Organic LETTERS

Letter

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Three Diketopiperazine Alkaloids with Spirocyclic Skeletons and One Bisthiodiketopiperazine Derivative from the Mangrove-Derived Endophytic Fungus *Penicillium brocae* MA-231

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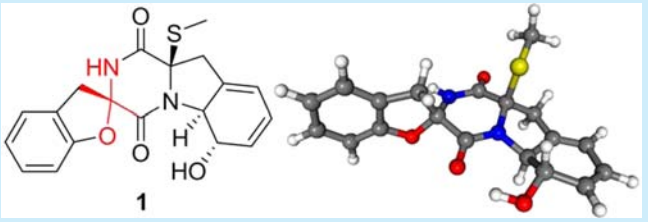
Supporting Information

ABSTRACT: Four new diketopiperazines including spirobrocazines A–C (**1**–**3**) and brocazine G (**4**) were characterized from the mangrove-derived *Penicillium brocae* MA-231. Compounds **1** and **2** had a 6/5/6/5/6 cyclic system with a rare spirocyclic center at C-2. Their structures and absolute configurations were determined by spectroscopic analysis, TDDFT-ECD calculations, and X-ray diffraction. Compound **4** exhibited potent cytotoxicity against both sensitive and cisplatin-resistant human ovarian cancer cells and strong antimicrobial activity against pathogenic *Staphylococcus aureus*.



The structurally diverse and biologically significant epithiodioxopiperazines (ETPs) that are mainly produced by marine fungi have drawn wide attention in recent years.[1] ETPs, characterized by a unique disulfide bridge or polysulfide six-membered dioxopiperazine ring, have been found to possess a broad spectrum of biological activities, such as antimicrobial, antitumor, antiviral, immunosuppressive, and enzyme inhibitory activities.[2] During our ongoing research on the discovery of bioactive metabolites from marine derived fungi,[3−5] a series of ETPs were previously isolated from the mangrove-derived endophytic fungus *Penicillium brocae* MA-231 that was cultured on the liquid potato−dextrose broth (PDB) medium.[6,7] Further work on this fungus using the OSMAC (One Strain, MAny Compounds) approach[8] revealed that alteration of the fermentation media strongly affected the chemical profiles of this fungal strain. As a result, spirobrocazines A–C (**1**–**3**), which possess very rare spirocyclic skeletons among reported ETPs, together with a new bisthiodiketopiperazine derivative, brocazine G (**4**), were isolated from the fungus that was cultured on Czapek medium. It is noteworthy that spirobrocazine C (**3**) possessed a different absolute configuration at the spirocyclic center C-2 from that of spirobrocazines A (**1**) and B (**2**). Herein, the isolation, structure elucidation, and biological evaluation of compounds **1**–**4** are presented (Figure 1).

Spirobrocazine A (**1**) was determined to have the molecular formula $C_{19}H_{18}N_2O_4S$ based on the positive HR-ESI-MS data,

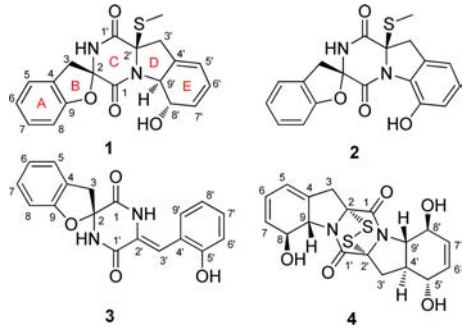


Figure 1. Chemical structures of compounds **1**–**4**.

indicating 12 degrees of unsaturation. Analysis of the ^{1}H NMR data (Table 1) as well as COSY and HSQC spectra (Supporting Information) revealed the presence of an *ortho*-substituted phenyl system, two sp^3-hybridized and three olefinic methines, two methylenes, and one tertiary methyl group in **1**. Its ^{13}C NMR and DEPT data (Table 1) showed the presence of 19 carbon resonances, including one methyl, two aliphatic methylenes, seven aromatic/olefinic methines, two oxygenated or heteroatom-bonded methines, two diagnostic amide carbonyls, three nonprotonated olefinic carbons, and two non-

