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SYNTHESIS AND BIOLOGICAL PROPERTIES OF A TRYPTOPHAN-CONTAINING FRAGMENT OF A MYELIN PROTEIN AND ITS ANALOGS

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UDC 547.466.1

It has been shown previously [1] that the main protein of brain myelin when administered to animals with an adjuvant causes a disease which has been given the name of experimental allergic encephalomyelitis (EAE) and which may be a good model of some autoimmune diseases of man (disseminated sclerosis, etc.) [1, 2].

The complete amino-acid sequences of the main proteins of human and bovine myelins have been determined, and it has been shown that the hydrolysis of these proteins by proteases can give a series of fragments possessing EAE activity [1]. One such fragments is a peptide corresponding to sequence 111-121 of the main protein of myelin, and also the peptide 113-121:

$$Ser^{111}\text{-}Arg^{112}\text{-}Phe^{113}\text{-}Ser^{114}\text{-}Try^{115}\text{-}Gly^{116}\text{-}Ala^{117}\text{-}Glu^{118}\text{-}Gly^{119}\text{-}Gln^{120}\text{-}Arg^{121}\text{-}Pro^{122}\text{-}Gly^{123}.$$

Westall et al. [3] have synthesized a number of peptide analogs of fragment 111-121 by the solid-phase method and have shown that the amino-acid residues tryptophan-115, glutamin 120, and arginine-121 (or lysine-121) are essential for the appearance of EAE activity. Later, Japanese workers synthesized peptides 112-121 and 113-121, and also a number of analog of them and showed that the minimum peptide inducing EAE is the nonapeptide 113-121 [4, 5].

Carrying out a program of structural-functional investigations including the synthesis and physicochemical and biological study of encephalitogenic peptides, we have synthesized the nonapeptide 113-121 and one of its glycine analogs [6, 7].

In the present paper we give experimental details of the synthesis of these compounds and a number of other analogs of the nonapeptide 113-121. Scheme 1 shows the synthesis of

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the nonapeptide obtained by the azide condensation (in the Rudinger modification [8]) of two fragments: the N-terminal pentapeptide and the C-terminal tetrapeptide. The reaction of the γ-benzyl ester of benzyloxycarbonylglutamic acid with the tert-butyl ester of glycine in the presence of dicyclohexylcarbodiimide readily formed the protected dipeptide (III). Its treatment with TFA led to the dipeptide (V). The latter was condensed with the amino component (VI) by the carbodiimide method. We prepared the p-nitrobenzyl ester of benzyloxycarbonylnitroarginine required for the synthesis of (VI) by an improved method (in comparison with [9]), which halved the reaction time and raised the yield. The protected tetrapeptide (VIII) was the initial block for the synthesis of the nonapeptide.

The N-terminal pentapeptide was synthesized in the following way. The amino component obtained by the deblockage of the ethyl ester of benzyloxycarbonylglycylalanine with hydrogen bromide in acetic acid was condensed by the carbodiimide method with benzyloxycarbonyltryptophan. Hydrogenation of the compound obtained over palladium black formed the tripeptide (VIIa). The azide condensation of (VIIa) with the hydrazide of benzyloxycarbonylphenylalanylserine [9] led to the completely protected N-terminal block. Its conversion into the hydrazide (X) followed by condensation with the tetrapeptide (XI) gave the completely protected nonapeptide (XII). The hydrogenation of (XII) permitted the simultaneous elimination of all the protective groups and the production of the desired peptide (XIII).

When an electrophoretogram of the peptide (XIII) was treated with Ehrlich's reagent, in addition to the main product, a yellow material was observed. According to the literature [4], this coloration shows the presence of 2,3-dihydrotryptophan. Furthermore, material more polar than the peptide (XIII) was detected on the electrophoretogram. In contrast to the peptide (XIII) it did not give a color with Ehrlich's reagent and possessed a different luminescence in ultraviolet light. It is possible that this material includes products of the oxidation of tryptophan, and therefore to protect the tryptophan residue sometimes 5% of indole was added to the reaction mixture on hydrogenation.

