Studies on Peptides. XLV.^{1,2)} Synthesis of the Bovine Type Corticotropin-like Intermediate Lobe Peptide (CLIP)

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Synthesis of the docosapeptide, H-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu-Phe-OH, corresponding to bovine type corticotropin-like intermediate lobe peptide (CLIP), was described.

Recently, Scott, *et al.*⁴⁾ reported the isolation of a new ACTH-like peptide from the pars intermedia of pig pituitary. This peptide, the sequence of which is corresponding to positions 18 to 39 of its origin, was termed as corticotropin-like intermediate lobe peptide (CLIP). They suggested that this immunoreactive peptide and α -melanocyte-stimulating hormone are formed by the intracellular cleavage of ACTH and added an important concept in endocrine chemistry that ACTH may be not only a hormone in its own right but the precursor (prohormone) of other biologically active peptides. Thus the above finding became relevant to the recent development in the field of proinsulin⁵⁾ and proglucagon.⁶⁾

ACTH's so far known in pig, man,^{7,8)} sheep and beef⁹⁾ are different each other, in part, in their C-terminal portions and the sequences around these areas have recently revised.

Fig. 1. Synthetic Route to the Bovine Type CLIP

- 1) Part XLIV: H. Yajima and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **22**, 1087 (1974).
- 2) Amino acids, peptides and their derivatives, except Gly, are of the L-configuration. Abbreviation used are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, ONP = *p*-nitrophenyl ester, OPCP = pentachlorophenyl ester, OSU = N-hydroxysuccinimide ester.
- 3) Location: Sakyo-ku, Kyoto.
- 4) A.P. Scott, J.G. Ratcliffe, L.H. Rees, J. Landon, H.P.J. Bennett, P.J. Lowry and C. McMartin, *Nature New Biol.*, **244**, 65 (1973).
- 5) R.E. Chance, R.M. Ellis and W.W. Bromer, *Science*, **161**, 165 (1968); J. Markussen and F. Sundby, *Eur. J. Biochem.*, **34**, 401 (1973). See other references thereof.
- 6) H.S. Tager and D.F. Steiner, *Proc. Nat. Acad. Sci. U.S.A.*, **70**, 2321 (1973).
- 7) L. Gráf, S. Bajusz, A. Patthy, E. Barat and G. Cseh, *Acta Biochim. Biophys. Acad. Sci. Hung.*, **6**, 415 (1971).
- 8) B. Riniker, P. Sieber, W. Rittel and H. Zuber, *Nature New Biol.*, **235**, 114 (1972).
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From the above informations, it is evident that mammalian CLIP's vary somewhat from species to species. Indeed Scott, *et al.*⁵⁾ confirmed the presence of different CLIP's in rat and human pituitaries respectively.

We expect to report a series of syntheses of CLIP's in various mammalian species and in this paper, we wish to describe the synthesis of docosapeptide which should be defined as the bovine CLIP.

Our synthesis was led by the newly revised sequence of bovine ACTH⁹⁾ as shown in Fig. 1, in which 3 subunits: Z-Arg(NO₂)-Pro-OH (I), Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH (II) and Z(OMe)-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, served as stems to construct the entire sequence of the bovine type CLIP. Among those, syntheses of Z-Arg(NO₂)-Pro-OH^{10,11)} and Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH¹²⁾ were previously reported.

Synthetic route to the C-terminal pentadecapeptide ester (III), abbreviated as Z(OMe)-(b-ACTH 25-39)-OBzl, is illustrated in Fig. 2. The tripeptide ester, Z(OMe)-Leu-Glu(OBzl)-Phe-OBzl (IV, positions 37 to 39) and the protected hexapeptide, Z(OMe)-Ser-Ala-Gln-Ala-Phe-Pro-OH (V, positions 31 to 36), were first prepared and after assembling these two fragments, elongation of the resulting nonapeptide chain was achieved in a stepwise manner to the pentadecapeptide (III) by the active ester procedure.¹³⁾

Fig. 2. Synthetic Route to the C-Terminal Pentadecapeptide Ester, Z(OMe)-(b-ACTH 25-39)-OBzl

The protected tripeptide ester, Z(OMe)-Leu-Glu(OBzl)-Phe-OBzl (IV) was synthesized by the dicyclohexylcarbodiimide (DCC) procedure¹⁴⁾ in a stepwise manner starting from H-Phe-OBzl. Chromatography on silica was effective in purifying this tripeptide (IV) and the

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12) H. Yajima and H. Kawatani, *Chem. Pharm. Bull.* (Tokyo), **19**, 1905 (1971).

