Solid-Phase Syntheses of $Glu_{20}Ala_{20}Phe$ and $Ala_{20}Glu_{20}Phe$ by the Step-by-step Coupling of Dipeptide and Tetrapeptide[1]

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$Glu_{20}Ala_{20}Phe$, (I), and $Ala_{20}Glu_{20}Phe$, (II), were synthesized by the step-by-step coupling of N^{α}-*t*-butoxycarbonyl (BOC-) derivatives of Ala–Ala and (γ-benzyl-Glu)$_4$ to phenylalanyl-1% crosslinked polystyrene resin using dicyclohexylcarbodiimide. The products were cleaved from the resin with hydrogen bromide in acetic acid and purified by gel filtration, followed by DEAE-cellulose chromatography, with yields of approximately 10% for both of the hentetracontapeptides. The optical purities of the glutamic acid and alanine incorporated in the peptides, I and II, were estimated as 1 and 5% respectively from the analysis of the diastereomeric mixture of dipeptides derived from the reaction between the total acid hydrolyzate of I or II with the *N*-hydroxysuccinimidyl ester of BOC-L-Leu.

The problem that an α-helix, one of the most important ordered secondary structure of a polypeptide chain, is uncoiled from one side, either the N- or the C-terminal, has not yet been studied experimentally. The available works on the problem have been limited to a few theoretical treatments.[2,3] The study of the effects exerted by the secondary structures of neighboring moieties in a polypeptide chain on a local α-helix has importance, especially in comprehending the stability of proteins, in which secondary structures are sequenced.

Block copolypeptides will serve as a good model to study the effects of a local conformation of a polypeptide chain on other parts of the chain. Previously we attempted to synthesize and to study the stability of the local α-helical conformation in two kinds of block copolypeptides, $Glu_{20}Ala_{20}$ and $Ala_{20}Glu_{20}$, in solution. The number of residues in a block, 20, was chosen as a compromise between the need for a stable α-helix and ease of syntheses. However, as has already been reported,[3] the attempted solid-phase peptide synthesis of these block copolypeptides by a step-by-step coupling of *t*-butoxycarbonyl-(BOC-)glutamic acid or -alanine was shown to be impractical due to the poor yield of the desired peptides. Furthermore, the difficulties in the solid-phase peptide synthesis of glutamyl peptides (including γ-peptides) have been well documented.[4–7] On the other hand, the coupling reactions of oligopeptides on a solid support have found their successful applications in syntheses of sequential peptides,[8,9] physiologically active peptides,[10–16] and some miscellaneous peptides.[17–19] We also tried a condensation of oligopeptides on a solid support as an alternative route to synthesizing $Glu_{20}Ala_{20}$ and $Ala_{20}Glu_{20}$ and obtained a satisfactory result, as will be described in this paper.

Results and Discussion

The synthesis of oligoglutamyl peptides by conventional solid-phase peptide synthesis has caused much trouble, as was initially documented by Bonora *et al.*[4] The yield of the desired peptide was drastically decreased with an increase in the number of residues, *n*, on their attempted synthesis of $(Glu)_n$, and the synthesis failed when *n* reached 5, where the desired peptide could not be isolated at all. As has also been reported previously,[3] we also attempted solid-phase syntheses of $(Glu)_n$ and $(Ala)_n$ by the step-by-step coupling of BOC-glutamic acid or -alanine. For both $(Glu)_n$ and $(Ala)_n$, it was observed that the elongation of a peptide chain proceeded smoothly with each cycle of the solid-phase synthesis if *n* was below about 5; then it became gradually sluggish and apparently stopped when *n* reached around 10. The failure of the syntheses might be ascribed to something other than the cleavage process of peptides from peptide-resin, because the yield of peptides was based on the incorporation of amino acids (glutamic acid or alanine), which was evaluated on the basis of the amino-acid analyses of hydrolyzates of peptide-resin. Some of the reasons for the failure of the synthesis by Bonora *et al.*[4] might be attributed to undesired side reactions of a glutamyl side chain which occurred during a cleavage reaction of peptides from peptide-resin, as has been reported by Sano and Kawanishi[5] and Feinberg and Merrifield,[6] but there seem to be other reasons inherent in the method itself in the case of solid-phase peptide syntheses of homologous peptides with enough residues to form a regular secondary structure.[3]

