificus*, with MIC values of 4, 8, and 8 μg/mL, respectively, while the MIC values of the positive control chloramphenicol for those three bacteria are all 0.5 μg/mL.

Table 2. Antimicrobial activities of compounds **1** and **2** (MIC, μg/mL).

Strains	**1**	**2**	Positive control[b]
E. tarda	–[a]	4	0.5
V. alginolyticus	-	8	0.5
V.vulnificus	-	8	0.5

[a] (–) = MIC > 64 μg/mL. [b] Chloramphenicol as positive control.

Conclusions

In summary, nine compounds (**1**–**9**), including two new phenol derivatives (**1** and **2**), were isolated from culture extract of the deep sea cold seep-derived fungus *A. insuetus* SD-512. Among them, compound **1** was the first report of isoprenyl phenol derivative with a unique 1-glycosylation from species *A. insuetus*. The absolute configuration was determined on the basis of the combination of coupling constant analysis, acid hydrolysis, chemical derivatization and HPLC analysis. The result of antibacterial activities exhibited that compound **2** could inhibit three aquatic pathogens with MIC values ranging from 4~8 μg/mL, which may be useful for the discovery of antibacterial agents. Due to the characteristic chemosynthetically-driven ecosystems in the cold seep area, secondary metabolites from deep-sea cold seep-derived fungi may be a new source of structurally unique and biologically active compounds.

Experimental Section

General

Optical rotations were measured by an Optical Activity AA-55 polarimeter. UV spectra were recorded on PuXi TU-1810 UV-visible spectrophotometer. CD spectra were acquired on a JASCO J-715 spectropolarimeter. The NMR spectra were recorded on Bruker Avance 500 MHz spectrometer, using solvent chemical shifts (DMSO-d_6: δ_H/δ_C 2.50/39.52; $CDCl_3$: δ_H/δ_C 7.26/77.16) as reference. Mass spectra were obtained on a VG Autospec 3000 mass spectrometer.

Fungal Material

The fungus *A. insuetus* SD-512 was isolated from the deep sea-sediment sample collected in September 2017, from the cold seep area of South China Sea at a depth of 1331 m. Besides, the sequenced data of ITS region derived from the fungal strain have been deposited in GenBank (accession no. MN696202), and the fungus was identified as *A. insuetus* according to a BLAST search result, which showed that the sequence was most similar (100%) to the sequence of *A. insuetus* (compared to accession no. MN 650839). The strain is preserved at the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences.

Fermentation

For chemical investigations, the fungus SD-512 was fermented statically at 28 °C in rice solid medium containing rice (70 g/flask), peptone (0.3 g/flask), yeast extract (0.5 g/flask), corn steep liquor (0.2 g/flask), monosodium glutamate (0.1 g/flask) and naturally sourced and filtered seawater (obtained from the Huiquan Gulf of the Yellow Sea near the campus of IOCAS, 100 mL/flask) in 1L flasks (×120).

Extraction and Isolation

After incubation of 35days, the fungal culture was extracted thoroughly with ethyl acetate (EtOAc) to give an extract (70.0 g). The EtOAc extract was fractionated by silica gel VLC (vacuum liquid chromatography) using a stepwise gradient of a mixture of petroleum ether (PE)/EtOAc (20:1 to 1:1), and then CH_2Cl_2/MeOH (20:1 to 1:1) to yield nine fractions (Frs. 1−9). Fr. 4 (2.9 g), eluted with PE/EtOAc (2:1), was subjected to CC over RP-18 eluting with MeOH/H_2O gradient (from 1:9 to 1:0) to afford nine subfractions (Frs. 4.1−4.9). Fr.4.1 (107 mg), Fr.4.3 (68 mg) and Fr.4.5 (115 mg) were combined and purified by preparative TLC as well as CC on Sephadex LH-20 (MeOH) to give **3** (11.3 mg), **2** (3.1 mg) and **9** (16.9 mg), respectively. Fr.4.4 (597 mg) was split by CC on silica gel (200–300 mesh) eluted with a CH_2Cl_2/EtOAc gradient, from 50:1 to 1:1, to acquire **4** (11.0 mg), **5** (98.8 mg), **8** (23.8 mg). Fr. 6 (10.1 g), eluted with CH_2Cl_2/MeOH (20:1), was further purified by CC over RP-18, preparative TLC and then CC on Sephadex LH-20 (MeOH) to obtain **6** (135.6 mg). Fr. 7 (4.3 g), eluted with CH_2Cl_2/MeOH (10:1), was applied to CC over RP-18, eluting stepwise from MeOH/H_2O 1:9 to 1:0, to give nine subfractions (Frs. 7.1−7.9). Fr.7.2 (381 mg) and Fr.7.6 (72 mg) was purified to preparative TLC and CC over Sephadex LH-20 (MeOH) to yield **7** (31.5 mg) and **1** (11.0 mg), respectively.

