SYNTHESIS AND PHYSICOCHEMICAL AND ENCEPHALITOGENIC PROPERTIES OF ANALOGS OF A TRYPTOPHAN-CONTAINING FRAGMENT OF MYELIN PROTEIN

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Studying the dependence of encephalitogenic activity on the structure of the nonapeptide
113 114 115 116 117 118 119 120 121
Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg — fragment 113-121 of the main protein of human myelin — we have shown that the replacement of the Glu-6 residue by glycine does not affect the encephalitogenic property, while the replacement of Ala-5 by glycine, as well, makes it inactive [4, 5]. Japanese workers have come to a similar conclusion concerning the necessity for alanine in position 5 [3]. It has also been found that the acetylation of the N-terminal phenylalanine of the synthetic nonapeptide does not affect its experimental allergic encephalitomyelitis (EAE) activity [4].

Synthetic investigations performed in a number of laboratories have been directed to the determination of the dependence of the EAE activity on the structure of the peptides [1-6], their possible use for the inhibition and treatment of EAE [7, 8], and the study of the connection between the cutaneous allergic reaction (CAR) of the synthetic peptides and their fragments and analogs, and their structures [6, 9]. The biological properties of the synthetic peptides and their analogs have been discussed in a review [10].

It is known that necessary amino acids for the appearance of EAE activity are Trp-115, Gln-120, and Arg(Lys)-121 [1], and also Ala-5 [3, 4]. In view of the fact that phenylalanine is not an essential amino acid and can be replaced by valine [1], it was difficult to explain the absence of activity in the synthetic peptide 114-121 [2], and also in the large pepsin fragment 114-132 of the myelin protein [11] lacking Phe-113. Our suggestion of the necessity for a positive charge on the amino group of the amino acid in position 1 was not justified, since the N-acetyl derivative of the nonapeptide was as active as the initial peptide [4]. On this basis, it might be assumed that the hydrophobicity of the N-terminal amino acid is important for the induction of the disease. To check this hypothesis, in the present communication we describe the synthesis and biological testing of analogs of the nonapeptide 113-121 with the replacement of phenylalanine by glycine (VIII, XII). We also describe shortened (XIV) and lengthened (XVI) analogs in which the distance between the essential amino acids has been changed. Since the replacement of glutamic acid by glycine does not affect activity [4], in this series of compounds we synthesized peptides with glycine on position 6.

The synthesis of these peptides was effected by the classical method of condensing fragments (Schemes 1 and 2). To block the amino groups we used benzyloxycarbonyl (Z) protection. The formation of the peptide bonds was performed by the azide and carbodiimide methods and also with the aid of activated esters — N-hydroxysuccinimide and p-nitrophenyl esters. The methyl, ethyl, and p-nitrobenzyl esters were used to protect the carboxy function. The complete deblocking of the protected peptides was brought about by hydrogenation over palladium, and purification after hydrogenation by ion-exchange chromatography on CM-cellulose.

Below we give the results of biological tests on guinea pigs. The encephalitogenic activity is expressed by the ratio of the number of animals with clinical symptoms of EAE to the total number of animals tested.

As can be seen from the facts given, the replacement of the hydrophobic phenylalanine by glycine or a change in the length of the encephalitogenic determinant by one amino-acid residue leads to the loss of EAE activity. Apparently, the receptors of the T-lymphocytes sensitized by peptides (VIII, XII, XIV, and XVI) are not complementary to the corresponding section

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Compound No.	Formula	Dose	EAE Activity
VIII	H-Gly-Ser-Trp-Gly-Ala-Gly-Gly-Gln-ArgOH	50	0/4
XII	H-Gly-Gly-Trp-Gly-Ala-Gly-Gly-Gln-ArgOH	50	0/4
XIV	H-Phe-Ser-Trp-Gly-Ala-[redacted]-Gly-Gln-ArgOH	50	0/4
XVI	H-Phe-Ser-Trp-Gly-Ala-$(Gly)_3$-Gln-ArgOH	50	0/4
T-27	H-Phe-Ser-Trp-Gly-Ala-Gly-Gly-Gln-ArgOH	50	3/4

