OF4949, NEW INHIBITORS OF AMINOPEPTIDASE B

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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New aminopeptidase B inhibitors that we named OF4949-**I**, **II**, **III** and **IV** were isolated from the culture broth of a fungus, *Penicillium rugulosum* OF4949. The molecular formula of **I** was $C_{23}H_{26}N_4O_8$ and that of **II**, $C_{22}H_{24}N_4O_8$, judging from elemental analysis and secondary ion mass spectrometry. The concentrations of **I**, **II**, **III** and **IV** required for 50% inhibition of aminopeptidase, using Ehrlich ascites carcinoma cells as the source of the enzyme, were 0.0054, 0.0048, 3.4 and 1.7 μg/ml, respectively. Components **I** and **II** augmented delayed-type hypersensitivity in mice to sheep red blood cells.

Aminopeptidase B (EC 3.4.11.6) hydrolyzes peptides containing L-arginine or L-lysine as a *N*-terminal amino acid, to yield *N*-terminal basic amino acids. This enzyme is widely distributed among mammalian tissues and cells including rat liver, bovine pituitary gland, tumor cells, polymorphonuclear leucocytes, macrophages, and lymphocytes.[1~4] Reports by AOYAGI *et al.* have also suggested that aminopeptidase, like phosphatase and esterase, is located not only in the cytoplasm but also on the cell membrane, without being released extracellularly.

The specific inhibitors of aminopeptidase B, such as bestatin, arphamenines A and B, and α-aminoacylarginine, bind to the cell surface and modify the functions of immunoresponsive cells, enhancing delayed-type hypersensitivity (DTH) and other cellular immune responses as well.[5~9]

We screened for aminopeptidase B inhibitors that modify cellular immune responses in the products of fungi, actinomycetes, and bacteria using as the source for this enzyme intact Ehrlich ascites carcinoma (EAC) cells, and found OF4949-**I**, **II**, **III** and **IV** in the product of a fungus, *Penicillium rugulosum* OF4949. These are the first inhibitors of aminopeptidase discovered in the products of fungi. This paper deals with the taxonomy of the organism, fermentation, isolation, physico-chemical properties, and biological properties of these compounds. The investigation of their structures is reported elsewhere.[10]

Materials and Methods

General

UV spectra were recorded on a Shimadzu double-beam UV-200 spectrometer, and IR spectra on a Hitachi 270-30 spectrometer. Optical rotation was measured by use of a Jasco DIP-181, and potentiometric titration was obtained on a Toa autotitrator HTS-10A. ^{13}C (50.3 MHz) and ^{1}H (200 MHz) NMR spectra were measured on a Varian XL-200. Chemical shifts were studied with reference to internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) for ^{1}H NMR and *p*-dioxane for ^{13}C

NMR. Secondary ion mass spectra were measured on a Hitachi M-80A.

For the analysis of the active fractions, high-performance liquid chromatography (HPLC) was done on a YMC-GEL A-312 ODS (Yamamura Chemical Co.) or Nucleosil $5C_{18}$ (Macherey-Nagel Co.) column using a Shimadzu Model LC-3A pump. Preparative HPLC was done on a Prep PAK $500/C_{18}$ (Waters Co.) or YMC-GEL ODS-1 column (Yamamura) using a Waters System 500 pump, or on an Aquasil SS-352N column (Senshu Kagaku Co.) using a Altex 100 A pump.

Screening

To test inhibition by culture broth, strains of fungi, actinomycetes, and bacteria isolated from soil samples were inoculated into a 100-ml Erlenmeyer flask containing 20 ml of sterile growth medium. The culture media were: for fungi, glucose 3.0%, Polypepton 0.5%, yeast extract 0.1%, KH_2PO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05% (pH 5.7); for actinomycetes, potato starch 1.0%, glucose 1.0%, soybean meal 1.5%, yeast extract 0.5%, NaCl 0.2% and $CaCO_3$ 0.3% (pH 7.2); and for bacteria, glucose 1.0%, glycerol 1.0%, Polypepton 1.0%, yeast extract 0.1%, KH_2PO_4 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05% (pH 7.2). Cultivation was at 27°C for 4~7 days on a rotary shaker. Culture broth was extracted by addition of an equal volume of methanol. The extract (10 μl) was added to the reaction mixture to measure its inhibition of aminopeptidase B from EAC. All together, 500 strains of fungi, 463 strains of actinomycetes, and 495 strains of bacteria were tested.

Taxonomic Studies

The taxonomic characterization of strain No. OF4949 was done using the media and methods described by RAPER and THOM[11] and ABE.[12] The fungal specimens were prepared for scanning electron microscopy as follows. An area of the colony having many spores, being cultivated on CZAPEK's agar or malt - extract agar for 5 to 10 days, was cut out and fixed in 1.0% glutaraldehyde solution for 2 hours. After being dehydrated by washing for 15 minutes each time in increasing concentrations of acetone, samples were rinsed in absolute isoamyl acetate, and dried to the critical point in CO_2 in an automatic critical-point apparatus (Hitachi Koki Co., Ltd., HCP-2). The specimens were coated with platinum - palladium in a vacuum evaporator and observed under a Hitachi S-501 scanning electron microscope at a voltage of 15 to 30 kV.

