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# Studies on Peptides. XXII.[1,2] Synthesis of the partially Protected Pentadecapeptide related to Monkey and Human β-Melanocyte-stimulating Hormones

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Synthesis of the partially protected pentadecapeptide related to monkey and human β-melanocyte-stimulating hormone, H-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys($N^{\varepsilon}$-formyl)-Asp-OH (III), was described. Condensation of Z-Glu(γ-OBzl)-OH with H-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys($N^{\varepsilon}$-formyl)-Asp-OH (IV) by the *p*-nitrophenyl ester method and subsequent hydrogenation gave H-Glu-IV, which was condensed with Boc-Met-OH by the same procedure. Treatment of the product with trifluoroacetic acid gave H-Met-Glu-IV. Boc-Arg($N^{G}$-nitro)-OH was condensed with this dodecapeptide by the mixed anhydride procedure and the product was treated with hydrogen fluoride to give H-Arg-Met-Glu-IV, to which Boc-Pro-Tyr-OH was added by the azide procedure to afford the protected III. Treatment of which with trifluoroacetic acid gave III. The MSH activity of the synthetic peptides was as follows; H-Glu-IV, $6.0\times10^{6}$: H-Met-Glu-IV, $1.9\times10^{6}$, $2.3\times10^{7}$: H-Arg-Met-Glu-IV, $1.8\times10^{8}$ and III, $2.2\times10^{12}$ units/g.

Recently we have reported in the preliminary communications the total syntheses of the octadecapeptide (I)[4] and the docosapeptide (II)[1] corresponding to the entire amino acid sequences of monkey[5] and human β-melanocyte-stimulating hormones (MSH).[6] In these syntheses, the partially protected pentadecapeptide, prolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-$N^{\varepsilon}$-formyllysylaspartic acid (III), which is common to these two hormones, was used in the final coupling reaction with the N-terminal portions of these MSHs which are different in part from each other. In this paper, we now describe in full the synthesis of III.

As has been mentioned previously,[4] choice of the protecting group for the α-amino and guanidino functions of arginine which is adjacent to the methionine residue and the ε-amino group of the lysine residue near the C-terminal portion of these molecules determined the main strategy for the total syntheses of these peptide hormones. In this paper, we describe the key problems involved in the total syntheses of these two MSHs.

The formyl group was selected for the protection of the ε-amino function of the lysine residue.[7] Thus, histidylphenylalanylarginyltryptophylglycylserylprolylprolyl-$N^{\varepsilon}$-formyllysylaspartic acid (IV) was prepared as described previouly.[8] The synthetic route from IV to III is illustrated in Chart 1.

1) Part XXI: H. Yajima, K. Kawasaki, H. Minami, H. Kowatani, N. Mizokami, and Y. Okada, *Biochim. Biophys. Acta*, **175**, 228 (1969).
2) The amino acids, peptides, and their derivatives (except glycine) mentioned in this communication are of the L-configuration. The customary L-designation has been eliminated for space conservation. Their abbreviated designation are those recommended by IUPAC–IUB commision on Biochemistry nomenclature in July, 1965 and July, 1966; *Biochemistry* 5, 2485 (1966); 6, 362 (1967).
3) Location: *Sakyo-ku, Kyoto.*
4) H. Yajima, Y. Okada, Y. Kinomura, and H. Minami, *J. Am. Chem. Soc.*, **90**, 527 (1968).
5) T.H. Lee, A.B. Lerner, and V.B. -Janusch, *J. Biol. Chem.*, **236**, 1390 (1961).
6) J.I. Harris, *Nature*, **184**, 167 (1959).
7) K. Hofmann, E. Stütz, G. Spühler, H. Yajima, and E.T. Schwartz, *J. Am. Chem. Soc.*, **82**, 3727 (1960).
8) H. Yajima, Y. Okada, Y. Kinomura, and E. Seto, *Chem. Pharm. Bull.* (Tokyo), **15**, 270 (1967).

