Cyclic Peptides from a *Ruegeria* Strain of Bacteria Associated with the Sponge *Suberites domuncula*

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Two new cyclic peptides, cyclo-(glycyl-L-seryl-L-prolyl-L-glutamyl) and cyclo-(glycyl-L-prolyl-L-glutamyl), have been isolated from the cell extract of a *Ruegeria* strain associated with cell cultures of *Suberites domuncula*. Three other cyclopeptides have been isolated for the first time from a natural source. Additionally, a new diastereoisomer of a known compound is reported. The structures of isolated compounds have been elucidated by means of spectroscopic data (1D-, 2D-NMR, HRESIMS) and chiral HPLC analysis. The new cyclopeptides exhibited moderate antimicrobial activity against *Bacillus subtilis.*

Cultured marine bacteria have emerged to be a rich source of structurally novel and biologically active secondary metabolites, which might be useful leads in the development of new pharmaceutical agents.[1,2] Relative to terrestrial strains, they yield a significantly improved ratio of new to known compounds, especially strains that are highly adapted to specific marine conditions such as symbionts or those inhabiting extreme environments.[1]

One of the major goals of sponge symbiont research is to isolate and culture the specific sponge-associated bacteria. Unfortunately, we know very little about the chemical and physical environment provided by the sponge host, and to date the culture of obligate symbionts has been an elusive goal.[3] Some studies concerning sponge symbionts have demonstrated the presence of certain bioactive metabolites in symbiontic microorganisms associated with sponges.[3] Other research has focused on the characterization of entire sponge microbial communities by 16S rDNA sequence analysis.[4−6] Since sponges are filter feeders, the study of specifically sponge-associated microorganisms by cultivation-based approaches using fresh collected sponge specimens could not exclude the isolation of non-specific bacteria. A possible way to reduce the numbers of these transient bacteria has been demonstrated recently. Thakur and co-workers[7] have shown the occurrence of characteristic 16S rDNA sequences corresponding to specific bacterial strains in sponge cell culture aggregates (primmorphs) of *Suberites domuncula* Olivi. They were also able to isolate and culture two new bacterial strains related to *Pseudomonas* sp. associated with sponge primmorphs. We have previously reported the isolation, identification by 16S rDNA sequence analysis, and characterization of fatty acid and exocellular peptide composition of a *Ruegeria* strain SDC-1 associated with cell cultures of *S. domuncula*.[8] It remains to be clarified whether these bacteria are specifically sponge-associated bacteria or are just survivors of the establishment of primary sponge cell culture. In both studies, new bacterial strains with antibacterial activities were isolated.

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Continuing our search for new bioactive compounds from marine bacteria,[8−10] we report the structural elucidation of novel peptides from the α-proteobacterium *Ruegeria* SDC-1.

The crude cell extract of strain SDC-1 was first fractionated by solvent/solvent partitioning. The aqueous fraction was purified repeatedly by Lobar RP-18, followed by reversed-phase HPLC (water/methanol) to yield compounds **1**−**3**. The *n*-BuOH fraction was subjected to RP-18 column chromatography (CC) followed by reversed-phase HPLC (water/acetonitrile) to yield compounds **4**−**7**. Compounds **1** and **2** appeared to be new peptides, while compounds **3**[11] and **4**[12] were found to be known synthetic compounds. Herein we report for the first time their isolation from a natural source. Peptide **6** is a new diastereoisomer of **5** that was previously isolated from a blocked mutant of *Streptomyces fradiae*.[13] Cyclodipeptide **7** was previously isolated from rabbit skin tissue extract[14] and showed activity as a plant growth regulator. Compound **8** is a widely distributed cyclodipeptide.