The encephalitogenic activities of the synthetic nonapeptides were as follows:

Number of the Peptide	Peptide	Dose, μg	Encephalitogenic activity
XIII	H-Phe-Ser-Try-Gly-Ala-Glu-Gly-Cln-ArgOH	50	3/4
XIV	CH_3CO-Phe-Ser-Try-Gly-Ala-Glu-Gly-Gln-ArgOH	50	3/4
XXIII	H-Phe-Ser-Try-Gly-Ala-Gly-Gly-Gln-ArgOH	50	2/4
XXVII	H-Phe-Ser-Try-Gly-Gly-Gly-Gly-Gln-ArgOH	50	0/4
	Main protein of myelin	50	4/4

Note. The encephalitogenic activity is expressed by the ratio of the number of diseased guinea-pigs to the total number of animals tested. The clinical symptoms of EAE appeared 12-18 days after the administration of the encephalitogenic materials. The tests were performed on guinea-pigs weighing 400-500 g by the method of Kozulina et al. [10].

As the facts given above show, the synthetic nonapeptide (XIII) possesses a high EAE activity.

The decapeptide with the sequence 112-121 (see formula 1) causes a more rapid development of the disease than the nonapeptide 113-121 [4]. This leads to the idea that the side chain of arginine-112, bearing a positive charge, may play a decisive role in the antigenic determinant (in addition to the essential tryptophan, glutamine, and lysine residues).

Taking this into account, the activity of the nonapeptide 113-121 could be explained by the assumption that the function of the charged guanidine grouping of the arginine-112 residue in the nonapeptide is played by the protonated NH_2 group of phenylalanine-113. In order to test this hypothesis, we synthesized the N-acetyl derivative of the peptide (XIII). Acylation was performed in an aqueous medium with N-acetoxysuccinimide. The acetyl derivative obtained (XIX) possessed EAE activity, which indicates the nonparticipation of the NH_2 terminal group of phenylalanine in the induction of the disease. This conclusion is valid only if no enzymatic deacetylation of the peptide (XIV) takes place in the animal organism.

Considerable interest is presented by the synthesis of glycine analogs of the nonapeptide 113-121. This is due to the fact that the introduction of glycine in place of any am-

TABLE 1. Protected Peptides*

Compound	Method of synthesis	Yield, %	mp, °C	$[\alpha]_D^{25}$ (c=1)	R_{f_1}	R_{f_2}	Found, %	Formula	Calculated, %
Z-Glu (OBzl)-Gly·OBu^t	A(CH_2Cl_2)	83	60–70 ‡	−3,9 (ethyl acetate)	0,75	0,78	C 65,10 H 6,70 N 5,75	$C_{26}H_{32}N_2O_7$	C 64,45 H 6,66 N 5,78
Z-Glu (OBzl)-Gly-OH· DCHA	D(TFA)	90	148–151 (water)	−5,5 (ethanol)	0,68	0,58	C 67,28 H 7,64 N 6,93	$C_{34}H_{47}N_3O_7$	C 67,00 H 7,77 N 6,93
Z-Try-Gly-Ala-OEt	A (DMF)	92	135–136 ‡	−2,7 (ethyl ecetate)	0,76	0,72	C 63,54 H 6,01 N 11,43	$C_{26}H_{30}N_4O_6$	C 63,25 H 6,07 N 11,32
Z-Glu (OBzl)-Gly-Gln-(NO_2) Arg-ONB	A (DMF)	80	142–145 (ethanol)	−7,0 (DMF)	0,55	0,75	C 52,89 H 5,74 N 15,13	$C_{40}H_{48}N_{10}O_{14}\cdot H_2O$	C 52,74 H 5,53 N 15,38
Z-Phe-Ser-Try-Gly-Ala-OEt	C(DMF–THF)	65	143–151 (ethanol–water)	−18,8 (ethanol)	0,63	0,65	C 61,92 H 6,20 N 10,85	$C_{38}H_{44}N_6O_9$	C 62,49 H 6,09 N 11,52
Z-Phe-Ser-Try-Gly-Ala-$NHNH_2$	B (methanol)	88	225–226 (DMF–water)	−9,5 (DMF)	0,48	0,64	C 59,08 H 5,70 N 14,97	$C_{36}H_{42}N_8O_8\cdot H_2O$	C 58,94 H 5,77 N 15,28
Z Gly-Gly-Gln-(NO_2) Arg-ONB	A (DMF)	69	126–128 (ethanol)	−8,0 (DMF)	0,53	0,78	C 49,43 H 5,78 N 18,65	$C_{30}H_{39}N_{10}O_{12}$	C 49,24 H 5,37 N 19,14