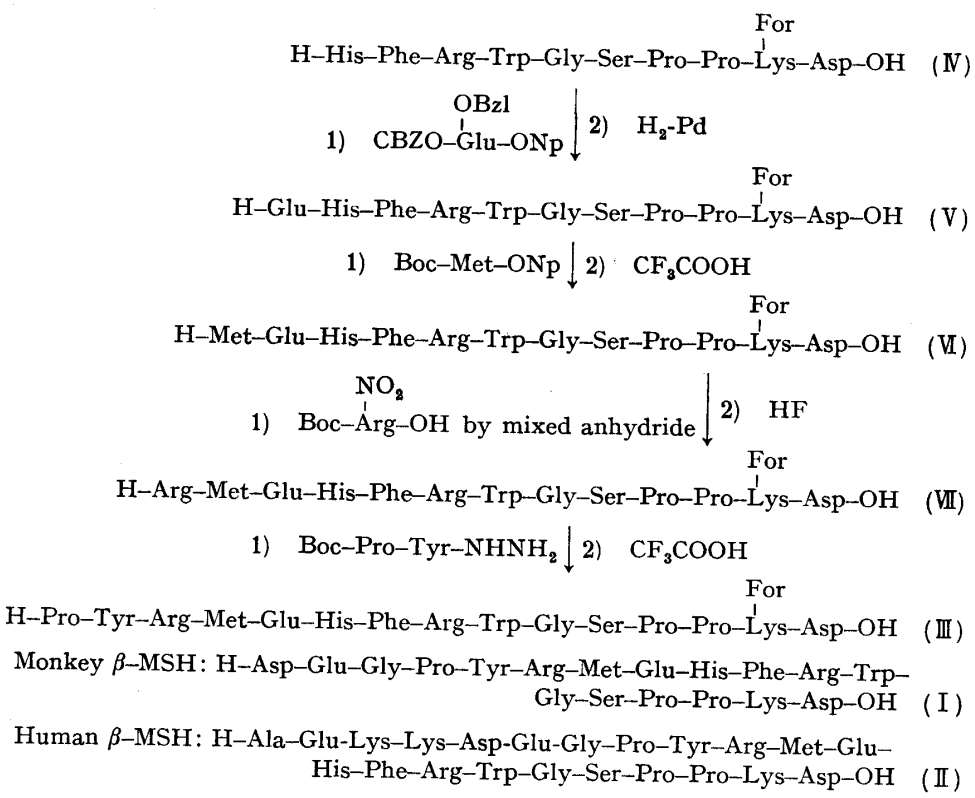


Chart 1. Synthetic Rout to the Partially Protected Pentadecapeptide related to Monkey and Human β–Melanocyte–stimulating Hormones

$N^{\alpha}$-Benzyloxycarbonyl-γ-benzyl-α-*p*-nitrophenylglutamate[9] was allowed to react with IV. Progress of the reaction was followed by examination of the reaction mixture by thin–layer chromatography until the ninhydrin test became negative. Overnight reaction at room temperature was sufficient for completion of the coupling reaction. The product, without further purification, was submitted to hydrogenolysis to remove the benzyloxycarbonyl and benzyl groups from the glutamyl residue. Reduction was started in 40% acetic acid and the solution was diluted with water as hydrogenation proceeded. This procedure avoided the formation of water–insoluble side reaction products. The cause of formation of which is yet unclear. The product, glutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-$N^{\varepsilon}$-formyllysylaspartic acid (V) was purified by column chromatography on carboxymethyl cellulose (CM–cellulose) using pyridine acetate buffers as eluent. Acid hydrolysis of the purified peptide gave the constituent amino acids in the ratios predicted by theory but the recovery of glutamic acid in leucine aminopeptidase (LAP)[10] digestion was somewhat low. Under the identical conditions, even the standard sample of glutamic acid was recovered in 64%. Though the reason is as yet unclear, this is presumably due to the conversion of glutamic acid to pyroglutamic acid in a tris buffer or the presence of an enzyme to consume glutamic acid, such as decarboxylase, in our LAP preparation.

The partially protected undecapeptide (V) was then allowed to react with $N^{\alpha}$-*t*-butoxycarbonylmethionine *p*-nitrophenyl ester[11] under the nitrogen atomosphere. The product was treated with trifluoroacetic acid to remove the *t*-butoxycarbonyl group. The resulting partially protected dodecapeptide, methionylglutamylhistidylphenylalanylarginyltryptophylgly-

9) G. Loose, H. Jeschkeit, and W. Langenbeck, *Chem. Ber.*, **96**, 204 (1963).
10) Hog kidney preparation, fractionated twice by ammonium sulfate according to the method of D.H. Spackman, E.L. Smith and D.M. Brown, *J. Biol. Chem.*, **212**, 255 (1955). Based on the recovery of standard Glu (64%), the corrected figure of Glu was given in the experimental section.
11) K. Hofmann, W. Haas, M.J. Smithers and G. Zanetti, *J. Am. Chem. Soc.*, **87**, 631 (1965).

cylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid (VI) was submitted to purification on CM-cellulose. Pyridine acetate buffers served here again to elute the desired compound. Prior to this experiment, we have confirmed that N$^{\varepsilon}$-formyllysine survived the action of trifluoroacetic acid mostly unchanged. The choice of the formyl group for the protection of the $\varepsilon$-amino group of the lysine residue and adoption of the *t*-butoxycarbonyl group for the $\alpha$-amino protection of forecomming amino acids have secured us to elongate the peptide chain toward the N-terminal portion of these two hormones. At this stage, the benzyloxycarbonyl group removable by catalytic hydrogenation is no longer applicable because of the presence of the methionine residue which poisons the catalyst.

