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Pyloricidins[†], Novel Anti-*Helicobacter pylori* Antibiotics Produced by *Bacillus* sp.

II. Isolation and Structure Elucidation

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Novel anti-*Helicobacter pylori* antibiotics, pyloricidins A, A_1, A_2, B, C and D were isolated from *Bacillus* sp. HC-70 and *Bacillus* sp. HC-72 by column chromatographies using adsorption and ion exchange resins. Their structures have been elucidated based on spectroscopic and degradation studies and shown to be peptide-like compounds. These compounds contained two unusual amino acids, *viz.*, 5-amino-2,3,4,6-tetrahydroxyhexanoic acid and 3-amino-3-phenylpropionic acid (β-phenylalanine). The structure-activity relationship studies suggested that 3-(5-amino-2,3,4,6-tetrahydroxyhexanoyl)amino-3-phenylpropionic acid moiety was essential for anti-*H. pylori* activity.

Peptic ulcer disease affects many people in the world. Although the disease has relatively low mortality, it is still a major health problem. In the past, this disease was believed to be related to stress and excess of the digestive secretions such as gastric acid. In 1983, WARREN and MARSHALL isolated a spiral Gram-negative bacterium *Helicobacter pylori*, from patients with gastric ulcer disease[1]. Recent studies revealed that *H. pylori* was strongly associated with peptic ulcer disease and eradication of *H. pylori* could cure and reduce its recurrence[2]. Various therapeutic regimens have been used in the treatment of peptic ulcer, but antibiotics alone could not achieve *H. pylori* eradication. Thus, triple therapy consisting of a proton pump inhibitor and antibiotics, such as amoxicillin and clarithromycin, is recommended now[3,4]. This therapy has yielded eradication rates of greater than 80%. However, side effects have been recorded in a significant number of patients and antibiotic resistance has also been shown to be prevalent[5]. Therefore, drugs which have strong anti-*H. pylori* activity and no significant side effects were required.

In a screening program designed to discover new anti-*Helicobacter pylori* antibiotics from microorganisms, pyloricidins A (**1**), A_1 (**1a**), A_2 (**1b**), B (**2**) and C (**3**) were isolated from *Bacillus* sp. HC-70, and pyloricidins C (**3**) and D (**4**) from *Bacillus* sp. HC-72 (Fig. 1). These compounds had specific activity against *H. pylori*. The taxonomy of the producing strain, fermentation and biological activity of pyloricidins were reported in another paper[6].

In this paper, we report the isolation and structural elucidation of pyloricidins.

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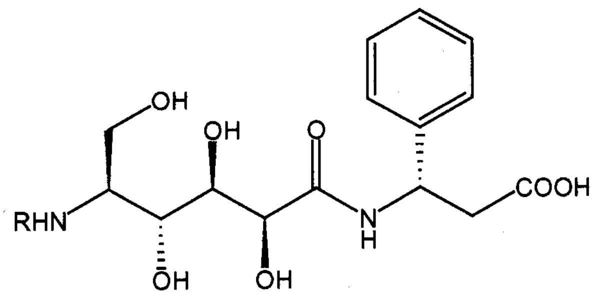
[†] Pyloricidins were presented as HC-70s in WO99/2549 (January 21, 1999).

Fig. 1. Structures of pyloricidins.



Pyloricidin A	**(1)**	:R=	H- L-Val- L-Val- L-Leu-
Pyloricidin A_1	**(1a)**	:R=	H- L-Val- L -Ile - L-Leu-
Pyloricidin A_2	**(1b)**	:R=	H- L-Val- L-Leu- L-Leu-
Pyloricidin B	**(2)**	:R=	H- L-Val- L-Leu-
Pyloricidin C	**(3)**	:R=	H- L-Leu-
Pyloricidin D	**(4)**	:R=	H-

Results

Isolation

A preliminary test showed that anti-*H. pylori* activity was found in the culture filtrate rather than in the cell mass. As pyloricidins were amphoteric, water-soluble substances, they were purified by column chromatographies using cation exchange resins such as Amberlite IR-120 and Lewatit CNP-80, anion exchange resin such as Amberlite IRA-67, and adsorption resins such as Diaion HP-20, Sepabeads SP-207 and Sepabeads SP-850.

Since the production ratio of pyloricidins varied with the fermentation time[6], pyloricidins A (**1**), A_1 (**1a**) and A_2 (**1b**) were isolated from the fermentation broth of *Bacillus* sp. HC-70 cultured for 30 hours, and pyloricidins B (**2**) and C (**3**) from the broth of the same strain cultured for 42 hours. The isolation procedure of **2** and **3** is outlined in Fig. 2. Separation of **2** and **3** was performed by SP-850 chromatography. Compound **1** was isolated according to a similar method to **2** and **3**. Compounds **1a** and **1b** were isolated as minor products from crude crystals of **1** using preparative HPLC.

In addition, pyloricidins C (**3**) and D (**4**) were isolated from the broth filtrate of *Bacillus* sp. HC-72 and separation of **3** and **4** was carried out by SP-207 chromatography. The fractions containing pyloricidins were detected by the antibacterial activity against *H. pylori* and HPLC analysis during the purification process. Details of the isolation procedures of pyloricidins are described in the experimental section.

Physico-chemical Properties

The physico-chemical properties of pyloricidins are summarized in Table 1. These compounds are soluble in water, DMF, DMSO and pyridine but insoluble in EtOAc and *n*-hexane. They showed positive color reactions with ninhydrin and Greig-Leaback reagents and negative color reactions with Ehrlich and Sakaguchi reagents.

The presence of amide groups was suggested by the characteristic absorption at $1630\sim1660\,cm^{-1}$ in the IR spectrum. In addition, characteristic NH signals at $7\sim9$ ppm in the ^1H NMR spectra and signals of carbonyl and their α-carbon in the ^{13}C NMR spectra indicated that **1**~**4** were peptide-like compounds. The results of amino acid analysis of the compounds upon acid hydrolysis are shown in Table 1.

Structure Elucidation

Structure of **3**

The molecular formula of **3** was determined to be $C_{21}H_{33}N_3O_8$ by molecular ion measurement in the FAB-MS, ^{13}C NMR spectra and elemental analysis. The molecular formula indicated seven degrees of unsaturation. The amino acid constitution of **3** was Leu (1 mol) and β-phenylalamine (β-Phe, 1 mol) by amino acid analysis. The structure of **3** was elucidated from analysis of ^1H and ^{13}C NMR (Table 2-2) and 2D NMR experiments such as ^1H-^1H COSY, ^1H-^{13}C COSY and HMBC. Twenty-one carbons in the ^{13}C NMR spectra were assigned to two methyl, three methylene, seven methine, five olefinic methine, one olefinic quarternary and three carbonyl carbons by DEPT experiments including overlapping signals at δ 126.46. Among them, fifteen carbon signals were easily assigned to the signals due to Leu and β-Phe moieties. The remaining six carbon signals were deduced to be one aminomethine (δ 51.22), one oxymethylene (δ 60.92), three oxymethine (δ 67.63, 70.74 and 71.21) and one carbonyl (δ 172.39) carbons. Considering the degrees of unsaturation, no additional ring system exists in this six-carbon moiety. All of these data suggested that these six carbons formed a new tetrahydroxyamino acid moiety. The ^1H-^1H COSY data revealed the coupling between 2-H (δ 4.13) and 3-H (δ 3.52); 3-H and 4-H (δ 3.77); 5-H (δ 3.99) and NH (δ 7.85); 5-H and 6-H (δ 3.43) (Fig. 3). This showed the partial structures, –CH(–O–)–CH(–O–)–CH(–O–)– (i) and –CH(–NH–)–CH_2O– (ii). In addition, the HMBC spectrum

Table 1. Physico-chemical properties of pyloricidins (**1**~**4**).

