PLIPASTATINS: NEW INHIBITORS OF PHOSPHOLIPASE A_2, PRODUCED BY *BACILLUS CEREUS* BMG302-fF67

III. STRUCTURAL ELUCIDATION OF PLIPASTATINS

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Plipastatins are new inhibitors of phospholipase A_2 produced by *Bacillus cereus* BMG302-fF67. Structures of the plipastatins have been determined by UV, mass and NMR spectrometries and chemical degradation. The carboxyl group of the *C*-terminal L-isoleucine of plipastatinic acid has been shown to form a lactone linkage with the hydroxyl group of L-tyrosine. The total structure of plipastatins has thus been established.

As reported in previous papers[1,2], plipastatins are new acylpeptides inhibiting phospholipase A_2 and are produced by *Bacillus cereus* BMG302-fF67. The taxonomy of the producing organism, production, isolation and characterization[1], structure of plipastatinic acid (a degradation product of plipastatin)[2] were reported in the preceding papers.

In this paper, we report on the total structure of plipastatins.

Materials and Methods

Production and Isolation

Plipastatins, plipastatins A1, A2, B1 and B2, were prepared as described in a previous paper[1].

Chemicals

Benzyloxycarbonyl-L-tyrosine was purchased from Kokusan Chemical Works. *N*-Acetyl-L-tyrosine amide was from Peptide Institute Inc. All other chemicals were of analytical grade.

Analytical Instruments

UV spectra were measured on a Beckman DU-8 spectrophotometer. Secondary ion mass spectrometry (SI-MS) was carried out by a Hitachi M-80H mass spectrometer. NMR spectra were recorded on a Jeol JNM-GX400 NMR spectrometer with ^1H NMR at 400 MHz and ^{13}C NMR at 100 MHz. Amino acid analysis was carried out on a Hitachi 835 automatic amino-acid analyzer.

Preparation of *O*-Acetyltyrosine and *O*,*N*-Diacetyltyrosine Amide

O-Acetyltyrosine was prepared from benzyloxycarbonyl-L-tyrosine by an acetylation (Ac_2O/pyridine) and a successive hydrogenation (H_2/Pd-C) to remove the amino-protecting group. *O*-Acetyltyrosine was crystallized from aq EtOH as colorless amorphous: MP 220~221°C.

Anal Calcd for $C_{11}H_{13}NO_4$: C 59.18, H 5.87, N 6.28.
Found: C 58.84, H 5.69, N 5.94.

O,*N*-Diacetyltyrosine amide was prepared from *N*-acetyl-L-tyrosine amide by an acetylation (Ac_2O/pyridine) and crystallized from MeOH as colorless plate: MP 214~216°C.

Anal Calcd for $C_{13}H_{16}N_2O_4$: C 59.08, H 6.10, N 10.60.
Found: C 59.05, H 6.04, N 10.35.

Reduction with Lithium Borohydride

Plipastatin A1 (PS-A1) was reduced with lithium borohydride ($LiBH_4$) in tetrahydrofuran (THF)

in three ways and analyzed for amino acid composition.

1) PS-A1 (10 mg) was suspended in THF (2 ml), added $LiBH_4$ (1.5 mg) and refluxed at 75~80°C for 3 hours and cooled to room temp. After quenching with small amount of 10% HCl - MeOH, the solution was evaporated. The residue was separated by silica gel TLC with PrOH - H_2O (75: 25). The product (Rf 0.45) on the plate was eluted with MeOH and the eluate was concd under reduced pressure and hydrolyzed with constant boiling hydrochloric acid at 105°C for 24 hours. The hydrolysate was analyzed on an amino acid analyzer.

2) PS-A1 (10 mg) was methylated with trimethyl orthoformate (100 μl) in MeOH (1 ml) and catalytic hydrochloric acid at room temp for 5 hours, reduced with $LiBH_4$ in THF by the same procedure mentioned above and hydrolyzed with HCl, successively. After the hydrolysis, the amino acid composition was analyzed.

3) Plipastatinic acid A1 (10 mg) was reduced with $LiBH_4$ in THF by the same procedure 1) and hydrolyzed with HCl. The hydrolysate was analyzed on an amino acid analyzer.

