FULL PAPER

Two New Cyclic Tetrapeptides of *Streptomyces rutgersensis* T009 Isolated from *Elaphodus davidianus* Excrement

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Two new cyclic tetrapeptides, cyclo(L-Val-L-Leu-L-Val-L-Ile) (**1**) and cyclo(L-Leu-L-Leu-L-Ala-L-Ala) (**2**), and 15 known compounds, cyclo(Gly-L-Leu-Gly-L-Leu) (**3**), cyclo(L-Ser-L-Phe) (**4**), cyclo(L-Leu-L-Ile) (**5**), cyclo(L-Tyr-L-Phe) (**6**), cyclo(Gly-L-Trp) (**7**), cyclo(L-Leu-L-Tyr) (**8**), cyclo(Gly-L-Phe) (**9**), cyclo(L-Phe-*trans*-4-hydroxy-L-Pro) (**10**), cyclo(L-Leu-L-Leu) (**11**), cyclo (L-Val-L-Phe) (**12**), cyclo(L-Val-L-Leu) (**13**), cyclo(L-Ile-L-Ile) (**14**), cyclo(L-Tyr-L-Tyr) (**15**), turnagainolide A (**16**), and bacimethrin (**17**) were isolated from the fermentation broth of *Streptomyces rutgersensis* T009 obtained from *Elaphodus davidianus* excrement. Their structures were identified on the basis of spectroscopic analysis. Meanwhile, the absolute configurations of the amino acid residues of compounds **1** and **2** were determined by advanced *Marfey* method. Compound **3** was obtained from a natural source for the first time. The X-ray single crystal diffraction data of bacimethrin (**17**) were also reported for the first time. Compounds **1** – **17** exhibited no antimicrobial activities with the *MIC*s > 100 μg/ml.

Keywords: Cyclic tetrapeptides, *Streptomyces rutgersensis*, *Elaphodus davidianus*, X-Ray crystallography

Introduction

Although an important number of drugs and analogs were successfully introduced in the market and still used today in clinical practice, finding and exploring highly bioactive compounds were still challenging tasks for chemists and pharmacologists [1 – 3]. Microorganism from animal excrement had been one of the most important natural sources for the discovery of novel compounds in recent years [4][5]. Our co-authors had investigated the antimicrobial activities of the fermentation broth of *Streptomyces rutgersensis* T009 and together with a zone of inhibition toward *Bacillus subtilis* of 12 mm at a concentration of 0.5 mg/ml in the agar diffusion assay. Chemical studies were deeply carried out subsequently. Two new compounds were isolated and identified as cyclo(L-Val-L-Leu-L-Val-L-Ile) (**1**) and cyclo(L-Leu-L-Leu-L-Ala-L-Ala) (**2**). The known compound **3** was isolated from a natural source for the first time (*Fig. 1*).

Results and Discussion

The AcOEt extract of the fermentation broth of *S. rutgersensis* T009 was fractionated by column chromatography (CC) on silica gel, *MCI*, *Sephadex LH-20* and reversed-phase (RP) silica gel to yield **1** – **3**, and 14 known compounds (*Fig. 1*).