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14) J.C. Sheehan and G.P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).

intermediate, Z(OMe)-Glu(OBzl)-Phe-OBzl as well. Deprotection of the Z(OMe) group of this intermediate was performed by the trifluoroacetic acid (TFA) treatment¹⁵⁾ in the usual manner.

The synthesis of the hexapeptide, Z(OMe)-Ser-Ala-Gln-Ala-Phe-Pro-OH (V) was achieved by the successive azide coupling of three dipeptide units. The reaction of Z-Phe-OSU with the triethylammonium salt of H-Pro-OH gave Z-Phe-Pro-OH,^{16,17)} which after hydrogenation, was condensed with Z-Gln-Ala-NHNH₂ by the modified azide procedure.¹⁸⁾ Hydrogenation of the product, Z-Gln-Ala-Phe-Pro-OH, gave the free tetrapeptide, H-Gln-Ala-Phe-Pro-OH, in crystalline form. Its physical constants are in good agreement with literature values of the sample prepared by Bajusz and Lázár in an alternate stepwise manner. With this tetrapeptide, Z(OMe)-Ser-Ala-NHNH₂ was condensed again by the azide procedure. The product, Z(OMe)-Ser-Ala-Gln-Ala-Phe-Pro-OH (V), was obtained in analytically pure form after batchwise washing with a citric acid solution and recrystallization from dimethylformamide (DMF) and ethyl acetate. Such batchwise washing procedure was effective in purifying the products which were no longer extractable in ethyl acetate in the latter stage of the synthesis.

To assemble the two peptide units thus obtained, Z(OMe)-Leu-Glu(OBzl)-Phe-OBzl (IV) was treated with TFA in the presence of anisole as usual to remove the Z(OMe) group.¹⁵⁾ However the deprotected tripeptide ester was not precipitable by addition of ether. The cooled reaction mixture was basified with sodium carbonate. The product was extracted with ethyl acetate and then submitted to the coupling reaction with the protected hexapeptide (V) with DCC in the presence of N-hydroxybenztriazole (HOBT) according to König and Geiger.¹⁹⁾ Purification of the product by batchwise washing followed by recrystallization afforded an analytically pure sample of the protected nonapeptide ester, Z(OMe)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, in fairly good yield.

Combination of TFA treatment for the deprotection of the Z(OMe) group and the *p*-nitrophenyl ester procedure¹³⁾ was applied to the elongation of the nonapeptide in a stepwise manner to the pentadecapeptide stage (III) as mentioned earlier. In every step, the deprotected peptide esters were precipitated as fine powder by the addition of ether and the protected peptide esters, after coupling reactions, were isolated in analytically pure form in more than 80% yield. The purity of the protected pentadecapeptide ester, Z(OMe)-(b-ACTH 25-39)-OBzl (III), was confirmed by thin layer chromatography, elemental and amino acid analyses.

For the condensation of Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH (II) and Z(OMe)-(b-ACTH 25-39)-OBzl (III), the Z(OMe) group of the latter was cleaved by TFA in the presence of anisole. The resulting TFA salt was converted to the corresponding hydrochloride and subsequently neutralized with triethylamine. All of the TFA, hydrochloride salts and the free base could be handled as fine powder by precipitation with ether. The deblocked pentadecapeptide ester was coupled with (II) by DCC in the presence of HOBT and the resulting protected eicosapeptide ester, Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, abbreviated as Z(OMe)-(b-ACTH 20-39)-OBzl, was purified by batchwise washing with acid, base and methanol followed by recrystallization from DMF and ethyl acetate.

The protected eicosapeptide ester, Z(OMe)-(b-ACTH 20-39)-OBzl, after treatment with TFA followed by neutralization with triethylamine, was submitted to the condensation with Z-Arg(NO₂)-Pro-OPCP.¹¹⁾ The reaction underwent smoothly to give the protected

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17) a) S. Sakakibara and M. Itoh, *Bull. Chem. Soc. Japan*, **40**, 656 (1967); b) R.E. Neuman and E.L. Smith, *J. Biol. Chem.*, **193**, 97 (1951).

