[Chem. Pharm. Bull. 21(12)2634—2638(1973)] *UDC 547.466.1.057 : 615.36.011.5*

Studies on Encephalitogenic Fragments of Myelin Protein. II.[1,2] Solid Phase Synthesis of Tryptophan-containing Decapeptide

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(Received February 6, 1973)

Solid phase synthesis of H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH was accomplished by two different approaches, stepwise chain elongation and fragment condensation. Two fragments, Boc-Ala-Glu(OBzl)-Gly-OH (II) and Boc-Phe-Ser-Trp-Gly-OH (IV), were used for fragment condensation. Overall yields of the decapeptide were 10% in stepwise chain elongation and 34% in fragment condensation respectively. A failure pentapeptide, H-Phe-Ser-Trp-Gly-Arg-OH, was isolated in the two procedures.

One of the active fragments of human encephalitogenic basic protein, which contains single tryptophan residue in the molecule, have been shown to be a pentadecapeptide, H–Ser–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg–Pro–Gly–Phe–Gly–OH.[4] Westall *et al.*[5] have synthesized several peptides related to the peptide and revealed that the undecapeptide, H–Ser–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg–OH, was encephalitogenic in guinea pigs. In a previous study[1] it was found that the length limit of peptide chain for the induction of experimental allergic encephalomyelitis (EAE) in guinea pigs was the nonapeptide, H–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg–OH. The encephalitogenic activity of the synthetic nonapeptide and decapeptide, H–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg–OH, was approximately equal.

This paper describes the solid phase synthesis[6] of the decapeptide, H–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg–OH. The decapeptide was prepared by two different synthetic approaches, those were procedure of stepwise chain elongation and fragment condensation. The yields were satisfactory as compared with that of the conventional method described in a previous communication.[1]

First, for the stepwise chain elongation the C-terminal amino acid, Boc–Arg(NO_2)–OH, was esterified onto chloromethylated copolymer of styrene with 2% divinylbenzene[6] in DMF at room temperature in the same manner as described by Sakakibara[7] and Marglin.[7] The N-terminal protecting group was removed with 4N HCl in dioxane and the resulting hydrochloride was neutralized with triethylamine. The free base on the resin was condensed with the forecoming amino acid derivative, Boc–Gln–ONp,[8] to yield Boc–Gln–Arg(NO_2)-resin. Addition of succeeding amino acids one at a time was carried out by repeating such cycle with

1) Part I: K. Suzuki, T. Abiko, N. Endo, Y. Sasaki and J. Arisue, *Chem. Pharm. Bull.* (Tokyo), **21**, 2627 (1973).
2) Symbols for amino acid derivatives and peptides used in this text are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature; *Biochem. J.*, **126**, 773 (1972). Other abbreviations: OAo=acetoxime ester, DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide.
3) Location: *Komatsushima, Sendai.*
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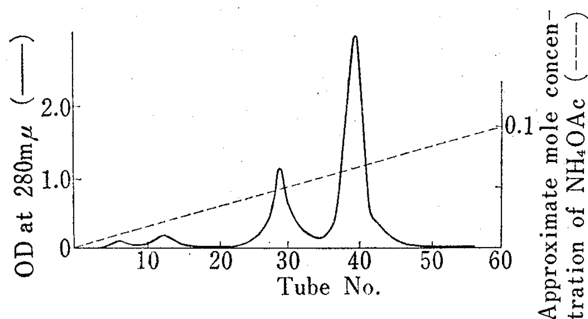
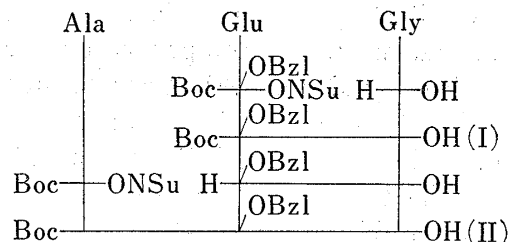


Fig. 1. Purification of Synthetic Decapeptide, obtained by Stepwise Procedure, on a Column of CM-Cellulose

The column (2×14 cm), applied with crude material (47.8 mg), was eluted with a linear gradient elution from H_2O (300 ml) in mixing chamber to 0.1M NH_4OAc buffer (pH 6.50, 300 ml). Fractions of 10 ml each were collected.



