Amino Acids and Peptides. XLIX. Synthesis of γ-2-Adamantylglutamate and Its Evaluation for Peptide Synthesis[1,2]

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Received August 28, 1996; accepted September 27, 1996

2-Adamantyl ester was examined for the selective protection of the γ-carboxyl function of the Glu residue, with the aim of preventing side reactions during peptide synthesis and increasing the solubility in organic solvents of peptide intermediates containing the Glu residue. Z–Glu(O-2-Ada)–OBzl was synthesized from Z–Glu–OBzl and adamantan-2-ol with the aid of dicyclohexylcarbodiimide (DCC) and 4-*N*,*N*-dimethylaminopyridine (DMAP) in AcOEt. The 2-adamantyl ester group was stable to TFA, 20% piperidine/DMF and 10% Et_3N/DMF up to 24 h and was easily removed by MSA, 1 M TFMSA–thioanisole in TFA, and HF. Therefore, H–Glu–(O-2-Ada)–OH could be applied for peptide synthesis in both solution and solid phase methods in combination with a Boc or Fmoc group as the N^{α}-protecting group. Boc–Glu(O-2-Ada)–OH was successfully employed for the synthesis of Bz–Ile–Glu–Gly–Arg–CH_2Cl, an irreversible inhibitor of factor Xa.

Key words γ-2-adamantylglutamate; 2-adamamntyl ester; synthetic procedure; property; peptide synthesis

The tendency of aspartic acid to form a five-membered ring (aspartimide)[3] during the peptide synthesis results in a series of side reactions. Its homolog, glutamic acid, similarly undergoes numerous side reactions, since it can form both five (pyrolidone)[4]- and six (glutarimide)[5]-membered rings, although the incorporation of glutamic acid into peptide chains is usually less troublesome than the introduction and further handling of the aspartyl residue. For the selective protection of the γ-carboxyl function of the Glu residue, benzyl ester,[6] *tert*-butyl ester,[7] cyclopentyl ester,[8] cyclohexyl ester[9] and cycloheptyl ester[10] have so far been employed.

We report here the synthesis of γ-2-adamantylglutamate (H–Glu(O-2-Ada)–OH, Fig. 1), and evaluation of its suitability for use in peptide synthesis.

Previously, we reported on the synthesis of H–Asp(O-2-Ada)–OH and its applicability to peptide synthesis, based on its ability to prevent aspartimide formation during peptide synthesis and to increase the solubility of peptide intermediates in organic solvents.[11] According to the synthetic procedure developed for H–Asp(O-2-Ada)–OH,[11] Z–Glu–OBzl[12] was esterified with adamantan-2-ol with the aid of DCC and DMAP in DMF. However, the reaction mixture showed several spots on thin-layer chromatograpy and the yield of Z–Glu(O-2-Ada)–OBzl was very low. From the reaction mixture, four products (A, B, C and D) were separated by silica gel column chromatography and the structure of each product was identified by elemental analysis. Compound A was the desired product, Z–Glu(O-2-Ada)–OBzl, B was the acyl urea of Z–Glu–OBzl, C was a pyrolidone derivative and D was the starting material, Z–Glu–OBzl. Compounds A, B, C and D were well separated on HPLC, as shown in Fig. 2. Therefore, by using these compounds as markers, suitable reaction conditions for the synthesis of Z–Glu(O-2-Ada)–OBzl were studied. In order to increase the yield of Z–Glu(O-2-Ada)–OBzl, various solvents for the reaction was examined. An equal amount of Z–Glu–OBzl was used to couple with adamantan-2-ol in various solvents, such as DMF, THF, AcOEt, dioxane and pyridine. Each reaction mixture was stirred at 4 °C for 14 h. After removal of the solvent, the residue was taken up in MeOH and analyzed by HPLC to measure the amounts of the products. The results are summarized in Table 1. The ratios of the amount of the desired compound A to the main side product B were as follows: 0.87 for DMF, 0.67 for THF, 0.04 for dioxane, 0.49 for pyridine, 1.26 for AcOEt. Therefore, AcOEt was the most favorable of the solvents so far examined for the coupling reaction from the viewpoints of increasing the yield of the

