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33. Haruaki Yajima, Yoshio Okada, Yasuhiko Kinomura,*1 and Eisuke Seto*2 : Studies on Peptides. XII.*3,4 Synthesis of the Decapeptide corresponding to the C-Terminal Portion of β-Melanocyte-stimulating Hormone.

(*Faculty of Pharmaceutical Sciences, Kyoto University*1)

Synthesis of the decapeptide, histidylphenylalanylarginyltryptophylglycylserylprolylprolyllysylaspartic acid (I) corresponding to the C-terminal portion of β-melanocyte-stimulating hormones (β-MSH) from pig, sheep, beef, monkey and human was described. I was obtained by deformylation of histidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid (II) with aqueous hydrazine. The MSH activity of I and II was examined *in vitro*. It was found that I and II exhibited the activity, 2.4×10^6 and 1.0×10^6 MSH U/g. respectively.

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Shortly after the isolation of homogeneous α-melanocyte-stimulating hormone (α-MSH)[1] from pig pituitary glands, β-MSH, the 2nd principle also capable to stimulate pigment cells, was discovered from the same organ in 1955.[2,3] Subsequently MSHs from various animals were isolated and the entire amino acid sequence of these hormones from pig, sheep, beef, horse, monkey and human were elucidated as shown in Chart 1. It was shown that β-MSH varies somewhat from species to species and contains either 18 or 22 amino acids.[4]

α-MSH

pig, beef, horse, monkey Ac. Ser. Tyr. Ser. Met. Glu. His. Phe. Arg. Try. Gly. Lys. Pro. Val. NH_2

β-MSH

pig Asp. Glu. Gly. Pro. Tyr. Lys. Met. Glu. His. Phe. Arg. Try. Gly. Ser. Pro. Pro. Lys. Asp.
beef Asp. Ser. Gly. Pro. Tyr. Lys. Met. Glu. His. Phe. Arg. Try. Gly. Ser. Pro. Pro. Lys. Asp.
horse Asp. Glu. Gly. Pro. Tyr. Lys. Met. Glu. His. Phe. Arg. Try. Gly. Ser. Pro. Arg. Lys. Asp.
monkey Asp. Glu. Gly. Pro. Tyr. Arg. Met. Glu. His. Phe. Arg. Try. Gly. Ser. Pro. Pro. Lys. Asp.
human Ala. Glu. Lys. Lys. Asp. Glu. Gly. Pro. Tyr. Arg. Met. Glu. His. Phe. Arg. Try. Gly. Ser. Pro. Pro. Lys. Asp.

Chart 1. Amino Acid Sequences of α- and β-Melanocyte-stimulating Hormone

Valuable informations on the biological function of α-MSH were obtained through studies of a number of synthetic peptides.[5] On the other hand, information on the structures and MSH activities of the β-MSH series is limited despite the announcement of the total synthesis of beef β-MSH by Schwyzer, *et al.*[6] They have evaluated the MSH activity of a few synthetic peptide subunits related to beef β-MSH.[7,8]

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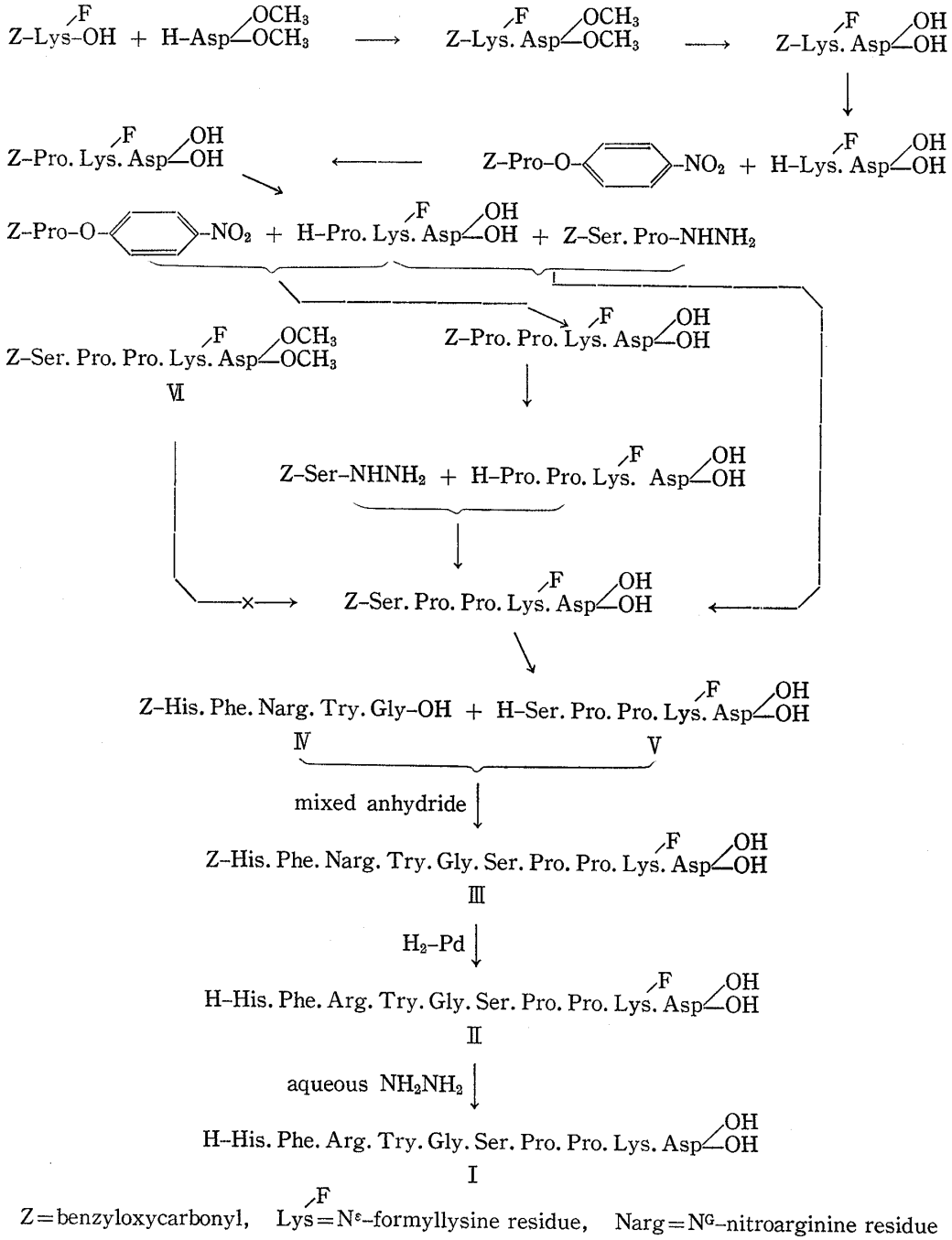
*1 Sakyo-ku, Kyoto (矢島治明, 岡田芳男, 木野村保彦).
*2 A research fellow from Nippon Shinyaku Co. (瀬戸英助).
*3 Part XI : Biochim. et Biophys. Acta, **127**, 545 (1966).
*4 The peptides and peptide derivatives mentioned in this communication (with exception of glycine) are of the L-configuration.

