STRUCTURES OF NEW PEPTIDE ANTIBIOTICS, PLUSBACINS $A_1 \sim A_4$ AND $B_1 \sim B_4$

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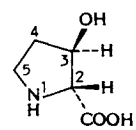
The constituent amino acids of plusbacins $A_1 \sim A_4$ were determined to be two moles of L-*trans*-3-hydroxyproline and one mole each of D-*threo*-β-hydroxyaspartic acid, L-*threo*-β-hydroxyaspartic acid, D-*allo*-threonine, D-serine, D-alanine and L-arginine. In plusbacins $B_1 \sim B_4$, one mole of L-*trans*-3-hydroxyproline is replaced by L-proline. The fatty acid residue of A_1 and B_1 was determined to be 3-hydroxy-tetradecanoic acid, for A_2 and B_2 to be 3-hydroxy-isopentadecanoic acid, for A_3 and B_3 to be 3-hydroxy-isohexadecanoic acid, and for A_4 and B_4 to be 3-hydroxy-hexadecanoic acid. A lactone linkage was suggested to reside between L-*threo*-β-hydroxyaspartic acid and 3-hydroxy-fatty acid residues by degradation experiments. The amino acid sequences of plusbacins A_2 and B_2 were confirmed by Edman degradation of their deacylated products, and supported by mass spectrometric studies. From the above, structures of all components of plusbacins were concluded.

In the preceding paper, the isolation and characterization of new peptide antibiotics, plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$, from a strain of *Pseudomonas* sp. have been reported. From their physico-chemical properties, these antibiotics are presumed to be acyloctapeptides containing a lactone linkage. An unknown amino acid was found in all the components and it could be assumed to be hydroxyproline. Further, it was presumed that the difference between A and B series antibiotics is caused by replacement of the unknown amino acid with proline. The differences among the antibiotics suffixed 1 to 4 in both A and B series occur in their fatty acid residues[1].

In the measurement of NMR spectroscopy with plusbacin A_2, sharp signals could not be observed, probably because of the presence of several conformers in solutions. This phenomenon occurred even in the alkali-treated plusbacin A_2, which lacked a lactone linkage. Therefore, we gave up structural analysis by NMR spectroscopy.

The unknown amino acid was isolated from the acid hydrolysate of plusbacin A_2. From the properties, the amino acid was identified with L-*trans*-3-hydroxyproline (Fig. 1). HR-LSI-MS; *m/z* 132.0661 (MH^+, Δ 0.1 mmu), molecular formula; $C_5H_9NO_3$. CD $[\theta]_{240}$ 0, $[\theta]_{220}$ +2,880, $[\theta]_{210}$ +5,380 (*c* 0.054, 0.5 N HCl). ^{1}H NMR (200 MHz, D_2O, DSS as an internal reference); δ 1.93~2.03 (m, 2H, H-4), 3.40~3.58 (m, 2H, H-5), 4.01 (d, J=1.57 Hz, H, H-2), 4.60~4.64 (m, H, H-3). ^{13}C NMR (36.5 MHz, D_2O, CH_3CN as an internal reference); δ 68.2 (d, C-2), 73.1 (d, C-3), 30.6 (t, C-4), 43.4 (t, C-5), 171.2 (s, C=O).

Fig. 1. L-*trans*-3-Hydroxyproline.



The L-configuration was deduced from the plus Cotton effect at 220 nm in the CD spectrum[2] and the *trans* relationship between H-2 and H-3 from their coupling constant[3].

Table 1. Amino acid analyses of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$.

Plusbacin	Found (μmoles/mg)						
	HyAsp	HyPro	aThr[a]	Ser	Pro	Ala	Arg
A_1	1.50	1.34	0.69	0.66	0.00	0.75	0.75
	(2)	(2)	(1)	(1)		(1)	(1)
A_2	1.58	1.55	0.71	0.71	0.00	0.77	0.77
	(2)	(2)	(1)	(1)		(1)	(1)
A_3	1.54	1.55	0.70	0.71	0.00	0.77	0.77
	(2)	(2)	(1)	(1)		(1)	(1)
A_4	1.45	1.38	0.66	0.64	0.00	0.71	0.71
	(2)	(2)	(1)	(1)		(1)	(1)
B_1	1.69	0.76	0.79	0.76	0.83	0.87	0.86
	(2)	(1)	(1)	(1)	(1)	(1)	(1)
B_2	1.54	0.71	0.70	0.69	0.74	0.77	0.75
	(2)	(1)	(1)	(1)	(1)	(1)	(1)
B_3	1.47	0.62	0.67	0.65	0.71	0.75	0.72
	(2)	(1)	(1)	(1)	(1)	(1)	(1)
B_4	1.49	0.67	0.68	0.66	0.70	0.76	0.74
	(2)	(1)	(1)	(1)	(1)	(1)	(1)

