

Studies on Encephalitogenic Fragments of Myelin Protein. IV.^{1,2)} Synthesis of Glycine Analogs of Tryptophan-containing Fragment

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Synthesis of five glycine analogs of H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH, in which amino acid residues at positions 3, 6 and 7 are replaced by glycine, is reported. Correlation of structure and activity of the encephalitogenic decapeptide fragment is discussed on the basis of the activity in guinea pigs and circular dichroism spectra of the synthetic glycine analogs.

For a study of the correlation of the structure and experimental allergic encephalomyelitis (EAE) activity of a dodecapeptide, H-Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys(Arg)-OH, corresponding to positions 112 to 122 of bovine EAE myelin protein, Westall, *et al.*⁴⁾ have synthesized a series of the peptide analogs in which each amino acid residue except for alanine and two glycine residues was replaced by amino acid residue having rather stereochemically similar side chain in the sequence. It was concluded that structural requirement for EAE activity was $\cdots\text{Trp}\cdots\text{Gln-Lys(Arg)-OH}$ in the sequence. In a previous communication,⁵⁾ it was explored that synthetic decapeptide, H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH, corresponding to positions 113 to 122 of human EAE myelin protein,⁶⁾ induced strongly EAE in guinea pigs.

In the present study, five glycine analogs of the human decapeptide were prepared, in which glycine replaced one or more of amino acids except for three essential amino acid residues in the sequence, in order to ascertain to what degree the side chains of amino acid residues contributed to biological activity. The result of the biological assay and the conformational study by circular dichroism (CD) spectra are also described. On the basis of these investigations, the correlation of the structure and the EAE activity in guinea pigs is discussed.

Amino acid sequences of five synthetic analogs are shown in Table I, together with their biological activity. The method of peptide synthesis used here is virtually similar with a previous communication⁷⁾ on the synthesis of the decapeptide, but in this study the protecting group for the carboxyl of the carboxyl terminal arginine residue is benzyl group which can be cleaved with anhydrous hydrogen fluoride (HF).⁸⁾ The key steps for the synthesis of the decapeptide analogs are coupling reaction of the amino terminal pentapeptide azide derivatives and carboxyl terminal pentapeptide ester derivatives. Synthetic schemes of the key intermediates of carboxyl and amino terminal pentapeptide derivatives are illustrated in Fig. 1 and 2 respectively. For the synthesis of 6-glycine decapeptide H-Arg-Phe-Ser-Trp-Gly-

1) Part III: Y. Sasaki, *Chem. Pharm. Bull.* (Tokyo), **22**, 2188 (1974).

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: OAc=acetoxime ester, DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide, CMC=carboxymethyl cellulose.

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

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

5) K. Suzuki and Y. Sasaki, *Chem. Pharm. Bull.* (Tokyo), **21**, 2634 (1973).

6) P.R. Carnegie, *Nature*, **229**, 25 (1971); *idem*, *Biochem. J.*, **123**, 57 (1971).

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

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

TABLE I. Encephalitogenic Activity of Synthetic Peptides^{a)}

Material	Encephalitogenic activity ^{b)}		
	Dose (μg)	Clinical	Clinical+histological
H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH	10	6/7	7/7
H-Arg-Phe-Ser-Trp-Gly-Gly-Glu-Gly-Gln-Arg-OH (V)	100	1/5 ^{c)}	0/4
H-Arg-Phe-Ser-Trp-Gly-Gly-Gly-Gly-Gln-Arg-OH (IX)	100	0/5	0/5
H-Arg-Phe-Gly-Trp-Gly-Gly-Glu-Gly-Gln-Arg-OH (XIII)	100	0/5	2/5
H-Arg-Phe-Gly-Trp-Gly-Ala-Gly-Gly-Gln-Arg-OH (XVI)	100	4/5	5/5
H-Arg-Phe-Gly-Trp-Gly-Gly-Gly-Gly-Gln-Arg-OH (XVIII)	100	0/5	0/5

a) The replaced amino acid residues are shown with underlines.

b) Encephalitogenic activity is expressed as the number of guinea pigs showing clinical and histological signs of EAE over the animals tested.

c) One out of five animals tested died after showing severe clinical signs, but the others did not show any clinical or histological signs.