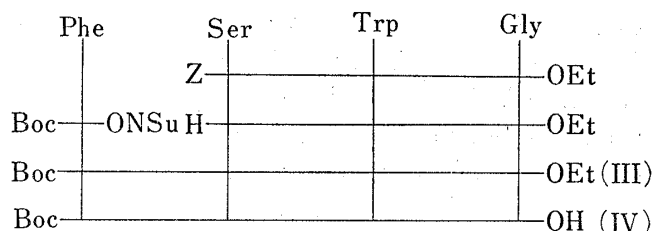


Fig. 2. Synthesis of Two Subunits, II and IV

minor modification until the decapeptide derivative, Boc–Arg(NO_2)–Phe–Ser(Bzl)–Trp–Gly–Ala–Glu(OBzl)–Gly–Gln–Arg(NO_2)-resin, was formed. All protecting groups including resin of the decapeptide derivative was removed by hydrogen fluoride.[9] The product was successively purified through carboxymethyl(CM-) cellulose column and preparative thin-layer chromatography(TLC). The elution pattern obtained after application of crude material to the CM-cellulose column is illustrated in Fig. 1. The last peak in this pattern contained the desired decapeptide and one minor failure peptide, as analysed by TLC. Overall yield of the decapeptide was 10% on the basis of the starting material, Boc–Arg(NO_2)-resin. The decapeptide so obtained was found to be a unity from the result of paper chromatography and amino acid analysis. The amino acid analysis of a failure peptide isolated in pure form from the others by TLC, revealed that the peptide was composed from equimole of Phe, Ser, Trp, Gly and Arg. Therefore, it may safely be assumed that the amino acid sequence of the failure peptide is H–Phe–Ser–Trp–Gly–Arg–OH. The formation of such a failure pentapeptide implied that some coupling failed but some subsequent steps succeeded again. Second, for the fragment condensation, two subunits having Gly residue in their C-terminal were prepared by classical method as illustrated in Fig. 2. The subunits and Boc–Arg(NO_2)–OH were condensed successively with H–Gln–Arg(NO_2)-resin to yield Boc–Arg(NO_2)–Phe–Ser–Trp–Gly–Ala–Glu(OBzl)–Gly–Gln–Arg(NO_2)-resin. For the synthesis of a subunit, Boc–Ala–Glu(OBzl)–Gly–OH(II), the following series of reactions was carried out. Boc–Glu(OBzl)–Gly–OH(I) was prepared from Boc–Glu(OBzl)–ONSu[10] and H–Gly–OH according to the procedure described by Anderson, *et al.*[11] *tert*-Butyloxycarbonyl group of I was removed by treatment with trifluoroacetic acid and the dipeptide thereby formed was condensed with Boc–Ala–ONSu[11] to yield Boc–Ala–Glu(OBzl)–Gly–OH(II). For the synthesis of the other subunit Boc–Phe–Ser–Trp–Gly–OH(IV), the following series of reactions was carried out. Benzyloxycarbonyl group of Z–Ser–Trp–Gly–OEt[1] was removed by catalytic hydrogenolysis and the tripeptide ester thereby formed was condensed with Boc–Phe–ONSu[11] to yield Boc–Phe–Ser–Trp–Gly–OEt(III). III was saponified with 2N NaOH to yield Boc–Phe–Ser–Trp–Gly–OH(IV). H–Gln–Arg(NO_2)-resin prepared as described above was condensed with

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10) C.B. Anfinsen, D. Ontjes, M. Ohno, L. Corley and A. Eastlake, *Proc. Natl. Acad. Sci. U. S.*, **58**, 1806 (1967).
11) G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

