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Note

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Gliotoxin Analogues from a Marine-Derived Fungus, *Penicillium* sp., and Their Cytotoxic and Histone Methyltransferase Inhibitory Activities

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Supporting Information

ABSTRACT: Seven gliotoxin-related compounds were isolated from the fungus *Penicillium* sp. strain JMF034, obtained from deep sea sediments of Suruga Bay, Japan. These included two new metabolites, bis(dethio)-10a-methylthio-3a-deoxy-3,3a-didehydrogliotoxin (**1**) and 6-deoxy-5a,6-didehydrogliotoxin (**2**), and five known metabolites (**3**−**7**). The structures of the new compounds were elucidated by analysis of spectroscopic data and the application of the modified Mosher's analysis. All of the compounds exhibited cytotoxic activity, whereas compounds containing a disulfide bond showed potent inhibitory activity against histone methyltransferase (HMT) G9a. None of them inhibited HMT SET7/9.

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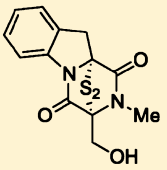

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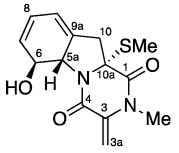
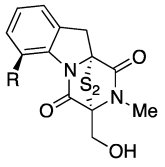
After intensive investigation of secondary metabolites of marine macroorganisms for almost half a century,[1] some marine natural product chemists concluded that new structural entities from these organisms were nearly exhausted. Therefore, they turned their attention to marine microorganisms as an untapped source of secondary metabolites.[2,3] With this trend in mind we have been searching for cytotoxic metabolites produced by marine-derived fungi. We found that a *Penicillium* sp. (strain JMF034) isolated from deep-sea sediments collected in Suruga Bay exhibited potent cytotoxic activity. From the culture medium we isolated a series of metabolites related to gliotoxin[4] and gliotoxin itself as the active constituents, among which two metabolites are new. Gliotoxin is a representative member of the epipolythiodioxopiperazine (ETP) class of fungal metabolites.[5] Due to its potent cytotoxicity toward cancer cell lines, this compound has been considered as a lead for anticancer agents.[5] Recently, dimeric ETPs were shown to inhibit histone methyltransferase (HMT).[6] These observations prompted us to examine the HMT-inhibitory activity of the metabolites, some of which showed significant activity. Here we describe structure elucidation of the new compounds.

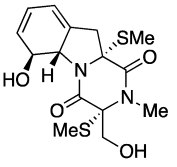
The culture medium of *Penicillium* sp. JMF034 was extracted with EtOAc, and the combined EtOAc layers were fractionated by solvent partitioning, gel permeation, and reversed-phase column chromatographies followed by ODS-HPLC to yield compounds **1**−**7**. The known compounds were identified as bis(dethio)bis(methylthio)gliotoxin (**3**),[7] bis(dethio)bis(methylthio)-5a,6-didehydrogliotoxin (**4**),[8] 5a,6-didehydrogliotoxin (**5**),[9] gliotoxin (**6**),[10] and gliotoxin G (**7**),[8] on the basis of ESIMS and ^{1}H NMR data.



1



2 R = H
5 R = OH



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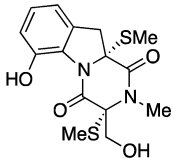

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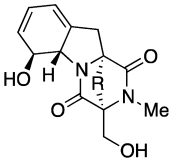

6 R = S_2
7 R = S_4

The molecular formula of compound **1** was determined to be $C_{14}H_{16}N_2O_3S$ on the basis of the HRESIMS data. Analysis of the ^{1}H NMR spectrum (Table 1) in conjunction with the HSQC data revealed two singlet methyls, a pair of non-equivalent methylene protons, two oxygen- or nitrogen-substituted methines, five olefinic protons, two of which were a pair of exomethylene protons, and an exchangeable proton. Even though the number of protons was small, interpretation of the COSY spectrum was complex due to the overlap of H-5a and H-6, the absence of coupling between H-6 and H-7, and the observation of long-range couplings between H-5a and H-9

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Table 1. ^{1}H and ^{13}C NMR Data for Compounds 1 and 2 (600 MHz, $CDCl_3$)