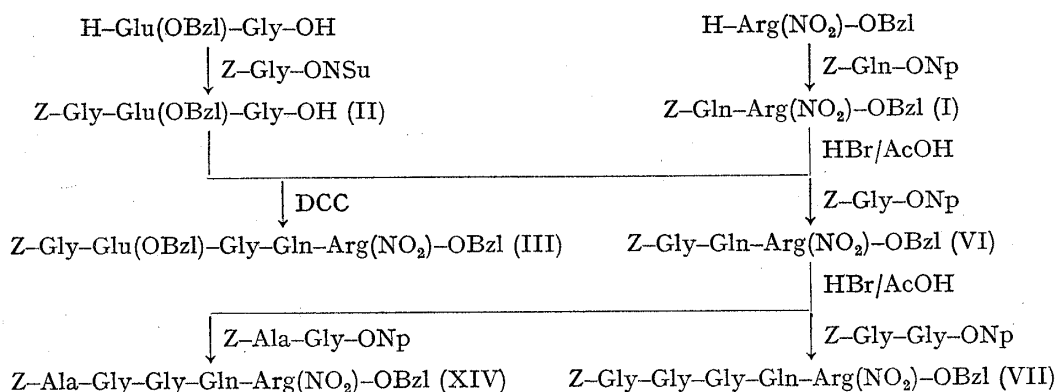


Fig. 1. Synthetic Scheme of C-Terminal Pentapeptide Derivatives

Gly-Glu-Gly-Gln-Arg-OH(V), Z-Gln-ONp⁹⁾ and H-Arg(NO₂)-OBzl ditosylate¹⁰⁾ were condensed to yield Z-Gln-Arg(NO₂)-OBzl (I). I was de-benzyloxycarbonylated with hydrogen bromide-acetic acid solution and the resulting dipeptide ester was condensed by DCC method with Z-Gly-Glu(OBzl)-Gly-OH (II), which was prepared from Z-Gly-ONSu¹¹⁾ and de-*tert*-butoxycarbonylated Boc-Glu(OBzl)-Gly-OH,⁵⁾ to yield the protected pentapeptide derivative, Z-Gly-Glu(OBzl)-Gly-Gln-Arg(NO₂)-OBzl (III). Coupling of the protected pentapeptide azide derived from Z-Arg(NO₂)-Phe-Ser-Trp-Gly-NHNH-Boc⁷⁾ and de-benzyloxycarbonylated III yielded Z-Arg(NO₂)-Phe-Ser-Trp-Gly-Gly-Glu(OBzl)-Gly-Gln-Arg(NO₂)-OBzl (IV). IV was treated with HF in the presence of anisole at 0° for one hour. The crude decapeptide thus obtained was purified through CMC column and subsequent cellulose column chromatography. The decapeptide so obtained was found to be a unity from the result of paper chromatography using two different solvent systems.

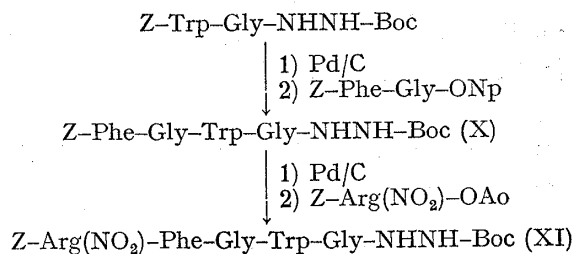


Fig. 2. Synthetic Scheme of N-Terminal Pentapeptide Derivative XI

9) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).

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

11) G.W. Anderson, J.E. Zimmerman and F.M. Callahan, *J. Am. Chem. Soc.*, **86**, 1839 (1964).

The amino acid ratios in the acid hydrolysate of V and aminopeptidase(AP)-M digest agreed with theoretical values. The synthesis of the 6,7-bis-glycine decapeptide (IX) followed the same pattern as described above. Z-Gly-ONp¹²⁾ was condensed with de-benzyloxycarbonylated II to yield Z-Gly-Gln-Arg(NO₂)-OBzl (VI). After removal of the benzyloxycarbonyl group of VI, the resulting tripeptide ester was condensed with Z-Gly-Gly-ONp¹³⁾ to yield Z-Gly-Gly-Gly-Gln-Arg(NO₂)-OBzl (VII). The amino terminal pentapeptide derivative and VII were coupled analogously to yield Z-Arg(NO₂)-Phe-Ser-Trp-Gly-Gly-Gly-Gly-Gln-Arg(NO₂)-OBzl (VIII). VIII was treated with HF followed by purification on CMC column to give homogeneous decapeptide, H-Arg-Phe-Ser-Trp-Gly-Gly-Gly-Gly-Gln-Arg-OH (IX).