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subunit II by means of dicyclohexylcarbodiimide in the presence of N-hydroxysuccinimide.[12] Unchanged amine component on the resin was determined according to the procedure described by Dorman.[13] Consequently, it was found that coupling period of 15 hours was satisfactory to minimize the unchanged amine component. Addition of the succeeding subunit IV and Boc–Arg(NO_2)–OH one at a time was accomplished essentially in the same manner as described above for the stepwise chain elongation. The resulting decapeptide derivative, Boc–Arg(NO_2)–Phe–Ser–Trp–Gly–Ala–Glu(OBzl)–Gly–Gln–Arg(NO_2)-resin, was treated with hydrogen fluoride and purified just in the same manner as described above. Overall yield of the decapeptide was 34% on the basis of the starting material Boc–Arg(NO_2)-resin. The decapeptide thus obtained was identical to that prepared by the stepwise chain elongation in regard to chemical, physical and biological properties. In this case, a failure pentapeptide was also isolated. This peptide was assumed to be identical to H–Phe–Ser–Trp–Gly–Arg–OH, isolated in the stepwise chain elongation, from the results of amino acid analysis and coincidence of chromatographic patterns of the both peptides.

Encephalitogenic activity of the decapeptide, which was synthesized by two different approaches, was shown in Table I. It is apparent that two samples of the decapeptide have almost same activity.

Table I. Encephalitogenic Activity of Synthetic Peptides and Encephalitogenic Protein

Material	Dose (μg)	Encephalitogenic activity[a]	
		clinical	clinical+ histological
Decapeptide (fragment condensation)	1.0[14]	2/6	5/6
	5.0[14]	4/6	6/6
	5.0	6/7	7/7
	100	6/6	
Decapeptide (stepwise elongation)	5.0	2/2	
	100	4/5	
Bovine encephalitogenic protein	20[14]	10/10	

a) Encephalitogenic activity is expressed as the number of guinea pigs showing clinical and histological signs of EAE over the animals tested in the manner as described by Eylar.[15]

Experimental

All melting points are uncorrected. For paper chromatography, Z-group of the protected peptides for the preparations of two subunits were deblocked by catalytic hydrogenation and Boc-group with trifluoroacetic acid and the resulting amine components were chromatographed on filter paper, Toyo Roshi No. 51, at room temperature. Rf^1 values refer to Partridge system[16] and Rf^2 values refer to the system of BuOH-pyridine-AcOH-H_2O (30 : 20 : 6 : 24).[17] For amino acid analysis, peptides were hydralized according to the directions given by Stewart, *et al.*[18] Amino acid analysis was carried out on Hitachi Model KLA-3B amino acid analyzer according to the directions given by Moore, *et al.*[19]

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13) L.C. Dorman, *Tetrahedron Letters*, **1969**, 2319.
14) Assayed by Dr. E.H. Eylar, Merck Institute, N.J. for the authors and the others were assayed by Drs. Y. Nagai and S. Otani *et al.*, Institute of Medical Science, University of Tokyo. The details will be published elsewhere by Dr. Nagai, *et al.*
15) E.H. Eylar, J. Salk, G.C. Beveridge and L.V. Brown, *Arch. Biochem. Biophys.*, **132**, 34 (1969).
16) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).
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18) J.M. Stewart, S.H. Ferreira and L.J. Green, *Biochem. Pharmcol.*, **20**, 1557 (1971).
19) S. Moore, D.H. Spackman and W.H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

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Boc-Arg(NO_2)-Phe-Ser(Bzl)-Trp-Gly-Ala-Glu(OBzl)-Gly-Gln-Arg(NO_2)-resin——Boc-Arg(NO_2)-resin was prepared from Boc-Arg(NO_2)-OH (5.11 g, 16 mmole) and dry chloromethylated polystyrene 2% divinylbenzene (10 g, chlorine content 1.6 mmole/g) in DMF according to the procedure described by Sakakibara[7] and Marglin,[7] yield 10.66 g. The amino acid content in the resin so obtained was 0.255 mmole/g from the result of the amino acid analysis in the acid hydrolysate of the dry resin.