As has been mentioned previously,[8] peptides containing N$^{\varepsilon}$-formyllysine are soluble in water as well as various buffers. Therefore, purification of these peptides on ion-exchanger is possible. This property permitted us to isolate every synthetic intermediate free from minor contaminants which presumably resulted from the coupling reaction of active esters with amino components possessing unprotected functional groups such as histidine and serine.

The partially protected dodecapeptide (VI) was condensed with N$^{\alpha}$-*t*-butoxycarbonyl-N$^{G}$-nitroarginine[12] by the mixed anhydride procedure.[13-15] The use of the active ester of N$^{G}$-nitroarginine, such as 2,4-dinitrophenyl[16] or pentachlorophenyl esters[17,18] is known in the literatures. However such procedures were unsuccessful in this case, since the reaction stopped in a half way and could not be duplicated in both cases. The product obtained by the mixed anhydride procedure was treated with anhydrous hydrogen fluoride, according to Sakakibara and Shimonishi,[19] to remove the *t*-butoxycarbonyl and the nitro groups from the arginyl residue.[19,20] Usefulness of this procedure was examined previously in the synthesis of a model peptide, arginylmethionine from N$^{\alpha}$-benzyloxycarbonyl-N$^{G}$-nitroarginylmethionine.[21] In addition to this model synthesis, it was confirmed that N$^{\varepsilon}$-formyllysine was not affected by this reagent. The above authors[19] described that the formyl group at $\alpha$-amino function of amino acids was stable to this reagent. Usefulness of the formyl group in our present synthesis was further evaluated.

The desired peptide, arginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid (VII) was purified by a column of CM-cellulose using ammonium acetate buffers as eluent. When absorbancy at 280 m$\mu$ in various fractions was examined, a broad peak was detected. Thin-layer chromatographic examination revealed that the first purified sample was still contaminated with a minor component which was positive to Pauly, Sakaguchi and Ehrlich tests but completely negative to the ninhydrin test. Despite the use of isobutyl chloroformate instead of ethyl chloroformate, we were unable to avoid the formation of such a side reaction product in this coupling procedure, though the formation of such a compound was not noticed during the synthesis of a model peptide, N$^{\alpha}$-*t*-butoxycarbonyl or N$^{\alpha}$-benzyloxycarbonyl-N$^{\alpha}$-nitroarginylmethionine methyl ester[21] by the mixed anhydride procedure.

12) K. Hofmann, W. Haas, M.J. Smithers, R.W. Wells, Y. Wolman, Y. Yanaihara, and Z. Zanetti, *J. Am. Chem. Soc.*, **87**, 620 (1965).
13) Th. Wieland and H. Bernhard, *Ann. Chem.*, **572**, 190 (1951).
14) J.R. Vaughan, Jr., *J. Am. Chem. Soc.*, **73**, 3547 (1951).
15) R.A. Boissonnas, *Helv. Chim. Acta*, **34**, 874 (1951).
16) M. Bodanszky and M.A. Ondetti, *Chem. Ind.* (London), **1966**, 26.
17) J. Kovacs and M.Q. Ceprini; *Chem. Ind.* (London), **1965**, 2100.
18) J. Kovacs, M.Q. Ceprini, C.A. Dupraz, and G.N. Schmit, *J. Org. Chem.*, **32**, 3696 (1967).
19) S. Sakakibara and S. Shimonishi, *Bull. Chem. Soc. Japan*, **38**, 1412 (1965); S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *ibid.*, **40**, 2164 (1967); S. Sakakibara, Y. Kishida, R. Nishizawa and Y. Shimonishi, *ibid.*, **41**, 438 (1968).
20) J. Leonard, and A.B. Robinson, *J. Am. Chem. Soc.*, **89**, 181 (1967).
21) H. Yajima, Y. Kinomura, T. Oshima, and Y. Okada, *Chem. Pharm. Bull.* (Tokyo), **15**, 1922 (1967).

Vaughan,[22] Albertson[23] and later Stewart[24] suggested briefly but without enough experimental proof, that the use of a relatively higher peptide as an amino component in the mixed anhydride procedure would result in a high molecular weight by-product, the so-called urethan, which is difficult to separate from the desired product. The introduced alkylurethan group can no longer be removed by mild acidolysis, such as by treatment with trifluoroacetic acid. It seems reasonable to assume that a minor contaminant in VII is the alkyloxycarbonyl derivative of dodecapeptide (VI), because of the negative color test with ninhydrin.

The 2nd chromatography on CM–cellulose in the buffer system of ammonium acetate allowed us to purify the partially protected tridecapeptide (VII) in a form free from the urethan. Complete removal of ammonium acetate from the eluents by repeated lyophilization was necessary for the purified peptide to produce a single spot on thin-layer chromatography. The homogeniety of the desired compound was further established by amino acid analysis.

