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Circulocins, new antibacterial lipopeptides from *Bacillus circulans*, J2154

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Abstract—A series of new lipopeptides, circulocins α–δ (**1**–**4**), was isolated from the fermentation broth of *Bacillus circulans*, J2154. These compounds exhibited potent antibiotic activity against the Gram-positive bacteria, including the piperacillin-resistant *Streptococci* and vancomycin-resistant *Enterococci*. Their structures were established using spectroscopic methods, and the absolute configurations were determined by amino acid analyses.

1. Introduction

Strains of *Bacillus* are known producers of bioactive cyclic lipopeptides,[1] which can be divided into two groups. The first group has a common feature that the N-terminal of the peptide chain is connected via an amide bond to a β-hydroxy or β-amino fatty acid and the carboxyl group on the C-terminal forms a lactone with the β-hydroxy group, such as surfactin[2] and lichenysins,[3] or forms a lactam with the β-amino group, such as iturins[4] and bacillomycins.[5] In this case, the fatty acid is incorporated into the cyclic system. The second group consists of plipastatins,[6] colistins,[7] and octapeptins,[8] in which the N-terminal of the cyclic peptide is connected by an amide bond to a fatty acid, lying outside the cyclic system.

In the course of screening for new antibiotics from microorganisms to overcome the increasing resistance in

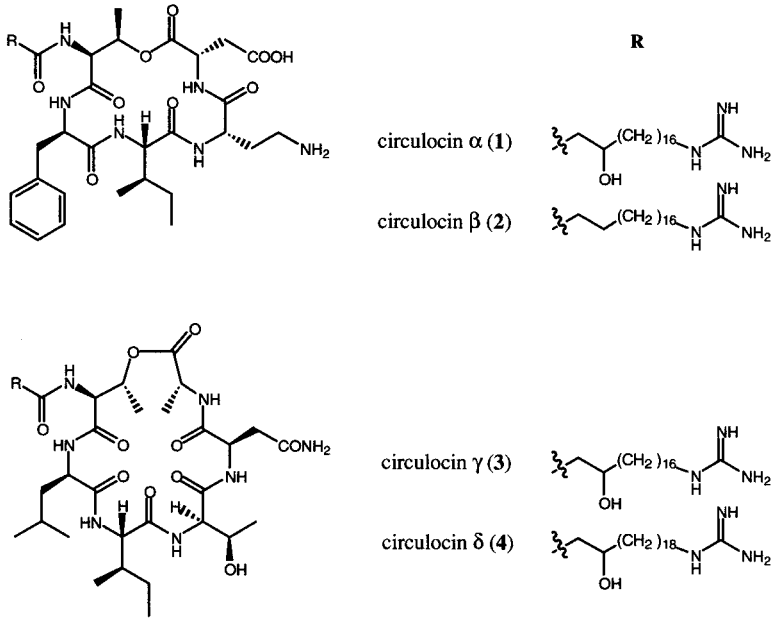

Figure 1. Structures of circulocins.

Keywords: circulocins; *Bacillus circulans*; lipopeptides.
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Table 1. ^{1}H and ^{13}C NMR data of circulocin α (**1**), in DMSO-d_6

