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

II. AMINO ACID SEQUENCE AND LOCATION OF LACTONE LINKAGE

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Bacillus subtilis C-756 produced three kinds of inhibitors of cyclic adenosine 3′,5′-monophosphate (cAMP) phosphodiesterase. Each was an acylpeptide consisting of a β-hydroxy fatty acid residue and heptapeptide. By the application of mass spectrometry, the amino acid sequence of peptide was determined to be β-hydroxy fatty acid-Glu-Leu-Leu-Val-Asp-Leu-Leu in all three cases. Each had a lactone linkage between the carboxyl group of *C*-terminal leucine and the β-hydroxyl group of the fatty acid moiety. The total structures of these inhibitors were thus established.

In a screening for inhibitors of cAMP phosphodiesterase, *Bacillus subtilis* C-756 was found to produce three kinds of inhibitors, designated as APD-I, -II and -III[1]. They were acylpeptides and had potent inhibitory activities compared with papaverine and theophylline. The inhibitor was composed of a heptapeptide and five kinds of β-hydroxy fatty acid residues, the structures of which were elucidated in a previous paper[2].

In this paper, we describe the amino acid sequence, location of the lactone linkage and the total structures of the inhibitors.

Materials and Methods

Microorganism

Bacillus subtilis C-756 isolated from soil[3] was used.

Production, Isolation and Purification

The inhibitors, APD-I, -II and -III, were prepared as described in a previous paper[2].

Analysis of Amino Acids

The inhibitor and its derivative were suspended in 6 N HCl and held at 110°C for 20 hours in a sealed tube. After the oily material was extracted with ether, the aqueous residues were subjected to a Hitachi 835 amino acid analyzer.

Permethylation of the Inhibitor

To open the lactone linkage, the inhibitor was dissolved in 0.1 N NaOH, allowed to stand overnight at room temperature and extracted with ethyl acetate at pH 3. The extract was concentrated under reduced pressure, and subjected to *N*-permethylation by the procedure of HAKOMORI[4]. DMSO (0.2 ml) was added to NaH obtained by rinsing NaH oil suspension (20 mg) three times with dry ether. The mixture was maintained at 100°C until H_2 evolution ceased and cooled to room temperature. An opened inhibitor (2 mg) and CH_3I (0.3 ml) were added to this reagent, and allowed to stand in a nitrogen stream for 1.5 hours at room temperature. The reaction mixture was diluted with H_2O and the permethylated product was extracted with chloroform. After the extract was concentrated under reduced pressure, the residue was subjected to mass spectrometry without further purification. The permethylated product was detected by vaporization at 290°C on a gas chromatograph with a steel column (φ 3 mm × 1.0 m) packed with 2% OV-1 on Chromosorb W.

Mass spectra of the permethylated inhibitors were obtained on a Jeol JMS-D300 mass spectrometer by the direct inlet system.

Preparation of *N*-Acyl Amino Acid Methyl Ester

Each inhibitor (100 mg) was dissolved in 30% HCl - methanol (12 N HCl - methanol, 30: 70) and held at 90°C for 15 hours in a sealed tube. After methanolysis, HCl - methanol was evaporated and the residue was extracted with petroleum ether. The extract was washed with H_2O, dried with anhydrous Na_2SO_4 and concentrated under reduced pressure. The oily material was chromatographed on a Silica Gel 60 column with chloroform and chloroform - methanol (5: 1), successively. After fatty acid methyl ester was eluted with chloroform, *N*-acyl amino acid methyl ester was eluted with chloroform - methanol (5: 1) and concentrated under reduced pressure. The eluate was hydrolyzed with 6 N HCl at 110°C for 20 hours in a sealed tube and subjected to an amino acid analyzer. The Rf value of this eluate on a Silica Gel 60 plate with chloroform - methanol (5: 1) was about 0.28.

Location of Lactone Linkage

The inhibitor was reduced with $LiBH_4$ in tetrahydrofuran in three ways and analyzed for amino acid composition.

1) Each inhibitor (5 mg) was added to $LiBH_4$ - THF solution, refluxed for 6 hours and cooled to room temperature. After addition of methanol, the solution was evaporated. The residue was extracted with ethyl acetate at pH 2, concentrated under reduced pressure and hydrolyzed with 6 N HCl at 110°C for 20 hours. The hydrolysate was analyzed on an amino acid analyzer.

2) Each inhibitor (5 mg) was methylated with diazomethane, reduced with $LiBH_4$ and hydrolyzed with HCl, successively. After that, the amino acid composition was analyzed.

3) Each inhibitor (5 mg) was dissolved in 0.5 N NaOH and allowed to stand for 1 hour at room temperature to open the lactone linkage. The opened inhibitor was extracted with ethyl acetate at pH 2, reduced with $LiBH_4$ and hydrolyzed with HCl. Then, the hydrolysate was analyzed on an amino acid analyzer.

