Synthesis of α-Helices Having a Positively Charged Random Coil-Block on Either N- or C-Terminal

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To study a charge-induced stabilization of α-helix, α-helical peptides having a positively charged random-coil block on either N- or C-terminal of the helix, and having a strictly defined length, $(Ala)_{20}(X)_{20}Phe$ and $(X)_{20}(Ala)_{20}Phe$ (X=Lys or Orn), were synthesized. Component dipeptides were coupled on *p*-(oxymethyl)-phenylacetamidomethyl polystyrene.

A peptide bond is not symmetric and a polypeptide chain has a direction. As a consequence, properties inherent to α-helix and extrinsic effects of surrounding groups exerting upon α-helix might be different at the N- and C- terminal parts of a helix. We intended to discriminate the difference, and tried to approach the problem by studying the stabilities of polyalanine helix having a negatively charged random coil block either on an N- or C-terminal side of the helix.[1,2] The result showed that the difference in stabilities of two polymers was not solely depended on an enthalpic term (electrostatic interaction between external charges and a helix dipole) but also contributed from an entropic term, suggesting a contribution of helix-intrinsic properties on helical stability was different at the parts near N- and C-terminals. Since the stabilities of secondary structures are fundamental problem to study protein folding, asymmetry in the stability of α-helix has been called much attention recently[3] and a study of a system similar to that stated above but with positive charges has been requested.[4] If we could estimate a stability of polyalanine helix having a positively charged block on either N- or C-terminal, a combined analysis with the previous result for the helix with negative charges would make a comparison of the effects of opposite electric charges on the stability of α-helix possible. Furthermore, an estimation of intrinsic stabilities of α-helix at N- and C-terminal parts will be possible, under an assumption that the effect of positive and negative charges on an electric dipole was symmetric.

From a standpoint of peptide synthesis, preparations of homooligopeptides meet troubles not encountered in synthesis of peptides which incorporate various amino acid residues as naturally occurring ones. An application of a solution method for the preparation of oligopeptides having identical amino acid residues is sometimes extremely difficult, as such a peptide became less and less soluble in most of the solvents used in peptide chemistry when the number of residues increased. The other choice, a solid-phase method, is free from such a problem, however, purification of the product is difficult since immature peptides (namely, peptides having lesser numbers of residues than that was intended) will have similar properties with the desired one. Since the number of residues included in a peptide is most critical to stabilities of peptide secondary structures, it is crucial to have peptides with strictly defined length for a study of peptide conformation. In the present paper, we tried a coupling of dipeptide unit with a solid-phase methodology: in this case, immature peptides lacked two or multiples of two residues were expected to be discriminated more readily from the desired peptide.

Results and Discussion

Synthesis of $(Ala)_{20}(Lys)_{20}Phe$, (1), $(Lys)_{20}(Ala)_{20}Phe$, (2), $(Ala)_{20}(Orn)_{20}Phe$, (3), and $(Orn)_{20}(Ala)_{20}Phe$, (4). Merrifield's solid-phase procedure with *p*-(oxymethyl)-phenylacetamidomethyl (PAM)-resin[6] was used to synthesize **1**—**4**. The peptides have a general formula, $(L\text{-}Ala)_n(L\text{-}X)_nPhe$ or $(L\text{-}X)_n(L\text{-}Ala)_nPhe$ (X=Lys or Orn), in which poly-L-alanine block assumes α-helix (at least partly), and a block of positively charged lysine or ornithine forms a random-coil. The number of residues, *n*, was 20, which was consistent with the previous work.[1] The side chain of lysine is long and a hydrophobic nature of alkyl chain sometimes induces ordered structures, for example, β-structure. As we hoped to minimize such a possibility in the following study, peptides containing ornithine, which had one less methylene group were also synthesized. C-Terminal phenylalanine was incorporated as an internal marker during the synthesis. As the length of α-helix is most critical to its stability, dipeptide coupling was employed to ensure the desired product free from immature peptides. With dipeptide coupling, the inmature peptides differed by two amino acid residues and separation from the desired peptide was expected easier.[5] PAM-resin[6] was prepared with a slight modification: *p*-(bromomethyl)phenylacetic acid phenacyl ester was obtained from phenacyl alcohol (readily prepared from styrene oxide and dimethyl sulfoxide[7]) and trifluoroacetic anhydride, the procedure simpler and with higher yield. Dipeptide coupling was carried out with dicyclohexyl-