Although we are not yet aware of the exact reasons for the difficulties encountered in the solid-phase peptide synthetic approaches to some of the homologous peptides, one way devoid of difficulties would be a modification of the solid-phase method to incorporate a coupling reaction using oligopeptides instead of amino acids. The advantages would be as follows: (1) an improvement in the yield of the final product because of the smaller number of steps, (2) discrete distribution of the residues, which promises an easy purification of the reaction products, and (3) the possibility that the reactivity of a growing peptide chain on a solid matrix is critically dependent on the chain length due to interaction between the reactive center of a chain and a supporting matrix, especially if a stable secondary structure is present in that chain. Interactions of that kind seem likely for solid-phase syntheses of homologous peptides because a unique secondary structure is to be expected. The use of oligopeptides instead of amino acids as the unit in the syntheses may surpass the critical chain length.

Syntheses. Taking these points into account, we attempted solid-phase syntheses of $Glu_{20}Ala_{20}Phe$, (I), and $Ala_{20}Glu_{20}Phe$, (II), using oligopeptides as the

TABLE 1. A CYCLE OF SOLID-PHASE PEPTIDE SYNTHESIS FOR THE ADDITION OF ONE OLIGOPEPTIDE[a)]

	Function	Solvent or reagent	Time (min)	Number of applications
(1)	wash	CH_2Cl_2	2	3
(2)	deprotection	CF_3CO_2H–CH_2Cl_2 (1 : 1)	30	1
(3)	wash	CH_2Cl_2	2	4
(4)	neutralization	triethylamine (20 mmol) in CH_2Cl_2	5	2
(5)	wash	CH_2Cl_2	2	2
(6)	wash[b)]	CH_2Cl_2–DMF[c)] (1 : 1)	2	1
(7)	wash[b)]	DMF	2	1
(8)	peptide[d)]	BOC–(γ-Bzl–Glu)$_4$ in CH_2Cl_2 or BOC–Ala_2 in 30% DMF in CH_2Cl_2	2	1
(9)	coupling	DCC in CH_2Cl_2[e)]	240	1
(10)	wash	CH_2Cl_2	2	3

a) The amount was for 5 g of resin containing 0.825 mmol of a reactive amino group. Volume of solvent, 60 ml. b) In the case of glutamyl peptide, these procedures were omitted and Step (5) was repeated. c) *N*,*N*-dimethylformamide. d) 3.3 mmol of oligopeptide in 25 ml of the solvent. e) 3.3 mmol of DCC in 5 ml of the solvent.

condensing unit. First, phenylalanine was anchored to resin as an internal marker. 1% cross-linked polystyrene resin was used, for its porosity is higher than that of the usual 2% cross-linked resin. BOC–(γ-benzyl–Glu)$_4$, (III), and BOC–(Ala)$_2$, (IV), were used as units for the syntheses of a Glu_{20}– and an $(Ala)_{20}$–block respectively. We attempted to use BOC–Ala_4 as a condensing unit, but the poor solubility of the tetrapeptide in most solvents made the attempt impractical.

All the peptides used for solid-phase synthesis were synthesized by the method of Anderson *et al.*[20] The reaction of the BOC-alanine *N*-hydroxysuccinimidyl-(–OSu) ester with alanine gave IV. The optical purity of the purified IV which was used in the solid-phase syntheses was shown, by an analysis to be described in a later section, to be 97% in the L-configuration. The γ-benzyl(γ-Bzl–) ester of BOC–Glu–OSu, (V), was reacted with γ-Bzl-glutamate to give BOC–(γ-Bzl–Glu)$_2$, (VI), a half of which was then converted into its *N*-hydroxysuccinimidyl ester, (VII), and the other half to (γ-Bzl–Glu)$_2$, (VIII). A reaction between VII and VIII gave the desired tetrapeptide, III, and the peptide was purified *via* its salt with dicyclohexylamine (DCHA). The optical purity of I that was used in the following solid-phase synthesis was greater than 99% in the L-configuration.

