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247. Haruaki Yajima, Yasuhiko Kinomura, Takao Oshima, and Yoshio Okada*1: Studies on Peptides. XV.*2,3 Synthesis of Prolyltyrosylarginylmethionine, the Tetrapeptide situated within the Amino Acid Sequences of Monkey and Human β-Melanocyte-stimulating Hormones.

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Arginylmethionine was prepared by three different methods, one from N^{α}-benzyloxycarbonyl-N^{G}-tosylarginylmethionine with sodium in liquid ammonia and others, from N^{α}-benzyloxycarbonyl-N^{G}-nitroarginylmethionine by treatment with hydrogen fluoride, and from N^{α}-*t*-butoxycarbonyl-N^{G}-nitroarginylmethionine by treatment with stannous chloride in 80% formic acid. Arginylmethionine was coupled with N^{α}-*t*-butoxycarbonylprolyltyrosine *via* the azide method and the resulting peptide was treated with trifluoroacetic acid to give prolyltyrosylarginylmethionine, the tetrapeptide situated within the structures of monkey and human β-melanocyte-stimulating hormones.

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The preparation of arginylmethionine has not been reported in the literature so far because of its difficulty in synthesis. The particular arrangement of these two amino acids occurs in nature as shown in the amino acid sequences of monkey[1] and human β-melanocyte-stimulating hormones (MSH).[2~5] We have recently synthesized a partially protected undecapeptide, glutamylhistidylphenylalanylarginyltryptophylglycylserylprolyl-prolyl-N^{ε}-formyllysylaspartic acid which embodies the common C-terminal portion of these hormones and a part of which was published.[6] When we started the total syntheses of these hormones, we felt it necessary to establish fundamental methodologies to build up this key dipeptide, arginylmethionine and its related peptides.

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. Sep. Pro. Pro. Lys. Asp.

Chart 1. Amino Acid Sequences of Monkey and Human β-MSH

It is now well documented that when arginine or arginine-containing peptides are used as an amino component, the peptide forming reaction proceeds without accompanying any side reactions at the guanidino group so long as it is protonated.[7] However, when the carboxyl group of arginine is activated, protection of the guanidino group is essential as well as its α-amino nitrogen. The N^{G}-nitro group is the first one invented

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*2 Part XIV. H. Yajima, O. Nishimura, K. Kawasaki, Y. Okada: This Bulletin, **15**, 854 (1967).

*3 Peptides and peptide derivatives mentioned in this communication are of the L-configuration.

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for this purpose by Kossel and Kennaway[8] and Bergmann, *et al.*[9] Later, Hofmann, *et al.*[10,11] successfully applied a mixed anhydride of N^{α}-benzyloxycarbonyl-N^{G}-nitroarginine for the syntheses of various arginyl peptides. Wide applicability of N^{G}-nitroarginine was systematically investigated by many other authors[12~19] and this compound became the most important N^{G}-substituted derivative of arginine because of its outstanding feature that the nitro group can be reduced off catalytically with the regeneration of the guanidino group.[9] However this feature becomes invalid when sulfur-containing amino acids such as methionine are adjacent to arginine because the nitro group can no longer be removed by catalytic hydrogenation. As an alternate method, Young, *et al.*[20,21] reported that the N^{G}-nitroarginine could be reduced to arginine by electrolytic reduction at mercury cathode. But there is an indication as mentioned by Gros, *et al.*[22] that this reduction frequently stopped in the middle, *i.e.*, at the level of aminoguanidino group. Therefore it seems difficult to prepare arginylmethionine by adopting N^{G}-nitroarginine as a starting material.

Quite recently Kung, *et. al.*[23] reported the regeneration of arginine from N^{G}-nitroarginine by the reduction with sodium in liquid ammonia at the last stage of their synthesis of the B-chain of beef insulin, in which catalytic reduction was not permitted because of the presence of two cysteine residues. As was noticed by these authors and also by others[24,25] unequivocal removal of the nitro group by this reduction is not possible. We have also found that treatment of N^{α}-benzyloxycarbonyl-N^{G}-nitroarginylmethionine with sodium in liquid ammonia formed a mixture of several compounds.

