solid had separated: wt, 4.60 g. Recrystallization from EtOH gave pure product: wt, 2.20 g; yield, 44%; mp 169–175 °C. Anal. ($C_{14}H_{15}NO_4S$) C, H, N, S.

Compound **45** was similarly obtained.

Biological Methods. The in vitro ACE inhibitory activity was determined by a radioassay procedure reported previously.[5] Activity is reported as the IC_{50}, which is the approximate molar concentration of test compound causing a 50% inhibition of the control converting enzyme activity.

The test solutions were prepared by dissolving 2–5 mg of test compound in 1 mL of Me_2SO and diluting to the desired concentration with a pH 8 buffer of 0.05 M Hepes (Calbiochem), 0.1 M NaCl, and 0.6 M Na_2SO_4 in H_2O.

Blood Pressure and Heart Rate Test in the Conscious Rat. Hypertension of renal origin was produced in rats by placing a silver clip (0.2-mm gap) around the left renal artery near the aorta and leaving the contralateral kidney intact. Four-week-old Sprague–Dawley male albino rats (Charles River, Wilmington, MA) were clipped soon after arrival, and the hypertension was allowed to develop for 4–8 weeks. The rats were then cannulated for blood pressure monitoring as described previously.[37] Only rats with mean aortic blood pressures of >160 mmHg were used. At the time of cannulation the rats weighed 280–320 g. The rats were given free access to a standard lab show (5012 Purina, Richmond, IN) and tap water and were maintained on a 12-h dark/12-h light cycle.

One-minute running average values of heart rate and aortic blood pressure (mean, systolic, and diastolic) for each rat were recorded every 30th minute by means of a computer-assisted data capture scheme.

Acknowledgment. We wish to thank the following chemists for their contributions of syntheses, development of procedures, and helpful suggestions: Dr. J. Kaltenbronn, Dr. O. Goel, Mr. U. Krolls, Mr. D. de John, Mr. D. Schweiss, Mr. N. Colbry, Dr. R. Meyer, Mr. T. Puls, Mr. L. Bennett, Mr. W. Pearlman, and Mr. D. Johnson. We also thank Dr. F. MacKellar and his staff for analytical data. In particular we thank Mr. E. Schoeb for infrared spectra, Mr. B. Scott and Ms. S. Uhlendorf for NMR spectra, Dr. H. Schneider, Mr. R. Leeds and Mr. M. Young for HPLC data, and Mr. G. Sekerak for elemental analyses and optical rotations. The contributions of the following pharmacologists are also appreciated: Dr. M. Ryan, Mr. T. C. Major, Mr. R. Singer, Ms. B. Olszewski, Ms. F. Bjork, and Dr. R. Smith.