	1			2		
position	δ_C,[a] type	δ_H (J in Hz)	HMBC	δ_C,[a] type	δ_H (J in Hz)	HMBC
1	164.5, C			166.1, C		
3	138.1, C			77.0, C		
3a	105.2, CH_2	H_Z: 5.90, d (1.8)	3, 4	61.0, CH_2	4.48, d (12.7)	3, 4
		H_E:5.09, d (1.8)	3, 4		4.32, d (12.7)	3, 4
4	161.5, C			161.5, C		
5a	69.2, CH	4.90, d (13.0)	6, 7	138.2, C		
6	74.1, CH	4.89, d (13.0)	5a, 7, 9a	115.8, CH	7.95, d (8.3)	8, 9a
7	130.8, CH	5.79, d (9.7)	5a, 9	125.1, CH	7.36, dd (8.3, 7.9)	5a, 9
8	123.2, CH	5.91, m	6, 9a	126.2, CH	7.24, dd (7.9, 7.5)	6, 9a
9	120.5, CH	5.95, br s	5a, 7	128.8, CH	7.37, d (7.5)	5a, 7
9a	132.0, C			128.1, C		
10	39.5, CH_2	3.07, d (16.2)	1, 9, 9a, 10a	36.7, CH_2	4.33, d (18.4)	1, 9
		3.00, d (16.2)	5a, 9, 9a, 10a		3.35, d (18.4)	5a, 10a, 9
10a	73.5, C			74.0, C		
S-Me	14.5, CH_3	2.13, s	10a	27.6, CH_3	3.27, s	1, 3
N-Me	30.5, CH_3	3.29, s	1, 3			
6-OH		6.07, s	5a, 6, 7			

[a]Carbon chemical shifts were assigned from HSQC and HMBC spectra.

and between H-5a and H-10″.[11] Two partial structures (units a and b, Figure 1) were assigned by interpretation of the 2D

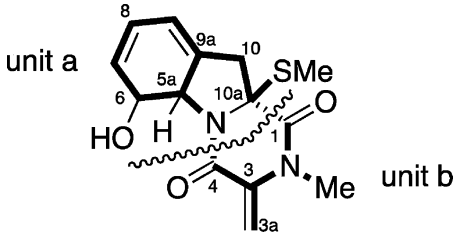


Figure 1. Partial structures of **1**.

NMR data (Table 1). In unit a, the connectivity of the three olefinic protons (H-7, H-8, and H-9) was unambiguously assigned from the COSY data. HMBC cross-peaks from OH-6 to C-5a, C-6, and C-7 showed that C-7 was connected to C-6, which was linked to C-5a. HMBC cross-peaks from H_2-10 to C-9, C-5a, C-9a, and C-10a indicated that C-9 was connected to C-9a. HMBC cross-peaks from H_2-10 to C-10a and from the S-Me to C-10a showed the connection between C-10 and C-10a. NMR data of this portion matched well with those of gliotoxin,[10] suggesting that C-5a was attached to a nitrogen atom, which was connected to C-10a. In unit b, an exomethylene (C-3 and C-3a) was flanked by an amide carbonyl (C-4) and the *N*-methyl group on the basis of the HMBC data. HMBC correlations from H-10″ to C-1 and from *N*-Me to C-1 implied the linkage of C-1 and C-10a, revealing the other linkage of C-4 and N-5. These final linkages are consistent with the established structures of gliotoxin-type metabolites and with recent biosynthetic evidence[11] for this class of compounds.

The relative configuration of **1** was determined by interpretation of the NOESY data (Figure 2). In the NOESY

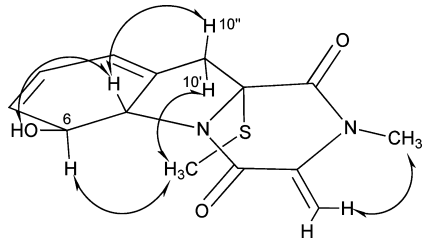
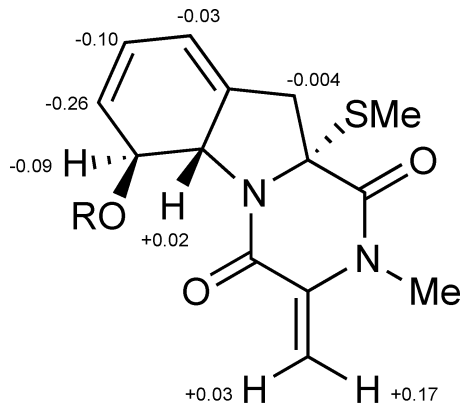