Addition of the dipeptide unit, prolyltyrosyl residue to VII was performed in essentially the same way as described in our model synthesis of prolyltyrosylarginylmethionine.[21] *t*-Butoxycarbonylprolyltyrosine azide was allowed to react with VII. The reaction proceeded smoothly as demonstrated in the above model synthesis. The product was subsequently treated with trifluoroacetic acid. Partial decomposition of the tryptophyl residue in our peptides can not be avoided during multiple treatments with this acid as we have adopted, however purification on CM–cellulose was found efficient to remove, in every step of the synthesis, such a decomposition product which exhibited a pink color.

The isolated partially protected pentadecapeptide, prolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-$N^{\varepsilon}$-formyllysylaspartic acid (III) exhibited a single spot on thin-layer chromatography and the acid hydrolysate contained every amino acids present in III except for tryptophan in the ratios predicted by theory. Complete digestion of the synthetic III with leucine aminopeptidase was also established. Nearly theoretical amount of $N^{\varepsilon}$-formyllysine, instead of lysine, was found in the hydrolysate indicating the formyl group survived indeed the action of trifluoroacetic acid and hydrogen fluoride treatment. The results cited above seem to demonstrate that our synthetic peptide (III) possessed the high degree of homogeniety and stereospecificity of the constituent amino acids.

In a routine preparation of III, it was found that some urethan contaminated in the tridecapeptide (VII) could be separated out in the purification stage of III, even if we employed such a sample of VII which was obtained by the first CM–cellulose purification.

An alternate synthetic route to III was taken into consideration. Coupling reaction of $N^{\alpha}$-*t*-butoxycarbonylprolyltyrosylarginylmethionine azide, if it can be prepared, with VI will lead to the synthesis of III, since the azide procedure of arginine containing peptides is known.[25] However the preparation of such a hydrazide involved in several problems. Conversion of methyl esters to the corresponding hydrazides is not practical when peptides contain the arginyl residue which is sensitive to the basicity.[26–28] The use of $N^{G}$-nitroarginine instead of arginine in the azide procedure is known to cause some side reaction.[29,30] Even this reaction goes well, subsequent removal of the nitro group with hydrogen fluoride may cause some side reac-

22) J.R. Vaughan, the 128th Am. Chem. Soc. Meeting, Minneapolis, Minn. Sept. 1955, Abstract, p. 27c.
23) N.F. Albertson, *Organic Reactions*, **12**, 157 (1962).
24) F.H.C. Stewart, *Aust. J. Chem.*, **18**, 887 (1965).
25) K. Hofmann, F.M. Finn, M. Limetti, J. Montibeller and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).
26) S.A. Walsman and M. Tishler, *J. Biol. Chem.*, **142**, 519 (1942).
27) H. Brookmann, N. Grubhofer, W. Kass, and H. Kalbe, *Chem. Ber.*, **84**, 26 (1951).
28) M.M. Shemyakin, T.A. Ovchinnikov, E.I. Vinogradova, M.Y. Feigina, A.A. Kiryushkin, N.A. Aldanova, Y.B. Alakhov, V.M. Lipkin, and B.V. Rosinov, *Experientia*, **23**, 428 (1967).
29) J.S. Fruton, *Advances in Protein Chem.*, **5**, 64 (1949).
30) A.F. McKay, *Chem. Rev.*, **51**, 301 (1952).

tion on the tyrosyl residue in this tetrapeptide unit. The use of $N^{\alpha}$,$N^{G}$-di-*t*-butoxycarbonylarginine[31] offers another possibility to prepare this tetrapeptide derivative, but this kind of urethan type protection for the guanidino function of arginine, such as tribenzyloxycarbonylarginine,[32] seems practical only in the synthesis of peptides with N-terminal arginine. This may be the reason that application of *t*-butoxycarbonyl derivative of arginine for the synthesis of relatively large peptides is as yet not known. Synthesis of the above tetrapeptide hydrazide starting from monosubstituted hydrazine[33] would be an alternate approach. For this, at least three different amino protecting groups would be required. Therefore, practical difficulty would not be avoided. With these consideration, the method which we employed for the synthesis of the partially protected pentadecapeptide (III) seems to be the method of choice.

The MSH activity of the synthetic peptides was examined *in vitro* according to the method of Shizume, *et al.*[34] using frog skins from *Rana pipiens*. The activity was expressed as units per gram as listed in Table I.