	^{13}C (75 MHz)	^{1}H (300 MHz, mult, *J* in Hz)	HMBC (*J*=8 Hz)
Threonine (*Thr*)			
1	168.7		H-2, H-3, 2-NH (Phe)
2	55.3	4.44 (dd, 9, 3.5)	H_3-4
2-NH		8.09 (d, 9)[a]	
3	70.3	5.10 (m)	H-2, H_3-4
4	15.4	1.25 (3H, d, 7)	H-2
Phenylalanine (*Phe*)			
1	170.9		H-2, 2-NH, H-2 (Ile), 2-NH (Ile)
2	54.6	4.55 (dt, 7, 7)	H_2-3
2-NH		7.76 (d, 7)[a]	
3	37.6	2.90 (m)	H-2′, H-4′
		2.82 (m)	
1′	136.6		H_2-3, H-3′, H-5′
2′, 6′	129.2	7.16 (2H, dd, 7.5, 2)	H_2-3, H-4′
3′, 5′	128.0	7.21 (2H, dd, 7.5, 7.5)	
4′	126.4	7.18 (m)	H-2′, H-6′
Isoleucine (*Ile*)			
1	171.1		H-2, 2-NH, 2-NH (Dba)
2	58.3	3.88 (dd, 7, 6)	2-NH, 3-CH_3
2-NH		8.12 (d, 7)[a]	
3	34.9	1.67 (m)	H-2, 3-CH_3, H_2-4, H_3-5
3-CH_3	15.5	0.60 (3H, d, 7)	H-2, H_2-4
4	24.2	1.15 (m)	H-2, 3-CH_3, H_3-5
		0.85 (m)	
5	11.2	0.73 (3H, t, 7.5)	
2,4-Diaminobutanoic acid (*Dba*)			
1	169.9		H-2, H-2 (Asp), 2-NH (Asp)
2	50.0	4.26 (dt, 8.5, 8.5)	H_2-3
2-NH		7.92 (d, 8.5)[a]	
3	29.9	1.95 (m)	H-2
		1.85 (m)	
4	36.2	2.70 (2H, m)	H-2, H_2-3
4-NH_3		7.80 (3H, m)[a]	
Aspartic acid (*Asp*)			
1	169.2		H-2, H2-3, 2-NH, H-3 (Thr)
2	51.3	4.35 (dt, 7)	H_2-3
2-NH		8.21 (d, 7)[a]	
3	35.0	2.83 (m)	H-2
		2.67 (m)	
4	171.7		H_2-3
19-Guanidino-3-hydroxy-nonadecanoic acid (*Ghn*)			
1	171.5		H-2, H_2-3, 2-NH (Thr)
2	43.4	2.35 (2H, m)	
3	67.4	3.85 (t, 7)	
4	37.0	1.37 (2H, m)	
5	25.2	1.36 (2H, m)	
6	28.5	1.30 (2H, m)	
7–16	29.0 (10C)	1.25 (20H, m)	
17	25.2	1.35 (2H, m)	H_2-19
18	28.4	1.45 (2H, m)	H_2-19
19	40.7	3.07 (dd, 7, 7)	
		3.05 (dd, 7, 7)	
NH*C*NHNH$_2$	156.6		H_2-19
N*H*CNHNH$_2$		7.69 (t, 7)[a]	

^{1}H and ^{13}C signals assigned by COSY and HMQC, etc.
[a] Exchangeable when D_2O was added.

antibacterial chemotherapy,[9] a series of novel cyclic lipopeptides, designated circulocins α–δ (**1**–**4**), were isolated from a strain of *Bacillus circulans*, J2154. These compounds, belonging to the second group, contain either a depsi-pentapeptide or depsi-hexapeptide core that is connected to a fatty acid, terminating in a guanidino function (Fig. 1).

2. Results and discussion

2.1. Structures of circulocins

The lipopeptides were produced by fermentation of strain J2154 in a liquid medium at 28°C for 3 days. The organic extract of the whole broth was separated by reverse phase

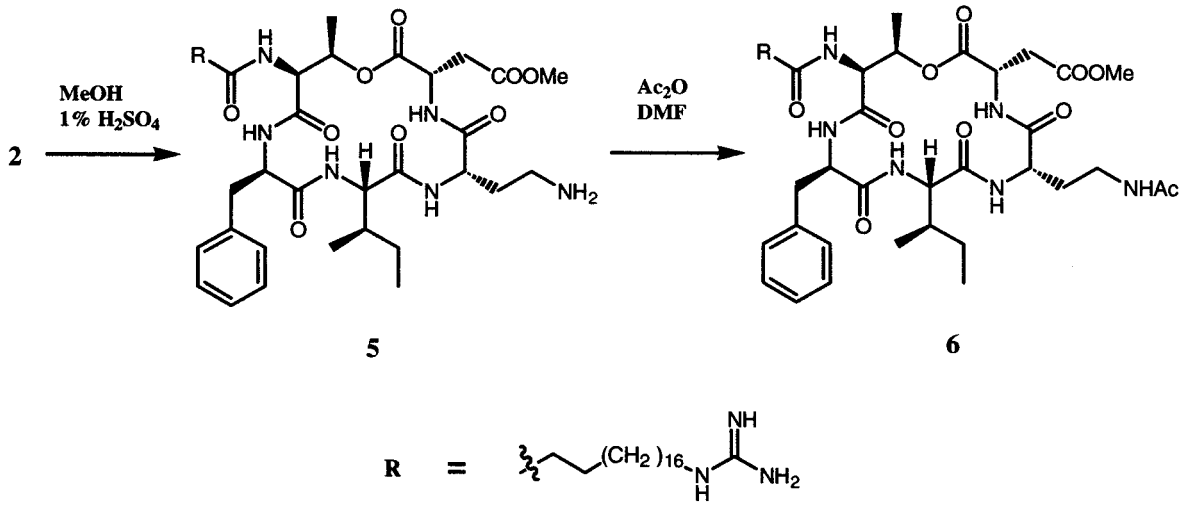

Figure 2. Derivatization of circulocin β (**2**).

