Meng, L. H., Li, X. M., Lv, C. T., Huang, C. G. & Wang, B. G. Brocazines A–F, cytotoxic bisthiodiketopiperazine derivatives from Penicillium brocae MA-231, an endophytic fungus der...

ARTICLE *in* JOURNAL OF NATURAL PRODUCTS · AUGUST 2014

Impact Factor: 3.8 · DOI: 10.1021/np500382k · Source: PubMed

JOURNAL OF NATURAL PRODUCTS

Article

pubs.acs.org/jnp

Brocazines A–F, Cytotoxic Bisthiodiketopiperazine Derivatives from *Penicillium brocae* MA-231, an Endophytic Fungus Derived from the Marine Mangrove Plant *Avicennia marina*

Ling-Hong Meng,[†,‡] Xiao-Ming Li,[†] Cui-Ting Lv,[§] Cai-Guo Huang,*[,§] and Bin-Gui Wang*[,†]

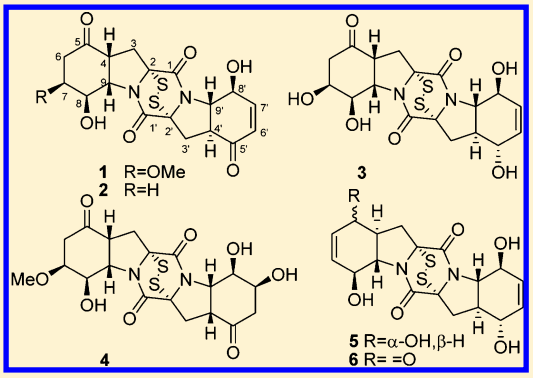
[†]Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Nanhai Road 7, Qingdao 266071, People's Republic of China

[‡]University of Chinese Academy of Sciences, Yuquan Road 19A, Beijing 100049, People's Republic of China

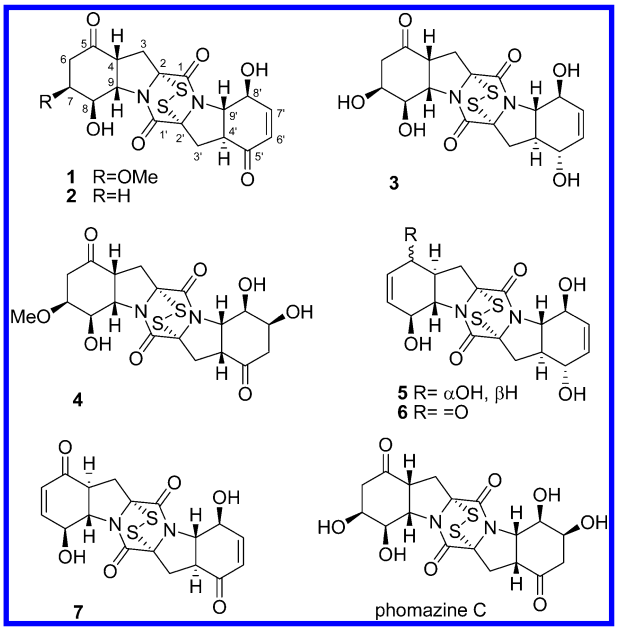
[§]Department of Biochemistry and Molecular Biology, Second Military Medical University, Xiangyin Road 800, Shanghai 200433, People's Republic of China

Supporting Information

ABSTRACT: Six new disulfide-bridged diketopiperazine derivatives, brocazines A–F (**1**–**6**), along with one known analogue (**7**), were isolated and identified from the cytotoxic extract of *Penicillium brocae* MA-231, a fungus obtained from the fresh tissue of the marine mangrove plant *Avicennia marina*. The structures of these compounds were established on the basis of detailed interpretation of NMR and mass spectroscopic data. X-ray crystallographic analysis confirmed the structure of **1** and established the structure and absolute configuration of **5**, while the absolute configurations for compounds **1**, **4**, and **6** were deduced by comparison of the CD data with those of **5**. Compounds **1**, **2**, **5**, and **6** showed cytotoxic activities against several tumor cell lines.



Thiodiketopiperazines (TDKPs) are mainly produced by fungal species of the genera *Epicoccum*,[1−3] *Exserohilum*,[4] and *Phoma*.[5] These compounds have been reported to possess various biological properties such as antibacterial activity,[1] inhibition of HIV-1 replication,[2] inhibition of the release of β-glucuronidase in rat polymorphonuclear leukocytes induced by platelet-activating factor,[3] and antitumor activity.[4−6] In continuation of our approach to identify new bioactive secondary metabolites from marine-derived fungi,[7−11] we performed chemical investigations on the cytotoxic extract of the fungal strain *Penicillium brocae* MA-231, an endophytic fungus isolated from the fresh tissue of the marine mangrove plant *Avicennia marina*. As a result, six new disulfide-bridged diketopiperazines, brocazines A–F (**1**–**6**), as well as one known analogue, epicorazine A (**7**),[1] were characterized from the culture extract of the fungus. The structures of compounds **1**–**7** were established on the basis of spectroscopic analysis, and compounds **1** and **5** were confirmed by single-crystal X-ray diffraction analysis. This paper describes the isolation, structure determination, stereochemical assignment, and cytotoxicity of the isolated compounds.



RESULTS AND DISCUSSION

The fermentation culture of *P. brocae* MA-231 was exhaustively extracted with EtOAc to afford an extract, which was further purified by a combination of column chromatography including silica gel, Sephadex LH-20, Lobar LiChroprep RP-18, and semipreparative HPLC, to yield seven disulfide diketopiperazines (**1**–**7**).