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19) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).

docosapeptide ester, Z-Arg(NO₂)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, which was purified by batchwise washing and recrystallization. This was then hydrogenated over a large excess of palladium catalyst to remove the Z, NO₂ and benzyl protecting groups. Especially, reductive removal of the NO₂ group from such a relatively large peptide required a long period. The deprotected product was then purified by column chromatography on CM-cellulose. Gradient elution consisting of pyridine acetate buffer was applied to elute the desired compound. The absorbancy due to the Tyr residue was the guide for this chromatographic purification. Homogeneity of the synthetic bovine type CLIP thus obtained was confirmed by thin-layer chromatography and elemental and amino acid analyses. Complete digestion of this peptide with leucine aminopeptidase was unsuccessful because of the presence of the Pro residue.²⁰⁾

Its biological properties will be reported together with those of other CLIP's of various mammalian species in future.

Experimental

General experimental methods employed are essentially the same as described in the Part XXII²¹⁾ of this series. Thin-layer chromatography was performed on silica gel (Kieselgel G, Merck). *R_f* values refer to the following solvent systems: *R_{f1}* CHCl₃-MeOH-H₂O (40:15:5), *R_{f2}* CHCl₃-MeOH-AcOH (9:1:0.5), *R_{f3}* *n*-butanol-pyridine-AcOH-H₂O (4:1:1:2).

Z(OMe)-Glu(OBzl)-Phe-OBzl—Z(OMe)-Glu(OBzl)-OH (12.0 g) and DCC (6.6 g) were added to a solution of H-Phe-OBzl (prepared from 10.1 g of the hydrobromide and 4.1 ml of Et₃N) in DMF (90 ml) under cooling with ice and the mixture was stirred at room temperature for 48 hr. After filtration, the filtrate was condensed *in vacuo* and the residue was dissolved in AcOEt, which was washed with 5% Na₂CO₃, 5% citric acid and H₂O, dried over Na₂SO₄ and then evaporated. Tritulation of the residue gave solid which was recrystallized from AcOEt and ether. For further purification, the product was applied to a column on silica (3 × 20 cm), which was eluted with CHCl₃. The first cup gave the solid which was recrystallized from AcOEt and ether; yield 13.4 g (70%), mp 107–111°, [α]_D²⁵ –3.5° (*c*=1.0, DMF). *R_{f1}* 1.0, *R_{f2}* 0.78. *Anal.* Calcd. for C₃₇H₃₈O₈N₂: C, 69.58; H, 6.00; N, 4.39. Found: C, 69.72; H, 6.06; N, 4.69.

Z(OMe)-Leu-Glu(OBzl)-Phe-OBzl (IV)—Z(OMe)-Glu(OBzl)-Phe-OBzl (13.0 g) was treated with TFA (13 ml) in the presence of anisole (6 ml) for 45 min. The product was precipitated with petroleum ether and the gummy precipitate was washed with dry ether. A solution of 1N HCl-dioxane (20 ml) was added and the solvent was evaporated *in vacuo*. The residue, after drying over KOH pellets *in vacuo* overnight, was dissolved in DMF (100 ml). To this solution, Z(OMe)-Leu-OH (5.8 g) and DCC (4.1 g) were added and the mixture was stirred for 48 hr. The solution was filtered and then evaporated to give an oily residue, which was dissolved in AcOEt. The organic phase, after washing with 5% Na₂CO₃, 5% citric acid and H₂O, was dried over Na₂SO₄ and then evaporated. The resulting semi-solid was applied to a column chromatography on silica (3 × 20 cm), which was eluted with CHCl₃. The desired compound emerged from the column was recrystallized from AcOEt and ether; yield 9.9 g (66%), mp 87–88°, [α]_D²⁵ –10.0° (*c*=1.0, MeOH), *R_{f1}* 1.0, *R_{f2}* 0.57. Amino acid ratios in an acid hydrolysate; Leu_{0.89} Glu_{1.00} Phe_{1.05} (average recovery 99%). *Anal.* Calcd. for C₄₃H₄₉O₉N₃: C, 68.68; H, 6.57; N, 5.59. Found: C, 68.42; H, 6.61; N, 5.61.