HPLC to afford circulocins α–δ (**1**–**4**), all as amorphous white powders.

The molecular formula of circulocin α (**1**) was determined to be $C_{47}H_{79}N_9O_{10}$ by high resolution Fourier transform ion cyclotron resonance (FTICR) mass spectrometry. The ^{13}C NMR spectrum contained seven carbonyl signals near δ 170, and five methine signals between δ 51 and 58, indicative of a peptide structure. The ^1H NMR spectrum displayed five exchangeable doublet signals in the range from δ 7.75 to 8.22, which were assigned to amide NH protons of the peptide.

Detailed analysis of 2D ^1H–^1H COSY, TOCSY, ^1H–^{13}C HMBC, and HMQC data for compound **1** revealed the presence of five amino acids, threonine (Thr), phenylalanine (Phe), isoleucine (Ile), 2,4-diaminobutanoic acid (Dba), and aspartic acid (Asp). In addition, a β-hydroxy fatty acid terminating in a guanidino group was identified from the NMR data. Both the ^{13}C and ^1H NMR spectra suggested the presence of a fatty chain, and the COSY and TOCSY data helped to delineate the spin system of the fatty acid. In the HMBC spectrum, the C-1 resonance at δ 171.5 was correlated to signals of methylene protons (H_2-2) at 2.35 and a methine proton (H-3) at 3.85, indicative of the β-hydroxy carbonyl portion of the fatty acyl moiety. The terminus of the fatty acyl chain was defined by HMBC correlations from methylene proton signals at 3.07 and 3.05 to two methylene carbons at 28.4 and 25.2, and to a carbon signal at 156.6, assigned to the guanidino group. The fatty acid was deduced to have 16 methylenes between the β-hydroxy and the guanidino groups on the basis of integration of the proton NMR signals and the molecular formula. Additional support for this fragment was obtained by ESIMS analysis of the butanol extract of an acid (6N HCl, 105°C, 15 h) hydrolysate of compound **1**. This spectrum contained a prominent ion at *m/z* 372, corresponding to the $(M+H)^+$ for 19-guanidino-3-hydroxy-nonadecanoic acid (Ghn).

The sequence of the lipopeptide was primarily determined by interpretation of the ^1H–^{13}C HMBC data. The respective two-bond correlations from α-amide NH's of phenylalanine, isoleucine, 2,4-diaminobutanoic acid, and aspartic acid to C-1 carbonyls of threonine, phenylalanine, isoleucine, and 2,4-diaminobutanoic allowed the sequence to be established as Thr-Phe-Ile-Dba-Asp. The three-bond correlation between H-3 (Thr) and C-1 (Asp) across the ester linkage was also observed, which established the cyclic depsi-pentapeptide system. The evidence for the attachment of 19-guanidino-3-hydroxy-nonadecanoic acid to the α-amino group on threonine was found in the two-bond correlation between α-amide NH of Thr and C-1 of Ghn. Additional two- or three-bond correlations in the HMBC spectrum and ROE cross peaks in the ROESY spectrum supported the structure (Table 1 and Fig. 3).

Circulocin β (**2**) differed from **1** only in the nature of the fatty acid where the β-hydroxy was lacking, which was indicated by the MS and NMR spectral data. Compound **2** was reacted with acidic methanol to give a methyl ester (**5**), which was then treated with acetic anhydride in *N*,*N*-dimethylformamide to give an acetamide (**6**) (Fig. 2). The successful methylation and acetylation under these mild conditions confirmed the presence of the free carboxyl and amino groups in both **1** and **2**.

The molecular formula of circulocin γ (**3**) was determined to be $C_{45}H_{82}N_{10}O_{11}$ by high-resolution FTICR mass spectrometry. Compound **3** was also a cyclic lipopeptide, but appeared to be different in amino acid composition, compared with **1** and **2**. The presence of six amino acids, threonine-I (ThrI), leucine (Leu), isoleucine (Ile), threonine-II (ThrII), asparagine (Asn), and alanine (Ala), together with the 19-guanidino-3-hydroxy-nonadecanoic acid (Ghn), was revealed by analysis of the NMR data. The sequence of these units was determined on the basis of ^1H–^{13}C HMBC data, similar to the analysis for **1**. The respective two-bond correlations from α-amide NH's of leucine, isoleucine, threonine-II, asparagine and alanine to C-1 carbonyls of threonine-I, leucine, isoleucine, threonine-II, and asparagine allowed the sequence to be established as ThrI-Leu-Ile-ThrII-Asn-Ala. The three-bond correlation between H-3 (ThrI) and C-1 (Ala) across the ester linkage was also observed, which established the cyclic depsi-hexapeptide system. The attachment of 19-guanidino-3-hydroxy-nonadecanoic acid (Ghn)

