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Synthesis of Tuftsin and Its Analogs

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Tuftsin, a phagocytosis stimulating peptide, was synthesized according to a liquid phase method together with its four analogs, H–Thr–Arg–Pro–Arg–OH (**2**), H–Thr–Lys–Pro–Lys–OH (**3**), H–Thr–Arg–Pro–Lys–OH (**4**), and H–Arg–Pro–Lys–Thr–OH (**5**).

Tuftsin (**1**), a tetrapeptide containing two basic amino acids, lysine and arginine, was isolated from the enzymatic digest of γ-globulin[1)] and synthesized according to Merrifield's solid phase method by Nishioka *et al.*[2)] They also synthesized two analogs of **1** lacking N- or C-terminal amino acid of the original peptide, *viz.*, H–Lys–Pro–Arg–OH and H–Thr–Lys–Pro–OH, and another analog having an additional proline, H–Thr–Lys–Pro–Pro–Arg–OH.[3)] None of these analogs exhibited the phagocytosis stimulating activity, but the pentapeptide, H–Thr–Lys–Pro–Pro–Arg–OH, strongly inhibited tuftsin activity. This indicates that a certain restriction of the primary structure is required for biological activity to appear. However, the relationship between structure and activity is still unclarified. In order to investigate the effect of the replacement of lysine by arginine, or arginine by lysine, we synthesized the following tuftsin analogs (**2**—**5**) as well as **1** by a liquid phase method. **2**, *i.e.*, [2-arginine]-tuftsin, and **3**, *i.e.*, [4-lysine]-tuftsin are analogs in which either one of the two basic amino acids is replaced by the other. **4**, *i.e.*, [2-arginine, 4-lysine]-tuftsin has a sequence in which the positions of lysine and arginine are exchanged. **5**, the analog with the retro sequence of **1**, was also synthesized in order to see the effect of alternation of the primary structure.

The synthesis of **1** and its analogs is summarized in Charts 1—5. Benzyl ester was employed for the protection of carboxyl functions of C-terminal amino acids. In each step of the peptide elongation, *t*-butoxycarbonyl amino acids were used as the acylating reagent except in the final stages of the elongation. The *t*-butoxycarbonyl group was removed by HCl/THF or trifluoroacetic acid treatment prior to the next coupling. The ε-amino group of lysine and the guanidyl group of arginine were protected by benzyloxycarbonyl and nitro group, respectively. A mixed anhydride method was employed for the coupling reaction except at the introduction of N^{α}-*t*-butoxycarbonyl, N^{g}-nitro arginine, when the dicyclohexyl carbodiimide/*N*-hydroxy-1*H*-benzotriazole method[4)] was used. Some of the intermediates were obtained as oily substance with a trace of impurities detectable

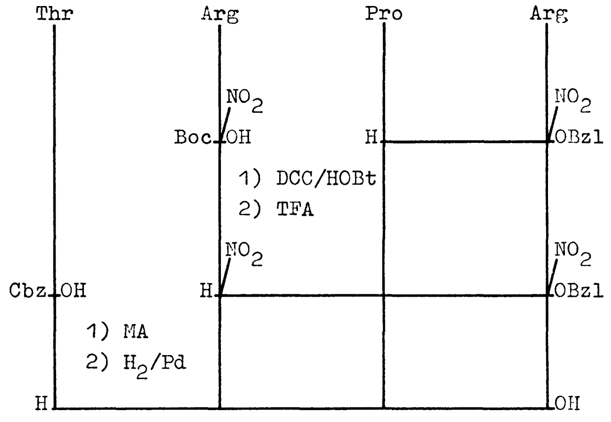

Chart 2. Preparation of **2**.

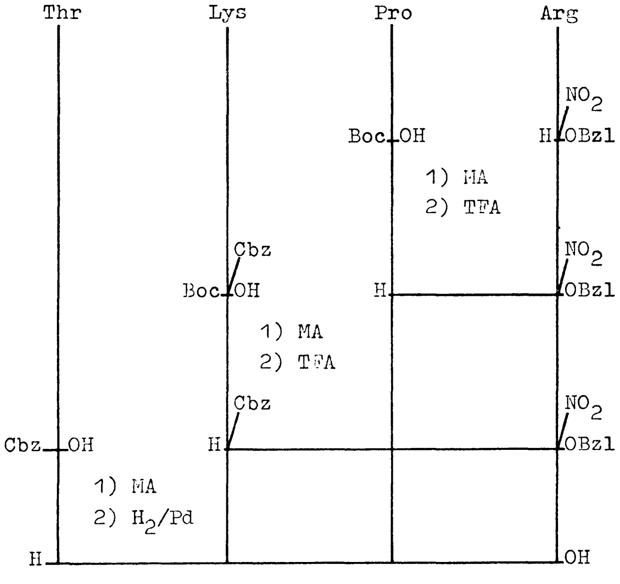

Chart 1. Preparation of **1**.

