SYNTHESIS AND PROPERTIES OF TUFTSIN, L-THREONYL-L-LYSYL-L-PROLYL-L-ARGININE*

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L-Thr-L-Lys-L-Pro-L-Arg (*Ia*) was prepared by classical procedures. The α-amino groups were protected by the 2-nitrobenzenesulfenyl group and the guanidino group of arginine was blocked by the nitro group; in the remaining cases, the benzyl-type residues were used as protecting groups. In the final stage, protecting groups were removed by the action of liquid hydrogen fluoride. The tetrapeptide *Ia* was characterised by means of circular dichroism and biological assays (stimulation of phagocytosis).

In connection with our investigations on basic histon-like peptides[1,2], attention has been now paid to two reported tetrapeptides, the structure of which resembles the histon fragments, namely, L-Thr-L-Lys-L-Pro-L-Arg** (*Ia*) (capable of inducing phagocytosis[4]) and L-Thr-L-Pro-L-Arg-L-Lys (active on hamster ovaries[5]). The former tetrapeptide was synthesised with the use of the solid-phase method without characterising chemically the product[6]. The recently published[7] synthesis of compound *Ia* by a classical route in solution prompted us to report the synthesis of the same compound as developed in this Laboratory.

The main aspects taken into consideration in the choice of tactics of the present synthesis consisted in continuity with the preparation of histon-like peptides and in a facile approach to the preparation of analogues. The synthesis is shown in Scheme 1. The starting protected dipeptide L-lysyl-L-proline was simultaneously used as the starting material in the synthesis[1] of histon-like polypeptides poly(L-Ala-L-Lys-L-Pro), poly(L-Ala-L-Lys-L-Pro-L-Lys), and poly(L-Ala-L-Lys-L-Lys-L-Pro-L-Lys). The attachment of the L-threonine residue to this dipeptide with the use of an activated ester (2,4,5-trichlorophenyl or pentachlorophenyl) cannot be considered successful because of the necessity of purifying chromatographically both the activated ester of L-threo-

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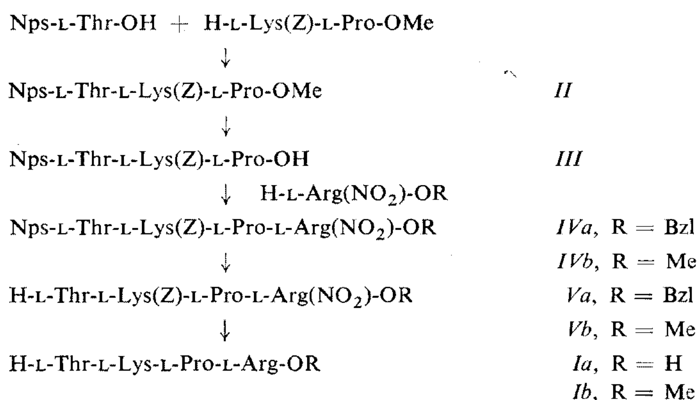
** The nomenclature and abbreviations follow suggestions reported elsewhere[3].

nine (*cf.*[8]) and the resulting protected tripeptide *II*. Also the direct condensation of 2-nitrobenzenesulfenyl-L-threonine with L-lysyl-L-proline methyl ester in the presence of N,N'-dicyclohexylcarbodiimide was far from being smooth. In addition to the required protected tripeptide *II* (isolated in 50% yield), a less polar by-product was obtained in 10–15% yield; this by-product had to be separated by column chromatography on silica gel. However, with the use of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline as condensing agent, the protected tripeptide resulted in a considerably higher yield and was free of the by-product.



SCHEME 1

In the condensation by means of N,N'-dicyclohexylcarbodiimide, the amino-acid analysis of the by-product indicated the presence of the same amino acids as in the case of the main product *II*, but the elemental analysis of the by-product exhibited a considerably higher content of carbon; an addition of N,N'-dicyclohexylcarbodiimide cannot thus be excluded[9].

