A new diketopiperazine derivative from a deep sea-derived *Streptomyces* sp. SCSIO 04496

Minghe Luo<sup>ab</sup>, Guiling Tang<sup>b</sup>, Jianhua Ju<sup>b</sup>, Laichun Lu<sup>a</sup>* and Hongbo Huang<sup>b</sup>*

<sup>a</sup>Department of Pharmacy, Institute of Surgery Research, Daping Hospital, Third Military Medical University, Chongqing 400042, P.R. China; <sup>b</sup>CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, RNAM Center for Marine Microbiology, Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, P.R. China

(Received 11 March 2015; final version received 18 April 2015)

A new diketopiperazine (DKP) derivative, (6<sup>R</sup>,3<sup>Z</sup>)-3-benzylidene-6-isobutyl-1-methyl piperazine-2,5-dione (1), as well as five known DKPs 2–6 was isolated from a deep sea-derived *Streptomyces* sp. SCSIO 04496. The structure of 1 was elucidated using a combination of 1D and 2D NMR, HR-ESI-MS and chiral-phase HPLC techniques. Compounds 1–6 did not show cytotoxic activity at a concentration of 100μM in bioactivity assay.

**Keywords:** diketopiperazine; cyclodipeptide; *Streptomyces* sp.

1. Introduction

Diketopiperazine is a small family among natural products. Nowadays, Diketopiperazine derivatives (DKPs) have received much attention because of their significant bioactivities including cytotoxicity (Wang et al. 2012), antibacterial activity (Graz et al. 1999) and antiviral activity (Wang et al. 2013). Natural DKPs have provided important inspiration for drug discovery (Borthwick 2012). For example, the vascular disrupting agent NPI-2358 (Plinabulin) is a synthetic analogue of natural diketopiperazine NPI-2350, which was isolated from *Aspergillus* sp. (Nicholson et al. 2006). NPI-2358 is now in phase II clinical trial as an anti-cancer agent (Mita et al. 2010). Marine-derived microorganisms have been looked upon as potential sources of bioactive compounds due to the unique and extreme environment characterised by high pressure, low temperature, lack of light and variable salinity and oxygen concentration (Blunt et al. 2013). As part of our ongoing research program to discover bioactive metabolites with novel structures from the South China Sea-derived actinomycetes, we have reported an antimicrobial and cytotoxic polythiazole cyclopeptide marthiapeptide A from

*Corresponding authors. Email: lulaicq@163.com (L. Lu); huanghb@scsio.ac.cn (H. Huang)

© 2015 Taylor & Francis
Marinactinospora thermotolerans SCSIO 00652 (Zhou et al. 2012), cytotoxic angucyclines grincamycins B-F from Streptomyces lusitanus SCSIO LR32 (Huang et al. 2012). Recently, an actinomycete strain was isolated from a marine sediment sample collected in the South China Sea. This strain was subsequently identified as Streptomyces sp. SCSIO 04496 on the basis of 16S rDNA sequence analysis. Chemical investigation of the culture of this strain resulted in the isolation and identification of six DKPs, including a new DKP 1 and five known DKPs 2–6 (Figure 1 and Figure S1). We reported herein the isolation, structure elucidation of the new compound 1, as well as the full assignment of the NMR data for the known compound 2.

2. Results and discussion

The structures of known compounds 3–6 were identified to be 1-N-methylalbonoursin (3) (Gurney & Mantal 1993), (3E,6Z)-6-benzylidene-1-methyl-3-(2-methylpro-pylidene) piperazine-2,5-dione (4) (Robins & Sefton 1984), albonoursin (5) (Fukushima et al. 1973) and (3Z,6E)-1-N-methyl-3-benzylidene-6-(methyl-3-hydroxypropylidene) piperazine-2,5-dione (6) (Wang et al. 2013) by comparing their NMR data with those previously reported, respectively.

