New C_{13} lipids from the marine-derived fungus *Trichoderma harzianum*

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(Received 26 December 2014; final version received 14 April 2015)

This paper is dedicated to the 80th birthday of Prof. Xin-Sheng Yao for his contribution to natural product chemistry.

Chemical examination of the fermentation broth of a sponge-associated fungus *Trichoderma harzinum* HMS-15-3 led to the isolation of four pairs of new C_{13} lipid enantiomers namely harzianumols A–H (1a–4b). Their structures were elucidated on the basis of extensive spectroscopic (IR, MS, 1D, and 2D NMR) data analysis, including the modified Mosher’s method for the assignment of their absolute configurations. The new compounds were evaluated for antihyperlipidemic effects in HepG2 cells.

**Keywords:** *Trichoderma harzianum*; harzianumols A–H; structural elucidation

1. Introduction

Fungal secondary metabolites are a rich source for mining new bioactive molecules with potential pharmaceutical applications, while some fungus-derived metabolites have been developed as antibiotics, antiviral, antitumor, and cholesterol-lowering drugs [1]. Polyketides encompass a large group of structurally diverse fungal natural products, while polyketide-based compounds are reported to be potential leads used for molecular probes, new drug candidates, in addition to the ecological functions such as toxins and virulence factors of pathogens [2,3]. During the past decades, a number of polyketide-derived drugs or lead compounds have been found from fungi, such as the cholesterol-lowering lovastatin [4], the immunosuppressive mycophenolic acid [5], the antifungal griseofulvin [6], and the histone deacetylase (HDAC) inhibitor depudecin [7,8].

In the course of our search for new bioactive secondary metabolites from marine-derived microorganisms, a fungus *Trichoderma harzianum* HNS-15-3 isolated from the sponge *Petrospongia nigra* HNS-15 was undertaken for cultivation. Screening the functional gene clusters using bioinformatics tools including antiSMASH [1,9] revealed the fungus *T. harzianum* possessing many synthetic genes related to PKS (polyketide synthase) biosynthetic pathways. The $^1$H NMR spectrum of the EtOAc extract of the cultured material featured the resonances for unsaturated lipids.

2. Results and discussion

Chromatographic separation of the EtOAc extract of the solid fermentation material
of *T. harzianum* HNS-15-3 resulted in the isolation of four HPLC (C18) pure components (1–4) (Figure 1). Each component displayed a pair of inseparable shoulder peaks in chiral column HPLC [10] and the duplicated NMR data, suggesting the presence of enantiomeric mixture.

The enantiomeric mixture of harzianumols A (1a) and B (1b) has the molecular formula of C13H22O2 based on the HR-ESI-MS data (m/z 233.1509 [M + Na]+), containing three degrees of unsaturation. The 13C NMR spectrum (Table 1) exhibited a total of 13 carbon resonances, including 6 olefinic carbons for 3 double bonds, 2 hydroxymethine, 3 methylene, and 2 methyl carbons. These functionalities accounted for all degrees of unsaturation in the molecule, indicating an acyclic lipid. The COSY correlations established a linear spin system from C-1 to C-13, while the correlations from the olefinic proton H-2 (δH 5.40, dq, J = 15.6, 5.8 Hz) to the methyl protons H3-1 (δH 1.61, d, J = 5.8 Hz) and from the olefinic proton H-12 (δH 5.55, dq, J = 15.3, 6.7 Hz) to the methyl protons H3-13 (δH 1.70, d, J = 6.7 Hz) indicated the terminals C-1 (δC 18.2) and C-13 (δC 18.2) to be ended by methyl groups. The olefinic couplings from H-10 (δH 5.99, dd, J = 14.8, 10.2 Hz) to H-9 (δH 5.61, dt, J = 14.8, 6.8 Hz) and H-11 (δH 6.00, dd, J = 15.3, 10.2 Hz) and between H-11 and H-12 concluded the conjugated double bonds to be resided at C-9 (δC 130.1)/C-10 (δC 131.8) and C-11 (δC 132.3)/C-12 (δC 126.6), while the remaining olefinic bond was located at C-2 (δC 124.4)/C-3 (δC 132.2). The COSY and HMBC correlations also assigned two hydroxyl groups positioned at C-6 (δC 73.3) and C-7 (δC 74.4), as evident from the COSY relationships from H-6 (δH 3.17–3.14, m) to H-7 (δH 3.21–3.17, m) and H 2-5 (δH 1.27–1.23, 1.58–1.53) and from H-7 to H2-8 (δH 2.05–2.00, 2.30–2.25), in association with the HMBC interactions from H-7 to C-5 (δC 33.0), C-6, C-8 (δC 36.7), and C-9 (Figure 2). The JH-2/H-3 (15.6 Hz), JH-9/H-10 (14.8 Hz), and JH-11/H-12 (15.3 Hz) values confirmed E geometry of the double bonds. Based on the modified Mosher’s method which is used for the assignment of the absolute configurations of vicinal diols [10,11], the (R)- and (S)-MPA diesters of 1 were prepared (Figure 3) [11], respectively. The (R)-MPA diester of 1 displayed two peaks with a ratio of 2:1 in the HPLC using chiral column (OD-H), which were able to be separated to afford (R)-MPA diesters of 1a and 1b. Subsequently, two peaks of (S)-MPA diester of 1 were also purified by the same protocol to yield (S)-MPA diesters of 1a and 1b. Calculation of the ΔδRS values

