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Studies on Peptides. CXXIV.[1,2] Solution Synthesis of the Hentetracontapeptide Amide Corresponding to the Entire Amino Acid Sequence of Human Corticotropin Releasing Factor (hCRF)

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The hentetracontapeptide amide corresponding to the entire amino acid sequence of human corticotropin releasing factor (hCRF), deduced from the complementary deoxyribonucleic acid (cDNA) sequence analysis, was synthesized by assembling nine peptide fragments in solution followed by deprotection with 1 M trifluoromethanesulfonic acid–thioanisole (molar ratio 1 : 1) in trifluoroacetic acid. The synthetic peptide significantly elevated the immunoreactive corticotropin level in rat plasma.

Keywords——human corticotropin releasing factor (hCRF) solution synthesis; rat CRF; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; Curtius azide rearrangement; immunoreactive corticotropin release

As described in the preceding paper, Numa and his associates[3] in 1983 determined the structure of human corticotropin releasing factor (hCRF) by sequence analysis of cloned deoxyribonucleic acid (DNA) complementary to the messenger ribonucleic acid (mRNA) encoding the precursor. This hypothalamic principle was found to be identical with the releasing factor from rat.[4] Because of the great physiological importance of hCRF, we undertook the synthesis of the hentetracontapeptide amide corresponding to the entire amino acid sequence of hCRF, H–(hCRF 1—41)–NH_2, and reported in the preceding paper the synthesis of nine peptide fragments, [1]—[9], which were selected as the building blocks. We wish to report in this paper that we succeeded in obtaining the biologically active hentetracontapeptide amide by successive azide condensations[5] of the above nine peptide fragments followed by thioanisole-mediated deprotection[6] of all protecting groups employed with 1 M TFMSA in TFA.[7] Among the nine fragments selected, four fragments containing Glu(OBzl) or Asp(OBzl), [5], [6], [8], and [9], were synthesized with the aid of a substituted hydrazine, Troc–$NHNH_2$.[8] Thus, prior to fragment condensation, the Troc groups attached to these fragments were removed by the use of Zn[9] or Cd[10] in AcOH. Removal of the Troc group with Cd/AcOH required a slightly longer time than with Zn/AcOH, but gave in most instances more homogeneous hydrazides than the latter treatment, as reported.[10] Thus, purification of the hydrazides was relatively easy.

Necessary fragments thus obtained were then assembled successively onto the C-terminal fragment [1], *via* the azide as shown in Fig. 1. The amount of the acyl component was increased from 1.4 to 5 eq according to the progress of the chain elongation. The fragment

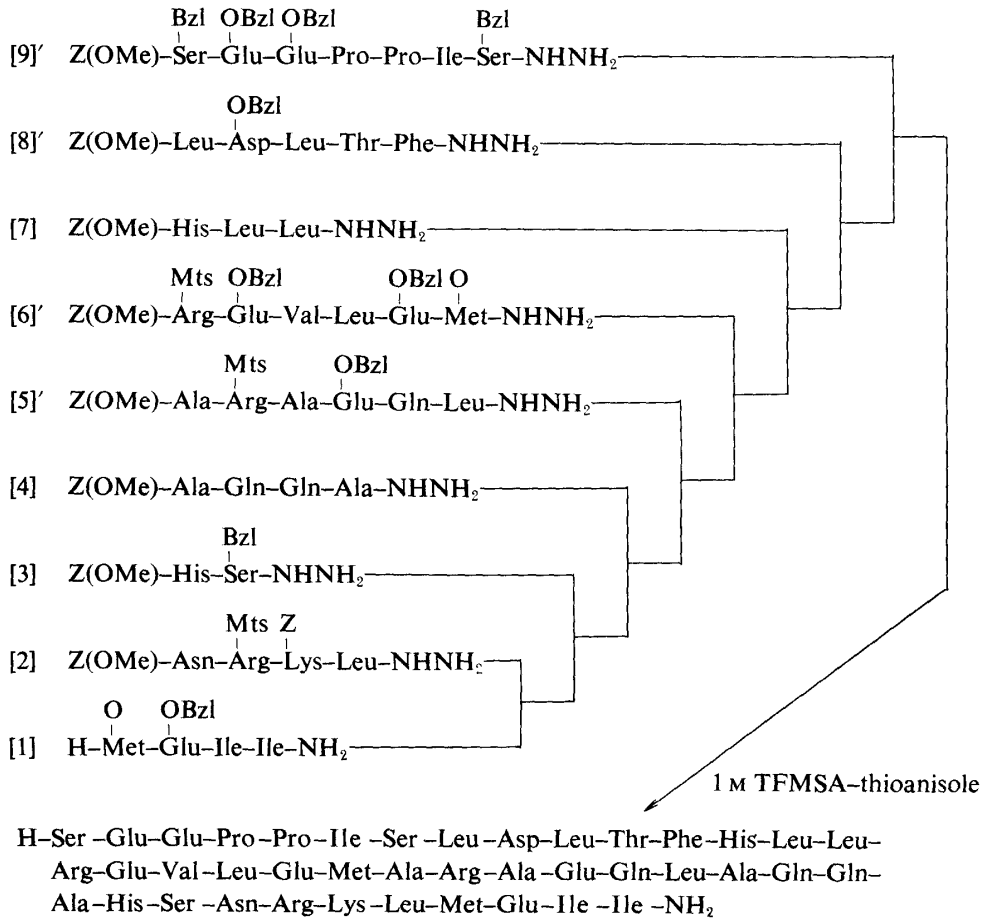

Fig. 1. Synthetic Route to Human Corticotropin Releasing Factor (hCRF)

