Communications to the Editor

CHITINOVORINS A, B AND C, NOVEL β-LACTAM ANTIBIOTICS OF BACTERIAL ORIGIN

Sir:

In recent several years, isolation of β-lactam antibiotics including monobactams, a carbapenem and a cephem compound from bacterial strains has been reported[1]. In this paper, we report the isolation of chitinovorins A, B and C from a bacterial strain identified as *Flavobacterium chitinovorum* sp. nov. which are characteristic in possessing a formylamino group at the C-7 position of the cephem nucleus.

The strain was cultivated in 30-liter jar fermentors each containing 20 liters of a medium consisting of glucose 1.0%, glycerol 0.25%, starch 0.25%, soybean meal 1.0%, corn steep liquor 0.5%, yeast extract 0.1%, NaCl 0.1%, $CaCO_3$ 0.1% (pH 7.0) for 2 days at 28°C under aeration of 20 liters per minute and agitation of 200 rpm. Antibiotic activity was assayed by the pulp disk agar diffusion method using *E. coli.* LS-1 (a supersensitive mutant to β-lactam antibiotics). The active compounds in the culture filtrate (150 liters) were adsorbed on an activated carbon (Wako Chemicals) at pH 7.0 and eluted with 60% acetone at pH 3.5. The eluate was concentrated and freeze-dried to give a crude powder. It was dissolved in water and passed through a column of SP-Sephadex C-25 (Pharmacia Fine Chemicals) at pH 2.0. Chitinovorins A and B were adsorbed on the column and eluted with 0.2 M NaCl, whereas chitinovorin C appeared in the effluent and recovered with a column of Amberlite IRA-68 (Cl^-) (Rohm and Haas Co., Ltd.). Chitinovorin A (a main product) was purified by column chromatography with MCI gel CHP-20P (Mitsubishi Kasei Kogyo) followed by Hitachi gel #3019 (Hitachi Seisakusho). Chitinovorin B was isolated from the enriched fraction obtained in the above chromatographic procedure by preparative HPLC on a Nucleosil $10C_{18}$ column (Macherey-Nagel). Chitinovorin C was purified by column chromatography with QAE-Sephadex A-25 (Pharmacia Fine Chemicals) and preparative HPLC on a Nucleosil $10C_{18}$ column. Finally, chitinovorin A hydrochloride (85 mg), B hydrochloride (4 mg) and C sodium salt (3 mg) were obtained.

Chitinovorins A (**1**) and B (**2**) are basic substances and the hydrochloric acid salts are obtained as hygroscopic colorless powders. They are readily soluble in water and show positive reactions with ninhydrin and SAKAGUCHI's reagents. Chitinovorin C (**3**) is an acidic substance. The sodium salt is a colorless amorphous powder which is soluble in water and posi-

Table 1. Physico-chemical properties of chitinovorins A, B and C.

	A (**1**)	B (**2**)	C (**3**)
Molecular formula	$C_{26}H_{41}N_9O_{11}S$[a]	$C_{29}H_{46}N_{10}O_{12}S$[b]	$C_{15}H_{20}N_4O_8S$[c]
SIMS *m/z*	688 (M+1)	759 (M+1)	417 (M+1)
UV $\lambda_{max}^{H_2O}$ nm (ε)	261 (8,000)	261 (8,000)	260 (8,300)
IR (KBr) cm^{-1}	3380, 3200, 1772, 1725, 1665, 1510, 1400, 1165	3380, 3200, 1772, 1725, 1660, 1525, 1400, 1160	3400~3200, 1764, 1670~1600, 1510, 1400, 1136, 1032, 990
CD (H_2O, pH 7.0)	300, 0	305, 0	300, 0
λ (nm), [θ]	258, +45,100	259, +47,300	257, +50,400
	243, 0	243, 0	242, 0
	231, −53,100	230, −54,900	228, −55,800
	210, −23,800	211, −22,500	210, −20,100
	202, −29,300	201, −37,700	200, −30,700
	195, −22,000	195, −33,900	195, −16,500

a) From elemental analysis of the diacetyl derivative.
b) From the results of SIMS.
c) From elemental analysis of the sodium salt.