Boc-Arg(NO_2)-resin (2 g, 0.51 mmole) was placed in a reaction vessel. Generally, the following cycle was used for condensation of amino acids onto the resin one at a time. 1) Washing with dioxane (15 ml×3). 2) Cleavage of the Boc group by suspension in 4N HCl in dioxane (15 ml) for 5 min. This operation was repeated for additional 30 min. 3) Washing with dioxane (15 ml×3). 4) Washing with chloroform (15 ml×3). 5) Neutralization of the hydrochloride with a 10% solution of Et_3N in DMF (15 ml) for 15 min. 6) Washing with chloroform (15 ml×3). 7) Washing with CH_2Cl_2 (15 ml×3). 8) Addition of Boc-amino acid (1.53 mmole) in CH_2Cl_2 (12 ml) and shaking 5 min. 9) Addition of DCC (1.53 mmole) in CH_2Cl_2 (3 ml) and shaking for 5 hr. 10) Washing with DMF (15 ml×3). 11) Acetylation of the unchanged amino group by addition of acetic anhydride (0.8 ml) and Et_3N (0.5 ml) in DMF (14 ml), followed by shaking for 20 min. 12) Washing successively with DMF (15 ml×3), absolute EtOH (15 ml), AcOH (15 ml×3) and absolute EtOH (15 ml×3).

Boc group of Boc-Gln residue was removed by trifluoroacetic acid (15 ml) for 15 min and neutralization of the trifluoroacetate was performed for 5 min. For introduction of Gln, Boc-Gln-ONp (2.55 mmole) was employed and the coupling period was for 15 hr. Boc-Trp-OH and Boc-Arg(NO_2)-OH were dissolved in DMF–CH_2Cl_2 (4:6) and DMF–CH_2Cl_2 (1:1) respectively. Boc-Gln-ONp was dissolved in DMF containing urea (1.35 g, 22.5 mmole).[20] After introduction of Trp, β-mercaptoethanol (0.15 ml) was added to 4N HCl-dioxane.[21] After introduction of N-terminal Arg (NO_2) and the following acetylation, the resulting protected peptidyl-resin was further washed with 15 ml portions of DMF, CH_2Cl_2, AcOH, EtOH 3 times each and dried over KOH pellets in vacuum; yield 2.548 g.

H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH——The protected peptidyl-resin (1.210 g) prepared as described above was treated with anhydrous HF (20 ml) in the presence of anisole (5 ml, 30 eq) under ice-cooling for 30 min. After evaporation of HF in vacuum for 5 hr. The residue was extracted with 1% AcOH (25 ml×2) for 30 min and the extracts were washed with EtOAc (10 ml×2). The solution was evaporated to small volume and added to a column of Dowex 1×2 (acetate type, 2×8 cm) which was washed with 2% AcOH. Sakaguchi test positive eluates were collected, evaporated in vacuum and lyophilized to give pale yellow powder; 193 mg. The product was dissolved in H_2O (10 ml) and added to a CM-cellulose column (2×14 cm) which was eluted with a linear gradient elution from H_2O (300 ml) in mixing chamber to 0.1M NH_4OAc buffer (pH 6.50, 300 ml) in reservoir. Fractions of 10 ml each were collected at a flow rate of 4 ml/min with an automatic fraction collector and absorbancy at 280 mμ was determined on each fraction. The eluates in tubes No. 37 to 44 were pooled, evaporated to small volume in vacuum and lyophilized. NH_4OAc was removed by repeated lyophilization to constant weight; yield 47 mg. The product was dissolved in H_2O (1 ml) and submitted to preparative TLC (Wakogel B-5, 20×20 cm×5) using Partridge system as a developing solvent. Zone corresponding to *Rf* 0.15 was separated and extracted with 1% AcOH. The extracts were evaporated to small volume in vacuum and submitted to CM-cellulose chromatography as described above; yield 35 mg; mp 196—215° (decomp.); $[\alpha]_D^{13}$ −16.8° (c=2, H_2O); Rf^1 0.15, Rf^2 0.35, single ninhydrin, Sakaguchi and Ehrlich positive spot; amino acid ratios in the acid hydrolysate: Phe 1.0, Ser 0.9, Gly 2.0, Ala 1.2, Glu 2.0, Trp 0.9, Arg 1.9; amino acid ratios in the AP-M digest[22]: Phe 0.8, Ser 1.0, Gly 2.0, Ala 1.0, Glu 0.8, Gln 1.0, Trp 0.9, Arg 1.9.

