ISOLATION AND CHARACTERIZATION OF BULGECINS, NEW BACTERIAL METABOLITES WITH BULGE-INDUCING ACTIVITY

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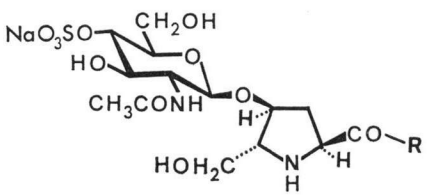
(Received for publication August 20, 1984)

Bulgecins A, B and C, new bacterial metabolites which induce the formation of bulges by cooperation with β-lactam antibiotics, were isolated from the culture broth of *Pseudomonas mesoacidophila*. The three components, separated by column chromatography on QAE-Sephadex A-25, are water-soluble acidic compounds containing a sulfate group in the molecule. Acid hydrolysis showed that D-glucosamine and a new proline derivative are common constituents of the three components. In addition, taurine and β-alanine are constituents of bulgecins A and B, respectively.

New monocyclic β-lactam antibiotics, sulfazecin and isosulfazecin, were isolated from the cultures of *Pseudomonas acidophila* and *Pseudomonas mesoacidophila*[1]. The same microorganisms were found to produce a substance, named bulgecin, which induced bulge formation in cooperation with β-lactam antibiotics[2]. Bulgecin consists of a major component, A (**1**), and two minor components, B (**2**) and C (**3**). The structures of **1**, **2** and **3** are shown in Fig. 1. The present paper deals with the isolation and chemical characterization of bulgecins.

Fig. 1. Structures of bulgecins.



1 R=$NHCH_2CH_2SO_3H$
2 R=$NHCH_2CH_2CO_2H$
3 R=OH

Isolation

Isolation of **1**, **2** and **3** from the culture broth was monitored by measurement of the bulge-inducing activity and lysis-enhancing activity[2]. In the later stage of purification, chemical methods involving thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) were also employed.

The culture broth of *P. mesoacidophila*, adjusted to pH 10, was kept at 25°C for 2 hours in order to decompose isosulfazecin. The active principles were adsorbed on a column of Amberlite IRA-68 (Cl^-). The column was washed with water and developed with 2% aqueous sodium chloride solution. Active fractions were desalted through a charcoal column, concentrated and the active principles were precipitated by addition of methanol - ethyl ether. The crude powder obtained was chromatographed on a column of QAE-Sephadex A-25 developed with 0.04 M phosphate buffer (pH 6.0). The minor component, **2**, was eluted first, followed by **3**. After desalination with a charcoal column, each was further purified by rechromatography on QAE-Sephadex to separate them completely and then subjected to gel filtration on Sephadex LH-20 with 10% aqueous methanol. Concentration and lyophilization afforded a white powder of **2** or **3**.

Table 1. Mobilities of bulgecins on TLC, HPLC and paper electrophoresis.

TLC

Solvent system	Adsorbent	Rf value		
		1	2	3
1-PrOH - H_2O (3: 1)	Silica gel*	0.33	0.27	0.26
1-BuOH - AcOH - H_2O (2: 1: 1)	Cellulose**	0.12	0.32	0.18

* Silica gel f spot film (Tokyo Kasei).
** Precoated TLC plate (Merck).

HPLC

System*	Retention time (minutes)			
	1	2	3	Sulfazecin
2% CH_3CN - 0.005 M $(n\text{-}C_4H_9)_4NHSO_4$, 0.05 M $(NH_4)_2SO_4$, pH 3.0	7.7	3.1	4.2	10.6
6% CH_3CN - 0.005 M $[(n\text{-}C_4H_9)_4N]_2SO_4$, 0.005 M CH_3COONa, pH 5.5	10.7	6.4	3.9	7.6

* Column: YMC-Pack (6×100 mm); Detection: UV, λ=214 nm; Flow rate: 2.0 ml/minute.

Paper Electrophoresis

System*	Mobility**			
	1	2	3	Glycine
0.05 M Phosphate buffer pH 6.0 (700 V, 80 minutes)	0.65	0.56	0.59	0.00
0.05 M Phosphate buffer pH 7.2 (800 V, 80 minutes)	1.00	0.94	0.82	−0.38

* LKB Multiphor, Toyo filter paper No. 51A.
** Mobilities relative to glutamic acid.

