Published By Guangdong Pharmaceutical University, Guangzhou
Journal of Holistic Integrative Pharmacy
2022, 3(3):227-231

EXPERIMENTAL RESEARCH

Novel phenylacetate derivatives isolated from the fungus *Penicillium canescens*

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[Abstract] Two undescribed phenylacetate derivatives (compounds **1** and **2**) with a known analog were isolated from a soil-derived fungus *Penicillium canescens* through the "one strain-many compounds" method. The new structures were assigned using extensive 1D and 2D NMR spectra, high-resolution electrospray ionization mass spectrometry, and the comparison of coupling cleavages of known compounds, which are two pairs of racemic mixtures of aromatic polyketides with a terminal butan-2,3-diol group. In the bioassay, the biological screening signifies no cytotoxic activities against several human cancer cell lines (HL-60, A549, SMMC-7721, MCF-7, and SW480) at a concentration of 40.0 μM.

[Key words] *Penicillium canescens*; Trichocomaceae; One strain-many compounds; Aromatic polyketides

1 Introduction

Fungal polyketides are a special family of natural products synthesized by polyketide synthases, many of which have diverse complicated structures and biological activities. Among them, the fungus *Penicillium canescens* Sopp O.J. (Trichocomaceae) was broadly distributed in natural resources and produced various bioactive secondary metabolites, such as aromatic polyketides, meroterpenoids, tetrapeptides, azaphilones, and alkaloids[1-8]. In our recent chemical investigations of *P. canescens* CGMCC 3.79658 (ATCC 10419), a series of novel dimeric polyketides featuring novel chemical architectures were discovered in a combination of "one strain-many compounds" (OSMAC) and MS/MS-based molecular networking strategies[6-8]. In addition, some dimeric and aromatic polyketides displayed a potential PTP1B inhibition effect and cytotoxicity against human cancer cell lines.

In our continuing research on bioactive polyketides, the fermentation of *P. canescens* CGMCC 3.79658 following the OSMAC method induced the isolation of two new aromatic polyketides and a known analog, methyl 2-acetyl-3,5-dihydroxyphenylacetate (compound **3**) (Fig. 1). Both compounds, (±)-penicanesins H (compound **1**) and I (compound **2**), are two pairs of phenylacetate enantiomers, comprising a phenylacetic acid core structure with a terminal butan-2,3-diol group.

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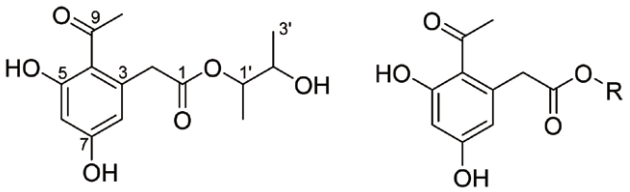

Fig. 1 Chemical structures of compounds 1–4.

Their structures and relative configurations are established by combining extensive spectroscopic data and comparing coupling cleavages with known compounds sharing the same butan-2,3-diol group. In this study, we report their isolation, structure elucidation, and cytotoxic activities.

2 Methods

2.1 General experimental procedures

Optical rotations (ORs) were determined with a Rudolph Autopol IV automatic polarimeter. The UV, IR, and electronic circle dichroism (ECD) spectra were obtained on a PerkinElmer LAMBDA 35 UV-vis spectrophotometer, a Bruker Vertex 70 instrument, and a JASCO-810 ECD spectrometer, respectively. NMR spectra were recorded on a Bruker Ascend^{TM} 400 MHz spectrometer, with ^{1}H and ^{13}C NMR chemical shifts referenced to the solvent or solvent impurity peaks for CD_3OD (δ_H 3.31 and δ_C 49.0). High-resolution electrospray ionization mass spectrometry (HRESIMS) data were acquired using a hybrid quadrupole time-of-flight (q-TOF) mass spectrometer (Model 6540, Agilent). The measurement temperature was 300 K. Semi-preparative HPLC utilized an Agilent 1200 quaternary system with a UV detector, using a reversed-phased C_{18} column (5 μm, 10 × 250 mm, Welch Ultimate XB-C_{18}) and chiral columns (5 μm, 4.6 × 250 mm, DAICEL CHIRALPAK IG and IC). Column chromatography (CC) was performed using silica gel (Qingdao Marine Chemical Inc., China), ODS (50 μm, Merck, Germany), and Sephadex LH-20 (Merck, Germany). Thin-layer chromatography was performed with silica gel 60 F_{254} (Yantai Chemical Industry Research Institute).

