A novel cyclic dipeptide from deep marine-derived fungus *Aspergillus* sp. SCSIOW2

Xiang Zhou\(^{a1}\), Pingyan Fang\(^{a1}\), Jianqiang Tang\(^{a}\), Zhiqin Wu\(^{a}\), Xiaofan Li\(^{a}\), Shuiming Li\(^{a}\), Yong Wang\(^{a}\), Gang Liu\(^{a}\), Zhendan He\(^{b}\), Deming Gou\(^{a}\), Xinsheng Yao\(^{c}\) and Liyan Wang\(^{a}\)*

\(^{a}\)College of Life Science, Shenzhen Key Laboratory of Marine Bioresource and Eco-environmental Science, Shenzhen Key Laboratory of Microbial Genetic Engineering, Shenzhen University, Shenzhen 518060, P.R. China; \(^{b}\)Department of Pharmacology, School of Medicine, Shenzhen University, Shenzhen 518060, P.R. China; \(^{c}\)Institute of Traditional Chinese Medicine & Natural Products, Jinan University, Guangzhou 510632, P.R. China

(Received 8 January 2015; final version received 15 March 2015)

A novel cyclic dipeptide, 14-hydroxy-cyclopeptine (1), was purified from a deep sea derived fungal isolate identified as an *Aspergillus* sp. The structure was elucidated by detailed spectroscopic analyses using 1D and 2D NMR experiments and high resolution mass spectrometry. The absolute configuration of the amino acid was determined by Marfey’s method. Two conformational isomers of 1 were established by ROE analyses. 1 inhibited nitric oxide production with IC\(_{50}\) values at 40.3 \(\mu\)g/mL in a lipopolysaccharide and recombinant mouse interferon-\(\gamma\)-activated macrophage-like cell line, RAW 264.7 and showed no cytotoxic effect in the tested dose range up to 100 \(\mu\)g/mL.

**Keywords:** marine fungi; nitric oxide inhibitory activity; cyclodipeptide; *Aspergillus* sp;

1. **Introduction**

Macrophages play major roles in the innate and adaptive immune responses by releasing various factors such as pro-inflammatory cytokines, oxygen and nitrogen species. One critical releasing factor of nitric oxide (NO) has been implicated in numerous physiological and pathological processes (Bogdan 2001; Dawn & Bolli 2002). Excessive production of NO appears to associate with many chronic or acute diseases related to inflammation such as rheumatoid arthritis, cancer and even Alzheimer’s disease. Therefore, inhibition of activation and NO production may be of therapeutic benefit against various types of diseases (Aktan et al. 2003).

*Corresponding author. Email: lixiaof@szu.edu.cn; lwang@szu.edu.cn
So far, more than 1000 structurally unique and biologically active compounds have been isolated from marine-derived fungi (Rateb & Ebel 2011). Accordingly, marine fungi have attracted increasing attention as a resource for drug discovery (Bhadury et al. 2006). Plinabulin, a synthetic cyclic dipeptide analogue of halimide, which is isolated from a marine fungus species, is now in phase II clinical trial for treatment of non-small cell lung cancer (Yamazaki et al. 2011). In our efforts to identify novel structures and bioactive metabolites from deep sea (over 1000 m) derived fungi, we screened the fermentation extracts of 20 fungi for NO production inhibitory activities using macrophage RAW 264.7 cells. Among them, the EtOAc extracts of Aspergillus sp. SCSIO-W2 showed strong potency to inhibit NO production without cytotoxic effects. A bioassay-guided chemical investigation resulted in the isolation of a novel cyclic dipeptide, cyclo-(L-N-MeTyr-anthranilic acid), which we have named 14-hydroxy-cyclopeptine (1).