Compound 1 was isolated as a white solid. The molecular formula of 1 was determined to be C_{16}H_{20}N_{2}O_{2} on the basis of HR-ESI-MS (m/z 273.1590, [M + H]^+) suggestive of eight degrees of unsaturation. Analysis of the 1D-NMR spectroscopic data disclosed three methyls, including a nitrogen-bearing methyl (δH 3.08, δC 33.5, N-Me), one methylene (δH 1.81, δC 42.1, C-7), two aliphatic and six aromatic methines, and four quaternary carbons. The singlet signal at δH 7.02 (H-11) in 1H NMR spectrum revealed the existence of a trisubsituted vinyl. A set of 1H NMR signals at δH 7.43 (2H, H-14, 16), δH 7.35 (2H, H-13, 17) and δH 7.33 (1H, H-15) suggested the presence of a monosubstituted benzene. In the HMBC spectrum, the correlations of H-13/C-11 (δC 116.2) and H-11/C-13 (δC 128.5) and C-2 (δC 159.1) (Figure S8) determined a dehydrophenylalanine (deh-Phe) unit. Furthermore, the HMBC correlations from methyl protons H-3-9 and H-3-10 to C-7 and C-8 (δC 24.5), from H-7 to C-8, C-6 (δC 61.7) and C-5 (δC 166.1), as well as from H-6 (δH 4.01) to C-5, C-7 and C-8 identified a Leucine (Leu) unit. The N-Me was attached to the Leu based on its HMBC correlation with C-7. The important HMBC correlations of H-6/C-2 and N-Me/C-2 established a diketopiperazine structure of 1. The NOESY correlation between 4-NH (δH 7.79) and H-13, 17 indicated the Z-configuration of the Δ^{3,11} double bond (Figure S8). The absolute configuration of the aliphatic amino acid unit was determined as N-Me-L-Leu by chiral-phase HPLC analysis of hydrolysate of compound 1 (Figure S10). Thus, compound 1 was identified as (6R, 3Z)-3-benzylidene-6-isobutyl-1-methylpiperazine-2, 5-dione.

Compound 2 had a molecular formula of C_{16}H_{18}N_{2}O_{3} as determined by HR-ESI-MS (m/z 287.1379 [M + H]^+). The 1H and 13C NMR spectroscopic data of 2 were similar with those of the known compound 3, except that the signals of the benzene unit were obviously different. Two pairs of ortho-coupled aromatic signals at δH 7.37 (2H, d, J = 8.5 Hz, H-13, 17), 6.86 (2H, d, J = 8.5 Hz, H-14, 16) combined with a set of 13C NMR signals at δC 159.2 (C-15), 116.9 (C-14, 16), 131.7 (C-13, 17), 125.7 (C-12) in 2 inferred the presence of a p-hydroxyl substituted phenyl instead of the monosubstituted benzene ring in 3. This elucidated structure was further

![Figure 1. Chemical structure of compounds 1 and 2.](image-url)
confirmed based on detailed inspection of the HMBC spectrum of 2 (Figure S11). The NOE correlations of H-4 ($\delta_H$ 7.85)/H-13,17 and of N-CH$_3$ ($\delta_H$ 3.27) to H-7($\delta_H$ 5.59) indicated Z- and E-configurations of the $\Delta^3$,11 and $\Delta^6$,9 double bonds, respectively. Therefore, compound 2 was determined to be (3Z,6E)-3-(4-hydroxybenzylidene)-1-methyl-6-(2-methylpropylidene)piperazine-2,5-dione. The assignments of NMR data for 2 were listed in Table S1.

Compounds 1–6 were evaluated for their cytotoxic activities against a panel of five tumour cells, including SF-268, MCF-7, NCI-H460, HepG-2 and LX-2 cells using the Sulforhodamine B (SRB) method (Chen et al. 2012). None of these compounds showed significant anti-proliferation activity at a concentration of 100 $\mu$M.