The further synthetic steps such as saponification to the acid *III* and condensation of this acid with N^G-nitro-L-arginine benzyl ester in the presence of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, afforded satisfactory yields of the required products. The removal of blocking groups from the protected tetrapeptide *IVa* by the action of liquid hydrogen fluoride yielded a free tetrapeptide retaining aromatic compounds obviously formed as by-products during the removal of blocking groups, probably under participation of the anisol added[10]. It proved advisable to remove the blocking groups in two steps. The first step consisted in the removal of the 2-nitrobenzenesulfenyl group by ethereal hydrogen chloride with the formation of the ester hydrochloride *Va*, in the next step, the remaining blocking groups were removed using liquid hydrogen fluoride. The complete removal of blocking groups was checked by means of UV and CD spectra of compounds *IVa*, *Va*, and *Ia*. Compound *Ia*

does not contain more than 2% of unremoved nitro groups. In biological assays, the tetrapeptide *Ia* induced phagocytosis in accordance with the literature[4].

Condensation of *III* with N^G-nitro-L-arginine methyl ester afforded 2-nitrobenzenesulfenyl-L-threonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-prolyl-N^G-nitro-L-arginine methyl ester (*IVb*), the blocking groups of which were removed in two steps (analogously to the preparation of compound *Ia*) with the formation of the methyl ester *Ib*.

In view of the presence of four different ionogenic groups, tuftsin (*Ia*) appears as an interesting object of investigations on chiroptical properties at various pH values. Furthermore, the methyl ester *Ib* is a suitable model compound with a blocked carboxylic function. From titrimetric curves in the pH range 3–10, equivalence points were determined (*Ia*, pH 8·5 and 4·9; *Ib*, 8·5 and 5·5). Table I shows CD parameters of compounds *Ia* and *Ib* in strongly alkaline medium, in equivalence points, and in strongly acidic medium. Three bands may be observed on the curves, a negative one ($n - \pi^*$ transition) at 240 nm, a positive one ($n - \pi^*$ transition) at 220 nm, and a negative one ($\pi - \pi^*$ transition) at 185 nm. In strongly acidic medium, the CD characteristics of compounds *Ia* and *Ib* are almost the same. The shift into the alkaline region is accompanied by insignificant quantitative changes in the CD spectrum of *Ib* while in the CD spectrum of tuftsin (*Ia*), the long-wavelength negative band intensifies and, simultaneously, the band at 220 nm almost disappears. In the strongly acidic medium, both compounds *Ia* and *Ib* may be assumed to exist in extended conformation in view of the repulsive interactions of the three positive charges.

TABLE I

CD Parameters of Tuftsin (*Ia*) and its Methyl Ester *Ib* in Aqueous Media of Various pH Values

Parameter	*Ia*			*Ib*		
	pH 2	4·9	8·5	pH 1	5·5	8·5
λ_{max}[a] ([θ])[b]	244 (−150)	241 (−340)	235 (−120)	243 (−230)	244 (−210)	238 (−480)
Cross[a]	241	236	226	239	239	233
λ_{max}[a] ([θ])[b]	218 (+18 000)	221 (+6 000)	223[d] (+250)	217 (+12 000)	218 (+15 800)	220 (+5 600)
Cross[c]	207	214	221	208	208	211
λ_{max}[a] ([θ])[b]	195 (−66 000)	198 (−58 000)	196 (−58 000)	195 (−62 000)	194 (−83 000)	196 (−47 000)

[a] Band maximum, wavelength in nm; *b* molar elipticity in deg cm^2 decimol^{-1}; [c] cross point of CD curve with zero elipticity line; [d] in 0·2M-NaOH: λ_{max} 223 nm, [θ] +2300.

The conformational arrangement changes after dissociation of the carboxylic function, possibly due to an ionic interaction between the carboxylate anion and the ammonium cation in the side chain of lysine. This idea on a pseudocyclic arrangement of the molecule in media of non-extreme pH values is not at variance with analysis of space--filling models.

EXPERIMENTAL

Melting points were taken on a heated microscope stage (Kofler microblock) and are not corrected. Samples for elemental analysis were dried over phosphorus pentoxide at 1 Torr and room temperature for 12 h. Thin-layer chromatography was carried out on silica gel plates (Kieselgel G, Merck) in the solvent systems S_1, 2-butanol–25% aqueous ammonia–water (85 : 7·5 : 7·5), and S_2, 2-butanol–90% aqueous formic acid–water (75 : 13·5 : 11·5). Electrophoresis was carried out on Whatman paper No 3 MM for 1 h at a potential drop of 20 V cm^{-1} in buffer solutions 1M acetic acid (pH 2·4) and pyridinium acetate (pH 5·7). Spots were detected by ninhydrin and by chlorination. Samples for amino acid analyses were hydrolysed at 105°C for 20 h in 6M-HCl (in ampoules sealed at 1 Torr). The analyses were carried out on an automatic two-column analyser (Type 6020; Development Workshops, Czechoslovak Academy of Sciences, Prague). Solutions were taken down under diminished pressure on a rotatory evaporator (bath temperature 40°C). Solutions were dried over anhydrous sodium sulfate. The UV spectra were measured on a Specord UV VIS apparatus (Carl Zeiss, Jena), the CD spectra on a Roussel-Jouan Dichrograph CD 185 Model II apparatus, and the IR spectra on a UR 10 apparatus (Carl Zeiss, Jena). Titrimetric curves were measured with 0·1M-HCl on a Titrator TTT 1c with a glass electrode and Titrigraph SBR 2c (Radiometer Copenhagen).