Table I. The MSH Activity of the Synthetic Peptides

| | MSH U/g |
|---|---|
| H–His–Phe–Arg–Trp–Gly–Ser–Pro–Pro–Lys(For)–Asp–OH | $3.0\times10^{6}$ |
| H–Glu–His–Phe–Arg–Trp–Gly–Ser–Pro–Pro–Lys(For)–Asp–OH | $6.0\times10^{6}$ |
| H–Met–Glu–His–Phe–Arg–Trp–Gly–Ser–Pro–Pro–Lys(For)–Asp–OH | $1.9\times10^{6}$, $2.3\times10^{7}$ |
| H–Arg–Met–Glu–His–Phe–Arg–Trp–Gly–Ser–Pro–Pro–Lys(For)–Asp–OH | $1.8\times10^{8}$ |
| H–Pro–Tyr–Arg–Met–Glu–His–Phe–Arg–Trp–Gly–Ser–Pro–Pro–Lys(For)–Asp–OH | $2.2\times10^{12}$ |
| Ac–Ser–Tyr–Ser–Met–Glu–His–Phe–Arg–Trp–Gly–Lys–Pro–Val–$NH_2$ (α-MSH) | $2.0\times10^{12}$ |

The activities of the deca, undeca and dodecapeptides are of the same magnitude within the limit of experimental tolerance. The addition of the basic arginine residue to dodecapeptide brought about a hundred-hold increase in activity. It seems significant to note that the activity of pentadecapeptide is equivalent to that of native α-MSH,[35] the most potent melanocytic principle in mammalian pituitary glands. If $N^{\varepsilon}$-formyllysylaspartic acid is eliminated from this pentadecapeptide, the resulting tridecapeptide is corresponding exactly the sequence of α-MSH except for a few alternation in the amino acid residues. The potent MSH activity of the synthetic pentadecapeptide can be fairly recognized by such comparison.

## Experimental

The melting points are uncorrected. Rotations were determined in a Rex Photoelectric Polarimeter Model NEP-2. Amino acid composition of the acid and enzymatic hydrolysates was determined with a Hitachi Amino Acid Analyser, Model KLA-2 according to the method of S. Moore, D.H. Spackmann, W.H.

31) Z. Paulay and S. Bajusz, *Acta Chim. Hung. Tomus*, **43**, 147 (1965).
32) L. Zervas, T.T. Otani, M. Winitz, and J.P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2878 (1959); L. Zervas, M. Winitz and J.P. Greenstein, *ibid.*, **83**, 3300 (1961); *J. Org. Chem.*, **22**, 1515 (1957).
33) K. Hofmann, M.Z. Magee and A. Lindenman, *J. Am. Chem. Soc.*, **72**, 2814 (1950); K. Hofmann, A. Lindenman, M.Z. Magee, and N.H. Khan, *ibid.*, **74**, 2814 (1950).
34) K. Shizume, A.B. Lerner, and J.S. Fitzpatrick, *Endocrinology*, **54**, 553 (1954).
35) S. Lande, A.B. Lerner, and G.V. Upton, *J. Biol. Chem.*, **240**, 4259 (1965).

Stein.[36] Unless stated otherwise, solvents were evaporated *in vacuo* at a bath temperature of 40 to 50° in a rotatory evaporator. For column chromatography, Toyo Fraction Collector Model SF-200-A was used. CM-cellulose was purchased from Bio-Rad Biochem. Co., Richimond California U.S.A. (Cellex-CM, 0.7 meq/g). On paper chromatography, $Rf_1$ values refer to the system of *n*-butanol, AcOH and $H_2O$ (4:1:5) and $Rf_2$ values to *sec*-butanol and 3% ammonia (3:1) and are expressed as a multiple distance traveled by a Phe marker under the identical condition. On thin-layer chromatography (Kieselgel G, Merck), $Rf_3$ and $Rf_4$ values refer to the system of *n*-butanol, pyridine, AcOH and $H_2O$ (4:1:1:2) and (15:10:3:12) respectively. The following abbreviations were used for solvents, dimethylformamide=DMF, and tetrahydrofuran =THF.

**Treatment of $N^{\varepsilon}$-Formyllysine with Anhydrous Trifluoroacetic Acid**——$N^{\varepsilon}$-Formyllysine (0.50 g) was treated with anhydrous trifluoroacetic acid (2 ml) at room temperature for 2 hr. The solvent was removed by evaporation and the residue was dissolved in EtOH and the solution was neutralized with triethylamine. The resulting crystalline powder was collected. Chromatographically pure $N^{\varepsilon}$-formyllysine, 0.45 g (90%), mp 216—218°, was recovered.

