

[Chem. Pharm. Bull.]
34(10)4362—4367(1986)

Studies on Peptides. CXLIV.^{1,2)} Synthesis and Immunological Properties of Formylmethionyl Human Adrenocorticotropin

KOICHI YASUMURA,^a KENJI OKAMOTO,^a HIDEKI ADACHI,^b
KENICHI AKAJI,^c and HARUAKI YAJIMA^{*,c}

Kyoto Pharmaceutical University,^a Yamashina-ku, Kyoto 607, Japan, Faculty of Medicine,
Kyoto University,^b Sakyo-ku, Kyoto 606, Japan and Faculty of Pharmaceutical
Sciences, Kyoto University,^c Sakyo-ku, Kyoto 606, Japan

(Received May 15, 1986)

Formylmethionyl human adrenocorticotropin (For-Met-hACTH) was synthesized by assembling 4 peptide fragments followed by deprotection with 1 M trifluoromethanesulfonic acid-thioanisole in trifluoroacetic acid. When the immunological cross reactivity against anti-ACTH sera was examined, some difference was observed between For-Met-hACTH and synthetic hACTH.

Keywords—For-Met-hACTH synthesis; *N*^G-mesitylenesulfonylarginine; thioanisole-mediated deprotection; trifluoromethanesulfonic acid deprotection; immunological cross reactivity

Recently the recombinant deoxyribonucleic acid (DNA) method³⁾ has opened a new approach to the synthesis of polypeptides. In many cases, a triplet code, ATG, must be added to the eukaryotic sequence in order to initiate translation in procaryotic cells.⁴⁾ Consequently, peptides expressed by *E. coli*, for example, possess unnatural formylmethionine (For-Met) or Met at the N-terminus.⁵⁾ The cyanogen bromide cleavage of Met⁶⁾ is impossible for peptides containing internal Met residues. Thus, it seems worthwhile to examine whether peptides possessing such extra For-Met or Met exhibit immunologically the same properties as the parent molecules. As an example we have synthesized chemically For-Met human adrenocorticotropin (For-Met-hACTH) and compared its immunological properties with those of hACTH.⁷⁾

The synthetic route to For-Met-hACTH is shown in Fig. 1. Four fragments, [1] to [4], were selected as building blocks to construct this 40-residue peptide. Of these, two fragments, [1] and [3], are known common intermediates used for our previous syntheses of hACTH⁸⁾