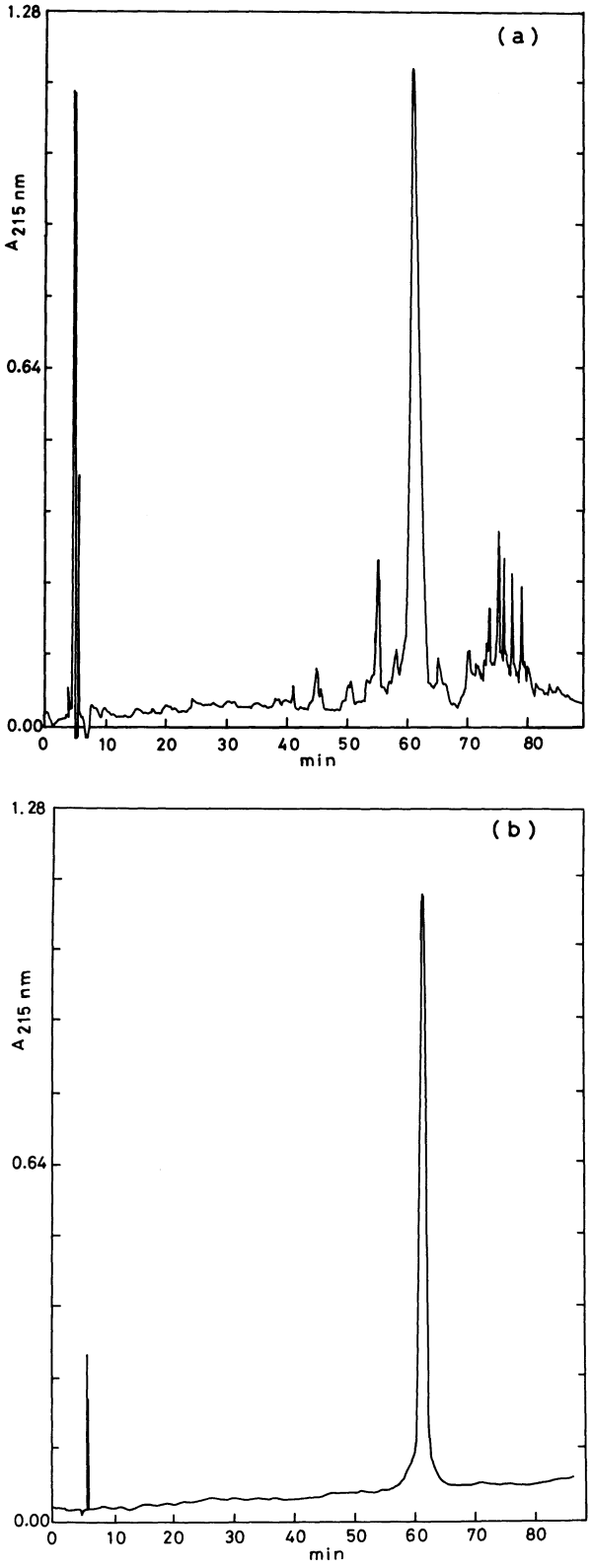

Fig. 1. HPLC analysis (Cosmosil $5C_{18}$-P, 4.6×150 mm) of **1**.
(a) Crude product obtained by HF-cleavage. (b) After purification with preparative HPLC.
Peptide was eluted with an H_2O–acetonitrile gradient containing 0.05% TFA (10 to 20% acetonitrile over a period of 0 to 60 min and 20 to 40% acetonitrile from 60 to 90 min). Flow rate, 1 ml min^{-1}.