Z-Phe-Pro-OH—Because of difficulty in purification, the title compound was prepared as follows instead of the ONP method.¹⁶⁾ To a solution of H-Pro-OH (11.1 g) and Et₃N (27 ml) in a mixture of H₂O (100 ml) and tetrahydrofuran (200 ml), Z-Phe-OSU (35.0 g) was added and the mixture was stirred at room temperature overnight. After evaporation of the solvent, the residue was dissolved in H₂O, which after washing with ether, was acidified with 1N HCl and the resulting precipitate was extracted with AcOEt. The organic phase was washed with H₂O-NaCl, dried over Na₂SO₄ and then evaporated. The solid formed by addition of petroleum ether was recrystallized from AcOEt and ether; yield 28.0 g (72%), mp 108–110°, [α]_D²⁵ –31.0° (*c*=1.1, DMF). (lit.¹⁶⁾ 109–110°, lit.^{17a)} mp 105–106.5°, [α]_D²⁵ –65.4° in pyridine, lit.^{17b)} mp 109.5–110.5°. *R_{f1}* 0.60. *Anal.* Calcd. for C₂₂H₂₄O₅N₂: C, 66.65; H, 6.10; N, 7.07. Found: C, 66.40; H, 6.23; N, 7.10.

H-Phe-Pro-OH—Z-Phe-Pro-OH was hydrogenated according to Bajusz and Lázár.¹⁶⁾ mp 127–129°, [α]_D²⁵ –26.2° (*c*=1.0, H₂O). (lit.¹⁶⁾ mp 127–129°, [α]_D²⁵ –22.05° in H₂O). *R_{f1}* 0.20. *Anal.* Calcd. for C₁₄H₁₈O₃N₂: C, 64.10; H, 6.92; N, 10.68. Found: C, 64.33; H, 6.93; N, 10.82.

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21) H. Yajima, Y. Okada, H. Kawatani and N. Mizokami, *Chem. Pharm. Bull.* (Tokyo), **17**, 1229 (1969).

Z-Gln-Ala-OMe—The title compound was prepared by the mixed anhydride procedure²²⁾ using ethyl chloroformate; yield 80%, mp 210–211°, $[\alpha]_D^{25} -12.2^\circ$ ($c=0.4$, DMF). R_f 0.65. *Anal.* Calcd. for $C_{17}H_{23}O_6N_3$: C, 55.88; H, 6.35; N, 11.50. Found: C, 55.66; H, 6.31; N, 11.63.

Z-Gln-Ala-NHNH₂—Z-Gln-Ala-OMe was converted to the corresponding hydrazide in the usual manner; yield 93%, mp 228–230°. R_f 0.50. *Anal.* Calcd. for $C_{16}H_{23}O_5N_5$: C, 52.59; H, 6.35; N, 19.17. Found: C, 52.40; H, 6.34; N, 19.44.

Z-Gln-Ala-Phe-Pro-OH—Z-Gln-Ala-NHNH₂ (12.6 g) was dissolved in DMF (80 ml) with an aid of 1.1N HCl-DMF (62 ml). To this solution, isoamylnitrite (4.6 ml) was added under cooling with ice-NaCl. The solution was stirred for 5 min, while the hydrazine test²³⁾ became negative. After addition of Et₃N (14 ml), the solution was combined with a solution of H-Phe-Pro-OH (9.2 g) and Et₃N (4.9 ml) in 50% aqueous DMF (80 ml). After stirring at 4° for 48 hr, the solution was condensed and the residue was dissolved in 3% NH₄OH, which was washed with AcOEt. The aqueous phase was acidified with 1N HCl and the resulting precipitate was extracted with AcOEt, which after washing with H₂O-NaCl, dried over Na₂SO₄ and then evaporated to give gelatinous mass. It was recrystallized from MeOH and AcOEt; yield 15.0 g (75%), mp 116–118°, $[\alpha]_D^{25} -27.2^\circ$ ($c=1.0$, DMF). R_f 0.45. *Anal.* Calcd. for $C_{30}H_{37}O_8N_5 \cdot 1/2H_2O$: C, 59.58; H, 6.34; N, 11.58. Found: C, 59.46; H, 6.53; N, 11.44.

H-Gln-Ala-Phe-Pro-OH—In the usual manner, Z-Gln-Ala-Phe-Pro-OH (30.0 g) in 98% MeOH (300 ml) was hydrogenated over a Pd catalyst. The white crystalline product formed during hydrogenolysis was dissolved by heating and the catalyst was removed by filtration. The filtrate was condensed and AcOEt was added to the residue. The crude product thus obtained was recrystallized from MeOH and AcOEt; yield 21.85 g (95%), mp 145–147°, $[\alpha]_D^{25} -62.9^\circ$ ($c=1.0$, H₂O). (lit.¹⁶⁾ mp 150–152°, $[\alpha]_D^{20} -68.8^\circ$ in H₂O). R_f 0.04, R_f 0.53. *Anal.* Calcd. for $C_{22}H_{31}O_6N_5 \cdot H_2O$: C, 55.10; H, 6.94; N, 14.61. Found: C, 55.14; H, 6.67; N, 13.42.