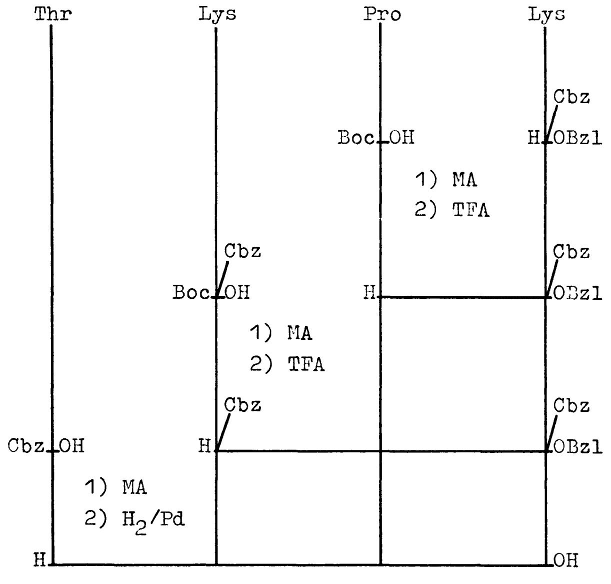

Chart 3. Preparation of **3**.

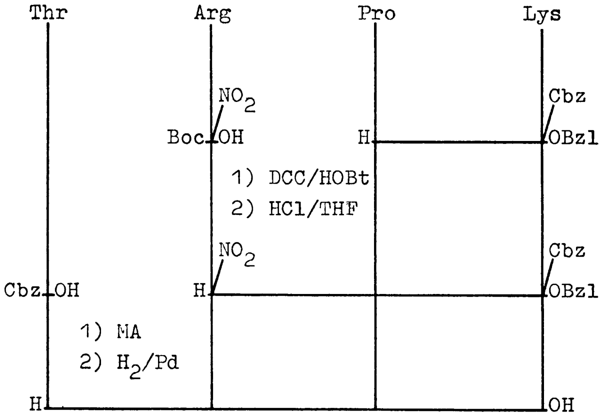

Chart 4. Preparation of **4**.

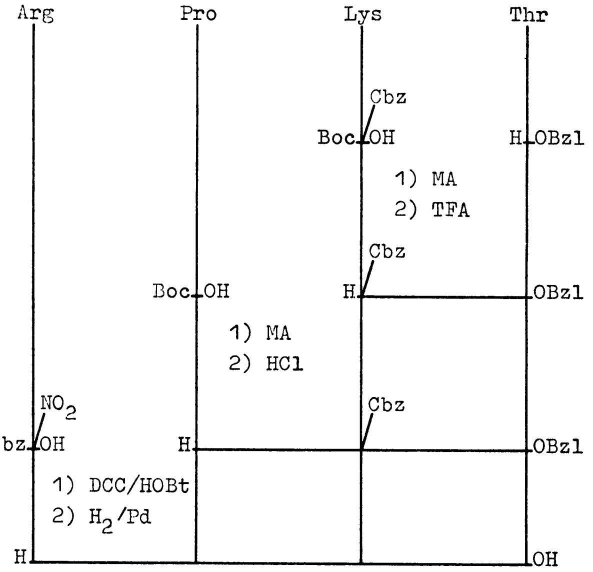

Chart 5. Preparation of **5**.

on TLC. However, no further trials for crystallization nor purification were carried out, since an ion exchange chromatography was expected to be effective for the purification of the basic peptides finally obtained. In the final stages of the construction of the peptides, benzyloxycarbonyl amino acids were used for acylation. The protected tetrapeptides were hydrogenated in the presence of palladium black in order to remove all the blocking groups.

The resulting free peptides were purified by carboxymethyl cellulose (Serva, or Whatman CM-52) chromatography using a linear gradient of ammonium acetate buffer (0—0.4 M, pH 7.0). **2** and **3** could be purified satisfactorily, but not the other analogs or **1**. The latter peptides gave no reasonable results on amino acid analysis after acid hydrolysis, although each of them showed individually a single spot on TLC (Avicel-cellulose, 1-butanol–pyridine–acetic acid–water, 16 : 10 : 3 : 12) and electrophoresis (Avicel-cellulose, pH 1.9, 40 V/cm). All efforts to remove the impurities by the carboxymethyl cellulose column were unsuccessful. Further purification of **4**, **5**, and **1** was successfully carried out on a Dowex 50 W×4 column with a linear gradient of pyridinium acetate buffer (0.6—2.0 M, pH 4.2—6.1). The highly purified products were desalted by a Biogel P-2 column and were lyophilized to give hygroscopic solids.