condensation reactions from fragment [1] to [7] proceeded smoothly without encountering any particular problems. However, the subsequent azide condensations of fragment [8] and [9] had to be performed at lower temperature (−18 °C) than usual (4 °C), otherwise in both cases, the product gave a low recovery of the C-terminal amino acid of the employed acyl component in acid hydrolysis, due to the Curtius rearrangement followed by urea formation.[11] The results imply that the α-amino groups participating in the above two condensations are both sterically hindered. A recent circular dichroism (CD) spectral survey[12] indicated that structurally related ovine CRF exhibited predominantly helical character in trifluoroethanol, especially in its middle portion, while this was less marked in water. Whether protected hCRF intermediates exhibit similar properties in DMF is not known, but steric factors can not be excluded in such unusual azide reactions. Completion of the coupling reaction was checked by means of the ninhydrin test, and most products were purified by precipitation from DMSO or DMF with appropriate solvents, such as MeOH or AcOEt. In some instances, gel-filtration on Sephadex LH-60 was found to be effective for purifying the protected intermediates, including the protected hCRF. Throughout this synthesis, Asp was taken as a diagnostic amino acid in acid hydrolysis. By comparison of the recovery of Asp with those of newly incorporated amino acids, satisfactory incorporation of each fragment, after each condensation reaction, was ascertained as shown in Table I.

Next, deprotection and subsequent purification were carried out according to the following scheme (Fig. 2). The protected hentetracontapeptide amide, Z(OMe)-(hCRF 1—41)-NH_2, was treated with 1 M TFMSA–thioanisole (molar ratio 1 : 1) in TFA in the presence of *m*-cresol in an ice bath for 90 min. This reagent system has the ability to cleave the Mts[13]

TABLE I. Amino Acid Ratios in a 6 N HCl (24 h) Hydrolysate of Synthetic hCRF and Its Intermediates

	Protected peptide								Synthetic hCRF	Residue
	34—41	32—41	28—41	22—41	16—41	13—41	8—41	1—41		
Asp	1.00	1.00	1.00	1.00	1.00	1.00	2.00	2.00	2.00	2
Thr							1.09	1.07	1.00	1
Ser		0.91	0.92	0.91	0.95	0.98	0.96	2.69	2.89	3
Glu	0.99	1.02	3.04	5.47	7.04	7.09	6.80	8.84	9.18	9
Pro								2.17	2.28	2
Ala			2.07	4.63	4.15	4.06	3.83	3.96	4.36	4
Val					0.96	1.02	0.91	0.90	0.98	1
Met[a]	0.74	0.77	0.73	0.76	1.47	1.55	1.31	1.48	1.71	2
Ile	1.79[b]	1.18	1.19	1.27	1.11	1.32	1.16	2.07	2.17	3
Leu	0.94	1.00	1.00	1.98	2.93	4.92	7.03	7.15	6.97	7
Phe							0.92	0.96	0.99	1
Lys	0.98	1.06	1.02	1.02	0.99	1.04	1.00	0.94	1.08	1
His		0.96	0.97	0.96	0.91	2.04	1.89	1.83	1.81	2
Arg	0.92	0.93	0.96	2.19	2.92	3.14	2.94	2.81	2.83	3
Recov. (%)	77	82	75	76	79	91	83	71	78	

a) Met(O) was not calculated. *b*) 72 h hydrolysis. Asp: diagnostic amino acid.