Z(OMe)-Ser-Ala-OMe—Under cooling (–5°), isoamylnitrite (13.3 ml) was added dropwise to a solution of Z(OMe)-Ser-NHNH₂ (28.3 g) in 1N HCl-DMF (100 ml). After stirring for 5 min, when the hydrazine test became negative, the solution was neutralized with Et₃N (27.6 ml). This solution was then combined with a solution of H-Ala-OMe (prepared from 13.9 g of the hydrochloride with 27.6 ml of Et₃N) in DMF (100 ml) and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 5% citric acid, 5% Na₂CO₃ and H₂O, dried over Na₂SO₄ and then evaporated. The resulting solid was recrystallized from MeOH and AcOEt; yield 21.7 g (61%), mp 146–148°, $[\alpha]_D^{25} -2.9^\circ$ ($c=0.9$, DMF). R_f 0.68. *Anal.* Calcd. for $C_{16}H_{22}O_7N_2$: C, 54.23; H, 6.26; N, 7.91. Found: C, 54.51; H, 6.41; N, 7.94.

Z(OMe)-Ser-Ala-NHNH₂—Z(OMe)-Ser-Ala-OMe (11.5 g) in MeOH (100 ml) was converted to the corresponding hydrazide in the usual manner. It was recrystallized from MeOH; yield 10.8 g (94%), mp 212–214°. *Anal.* Calcd. for $C_{15}H_{22}O_6N_4$: C, 50.84; H, 6.26; N, 15.81. Found: C, 50.60; H, 6.04; N, 15.66.

Z(OMe)-Ser-Ala-Gln-Ala-Phe-Pro-OH (V)—Under cooling with ice-NaCl, 1N HCl-DMF (4.8 ml) and isoamylnitrite (3.2 ml) were added successively to a solution of Z(OMe)-Ser-Ala-NHNH₂ (8.50 g). After stirring for 10 min, when the hydrazine test of the solution became negative, Et₃N (6.7 ml) was added. This solution was then combined with a solution of H-Gln-Ala-Phe-Pro-OH (9.23 g) in H₂O (60 ml) containing Et₃N (5.6 ml) and the mixture was stirred at 4° for 48 hr. A few drop of AcOH was added and the solvent was evaporated *in vacuo*. The solid formed by addition of ether to the residue, was collected by filtration, washed batchwisely with AcOEt, 5% citric acid and H₂O and recrystallized from DMF and AcOEt; yield 10.2 g (64%), mp 187–189°, $[\alpha]_D^{25} -22.1^\circ$ ($c=0.9$, DMF), R_f 0.28. Amino acid ratios in an acid hydrolysate; Ser_{0.91} Ala_{2.34} Glu_{1.20} Phe_{1.00} Pro_{1.05} (average recovery 92%). *Anal.* Calcd. for $C_{37}H_{49}O_{12}N_7 \cdot H_2O$: C, 55.42; H, 6.41; N, 12.23. Found: C, 55.49; H, 6.53; N, 12.34.

Z(OMe)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl—Z(OMe)-Leu-Glu(OBzl)-Phe-OBzl (5.64 g) was treated with TFA (6 ml) in the presence of anisole (2.5 ml) at 0° for 60 min. Under cooling with ice-NaCl, 5% Na₂CO₃ was added and the resulting precipitate was extracted with AcOEt, which was washed with 5% Na₂CO₃ and H₂O-NaCl and dried over Na₂SO₄. After filtration, the filtrate was combined with a solution of Z(OMe)-Ser-Ala-Gln-Ala-Phe-Pro-OH (3.92 g) in DMF (40 ml). To this solution, DCC (1.85 g) and HOBT (1.57 g) were added and the mixture was stirred at room temperature for 48 hr. The solvent, after filtration, was evaporated *in vacuo*. Addition of AcOEt to the residue afforded the solid, which was washed batchwisely with 5% citric acid, 5% Na₂CO₃, H₂O and ether and then recrystallized from DMF and AcOEt; yield 5.03 g (71%), mp 205–207°, $[\alpha]_D^{25} -29.2^\circ$ ($c=1.2$, DMF), R_f 0.61. Amino acid ratios in an acid hydrolysate; Ser_{0.76} Ala_{2.00} Glu_{2.00} Phe_{1.73} Pro_{0.85} Leu_{1.00} (average recovery 100%). *Anal.* Calcd. for $C_{71}H_{88}O_{17}N_{10} \cdot 3H_2O$: C, 60.56; H, 6.73; N, 9.95. Found: C, 60.74; H, 6.56; N, 10.02.