Methylation-Reduction Procedure on Plipastatin A1

PS-A1 (10 mg) was dissolved in MeOH (1 ml). Trimethyl orthoformate (0.2 ml) and catalytic hydrochloric acid were added to the solution, which was stood at room temp for 3 hours. The reaction mixture was concd under reduced pressure to dryness after neutralizing with a sodium bicarbonate. The dimethyl ester of PS-A1 was purified by silica gel TLC with PrOH - H_2O (75: 25), which showed the molecular ion at m/z 1,491 ($M^+ + 1$) and two carbomethoxy groups at δ 3.57 and 3.56 ppm in the ^1H NMR (DMSO-d_6). The dimethyl ester (4 mg) suspended in THF (1 ml) was refluxed at 75~80°C for 3 hours with $LiBH_4$. The reaction mixture was quenched with HCl and concd to dryness. The product was hydrolyzed with constant boiling hydrochloric acid at 105°C for 20 hours and the hydrolysate was analyzed with an amino acid analyzer. The synthesized samples of δ-hydroxynorvaline (α-amino-δ-hydroxyvaleric acid, α-AHV) and γ-amino-δ-hydroxyvaleric acid (γ-AHV) were used as reference for the analysis.

Oxidation with Dimethyl Sulfoxide-Acetic Anhydride[3] of Plipastatin A1

PS-A1 (10 mg) was dissolved in a mixture (0.8 ml) of DMSO - Ac_2O (3: 1) and allowed to stand at room temp for 20 hours. The reaction mixture was evaporated *in vacuo* on hot plate (40~50°C). The residue was applied on a preparative silica gel TLC with PrOH - H_2O (75: 25). The product on the plate was eluted with MeOH and the eluate was evaporated. The residue was hydrolyzed with HCl at 105°C for 24 hours. The hydrolysate was analyzed with an amino acid analyzer.

Dinitrophenylation of Plipastatin A1

PS-A1 (10 mg) and sodium bicarbonate (10 mg) was dissolved in 0.2 ml of H_2O and then added 0.4 ml of 5% solution (w/v) of 2,4-dinitrofluorobenzene (FDNB) in EtOH to the solution. After 2 hours at 37°C, the reaction mixture was diluted with 2 ml of H_2O and extracted with ether to remove excess FDNB. The dinitrophenyl(DNP)·PS-A1 was extracted with EtOAc after acidification with HCl, purified by silica gel TLC with PrOH - H_2O (9: 1). Two DNP-residues were found in ^1H NMR (DMSO-d_6). After the hydrolysis with HCl at 105°C for 24 hours, the hydrolysate was analyzed with an amino acid analyzer. The aliquot hydrolysate was also determined for either D- or L-form of each amino acid components by the method of HPLC after the preparation of [L-Phe]dipeptide diastereomers as mentioned in the preceding paper[2]. The following aminoacids as L-Glu, D-allo-Thr, D-Ala, L-Tyr, L-Ile, L-Pro, *N*-δ-DNP-D-Orn and *O*-DNP-D-Tyr in the order eluted were detected.

Acetylation of Plipastatin A1

PS-A1 (25 mg) was acetylated with acetic anhydride (0.1 ml) in pyridine (0.5 ml) at room temp for 16 hours. The reaction mixture poured into chilled water. The product was extracted with EtOAc after adjusting pH to 4. The extract was washed with H_2O, dried over Na_2SO_4 and concd to dryness. The acetylated PS-A1 was purified by silica gel TLC with $CHCl_3$ - MeOH - AcOH (8: 2: 0.3, Rf 0.57) and crystallized in colorless prisms from EtOAc: MP 154~155°C, four signals of acetyl residues were found at 2.29 Tyr(Ac), 2.08 fatty acid(Ac), 1.96 Orn(Ac) and 1.82 ppm Thr(Ac) on the ^1H NMR spectrum in $CDCl_3$.

Results and Discussion

Location of Lactone Linkage

The IR spectra of plipastatins (PS) indicated the presence of a lactone linkage (1760 cm^{-1}), which was easily opened after treatment with dilute alkali and PS were converted to plipastatinic acid (PSA). Thus, a lactone linkage was presumed to be formed between one of the hydroxyl groups of the fatty acid, allo-threonine, D- or L-tyrosine and the carboxyl group of either glutamic acid or the *C*-terminal isoleucine.

The UV spectra of plipastatin A1 (PS-A1) and plipastatinic acid A1 (PSA-A1) are shown in Fig. 1. The UV absorption of plipastatin A1 was due to the tyrosine residue. The molecular extinction coefficients (ε) of PS-A1 and PSA-A1 were determined at absorption maximum, and showed approximately one equivalent of tyrosine for PS-A1 and two equivalents for PSA-A1, in comparison with tyrosine and *O*-acetyltyrosine (Table 1), respectively. A hypochromic effect of acetylation of the hydroxyl group of tyrosine was observed in the UV spectra (Fig. 2). The UV absorption of intact plipastatins showed the presence of one free tyrosine residue in the peptide, and the absorption of the lactone-opened plipastatinic acid showed two. This was evidence that the hydroxyl group of one of the tyrosine residues should be involved in the lactone linkage.