3. Experimental

3.1. General experimental procedures

Materials for column chromatography were silica gel (100–200 mesh; Jiangyou Silica Gel Development, Inc.). Thin layer chromatography (TLC) was conducted with precoated glass plates (0.1–0.2 mm; silica gel GF254, 10–40 nm). Semi-preparative HPLC were performed with L-2000 system (Hitachi) using a YMC-Pack ODS-A column (250 $\times$ 10 mm, 5 $\mu$m). The analytical chiral-packed column (MCIGEL CRS10W, 4.6 $\times$ 50 mm), and a 1260 infinity HPLC system (Agilent) were used for the chirality analysis. Low and high resolution mass spectral data were obtained on amazon SL ion trap mass spectrometer and MaXis quadrupole-TOF mass spectrometer (Bruker), respectively. Optical rotations were recorded with a MCP 300 polarimeter (Anton Paar). NMR spectra were recorded on an Aonvance 500 spectrometer (Bruker) at 500 MHz for $^1$H and 125 MHz for $^{13}$C.

3.2. Strain material

Strain SCSIO 04496 was isolated from a sediment sample collected from the South China Sea (E 120°0.250′ and N 20°22.971′) at a depth of 3536 m using isolation media plates containing Gauze’s medium No. 1 (soluble starch 2.0%, KNO$_3$ 0.1%, K$_2$HPO$_4$ 0.05%, MgSO$_4$·7H$_2$O 0.05%, FeSO$_4$·7H$_2$O 0.01%, pH 7.4) after incubation at 28°C for 2 weeks. This strain was deposited in the type culture collection of the Center for Marine Microbiology, Research Network of Applied Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China.

The almost full 16S rDNA gene sequence (1422bp) of the strain SCSIO 04496 was submitted to GenBank under the accession number KM077017, which showed the highest similarity (98.2%) with that of Streptomyces flocculus NBRC 13041T (AB184272). The phylogenetic tree generated by a neighbour-joining method clearly revealed the evolutionary relationship of the strain SCSIO 04496 with a group of Streptomyces species (Figure S2). On the basis of the 16S rDNA gene sequence analysis, the strain SCSIO 04496 was designated as a species of Streptomyces. Pure culture in dish and scanning electron micrographs of spore chains and aerial mycelia of strain SCSIO 04496 see Figure S3.