Preparation of EAC Cells

EAC cells were maintained in *ddY* mice (Shizuoka Agricultural Cooperative Association) by ip injection of 2×10^6 cells/mouse. After 7 days, EAC cells were obtained from the mice and were treated with Tris buffer (pH 7.2) and ammonium chloride to burst the red blood cells. After being washed three times with HANKS' balanced salt solution (Nissui Pharmaceutical Co.), the EAC cells were suspended in the same buffer at a concentration of 2.5×10^7 cells/ml. Microscopic examination of the washed cells showed that 96% or more of the cells were viable EAC cells; the rest were leucocytes.

Assay of Aminopeptidase B Inhibition

We used a modification of the method of HOPUS *et al.*[13] to measure aminopeptidase B activity. The incubation mixture consisted of 0.1 ml of 3 mM L-arginine-β-naphthylamide (Sigma Chemical Co.), 0.7 ml of HANKS' balanced salt solution, and 10 μl of water, with or without the inhibitor. After 3 minutes of incubation at 37°C, 0.2 ml of an EAC cell suspension was added. Exactly 30 minutes later, incubation was stopped by adding 3 ml of the stabilized diazonium salt Garnet GBC (0.3 mg/ml; Sigma) in 1.0 M acetate buffer at pH 4.2 containing 3% Tween 20. The mixture was left for 15 minutes at room temperature and centrifuged; then its absorbance was measured at 525 nm using a Shimadzu UV-730 colorimeter. The percentage of inhibition can be calculated from the absorbance with (A) and without (B) inhibitor, by the following equation:

$$\text{Percentage of inhibition} = \frac{B-A}{B} \times 100$$

DTH Response

CDF_1 mice (BALB/C×DBA/2, males 10 weeks old, Shizuoka Agricultural Cooperative) were immunized by an iv injection of 10^5 sheep red blood cells. OF4949-I and II were given by ip injection at the time of immunization. After 96 hours, DTH was elicited by injecting 10^8 sheep red blood cells

Table 1. Morphology of the organism.

Penicilli	Biverticillate and usually symmetrical	30~80×2.5~3.0 μm
Conidiophores metulae	Sometimes branched	10~13×2.5~3.0 μm
Sterigmata	Lanceolated with accuminate tips	10~13×2.5~3.0 μm
Conidia	Elliptical or ovalate	3.0~4.0×2.5~3.5 μm

Table 2. Cultural characteristics at 27°C after 14 days of growth.

	Growth (colony size)	Color	Remarks
CZAPEK's agar	10~16 mm, velvety	Pale yellow or dusty yellow reverse, pale yellow	Grew more slowly than on other media, no soluble pigments
Malt extract agar	20~24 mm, velvety	Dusty dull green reverse, lemon yellow	No soluble pigments
Potato dextrose agar	27~29 mm, velvety	Dusty yellowish green reverse, orange citrin	No soluble pigments
Malt - juice agar	33~35 mm, velvety	Dull green in the central area, pale yellow in the marginal area reverse, raw sienna	Grew more quickly than on other media, strongly wrinkled, no soluble pigments
Steep agar	20~23 mm, velvety	Dusky dull green or dusky yellowish green reverse, orange yellow in the central area, or colorless	No soluble pigments

into the footpad of the left hind paw, and 24 hours thereafter, the resulting edema of that paw was measured with calipers.

Results

Screening

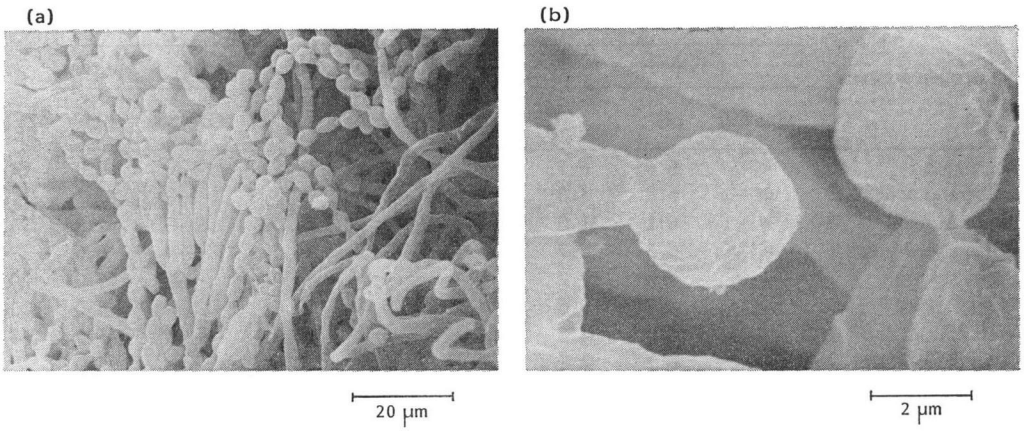
Of the many organisms tested, ten strains of fungi, one strain of actinomycetes, and five strains of bacteria inhibited the aminopeptidase B from EAC by 50% or more. Among these 16 strains, strain No. OF4949 was selected for further study because it produced the strongest inhibitor of low molecular weight.