Received: May 6, 2014
Published: August 8, 2014

dx.doi.org/10.1021/np500382k | *J. Nat. Prod.* 2014, 77, 1921−1927

Table 1. ^{13}C NMR Spectroscopic Data for Compounds 1−6 in DMSO-d_6

no.	1	2	3	4	5	6
1	164.0, C	162.4, C	164.1, C	162.1, C	164.6, C	164.3, C
2	76.1, C	75.3, C	75.9, C	76.1, C	76.2, C	75.6, C
3	32.7, CH_2	32.3, CH_2	32.4, CH_2	32.2, CH_2	33.7, CH_2	29.9, CH_2
4	46.4, CH	46.7, CH	46.3, CH	46.3, CH	46.3, CH	48.2, CH
5	207.7, C	208.6, C	208.3, C	207.8, C	69.9, CH	194.8,C
6	40.6, CH_2	33.8, CH_2	43.6, CH_2	40.7, CH_2	128.7, CH	128.6, CH
7	75.4, CH	25.3,CH_2	65.8, CH	75.5,CH	133.9, CH	151.1, CH
8	62.0, CH	60.7, CH	65.8, CH	62.0, CH	68.5, CH	70.5, CH
9	63.3, CH	65.8, CH	63.6, CH	63.6, CH	68.2, CH	68.2, CH
1′	162.3, C	164.1, C	162.6, C	162.1, C	164.6, C	164.5, C
2′	75.5, C	76.3, C	76.3, C	76.2, C	76.2, C	76.0, C
3′	29.6, CH_2	29.6, CH_2	33.5, CH_2	32.6, CH_2	33.7, CH_2	33.7, CH_2
4′	48.1, CH	48.1,CH	46.3, CH	46.4, CH	46.3, CH	46.3, CH
5′	194.8, C	194.8, C	69.9, CH	208.1, C	69.9, CH	68.2, CH
6′	128.6, CH	128.6, CH	128.7, CH	43.6, CH_2	128.7, CH	128.6, CH
7′	151.1, CH	151.1,CH	133.8, CH	65.9,CH	133.9, CH	133.8, CH
8′	70.4, CH	70.5, CH	68.5, CH	65.9, CH	68.5, CH	68.4, CH
9′	68.2, CH	68.1, CH	68.1, CH	63.2, CH	68.2, CH	68.2, CH
7-OMe	56.0, CH_3			56.0, CH_3		

Table 2. ^{1}H NMR Spectroscopic Data for Compounds 1−6 in DMSO-d_6

no.	1	2	3	4	5	6
3α	2.75, d (14.7)	2.81, d (14.7)	2.75, dd (1.5, 14.6)	2.73, m (overlap)	2.56, dd (5.5, 14.0)	2.61, dd (5.2, 13.8)
3β	3.01, dd (8.3, 14.7)	2.95, dd (8.2, 14.7)	2.99, dd (8.3, 14.6)	2.99, dd (8.3, 14.3)	2.74, d (13.4)	2.86, dd (12.8, 13.8)
4	3.21, m	3.25, m	3.16, m	3.17, m (overlap)	2.23, m	3.38, ddd (5.6, 12.6, 12.8)
5					4.21, d (8.0)	
6	α 2.65, dd (4.6, 16.1)	α 2.29, dt (3.4, 16.8)	α 2.71, m (overlap)	α 2.70, m (overlap)	5.72, d (10.0)	6.08, dd (2.0, 10.2)
	β 2.74, d (16.1)	β 2.70, ddd (4.7, 11.3, 17.8)	β 2.54, m (overlap)	β 2.63, m (overlap)		
7	3.34, m (overlap)	α 1.68, ddd (2.5, 8.4, 17.2)	3.70, m	3.30, m	5.55, d (10.0)	6.91, dd (1.4, 10.2)
		β 1.91, m				
8	5.07, d (2.4)	4.87, d (2.9)	4.78, brs	5.02, brs	4.27, d (7.0)	4.71, d (8.5)
9	4.42, dd (4.4, 7.3)	4.38, dd (2.1, 7.1)	4.40, dd (4.5, 7.2)	4.40, m	3.54, dd (8.1, 11.9)	4.00, dd (8.6, 13.0)
3′α	2.57, dd (5.7, 14.0)	2.54, dd (5.6, 14.3)	2.48, m (overlap)	2.73, m (overlap)	2.56, dd (5.5, 14.0)	2.58, dd (5.5, 13.9)
3′β	2.83, dd (12.6, 14.0)	2.81, m	2.71, m	2.99, dd (8.3, 14.3)	2.74, d (13.4)	2.74, t (13.4)
4′	3.34, ddd (5.7, 12.6,13.0)	3.35, m	2.19, m	3.17, m	2.23, m	2.24, m
5′			4.22, d (6.5)		4.21, d (8.0)	4.22, brs
6′	6.06, dd (2.1, 10.1)	6.06, dd (2.0, 10.2)	5.54, d (10.0)	α 2.53, m (overlap)	5.72, d (10.0)	5.72, d (10.0)
				β 2.66, m (overlap)		
7′	6.90, dd (1.5, 10.1)	6.90, dd (1.3, 10.2)	5.71, d (10.1)	3.66, d (4.1)	5.55, d (10.0)	5.55, d (10.0)
8′	4.67, dt (8.5, 1.5)	4.68, d (8.5)	4.22, d (6.5)	4.73, brs	4.27, d (7.0)	4.28, d (7.2)
9′	3.98, dd (8.6, 13.0)	4.00, dd (8.6, 13.0)	3.52, dd (8.0, 11.8)	4.40, m	3.54, dd (8.1, 11.9)	3.53, dd (8.1, 12.0)
5-OH					5.39, brs	
7-OH			5.15, d (4.9)			
7-OMe	3.24, s			3.21, s		
8-OH	5.66, brs	5.51, d (4.0)	5.48, d (4.3)	5.63, brs	5.61, s	5.91, s
5′-OH			5.57, s		5.39, brs	5.39, d (5.7)
7′-OH				5.16, brs		
8′-OH	5.85, s	5.88, s	5.37, d (5.4)	5.47, brs	5.61, s	5.58, s