H-Phe-Ser-Trp-Gly-Arg-OH——Zone corresponding to *Rf* 0.35 on TLC described above was separated and treated as described above; yield 1.7 mg; Rf^1 0.35, Rf^2 0.55, single ninhydrin, Sakaguchi and Ehrlich positive spot; amino acid ratios in the acid hydrolysate: Phe 1.0, Ser 1.0, Gly 0.9, Trp 0.8, Arg 1.0.

Boc-Glu(OBzl)-Gly-OH (I)——A solution of Boc-Glu(OBzl)-ONSu (434.2 mg, 1 mmole) in EtOH (4 ml) was added to a solution of H-Gly-OH (75.0 mg, 1 mmole) and $NaHCO_3$ (168 mg, 2 mmole) in H_2O (3 ml) and the mixture was allowed to stand overnight at room temperature, when EtOH was evaporated in vacuum. The residue was washed with EtOAc two times and the precooled water layer was acidified to Congo red with powder of citric acid. The precipitate thereby formed was extracted with EtOAc three times and the extracts were washed successively with 1N citric acid and H_2O. The solution was dried over $MgSO_4$ and evaporated to dryness in vacuum. The residue was recrystallized from EtOAc; columns, yield 346.7 mg (88%); mp 114—115°; $[\alpha]_D^{13}$ −4.5° (c=1, DMF); Rf^1 0.61, Rf^2 0.64; *Anal.* Calcd. for $C_{19}H_{26}O_7N_2$: C, 57.86; H, 6.64; N, 7.10. Found: C, 57.45; H, 6.62; N, 7.04.

20) C. Westall and A.B. Robinson, *J. Org. Chem.*, 35, 2842 (1970).
21) J.M. Stewart and J.D. Young, "Solid Phase Peptide Synthesis," W.H. Freeman and Co., 1969, p. 48.
22) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, 339, 186 (1963); G. Pfleiderer, P.G. Celliers, M. Stanulovic, E.D. Washmuth, H. Determan and G. Braunitzer, *ibid.*, 340, 552 (1964). Röhm and Haas (Darmstadt) preparation was used.

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Boc-Ala-Glu(OBzl)-Gly-OH (II)——I (394.4 mg, 1 mmole) was dissolved in trifluoroacetic acid (2 ml, 30 eq) and the solution was kept at room temperature for 15 min. Trifluoroacetic acid was evaporated in vacuum and the residue was dried over KOH pellets in vacuum. A solution of the residue and $NaHCO_3$ (168 mg, 2 mmole) in H_2O was added to a solution of Boc-Ala-ONSu (286.7 mg, 1 mmole) in EtOH (3 ml) and DMF (1 ml). The mixture was kept at room temperature for 18 hr and treated just in the same manner as described above. The product was recrystallized from EtOAc; needles, yield 231 mg (49%); mp 156—159°; $[\alpha]_D^{18}$ −26.4° (c=1, DMF); *Rf*[1] 0.62, *Rf*[2] 0.66; *Anal.* Calcd. for $C_{22}H_{31}O_8N_3$: C, 56.76; H, 6.71; N, 9.03. Found: C, 56.47; H, 6.79; N, 8.74.

Boc-Phe-Ser-Trp-Gly-OEt (III)——Z-Ser-Trp-Gly-OEt[1)] (2.55 g, 5 mmole) was hydrogenated in MeOH (25 ml), H_2O (3 ml) and AcOH (0.6 ml) over 5% Pd-C in the usual manner until the evolution of CO_2 ceased. The catalyst was removed by filtration and the filtrate was evaporated in vacuum. To the solution of this tripeptide ester in DMF (30 ml), Boc-Phe-ONSu (1.81 g, 5 mmole) and Et_3N (0.7 ml, 5 mmole) were added. After 24 hr, the reaction mixture was diluted with H_2O (100 ml) and extracted with EtOAc. The EtOAc solution was washed successively with 1N NH_4OH, H_2O, 1N citric acid and H_2O. The solution was dried over $MgSO_4$ and evaporated in vacuum. The residue was reprecipitated from EtOAc and petroleum ether; amorphous powder, yield 2.79 g (85%); mp 120—122°; $[\alpha]_D^{18}$ −6.4° (c=1, DMF); *Rf*[1] 0.70, *Rf*[2] 0.86; *Anal.* Calcd. for $C_{32}H_{41}O_8N_5$: C, 61.62; H, 6.63; N, 11.23. Found: C, 61.53; H, 6.64; N, 10.94.

