SYNTHESIS OF BLOOD SERUM THYMIC FACTOR AND ITS ANALOGS

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The blood serum thymic factor (FTS) and its two analogs (desGlp[1])-FTS and (Gln[1])-FTS were synthesized by a novel procedure. The biological activity of these compounds was assayed by the E-rosette test. The activity of (desGlp[1])-FTS is decreased and (Gln[1])-FTS shows an inhibitory effect.

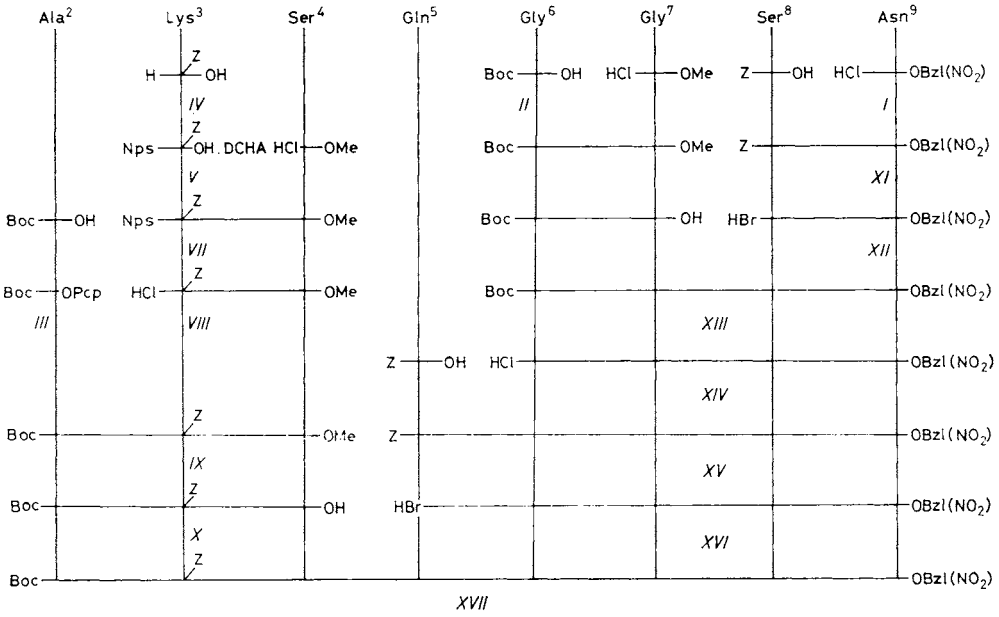
Thymus produces a great number of peptide hormones affecting the multiplication, development, maturation, and differentiation of T-lymphocytes in the thymus and their behavior outside the gland[1,2]. The hormone responsible for the activation of T-lymphocytes[3], the blood serum thymus factor (FTS) discovered and isolated by Bach and coworkers[4,5] is a nonapeptide*

$$\overset{1}{\text{Glp}}\text{-}\overset{2}{\text{Ala}}\text{-}\overset{3}{\text{Lys}}\text{-}\overset{4}{\text{Ser}}\text{-}\overset{5}{\text{Gln}}\text{-}\overset{6}{\text{Gly}}\text{-}\overset{7}{\text{Gly}}\text{-}\overset{8}{\text{Ser}}\text{-}\overset{9}{\text{Asn}}$$

Its amino acid sequence has been determined[8] and confirmed by synthesis[9,10]. A series of papers has been devoted to the biological response[11,12] brought about by synthetic fragments of FTS. The elimination of the pyroglutamic residue in position 1 has no effect on the activity of the peptide[13]. Additional eliminations of amino acid residues at the C- and N-terminus result in an activity decrease[13]. The smallest active part of the peptide has been identified[13] with pentapeptide Lys-Ser-Gln-Gly-Gly. Except for glycine in position 7 all the amino acid residues were substituted[14–16]. The substitution of the residue in position 1 is without any effect on the biological activity of the peptide[17,18]. A prolonged effect has been observed in some cases[18]. The peptide retains its full biological activity even after the replacement of the residue in position 3 by D-lysine or homoarginine and of the residue in position 5 by glutamic acid[18]. Substitutions of residues in other positions lead to a decrease of biological activity paralleled by protracted action in some cases[18].

