BACITHROCINS A, B AND C, NOVEL THROMBIN INHIBITORS

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Novel thrombin inhibitors, bacithrocins A, B and C, have been isolated from the culture broth of *Bacillus laterosporus* Laubach NR 2988. The structures of these inhibitors have been determined to be *N*-acyl-L-phenylalanyl-DL-arginal by the 2D-NMR experiments on their oxidation products and by amino acid analysis. Bacithrocin A inhibits thrombin, factor Xa and trypsin with IC_{50}s of 48, 13 and 0.65 μM, respectively, which are similar to those of bacithrocins B and C. Bacithrocins prolong the clotting time induced by thrombin and factor Xa.

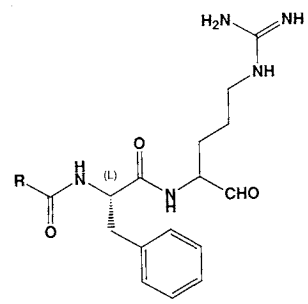
Thrombin, one of the serine proteases, plays a key role in blood coagulation. It converts fibrinogen into fibrin and activates the cofactors V and VIII, and also induces platelet aggregation itself. Therefore, a specific inhibitor of thrombin can be an effective anticoagulant agent for the treatment of diseases caused by an aberration of this enzyme activity. In our screening program for thrombin inhibitors, we isolated novel thrombin inhibitors, bacithrocins A (**1**), B (**2**) and C (**3**) from the culture broth of a strain, NR 2988 (Fig. 1). The strain was isolated from a soil sample collected at Iruma, Saitama Prefecture, Japan and was identified as *Bacillus laterosporus* Laubach based on its morphological and physiological properties[1]. Another component, bacithrocin D (**4**), which was characterized and identified as thiolstatin D[2,3] was also isolated with these inhibitors. In the present paper, we describe the fermentation, isolation, structural elucidation and biological activity of these four inhibitors.

Results

Fermentation

The stock culture of *B. laterosporus* NR 2988, stored at −80°C, was thawed, and 200 μl of the culture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a seed medium consisting of glucose 2.0%, dextrin 2.0%, S-III meat (Ajinomoto Co. Inc.) 1.5%, Pharmamedia (Traders Protein) 1.5%, K_2HPO_4 0.06% and KH_2PO_4 0.025%. The flask was agitated at 190 rpm on a rotary shaker at 27°C for 1 day. Then, 30 ml of the culture was transferred to a 50-liter jar fermentor containing 30 liters of production medium having the same composition as the seed medium. The fermentation was carried out at 27°C for 3 days with aeration of 30 liters/minute and with agitation of 300 rpm.

Fig. 1. Structures of bacithrocins A (**1**), B (**2**), C (**3**) and D (**4**).

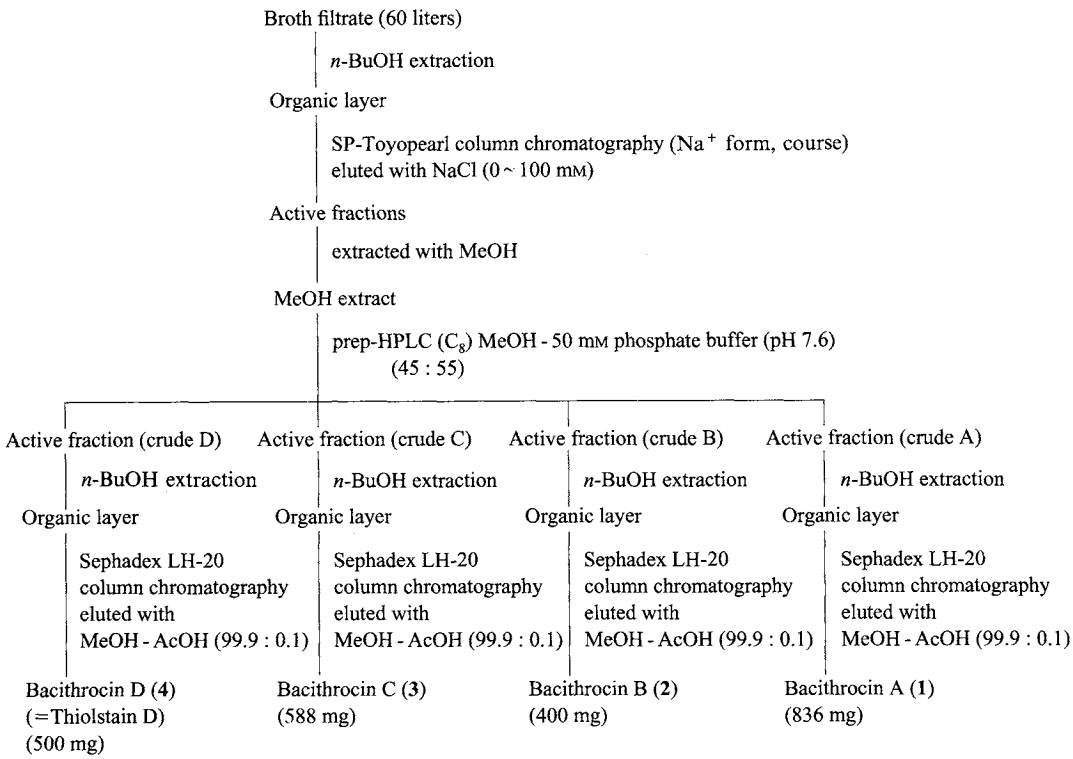


Bacithrocin A (**1**) R = $CH_2CH(CH_3)_2$
Bacithrocin B (**2**) R = $CH(CH_3)_2$
Bacithrocin C (**3**) R = CH_2CH_3
Bacithrocin D (**4**) R = CH_3
(= Thiolstatin D)