2-Nitrobenzenesulfenyl-L-threonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-proline Methyl Ester (*II*)

A. A solution of 2-nitrobenzenesulfenyl-L-threonine dicyclohexylammonium salt[11] (9 g) in chloroform (150 ml) was treated at room temperature with N^{ε}-benzyloxycarbonyl-L-lysyl--L-proline methyl ester bis(4-toluenesulfonyl)imide salt[1] (14 g) and then at 0°C with N,N′-dicyclohexylcarbodiimide (4·6 g). The mixture was kept at 0°C overnight, evaporated, the residue triturated with ethyl acetate, and the solid filtered off. The filtrate was washed with 0·5M-H_2SO_4, 0·5M-$NaHCO_3$, and water, dried, and evaporated. The residue was dissolved in chloroform and the solution applied to a column of silica gel (350 g; particle size, 30—60 μm). Elution with chloroform (750 ml total) yielded a fraction containing a yellow foamy substance (0·40 g), homogeneous on chromatography. Amino acid analysis: Thr 0·8, Lys 1·0, Pro 1·1. The remaining chloroform fractions (1 800 ml total) contained 6·5 g (50%) of the non-crystalline compound *II*, homogeneous on chromatography in the solvent system S_1. For $C_{30}H_{39}N_5O_9S$ (645·7) calculated: 55·80% C, 6·08% H, 10·84% N; found: 56·04% C, 6·31% H, 10·55% N. Amino-acid analysis: Thr 0·97, Lys 0·97, Pro 1·00. $[\alpha]_D$ −72·7° (*c* 0·51, dimethylformamide).

B. 1-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (2·0 g) was added to a solution of 2-nitrobenzenesulfenyl-L-threonine dicyclohexylammonium salt (2·8 g) and N^{ε}-benzyloxycarbonyl--L-lysyl-L-proline methyl ester bis(4-toluenesulfonyl)imide salt (4·4 g) in chloroform (100 ml). The mixture was stirred at room temperature overnight, evaporated, the residue dissolved in ethyl acetate, the solution washed with 0·5M-H_2SO_4, 0·5M-$NaHCO_3$, and water, dried, and evaporated. The residue was chromatographed on a column of silica gel (100 g) with chloroform as eluant. The first fraction (380 ml) of the effluent was followed by the main fraction (720 ml) containing

exclusively the required product *II* as determined by thin-layer chromatography of a sample. Evaporation yielded 2·95 g (77%) of a non-crystalline substance, identical with the specimen prepared by procedure *A*. The identity of the two compounds was also confirmed by removal of the 2-nitrobenzenesulfenyl group and electrophoresis at pH 5·7.

2-Nitrobenzenesulfenyl-L-threonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-proline (*III*)

A solution of the protected tripeptide *II* (6·4 g) in acetone (50 ml) was treated with 1M-NaOH (11 ml), the mixture stirred at 20°C for 1 h, diluted with water (50 ml), and acetone evaporated. The remaining solution was filtered, the filtrate acidified with 0·5M-H_2SO_4, and extracted with ethyl acetate. The extract was dried, evaporated, and the residue triturated with ether to afford 4·9 g (79%) of a yellow noncrystalline substance *III*, free of any ester *II* on chromatography in the solvent system S_1. For $C_{29}H_{37}N_5O_9S$ (631·7) calculated: 55·15% C, 5·89% H, 11·08% N; found: 55·48% C, 6·24% H, 10·66% N. Amino acid analysis: Thr 0·95, Lys 1·06, Pro 1·00. $[\alpha]_D$ −69·5° (*c* 0·49, dimethylformamide).