Compound **1** was obtained as colorless crystals, and its molecular formula was determined as $C_{19}H_{20}N_2O_7S_2$ on the basis of positive HRESIMS, indicating 11 degrees of unsaturation. The ^{1}H and ^{13}C NMR data of **1** suggested the presence of one methoxy, three methylenes, nine methines (five oxygenated or nitrogenated and two olefinic), six quaternary carbons (two ketone and two amide carbonyls), and two exchangeable protons (δ_H 5.66 and 5.85) (Tables 1 and 2). Detailed analysis of the NMR data disclosed the structure of **1** to possess a disulfide diketopiperazine skeleton with a 6−5−6−5−6 ring system. Specifically, the ^{1}H and ^{13}C NMR chemical shifts for the right portion of **1** were nearly identical to those of the symmetrical epicorazine A (**7**), an epidithiodiketopiperazine identified from the fungus *Epicoccum nigrum*.[1] For the left portion of **1**, the primary difference in the NMR spectroscopic data was that the two olefinic methine signals at δ_H 6.09/6.91 and δ_C 129.5/151.3 (C-2/C-3) in epicorazine A (**7**)[1] were replaced by methylene and oxymethine signals at δ_H 2.74/2.65 (H-6) and 3.34 (H-7) and δ_C 40.6/75.4 (C-6/C-7) in **1**, respectively. These observations were supported by the relevant COSY and HMBC correlations (Figure 1). In addition, a C-7

dx.doi.org/10.1021/np500382k | J. Nat. Prod. 2014, 77, 1921−1927

methoxy group (δ_H 3.24 and δ_C 56.0) was assigned, which was confirmed by an HMBC experiment (Figure 1).

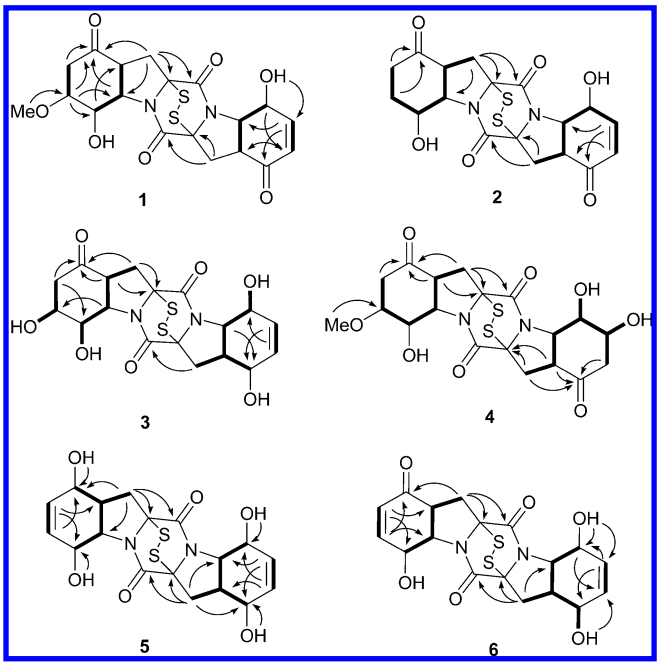


Figure 1. Key HMBC (arrows) and COSY (bold lines) correlations for compounds **1**–**6**.

The relative configuration of **1** was determined by analysis of *J*-values and NOESY data (Figure 2). The large coupling constant between H-4′ and H-9′ (J = 13.0 Hz) revealed that they were *trans* oriented, and the key NOE correlations from H-4′ to H-8′ suggested the cofacial orientation of the two hydrogens. Moreover, NOE correlations from H-4, OH-8, and OMe-7 to H-9 suggested the same orientation of these groups. However, the relative configuration of the C-2(2′) disulfide bridge and that of the whole molecule could not be directly established by spectroscopic methods. Upon slow evaporation of the solvent (MeOH) by storing the sample in a refrigerator, a single crystal of **1** was cultivated, making feasible an X-ray diffraction analysis (Figure 3) that unequivocally confirmed the structure and relative configuration of **1**.

Although two sulfur atoms were present in **1**, the Flack parameter 0.02(17) could not be used to unambiguously assign the absolute configuration because of the large uncertainty,[12] but considering that the CD spectrum of **1** exhibited a positive Cotton effect (CE) at 264 nm (characteristic for the 2*R*/2′R configurations in TDKPs),[6] which is the same as that of **5** (see later in the discussion for compound **5**, where X-ray data firmly established the absolute configuration), along with biogenetic arguments, the absolute configuration of **1** was assigned as 2*R*, 4*R*, 7*S*, 8*R*, 9*S*, 2′*R*, 4′*S*, 8′*S*, and 9′*S*. On the basis of the above evidence, the structure of **1** was determined, and the trivial name brocazine A was assigned to this compound.

Compound **2** was assigned the molecular formula $C_{18}H_{18}N_2O_6S_2$ (11 unsaturations), having one OCH_2 unit less than **1**, on the basis of HRESIMS data. Its NMR spectroscopic data were very similar to those of **1**. However, signals for the methoxy group resonating at δ_H 3.24 and δ_C 56.0 and that of the oxymethine group resonating at δ_H 3.34 (H-7) and δ_C 75.4 (C-7) in **1** disappeared in the NMR spectra of **2**. Instead, methylene signals at δ_H 1.91/1.68 (H-7) and δ_C 25.3 (C-7) were observed (Tables 1 and 2). The cross-peaks from H-7 to H-6 and H-8 in the COSY spectrum as well as the correlations from H-7 to C-5 and C-9 in the HMBC spectrum confirmed the above deduction (Figure 1). The relative configuration of **2** was also deduced from $^1H-^1H$ coupling constants and NOESY data. The coupling patterns for the relevant protons on the right portion of **2** were identical to their counterparts of **1**, while NOE correlations of H-9 with H-4 and OH-8 placed these protons on the same face of the molecule,

