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

## II. STRUCTURE OF FATTY ACID RESIDUE AND AMINO ACID SEQUENCE

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Plipastatins, new inhibitors of phospholipase $A_2$, were produced by a strain classified as *Bacillus cereus* BMG302-fF67. The plipastatins are a family of acylated decapeptides which differ from each other by amino acid composition and the nature of the fatty acid side chain. The fatty acids have been shown to be 3(*R*)-hydroxyhexadecanoic acid (*n*-$C_{16}h^3$) and 14(*S*)-methyl-3(*R*)-hydroxyhexadecanoic acid (*a*-$C_{16}h^3$) by mass, NMR and optical rotation. Amino acid sequence analysis by secondary ion mass spectrometry and additional physico-chemical evidence indicate that the structures of plipastatinic acids, the lactone-opened peptides are as follows: β-Hydroxy fatty acid→L-Glu→D-Orn→L-Tyr→D-allo-Thr→L-Glu→D-Ala(Val)→L-Pro→L-Gln→D-Tyr→L-Ile·OH.

Plipastatins produced by *Bacillus cereus* BMG302-fF67 are new acylpeptides which inhibit phospholipase $A_2$. The isolation and characterization of plipastatins have been described in a previous paper[1]. In this paper, we report on the structures of the fatty acid residues and the amino acid sequences of plipastatinic acids.

## Materials and Methods

### Production and Isolation

Plipastatins, plipastatins A1, A2, B1 and B2, were prepared from the fermentation broths of *B. cereus* BMG302-fF67 as described in a previous paper[1].

### Analytical Instruments

Amino acid analysis was determined on a Hitachi 835 automatic amino-acid analyzer. Optical rotations at 589 nm were measured by a Perkin-Elmer model 241 polarimeter. Secondary ion mass spectra (SI-MS) were measured 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. HPLC was performed by a Waters Model ALC/GPC 244 system.

### Methanolysis of Plipastatins

Each plipastatin (100 mg) was subjected to methanolysis in 30% HCl - MeOH (10 ml) at 90°C for 20 hours or 50°C for 48 hours in a sealed tube.

### Isolation of Fatty Acid Methyl Ester and Dimethyl *N*-Acyl Glutamate

After methanolysis, the solvents were removed by evaporation under reduced pressure and the residue was extracted with ether. The ethereal extract was washed with $H_2O$, dried ($Na_2SO_4$) and concd under reduced pressure. The oily material was subjected to silica gel TLC with $CHCl_3$ - MeOH (50: 1). The extract from the Rf 0.58 fraction, with the developing solvent, was concd to give the fatty acid methyl ester. The other extract from the Rf 0.20 fraction with MeOH was concd to give the dimethyl

*N*-acyl glutamate.

Isolation of *N*-Acyl Tripeptide Methyl Ester and Dipeptide Methyl Ester

After the ethereal extraction of the methanolysis product, the residue (*ca.* 100 mg) was chromatographed on a Sephadex LH-20 column (2.5×120 cm) with MeOH. All fractions were monitored by their absorption at 254 nm unless otherwise noted. The fraction of *N*-acyl tripeptide methyl ester was further separated by HPLC (LiChrosorb RP-2) with a 1: 1 mixture of MeCN and acetate buffer (2% potassium acetate plus 6% acetic acid, pH 4). The methyl ester was finally purified on a Sephadex LH-20 column (1×100 cm). The fraction of dipeptide methyl ester obtained from the former Sephadex LH-20 column was further purified by TLC (silica gel) with butanol - acetic acid - $H_2O$ (4: 1: 1) and by a successive Sephadex LH-20 column (1×100 cm) chromatography.

Hydrolysis of Plipastatins

Each plipastatin (4 mg) was hydrolyzed at 105°C for approximately 20 hours with constant boiling hydrochloric acid (1 ml) in a sealed tube. The hydrolysate was diluted with $H_2O$ (5 ml), extracted with ether to remove fatty acid residue and evaporated several times with $H_2O$ to remove hydrochloric acid.

Preparation of L-Phenylalanyl Amino Acid Diastereomer

The modification of the procedure by MITCHELL *et al.*[2] was carried out with L-phenylalanine instead of L-leucine.