Taxonomy of the Organism

The organism that produced the OF4949 complex was obtained from a soil sample collected in Kyoto Prefecture, Japan. For the identification of the fungus, we prepared CZAPEK's agar, malt - extract agar, potato agar, malt - juice agar and steep agar. A stock culture of the isolate was inoculated on to these media and incubated at 24, 27 and 37°C, and the cultures were observed for about 30 days. The cultural characteristics and morphology of the organism are summarized in Tables 1 and 2, respectively. Conidiophores arose from the basal felt, and were sometimes branched. Penicilli were typically biverticillate and symmetrical but were sometimes irregular, consisting of a terminal verticile of 5 to 9 metulae. Each metulae bore 5 to 8 terminal sterigmata that lanceolated with an acuminate tip. The conidia had echinulate or verruculated walls and were elliptical or oval, in tangled chains 30 to 80 μm in length (Fig. 1).

This organism, strain No. OF4949, was identified as *Penicillium rugulosum* Thom by comparison

Fig. 1. Scanning electron micrograph of Penicillia (a) and spore (b) of *Penicillium rugulosum* OF4949.



of the cultural characteristics and morphology with those given by RAPER and THOM[11] and also by ABE[12] for the type species, and with *P. rugulosum* Thom IFO 4683, 5746 and 6965 from the Institute for Fermentation, Osaka, Japan. The specific strain has been deposited in the Fermentation Research Institute Collection, at the Agency of Industrial Science and Technology, Japan, under the name *P. rugulosum* OF4949, with the accession No. FERM BP-203.

Fermentation

When fermentation was done in 500-ml Erlenmeyer flasks using basal medium containing glucose 2.0%, Polypepton 1.0%, yeast extract 0.1%, KH_2PO_4 0.05% and $MgSO_4 \cdot 7H_2O$ 0.05%, pH 5.7, the productivity of the *P. rugulosum* OF4949 was very low, about 5 μg/ml of culture broth. Productivity was improved by modifying the fermentation medium and the culture conditions. Examples of the experiments on the influence of nitrogen and carbon sources by modification of the basal medium are in Table 3. The amount of the inhibitors of aminopeptidase B in the culture broth was measured by enzyme inhibition assays using purified OF4949-**I** as the standard. We selected as the optimum production medium, one consisting of maltose 12.0%, soybean meal 2.0%, yeast extract 0.1%, KH_2PO_4 0.05% and $MgSO_4 \cdot 7H_2O$ 0.05% (pH 5.9).

The effect of temperature on OF4949 production was investigated within the temperatures of 3 to 40°C, using a temperature gradient incubator. Production of OF4949 increased with rising temperature, reaching a maximum at 27~30°C.

A seed culture shaken for 4 days in a 2-liter Erlenmeyer flask containing 500 ml of the basal medium was inoculated into a 30-liter jar fermentor containing 15 liters of the production medium. Fermentation was carried out at 27°C for 7 to 9 days. The optimal aeration was 1.0 vol/vol/minute and the optimal agitation, 450 rpm.

Fig. 2 shows the typical course of OF4949 production when *P. rugulosum* OF4949 was cultured using production medium in a jar fermentor under optimum culture conditions. OF4949 accumulation reached a maximum of about 63 μg/ml on the eighth day of incubation.

Isolation

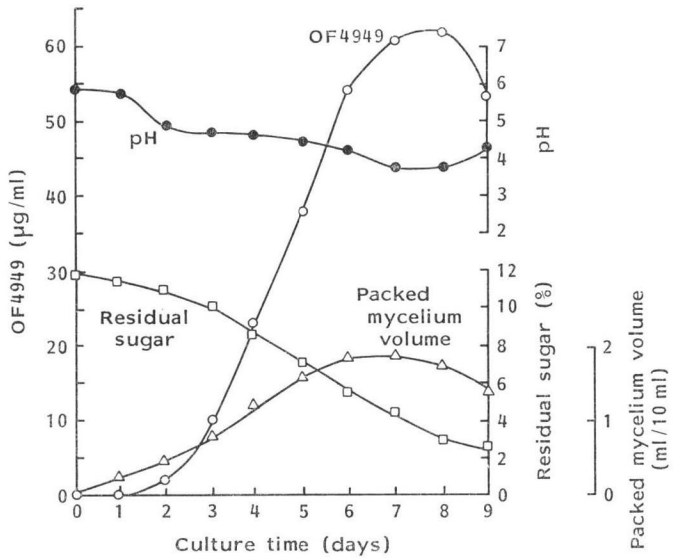
The extraction and purification processes are shown in Scheme 1. The OF4949 was isolated from the culture filtrate and from the mycelia as follows. After 8 days of fermentation, 700 liters of

Table 3. Effect of nitrogen and carbon sources on OF4949 production by *Penicillium rugulosum* OF4949.

Nitrogen sources	Carbon sources								
	Glucose			Galactose			Maltose		
	Growth	pH	OF4949 (μg/ml)	Growth	pH	OF4949 (μg/ml)	Growth	pH	OF4949 (μg/ml)
Polypepton	++	6.6	6.1	++	6.0	9.1	++	6.0	13.4
Casamino acid	++	6.8	3.6	++	6.7	12.5	++	5.2	14.5
Soybean meal	++	6.1	21.0	++	5.3	28.3	++	4.9	26.8
Cotton seed meal	++	6.3	15.7	++	7.3	16.5	++	6.6	17.9
Bacto soytone	++	6.0	17.2	++	5.8	24.0	++	5.0	20.2
Meat extract	++	5.7	6.5	++	5.4	12.6	++	6.0	13.6
Malt extract	++	6.0	4.3	++	5.8	2.9	++	5.9	4.1
Corn steep liquor	++	6.2	2.1	++	6.0	3.6	++	6.0	4.7
Na-glutamate	+	6.4	2.1	++	6.2	3.1	+	5.7	2.9
L-Aspartic acid	+	6.0	2.7	+	6.0	2.1	+	5.8	4.4
L-Tyrosine	+	6.3	3.6	+	6.3	4.7	+	6.0	6.1
L-Phenylalanine	+	6.5	2.2	+	6.3	3.7	+	6.2	6.8
NH_4NO_3	±	6.0	1.6	±	6.0	4.3	±	6.1	6.5
None	±	5.9	3.5	±	6.0	3.7	±	6.0	2.9