2. Results and discussion

14-Hydroxy-cyclopeptine (1) was obtained as a colourless oil, its molecular formula was determined as C_{17}H_{16}N_{2}O_{3}, according to its HR-ESI-MS peak at m/z 297.1211 [M + H]^+ and m/z 593.2375 [2M + H]^+, requiring 11 degrees of unsaturation. Interestingly, all the proton and carbon signals of 1 recorded in DMSO-d6 were observed in pairs at room temperature. The temperature dependency of ^1H NMR was then analysed, at +50°C, the two sets of signals began to merge, at +85°C, coalesced into single resonances (Figure S1–S3), this indicated the paired signals resulted from two different stable conformations in chemical exchange. Analysis of ^1H and ^13C NMR data revealed the presence of four carbonyl signals (δ_c 171.9, 171.1, 169.6, and 167.2), two amide protons (δ_H 10.5 and 10.6) and two N-methyl signals (δ_H 2.94 and 2.87). Comprehensive analysis of 2D (COSY, HSQC and HMBC) NMR spectroscopic data constructed four amino acid residues as two N-MeTyr and two anthranilic acids (AAs). Two partial sequences of N-MeTyr-AA were established by HMBC correlations of the carbonyl carbons of each AA with the two N-methyl protons, respectively (Figure 1). However, N-MeTyr-

Figure 1. Left: Planner structure of 1 with key ^1H–^1H COSY and HMBC correlations; Right: Conformers A and B of 1 in solution and key ROE correlations.
AA only accounted for 10 degrees of unsaturation, which suggested a cyclodipeptide of 1. Finally, HMBC correlations of each amide proton of AA with carbonyl carbon of N-MeTyr confirmed the final planar structures as cyclo-(N-MeTyr-AA). 1 had a specific optical rotation of $[\alpha]_{D}^{20} = 93.5^\circ$, cyclopeptin has a specific optical rotation of $[\alpha]_{D}^{20} = 93.5^\circ$, 4′-methoxycyclopeptin had a specific optical rotation of $[\alpha]_{D}^{20} = 90.2^\circ$ (Kusano et al. 2000), indicating the same absolute configuration for the sole chiral centre at C-3 for the three molecules. This result supported stereochemistry at C-3 most probably being S. The absolute configuration of N-MeTyr was finally determined as L-configuration based on hydrolysis and Marfey’s analysis (Figure S12). $^1$H NMR integral area shows that the two distinct sets of conformers can be observed in about 4:5 ratio at room temperature. Small cyclic peptides have very different conformational behaviour to acyclic peptides, most significantly with respect to cis/trans isomerisation of the peptide groups. However, cyclic dipeptides usually have both peptide bonds in the cis conformation because this is the only configuration that allows for closure of the ring (Oakley & Johnston 2013). Preferred conformations for 1 were deduced by ROE spectral data. ROE correlations between N-methyl group and H-10 (tyrosine’s β proton), H-12/16 in the major conformer (conformer A) indicated that the β-C of tyrosin (C-10) was in a pseudoequatorial direction. ROE correlation between N-methyl and H-3 in the minor conformer (conformer B) supported that the β-C of tyrosin was in a pseudoaxial direction (Figure 1). Up-field shifts of 10a and 10b in B form depend on the shielding effect of the benzene ring. Changing the structure from A to B can be done simply by ring flipping of the seven-membered cyclodipeptide ring with two peptide bonds remaining in cis conformation.

Cyclodipeptides constitute a large class of natural products that exhibit various biological activities including phytotoxin (thaxtomin A), antifungal [cyclo(l–Phe–l–Pro), cyclo(l–Phe–trans–4–OH–l–Pro)], siderophores (erythrochelin, coprogen and dimeramic acid), mycotoxins (roquefortine C and acetylaszonalenin), antibacterial (bicyclomycin, albonoursin brevianamide S), antitumour (ambewelamides A, B, phenylahistin and verticillin A), antiviral and immunosuppressive activities (gliotoxin and sirodesmin PL) (Gu et al. 2013). Structurally, 14-hydroxy-cyclopeptine (1) also belongs to benzodiazepine alkaloids of the cyclopenin group. Benzodiazepines are a class of psychoactive drugs whose core chemical structure is the fusion of a benzene ring and a diazepine ring.