Table 2. ^{1}H and ^{13}C NMR data of circulocin γ (**3**), in DMSO-d_6

	^{13}C (75 MHz)	^{1}H (300 MHz, mult, *J* in Hz)	HMBC (*J*=8 Hz)
Threonine-I (*ThrI*)			
1	168.0		H-2, H-2 (Leu), 2-NH (Leu)
2	56.6	4.39 (dd, 8.5, 2.5)	2-NH, H_3-4
2-NH		8.05 (d, 8.5)[a]	
3	70.3	5.30 (dq, 2.5, 6.5)	H_3-4
4	16.0	1.12 (3H, d, 6.5)	
Leucine (*Leu*)			
1	171.6		H-2, H-2 (Ile), 2-NH (Ile)
2	50.1	4.65 (br dd, 7.8, 7.8)	
2-NH		7.48 (d, 7.8)[a]	
3	42.3	1.39 (2H, m)	H-2, H-4, H_3-5, H_3-6
4	24.0	1.43 (m)	H_3-5, H_3-6
5	22.6	0.83 (3H, d, 6.5)	H_3-6
6	22.2	0.83 (3H, d, 6.5)	H_3-5
Isoleucine (*Ile*)			
1	173.0		H-2, 2-NH (ThrII)
2	56.7	4.20 (dd, 7.7, 7.7)	3-CH_3
2-NH		8.36 (d, 7.7)[a]	
3	35.8	1.76 (m)	H-2, 3-CH_3, H_2-4, H_3-5
3-CH_3	15.3	0.80 (3H, d, 7)	H-2, H_2-4
4	24.2	1.40 (m)	H-2, 3-CH_3, H_3-5
		1.17 (m)	
5	10.6	0.79 (3H, t, 7)	H_2-4
Threonine-II (*ThrII*)			
1	170.2		H-2, H-3, 2-NH (Asn)
2	60.1	3.92 (m)	H_3-4
2-NH		8.38 (d, 5.8)[a]	
3	65.6	3.92 (m)	H-2, H_3-4
4	19.3	1.09 (3H, d, 7)	H-2, H-3
Asparagine (*Asn*)			
1	169.7		H-2, H_2-3, H-2 (Ala), 2-NH (Ala)
2	50.4	4.28 (dt, 7, 7)	H_2-3
2-NH		8.09 (d, 7)[a]	
3	36.5	2.77 (dd, 15, 6.2)	H-2
		2.59 (dd, 15, 7.2)	
4	172.4		H-2, H_2-3, 4-NH_2
4-NH_2		7.41 (br s)[a]	
		7.00 (br s)[a]	
Alanine (*Ala*)			
1	170.6		H-2, H_3-3, H-3 (ThrI)
2	47.8	3.95 (dq, 7, 7)	H_3-3
2-NH		7.32 (d, 7)[a]	
3	16.9	1.17 (3H, d, 7)	H-2
19-Guanidino-3-hydroxy-nonadecanoic acid (*Ghn*)			
1	171.6		H_2-2, H-3, H-2 (ThrI), 2-NH (ThrI)
2	42.8	2.45 (dd, 13.2, 4.9)	
		2.32 (dd, 13.2, 6.2)	
3	67.5	3.80 (m)	
4	36.4	1.36 (2H, m)	
5	25.3	1.35 (2H, m)	
6	28.5	1.25 (2H, m)	
7–16	28.9-29.0 (10C)	1.25 (20H, m)	
17	25.9	1.25 (2H, m)	H_2-19
18	28.3	1.42 (2H, m)	H_2-19
19	40.6	3.07 (dd, 7, 7)	
		3.05 (dd, 7, 7)	
NH*C*NHNH_2	156.6		H_2-19, N*H*CNHNH_2
N*H*CNHNH_2		7.52 (t, 7)[a]	

^{1}H and ^{13}C signals assigned by COSY and HMQC etc.
[a] Exchangeable when D_2O was added.

to the α-amino group on threonine-I was confirmed by the two-bond correlation between the α-amide NH of ThrI and C-1 of Ghn. Additional two- or three-bond correlations in the HMBC spectrum and ROE cross peaks in the ROESY spectrum supported the structure (Table 2 and Fig. 3).