Chemicals

All the chemicals were of analytical grade.

Results and Discussion

Amino Acid Sequences

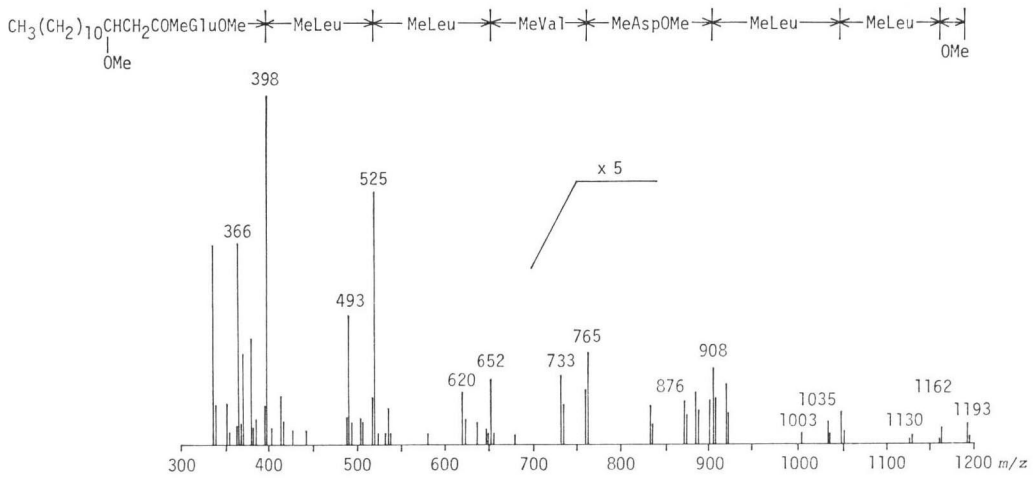
The inhibitors were composed of five kinds of β-hydroxy fatty acid residues and heptapeptide. The structures of these fatty acid residues were reported in a previous paper. The aqueous residue of hydrolysates was analyzed on an amino acid analyzer and the results showed the same compositions (Table 1). Glutamic acid, aspartic acid, valine and leucine were detected in the molecular ratio of 1: 1: 1: 4.

Table 1. Amino acid compositions of inhibitors.

Inhibitor	Asp	Glu	Val	Leu
APD-I	1.06	0.96	1.00	3.62
APD-II	1.10	0.98	1.00	3.92
APD-III	1.15	1.01	1.00	3.94

The sequence of these amino acids was determined by the application of mass spectrometry. The inhibitor was changed into permethylated derivative, which was confirmed to vaporize on a gas chromatograph run at 290°C. In the spectrum of APD-II derivative (Fig. 1), the parent peak (*m/z* 1,193) and the demethoxy peak (*m/z* 1,162) are recognized. The next peak at *m/z* 1,130 is due to the further loss of MeOH from the fatty acid moiety of permethylated derivative. The mass difference of 127 between *m/z* 1,162 and *m/z* 1,035 corresponds to the loss of an *N*-methylated leucine (MeLeu) and indicates that the *C*-terminal amino acid is leucine. The peaks at *m/z* 908, 765, 652, 525 and 398 can be regarded to be derived from the further successive elimination of MeLeu, MeAspOMe, MeVal, MeLeu and MeLeu from *m/z* 1,035, respectively. The remaining amino acid, Glu, is therefore directly linked

Fig. 1. Mass spectrum of permethylated APD-II.



to the fatty acid moiety. Furthermore, fragmentations of the APD-I derivative were all 14 mass less than those of the APD-II derivative and fragmentations of the APD-III derivative were all 14 mass more. On the other hand, *N*-acyl amino acid methyl ester was hydrolyzed with HCl and glutamic acid was detected in all three inhibitors by an amino acid analyzer. This result suggested that there was an amide linkage between the carboxyl group of the fatty acid and the amino acid group of glutamic acid. From the fragmentation pattern shown in the mass spectra and the analyses of acyl amino acid methyl ester, the linkage of the amino acid sequence in all inhibitors was found to be β-hydroxy fatty acid-Glu-Leu-Leu-Val-Asp-Leu-Leu.

Location of Lactone Linkage

The IR spectra of these inhibitors similarly indicated the presence of a lactone linkage (1730 cm^{-1}). A lactone linkage was presumed to be formed between the β-hydroxyl group of the fatty acid and one carboxyl group of either glutamic acid, aspartic acid or *C*-terminal leucine. After the inhibitor was reduced with $LiBH_4$ in three ways, that is (1) without any pretreatment, (2) after methylation with diazomethane, and (3) after treatment with alkali to open the linkage, the respective products were hydrolyzed with HCl and analyzed for amino acid composition. It is well known that a lactone or ester group is reduced to an alcohol with $LiBH_4$, but the carboxyl group is not reduced. The data on

Table 2. Determination of the location of lactone linkage in inhibitors.