We examined the inhibitory effects of 1 on the production of NO induced by lipopolysaccharide (LPS)/INF-γ. 1 showed weak NO production inhibitory activity [IC$_{50}$ = 40.3 µg/mL (68.0 µM)]. The cytotoxic effect of 1 was measured with MTT assay. 1 did not show any cytotoxic effect at the tested dose range (30–100 µg/mL). Quercetin was used as positive control which showed stronger NO inhibitory effect (inhibitory rate 97.4% at 100 µg/mL).

3. Experimental
3.1. General experimental procedures

Optical rotations were determined on a Jasco P-1020 polarimeter (Jasco, Hachioji, Tokyo, Japan). UV data were recorded on a Perkin Elmer Lambda 25 UV/vis spectrometer (Perkin Elmer, Boston, MA, USA). IR data were recorded using a Nicolet Avatar 330 FT-IR spectrometer (Thermo Scientific, Waltham, MA, USA). NMR spectra were acquired on a Bruker AVANCE-400 (400 MHz) or a Bruker ASCEND 600 MHz (Bruker, Ettlingen, Germany) NMR magnet system using TMS as internal standard. HR-ESI-MS were recorded on an AB SCIEX TOF/TOF™ 5800 system (AB Sciex, Redwood City, CA, USA). Column chromatography (CC) was carried out with silica gel (100–200 mesh, Qingdao Marine Chemical Factory) and Sephadex LH-20 (Amersham Pharmacia Biotech). TLC was performed on Merck TLC plates (silica gel 60 RP-18 F$_{254}$S and silica gel 60 F254), with compounds were visualised by spraying
with 5% (v/v) H$_2$SO$_4$ in EtOH and then heating on a hot plate. HPLC was performed on a Shimadzu LC-20AT pump equipped with a SPD-20A UV–vis detector. A YMC-Pack Pro C18 column (10 × 250 mm I.D. 5 µ) and a YMC-Pack Pro C18 column (4.6 × 250 mm I.D. 5 µ) were used for semi-preparative and analysis purposes, respectively.

3.2. Strains

Fungus SCSIOW2 was isolated from deep marine sediment sample collected in the South China Sea (112°30.203E, 18°1.654N) at a depth of 2439 m. This fungus was characterised as *Aspergillus* sp. by the analysis of the ITS region sequence with GenebankS1. This fungus was deposited in Marine Microbial Lab., College of Life Science, Shenzhen University (Shenzhen, China).

3.3. Fermentation, extraction and isolation

*Aspergillus* sp. SCSIOW2 was cultured in 250 mL Erlenmeyer flasks containing 50 mL of the seed medium (2.0% glucose, 1.0% peptone, 0.5% yeast extract, 3.0% sea salt, with the pH adjusted to 7.5). After growth at 28°C, 220 rpm for 2 days, 5 mL of seed cultures was inoculated into 60 Erlenmeyer flasks (2000 mL), each containing solid media (150 g rice, 7.5 g sea salt, 250 mL H$_2$O, and the flasks were sterilised by autoclave). The resulting cultures were fermented at 28°C under static condition for 40 days. The fermented broth of each flask was extracted with 400 mL EtOAc three times. The combined extract (50.0 g) was applied to a Silica gel CC, gradient elution with CHCl$_3$–MeOH afforded 23 fractions (Fr.1–Fr.23); Fr.11 (3.4 g), eluted with CHCl$_3$–MeOH (9:1), was further isolated by Sephadex LH-20 CC with CHCl$_3$–MeOH (1:1) to give 13 fractions (Fr.11-1–Fr.11-13); Fr.11-8 was purified by HPLC with an ODS column eluted with MeOH–H$_2$O (40:60–100:0 over 30 min, 2.5 mL/min) to yield 14-hydroxy-cyclopeptine (1) (14.7 mg, $t_R$ 10.7 min).