113-121 of the main myelin protein which is subjected to immunological attack [1, 12]. The immunological specificity of the proteins and peptides is determined by their conformation [13], and therefore it was of interest to investigate the conformational lability of the peptides synthesized. We recorded their CD spectra in various solvents and at various temperatures. It follows from literature information on the CD spectra of various analogs of sections 112-121 and 113-121 in water and in 0.1 N hydrochloric acid [3] that they lack any regular structure whatever of the α-helix type or β structure. Information on the conformation of the protein itself is contradictory [14, 15]. It can only be stated that under the influence of sodium dodecyl sulfate (SDS) or 6 M quanidine hydrochloride the CD spectra of the protein change, while the temperature has little influence on the circular dichroism of the protein (0-80°C) [15].

We have investigated the CD spectra of the octapeptide (XIV), the nonapeptide T-27, and the decapeptide (XVI) in water, 1% SDS, and 8 M urea at temperatures of 25, 42, and 50°C. In all the curves (Fig. 1), in the 200-240 nm region there is one peak of dichroic absorption at 222-224 nm. The ellipticity of the peptides investigated in the region mentioned is due to the dichroic absorption of the peptide chromophore and of the chromophores of the tryptophan and phenylalanine side chains. It is known that in a nonpolar medium a "red" shift of the Cotton effect is characteristic for the $n\pi^*$ transition of a carbonyl group. As can be seen from Fig. 1, the CD spectra of the peptides no not change in the presence of 8 M urea and in 1% SDS. This is obviously due to the fact that the ellipticity of the compounds under investigation arises mainly from the contributions of the aromatic side chains of phenylalanine and tryptophan. The marked decrease in the dichroic absorption of peptide (VIII) is therefore due to the absence of phenylalanine and not to conformational changes.

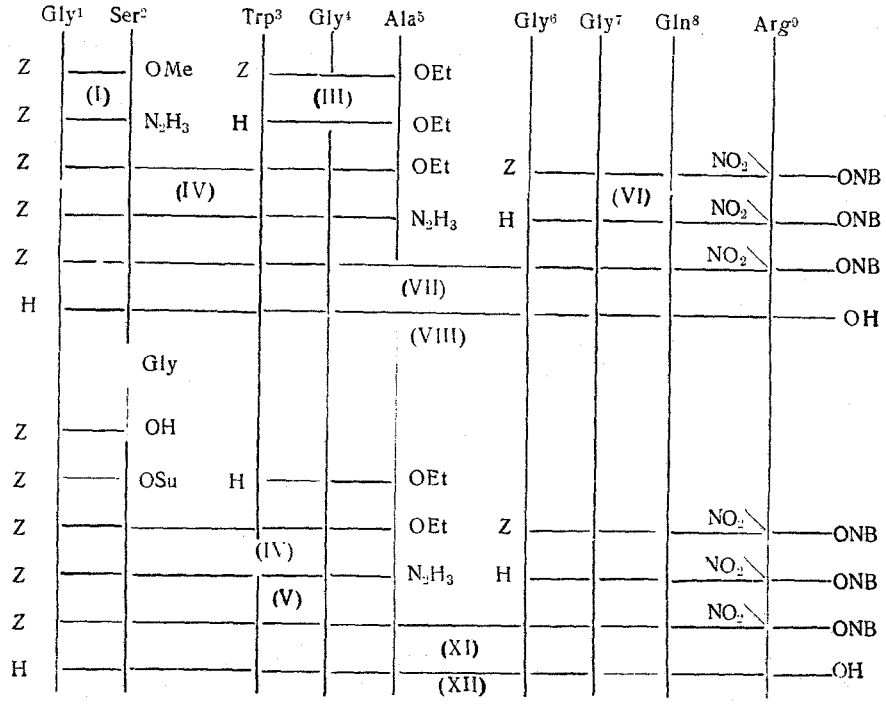
The CD spectra of the peptides under consideration in the 250-300 nm region are characterized by the presence of a small negative band at 290 nm which is connected with the presence of tryptophan. The dichroic absorption at 290 nm does not change when the solutions of the peptides are heated to 50°C.

