STUDIES ON A NEW IMMUNOACTIVE PEPTIDE, FK-156

IV. SYNTHESIS OF FK-156 AND ITS GEOMETRIC ISOMER

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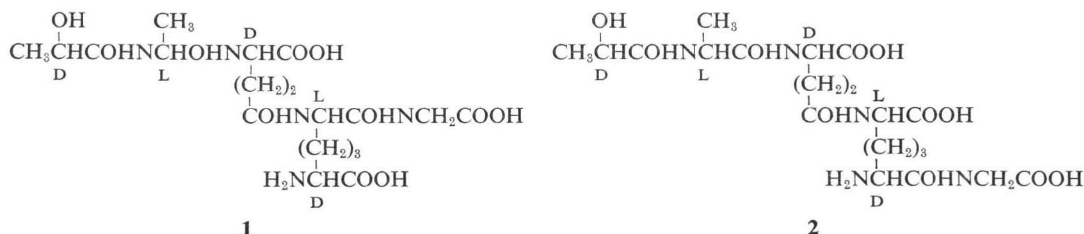
For the structural confirmation of FK-156, two possible structures, **1** and its geometric isomer **2**, were synthesized. Di-Z-*meso*-diaminopimelic acid (**4**) was converted into **14** *via* a sequence of reactions involving, as key steps, an enzyme-mediated asymmetric hydrolysis (**6**→**7**), followed by carbobenzyloxylation using a copper chelate procedure (**7**→**8**). Condensation of **14** and the appropriately protected lactoyl dipeptide **17** and removal of the protecting groups of the resulting **18** afforded **1**. Protection of **7** to **22**, followed by coupling to glycine *via* an azide method, gave **25**. Derivatization of **25** to **29** and condensation with **17** gave **30**, which was deprotected to yield **2**. Compound **1** proved to be identical in all respects with the natural product.

FK-156 (**1**) is a new adjuvant-active and immunostimulating peptide isolated from *Streptomyces olivaceogriseus* sp. nov. Its discovery, isolation and structure elucidation were described in the preceding papers of this series[1~3]. In the previous communication[4], we reported the total synthesis of **1**. This paper is devoted to a full account of that work. As described in one of the preceding papers[3], the structure of FK-156 was deduced as being **1** on the basis of its physical properties and by analogy with the bacteria cell wall peptidoglycan structures. At that time, however, there still remained another possibility of being **2**, which was ruled out only from the fact that, in the ^1H NMR analysis, the signal at δ 3.88 (in D_2O) attributed to the D asymmetric methine proton of *meso*-2,2′-diaminopimelic acid moiety was shifted high-field to δ 3.36 in D_2O-NaOD. For ascertainment of the structure of FK-156, we synthesized both **1** and **2**.

A crucial problem in the syntheses of **1** and **2** was to selectively form the peptide bonds on *meso*-2,2′-diaminopimelic acid (**3**) with proper differentiation between its two pairs of the amino acid moieties. Fortunately, a clue for this differentiation was obtained from the experiment of BRICAS *et al.*[5], who had previously performed the asymmetric hydrolysis of the bis-Boc-hydrazide derivative **6** to the mono-Boc-hydrazide **7** using leucine aminopeptidase (calf lens). Therefore, we decided to start from following their procedure.



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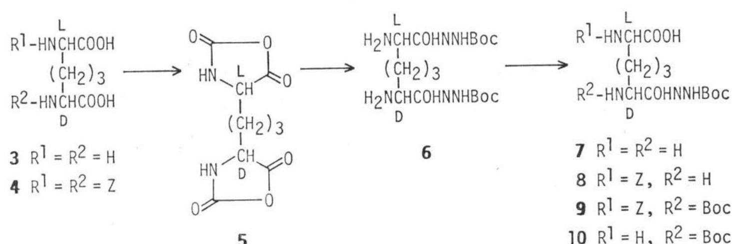
The synthetically available mixture of *meso*- and DL-2,2′-diaminopimelic acids[6] was separated by carbobenzyloxylation, followed by fractional crystallization, according to the known procedure[7], to give the pure di-Z-*meso*-2,2′-diaminopimelic acid (**4**). Although BRICAS *et al.*[5] had prepared the enzyme substrate **6** from **4** *via* mixed anhydride coupling with *t*-butyl carbazate followed by removal of the Z group by hydrogenolysis, we prepared **6** by an alternative and considerably improved procedure as follows. On treatment of **4** with phosphorus pentachloride in methylene chloride at reflux, we observed that the bis-*N*-carboxyanhydride **5** was crystallized out from the reaction mixture. The simple filtration after cooling gave **5** of adequate purity in 95% yield. This anhydride was then allowed to react with *t*-butyl carbazate in the presence of oxalic acid in acetonitrile-methanol, giving **6** as the crystalline dioxalate, which was isolated by filtration from the reaction mixture in 86% yield.

Scheme 1.



After conversion of the oxalate into the free base, **6** was subjected to the asymmetric hydrolysis using hog kidney leucine aminopeptidase according to the method of BRICAS *et al.*[5] and the product was purified by Diaion HP-20 column chromatography to give **7** in 80% yield. This asymmetric hydrolysis was subsequently found to be well mediated also by an aminopeptidase isolated from *Streptomyces sapporonensis*.* Thus, **6** (free base), incubated in the presence of the enzyme at pH 7.5 and 37°C for 1.5 hours, provided, after purification by chromatography, **7** in 84% yield. This microbial enzyme is more readily available than the hog kidney enzyme and thus more efficient for a large-scale preparation of **7**.

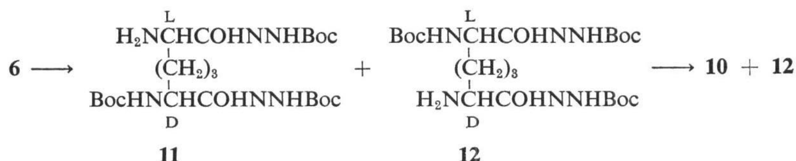
For the synthesis of **1**, we next needed to differentially protect the two amino groups in **7**, because it is necessary, after coupling glycine to the L-carboxyl group, to selectively remove the L-amino protecting group. For this purpose, we examined an approach utilizing the peculiarity of the hydrazide group on the D asymmetric center. When **7** was acylated with 1.1 equivalent of carbobenzoxy chloride in the presence of cupric chloride (0.5 equivalent) at pH 9~10, we found that the carbobenzyloxylation occurred selectively on the L-amino acid moiety, giving **8** in 85% yield after Diaion HP-20 column chromatography. The structure of **8** was deduced on the basis of its *pKa* values, 3.4 and 7.5, which correspond to those of the carboxyl group of an acylated α-amino acid and the amino group of an amidated α-amino acid, respectively.[8]

This structural assignment was corroborated as follows. *t*-Butoxycarbonylation of **8** with di-*t*-butyl dicarbonate to **9** in 93% yield, followed by removal of the Z group by hydrogenolysis over 10% palladium-charcoal, gave **10**, which showed *pKa* values, 2.3 and 9.4, corresponding to those of an unprotected α-amino acid moiety. A final conclusion on the structure of **10** was obtained by its synthesis *via* an alternative pathway as follows. The bis-Boc-hydrazide **6** (free base) was partially protected

* Isolation and characterization will be reported by IMANAKA *et al.* in due course.

with the Boc group using di-*t*-butyl dicarbonate to give the racemic mono-Boc derivatives **11** and **12**. This racemic mixture was then subjected to the leucine aminopeptidase-mediated hydrolysis. Only **11**, possessing a free amino group at the L-moiety, underwent enzymatic hydrolysis to give **10**, while **12** was recovered unchanged, establishing the structure of **10** and, consequently, that of **8**. It is particularly noteworthy that, in the above copper chelate-controlled carbobenzyloxylation, the chelation did occur preferentially at the amino acid hydrazide side.*

Scheme 2.

