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Studies on Peptides. XLVIII.[1,2] Application of the Trifluoromethanesulphonic Acid Procedure to the Synthesis of Tuftsin

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Trifluoromethanesulphonic acid removed all protecting groups from Z–Thr–Lys(Z)–Pro–Arg(Tos)–OH to give the phagocytosis-stimulating tetrapeptide, H–Thr–Lys–Pro–Arg–OH, named tuftsin. To this synthesis, the 5-chloro-8-quinolyl ester procedure was applied. With this connection, a number of analogous active esters was prepared.

Recently we reported that trifluoromethanesulphonic acid has an enough acidity to cleave a number of protecting groups currently employed in peptide synthesis.[4] We wish to describe in this paper the first application of this reagent to peptide synthesis. As an example, the synthesis of tuftsin, H–Thr–Lys–Pro–Arg–OH, was selected. This phagocytosis-stimulating tetrapeptide was isolated by Najjar, *et al.*[5,6] from γ-globulin and its solid phase synthesis was achieved by the same group of investigators.[6]

Our synthetic scheme of tuftsin in the conventional manner is illustrated in Fig. 1. This peptide contains two basic amino acid residues, Lys and Arg, which both require protecting groups during the synthesis. Two different protecting groups, the Z and Tos groups, were selected respectively for this synthesis in order to examine the usefulness of the above deblocking reagent.

With this convenience, we applied the active ester procedure of 5-chloro-8-hydroxyquinoline to the present synthesis. This type of active ester procedure was first introduced by Jakubke,

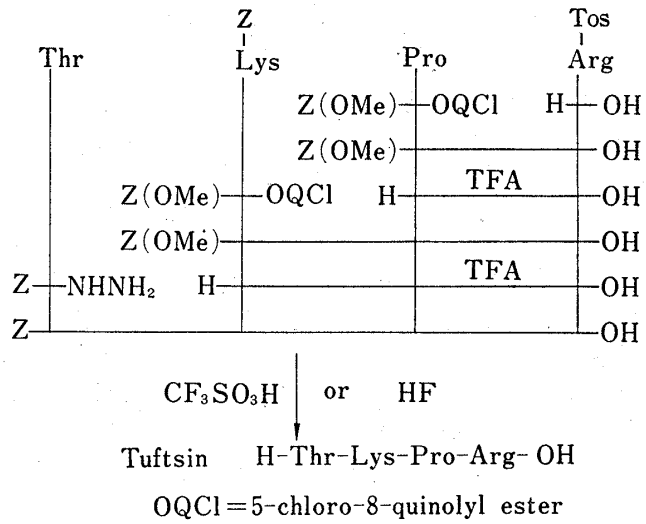

Fig. 1. Synthetic Scheme of Tuftsin

1) Part XLVII: H. Watanabe, M. Kubota, H. Yajima, A. Tanaka, M. Nakamura and T. Kawabata, *Chem. Pharm. Bull.* (Tokyo), **22**, 1889 (1974).
2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration. Abbreviations used are those recommended by IUPAC–IUB Commission of Biochemical Nomenclature: *Biochemistry*, **5**, 2485 (1966), *ibid.*, **6**, 362 (1967), *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, Z(OMe)=*p*-methoxybenzyloxycarbonyl.
3) Location: *Sakyo-ku, Kyoto*; *a*) Present address: *Gifu College of Pharmacy*; *b*) Present address: *Otsuka Pharm. Co.*
4) H. Yajima, N. Fujii, H. Ogawa and H. Kawatani, *Chem. Commun.*, **1974**, 107.
5) K. Nishioka, A. Constantopoulos, P.S. Satoh and V.A. Najjar, *Biochim. Biophys. Acta*, **310**, 217 (1973); A. Constantopoulos and V.A. Najjar, *J. Biol. Chem.*, **248**, 3819 (1973).
6) K. Nishioka, A. Constantopoulos, P.S. Satoh and V.A. Najjar, *Biochem. Biophys. Res. Commun.*, **47**, 172 (1972); K. Nishioka, P.S. Satoh, A. Constantopoulos and V.A. Najjar, *Biochim. Biophys. Acta*, **310**, 230 (1973).

et al.[7,8] but its information in general peptide synthesis is limited.[9] As pointed out by these authors,[7] 5-chloro-8-hydroxyquinoline is a compound soluble in most of organic solvents with low boiling points. Tetrahydrofuran seems to be a preferable solvent for the preparation of these esters by dicyclohexylcarbodiimide (DCC). Thus the 5-chloro-8-quinolyl esters of Z(OMe)–Pro–OH and Z(OMe)–Lys(Z)–OH, the necessary starting material of tuftsin, were prepared.