Received: September 1, 2016
Published: October 10, 2016

DOI: 10.1021/acs.orglett.6b02620
Org. Lett. 2016, 18, 5304−5307

Table 1. NMR Data for Compounds 1−4

	1 (acquired in acetone-d_6)		**2** (acquired in DMSO-d_6)		**3** (acquired in DMSO-d_6)		**4** (acquired in $CDCl_3$)	
no.	δ_C [a]	δ_H [b] (mult, *J*, Hz)	δ_C [a]	δ_H [b] (mult, *J*, Hz)	δ_C [a]	δ_H [b] (mult, *J*, Hz)	δ_C [a]	δ_H [b] (mult, *J*, Hz)
1	167.1		163.3		161.7		164.6	
2	94.1		93.2		92.6		78.2	
3α	40.5	4.14, d (16.9)	41.2	4.04, d (17.0)	36.5	3.97, d (16.6)	36.4	3.69, d (17.8)
3β		3.33, d (16.9)		3.40, d (17.0)		3.26, d (16.6)		2.93, d (17.8)
4	126.2		125.3		125.3		131.2	
5	125.4	7.24, d (7.9)	124.4	7.30, d (7.2)	124.6	7.27, d (7.3)	120.6	5.98, d (2.3)
6	122.4	6.92, t (7.9)	121.4	6.95, dd (7.2, 6.9)	121.2	6.91, dd (7.7, 7.3)	123.5	5.92, d (9.8)
7	129.2	7.16, t (7.9)	128.1	7.16, dd (8.0, 6.9)	128.0	7.14, t (7.7)	130.5	5.76, d (9.8)
8	110.3	6.80, d (7.9)	109.0	6.82, d (8.0)	108.9	6.79, d (7.7)	73.4	4.77, d (14.5)
9	158.2		156.6		156.5		69.9	4.80, d (14.5)
1′	167.4		165.5		161.2		165.7	
2′	73.9		72.5		125.1		76.7	
3′α	39.8	3.14, d (15.1)	40.7	3.70, d (16.6)	114.2	6.95, s	34.8	2.77, t (13.6)
3′β		3.08, d (15.1)		3.35, d (16.6)				2.67, dd (13.6, 5.6)
4′	134.1		132.5		120.1		46.7	2.47, m
5′	120.3	6.02, d (2.0)	116.5	6.93, d (7.0)	154.7		70.4	4.34, d (9.0)
6′	124.0	5.90, dd (9.1, 2.0)	128.8	7.20, dd (8.1, 7.0)	116.1	6.92, d (8.2)	132.6	5.82, d (10.1)
7′	131.6	5.66, dd (9.1, 1.3)	117.1	6.85, d (8.1)	130.2	7.22, t (8.2)	130.6	5.76, d (10.1)
8′	75.1	4.81, dd (12.9, 1.3)	146.1		119.5	6.89, t (8.0)	70.4	4.50, d (7.4)
9′	70.4	4.92, d (12.9)	127.0		131.2	7.44, d (8.0)	69.3	3.47, dd (12.5, 7.4)
2′-SMe	14.6	2.30, s	13.4	2.25, s				
2-NH		8.71, s		9.94, s		10.42, br s		
2′-NH						9.76, s		
5′−OH						10.66, br s		5.65, s
8-OH								5.27, s
8′−OH		5.36, s		10.00, s				5.63, s

[a]Measured at 125 MHz and multiplicities were determined by DEPT and HSQC experiments. [b]Measured at 500 MHz.

protonated heteroatom-bonded carbons. These data along with biogenetic reasoning suggested that compound **1** is an ETP derivative.

The planar structure of compound **1** was assigned by detailed analysis of its COSY and HMBC data (Figure S29). COSY correlations from H-6 to H-5 and H-7 and from H-7 to H-8, along with HMBC correlations from H-6 to C-4 and from H-7 to C-9, confirmed the presence of the *ortho*-substituted phenyl unit (ring A), while ring B was established as a 2,3-dihydrofuran unit by HMBC correlations from H-3 to C-1, C-2, C-4, C-5, and C-9. Ring E was assembled as a 1,3-cyclohexadiene unit by the COSY correlations from H-6′ to H-5′ and H-7′, from H-7′ to H-8′, and from H-8′ to H-9′, along with HMBC correlations from H-6′ to C-4′ and from H-7′ to C-9′. Subsequently, HMBC correlations from H-3′ to C-2′, C-4′, C-5′, and C-9′ led to the construction of the pyrrolidine unit (ring D) and the correlation from the protons of *S*-methyl to C-2′ attached the *S*-methyl to C-2′. These overall data combined with the molecular formula defined a thiodiketopiperazine with an unprecedented spirocyclic skeleton as compound **1**.