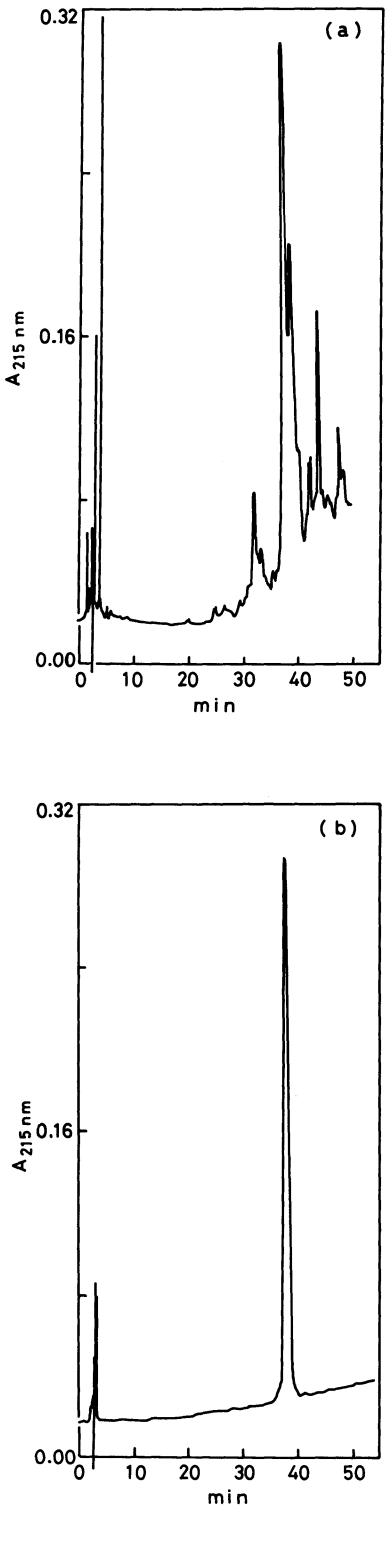

Fig. 2. HPLC analysis (Cosmosil $5C_{18}$-P, 4.6×150 mm) of **2**.
Peptide was eluted with an H_2O–acetonitrile gradient containing 0.05% TFA (20 to 30% acetonitrile over a period of 0 to 30 min and 30 to 40% acetonitrile from 30 to 45 min). Flow rate, 1 ml min^{-1}.
(a) Crude product obtained by HF-cleavage. (b) After purification with preparative HPLC.

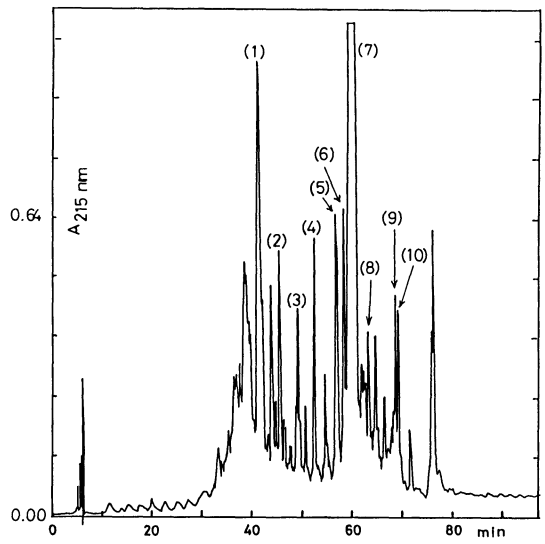

Fig. 3. Demonstration of resolution in HPLC of crude **1**.
Column: Cosmosil $5C_{18}$-P, 10×250 mm, flow rate, 3 ml min^{-1}. Linear gradient elution: 0.05% aq.TFA: CH_3CN (100:0)→(67:23) for 0→80 min. About 2 mg of peptide was loaded. Peak assignment follows amino acid analysis: (1) $Ala_{2\,or\,4}Lys_{14\,or\,16}Phe$, (2) $Ala_6Lys_{18}Phe$, (3) $Ala_{8\,or\,10}Lys_{18}Phe$, (4) $Ala_{12}Lys_{18}Phe$, (5) $Ala_{16}Lys_{18}Phe$, (6) $Ala_{18}Lys_{20}Phe$, (7) peptide, **1**, (8) $Ala_{18}Lys_{16}Phe$, (9) $Ala_{20}Lys_{18}Phe$, (10) $Ala_{20}Lys_{16}Phe$.