* All amino acids with the exception of glycine are of L-configuration unless stated otherwise. The nomenclature and symbols for compounds follow the suggestions of the IUPAC-IUB Commission for biochemical nomenclature[6,7]. Glp = pyroglutamic acid.

This study was undertaken in an effort to reevaluate the importance of position 1 either by elimination of the pyroglutamic residue or by its replacement by a glutamine residue. Our plans were based on the known fact[19] that the pyroglutamic acid residue is irreplaceable by a glutamine residue, *e.g.* in TRF and also that the N-terminal pyroglutamic acid cannot be substituted[20] by other amino acid residues without a simultaneous decrease of activity as, *e.g.* in LRF.

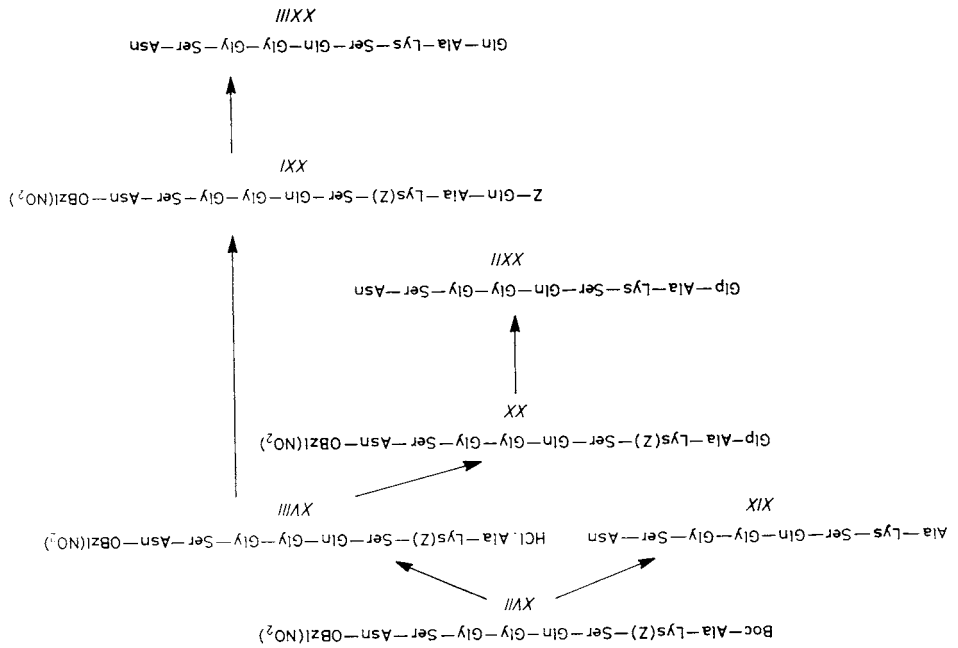


SCHEME 1

The procedure of the synthesis of FTS and of its two analogs, *i.e.* of (desGlp[1])-FTS and of (Gln[1])-FTS is shown in Schemes 1 and 2. We started the synthesis with protected octapeptide *XVII* which represented a common intermediary product for the preparation of final products *XIX*, *XXII*, and *XXIII*. Intermediate *XVII* was prepared by condensation of tripeptide *X* with pentapeptide *XVI* by the carbodiimide method in the presence of 1-hydroxybenztriazole[21].

The biological activities of FTS and its analogs were examined by the recovery assay of T-lymphocytes[22]. By this assay the increase is determined of the number of certain receptors for sheep erythrocytes on the surface of T-lymphocytes after their preceding treatment with trypsin. Using this assay we have been able to show that the analog with N-terminal glutamine in position 1, *i.e.* (Gln[1])-FTS has an inhibitory power. According to recorded data, however, some authors[17,23] using

the method of total E-rosettes[24] or of active E-rosettes[25] were unable to show similar differences between FTS and (Gln[1])-FTS. Neither was a difference in the effect of these products demonstrated by Tsukamoto and coworkers[26] who compared the action of FTS and its analogs by using the assay based on an inflammatory reaction of the late hypersensitivity type in mice. We decided therefore to assay



SCHEME 2

the FTS and its analogs by a biologically more sensitive test using trypsin-treated lymphocytes[22]. The principle of the method is based on the ability of the peripheral blood T-lymphocyte to restore the cell membrane receptor for sheep red blood cells. The treatment of T-lymphocytes with trypsin leads to the loss of this receptor; spontaneous restoration of this receptor can be enhanced by some immunomodulators. The difference of 5% between the newly formed E-rosettes in the sample cultivated with the immunomodulator and in the sample without the active substance can be regarded as a reliable sign of the stimulating effect. The determined biological activities of the FTS and its analogs are given in Table I also showing the thymosine activity of fraction V (isolated from calf thymus) for reasons of comparison.