Phagocytosis stimulating activities of the synthetic peptides were measured according to the literature.[5] Compounds **2**, **3**, **4**, and **1** showed biological activity. For the appearance of biological activity, some alternation might be allowed in the primary structure of tuftsin, where the basic amino acids at positions 2 and 4 are exchangeable with each other without abolishing the biological potency.

Experimental[6]

Amino acid analyses were carried out with a JLC-6AS automatic analyzer (JEOL) after acid hydrolysis in HCl of constant bp (110 °C, 16 h). The theoretical values of amino acid ratios are shown in parentheses after each result. In TLC, Merck silicagel-precoated plate (S) and Avicel cellulose-precoated plate (C) were used with the following solvent systems: chloroform–methanol, 99 : 1 (a), 98 : 2 (b), 95 : 5 (c), 90 : 10 (d); 1-butanol–acetic acid–water 4 : 1 : 1 (e); 2-butanol–7M ammonia–ethanol, 1 : 4 : 2 (f), 1-butanol–pyridine–acetic acid–water, 16 : 10 : 3 : 12 (g). All the R_f values are the results of TLC. The properties and analytical results of the intermediates obtained in solid are as follows: Boc–Arg(NO_2)–Pro–Arg(NO_2)–OBzl: mp 89—111 °C, yield 65%, R_f (S, c) 0.34. $[\alpha]_D^{24}$ −31.1° (*c* 1.00, DMF). Found: C, 48.95; H, 6.64; N, 21.37%. Calcd for $C_{29}H_{45}O_{10}N_{11}$; C, 49.22; H, 6.41; N, 21.77%. Boc–Thr–Arg(NO_2)–Pro–Arg(NO_2)–OBzl (**6**); mp 88—124 °C, yield 92%, R_f(S, d) 0.50. $[\alpha]_D^{19}$ −29.0° (*c* 1.00, DMF). Found: C, 50.50; H, 6.13; N, 19.61%. Calcd for $C_{36}H_{50}O_{12}N_{12}\cdot H_2O$: C, 50.23; H, 6.09; N, 19.52%. The following intermediates were obtained as oily products and were used without crystallization (yield, R_f).

Boc-Pro–Arg(NO_2)–OBzl: 98%, (S, e) 0.44. Boc–Pro–Lys(Cbz)–OBzl: 95%, (S, a) 0.48. Boc–Lys(Cbz)–Pro–Lys(Cbz)–OBzl: 74%, (S, a) 0.19. Cbz–Thr–Lys(Cbz)–Pro–Lys(Cbz)–OBzl (**7**): 85%, (S, b) 0.29, (S, c) 0.82. Boc–Arg(NO_2)–Pro–Lys(Cbz)–OBzl: 100%, (S, c) 0.79. Cbz–Thr–Arg(NO_2)–Pro–Lys(Cbz)–OBzl (**8**): 90%, (S, c) 0.30. Boc–Lys(Cbz)–Thr–OBzl: 99%, (S, b) 0.70. Boc–Pro–Lys(Cbz)–Thr–OBzl: 98%, (S, b) 0.33. Cbz–Arg(NO_2)–Pro–Lys(Cbz)–Thr–OBzl (**9**): 85%, (S, c) 0.36. Boc–Lys(Cbz)–Pro–Arg(NO_2)–OBzl: 68%, (S, c) 0.49. Cbz–Thr–Lys(Cbz)–Pro–Arg(NO_2)–OBzl (**10**): 90%, (S, c) 0.41.

Preparation of 1. **10** (12.0 g) was hydrogenated in a mixture of DMF (100 ml), water (20 ml) and acetic acid (3.5 ml) in the presence of Pd black. After hydrogenation for 12 h, the mixture was diluted with additional solvent; DMF (200 ml), water (50 ml) and acetic acid (3 ml). Hydrogenation was continued for 17 h more, the catalyst being removed after the addition of DMF (300 ml). The solvent was evaporated *in vacuo* and the residue was dissolved in water. The water-insoluble parts were removed by filtration. A half of the filtrate was applied to a CM-cellulose (Serva) column (3.7×42 cm), which was eluted with a linear gradient of ammonium acetate (pH 7.0, 0—0.4 M, 800 ml each). The fractions from 1092 ml to 1248 ml were combined, concentrated and desalted by means of a Biogel P-2 column (3.5×35 cm, 4% acetic acid). Lyophilization of the desalted fractions gave 1.01 g of the product. A part of the product was applied to a Dowex 50 W×4 column (2.2×30 cm), which was eluted with a linear gradient of pyridinium acetate