Isolation

Isolation of bacithrocins was carried out by

Fig. 2. Isolation procedure of bacithrocins.



monitoring the inhibitory activity against thrombin. The isolation procedure is outlined in Fig. 2. Bacithrocins A (836 mg), B (400 mg), C (588 mg) and D (500 mg) were isolated as colorless powders from the broth filtrate (60 liters) by *n*-butanol extraction followed by chromatography with SP-Toyopearl and Sephadex LH-20. Details of the isolation procedure are described in the experimental section.

Physico-chemical Properties

The physico-chemical properties of **1**, **2**, **3** and **4** are summarized in Table 1. The molecular formulae of **1**, **2**, **3** and **4** were determined to be $C_{20}H_{31}N_5O_3$, $C_{19}H_{29}N_5O_3$, $C_{18}H_{27}N_5O_3$ and $C_{17}H_{25}N_5O_3$ respectively, from HRFAB-MS data. The IR spectra (Fig. 3) of bacithrocins suggested that these inhibitors are peptidic compounds and structurally related to thiolstatin D, *N*-acetylphenylalanylarginal[2,3]. Acid hydrolysis of the bacithrocins in 6 N HCl at 120°C for 15 hours followed by TLC and chiral HPLC analysis indicated that the compounds contained L-phenylalanine. GC-MS analysis of the hydrolysates of **1**, **2**, **3** and **4** indicated the presence of isovaleric acid, isobutyric acid, propionic acid and acetic acid, respectively.

Since ^1H and ^{13}C NMR spectra of **1** were very complicated due to its tautomerism, analysis of the NMR spectra was quite difficult. However, a weak signal at δ_H 9.27 suggested the presence of an aldehyde functionality, and that was further supported by a blue coloration in the blue tetrazolium reaction. The presence of a guanidino group was also indicated by an orange coloration in the Sakaguchi reaction. Taking the molecular formula, $C_{20}H_{31}N_5O_3$, into consideration, it is reasonable to assign the structural unit other than the phenylalanine residue (C_9H_9NO) and the isovaleric acid residue (C_5H_9O) to an arginal unit ($C_6H_{13}N_4O$) containing the aldehyde and guanidino groups.

Table 1. Physico-chemical properties of bacithrocins A (**1**), B (**2**), C (**3**) and D (**4**).

	1	**2**	**3**	**4**
Appearance	Colorless powder	Colorless powder	Colorless powder	Colorless powder
HRFAB-MS (*m/z*) $(M+H)^+$				
Calcd:	390.2506	376.2350	362.2193	348.2037
Found:	390.2512	376.2363	362.2205	348.2047
Molecular formula	$C_{20}H_{31}N_5O_3$	$C_{19}H_{29}N_5O_3$	$C_{18}H_{27}N_5O_3$	$C_{17}H_{25}N_5O_3$
UV $\lambda_{max}^{H_2O}$ nm ($\log\varepsilon$)	252 (2.13), 257 (2.23), 263 (2.24)	252 (2.07), 257 (2.17), 263 (2.09)	252 (2.04), 257 (2.17), 263 (2.04)	252 (2.06), 257 (2.17), 263 (2.02)
$[\alpha]_D^{24}$ (*c* 1.0, H_2O)	+9.4	+11.9	+15.5	+20.5
IR (KBr) cm^{-1}	3400~3200, 1670, 1540	3400~3200, 1670, 1540	3400~3200, 1670, 1540	3400~3200, 1670, 1540
Color reaction				
Positive	Lydon-Smith, Sakaguchi, Blue tetrazolium	Lydon-Smith, Sakaguchi, Blue tetrazolium	Lydon-Smith, Sakaguchi, Blue tetrazolium	Lydon-Smith, Sakaguchi, Blue tetrazolium
Negative	Ninhydrin	Ninhydrin	Ninhydrin	Ninhydrin
Retention time (t_R)*	19.6 minutes	12.6 minutes	9.4 minutes	7.4 minutes

* HPLC conditions: column YMC-pack A-203 (4.6 mm i.d. × 250 mm); carrier MeOH - 50 mM phosphate buffer (pH 7.6) = 45 : 55; flow rate 1.0 ml/minute; detection UV 210 nm.

Fig. 3. IR spectra (KBr) of bacithrocins.