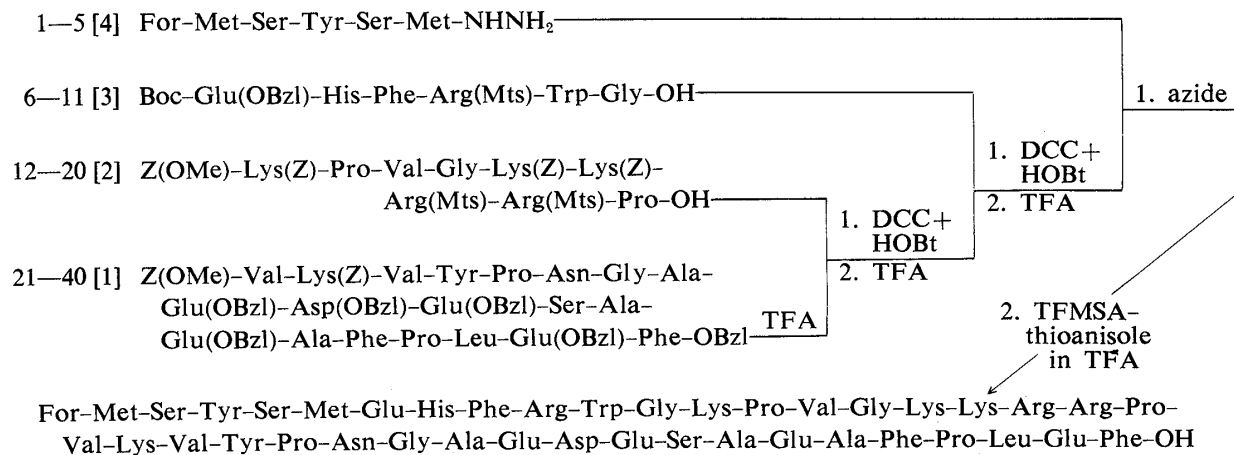


Fig. 1. Synthetic Route to For-Met-human ACTH

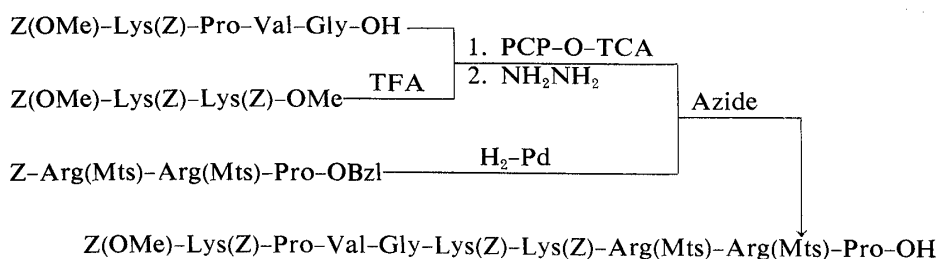


Fig. 2. Synthetic Scheme for the Protected Nonapeptide (Positions 12–20)

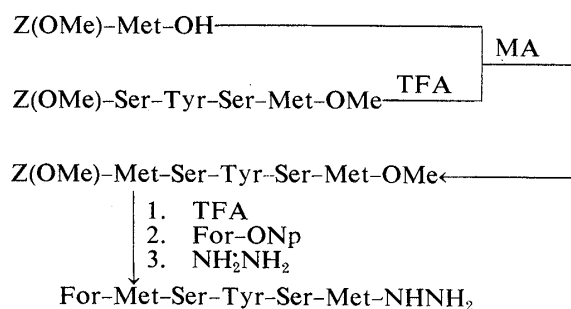


Fig. 3. Synthetic Scheme for the N-Terminal Pentapeptide Hydrazide

and ostrich ACTH.⁹⁾ Fragment [2] was synthesized by assembling three known intermediates, $\text{Z(OMe)-Lys(Z)-Pro-Val-Gly-OH}$,^{9,10)} $\text{Z(OMe)-Lys(Z)-Lys(Z)-OMe}$ ¹⁰⁾ and $\text{Z-Arg(Mts)-Arg(Mts)-Pro-OBzl}$ ⁹⁾ as shown in Fig. 2. First, the two former peptides were condensed by using pentachlorophenyl trichloroacetate (PCP-O-TCA)¹¹⁾ and the product, after being converted to the corresponding hydrazide, was coupled with a hydrogenated sample of the above Arg(Mts)-peptide by the azide procedure¹²⁾ to give [2]. Fragment [4] was synthesized as illustrated in Fig. 3. $\text{Z(OMe)-Ser-Tyr-Ser-Met-OMe}$ ¹³⁾ was treated with TFA in the presence of anisole containing 2% EDT, then coupled with Z(OMe)-Met-OH by the mixed anhydride (MA) procedure¹⁴⁾ to afford the protected pentapeptide ester, $\text{Z(OMe)-Met-Ser-Tyr-Ser-Met-OMe}$. Its Z(OMe) group was replaced with the For group by TFA treatment, followed by reaction with *p*-nitrophenyl formate.¹⁵⁾ The resulting $\text{For-Met-Ser-Tyr-Ser-Met-OMe}$ was converted to [4] by the usual hydrazine treatment.

