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NEW DIKETOPIPERAZINE DERIVATIVES FROM CULTURE BROTH OF *STAPHYLOCOCCUS* SP. ISOLATED FROM *CORALLINA OFFICINALIS* LINEAUS

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Abstract – Two new diketopiperazine derivatives, staphyloamides A (**1**), and B (**2**), have been isolated from the culture broth of *Staphylococcus* sp. (No. P-100826-4-6) derived from *Corallina officinalis* Lineaus, along with the known cyclo (L-pro-D-phe) (**3**), cyclo (D-6-Hypro-L-phe) (**4**), cyclo (L-pro-L-val) (**5**), cyclo (L-pro-L-phe) (**6**), cyclo (L-pro-L-tyr) (**7**), cyclo (L-pro-L-leu) (**8**), cyclo (L-pro-L-ala) (**9**), and bacillusamide B (**10**). These structures were elucidated by extensive spectroscopic methods. Antimicrobial activities of compounds **3**–**10** were done.

INTRODUCTION

Natural products are a major resource for drug development. A large number of plants, microbes, and marine animals have been examined for bioactive secondary metabolites.[1] Marine microorganisms are of considerable current interest as a new and promising source of biologically active compound, many of which can be used for drug development.[2,3] During the past two decades, research on marine bacteria has highlighted the tremendous potential of this microorganism as a source of new bioactive secondary metabolites.[4,5] Recently, special interest has pointed to diketopiperazines which are an important class of compounds displaying a variety of biological effects, such as antimicrobial, herbicidal, antiviral, immunosuppressive and cytotoxic activities.[6-8] As a part of ongoing effort to discover biologically active metabolites from marine bacteria, *Staphylococcus* sp. (No. P-100826-4-6) was selected from our

screening program for further studies. It has been observed that culture broth of *Staphylococcus* sp. (No. P-100826-4-6) derived from *Corallina officinalis* Lineaus family Corallinaceae, showed antifungal activity against *Aspergillus niger, Penicillium crustosum* and *Schizophyllum commune*. Therefore, we have researched the active constituents of this bacterium. The marine bacterium *Staphylococcus* sp**.** was obtained from *Corallina officinalis* Lineaus family Corallinaceae, collected in Nagasaki Shitsu coast of Japan in 2010. The strain was cultured at 25 ºC on rotary shakers using a seawater-based medium. The fermentation broth (32L) was successively partitioned with EtOAc to give an EtOAc extract (5.2 g) and the aqueous layer was subjected to Diaion HP-20 using H_2O, 60% MeOH, 100% MeOH and acetone to give 60% MeOH (28.3 g), 100% MeOH (8 g) and acetone (3.9 g) elution fractions respectively. Of them, the EtOAc extract and 100% MeOH elution fraction showed antifungal activity against the fungi stated above. The EtOAc extract and 100% MeOH elution fraction were subjected to Sephadex LH-20, silica gel column chromatography and octa decyl silyl (ODS) column chromatography followed by reversed phase HPLC. As a result, two new diketopiperazines, staphyloamides A (**1**) and B (**2**), have been isolated along with eight known compounds: **3**,[9] **4**,[10] **5**,[11] **6**,[12] **7**,[13] **8**,[14] **9**,[15] and **10**[16] (Figure 1). The structures of these compounds were elucidated by using extensive spectroscopic methods.

