

ORIGINAL ARTICLE

New diketopiperazine derivatives from a deep-sea-derived *Nocardiopsis alba* SCSIO 03039

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The strain SCSIO 03039 was isolated from a sediment sample in the Indian Ocean and was characterized as a *Nocardiopsis alba* species on the basis of its 16S rRNA gene sequence. Seven diketopiperazines (DKPs), including two new DKPs nocazines D (2a) and E (2b), and five known DKPs (1, 3–6), were isolated from *N. alba* SCSIO 03039, along with two known compounds 2-methoxy-1,4-naphthoquinone (7) and 1-hydroxy-4-methoxy-2-naphthoic acid (8). Their structures were elucidated by mass and NMR spectroscopic analyses. The structure of methoxyneihumicin (1), previously proposed in a conference poster lacking publicly available experimental data, was validated for the first time by detailed NMR analyses and X-ray diffraction study. The two enantiomers nocazines D (2a) and E (2b) were isolated as a mixture. Compounds 3 and 4 were only known as synthetic compounds before. Methoxyneihumicin (1) exhibited *in vitro* cytotoxicities against MCF-7 and SF-268 with IC₅₀ values of 4.6 and 12.7 μM, respectively, better than those of 6 (22.0 and 20.6 μM). The other compounds showed less pronounced cytotoxicities against three tested human cancer cell lines and no compounds displayed antibacterial activities toward four indicator strains.

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INTRODUCTION

Marine actinomycetes have been proven as a prolific resource for drug discovery.^{1–3} However, reports of natural products derived from deep-sea (>1000 m) culturable actinomycetes are still scarce, probably because of the difficulty in accessing the deep-sea samples and the unculturability of deep-sea-derived actinomycetes.⁴ Our recent investigations on deep-sea-derived actinomycetes have led to the discovery of culturable new actinobacterial taxa,^{5–9} and the isolation of novel natural products, including spiro-tetronates,¹⁰ β-carboline and indolactam alkaloids,¹¹ spiro-bisindole alkaloids,¹² diaza-anthraquinone derivatives¹³ and angucycline glycosides,¹⁴ which exhibit antibacterial, anti-malarial and antitumor properties. From our numerous actinomycetes derived from deep-sea sediments, the isolate SCSIO 03039, identified as a *Nocardiopsis alba*, was found to produce seven diketopiperazines (DKPs), including two new DKPs nocazines D (2a) and E (2b), and five known DKPs (1, 3–6). Compound 1, methoxyneihumicin, previously reported in a conference poster without available experimental data,¹⁵ was structurally validated herein for the first time by NMR analyses and the X-ray diffraction study. Compound 3 (*S,Z*)-3-benzylidene-6-methylpiperazine-2,5-dione and 4 (*S,Z*)-3-benzylidene-6-isopropylpiperazine-2,5-dione

were only known as synthetic compounds before.¹⁶ The two known DKPs, (3*Z*,6*Z*)-3-(4-methoxybenzylidene)-6-(2-methylpropylidene)-piperazine-2,5-dione (5)^{17,18} and XR334 (6),¹⁹ were reisolated from *N. alba* SCSIO 03039. In addition, another two known compounds, 2-methoxy-1,4-naphthoquinone (7)^{20,21} and 1-hydroxy-4-methoxy-2-naphthoic acid (8),²² were also isolated. We reported herein the isolation, structure elucidation and biological activities of these compounds.

RESULTS AND DISCUSSION

Taxonomy of the producing strain

The almost full 16S rRNA gene sequence (1447 bp) of the producing strain SCSIO 03039 was submitted to GenBank under the accession number JX195181, which showed the highest similarity (99.3%) to that of *N. alba* DSM 43377^T (X97883). The phylogenetic tree generated by a neighbor-joining method clearly revealed the evolutionary relationship of the strain SCSIO 03039 with a group of *Nocardiopsis* species (Supplementary Figure S1). On the basis of the 16S rRNA gene sequence analysis,²³ the strain SCSIO 03039 was designated as a species of *N. alba*.

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Isolation of compounds 1-8

The fermentation broths of 201 culture of *N. alba* SCSIO 03039 were extracted with butanone and the mycelia cakes were extracted with methanol, respectively. The combined crude extracts were subjected to various chromatographic methods (such as silica gel column, Sephadex LH-20 chromatography and semipreparative HPLC) and led to the isolation of two new DKPs nocazines D (**2a**) and E (**2b**), five known DKPs (**1**, **3–6**), along with two known compounds 2-methoxy-1,4-naphthoquinone (**7**) and 1-hydroxy-4-methoxy-2-naphthoic acid (**8**).

