

ORIGINAL ARTICLE

Two new amides from a halotolerant fungus, *Myrothecium* sp. GS-17

Tao Liu¹, Songya Zhang², Jing Zhu³, Huaqi Pan⁴, Jiao Bai², Zhanlin Li², Liping Guan², Guyue Liu⁵, Chunmao Yuan², Xin Wu⁵ and Huiming Hua²

Two new amides, named *N*-acetyl-2,4,10,17-tetrahydroxyheptadecylamine (1) and *N*-acetyl-3,5,11,18-tetrahydroxyoctadecyl-2-amine (2), were isolated from a halotolerant fungus, *Myrothecium* sp. GS-17. Their structures were identified on the basis of spectroscopic characteristics. The cancer cell cytotoxicities of two compounds were evaluated, and compound 2 exhibited weak cytotoxicity in HL-60 cell line.

The Journal of Antibiotics (2015) 68, 267–270; doi:10.1038/ja.2014.136; published online 1 October 2014

INTRODUCTION

Recent investigations of cultured extreme environmental microbes revealed numerous bioactive natural products.^{1,2} Diverse secondary metabolites have been isolated from various cultured extreme-tolerant microbes, extremophiles and deep-sea microbes, which exhibited interesting biological activities, such as antitumor, antifungal and antibacterial activity.^{3–6}

In the course of our ongoing studies of special environmental microorganisms as sources of new bioactive secondary metabolites, a halotolerant fungus *Myrothecium* sp. GS-17 was isolated from the soil sample of a salina at Gansu province in China. In the preliminary screening of the extracts from liquid fermentation cultures for *in vitro* activity, the fungal GS-17 was found to produce metabolites with strong growth inhibition activity against human leukemia (HL-60) cell line. Chemical investigation of the extract led to the isolation of two new amide derivatives, *N*-acetyl-2,4,10,17-tetrahydroxy-heptadecylamine (1) and *N*-acetyl-3,5,11,18-tetrahydroxyoctadecyl-2-amine (2). By means of spectroscopic analysis (UV, IR, NMR and MS) and physico-chemical properties, their chemical structures were determined respectively. Herein, we describe the isolation, structure elucidation and biological activities of the two compounds.

RESULTS

The extract of fermented broth of *Myrothecium* sp. GS-17 was fractionated by repeated column chromatography to yield compounds 1 and 2 (Figure 1). Their structures were elucidated on the basis of spectroscopic methods.

Physicochemical properties

N-acetyl-2,4,10,17-tetrahydroxyheptadecylamine (1). White amorphous powder; mp 143 °C, $[\alpha]_{\text{D}}^{20} = -11.5$ (*c* 0.13, MeOH); UV (MeOH) λ_{max}

(log ϵ) 209 nm (1.67); IR (KBr) ν_{max} 3290, 2922, 2849, 1623 cm^{-1} ; ESI-MS m/z 384.3 $[\text{M}+\text{Na}]^+$, HRESI-MS m/z 384.2730 $[\text{M}+\text{Na}]^+$ (calcd. for $\text{C}_{19}\text{H}_{39}\text{NaNO}_5^+$, 384.2720); EI-MS m/z 228, 146, 128, 102, 73, 43. ¹H-NMR (300 MHz, pyridine-*d*₅) and ¹³C-NMR (150 MHz, pyridine-*d*₅) data are shown in Table 1.

N-acetyl-3,5,11,18-tetrahydroxyoctadecyl-2-amine (2). White amorphous powder; mp 142 °C, $[\alpha]_{\text{D}}^{20} = -40$ (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ϵ) 207 nm (1.56); IR (KBr) ν_{max} 3331, 2924, 2850, 1619 cm^{-1} ; ESI-MS m/z 376.1 $[\text{M}+\text{H}]^+$, HRESI-MS m/z 398.2886 $[\text{M}+\text{Na}]^+$ (calcd. for $\text{C}_{20}\text{H}_{41}\text{NaNO}_5^+$, 398.2877); EI-MS m/z 242, 160, 142, 116, 87, 43. ¹H-NMR (300 MHz, pyridine-*d*₅) and ¹³C-NMR (150 MHz, pyridine-*d*₅) data are shown in Table 1.