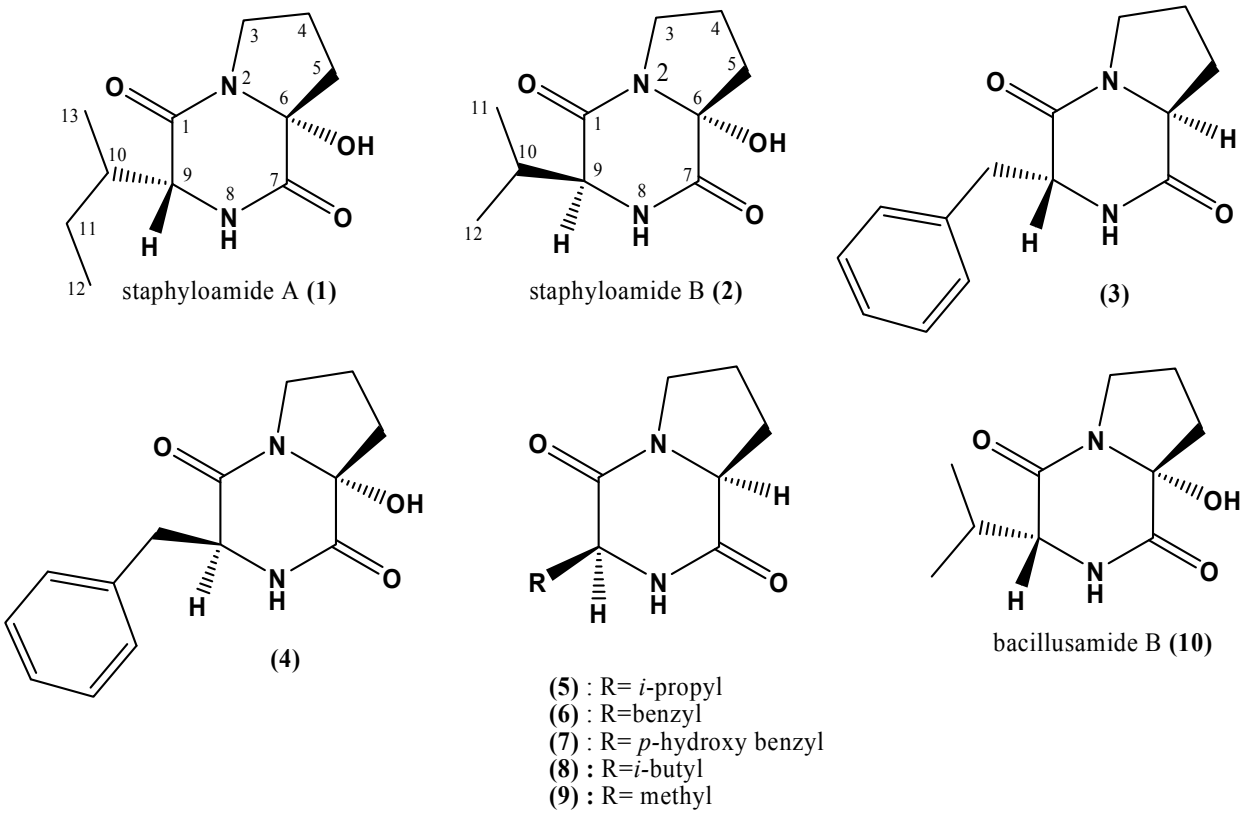

Figure 1. Structures of compounds **1**–**10**

RESULTS AND DISCUSSION

Staphyloamides A (**1**) was obtained as a white amorphous powder. The molecular formula was assigned as $C_{11}H_{19}N_2O_3$ by HR-FAB-MS (*m/z* 227.1396 $[M+H]^+$, calcd 227.1391, Δ + 0.5 mmu), indicating 4 degrees of unsaturation. The IR spectrum of **1** suggested the presence of a hydroxy group (3229 cm^{-1}) and amide carbonyl groups (1699 cm^{-1} and 1652 cm^{-1}). The ^{1}H-NMR spectrum showed signals for an isobutyl group [δ_H 0.79 (3H, t, *J*= 7.5 Hz, H-12), 1.20 (3H, d, *J*= 6.8 Hz, H-13), 1.37, 1.93 (1H, each m, H-11) and 2.51 (1H, m, H-10)] in addition to an amide proton [δ_H 9.54 (1H, brs, H-8)] (Table 1). The ^{13}C-NMR spectrum displayed 11 carbon signals, including two methyl carbons [δ_C 11.0 (C-12), 16.0 (C-13)], three methylene carbons [δ_C 19.9 (C-4), 25.8 (C-11), 38.1(C-5)], one methylene carbon bearing nitrogen [δ_C 45.9 (C-3)], one methine carbon [δ_C 40.1 (C-10)], one methine carbon bearing nitrogen [δ_C 63.0 (C-9)], one quaternary carbon bearing oxygen [δ_C 87.4 (C-6)] and two carbonyl carbons [δ_C 167.4 (C-1), 169.5 (C-7)] (Table 1). These data together with degree of unsaturation revealed that **1** contained two rings in the molecule, thus **1** was found to have a bicyclic skeleton. Analysis of ^{1}H-^{1}H COSY spectrum of **1** showed extended ^{1}H-^{1}H spin system from H-9 to H_3-12 via H-10 and H-11, as well as a vicinal correlation from H-10 to H_3-13, in addition to ^{1}H-^{1}H COSY correlations of H-3 – H-5. The hetero nuclear multiple bond correlation (HMBC) of NH-8 to C-1 and C-10 were observed, as well as H_3-13 to C-9, C-10, C-11, and H-9 to C-10, in addition to the correlation of H-12 to C-10 and C-11and H-9 and NH-8 to C-1 carbonyl, giving rise to an isoleucine moiety (**b**) (Figure 2), Moreover HMBC correlations of H-5 to C-3, C-6, and C-4 indicated the presence of proline moiety (**a**) (Figure 2). The connectivity of these two rings was revealed by HMBC correlation of NH-8 to C-6, C-1 and C-10 (Figure 2). The relative stereochemistry of C-6 and C-9 of **1** was confirmed on the basis of comparing the ^{13}C- NMR data of **1** with those of known compound, notoamide M and other known compounds containing hydroxyproline moiety.