N^G-Nitro-L-arginine Benzyl Ester

Thionyl chloride (2·0 ml) was added dropwise at −30°C to a stirred suspension of N^G-nitro-L-arginine (2·20 g) in benzyl alcohol (15 ml). Stirring was continued at room temperature for 2 days. Thionyl chloride was then evaporated and the residue poured into ether. The solid was collected, dissolved in water, the aqueous solution washed with ether, made alkaline with aqueous ammonia, and extracted with chloroform. Ammonia was then evaporated from the aqueous phase and the residual solution (pH 8) kept at 0°C overnight to deposit 0·61 g (28%) of N^G-nitro-L-arginine (m.p. 254−256°C). The above chloroform extracts were washed with three portions of aqueous ammonia, dried, and evaporated. The residue solidified on trituration with ether at 0°C. The solid was collected and washed with ether; yield, 1·50 g (67%, referred to the reacted N^G-nitro-L-arginine); m.p. 54−56°. Recrystallisation from ethyl acetate–ether yielded 1·25 g of the title benzyl ester, m.p. 59−62°C, $[\alpha]_D$ −2·8° (*c* 0·49, 50% aqueous methanol). For $C_{13}H_{19}N_5O_4$ (309·3) calculated: 50·48% C, 6·19% H, 22·64% N; found: 50·11% C, 6·38% H, 22·83% N. The substance is not hygroscopic in contrast to the hydrochloride or 4-toluenesulfonate reported in literature[13,14].

2-Nitrobenzenesulfenyl-L-threonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-prolyl-N^G-nitro-L-arginine Benzyl Ester (*IVa*)

A mixture of the acid *III* (1·86 g), N^G-nitro-L-arginine benzyl ester (1·02 g), 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (1·0 g), and chloroform (15 ml) was stirred at room temperature for 24 h, treated with additional 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (0·30 g), and stirred for 24 h more. The mixture was washed with three portions of 0·15M-H_2SO_4, water, two portions of 0·5M-$NaHCO_3$, water again, dried, and evaporated. As shown by thin-layer chromatography (in 95 : 5 chloroform–methanol), the residual yellow powder (2·85 g) contains the required product along with two by-products of lower and higher R_F values. A portion of the residue (2·0 g) was dissolved in chloroform (10 ml) and the solution applied to a column of silica gel (190 g; particle size, 30−60 μm). The column was washed with chloroform (200 ml; 0·6% of ethanol as stabilizer) and eluted with 96 : 4 (v/v) chloroform–methanol. The content of eluates was checked by thin-layer chromatography. The fraction containing the main product was evaporated, the residue dissolved in chloroform (15 ml), the solution filtered, and the filtrate evaporated to afford 1·12 g (59%) of compound *IVa*, homogeneous on chromatography. For

$C_{42}H_{54}N_{10}O_{12}S$ (923·0) calculated: 54·66% C, 5·90% H, 15·18% N; found: 54·71% C, 6·06% H, 14·70% N. Amino acid analysis: Thr 0·93, Lys 1·18, Pro 1·00, Arg 0·75. $[\alpha]_D$ −52·8° (*c* 0·53, dimethylformamide). UV spectrum (methanol): λ_{max} 385 nm (log ε 3·62), λ_{max} 270 nm (log ε 4·00), λ_{max} 250 nm (log ε 4·27). CD spectrum (methanol): λ_{max} 387 nm, [θ] 1870°; 360 nm [θ] 1 920°; 306 nm, [θ] 2520° ([θ] molar elipticities in deg cm^2 $dmol^{-1}$).

L-Threonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-prolyl-N^G-nitro-L-arginine Benzyl Ester Hydrochloride (*Va*)

Ethereal hydrogen chloride (0·5 ml of 2·2M solution) was added to a solution of the tetrapeptide *IVa* (250 mg) in ethyl acetate (5 ml) and the suspension was evaporated to remove hydrogen chloride and ether. The residue was triturated with ether, the solid collected with suction, dissolved in a mixture of ethyl acetate and methanol, and precipitated with ether. Yield, 200 mg (92%) of compound *Va* containing according to electrophoresis (pH 5·7) a trace of another less mobile compound. For $C_{36}H_{52}ClN_9O_{10}.2\,H_2O$ (842·4) calculated: 51·33% C, 6·70% H, 14·96% N; found: 51·96% C, 6·63% H, 14·71% N. $[\alpha]_D$ −25·2° (*c* 0·31, methanol). UV spectrum (water): λ_{max} 269 nm (log ε 4·20), λ_{max} 204 nm (log ε 4·53). CD spectrum (methanol): λ_{max} 308nm, [θ] 1 450° (deg cm^2 $dmol^{-1}$).

