ACYLPEPTIDES, THE INHIBITORS OF CYCLIC ADENOSINE 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE

Sir:

In a screening for inhibitors of cyclic adenosine 3′,5′-monophosphate phosphodiesterase (PDE) [EC 3.1.4.17], inhibitors were isolated from the culture filtrate of *Bacillus subtilis* C-756. The isolated inhibitors were acylpeptides and had potent inhibitory activities compared with papaverine and theophylline, which are known as PDE inhibitors.

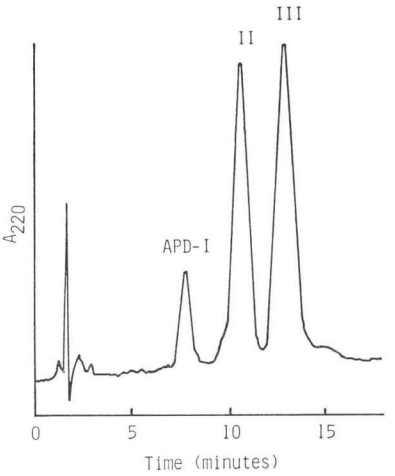
This communication deals with the isolation, characterization and some properties of the inhibitors.

To assay for PDE activity and inhibitory activity, a reaction mixture (1.0 ml) containing 40 mM tris-HCl (pH 7.5), 2 mM $MgSO_4 \cdot 7H_2O$, 0.2 mM cyclic adenosine 3′,5′-monophosphate (cAMP), PDE (*ca.* 140 μg-protein, Boehringer Mannheim GmbH), alkaline phosphatase (*ca.* 70 μg-protein, Boehringer Mannheim GmbH) and an inhibitor was incubated at 38°C for 20 minutes. The reaction was stopped by addition of 5% trichloroacetic acid and the amount of inorganic phosphorus which was liberated from cAMP (cAMP →adenosine 5′-monophosphate→adenosine+Pi) was spectroscopically measured[1]. PDE activity is proportional to the amount of liberated phosphorus under this condition. The percent inhibition was calculated by the formula (A–B)/A×100, wherein A is the phosphorus amount without an inhibitor and B is the amount with an inhibitor.

The microorganism, which was isolated from soil and identified as *Bacillus subtilis*[2], was cultured in Erlenmeyer flasks at 30°C for 2 days on a rotary shaker to produce the inhibitor. The production medium contained 1% glucose, 1% peptone, 0.3% yeast extract, 0.3% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.1% K_2HPO_4 (pH 6.8).

The inhibitor in culture filtrate (40 liters) was precipitated by addition of concentrated HCl or 0.6% $CuSO_4 \cdot 5H_2O$, and collected by centrifugation. The collected inhibitor was extracted with ethyl acetate (pH 3), washed with diluted $NaHCO_3$ and dried with anhydrous Na_2SO_4. The ethyl acetate extract was concentrated under reduced pressure to a crude powder (14 g). The crude material was chromatographed on a Sephadex G-50 column with 80 mM tris-HCl (pH 7.5) and a Silica gel 60 column with chloroform - methanol (8: 1, v/v), successively. Furthermore, the active fraction (8 g) was chromatographed on a Sephadex LH-20 column with acetone, and a Silica gel 60 column with chloroform - methanol (5: 1). The active eluate was concentrated under reduced pressure to a colorless amorphous powder (3 g). This preparation showed a single spot on a Merck silica gel 60 plate with various solvent systems such as chloroform - methanol (5: 1, Rf 0.15), ethyl acetate - methanol (7: 3, Rf 0.34), chloroform - methanol - acetic acid (85: 15: 2, Rf 0.46) and ethanol (Rf 0.55), but it was further separated into three fractions by reverse HPLC (μBondapak C_{18}) with acetonitrile - 1% acetic acid (68: 32). The respective fractions were designated as APD-I, -II and -III in the order eluted, and the relative quantities were approximately estimated as 10%, 40% and 50% from their respective peak areas (Fig. 1).

Fig. 1. HPLC of inhibitors.
Column: μBondapak C_{18}.
Eluent: acetonitrile - 1% acetic acid (68: 32).
Flow: 1.5 ml/minute. Detector: UV 220 nm.