Structure elucidation

Compounds **5–8** (Figure 1) were found to be known compounds (3*Z*,6*Z*)-3-(4-methoxybenzylidene)-6-(2-methylpropylidene)-piperazine-2,5-dione (**5**),^{17,18} XR334 (**6**),¹⁹ 2-methoxy-1,4-naphthoquinone (**7**)^{20,21} and 1-hydroxy-4-methoxy-2-naphthoic acid (**8**),²² respectively, by comparing their NMR data with those previously reported (Supplementary Figures S6–S9).

The molecular formula of compound **1** was determined to be C₂₀H₁₈N₂O₃ on the basis of high-resolution fast atom bombardment MS (HRFABMS) (m/z 335.1393 [M + H]⁺, calcd. for 335.1396). The ¹H and ¹³C NMR data of **1** were very similar to those of nocazine A,²⁴ a metabolite isolated from the marine-derived *Nocardiopsis dassionvillei* HR10-5. The comparison of ¹H and ¹³C NMR data between **1** and nocazine A revealed the loss of signals for one N-Me and one methoxy group in **1**. The sets of coupled ¹H NMR signals at δ_{H} 7.43 (2H, ddd, $J = 8.0, 7.5, 1.8$ Hz), 7.37 (2H, brd, $J = 7.5$ Hz) and 7.32 (1H, tt, $J = 7.8, 1.5$ Hz) revealed the presence of one 1-substituted benzene in **1**. HMBC correlations from H-7 to C-9/C-13 located the 1-substituted benzene at C-7 (Figure 2). Thus, these comparisons indicated that **1** differed from nocazine A by losing N-1 methyl group and C-11 methoxy group (Figure 1). The X-ray crystal diffraction analysis of **1** also supports this conclusion (Figure 3), and further determined the *Z* configuration of both $\Delta^{6,7}$ and $\Delta^{3,14}$ double bonds. Thus, compound **1** was identified as (3*Z*, 6*Z*)-6-benzylidene-5-methoxy-3-(4-methoxybenzylidene)-1,6-dihydropyrazin-2(3H)-one (methoxyneihumicin, Figure 1). It should be noted that the structure of **1** was previously proposed in a conference poster without publicly available experimental data.¹⁵

The molecular formula of compound **2** was determined to be C₁₉H₁₈N₂O₃ on the basis of HRFABMS (m/z 345.1215 [M + Na]⁺, calcd for C₁₉H₁₈N₂O₃Na 345.1215). The ¹H and ¹³C NMR data of **2** were classified into one methoxy group ($\delta_{\text{H}}/\delta_{\text{C}}$ 3.83/55.4), ten sp² methines, four sp² quaternary carbons, one sp² methylene, one sp³ methine and two amidocarbonyl carbons (δ_{C} 159.9, 165.0) (Table 1).

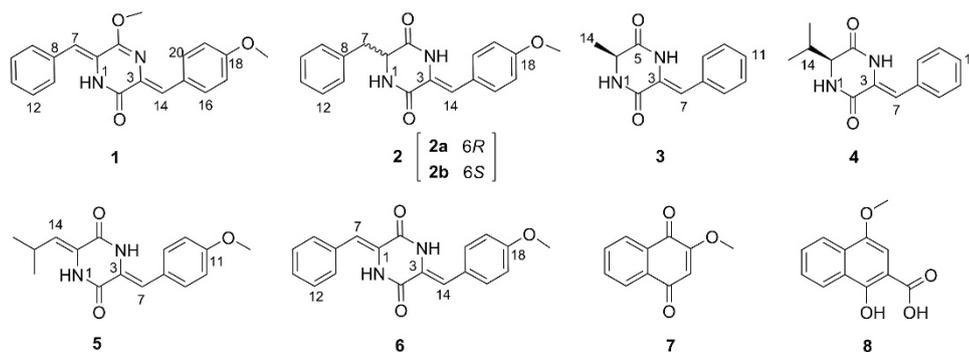


Figure 1 Chemical structures of compounds 1–8.