The major component, **1**, was eluted with the phosphate buffer containing 0.1 M sodium chloride by the QAE-Sephadex chromatography described above. Active fractions were desalted through a charcoal column, concentrated and lyophilized to a powder, which was purified by Sephadex LH-20 chromatography. Crystallization of the powder from aqueous methanol gave colorless needles of **1**.

Chemical Characterization

Components **1**, **2** and **3** obtained by the purification procedure mentioned above proved to be homogeneous on TLC, HPLC and paper electrophoresis. Their mobilities are shown in Table 1.

Bulgecins are acidic compounds as indicated by their behavior on ion-exchange chromatography and paper electrophoresis. They are soluble in water, dimethylformamide and dimethyl sulfoxide and slightly soluble in methanol, but insoluble in ethyl acetate and chloroform. They show a positive color reaction to Greig-Leaback reagent, but only **3** is positive to the ninhydrin test. The physico-chemical properties of **1**, **2** and **3** are summarized in Table 2.

Bulgecins showed no UV absorption above 210 nm. The IR spectrum of **1** (Fig. 2) showed intense amide peaks at 1660 and 1560 cm^{-1}, and absorptions at 1220 and 820 cm^{-1}, indicative of the presence of sulfate (OSO_3^-),[3] which agrees with the acidic nature of **1**.

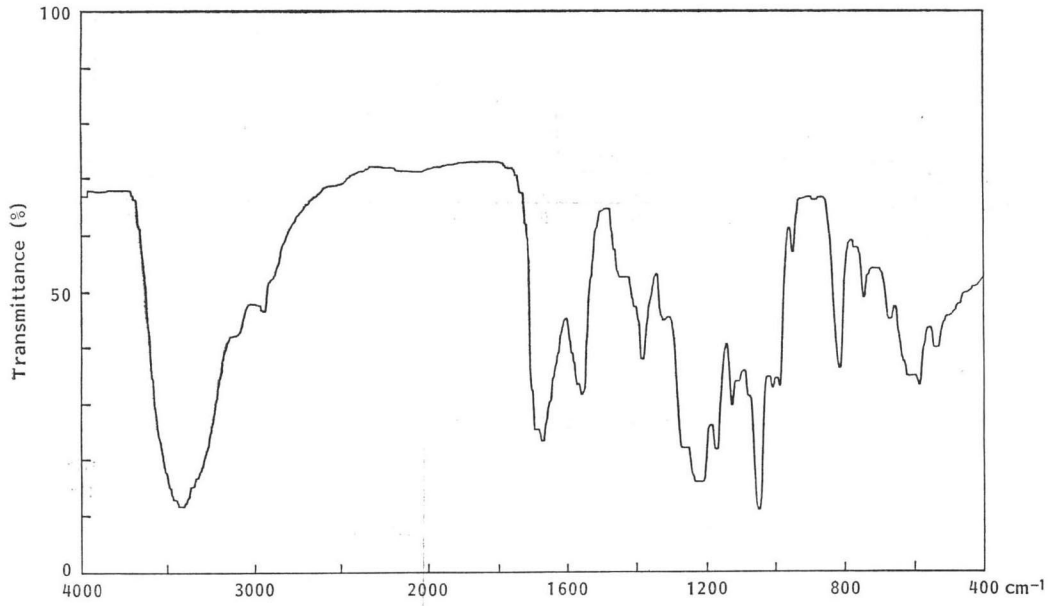
The ^1H NMR spectrum of **1** (Fig. 3) revealed the presence of an anomeric proton (4.45 ppm), two amide protons (7.76, 8.36 ppm) and an *N*-acetyl group. The ^{13}C NMR spectrum of **1** (Fig. 4) showed 16 carbon signals, including the signal at 101.1 ppm (d) assignable to an anomeric carbon. Other com-

Table 2. Physico-chemical properties of bulgecins.