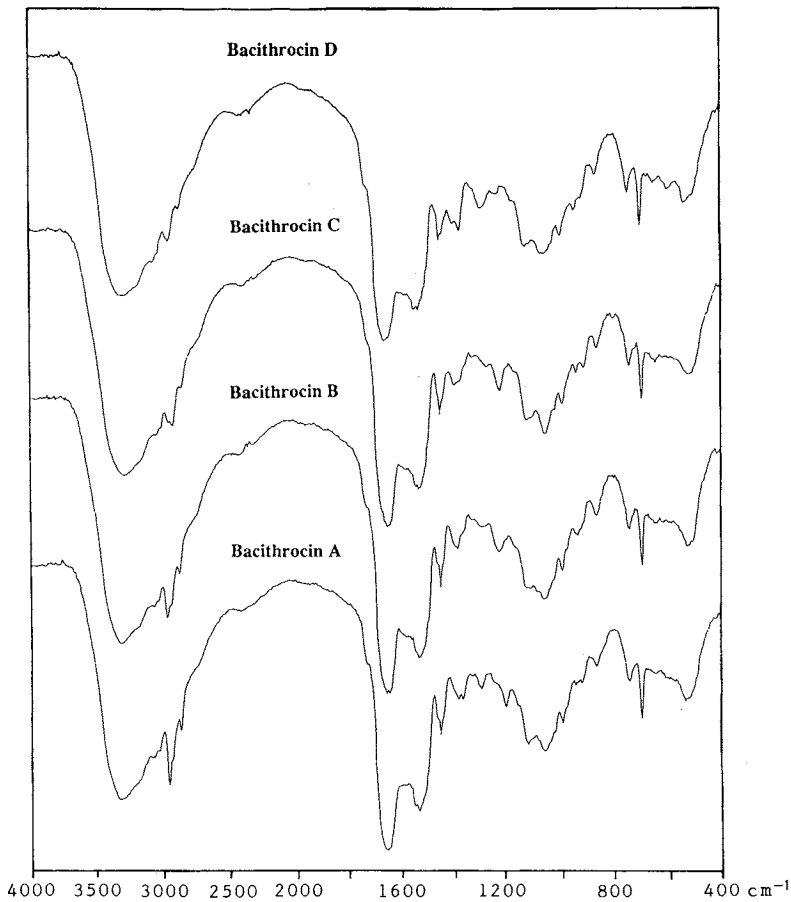
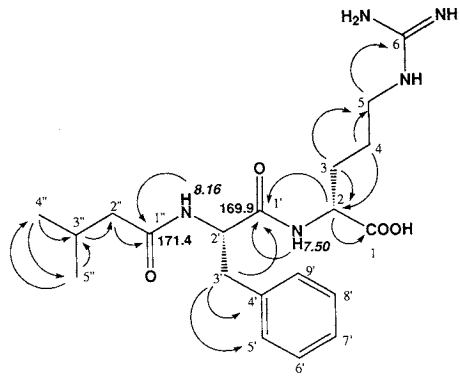


Table 2. NMR spectral data for **1b** in DMSO-d_6.

	^{13}C[a]	^{1}H
Arginine		
1	175.0	—
2	53.5	3.89 (1H, br q, J=6.4 Hz)
3	24.9	1.57 (2H, m)
4	29.6	1.34 (2H, m)
5	40.4	3.00 (2H, t, J=7.0 Hz)
6	157.3	—
2-NH		7.50 (1H, d, J=6.8 Hz)
Phenylalanine		
1′	169.9	—
2′	54.3	4.48 (1H, m)
3′	37.5	a 2.70 (1H, dd, J=10.4, 13.6 Hz)
		b 3.10 (1H, dd, J=4.8, 13.6 Hz)
4′	138.4	—
5′, 9′	129.1	7.23 (2H)
6′, 8′	127.9	7.23 (2H)
7′	126.0	7.18 (1H, m)
2′-NH		8.16 (1H, br)
Isovaleric acid		
1″	171.4	—
2″	44.5	1.88 (2H)
3″	25.4	1.88 (1H)
4″	22.1	0.68 (3H, d, J=6.4 Hz)
5″	22.2	0.75 (3H, d, J=6.4 Hz)

[a] Assigned by DEPT and HMBC experiments.

Fig. 4. ^{13}C-^{1}H long range couplings (arrows) obtained from the HMBC experiments on **1b** (partial, in DMSO-d_6).



1b

Since an aldehyde proton signal was observed at δ_H 9.27 in the ^{1}H NMR spectrum of **1** and its intensity was weak, the aldehyde functionality was suggested to be involved in the tautomerism similar to the case with leupeptin[4,5]. In order to avoid this tautomerism, the aldehyde was oxidized to a carboxylic acid. Bacithrocin A was treated with platinum black under O_2 at pH 7.4 to give the oxidation products, **1a** ($C_{20}H_{31}N_5O_4$) and **1b** ($C_{20}H_{31}N_5O_4$). The TLC and chiral HPLC analysis of the hydrolysate of **1a** and **1b** indicated that **1a** and **1b** possessed L- and D-arginine residues, respectively, in addition to L-phenylalanine. This result suggested that the *C*-terminal end of **1** was a mixture of L- and D-arginal residues.

The structural elucidation of **1b** was accomplished by the NMR experiments. The ^{1}H and ^{13}C NMR spectral data of **1b** are summarized in Table 2. The ^{1}H signals were assigned by homo decoupling and COSY experiments. The sequence of isovaleric acid and amino acid residues was determined based on the following ^{13}C-^{1}H long range couplings obtained by an HMBC experiment on **1b**: between 2-H (δ_H 3.98) and C-1′ (δ_C 169.9) and between 2-NH (δ_H 7.50) and C-1′, and also between 2′-NH (δ_H 8.16) and C-1″ (δ_C 171.4), as shown in Fig. 4. Thus, the structure of **1b** was determined to be *N*-isovaleryl-L-phenylalanyl-D-arginine.