(0.6 M pyridine, pH 4.2—2.0 M pyridine, pH 6.1, 800 ml each). The fractions from 954 ml to 1425 ml were combined, desalted by means of Biogel column (3.5 × 35 cm) and lyophilized. R_f(S, f) 0.28, (C, g) 0.20. $[\alpha]_D^{24}$ −60.5° (*c* 0.95, 5% acetic acid). (lit,[7] $[\alpha]_D^{22}$ −60.8°, 5% acetic acid). Amino acid ratios: Thr 1.01 (1), Pro 0.99 (1), Lys 1.02 (1), Arg 0.97 (1).

Found: C, 47.36; H, 8.00; N, 17.27%. Calcd for $C_{21}H_{40}O_6N_8 \cdot 2CH_3COOH \cdot H_2O$: C, 47.01; H, 7.89; N, 17.54%.

Preparation of **2**. **6** (0.50 g) was hydrogenated on a mixture of ethanol (60 ml), water (60 ml) and 1 M acetic acid (6 ml) in the presence of Pd black. After removal of the catalyst, the solvent was evaporated *in vacuo*. The crude tetrapeptide obtained was applied to a CM-cellulose (Whatman CM-52) column (2.0 × 44 cm), which was eluted with a linear gradient of ammonium acetate (pH 7.0, 0—0.4 M, 400 ml each). The fractions from 470 ml to 520 ml were combined and concentrated. After being desalted with Biogel P-2 column (3.5 × 35 cm) and lyophilized, 0.22 g of the desired peptide was obtained. R_f (S, f) 0.04, (C, g) 0.27, (S, g) 0.12. $[\alpha]_D^{24}$ −56.6° (c 0.90, 5% acetic acid). Amino acid ratios: Thr 1.06 (1), Pro 0.96 (1), Arg 1.98 (2).

Found: C, 45.22; H, 7.83; N, 20.58%. Calcd for $C_{21}H_{40}O_6N_{10} \cdot 2CH_3COOH \cdot H_2O$: C, 45.04; H, 7.56; N, 21.01%.

Preparation of **3**. **7** was treated in a way similar to that for the preparation of **2**, CM-cellulose (Serva) being used in the ion exchange chromatography. R_f (S, f) 0.57, (C, g) 0.21, (S, g) 0.10. $[\alpha]_D^{24}$ −66.7° (*c* 1.02, 5% acetic acid). Amino acid ratios: Thr 1.01 (1), Pro 0.95 (1), Lys 2.04 (2).

Found: C, 49.30; H, 8.66; N, 13.55%. Calcd for $C_{21}H_{40}O_6N_6 \cdot 2CH_3COOH \cdot H_2O$: C, 49.17; H, 8.25; N, 13.76%.

Preparation of **4**. **8** was hydrogenated in a mixture of ethanol, water and acetic acid. The product was purified as in the preparation of **1**. R_f (S, f) 0.27, (C, g) 0.19. $[\alpha]_D^{24}$ −62.6° (*c* 0.92, 5% acetic acid). Amino acid ratios: Thr 0.99 (1), Pro 1.00 (1), Lys 1.00 (1), Arg 1.00 (1).

Found: C, 47.02; H, 8.19; N, 17.25%. Calcd for $C_{21}H_{40}O_6N_8 \cdot 2CH_3COOH \cdot H_2O$: C, 47.01; H, 7.89; N, 17.54%.

Preparation of **5**. **9** was treated as described in the preparation of **4**. R_f (S, f) 0.26, (C, g) 0.25, (S, g) 0.04. $[\alpha]_D^{24}$ −58.0° (*c* 0.87, 5% acetic acid). Amino acid ratios: Thr 0.99 (1), Pro 1.00 (1), Lys 1.02 (1), Arg 0.98 (1).

Found: C, 47.14; H, 7.79; N, 17.56%. Calcd for $C_{21}H_{40}O_6N_8 \cdot 2CH_3COOH \cdot H_2O$: C, 47.01; H, 7.89; N, 17.54%.

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5) V. A. Najjar and A. Constantopoulos, *J. Reticuloendothel. Soc.*, **12**, 197 (1972).

6) All melting points are uncorrected. Amino acids used are of the L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature. Other abbreviations are as follows. Boc = *t*-butoxycarbonyl, Cbz = benzyloxycarbonyl, OBzl = benzyl ester, TFA = trifluoroacetic acid, MA = mixed anhydride method, DCC / HOBt = dicyclohexylcarbodiimide / *N*-hydroxy-1*H*-benzotriazole method.

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