EXPERIMENTAL

The melting points were determined in a Kofler block and were not corrected. The optical rotations were measured in a Perkin–Elmer 141 polarimeter. The samples for analysis were dried *in vacuo* at 130 Pa over phosphorus pentoxide at room temperature (products with melting points below 115°C) or at 105°C (products with melting points higher than 115°C). Thin-layer chromatography was performed on silica gel (Kieselgel G, Merck) in the systems butanol–acetic acid–water (4 : 1 : 1) (S_1), butanol–acetic acid–pyridine–water (15 : 3 : 10 : 6) (S_2), and 2-propanol–water (2 : 1) (S_3). Electrophoresis was effected in the same silica gel layer at a potential gradient of 38 V/cm in the buffer pyridine–acetic acid, pH 5·0 (E_1). Free amino acids and peptides were stained with ninhydrin, the protected compounds were detected by chlorination and 2-tollidine. Standard procedure *A* involves the dissolving of the reaction mixture, washing with 1% citric acid, 5% sodium bicarbonate, water, drying over anhydrous sodium sulfate and evaporation. Standard procedure *B* involves the dissolving of the reaction mixture, washing with 1 mol l^{-1} hydrochloric acid, water, 5% sodium bicarbonate, water, drying over anhydrous sodium sulfate and evaporation. Standard procedure *C* involves trituration of the dry residue of the reaction mixture with 1 mol l^{-1} hydrochloric acid, water, 5% sodium bicarbonate, water and drying over phosphorus pentoxide in a desiccator. The values of weight percent are given. The solutions were evaporated *in vacuo* in a rotary vacuum evaporator. The hydrochlorides of amino acid methyl esters were prepared by esterification according to Brenner[27], the N^{α}-benzyloxycarbonyl-amino acids were prepared according to Bergman and Zervas[28].

Starting Material

Asparagine 4-nitrobenzyl ester hydrochloride (*I*) was prepared according to Maclaren[29] in a yield of 60%. M.p. 188–189°C (ref.[29] m.p. 182·5–184°C). Tert.-butyloxycarbonylglycine (*II*) was prepared according to Moroder and coworkers[30] in a yield of 73%. M.p. 85–85°C (ref.[31] m.p. 88·5–89°C). Tert.-butyloxycarbonylalanine pentachlorophenyl ester (*III*) was prepared according to Kovacs and coworkers[32] in a yield of 58%. M.p. 166–168°C (ref.[33] m.p. 166°C). N^{ε}-Benzyloxycarbonyllysine (*IV*) was prepared according to Neuberger and Sanger[34] in a yield of 86%. M.p. 261–263°C (ref.[35] m.p. 262°C). N^{α}-2-Nitrobenzenesulfenyl-N^{ε}-benzyloxycarbonyl-lysine dicyclohexylammonium salt (*V*) was prepared according to Zervas and coworkers[36] in a yield of 55%. M.p. 178–181°C (ref.[36] m.p. 184–187°C). Pyroglutamic acid (*VI*) was prepared according to Beecham[37] in a yield of 60%. M.p. 157–160°C (ref.[37] m.p. 159–160·5°C).

TABLE I
Results of E-rosette test

Compound	Difference in number of newly formed rosettes (%)
FTS (*XXII*)	9·20
(desGlp[1])-FTS (*XIX*)	3·18
(Gln[1])-FTS (*XXIII*)	−1·64
Thymosin fraction V	3·80

N$^{\alpha}$-2-Nitrobenzenesulfenyl-N$^{\varepsilon}$-benzyloxycarbonyllysyl-serine Methyl Ester (*VII*)