[10,16-19] Because the chemical chifts of C-3, C-4, C-5, C-6 and C-7 showed the same data with those of notoamide M, the relative stereochemistry of C-6 and C-9 as shown in Figure 1. The absolute configuration of the amino acid of **1** was determined by acid hydrolysis and Marfay's method,[20] using standard amino acids. The absolute configuration of the isoleucine was determined as D-form (Table 3). Thus, the absolute configuration was defined as 6 *R*, 9 *R*.

Staphyloamide B (**2**) was obtained as white amorphous powder. The molecular formula was assigned as $C_{10}H_{17}N_2O_3$ by HR-FAB-MS (*m/z* 213.1266 $[M+H]^+$, calcd 213.1240, Δ+2.6 mmu), indicating 4 degrees of unsaturation. The IR spectrum of **2** suggested the presence of a hydroxy group (3244 cm^{-1}) and amide carbonyl groups (1700 cm^{-1} and 1652 cm^{-1}). The ^{1}H-NMR spectrum showed signals for an isopropyl group [δ_H 1.12 (3H, d, *J*= 7.1 Hz, H-12), 1.16 (3H, d, *J*= 7.1 Hz, H-11), 2.83 (1H, dsept, *J*= 7.1, 4.6 Hz, H-10)] and amide proton [δ_H 8.73 (1H, brs, H-8)] (Table 2). The ^{13}C-NMR spectrum displayed 10 carbon signals, including two methyl carbons [δ_C 16.8 (C-12), 18.8 (C-11)], two methylene carbons [δ_C 20.5

(C-4), 37.1 (C-5)], one methylene carbon bearing nitrogen [δ_C 45.3 (C-3)], one methine carbon [δ_C 29.2 (C-10)], one methine carbon bearing nitrogen [δ_C 60.7 (C-9)], one quaternary carbon bearing oxygen [δ_C 88.0 (C-6)] and two carbonyl carbons [δ_C 167.5 (C-1), 170.4 (C-7)] (Table 2). These data showed the presence of two amide groups and thus **2** was found to have a bicyclic skeleton in the same manner as **1.** Detailed analysis of 2D NMR spectral data such as COSY and HMBC spectrum showed the presence of two partial structures including 6-hydroxyproline moiety (**a**) and valine moiety (**b**) (Figure 2). Furthermore, the connectivity of these partial structures was revealed by HMBC correlation of NH-8 to C-6, C-1 and H-5 to C-7. The ^{1}H- and ^{13}C-NMR signals of compound **2** and previously isolated compound bacillusamide B (**10**)[16] showed close correspondence, except the chemical shift of H-9 [**2**: δ_H 4.50 (1H, d, *J*= 2.5 Hz), **10**: 3.87 (1H, dd, *J*= 6.8, 4.0 Hz)]. This fact described that **2** was diastereoisomer to bacillusamide B (**10**) at position C-9 as shown in Figure 1.

