ATRIOPEPTINS. I. SYNTHESIS OF C-TERMINAL FRAGMENTS

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Peptides, corresponding to the C-terminal sequence in atriopeptins, have been synthesized using classical methods of peptide synthesis in solution and characterized by various physicochemical methods. The synthetic strategy and methods are discussed.

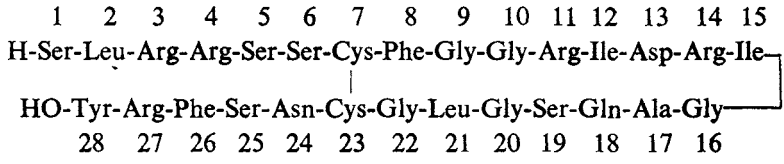
In 1981 it has been shown[1] that application of rat atrial extracts increased excretion of sodium ions and water in experimental animals. The discovery that the atrium contains a factor exhibiting strong natriuretic effect has given impetus to many investigations resulting in the isolation of several so-called atriopeptins and determination of their structure. Several recent reviews and papers[2-5] discuss the physiological function of these compounds. It is assumed that atriopeptins are involved in the blood volume regulation and in the water-salt exchange[6].

There are, however, contradictory data on the relative activity of various atriopeptins, precluding dependable predictions concerning the utilization of these compounds in the medicinal practice[7]. We therefore have set out to synthesize atriopeptins in the chemical way and investigate their biological role.

So far, great majority of the described syntheses of these compounds are based on the solid-phase method[8-10]. In one of the syntheses the fragments were obtained by the solid-phase method and the further condensations were performed using classical solution methods of the peptide chemistry[11]. In another case, classical methods of peptide synthesis in solution were used throughout the whole synthesis[12], however, no chemical details on this synthesis are given.

The series of our investigations concerns the synthesis of the peptide of the sequence 123 – 157 in proatriopeptine[1], denoted* as α-r-ANF, and its three analogues:

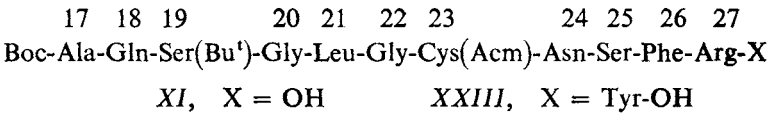
* The nomenclature and symbols in this and the following papers obey the published recommendations (Eur. J. Biochem. *138*, 9 (1984)). Other symbols used are: DMF N,N-dimethylformamide, DCC N,N′-dicyclohexylcarbodiimide, HONB N-hydroxy-5-norbornene-2,3-dicarboxyimide, "complex F" crystalline adduct of DCC (1 mol) and pentafluorophenol (3 mol), Pfp pentafluorophenyl, α-r-ANF rat atrial natriuretic peptide, AP-II α-r-ANF_{5-27}, AP-III α-r-ANF_{5-28}.



Sequence
1 – 28 α-r-ANF
5 – 28 AP-III
5 – 27 AP-II
7 – 27 des-Ser[5]-des-Ser[6]-AP-II

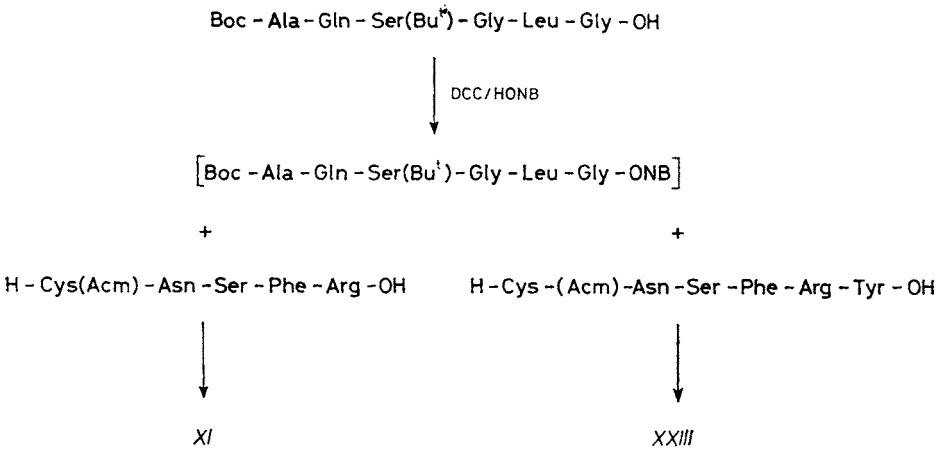
Our strategy of synthesis is based on the structural features of these compounds. All the peptide chain of α-r-ANF was divided into five approximately equal fragments: 1 – 6, 7 – 10, 11 – 16, 17 – 22 and 23 – 28. It is important to note that, except the fragment 1 – 6, all the remaining fragments contain the optically inactive glycine residues at the C-terminal. The final synthetic step consists in condensation between the Gly[16] and Ala[17] residues of two large fragments.

