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Studies on Peptides. CXXIII.[1,2] Preparations of Nine Peptide Fragments for the Synthesis of Human Corticotropin Releasing Factor (hCRF)

NOBUTAKA FUJII, MOTOYOSHI NOMIZU, KENICHI AKAJI, MASANORI SHIMOKURA, SHINICHI KATAKURA and HARUAKI YAJIMA*

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606, Japan

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Nine peptide fragments were synthesized by known amide-forming reactions as building blocks for the solution synthesis of the hentetracontapeptide amide corresponding to the entire amino acid sequence of human corticotropin releasing factor (hCRF). Amino acid derivatives bearing protecting groups removable by 1 M trifluoromethanesulfonic acid–thioanisole in TFA were employed.

Keywords——human corticotropin releasing factor solution synthesis; rat corticotropin releasing factor; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; 2,2,2-trichloroethyloxycarbonyl hydrazine; mesitylene-2-sulfonyl arginine; methionine sulfoxide reduction

This is the first of two consecutive papers in which we wish to report the first solution synthesis of human corticotropin releasing factor (hCRF). Much research has been done on the structural elucidation of hCRF. As early as 1948, Green and Harris[3] proposed that the hypothalamus plays a key role in the control of the secretion of pituitary hormones. In 1955, Guillemin and Rosenberg[4] and Saffran and Schally[5] independently offered the first direct evidence for the presence in the hypothalamus of such a factor stimulating corticotropin (ACTH) secretion from the pituitary glands. Among various hormone-releasing factors predicted to be present in the hypothalamus, CRF thus became the subject of intensive chemical investigations in the field of neuroendocrinology. Several peptides isolated from hypothalamic extracts were proposed as candidates for CRF.[6] However, none fulfilled the criteria expected for physiological CRF.

Even after the structural elucidations of thyrotropin-releasing factor (TRH),[7] luteinizing hormone–releasing hormone (LH–RH)[8] and growth hormone release-inhibiting factor (somatostatin),[9] the chemical nature of CRF continued to elude investigators. In 1979, Montecucchi *et al.*[10] found that sauvagine, a 40-residue amidated peptide, isolated from the skin of South American frog, potently stimulates the release of ACTH and β-endorphin as well.

Further, in 1981, Vale *et al.*[11] succeeded in characterizing a 41-residue ovine hypothalamic peptide that stimulates the secretion of corticotropin and β-endorphin. Subsequently, a similar rat hypothalamic peptide, now called rCRF, was characterized by the same group of investigators.[12] Recently, two fish caudal neurosecretory peptides, sucker urotensin I[13] and carp urotensin I,[14] have been added to the list of the CRF-family peptides, because of their structural similarities.

In 1983, Furutani *et al.*[15] succeeded in sequencing complementary deoxyribonucleic acid (cDNA) of the oCRF precursor and their results supported the structure of oCRF deduced by

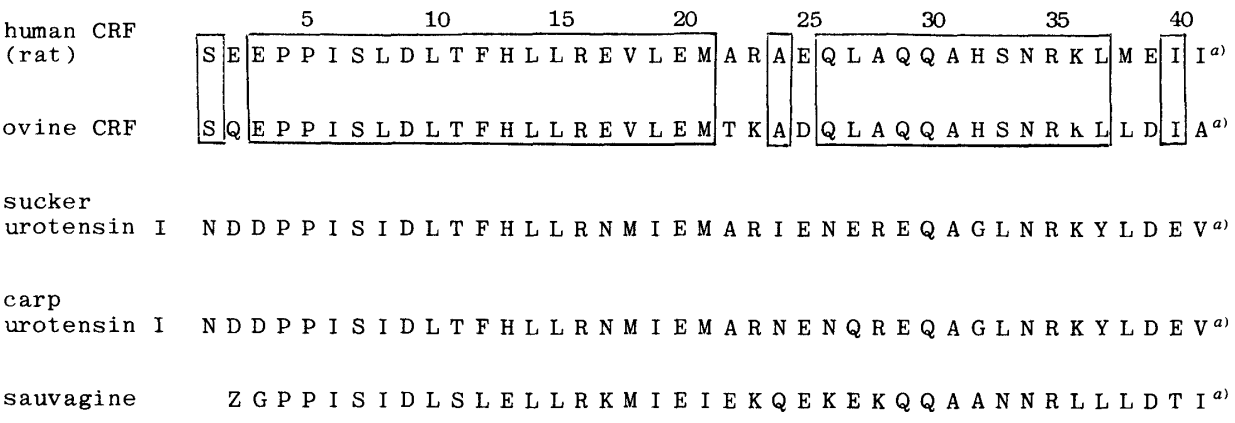

Fig. 1. Structure of CRF and Related Peptides

A = Ala, D = Asp, E = Glu, F = Phe, G = Gly, H = His, I = Ile, K = Lys, L = Leu, M = Met, N = Asn, Q = Gln, R = Arg, S = Ser, T = Thr, V = Val, Y = Tyr, Z = Pyr.
a) Amidated C terminus.

chemical sequence analysis. By means of a similar technique, the hCRF precursor gene was soon sequenced by the same group of investigators.[16] It was found that hCRF has seven amino acid substitutions in comparison with oCRF as shown in Fig. 1. Thus, the peptide structure of hCRF was convincingly determined prior to the isolation of hCRF from human hypothalamus. The structure of hCRF was found to be identical with that of rCRF.

We have synthesized the hentetracontapeptide amide corresponding to the entire amino acid sequence of hCRF deduced from the cDNA sequence by methods different from those employed for the syntheses of structurally related oCRF by other authors.[11,17] The deprotecting procedure with 1 M TFMSA–thioanisole in TFA that we employed in this synthesis has several advantageous features, as reported previously[18] and reviewed recently.[19]

Our synthetic route to hCRF is illustrated in Fig. 2, which shows the nine peptide fragments selected as building blocks to construct the entire peptide backbone of hCRF. In this paper, we wish to describe the syntheses of these fragments.

The Z(OMe) group,[20] removable by TFA, was adopted as a temporary protecting group for every intermediate, and amino acid derivatives bearing protecting groups removable by 1 M TFMSA–thioanisole in TFA were employed; *i.e.*, Lys(Z), Glu(OBzl), Asp(OBzl) and Arg(Mts).[21] In addition, we employed Ser(Bzl) in connection with our parallel synthesis of oCRF.

The Met residue was reversibly protected as its sulfoxide[22] in order to prevent partial S-alkylation during the N^{α}-TFA deprotection as well as partial air-oxidation during the synthesis. The substituted hydrazine, Troc-$NHNH_2$,[23] was employed for the preparation of four fragments containing the Glu(OBzl) or the Asp(OBzl) residue. This protecting group is known to be cleaved by Zn[24] or Cd[25] in the presence of AcOH without affecting other functional protecting groups.