Compound **1** was obtained as colorless needles, and the molecular formula was determined as $C_{22}H_{40}O_4N_4$ by HR-ESI-MS (*m/z* 443.3272 ($[M + H_3O]^+$)). The ^{13}C-NMR and DEPT spectra showed the presence of four C=O groups at 171.5, 170.5, 170.4, and 170.0 ppm, eight CH signals at 63.1, 62.6, 62.1, 55.8, 41.1, 34.7, 34.1, and 26.6 ppm, two CH_2 signals at 47.3 and 27.1 ppm, and eight Me signals at 25.4, 23.7, 21.4, 21.3, 19.8, 19.6, 17.7, and 14.2 ppm. In the ^{1}H-NMR spectrum, four NH groups at 9.23 (1 H, *s*), 9.12 (1 H, *s*) and 8.88 ppm (2 H, *s*), four amide α-H atoms at 4.27 (1 H, *d*), 4.17 (1 H, *s*), and 4.12 (2 H, *m*) ppm, and signals at 0.93 – 2.70 ppm from amino acid residues appeared. The spectra suggested the presence of a cyclotetrapeptide for compound **1**. The amino acid residues were identified as two valine groups (Val), one leucine group (Leu), and one isoleucine group (Ile) by ^{1}H,^{1}H-COSY, HSQC, and HMBC spectra (*Fig. 2*). The sequence of the amino acid residues of **1** was confirmed by HMBC, which showed correlations from the amide protons to the α-CH C-atoms of the adjacent residues. In the HMBC spectrum, the correlations between Leu-α-C (55.8 ppm) and NH-Val (1) (9.11 ppm), Val(2)-α-C (63.1 ppm) and NH (Leu) (9.23 ppm) were distinct, and the correlations between Ile-α-C (62.6 ppm) and

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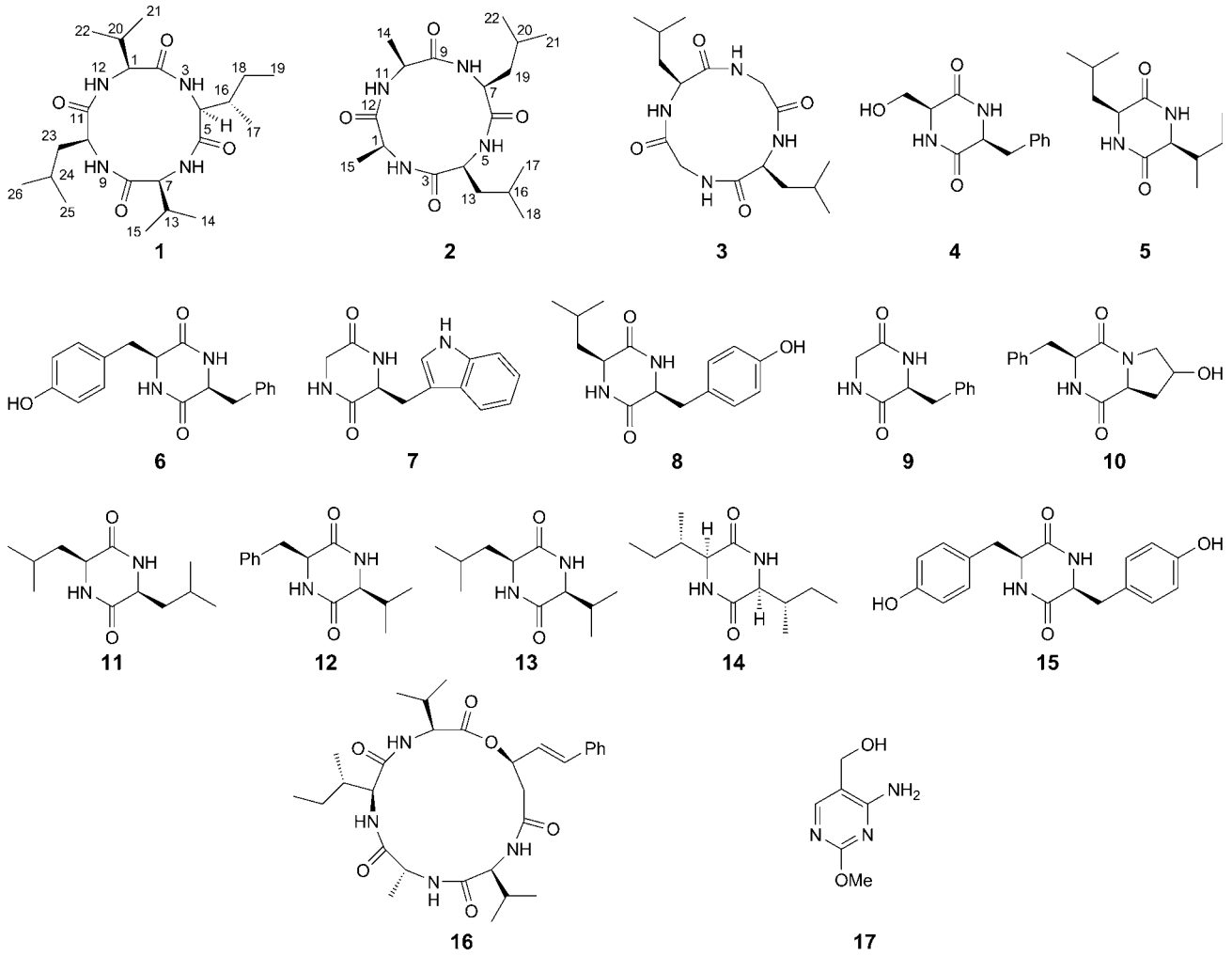