In the present paper we describe the synthesis of two C-terminal fragments of the sequence 17 – 27 and 17 – 28,



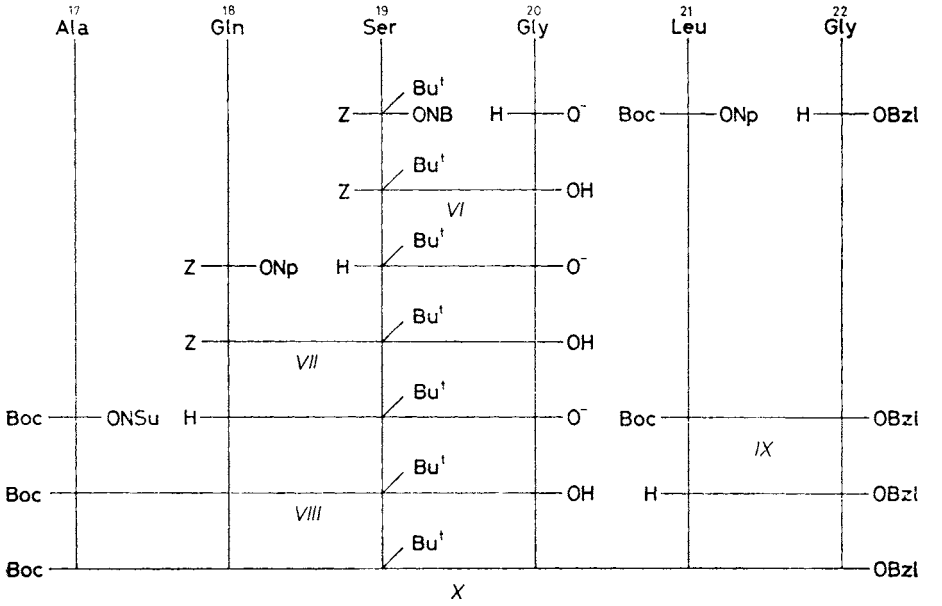
which are common for all natural atriopeptins, excepting AP-I, the peptide chain of which ends by the Asn[24] residue. The mentioned undeca- and dodecapeptide were obtained by condensation of fragment 17 – 22 with the respective fragments 23 – 27 and 23 – 28 as shown in Scheme 1.

Tyrosine, serine and aspartate moieties were protected with the tert-butyl group, α-amino groups were blocked with carbobenzoxy- or tert-butyloxycarbonyl groups. The thiol group of cysteine was converted into an acetamidomethyl one. Arginine was used either as its N-α-Boc(N^{ω}-NO_2) or N-α-Z-(N^{ω}-NO_2) derivative or as the free base, the carboxyl groups were protected as benzyl esters or salts. The peptide bonds were synthesized using various activated esters. Originally, we planned to synthesize the fragment 17 – 22 by stepwise construction of the peptide chain starting from the glycine residue with protection of the carboxyl function by salt formation. However, with longer peptide chain the solubility of the peptides strongly decreased and, moreover, impurities were gradually cumulated that were separable only with difficulty. Therefore, we decided to perform the synthesis by condensation of fragments Boc-Ala-Gln-Ser(Bu[t])-Gly-OH (*VIII*) and H-Leu-Gly-OBzl. The best results were obtained using the complex F. The dipeptide Boc-Leu-Gly-OBzl (*IX*) was



SCHEME 1

obtained by condensation of tert-butyloxycarbonylleucine *p*-nitrophenyl ester with glycine benzyl ester. The Boc protecting group was removed by treatment with trifluoroacetic acid and the trifluoroacetate ions were removed by treatment of the



SCHEME 2

peptide in aqueous isopropyl alcohol with an ion-exchanging resin (OH^- form) at −40°C. The protected tetrapeptide Boc-Ala-Gln-Ser(Bu^t)-Gly-OH (*VIII*) was synthesized by stepwise addition of amino acid moieties using the activated ester method. The glycine carboxyl groups were protected by salt formation. The pathway to the fragment 17−22 (*X*) is depicted in Scheme 2.

The C-terminal hexapeptide was obtained in several ways. Originally, the synthesis started from free arginine. Stepwise construction of the peptide chain (one amino acid moiety in one step) afforded the protected tetrapeptide Boc-Asn-Ser(Bu^t)-Phe--Arg-OH (*III*) which reacted with Tyr(Bu^t)-OBu^t in the presence of phosphoryl azide. After removal of the protecting groups and chromatography (a Lobar column, Lichroprep RP-8) the pentapeptide H-Asn-Ser-Phe-Arg-Tyr-OH (*V*) was obtained in a 20% yield. Attachment of cysteine moiety gave the C-terminal protected hexapeptide Boc-Cys-(Acm)-Asn-Ser-Phe-Arg-Tyr-OH (Scheme 3, pathway A). This synthetic scheme was chosen for several reasons: Firstly, the synthesis of peptides containing the C-terminal arginine is substantially simpler thanks to the intramolecular salt formation in the arginine molecule, secondly, the obtained protected tetrapeptide requires no addition of base during the condensation in the presence of diphenylphosphoryl azide, and thirdly, the obtained tetrapeptide represents a universal product suitable for the synthesis of the fragment 23−27 as well as the fragment 23−28. However, as already mentioned, the yield of the pentapeptide *V* was very low (20%) and therefore the fragment 23−28 was prepared by stepwise synthesis, starting from O-tert-butyltyrosine tert-butyl ester or from O-tert-butyl-

