EBELACTONE, AN INHIBITOR OF ESTERASE, PRODUCED BY ACTINOMYCETES

Sir:

We have demonstrated that aminopeptidases, phosphatases and esterases are located not only in cells but also on the cellular membrane of various kinds of animal cells[1~3] and that inhibitors of these enzymes modified immune responses[4,5]. Among these inhibitors, bestatin, amastatin and forphenicine enhanced immune responses, but esterastin which inhibited esterase suppressed immune responses[6,7]. We continued the screening of esterase inhibitors and found another group of inhibitors which we named ebelactone. Ebelactone inhibited also N-formylmethionine aminopeptidase. This inhibitor enhanced immune responses. In this paper, we report on the isolation and characterization of ebelactone.

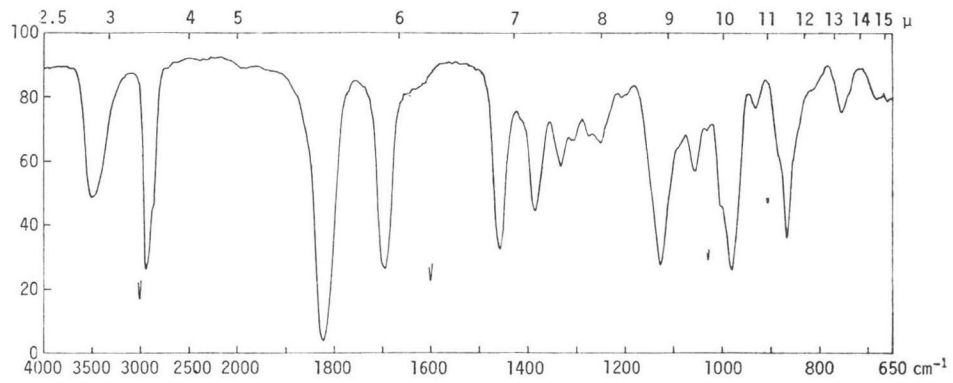
In the screening study, culture filtrates of many strains of various species of soli actinomycetes showed the activity to inhibit esterase and we were successful in the isolation of an inhibitor from the strain MG7-G1. This strain, closely related to *Streptomyces aburaviensis*, was isolated from a soil sample collected in Rissho University, Kumagaya City, Saitama Prefecture.

In determining the inhibitory activity against esterase, the method used previously[6] was modified as follows: 1 ml of 0.1 M phosphate buffer containing 0.06% Triton X-100 (pH 7.0), 0.95 ml of water with or without a test sample and 0.025 ml of esterase solution (from hog liver, Sigma Chemical Co., U.S.A.) were pipetted into a series of test tubes (1.5×10 cm) at room temperature; after 3 minutes, 0.025 ml of 40 mM *p*-nitrophenyl acetate (PNPA, Sigma Chemical Co., U.S.A.) was added and well mixed. Exactly 15 minutes thereafter, the absorbance of the reaction mixture was read at 400 nm. The concentration of enzyme in the solution was adjusted to yield 50 nmoles of *p*-nitrophenol. The reaction was also carried out without the enzyme solution to obtain the blank value.

Ebelactone was produced by shaken culture and tank fermentation of the strain MG7-G1 in media containing various carbon and nitrogen sources. Glycerol, glucose, lactose, maltose and starch are examples of carbon sources and fish meal, N-Z amine, yeast extract and soy-bean meal are examples of nitrogen sources suitable for the production of ebelactone. A typical medium used for production contained 3.0% glycerol, 2.0% fish meal and 0.2% $CaCO_3$. The maximum production was attained in 2 days in the shaken culture.

Ebelactone was extracted from the whole broth with same volume of butyl acetate. pH was not adjusted. The butyl acetate extract was concentrated under reduced pressure and the brownish oily residue thus obtained was chromatographed on a silica gel column using *n*-hexane - chloroform - ethyl acetate (5: 5: 1). The active fractions were combined and concentrated under reduced pressure. The concentrate was subjected to reversed phase silica gel column chromatography with methanol - water (1: 1). Two active peaks were obtained. The active compound in the first peak was named ebelactone A and that in the second peak was named ebelactone B. The yield of ebelactone A is twice of ebelactone B. The two compounds obtained

Fig. 1.



from each peak as colorless powders were recrystallized from methanol-water. Ebelactones A and B showed 50% esterase inhibition at 0.056 μg/ml and 0.00035 μg/ml, respectively.