1) A. B. Lerner, T. H. Lee : J. Am. Chem. Soc., **77**, 1066 (1955).
2) B. J. Benfey, J. L. Purvis : *Ibid.*, **77**, 5167 (1955).
3) J. Porath, P. Roos, F. W. Landgrebe, G. M. Mitchell : Biochim. et Biophys. Acta, **17**, 598 (1955).
4) See review article of A. B. Lerner, T. H. Lee : Vitamines and Hormones, **20**, 337 (1963).
5) See review article of K. Hofmann, H. Yajima : "Recent Prog. in Hormone Res.," **18**, 41 (1962), ed. by G. Pincus, Academic Press, New York, N. Y.
6) R. Schwyzer, B. Iselin, H. Kappeler, B. Riniker, W. Rittel, H. Zuber : Helv. Chim. Acta, **46**, 1975 (1963).
7) R. Schwyzer, H. Kappeler, B. Iselin, W. Rittel, H. Zuber : *Ibid.*, **42**, 1702 (1959).
8) B. Iselin, R. Schwyzer : *Ibid.*, **45**, 1499 (1962).

Recently it has become apparent that in addition to the effect on melanocytes, β-MSH elicits other physiological responses. For example, β-MSH appears to possess central-nervous system stimulating activity in cat[9,10] and is reported to accelerate the rate of disappearance of radioactivity from the rabit thyroid following a tracer dose of ^{131}I.[11] It was reported that extended treatment by β-MSH induced a significant decrease of the weight of the testicles of mice.[12] In fact, reason for the presence of two principles in one mammalian pituitary gland capable of bringing about the same pigmentation is still a mystery. With these consideration, we undertook the systematic



Z=benzyloxycarbonyl, Lys(F)=N^{ε}-formyllysine residue, Narg=N^{G}-nitroarginine residue

Chart 2. Synthetic Route of I

9) R. Guillemin, W. A. Krivoy : Proc. Natl. Acad. Sci., **250**, 1117 (1960).
10) W. A. Krivoy, R. Guillemin : Endocrinology, **69**, 170 (1961).
11) G. Cehovic : Compt. rend. (France), **251**, 832 (1960).
12) G. Cehovic : *Ibid.*, **261**, 1405 (1965).

synthesis of the peptides related to β-MSH in order to evaluate its physiological functions. Here, we wish to describe the synthesis and assay result of the decapeptide corresponding to the C-terminal portion of pig, sheep, beef, monkey and human β-MSH.

Synthesis of the decapeptide (I), histidylphenylalanylarginyltryptophylglycylseryl prolylprolyllysylaspartic acid, resulted from deformylation of histidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysyl aspartic acid (II) as shown in Chart 2. This partially protected decapeptide (II) was prepared by hydrogenolysis of N^{α}-benzyloxycarbonylhistidylphenylalanyl-N^{G}-nitroarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid (III) which was synthesized by coupling N^{α}-benzyloxycarbonylhistidylphenylalanyl-N^{G}-nitroarginyltryptophylglycine (IV) with serylprolylprolyl-N^{ε}-formyllysylaspartic acid (V). The former pentapeptide (IV) was prepared by the known method described by Hofmann and Lande.[13] We have synthesized the latter partially protected pentapeptide (V) by a method different from that of the Swiss group.[6~8] The formyl group[14] served to protect the ε-amino group of the lysine residue of this fragment. Generally N^{ε}-formyllysine peptides are very soluble in aqueous buffers. This property permits one to purify these compounds, such as the partially protected decapeptide (II) by column chromatography on an ion-exchanger. We found that the formyl group could be removed by aqueous hydrazine in the final step of the synthesis without accompanying serious cleavage of peptide bonds.

We have first attempted to prepare V through the fully protected pentapeptide, N^{α}-benzyloxycarbonylserylprolylprolyl-N^{ε}-formyllysylaspartic acid dimethyl ester (VI). N^{α}-benzyloxycarbonyl-N^{ε}-formyllysine[14] was condensed with aspartic acid dimethyl ester to form N^{α}-benzyloxycarbonyl-N^{ε}-formyllysylaspartic acid dimethyl ester which was hydrogenated over a palladium catalyst. The resulting dipeptide ester has, as usually, a great tendency to form the diketopiperazine. Therefore it was isolated as its hydrochloride. This was condensed with N^{α}-benzyloxycarbonylproline *via* the mixed anhydride procedure[15] to form N^{α}-benzyloxycarbonylprolyl-N^{ε}-formyllysylaspartic acid dimethyl ester. After hydrogenolysis, the resulting tripeptide ester was condensed with a mixed anhydride of N^{α}-benzyloxycarbonylserylproline[16] to give the fully protected pentapeptide (VI). Attempt to saponify the dimethyl ester by sodium hydroxide at this stage resulted in the marked cleavage of the particular peptide bond between the serine and proline residues. Examination of the reaction mixture by paper chromatography revealed the presence of free serine and a yellow spot by ninhydrin test. The formation of free serine indicated the N^{α}-benzyloxycarbonyl group of serine was also cleaved by the action of sodium hydroxide. Previously Schwyzer, *et al.*[7] treated the protected octadecapeptide related to beef β-MSH with sodium in liquid ammonia and reported that this treatment caused serious fragmentation of the constructed peptide bonds, though where was not specified. This result suggested that alkaline-labile bonds are present within the molecule of beef β-MSH. We have now noticed that cleavage of the serylprolyl bond proceeded with dilute alkali even in an ice-cold condition.