Compound **1** was isolated as an amorphous solid with $[\alpha]_D$ −26.9° (*c* 0.0018, MeOH) and showed a pseudomolecular ion peak at *m*/*z* 393.1350 (M + Na^+, calculated 393.1325) in HRESIMS (positive ion mode), consistent with a molecular formula $C_{15}H_{22}O_7N_4$. Its peptide nature was suggested by the molecular formula itself and from analysis of the ^{1}H and ^{13}C NMR spectra (Table 1). Compound **1** did not react in a ninhydrine test, indicating a cyclic or an *N*-terminus-blocked peptide. Because of the overlap of ^{1}H NMR signals in DMSO-d_6, the NMR data for **1** were obtained using D_2O + CD_3OD (1:3). The ^{1}H NMR spectrum of **1** showed three α-methine protons and a pair of α-methylene protons of amino acid residues. Furthermore, the ^{13}C NMR spectrum showed four signals for α-methine carbons and five signals for carbonyls, suggesting that **1** was a tetrapeptide, containing a dicarboxylic amino acid. The COSY-45 spectrum showed that the most downfield α-methine proton (δ 4.58) was correlated with two geminal protons, forming an ABX system (δ 4.03 and 3.94). An HMQC experiment showed correlation between these geminal protons and an O-bearing carbon (δ 61.8). Thus, a seryl residue was identified. The α-methine proton at δ 4.55 was correlated with nonequivalent methylene protons (δ 2.35 and 2.06). Since the integration of signal at δ 2.06−2.08 showed three protons, the diastereotopic β-proton at

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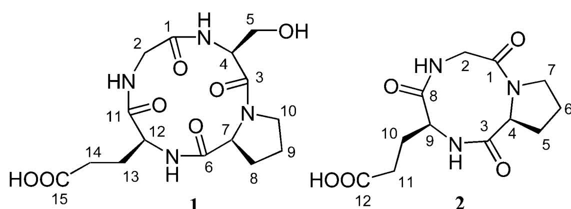
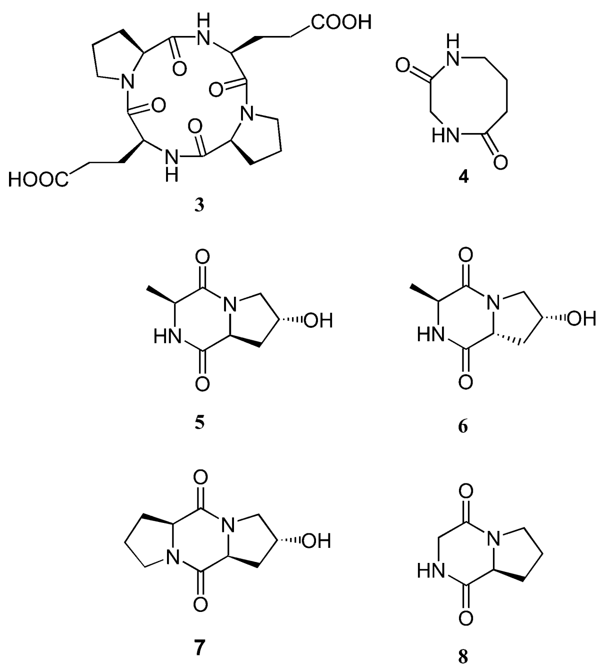
Table 1. NMR Spectra Data of Peptides **1** and **2** in D_2O + CD_3OD[a]

1	^{13}C δ	^{1}H δ	HMBC (J_{C-H} = 10 Hz)	**2**	^{13}C δ	^{1}H δ	HMBC (J_{C-H} = 10 Hz)
Gly 1	169.9 s		H2	Gly 1	169.8 s		H2
2	42.3 t	4.23 d (17.2)		2	42.2 t	4.20 d (17.3)	
		4.13 d (17.2)				4.13 d (17.3)	
Ser 3	174.2 s		H7, H5	Pro 3	174.8 s		H5
4	55.6 d	4.58 dd (4.8; 3.8)		4	60.0 d	4.49 dd (8.6; 3.5)	H5, H6
5	61.8 t	4.03 dd (11.8; 4.8)		5	29.5 t	2.34 m	H4, H6, H7
		3.94 dd (11.8; 3.8)				2.08 m	
Pro 6	175.2 s		H8	6	24.9 t	2.08 m	H5, H7
7	61.2 d	4.55 m		7	47.6 t	3.67 m	H5, H6
8	30.2 t	2.35 m	H7, H9, H10	Glu 8	176.2 s		H2, H10
		2.06 m					
9	25.1 t	2.08 m	H7, H8, H10	9	57.5 d	4.42 dd (9.1; 4.9)	H11
10	47.8 t	3.68 m	H8	10	25.8 t	2.58 m	H11
						2.14 m	
Glu 11	176.3 s		H2, H13, H14	11	29.5 t	2.47 m	H10
12	57.8 d	4.43 dd (9.1; 4.9)	H13, H14	12	183.1 s		H10, H11
13	26.0 t	2.60 m	H12, H14				
		2.17 m					
14	30.0 t	2.49 m	H13				
15	183.2 s		H13, H14				

[a] Chemical shifts are referred to as residual MeOH resonance. Multiplicities are indicated by usual symbols. Coupling constants (hertz) are in parentheses.