HyAsp; β-hydroxyaspartic acid, HyPro; L-*trans*-3-hydroxyproline, aThr; *allo*-threonine.
[a] Calculation is based on Thr as a standard.

All of the constituent amino acids of plusbacins were re-examined by amino acid autoanalysis using the above-prepared specimen of L-*trans*-3-hydroxyproline as a reference. The results are shown in Table 1.

The chiralities of amino acids other than L-*trans*-3-hydroxyproline were determined by comparison with reference amino acids in HPLC using a chiral column, MIC GEL CRS-10W (Mitsubishi Kasei Co., Ltd.)[4]. The retention volumes of reference amino acids used in the experiment are listed in Table 2. Consequently, β-hydroxyaspartic acids were determined to be one mole each of D-*threo* and L-*threo* forms, threonine to be D-*allo* form, serine to be D-form, proline to be L-form, alanine to be D-form, and arginine to be L-form.

Table 2. Retention volumes of amino acids used as references.

Amino acid	Mobile phase (flow rate in ml/minute)	Retention volume (ml)
D-Alanine	I (0.45)	3.6
L-Alanine		5.0
D-Threonine	I (0.90)	3.3
L-Threonine		4.9
D-*allo*-Threonine		5.6
L-*allo*-Threonine		8.2
D-Serine	I (0.45)	3.7
L-Serine		4.5
D-Arginine	I (0.45)	1.8
L-Arginine		3.0
D-Proline	II (0.90)	3.2
L-Proline		5.7
D-*threo*-β-Hydroxyaspartic acid	III (0.90)	8.2
L-*threo*-β-Hydroxyaspartic acid		11.9
D-*erythro*-β-Hydroxyaspartic acid		10.7
L-*erythro*-β-Hydroxyaspartic acid		16.4

Column; MIC GEL CRS-10W (5.4 × 50 mm). Mobile phase; I. 0.1 M $CuSO_4$, II. 1.0 M $CuSO_4$, III. CH_3CN-2 mM $CuSO_4$ (4:96). Detection; OD at 254 nm.

Thus the identity and stereochemistry of all the amino acids were determined. Plusbacins $A_1 \sim A_4$ contain two moles of L-*trans*-3-hydroxyproline (L-HyPro) and one mole each of D-*threo*-β-hydroxyaspartic acid (D-HyAsp), L-*threo*-β-hydroxyaspartic acid (L-HyAsp), D-*allo*-threonine (D-aThr), D-serine (D-Ser), D-alanine (D-Ala) and L-arginine (L-Arg). In plusbacins $B_1 \sim B_4$, one mole of L-*trans*-3-hydroxyproline is replaced by L-proline (L-Pro).

From the previous experiments by gas-liquid chromatography, it has been already reported that the

fatty acid residues of plusbacins A_1 and B_1 are the same one, and it is presumed to be 3-hydroxy-tetradecanoic acid by comparison of retention times of the methyl esters with reference fatty acid methyl esters. Similarly, the fatty acid residue of A_2 and B_2 is presumed to be 3-hydroxy-isopentadecanoic acid, that of A_3 and B_3 to be 3-hydroxy-isohexadecanoic acid, and that of A_4 and B_4 to be 3-hydroxy-hexadecanoic acid[1].

The fatty acid residue of plusbacin A_2, a main component of the complex, was isolated from the hydrolysate with constant boiling HCl at 110°C for 3 hours. The results of LSI-MS and ^1H NMR verified the above presumed structure.