In a similar manner, the structure of **1a** was determined to be *N*-isovaleryl-L-phenylalanyl-L-arginine. The structure of **1a** was also confirmed by comparing its physico-chemical data with those of the corresponding synthesized sample (see experimental section). Therefore, the structure of **1** was deduced to be *N*-isovaleryl-L-phenylalanyl-DL-arginal (Fig. 1).

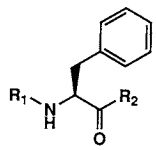
Structural Elucidation of **2**, **3** and **4**

The structures of **2**, **3** and **4** were also determined based on the structural elucidation of their oxidation products. The oxidation of **2**, **3** and **4** gave **2a** and **2b**, **3a** and **3b** and **4a** and **4b**, respectively (Fig. 5). The TLC and chiral HPLC analysis of the hydrolysate of these oxidation products indicated that the compounds

possessed L- or D-arginine and L-phenylalanine, whereas **2**, **3** and **4** possessed only L-phenylalanine. This result indicated that the *C*-terminal ends of **2**, **3** and **4** were also L- and D-arginal. Since their ^{1}H NMR spectral data were quite similar to those of **1a** and **1b** except for the signals assigned to the carboxylic acid residues and hydrolysis of **2**, **3** and **4** gave isobutyric acid, propionic acid and acetic acid, respectively, the structures of **2a**, **2b**, **3a**, **3b**, **4a** and **4b** were suggested as shown in Fig. 5. To confirm the structures, we synthesized *N*-isobutyryl-L-phenylalanyl-L-arginine, *N*-propionyl-L-phenylalanyl-L-arginine and *N*-acetyl-L-phenylalanyl-L-arginine and compared their physico-chemical data with those of the oxidation products **2a**, **3a** and **4a**. The physico-chemical properties of **2a**, **3a** and **4a** were identical with those of the synthesized authentic samples. Therefore, the structures of **2**, **3** and **4** were deduced to be *N*-isobutyryl-L-phenylalanyl-DL-arginal, *N*-propionyl-L-phenylalanyl-DL-arginal and *N*-acetyl-L-phenylalanyl-DL-arginal, respectively (Fig. 1).

The structures of **1**, **2**, **3** and **4** were further confirmed by the analysis of their FAB-MS data. Common fragment peaks attributable to the arginal and phenylalanine residues were observed at *m/z* 185, 142 and 120 in the FAB-MS of **1**, **2**, **3** and **4** (Fig. 6). Successive fragmentations caused by the successive loss of amino acids from the *C*-terminal end revealed the order of the linkages and also the alteration of carboxylic acids (Fig. 6). Therefore, the structures of bacithrocins were unambiguously established, as shown in Fig. 1, and bacithrocin D was revealed to be identical to thiolstatin D[2,3]. Bacithrocins and leupeptin are characterized by the tautomerism caused by having an arginal moiety in the molecule. Thiolstatin D was reported as a single isomer, but it is likely that thiolstatin D exists in solution as a mixture of tautomers as do the bacithrocins.

Fig. 5. Structures of oxidation products of bacithrocins.



Compound	R_1	R_2
1a	$CH_2CH(CH_3)_2$	L-Arg
1b	$CH_2CH(CH_3)_2$	D-Arg
2a	$CH(CH_3)_2$	L-Arg
2b	$CH(CH_3)_2$	D-Arg
3a	CH_2CH_3	L-Arg
3b	CH_2CH_3	D-Arg
4a	CH_3	L-Arg
4b	CH_3	D-Arg

Fig. 6. FAB-MS fragmention of bacithrocins.

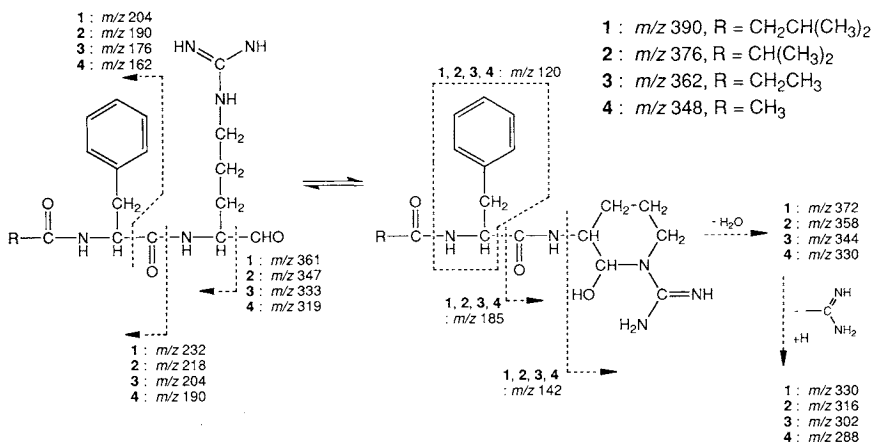


Table 3. Biological activity of bacithrocins.