δ 2.06 overlapped with a methylene group. These methylene protons were correlated in the COSY spectrum with a methylene group close to a heteroatom (δ 3.68), thus clarifying the spin system of a proline residue that was further confirmed by the analysis of TOCSY, HMQC, DEPT, and HMBC spectra. The α-methine proton at δ 4.43 was coupled to diastereotopic methylene protons (δ 2.60 and 2.17) that were further correlated with methylene protons at δ 2.49. HMBC correlations observed between this set of protons and a carboxyl group at δ 183.2 revealed the side chain of the glutamic acid residue. The COSY spectrum showed that the remaining protons at δ 4.23 and 4.13 were correlated between themselves, and they were correlated with a carbonyl at δ 169.9 (HMBC), defining a glycyl moiety.

The amino acid sequence of **1** was deduced by detailed interpretation of HMBC and ESIMS data. The long-range correlation of αH(Gly)/CO(Glu) and αH(Pro)/CO(Ser) revealed the presence of segments Glu-Gly and Ser-Pro, respectively. In addition to the pseudomolecular ion in the ESIMS spectrum, fragments at m/z 226 [Glu − Pro]$^+$ and 211 [Gly − Glu + Na]$^+$ were observed.

The stereochemistry of **1** was determined by chiral HPLC analysis of the acid hydrolysate, which established the L configuration for all amino acid residues. Thus, the structure of peptide **1** was elucidated as cyclo-(glycyl-L-seryl-L-prolyl-L-glutamyl).

Peptide **1** existed as primarily a single conformation in D_2O. The cis and trans conformations of that bound to nitrogen in proline containing peptides can be distinguished in solution by the pattern of dipolar couplings in a ROESY spectrum[15] and by the chemical shift difference between the β- and γ-carbon atoms of proline.[16] The chemical shift difference $\Delta\delta_{C\beta-C\gamma} = 5.3$ was in the border range of cis and trans conformers. The ROESY cross-peak (mixing time 200 ms) between αH (Ser) and δH_2 (Pro) supported a trans geometry for the Ser-Pro linkage.

Compound **2** was isolated as a colorless amorphous solid with an optical rotation of $[\alpha]_D$ −27.8° (c 0.001, MeOH). The analysis of NMR and ESIMS data showed that the structure of **2** was closely related to **1**. HRESIMS analysis displayed a pseudomolecular ion at m/z 306.1023 (M + Na$^+$, calculated 306.1005 for $C_{12}H_{17}O_5N_3Na$) corresponding to one serine unit less than the pseudomolecular ion of **1**. In support of the mass spectral analysis, the serine signals were not observed in the NMR spectrum of **2**. An HMBC correlation between the αH of the glycine residue and carbonyl group of the glutamic acid moiety confirmed the presence of the fragment Gly-Glu. The interresidue ROESY correlation (mixing time 200 ms) observed between δH_2(Pro) and αH(Gly) suggested an amide bond involving the carbonyl of glycine and the nitrogen of proline and a trans conformation for the Gly-Pro linkage, which was further confirmed by $\Delta\delta_{C\beta-C\gamma} = 4.6$. The chiral HPLC analysis of the acid hydrolysate of compound **2** suggested the presence of L-Glu and L-Pro. Hence, the structure of compound **2** was elucidated as cyclo-(glycyl-L-prolyl-L-glutamyl).

Examination of the NMR data of compound **3** revealed a structure similar to that of peptide **1** but without glycyl and seryl moieties. HRESIMS analysis provided a pseudomolecular ion at *m/z* 475.1772 ($M + Na^+$, calculated 475.1744 for $C_{20}H_{28}O_8N_4Na$) that is twice the expected mass for a cyclic dipeptide (Glu-Pro), suggesting a dimer. The NMR data therefore showed only half of the molecule, indicating a symmetric structure for peptide **3**. ESIMS displayed additional ions at *m/z* 322 $[M - Glu + Na]^+$ and 249 $[Glu - Pro + Na]^+$, with the latter signal due to the preferential cleavage of the peptide bond at proline residues.[17] These data supported the structure of a cyclic tetrapeptide. The chiral HPLC analysis of the acid hydrolysate of compound **3** gave L-Glu and L-Pro. Hence, the structure of compound **3** was elucidated as cyclo-(L-prolyl-L-glutamyl)$_2$.[11] Peptide **3** was shown to exist as primarily a single conformation in D_2O with a trans conformation for the Glu-Pro linkage elucidated by the ROESY correlation (mixing time 200 ms) between αH (Glu)/δH_2 (Pro) and by $\Delta\delta_{C\beta-C\gamma} = 3.6$.

