Notes

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Studies on Encephalitogenic Fragments of Myelin Protein. III.[1,2] Synthesis of H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Asn-Arg-OH as an Analog of Encephalitogenic Decapeptide

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Westall, *et al.*[4] have shown that Trp, Gln and Lys(Arg) residues in an encephalitogenic tryptic peptide, H–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Lys(Arg)–OH, of myelin basic proteins are essential for induction of experimental allergic encephalomyelitis (EAE) in animals. On the other hand, Shapira, *et al.*[5] described as follows: –Gln–Arg– or –Gln–Lys– sequence has been found only rarely in proteins other than myelin basic protein and it may be of significance that the three –Gln–Lys– sequences in the basic proteins all reside in the active regions. Furthermore, two sequences are found in the human hemoglobin beta chain: H–$\overset{31}{\mathrm{Leu}}$–Leu–Val–Val–Tyr–Pro–Trp–Thr–Gln–$\overset{40}{\mathrm{Arg}}$–OH, H–$\overset{121}{\mathrm{Glu}}$–Phe–Thr–Pro–Pro–Val–Gln–Ala–Ala–Tyr–Gln–$\overset{132}{\mathrm{Arg}}$–OH. If one of these tryptic peptides were found to be encephalitogenic, demyelinating diseases, such as multiple sclerosis, might be initiated by proteins containing –Gln–Arg(Lys)– sequence that are located elsewhere in the body or in the environment, Shapira, *et al.* assumed.

In this context, it is of interest to study further the significance of the –Gln–Arg– sequence on encephalitogenic activity. In the present communication, on the basis of these consideration, synthesis of H–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Asn–Arg–OH in which the Gln residue in an encephalitogenic fragment[6] of human myelin protein is replaced by Asn residue having chemical properties similar to those of Gln and the result of the biological assay is described.

The key steps of the synthesis of the decapeptide (IV), H–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Asn–Arg–OH, are illustrated in Fig. 1. H–Arg(NO_2)–OBzl ditosylate[7] was condensed with Z–Asn–ONp[8] to form crystalline Z–Asn–Arg(NO_2)–OBzl (I). After removal of the Z–group of I with 5N HBr in AcOH, the resulting dipeptide ester was condensed with Boc–Ala–Glu(OBzl)–Gly–OH[1] by DCC method to yield Boc–Ala–Glu(OBzl)–Gly–Asn–Arg(NO_2)–OBzl (II). After removal of the Boc–group of II with trifluoroacetic acid, the resulting pentapeptide ester was condensed with Z–Arg(NO_2)–Phe–Ser–Trp–Gly–$NHNH_2$, derived from Z–Arg(NO_2)–Phe–Ser–Trp–Gly–NHNH–Boc,[6] according to Rudinger's procedure[9] to yield Z–Arg(NO_2)–Phe–Ser–Trp–Gly–Ala–Glu(OBzl)–Gly–Asn–Arg(NO_2)–OBzl (III). In

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[1] Part II: K. Suzuki and Y. Sasaki, *Chem. Pharm. Bull.* (Tokyo), **21**, 2634 (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: DCC = dicyclohexylcarbodiimide, DMF = dimethylformamide.

[3] Location: *Komatsushima, Sendai.*

[4] F.C. Westall, A.B. Robinson, J. Caccam, J. Jackson and E.H. Eylar, *Nature*, **229**, 22 (1971).

[5] R. Shapira, F.C-H. Chou, S. Mckneally, E. Urban and R.F. Kibler, *Science*, **173**, 736 (1971).

[6] K. Suzuki, T. Abiko, N. Endo, Y. Sasaki and J. Arisue, *Chem. Pharm. Bull.* (Tokyo), **21**, 2627 (1973).

[7] S. Sakakibara and N. Inukai, *Bull. Chem. Soc. Japan*, **39**, 1567 (1966).

[8] E. Wünsch, F. Drees and J. Jentsch, *Chem. Ber.*, **98**, 803 (1965).

[9] J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961)

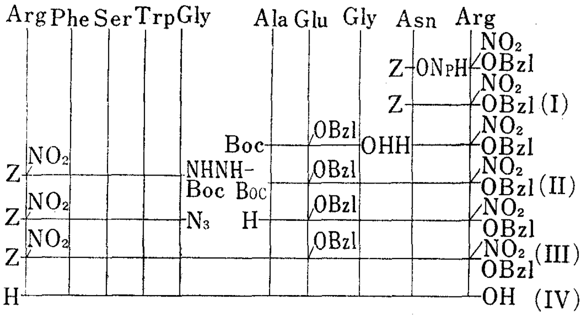

Fig. 1. Synthesis of Decapeptide (IV), H–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Asn–Arg–OH