Z-Phe-Ser-Try-Gly-OH	E (methanol)	71	135—145 (ethanol–acetate)	−21,0 (ethanol)	0,77	0,62	C 63,67 H 6,25 N 11,29	$C_{33}H_{36}N_5O_9$	C 63,12 H 5,76 N 11,15
Z-Gly-Gly-Gly-Gln-(NO_2) Arg-ONB	A (DMF)	70	130—140 (ethanol)	−8,8 (DMF)	0,50	0,70	C 58,83 H 5,48 N 19,37	$C_{32}H_{42}N_{11}O_{13}$	C 48,79 H 5,37 N 19,56
Z-Phe-Ser-Try-Gly-Ala-Glu (OBzl)-Gly-Gln-(NO_2) ArgONB †	C (DMF)	50	200—210 (ethanol)	−16,5 (acetic acid)	0,54	0,75	C 55,44 H 5,65 N 14,89	$C_{68}H_{80}N_{16}O_{20}\cdot 2H_2O$	C 55,28 H 5,73 N 15,17
Z-Phe-Ser-Try-Gly-Ala-Gly-Gly-Gln-(NO_2) ArgONB **	C (DMF)	50	—	—	—	—	—	$C_{58}H_{70}N_{16}O_{18}$	—
Z-Phe-Ser-Try-Gly-Gly-Gly-Gly-Gln-(NO_2) ArgONB	A (DMF)	70	160—170 (ethanol)	−10,5 (DMF)	0,49	0,71	C 57,55 H 5,48 N 16,97	$C_{67}H_{68}N_{16}O_{18}$	C 57,32 H 5,49 N 17,28

*Abbreviations used: ONp) p-nitrophenyl ester; ONB) p-nitrobenzyl ester; OBzl) benzyl ester; $-OBu^t$) tert-butyl ester; DMF) dimethylformamide; THF) tetrahydrofuran; TFA) trifluoroacetic acid; DCHA) dicyclohexylamine.

†Literature data [3]: mp 165-175°C, $[\alpha]_D^{25}$ − 14.0 (c 1, CH_3COOH).

††Crystallized from a mixture of ethyl acetate and petroleum ether.

