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

I. PURIFICATION, PHYSICOCHEMICAL PROPERTIES AND STRUCTURES OF FATTY ACID RESIDUES

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(Received for publication February 28, 1983)

An inhibitor of cyclic adenosine 3′,5′-monophosphate (cAMP) phosphodiesterase was isolated from the culture filtrate of *Bacillus subtilis* C-756 isolated from soil. It was purified and finally separated into three fractions by reverse-phase HPLC. The respective fractions were designated as APD-I, -II and -III in the order eluted and the relative quantities of APD-I, -II and -III were approximately 10%, 40% and 50%, respectively. They were acylpeptides composed of β-hydroxy fatty acid residues and heptapeptide. Though the amino acid compositions of the peptides were the same, the fatty acid residues were all different. APD-I contained a mixture of 3-hydroxy-11-methyldodecanoic acid (*i*-$C_{13}h^3$) and 3-hydroxy-10-methyldodecanoic acid (*a*-$C_{13}h^3$). APD-II contained 3-hydroxytetradecanoic acid (*n*-$C_{14}h^3$). APD-III contained a mixture of 3-hydroxy-13-methyltetradecanoic acid (*i*-$C_{15}h^3$) and 3-hydroxy-12-methyltetradecanoic acid (*a*-$C_{15}h^3$).

Since cyclic adenosine 3′,5′-monophosphate (cAMP) was discovered by SUTHERLAND and RALL[1], its action has been widely studied. Now it has been established as an intracellular second messenger mediating the actions of various hormones[2] and has been suggested to be concerned with the control of many cellular functions[3]. In living cells, cAMP is synthesized from adenosine triphosphate (ATP) by adenylate cyclase [EC 4. 6. 1. 1] and degradated to adenosine 5′-monophosphate (5′-AMP) by cAMP phosphodiesterase [EC 3. 1. 4. 17].

Papaverine and methylxanthines such as caffeine and theophylline are well-known inhibitors of cAMP phosphodiesterase. As to inhibitors produced by a microorganism, UMEZAWA *et al.* isolated reticulol[4], PDE-I and PDE-II[5] from the culture filtrate of Streptomyces. We screened microbial culture filtrates for inhibitors of cAMP phosphodiesterase and, reported in a previous communication[6], isolated inhibitors from the culture filtrate of *Bacillus subtilis* C-756 isolated from soil. These inhibitors were three acylpeptides which had potent inhibitory activities compared with papaverine and theophylline.

This paper deals with the purification, physicochemical properties and structural elucidation of the fatty acid residues of the inhibitors.

Materials and Methods

Microorganism and Medium

Bacillus subtilis C-756 isolated from soil[7] was used.

The production medium for the inhibitor was composed of 1% glucose, 1% peptone, 0.3% yeast extract, 0.3% NaCl, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.1% K_2HPO_4 at pH 6.8.

HPLC

HPLC was carried out using a Waters Model 6000A pump with a Waters Model U6K injector and a

UV detector, Shimadzu SPD-2A. Column: μBondapak C_{18} (φ 3.9 mm × 30 cm). Eluent: acetonitrile - 1% acetic acid (68: 32). Flow: 1.5 ml/minute. Detection: UV 220 nm.

Physicochemical Properties

Melting points were taken using a Yanaco MP-S3 apparatus and are uncorrected. UV spectra were recorded on a Hitachi 124 spectrophotometer, and IR spectra on a Jasco IRA-1 spectrophotometer. Optical rotations were measured on a Union PM101 polarimeter.

Hydrolysis of the Inhibitor

The inhibitor was suspended in 6 N HCl and maintained at 110°C for 20 hours in a sealed tube. The reaction mixture was diluted with water and evaporated several times to remove HCl.

Gas Chromatography of Fatty Acid Methyl Esters

Fatty acid methyl esters were analyzed on a gas chromatograph equipped with a hydrogen flame detector and a steel column (φ 3 mm × 1.5 m) packed with 15% diethylene glycol succinate polymer on Chromosorb W under condition of carrier gas (N_2), 45 ml/minute; temperature, 170°C.