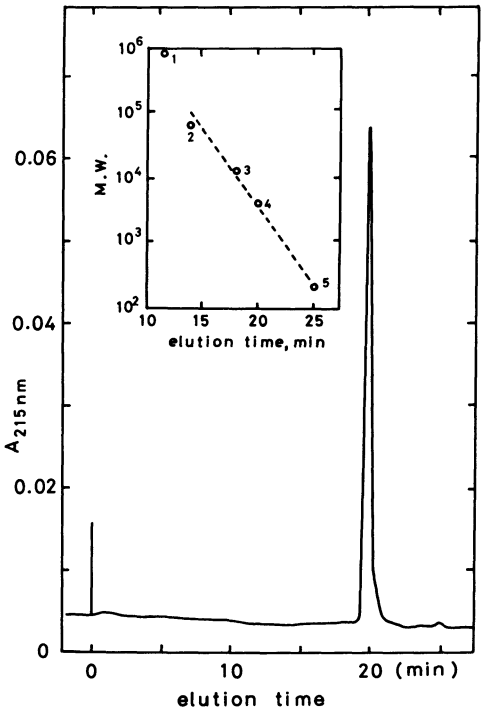

Fig. 4. Size exclusion chromatography of peptide **2**. Column: TSK Gel G2000SWXL (7.5×300 mm), elution with 0.1% TFA-0.2M KCl at a flow rate of 0.5 ml min^{-1}, at room temperature. Sample: 2 μg in 1 μl of 0.1% of TFA. Inset, Calibration of the column. 1, Blue Dextran (Pharmacia); 2, bovine serum albumin; 3, bovine pancreatic ribonuclease A; 4, peptide **2**, 5, GlyHis. M.W.: molecular weight, in logarithmic scale.

carbodiimide in the presence of 1-hydroxybenzotriazole. After the desired number of coupling, the products were cleaved from resin support with HF and were purified with high-performance liquid chromatography (HPLC). The results for peptides **1** and **2** are shown in Figs. 1 and 2, respectively. We characterized some of the satellite products and confirmed separation of the desired 41-residue peptides from immature peptides. As an example, the case of peptide **1** is shown in Fig. 3. Ion-exchange chromatography with carboxymethylcellulose was also employed to check the purity of HPLC-purified materials. We found only a single symmetric peak in every case. The overall yield of peptide **1**—**4** was from 9 to 19%.

Gel filtraion profiles of peptides **1**—**4** with TSK Gel G2000 (or 3000) SW may need some remarks. Each of the peptides gave a single peak at the position of expected elution position (as a typical example, Fig. 4 shows a chromatogram for peptide **2**, nearly identical results were obtained for the other peptides), but it was much retarded at higher pH. In the case of polylysines having molecular weights higher than 50000, such retardation of peak position as observed even at pH 3.0. The observed molecular weights of peptides **1** and **2** in solution derived from sedimentation equilibrium analysis at pH 4.65 (0.01 M KH_2PO_4–0.1 M KCl 1 M=1 mol dm^{-3}) were 4100 (c=0.668 mg ml^{-1}) and 4130 (c=0.789 mg ml^{-1}), respectively.

Check of Racemization. Athough 1-hydroxybenzotriazole[8] was included in dicyclohexylcarbodiimide-mediated coupling of dipeptide, racemization at the C-terminal amino acid of dipeptide might be a problem. We checked the content of D-amino acids with a modified[5] Manning-Moore procedure,[9] coupled with an HPLC separation of diastereomeric peptides.[10] The amounts (± 0.5%) of D-amino acids detected were: Ala in **1** or **3**, 0.7%; Ala in **2**, 1.0%; Ala in **4**, 0.6%; Orn in **3**, 1.5%; Orn in **4**, 1.9%; Lys in **1**, 1.0%; Lys in **2**, 0.8%.

Experimental†

All amino acids are of L-configuration. Melting points were measured on a micro hotplate and described without correction. Dipeptides, N^{α}-Boc-Lys(N^{ε}-*o*-Cl-Z)-Lys(N^{ε}-*o*-Cl-Z)-OH, N^{α}-Boc-Orn(N^{ε}-*o*-Cl-Z)-Orn(N^{ε}-*o*-Cl-Z)-OH, and Boc-AlaAla-OH, were prepared from Boc-amino acid succinimido esters. Elemental analysis and melting points are summarized in Table 1.