The solution of compound *V* (18·4 g; 30 mmol) and serine methyl ester hydrochloride (4·6 g; 30 mmol) in dimethylformamide (200 ml) was cooled down to 0°C and treated with N,N'-dicyclohexylcarbodiimide (6·6 g). N,N'-Dicyclohexylurea which had separated after 3 h of stirring at room temperature was filtered off, the solution was evaporated, the dry residue dissolved in ethyl acetate and treated according to procedure *A*. The dry residue was crystallized from ethyl acetate by the addition of petroleum ether. The yield was 15·3 g (95%) of product *VII* melting at 111—114°C. The sample for analysis was crystallized by the same procedure, m.p. 118—119°C, $[\alpha]_D^{20}$ −19·8° (*c* 0·2, methanol). The product was chromatographically homogeneous: R_F 0·85 (S_1), 0·76 (S_2). For $C_{24}H_{30}N_4O_8S$ (534·6) calculated: 53·92% C, 5·67% H, 10·40% N; found: 54·03% C, 5·71% H, 10·40% N.

N$^{\varepsilon}$-Benzyloxycarbonyllysyl-serine Methyl Ester Hydrochloride (*VIII*)

A 4·3 mol l^{-1} solution of hydrogen chloride in acetic acid (12 ml) was added to the solution of protected dipeptide *VII* (13·4 g; 25 mmol) in methanol and the mixture was stirred 45 min at room temperature. The hydrochloride precipitated by ether after evaporation was filtered off and crystallized from methanol by the addition of ether. The yield was 8·4 g (80%) of product *VIII* melting at 168—171°C. The sample for analysis was crystallized by the same procedure, m.p. 171—175°C, $[\alpha]_D^{20}$ +5·7° (*c* 0·2, methanol). The product was chromatographically homogeneous: R_F 0·45 (S_1), 0·69 (S_2). For $C_{18}H_{28}N_3O_6Cl$ (418·0) calculated: 51·77% C, 6·77% H, 10·06% N; found: 52·07% C, 6·80% H, 10·01% N.

Tert.-Butyloxycarbonylalanyl-N$^{\varepsilon}$-benzyloxycarbonyllysyl-serine Methyl Ester (*IX*)

The solution of N$^{\varepsilon}$-benzyloxycarbonyllysyl-serine methyl ester in dimethylformamide (25 ml), liberated from the hydrochloride of *VIII* (3·5 g; 8·4 mmol) by the addition of N-ethylpiperidine (1·2 ml), was treated with the solution of tert.-butyloxycarbonylalanine pentachlorophenyl ester (3·6 g; 8·4 mmol) in dimethylformamide (30 ml). The mixture was stirred 12 h at room temperature, evaporated, the dry residue was dissolved in ethyl acetate and treated according to procedure *A*. The product was crystallized from ethyl acetate by addition of ether. The yield was 4·3 g (91%) of *IX* melting at 110—112°C (ref.[16] m.p. 103—111°C). The sample for analysis was crystallized by the same procedure, the melting point remained unchanged, $[\alpha]_D^{20}$ −28·5° (*c* 0·2, methanol). The product was chromatographically homogeneous: R_F 0·87 (S_1), 0·83 (S_2). For $C_{26}H_{40}N_4O_9 . H_2O$ (570·7) calculated: 54·71% C, 7·26% H, 9·82% N; found: 54·63% C, 7·01% H, 9·55% N.

Tert.-Butyloxycarbonylalanyl-N$^{\varepsilon}$-benzyloxycarbonyllysyl-serine (*X*)

1M-NaOH (5·5 ml) was added to a solution of ester *IX* (2·0 g; 3·6 mmol) in methanol (20 ml). The mixture was stirred 30 min at room temperature, the pH of the solution was adjusted to pH 7 by 1 mol l^{-1} hydrochloric acid afterwards and methanol was distilled off from the solution. The pH of the aqueous evaporate was adjusted to pH 3 by 1 mol l^{-1} hydrochloric acid and the product was extracted with ethyl acetate (3 × 100 ml). The pooled organic extracts were dried by anhydrous sodium sulfate, taken to dryness and the dry residue crystallized from ethyl acetate by the addition of petroleum ether. The yield was 1·7 g (88%) of *X*, m.p. 132—134°C. The sample for analysis was crystallized in the same manner, the melting point remained the same, $[\alpha]_D^{20}$ −23·2° (*c* 0·2, methanol). The product was chromatographically homogeneous: R_F 0·75 (S_1), 0·75 (S_2). For $C_{25}H_{38}N_4O_9$ (538·6) calculated: 55·74% C, 7·13% H, 10·40% N; found: 56·13% C, 7·46% H, 10·40% N.