L-Threonyl-L-lysyl-L-prolyl-L-arginine (*Ia*)

The hydrochloride of compound *Va* (200 mg) was deblocked according to Sakakibara and coworkers[12] by the action of liquid hydrogen fluoride at 0°C for 40 min in the presence of anisol (1 ml). The mixture was evaporated, the residue dried, and dissolved in 10% aqueous acetic acid. The solution was washed with the solvent mixture ethyl acetate–ether and concentrated. The concentrate was applied to a column (10 ml) of Amberlite IR-4B (acetate) ion exchange resin (pH 2·4) and the column eluted with water (30 ml). The effluent was freeze-dried to afford 107 mg (52%) of compound *Ia*, homogeneous on electrophoresis at pH 5·7. For $C_{21}H_{40}N_8O_6.3\,CH_3.COOH.3\,H_2O$ (734·8) calculated: 44·13% C, 7·96% N, 15·25% N; found: 44·28% C, 8·35% H, 15·80% N. Amino acid analysis: Thr 0·98, Lys 1·02, Pro 1·00, Arg 1·01. $[\alpha]_D$ −59·9° (*c* 0·50, 1M acetic acid); reported[7] for a compound formulated on the basis of elemental analysis as $C_{21}H_{40}.N_8O_6.2\,CH_3COOH.1{\cdot}5\,H_2O$, $[\alpha]_D$ −60·8° (*c* 0·6, 5% aqueous acetic acid). CD spectrum (methanol): λ_{max} 217 nm, [θ] −61 000° (deg cm^2 $dmol^{-1}$).

2-Nitrobenzenesulfenyl-L-threonyl-N^{ε}-benzyloxycarbonyl-L-lysyl-L-prolyl-N^G-nitro-L-arginine Methyl Ester (*IVb*)

A mixture of the acid *III* (632 mg), N^G-nitro-L-arginine methyl ester hydrochloride (270 mg), N-ethylpiperidine (0·137 ml), 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (280 mg), and dimethylformamide (5 ml) was stirred for 24 h at room temperature, treated with another portion of 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (100 mg), and the stirring continued for 24 h more. The mixture was then evaporated, the residue dissolved in chloroform, the solution washed with three portions of 0·15M-H_2SO_4, water, two portions of 0·5M-$NaHCO_3$, water again, dried, and evaporated. According to the thin-layer chromatography (in chloroform–methanol 92 : 8) of a sample of the residue, the main component is accompanied by two compounds, one of a lower R_F value and the other of a higher R_F value. The residue was dissolved in chloroform (3 ml) and the solution applied to a column of silica gel (50 g; particle size, 30–60 μm). The elution was performed with chloroform–methanol 96 : 4 (the chloroform was stabilised with 0·6% of ethanol). The content of the eluate was checked by thin-layer chromatography.

The fraction containing the main product was evaporated, the residue dissolved in chloroform (5 ml), the solution filtered, and the filtrate evaporated to afford 506 mg (60%) of compound *IVb* as a yellow solid foam, homogeneous on chromatography. For $C_{36}H_{50}O_{12}S.H_2O$ (864·9) calculated: 50·02% C, 6·09% H, 15·92% N; found: 49·99% C, 6·06% H, 16·19% N.

L-Threonyl-L-lysyl-L-prolyl-L-arginine Methyl Ester (*Ib*)

Ethereal hydrogen chloride (0·3 ml of 2M solution) was added to a solution of compound *IVb* (120 mg) in ethyl acetate (3 ml), the precipitate decanted with four 30 ml portions of ether, triturated with ether, and dried; yield, 95 mg of compound *Vb*. A portion of the product *Vb* (65 mg) was treated at −60°C with 0·3 ml of anisol and 5 ml of liquid hydrogen fluoride, the mixture kept at 0°C for 35 min, dried at 30°C/1 Torr. The residual liquid was precipitated with ether (150 ml), the precipitate decanted with two 150 ml portions of ether, dried at 20°C/15 Torr, and repeatedly dissolved in water and freeze-dried. Yield, 53 mg of compound *Ib*. For $C_{22}H_{42}.N_8O_6.3\ HF.3\ H_2O$ (514·6) calculated: 41·61% C, 7·46% H, 17·69% N; found: 41·99% C, 7·69% H, 17·26% N.

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