A sodium bicarbonate stock solution (100 $\mu$l, 23 $\mu$mol) was added to a 1 ml reaction tube containing the hydrolysate (*ca.* 2 mg) or known free amino acid (1 mg). The stock solution (100 $\mu$l, 22 $\mu$mol) of *tert*-butyloxycarbonyl-L-phenylalanine-*N*-hydroxysuccinimide ester (Boc·L-Phe·OSu) in dioxane was added to the amino acid solution. The mixture was allowed to stand at room temp for 16 hours, and evaporated to dryness *in vacuo* at 40~50°C. The residue was dissolved in trifluoroacetic acid (TFA, 10 $\mu$l) and allowed to stand at room temp for 1 hour to remove the Boc group. The TFA was removed by evaporation *in vacuo*. The final residue was extracted with $H_2O$ (200 $\mu$l) and filtered through a sintered glass funnel. An aliquot of the dipeptide solution thus prepared was injected onto the reverse phase column on HPLC.

Determination of D- and L-Amino Acids by HPLC for Diastereomeric Dipeptide

The reverse phase column (Nucleosil $5C_{18}$, 0.46×10 cm) was operated at a flow rate of 1 ml/minute with a linear gradient from the solvent mixture of A to B per 40 minutes (A; 15% ammonium acetate - acetic acid - $H_2O$ - MeCN 80: 1: 1,600: 0, B; 80: 1: 1,000: 600) using a Waters ALC/GPC 200 system. The detection was carried out on OD at 254 nm.

Preparation of Plipastatinic Acid A1

Plipastatin A1 (50 mg) was dissolved in 5 ml of 0.1 N NaOH and allowed to stand at 37°C for 2 hours. It was then neutralized with HCl and lyophilized. The plipastatinic acid A1 was purified by HPLC (Nucleosil $5N_{18}$, 2×30 cm) with a 45: 55 mixture of MeCN and acetate buffer (2% potassium acetate plus 6% acetic acid, pH 4), and crystallized from MeOH to give colorless prisms.

Carboxypeptidase Digestion of Plipastatinic Acid A1

Plipastatinic acid A1 (2 mg) was hydrolyzed with a carboxypeptidase Y[3] (EC 3.4.12), purchased from Oriental Yeast Co., at 37°C on a substrate concentration of 0.67% (w/v) in 0.1 M pyridium - acetate buffer, pH 5, at a substrate/enzyme ratio of 10: 1 (w/w). Aliquots (50 $\mu$l, each containing *ca.* 300 $\mu$g of sample material) were taken from the digest at appropriate times (0, 6, 23, 48 hours) and lyophilized. The freeze - dried sample was directly submitted to mass measurement using SI-MS.

Chemicals

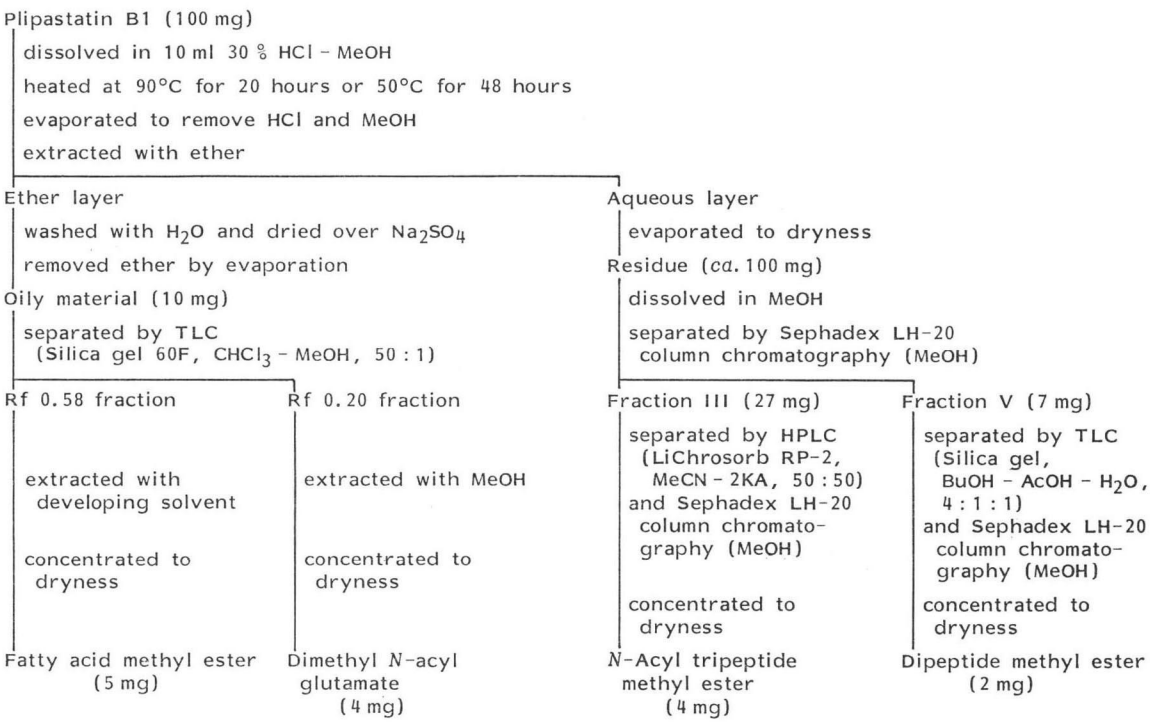
Boc·L-Phe·OSu was purchased from Kokusan Chemical Works. All other chemicals were of analytical grade.