Insphenol A (1). White amorphous powder; $[\alpha]_D^{25}$ +46.2 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.95) nm, 220 (3.36) nm, 269 (2.30) nm; ECD (3.8 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 203 (–2.11), 226 (–0.52); IR ν_{max} 3239, 2959, 2922, 2870, 1425, 1379, 1196, 1085,1043, 1020, 990 cm^{-1}; ^{1}H- and ^{13}C-NMR data, *Table 1*; HR-ESI-MS *m/z* 419.2038 $[M + Na]^+$ (calcd for $C_{21}H_{32}O_7Na$, 419.2040).

Acetylpeniciphenol (2). Colorless oil; UV (MeOH) λ_{max} (logε) 209 (4.07) nm, 286 (3.08) nm; IR ν_{max} 3348, 2917, 1716, 1582, 1466, 1374, 1256, 1023, 993 cm^{-1}; ^{1}H- and ^{13}C-NMR data, *Table 1*; HR-ESI-MS *m/z* 245.0791 $[M+Na]^+$ (calcd for $C_{12}H_{14}O_4Na$, 245.0784).

Acid hydrolysis and determination of the absolute configuration of glucose

Compound **1** (1.0 mg) was dissolved in 3 N HCl (0.5 mL) and heated to 100 °C for 2 h. Then, the sugar residue was obtained from the aqueous layer after extracting with EtOAc. The sugar residue was dissolved in 0.5 mL pyridine with the presence of 0.5 mg of L-cysteine methyl ester hydrochloride and heated to 60 °C for 1 h. Then, 10 μL of σ-tolylisothiocyanate was added to the mixture and the heating was continued for 1 h. After that, the reaction mixture was subjected to HPLC analysis and the HPLC condition was 10% to 100% MeCN gradient with 0.1% TFA over 40 min (Elite, SinoChrom ODS-BP, 250 mm × 4.60 mm, 5 μm; UV detection 254nm; flow rate 1.0 mL/min).[16]

Antibacterial Assays

The antibacterial activities against human pathogenic bacteria (*Escherichia coli* QDIO-1 and *Pseudomonas aeruginosa* QDIO-2) and aquatic pathogens (*Edwardsiella tarda* QDIO-8, *Vibrio alginolyticus* QDIO-7, *V. anguillarum* QDIO-9, and *V. vulnificus* QDIO-4 were determined by a serial dilution technique using 96-well microtiter plates.[17] The human or aquatic pathogenic strains were offered by IOCAS. Tested compounds and positive control (chloramphenicol) were dissolved in DMSO to give a stock solution.

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.2021xxxxx

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Author Contribution Statement

L.P. C. performed the experiments for the isolation, structure elucidation, acid hydrolysis and chemical derivatization, antibacterial evaluation and prepared the manuscript; X.M. L. performed the 1D and 2D NMR experiments; Y.P. W. contributed to the isolation of compounds from the fungal strain SD-512; Y.H. L. contributed to the acid hydrolysis and chemical derivatization of compound **1**. X. L. revised the manuscript; B.G. W. supervised the research work and revised the manuscript.

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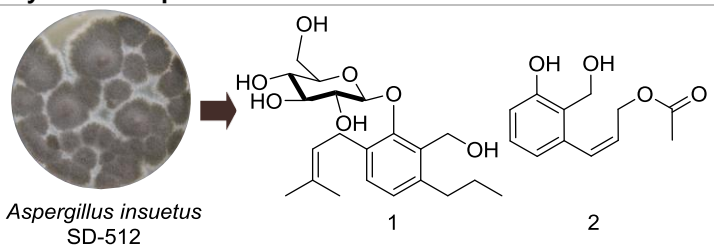
Entry for the Graphical Illustration



Twitter Text

Two New Phenol Derivatives, Insphenol A and Acetylpeniciphenol, with Unique 1-Glycosylation and Antibacterial Activities from Cold Seep Derived Fungus *Aspergillus insuetus* SD-512 by B.-G. Wang et al., Institute of Oceanology, CAS, China