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Studies on Peptides. CLIII.[1,2] Application of the Hard-Acid Deprotecting Procedure to the Synthesis of Ovine Corticotropin Releasing Factor (oCRF)

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A 41-residue peptide corresponding to the entire amino acid sequence of ovine corticotropin releasing factor (oCRF) was synthesized by assembling ten peptide fragments of established purity, followed by hard-acid deprotection with 1 M trimethylsilyl trifluoromethanesulfonate in trifluoroacetic acid. β-Cycloheptylaspartate was employed to minimize base-catalyzed succinimide formation. When tested by *in vivo* assay, synthetic oCRF was as active as synthetic hCRF in terms of ability to release immunoreactive corticotropin.

Keywords——ovine corticotropin releasing factor synthesis; β-cycloheptylaspartate; thioanisole-mediated deprotection; hard-acid deprotection; trimethylsilyl trifluoromethanesulfonate deprotection; base-catalyzed succinimide formation; immunoreactive corticotropin release

Following the synthesis of human corticotropin releasing factor (hCRF),[3] we wish to present a detailed account of our solution phase synthesis of ovine CRF (oCRF), the structure of which was elucidated by Vale *et al.*[4] in 1981. Several research groups have reported solid-phase[4,5] or, preliminarily, conventional solution-phase syntheses[6] of this hypothalamic peptide. However, no detailed information on the solution-phase method is available at the present time. From a synthetic viewpoint, especially in the solution-phase synthesis, CRF offers particular difficulty in the elongation of the peptide chain, presumably due to its high helical content.[7]

Different from our previous synthesis of hCRF,[3] our newly developed hard-acid deprotecting procedure[8] was employed in the present oCRF synthesis. The TFA-labile Z(OMe)[9] or the Boc group was employed for N^{α}-protection. In the final step of the synthesis, all side-chain protecting groups were cleaved by treatment with 1 M TMSOTf–thioanisole/TFA, *i.e.*, Z from Lys, Bzl from Glu, Asp and Ser, Mts[10] from Arg, and Chp from Asp (position 25). Asp(OChp)[11] was employed for preparation of the Asp–Gln sequence in order to minimize base-catalyzed succinimide formation.[12] Ten peptide fragments were selected as building blocks to construct the entire peptide backbone of oCRF. This factor possesses the same sequence as hCRF,[13] except for replacement of seven residues at positions 2, 22, 23, 25, 38, 39, and 41, *i.e.*, Glu, Ala, Arg, Glu, Met, Glu, and Ile of hCRF with Gln, Thr, Lys, Asp, Leu, Asp, and Ala, respectively. Thus, of the ten fragments, six, [2], [3], [4], [7], [8], and [9], are identical with those employed for the hCRF synthesis and four, [1], [5], [6], and [10], which cover the area of species variation, were newly synthesized.