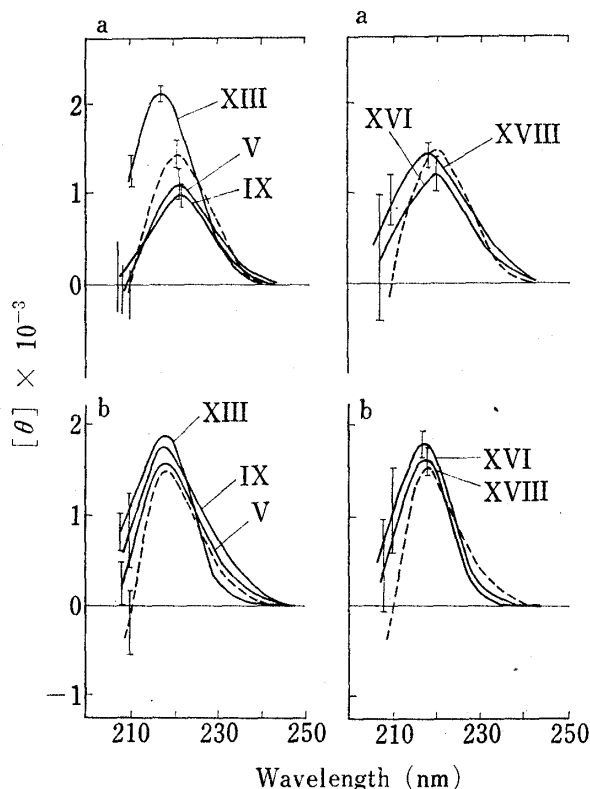


Fig. 3. CD Spectra of Synthetic Decapeptide (-----) and Glycine Analogs (—) in Water (a) and in 0.1N HCl (b)

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

Arg-Phe-Gly-Trp-Gly-Gly-Gly-Gly-Gln-Arg-OH (XVIII) was prepared *via* Z-Arg(NO₂)-Phe-Gly-Trp-Gly-Gly-Gly-Gly-Gln-Arg(NO₂)-OBzl (XVII).

The EAE activity¹⁶⁾ of the five synthetic glycine analogs was assayed using guinea pigs by the procedure given by Eylar, *et al.*¹⁷⁾ and compared with that of the decapeptide, H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH.^{5,7)} The result is shown in Table I. Four 6-glycine analogs, V, IX, XIII, XVIII, in which alanine residue at position 6 was replaced,

Synthesis of a key amino terminal pentapeptide derivative XI is illustrated in Fig. 2. Z-Trp-Gly-NHNH-Boc⁷⁾ was hydrogenated over Pd-carbon and the resulting H-Trp-Gly-NHNH-Boc was condensed with Z-Phe-Gly-ONp¹⁴⁾ to yield Z-Phe-Gly-Trp-Gly-NHNH-Boc (X). De-benzyloxycarbonylated X was condensed with Z-Arg(NO₂)-OAc¹⁵⁾ to yield Z-Arg(NO₂)-Phe-Gly-Trp-Gly-NHNH-Boc (XI). Coupling of the protected pentapeptide azide derived from XI and de-benzyloxycarbonylated III yielded Z-Arg(NO₂)-Phe-Gly-Trp-Gly-Gly-Glu(OBzl)-Gly-Gln-Arg(NO₂)-OBzl (XII). XII was treated with HF followed by purification on CMC column to give homogeneous 3,6-bis-glycine analog, H-Arg-Phe-Gly-Trp-Gly-Gly-Glu-Gly-Gln-Arg-OH (XIII). For the synthesis of 3,7-bis-glycine analog XVI, Z-Ala-Gly-ONp¹⁴⁾ was condensed with de-benzyloxycarbonylated VI to yield Z-Ala-Gly-Gly-Gln-Arg(NO₂)-OBzl (XIV). 3,7-Bis-glycine analog, H-Arg-Phe-Gly-Trp-Gly-Ala-Gly-Gly-Gln-Arg-OH (XVI) was prepared analogously *via* Z-Arg(NO₂)-Phe-Gly-Trp-Gly-Ala-Gly-Gly-Gln-Arg(NO₂)-OBzl (XV). 3,6,7-Tri-glycine analog, H-

12) B. Iselin, W. Rittel, P. Sieber and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).

13) M. Bodanszky, J.T. Sheehan, M.A. Ondetti and S. Lande, *J. Am. Chem. Soc.*, **85**, 991 (1963).

14) K. Suzuki and T. Abiko, *Chem. Pharm. Bull.* (Tokyo), **15**, 1212 (1967).

15) M. Fujino and O. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1937 (1969).

16) Assayed by Dr. Y. Nagai, *et al.*, 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.

17) E.H. Eylar, J. Salk, G.C. Beveridge and L.V. Brown, *Arch. Biochem. Biophys.*, **132**, 34 (1969).

were inactive or slightly active at a dose of 100 μ g. On the other hand, 3,7-bis-glycine analog XVI, in which the alanine residue was not replaced, was highly active. On the basis of these findings, it can be concluded that the methyl group in alanine residue at position 6 is very important to impart EAE activity to a decapeptide and the side chains of serine and glutamic acid residue are not required for the activity.