EXPERIMENTAL

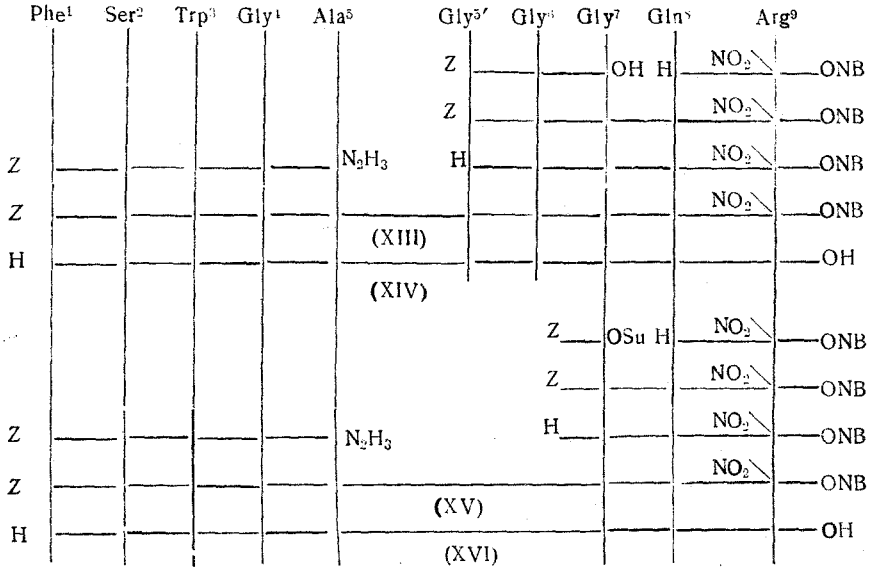
All the amino acids used were the L isomers. The melting points were determined in open capillaries and their uncorrected values are given. The homogeneity of the protected peptides was determined by thin-layer chromatography on Silufol plates in the following solvent systems: butan-1-ol—acetic acid—water (4:1:1, R_{f_1}) and butan-1-ol—acetic acid—pyridine—water (30:20:6:10, R_{f_2}). The chromatograms were revealed with iodine vapor or with Ehrlich's reagent. The homogeneity of the free peptides was confirmed by chromatography on paper in two solvent systems: butan-1-ol—acetic acid—water (4:1:5, R_{f_A}) and isomyl alcohol—pyridine—water (35:35:30, R_{f_B}). For high-voltage electrophoresis we used FN-17 paper (GDR). The chromatograms were revealed with the Ehrlich of Sakaguchi reagent or by the Rydon-Smith method. The amino-acid compositions of the peptides were determined on an AAA-881 amino-acid analyzer (Mikrotechnica, Prague).

The angles of rotation and the circular dichroism spectra were recorded on a "Spectropol-1" spectropolarimeter (Sofica, France) fitted with a special attachment for recording CD. The measurements were performed in a thermostated cell at 25°C with an optical path length of 0.1 cm. The curves were recorded 2-3 times at the rate of 5 nm/min. The CD results are shown in the form of molecular ellipticities. The results of the analyses of all the compounds corresponded to the calculated figures.

Methyl Ester of Benzyloxycarbonylglycylserine (I) [16]. A solution of 7 g (0.033 mole) of benzyloxycarbonylglycine and 4.6 ml (0.033 mole) of triethylamine in 30 ml of methylene chloride was treated with 2.48 g (0.033 mole) of serine methyl ester hydrochloride and was cooled to 0°C. Then 7 g (0.033 mole) of DCHC was added and the mixture was stirred at the



Scheme 1. Synthesis of glycine analogs of the nonapeptide T-27.



Scheme 2. Synthesis of the decapeptide (XVI) and the octapeptide (XIV).

same temperature for 1 h and was left at 10°C for 20 h. The dicyclohexylurea was filtered off, the solvent was distilled off, the residue was dissolved in ethyl acetate, and the solution was washed with 1 N hydrochloric acid, water, 1 N sodium bicarbonate, and 20% sodium chloride solutions and was dried over magnesium sulfate. After filtration and the distillation of the solvent, the product was crystallized by triturating it with petroleum ether. It was further purified by recrystallization from ethyl acetate—petroleum ether. Yield 8 g (80%), mp 97°C (literature information: mp 96°C).