Structure determination

Compound 1 was isolated as an amorphous powder, and its molecular formula was determined as $\text{C}_{19}\text{H}_{39}\text{NO}_5$ by HRESI-MS (m/z 384.2730 $[\text{M}+\text{Na}]^+$, calcd. 384.2720), with one degree of unsaturation (Supplementary Figure S2). The ¹H-NMR (300 MHz, pyridine-*d*₅) spectrum (Table 1, Supplementary Figure S5) of 1 displayed signals for one amide proton at δ_{H} 8.68 (1H, t, *J* = 5.6 Hz), four hydroxyl protons at δ_{H} 6.40 (1H, br s), 5.95 (1H, br s), 5.87 (1H, br s) and 5.59 (1H, br s), three oxymethines at δ_{H} 4.66 (1H, m), 4.38 (1H, br s) and 3.81 (1H, m). Inspection of ¹³C-NMR and the DEPT spectra (Table 1, Supplementary Figures S6 and S7) revealed the existence of 19 carbons including one carbonyl carbon at δ_{C} 170.5, three oxymethines at δ_{C} 70.9, 68.4, 68.1, one oxymethylene δ_{C} 62.1, one nitrogen-bearing sp³ methylene at δ_{C} 47.2 and one methyl group at δ_{C} 23.1. Six aliphatic methylene signals [δ_{H} 1.37–1.78 (12H, m), δ_{C} 26.3–30.4] suggested the presence of aliphatic chain. The proton and carbon signals

¹Department of Natural Products Chemistry, School of Pharmacy, China Medical University, Shenyang, China; ²Key Laboratory of Structure-Based Drug Design & Discovery, Ministry of Education, Shenyang Pharmaceutical University, Shenyang, China; ³State Key Laboratory of Magnetic Resonance and Atomic and Molecular Physics, Wuhan Institute of Physics and Mathematics, Chinese Academy of Sciences, Wuhan, China; ⁴Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang, China and ⁵School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, Shenyang, China
Correspondence: Dr T Liu, Department of Natural Products Chemistry, School of Pharmacy, China Medical University, Shenyang 110001, China.
E-mail: taoliu0307@163.com

Received 22 June 2014; revised 30 August 2014; accepted 7 September 2014; published online 1 October 2014

(Table 1) were assigned from the 2D-NMR (^1H - ^1H COSY, HSQC and HMBC).

The ^1H - ^1H COSY spectra (Figure 2 and Supplementary Figure S8) of **1** disclosed two structural units of NH-C1-C2-C3-C4-C5, C2-OH, C4-OH and C16-C17-OH, suggesting the presence of 2,4,17-trihydroxy groups. In the HMBC spectrum (Figure 2 and Supplementary Figure S10), the correlations from N-H (δ_{H} 8.68) and methyl protons H-2' (δ_{H} 2.07) to a carbonyl carbon C-1' (δ_{C} 170.5), and from N-H (δ_{H} 8.68) to C-1 (δ_{C} 47.2) indicated that C-1 was substituted by an acetylamino group.

The position of the fourth hydroxyl group was determined to be located at C-10 by a significant fragment ion peak at m/z 228 [$\text{M} - \text{C}_7\text{H}_{14}\text{OH} - \text{H}_2\text{O}$] $^+$ in EI-MS (Figure 3 and Supplementary Figure S11). Thus, the structure of **1** was determined to be *N*-acetyl-2,4,10,17-tetrahydroxyheptadecylamine.⁷

Compound **2** was isolated as an amorphous powder, and its molecular formula was determined as $\text{C}_{20}\text{H}_{41}\text{NO}_5$ by HRESI-MS (m/z 398.2886 [$\text{M} + \text{Na}$] $^+$, calcd. 398.2877), with one degree of unsaturation (Supplementary Figure S13). Compared with the

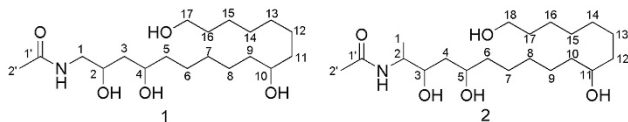


Figure 1 The structures of compounds **1** and **2**.

