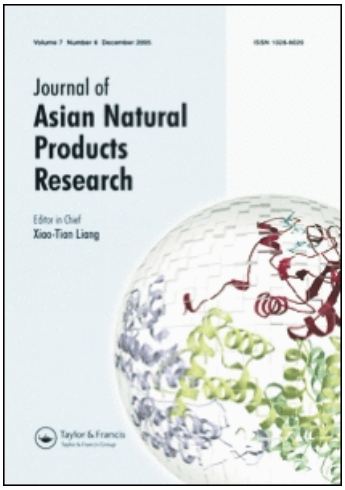

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Two new compounds from the broth of the marine fungus *Penicillium griseofulvum* Y19-07

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Two new compounds, 4-hydroxyphenethyl methyl succinate (**1**) and 4-hydroxyphenethyl 2-(4-hydroxyphenyl)acetate (**2**), were isolated from the EtOAc extract of the broth of the marine fungus *Penicillium griseofulvum* Y19-07. Five known compounds were also obtained in this study. The structures of the new compounds were elucidated by 1D and 2D NMR spectroscopy and mass spectrometry. All of the isolates were evaluated for their scavenging properties toward the 2,2-diphenyl-1-picrylhydrazyl free radical by spectroscopic assays. Also, in the cytotoxicity assay of the two new compounds against HL-60 and PC-3 prostate cancer cell lines, compound **2** showed potential activity with an IC_{50} value of 64.5 μM against human HL-60 cancer cells.

Keywords: *Penicillium griseofulvum*; marine fungus; cytotoxicity

1. Introduction

As is well known, many active compounds have been found in marine fungi in the past years [1]. Marine micro-organisms have proven to be a promising source for the production of novel antitumor agents [2]. Marine-derived fungi also represent the potential for the discovery of new cytotoxic metabolites [3]. To discover new cytotoxic and anti-oxidant compounds, we have investigated the chemical constituents of the marine fungus *Penicillium griseofulvum* Y19-07, and report here the isolation, characterization, and biological activity of two new compounds: 4-hydroxyphenethyl methyl succinate (**1**) and 4-hydroxyphenethyl 2-(4-hydroxyphenyl)acetate (**2**) (Figure 1).

2. Results and discussion

Compound **1** was obtained as a colorless oil. The molecular formula was determined to be $C_{13}H_{16}O_5$ by HR-TOF-MS at *m/z* 275.0899 $[M+Na]^+$. IR absorptions were observed at 3409, 1715, 1612, 1512, and 1450 cm^{-1}, indicating the presence of hydroxyl, ester carbonyl groups, and a benzene ring. The UV spectrum showed a maximum absorption at 277 nm, and a bathochromic shift in alkaline solution, indicating the presence of a phenol derivative [4]. This was confirmed by the ^1H NMR spectrum, which showed one proton at δ_H 9.23 (1H, br s) assigned to 4-OH, which disappeared upon addition of D_2O. The ^1H NMR spectrum of compound **1** exhibited a 1,4-disubstituted benzene ring at δ_H 7.01 and 6.66 (each 2H, d,

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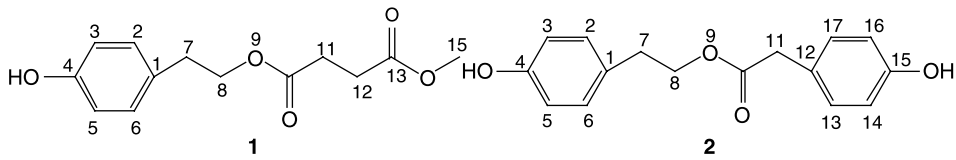

Figure 1. The structures of compounds **1** and **2**.

$J = 8.4$ Hz, H-2, 6 and H-3, 5), and this paratope-substituted phenyl moiety can also be observed from the ^{13}C NMR spectrum at δ_C 155.9, 129.8, 127.9, and 115.2. An oxymethylene unit [δ_H 4.12 (2H, t, $J = 7.2$ Hz, H-8)] split into a triplet due to coupling with another methylene group [δ_H 2.73 (2H, t, $J = 7.2$ Hz, H-7)]. In addition, the correlations of the methylene group (δ_H 2.73, H-7) with the carbon signals at δ_C 127.9 (C-1), 129.8 (C-2, C-6) and the correlation of the oxymethylene unit signal at δ_H 4.12 (H-8) with the carbonyl group signal at δ_C 171.9 (C-10) were observed from the HMBC spectrum. Therefore, the above information can establish the presence of the partial substructure, fragment **1a** (Figure 2) of compound **1**. A methoxyl signal [δ_H 3.55 (3H, s, 15-OCH_3)] was observed to correlate with the other carbonyl group signal at δ_C 172.4 (C-13) from the HMBC spectrum, which established the partial substructure, fragment **1b** (Figure 2). In addition, the ^1H NMR and HSQC spectra revealed the presence of an ethyl group [δ_H 2.52 (4H, s, H-11, H-12)], fragment **1c** (Figure 2) of compound **1**. Then, the entire skeleton of compound **1** was constructed from the HMBC spectrum (Figure 3). On the basis of the above evidence, compound **1** was elucidated as 4-hydroxyphenethyl methyl succinate.