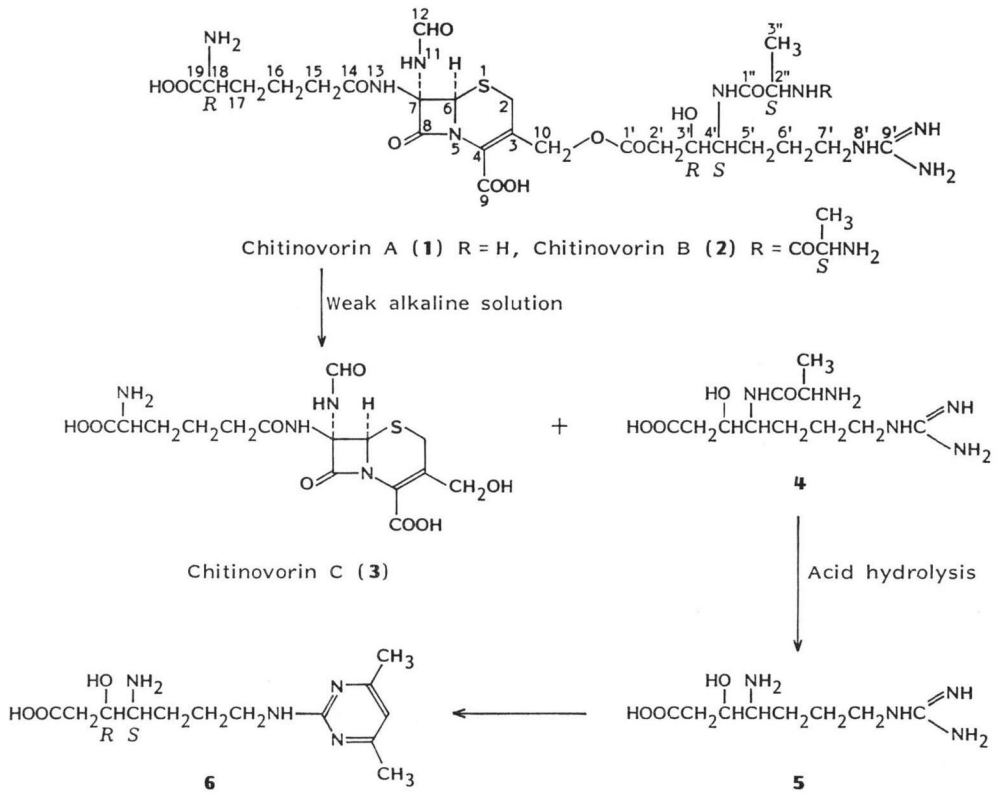
Table 2. ^{1}H NMR data of chitinovorins A and C and **4**[a] (D_2O, ref: external TMS).

Position[b]	δ (multiplicity, J)		
	Hydrochloride of A (**1**)	Sodium salt of C (**3**)	Hydrochloride of **4**
2-CH_2	2.67 (d, 17.8)	2.69 (d, 17.6)	
	2.99 (d, 17.8)	2.96 (d, 17.6)	
6-CH	4.67 (s)	4.65 (s)	
10-CH_2	4.03 (d, 12.4)	3.53 (d, 12.5)	
	4.21 (d, 12.4)	3.55 (d, 12.5)	
12-CH	7.48 (s)	7.47 (s)	
15-CH_2	1.77 (t-like)	1.76 (t-like)	
16-CH_2	~1.0 (m)	1.06 (m)	
17-CH_2	~1.25 (m)	1.23 (m)	
18-CH	3.07 (t-like)	3.05 (t-like)	
2′-CH_2	1.84 (dd-like)		1.79 (dd-like)
	2.00 (dd-like)		1.96 (dd-like)
3′-CH	~3.37 (m)		3.37 (m)
4′-CH	3.21 (m)		3.21 (m)
5′-CH_2 / 6′-CH_2	0.7~1.2 (m)		0.7~1.2 (m)
7′-CH_2	2.53 (t-like)		2.53 (t-like)
2″-CH	~3.39 (m)		3.42 (q, 7.6)
3″-CH_3	0.83 (d, 6.6)		0.87 (d, 7.6)

a) See text and Scheme 1.