Fig. 1. Structures of compounds **1** – **17**

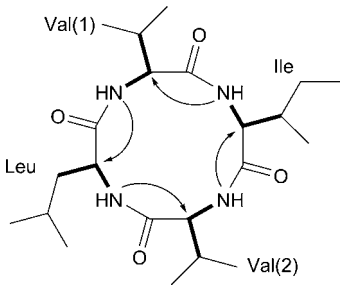

Fig. 2. Key HMBC (H→C) and ^{1}H,^{1}H-COSY (▬) correlations of compound **1**

NH-Val(2) (8.88 ppm), Val(1)-α-C (62.1 ppm) and NH (Ile) (8.88 ppm) were observed. Consequently, the sequence of amino acid was identified as Leu-Val-Ile-Val.

Compound **2** was isolated as white amorphous powder, and the molecular formula was determined as $C_{18}H_{32}O_4N_4$ by HR-ESI-MS (*m/z* 391.2247 ([*M* + Na]$^+$)). The ^{13}C-NMR spectrum showed four signals for C=O groups at 171.1, 170.9 C(2) and 170.4 ppm, six CH signals at 55.8, 54.9, 52.2, 51.2, and 25.5 C(2) ppm, two CH_2 signals at 44.8 and 44.2 ppm, six Me signals at 24.2, 24.1, 22.7, 22.6, 21.4, and 19.4 ppm. The ^{1}H-NMR spectrum showed signals of four NH groups at 9.40 (1 H, *s*), 9.33 (1 H, *s*), 9.31 (1 H, *s*), and 9.22 (1 H, *s*) ppm, four amide α-H atoms at 4.39 – 4.19 (4 H, *m*) ppm, 24 H-atom signals at 0.88 – 2.10 ppm from amino acid residues. The spectra suggested also a cyclotetrapeptide for compound **2**. The amino acid residues were identified as two leucine groups (Leu) and two alanine groups (Ala) by 2D-NMR spectra. In the HMBC spectrum of **2** (*Fig. 3*), the correlations between C(1) (52.2 ppm) and H–N(11) (9.40 ppm), C(10) (51.2 ppm) and H–N(8) (9.22 ppm), C(4) (54.9 ppm) and H–N(2) (9.33 ppm), C(7) (55.8 ppm) and H–N(5) (9.31 ppm) were clearly observed. The sequence of amino acid was unambiguously confirmed as Ala-Ala-Leu-Leu.