	Enzyme inhibition (IC_{50}: μM)				Clotting time (IC_{50}: μM)*	
	Thrombin	Factor Xa	Trypsin	Papain	Thrombin	Factor Xa
Bacithrocin A (**1**)	48	13	0.65	0.02	360	77
Bacithrocin B (**2**)	84	17	1.7	0.02	590	150
Bacithrocin C (**3**)	80	15	1.3	0.02	550	120
Bacithrocin D (**4**)	124	9	0.85	0.01	>690	120
Antipain	13	5.1	0.06	0.05	74	15
Leupeptin	16	7.9	0.05	0.01	49	18

* IC_{50} value indicates the concentration of the compound leading to a clotting time twice that of the control.

Biological Activity

The inhibitory activities of the bacithrocins against three serine proteases, thrombin, factor Xa and trypsin, and a thiol protease, papain, are shown in Table 3. The effects of bacithrocins on prolonging clotting time are also characterized by the IC_{50} value (μM) indicating the concentration of the compound leading to a clotting time twice that of the control (Table 3). It is well known that the factor Xa-induced plasma clotting involves enzymatic reactions of both thrombin and factor Xa. The result that bacithrocins show a more potent prolongation effect on the factor Xa-induced clotting time than on the thrombin-induced clotting time may be attributable to the stronger inhibitory action against factor Xa than against thrombin.

Discussion

Bacithrocins A, B and C are novel thrombin inhibitors produced by *Bacillus laterosporus*. The structural studies revealed that bacithrocins share the same units of L-phenylalanine and DL-arginal, and differ from each other in the type of *N*-acyl unit at the *N*-terminal end. Bacithrocins are active against thrombin, factor Xa, trypsin and papain, and prolong the clotting time induced by thrombin and factor Xa.

MURAO *et al.* previously reported closely related compounds, thiolstatins A, B, C and D produced by *Bacillus cereus*[2,3], and thiolstatin D was obtained together with bacithrocins. However, bacithrocins A, B and C are completely different from thiolstatins A, B and C. Thiolstatin A, B, C and D contain *N*-acetyl unit at the *N*-terminal end[3]. On the other hand, bacithrocins A, B and C contain *N*-isovaleryl, *N*-isobutyryl and *N*-propionyl units, respectively. *B. laterosporus*, the producing organism of bacithrocins, is readily distinguished from *B. cereus* morphologically. Namely *B. laterosporus* has a canoe-shaped body attached to the side of the spore while B. cereus doesn't have. From these reasons, it was concluded that bacithrocins are novel thrombin inhibitors.

Experimental

General Procedures

UV spectra were recorded on a KONTRON Uvikon 860 UV spectrometer, and IR spectra on a Hitachi 270-30 or Perkin Elmer 1600 IR spectrometer. FAB-MS, HRFAB-MS and GC-MS were measured on JEOL JMS-DX303, JMS-SX102 and JMS-AX505 mass spectrometers, respectively. ^1H and ^{13}C NMR were recorded on a JMN-GSX-400 NMR spectrometer at 400 and 100 MHz, respectively, using TMS as an internal standard. Optical rotations were measured on a JASCO DIP-140 digital polarimeter.

Isolation of Bacithrocins

In the following procedure, the fractions were monitored by the thrombin inhibitory activities measured. The broth filtrate (60 liters) was adjusted to pH 8.0 with 6N NaOH and extracted with *n*-butanol. The organic layer was concentrated under reduced pressure. The residue was dissolved in H_2O (5 liters) and

then applied to a SP-Toyopearl (Na^+ form, course grade) column (7 × 130 cm) and the column was washed with H_2O (20 liters) and with 50 mM NaCl solution (25 liters), and then eluted with 100 mM NaCl solutions (50 liters). The active fractions (100 mM NaCl fractions) were collected, adjusted to pH 7.0, concentrated and lyophilized. The residual powder was suspended in methanol and stirred for 30 minutes. Insoluble substances were removed by filtration, and the filtrate was concentrated under reduced pressure to give the crude mixture of bacithrocins. Then, these inhibitors were separated by preparative HPLC over a C_8 reversed-phase column of YMC pack SH-263-10 (30 mm i.d. × 250 mm) using methanol - 50 mM phosphate buffer, pH 7.6 (45 : 55). Each fraction was evaporated to remove the methanol and extracted with *n*-butanol at pH 8.0. The *n*-butanol extracts were concentrated to dryness. The crude bacithrocins A, B, C and D were separately dissolved in 5 ml of methanol and then applied to a Sephadex LH-20 column (2 × 140 cm) eluted with methanol - acetic acid (99.9 : 0.1). The active fractions were collected, concentrated under reduced pressure and lyophilized to give **1** (836 mg), **2** (400 mg), **3** (588 mg) and **4** (500 mg) as white powders.