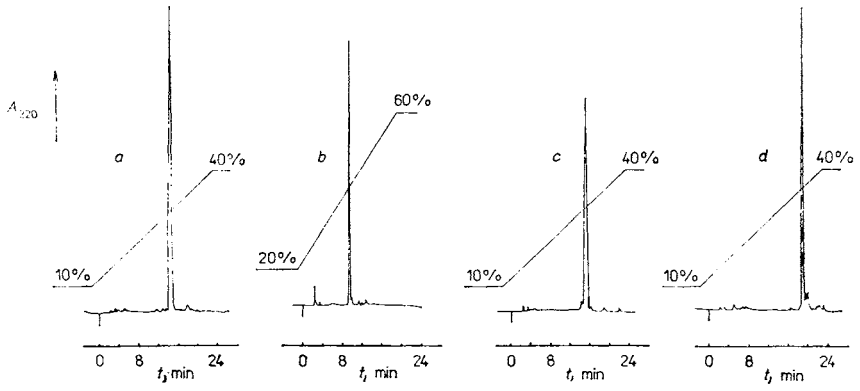
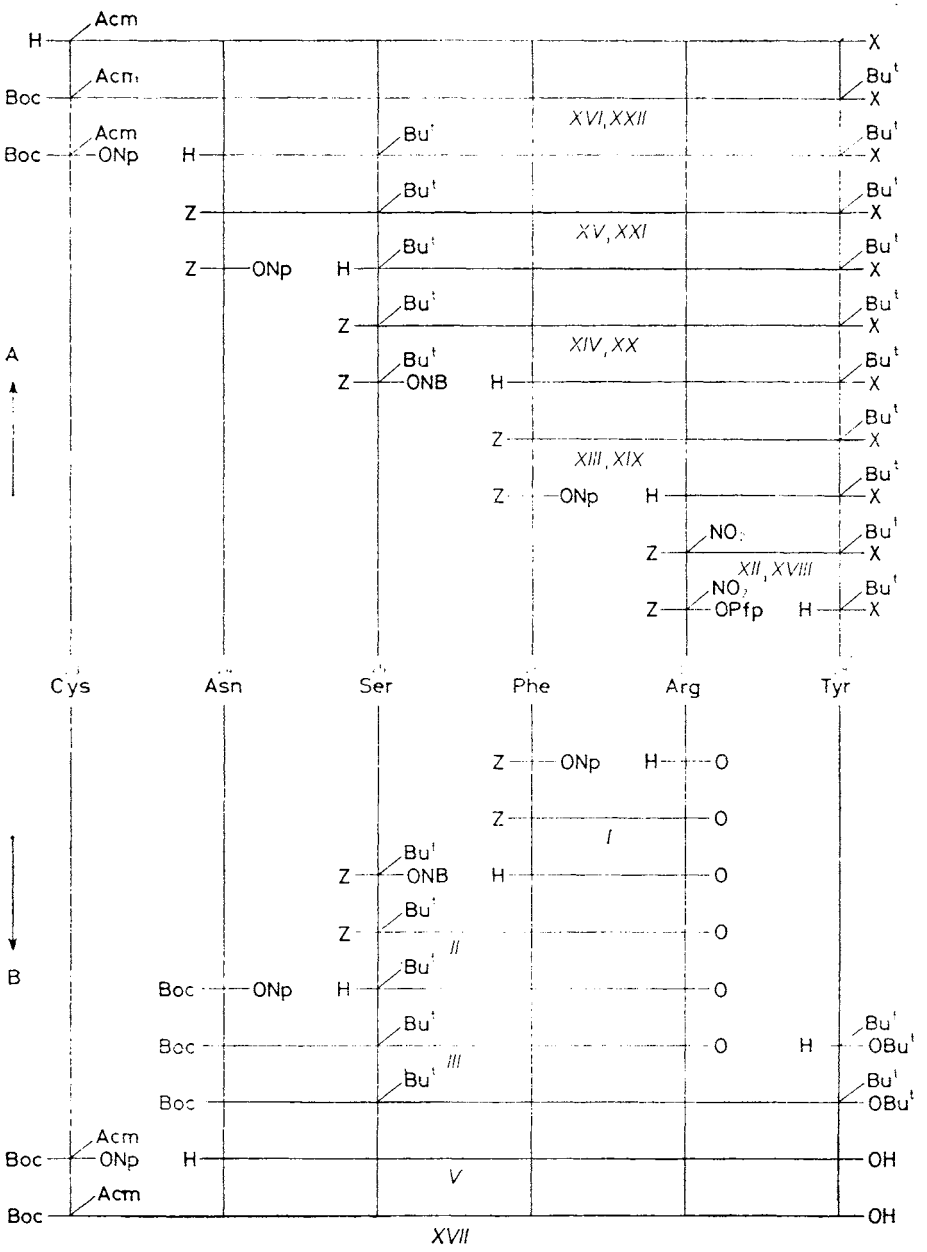


FIG. 1

HPLC of fragments of atriopeptins: *a* α-r-ANF(24−28)-peptide, *b* α-r-ANF(23−28)-peptide, *c* α-r-ANF(17−27)-peptide, *d* α-r-ANF(17−28)-peptide (on a Spherisorb ODS column (4·6 × 250 mm); gradient of acetonitrile in 0·05M-KH_2PO_4 (pH 3·0); elution rate 1 ml/min)



X = OH, Bu^t

SCHEME 3

tyrosine (Scheme 3, pathway B). After deprotection, the hexapeptide H-Cys(Acm)--Asn-Ser-Phe-Arg-Tyr-OH was obtained in satisfactory yield in both cases.

Further condensation of the fragments 17–22 and 23–27 or 23–28 was carried out according to Scheme 2, the yield of the protected peptides 17–27 and 17–28 being 75% and 86%, respectively.

Structure of the obtained compounds was confirmed by ^{1}H NMR spectra and amino acid analysis and purity of the intermediates was checked by TLC (in three different chromatographic systems). After removal of the tert-butyl protecting groups by standard treatment with trifluoroacetic acid, the fragments 17–27, 17–28, 23–28 and 24–28 contained not less than 90% of the desired compound (according to HPLC, see Fig. 1) and were employed without purification in the further synthesis.

EXPERIMENTAL

The employed amino acids and their derivatives were purchased from Reanal (Hungary) and Fluka (Switzerland). Purity of the obtained compounds was checked by TLC on Kieselgel 60 (Merck, F.R.G.) plates in the following systems: chloroform–methanol–32% acetic acid (60 : 45 : 20, A); 1-butanol–acetic acid–water (3 : 1 : 1, B); 1-butanol–acetic acid–pyridine–water (10·5 : 6 : 1 : 7·5, C); ethyl acetate–pyridine–acetic acid– water (45 : 20 : 6 : 11, D); ethyl acetate–-pyridine–acetic acid–water (120 : 40 : 6 : 4, E); chloroform–methanol–25% NH_4OH (30 : 22 : 10, F); chloroform–methanol–acetic acid (32 : 2 : 1, G); chloroform–methanol–acetic acid (9 : 1 : 0·5, H); ethyl acetate (I). Spots were detected with ninhydrin or chlorobenzidine. The peptides were hydrogenated over a 10% Pd/C catalyst (Fluka or Merck, 5–10 wt%). Melting points were determined on a Boetius block (G.D.R.) and are uncorrected. Optical rotations were measured with a Perkin–Elmer (U.S.A.) polarimeter. Amino acid analysis was performed on a Labotron Liquimat (F.R.G.) instrument after hydrolysis of the peptides with 6M-HCl (with 2% of phenol) at 110°C for 24 h. Cysteine was not determined.

