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A New Acetylenic Compound and Other Bioactive Metabolites from a Shark Gill-derived *Penicillium* Strain

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Abstract: Nine chiral compounds (**1**−**9**) were isolated from the static fermentation culture of a shark gill-derived fungus *Penicillium polonicum* AP2T1. These compounds include a new acetylenic aromatic ether (**1**, (-)-WA), four alkaloids (aurantiomide C (**2**), fructigenine A (**3**), cyclopenin (**4**) and cyclopenol (**5**)) and four oxygenated compounds ((*R*)-penipratynolene (**6**), (3*S*,4*S*)-3,4-dihydro-3,4,8-trihydroxyl-naphthalenone (**7**), verrucosidin (**8**) and norverrucosidin (**9**)). Their structures were elucidated by MS, NMR, optical rotation and circular dichroism (CD). In antimicrobial tests, compounds **1**–**4**, **6** and **8**–**9** showed weak antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, and/or *Escherichia coli*. Compounds **3**, **8** and **9** also exhibited moderate toxicity against *Artemia salina* larva, and showed cytotoxicity against human colon cancer cell line HCT116.

Keywords: Secondary metabolites; shark; *Penicillium polonicum*; bioactivity.

1. Introduction

As a promising resource, marine fungi can produce diverse leading compounds with attractive bioactivities [1]. However, most of the chemically characterized marine fungi have been so far isolated from marine plants or settled invertebrates, and seldom from fishes [1, 2]. The ocean dominator, sharks, are even less explored in terms of chemistry, bioactivity and chemical ecology of their symbiotic microbes [3, 4], despite the fact that sharks are believed to have powerful resistance to diseases like infections and cancers [5-7]. In this paper, a new acetylenic aromatic ether together with eight known natural products was characterized from a shark gill-derived fungus *Penicillium polonicum* AP2T1. The bioactivity of these compounds were also evaluated.

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2. Materials and Methods

2.1. Microorganism Material

The strain *Penicillium polonicum* AP2T1 is deposited in China General Microbiological Culture Collection Center (CGMCC accession number 8516). It was isolated from gills of shark *Isurus oxyrinchus* and classified as *Penicillium polonicum* by sequencing, online BLAST comparison, and offline phylogenic tree construction of both its ITS1-5.8S-ITS2 rDNA (accession number JN368448 in Genbank) and β-tubulin gene partial sequence (accession number KT633935).

2.2. Fermentation and Isolation

A 20-l static fermentation was carried out at 28 °C for 30 days in 5-l glass bottles each filled with 1 l of autoclaved potato sucrose broth containing natural sea salts (20 g/l). After fermentation, the mycelium was obtained by filtration and then extracted with MeOH, while fermentation broth was extracted with ethyl acetate (EtOAc), both at room temperature. After TLC comparison, the two extracts were finally combined and condensed *in vacuo* under 45 °C to yield a crude extract (11.2 g).