The C-terminal tetrapeptide fragment, Z(OMe)–Met(O)–Glu(OBzl)–Ile–Ile–NH_2[1] was synthesized according to the scheme illustrated in Fig. 3. The mixed anhydride method[26] was preferentially employed for preparation of Z(OMe)–Ile–Ile–NH_2, since the usual DCC condensation[27] between Ile and Ile is known to give a sizable amount of the acyl-urea compound, as reported in our previous syntheses of bovine pancreatic trypsin inhibitor[28] and RNase A.[29] Z(OMe)–Ile–Ile–NH_2 was treated with TFA to remove the Z(OMe) group as usual, then Z(OMe)–Glu(OBzl)–OH was condensed with it by the Np method.[30] As a solvent, a mixture of DMF and DMSO had to be employed owing to the poor solubility of the amino

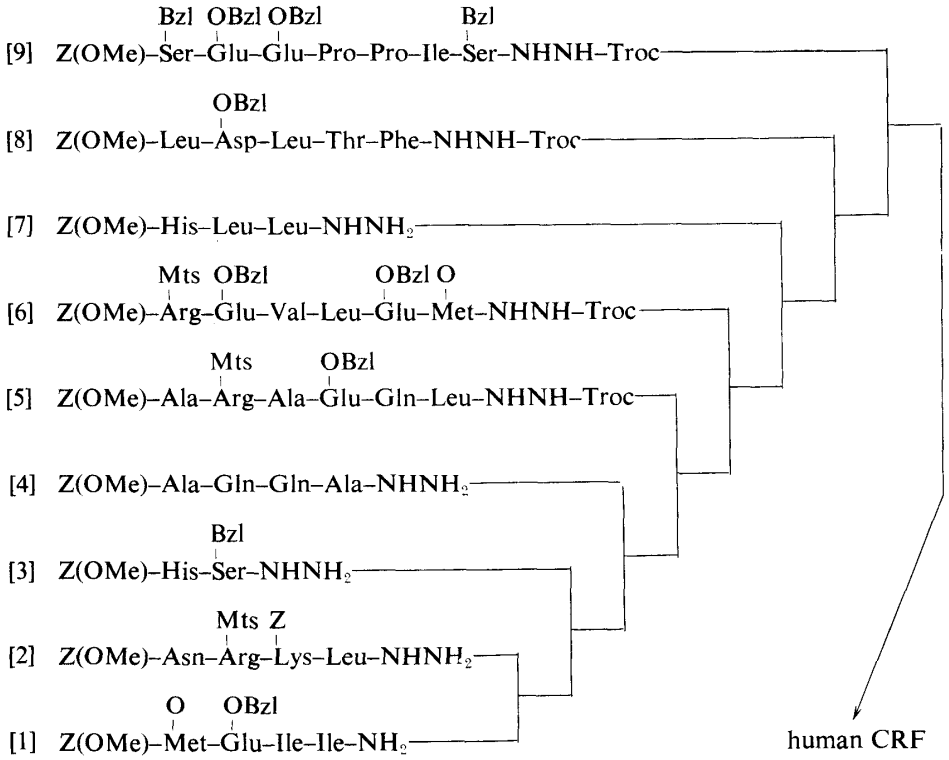

Fig. 2. Synthetic Route to Human CRF

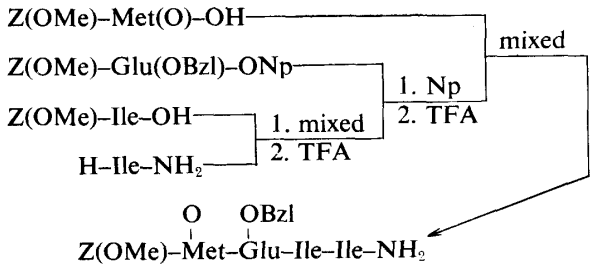

Fig. 3. Synthetic Scheme for the Protected Tetrapeptide Amide, Z(OMe)-(hCRF 38—41)-NH_2 [1]

component in DMF. The next mixed anhydride condensation of Z(OMe)–Met(O)–OH was also performed in the same solvent system and the purity of the product [1] was confirmed by thin layer chromatography (TLC) and amino acid analysis after 6 N HCl hydrolysis, as was also done with other fragments. In this instance, hydrolysis for 72 h was required to obtain a satisfactory recovery of Ile, because of steric hindrance.[28,29]

The next fragment, Z(OMe)–Asn–Arg(Mts)–Lys(Z)–Leu–$NHNH_2$ [2], was synthesized using the known tripeptide, Z(OMe)–Arg(Mts)–Lys(Z)–Leu–OMe.[31] Z(OMe)–Asn–OH was condensed with a TFA treated sample of the above tripeptide by the Np method, according to the scheme illustrated in Fig. 4. The resulting protected tetrapeptide ester was smoothly converted to the corresponding hydrazide [2] by the usual hydrazine treatment.

Fragment [3], Z(OMe)–His–Ser(Bzl)–$NHNH_2$, is a known peptide used for our previous synthesis of VIP (vasoactive intestinal polypeptide).[32] The tetrapeptide, Z(OMe)–Ala–Gln–Gln–Ala–$NHNH_2$ [4], was prepared in a stepwise manner starting with H–Ala–OMe as shown in Fig. 5. Two residues of Z(OMe)–Gln–OH were successively introduced onto H–Ala–OMe by the Np method as usual. The N-terminal Gln residue has a tendency to undergo cyclization to form a pyroglutaminyl residue[33] when exposed to amine during the fragment condensation reaction. Thus, this fragment was terminated by the mixed anhydride in-

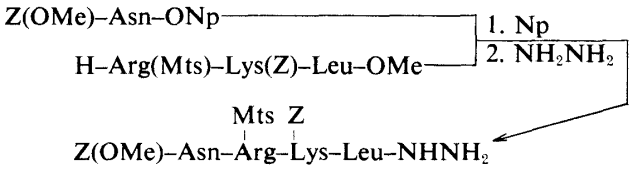

Fig. 4. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)-(hCRF 34—37)-$NHNH_2$ [2]

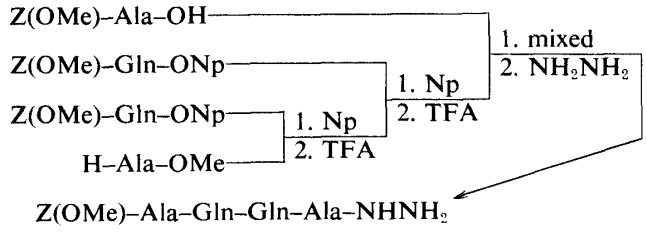

Fig. 5. Synthetic Scheme for the Protected Tetrapeptide Hydrazide, Z(OMe)-(hCRF 28—31)-$NHNH_2$ [4]

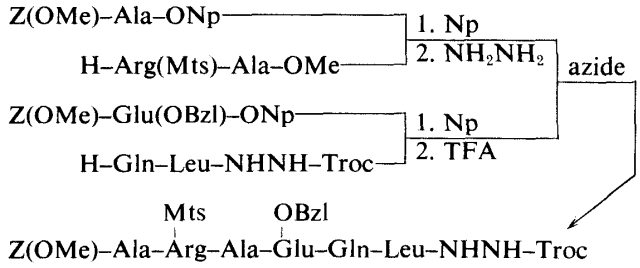

Fig. 6. Synthetic Scheme for the Protected Hexapeptide Troc-Hydrazide, Z(OMe)-(hCRF 22—27)-NHNH-Troc [5]

troduction of Z(OMe)-Ala-OH. The intermediates, H-Gln-Ala-OMe and H-Gln-Gln-Ala-OMe, were less soluble in DMF and therefore the acylations were performed with the aid of DMSO or HMPA. Conversion of Z(OMe)-Ala-Gln-Gln-Ala-OMe to [4] by hydrazine treatment was also conducted in DMF-HMPA.