	Pyloricidin A (**1**)	Pyloricidin B (**2**)	Pyloricidin C (**3**)	Pyloricidin D (**4**)
Appearance	Colorless needles	Colorless needles	Colorless needles	Colorless needles
$[\alpha]_D^{24}$	-89° (c 0.53, 0.1N HCl)	-69° (c 0.50, 0.1N HCl)	-67° (c 0.55, 0.1N HCl)	-89° (c 0.53, 0.1N HCl)
UV $\lambda_{max}(H_2O)$ nm (ε)	258 (310)	257 (270)	257 (350)	257 (310)
IR ν_{max}(KBr) cm^{-1}	3300, 2960, 1640, 1540, 1400, 1050	3370, 2970, 1630, 1520, 1400, 1050	3390, 2970, 1660, 1540, 1400, 1050	3370, 2940, 1650, 1540, 1400, 1050
Elemental analysis				
Found	C 55.23 H 8.03 N 10.47	C 51.44 H 7.84 N 9.32	C 53.14 H 7.14 N 8.98	C 49.11 H 6.78 N 7.89
Calcd	C 55.43 H 7.95 N 10.42	C 51.30 H 7.95 N 9.20	C 53.27 H 7.45 N 8.87	C 48.78 H 6.82 N 7.58
Molecular formula	$C_{31}H_{51}N_5O_{10}$ (H_2O)	$C_{26}H_{42}N_4O_9$ ($3H_2O$)	$C_{21}H_{33}N_3O_8$ (H_2O)	$C_{15}H_{22}N_2O_7$ ($1.5H_2O$)
FAB-MS (m/z)	654 $(M+H)^+$	555 $(M+H)^+$	456 $(M+H)^+$	342 $(M+H)^+$
Amino acid analysis*1	L-Val (2mol) L-Leu (1mol) β-Phe (1mol)	L-Val (1mol) L-Leu (1mol) β-Phe (1mol)	L-Leu (1mol) β-Phe (1mol)	β-Phe (1mol)
Edman degradation	Val→Val→Leu	Val→Leu		
Rf.(Silica gel TLC)*2	0.45	0.41	0.35	0.30
Rt. (minutes)*3	16.8	8.1	6.0	4.0

*1 6N HCl, 110℃, 24~72hours
*2 Merck Kieselgel 60 F_{254}: n-BuOH-AcOH-H_2O (12:3:5)
*3 Solvent system: CH_3CN-20 mM KH_2PO_4 15:85 (v/v), 1.0 ml/min
Column YMC-Pack ODS-A, A-312 ϕ6.0 mm × 150 mm

indicated the following two- and three-bonds connectivities; from 2-H to carbonyl carbon C-1 (δ 172.39), C-3 (δ 71.21) and C-4 (δ 67.63), from 3-H to C-1, C-2 (δ 70.74) and C-5 (δ 51.22), from 4-H to C-6 (δ 60.92), from 5-H to C-6, and from 6-H to C-4 and C-5. This result combined the partial structures (i) and (ii), and clarified the unidentified tetrahydroxyamino acid to be 5-amino-2,3,4,6-tetrahydroxyhexanoic acid (5-ATHH).

The sequence of these amino acids was revealed by HMBC experiment. As shown in Fig. 3, the amide protons of 5-ATHH (δ 7.85) and β-Phe (δ 8.34) gave cross peaks with carbonyl carbons of leucine (δ 174.27) and 5-ATHH (δ 172.39), respectively. Finally, the structure of **3** was determined to be 3-(5-leucylamino-2,3,4,6-tetrahydroxyhexanoyl)amino-3-phenylpropionic acid.

Structures of **1**, **1a**, **1b**, **2** and **4**

The NMR data (Table 2) indicated that **1** and **2** also contained Leu, 5-ATHH and β-Phe moieties as **3**. Furthermore, **1** and **2** yielded **3** by enzymatic conversion using leucine aminopeptidase. This result suggested that **1** and **2** had peptide or amino acid binding to *N*-terminus of **3**.

The molecular formula of **1** was determined to be $C_{31}H_{51}N_5O_{10}$. The amino acid constitution except 5-ATHH was Val (2 mol), Leu (1 mol) and β-Phe (1 mol). The sequence of Val-Val-Leu was clarified by Edman's degradation. Therefore, it was estimated that **1** had Val-Val binding to *N*-terminus of **3**. The sequence of five amino acids was confirmed by HMBC experiments and ^1H-^{13}C long range coupling analysis. From these results, **1** was

Fig. 2. Purification procedure for pyloricidins B (**2**) and C (**3**).

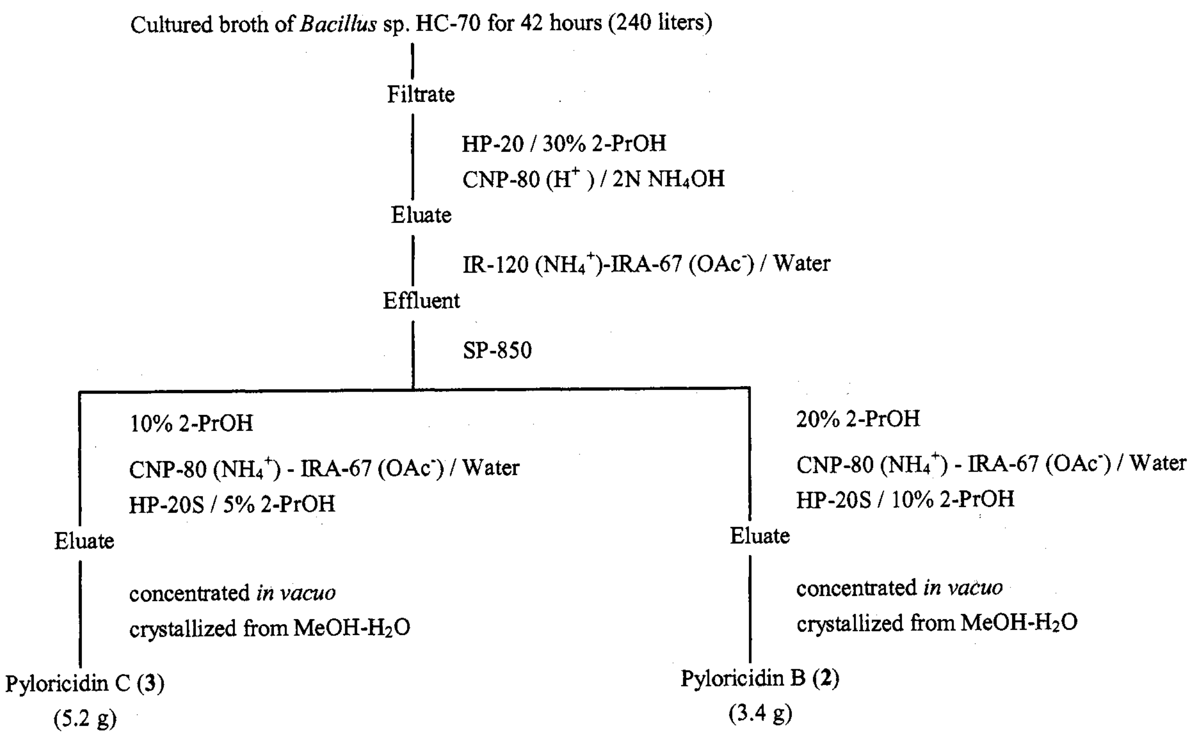
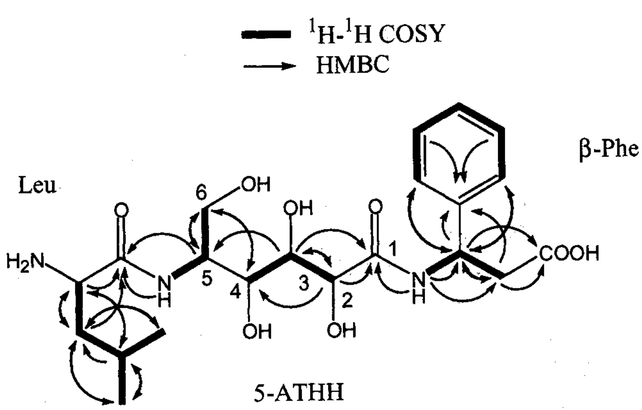


Fig. 3. 2D NMR data of pyloricidin C (**3**).



determined to be 3-[5-(valyl-valyl-leucyl)amino-2,3,4,6-tetrahydroxyhexanoyl]amino-3-phenylpropionic acid.