Plipastatin A1 was reduced with $LiBH_4$ in three ways, namely, (1) without any pretreatment, (2) after methylation with trimethyl orthoformate to convert a carboxyl group to a methyl ester, and (3) after treatment with alkali to open the lactone linkage. The respective products from each reaction were then completely hydrolyzed with hydrochloric acid and analyzed for the amino acid composition. The data from the amino acid compositions analyses are summarized in Table 2. PSA-A1 (control 2) revealed the presence of all amino acid residues found in the intact PS-A1 (control 1). When PS-A1 was reduced without any pretreatment (1), isoleucine residue disappeared; when PS-A1 was methylated prior to reduction (2), isoleucine and two of the three glutamic acid residues disappeared; and when a lactone linkage was previously opened by alkali treatment (3), no change in amino acid composition occurred. From these results, it was presumed that the carboxyl group of *C*-terminal isoleucine formed a lactone ring and one of the three glutamic acid residues existed as glutamine. Liberation of an equivalent mol of ammonia by acid hydrolysis coincides well with the above.

By methylation-reduction, the glutamyl

Fig. 1. UV spectra of plipastatin A1 and plipastatinic acid A1 (in MeOH).

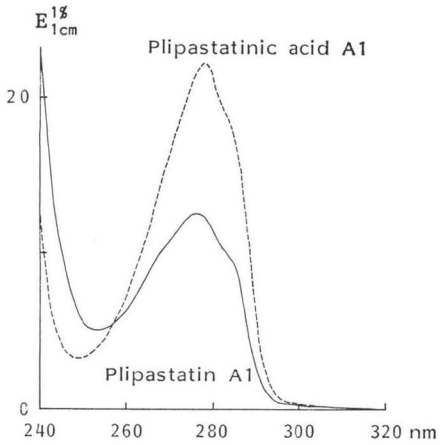


Table 1. Comparison of molar extinction coefficients between plipastatin A1 and plipastatinic acid A1, tyrosine and *O*-acetyltyrosine.

	λ_{max}	Solvent	ε
Plipastatin A1	276	MeOH	1,847
Plipastatinic acid A1	278	MeOH	3,301
Tyr	275	H_2O	1,360
Tyr(Ac)*	269	H_2O	215
Ac·Tyr·NH_2**	278	MeOH	1,666
Ac·Tyr(Ac)·NH_2***	264	MeOH	366

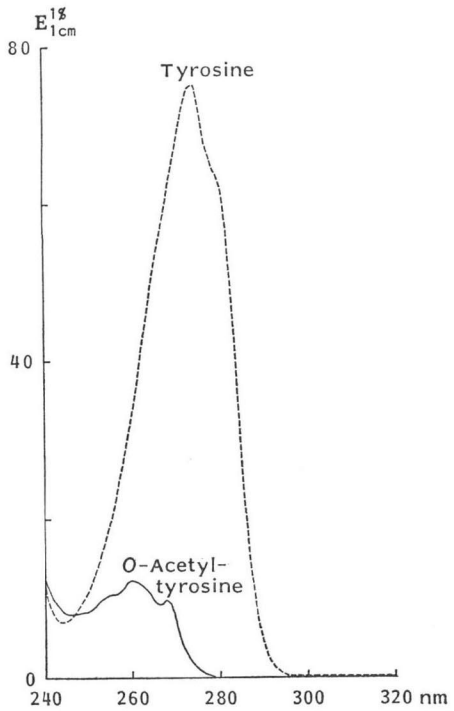
* *O*-Acetyltyrosine.
** *N*-Acetyltyrosine amide.
*** *O*,*N*-Diacetyltyrosine amide.

Table 2. Change in amino acid composition of plipastatin A1 after reduction with or without prior methylation.