b) Numbering is illustrated in Scheme 1.

Scheme 1.



tive to ninhydrin but negative to SAKAGUCHI's reaction. Some other physico-chemical properties are shown in Table 1.

By acid hydrolysis, **1** produced one mole each of α-aminoadipic acid (Aad) and Ala, *ca.* 0.5 mol of Gly and a basic compound (**5**) positive to ninhydrin and SAKAGUCHI's reactions. **2** produced Aad (1 mol), Ala (2 mol), Gly (*ca.* 0.5 mol) and **5**. **3** produced only Aad (1 mol) and Gly (*ca.* 0.5 mol). The chiralities of Aad and Ala were determined to be D and L forms, respectively, by their CD spectra. By acid hydrolysis after dinitrophenylation, **1** produced DNP-Aad and DNP-Ala. EDMAN degradation reaction on **2** indicated the presence of Ala→Ala sequence. When **1** was treated with carbonate buffer solution, pH 9.0 at 27°C for 20 hours, most of **1** disappeared and the appearance of **3** and a basic compound (**4**) was observed in appreciable yield. They were separated by chromatography on a MCI gel CHP-20P column, and the sodium salt of **3** and the hydrochloric acid salt of **4** were prepared.

The characteristic UV absorptions (261, 260 nm) and the IR absorptions (1772, 1764 cm^{-1}) of these antibiotics (Table 1) and the production of some one half mol of Gly by acid hydrolysis[2] indicated these antibiotics (**1**, **2** and **3**) have a 7-substituted cephem nucleus. Furthermore, the above degradative studies suggested that **1** is composed of two moieties, **3** and **4**, and **2** is composed of **1** and one additional Ala residue.

The ^1H NMR and ^{13}C NMR data of **1**, **3** and **4** recorded with a Varian XL-200 spectrometer are listed in Tables 2 and 3. The signals in the ^1H NMR of **3** were easily assigned by comparison with those of cephamycin C and deacetylcephalosporin C except for a singlet at 7.47. From single-frequency selective decoupling (SFSD) experiments and the ^1H-undecoupled spectrum in the ^{13}C NMR study of **3**, a ^{13}C signal at 164.3 (d, $^1J_{C-H}$=200 Hz) was observed to be coupled with the ^1H signal at 7.47. The chemical shifts and large $^1J_{C-H}$ value suggested a formylamino structure. It was also noticed that a ^{13}C signal at 77.5 (s) which should be assigned to C-7 is located at significantly higher field than the corresponding signal (95.7) observed with cephamycin C. Thus, the presence of a formylamino group at C-7 position was deduced, and all the signals in the ^1H NMR and ^{13}C NMR of **3** were assigned exactly as shown in Tables 2 and 3 based on the postulated structure in Scheme 1. The molecular formula (Table 1) agreed with the assigned structure.

The configuration at C-6 was deduced to be *R* from the CD spectrum (Table 1) which showed similar Cotton effects to that observed with cephamycin C. In order to determine the configuration at C-7, a model compound, 7β-(*R*-5-amino-5-

Table 3. ^{13}C NMR data of chitinovorins A and C and **4**[a] (D_2O, ref: internal dioxane δ=67.4).