2.2 Fungal material

The fungus *P. canescens* Sopp O.J. (Trichocomaceae), strain CGMCC 3.795 8 (ATCC 10419), was bought from the China General Microbiological Culture Collection Center (CGMCC). An ITS-rDNA sequence amplified by conserved primer ITS1 and ITS4 showed 100% identity with *P. canescens* ATCC 10419 v1.0 by JGI BLAST (https://mycocosm.jgi.doe.gov/pages/blast-query.jsf?db=Penca1).

2.3 Fermentation and extraction

The fungal strain was maintained on a potato dextrose agar medium at 28 ºC for five days and was used as the seed culture. The fermentation was carried out on a solid rice medium at 25 ºC for 30 days in 250 Erlenmeyer flasks (1 L), with 200 g of rice and 200 mL of distilled water in each flask. After incubation, the moldy rice was extracted with ethanol at room temperature. The entire crude extract was suspended in H_2O and extracted with ethyl acetate (EtOAc) to yield 350.0 g of residue. Moreover, we also tried a liquid medium (10 g maltose, 10 g glucose, 5 g peptone, 10 g mannitol, 2.5 g yeast extract, 0.25 g K_2HPO_4, 0.25 g $FeSO_4 \cdot 7H_2O$, and 500 mL distilled water per 1 L Erlenmeyer flask) to culture this fungus under shaking for nine days (27 ºC, 160 rpm).

2.4 Isolation and purification

The EtOAc extract was subjected to a flash CC over silica gel with gradient elution using petroleum ether/EtOAc (50 : 1 to EtOAc neat, *v*/*v*) and EtOAc/MeOH gradient (10 : 1 to MeOH, *v*/*v*) to afford five fractions (Fr. 1–Fr. 5). The Fr. 3 was subjected to an RP-C18 gel column with gradient elution using MeOH/H_2O (20%–100%, *v*/*v*) to yield eight fractions (Fr. 3A–Fr. 3J). Fr. 3D was separated by a silica CC using CH_2Cl_2/MeOH (60 : 1, *v*/*v*) to obtain five subfractions (Fr. 3D-1–5). From Fr. 3D-1 and 3D-2,

compound **3** (11.0 mg) was purified using semi-preparative HPLC (42% MeOH in H_2O for 40 min; flow rate: 2.0 mL/min; compound **3**: t_R = 30.9 min). After CC using silica CC with CH_2Cl_2/MeOH (60 : 1, *v*/*v*) and Sephadex LH-20 with MeOH, Fr. 3E was divided into four subfractions (Fr. 3E-1–4). From Fr. 3E-3, compounds **1** (14.4 mg) and **2** (9.4 mg) were purified by semi-preparative HPLC using a DAICEL CHIRALPAK IC column (89% hexane in isopropyl for 70 min; flow rate: 3.0 mL/min; compound **1**: t_R = 43.8 min; compound **2**: t_R = 66.3 min).

2.4.1 Penicanesin H (compound 1)

Pale-yellow oil; UV (MeCN) λ_{max} nm (log ε): 220 (4.4), 269 (3.8), and 303 (3.7); IR (KBr) ν_{max} cm^{-1}: 3 427, 2 984, 2 929, 1 711, 1 614, 1 464, 1 269, 1 170, 1 094, and 849; ^1H (400 MHz) and ^{13}C (100 MHz) NMR data (CD_3OD), see Table 1; (+) ESI-MS: *m*/*z* 305.100 4 $[M + Na]^+$ (calcd. for $C_{14}H_{18}NaO_6$, 305.099 6).