The above experiment indicated that the protecting group of the carboxyl function should be removed in the early stage of the synthesis. N^{α}-Benzyloxycarbonyl-N^{ε}-formyllysylaspartic acid dimethyl ester was found to be saponified smoothly. N^{α}-Benzyloxycarbonyl-N^{ε}-formyllysylaspartic acid thus obtained was hydrogenated to give N^{ε}-formyllysylaspartic acid. This was condensed with N^{α}-benzyloxycarbonylproline either by means of the *p*-nitrophenyl ester procedure[17] or by the mixed anhydride method[15]

13) K. Hofmann, S. Lande : J. Am. Chem. Soc., **83**, 2286 (1961).
14) K. Hofmann, E. Stütz, G. Spühler, H. Yajima, E. T. Schwartz : *Ibid.*, **82**, 3727 (1960).
15) J. R. Vaughan, Jr., R. L. Osato : J. Am. Chem. Soc., **73**, 3547 (1951).
16) H. Yajima, Y. Okada, T. Oshima : This Bulletin, **14**, 707 (1966).
17) M. Bodanszky, V. du Vigneaud : J. Am. Chem. Soc., **81**, 5688 (1959).

to form N^{α}-benzyloxycarbonylprolyl-N^{ε}-formyllysylaspartic acid. This was also prepared by saponification of N^{α}-benzyloxycarbonylprolyl-N^{ε}-formyllysylaspartic acid dimethyl ester, but the yield was extremely poor. After hydrogenolysis, the resulting tripeptide, prolyl-N^{ε}-formyllysylaspartic acid was again condensed with N^{α}-benzyloxycarbonylproline *p*-nitrophenyl ester to give N^{α}-benzyloxycarbonylprolylprolyl-N^{ε}-formyllysylaspartic acid. After hydrogenolysis, the resulting tetrapeptide, prolylprolyl-N^{ε}-formyllysylaspartic acid reacted with N^{α}-benzyloxycarbonylserine azide[18] to yield N^{α}-benzyloxycarbonylserylprolylprolyl-N^{ε}-formyllysylaspartic acid. This protected pentapeptide was also prepared by coupling N^{α}-benzyloxycarbonylserylproline azide with the tripeptide, prolyl-N^{ε}-formyllysylaspartic acid, in lesser yield than the former procedure. Hydrogenolysis of this peptide gave the partially protected pentapeptide, serylprolylprolyl-N^{ε}-formyllysylaspartic acid (V). The product produced a gingle spot on paper chromatogram in two different solvent systems and the acid hydrolysis gave the constituent amino acids in the ratios predicted by theory. Digestion of the peptide with leucine amino peptidase (LAP)[19] demonstrated the presence of one mole of N^{ε}-formyllysine in the hydrolysate. The partially protected pentapeptide (V), a subunit for the synthesis of I, thus obtained appears to be homogeneous and to possess the well defined L-configuration of the constituent amino acids.

This N^{ε}-lysine blocked pentapeptide (V) was then condensed with N^{α}-benzyloxycarbonylhistidylphenylalanyl-N^{G}-nitroarginyltryptophylglycine (IV)[13] by means of the mixed anhydride procedure to give the protected decapeptide, N^{α}-benzyloxycarbonylhistidylphenylalanyl-N^{G}-nitroarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid (III) which was subsequently subjected to hydrogenolysis without further purification. The resulting crude product was purified by column chromatography on carboxymethylcellulose (CMC).[20] The partially protected decapeptide (II), histidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid, emerged from the column with 0.05*M* pyridine acetate buffer at pH 5.0, while the contaminated pentapeptide, histidylphenylalanylarginyltryptophylglycine was found in the 0.1*M* eluate. Examination by paper chromatography and the amino acid analysis of the acid hydrolysate seems enough to verify the conclusion that this synthetic partially protected decapeptide (II) is homogeneous.

Next deformylation of the partially protected decapeptide (II) was investigated. Removal of the formyl group from α-amino function of amino acids or peptides by methanolic hydrogen chloride was examined previously by Sheehan, *et al.*[21] However the cleavage of the formyl group attached at ε-amino group of lysine proceeds somewhat slowly than that of N^{α}-group under the identical conditions. When Hofmann and his colleagues[22,23] removed a number of formyl groups attached at ε-amino groups of lysine residues present in their synthetic eicosapeptide or tricosapeptide related to adrenocorticotropic hormone, certain degree of acid hydrolysis of peptide bonds within the sequence was recorded. We have noticed that the same treatment resulted in serious destruction of the peptide bonds of V and II. In preliminary experiments, we have now found that the treatment of N^{ε}-formyllysine with dilute aqueous hydrazine at 37° for 48 hours generated free lysine in satisfactory yield. Exposure of protected peptide esters to 80~85% hydrazine hydrate is commonly used in peptide chemistry

18) R. W. Holley, E. Sondheimer : *Ibid.*, **76**, 1326 (1954).
19) Partially purified LAP was prepared according to the method of D. H. Spackman, E. L. Smith, D. M. Brown : J. Biol. Chem., **212**, 244 (1955). Some prolinase activity was found in this preparation.
20) E. A. Peterson, H. A. Sober : J. Am. Chem. Soc., **78**, 751 (1956). Commercial preparation (Cellex CM, 0.8 meq/g.) was purchased from Bio. Rad. Laboratories.
21) J. C. Sheehan, D. D. H. Yang : J. Am. Chem. Soc., **80**, 1154 (1958).
22) K. Hofmann, H. Yajima, T. Y. Liu, N. Yanaihara : *Ibid.*, **84**, 4475 (1962).
23) K. Hofmann, H. Yajima, T. Y. Liu, N. Yanaihara, C. Yanaihara, J. H. Humes : *Ibid.*, **84**, 4481 (1962).

for preparation of corresponding hydrazides without affecting polyfunctional groups of constituent amino acids. Therefore it is reasonable to assume that the functional groups of Ⅱ remain intact under the above stated, rather mild conditions. The protected decapeptide (Ⅱ) was incubated under the identical conditions and the product was again purified by ion-exchange chromatography on CMC and 0.1*M* pyridine acetate buffer served to elute the desired compound (I). Chromatographic pattern indicated that very little cleavage of the peptide bond occurred between the glycine and serine residues. Geschwind and Li[24] mentioned that dilute sodium hydroxide cleaved the glycylseryl peptide bond of pig and beef β-MSH. It is obvious that, in addition to the serylprolyl bond as mentioned above, another alkali labile peptide bond is present in the C-terminal portion of β-MSH.