Introduction of N^{G}-tosylarginine[26~28] offers another reversible protection of the guanidino group. In this case, the tosyl group can be removed only by reduction with sodium in liquid ammonia. If unexpected side reactions would not occur during the treatment of the peptide with sodium in liquid ammonia, the synthesis of arginylmethionine from N^{α}-benzyloxycarbonyl-N^{G}-tosylarginylmethionine seems to be promissing. Indeed, by this method, we have prepared arginylmethionine as its acetate. Although desalting procedure by an ion-exchanger was required, this synthetic peptide exhibited a sharp single spot positive to ninhydrin, Sakaguchi and methionine tests on paper and thin-layer chromatography and acid hydrolysis gave arginine and methionine in nearly

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equal amounts. Its optical purity was established by leucine aminopeptidase (LAP)*4 digestion.

It should be recalled that there are indications that the action of sodium in liquid ammonia cleaves the peptide bonds preferentially at the prolyl bond.[29~31] It is noted that both human and monkey β-MSHs possess the amino acid sequence of prolyltyrosylarginylmethionine. We have treated N^{α}-benzyloxycarbonylprolyltyrosine[32] with sodium in liquid ammonia and observed that certain degree of fission of peptide bond occurred under the condition we employed. Therefore, application of this procedure to the syntheses of peptides related to β-MSHs is not promissing.

Sakakibara, *et al.*[33] reported in 1965 that hydrogen fluoride removed the N^{G}-nitro group of arginine as well as benzyloxycarbonyl group very smoothly. When N^{α}-benzyloxycarbonyl-N^{G}-nitroarginylmethionine was treated with hydrogen fluoride in a dry ice-methanol bath for 30 min., practically pure arginylmethionine was obtained. The arginylmethionine acetate was isolated in satisfactory yield after treatment of the product with Amberlite IRA-400 (acetate cycle). Two samples thus obtained by two alternate methods, the one from N^{α}-benzyloxycarbonyl-N^{G}-tosylarginylmethionine and the other from N^{α}-benzyloxycarbonyl-N^{G}-nitroarginylmethionine, exhibited the identical Rf values on paper and thin-layer chromatography and gave essentially the identical rotation values.

The other interesting finding offered by Noguchi, *et al.*[34] was that the nitro group of arginine could be reduced off by treatment with stannous chloride in formic acid although the reduction by zinc was known to be unsuccessful.[25] When N^{α}-*t*-butoxycarbonyl-N^{G}-nitroarginylmethionine was treated with stannous chloride in 80% formic acid at 50° for 14 hr., this nitro group was reduced off and at the same time N^{α}-*t*-butoxycarbonyl group was cleaved under these conditions. After treatment of the reaction mixture with hydrogen sulfide followed by Amberlite IRA-400 (acetate cycle), arginylmethionine acetate was obtained.

The findings of these methods by Sakakibara, *et al.* and Noguchi, *et al.* for the regeneration of arginine from N^{G}-nitroarginine under relatively mild conditions, promised further wide applicability of N^{G}-nitroarginine in the synthesis of arginine-peptides containing sulfur atom, although other N^{G}-protecting groups are currently being investigated.[22,35~38]

Arginylmethionine obtained above was allowed to react with N^{α}-*t*-butoxycarbonylprolyltyrosine azide. The resulting product was treated with trifluroacetic acid. The ensuing prolyltyrosylarginylmethionine was obtained in pure form by purification on carboxymethyl (CM-) cellulose. It is known that in the synthesis of methionine-peptides, acid-catalyzed debenzyloxycarbonylation with hydrogen bromide in acetic acid[39,40] or in

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nitromethane[41] as well as concentrated hydrochloric acid[42,43] may accompany the cleavage of the methyl thioether of the methionine residue. The above experimental results demonstrated that the syntheses of arginylmethionine and the tetrapeptide containing this sequence were achieved avoiding such a side reaction as mentioned above. Furthermore, in order to prevent the possible oxidation of the methionine residue to the corresponding sulfoxide,[32,44] each reaction was carried out under nitrogen gas.