Solid-phase peptide syntheses were carried out using a cycle summarized in Table 1. After each cycle had finished, an aliquot of peptide resin was hydrolyzed with acid and analyzed for its amino-acid composition. The results are shown in Fig. 1, where a tendency for the yield of peptide-chain elongation to decrease was observed when the number of residues reached about 10. Even under the assumption that the yield of a reaction needed to extend one oligopeptide unit is uniformly α, irrespective of the length of the peptide chain, there are two extreme possibilities. (1) At the one extreme, α is considered simply as the possibility of a reaction occurring at a reactive center, and the unreacted peptide fraction (1-α) remains reactive and will react with the same probability in the next reaction cycle (for instance, an incomplete removal of the BOC group, *etc.*). The total amount of alanine and glutamic acid incorporated

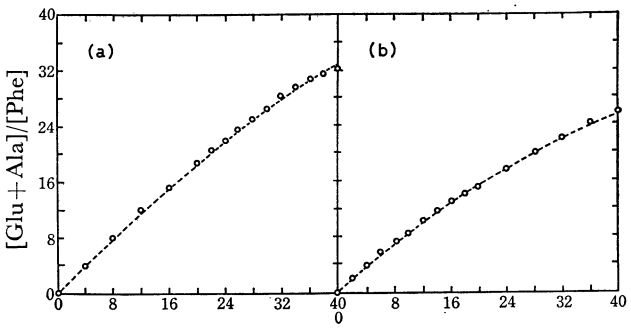

Fig. 1. The amount of amino acids incorporated with the progress of solid-phase peptide syntheses of: (a) $Ala_{20}Glu_{20}Phe$, and (b) $Glu_{20}Ala_{20}Phe$. The ordinate is the total amount of glutamic acid and alanine per mol of phenylalanine, and the abscissa is the repeated number of cycles of solid-phase synthesis. Circles represent the observed values and dotted curves for the calculation based on the dead-end products assumption. α(see text) was chosen as 0.97 for (a) and 0.95 for (b).

in the products will be linearly proportional to α. (2) At the other extreme, the peptide fraction, α, is considered living and reactive, and the fraction, (1-α), is a dead-end product which is no more reactive. In this case, if we start from 1 mol of phenylalanyl resin, the amount of such a dead-end product, $Glu_{4i}Ala_{2j}$, will be given by $\alpha^{i+j}(1-\alpha)$ moles, and the amounts of alanine and glutamic acid incorporated after m times coupling of IV and n times coupling of III are given by $2\sum_{i=1}^{m}\alpha^i$ and $4\sum_{i=1}^{n}\alpha^i$ moles respectively (after 10 times coupling of IV, followed by 5 times coupling of III, the number is $2\sum_{i=1}^{10}\alpha^i+4\alpha^{10}\sum_{i=1}^{5}\alpha^i$). The actual amounts of glutamic acid and alanine found in the acid-hydrolyzates of peptide resin with the progress of the number of coupling reactions (Fig. 1) deviate from a line, thus showing the (1) assumption to be unlikely. For a comparison, theoretical curves were calculated under the (2) assumption and for various values of α. α=0.97 and 0.95 for the

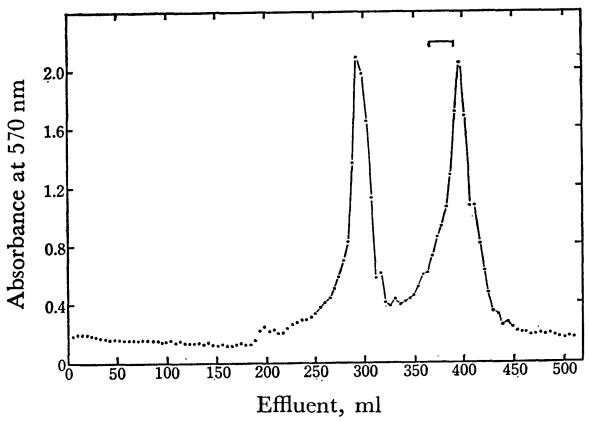

Fig. 2. Sephadex G-50 gel filtration of the product of solid-phase synthesis of $Glu_{20}Ala_{20}Phe$. The ordinate is absorption at 570 nm after the ninhydrin reaction of alkaline hydrolyzates of 0.01 ml aliquots from chromatographic fractions. The inserted bar shows the position of fractions which gave the amino acid composition was Glu: Ala: Phe= >19: >19: 1.

syntheses of Ala_mGlu_nPhe and Glu_mAla_nPhe respectively gave the best fit with the experimentally observed values, as shown in Fig. 1. Although the efficiency of a real peptide coupling reaction might depend on the length of the peptide chain, the comparison of the experimentally obtained plot and the theoretical ones for the extreme cases suggests that the experiments support, rather, the dead-end product assumption.