3.3. Fermentation, extraction and isolation

A single colony of SCSIO 04496 on ISP4 medium (soluble starch 1.0%, K$_2$HPO$_4$ 0.1%, MgSO$_4$·7H$_2$O 0.1%, (NH$_4$)$_2$SO$_4$ 0.2%, CaCO$_3$ 0.2%, sea salt 3.0%, pH 7.2 before sterilisation) was inoculated into a 250 mL Erlenmeyer flask containing 50 mL of the seed medium (soluble starch 2%, soybean powder 0.5%, yeast exact power 0.5%, bacterial peptone 0.2%, sea salt 3.0% CaCO$_3$ 0.2%, pH 7.2 before sterilisation). After incubating on a rotary shaker (200 rpm) for 2 days at 28°C, the seed cultures were transferred to 1000 mL Erlenmeyer flask containing 200 mL of the
seed medium. These flasks were incubated at 28°C on a rotary shaker at 200 rpm for 7 days. A total of 6 L culture was made by this way. After fermentation, the culture was centrifuged to yield supernatant and mycelia cakes. The fermentation broths were extracted three times with 6 L butanone, and the mycelia cakes were extracted three times with 1.5 L acetone. After removing solvents, both fermentation broths and mycelia cakes were combined, according to the results of HPLC-DAD analyses, to afford 3.21 g crude extracts. The crude extracts were subjected to normal phase silica gel (100–200 mesh) column chromatography (CC) and eluted with CHCl$_3$/MeOH (100/0, 98/2, 96/4, 94/6, 90/10, v/v, each of 200 mL) to yield five fractions (Fr.1–Fr.5). Fr.1 was subjected to silica gel CC using gradient elution with a mixture of petroleum ether and ethyl acetate (100:0, 90:5, 90:10, 85:15, 80:20, v/v, each of 200 mL) to give eight fractions (Fr.1-1–Fr.1-8). Fr.1-3 and Fr.1-4 were combined and subjected to silica gel CC using gradient elution with mixture of petroleum ether and chloroform (8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10, v/v, each of 100 mL) to give nine fractions (Fr.1-(3-4)-1–Fr.1-(3-4)-9). Fr.1-(3-4)-3 was further purified by recrystallisation to get rid of impurity materials, and finally purified compound 3 is obtained. Fr.1-5 and Fr.1-6 were combined and subjected to semi-preparative HPLC with an ODS column using elution system consisting of solvent A (H$_2$O) and solvent B (CH$_3$CN), eluted with a linear gradient of 60–80% solvent B over 20 min, and UV detection was at 320 nm, to give compounds 1, 4 and 5 at retention time of 13.7 min, 17.9 min and 15.7 min, respectively. Fr.1-7 and Fr.1-8 were combined and further purified by semi-preparative HPLC using elution system consisting of solvent A (H$_2$O) and solvent B (CH$_3$CN), eluted with a linear gradient of 30–80% solvent B over 20 min. The eluting peak at 16.9 min was compound 6. Fr.2 was subjected to silica gel CC and eluted with CHCl$_3$/CH$_3$OH (100/0, 99/1, 98/2, 97/3, v/v, each of 100 mL) to give Fr.2-2. Fr.2-2 was further purified by semi-preparative HPLC using elution system consisting of solvent A (H$_2$O) and solvent B (CH$_3$CN), eluted with a linear gradient of 30–80% solvent B over 20 min to yield compound 2 at 18.2 min.

3.4. Acid hydrolysis

Compound 1 was dissolved in 6 N HCl (1 mL) and heated at 110°C for 18 h. After cooling to room temperature, the solvent was removed under reduced pressure, and the standard amino acids were prepared according to the published method (Chen et al. 2012). The dried hydrolysate was dissolved in 100 µL of 2 mM CuSO$_4$–H$_2$O solution. Ten microlitres of this sample were then analysed by HPLC with a chiral column (MCigel CRS10W) using 2 mM CuSO$_4$–H$_2$O solution as the mobile phase at a flow rate of 0.5 mL/min with UV detection at 254 nm. The retention times of the N-Me-d-Leu and N-Me-l-Leu were 14.8 and 22.5 min, respectively. Thus, the N-Me-Leu residue in compound 1 was assigned as N-Me-d-Leu (14.7 min) (Figure S10).

3.5. Cytotoxic activity test

Compounds 1–6 were evaluated for their cytotoxic activities against five tumour cell lines, including SF-268, MCF-7, NCI-H460, HepG-2 and LX-2 cells using RSB method (Chen et al. 2012) according to published protocols.
4. Conclusions
A new diketopiperazine derivative, (6R,3Z)-3-benzylidene-6-isobutyl-1-methyl piperazine-2,5-dione (1), as well as five known DKPs 2-6 was isolated from a deep sea-derived Streptomyces sp. SCSIO 04496. Compounds 1–6 did not show cytotoxic activity at the concentration of 100 μM.

Supplementary material
Supplementary material relating to this article is available online at http://dx.doi.org/10.1080/14786419.2015.1045509, alongside Figures S1–S11 and the original spectra of compound 1.

Acknowledgement
We are grateful to Mr Li, Mrs Sun, Mrs Xiao and Miss Zhang of the South China Sea Institute of Oceanology for recording NMR and MS data.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This study was supported in part by MOST [grant number 2012AA092104], the National Natural Science Foundation of China for Young Scientists [grant number 41106138], international S&T cooperation program of China [grant number 2011DFA32540] and the Knowledge Innovation Programs of CAS [grant numbers KZCX2-YW-JC202; KSCX2-EW-G-12].