![Figure 1. Structures of compounds 1a–4b.](image-url)
| Position | δH  | δC  | δH  | δC  | δH  | δC  | δH  | δC  |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|
| 1        | 1.61 (d, 5.8) | 18.2 | 1.61 (d, 5.2) | 18.1 | 1.61 (d, 5.6) | 18.2 | 3.86 (dd, 5.8, 5.5) | 62.0 |
| 2        | 5.40 (dq, 15.6, 5.8) | 124.4 | 5.39 (dq, 16.0, 5.2) | 124.7 | 5.40 (dq, 16.0, 7.0) | 124.4 | 5.54 (dt, 16.0, 5.8) | 130.7 |
| 3        | 5.41 (dt, 15.6, 7.0) | 132.2 | 5.41 (dt, 16.0, 7.0) | 131.7 | 5.42 (dt, 16.0, 6.5) | 132.2 | 5.56 (dt, 16.0, 7.0) | 130.7 |
| 4        | 2.12–2.08 (m); 1.94–1.90 (m) | 28.8 | 2.09–2.04 (m); 1.94–1.90 (m) | 28.5 | 2.12–1.98 (m); 1.95–1.90 (m) | 28.9 | 2.10 (m); 1.98 (m) | 28.8 |
| 5        | 1.58–1.53 (m); 1.27–1.23 (m) | 33.0 | 1.45–1.40 (m); 1.37–1.33 (m) | 33.0 | 1.57–1.52 (m); 1.28–1.24 (m) | 33.2 | 1.48 (m); 1.35 (m) | 32.3 |
| 6        | 3.17–3.14 (m) | 73.3 | 3.54–3.49 (m) | 71.8 | 3.23–3.19 (m) | 73.6 | 3.26–3.21 (m) | 72.9 |
| 7        | 3.21–3.17 (m) | 74.4 | 3.74 (t, 5.7) | 80.5 | 3.19–3.14 (m) | 73.6 | 3.25–3.20 (m) | 72.9 |
| 8        | 2.30–2.25 (m); 2.05–2.00 (m) | 36.7 |                  |      |                  |      |                  |      |
| 9        | 5.61 (dt, 14.8, 6.8) | 130.1 | 2.66–2.61 (m); 2.52–2.48 (m) | 38.9 | 2.23–2.19 (m); 2.07–2.03 (m) | 28.9 |                  |      |
| 10       | 5.99 (dd, 14.8, 10.2) | 131.8 | 2.14–2.08 (m) | 26.1 | 5.76 (dt, 15.2, 7.0) | 136.2 | 5.75 (dt, 15.6, 7.0) | 136.0 |
| 11       | 6.00 (dd, 15.3, 10.2) | 132.3 | 5.39 (dt, 16.0, 7.0) | 130.8 | 6.06 (dd, 15.2, 10.5) | 131.0 | 6.05 (dd, 15.6, 10.2) | 131.1 |
| 12       | 5.55 (dt, 15.3, 6.7) | 126.6 | 5.41 (dq, 16.0, 5.2) | 125.1 | 6.31 (ddd, 17.0, 10.5, 10.2) | 137.8 | 6.30 (ddd, 17.0, 10.2, 10.0) | 137.7 |
| 13       | 1.70 (d, 6.7) | 18.2 | 1.60 (d, 6.5) | 18.1 | 5.09 (brd, 17.0); 4.95 (brd, 10.2) | 115.2 | 5.08 (brd, 17.0); 4.95 (brd, 10.0) | 115.3 |
| OH-1     |                  |      |                  |      |                  |      |                  |      |
| OH-6     | 4.34 (d, 5.0) | 4.71 (d, 5.0) | 4.34 (d, 5.0) | 4.27 (d, 5.0) |
| OH-7     | 4.31 (d, 5.0) | 5.36 (d, 5.7) | 4.40 (d, 5.0) | 4.25 (d, 5.0) |
(Table 2) resulted in 6R and 7S configurations for 1a, whereas 1b was assigned to 6S and 7R configurations.

