SYNTHESIS OF THREE ANALOGUES OF TUFTSIN WITH 1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID*

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Using 1-aminocyclopropane-1-carboxylic acid**, three analogues of tuftsin were synthesized in which this amino acid is in position 1 (replacement of threonine) or 3 (replacement of proline) or in both these positions, i.e. [Acc^1]tuftsin, [Acc^3]tuftsin and [Acc^1, Acc^3]tuftsin, respectively. The analogues were prepared by the stepwise methodology. During the synthesis, only the amino group in the lysine side chain was protected (with benzyloxycarbonyl group), the guanidine group of arginine was only protonated and the threonine hydroxyl remained free. Since the carboxy group at the carboxyl end was also unprotected, the peptide chain was constructed using the method of active pentafluorophenyl esters. The α-amino groups were protected with tert-butyloxycarbonyl group, except for 1-aminocyclopropane-1--carboxylic acid which was protected with benzyloxycarbonyl group. The former protecting group was removed by treatment with 70% aqueous trifluoroacetic acid, the latter with hydrobromic acid in acetic acid. The final products were purified by HPLC. The whole synthesis is shown in Scheme 1.

EXPERIMENTAL

Melting points were determined on a Kofler block or a Digital Melting Point Analyzer, Model 355 (Fisher) and are uncorrected. Thin-layer chromatography was performed on Merck plates (Kieselgel 60 F-254) in the following solvent systems: A 2-propanol–pyridine–acetic acid–water (10 : 5 : 4 : 4), B 1-propanol–ammonia (7 : 3), C 1-butanol–ethanol–acetic acid–water (80 : 10 : 5 : : 30), D benzene–ethyl acetate–acetic acid (60 : 20 : 1), E 1-butanol–pyridine–acetic acid–water (4 : 1 : 1 : 2), F 1-butanol–pyridine–acetic acid–water (15 : 10 : 3 : 12). Paper electrophoresis was performed in a moist chamber in 5M acetic acid, pH 1·7 on a paper FN-15 (Filtrak,G.D.R.), potential gradient 20 V/cm, 90 min. Spots in TLC and electrophoresis were detected with ninhydrin or by the chlorination method using N,N,N′,N′-tetramethyldiaminodiphenylmethane[2].

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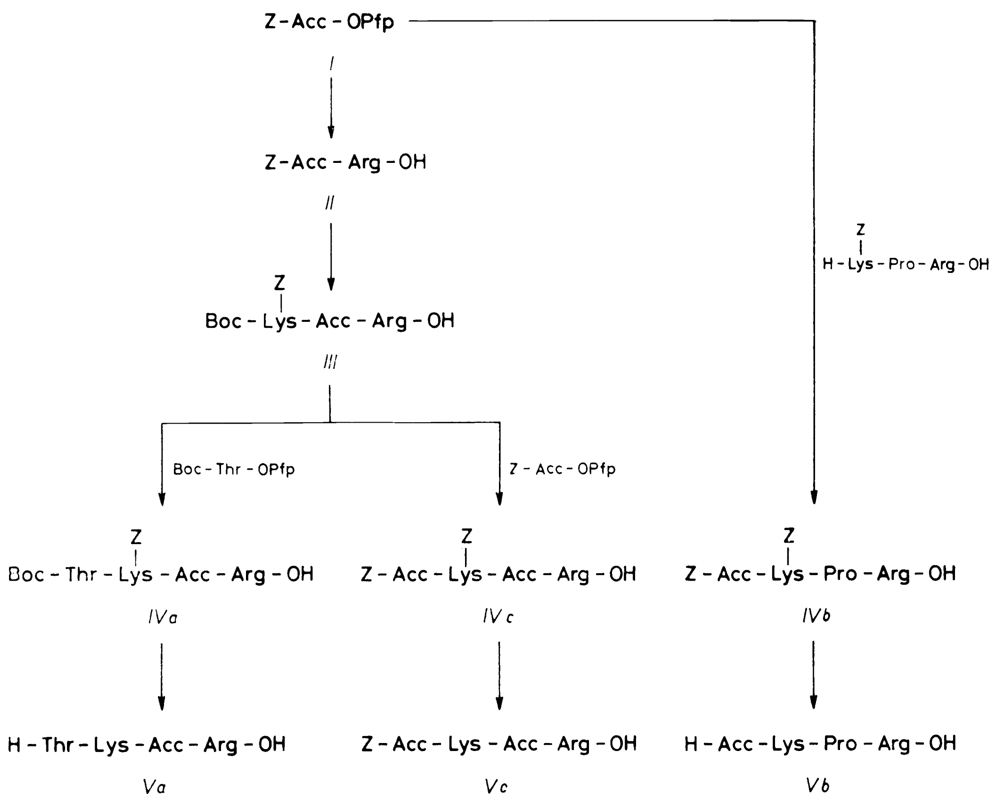
** All the chiral amino acids, mentioned in this work, are of the L-series. The nomenclature and symbols of the amino acids and peptides obey the published recommendations[1]; Acc denotes the 1-aminocyclopropane-1-carboxylic acid, Pfp pentafluorophenol.

