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Novel Cyclic Hexapeptides from Marine-Derived Fungus, *Aspergillus sclerotiorum* PT06-1

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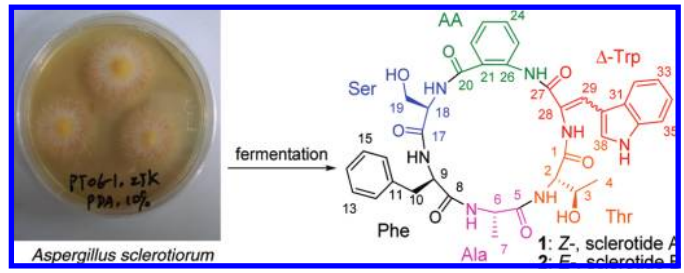
ABSTRACT



Two novel cyclic hexapeptides containing both anthranilic acid and dehydroamino acid units, sclerotides A (1) and B (2), were isolated from the marine-derived halotolerant *Aspergillus sclerotiorum* PT06-1 in a nutrient-limited hypersaline medium. Both 1 and 2 are photointerconvertible and could be interconverted via a radical reaction initiated by direct photoisomerization. Both compounds showed moderate antifungal activity. Compound 2 also showed weak cytotoxicity and antibacterial activity.

Cyclopeptides derived from natural sources have been chiefly found in plants, in lower sea animals, and in microorganisms, possessing many interesting bioactivities, such as antimicrobial, insecticidal, antimalarial, estrogenic, sedative, nematicidal, cytotoxic, and immunosuppressive activities.[1,2] As part of our ongoing efforts to discover structurally novel and bioactive natural compounds from microorganisms, a marine-derived halotolerant fungal strain PT06-1, identified as *Aspergillus sclerotiorum*, was isolated from the Putian Sea Salt Field, Fujian, China. Only five secondary metabolites, sclerotiamide,[3] scleramide,[4] penicillic acid,[5] ochratoxin A,[6] and Ro 09-1469,[7] had been previously reported from *A. sclerotiorum*. The metabolite pattern of this fungus exhibited distinct different TLC and HPLC profiles in different salty media. Two novel cyclic hexapeptides containing both anthranilic acid and dehydrotryptophan residues, sclerotides

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A (**1**) and B (**2**), were identified from the metabolites in a nutrient-limited medium containing 10% NaCl. To the best of our knowledge, this is the first example of cyclopeptides containing both anthranilic acid and dehydroamino acid residues in natural products, and the microbial secondary metabolites under high salt culture conditions were rarely reported.

A. sclerotiorum PT06-1 was grown under static conditions at 28 °C for 30 days and then was harvested by extraction with EtOAc. The EtOAc extract was concentrated under reduced pressure to give a dark brown gum (8.0 g). The crude residue was separated into five fractions on a silica gel vacuum liquid chromatograph using a step gradient elution with MeOH−$CHCl_3$ (0−100%). Fraction 3 (1.2 g) from the 20:1 of $CHCl_3$/MeOH eluents was subjected to silica gel column chromatography using a step gradient elution of acetone/petroleum ether (40−100%) to afford three subfractions (Fr.3-1∼3-3). Fr.3-3 (73 mg) was combined with fraction 4 (0.8 g) from the 10:1 of $CHCl_3$/MeOH eluents, and the mixture was further separated into four subfractions (Fr.4-1∼4-4) by Sephadex LH-20, eluting with MeOH. Fr.4-1 (240 mg) was then purified by semipreparative HPLC (57% MeOH/H_2O, 4.0 mL/min) to yield compounds **2** (14 mg, t_R = 13 min) and **1** (90 mg, t_R = 15 min). Both compounds **1** and **2** were kept away from radiation by silver paper.