Benzyloxycarbonylserine Hydrazide (II). A solution of 8 g (0.0258 mole) of (I) in 30 ml of methanol was treated with 5 ml of hydrazine hydrate and the mixture was kept at 20°C for 24 h. The precipitate that deposited was filtered off, washed with methanol and with ether, and dried in a vacuum desiccator over sulfuric acid. Yield 6 g (75%), mp 211-212°C. $[\alpha]_D^{25}$

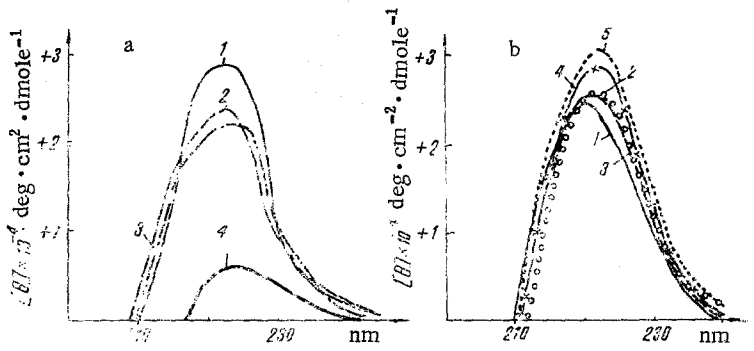


Fig. 1. CD spectra of the synthetic peptides: a) in water: 1) T-27; 2) (XIV); 3) (XVI); 4) (VIII); b) 1) (XIV) in water at 25°C; 2) (XIV) in water at 50°C; 3) (XIV) in 8 M urea; 4) T-27 in water; 5) T-27 in 1% SDS (in those cases where the temperatures are not shown, the measurements were performed at 25°C).

$-11°$ (c 1; acetic acid), R_{f_1} 0.53, R_{f_2} 0.64, $C_{13}H_{18}O_5N_4$.

Benzyloxycarbonylglycylseryltryptophanylglycylalanine Hydrazide (V). A solution of 4 g (0.0129 mole) of (II) and 20 ml of DMF was cooled to −20°C and 2.3 ml of a 2.4 N solution of hydrogen chloride in tetrahydrofuran and 1.46 ml (0.0129 mole) of tert-butyl nitrite were added. After stirring at −20°C for 30 min, the mixture was neutralized with triethylamine and a cooled solution of the amino component obtained by the hydrogenation of 7 g (0.014 mole) of the ethyl ester of benzyloxycarbonyltryptophanylglycylalanine [4] was added. The reaction mixture was stirred at −10°C for 1 h and was left at +10°C for 20 h. The salts were filtered off, the solvent was evaporated, the residue was dissolved in ethyl acetate, and the solution was washed in the usual way and was dried over magnesium sulfate. After the solvent had been distilled off, the residue could not be crystallized, and therefore the pentapeptide ester obtained was dissolved in methanol and was converted into the hydrazide as described for (II).

Yield 5 g (67%), mp 192-195°C, $[\alpha]_D^{25}$ − 19.8° (c 1; DMF), R_{f_1} 0.6, R_{f_2} 0.62; $C_{29}H_{36}O_8N_8 \cdot H_2O$.

p-Nitrobenzyl Ester of Benzyloxycarbonylglycylseryltryptophanylglycylalanylglycylglycylglutaminylnitroarginine (VII). The completely protected peptide (VII) was obtained from 2 g (0.0032 mole) of the hydrazide (V) and the amino component obtained by the deblocking of 3.2 g (0.0045 mole) of the p-nitrobenzyl ester of benzyloxycarbonylglycylglycylglutaminylnitroarginine [4] with hydrogen bromide in acetic acid. The fragments were linked by the azide method as described for (V). After the solvent had been distilled off, the residual oil was dissolved in 3 ml of DMF and this solution was poured with stirring into a cooled solution of 1 N hydrochloric acid. The precipitate that deposited was washed on the filter with water, with 1 N sodium bicarbonate solution, and with water again and was dried in a desiccator over phosphorus pentoxide. After recrystallization from ethanol, the yield was 1.7 g (45%), mp 160-170°C, $[\alpha]_D^{25}$ − 15° (c 1, DMF), R_{f_1} 0.41, R_{f_2} 0.43, $C_{51}H_{64}N_{16}O_{18}$.