Table 1. ^{1}H-(500 MHz) and ^{13}C-NMR (125 MHz) data of 1 in pyridine-d_5[a]

No.	**1**	
	δ_H	δ_C
1	-	167.4
2	-	-
3	3.83 (2H, m)	45.9
4	1.76 (1H, m) 2.19 (1H, m)	19.9
5	2.24 (1H, ddd, *J*= 12.8, 12.2, 7.3 Hz) 2.57 (1H, dd, *J*= 12.8, 5.5 Hz)	38.1
6	-	87.4
7	-	169.5
8-NH	9.54 (1H, brs)	-
9	4.01 (1H, dd, *J*= 8.0, 4.1 Hz)	63.0
10	2.51 (1H, m)	40.1
11	1.37 (1H, m) 1.93 (1H, m)	25.8
12	0.79 (3H, t, *J*= 7.5 Hz)	11.0
13	1.20 (3H, d, *J*= 6.8 Hz)	16.0

[a]Spectra were acquired at 23 ºC. Chemical shifts were given in δ (ppm) and referenced to internal solvent for pyridine-d_5 at 7.19 (δ_H) and 123.5 (δ_C) ppm

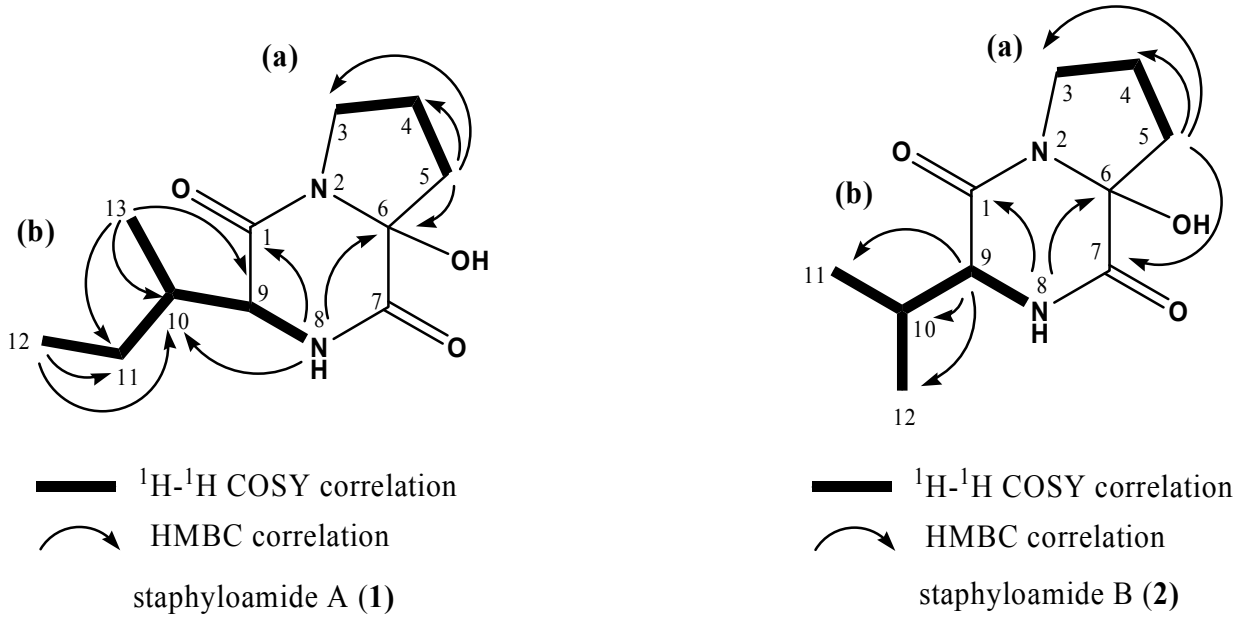

Figure 2. Partial structures (**a**) and (**b**) of staphyloamides A (**1**) and B (**2**)