First, Z(OMe)–Pro-OQCl, which was obtained as an oily compound, was allowed to react with the triethylammonium salt of H–Arg(Tos)-OH[10] in aqueous tetrahydrofuran to give Z(OMe)–Pro–Arg(Tos)–OH in 63% yield. It was shown that 5-chloro-8-hydroxyquinoline liberated from the coupling reaction could be removed easily from the reaction mixture by washing with dilute hydrochloric acid but not completely with citric acid. The usual treatment of the above protected dipeptide with trifluoroacetic acid (TFA) in the presence of anisole removed the α-amino protecting group and the resulting dipeptide, after neutralization with triethylamine, was condensed with Z(OMe)–Lys(Z)–OQCl to give the protected tripeptide, Z(OMe)–Lys(Z)–Pro–Arg(Tos)–OH, in 66% yield. This, after the similar treatment with TFA, followed by neutralization with triethylamine, was condensed with Z–Thr–$NHNH_2$ by the modified azide procedure.[11] Since the hydroxyl group of Thr did not protected, the azide procedure was judged as practical. The resulting Z–Thr–Lys(Z)–Pro–Arg(Tos)–OH was obtained in analytically pure form after recrystallization from ethanol and ether.

The protected tetrapeptide thus obtained was then submitted to the final deblocking reaction. In previous model experiments, removal of the Z(OMe) and Z groups by trifluoromethanesulphonic acid required the treatment at 0° for 15 min., while the Tos group at 40° for 45 to 60 min. Leading from these observations, the sample was treated with 20 equimoles of this reagent at 40° for 45 min and the progress of the reaction was pursued by thin-layer chromatography. The excess reagent played a role as a solvent also.

The deblocked peptide, which exhibited apparently a single spot on thin-layer chromatography, was precipitated by addition of dry ether as fine powder and then treated with Amberlite IR-4B (acetate form). The product was applied to a column of CM-cellulose, which was eluted with ammonium acetate buffers. The homogeneous tetrapeptide, H–Thr–Lys–Pro–Arg–OH, was thus obtained with satisfactory elemental and amino acid analyses. Complete digestion of synthetic tuftsin was achieved by exposure to action of aminopeptidase (AP-M), though this peptidase was reported not to cleave the Pro bond.[12] The parent amino acids were recovered in ratios predicted by theory indicating that the L-configuration of constituent amino acids was maintained throughout this synthesis and further the deblocking of protecting groups by this reagent was completed without sizable side reactions. The yield of this deblocking step was 88% and this was comparable to that of the hydrogen fluoride treatment.[13] Activity of the synthetic peptide was examined by Prof. Shozo Nakasawa and his associates of Kyoto College of Pharmacy. They have found that the synthetic peptide exhibited the significant stimulation of chemotaxis in the *E. coli* system. A detailed account

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Table I. 5-Chloro-8-quinolyl Esters of N-Protected Amino Acids