The concentrations of the carbon and nitrogen sources were 2.0% and 1.0%, respectively.
Degree of growth: ++; abundant growth, +; moderate growth, ±; poor growth.

Fig. 2. OF4949 production using *Penicillium rugulosum* OF4949.



culture broth of *P. rugulosum* OF4949 was filtered with the aid of Silica No. 600S (Chuo Silica Co.). The mycelial cake was extracted three times with three volumes of 50% acetone. The extracts were combined and partially evaporated under reduced pressure to give an aqueous concentrate. After the pH was adjusted to 2.0, both the culture filtrate and the mycelial extract were put on a Diaion SK-104 column and eluted with 1 N ammonia. The active fractions of the eluate were adjusted to pH 5.0 with 20% H_2SO_4 and adsorbed on a Diaion HP-20 column. The greater part of the active substances was eluted with 50% methanol. Active fractions were concentrated under reduced pres-

Scheme 1. Purification procedure.

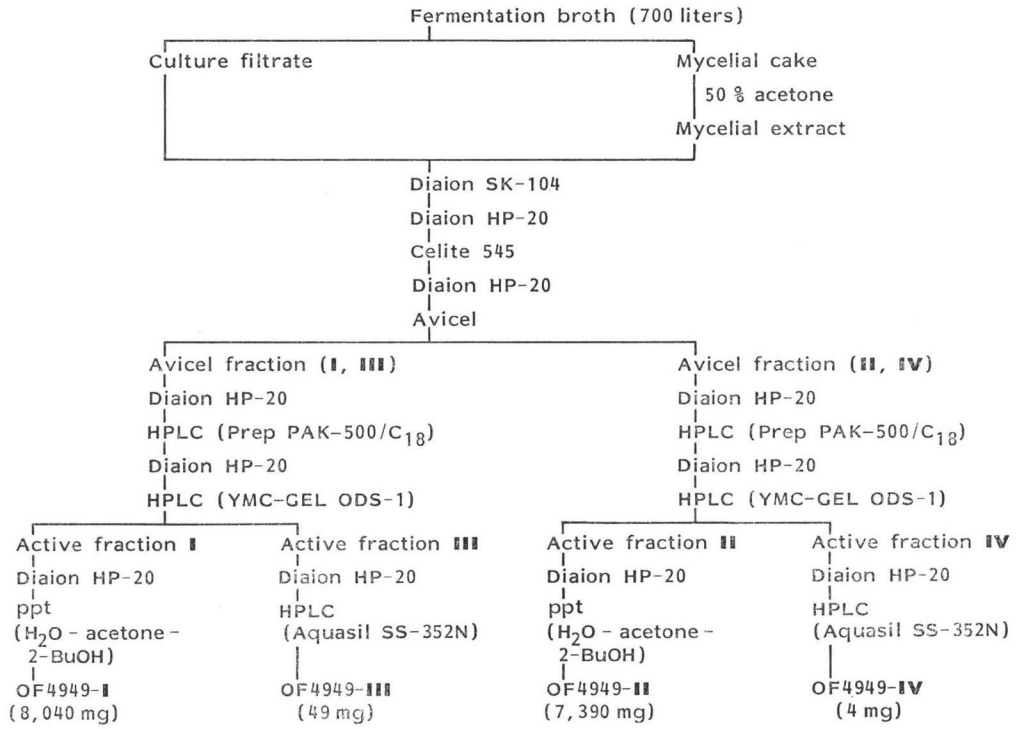


Table 4. Silica gel TLC of OF4949 complex.

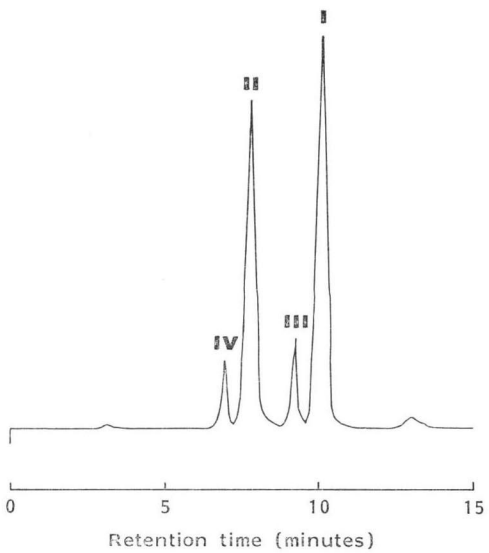
Component	Rf value	
	PrOH - 28% NH_4OH (2:1)	EtOH - 50 mM NaOAc (3:1)
OF4949-I	0.41	0.28
OF4949-II	0.32	0.24
OF4949-III	0.45	0.38
OF4949-IV	0.37	0.35

TLC on a precoated Merck 60 F_{254} TLC plate 0.25 mm thick.