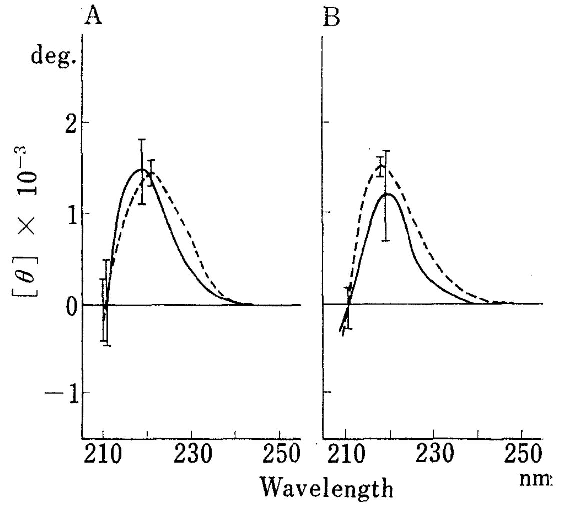

Fig. 2. CD Spectra of Synthetic Decapeptide (IV) (——) and [Gln^9]-Decapeptide[1,6] (------) in water (A) and in 0.1N HCl (B)

The measurements were done at about 0.1% of the peptides with 0.1 cm-cell at room temperature.

the course of this investigation, amino deblocked derivatives of the fully protected intermediary peptides were submitted to paper chromatography to assess the purity of these intermediates, since Hofmann, *et al.*[10] represented that thin-layer chromatography (TLC) of amino deblocked peptide were more valuable as an index of purity than that of the fully protected peptides. III was treated with anhydrous HF[11] to remove all protecting groups and passed through a carboxymethyl (CM)-cellulose column. Analysis of the eluates containing the desired peptide revealed the presence of a minor contaminant. The product was purified by preparative TLC to give H–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Asn–Arg–OH (IV).

Encephalitogenic activity[12] of the synthetic decapeptide (IV) was assayed using guinea pigs in the manner as described by Eylar.[13] Four out of five animals tested showed paralysis of hind foot at a dose of 100 μg in about three weeks after injection of the peptide. One out of four animals paralyzed thus showed weight loss as well, to be assigned as typical clinical signs of EAE and showed histological signs of EAE, too. Thus, it was judged that one out of five animals tested was induced EAE by injection of the synthetic peptide.

In order to assess the influence on conformation of Asn residue of IV, circular dichroism (CD) was measured. Fig. 2 shows CD spectra in water and in 0.1N HCl. The CD curves of IV were very similar to those of the synthetic H–Arg–Phe–Ser–Trp–Gly–Ala–Glu–Gly–Gln–Arg–OH and its analogs[14] containing essential three residues, Trp, Gln and Arg. From this point of view, it seems that a specific backbone conformation, such as α-helix or β-pleated sheet structure, is not required for induction of EAE. Consequently, it seems that the acid amide group of the side chain of Asn or Gln residue in the peptides plays an important part as one of the essential groups. Furthermore, the hydrophobic methylene carbon of Gln as

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[10] H.T. Storey and K. Hofmann, "Peptides 1971," North-Holland, Amsterdam, 1973, p. 227.

[11] S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada and H. Sugihara, *Bull. Chem. Soc. Japan*, **40**, 2164 (1967).

[12] Assayed by Drs. Y. Nagai, K. Nakano and T. Yasuda, Institute of Medical Science, University of Tokyo and Dr. T. Yonezawa, Department of Pathology, Kyoto Prefectural University of Medicine. A part of the biological study was presented at the 4th International Meeting of the International Society for Neurochemistry, Tokyo, August 26—31, 1973 by Dr. Y. Nagai, *et al.*, by whom the details will be published elsewhere.

[13] E.H. Eylar, J. Salk, G.C. Beveridge and L.V. Brown, *Arch. Biochem. Biophys.*, **132**, 34 (1967).

[14] K. Suzuki and Y. Sasaki; A part of this work was presented at the 11th Symposium on Peptide Chemistry, Kanazawa, Japan, October 24—25, 1973.

well as its acid amide group may affect the induction of EAE. On the basis of these findings, together with the fact that H–Arg–Phe–Gly–Trp–Gly–Gly–Gly–Gly–Gln–Arg–OH and H–Arg–Phe–Ser–Trp–Gly–Gly–Gly–Gly–Gln–Arg–OH do not exhibit any activity for the induction of EAE,[14] it seems that Shapira's assumption described above is not supported so strongly. Incidentally, distribution of the –Gln–Arg(Lys)– or –Asn–Arg(Lys)– sequence is not so rare in other proteins according to up–to–date sequencial studies of proteins.[15]