3.3.1. 14-Hydroxy-cyclopeptine (I)

Colourless oil; [α]$_D^{20}$ − 93.5° (c = 0.9, CHCl$_3$); UV (MeOH) $\lambda_{max}$ (log ε) 216 (4.9), 278 (3.8) nm; IR (film) $\nu_{max}$: 3415, 1682, 1624, 1512, 1022 and 761 cm$^{-1}$; $^1$H NMR (400 MHz, DMSO-d$_6$) $\delta$: 10.6 (1H, s, 1-NH, B-form), 10.5 (1H, s, 1-NH, A-form), 9.36 (1H, s, 14-OH, B-form), 9.28 (1H, s, 14-OH, A-form), 7.83 (1H, d, $J$ = 7.6 Hz, 6-H, B-form), 7.71 (1H, d, $J$ = 7.6 Hz, 6-H, A-form), 7.70 (1H, dd, $J$ = 7.6, 7.6, 8-H, B-form), 7.51 (1H, dd, $J$ = 7.6, 7.6, 8-H, A-form), 7.27 (1H, m, 7-H, B-form), 7.24 (1H, m, 7-H, A-form), 7.18 (1H, m, 9-H, B-form), 7.07 (1H, m, 9-H, A-form), 7.03 (1H, d, $J$ = 7.6, 12/16-H, A-form), 6.78 (1H, d, $J$ = 7.6, 12/16-H, B-form), 6.65 (1H, d, $J$ = 7.6 Hz, 13/15-H, B-form), 6.63 (1H, d, $J$ = 7.6–Hz, 13/15-H, A-form), 4.19 (2H, m, 3-H, A and B-form), 2.94 (3H, s, 17-Me, A-form), 2.87 (3H, s, 17-Me, B-form), 3.16 (1H, dd, $J$ = 14.0, 7.2, 10b, A-form), 3.05 (1H, dd, $J$ = 14.0, 7.2, 10a, A-form), 2.57 (1H, dd, $J$ = 13.6, 6.8, 10b, B-form), 2.50 (1H, m, 10a, A-form) and $^{13}$C NMR data (100 MHz, DMSO-d$_6$) $\delta$: 171.9 (s, C-2, B-form), 171.1 (s, C-2, A-form), 169.6 (s, C-5, A-form), 167.2 (s, C-5, B-form), 158.2 (s, C-14, A-form), 157.8 (s, C-14, B-form), 138.5 (s, C-9a, A-form), 137.6 (s, C-9a, B-form), 134.3 (d, C-8, B-form), 133.9 (d, C-8, A-form), 132.8 (d, C-6, B-form), 132.6 (d, C-6, A-form), 131.8 (d, C-12/16, A-form), 131.7 (d, C-12/16, B-form), 129.1 (s, C-11, B-form), 129.0 (s, C-5a, A-form), 128.5 (s, 5a, B-form), 127.7 (s, C-11, A-form), 126.0 (d, C-7, A-form), 125.8 (d, C-7, B-form), 122.6 (d, C-9, A-form), 122.1(d, C-9, B-form), 117.2 (d, C-13/15, B-form), 117.1 (d, C-13/15, A-form), 69.5 (d, C-3, B-form), 58.2 (d, C-3, A-form), 50.5 (q, C-17, B-form), 34.9 (t, C-10, B-form), 32.6 (t, C-10, A-form), 30.6 (q, C-17, A-form); HR-ESI-MS $m/z$ 297.1211 [M + H]$^+$ (Calcd for C$_{17}$H$_{17}$N$_2$O$_3$, 297.1239).
3.4. **NO inhibitory assay**