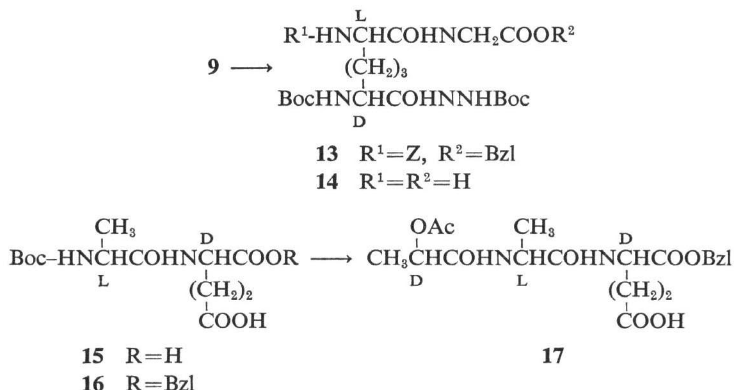


With the assurance afforded by the above favorable finding, we then focused on the synthesis of **1** using **9** as the key intermediate. Coupling **9** to benzyl glycinate was carried out *via* mixed anhydride activation using isobutyl chloroformate to produce **13** in 90% yield, which in turn was subjected to hydrogenolysis, providing **14** in 89% yield.

The remaining fragment necessary for constructing the framework of **1** is the appropriately protected D-lactoyl dipeptide **17**. Although α-benzyl Boc-L-alanyl-D-glutamate (**16**), an inevitable intermediate for reaching **17**, had been prepared by the stepwise procedure *via* α-benzyl D-glutamate[7], we preferred to synthesize it by a more direct and shorter route. Thus, Boc-L-alanyl-D-glutamic acid (**15**), prepared by the standard manner, was selectively alkylated by treatment with benzyl bromide (1.2 equivalent) in the presence of triethylamine in dimethylformamide[8] to yield the α-benzyl ester **16** contaminated with small amounts of the γ-benzyl ester and the dibenzyl ester. Purification of this mixture by recrystallization after a somewhat modified extraction procedure secured a 50% yield of the desired **16**. Removal of the Boc protecting group in **16** by treatment with trifluoroacetic acid and subsequent acylation with *O*-acetyl-D-lactoyl chloride, prepared from D-alanine according to the procedure reported in the literature[9], provided the requisite fragment **17** in 87% yield.

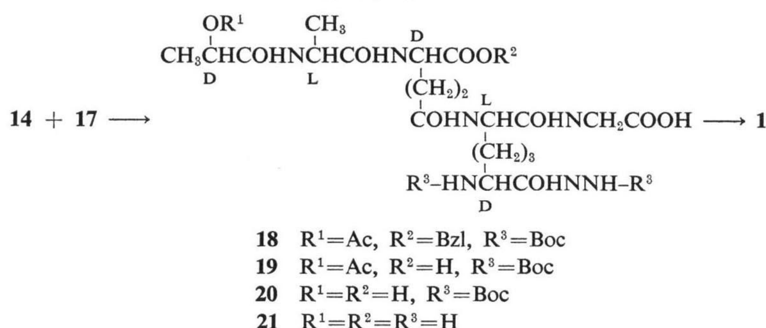
The final steps in the synthesis of **1** were the condensation of **14** and **17** and the removal of the pro-

Scheme 3.



* Details will be reported elsewhere.

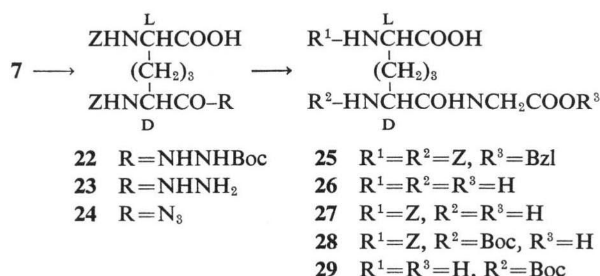
Scheme 4.



tecting groups. The fragment **17** was converted to the mixed anhydride *in situ* with isobutyl chloroformate and allowed to react with the silylated **14**, prepared *in situ* by treatment with bis(trimethylsilyl)-acetamide, resulting in a 89% yield of the condensation product **18**. For removal of the protecting groups in **18**, catalytic hydrogenation over 10% palladium-charcoal was first conducted and the product **19** was subsequently subjected to alkaline hydrolysis using aqueous potassium carbonate to yield **20**, which in turn was treated with trifluoroacetic acid to give **21**. Finally, **21** was subjected to oxidation with sodium metaperiodate in dilute sulfuric acid, followed by a spontaneously occurring hydrolysis, to yield the crude product **1**, which was purified by column chromatography of Diaion HP-20 and lyophilized to provide the pure compound in 61% yield based on **18**. This synthetic product was shown to be identical with the naturally occurring material in all respects.

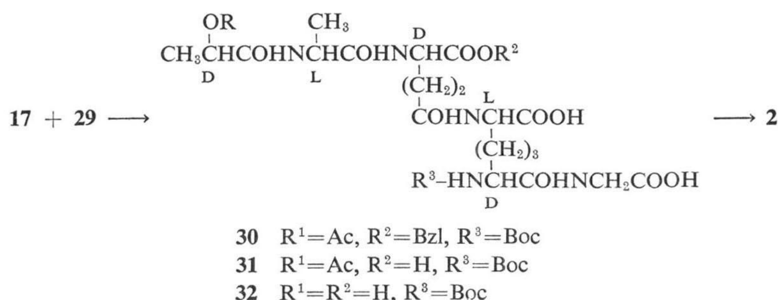
Parallel with this synthesis of **1**, we carried forward a scheme for preparing **2**. In approaching the synthetic problems for **2**, we first aimed at the selective coupling of glycine to the D-carboxyl group of *meso*-diaminopimelic acid (**3**). For this purpose, we chose the azide method starting from **7**. Carbobenzyloxylation of **7** by the standard manner gave the di-Z derivative **22**, which was followed, without purification, by removal of the Boc protecting group with hydrogen chloride in ethyl acetate to give **23** as hydrochloride in 83% yield from **7**. To prepare the azide **24**, **23** was dissolved in a mixture of ethyl acetate and 1 N hydrochloric acid and treated with aqueous sodium nitrite. The resulting **24** in the organic layer was washed with a chilled brine and immediately coupled to benzyl glycinate to produce a 51% yield of **25**. Removal of all the Z protecting groups in **25** by hydrogenolysis provided the dipeptide **26** in 90% yield.