The C-terminal fragment, Boc–Leu–Asp(OBzl)–Ile–Ala–NH_2 [1], was prepared in a

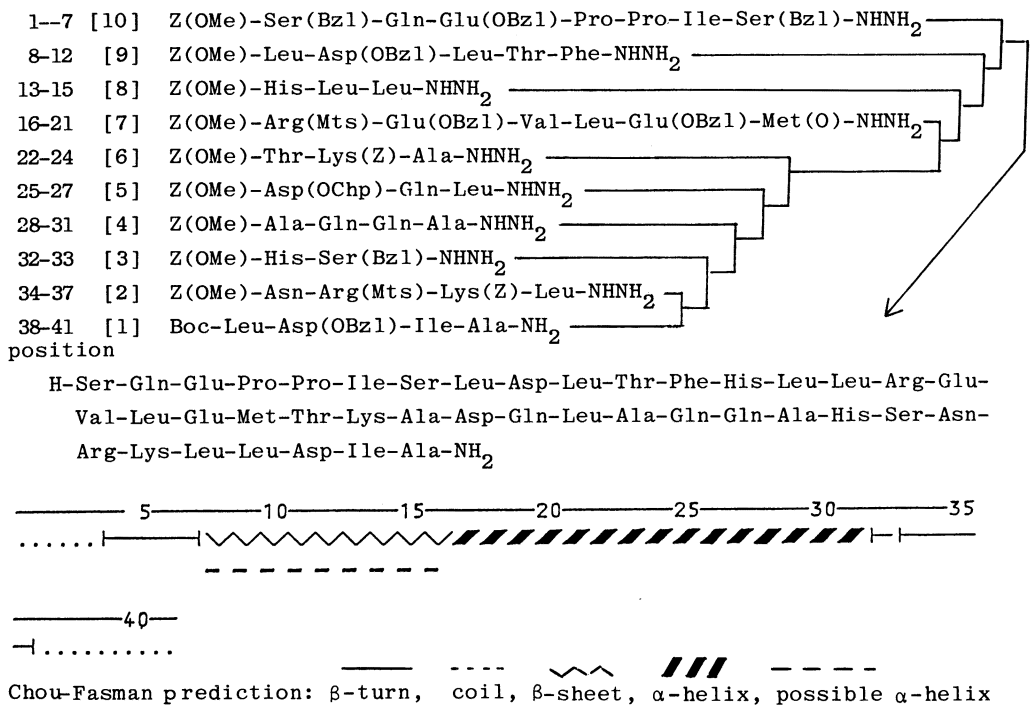

Fig. 1. Synthetic Route to oCRF and Prediction of Its Conformation

stepwise manner starting from H–Ala–NH_2, onto which the respective three amino acid residues were condensed by the mixed anhydride (MA),[14] the Np[15] and again the MA procedures, respectively. Fragment [5] contains the Asp–Gln sequence, which is somewhat susceptible to base-catalyzed succinimide formation.[12] In order to minimize this side reaction, Z(OMe)–Asp(OChp)–OH, instead of Z(OMe)–Asp(OBzl)–OH, was condensed with a TFA-treated sample of Z(OMe)–Gln–Leu–NHNH–Troc[16] by the Su procedure[17] and from the resulting tripeptide derivative, the Troc group[18] was removed by treatment with Cd/AcOH[19] to afford [5]. Fragment [6], Z(OMe)–Thr–Lys(Z)–Ala–$NHNH_2$, was easily prepared by condensation of Z(OMe)–Thr–$NHNH_2$ with a TFA-treated sample of Z(OMe)–Lys(Z)–Ala–OMe[20] *via* the azide,[21] followed by the usual hydrazine treatment. Fragment [10], Z(OMe)–Ser(Bzl)–Gln–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–$NHNH_2$, was prepared by stepwise condensations of Z(OMe)–Gln–OH and Z(OMe)–Ser(Bzl)–OH onto a TFA-treated sample of Z(OMe)–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc[3] *via* the Np ester and then the MA procedures, respectively, followed by the usual Zn/AcOH treatment.[22]

Ten fragments were then assembled successively, as shown in Fig. 1, by the azide procedure to minimize racemization. As a solvent, DMF or DMF–DMSO (1 : 1) was employed and the amount of the acyl component was increased from 1.1 to 7 eq as chain elongation progressed. Condensations of fragments from [1] to [7] proceeded as usual without particular difficulty. However, in the subsequent azide condensations of fragments from [8] to [10], each acyl component had to be used in a large excess (7 eq) in order to bring the reaction to completion and every reaction had to be performed at a lower temperature (−18 °C) than usual (+4 °C) in order to minimize Curtius rearrangement.[23] As reported in our previous hCRF synthesis, helical or β-sheet conformation of CRF may be responsible for such unusual phenomena in the chain elongation reactions. Each protected product was purified either by precipitation from DMF with MeOH or by gel-filtration on Sephadex LH-60 using DMF as

TABLE I. Amino Acid Ratios in 6 N HCl Hydrolysates of Synthetic oCRF and Its Intermediates

	Protected peptides									Syn. oCRF	Residue
	34—41	32—41	28—41	25—41	22—41	16—41	13—41	8—41	1—41		
Asp	2.01	2.05	2.09	3.00	3.04	3.23	3.15	4.40	4.27	3.85	(4)
Thr					0.86	0.91	0.94	2.01	2.29	1.99	(2)
Ser		0.95	0.90	1.01	1.00	1.03	1.10	1.18	3.34	3.03	(3)
Glu			2.08	2.80	3.01	5.23	4.88	5.06	7.25	7.29	(7)
Pro									1.75	1.70	(2)
Ala	1.07	1.05	3.32	2.96	4.04	4.41	4.12	4.19	4.32	4.09	(4)
Val						0.73	0.81	0.64	1.12	1.02	(1)
Met						0.68	0.79	0.67	0.83	1.06	(1)[a]
Ile	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	2.00	2.00	(2)
Leu	2.02	2.01	2.05	2.81	3.03	3.95	6.07	8.27	7.60	7.58	(8)
Phe								0.87	1.00	0.95	(1)
Lys	1.01	1.06	0.95	1.06	1.86	2.02	1.97	1.99	2.12	2.00	(2)
His		1.06	0.98	0.95	1.00	1.07	1.99	1.85	1.69	1.89	(2)
Arg	1.04	0.99	0.89	0.98	0.96	2.05	1.87	1.82	1.91	1.81	(2)
Rec. %	76	78	82	83	83	73	84	70	71	80	

a) Met + Met(O).