Glycylseryltryptophanylglycylalanylglycylglycylglutaminylarginine (VIII). In the form of a suspension of 10 ml of a mixture of methanol, acetic acid, and water (6:1:1), 0.5 g of the protected peptide (VII) was hydrogenated over palladium for 16 h. The solvent was driven off in vacuum, the residue was dissolved in methanol, and the product was precipitated with ether. Yield 0.32 g (87%).

The peptide was purified by ion-exchange chromatography on CM-cellulose equilibrated with 0.05 M ammonium acetate buffer, pH 6. Elution was performed with a concentration gradient of this buffer from 0.05 to 0.5 M. The optical densities of the fractions were determined at 280 nm. The fraction containing the main substance was freeze-dried to constant weight. Yield 71%, mp 165-170°C, R_{f_A} 0.35, R_{f_B} 0.15, E_{Lys} 0.4 (pH 6.5), $[\alpha]_D^{25}$ − 22.9° (c 0.45, water). Amino-acid composition: alanine, 1.0; glutamic acid, 1.16; glycine, 4.25; serine, 0.94; arginine, 1.1.

Ethyl Ester of Benzyloxycarbonylglycylglycyltryptophanylglycylalanine (IX). A solution of 20 ml of DMF of the amino component obtained by hydrogenating 3.2 g (0.0065 M) of the protected tripeptide (III) [4] was added to a solution of 2.55 g (0.0061 M) of the N-hydroxysuccinimide ester of benzyloxycarbonylglycylglycine in 30 ml of DMF. The reaction mixture was stirred at 20°C for 1 h and was left at the same temperature for 20 h. The solvent was evaporated, the residue was dissolved in ethyl acetate, and the solution was washed and dried in the usual way. After distillation of the solvent, the residue was recrystallized from ethanol. Yield 2.0 g (55%), mp 158-162°C, $[\alpha]_D^{25} - 3.6°$ (c 0.5; ethyl acetate), R_{f_1} 0.6, R_{f_2} 0.61, $C_{30}H_{36}O_8N_6$.

Hydrazide of Benzyloxycarbonylglycylglycyltryptophanylglycylalanine (X). This was obtained by the treatment of 3 g (0.01 mole) of (IX) with 0.7 ml of hydrazine hydrate in methanol at 20°C for 20 h and was isolated in the same way as (II). The reaction product was crystallized from ethanol. Yield 2.4 g (83%), mp 200-202°C, $[\alpha]_D^{25} - 11.5°$ (c 1; DMF), R_{f_1} 0.43, R_{f_2} 0.52, $C_{28}H_{34}O_7N_8$.

p-Nitrobenzyl Ester of Benzyloxycarbonylglycylglycyltryptophanylglycylalanylglycylglycylglutaminylnitroarginine (XI). This peptide was obtained from 1.65 g (0.0023 mole) of (X) and 2.2 g (0.003 mole) of the protected peptide (VI) as described for (VII). Yield 1.5 g (50%), mp 142-151°C, $[\alpha]_D^{25} - 9.0°$ (c 1; DMF), R_{f_1} 0.5, R_{f_2} 0.58, $C_{50}H_{62}O_{17}N_{16} \cdot 4H_2O$.