Compound **2**, obtained as a colorless oil, was assigned the molecular formula $C_{16}H_{16}O_4$ by its HR-TOF-MS at *m/z* 295.0949 $[M+Na]^+$. IR absorptions were observed at 3410, 1716, 1615, 1510, and 1450 cm^{-1}, indicating the presence of a hydroxyl group, an ester carbonyl group, and a benzene ring. The UV spectrum showed a maximum absorption at 277 nm, and a bathochromic shift in alkaline solution also indicated the presence of a phenol derivative. This was confirmed by the ^1H NMR spectrum, which showed two protons at δ_H 9.31 (2H, br s) assigned to 4- and 15-OH, which also disappeared upon addition of D_2O. Analysis of the ^1H and ^{13}C NMR spectra revealed that compound **2** had two paratope-substituted phenyl fragments, one fragment showed four aromatic proton signals at δ_H 7.00 (2H, d, $J = 8.4$ Hz, H-2, H-6) and 6.66 (2H, d, $J = 8.4$ Hz, H-3, H-5). The HSQC spectra showed its aromatic carbon signals at δ_C 155.9, 129.8 × 2, 127.9, 115.2 × 2, two methylene groups [δ_H 2.73 (2H, t, $J = 7.2$ Hz, H-7), δ_H 4.12 (2H, t, $J = 7.2$ Hz, H-8)], and a carbonyl carbon signal at δ_C 171.5 (C-10), which revealed that it was similar to the above fragment **1a** (Figure 2) of compound **1**. While the other fragment showed four aromatic proton signals at δ_H 6.98 (2H, d, $J = 8.4$ Hz, H-14, H-16) and 6.68 (2H, d, $J = 8.4$ Hz, H-13, H-17), the ^{13}C NMR and HSQC spectra

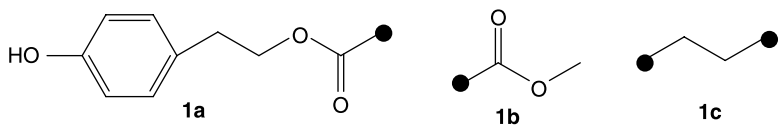

Figure 2. The fragments of compound **1**.

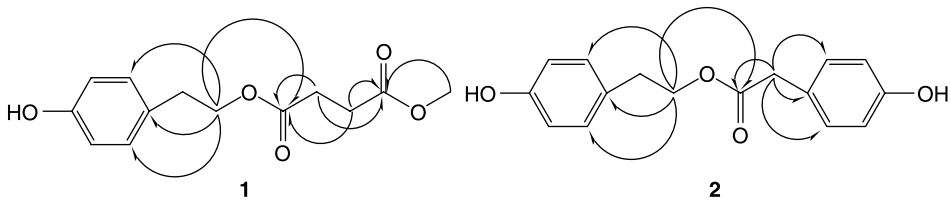

Figure 3. The key HMBC correlations of compounds **1** and **2**.

showed six aromatic carbon signals at δ_C 156.3, 130.3 × 2, 124.5, and 115.2 × 2. In addition, the ^{1}H NMR and HSQC spectra of **2** exhibited one methylene group [δ_H 3.48 (2H, s, H-11), δ_C 39.7 (C-11)]. Moreover, the HMBC spectra revealed that this methylene group at δ_H 3.48 (2H, s, H-11) correlated with three aromatic carbon signals at δ_C 124.5 (C-12), 130.3 (C-13, C-17) and the carbonyl carbon signal at δ_C 171.5 (C-10) (Figure 3). Therefore, on the basis of the above evidence, compound **2** was identified as 4-hydroxyphenethyl 2-(4-hydroxyphenyl)acetate (**2**).

The other known isolates, 3-benzyl-pyrrolopiperazine-1,4-dione (**3**) [5], 4-hydroxyphenethyl acetate (**4**) [6], 4-methyl-pyrocatechol (**5**), methyl(*p*-hydroxy-phenyl)acetate (**6**), and *p*-hydroxyphenethyl alcohol (**7**), were readily identified by comparison of their physico-chemical values with those in the literature. The compounds described above were isolated from *P. griseofulvum* for the first time.