Scheme 5.



The next step was the selective protection of the L-amino group in **26**, which was found to be well controlled again by employing the copper-chelate method. Thus, **26** was carbobenzyloxylated in the presence of cupric chloride at pH 11~12 to afford selectively the mono-Z derivative **27** in 78% yield.

Scheme 6.



The structural assignment of **27** was based on its *pKa* values, 3.2 and 3.7, corresponding to those of the carboxyl groups of acylated α-amino acids and 8.2, corresponding to the amino groups of amidated α-amino acids[8]. It is noteworthy that, in analogy with the case of **7**, the copper chelation occurred preferentially at the dipeptide function in **26**.

Protection of the free D-amino group of **27** with the Boc group by using di-*t*-butyl dicarbonate and subsequent deprotection of the Z group of the resulting **28** by hydrogenolysis gave **29** in 83% yield.

Finally, the lactoyl dipeptide fragment **17** was coupled to **29** using the *N*-hydroxysuccinimide active-ester procedure, affording a 72% yield of the condensation product **30**, which was deprotected by the following successive treatments. Hydrogenolysis over 10% palladium-charcoal (**30**→**31**), alkaline hydrolysis with 1 N sodium hydroxide (**31**→**32**) and treatment with trifluoroacetic acid yielded, after Diaion HP-20 column chromatography, the final product **2** in 51% yield based on **30**. This product was shown to be different from FK-156 by comparison of their TLC and spectral properties. In ^{1}H NMR spectrum of **2**, the methine proton of the D asymmetric center of the diaminopimelic acid moiety appeared at δ 4.09 in contrast to the corresponding signal of FK-156 at δ 3.88. Furthermore, the biological activity of **2** was found to be considerably less than that of FK-156*.

In conclusion, the structure of FK-156 was confirmed to be **1** as described above. That synthetic route to **1** is sufficiently capable of providing the sample necessary for more detailed biological testing. It should be also noted that the syntheses of **1** and even the geometric isomer **2** could serve for the preparation of various compounds related to FK-156.

Experimental**

Melting points were measured on a Thomas-Hoover apparatus and are uncorrected. Infrared and ^{1}H NMR spectra were recorded using a Hitachi 260-10 spectrophotometer and a JEOL PS-100 spectrophotometer, respectively. Optical rotations were measured on a JASCO automatic polarimeter. Thin-layer chromatography (TLC) was carried out on silica gel 60-F_{245} (E. Merck AG) using the following solvent systems: A, *n*-BuOH - AcOH - H_2O (5: 1: 4, upper phase); B, *n*-BuOH - AcOH - H_2O (2: 1: 1); C, *n*-BuOH - AcOH - H_2O (5: 2: 3); D, AcOEt - AcOH (10: 1); E, *n*-PrOH - H_2O (3: 2): the spots were detected by visualization with UV light or spraying with 0.5% ninhydrin solution in EtOH.

Bis-*N*-carboxyanhydride (**5**) of *meso*-A_2pm (**3**)

A solution of **4** (6.96 g, 15.2 mmole) in CH_2Cl_2 (140 ml) was cooled to 0°C and PCl_5 (7.00 g, 33.6

* The biological property of **2** will be reported elsewhere.

** The usual symbols of peptide chemistry were used in this experimental section. For the designation of the substitutions on diaminopimelic acid we used the abbreviation advocated by BRICAS *et al.*[12]

mmole) was added. The mixture was stirred at the same temperature for 15 minutes, at room temperature for 15 minutes and at reflux for 30 minutes. After standing at 0°C for 20 minutes, the resulting crystalline solid was filtered and washed with CH_2Cl_2 to give 3.50 g (95%) of **5**: mp>250°C; IR (Nujol) 3250, 1840, 1765 cm^{-1}; NMR (DMSO-d_6) δ 1.2~2.0 (m, 6H), 4.43 (t, J = 5 Hz, 2H), 9.06 (s, 2H).

Anal. Calcd. for $C_9H_{10}O_6N_2$: C 44.62, H 4.16, N 11.57.
Found: C 44.29, H 4.06, N 11.34.

meso-A_2pm-bis-NHNHBoc (**6**)

A solution of **5** (2.91 g, 12 mmole) in dry MeCN (20 ml) was added dropwise to a stirring solution of *t*-butyl carbazate (3.17 g, 24 mmole) and oxalic acid (3.06 g, 24 mmole) over a period of 10 minutes at room temperature and the mixture was stirred for an additional 30 minutes. The reaction mixture was then cooled to 0°C and, after 30 minutes, the precipitated crystals were collected by filtration and washed with MeCN to give 6.55 g (86%) of **6**: mp 130~135°C (dec); Rf 0.39 (A); IR (Nujol) 1720, 1680, 1610 cm^{-1}; NMR (D_2O) δ 1.47 (s, 18H), 1.3~2.3 (m, 6H), 4.10 (t, J=6 Hz, 2H).

Anal. Calcd. for $C_{17}H_{34}O_6N_6 \cdot 2C_2H_2O_4 \cdot 2H_2O$: C 39.74, H 6.67, N 13.24.
Found: C 39.42, H 6.48, N 13.24.

meso-A_2pm(D)-NHNHBoc (**7**)

(a) A sample (3.17 g, 5 mmole) of **6** (dioxalate) was dissolved in a mixture of MeOH (30 ml) and H_2O (18 ml) and cooled to 0°C. This solution was stirred and a solution of NaOH (1.60 g, 20 mmole) in H_2O (5 ml) was added. The precipitated sodium oxalate was filtered off and the filtrate was concentrated. The residue was dissolved in H_2O (15 ml) and adjusted to pH 8.5 with 2 N HCl. To this solution was added 0.2 ml of leucine aminopeptidase from hog kidney (5 mg protein/ml; Boehringer Mannheim GmbH) and the mixture was incubated at 37°C for 5 hours during which time the pH was maintained at 8.5 with 1 N NaOH. The reaction mixture was washed with AcOEt and the aqueous layer was brought to pH 7.8 with 1 N HCl and concentrated to about 10 ml. This concentrate was chromatographed on Diaion HP-20 (100 ml) eluting with H_2O and the eluate was evaporated to give an oil which was pulverized from MeOH - ether to give 1.29 g (80%) of **7** as powder: $[\alpha]_D$ −19.8° (*c* 1.0, H_2O); *pKa* 2.0, 7.1, 9.6 (H_2O); Rf 0.25 (A); IR (Nujol) 1730, 1690, 1610, 1580 cm^{-1}; NMR (CD_3OD) δ 1.47 (s, 9H), 1.4~2.1 (m, 6H), 3.3~3.7 (m, 2H).