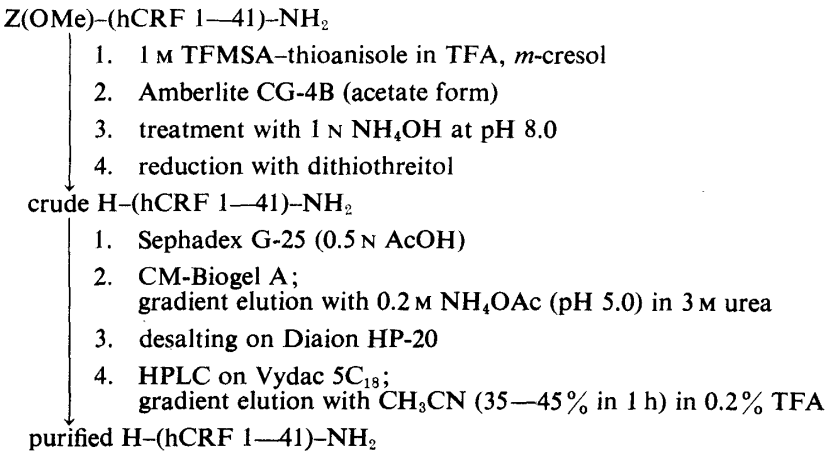

Fig. 2. Deprotection and Purification of H-(hCRF 1—41)-NH_2

group from the Arg residue within 60 min at 0 °C, as well as other protecting groups based on benzyl alcohol, without any marked side reaction.[14] After precipitation of the deprotected peptide with ether, this treatment was repeated twice more to ensure complete deprotection. The deprotected peptide was converted to the corresponding acetate by treatment with Amberlite CG-4B and then treated with dilute ammonia at pH 8.0 in order to reverse the possible N→O shift[15] at the Ser and Thr residues. As reported previously,[16] the thioanisole-mediated deprotection we employed has the ability to reduce the Met(O) residue. To ensure its complete reduction, the above treated peptide was incubated with dithiothreitol.[17] After removal of the reducing reagent by gel-filtration on Sephadex G-25, the product was purified by ion-exchange chromatography on CM-Biogel A, using gradient elution with pH 5.0, 0.2 M ammonium acetate buffer containing 3 M urea. Since CRF-related peptides show a tendency to self aggregate,[18] addition of urea is necessary to obtain a better recovery in this chromatographic purification step. When monitored by the Folin–Lowry test,[19] a front shoulder peak was detected besides the main peak. After high performance liquid chromatog-

raphy (HPLC) examination, this minor peak was found to be attributable to the di-Met(O) derivative of hCRF which contains two Met residues, since the main product was converted to this minor component by excess H_2O_2 treatment.

The main product, obtained after desalting on Diaion HP-20, gave a single spot on TLC, but showed small side peaks on HPLC. To remove these impurities, preparative reverse phase HPLC on a Vydac $5C_{18}$ column[20] was effective. The purity of the product as estimated by HPLC was more than 95%. The minor impurity (approximately 5%) was found to be the Met(O) derivatives. The product was so sensitive to air oxidation that rechromatography of the product obtained from the main peak gave a small side peak corresponding to the sulfoxide. The synthetic peptide with this degree of purity exhibited a single band on disk-isoelectrofocusing (Ampholine, pH 3—10), and its acid hydrolysis gave the amino acid ratios predicted by theory. Its purity was further confirmed by enzymic digestion.

When tested in an *in vivo* assay according to Rivier *et al.*,[21] our synthetic hCRF (1—10 μg/kg) significantly stimulated the secretion of immunoreactive corticotropin in rats.

Experimental

General experimental procedures employed in this investigation were essentially the same as described in the preceding paper.[1] Thin layer chromatography (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–AcOH (9:1:0.5), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24). HPLC was conducted with a Waters 204 compact model equipped with a Vydac $5C_{18}$ (4.6 × 250 mm) column.

Papain (Lot. 62F-8000) and leucine aminopeptidase (Lot. 102F-8160) were purchased from Sigma Chemical Co.

Z(OMe)–Ala–Arg(Mts)–Ala–Glu(OBzl)–Gln–Leu–NHNH$_2$ [5]′——Z(OMe)–Ala–Arg(Mts)–Ala–Glu(OBzl)–Gln–Leu–NHNH–Troc (3.00 g, 2.29 mmol) in a mixture of DMF–AcOH (30 ml–3.9 ml) was treated with Zn powder (3.00 g, 20 eq) at room temperature for 5 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 2% EDTA to form a powder, which was washed with 5% $NaHCO_3$ and H_2O. The product was precipitated from DMF with EtOH; yield 1.74 g (67%), mp 236—237 °C, $[\alpha]_D^{20}$ −66.2° (*c* = 1.0, DMF), Rf_1 0.69, Rf_2 0.05. Amino acid ratios in 6 N HCl hydrolysate: Glu 2.10, Ala 2.18, Leu 1.00, Arg 1.03 (recovery of Leu 89%). *Anal.* Calcd for $C_{53}H_{76}N_{12}O_{14}S \cdot 2H_2O$: C, 54.25; H, 6.87; N, 14.33. Found: C, 54.23; H, 6.78; N, 14.26.

Z(OMe)–Arg(Mts)–Glu(OBzl)–Val–Leu–Glu(OBzl)–Met(O)–NHNH$_2$ [6]′——Z(OMe)–Arg(Mts)–Glu(OBzl)–Val–Leu–Glu(OBzl)–Met(O)–NHNH–Troc (2.68 g, 1.77 mmol) dissolved in DMF–AcOH (10 ml–2 ml) was treated with Cd powder (1.99 g, 10 eq) at room temperature overnight. The solution was filtered, the filtrate was concentrated, and the residue was isolated as stated above then precipitated from DMF with MeOH; yield 1.99 g (84%), mp 233—234 °C, $[\alpha]_D^{20}$ −8.0° (*c* = 0.8, DMF), Rf_1 0.72, Rf_2 0.39. Amino acid ratios in 6 N HCl hydrolysate: Glu 2.13, Val 0.96, Met 0.94, Leu 1.00, Arg 1.03. (recovery of Leu 91%). *Anal.* Calcd for $C_{64}H_{91}N_{11}O_{16}S_2 \cdot H_2O$: C, 56.91; H, 6.79; N, 11.41. Found: C, 56.81; H, 6.70; N, 11.23.

Z(OMe)–Leu–Asp(OBzl)–Leu–Thr–Phe–NHNH$_2$ [8]′ ——Z(OMe)–Leu–Asp(OBzl)–Leu–Thr–Phe–NHNH–Troc (1.03 g, 0.98 mmol) in a mixture of DMF–AcOH (10 ml–2 ml) was treated with Cd powder (1.11 g, 10 eq) at room temperature for 48 h. The solution was filtered and the filtrate was concentrated. The residue was treated with 2% EDTA to form a powder, which was isolated as stated above and precipitated from DMSO with AcOEt; yield 0.68 g (79%), mp 268—269 °C, $[\alpha]_D^{15}$ −8.9° (*c* = 1.0, DMSO), Rf_1 0.74. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.06, Thr 1.03, Leu 2.13, Phe 1.00 (recovery of Phe 89%). *Anal.* Calcd for $C_{45}H_{61}N_7O_{11}$: C, 61.70; H, 7.02; N, 11.19. Found: C, 61.58; H, 7.06; N, 11.37.