Compound **1**, $[\alpha]^{25}_D$ −73 (*c* 0.3, MeOH), UV (MeOH) λ_{max} (log ε) 203 (3.9), 222 (3.8), 268 (3.3), 350 (3.6) nm, was obtained as a yellow amorphous powder. Its molecular formula was determined as $C_{37}H_{39}N_7O_8$ according to the HRESIMS at *m/z* 710.2962 $[M + H]^+$, requiring 22 degrees of unsaturation. Analysis of ^1H and ^{13}C NMR data (Table 1) revealed the presence of six carbonyl signals (δ_C 172.6, 171.7, 170.4, 170.3, 169.0, and 163.6) and six amide protons (δ_H 7.37, 8.37, 8.62, 8.76, 8.77, and 10.92) coupling with signals between 3.6 and 4.6 ppm, indicating the hexapeptide nature of **1**. $^1H-^1H$ COSY experiment (Figure 1) constructed the six amino acid residues as threonine, alanine, phenylalanine, serine, anthranilic acid (AA), and dehydrotryptophan (Δ-Trp). These residues accounted for 21 of the 22 degrees of unsaturation, indicating that **1** is a cyclopeptide. The amino acid sequence was determined mainly by HMBC correlations of the carbonyl carbon of one amino acid residue with the amide protons of the neighboring residue, and the carbonyl carbon signals in each amino acid unit were deduced by their HMBC correlations with the β-proton of respective amino acids (Figure 1). Thus, the constitution of **1** was assigned as *cyclo* (Thr-Ala-Phe-Ser-AA-Δ-Trp). Compound **2**, $[\alpha]^{25}_D$ −111 (*c* 0.3, MeOH), UV (MeOH) λ_{max} (log ε) 203 (3.9), 222 (3.8), 274 (3.3), 352 (3.5) nm, showed a pseudomolecular ion peak at *m/z* 710.2929 $[M + H]^+$, indicating that **2** was an isomer of **1**. As expected, UV absorption and 1D NMR data (Table 1) of **2** were very similar to those of **1**. Further analysis of 2D NMR data of **2** (Figure 1) revealed that **2** shared the same constitution as **1**. The obvious differences between H-29, H-38, and NH in dehydrotryptophan residue revealed that **1** and **2** are a pair of geometric isomers of C_{28}═C_{29}. The downfield chemical shift of H-28 (δ_H 7.96 vs 7.03) implied *Z*-configuration of C_{28}═C_{29} in **1** due to the deshielding effect of the 27-carbonyl,[8,9] while **2** was *E*-configuration. The absolute configurations of **1** and **2** were determined by amino acids analyses of acidic hydrolysates through a chiral Crownpak CR(+) HPLC column.[9,10] Mix HPLC analyses of the hydrolysates with authentic samples (co-injection) confirmed that the amino acids both in **1** and **2** were L-Thr, L-Ala, D-Phe, and D-Ser.

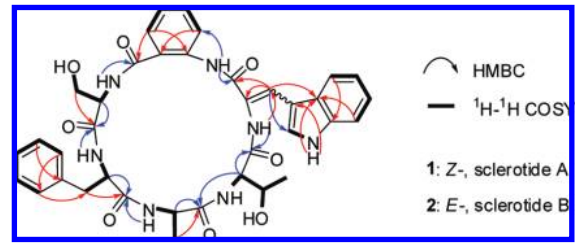

Figure 1. Key $^1H-^1H$ COSY and HMBC correlations of **1** and **2**.

To further confirm the geometry of the double bond, conformational searches were performed by the HyperChem package using an Amber force field. The conformations with low energy from 0 to 3 kcal/mol were optimized again at the B3LYP/3-21G* level.[11] The B3LYP/3-21G*-optimized geometries with energy from 0 to 1.5 kcal/mol were optimized at the B3LYP/6-31G(d) level to look for the most stable geometries.[12] Both structures were found that have 1.0 kcal/mol energy lower than the second stable conformation. Thus, only two conformations were used in ^{13}C NMR computations at the B3LYP/6-311+G(2d,p) level for **1** and **2**. The magnetic shielding values were converted into chemical shifts after the corrections using slope and intercept of the linear-square functions.[13] The relative errors of chemical shifts were computed by subtracting the calculated ^{13}C NMR from the experimental shifts. Two cases should be considered in the data treatments. The first case is that

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Table 1. ^{1}H and ^{13}C NMR Data for **1** and **2** (600, 150 MHz, DMSO-d_6, TMS, δ ppm)