Registry No. 3, 82717-96-2; 4, 84324-12-9; 5, 82586-60-5; 5 (acid), 74163-81-8; 6, 103733-29-5; 6 (acid), 103733-65-9; 7, 77497-96-2; 7·HCl, 103733-30-8; 8, 103733-31-9; 8 (acid), 103733-66-0; 8·HCl (acid), 82586-62-7; 9, 103733-32-0; (±)-10, 103733-33-1; (±)-11, 96325-07-4; (±)-12, 103733-34-2; 13, 82586-56-9; 14, 103733-35-3; 14·$C_4H_4O_4$, 103733-36-4; 15, 82586-54-7; 16, 103775-05-9; 17, 103733-37-5; 17·$C_4H_4O_4$, 103733-38-6; 18, 103733-40-0; 19, 82637-57-8; 19·$C_4H_4O_4$, 82637-58-9; 19·HCl, 82586-51-4; 20, 103733-42-2; (*S,S,S*)-21, 103733-43-3; (*S,S,R*)-21, 103775-15-1; 22, 85441-61-8; 22·HCl, 82586-55-8; 23, 103775-09-3; 23·HCl, 89300-89-0; 24, 103833-16-5; 24·HCl, 103775-06-0; 25, 103775-10-6; 25·HCl, 82586-52-5; 26, 103775-11-7; 26·HCl, 103833-14-3; 27, 103775-12-8; 27·HCl, 103833-15-4; 28, 103775-13-9; 28·HCl, 103733-44-4; 29, 103833-17-6; 29·HCl, 103775-07-1; 30, 103733-45-5; 31, 103733-46-6; 32, 103775-08-2; 33, 85441-60-7; 34, 103775-14-0; 34·HCl, 82586-57-0; 35, 103733-47-7; 36, 103733-48-8; 37, 103733-49-9; 38, 103733-50-2; 39, 103733-51-3; (*S,R*)-40, 100486-65-5; (*S,S*)-40, 100486-33-7; (*S,S*)-40·$C_{12}H_{23}N$, 103733-52-4; (*S,R*)-40·$C_{12}H_{23}N$, 103733-67-1; 41, 77832-18-9; (±)-42, 103733-53-5; (±)-43, 103733-54-6; (±)-44, 103733-55-7; (±)-44·$C_{12}H_{23}N$, 103733-56-8; (±)-(*R**,*R**)-45, 103733-57-9; (±)-(*R**,*R**)-45·$C_{12}H_{23}N$, 103733-58-0; (±)-(*R**,*R**)-46, 103733-59-1; (±)-(*R**,*R**)-47, 103733-60-4; (±)-(*R**,*R**)-48, 103733-61-5; (±)-(*R**,*R**)-48·$C_{12}H_{23}N$, 103733-62-6; (±)-49, 103733-63-7; (±)-50, 103733-64-8; (±)-$H_3CCOSCH_2CH(CH_3)COCl$, 70354-87-9; isoquinoline-1-carboxylic acid ethyl ester, 50458-78-1; 1,2,3,4-tetrahydroisoquinoline, 91-21-4.

(37) Smith, R. D.; Wood, T. J.; Tessman, D. K.; Olszewski, B. J.; Currier, G.; Kaplan, H. R.; *NIH* 1980, *41*, 1473.

Tuftsin Analogues: Synthesis, Structure–Function Relationships, and Implications for Specificity of Tuftsin's Bioactivity

Shlomo Dagan,[†‡] Philip Gottlieb,[†] Esther Tzehoval,[‡] Michael Feldman,[‡] Mati Fridkin,*[†] Koichi Yasumura,[§] Kenji Okamoto,[§] and Haruaki Yajima*[∥]

Departments of Organic Chemistry and Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel, and Kyoto College of Pharmacy, Yamashina-Ku, Kyoto, 607 Japan, and Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-Ku, Kyoto, 606 Japan. *Received June 14, 1985*

Thirteen analogues of the natural macrophage activator peptide tuftsin, ten of which are novel, were synthesized with the aim of exploring the relation between their biological potency and their capacity to attach specifically to cellular tuftsin's receptors. The analogues representing modifications and chain extensions at various parts of the parent tuftsin molecule can be classified as (a) N-terminal analogues, (b) C-terminal analogues, (c) "within-chain" derivatives, or (d) dimers of tuftsin and retrotuftsin. The various synthetic routes employed to prepare the analogues are described. A direct correlation was found between the ability of analogues to inhibit [^{3}H-Arg^4]tuftsin specific binding to mice peritoneal macrophages and their capacity to enhance phagocytosis or to inhibit tuftsin-mediated phagocytosis by the cells and to potentiate the cell's immune response.

Tuftsin[1] is an immunoglobulin G associated tetrapeptide of the sequence L-Thr-L-Lys-L-Pro-L-Arg, located in the Fc domain of the protein's heavy chain (residues 289–292).[2] It is released from a unique Ig fraction, leukokinin, through an enzymatic processing.[3] Tuftsin possesses a wide range of activities that it exerts on the phagocytic cells. In fact, the peptide is capable of potentiating most functions of blood granulocytes and tissue macrophages including phagocytosis,[1,4–6] motility,[4,7,8] immunogenic response,[9]

† Department of Organic Chemistry, The Weizmann Institute of Science.