Compound **4** was isolated as an optically inactive colorless solid. The analysis of its ESIMS and NMR data suggested a structure consistent with cyclo-(glycyl-γ-aminobutyryl), previously described as a synthetic compound.[12]

The nearly identical spectral data of compounds **5** and **6** showed their close resemblance. HREIMS analysis revealed almost identical molecular ions at *m/z* 184.0836 and 184.0853, respectively (calculated 184.0848), consistent with the molecular formula $C_8H_{12}O_3N_2$. Detailed analyses of the ^1H and ^{13}C NMR spectral data (COSY-45, HMQC, and HMBC) of **5** and **6** established the presence of alanine and 4-hydroxy-proline residues. The chiral HPLC analysis of acid hydrolysates of **5** and **6** gave L-Ala for both compounds, *trans*-4-hydroxy-L-proline for **5**, and *cis*-4-hydroxy-D-proline for **6**. Hence, the structures were elucidated as cyclo-(L-alanyl-*trans*-4-hydroxy-L-prolyl) (**5**) and cyclo-(L-alanyl-*cis*-4-hydroxy-D-prolyl) (**6**).

The spectral data of **7** and **8** and chiral HPLC analysis of their acid hydrolysates are consistent with the known structures cyclo-(L-prolyl-*trans*-4-hydroxy-L-prolyl) and cyclo-(glycyl-L-prolyl) for **7** and **8**, respectively.

The isolated compounds **1**−**8** were tested in antimicrobial and antifungal assay (*Bacillus subtilis* subsp. *spizizenii*, *Escherichia coli*, and *Saccharomyces cerevisiae*). Cyclopeptides **3**−**8** were inactive. Cyclopeptides **1** and **2** showed moderate effects against *B. subtilis* (minimum inhibitory concentration (MIC) of 25 and 50 μg/mL, respectively) but were not active against *E. coli* and *S. cerevisiae*.

It is intriguing to speculate about the possible function of the isolated compounds. Since cyclopeptides **1** and **2** exhibited activity against one species of Gram-positive bacteria, they could play some role in a chemical defense of the Gram-negative strain *Ruegeria* SDC-1. Cyclotetrapeptide **3** has been shown to form complexes selectively with Ca^{2+} and Ba^{2+} ions.[11] Cation affinity of other cyclotetrapeptides has been reported,[18] and on the basis of structural homology, this could be also be hypothesized for cyclotetrapeptide **1**. Further studies are needed to better understand the possible functions of cyclopeptides **1**−**3**. Cyclodipeptides **5** and **7** are known to show activity as plant growth regulators.[13,14] Cyclodipeptide **8** was found to stimulate antibiotic production of *Pseudoalteromonas luteoviolacea*.[19] Recently, it has been shown that cyclodipeptides are able to activate or antagonize LuxR-mediated quorum sensing system of bacteria, and they are thus considered members of this family of cell−cell signaling compounds.[20] Compounds **5**−**8** have structural similarities to ligands of LuxR-based biosensors[13,14,19] and could be potentially used as signal molecules. It is interesting to note that the cell-associated peptides described here are structurally different from previously reported extracellular diketopiperazines isolated from the culture broth of *Ruegeria* SDC-1.[8]

Experimental Section

General Experimental Procedures. Optical rotation was measured on a JASCO DIP 370 polarimeter, using a 10-cm microcell. ^1H and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively, on a Bruker AMX-600 spectrometer in D_2O/CD_3OD using the residual MeOH resonances at δ_H 3.48 ppm and δ_C 49.0 ppm as internal references, respectively. EIMS were obtained on a Fisons TRIO 2000 spectrometer. HRESIMS was obtained on a Q-TOF micro Micromass instrument equipped with an ESI (positive ion) source.