an eluant. Throughout this synthesis, Ile was used as a diagnostic amino acid (Table I). After each condensation, each product was subjected to 6 N HCl hydrolysis and the recovery of Ile was compared with those of newly added amino acids to assure satisfactory incorporation. The homogeneity of each product was further ascertained by elemental analysis and thin layer chromatography (TLC).

In the final step, protected oCRF was treated with 1 M TMSOTf–thioanisole/TFA in the presence of *m*-cresol to remove all protecting groups employed, except Met(O), which is known to be partially reduced under this deprotecting condition.[16] The deprotected peptide was purified in essentially the same manner as described in the final step of our hCRF synthesis: 1) Treatment with dil. ammonia containing NH_4F to reverse any possible N→O shift[24] and ensure the complete hydrolysis of trimethylsilyl residues. 2) Incubation with dithiothreitol to ensure the complete reduction of Met(O). 3) Gel-filtration on Sephadex G-50. 4) Ion-exchange chromatography on CM-cellulose. 5) Desalting on Diaion HP-20. 6) High performance liquid chromatography (HPLC) on Vydac 5C18. The peptide thus purified exhibited a sharp single spot on TLC and a single band in disk isoelectrofocusing (Pharmalyte, pH 3—10). Its purity was further confirmed by acid hydrolysis and leucine aminopeptidase (LAP) digestion.

When tested in rats anesthetized with sodium pentobarbital (50 mg/kg) according to Rivier *et al.*,[25] our synthetic oCRF (5.0 μg/kg) significantly elevated the plasma immunoreactive corticotropin (ACTH) level (76% ± 23) and its potency was judged to be equivalent to that of synthetic hCRF.

Experimental

General experimental methods employed here are essentially the same as described in our hCRF synthesis.[3]

Unless otherwise stated, 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.

HPLC was conducted with a Waters 204 compact model. TLC was performed on silica gel (Kieselgel G, Merck). *Rf* values refer to the following solvent systems (v/v). Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 $CHCl_3$–MeOH (10:0.5), Rf_3 $CHCl_3$–MeOH (9:1), Rf_4 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24).

An ACTH immunoassay kit was purchased from Japan Radioisotope Association (Bunkyo-ku, Tokyo).

Z(OMe)–Ile–Ala–NH_2——The title compound was prepared by the MA procedure and purified by procedure B followed by recrystallization from DMF with MeOH; yield 62%, mp 229—231 °C, $[\alpha]_D^{20}$ +3.9° (c=1.0, DMSO), Rf_1; 0.75. *Anal.* Calcd for $C_{18}H_{27}N_3O_5$: C, 59.15; H, 7.45; N, 11.50. Found: C, 58.86; H, 7.48; N, 11.33.

Boc–Asp(OBzl)–Ile–Ala–NH_2——A mixture of Boc–Asp(OBzl)–ONp (20.1 g, 45.2 mmol) and a TFA-treated sample of the above dipeptide (15.0 g, 41.0 mmol) in DMF (150 ml) containing Et_3N (11.5 ml, 82.1 mmol) was stirred at 4 °C for 12 h, then concentrated. The residue was purified by procedure B followed by precipitation from DMF with MeOH; yield 16.6 g (80%), mp 198—200 °C, $[\alpha]_D^{20}$ −26.1° (c=1.1, DMSO), Rf_1 0.81. *Anal.* Calcd for $C_{25}H_{38}N_4O_7$: C, 59.27; H, 7.56; N, 11.06. Found: C, 59.00; H, 7.50; N, 10.98.

