STUDIES ON A NEW IMMUNOACTIVE PEPTIDE, FK-156

III. STRUCTURE ELUCIDATION

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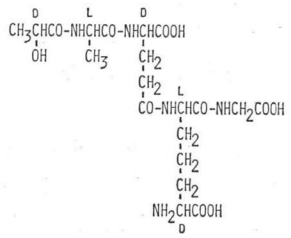
FK-156, $C_{20}H_{33}N_5O_{11}$, mp 143~148°C (dec.) is a new immunostimulant produced by *Streptomyces olivaceogriseus* sp. nov. and *S. violaceus*. The structure has been elucidated to be D-Lac-L-Ala-γ-D-Glu-(L)*meso*-α,ε-A_2pm(L)-GlyOH on the basis of chemical and spectroscopic properties.

FK-156 (Fig. 1) was isolated in the course of our screening program for novel immunostimulants from the culture filtrate of strains of *Streptomyces olivaceogriseus* sp. nov. and *S. violaceus*. Taxonomy, isolation and characterization of FK-156 are reported in the preceding papers[1,2]. This report describes the structure elucidation of this immunostimulant.

Fig. 1.



FK-156 (**1**)

FK-156 (**1**) is a water-soluble acidic peptide, mp 143~148°C (dec.), $[\alpha]_D^{25}$ −30.0° (*c* 0.5, H_2O). **1** has the molecular formula $C_{20}H_{33}N_5O_{11}$ as confirmed by microanalysis and its FD mass spectrum, these *pKa'* values 4.3, 5.8, 6.3, and 10.9 (67% DMSO) indicate the presence of three carboxyls and one amino group in its molecule. Its UV spectrum showed only end absorption and its IR spectrum (Fig. 2) exhibited bands at 1700 (COOH) and 1655 cm^{-1}(CONH). Hydrolysis of **1** to determine the amino

Fig. 2. IR spectrum of FK-156 (KBr).

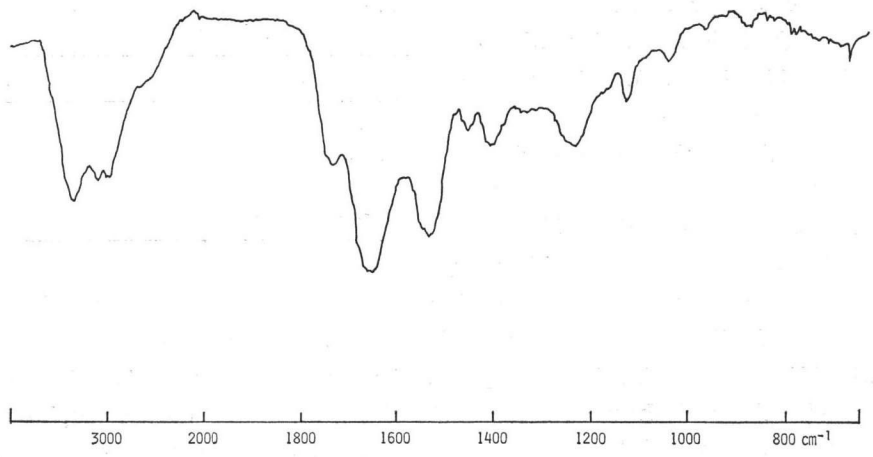
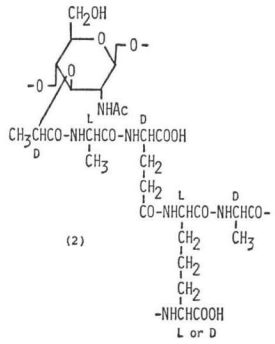


Table 1. Amino acid ratios and content (%) of FK-156.

Amino acid	Ratio
Alanine	1.00
Glutamic acid	1.01
Glycine	0.96
α,ε-Diaminopimelic acid	0.99
Recovery	88.8%
Lactic acid	5.9%

Fig. 3. Structure of bacterial peptidoglycan.



acids and other contents was carried out according to the usual procedure for peptides (6 N HCl at 110°C in a sealed tube for 18 hours). Analysis of the hydrolysate by an automatic amino acid analyzer and an isotacophoresis analyzer indicated that **1** contained glycine, alanine, glutamic, α,ε-diaminopimelic and lactic acids (Table 1). Alanine was assigned a L-configuration because it was not oxidized by D-amino acid oxidase[3]. The α,ε-diaminopimelic acid was identified as being *meso* by direct comparison of its Rf value with authentic samples on paper chromatography with methanol - water - 10 N HCl - pyridine (80: 17.5: 2.5: 10)[4]. No L,L-diaminopimelic acid could be detected in the hydrolysate. The configurations of glutamic and lactic acids could not be established by means of enzymatic experiment or other methods. The D-configuration of lactic and glutamic acids in FK-156 were assumed since both are D in the bacterial peptidoglycan (Fig. 3)[5]. This assumption was later confirmed by chemical synthesis.