‡ Department of Cell Biology, The Weizmann Institute of Science.

§ Kyoto College of Pharmacy.

∥ Kyoto University.

(1) Najjar, V. A.; Nishioka, K. *Nature (London)* 1970, *228*, 672.
(2) Edelman, G. M.; Cunningham, B. A.; Gall, W. E.; Gottlieb, P. D.; Rutishauser, U.; Waxdal, M. *Proc. Natl. Acad. Sci. U.S.A.* 1969, *63*, 78.
(3) Najjar, V. A. In *Macrophages and Lymphocytes*; Escobar, M. R., Friedman, H., Eds.; Plenum: New York, 1980; Part A, p 131.

0022-2623/86/1829-1961$01.50/0 © 1986 American Chemical Society

bactericidal activity,[10] and, very notably, tumoricidal activity.[11-15]

Structure–function studies with numerous synthetic analogues of tuftsin have demonstrated that rather strict structural integrity of the peptide should be preserved in order to achieve full biological potency.[15] These findings suggested the existence of specific cellular receptors for tuftsin. Direct binding studies, employing radiolabeled derivatives of tuftsin, revealed unique and single populations of sites for the peptide on phagocytic cell surfaces.[15-19] The multitude and diversity of activities of tuftsin raise, however, the question whether only one receptor suffices to translate the signals generated by the peptide into potentiation of the corresponding function, or whether perhaps several tuftsin-related receptors do exist on phagocytes.

The present study is aimed at shedding some additional and greater light on the relationships between the biological activities and structure of several analogues of tuftsin, thus, to gain better understanding of the specificity of phagocytes stimulation by tuftsin.

Thirteen analogues of tuftsin, representing structural alterations at various positions of the tuftsin molecule, were thus chemically synthesized. The compounds fall into four categories: analogues based on modifications at the peptides' N-terminal ends, H-Arg-Lys-Pro-Arg-OH, H-D-Thr-Lys-Pro-Arg-OH, H-Thr-Thr-Lys-Pro-Arg-OH, H-Val-Thr-Lys-Pro-Arg-OH, H-Lys-Thr-Lys-Pro-Arg-OH, and H-Arg-Thr-Lys-Pro-Arg-OH; C-terminal analogues, H-Thr-Lys-Pro-Cit-OH (Cit, citrulline) and H-Thr-Lys-Pro-Arg-Leu-OH; "within-chain" derivatives, H-Thr-Lys-β-Ala-Arg-OH and H-Thr-Lys-Lys-Pro-Arg-OH; and peptides composed of double sequences of tuftsin and retro-tuftsin, H-Arg-Pro-Lys-Thr-Thr-Lys-Pro-Arg-OH, H-Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg-OH, and H-Thr-Lys-Pro-Arg-Arg-Pro-Lys-Thr-OH.

Ten of the analogues synthesized are novel. Three, H-Lys-Thr-Lys-Pro-Arg-OH,[20] H-Arg-Thr-Lys-Pro-Arg-OH,[21] and H-Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg-OH,[22,23] have been described. A comparative evaluation of the peptides' capacities in two distinct assay systems, enhancement of phagocytosis and potentiation of the immune response of macrophages, was, for the first time, undertaken. Moreover, in parallel, the peptide's ability to inhibit [^{3}H-Arg^4]tuftsin specific binding to cells was examined.

Chemistry. Analytical data of protected intermediate peptides and protected tuftsin analogues are listed in Table I. Table II summarizes analytical data of unprotected tuftsin analogues. The amino acid composition of tuftsin analogues is given in Table III.

The synthesis of fully protected derivatives of tuftsin analogues is briefly outlined as follows.