Acid Hydrolysis of Bacithrocins and Their Derivatives

A solution of the sample (3 mg) in 6 N HCl (1 ml) was stirred at 120°C for 15 hours. The reaction mixture was evaporated to dryness. The residue was dissolved in H_2O (4 ml) and $CHCl_3$ (4 ml). The aqueous layer was concentrated and applied to the TLC and chiral HPLC analysis for the determination of amino acid composition and its chirality. The organic layer was concentrated and applied to the GC-MS analysis for the determination of carboxylic acid residue. The following conditions were used. TLC analysis: plate, silica gel (Merck, Kieselgel $60F_{254}$); solvent, *n*-butanol - acetic acid - H_2O (4 : 1 : 2); detection, ninhydrin. Chiral HPLC analysis: column, TSK-GEL Enantio L-1 (4.6 mm i.d. × 250 mm); solvent, 1 mM copper sulphate; flow rate, 1.0 ml/minute; detection, UV 230 nm. GC-MS analysis: column, Unisole 30 T 3% (3 mm i.d. × 1 m); column temperature, 80 ~ 170°C (10°C/minute); carrier gas, helium; flow rate, 20 ml/minute; ionization mode, EI (positive); ionization volt, 30 eV.

Oxidation of **1**

Bacithrocin A (100 mg) was dissolved in 20 ml of 50 mM phosphate buffer (pH 7.4) at room temperature. To this solution was added Pt-black (10 mg) under O_2 with stirring. After stirring the mixture for 2 hours at room temperature, Pt-black was removed by filtration. The filtrate was concentrated under reduced pressure. The concentrate was purified by preparative HPLC over a C_8 reversed-phase silica gel column (YMC pack SH-243-15, 20 mm i.d. × 250 mm: YMC Co., Ltd.) with MeOH - H_2O (35 : 65) at a flow rate of 25 ml/minute to give 20.4 mg of **1a** (retention time, 22.6 minutes) and 24.5 mg of **1b** (retention time, 25.6 minutes) as powders. **1a** amino acid composition: L-phenylalanine and L-arginine; HRFAB-MS *m/z* calcd for $C_{20}H_{32}N_5O_4$: 406.2454, found 406.2455 $(M+H)^+$; IR ν_{max} (KBr): 3500 ~ 3100, 2960, 1650, 1400, 700 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.67 (3H, d, J = 6.4 Hz), 0.75 (3H, d, J = 6.4 Hz), 1.44 (2H, m), 1.63 (2H, m), 1.89 (3H, m), 2.71 (1H, dd, J = 10.4, 14.0 Hz), 3.04 (2H, br t, J = 6.5 Hz), 3.08 (1H, dd, J = 4.0, 14.0 Hz), 3.87 (1H, br q, J = 6.5 Hz), 4.44 (1H, dd, J = 4.0, 10.4 Hz), 7.18 (1H, m), 7.20 (4H, m), 7.49 (1H, d, J = 6.8 Hz), 8.20 (1H, br). **1b** amino acid composition: L-phenylalanine and D-arginine; HRFAB-MS *m/z* calcd for $C_{20}H_{32}N_5O_4$: 406.2454, found 406.2459 $(M+H)^+$; IR ν_{max} (KBr): 3500 ~ 3100, 2960, 1650, 1400, 700 cm^{-1}; ^1H and ^{13}C NMR spectral data are shown in Table 2.

Oxidation of **2**

Bacithrocin B (100 mg) was treated with Pt-black under the same conditions for **1**. After the removal of Pt-black, the reaction mixture was concentrated under reduced pressure. The concentrate was purified by preparative HPLC over a C_8 reversed-phase silica gel column with MeOH - H_2O (20 : 80) at a flow rate of 30 ml/minute to give 12.1 mg of **2a** (retention time, 37.4 minutes) and 17.4 mg of **2b** (retention time, 44.2 minutes) as powders. **2a** amino acid composition: L-phenylalanine and L-arginine; HRFAB-MS *m/z* calcd for $C_{19}H_{30}N_5O_4$: 392.2298, found 392.2295 $(M+H)^+$; IR ν_{max} (KBr) : 3500 ~ 3100, 2960, 1650, 1400, 700 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.80 (3H, d, J = 6.4 Hz), 0.94 (3H, d, J = 6.4 Hz), 1.44 (2H, m), 1.64 (2H, m), 2.34 (1H, seven-lines, J = 6.4 Hz), 2.75 (1H, dd, J = 10.8, 14.0 Hz), 3.06 (2H, br t, J = 7 Hz), 3.10 (1H, dd, J = 3.6, 14.0 Hz), 3.86 (1H, q, J = 6.0 Hz), 4.38 (1H, m), 7.18 (1H, m), 7.23 (4H, m), 7.48 (1H, d, J = 6.8 Hz), 8.08 (1H, d, J = 8.8 Hz), 9.45 (1H, br). **2b** amino acid composition: L-phenylalanine and D-arginine; HRFAB-MS *m/z* calcd for $C_{19}H_{30}N_5O_4$: 392.2298, found 392.2293

$(M+H)^+$; IR ν_{max} (KBr): 3500~3100, 2960, 1650, 1400, 700 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.80 (3H, d, J=6.4 Hz), 0.94 (3H, d, J=6.4 Hz), 1.35 (2H, m), 1.59 (2H, m), 2.36 (1H, seven-lines, J=6.4 Hz), 2.72 (1H, dd, J=10.4, 14.0 Hz), 3.00 (2H, br t, J=6.8 Hz), 3.06 (1H, dd, J=4.4, 14.0 Hz), 3.89 (1H, q, J=6.0 Hz), 4.43 (1H, m), 7.18 (1H, m), 7.23 (4H, m), 7.52 (1H, d, J=7.2 Hz), 8.11 (1H, d, J=8.8 Hz), 9.34 (1H, br); ^{13}C NMR (100 MHz, DMSO-d_6): 19.2×2, 24.8, 29.5, 33.7, 37.2, 40.3, 53.4, 54.1, 125.9, 127.8×2, 129.0×2, 138.3, 157.2, 169.8, 174.9, 175.9.