The fundamental experiments described herein and some investigations for catalytic hydrogenolysis of methionine-containing peptides in which we have recently engaged are now serving to prepare the peptides related to monkey and human β-MSHs which contain the particular amino acid sequence of arginylmethionine. These results will be described in the future.

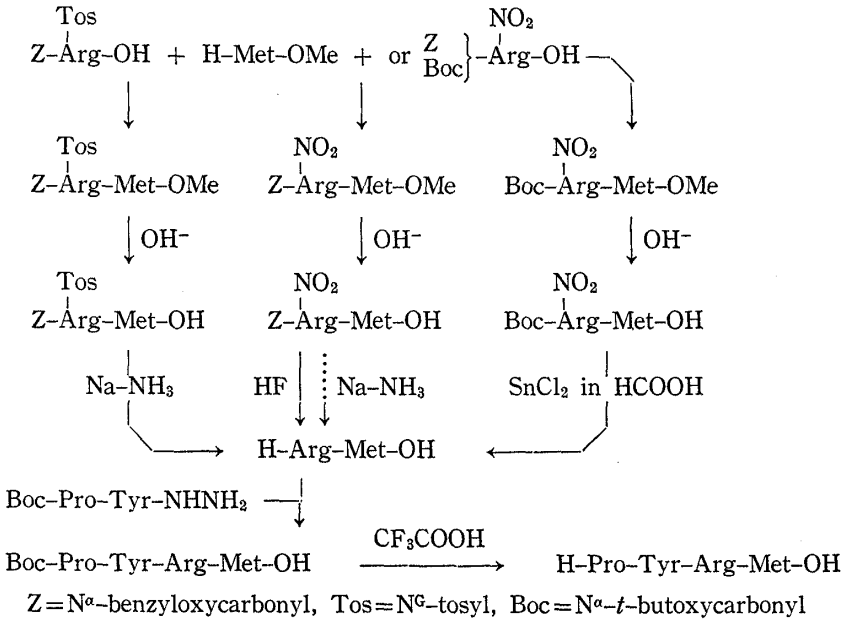

Chart 2. Synthetic Scheme of H-Pro-Tyr-Arg-Met-OH

Experimental

The general experimental methods are essentially the same as described in the part IV[45] of this series. Every reaction of methionine-peptides was carried out under nitrogen gas. The following abbreviations for amino acids were used: Pro=proline, Tyr=tyrosine, Arg=arginine and Met=methionine. On paper chromatography, Rf^1 values refer to the system of Partridge[46] and Rf^2 values refer to the system of 2-butanol–3% ammonia[47] using a phenylalanine marker. In thin-layer chromatography, Rf^3 values refer to the system of *n*-butanol, pyridine, acetic acid, H_2O (4:1:1:2) on silica (Kieselgel G. Merck) plate and Rf^4 values refer to the same solvent system on alumina (Aluminumoxid G. Merck) plate.

N^{α}-Benzyloxycarbonyl-N^{G}-tosylarginylmethionine Methyl Ester——A mixed anhydride, prepared in the usual manner from N^{α}-benzyloxycarbonyl-N^{G}-tosylarginine[27] (8.55 g.) in tetrahydrofuran (THF) (20 ml.) with ethyl chloroformate (1.8 ml.) and triethylamine (2.5 ml.) was added to an ice-cold solution of methionine methyl ester (prepared from 3.66 g. of the hydrochloride[48,49] with 2.5 ml. of triethylamine) in dimethylform-

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amide (DMF) (30 ml.). After the mixture was stirred in an ice-bath for 2 hr., the solvent was evaporated and the residue was dissolved in AcOEt, which was washed successively with 5% NH_4OH, 2*N* HCl and H_2O, dried over Na_2SO_4 and then evaporated to give an oily residue; Yield 10.58 g. (95%), $[\alpha]_D^{20}$ +1.5° (c=1.4, MeOH). Rf^3 0.70, Rf^4 0.84, single spot positive to iodine and methionine tests.