Removal of ammonium acetate by freeze-drying was carried out at 50°C and 150 Pa. Samples for amino acid analysis were hydrolyzed with 6M-HCl at 105°C for 20 h and were analyzed on an automatic two-column analyzer 6020 (Developmental Workshops of Czechoslovak Academy of Sciences) or on a D-500 (Durrum, U.S.A.) analyzer. Optical rotations were measured on



a Perkin–Elmer 141 MCA instrument (Norwalk, U.S.A.). High performance liquid chromatography (HPLC) was carried out on an SP-8700 instrument equipped with an SP-8400 detector and an SP-4100 integrator (all from Spectra-Physics), using a Separon SIX C18 (7 μm, 0·32 × 15 cm) column (Laboratorní přístroje, Prague), or on a Du Pont 830 instrument using a Zorbax C_8 column (0·46 × 15 cm). Unless stated otherwise, preparative chromatography was performed on a Du Pont 830 instrument using a 2·12 × 25 cm Zorbax column.

Pentafluorophenyl Benzyloxycarbonyl-1-aminocyclopropane-1-carboxylate (*I*)

Dicyclohexylcarbodiimide (2·2 g; 10·7 mmol) was added at −10°C to a solution of benzyloxycarbonyl-1-aminocyclopropane-1-carboxylic acid[3] (2·1 g; 9·0 mmol) and pentafluorophenol (2·5 g; 13·5 mmol) in ethyl acetate (75 ml) and the reaction mixture was stirred at −10°C for 1 h. After standing overnight at room temperature, the separated dicyclohexylurea was removed by filtration. The filtrate was concentrated, the residue dissolved in a small amount of ether, diluted

with hexane and set aside overnight. The crystalline product was filtered and washed with hexane to give 3·28 g (91%) of ester *I*, m.p. 114·5—115·5°C. TLC: R_F 0·41 (B), 0·81 (D). For $C_{18}H_{12}$.NO_4F_5 (401·3) calculated: 53·88% C, 3·01% H, 23·67% F, 3·49% N; found: 53·80% C, 3·13% H, 23·54% F, 3·35% N.

