

ORIGINAL ARTICLE

Two acid sorbicillin analogues from saline lands-derived fungus *Trichoderma* sp.

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Two new acid sorbicillin analogues, (*E*)-6-(2,4-dihydroxyl-5-methylphenyl)-6-oxo-2-hexenoic acid (1) and 6-(2,4-dihydroxyl-5-methylphenyl)-6-oxohexanoic acid (2), together with 12 known compounds, were isolated from a saline lands-derived fungus *Trichoderma* sp. The structures of the new compounds were established by interpretation of their spectroscopic data. Their cytotoxic effects on P388 and HL-60 cell lines were preliminarily evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) method. Furthermore, the new compounds exhibited weak radical scavenging activity against DPPH (1,1-diphenyl-2-picrylhydrazyl).

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INTRODUCTION

Fungi of the genus *Trichoderma* are ubiquitous in the environment. In recent years, more and more attention has been paid to them because of a wide range of prospective utility¹ as biological control agents to suppress different fungal plant pathogens and to promote plant growth and for producing commercial enzymes, such as cellulases, hemicellulases, proteases and β-1,3-glucanase.² At the same time, many structurally novel and biologically active secondary metabolites have been reported from the species. For example, peptibols,³ which are linear peptides characterized by an acetylated N-terminus, showed cytotoxic, membrane-perturbing and antibiotic activities. Trichocaranes⁴ A-D, isolated from *Trichoderma virens*, significantly inhibited the growth of etiolated wheat coleoptiles. In addition, a series of sorbicillin-related compounds^{5–7} from *Trichoderma* showed moderate radical scavenging activity.

In our search for cytotoxic compounds from fungi, a *Trichoderma* sp. was isolated from the saline lands soil collected along the coast of Bohai Bay in Zhanhua, Shandong Province, China. Its extract showed moderate activity to brine shrimp. The isolation work led to the finding of two new compounds (*E*)-6-(2,4-dihydroxyl-5-methylphenyl)-6-oxo-2-hexenoic acid (1) and 6-(2,4-dihydroxyl-5-methylphenyl)-6-oxohexanoic acid (2), along with 12 known ones, which were trichodimerol, bisvertinolone, adenosine, ergosterol, ergosterol peroxide, cerevisterol, 3β,5α-dihydroxy-6β-methoxyergosta-7,22-diene, (22*E*,24*R*)-5α,6α-epoxy-ergosta-8,22-diene-3β,7α-diol, cyclo-(L-prolyl-L-valine), cyclo-(L-leucyl-L-proline), cyclo-(L-isoleucyl-L-proline) and cyclo-(L-phenylalanyl-L-proline), respectively. The known compounds were identified by comparison with previously reported physical and spectral data. In this paper, we reported the new compounds' isolation, structure elucidation, cytotoxic activities to P388 and HL-60 cell lines and radical scavenging activity against DPPH (1,1-diphenyl-2-picrylhydrazyl) (Figure 1).

RESULTS AND DISCUSSION

Structure determination

Compound 1 was isolated as colorless needles. Its molecular formula was determined as C₁₃H₁₄O₅ by high resolution electrospray ionization mass spectra. Its IR spectrum showed a broad absorption band for hydroxyl (about 3000 cm⁻¹), characteristic absorptions for aromatic ring (1604, 1538 and 1512 cm⁻¹) and carbonyl (1668 and 1628 cm⁻¹). The UV spectrum at 327 (3.89), 279 (4.12), 233 (4.02) and 211 (4.39) nm, displayed typical absorptions of benzene ring connected with chromophore and auxochrome.

Analysis of the ¹H NMR data in conjunction with the HSQC spectrum disclosed the presence of one methyl, two aliphatic methylenes, two aromatic and two olefinic methines. The ¹³C NMR spectrum further displayed the presence of one carboxyl, one ketone carbonyl and four aromatic quaternary carbons, two of which should be oxygenated in view of their chemical shifts (δ_{C} 164.63 and 164.58). The above evidence revealed the presence of a benzene ring with four substituents in 1, and two aromatic proton singlets strongly suggested that the two aromatic methine were in para positions. The upfield shift of the carboxyl carbon (δ_{C} 170.1) indicated conjugation with the double bond. In the HMBC spectrum, the correlations of H-12 with C-13 and C-6, and H-5 with C-12 demonstrated proximity of the methyl and the ketone carbonyl with the ortho aromatic carbon, C-12. The triplet of one aliphatic methylene proton signal (δ_{H} 3.10) and the quartet of another (δ_{H} 2.58) demonstrated the former linked with the carbonyl and the latter with an olefinic methine (δ_{H} 6.98), which was observed a multiplet. The *E*-geometry of the double bond was assigned on the basis of the coupling constant (14.9 Hz). The whole structure of 1 was confirmed by the HMBC spectrum (Figure 2).