**Treatment of $N^{\varepsilon}$-Formyllysine with Anhydrous Hydrogen Fluoride**——Hydrogen fluoride (approximately 5 ml) was collected in a container of difuron containing $N^{\varepsilon}$-formyllysine (0.50 g) and anisol (0.5 ml) and the solution was stirred in an ice-bath for 1.5 hr. The hydrogen fluoride was then evaporated and the residue, after drying over KOH pellets *in vacuo*, was dissolved in $H_2O$, which was treated with Dowex-50 (ammonium cycle, 3 g) for 2 hr. The resin was removed by filtration and the filtrate was evaporated. Addition of acetone to the residue afforded crystalline powders; yield 0.46 g (92%), mp 216—218° identical with the starting material. Paper chromatographic examination of the reaction mixture did not indicate the presence of lysine.

**Glutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-$N^{\varepsilon}$-formyllysylaspartic Acid Acetate Octahydrate (V)**——Histidylphenyalanylarginyltryptophylglycylserylprolylprolyl-$N^{\varepsilon}$-formyllysylaspartic acid (1.42 g) was dissolved in $H_2O$ (10 ml) and the solution, after addition of 1N HCl (6.0 ml), was lyophilized. To a solution of the hydrochloride thus formed in 95% aqueous DMF (20 ml), 1M triethylamine in DMF (4 ml) and $N^{\alpha}$-benzyloxycarbonyl-$\gamma$-benzyl-$\alpha$-*p*-nitrophenylglutamate (1.23 g) were added. The solution was stirred at room temperature for 20 hr and the solvent was evaporated *in vacuo*. The residue was triturated with AcOEt the resulting solid powder was collected by filtration and washed with AcOEt; yield 1.50 g. The crude protected peptide thus obtained was dissolved in 40% AcOH (100 ml) and hydrogenated over a Pd catalyst. The solution, after 1 hr's hydrogenation, was diluted with $H_2O$ (60 ml) and hydrogenation was continued for an additional 6 hr. The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residue was lyophilized and kept over KOH pellets *in vacuo* overnight. The resulting powder was dissolved in $H_2O$ (500 ml) and the solution was applied to a column of CM-cellulose (3×23 cm), which was eluted with the following pH 5.0 pyridine acetate buffers; 0.01M (1800 ml) and 0.025M (1400 ml). Individual fractions (20 ml each) were collected and absorbancy at 280 mμ was determined in each fraction. The desired product was present in the 0.025M eluates (tube 139—210), these were pooled, the solvent was evaporated *in vacuo* and the residue was lyophilized to give a colorless fluffy powder. Yield 0.92 g (60%). $[\alpha]_D^{20}$ −73.6° ($c$=0.4, $H_2O$). $Rf_1$ 0.20, $Rf_2$ 0.22×Phe, $Rf_3$ 0.28 and $Rf_4$ 0.37, single spot positive to ninhydrin, Pauly, Sakaguchi and Ehrlich tests. Amino acid ratios in an acid hydrolysate; $Glu_{1.00}His_{0.92}Phe_{0.89}Arg_{0.87}Gly_{1.03}Ser_{1.01}Pro_{1.98}Lys_{1.12}Asp_{1.04}$ (average recovery 100%). Amino acid ratios in a LAP digest; $Glu_{0.68}His_{0.76}Phe_{1.06}Arg_{1.27}Trp_{0.97}Gly_{1.26}Ser_{0.72}Pro_{2.15}N^{\varepsilon}$-$Formyllys_{1.00}Asp_{0.93}$ (average recovery 96%, corrected Glu 1.06).[10] Mobility on paper electrophoresis was −1.7 cm at pH 6.5 of 0.1M pyridine acetate buffer (1150 V, 4 mA for 2 hr). *Anal.* Calcd. for $C_{63}H_{86}O_{18}N_{18}\cdot CH_3COOH\cdot 8H_2O$: C, 49.2; H, 6.7; N, 15.9. Found: C, 49.3; H, 7.1; N, 15.6.

**Methionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-$N^{\varepsilon}$-formyllysylaspartic Acid Acetate Hexahydrate (VI)**——Glutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-$N^{\varepsilon}$-formyllysylaspartic acid (V) was converted to its hydrochloride as described above. This hydrochloride (0.79 g) was dissolved in DMF (10 ml). Triethylamine (0.22 ml) and $N^{\alpha}$-*t*-butoxycarbonylmethionine *p*-nitrophenyl ester (0.39 g) were added to the above solution, which was stirred under $N_2$ at room temperature for 12 hr. After evaporation of the solvent, the residue was treated with AcOEt and the resulting solid was collected by filtration and washed throughly with AcOEt. This solid powder was subsequently treated with anhydrous trifluoroacetic acid (1 ml) at room temperature for 30 min. Dry ether (150 ml, stored over ferrous sulfate) was added and the resulting white powder was collected by filtration, dried over KOH pellets *in vacuo* and then lyophilized. The product was dissolved in $H_2O$ (300 ml) and the solution, after the pH of the solution was adjusted to 4.5 with 3% $NH_4OH$, was applied to a column of CM-cellulose (3×10 cm), which was eluted with first with $H_2O$ (700 ml) and then pH 5.0 pyridine acetate buffers; 0.01M (2200 ml) and 0.05M (700 ml). Individual fractions (20 ml each) were collected with a flow rate of 3 ml per min and the absorbancy of each fraction was determined at 280 mμ. The desired fractions (tube 85 to 137) present in the 0.01M eluate were collected. The solvent was removed by first evaporation and finally by lyophiliza-