Z(OMe)–Ser(Bzl)–Glu(OBzl)–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH$_2$ [9]′——Z(OMe)–Ser(Bzl)–Glu(OBzl)–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc (1.66 g, 1.13 mmol) in MeOH–AcOH (1:1, 20 ml) was treated with Zn powder (0.74 g, 10 eq) for 2 h. The solution was filtered and the filtrate was concentrated. The residue, after being treated with 2% EDTA and 5% $NaHCO_3$, was extracted with *n*-BuOH. The organic phase was washed with H_2O, dried over $MgSO_4$, and concentrated. Treatment of the residue with isopropyl ether afforded a powder, which was recrystallized from MeOH and ether; yield 1.39 g (95%), mp 99—100 °C, $[\alpha]_D^{20}$ −38.7° (*c* = 1.0, DMF), Rf_1 0.75. Amino acid ratios in 6 N HCl hydrolysate: Ser 1.71, Glu 2.03, Pro 2.15, Ile 1.00 (recovery of Ile 86%). *Anal.* Calcd for $C_{69}H_{85}N_9O_{16} \cdot 4H_2O$: C, 60.55; H, 6.85; N, 9.21. Found: C, 60.44; H, 6.67; N, 8.77.

Z(OMe)–Asn–Arg(Mts)–Lys(Z)–Leu–Met(O)–Glu(OBzl)–Ile–Ile–NH$_2$, Z(OMe)–(hCRF 34—41)–NH$_2$—— Z(OMe)–Met(O)–Glu(OBzl)–Ile–Ile–NH_2 [1] (4.52 g, 5.84 mmol) was treated with TFA–anisole (10 ml–3 ml) in an ice-bath for 60 min, then TFA was removed by evaporation and dry ether was added. The resulting powder was dried over KOH pellets *in vacuo* for 3 h and dissolved in DMSO–DMF (1:1, 40 ml) containing Et_3N (0.81 ml, 5.84 mmol).

The azide [prepared from 8.97 g (8.76 mmol) of Z(OMe)-Asn-Arg(Mts)-Lys(Z)-Leu-$NHNH_2$ [2]] in DMF (40 ml) and Et_3N (1.22 ml, 8.76 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 20 h. H_2O (200 ml) was added to the reaction mixture. The resulting powder was washed with 5% citric acid and H_2O and precipitated from DMSO with MeOH; yield 8.29 g (89%), mp 258—260 °C, $[\alpha]_D^{20}$ −14.1° (c = 1.0, DMSO), Rf_1 0.68. *Anal.* Calcd for $C_{77}H_{112}H_{14}O_{19}S_2 \cdot H_2O$: C, 57.09; H, 7.09; N, 12.11. Found: C, 56.89; H, 7.03; N, 12.14.

Z(OMe)-His-Ser(Bzl)-Asn-Arg(Mts)-Lys(Z)-Leu-Met(O)-Glu(OBzl)-Ile-Ile-NH_2, Z(OMe)-(hCRF 32—41)-NH_2——The above protected octapeptide amide (1.92 g, 1.20 mmol) was treated with TFA-anisole (5 ml–0.9 ml) and the N^{α}-deprotected peptide, isolated as stated above, was dissolved in DMSO-DMF (1 : 1, 20 ml) containing Et_3N (0.17 ml, 1.20 mmol). The azide [prepared from 0.92 g (1.80 mmol) of Z(OMe)-His-Ser(Bzl)-$NHNH_2$[22] [3]] in DMF (5 ml) and Et_3N (0.30 ml, 2.16 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 14 h, was diluted with H_2O (100 ml). The resulting powder was washed with H_2O and precipitated from DMSO with MeOH; yield 1.88 g (82%), mp 235—237 °C, $[\alpha]_D^{20}$ −14.8° (c = 1.0, DMSO), Rf_1 0.59. *Anal.* Calcd for $C_{93}H_{130}N_{18}O_{22}S_2 \cdot 3H_2O$: C, 56.69; H, 6.96; N, 12.80. Found: C, 56.39; H, 6.82; N, 12.55.

Z(OMe)-Ala-Gln-Gln-Ala-His-Ser(Bzl)-Asn-Arg(Mts)-Lys(Z)-Leu-Met(O)-Glu(OBzl)-Ile-Ile-NH_2, Z-(OMe)-(hCRF 28—41)-NH_2——The above protected decapeptide amide (1.88 g, 0.99 mmol) was treated with TFA-anisole (5 ml–0.5 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMSO-DMF (1 : 1, 20 ml) containing Et_3N (0.27 ml, 1.96 mmol). The azide [prepared from 0.84 g (1.41 mmol) of Z(OMe)-Ala-Gln-Gln-Ala-$NHNH_2$ [4]] in DMSO-DMF (20 ml, 1 : 1) and Et_3N (0.24 ml, 1.69 mmol) were added to the above ice-chilled solution. After being stirred at 4 °C for 14 h, the reaction mixture was diluted with H_2O (100 ml). The resulting powder was washed with H_2O and precipitated twice from DMSO with MeOH; yield 1.48 g (65%), mp 262—264 °C, $[\alpha]_D^{20}$ −18.0° (c = 1.0, DMSO), Rf_1 0.45. *Anal.* Calcd for $C_{109}H_{159}N_{24}O_{28}S_2 \cdot 2H_2O$: C, 55.69; H, 6.86; N, 14.30. Found: C, 55.80; H, 6.91; N, 14.04.