Plusbacins $A_1 \sim A_4$ were hydrolyzed with constant boiling HCl at 110°C for 1 hour. The fatty acids liberated were extracted from the hydrolysates and methylated to their methyl esters, which were then analyzed by GC-MS. A base peak at m/z, 103, which is caused by β, γ fragmentation characteristic for 3-hydroxy-fatty acid methyl esters[5], and peaks corresponding to $(M-H_2O)$ were observed with the above four esters.

Plusbacins $A_1 \sim A_4$ were also hydrolyzed for 24 hours. The longer hydrolysis time should produce α,β-unsaturated fatty acids from destruction of 3-hydroxy-fatty acids[5]. These fatty acids were extracted, methylated, hydrogenated and then subjected to gas-liquid chromatography. By comparison of retention times with reference fatty acid esters, the saturated fatty acid derived from plusbacin A_1 directly coincided with an authentic specimen of tetradecanoic acid, that from A_2 with isopentadecanoic acid, that from A_3 with isohexadecanoic acid and that from A_4 with hexadecanoic acid. These data confirmed the tentative identification of the fatty acid residues of plusbacins mentioned in the preceding paper[1].

The IR spectra of plusbacins A_2 and B_2 suggested the presence of a lactone linkage[1]. By treatment with dil NaOH, plusbacin A_2 gave a product, which lacked an absorption at 1735 cm^{-1}, indicating it to be a lactone ring-opened product.

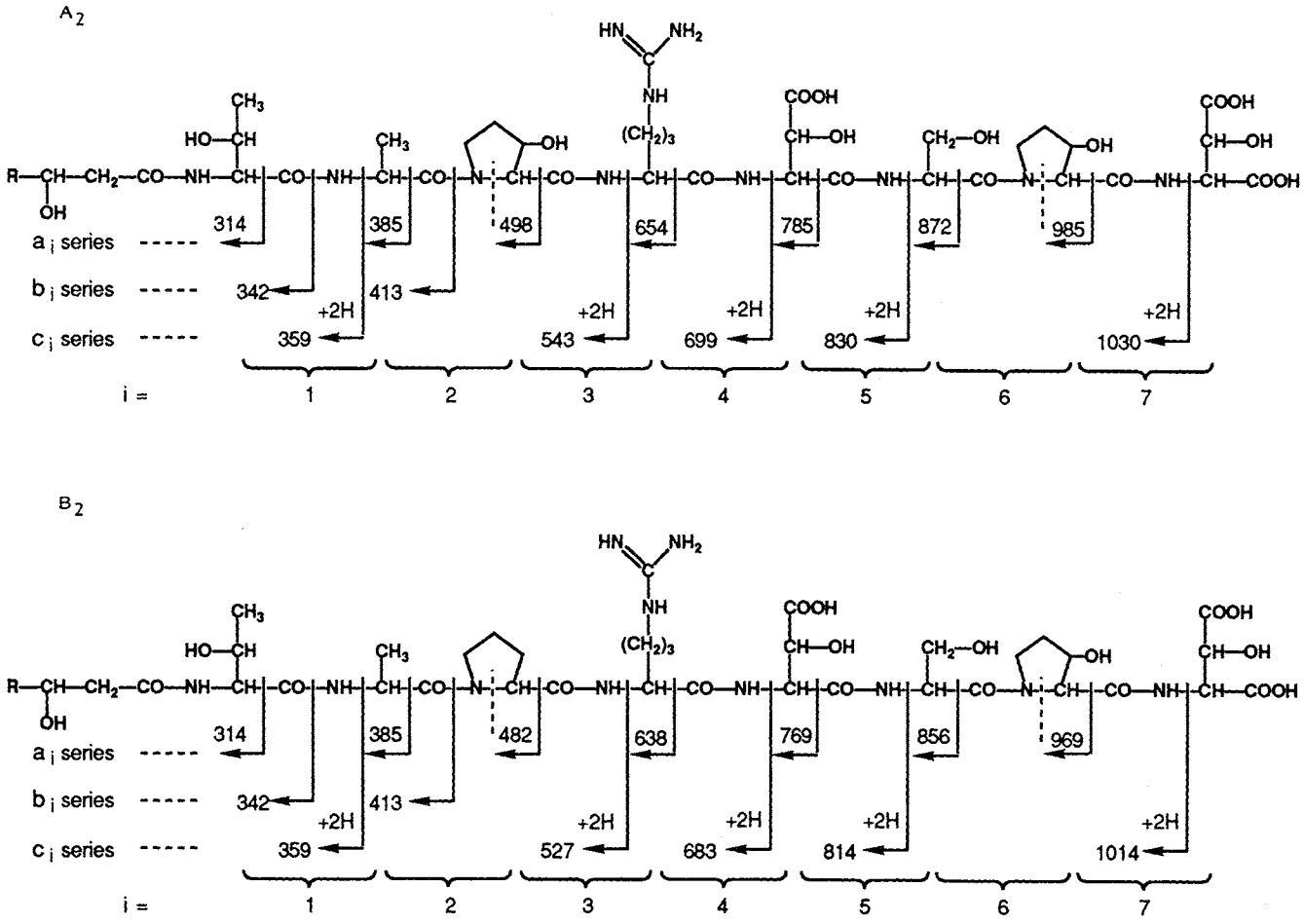
When plusbacin A_2 and alkali-treated plusbacin A_2 were oxidized with chromic acid, and the products were hydrolyzed and subjected to analyses for their constituent amino acids, disappearance of all β-hydroxyamino acids, *i.e.*, D-HyAsp, L-HyAsp, D-aThr and D-Ser, was observed in both specimens. This implied that the hydroxy group involved in the lactone linkage is not any of the β-hydroxyamino acids, but that of the 3-hydroxy-fatty acid.