OQCl ester	mp (°C)	Yield (%)	Recryst. from	Formula	Analysis (%) Calcd. (Found) C	H	N
Z-Val-[a]	93— 94	76	AcOEt–P.A.	$C_{22}H_{21}O_4N_2Cl$	64.00 (63.73)	5.13 (5.24)	6.79 (6.81)
Z-Trp-	122—123	93	AcOEt–P.A.	$C_{28}H_{22}O_4N_3Cl \cdot 1/2H_2O$	66.07 (66.08)	4.56 (4.57)	8.26 (8.22)
Z(OMe)-Trp-	96— 97	92	AcOEt–P.A.	$C_{29}H_{24}O_5N_3Cl$	65.72 (65.50)	4.56 (4.69)	7.93 (7.74)
Z-Gln-	216—217	66	THF–EtOH	$C_{22}H_{20}O_5N_3Cl$	59.80 (59.89)	4.56 (4.48)	9.51 (9.41)
Z(OMe)-Gln-	152—154	79	THF–ether	$C_{23}H_{22}O_6N_3Cl$	58.54 (58.54)	4.70 (4.63)	8.91 (9.13)
Z(OMe)-Glu(OBzl)-	92— 93	85	AcOEt–ether	$C_{30}H_{27}O_7N_2Cl$	64.00 (63.99)	4.83 (4.96)	4.98 (4.98)
Z-Asn-[b]	176—179	25	EtOH–ether	$C_{21}H_{18}O_5N_3Cl$	58.95 (58.92)	4.24 (4.01)	9.82 (9.69)
Z(OMe)-Asn-[b]	173—176	27	MeOH–ether	$C_{22}H_{20}O_6N_3Cl$	57.71 (57.45)	4.40 (4.42)	9.18 (9.25)
Z(OMe)-Asp(OBzl)-	100—101	71	AcOEt–ether	$C_{29}H_{25}O_7N_2Cl$	63.44 (63.49)	4.59 (4.70)	5.10 (5.24)
Z(OMe)-Met-	128—129	82	AcOEt–ether	$C_{23}H_{23}O_5N_2SCl$	58.16 (57.95)	4.88 (4.76)	5.90 (5.74)
Z(OMe)-Lys(Z)-	136—137	78	THF–EtOH	$C_{32}H_{32}O_7N_3Cl$	63.41 (63.57)	5.32 (5.56)	6.93 (7.19)
Z(OMe)-His(Bzl)-	147—148	79	AcOEt–ether	$C_{31}H_{27}O_5N_4Cl$	65.20 (65.94)	4.77 (5.40)	9.81 (9.96)
Z(OMe)-Cys(Bzl)-	125—126	94	ether	$C_{28}H_{25}O_5N_2SCl$	62.62 (62.10)	4.69 (4.60)	5.22 (5.20)

a) DL form,[6] mp 98—99.5°
b) Low yield is due to the formation of a impurity (presumably a dehydro compound).

of their investigations will be published in future. Further application of this deblocking reagent to the synthesis of more complex peptides is under investigations.

Experimental

Thin-layer chromatography was performed on silicagel (Kieselgel, G. Merck). *Rf* values refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH–H_2O (8:3:1), Rf_2 *n*-BuOH–AcOH–pyridine–H_2O (30:6:20:24), Rf_3 *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2).

Preparation of the 5-Chloro-8-Hydroxyquinolyl Ester of N-Protected Amino Acids——These esters were prepared in essentially the same manner as described by Jakubke, *et al.*[6] To an equimolar mixture (5 mM) of 5-chloro-8-hydroxyquinoline and a N-protected amino acid in tetrahydrofuran (20 ml), DCC (6 mM) was added under cooling with ice. After stirring at room temperature overnight, the solution was filtered and condensed *in vacuo*. The residue was dissolved in AcOEt, which was washed with 5% $NaHCO_3$, 0.05N HCl and H_2O, dried over Na_2SO_4 and then evaporated. The product was recrystallized from appropriate solvents. The results are listed in Table I.

Z(OMe)-Pro-Arg(Tos)-OH——Z(OMe)–Pro–OQCl (13.23 g) in THF (100 ml) was added to a solution of H–Arg(Tos)–OH (9.85 g) in H_2O (100 ml) containing Et_3N (8.3 ml) and the mixture was stirred at room temperature for 24 hr. After evaporation of the solvent, the residue was dissolved in 3% NH_4OH, which was washed with ether. The aqueous phase was acidified with citric acid and the resulting precipitate was extracted with AcOEt, which was washed with 0.5N HCl and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from MeOH and ether; yield 11.16 g (63%), mp 60—62°, $[\alpha]_D^{21}$ −14.3° (c=0.8, DMF), Rf_1 0.42. *Anal.* Calcd. for $C_{27}H_{35}O_8N_5S$: C, 54.99; H, 5.98; N, 11.88. Found: C, 55.03; H, 5.94; N, 11.67.