Boc–Leu–Asp(OBzl)–Ile–Ala–NH_2 [1]——An MA [prepared from 9.6 g (41.5 mmol) of Boc–Leu–OH] in THF was added to an ice-chilled solution of a TFA-treated sample of the above tripeptide amide (14.0 g, 27.6 mmol) in DMF (100 ml) containing Et_3N (3.8 ml, 27.6 mmol) and the mixture was stirred at 4 °C for 5 h. The product was purified by procedure B followed by precipitation from DMF with AcOEt; yield 10.33 g (60%), mp 222—225 °C, $[\alpha]_D^{20}$ −32.3° (c=1.1, DMSO), Rf_1 0.83. Amino acid ratios in 6 N HCl hydrolysate: Leu 1.10, Asp 1.04, Ile 1.00, Ala 1.00 (recovery of Ala, 69%). *Anal.* Calcd for $C_{31}H_{49}N_5O_8 \cdot 1/2H_2O$: C, 59.21; H, 8.01; N, 11.14. Found: C, 59.24; H, 7.96; N, 11.19.

Z(OMe)–Asp(OChp)–Gln–Leu–NHNH–Troc——A mixture of Z(OMe)–Asp(OChp)–OSu (4.80 g, 9.79 mmol), Et_3N (2.5 ml, 17.9 mmol) and a TFA-treated sample of Z(OMe)–Gln–Leu–NHNH–Troc[16] (5.0 g, 8.16 mmol) in DMF (50 ml) was stirred at room temperature for 12 h. The product was purified by procedure A followed by recrystallization from AcOEt and ether; yield 4.85 g (72%), mp 164—166 °C, $[\alpha]_D^{20}$ −15.8° (c=0.8, DMF), Rf_1 0.74. *Anal.* Calcd for $C_{34}H_{49}Cl_3N_6O_{11}$: C, 49.55; H, 5.99; N, 10.20. Found: C, 49.25; H, 6.02; N, 10.23.

Z(OMe)–Asp(OChp)–Gln–Leu–$NHNH_2$——The above tripeptide derivative (3.50 g, 4.25 mmol) in MeOH–AcOH (20 ml—5 ml) was treated with Cd powder (4.8 g, 10 eq) at room temperature for 12 h. The solution was filtered, the filtrate was concentrated and the residue was treated with 5% EDTA. The resulting powder was washed with 5% EDTA and H_2O and then precipitated from MeOH with ether; yield 2.02 g (73%); mp 191—193 °C, $[\alpha]_D^{15}$ −11.7° (c=1.0, DMF), Rf_1 0.62. Amino acid ratios in 6 N HCl hydrolysate: Asp 1.01, Glu 0.99, Leu 1.00 (recovery of Leu, 86%). *Anal.* Calcd for $C_{31}H_{48}N_6O_9$: C, 57.39; H, 7.46; N, 12.96. Found: C, 57.27; H, 7.61; N, 12.85.

Z(OMe)–Thr–Lys(Z)–Ala–OMe——The azide [prepared from 3.37 g (11.3 mmol) of Z(OMe)–Thr–$NHNH_2$] in DMF (10 ml) and Et_3N (2.9 ml, 20.8 mmol) were added to an ice-chilled solution of a TFA-treated sample of Z(OMe)–Lys(Z)–Ala–OMe[20] (5.00 g, 9.44 mmol) in DMF (50 ml) and the mixture was stirred at 4 °C for 12 h. The product was purified by procedure B followed by precipitation from DMF with MeOH; yield 4.70 g (79%), mp 153—156 °C, $[\alpha]_D^{20}$ −1.0° (c=1.0, DMF), Rf_2 0.40. *Anal.* Calcd for $C_{31}H_{42}N_4O_{10}$: C, 59.03; H, 6.71; N, 8.89. Found: C, 58.89; H, 6.74; N, 8.80.

Z(OMe)–Thr–Lys(Z)–Ala–$NHNH_2$ [6]——The above tripeptide ester (2.50 g, 3.96 mmol) in DMF (30 ml) was treated with 80% hydrazine hydrate (2.4 ml, 10 eq) at room temperature for 12 h, then the solution was concentrated and the residue was precipitated from DMSO with MeOH; yield 2.46 g (98%), mp 214—217 °C, $[\alpha]_D^{20}$ +3.9° (c=1.0, DMSO), Rf_1 0.49. Amino acid ratios in 6 N HCl hydrolysate: Thr 1.02, Lys 1.00, Ala 1.12 (recovery of Lys, 88%). *Anal.* Calcd for $C_{30}H_{42}N_6O_9$: C, 57.13; H, 6.71; N, 13.33. Found: C, 56.96; H, 6.72; N, 13.43.