Compounds **3** – **17** were identified as cyclo(Gly-L-Leu-Gly-L-Leu) (**3**) [6], cyclo(L-Ser-L-Phe) (**4**) [7], cyclo(L-Leu-L-Ile) (**5**) [8], cyclo(L-Tyr-L-Phe) (**6**) [9], cyclo(Gly-L-Trp) (**7**) [10], cyclo(L-Leu-L-Tyr) (**8**) [7], cyclo(Gly-L-Phe) (**9**)

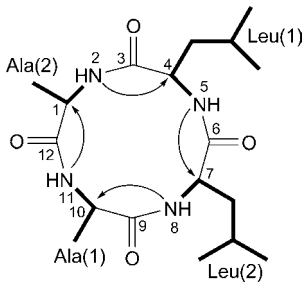

Fig. 3. Key HMBC (H→C) and ^{1}H, ^{1}H-COSY (▬) correlations of compound **2**

[8], cyclo(L-Phe-*trans*-4-hydroxy-L-Pro) (**10**) [11], cyclo(L-Leu-L-Leu) (**11**) [8], cyclo(L-Val-L-Phe) (**12**) [12], cyclo (L-Val-L-Leu) (**13**) [13], cyclo(L-Ile-L-Ile) (**14**) [14], cyclo (L-Tyr-L-Tyr) (**15**) [9], turnagainolide A (**16**) [15], and bacimethrin (**17**) [16] on the basis of NMR and HR-ESI-MS data. Fortunately, compound **17** yielded a crystal suitable for X-ray analysis, and a crystallographic study was conducted (*Fig. 4*).

Because most of the metabolites from this strain were cyclopeptides, so *S. rutgersensis* T009 showed the latent capacity of production of cyclopeptide, and this strain may be used as the bioreactor producing cyclopeptide.

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Experimental Part

General

Column chromatography (CC): silica gel (SiO_2; 200 – 300 mesh; *Qingdao Marine Chemical Group Co.*, Qingdao, China), *Sephadex LH-20* (*GE Healthcare Co.*, Buckinghamshire, UK). M.p.: *XRC-1 Melting Point Apparatus* (*Sichuan University Science Instrument Co.*, Chengdu, China); uncorrected. 1D- and 2D-NMR spectra: *Bruker DRX-500* MHz instruments (*Bruker Co.*, Karlsruhe, Germany); δ in ppm rel. to Me_4Si as internal standard, J in Hz. HR-ESI-MS: *Agilent G3250AA* (Agilent, Santa Clara, USA) and *AutoSpec Premier P776* spectrometers (Waters, Milford, USA); in m/z.

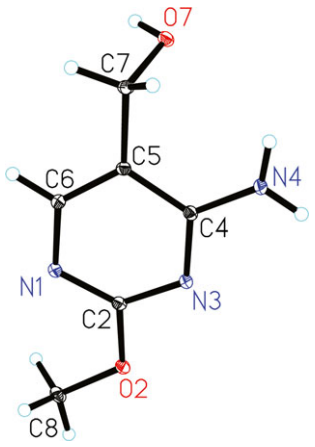

Fig. 4. ORTEP Plot of bacimethrin (**17**)

Streptomyces Isolate

The bacterial strain *Streptomyces rutgersensis* T009 was isolated from fresh fecal samples of healthy *Elaphodus davidianus* living in Yunnan Wild Animal Park, Kunming, Yunnan Province, P. R. China. The strain was assigned as *Streptomyces rutgersensis* by characterization and complete 16S rRNA gene sequence was preserved at Yunnan Institute of Microbiology, Yunnan University, China.

