ARPHAMENINES A AND B, NEW INHIBITORS OF AMINOPEPTIDASE B, PRODUCED BY BACTERIA

Sir:

We have previously reported that aminopeptidases, alkaline phosphatase, esterase and glycosidases are located on the surface of mammalian cells including macrophages and lymphocytes[1~4]. The specific inhibitors of these enzymes are produced by Actinomycetes, and they, bestatin[5], amastatin[6], forphenicine[7], esterastin[8] and ebelactones A and B[9], have been discovered. These inhibitors were proved to bind to cells and to modify the functions of immunoresponsive cells; *e.g.* bestatin and ebelactones enhance cellular immunity, amastatin increases humoral immunity, forphenicine enhances both cellular and humoral immune responses, but esterastin suppressed immune responses[2,10].

We have further continued the screening for inhibitors of aminopeptidase B, and discovered another group of the inhibitors which we named arphamenines A and B. Arphamenines are completely specific inhibitors of aminopeptidase B. They also enhanced immune responses. In this paper, the isolation and characterization of arphamenines are reported.

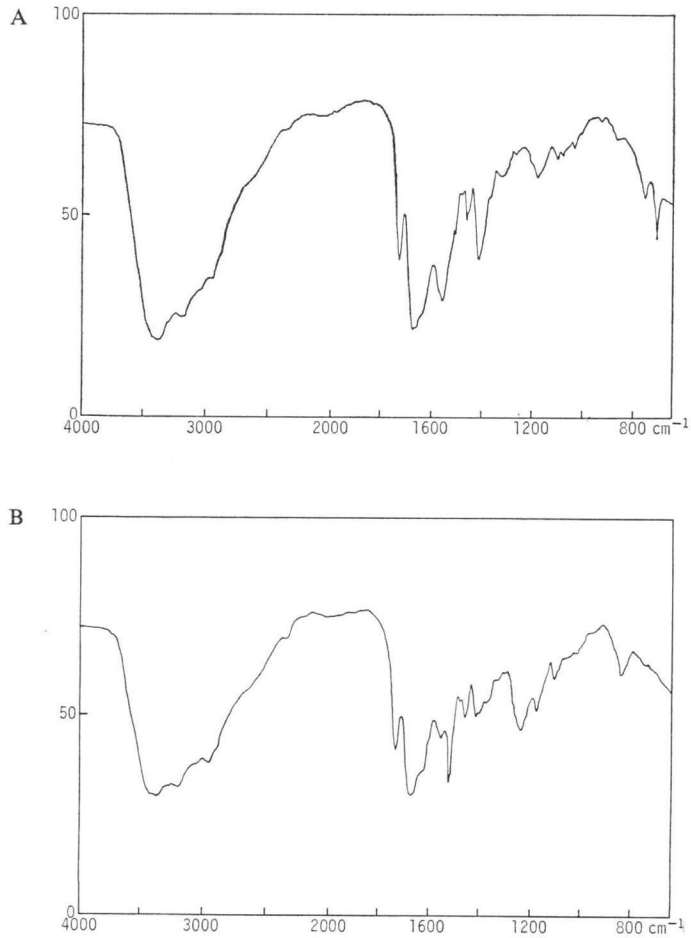
The producing strain, which has the strain number BMG361-CF4 in Institute of Microbial Chemistry, was classified as *Chromobacterium violaceum*. Aminopeptidase B prepared from rat liver by the method of HOPSU *et al.*[11] was used. The activity was measured as reported previously[5]. The concentration of an inhibitor required for 50% inhibition (IC_{50}) was determined.

Arphamenines were produced by shaken culture of BMG361-CF4 in a medium containing 3.0% soluble starch, 0.5% soy-bean meal, 1.2% corn gluten meal and 0.2% $CaCO_3$, pH 7.4 with 5 N NaOH before sterilization. The maximum production was attained after 2 days of culture at 27°C. Nine liters of a culture filtrate were adjusted to pH 5.0 and passed through a column of Amberlite XAD-4 (900 ml) and the adsorbed material was eluted with 50% aqueous acetone. The active eluate was concentrated under reduced pressure to give a brownish powder (19.6 g, IC_{50} 0.2 μg/ml). It was placed on a column of CM-Sephadex C-25 which was equilibrated with 0.05 M sodium citrate buffer at pH 4.5, and the chromatography was developed by a linear gradient of NaCl from 0 to 0.55 M in the same buffer. Under these conditions, the activity appeared in two separate fractions. Each of them was desalted by Amberlite XAD-4 adsorption followed by elution with 50% aqueous acetone. The active compound from the earlier eluate (188 mg) was named arphamenine A and that from the later eluate (79 mg) was named arphamenine B.

Further purification was achieved by the following procedure. The crude arphamenine A was rechromatographed on CM-Sephadex C-25 developed by a linear gradient of NaCl from 0 to 0.5 M. The purified material thus obtained was desalted by a column of Sephadex LH-20 eluted with water to give 92 mg of arphamenine A (IC_{50} 0.006 μg/ml). The arphamenine B was purified by CM-Sephadex C-25 chromatography developed by a linear gradient from 0.15 to 0.6 M NaCl followed by Sephadex LH-20 chromatography developed with 0.01 N HCl. The active eluate was lyophilized after adjusting the pH to 5.0 with Dowex WGR (OH^-). The purified arphamenine B (32 mg) thus obtained showed IC_{50} 0.002 μg/ml.