Fragment [5], Z(OMe)-Ala-Arg(Mts)-Ala-Glu(OBzl)-Gln-Leu-NHNH-Troc, was prepared with the aid of Troc-$NHNH_2$. Two units, Z(OMe)-Ala-Arg(Mts)-Ala-$NHNH_2$ and Z(OMe)-Glu(OBzl)-Gln-Leu-NHNH-Troc, served to construct this peptide fragment as shown in Fig. 6. The former hydrazide was prepared by the Np condensation of Z(OMe)-Ala-OH with a TFA-treated sample of the known dipeptide, Z(OMe)-Arg(Mts)-Ala-OMe,[34] followed by the usual hydrazine treatment of the resulting protected tripeptide ester, Z(OMe)-Ala-Arg(Mts)-Ala-OMe. The latter substituted hydrazide derivative was prepared by the Np condensation of Z(OMe)-Glu(OBzl)-OH with a TFA-treated sample of the known dipeptide, Z(OMe)-Gln-Leu-NHNH-Troc.[35] The azide condensation[36] of these two fragments proceeded smoothly without particular difficulty.

Fragment [6] containing two Glu(OBzl) residues, Z(OMe)-Arg(Mts)-Glu(OBzl)-Val-Leu-Glu(OBzl)-Met(O)-NHNH-Troc, was also prepared with the aid of Troc-$NHNH_2$. This peptide backbone was constructed in a stepwise manner starting with H-Met(O)-NHNH-Troc[37] as shown in Fig. 7. The Np method was employed for condensation of two residues of Z(OMe)-Glu(OBzl)-OH and the mixed anhydride method for Z(OMe)-Leu-OH, Z(OMe)-Val-OH and Z(OMe)-Arg(Mts)-OH *in situ*. By this approach, the intermediates and [6] could be purified easily by simple recrystallization or precipitation without encountering any solubility problem.

Fragment [7], Z(OMe)-His-Leu-Leu-$NHNH_2$, was prepared easily by the azide condensation of Z(OMe)-His-$NHNH_2$ with a TFA-treated sample of the known dipeptide, Boc-Leu-Leu-OMe,[38] followed by the usual hydrazine treatment of the resulting tripeptide ester, Z(OMe)-His-Leu-Leu-OMe.

Initially, we attempted to elongate the hCRF peptide chain by using the following two fragments, Z(OMe)-Thr-Phe-His-Leu-Leu-$NHNH_2$ (positions 11—15) [A] and Z(OMe)-

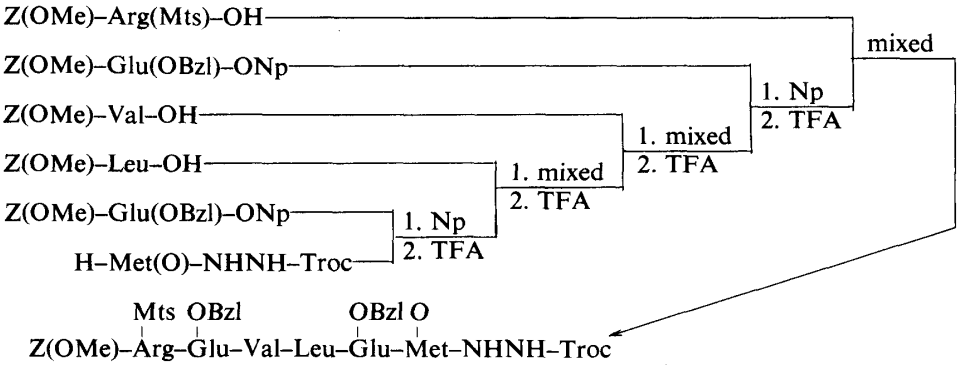

Fig. 7. Synthetic Scheme for the Protected Hexapeptide Troc-Hydrazide, Z(OMe)-(hCRF 16—21)-NHNH-Troc [6]

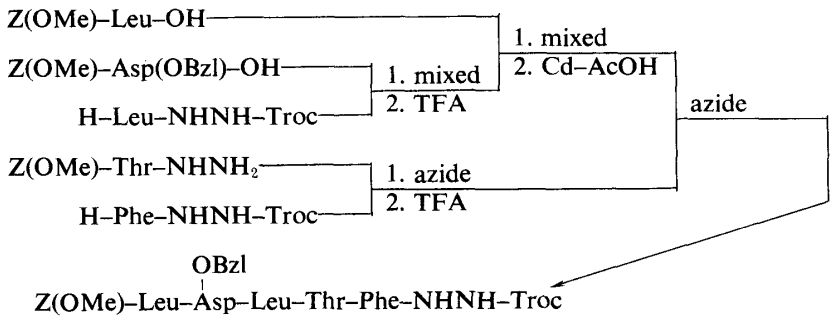

Fig. 8. Synthetic Scheme for the Protected Pentapeptide Troc-Hydrazide, Z(OMe)-(hCRF 8—12)-NHNH-Troc [8]

Leu-Asp(OBzl)-Leu-$NHNH_2$ (positions 8—10) [B]. The azide condensation of [A] with the amino component (hCRF 16—41) went smoothly. The acid hydrolysate of the product gave constituent amino acids in the ratios predicted by theory, but the acid hydrolysate of the azide condensation product of [B] (hCRF 8—41) gave a low recovery of Leu, despite a satisfactory recovery of Asp. Thus, the possibility cannot be excluded that the product may be contaminated with the urea-derivative[39] of Leu (position 10) formed during the azide condensation of [B]. We therefore decided to construct the peptide bond between Phe (12) and His (13), instead of between Leu (10) and Thr (11) as stated above.

Fragment [8], Z(OMe)-Leu-Asp(OBzl)-Leu-Thr-Phe-NHNH-Troc, which contains the Asp(OBzl) residue, was also prepared with the aid of Troc-$NHNH_2$. Two fragments served to prepare this peptide unit, *i.e.*, Z(OMe)-Leu-Asp(OBzl)-Leu-NHNH-Troc and Z(OMe)-Thr-Phe-NHNH-Troc, as shown in Fig. 8. Initially, we expected to use the former tripeptide derivative as one of the fragments for hCRF synthesis. However, for the reason stated above, we decided to join this fragment with the latter dipeptide unit. Removal of the Troc group from the former tripeptide derivative with Cd-AcOH required a much longer time than with Zn-AcOH, but a homogeneous hydrazide was easily obtained. The resulting hydrazide, Z(OMe)-Leu-Asp(OBzl)-Leu-$NHNH_2$ was then condensed, *via* the azide, with a TFA-treated sample of Z(OMe)-Thr-Phe-NHNH-Troc, which was prepared by means of the same azide reaction. Acid hydrolysis of [8] gave a satisfactory recovery of Leu.