The four fragments obtained as outlined above were then assembled according to the scheme illustrated in Fig. 1. The DCC-HOBt procedure¹⁶⁾ was employed to condense the Pro- and Gly- terminal fragments, [2] and [3], since no risk of racemization was involved in these coupling reactions. The DCC moiety attached at the unmasked imidazole ring of His is known to be cleaved by treatment with MeOH and AcOH.¹⁷⁾ Anisole containing 2% EDT¹⁸⁾ was employed in TFA deprotection of the Boc group from the Trp-containing peptide to minimize indole alkylation. The N-terminal fragment [4] was introduced by the azide procedure. Products were purified either by column chromatography on silica gel or by precipitation from DMF with MeOH. The homogeneity of these protected peptides was confirmed by thin-layer chromatography (TLC), elemental analysis and amino acid analysis after acid hydrolysis (Table 1).

Finally, protected For-Met-ACTH was treated with 1 M TFMSA-thioanisole in TFA¹⁹⁾ to remove all protecting groups employed, except the For group. *m*-Cresol, skatole and EDT were used as the additional scavengers to minimize side reactions, such as *O*-sulfonylation of Tyr²⁰⁾ and indole-alkylation.^{18,21)} The deprotected peptide was purified by the following steps as described in the hACTH synthesis⁹⁾: 1. Conversion to the corresponding acetate by treatment with Amberlite IR-45 (acetate form), 2. Incubation with 2-mercaptoethanol to

TABLE I. Amino Acid Ratios in 6N HCl Hydrolysates of For-Met-hACTH and Intermediates

	Protected peptides			Synthetic For-Met-hACTH	Theory
	12-40	6-40	1-40		
Asp	2.11	2.12	1.98	2.11	(2)
Ser	0.94	0.95	2.43	2.77	(3)
Glu	4.21	5.24	4.59	5.16	(5)
Pro	4.30	4.27	4.17	4.47	(4)
Gly	2.05	3.12	3.10	3.00	(3)
Ala	3.16	3.19	3.43	2.95	(3)
Val	3.15	3.14	2.94	3.03	(3)
Met			1.80	1.92	(2)
Leu	1.00	1.00	1.00	1.00	(1)
Tyr	0.88	0.95	1.79	1.96	(2)
Phe	1.95	2.97	2.71	3.01	(3)
Trp ^{a)}		0.70	0.61	0.78	(1)
Lys	4.26	4.17	3.70	4.28	(4)
His		0.99	0.90	0.93	(1)
Arg	2.04	3.06	2.76	2.99	(3)
Recov.	76%	80%	86%	88%	

a) 4N MeSO₃H hydrolysis.

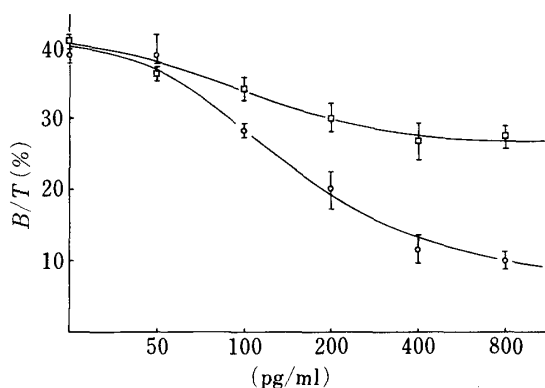


Fig. 4. Cross-Reactivity of For-Met-hACTH

Each value plotted is the mean for 3 duplicate determinations with the standard deviation (S.D.) shown by the vertical line.

○, kit ACTH; □, For-Met-hACTH.

reduce the Met sulfoxide possibly formed during manipulation, 3. Gel-filtration on Sephadex G-25 to remove scavengers, and 4. Ion-exchange chromatography on CM-cellulose. The product thus obtained exhibited a single spot on TLC and behaved as a single component on polyacrylamide gel disc electrophoresis. Its acid hydrolysate contained the constituent amino acids in the ratios predicted by theory.