For a study of the correlation of the conformation and EAE activity of the synthetic glycine analogs, CD of the peptides was measured and compared with that of H-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg-OH.¹⁾ Fig. 3 shows CD spectra of the synthetic peptides in water and 0.1N HCl. These CD spectra show that the decapeptide and synthetic glycine analogs do not exist in solution as a specific backbone conformation, such as α -helix or β -pleated sheet structure. In conclusion, it can be stated that a specific backbone conformation is not required for the EAE activity of the decapeptide but the side chain of the alanine residue as well as those of three essential amino acids, tryptophan, glutamine and carboxyl terminal arginine, play an important role for the EAE activity and side chains of serine and glutamic acid are not required for the activity.

Experimental

All melting points are uncorrected. For paper chromatography, Z-group of the protected intermediates were deblocked with HBr in AcOH and the resulting amino components were chromatographed on filter paper, Toyo Roshi No. 51, by ascending procedure using Partridge system¹⁸⁾ (A) and BuOH-pyridine-AcOH-H₂O (30:20:6:24)¹⁹⁾ (B) as developing solvents. The CD spectra were recorded on a JASCO ORD/UV-5 Recording Polarimeter with CD attachment and the molecular ellipticity ($[\theta]$) 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 hydrolysates and the aminopeptidase (AP)-M digests²⁰⁾ were determined with Hitachi Model KLA-3B amino acid analyzer according to the directions given by Moore, *et al.*²¹⁾

Z-Gln-Arg(NO₂)-OBzl (I)—To a solution of H-Arg(NO₂)-OBzl ditosylate (7.84 g) and Z-Gln-ONp (4.82 g) in DMF (30 ml), Et₃N (1.82 ml) was added portionwise and the solution was stirred for 20 hr at room temperature when the solution was poured into cooled 1N NH₄OH (300 ml) with stirring. The precipitate thereby formed was collected on filter and washed successively with 1N NH₄OH, 1N HCl and H₂O. After dried over P₂O₅ under vacuum, the product was recrystallized from DMF-EtOAc; amorphous powder; yield 4.21 g (61%); mp 191.5–193°; $[\alpha]_D^{25} -8.3^\circ$ ($c=1.1$, DMF); de-Z peptide ester HBr salt, *Rf* 0.42 (A), 0.75 (B), single ninhydrin positive spot; *Anal.* Calcd. for C₂₆H₃₃O₈N₇: C, 54.63; H, 5.82; N, 17.16. Found: C, 54.32; H, 5.74; N, 16.97.

Z-Gly-Glu(OBzl)-Gly-OH (II)—Boc-Glu(OBzl)-Gly-OH (3.16 g) was treated with trifluoroacetic acid (15 ml) for 20 min at room temperature. After evaporation of trifluoroacetic acid under vacuum, the residue was dissolved in H₂O (10 ml) containing NaHCO₃ (1.60 g). To the solution, Z-Gly-ONSu (2.45 g) in EtOH (15 ml) was added and stirred for 30 hr at room temperature. The reaction mixture was concentrated to small volume under vacuum and washed with EtOAc. The aqueous phase was cooled, acidified with 5N HCl to approximately pH 3 and extracted three times with EtOAc. The extracts were washed with 1N HCl and H₂O. The combined extract was dried over MgSO₄ and evaporated under vacuum to give an oily residue which crystallized on standing in a cold. The product was recrystallized from EtOH-ether; amorphous powder; yield 2.91 g (77%); mp 115–117°; $[\alpha]_D^{25} -9.5^\circ$ ($c=0.9$, EtOH); de-Z peptide HBr salt, *Rf* 0.52 (A), 0.60 (B), single ninhydrin positive spot; *Anal.* Calcd. for C₂₄H₂₇O₈N₃: C, 59.37; H, 5.61; N, 8.66. Found: C, 59.00; H, 5.59; N, 8.35.

Z-Gly-Glu(OBzl)-Gly-Gln-Arg(NO₂)-OBzl (III)—I (572 mg) in AcOH (2 ml) was added 5N HBr in AcOH (3 ml). After 30 min at room temperature, the solution was diluted with dry ether. The precipitate thereby formed was collected by centrifugation, washed with dry ether and dried over KOH pellets under vacuum. The product was dissolved in DMF (1 ml) and acetonitrile (2 ml) containing Et₃N (0.15 ml) and the solution was combined with a solution of II (470 mg) and N-hydroxysuccinimide (115 mg) in DMF (1 ml) and acetonitrile (2 ml). The mixture was cooled in an ice-NaCl bath and DCC (210 mg) was added. After stirring at 5° for 48 hr, dicyclohexylurea formed was filtered off and the mixture was poured into cooled 1N

18) S.M. Partridge, *Biochem. J.*, **42**, 238 (1948).