Benzyloxycarbonylseryl-asparagine 4-Nitrobenzyl Ester (*XI*)

The solution of benzyloxycarbonylserine (12·0 g; 50 mmol) and N-ethylpiperidine (7·0 ml) in dimethylformamide (100 ml), cooled down to −20°C, was treated with ethyl chloroformate (5·0 ml) with stirring. Stirring was continued for 10 min at −10°C, the solution was then cooled down to −20°C and asparagine 4-nitrobenzyl ester in dimethylformamide (100 ml), which had been liberated from hydrochloride *I* (15·2 g; 50 mmol) by the addition of N-ethylpiperidine (7·0 ml), was added within 2–3 min. The mixture was stirred 30 min with cooling at 0°C, then 2 h at room temperature, taken to dryness afterwards and treated according to procedure *B*. The dry residue was crystallized from ethyl acetate. The yield was 18·8 g (77%) of *XI*, m.p. 174–178°C. The sample for analysis was crystallized from a mixture of dimethylformamide and 2-propanol by the addition of petroleum ether, m.p. 179–180°C, $[\alpha]_D^{20}$ −8·6° (*c* 0·2, dimethylformamide). The product was chromatographically homogeneous: R_F 0·73 (S_1), 0·76 (S_2). For $C_{22}H_{24}N_4O_9.H_2O$ (506·5) calculated: 53·11% C, 5·08% H, 11·26% N; found: 53·49% C, 5·13% H, 11·54% N.

Seryl-asparagine 4-Nitrobenzyl Ester Hydrobromide (*XII*)

A 38% (by weight) solution of hydrogen bromide in acetic acid (110 ml) was added to a solution of dipeptide *XI* (21·1 g, 43·2 mmol) in glacial acetic acid. The mixture was set aside for 1 h at room temperature with occasional stirring. Product *XII*, precipitated after this period by the addition of ether was filtered off, washed, and dried 2 h over phosphorus pentoxide and sodium hydroxide in a desiccator. The yield was 17·6 g (95%) of *XII*. The compound was chromatographically and electrophoretically homogeneous: R_F 0·36 (S_1), 0·65 (S_2).

Tert.-Butyloxycarbonylglycyl-glycyl-seryl-asparagine 4-Nitrobenzyl Ester (*XIII*)

A solution of seryl-asparagine 4-nitrobenzyl ester in dimethylformamide (50 ml), liberated from hydrobromide *XII* (8·16 g; 19·0 mmol) by the addition of triethylamine (2·7 ml), was added to the solution of tert.-butyloxycarbonylglycyl-glycine[38] (4·4 g; 19·0 mmol) in dimethylformamide (50 ml). The mixture was cooled down to −15°C and N,N′-dicyclohexylcarbodiimide (4·2 g) was added. The mixture was stirred 2 h at 0°C, then set aside for 8 h at room temperature and taken to dryness afterwards. The dry residue was dissolved in ethyl acetate (100 ml), N,N′-dicyclohexylurea which had separated was filtered off and the filtrate was treated according to procedure *A*. The product was crystallized from 2-propanol by the addition of petroleum ether. The yield was 4·8 g (40%) of *XIII* melting at 105–109°C (ref.[16], m.p. 104–107°C), $[\alpha]_D^{20}$ −11·8° (*c* 0·2, dimethylformamide). The compound was chromatographically homogenous: R_F 0·69 (S_1), 0·83 (S_2).

Glycyl-glycyl-seryl-asparagine 4-Nitrobenzyl Ester Hydrochloride (*XIV*)

The solution of *XIII* (3·90 g; 6·2 mmol) in glacial acetic acid (13·0 ml) was treated with 1 mol l^{-1} hydrochloric acid in acetic acid (13·0 ml) and then stirred 1 h at room temperature. The product precipitated after this period by the addition of ether was filtered off, washed and dried 5 h over phosphorus pentoxide and sodium hydroxide in a desiccator. The yield was 3·3 g (96%) of *XIV*. The compound was electrophoretically and chromatographically homogeneous: R_F 0·10 (S_1), 0·62 (S_2).