Figure 2. Key NOESY correlations of **1**.

spectrum, 10a-*S*-Me was correlated to H-6 and H-10′ (δ 3.00), whereas H-5a was correlated to H-10″ (δ 3.07) and OH-6. Additionally, a coupling constant of 13.0 Hz between H-5a and H-6 indicated that the two protons were antiperiplanar. Therefore, H-6 and 10a-*S*-Me were on the same face of the tetrahydroindole ring system, whereas H-5a was on the other face.

The absolute configuration of **1** was determined by the modified Mosher's method.[12] Esterification of **1** with (*R*)- and (*S*)-MTPA-Cl afforded the (*S*)- and (*R*)-MTPA esters, **1a** and **1b**, respectively. The distribution of $\Delta\delta$ values indicated the 6*S* configuration (Figure 3), the same configuration as is known



1a R=(S)-MTPA
1b R=(R)-MTPA

Figure 3. Values of $\Delta\ \delta_S - \delta_R$ of the MTPA esters of **1**.

for gliotoxin.[10] Therefore, compound **1** is 5a*S*,6*S*,10a*R*-bis(dethio)-10a-methylthio-3a-deoxy-3,3a-didehydrogliotoxin.

Compound **2** had the molecular formula $C_{13}H_{12}N_2O_3S_2$ as determined by HR ESIMS and NMR data (Table 1). The ^{1}H NMR data in conjunction with the HSQC data suggested the presence of four aromatic protons, a pair of nonequivalent methylene protons, an *N*-methyl, and a hydroxymethyl group. The COSY data suggested that the benzene ring was *ortho* disubstituted, whereas the remaining signals coincided well with those of the corresponding parts of dehydrogliotoxin (**5**).[9]

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Table 2. Inhibitory Activity against HMT G9a and Cytotoxicity on P388 Cells of Compounds 1−7 (IC_{50})

	1	2	3	4	5	6	7
HMT G9a (μM)	>100	55	>100	58	2.6	6.4	2.1
cytotoxicity (μM)	3.4	0.058	0.11	0.11	0.056	0.024	0.020

Therefore, **2** was suggested to be the deoxy derivative of **5**, which was supported by the HMBC data (Table 1).[13] Both compound **1** and the co-isolated gliotoxin **6**[14] have a 10a*R* configuration, with the configuration of **6** being assigned by comparison of specific rotation data. On the basis of biogenetic considerations we also presume a 10a*R* configuration for **2**.

The cytotoxic activities of compounds **1**−**7** were examined against P388 murine leukemia cells. Gliotoxin (**6**) and gliotoxin G (**7**) exhibited the most potent activity, whereas compounds **2**−**5** also showed significant activity (Table 2). However, compound **1** had only marginal activity. We also examined the inhibitory activity of **1**−**7** against HMT G9a and HMT Set7/9 (lysine-specific histone methyltransferase for lysine 4 in histone H3). As expected from the previous report,[4] compounds with a disulfide or tetrasulfide bond (**5**, **6**, and **7**) exhibited potent inhibitory activity. The weaker activity observed for **2**, which also has a disulfide bond, suggested that the C-6 hydroxy group interfered with the G9a inhibitory activity. None of **1**−**7** inhibited HMT Set7/9 at 100 μM.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. UV spectra were recorded on a Shimadzu Biospec 1600. NMR spectra were recorded on a JEOL alpha 600 NMR spectrometer at 300 K. Chemical shifts were referenced to solvent peaks: δ_H 7.27 and δ_C 77.2 for $CHCl_3$. ESI mass spectra were measured on a JEOL JMS-T 100LC. HPLC was carried out on a Shimadzu LC 20AT with a SCL-10Avp controller and a SPD-10Avp detector.