The enantiomeric mixture of harzianumols C (2a) and D (2b) has a molecular formula of C_{13}H_{22}O_{3} as determined by the HR-ESI-MS data (249.1456 [M + Na]^+) bearing three degrees of unsaturation. The COSY correlations established two spin systems, of which the moiety from C-1 to C-7 was identical to that of 1. The second spin system afforded a linear chain from C-9 to C-13, in which a double bond was resided at C-11 (δC 130.8) and C-12 (δC 125.1) according to the terminal methyl protons H3-13 (δH 1.60, d, J = 6.5 Hz) correlated to C-11 and C-12 in the HMBC spectrum. In addition, a carbonyl carbon was located at C-8 based on the HMBC interactions from C-8 (δC 212.4) to H-6 (δH 3.54–3.49), H-7 (δH 3.74, t, J = 5.7 Hz), H2-9 (δH 2.52–2.48, 2.66–2.61), and H2-10 (δH 2.14–2.08). The JH-2/H-3 (16.0 Hz) and JH-11/H-12 (16.0 Hz) values of olefinic protons were characteristic of E geometries of the double bonds C-2 (δC 124.7)/C-3 (δC 131.7) and C-11/C-12. Following the same protocol

| Protons | 1a | 1b | 2a | 2b | 3a | 3b | 4a | 4b |
|---------|----|----|----|----|----|----|----|----|
| H-5     | +0.31 | -0.31 | +0.23 | -0.23 | +0.36 | -0.36 | +0.08 | -0.08 |
| H-6     | +0.14 | -0.14 | +0.17 | -0.17 | +0.39 | -0.39 | +0.13 | -0.13 |
| H-7     | -0.25 | +0.25 | -0.29 | +0.29 | -0.30 | +0.30 | +0.13 | -0.13 |
| H-8     | -0.18 | +0.18 | -0.21 | +0.22 | -0.36 | +0.36 | +0.29 | -0.29 |
as for 1a and 1b, (R)- and (S)-MPA diesters of 2 were achieved. On the basis of HPLC separation using chiral column, the (R)-diesters of 2a and 2b in addition to the (S)-MPA diesters of 2a and 2b were purified in a ratio of 3:1. Calculation of the Δδ^{RS} values (Table 2) resulted in 6R and 7R configurations of 2a as well as 6S and 7S configurations of 2b. The enantiomeric mixture of harzianumol E and F (3a and 3b) has the same molecular formula as that of 1, as provided by the HR-ESI-MS data (m/z 233.1507 [M + Na]^+). Interpretation of COSY, HMQC, and HMBC data (Figure 2) revealed the structure of 3 closely related to that of 1, except for the conjugated double bonds which were migrated to the terminal at C-10 (δ_C 136.2)/C-11 (δ_C 131.0) and C-12 (δ_C 137.8)/C-13 (δ_C 115.2) on the basis of the terminal olefinic methylene H_2-13 (δ_H 5.09, brd, J = 17.0 Hz; 4.95, brd, J = 10.2 Hz) correlated to H-12 (δ_H 6.31, ddd, J = 17.0, 10.5, 10.2 Hz) and the correlations from the olefinic proton H-11 (δ_H 6.06, dd, J = 15.2, 10.5 Hz) to H-10 (δ_H 5.76, dt, J = 15.2, 7.0 Hz) and H-12. Based on the J values of the olefinic protons, the geometries of the double bonds were assigned to be E form. As mentioned in 1 and 2, the (R)- and (S)-MPA esterification of 3 generated the derivatives including (R)-diesters of 3a and 3b and (S)-MPA diesters of 3a and 3b, which were separated by HPLC using chiral column. The configurations of C-6 and C-7 in 3a and 3b were the same as those of 1a and 1b, respectively, based on the Δδ^{RS} values (Table 2) of 3a and 3b using the Mosher method.