The purified compound (I) exhibited a single ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot on paper chromatogram in two different solvent systems and produced a single ninhydrin positive spot on paper electropherograms at two different pH values. Although the elemental analysis of the product (I) was matched with the values calculated for relatively high hydration, this seems responsible to the very hygroscopic property of this compound. Acid hydrolysate contained the constituent amino acids in the ratios predicted by theory. Usual LAP digestion gave somewhat low recovery of the amino acids present in the last pentapeptide of the C-terminus. It is known that LAP treatment of larger peptides showed similar phenomena.[22,23,25] These rather extensive analytical evaluations appear to justify the conclusion that our synthetic I exhibits a high degree of homogeneity.

The MSH activity of I and Ⅱ were assayed *in vitro* according to the procedure described by Lerner and Wright[26] using frog-skins from *Rana pipiens*. It was found that the free decapeptide (I) and formyldecapeptide (Ⅱ) exhibited the activity equivalent to 2.4×10^6 and 1×10^6 MSH U/g. respectively. From this observation that Ⅱ possesses the activity at the level of 10^6 MSH U/g., it can be seen that the free ε-amino group of the lysine residue is not essential for the intrinsic MSH activity of this compound.

The MSH activity of the octapeptide and the partially protected octapeptide of the α-MSH series, histidylphenylalanylarginyltryptophylglycyllysylprolylvaline amide and histidylphenylalanylarginyltryptophylglycyl-N^{ε}-formyllysylprolylvaline amide, was reported to be 8×10^6 MSH U/g.[27] Comparison of this value with those of the above decapeptides (I and Ⅱ) indicated that these compounds possess nearly equal level of the MSH activity as shown in Table I. The free ε-amino group of the lysine residue of the octapeptide amide does not seem to be essential. This relation seems to be similar to the lysine residue located at the 2nd position from the C-terminal of the decapeptide (I). When the structure of the above stated octapeptide amide and the decapeptide (I),

Table I. Melanocyte-stimulating Hormone Activity of Synthetic Peptides

		MSH U/g.
α-MSH series[27]	H-His. Phe. Arg. Try. Gly. Lys(F). Pro. Val-NH_2	8×10^6
	H-His. Phe. Arg. Try. Gly. Lys. Pro. Val-NH_2	8×10^6
β-MSH series	H-His. Phe. Arg. Try. Gly. Ser. Pro. Pro. Lys(F). Asp(OH)-OH	1×10^6
	H-His. Phe. Arg. Try. Gly. Ser. Pro. Pro. Lys. Asp(OH)-OH	2.4×10^6

24) I. I. Geschwind, C. H. Li : Arch. Biochem. Biophys., **106**, 200 (1964).
25) W. F. White : J. Am. Chem. Soc., **77**, 4691 (1955).
26) A. B. Lerner, M. R. Wright : Methods Biochem. Anal., **8**, 295 (1960).
27) K. Hofmann, H. Yajima : J. Am. Chem. Soc., **83**, 2289 (1961).

was compared, the proline residue both at the 7th position from the N-terminus can be judged as a common structural feature of these two peptides in addition to histidyl-phenylalanylarginyltryptophylglycine, an active fragment of α-MSH.[5] This seems to give an important conformational effect to the C-terminal portion of both α- and β-MSH.

Synthesis of the N-terminal portion of β-MSHs and detail examination of extra-MSH activity of the synthetic peptides will be reported in the future.

Experimental

Analytical procedures and general experimental methods employed in this investigation are essentially the same as recorded in the IV of this series.[28] Rf^1 refers to the system of Partridge[29]; Rf^2 values refer to the 2-butanol–ammonia system[30] and are expressed as multiples of the distance traveled by a phenylalanine marker. The following abbreviations are used : His=histidine, Phe=phenylalanine, Arg=arginine, Try=tryptophan, Gly=glycine, Ser=serine, Pro=proline, Lys=lysine, Asp=aspartic acid.

N^{α}-Benzyloxycarbonyl-N^{ε}-formyllysylaspartic Acid Dimethyl Ester——A mixed anhydride was prepared in the usual manner[15] from N^{α}-benzyloxycarbonyl-N^{ε}-formyllysine[14] (10.83 g.) in dry tetrahydrofuran (75 ml.) with triethylamine (4.9 ml.) and ethyl chloroformate (3.4 ml.). This solution was added slowly with stirring to a chilled solution of dimethyl aspartate (prepared from 7.52 g. of the hydrochloride with 4.9 ml. of triethylamine) in dimethylformamide (25 ml.). The mixture was stirred at 0° for 3 hr. and the solvent was evaporated *in vacuo*. The sparingly soluble white precipitate formed by addition of ether was purified by suspending it three times in ice-cold 10% citric acid followed by three similar treatments with 5% NH_4OH. The compound was finally washed with H_2O, dried and recrystallized from AcOEt; yield 10.36 g. (65%), m.p. 141~143°, $[\alpha]_D^{27}$ −11.3° (c=0.7, MeOH). *Anal.* Calcd. for $C_{21}H_{29}O_8N_3$: C, 55.9; H, 6.5; N, 9.3. Found : C, 55.8; H, 6.7; N, 9.3.

N^{ε}-Formyllysylaspartic Acid Dimethyl Ester Hydrochloride——N^{α}-Benzyloxycarbonyl-N^{ε}-formyllysylaspartic acid dimethyl ester (5.67 g.) was dissolved in a mixture of MeOH (30 ml.), glacial AcOH (1.8 ml.) and H_2O (5 ml.), a Pd catalyst was added and the mixture was stirred vigorously in a stream of H_2 until evolution of CO_2 ceased. The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The resulting sirup was dissolved in ice-cold 1*N* HCl (12.6 ml.) and the solution was lyophilized to give an oily product; yield 5.80 g. (100%) $[\alpha]_D^{20}$ +12.6° (c=1.0, MeOH), Rf^1 0.65.

Diketopiperazine of N^{ε}-Formyllysylaspartic Acid Dimethyl Ester——N^{α}-Benzyloxycarbonyl-N^{ε}-formyllysylaspartic acid dimethyl ester (2.26 g.) in MeOH (30 ml.) containing 13 v/v% aqueous AcOH (7 ml.) was hydrogenated as described above. The catalyst was removed by filtration and the filtrate was condensed *in vacuo*. Anhydrous benzene was added to the residue and then evaporated. This procedure was repeated five times to give the crystalline product which was recrystallized from MeOH with ether; yield 1.15 g. (81%), m.p. 179~180°. The mass spectra exhibited the parent peak at m/e 285. *Anal.* Calcd. for $C_{12}H_{19}O_5N_3$: C, 50.5; H, 6.9; N, 14.7. Found : C, 50.3; H, 6.8; N, 14.6.