HPLC analyses were performed on an Altex 332 instrument with an Altex/Hitachi 155-40 detector (at 220 nm). Acetonitrile (Merck Lichrosolve), water, trifluoroacetic acid (three times distilled in glass), and KH_2PO_4 (Sigma) were used for the separation. The homogeneity of the prepared compounds was confirmed by ^{1}H NMR spectra, obtained at 37°C on a Bruker WH-500 instrument (F.R.G.) (500 MHz, sodium 2,2-dimethyl-2-silapentane-5-sulfonate as internal standard) by the double resonance method. The samples were made by dissolution of the compound (1 mg) in hexadeuterodimethyl sulfoxide (0·5 ml). After extraction the organic solutions were washed with water, dried over anhydrous sodium sulfate and the solvents were evaporated on a rotatory evaporator at 40°C. Column chromatography was carried out on silica gel L 40-100 (Czechoslovakia).

Z-Phe-Arg-OH (*I*)

Arginine (3·48 g; 20 mmol) was added to a solution of Z-Phe-ONp (10·10 g; 24 mmol) in DMF (100 ml). After stirring at 20°C for 20 h, the mixture was concentrated to a small volume and treated with ether (150 ml). The precipitate was filtered, washed on the filter with ether and twice reprecipitated with ether from ethanol; yield 7·40 g (81%) of *I*, m.p. 131–133°C; $[\alpha]_D^{15}$ −15·2° (*c* 1; DMF); R_F 0·77 (A), 0·65 (B), 0·63 (C).

Z-Ser(Bu^t)-Phe-Arg-OH (*II*)

Compound *I* (6·20 g; 13·6 mmol) was hydrogenated in methanol (100 ml) over a Pd/C catalyst. The catalyst was filtered off, the solvent was evaporated and the residue was dissolved in DMF (40 ml). The obtained solution was stirred with Z-Ser(Bu^t)-ONB (7·00 g; 15·3 mmol) at 20°C for 24 h, concentrated to a small volume and the residue was dissolved in ethyl acetate and reprecipitated with ether. The precipitate was collected on filter, washed with ether, dried, dissolved in chloroform (20 ml) and chromatographed on a 2·5 × 40 cm column of silica gel in chloroform–methanol (4 : 1). The product-containing fractions (TLC) were combined, the solvent was evaporated and the product was reprecipitated with ether from ethanol solution. Yield 6·77 g (83%) of *II*, m.p. 107—109°C; $[\alpha]_D^{15}$ −6·4° (*c* 1; DMF); R_F 0·85 (A), 0·30 (D), 0·68 (B).

Boc-Asn-Ser(Bu^t)-Phe-Arg-OH (*III*)

Compound *II* (3·0 g) was hydrogenated in methanol (70 ml) over a Pd/C catalyst. The catalyst was filtered off, the solvent was evaporated, the residue was dissolved in DMF (30 ml) and Boc-Asn-ONp (2·2 g; 5·7 mmol) was added. The mixture was kept at 20°C for 15 h, the solvent was evaporated and the residue was treated with ether. The precipitate was filtered, washed with ether and reprecipitated with ether from chloroform to give 3·00 g (88%) of *III*, m.p. 152—155°C; $[\alpha]_D^{15}$ −20·5 (*c* 1; DMF); R_F 0·81 (A), 0·46 (D), 0·63 (C).

Boc-Cys(Acm)-Asn-Ser-Phe-Arg-OH (*IV*)

Compound *III* (1·7 g; 2·5 mmol) was treated with trifluoroacetic acid (40 ml) for 1 h. The acid was evaporated, the residue was mixed with ether (120 ml) and the precipitate was filtered, washed with ether, dried, dissolved in water (80 ml) and treated with an ion-exchanging resin (OH^- form) to pH 10. The resin was filtered off, washed on the filter with water and the combined filtrates were coevaporated with isopropyl alcohol. The residue was dissolved in DMF (20 ml) and Boc-Cys(Acm)-ONp (1·1 g; 2·7 mmol) was added. After standing for 20 h, the mixture was diluted with ether (100 ml), the precipitate was filtered and twice crystallized from chloroform. The obtained compound *IV* (2·1 g; 89%) melted at 153—155°C; $[\alpha]_D^{15}$ −7·8° (*c* 1; DMF); R_F 0·58 (A), 0·52 (B), 0·57 (C). Amino acid analysis: Asp 0·96; Ser 0·92; Phe 1·08; Arg 1·04. ^{1}H NMR, δ: Acm — 8·62, 1 H (NH), 4·28, 4·15, 2 H (CH_2), 1·85, 3 H (CH_3); Cys — 6·97, 1 H (NH), 4·17, 1 H (α-CH), 2·88, 2·62, 2 H (β-CH_2); Asn — 8·13, 1 H (NH), 4·55, 1 H (α-CH), 2·52, 2·43, 2 H (β-CH_2), 7·40, 6·93, 2 H ($CONH_2$); Ser — 7·68, 1 H (NH), 4·23, 1 H (α-CH), 3·58, 3·48, 2 H (β-CH_2), 5·79, 1 H (OH); Phe — 8·38, 1 H (NH), 4·37, 1 H (α-CH), 3·09, 2·84, 2 H (β-CH_2); Arg — 7·52, 1 H (NH), 3·89, 1 H (α-CH), 1·62. 1·55, 2 H (β-CH_2), 1·44 m, 2 H (γ-CH_2), 1·38, 9 H (Boc).