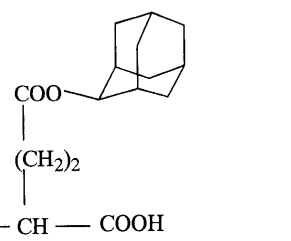

Fig. 1. Structure of γ-2-Adamantylglutamate

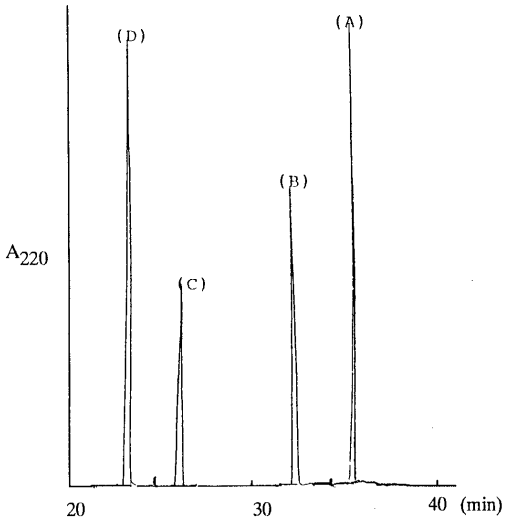

Fig. 2. HPLC Profiles of Compounds A [Z–Glu(O-2-Ada)–OBzl], B [*N*–(Z–Glu–OBzl),*N*,*N*′-dicyclohexylurea], C (Z–Pyr–OBzl) and D (Z–Glu–OBzl)

The column and solvent system are described in Experimental.

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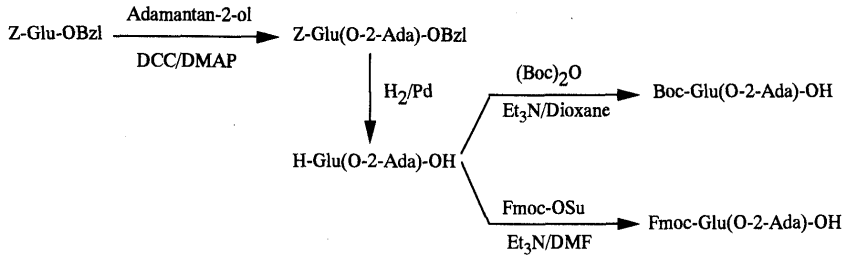

Chart 1. Synthetic Scheme for γ-2-Adamantylglutamate and Its Derivatives

Table 1. Effect of the Solvent on the Production of Compound A, B, C or D in the Coupling Reaction

Compound Solvent	(A) Z–Glu(2-O-Ada)–OBzl (nmol)	(B) Acyl urea (nmol)	(C) Z–Pyr–OBzl (nmol)	(D) Z–Glu–OBzl (nmol)	(A/B) Z–Glu(2-O-Ada)–OBzl/Acyl urea
DMF	207.76	237.89	21.84	21.55	0.87
THF	169.93	255.29	15.88	57.65	0.67
AcOEt	233.72	177.65	7.26	110.87	1.26
Dioxane	15.79	392.48	1.34	82.46	0.04
Pyridine	153.55	314.68	17.52	1.27	0.49

Table 2. Stability of γ-2-Adamantylglutamate under Various Acidic and Basic Conditions

Condition	Method	% cleavage					
		1 min	10 min	30 min	1 h	2 h	24 h
1 N HCl (100 eq)	A	0	0	0	0	3	83
7.2 N HCl/dioxane (200 eq)	A	0	0	4	4	9	24
TFA (300 eq)	A	0	0	0	0	0	0
MSA (400 eq)	A	30	70	100			
1 M TFMSA/TFA	A	96	100				
20% piperidine/DMF (500 eq)	A	0	0	0	0	0	1.1
10% Et_3N/H_2O + dioxane (50 eq)	A	0	0	1	1	2	2.5
0.1 N NaOH (10 eq)	B		14.7	31.7	72.7		
1 N NaOH (100 eq)	B	23.1	96	98	100		
10% Et_3N/DMF	B	0	0	0	0	0	0

Method A: determined by the measurement of H–Glu–OH regenerated from H–Glu(O-2-Ada)–OH with an amino acid analyzer. Method B: determined by the measurement of Boc–Glu–OH generated from Boc–Glu(O-2-Ada)–OH by HPLC using the column and solvent system described in Experimental.

desired compound and suppressing side reactions. Since some starting material, Z–Glu–OBzl, still remained in AcOEt, the reaction time was extended to 24 h to obtain a better yield of the desired compound.

Z–Glu(O-2-Ada)–OBzl was prepared according to Chart 1. Removal of the Z and benzyl groups by catalytic hydrogenation gave γ-2-adamantylglutamate (H–Glu(O-2-Ada)–OH), quantitatively. Boc–Glu(O-2-Ada)–OH and Fmoc–Glu(O-2-Ada)–OH were easily derived as shown in Chart 1.