Anal. Calcd. for $C_{12}H_{24}O_5N_4 \cdot H_2O$: C 44.71, H 8.13, N 17.38.
Found: C 45.01, H 7.98, N 17.73.

(b) A sample (6.35 g, 10 mmole) of **6** (dioxalate) was converted into the free base as described above and dissolved in H_2O (200 ml). The pH of the solution was adjusted to 7.5 and the aminopeptidase isolated from *Streptomyces sapporonensis* [70 mg, activity 5.9 units/mg (protein)*] was added. The mixture was incubated at 37°C for 1.5 hours during which time the pH was maintained at 7.5. The reaction mixture was filtered and the filtrate was washed with AcOEt. The aqueous layer was concentrated to about 20 ml and purified as described above to give 2.71 g (84%) of **7**.

Z-(L)*meso*-A_2pm(D)-NHNHBoc (**8**)

To a solution of **7** (5.74 g, 17.8 mmole) in 0.5 N NaOH (75 ml) was added $CuCl_2 \cdot 2H_2O$ (1.52 g, 8.9 mmole) and the mixture was stirred for 30 minutes. The mixture was then cooled to 0°C and, with stirring, carbobenzoxy chloride (3.34 g, 19.6 mmole) was added dropwise over a period of 20 minutes during which time the pH was maintained at 10 with 2 N NaOH. After stirring for an additional 1 hour at pH 9~10 at the same temperature, the reaction mixture was brought to pH 7 with 5% HCl and washed with AcOEt. To the aqueous layer was added *n*-BuOH (50 ml) and H_2S was bubbled for 5 minutes. After adjusting the pH to 3, the *n*-BuOH layer was separated and the aqueous layer was extracted with *n*-BuOH. The combined extract was filtered through a pad of Celite and the filtrate was evaporated to leave a powder, which was washed with ether to give 6.91 g (85%) of **8**. An analytically pure sample was prepared by recrystallization from H_2O: mp 194~196°C (dec); $[\alpha]_D$ −20.8° (*c* 0.5, MeOH); *pKa* 3.4, 7.5 (H_2O); Rf 0.62 (A); IR (Nujol) 1735, 1705, 1690 cm^{-1}; NMR (CD_3OD) δ 1.67 (s, 9H), 1.6~2.1 (m, 6H), 3.6~4.2 (m, 2H), 5.12 (s, 2H), 7.43 (s, 5H).

* One unit was defined as the quantity capable of hydrolyzing 1.0 μ mole of L-leucine *p*-nitroanilide per minute at pH 7.0 and 37°C.

Anal. Calcd. for $C_{20}H_{30}O_7N_4 \cdot H_2O$: C 52.62, H 7.07, N 12.27.
Found: C 52.41, H 6.91, N 12.23.

Z-(L)-Boc-(D)*meso*-A_2pm(D)-NHNHBoc (**9**)

Di-*t*-butyl dicarbonate (2.99 g, 13.7 mmole) and triethylamine (2.54 g, 25.1 mmole) were added to a solution of **8** (5.20 g, 11.4 mmole) in 50% aqueous dioxane (100 ml) and the mixture was stirred for 3 hours at room temperature. After evaporation of dioxane, the residual aqueous layer was washed with ether and, after acidification to pH 2 with 5% HCl, extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$ and evaporated to give 5.71 g (93%) of **9** as crystalline mass. An analytical sample was prepared by recrystallization from isopropyl ether: mp 146~148°C; $[\alpha]_D$+18.8° (*c* 1.0, MeOH); IR (Nujol) 1745, 1720, 1700, 1690, 1645 cm^{-1}; NMR ($CDCl_3$) δ 1.4~2.1 (m, 6H), 1.67 (s, 18H), 4.1~4.5 (m, 2H), 5.13 (s, 2H), 5.50 (br s, 1H), 6.00 (br s, 1H), 7.05 (br s, 1H), 7.35 (s, 5H), 8.37 (br s, 1H), 8.90 (br s, 1H).

Anal. Calcd. for $C_{25}H_{38}O_9N_4$: C 55.75, H 7.11, N 10.40.
Found: C 55.68, H 7.16, N 10.21.

Boc-(D)*meso*-A_2pm(D)-NHNHBoc (**10**)

(a) A solution of **9** (1.06 g, 2 mmole) in AcOH (10 ml) was hydrogenated over 10% Pd-C (80 mg) under H_2 at atmospheric pressure. After removal of the catalyst by filtration, the filtrate was evaporated and the residue was dissolved in H_2O (5 ml) and purified by chromatography on Diaion HP-20 (30 ml) eluting with MeOH - H_2O (1: 1) to give 0.80 g (95%) of **10**. An analytically pure sample was prepared by recrystallization from H_2O: mp 197~200°C (dec); $[\alpha]_D$+29.5° (*c* 0.5, MeOH); Rf 0.55 (A); IR (Nujol) 1730, 1680 (sh), 1665 cm^{-1}; NMR (CD_3OD-D_2O) δ 1.5~2.2 (m, 24H), 3.75 (m, 1H), 4.10 (m, 1H).

Anal. Calcd. for $C_{17}H_{32}O_7N_4 \cdot 2H_2O$: C 46.35, H 8.23, N 12.72.
Found: C 46.59, H 8.09, N 12.93.

(b) A solution of the racemic mixture of **11** and **12** (2.44 g, 4 mmole) in 10% aqueous MeOH (200 ml) was adjusted to pH 8.5 and 0.1 ml of leucine aminopeptidase from hog kidney (5 mg protein/ml) was added. The mixture was incubated at 37°C for 15 hours during which time the pH was maintained at 8.5 with 1 N NaOH. After evaporation of MeOH, the resulting aqueous solution was extracted with AcOEt and the extract was, after drying over $MgSO_4$, evaporated to an oil, which was purified by silica gel column chromatography eluting with 5% MeOH in AcOEt to give 0.68 g of **12**. An analytically pure sample was prepared by conversion into the oxalate and trituration with AcOEt - *iso*-propyl ether: powder; $[\alpha]_D$−2.3° (*c* 0.2, MeOH).

Anal. Calcd. for $C_{22}H_{42}O_8N_6 \cdot C_2H_2O_4$: C 47.36, H 7.29, N 13.81.
Found: C 47.51, H 7.20, N 13.65.

The aqueous layer was concentrated to about 20 ml and, after acidification to pH 4 with 2% HCl, applied to a column of Diaion HP-20 (50 ml) eluting with MeOH - H_2O (3: 7). The eluate was evaporated and the residue was purified by recrystallization from H_2O to give 0.56 g (32%) of **10**: mp 197~200°C (dec); $[\alpha]_D$+29.4° (*c* 0.4, MeOH).