Compounds **1a** and **1b**, the minor products obtained from the crude crystals of **1**, had an identical molecular formula of $C_{32}H_{53}N_5O_{10}$. The amino acid constitutions except 5-ATHH were Val (1 mol), Ile (1 mol), Leu (1 mol) and β-Phe (1 mol) in **1a**, and Val (1 mol), Leu (2 mol) and β-Phe (1 mol) in **1b**. Therefore, it was suggested that one mole of Val in **1** was substituted by one mole of Ile in **1a** and by one mole of Leu in **1b**. The sequences of these amino acids were elucidated by similar methods as described above and the structures of **1a** and **1b** were determined, as shown in Fig. 1.

The molecular formula of **2** was determined to be $C_{26}H_{42}N_4O_9$. The amino acid constitution except 5-ATHH was Val (1 mol), Leu (1 mol) and β-Phe (1 mol). The sequence of the four amino acids was elucidated by ^{1}H-^{13}C long range coupling analysis and the sequence of Val-Leu was confirmed by Edman's degradation analysis. These experiments clarified that **2** was 3-[5-(valyl-leucyl)amino-2,3,4,6-tetrahydroxyhexanoyl]amino-3-phenylpropionic acid.

The molecular formula of **4** was determined to be $C_{15}H_{22}N_2O_7$. The NMR data (Table 2) showed that **4** also contained 5-ATHH and β-Phe moieties. The amino acid analysis indicated that Leu observed in **3** was absent in **4**. Upon treatment with pronase (Actinase E), **3** gave **4**. These results suggested that *N*-terminal Leu in **3** was detached in **4**. The ^{1}H-^{13}C long range coupling analysis confirmed the structure of **4** to be 3-(5-amino-2,3,4,6-tetrahydroxyhexanoyl)amino-3-phenylpropionic acid.

Table 2-1. ^{1}H and ^{13}C NMR spectral data of pyloricidins A (**1**) and B (**2**) in DMSO-d_6.

		Pyloricidin A (**1**)			Pyloricidin B (**2**)		
Moiety	Position	δC	δH	*J* (Hz)	δC	δH	*J* (Hz)
L-Val	C=O	172.64					
	2	59.59	3.11	(1H, d, *J*=4.8)			
	3	31.07	1.95	(1H, m)			
	4	16.81	0.79	(3H, d, *J*=6.8)			
	4'	19.26	0.88	(3H, d, *J*=6.2)			
	NH_2		nd				
L-Val	C=O	170.86			173.00		
	2	57.35	4.19	(1H, d, *J*=6.6)	58.01	3.69	(1H, m)
	3	30.67	1.97	(1H, m)	30.52	2.14	(1H, m)
	4	18.04	0.84	(3H, d, *J*=6.8)	17.90	0.98	(3H, d, *J*=7.5)
	4'	19.33	0.871	(3H, d, *J*=6.2)	18.78	1.01	(3H, d, *J*=6.8)
	NH		8.00	(1H, br s)	(NH_3^+)	8.07	(3H, br d, *J*=4.2)
L-Leu	C=O	172.42			168.30		
	2	51.15	4.33	(1H, m)	51.98	4.52	(1H, m)
	3	40.59	1.50	(2H, dd, *J*=6.4, 7.1)	41.47	1.57	(2H, t like)
	4	24.14	1.60	(1H, m)	24.60	1.72	(1H, m)
	5	21.49	0.83	(3H, d, *J*=6.3)	21.93	0.93	(3H, d, *J*=6.0)
	5'	22.96	0.869	(3H, d, *J*=7.5)	23.36	0.96	(3H, d, *J*=6.0)
	NH		8.02	(1H, d, *J*=8.2)		8.51	(1H, d, *J*=8.1)
5-ATHH	C=O	172.33			172.69		
	2	71.07	4.13	(1H, br d, *J*=1)	71.46	4.24	(1H, br s)
	3	71.07	3.52	(1H, dd, *J*=1, 6.6)	71.88	3.62	(1H, d, *J*=9.6)
	4	67.75	3.77	(1H, d, *J*=6.6)	68.10	3.87	(1H, d, *J*=9.6)
	5	51.30	4.00	(1H, m)	51.98	4.10	(1H, m)
	6	60.72	3.39	(1H, dd, *J*=6.0, 10.1)	62.00	3.48	(1H, dd, *J*=6.2, 9.8)
			3.44	(1H, dd, *J*=8.1,10.1)		3.53	(1H, dd, *J*=9.2, 9.8)
	NH		7.14	(1H, d, *J*=8.7)		7.58	(1H, d, *J*=8.7)
D-β-Phe	C=O	173.68			173.26		
	2	41.34	2.64	(1H, dd, *J*=6.9, 15.5)	40.79	2.81	(1H,dd,J=7.1, 16.0)
			2.71	(1H, dd, *J*=6.1, 15.5)		2.89	(1H,dd,J=6.2, 16.0)
	3	49.22	5.20	(1H, m)	49.50	5.31	(1H, m)
	4	142.61			142.65		
	5,9	126.47	7.34	(2H, m)	127.41	7.34	(2H, m)
	6,8	127.98	7.38	(2H, m)	128.68	7.41	(2H, m)
	7	126.56	7.19	(1H, m)	127.09	7.25	(1H, m)
	NH		8.22	(1H, d, *J*=8.6)		8.24	(1H, d, *J*=8.8)

* pyloricidin B (**2**) was measured in DMSO-d_6 : trifluoroacetic acid (9:1)

Absolute Stereochemistry

Absolute configurations of Val, Leu and Ile were determined by HPLC analysis using a chiral column. The results showed that all α-amino acids were L-forms. Acid hydrolysis of **1** gave β-Phe (**5**) in addition to a tripeptide L-Val-L-Val-L-Leu (**6**). The optical rotation of **5** was levorotatory: $[\alpha]_D^{24}$ $-7.5°$ (c 0.65, H_2O). Therefore, the

Table 2-2. ^{1}H and ^{13}C NMR spectral data of pyloricidins C (**3**) and D (**4**) in DMSO-d_6.

Moiety	Position	Pyloricidin C (3) δC	δH	J (Hz)	Pyloricidin D (4) δC	δH	J (Hz)
L-Leu	C=O	174.27					
	2	52.30	3.35	(1H, m)			
	3	42.71	1.29	(1H, ddd, J=5.2, 9.0, 13.6)			
			1.48	(1H, ddd, J=4.9, 8.6, 13.6)			
	4	23.86	1.72	(1H, m)			
	5	21.66	0.87	(3H, d, J=6.6)			
	5'	23.04	0.88	(3H, d, J=6.7)			
	NH_2		nd				
5-ATHH	C=O	172.39			172.32		
	2	70.74	4.13	(1H, br s)	70.82	4.15	(1H, br s)
	3	71.21	3.52	(1H, d, J=9.8)	71.38	3.78	(1H, d, J=9.4)
	4	67.63	3.77	(1H, d, J=9.8)	66.59	3.65	(1H, d, J=9.4)
	5	51.22	3.99	(1H, m)	53.34	3.26	(1H, m or t like)
	6	60.92	3.43	(2H ,m)	60.89	3.49	(1H, dd, J=6.3, 11.0)
						3.53	(1H, dd, J=5.8, 11.0)
	NH		7.85	(1H, d, J=8.3)		nd	
D-β-Phe	C=O	172.69			175.06		
	2	41.30	2.67	(1H, dd, J=6.7, 15.6)	43.11	2.49	(2H, m)
			2.72	(1H, dd, J=6.3, 15.6)			
	3	49.01	5.19	(1H, m)	49.69	5.08	(1H, m)
	4	142.66			143.43		
	5,9	126.46	7.32	(2H, m)	126.32	7.32	(2H, m)
	6,8	127.92	7.28	(2H, m)	127.85	7.20	(2H, m)
	7	126.46	7.21	(1H, m)	126.20	7.14	(1H, m)
	NH		8.34	(1H, d, J=8.7)		8.94	(1H, d, J=8.7)

configuration of **5** was determined to be D-form in comparison with the optical rotation values of the reference compounds, L(+)-β-phenylalanine; $[\alpha]_D^{22}$ $+10.6°$ (c 0.65, H_2O)[7].