19) S.G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953).

20) G. Pfeleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963); G. Pfeleiderer, P.G. Celliers, M. Stanulovic, E.D. Washmuth, H. Determan and G. Braunitzer, *ibid.*, **340**, 552 (1964). Rörm and Haas (Darmstadt) preparation was used.

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

HCl (30 ml) with stirring. The precipitate thereby formed was collected on filter, washed with 1N NH_4OH and H_2O , and dried over P_2O_5 under vacuum. The product was recrystallized from DMF-EtOAc; amorphous powder; yield 616 mg (68%); mp 113–126°; de-Z peptide ester HBr salt, R_f 0.30 (A), 0.55 (B), single ninhydrin positive spot; the elemental analysis of this product gave no correct values but the crude product was used for the next coupling reactions without further purification.

Z-Arg(NO_2)-Phe-Ser-Trp-Gly-Gly-Glu(OBzl)-Gly-Gln-Arg(NO_2)-OBzl (IV)—Z-Arg(NO_2)-Phe-Ser-Trp-Gly-NHNH-Boc (273 mg, 0.288 mmoles) was treated with 80% trifluoroacetic acid in CH_2Cl_2 (1 ml) for 20 min at room temperature under N_2 gas. The solution was diluted with peroxide free dry ether. The precipitate thereby formed was collected by centrifugation, washed with peroxide free dry ether and dried over KOH pellets under vacuum. The hydrazide was dissolved in DMF (2 ml) and cooled to -50° with a dry ice-70% EtOH bath. 4N HCl in dioxane (0.36 ml) was added to the stirred solution, followed by addition of 9.28% (W/V) isoamyl nitrite in DMF (0.364 ml, 0.288 mmoles). The solution was stirred for 30 min at -30° , cooled again to -50° and Et_3N (0.266 ml, 1.90 mmoles) was added. H-Gly-Glu(OBzl)-Gly-Gln-Arg(NO_2)-OBzl·HBr, prepared from III (261 mg, 0.288 mmoles) in the same manner as described for the preparation of II, was dissolved in DMF (2 ml) containing Et_3N (0.05 ml) and this cooled solution was combined with the azide solution. The mixture was stirred for 48 hr at 5° . The reaction mixture was poured into cooled 1N citric acid (20 ml). The precipitate thereby formed was collected on filter, washed with 1N NaHCO_3 and H_2O . After dried over P_2O_5 under vacuum, the product was recrystallized from DMF-EtOAc; amorphous powder; yield 305 mg (67%); mp 141–146°; $[\alpha]_D^{25} + 29.5^\circ$ ($c=0.5$, DMF); *Anal.* Calcd. for $\text{C}_{73}\text{H}_{90}\text{O}_{21}\text{N}_{20}$: C, 55.36; H, 5.73; N, 17.69. Found: C, 55.13; H, 5.73; N, 17.78.

H-Arg-Phe-Ser-Trp-Gly-Gly-Glu-Gly-Gln-Arg-OH (V)—IV (262 mg) was treated with anhydrous HF (15 ml) in the presence of anisole (2 ml) for 60 min at 0° . After evaporation of the excess HF under vacuum, the residue was extracted with 2 portions of 1% AcOH (30 ml). The combined extract was washed with EtOAc and concentrated to small volume under vacuum. The clear solution thus obtained was submitted to a column of Dowex 1 $\times$ 2 (acetate form, 1.8×12 cm), which was eluted with 1% AcOH. Sakaguchi positive eluates were pooled and lyophilized to yield slightly yellow fluffy material (163 mg). The product was dissolved in H_2O (1 ml) and submitted to a column of CMC (1.8×20 cm) which was eluted with a linear gradient elution from H_2O (300 ml) in mixing chamber to 0.1M NH_4OAc buffer (pH 6.42, 300 ml) in reservoir. Fractions of 10 ml each were collected at a flow rate of 3 ml/min with an automated fraction collector and absorbancy at 280 nm was determined on each fraction. The eluates in tubes No. 38–48 were pooled, evaporated under vacuum and lyophilized to constant weight to yield colorless fluffy material (110 mg) which was detected a minor contaminant on paper chromatography. A portion (68 mg) of the material was submitted to a cellulose column (2.5×32 cm) which was eluted with a solvent of $n\text{-BuOH}$ -pyridine-AcOH- H_2O (30:20:6:24). Fractions 5 ml each were collected at a flow rate of 1 ml/2 min and absorbancy at 280 nm was determined on each fraction. Tubes No. 28–35 were pooled, evaporated to dryness under vacuum and lyophilized from H_2O ; colorless fluffy material; yield 42 mg (43%); mp 168–181° (decomp.); $[\alpha]_D^{25} - 16.4^\circ$ ($c=2.0$, H_2O); R_f 0.15 (A), 0.35 (B), single ninhydrin, Ehrlich, Sakaguchi and chlorine positive spot; amino acid ratios in the acid hydrolysate: Trp 0.55, Arg 1.94, Ser 0.89, Glu 2.02, Gly 3.16, Phe 1.00; amino acid ratios in the AP-M digest: Trp, 0.83, Arg 1.92, Gln 0.86, Ser 0.97, Glu 1.03, Gly 3.28, Phe 1.12.