Next, the stability and susceptibility of the 2-adamantyl ester group to various acids were examined by measuring the amount of Glu residue regenerated from H–Glu(O-2-Ada)–OH with an amino acid analyzer, and to various bases by measuring the amount of Boc–Glu–OH generated from Boc–Glu(O-2-Ada)–OH by HPLC; the results are summarized in Table 2. The 2-Ada group is stable to trifluoroacetic acid (TFA) up to 24 h and easily removable by methanesulfonic acid (MSA) and 1 M trifluoromethanesulfonic acid (TFMSA)–thioanisole/TFA. This group can survive treatment with 20% piperidine/DMF and 10% Et_3N/DMF up to 24 h, indicating the possibility of application of H–Glu(O-2-Ada)–OH derivatives to peptide synthesis in combination with a Boc or Fmoc group as the N^{α}-protecting group.

Finally, an irreversible inhibitor (Bz–Ile–Glu–Gly–Arg–CH_2Cl) of factor Xa, which is a factor involved in both intrinsic and extrinsic pathways of blood coagulation, was synthesized using γ-2-adamantylglutamate by the route shown in Fig. 3. Each coupling yield in the synthesis of Boc–Ile–Glu(O-2-Ada)–Gly–OBzl was approximately 80%, indicating that the steric effect of the 2-Ada group did not affect the coupling yields. Peptide intermediates containing Glu(O-2-Ada) are more soluble in organic solvents than those containing Glu(O-Chx). At the final step, the protecting groups were removed by the HF method[13] to give the desired tetrapeptide chloromethyl ketone. After purification of the crude product by preparative HPLC, the Bz–Ile–Glu–Gly–Arg–CH_2Cl exhibited a single peak on analytical HPLC, as shown in Fig. 4. This chloromethyl ketone exhibited selective inhibitory activity against factor Xa with a K_i value of 1×10^{-6} M, while it inhibited thrombin with a K_i value of 3.0×10^{-3} M. The details of the inhibitory activity of this

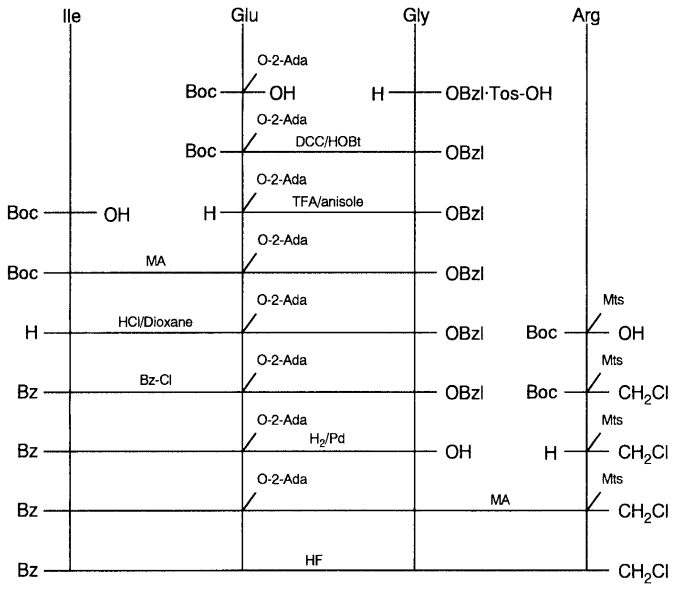

Fig. 3. Synthetic Scheme for Bz–Ile–Glu–Gly–Arg–CH_2Cl

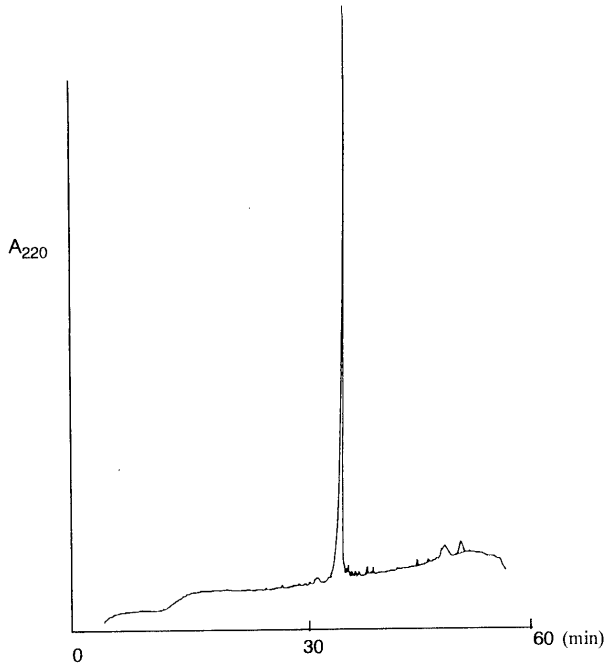

Fig. 4. HPLC Profile of Bz–Ile–Glu–Gly–Arg–CH_2Cl

The column and solvent system are described in Experimental.

compound will be published elsewhere.