Fermentation, Extraction, and Isolation

This strain was cultivated on 60 L scale using 500 ml *Erlenmeyer* flasks containing 100 ml of the seed medium (yeast extract 0.4%, glucose 0.4%, malt extract 0.5%, decavitamin 0.01%, pH 7.5) for 3 d and the fermentation medium (soluble starch 2.4%, beef extract 0.3, glucose 0.1%, peptone 0.3%, yeast extract 0.5%, $CaCO_3$ 0.4%, pH 7.5) at 28° for 7 d on rotary shaker (250 rpm). The fermentation broth was extracted with AcOEt (3 × 60 l) and the solvent was removed under vacuum. The AcOEt extract (29 g) was separated into 11 fractions (Fr. 1 – Fr. 11) on a chromatographic column (10 × 120 cm) on silica gel (200 – 300 mesh) with a $CHCl_3$/MeOH solvent system (from 100:0 to 1:1 and finally 0:100). Fraction 2 (3.1 g) was separated on *Sephadex LH-20* (MeOH) to give four fractions (Fr. 2.1 – 2.4). In the further purification, CC on SiO_2 with petroleum ether/AcOEt, $CHCl_3$/MeOH, reversed-phase (RP) silica gel C_{18} with H_2O/MeOH, and RPHPLC with H_2O/MeCN were used. Finally, **2** (21 mg), **3** (11 mg), and **4** (8 mg) were obtained from Fr. 2.2, **5** (30 mg), **6** (15 mg), **7** (3.5 mg), **8** (9 mg), **9** (13 mg), and **10** (4 mg) were obtained from Fr. 2.3. Fraction 5 (5 g) was subjected to chromatography on *MCI* with MeOH/H_2O system (from 20:80 to 75:25 and finally 100:0) and purified into seven subfractions (Fr. 5.1 – 5.7). Fr. 5.2 was further separated by gel chromatography on *Sephadex LH-20* (MeOH) to obtain **1** (15 mg), **11** (25 mg), **12** (8 mg), and **13** (11 mg), and Fr. 5.3 was further separated by gel chromatography on *Sephadex LH-20* (MeOH) to obtain **14** (16 mg), **15** (7 mg), **16** (45 mg), and **17** (53 mg).

Cyclo(L-Val-L-Leu-L-Val-L-Ile) (**1**). Amorphous powder. $[\alpha]_D^{20} = -14.8$ (c = 0.1, $CHCl_3$). M.p. 234 – 236°. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 443.3272 ($[M + H_3O]^+$, $C_{22}H_{43}N_4O_5^+$; calc. 443.3228).

Cyclo(L-Leu-L-Leu-L-Ala-L-Ala) (**2**). Amorphous powder. $[\alpha]_D^{20} = -5$ (c = 0.1, $CHCl_3$). M.p. 218 – 220°. ^{1}H- and

Table 1. ^{1}H- and ^{13}C-NMR data of compounds **1** and **2** in (D_5)pyridine. δ in ppm, J in Hz

Position	**1**		**2**	
	δ(H)[a]	δ(C)[b]	δ(H)[a]	δ(C)[b]
1	4.12 (*m*)	63.1	4.30 – 4.35 (*m*)	52.2
2		170.5	9.33 (*s*)	
3	8.88 (*s*)			170.9
4	4.12 (*m*)	62.6	4.19 – 4.22 (*m*)	54.9
5		170.4	9.31 (*s*)	
6	8.88 (*s*)			171.1
7	4.17 (*s*)	62.1	4.26 – 4.29 (*m*)	55.8
8		171.5	9.22 (*s*)	
9	9.23 (*s*)			170.4
10	4.27 (*d*, *J* = 9.2)	55.8	4.36 – 4.39 (*m*)	51.2
11		170.0	9.38 (*s*)	
12	9.12 (*s*)			170.9
13	2.34 – 2.37 (*m*)	41.1	1.81 – 1.95 (*m*)	44.2
14	1.10 (*d*, *J* = 5.0)	19.8	1.66 (*d*, *J* = 5.5)	21.4
15	1.11 (*d*, *J* = 5.0)	19.6	1.66 (*d*, *J* = 5.5)	19.4
16	2.68 – 2.70 (*m*)	34.1	1.85 – 1.94 (*m*)	25.5
17	1.19 (*d*, *J* = 2.5)	17.7	0.90 (*d*, *J* = 4.5)	24.1
18	1.80 – 1.91 (*m*)	27.1	0.90 (*d*, *J* = 4.5)	22.6
19	0.89 (*t*, *J* = 7.5)	14.2	2.06 – 2.12 (*m*)	44.8
20	2.59 – 2.62 (*m*)	34.7	2.00 – 2.12 (*m*)	25.5
21	1.18 (*d*, *J* = 4.8)	21.4	0.90 (*d*, *J* = 4.5)	24.2
22	1.17 (*d*, *J* = 4.8)	21.3	0.90 (*d*, *J* = 4.5)	22.7
23	2.13 – 2.17 (*m*)	47.3		
24	1.40 – 1.48 (*m*)	26.6		
25	0.93 (*d*, *J* = 6.3)	25.4		
26	0.93 (*d*, *J*= 6.3)	23.7		

