ISOLATION AND CHARACTERIZATION OF I5B2, A NEW PHOSPHORUS CONTAINING INHIBITOR OF ANGIOTENSIN I CONVERTING ENZYME PRODUCED BY *ACTINOMADURA* SP.

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A new inhibitor of angiotensin I converting enzyme, I5B2, was isolated from the culture broth of *Actinomadura* sp. No. 937ZE-1. This compound contains *N*-methylvaline, tyrosine and 1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid. The microorganism also produced another inhibitor, I5B1, which is identical with K-4 isolated from *Actinomadura* sp. as an antihypertensive agent.

In our previous paper, we reported that *Streptomyces* sp. No. A467P-2 produced ancovenin, a new oligopeptide inhibitor of angiotensin I converting enzyme (ACE), which contains unique amino acids such as *threo*-β-methyllanthionine, *meso*-lanthionine and dehydroalanine in its peptide sequence[1]. In subsequent screening, two inhibitors designated as I5B1 and I5B2 were produced by *Actinomadura* sp. No. 937ZE-1 isolated from a soil sample collected at Onoda City, Yamaguchi Prefecture, Japan. The present paper describes the production, isolation, and physico-chemical properties as well as the structures and activities of these two inhibitors.

Materials and Methods

General

The IR spectra were obtained in KBr disc using a Hitachi IR spectrophotometer Model 260-10. UV absorption spectra were taken with a Hitachi UV spectrophotometer Model 124. Amino acid analyses were carried out with a Hitachi KLA-5 analyzer. Fast atom bombardment mass spectra (FAB-MS) were obtained with the Matsuda type mass spectrometer of Osaka University. Melting points are uncorrected.

Assay for ACE Inhibitory Activity

Inhibition activities in each chromatography were determined by the method described in the previous paper[1]. IC_{50} values were determined using hippurylhistidylleucine as a substrate.

Preparative HPLC of I5B1 and I5B2

The chromatographic conditions of preparative HPLC were as follows; column: NOVA-PAK Cartridge (Waters Associates, Inc.), mobile phase: CH_3CN - H_2O - 1 N HCl (30: 1,000: 1 for I5B1 and 20: 1,000: 1 for I5B2), flow rate: 2 ml/minute, detection: UV absorbance detector at 210 nm.

Production of I5B1 and I5B2

The producing organism, grown on the agar medium, was inoculated in a 500-ml Erlenmeyer

Fig. 1. Isolation and purification of I5B1 and I5B2.