Oxidation of **3**

Bacithrocin C (100 mg) was treated with Pt-black under the same conditions for **1**. After the removal of Pt-black, the reaction mixture was concentrated under reduced pressure. The concentrate was purified by preparative HPLC over a C_8 reversed-phase silica gel column with MeOH - H_2O (20 : 80) at a flow rate of 30 ml/minute to give 14.4 mg of **3a** (retention time, 17.6 minutes) and 21.4 mg of **3b** (retention time, 22.2 minutes) as powders. **3a** amino acid composition: L-phenylalanine and L-arginine; HRFAB-MS m/z calcd for $C_{18}H_{28}N_5O_4$: 378.2141, found 378.2147 $(M+H)^+$; IR ν_{max} (KBr): 3500~3000, 1650, 1400, 700 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.88 (3H, t, J=8.0 Hz), 1.43 (2H, m), 1.63 (2H, m), 2.00 (1H, dq, J=16.0, 8.0 Hz), 2.06 (1H, dq, 16.0, 8.0 Hz), 2.73 (1H, dd, J=10.0, 14.0 Hz), 3.05 (2H, br t, J=7 Hz), 3.07 (1H, dd, J=3.6, 14.0 Hz), 3.86 (1H, q, J=6.8 Hz), 4.41 (1H, dd, J=3.6, 10.4 Hz), 7.18 (1H, m), 7.22 (4H, m), 7.51 (1H, d, J=6.4 Hz), 8.10 (1H, br). **3b** amino acid composition: L-phenylalanine and D-arginine; HRFAB-MS m/z calcd for $C_{18}H_{28}N_5O_4$: 378.2141, found 378.2141 $(M+H)^+$; IR ν_{max} (KBr): 3500~3000, 1650, 1400, 700 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 0.89 (3H, t, J=8.0 Hz), 1.32 (2H, m), 1.57 (2H, m), 1.99 (1H, dq, J=16.0, 8.0 Hz), 2.07 (1H, dq, 16.0, 8.0 Hz), 2.71 (1H, dd, J=10.0, 14.0 Hz), 3.00 (2H, br t, J=7 Hz), 3.03 (1H, dd, J=5.2, 14.0 Hz), 3.89 (1H, q, J=6.4 Hz), 4.47 (1H, m), 7.18 (1H, m), 7.22 (4H, m), 7.53 (1H, d, J=6.4 Hz), 8.15 (1H, br); ^{13}C NMR (100 MHz, DMSO-d_6): 9.7, 24.8, 28.3, 29.5, 37.5, 40.3, 53.4, 54.2, 126.0, 128.1×2, 129.0×2, 138.2, 144.2, 157.2, 169.8, 172.8, 175.0.

Oxidation of **4**

Bacithrocin D (100 mg) was treated with Pt-black under the same conditions for **1**. After the removal of Pt-black, the reaction mixture was concentrated under reduced pressure. The concentrate was purified by preparative HPLC over a C_8 reversed-phase silica gel column with MeOH - H_2O (15 : 85) at a flow rate of 30 ml/minute to give 13.5 mg of **4a** (retention time, 14.0 minutes) and 20.1 mg of **4b** (retention time, 19.8 minutes) as powders. **4a** amino acid composition: L-phenylalanine and L-arginine; HRFAB-MS m/z calcd for $C_{17}H_{26}N_5O_4$: 364.1985, found 364.1982 $(M+H)^+$; IR ν_{max} (KBr) : 3500~3000, 1650, 1400, 700 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.44 (2H, m), 1.63 (2H, m), 1.75 (3H, s), 2.72 (1H, dd, J=10.4, 14.0 Hz), 3.03 (3H, m), 3.87 (1H, q, J=6.8 Hz), 4.43 (1H, m), 7.18 (1H, m), 7.23 (4H, m), 7.53 (1H, d, J=7.2 Hz), 8.18 (1H, br d, 8.0 Hz), 9.40 (1H, br). **4b** amino acid composition: L-phenylalanine and D-arginine; HRFAB-MS m/z calcd for $C_{17}H_{26}N_5O_4$: 364.1985, found 364.1980 $(M+H)^+$; IR ν_{max} (KBr): 3500~3000, 1650, 1400, 700 cm^{-1}; ^1H NMR (400 MHz, DMSO-d_6): 1.31 (2H, m), 1.56 (2H, m), 1.76 (3H, s), 2.71 (1H, dd, J=10.4, 14.0 Hz), 3.03 (3H, m), 3.89 (1H, q, J=6.8 Hz), 4.48 (1H, m), 7.18 (1H, m), 7.23 (4H, m), 7.54 (1H, d, J=6.8 Hz), 8.23 (1H, br d, 8.4 Hz), 9.26 (1H, br); ^{13}C NMR (100 MHz, DMSO-d_6): 22.3, 24.8, 29.5, 37.6, 40.3, 53.3, 54.4, 126.2, 127.9×2, 129.2×2, 138.2, 157.2, 169.0, 169.7, 175.0.