Inhibitor	Treatment			Amino acid compositions			
	Hydrolysis of lactone	Methylation	Reduction	Asp	Glu	Val	Leu
APD-I	−	−	+	0.81	0.85	1.00	2.76
	−	+	+	0.03	0.17	1.00	2.78
	+	−	+	1.03	1.01	1.00	3.91
APD-II	−	−	+	0.86	0.96	1.00	2.79
	−	+	+	0.02	0.17	1.00	2.72
	+	−	+	1.08	0.86	1.00	3.85
APD-III	−	−	+	0.77	0.83	1.00	2.81
	−	+	+	0.04	0.18	1.00	2.79
	+	−	+	1.05	1.18	1.00	4.12

the amino acid compositions are summarized in Table 2. When the inhibitor was reduced without any pretreatment, one of the four leucine residues disappeared; when the inhibitor was methylated prior to reduction, glutamic acid, aspartic acid and one of the four leucine residues disappeared; and when a lactone linkage was previously opened by alkali treatment, no change in amino acid composition occurred. From these results, it was concluded that a lactone linkage was formed between the carboxyl group of *C*-terminal leucine and the β-hydroxyl group of the fatty acid moiety of the inhibitor.

Total Structures of Inhibitors

The total structures of the inhibitors were established and grouped into five kinds of acylpeptides from the constitutive fatty acids. These are summarized in Table 3. APD-I was a mixture of compound I and II, which was not separated by HPLC at this stage, and the relative quantities of compound I and II were approximately 70% and 30% from their peak areas of fatty acid methyl esters. Similarly, APD-III was a mixture of compound IV and V, and their quantities were approximately 60% and 40% from the gas chromatogram and the ^{13}C NMR spectrum as shown in a previous paper[2]. But APD-II (compound III) was isolated as a pure preparation and its proportion was the highest among the five compounds. Furthermore, as shown in an accompanying paper[9], the concentrations of APD-I, -II and -III required for 50% inhibition (IC_{50}) of cAMP phosphodiesterase in our assay system were 8.5×10^{-5} M, 4.5×10^{-5} M and 8.1×10^{-5} M. APD-II had the most potent activity, and the activities of APD-I, -II and -III were comparable to those of reticulol[5], PDE-I and PDE-II[6] which have been reported as inhibitors. However, the inhibitor was an acylpeptide and seemed to have no structural resemblance with the substrate.

Table 3. Total structures of inhibitors.

R-$CHCH_2CO$-Glu-Leu-Leu-Val-Asp-Leu-Leu
(lactone: O linking the CH of R-CH to the C-terminal Leu)

Compound	R	Fatty acid residue
I	$CH_3CH(CH_2)_7-$ CH_3	3-Hydroxy-11-methyldodecanoic acid
II	$CH_3CH_2CH(CH_2)_6-$ CH_3	3-Hydroxy-10-methyldodecanoic acid
III	$CH_3(CH_2)_{10}-$	3-Hydroxy-tetradecanoic acid
IV	$CH_3CH(CH_2)_9-$ CH_3	3-Hydroxy-13-methyltetra-decanoic acid
V	$CH_3CH_2CH(CH_2)_8-$ CH_3	3-Hydroxy-12-methyltetra-decanoic acid

Compound IV has been reported as surfactin[7], which is a potent surfactant and clotting inhibitor in the thrombin fibrinogen system. Surfactin was hydrolyzed with HCl in the same manner as our experiment and its fatty acid residue was examined. It contained compound V besides compound IV, but did not contain compound III. So the strain of *Bacillus subtilis* C-756 is different from the strain, *Bacillus subtilis* producing surfactin.

Esperin[8], antibacterial substance isolated from *Bacillus mesentricus*, has been reported to be a mixture of three acylpeptides and to have a structure similar to our inhibitors as shown in Fig. 2. But the location of the lactone linkage is between the β-hydroxy fatty acid moiety and the carboxyl group of aspartic acid, and the *C*-terminal amino acid replaced to 30% by valine.

Fig. 2. Structures of esperine.

R-$CHCH_2CO$-Glu-Leu-Leu-Val-Asp-Leu-Leu(Val)OH
(lactone: O linking the CH of R-CH to Asp)

R=$C_{12}H_{25}$(45%), $C_{11}H_{23}$(35%), $C_{10}H_{21}$(20%).

Our inhibitors, surfactin and esperin are all analogous compounds and have several biological activities. We were interested in the relation between structure and inhibition. The results of the inhibition of cAMP phosphodiesterase will be reported in a later paper[9].

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