The absolute configurations of 5-ATHH moiety were still unknown. The crystals of pyloricidins and their derivatives, such as N-4-bromobenzoates were not suitable for a single X-ray diffraction analysis. However, a degradation experiment provided crystals suitable for X-ray analysis. The acid hydrolysis of **4** afforded 5-ATHH (**7**) and its δ-lactam compound (**8**) as shown in Fig. 4. Crystallization of the mixture containing **7** and **8** from EtOH-H_2O gave colorless prisms of **8**. The structure of **8** including the relative stereochemistry and the absolute configuration was determined by X-ray analyses. As this compound contains only light elements up to oxygen, the intensity data were carefully measured to determine the right enantiomer from their small anomalous differences. Three crystals grown from the same batch were reshaped to spheres, and subjected to the analyses. Table 3 summarizes the crystal data and the structure refinement parameters. Although the estimated standard deviations exceeded the ideal range, the Flack parameters[8] in the three independent analyses fell into the same trend. Thus the absolute structure of **8** depicted in Fig. 5 was supported.

In addition, the absolute configuration of C-4 position was confirmed by an application of modified Mosher's method[9]. 4-Hydroxy derivative (**10**) was prepared from **7** through a γ-lactone compound (**9**), as shown in Fig. 6. Then (S) and (R)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) esters (**11a** and **11b**, respectively) of **10** were synthesized. By analysis of

Fig. 4. Degradation and modification studies of pyloricidins.

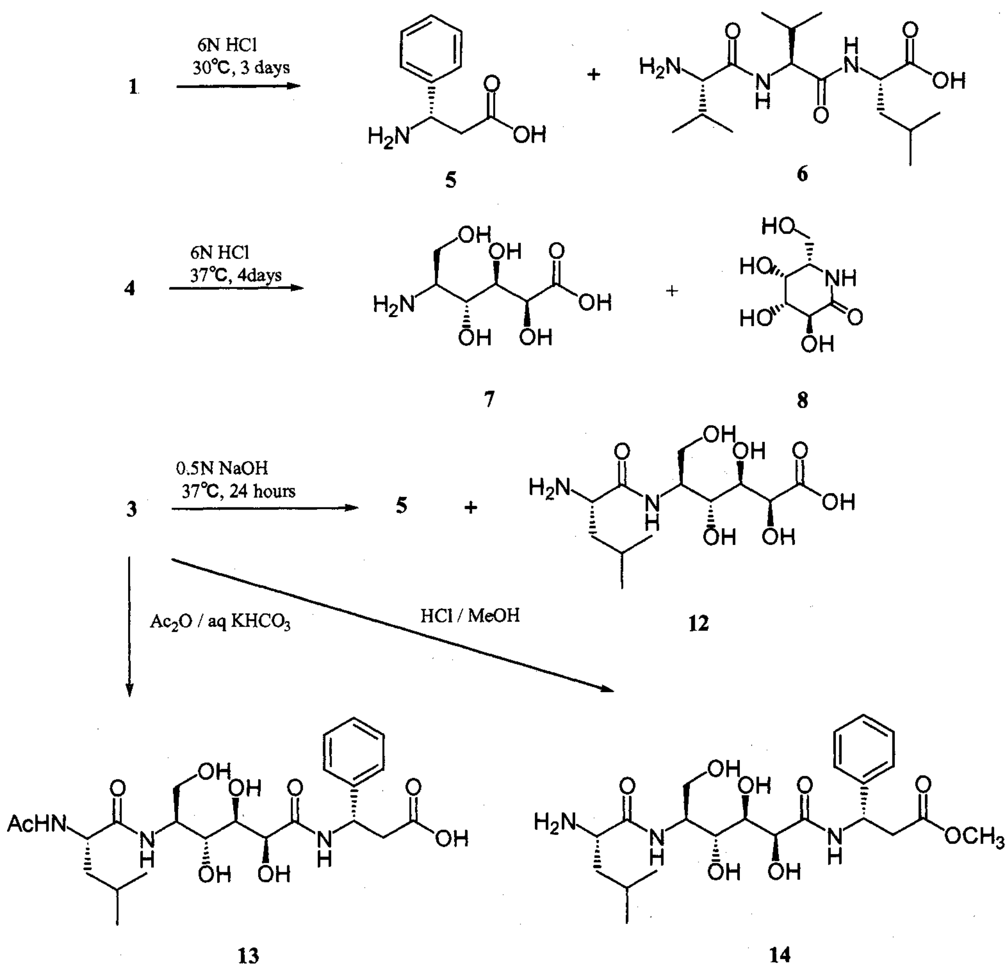


Table 3. Crystal data of **8**.

Crystal		# 1	# 2	# 3
Crystal Color, Habit			colorless, prismatic	
Crystal Dimensions (mm)		0.28x0.24x0.22	0.40x0.40x0.38	0.16x0.16x0.16
Crystal System			orthorhombic	
Space Group			P212121	
Lattice Parameters	a (Å)	11.987(2)	11.986(1)	11.986(3)
	b (Å)	12.933(2)	12.928(1)	12.934(2)
	c (Å)	4.992(1)	4.991(1)	4.992(2)
Z			4	
No. of Reflections Measured				
Total		4641	4643	4648
Unique		1161	1157	1161
No. of Observations (I>2σ(I))		1129	1142	1090
No. of Variables			114	
Residuals: R1, wR2		0.025, 0.063	0.029, 0.075	0.030, 0.077
Goodness of Fit Indicator		1.17	1.25	1.21
Flack Parameter		-0.09(23)	-0.09(25)	0.01(29)

chemical shift differences (Fig. 7) in ^{1}H NMR spectra of **11a** and **11b**, the absolute configuration of C-4 was assigned to *R*. This result was coincident with that of the X-ray analyses. Thus, the absolute structure of **8** was determined to be (3*S*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-hydroxymethylpiperidine-2-one. Finally, the absolute structure of **7** was determined to be (2*S*,3*R*,4*R*,5*S*)-5-amino-2,3,4,6-tetrahydroxyhexanoic acid.

Aniti-*H. pylori* Activity of Degradation and Modification Products

Degradation products (**5**~**8**) described in the section of structure elucidation did not show anti-*H. pylori* activity (MIC >128 μg/ml). Furthermore, 5-leucylamino-2,3,4,6-tetrahydroxyhexanoic acid (**12**) which was obtained by mild alkaline hydrolysis of **3** was also inactive (Fig. 4). All of these data indicated that **4** was a minimum structure for anti-*H. pylori* activity. In addition, an *N*-acetylated derivative (**13**) and a methyl ester derivative (**14**) of **3** did not show activity. This data suggested that both a primary amine at *N*-terminal and a carboxyl at *β*-Phe are essential for anti-*H. pylori* activity.

Discussion

Quinolone derivatives[10,11], brefedlin A[12] and CJ-

Fig. 5. ORTEP drawing of **8**.

Displacement ellipsoids are plotted at the 50% probability level for non-hydrogen atoms.