FK-156 (**1**) was treated with dansyl chloride in the usual manner and resulting dansyl derivative was hydrolyzed to afford mono-dansyl α,ε-diaminopimelic acid, identical to an authentic sample prepared from mono-benzyloxycarbonyl diaminopimelic acid[6]. This fact suggested that the *N*-terminus of **1** was α,ε-diaminopimelic acid. *C*-Terminal determination of **1** using ^{3}H-labelling reaction according to MATSUO's method[7] revealed that glycine, glutamic acid and α,ε-diaminopimelic acid contained the free carboxyl groups of FK-156 (**1**) (Table 2), the result being consistent with the *pKa'* values described above. At present there is no plausible explanation for the high incorporation of ^{3}H (tritium) to α,ε-diaminopimelic acid. With MATSUO's method, acetylation of the free amino group of α,ε-diaminopimelic acid residue might be fast and the *N*-acetyl group might facilitate the formation of oxazolone ring which is a basic moiety causing ^{3}H incorporation. Based on these results, the α-carboxyl group of glutamic acid residue should be a free acid, whereas the γ-carboxyl group should be bonded to another amino acid.

Table 2. *C*-Terminal determination of FK-156 using ^{3}H-labelling reaction.

Amino acid	Ratio	
Alanine	0.04	0.009
Glutamic acid	0.47	0.57
Glycine	0.33	0.35
α,ε-Diaminopimelic acid	1.00	1.00

The PMR spectrum (Fig. 4) of **1** exhibited signals at δ 4.1 ~ 4.6 (4H, m) due to α-methine protons of amino acids and lactic acid residues. One triplet (J = 6 Hz) at δ 3.88 was attributed to a methine proton of diaminopimelic acid residue bearing a free amino moiety and a carboxyl group. The 0.52 ppm up-field shift (δ 3.35) of this signal in D_2O containing NaOD (Fig. 5) lended additional support to the assumption that both the free carboxyl and amino groups of the α,ε-diaminopimelic acid are on the same carbon. The methylene protons of glycine appeared as a singlet at δ 4.02. The doublets

Fig. 4. PMR of FK-156 (D_2O).

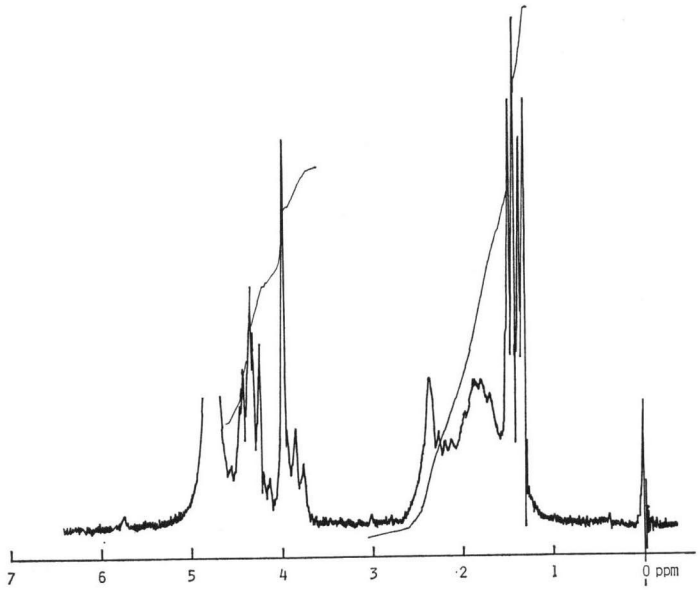
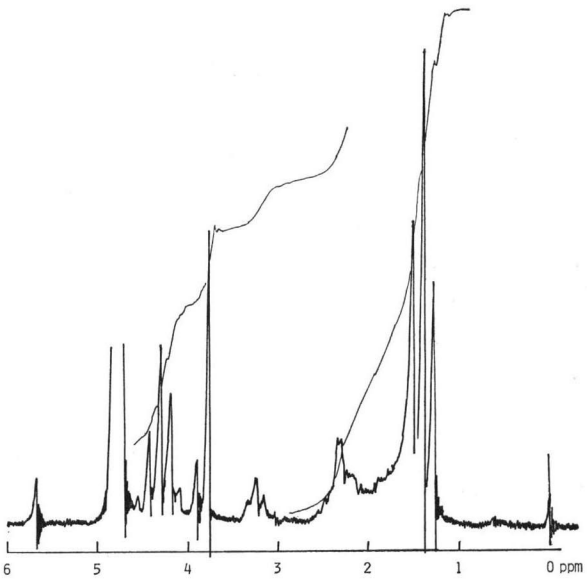


Fig. 5. PMR of FK-156 (NaOD-D_2O).