Z-Thr-Lys(Z)-β-Ala-Arg(NO_2)-OBzl (II) was synthesized by the azide condensation[23] through coupling of Z-Thr-Lys(Z)-N_3, prepared from Z-Thr-Lys(Z)-$NHNH_2$,[24] with H-β-Ala-Arg(NO_2)-OBzl, prepared by HBr/AcOH treatment of the corresponding Z-derivative (I). The latter peptide (I) was prepared by the usual *N,N'*-dicyclohexylcarbodiimide (DCC) method.[25] Similarly, Z-Thr-Lys(Z)-Pro-Cit-OBzl (V) was synthesized from Z-Thr-Lys(Z)-$NHNH_2$ and a TFA-treated[27] Z(OMe)-Pro-Cit-OBzl (IV), prepared by the usual DCC method. Z-Arg(NO_2)-Lys(Z)-Pro-Arg(NO_2)-OBzl (IX) was prepared starting with H-Arg(NO_2)-OBzl following successive condensations with Z(OMe)-Pro-OH, Z(OMe)-Lys(Z)-OH, and Z-Arg(NO_2)-OH by the usual DCC procedure, the 5-chloro-8-quinolyl active ester (QCl) procedure,[28] and the mixed anhydride procedure,[29] respectively. An intermediate, Z(OMe)-Lys(Z)-Pro-Arg(NO_2)-OBzl (VIII), used for the preparation of IX was also condensed, after selective N-terminal deprotection, with each of Z-D-Thr-$NHNH_2$, Z-Thr-Thr-$NHNH_2$ (XIV), Z-Val-Thr-$NHNH_2$,[30] and Z-Lys(Z)-Thr-$NHNH_2$[31] by the azide procedure to afford Z-D-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl (XI), Z-Thr-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl (XV), Z-Val-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl (XVII), and Z-Lys(Z)-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl (XIX), respectively. Peptide XIV was prepared by the azide procedure, followed by the usual hydrazine treatment. Z-(OMe)-Arg(NO_2)-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl (XXII) was prepared, starting with a TFA-treated sample of VIII, by successive condensation with Z(OMe)-Thr-$NHNH_2$ and Z(OMe)-Arg(NO_2)-OH by the azide procedure and the DCC/*N*-hydroxybenzotriazole (HOBT) procedure,[32] respectively. Z-Thr-Lys(Z)-Lys(Z)-Pro-Arg(NO_2)-OBzl