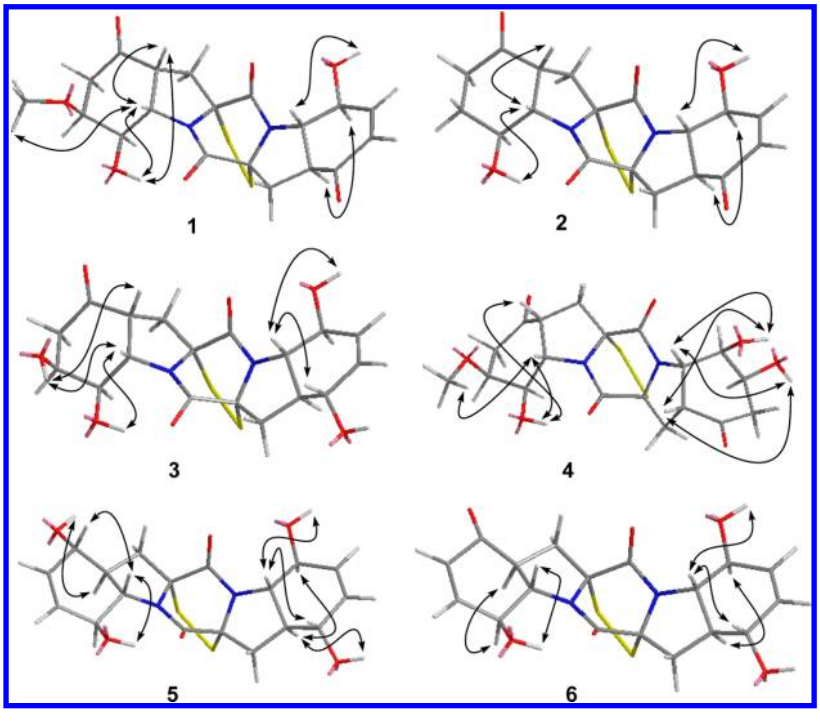


Figure 2. Key NOESY correlations for compounds **1**–**6**.

dx.doi.org/10.1021/np500382k | *J. Nat. Prod.* 2014, 77, 1921–1927

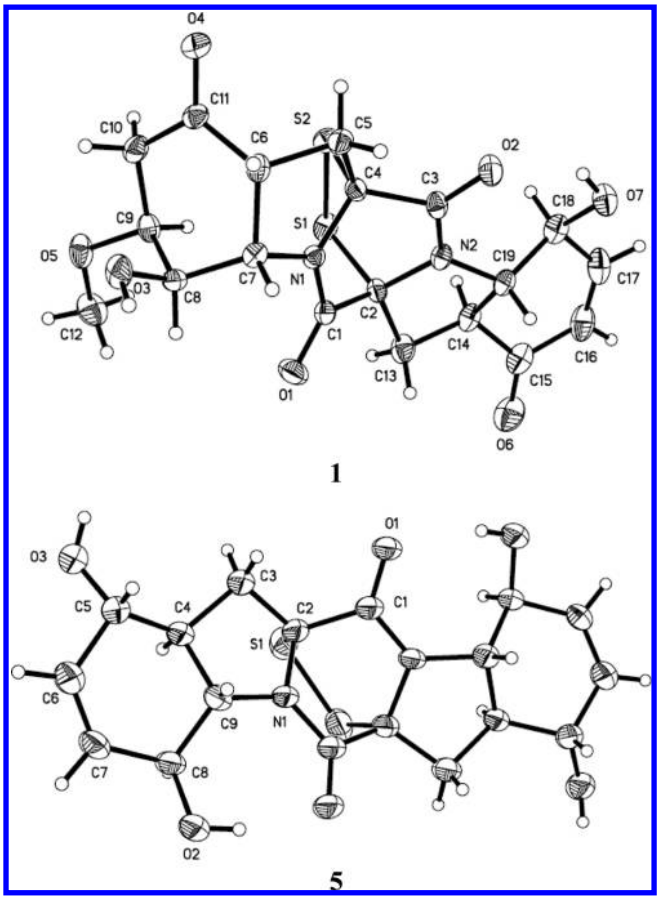


Figure 3. X-ray crystallographic structures of compounds **1** and **5**. (Note: different numbering systems are used for the structures in the text.)

the same as that of **1**. As might be expected, the CD spectrum of **2** was in full agreement with that of **1** (Experimental Section and Supporting Information Figures S7 and S14), which exhibited negative CEs at approximately 203 and 222 nm and a positive CE at 264 nm. Therefore, the absolute configuration of compound **2** was also assigned as 2*R*, 4*R*, 8*S*, 9*S*, 2′*R*, 4′*S*, 8′*S*, and 9′*S*. The absolute configurations (ACs) of the α/α' stereogenic carbons 2*R*/2′*R* obeyed the rule that the CD band around 270 nm can be used to determine the ACs of the asymmetric carbons at the α/α' positions in TDKPs, with a positive CE representing the 2*R*/2*R*′ configurations.[6] On the basis of the above data, the structure of **2** was determined, and the trivial name brocazine B was assigned to this compound.

Brocazine C (**3**) had the molecular formula $C_{18}H_{20}N_2O_7S_2$ (10 unsaturations) as determined by HRESIMS data, having one carbon atom less than **1**. Detailed comparison of the NMR data of **3** with those of **1** suggested that compound **3** had the same basic structure as **1**. The methoxy signal resonating at δ_H 3.24 and δ_C 56.0 (OCH_3-7) and one of the two carbonyl carbons resonating at δ_C 194.8 (C-5′) in the NMR spectra of **1** were absent in those of **3**. Instead, an oxymethine signal resonating at δ_H 4.22 (H-5′) and δ_C 69.9 (C-5′) was observed. This observation suggested the C-5′ ketone functionality in **1** was reduced in **3**. In addition, the chemical shift of the olefinic carbon C-7′ shifted upfield (δ_C 151.1 in **1** vs δ_C 133.8 in **3**) due to the lack of conjugation in **3**. These observations were further supported by the COSY and HMBC correlations (Figure 1).

