Sulfurated diketopiperazines from an algicolous isolate of *Trichoderma viride*

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**ABSTRACT**

Two new naturally occurring sulfurated diketopiperazines, dehydroxymethylbis(dethio)bis(methylthio)gliotoxin (1) and (3S,6R)-6-(para-hydroxybenzyl)-1,4-dimethyl-3,6-bis(methylthio)piperazine-2,5-dione (2), along with three known analogues (3–5) were isolated from the culture extract of *Trichoderma viride* Y13-3, obtained from the surface of the marine red alga *Gracilaria vermiculophylla*. The structures and relative configurations of 1 and 2 were determined by extensive 1D/2D NMR, MS, and IR spectroscopic data, and their absolute configurations were established by analysis of ECD spectra aided by quantum chemical calculations. Compounds 1–5 were evaluated for the inhibition of some marine-derived organisms.

1. Introduction

Sulfur-containing secondary metabolites have been discovered from various organisms, including terrestrial and marine species (Duan et al., 2007; Li et al., 2013, 2017; Liu et al., 2015; Meng et al., 2013, 2014, 2016; Petkowshi et al., 2018). Among them, marine-derived filamentous fungi have given a number of sulfur-containing compounds with high structural diversity and intriguing bioactivities, which have attracted a great attention for marine natural product research (Ji and Wang, 2016; Meng et al., 2013, 2014, 2016). Gliotoxin with a unique disulfide bridge is a representative fungal toxin that has been obtained from *Trichoderma viride* for the first time (Brian, 1944), and later this compound along with its analogues have also been found in some marine-derived fungi (Chen et al., 2013, 2014, 2016). Gliotoxin with a unique disulfide bridge is a representative fungal toxin that has been obtained from *Trichoderma viride* for the first time (Brian, 1944), and later this compound along with its analogues have also been found in some marine-derived fungi (Chen et al., 2013, 2014, 2016). Gliotoxin with a unique disulfide bridge is a representative fungal toxin that has been obtained from *Trichoderma viride* for the first time (Brian, 1944), and later this compound along with its analogues have also been found in some marine-derived fungi (Chen et al., 2013, 2014, 2016).

2. Results and discussion

Compound 1 was obtained as a colorless oil, and a molecular formula of C\(_{14}\)H\(_{18}\)N\(_2\)O\(_3\)S\(_2\) was determined by interpretation of HREIMS (m/z 326.0760 [M]+, calcd for C\(_{14}\)H\(_{18}\)N\(_2\)O\(_3\)S\(_2\), 326.0759), requiring seven degrees of unsaturation. The IR spectrum gave absorption bands at 3424 and 1646 cm\(^{-1}\), demonstrating the presence of hydroxy and carbonyl groups. The \(^1H\) NMR spectrum (in CDCl\(_3\), Table 1) in conjunction with HSQC data displayed three methyl singlets, two doublets ascribable to a pair of nonequivalent methylene protons, one singlet and two doublets attributable to three oxygenated or nitrogenated methines, one broad singlet assignable to an exchangeable proton, and one doublet, one doublet of double doublets, and one multiplet due to three olefinic protons. The \(^13C\) NMR spectrum (Table 1) showed 14 resonances, classified into three methyls, one methylene, six methines, and four nonprotonated carbons by DEPT experiments. A detailed comparison of NMR data with those reported for bis(dethio)bis(methylthio)gliotoxin (4) (Lee et al., 2001; Sun et al., 2012) revealed their similarity, except for the lack of signals for a hydroxymethyl group in 1. Thus, 1 was proposed to be a dehydroxymethyl derivative of 4, and its planar structure was further verified by the COSY correlations of H-9/H-10/H-11/H-12/H-13 and HMBC correlations from H-3 to C-2 and C-5, H-7 to C-8, C-9, and C-13, from MeS-3 to C-3, from MeS-4 to C-3 and C-5, and from MeS-6 to C-6 (Fig. 2).