(4) Constantopoulos, A.; Najjar, V. A. *Cytobios* **1972**, *6*, 97.
(5) Nishioka, K.; Constantopoulos, A.; Satoh, P. S.; Mitchell, W. M.; Najjar, V. A. *Biochim. Biophys. Acta* **1973**, *310*, 217.
(6) Fridkin, M.; Stabinksy, Y.; Zakuth, V.; Spirer, Z. *Biochim. Biophys. Acta* **1977**, *496*, 203.
(7) Nishioka, K. *Gann* **1978**, *69*, 569.
(8) Horsmanheimo, A.; Horsmanheimo, M.; Fudenberg, H. H. *Clin. Immunol. Immunopathol.* **1978**, *11*, 251.
(9) Tzehoval, E.; Segal, S.; Stabinsky, Y.; Fridkin, M.; Spirer, Z.; Feldman, M. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 3400.
(10) Martinez, J.; Winternitz, F. *Mol. Cell. Biochem.* **1981**, *41*, 123.
(11) Nishioka, K. *Br. J. Cancer* **1979**, *39*, 342.
(12) Najjar, V. A.; Chaudhuri, M. K.; Konopinska, D.; Beck, B. D.; Layne, P. P.; Linehan, L. In *Augmenting Agents in Cancer Therapy*; Chirigos, M. A., Hersh, E. M., Mastrangelo, M. J., Eds.; Raven: New York, 1981; p 459.
(13) Catane, R.; Schlanger, S.; Weiss, L.; Penchas, S.; Fuks, Z.; Treves, A. J.; Gottlieb, P.; Fridkin, M. In *Antineoplastic, Immunogenic and Other Effects of the Tetrapeptide Tuftsin: A Natural Macrophage Activator*; Najjar, V. A., Fridkin, M., Eds.; Annals of the New York Academy of Sciences: New York, 1983; Vol. 419, p 251.
(14) Knyszynski, A.; Gottlieb, P.; Fridkin, M. *J. Natl. Cancer Inst.* **1983**, *71*, 87.
(15) Fridkin, M.; Gottlieb, P. *Mol. Cell. Biochem.* **1981**, *41*, 73.
(16) Stabinsky, Y.; Gottlieb, P.; Zakuth, V.; Spirer, Z.; Fridkin, M. *Biochem. Biophys. Res. Commun.* **1978**, *83*, 599.
(17) Bar-Shavit, Z.; Stabinsky, Y.; Fridkin, M.; Goldman, R. *J. Cell. Physiology* **1979**, *100*, 55.
(18) Nair, R. M. G.; Ponce, B.; Fudenberg, H. H. *Immunochemistry* **1978**, *15*, 901.
(19) Bump, N.; Najjar, V. A. *Mol. Cell. Biochem.* **1984**, *63*, 137.
(20) Sucharda-Sobczyk, A.; Siemion, I. Z.; Konopinska, D. *Eur. J. Biochem.* **1979**, *96*, 131.
(21) Konopinska, D.; Kazanowska, B.; Boguslawska-Jaworska, J. *Int. J. Peptide Protein Res.* **1984**, *24*, 267.
(22) Konopinska, D.; Najjar, V. A.; Callery, M. *Pol. J. Chem.* **1982**, *56*, 1063.
(23) Najjar, V. A.; Linehan, L.; Konopinska, D. In *Antineoplastic, Immunogenic and Other Effects of the Tetrapeptide Tuftsin: A Natural Macrophage Activator*; Najjar, V. A., Fridkin, M., Eds.; Annals of the New York Academy of Sciences: New York, 1983; Vol. 419, p 261.
(24) Honzl. J.; Rudinger, J. *Collect. Czech. Chem. Commun.* **1961**, *26*, 2333.
(25) Yasumura, K.; Okamoto, K.; Shimamura, S. *Yakugaku* Zasshi **1977**, *97*, 324.
(26) Sheehan, J. C.; Hess, G. P. *J. Am. Chem. Soc.* **1955**, *77*, 1067.
(27) Weygand, F.; Hunger, K. *Chem. Ber.* **1962**, *95*, 1.
(28) Yajima, H.; Ogawa, H.; Watanabe, H.; Fujii, N.; Kurobe, M.; Miyamoto, S. *Chem. Pharm. Bull.* **1975**, *23*, 371.
(29) Vaughan, J. R., Jr.; Osato, R. L. *J. Am. Chem. Soc.* **1952**, *74*, 676.
(30) Hiramoto, M.; Okada, K.; Nagai, S. *Chem. Pharm. Bull.* **1971**, *14*, 1315.
(31) Okamoto, K.; Shimamura, S. *Yakugaku Zasshi* **1976**, *96*, 315.
(32) Konig, W.; Geiger, R. *Chem. Ber.* **1970**, *103*, 788.