H-Asn-Ser-Phe-Arg-Tyr-OH (*V*)

Diphenylphosphoryl azide (0·27 ml; 1·25 mmol) was added at 0°C to a solution of *III* (0·68 g; 1·0 mmol) and H-Tyr(Bu^t)-OBu^t (0·30 g; 1·0 mmol) in DMF (5 ml). After stirring at 20°C for 20 h, the solvent was evaporated and the residue was treated with water (50 ml). The separated precipitate was collected on filter, washed with water, dissolved in methanol (10 ml) and precipitated with 2% sulfuric acid. The precipitate was filtered, washed with water and dried in vacuo over phosphorus pentoxide in a desiccator. The obtained compound was treated with trifluoroacetic acid (30 ml) for 1 h, the acid was evaporated and ether (60 ml) was added to the residue. The precipitated material was filtered, washed with ether, dissolved in a methanol–water mixture (1 : 1) and treated with an ion-exchanging resin (acetate form). The resin was filtered off, washed

on the filter with methanol and the combined filtrates were taken down. The residue was dissolved in methanol and purified by HPLC (column Lobar, size B (310-25), Lichroprep RP-8 (50–63 μm)), elution with a gradient water–methanol (both solvents contained 0·1% of CF_3COOH). Fractions, corresponding to the principal peak, were combined, the solvents were removed and the product was precipitated with ether from methanol. Yield 0·25 g (20%) of *V* as amorphous powder, homogeneous according to HPLC (see Fig. 1*b*); R_F 0·30 (C), 0·22 (A).

Z-Ser(Bu^t)-Gly-OH (*VI*)

A solution of Z-Ser(Bu^t)-ONB (4·72 g; 10 mmol) in DMF (60 ml) was added to a solution of glycine (0·75 g; 10 mmol) in 1M-NaOH (10 ml). After standing for 15 h, the mixture was taken down, the residue was diluted with 2% sulfuric acid (100 ml) and extracted three times with ether. The organic layer was washed with water to neutrality, dried over sodium sulfate and the solvent was evaporated. Crystallization from ether afforded 3·00 g (85%) of *VI*, m.p. 80–84°C; $[\alpha]_D^{15}$ −13·1° (*c* 1; DMF); R_F 0·47 (E), 0·68 (H), 0·87 (C).

Z-Gln-Ser(Bu^t)-Gly-OH (*VII*)

Compound *VI* (2·8 g; 8 mmol) was hydrogenated in methanol (45 ml) over Pd/C catalyst. The catalyst was filtered off, washed with methanol, the filtrate was mixed with 40% solution of Triton B (4 ml; 8 mmol) in methanol and the solvent was evaporated. The dry residue was dissolved in DMF (50 ml) and Z-Gln-ONP (3·2, g; 8 mmol) was added to the solution. After 15 h the reaction mixture was concentrated to a small volume, the residue was diluted with water (100 ml) and extracted three times with ether. The aqueous layer was acidified with 2% sulfuric acid to pH 2 and extracted with 1-butanol (90 ml). The organic phase was washed with water, the solvent was evaporated and the product was precipitated with ether from methanol; yield 3·4 g (88%) of *VII*, m.p. 165–170°C; $[\alpha]_D^{15}$ +12·8° (*c* 1; DMF); R_F 0·75 (A), 0·67 (B), 0·65 (C).

Boc-Ala-Gln-Ser(Bu^t)-Gly-OH (*VIII*)

The title tetrapeptide was obtained from *VII* (4·2 g; 8·7 mmol), 40% methanolic Triton B (4·35 ml) and Boc-Ala-OSu (2·5 g; 8·7 mmol) analogously as described for *VII*. Yield 2·92 (65%) of *VIII*, m.p. 190–192°C; $[\alpha]_D^{15}$ +10·6° (*c* 1; DMF); R_F 0·61 (A), 0·64 (B), 0·68 (C). ^{1}H NMR, δ: Ala — 6·96, 1 H (NH), 3·97, 1 H (α-CH), 1·17, 3 H (CH_3); Gln — 7·89, 1 H (NH), 4·29, 1 H (α-CH), 1·72, 1·87, 2 H (β-CH_2), 2·09, 2 H (γ-CH_2), 6·70, 7·22, 2 H (NH_2); Ser — 7·82, 1 H (NH), 4·35, 1 H (α-CH), 3·46, 2 H (β-CH_2); Gly — 8·10, 1 H (NH), 3·76, 2 H (α-CH_2), 1·37, 9 H (Boc), 1·100, 9 H (Bu^t).

Boc-Leu-Gly-OBzl (*IX*)

A solution of glycine benzyl ester tosylate (1·83 g; 5·43 mmol), and triethylamine (0·75 ml; 5·43 mmol) in DMF (20 ml) was added to a solution of Boc-Leu-ONB (2·13 g; 5·43 mmol) in DMF (30 ml). After standing for 12 h the reaction mixture was taken down, the residue was diluted with 2% sulfuric acid (60 ml) and extracted with ethyl acetate. The organic phase was washed with water to neutrality, dried, the solvent was evaporated and the product was dried in vacuo; yield 2·01 g (96%) of *IX* as an oil; R_F 0·76 (G), 0·91 (D), 0·94 (A).

Boc-Ala-Gln-Ser(Bu^t)-Gly-Leu-Gly-OBzl (*X*)

Compound *IX* (3·2 g; 8·4 mmol) was treated with trifluoroacetic acid (100 ml) for 1 h. The acid was evaporated, the residue dissolved in a small volume of ether and precipitated with hexane.