As readily inferred from the structures elucidated in the following paper[12], arphamenines A and B are easily epimerized. The purified arphamenines A and B described above contained a small amount of *epi*-arphamenines A and B, respectively. They were detected by thin-layer chromatography on a silica gel G (E. Merck) developed with phenol - water (75: 25). The Rf values were 0.43 (A), 0.33 (*epi* A), 0.38 (B) and 0.28 (*epi* B). Arphamenine A and *epi*-arphamenine A were separated by reverse phase column chromatography (SSC-ODS-272, Senshu Kagaku Co., Tokyo) with a linear gradient of CH_3CN from 0 to 16% in the 0.17 M pyridine - formic acid buffer at pH 2.95 using Waters' HPLC system. The separated arphamenine A was further purified by CM-Sephadex C-25 (H^+) chromatography developed with 0.1 N HCl, followed by Sephadex LH-20 chromatography developed with water. Arphamenine B and *epi*-arphamenine B were also separated by the same column with a linear gradient of CH_3CN from 0 to 16% in the 0.03 M pyridine - formic acid buffer at pH 2.95 using the same system described above. The separated arphamenine B was purified by CM-Sephadex C-25 (H^+) chromatography developed with 0.1 N

Fig. 1. The IR spectra of arphamenines A and B.



HCl, followed by Sephadex LH-20 chromatography developed with 0.01 N HCl. The active eluate was lyophilized after adjusting the pH to 5 with Dowex WGR (OH^-).

Arphamenines A and B are obtained as a colorless powder with the following properties. Arphamenine A; mp 117~119°C (dec.), $[\alpha]_D^{20}$ +46° (*c* 0.3, 0.1 N HCl), *Anal.* Calcd. for $C_{16}H_{24}N_4O_8 \cdot HCl$: C 53.85, H 7.06, N 15.70, O 13.45, Cl 9.93; found: C 53.45, H 7.11, N 14.91, O 14.20, Cl 10.49. The molecular formula was assigned to be $C_{16}H_{24}N_4O_8$ by the elemental analysis and FD mass spectrometry, [*m/z* 321 (M+1)]. Arphamenine B; mp 117~119°C (dec.), $[\alpha]_D^{20}$ +49° (*c* 0.3, 0.1 N HCl), *Anal.* Calcd. for $C_{16}H_{24}N_4O_4 \cdot HCl \cdot H_2O$: C 49.17, H 6.96, N 14.33, O 20.47, Cl 9.07: found: C 49.30, H 6.88, N 13.77, O 20.82, Cl 8.73. The molecular formula was assigned to be $C_{16}H_{24}N_4O_4$ by the elemental analysis and FD mass spectrometry, [*m/z* 337 (M+1)]. The IR and UV spectra of arphamenines A and B are shown in Figs. 1 and 2, respectively.

Arphamenines A and B are soluble in water and methanol, but insoluble in butanol, chloroform and ether. They give possitive ninhydrin, RYDON-SMITH, SAKAGUCHI and 2,3,5-triphenyltetrazolium chloride reactions. On thin-layer chromatograms of silica gel G (E. Merck), arphamenines A and B give a single spot at Rf 0.27 and 0.23 (BuOH - AcOH - H_2O, 4: 1: 1) and Rf 0.63 and 0.62 (PrOH - Pyridine - AcOH - H_2O, 15: 10: 3: 12), respectively.

As described in the following paper, the structures of arphamenines A and B were determined as 5-amino-8-guanidino-4-oxo-2-phenylmethyloctanoic acid and 5-amino-8-guanidino-2-(4-

Fig. 2. The UV spectrum of arphamenines A and B.

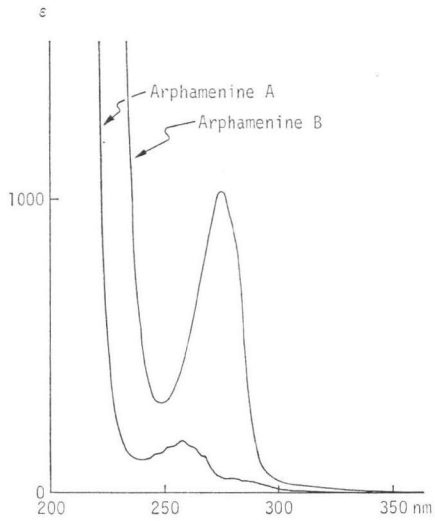


Table 1. Inhibitory activity of arphamenines and bestatin.

Inhibitor	IC_{50} (μg/ml)		
	Amino-peptidase A	Amino-peptidase B	Leucine amino-peptidase
Arphamenine A	>100	0.006	>100
Arphamenine B	>100	0.002	>100
Bestatin	>100	0.05	0.01

hydroxyphenylmethyl)-4-oxooctanoic acid, respectively.

Activities of arphamenines A and B in inhibiting aminopeptidases are shown in Table 1 in comparison with bestatin. Arphamenines A and B show strong inhibition against aminopeptidase B, but they do not inhibit leucine aminopeptidase and aminopeptidase A. Arphamenines are competitive with the substrate. The *Ki* value of arphamenine A is 2.5×10^{-9} M and that of arphamenine B is 8.4×10^{-10} M.

Oral administration of 0.005~5.0 μg/mouse of arphamenines A and B augmented delayed type hypersensitivity (DTH) to sheep red blood cells in footpad test using CDF_1 mice older than 10 weeks[13]. Arphamenines A and B at 100 μg/ml had no antimicrobial activity. They have low toxicity; no death after intraperitoneal injection of 500 mg/kg to mice. Arphamenines inhibit sarcoma 180 and IMC carcinoma. They inhibit the generation of suppressor cells.

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