Thus, we have been able to develop the 2-adamantyl ester as a protecting group for the γ-carboxyl function of the Glu residue. It was confirmed that this protecting group could be applied to peptide synthesis and, moreover, as expected, it had the ability to increase the solubility of the peptide intermediates in organic solvents.

Experimental

The melting points were determined with a Yanagimoto micro apparatus and are uncorrected. On TLC (Kieselgel G, Merck), Rf^1 and Rf^2 values refer to the systems of $CHCl_3$, MeOH, H_2O (8:3:1, lower phase), and $CHCl_3$, MeOH, AcOH (90:8:2), respectively. Optical rotations were measured with an automatic DIP-360 polarimeter (Japan Spectroscopic Co., Ltd., Japan). Amino acid compositions of acid hydrolysates were determined with an automated amino acid analyzer (K-101 AS or K-202 SN, Kyowa Seimitsu Co., Ltd., Japan). ^{1}H- (400, 500 MHz) and ^{13}C- (100, 125 MHz) NMR spectra were recorded on either a Bruker AM400 or a ARX500 spectrometer. Chemical shift values are expressed as ppm down field from tetramethylsilane used as an internal standard (δ-value). The J values are indicated in Hz. Attribution of ^{13}C signals was made with the aid of distortionless enhancement by polarization transfer (DEPT) experiments, and multiplicities are indicated by *prim* (primary), *sec* (secondary), *tert* (tertiary) or *quart* (quaternary). Mass spectra were measured with a Hitachi M-2000 mass spectrometer. IR spectra were measured on a Hitachi 260-30 spectrophotometer. As regards the mobile-phase system for HPLC, A and B refer to water and MeCN, respectively, both containing 0.05% TFA.

Reaction of Z–Glu–OBzl and Adamantan-2-ol An ice-cold solution of Z–Glu–OBzl (3.2 g, 8.6 mmol), adamantan-2-ol (1.30 g, 8.6 mmol) and DMAP (0.1 g, 0.9 mmol) in DMF (50 ml) was treated with DCC (1.95 g, 9.5 mmol), and the reaction mixture was stirred at 4 °C overnight. After removal of dicyclohexylurea and the solvent, the residue in $CHCl_3$ (5 ml) was applied to a column of silica gel (2 cm × 35 cm), equilibrated and eluted with $CHCl_3$. Separated products (A, B, C and D) were identified as follows: A, Z–Glu(O-2-Ada)–OBzl: oily material, $[\alpha]_D^{25}$ −15.2° (c=1.0, MeOH), Rf^1 0.82. MS (SIMS) m/z: 506 (M^++1). NMR ($CDCl_3$) δ_H: 1.51—1.95 (14H, m, adamantyl), 1.99 (2H, m, Glu-β-CH_2), 2.38 (2H, m, Glu-γ-CH_2), 4.46 (1H, m, Glu-α-CH), 4.90 (1H, s, O–CH–), 5.09 (2H, s, $PhCH_2OCON$), 5.16 (2H, m, $PhCH_2OCOC$), 5.53 (1H, d, J=1.2, NH), 7.33—7.37 (10H, m, 2 × Ph); ($CDCl_3$) δ_C 26.95, 27.04, 31.79 (*tert*, adamantyl), 31.73, 36.28, 37.34 (*sec*, adamantyl), 27.67 (*sec*, Glu-β-CH_2), 30.63 (*sec*., Glu-γ-CH_2), 53.56 (*tert*, Glu-α-CH), 67.03 (*sec*, $PhCH_2OCON$), 67.28 (*sec*, $PhCH_2OCOC$), 77.39 (*tert*, O–CH), 128.06, 128.14, 128.26, 128.47, 128.50, 128.61 (*tert*, Ph), 135.22, 136.21 (*quart*, Ph). *Anal.* Calcd for $C_{30}H_{35}NO_6 \cdot 1/2H_2O$: C, 70.0; H, 7.05; N, 2.92. Found: C, 70.3; H, 7.07; N, 2.75. B, *N*–(Z–Glu–OBzl),*N*,*N*′-dicyclohexylurea: mp 79—83 °C, $[\alpha]_D^{25}$ −8.6° (c=1.0, MeOH). *Anal.* Calcd for $C_{33}H_{43}N_3O_6$: C, 68.6; H, 7.50; N, 7.27. Found: C, 68.4; H, 7.38; N, 7.23. C, Z–Pyr–OBzl: identified by comparison with an authentic sample. D, Z–Glu–OBzl: identified by comparison with an authentic sample. HPLC profiles are shown in Fig. 2. Column, μ Bondasphere C18 (3.9 × 150 mm); eluent, A : B, 83 : 17 for 5 min, to 20 : 80 for 25 min and to 83 : 17 for 10 min; flow rate, 1 ml/min.