	1	2	3
Appearance	Colorless needles	White powder	White powder
MP	211~212°C		
$[\alpha]_D^{20}$ (1 N AcOH)	+6.5° (*c* 0.5)	−2.6° (*c* 0.5)	+2.9° (*c* 0.6)
MS $(M+H)^+$ *m/z*	574*	538**	467*
Molecular formula	$C_{16}H_{28}N_3O_{14}S_2Na$	$C_{17}H_{28}N_3O_{13}SNa$	$C_{14}H_{23}N_2O_{12}SNa$
IR (KBr) (cm^{-1})	3420, 1660, 1560, 1220, 1050, 820	3420, 1670, 1570, 1250, 1050, 820	3420, 1650, 1560, 1250, 1050, 820
^1H NMR (90 MHz, DMSO-d_6) δ(ppm)			
$NCOCH_3$	1.83	1.82	1.83
Anomeric H	4.45	4.35	4.40
Amide H	7.76, 8.36	7.75, 8.00	7.84
^{13}C NMR (25 MHz, D_2O) Number of carbon	16	17	14

* FAB-MS, ** SIMS.

Fig. 2. IR spectrum of **1** (KBr).



ponents showed spectra similar to those of **1** (Table 2).

The molecular formulae of $C_{16}H_{28}N_3O_{14}S_2Na$ for **1**, $C_{17}H_{28}N_3O_{13}SNa$ for **2** and $C_{14}H_{23}N_2O_{12}SNa$ for **3** were determined by elemental analysis and FAB mass spectrometry.

Acid Hydrolysis

Acid hydrolysis of **1** with 5.5 N HCl at 110°C for 8 hours yielded three ninhydrin-positive compounds, which were isolated by ion-exchange chromatography. Two of them were identified as D-glucosamine and taurine by direct comparison with authentic samples. Crystallization of the unidentified compound from aqueous methanol afforded colorless prisms (**4**). Compound **4**, mp 182°C,

Fig. 3. ^{1}H NMR spectrum of **1** (90 MHz, DMSO-d_6).

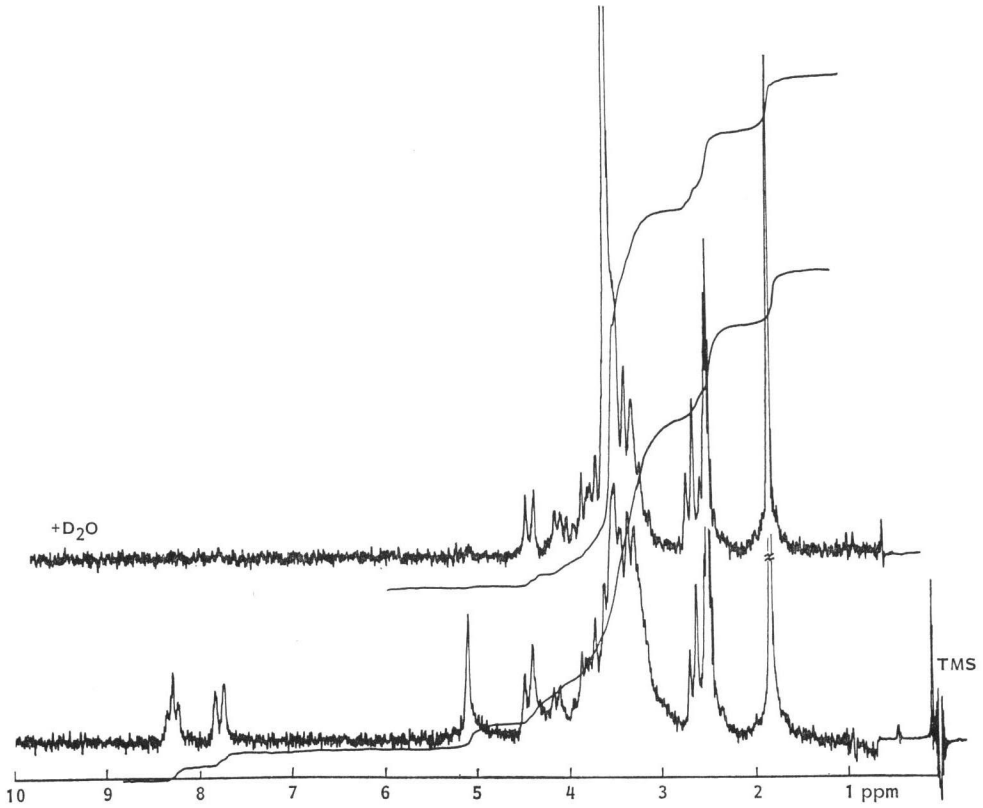


Fig. 4. ^{13}C NMR spectrum of **1** (25 MHz, D_2O).