In the N-terminal portion of hCRF, there are two Glu residues. Thus, fragment [9] was prepared starting with H-Ser(Bzl)-NHNH-Troc. Among various synthetic routes that we examined, a homogeneous peptide derivative, Z(OMe)-Glu(OBzl)-Pro-Pro-Ile-Ser(Bzl)-NHNH-Troc containing two Pro residues, could only be obtained by the stepwise active ester

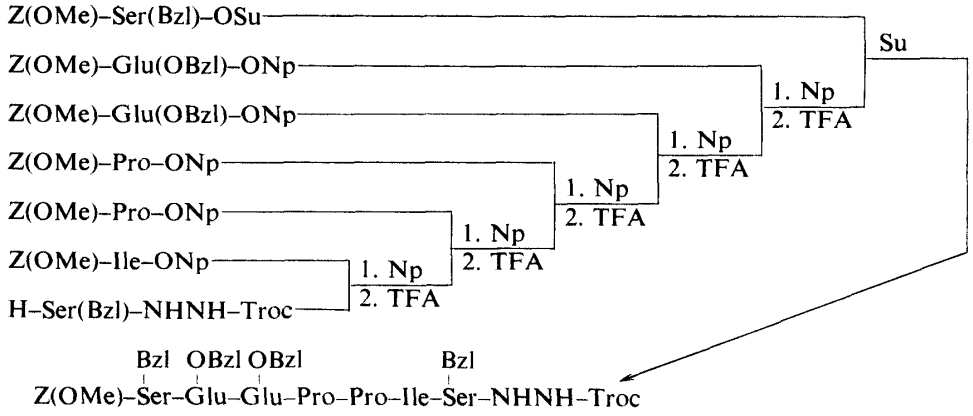

Fig. 9. Synthetic Scheme for the Protected Heptapeptide Troc-Hydrazide, Z(OMe)-(hCRF 1—7)-NHNH-Troc [9]

procedure. Thus the entire heptapeptide was obtained in a stepwise manner as shown in Fig. 9.

Nine peptide fragments were thus prepared, and were used to construct the entire peptide backbone of hCRF as will be reported in the following paper.

Experimental

General experimental procedures employed in this investigation are essentially the same as described in part 88[40] of this series.

N^{α}-Deprotection——The N^{α}-protecting group, Z(OMe) or Boc, was cleaved by TFA (*ca.* 10 ml per 1 g of a peptide) in the presence of anisole (2 mol eq or more) at ice-bath temperature for 60 min. After evaporation of TFA *in vacuo* at 30 °C or less, the residue was treated with dry ether. If a powder was obtained, it was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction. If an oily precipitate was obtained, it was washed with *n*-hexane, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction.

Condensation Reactions——The DCC and the active ester condensations were performed at room temperature (17—25 °C). The azide condensation was performed using isoamyl nitrite. A mixed anhydride was prepared using isobutyl chloroformate.

Purification——Unless otherwise mentioned, products were purified by one of the following procedures. Procedure A: For purification of protected peptide esters soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$, and H_2O–NaCl, dried over Na_2SO_4 and concentrated. The residue was crystallized or precipitated from appropriate solvents. Procedure B: For purification of protected peptides less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O and crystallized or precipitated from appropriate solvents. For purification of His-containing peptides, 3% AcOH was used for washing instead of 5% citric acid.

TLC was performed on silica gel (Kieselgel, G, Merck). *Rf* values refer to the following v/v solvent systems: Rf_1 $CHCl_3$: MeOH : H_2O (8 : 3 : 1); Rf_2 $CHCl_3$: MeOH (9 : 1); Rf_3 $CHCl_3$: MeOH (10 : 0.5); Rf_4 $CHCl_3$: MeOH : AcOH (9 : 1 : 0.5); Rf_5 *n*-BuOH : AcOH : AcOEt : H_2O (1 : 1 : 1 : 1).

Z(OMe)-Ile-Ile-NH_2——A mixed anhydride [prepared from 7.03 g (23.78 mmol) of Z(OMe)-Ile-OH] in DMF (50 ml) was added to an ice-chilled solution of H-Ile-NH_2[41] [prepared from 7.00 g (23.78 mmol) of Z(OMe)-derivative] in DMF (50 ml) and the mixture, after being stirred in an ice-bath for 3 h, was concentrated. The product was purified by procedure B, followed by washing with MeOH; yield 7.45 g (77%), mp 278—279 °C, $[\alpha]_D^{20}$ −5.0° (c = 0.2, DMSO), Rf_5 0, *Anal.* Calcd for $C_{21}H_{33}N_3O_5 \cdot 1/4H_2O$: C, 61.22; H, 8.20; N, 10.20. Found: C, 60.93; H, 8.25; N, 10.54.

Z(OMe)-Glu(OBzl)-Ile-Ile-NH_2——A TFA-treated sample of Z(OMe)-Ile-Ile-NH_2 (5.00 g, 12.27 mmol) was dissolved in DMF–DMSO (1 : 1, 50 ml) together with Et_3N (3.42 ml, 24.54 mmol) and Z(OMe)-Glu(OBzl)-ONp (7.05 g, 13.50 mmol). The mixture was stirred at room temperature for 13 h and H_2O (150 ml) was added. The resulting powder was purified by procedure B followed by precipitation from DMSO with EtOH; yield 7.10 g (92%), mp 258—259 °C, $[\alpha]_D^{20}$ −20.7° (c = 1.0, DMSO), Rf_5 0.89, *Anal.* Calcd for $C_{33}H_{46}N_4O_8$: C, 63.24; H, 7.40; N, 8.94. Found: C, 63.47; H, 7.33; N, 9.14.

Z(OMe)-Met(O)-Glu(OBzl)-Ile-Ile-NH_2 [1], Z(OMe)-(hCRF 38—41)-NH_2——A TFA-treated sample of

Z(OMe)–Glu(OBzl)–Ile–Ile–NH_2 (3.00 g, 4.79 mmol) was dissolved in DMF–DMSO (1 : 1, 50 ml) containing Et_3N (0.67 ml, 4.79 mmol). A mixed anhydride [prepared from 1.74 g (5.27 mmol) of Z(OMe)–Met(O)–OH] in DMF (20 ml) was added to the above ice-chilled solution and the mixture, after being stirred in an ice-bath for 3 h, was poured into H_2O (150 ml). The resulting powder was purified by procedure B followed by precipitation from DMSO with EtOH; yield 3.21 g (87%), mp 261—262 °C, $[\alpha]_D^{20}$ −9.0° (c = 1.0, DMSO), Rf_1 0.65, *Anal.* Calcd for $C_{38}H_{55}N_5O_{10}S$: C, 58.97; H, 7.16; N, 9.05. Found: C, 58.39; H, 7.04; N, 8.96. Amino acid ratios in 6 N HCl hydrolysate (72 h): Glu 1.00, Ile 1.79, Met 0.75; Met(O) was not determined (recovery of Glu 99%).