N^{α}-Benzyloxycarbonyl-N^G-tosylarginylmethionine Monohydrate——N^{α}-Benzyloxycarbonyl-N^G-tosylarginylmethionine methyl ester (2.10 g.) in MeOH (10 ml.) was treated with 1*N* NaOH (4.1 ml.) with stirring at room temperature for 1 hr. The solvent was evaporated *in vacuo* at 25° and the residue was dissolved in H_2O, which was washed with AcOEt. The aqueous phase was acidified with 2*N* HCl and the precipitate thereby formed was extracted with AcOEt, which was washed with H_2O, dried over Na_2SO_4 and then evaporated to give an oily residue. After drying, this turned to an amorphous powder. Recrystallization of this powder with various organic solvents was unsuccessful; Yield 1.80 g. (93%). $[\alpha]_D^{23}$ −48.5° (c=1.4, MeOH). Rf^3 0.65, Rf^4 0.51, single spot positive to iodine and methionine tests. *Anal.* Calcd. for $C_{26}H_{35}O_7N_5S \cdot H_2O$: C, 51.1; H, 6.1; N, 11.4. Found: C, 50.9; H, 6.2; N, 11.4.

N^{α}-Benzyloxycarbonyl-N^G-nitroarginylmethionine Methyl Ester——A mixed anhydride was prepared in the usual manner from N^{α}-benzyloxycarbonyl-N^G-nitroarginine (5.30 g.) in THF (25 ml.) with triethylamine (2.3 ml.) and ethyl chloroformate (1.4 ml.). This solution was added to an ice-cold solution of methionine methyl ester (prepared from 2.97 g. of the hydrochloride with 2.3 ml. of triethylamine) in DMF (20 ml.). The mixture was stirred in an ice-bath for 2.5 hr. and the solvent was evaporated *in vacuo*. The residue in AcOEt was washed successively with 5% NH_4OH, 2*N* HCl and H_2O. The organic phase was dried over Na_2SO_4 and then evaporated to give a crystalline solid, which was recrystallized from MeOH; Yield 5.27 g. (70%), m.p. 137~138°, $[\alpha]_D^{26}$ −25.6° (c=1.2, MeOH), Rf^3 0.72, Rf^4 0.85, single spot positive to iodine and methionine tests. *Anal.* Calcd. for $C_{20}H_{30}O_7N_6S$: C, 48.2; H, 6.4; N, 16.9. Found: C, 48.3; H, 6.1; N, 16.9.

N^{α}-Benzyloxycarbonyl-N^G-nitroarginylmethionine——To a solution of N^{α}-benzyloxycarbonyl-N^G-nitroarginylmethionine methyl ester (2.40 g.) in MeOH (40 ml.), 1*N* NaOH (15 ml.) was added and the solution was stirred at room temperature for 1 hr. The pH of the solution was adjusted to 6 with AcOH and the solvent was evaporated. The residue was dissolved in AcOEt, which was extracted with 3 portions of 5% NH_4OH. The aqueous phase was acidified with 5*N* HCl and the resulting precipitate was extracted with AcOEt, which was washed with H_2O, dried over Na_2SO_4 and evaporated. The residue was solidified with MeOH and recrystallized from the same solvent; Yield 2.10 g. (87%), m.p. 161~162°, $[\alpha]_D^{24}$ −13.3° (c=0.4, MeOH), Rf^3 0.63, Rf^4 0.71, single spot positive to methionine and iodine tests. *Anal.* Calcd. for $C_{19}H_{28}O_7N_6S$: C, 47.4; H, 5.9; N, 17.1. Found: C, 47.1; H, 5.8; N, 17.4.