The relative configuration of 1 was established to be identical to that of 4 by analysis of NOESY spectra and coupling constants. The NOE correlations of MeS-6 with H-7a and MeS-5 located them on the same face of the molecule, while those of H-13 with H-7b and OH-12...
positioned them on the other face (Fig. 3). The large coupling constants of H-12 and H-13 also suggested their opposite orientation. Regardless of the rotations of OH, MeN, and MeS groups, only one energy-minimized conformer (1a) (Fig. 3) optimized at the B3LYP/6-31 G(d) level in MeOH with the integral equation formalism variant (IEF) of the polarizable continuum model (PCM) via Gaussian 09 software (Frisch et al., 2010) was obtained. Its electronic circular dichroism (ECD) spectrum was computed at the same level through the time-dependent density function theory (TD-DFT) method and depicted by SpecDis software with sigma = 0.2 (Bruhn et al., 2011). Based on the comparison of experimental and calculated ECD spectra (Fig. 4), the absolute configuration of 1 was assigned to be 3R, 6R, 12S, and 13S. The structure of 1 was previously reported as a synthetic derivative of 4, but its stereochemistry at C-3 was not determined (Okamoto et al., 1986). It is also worth to mention that H2-7 of the synthetic analogue showed only a broad singlet, rather than two doublets, in the 1H NMR spectrum recorded in CDCl3.

Compound 2 was isolated as a colorless oil with a molecular formula of C15H20N2O3S2 given by HREIMS (m/z 340.0911 [M]+, calcld for C15H20N2O3S2, 340.0915), implying seven degrees of unsaturation. The IR absorption peaks at 3422 and 1647 cm⁻¹ were slated for hydroxy and carbonyl groups, respectively. The 1H NMR spectrum (Table 1) exhibited four methyl singlets, two doublets due to geminal protons of a methylene, one singlet attributable to a deshielded methine, and two doublets assignable to four aromatic protons. The 13C NMR spectrum (Table 1) gave only 13 signals, rather than 15 ones as shown in the molecular formula. However, those at δC 115.9 and 131.8 corresponded to two pairs of methines by analysis of HSQC data. The above NMR data, except for the deshielded 1H NMR signal for H-3, closely resembled those of (3R,6R)-6-(para-hydroxybenzyl)-1,4-dimethyl-3,6-bis (methylthio)piperazine-2,5-dione (3) (Hanson and O’Leary, 1981).

### Table 1

| pos | 1 (in CDCl3) | 2 (in CDCl3) | 1 (in DMSO-d6) | 2 (in DMSO-d6) |
|-----|--------------|--------------|----------------|--------------|
| 2   | 167.3, C     | 167.4, C     | 166.1, CH      | 4.61, s      |
| 3   | 67.8, CH     | 66.1, CH     | 164.0, C       | 2.76, s      |
| 5   | 164.6, C     | 71.7, C      | 71.7, C        | 65.9, C      |
| 6   | 71.9, C      | 164.0, C     | 71.7, C        | 164.8, C     |
| 7a  | 3.04, d (16.0) | 37.9, CH2   | 37.7, CH2      | 76.5, C      |
| 7b  | 2.94, d (15.9) | 131.5, C    | 133.3, C       | 126.3, C     |
| 8   | 131.5, C     |              | 131.5, C       |              |
| 9   | 5.95, m      | 120.6, CH    | 119.0, CH      | 7.02, d (8.2) |
| 10  | 12.3, CH     | 123.1, CH    | 123.4, CH      | 131.8, CH    |
| 11  | 130.5, CH    | 5.89, d (9.8) | 130.1, CH      | 115.9, CH    |
| 12  | 74.4, CH     | 4.60, d (13.4)| 73.7, CH      | 115.9, CH    |
| 13  | 69.3, CH     | 4.75, d (13.3) | 68.5, CH    | 115.9, CH    |
| a   | 131.0, C     |              | 131.8, CH      |              |
| MeN-1 | 3.11, s | 32.4, CH2 | 31.4, CH2 | 3.04, s |
| MeN-4 | 3.24, s | 18.0, CH2 | 17.1, CH2 | 3.45, s |
| MeS-3 | 2.45, s | 15.0, CH2 | 14.2, CH2 | 1.67, s |
| MeS-6 | 2.20, s | 5.58, br s | 1.96, s | 12.7, CH3 |
| OH-12 | 5.61, br s | 10.0, br s | 3.26, s | 30.7, CH3 |

Fig. 1. Structures of compounds 1–5.