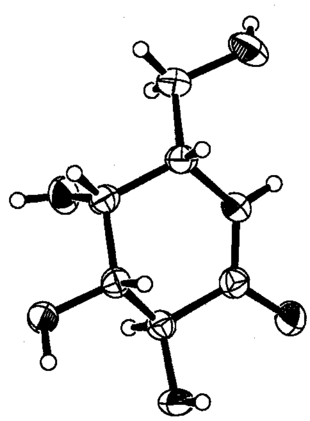
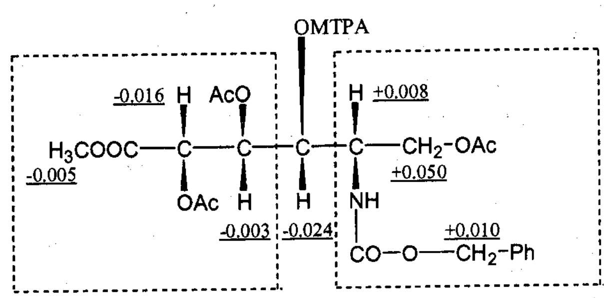
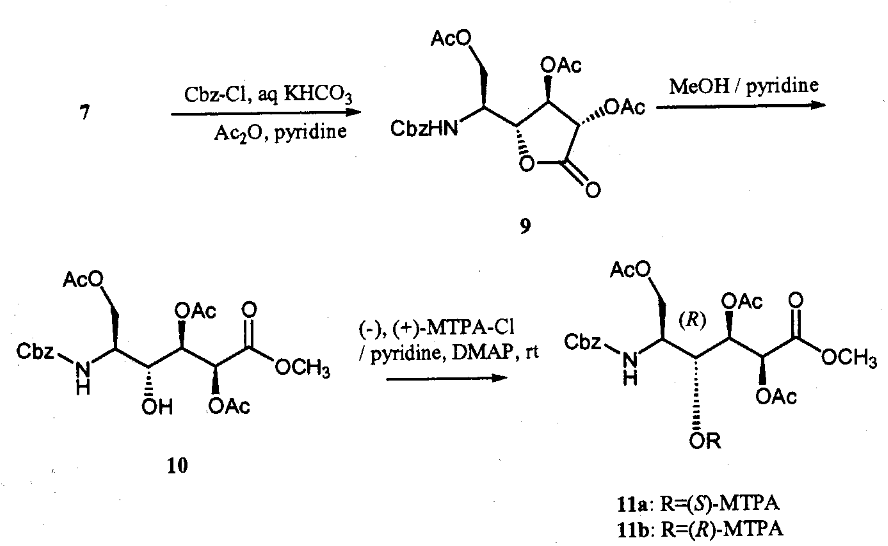


Fig. 7. Chemical shift differences obtained from **11a** and **11b**.



Figures showed Δδ (δ_{11a}-δ_{11b})

Fig. 6. Synthesis of MTPA esters (**11a** and **11b**).



12,954[13] were already reported as anti-*H. pylori* compounds. Pyloricidins were peptide-like compounds that contained two unusual amino acids, *viz.*, (2*S*,3*R*,4*R*,5*S*)-5-amino-2,3,4,6-tetrahydroxyhexanoic acid and D-*β*-Phe. Pyloricidins are the first peptide-like antibiotics active against *H. pylori*.

The structure-activity relationship of degradation products indicated that **4** was a minimum structure for anti-*H. pylori* activity. Comparing activities of **1**, **1a**, **1b** and **2** with those of **3** and **4**, the addition of di- or tripeptide at *N*-terminal of **4** increased the activity[6]. This suggests that modification of *N*-terminal moiety with oligopeptides is promising. Pyloricidins in this study could provide useful information for the synthesis of new anti-*H. pylori* agents.

Experimental

General

IR spectra were obtained on a HORIBA FT-200 FT-IR infrared spectrometer. UV spectra were recorded on a HITACHI U-3200 spectrophotometer. Optical rotations were measured on a JASCO DIP-360 digital polarimeter. ^{1}H (300 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on a BRUKER AC300 NMR spectrometer or a BRUKER DPX300 NMR spectrometer in DMSO-d_6 solution unless otherwise stated. Trimethylsilane was used as an internal standard (0 ppm). TLC was performed on Kieselgel 60 F_{254}, using *n*-BuOH - AcOH - H_2O 12 : 3 : 5 (v/v/v). HPLC analyses were carried out using a column of YMC-Pack ODS-A, A-312 (i.d. 6.0 mm×150 mm) with a flow rate of 1 ml/minute. Evaporations were carried out *in vacuo* at ≦40°C.

Isolation of 1, 1a and 1b from *Bacillus* sp. HC-70

The filtrate of the fermentation broth (250 liters) of *Bacillus* sp. HC-70 cultured for 30 hours was adjusted to pH 6.0 and applied to a column of Diaion HP-20 (15 liters, Mitsubishi Chemical Industries). The column was washed with water (45 liters) and eluted with 30% aqueous 2-PrOH (60 liters). The eluate was successively loaded onto a column of Lewatit CNP-80 (H^+ form, 20 liters, Bayer). After washing with water (60 liters), active substances were eluted with 2 N aqueous NH_4OH (80 liters). The eluate was concentrated to *ca.* 9.5 liters and applied to columns of Amberlite IR-120 (NH_4^+ form, 1.5 liters, Rohm & Haas company), Amberlite IRA-67 (OAc^- form, 1.5 liters, Rohm & Haas company) and Sepabeads SP-850 (2 liters, Mitsubishi Chemical Industries) in series. After washing with water (8 liters), the column of SP-850 was further washed with 0.2 N aqueous NH_4OH (2 liters), water (6 liters), 0.1 N aqueous HCl (2 liters), water (6 liters) and 5% aqueous 2-PrOH (6 liters) successively, and then eluted with 20% aqueous 2-PrOH (8 liters) and 30% aqueous 2-PrOH (6 liters). The combined active fraction was passed through columns of IR-120 (NH_4^+ form, 0.5 liter) and of IRA-67 (OAc^- form, 0.5 liter) and washed with water (2 liters). The effluent (12.5 liters) was concentrated *in vacuo* to give the crude crystals (9.6 g) of **1**, which contained **1a** and **1b** as minor products. The crude crystals of **1** were further purified by preparative reversed phase HPLC (column, YMC·GEL KE-ODS-10S i.d. 50 mm×300 mm; mobile phase, CH_3CN - 20 mM KH_2PO_4 17 : 83; flow rate, 30 ml/minute; detection, UV 214 nm). (i) Fractions containing **1** were loaded on a column of HP-20 (250 ml) and the column was washed with water (750 ml), and then eluted with 20% aqueous 2-PrOH (250 ml) and 0.1 N NH_4OH / 20% aqueous 2-PrOH (750 ml). The eluate containing **1** was concentrated *in vacuo* and recrystallized from water to yield crystals of **1** (4.49 g).

(ii) Fractions containing **1a** were desalted by almost the same procedure as described in (i) to give crystals of **1a** (249 mg); *Anal.* Calcd for $C_{32}H_{53}N_5O_{10}\cdot 2H_2O$: C 54.61, H 8.16, N 9.95. Found: C 54.54, H 8.16, N 10.12; FAB-MS *m/z* 668 $[M+H]^+$; ^{13}C NMR δ 10.84 (q), 15.32 (q), 16.90 (q), 18.98 (q), 21.28 (q), 23.06 (q), 23.98 (d), 24.30 (t), 30.85 (d), 36.72 (d), 40.48 (t), 41.16 (t), 48.99 (d), 50.82 (d), 51.11 (d), 56.49 (d), 58.82 (d), 60.46 (t), 67.18 (d), 70.83 (d), 71.03 (d), 126.47 (d×3), 127.95 (d×2), 142.60 (s), 170.78 (s), 172.22 (s), 172.35 (s), 172.38 (s), 172.71 (s); TLC, Rf=0.51. HPLC, Rt=27.0 minutes. Solvent system: CH_3CN - 20 mM KH_2PO_4 15 : 85.

(iii) Fractions containing **1b** were concentrated and recrystallized from water to yield crystals of **1b** (400 mg); *Anal.* Calcd for $C_{32}H_{53}N_5O_{10}\cdot 3.5H_2O$: C 52.59, H 8.27, N 9.58. Found: C 52.59, H 8.03, N 9.78; FAB-MS *m/z* 668 $[M+H]^+$; ^{13}C NMR δ 16.95 (q), 19.01 (q), 21.25 (q), 21.58 (q), 22.92 (q), 23.06 (q), 24.07 (d×2), 30.85 (d), 40.43 (t), 40.94 (t), 41.24 (t), 49.07 (d), 50.65 (d), 51.03 (d×2), 59.00 (d), 60.47 (t), 67.20 (d), 70.77 (d), 71.00 (d), 126.45 (d×2), 126.50 (d), 127.95 (d×2), 172.30 (s×2), 172.45 (s), 172.78 (s), 172.86 (s); TLC, Rf=0.54. HPLC, Rt=39.0 minutes. Solvent system: CH_3CN - 20 mM KH_2PO_4 15 : 85.