The comparison of 1D NMR data of **2** with those of nocazine C²⁴ showed that **2** only differed from nocazine C by lacking signals for N-4 methyl group (N-1 methyl group in nocazine C). Correspondingly, C-2, C-3, C-5 and C-14 (C-5, C-6, C-2 and C-7, respectively, in nocazine C²⁴) shifted upfield for 4.5, 4.7, 2.5 and 5.0 p.p.m., respectively. The planar structure of **2** (Figure 1) was further confirmed by HMBC correlations from H-6 to C-2/C-5, from H-14 to C-2/C-5 and from H-7 to C-5/C-6 (Figure 2). The $\Delta^{3,14}$ double bond in **2** was deduced to be *Z* configuration from the relative downfield shift of H-14 (δ_{H} 6.81), in comparison with the vinyl proton chemical shifts of H-7 (δ_{H} 7.03) in nocazine C,²⁴ and H-7 (δ_{H} 6.54) in (3*S*,6*E*)-3-benzyl-6-benzylidenepiperazine-2,5-dione,²⁵ based on previous reports that the (*Z*)-vinyl proton was farther downfield than the (*E*)-vinyl proton because of the deshielding effect of the 5-ketone in DKP compounds.^{24–27} Consistent with the *Z* configuration of $\Delta^{3,14}$, the NOESY correlation was observed between 4-NH (δ_{H} 9.65 in DMSO-*d*₆) and H-20 (δ_{H} 7.18 in DMSO-*d*₆), and no NOESY correlation between 4-NH (δ_{H} 9.65 in DMSO-*d*₆) and H-14 (δ_{H} 6.30 in DMSO-*d*₆) was observed (Figure 2, Supplementary Figure S3). Marfey's method was applied to determine the absolute configuration of the phenylalanine moiety in **2**.²⁸ Unexpectedly, both L- and D-phenylalanine derivatives were found in HPLC analysis of the 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) derivatives of the acid hydrolysates of **2**, when compared with those prepared from authentic samples of both L- and D-phenylalanine (FDAA $t_{\text{R}} = 11.9$ min; L-phenylalanine derivative $t_{\text{R}} = 14.5$ min;

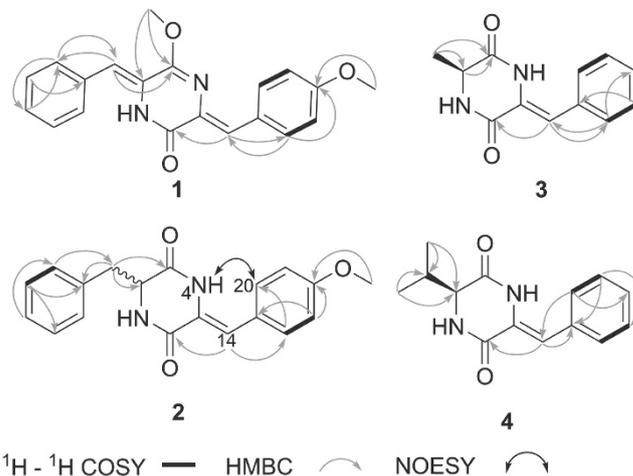


Figure 2 Selected key ¹H-¹H COSY and HMBC correlations for compounds 1–4.

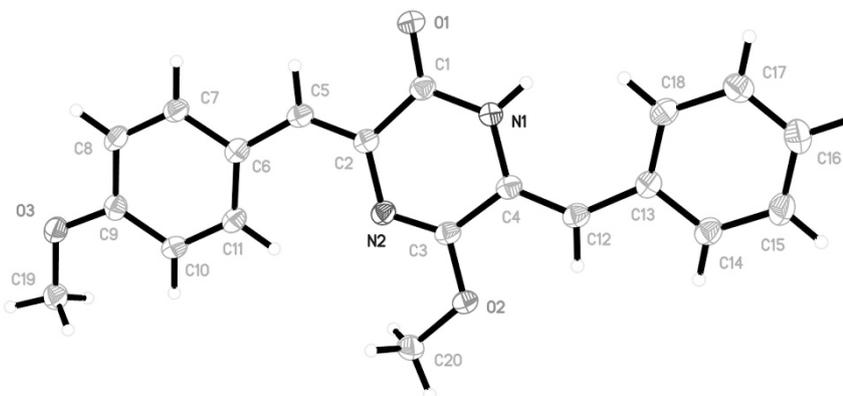


Figure 3 X-ray crystal structure of methoxyneihumicin (**1**).

Table 1 ^1H and ^{13}C NMR assignment of compound **1–4** (J in Hz within parentheses) (500 MHz for ^1H and 125 MHz for ^{13}C , CDCl_3 and tetramethylsilane)