The peptides were cleaved from the resin by a conventional method, using hydrogen bromide in a mixture of acetic acid and trifluoroacetic acid. The products, (IX) and (X), obtained from the syntheses of I and II respectively were treated with aq. sodium hydrogencarbonate, and a fraction soluble in hydrogencarbonate solution was chromatographed on Sephadex G-50 (Figure 2 shows the results for IX). A fast eluting component appeared for both IX and X; it is considered to consist of peptide aggregates which are composed of alanine-rich peptides, as shown by their amino-acid analyses. All the chromatographic fractions were analyzed for their amino-acid compositions: the fractions containing Glu: Ala: phe=>19:>19: 1 were collected, desalted on a column Sephadex G-10, and lyophilized. The lyophilized material was chromatographed on DEAE-cellulose, and the fractions which gave the amino acid composition of: Glu: Ala: Phe= >19: >19: 1 were considered to be the final product, I or II (Fig. 3). These final products were analyzed by means of 2,4-dinitrophenylation for their free amino groups. The yield of 92—94% 2,4-dinitrophenylalanine or -glutamic acid suggested that only small amounts of pyroglutamate and/or other N-blocked products were present. The final yields of I and II were 9 and 11% respectively.

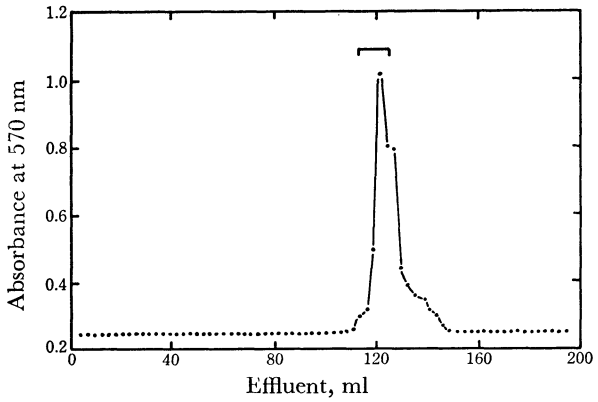

Fig. 3. DEAE-cellulose chromatography of $Glu_{20}Ala_{20}$-Phe. Aliquot of 0.025 ml from each fraction was alkaline hydrolyzed and absorbance at 570 nm was recorded after the ninhydrin reaction. The inserted bar has the same meaning as used in Fig. 2.

Degree of Racemization in the Products. The chromatographically purified products, I and II, were analyzed for their degrees of racemization caused by the peptide-coupling reactions. The racemization detection method by Manning[21] was modified and used. The peptide hydrolyzates, which were obtained by the treatment of the peptides with 6 M hydrochloric acid at 110 °C for 24 h, were reacted with BOC–L-Leu–OSu in the presence of sodium hydrogencarbonate. The products, after an acid treatment to remove the BOC group, were analyzed with an amino-acid analyzer. The authentic samples of D,L-glutamic acid and D,L-alanine were reacted with BOC–L-Leu–OSu under the same conditions and the ninhydrin colour value for each dipeptide separated on an amino-acid analyzer was used as the reference standard. The overall (because they included the difference in the reactivities of L- or D-amino acids towrd BOC–L-Leu–OSu) average ratio of ninhydrin colour values obtained from several experiments were: (L-Leu–D-Ala)/(L-Leu–L-Ala)=0.74, (L-Leu–D-Glu)/(L-Leu–L-Glu)=0.60, starting from a racemic mixture. The authentic samples of each dipeptide were also synthesized and used to identify the peaks in the chromatograms. These analyses showed the amounts of D-glutamic acid and D-alanine present in the hydrolyzate of the peptide I to be less than 1 and 5% respectively. The peptide II gave the same values.

The starting materials, III and IV, for the solid-phase syntheses were also tested for their degrees of racemization by the same method. The content of D-glutamic acid in III was shown to be less than 1%, and that of D-alanine in IV, to be 3%. Unfortunately, the original L-alanine used in the syntheses was found to contain 3% D-alanine. A comparison of the ORD curves of the hydrolyzate of IV and another sample of pure L-alanine confirmed the result ($[\alpha]_{230}$ were 1750° and 1860° (1%, 1 M HCl) for the hydrolyzate of IV and pure L-alanine respectively). Recrystallization seemed not to be able to separate a diastereomeric mixture, and the origin of a half of the D-alanine found in I and II was explained from the purity of the starting material. Therefore, the racemization accompanied by one step of the peptide-coupling reaction was about 0.3% or lower for the coupling of BOC–$(Ala)_2$ or BOC–$(\gamma$-Bzl–Glu$)_4$ respectively.