Z(OMe)-Ala-Arg(Mts)-Ala-Glu(OBzl)-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser(Bzl)-Asn-Arg(Mts)-Lys(Z)-Leu-Met(O)-Glu(OBzl)-Ile-Ile-NH_2, Z(OMe)-(hCRF 22—41)-NH_2——The above protected tetradecapeptide amide (0.99 g, 0.43 mmol) was treated with TFA-anisole (5 ml–0.5 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMSO-DMF (1 : 1, 30 ml) containing Et_3N (0.12 ml, 0.85 mmol). The azide [prepared from 1.46 g (1.28 mmol) of Z(OMe)-Ala-Arg(Mts)-Ala-Glu(OBzl)-Gln-Leu-$NHNH_2$ [5]] in DMSO-DMF (1 : 1, 10 ml) and Et_3N (0.21 ml, 1.54 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 14 h. Then the reaction mixture was diluted with H_2O (100 ml) to afford a powder, which was washed with H_2O and precipitated three times from DMSO with aqueous MeOH; yield 1.04 g. A part of this partially purified product (452 mg) was dissolved in DMSO (5 ml) and the solution was applied to a column of Sephadex LH-60 (3 × 136 cm), which was eluted with DMSO-DMF (3 : 7). Each fraction (11 ml) was examined for ultraviolet (UV) absorption at 275 nm. The fractions corresponding to the main peak (tube Nos. 46—57) were combined and the solvent was removed by evaporation. Treatment of the residue with AcOEt afforded a powder; yield 417 mg. The rest of the sample was similarly purified; total yield 922 mg (66%), mp 280 °C (dec.), $[\alpha]_D^{20}$ −6.4° (c = 0.9, DMSO), Rf_1 0, Rf_3 0.88. *Anal.* Calcd for $C_{153}H_{220}N_{34}O_{39}S_3 \cdot 4H_2O$: C, 55.22; H, 6.91; N, 14.31. Found: C, 54.98; H, 6.71; N, 14.17.

Z(OMe)-Arg(Mts)-Glu(OBzl)-Val-Leu-Glu(OBzl)-Met(O)-Ala-Arg(Mts)-Ala-Glu(OBzl)-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser(Bzl)-Asn-Arg(Mts)-Lys(Z)-Leu-Met(O)-Glu(OBzl)-Ile-Ile-NH_2, Z(OMe)-(hCRF 16—41)-NH_2——The above protected eicosapeptide amide (600 mg, 0.18 mmol) was treated with TFA-anisole (4 ml–0.6 ml) and the N^{α}-deprotected peptide, isolated as described above, was dissolved in DMSO-DMF (6 ml) containing Et_3N (51 μl, 0.37 mmol). The azide [prepared from 614 mg (0.46 mmol) of Z(OMe)-Arg(Mts)-Glu(OBzl)-Val-Leu-Glu(OBzl)-Met(O)-$NHNH_2$ [6]] in DMF (1 ml) and Et_3N (64 μl, 0.46 mmol) were added to the above ice-chilled solution. After being stirred at 4 °C for 36 h, the reaction mixture was diluted with H_2O (100 ml). The resulting powder was washed with H_2O and precipitated from DMSO with AcOEt; yield 805 mg. This partially purified product was dissolved in DMSO (5 ml) and the solution was applied to a column of Sephadex LH-60 (3 × 135 cm), which was eluted with DMSO-DMF (3 : 7). Each fraction (10 ml) was examined for UV absorption at 288 nm. The fractions corresponding to the main peak (tube Nos. 45—54) were combined and the solvent was removed by evaporation. Treatment of the residue with AcOEt afforded a powder; yield 497 mg (61%), mp 288 °C (dec.), $[\alpha]_D^{20}$ −10.0° (c = 0.5, DMSO), Rf_1 0.71. *Anal.* Calcd for $C_{208}H_{297}N_{43}O_{52}S_5 \cdot 3H_2O$: C, 56.19; H, 6.87; N, 13.55. Found: C, 56.02; H, 6.75; N, 13.28.

Z(OMe)-His-Leu-Leu-Arg(Mts)-Glu(OBzl)-Val-Leu-Glu(OBzl)-Met(O)-Ala-Arg(Mts)-Ala-Glu(OBzl)-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser(Bzl)-Asn-Arg(Mts)-Lys(Z)-Leu-Met(O)-Glu(OBzl)-Ile-Ile-NH_2, Z(OMe)-(hCRF 13—41)-NH_2——The above protected hexacosapeptide amide (467 mg, 0.106 mmol) was treated with TFA-anisole (3 ml–0.5 ml) and the N^{α}-deprotected peptide, isolated as stated above, was dissolved in DMSO-DMF (1 : 1, 5 ml) containing Et_3N (30 μl, 0.212 mmol). The azide [prepared from 298 mg (0.530 mmol) of Z(OMe)-His-Leu-Leu-$NHNH_2$ [7]] in DMF (2 ml) and Et_3N (74 μl, 0.530 mmol) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. Then the reaction mixture was diluted with H_2O (100 ml) to afford a powder, which was washed with H_2O and twice precipitated from DMSO with AcOEt; yield 504 mg. This partially purified product was dissolved in DMF and the solution was applied to a column of Sephadex