Table 3. Edman degradation of deacylated plusbacin A_2.

	PTH-amino acid	Amino acid found (in ratio)					
		HyAsp	HyPro	aThr[a]	Ser	Ala	Arg
Original peptide		1.68	1.35	1.03	1.00	1.13	1.21
Step 1	aThr[b], ΔThr	1.80	1.05	0.03	1.00	1.14	1.25
Step 2	Ala	1.79	1.25	0.02	1.00	0.11	1.21
Step 3	HyPro	1.89	0.65	0.02	1.00	0.08	1.14
Step 4	Arg	1.83	0.66	0.02	1.00	0.07	0.39
Step 5	HyAsp	1.38	0.54	0.02	1.00	0.06	0.18
		(1.00	0.39	0.01	0.72	0.05	0.13)
Step 6	Ser	1.00	0.43	0.02	0.31	0.06	0.19
Step 7	HyPro	1.00[c]	0.13	0.02	0.21	0.05	0.10

[a] Calculation is based on Thr as a standard.
[b] PTH-Thr was used as a reference for the identification.
[c] When analyzed before hydrolysis, only HyAsp was detected.

Fig. 2. Fragment ions of alkali-treated plusbacin A_2 and B_2.



When plusbacin A_2 and alkali-treated plusbacin A_2 were reduced with lithium borohydride and the reduced products were hydrolyzed and analyzed for their amino acids, the relative content of HyAsp in plusbacin A_2 was reduced approximately to a half, whereas no change occurred in alkali-treated plusbacin A_2. This meant that the carboxyl group of D- or L-HyAsp residue was involved in the lactone linkage.

Alkali-treated plusbacin A_2 was deacylated with polymyxin acylase[6] to a deacylated product (tentatively called deacylated plusbacin A_2) in good yield. Edman degradation on deacylated plusbacin A_2 proceeded to the *C*-terminus. The result (Table 3) indicated the amino acid sequence as below.

aThr-Ala-HyPro-Arg-HyAsp-Ser-HyPro-HyAsp

The stereochemistry of the *C*-terminal HyAsp, the remaining amino acid after 7th step of the Edman degradation, was confirmed to be L-*threo* form by the HPLC method described previously.

Examination of deacylated plusbacin B_2 by an automatic amino acid sequencer disclosed that the 3rd HyPro residue in A_2 was replaced by Pro in B_2.