General Procedure for Studies on the Effect of Solvent on the Coupling Reaction of Z–Glu–OBzl with Adamantan-2-ol Z–Glu–OBzl (3.2 g, 8.6 mmol) and adamantan-2-ol (1.30 g, 8.6 mmol) and DMAP (0.1 g, 0.9 mmol) were dissolved in a solvent (50 ml, DMF, AcOEt, THF, dioxane, or pyridine). DCC (1.95 g, 9.05 mmol) was added to the cold solution and the reaction mixture was stirred at 4 °C for 14 h. After removal of the dicyclohexylurea and the solvent, the residue was dissolved in MeOH. The solution (20 μl) was examined by HPLC and the amounts of the products (A, B, C and D) were determined by using A and B isolated above and C and D prepared in our laboratory as standards; the results are summarized in Table 1.

Z–Glu(O-2-Ada)–OBzl Z–Glu–OBzl (3.2 g, 8.6 mmol), adamantan-2-ol (1.30 g, 8.6 mmol) and DMAP (0.10 g, 0.9 mmol) were dissolved in AcOEt (50 ml). DCC (1.95 g, 9.05 mmol) was added to the above solution under cooling with ice-salt. The reaction mixture was stirred at 4 °C for 24 h. After removal of the dicyclohexylurea, the AcOEt solution was washed with 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down to give an oily material. This product in $CHCl_3$ (5 ml) was applied to a silica gel column (2 cm × 35 cm), equilibrated and eluted with $CHCl_3$. The eluate was evaporated to give an oily material (3.4 g, 80.0%). This oily material was identified as Z–Glu(O-Ada)–OBzl by comparing the NMR and MS data and rotation value of this oily material with those of an authentic sample obtained as described above.

H–Glu(O-2-Ada)–OH Z–Glu(O-2-Ada)–OBzl (3.4 g, 6.73 mmol) in methanol (50 ml) was hydrogenated over 10% Pd–C for 4 h. After removal of Pd and the solvent, petroleum ether was added to the residue to afford crystals, which were collected by filtration (1.42 g, 75.4%), mp 183—186 °C, $[\alpha]_D^{25}$ +4.2° (c=1.0, MeOH), Rf^2 0.51. MS (SIMS) m/z: 282 (M^++1). *Anal.* Calcd for $C_{15}H_{23}NO_4 \cdot 1/2H_2O$: C, 64.0; H, 8.33; N, 4.97. Found: C, 64.2; H, 8.24; N, 4.97.

Boc–Glu(O-2-Ada)–OH·DCHA An ice-cold solution of H–Glu(O-2-Ada)–OH (1 g, 3.55 mmol) in dioxane (10 ml) and water (20 ml) containing Et_3N (0.49 ml, 3.55 mmol) was treated with $(Boc)_2O$ (0.85 g, 3.9 mmol) in dioxane (10 ml). The reaction mixture was stirred at room temperature for 2 h. After removal of the solvent, the residue was dissolved in water (10 ml). The solution was acidified with 10% citric acid and the oily product was extracted with AcOEt. The extract was

washed with water, dried over Na_2SO_4 and evaporated down. The residue was dissolved in ether (20 ml), and dicyclohexylamine (0.71 ml, 3.55 mmol) was added to the solution to give crystals, which were collected by filtration and washed with ether (1.2 g, 60%), mp 144—148 °C, $[\alpha]_D^{25}$ +12.3° (c=1.0, MeOH), Rf^2 0.66. *Anal.* Calcd for $C_{32}H_{54}N_2O_6$: C, 68.35; H, 9.50; N, 4.97. Found: C, 68.0; H, 9.54; N, 4.91.

Examination of Stability and Susceptibility of the 2-Ada Group Method A: H–Glu(O-2-Ada)–OH (0.02 mol) was dissolved in the test acid solution at room temperature. A 10 μl aliquot of solution was collected at 5, 20, 40, 60 and 120 min and 24 h. The solution was neutralized with 0.5 M Na_2CO_3 and diluted with 0.1 M HCl [H–Glu(O-2-Ada)–OH, 1×10^{-8} M]. This solution (0.1 ml) was injected into the amino acid analyzer and regenerated Glu was measured.