Table 2. ^{1}H-(500 MHz) and ^{13}C-NMR (125 MHz) data of **2** and bacillusamide B in pyridine-d_5[a]

No.	**2**		**bacillusamide B**	
	δ_H	δ_C	δ_H	δ_C
1	-	167.5	-	167.5
2	-	-	-	-
3	3.69 (1H, ddd, *J*= 14.2, 9.4, 2.5 Hz) 3.91 (1H, ddd, *J*= 14.2, 9.4, 2.5 Hz)	45.3	3.82 (2H, t, *J*= 9.2Hz)	45.8
4	1.78 (1H, m) 2.18 (1H, m)	20.6	1.74 (1H, m) 2.23 (1H, m)	19.8
5	2.45 (1H, ddd, *J*= 11.9, 8.2, 3.9 Hz) 2.53 (1H, dd, *J*= 13.0, 5.7 Hz)	37.1	2.27 (1H, m) 2.56 (1H, m)	37.9
6	-	88.0	-	87.3
7	-	170.4	-	169.5
8	8.73 (1H, brs)	-	9.58 (1H, m)	-
9	4.50 (1H, d, *J*= 2.5 Hz)	60.7	3.87 (1H, dd, *J*= 6.8, 4.0 Hz)	64.3
10	2.83 (1H, dsept, *J*= 7.1, 4.6 Hz)	29.2	2.69 (1H, m)	33.8
11	1.16 (3H, d, *J*= 7.1 Hz)	18.8	1.18 (3H, d, *J*= 6.8 Hz)	19.9
12	1.12 (3H, d, *J*= 7.1 Hz)	16.8	1.12 (3H, d, *J*= 6.8 Hz)	19.6

[a]Spectra were acquired at 23 ºC. Chemical shifts were given in δ (ppm) and referenced to internal solvent for pyridine-d_5 at 7.19 (δ_H) and 123.5 (δ_C) ppm

Table 3. Retention times for amino acids obtained from **1** and **2** as their N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (FDLA) derivertives

residue	Standards retention time (min)	acid hydrolysate of **1** retention time (min)	acid hydrolysate of **2** retention time (min)
L-Ile	3.1		
D-Ile	4.3	3.8	
L-Val	3.1		3.2
D-Val	3.7		

Retention times were determined by HPLC analyses [Mightysil RP-18 (250 x 4.6 mm i.d., Kanto Chemical Co. Inc.), mobile phase; 40%MeCN-H_2O, flow rate; 1 mL/min, detection; UV 340 nm]

The absolute configuration of the valine resdue was determined as L-form (Table 3). Thus, the absolute configuration was defined as 6 *R*, 9 *S*.

The antimicrobial activities of compounds **3**–**10** were tested for the growth inhibition of 11 microbes including gram positive, negative bacteria and fungi using a paper disk method.[21] The growth inhibition was studied in a concentration of 100 and 50 μg /disk. As result, compound **4** and **8** slightly showed an inhibition circle against *Schizophyllum commune, Staphylococcus aureus* subsp. *aureus,* and *Escherichia coli,* while other compounds did not show antimicrobial activity at this concentration. The bioactivity of compounds **1** and **2** couldn't be done due to small amounts.