The free radical scavenging properties of the seven compounds were evaluated against the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical [7]. The free radical scavenging effects of these isolates, corresponding to the intensity of quenching of the DPPH free radical, were evaluated by spectroscopic assay. In this study, vitamin E showed an IC_{50} value of 26.5 μM. The new compounds **1** and **2** showed a moderate effect with IC_{50} values of 58.6 and 56.2 μM, respectively, while compound **5** showed stronger activity in this study with an IC_{50} value of 7.1 μM. The others showed weaker activity than compounds **1** and **2**. From this study, we find that those which have no phenol group nearly showed no activity. Therefore, it implies that the presence of a phenol group is significant for the free radical scavenging effect. In addition, the higher DPPH free radical scavenging activity of compound **5** seems to suggest that the structure having two —OH groups in near space may catch free radical easily. In addition, the cytotoxicities of compounds **1** and **2** were evaluated against PC-3 (prostate cancer) cell lines using the MTT assay [8]. Compound **2** showed a moderate effect with an IC_{50} value of 64.5 μM, but compound **1** had very low activity ($IC_{50} > 100$ μM). Meanwhile, in the tumor cell HL-60 growth inhibition assay, the two compounds had very low activity ($IC_{50} > 80$ μM).

3. Experimental

3.1 General experimental procedures

UV spectra were obtained on a Shimadzu UV-2201. IR spectra were recorded on a Bruker IFS-55 infrared spectrophotometer. The NMR spectral data were recorded on Bruker AV-600 (600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR; Bruker, Fallanden, Switzerland) in dimethyl sulfoxide (DMSO)-d_6 with TMS as the internal standard. The HR-FAB-MS data were obtained on the Micross Mass Autospec-UltimaE TOF mass spectrophotometer. Chromatography was performed on silica gel (200–300 mesh; Qingdao Haiyang Chemical Factory, Qingdao, China), Sephadex LH-20 (Pharmacia, Piscataway, NJ, USA), and reversed-phase HPLC (Shimadzu LC-10 AVP, Kyoto, Japan).

3.2 Fungal material

P. griseofulvum Y19-07 was isolated from the mangrove *Lumnitzera racemosa* collected from South China Sea, in 2007 (accession number HTTM-Z07001), which was deposited in the First Institute of Oceanography, SOA, Qingdao, China. The stain is recorded at GenBank with the code number FJ481096.

3.3 Cultivation and methods

The initial cultures were maintained on the seawater agar. Then, the mycelia were cut and aseptically transferred to a 250 ml Erlenmeyer flask containing 100 ml of culture media (extract of potato in 20% seawater, 0.2% peptone, 0.1% yeast extract, 1.7% glucose, 2% NaCl, 0.13% $MgCl_2-6H_2O$, 0.02% KCl, 0.001% $FePO_4$, pH 6.5). The flask was incubated at 25°C on a rotary shaker, with 150 rpm, for 15 days.

3.4 Extraction and isolation

The cultures (80 liters) were centrifuged at 4000 rpm to separate the mycelial mass from the aqueous layer. The aqueous layer of *P. griseofulvum* was concentrated to 1200 ml. The concentrate was extracted with ethyl acetate to get the crude extract (11 g). The ethyl acetate-soluble fraction was subjected to a silica gel column, eluted with $CHCl_3-CH_3OH$ (100:1–0:1), yielding 10 fractions. Fraction 2 (1.2 g) was purified by Sephadex LH-20 column chromatography (MeOH) and preparative HPLC (Chromatorex C_{18}) to obtain compounds **1** (9 mg, MeOH–H_2O 4.5:5.5), **6** (3.2 mg, MeOH–H_2O 11:9), and **7** (3.6 mg, MeOH–H_2O 11:89). Fraction 3 (2 g) was subjected to a silica gel column, and then purified by Sephadex LH-20 column chromatography (MeOH) and preparative HPLC (Chromatorex C_{18}, MeOH–H_2O 1:1) to obtain compounds **2** (12 mg), **3** (6.4 mg), **4** (19.7 mg), and **5** (4.3 mg).

*Compound (**1**)*: Colorless oil. UV λ_{max} (MeOH) nm: 277. IR (KBr) ν_{max} (cm^{-1}): 3409, 1715, 1612, 1512, and 1450; ^1H NMR (DMSO, 600 MHz) and ^{13}C NMR (DMSO, 150 MHz) spectral data: see Table 1. HR-TOF-MS *m/z*: 275.0899 $[M+Na]^+$ (calcd for $C_{13}H_{16}O_5Na$, 275.0890).