N^{α}-*t*-Butoxycarbonyl-N^G-nitroarginylmethionine Dicyclohexylamine Salt——A mixed anhydride, prepared in the usual manner from N^{α}-*t*-butoxycarbonyl-N^G-nitroarginine[50] (2.0 g.) in THF (40 ml.) with triethylamine (1.2 ml.) and ethyl chloroformate (0.8 ml.) was added to a solution of methionine methyl ester (prepared from 1.40 g. of the hydrochloride and 1.2 ml. of triethylamine) in DMF (20 ml.) and the solution was stirred in an ice-bath for 2.5 hr. The solvent was evaporated and the residue was extracted with AcOEt. The organic phase was washed successively with 5% NH_4OH, 10% citric acid and H_2O, dried over Na_2SO_4 and then evaporated to give an oily residue; Yield 2.36 g. (74%), Rf^3 0.75, Rf^4 0.79. This product (2.3 g.) in MeOH (40 ml.) was treated with 1*N* NaOH (6.7 ml.) in an ice-bath for 40 min. The pH of the solution was adjusted to 6 with AcOH and the solvent was evaporated. The residue was dissolved in 5% NH_4OH, which was washed with AcOEt and then acidified with 10% citric acid. The resuting oil was extracted with AcOEt, which after washing with H_2O, was dried over Na_2SO_4 and then evaporated to give an oily residue; Yield 2.06 g. (90%), Rf^3 0.63, Rf^4 0.67. N^{α}-*t*-butoxycarbonyl-N^G-nitroarginylmethionine (2.0 g.) thus obtained was dissolved in acetone (4 ml.) and dicyclohexylamine (2.0 ml.) was added. The crystalline solid formed on standing was collected by filtration and recrystallized from acetone and ether; Yield 2.38 g. (79%), m.p. 117~118°, $[\alpha]_D^{25}$ +6.4° (c=0.7, MeOH). *Anal.* Calcd. for $C_{16}H_{30}O_7N_6S \cdot C_{12}H_{23}N$: C, 53.3; H, 8.4; N, 15.5. Found: C, 53.1; H, 8.7; N, 15.4.

Arginylmethionine Acetate——a) N^{α}-Benzyloxycarbonyl-N^G-tosylarginylmethionine (1.20 g.) was dissolved in liquid NH_3 (approximately 200 ml.). Sodium was added in small pieces until a permanent blue color was obtained. Stirring was continued in a dry-ice acetone bath for 30 min. NH_4Cl was added to discharge the excess of Na and the NH_3 was allowed to evaporate. The last trace of NH_3 was removed by passing N_2 gas over the residue. The flask was kept in a desiccator over H_2SO_4. Examination of the residue by paper chromatography in the system of Partridge revealed the presence of one major spot with Rf^1 0.40 and two very minor components with Rf^1 0.17 and 0.35 (ninhydrin stain). The residue was dissolved in H_2O (500 ml.) and the pH of the solution was adjusted to 7 with AcOH. The solution was applied to a column of Amberlite CG-50 (H^+ form), which was eluted first with H_2O (2000 ml.) and then 0.01*M* pyridine acetate buffer at pH 5.0. Individual fractions (23 ml. each) were collected. Ninhydrin, Sakaguchi and methionine tests of the effluent served to locate the desired compound. The contents of the tubes (No. 151~280) were

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combined and the solvent was evaporated. The resulting solid was recrystallized from H_2O and EtOH; Yield 0.60 g. (81%), m.p. 185~186°, $[\alpha]_D^{22}$ +18.9°(c=1.2, H_2O). The Rf value of arginylmethionine was greatly influenced by the solvents (indicated in the bracket) in which the sample was dissolved, Rf^1 0.40, Rf^2 0.53, Rf^3 0.28 (H_2O), Rf^1 0.40 (5% AcOH), Rf^1 0.20 (0.1*N* HCl), single spot positive to ninhydrin, Sakaguchi and methionine tests. Rf^1 0.40 and 0.75 (5% trichloroacetic acid), two spots presumably due to partial dissociation. Amino acid ratios in acid hydrolysate $Arg_{1.07}$ $Met_{1.00}$ (average recovery 92%), amino acid ratios in LAP digest $Arg_{1.05}$ $Met_{1.00}$ (average recovery 95%). *Anal.* Calcd. for $C_{11}H_{23}O_3N_5S \cdot CH_3COOH$: C, 42.7; H, 7.7; N, 19.2. Found: C, 42.6; H, 7.9; N, 19.0.