Table I. Synthetic Data of Protected Intermediate Peptides

compd	synthetic method[a]	yield, %	mp,[b] °C	$[\alpha]_D$ (temp, °C; concn, %, solvent), deg	mobility (R_{f1})[c]	formula	anal.
(I) Z-β-Ala-Arg(NO$_2$)-OBzl	DCC	85	142–144	−12.3 (13; 1.06, MeOH)	0.34	$C_{24}H_{30}N_6O_7$	C, H, N
(II) Z-Thr-Lys(Z)-β-Ala-Arg(NO$_2$)-OBzl	azide	50	144–146	−11.8 (29; 0.51, MeOH)	0.40	$C_{42}H_{55}N_9O_{12}\cdot 1.5H_2O$	C, H, N
(IV) Z(OMe)-Pro-Cit-OBzl	DCC	76	141–144	−43.3 (33; 1.04, EtOH)	0.43	$C_{27}H_{34}N_4O_7$	C, H, N
(V) Z-Thr-Lys(Z)-Pro-Cit-OBzl	azide	50		−21.8 (30; 1.01, CHCl$_3$)	0.40	$C_{44}H_{57}N_7O_{11}$	C, H, N
(VII) Z(OMe)-Pro-Arg(NO$_2$)-OBzl	DCC	83		−54.6 (26; 1.19, CHCl$_3$)	0.50	$C_{27}H_{34}N_6O_8$	C, H, N
(VIII) Z(OMe)-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	QCl	73		−26.2 (12; 1.07, CHCl$_3$)	0.44	$C_{41}H_{52}N_8O_{11}$	C, H, N
(IX) Z-Arg(NO$_2$)-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	MA	50		−21.0 (26; 1.00, CHCl$_3$)	0.47	$C_{46}H_{61}N_{13}O_{13}$	C, H, N
(XI) Z-D-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	azide	80		+3.3 (20; 1.21, CHCl$_3$)	0.48	$C_{44}H_{57}N_9O_{12}$	C, H, N
(XIII) Z-Thr-Thr-OMe	azide	49	67–68	−16.1 (12; 1.12, CHCl$_3$)	0.50	$C_{17}H_{24}N_2O_7$	C, H, N
(XIV) Z-Thr-Thr-NHNH$_2$	NH$_2$NH$_2$·H$_2$O	70	213–214	+17.0 (14; 1.00, DMF)	0.40[d]	$C_{16}H_{24}N_4O_6$	C, H, N
(XV) Z-Thr-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	azide	61		−22.0 (26; 1.09, CHCl$_3$)	0.44[d]	$C_{48}H_{64}N_{10}O_{14}$	C, H, N
(XVII) Z-Val-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	azide	76		−17.6 (26; 1.02, CHCl$_3$)	0.51	$C_{49}H_{66}N_{10}O_{13}$	C, H, N
(XIX) Z-Lys(Z)-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	azide	75		−13.3 (26; 1.20, CHCl$_3$)	0.49	$C_{58}H_{75}N_{11}O_{15}$	C, H, N
(XXI) Z(OMe)-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	azide	87		−9.4 (18; 1.06, CHCl$_3$)	0.44	$C_{45}H_{59}N_9O_{13}$	C, H, N
(XXII) Z(OMe)-Arg(NO$_2$)-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	DCC/HOBT	83		−40.8 (12; 0.20, MeOH)	0.30	$C_{51}H_{70}H_{14}O_{16}\cdot 0.5H_2O$	C, H, N
(XXIV) Z(OMe)-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	QCl	78		−6.8 (20; 1.03, CHCl$_3$)	0.46	$C_{55}H_{70}N_{10}O_{14}$	C, H, N
(XXV) Z-Thr-Lys(Z)-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	azide	90		−14.5 (26; 1.10, CHCl$_3$)	0.50	$C_{58}H_{75}N_{11}O_{15}$	C, H, N
(XXVII) Z(OMe)-Arg(NO$_2$)-Leu-OBzl	DCC/HOBT	97		−31.0 (10; 1.13, CHCl$_3$)	0.50		
(XXVIII) Z(OMe)-Lys(Z)-Pro-Arg(NO$_2$)-Leu-OBzl	MA	85		−34.2 (26; 1.11, CHCl$_3$)	0.52	$C_{47}H_{63}N_9O_{12}$	C, H, N
Z-Thr-Lys(Z)-Pro-Arg(NO$_2$)-Leu-OBzl	azide	50		−25.7 (25; 0.35, CHCl$_3$)	0.42	$C_{50}H_{68}N_{10}O_{13}$	C, H, N
(XXXI) Z(OMe)-Lys(Z)-Thr-OMe	DCC	93	131–132	−3.7 (13; 5.34, CHCl$_3$)	0.50	$C_{28}H_{37}N_3O_9$	C, H, N
(XXXII) Z(OMe)-Lys(Z)-Thr-NHNH$_2$	NH$_2$NH$_2$·H$_2$O	88	158–161	−4.3 (17; 0.47, DMF)	0.60[a]	$C_{27}H_{37}N_5O_8$	C, H, N
(XXXIII) Z(OMe)-Lys(Z)-Thr-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	azide	78		−36.5 (14; 0.52, MeOH)	0.44	$C_{63}H_{84}N_{12}O_{18}$	C, H, N
(XXXIV) Z-Arg(NO$_2$)-Pro-Lys(Z)-Thr-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	PCP	73		−64.3 (16; 0.28, MeOH)	0.44	$C_{77}H_{100}N_{18}O_{21}$	C, H, N
(XXXVI) Z(OMe)-Lys(Z)-Pro-Arg(NO$_2$)-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	MA	78		−30.0 (11; 0.50, CHCl$_3$)	0.22	$C_{70}H_{95}N_{17}O_{20}$	C, H, N
(XXXVII) Z-Thr-Lys(Z)-Pro-Arg(NO$_2$)-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl	azide	82		−57.9 (17; 0.57, MeOH)	0.41	$C_{73}H_{100}N_{18}O_{21}$	C, H, N
(XXXIX) Z(OMe)-Pro-Lys(Z)-Thr-OBzl	DCC/HOBT	71	180–181	−34.0 (14; 1.03, DMF)	0.82	$C_{39}H_{48}N_4O_{10}$	C, H, N
(XL) Z(OMe)-Arg(NO$_2$)-Pro-Lys(Z)-Thr-OBzl	DCC/HOBT	55		−25.0 (15; 0.56, CHCl$_3$)	0.50	$C_{45}H_{59}N_9O_{13}$	C, H, N
(XLI) Z(OMe)-Arg(NO$_2$)-Arg(NO$_2$)-Pro-Lys(Z)-Thr-OBzl	DCC/HOBT	68		−44.4 (17; 0.54, MeOH)	0.40	$C_{51}H_{70}N_{14}O_{16}$	C, H, N
(XLII) Z(OMe)-Lys(Z)-Pro-Arg(NO$_2$)-Arg(NO$_2$)-Pro-Lys(Z)·Thr-OBzl	MA	72		−77.8 (16; 0.54, MeOH)	0.40	$C_{70}H_{95}N_{17}O_{20}$	C, H, N
(XLIII) Z-Thr-Lys(Z)-Pro-Arg(NO$_2$)-Arg(NO$_2$)-Pro-Lys(Z)-Thr-OBzl	azide	67		−51.6 (14; 0.62, MeOH)	0.31	$C_{73}H_{100}N_{18}O_{21}$	C, H, N