Position	1		2		3		4	
	δ_{C}	δ_{H} (multi, J in Hz)	δ_{C}	δ_{H} (multi, J in Hz)	δ_{C}	δ_{H} (multi, J in Hz)	δ_{C}	δ_{H} (multi, J in Hz)
1-NH								6.39 br s
2	160.4 C		159.9 C		159.7 C		160.1 C	
3	129.7 C		123.8 C		125.4 C		125.2 C	
5	153.6 C		165.0 C		166.2 C		165.3 C	
5-OCH ₃	55.3 CH ₃	3.85 (s)						
6	124.5 C		57.1 CH	4.40 (m)	51.6 CH	4.30 (m)	61.3 CH	4.07 (dd, 3.0, 2.5)
7	110.1 CH	6.56 (s)	41.0 CH ₂	3.05 (dd, 13.8, 8.8) 3.39 (dd 13.8, 3.8)	116.2 CH	6.99 (s)	116.0 CH	7.00 (s)
8	133.6 C		134.7 C		132.8 C		132.8 C	
9, 13	128.3 CH	7.37 (brd, 7.5)	129.7 CH	7.24 overlapped	128.4 CH	7.35 (d, 8.0)	128.8 CH	7.36 (d, 7.5)
10, 12	129.5 CH	7.43 (ddd, 8.0, 7.5, 1.8)	129.1 CH	7.35 (t, 7.3)	129.5 CH	7.45 (dd, 8.0, 7.5)	129.5 CH	7.44 (dd, 8.5, 7.5)
11	128.3 CH	7.32 (tt, 7.8, 1.5)	127.8 CH	7.28 (d, 7.0)	128.8 CH	7.36 overlapped	128.4 CH	7.34 (m, overlapped)
14	128.6 CH	7.27 (s)	116.3 CH	6.81 (s)	21.9 CH ₃	1.60 (d, 7.0)	33.3 CH	2.43 (ddd, 7.0, 6.8, 3.0)
15	128.0 C		125.0 C				16.2 CH ₃	1.01 (d, 6.8)
16, 20	133.4 CH	8.12 (d, 9.0)	129.9 CH	7.22 (d, 8.5)			18.5 CH ₃	1.10 (d, 7.0)
17, 19	113.9 CH	6.93 (d, 9.0)	114.9 CH	6.95 (d, 8.5)				
18	160.4 C		159.8 C					
18-OCH ₃	54.5 CH ₃	4.06 (s)	55.4 CH ₃	3.83 (s)				

D-phenylalanine derivative $t_{\text{R}} = 15.3$ min) (Supplementary Figure S10A). Further HPLC analysis revealed that the two isomers were separable on an analytical chiral column (Supplementary Figure S10B). These data indicated that **2** was actually a mixture of two enantiomers of **2a** (6R, containing a D-phenylalanine) and **2b** (6S, containing an L-phenylalanine) (Figure 1). However, compound **2** was only isolated in minor amounts, preventing further efforts to obtain pure **2a** and **2b** by preparative chiral HPLC. Thus, the structures of enantiomers **2a** and **2b** were determined as (R, Z)-3-benzylpiperazine-6-(4-methoxybenzylidene)-2,5-dione and (S, Z)-3-benzylpiperazine-6-(4-methoxybenzylidene)-2,5-dione, designated nocazines D (**2a**) and E (**2b**), respectively (Figure 1).

Compound **3** was obtained as white powder. The inspection of the ^1H and ^{13}C NMR data of **3** revealed the presence of one methyl group ($\delta_{\text{H}}/\delta_{\text{C}}$ 1.60/21.9), six sp^2 methines, one sp^3 methine, two sp^2 quaternary carbons and two amidocarbonyl carbons (δ_{C} 159.7, 166.2) (Table 1). The comparison of its NMR data with that of **2** revealed the existence of 3-benzylidenepiperazine-2,5-dione moiety, which were further identified by the HMBC correlations from H-7 to C-2/C-9, from H-9 to C-7/C-11 and from H-10 to C-8. In addition,

the HMBC correlations from H-14 to C-5/C-6 located the methyl group ($\delta_{\text{H}}/\delta_{\text{C}}$ 1.60/21.9) at C-6. Therefore, the planar structure of **3** was determined to be 3-benzylidene-6-methylpiperazine-2,5-dione (Figure 1). The Z configuration of $\Delta^{3,7}$ double bond in **3** was deduced from the chemical shift of H-7 (δ_{H} 6.99), similar to that of **2** (δ_{H} 6.81, H-14) (Table 1). The absolute configuration of the alanine moiety in **3** was determined as L by Marfey's method (FDAA $t_{\text{R}} = 15.9$ min; L-alanine derivative $t_{\text{R}} = 14.5$ min; D-alanine derivative $t_{\text{R}} = 16.2$ min) (Supplementary Figure S11A). Therefore, the structure of compound **3** was elucidated as (S, Z)-3-benzylidene-6-methylpiperazine-2,5-dione. The compound **3** was chemically synthesized before,¹⁶ however, only ^1H NMR data were available.¹⁶

The molecular formula of compound **4** was assigned to be $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_2$ by HRFABMS (m/z 245.1294 $[\text{M} + \text{H}]^+$, calcd. for $\text{C}_{14}\text{H}_{17}\text{N}_2\text{O}_2$ 245.1290). The ^1H and ^{13}C NMR data of **4** was consistent with those of the synthetic compound 6-benzylidene-3-isopropylpiperazine-2,5-dione. (Supplementary Figure S5). The Z configuration of $\Delta^{3,7}$ double bond in **4** could be deduced from the similarity of the chemical shift of H-7 (δ_{H} 7.00) to that of **3** (δ_{H} 6.99). The absolute configuration of the valine moiety in **4** was determined

as L by Marfey's method (FDAA t_R = 11.9 min; L-valine derivative t_R = 12.9 min; D-valine derivative t_R = 14.1 min) (Supplementary Figure S11B). Thus, compound **4** was elucidated as (S, Z)-3-benzylidene-6-isopropylpiperazine-2,5-dione (Figure 1).