**Not isolated in the analytically pure state.

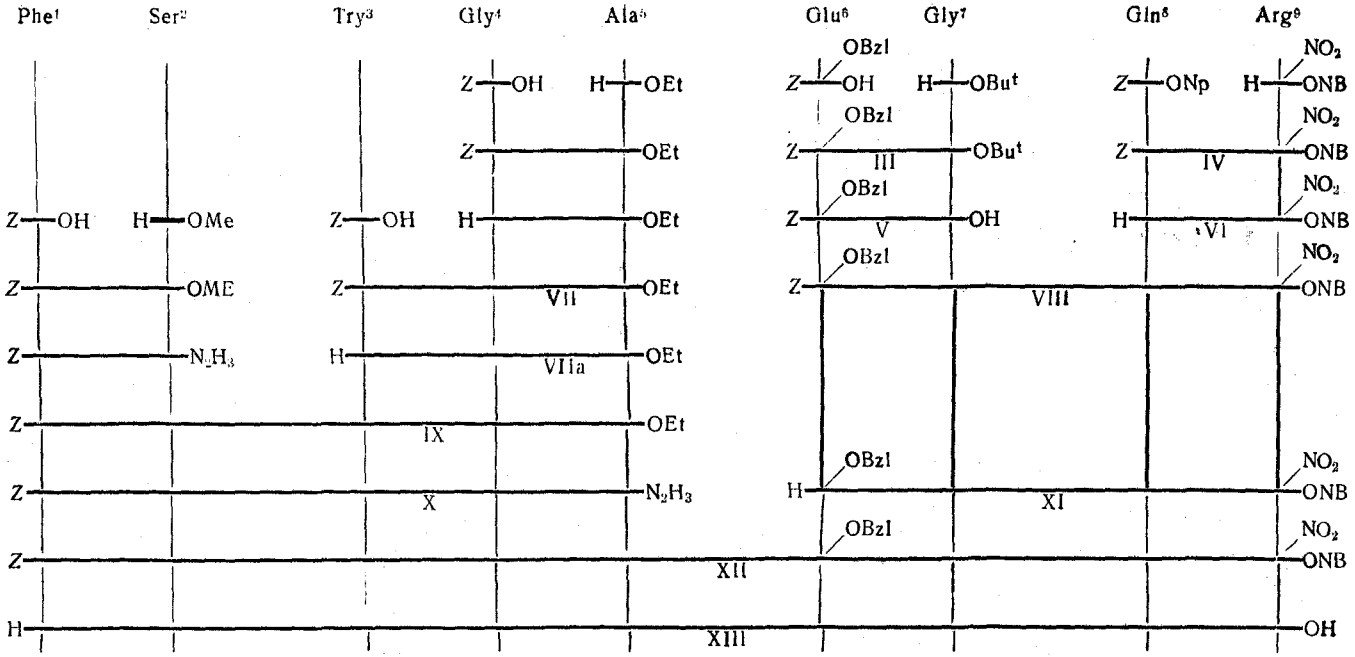
TABLE 2. Free Peptides

Compound	Yield, %		mp, °C	Method of purification	$[\alpha]_D^{25}$	Mobility, E	R_{fA}	R_{fB}
	before purification	after purification stage						
H-Phe-Ser-Try-Gly-Ala-Glu-Gly-Gln-ArgOH*	60	60	155—164	Electrophoresis pH 1,7	—30,0 (c=1, water)	0,33 (Lys) pH 3,5	0,18	0,21
CH_3CO-Phe-Ser-Try-Gly-Ala-Glu-Gly-Gln-ArgOH	—	30	—	Electrophoresis pH 6,5	—	0,14 (Glu) pH 6,5	0,33	0,08
H-Phe-Ser-Try-Gly-Ala-Gly-Gly-Gln-ArgOH	50	80	130—153	Electrophoresis pH 6,5	—9,1 (c=1, water)	0,4 (Lys) pH 3,5	0,79	0,83
H-Phe-Ser-Try-Gly-Gly-Gly-Gly-Gln-ArgOH	60	76	148—160	CM-cellulose pH 6,0	—6,3 (c=0,3 water)	0,4 (Lys) pH 3,5	0,37	0,12

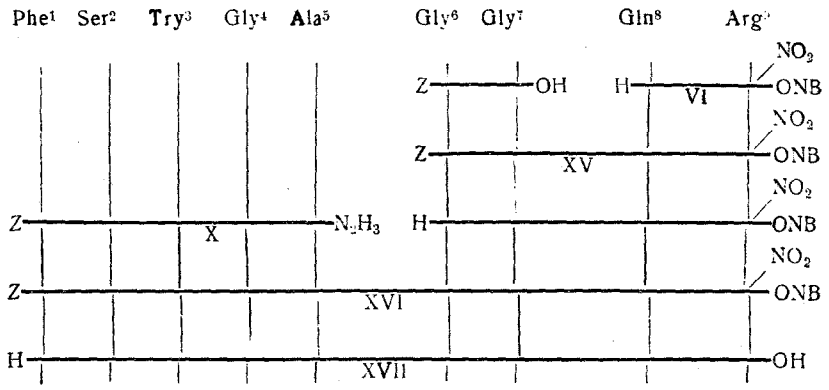
Compound	Found, %	Formula	Calculated, %	Amino-acid composition†					
				Phe	Ser	Gly	Ala	Glu	Arg
H-Phe-Ser-Try-Gly-Ala-Glu-Gly-Gln-ArgOH*	C 46,73 H 7,25 N 15,72	$C_{46}H_{63}N_{14}O_{14}\cdot CH_3COOH\cdot 7H_2O$	C 47,02, H 6,70, N 16,00	1,1	0,8	1,7	1,0	2,0	0,8
CH_3CO-Phe-Ser-Try-Gly-Ala-Glu-Gly-Gln-ArgOH	—	—	—	1,1	1,0	1,9	1,0	2,2	1,1
H-Phe-Ser-Try-Gly-Ala-Gly-Gly-Gln-ArgOH	C 48,12 H 7,07 N 16,30	$C_{43}H_{60}N_{14}O_{12}\cdot 2CH_3COOH\cdot 5H_2O$	C 48,08, H 6,61, N 16,70	1,0	0,98	3,4	1,18	1,07	0,89
H-Phe-Ser-Try-Gly-Gly-Gly-Gly-Gln-ArgOH	C 48,91 H 6,00 N 17,55	$C_{42}H_{57}N_{14}O_{12}\cdot 2CH_3OH\cdot 3H_2O$	C 49,15, H 6,55, N 17,44	1,0	0,6	4,3	—	1,1	0,8

*Literature data [3]: mp 147-156°C, $[\alpha]_D^{25}$ — 47.2 (c 1; water).