NOE correlations from the protons of *S*-methyl to H_2-3 and H-8′ revealed the cofacial orientation of these groups (Figure S30) and indicated that rings B and C were perpendicular to each other, while NOEs from H-3 to H-8′ confirmed the same side of them and from revealed H-3′ to H-9′. In addition, the large coupling constant between H-8′ and H-9′ (J = 12.9 Hz) suggested that they had a *trans*-diaxial relationship. The (2*S**,2′*R**,8′*S**,9′*S**) relative configuration could thus be deduced for **1**.

In order to determine the absolute configuration, a conformational search, DFT optimizations, and TDDFT-ECD calculations were performed on the arbitrarily chosen (2*S*,2′*R*,8′*S*,9′*S*)-enantiomer of **1**. B3LYP/6-31G(d) reoptimization of the initial three conformers yielded a single major conformer with 99.8% Boltzmann population (Figure S31). ECD spectra computed for this conformer at various levels reproduced well the experimental ECD spectrum, allowing the elucidation of the absolute configuration as (2*S*,2′*R*,8′*S*,9′*S*) (Figure 2).

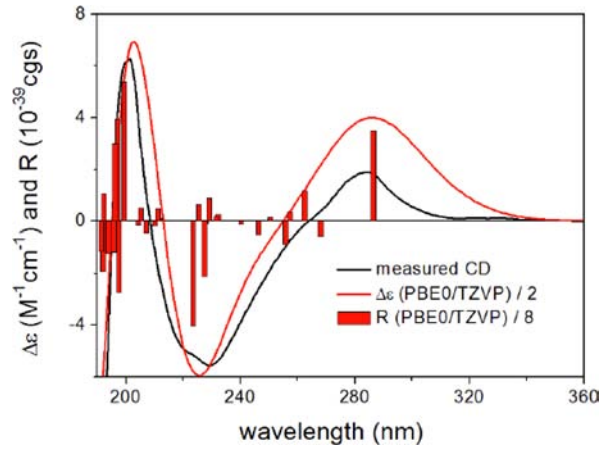


Figure 2. Experimental ECD spectrum of **1** in MeCN compared with the PBE0/TZVP ECD spectrum computed for the B3LYP/6-31G(d)-optimized single major conformer of (2*S*,2′*R*,8′*S*,9′*S*)-**1**. Bars represent computed rotational strengths of this conformer.

DOI: 10.1021/acs.orglett.6b02620
Org. Lett. 2016, 18, 5304−5307

Spirobrocazine B (**2**) had a molecular formula $C_{19}H_{16}N_2O_4S$ on the basis of positive HR-ESI-MS data, with two protons less than that of **1**. Comparison of its NMR data (Table 1) with those of **1** revealed that their structures were closely related. The primary difference was that the two oxygenated or heteroatom-bonded methine signals at δ_H 4.81/4.92 and δ_C 75.1/70.4 (CH-8′/CH-9′) in **1** were replaced by two nonprotonated aromatic carbon signals at δ_C 146.1/127.0 (C-8′/C-9′) in **2**. These observations were further confirmed by the relevant COSY and HMBC correlations (Figure S29). The relative configurations of **2** were assigned similar to those of **1** on the basis of NOE correlation from the protons of *S*-methyl to H_2-3 (Figure S30). The (2*S*,2′*R*) absolute configuration of **2** was determined by TDDFT-ECD calculations, the details of which are shown in the Supporting Information.