Z-Gly-Gln-Arg(NO_2)-OBzl (VI)—I (2.86 g) in AcOH (10 ml) was treated with 5N HBr in AcOH (15 ml). After 30 min at room temperature, the mixture was diluted with dry ether. The precipitate thereby formed was collected by centrifugation, washed with dry ether and dried over KOH pellets under vacuum. To a solution of this product in DMF (25 ml), Z-Gly-ONp (1.65 g) was added, followed by addition of Et_3N (0.75 ml). After 40 hr at room temperature, the reaction mixture was poured into cooled 1N NH_4OH (200 ml) with stirring. The precipitate thereby formed was collected on filter, washed successively with 1N NH_4OH , H_2O , 1N HCl and H_2O . The dried product was recrystallized from DMF-EtOAc-pet. ether; amorphous powder; yield 1.41 g (45%); mp 184–186°; $[\alpha]_D^{25} - 12.4^\circ$ ($c=0.9$, DMF); de-Z peptide ester HBr salt, R_f 0.35 (A), 0.59 (B), single ninhydrin positive spot; *Anal.* Calcd. for $\text{C}_{28}\text{H}_{36}\text{O}_9\text{N}_8$: C, 53.49; H, 5.77; N, 17.83. Found: C, 53.37; H, 5.70; N, 17.93.

Z-Gly-Gly-Gly-Gln-Arg(NO_2)-OBzl (VII)—The compound was prepared from Z-Gly-Gly-ONp (291 mg) and VI (471 mg) essentially in the same manner as described for the preparation of VI. The product was recrystallized from DMF-EtOAc; amorphous powder; yield 465 mg (83%); mp 137–139°; $[\alpha]_D^{25} - 10.0^\circ$ ($c=1.0$, DMF); de-Z peptide ester HBr salt, R_f 0.32 (A), 0.62 (B), single ninhydrin positive spot; *Anal.* Calcd. for $\text{C}_{32}\text{H}_{42}\text{O}_{11}\text{N}_{10}$: C, 51.74; H, 5.70; N, 18.86. Found: C, 51.65; H, 5.78; N, 18.96.

Z-Arg(NO_2)-Phe-Ser-Trp-Gly-Gly-Gly-Gln-Arg(NO_2)-OBzl (VIII)—The compound was prepared from Z-Arg(NO_2)-Phe-Ser-Trp-Gly-NHNH-Boc (95 mg) and VII (74 mg) essentially in the same manner as described for the preparation of IV. The product was recrystallized from DMF-EtOAc; slightly pale yellow amorphous powder; yield 88 mg (66%); mp 141–144°; $[\alpha]_D^{25} + 9.0^\circ$ ($c=0.5$, DMF); *Anal.* Calcd. for $\text{C}_{63}\text{H}_{80}\text{O}_{19}\text{N}_{20} \cdot \text{H}_2\text{O}$: C, 52.56; H, 5.74; N, 19.46. Found: C, 52.58; H, 5.42; N, 19.02.

H-Arg-Phe-Ser-Trp-Gly-Gly-Gly-Gln-Arg-OH (IX)—The compound was prepared from VIII (38 mg) essentially in the same manner as described for the preparation of V. CMC column (1.8×7 cm) chromatography was carried out using linear gradient elution from H_2O (300 ml) in mixing chamber to 0.15M NH_4OAc buffer (pH 6.42, 300 ml) in reservoir. The eluates in tubes No. 33–40 were pooled and

lyophilized. Rechromatography on CMC gave homogeneous IX; colorless fluffy material; yield 24 mg (80%); mp 153—166° (decomp.); $[\alpha]_D^{18} + 11.0^\circ$ ($c=0.9$, H_2O); R_f 0.12 (A), 0.39 (B), single ninhydrin, Sakaguchi, Ehrlich and chlorine positive spot; amino acid ratios in the acid hydrolysate: Trp 0.68, Arg 2.00, Ser 1.05, Glu 0.95, Gly 4.14, Phe 1.18; amino acid ratios in the AP-M digest: Trp 1.14, Arg 2.04, Gln 0.76, Ser 0.97, Gly 3.96, Phe 1.13.