LH-60 (3 × 122 cm), which was eluted with DMF. Each fraction (8.2 ml) was examined for UV absorption at 275 nm. The fractions corresponding to the main peak (tube Nos. 40—61) were combined and the solvent was removed by evaporation. Treatment of the residue with AcOEt afforded a powder; yield 429 mg (85%), mp 148—150 °C, $[\alpha]_D^{20}$ −10.2° (c=0.5, DMSO), Rf_1 0.49. *Anal.* Calcd for $C_{226}H_{326}N_{48}O_{55}\cdot 8H_2O$: C, 55.40; H, 7.04; N, 13.72. Found: C, 55.49; H, 6.91; N, 13.45.

Z(OMe)-Leu-Asp(OBzl)-Leu-Thr-Phe-His-Leu-Leu-Arg(Mts)-Glu(OBzl)-Val-Leu-Glu(OBzl)-Met(O)-Ala-Arg(Mts)-Ala-Glu(OBzl)-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser(Bzl)-Asn-Arg(Mts)-Lys(Z)-Leu-Met(O)-Glu(OBzl)-Ile-Ile-NH_2, Z(OMe)-(hCRF 8—41)-NH_2——The above protected nonacosapeptide amide (691 mg, 145 μmol) was treated with TFA–anisole (5 ml–0.7 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMSO–DMF (1 : 1, 5 ml) containing Et_3N (60 μl, 435 μmol). The azide [prepared from 635 mg (725 μmol) of Z(OMe)-Leu-Asp(OBzl)-Leu-Thr-Phe-$NHNH_2$ [8]′] in DMSO–DMF (1 : 1, 4 ml) and Et_3N (101 μl, 725 μmol) were added to the above ice-chilled solution and the mixture was stirred at −18 C for 48 h and then at 4 C for 24 h. The reaction mixture was diluted with H_2O (100 ml), and the resulting powder was washed with H_2O then precipitated from DMF with AcOEt; yield 801 mg. This partially purified product was purified by gel-filtration on Sephadex LH-60 (3 × 122 cm column) as stated above; yield 680 mg (86%), mp 149—151 °C, $[\alpha]_D^{20}$ −2.5° (c=0.4, DMSO), Rf_1 0.52. *Anal.* Calcd for $C_{262}H_{375}N_{53}O_{63}S_5$: C, 57.89; H, 6.95; N, 13.66. Found: C, 57.68; H, 6.95; N, 13.36.

Z(OMe)-Ser(Bzl)-Glu(OBzl)-Glu(OBzl)-Pro-Pro-Ile-Ser(Bzl)-Leu-Asp(OBzl)-Leu-Thr-Phe-His-Leu-Leu-Arg(Mts)-Glu(OBzl)-Val-Leu-Glu(OBzl)-Met(O)-Ala-Arg(Mts)-Ala-Glu(OBzl)-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser(Bzl)-Asn-Arg(Mts)-Lys(Z)-Leu-Met(O)-Glu(OBzl)-Ile-Ile-NH_2, Z(OMe)-(hCRF 1—41)-NH_2——The above protected tetratriacontapeptide amide (673 mg, 124 μmol) was treated with TFA–anisole (5 ml–0.7 ml) and the N^{α}-deprotected peptide, isolated as usual, was dissolved in DMF (6 ml) containing Et_3N (52 μl, 372 μmol). The azide [prepared from 804 mg (620 μmol) of Z(OMe)-Ser(Bzl)-Glu(OBzl)-Glu(OBzl)-Pro-Pro-Ile-Ser(Bzl)-$NHNH_2$ [9]′] in DMF (6 ml) and Et_3N (86 μl, 620 μmol) were added to the above ice-salt-chilled solution. The reaction mixture was stirred at −18 C for 48 h and at 4 C for 24 h, and then diluted with H_2O (100 ml). The resulting powder was washed with H_2O and twice precipitated from DMF with AcOEt; yield 799 mg. This partially purified product was purified by gel-filtration on Sephadex LH-60 (3 × 122 cm column) using DMF as the eluant as described above; yield 559 mg (69%), mp 146—148 C, $[\alpha]_D^{20}$ −5.9 (c=0.4, DMSO), Rf_1 0.62. *Anal.* Calcd for $C_{322}H_{448}N_{60}O_{76}S_5\cdot 7H_2O$: C, 58.05; H, 6.99; N, 12.62. Found: C, 57.76; H, 6.83; N, 12.64.