Detection by color reaction with ninhydrin, or UV.

Fig. 3. HPLC of OF4949 complex.

Conditions: Column; YMC-GEL A-312 ODS, 4×150 mm. Mobile phase; 0.1 M citrate buffer (pH 5.4) - acetonitrile (95:5). Flow rate; 1.0 ml/minute. Detection; UV 275 nm.



sure, giving 162 g of crude material. This was put on a Celite 545 column equilibrated with butanol, and developed with a mixture of butanol and 1 N ammonia (15: 85). After being desalted on a Diaion HP-20 column, the active fractions were concentrated, giving 34.5 g of crude material. The crude material was put on a column of Avicel, and was chromatographed with the same solvent system as that for the

Table 5. UV spectra of OF4949-I, II, III and IV.

λ_{max} nm (ε)	OF4949			
	I	II	III	IV
H_2O	213 (14,770),	214 (13,600),	213 (14,760),	214 (13,560),
	230 (sh, 9,840),	—	231 (sh, 9,760),	—
	273 (2,240),	272 (2,870),	273 (2,360),	272 (2,800),
	283 (sh, 1,940)	279 (2,880)	282 (sh, 1,980)	279 (2,840)
0.05 N HCl	213 (14,570),	213 (13,170),	213 (14,520),	214 (13,150),
	228 (sh, 9,840),	—	230 (sh, 9,760),	—
	273 (2,240),	272 (2,640),	273 (2,360),	272 (2,620),
	283 (sh, 1,940)	279 (2,690)	283 (sh, 1,940)	279 (2,640)
0.05 N NaOH	214 (14,970),	218 (14,740),	214 (14,830),	218 (14,650),
	—	242 (sh, 8,260),	—	243 (sh, 8,200),
	273 (2,280),	—	273 (2,380),	—
	283 (sh, 1,940)	297 (3,210)	283 (sh, 1,980)	297 (3,180)

Celite 545 column. Inhibitory activity appeared in two separate fractions. The active fraction that eluted first contained **I** and a trace amount of **III**; the second active fraction contained **II** and a trace amount of **IV**. The fractions were desalted separately on a Diaion HP-20 column; the first fraction gave 13.2 g and the second, 12.5 g of a crude mixture.

The crude mixture containing **I** and **III** was chromatographed on a Prep PAK-500/C_{18} column with 0.1 M citrate buffer, pH 5.7, containing 7.0% acetonitrile, then on a YMC-GEL ODS-1 column with 0.05 M sodium acetate containing 5.0% acetonitrile. The eluted fractions were assayed for enzyme inhibition and examined by HPLC. The fractions containing **I** and **III** were desalted separately on a Diaion HP-20 column and freeze-dried. In the final step, **I** was purified by precipitation with a mixture of water, acetone, and 2-butanol (1 : 3 : 2) to afford **I** as a colorless powder (8.04 g). Component **III** was purified by preparative HPLC on an Aquasil SS-352N column with ethanol - 0.05 M sodium acetate (5 : 1), giving pure **III** (49 mg).

The crude mixture containing **II** and **IV** was put on a Prep PAK-500/C_{18} column, with 0.05 M citrate buffer, pH 5.7, containing 2.0% acetonitrile. The active fraction was desalted on a Diaion HP-20 column and concentrated, and then fractionated by chromatography on a YMC-GEL ODS-1 column with 0.05 M sodium acetate containing 2.0% acetonitrile. The fractions containing **II** and **IV** were separately collected, and desalted on a Diaion HP-20 column. Component **II** was then precipitated from a mixture of water, acetone and 2-butanol (1 : 3 : 2), giving pure **II** (7.39 g). The fractions containing **IV** were finally purified by HPLC on an Aquasil SS-352N column. The peak fraction of the component was collected, desalted, and concentrated, giving essentially pure **IV** (4.3 mg).

Physico-chemical Properties

The four components of OF4949 had similar physico-chemical and spectroscopic properties. They were all soluble in dimethyl sulfoxide, ammonia and water, slightly soluble in methanol, and insoluble in ethanol, butanol, acetone, ethyl acetate, chloroform, benzene and *n*-hexane. They were positive for ninhydrin, $KMnO_4$, Rydon-Smith, and iodine color reactions, but not for the Sakaguchi, Prochazka, Harnes, and anthrone reactions. In the color reaction with ferric chloride, **II** and **IV** were positive, and **I** and **III** were negative. TLC and HPLC made it possible to distinguish clearly among the four components (Table 4 and Fig. 3). The UV absorption maxima of both **II** and **IV** were at 214, 272 and 279 nm in neutral and acidic solutions, with a bathochromic shift to 218, 243

Fig. 4. IR spectra of OF4949-I, II, III and IV (KBr).