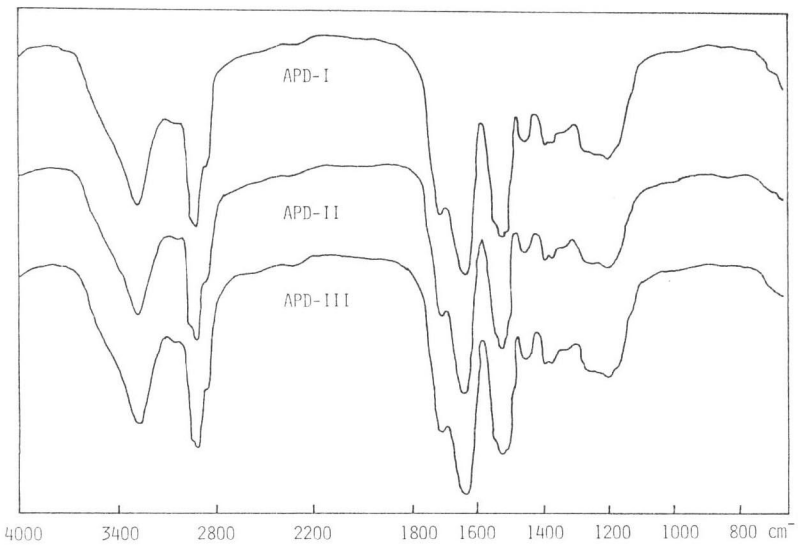


Physicochemical properties such as melting point, elemental analysis and $[\alpha]_D$ value are listed in Table 1. Ultraviolet absorption spectra showed the absence of absorption maximum in the range from 220 nm to 400 nm. But their infrared absorption spectra indicated the presence of peptide bonds (1650, 1520 cm^{-1}), and an ester or lactone linkage (1730 cm^{-1}) as shown in Fig. 2. They are soluble in alkaline water, methanol, ethanol, *n*-butanol, acetone, ethyl acetate, chloroform, acetonitrile, tetrahydrofuran and acetic

Table 1. Physicochemical properties of inhibitors.

	APD-I	APD-II	APD-III
Melting point (°C)	136~137	137~138	139~140
Elemental analysis	C 59.93	C 60.23	C 60.89
	H 8.71	H 8.76	H 8.89
	N 9.44	N 9.19	N 9.10
$[\alpha]_D^{25}$ ($CHCl_3$)	+38.5° (*c* 0.26)	+38.2° (*c* 0.34)	+37.5° (*c* 0.37)
(CH_3OH)	−33.3° (*c* 0.24)	−38.3° (*c* 0.44)	−37.2° (*c* 0.38)

Fig. 2. Infrared absorption spectra of inhibitors (KBr).



acid, but insoluble in water, petroleum ether and *n*-hexane.

The hydrolysis of the inhibitors was carried out with 6 N HCl at 110°C for 20 hours and the amino acid composition was determined with an amino acid analyzer. Amino acid analyses on their hydrolysates showed the same amino acid compositions to be glutamic acid (1), aspartic acid (1), valine (1) and leucine (4).

The ether extracts of the hydrolysates were methylated with diazomethane and subjected to GLC, using a column packed with 1.5% diethyleneglycol succinate on Chromosorb W, for analyses of fatty acid residues. The ether extract of APD-II hydrolysate showed a single peak of fatty acid residue (*m/z* 258, M^+) and those of APD-I hydrolysate and APD-III hydrolysate each showed two neighboring peaks (*m/z* 244, M^+ and *m/z* 272, M^+, respectively), which suggested a mixture of fatty acid residues. But APD-I and APD-III were not throughly separated by HPLC at this stage. In each mass spectrum (unpublished data), a base peak at *m/z* 103, which is attributed to be the fragment ion caused by β,γ-fragmentation and thought to be the common base peak of β-hydoxyl fatty acid methyl esters[8], was observed.

Table 2. IC_{50} for cyclic AMP phosphodiesterase.

Inhibitor	IC_{50}
APD-I	8.5×10^{-5} M
APD-II	4.5×10^{-5} M
APD-III	8.1×10^{-5} M
Theophylline	2.8×10^{-3} M
Papaverine	1.1×10^{-4} M

IC_{50}: Inhibitor concentration in the reaction mixture for 50% inhibition.
Substrate: 200 μM cAMP.
Enzyme: 140 μg-protein PDE.

Calculating from the values obtaind through the analyses of the amino acid compositions and β-hydroxyl fatty acid residues, the molecular

weights of APD-I, -II and -III are approximately 1,008, 1,022 and 1,036, respectively.

The concentrations of APD-I, -II, -III, theophylline and papaverine-HCl required for 50% inhibition (IC_{50}) of PDE in our assay system are shown in Table 2. Besides theophylline and papaverine, reticulol[4], PDE-I and II[5] have been reported as PDE inhibitors. Our inhibitors had potent inhibitory activity comparable to those of reported inhibitors, and were acylpeptides which seemed to have no structural relationship with cAMP.

Details on structures and inhibition will be soon reported.

KUNIAKI HOSONO
HIDEO SUZUKI
Fermentation Research Institute
Yatabe machi, Ibaraki, Japan

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