Characterization of Fatty Acid Methyl Esters

Each inhibitors (100 mg) was dissolved in 30% HCl - methanol (12 N HCl - methanol, 30: 70) and maintained at 90°C for 15 hours in a sealed tube. After methanolysis, HCl-methanol was removed by evaporation 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. Fatty acid methyl esters were eluted with chloroform and acyl amino acid methyl ester was eluted with chloroform - methanol. The chloroform eluate was concentrated under reduced pressure to obtain the fatty acid methyl esters (*ca.* 17 mg). The Rf value of the chloroform eluate on a Silica Gel 60 plate with chloroform - methanol (5: 1) was about 0.69.

Mass spectra were obtained on a Jeol JMS-D300 gas chromatograph-mass spectrometer (GC-MS), using a glass column (φ 3 mm × 1.0 m) packed with 5% diethylene glycol adipate on Chromosorb W at 170°C.

NMR spectra were recorded on a Jeol FX-400 spectrometer with ^1H NMR at 400.05 MHz and a Jeol FX-90Q spectrometer with ^{13}C NMR at 22.50 MHz, using TMS (0 ppm) as the internal reference.

Chemicals

Fatty acid methyl esters of 3-hydroxydodecanoic acid and 3-hydroxyhexadecanoic acid were obtained from Applied Science Laboratories Inc. (U.S.A.). All the other chemicals were of the highest grade in purity.

Results and Discussion

Production, Isolation and Purification

The strain of *Bacillus subtilis* C-756 was cultured in Erlenmeyer flasks at 30°C for 2 days on a rotary shaker to produce the inhibitor. The inhibitor produced in the 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 at 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), successively. The active fraction (8 g) was then 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 on 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 10%, 40% and 50% from their respective peak areas, respectively (Fig. 1).

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

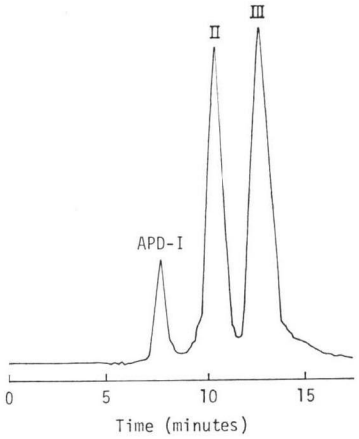


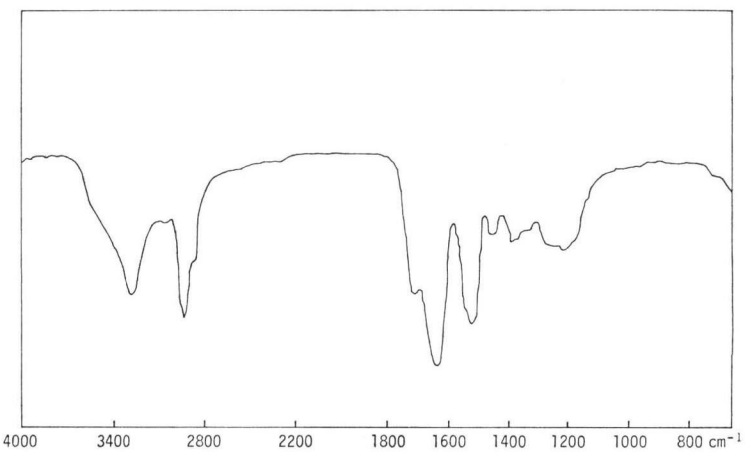
Table 1. Physicochemical properties of inhibitors.

		APD-I	APD-II	APD-III
Melting point (°C)		136~137	137~138	139~140
Elemental analysis	C	59.93	60.23	60.89
	H	8.71	8.76	8.89
	N	9.44	9.19	9.10
$[\alpha]_D^{22}$	°($CHCl_3$)	+38.5	+38.2	+37.5
		(*c* 0.26)	(*c* 0.34)	(*c* 0.37)
	(CH_3OH)	−33.3	−38.3	−37.2
		(*c* 0.24)	(*c* 0.44)	(*c* 0.38)

Physicochemical Properties

The physicochemical properties of APD-I, -II and -III are listed in Table 1. The UV spectra of the inhibitors showed the absence of an absorption maximum in the range from 220 nm to 400 nm. But the IR spectra of APD-I, -II and -III were similar, and indicated the presence of peptide bonds (1650, 1520 cm^{-1}) and a lactone linkage (1730 cm^{-1}) in the molecules (Fig. 2). APD-I, -II and -III are soluble in alkaline water, methanol, ethanol, 1-butanol, acetone, ethyl acetate, chloroform, acetonitrile, tetrahydrofuran and acetic acid, but insoluble in water, petroleum ether and *n*-hexane.