†The presence of Try was determined spectroscopically and by means of Ehrlich's reagent.



Scheme 1. Synthesis of an encephalitogenic nonapeptide.



Scheme 2. Synthesis of the 6-lysine analog.

ino acid leads to the elimination of a side chain. Furthermore, the glycine analogs of peptides possess greater conformational mobility. Since the opinion exists that the serologic specificity of the antigenic determinant depends on the tertiary structure, the synthesis of peptides with a modified conformation is important for studying this question.

The 6-glycine analog of the peptide (XIII) was synthesized by the azide condensation of the N-terminal pentapeptide and the corresponding C-terminal tetrapeptide (XVa) (Scheme 2). The fully protected tetrapeptide (IV) was obtained by the carbodiimide method from benzyloxycarbonylglycylglycine and the dipeptide (VI). After the removal of the N-protection with hydrogen bromide in acetic acid, the amino component (XVa) was subjected to azide condensation with the pentapeptide (X), and the protected peptide (XVI) was obtained; hydrogenation of the latter over palladium black yielded the required analog (XVII). Biological tests showed that the elimination of the side chain of the 6-glutamic acid does not affect the EAE activity of the compound. Another glycine analog of the peptide (XIII), namely a peptide containing glycine residues in place of the alanine-5 and glutamic acid-6 residues — the peptide (XXVII) — was synthesized in accordance with Scheme 3.

Peptides (XIII), (XIV), (XXIII), and (XXVII) were purified by high-voltage electrophoresis on paper at pH 1.7 or 3.5 or by ion-exchange chromatography on CM-cellulose.

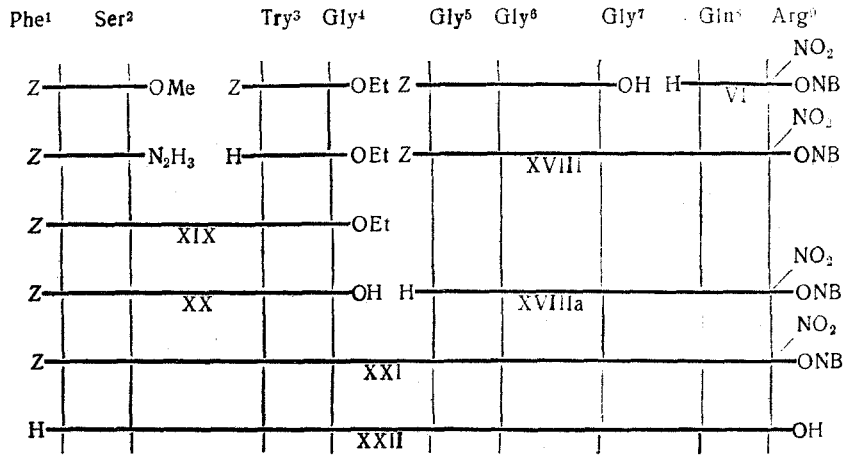
To investigate the characteristics of the conformation of the encephalitogenic nonapeptide we recorded the circular dichroism (CD) spectrum of this compound in aqueous solution. Analysis of the curve obtained (Fig. 1) permits the conclusion that the peptide (XIII) does not possess a regular structure of the α-helix type or the β structure in aqueous solution.

EXPERIMENTAL

The initial amino acids were the L isomers. The melting points were determined in open capillaries and are uncorrected. The homogeneity of the protected peptides was determined by thin-layer chromatography on "silufol" plates in the following solvent systems: 1-butanol—acetic acid—water (4:1:1) (R_{f_1}), and 1-butanol—pyridine—acetic acid—water (30:20:6:1) (R_{f_2}). The chromatograms were revealed with iodine and with Ehrlich's reagent. The homogeneity of the free peptides was checked by chromatography on paper in the following two solvent systems: 1-butanol—acetic acid—water (4:1:5) (R_{fA}), and isoamyl alcohol—pyridine—water (35:35:30) (R_{fB}). Filtrak FN-17 paper (GDR) was used.