NMR data of compound **1**, the ^1H -, ^{13}C -NMR and DEPT data (Supplementary Figures S16–S18) indicated **2** was an analog of compound **1**. Inspection of ^{13}C -NMR (Table 1) and the DEPT spectra of **2** revealed that compound **2** possessed one more methyl than compound **1**. The nitrogen-bearing methylene at δ_{C} 47.2 of compound **1** was replaced by a nitrogen-bearing methine at δ_{C} 50.4. The HMBC correlations (Figure 2 and Supplementary Figure S21) from H-1 to C-2 and C-3 suggested an acetyl amino at C-2. 11-Hydroxy group location was determined by a fragment ion peak at m/z 242 [$\text{M} - \text{C}_7\text{H}_{14}\text{OH} - \text{H}_2\text{O}$] $^+$ in EI-MS (Figure 3 and Supplementary Figure S22). Thus, the structure of **2** was determined to be *N*-acetyl-3,5,11,18-tetrahydroxyoctadecyl-2-amine.⁷

Biological activity

The cancer cell cytotoxicities of compounds **1** and **2** were evaluated. The cytotoxic potential of the isolated compounds was investigated by determining their concentrations required for 50% growth inhibition (IG₅₀ value) for three human cancer cell lines (HL-60, MCF-7, PC-3). Compound **2** shows a weak activity against human leukemia (HL-60) cancer cell with IG₅₀ value of 63.61 μM .

DISCUSSION

Our investigation on the metabolites of the halotolerant fungus *Myrothecium* sp. GS-17 has resulted in the isolation of two new amides, *N*-acetyl-2,4,10,17-tetrahydroxyheptadecylamine (**1**) and *N*-acetyl-3,5,11,18-tetrahydroxyoctadecyl-2-amine (**2**). Compound **2**

Table 1 NMR spectroscopic data of compounds **1** and **2** (in pyridine- d_5)

Compound 1				Compound 2			
Position	δ_{H} mult. (JHz)	δ_{C}	HMBC	Position	δ_{H} mult. (JHz)	δ_{C}	HMBC
1	3.87, m 3.67, m	47.2	C-2, C-3, C-1'	1	1.40, d (6.7)	18.0	C-2, C-3
2	4.66, m	68.4	C-1, C-3, C-4	2	4.60, m	50.4	
3	1.94, t (6.0)	43.3	C-1, C-4, C-5	3	4.52, m	71.1	
4	4.38, br s	68.1	C-2, C-6	4	1.99, 2.01, ddd (16.7, 8.9, 3.0)	42.4	C-3, C-5, C-6
5	1.76, m 1.64, m	39.1	C-4	5	4.37, m	68.4	
				6	1.79, m 1.67, m	39.1	C-5
6–8 or 12–14	1.37–1.78	26.3 26.5 26.5 30.0 30.3 30.4		7–9 or 13–15	1.43–1.81	26.3 26.5 26.5 30.0 30.2 30.4	
9	1.60, m	38.5		10	1.63, m	38.5	
10	3.81, m	70.9		11	3.81, m	70.9	
11	1.60, m	38.5		12	1.63, m	38.5	
15	1.52, 2 H, m	26.5		16	1.55, m	26.4	
16	1.74, 2 H, m	33.8		17	1.75, m	33.8	
17	3.86, m	62.1	C-15, C-16	18	3.86, m	62.1	C-16, C-17
-HN	8.68, t (5.6)		C-1, C-1'	-HN	8.19, d (8.8)		C-1'
1'		170.5		1'		169.7	
2'	2.07, s	23.1	C-1'	2'	2.11, s	23.3	C-1'
2-OH	6.40, br s			-OH	6.37, br s		
4-OH	5.95, br s			-OH	5.90, m		
10-OH	5.59, br s			-OH	5.58, m		
17-OH	5.87, br s			-OH	5.68, m		

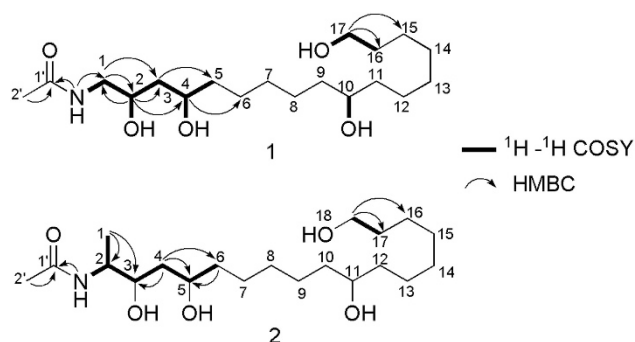


Figure 2 Key ^1H - ^1H COSY and HMBC correlations of compounds **1** and **2**.