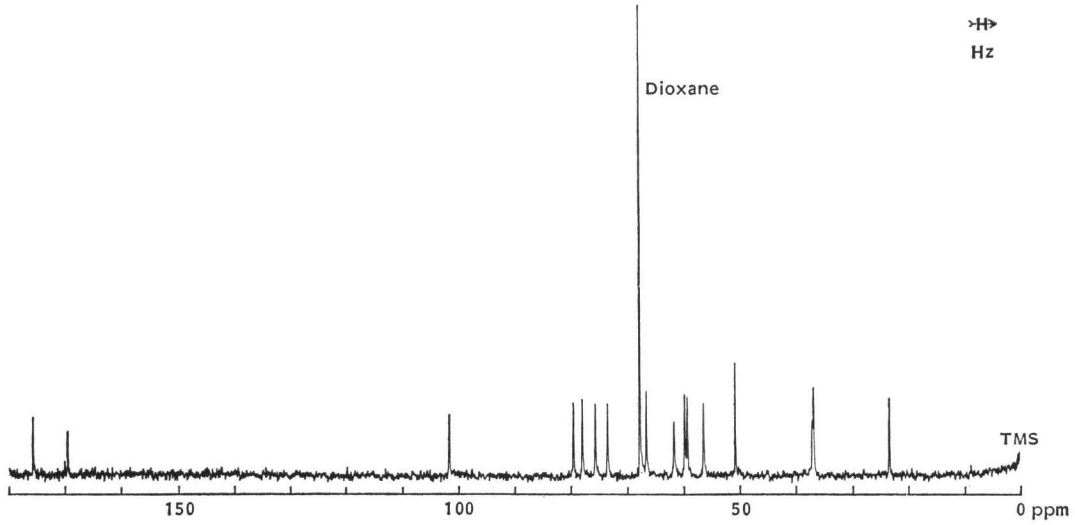
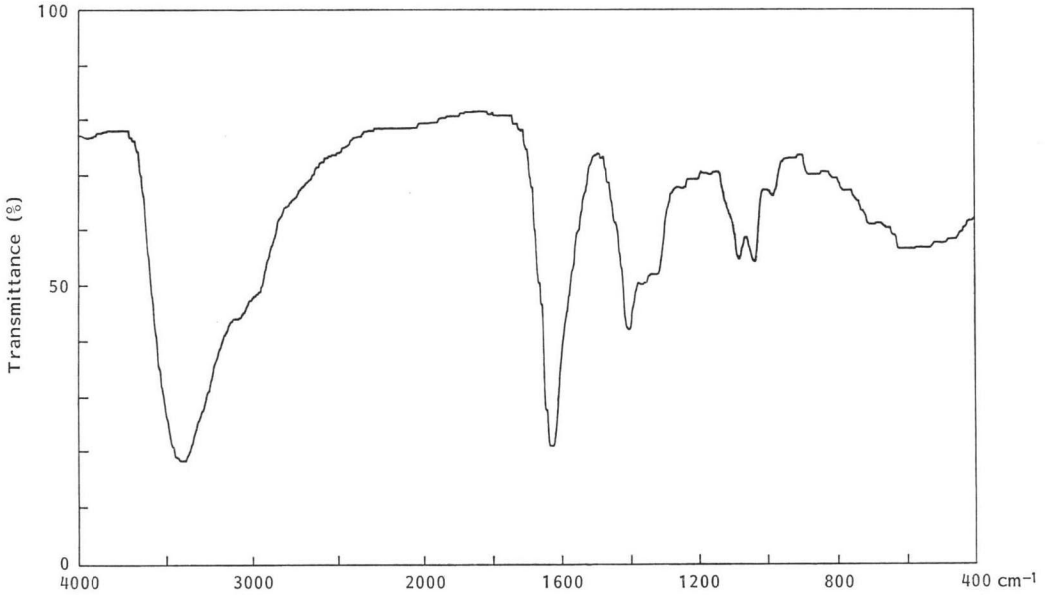


Fig. 5. IR spectrum of **4** (KBr).