The oily material was separated by decantation, dissolved in a mixture of isopropyl alcohol–water (1 : 1; 100 ml) and treated with an ion-exchanging resin (OH^- form) at −40°C. The resin was filtered off, washed with a mixture of isopropyl alcohol and water (1 : 1) and the combined filtrates were evaporated to dryness. The residue was twice coevaporated with isopropyl alcohol and dried in vacuo over sodium hydroxide in a desiccator. The dry material was dissolved in DMF (50 ml), compound *VIII* (2.9 g; 6·4 mmol) was added and the obtained solution was cooled to −10°C. Complex F (6·4 g; 8·4 mmol) was added and the reaction mixture was kept at −4°C for 1 h and then at room temperature for 5 h. The separated gel was dissolved by warming, and N,N′-dicyclohexylurea was filtered off. The filtrate was concentrated to half of the original volume, ethyl acetate was added and the precipitate was filtered, dried and crystallized from ethanol to give 3·3 g (75%) of compound *X*, m.p. 224–226°C; $[\alpha]_D^{1.5}$ +74° (*c* 1; DMF); R_F 0·73 (A), 0·88 (B), 0·91 (C). ^{1}H NMR, δ: 1·37, 9 H (Boc); 1·08, 9 H (Bu^t); Ala — 6·96, 1 H (NH), 3·97, 1 H (α-CH), 1·17, 3 H (CH_3); Gln — 7·89, 1 H (NH), 4·29, 1 H (α-CH), 1·88, 1·73, 2 H (β-CH_2), 2·09 m, 2 H (γ-CH_2), 7·19, 6·73, 2 H ($CONH_2$); Ser — 7·86, 1 H (NH), 4·31, 1 H (α-CH), 3·46, m, 2 H (β-CH_2); Gly — 8·04, 1 H (NH), 3·75, m, 2 H (CH_2); Leu — 7·94; 1 H (NH), 4·34, 1 H (α-CH), 1·45, 2 H (β-CH_2), 1·59, 2 H, (γ-CH_2), 0·81. 3 H (δ-CH_3), 0·85; 3 H (δ-CH_3), 5·11, 2 H (CH_2-Ph). Amino acid analysis: Ser 0·85; Glu 1·03; Gly 2·12; Ala 0·96, Leu 1·06.

Boc-Ala-Gln-Ser(Bu^t)-Gly-Leu-Gly-Cys(Acm)-Asn-Ser-Phe-Arg-OH (*XI*)

A) Compound *X* (0·58 g; 0·75 mmol) was hydrogenated in DMF (20 ml) over a Pd/C catalyst. The catalyst was filtered off, washed on the filter with DMF and the filtrate was concentrated to 10 ml. HONB (0·18 g; 0·98 mmol) was added followed, under stirring, with N,N′-dicyclohexylcarbodiimide. The reaction mixture was stirred at −10°C for 1 h, at −4°C for 2 h and at 20°C for 10 h. The precipitated N,N′-dicyclohexylurea was filtered off, the filtrate was concentrated to a small volume and the residue was mixed with ether (40 ml). The precipitate was collected on a filter, washed with ether and dried in vacuo in a desiccator to afford 0·58 g (90%) of Boc-Ala-Gln-Ser(Bu^t)-Gly-Leu-Gly-ONB. A part (0·34 g) of this product was dissolved in DMF and the solution was used in the procedure *B*.

B) Compound *IV* (0·45 g; 0·5 mmol) was mixed with trifluoroacetic acid (25 ml). After standing for 1 h, the acid was evaporated, the residue was mixed with ether, the precipitate was filtered, washed with ether and dried. The residue was dissolved in water (30 ml) and treated with an ion-exchanging resin (OH^- form) to pH 10. The resin was filtered off, washed on the filter with water, the combined filtrates were codistilled with isopropyl alcohol and the dry residue was triturated with ether, filtered and dried. A solution of this material in DMF(5 ml) was added under stirring to the solution obtained under *A*). The reaction mixture was stirred at 20°C for 15 h and the product was precipitated with ethyl acetate. Reprecipitation with ethyl acetate from DMF afforded 0·54 g (75%) of *XI* as amorphous powder, $[\alpha]_D^{2.0}$ +13·5° (*c* 1; DMF); R_F 0·67 (A), 0·47 (B), 0·20 (D). Amino acid analysis: Asp 1·16; Ser 1·86; Glu 1·09; Gly 2·04; Ala 1·00; Leu 1·08; Phe 1·17; Arg 0·99.

Z-Arg(NO_2)-Tyr(Bu^t)-OH (*XII*)

A solution of H-Tyr(Bu^t)-OH (1·3 g; 5·5 mmol) in 40% solution of Triton B in methanol (2·75 ml) was evaporated and a solution of Z-Arg(NO_2)-OPfp (3·0 g; 5·5 mmol) in DMF (30 ml) was added to the residue. The reaction mixture was kept at 20°C for 8 h, the solvent was evaporated, the residue was dissolved in ethyl acetate (120 ml), washed twice with 2% sulfuric acid and water

and dried. Removal of the solvent and crystallization from ether afforded 2·0 g (63%) of compound *XII*, m.p. 123–125°C; $[\alpha]_D^{20}$ −4·3° (*c* 1; DMF); R_F 0·24 (F), 0·81 (B), 0·76 (C).