Mouse RAW264.7 macrophage cells purchased from American Type Culture Collection were cultured in RPMI-1640 medium with 10% foetal bovine serum (Hyclone). It is essential to subculture the cells before reaching 90% confluence. Cells were plated in a 96-well plate at a density of $1.0 \times 10^5$ cells/well and grown for 2 h to allow cells to attach to the plate. Samples tested were dissolved in DMSO, and then made two fold serial dilutions in RPMI-1640. The final concentrations of dried extracts in the culture medium were 100, 30 and 10 $\mu$g/mL. Quercetin (Sigma) was used as the positive control. The extracts were added to the culture simultaneously with both *Escherichia coli* LPS (1.5 $\mu$g/mL, Sigma) and recombinant mouse interferon-$\gamma$ IFN-$\gamma$; 10 ng/mL, Peptidech). Cell viability and nitrite concentration in the culture medium were determined 24 h after incubation. Control cells were grown under identical conditions without the test extracts. Cell viability was determined using the Cell Titer 96 Aqueous One Solution Proliferation Assay Kit (Promega). NO levels in the culture media were determined by Griess Reagent System according to manufacturer’s instructions (Promega). Details of the protocol could be found in our previous report (Li et al. 2014).

**Supplementary material**

Supplementary material relating to this paper is available online.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**Funding**

This work was supported by the National High Technology Research and Development Program of China (863 program) [grant number 2012AA092104]; the National Natural Science Foundation of China (NSFC) [grant number 41276136]; and Science and Technology Project of Shenzhen city, Shenzhen Bureau of Science, Technology and Information [grant number JCYJ20130408172946974].

**Note**

1. These authors contributed equally.

**References**

Aktan F, Henness S, Roufogalis BD, Ammit AJ. 2003. Nitric Oxide. 8:235–242. doi:10.1016/S1089-8603(03)00032-6.

Bhadury P, Mohammad BT, Wright PC. 2006. The current status of natural products from marine fungi and their potential as anti-infective agents. J Ind Microbiol Biotechnol. 33:325–337. doi:10.1007/s10295-005-0070-3.

Bogdan C. 2001. Nitric oxide and the immune response. Nat Immunol. 2:907–916. doi:10.1038/ni1001-907.

Dawn B, Bolli R. 2002. Role of nitric oxide in myocardial preconditioning. Ann New York Acad Sci. 962:18–41. doi:10.1111/j.1749-6632.2002.tb04053.x.

Gu B, He S, Yan X, Zhang L. 2013. Tentative biosynthetic pathways of some microbial diketopiperazines. Appl Microbiol Biotechnol. 97:8439–8453. doi:10.1007/s00253-013-5175-4.

Kusano M, Koshino H, Uzawa J, Fujioka S, Kawano T, Kimura Y. 2000. Nematicidal alkaloids and related compounds produced by the fungus *Penicillium cf. Simplicissimum*. Biosci Biotechnol Biochem. 64:2559–2568. doi:10.1271/bbb.64.2559.

Li L, Wang L, Wu Z, Yao L, Wu Y, Huang L, Liu K, Zhou X, Gou D. 2014. Anthocyanin-rich fractions from red raspberries attenuate inflammation in both RAW264.7 macrophages and a mouse model of colitis. Sci Rep. 1–11. doi:10.1038/srep06234.

Oakley MT, Johnston RL. 2013. Exploring the energy landscapes of cyclic tetrapeptides with discrete path sampling. J Chem Theory Comput. 9:650–657. doi:10.1021/ct3005084.

Rateb ME, Ebel R. 2011. Secondary metabolites of fungi from marine habitats. Nat Prod Rep. 28:290–344. doi:10.1039/c0np00061b.
Yamazaki Y, Kido Y, Hidaka K, Yasui H, Kiso Y, Yakushiji F, Hayashi Y. 2011. Tubulin photoaffinity labeling study with a plinabulin chemical probe possessing a biotin tag at the oxazole. Bioorg Med Chem. 19:595–602. doi:10.1016/j.bmc.2010.10.055.