Mono-Boc-*meso*-A_2pm-bis-NHNHBoc (**11** and **12**)

A sample (11.46 g, 18 mmole) of **6** (dioxalate) was dissolved in a mixture of MeOH (60 ml) and H_2O (36 ml) and, under ice-bath cooling, a solution of NaOH (2.88 g, 72 mmole) in H_2O (15 ml) was added. The precipitated sodium oxalate was filtered off and the filtrate was concentrated. The residue was dissolved in a mixture of dioxane (80 ml) and H_2O (150 ml) and, under ice-bath cooling, di-*t*-butyl dicarbazate (4.52 g, 21.6 mmole) was added. After stirring at 0°C for 30 minutes and at room temperature for 5 hours, dioxane was evaporated and the resulting aqueous layer was extracted with AcOEt. The extract was washed with H_2O and extracted with 1% aqueous solution of oxalic acid. The organic layer was washed with H_2O, dried over $MgSO_4$ and evaporated to give 4.70 g (42%) of di-Boc-*meso*-A_2pm-bis-NHNHBoc[powder; *m/z* 518 (M−100); IR (Nujol) 1710, 1670 cm^{-1}]. The aqueous layer was washed with AcOEt, adjusted to pH 10 with 5% NaOH and extracted with AcOEt. The extract was washed with H_2O, dried over $MgSO_4$ and evaporated to give 2.96 g (27%) of **11** and **12** (racemic mixture) which was converted into the oxalate: mp 125~129°C (dec) (oxalate) [Ref. 5, mp 133~134°C].

Anal. Calcd. for $C_{22}H_{42}O_8N_6 \cdot C_2H_2O_4 \cdot H_2O$: C 46.00, H 7.40, N 13.41.
Found: C 46.06, H 7.20, N 13.01.

Z-(L)-Boc-(D)*meso*-A_2pm(D)-NHNHBoc-(L)-GlyOBzl (**13**)

A solution of **9** (2.69 g, 5 mmole) and *N*-methylmorpholine (0.51 g, 5 mmole) in dry CH_2Cl_2 (30 ml) was cooled to $-15°C$ and a solution of isobutyl chloroformate (0.69 g, 5 mmole) in dry CH_2Cl_2 (2 ml) was added dropwise with stirring and the mixture was stirred for 30 minutes at $-15\sim-10°C$. To this mixture was added a solution of benzyl glycinate (TsOH salt) (1.69 g, 5 mmole) and *N*-methylmorpholine (0.51 g, 5 mmole) in CH_2Cl_2 (30 ml) and the mixture was stirred for 1 hour at $-10°C$ and for 30 minutes at 0°C. After evaporation of the reaction mixture, the residue was dissolved in AcOEt and washed successively with 1% HCl, H_2O, 2% $NaHCO_3$ and brine. Drying over $MgSO_4$ and evaporation gave the residue, which was recrystallized from ether to give 3.08 g (90%) of **13**: mp 85~87°C; $[\alpha]_D+6.4°$ (*c* 1.0, MeOH); IR (Nujol) 1750, 1730, 1685, 1670, 1645 cm^{-1}; NMR ($CDCl_3$) δ 1.43 (s, 18H), 1.5~2.2 (m, 6H), 4.10 (d, J = 6 Hz, 2H), 4.1~4.5 (m, 2H), 5.10 (s, 2H), 5.17 (s, 2H), 5.40 (d, J = 8 Hz, 1H), 5.90 (d, J = 8 Hz, 1H), 6.73 (br s, 1H), 7.33 (s, 10H), 7.73 (br s, 1H), 8.50 (m, 1H).

Anal. Calcd. for $C_{34}H_{47}O_{10}N_5$: C 59.55, H 6.91, N 10.21.
Found: C 59.28, H 6.80, N 10.04.

Boc-(D)*meso*-A_2pm(D)-NHNHBoc-(L)-Gly (**14**)

A solution of **13** (2.06 g, 3 mmole) in MeOH (20 ml) containing AcOH (0.5 ml) was hydrogenated over 10% Pd-C (500 mg) under H_2 at atmospheric pressure. After removal of the catalyst by filtration, the filtrate was evaporated and the residue was triturated with ether to give 1.33 g (89%) of **14**: mp 130~138°C (dec); $[\alpha]_D+46.2°$ (*c* 0.5, MeOH); Rf 0.50 (A); NMR (CD_3OD) δ 1.60 (s, 9H), 1.63 (s, 9H), 1.7~2.0 (m, 6H), 3.92 (s, 2H), 3.8~4.1 (m, 2H).

Anal. Calcd. for $C_{19}H_{35}O_8N_5\cdot 2H_2O$: C 45.86, H 7.92, N 14.07.
Found: C 45.95, H 7.63, N 13.85.

Boc-L-Ala-D-Glu (**15**)

A solution of D-Glu (7.36 g, 50 mmole) and triethylamine (10.12 g, 100 mmole) in 50% aqueous acetone (140 ml) was cooled to 0°C and a solution of Boc-L-AlaOSu (14.32 g, 50 mmole) was added. After stirring the mixture for 1 hour at the same temperature and for 15 hours at room temperature, acetone was evaporated and the resulting aqueous layer was acidified with 1 N HCl and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$ and evaporated to leave a crystalline mass, which was washed with AcOEt to give 14.16 g (89%) of **15**: mp 152~154°C (dec); $[\alpha]_D-17.6°$ (*c* 1.0, MeOH); IR (Nujol) 1750, 1705, 1660, 1640 cm^{-1}; NMR (DMSO-d_6) δ 1.16 (d, J = 7 Hz, 3H), 1.40 (s, 9H), 1.7~2.5 (m, 4H), 3.8~4.3 (m, 2H), 6.85 (br d, J = 7 Hz, 1H), 7.95 (d, J= 8 Hz, 1H), 12.33 (br s, 2H).

Anal. Calcd. for $C_{13}H_{22}O_7N_2$: C 49.05, H 6.97, N 8.80.
Found: C 48.76, H 7.12, N 8.61.

Boc-L-Ala-D-Glu(OH)OBzl (**16**)

A solution of **15** (5.10 g, 16 mmole) and triethylamine (1.78 g, 17.6 mmole) in DMF (12 ml) was cooled to 0°C and benzyl bromide (3.28 g, 19.2 mmole) was added. The mixture was stirred at the same temperature for 1 hour, at room temperature for 1 hour and then at 50°C for 1 hour. After cooling, the reaction mixture was poured into H_2O (50 ml) containing $NaHCO_3$ (1.68 g, 20 mmole) and washed with isopropyl ether (20 ml). The aqueous layer was, after removal of the powder precipitated during washing, acidified to pH 3 with 1 N HCl and extracted with AcOEt. The extract was washed twice with 0.1 M AcONa (20 ml each), dried over $MgSO_4$ and evaporated and the residue was crystallized from ether - isopropyl ether to give 3.27 (50%) of **16**: mp 84~86°C; $[\alpha]_D-3.1°$ (*c* 0.4, MeOH); Rf 0.56 (D); IR (Nujol) 1750, 1725, 1690, 1660 cm^{-1}; NMR ($CDCl_3$) δ 1.1~1.7 (m, 12H), 1.9~2.6 (m, 4H), 4.1~4.9 (m, 2H), 5.15 (s, 2H), 5.64 (m, 1H), 7.35 (s, 5H), 9.05 (s, 1H).