## Results and Discussion

Structures of Fatty Acid Residue

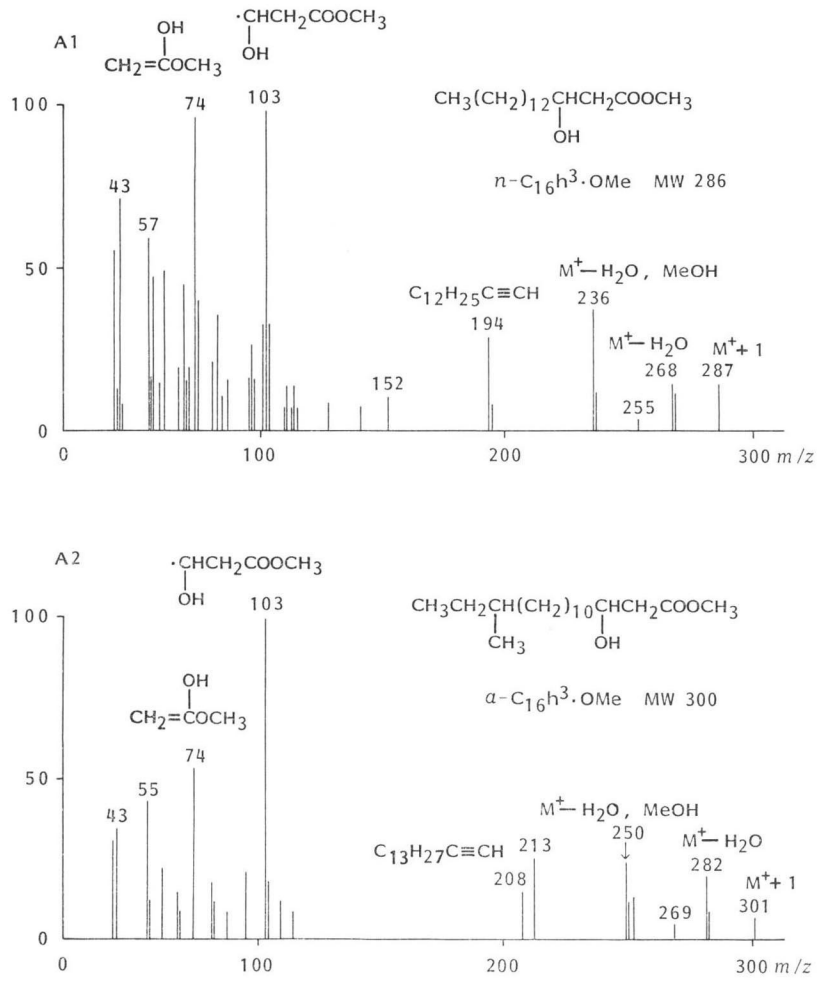
The fatty acid methyl esters were isolated by preparative TLC (silica gel) from an ethereal extract

Fig. 1. Methanolysis of plipastatin B1.




Fraction I, II, IV, VI and VII were not identified.
2KA; 2% KOAc+6% AcOH.

Fig. 2. Mass spectra of fatty acid methyl esters from plipastatins A1 and A2 preparations.

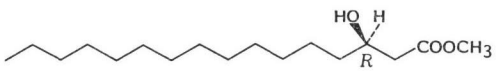



of the methanolysis product. The typical procedure and results of the methanolysis of plipastatin B1 are shown in Fig. 1. Two different fatty acid methyl esters were observed after methanolysis of the plipastatins; from plipastatins A1 and B1, 3-hydroxyhexadecanoyl acid methyl ester was found, and from plipastatins A2 and B2, 3-hydroxyheptadecanoyl acid methyl ester was found.