Biological activity

Eight compounds (**1–8**) were evaluated for *in vitro* cytotoxic activities against three human cancer cell lines SF-268, MCF-7 and NCIH460, and antibacterial activities against four indicator strains *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *Bacillus subtilis* SCSIO BS01 and *Bacillus thuringiensis* SCSIO BT01. Methoxyneihumicin (**1**) showed moderate activities against human tumor cell lines MCF-7 (IC₅₀ 4.6 μM) and SF-268 (IC₅₀ 12.7 μM). Compound **6** (XR334), lacking an O-methyl group at C-5 (Figure 2), exhibited less cytotoxic activities than **1**. However, when the Δ^{6,7} double bond in compound **6** was reduced to afford a mixture of **2a** and **2b**, the latter compounds showed little cytotoxic activities (Table 2). The DKPs **3–5** and the compounds **7** and **8** exhibited little antiproliferative activities against the three tested cancer cell lines (Table 2). And no compounds displayed antibacterial activities (MIC > 128 μg ml⁻¹) against four indicator strains.

DKPs are the smallest cyclic peptides known and exhibit a wide spectrum of biological activities with various therapeutic possibilities.^{29,30} Recently, marine actinomycetes have been emerged as producers for novel DKPs, such as cyclomarazines,³¹ dimeric DKP naseseazines,³² neihumicins,³³ nocardioazines (containing a novel bridged DKP scaffold),³⁴ nocazines,²⁴ and cyclo(2-hydroxy-Pro-R-Leu).³⁵ However, none of the described producers were isolated from a deep-sea environment greater than the depth of 1000 m. In contrast, the strain *N. alba* SCSIO 03039 was isolated from the abyssal deep sedimental sample (–3412 m) in the Indian Ocean. In this study, we showed that this deep-sea-derived actinomycete was capable of producing seven DKPs. Nocazines D and E (**2a** and **2b**) were new. Methoxyneihumicin (**1**) was structurally validated for the first time by NMR and X-ray analyses. Compounds **3** and **4**, previously known only as synthetic compounds, were isolated for the first time from a natural source. Interestingly, methoxyneihumicin (**1**) displayed cytotoxicities against MCF-7 and SF-268 with IC₅₀ values of 4.6 and 12.7 μM, significantly better than other DKPs isolated in this study.

METHODS

General experimental procedures

Materials for column chromatography were silica gel (100–200 mesh; 300–400 mesh; Jiangyou Silica Gel Development, Inc., Yantai, China), Sephadex LH-20

(40–70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden) and YMC*-GEL ODS-A (12 nm S-50 μm; YMC Company Ltd., Kyoto, Japan). TLC (0.1–0.2 mm or 0.3–0.4 mm) was conducted with precoated glass plates (silica gel GF₂₅₄, 10–40 nm; Jiangyou Silica Gel Development, Inc.). Medium-pressure liquid chromatography was performed on automatic flash chromatography (EZ Purifier III; Leisure Science Co., Ltd., Shanghai, China). ESI-MS was acquired on a MDS SCIEX API 2000 liquid chromatography/tandem MS (LC/MS/MS) instrument (Applied Biosystems, Inc., Forster, CA, USA). HRFABMS data were obtained on a quadrupole-time-of-flight MS (Waters, Milford, MA, USA). The optical rotation was recorded on a 341 Polarimeter (PerkinElmer, Inc., Norwalk, CT, USA). UV spectra were recorded on an U-2900 spectrophotometer (Hitachi, Tokyo, Japan). The analytical chiral packed column Chiralpak AD-H (250 × 4.6 mm² ID, 5 μm) (Daicel Chemicals, Tokyo, Japan), and an Agilent 1200 series HPLC system (Agilent, Santa Clara, CA, USA) were used for the chirality analysis. ¹H, ¹³C NMR and 2D NMR spectra were recorded on a Bruker AV-500 NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) with tetramethylsilane (δ 0.0 p.p.m.) as the internal standard. ¹H NMR data were reported as follows: chemical shift (multiplicity: singlet (s), doublet (d), triplet (t) and multiplet (m)), coupling constants (Hz); ¹³C NMR data were reported as follows: chemical shift (quaternary carbon (C), methine (CH), methylene (CH₂), methyl (CH₃)). Deuterated NMR solvents were purchased from Cambridge Isotopes (Andover, MA, USA).