Benzyloxycarbonyl-1-aminocyclopropane-1-carbonyl-arginine (*II*)

A solution of arginine (0·52 g; 3·0 mmol) in water (7 ml) was added to a vigorously stirred solution of ester *I* (1·3 g; 3·25 mmol) in dioxane (14 ml). The reaction mixture was stirred at room temperature for 2 h, the solvent evaporated and the residue suspended in dimethylformamide (10 ml). The suspension was stirred for 24 h (no free arginine present according to TLC), diluted with ethyl acetate (100 ml) and set aside in a refrigerator overnight. The product was filtered and washed with ethyl acetate and ether; yield 1·07 g (92%) of dipeptide *II*, m.p. 148—150°C, $[\alpha]_D$ −22·5° (*c* 0·48, 10% acetic acid). TLC: R_F 0·76 (A), 0·49 (B), 0·46 (C). For $C_{18}H_{25}N_5O_5$.H_2O (409·4) calculated: 52·80% C, 6·65% H, 17·10% N; found: 53·13% C, 6·65% H, 17·17% N.

N^α-Tert-butyloxycarbonyl-N^ε-benzyloxycarbonyllysyl-
-1-aminocyclopropane-1-carbonyl-arginine (*III*)

To a solution of dipeptide *II* (0·98 g; 2·5 mmol) in acetic acid (4 ml) was added 35% HBr in acetic acid (4 ml). After standing for 1 h at room temperature, the reaction mixture was diluted with ether. The separated hydrobromide was triturated several times with ether, filtered, dried in a desiccator (NaOH) overnight, dissolved in water (20 ml) and the solution was adjusted to pH 9·0 with Dowex 1 × 2 (OH^- form). The ion exchanger was filtered off and washed with water. Water was evaporated from the combined filtrates, the remaining dipeptide was dissolved in water (5 ml) and the solution poured into a stirred solution of pentafluorophenyl ester of N^α-tert-butyloxycarbonyl-N^ε-benzyloxycarbonyllysine[4] (1·7 g; 3 mmol) in dioxane (10 ml). The reaction mixture was stirred at room temperature for 2 h, the solvent evaporated and the residue dissolved in dimethylformamide (4 ml). After stirring for 24 h (TLC in system C detected no starting dipeptide), the solvent was evaporated and the residue crystallized from ethanol (3 ml), ethyl acetate (9 ml) and ether (90 ml). Filtration and washing with ether afforded the crude product which consisted of three compounds (TLC in (C): R_F 0·86, 0·52 and 0·33; HPLC in methanol–0·05% aqueous trifluoroacetic acid (70 : 30): *k* 0·29, 3·00, 5·43). The crude product was purified by HPLC on a reversed phase (350 mg batches; 1·2 × 25 cm column, Separon SIC_{18}; methanol-0·2M-CH_3COONH_4 55 : 45; flow rate 4·5 ml/min). Three fractions were obtained: 1) fraction 19·5—44·5 ml, R_F 0·33 (C), contained Acc and Arg in the ratio 1 : 1 (amino acid analysis). According to IR spectrum the compound is likely a diketopiperazine: 3 220, 1 680, 1 670 cm^{-1} (*cis*-CONH); *2*) fraction 84 – 100 ml, R_F 0·86 (C), according to amino acid analysis it contained only Lys; *3*) fraction 108—138 ml; R_F 0·52 (C), 0·91 (A), contained the desired product. The solvent was evaporated, the ammonium acetate removed by freeze-drying and the residue crystallized from ethanol–ethyl acetate–ether (1 : 3 : 30). Total yield of the pure tripeptide *III* was 410 mg (24%); m.p. 128—131°C, $[\alpha]_D$ −9·9° (*c* 0·3, 10% acetic acid). Amino acid analysis: Lys 1·02, Arg 0·97, Acc 1·03. For $C_{29}H_{45}N_7O_8$.1·5 H_2O (646·7) calculated: 53·86% C, 7·48% H, 15·16% N; found: 54·22% C, 7·12% H, 14·87% N.