		sclerotide A (1)		sclerotide B (2)	
position		δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
Thr	1	170.3, qC		170.5, qC	
	2	59.0, CH	4.31, dd (3.2, 8.7)	59.4, CH	4.13, dd (2.2, 7.0)
	3	65.6, CH	4.43, m	65.8, CH	4.33, m
	4	20.8, CH_3	1.16, d (6.4)	20.5, CH_3	1.12, d (6.2)
	NH		7.37, d (8.2)		7.19, br s
	3-OH		5.14, br s		5.06, d (5.9)
Ala	5	172.6, qC		172.2, qC	
	6	49.6, CH	4.20, dq (6.3, 7.3)	49.8, CH	4.03, dq (6.1, 7.3)
	7	16.6, CH_3	1.26, d (7.3)	16.7, CH_3	1.21, d (7.3)
	NH		8.77, d (6.4)		8.86, d (5.9)
Phe	8	171.7, qC		172.1, qC	
	9	54.5, CH	4.57, "q" like (7.6)	54.1, CH	4.65, "q" like (7.6)
	10	35.6, CH_2	2.93, dd (7.3, 13.7); 2.87, dd (8.2, 13.7)	36.7, CH_2	2.89, dd (7.2, 13.2); 2.86, dd (8.1, 13.2)
	11	137.5, qC		137.2, qC	
	12	129.1, CH	7.20, d (7.8)	129.1, CH	7.20, d (8.0)
	13	128.1, CH	7.24, t (7.3)	128.1, CH	7.25, t (7.7)
	14	126.4, CH	7.17, t (7.8)	126.4, CH	7.19, t (8.0)
	15	128.1, CH	7.24, t (7.3)	128.1, CH	7.25, t (7.7)
	16	129.1, CH	7.20, t (7.8)	129.1, CH	7.20, d (8.0)
	NH		8.37, d, (7.3)		8.09, d (7.7)
Ser	17	170.4, qC		169.9, qC	
	18	58.0, CH	4.23, "q" like (7.0)	57.8, CH	4.28, "q" like (7.2)
	19	60.9, CH_2	3.67, m	61.3, CH_2	3.64, ddd (3.7, 6.2, 12.1); 3.61, ddd (5.5, 6.2, 12.1)
	NH		8.62, br s		8.62, d (7.3)
	19-OH		5.15, br s		5.12, t (6.2)
anthranilic acid	20	169.0, qC		168.7, qC	
	21	122.0, qC		123.4, qC	
	22	129.2, CH	7.85, dd (1.4, 7.8)	128.9, CH	7.79, d (7.7)
	23	122.9, CH	7.20, dt (1.1, 7.8)	122.9, CH	7.24, dt (1.1, 7.7)
	24	132.1, CH	7.58, ddd (1.4, 7.8, 8.2)	131.8, CH	7.57, ddd (1.4, 7.7, 8.2)
	25	121.2, CH	8.53, d (8.2)	121.4, CH	8.37, d (8.2)
	26	138.4, qC		137.7, qC	
	NH		10.92, s		10.79, s
Δ-Trp	27	163.6, qC		163.8, qC	
	28	122.5, qC		123.1, qC	
	29	126.0, CH	7.96, s	124.3, CH	7.03, s
	30	108.6, qC		108.5, qC	
	31	127.4, qC		127.7, qC	
	32	117.7, CH	7.75, d (7.8)	117.5, CH	7.60, d (7.7)
	33	120.4, CH	7.16, dt (1.3, 7.8)	120.0, CH	7.13, dt (1.1, 7.7)
	34	122.2, CH	7.18, dt (1.4, 7.8)	121.9, CH	7.17, dt (1.5, 7.7)
	35	112.0, CH	7.42, d (7.8)	112.0, CH	7.44, d (7.7)
	36	135.6, qC		135.6, qC	
	37		11.96, s		11.61, s
	38	128.7, CH	7.98, d (2.3)	128.9, CH	8.39, br s
	NH		8.76, s		9.07, s

the structure **1** calculated is the experimental structure **1**; the second case is that the computed **1** is the experimental structure **2**. In the first case, all of the maximum of relative errors of chemical shift were below 8.0 ppm. However, in the second case, the maximum errors at C-21 were 8.9 ppm. Thus, the second assumption was wrong. The calculated results agreed with the experimental structures **1** and **2**. Thus, the structures of compounds **1** and **2** were determined as *cyclo* (L-Thr-L-Ala-D-Phe-D-Ser-AA-*Z*-Δ-Trp) and *cyclo* (L-Thr-L-Ala-D-Phe-D-Ser-AA-*E*-Δ-Trp), respectively.

Compounds **1** and **2** were assayed for their cytotoxic effects on HL-60 cell lines by the MTT method[14] and A-549 cell lines using the SRB method.[15] The antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Bacillus subtilis*, *Pseudomonas*

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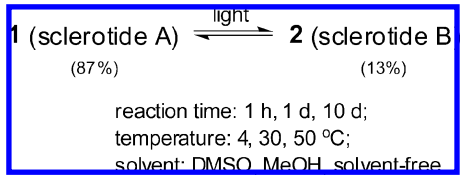
aeruginosa, and *Candida albicans* were evaluated by an agar dilution method.[16] Both **1** and **2** exhibited moderate antifungal activity against *Candida albicans* with the MIC values of 7.0 and 3.5 μM, respectively. Compound **2** also showed weak cytotoxic activity against the HL-60 cell line (IC_{50}, 56.1 μM) and selective antibacterial activity against *Pseudomonas aeruginosa* (MIC, 35.3 μM).

Scheme 1. Photointerconversion of **1** and **2**



Compounds **1** and **2** were stable in the dark, while they changed into each other in light, indicating that **1** and **2** are photointerconvertible (Scheme 1). HPLC analysis showed that the ratio of **1** and **2** was 87:13 in the equilibrium, and both temperature and solvent had little influence on the photoequilibrium. The time for **1** achieving the equilibrium was only several hours, but it was several days for **2**. Thus, the photointerconversion was a radical mechanism. The foregoing results also suggested that the *Z*-isomer (**1**) was lower in energy and the main component. It was possible that the sclerotide A was really produced by *A. sclerotiorum*, while sclerotide B was formed from the photoreaction of sclerotide A during the fermentation or subsequent isolation steps.[17]

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Supporting Information Available: Experimental details, NMR spectra of **1** and **2**, HPLC profiles of acidic hydrolysates and photointerconverting mixtures of **1** and **2**, 18S rRNA and ITS sequences of *A. sclerotiorum* PT06-1, and the calcd ^{13}C NMR by the HyperChem package. This material is available free of charge via the Internet at http://pubs.acs.org.

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