Z(OMe)–Asn–Arg(Mts)–Lys(Z)–Leu–OMe——A TFA-treated sample of Z(OMe)–Arg(Mts)–Lys(Z)–Leu–OMe[31] (18.00 g, 19.78 mmol) was dissolved in DMF (150 ml), together with Et_3N (6.1 ml, 43.52 mmol) and Z(OMe)–Asn–ONp (9.91 g, 23.74 mmol). The solution, after being stirred overnight, was concentrated and the residue was purified by procedure B followed by precipitation from DMF with MeOH; yield 14.52 g (72%), mp 180—183 °C, $[\alpha]_D^{20}$ −10.1° (c = 1.1, DMF), Rf_1 0.60. *Anal.* Calcd for $C_{49}H_{69}N_9O_{13}S$: C, 57.46; H, 6.79; N, 12.31. Found: C, 57.20; H, 6.79; N, 12.49.

Z(OMe)–Asn–Arg(Mts)–Lys(Z)–Leu–$NHNH_2$ [2], Z(OMe)–(hCRF 34—37)–$NHNH_2$——Z(OMe)–Asn–Arg(Mts)–Lys(Z)–Leu–OMe (14.00 g, 13.67 mmol) in DMF (70 ml) was treated with 80% hydrazine hydrate (8.3 ml, 10 eq) overnight. After evaporation of the solvent, the residue was treated with H_2O to form a solid, which was precipitated from DMF with MeOH; yield 13.90 g (99%), mp 184 – 187 °C, $[\alpha]_D^{20}$ −8.0° (c = 1.0, DMF), Rf_1 0.50, Rf_2 0.13. *Anal.* Calcd for $C_{48}H_{69}N_{11}O_{12}S$: C, 56.29; H, 6.79; N, 15.04. Found: C, 56.07; H, 6.82; N, 14.79. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.02, Arg 1.00, Lys 0.89, Leu 1.11 (recovery of Arg 90%).

Z(OMe)–Gln–Ala–OMe——A mixture of Z(OMe)–Gln–ONp (15.00 g, 34.77 mmol) and H–Ala–OMe [prepared from 7.28 g (52.16 mmol) of the HCl salt] in DMF (150 ml) was stirred overnight. After evaporation of the solvent, the product was purified by procedure B followed by precipitation from DMSO with MeOH; yield 10.62 g (77%), mp 184—187 °C, $[\alpha]_D^{20}$ −6.1° (c = 1.0, DMSO), Rf_1 0.70, Rf_2 0.35. *Anal.* Calcd for $C_{18}H_{25}N_3O_7$: C, 54.67; H, 6.37; N, 10.63. Found: C, 54.51; H, 6.36; N, 10.40.

Z(OMe)–Gln–Gln–Ala–OMe——A TFA-treated sample of Z(OMe)–Gln–Ala–OMe (7.40 g, 18.72 mmol) was dissolved in DMF–DMSO (2 : 1, 150 ml) together with Et_3N (5.5 ml, 39.31 mmol) and Z(OMe)–Gln–ONp (8.88 g, 20.59 mmol). After being stirred overnight, the solution was concentrated and the product was purified by procedure B followed by precipitation from DMSO with MeOH; yield 8.08 g (83%), mp 243—246 °C, $[\alpha]_D^{20}$ −5.0° (c = 1.0, DMSO), Rf_1 0.46. *Anal.* Calcd for $C_{23}H_{33}N_5O_9$: C, 52.76; H, 6.35; N, 13.38. Found: C, 52.58; H, 6.27; N, 13.21.

Z(OMe)–Ala–Gln–Gln–Ala–OMe——A TFA-treated sample of Z(OMe)–Gln–Gln–Ala–OMe (6.20 g, 11.84 mmol) was dissolved in DMF–DMSO (1 : 1, 100 ml) containing Et_3N (3.3 ml, 23.68 mmol). A mixed anhydride [prepared from 4.50 g (17.76 mmol) of Z(OMe)–Ala–OH] in THF (70 ml) was added to the above ice-chilled solution and the mixture, after being stirred in an ice-bath for 5 h, was concentrated. The product was purified by procedure B followed by precipitation from DMSO with MeOH; yield 6.73 g (90%), mp 264—266 °C, $[\alpha]_D^{20}$ −10.7° (c = 1.0, DMSO), Rf_1 0.42. *Anal.* Calcd for $C_{26}H_{38}N_6O_{10}$: C, 52.51; H, 6.44; N, 14.14. Found: C, 52.30; H, 6.39; N, 13.98.

Z(OMe)–Ala–Gln–Gln–Ala–$NHNH_2$ [4], Z(OMe)–(hCRF 28—31)–$NHNH_2$——Z(OMe)–Ala–Gln–Gln–Ala–OMe (6.00 g, 10.09 mmol) in HMPA–DMF (20 ml–60 ml) was treated with 80% hydrazine hydrate (6.1 ml, 10 eq) overnight. MeOH (200 ml) was added to form a gelatinous solid, which was collected by filtration and precipitated from DMSO with MeOH; yield 5.58 g (93%), mp 254—257 °C, $[\alpha]_D^{20}$ −11.6° (c = 0.5, DMSO), Rf_1 0.08, Rf_5 0.48. *Anal.* Calcd for $C_{25}H_{38}N_8O_9 \cdot 1/2H_2O$: C, 49.74; H, 6.51; N, 18.57. Found: C, 49.94; H, 6.47; N, 18.65. Amino acid ratios in 6 N HCl hydrolysate: Ala 2.00, Glu 1.97 (recovery of Ala 93%).

Z(OMe)–Glu(OBzl)–Gln–Leu–NHNH–Troc——A TFA-treated sample of Z(OMe)–Gln–Leu–NHNH–Troc[35] (5.40 g, 8.81 mmol) was dissolved in DMF (80 ml) together with Et_3N (2.5 ml, 17.62 mmol) and Z(OMe)–Glu(OBzl)–ONp (5.98 g, 11.45 mmol). The mixture, after being stirred overnight, was concentrated and the product was purified by procedure B followed by precipitation from DMF with EtOH; yield 6.12 g (84%), mp 178—179 °C, $[\alpha]_D^{23}$ +11.1° (c = 1.0, DMF), Rf_1 0.68. *Anal.* Calcd for $C_{35}H_{45}Cl_3N_6O_{11}$: C, 50.52; H, 5.45; N, 10.10. Found: C, 50.95; H, 5.53; N, 10.17.

Z(OMe)–Ala–Arg(Mts)–Ala–OMe——A TFA-treated sample of Z(OMe)–Arg(Mts)–Ala–OMe[34] (11.50 g, 18.99 mmol) was dissolved in DMF (70 ml) together with Et_3N (5.3 ml, 37.98 mmol) and Z(OMe)–Ala–ONp (7.82 g, 20.89 mmol). The mixture, after being stirred overnight, was concentrated and the oily product was purified by procedure A; yield 11.32 g (88%), Rf_1 0.67.