The relative configuration of **3** was deduced to be the same as that of **1** and **2**, according to the NOESY experiment (Figure 2) as well as the coupling patterns of the relevant protons (Table 2). The additional OH group (at C-5′) was deduced to be α-orientated based on the NOESY correlations from H-9′ to H-5′ and OH-8′ (Figure 2). The CD spectrum exhibited negative CEs at approximately 202 and 229 nm and a positive CE at 265 nm, similar to those of **1** and **2**. Therefore, the absolute configuration of compound **3** was assigned as 2*R*, 4*R*, 7*S*, 8*R*, 9*S*, 2′*R*, 4′*S*, 5′*S*, 8′*S*, and 9′*S*.[6]

The molecular formula of brocazine D (**4**) was determined to be $C_{19}H_{22}N_2O_8S_2$ by HRESIMS, having one CH_2 unit more than that of phomazine C, a TDKP derivative that was very recently isolated from the marine-derived fungus *Phoma* sp. OUCMDZ-1847.[5] The ^1H and ^{13}C NMR spectroscopic data of **4** were similar to those of **1** and phomazine C.[5] The NMR chemical shifts for the left portion of **4** were nearly identical to those of the left half of **1**, whereas those for the right portion of **4** matched well with that of the symmetrical phomazine C. These observations were confirmed by relevant COSY and HMBC correlations (Figure 1).

The relative configuration of **4** was deduced by a NOESY experiment. No significant coupling was observed between H-7 and H-8, nor H-7′and H-8′, indicating that the vicinal angle between the protons in each pair was close to 90°. NOE correlations from OH-8 to H-4 and H-9 and from H-9 to OMe-7 placed them on the same face, opposite that of H-7 and H-8. Similarly, NOE correlations from OH-7′ and OH-8′ to H-4′ and H-9′ indicated the same face of these groups. Compound **4** had an almost identical CD spectrum to that of phomazine C.[5] Consequently, the absolute configuration at C-2(2′), C-4(4′), C-7(7′), C-8(8′), and C-9(9′) was assigned as *R*, *R*, *S*, *R*, and *S*.

Brocazine E (**5**) was obtained as colorless crystals, and its molecular formula was determined as $C_{18}H_{20}N_2O_6S_2$ by HRESIMS. Its ^1H and ^{13}C NMR data (Tables 1 and 2) revealed one methylene, six methines (two oxygenated, two nitrogenated, and two olefinic) and two quaternary carbons (one amide carbonyl). These data accounted for only half of the elemental composition, indicating a symmetrical nature of **5**. The structure of **5** was deduced from exhaustive analyses of the COSY and HMBC spectra. The correlations from H-3 to H-4, from H-4 to H-5 and H-9, from H-9 to H-8, and from H-6 to H-7 were observed in the COSY experiment, while in the HMBC spectrum, a full set of all possible ^2J and ^3J correlations from H-3 to C-1, C-2, C-5, and C-9, from H-6 to C-4 and C-8, from H-7 to C-5, from OH-5′ to C-5, and from OH-8′ to C-8 allowed the construction of the C-1 to C-9 fragment, half of the symmetrical 6−5−6−5−6 TDKP skeleton for **5**. The relative configuration was also assigned by analysis of its $^1H-^1H$ coupling constants and NOESY data. The large coupling constants between H-4 and H-9 as well as of H-4′ and H-9′ (J = 11.9 Hz) revealed the *trans*-relationships for the proton pairs. NOE correlations from OH-8/8′ to H-9/9′ and from H-9/9′ to H-5/5′ in the NOESY spectrum indicated the cofacial orientation relative to these protons, while the correlation from OH-5/5′ to H-4/4′ placed these protons on the opposite face. An X-ray crystallographic experiment confirmed the structure and relative configuration of **5** as depicted (Figure 3). The Cu/Kα radiation used for the X-ray diffraction allowed the assignment of the absolute configuration of all of the stereogenic centers in **5** as 2*R*, 4*S*, 5*S*, 8*S*, 9*S*, 2′*R*,4′*S*, 5′*S*, 8′*S*, and 9′*S*.

dx.doi.org/10.1021/np500382k | *J. Nat. Prod.* 2014, 77, 1921−1927

Table 3. Cytotoxicity of Compounds 1−6 against Nine Tumor Cell Lines (IC_{50}, μM)

	Du145[a]	HeLa[b]	HepG2[c]	MCF-7[d]	NCI-H460[e]	SGC-7901[f]	SW1990[g]	SW480[h]	U251[i]
1	4.2	6.8	6.4	5.5	4.9	2.6	6.0	2.0	5.2
2	3.6	5.3	5.5	6.1	4.0	2.4	6.4	1.2	3.5
3	n.a.[j]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	11.2	4.3	5.6	9.0	12.4	3.3	2.1	n.t.[k]	6.1
6	1.7	6.9	2.9	3.0	0.89	8.0	5.9	n.t.	5.3
positive control	1.5[l]	5.0[l]	5.1[m]	1.8[l]	7.6[n]	2.9[o]	2.2[p]	11.3[m]	10.8[n]

[a]Human prostate cancer. [b]Human cervix carcinoma. [c]Human hepatoma cells. [d]Human breast carcinoma. [e]Human large-cell lung carcinoma. [f]Human gastric carcinoma. [g]Human pancreatic cancer. [h]Human colon carcinoma cancer. [i]Human glioma cells. [j]n.a.: no activity (>20 μM). [k]n.t.: not tested. [l]Paclitaxel. [m]Cisplatin. [n]Cefitinib. [o]Doxorubicin. [p]Gemcitabine.