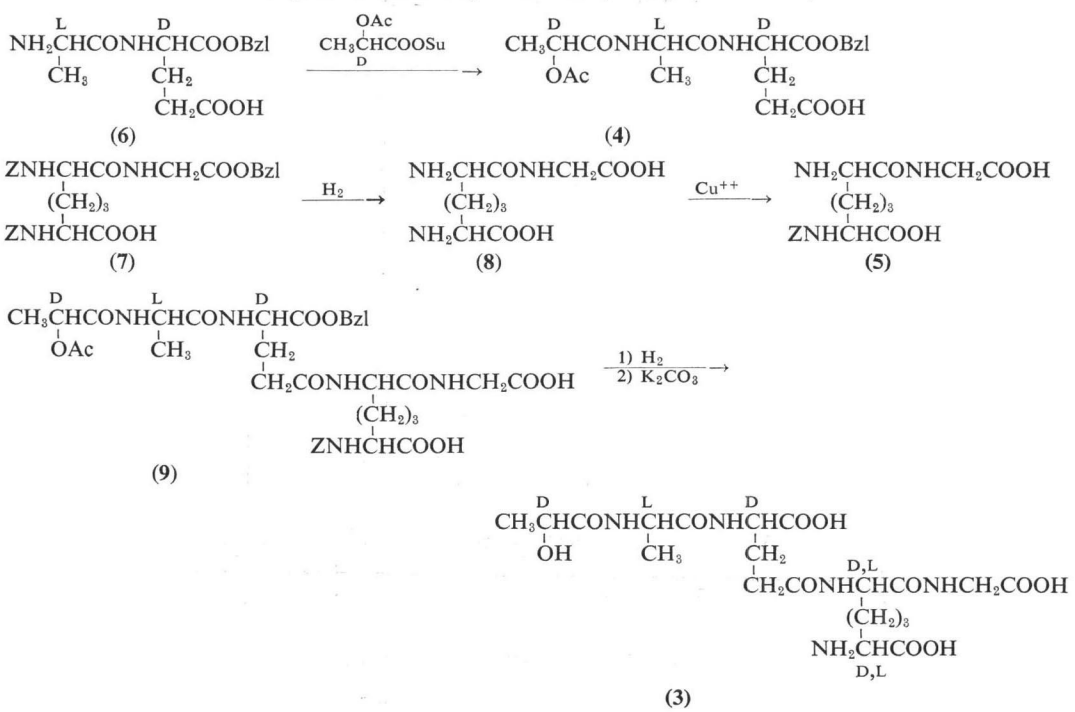


at δ 1.40 (J = 7 Hz) and 1.46 (J = 7 Hz) were assigned to methyl protons of the alanine and lactic acid residues, respectively.

In order to confirm the amino acid and lactic acid sequences of FK-156(**1**), the synthesis of D-lactyl-L-alanyl-γ-D-glutamyl-(α)-α,ε-D,L-diaminopimelyl* (α)-glycine (**3**) was undertaken. The synthesis of **3** (Fig. 6) was based on the coupling of D-Lac(Ac)-L-Ala-D-Glu(OH)OBzl (**4**) and H-(α), Z-(ε)-D,L-A_2pm* (α)-GlyOH (**5**) using active ester procedure followed by removal of protective groups. Fragment

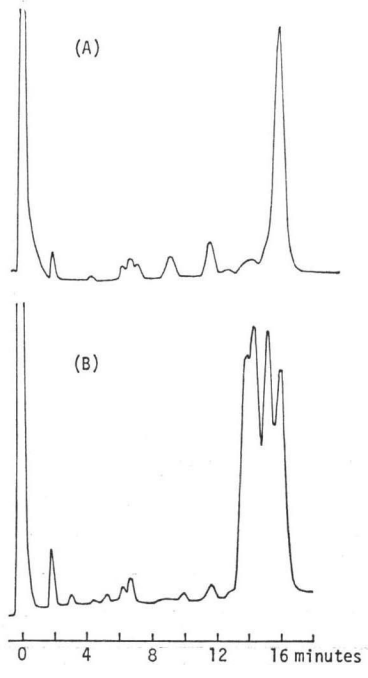
* D,L-Diaminopimelic acid (D,L-A_2pm) is a mixture consisting of *d,l-* and *meso*-acids.