MS and NMR spectral data identified circulocin δ (**4**) as a

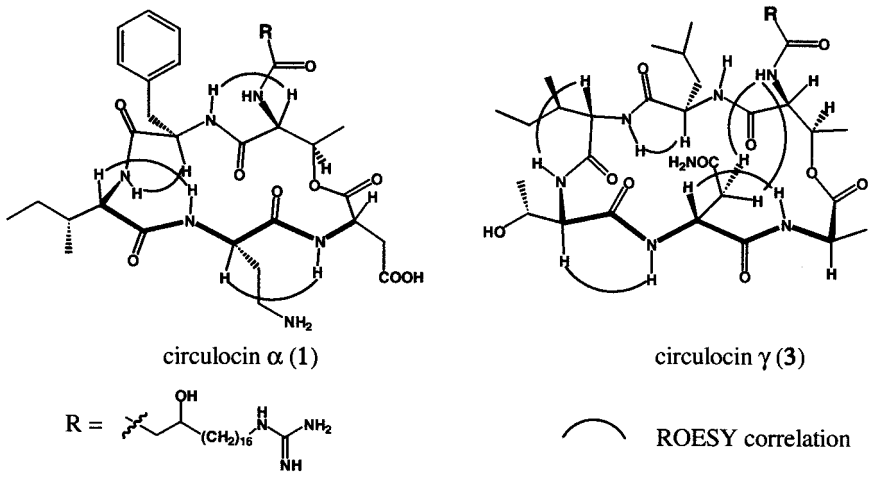

Figure 3. Stereo perspectives of circulocins α (**1**) and γ (**3**).

homologue of γ (**3**) with two additional methylenes in the fatty acid.

Circulocins α (**1**) and γ (**3**) were hydrolyzed by treatment of 6N HCl at 100°C, and the absolute stereochemistry of the amino acids was established by LC–MS analysis of Marfey's derivatives. For compound **1**, the analysis indicated the presence of L-threonine, D-phenylalanine, L-isoleucine, L-2,4-diaminobutanoic acid, and L-aspartic acid. This both confirmed the amino acid composition and defined the configuration for the cyclic system.

On the other hand, the LC–MS analysis on the hydrolysate of compound **3** indicated the presence of D-leucine, L-isoleucine, D-aspartic acid, and D-alanine, in addition to L-threonine and D-*allo*-threonine. Threonine-I between D-alanine and D-leucine, which had chemical shift data and homonuclear coupling constants comparable to those of L-threonine in **1** (Tables 1 and 2), was assigned to L- and threonine-II to D-*allo*. The configurations of the cyclic peptide were thus defined. Circulocins γ (**3**) and δ (**4**) are closely related to the recently reported depsi-hexapeptide LI-F antibiotics isolated from *Bacillus polymyxa*.[10]

The predominant conformations of these molecules could be deduced from the ROESY data and the homonuclear coupling constants, as shown in Fig. 3. For both compounds **1** and **3**, the strong cross peaks between the α-protons of the amino acid residues and the NH protons of the adjacent residues (towards the C-termini) were observed in ROESY spectra. The coupling constants between the α-protons and the annular NH protons were measured from 5.8 to 8.5 Hz (most of them from 7 to 8 Hz), indicative of *trans* configurations between the α-protons and NH protons in all amino acid residues. Moreover, the coupling constants between the α- and β-protons of the L-threonine residues of compounds **1** and **3** were small, suggesting *gauche* orientations between these protons in both compounds. Finally, in circulocin γ (**3**), the transannular ROEs between H_2-3 (Asn) at δ 2.77 and 2.59, and 2-NH (ThrI) at 8.05 in the ROESY spectrum suggested that the side chain (C-3, C-4) on the asparagine and the fatty acid connecting to threonine-I are both facing inward with respect to the cyclic peptide ring. The observed transannular ROEs also confirmed the assignments of the L-configuration for threonine-I and D-*allo* for threonine-II. This conclusion was arrived at by the inability to establish a molecular model for compound **3** using the alternative assignments for threonine-I (D-*allo*) and threonine-II (L), in which the asparagine side chain and the fatty acid were spatially proximate while the α-protons and NH protons in all amino acid residues remained in the *trans* orientations, and the α-protons of Leu, Ile, ThrII, Asn were, respectively, close through space to the NH protons of Ile, ThrII, Asn and Ala.