The enantiomeric mixture of harzianumols G and H (4a and 4b) was determined to be 1-hydroxylated analogs of 3a and 3b, on the basis of the closely similar NMR data with the exception of C-1 of 4 to be a hydroxymethylene (δ_H 3.86, δ_C 62.0) instead of methyl resonances of 3. The molecular formula of 4 (C_{13}H_{22}O_{3}) exhibiting an additional oxygen atom in comparison with that of 3 further supported the structural assignment. The enantiomeric mixture of 4 was recognized by the chiral HPLC spectrum. Following the same protocol as 3, the (R)-diesters of 4a and 4b and (S)-MPA diesters of 4a and 4b were purified. However, calculation of the Δδ^{RS} values (Table 2) revealed R configuration for C-6 and C-7 of 4a, and S configuration for C-6 and C-7 of 4b. These lipids were tested for antihyperlipidemic effects in HepG2 cells, whereas none of them showed significant lipid-lowering activity. The C_{13}-polyketide metabolites are rarely found from nature. Oshima and coworkers first reported a number of C_{13} polyketides from a fungus Chaetomium mollipilium through the epigenetic regulation approach to inhibit NAD^{+}-dependent HDAC activity by the inhibitor nicotinamide [12]. This finding implied that C_{13} polyketides and relative polyketides are a group of unexplored metabolites embedded in fungi. Uncovering structurally unprecedented metabolites by genomics guidance and by stimulation of silent biosynthetic gene clusters may provide additional approaches to discover chemical diversity.

3. Experimental

3.1 General experimental procedures

IR spectra were determined on a Thermo Nicolet Nexus 470 FT-IR spectrometer (Thermo Fisher Scientific Inc., New York, NY, USA).[^1]H, [^13]C, and 2D NMR spectra were recorded on a Bruker Advance 400 NMR spectrometer (400 MHz for [^1]H and 100 MHz for [^13]C, respectively) (Bruker Corporation, Zurich, Switzerland). Chemical shifts are expressed in δ (ppm) referenced to the solvent peaks at δ_H 2.50 and δ_C 39.8 for DMSO-d_6, and δ_H 7.28 and δ_C 77.0 for CDCl_3, respectively, and coupling constants are in Hz. ESI-MS and HR-ESI-MS spectra were obtained from a Thermo Scientific LTQ Orbitrap.
XL instrument (Thermo Fisher Scientific Inc., New York, NY, USA). Silica gel (160–200 and 200–300 mesh, Qingdao Marine Chemistry Co., Ltd, Qingdao, China) and ODS (50 μm, YMC Co., Ltd, Kyoto, Japan) were used for column chromatography. Precoated silica gel plates (Kieselgel 60 F254, 0.25 mm, Merck, Darmstadt, Germany) were used for TLC analysis. HPLC chromatography was performed on an Alltech instrument (426-HPLC pump) equipped with an Alltech uvis-200 detector (Alltech Inc., Chicago, IL, USA) at 210 nm and semi-preparative reversed-phased columns (YMC-packed C18, 5 μm, 250 mm × 10 mm).

### 3.2 Fungal material

Fungus *T. harzinum* HNS-15-3 was isolated from the sponge *P. nigra* HNS-15 collected from South China Sea. The species was identified by Prof. Tian Li from the Qingdao Chemical and Technology University using morphological method and analysis of the ITS region of the rDNA, whose sequence data have been deposited at GenBank with the accession number KJ716469. The voucher specimen is deposited in State Key Laboratory of Natural and Biomimetic Drugs of China at −80°C. The producing strain was prepared on potato dextrose agar (PDA) slants and stored at 4°C.

### 3.3 Fermentation and extraction

The fungal strain was cultured on slants of PDA at 25°C for 7 days. Fermentation was carried out in fifty 500 ml Erlenmeyer flasks each containing 100 g of rice for 30 days at 25°C. The fermentation broth was extracted three times with EtOAc, and then was concentrated under reduced pressure to give an extract (23.2 g).