Isolation of 2 and 3 from *Bacillus* sp. HC-70

The procedure for isolation of **2** and **3** is shown in Fig. 2. The filtrate of the fermentation broth (240 liters) of *Bacillus* sp. HC-70 cultured for 42 hours was adjusted to pH 7.0 and loaded onto a column of HP-20 (15 liters). After washing with water (45 liters), the active compounds were eluted with 30% aqueous 2-PrOH (60 liters). Then the eluate was subjected to a column of CNP-80 (H^+ form, 20 liters) and the column was washed with water (60 liters) and eluted with 2 N aqueous NH_4OH (80 liters). The eluate was successively concentrated to *ca.* 8.8 liters and applied to columns of IR-120 (NH_4^+ form, 1.5 liters), IRA-67 (OAc^- form, 1.5 liters) and SP-850 (2 liters) in series. After washing the columns with water (8 liters), the column of SP-850 was further washed with 0.1 N aqueous HCl (2 liters), water (6 liters) and 5% aqueous 2-PrOH (6 liters) successively, and then eluted with 10% aqueous 2-PrOH (6 liters) to give fractions containing **3**, and 20% aqueous 2-PrOH (4 liters) to give fractions containing **2**.

(i) Fractions containing **3** were concentrated to *ca.* 1.3 liters and passed through columns of CNP-80 (NH_4^+ form, 100 ml) and IRA-67 (OAc^- form, 100 ml) and washed with water (400 ml). The effluent (1.7 liters) was applied to a column of Diaion HP-20S (1 liter, Mitsubishi Chemical Industries). After washing with water (3 liters) and 2% aqueous 2-PrOH (3 liters) successively, **3** was eluted with 5% aqueous 2-PrOH (4 liters). The eluate was concentrated and crystallized from MeOH - H_2O (2 : 1) to give crystals of **3** (5.2 g).

(ii) Fractions containing **2** were purified by almost the same procedure as described in (i) to afford crystals of **2** (3.4 g).

Isolation of **3** and **4** from *Bacillus* sp. HC-72

The filtrate of the fermentation broth (1620 liters) of *Bacillus* sp. HC-72 cultured for 90 hours was applied to a column of Sepabeads SP-207 (75 liters, Mitsubishi Chemical Industries). The column was washed with water (225 liters), and eluted with 30% aqueous 2-PrOH (300 liters). The eluate was subjected to columns of IRA-67 (OAc^- form, 8 liters) and CNP-80 (H^+ form, 45 liters) in series. After washing the columns with water (300 liters), the column of CNP-80 was eluted with 2 N aqueous NH_4OH (135 liters). The eluate was concentrated to *ca.* 10 liters and applied to columns of IRA-67 (OAc^- form, 2 liters) and SP-207 (3 liters) successively. After the columns were washed with water (9 liters), the column of SP-207 was eluted with 5% aqueous 2-PrOH (8 liters) to give a fraction containing mainly **4** and then with 15% aqueous 2-PrOH (15 liters) to give a fraction containing mainly **3**. Both fractions were separately re-chromatographed on SP-207 to afford a fraction containing **4** and a fraction containing **3**. The former fraction was concentrated and crystallized from EtOH - H_2O (5 : 1) to give colorless needles of **4** (28.4 g).

The latter fraction was concentrated and crystallized from MeOH - H_2O (2 : 1) to give colorless needles of **3** (8.4 g).

Preparation of **4** by Enzymatic Conversion from **3**

To a solution of **3** (3.0 g, 6.59 mmol) in 40 mM potassium phosphate buffer (pH 8.0, 750 ml) containing 4 mM $CoCl_2$, Actinase E (300 mg, 300,000 units, Kaken Pharmaceutical Co., Ltd.) was added. The reaction mixture was incubated at 37°C for 2 hours, and the insoluble material was removed by filtration. The filtrate was adjusted to pH 6.0 and applied to a column of SP-207 (200 ml). The column was washed with water (600 ml), and eluted with 10% aqueous 2-PrOH (600 ml). The eluate was concentrated and crystallized from EtOH - H_2O to give colorless needles of **4** (1.66 g, yield 74%). The physico-chemical data were identical with those of the natural product (**4**).

Preparation of **5** and **6** from **1**

A solution of **1** (653 mg, 1.0 mmol) in 6 N HCl (65 ml) was maintained at 30°C for 3 days. The reaction mixture was adjusted to pH 6.0 and applied to a column of SP-207 (100 ml). The column was washed with water (300 ml) and then eluted with 5% aqueous 2-PrOH (300 ml), 10% aqueous 2-PrOH (300 ml) and 30% aqueous 2-PrOH (300 ml) successively. (i) The elute (300 ml) of 5% 2-PrOH was concentrated and crystallized from water to give colorless needles of **5** (63 mg, 38% yield); $[\alpha]_D^{24}$ $-7.5°$ (*c* 0.65, H_2O); TLC, Rf=0.41. HPLC, Rt=4.3 minutes. Solvent system: CH_3CN - 20 mM KH_2PO_4 7.5 : 92.5 (v/v).

(ii) The eluate (600 ml) of 10% and 30% aqueous 2-PrOH was concentrated and chromatographed on a column of Sephadex G-10 (550 ml, Pharmacia) eluting with water. Fractions containing **6** were concentrated and then crystallized from water to give colorless needles of **6** (108 mg, 33% yield); ^1H NMR δ 0.78 (3H, d, J=6.8 Hz), 0.82 (3H, d, J=6.4 Hz), 0.83 (3H, d, J=6.7 Hz), 0.87 (6H, d, J=6.5 Hz), 0.88 (3H, d, J=6.9 Hz), 1.49 (2H, m), 1.60 (1H, m), 1.95 (1H, m), 1.97 (1H, m), 3.12 (1H, d, J=5.0 Hz), 4.15 (1H, m), 4.20 (1H, m), 8.01 (1H, br s), 8.06 (1H, d, J=7.9 Hz); FAB-MS *m/z* 330 $[M+H]^+$; TLC, Rf=0.55. HPLC, Rt=15.8 minutes. Solvent system: CH_3CN - 20 mM KH_2PO_4 7.5 : 92.5.

Preparation of **5** and **12** from **3**

A solution of **3** (910 mg, 2.0 mmol) in 0.5 N NaOH (200 ml) was allowed to stand for 24 hours at 37°C. The reaction mixture was adjusted to pH 5.0 and loaded onto a column of SP-207 (100 ml). The column was washed with water (300 ml) and then eluted with 5% aqueous 2-PrOH (300 ml). (i) The passed and washed fractions were combined and applied to a column of activated charcoal LH_2C (70 ml, Takeda Chemical Industries). The column was washed with water (210 ml) and eluted with 10% aqueous 2-PrOH (210 ml). The eluate was concentrated and chromatographed on a column of Sephadex G-10 (550 ml) developing with water. Fractions containing **12** were concentrated and then crystallized from EtOH - H_2O to give colorless needles of **12** (301 mg, 49% yield); ^{1}H NMR δ 0.86 (3H, d, J=7.1 Hz), 0.89 (3H, d, J=7.3 Hz), 1.36 (1H, m), 1.49 (1H, m), 1.67 (1H, m), 3.30 (1H, dd, J=3.9, 9.3 Hz), 3.38 (1H, dd, J=6.3, 9.8 Hz), 3.43 (1H, dd, J=9.3, 9.8 Hz), 3.58 (1H, m), 3.70 (1H, d, J=9.3 Hz), 3.85 (1H, d, J=3.9 Hz), 4.05 (1H, d like), 7.90 (1H, d, J=8.6 Hz); FAB-MS m/z 309 $[M+H]^+$; TLC, Rf=0.09. HPLC, Rt=2.8 minutes. Solvent system: CH_3CN - 20 mM KH_2PO_4 7.5 : 92.5 (v/v).