N^α-Tert-butyloxycarbonylthreonyl-N^ε-benzyloxycarbonyllysyl-
-1-aminocyclopropane-1-carbonyl-arginine (*IVa*)

The protected tripeptide *III* (270 mg; 0·437 mmol) was dissolved in 70% aqueous trifluoroacetic acid (3 ml). After 90 min, the mixture contained (according to TLC) exclusively N^ε-benzyloxy-

carbonyllysyl-1-aminocyclopropane-1-carbonyl-arginine (R_F 0·59 in (A)) and no N$^{\alpha}$-protected tripeptide. After 2 h the trifluoroacetic acid was evaporated, and the residue coevaporated with ethanol and benzene. The obtained material was dried in a desiccator (NaOH, P_2O_5) overnight, then dissolved in water (15 ml) and the solution adjusted to pH about 9 by addition of Dowex 1×8. The ion-exchanger was filtered off, washed with water and the filtrates were taken down. The residue was dissolved in water (2 ml) and added to a stirred solution of pentafluorophenyl ester of tert-butyloxycarbonylthreonine[4] (200 mg; 0·52 mmol) in dioxane (5 ml). After stirring at room tempeature for 2 h, the dioxane was evaporated and the residue stirred with dimethylformamide (1 ml) overnight. According to TLC in the system (A), no free tripeptide was already present. The reaction mixture was diluted with ethyl acetate and kept in a refrigerator. The separated tetrapeptide *IVa* was collected and washed with ethyl acetate; yield 153 mg (49%), m.p. 138–141°C, $[\alpha]_D$ −15·5° (*c* 0·4, 10% acetic acid). R_F 0·92 (A), 0·39 (C), 0·68 (F). For $C_{33}H_{52}N_8O_{10}.3\,H_2O$ (774·9) calculated: 51·15% C, 7·54% H, 14·46% N; found: 50·82% C, 7·01% H, 14·60% N.

Benzyloxycarbonyl-1-aminocyclopropane-1-carbonyl-N$^{\varepsilon}$-
-benzyloxycarbonyllysyl-prolyl-arginine (*IVb*)

The Boc group in N$^{\alpha}$-tert-butyloxycarbonyl-N$^{\varepsilon}$-benzyloxycarbonyllysyl-prolyl-arginine[5] (0·32 g; 0·5 mmol) was removed in the same manner as described for the tripeptide *III*. The obtained free tripeptide (233 mg; 0·44 mmol) was dissolved in water (1·5 ml) and added to a solution of active ester *I* (200 mg; 0·5 mmol) in dioxane (4·5 ml). After stirring at room temperature for 2 h, the solvents were evaporated, and the residue was stirred with dimethylformamide (1 ml) overnight (according to TLC in the system (A), no free tripeptide was then present). The reaction mixture was diluted with ethyl acetate, set aside in a refrigerator for several hours and the separated tetrapeptide collected and washed with ethyl acetate. Yield 0·31 g (94%) of tetrapeptide *IVb*, m.p. 126–129°C, $[\alpha]_D$ −43·6° (*c* 0·8, 10% acetic acid). R_F 0·64 (A), 0·52 (B), 0·30 (C). HPLC in acetonitrile–0·2M ammonium acetate (30 : 70): *k* 6·62. For $C_{37}H_{50}N_8O_9.2{\cdot}5\,H_2O$ (795·9) calculated: 55·84% C, 6·96% H, 14·08% N; found: 55·71% C, 6·48% H, 14·12% N.

Benzyloxycarbonyl-1-aminocyclopropane-1-carbonyl-N$^{\varepsilon}$-
-benzyloxycarbonyllysyl-1-aminocyclopropane-1-carbonyl-arginine (*IVc*)

The Boc group in protected tripeptide *III* (270 mg; 0·437 mmol) was removed in the same manner as described for the preparation of tetrapeptide *IVa*. After evaporation of solvents, the remaining free tripeptide was dissolved in water (1·5 ml) and added to a stirred solution of the active ester *I* (200 mg; 0·5 mmol) in dioxane (4 ml). The further reaction course and work-up procedure were analogous as in the case of compound *IVa*. The separated tetrapeptide was filtered, washed with ethyl acetate and dried in a desiccator. Yield 200 mg (62% of tetrapeptide *IVc*, m.p. 132–134°C, $[\alpha]_D$ −5·6° (*c* 0·32, 10% acetic acid)). TLC: R_F 0·92 (A), 0·37 (C), 0·64 (F). For $C_{36}H_{48}N_8O_9$. .CF_3COOH (850·9) calculated: 53·64% C, 5·80% H, 13·17% N; found: 53·63% C, 6·05% H, 13·40% N.