Z(OMe)–Ala–Arg(Mts)–Ala–$NHNH_2$——The above protected tripeptide ester (11.00 g, 16.25 mmol), dissolved in MeOH (100 ml), was treated with 80% hydrazine hydrate (4.9 ml, 5 eq) overnight. The solvent was removed by evaporation and the residue was treated with H_2O to afford a powder, which was recrystallized from MeOH and ether; yield 10.28 g (92%), mp 133—134 °C, $[\alpha]_D^{23}$ +22.7° (c = 1.0, DMF), Rf_1 0.62. *Anal.* Calcd for $C_{30}H_{44}N_8O_8S \cdot 1/2H_2O$: C, 52.54; H, 6.61; N, 16.34. Found: C, 52.55; H, 6.81; N, 16.22.

Z(OMe)–Ala–Arg(Mts)–Ala–Glu(OBzl)–Gln–Leu–NHNH–Troc [5], Z(OMe)–(hCRF 22—27)–NHNH–Troc——A TFA-treated sample of Z(OMe)–Glu(OBzl)–Gln–Leu–NHNH–Troc (2.5 g, 3.00 mmol) was dissolved in DMF (30 ml) containing Et_3N (0.42 ml, 3.00 mmol). The azide [prepared from 2.45 g (3.60 mmol) of Z(OMe)–Ala–Arg(Mts)–Ala–$NHNH_2$] in DMF (10 ml) was added to the above ice-chilled solution and the mixture, after being

stirred at 4 °C for 14 h, was neutralized with AcOH and concentrated. The product was purified by procedure B followed by precipitation from DMF with EtOH; yield 3.04 g (77%), mp 209—211 °C, $[\alpha]_D^{20}$ −13.0° (c = 1.0, DMF), Rf_1 0.65, Rf_4 0.23. *Anal.* Calcd for $C_{56}H_{77}Cl_3N_{12}O_{16}S$: C, 51.23; H, 5.91; N, 12.81. Found: C, 51.12; H, 6.02; N, 13.04.

Z(OMe)–Glu(OBzl)–Met(O)–NHNH–Troc——A TFA-treated sample of Z(OMe)–Met(O)–NHNH–Troc[37] (15.00 g, 28.91 mmol) was dissolved in DMF (80 ml) together with Et_3N (8.9 ml, 63.60 mmol) and Z(OMe)–Glu(OBzl)–ONp (18.13 g, 34.69 mmol). The mixture, after being stirred for 15 h, was concentrated and the product was purified by procedure A followed by recrystallization from MeOH and ether; yield 15.50 g (73%), mp 138—139 °C, $[\alpha]_D^{20}$ +7.0° (c = 1.0, DMF), Rf_2 0.46. *Anal.* Calcd for $C_{29}H_{35}Cl_3N_4O_{10}S$: C, 47.19; H, 4.78; N, 7.59. Found: C, 47.28; H, 4.66; N, 7.70.

Z(OMe)–Leu–Glu(OBzl)–Met(O)–NHNH–Troc——A TFA-treated sample of Z(OMe)–Glu(OBzl)–Met(O)–NHNH–Troc (11.00 g, 14.90 mmol) was dissolved in DMF (80 ml) containing Et_3N (2.1 ml, 14.90 mmol). A mixed anhydride [prepared from 10.65 g (22.35 mmol) of Z(OMe)–Leu–OH·DCHA] in THF (100 ml) was added to the above ice-chilled solution and the mixture, after being stirred in an ice-bath for 4 h, was concentrated. The product was purified by procedure A, followed by recrystallization from MeOH and ether; yield 9.56 g (75%), mp 127—128 °C, $[\alpha]_D^{20}$ −22.9° (c = 1.0, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{35}H_{46}Cl_3N_5O_{11}S$: C, 49.38; H, 5.45; N, 8.23. Found: C, 49.41; H, 5.37; N, 8.25.

Z(OMe)–Val–Leu–Glu(OBzl)–Met(O)–NHNH–Troc——A TFA-treated sample of Z(OMe)–Leu–Glu(OBzl)–Met(O)–NHNH–Troc (9.50 g, 11.16 mmol) was dissolved in DMF (30 ml) containing Et_3N (1.5 ml, 11.16 mmol). A mixed anhydride [prepared from 3.77 g (13.39 mmol) of Z(OMe)–Val–OH] in THF (100 ml) was added to the above ice-chilled solution and the mixture, after being stirred in an ice-bath for 4 h, was concentrated. The product was purified by procedure B followed by precipitation from DMF with AcOEt; yield 6.70 g (63%), mp 194—195 °C, $[\alpha]_D^{20}$ −27.0° (c = 1.0, DMF), Rf_1 0.71. *Anal.* Calcd for $C_{52}H_{68}Cl_3N_7O_{15}S$: C, 50.55; H, 5.83; N, 8.84. Found: C, 50.71; H, 5.92; N, 9.14.

Z(OMe)–Glu(OBzl)–Val–Leu–Glu(OBzl)–Met(O)–NHNH–Troc——A TFA-treated sample of the above protected tetrapeptide (4.00 g, 4.21 mmol) was dissolved in DMF (30 ml) together with Et_3N (1.3 ml, 9.26 mmol) and Z(OMe)–Glu(OBzl)–ONp (2.64 g, 5.05 mmol) and the mixture, after being stirred for 15 h, was concentrated. The product was purified by procedure B followed by precipitation from DMF with MeOH; yield 3.60 g (73%), mp 194—195 °C, $[\alpha]_D^{20}$ −27.0° (c = 1.0, DMF), Rf_1 0.72. *Anal.* Calcd for $C_{52}H_{68}Cl_3N_7O_{15}S$: C, 53.40; H, 5.86; N, 8.38. Found: C, 53.65; H, 6.01; N, 8.65.

Z(OMe)–Arg(Mts)–Glu(OBzl)–Val–Leu–Glu(OBzl)–Met(O)–NHNH–Troc [6], Z(OMe)–(hCRF 16—21)–NHNH–Troc——A TFA-treated sample of the above protected pentapeptide (3.00 g, 2.57 mmol) was dissolved in DMF (20 ml) containing Et_3N (0.36 ml, 2.57 mmol). A mixed anhydride [prepared from 1.86 g (3.08 mmol) of Z(OMe)–Arg(Mts)–OH·CHA] in THF (30 ml) was added and the mixture, after being stirred in an ice-bath for 4 h, was concentrated. The product was purified by procedure B followed by precipitation from DMF with MeOH; yield 2.45 g (63%), mp 222—223 °C, $[\alpha]_D^{20}$ −9.8° (c = 1.0, DMF), Rf_1 0.66. *Anal.* Calcd for $C_{67}H_{90}Cl_3N_{11}O_{18}S_2$: C, 53.48; H, 6.33; N, 10.25. Found: C, 53.50; H, 6.08; N, 10.32.