The immunological properties of the synthetic ACTH analog with this purity were examined by using an ACTH antisera kit (IM 66, Amersham International plc, Buckinghamshire, England). The cross reactivity of For-Met-ACTH was found to be *ca.* 25–30% of that of standard hACTH (kit purchased from the same company), as shown in Fig. 4. The result indicated that For-Met-ACTH is an immunologically different molecule from hACTH, and thus the immune system has the ability to distinguish even a slightly modified compound from the parent compound.

Experimental

General experimental methods employed in this investigation are essentially the same as described in the hACTH

synthesis.⁸⁾

TLC was performed on silica gel (CD-alurol, Kieselgel 60 F254, Merck). *R_f* values refer to the following solvent systems: *R_f1* CHCl₃–MeOH (29:1), *R_f2* CHCl₃–MeOH (9:1), *R_f3* CHCl₃–MeOH–H₂O (18:3:1, lower phase), *R_f4* CHCl₃–MeOH–H₂O (8:3:1), *R_f5* *n*-BuOH–AcOH–pyridine–H₂O (4:1:1:2).

Z(OMe)–Lys(Z)–Pro–Val–Gly–Lys(Z)–Lys(Z)–OMe—A mixture of Z(OMe)–Lys(Z)–Pro–Val–Gly–OH^{9,10)} (7.3 g, 10.5 mmol), PCP–O–TCA (4.8 g, 11.6 mmol) and Et₃N (1.5 ml, 10.5 mmol) in DMF (20 ml) was stirred in an ice-bath for 3.5 h, then added to a solution of a TFA-treated sample of Z(OMe)–Lys(Z)–Lys(Z)–OMe¹⁰⁾ (7.6 g, 10.6 mmol) and Et₃N (1.5 ml, 10.5 mmol) in DMF (20 ml). The mixture was stirred at room temperature for 18 h and concentrated, then the residue was dissolved in AcOEt. The organic phase was washed with 5% citric acid, 5% NaHCO₃, and H₂O–NaCl, dried over Na₂SO₄, and concentrated (extraction procedure). The residue was recrystallized from AcOEt and ether; yield 9.8 g (75%), mp 133–135 °C, $[\alpha]_D^{27} - 28.4^\circ$ (*c* = 0.9, DMF), *R_f2* 0.80. *Anal.* Calcd for C₆₄H₈₅N₉O₁₆: C, 62.17; H, 6.93; N, 10.20. Found: C, 62.25; H, 6.95; N, 10.13.

Z(OMe)–Lys(Z)–Pro–Val–Gly–Lys(Z)–Lys(Z)–NHNH₂—The above protected hexapeptide ester (3.0 g, 2.4 mmol) in MeOH–DMF (10 ml–5 ml) was treated with 80% hydrazine hydrate (1.0 ml) at room temperature for 24 h and the solvent was removed by evaporation. Treatment of the residue with H₂O (100 ml) afforded a powder, which was washed with MeOH; yield 3.0 g (99%), mp 167–170 °C, $[\alpha]_D^{29} - 28.0^\circ$ (*c* = 0.5, DMF), *R_f2* 0.71. *Anal.* Calcd for C₆₃H₈₅N₁₁O₁₅: C, 61.20; H, 6.93; N, 12.46. Found: C, 61.09; H, 6.91; N, 12.40.