36) S. Moore, D.H. Spackmann, and W.H. Stein, *Anal. Chem.*, 30, 1185 (1958).

tion to give a colorless fluffy powder. Yield 0.49 g (56%). $[\alpha]_D^{20}$ −66.6° ($c$=0.8, $H_2O$). $Rf_1$ 0.11, $Rf_2$ 0.33 × Phe, $Rf_3$ 0.25, single spot positive to methionine, Sakaguchi, Pauly and Ehrlich positive spot. Amino acid ratios in an acid hydrolysate; $Met_{1.05}Glu_{1.01}His_{1.04}Phe_{0.97}Arg_{0.92}Gly_{1.00}Ser_{0.97}Pro_{1.97}Lys_{1.14}Asp_{1.02}$ (average recovery 100%). *Anal.* Calcd. for $C_{68}H_{95}O_{19}N_{19}S \cdot CH_3COOH \cdot 6H_2O$: C, 50.0; H, 6.6; N, 15.8. Found: C, 50.1; H, 7.2; N, 15.5.

**Arginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic Acid Diacetate Octahydrate (VII)**——A mixed anhydride was prepared in the usual manner from N$^{\alpha}$-*t*-butoxycarbonyl-N$^{G}$-nitroarginine (0.19 g) and triethylamine (0.1 ml) with isobutyl chloroformate (0.09 ml) in anhydrous THF (10 ml). The solution containing the above mixed anhydride was added to a solution of methionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid acetate (VI) (0.48 g) and triethylamine (0.04 ml) in 90% aqueous DMF (6.5 ml). The mixture was stirred in an ice–bath for 0.5 hr and at room temperature for 2 hr. The solvent was then removed *in vacuo* and the residue was treated with AcOEt to form a solid powder which was collected by filtration and dried over $P_2O_5$. Examination of this powder by thin layer chromatography revealed the presence of 2 spots (Ehrlich stain); a minor spot of $Rf_3$ 0.47 and a major spot of $Rf_3$ 0.56.

This powder (0.66 g) was placed in a container of difuron with anisol (0.7 ml) and treated with anhydrous hydrogen fluoride (approximately 10 ml) in an ice–bath for 2 hr. The hydrogen fluoride was removed under reduced pressure. The residue was dried over KOH pellets *in vacuo* overnight and then dissolved in $H_2O$ (30 ml), which after filtration with an aid of filter cell was lyophilized (two spots: $Rf_4$ 0.17 and 0.40 by Ehrlich stain). The resulting powder was again dissolved in $H_2O$ (50 ml), which was treated with Amberlite CG–4B (acetate cycle, approximately 4 g). The resin was removed by filtration and the filtrate was lyophilized. The product was dissolved in $H_2O$ (500 ml) and the solution was applied to a column of CM–cellulose (2 × 13 cm), which was eluted with the following pH 6.9 ammonium acetate buffers; 0.005M (700 ml), 0.01M (1400 ml), 0.02M (800 ml), 0.04M (800 ml), 0.06M (800 ml) and 0.1M (800 ml). Individual fractions (15 ml each) were collected and the absorbancy of each fraction was determined at 280 m$\mu$. The compound emerged from the column with a broad peak which present in mainly 0.02 to 0.06M eluates (tube 130 to 280). The contents of these tubes were collected and the bulk of the solvent was removed by evaporation. The residue was repeatedly lyophilized to constant weight to give a colorless powder. Yield 0.45 g (84%). $[\alpha]_D^{20}$ −36.7° ($c$=0.2, 1N AcOH). $Rf_4$ 0.44 (positive to ninhydrin, methionine, Sakaguchi, Ehrlich and Pauly tests. The $Rf$ value increased from 0.17 to 0.44 after purification), contaminated with a faint spot of $Rf_4$ 0.50 (ninhydrin negative, but positive to Sakaguchi, methionine, Pauly and Ehrlich tests).