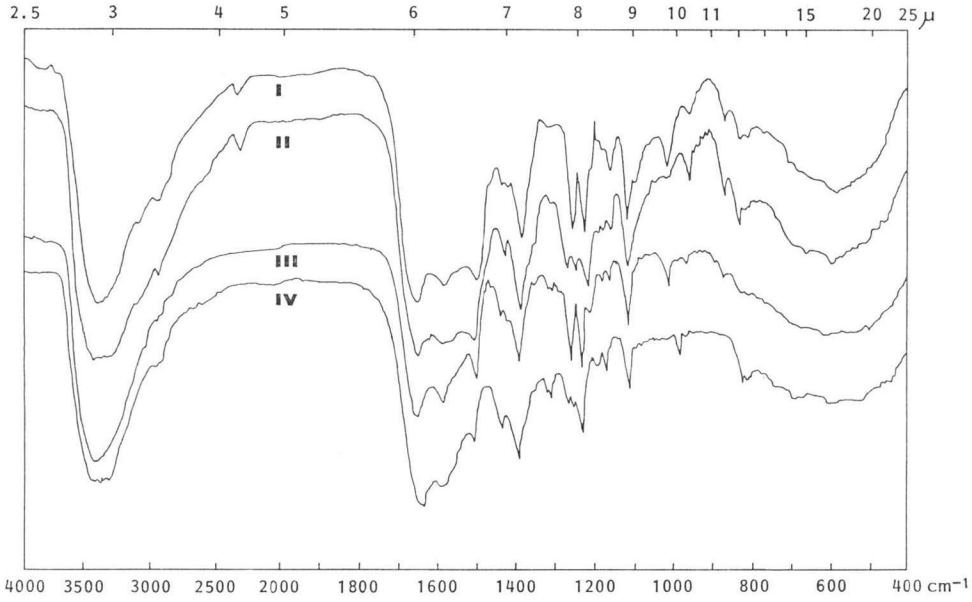
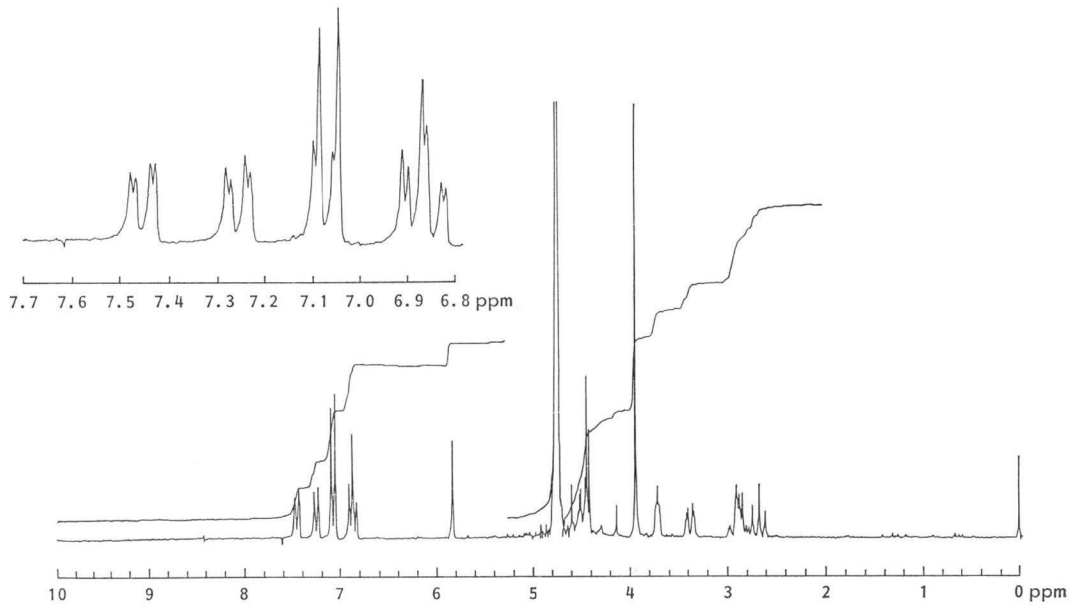


Fig. 5. ^{1}H NMR spectrum of OF4949-I (200 MHz, 0.06 N ND_4OD).



and 297 nm in alkaline solution, suggesting the presence of a phenolic hydroxyl group (Table 5). The UV absorption maxima of **I** and **III** were at 213, 273 and 283 nm, whatever the pH. The IR spectra of the four components had characteristic peaks at 3200~3500 cm^{-1} (amide group, hydroxyl group, or both), 1570~1580 and 1650 cm^{-1} (amide bond), and 1015 and 1260 cm^{-1} (ether bond), with the only difference being a peak at 1020 and 1260 cm^{-1} (methoxyl group on an aromatic ring) present in **I** and **III** only (Fig. 4).

Fig. 6. ^1H NMR spectrum of OF4949-II (200 MHz, 0.06 N ND_4OD).