Experimental

The melting points were determined on a micro hot plate and are uncorrected. The elementary analyses were carried

out in the elementary analysis section in the Institute for Chemical Research, Kyoto University. The amino-acid analyses were carried out with a JEOL liquid chromatographic system under the conditions of Spackman *et al.*[22] A JASCO J-20 spectropolarimeter was used to measure the optical rotations. The BOC-hydrazide, trifluoroacetic acid, dicyclohexylcarbodiimide (DCC), and HOSu were purchased from the Protein Research Foundation, Osaka, Japan. The polystyrene beads (SX-1) were products of BioRad, Calif., U.S.A. The amino acids and other reagents were purchased from Nakarai Chemical Co., Kyoto, Japan. Reagent-grade dichloromethane was stored over sodium hydrogencarbonate, dried over molecular sieves (Type 4A, Linde), and distilled before use. The *N,N*-dimethylformamide was passed through a freshly packed column of silica gel G, dried over a molecular sieve (Type 4A, Linde), and distilled *in vacuo*. The trifluoroacetic acid was used after distillation over P_2O_5. The triethylamine was treated with 2,4-dinitrofluorobenzene and distilled over sodium.

N^{α}-t-Butoxycarbonyl-L-alanyl-L-alanine, (IV). The dipeptide was synthesized from 143 g of BOC-Ala-OSu,[20] 53.5 g of alanine, and 50.5 g of $NaHCO_3$ in a mixture of 900 ml of tetrahydrofuran and 600 ml of water, according to the general method of Anderson *et al.*[20] Yield, 123 g (95%); mp 128—129 °C in a sealed tube (lit mp 85.5—87 °C,[23] 132—133 °C[24]). The compound could not be distinguished in melting point or infrared spectrum from the specimen obtained by the coupling of BOC-alanine with methyl alaninate employing isobutoxycarbonyl chloride, followed by saponification.[24] IV was hydrolyzed in 6M HCl at 110 °C for 24 h, and the hydrolyzate was submitted to measurements of the optical rotation and the racemization-detection reaction.

N^{α}-t-Butoxycarbonyl-γ-benzyl-L-glutamic Acid, N-Hydroxysuccinimidyl Ester (V). 90 g of BOC-(γ-Bzl-Glu)[25] was allowed to react with 19.9 g of HOSu and 39.2 g of DCC at −8 °C in a mixture of 600 ml of ethyl acetate and 100 ml of tetrahydrofuran. Yield of V after recrystallization from 2-propanol, 68.3 g (91%); mp 106 °C (lit mp 100—101 °C[26]).

N^{α}-t-Butoxycarbonyl-γ-benzyl-L-glutamyl-γ-benzyl-L-glutamic Acid (VI), and Its Dicyclohexylammonium Salt (VIa). 71.7 g of V, 59 g of γ-benzyl-L-glutamate, and 34.6 ml of triethylamine were stirred in 600 ml of *N,N*-dimethylformamide at room temperature for 10 h. Oily VI, obtained by the evaporation of the solvent *in vacuo*, was treated with 30 g of DCHA to give VIa, which was then recrystallized from ethyl acetate-cyclohexane. The VIa had a mp of 108—109 °C. Found: C, 66.65; H, 8.27; N, 5.72%. Calcd for $C_{41}H_{59}O_9N_3$ (VIa): C, 66.73; H, 8.06; N, 5.69%. VI has been known as oil.[26]

N-Hydroxysuccinimidyl Ester of VI(VII). 100 g of VIa was suspended in 400 ml of ethyl acetate and washed with 20% citric acid to convert the VIa into VI. The VI was reacted with 18.7 g of HOSu and 33.4 g of DCC at −8 °C for 10 h, and then at 5 °C for 25 h. The product, VII, was recrystallized from 2-propanol. Yield, 81 g (91%); mp 112—114 °C. Found: C, 60.88; H, 6.18; N, 6.42%. Calcd for $C_{33}H_{39}O_{11}N_3$: C, 60.63; H, 6.01; N, 6.43%.