Experimental

Melting points are uncorrected. For paper chromatography, Z-group of I and Boc-group of II were deblocked with 5N HBr in AcOH and trifluoroacetic acid respectively and the resulting amino components were chromatographed on filter paper, Toyo Roshi No. 51, with Partridge system[16] (A) and BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24)[17] (B) as solvent. The CD spectra were recorded on a JASCO ORD–UV-5 Recording Polarimeter with CD attachment and the molecular ellipticity ([θ]) was corrected relatively using D-pantolactone ($[\theta]_{221} = -12.8 \times 10^3$ in 95% EtOH) at 25°. The amino acid compositions of the acid hydrolysate and aminopeptidase (AP)-M digest[18] were determined with Hitachi Model KLA-3B amino acid analyzer according to the directions given by Moore, *et al.*[19]

Z-Asn-Arg(NO_2)-OBzl (I)——To a solution of H–Arg(NO_2)–OBzl ditosylate (6.54 g) and Z–Asn–ONP (3.87 g) in dimethylformamide (DMF) (10 ml) was added Et_3N (1.4 ml) portionwise and the solution was stirred for 24 hr at room temperature, when the reaction mixture was poured into cooled 1N NH_4OH (100 ml) with stirring. The precipitate thereby formed was collected on filter and washed successively with 1N HCl and H_2O. After dried over P_2O_5 in vacuum, the product was recrystallized from EtOH; beads, yield 3.65 g (63%); mp 180—182°; $[\alpha]_{\rm D}^{21}$ −20.3° (c=2, DMF); de-Z peptide ester HBr salt, *Rf* (A) 0.32, *Rf* (B) 0.66, single ninhydrin positive spot; *Anal.* Calcd. for $C_{25}H_{31}O_8N_7$: C, 53.85; H, 5.60; N, 17.59. Found: C, 53.85; H, 5.61; N, 17.52.

Boc-Ala-Glu(OBzl)-Gly-Asn-Arg(NO_2)-OBzl (II)——I (558 mg) in AcOH (2 ml) was treated with 5N HBr in AcOH (4 ml) at room temperature for 40 min. The solution was diluted with anhydrous ether. The precipitate thereby formed was separated by centrifugation and dried over KOH pellets in vacuum. The product was dissolved in acetonitrile (10 ml), to which Et_3N (0.15 ml) was added. Boc–Ala–Glu(OBzl)–Gly–OH (466 mg) in acetonitrile (5 ml) and DMF (2 ml) was combined with the H–Asn–Arg(NO_2)–OBzl solution and cooled to 0°, when DCC (231 mg) was added. After standing at 5° for 40 hr, a few drops of AcOH were added and the mixture was stirred at room temperature for 1 hr. The precipitate was filtered off and the filtrate was evaporated in vacuum. The residue was washed successively with 1N NH_4OH, 1N AcOH and H_2O. The dried product was recrystallized from MeOH and petroleum ether; amorphous powder, yield 621 mg (71%); mp 162—166°; $[\alpha]_{\rm D}^{20}$ −18.0° (c=0.8, DMF); de-Boc peptide ester, *Rf* (A) 0.66, *Rf* (B) 0.83, single ninhydrin positive spot; *Anal.* Calcd. for $C_{39}H_{54}O_{13}N_{10}$: C, 53.78; H, 6.25; N, 16.08. Found: C, 53.68; H, 6.40; N, 15.94.

Z-Arg(NO_2)-Phe-Ser-Trp-Gly-Ala-Glu(OBzl)-Gly-Asn-Arg(NO_2)-OBzl (III)——II (87 mg) was treated with trifluoroacetic acid (0.4 ml) at room temperature for 20 min and the reaction mixture was diluted with anhydrous ether. The precipitate thereby formed was separated by centrifugation and dried over P_2O_5 in vacuum. The product was dissolved in DMF (1 ml) and added Et_3N (0.015 ml).

A solution of Z–Arg(NO_2)–Phe–Ser–Trp–Gly–$NHNH_2$ in DMF (2 ml), prepared from Z–Arg(NO_2)–Phe–Ser–Trp–Gly–NHNH–Boc[6] (95 mg) in the same manner as described in a previous communication,[6] was chilled in dry ice–80% EtOH bath to −50°. To the solution was added 4N HCl in dioxane (0.125 ml) with stirring, followed by addition of isoamylnitrite (0.013 ml). The mixture was stirred at −30° for 20 min and then chilled to −50° and Et_3N (0.084 ml) was added. After stirring for 15 min, the pentapeptide ester solution described above was added. The mixture was stirred at 5° for 48 hr and poured into 1N citric acid (20 ml) with stirring. The precipitate thereby formed was collected on filter and washed successively with H_2O, 1N $NaHCO_3$ and H_2O. The dried product was recrystallized from DMF and EtOAc; slightly pale yellow amorphous powder, yield 113 mg (71%); mp 138—145°; $[\alpha]_{\rm D}^{20}$ +12.2° (c=1, DMF); fully protected

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[15] M.O. Dayhoff, (ed.), "Atlas of Protein Sequence and Structure," Vol. 5, National Biomedical Research Foundation, Silver Spring, Md., 1972.