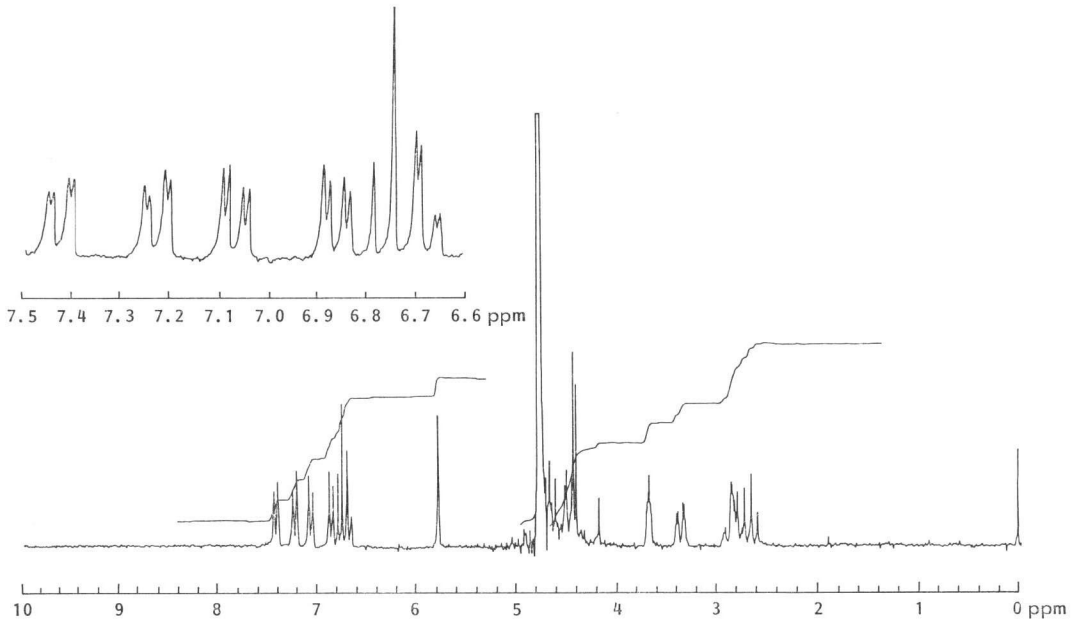
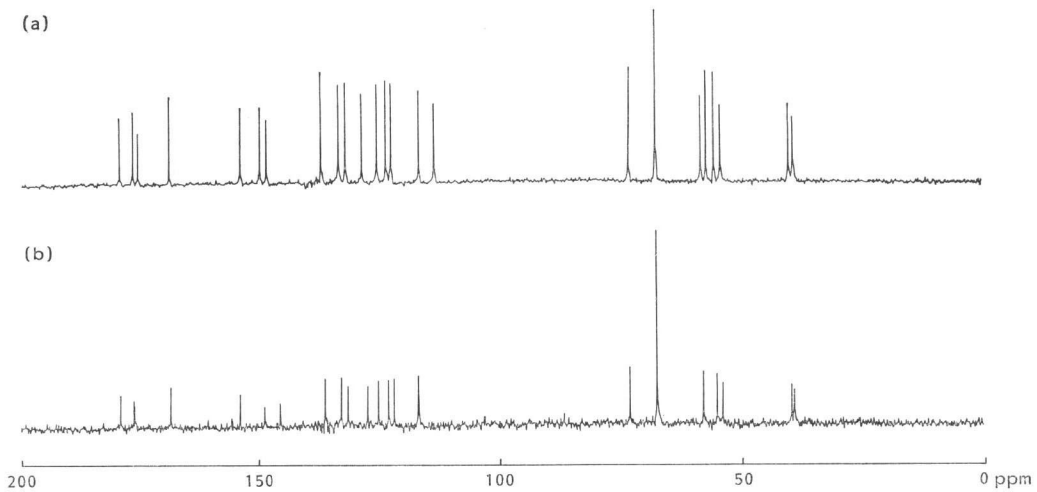


Fig. 7. ^{13}C NMR spectra of OF4949-I (a) and II (b) (50.3 MHz, 0.06 N ND_4OD).



Other properties of the two major components of OF4949 were as follows. **I**: MP 280~285°C (dec); $[\alpha]_D^{25}$ −64.8° (*c* 1.0, H_2O); *Anal* calcd for $C_{23}H_{26}N_4O_8 \cdot 2H_2O$; C 52.87, H 5.79, N 10.72; found, C 52.30, H 5.76, N 10.64; no halogen, sulfur, or phosphorus. The molecular formula was decided from the elemental analysis and secondary ion mass spectrometry [*m/z* 487 (MH^+)] to be $C_{23}H_{26}N_4O_8$. Potentiometric titration showed the presence of two dissociable groups, at *pKa'* 3.12 (carboxyl) and 7.56 (amino). **II**: MP 280~285°C (dec); $[\alpha]_D^{25}$ −42.6° (*c* 1.0, H_2O); *Anal* calcd for $C_{22}H_{24}N_4O_8 \cdot 2H_2O$; C 51.97, H 5.55, N 11.06; found, C 51.60, H 5.76, N 11.06; no halogen, sulfur, or phosphorus. The molecular formula was decided from the elemental analysis and secondary ion mass spectrometry [*m/z* 473 (MH^+)] to be $C_{22}H_{24}N_4O_8$. Potentiometric titration showed the presence of three dissociable

Table 6. Inhibition by OF4949 of aminopeptidase B from EAC.

Inhibitor	IC_{50} (μg/ml)
OF4949-I	0.0054
OF4949-II	0.0048
OF4949-III	3.4
OF4949-IV	1.7

Table 7. Effect of OF4949 on DTH to sheep red blood cells.

Compound	Dose (μg/kg)	Increase in footpad thickness ($\bar{X}\pm$SD, $\times$0.1 mm)	T/C (%)
OF4949-I	—	7.2±0.3	100
	5	9.5±0.6	132
	50	11.7±1.5	163
	500	9.1±0.5	126
OF4949-II	—	9.2±1.0	100
	5	11.7±1.9	127
	50	13.5±1.2	147
	500	11.4±2.6	124

groups, at *pKa'* 3.12 (carboxyl), 7.35 (amino) and 9.60 (phenolic hydroxyl).