Z(OMe)–His–Leu–Leu–OMe——A TFA-treated sample of Boc–Leu–Leu–OMe[38] (11.00 g, 30.69 mmol) was dissolved in DMF (100 ml) containing Et_3N (4.3 ml, 30.69 mmol). The azide [prepared from 11.25 g (33.76 mmol) of Z(OMe)–His–$NHNH_2$] in DMF (60 ml) and Et_3N (4.7 ml, 33.76 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C overnight, was concentrated. Treatment of the residue with 5% $NaHCO_3$ afforded a powder, which was purified by procedure B followed by recrystallization from MeOH and ether; yield 13.07 g (76%), mp 164—167 °C, $[\alpha]_D^{20}$ −41.7° (c = 1.0, MeOH), Rf_1 0.66. *Anal.* Calcd for $C_{28}H_{41}N_5O_7$: C, 60.09; H, 7.38; N, 12.52. Found: C, 59.82; H, 7.35; N, 12.48.

Z(OMe)–His–Leu–Leu–$NHNH_2$ [7], Z(OMe)–(hCRF 13—15)–$NHNH_2$——Z(OMe)–His–Leu–Leu–OMe (2.00 g, 3.57 mmol) in MeOH (20 ml) was treated with 80% hydrazine hydrate (2.2 ml, 10 eq) for 12 h and the solvent was removed by evaporation. Treatment of the residue with H_2O afforded a powder, which was precipitated from DMF with AcOEt; yield 1.56 g (78%), mp 177—178 °C, $[\alpha]_D^{20}$ −18.3° (c = 1.0, DMF), Rf_1 0.67. *Anal.* Calcd for $C_{27}H_{41}N_7O_6 \cdot 1/2H_2O$: C, 57.02; H, 7.44; N, 17.24. Found: C, 57.07; H, 7.32; N, 17.33. Amino acid ratios in 6 N HCl hydrolysate: Leu 2.00, His 0.96 (recovery of Leu 95%).

Z(OMe)–Phe–NHNH–Troc——DCC (15.03 g, 72.84 mmol) was added to a solution of Z(OMe)–Phe–OH (20.00 g, 60.72 mmol) and Troc–$NHNH_2$ (15.11 g, 72.84 mmol) in THF (200 ml) and the mixture, after being stirred overnight, was filtered. The filtrate was concentrated and the product was purified by procedure A followed by recrystallization from AcOEt and ether; yield 23.06 g (73%), mp 134—136 °C, $[\alpha]_D^{15}$ −18.3° (c = 0.9, DMF), Rf_1 0.93. *Anal.* Calcd for $C_{21}H_{22}Cl_3N_3O_6$: C, 48.62; H, 4.28; N, 8.10. Found: C, 48.90; H, 4.53; N, 8.15.

Z(OMe)–Thr–Phe–NHNH–Troc——The azide [prepared from 2.87 g (9.64 mmol) of Z(OMe)–Thr–$NHNH_2$] in DMF (30 ml) and Et_3N (1.6 ml, 11.57 mmol) were added to an ice-chilled solution of H–Phe–NHNH–Troc [prepared from 5.00 g (9.64 mmol) of Z(OMe)–Phe–NHNH–Troc] in DMF (30 ml) and the mixture, after being stirred at 4 °C for 15 h, was concentrated. The product was purified by procedure A followed by recrystallization from AcOEt and isopropyl ether; yield 4.60 g (77%), mp 69—71 °C $[\alpha]_D^{20}$ +1.0° (c = 1.0, DMF), Rf_3 0.30. *Anal.* Calcd for

$C_{25}H_{29}Cl_3N_4O_8$: C, 48.44; H, 4.72; N, 9.04. Found: C, 48.76; H, 4.85; N, 8.96.

Z(OMe)–Asp(OBzl)–Leu–NHNH–Troc——A mixed anhydride [prepared from 14.40 g (37.17 mmol) of Z(OMe)–Asp(OBzl)–OH] in THF (100 ml) was added to an ice-chilled solution of H–Leu–NHNH–Troc [prepared from 15.00 g (30.94 mmol) of the Z(OMe) derivative][35] in DMF (60 ml) and the mixture, after being stirred in an ice-bath for 4 h, was concentrated. The product was purified by procedure A followed by recrystallization from AcOEt and ether; yield 13.50 g (70%), mp 130—132 °C, $[\alpha]_D^{20}$ −9.0° (c = 1.0, DMF), Rf_3 0.72. *Anal.* Calcd for $C_{29}H_{35}Cl_3N_4O_8$: C, 50.48; H, 5.11; N, 8.12. Found: C, 50.55; H, 5.17; N, 8.10.

Z(OMe)–Leu–Asp(OBzl)–Leu–NHNH–Troc——A TFA-treated sample of Z(OMe)–Asp(OBzl)–Leu–NHNH–Troc (14.00 g, 20.29 mmol) was dissolved in DMF (60 ml) containing Et_3N (2.8 ml, 20.29 mmol). A mixed anhydride [prepared from 11.61 g (24.35 mmol) of Z(OMe)–Leu–OH·DCHA] in THF (100 ml) was added to the above ice-chilled solution and the mixture, after being stirred in an ice-bath for 5 h, was concentrated. The product was purified by procedure A followed by recrystallization from MeOH and ether; yield 11.30 g (69%), mp 87—88 °C, $[\alpha]_D^{20}$ −25.6° (c = 1.0, DMF), Rf_3 0,65. *Anal.* Calcd for $C_{35}H_{46}Cl_3N_5O_{10}$: C, 52.34; H, 5.77; N, 8.72. Found: C, 52.40; H, 5.77; N, 8.80.

Z(OMe)–Leu–Asp(OBzl)–Leu–NHNH$_2$——The above Troc-derivative (4.00 g, 4.98 mmol) in DMF–MeOH (1 : 1, 40 ml) was treated with Cd powder (4.47 g, 8 eq) in the presence of AcOH (5.70 ml) for 14 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 2% EDTA. The resulting powder was washed with 5% $NaHCO_3$ and H_2O, and precipitated from DMF with ether; yield 2.50 g (80%), mp 184—185 °C, $[\alpha]_D^{20}$ −27.7° (c = 1.0, DMF), Rf_3 0.55. *Anal.* Calcd for $C_{32}H_{45}N_5O_8 \cdot 1/2H_2O$: C, 60.36; H, 7.28; N, 11.00. Found: C, 60.31; H, 7.16; N, 11.04.

Z(OMe)–Leu–Asp(OBzl)–Leu–Thr–Phe–NHNH–Troc [8], Z(OMe)–(hCRF 8—12)–NHNH–Troc——Z(OMe)–Thr–Phe–NHNH–Troc (3.50 g, 5.65 mmol), after TFA treatment, was dissolved in DMF (20 ml) containing Et_3N (0.79 ml, 5.65 mmol). The azide [prepared from 3.55 g (5.65 mmol) of Z(OMe)–Leu–Asp(OBzl)–Leu–NHNH$_2$] in DMF (30 ml) and Et_3N (0.94 ml, 6.78 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 40 h, was concentrated. The product was purified by procedure B followed by recrystallization from DMF and ether; yield 2.85 g (48%), mp 179—181 °C, $[\alpha]_D^{20}$ −16.8° (c = 1.0, DMF), Rf_2 0.56. *Anal.* Calcd for $C_{48}H_{62}Cl_3N_7O_{13}$: C, 54.83; H, 5.94; N, 9.33. Found: C, 54.57; H, 6.09; N, 9.40.