Z(OMe)–Gln–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc——A mixture of Z(OMe)–Gln–ONp (1.19 g, 2.76 mmol), Et_3N (0.74 ml, 5.27 mmol) and a TFA-treated sample of Z(OMe)–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc[3] (2.70 g, 2.51 mmol) in DMF (20 ml) was stirred at 4 °C for 24 h. The product was purified by procedure A followed by column chromatography on silica gel (4.3 × 9 cm) using $CHCl_3$–MeOH (10:0.5) as an eluant. The product was recrystallized from MeOH and isopropyl ether; yield 1.91 g (63%), mp 98—100 °C, $[\alpha]_D^{15}$ −23.8° (c=1.0, DMF), Rf_3 0.42. *Anal.* Calcd for $C_{55}H_{70}Cl_3N_9O_{15}$: C, 54.88; H, 5.86; N, 10.47. Found: C, 54.87; H, 5.93; N, 10.46.

Z(OMe)–Ser(Bzl)–Gln–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–NHNH–Troc——An MA [prepared from 0.68 g (1.90 mmol) of Z(OMe)–Ser(Bzl)–OH] in THF (20 ml) was added to an ice-chilled solution of a TFA-treated sample of the above protected hexapeptide (1.90 g, 1.58 mmol) in DMF (15 ml) containing Et_3N (0.22 ml, 1.58 mmol) and the mixture was stirred at 4 °C for 4 h. The product was purified by procedure A followed by recrystallization from MeOH and ether; yield 1.72 g (79%), mp 89—91 °C, $[\alpha]_D^{15}$ −22.9° (c=1.0, DMF), Rf_3 0.40. *Anal.* Calcd for $C_{65}H_{81}Cl_3N_{10}O_{17}$: C, 56.54; H, 5.91; N, 10.15. Found: C, 56.51; H, 5.78; N, 10.05.

Z(OMe)–Ser(Bzl)–Gln–Glu(OBzl)–Pro–Pro–Ile–Ser(Bzl)–$NHNH_2$ [10]——The above protected heptapeptide derivative (1.40 g, 1.01 mmol) in MeOH–AcOH (5 ml–5 ml) was treated with Zn powder (0.66 g, 10 eq) at room temperature for 18 h. The mixture was filtered, the filtrate was concentrated and the residue was dissolved in *n*-BuOH. The organic phase was washed with 5% EDTA and H_2O and then concentrated. The residue was treated with

TABLE II. Characterization of Protected oCRF and Its Intermediates

	Puri. proc. Yield (%)	Rf_1	mp (°C)	$[\alpha]_D^{15}$ (DMF)	Formula	Analysis (%) Calcd (Found) C	H	N
Z(OMe)-(34—41)-NH_2	C 83	0.74	253—256	−26.6°	$C_{74}H_{106}N_{14}O_{18}S$	58.79 (58.50	7.07 7.09	12.97 13.06)
Z(OMe)-(32—41)-NH_2	C 93	0.63	232—234	−19.2°	$C_{90}H_{124}N_{18}O_{21}S \cdot 4H_2O$	56.94 (56.93	7.01 6.98	13.28 13.17)
Z(OMe)-(28—41)-NH_2	C 76	0.25	214—216	−16.9°	$C_{106}H_{150}N_{24}O_{27}S$	57.23 (56.95	6.80 6.93	15.11 15.32)
Z(OMe)-(25—41)-NH_2	C 69	0.57	250—252	−48.5°	$C_{128}H_{186}N_{28}O_{33}S \cdot 6H_2O$	55.20 (55.13	7.17 6.92	14.08 13.86)
Z(OMe)-(22—41)-NH_2	C 82	0.46	263—264	−47.5°	$C_{149}H_{216}N_{32}O_{39}S \cdot 7H_2O$	55.27 (55.09	7.16 6.87	13.85 13.68)
Z(OMe)-(16—41)-NH_2	D 43	0.64	238—239	−15.2°	$C_{204}H_{293}N_{41}O_{52}S_3 \cdot 8H_2O$	55.78 (56.00	7.09 6.83	13.08 12.80)
Z(OMe)-(13—41)-NH_2	D 73	0.60	230—231	−8.2°	$C_{222}H_{322}N_{46}O_{55}S_3 \cdot 3H_2O$	57.15 (57.07	7.09 7.12	13.81 13.92)
Z(OMe)-(8—41)-NH_2	D 78	0.58	226—228	−5.9°	$C_{258}H_{371}N_{51}O_{63}S_3 \cdot 7H_2O$	57.20 (57.25	7.16 7.08	13.19 13.28)
Z(OMe)-(1—41)-NH_2	D 66	0.65	185—186	−7.8°	$C_{311}H_{439}N_{59}O_{75}S_3 \cdot 4H_2O$	58.61 (58.53	7.07 7.22	12.97 12.93)