Z(OMe)–Lys(Z)–Pro–Val–Gly–Lys(Z)–Lys(Z)–Arg(Mts)–Arg(Mts)–Pro–OH [2]—Z–Arg(Mts)–Arg(Mts)–Pro–OBzl⁹⁾ (6.0 g, 5.9 mmol) in MeOH (60 ml) containing AcOH (2 ml) was hydrogenated over a Pd catalyst for 6 h. The catalyst was removed by filtration and the filtrate was concentrated, then the residue was dissolved in DMF (10 ml) containing Et₃N (0.8 ml, 5.9 mmol). The azide [prepared from 6.3 g (5.1 mmol) of the above hexapeptide hydrazide] in DMF (20 ml) and Et₃N (0.7 ml, 5.1 mmol) were added to the above ice-chilled solution and the mixture, after being stirred at 4 °C for 65 h, was concentrated. The residue was dissolved in CHCl₃. The organic solution was washed with 5% citric acid and H₂O–NaCl, dried over Na₂SO₄ and concentrated. The residue was precipitated from MeOH with ether; yield 8.3 g (81%), mp 127–130 °C, $[\alpha]_D^{29} - 25.8^\circ$ (*c* = 0.5, DMF). *R_f4* 0.54. Amino acid ratios in 6 N HCl hydrolysate: Lys 2.88; Pro 1.93; Val 0.98; Gly 1.00, Arg 1.94 (recovery of Gly, 88%). *Anal.* Calcd for C₉₈H₁₃₄N₁₈O₂₃S₂: C, 58.96; H, 6.77; N, 12.63. Found: C, 58.67; H, 6.65; N, 12.45.

Z(OMe)–Met–Ser–Tyr–Ser–Met–OMe—A mixed anhydride [prepared from 1.6 g (5.0 mmol) of Z(OMe)–Met–OH] in THF (20 ml) was added to a solution of a TFA-treated sample of Z(OMe)–Ser–Tyr–Ser–Met–OMe¹³⁾ (3.0 g, 4.5 mmol) and Et₃N (0.6 ml, 4.5 mmol) in DMF (10 ml) and the mixture was stirred in an ice-bath for 6 h. The solution was concentrated and the residue was recrystallized from MeOH and ether; yield 3.1 g (86%), mp 189–190 °C, $[\alpha]_D^{30} - 12.1^\circ$ (*c* = 1.1, DMF), *R_f4* 0.59. *Anal.* Calcd for C₃₅H₄₉N₅O₁₂S₂: C, 52.82; H, 6.21; N, 8.80. Found: C, 52.52; H, 6.36; N, 8.82.

For–Met–Ser–Tyr–Ser–Met–OMe—A mixture of *p*-nitrophenyl formate¹⁵⁾ (0.6 g, 3.8 mmol) and a TFA-treated sample of Z(OMe)–Met–Ser–Tyr–Ser–Met–OMe (3.0 g, 3.8 mmol) in DMF (6 ml) containing Et₃N (0.5 ml, 3.8 mmol) was stirred in an ice-bath for 30 min and concentrated. Treatment of the residue with ether afforded a powder, which was recrystallized from MeOH and ether. The product was further purified by column chromatography on silica gel (5.5 × 13 cm) using CHCl₃–MeOH–H₂O (18:3:1) as an eluant; yield 1.1 g (42%), mp 212–214 °C, $[\alpha]_D^{30} - 15.4^\circ$ (*c* = 1.0, DMF), *R_f4* 0.44. *Anal.* Calcd for C₂₇H₄₁N₅O₁₀S₂: C, 49.15; H, 6.26; N, 10.61. Found: C, 48.87; H, 6.49; N, 10.64.

For–Met–Ser–Tyr–Ser–Met–NHNH₂—The above pentapeptide ester (0.6 g, 1 mmol) in DMF (5 ml) was treated with 80% hydrazine hydrate (0.3 ml) at room temperature for 15 h. The residual solid was washed with MeOH and H₂O; yield 0.6 g (94%), mp 219–222 °C, $[\alpha]_D^{30} - 11.5^\circ$ (*c* = 0.6, DMF), *R_f4* 0.21. Amino acid ratios in a 6 N HCl (with phenol) hydrolysate: Met 2.12, Ser 1.90, Tyr 1.00 (recovery of Tyr, 73%). *Anal.* Calcd for C₂₆H₄₁N₇O₉S₂: C, 47.35; H, 6.26; N, 14.86. Found: C, 47.14; H, 6.54; N, 15.13.