### 3.4 Purification

The EtOAc extract (23.0 g) was subjected to silica gel vacuum liquid chromatography, eluting with petroleum ether (PE)–EtOAc (from 50:1 to 1:1 gradient) to obtain eight fractions (F1–F8). F5 (3.2 g, PE–EtOAc 1:1) was chromatographed over a Sephadex LH-20 column, using MeOH–CH2Cl2 (1:1, v/v) as a mobile phase, to afford seven portions (P1–P7). P4 (650 mg) was separated by semipreparative HPLC eluting with MeCN–H2O (58:42, flow rate: 2 ml/min, detection: UV 210 nm) to afford 1 (18.4 mg, Rt 26.3 min), 2 (16.2 mg, Rt 25.8 min), and 3 (21.6 mg, Rt 26.7 min), while 4 (4.8 mg, Rt 26.9 min) was prepared by the same protocol as for 1 using MeCN–H2O (47:53) from P3 (270 mg).

#### 3.4.1 Compound 1

Colorless oil; IR (KBr) νmax 3288, 1447, 1212, 1049 cm⁻¹; 1H and 13C NMR spectral data, see Table 1; HR-ESI-MS m/z 233.1509 [M + Na][+] (calcd for C13H22O2Na, 233.1512).

#### 3.4.2 Compound 2

Colorless oil; IR (KBr) νmax 3329, 1708, 1449, 1376, 1238, 1203 cm⁻¹; 1H and 13C NMR spectral data, see Table 1; HR-ESI-MS m/z 249.1456 [M + Na][+] (calcd for C13H22O3Na, 249.1461).

#### 3.4.3 Compound 3

Colorless oil; IR (KBr) νmax 3312, 2939, 2156, 1747, 1678, 1509, 1441, 1376, 1258 cm⁻¹; 1H and 13C NMR spectral data, see Table 1; HR-ESI-MS m/z 233.1507 [M + Na][+] (calcd for C13H22O2Na, 233.1512).

#### 3.4.4 Compound 4

Colorless oil; IR (KBr) νmax 3345, 2918, 2157, 1745, 1677, 1434, 1376, 1267 cm⁻¹; 1H and 13C NMR spectral data, see Table 1; HR-ESI-MS m/z 249.1459 [M + Na][+] (calcd for C13H22O3Na, 249.1461).
3.5 Mosher’s reaction

The enantiomeric mixture of 1a and 1b (0.01 mmol), 4-dimethylaminopyridine, cat., and dicyclohexylcarbodiimide, 0.01 mmol were dissolved in methylene dichloride (0.5 ml), and then (R)- or (S)-MPA (0.01 mmol) was added to the solution [10,11]. After stirring at room temperature for 24 h, the mixture was evaporated under reduced pressure to obtain a residue, which was separated using a reversed phase semipreparative HPLC (OD-H) with 83% CH3OH–H2O as mobile phase to yield (R)-MPA esters of 1a and 1b, and (S)-MPA esters of 1a and 1b. Following the same protocol as for compounds 1a and 1b, (R)- and (S)-MPA esters of compounds 2a/2b, 3a/3b, and 4a/4b were prepared.

3.6 Cell-based lipid accumulation assay

HepG2 cells were maintained in DMEM (Dulbecco’s modification of Eagle’s medium) supplemented with 10% fetal bovine serum and penicillin/streptomycin (100 μg/ml). The cells with 70–80% confluence were incubated in DMEM plus oleic acid (100 μM) for 12 h, then were treated with the compounds (each, 10 μM), while the positive control lovastatin in DMEM/oleic acid (100 μM) was used as a blank for an additional 6 h. Subsequently, the cells were subjected to Oil Red O staining or total cholesterol (TC) and triglyceride (TG) determination as described previously [13]. Each experiment (n = 8 for Oil Red O staining or n = 3 for TC and TG determination) was repeated in triplicate.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was financially supported by grants from the National Basic Research Program 973 [grant number 2015CB755900]; NSFC-Shangdong Joint Fund for Marine Science [grant number U1406402]; COMRA [grant number DY125-15-T-01]; and Sino-German Project [GZ816].

Note

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