[a]) Recorded at 500 MHz. [b]) Recorded at 125 MHz.

^{13}C-NMR: see *Table 1*. HR-ESI-MS: 391.2247 ([M + Na]$^+$, $C_{18}H_{32}N_4NaO_4^+$; calc. 391.2316).

Cyclo(Gly-L-Leu-Gly-L-Leu) (**3**). ^{1}H-NMR (CD_3OD, 400 MHz): 1.03 (*d*, *J* = 8.0, 6 H, H_δ-Leu); 0.99 (*d*, *J* = 8.0, 6 H, H_δ-Leu); 1.53 – 1.72 (*m*, 4 H, H_β-Leu); 1.78 – 1.94 (*m*, 2 H, H_γ-Leu); 4.02 (*d*, *J* = 18.0, 2 H, H-Gly), 3.88 (*t*, *J* = 6.8, 2 H, H_α-Leu), 3.83 (*d*, *J* = 18.0, 2 H, H-Gly). ^{13}C-NMR (CD_3OD, 100 MHz): 22.9 (C(δ)-Leu); 24.3 (C(δ)-Leu); 26.1 (C(γ)-Leu); 44.7 (C($_\beta$)-Leu); 46.1 (C(α)-Gly); 55.7 (C(α)-Leu); 169.7 (C=O Leu); 172.4 (C=O Gly). HR-ESI-MS: 339.2246 ([M – H]$^-$, $C_{16}H_{27}N_4O_4^-$; calc. 339.2038).

X-Ray Crystallographic Analysis of **17**

A crystal of compound **17** was obtained from a solution of $CHCl_3$/MeOH. All crystallographic data were collected on a *Bruker APEX-II CCD* diffractometer with graphite monochromated MoK_α radiation (λ = 0.71073 Å). Empirical absorption corrections were carried out using the SADABS program [17]. The structure was solved by direct methods and refined on F^2 by full-matrix least-squares technique using SHELXL-97 software [18]. Crystal data: $C_6H_9N_3O_2$, M = 155.16, monoclinic, a = 8.3990(8) Å, b = 8.2326(8) Å, c = 10.1314(9) Å, β = 94.5120(10)°, V = 698.37(11) Å^3, T = 100(2) K, space group $P2/n$, Z = 4, μ(MoK_α) = 0.114 mm^{-1}, 7301 reflections measured, 1978 independent reflections (R_{int} = 0.0217). The final R_1 values were 0.0362 ($I > 2\sigma(I)$). The final $wR(F^2)$ values were 0.1000 ($I > 2\sigma(I)$). The final R_1 values were 0.0409 (all data). The goodness of fit on F^2 was 1.104. Crystallographic data for **17** had been deposited in the *Cambridge Crystallographic Data Centre* (deposition No.: CCDC-1415864).