Purification procedure: C, precipitation from DMF with MeOH; D, gel-filtration on Sephadex LH-60.

isopropyl ether and the resulting powder was precipitated from MeOH with ether; yield 0.76 g (62%), mp 93—95 °C, $[\alpha]_D^{15}$ −24.2° (c = 1.0, DMF), Rf_1 0.46. Amino acid ratios in 6 N HCl hydrolysate: Ser 2.00, Glu 2.05, Pro 2.22, Ile 1.00 (recovery of Ile, 97%). *Anal.* Calcd for $C_{62}H_{80}N_{10}O_{15} \cdot H_2O$: C, 60.87; H, 6.76; N, 11.45. Found: C, 60.73; H, 6.59; N, 11.19.

Synthesis of Protected oCRF——Successive azide condensations of the ten fragments were carried out according to the indicated route (Fig. 1). Prior to condensation, the Z(OMe) group was removed from the respective amino component by treatment with TFA (*ca.* 1 ml per 0.1 g of the peptide) in the presence of anisole (*ca.* 10 eq) in an ice-bath for 60 min. The TFA-treated sample was precipitated with dry ether, dried over KOH pellets *in vacuo* for 2 h and dissolved in DMF or DMF–DMSO (1 : 1, for condensations of [2], [3], [4], and [6]) containing Et_3N (1 eq). The corresponding azide (the amount was increased from 1.1 to 7 eq as chain elongation progressed) in DMF and Et_3N (1 eq) were added to the above ice-chilled solution and the mixture was stirred at 4 °C (condensations from [1] to [7]) or −18 °C (condensations from [8] to [10]) as described in the text, until the solution became negative to the ninhydrin test. H_2O was added and the resulting powder was purified by precipitation from DMF with MeOH (procedure C) or by gel-filtration on Sephadex LH-60 using DMF as an eluant (procedure D). In the latter case, eluates (10 ml each) were examined by measuring the ultraviolet absorption (UV) at 280 nm and the fractions corresponding to the front main peak were combined. The solvent was removed by evaporation and the residue was treated with ether to form a powder. Purification procedures, yields, physical constants and analytical data of protected oCRF and its protected intermediates are listed in Table II.

H–Ser–Gln–Glu–Pro–Pro–Ile–Ser–Leu–Asp–Leu–Thr–Phe–His–Leu–Leu–Arg–Glu–Val–Leu–Glu–Met–Thr–Lys–Ala–Asp–Gln–Leu–Ala–Gln–Gln–Ala–His–Ser–Asn–Arg–Lys–Leu–Leu–Asp–Ile–Ala–NH_2 (oCRF)——Protected oCRF (55 mg, 8.7 μmol) was treated with 1 M TMSOTf–thioanisole in TFA (3.9 ml) in the presence of *m*-cresol (128 μl, 140 eq) in an ice-bath for 2 h, then dry ether was added. The resulting powder was collected by centrifugation, dried over KOH pellets *in vacuo* for 2 h and dissolved in H_2O–MeOH (5 ml–1 ml). The pH of the ice-chilled solution was adjusted to 8.0 with 5% NH_4OH containing NH_4F (35 eq) and after 30 min, to 6.0 with 1 N AcOH. Dithiothreitol (135 mg, 100 eq) was added and the solution, after being incubated at 37 °C for 12 h, was applied to a column of Sephadex G-50 (2.4 × 133 cm), which was eluted with 1 N AcOH. Individual fractions (5.5 ml) was examined by the Folin–Lowry test[26] (optical density at 750 nm). The fractions corresponding to the front main peak (tube Nos. 34—