Anal. Calcd. for $C_{20}H_{28}O_7N_2\cdot\frac{1}{2}H_2O$: C 57.54, H 7.00, N 6.71.
Found: C 57.68, H 6.98, N 6.71.

O-Acetyl-D-lactoyl Chloride

Thionyl chloride (14.5 ml, 200 mmole) was added to *O*-acetyl-D-lactic acid (12.25 g, 100 mmole), prepared from D-Ala according to the method reported in the reference 11, and the mixture was refluxed for 1 hour. After removal of the excess of thionyl chloride by evaporation, the residue was distilled

to give 12.06 g (67%) of *O*-acetyl-D-lactoyl chloride: bp 64~65°C/22 torr; $[\alpha]_D$ +31.0° (*c* 4.0, $CHCl_3$); IR (film) 1790, 1745 cm^{-1}; NMR ($CDCl_3$) δ 1.58 (d, *J* = 7 Hz, 3H), 2.13 (s, 3H), 5.18 (q, *J* = 7 Hz, 1H).

D-Lactoyl(OAc)-L-Ala-D-Glu(OH)OBzl (**17**)

Hydrogen chloride gas was bubbled into a cooled and stirred solution of **16** (2.09 g, 5 mmole) in AcOEt (20 ml) during a period of 15 minutes. After stirring for an additional 15 minutes, the reaction mixture was purged with N_2 and evaporated to leave an oil, which was dissolved in CH_2Cl_2 (10 ml) and, after addition of bis(trimethylsilyl)acetamide (3.36 g, 16.5 mmole), the mixture was cooled to −40°C. *O*-Acetyl-D-lactoyl chloride (1.05 g, 7 mmole) was added with stirring and the mixture was stirred at −15~−10°C for 1 hour and then allowed to warm to room temperature. After evaporation of the reaction mixture, the residue was dissolved in AcOEt and washed successively with dilute HCl and brine. The organic layer was dried over $MgSO_4$ and evaporated to give an oil, which was crystallized from isopropyl ether to give 1.84 g (87%) of **17**: mp 105~106°C; $[\alpha]_D$−16.1° (*c* 1.0, MeOH); IR (Nujol) 1740, 1660, 1640 cm^{-1}; NMR ($CDCl_3$) δ 1.40 (d, *J* = 7 Hz, 3H), 1.47 (d, *J* = 7 Hz, 3H), 2.13 (s, 3H), 2.1~2.5 (m, 4H), 4.5~5.3 (m, 3H), 5.20 (s, 2H), 7.40 (s, 5H).

Anal. Calcd. for $C_{20}H_{26}O_8N_2$: C 56.86, H 6.20, N 6.63.
Found: C 56.75, H 6.28, N 6.61.

D-Lactoyl(OAc)-L-Ala-γ-D-Glu(α-OBzl)-(L)-Boc(D)*meso*-A_2pm(D)-NHNHBoc-(L)-Gly (**18**)

A solution of **17** (5.07 g, 12 mmole) and *N*-methylmorpholine (1.11 g, 11 mmole) in CH_2Cl_2 (60 ml) was cooled to −15°C and isobutyl chloroformate (1.50 g, 11 mmole) was added dropwise with stirring at −15~−10°C. After stirring for 30 minutes at the same temperature, a solution of the silyl ester of **14**, prepared from **14** (4.97 g, 10 mmole) and bis(trimethylsilyl)acetamide (7.41 g, 36 mmole) in a mixture of CH_2Cl_2 (50 ml) and DMF (10 ml) by stirring for 15 minutes at room temperature, was added dropwise and the mixture was stirred at −10°C for 2 hours and at 0°C for 1 hour. After concentration of the reaction mixture to about 30 ml, the concentrate was poured into AcOEt (200 ml) and washed with 2% HCl and H_2O. Drying over $MgSO_4$ and evaporation gave an amorphous material, which was crystallized from $CHCl_3$ - ether to give 7.68 g (89%) of **18**: mp 105~113°C (dec); $[\alpha]_D$−4.5° (*c* 0.5, MeOH); IR (Nujol) 1730 (br), 1650 (br) cm^{-1}; NMR (CD_3OD) δ 1.3~1.8 (m, 30H), 2.08 (s, 3H), 2.1~2.4 (m, 4H), 3.92 (s, 2H), 4.2~4.6 (m, 4H), 4.95 (m, 1H), 5.17 (s, 2H), 7.33 (s, 5H).

Anal. Calcd. for $C_{39}H_{59}O_{15}N_7 \cdot \frac{1}{2}H_2O$: C 53.54, H 6.91, N 11.21.
Found: C 53.79, H 6.70, N 10.93.

D-Lactoyl-L-Ala-γ-D-Glu(α-OH)-(L)*meso*-A_2mp(L)-Gly (FK-156) (**1**)

A solution of **18** (4.37 g, 5 mmole) in AcOH (50 ml) was hydrogenated over 10% Pd-C (1.00 g) under H_2 at atmospheric pressure. After removal of the catalyst by filtration, the filtrate was evaporated and the residue was pulverized with ether to give 3.44 g of **19**.

A 1.93 g (2.5 mmole) sample of **19** was dissolved in 50% aqueous MeOH (35 ml) and, with stirring, adjusted to pH 9 with 5% aqueous K_2CO_3. The stirring was continued for 2.5 hours at pH 9 at room temperature. After evaporation of MeOH, the resulting aqueous solution was acidified to pH 3 with 5% HCl and put on a column of Diaion HP-20 (50 ml). Elution with MeOH - H_2O (1:1) and evaporation gave 1.57 g of **20**.

A 1.10 g (1.5 mmole) sample of **20** was dissolved in trifluoroacetic acid (5 ml) and stirred at room temperature for 15 minutes. After evaporation of trifluoroacetic acid, the residue was pulverized with ether and the resulting powder was dissolved in H_2O (25 ml). This solution was cooled to 0°C and acidified to pH 1 with 0.1 N H_2SO_4. A solution of $NaIO_4$ (0.80 g, 3.75 mmole) in H_2O (10 ml) was added with stirring and the mixture was stirred for 1 hour at the same temperature. After decomposition of the excess oxidant with $NaHSO_3$, the reaction mixture was brought to pH 3 with saturated $NaHCO_3$ and concentrated to about 5 ml. The concentrate was applied to column chromatography of Diaion HP-20 eluting with H_2O and the eluate was concentrated and lyophilized to give 0.66 g (61% from **18**) of **1** as powder: $[\alpha]_D$ −30.0° (*c* 0.4, H_2O); Rf 0.21 (B), 0.60 (E); retention time, 13.5 minutes (high pressure LC); IR (Nujol) 1720 (br), 1640 (br) cm^{-1}; NMR (D_2O) δ 1.2~2.5 (m, 10H), 1.40 (d, *J* = 7 Hz, 3H), 1.46 (d, *J* = 7 Hz, 3H), 3.88 (*t*, *J* = 6 Hz, 1H), 4.02 (s, 2H); Amino acid anal. Ala 1.00,

Glu 1.01, Gly 0.97, *meso*-A_2pm 1.06.