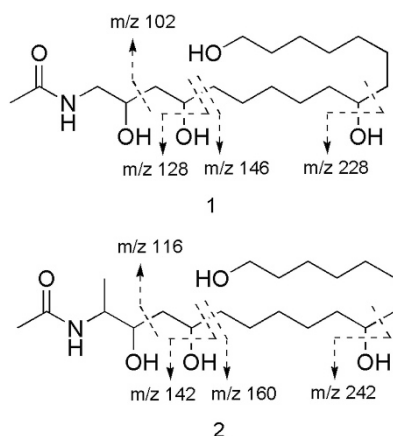


Figure 3 Key fragment ions in EI-MS of compounds **1** and **2**.

shows a weak activity against HL-60 cancer cells. The notable growth inhibition activity of the original extract against HL-60 cell line could be attributed to the presence of trichothecenes, which was reported to show various remarkable cytotoxic activities.⁸ Compound **2** has one more methyl than **1**, which increased the cytotoxic activity against human leukemia (HL-60) cancer cell in this kind of amide structure. These two compounds belong to ceramide (or sphingolipid) analogs, which are a family of important lipid in skin, hair and nail of human and animal's brain.^{9,10} In addition, people found that ceramides have relatively potent antitumor activity.¹¹

METHODS

General experimental procedures

The UV spectra were conducted on a UV-2201 spectrometer (Shimadzu, Kyoto, Japan). The FT-IR spectra were obtained on a Bruker IFS-55 spectrometer (Bruker, Ettlingen, Germany) by a KBr disk method. ESI-MS spectra were conducted on an Agilent 1100 ion trap spectrometer (Agilent Technologies, Palo Alto, CA, USA). HRESI-MS was recorded on a QFT-ESI mass spectrometer (Varian, Palo Alto, CA, USA). ^1H -NMR and ^{13}C -NMR spectra were recorded on Bruker ARX-300 and AV-600 NMR spectrometers (Bruker, Ettlingen, Germany) using pyridine- d_5 as an internal standard [$\delta_{\text{H/C}}$ 7.20, 7.57, 8.72/123.44, 135.43, 149.84 ($\text{C}_5\text{D}_5\text{N}$)]. Optical rotations were measured with a Perkin-Elmer 241 polarimeter (Perkin-Elmer, Boston, MA, USA). The chromatographic silica gel (200–300 mesh) was purchased from Qingdao Ocean Chemical Factory (Qingdao, China) and ODS (50 μm) was purchased from YMC Co. Ltd, Kyoto, Japan. Sephadex LH-20 was purchased from GE Healthcare, Uppsala, Sweden. RP-HPLC analysis and semi-preparation were conducted using HITACHI L2130 series pumping system equipped with a HITACHI L2400 UV detector (Tokyo, Japan) and performed with a C-18 column (10 \times 250 mm, 10 μm ; YMC Co. Ltd, Kyoto, Japan).

Strain

The halotolerant fungal strain GS-17 was separated by dilution isolation method from the soil sample of a salina at Gansu province. The fungus was identified as *Myrothecium* sp. on the basis of morphological and blast search of ITS sequence using the Genbank database. The morphology of the strain GS-17 displays that the colonies are white, ball and flocculent with black oil secretions on the surface. The sizes of strains are 0.5–2.5 μm \times 6–10 μm , and they are round and opaque with irregular edge and surface projection. In addition, colonies are beige granules in liquid nutrient medium. ITS sequence of strain GS-17 showed 100% homology with those of *Myrothecium verrucaria* (GenBank accession number HQ625520) and *Myrothecium atroviride* (GenBank accession number AJ302002; Supplementary Figure S23). The voucher specimen was deposited in Department of Natural Products Chemistry, Shenyang Pharmaceutical University, China. The initial cultures were maintained on the potato dextrose agar (PDA) plate.

Fermentation

A small loop of spores growing on a PDA slant was inoculated into a 250 ml Erlenmeyer flask containing 75 ml sea-water-based culture medium (maltose 2%, monosodium glutamate 1%, KH_2PO_4 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.03%, glucose 1%, yeast extract 0.3%, corn steep liquor 0.1%, mannitol 2%, sea water, pH 6.5) and cultured at 28 $^\circ\text{C}$ for 2 days on a rotary shaker at 180 rotations per minute. Then, 10 ml of the resultant seed culture was inoculated into a 500 ml Erlenmeyer flask containing 150 ml of the above culture medium and incubated (500 flasks) for 8 days at the same conditions.