Z(OMe)–Ser(Bzl)–NHNH–Troc——A mixed anhydride [prepared from 15.00 g (32.71 mmol) of Z(OMe)–Ser(Bzl)–OH·CHA] in THF (100 ml) was added to an ice-chilled solution of Troc–NHNH$_2$ (7.43 g, 35.81 mmol) in THF (50 ml) and the mixture, after being stirred in an ice-bath for 5 h, was concentrated. The product was purified by procedure A followed by recrystallization from MeOH and ether; yield 13.50 g (75%), mp 91—92 °C, $[\alpha]_D^{15}$ −3.5° (c = 0.6, MeOH), Rf_3 0.76. *Anal.* Calcd for $C_{22}H_{24}Cl_3N_3O_7$: C, 48.14; H, 4.41; N, 7.66. Found: C, 48.06; H, 4.23; N, 7.75.

Z(OMe)–Ile–Ser(Bzl)–NHNH–Troc——A TFA-treated sample of Z(OMe)–Ser(Bzl)–NHNH–Troc (9.45 g, 17.22 mmol) was dissolved in DMF (70 ml) together with Et_3N (5.4 ml, 38.74 mmol) and Z(OMe)–Ile–ONp (7.89 g, 18.95 mmol) and the mixture, after being stirred for 12 h, was concentrated. The product was purified by procedure A followed by recrystallization from DMF and ether; yield 9.28 g (81%), mp 124—125 °C, $[\alpha]_D^{20}$ +2.98° (c = 1.0, DMF), Rf_3 0.82. *Anal.* Calcd for $C_{28}H_{35}Cl_3N_4O_8 \cdot 1/2H_2O$: C, 50.12; H, 5.41; N, 8.35. Found: C, 50.20; H, 5.55; N, 8.60.

Z(OMe)–Pro–Ile–Ser(Bzl)–NHNH–Troc——A TFA-treated sample of Z(OMe)–Ile–Ser(Bzl)–NHNH–Troc (9.02 g, 13.63 mmol) was dissolved in DMF (100 ml) together with Et_3N (4.8 ml, 34.08 mmol), HOBT (0.92 g, 6.82 mmol) and Z(OMe)–Pro–ONp (8.19 g, 20.45 mmol) and the mixture, after being stirred overnight, was concentrated. The product was purified by procedure A followed by precipitation from DMF with ether; yield 8.05 g (78%), mp 188—189 °C, $[\alpha]_D^{20}$ −29.4° (c = 1.0, DMF), Rf_3 0.43. *Anal.* Calcd for $C_{33}H_{42}Cl_3N_5O_9$: C, 52.21; H, 5.58; N, 9.23. Found: C, 52.28; H, 5.87; N, 9.20.

Z(OMe)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc——A TFA-treated sample of Z(OMe)–Pro–Ile–Ser(Bzl)–NHNH–Troc (7.90 g, 10.41 mmol) was dissolved in DMF (80 ml) together with Et_3N (3.7 ml, 26.03 mmol), HOBT (0.70 g, 5.21 mmol) and Z(OMe)–Pro–ONp (6.25 g, 15.61 mmol). The mixture, after being stirred for 12 h, was concentrated and the product was purified by procedure A followed by recrystallization from MeOH and ether; yield 7.76 g (87%), mp 127—128 °C, $[\alpha]_D^{20}$ −39.2° (c = 1.0, DMF), Rf_3 0.35. *Anal.* Calcd for $C_{38}H_{49}Cl_3N_6O_{10}$: C, 53.30; H, 5.77; N, 9.82. Found: C, 53.56; H, 5.95; N, 9.93.

Z(OMe)–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc——A TFA-treated sample of the above protected tetrapeptide (7.66 g, 8.95 mmol) was dissolved in DMF (70 ml) together with Et_3N (2.3 ml, 18.80 mmol), HOBT (0.60 g, 4.48 mmol) and Z(OMe)–Glu(OBzl)–ONp (5.14 g, 9.85 mmol). The mixture, after being stirred at 4 °C for 12 h, was concentrated and the product was purified by procedure A followed by column chromatography on silica gel (4.3 × 17 cm) using $CHCl_3$–MeOH (10 : 0.5) as an eluant. The product was finally recrystallized from MeOH and ether; yield 5.02 g (52%), mp 107—108 °C, $[\alpha]_D^{20}$ −56.9° (c = 1.0, DMF), Rf_3 0.40. *Anal.* Calcd for $C_{50}H_{62}Cl_3N_7O_{13} \cdot H_2O$: C, 54.92; H, 5.90; N, 8.97. Found: C, 54.96; H, 5.73; N, 9.42.

Z(OMe)–Glu(OBzl)–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc——A TFA-treated sample of the above protected pentapeptide (4.88 g, 4.54 mmol) was dissolved in DMF (50 ml) together with Et_3N (0.64 ml, 4.54 mmol), HOBT (0.31 g, 2.27 mmol) and Z(OMe)–Glu(OBzl)–ONp (2.49 g, 4.77 mmol). After addition of NMM (0.60 ml,

5.90 mmol), the mixture was stirred at 4 °C overnight and concentrated. The product was purified by procedure A followed by column chromatography on silica gel (4.3 × 12 cm) using $CHCl_3$–MeOH (10:0.5) as an eluant. The product was finally recrystallized from AcOEt and isopropyl ether; yield 3.00 g (51%), mp 81—83 °C, $[\alpha]_D^{20}$ −35.9° (c = 1.0, DMF), Rf_3 0.42. *Anal.* Calcd for $C_{62}H_{75}Cl_3N_8O_{16}$: C, 57.51; H, 5.84; N, 8.66. Found: C, 57.27; H, 5.87; N, 8.49.

Z(OMe)–Ser(Bzl)–Glu(OBzl)–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc [1], Z(OMe)–(hCRF 1—7)–NHNH–Troc——A TFA-treated sample of the above protected hexapeptide (2.10 g, 1.62 mmol) was dissolved in DMF (30 ml) together with Et_3N (0.57 ml, 4.05 mmol), HOBT (0.11 g, 0.81 mmol) and Z(OMe)–Ser(Bzl)–OSu (1.11 g, 2.43 mmol) and the mixture, after being stirred at 4 °C overnight, was concentrated. The product was purified by procedure A followed by recrystallization from MeOH and ether; yield 1.80 g (75%), mp 96—98 °C, $[\alpha]_D^{20}$ −41.3° (c = 1.0, DMF), Rf_3 0.33. *Anal.* Calcd for $C_{72}H_{86}Cl_3N_9O_{18} \cdot 2H_2O$: C, 57.35; H, 6.02; N, 8.36. Found: C, 57.20; H, 5.80; N, 8.90.

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References and Notes

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