	Treatment			Amino acid compositions							
	Hydrolysis of lactone	Methylation	Reduction	Thr	Glu	Pro	Ala	Ile	Tyr	Orn	NH_4
Control 1	−	−	−	0.99 (1)	3.17 (3)	1.21 (1)	1.21 (1)	0.80 (1)	1.77 (2)	1.00 (1)	0.76 (1)
Control 2	+	−	−	1.00 (1)	3.21 (3)	1.16 (1)	1.07 (1)	0.84 (1)	1.77 (2)	1.00 (1)	0.95 (1)
(1)	−	−	+	0.88 (1)	2.63 (3)	0.81 (1)	0.83 (1)	0.15 (0)	1.56 (2)	1.00 (1)	0.85 (1)
(2)	−	+	+	0.93 (1)	1.29 (1)	0.89 (1)	0.82 (1)	0.23 (0)	1.86 (2)	1.00 (1)	0.83 (1)
(3)	+	−	+	0.90 (1)	2.81 (3)	0.87 (1)	0.94 (1)	0.81 (1)	1.71 (2)	1.00 (1)	0.88 (1)

+; With, −; without.

Fig. 2. UV spectra of tyrosine and *O*-acetyltyrosine (in H_2O).



residue which forms the α-carboxyl peptide bond gives rise to the δ-hydroxynorvaline (α-AHV), whereas the residue which forms the δ-carboxyl peptide bond gives rise to γ-amino-δ-hydroxyvaleric acid (γ-AHV)[3]. Two model compounds, α-AHV and γ-AHV, were synthesized and used as the reference materials in the amino acid analysis. When these reactions were applied to PS-A1, the results were complicated due to extensive side reactions. However, with respect to the fate of the glutamyl residues, we found a large amounts of α-AHV (0.83) in addition to a small amount of γ-AHV (0.07); this small amount should be due to the glutaminyl residue modified during reduction[3]. This indicates that both of the glutamyl residues in plipastatin really exist as glutamyl residues, forming α-carboxyl peptide bond, but the δ-carboxyl of the residue is not involved in the peptide bond.

Furthermore, when plipastatinic acid A1 was digested by carboxypeptidase Y, the release of a glutaminyl residue was observed by mass spectrometry after the release of Ile and Tyr residues from the *C*-terminus of the peptide (see Part II.[2]). This suggests that the glutaminyl residue links to the next amino acid through its carboxyl group.

Plipastatin A1 was oxidized in dimethyl sulfoxide-acetic anhydride mixtures by the method of ALBRIGHT and GOLDMAN[4], and the product was hydrolyzed with hydrochloric acid and analyzed for amino acid composition. When it was oxidized the allo-threonine residue and one of tyrosine residues disappeared (Table 3). The free hydroxyl group of allo-threonine should be converted to the carbonyl[4,5], which is destroyed by hydrolysis, and the tyrosine residue should also be destroyed.

Table 3. Amino acid composition of plipastatin A1 after oxidation or dinitrophenylation.

Treatment	Amino acid composition							
	Thr	Glu	Pro	Ala	Ile	Tyr	Orn	NH_4
Oxidation	0.07	3.06	1.10	1.03	0.91	1.15	1.00	0.93
	(0)	(3)	(1)	(1)	(1)	(1)	(1)	(1)
Dinitrophenylation	1.19	2.92	1.24	1.46	0.73	1.00*	—*	3.00
	(1)	(3)	(1)	(1)	(1)	(1)	0	(3)

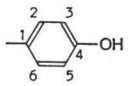
* *O*-DNP-Tyr and *N*-δ-DNP-Orn were not detected by the automatic amino acid analyzer.

Table 4. Chemical shifts of participated proton and carbon in lactone linkage.

Residues		Chemical shift (ppm)					
		^{1}H NMR			^{13}C NMR		
		PS[a]	PSA[b]	4AcPS[c]	PS[a]	PSA[b]	4AcPS[c]
D-Tyrosine	*C-1	—	—	—	127.3	128.2	134.2
	C-2, 6	7.10	7.05	7.27	130.3	130.0	130.2
	C-3, 5	6.65	6.61	7.05	114.9	114.8	121.8
	C-4	—	—	—	155.9	155.7	149.3
L-Tyrosine	*C-1	—	—	—	132.7	128.1	133.8
	C-2, 6	6.91	6.95	7.24	130.7	130.0	130.7
	C-3, 5	6.85	6.61	6.99	120.5	114.7	120.8
	C-4	—	—	—	149.1	155.6	149.9
allo-Threonine	βCH	3.68	3.83	5.08	66.9	67.4	68.2
Fatty acid	βCH	3.76	3.79	5.23	67.4	67.4	70.4

[a] Plipastatin A1 in DMSO-d_6 at 50°C.
[b] Plipastatinic acid A1 in DMSO-d_6 at 50°C.
[c] Tetraacetyl plipastatin A1 in $CDCl_3$ at 35°C.