Acid Hydrolysis and Advanced Marfey Analysis

Compound **1** (1 mg) was subjected to acid hydrolysis at 110° for 24 h with 6N HCl (1.0 ml), and then the hydrolysate was evaporated to dryness and resuspended in acetone (100 µl) [19][20]. To each half portion (50 µl) were added 100 µl of 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide or 1-fluoro-2,4-dinitrophenyl-5-D-leucin amide (L-FDLA or D-FDLA, 1% in acetone) and 20 µl of $NaHCO_3$ (1M), and the mixture was heated at 40° for 1.5 h. The mixtures were cooled, neutralized with 2N HCl (10 µl), dried in vacuum for 3 h and dissolved in 50% aqueous MeCN. Of each solution of FDLA derivatives, 5 µl was analyzed by LC/MS. The analysis of the L- and L,D-FDLA (mixture of L- and D-FDLA) derivatives was carried out using an *Agilent Eclipse XDB-C18* column (4.6 × 150 mm, 5 µm) maintained at 40°. Aq. MeCN containing 0.1% HCOOH was used as a mobile phase with a linear gradient elution mode (MeCN 30 – 50% for 50 min) at a flow rate of 1.0 ml/min. A *Waters Xevo TQ-S* mass spectrometer (*Waters*, Milford, MA, USA) was used for detection in ESI (negative) mode. The capillary voltage was kept at 2.5 kV, and the ion source at 350°. N_2 was used as a sheath gas at 400 l/h. A mass range of m/z 100 – 1000 was scanned in 0.2 s. The absolute configurations of amino acids in compound **2** were identified on the basis of the same method as mentioned above. The retention times (t_R, min) of the *Marfey* derivatized amino acids were summarized in *Tables 2* and *3*.

Table 2. Analysis of FDLA Derivates of Acid Hydrolysate of Compound **1**

Amino acid	L-FDLA Derivatives		L,D-FDLA Derivatives			Absolute configuration
	t_R [min]	[M – H]$^-$	t_{R1} [min]	t_{R2} [min]	[M – H]$^-$	
Ile	2.12	424	2.09	11.79	424	L
Val	5.89	410	5.90	11.31	410	L
Leu	7.68	424	7.80	17.10	424	L

Table 3. Analysis of FDLA Derivates of Acid Hydrolysate of Compound **2**

Amino acid	L-FDLA Derivatives		L,D-FDLA Derivatives			Absolute configuration
	t_R [min]	$[M - H]^-$	t_{R1} [min]	t_{R2} [min]	$[M - H]^-$	
Ala	4.51	382	4.57	5.99	382	L
Leu	7.66	424	7.70	17.09	424	L

Antimicrobial Assays

Antimicrobial assays were carried out in 96-well sterilized microplates using a microdilution method. Briefly, 4-day-old spores from *Monilia albican* were grown on PDB medium (potato 200 g, glucose 20 g, distilled H_2O 1000 ml), and the test concentration was 1×10^3 spores/ml. The 18-h-old bacterial cultures from *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Mycobacterium tuberculosis* were grown on LB medium (yeast extract 5 g, tryptone 10 g, NaCl 10 g, distilled H_2O 1000 ml, pH 7.0) to reach 1×10^5 colony-forming units per milliliter. The test samples were dissolved in DMSO, and their final concentrations ranged from 512 to 1 μg/ml by using a twofold serial dilution method. The final concentration of DMSO did not exceed 5%. The wells containing test strains and diluted samples were incubated at 28° (4 days) for fungi and 37° (24 h) for bacteria. The wells containing a culture suspension and DMSO were run as negative controls. As a positive control, nystatin (*Taicheng Pharmaceutical Co., Ltd*., Guangdong, China) had antifungal activity against *M. albican* with a *MIC* of 8 μg/ml, kanamycin (*Yunke Biotechnology*, Kunming, China) showed antibacterial activity against *E. coli*, *S. aureus*, *B. subtilis*, and *M. tuberculosis* with *MIC*s of 4, 8, 8, and 16 μg/ml, respectively. All experiments were repeated three times. The growth of test strains was observed with a *CX21BIM-set5* microscope (*Olympus Corp.*, Tokyo, Japan). *MIC*s were determined as the lowest concentrations that produce complete growth inhibition of the tested microorganisms. Compounds **1** – **17** did not show antimicrobial activities against *E. coli*, *S. aureus*, *M. albican*, and *B. subtilis*, *M. tuberculosis* with the *MIC*s > 100 μg/ml.

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