Position[b]	δ (multiplicity)			Position[b]	δ (multiplicity)		
	Hydrochloride of A (**1**)	Sodium salt of C (**3**)	Hydrochloride of **4**		Hydrochloride of A (**1**)	Sodium salt of C (**3**)	Hydrochloride of **4**
2	26.5 (t)	26.2 (t)		18	55.3 (d)	55.3 (d)	
3	115.3 (s)	120.4 (s)		19	175.2 (s)	175.4 (s)	
4	132.9 (s)	130.7 (s)		1′	173.9 (s)		176.4 (s)
6	63.9 (d)	64.0 (d)		2′	39.2 (t)		39.1 (t)
7	77.6 (s)	77.5 (s)		3′	70.5 (d)		70.6 (d)
8	160.0 (s)	160.0 (s)		4′	54.3 (d)		54.3 (d)
9	168.9 (s)	169.4 (s)		5′	26.9 (t)[c]		26.9 (t)[c]
10	65.1 (t)	61.7 (t)		6′	25.4 (t)[c]		25.4 (t)[c]
12	164.3 (d)	164.3 (d)		7′	41.5 (t)		41.5 (t)
14	177.6 (s)	177.7 (s)		9′	157.5 (s)		157.5 (s)
15	35.3 (t)	35.3 (t)		1″	171.6 (s)		171.6 (s)
16	21.4 (t)	21.4 (t)		2″	50.0 (d)		49.9 (d)
17	30.7 (t)	30.8 (t)		3″	17.6 (q)		17.5 (q)

[a] See text and Scheme 1.
[b] Numbering is illustrated in Scheme 1.
[c] May be interchanged.

carboxyvarelylamino)-7α-formylamino-3-methyl-3-cephem-4-carboxylic acid, was stereospecifically synthesized from 7β-amino-3-methyl-3-cephem-4-carboxylic acid (data will be published elsewhere). The ^{1}H NMR and ^{13}C NMR of the model compound including a ^{1}H signal at 7.43 and a ^{13}C signal at 164.2 which arise from the 7α-formylamino group were quite similar to those of **3** except for signals due to the 3-methyl group and 2-CH_2. Thus, the stereochemistry of **3** was confirmed.

The basic compound (**4**), already shown to be derived from **1** by treatment with weak alkaline solution, was also obtained by partial acid hydrolysis of **1**. The hydrochloric acid salt was obtained as a hygroscopic colorless powder, positive to ninhydrin and SAKAGUCHI's reactions. **4** was cleaved to **5** and Ala by complete acid hydrolysis. **4** produced **5** and DNP-Ala by acid hydrolysis after 2,4-dinitrophenylation, indicating that **4** is composed of **5** and an Ala residue which linked to an amino group of **5**. **5** was considered to have an amino group and a guanidino group from its color reactions. Further, the presence of a carboxyl group was suggested by paper electrophoresis using carbonate buffer, pH 10.0, where **5** migrated to the anode. In the ^{1}H NMR study of **4**, the presence of a proton-proton coupling system (C-2′ to C-7′ in Scheme 1) was determined by detailed decoupling experiments in addition to an Ala unit. Analysis of the ^{13}C NMR of **4** and taking into account the above results lead to the postulated structure shown in Scheme 1. In the ^{1}H NMR of **5**, signals due to the methyne protons of C-3′ and C-4′ shifted to 3.70 and 2.76, but other signals did not shift appreciably, confirming that the alanylamino group is attached to C-4′ and the guanidino group to C-7′.

5 was converted to **6** by reacting with acetylacetone. **6** was obtained as a colorless crystal whose elemental analysis and mass spectral data, m/z 283 (M+1), agreed with the structure. Furthermore, X-ray diffraction analysis of **6** revealed that the relative configurations at C-3′ and C-4′ are (R*) and (S*). In order to determine the absolute configuration, the (C-3′ R, C-4′ S) compound was derived from L-ornithine (data will be published elsewhere). When the CD spectra of N-p-bromobenzoyl derivatives of the both compounds (natural and synthetic) were compared, identical Cotton effects, negative at 246 nm and positive at 228 nm, were observed.