$[\alpha]_D^{24}$ −13.1° (*c* 0.95, H_2O), *pKa'* 2.6 and 8.5, had an IR spectrum indicating the presence of carboxylate (1620 and 1410 cm^{-1}) as shown in Fig. 5. The molecular formula of $C_6H_{11}NO_4$ for **4** was determined by microanalysis and mass spectrometry (*m/z*, MH^+ 162). The pink yellow color-reaction with the ninhydrin reagent suggested that **4** was an iminocyclic acid. Furthermore, the ninhydrin complex of **4** in acetic acid showed a characteristic absorption spectrum with a maximum at 350 nm, indicating **4** to be a proline derivative, not a pipecolic acid derivative[4].

Table 3. Ratios of constituents of bulgecins.

Constituent	1	2	3
D-Glucosamine	1.07	1.00	1.00
4	0.82	0.94	0.92
Taurine	1.00	0.00	0.00
β-Alanine	0.00	1.00	0.00
Recovery	88	85	87

Bulgecins were hydrolyzed with 6 N HCl at 110°C for 18 hours. Analyses of hydrolysates were carried out with a Hitachi 835 amino acid analyzer.

Consideration of all of these findings showed that **4** is a new proline derivative. Its molecular formula differs from those of other reported proline derivatives of natural origin including 4-hydroxyproline[5], *allo*-4-hydroxyproline[6], 3-hydroxyproline[7], 4-hydroxymethylproline[8], 3,4-dihydroxyproline[9,10], 3-hydroxy-5-methylproline[11] and 3-hydroxy-4-methylproline[12].

Acid hydrolysis of both **2** and **3** gave D-glucosamine and **4**. Moreover, β-alanine was recovered from acid hydrolysates of **2** as shown in Table 3.

Discussion

In addition to inducing the formation of bulges, bulgecin A (**1**) enhanced the lytic activity of some β-lactam antibiotics, although showing no antibacterial activity by itself[2]. Bulgecins B (**2**) and C (**3**) were found to have similar but slightly weaker activity than bulgecin A. Bulgecin A is not toxic; the LD_{50} was estimated to be more than 1,000 mg/kg by intravenous injection in mice.

The physico-chemical properties and acid hydrolysis of **1**, **2** and **3** indicated that **1** is a new sulfated glycopeptide composed of D-glucosamine, taurine and a novel amino acid, and that **2** and **3** are analogs of

1. Structural studies of bulgecins, including the new proline derivative will be described elsewhere[13].

Experimental

Melting point determinations were performed with a Yamato Model MP-21 melting point apparatus. IR spectra were recorded with a Hitachi 285 grating infrared spectrophotometer. Rotations were determined with a Jasco Dip-181. NMR spectra were obtained using a Varian XL-100 or EM-390 instrument; chemical shifts (δ) are reported in ppm downfield from an internal TMS reference. Mass spectra were determined with a Jeol LMS-DX 300 and a Hitachi M-80A instrument.

Isolation of 1, 2 and 3

P. mesoacidophila SB-72310 was cultivated in a 200-liter fermentor in the same manner as described for the production of isosulfazecin[14]. The culture broth (103 liters) was adjusted to pH 10 with 4 N NaOH and kept to stand at room temperature for 2 hours. The mixture was applied to a column (30 liters) of Amberlite IRA-68 (Cl^-). The column was washed with water (45 liters) and elution was carried out with 2% aq NaCl (240 liters). The eluate was desalted through a charcoal column (15 liters), which was washed with H_2O (45 liters) and the active materials were eluted with 7% aq 2-BuOH (75 liters). The eluate was concentrated to a small volume and the product was precipitated as a powder by addition of MeOH - ether (1 : 1). This powder (24 g) was dissolved in H_2O (200 ml) and applied on a column (1 liter) of QAE-Sephadex A-25 (Cl^-). Elution was effected with 0.04 M phosphate buffer (pH 6.0) (11 liters) and then with the same buffer containing 0.1 M NaCl. Each fraction (1 liter) of eluate was tested for activity and by HPLC. **2**, **3** and **1** were eluted from the column in fractions of 3~4, 7~9 and 12~14, respectively.

The fractions containing **2** were desalted through a charcoal column (400 ml), concentrated and lyophilized to give a powder (810 mg). This was rechromatographed on QAE-Sephadex A-25 (100 ml) in 0.04 M phosphate buffer (pH 6.0). The active fractions were desalted through a charcoal column (50 ml) and concentrated to a small volume, which was applied to a column of Sephadex LH-20 (550 ml). The column was developed with 10% aq MeOH. The fractions giving a single peak by HPLC were concentrated and lyophilized to give 37 mg of **2** as a white powder. Component **3** was also obtained as a white powder from the fractions containing **3** in the same way as described for **2**; yield, 155 mg.