Isolation and taxonomic studies of the strain SCSIO 03039

A sediment sample was collected aseptically with a grab-bucket collection sampler from the location (E 87°59.7', N 9°59.3') at the depth of 3412 m in the Indian Ocean. The surface layer (0–10 cm) of the sediment sample was obtained as a subsample. The wet subsample was first air-dried aseptically in a laminar flow hood. Then, 2 g air-dried sample was suspended in 18 ml sterile seawater. Finally, 0.1 ml of such seawater suspensions was spread on isolation media plates containing Gauze's medium No.1 (soluble starch 2%, KNO₃ 0.1%, K₂HPO₄ 0.05%, MgSO₄ · 7 H₂O 0.05%, FeSO₄ · 7 H₂O 0.01%, pH 7.4). After incubation at 28 °C for 2 weeks, one gray white colony, designated SCSIO 03039, was isolated and deposited in the type culture collection of Center for Marine Microbiology, Research Network of Applied Microbiology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China. Isolation of genomic DNA, amplification of 16S rRNA gene by PCR, sequence comparison and phylogenetic tree construction of the strain SCSIO 03039 (Supplementary Figure S1) were performed as described previously.⁵

Fermentation of the strain SCSIO 03039, extraction and isolation of secondary metabolites

A single colony of SCSIO 03039 on ISP 4 medium (soluble starch 1%, K₂HPO₄ 0.1%, MgSO₄ · 7 H₂O 0.1%, (NH₄)₂SO₄ 0.2%, CaCO₃ 0.2%, sea salt 3%, pH 7.0–7.4 before sterilization) was inoculated into a 250-ml Erlenmeyer flask containing 30 ml of the seed medium (soluble starch 2%, glucose 1%, bacterial protein 0.5%, yeast exact 0.5%, CaCO₃ 0.2%, sea salt 3%, pH 7.0 before sterilization). After incubating on a rotary shaker (200 r.p.m.) for 3 days at 28 °C, the pre-cultures were transferred to 1000-ml Erlenmeyer flask containing 200 ml of the seed medium. A total of 201 cultures were made by this way. Fermentations were done on a rotary shaker (200 r.p.m.) for 7 days at 28 °C. The 20-l fermentation broths were extracted four times with 20 l butanone, and the mycelia cakes were extracted four times with 4 l methanol. After removing solvents, both residues from fermentation broths and mycelia cakes were combined to afford 11.86 g crude extracts.

The crude extracts were subjected to normal phase silica gel column (100–200 mesh, 90 g) and eluted with CHCl₃/CH₃OH (100/0, 100/1, 100/2, 100/4, 100/8, 100/16, 100/32 and 0/100, v/v, each of 360 ml) to yield eight fractions (Fr.1–Fr.8). Fr.1 and Fr.2 were combined and subjected to Sephadex LH-20 chromatography and were eluted with CHCl₃/CH₃OH (1:1) to give six subfractions (Fr.1-1–Fr.1-6). Fr.1-2 (23.7 mg) was further purified by semi-preparative HPLC on a Varian Star Workstation, using a reversed phase column C18 (YMC*GEL ODS-A, 120 Å S-5 μm, 250 × 10 mm²; solvent A, 0.1% formic acid in water; solvent B, 90% acetonitrile in water; eluted with constant 70% solvent B) to obtain compounds **7** (2.9 mg) and **8** (3.0 mg).

Table 2 Cytotoxicities of compounds **1–8**

Compounds	IC ₅₀ (μM)		
	SF-268	MCF-7	NCI-H460
1	12.7	4.6	87.7
2	> 100	> 100	> 100
3	> 100	> 100	> 100
4	> 100	> 100	> 100
5	> 100	75.0	99.4
6	20.6	22.0	97.0
7	93.2	95.0	97.6
8	> 100	> 100	> 100
Cisplatin	3.0	13.9	6.7