Z-Phe-Arg(NO_2)-Tyr(Bu^t)-OH (*XIII*)

Compound *XII* (2·5 g; 4·32 mmol) was hydrogenated in glacial acetic acid (50 ml). The catalyst was filtered off, the filtrate was taken down and the residue was mixed with ether (150 ml). The precipitate was filtered, washed with ether, dried over NaOH in vacuo in a desiccator and dissolved in DMF (30 ml). To this solution Z-Phe-ONp (2·1 g; 5 mmol) was added. The reaction mixture was kept at 20°C for 10 h, the solvent was evaporated, the residue was treated with ether (120 ml), the precipitate was filtered, washed with ether and dried. Crystallization from ethanol gave 2·0 g (67%) of compound *XIII*, m.p. 152–155°C; $[\alpha]_D^{20}$ −8·6° (*c* 1; DMF); R_F 0·84 (A), 0·47 (B), 0·67 (C).

Z-Ser(Bu^t)-Phe-Arg-Tyr(Bu^t)-OH (*XIV*)

Compound *XIII* (1·96 g; 2·9 mmol) was hydrogenated in methanol (50 ml), the catalyst was filtered off, and the solvent was evaporated. The residue was dissolved in DMF (20 ml) and mixed with Z-Ser(Bu^t)-ONB (1·50 g; 3·2 mmol). The reaction mixture was kept at 20°C for 10 h, the solvent was evaporated, the residue was dissolved in ethyl acetate (100 ml) and washed three times with water. Evaporation of the solvent and crystallization from ether gave 1·40 g (59%) of compound *XIV*, m.p. 146–148°C; $[\alpha]_D^{20}$ −5·6° (*c* 1; DMF); R_F 0·82 (A), 0·49 (B), 0·70 (C).

Z-Asn-Ser(Bu^t)-Phe-Arg-Tyr(Bu^t)-OH (*XV*)

The title pentapeptide was obtained from compound *XIV* (1·4 g; 1·7 mmol) and Z-Asn-ONp (0·8 g; 1·9 mmol) as described for compound *XIV*. Crystallization from chloroform furnished 1·5 g (1·7 mmol) of *XV*, m.p. 171–173°C; $[\alpha]_D^{20}$ −12·6 (*c* 1; DMF); R_F 0·71 (A), 0·39 (B), 0·69 (C).

Boc-Cys(Acm)-Asn-Ser(Bu^t)-Phe-Arg-Tyr(Bu^t)-OH (*XVI*)

The title compound was obtained from compound *XV* (1·46 g; 1·56 mmol) and Boc-Cys(Acm)--ONp (0·67 g; 1·62 mmol) analogously as described for the preparation of *XIV*. Yield 1·29 g (75%) of *XVI*, m.p. 198–200°C; $[\alpha]_D^{20}$ −19·6° (*c* 1; DMF); R_F 0·76 (A), 0·37 (B), 0·66 (C). Amino acid analysis: Asp 1·00; Ser 0·79; Tyr 1·00; Phe 1·01; Arg 0·99. ^{1}H NMR, δ: Cys — 6·57, 1 H (NH), 4·17, 1 H (α-CH), 2·88, 2·62, 2 H (β-CH_2); Asn — 8·14, 1 H (NH), 4·57, 1 H (α-CH), 2·54, 2·42, 2 H (β-CH_2), 7·46, 6·98, 2 H ($CONH_2$); Ser — 7·84, 1 H (NH), 4·18, 1 H (α-CH), 3·54, 2 H (β-CH_2); Phe — 7·93, 1 H (NH), 4·51, 1 H (α-CH), 3·00, 2·83, 2 H (β-CH_2), 7·17, 5 H (aromatic H); Arg — 7·81, 1 H (NH), 4·28, 1 H (α-CH), 1·76, 1·70, 2 H (β-CH_2), 1·49, 1·35, 2 H (γ-CH_2), 3·06, 2·98, 2 H (δ-CH_2); Tyr — 7·81, 1 H (NH), 4·124, 1 H (α-CH), 3·07, 2·73, 2 H (β-CH_2), 7·09, 6·78, 4 H (aromatic H); Acm — 8·55, 1 H (NH), 4·28, 2 H (CH_2), 1·84, 3 H (CH_3), 1·04, 1·23, 2 × 9 H (Bu^t), 1·38, 9 H (Boc).

Boc-Cys(Acm)-Asn-Ser-Phe-Arg-Tyr-OH (*XVII*)

Boc-Cys(Acm)-ONp (0·17 g; 0·40 mmol) was added to a solution of compound *V* (0·40 g; 0·42 mmol) in DMF (5 ml). After 3 h the solvent was evaporated and the remaining oil was precipitated with ether from isopropyl alcohol to give 0·2 g (56%) of *XVII* as an amorphous powder, R_F 0·56 (A), 0·32 (B), 0·60 (D).

Z-Arg(NO_2)-Tyr(Bu^t)-OBu^t (*XVIII*)

A) Triethylamine (0·83 ml, 6 mmol) was added to a solution of HCl.H-Tyr(Bu^t)-OBu^t (1·96 g; 6 mmol) in DMF (30 ml). The precipitate was filtered off, washed with DMF, the combined filtrates were taken down in vacuo and the residue was dissolved in DMF (30 ml).

B) N-Methylmorpholine (0·56 ml; 5 mmol) was added to a solution of Z-Arg(NO_2)-OH (1·76 g; 5 mmol) in DMF (25 ml), the mixture was cooled to −20°C and isobutyl chloroformate (0·65 ml; 5 mmol) was added, followed after 3 min by the solution prepared ad *A*) (precooled to −20°C). After 2 h the solvent was evaporated, the residue was dissolved in ethyl acetate (100 ml) and the solution was successively washed with 2% sulfuric acid, water, 5% solution of sodium hydrogen carbonate, and water. After drying, the solvent was evaporated and the product was crystallized from ethanol; yield 2·17 g (70%) of compound *XVIII*, m.p. 167−168°C; $[\alpha]_D^{20}$ −0·6° (*c* 1; DMF); R_F 0·46 (H), 0·74 (D), 0·50 (I).