[16] S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).

[17] S.G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953).

[18] 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). Rörm and Haas (Darmsatdt) preparation was used.

[19] S. Moore, D.H. Spackman and W.H. Stein, *Anal. Chem.*, **30**, 1185 (1958).

peptide, *Rf* (A) 0.77, *Rf* (B) 0.93, single Ehrlich positive spot; *Anal.* Calcd. for $C_{73}H_{90}O_{21}N_{20}$: C, 55.36; H, 5.73; N, 17.69. Found: C, 55.27; H, 5.63; N, 17.88.

H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Asn-Arg-OH (IV)——III (74 mg) was treated with anhydrous HF (5 ml) in the presence of anisole (0.7 ml) under ice-cooling for 50 min. After evaporation of the excess HF in vacuum, the residue was extracted with 1% AcOH (10 ml × 2) and the combined extracts were washed with peroxide free ether. The clear solution thus obtained was passed through a column of Dowex 1 × 2 (acetate form, 1.8 × 7 cm) and the column was washed with 2% AcOH. Sakaguchi reaction positive eluates were collected and lyophilized. The crude decapeptide (59 mg) was dissolved in H_2O (2 ml). A small amount of insoluble material was removed by centrifugation and the supernatant was applied on a column of CM-cellulose (1.8 × 15 cm), which was eluted with a linear gradient elution from H_2O (300 ml) in mixing chamber to 0.1 M NH_4OAc (pH 6.5, 300 ml) in reservoir at a flow rate of 5 m/4 min with an automated fraction collector. Fractions of 5 ml each were collected and the absorbancy of each fraction was determined at 280 nm. The fraction of tubes No. 40 to 51 were pooled and lyophilized to constant weight (41 mg). The product was dissolved in H_2O (0.6 ml) and submitted to preparative TLC (Wakogel B-5, 20 × 20 cm × 5) using the system of BuOH–pyridine–AcOH–H_2O (30: 20: 6: 24) as a developing solvent. Zone corresponding to *Rf* 0.35 was separated and extracted with 1% AcOH. The extract was evaporated to small volume. The solution was applied on a column of CM-cellulose (1.8 × 12 cm), which was eluted with H_2O (200 ml) and then with 2% AcOH (200 ml). The Sakaguchi reaction positive fractions were pooled and lyophilized; amorphous powder, yield 21 mg (38%); mp 193—199° (decomp.); $[\alpha]^{21}_{D}$ +40.2° (c=0.5, H_2O); *Rf* (A) 0.13, *Rf* (B) 0.34, single ninhydrin, Ehrlich and Sakaguchi positive spot; amino acid ratios in the acid hydrolysate: Arg 1.92, Asp 1.02, Ser 0.96, Glu 1.03, Gly 2.14, Ala 1.00, Phe 1.02; amino acid ratios in the AP-M digest: Trp 1.05, Arg 1.95, (Asn+Ser) 1.72, Glu 0.93, Gly 2.10, Ala 1.00, Phe 0.96.

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Syntheses of [Phe(4NO$_2$)^5, Tyr(Me)^8]- and [Tyr(Me)^5, Phe(4NO$_2$)^8]- Bradykinin[1]

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Synthesis of [Tyr(Me)^5, Tyr(Me)^8]-bradykinin showing relatively high bradykinin-like activity and antibradykinin activity as well has been reported by Stewart, *et al.*[3] Such a peptide exhibiting antibradykinin activity is a quite few among many synthetic bradykinin analogs.[4] Seeking for antibradykinin peptides exhibiting no bradykinin-like activity is of interest. In this context, it is of interest to examine further the biological activity of bradykinin analogs in which two phenylalanine residues of bradykinin are substituted with another

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1) The amino acid residues except glycine are of the L-configuration. The abbrebiations used to denote amino acid derivatives and peptides are those recommended by IUPAC-IUB Commission on Biochemical Nomenclature; *Biochim. Biophys. Acta*, **263**, 205 (1972). Other abbreviations: DMF=dimethylformamide, Et_3N=triethylamine, TFA=trifluoroacetic acid, DCC=dicyclohexylcarbodiimide.

2) Location: *Komatsushima, Sendai, 983, Japan.*

3) J.M. Stewart, and D.H. Wooly, "Hypotensive peptides," ed. by E.G. Erdös, N. Back, F. Sicuteri, and A.F. Wilde, Springer-Verlag, New York Inc., 1966, p. 23.

4) E. Schröder, "Handbook of Experimental Pharmacology," Vol. 25, ed., E.G. Erdös, Springer-Verlag, Berlin-New York, 1970, p. 324.