Method B: Boc–Glu(O-2-Ada)–OH (13 mmol) was dissolved in a base solution at room temperature. A 50 μl aliquot of the solution was collected at 5, 20, 40, 60 and 120 min and 24 h, then the solution was adjusted with 0.1 M HCl to pH 7 and diluted with MeOH (100 μl). An aliquot (20 μl) was subjected to HPLC and the generated Boc–Glu–OH was determined. Column, μ Bondasphere C18 (3.9 × 150 mm); eluent, A : B, 83 : 17 for 5 min, to 20 : 80 for 25 min and to 83 : 17 for 10 min; flow rate, 1 ml/min. These results are summarized in Table 2.

Fmoc–Glu(O-2-Ada)–OH Fmoc–OSu (0.54 g, 1.6 mmol) in DMF (10 ml) was added to a solution of H–Glu(O-2-Ada)–OH (0.45 g, 1.6 mmol) in DMF (10 ml) containing Et_3N (0.23 ml, 1.6 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was dissolved in water (5 ml) and the solution was adjusted to pH 2 with 1 M HCl. The resultant oily material was extracted with AcOEt. The extract was washed with water, and dried over Na_2SO_4 and evaporated down. The residue was purified by silica gel column chromatography to afford an amorphous powder (0.48 g, 61%), $[\alpha]_D^{25}$ +3.2° (c=0.5, MeOH), Rf^2 0.68. *Anal.* Calcd for $C_{30}H_{33}NO_6 \cdot H_2O$: C, 69.1; H, 6.76; N, 2.68. Found: C, 69.2; H, 6.91; N, 2.48.

Boc–Glu(O-2-Ada)–Gly–OBzl An ice-cold solution of Boc–Glu(O-2-Ada)–OH (2.5 g, 6.56 mmol), H–Gly–OBzl · TosOH (2.87 g, 8.53 mmol), HOBt (0.88 g, 6.56 mmol) in DMF (50 ml) containing Et_3N (0.49 ml, 8.53 mmol) was treated with DCC (1.49 g, 7.22 mmol). The reaction mixture was stirred at 4 °C overnight. After removal of dicyclohexylurea and the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. The residue in $CHCl_3$ (5 ml) was applied to a silica gel column (2.3 × 50 cm), equilibrated and eluted with $CHCl_3$ and hexane (3 : 2). The eluate (2000 to 2700 ml) was evaporated to give an oily material (3.1 g, 83%), $[\alpha]_D^{25}$ −14.1° (c=1.0, MeOH), Rf^2 0.66. *Anal.* Calcd for $C_{29}H_{40}N_2O_7$: C, 65.9; H, 7.62; N, 5.29. Found: C, 65.7; H, 7.62; N, 5.17.

Boc–Ile–Glu(O-2-Ada)–Gly–OBzl H–Glu(O-2-Ada)–Gly–OBzl · TFA [prepared from Boc–Glu(O-2-Ada)–Gly–OBzl (1.8 g, 3.4 mmol) and TFA (5 ml) in the usual manner] was dissolved in DMF (20 ml) containing Et_3N (0.5 ml, 3.4 mmol). A mixed anhydride [prepared from Boc–Ile–OH (0.79 g, 3.4 mmol), isobutyl chloroformate (0.5 ml, 3.74 mmol) and Et_3N (0.5 ml, 3.4 mmol) in the usual manner] in THF (15 ml) was combined with the above solution. The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. The residue in $CHCl_3$ (5 ml) was applied to a silica gel column (2.3 × 50 cm), equilibrated and eluted with $CHCl_3$ and hexane (5 : 1). The eluate (770—1150 ml) was evaporated down to give an amorphous powder (1.7 g, 76%), $[\alpha]_D^{25}$ −32.7° (c=1.0, MeOH), Rf^2 0.61. *Anal.* Calcd for $C_{35}H_{51}N_3O_8$: C, 65.5; H, 8.01; N, 6.55. Found: C, 65.6; H, 8.12; N, 6.37.

Bz–Ile–Glu(O-2-Ada)–Gly–OBzl Benzoyl chloride (0.1 ml, 0.87 mmol) was added to a cold solution of H–Ile–Glu(O-2-Ada)–Gly–OBzl · TFA [prepared from Boc–Ile–Glu(O-2-Ada)–Gly–OBzl (0.48 g, 0.73 mmol) and TFA (8.3 ml, 7.3 mmol) in the usual manner] in DMF (30 ml) containing Et_3N (1.0 ml, 0.73 mmol). The reaction mixture was stirred at room temperature overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. The residue in $CHCl_3$ (5 ml) was applied to a silica gel column (2.0 × 30 cm), equilibrated and eluted with $CHCl_3$. The eluate (180 to 540 ml) was evaporated down. Petroleum ether was added to the residue to afford crystals (0.41 g, 84.6%), mp 89—92 °C, $[\alpha]_D^{25}$ −26.3° (c=1.0, MeOH), Rf^2 0.62. *Anal.* Calcd for $C_{30}H_{41}N_3O_7$: C, 68.8; H, 7.47; N, 6.31. Found: C, 68.5; H, 7.31; N, 6.31.