b) N^{α}-Benzyloxycarbonyl-N^G-nitroarginylmethionine (1.41 g.) and anisol (2.1 ml.) were placed in a container of difurone (polytrifluoro monochloroethylene). Under cooling with dry ice-MeOH, anhyrous HF (approximately 50 ml.) was collected in this flask and the solution was stirred for 30 min. in the same bath. Cooling was replaced to an ice-bath and the solution was stirred for 30 min. The HF was allowed to evaporate and finally removed *in vacuo*. The residue was kept over KOH pellets in an evacuated desiccator. The residue was dissolved in H_2O and the solution was treated with Amberlite IRA-400 (acetate cycle). After filtration, the filtrate was evaporated and the residue was recrystallized from H_2O and EtOH; Yield 0.89 g. (87%), m.p. 184~185°, mixed m.p. with the sample obtained in (a) was 185~186°, $[\alpha]_D^{22}$ +18.8°(c=0.9, H_2O). Rf^1 0.40, Rf^2 0.53, Rf^3 0.28, single spot positive to ninhydrin, Sakaguchi and methionine tests.

c) To a solution of N^{α}-*t*-butoxycarbonyl-N^G-nitroarginylmethionine (0.50 g.) in 80% formic acid (10 ml.), $SnCl_2 \cdot 2H_2O$ (1.45 g.) was added. The solution was stirred at 50° for 14 hr. and then filtered. The filtrate was evaporated and the residue was dissolved in H_2O (50 ml.). Hydrogen sulfide was bubbled into the solution and the resulting precipitate was removed by filtration. The filtrate was washed once with AcOEt and then treated with Amberlite IRA-400 (acetate cycle, approximately 10 g.) for 4 hr. After filtration, the solution was evaporated and the residue was crystallized from EtOH and recrystallized from H_2O and EtOH; Yield, 0.15 g. (35%), m.p. 180~184°, Rf^1 0.40, ninhydrin, Sakaguchi and methionine tests positive spot. $[\alpha]_D^{20}$ +16.9° (c=1.1, H_2O), Mixed m.p. with the sample obtained in (a) was 182~186°.

Treatment of N^{α}-benzyloxycarbonyl-N^G-nitroarginylmethionine with Sodium in Liquid Ammonia——N^{α}-Benzyloxycarbonyl-N^G-nitroarginylmethionine (0.30 g.) was dissolved in liquid NH_3 (approximately 50 ml.). Small pieces of Na were added to the solution until a permanent blue color was obtained. Stirring was continued in a dry ice-acetone bath for 20 min. After NH_4Cl was added to discharge the excess of Na, the NH_3 was allowed to evaporate. The flask was kept over H_2SO_4 *in vacuo*. Examination of the residue by paper chromatography showed various ninhydrin positive spots, Rf^1 0.06, 0.28, 0.32 and 0.59.

N^{α}-Benzyloxycarbonylprolyltyrosine Methyl Ester——Dicyclohexylcarbodiimide (1.30 g.) was added to an ice-cold solution of tyrosine methyl ester (prepared from 1.44 g. of the hydrochloride[51]) and 0.84 ml. of triethylamine) and N^{α}-benzyloxycarbonylproline (1.23 g.) in DMF (10 ml.). After stirring overnight, the solution was filtered and the filtrate was condensed *in vacuo*. The residue was dissolved in AcOEt. The organic phase was washed successively with 10% $NaHCO_3$, 2*N* HCl and H_2O, dried over Na_2SO_4 and then evaporated to give a solid which was recrystallized from AcOEt and ether; Yield 2.20 g. (93%), m.p. 77~79°, $[\alpha]_D^{15}$ −20.0° (c=1.0, EtOH). (lit.[32] m.p. 72~74°, $[\alpha]_D^{27}$ −24° in EtOH). *Anal.* Calcd. for $C_{23}H_{26}O_6N_2$: C, 64.8; H, 6.2; N, 6.6. Found: C, 64.5; H, 6.4; N, 6.4.