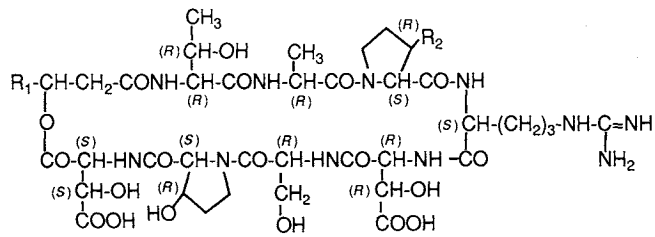
These amino acid sequences were also supported by analyses of mass fragmentation in LSI-MS with alkali-treated plusbacins A_2 and B_2 as shown in Fig. 2. All the fragment ions of a_i series due to C–CO bond cleavage were observed. While N–C bond cleavage caused the fragment ions of c_i series which appeared, it did not cause fragmentation at HyPro and Pro residues. Further in LSI-MS with all components of plusbacins, fragment ion peaks of MH − 74 and MH − 131 were observed. These fragment ions were considered to be caused by breaking away of [CH(OH)–COOH − H] and CO–CH(NH)–CH(OH)–COOH from $(M+H)^+$, respectively, supporting the lactone structure linked by α-carboxyl group of HyAsp residue.

Thus, the structures of plusbacins A_2 and B_2 were determined without any ambiguity. From their amino acid and fatty acid constitutions, we proposed the structures of all the components of plusbacins as shown in Fig. 3.

Experimental

The IR spectra were measured with a Jasco DS-403G spectrometer. CD curves were recorded with a Jasco J-40C automatic recording spectropolarimeter, ^{1}H NMR spectra with a Varian VXR-200 spectrometer. LSI-MS and HR-LSI-MS were observed with a Hitachi M-90 mass spectrometer. Amino acid

Fig. 3. Structures of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$.



A_1	$R_1 = CH_3-(CH_2)_{10}-$	$R_2 = -OH$
A_2	$R_1 = CH_3-CH(CH_3)-(CH_2)_9-$	$R_2 = -OH$
A_3	$R_1 = CH_3-CH(CH_3)-(CH_2)_{10}-$	$R_2 = -OH$
A_4	$R_1 = CH_3-(CH_2)_{12}-$	$R_2 = -OH$
B_1	$R_1 = CH_3-(CH_2)_{10}-$	$R_2 = -H$
B_2	$R_1 = CH_3-CH(CH_3)-(CH_2)_9-$	$R_2 = -H$
B_3	$R_1 = CH_3-CH(CH_3)-(CH_2)_{10}-$	$R_2 = -H$
B_4	$R_1 = CH_3-(CH_2)_{12}-$	$R_2 = -H$

analysis was carried out with a Hitachi amino acid autoanalyzer 835, gas-liquid chromatography with a Shimadzu gas chromatography GC-7AG and gas chromatography/mass spectrometry with a Hitachi M-68 mass spectrometer. The automated Edman degradation was performed with a model 477A Applied Biosystems gas phase sequencer equipped with a 120A PTH amino acid analyzer.

Identification of L-*trans*-3-Hydroxyproline

Plusbacin A_2 hydrochloride (110 mg) was hydrolyzed with 6 N HCl at 110°C for 3 hours in a vacuum sealed tube. The hydrolysate was extracted with ethyl ether, and the extract was used for analyses of fatty acids as given below. The residual part was concentrated and further hydrolyzed with 6 N HCl at 110°C for 20 hours. The hydrolysate was concentrated and subjected to preparative paper chromatography on a paper (Toyo Roshi No. 51) with *n*-BuOH - AcOH - H_2O (4 : 1 : 2). An unknown amino acid was separated as a zone of Rf 0.34 detectable by ninhydrin coloration (yellow). The amino acid was extracted with slightly acidified aq MeOH. The extract was further purified by paper chromatography on a paper (Toyo Roshi No. 51) with *n*-BuOH - pyridine - AcOH - H_2O (10 : 6 : 1 : 4) and then by TLC on cellulose plates (Avicel SF) with phenol - water (4 : 1). The extract from the plates was diluted with water of pH 2.0 (HCl) and passed through a short column of Dowex 50X2 (H^+), which was then eluted with 0.3 N NH_4OH. The eluate was concentrated and freeze-dried to give a colorless powder (8 mg) of the amino acid.

The physico-chemical properties, which indicated the amino acid to be L-*trans*-3-hydroxyproline, are cited in the text.

Amino Acid Analysis

Hydrochlorides of plusbacins $A_1 \sim A_4$ and $B_1 \sim B_4$ (1 ~ 2 mg) were hydrolyzed with constant boiling HCl at 110°C for 20 hours. The hydrolysates were analyzed by an automatic amino acid analyzer using a synthesized specimen of D,L-*threo*-β-hydroxyaspartic acid and the above-prepared specimen of L-*trans*-3-hydroxyproline as references. The results obtained are shown in Table 1.