N^{α}-Benzyloxycarbonylprolyl-N^{ε}-formyllysylaspartic Acid Dimethyl Ester——A mixed anhydride was prepared in the usual manner from N^{α}-benzyloxycarbonylproline (2.92 g.) in ice-cold tetrahydrofuran (17 ml.) with triethylamine (1.8 ml.) and ethyl chloroformate (1.2 ml.). This solution was added to an ice-cold solution of N^{ε}-formyllysylaspartic acid dimethyl ester (prepared from 5.80 g. of the hydrochloride with 1.8 ml. of triethylamine) in dimethylformamide (12 ml.) and the mixture was stirred in an ice-bath for 2 hr. The solvent was removed *in vacuo*, the residue was dissolved in AcOEt which was washed successively with 10% citric acid, 5% NH_4OH and H_2O and dried over Na_2SO_4. The solvent was evaporated and the residue was recrystallized from AcOEt; yield 4.62 g. (64%), m.p. 133~135°, $[\alpha]_D^{27}$ −55.2° (c=1.0, MeOH). Amino acid ratios in an acid hydrolysate $Pro_{1.03}Lys_{1.00}Asp_{0.98}$ (average recovery 94%). *Anal.* Calcd. for $C_{26}H_{36}O_9N_4$: C, 56.9; H, 6.6; N, 10.2. Found : C, 57.0; H, 6.9; N, 9.9.

Prolyl-N^{ε}-formyllysylaspartic Acid Dimethyl Ester Hydrochloride——N^{α}-Benzyloxycarbonylprolyl-N^{ε}-formyllysylaspartic acid dimethyl ester (0.57 g.) in MeOH (20 ml.) containing 27 v/v% AcOH (0.7 ml.) was hydrogenated over a Pd catalyst in the usual manner. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in ice-cold 1*N* HCl (1 ml.) and the solution was lyophilized to give an oily product; yield 0.47 g. (100%), $[\alpha]_D^{27}$ −35.6° (c=0.7, MeOH), Rf^1 0.65.

N^{σ}-Benzyloxycarbonylserylprolylprolyl-N^{ε}-formyllysylaspartic Aicd Dimethyl Ester (VI)——A mixed anhydride, prepared in the usual manner from N^{α}-benzyloxycarbonylserylproline[16] (0.34 g.) in a mixture of dry tetrahydrofuran (4.0 ml.) and dioxane (4.0 ml.) with *n*-tributylamine (0.24 ml.) and ethyl chloroformate (0.10

28) H. Yajima, K. Kubo : This Bulletin, **13**, 759 (1965).
29) S. M. Partridge : Biochem. J., **42**, 238 (1948).
30) J. F. Roland, A. M. Gross : Anal. Chem., **26**, 502 (1954).

ml.), was added slowly with stirring to a chilled solution of prolyl-N^{ε}-formyllysylaspartic acid dimethyl ester (prepared from 0.51 g. of the hydrochloride and 0.14 ml. of triethylamine) in dimethylformamide (5 ml.). The mixture was stirred in an ice-bath for 2.5 hr., when the bulk of the solvent was removed *in vacuo*. The residue was extracted into AcOEt and the extract was washed with three portions of 10% citric acid, 5% NH_4OH and H_2O. Evaporation of the dried AcOEt solution (over Na_2SO_4) gave an oil; yield 0.25 g. (34%), $[\alpha]_D^{15}$ −74.1° (c=0.9, MeOH). Amino acid ratios in an acid hydrolysate $Ser_{0.76}Pro_{1.74}Lys_{1.04}Asp_{1.00}$ (average recovery 82%).

The product (10 mg.) was dissolved in MeOH (0.2 ml.) and 1*N* NaOH (0.1 ml.) was added. After standing in an ice-bath for 40 min., the solution was neutralized with AcOH. Examination of the solution by paper chromatography in the system of Partridge revealed the presence of ninhydrin positive spots; Rf^1 0.17 (Ser) and 0.22 (yellow).

The product (10 mg.) in 0.5*N* HCl (0.2 ml.) was heated in a boiling water-bath for 30 min., paper chromatogram of the solution showed a number of ninhydrin positive spots; Rf^1 0.22, 0.32 and 0.64.

N^{α}-Benzyloxycarbonylserylproline Methyl Ester——A solution of N^{α}-benzyloxycarbonylserylproline[16] (1.00 g.) in MeOH (15 ml.) was cooled in an ice-bath and diazomethane (prepared from 5.0 g. of nitrosomethylurea) in ether was added until a faint yellow color persisted. The mixture was kept for 1 hr. at room temperature, the excess of diazomethane was destroyed by addition of a few drops of glacial AcOH and the solvent was evaporated to dryness *in vacuo*. The residue was recrystallized from MeOH; yield 0.70 g. (65%), m.p. 117~121°, $[\alpha]_D^{15}$ −68.2° (c=0.9, MeOH). *Anal.* Calcd. for $C_{17}H_{22}O_6N_2$: C, 58.3; H, 6.3; N, 8.0. Found : C, 58.2; H, 6.6; N, 7.9.

N^{α}-Benzyloxycarbonylserylproline Hydrazide——N^{α}-Benzyloxycarbonylserylproline methyl ester (0.60 g.) was dissolved in MeOH (5 ml.) and 80% hydrazine hydrate (0.6 ml.) was added. The solution was kept at room temperature for 24 hr. The solvent was evaporated and the residue was recrystallized from MeOH; yield 0.49 g. (79%), m.p. 137~141°, $[\alpha]_D^{24}$ −93.9° (c=1.0, 40% AcOH). *Anal.* Calcd. for $C_{16}H_{22}O_5N_4$: C, 54.8; H, 6.3; N, 16.0. Found : C, 55.1; H, 6.5; N, 15.7.

N^{α}-Benzyloxycarbonyl-N^{ε}-formyllysylaspartic Acid——N^{α}-Benzyloxycarbonyl-N^{ε}-formyllysylaspartic acid dimethyl ester (15.09 g.) was dissolved in hot MeOH (40 ml.), the solution was chilled at room temperature and 1*N* NaOH (136 ml.) was added. The mixture was kept at room temperature for 2 hr. and then neutralized with glacial AcOH. The solution was condensed *in vacuo* to one half of the volume and washed with AcOEt. The aqueous phase was acidified to pH 4.0 with 1*N* HCl. Storage of the solution gave a solid which was collected by filtration, washed with H_2O and recrystallized from a mixture of MeOH and AcOEt; yield 11.03 g. (78%), m.p. 154~156°, $[\alpha]_D^{20}$ +0.7° (c=0.6, MeOH). *Anal.* Calcd. for $C_{19}H_{25}O_8N_3$: C, 53.9; H, 6.0; N, 9.9. Found : C, 53.6; H, 6.3; N, 9.7.