Z(OMe)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl—In the usual manner, the above protected nonapeptide ester (4.06 g) was treated with TFA (5 ml) in the presence of anisole (3 ml)

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23) H.E. Ertel and L. Horner, *J. Chromatog.*, **7**, 268 (1962).

at 0° for 60 min, when dry ether was added. The resulting powder was collected by filtration, washed thoroughly with ether and then dissolved in DMF (40 ml). To this solution, Z(OMe)-Glu(OBzl)-ONP (3.32 g) and Et₃N (0.8 ml) were added, the mixture was stirred at room temperature for 48 hr and then the solvent was evaporated *in vacuo*. Addition of ether to the residue afforded the solid, which was washed batchwisely with 5% citric acid, 5% Na₂CO₃, H₂O and ether and then recrystallized from DMF and AcOEt; yield 4.56 g (95%), mp 132–136°, $[\alpha]_D^{25}$ –27.5° ($c=1.2$, DMF), R_f 0.63. *Anal.* Calcd. for C₈₃H₁₀₁O₂₀N₁₁·2H₂O: C, 61.95; H, 6.58; N, 9.35. Found: C, 61.84; H, 6.34; N, 9.64.

Z(OMe)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl—The above protected decapeptide ester (3.15 g) was treated with TFA (3 ml) in the presence of anisole (2 ml) as stated above. The TFA salt precipitated by addition of dry ether was collected by filtration, washed with ether and then dissolved in DMF (30 ml). Z(OMe)-Asp(OBzl)-ONP (1.53 g) and Et₃N (0.28 ml) were then combined and the mixture was stirred at room temperature for 48 hr, when the solvent was evaporated *in vacuo*. Addition of ether to the residue gave the solid, which was washed batchwisely with base, acid, H₂O and ether as stated above and then recrystallized from DMF and ether; yield 3.67 g (84%), mp 149–152°, $[\alpha]_D^{25}$ –21.7° ($c=0.9$, DMF), R_f 0.62. *Anal.* Calcd. for C₉₄H₁₁₂O₂₃N₁₂·3H₂O: C, 61.62; H, 6.31; N, 9.18. Found: C, 61.77; H, 6.49; N, 9.26.

Z(OMe)-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl—The above protected undecapeptide ester (3.02 g) was treated with TFA (4 ml) in the presence of anisole (2 ml) in the usual manner. The TFA salt precipitated by addition of dry ether was collected by filtration, washed with ether and then dissolved in DMF (30 ml). To this solution, Z(OMe)-Glu(OBzl)-ONP (1.88 g) and Et₃N (0.48 ml) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated *in vacuo*. Addition of ether and AcOEt to the residue gave the solid, which was washed batchwisely with base, acid, H₂O and EtOH as stated above and then recrystallized from DMF and ether; yield 2.80 g (79%), mp 165–168°, $[\alpha]_D^{25}$ –23.9° ($c=1.0$, DMF). R_f 0.60. *Anal.* Calcd. for C₁₀₆H₁₂₅O₂₆N₁₃·5H₂O: C, 61.00; H, 6.52; N, 8.72. Found: C, 61.16; H, 6.29; N, 9.12.

