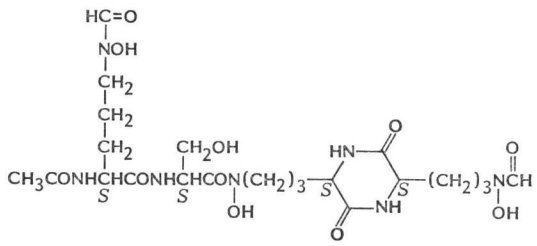
FOROXYMITHINE, A NEW INHIBITOR OF ANGIOTENSIN-CONVERTING ENZYME, PRODUCED BY ACTINOMYCETES

Sir:

Ancovenin[1], muraceins[2], L-681,176[3], I5B2[4] and phenacein[5] which inhibit an angiotensin-converting enzyme have been found in culture broths of actinomycetes. Moreover, aspergillomarasmines[6], products of fungi have also been reported to inhibit this enzyme. Testing the activity of culture filtrates of freshly isolated actinomycetes strains in inhibiting an angiotensin-converting enzyme (ACE, dipeptidyl carboxypeptidase EC 3.4.15.1), we found a new peptide and we named this inhibitor foroxymithine. The strain MG329-CF56 producing foroxymithine was found to belong to *Streptomyces nitrosporeus*. In this paper, the isolation, physico-chemical properties and biological activities of foroxymithine are reported. The structure of foroxymithine was determined and is shown in Fig. 1. The structural studies will soon be reported elsewhere.

ACE was prepared from bovine lung homogenates by solubilizing with Triton X-100 (0.1%, w/v). ACE and its inhibition was measured using a slightly modified HAYAKARI's method[7]. A reaction mixture was prepared by adding 0.05 ml of 12 mM hippuryl-L-histidyl-L-leucine (Peptide Institute, Japan) dissolved in 0.5 M Tris-HCl buffer solution (pH 8.0, containing 0.3 M NaCl) to 0.4 ml of water or a test solution. The mixed solution was incubated for 3 minutes at 37°C, and 0.05 ml of the enzyme solution (2 mg protein/ml) was added. The resulting mixture was incubated for 30 minutes at 37°C. Thereafter, 0.03 ml of 1 N sodium hydroxide was added to terminate the reaction. Fifteen minutes later, 2 ml of 0.06 M phosphate buffer (pH 7.2) and 2 ml of 1% cyanuric chloride (2,4,6-trichloro-*s*-triazine), freshly dissolved in 2-methoxyethanol, were added. After the mixture was allowed to stand for 15 minutes at room temperature, the absorbance at 382 nm was measured. In one of blanks the sodium hydroxide solution was added prior to the addition of the enzyme solution. In the other blank the test solution was not added. From the data thus obtained, the concentration of inhibitor required for 50% inhibition (IC_{50}) was calculated.

Fig. 1. The structure of foroxymithine.



Foroxymithine is produced by shake culture of strain MG329-CF56 in Sakaguchi flasks (500-ml) in a medium consisting of glycerol 1.5%, Pharma media (Traders Protein Division of Traders Oil Mill Company, U.S.A.) 1.5%, NaCl 0.3%, and L-asparagine monohydrate 0.2%, adjusted to pH 7.4 with 5 N NaOH before sterilization. Maximum production was attained in 3 to 4 days (27°C, 130 strokes per minute) and maintained for 3~6 days thereafter.

The inhibitor in 5.5 liters of the culture filtrate was adsorbed on activated carbon (100 g) and eluted with 2.4 liters of 50% aqueous acetone. The eluate was concentrated under reduced pressure to yield a crude powder, 16.5 g. It was dissolved in 250 ml of distilled water and passed through a column of DEAE-Sephadex A-25 (Cl^- form, 100 ml) and the resin bed was washed with distilled water. The active effluent and washings were combined and concentrated under reduced pressure to give 11.1 g of a brownish powder. It was subjected to a column chromatography on silanised silica gel 60 (E. Merck, 600 ml) and developed with distilled water. Active fractions were combined and concentrated under reduced pressure to give 3.5 g of crude foroxymithine. The crude foroxymithine thus obtained was dissolved in 80 ml of distilled water and the solution was divided into eight equal portions. Each portion was applied to a Nucleosil $_5C_{18}$ (20×300 mm, Machery-Nagel, Germany) preparative HPLC column which had been equilibrated with a 1:9 mixture of methanol and an aqueous solution of 0.4% acetic acid. The column was developed with the same solution at a flow rate of 6 ml per minute. The active eluate was concentrated under reduced pressure and lyophilized, yielding 1.38 g of pure foroxymithine dihydrate.

Physico-chemical properties of foroxymithine dihydrate are as follows: Foroxymithine dihydrate was obtained as a colorless powder, mp

Fig. 2. IR spectrum of foroxymithine dihydrate (KBr).