Fig. 6. Schema of synthesis of **3** (four diastereoisomers).



(**4**) was prepared by the active ester procedure. Thus, treatment of D-Glu(OH)OBzl with Boc-L-AlaOSu followed by TFA in the presence of anisole afforded the N^{α}-deprotected peptide (**6**). Condensation of the peptide (**6**) with D-Lac(Ac)OH[9] as its *N*-hydroxysuccinimide ester furnished the fragment (**4**) in satisfactory yield. The fragment (**5**) was synthesized by the copper-chelate method. The condensation of equimolar Z,Z-A_2pmOH* and H-GlyOBzl with DCC in the presence of HOSu gave the half-peptide (**7**). After removal of the Z- and benzyl group by hydrogenation, the resulting dipeptide (**8**) was converted to its copper complex[6] with $CuCO_3Cu(OH)_2 \cdot nH_2O$, which was subjected to benzyloxycarbonylation with Z-Cl, giving the mono-Z-peptide copper complex. Treatment of the complex with hydrogen sulfide furnished the fragment (**5**) in acceptable yield. Condensation of the fragment (**5**) with the fragment (**4**) in an active ester method using *N*-hydroxysuccinimide afforded the protected peptide (**9**) in acceptable yield.

Fig. 7. HPLC of FK-156 and synthetic sample.
(A) FK-156, (B) Synthetic sample (**3**).
Column Packing: Nucleosil C_{18}(5 m).
Mobile Phase: 1/40 M KH_2PO_4- H_3PO_4 (250: 1).
Flow Rate: 1 ml/minute.



Removal of the protecting groups by hydrogenation followed by hydrolysis with 0.05 M potassium carbonate gave peptide (**3**). Comparison of its PMR spectrum (D_2O) and TLC behavior [cellulose sheet, butanol - acetic acid - water, 4: 1: 2] with those of natural FK-156 did not apparently give any significant difference between them, though synthetic peptide (**3**) should consist of four diastereomers due to a mixture of the starting material, α,ε-diaminopimelic acid. However, HPLC of the peptide (**3**) showed four peaks (Fig. 7) and one of them was coincided with the peak of FK-156 (**1**). The results confirmed that the synthetic preparation is compositionally homogeneous, the products differing only in the optical configuration of the α,ε-diaminopimelic acid, and supported the foregoing assignments of the sequence of FK-156 and of optical configuration of other amino acids and lactic acid.

The above results and the analogy to the structure of bacterial cell wall peptidoglycan (**2**)[5] allowed assignment of the structure of FK-156 to **1**. Establishment of the structure (**1**) has been provided by the total synthesis of optically active FK-156, which will be described in the succeeding paper[10].

Experimental

All melting points were determined with a Yanagimoto microscope hot-stage apparatus and uncorrected. IR spectra were recorded with a JASCO IRA-2 grating spectrophotometer. UV spectra were recorded by a Hitachi model 200-20 spectrophotometer. PMR spectra were obtained with a JEOL-JMN-PMX 60 NMR spectrometer (tetramethyl silane and sodium 3-trimethylsilyl propionate-d_4 as an internal standard). FD-mass spectrum was determined with a JEOL JMS-D-300 Mass spectrometer. Amino acid analysis was carried out with a Hitachi Type 835 Amino acid analyzer. Isotacophoresis was done with a Shimadzu IP-1B isotacophoresis analyzer. HPLC was recorded by a Waters Associate Model M 6000A [Column: nucleosil C_{18} (5 μm), Mobile phase: 1/40 M KH_2PO_4 - H_3PO_4 (250: 1), Flow rate: 1 ml/minute].

Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature [Biochem. J. 102: 23 (1967), 104: (1967), 126: (1972)].

Amino acid protecting groups: Z=benzyloxycarbonyl, Boc=*tert*-butyloxycarbonyl.

Carboxyl protecting groups: Bzl=benzyl.

Amino acids: Ala=alanine, Gly=glycine, Glu=glutamic acid, A_2pm=diaminopimelic acid.