N^{α}-Benzyloxycarbonylprolyltyrosine Hydrazide——To a solution of N^{α}-benzyloxycarbonylprolyltyrosine methyl ester (2.30 g.) in MeOH (10 ml.), 80% hydrazine hydrate (0.8 ml.) was added. The solid mass formed on standing overnight was recrystallized from MeOH; Yield 2.0 g. (87%), m.p. 204~205°, $[\alpha]_D^{22}$ −64.0°(c=1.0, MeOH). *Anal.* Calcd. for $C_{22}H_{26}O_5N_4$: C, 62.0; H, 6.1; N, 13.1. Found: C, 61.7; H, 6.2; N, 13.3.

N^{α}-Benzyloxycarbonylprolyltyrosine Hemihydrate——N^{α}-Benzyloxycarbonylprolyltyrosine methyl ester (1.0 g.) in ice-cold MeOH (4 ml.) was treated with 1*N* NaOH (5.1 ml.) for 30 min. The pH of the solution was adjusted to 6 with AcOH. The solvent was evaporated and the residue was dissolved in 5% NH_4OH, which after washing with ether, was acidified with 5*N* HCl. The resulting product was extracted with AcOEt, which was washed with H_2O, dried over Na_2SO_4 and evaporated. Addition of petroleum ether (b.p. 30~50°) to the residue afforded a solid; Yield 0.91 g. (96%), m.p. 59~68°. $[\alpha]_D^{17}$ −6.2° (c=0.7, MeOH). (lit.[32] oil). *Anal.* Calcd. for $C_{22}H_{24}O_6N_2 \cdot \frac{1}{2}H_2O$: C, 62.7; H, 5.9; N, 6.6. Found: C, 63.2; H, 6.3; N, 6.2.

Prolyltyrosine——N^{α}-Benzyloxycarbonylprolyltyrosine (5.0 g.) in MeOH (18 ml.) containing AcOH (0.3 ml.) was hydrogenated over a palladium catalyst in the usual manner. The catalyst was removed by filtration and the filtrate was condensed *in vacuo*. The residue was recrystallized from EtOH; Yield 1.80 g. (54%), m.p. 213~215° (c=1.0, 1*N* HCl), $[\alpha]_D^{18}$ −8.2° (c=1.0, H_2O) (lit.[52] m.p. 222~223°, $[\alpha]_D^{23}$ −10.5° in H_2O). Rf^1 0.53, Rf^2 0.83, single ninhydrin and Pauly positive spot. *Anal.* Calcd. for $C_{14}H_{18}O_4N_2$: C, 60.4; H, 6.5; N, 10.1. Found: C, 60.5; H, 7.2; N, 10.1.

Treatment of N^{α}-Benzyloxycarbonylprolyltyrosine with Sodium in Liquid Ammonia——N^{α}-Benzyloxycarbonylprolyltyrosine (0.30 g.) in liquid NH_3 (approximately 70 ml.) was treated with sodium until a permanent blue color was obtained. The stirring was continued in a dry-ice acetone bath for 5 min. After

51) R. A. Boissonnas, St. Guttmann, P. A. Jaquenoud, J. P. Waller: Helv. Chim. Acta, **38**, 1491 (1955).

addition of NH_4Cl, the NH_3 was allowed to evaporate. Examination of the residue by paper chromatography (ninhydrin stain) showed three spots, Rf^1 0.34 (faint yellow spot), 0.37 (faint spot) and 0.53 (corresponding to prolyltyrosine).