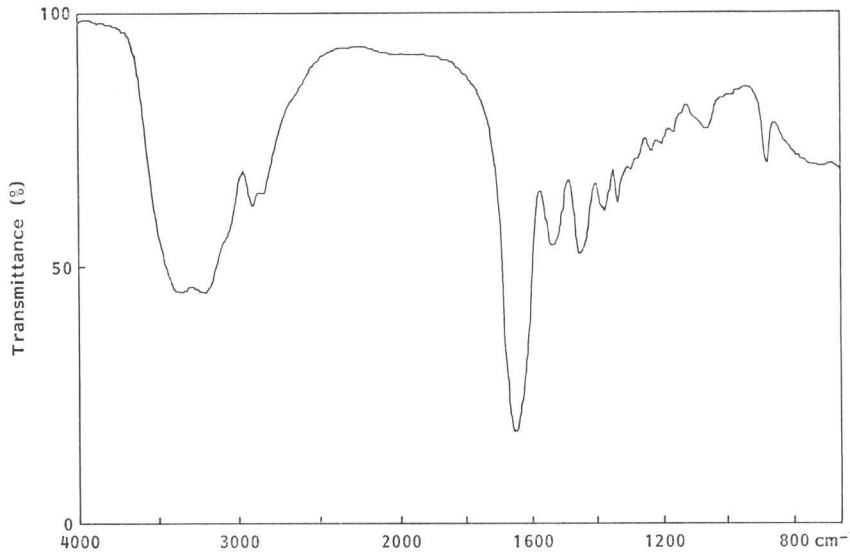
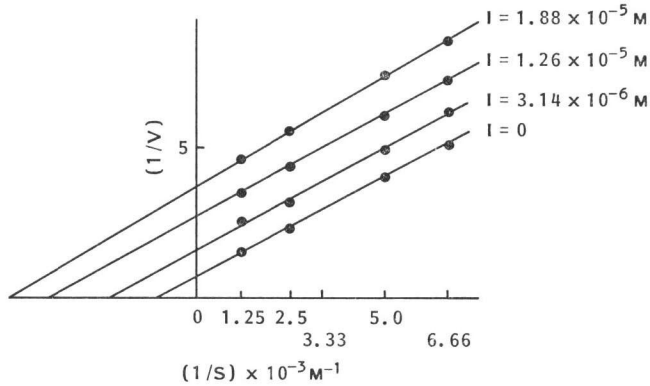


Fig. 3. Lineweaver-Burk plots of angiotensine-converting enzyme activity in the presence of foroxymithine.



Substrate (S): Hip-His-Leu, $Km = 0.5 \times 10^{-4}$ M.
Inhibitor (I): Foroxymithine dihydrate.

106~130°C; $[\alpha]_D^{22}$ −44.5° (*c* 1, H_2O). It is soluble in H_2O, dimethylsulfoxide, partially soluble in methanol but hardly soluble in acetone, 2-propanol, EtOAc, $CHCl_3$ and benzene. It gives positive Rydon-Smith, $KMnO_4$ and $FeCl_3$ reactions. On silica gel thin-layer chromatogram, it gives a single spot at Rf 0.33 (EtOH - 25% NH_4OH, 2: 1). The molecular formula of foroxymithine was established as $C_{22}H_{37}N_7O_{11}$ (MW 575.57) by elemental analysis and secondary ion mass spectrometry Calcd for $C_{22}H_{37}N_7O_{11} \cdot 2H_2O$: C 43.20, H 6.75, N 16.03; Found C 43.46, H 6.31, N 16.12; $[M+H]^+$ *m/z* 576 (SI-MS). Potentiometric titration gave *pK*a′ values of 7.7, 8.5 and 9.2. The IR spectrum of foroxymithine dihydrate is shown in Fig. 2.

Determination of the chemical structure of foroxymithine as (3*S*,6*S*)-3-[3-[*N*-[*N*-(N^{α}-acetyl-N^{δ}-formyl-N^{δ}-hydroxy-L-ornithyl)-L-seryl]-*N*-(hydroxy)amino]propyl]-6-[3-(*N*-formyl-*N*-hydroxyamino)propyl]-2,5-piperazinedione (Fig. 1) will be reported elsewhere.

Foroxymithine dihydrate showed an IC_{50} value of 7 μg/ml against ACE (bovine lung). Inhibition caused by foroxymithine is uncompetitive with the substrate (Fig. 3). Activity of foroxymithine in inhibiting peptidases is shown in Table 1. Except for angiotensin-converting

Table 1. Inhibitory activity of foroxymithine dihydrate against peptidases.

	IC_{50} (μg/ml)
Angiotensin-converting enzyme	7.0
Carboxypeptidase A	>100
Carboxypeptidase B	>100
Aminopeptidase A	>100
Aminopeptidase B	>100
Dipeptidyl aminopeptidase IV	>100
Thermolysin	>100
Trypsin	>100
Papain	>100
Chymostatin	>100

enzyme, metaloenzymes such as carboxypeptidase A and B and thermolysin are not significantly inhibited by 100 μg/ml of foroxymithine dihydrate.

As readily inferred from the structure containing hydroxamic siderophore's, foroxymithine should have a metal chelating ability. The ACE inhibitory effect of foroxymithine (1.6×10^{-4} M) is completely abolished by addition of $FeCl_3$ and partially abolished with $ZnCl_2$, but the addition of other metal salts such as $CaCl_2$, $MgCl_2$, $MnCl_2$ or $CoCl_2$ do not significantly alter the inhibitory activity of foroxymithine (all at 2×10^{-4} M).

The effect of foroxymithine on blood pressure of spontaneously hypertensive rats were examined. The oral administration of foroxymithine dihydrate (25 mg/kg, 50 mg/kg) significantly reduced the systolic blood pressure from 1 to 3 hours after the administration. For the blood pressure measurement, the tail-water-plethysmographic method of OKAMOTO *et al.*[8] was used.

Foroxymithine dihydrate has low acute toxicity. No deaths occurred after intravenous injection of 800 mg/kg in mice. The other strain (MG325-CF7) which was isolated from a soil sample collected at Mt. Kurama (Kyoto, Japan) and classified as *Streptomyces zaomyceticus*, was also found to produce foroxymithine in the same medium described above.

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