Anal. Calcd. for $C_{20}H_{33}O_{11}N_5 \cdot 2H_2O$: C 43.24, H 6.71, N 12.61.
Found: C 43.47, H 6.57, N 12.57.

Di-Z-*meso*-A_2pm(D)-NHNHBoc (**22**)

A sample (6.09 g, 20 mmole) of **7** was dissolved in a cooled mixture of dioxane (20 ml) and H_2O (30 ml) containing NaOH (3.20 g, 80 mmole). Carbobenzoxy chloride (8.50 g, 50 mmole) was added dropwise during a period of 20 minutes at 0°C with stirring. After stirring for 1 hour at the same temperature, the reaction mixture was concentrated in order to remove dioxane. The concentrate was then acidified to pH 2 with 10% HCl and extracted with AcOEt. The extract was washed with brine, dried over $MgSO_4$ and evaporated to leave an amorphous solid, which was washed with isopropyl ether to give 10.54 g (92%) of **22** as a crude powder. This product was used for the next step without purification.

Di-Z-*meso*-A_2pm(D)-$NHNH_2$ (**23**)

A 8.59 g (15 mmole) portion of **22** obtained as described above was dissolved in AcOEt (100 ml) and cooled to 0°C. Hydrogen chloride gas was bubbled into the solution for 30 minutes and stirred for an additional 1 hour at the same temperature. After the mixture was purged with N_2, the resulting crystalline precipitate was collected by filtration and washed with ether to give 6.86 g (90%) of **23** as hydrochloride: mp 114~120°C (dec); $[\alpha]_D$ +9.4° (*c* 0.4, AcOH); IR (Nujol) 1680, 1520 cm^{-1}; NMR (DMSO-d_6) δ 1.3~1.7 (m, 6H), 3.8~4.3 (m, 2H), 5.10 (s, 4H), 7.40 (s, 10H), 7.3~7.8 (m, 3H), 11.30 (br signal, 1H).

Anal. Calcd. for $C_{23}H_{28}O_7N_4 \cdot HCl \cdot \frac{1}{2}H_2O$: C 53.33, H 5.83, N 10.81.
Found: C 53.18, H 5.80, N 10.76.

Di-Z-*meso*-A_2pm(D)-GlyOBzl (**25**)

A sample (8.44 g, 10 mmole) of GlyOBzl·TsOH was partitioned between CH_2Cl_2 and 5% aqueous K_2CO_3 and the organic layer was separated, dried over $MgSO_4$ and concentrated to about 20 ml. A solution of **23** (5.80 g, 10 mmole) in a mixture of AcOEt (50 ml) and 1 N HCl (33 ml) was cooled to 0°C and a solution of $NaNO_2$ (0.76 g, 11 mmole) in H_2O (10 ml) was added dropwise with stirring. The stirring was continued for 10 minutes and the organic layer was separated, washed with a chilled brine, dried over $MgSO_4$ and filtered. The filtrate containing the resulting **24** was then added to the above CH_2Cl_2 solution of GlyOBzl at 0°C and the mixture was kept at 4°C for 20 hours. After washing with dilute HCl and brine, the mixture was dried over $MgSO_4$ and evaporated to leave an oil, which was chromatographed on silica gel (40 g) eluting with CH_2Cl_2 - MeOH (20: 1) to give 3.09 g (51%) of **25** as amorphous solid: IR (Nujol) 1740, 1695, 1650 cm^{-1}; NMR (Me_2CO-d_6) δ 1.3~2.4 (m, 6H), 3.9~4.6 (m, 2H), 4.06 (d, J = 6 Hz, 2H), 5.12 (s, 4H), 5.18 (s, 2H), 6.3~6.9 (br signal, 2H), 7.38 (s, ~15H), 7.77 (t, J = 6 Hz, 1H). This material was used for the next reaction without further purification.

meso-A_2pm(D)-Gly (**26**)

A solution of **25** (3.03 g, 5 mmole) in AcOH (50 ml) was hydrogenated over 10% Pd-C (0.50 g) under H_2 at atmospheric pressure. After removal of the catalyst by filtration, the filtrate was evaporated to leave an oily material, which was purified by column chromatography of Diaion HP-20 eluting with H_2O, followed by recrystallization from MeOH - H_2O to give 1.11 g (90%) of **26**: mp 235°C (dec.); $[\alpha_D]$ −60.6° (*c* 0.5, H_2O); Rf 0.19 (C); IR (Nujol) 1655 (sh), 1580 (br) cm^{-1}; NMR (D_2O) δ 1.3~2.3 (m, 10H), 3.73 (t, J = 6 Hz, 1H), 3.80 (s, 2H), 4.00 (t, J = 6 Hz, 1H).

Anal. Calcd. for $C_9H_{17}O_5N_3 \cdot H_2O$: C 40.75, H 7.22, N 15.84.
Found: C 40.52, H 7.14, N 15.58.

Z-(L)*meso*-A_2pm(D)-Gly (**27**)

A solution of **26** (1.06 g, 4 mmole) and $CuCl_2 \cdot 2H_2O$ (0.68 g, 4 mmole) in H_2O (100 ml) was cooled to 5°C and the pH was brought to 12 with 3 N NaOH. Carbobenzoxy chloride (2.85 ml, 20 mmole) was added with stirring and the mixture was maintained at pH 11~12 with 3 N NaOH. After 4 hours, an additional 1.70 g (12 mmole) of carbobenzoxy chloride was added and the mixture was stirred for 5 hours during which time the pH was maintained at 11~12. The reaction mixture was acidified to pH 2 with 3 N HCl and washed with ether. The aqueous layer was brought to pH 4.5 and treated with

H_2S gas. The resulting precipitate was filtered off and the filtrate was concentrated to about 50 ml. The concentrate was applied to a Diaion HP-20 column chromatography and eluted with aqueous MeOH to give a crystalline material, which was recrystallized from MeOH - H_2O and washed with *i*-PrOH to give 1.19 g (78%) of **27**: mp 161~162°C (dec); $[\alpha]_D$ −25.3° (*c* 0.5, MeOH); *pKa* 3.2, 3.7, 8.2 (H_2O); Rf 0.56 (E); IR (Nujol) 1695, 1660 (sh), 1650 cm^{-1}; NMR (CD_3OD) δ 1.2~2.2 (m, 6H), 3.7~4.3 (m, 4H), 5.10 (s, 2H), 7.40 (s, 5H).