Table 4. Antimicrobial spectra of chitinovorins A, B and C.

Organism	MIC (μg/ml)[a]		
	A	B	C
Staphylococcus aureus 209P JC-1	>200	>100	>200
S. pyogenes C-203	100	100	>200
S. pneumoniae I	50	100	>200
Escherichia coli NIHJ JC-2	25	50	100
E. coli 377 (R)[b]	50	50	100
E. coli 73 (R)[c]	50	50	100
Klebsiella pneumoniae SRL-1	50	50	200
Proteus mirabilis PR-4	50	50	100
P. vulgaris CN-329	100	100	100
Enterobacter cloacae A13047	50	50	100
Serratia marcescens A13880	200	100	200
Pseudomonas aeruginosa 25619	>200	>100	>200

[a] Agar dilution method: inoculum= one loopful of 10^6 cells/ml.
[b] Cephalosporinase producing strain.
[c] Penicillinase producing strain.

For the structure elucidation of **1**, only the problem of the linkage of **3** and **4** was left. From the difference of the chemical shifts of the ^{1}H signals attributed to the C-10 methylene between **1** and **3**, an ester linkage between the hydroxymethyl of **3** and the carboxyl of **4** was postulated (Scheme 1). An absorption at 1725 cm^{-1} shown in the IR of **1** and **2** but not in **3** (Table 1) was attributed to the ester carbonyl hydrogen-bonded to the hydroxyl group at C-3′. The molecular formula (Table 1) agreed with the postulated structure. In the ^{1}H NMR study of **1**, the proton-proton coupling systems due to Aad, Ala and **5** units were confirmed by decoupling technique (Table 2). All the ^{13}C signals of **1** were exactly assigned by SFSD experiments (Table 3), supporting the structure.

As has already been mentioned, **2** has the structure in which one additional L-Ala residue links to the L-Ala residue of **1**.

Chitinovorins A, B and C show weak inhibitory activities against Gram-negative bacteria as shown in Table 4.

Although the synthesis of a variety of β-lactam compounds possessing a formylamino group in their β-lactam rings has been reported[3,4], the occurrence of such compounds in nature has not

been reported yet.

Addendum in Proof

After submission of this paper, the isolation of two 7-formylaminocephem compounds, SQ 28,516 and SQ 28,517, from *Flavobacterium* sp. SC 12,154 has been reported[5].

Addendum from The Editorial Office

Cephabacins F_1 and F_2 (will appear in HARADA, S., *et al.*, J, Antibiotics 38(12), 1984) are identical with chitinobolins A and B.

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References

1) SYKES, R. B.; W. L. PARKER & J. S. WELLS: β-Lactam antibiotics produced by bacteria. *In* Trends in Antibiotic Research. Genetics, Biosyntheses, Actions & New Substances. *Ed.*, H. UMEZAWA *et al.*, pp. 115~124, Japan Antibiotics Res. Assoc., Tokyo, 1982
2) BRANNON, D. R.; J. A. MABE, R. ELLIS, J. G. WHITNEY & R. NAGARAJAN: Origin of glycine from acid hydrolysis of the β-lactam antibiotic A16886B. Antimicrob. Agents Chemother. 1: 242~246, 1972
3) MILNER, P. H. (Beecham): α-Formamido-substituted β-lactam compounds — Useful as broad spectrum antibacterials and intermediate. Japan Kokai 58-38,288, Mar. 5, 1983
4) PONSFORD, R. J. (Beecham): 1-Acyl-2-azetidinones. Japan Kokai 58-134,074, Aug. 10, 1983
5) SINGH, P. D.; M. G. YOUNG, J. H. JOHNSON, C. M. CIMARUSTI & R. B. SYKES: Bacterial production of 7-formamidocephalosporins. Isolation and structure determination. J. Antibiotics 37: 773~780, 1984