The crude extract was subjected to column chromatography (CC) (sephadex LH-20; MeOH) to give six fractions (Frs. I–VI). In them, Frs. II and III showed potent toxicity against brine shrimp larva. Fr. III (1.42 g) was subsequently separated by CC on RP-18 (MeOH/H_2O, 70:30) into 5 subfractions (sFrs.1–5). The sFr. 1 (1.0 g) was further fractioned by CC on RP-18 (MeOH/H_2O, 20:80−100:0, stepwise) into 9 secondary subfractions (ssFrs. 1–9). The ssFr. 5 (47 mg) was purified by preparative RP-HPLC (MeOH/H_2O containing 0.05% formic acid, 25:75, 12 mL/min) to yield compound **1** (14.6 mg). The ssFr. 3 (59.0 mg) was separated by preparative RP-HPLC (MeOH/H_2O containing 0.05% formic acid, 10:90, 15 mL/min) to produce compound **7** (4.6 mg). The ssFr. 6 (217 mg) was refined by CC on silica gel ($CHCl_3$/MeOH, 25:1) to give compound **5** (183.6 mg). The ssFr. 8 (55.0 mg) was separated by preparative RP-HPLC (MeOH/H_2O containing 0.05% formic acid, 35:65, 12 mL/min) to yield compound **4** (27.1 mg) and **6** (3.0 mg). The sFr. 2 (106 mg) was purified by CC on silica gel ($CHCl_3$/MeOH, 20:1−2:1, stepwise), preparative TLC (silica gel, cyclohexane/acetone, 3:4), and preparative RP-HPLC (MeOH/H_2O containing 0.05% formic acid, 50:50, 12 mL/min) to give compound **2** (29.7 mg). Fr. II (5.36 g) was partitioned with petroether to give a low polar oil (2.19 g), which was fractioned by CC on RP-18 (MeOH/H_2O, 60:40−100:0, stepwise) into 22 parts (Parts 1−22). By preparative TLC (silica gel, $CHCl_3$/MeOH, 50:1), compounds **3** (12.4 mg) and **9** (11.7 mg) from Part 7 (33.7 mg) and compound **8** (116 mg) from Part 8 (178 mg) were obtained, respectively.

3. Results and Discussion

3.1. Structure Elucidation

Compounds **2**−**9** were elucidated to be known compounds aurantiomide C(**2**)[8], fructigenine A (**3**)[9], cyclopenin (**4**)[10], cyclopenol (**5**)[11], (*R*)-penipratynolene (**6**)[12], *trans*-3,4-dihydro-3,4,8-trihydroxyl-naphthalenone (**7**)[13, 14], verrucosidin (**8**)[15] and norverrucosidin (**9**)[15] (Figure 1), respectively, by interpretation of MS, 1D and 2D NMR including NOESY and specific optical rotation as well as by comparison with data from literatures.

The new compound **1**, m.p. 113−114 ºC, UV λ_{max} nm (logε) (MeOH) 248 (3.66) and $[\alpha]_D^{20}$ −9.4 (*c* 0.4, MeOH), was obtained as an amorphous powder and gave a molecular formula of $C_{11}H_{10}O_4$, as revealed by quasi-molecular ion peak at m/z 229.0461 ($[M+Na]^+$, $C_{11}H_{10}NaO_4^+$; calcd. 229.0471) in HR-ESI-MS, indicating 7 degrees of unsaturation. Its IR showed main absorption at 3276 (OH and/or acetylenyl), 2936, 2556, 1676 (CO), 1604, 1581, 1512, 1429 (Ar), 1322, 1301, 1247, 1168, and 1028 cm^{-1}. Its ^1H and ^{13}C NMR data were attributed in Table 1 by 2D NMR, displaying the presence of one *p*-disubstituted benzene ring (δ_H 7.03 (H-3 and H-5, d, J = 8.9 Hz, 2H) and 7.88 (H-2 and H-6, d, J =

8.9 Hz, 2H); six sp^2 carbons), two hydroxyls (δ_H 5.87 and 12.64), one acetylenyl (δ_H 3.41 (H-4', d, J = 2.2 Hz); δ_C 83.5 (C-3') and 75.7 (C-4')), two sp^3 carbons, and one carbonyl at δ_C 167.1 (C-7). The interpretation to the 1D and 2D (Fig. 2) NMR data suggested that **1** has the same planar structure as antibiotic WA (4-((*S*)-2-hydroxybut-3-ynyloxy)-benzoic acid), which was produced by an unidentified soil fungus 38[16, 17]. However, it displayed opposite optical rotation direction to antibiotic WA with comparable absolute value [for compound **1**: $[\alpha]_D^{20}$ −9.4 (*c* 0.4, MeOH); for antibiotic WA [16]: $[\alpha]_D^{20}$ +10.85 (*c* 0.4, MeOH)]. Besides, in circular dichroism (CD) measurement, compound **1** showed positive Cotton effect near 250 nm as same as the known **6**, whose configuration at C-2' is *R*-type and was verified by same optical property with previously reported data [12] (Figure 2). Therefore, compound **1** was deduced to have *R*-configuration at C-2' too. A natural compound under product number Amb8398639 was ever listed with the same planar structure in the online catalogue of Ambinter company. However, no stereochemistry or optical data was provided and actually it is not produced any more by Ambinter. So, compound **1** was named as (-)-WA and cautiously determined as a new compound. Considering the co-existence of compounds **1** and **6** and their identical stereo-configurations, we speculate that **6** may be biosynthesized by **1** through a methylation reaction.