Benzyloxycarbonylglutaminyl-glycyl-glycyl-seryl-asparagine 4-Nitrobenzyl Ester (*XV*)

The solution of benzyloxycarbonylglutamine (2·0 g; 7·0 mmol) and N-ethylpiperidine (1·0 ml) in dimethylformamide (50 ml), cooled down to −20°C, was treated with ethyl chloroformate (0·7 ml) with stirring. Stirring was continued for 10 min at −10°C, the mixture was cooled down to −20°C afterwards and a solution was added to a solution of glycyl-glycyl-seryl-asparagine in dimethylformamide (50 ml), which had been liberated from hydrochloride *XIV* (3·5 g; 7·0 mmol) by the addition of N-ethylpiperidine (1·0 ml). The mixture was stirred 30 min at 0°C, 2 h at room temperature, evaporated and treated according to procedure *C*. The product was crystallized from a mixture of dimethylformamide and 2-propanol by the addition of petroleum ether. The yield was 2·9 g (55%) of *XV* melting at 174–177°C (ref.[23] m.p. 176–179°C), $[\alpha]_D^{20}$ −29·0° (*c* 0·2, dimethylformamide). The compound was chromatographically homogeneous: R_F 0·60 (S_1), 0·82 (S_2).

Glutaminyl-glycyl-glycyl-seryl-asparagine 4-Nitrobenzyl Ester Hydrobromide (*XVI*)

A 38% (by weight) solution of hydrogen bromide in acetic acid (4·6 ml) was added to a solution of *XV* (3·4 g; 4·5 mmol) in glacial acetic acid. The mixture was treated as described for *XII*. The product was electrophoretically and chromatographically homogeneous: R_F 0·07 (S_1), 0·52 (S_2). The yield was 2·7 g (90%) of *XVI*.

Tert.-Butyloxycarbonylalanyl-N$^{\varepsilon}$-benzyloxycarbonyl-
-lysyl-seryl-glutaminyl-glycyl-glycyl-seryl-asparagine 4-Nitrobenzyl Ester (*XVII*)

A solution of *X* (2·5 g; 4·6 mmol), 1-hydroxybenztriazole (0·64 g) and N,N′-dicyclohexylcarbodiimide (1·0 g) in dimethylformamide (30 ml) was stirred 10 min at −10°C. A precooled (−10°C) solution of glutaminyl-glycyl-glycyl-seryl-asparagine 4-nitrobenzyl ester, which had been liberated from hydrobromide *XVI* (3·1 g; 4·6 mmol) by the addition of triethylamine (0·7 ml) was added afterwards. The mixture was stirred 2 h at 0°C, then set aside for 8 h at room temperature and evaporated. The dry residue was dissolved in butanol, N,N′-dicyclohexylurea which had separated was filtered off and the filtrate was treated according to procedure *A*. The product was crystallized from dimethylformamide by the addition of a mixture of 2-propanol and petroleum ether (1 : 1). The yield was 1·6 g (31%) of *XVII* melting at 189–192°C. The sample for analysis was crystallized in the same manner, m.p. 198–199°C, $[\alpha]_D^{20}$ −9·9° (*c* 0·2, dimethylformamide), −11·2° (*c* 0·2, acetic acid). The product was chromatographically homogeneous: R_F 0·38 (S_1), 0·82 (S_2). For $C_{48}H_{68}N_{12}O_{19}.3\,C_3H_8O$ (1 297) calculated: 52·76% C, 7·16% H, 12·95% N; found: 52·62% C, 6·87% H, 12·61% N. Amino acid analysis: Ala 1·00, Lys 0·93, Ser 1·64, Glu 1·02, Gly 1·93, Asp 1·12.

Alanyl-N$^{\varepsilon}$-benzyloxycarbonyllysyl-seryl-glutaminyl-glycyl-glycyl-seryl-asparagine
4-Nitrobenzyl Ester Hydrochloride (*XVIII*)

A 1·9 mmol l^{-1} solution of hydrogen chloride in acetic acid (2·0 ml) was added to a solution of XVII (570 mg; 0·5 mmol) in glacial acetic acid (3·5 ml) and the mixture was stirred 45 min at room temperature. *XVIII* was then precipitated with ether, filtered off, washed, and dried 2 h over phosphorus pentoxide and sodium hydroxide in a desiccator. The product was electrophoretically and chromatographically homogeneous: R_F 0·34 (S_1), 0·78 (S_2). The yield was 49 mg (93%) of *XVIII*.