Fig. 2. IR spectrum of APD-II (KBr).



Structures of Fatty Acid Residues

The inhibitor was hydrolyzed with HCl and yellowish oily material was liberated. The oily material of hydrolysates was extracted with ether, and subjected to gas chromatography after esterification with diazomethane. The extract of APD-II hydrolysate showed a single peak of fatty acid residue and those of APD-I and -III hydrolysates each showed two neighboring peaks, suggesting a mixture of fatty acid residues. The retention times of these five fatty acid methyl esters were compared with those of reference fatty acid methyl esters under the same conditions. A commercial preparation of 3-hydroxydodecanoic acid (n-$C_{12}h^3$) and 3-hydroxyhexadecanoic acid (n-$C_{16}h^3$) methyl esters, and a mixture of 3-hydroxy-8-methylnonanoic acid (i-$C_{10}h^3$), 3-hydroxydecanoic acid (n-$C_{10}h^3$), 3-hydroxy-9-methyldecanoic acid (i-$C_{11}h^3$) and 3-hydroxy-8-methyldecanoic acid (a-$C_{11}h^3$) methyl esters prepared from cerexine D[8] were used as references. Well paralleled linear relationships between retention times and carbon numbers were observed with respective series of homologous fatty acid methyl esters (Fig. 3). The linear relationships with methyl esters of branched chain fatty acids have been shown to provide tentative identifications of their structures[9].

Fig. 3. Relationships between retention times and carbon numbers of β-hydroxy fatty acid methyl esters.

1: normal fatty acid esters, 2: *anteiso* fatty acid esters, 3: *iso* fatty acid esters.

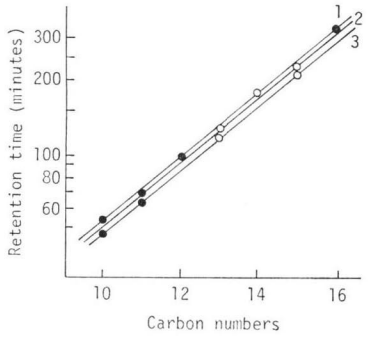
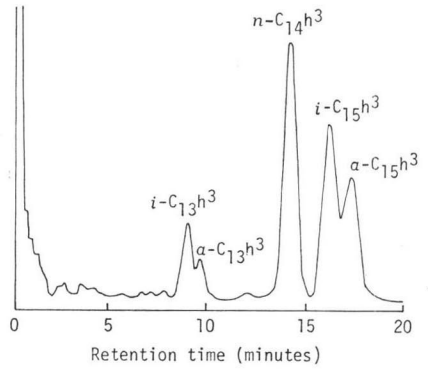


Fig. 4. Gas chromatogram of fatty acid methyl esters from inhibitors.

Column: a steel column (ϕ 3 mm × 1.5 m) packed with 15% diethylene glycol succinate polymer on Chromosorb W. Carrier gas: nitrogen, 45 ml/minute. Temperature: 170°C.



On the basis of the relationships between retention times and carbon numbers, the five fatty acids liberated from APD-I, -II and -III were assigned as 3-hydroxy-11-methyldodecanoic acid (i-$C_{13}h^3$), 3-hydroxy-10-methyldodecanoic acid (a-$C_{13}h^3$), 3-hydroxytetradecanoic acid (n-$C_{14}h^3$), 3-hydroxy-13-methyltetradecanoic acid (i-$C_{15}h^3$) and 3-hydroxy-12-methyltetradecanoic acid (a-$C_{15}h^3$) in the order eluted (Fig. 4). Furthermore, the relative quantities of i-$C_{13}h^3$ and a-$C_{13}h^3$ were approximately 70% and 30%, and those of i-$C_{15}h^3$ and a-$C_{15}h^3$ were approximately 60% and 40%, from their respective peak areas.

To obtain methyl esters without destruction of the β-hydroxy fatty acids, and formation of a mixture of α,β-unsaturated fatty acids and butyrolactones, the inhibitors were subjected to methanolysis with 30% HCl - methanol. The products were extracted with ether and chromatographed on a Silica Gel 60 column with chloroform and chloroform - methanol (5: 1), successively. Fatty acid methyl esters were eluted with chloroform and acyl amino acid methyl ester was eluted with chloroform - me-

thanol. The chloroform eluates did not contain N and showed in the IR spectra the typical feature characteristic of a fatty acid.