Spirobrocazine C (**3**), obtained as colorless crystals, was assigned a molecular formula $C_{18}H_{14}N_2O_4$ by the positive HR-ESI-MS data. The NMR data indicated the presence of two *ortho*-substituted phenyl groups, one methylene, one olefinic methine, four nonprotonated (two amide carbonyls, one heteroatom-bonded, and one olefinic) carbons, and three exchangeable protons in **3** (Table 1). Detailed analysis of the NMR data disclosed the structure of **3** to possess a spirocyclic diketopiperazine skeleton. These observations were supported by the relevant correlations from H-5/H-6′ through H-6/H-7′ to H-7/H-8′ and then to H-8/H-9′ in the COSY spectrum and by the key HMBC correlations from H-3 to C-1, C-2, C-4, C-5, and C-9, from H-5 to C-9, from H-8 to C-4, from H-3′ to C-1′ and C-5′, from H-4′ to C-6′, and from H-9′ to C-5′ (Figure S29). The chemical shift of H-3′ was observed at δ_H 6.95, which was downfield-shifted by the deshielding effect of the C═O group, suggesting a *Z*-configuration of the double bond at C-3′.[9] However, the stereochemistry at the spirocyclic center C-2 was not assigned due to the absence of reliable data. Finally, single-crystal X-ray diffraction analysis (Figure 3) confirmed the planar structure of **3**, and the final refinement on the Cu Kα data resulted in a Flack parameter of 0.0(7), which gave an unambiguous assignment of the absolute configuration as (2*R*). It is noteworthy that compound **3** possesssed a different absolute configuration at the spirocyclic center C-2 from that of compounds **1** and **2**, indicating that it might be a shunt product in the biosynthesis of the fungus *P. brocae* MA-231.

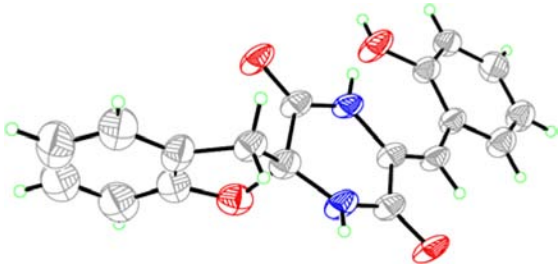
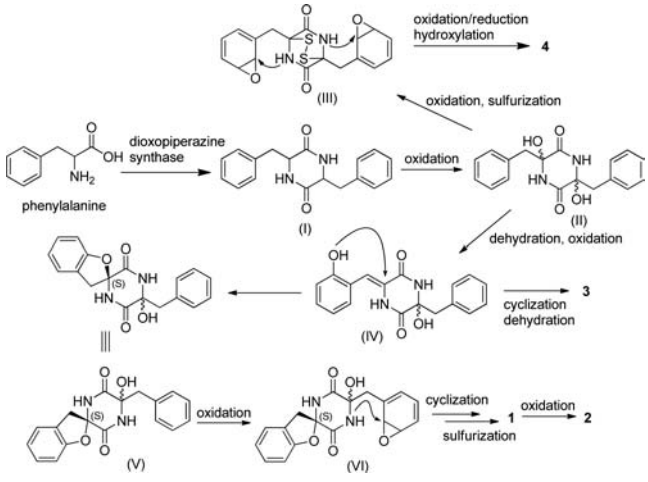


Figure 3. ORTEP diagram of compound **3** from the X-ray diffraction analysis showing the relative orientation of two neighboring molecules in the lattice.

Brocazine G (**4**) was assigned the molecular formula $C_{18}H_{18}N_2O_5S_2$ on the basis of positive HR-ESI-MS experiment. Detailed analysis of the NMR data disclosed the structure of **4** to possess a disulfide diketopiperazine skeleton. Specifically, the ^1H and ^{13}C NMR chemical shifts for the right portion of **4** were nearly identical to those of brocazine F, an epidithiodiketopiperazine identified from *P. brocae* MA-231 that was cultured on PDB medium.[6] For the left portion of **4**, the primary difference in the NMR spectroscopic data (Table S4) was that the aliphatic methine and ketone signals at $\delta_{C/H}$ 48.2/3.38 (CH-4) and δ_C 194.8 (C-5) in brocazine F were replaced by olefinic quartnary carbon and methine signals at δ_C 131.2 (C-4) and $\delta_{C/H}$ 120.6/5.98 (CH-5) in **4**, respectively. These observations were supported by the relevant COSY and HMBC correlations (Figure S29). The relative configuration of **4** was deduced from *J* values and NOESY data. The coupling patterns for the relevant protons on the right portion of **4** were nearly identical to their counterparts of brocazine F, while the large coupling constant between H-8 and H-9 (14.5 Hz) revealed the *trans*-relationship for the proton pair. NOE correlations from the proton of 8-OH to H-9, from H-9′ to the proton of 8′ßOH and H-5′, and from H-8′ to H-4′ confirmed the relative configuration of **4**. As might be expected, the ECD spectrum of **4** was in full agreement with that of brocazine F,[6] which exhibited negative CEs at approximately 203 and 230 nm and a positive CE at 270 nm. Furthermore, Therefore, the absolute configuration of compound **4** was assigned as (2*R*,8*S*,9*S*,2′*R*,4′*S*,5′*S*,8′*S*,9′*S*), which was also confirmed by TDDFT-ECD calculations (Supporting Information).