Z(OMe)–Lys(Z)–Pro–Val–Gly–Lys(Z)–Lys(Z)–Arg(Mts)–Arg(Mts)–Pro–Val–Lys(Z)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBzl)–Asp(OBzl)–Glu(OBzl)–Ser–Ala–Glu(OBzl)–Ala–Phe–Pro–Leu–Glu(OBzl)–Phe–OBzl—The protected eicosapeptide ester [1] (3.6 g, 1.2 mmol) was treated with TFA–anisole (10 ml–2 ml) in an ice-bath for 60 min, then dry ether was added. The resulting TFA salt was dissolved in DMF (10 ml) and the solution was neutralized with Et₃N (0.2 ml), then dry ether was added. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 1 h and dissolved in DMF (10 ml). A mixture of DCC (0.7 g, 3.5 mmol), HOBT (0.8 g, 5.3 mmol) and fragment [2] (3.5 g, 1.8 mmol) in DMF (10 ml) was stirred in an ice-bath for 3 h and filtered. This filtrate was added to the above solution containing a free amino component and the mixture was stirred at room temperature for 45 h. Ether was added and the resulting powder was purified by column chromatography on silica gel (4 × 20 cm), which was eluted with CHCl₃–MeOH–H₂O (120:10:1). The desired product was precipitated from DMF with ether; yield 4.2 g (73%), mp 194–203 °C, $[\alpha]_D^{25} - 22.0^\circ$ (*c* = 0.4, DMF), *R_f3* 0.25, *R_f4* 0.61. *Anal.* Calcd for C₂₄₉H₃₂₀N₄₀O₅₈S₂·3H₂O: C, 60.79; H, 6.68; N, 11.39. Found: C, 60.64; H, 6.65; N, 11.33.

Boc–Glu(OBzl)–His–Phe–Arg(Mts)–Trp–Gly–Lys(Z)–Pro–Val–Gly–Lys(Z)–Lys(Z)–Arg(Mts)–Arg(Mts)–Pro–Val–Lys(Z)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBzl)–Asp(OBzl)–Glu(OBzl)–Ser–Ala–Glu(OBzl)–Ala–Phe–Pro–Leu–Glu(OBzl)–Phe–OBzl—The above protected nonacosapeptide ester (1.0 g, 0.2 mmol) was treated

with TFA and converted to a free amino component as stated above. Next, a mixture of DCC (0.1 g, 0.6 mmol), HOBt (0.1 g, 0.6 mmol) and fragment [3] (0.4 g, 0.3 mmol) in DMF (5 ml) was stirred in an ice-bath for 2 h and filtered. This filtrate was added to a solution of the above amino component in DMF (5 ml) and the mixture was stirred at room temperature for 20 h, then ether was added. The resulting powder was dissolved in DMF–MeOH–2 N AcOH (10 ml–8 ml–2 ml) and the solution was heated at 65 °C for 4 h to remove the DCC moiety presumably attached at the His residue. Ether was added and the resulting powder was purified as stated above; yield 0.88 g (73%), mp 175–180 °C, $[\alpha]_D^{25} - 24.7^\circ$ ($c=0.4$, DMF), R_f 0.22, R_f 0.59. *Anal.* Calcd for $C_{300}H_{384}N_{52}O_{67}S_3 \cdot 3H_2O$: C, 60.65; H, 6.62; N, 12.26. Found: C, 60.47; H, 6.67; N, 12.35.