Fungal Material. Deep-sea sediments were collected by the unmanned ROV KAIKO system from Fujikawa, Suruga-Bay, Japan, at a depth of 1151 m, in July 1996. The sediment sample was stored in a sterilized sampler and frozen with liquid nitrogen. Then the sample was transported to the laboratory, where it was kept frozen until processed. The *Penicillium* sp. JMF034 strain was isolated from this sample. To investigate the taxonomic position of the strain, the 28S rDNA-D1/D2 gene was amplified using the PCR method with primers NL1 and NL4.[15] The PCR product was sequenced with the dideoxynucleotide chain-termination method, using a BigDye Terminator v3.1 kit (Applied Biosystems) and ABI PRISM 3130xl genetic analyzer system (Applied Biosystems). The 28S rDNA-D1/D2 gene sequence of the isolated fungus (DDBJ accession no. AB684325) was compared with other sequences in the public database using the BLAST program. Strain JMF034 showed the highest similarities to strain *Penicillium angulare* NRRL28157^T (sequence identity 99.1%), *P. adametzioides* NRRL3405^T (98.9%), and *P. brocae* NRRL31472^T (sequence identity 98.8%). Therefore JMF034 is included in the *Penicillium* genus..

Fermentation, Extraction, and Isolation. The fungal strain was cultured in 20 × 500 mL Erlenmeyer flasks each containing 100 mL of production medium (0.3 g yeast extract, 0.3 g malt extract, 0.5 g peptone, 1 g glucose, pH 7.2−7.4) at 27 °C. After 14 days of static culture, the fermentation broth, including cells, was harvested and then centrifuged to separate the mycelial mass from the aqueous layer.

The mycelial mass and the aqueous layer were exhaustively extracted with acetone and EtOAc, respectively. Then each extract was concentrated in vacuo. The EtOAc extract was subjected to C_{18} flash column chromatography (5 × 30 cm), eluting with a stepwise gradient of 20%, 40%, 60%, 80%, and 100% (v/v) MeOH in H_2O (2 L each). The fraction that eluted with 60% MeOH was further fractionated with a Sephadex LH-20 column using $CHCl_3$/MeOH (1:1), followed by ODS-HPLC using gradient elution from 50% to 80% aqueous MeOH to yield five fractions (A−E). Fraction B was then purified by semipreparative ODS-HPLC using a gradient of MeCN in H_2O (30−50%) to afford **3** (5.4 mg), **4** (2.6 mg), **5** (2.8 mg), and **6** (4.2 mg). Fractions C and D were combined and purified by HPLC using gradient elution from 35% to 55% MeCN in H_2O to yield **1** (1.5 mg), **2** (2.6 mg), and **7** (3.0 mg).

*Bis(dethio)-10a-methylthio-3a-deoxy-3,3a-didehydrogliotoxin (**1**):* white solid; $[\alpha]^{25}_D$ −4.6 (*c* 0.03, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.20), 265 (4.07) nm; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m*/*z* 315.0759 $[M + Na]^+$ (calcd for $C_{14}H_{16}N_2O_3S$, 315.0779).

*6-Deoxy-5a,6-didehydrogliotoxin (**2**):* colorless oil; $[\alpha]^{25}_D$ −20 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.26), 265 (3.91) nm; ^{1}H NMR ($CDCl_3$) and ^{13}C NMR ($CDCl_3$), see Table 1; HRESIMS *m*/*z* 331.0315 $[M + Na]^+$ (calcd for $C_{13}H_{12}N_2O_3S_2$, 331.0338).

*Gliotoxin (**6**):* $[\alpha]^{25}_D$ −440 (*c* 0.11, MeOH). The sign and magnitude of the specific rotation value were comparable to those of gliotoxin in the literature,[14] which was $[\alpha]^{25}_D$ −290 (*c* 0.08, EtOH).

*Preparation of MTPA Esters of **1a** and **1b**.* Compound **1** (100 μg for each) was reacted with either *R*-(−)- or *S*-(+)-MTPA Cl (5 μL) in 50 μL of CH_2Cl_2 and 50 μL of pyridine for 2 h. The reaction mixture was diluted with H_2O and extracted with CH_2Cl_2 three times. The organic layers were combined and separated by ODS-HPLC (C_{18}-stationary phase, 10 × 50 mm; 40−60% MeCN in H_2O) to afford the *S*-(−)- or *R*-(+)-MTPA esters **1a** and **1b**.