Fr.1-5 (1.42 g) was subjected to silica gel column (100–200 mesh) and was eluted with petroleum ether/ethyl acetate (100/0, 100/10, 100/20, v/v, each of 100 ml) to give three subfractions: Fr.1-5-1 (100/0, v/v), Fr.1-5-2 (100/10, v/v) and Fr.1-5-3 (100/20, v/v). The elutions from petroleum ether/ethyl acetate (100/30, 100 ml) were collected with 10 ml each and combined on the basis of TLC analysis to give four subfractions Fr.1-5-4–Fr.1-5-7. Fr.1-5-4 (87.2 mg) was purified by semipreparative HPLC (YMC*GEL ODS-A, 120 Å S-5 µm, 250 × 10 mm²; solvent A, 0.1% formic acid in water; solvent B, 90% acetonitrile in water; eluted with constant 85% solvent B) to obtain compound **1** (8.2 mg). Fr.1-5-7 (185.5 mg) was purified by semipreparative HPLC (YMC*GEL ODS-A, 120 Å S-5 µm, 250 × 10 mm²; solvent A, 0.1% formic acid in water; solvent B, 90% acetonitrile in water, eluted with constant 70% solvent B) to obtain the compounds **5** (10.1 mg) and **6** (3.6 mg). Fr.3 was subjected to Sephadex LH-20 chromatography and was eluted with CHCl₃/CH₃OH (1:1) to give two subfractions (Fr.3-1 and Fr.3-2). Fr.3-2 (1.79 g) was separated by silica gel column chromatography (100–200 mesh) and was eluted with CHCl₃/CH₃OH (30:1) to give two subfractions (Fr.3-1-1 and Fr.3-1-2). Fr.3-1-2 (531 mg) was separated by normal phase silica gel column chromatography (100–200 mesh) and was eluted with petroleum ether/ethyl acetate (100/0, 100/10, 100/20, 100/30) to give four subfractions (Fr.3-1-2-1–Fr.3-1-2-4). Fr.3-1-2-2 (90.2 mg), Fr.3-1-2-3 (111 mg) and Fr.3-1-2-4 (18.1 mg) were further purified by semipreparative HPLC (YMC*GEL ODS-A, 120 Å S-5 µm, 250 × 10 mm²; solvent A, 0.1% formic acid in water; solvent B, 90% acetonitrile in water) to yield compounds **2** (3.4 mg) from Fr.3-1-2-2 (eluted with constant 50% solvent B), **3** (6.7 mg) from Fr.3-1-2-3 (eluted with constant 50% solvent B) and **4** (2.6 mg) from Fr.3-1-2-4 (eluted with constant 55% solvent B), respectively.

Determining the absolute configuration of amino acids in compounds 2–4 by Marfey's method

The FDAA derivatized hydrolysates of compounds **2** (0.50 mg), **3** (0.50 mg) and **4** (0.58 mg), and the standard amino acids were prepared according to the published method.²⁴ The FDAA derivatives were then subjected to HPLC analysis (Luna C₁₈ column; 5 µm, 4.6 × 150 mm²) using the following solvent system: solvent A, 10% acetonitrile in water supplementing with 0.1% formic acid; solvent B, 90% acetonitrile in water. The HPLC program for detecting FDAA derivatives of compounds **2** and **4** was set as: 5–100% solvent B (0–20 min, linear gradient), 100% solvent B (20–23 min), 100–5% solvent B (23–24 min) and 5% solvent B (24–30 min). The HPLC program for detecting FDAA derivatives of compound **3** was set as: 5–55% solvent B (0–20 min, linear gradient), 55–100% solvent B (20–24 min), 100% solvent B (24–25 min), 5–100% solvent B (25–26 min) and 5% solvent B (26–30 min). The flow rate was set at 1 ml min⁻¹ with UV detection at λ = 340 nm.

Chiral analysis of compound 2

The solution of **2** in methanol was subjected to HPLC analysis (Chiralpak AD-H, 250 × 4.6 mm², ID 5 µm) (Daicel Chemicals) on an Agilent 1200 series HPLC system, eluted with 20% 2-propanol in n-hexane, UV detection at λ = 254 nm.

Methoxyneihumicin (1): yellow needle crystal, UV/vis (in CH₃OH) λ_{max} nm (log ε): 202 (3.96), 227 (3.76), 310 (3.68) nm, ESI-MS *m/z* 357.0 [M + Na]⁺, 333.1 [M – H][–], HRFABMS *m/z* 335.1393 (calcd. for C₂₀H₁₉N₂O₃ [M + H]⁺, 335.1396), see Table 1 and Supplementary Figure S2 for ¹H NMR and ¹³C NMR. X-ray crystal structure analysis of compound **1**: Yellow needle crystal, C₂₀H₁₈N₂O₃, *M* = 334.36, *T* = 150(2) K, λ = 1.54178 Å; crystal system, monoclinic, space group P 1 2₁/c 1, *a* = 6.39230(10) Å, *b* = 14.9704(3) Å, *c* = 16.7559(3) Å, α = 90°, β = 91.785(2)°, γ = 90°, *V* = 1602.68(5) Å³, *Z* = 4, *D_c* = 1.386 Mg m⁻³; absorption coefficient, 0.766 mm⁻¹, *F*(000) = 704; and crystal size, 0.43 × 0.33 × 0.22 mm³. Theta range for data collection, 3.96–67.01°; index ranges, –7 ≤ *h* ≤ 5, –17 ≤ *k* ≤ 17, –19 ≤ *l* ≤ 20; reflections collected, 9663; independent reflections, 2858 (*R* (int) = 0.0165); completeness to theta = 67.01°, 99.8%; absorption correction, semi-empirical from equivalents; maximum and minimum transmission, 0.8496 and 0.7343; refinement method, full-matrix least-squares on *F*²; data/restraints/parameters, 2858/0/228; goodness-of-fit on *F*², 1.042; NFinal *R* indices (*I* > 2 sigma (*I*)), *R*₁ = 0.0342, *wR*₂ = 0.0856; *R* indices (all data), *R*₁ = 0.0376, *wR*₂ = 0.0882;