For–Met–Ser–Tyr–Ser–Met–Glu(OBzl)–His–Phe–Arg(Mts)–Trp–Gly–Lys(Z)–Pro–Val–Gly–Lys(Z)–Lys(Z)–Arg(Mts)–Arg(Mts)–Pro–Val–Lys(Z)–Val–Tyr–Pro–Asn–Gly–Ala–Glu(OBzl)–Asp(OBzl)–Glu(OBzl)–Ser–Ala–Glu(OBzl)–Ala–Phe–Pro–Leu–Glu(OBzl)–Phe–OBzl—The above protected pentatriacontapeptide ester (156 mg, 26.4 μ mol) was treated with TFA (0.5 ml)–anisole containing 2% EDT (0.1 ml) in an ice-bath for 60 min, then dry ether was added. The resulting powder was dissolved in DMF (1.0 ml) and then solution was neutralized with 5% Et_3N /DMF (74 μ l). The azide [prepared from 35 mg (52.8 μ mol) of For–Met–Ser–Tyr–Ser–Met–NHNH₂] in DMF (1.0 ml) and 5% Et_3N /DMF (0.15 ml) were added to the above ice-chilled solution and the mixture was stirred at 4 °C for 48 h. Ether was added and the resulting powder was precipitated from DMF with MeOH; yield 167 mg (99%), mp 206–210 °C, $[\alpha]_D^{20} - 26.2^\circ$ ($c=0.3$, DMF), R_f 0.14, R_f 0.50. *Anal.* Calcd for $C_{321}H_{413}N_{57}O_{74}S_5 \cdot 5H_2O$: C, 59.27; H, 6.56; N, 12.27. Found: C, 59.09; H, 6.49; N, 12.39.

For–Met–hACTH—The above protected tetracontapeptide ester (166 mg, 25.8 μ mol) was treated with 1 M TFMSA–thioanisole in TFA (7.22 ml, 280 eq) in the presence of *m*-cresol (0.76 ml), EDT (0.04 ml) and skatole (20 mg) in an ice-bath for 2.5 h, then *n*-hexane was added. The resulting oily precipitate was treated with dry ether to form a powder, which was washed with ether and dissolved in H₂O (20 ml). The solution was treated with Amberlite IR-45 (acetate form, approximately 5 g) for 30 min and filtered. The filtrate was incubated with 2-mercaptoethanol (0.5 ml) at 50 °C for 15 h and concentrated. The residue was dissolved in 3% AcOH (5 ml) and the solution was applied to a column of Sephadex G-25 (2 $\times$ 150 cm), which was eluted with 3% AcOH. The fractions (5 ml each) corresponding to the front main peak (tube Nos. 34–50, monitored by ultraviolet (UV) absorption measurement at 280 nm) were combined and the solvent was removed by lyophilization. Next, the residue was dissolved in H₂O (3 ml) and the solution was applied to a column of CM-cellulose (1.8 $\times$ 15.7 cm), which was eluted with a gradient prepared from pH 6.9, 0.2 M AcONH₄ (500 ml) through a mixing flask containing pH 6.9, 0.05 M AcONH₄ (300 ml). The fractions (5 ml each) corresponding to the main peak (tube Nos. 84–98, monitored by UV absorption measurement at 280 nm) were combined and the solvent and the salt were removed by repeated lyophilization to give a fluffy white powder; yield 26.4 mg (19%), $[\alpha]_D^{17} - 76.7^\circ$ ($c=0.4$, 1% AcOH), R_f 0.34. The product exhibited a single band (stained by Amido Schwarz) in disc electrophoresis on 15% polyacrylamide gel (0.55 $\times$ 7.2 cm) at pH 4.0, 0.3 M glycine–AcOH buffer. Its mobility was 1.3 cm from the origin toward the cathode after running at 5 mA per tube for 80 min. *Anal.* Calcd for $C_{213}H_{317}N_{57}O_{60}S_2 \cdot 7CH_3COOH \cdot 20H_2O$: C, 49.74; H, 7.08; N, 14.57. Found: C, 49.43; H, 6.78; N, 14.17.