*(S)-MTPA Ester of **1** (**1a**):* ^{1}H NMR ($CDCl_3$) δ 6.32 (H-6), 5.99 (H-9), 5.94 (H-8), 5.67 (H_Z-3a), 5.43 (H-7), 5.36 (H-5a), 4.94 (H_E-3a), 3.28 (N-CH_3), 3.02 (H-10), 2.23 (S-CH_3); ESIMS *m*/*z* 531 $[M + Na]^+$.

*(R)-MTPA Ester of **1** (**1b**):* ^{1}H NMR ($CDCl_3$) δ 6.41 (H-6), 6.04 (H-8), 6.03 (H-9), 5.69 (H-7), 5.50 (H_Z-3a), 5.34 (H-5a), 4.91 (H_E-3a), 3.26 (N-CH_3), 3.03 (H-10), 2.21 (S-CH_3); ESIMS *m*/*z* 531 $[M + Na]^+$.

Assay for Cytotoxicity against P388 Cells. P388 murine leukemia cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 μg/mL kanamycin, and 10 μg/mL 2-hydroxyethyl disulfide at 37 °C under an atmosphere of 5% CO_2. To each well of the 96-well microplate containing 100 μL of tumor cell suspension (1 × 10^4 cells/mL) was added 100 μL of test solution dissolved in RPMI-1640 medium, and the plate was incubated in a CO_2 incubator at 37 °C for 96 h. After the addition of 50 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide saline solution (1 mg/mL) to each well, the plate was incubated for 3 h under the same conditions to stain live cells. After the incubation, the plate was centrifuged, the supernatants were removed, and the cells were dissolved in 150 μL of DMSO to determine the IC_{50} values.

Histone Methyltransferase Inhibitory Activity Assay. A mixture of 1 μL of GST (glutathione *S*-transferase)-G9a (200 ng) or His (histone)-Set7/9 (931 ng), 1 μL of BSA (150 ng), 25 μL of 2× HMT activity buffer (100 mM Tris-HCl (pH 8.5), 20 mM $MgCl_2$, 40 mM KCl, 20 mM 2-mercaptoethanol, 500 mM sucrose), 1 μL of a DMSO solution of the compound of interest, and 20 μL of H_2O was incubated at room temperature (rt) for 1 h in a total volume of 48 μL/well. After the addition of 1 μL of *S*-adenosylmethionine (50 ng) and 1 μL of biotinylated H3 peptide (50 ng) into this reaction mixture, the resulting mixture was further incubated at 37 °C for 2 h followed by boiling at 96 °C for 30 min. The supernatant of the mixture was transferred to a streptavidin-coated plate and incubated at rt for 1 h. The supernatant was removed, and the remaining plate was washed with 300 μL of PBS containing 0.5% Tween20 (PBST) three times. Then, the plate was treated with an antimethylated-lysine antibody[16] for G9a inhibitory activity assay or an antidimethyl-Histone H3 (Lys4)

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antibody (Upstate, 05-790) for a Set7/9 inhibitory activity assay in PBST. After incubation at rt for 1 h, the supernatant was removed, and the remaining plate was washed three times with 300 μL of PBST. Subsequently, the secondary antibody conjugated with mouse HRP (horseradish peroxidase) for the G9a inhibitory activity assay or rabbit HRP for the Set7/9 inhibitory activity assay in PBST (100 μL/well) was added to the plate, and the resulting mixture was incubated at rt for 1 h. After the removal of the supernatant, the plate was washed five times with 300 μL of PBST. A substrate of 3,3′,5,5′-tetramethylbenzidine peroxidase was added into the plate, and the reaction was run at rt for 40 min. Finally, the inhibitory activity was evaluated by measuring absorption of each well at 650 nm using a plate reader.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^{1}H NMR, ^{1}H−^{1}H COSY, HSQC, and HMBC data for compounds **1** and **2** and ^{1}H NMR data for the MTPA esters of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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