$BrCH_2C_6H_4CH_2COOH$. A solution of *p*-tolylacetic acid (25 g, 0.17 mol) and Br_2 (29.5 g, 1.1 equiv) in 200 ml CCl_4

† Abbreviations used: Boc, *t*-butyloxycarbonyl; *o*-Cl-Z, *o*-chlorobenzyloxycarbonyl; DCC, dicyclohexylcarbodiimide; DCHA, dicyclohexylamine; DIEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; HOBT, 1-hydroxybenzotriazole; TLC, thin-layer chromatography.

Table 1.

Compound	Melting point/°C[a]	Formula
N^{α}-Boc-Lys(N^{ε}-*o*-Cl-Z)-OSu	102—103 (AcOEt-Et_2O)	$C_{23}H_{30}O_8N_3Cl$
N^{α}-Boc-[Lys(N^{ε}-*o*-Cl-Z)]$_2$-OH·DCHA	142—143 (EtOH-AcOEt)	$C_{45}H_{67}O_9N_5Cl_2$
H-Orn(N^{δ}-*o*-Cl-Z)-OH	245—250 decomp (H_2O)	$C_{13}H_{17}O_4N_2Cl$
N^{α}-Boc-Orn(N^{δ}-*o*-Cl-Z)-OH	122—123 (AcOEt)	$C_{18}H_{25}O_6N_2Cl$
N^{α}-Boc-Orn(N^{δ}-*o*-Cl-Z)-OSu	152—156 (EtOH)	$C_{22}H_{28}O_8N_3Cl$
N^{α}-Boc-[Orn(N^{δ}-*o*-Cl-Z)]$_2$-OH	130 (MeOH-H_2O)	$C_{31}H_{40}O_9N_4Cl$
N^{α}-Boc-[Orn(N^{δ}-*o*-Cl-Z)]$_2$-OH·DCHA	166—169 (AcOEt)	$C_{43}H_{63}O_9N_5Cl_2$
Boc-Phe-$OCH_2C_6H_4CH_2CO_2CH_2COC_6H_5$	80—82 (AcOEt)	$C_{31}H_{33}O_7N$
Boc-Phe-$OCH_2C_6H_4CH_2CO_2H$	100—102 (Et_2O-pet.ether)	$C_{23}H_{27}O_6N$

Compound	Calcd			Found		
	C%	H%	N%	C%	H%	N%
N^{α}-Boc-Lys(N^{ε}-*o*-Cl-Z)-OSu	53.96	5.91	8.21	54.01	5.95	8.25
N^{α}-Boc-[Lys(N^{ε}-*o*-Cl-Z)]$_2$-OH·DCHA	60.46	7.67	7.83	60.33	7.55	7.52
H-Orn(N^{δ}-*o*-Cl-Z)-OH	51.92	5.70	9.32	51.94	5.59	9.37
N^{α}-Boc-Orn(N^{δ}-*o*-Cl-Z)-OH	53.93	6.29	6.99	53.91	6.18	7.02
N^{α}-Boc-Orn(N^{δ}-*o*-Cl-Z)-OSu	53.06	5.67	8.44	53.00	5.70	8.42
N^{α}-Boc-[Orn(N^{δ}-*o*-Cl-Z)]$_2$-OH	54.47	5.90	8.20	54.54	5.88	8.12
N^{α}-Boc-[Orn(N^{δ}-*o*-Cl-Z)]$_2$-OH·DCHA	59.71	7.34	8.10	59.44	7.42	8.06
Boc-Phe-$OCH_2C_6H_4CH_2CO_2CH_2COC_6H_5$	70.04	6.26	2.64	70.31	6.27	2.64
Boc-Phe-$OCH_2C_6H_4CH_2CO_2H$	66.81	6.58	3.39	66.93	6.43	3.30

a) Solvent for recrystallization in parentheses.

was stirred at room temperature and illuminated with a 60-W reflector lamp. The distance between the lamp and the reaction vessel was determined to give a moderate reaction. As the separated crystals of the product inhibited the reaction by reflecting back the incident light, the reaction mixture was filtered three times to collect crystals during 24 h of the photoreaction. If the solution became colorless, up to 3 ml of Br_2 was added to the reaction mixture. After the reaction had been completed (checked with TLC), the solvent was evaporated to give an additional amount of *p*-(bromomethyl)phenylacetic acid, which was recrystallized from acetone-hexane. Yield, 72%; mp 179—180 °C.