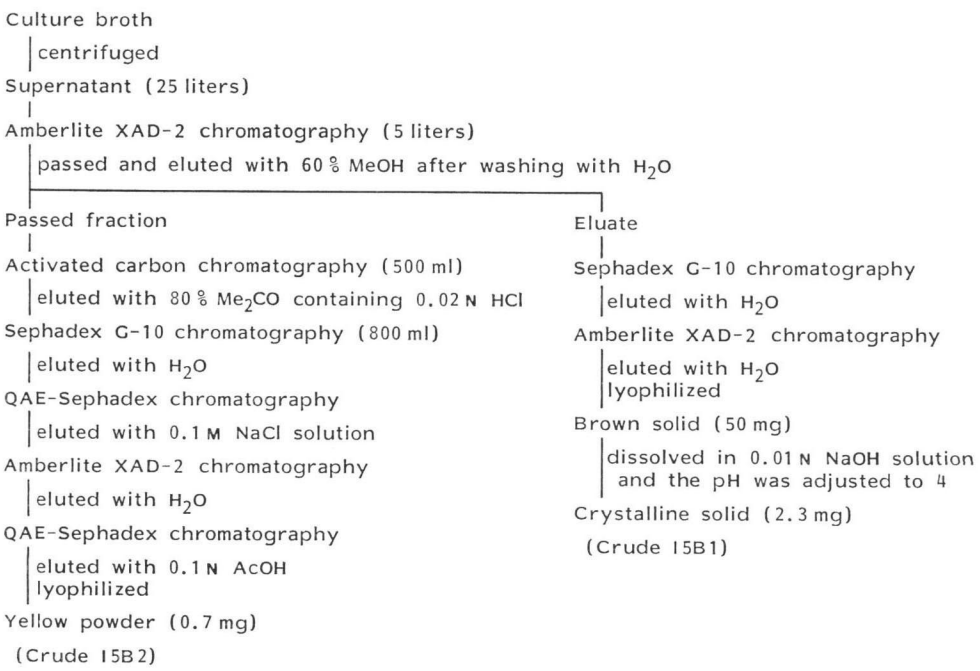
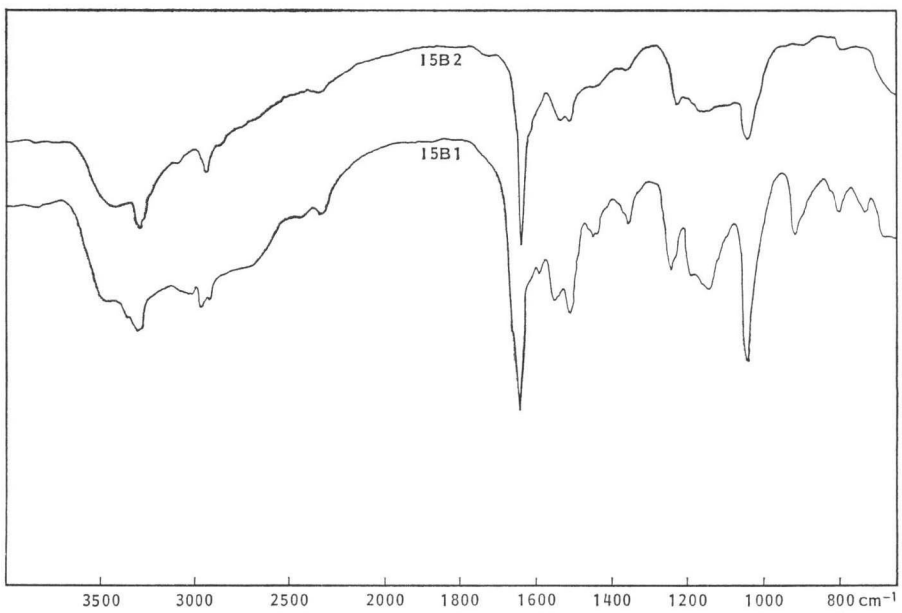


Fig. 2. IR spectra of I5B1 and I5B2.



flask containing 50 ml of a seed medium composed of glucose 1%, glycerol 1%, soybean meal 1%, yeast extract 0.25%, meat extract 0.1%, $(NH_4)_2SO_4$ 0.5%, NaCl 0.4%, $CaCO_3$ 0.4%, K_2HPO_4 0.05% and $MgSO_4 \cdot 7H_2O$ 0.05%, the pH being adjusted to 7.2 before sterilization. The seed culture was then incubated at 28°C for 7 days on a rotary shaker, and the culture (2 ml) was transferred into a 500-ml Erlenmeyer flask containing 100 ml of a medium composed of lactose 3%, soybean meal 1.5%, yeast extract 0.25%, meat extract 0.1%, $(NH_4)_2SO_4$ 0.5%, NaCl 0.4%, $CaCO_3$ 0.4%, K_2HPO_4 0.05 % and

Table 1. Physico-chemical properties of I5B1 and I5B2.

	I5B1	I5B2
$[\alpha]_D$	−110° (*c* 0.075, 0.01 N NaOH)	−55° (*c* 0.0186, 0.01 N NaOH)
Mp	>300°C	>300°C
Color reaction		
Positive	I_2, HANES-ISHERWOOD, PAULY, (ninhydrin[a])	I_2, HANES-ISHERWOOD, PAULY, (ninhydrin[a])
Negative	MOLISH	MOLISH
UV (λ_{max} nm)	238, 293 (0.01 N NaOH)	240, 292 (0.01 N NaOH)
	275, 281 (sh) (0.02 N HCl)	222, 274, 280 (sh) (0.01 N HCl)
Rf value on TLC		
Silica gel plate[b]	0.38[c], 0.40[d]	0.36[c], 0.40[d]
Cellulose plate[e]	0.52[c]	0.38[c]
Composition	*N*-Methylvaline, Phe, 1-amino-2-(4-hydroxyphenyl)-ethylphosphonic acid	*N*-Methylvaline, Tyr, 1-amino-2-(4-hydroxyphenyl)-ethylphosphonic acid
Mw[f]	477	493

[a] Staining was very weak even though γ-collidine was used as sensitizer.
[b] Silica gel $60F_{254}$ (Merck).
[c] 1-BuOH - AcOH - H_2O (4: 1: 1).
[d] 1-BuOH - MeOH - H_2O (4: 1: 2).
[e] Cellulose plate (Merck).
[f] The value was determined by FAB-MS.