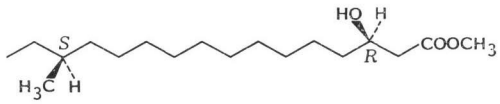
The mass spectra of these fatty acid methyl esters showed similar fragment ion peaks such as $M^+ - H_2O$, $M^+ - H_2O - MeOH$, $\cdot CH(OH)CH_2COOCH_3$ ($m/z$ 103) and $CH_2=C(OH)OCH_3$ ($m/z$ 74), however, the molecular ion peaks ($M^+ + 1$) were different. A common base peak was observed at $m/z$ 103, which is attributed to the fragment ion caused by $\beta, \gamma$-fragmentation, and thought to be the base peak of $\beta$-hydroxy fatty acid methyl esters[4,5]. The mass spectra of fatty acid methyl esters from plipastatins A1 and A2 preparations having their molecular ion peaks at $m/z$ 287 ($M^+ + 1$) and 301 ($M^+ + 1$), respectively, are shown in Fig. 2. The respective molecular ion peaks suggested the ester from plipastatins A1 and B1 to be $\beta$-hydroxy $C_{16}$ carboxylic acid, and the ester from plipastatins A2 and B2 to be $\beta$-hydroxy $C_{17}$ carboxylic acid. Physico-chemical properties of these fatty acid methyl esters from plipastatins are summarized in Table 1.

Table 1. Physico-chemical properties of fatty acid methyl esters from plipastatins.

| | | Plipastatin | | | |
|---|---|---|---|---|---|
| | | A1 | A2 | B1 | B2 |
| Structure | | **1** | **2** | **1** | **2** |
| MP (°C) | | 49~50 | | 48~49 | |
| MW ($m/z$, $M^+ + 1$) | Calcd | 287.2585 | 301.2741 | 287.2584 | 301.2740 |
| | for | $C_{17}H_{35}O_3$ | $C_{18}H_{37}O_3$ | $C_{17}H_{35}O_3$ | $C_{18}H_{37}O_3$ |
| | Found | 287.2585 | 301.2743 | 287.2568 | 301.2727 |
| MF | | $C_{17}H_{34}O_3$ | $C_{18}H_{36}O_3$ | $C_{17}H_{34}O_3$ | $C_{18}H_{36}O_3$ |
| Elemental analysis | Calcd | C 71.28, H 11.96 | C 71.95, H 12.08 | C 71.28, H 11.96 | C 71.95, H 12.08 |
| | for | $C_{17}H_{34}O_3$ | $C_{18}H_{36}O_3$ | $C_{17}H_{34}O_3$ | $C_{18}H_{36}O_3$ |
| | Found | C 71.20, H 12.11 | C 71.61, H 12.32 | C 70.85, H 11.59 | C 71.49, H 11.98 |
| $[M]_D$ ($CHCl_3$) | | −35.0 ($c$ 0.42) | −21.8 ($c$ 0.52) | −34.0 ($c$ 0.37) | −21.0 ($c$ 0.6) |




**1**




**2**

Except for the configuration, structures **1** and **2** can be immediately assigned to the two esters on the basis of NMR spectra as shown in Fig. 3. The $^1H$ NMR spectra of these esters were similar except for the shape of signals corresponding to the methyl substituent at high field. The methyl bond present in plipastatin A1 or B1 preparations was observed as one methyl triplet signal at 0.88 ppm which suggested the structure of 3-hydroxyhexadecanoic acid ($n$-$C_{16}h^3$) to be a straight chain without any branches. However, the methyl bond in plipastatin A2 or B2 preparations was observed as two methyl signals, the one (triplet) at 0.85 ppm and the other (doublet) at 0.84 ppm which seemed to point to an *anteiso* terminus. None the less, because methyl branching at other points along the chain would give nearly identical absorption in this region[6], the presence of an *anteiso* terminus cannot be conclusively confirmed by these signals alone.

The $^{13}C$ NMR spectra were examined, proving the structure to be *anteiso* terminus. The methyl substituent at the 14-position of 14-methyl-3-hydroxyhexadecanoic acid ($a$-$C_{16}h^3$) methyl ester was detected at 19.25 ppm in the $^{13}C$ NMR spectrum of plipastatin A2 or B2 preparation. This result was in good agreement with the value (19.14 ppm) calculated by the methods of LINDEMAN and ADAMS[7], DOSTOVALOVA *et al.*[8]. Assignments of the signals of both $n$-$C_{16}h^3$ and $a$-$C_{16}h^3$ are shown in Table 2.