Bz–Ile–Glu(O-2-Ada)–Gly–OH Bz–Ile–Glu(O-2-Ada)–Gly–OBzl (0.4 g, 0.6 mmol) in MeOH (15 ml) was hydrogenated over a Pd catalyst. After 6 h, the catalyst and the solvent were removed. Ether was added to the residue to afford crystals (0.27 g, 77.1%), mp 179—181 °C, $[\alpha]_D^{25}$ −26.4° (c=1.0, MeOH), Rf^1 0.44, Rf^2 0.40. *Anal.* Calcd for $C_{30}H_{31}N_3O_7 \cdot 1/2H_2O$: C, 63.7; H, 7.47; N, 7.43. Found: C, 63.8; H, 7.47; N, 7.41.

Boc–Arg(Mts)–CH_2Cl Diazomethane [prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (2.84 g, 13.2 mmol) and KOH (0.86 g, 24 mmol) in the usual manner] was added to a mixed anhydride [prepared from Boc–Arg(Mts)–OH (2.0 g, 4.4 mmol), Et_3N (0.62 ml, 4.4 mmol) and isobutyl chloroformate (0.5 ml, 4.4 mmol) in the usual manner] in THF (100 ml) at −15 °C and the reaction mixture was stirred at 4 °C for 15 h. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. The residue in $CHCl_3$ (5 ml) was applied to a silica gel column (2.0 × 50 cm), equilibrated and eluted with $CHCl_3$. The eluate (1900 to 3800 ml) was concentrated to a small volume. This residue (Boc–Arg(Mts)–CH_2N_2) was dissolved in THF (150 ml) and to the solution, 8.3 M HCl in dioxane was added under cooling with ice-salt. The reaction mixture was stirred at the same temperature for 3 h, then neutralized with Et_3N. The solvent was removed by evaporation and the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. Petroleum ether was added to the residue to afford crystals, which were collected by filtration (0.8 g, 37.2%), mp 122—125 °C, $[\alpha]_D^{25}$ −18.2° (c=1.0, MeOH). Rf^1 0.47, MS (SIMS) m/z: 489 (M^+). IR cm^{-1}: ν_{SO_2} 1134, 1360, ν_{Ph}1625, ν_{CON} 1706, ν_{COCH_2} 1758. *Anal.* Calcd for $C_{21}H_{33}ClN_4O_5S$: C, 51.6; H, 6.76; Cl 7.27; N, 11.5, Found: C, 51.6; H, 6.99; Cl, 7.02; N, 11.3.

Bz–Ile–Glu(O-2-Ada)–Gly–Arg(Mts)–CH_2Cl A mixed anhydride [prepared from Bz–Ile–Glu(O-2-Ada)–Gly–OH (0.1 g, 0.18 mmol), isobutyl chloroformate (0.021 ml, 0.18 mmol) and Et_3N (0.025 ml, 0.18 mmol) in the usual manner] in THF (15 ml) was added to a cold solution of H–Arg(Mts)–CH_2Cl · TFA [prepared from Boc–Arg(Mts)–CH_2Cl (0.12 g, 0.24 mmol) and TFA (5.0 ml) in the usual manner] in DMF (15 ml) containing Et_3N (0.025 ml, 0.18 mmol). The reaction mixture was stirred at 4 °C overnight. After removal of the solvent, the residue was extracted with AcOEt. The extract was washed with 10% citric acid, 5% $NaHCO_3$ and water, dried over Na_2SO_4 and evaporated down. The residue in $CHCl_3$ (5 ml) was applied to a silica gel column (2.0 × 30 cm), equilibrated and eluted with $CHCl_3$ and MeOH (100 : 1). The eluate (1000 to 3200 ml) was evaporated down. Petroleum ether was added to the residue to afford crystals (0.08 g, 49.2%), mp 113—117 °C, $[\alpha]_D^{25}$ −34.7° (c=1.0, MeOH). *Anal.* Calcd for $C_{46}H_{64}ClN_7O_9S \cdot 13/4H_2O$: C, 57.0; H, 7.39; N, 10.1. Found: C, 57.3; H, 7.65; N, 10.2.