2.2. Biological activity

Circulocins α (**1**), β (**2**) and γ (**3**), exhibited potent antibiotic

Table 3. Antibacterial activity of circulocins α (**1**), β (**2**), and γ (**3**)

Test organism	MIC (μg/mL)[a]		
	Circulocin α (**1**)	β (**2**)	γ (**3**)
Staphylococcus aureus (9 strains, including two piperacillin-resistant strains)	2–4	2–4	0.5–1
S. haemolyticus GC 4546	4	2	1
Enterococcus faecalis (9 strains, including drug-resistant strains)	4–8	2–4	1–2
E. faecium (4 strains, including vancomycin-resistant strains)	4–8	4	1–2
Streptococcus pyogenes GC 4563	32	32	2
S. pneumoniae (3 strains)	32	32	1–2
Escherichia coli (2 strains)	>64	>64	>64
Micrococcus luteus GC 4562	4	2	0.12
Candida albicans GC 3066	4	4	2

[a] Agar dilution method in Mueller–Hinton AII, incubated at 35°C for 18 h.

activity against the Gram-positive bacteria, including the piperacillin-resistant *Streptococci* and vancomycin-resistant *Enterococci*, but showed poor activity against the Gram-negative bacteria. Among these compounds, the depsi-hexapeptide, circulocin γ (**3**), showed the best activity against all tested organisms. The MIC data obtained from agar dilution method are listed in Table 3.

3. Conclusions

Circulocins α–δ (**1**–**4**), a series of new cyclic lipopeptides, were isolated from the fermentation broth of *Bacillus circulans*, J2154, and their structures characterized. Although some long-chain ω-guanidino fatty acids (up to C12) have been synthesized by amidation of the corresponding amino acids,[11] only a few such examples have been found in natural products.[10] To our knowledge, those encountered in the herein described circulocins have not been reported previously. Circulocins, possessing both hydrophilic and hydrophobic substructures, are active against the yeast *Candida albicans*, in addition to the Gram-positive bacteria. The structural features and antimicrobial profiles imply that these new peptides may also have surface-active properties observed in other *Bacillus*-produced lipopeptides.[12]

4. Experimental

4.1. Fermentation

A defrosted cell suspension (0.5 mL) of strain J2154 was streaked onto a Bennett agar plate, and the cells were allowed to grow at 28°C for 7 days. Several loops of the surface growth were transferred into a 250-mL Erlenmeyer flask that contained 50 mL of a seed medium consisting of artificial sea water (500 mL/L), K_2HPO_4 (0.5 g/L), yeast extract (4 g/L), and soluble starch (10 g/L). The flask was shaken at 200 rpm at 28°C for 7 days, and the first stage seed thus produced was inoculated into a 2.8-L fernbach flask containing 0.5 L of the seed medium. After growing at 28°C for 6 days on a 200 rpm rotary shaker, the second stage seed (200 mL) was added to a 10-L Bio-flo 3000 fermentor that contained 10 L of a production medium consisting of dextrose (10 g/L), soluble starch (20 g/L), yeast extract (5 g/L), *N-Z* amine A (5 g/L), $CaCO_3$ (1 g/L), and agar (0.4 g/L). The pH was adjusted to 7.3 with 1N NaOH; temperature was maintained at 28°C; air was sparged at 10 L/min; agitation was set at 200 rpm; prior to autoclaving, lard oil (3 mL) was added to prevent foaming. The whole mash was harvested after incubation for 3 days.

4.2. Isolation and purification

A portion of the viscous whole fermentation broth (2 L) was triturated with methanol (2 L), and the resulting mixture was sonicated at ambient temperature for 30 min and filtered over celite. The filtrate was concentrated to an aqueous suspension (~2 L), followed by extraction with *n*-butanol (2×1 L). The combined organic layer, washed with water, was evaporated under reduced pressure to a gum, which was then chromatographed by reverse phase HPLC on a C18 column (YMC ODS-A, 10 μm, 70×500 mm). The mobile phase, monitored by UV detection at 210 nm, was a gradient of 5–95% acetonitrile in water containing 0.01% trifluoroacetic acid (TFA) over 60 min. The broad peak at about 40 min was active against *Staphylococcus aureus* in agar plate assay. Upon concentration, the residue (210 mg) was further separated by reverse phase HPLC (26–60% acetonitrile in water with 0.01% TFA, 45 min) to afford circulocins α (35.7 min, 27 mg), β (37.8 min, 23 mg), γ (39.7 min, 30 mg), and δ (43.9 min, 10.5 mg).

4.2.1. Circulocin α (1). $[\alpha]_D=-41.6°$ (*c* 0.63, MeOH); HRESIMS *m/z* 930.6028 (MH^+, $C_{47}H_{80}N_9O_{10}$ requires 930.6022); ^1H and ^{13}C NMR data: see Table 1.