Extraction and isolation

The fermented broth (56 l) was filtered through cheese cloth to yield the supernatant and mycelia. The supernatant was subjected to HPD100 macroporous absorption resin, eluted in gradient with EtOH/ H_2O 30%, 70%, 100% to yield three fractions (1–3). Fraction 2 was fractionated on a silica gel column eluting with CH_2Cl_2 –MeOH (from 100:0 to 0:100) and was further separated by a flash silica gel column (eluted with petroleum ether/acetone, 10:1) to give three subfractions (A–C). Subfraction A was further purified by RP-HPLC [$\text{MeCN}/\text{H}_2\text{O}$ (65%), 2.0 ml min^{-1}] to yield **1** (10.0 mg) and **2** (6.0 mg).

In vitro cytotoxic assay

The *in vitro* cell growth inhibition activities of crude extracts and the isolated compounds in human promyelocytic leukemia HL-60 cell lines were evaluated by the Trypan blue method. In addition, the cell growth inhibition activities of compounds were assayed by the Trypan blue method using human breast adenocarcinoma MCF-7 cell line, and the MTT method using human prostate cancer PC-3 cell line as previously described.^{12,13} The cell lines 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^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin, 1 mmol glutamine and 10% heat-inactivated fetal bovine serum.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This project was supported by the National Natural Science Foundation of China (Grant No. 20872094).

- Ding, Z. G. *et al*. Naphthosporinone A: an unprecedented and highly functionalized polycyclic metabolite from an alkaline mine waste extremophile. *Chem. Eur. J.* **16**, 3902–3905 (2010).
- Wilson, Z. E., Brimble, M. A. Molecules derived from the extremes of life. *Nat. Prod. Rep.* **26**, 44–71 (2009).
- Pettit, R. K. Culturability and secondary metabolite diversity of extreme microbes: expanding contribution of deep sea and deep-sea vent microbes to natural product discovery. *Mar. Biotechnol.* **13**, 1–11 (2011).
- Chen L. *et al*. A new cytotoxic metabolite from a deep sea derived fungus, *Phialocephala* sp. *Yao. Xue. Xue. Bao.* **45**, 1275–1278 (2010).

- 5 Yonezawa, K., Yamada, K., Kouno, I. New diketopiperazine derivatives isolated from sea urchin-derived bacillus sp. *Chem. Pharm. Bull. (Tokyo)* **59**, 106–108 (2011).
- 6 Rhee, K. H. Cyclic dipeptides exhibit synergistic, broad spectrum antimicrobial effects and have anti-mutagenic properties. *Int. J. Antimicrob. Agents* **24**, 423–427 (2004).
- 7 Yang, G. *et al.* Flavusides A and B, antibacterial cerebrosides from the marine-derived fungus *Aspergillus Flavus*. *Chem. Pharm. Bull. (Tokyo)* **59**, 1174–1177 (2011).
- 8 Namikoshi, M. *et al.* A new macrocyclic trichothecene, 12,13-deoxyroridin E, produced by the marine-derived fungus *Myrothecium roridum* collected in Palau. *J. Nat. Prod.* **64**, 396–398 (2001).
- 9 Školová, B. *et al.* The role of the trans double bond in skin barrier sphingolipids: permeability and infrared spectroscopic study of model ceramide and dihydroceramide membranes. *Langmuir* **30**, 5527–5535 (2014).
- 10 Kulmacz, R. J., Kisić, A., Schroeffer, G. J. Jr. Sphingolipid base metabolism. Chemical syntheses and properties of N-acetyl derivatives of 4R-, 4S-, 5R-, and 5S-hydroxysphinganine. *Chem. Phys. Lipids* **23**, 291–319 (1979).
- 11 Liu, J. W., Beckman, B. S., Foroozesh, M. A review of ceramide analogs as potential anticancer agents. *Future Med. Chem.* **5**, 1405–1421 (2013).
- 12 Wang, F., Hua, H. M., Pei, Y. H., Chen, D., Jing, Y. K. Triterpenoids from the resin of *Styrax tonkinensis* and their antiproliferative and differentiation effects in human leukemia HL-60 cells. *J. Nat. Prod.* **69**, 807–810 (2006).
- 13 Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55–63 (1983).



This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>

Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)