Z(OMe)-Lys(Z)-Pro-Arg(Tos)-OH——Z(OMe)–Pro–Arg(Tos)–OH (5.90 g) was treated in the usual manner with TFA (15 ml) in the presence of anisole (5 ml) at 0° for 2 hr. The fine powder formed by addition of dry ether was collected by filtration, washed with ether and then dissolved in 70% aqueous THF (70 ml). To this solution, Et_3N (4.2 ml) and Z(OMe)–Lys(Z)–OQCl (7.27 g) in THF (200 ml) were combined

and the mixture was stirred at room temperature for 24 hr. After evaporation of the solvent, the residue was dissolved in 3% NH_4OH, which was washed with AcOEt and then acidified with citric acid. The resulting precipitate was extracted with AcOEt, which after washing with 0.5N HCl and H_2O–NaCl, was dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and then recrystallized from THF and ether; yield 5.61 g (66%), mp 62—65°, $[\alpha]_D^{21}$ −16.6° (c=0.9, DMF). Rf_1 0.45. *Anal.* Calcd. for $C_{41}H_{53}O_{11}N_7S$: C, 57.80; H, 6.27; N, 11.51. Found: C, 57.83; H, 6.37; N, 11.22.

Z-Thr-Lys(Z)-Pro-Arg(Tos)-OH——As stated above, Z(OMe)–Lys(Z)–Pro–Arg(Tos)–OH (3.53 g) was treated with TFA (15 ml) in the presence of anisole (2.7 ml) for 1.5 hr, when dry ether was added. The resulting powder was collected by filtration, washed with ether and then dissolved in DMF (20 ml). To this ice-cold solution, Et_3N (2.1 ml) and the azide (derived from 1.31 g of Z–Thr–$NHNH_2$ with 2.9 ml of 3.6N HCl–DMF, 0.72 ml of isoamylnitrite and 2.1 ml of Et_3N) in DMF (20 ml) were added and the mixture was stirred at 4° for 48 hr. The solvent was evaporated and the residue was dissolved in 3% NH_4OH, which after washing with AcOEt, was acidified with citric acid. The resulting precipitate was extracted with AcOEt, which was washed with 0.5N HCl and H_2O–NaCl, dried over Na_2SO_4 and then evaporated. The residue was triturated with ether and recrystallized from EtOH and ether; yield 3.25 g (86%), mp 114—117°, $[\alpha]_D^{22}$ −14.0° (c=1.0, DMF). Rf_1 0.29. *Anal.* Calcd. for $C_{44}H_{58}O_{12}N_8S$: C, 57.25; H, 6.33; N, 12.14. Found: C, 56.96; H, 6.36; N, 11.98.

H-Thr-Lys-Pro-Arg-OH——Z–Thr–Lys(Z)–Pro–Arg(Tos)–OH (461 mg) was treated with trifluoromethanesulphonic acid (2 ml) in the presence of anisole (1.1 ml) at 40° for 45 min, when dry ether was added. The resulting fine powder was collected by filtration and washed with ether. This was then dissolved in H_2O (10 ml), which was stirred with Amberlite IR-4B (acetate form, approximately 3 g) for 30 min. The resin was removed by filtration and the filtrate was lyophilized. The residue was then dissolved in H_2O (50 ml) and applied to a column of CM-cellulose (2.4×20 cm), which was eluted with H_2O (100 ml) and then with 0.25M ammonium acetate buffer (pH 6.9, 600 ml) through a mixing flask containing H_2O (300 ml). Individual fractions (5 ml each) were collected and the desired peptide was located by Sakaguchi and ninhydrin tests. Fractions (tube No. 31—33) which contained the desired compound were pooled and the solvent was evaporated *in vacuo*. The residue was lyophilized to give fine powder; yield 300 mg (88%), $[\alpha]_D^{22}$ −60.8° (c=0.6, 5% AcOH). Rf_2 0.25, Rf_3 0.13. Amino acid ratios in acid hydrolysate and AP-M digest (number in blacket): $Thr_{1.03(0.98)}Lys_{0.96(1.00)}Pro_{1.00(1.17)}Arg_{0.98(0.94)}$ [average recovery 98% (97%)]. *Anal.* Calcd. for $C_{21}H_{40}O_6N_8 \cdot 2CH_3COOH \cdot 1.5H_2O$: C, 46.35; H, 7.93; N, 17.30. Found: C, 46.35; H, 8.41; N, 17.38. For comparison, the protected tetrapeptide (277 mg) was treated with HF[13] and the product was similarly purified; yield 64%, Rf_2 0.27, Rf_3 0.13. Amino acid ratios in an acid hydrolysate: $Thr_{0.92}Lys_{1.04}Pro_{1.00}$-$Arg_{1.05}$ (average recovery 97%).