(ii) The eluate of 5% aqueous 2-PrOH was concentrated and crystallized from water to give colorless needles of **5** (182 mg, 55% yield).

Analysis of Amino Acids

(a) Amino acid analysis

Each compound of pyloricidins (*ca.* 0.5 mg) was completely hydrolyzed with 6 N HCl for 24~72 hours. The hydrolysates were evaporated to dryness and dissolved in 0.5 ml of 0.02 N aqueous HCl, and then 20 μl of them were separately applied to HITACHI L-8500A Amino Acid Analyzer.

(b) Absolute configuration of the α-amino acids

The approximately 1 mg of each compound was hydrolyzed with 6 N HCl for 24 hours. The hydrolysate was concentrated and dissolved in water (0.5 ml), and then 20 μl of the resulting solution was applied to the chiral HPLC analysis. The chiral HPLC analysis was carried out with the following general conditions: column; OA-5000 (Sumika Chemical Analysis Service, Ltd.), flow rate; 1.0 ml/minute, detection; UV 254 nm, temperature; ambient temperature.

The HPLC analysis for Ile and Leu was carried out with MeOH - 3 mM $CuSO_4$ 15 : 85 (v/v). For this HPLC analysis, D-Ile and L-Ile were eluted at 15.2 minutes and 11.8 minutes and D-Leu and L-Leu were eluted at 19.7 minutes and 13.3 minutes, respectively.

The HPLC analysis for Val was carried out with 1 mM $CuSO_4$. For this HPLC analysis, D-Val and L-Val were eluted at 21.1 minutes and 12.3 minutes, respectively.

The HPLC analysis for β-Phe was carried out with MeOH - 3 mM $CuSO_4$ 8 : 92 (v/v). For this HPLC analysis, D-β-Phe and L-β-Phe were eluted at 26.9 minutes and 23.8 minutes, respectively.

Protein Sequence Analysis (Edman Degradation Method)

The *ca.* 1 mg of each compound was dissolved in 10 ml of 0.1% TFA and then 20 μl of the solution was analyzed on a Protein Sequencer 473A (Applied Biosystems).

Preparation of **7** and **8** from **4**

(i) A solution of **4** (5.0 g, 14.6 mmol) in 6 N HCl (500 ml) was kept at 37°C for 4 days. The reaction mixture was cooled with an ice bath, and 10 N NaOH was cautiously added to attain pH 5. The adjusted solution was passed through a column of SP-207 (250 ml) and the column was successively washed with water (750 ml). The effluent was applied to a column of activated charcoal LH_2C (200 ml, Takeda Chemical Industries). After washing with water (600 ml), the column was eluted with 10% aqueous 2-PrOH (600 ml). The eluate was concentrated to give 1.87 g of crude gum. The gum was then crystallized in 3 ml of EtOH - H_2O (2 : 1) to give colorless prisms of **8** (412 mg, 16% yield); ^{1}H NMR δ 3.26 (1H, m), 3.44 (2H, m), 3.54 (1H, ddd, J=2.2, 4.8, 9.5 Hz), 3.83 (1H, dd, J=4.6, 9.5 Hz), 3.87 (1H, br), 4.68 (1H, t, J=5.8), 4.92 (1H, d, J=4.3), 5.03 (1H, d, J=4.6), 5.05 (1H, d, J=4.8), 7.00 (1H, s); IR; ν_{max} (KBr) cm^{-1} 3317, 1633, 1421, 1338, 1103; FAB-MS m/z 178 $[M+H]^+$.

(ii) The mother liquid of **8** was concentrated and chromatographed on a column of Sephadex G-10 (550 ml) eluting with water. Fractions containing **7** were concentrated and lyophilized to give 638 mg of a crude powder, and then crystallized from EtOH - H_2O (2 : 1) to give colorless needles of **7** (505 mg, 18% yield); ^{1}H NMR δ 3.36 (1H, br), 3.56 (2H, d, J=6.8 Hz), 3.70 (1H, d, J=9.4 Hz), 3.83 (1H, d, J=9.4 Hz), 4.27 (1H, s), 7.62 (3H, br s); IR; ν_{max} (KBr) cm^{-1} 3421, 1589, 1419, 1358, 1066; FAB-MS m/z 196 $[M+H]^+$.

Crystal Analysis of **8**

Colorless prism crystals obtained from EtOH - H_2O solution were subjected to X-ray crystal analyses. Appropriately sized crystals were cut into cubes and were gently shaken in an aliquot of 50% ethanol aqueous solution to obtain sphere like crystals. Intensity data were collected for all the reflections in the reciprocal space within a resolution limit by a four-circle diffractometer, RIGAKU AFC5R, using Cu-Kα radiation. Spherical absorption correction was applied. The structure was solved by direct methods (SIR92[14] in teXsan[15] software package) and refined by SHELX-97[16].

Preparation of **9** from **7**

To a solution of **7** (280 mg, 1.44 mmol) in 0.3 M aqueous

$KHCO_3$ (28 ml), carbobenzoxy chloride (432 μl, 2.88 mmol) was added and the mixture was stirred at room temperature for 3.5 hours while cautiously adding 0.3 M aqueous $KHCO_3$ to maintain pH above 8. The mixture was adjusted to pH 7 and then washed twice with EtOAc. The aqueous layer was applied to a column of HP-20S (50 ml) and eluted with 5~30% aqueous 2-PrOH (150 ml). The eluate was concentrated and lyophilized to give crude powder of 5-benzyloxycarbonylamino-2,3,4,6-tetrahydroxyhexanoic acid (404 mg, 86%).

The obtained compound (390 mg, 1.18 mmol) was dissolved in abs. pyridine (5 ml), and acetic anhydride (5 ml) was added gradually to the solution. After being stirred for 20 hours at room temperature, the reaction mixture was concentrated to give an oily residue (530 mg). To a solution of the residue in acetone (20 ml), acetic anhydride (240 μl) and TFA (240 μl) were added and the mixture was stirred for 2 hours at room temperature. The reaction mixture was concentrated, poured into H_2O and extracted with EtOAc. The organic layer was washed with 3% aqueous $NaHCO_3$, saturated aqueous NaCl, dried over Na_2SO_4, and evaporated to dryness. The residue (554 mg) was purified by preparative HPLC (column: YMC-Pack ODS SH-363-15, i.d. 30×250 mm) using CH_3CN : 50 mM H_3PO_4 - KH_2PO_4 (pH 3.0) (40 : 60). Fractions containing **9** were combined, concentrated and then extracted with EtOAc. The extract was washed with saturated aqueous NaCl, dried over Na_2SO_4 and concentrated to give **9** (259 mg, 49%); ^{1}H NMR δ 2.04 (3H, s), 2.11 (3H, s), 2.14 (3H, s), 4.14 (1H, dd, J=5.8, 10.8 Hz), 4.34 (2H, m), 5.10 (1H, d, J=10.8 Hz), 5.13 (2H, m), 5.50 (1H, br s), 5.51 (1H, d, J=5.3), 7.3~7.4 (5H, m). IR; ν_{max} (KBr) cm^{-1} 3399, 1778, 1722, 1535, 1227, 1108. FAB-MS m/z 438 $[M+H]^+$.

Preparation of **10** from **9**

Compound **9** (258 mg, 0.59 mmol) was dissolved in MeOH (15 ml), and pyridine (50 μl, 0.62 mmol) was added to the solution. After being stirred at room temperature for 26 hours, the reaction mixture was concentrated, poured into water and then extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4 and concentrated to afford **10** (259 mg, 94%); ^{1}H NMR δ 2.06 (3H, s), 2.08 (3H, s), 2.20 (3H, s), 3.73 (3H, s), 3.85 (1H, d, J=9.6), 4.08 (2H, m), 4.31 (1H, dd, J=7.2, 10.7 Hz), 5.04 (1H, d, J=12.2), 5.12 (1H, br s), 5.13 (1H, d, J=12.2), 5.30 (1H, d, J=9.6), 5.41 (1H, d, J=1.8), 7.3~7.4 (5H, m). IR; ν_{max} (KBr) cm^{-1} 3437, 1751, 1527, 1228, 1055. FAB-MS m/z 470 $[M+H]^+$.