and largest difference peak and hole, 0.206 and –0.230 e.Å⁻³. Crystallographic data (excluding structure factors) for compound **1** in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary publication number CCDC 888963. Copies of the data can be obtained free of charge on application to CCDC.

3-Benzylpiperazine-6-(4-methoxybenzylidene)-2,5-dione (a mixture of nocazine D (**2a**, 6*R*) and nocazine E (**2b**, 6*S*)): white powder, UV/vis (in CH₃OH) λ_{max} nm (log ε): 204 (3.85), 311 (3.44) nm, ESI-MS *m/z* 345.1 [M + Na]⁺, 321.3 [M – H][–], HRFABMS *m/z* 345.1215 (calcd. for C₁₉H₁₈N₂O₃Na [M + Na]⁺, 345.1215; see Table 1 and Supplementary Figure S3 for ¹H and ¹³C NMR data.

(**S**, **Z**)-**3-Benzylidene-6-methylpiperazine-2,5-dione (3)**: white powder, UV/vis (in CH₃OH) λ_{max} nm (log ε): 219 (4.52), 292 (3.75) nm, ESI-MS *m/z* 217.4 [M + H]⁺, 215.1 [M – H][–]; HRFABMS *m/z* 215.0817 (calcd. for C₁₂H₁₁N₂O₂ [M – H][–], 215.0815); [α]_D²⁵ = –239° (*c* = 0.14, CH₃OH); see Table 1 and Supplementary Figure S4 for ¹H and ¹³C NMR data.

(**S**, **Z**)-**3-Benzylidene-6-isopropylpiperazine-2,5-dione (4)**: white needle crystal, UV/vis (in CH₃OH) λ_{max} nm (log ε): 224 (3.65), 296 (3.74) nm, ESI-MS *m/z* 267.3 [M + Na]⁺, 245.2 [M + H]⁺, 243.2 [M – H][–], HRFABMS *m/z* 245.1294 (calcd. for C₁₄H₁₇N₂O₂ [M + H]⁺, 245.1290), 267.1115 (calcd. for C₁₄H₁₆N₂O₂Na, [M + Na]⁺, 267.1109); [α]_D²⁵ = –2.5° (*c* = 0.08, CH₃OH); see Supplementary Figure S5 for ¹H NMR data.

(**3Z**, **6Z**)-**3-(4-Methoxybenzylidene)-6-(2-methylpropylidene)piperazine-2,5-dione (5)**: white needle crystal, ESI-MS *m/z* 309.2 [M + Na]⁺, 285.3 [M – H][–]; see Supplementary Figure S6 for ¹H and ¹³C NMR data.

XR334 (6): yellow powder, ESI-MS *m/z* 321.1 [M + H]⁺, 343.1 [M + Na]⁺; see Supplementary Figure S7 for ¹H and ¹³C NMR data.

2-Methoxynaphthalene-1,4-dione (7): yellow powder, ESI-MS *m/z* 211.2 [M + H]⁺, 243.2 [M + Na]⁺; see Supplementary Figure S8 for ¹H and ¹³C NMR data.

1-Hydroxy-4-methoxy-2-naphthoic acid (8): brown powder, ESI-MS *m/z* 241.0 [M + Na]⁺, 217.1 [M – H][–]; see Supplementary Figure S9 for ¹H and ¹³C NMR data.

Biological activity

Antibacterial activities were evaluated against bacterial strains *S. aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *B. subtilis* SCSIO BS01 and *B. thuringiensis* SCSIO BT01 according to previously described methods.³⁶ Cytotoxic activities were determined against three tumor cell lines, including MCF-7 cells (human breast adenocarcinoma cell line), NCI-H460 (human non-small cell lung cancer cell line) and SF-268 (human glioma cell line), according to published protocols.³⁷

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