EXPERIMENTAL

General

IR spectra were obtained with JASCO FT/IR-410 spectrophotometers. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. ^{1}H- and ^{13}C-NMR, ^{1}H-^{1}H COSY, NOESY, HSQC and HMBC spectra were recorded with a Unity plus 500 spectrometer (Varian Inc., U.S.A.) operating at 500 MHz for ^{1}H, and 125 MHz for ^{13}C, respectively.^{1}H-NMR chemicals hifts are expressed in δ values referring to the solvent peak δ_H 7.19 for pyridine-d_5, and coupling constants are expressed in Hz. ^{13}C-NMR chemical shifts are expressed in δ values referring to the solvent peak δ_C 123.5 for pyridine-d_5. Sephadex LH-20 (Pharmacia Fine Chemical Co.Ltd),Wakogel C-300 (Wako Pure Chemical Industries, Ltd, Japan, 45~75 μm) and Cosmosil 5C18-140 PREP (Nakarai tesque, No.379-34) were used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) and precoated RP-18 F_{254s} plates (Merck) were used for thin-layer chromatography (TLC) analysis. FAB-MS were recorded on JMS DX-303 spectrometer (JEOL Ltd., Japan), and *m*-nitrobenzyl alcohol or Magic bullet as a matrix. Preparative HPLC was peroformed on a Develosil C-30-UG-5 (250 x 4.6 mm i.d Nomura

Chemical Co., Aichi, Japan) and a Wakosil-II 5sil-100 (150 x 4.6 mm "B" Wako pure chemical industries, Ltd, Japan) at a flow rate of 1.5 mL/min, equipped with a TOSOH RI-8020 detector and a JASCO BIP-I HPLC pump.

Bacterial Material and Fermentation

The marine *Staphylococcus* sp. (strain number P-100826-4-6) was isolated from *Corallina officinalis* Linnaeus family Corallinaceae, collected in Nagasaki Shitsu coast of Japan in 2010. The subcultures of the bacterium are deposited at the Garden for Medicinal Plants, Graduated School of Biomedical Sciences, Nagasaki University. The bacterium was grown in a seawater-based medium (D-glucose 1%; polypeptone 0.5%; yeast extract 0.3%; KH_2PO_4 0.3%; $MgSO_4$ 0.1%; pH 7.5), using rotary-shaking at 120 rpm for 28 days at 25 °C. The culture broth (32L) was sonicated then filtrated.

Extraction and Isolation

The filtered broth was extracted by EtOAc (10 L x3). The EtOAc extract was concentrated under a reduced pressure to dryness to produce EtOAc extract (5.2 g). The aqueous layer were chromatographed on Diaion HP-20 column eluting with H_2O, 60% MeOH, 100% MeOH and acetone respectively to give elution fractions of 60% MeOH (28.3 g), 100% MeOH (8.0 g) and acetone (3.9 g). The dried residue of EtOAc extract (5.2 g) was subjected to silica gel column chromatography ($CHCl_3$ - MeOH = 10:0 ~ 0:10) to yield 25 fractions. The 13th fraction (1.1 g) was chromatographed on Sephadex LH-20 ($CHCl_3$ - MeOH = 1:1) to yield four fractions (fractions A – D). Fraction B (143 mg) was subjected to silica gel column chromatography (*n*-hexane- acetone = 10:0 ~ 0:10) to yield six fractions. Fraction B-2 was subjected to reversed phase HPLC Develosil C-30 (30% MeOH - H_2O) to give compounds **5** (12 mg) and **9** (20 mg). Fraction D (380 mg) was subjected to silica gel column chromatography (*n*-hexane - acetone = 10:0 ~ 0:10 followed by (acetone - MeOH = 5:5 ~ 0:10) to yield four fractions (fractions D-1 – D-4). Fraction D-2 was purified on Sephadex LH-20 ($CHCl_3$ - MeOH = 1:1) to give compound **10** (10 mg). Fraction D-1 (30 mg) was subjected to reversed phase HPLC Wakosil 5C-18 (40% MeOH - H_2O) to give compounds **2** (2.3 mg) and **1** (1.7 mg). The 100% MeOH elution fraction (8.0 g) was dissolved in a mixture of $CHCl_3$ - MeOH - H_2O = 5:5:1 to give soluble part (4.7 g) and an insoluble part (2.3 g). The soluble part was subjected to Sephadex LH-20 ($CHCl_3$ - MeOH = 1:1) to yield seven fractions (fractions from A – G). Fraction D (587 mg) was chromatographed on ODS column chromatography (MeOH - H_2O = 20:80 ~100:0) to give seven fractions. Fraction D-3 (105 mg) was chromatogaphed on silica gel column chromatography ($CHCl_3$ - MeOH = 10:0 ~ 0:10) to give four fractions (fractions D-3a – D-3d). Fraction D-3a (73 mg) was subjected to Sephadex LH-20 (100% MeOH) followed by silica gel column chromatography ($CHCl_3$ - MeOH = 99:1) to give compounds **8** (4 mg) and **6** (37 mg). Fraction D-3c (13 mg) was subjected to reversed phase HPLC