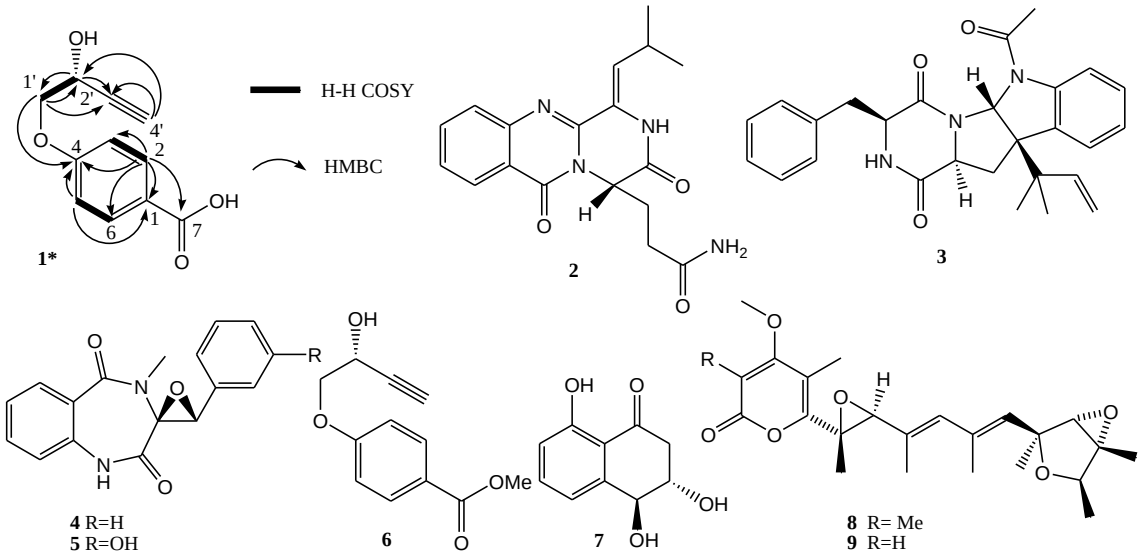

Figure 1. Structures of compounds **1–9** (**1** is new) and selected HMBC and H-H COSY correlations of **1**.

3.2. Antimicrobial, Larvicidal and Cytotoxic Activities

All the isolated nine compounds were screened for cytotoxicity, antimicrobial activity, and brine shrimp larva lethality.

Antimicrobial assay was carried out by standard paper disk method (25 μg/disk)[18], using *Staphylococcus aureus* DSM-20231, *Bacillus subtilis* DSM-10, *Escherichia coli* AB3027 (from Yale Univeristy's Coli Genetic Stock Center), *Candida albicans* Tü-164 (from Tübinger collection), and *Rhodotorula rubra* Tü-136 (from Tübinger collection) as indicator organisms. Compounds **1**–**4**, **6**, and **8**–**9** displayed weak inhibition zones of 7.5−8.5 mm in diameter against the tested bacteria (to *B. subtilis*, **1**: 8.0 mm, **4**, **6** and **9**: 7.5 mm, penicillin G as control: 7.0 mm; to *S. aureus*, **2** and **8**: 7.5 mm, **3** and **9**: 8.0 mm, penicillin G: 40.0 mm; to *E. coli*, **1** and **6**: 8.5 mm, **2**: 8 mm, penicillin G: 6.5 mm). Compared with its previously characterized stereo isomer WA inhibiting fungus *Pyricularia oryzae* [17], the new compound **1** showed different antimicrobial spectrum.