A plausible biosynthetic pathway for compounds **1**−**4** is proposed as shown in Scheme 1.[1,2,10] The biosynthesis of **4** likely starts with the cyclodipeptide (I) composed of two phenylalanines followed by oxidation (bishydroxylation) as catalyzed by some oxygenase, e.g., GliC (a cytochrome P450 enzyme),[10] to afford the key intermediate II,[1] the hydroxyl groups of which could be replaced in the following step(s) by thiol groups to yield III.[1,2,10] In contrast, the shunt products **1**−**3** might be from a different pathway, in which the intermediate II could be easily transferred to IV by dehydration and oxidation, which undergo cyclization and dehydration to form **3**. Cyclization and oxidation of IV via intermediate V would produce VI, and compounds **1** and **2** could be obtained from VI by cyclization, sulfurization, and oxidation.[1,10]

Scheme 1. Proposed Biosynthetic Pathway for Compounds 1−4



Compounds **1**−**4** were evaluated for anticancer and antimicrobial activities. Anticancer cytotoxicity was tested at sensitive and cisplatin-resistant human ovarian cancer cell lines A2780 and A2780 CisR.[11] Whereas compound **3** only showed moderate activity against A2780 cells (IC_{50} 59 μM), compound

DOI: 10.1021/acs.orglett.6b02620
Org. Lett. 2016, 18, 5304−5307

4 showed strong activity not only to A2780 but also to A2780 CisR cells, with IC_{50} values of 664 and 661 nM, respectively, which are stronger than that of the positive control cisplatin (with IC_{50} values of 1.67 and 12.63 μM, respectively). In addition, compound **4** showed strong and selective activity against human pathogen *Staphylococcus aureus* with MIC value of 0.25 μg/mL, which is stronger than that of the positive control, chloromycetin (MIC = 0.5 μg/mL). Compound **1** exhibited moderate antibacterial activities against *Escherichia coli*, *S. aureus*, and *Vibrio harveyi*, with MIC values of 32.0, 16.0, and 64.0 μg/mL, respectively, while the positive control chloromycetin has MIC values of 2.0, 0.5, and 2.0 μg/mL, respectively. Moreover, compound **3** also showed antibacterial activities against *E. coli*, *Aeromonas hydrophilia*, and *V. harveyi*, each with an MIC value of 32.0 μg/mL.

In conclusion, spirobrocazines A–C (**1**–**3**) with a very rare spirocyclic skeleton were isolated and identified from *P. brocae* MA-231, indicating that additional shunt pathways involved in the biosynthesis of ETPs were activated in optimal culture conditions. It is noteworthy that compound **3** possessed a different configuration at the spirocyclic center C-2 from those of spirobrocazines A (**1**) and B (**2**). Compound **4** may prove useful as cytotoxic agent against human ovarian cancer, even if chemoresistance against clinically used platinum compounds has occurred.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b02620.

Detailed experimental procedures; full spectroscopic data (1D and 2D NMR and ECD) of compounds **1**–**4**; ECD calculations of compounds **1**, **2**, and **4** (PDF)

X-ray data for compound **3** (CIF)

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported by the Natural Science Foundation of China (81673351) and by the Scientific and Technological Innovation Project of Qingdao National Laboratory for Marine Science and Technology (No.2015ASKJ02). M.U.K. acknowledges a grant from the Bundesministerium für Wirtschaft (BMWi) AiF/ZIM project KF2388801UL9. T.K. thanks the Hungarian National Research Foundation (OTKA K105871) for financial support and the National Information Infrastructure Development Institute (NIIFI 10038) for CPU time. L.-H.M. thanks the China Postdoctoral Science Fundation for project funding (2016M590667), and B.-G.W. acknowledges the support of Taishan Scholar Project from Shandong Province.

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DOI: 10.1021/acs.orglett.6b02620
Org. Lett. 2016, 18, 5304–5307