Bz–Ile–Glu–Gly–Arg–CH_2Cl Bz–Ile–Glu(O-2-Ada)–Gly–Arg(Mts)–CH_2Cl (0.08 g, 0.088 mmol) was placed into an HF-resistant reaction vessel with thioanisole (0.5 ml). Anhydrous HF (5 ml) was distilled into the vessel and the solution was kept at 0 °C for 1 h. After removal of HF, ether was added to the residue to give a solid. This crude product was purified by preparative HPLC [column, Cosmosil packed column 5C18-AR (4.6 × 250 mm); eluent, A : B 90 : 10 to 50 : 50 for 40 min, 50 : 50 for 5 min and to 90 : 10 for 10 min; flow rate, 2.0 ml/min]. The purified compound (0.024 g, 46.8%), $[\alpha]_D^{25}$ −56.3° (c=1.0, MeOH), amino acid analysis of acid hydrolysate, Ile : Glu : Gly = 0.99 : 1.00 : 1.17 (average re covery 75%), exhibited a single peak on analytical HPLC, as shown in Fig. 4. Column, Cosmosil pack 5C18-AR (4.6 × 250 mm); eluent, A : B, 90 : 10 to 50 : 50 for 40 min, 50 : 50 for 5 min and to 90 : 10 for 10 min; flow rate, 1 ml/min.

Assay Procedure Bz–Ile–Glu–Gly–Arg–pNA (S-2222) was used as a substrate for factor Xa. The K_i value was calculated from Dixon plot.[14]

Acknowledgement The authors are grateful to Professor J. Yamamoto, Faculty of Nutrition, Kobe Gakuin University, for the biological test of the tetrapeptide chloromethyl ketone, Dr. T. Yokoi, Faculty of Pharmaceutical Sciences, Kobe Gakuin University for valuable advice on NMR data and Dr. T. Yamamoto, New Drug Research Laboratories, Kanebo Ltd., for measurement of MS. This work was supported in part by a grant from the Science Research Promotion Fund of the Japan Private School Foundation.

References

1) Part XLVIII: Yokoi T., Taguchi H., Nishiyama Y., Igarashi K., Kasuya F., Okada Y., *J. Chem. Res.*, in press.
2) Abbreviations used in this report for amino acids, peptides and their derivatives are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485—2489 (1966); **6**, 362—369 (1967); **11**, 1726—1732 (1972). All amino acid residues except Gly are of L-configuration, unless otherwise specified. The following additional abbreviations are used: AcOEt, ethyl acetate; 2-Ada, 2-adamantyl; Boc, *tert*-butyloxycarbonyl; Bz, benzoyl; Z, benzyloxycarbonyl; Mts, mesitylene-2-sulfonyl; Boc_2O, di-*tert*-butyldicarbonate; Fmoc, fluoren-9-ylmethyloxycarbonyl; Bzl, benzyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DMAP, 4-*N,N*-dimethylaminopyridine; –OSu, *N*-hydroxysuccinimidyl ester; TFA, trifluoroacetic acid; TFMSA, trifluoromethanesulfonic acid; MSA, methanesulfonic acid; DMF, *N,N*-dimethylformamide; THF, tetrahydrofuran; DCHA, dicyclohexylamine; Pyr, pyroglutamic acid; Chx, cyclohexyl.
3) Bodanszky M., Tolle J. C., Deshmane S. S., Bodanszky A., *Int. J. Peptide Protein Res.*, **12**, 57—68 (1978).
4) Schneider F., *Hoppe-Seylers Z. Physiol. Chem.*, **323**, 38—53 (1963).
5) Batersby A. R., Robinson J. C., *J. Chem. Soc.*, **1955**, 259—269.
6) Schwyzer R., Sieber P., *Helv. Chim. Acta*, **42**, 972—977 (1959).
7) Schroeder E., Klieger E., *Justus Liebigs Ann. Chem.*, **673**, 196—207 (1955).
8) Yamashiro D., Garzia R., Hammonds R. G., Jr., Li C. H., *Int. J. Peptide Protein Res.*, **19**, 284—289 (1982).
9) Tam J. P., Wong T. W., Riemen M. W., Jioeng F. S., Merrifield R. B., *Tetrahedron Lett.*, **42**, 4033—4036 (1979).
10) Fujii N., Sakurai M., Akaji K., Nomizu M., Yajima H., Mizuta K., Ando A., Moriga M., Inoue K., Hosotani R., Tobe T., *Chem. Pharm. Bull.*, **34**, 2397—2410 (1986).
11) Okada Y., Iguchi S., *J. Chem. Soc., Perkin Trans. 1*, **1988**, 2129—2136.
12) Weygand F., Hunger K., *Chem. Ber.*, **95**, 7—16 (1962).
13) Sakakibara S., Shimonishi Y., Kishida Y., Okada M., Sugihara H., *Bull. Chem. Soc. Jpn.*, **40**, 2164—2167 (1967).
14) Dixon M., *Biochem. J.*, **55**, 170—177 (1953).