Optical rotations for the esters in chloroform are shown in Table 1. The ($R$) configuration at the $\beta$-carbon atom for each of the esters can be inferred from the negative sign of the molecular rotation in chloroform[9~11]. The carboxylic acid corresponding to **1**, 3($R$)-hydroxyhexadecanoic acid, has previously been obtained from other bacterial species[12]. Its absolute configuration was inferred from its optical rotation[13] and subsequently proved by synthesis of the optical antipode from a starting material of known absolute configuration[14]. The absolute value of the molecular rotation of **2** in chloroform is smaller than that of **1** by 13°. This value is in excellent agreement with the limiting value (*ca.* 14°) of $[M]_D$ for long-chain *anteiso* acids[15,16]. The sign of this contribution to the rotation of **2** (+) allows the assignment of the ($S$) configuration at C-14.

Thus, the constitutive fatty acid of plipastatins A1 and B1 was proposed to be 3($R$)-hydroxyhexadecanoic acid ($n$-$C_{16}h^3$), and that of plipastatins A2 and B2 was 14($S$)-methyl-3($R$)-hydroxyhexadecanoic acid ($a$-$C_{16}h^3$).

Fig. 3. $^1H$ NMR spectra of fatty acid methyl esters from plipastatins A1 and A2 preparations (in $CDCl_3$).

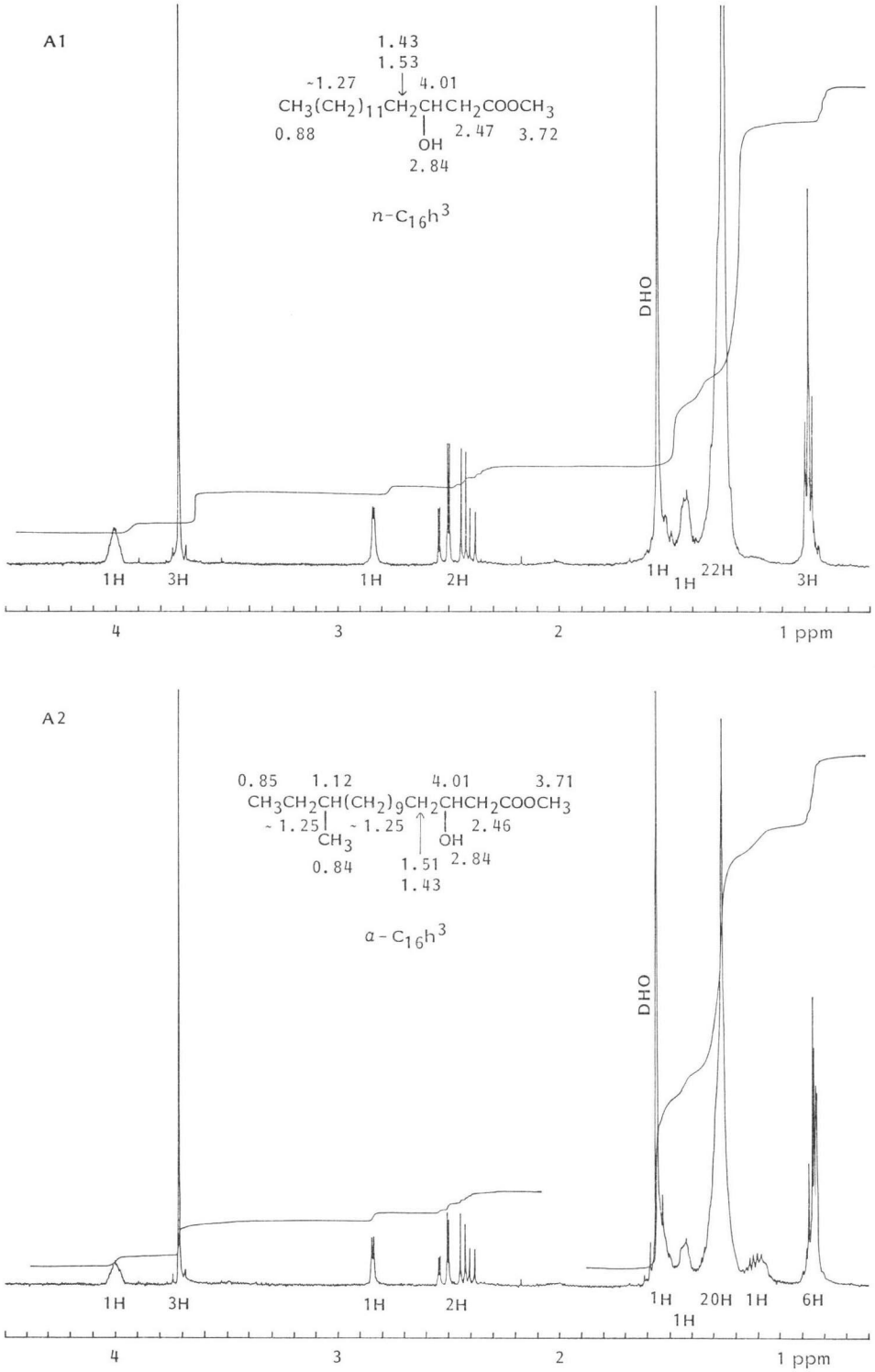
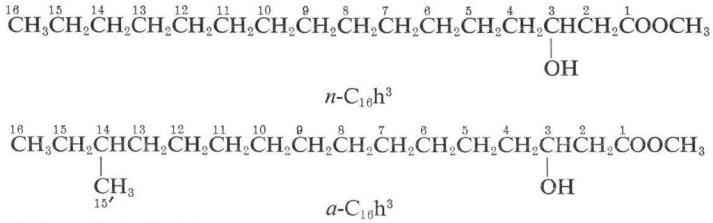