Fig. 2. Key COSY (bold lines) and HMBC (arrows) correlations of 1 and 2.

Fig. 3. Energy-minimized conformers and NOE correlations of 1 and 2 (Boltzmann populations).

Fig. 4. Key COSY (bold lines) and HMBC (arrows) correlations of 1 and 2.
Thus, 2 was speculated to be a C-3 epimer of 3, which was corroborated by the NOE correlation between H-3 and MeS-6 (Fig. 3). The COSY and HMBC correlations as illustrated in Fig. 2 further validated the structure of 2. Its ECD spectrum was simulated at the B3LYP/6-31 G(d) level in MeOH using the TD-DFT method based on three energy-minimized conformers (2a–2c) (Fig. 3). In view of the similarity between experimental and calculated ECD curves (Fig. 5), the absolute configuration of 2 was deduced to be 3S and 6R, which was further confirmed by comparison of the specific optical rotation with that of trans-bis(methylthio)silvatin (α, α-D +19.9) (Kawahara et al., 1987).

Compounds 1–5 were evaluated for the inhibition of four marine phytoplankton species (Chattonella marina, Heterosigma akashiwo, Karlodinium veneficum, and Prorocentrum donghaiense), one marine zooplankton species (Artemia salina), and five marine-derived pathogenic bacteria (Vibrio parahaemolyticus, V. anguillarum, V. harveyi, V. splendidus, and Pseudoalteromonas cincta) (Chen et al., 1996; Miao et al., 2012). However, none of them could inhibit the five plankton species tested at 100 μg/mL and the five bacteria tested at 20 μg/disk.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Jasco P-1020 polarimeter (JASCO, Tokyo, Japan). NMR spectra were recorded on a Bruker Avance III 500 NMR spectrometer (500 and 125 MHz for 1H and 13C, respectively) using tetramethylsilane (TMS) as an internal standard (Bruker Corp., Billerica, MA, USA). Low and high resolution EI mass spectra were obtained on an Autospec Premier P776 mass spectrometer (Waters Corp., Milford, MA, USA). Column chromatography (CC) was carried out with silica gel (200–300 mesh, Qingdao Haiyang Chemical Co., Qingdao, China), RP-18 (AAG12550, YMC Co. Ltd., Kyoto, Japan), and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden). Thin-layer chromatography (TLC) was performed with precoated silica gel plates (GF-254, Qingdao Haiyang Chemical Co., Qingdao, China).

3.2. Fungal material and fermentation

Trichoderma virens Y13-3, as an algal epiphyte, was obtained from the surface of the marine red alga Gracilaria vermiculophylla collected from Yangma Island in July 2015. Following previous procedures (Abdel-Gawad et al., 2014; Jenkins et al., 1998), the alga sample was washed with sterile seawater thrice and then put onto the PDA plate (200 mg/L chloramphenicol) for 2–3 days at 28°C until some colonies appeared. The colonies were purified by repeated inoculation on PDA plates until the single colony was got. The species was identified by morphological observation and by analysis of the ITS regions of its rDNA, deposited at GenBank (no. MG828822). The initial cultures were maintained on potato dextrose agar plates, and the mass cultures were performed statically at room temperature for 30 days in 200 × 1 L Erlemmeyer flasks, each containing 300 mL media with 500 mL potato (100 g) broth, 20 g glucose, 5 g peptone, 5 g yeast extract powder, and 500 mL natural seawater from the coast of Yantai.