*Compound (**2**)*: Colorless oil. UV λ_{max} (MeOH) nm: 277. IR (KBr) ν_{max} (cm^{-1}): 3410, 1716, 1615, 1510, and 1450. ^1H NMR (DMSO, 600 MHz) and ^{13}C NMR (DMSO, 150 MHz) spectral data: see Table 1. HR-TOF-MS *m/z*: 295.0949 $[M+Na]^+$ (calcd for $C_{16}H_{16}O_4Na$, 295.0941).

3.5 Free radical scavenging activity (DPPH scavenging activity)

The free radical scavenging activity of the test compounds was examined with the DPPH free radical, as described previously [9]. α-Tocopherol (vitamin E) was used as the control. The reaction was performed in 100 μl solution containing 0.1 mM freshly prepared DPPH in alcohol and various concentrations of the tested samples (in alcohol). The reaction mixture was shaken vigorously, and its absorbance at 517 nm was determined after 30 min incubation in a dark area. The scavenging effect was calculated against a vehicle control (alcohol). All tests were run in triplicate and were averaged. Decreasing the DPPH solution absorbance indicates an increase in DPPH free radical scavenging activity.

3.6 Cytotoxicity of compounds 1 and 2

3.6.1 Tumor cell HL-60 growth inhibition assay

Human leukemia HL-60 cells (obtained from American Type Culture Collection, Rockville, MD, USA) were cultured in RPMI-1640 medium (Gibco, New York, NY, USA) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mmol glutamine, and 10% heat-inactivated fetal bovine serum (Gibco).

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data for compounds **1** and **2** in DMSO-d_6.

	1		**2**	
No.	δ_H (mult.; *J*, Hz)	δ_C	δ_H (mult.; *J*, Hz)	δ_C
1	–	127.9	–	127.9
2	7.01 (d, $J = 8.4$)	129.8	7.00 (d, $J = 8.4$)	129.8
3	6.66 (d, $J = 8.4$)	115.2	6.66 (d, $J = 8.4$)	115.2
4	–	155.9	–	155.9
4-OH	9.23 (br s)	–	9.31 (br s)	–
5	6.66 (d, $J = 8.4$)	115.2	6.66 (d, $J = 8.4$)	115.2
6	7.01 (d, $J = 8.4$)	129.8	7.00 (d, $J = 8.4$)	129.8
7	2.73 (t, $J = 7.2$)	33.6	2.73 (t, $J = 7.2$)	33.6
8	4.12 (t, $J = 7.2$)	65.1	4.12 (t, $J = 7.2$)	65.1
10	–	171.9	–	171.5
11	2.52 (s)	28.7	3.48 (s)	39.7
12	2.52 (s)	28.5	–	124.5
13	–	172.4	6.98 (d, $J = 8.4$)	130.3
14	–	–	6.68 (d, $J = 8.4$)	115.2
15	3.55 (s)	51.5	–	156.3
15-OH	–	–	9.31 (br s)	–
16	–	–	6.68 (d, $J = 8.4$)	115.2
17	–	–	6.98 (d, $J = 8.4$)	130.3

Cell growth inhibition assay was performed as reported previously. Cells were seeded at a density of 5×10^4 cells/ml and incubated with various concentrations of the tested compounds for 3 days. The compounds were dissolved in DMSO, and the amount of DMSO was controlled lower than 0.1% in the final concentration. The number of cells in each group was determined by hemocytometer, and the cell viability was determined using trypan blue staining. The growth inhibitory ability of the new compounds was calculated and expressed as the ratio of the cell number of the treated group to that of the untreated group. The concentration (IC_{50}) that inhibited half of the cell growth was calculated. 5-Fluorouracil (5-FU) was used as a positive control, and 0.1% DMSO was used as a negative control.

3.6.2 Tumor cell PC-3 growth inhibition assay

The two compounds were tested for cytotoxicity using the MTT-cell culture assay, described by Mosmann [8]. Briefly, 2×10^4 cells/100 μl were seeded in 96-well microplates and pre-incubated for 24 h in order to allow the attachment of cells. After plating the cells, fresh medium (100 μl) containing various concentrations of the test compounds was added to the cultures. The cells were incubated with each compound for 4 days. Cell survival was evaluated by adding MTT tetrazolium salt solution with fresh medium. After 3 h incubation at 37°C, 200 μl of DMSO was added to dissolve the precipitate of reduced MTT. Microplates were then shaken for 15 min and the absorbance was determined at 550 nm with a multiwell scanning spectrophotometer.

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