Table 2. $^{13}C$ NMR data for plipastatins A1 and A2 preparation in $CDCl_3$.



| Carbons | Chemical shift (ppm) | | | | Carbons | Chemical shift (ppm) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *n*-$C_{16}h^3$ | | *a*-$C_{16}h^3$ | | | *n*-$C_{16}h^3$ | | *a*-$C_{16}h^3$ | |
| | Calcd | Found | Calcd | Found | | Calcd | Found | Calcd | Found |
| $OCH_3$ | — | 51.69 | — | 51.68 | C-11 | 29.96 | 29~30 | 30.21 | 30.04 |
| C-1 | — | 173.40 | — | 173.40 | C-12 | | | 27.27 | 27.13 |
| C-2 | 41.86 | 41.20 | 41.86 | 41.19 | C-13 | 29.71 | | 36.66 | 36.68 |
| C-3 | 66.65 | 68.12 | 66.65 | 68.10 | C-14 | 32.4 | 31.95 | 34.59 | 34.44 |
| C-4 | 38.40 | 36.63 | 38.40 | 36.61 | C-15 | 22.65 | 22.71 | 29.60 | ~30 |
| C-5 | 24.71 | 25.50 | 24.71 | 25.49 | C-15′ | — | — | 19.14 | 19.25 |
| C-6~10 | 29.96 | 29~30 | 29.96 | 29~30 | C-16 | 13.86 | 14.10 | 10.87 | 11.39 |

Table 3. Amino acid compositions of plipastatins.

| Plipastatin | Thr | Glu | Pro | Ala | Val | Ile | Tyr | Orn | $NH_4$ |
|---|---|---|---|---|---|---|---|---|---|
| A1 | 0.99 | 3.17 | 1.21 | 1.12 | — | 0.80 | 1.77 | 1.00 | 0.76 |
| A2 | 0.98 | 3.22 | 1.17 | 1.05 | — | 0.83 | 2.10 | 1.00 | 0.93 |
| B1 | 0.97 | 3.06 | 1.10 | — | 1.10 | 0.85 | 1.86 | 1.00 | 1.02 |
| B2 | 0.96 | 3.02 | 1.09 | — | 1.01 | 0.91 | 1.77 | 1.00 | 1.12 |

Table 4. Determination of D- or L-amino acids composed plipastatins.