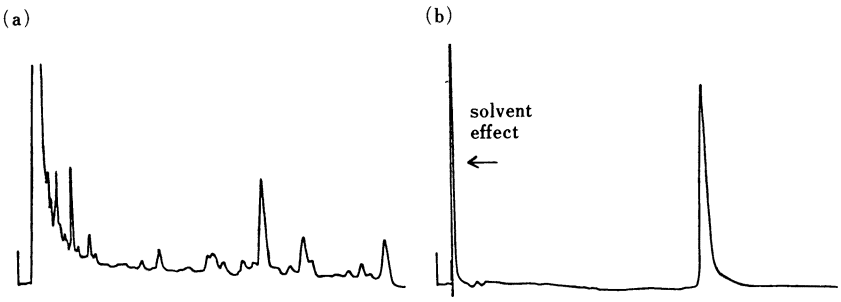

Fig. 2. HPLC of Synthetic oCRF (OD at 233 nm)
(a) CM-Purified sample. (b) Purified synthetic oCRF.

47) were combined and the solvent was removed by lyophilization to give a fluffy powder; yield 37 mg (91%).

The crude product thus obtained (15 mg) was dissolved in pH 5.0, 0.02 M $AcONH_4$ buffer (5 ml) containing 3 M urea and the solution was applied to a column of CM-cellulose (1.6 × 4 cm), which was eluted with pH 5.0, 0.2 M $AcONH_4$ (200 ml) containing 3 M urea through a mixing flask containing the starting buffer (100 ml). The fractions (4 ml each) corresponding to the main peak (tube Nos. 23—27, monitored by the Folin–Lowry test) were combined and the solvent was removed by lyophilization. The residue was dissolved in 5% acetonitrile in 1 N AcOH and the solution was applied to a column of Diaion HP-20 (1.6 × 5 cm), which was first washed with the same solvent (120 ml) to remove urea and the salt and then eluted with a gradient of acetonitrile (5 to 50%) in 1 N AcOH (300 ml). The fractions (4 ml each) corresponding to the main peak (tube Nos. 35—60, monitored by the Folin–Lowry test) were combined and the solvent was removed by lyophilization to give a white fluffy powder. The rest of the crude sample was similarly purified; total yield 15.0 mg (37%).

Subsequent purification was performed by reverse-phase HPLC on a Vydac 5C18 column (4.6 × 250 mm). The above CM-purified sample was dissolved in 0.5 N AcOH (0.7 ml) containing 5% 2-mercaptoethanol and the solution was incubated at 37 °C for 12 h in order to reduce the Met(O) residue formed during manipulation. The solution (100 μl each) was applied to the HPLC column, which was eluted with a gradient of acetonitrile (32 to 37% in 60 min) in 0.1% TFA at a flow rate of 0.8 ml/min. The eluate corresponding to the main peak (Fig. 2a; retention time, 45.3 min) was collected and the solvent was removed by lyophilization to give a white fluffy powder; yield 0.3 mg. The rest of the sample was similarly purified; total yield, 4.1 mg; overall yield from protected oCRF, 10.1%. $[\alpha]_D^{15} -136.4°$ ($c=0.03$, 1 N AcOH); Rf_4 0.43, Rf_5 0.36; retention time 45.3 min in HPLC on a Vydac 5C18 column (4.6 × 250 mm) by gradient elution as stated above (Fig. 2b). Amino acid ratios in a 6 N HCl hydrolysate are listed in Table I. Amino acid ratios in an LAP digest (numbers in parentheses are theoretical): Asp 3.23 (3), Thr 1.78 (2), Ser 1.86 (2), Glu 2.41 (3), Pro 0.75 (2), Ala 3.80 (4), Val 1.09 (1), Met 0.81 (1), Ile 1.58 (2), Leu 7.47 (8), Phe 1.10 (1), Lys 2.00 (2), His 1.67 (2), Arg 1.95 (2), Asn (1) and Gln (4) were not determined (recovery of Lys 70%, incomplete digestion of the Glu–Pro–Pro–Ile sequence was presumably due to poor prolidase activity of this enzyme preparation).

References and Notes

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2) Amino acids used in this investigation are of the L-configuration. The following abbreviations are used. Bzl = benzyl, Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Mts = mesitylenesulfonyl, Chp = cycloheptyl, Troc = 2,2,2-trichloroethyloxycarbonyl, TFA = trifluoroacetic acid, TMSOTf = trimethylsilyl trifluoromethanesulfonate, THF = tetrahydrofuran, DMF = dimethylformamide, DMSO = dimethylsulfoxide, EDTA = ethylenediaminetetraacetic acid disodium salt, CM = carboxymethyl.
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