References
Blunt JW, Copp BR, Keyzers RA, Munro MH, Prinsep MR. 2013. Marine natural products. Nat Prod Rep. 30:237–323. doi:10.1039/C2NP20112G.
Borthwick AD. 2012. 2,5-Diketopiperazines: synthesis, reactions, medicinal chemistry, and bioactive natural products. Chem Rev. 112:3641–3716. doi:10.1021/cr200398y.
Chen Z, Song Y, Chen Y, Huang H, Zhang W, Ju J. 2012. Cyclic heptapeptides, cordyheptapeptides C–E, from the marine-derived fungus Acremonium persicinum scsio 115 and their cytotoxic activities. J Nat Prod. 75:1215–1219. doi:10.1021/np300152d.
Fukushima K, Yazawa K, Arai T. 1973. Biological activities of albonoursin. J Antibiot. 26:175–176. doi:10.7164/antibiotics.26.175.
Graz M, Hunt A, Jamie H, Grant G, Milne P. 1999. Antimicrobial activity of selected cyclic dipeptides. Pharmazie. 54:772–775.
Gurney KA, Mantle PG. 1993. Biosynthesis of 1-N-methylalbonoursin by an endophytic Streptomyces sp. isolated from perennial reygrass. J Nat Prod. 56:1194–1198. doi:10.1021/np50097a031.
Huang H, Yang T, Ren X, Liu J, Song Y, Sun A, Ma J, Wang B, Zhang Y, Huang C, et al. 2012. Cytotoxic angucycline class glycosides from the deep sea actinomycete Streptomyces lusitanus SCSIO LR32. J Nat Prod. 75:202–208. doi:10.1021/np2008335.
Mita AC, Heist RS, Aren O, Mainwaring PN, Bazhenova L, Gadgeel SM, Blum RH, Polikoff J, Biswas J, Spear MA. 2010. Phase II study of docetaxel with or without plinabulin (NPI-2358) in patients with non-small cell lung cancer (NSCLC). 2010 ASCO Annual Meeting Abstracts Part 1. J Clin Oncol. 28:7592.
Nicholson B, Lloyd GK, Miller BR, Palladino MA, Kiso Y, Hayashi Y, Neuteboom ST. 2006. NPI-2358 is a tubulin-depolymerizing agent: in-vitro evidence for activity as a tumor vascular-disrupting agent. Anti-Cancer Drugs. 17:25–31. doi:10.1097/01.cad.0000182745.01612.8a.
Robins DJ, Sefton MA. 1984. 1-N-methyl-(6E)-(2-methylpropyldiene)-(3Z)-3-(phenylmethylene)-2,5-piperazinedione, a metabolite from Streptomyces albus. Phytochemistry. 23:200–201. doi:10.1016/0031-9422(84)83115-5.
Wang FZ, Huang Z, Shi XF, Chen YC, Zhang WM, Tian XP, Li J, Zhang S. 2012. Cytotoxic indole diketopiperazines from the deep sea-derived fungus Acrostalagmus luteoalbus SCSIO F457. Bioorg Med Chem Lett. 22:7265–7267. doi:10.1016/j.bmcl.2012.08.115.

Wang P, Xi L, Liu P, Wang Y, Wang W, Huang Y, Zhu W. 2013. Diketopiperazine derivatives from the marine-derived actinomycete Streptomyces sp. FXJ7.328. Mar Drugs. 11:1035–1049. doi:10.3390/md11041035.

Zhou X, Huang H, Chen Y, Tan J, Song Y, Zou J, Tian X, Hua Y, Ju J. 2012. Marthiapptide A, an anti-infective and cytotoxic polythiazole cyclopeptide from a 60 L scale fermentation of the deep sea-derived Marinactinospora thermotolerans SCSIO 00652. J Nat Prod. 75:2251–2255. doi:10.1021/np300554f.