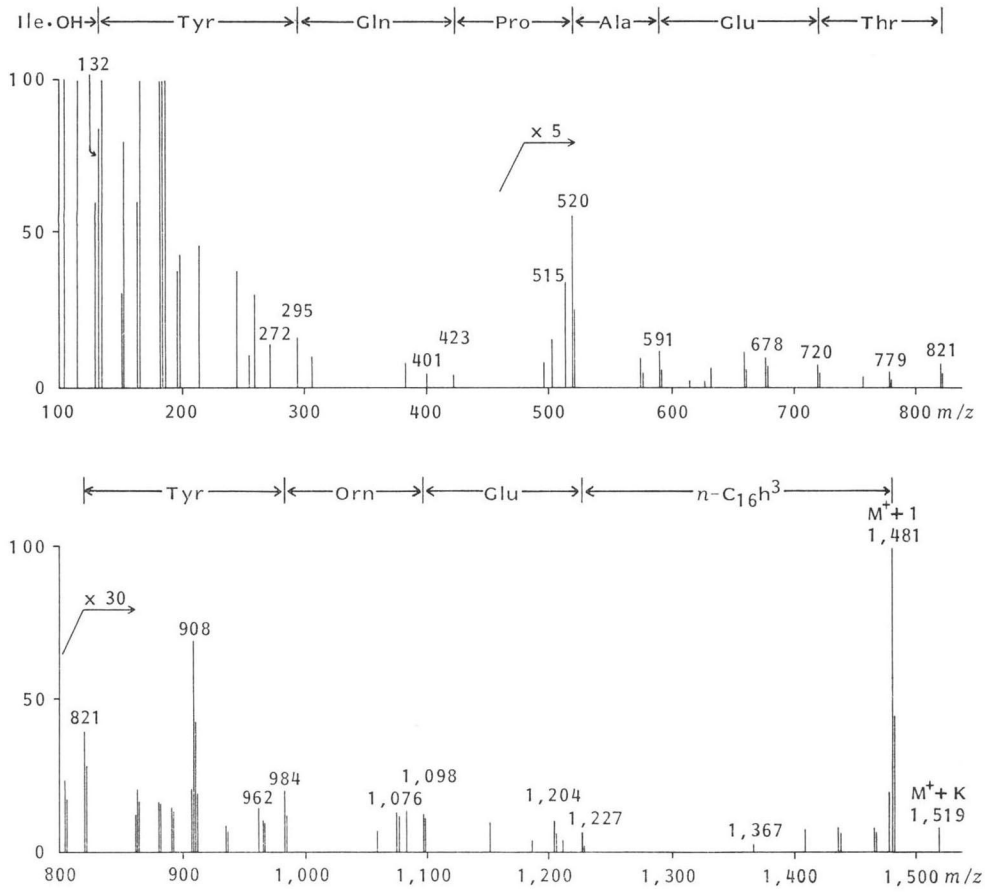
| Amino acid | Elution time (minutes) [L-Phe]Dipeptide | | Identification of amino acid composed plipastatin | | | |
|---|---|---|---|---|---|---|
| | L | D | A1 | A2 | B1 | B2 |
| Glu | 7.37 | 13.18 | L | L | L | L |
| Thr | 11.20 | 19.56 | — | — | — | — |
| allo-Thr | 12.01 | 16.94 | D | D | D | D |
| Ala | 15.35 | 19.30 | D | D | — | — |
| Val | 16.60 | 26.77 | — | — | D | D |
| Tyr | 19.87 | 25.07 | L, D | L, D | L, D | L, D |
| Ile | 21.67 | 30.73 | L | L | L | L |
| Pro | 22.73 | 23.08 | L | L | L | L |
| Orn | 24.80 | 29.83 | D | D | D | D |

—: Not detected.

## Amino Acid Sequences

The results of amino acid analyses of the plipastatins, as shown in Table 3, indicate the presence of two different decapeptides; a plipastatin A group comprised of Thr(1), Glu(3), Pro(1), Ala(1), Ile(1), Tyr(2) and Orn(1) residues, and a plipastatin B group comprised of Thr(1), Glu(3), Pro(1), Val(1), Ile(1), Tyr(2) and Orn(1) residues. One mol of ammonia from each peptide suggests that each should contain one mol of glutamine residue.

Fig. 4. Mass spectrum of plipastatinic acid A1.




To determine the configurations, the free amino acids obtained from plipastatin hydrolysate have been treated with Boc·L-Phe·OSu to form, after acid-lytic cleavage of the Boc group, the diastereomeric dipeptides L-Phe-D-amino acid or L-Phe-L-amino acid. The elution times of the [L-Phe]dipeptide diastereomers of each amino acid contained in plipastatins on HPLC are listed in Table 4. The qualitative analyses of the resulting dipeptide mixture obtained from plipastatin hydrolysates showed that group A (plipastatins A1 and A2), was composed of D-allo-Thr, L-Glu, L-Pro, D-Ala, L-Ile, L-Tyr, D-Tyr and D-Orn; group B (plipastatins B1 and B2) was composed of D-allo-Thr, L-Glu, L-Pro, D-Val, L-Ile, L-Tyr, D-Tyr and D-Orn (Table 4).

The presence of a lactone linkage in plipastatin A1 was suggested by the absorption at 1760 $cm^{-1}$ in IR spectrum[1]. When the plipastatins were treated with dilute sodium hydroxide at room temperature, they were easily converted into the products named plipastatinic acids. The plipastatinic acids showed the disappearance of the absorption at 1760 $cm^{-1}$ in IR spectra and the increase of 18 ($H_2O$) mass units over the corresponding plipastatins in mass spectra. The amino acid analyses of plipastatinic acids revealed the presence of all amino acid residues found in the intact plipastatins. These data indicate that plipastatinic acids are the product of opening of the lactone ring present in plipastatins.