$BrCH_2C_6H_4CH_2COOCH_2COC_6H_5$. *p*-(Bromomethyl)-phenylacetic acid, 2.29 g, and 2.5 ml of trifluoroacetic anhydride were dissolved in 5 ml of benzene with warming. The mixture was kept at room temperature for 1 h and evaporated. The residue was dissolved in a mixture of 5 ml benzene and 1.55 g of phenacyl alcohol[7] with warming. Unreacted bromo acid was recovered with filtration after 3 h at room temperature and the filtrate was washed with aq. $NaHCO_3$ and concentrated to afford the phenacyl ester, which was recrystallized from ethyl acetate-cyclohexane, mp 88—90 °C. Yield, 79% (after correction of recovered acid).

Boc-Phe-$OCH_2C_6H_4CH_2COOCH_2COC_6H_5$. A solution of 2.8 g (8 mmol) of *p*-(bromomethyl)phenylacetic acid phenacyl ester, 4.25 g (16 mmol) of Boc-Phe-OH, and 2.8 ml (16 mmol) of DIEA in 60 ml DMF was stirred at room temperature for 24 h. After 8 h from the start of the reaction, TLC showed the bromo ester was consumed. Evaporation of DMF afforded an oil, which was dissolved in AcOEt (filtration was sometimes necessary to remove a mass of DIEA·HBr) and washed successively with 5% aq. citric acid and 0.5 M carbonate buffer, pH 10. Concentration of AcOEt layer gave needles, which were recrystallized from AcOEt. Yield, 93%.

Boc-Phe-$OCH_2C_6H_4CH_2COOH$. The compound was synthesized by a Zn-reduction of *p*-[(Boc-phenylalanyl)oxymethyl]phenylacetic acid phenacyl ester in a similar way as described in Ref. 6. Yield, 69%.

Boc-Phe-$OCH_2C_6H_4CH_2CONHCH_2$-Resin. Polystyrene beads (BioRad SX-1, 200—400 mesh) were aminomethylated according to the method described.[6] The content of amino group was 0.3 mmol g^{-1} resin from picric acid titration of the product. The resin was coupled[6] to Boc-Phe-OCH_2-$C_6H_4CH_2COOH$ and treated with 1-acetylimidazole (3 g/20 g resin) to block the residual free amino groups, if any. The product, which showed 0.285 mmol phenylalanine per g of resin on amino acid analysis, was used for the solid-phase peptide synthesis.

Solid-Phase Synthesis of H-$(Lys)_{20}(Ala)_{20}$Phe-OH, (1); H-$(Ala)_{20}(Lys)_{20}$Phe-OH, (2); H-$(Orn)_{20}(Ala)_{20}$Phe-OH, (3); and H-$(Ala)_{20}(Orn)_{20}$Phe-OH, (4). Solid-phase peptide synthesis was carried out according to the schedule outlined in Table 2. Double coupling was applied in every cycle except first 3 cycles of **1** and **3**. After each cycle was finished, an aliquot of the peptide resin was removed and checked by the Kaiser test.[11] Another aliquot was hydrolyzed and analyzed on an amino acid analyzer. After completion of required cycles, peptide resin was first treated with 50% TFA in dichloromethane, then with HF-20% anisole at 0 °C for 1 h. The residue was extracted with water (peptides **2** and **4**) or with 30% AcOH (peptides **1** and **3**). In the latter case, extracts were dialyzed against water and centrifuged to remove insolubles. The lyophilized peptides were dissolved in 0.05% TFA and purified with HPLC on a C_{18} column, with a linear gradient of acetonitrile containing 0.05% of TFA. The final yield of the purified peptide: **1**, 10%; **2**, 19%; **3**, 8.6%; **4**, 13.4%. Amino acid composition: **1**, Ala:Lys: Phe=21.4:21.0:1.0; **2**, Ala:Lys:Phe=20.6:19.3:1.0; **3**, Ala: Orn:Phe=20.8:20.2:1.0; **4**, Ala:Orn:Phe=19.3:19.4:1.0.

Check of Racemization. Peptide-resin and free peptides (about 1 μ mol peptide) were hydrolyzed with concd HCl-propionic acid (1:1, v/v, 110 °C, 24—48 h) or 6M HCl-0.15% phenol (110 °C, 24—48 h), respectively. To the hydrolyzate

Table 2.