* Numbering of tyrosine carbon.

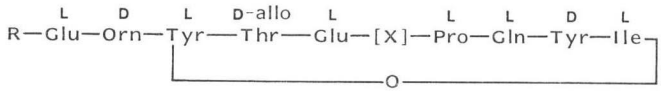


Plipastatin A1 was dinitrophenylated in the usual way, and the product was hydrolyzed and analyzed with an amino acid analyzer. The data from the amino acid composition analyses of these experiments are summarized in Table 3. When PS-A1 was exhaustively dinitrophenylated and hydrolyzed, free L-tyrosine was found in addition to *O*-DNP-D-tyrosine, *N*-δ-DNP-D-Orn and other free amino acids by the analysis of [L-Phe]dipeptide diastereomers as described in the preceding paper[2]. These facts indicate that the hydroxyl group of the L-tyrosine residue, located in the third residue from *N*-terminal amino acid of the peptide, is involved in the lactone linkage, but allo-threonine and D-tyrosine are not.

NMR studies of PS-A1 and its derivatives support the hypothesis that a lactone linkage between the carboxyl group of *C*-terminal isoleucine and the hydroxyl group of tyrosine exists. The comparable chemical shifts of the appropriate proton and carbon of tyrosine, allo-threonine and β-hydroxy fatty acid residues on the lactone linkage are listed in Table 4. Two tyrosine residues were observed by the different chemical shift of the carbons in the intact PS. One was a tyrosine residue having free hydroxyl group (in case of C-4 at 155.9 ppm) which was similar to the lactone-opened tyrosine residue (C-4 at 155.6 ppm) in PSA and the another was a lactonized tyrosine residue (C-4 at 149.1 ppm) which was similar to the acetylated tyrosine (C-4 at 149.3 ppm) in tetraacetyl PS (4AcPS).

In addition, the β-methine protons of allo-threonine and β-hydroxy fatty acid residues (δ_H 3.68

Table 5. Total structure of plipastatins.



Plipastatin	R	[X]
A1	$CH_3(CH_2)_{12}CHCH_2CO-$ (OH)	3(*R*)-hydroxyhexadecanoic acid	D-Ala
A2	$CH_3CH_2CH(CH_3)(CH_2)_{10}CHCH_2CO-$ (OH)	14(*S*)-methyl-3(*R*)-hydroxyhexadecanoic acid	D-Ala
B1	$CH_3(CH_2)_{12}CHCH_2CO-$ (OH)	3(*R*)-hydroxyhexadecanoic acid	D-Val
B2	$CH_3CH_2CH(CH_3)(CH_2)_{10}CHCH_2CO-$ (OH)	14(*S*)-methyl-3(*R*)-hydroxyhexadecanoic acid	D-Val

and 3.76 ppm in DMSO-d_6) in the intact PS appeared about 1 ppm up-field compared with those of the acetylated compound 4AcPS (δ_H 5.08 and 5.23 ppm in $CDCl_3$). The hydroxyl groups of allo-threonine and β-hydroxy fatty acid residues are therefore indicated to be free from the lactone linkage. From these results, it was concluded that a lactone linkage was formed between the carboxyl group of *C*-terminal L-isoleucine and the hydroxyl group of L-tyrosine.

Total Structures of Plipastatins

The total structures of all plipastatins were established as summarized in Table 5. The group A plipastatins had D-Ala at the 6-position of the amino acid sequence from the *N*-terminus, and group B had D-Val in this position. The plipastatins numbered 'one' (*i.e.* A1 and B1) had 3(*R*)-hydroxyhexadecanoic acid (β-hydroxypalmitic acid) as the fatty acid residue and those numbered 'two' (A2 and B2) had 14(*S*)-methyl-3(*R*)-hydroxyhexadecanoic acid (β-hydroxy-*anteiso*-palmitic acid) as the fatty acid residue.

Several acylpeptides having a lactone linkage between the carboxyl group of amino acid residue and the β-hydroxyl group of fatty acid moiety have been reported such as esperin[6], surfactin[7], globomycin[8] and a phosphodiesterase inhibitors[9]. And some acylpeptides which have a lactone linkage between the carboxyl group and the hydroxyl group of the amino acid residues have been also reported such as brevistin[10], lipopeptin A[11] and various neopeptins[12]. So far as we know, plipastatins are the first case that the hydroxyl group of tyrosine is involved in a lactone linkage.

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