The homogeneous compound was obtained by the 2nd chromatographic purification. An example was given. The peptide obtained in the first purification (150 mg) was dissolved in $H_2O$ (500 ml) and the solution was applied to a column of CM–cellulose (2 × 10 cm), which was eluted with the following pH 6.9 ammonium acetate buffers; 0.01M (750 ml), 0.02M (750 ml), 0.04M (900 ml) and 0.06M (750 ml). Individual fractions of 15 ml were collected and absorbancy was determined as stated above. A peak present in the 0.01M eluates was discarded. The desired material was present in the 0.02M eluates (tube 115 to 135), which were pooled, evaporated to a small volume and lyophilized to constant weight. A fluffy colorless powder was obtained. Yield 70 mg (46%). $[\alpha]_D^{20}$ −55.6° ($c$=0.2, 1N AcOH). $Rf_4$ 0.44, Amino acid ratios in an acid hydrolysate $Arg_{1.98}Met_{0.97}Glu_{1.01}His_{1.03}Phe_{0.97}Gly_{1.00}Ser_{0.87}Pro_{1.92}Lys_{1.03}Asp_{1.01}$ (average recovery 95%). *Anal.* Calcd. for $C_{74}H_{107}O_{20}N_{23}S \cdot 2CH_3COOH \cdot 8H_2O$: C, 48.4; H, 6.8; N, 16.6. Found: C, 48.4; H, 7.1; N, 16.0.

**Prolyltyrosylarginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic Acid Diacetate Nonahydrate (III)**——The entire reaction was performed in a cold room at 4°. A solution of N$^{\alpha}$-*t*-butoxycarbonylprolyltyrosine hydrazide (0.24 g) in DMF (10 ml) and an aqueous solution of 1M $NaNO_2$ (0.8 ml) were combined. Under cooling with ice-NaCl, 1N HCl (1.6 ml) was added. The solution was stirred for 5 min. The pH of the solution was then adjusted to 8 with 1M triethylamine in DMF (1 ml). This solution containing the above azide was combined to a solution of arginylmethionylglutamylhistidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid diacetate (0.51 g) obtained in the first purification and triethylamine (0.07 ml) in 80% aqueous DMF (11 ml). After the solution was stirred for 12 hr, an additional azide (prepared from 0.17 g of the hydrazide) was added and the solution was further stirred for 12 hr. During this period, the ninhydrin positive spot disappeared completely and a new spot (Ehrlich stain) with $Rf_4$ 0.71 was detected. After evaporation of the solvent, AcOEt was added to the residue to form a solid powder, which was collected by filtration and washed thoroughly with AcOEt. The protected peptide thus obtained was treated with anhydrous trifluoroacetic acid (1 ml) at room temperature for 30 min. Dry ether (stored over ferrous sulfate) was added and the resulting solid powder was collected by filtration and dried over KOH pellets *in vacuo*. The product was dissolved in $H_2O$ (30 ml) and the solution was treated with Amberlite CG-4B (type I, approximately 5 g). The resin was removed by filtration and the filtrate was lyophilized. The resulting powder was again dissolved in $H_2O$ (600 ml) and the solution was applied to a column of CM–cellulose (2 × 13 cm) which was eluted with the following pH 6.9 ammonium acetate buffers; 0.005M (750 ml), 0.01M (600 ml), 0.05M (800 ml) and 0.1M (700 ml). Individual fractions of 15 ml each were collected and absorbancy at 280 m$\mu$ was determined in each fraction. In the 0.01M eluate, the urethan of VI contaminated in the starting material emerged from

the column. The main peak present in the 0.05M eluates (tube 130 to 190) was collected and the solvent was removed by evaporation. The residue was repeatedly lyophilized to constant weight to give a white powder. Yield 0.49 g (86%). $[\alpha]_D^{30}$ $-60.7°$ ($c=0.3$, 1N AcOH). $Rf_4$ 0.47, single spot positive to ninhydrin, Sakaguchi and methionine and Ehrlich tests; homogeneous on paper electrophoresis at pH 4.0 of 0.1M pyridine acetate buffer under the condition of 900 V, 18 mA for 2 hr (mobility-1.5 cm, Ehrlich stain). Amino acid ratios in an acid hydrolysate $Pro_{3.17}Tyr_{0.88}Arg_{1.82}Met_{0.94}Glu_{1.01}His_{0.97}Phe_{1.00}Gly_{0.97}Ser_{0.84}Lys_{1.06}Asp_{1.00}$ (average recovery 89%). Amino acid ratios in a LAP digest; $Pro_{2.90}Tyr_{1.00}Arg_{2.08}Met_{1.26}Glu_{0.68}His_{1.35}Phe_{1.28}$-$Trp_{0.75}Gly_{1.15}Ser_{0.93}N^{\varepsilon}$-$Formyllys_{1.11}Asp_{0.83}$ (average recovery 82%, corrected Glu 1.06).[10] *Anal.* Calcd. for $C_{88}H_{123}O_{23}N_{25}S \cdot 2CH_3COOH \cdot 9H_2O$: C, 49.9; H, 6.8; N, 15.8. Found: C, 50.6; H, 6.6; N, 15.0.

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