Z(OMe)-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl—The above protected dodecapeptide ester (3.43 g) was treated with TFA (5 ml) in the presence of anisole (2 ml) in the usual manner. The TFA salt precipitated by addition of dry ether was collected by filtration, washed with ether, and then dissolved in DMF (30 ml). To this solution, Z(OMe)-Ala-ONP (1.35 g) and Et₃N (0.5 ml) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated *in vacuo* and the residue was treated with ether to afford the solid, which was washed batchwisely with base, acid, H₂O and EtOH as stated above and then recrystallized from DMF and ether; yield 3.21 g (84%), mp 192–196°, $[\alpha]_D^{25}$ –22.2° ($c=0.9$, DMF), R_f 0.67. Amino acid ratios in an acid hydrolysate; Ala_{3.12}Glu_{3.89}Asp_{1.09}Ser_{0.75}Phe_{1.92}Pro_{1.41}Leu_{1.00} (average recovery 99%). *Anal.* Calcd. for C₁₀₉H₁₃₀O₂₇N₁₄·3H₂O: C, 61.69; H, 6.46; N, 9.24. Found: C, 61.59; H, 6.29; N, 9.47.

Z(OMe)-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl—The above protected tridecapeptide ester (3.04 g) was treated with TFA (4 ml) in the presence of anisole (2 ml) in the usual manner. The TFA salt precipitated by addition of dry ether, was collected by filtration, washed thoroughly with ether and then dissolved in DMF (30 ml). To this solution, Z(OMe)-Gly-ONP (1.08 g) and Et₃N (0.4 ml) were added and the mixture was stirred at room temperature for 48 hr. The solvent was evaporated *in vacuo*. Addition of ether to the residue gave the solid, which was washed batchwisely with base, acid, H₂O and ether and then recrystallized from DMF and EtOH; yield 2.79 g (86%), mp 199–201°, $[\alpha]_D^{25}$ –20.9° ($c=1.0$, DMF), R_f 0.55. *Anal.* Calcd. for C₁₁₁H₁₃₃O₂₈N₁₅·4H₂O: C, 60.67; H, 6.19; N, 9.56. Found: C, 60.72; H, 6.46; N, 10.00.

Z(OMe)-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, **Z(OMe)-(b-ACTH 25-39)-OBzl**—In the usual manner, the above protected tetradecapeptide ester (2.55 g) was treated with TFA (5 ml) in the presence of anisole (2 ml). The TFA salt precipitated by addition of dry ether, was washed with ether and then dissolved in DMF (25 ml). Z(OMe)-Asn-ONP (1.0 g) and Et₃N (0.34 ml) were added to the solution, which was stirred at room temperature for 48 hr. The solvent was evaporated and the residue was treated with ether and AcOEt. The resulting powder was washed batchwisely with base, acid, H₂O and EtOH and recrystallized from tetrahydrofuran and ether; yield 2.34 g (87%), mp 199.5–202°, $[\alpha]_D^{25}$ –24.5° ($c=0.9$, DMF), R_f 0.53. Amino acid ratios in an acid hydrolysate; Asp_{2.08}Gly_{1.23}Ala_{3.00}Glu_{3.87}Ser_{0.87}Phe_{2.24}Leu_{1.00}Pro_{0.98} (average recovery 99%). *Anal.* Calcd. for C₁₁₅H₁₃₆O₃₀N₁₇: C, 58.87; H, 5.87; N, 10.64. Found: C, 58.86; H, 5.99; N, 10.92.

Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, **Z(OMe)-(b-ACTH 20-39)-OBzl**—The above protected pentadecapeptide ester (2.24 g) was treated with TFA (5 ml) in the presence of anisole (2 ml) at 0° for 60 min, when dry ether was added. The resulting powder was collected by filtration, washed with ether and dissolved in 0.5N HCl-DMF (4 ml). The solvent was evaporated *in vacuo*. Addition of dry ether afforded the powder, which was dissolved in a small amount of DMF and Et₃N (0.4 ml) was added. The solvent was again evaporated *in vacuo* and dry ether was added to give the solid, which was collected by filtration, washed with ether and then dissolved in DMF (20 ml). To this solution, Z(OMe)-Val-Lys(Z)-Val-Tyr-Pro-OH⁽¹²⁾ (1.36 g), HOBT (0.35 g) and DCC (0.41 g) were combined. The mixture was stirred at room temperature for 48 hr

and then filtered. The filtrate was condensed *in vacuo*. Addition of AcOEt to the residue afforded the powder, which was washed batchwisely with 5% citric acid, 5% Na₂CO₃, H₂O, AcOEt and hot MeOH and then recrystallized from DMF and AcOEt; yield 2.47 g (83%), mp 177—179°, $[\alpha]_D^{25} -24.1^\circ$ ($c=1.1$, DMF), Rf_1 0.56. Amino acid ratios in an acid hydrolysate; Val_{1.98}Lys_{1.05}Tyr_{0.25}Pro_{2.23}Asp_{2.21}Gly_{1.04}Ala_{3.00}Glu_{3.80}Ser_{0.83}Phe_{1.83}Leu_{0.93} (average recovery 84%). *Anal.* Calcd. for C₁₅₃H₁₉₁O₃₈N₂₃·7H₂O: C, 59.54; H, 6.70; N, 10.50. Found: C, 59.47; H, 6.53; N, 10.94.