γ-Benzyl-L-glutamyl-γ-benzyl-L-glutamic Acid(VIII). A 5.1-g portion of VIa was dissolved in 20 ml of dichloromethane and washed with 20% citric acid. The organic phase was washed with H_2O, dried over drierite, and then added to 10 ml of trifluoroacetic acid. The mixture was kept at room temperature for 1 h and then evaporated *in vacuo*. The residue was treated with 3% aq $NaHCO_3$ to crystallize the dipeptide acid VIII, which was subsequently recrystallized from ethanol. Yield, 2.1 g (65%); mp 124—125 °C. Found: C, 62.99; H, 6.22; N, 5.92%. Calcd for $C_{24}H_{28}O_7N_2$: C, 63.14; H, 6.18; N, 6.14%. The washing procedure with citric acid could be eliminated without any trouble. In that case, the amount of dichloromethane was reduced to 10 ml.

N^{α}-t-Butoxycarbonyl-(γ-benzyl-L-glutamyl-)$_3$-γ-benzyl-L-glutamic-Acid, (III), and Its Dicyclohexylammonium Salt(IIIa). 52 g of VIII, 74 g of VII, 22.8 g of triethylamine, and 500 ml of *N,N*-dimethylformamide were mixed and stirred at room temperature for 24 h. The residues which were obtained after the evaporation of the solvent *in vacuo* were dissolved in ethyl acetate and washed thoroughly with 3% aq $NaHCO_3$. In the washing procedure, the sodium salt of tetrapeptide acid, III, was not transferred to the aqueous phase, but remained in the organic phase. The organic layer was washed with 5% citric acid and then with water, and dried over Na_2SO_4. A 20.4-g portion of DCHA was added to convert the III into IIIa, which was recrystallized several times from ethyl acetate. Yield, 105 g (79%); mp 129—130 °C. Found: 66.06; H, 7.24; N, 5.90%. Calcd for $C_{65}H_{85}O_{15}N_5$: C, 66.36; H, 7.28; N, 5.95%.

Solid-phase Peptide Synthesis. Polystyrene beads (BioBeads SX-1) which had been crosslinked with 1% divinylbenzene were chloromethylated[27] to a substitution level of 0.18 mmol Cl/g resin. The chloromethylated resin was reacted with BOC-phenylalanine to give the starting resin, BOC-Phe-resin. Acid hydrolysis, followed by amino-acid analysis, showed that 0.165 mmol of phenylalanine was incorporated per g of resin. 15 cycles of solid-phase peptide synthesis (the cycle is summarized in Table 1) were manually performed with a home-made shaker, starting with 5 g of BOC-Phe-resin for each block copolypeptide. After Step (11) in Table 1, an aliquot of peptide resin was removed, acid-hydrolyzed and analyzed for its amino-acid composition. Hydrolyses of the resin-bound peptides were carried out in a mixture of propionic acid and concd HCl (1: 1) or, for alanine-rich peptide-resin, in 95% trifluoroacetic acid, both in a sealed and evacuated tube at 110 °C for 24 h.

Cleavage of Peptide from Peptide-resin and Gel Chromatography of Peptides. A 20-ml portions of 20% HBr in acetic acid was added to 3 g of peptide-resin suspended in 10 ml of trifluoroacetic acid. The mixture was kept at room temperature for 1 h and then filtered to separate the resin. The resin was washed with a 1: 1 mixture of acetic acid and trifluoroacetic acid, and the combined filtrate was evaporated *in vacuo*. The residue was thoroughly washed with ether and dissolved in aq hydrogencarbonate solution. The peptides precipitated by acidification with HCl were redissolved in a small amount of aq $NaHCO_3$, and chromatographed on Sephadex G-50 (medium, 2.8×130 cm). Elution was achieved with 0.1 M NaCl-0.05M $NaHCO_3$-0.005M Na_2CO_3, and each fraction was analyzed for its peptide content by ninhydrin analysis after alkaline hydrolysis[28] or by UV absorption at 230—240 nm. An aliquot from each peptide-containing fraction was acid-hydrolyzed and analyzed for its amino-acid composition. Fractions containing a peptide whose amino acid composition was Glu: Ala: Phe= >19: >19: 1 were pooled and lyophilized. The lyophilized material was then dissolved in 2 ml of H_2O and placed on a column (1.5×130 cm) of Sephadex G-10. Elution was achieved with H_2O, and the peptide-containing fractions were combined and lyophilized.