The sequence of these amino acids was determined by the application of SI-MS. The intact

Table 5. Properties of dimethyl *N*-acyl glutamate from plipastatins.

| | | Plipastatin | | | |
|---|---|---|---|---|---|
| | | A1 | A2 | B1 | B2 |
| MP (°C) | | | | 82~82.5 | |
| MW (*m/z*, $M^+$) | Calcd | 429.3087 | 443.3244 | 429.3088 | 443.3244 |
| | for | $C_{23}H_{43}NO_6$ | $C_{24}H_{45}NO_6$ | $C_{23}H_{43}NO_6$ | $C_{24}H_{45}NO_6$ |
| | Found | 429.3075 | 443.3257 | 429.3098 | 443.3261 |
| $[\alpha]_D$ ($CHCl_3$) | | | | +2.6 (*c* 0.34) | |
| Amino acid detected after hydrolysis | | Glu | Glu | Glu | Glu |

plipastatins showed the respective parent peaks but not good fragments for interpretation of the amino acid sequence. In the spectrum of plipastatinic acid A1 (Fig. 4), the parent peak (*m/z* 1,481, $M^+ + 1$) and deacylpeptide peak (*m/z* 1,227, $M^+ - 255 + 2$) are recognized. The mass difference of 129 between *m/z* 1,227 and 1,098 corresponds to the loss of a glutamic acid molecule, indicating that the *N*-terminal amino acid is Glu. The peaks at *m/z* 984, 821, 720, 591, 520, 423, 295 and 132 can be regarded as being derived from the further successive elimination of Orn, Tyr, Thr, Glu, Ala, Pro, Gln and Tyr from *m/z* 1,098, respectively. The residual amino acid, Ile, is therefore the *C*-terminal amino acid. Furthermore, the fragmentation of plipastatinic acid A2 was the same as that of A1, except for the parent peak (*m/z* 1,495, $M^+ + 1$) which was 14 mass more than that of plipastatinic acid A1. Similar fragmentations of plipastatinic acids B1 and B2 were observed with the fragment of Val replacing Ala, corresponding to plipastatinic acids A1 and A2, respectively.

In addition, dimethyl *N*-acyl glutamate was isolated by silica gel TLC of the ethereal extract of the methanolysis product (see Fig. 1). Some properties of dimethyl *N*-acyl glutamate are summarized in Table 5. The glutamate showed an amide linkage between the carbonyl group of the fatty acid and the amino group of glutamic acid, which was suggested from the mass and NMR spectrometries. Furthermore, *N*-acyl tripeptide and dipeptide methyl esters were isolated from the residue which was obtained after ethereal extraction of methanolysis product (Fig. 1). The amino acid linkages of these methyl esters were found to be β-hydroxy fatty acid (*n*-$C_{16}h^3$)-(L-Glu)-(D-Orn)-(L-Tyr) and (D-Tyr)-(L-Ile), respectively, according to the mass spectrometry and the analyses of [L-Phe]dipeptide diastereomers obtained from their acid-hydrolyses.

Several methods were reported for sequencing a peptide from its *C*-terminal end in combination with mass spectrometry and digestion of a peptide by carboxypeptidase[17,18]. When plipastatinic acid A1 was digested by carboxypeptidase Y during 6 hours, mass peaks at *m/z* 1,368, 1,205 and 1,077 were observed together with the mass peak of the undigested starting peptide (*m/z* 1,481). The mass differences between 1,481 and 1,368, 1,368 and 1,205, and 1,205 and 1,077 correspond to the residual weights of Ile(113), Tyr(163) and Gln(128), respectively, indicating that the *C*-terminal sequence of the peptide is -Gln-Tyr-Ile·OH. After digestion for 6 hours, no new mass peaks were observed, suggesting that the digestion had practically stopped at this stage. However, the digestion of plipastatins by this carboxypeptidase Y was not observed because of the absence of a *C*-terminal amino acid residue.

From the fragmentation pattern shown in the mass spectra of plipastatinic acids and carboxypeptidase digestion, the identifications of *N*-acyl glutamic acid, *N*-acyl tripeptide and dipeptide methyl esters, and the analyses of amino acid configuration by [L-Phe]dipeptide diastereomers, the linkage of amino acid sequence in all plipastatinic acids were elucidated to be as follows: β-Hydroxy fatty acid-(L)Glu-

(D)Orn-(L)Tyr-(D-allo)Thr-(L)Glu-(D)Ala(Val)-(L)Pro-(L)Gln-(D)Tyr-(L)Ile·OH.

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