Bacterial Cultivation. The strain SDC-1 was isolated from the cell culture of *S. domuncula* and identified by 16S rRNA gene sequence analysis as an α-proteobacterium of the genus *Ruegeria* as previously reported.[8] The voucher specimen of the strain SDC-1 was maintained in the ICB-CNR collection. The strain was grown in Marine Broth 2216 (Difco) (8 × 3 L batch cultures) at 19 °C and pH 7.6 for 5 days with rotator shaking. The cells were harvested by centrifugation at 15 000 × g for 15 min, recovering the pellet.

Isolation of Bacterial Metabolites. The pellet was sonicated and extracted with EtOH (3 × 250 mL). The solvent was removed under reduced pressure, and the crude extract (2.5 g dry weight) was dissolved in water (100 mL) and subsequently extracted with $CHCl_3$ (3 × 100 mL; 400 mg dry weight) and *n*-BuOH (3 × 100 mL, 168 mg dry weight). The aqueous residue provided 1.3 g of extract (dry weight). The aqueous fraction was chromatographed on a Lobar RP-18 column (LiChroprep RP-18, 40−63 μm, 310 × 25 mm, Merck) with a water/methanol gradient (flow rate: 3 mL/min, 9 mL × fraction). Fractions with typical ^1H NMR signals for peptides were further purified by reversed-phase HPLC (Kromasil RP-18, 5 μm, 10 × 250 mm) with a water/acetonitrile gradient containing 0.05% trifluoracetic acid (flow: 2.5 mL/min; detection: UV at 215 nm). The following peptides were recorded in order of elution from the RP-18 column: cyclo-(glycyl-γ-aminobutyryl) (**4**, 1.0 mg), cyclo-(glycyl-L-seryl-L-prolyl-L-glutamyl) (**1**, 1.8 mg), cyclo-(glycyl-L-prolyl-L-glutamyl) (**2**, 1.0 mg), and cyclo-(L-prolyl-L-glutamyl)$_2$ (**3**, 2.4 mg). The *n*-butanol extract was subjected to RP-18 flash chromatography (LiChroprep RP-18, 40−63 μm, 500 × 30 mm, Merck) using a water/methanol gradient. The peptide fractions were further purified by RP-18 HPLC. In order of elution from RP-18 column, the following peptides were yielded: cyclo-(L-alanyl-*trans*-4-hydroxy-L-prolyl) (**5**, 3.0 mg), cyclo-(L-alanyl-*cis*-4-hydroxy-D-prolyl) (**6**, 1.4 mg), cyclo-(glycyl-L-prolyl) (**8**, 3.4 mg), and cyclo-(L-prolyl-*trans*-4-hydroxy-L-prolyl) (**7**, 3.5 mg).

Compound 1: amorphous solid: $[\alpha]^{25}_D$ −26.9° (*c* 0.0018, MeOH); ^1H and ^{13}C NMR (D_2O/CD_3OD) data are reported in Table 1; ESIMS *m/z* (%) 393 $[M + Na]^+$ (100%), 226 $[Glu - Pro]^+$ (50%), 209 $[Gly - Glu + Na]^+$ (23%), 143 $[Gly - Ser - H]^+$ (20%); HRESIMS *m/z* 393.1350 (calcd for $C_{15}H_{22}O_7N_4Na$ 393.1325).

Compound 2: amorphous solid: $[\alpha]^{25}_D$ −27.8° (*c* 0.0010, MeOH); ^1H and ^{13}C NMR (D_2O/CD_3OD) data are reported in Table 1; ESIMS *m/z* (%) 306 $[M + Na]^+$ (100%), 130 $[Glu + H]^+$ (5%); HRESIMS *m/z* 306.1023 (calcd for $C_{12}H_{17}O_5N_3Na$ 306.1005).

Compound 3: amorphous solid: $[\alpha]^{25}_D$ −14.9° (*c* 0.0024, MeOH); ESIMS *m/z* (%) 475 $[M + Na]^+$ (28%), 322 $[M - Glu + Na]^+$ (5%), 306 $[M - Glu - NH_2 + Na]^+$ (25%), 249 $[Glu - Pro + Na]^+$ (100%), 227 $[Glu - Pro + H]^+$ (5%), 152 $[Glu + Na]^+$ (5%); HRESIMS *m/z* 475.1772 (calcd for $C_{20}H_{28}O_8N_4Na$ 475.1744).