H-Ser-Glu-Glu-Pro-Pro-Ile-Ser-Leu-Asp-Leu-Thr-Phe-His-Leu-Leu-Arg-Glu-Val-Leu-Glu-Met-Ala-Arg-Ala-Glu-Gln-Leu-Ala-Gln-Gln-Ala-His-Ser-Asn-Arg-Lys-Leu-Met-Glu-Ile-Ile-NH_2, H-(hCRF 1—41)-NH_2——The above protected hentetracontapeptide amide (200 mg, 31 μmol) was treated with 1 M TFMSA–thioanisole (molar ratio 1 : 1) in TFA (9.2 ml) in the presence of *m*-cresol (239 μl, 75 eq) in an ice bath for 90 min, then dry ether was added. The resulting powder was collected by centrifugation, and dried over KOH pellets *in vacuo* for 30 min. This treatment was repeated twice more to ensure complete deprotection. The deprotected peptide thus obtained was dissolved in H_2O (15 ml) containing dithiothreitol (236 mg, 50 eq), treated with Amberlite CG-4B (acetate form, approximately 2 g) for 30 min with stirring and then filtered. The pH of the solution was adjusted to 8.0 with 1 N NH_4OH. The solution was stirred for 30 min in an ice bath, and the pH was readjusted to 6.5 with 1 N AcOH. The solution was lyophilized to give a hygroscopic powder, which was dissolved in 4 ml of 0.5 N AcOH and incubated with dithiothreitol (236 mg, 50 eq) under an argon atmosphere at 37 C for 24 h. Then the solution was applied to a column of Sephadex G-25 (2 × 147 cm), which was eluted with 0.5 N AcOH. A part (150 μl) of each fraction (7 ml) was subjected to the Folin–Lowry test, and the optical density (OD) at 750 nm was determined. The fractions corresponding to the front main peak (tube Nos. 34—50) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 140 mg (96%).

The above crude product (40.12 mg) was dissolved in pH 4.5, 0.02 M NH_4OAc buffer containing 3 M urea and the solution was applied to a column of CM-Biogel A (1.8 × 8.6 cm), which was first eluted with the same buffer, and then with a gradient formed from the starting buffer (275 ml) and pH 5.0, 0.2 M NH_4OAc buffer containing 3 M urea (275 ml). The OD at 750 nm in the Folin–Lowry test of each fraction (6 ml) was determined as described above (Fig. 3). The fractions corresponding to the main peak (tube Nos. 69—94) were combined and the solvent was removed by lyophilization. The residue was dissolved in 5% acetonitrile in 1 N AcOH and applied to the column of Diaion HP-20 (1.8 × 8.2 cm), which was first washed with the same solvent (300 ml) to remove urea and the salt and then eluted with a gradient formed from 50% acetonitrile in 1 N AcOH (125 ml) through a mixing flask containing the starting solvent (125 ml). Each fraction (6 ml each) was examined by means of the Folin–Lowry test. The fractions corresponding to the main peak (tube Nos. 64—89) were combined and the solvent was removed by lyophilization to give a white fluffy powder; yield 23.28 mg (58%).

Subsequent purification was performed by reverse phase HPLC on Vydac $5C_{18}$ (4.6 × 250 mm column). The above CM-purified sample (20.01 mg) was dissolved in 0.5 N AcOH containing 5% β-mercaptoethanol (2 ml) and incubated at 37 °C for 48 h in order to reduce the Met(O) residues, which were formed during the above manipulations. Portions of the solution (500 μl each) were applied to the above column, which was eluted with a gradient of acetonitrile (35 to 45% in 1 h) in 0.2% TFA, at a flow rate of 0.7 ml per min. The eluate corresponding to

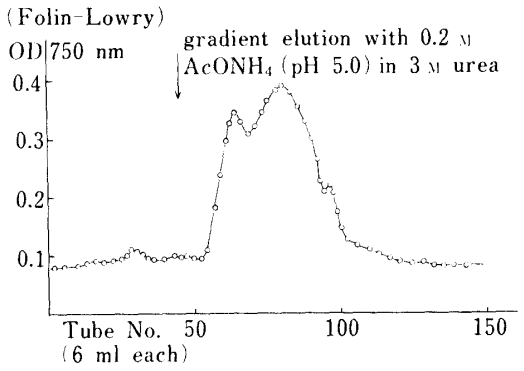

Fig. 3. Purification of Synthetic H-(hCRF 1—41)-NH_2 by Ion-exchange Chromatography on CM-Biogel A

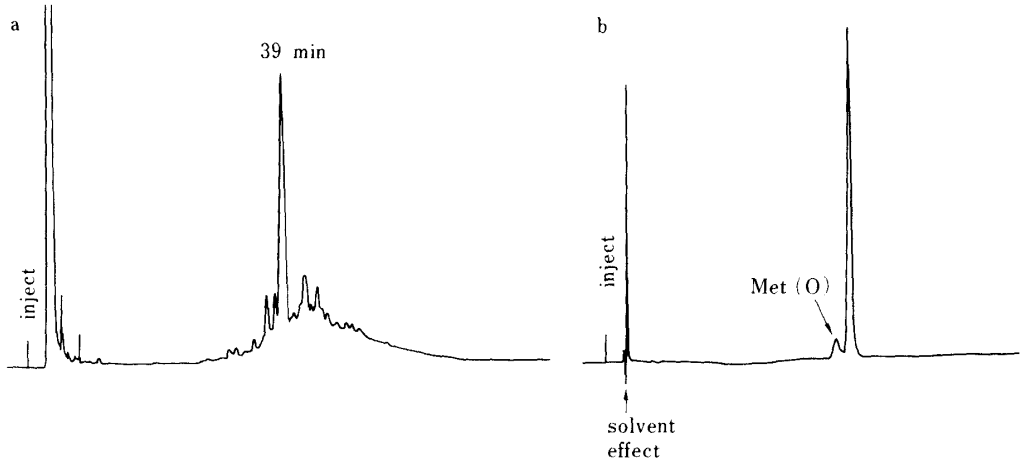

Fig. 4. HPLC of H-(hCRF 1—41)-NH_2
a, purification of CM-purified sample. b, purified sample.