Alanyl-lysyl-seryl-glutaminyl-glycyl-glycyl-seryl-asparagine (*XIX*)

Pd-Black (approximately 50 mg) was added to a solution of *XVII* (220 mg; 0·21 mmol) in 10 vol. % acetic acid (100 ml). Hydrogenolysis was carried out at normal pressure 8 h at room temperature and 8 h at 40°C. After Pd-black had been filtered off the solution was evaporated, the residue was dried over phosphorus pentoxide and sodium hydroxide in a desiccator and then dissolved in glacial acetic acid (3·5 ml). After 1 mol l^{-1} solution of hydrogen chloride in acetic acid (0·6 ml) had been added, the solution was stirred 2 h at room temperature. The dihydrochloride precipitated by the addition of ether was filtered off, dissolved in 50 vol. % methanol (25 ml) and deionized on Wofatite L 150 (in OH^--form) in 50 vol. % methanol, first batchwise and then on a column (1·8 × 25 cm). The pooled methanolic extracts were evaporated, the residue was dissolved in 0·2 mol l^{-1} acetic acid (5·0 ml), desalted by gel filtration on a Sephadex G-15 column (1·8 × 90 cm) and eluted by the same solvent. The course of the separation was monitored by thin-layer electrophoresis, homogeneous fractions were pooled, concentrated and lyophilized. The yield was 50 mg (27%) of *XIX*. The product was electrophoretically and chromatographically homogeneous: R_F 0·02 (S_1), 0·07 (S_2), 0·05 (S_3), $[\alpha]_D^{20}$ −19·5° (*c* 0·1, 0·1 mol l^{-1} acetic acid). For $C_{28}H_{49}N_{11}O_{13}.3\,CH_3COOH.2\,H_2O$ (964·1) calculated: 42·36% C, 6·80% H, 15·98% N; found: 42·22% C, 6·31% H, 15·81% N. Amino acid analysis: Glu 1·00, Lys 0·98, Ser 1·59, Gly 1·93, Asp 0·92, Ala 1·03.

Pyroglutamyl-alanyl-N^{ε}-benzyloxycarbonyllysyl-seryl-
-glutaminyl-glycyl-glycyl-seryl-asparagine 4-Nitrobenzyl Ester (*XX*)

A solution of pyroglutamic acid (72 mg; 0·56 mmol), 1-hydroxybenztriazole (70 mg) and N,N′-dicyclohexylcarbodiimide (120 mg) in dimethylformamide (10 ml) was stirred 10 min at −10°C. After this period a precooled (−10°C) solution of alanyl-N^{ε}-benzyloxycarbonyllysyl-seryl-glutaminyl-glycyl-glycyl-seryl-asparagine 4-nitrobenzyl ester, which had been liberated from hydrochloride *XVIII* (590 mg; 0·56 mmol) by the addition of N-ethylpiperidine (0·08 ml), was added. The mixture was stirred 2 h at 0°C, then set aside for 8 h at room temperature. After concentration and cooling, N,N′-dicyclohexylurea which had separated was filtered off and the solution was mixed with a saturated solution of sodium chloride. The product which had separated after 2 h of standing at room temperature was filtered off and crystallized from butanol (saturated with water at 20°C). The yield was 185 mg (28%) of *XX* melting at 193—196°C. The sample for analysis was crystallized in the same manner, the melting point remained the same, $[\alpha]_D^{20}$ −15·3° (*c* 0·2, dimethylformamide). The product was chromatographically homogeneous: R_F 0·26 (S_1), 0·78 (S_2). For $C_{48}H_{65}N_{13}O_{19}.C_4H_{10}O.2\,H_2O$ (1 238) calculated: 50·44% C, 6·44% H, 14·71% N; found: 50·37% C, 6·83% H, 14·41% N. Amino acid analysis: Glu 1·87, Ala 1·19, Lys 1·13, Ser 1·88, Gly 2·00, Asp 1·17.