Ebelactones A and B had the following properties.

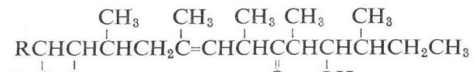
Ebelactone A: colorless needles, mp. 86.0°C, $[\alpha]_D^{20}$ −221° (*c* 1, MeOH), calcd. for $C_{20}H_{34}O_4$: C 70.97, H 10.12, O 18.91, found: C 70.97, H 10.20, O 19.07. This molecular formula was confirmed by the high resolution mass spectrometry of acetylebelactone A: $C_{22}H_{36}O_5$, M^+ *m/z* 380.2522 (calcd. 380.2560).

Ebelactone B: colorless needles, mp. 77.0°C, $[\alpha]_D^{26}$ −203° (*c* 1, MeOH), calcd. for $C_{21}H_{36}O_4$: C 71.59, H 10.23, O 18.18, found: C 71.72, H 10.31, O 18.37. The molecular formula of acetyl ebelactone B by high resolution mass spectrometry was $C_{23}H_{38}O_5$: M^+ *m/z* 394.2760 (calcd. 394.2717).

Ebelactone A and B have a similar IR spectrum and the spectrum of A is shown in Fig. 1. Both compounds had a very weak maximum at 291 nm in UV.

Ebelactones A and B are soluble in methanol, ethanol, ethyl acetate and chloroform, but insoluble in water and *n*-hexane. They give positive anisaldehyde-H_2SO_4 reaction and negative RYDON-SMITH and ninhydrin reactions. On thin-layer chromatography on silica gel G (E. Merck), ebelactones A and B give single spots at Rf 0.72 and 0.80 with *n*-hexane - chloroform - ethyl acetate (5: 5: 1) and Rf 0.38 and 0.42 with benzene - ethyl acetate (85: 15), respectively.

As we will report in the next paper, the structures of ebelactone A and B were determined to be 3,11-dihydroxy-2,4,6,8,10,12-hexamethyl-9-oxo-6-tetradecenoic 1,3-lactone and 2-ethyl-3,11-dihydroxy-4,6,8,10,12-pentamethyl-9-oxo-tetradecenoic 1,3-lactone, respectively as follows:



Ebelactone A: R=CH_3–
Ebelactone B: R=CH_3CH_2–

Activities of ebelactones A and B in inhibiting esterase, lipase and N-formylmethionine aminopeptidase are shown in Table 1 in comparison with esterastin. Intraperitoneal injection and oral administration of 0.5 ~ 50 μg/mouse of ebelactones enhanced delayed-type hypersensitivity against sheep red blood cells in the footpad test using CDF_1 female mice older than 8 weeks[3]. Ebelactones at 100 μg/ml had no antimicrobial activity. Both ebelactones have low toxicity: no death after intraperitoneal injection of 250 mg/kg to mice.

Table 1. Enzyme-inhibitory activity of ebelactones and esterastin.

Inhibitor	IC_{50} (μg/ml)[3]		
	Esterase Hog liver	Lipase[1] Hog pancreas	fMet AP[2] Rat liver
Ebelactone A	0.056	0.003	0.08
Ebelactone B	0.00035	0.0008	0.02
Esterastin	5	0.0002	75

1) The assay of lipase activity was carried out similarly to that of esterase.
2) N-Formylmethionine aminopeptidase (fMet AP) was measured by the hydrolysis of fMet β-naphthylamide.
3) IC_{50} is 50% inhibition concentration.

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HAMAO UMEZAWA
TAKAAKI AOYAGI
KAZUMICHI UOTANI
MASA HAMADA
TOMIO TAKEUCHI
SAKIKO TAKAHASHI*

Institute of Microbial Chemistry
14–23 Kamiosaki 3-chome, Shinagawa-ku, Tokyo, Japan
*The National Institute of Health
10–35 Kamiosaki 2-chome, Shinagawa-ku, Tokyo, Japan

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