Boc-Phe-Ser-Trp-Gly-OH (IV)——III (3.13 g, 5 mmole) was saponified in MeOH (30 ml) with 2N NaOH (3 ml, 1.2 eq) at room temperature for 1.5 hr. The solution was evaporated in vacuum and the residue was diluted with cold H_2O. The solution was acidified to Congo red with powder of citric acid and extracted with hot EtOAc. The EtOAc solution was washed with H_2O and dried over $MgSO_4$, when evaporated to dryness in vacuum. The residue was reprecipitated from EtOAc and petroleum ether; amorphous powder, yield 2.8 g (94%); mp 125—130°; $[\alpha]_D^{18}$ +39.2° (c=1, DMF); *Rf*[1] 0.61, *Rf*[2] 0.66; *Anal.* Calcd. for $C_{30}H_{37}O_8N_5$: C, 60.49; H, 6.26; N, 11.76. Found: C, 59.98; H, 6.48; N, 11.35.

Boc-Arg(NO_2)-Phe-Ser-Trp-Gly-Ala-Glu(OBzl)-Gly-Gln-Arg(NO_2)-resin——Boc-Arg(NO_2)-resin (2 g, 0.51 mmole) prepared in the same manner as described above was placed in a reaction vessel. Boc-Gln-ONp (936 mg, 2.55 mmole), N-protected tripeptide II (712 mg, 1.53 mmole), N-protected tetrapeptide IV (912 mg, 1.53 mmole) and Boc-Arg(NO_2)-OH (489 mg, 1.53 mmole) were condensed in this order onto the resin one at a time. For the condensation, steps 1—12 of the reaction cycle, described above for the stepwise peptide chain elongation, was carried out with the following modifications. Condensation of Boc-Gln-ONp and cleavage of the Boc group was carried out just in the same manner as described above. II in CH_2Cl_2 and IV in DMF–CH_2Cl_2 (1:1) was condensed in the presence of N-hydroxysuccinimide (176 mg, 1.53 mmole) for 15 hr. After introduction of the Trp-containing IV, β-mercaptoethanol (0.15 ml) was added to 4N HCl in dioxane. After introduction of the Ser-containing IV, step II was eliminated. After introduction of the N-terminal Arg (NO_2) the resulting protected peptidyl-resin was further washed with 15 ml portions of DMF, CH_2Cl_2, AcOH, EtOH 3 times each and dried over KOH pellets in vacuum; yield 2.475 g.

H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH——The protected peptidyl-resin (1.210 g) prepared by fragment condensation manner was treated with HF and purified just in the same manner as described above; yield 83 mg; mp 198—220° (decomp.); $[\alpha]_D^{18}$ −18.4° (c=2, H_2O); *Rf*[1] 0.15, *Rf*[2] 0.35, single ninhydrin, Sakaguchi and Ehrlich positive spot; amino acid ratios in the acid hydrolysate: Phe 1.0, Ser 1.0, Gly 2.1, Ala 1.1, Glu 2.2, Trp 0.7, Arg 1.8; amino acid ratios in the AP-M digest: Phe 1.0, Ser 1.1, Gly 2.0, Ala 1.1, Glu 1.1, Gln 1.1, Trp 0.9, Arg 1.8.

H-Phe-Ser-Trp-Gly-Arg-OH was isolated just in the same manner as described above; yield 1.6 mg; *Rf*[1] 0.35, *Rf*[2] 0.55, single ninhydrin, Sakaguchi and Ehrlich positive spot; amino acid ratios in the acid hydrolysate: Phe 1.0, Ser 1.1, Gly 1.2, Trp 0.8, Arg 0.8.

Acknowledgement The authors thank Mr. N. Endo and Miss K. Kudo for amino acid analysis and the staffs of Central Analysis Laboratory, Department of Chemistry, University of Tohoku, for elemental analysis.

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