Z-Phe-Gly-Trp-Gly-NHNH-Boc (X)—Z-Trp-Gly-NHNH-Boc (1.54 g) was hydrogenated in MeOH (30 ml) over 5% Pd/C for 5 hr at room temperature. After removal of the catalyst by the aid of Cellite, the solvent was evaporated under vacuum. To a solution of the residue in DMF (30 ml), Z-Phe-Gly-ONp (1.48 g) was added and the reaction mixture was stirred for 24 hr at room temperature, when EtOAc (50 ml) was added. The solution was washed successively with 1N NH_4OH , H_2O , 1N citric acid and H_2O saturated NaCl and dried over $MgSO_4$. A small amount of MeOH was added the extract in order to prevent precipitation of the product during the washings. The EtOAc solution was evaporated under vacuum and the residue was recrystallized from EtOAc-pet. ether; amorphous powder; yield 1.71 g (80%); mp 120—123°; $[\alpha]_D^{20} - 2.0^\circ$ ($c=1.0$, MeOH); de-Z peptide derivative, R_f 0.92 (A), 0.96 (B), single ninhydrin and Ehrlich positive spot; *Anal.* Calcd. for $C_{37}H_{48}O_8N_7$: C, 62.26; H, 6.07; N, 13.74. Found: C, 62.08; H, 6.31; N, 13.46.

Z-Arg(NO_2)-Phe-Gly-Trp-Gly-NHNH-Boc (XI)—X (1.71 g) was de-benzyloxycarbonylated in the same manner as described above. To a solution of the resulting tetrapeptide derivative in DMF (10 ml), was added Z-Arg(NO_2)-OAc (0.98 g) and glacial AcOH (0.21 ml) and the mixture was stirred for 48 hr at room temperature. The reaction mixture was poured into cooled 1N NH_4OH with stirring. The precipitate thereby formed was collected on filter, washed with 1N NH_4OH , 1N citric acid and H_2O , and dried over P_2O_5 under vacuum. The product was recrystallized from MeOH-EtOAc-pet. ether; amorphous powder; yield 1.62 g (80%); mp 157—160° (decomp.); $[\alpha]_D^{20} - 11.0^\circ$ ($c=1.0$, MeOH); fully protected peptide, R_f 0.95 (A), single Ehrlich positive spot; *Anal.* Calcd. for $C_{42}H_{52}O_{11}N_{12}$: C, 55.99; H, 5.82; N, 18.66. Found: C, 55.78; H, 5.89; N, 18.60.

Z-Arg(NO_2)-Phe-Gly-Trp-Gly-Gly-Glu(OBzl)-Gly-Gln-Arg(NO_2)-OBzl (XII)—This compound was prepared from XI (92 mg) and III (90 mg) essentially in the same manner as described for the preparation of IV. The reaction mixture was stirred for 40 hr at 5°. BuOH (20 ml) was added to the mixture and the BuOH layer was washed with 1N NH_4OH , 1N AcOH and H_2O which were saturated respectively with BuOH. The solvent was evaporated under vacuum and the residue was dried over P_2O_5 . The product was recrystallized from DMF-EtOAc; amorphous powder; yield 98 mg (62%); mp 136—139°; $[\alpha]_D^{18} + 14.0^\circ$ ($c=0.4$, DMF); *Anal.* Calcd. for $C_{72}H_{88}O_{20}N_{20} \cdot 2H_2O$: C, 54.39; H, 5.83; N, 17.62. Found: C, 54.03; H, 6.12; N, 17.75.

H-Arg-Phe-Gly-Trp-Gly-Gly-Gly-Gln-Arg-OH (XIII)—This compound was prepared from XII (42 mg) essentially in the same manner as described for the preparation of V. CMC column (1.8 × 9 cm) chromatography was carried out using a linear gradient elution from H_2O (300 ml) in mixing chamber to 0.1M NH_4OAc buffer (pH 6.42, 300 ml) in reservoir. The eluates in tubes No. 22—28 were pooled and lyophilized from H_2O . Rechromatography on CMC gave homogeneous XIII; colorless fluffy material; yield 17 mg (56%); mp 182—191° (decomp.); $[\alpha]_D^{18} + 11.9^\circ$ ($c=0.7$, H_2O); R_f 0.18 (A), 0.37 (B), single ninhydrin, Sakaguchi, Ehrlich and chlorine positive spot; amino acid ratios in the acid hydrolysate: Trp 0.72, Arg 2.04, Glu 2.13, Gly 3.96, Phe 1.08; amino acid ratios in the AP-M digest: Trp 0.96, Arg 1.90, Gln 0.95, Glu 0.95, Gly 4.09, Phe 1.15.