N^{α}-*t*-Butoxycarbonylprolyltyrosine Methyl Ester——A mixed anhydride was prepared in the usual manner from N^{α}-*t*-butoxycarbonylproline[53] (4.30 g.) in THF (30 ml.) with triethylamine (2.8 ml.) and ethyl chloroformate (1.9 ml.) was added to an ice-cold solution of tyrosine methyl ester (prepared from 4.63 g. of the hydrochloride with 2.8 ml. of triethylamine) in THF. The mixture was stirred in an ice-bath for 3 hr., the solvent was evaporated and the residue was dissolved in AcOEt, which was washed with 1*N* $NaHCO_3$, 10% citric acid and H_2O, dried over Na_2SO_4 and then evaporated. The oily residue turned to a solid by drying; 7.09 g. (90%), m.p. 63~64°, $[\alpha]_D^{19}$ −36.2° (c=1.3, MeOH). *Anal.* Calcd. for $C_{20}H_{28}O_6N_2$: C, 61.2; H, 7.2; N, 7.1. Found: C, 60.6; H, 7.5; N, 7.0.

N^{α}-*t*-Butoxycarbonylprolyltyrosine Hydrazide——N^{α}-*t*-Butoxycarbonylprolyltyrosine methyl ester (11.03 g.) was dissolved in EtOH (10 ml.) and 80% hydrazine hydrate (6.0 ml.) was added. The solution was kept at room temperature overnight. The solvent was evaporated and the residue was triturated with ether to give a solid, which was recrystallized from EtOH and ether; Yield 10.05 g. (95%), m.p. 174~176°, $[\alpha]_D^{22}$ −61.7° (c=0.6, MeOH). *Anal.* Calcd. for $C_{19}H_{28}O_5N_4$: C, 58.2; H, 7.2; N, 14.3. Found: C, 57.6; H, 7.3; N, 14.3.

Prolyltyrosylarginylmethionine Acetate Trihydrate——The entire operation was carried out in a cold room at 4°. N^{α}-*t*-Butoxycarbonylprolyltyrosine hydrazide (0.94 g.) was dissolved in DMF (6 ml.) and a solution of $NaNO_2$ (0.18 g.) in H_2O was combined. Under ice-NaCl cooling, 1*N* HCl (4.8 ml.) was added slowly and the solution was stirred for 4 min. The pH of the solution was adjusted to 8 with triethylamine. This solution was added to a solution of arginylmethionine (0.73 g.) and triethylamine (0.28 ml.) in H_2O (1.5 ml.). After stirring was continued at 0° for 24 hr. the second azide (prepared from 0.94 g. of the hydrazide) was added. The mixture was stirred for an additional 24 hr. The solvent was evaporated and the residue was distributed between AcOEt and H_2O. An oily layer was separated between the water and AcOEt layers. The AcOEt layer was removed and the rest was extracted with *n*-butanol saturated previously with H_2O. The *n*-butanol phase was separated and the solvent was evaporated and the residue was dried over P_2O_5 and KOH pellets *in vacuo*. This residue was treated with trifluoroacetic acid (3 ml.) at room temperature for 40 min. Dry ether was added and the resulting precipitate, after drying, was dissolved in H_2O (500 ml.) and the solution was applied to a column of CM-cellulose (3×30 cm.), which was eluted with the following pH 5.0 pyridine acetate buffers: 0.01*M* (500 ml.), 0.02*M* (1000 ml.), 0.1*M* (500 ml.) and 0.2*M* (300 ml.). Individual fractions (20 ml. each) were collected and absorbancy at 275 mμ was determined in each fraction. The desired fraction present in 0.02*M* eluate was collected, the solvent was evaporated and the residue was lyophilized to give a fluffy powder; Yield 0.72 g. (50%), $[\alpha]_D^{22}$ −23.2° (c=0.2, H_2O). Rf^1 0.30, single spot positive to ninhydrin, Pauly, Sakaguchi and methionine tests. Amino acid ratios in acid hydrolysate $Pro_{1.03}$ $Tyr_{0.94}$ $Arg_{1.00}$ $Met_{0.76}$ (average recovery 91%), amino acid ratios in LAP digest $Pro_{1.04}$ $Tyr_{1.01}$ $Arg_{1.00}$ $Met_{1.00}$ (average recovery 87%). *Anal.* Calcd. for $C_{25}H_{39}O_6N_7S \cdot CH_3COOH \cdot 3H_2O$: C, 47.7; H, 7.3; N, 14.4. Found: C, 47.5; H, 6.7; N, 13.9.

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