Glycylglycyltryptophanylglycylalanylglycylglycylglutaminylarginine (XII). The peptide (XI) (0.8 g) was hydrogenated over palladium for 16 h. The deblocked peptide was isolated in a similar manner to (VIII). Yield 0.63 g (85%). It was purified on CM-cellulose. The yield after purification was 70%, mp 161-188°C, R_{f_A} 0.41, R_{f_B} 0.12, E_{Lys} 0.37 (pH 6.5). Amino-acid composition: glycine, 4.7; alanine, 1.26; glutamic acid, 1.02; arginine, 1.07.

p-Nitrobenzyl Ester of Phenylalanylseryltryptophanylglycylalanylglycylglutaminylnitroarginine (XIII). This compound was synthesized by the azide method similarly to (VII) from 0.73 g (0.001 mole) of the hydrazide of benzyloxycarbonylphenylalanylseryltryptophanylglycylalanine [4] and 0.7 g (0.001 mole) of the p-nitrobenzyl ester of benzyloxycarbonylglycylglutaminylnitroarginine [2] that had been deblocked with hydrogen bromide in acetic acid and neutralized with triethylamine to pH 8 in DMF solution. The product was isolated and purified similarly to (VII). Yield 0.72 g (60%), mp 104-115°C (from ethanol), $[\alpha]_D^{25} - 7.0°$ (c 1; DMF), R_{f_1} 0.56, R_{f_2} 0.62, $C_{56}H_{67}O_{17}N_{15} \cdot 1.5H_2O$.

Phenylalanylseryltryptophanylglycylalanylglycylglutaminylarginine (XIV). The protected peptide (XII) (1 g) was hydrogenated for 16 h and isolated in a similar manner to (VIII). Yield 0.8 g (75%). After purification on CM-cellulose, yield 60%, mp 141-151°C, $[\alpha]_D^{25} -$ 13.2° (c 0.41; water), R_{f_A} 0.33, R_{f_B} 0.11, E_{Lys} 0.37 (pH 6.5). Amino-acid composition: phenylalanine, 1.14; serine, 1.08; glycine, 1.77; alanine, 0.8; glutamic acid, 0.9; arginine, 1.14.

p-Nitrobenzyl Ester of Phenylalanylseryltryptophanylglycylalanyltriglycylglutaminylnitroarginine (XV). This was obtained by the azide method from 1.39 g (0.0029 mole) of benzyloxycarbonylphenylalanylseryltryptophanylglycylalanine [4] and 1.5 g (0.0019 mole) of the p-nitrobenzyl ester of benzyloxycarbonyltriglycylglutaminylnitroarginine [4] deblocked with hydrogen bromide as for (VII). Yield 1.5 g (60%), mp 160-167°C, $[\alpha]_D^{25} - 9.0°$ (c 1; DMF), R_{f_1} 0.61, R_{f_2} 0.7, $C_{60}H_{73}O_{19}N_{17} \cdot 3H_2O$.

Phenylalanylseryltryptophanylglycylalanyltriglycylglutaminylarginine (VI). The protected peptide (XV) (1 g) was hydrogenated over palladium for 14 h. The product was isolated in a similar manner to (VIII) with a yield of 0.7 g (80%). After purification on CM-cellulose, the yield was 70%, mp 110-135°C, $[\alpha]_D^{25} - 15.7°$)c 0.42; water), R_{f_A} 0.37, R_{f_B} 0.17, E_{Lys} 0.35 (pH 6.5). Amino-acid composition: phenylalanine, 1.25; serine, 1.1; glycine, 4.2; alanine, 1.25; glutamic acid, 1.1; arginine, 1.2.

SUMMARY

1. Analogs of the encephalitogenic nonapeptide with the substitution of glycine in the first and in the first and second positions and also with the deletion of the glutamic acid

residue and the insertion of a glycine residue between the fifth and sixth positions have been synthesized.

2. An investigation of the encephalitogenic activity of the compounds synthesized has shown the necessity for a hydrophobic N-terminal amino acid, since the replacement of phenylalanine by glycine leads to the inactivation of the compound. A change in the length of the encephalitogenic determinant also leads to the loss of EAE activity.

3. The CD spectra of the peptides synthesized are identical in water, a 1% solution of SDS, and an 8 M solution of urea and do not change on heating to 50°C. It is obvious that under the conditions mentioned these compounds do not possess an ordered structure of the α-helical type of a β structure, and their dichroic absorption is due to the contribution of the aromatic side chains.

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