Fig. 3. Comparison of acid hydrolysate of I5B1 and I5B2 on TLC.

Support, silica gel; solvent, 1-BuOH - AcOH - H_2O (4: 1: 1); detection, ninhydrin reagent.

Sample: 1, acid hydrolysis product of I5B1; 2, acid hydrolysis product of I5B2; 3, tyrosine; 4, phenylalanine; 5, *N*-methylvaline; 6, 1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid.

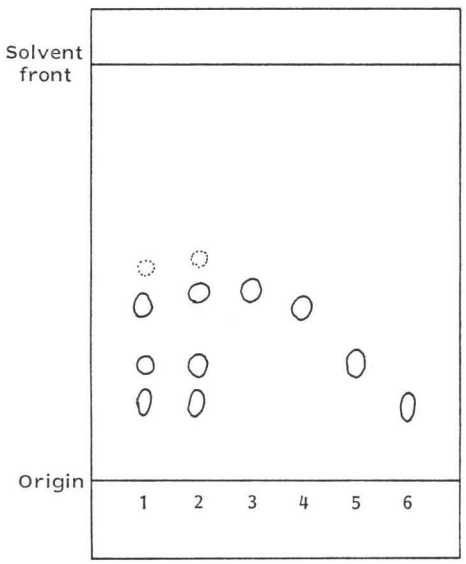


Table 2. Inhibitory activities of I5B1 and I5B2 against ACE.

Compound	IC_{50} (μM)[a]
I5B1	0.34
I5B2	0.091
Captopril	0.029

[a] Hippurylhistidylleucine was used as a substrate.

$MgSO_4 \cdot 7H_2O$ 0.05% (pH 7.2). The cultivation was then carried out at 28°C for 4 days on a rotary shaker.

Results

Cultivation, Purification and Physico-chemical Properties of I5B1 and I5B2

The cultivation was carried out in a 500-ml Erlenmeyer flask as described. Crude I5B1 and I5B2 were isolated as shown in Fig. 1 and final purification was achieved by preparative HPLC on reversed phase. Both I5B1 and I5B2 were obtained as white powders which were soluble in alkaline water but slightly soluble in water and insoluble in ether and acetone. The IR spectra of I5B1 and I5B2 are illustrated in Fig. 2 and the other physico-chemical properties of these

substances are summarized in Table 1.

Composition of I5B1 and I5B2

On acid hydrolysis with 6 N HCl (110°C, 20 hours), I5B2 gave tyrosine (Tyr), *N*-methylvaline (*N*-MeVal) and 1-amino-2-(4-hydroxyphenyl)ethylphosphonic acid (Ahp) as shown in Fig. 3. The time course of the hydrolysis of I5B2 showed that a molar ratio of Tyr and Ahp was 1: 1, although *N*-MeVal was not detected under the analytical conditions. Ahp was characterized by coloring tests such as HANES-ISHERWOOD and PAULY reactions. Moreover, Rf value on TLC and retention time on amino acid analysis of this substance agreed with those of authentic sample. The molecular weight (493) of I5B2 was determined by FAB-mass spectrometry. These results revealed that I5B2 was composed of each one mol of Tyr, *N*-MeVal and Ahp. On the other hand, I5B1 consists of each one mol of Phe, *N*-MeVal and Ahp.

Inhibitory Activities of I5B1 and I5B2

IC_{50} values were obtained in the tests using ACE of rat lung and hippurylhistidylleucine as a substrate. As shown in Table 2, I5B2 was 3.7 times more active than I5B1, while 3.1 times less active than captopril.

Discussion

Recently, several ACE inhibitors[2~5] of microbial origin and many synthetic inhibitors[6] have been reported. Among them, some inhibitors are known to contain P-N bonds or phosphonic acid function in the molecules[7,5]. Furthermore, two antihypertensive agents containing aminophosphonic acid, K-4[9] and K-26[10], had been isolated from the culture broth of actinomycetes. From the results of comparison of chemical composition and spectral data, I5B1 is now found to be identical with K-4 possessing the amino acid sequence of *N*-MeVal-Phe-Ahp. Since I5B2 is very similar to I5B1 (K-4) in many respects except for the difference of one amino acid residue, the structure of this new ACE inhibitor is assumed to be *N*-MeVal-Tyr-Ahp.

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