4.2.2. Circulocin β (2). $[\alpha]_D=46.5°$ (*c* 0.54, MeOH); HRESIMS *m/z* 914.6070 (MH^+, $C_{47}H_{80}N_9O_9$ requires 914.6073); ^1H NMR (DMSO-d_6, the chemical shift data assigned by 2D NMR spectra, the coupling constants not measurable owing to the broadening of the signals) δ Thr 8.13 (NH), 4.42 (H-2), 5.09 (H-3), 1.21 (H_3-4), Phe 7.64 (NH), 4.56 (H-2), 2.87 (2H, H_2-3), 7.15–7.20 (5H, H-2′–H-6′), Ile 8.15 (NH), 3.89 (H-2), 1.71 (H-3), 1.14, 0.94 (H_2-4), 0.72 (H_3-5), 0.64 (3-CH_3), Dba 7.95 (NH), 4.28 (H-2), 1.98, 1.85 (H_2-3), 2.75 (2H, H_2-4), 7.78 (4-NH_3), Asp 8.20 (NH), 4.35 (H-2), 2.85-2.66 (2H, H_2-3), 12.37 (COOH), 19-guanidino-nonadecanoic acid 2.25 (2H, H_2-2), 1.43 (H_2-3), 1.22 (H_2-4–H_2-17), 1.45 (H_2-18), 3.07 (H_2-19); ^{13}C NMR (DMSO-d_6) δ Thr 168.8 (s, C-1), 55.3 (d, C-2), 70.3 (d, C-3), 15.4 (q, C-4), Phe 170.9 (s, C-1), 54.5 (d, C-2), 37.6 (t, C-3), 136.6 (s, C-1′), 129.2 (2 C, d, C-2′, C-6′), 128.0 (2C, d, C-3′, C-5′), 126.3 (d, C-4′), Ile 171.1 (s, C-1), 58.3 (d, C-2), 35.0 (d, C-3), 15.5 (q, 3-CH_3), 24.3 (t, C-4), 11.2 (q, C-5), Dba 170.0 (s, C-1), 50.0 (d, C-2), 29.8 (t, C-3), 36.2 (t, C-4), Asp 169.2 (s, C-1), 51.3 (d, C-2), 35.1 (t, C-3), 171.1 (s, C-4), 19-guanidino-nonadecanoic acid 172.9 (s, C-1), 28.7 (13 C, t), 28.5 (t), 28.4 (t), 26.0 (t), 25.1 (t), 40.7 (t, C-19), 156.8 (s, $NHCNHNH_2$).

4.2.3. Circulocin γ (3). $[\alpha]_D=+12.3°$ (*c* 0.31, MeOH); HRESIMS *m/z* 939.6228 (MH^+, $C_{45}H_{83}N_{10}O_{11}$ requires 939.6238); ^1H and ^{13}C NMR data: see Table 2.

4.2.4. Circulocin δ (4). $[\alpha]_D=+12.9°$ (*c* 0.24, MeOH); HRESIMS *m/z* 967.6550 (MH^+, $C_{47}H_{87}N_{10}O_{11}$ requires 967.6551); ^1H NMR (DMSO-d_6, mult, *J* in Hz) δ ThrI 8.05 (d, 8.7, NH), 4.39 (dd, 8.7, 2.2, H-2), 5.30 (dq, 2.2, 6.5, H-3), 1.12 (3H, q, 6.5, H_3-4), Leu 7.45 (d, 7.9, NH), 4.64 (br dd, 7.9, 7.9, H-2), 1.39 (2H, m, H_2-3), 1.43 (m, H-4), 0.83 (6H, d, 6.5, H_3-5, H_3-6), Ile 8.35 (d, 7.7, NH), 4.21 (dd, 7.7, 7.7, H-2), 1.76 (m, H-3), 0.80 (3H, d, 6.5, 3-CH_3), 1.40, 1.17 (m, H_2-4), 0.79 (3H, t, 7.8, H_3-5), ThrII 8.37 (d, 5.6, NH), 3.92 (2H, m, H-2, H-3), 1.10 (3H, d, 7), Asn 8.05 (d, 7.2, NH), 4.27 (dt, 7.2, 7), 2.77 (dd, 15.3, 6.3), 2.59 (dd, 15.3, 7, H_2-3), 7.40 (br s), 6.99 (br s, 4-NH_2), Ala 7.30 (d, 7.2, NH), 3.96 (dq, 7.2, 7, H-2), 1.17 (3H, d, 7, H_3-3), 19-guanidino-3-hydroxy-henicosanoic acid 2.46 (dd, 13.2, 5.1), 2.30 (dd, 13.2, 6.3, H_2-2), 3.80 (m, H-3), 1.30–1.35 (12H, m), 1.24 (22H, H_2-4–H_2-20), 3.07, 3.05 (dd, 7, 7, H_2-21), 7.48 (t, 7, 21-NH).