Compound 5: amorphous solid: $[\alpha]^{25}_D$ −4.2° (*c* 0.0023, MeOH); ^1H NMR (D_2O/CD_3OD) δ 4.58 (2H, m, H-6 and H-8), 4.34 (1H, q, *J* = 7 Hz, H-3), 3.72 (1H, dd, *J* = 13.4 and 4.6 Hz,

H-9a), 3.47 (1H, dd, J = 13.4 and 0.8 Hz, H-9b), 2.31 (1H, ddd, J = 13.5, 6.4, and 0.9 Hz, H-7a), 2.16 (1H, ddd, J = 13.5, 11.8, and 4.1 Hz, H-7b), 1.38 (3H, d, J = 7.0, H-10); ^{13}C NMR (D_2O/CD_3OD) δ 173.0 (s, C-5), 169.4 (s, C-2), 68.6 (d, C-8), 58.2 (d, C-6), 54.5 (t, C-9), 51.5 (d, C-3), 36.9 (t, C-7), 15.2 (q, C-10); EIMS *m*/*z* (%) 184 $[M]^+$ (68), 166 (7), 113 (42), 71 (58); HREIMS *m*/*z* 184.0836 (calcd for $C_8H_{12}O_3N_2$, 184.0848).

Compound 6: amorphous solid: $[\alpha]^{25}_D$ +17.8° (*c* 0.0014, MeOH); ^{1}H NMR (D_2O/CD_3OD) δ 4.54 (1H, m, H-8), 4.50 (1H, dd, J = 8.9 and 6.7 Hz, H-6), 4.08 (1H, q, J = 7.0 Hz, H-3), 3.67 (1H, dd, J = 12.6 and 3.7 Hz, H-9a), 3.52 (1H, dd, J = 12.6 and 5.6 Hz, H-9b), 2.58 (1H, ddd, J = 14.0, 8.9 and 5.7 Hz, H-7a), 2.18 (1H, ddd, J = 14.0, 6.7 and 6.0 Hz, H-7b), 1.43 (3H, d, J = 7.0, H-10); ^{13}C NMR (D_2O/CD_3OD) δ 170.8 (s, C-5), 169.7 (s, C-2), 67.7 (d, C-8), 56.2 (d, C-6), 53.2 (t, C-9), 52.8 (d, C-3), 35.9 (t, C-7), 18.4 (q, C-10); EIMS *m*/*z* (%) 184 $[M]^+$ (78), 113 (57), 71 (83); HREIMS *m*/*z* 184.0853 (calcd for $C_8H_{12}O_3N_2$, 184.0848).

Stereochemistry of the Amino Acids. The stereochemistry of amino acids was determined by analysis of 100 μg of acid hydrolysate from compounds **1**−**3** and **5**−**8** (500 μL of 6 N HCl at 110 °C for 2 h) by chiral HPLC (Chirex D-Penicillamine, 250 × 4.6 mm; flow: 1 mL/min; detection: UV at 254 nm). The amino acid stereochemistry was determined by co-injection with standard amino acids using the following eluents: 2 mmol $CuSO_4$ in H_2O−MeOH (17:3) for Pro and Glu, 2 mmol $CuSO_4$ in H_2O−MeOH (7:3) for Ala and Hyp, and 2 mmol $CuSO_4$ in H_2O−^{i}PrOH (19:1) for Ser.

Antimicrobial and Antifungal Assay. The following strains were used in the antimicrobial assays: Gram-positive bacterium *B. subtilis* subsp. *spizizenii* (DSM 347), Gram-negative bacterium *E. coli* (DSM 498), and yeast *S. cerevisiae* (DSM 70449). The MIC was determined by twofold serial dilution, in duplicate, starting from 50 μg/mL. Gentamycin (MIC = 0.8 μg/mL) and nystatin (MIC = 100 μg/mL) were used as controls. The bacterial and yeast growth was observed after 48 h of incubation at optimal growth temperature for each strain. Cyclopeptides **3**−**8** exhibited no antimicrobial or antifungal activity. Cyclopeptide **1** and **2** showed moderate activity against *B. subtilis*, with an MIC of 25 μg/mL and 50 μg/mL, respectively. Both compounds were inactive against *E. coli* and *S. cerevisiae*.

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