References and Notes

- 1) Part CXLIII: H. Yajima, S. Futaki, A. Otaka, T. Yamashita, S. Funakoshi, K. Bessho, N. Fujii, and K. Akaji, *Chem. Pharm. Bull.*, **34**, 4356 (1986).
- 2) Amino acids (except Gly) used in this investigation are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Z(OMe) = *p*-methoxybenzyloxycarbonyl, Bzl = benzyl, Boc = *tert*-butoxycarbonyl, Mts = mesitylenesulfonyl, DCC = dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, THF = tetrahydrofuran, EDT = ethanedithiol, DMF = dimethylformamide.
- 3) R. Wetzel and D. V. Goeddel, "The Peptides, Analysis, Synthesis, Biology," Vol. 5, ed. by E. Gross and J. Meienhofer, Academic Press, New York, 1983, p. 1.
- 4) J. G. Files, K. Weber, and J. H. Miller, *Proc. Natl. Acad. Sci. U.S.A.*, **71**, 667 (1974).
- 5) N. Stebbing, K. Olson, N. Lin, R. N. Harkins, C. Snider, M. J. Ross, F. Fields, L. May, J. Fenno, D. Fodge, and G. Prender, "Insulins, Growth Hormones, and Recombinant DNA Technology," ed. by J. L. Gueriguian, Raven Press, New York, 1981, p. 71.
- 6) E. Gross and B. Witkop, *J. Biol. Chem.*, **237**, 1856 (1962).
- 7) B. Riniker, P. Sieber, W. Rittel, and H. Zuber, *Nature, New Biol.*, **235**, 114 (1972).
- 8) K. Koyama, H. Watanabe, H. Kawatani, J. Iwai, and H. Yajima, *Chem. Pharm. Bull.*, **24**, 2558 (1976).
- 9) K. Yasumura, K. Okamoto, S. Shimamura, M. Nakamura, K. Odaguchi, A. Tanaka, and H. Yajima, *Chem. Pharm. Bull.*, **30**, 866 (1982).
- 10) H. Yajima, K. Koyama, Y. Kiso, A. Tanaka, and M. Nakamura, *Chem. Pharm. Bull.*, **24**, 492 (1976).
- 11) M. Fujino and C. Hatanaka, *Chem. Pharm. Bull.*, **16**, 929 (1968).
- 12) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
- 13) K. Okamoto, K. Yasumura, N. Yamamura, S. Shimamura, K. Miyata, A. Tanaka, M. Nakamura, H.

-
- Kawauchi, and H. Yajima, *Chem. Pharm. Bull.*, **30**, 2595 (1982).
- 14) J. R. Vaughan Jr. and R. L. Osato, *J. Am. Chem. Soc.*, **74**, 676 (1952).
- 15) K. Okawa and S. Hase, *Bull. Chem. Soc. Jpn.*, **36**, 754 (1963).
- 16) W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
- 17) H. Rink and B. Riniker, *Helv. Chim. Acta*, **57**, 831 (1974).
- 18) J. J. Sharp, A. B. Robinson, and M. D. Kamen, *J. Am. Chem. Soc.*, **95**, 6097 (1973); H. Ogawa, T. Sasaki, H. Irie, and H. Yajima, *Chem. Pharm. Bull.*, **26**, 3144 (1978).
- 19) H. Yajima, N. Fujii, H. Ogawa, and H. Kawatani, *J. Chem. Soc., Chem. Commun.*, **1974**, 107; Y. Kiso, S. Nakamura, K. Ito, K. Ukawa, K. Kitagawa, T. Akita, and H. Moritoki, *ibid.*, **1979**, 971.
- 20) H. Yajima, M. Takeyama, J. Kanaki, O. Nishimura, and M. Fujino, *Chem. Pharm. Bull.*, **26**, 3572 (1978).
- 21) E. Wünsch, E. Jaeger, L. Kisfaludy, and M. Löw, *Angew. Chem.*, **89**, 330 (1977); Y. Masui, N. Chino, and S. Sakakibara, *Bull. Chem. Soc. Jpn.*, **53**, 464 (1980).