Table 1 ^1H and ^{13}C NMR data of compounds 1 and 2 recorded in CD_3OD (δ in ppm; Mult. *J* in Hz)

No.	1		2	
	δ_H	δ_C	δ_H	δ_C
1		173.1		173.2
2	3.66 s	41.2	3.66 s	41.1
3		137.8		137.8
4		120.4		120.4
5		161.1		161.0
6	6.27 d 2.3	102.8	6.26 d 2.2	102.7
7		161.9		161.9
8	6.19 d 2.3	112.2	6.19 d 2.2	112.2
9		205.6		205.6
1′	4.73 dq 6.5, 4.5	75.9	4.75 p 6.3	75.8
2′	3.75 dq 6.5, 4.5	70.1	3.73 p 6.3	70.1
3′	1.12 d 6.5	18.6	1.12 d 6.3	18.6
4′	1.19 d 6.5	15.2	1.18 d 6.3	15.8
9-Me	2.51 s	32.6	2.50 s	32.5

2.4.2 Penicanesin I (compound 2)

Pale-yellow oil; UV (MeCN) λ_{max} nm (log ε): 219 (4.2), 270 (3.9), and 301 (3.8); IR (KBr) ν_{max} cm^{-1}: 3 423, 2 983, 2 930, 1 710, 1 677, 1 613, 1 463, 1 356, 1 271, 1 172, 1 102, 1 044, and 848; ^1H (400 MHz) and ^{13}C (100 MHz) NMR data (CD_3OD), see Table 1; (+) ESI-MS: *m*/*z* 305.099 4 $[M + Na]^+$ (calcd. for $C_{14}H_{18}NaO_6$, 305.099 6).

2.5 Cytotoxicity assay

Cytotoxicity was determined using an MTS assay. Colorimetric assays were performed to evaluate each compound's activity. HL-60 (human promyelocytic leukemia cells), A549 (human lung cancer cells), SMMC-7721 (human hepatocellular carcinoma cells), MCF-7 (human breast adenocarcinoma cells), and SW480 (human colon cancer cell) tumor cells were purchased from the National Collection of Authenticated Cell Cultures of China. All cells were cultured in an RPMI-1640 or DMEM medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone) at 37 ºC in a humidified atmosphere with 5% CO_2. Cell viability was assessed by conducting colorimetric measurements of the amount of insoluble formazan formed in living cells based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2*H*-tetrazolium (MTS) (Sigma, St. Louis, MO). Briefly, 100 μL of suspended adherent cells were seeded into each well of a 96-well cell culture plate and allowed to adhere for 12 h to 24 h before the addition of the drug. Suspended cells were seeded before the addition of the drug with an initial density of 1 × 10^5 cells/mL in 100 μL of the medium. Each tumor cell line was exposed to each test compound at various concentrations in triplicate for 48 h. After the incubation, MTS solution (20 μL) was added to each well, and the incubation was continued for 2 h to 4 h at 37 ºC. The optical density of the lysate was measured at 492 nm in a 96-well microtiter plate reader (MULTISKAN FC). The purity of all compounds used for the biological assays was over 98% as assessed from the NMR spectra and HPLC traces.

3 Results and discussion

compound **1** was obtained as a pale-yellow oil. The positive mode HRESIMS of **1** exhibited

an $[M + Na]^+$ ion peak at *m/z* 305.100 4 with a molecular formula of $C_{14}H_{18}NaO_6$ (six unsaturation equivalents). The absorption bands of the IR spectrum at 3 427, 1 711, 1 614, 1 464, 1 271, and 1 171 cm^{-1} indicated the existence of phenolic hydroxyl groups, carbonyl groups, and a benzene ring. Analyses of the ^1H NMR spectrum of compound 1 (Table 1) showed two metacoupled aromatic protons at δ_H 6.27 (d, J = 2.3 Hz, H-6) and δ_H 6.19 (d, J = 2.3 Hz, H-8) and three methyls with a singlet at δ_H 2.51 (Me-9) and two doublets at δ_H 1.19 (J = 6.5 Hz, Me-4′) and δ_H 1.12 (J = 6.5 Hz, Me-3′). The ^{13}C NMR data (Table 1) and HSQC spectra showed 14 carbons, including three methyls, one methylene, four methines containing two oxygenated at δ_C 75.9 (C-1′) and δ_H 70.1 (C-3′) and two olefinic carbons resonated at δ_C 102.8 (C-6) and δ_H 112.2 (C-8), six unprotonated carbons with two carbonyls (acetyl at δ_C 205.6 (C-9) and an ester group at δ_C 173.1 (C-1)). Key correlations from H-2 to C-3/C-4/C-8, H-6 to C-4/C-7/C-8, and H-8 to C-2/C-4/C-6/C-7 in the HMBC spectrum suggested the presence of a tetrasubstituted benzene ring (Fig. 2), where the acetyl group was attached to the benzyl ring at C-4, supported by an HMBC correlation from 9-Me to C-4. The above spectroscopic information and a key HMBC correlation from H-2 to C-1 (Fig. 2) corroborated a structure of compound **1** similar to known compounds, compound **3**[8] and 2-acetyl-3,5-dihydroxyphenylacetic acid (compound **4**)[9-10], with a phenylacetic acid core. The only difference between compounds **1** and **3** was the substructure linked to the phenylacetic acid core to form phenylacetate. Here, ^1H–^1H COSY and HMBC