4.2.5. Methylation. A solution of **2** (5.0 mg) in methanol (0.5 mL) containing 1% H_2SO_4 was stirred at ambient

temperature for 15 h. The reaction mixture was neutralized by aqueous Na_2CO_3 and separated by reverse phase HPLC (C18 column, gradient solvent of acetonitrile in water with 0.01% TFA) to afford the methyl ester **5** (3.5 mg). ESIMS *m/z* 928.4 (MH^+, 10%), 464.9 ($(M+H_2)^{2+}$, 100%).

4.2.6. Acetylation. A solution of **5** (3.5 mg) in *N*,*N*-dimethylformamide (0.5 mL) and acetic anhydride (2 drops) was stirred at ambient temperature for 15 h. The reaction mixture was evaporated in vacuo and the residue separated by reverse phase HPLC to afford the acetamide **6** (3.3 mg). ESIMS *m/z* 970.8 (MH^+, 100%); ^1H NMR (DMSO-d_6, mult, *J* in Hz) δ Thr 8.13 (d, 9.0, NH), 4.42 (dd, 9.0, 2.8, H-2), 5.09 (dq, 2.8, 6.5, H-3), 1.21 (3H, m, H_3-4), Phe 7.70 (d, 8.7, NH), 4.57 (dt, 8.7, 6.5, H-2), 2.88 (2H, m, H_2-3), 7.16–7.25 (5H, m, H-2′–H-6′), Ile 8.09 (d, 8.2, NH), 3.85 (dd, 8.2, 7.0, H-2), 1.70 (m, H-3), 1.15 (m), 0.94 (2H, m, H_2-4), 0.73 (3H, t, 6.5, H_3-5), 0.67 (3H, d, 6.5, 3-CH_3), Dba 7.60 (d, 7.8, NH), 4.18 (dt, 7.8, 7.0, H-2), 1.72 (2H, m, 1.85, H_2-3), 2.92 (2H, m, H_2-4), 7.75 (t, 6.5, 4-NH), 1.76 (3H, s, CH_3CO), Asp 8.08 (d, 8.0, NH), 4.46 (m, H-2), 2.80–2.77 (2H, m, H_2-3), 3.56 (3H, s, OCH_3), 19-guanidinononadecanoic acid 2.22 (2H, t, 6.5, H_2-2), 1.45 (2H, m, H_2-3), 1.22 (H_2-4–H_2-17), 1.47 (H_2-18), 3.06, 3.08 (t, 6.5, H_2-19).

4.3. Determination of the absolute stereochemistry

4.3.1. Peptide hydrolysis. Peptide samples (1.0 mg) were dissolved in degassed 6N HCl (0.5 mL) in sealed vials and heated at 105°C for 15 h. The solvent and the acid were removed in vacuo.

4.3.2. LC–MS analysis of Marfey's (FDAA) derivatives[13] for circulocin α (1). An aqueous solution of the hydrolysate of **1** (0.1 mL) was treated with 6% triethylamine (30 μL) and 1% 1-fluoro-2,4-dinitrophenyl-5-L-alanineamide (FDAA) in acetone (0.1 mL) at 40°C for 1 h. The reaction mixture was diluted with 1:1 acetonitrile/water (0.2 mL) and the resulting solution was analyzed by HPLC. C18 column: YMC ODS-A, 4.6×150 mm, linear gradient solvent: 20–50% MeCN/H_2O with 0.01% TFA in 25 min, flow rate: 1 mL/min. The mobile phase was monitored by both UV absorption at 254 nm and negative ESIMS. The retention times in minutes and the negative ions, $(M-H)^-$, are given in parentheses: L-Thr (11.1, 370), L-Asp (11.5, 384), L-2,4-diaminobutanoic acid (20.5, 621), L-Ile (21.0, 382), D-Phe (23.8, 416).

4.3.3. LC–MS analysis of Marfey's derivatives for circulocin γ (3). An aqueous solution of the hydrolysate of **3** (0.1 mL) was treated with 6% triethylamine (30 μL) and 1% FDAA in acetone (0.1 mL) at 40°C for 1 h. The reaction mixture was diluted with 1:1 acetonitrile/water (0.2 mL) and the resulting solution was analyzed by HPLC, using the same system as in Section 4.3.2. The retention times in minutes and the negative ions, $(M-H)^-$, are given in parentheses: L-Thr (11.2, 370), D-*allo*-Thr (12.2, 370), D-Asp (12.3, 384), D-Ala (16.2, 340), L-Ile (21.1, 382), D-Leu (24.6, 382).

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