Stereochemistries of Constituent Amino Acids

The hydrolysate of plusbacin B_2 hydrochloride (2 mg) by the above conditions was chromatographed on a paper (Toyo Roshi No. 51) with *n*-BuOH - AcOH - H_2O (4 : 1 : 2). The amino acids were roughly separated as zones of Rf 0.16 (HyAsp), Rf 0.21 (Arg), Rf 0.25 (Ser), Rf 0.30 (HyPro), Rf 0.40 (Thr), Rf 0.45 (Ala) and Rf 0.50 (Pro). These amino acids were extracted and subjected to HPLC on a chiral column to compare with reference amino acids. The experimental condition and the retention volumes of reference amino acids used are cited in Table 2.

Isolation and Identification of Fatty Acid from Plusbacin A_2

The ether extract from the 3 hours hydrolysis of plusbacin A_2 (110 mg) as described in the former was purified by preparative TLC on silica gel plates (Merck, $60F_{254}$) developed with $CHCl_3$ - MeOH (5 : 1). A hydrophobic zone of Rf 0.23 detected by spraying water was extracted with $CHCl_3$ - MeOH (1 : 1). The extract was concentrated and dissolved in ether. The etherial solution was washed with dil HCl and water, dehydrated with sodium sulfate and concentrated to a colorless residue (6.5 mg). This was confirmed to be 3-hydroxy-isopentadecanoic acid from the following data. LSI-MS; *m/z* 259 $(M+H)^+$, molecular formula; $C_{15}H_{30}O_3$, ^1H NMR (200 MHz, $CDCl_3$, TMS as a reference); δ 0.87 (d, $J=6.5$ Hz, isopropyl) 1.27 (s-like, methylenes), 1.39 ~ 1.55 (m, γ-methylene), 2.47 (dd, $J=16.6$, 8.4 Hz) and 2.60 (dd, $J=16.6$, 2.6 Hz, α-methylene), 3.96 ~ 4.00 (m, β-methine).

Analysis of Fatty Acids by GC and GC-MS

Hydrochlorides of plusbacins $A_1 \sim A_4$ (2 ~ 3 mg) were hydrolyzed with constant boiling HCl at 110°C for 1 hour, respectively. Etherial extracts of the hydrolysates were methylated with trimethylsilyldiazomethane and subjected to GC-MS under the following conditions. Column; 5% DEGS HIEFF (3 mm × 1 m), carrier gas; He, 25 ml/minute, column temperature; 140°C (for A_1 and A_2), 160°C (for A_3 and A_4), ion sours temperature; 170°C, ion. Volt.; 30 V.

With the fatty acid methyl esters from plusbacins $A_1 \sim A_4$, a common base peak, *m/z* 103, was observed. Further, the methyl ester from plusbacin A_1 gave a peak due to $(M-H_2O)^+$ at *m/z* 240, and similarly

A_2 at *m/z* 254, A_3 at *m/z* 268, and A_4 at *m/z* 268, respectively.

Hydrochlorides of plusbacins $A_1 \sim A_4$ (2 ~ 3 mg) were hydrolyzed with constant boiling HCl at 110°C for 24 hours. Etherial extracts of the hydrolysates were methylated and then hydrogenated with PtO_2 in MeOH. The products were subjected to gas chromatography and compared with authentic fatty acid methyl esters. Gas chromatography was carried out in the following conditions: Column; SPB-1 fused capillary column (0.25 mm × 30 m) (film thickness 0.25 μm), carrier gas; He, temperature programing; 4°C/minute from 150°C to 250°C, detection; FID at 250°C. The sample prepared from plusbacin A_1 showed a peak at a retention time of 11.35 minutes which coincided with that of an authentic tetratradecanoic acid methyl ester. Similarly, the retention times of the sample from A_2 and isopentadecanoic acid methyl ester were the same (12.28 minutes), those from A_3 and isohexadecanoic acid methyl ester (14.43 minutes), and those from A_4 and hexadecanoic acid (15.10 minutes).