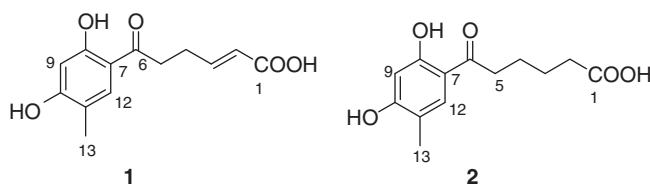
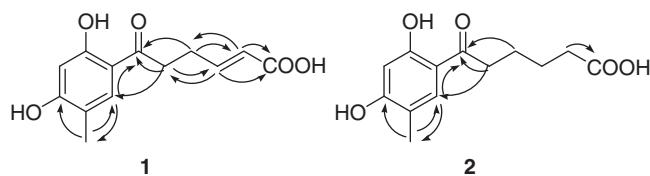
Compound 2 was isolated as colorless needles. The high resolution electrospray ionization mass spectra indicated a molecular formula of

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**Figure 1** Structures of **1** and **2**.**Figure 2** Key HMBC correlations for **1** and **2**.

$C_{13}H_{16}O_5$, which is 2 amu larger than **1**; that is, a difference of a H_2 in the molecular formula. Its IR and UV spectra were almost the same as **1**. Detailed analysis and comparison of the 1D NMR data, with those of **1**, revealed the appearance of two additional aliphatic methylenes and the disappearance of two olefinic methines, which demonstrated that **2** is the hydrogenated derivative of **1**. In addition, the disappearance of the double bond moved the chemical shift of the carboxyl downfield in **2**, which unambiguously confirmed the above conclusion. Finally, the HMBC data strongly supported the structure of **2**.

Biological activities

The cytotoxic effects of compounds **1** and **2** were preliminarily evaluated against P388 and HL-60 cell lines by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) method. The IC_{50} values of **1** and **2** against P388 and HL-60 cells were 72.8 and 44.5, and 52.5 and 81.2 μM , respectively (fluorouracil as positive control, IC_{50} 3.8 and 18.6 μM against P388 and HL-60 cells, respectively). The results showed that they were slightly active against the two cell lines, and the double bond had little effect on the activity.

In addition, compounds **1** and **2** showed weak radical scavenging activity against DPPH with IC_{50} values of 175 and 142 μM , respectively (ascorbic acid as positive control, IC_{50} 21 μM).

These two new compounds belong to 'sorbicillinoids' in view of structure and likely biogenesis. 'Sorbicillinoids' and 'bisobricillinoids' comprise a group of more than 30 monomeric and dimeric sorbicillin-related compounds,⁸ which exhibit a wide range of biological activities. To our best knowledge, this is the first time that acid analogues of 'sorbicillinoids' are reported. This discovery enriches the range of this kind of compound and provides some materials for studies of structure–activity relationships.

METHODS

General

Melting points were measured on an XRC-1 micro-melting point apparatus (Sichuan University Scientific Instrument Factory, Chengdu, China) and were uncorrected. IR spectra were taken on a Nicolet 6700 spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA) using attenuated total reflection method. UV spectra were recorded on Beckman DU 640 spectrophotometer (Global Medical Instrumentation, Inc., Ramsey, MN, USA). ESI-MS was measured on a Q-TOF Ultima GLOBAL GAA076 LC mass spectrometer (Waters Corporation, Milford, MA, USA). ^1H , ^{13}C NMR, DEPT, and 2D-NMR were recorded on a Bruker Avance 400 spectrometer (Bruker BioSpin AG,

Table 1 ^1H and ^{13}C NMR data for **1** and **2** (MeOH- d_4 , 400 and 100 MHz, TMS, δ in p.p.m.)

No.	1		2	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		170.7 s		177.8 s
2	5.88 (1H, d, 14.9)	123.9 d	2.34 (2H, t, 6.5)	35.1 t
3	6.98 (1H, m)	148.9 d	1.72 (2H, m)	25.5 t
4	2.58 (2H, q, 6.9)	27.7 t	1.72 (2H, m)	25.9 t
5	3.10 (2H, t, 6.9)	36.9 t	2.94 (2H, t, 6.9)	38.3 t
6		204.2 s		206.0 s
7		118.3 s		118.2 s
8		164.6 s		164.7 s
9	6.25 (1H, s)	102.9 d	6.24 (1H, s)	102.9 d
10		164.6 s		164.5 s
11		113.5 s		113.6 s
12	7.57 (1H, s)	133.2 d	7.58 (1H, s)	133.4 d
13	2.11 (3H, s)	15.5 q	2.12 (3H, s)	15.5 q

Fällanden, Switzerland) using tetramethylsilane (TMS) as internal standard. Semipreparative HPLC was performed on a SHIMADZU LC-6AD Liquid Chromatograph (SHIMADZU Corporation, Kyoto, Japan) with SPD-20A Detector (SHIMADZU Corporation), using an ODS column (HyperClone 5 μ ODS (C_{18}) 120A, 10 \times 250 mm, Phenomenex, Torrance, CA, USA).