Preparation of **1a**, *N*-isovaleryl-L-phenylalanyl-L-arginine

L-phenylalanyl-L-arginine·2TFA·1/2H_2O (20 mg) was dissolved in pyridine (1 ml). To this solution was added 20 μl of isovaleric anhydride with stirring at room temperature. The solution was stirred for 5 hours, and the solvent was evaporated under reduced pressure. The residue was dissolved in 2 ml of 100 mM phosphate buffer (pH 8.0) and purified first by preparative HPLC over a C_{18} reversed-phase silica gel column (YMC R-ODS-10, 4.6 mm i.d. × 250 mm, YMC Co., Ltd.) with 50% aqueous MeOH at a flow rate of 3.0 ml/minute (retention time, 4.1 minutes) and second by preparative HPLC over a C_8 reversed-phase silica gel column (Capcell pak C_8 SG, 10 mm i.d. × 250 mm, Shiseido) with 35% aqueous MeOH at a flow rate of 5.0 ml/minute to give 3.5 mg of *N*-isovaleryl-L-phenylalanyl-L-arginine (retention time, 15.9 minutes).

Preparation of **2a**, *N*-isobutyryl-L-phenylalanyl-L-arginine

L-Phenylalanyl-L-arginine·2TFA·1/2H_2O (20 mg) and isobutyric anhydride (20 μl) were stirred in

pyridine (1 ml) at room temperature for 5 hours. The reaction mixture was purified first by preparative HPLC over a C_{18} reversed-phase silica gel column with 50% aqueous MeOH at a flow rate of 2.0 ml/minute (retention time, 3.8 minutes) and second by preparative HPLC over a C_8 reversed-phase silica gel column with 30% aqueous MeOH at a flow rate of 5.0 ml/minute to give 4.2 mg of *N*-isobutyryl-L-phenylalanyl-L-arginine (retention time, 15.5 minutes).

Preparation of **3a**, *N*-propionyl-L-phenylalanyl-L-arginine

L-Phenylalanyl-L-arginine·2TFA·1/2H_2O (20 mg) and propionic anhydride (20 μl) were stirred in pyridine (1 ml) at room temperature for 5 hours. The reaction mixture was purified first by preparative HPLC over a C_{18} reversed-phase silica gel column with 40% aqueous MeOH at a flow rate of 2.0 ml/minute (retention time, 4.6 minutes) and second by preparative HPLC over a C_8 reversed phased silica gel column with 20% aqueous MeOH at a flow rate of 5.0 ml/minute to give 2.6 mg of *N*-propionyl-L-phenylalanyl-L-arginine (retention time, 10.2 minutes).

Preparation of **4a**, *N*-acetyl-L-phenylalanyl-L-arginine

L-Phenylalanyl-L-arginine·2TFA·1/2H_2O (20 mg) and acetic anhydride (10 μl) were stirred in pyridine (1 ml) at room temperature for 5 hours. The reaction mixture was purified first by preparative HPLC over a C_{18} reversed-phase silica gel column with 40% aqueous MeOH at a flow rate of 2.0 ml/minute (retention time, 3.3 minutes) and second by preparative HPLC over a C_8 reversed-phase silica gel column with 20% aqueous MeOH at a flow rate of 5.0 ml/minute to give 2.9 mg of *N*-propionyl-L-phenylalanyl-L-arginine (retention time, 10.2 minutes).

Enzyme Assays Using Fluorogenic Substrates[6]

The mixture of 195 μl of an enzyme solution (final concentration ; 0.08 u/ml thrombin, 0.015 u/ml factor Xa, 0.03 u/ml trypsin and 0.076 u/ml papain) and an inhibitor solution (50 μl) was incubated at 37°C. After 1 minute, a substrate solution (5 μl) in DMSO (final concentration; 20 μM Boc-Val-Pro-Arg-methylcoumarylamide (MCA) for thrombin, 120 μM Boc-Ile-Glu-Gly-Arg-MCA for factor Xa and 120 μM Bz-Arg-MCA for trypsin and papain) was added. Fluorescence of 7-amino-4-methylcoumarin produced was monitored in 30 seconds intervals for up to 180 seconds using a spectrophotometric centrifugal analyzer (Cobas Bio, Roche Diagnostica Inc.) at 380 nm extention and 460 nm emission wavelength.

Clotting Assays

The two clotting assays (thrombin-induced fibrinogen clotting time, factor Xa-induced clotting time) were performed using a fibrometer (Cobas Fibro, Roche Diagnostica Inc.) as described by H. P. WESSEL *et al.*[7]. Briefly, the time required to form fibrin fiber was measured after 2.5 NIH units of bovine thrombin was added to 1 ng/ml of fibrinogen for thrombin-induced clotting time. For factor Xa-induced clotting time, factor Xa (0.2 mU/ml) was incubated for 2 minutes at 37°C in the presence of 10 mM $CaCl_2$. Clotting time was determined after the addition of mixture of factor Xa-deficient plasma and rabbit brain cephalin (Sigma).

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