Z(OMe)-Arg(NO₂)-Pro-Val-Lys(Z)-Val-Tyr-Pro-Asn-Gly-Ala-Glu(OBzl)-Asp(OBzl)-Glu(OBzl)-Ser-Ala-Gln-Ala-Phe-Pro-Leu-Glu(OBzl)-Phe-OBzl, **Z(OMe)-(b-ACTH 18-39)-OBzl**—Z(OMe)-(b-ACTH 20-39)-OBzl (0.44 g) was treated with TFA (1.3 ml) in the presence of anisole (0.3 ml) at 0° for 45 min, when dry ether was added. The resulting powder was dissolved in DMF (10 ml) and to this solution, Et₃N (0.04 ml) and Z-Arg(NO₂)-Pro-OPCP (0.13 g) were added. The solution, after stirring at room temperature for 72 hr, was condensed *in vacuo* and the residue was treated with ether. The resulting powder was washed with 5% citric acid and H₂O and then recrystallized from tetrahydrofuran and AcOEt; yield 0.31 g, mp 148—150°, $[\alpha]_D^{25} -30.4^\circ$ ($c=0.6$, DMF). Rf_1 0.45, Rf_3 0.83. Amino acid ratios in an acid hydrolysate: Arg_{0.77}Pro_{3.01}Val_{2.03}Lys_{1.32}Tyr_{0.38}Asp_{2.19}Gly_{1.29}Ala_{3.00}Glu_{4.22}Ser_{0.87}Phe_{1.74}Leu_{0.98} (average recovery 91%).²⁴⁾ *Anal.* Calcd. for C₁₆₃H₂₀₇O₄₁N₂₉·10H₂O: C, 57.43; H, 6.71; N, 11.92. Found: C, 57.30; H, 6.71; N, 11.42.

H-Arg-Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Ser-Ala-Glu-Ala-Phe-Pro-Leu-Glu-Phe-OH (Bovine Type CLIP)—Z(OMe)-(b-ACTH 18-39)-OBzl (0.34 g) obtained above in 70% aqueous MeOH containing a few drop of AcOH, was hydrogenated over a Pd catalyst (approximately 0.6 g) at room temperature for 40 hr, until a ninhydrin and Sakaguchi positive spot appeared on thin-layer chromatography. The solution was filtered, the filtrate was evaporated to dryness and the residue, after lyophilization, was applied to a column of CM-cellulose (1.7 × 14 cm), which was eluted with H₂O (160 ml) and then 0.05N pyridine acetate buffer (450 ml) through a mixing flask containing H₂O (100 ml). Absorbancy at 275 mμ in individual fractions (10 ml each) was examined. A small peak present in H₂O eluates (ninhydrin positive, but Sakaguchi negative) was discarded. Fractions corresponding to the main peak, present in the gradient eluates (tube No. 20—30) were pooled, the solvent was evaporated *in vacuo* and the residue was lyophilized to give fluffy powder; yield 103 mg (40%), $[\alpha]_D^{20} -108.9^\circ$ ($c=0.1$, H₂O), Rf_3 0.54. Amino acid ratios in an acid hydrolysate: Arg_{1.04}Pro_{3.14}Val_{2.38}Lys_{1.01}Tyr_{0.93}Asp_{2.03}Gly_{0.95}Glu_{4.26}Ser_{0.75}Ala_{3.00}Phe_{1.91}Leu_{0.90} (average recovery 90%). *Anal.* Calcd. for C₁₁₂H₁₆₆O₃₅N₂₈·3CH₃COOH·11H₂O: C, 49.84; H, 7.09; N, 13.80. Found: C, 49.71; H, 6.58; N, 13.65.

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24) Low recovery of Tyr in an acid hydrolysate of protected peptides was mentioned by B. Iselin, *Helv. Chim. Acta*, **45**, 1510 (1962). By acid, Arg(NO₂) was converted to Arg and Orn, which overlapped with the Lys peak. Recovery of Lys was uncorrected.