The ^{1}H NMR and ^{13}C NMR spectra of **I** and **II** are given in Figs. 5, 6 and 7. The ^{1}H NMR spectra in 0.06 N ND_4OD suggested the presence in **I** and **II** of a di-substituted benzene ring and a tri-substituted benzene ring at δ 5.78~7.44, and the presence of a methoxyl group at δ 3.93, this last absent in **II**. The ^{13}C NMR spectra suggested the same. We concluded from these results that the phenolic hydroxyl group of **II** was methylated in **I**. Details about structure is reported elsewhere.[10]

Biological Properties

OF4949-I and II inhibited aminopeptidase B from EAC cells strongly, while III and IV inhibited more weakly (Table 6). Both **I** and **II** markedly augmented DTH response in doses of 5~500 μg/kg (Table 7). OF4949-I and II had no toxicity when given ip in ICR mice at 300 mg/kg. At 200 μg/ml, **I** and **II** had no antibacterial or antifungal activity.

Discussion

To make available large amounts of OF4949 for biological tests to evaluate these inhibitors and for industrial applications, we tried to improve the production of this complex. Maximum production by the *P. rugulosum* OF4949 was 63 μg/ml when cultured with a modified production medium under high aeration, 13 times the productivity of the original culture conditions.

As described in the following paper,[10] the components of OF4949 are novel cyclic peptides containing a diphenyl ether as a chromophore. In their inhibition of EAC aminopeptidase B, OF4949-I and II were about 100 times as effective as bestatin.[2] OF4949-I and II enhanced DTH, as do as other aminopeptidase B inhibitors such as bestatin,[14] arphamenines A and B,[6] and α-aminoacylarginine.[7] The results suggest that **I** and **II** may affect the immune system by binding to the surfaces of immunologically competent cells, as does bestatin.

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References

1) Aoyagi, T.; H. Suda, M. Nagai, K. Ogawa, J. Suzuki, T. Takeuchi & H. Umezawa: Aminopeptidase activities on the surface of mammalian cells. Biochim. Biophys. Acta 452: 131~143, 1976
2) Aoyagi, T.; M. Ishizuka, T. Takeuchi & H. Umezawa: Enzyme inhibitors in relation to cancer therapy. Jpn. J. Antibiotics 30 (Suppl.): S121~S132, 1977
3) Aoyagi, T. & H. Umezawa: Hydrolytic enzymes on the cellular surface and their inhibitors found in microorganisms. FEBS Feder., Europ. Biochem. Soc. 61: 89~99, 1980

4) AOYAGI, T.; M. NAGAI, M. IWABUCHI, W. S. LIAW, T. ANDOH & H. UMEZAWA: Aminopeptidase activities on the surface of mammalian cells and their alterations associated with transformation. Cancer Res. 38: 3505~3508, 1978
5) UMEZAWA, H.; T. AOYAGI, H. SUDA, M. HAMADA & T. TAKEUCHI: Bestatin, an inhibitor of aminopeptidase B, produced by actinomycetes. J. Antibiotics 29: 97~99, 1976
6) UMEZAWA, H.; T. AOYAGI, S. OHUCHI, A. OKUYAMA, H. SUDA, T. TAKITA, M. HAMADA & T. TAKEUCHI: Arphamenines A and B, new inhibitors of aminopeptidase B, produced by bacteria. J. Antibiotics 36: 1572~1575, 1983
7) YAMAMOTO, K.; H. SUDA, M. ISHIZUKA, T. TAKEUCHI, T. AOYAGI & H. UMEZAWA: Isolation of α-aminoacyl arginines in screening of aminopeptidase B inhibitors. J. Antibiotics 33: 1597~1599, 1980
8) UMEZAWA, H. (*Ed.*): Small Molecular Immunomodifiers of Microbial Origin—Fundamental and Clinical Studies of Bestatin. Japan Scientific Societies Press, Tokyo, 1981
9) UMEZAWA, H. & T. AOYAGI: Trends in research of low molecular weight protease inhibitor of microbial origin. *In* Protease Inhibitors—Medical and Biological Aspects. *Ed.*, N. KATSUNUMA *et al.*, pp. 3~15, Japan Scientific Societies Press, Tokyo, 1983
10) SANO, S.; K. IKAI, K. KATAYAMA, K. TAKESAKO, T. NAKAMURA, A. OBAYASHI, Y. EZURE & H. ENOMOTO: OF4949, new inhibitors of aminopeptidase B. II. Elucidation of structure. J. Antibiotics 39: 1685~1696, 1986
11) RAPER, K. B. & C. THOM: *Penicilllium rugulosum* series. *In* A Manual of the Penicillia. *Ed.*, K. RAPER *et al.*, pp. 646~650, Hafner Publishing Co., New York, 1968
12) ABE, S.: Studies on the classification of the Penicillia. J. Gen. Appl. Microbiol. 2: 126~128, 1956
13) HOPUS, V. K.; K. K. MAKINEN & G. G. GLENNER: Purification of a mammalian peptidase selective for N-terminal arginine and lysine residues: Aminopeptidase B. Arch. Biochem. Biophys. 114: 557~566, 1966
14) ISHIZUKA, M.; T. MASUDA, N. KANBAYASHI, S. FUKASAWA, T. TAKEUCHI, T. AOYAGI & H. UMEZAWA: Effect of bestatin on mouse immune system and experimental murine tumors. J. Antibiotics 33: 642~652, 1980