Others: Lac=lactic acid, Ac=acetyl, Su=succinimidyl, TFA=trifluoroacetic acid.

Z,Z-A_2pm(OH)GlyOBzl (**7**)

To a solution of Z,Z-A_2pm$(OH)_2$ (4.58 g), H-GlyOBzl[prepared from TsOH·H-GlyOBzl (3.21 g) and triethylamine (1.4 ml) in tetrahydrofuran (10 ml)] and HOSu (1.15 g) in tetrahydrofuran (100 ml) was added DCC (2.06 g) at 0°C and the mixture was stirred at room temperature overnight. The precipitate was filtered off and the filtrate was concentrated under reduced pressure to a residue which was taken up in ethyl acetate, washed with dilute hydrochloric acid, water, aqueous sodium bicarbonate and water, dried and evaporated under reduced pressure. The residue was chromatographed on silica gel in chloroform. Elution with chloroform - methanol (10: 1) gave Z,Z-A_2pm(OH)GlyOBzl (2.67 g) as prisms, mp 103~104°C: *Anal.*; Calcd. for $C_{32}H_{35}N_3O_9$: C 63.46, H 5.83, N 6.94, Found: C 63.20, H 6.00, N 7.02%: IR (KBr) 1730 (sh.), 1685 and 1645 cm^{-1}: PMR ($CDCl_3$): δ 0.83~2.0 (6H, m), 3.3~4.5 (4H, m), 4.97 (6H, br.s), 5.0~5.6 (2H, br.), 5.8~6.6 (2H,br.) and 7.18 (15H, m).

H,H-A_2pm(OH)GlyOH (**8**)

Z,Z-A_2pm(OH)GlyOBzl (5.1 g) was hydrogenated in methanol (80 ml) and water (20 ml) with 5% Pd-C (2 g) as catalyst. The reaction mixture was filtered and the filtrate was concentrated to dryness to afford H,H-A_2pm(OH)GlyOH (1.77 g) as powder. *Anal.*; Calcd. for $C_9H_{17}N_3O_5$: C 40.75, H 7.22, N 15.84, Found: C 40.51, H 7.48, N 15.75%: IR (KBr) 3400~2400, 1685 and 1630 cm^{-1}: PMR (D_2O) δ 1.1~2.2 (6 H, m), 3.5~4.2 (2H, m) and 3.80 (2H, s).

H-(α), Z-(ε)-D,L-A_2pm(α)-GlyOH (**5**)

A mixture of H,H-A_2pm(OH)GlyOH (1.7 g) and $CuCO_3$ - $Cu(OH)_2 \cdot nH_2O$ (1.173 g) was refluxed in water (50 ml) for 2 hours. The precipitate was filtered off and the filtrate was adjusted to pH 11.5 with 2 N NaOH. To this solution was added benzyloxycabonyl chloride (3.23 ml) at 0°C and the mixture was stirred at 0°C for 6 hours at pH 11.5. The reaction mixture was neutralized with dilute hydrochloric acid and treated with H_2S. After filtration of Cu-salt, the filtrate was subjected to a column of Diaion HP-20. Elution with 80% methanol afforded H-(α),Z-(ε)-D,L-A_2pm(α)-GlyOH (1.4 g) as powder. *Anal.*; Calcd. for $C_{17}H_{23}N_3O_7 \cdot 4H_2O$: C 45.03, H 6.89, N 9.27, Found: C 45.31, H 6.72, N 9.26%: IR(KBr) 1700, 1660(sh) and 1600 cm^{-1}: PMR(CD_3OD) δ 1.1~2.2 (6H, m), 3.6~4.4 (4H, m), 5.12 (2H, s) and 7.37 (5H, s).

D-Lac(Ac)OSu

To a solution of D-Lac(Ac)OH (1 g) and HOSu (870 mg) in dry dioxane (10 ml) was added DCC (1.56 g) at 0°C. The mixture was stirred at room temperature overnight. The precipitate was filtered off and the filtrate was evaporated under reduced pressure to give D-Lac(Ac)OSu (1.7 g) which was without further purification subjected to the next reaction.