The chromatograms were stained with the Ehrlich or the Sakaguchi reagent or by the Raidon—Smith method. The amino-acid compositions of acid hydrolyzates of the peptides were determined on an AAA-881 analyzer (Mikrotechna, Prague). The angles of rotation and the circular dichroism spectra were recorded on a "Spektrapol-1" spectropolarimeter (Sofica, France fitted with a special attachment for recording CD. The measurements were performed in a cell thermostatted at 25°C with a path for the beam of light of 0.1 cm. The concentration of the peptides was 0.25 mg/ml. The curves were recorded two or three times at the rate of 5 nm/min. The CD results are presented in the form of molecular ellipticities.

p-Nitrobenzyl Ester of Benzyloxycarbonylnitroarginine. A mixture of 15.5 g (44 mmole) of benzyloxycarbonylnitroarginine, 9.3 g (71 mmole) of triethylamine, 14.3 g (71 mmole) of p-nitrobenzyl bromide, and 0.5 g of sodium iodide was boiled in 70 ml of dry acetone under



Scheme 3. Synthesis of the 5-Gly,6-Gly analog.

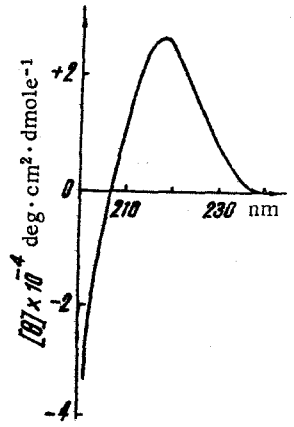


Fig. 1. Circular dichroism of the nonapeptide.

reflux for 7 h. The salts were filtered off, the solvent was evaporated off, and the residue was mixed with 80 ml of ethyl acetate and 20 ml of water, was washed in a separating funnel with 1 N hydrochloric acid, 1 N sodium bicarbonate, and water, and was dried over magnesium sulfate. The solvent was distilled off, the residue was dissolved in the minimum amount of hot ethyl acetate, and the solution was left in the refrigerator. The crystals that deposited were filtered off. Yield 15 g (70%), mp 118°C, $[\alpha]^{25}$ — 9.0 [c, 1; DMF]. Literature information [7]: mp 122°C (corr.), $[\alpha]^{25}$ —9.0 ± 1.0 (c 1; DMF).

Ethyl Ester of Benzyloxycarbonyltryptophanylglycylalanine (VII). Method A. A solution of 7.0 g (29 mmole) of the hydrobromide of the ethyl ester of glycyclalanine (obtained by the treatment of 10 g of benzyloxycarbonylglycylalanine [11] with hydrogen bromide in acetic acid for 40 min) in 10 ml of DMF was cooled, and triethylamine was added to pH 8. The salt that deposited was filtered off and the filtrate was treated with a solution of 9 g (27 mmole) of benzyloxycarbonyltryptophan in a mixture of 20 ml of DMF and 10 ml of methylene chloride. With stirring at 0°C, 5.7 g (27 mmole) of dicyclohexylcarbodiimide was added and the resulting mixture was kept at 0°C for 1 h and was left overnight in the refrigerator. The dicyclohexylurea was filtered off, the filtrate was evaporated, the residue was dissolved in ethyl acetate, and the solution was washed with hydrochloric acid, 1 N sodium bicarbonate, and water, and was dried over magnesium sulfate. The solvent was evaporated off, the residue was triturated with petroleum ether until it solidified, and it was filtered off, dried, and crystallized from a mixture of ethyl acetate and petroleum ether.

Hydrazide of Benzyloxycarbonylphenylalanylseryltryptophanylglycylalanine (X). Method B. A solution of 6.5 g (9 mmole) of the ester (IX) in 30 ml of methanol was treated with 1 ml (20 mmole) of hydrazine hydrate and the mixture was kept at room temperature for 24 h.

The precipitate was filtered off, washed with methanol, and dried in a desiccator over sulfuric acid.

Ethyl Ester of Benzyloxycarbonylphenylalanylseryltryptophanylglycylalanine (IX). Method C. A solution of 80 mmole of hydrogen chloride in tetrahydrofuran (THF) and 2.3 ml (20 mmole) of tert-butyl nitrite were added to a solution of 7.8 g (19.5 mmole) of the hydrazide of benzyloxycarbonylphenylalanylserine [9] in 20 ml of DMF cooled to −20°C. The mixture was stirred at −20°C for 30 min, and then 11 ml (80 mmole) of triethylamine and a solution of the amino component obtained by hydrogenating 9.8 g (20 mmole) of the protected tripeptide (VII) in a mixture of methanol, acetic acid, and water (6:1:1) over palladium for 6 h were added. The solution was stirred at −10°C for 1 h and was left overnight in the refrigerator. The salts were filtered off, the solvent was evaporated off in vacuum, the residue was dissolved in ethyl acetate, and the solution was washed with acid, sodium carbonate solution, and water, and dried over magnesium sulfate. After the solvent had been distilled off, the oil was treated with petroleum ether and the resulting precipitate was filtered off, dried, and crystallized from aqueous ethanol.