The mass spectra of these fatty acid methyl esters showed similar fragment ion peaks such as $M-H_2O$, $M-H_2O-CH_3$, $M-H_2O-OCH_3$, $CH(OH)CH_2COOCH_3$ (*m/z* 103) and $CH_2=C(OH)OCH_3$ (*m/z* 74) except for molecular ion peaks. A common base peak was observed at *m/z* 103, which is attributed to the fragment ion caused by β,γ-fragmentation and thought to be the common base peak of β-hydroxy fatty acid methyl esters[10]. The spectrum of the APD-II preparation, having its molecular ion peak at *m/z* 258, is shown in Fig. 5. Two neighboring peaks of the APD-I preparation had the same molecular ion of *m/z* 244, and those of the APD-III preparation had the molecular ion of *m/z* 272, similarly. The respective pairs of neighboring peaks suggested the APD-I preparation to be a mixture of β-hydroxy tridecanoic acids and APD-III preparation to be a mixture of β-hydroxy pentadecanoic acids.

The ^1H NMR spectra of these methyl esters were similar except for the shape of signals corresponding to the methyl substituent at high field. It has been reported that the methyl band of an *iso* fatty acid is sharply split and that the band of an *anteiso* fatty acid is a combined band of ill-defined ap-

Fig. 5. Mass spectrum of APD-II preparation.

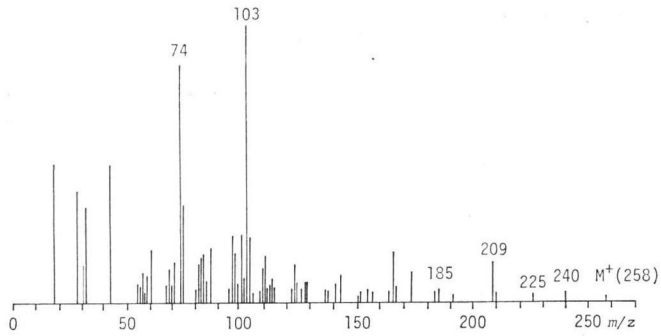
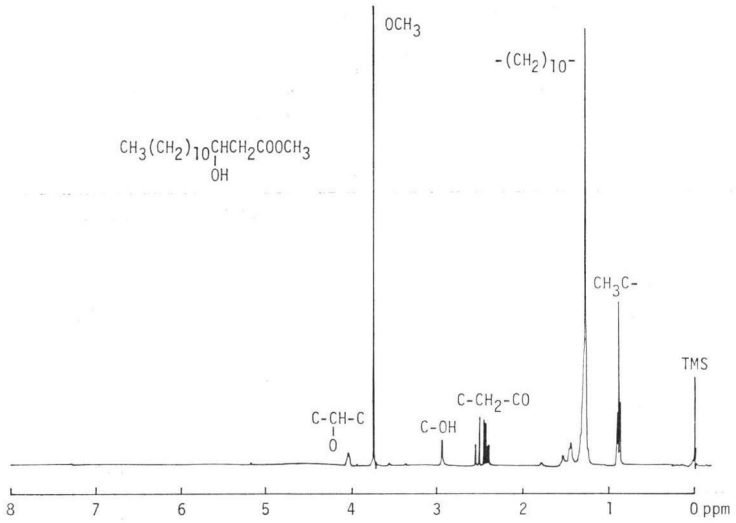


Fig. 6. ^1H NMR spectrum of APD-II preparation in $CDCl_3$.



pearance[11]. The methyl bands of the APD-I and -III preparations were more sharply split than that of the APD-II preparation. But from the ^{1}H NMR spectra, it could not be confirmed that the APD-I and -III preparations were each mixtures of *iso* and *anteiso* fatty acids. Assignment of signals from the APD-II preparation is cited in Fig. 6.