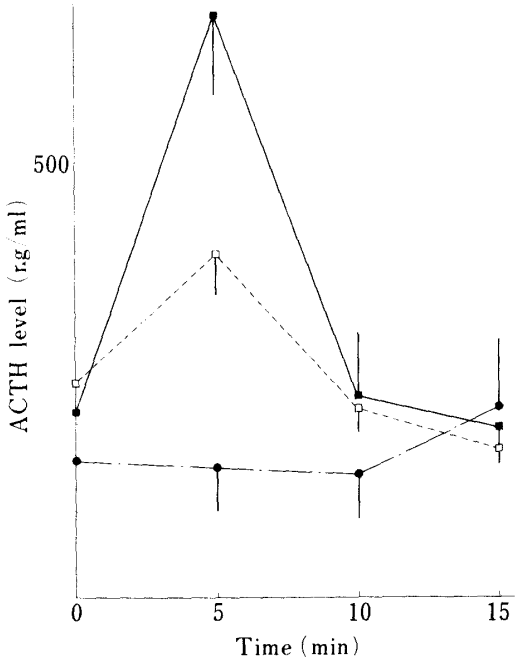

Fig. 5. Effect of Synthetic hCRF on Rat Plasma ACTH level
□, 1 μg; ■, 10 μg; ●, saline.

the main peak (retention time 39 min, Fig. 4a) was collected. The rest of the sample was similarly purified. The combined eluates, after addition of β-mercaptoethanol (0.5 ml), were concentrated by rotary evaporation *in vacuo* and rechromatographed under the same conditions. The desired eluates were again concentrated and applied to a column of Sephadex G-25 (1.8 × 40 cm), which was eluted with 1 N AcOH. The desired fractions were collected and

the solvent was removed by lyophilization to give a white fluffy powder; yield 9.33 mg (47%), total yield from the protected hCRF (1—41)-NH_2, 26%; $[\alpha]_D^{16}$ −84.1° (c=0.12 in 1 N AcOH), Rf_4 0.41, Rf_5 0.34. The purity of the final product was estimated by HPLC to be more than 95% (Fig. 4b). Removal of the *ca.* 5% impurities (Met(O) derivative of hCRF) was not an easy task when open column systems were employed for the purification because, even in the HPLC purification, purified hCRF was found to be oxidized to some extent.

The synthetic peptide exhibited a single band in disk isoelectrofocusing on 7.5% polyacrylamide gel (0.5 × 6.3 cm) containing Pharmalyte (pH 3—10): mobility, 2.1 cm from the origin toward the cathodic end of the gel, after running at 200 V for 4 h (stained with Coomassie Brilliant Blue G-250, Sigma). Amino acid ratios in 6 N HCl hydrolysate are shown in Table I. Amino acid ratios in papain plus leucine aminopeptidase digestion: Asp 1.00 (1), Thr 0.98 (1), Ser 2.90 (3), Glu 6.27 (6), Pro 1.81 (2), Ala 4.22 (4), Val 1.00 (1), Met 1.86 (2), Ile 3.13 (3), Leu 7.31 (7), Phe 0.96 (1), Lys 0.98 (1), His 2.10 (2), Arg 3.12 (3); Asn (1) and Gln (3) were not determined (recovery of Asp 78%). *Anal.* Calcd for $C_{208}H_{344}N_{60}O_{63}S_2 \cdot 7CH_3COOH \cdot 5H_2O$: C, 50.61; H, 7.31; N, 15.95. Found: C, 50.45; H, 7.36; N, 16.16.

Effect of Synthetic hCRF on Rat ACTH——An ACTH immunoassay kit was purchased from Amersham International plc (Buckinghamshire, England). Male Sprague–Dawley rats weighing 250—280 g were used in these experiments. The animals were obtained from Shizudokyo (Shizuoka) and fed a purina chow diet and water *ad libitum* unless otherwise mentioned. Experiments were conducted with groups of four rats per treatment.

The procedure described by River *et al.*[21] was used for catheterization and collection of blood. The animals were anesthetized with ether and implanted with an indwelling heparinized silicone *i.v.* tube. They were allowed to recover, then placed in individual cages in a quiet room. After 14 to 16 h, synthetic hCRF (10 or 1 μg in 0.1 ml of saline) or saline (0.1 ml) was injected through the cannula. The animals were bled through the same cannula and the blood was collected in heparinized sample tubes at 5, 10 and 15 min. Plasma concentrations of ACTH in rats were determined as shown in Fig. 5. Each point represents the mean ± S.E. of 4 rats.

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2) Amino acids and peptide derivatives mentioned in this investigation are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Mts = mesitylene-2-sulfonyl, Bzl = benzyl, Troc = 2,2,2-trichloroethyloxycarbonyl, EDTA = ethylenediaminetetraacetic acid disodium salt, DMF = dimethylformamide, DMSO = dimethylsulfoxide, TFMSA = trifluoromethanesulfonic acid, TFA = trifluoroacetic acid, ACTH = adrenocorticotropin.
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