Fungal material

The working strain, *Trichoderma* sp JH8 (GenBank accession numbers: HQ717798), was isolated from the soil of saline lands collected along the coast of Bohai Bay in Zhanhua, Shandong Province, China. It was preserved in our laboratory. Working stocks were prepared on potato dextrose agar slants stored at 4 °C.

Fermentation, extraction and isolation

Spores growing on petri dishes of potato dextrose agar were inoculated into 500-ml Erlenmeyer flasks containing 180 ml sea water-based culture medium (potato 200 g, glucose 20 g, mannitol 10 g, maltose 10 g, yeast extract 3 g, KH_2PO_4 0.5 g, $MgSO_4 \cdot 7H_2O$ 0.3 g, dissolved in 1 l seawater), and cultured at 28 °C for 8 days on a rotary shaker at 170 r.p.m.

The culture broth (50 l) was filtered through cheesecloth to separate the mycelia. The filtrate was extracted three times with ethyl acetate, whereas the mycelia were extracted three times with methanol. The methanol solution was evaporated under reduced pressure to afford an aqueous solution, which was extracted three times with ethyl acetate. The ethyl acetate extracts were combined and concentrated *in vacuo* to give a crude extract (56 g).

The crude extract was subjected to silica gel (200–300 mesh) column chromatography, eluting with a step gradient of petroleum ether–chloroform and chloroform–methanol mixtures. The fraction that was obtained with chloroform–methanol (40:1) was rechromatographed on a column of reverse phase silica gel (RP18, 25–40 μm , Merck KGaA, Darmstadt, Germany) with an $H_2O/MeOH$ gradient. The subfraction obtained from $H_2O/MeOH$ (8:1) was further separated by semipreparative HPLC on an ODS column (Phenomenex), using methanol–water (2:3) as eluting solvent to yield compounds **1** (25 mg) and **2** (36 mg).

Biological assay

The cell lines were grown in RPMI-1640 supplemented with 10% fetal bovine serum under a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Cell suspensions, 200 μl , at a density of 5×10^4 cell ml^{-1} were plated in 96-well microtiter plates and incubated for 24 h under the above conditions. Then 2 μl portions of the test compound solutions at different concentrations in MeOH were added to each well, and incubation continued for 72 h under the same conditions. Finally, 20 μl of MTT solution (5.0 mg ml^{-1}) in RPMI-1640

medium) was added to each well and incubation continued for 4 h. Then, the medium containing MTT, 150 µl, was removed from each well and gently replaced by dimethyl sulfoxide to dissolve any formazan crystals that formed. Absorbance was then determined on SPECTRAMAX PLUS plate reader (Molecular Devices Corporation, Sunnyvale, CA, USA) at 540 nm. The IC₅₀ values were obtained using the Bliss method.

In the DPPH scavenging assay, the samples to be tested were dissolved in methanol and the solution (160 µl) was dispensed into wells of a 96-well microtiter tray. DPPH solution in methanol (3.0×10^{-4} mol l⁻¹), 40 µl, was added to each well. After thorough mixing, the mixture was kept in the dark for 30 min. Thereafter, the absorbance was measured at 517 nm using methanol as the blank reference, and the percent inhibition was calculated. IC₅₀ values denote the concentration of sample required to scavenge 50% of the DPPH free radicals.

Physico-chemical properties

Compound 1: colorless needle (MeOH); mp 220~222 °C; high resolution electrospray ionization mass spectra m/z 249.0767 for [M-H]⁻ (calcd for C₁₃H₁₃O₅, 249.0763); IR (attenuated total reflection) ν_{max} cm⁻¹ 3500~2800, 1668, 1628, 1604, 1539, 1512, 1464, 1381, 1267, 1227, 1183, 1142, 1003, 966, 882, 858, 842, 794, 767, 747, 711; UV-vis (MeOH) λ_{max} nm (log ε) 327 (3.89), 279 (4.12), 233 (4.02), 211 (4.39); ¹H and ¹³C NMR see Table 1.

Compound 2: colorless needle (MeOH); mp 210~212 °C; high resolution electrospray ionization mass spectra m/z 251.0908 for [M-H]⁻ (calcd for C₁₃H₁₅O₅, 251.0919); IR (attenuated total reflection) ν_{max} cm⁻¹ 3390~2800, 1633, 1591, 1513, 1462, 1416, 1378, 1316, 1278, 1204, 1183, 1151, 1004, 973, 931, 919, 887, 844, 813, 744, 690; UV-vis (MeOH) λ_{max} nm (log ε) 326 (3.82), 278 (4.05), 234 (3.94), 213 (4.16); ¹H and ¹³C NMR see Table 1.

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