Brocazine F (**6**) was assigned the molecular formula $C_{18}H_{18}N_2O_6S_2$ by HRESIMS, with two protons less than that of **5**. Its NMR spectroscopic data were very similar to those of **5**. However, signals for the oxymethine group resonating at δ_H 4.21 (H-5) and δ_C 69.9 (C-5) in the NMR spectra of **5** disappeared in those of **6**. Instead, a carbonyl group resonating at δ_C 194.8 (C-5) was observed in the ^{13}C NMR spectrum of **6**. In addition, the chemical shift of C-7 shifted downfield from δ_C 133.9 (C-7) in **5** to δ_C 151.1 (C-7) in the ^{13}C NMR spectrum of **6**, due to the inductive and conjugative effects. This observation was further supported by the COSY and HMBC correlations (Figure 1). The ^1H and ^{13}C NMR data for the right portion of **6** were nearly identical to those of **5** (Tables 1 and 2).

The relative configuration of **6** was deduced from *J*-coupling constants and NOESY data. The coupling patterns for the relevant protons of **6** were identical to their corresponding ones of **5**, indicating that the two compounds possessed the same relative configuration. The observation was further confirmed by the NOESY correlations (Figure 2). As might be expected, compound **6** had an almost identical CD spectrum and the same sign of the optical rotation as that of **5**. Consequently, the absolute configuration of **6** was assigned as 2*R*, 4*S*, 8*S*, 9*S*, 2′*R*, 4′*S*, 5′*S*, 8′*S*, and 9′*S*.

The new compounds **1**−**6** were assayed for their cytotoxic activities against nine tumor cell lines, Du145, Hela, HepG2, MCF-7, NCI-H460, SGC-7901, SW1990, SW480, and U251 (Table 3). Compounds **1**, **2**, **5**, and **6** displayed cytotoxic activities against most of the tested cell lines, with IC_{50} values ranging from 0.89 to 9.0 μM. Surprisingly, compounds **3** and **4** did not show any activity toward all of the nine tested cell lines. These data indicated that compounds possessing two double bonds at C-6 and C-6′ (**5** and **6** vs **3** and **4**), or having one double bond at C-6/6′ conjugating with a keto group at C-5/5′ (**1** and **2** vs **3** and **4**), generally showed higher cytotoxicity. Notably, compounds **1** and **2** exhibited potent activity against the SW480 tumor cell line, with IC_{50} values of 2.0 and 1.2 μM, respectively, while compound **6** showed strong activity against the DU145 and NCI-H460 cell lines, with IC_{50} values of 1.7 and 0.89 μM, respectively. Literature search results indicated that TDKPs with a disulfide bridge usually have cytotoxic activity, whereas those with two thiomethyl groups generally have no such activity,[1−5] indicating that the disulfide bridge at the α/α′ positions in TDKPs is essential for the cytotoxicity.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined with an SGW X-4 micromelting-point apparatus. Optical rotations were measured on an Optical Activity AA-55 polarimeter. UV spectra were measured on a PuXi TU-1810 UV−visible spectrophotometer. CD spectra were acquired on a Chirascan spectropolarimeter. 1D and 2D NMR spectra were recorded at 500 and 125 MHz for ^1H and ^{13}C, respectively, on a Bruker Avance 500 MHz spectrometer with TMS as internal standard. Mass spectra were determined on a VG Autospec 3000 or an API QSTAR Pulsar 1 mass spectrometer. Analytical and semipreparative HPLC were performed using a Dionex HPLC system equipped with a P680 pump, an ASI-100 automated sample injector, and a UVD340U multiple wavelength detector controlled by Chromeleon software (version 6.80). Commercially available Si gel (200−300 mesh, Qingdao Haiyang Chemical Co.), Lobar LiChroprep RP-18 (40−63 μm, Merck), and Sephadex LH-20 (Pharmacia) were used for open column chromatography. All solvents used were distilled prior to use.

Fungal Material. The fungus *Penicillium brocae* MA-231 was isolated from the fresh tissue of the marine mangrove plant *Avicennia marina* that was collected at Hainan Island, P. R. China, in August 2012, and fungal identification was performed by analysis of its ITS region of the rDNA as described previously.[13] The resulting sequence data obtained from the fungal strain have been deposited in GenBank (with accession no. KM191342). A BLAST search result indicated that the sequence was most similar (99%) to the sequence of *Penicillium brocae* (compared to AF484393). The strain is preserved at Key Laboratory of Experimental Marine Biology, Institute of Oceanology of the Chinese Academy of Sciences.

Fermentation, Extraction, and Isolation. For chemical investigations, the fresh mycelia of *P. brocae* MA-231 were grown on PDA medium at 28 °C for 4 days and then inoculated for 30 days at room temperature in 60 × 1 L conical flasks containing 300 mL of potato-dextrose broth medium (20 g glucose, 5 g peptone, 3 g yeast extract, and 1 L naturally sourced and filtered seawater, which was obtained from the Huiquan Gulf of the Yellow Sea near the campus of the author's institution, pH 6.5−7.0). The whole fermented cultures (18 L) were filtered to separate the broth from the mycelia. The former was extracted three times with EtOAc, while the latter were extracted three times with a mixture of 80% acetone and 20% H_2O. The acetone solution was evaporated under reduced pressure to afford an aqueous solution, which was then extracted three times with EtOAc. Because the TLC and HPLC profiles of the two EtOAc solutions were almost identical, they were combined and concentrated under reduced pressure to give an extract (20.0 g), which was fractionated by silica gel vacuum liquid chromatography using different solvents of increasing polarity from petroleum ether to MeOH to yield nine fractions (Frs. 1−9) based on TLC analysis. Purification of Fr. 4 (1.27 g) by reversed-phase column chromatography (CC) over Lobar LiChroprep RP-18 with a MeOH−H_2O gradient (from 20:80 to 100:0) and then on Sephadex LH-20 (MeOH) yielded compound **7** (5.2 mg). Fr. 6 (2.5 g) was also purified by CC over Lobar LiChroprep RP-18 with a MeOH−H_2O gradient (from 20:80 to 100:0) to afford six subfractions (Fr. 6.1−6.6). Fr. 6.2 was further purified by CC on Sephadex LH-20 (MeOH) and then by preparative TLC (plate: 20 × 20 cm, developing solvents: $CHCl_3$−MeOH, 10:1) to yield compound **4** (6.0 mg). Fr. 6.2 was further purified by CC on silica gel eluting with a $CHCl_3$−MeOH gradient (from 50:1 to 2:1) to obtain compounds **2** (6.0 mg) and **6** (59.1 mg). Fr. 6.3 was further purified by CC on