Wakosil 5C-18 (40% MeOH - H_2O) to give compounds **3** (4 mg) and **4** (1.7 mg). Fraction E (465 mg) was chromatographed on silica gel column chromatography ($CHCl_3$ - MeOH = 10: 0 ~ 0:10) to give eight fractions. Fraction E-5 (32 mg) was subjected to Sephadex LH-20 ($CHCl_3$ -MeOH = 1:1) followed by ODS column chromatography (20% MeOH - H_2O) to yield compound **7** (13 mg).

Staphyloamide A (1): White amorphous powder $[\alpha]^{27}_D$ **-** 21.5 (*c* 0.085, MeOH); IR ν_{max} (dry film) 3229, 2974, 1699, 1652, 1429 cm^{-1}, ^{1}H- and ^{13}C-NMR data (see Table 1); (+) FAB-MS *m/z*: 227 $[M+H]^+$, (+) HR- FAB-MS *m/z*: 227.1396 $[M+H]^+$ (calcd for $C_{11}H_{19}N_2O_3$, 227.1391, Δ + 0.5 mmu).

Staphyloamide B (2): White amorphous powder $[\alpha]^{27}_D$ **-**146.5 (*c* 0.1, MeOH); IR ν_{max} (dry film) 3244, 2924, 1700, 1652, 1436 cm^{-1}, ^{1}H- and ^{13}C-NMR data (see Table 2); (+) FAB-MS *m/z*: 213 $[M+H]^+$, (+) HR- FAB-MS *m/z*: 213.1266 $[M+H]^+$ (Calcd for $C_{10}H_{17}N_2O_3$, 213.1240, Δ + 2.6 mmu).

Determination of Amino Acids Configuration

A sample of **1** or **2** (0.5 mg) was hydrolyzed in 6 N HCl (1 mL) at 110 °C for 16 h. After concentration to dryness, the residue was dissolved in 50 μL of pure H_2O and 40 μL of 1M $NaHCO_3$ aq. and 100 μL of 1% of N^{α}-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (FDLA) in acetone were added. The mixture was heated at 37 °C for 1 h and 20 μL of 1N HCl was added. The solution was evaporated to dryness to furnish a yellow solid. The residue was dissolved in H_2O/MeCN (500 μL) and analyzed by reversed-phase HPLC (Mightysil RP-18 (250 x 4.6 mm i.d., Kanto Chemical Co., Inc.), mobile phase; 40% MeCN–H_2O, flow rate; 1 mL/min, detection; UV 340 nm). and comparing with standard L- and D- amino acid in Table 3.

Antimicrobial activity assay

Activities of compounds were tested by paper disk methods against *Bacillus subtilis* subsp. *subtilis*, *Staphylococcus aureus* subsp. *aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus niger*, *Penicillium crustosum*, *Schizophyllum commune*, *Trichophyton concentrieum*, *Saccharomyces cerevisae*, *Serratia marcescens* subsp. *marcescens* and *Candida albicans* with 100 and 50 μg/ disk as result, compound **4** and **8** slightly showed an inhibition circle against *Schizophyllum commune, Staphylococcus aureus* subsp. *aureus,* and *Escherichia coli,* while other compounds showed no inhibition circle.

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