To elucidate the structure of the fatty acids, the ^{13}C NMR spectra were examined and proved them to be a mixture of *iso* and *anteiso* fatty acids. The methyl substituent at 13-position of *i*-$C_{15}h^3$ was detected at 22.67 ppm, and the methyl substituent at 12-position of *a*-$C_{15}h^3$ was at 19 26 ppm, in the ^{13}C NMR spectrum of APD-III preparation. Furthermore, calculating from the intensities of two methyl substituents (22.67 ppm) and one methyl substituent (19.26 ppm), the relative quantities of *i*-$C_{15}h^3$ and *a*-$C_{15}h^3$ were approximately 60% and 40%, respectively. This result was in good agreement with the relative quantities calculated from the peak areas of the gas chromatogram. Assignments of the signals from APD-II and -III preparations are shown in Tables 2 and 3, respectively.

Thus, the tentative identification of fatty acids presumed from the retention times of their methyl esters was finally demonstrated. The constitutive fatty acids of APD-I, -II and -III were a mixture of 3-hydroxy-11-methyldodecanoic acid (*i*-$C_{13}h^3$) and 3-hydroxy-10-methyldodecanoic acid (*a*-$C_{13}h^3$), 3-

Table 2. ^{13}C NMR data for APD-II preparation in $CDCl_3$.

$$\overset{14}{C}H_3\overset{13}{C}H_2\overset{12}{C}H_2\overset{11}{C}H_2\overset{10}{C}H_2\overset{9}{C}H_2\overset{8}{C}H_2\overset{7}{C}H_2\overset{6}{C}H_2\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H(OH)\overset{2}{C}H_2\overset{1}{C}OOCH_3$$

Carbon	Chemical shift (ppm)	Carbon	Chemical shift (ppm)
OCH_3	51.66 q	C-5	25.49 t
C-1	173.41 s	C-6,7,8,9,10,11	29.58 t
C-2	36.63 t	C-12	31.94 t
C-3	68.08 d	C-13	22.67 t
C-4	41.23 t	C-14	14.09 q

Assignments, s, d, t and q show multiplicity of off-resonance experiments.

Table 3. ^{13}C NMR data for APD-III preparation in $CDCl_3$.

$$\overset{14}{C}H_3\overset{13}{C}H(CH_3)\overset{12}{C}H_2\overset{11}{C}H_2\overset{10}{C}H_2\overset{9}{C}H_2\overset{8}{C}H_2\overset{7}{C}H_2\overset{6}{C}H_2\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H(OH)\overset{2}{C}H_2\overset{1}{C}OOCH_3$$

i-$C_{15}h^3$

$$\overset{14}{C}H_3\overset{13}{C}H_2\overset{12}{C}H(CH_3)\overset{11}{C}H_2\overset{10}{C}H_2\overset{9}{C}H_2\overset{8}{C}H_2\overset{7}{C}H_2\overset{6}{C}H_2\overset{5}{C}H_2\overset{4}{C}H_2\overset{3}{C}H(OH)\overset{2}{C}H_2\overset{1}{C}OOCH_3$$

a-$C_{15}h^3$

Chemical shift (ppm)	*i*-$C_{15}h^3$	*a*-$C_{15}h^3$	Chemical shift (ppm)	*i*-$C_{15}h^3$	*a*-$C_{15}h^3$
173.41 s	C-1	C-1	29.96 t	C-11	—
68.08 d	C-3	C-3	29.58 t	C-6,7,8,9,10	C-6,7,8,9,10,11,13
51.66 q	OCH_3	OCH_3	27.42 d	C-13	—
41.20 t	C-4	C-4	25.52 t	C-5	C-5
39.09 t	C-12	—	22.67 q	C-14, 13-CH_3	—
36.63 t	C-2	C-2	19.26 q	—	12-CH_3
34.46 d	—	C-12	11.40 q	—	C-14

Assignments, s, d, t and q show multiplicity of off-resonance experiments.

hydroxytetradecanoic acid (n-$C_{14}h^3$) and a mixture of 3-hydroxy-13-methyltetradecanoic acid (i-$C_{15}h^3$) and 3-hydroxy-12-methyltetradecanoic acid (a-$C_{15}h^3$), respectively.

The results demonstrating the amino acid sequence, the total structure of the inhibitor and the characteristics of the inhibition will be reported in later papers.

Acknowledgments

We thank Dr. J. SHOJI of Shionogi & Co., Ltd., for the gift of cerexine D. We are also indebted to Dr. H NAKANISHI of National Chemical Laboratory for Industry, Japan, for NMR spectrometric analyses and his useful discussion.

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