dx.doi.org/10.1021/np500382k | J. Nat. Prod. 2014, 77, 1921−1927

Sephadex LH-20 (MeOH) to get compound **1** (11.7 mg). Further purification of Fr. 7 (1.9 g) by CC over Lobar LiChroprep RP-18 with a MeOH–H_2O gradient (from 20:80 to 100:0) afforded two subfractions, Fr. 7-1 and Fr. 7-2. Fr. 7-1 was further purified by CC on Sephadex LH-20 (MeOH) and then by semipreparative HPLC (Elite ODS-BP column, 10 μm; 20 × 250 mm; 40% MeOH–H_2O, 16 mL/min) to afford compound **3** (10.3 mg, t_R 13.0 min). Fr. 7-2 was further purified by semipreparative HPLC (Elite ODS-BP column, 10 μm; 20 × 250 mm; 35% MeOH–H_2O, 16 mL/min) to afford compound **5** (54.1 mg, t_R 12.0 min).

*Brocazine A (**1**):* colorless crystals (MeOH); mp 230–232 °C; $[\alpha]_D^{25}$ −180 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 201 (4.47) nm; CD (*c* 0.32 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 203 (−16.7), 222 (−32.1), 264 (+5.59), 294 (−1.32) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m*/*z* 453 $[M + H]^+$; HRESIMS *m*/*z* 453.0773 $[M + H]^+$ (calcd for $C_{19}H_{21}N_2O_7S_2$, 453.0785).

*Brocazine B (**2**):* white powder; $[\alpha]_D^{25}$ −206 (*c* 0.31, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.00), 262 (2.98) nm; CD (*c* 0.38 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 223 (−26.8), 264 (+4.81), 294 (−0.74) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; ESIMS *m*/*z* 423 $[M + H]^+$; HRESIMS *m*/*z* 423.0669 $[M + H]^+$ (calcd for $C_{18}H_{19}N_2O_6S_2$, 423.0679).

*Brocazine C (**3**):* white powder; $[\alpha]_D^{25}$ −118 (*c* 0.54, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.16) nm; CD (*c* 0.47 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 202 (−5.07), 208 (−1.73), 229 (−22.9), 265 (+3.68) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 441.0786 $[M + H]^+$ (calcd for $C_{18}H_{21}N_2O_7S_2$, 441.0785).

*Brocazine D (**4**):* white powder; $[\alpha]_D^{25}$ −90.5 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.42) nm; CD (*c* 1.0 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 210 (+10.83), 240 (−20.88), 265 (+6.31), 290 (−0.84) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 471.0847 $[M + H]^+$ (calcd for $C_{19}H_{23}N_2O_8S_2$, 471.0850).

*Brocazine E (**5**):* colorless crystals (MeOH); mp 240–242 °C; $[\alpha]_D^{25}$ −208 (*c* 0.24, MeOH); UV (MeOH) λ_{max} (log ε) 201 (5.25) nm; CD (*c* 0.77 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 201 (−21.68), 208 (−12.79), 226 (−64.67), 266 (+18.77) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 425.0836 $[M + H]^+$ (calcd for $C_{18}H_{21}N_2O_6S_2$, 425.0836).

*Brocazine F (**6**):* white powder; $[\alpha]_D^{25}$ −210 (*c* 1.35, MeOH); UV (MeOH) λ_{max} (log ε) 202 (4.46), 267 (3.12) nm; CD (*c* 0.75 mM, MeOH) λ_{max} ($\Delta\varepsilon$) 202 (−28.47), 222 (−56.81), 266 (+10.28) nm; ^{1}H and ^{13}C NMR data, see Tables 1 and 2; HRESIMS *m*/*z* 423.0676 $[M + H]^+$ (calcd for $C_{18}H_{19}N_2O_6S_2$, 423.0679).

X-ray Crystallographic Analysis of Compounds 1 and 5.[14] All crystallographic data were collected on a Bruker Smart-1000 CCD diffractometer equipped with a graphite-monochromatic Mo Kα radiation (λ = 0.710 73 Å) source for **1** and Cu Kα radiation (λ = 1.541 78 Å) for **5** at 293(2) K. The data were corrected for absorption by using the program SADABS.[15] The structures were solved by direct methods with the SHELXTL software package.[16] All non-hydrogen atoms were refined anisotropically. The H atoms were located by geometrical calculations, and their positions and thermal parameters were fixed during the structure refinement. The structures were refined by full-matrix least-squares techniques.[17]

*Crystal data for compound **1**:* $C_{19}H_{20}N_2O_7S_2$, fw = 452.49, orthorhombic space group *P*2(1)2(1)2(1), unit cell dimensions *a* = 7.2595(7) Å, *b* = 7.5636(5) Å, *c* = 36.246(3) Å, *V* = 1990.2(3) Å^3, α = β = γ = 90°, *Z* = 4, d_{calcd} = 1.510 mg/m^3, crystal dimensions 0.21 × 0.20 × 0.11 mm, μ = 0.314 mm^{-1}, *F*(000) = 944. The 4734 measurements yielded 3273 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave R_1 = 0.0650 and wR_2 = 0.1352 [$I > 2\sigma(I)$]. The Flack parameter was 0.02(17) in the final refinement for all 4734 reflections with 3273 Friedel pairs.