Preparation of **11a** and **11b** from **10**

(i) To a solution of **10** (10 mg, 0.0213 mmol) in pyridine (0.5 ml), (−)-α-methoxy-α-trifluoromethylphenylacetyl chloride (100 mg, 0.40 mmol) and dimethylaminopyridine (10 mg) were added and the mixture was stirred at room temperature for 100 hours. The reaction mixture was concentrated, poured into water and then extracted with EtOAc. The extract was washed with 0.1 N aqueous HCl, 3% aqueous $NaHCO_3$ and water. The organic layer was dried over Na_2SO_4, concentrated and purified by preparative HPLC (column: YMC-Pack ODS D-ODS-5, i.d. 30×250 mm) using CH_3CN : 20 mM KH_2PO_4 (pH 4.5) (40 : 60~60 : 40). Fractions containing **11a** were combined, concentrated and then extracted with EtOAc. The organic layer was washed with water, dried over Na_2SO_4 and concentrated to give **11a** (5.6 mg, 38% yield); ^{1}H NMR (5.6 mg/0.5 ml, $CDCl_3$) δ 3.454 (3H, s), 3.690 (3H, s), 3.887 (2H, dd, J=1.5, 7.3 Hz), 4.440 (1H, m), 4.595 (1H, d, J=10.4 Hz), 4.785 (1H, d, J=1.6 Hz), 4.984 (1H, d, J=12.1 Hz), 5.130 (1H, d, J=12.1 Hz), 5.444 (1H, dd, J=1.6, 9.3 Hz), 5.634 (1H, dd, J=1.7, 9.3 Hz), 7.32~7.39 (5H, m), 7.43~7.50 (5H, m). ^{13}C NMR δ 20.33 (q, 3-OCOCH_3), 20.47 (q, 2-OCOCH_3), 20.53 (q, 6-OCOCH_3), 48.99 (d, C-5), 52.79 (q, COOCH_3), 55.26 (q), 62.52 (t, C-6), 67.72 (d, C-3), 69.55 (d, C-2), 70.63 (d, C-4), 127.35 (d), 128.30 (d), 128.38 (d), 128.59 (s), 128.82 (d), 129.04 (d), 130.18 (s), 130.67 (s), 130.89 (s), 135.90 (s), 155.82 (s), 165.32 (s), 167.24 (s, C-1), 169.12 (s, 3-OCOCH$_3$), 169.84 (s, 2-OCOCH$_3$), 170.37 (s, 6-OCOCH$_3$).

(ii) Compound **11b** was obtained from **10** (10 mg) and (+)-α-methoxy-α-trifluoromethylphenylacetyl chloride (100 mg, 0.40 mmol) in a manner similar to that described for the synthesis of **11a** (14.4 mg, 98% yield); ^{1}H NMR (14.4 mg/1.2 ml, $CDCl_3$) δ 3.489 (3H, s), 3.695 (3H, s), 3.837 (2H, d, J=7.3 Hz), 4.432 (1H, m), 4.636 (1H, d, J=10.6 Hz), 4.801 (1H, d, J=1.6 Hz), 4.975 (1H, d, J=12.1 Hz), 5.119 (1H, d, J=12.1 Hz), 5.447 (1H, dd, J=1.6, 9.0 Hz), 5.658 (1H, dd, J=1.6, 9.0 Hz), 7.32~7.38 (5H, m), 7.43~7.52 (5H, m). ^{13}C NMR δ 20.32 (q, 3-OCOCH_3), 20.45 (q, 2-OCOCH_3), 20.54 (q, 6-OCOCH_3), 48.95 (d, C-5), 52.81 (q, COOCH_3), 55.39 (q), 62.48 (t, C-6), 67.90 (d, C-3), 69.62 (d, C-2), 70.66 (d, C-4), 127.21 (d), 128.33 (d), 128.38 (d), 128.58 (s), 128.81 (d), 129.04 (d), 130.21 (s), 130.71 (s), 130.92 (s), 135.86 (s), 155.82 (s), 165.36 (s), 167.19 (s, C-1), 169.15 (s, 3-OCOCH$_3$), 169.92 (s, 2-OCOCH$_3$), 170.37 (s, 6-OCOCH$_3$).

Preparation of **13** from **3**

To a solution of **3** (50 mg, 0.11 mmol) in aqueous 0.05 M $KHCO_3$ (20 ml), Ac_2O (22 μl, 0.23 mmol) was added and the mixture was stirred at room temperature for an hour while cautiously adding aqueous 1 M $KHCO_3$ to maintain pH above 8. To the reaction mixture, 1 N aqueous HCl was added to attain pH 6.5. The resulting solution was applied to a column of HP-20 (5 ml) and washed with water (15 ml). Compound **13** was eluted with 30% aqueous 2-PrOH (40 ml) and the eluate was concentrated and freeze-dried to give a powder of **13** (49 mg, 90%) as potassium salt; ^{1}H NMR δ 0.84 (3H, d, J=6.4 Hz), 0.87 (3H, d, J=6.5 Hz), 1.44 (2H, br), 1.57 (1H, m), 1.83 (3H, s), 2.53 (2H, m), 3.45 (2H, m), 3.48 (1H, d, J=9.8 Hz), 3.76 (1H, d, J=9.8 Hz), 3.95 (1H, m), 4.12 (1H, br s), 4.30 (1H, q like), 5.11 (1H, m), 7.16 (1H, m), 7.23 (2H, m), 7.32 (1H, d, J=7.0 Hz), 7.33 (2H, m), 8.05 (1H, d, J=8.3 Hz), 8.75 (1H, d, J=7.7 Hz, NH); FAB-MS m/z 498 $[M+H]^+$; HPLC, Rt=15.5 minutes. Solvent system: CH_3CN - 20 mM KH_2PO_4 15 : 85 (v/v).

Preparation of **14** from **3**

To a solution of **3** (50 mg, 0.11 mmol) in MeOH (10 ml), 10% HCl - MeOH (10 ml, Tokyo Chemical Industries Co., Ltd.) was added and the mixture was stirred at room temperature for 16 hours. The reaction mixture was concentrated, poured into water (10 ml) and adjusted to pH 6.5 with aqueous 1 M $KHCO_3$. The resulting solution was subjected to a column of HP-20 (10 ml). After washing with water (30 ml), the column was eluted with 30% aqueous 2-PrOH (30 ml) and 50% aqueous 2-PrOH (30 ml). Fractions containing **14** were combined, concentrated and lyophilized to give a powder of **14** (34 mg, 66%) as hydrochloride salt; ^{1}H NMR δ 0.86 (3H, d, J=6.6 Hz), 0.89 (3H, d, J=6.7 Hz), 1.24 (1H, ddd, J=4.8, 9.2, 13.5 Hz), 1.46 (1H, ddd, J=4.4, 9.0, 13.5 Hz), 1.75 (1H, m), 2.84 (1H, dd, J=7.4, 15.8 Hz), 2.91 (1H, dd, J=6.8, 15.8 Hz), 3.40~3.48 (3H, m), 3.51 (3H, s), 3.76 (1H, br), 3.97 (1H, br), 4.11 (1H, br d, J=4.3 Hz), 4.56 (1H, d, J=6.3 Hz), 4.63 (1H, br s), 4.90 (1H, br d, J=6.0 Hz), 5.24 (1H, br d, J=7.0 Hz), 5.27 (1H, m), 7.20~7.37 (5H, m), 7.78 (1H, d, J=7.8 Hz), 8.12 (1H, d, J=8.9 Hz); FAB-MS m/z 470 $[M+H]^+$; HPLC; Rt=9.4 minutes. Solvent system: CH_3CN - 20 mM KH_2PO_4 - H_3PO_4 (pH3.0) 20 : 80 (v/v).

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