D-Lac(Ac)-L-Ala-D-Glu(OH)OBzl (**4**)

Boc-L-Ala-D-Glu(OH)OBzl (300 mg) was treated with TFA (1 ml) in the presence of anisole (0.2 ml) after stirring for 2 hours, excess TFA was evaporated off under reduced pressure. The residue was triturated with ether and decanted to give N^{α}-deprotected peptide which was dissolved in water (3 ml) containing triethylamine (0.4 ml). To this solution was added a solution of D-Lac(Ac)OSu (167 mg) in dimethylformamide (3 ml) at 0°C. The whole was stirred at room temperature overnight. The reaction mixture was acidified with dilute hydrochloric acid solution, extracted with ethyl acetate, washed with water and then dried. Evaporation of the solvent gave a residue which was subjected to a preparative thin-layer chromatography with methanol - chloroform (2: 8) to afford D-Lac(Ac)-L-Ala-D-Glu(OH)OBzl (270 mg). *Anal.*; Calcd. for $C_{20}H_{26}N_2O_8$: C 58.64, H 6.20, N 6.63, Found: C 58.64, H 6.47, N 6.58 %: IR ($CHCl_3$) 1730 and 1655 cm^{-1}: PMR ($CDCl_3$) δ 1.38, (3H, d, J = 7 Hz), 1.44 (3H, d, J = 7 Hz), 2.12 (3H, s), 2.0~2.7 (4H, m), 4.4~4.8 (2H, m), 5.0~5.3 (1H, m), 5.17 (2H, s), 6.80 (2H, br.s) and 7.3~7.4 (6H, m).

D-Lac(Ac)-L-Ala-γ-D-GluOBzl-(α),Z-(ε)A_2pm(α)-GlyOH (**9**)

D-Lac(Ac)-L-Ala-D-Glu(OSu)OBzl [prepared from D-Lac(Ac)-L-Ala-D-Glu(OH)OBzl (65 mg), HOSu (18 mg) and DCC (32 mg)] and H-(α), Z-(ε)-D,L-A_2pm(α)-GlyOH (59 mg) were dissolved in dimethylformamide (4 ml) containing triethylamine (0.03 ml) at 0°C. The mixture was stirred at room temperature overnight and the above procedure gave D-Lac(Ac)-L-Ala-γ-D-GluOBzl-(α), Z-(ε)-A_2pm(α)-GlyOH (86 mg) which was crystallized from *n*-hexane - chloroform. mp 210~213°C: *Anal.*; Calcd. for $C_{37}H_{47}N_5O_{14} \cdot H_2O$: C 55.29, H 6.14, N 8.71, Found: C 55.31, H 6.35, N 8.54%: IR (KBr) 1725, 1680 and 1620 cm^{-1}: PMR (CD_3OD) δ 1.35 (3H, d, J=7 Hz), 1.43 (3H, d, J=7 Hz), 1.3~2.2 (8H, m), 2.12 (3H, s), 2.30 (2H, m), 3.95 (2H, s), 4.0~5.0 (5H, m), 5.12 (2H, s), 5.18 (2H, s) and 7.36 (10H, s).

D-Lac(Ac)-L-Ala-γ-D-Glu-(α)-α,ε-D,L-A_2pm(α)-GlyOH

D-Lac(Ac)-L-Ala-γ-D-GluOBzl-(α),Z-(ε)-D,L-A_2pm(α)-GlyOH (20 mg) was hydrogenated in methanol (5 ml) and water (5 ml) with 10% Pd-C (50 mg) as catalyst. The reaction mixture was filtered and the filtrate was concentrated to dryness to afford D-Lac(Ac)-L-Ala-γ-D-Glu-(α)-α,ε-A_2pm(α)-GlyOH (10 mg). mp 145~150°C; IR (KBr) 1740 and 1660 cm^{-1}: PMR (D_2O) δ 1.40 (3H, d, J=7 Hz), 1.46 (3H, d, J=7 Hz), 1.4~2.2 (8H, m), 2.14 (3H, s), 2.30 (2H, m), 3.80 (1H, t, J=4.2 Hz), 3.94 (2H, s), 4.52 (3H, m) and 5.04 (1H, q, J=7Hz).

D-Lac-L-Ala-γ-D-Glu-(α)-α,ε-D,L-A_2pm(α)-GlyOH (**3**)

D-Lac(Ac)-L-Ala-γ-D-Glu-(α)-α,ε-D,L-A_2pm(α)-GlyOH (10 mg) was dissolved in 0.05 M K_2CO_3 (0.2 ml) and methanol (0.4 ml) at 0°C. This solution was stirred at room temperature for 1 hour. After neutralization with acetic acid, the solvent was evaporated under reduced pressure to afford a residue which was applied to an activated carbon column. Elution with 50% aqueous acetone gave **3** (7.0 mg) which was identical with natural FK-156 in PMR (D_2O) and TLC (cellulose sheet, butanol -

acetic acid - water, 4: 1: 2).

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