Z-Ala-Gly-Gly-Gln-Arg(NO_2)-OBzl (XIV)—This compound was prepared from VI (630 mg) and Z-Ala-Gly-ONp (402 mg) essentially in the same manner as described for the preparation of VI. The product was recrystallized from EtOH-EtOAc; amorphous powder; yield 504 mg (67%); mp 191—192°; $[\alpha]_D^{18} - 8.0^\circ$ ($c=1.0$, DMF); de-Z peptide ester HBr salt, R_f 0.31 (A), 0.64 (B), single ninhydrin positive spot; *Anal.* Calcd. for $C_{33}H_{44}O_{11}N_{10}$: C, 52.37; H, 5.86; N, 18.51. Found: C, 52.48; H, 5.98; N, 18.64.

Z-Arg(NO_2)-Phe-Gly-Trp-Gly-Ala-Gly-Gly-Gln-Arg(NO_2)-OBzl (XV)—This compound was prepared from XI (92 mg) and XIV (68 mg) essentially in the same manner as described for the preparation of IV. The product was recrystallized from DMF-EtOAc; amorphous powder; yield 87 mg (62%); mp 140—143°; $[\alpha]_D^{18} - 13.9^\circ$ ($c=0.9$, DMF); *Anal.* Calcd. for $C_{63}H_{80}O_{18}N_{20}$: C, 53.84; H, 5.74; N, 19.44. Found: C, 53.84; H, 5.86; N, 19.63.

H-Arg-Phe-Gly-Trp-Gly-Ala-Gly-Gly-Gln-Arg-OH (XVI)—The compound was prepared from XV (37 mg) essentially in the same manner as described for the preparation of V. CMC column (1.5 × 6 cm) chromatography was carried out using a linear gradient elution from H_2O (300 ml) in mixing chamber to 0.17M NH_4OAc buffer (pH 6.42, 300 ml) in reservoir. The eluates in tubes No. 35—44 were pooled and lyophilized repeatedly from H_2O to constant weight; colorless fluffy material; yield 26 mg (70%); mp 159—168° (decomp.); $[\alpha]_D^{18} + 3.6^\circ$ ($c=0.9$, H_2O); R_f 0.17 (A), 0.38 (B), single ninhydrin, Sakaguchi, Ehrlich and chlorine positive spot; amino acid ratios in the acid hydrolysate: Trp 0.56, Arg 2.21, Glu 0.93, Gly 3.84, Ala 0.82, Phe 0.96; amino acid ratios in the AP-M digest: Trp 1.11, Arg 2.32, Gln 0.94, Gly 4.15, Ala 0.88, Phe 1.01.

Z-Arg(NO_2)-Phe-Gly-Trp-Gly-Gly-Gly-Gly-Gln-Arg(NO_2)-OBzl (XVII) This compound was prepared from XI (110 mg) and VII (74 mg) essentially in the same manner as described for the preparation of IV. The product was recrystallized from DMF-EtOAc; amorphous powder; yield 60 mg (45%); mp

142—146°; $[\alpha]_D^{18} -16.0^\circ$ ($c=0.5$, DMF); *Anal.* Calcd. for $C_{62}H_{78}O_{18}N_{20} \cdot 4H_2O$: C, 50.88; H, 5.92; N, 19.14. Found: C, 51.11; H, 6.31; N, 18.98.

H-Arg-Phe-Gly-Trp-Gly-Gly-Gly-Gln-Arg-OH (XVIII)—The compound was prepared from XVII (60 mg) essentially in the same manner as described for the preparation of V. CMC column (1.5×10 cm) chromatography was carried out using a linear gradient elution from H_2O (300 ml) in mixing chamber to 0.15M NH_4OAc buffer (pH 6.42, 300 ml) in reservoir. The eluates in tubes No. 43—54 were pooled and lyophilized repeatedly from H_2O . Rechromatography on CMC gave homogeneous XVIII; colorless fluffy material; yield 32 mg (68%); $[\alpha]_D^{18} +5.6^\circ$ ($c=0.3$, H_2O); mp 152—163° (decomp.); *Rf* 0.12 (A), 0.42 (B), single ninhydrin, Sakaguchi, Ehrlich and chlorine positive spot; amino acid ratios in the acid hydrolysate: Trp 0.75, Arg 2.19, Glu 0.99, Gly 5.07, Phe 1.00; amino acid ratios in the AP-M digest: Trp 1.04, Arg 2.07, Glu 0.82, Gly 4.60, Phe 0.92.

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