The brine shrimp (*Artemia salina*) larva lethality test was performed by a 96-microwell plate method [19]. The brine shrimp eggs were purchased from JBL GmbH & CoKG. In the test, the concentration range was set to be 0.04−125 μg/mL. Compounds **3**, **8**, **9** and $Hg(NO_3)_2$ (control)

displayed moderate toxicity against *Artemia salina* larva with semi-lethal concentration (LC_{50}) of 125 μg/mL, 5 μg/mL, 17.2 μg/mL and 25 μg/mL in 24 h, respectively.

The cytotoxicity assay against human colon cancer cell line HCT116 wt (wild type, from Prof. Bert Vogelstein's laboratory in Johns Hopkins University) was performed by crystal violet method [20]. Compounds **3**, **8**, **9** and taxol (control) exhibited antiproliferatory activity with the half maximal inhibitory concentrations (IC_{50}) of 40.5 μg/mL, 30.8 μg/mL, 5.7 μg/mL and 0.02 μg/mL at 24 h, respectively. Compounds **2**, **5**, and **6** showed weaker activity.

Table 1. ^{1}H and ^{13}C NMR data for compound **1** (measured in DMSO-d_6 at 400 MHz for ^{1}H and 100 MHz for ^{13}C, δ in ppm).

Position	**^{13}C**	**^{1}H (*J* in Hz)**
1	123.6 (C)	
2,6	131.3 (CH)	7.88 (2H, *d*, *J* = 8.9)
3,5	114.4 (CH)	7.03 (2H, *d*, *J* = 8.9)
4	161.7 (C)	
7	167.1 (C)	
1'	71.4 (CH_2)	4.07 (2H, *m*)
2'	59.4 (CH)	4.59 (1H, *m*)
3'	83.5 (C)	
4'	75.7 (CH)	3.41 (1H, *d*, *J* = 2.2)
COOH		12.64 (1H, *brs*)
2'-OH		5.87 (1H, *brs*)

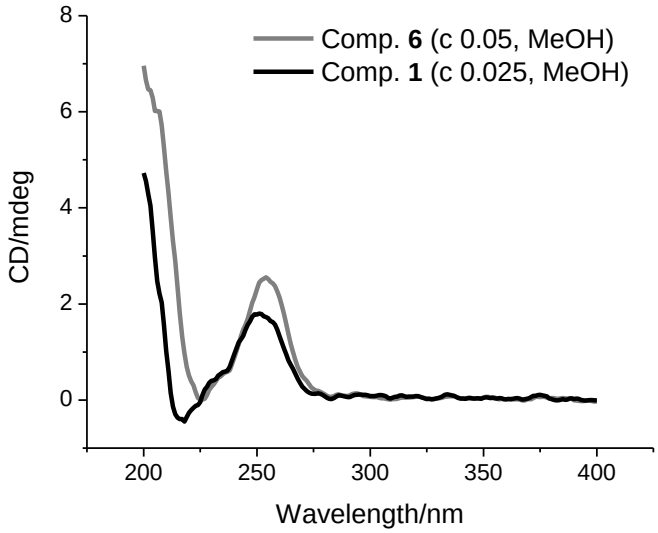

Figure 2. The circular dichroism (CD) spectra of compounds **1** and **6**.

In this study, nine chiral compounds were characterized. Most of them are frequently isolated or detected from strains of *Penicillium polonicum* and related *P. aurantiogriseum* [9, 21]. However, to the best of our knowledge, new compound **1** is the first example of acetylenic aromatic ethers in marine derived *Penicillium* and compound **7** is reported here from this genus for the first time.

Overall, *P. polonicum* strain AP2T1 from shark gill displayed impressive versatility in producing different types of bioactive metabolites, including acetylenic compounds, polyketides, and diverse alkaloids, which may be of value for its survival in shark's gill microenvironment, as well as benefit the host organism.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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