*Crystal data for compound **5**:* $C_{18}H_{20}N_2O_6S_2$, fw = 424.48, monoclinic space group, *C*2, unit cell dimensions *a* = 16.7824(11) Å, *b* = 8.1768(4) Å, *c* = 7.5091(3) Å, *V* = 982.74(9) Å^3, α = γ = 90°, β = 107.502(2)°, *Z* = 2, d_{calcd} = 1.434 mg/m^3, crystal dimensions 0.15× 0.11× 0.08 mm, μ = 2.797 mm^{-1}, *F*(000) = 444. The 2108 measurements yielded 1256 independent reflections after equivalent data were averaged, and Lorentz and polarization corrections were applied. The final refinement gave R_1 = 0.0535 and wR_2 = 0.1394 [$I > 2\sigma(I)$]. The Flack parameter was 0.02(6) in the final refinement for all 2108 reflections with 1256 Friedel pairs.

Cytotoxicity Assay. The cytotoxic activities of the isolated compounds against nine tumor cell lines including Du145 (human carcinoma of prostate cell line), HeLa (human cervix carcinoma cell line), HepG2 (human liver hepatocellular cells), MCF-7 (human breast carcinoma cell line), NCI-H460 (human large cell lung carcinoma cell line), SGC-7901 (human gastric carcinoma cell line), SW1990 (human pancreatic cancer cell line), SW480 (human colon carcinoma cancer), and U251 (human glioma cells) were determined according to previously reported methods.[18,19]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Selected 1D and 2D NMR and CD spectra of compounds **1**–**6**, as well as X-ray crystallographic files of compounds **1** and **5** (in CIF format). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*(C.-G. Huang) E-mail: huangcaig@hotmail.com.
*(B.-G. Wang) E-mail: wangbg@ms.qdio.ac.cn. Tel and Fax: +86-532-82898553.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support from the Natural Science Foundation of China (31270403 and 30910103914) and from the Ministry of Science and Technology of China (2013AA092901 and 2010CB833800) is gratefully acknowledged.

■ REFERENCES

(1) Deffieux, G.; Baute, M. A.; Baute, R.; Filleau, M. J. *J. Antibiot.* **1978**, *31*, 1102–1105.
(2) Guo, H. J.; Sun, B. D.; Gao, H.; Chen, X. L.; Liu, S. C.; Yao, X. S.; Liu, X. Z.; Che, Y. S. *J. Nat. Prod.* **2009**, *72*, 2115–2119.
(3) Wang, J. M.; Ding, G. Z.; Fang, L.; Dai, J. G.; Yu, S. S.; Wang, Y. H.; Chen, X. G.; Ma, S. G.; Qu, J.; Xu, S.; Du, D. *J. Nat. Prod.* **2010**, *73*, 1240–1249.
(4) Tan, R. X.; Jensen, P. R.; Williams, P. G.; Fenical, W. *J. Nat. Prod.* **2004**, *67*, 1374–1382.
(5) Kong, F. D.; Wang, Y.; Liu, P. P.; Dong, T. H.; Zhu, W. M. *J. Nat. Prod.* **2014**, *77*, 132–137.
(6) Wang, J. M.; Jiang, N.; Ma, J.; Yu, S. S.; Tan, R. X.; Dai, J. G.; Si, Y. K.; Ding, G. Z.; Ma, S. G.; Qu, J.; Fang, L.; Du, D. *Tetrahedron* **2013**, *69*, 1195–1201.
(7) Meng, L. H.; Li, X. M.; Lv, C. T.; Li, C. S.; Xu, G. M.; Huang, C. G.; Wang, B. G. *J. Nat. Prod.* **2013**, *76*, 2145–2149.
(8) An, C. Y.; Li, X. M.; Luo, H.; Li, C. S.; Wang, M. H.; Xu, G. M.; Wang, B. G. *J. Nat. Prod.* **2013**, *76*, 1896–1901.
(9) Wang, M. H.; Li, X. M.; Li, C. S.; Ji, N. Y.; Wang, B. G. *Mar. Drugs* **2013**, *11*, 2230–2238.
(10) Li, C. S.; Li, X. M.; Gao, S. S.; Lu, Y. H.; Wang, B. G. *Mar. Drugs* **2013**, *11*, 3068–3076.
(11) Liu, D.; Li, X. M.; Li, C. S.; Wang, B. G. *Helv. Chim. Acta* **2013**, *96*, 1055–1061.
(12) Flack, H. D. *Acta Crystallogr.* **1983**, *A39*, 876–881.
(13) Wang, S.; Li, X. M.; Teuscher, F.; Li, D. L.; Diesel, A.; Ebel, R.; Proksch, P.; Wang, B. G. *J. Nat. Prod.* **2006**, *69*, 1622–1625.
(14) Crystallographic data of compounds **1** and **5** have been deposited in the Cambridge Crystallographic Data Centre as CCDC 999912 (for **1**) and CCDC 999913 (for **5**). The data can be obtained

free of charge via http://www.ccdc.cam.ac.uk/data_request/cif (or from the CCDC, 12 Union Road, Cambridge CB21EZ, U.K.; fax: +44-1223-336-033; e-mail: deposit@ccdc.cam.ac.uk).
(15) Sheldrick, G. M. *SADABS, Software for Empirical Absorption Correction*; University of Gottingen: Germany, 1996.
(16) Sheldrick, G. M. *SHELXTL, Structure Determination Software Programs*; Bruker Analytical X-ray System Inc.: Madison, WI, 1997.
(17) Sheldrick, G. M. *SHELXL-97* and *SHELXS-97, Program for X-ray Crystal Structure Solution and Refinement*; University of Gottingen: Germany, 1997.
(18) Bergeron, R. J.; Cavanaugh, P. F., Jr.; Kline, S. J.; Hughes, R. G., Jr.; Elliott, G. T.; Porter, C. W. *Biochem. Biophys. Res. Commun.* **1984**, *121*, 848−854.
(19) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55−63.