$Glu_{20}Ala_{20}Phe$, (I). The yield of the material obtained after a gel filtration was 280 mg from 3 g of peptide-resin (14%). A 40-mg portion of the lyophilized sample was chromatographed on a DEAE-cellulose (Whatman DE-32) column (1.3×17 cm). The linear gradient of the concentration from 0M to 1M (in a 0.05M Na-phosphate buffer at pH 6.67) was applied for elution (total 200 ml). Each fraction was analyzed by ninhydrin analysis after alkaline hydrolysis,[28] and the peptide-containing fractions were analyzed for

their amino-acid compositions after total hydrolysis. Peptide-containing fractions, for which the amino-acid composition after total hydrolysis was given as Glu: Ala: Phe= >19: >19: 1, were pooled, desalted by Sephadex G-10, and lyophilized. Yield, 26 mg (the total yield was calculated as 306 mg (9%) starting from 5 g of BOC–Phe-resin).

$Ala_{20}Glu_{20}Phe$, (II). The gel-filtrated material was chromatographed on DEAE-cellulose and analyzed in a way similar to that described for I.

N-Terminal Analysis. Aliquots from I and II were 2,4-dinitrophenylated, hydrolyzed in 6M HCl at 110 °C for 18 h, and analyzed for the yield of 2,4-dinitrophenyl(DNP-)alanine or DNP-glutamic acid. After the necessary correction for the loss of DNP-amino acids during the acid hydrolysis, the yields of DNP-glutamic acid from I and DNP-alanine from II were found to be 92% and 94% respectively. No other DNP spots were detected on a TLC plate.

Detection of Racemization. The peptides in question (2 to 5 mg) were hydrolyzed in 2 ml of 6M HCl at 110 °C for 24 h. The hydrolyzates, which were completely free from acid by drying over KOH *in vacuo*, were dissolved in a mixture of 1 ml of H_2O and $NaHCO_3$ (5 equivalents for amino acids). 5 equivalents of BOC–L-Leu–OSu in 0.5 ml of acetonitrile were added to the mixture, and the reaction was carried out at room temperature for 1 h under shaking. The reaction mixture was then washed with ethyl acetate several times and evaporated to dryness. The residue was treated with a mixture of 1 ml of acetic acid and 1 ml of trifluoroacetic acid at room temperature 1 h, and then evaporated to afford a mixture of dipeptides, excess leucine, and some unreacted amino acids. The mixture was dissolved in a 0.2M sodium citrate buffer at pH 2.2 and placed in a 50-cm column of an amino-acid analyzer operated at 55 °C. A 0.2M sodium citrate buffer which had been adjusted to pH 3.10 by adding concd HCl to the regular pH 3.35 sodium citrate (0.2M) buffer[29] was used to separate L-Leu–D-Glu (425 min), L-Leu–L-Glu (488 min), and leucine (545 min), while the regular pH 4.25 buffer (0.2M sodium citrate)[29] was used to separete L-Leu–D-Ala (130 min) and L-Leu–L-Ala (157 min). The retention times are shown in parentheses. For the reference standard, authentic D,L-amino acids were treated under the same conditions. The product, the diastereomeric dipeptide mixture, was analyzed with an amino-acid analyzer to give the standard ratio of the ninhydrin colour values. The positions of the peptides were determined by the analysis of authentic peptides which had been synthesized from BOC–L-Leu–OSu and D- or L-amino acids. BOC–L-Leu–D-Ala (mp 166—168 °C from methanol. Found: C, 55.57; H, 8.67; N, 9.19%. Calcd for $C_{14}H_{26}O_5N_2$: C, 55.61; H, 8.67; N, 9.27%.) and BOC–L-Leu–L-Ala·DCHA (crystallized from ethyl acetate, mp 159—162 °C; the crystals turn unclear at 105—110 °C, presumably because of a loss of solvent for crystallization. Found: C, 63.71; H, 10.22; N, 7.78%. Calcd for $C_{26}H_{49}O_5N_3 \cdot 1/2CH_3CO_2C_2H_5$: C, 63.72; H, 10.12; N, 7.96%) were obtained as crystals, but the corresponding glutamyl peptides were oils.

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