Benzyloxycarbonylglutaminyl-alanyl-N^{ε}-benzyloxycarbonyllysyl-seryl-
-glutaminyl-glycyl-glycyl-seryl-asparagine 4-Nitrobenzyl Ester (*XXI*)

A solution of benzyloxycarbonylglutamine (160 mg; 0·57 mmol), 1-hydroxybenztriazole (72 mg) and N,N′-dicyclohexylcarbodiimide (125 mg) was stirred 10 min at −10°C. The subsequent treatment was identical with the procedure described for compound *XX*. The yield was 190 mg (25%) of *XXI* melting at 201—207°C. The sample for analysis was crystallized from butanol (saturated with water at 20°C), the melting point remained the same, $[\alpha]_D^{20}$ −22·8° (*c* 0·5, acetic acid) (ref.[23], m.p. 220—222°C, $[\alpha]_D^{20}$ −19·6° (*c* 0·5, acetic acid)). The product was chromatographically homogeneous: R_F 0·39 (S_1), 0·80 (S_2). For $C_{56}H_{74}N_{14}O_{21}.C_4H_{10}O.H_2O$ (1 389)

calculated: 51·86% C, 6·25% H, 14·12% N; found: 51·92% C, 5·99% H, 14·12% N. Amino acid analysis: Glu 2·00, Ala 1·00, Lys 1·09, Ser 1·77, Gly 2·00, Asp 1·09.

Pyroglutamyl-alanyl-lysyl-seryl-glutaminyl-glycyl--glycyl-seryl-asparagine (*XXII*)

Pd-Black (approximately 10 mg) was added to a solution of *XX* (90 mg; 0·08 mmol) in 10 mol. % acetic acid (20 ml) and hydrogenolysis was carried out 12 h at normal pressure and room temperature. After Pd-black had been filtered off the solution was evaporated, the residue dissolved in water (20 ml) and again evaporated (repeated twice), The crude product was dissolved in 0·2 mol l^{-1} acetic acid (5·0 ml) and treated by the procedure described for *XIX*. The yield was 65 mg (84%) of *XXII*. The product was chromatographically and electrophoretically homogeneous: R_F 0·02 (S_1), 0·20 (S_2), 0·20 (S_3), $[\alpha]_D^{20}$ −42·8° (*c* 0·1, 0·1 mol l^{-1} acetic acid), −45·9° (*c* 0·65, water) (ref.[26] $[\alpha]_D^{22}$ −36·0° (*c* 0·45, water)). For $C_{33}H_{54}N_{12}O_{14}.4\,CH_3COOH.2\,H_2O$ (1 135) calculated: 43·37% C, 6·58% H, 14·81% N; found: 43·17% C, 5·90% H, 14·89% N. Amino acid analysis: Glu 2·00, Ala 1·07, Lys 1·09, Ser 1·76, Gly 2·00, Asp 1·11.

Glutaminyl-alanyl-seryl-lysyl-glutaminyl-glycyl-glycyl-seryl-asparagine (*XXIII*)

Pd-Black (approximately 50 mg) was added to a solution of *XXI* (290 mg; 0·22 mmol) in 10 vol. % acetic acid (90 ml). Hydrogenolysis was carried out for 2·5 h at normal pressure and room temperature (the pH was adjusted by 1 mol l^{-1} hydrochloric acid (1·0 ml) to pH 2). After Pd-black had been filtered off the residue was dissolved in water (20 ml) and the dihydrochloride was converted into diacetate *XXIII* by Zerolit FF (in CH_3COO^--form) first batchwise (approximately 20 ml of resin) then on a column (1·8 × 25 cm). The pooled aqueous effluents were evaporated, the residue was dissolved in 0·2 mol l^{-1} acetic acid (5·0 ml) and treated by the procedure described for *XIX*. The yield was 70 mg (32%) of *XXIII*. R_F 0·02 (S_1), 0·25 (S_2), 0·20 (S_3) and a minor spot of R_F 0·05, $[\alpha]_D^{20}$ −54·0° (*c* 0·1, 0·1 mol l^{-1} acetic acid). For $C_{33}H_{57}N_{13}O_{15}.4\,CH_3COOH.H_2O$ (1 134) calculated: 43·42% C, 6·68% H, 16·06% N; found: 43·54% C, 6·55% H, 16·26% N. Amino acid analysis: Glu 1·90, Ala 1·06, Lys 1·03, Ser 1·72, Gly 1·88, Asp 1·00.

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