1. Wash. CH_2Cl_2, 2 min×3.
2. Deprotection. 50% TFA in CH_2Cl_2, 30 min×1.
3. Wash. CH_2Cl_2, 2 min×5.
4. Neutralization. 5% DIEA in CH_2Cl_2, 2 min×2 (or 3[a]) for first coupling, 2 min×1 for second coupling.
5. Wash. CH_2Cl_2, 2 min×5.
6. Dipeptide,[b] 2 min.
7. HOBT[c] in DMF, 2 min.
8. DCC[d] in CH_2Cl_2. 6—18 h[e] for first coupling, 7—9 h for second coupling.
9. Wash. CH_2Cl_2–DMF(1:1) 2 min×2; CH_2Cl_2, 2 min×3.
10. Kaiser test.

The synthesis was started with 5 g (equivalent to 1.5 mmol of Boc-Phe groups) of Boc-Phe-PAM-resin. The volume of solvent was gradually increased from 50 to 80 ml according to swelling of the resin. First and second coupling mean the respective cycles when double coupling was employed. When a second coupling was employed, the step 4 and so on followed the step 10.

a) At the cycle later than the 5th. b) 4.5 mmol in the first coupling, 2.3 mmol in the second coupling. Boc-AlaAla-OH was dissolved in 30 ml of 30% DMF in CH_2Cl_2, lysine- and ornithine-dipeptides were dissolved in CH_2Cl_2. c) One and a half equivalent to dipeptide was used as a solution in 5 ml. d) An equivalent amount to dipeptide was used as a solution in 5 ml. e) 6—9 h for the first 5 cycles for **1** and **3** (coupling of BocAlaAlaOH) and first 10 cycles for **2** (coupling of BocLys(*o*-Cl-Z)Lys(*o*-Cl-Z)OH); 12—18 h for the other cycles.

were added 1.2 ml of 1 M $NaHCO_3$ and 50 μl of Boc-L-leucine succinimido ester (0.4 g/5 ml CH_3CN or 0.25 mmol ml^{-1}. 0.25 equiv to amino groups) and the mixture was evaporated after standing at room temperature for 2 h. The residue was treated with TFA or HCl and analyzed by HPLC in a similar way as described by Takaya et al.[10] Separation of diastereomeric peptides was achieved with Cosmosil 5C_{18}-P (4.6×150 mm) at a flow rate of 1 ml min^{-1}. Elution times (min) of L-Leu-(X) were: (with 0.01 M potassium phosphate, pH 3.5) X=L-Lys, 16.5; D-Lys, 18.1; L-Orn, 8.5; D-Orn, 9.5; (with 0.01 M potassium phosphate, pH 3.5–CH_3CN=97:3) L-Ala, 5.5; D-Ala, 16.0. After a necessary correction for racemization during acid hydrolysis (estimated from free amino acids exposed to the same hydrolytic conditions), the degrees of racemization were estimated.

Chromatography. A Jasco Trirotar liquid chromatograph system was used for HPLC. For purification of peptides, Cosmosil 5C_{18}-P (10×250 mm, Nakarai Chem. Co.) or YMC-ODS 5 μm (20×250 mm, Yamamura Kagaku), for analytical purpose, Cosmosil 5C_{18}-P (4.6×150 mm), and for gel filtration, TSK G2000SWXL (7.5×300 mm, TOSO Co.) or G3000SW (7.5×600 mm) columns were used. Peptides were detected by UV absorption at 215 nm. For carboxymethylcellulose chromatography, the procedure described by Erickson and Merrifield[12] was followed.

Amino Acid Analysis. Peptide-resin and peptides were hydrolyzed at 130 °C for 12—24 h in concd HCl–propionic acid (1:1, v/v) or in TFA–concd HCl (9:1, v/v). In the latter case, a correction of amino acid recovery under the conditions was made: Ala, 98.4%; Phe, 96.4%; Orn, 75%; Lys, 97%, after 24 h. Amino acid analysis was carried out with a Jasco amino acid analyzer (single column operation, pH 5.25 ($[Na^+]$=0.2 M) and pH 5.0 ($[Na^+]$=1.6 M) buffers used).

Ultracentrifuge Analysis. Sedimentation equilibrium analysis was carried out with a Beckman Spinco E analytical ultracentrifuge, at 29500 rpm.

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