3.3. Extraction and isolation

The whole fermented cultures (60 L) were filtered to separate the mycelia from the broth, and the former were exhaustively extracted with CH2Cl2 and MeOH (1:1, v/v). After removing organic solvents, the residue was partitioned between EtOAc and H2O to afford an EtOAc-soluble extract (12.3 g). Additionally, the broth was thoroughly extracted with EtOAc and then concentrated to give an extract (14.9 g). Because of the identical TLC profiles, the two parts were combined and then subjected to silica gel CC with step-gradient solvent systems consisting of petroleum ether (PE)/EtOAc and CH2Cl2/MeOH to yield 10 fractions (Fr s 1–10). Fr. 6 eluted with PE/EtOAc (1:1) and was further purified by CC on RP-18 (MeOH/H2O, 3:2) and Sephadex LH-20 (MeOH) and preparative TLC (PE/EtOAc, 1:1, Rf = 0.5) to give 5 (9.2 mg). Fr. 7 eluted with CH2Cl2/MeOH (20:1) and was further purified by RP-18 CC (MeOH/H2O, 1:1), preparative TLC (CH2Cl2/MeOH, 20:1, Rf = 0.5 for 1, 0.8 for 2, and 3, and 0.3 for 4), and Sephadex LH-20 CC (MeOH) to produce 1 (3.1 mg), 3 (6.4 mg), and 4 (3.2 mg). Fr. 8 eluted with CH2Cl2/MeOH (10:1) and was further purified by RP-18 CC (MeOH/H2O, 2:3), preparative TLC (CH2Cl2/MeOH, 20:1, Rf = 0.7), and Sephadex LH-20 CC (MeOH) to afford 2 (2.0 mg).

3.3.1. Dehydroxymethylbis(dethio)bis(methylthio)gliotoxin (1)

Colorless oil; [α]D20 +22 (c 0.16, MeOH); IR (KBr) νmax 3422, 2917, 1674, 1647, 1614, 1516, 1434, 1388, 1269, 1244, 734 cm−1; 1H and 13C NMR data, Table 1; EIMS m/z (%) 326 [M]+ (10), 279 (8), 231 (17), 133 (10), 107 (15), 81 (20), 44 (100); HREIMS m/z 326.0760 [M]+ (calcd for C14H18N2O3S2, 326.0759).

3.3.2. (3S,6R)-6-(para-Hydroxybenzyl)-1,4-dimethyl-3,6-bis(methylthio)piperazine-2,5-dione (2)

Colorless oil; [α]D20 +11 (c 0.10, MeOH); IR (KBr) νmax 3422, 2917, 1647, 1614, 1516, 1434, 1388, 1269, 1244, 734 cm−1; 1H and 13C NMR data, Table 1; EIMS m/z (%) 340 [M]+ (12), 293 (100), 246 (34), 218 (38), 187 (19), 107 (45), 43 (28); HREIMS m/z 340.0911 [M]+ (calcd for C14H18N2O3S2, 340.0915).
Inhibition rate (IR) = (NCK – NT)/NCK × 100%

(NCK: the number of live cells under negative control. NT: the number of live cells under treatment)

The inhibition of one marine zooplankton species (Artemia salina) and five marine-derived pathogenic bacteria (Vibrio parahaemolyticus, V. anguillarum, V. harveyi, V. splendidus, and Pseudoalteromonas citrea) was assayed according to the procedures described by our group (Miao et al., 2012).

3.5. Computational details

Regardless of the rotations of OH, MeN, and MeS groups, one energy-minimized conformer (1a) of 1 and three energy-minimized conformers (2a–2c) of 2 (Fig. 3) without vibrational imaginary frequencies were obtained after optimization at the B3LYP/6–31 G(d) level in MeOH via Gaussian 09 software (Frisch et al., 2010). Subsequently, the ECD spectrum of each conformer was simulated at the same level through the TD-DFT method and then drawn by SpecDis software with a = 0.2 (Bruhn et al., 2011). The overall calculated ECD spectrum of 2 was generated by Boltzmann weighting of curves of the three lowest energy conformers with 79.2, 15.5, and 5.3% populations, respectively. All the above calculations were performed with the IEF-PCM as implemented in Gaussian 09.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:10.1016/j.phytol.2018.07.005.

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