Benzyloxycarbonyl-γ-benzylglutamylglycine (V). Method D. A solution of 8 g of the protected peptide (III) in 20 ml of TFA was kept at room temperature for 30 min. Then the solvent was distilled off and the residue was kept in vacuum over caustic potash, after which it was dissolved in ether, a slight excess of dicyclohexylamine was added, and the mixture was left in the refrigerator for a day. The salt of the peptide (V) that precipitated was filtered off, washed with ether, and crystallized from water.

Benzyloxycarbonylphenylalanylseryltryptophanylglycine (XX). Method E. A suspension of 2.3 g (3.5 mmole) of the peptide ester (XIX) in 25 ml of methanol, 2.5 ml of 2 N caustic soda solution was added, and the mixture was stirred at room temperature for 1.5 h. Then the methanol was distilled off, the residue was dissolved in water, and the solution was cooled and was acidified with 1 N hydrochloric acid. The product was extracted with ethyl acetate, washed with water to neutrality, and dried over magnesium sulfate. The solvent was distilled off, the residue was treated with petroleum ether, and the precipitate was filtered off and crystallized from a mixture of ethyl acetate and petroleum ether.

Isolation and Purification of the Protected Nonapeptide (XIII), (XXIII), and (XXVII). In view of the poor solubility of the protected nonapeptides, they were purified in the following way. After synthesis, the solvent (DMF) was evaporated to small volume and poured with stirring into a cooled solution of 1 N hydrochloric acid. The precipitate that deposited was filtered off and washed on the filter with water, sodium carbonate solution, and water again. Then it was crystallized from a large volume of ethanol. This gave a colorless or yellowish amorphous powder.

N-Acetyl Derivative of the Encephalitogenic Nonapeptide (XIV). A solution of 16 mg of N-acetoxysuccinimide in 1 ml of water was treated with 9 mg of the peptide (XIII), the pH was brought to 9.0 with N-ethylmorpholine, and the mixture was kept at room temperature for 20 h. Then it was acidified with acetic acid and evaporated to dryness, and the residue was triturated with hot ethyl acetate and was then dissolved in 1 ml of water and freeze-dried. The compound obtained was purified by electrophoresis on GP-17 paper at a voltage gradient of 60 V/cm, pH 6.5, for 2 h. The material was eluted from the paper with a 0.1 N solution of ammonia and was freeze-dried. Yield 3 mg.

Deblocking, Isolation, and Purification of the Nonapeptides. A completely protected nonapeptide (0.5 g) was hydrogenated over platinum black in a mixture of methanol, acetic acid, and water (6:1:1) for 16 h, the degree of elimination of the protective groups being monitored by paper electrophoresis. After the end of the reaction, the catalyst was filtered off, the solvent was distilled off in vacuum in a rotary evaporator, and the residue was triturated with methanol. The peptide was purified by electrophoresis on FN-17 paper with a voltage gradient of 70 V/cm at pH 1.7 for 1.5 h. After the staining of guiding strips with ninhydrin and the Ehrlich and Sakaguchi reagents, the material giving positive reactions with all these reagents was eluted with 10% acetic acid and freeze-dried. Purification on CM-cellulose was performed on a column 20 × 1.8 cm equilibrated with 0.05 M ammonium acetate buffer, pH 6.0. Elution was performed with a concentration gradient of this buffer from 0.05 to 0.5 M at the rate of 60 ml/h.

SUMMARY

1. By the azide condensation of fragments followed by the hydrogenolysis of the completely protected nonapeptides formed, the encephalitogenic nonapeptides with the structure Phe-Ser-Try-Gly-Ala-Glu-Gly-Gln-Arc and two of its analogs — the 6-glycine and 5,6-diglycine compounds, have been synthesized.

2. It has been shown that the 6-glycine analog induces EAE, i.e., the side chain in position 6 is not essential for the appearance of EAE activity.

3. The absence of an encephalitogenic effect in the 5,6-diglycine analog permits the conclusion that the side chain of the alanine-5 makes a contribution to the induction of the disease.

4. The N-acetyl derivative of the encephalitogenic nonapeptide retains a high EAE activity.

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