Anal. Calcd. for $C_{17}H_{23}O_7N_3$: C 52.30, H 6.20, N 10.77.
Found: C 52.21, H 6.32, N 10.68.

Z-(L)-Boc-(D)*meso*-A_2pm(D)-Gly (**28**)

Triethylamine (1.35 ml, 9.6 mmole) was added to a suspension of **27** (1.17 g, 3 mmole) in H_2O (15 ml) and the resulting clear solution was cooled to 0°C. To this solution was added a solution of di-*t*-butyl dicarbonate (0.72 g, 3.3 mmole) in dioxane (15 ml) and the mixture was stirred for 6 hours at room temperature. After evaporation, the residue was dissolved in H_2O, acidified to pH 2 with dilute HCl under cooling and extracted with AcOEt. The extract was washed with H_2O and brine, dried over $MgSO_4$ and evaporated to leave 1.33 g (92%) of **28** as amorphous solid: $[\alpha]_D$ +9.1° (*c* 0.4, $CHCl_3$); IR (Nujol) 1700 (br), 1650 (sh), 1520 cm^{-1}; NMR (DMSO-d_6) δ 1.37 (s, 9H), 1.1~1.9 (m, 6H), 3.6~4.2 (m, 2H), 3.73 (d, J = 6 Hz, 2H), 5.02 (s, 2H), 6.75 (m, 1H), 7.33 (s, 5H). This sample was used for the next reaction without further purification.

Boc-(D)*meso*-A_2pm(D)-Gly (**29**)

A solution of **28** (1.20 g, 2.5 mmole) in 90% aqueous MeOH (30 ml) was hydrogenated over 10% Pd-C (0.4 g) under H_2 at atmospheric pressure. After removal of the catalyst by filtration, the filtrate was concentrated and the concentrate was diluted with *i*-PrOH and refrigerated. The resulting crystalline precipitate was filtered and washed with ether to give 0.78 g (90%) of **29**: mp ~158°C (dec); $[\alpha]_D$ +12.2° (*c* 0.5, MeOH); Rf 0.60 (C); IR (Nujol) 1720 (sh), 1680, 1660, 1535 cm^{-1}; NMR (CD_3OD+D_2O) δ 1.3~2.2 (m, 6H), 1.40 (s, 9H), 3.66 (t, J = 6 Hz, 1H), 3.88 (s, 2H).

Anal. Calcd. for $C_{14}H_{25}O_7N_3 \cdot 1.5H_2O$: C 44.91, H 7.53, N 11.22.
Found: C 44.89, H 7.45, N 11.23.

D-Lactoyl(OAc)-L-Ala-γ-D-Glu(α-OBzl)-(L)-Boc-(D)*meso*-A_2pm(D)-Gly (**30**)

A solution of **17** (1.05 g, 2.5 mmole) and *N*-hydroxysuccinimide (0.32 g, 2.8 mmole) in a mixture of dioxane (10 ml) and THF (2 ml) was cooled to 5°C and dicyclohexylcarbodiimide (0.56 g, 2.7 mmole) was added. After stirring at room temperature overnight, the mixture was filtered in order to remove the resulting precipitate of the urea and the filtrate was evaporated to leave an oil of the *N*-hydroxysuccinimide ester of **17**. This was dissolved in dioxane (6 ml) and added to a cooled solution of **29** (0.64 g, 1.7 mmole) and *N*-methylmorpholine (0.67 ml, 6.1 mmole) in a mixture of DMF (6 ml) and H_2O (1 ml). The mixture was stirred at 0°C for 30 minutes and at room temperature for 4 hours. The reaction mixture was concentrated and diluted with H_2O. The resulting solution was washed with ether and, after acidified to pH 2 with 1 N HCl, extracted with CH_2Cl_2 - AcOEt (1:1). The extract was washed with H_2O and brine, dried over $MgSO_4$ and evaporated to leave an amorphous solid, which was dissolved in CH_2Cl_2 and triturated with ether to give 0.92 g (72%) of **30** as amorphous solid: $[\alpha]_D$ −2.2° (*c* 0.5, $CHCl_3$); IR (Nujol) 1735, 1655, 1525 cm^{-1}; NMR ($CDCl_3$-CD_3OD) δ 1.38 (d, J = 7 Hz, 3H), 1.47 (s, 9H), 1.49 (d, J = 7 Hz, 3H), 1.2~2.0 (m, 6H), 2.15 (s, 3H), 2.0~2.5 (m, 4H), 3.99 (br s, 2H), 5.02 (q, J = 7 Hz, 1H), 5.20 (s, 2H), 7.39 (s, 5H).

Anal. Calcd. for $C_{24}H_{49}O_{14}N_5 \cdot 1.5H_2O$: C 53.39, H 6.85, N 9.15.
Found: C 53.08, H 7.20, N 8.79.

D-Lactoyl-L-Ala-γ-D-Glu(α-OH)-(L)*meso*-A_2pm(D)-Gly (**2**)

A solution of **30** (0.90 g, 1.2 mmole) in 70% aqueous MeOH (20 ml) was hydrogenated over 10% Pd-C (0.2 g) under H_2 at atmospheric pressure. After removal of the catalyst by filtration, the filtrate was concentrated to leave an amorphous solid (**31**).

This solid was dissolved in 70% aqueous MeOH (12 ml) and cooled to 0°C. 1 N NaOH (2.3 ml) was added dropwise to the above solution with stirring and the stirring was continued for 1 hour at room temperature. The reaction mixture was neutralized to pH 7 with 1 N HCl and concentrated.

The residue was dissolved in H_2O and washed with ether. The aqueous layer was concentrated and, after acidification to pH 2 with 1 N HCl, applied to a Diaion HP-20 (40 ml) column chromatography eluting with 50% aqueous MeOH to give a powder (**32**).

This powder was dissolved in trifluoroacetic acid (5 ml) and stirred for 15 minutes at room temperature. After evaporation of trifluoroacetic acid, the residue was triturated with ether and the solid so obtained was dissolved in H_2O. The solution was adjusted to pH 3 with dilute NaOH and chromatographed on Diaion HP-20 (40 ml) eluting with H_2O. The fractions containing **2** were combined, concentrated and lyophilized to give 0.36 g (51%) of **2** as powder: $[\alpha]_D$ −35.4° (*c* 0.3, H_2O); Rf 0.56 (E); IR (Nujol) 1730 (sh), 1655 (br), 1540 cm^{-1}; NMR (D_2O) δ 1.1~2.5 (m, 10H), 1.37 (d, *J* = 7 Hz, 3H), 1.43 (d, *J* = 7 Hz, 3H), 4.01 (s, 2H), 4.2~4.6 (m, 4H); Amino acid anal. Ala 1.00, Glu 0.98, Gly 0.94, *meso*-A_2pm 1.10.

Anal. Calcd. for $C_{20}H_{33}O_{11}N_5 \cdot 4H_2O$: C 40.61, H 6.99, N 11.84.
Found: C 40.22, H 6.83, N 11.46.

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