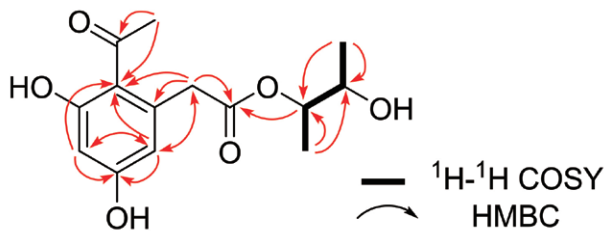

Fig. 2 ^{1}H–^{1}H COSY and key HMBC correlations of compounds 1 and 2.

spectra of compound **1** demonstrated the fragment of a butan-2,3-diol group CH_3–CH(OH)–CH(O)–CH_3, which was linked to the ester group at C-1 through the oxygen atom determined by a key correlation from H-1′ to C-1 (Fig. 2). Therefore, the planar structure of compound **1** was elaborated as mentioned above to be a (3-hydroxybutan-2-yl)-2-acetyl-3,5-dihydroxyphenyl acetate.

compound **2** was isolated, featuring a molecular formula of $C_{14}H_{18}NaO_6$ the same as that of compound **1**. Further spectroscopic analyses of 1D and 2D NMR spectra of compound **2** (Table 1) indicated that isolate compound **2** featured an identical planar structure to compound **1.** Therefore, the only difference between compounds **1** and **2** was the stereochemistry of the butan-2,3-diol group.

To determine their stereochemistry, we first tested their OR and ECD. However, the OR and ECD results showed that compounds **1** and **2** were both racemic mixtures, which means both compounds are four different stereoisomers. The separation of two pairs of enantiomers using several chiral columns was subsequently tried and failed, induced by tautomerization between those enantiomers. As a result, we merely determined their relative stereochemistry in this study. The ^1H NMR spectra of compounds **1** and **2** (Table 1) showed that the differences between them were coupling constants and cleavages of H-1′ and H-2′, where dq or p peaks belonged to H-1′ and H-2′ of compounds **1** or **2**, respectively. By comparing their coupling constants and cleavages with the reported compounds talaropyrazines B and C possessing the same butan-2,3-diol terminal group[11], the relative configurations of H-1′ and H-2′ in compounds **1** and **2** were determined to be *trans*- and *cis*-oriented, respectively. Finally, both compounds were constructed and designated as (±)-penicanesins H (compound **1**) and I (compound **2**).

As reported in our last study, some aromatic polyketides exhibited antitumor activity against human cancer cell lines[8]. Therefore, cytotoxicity was evaluated for all compounds against HL-60,

A549, SMMC-7721, MCF-7, and SW480 *in vitro* using an MTS assay, where all tested compounds showed no cytotoxicity at a 40 μM concentration.

4 Conclusion

In summary, two pairs of unreported stereoisomers of phenylacetate analogs, namely penicanesins H (compound **1**) and I (compound **2**), along with a known metabolite, were isolated from the culture broth of *P. canescens*. Their structures were established using detailed spectroscopic analysis and comparisons of coupling cleavages with known compounds. Investigation of the activity of the new products shows that there are no cytotoxic activities against human cancer cell lines at a 40 μM concentration.

5 Conflicts of interest

The authors have no conflicts of interest to declare.

6 Acknowledgments

We thank the Analytical and Testing Center at Huazhong University of Science and Technology for assistance in conducting the ECD, UV, and IR analyses. This work was supported by the Program for GDAS's project for science and technology development (No. 2021GDASYL-20210102009) and the National Natural Science Foundation for Young Scientists of China (No. 31800285).

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