The fractions containing **1** on the above QAE-Sephadex A-25 chromatography were passed through a charcoal column (500 ml). The column was washed with H_2O and **1** was eluted with 8% 2-BuOH (2 liters). The eluate was concentrated and solidified by addition of MeOH - ether. The solid (2.6 g) was chromatographed on a column of Sephadex LH-20 (1,500 ml) with 10% aq MeOH. The fractions containing **1** were concentrated and lyophilized to give a powder, which was crystallized from MeOH - H_2O to afford 1.39 g of analytically pure **1** as colorless crystals.

1: *Anal* Calcd for $C_{16}H_{28}N_3O_{14}S_2Na \cdot H_2O$: C 32.48, H 5.11, N 7.10, S 10.84, Na 3.88.
Found: C 32.25, H 5.47, N 7.20, S 10.86, Na 3.70.

2: *Anal* Calcd for $C_{17}H_{28}N_3O_{13}SNa \cdot H_2O$: C 36.76, H 5.44, N 7.56, S 5.77, Na 4.14.
Found: C 36.40, H 5.78, N 7.70, S 5.76, Na 4.00.

3: *Anal* Calcd for $C_{14}H_{23}N_2O_{12}SNa \cdot \frac{1}{2}H_2O$: C 35.37, H 5.09, N 5.89, S 6.74, Na 4.84.
Found: C 35.31, H 5.35, N 5.96, S 6.72, Na 4.80.

Acid Hydrolysis

A solution of **1** (1.2 g) in 5.5 N HCl (50 ml) was refluxed for 8 hours. The mixture was evaporated to dryness. The residue was dissolved in water (20 ml) and the solution, adjusted to pH 7.0, was applied to a column (3.3 × 50 cm) of CM-Sephadex C-25 (H^+). The column was developed with 0.02 M ammonium acetate buffer (pH 6.8). The fractions from 180 to 280 ml were combined and passed through an activated charcoal column (3.5 × 30 cm). After elution with water, the fractions (350~550 ml) were collected and evaporated, and the resulting residue was crystallized from MeOH - H_2O to afford 273 mg of **4**; mp 182°C, $[\alpha]_D^{24}$ −13.1° (*c* 0.95, H_2O).

Anal Calcd for $C_6H_{11}NO_4$: C 44.71, H 6.88, N 8.69.
Found: C 44.78, H 6.92, N 8.62.

The fractions from 300 to 380 ml from the above CM-Sephadex column were combined and passed through an activated charcoal column (3.5×25 cm). Elution was carried out with water and the fractions from 200 to 450 ml were collected and evaporated. The residue was crystallized from MeOH and recrystallized from MeOH - H_2O to give needles of taurine; 292 mg, mp 306~308°C (dec).

Anal Calcd for $C_2H_7NO_3S$: C 19.19, H 5.64, N 11.19, S 25.62.
Found: C 19.09, H 5.52, N 11.15, S 25.17.

Further material was eluted from the column of CM-Sephadex C-25 with 0.2 N AcOH (1 liter) and the eluate was evaporated. The residue was dissolved in water (50 ml) and applied to an activated charcoal column (3.5×30 cm). After elution with H_2O, the fractions from 400 to 1,000 ml were collected and concentrated to a small volume, to which 1 N HCl (2 ml) was added. The mixture was evaporated to give crystals, which were recrystallized from MeOH - H_2O to give 137 mg of D-glucosamine·HCl, mp 200~202°C, $[\alpha]_D^{22}$ +88.5° to 76.6° (*c* 1.0, H_2O).

Anal Calcd for $C_6H_{13}NO_5 \cdot HCl$: C 33.41, H 6.54, N 6.49, Cl 16.46.
Found: C 33.36, H 6.54, N 6.51, Cl 16.38.

Acknowledgment

We are grateful to Drs. K. MORITA, M. YONEDA and T. KISHI for their interest throughout this work. Thanks are also due to the members of pilot plant and physical analysis sections for their support, to Miss K. TERAO for amino acid analysis and to Mr. Y. NOHARA for his excellent assistance.

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