Z-Phe-Arg-Tyr(Bu^t)-OBu^t.HBr (*XIX*)

Compound *XVIII* (1·76 g; 2·8 mmol) was hydrogenated in glacial acetic acid (50 ml) over a Pd/C catalyst. The catalyst was filtered off, the filtrate was taken down and the residue was dissolved in DMF (15 ml). Pyridine hydrobromide (0·45 g; 2·8 mmol), followed by Z-Phe-ONp (1·18 g; 2·8 mmol), was added, the reaction mixture was kept at 20°C for 15 h, the solvent was evaporated, the residue was dissolved in l-butanol (70 ml) and washed with water. After evaporation of the organic layer, the residue was precipitated with ether from isopropyl alcohol to give 1·52 g (67%) of *XIX*, m.p. 98−99°C; $[\alpha]_D^{20}$ −9·7 (*c* 1; DMF); R_F 0·76 (B), 0·71 (D), 0·83 (A).

Z-Ser(Bu^t)-Phe-Arg-Tyr(Bu^t)-OBu^t.HBr (*XX*)

Compound *XIX* (1·47 g; 1·8 mmol) was hydrogenated in methanol (40 ml) over Pd/C catalyst. The catalyst was filtered off, the solvent was evaporated, the residue was dissolved in DMF (20 ml) and Z-Ser(Bu^t)-ONB (0·83 g; 1·8 mmol) was added. After 2 h the solvent was evaporated and the residue was dissolved in l-butanol (50 ml). The organic layer was washed with water, the solvent was evaporated and the residue was precipitated with ether from isopropyl alcohol, affording 1·46 g (83%) of *XX*, m.p. 97−98°C; $[\alpha]_D^{20}$ −10·5° (*c* 1; DMF); R_F 0·78 (B), 0·86 (A), 0·71 (D).

Z-Asn-Ser(Bu^t)-Phe-Arg-Tyr(Bu^t)-OBu^t.HBr (*XXI*)

Compound *XX* (1·40 g) was hydrogenated in methanol (50 ml) over Pd/C catalyst. After removal of the catalyst by filtration, the solvent was removed and the residue was dissolved in DMF (20 ml). To this solution was added Z-Asn-ONp (0·57 g; 1·5 mmol). After 3 h the reaction mixture was taken down, the residue was dissolved in 1-butanol (60 ml) and washed with water. The butanol was evaporated and the residue was precipitated with hexane from isopropyl alcohol to afford 1·28 g (82%) of compound *XXI*, m.p. 143−144°C; $[\alpha]_D^{20}$ −21·7° (*c* 1; DMF); R_F 0·68 (B), 0·80 (A), 0·62 (D).

Boc-Cys(Acm)-Asn-Ser(Bu^t)-Phe-Arg-Tyr(Bu^t)-OBu^t.HBr (*XXII*)

Compound *XXI* (1·25 g; 1·17 mmol) was hydrogenated in methanol (40 ml) over a Pd/C catalyst. The catalyst was filtered off, the solvent was evaporated and the residue was dissolved in DMF (15 ml). To the resulting solution was added Boc-Cys(Acm)-ONp (0·48 g; 1·17 mmol). After 4 h

the solvent was evaporated, the residue was dissolved in 1-butanol and washed with water. The butanol was evaporated and the residue was precipitated with hexane from isopropyl alcohol, giving 1·25 g (92%) of compound *XXII*, m.p. 129–131°C; $[\alpha]_D^{20}$ −37·2 (*c* 1; DMF); R_F 0·71 (A), 0·56 (B), 0·75 (D). ^{1}H NMR, δ: Cys — 6·97, 1 H (NH), 4·19, 1 H (α-CH), 2·85, 2·63, 2 H (β-CH_2); Acm — 8·51, 1 H (NH), 4·29, 4·14, 2 H (CH_2), 1·84, 3 H (CH_3); Asn — 8·05, 1 H (NH), 4·59, 1 H (α-CH), 2·56, 2·45, 2 H (β-CH_2), 7·48, 7·01 2 H ($CONH_2$); Ser — 7·87, 1 H (NH), 4·18, 1 H (α-CH), 3·39, 3·34, 2 H (β-CH_2); Phe — 7·97, 1 H (NH), 4·52, 1 H (α-CH), 3·00, 2·86, 2 H (β-CH_2), 7·20, 5 H (aromatic H); Arg — 7·98, 1 H (NH), 4·34, 1 H (α-CH), 1·70, 1·49, 2 H (β-CH_2), 7·52, 1 H (NH guanidine); Tyr — 8·19, 1 H (NH), 4·32, 1 H (α-CH), 2·90, 2 H (β-CH_2), 6·87, 4 H (aromatic H), 1·04, 1·26, 1·24, 3 × 9 H (Bu^t), 1·38, 9 H (Boc).

Boc-Ala-Gln-Ser(Bu^t)-Gly-Leu-Gly-Cys(Acm)-Asn-Ser-Phe-Arg-Tyr-OH (*XXIII*)

The title dodecapeptide was prepared from H-Cys(Acm)-Asn-Ser-Phe-Arg-Tyr-OH (0·2 g; 0·23 mmol) and Boc-Ala-Gln-Ser-(Bu^t)-Gly-Leu-Gly-ONB (0·2 g; 0·23 mmol) as described for the preparation of *XI*; compound *XXIII* was obtained as an amorphous powder, yield 0·31 g (86%); $[\alpha]_D^{20}$ −2·0° (*c* 1; DMF); R_F 0·58 (A), 0·33 (B), 0·57 (C). Amino acid analysis: Asp 0·92; Ser 1·74; Glu 1·09; Gly 2·00; Ala 1·07; Leu 1·05; Tyr 0·89; Phe 0·97; Arg 0·91.

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