Threonyl-lysyl-1-aminocyclopropane-1-carbonyl-arginine ([Acc^3]tuftsin *Va*)

To a solution of tetrapeptide *IVa* (135 mg) in acetic acid (1 ml) was added 3·8M-HBr in acetic acid (1 ml). After standing at room temperature for 1 h, the reaction mixture was diluted with ether. The precipitated hydrobromide was collected on filter, dried in a desiccator over sodium hydroxide, dissolved in water and the solution adjusted to pH 9 by addition of Dowex 1×8.

The ion exchanger was filtered, washed with water and the combined filtrates were concentrated. The residue consisted of two compounds (TLC: R_F 0·41 and 0·55 (A); HPLC in 0·2M ammonium acetate–acetonitrile (99 : 1): k 1·12 and 2·0). The material was purified by HPLC on a Zorbax C_8 column in 0·2M ammonium acetate. The solvent was evaporated and ammonium acetate removed by freeze-drying. The residue was dissolved in water and again freeze-dried to give 42 mg (46%) of tuftsin *Va*, m.p. 110–120°C, $[\alpha]_D$ −5·1° (*c* 1·0, 10% acetic acid). TLC: R_F 0·41 (A), 0·07 (E), 0·17 (F); $E_{1.7}^{His}$ 1·03. Amino acid analysis: Lys 1·01, Arg 1·03, Thr 1·00, Acc 0·92.

1-Aminocyclopropane-1-carbonyl-lysyl-prolyl-arginine ([Acc^1]tuftsin *Vb*)

Tetrapeptide *IVb* (280 mg) was deprotected as described for tetrapeptide *IVa*. The obtained deprotected product consisted of two compounds as shown by TLC (R_F 0·44, 0·56 (A)) as well as HPLC (k 1·57, 2·86 in 0·2M ammonium acetate–acetonitrile 99 : 1); only one peak in a mixture of 0·1M ammonium acetate and acetonitrile. The material was purified by HPLC in 0·2M ammonium acetate–acetonitrile (99 : 1). The solvent was evaporated, the ammonium acetate removed by freeze-drying, the residue dissolved in water and again freeze-dried to give 40 mg (22%) of tuftsin *Vb*, m.p. above 185–195°C. TLC: R_F 0·44 (A), 0·07 (E), 0·19 (F); $E_{1.7}^{His}$ 1·10; $[\alpha]_D$ −57·9° (*c* 0·75, 10% acetic acid). Amino acid analysis: Lys 1·06, Arg 1·02, Pro 0·99, Acc 0·92.

1-Aminocyclopropane-1-carbonyl-lysyl-1-aminocyclopropane-
-1-carbonyl-arginine ([Acc^1, Acc^3]tuftsin *Vc*)

Tetrapeptide *IVc* (180 mg) was deprotected in the same manner as described for tetrapeptide *IVa*. Since the deprotection product was completely pure (TLC and HPLC), it was only freeze-dried from its aqueous solution to afford 100 mg (88%) of product melting above 215–220°C, $[\alpha]_D$ −17·2° (*c* 0·16, 10% acetic acid); HPLC in 0·2M ammonium acetate–acetonitrile (99 : 1): k 1·62; TLC: R_F 0·41 (A), 0·09 (E), 0·13 (F); $E_{1.7}^{His}$ 1·06. Amino acid analysis: Lys 1·03, Arg 1·10, Acc 1·85.

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