N^{ε}-Formyllysylaspartic Acid——A sample of N^{α}-benzyloxycarbonyl-N^{ε}-formyllysylaspartic acid (9.30 g.) was hydrogenated over a Pd catalyst in a mixture of MeOH (40 ml.), H_2O (10 ml.) and glacial AcOH (4.2 ml.). The product was isolated in the usual manner and recrystallized from H_2O by addition of EtOH; yield 5.83 g. (91%), m.p. 173~176°, $[\alpha]_D^{15}$ +24.0° (c=1.1, H_2O), Rf^1 0.21, Rf^2 0.22. *Anal.* Calcd. for $C_{11}H_{19}O_6N_3$: C, 45.7; H, 6.6; N, 14.5. Found : C, 46.0; H, 6.8; N, 14.2.

N^{α}-Benzyloxycarbonylprolyl-N^{ε}-formyllysylaspartic Acid——a) A mixed anhydride, prepared from N^{α}-benzyloxycarbonylproline (0.36 g.) in ice-cold anhydrous tetrahydrofuran (5 ml.) with triethylamine (0.22 ml.) and ethyl chloroformate (0.15 ml.), was added to a chilled solution of N^{ε}-formyllysylaspartic acid (0.45 g.) and triethylamine (0.43 ml.) in 50 v/v% aqueous dimethylformamide (6 ml.). The mixture was stirred in an ice-bath for 3 hr. then the solvent was removed *in vacuo*. The residue was dissolved in H_2O which was washed with AcOEt. The aqueous phase was acidified to pH 4 with 1*N* HCl and the resulting precipitate was extracted with AcOEt, which was washed with a solution saturated with NaCl and dried over Na_2SO_4. Evaporation of the solvent gave a powder which was recrystallized from a mixture of MeOH and AcOEt; yield 0.30 g. (50%), m.p. 170~173°, $[\alpha]_D^{25}$ −43.4° (c=0.9, MeOH). *Anal.* Calcd. for $C_{24}H_{32}O_9N_4$: C, 55.4; H, 6.2; N, 10.8. Found : C, 55.4; H, 6.4; N, 10.5.

b) N^{ε}-formyllysylaspartic acid (6.39 g.) was dissolved in 75 v/v% aqueous dioxane (160 ml.) and triethylamine (6.2 ml.) was added. To this solution was added N^{α}-benzyloxycarbonylproline *p*-nitrophenyl ester[17] (10.24 g.) and the solution was stirred at room temperature for 48 hr. The bulk of the solvent was evaporated *in vacuo*. The residue was acidified to pH 4.0 with 1*N* HCl and AcOEt (10 ml.) was added. The crystalline product formed during storage in a refrigerator was collected by filtration and washed with 10% citric acid, H_2O and AcOEt and recrystallized from a mixture of MeOH and AcOEt; yield 9.0 g. (93%), m.p. 178~180°, mixed-melting point with the sample obtained in (a) was 178~180°, $[\alpha]_D^{25}$ −48.6° (c=1.0, MeOH). *Anal.* Calcd. for $C_{24}H_{32}O_9N_4$: C, 55.4; H, 6.2; N, 10.8. Found : C, 55.3; H, 6.3; N, 10.5.

c) N^{α}-Benzyloxycarbonylprolyl-N^{ε}-formyllysylaspartic acid dimethyl ester (0.28 g.) in MeOH (2 ml.) was treated with 1*N* NaOH (3 ml.) at 20° for 40 min. The solution was neutralized with 10% citric acid, condensed *in vacuo*, washed with AcOEt and then acidified to pH 4.0 with ice-cold 1*N* HCl. The product was extracted with AcOEt. The extract was washed with a solution saturated with NaCl, dried over Na_2SO_4 and condensed *in vacuo*. Tritulation of the residue with AcOEt gave a white powder; yield 0.03 g. (10%), m.p. 168~171°.

Prolyl-N$^{\varepsilon}$-formyllysylaspartic Acid——N$^{\alpha}$-Benzyloxycarbonylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid (7.92 g.) was hydrogenated over a Pd catalyst in a mixture of MeOH (40 ml.), H_2O (7 ml.) and glacial AcOH (3.3 ml.). The catalyst was removed by filtration and the filtrate was evaporated *in vacuo*. The resulting gelatinous product was purified by reprecipitation from H_2O with EtOH; yield 5.64 g. (80%), m.p. 143°(decomp.), $[\alpha]_D^{22}$ −42.1° (c=1.0, H_2O), Rf^1 0.21, Rf^2 0.25. *Anal.* Calcd. for $C_{16}H_{26}O_7N_4 \cdot 2H_2O$: C, 46.6; H, 7.5; N, 12.5. Found : C, 46.4; H, 7.6; N, 12.9.

N$^{\alpha}$-Benzyloxycarbonylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic Acid——To a solution of prolyl-N$^{\varepsilon}$-formyllysylaspartic acid (5.30 g.) in 55 v/v% aqueous dioxane (47 ml.) was added triethylamine (3.8 ml.) and N$^{\alpha}$-benzyloxycarbonylproline *p*-nitrophenyl ester (7.10 g.) in dioxane (40 ml.). The mixture was stirred at room temperature for 48 hr. After evaporation of the solvent, H_2O was added to the residue, the solution was neutralized with AcOH and washed with AcOEt. The aqueous phase was condensed to a small volume and then acidified to pH 4.0 with 1*N* HCl. The resulting precipitate was extracted with *n*-butanol, which was washed with H_2O saturated with *n*-butanol, and evaporated *in vacuo*. Tritulation of the residue with AcOEt furnished a semisolid material which was collected and dried over P_2O_5; yield 7.30 g. (90%). $[\alpha]_D^{21}$ −96.3° (c=1.0, MeOH). *Anal.* Calcd. for $C_{29}H_{39}O_{10}N_5 \cdot H_2O$: C, 54.8; H, 6.5; N, 11.0. Found : C, 54.9; H, 7.0; N, 11.1. Di-dicyclohexylamine salt : m.p. 230°(decomp.). *Anal.* Calcd. for $C_{29}H_{39}O_{10}N_5 \cdot 2C_{12}H_{23}N \cdot 2H_2O$: C, 62.6; H, 8.8; N, 9.7. Found : C, 62.8; H, 8.9; N, 9.9.