Alkali-treated Plusbacins A_2 and B_2

Plusbacin A_2 hydrochloride (15 mg) was dissolved in 2.7 ml of MeOH. To the solution, 0.3 ml of 1 N NaOH was added and the solution was stirred at room temperature for 30 minutes. The reaction mixture was neutralized with a dry ice (CO_2) and concentrated to dryness. The residue was dissolved in water (1.5 ml) and adsorbed on a Diaion CHP-20P column (10 ml). The column was washed with water and eluted with 40% aq acetone. The eluate was concentrated to dryness, giving a colorless powder (13 mg).

All the amino acid residues in the intact antibiotic were found by hydrolysis followed by amino acid analysis, and a molecular formula of $C_{49}H_{83}N_{11}O_{21}$ was indicated by HR-LS-MS, *m/z* 1,162.5850 $(M+H)^+$, Δ 1.1 mmu.

Similar procedures afforded alkali-treated plusbacin B_2, $C_{49}H_{83}N_{11}O_{20}$ HR-LSI-MS, *m/z* 1,146.5876 $(M+H)^+$, Δ −1.5 mmu.

Chromic Acid Oxidation

Chromic acid (100 mg) was dissolved in a mixture of pyridine (0.1 ml) and AcOH (3 ml), and a small residue was filtered off. Approximately 1 mg portions of plusbacin A_2 and alkali-treated plusbacin A_2 were dissolved in the chromic acid solution (0.1 ml) and allowed to stand for 20 hours at room temperature. After addition of MeOH (1.5 ml), the reaction mixtures were dried to residues, which were hydrolyzed and analyzed with an amino acid analyzer.

	Amino acid found (in ratio)					
	HyAsp	aThr[a]	Ser	Ala	Arg	HyPro
Plusbacin A_2	0.05	0.02	0.13	1.00	0.98	0.00
Alkali-treated plusbacin A_2	0.16	0.02	0.11	1.00	1.05	0.00

[a] Calculation was based on Thr as a standard.

Reduction with Lithium Borohydride

Approximately 2 mg portions of plusbacin A_2 and alkali-treated plusbacin A_2 were dissolved in MeOH (0.5 ml), and lithium borohydride (5 mg) was added. The solution was allowed to stand at room temperature for 24 hours. A few drops of 1 N HCl were added to the solution, which was then concentrated to dryness and hydrolyzed in the usual manner. The results of amino acid analysis are shown below.

	Amino acid found (in ratio)					
	HyAsp	aThr[a]	Ser	Ala	Arg	HyPro
Plusbacin A_2	0.91	0.95	0.78	1.00	1.00	1.35
Alkali-treated plusbacin A_2	1.54	0.94	0.43	1.00	1.05	1.30

[a] Calculation was based on Thr as a standard.

Deacylation of Plusbacin A_2 and B_2 with Polymyxin Acylase

About 31 mg of alkali-treated plusbacin A_2 was dissolved in 5 ml of 20 mM phosphate buffer, pH 7.6. Thirty mg of polymyxin acylase was added to the solution, which was then stirred at 37°C for 72 hours. The reaction mixture was centrifuged (3,000 rpm, 10 minutes) and the supernatant was subjected to preparative paper chromatography on a paper (Toyo Roshi, No. 51) with *n*-BuOH - AcOH - H_2O (4 : 1 : 2). A zone of Rf *ca.* 0.1 positive to Sakaguchi reaction was cut out and extracted with slightly acidified water. The extract was concentrated and freeze-dried to give a colorless powder (21 mg) (deacylated plusbacin A_2). Acid hydrolysis followed by amino acid analysis showed all the amino acids contained in the intact antibiotic.

Similar procedures afforded deacylated plusbacin B_2.

Edman Degradation

When deacylated plusbacin A_2 was analyzed with an automatic amino acid sequencer, the amino acid sequence was partially revealed as aThr-Ala-U-Arg (U: Later, clarified to be HyPro) from the *N*-terminus. Similar analysis on deacylated plusbacin B_2 clarified the sequence as aThr-Ala-Pro-Arg.

Then, Edman degradation on deacylated plusbacin A_2 was carried out in the usual manner using HyAsp and HyPro as references. Identification of PTH-amino acids was based on TLC experiments, in which PTH-HyAsp and PTH-HyPro were used as references. The result is shown in Table 3.

Acknowledgments

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