Prolylprolyl-N$^{\varepsilon}$-formyllysylaspartic Acid——N$^{\alpha}$-Benzyloxycarbonylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid (7.30 g.) in MeOH (30 ml.) containing 35 v/v% AcOH (6.8 ml.) was hydrogenated over a Pd catalyst. The catalyst was removed by filtration. After evaporation of the solvent, the residue was lyophilized twice to give a fluffy powder; yield 4.90 g. (86%), $[\alpha]_D^{24}$ −109.5° (c=0.9, H_2O), Rf^1 0.30, Rf^2 0.18. *Anal.* Calcd. for $C_{21}H_{33}O_8N_5 \cdot 3H_2O$: C, 46.9; H, 7.3; N, 13.0. Found : C, 46.9; H, 6.9; N, 13.1.

N$^{\alpha}$-Benzyloxycarbonylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic Acid——a) The entire operation was performed in a cold room at 4°. An AcOEt solution (approximately 40 ml.) of N$^{\alpha}$-benzyloxycarbonylserine azide[18] (prepared from 5.60 g. of the corresponding hydrazide) was added to a solution of prolylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid (4.90 g.) in H_2O (15 ml.) and triethylamine (4.2 ml.). The mixture was stirred for 24 hr., when an additional azide (prepared from 2.80 g. of the hydrazide) was added and the reaction was continued for an additional 24 hr. The aqueous phase was separated from the AcOEt layer which was extracted with 5% NH_4OH. This alkaline extract was combined with the above aqueous solution, the combined solution was washed with AcOEt, then neutralized with AcOH, condensed to one third of the volume and acidifled to pH 4.0 with 1*N* HCl. The resulting precipitate was extracted with *n*-butanol, which was washed with H_2O saturated with *n*-butanol and evaporated to dryness. The residue was tritulated with AcOEt to form a solid material which was re-dissolved in *n*-butanol and precipitated by addition of AcOEt; yield 5.52 g. (75%), $[\alpha]_D^{22}$ −115.0° (c=0.8, MeOH). Amino acid ratios in an acid hydrolysate $Ser_{0.90}Pro_{1.87}Lys_{1.01}Asp_{1.00}$ (average recovery 81%). *Anal.* Calcd. for $C_{32}H_{44}O_{12}N_6 \cdot H_2O$: C, 53.1; H, 6.4; N, 11.6. Found : C, 53.6; H, 7.1; N, 11.4.

b) The entire operation was carried out in a cold room at 4°. N$^{\alpha}$-Benzyloxycarbonylserylproline hydrazide (0.42 g.) was dissolved in 1*N* HCl (3.6 ml.) and $NaNO_2$ (0.08 g.) in H_2O (1 ml.) was added. The resulting azide was extracted with AcOEt which was washed successively with 1*N* HCl, a solution saturated with $NaHCO_3$ and a solution saturated with NaCl. This solution was added to a solution of prolyl-N$^{\varepsilon}$-formyllysylaspartic acid (0.30 g.) and triethylamine (0.28 ml.) in H_2O (1 ml.). The mixture was stirred vigorously for 24 hr., when an additional azide (prepared from 0.18 g. of the hydrazide) was added. After the reaction was continued for an additional 24 hr., the aqueous layer was separated from the AcOEt, which was extracted with 5% NH_4OH. The extract and the above aqueous solution were combined. The aqueous solution was washed with fresh AcOEt, then acidified to pH 4.0 with 1*N* HCl and the resulting precipitate was extracted with *n*-butanol equilibrated with H_2O. The extract was washed with H_2O saturated with *n*-butanol, and evaporated to dryness *in vacuo*. The resulting powder formed by addition of AcOEt to the residue was reprecipitated from MeOH with AcOEt; yield 0.11 g. (16%), $[\alpha]_D^{22}$ −114.6° (c=0.9, MeOH). Amino acid ratios in an acid hydrolysate $Ser_{0.94}Pro_{2.00}Lys_{0.91}Asp_{1.00}$ (average recovery 78%). *Anal.* Calcd. for $C_{32}H_{44}O_{12}N_6 \cdot H_2O$: C, 53.1; H, 6.4; N, 11.6. Found : C, 52.8; H, 6.6; N, 11.7.

Serylprolylprolyl-N$^{\varepsilon}$-formyllysylaspatic Acid (V)——N$^{\alpha}$-Benzyloxycarbonylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid (1.44 g.) in 80% MeOH (25 ml.) containing AcOH (0.5 ml.) was hydrogenated over a Pd catalyst. The catalyst was removed by filtration and the filtrate was condensed to a small volume and finally lyophilized to give a fluffy powder; yield 1.03 g. (91%), $[\alpha]_D^{21}$ −126.6° (c=0.9, H_2O), Rf^1 0.31, Rf^2 0.22. Amino acid ratios in an acid hydrolysate $Ser_{0.97}Pro_{2.01}Lys_{1.00}Asp_{1.06}$ (average recovery 91%). Amino acid ratios in a LAP digest $Ser_{1.09}Pro_{1.95}N^{\varepsilon}$-formyllys$_{1.00}Asp_{0.85}$ (average recovery 88%). *Anal.* Calcd. for $C_{24}H_{38}O_{10}N_6 \cdot 2H_2O$: C, 47.5; H, 6.9; N, 13.9. Found : C, 47.3; H, 7.0; N, 13.6.

Histidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic Acid (II)—— A mixed anhydride, prepared from N$^{\alpha}$-benzyloxycarbonylhistidylphenylalanyl-N^{G}-nitroarginyltryptophylglycine[13] (0.58 g. dried over P_2O_5 at 50° for 8 hr. *in vacuo*) in dry dimethylformamide (5 ml.) with triethylamine (0.14 ml.) and isobutyl chloroformate (0.13 ml.) was added to a solution of serylprolylprolyl-N$^{\varepsilon}$-formyllysylaspartic acid (0.37 g.) in H_2O (1 ml.) and dimethylformamide (2 ml.) and triethylamine (0.19 ml.). The mixture was stirred in an ice-bath for 30 min. and then at room temperature for 2 hr. After evaporation of the solvent,

the resulting powder formed by addition of H_2O to the residue was collected by filtration, washed with H_2O and dried. The product (0.99 g.) was subsequently hydrogenated over a Pd catalyst in 50% AcOH (25 ml.). Examination of the solution by paper chromatography revealed the presence of two ninhydrin positive spots with Rf^1 0.50 (histidylphenylalanylarginyltryptophylglycine) and 0.27. The solvent, after filtration, was evaporated to dryness *in vacuo*, the residue was dried over KOH pellets and then dissolved in H_2O (300 ml.). The solution was applied to a column of CMC (3×15 cm.) which was eluted with the following pyridine acetate buffers (pH 5.0); 0.01*M* (750 ml.), 0.02*M* (600 ml.), 0.05*M* (1200 ml.), and 0.1*M* (1500 ml.). Individual fractions of 17 ml. were collected with a flow rate of 5 to 6 ml. per min. Absorbancy at 280 mμ was determined in each fraction. The desired fraction present in the 0.05*M* eluate was collected and the solvent was removed *in vacuo* and the residue was lyophilized to give a fluffy powder; yield 0.28 g. (31.2%), $[\alpha]_D^{22}$ −65.8° (c=0.4, H_2O), Rf^1 0.27, Rf^2 0.56, sharp single spot, ninhydrin, Pauly, Sakaguchi and Ehrlich positive. Amino acid ratios in an acid hydrolysate $His_{0.92}Phe_{1.06}Arg_{0.96}Gly_{1.11}Ser_{0.94}Pro_{2.04}Lys_{0.88}Asp_{1.00}$ (average recovery 96%). Amino acid ratios in a LAP digest $His_{1.00}Phe_{0.99}Arg_{1.04}Try_{0.93}Gly_{0.96}Ser_{0.90}Pro_{2.06}N^{\varepsilon}$-formyllys$_{1.06}Asp_{0.75}$ (average recovery 85%). *Anal.* Calcd. for $C_{58}H_{79}O_{15}N_{17}\cdot CH_3COOH\cdot 7H_2O$: C, 50.0; H, 6.8; N, 16.5; CH_3COOH, 4.2. Found: C, 50.0; H, 6.9; N, 16.9; CH_3COOH, 4.1.

Treatment of N^{ε}-formyllysine with Aqueous Hydrazine——N^{ε}-Formyllysine (1.0 g.) was dissolved in H_2O (20 ml.) and 80% hydrazine hydrate (5 ml.) was added. The solution was incubated at 37° for 48 hr., when the solvent was evaporated *in vacuo*. The residue was lyophilized and kept over H_2SO_4 in an evacuated desiccator. Paper chromatogram of the residue revealed the presence of two ninhydrin positive spots, Rf^1 0.22 (heavy spot) and 0.44 (very faint spot, N^{ε}-formyllysine). The residue was dissolved in 1*N* HCl (4 ml.) and pH of the solution was adjusted to 7 with pyridine. After evaporation of the solvent, ethanol was added to form a crystalline solid; yield 0.84 g. (80%), m.p. 255~257°, $[M]_D^{29}$ +27.2° (c=1.4, 5*N* HCl), Rf^1 0.22 (lit.[31] m.p. 263~264°, $[M]_D$ +30.4 in 5*N* HCl). Identity was confirmed by mixed melting point and comparison of IR spectra with the authentic lysine monohydrochloride.

Histidylphenylalanylarginyltryptophylglycylserylprolylprolyllysylaspartic Acid (I)——Histidylphenylalanylarginyltryptophylglycylserylprolylprolyl-N^{ε}-formyllysylaspartic acid (0.16 g.) was dissolved in H_2O (1.2 ml.) and 80% hydrazine hydrate (0.2 ml.) was added. The solution was incubated at 37° for 48 hr., when the solution was lyophilized and the flask was kept over H_2SO_4 *in vacuo*. Examination of the product by paper chromatography revealed the presence of three ninhydrin positive spots, Rf^1 0.22 (heavy spot), 0.27 and 0.49 (faint spots). The mixture was dissolved in H_2O (100 ml.) and the solution was applied to a column of CMC (2×5.5 cm.) which was eluted with the following pyridine acetate buffers (pH 5.0); 0.02*M* (200 ml.), 0.05*M* (450 ml.), 0.1*M* (700 ml.), and 0.2*M* (200 ml.). Individual fractions (15 ml. each) were collected and absorbancy at 280 mμ was determined in each fraction. The desired fraction in 0.1*M* eluate was pooled and the solvent was first removed by evaporation and finally by lyophylization to give a fluffy powder; yield 0.07 g. (45%), $[\alpha]_D^{27}$ −62.5° (c=0.3, H_2O), Rf^1 0.22, Rf^2 0.42, single ninhydrin, Pauly, Sakaguchi and Ehrlich positive spot, homogeneous on paper electrophoresis at pH 3.8 and 6.5 in pyridine acetate buffers (1000 volt, 2 hr.). Amino acid ratios in an acid hydrolysate $His_{1.00}Phe_{0.98}Arg_{0.97}Gly_{0.92}Ser_{0.93}Pro_{2.01}Lys_{1.03}Asp_{1.00}$ (average recovery 83%). Amino acid ratios in an usual LAP digest; $His_{1.00}Phe_{1.02}Arg_{1.01}Try_{0.92}Gly_{0.92}Ser_{0.42}Pro_{0.77}Lys_{0.44}Asp_{0.44}$ when the 2nd shot of LAP was applied, the amino acid ratios were $His_{1.13}Phe_{1.09}Arg_{0.86}Try_{1.00}Gly_{1.19}Ser_{0.71}Pro_{1.90}Lys_{0.79}Asp_{0.61}$ (average recovery 83%). *Anal.* Calcd. for $C_{57}H_{79}O_{14}N_{17}\cdot 2CH_3COOH\cdot 11H_2O$: C, 47.4; H, 7.1; N, 15.4; CH_3COOH, 7.8. Found: C, 47.2, 47.8; H, 7.1, 7.1; N, 15.5, 14.7; CH_3COOH, 5.1.

In 0.05*M* eluate, II (33 mg.) was recovered unchanged. Histidylphenylalanylarginyltryptophylglycine (15 mg.) was isolated from 0.2*M* eluate, Rf^1 0.49, amino acid ratios in an acid hydrolysate $His_{1.07}Phe_{0.96}Arg_{0.93}Gly_{1.00}$ (average recovery 75%).

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