[a] DCC, *N,N'*-dicyclohexylcarbodiimide; DCC/HOBT, coupling in presence of *N*-hydroxybenzotriazole; QCl, 5-chloro-8-quinolyl active ester; MA, mixed anhydride; PCP, pentachlorophenyl active ester. [b] Melting points are given for crystalline products. [c] Solvent system 1, CHCl$_3$–MeOH (9:1, v/v). [d] Solvent system 2, R_{f2}, CHCl$_3$–MeOH–H$_2$O (8:3:1, v/v, lower phase).

(XXV) was synthesized by condensation of a TFA-treated sample of VIII with Z(OMe)-Lys(Z)-OQCl[28] and subsequently with Z-Thr-NHNH$_2$. Z-Thr-Lys(Z)-Pro-Arg(NO$_2$)-Leu-OBzl (XXIX) was prepared starting with H-Leu-OBzl by successive introduction of Z(OMe)-Arg(NO$_2$)-OH, Z(OMe)-Lys(Z)-Pro-OH,[33] and Z-Thr-NHNH$_2$ by the DCC/HOBT procedure, the mixed anhydride method, and the azide procedure, respectively. An intermediate, Z(OMe)-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl (XXI), for the preparation of XXII, after TFA treatment, was condensed with Z(OMe)-Lys(Z)-Thr-NHNH$_2$ (XXXII) by the azide procedure and subsequently with Z-Arg(NO$_2$)-Pro-pentachloro phenyl ester (PCP)[34] to afford Z-Arg(NO$_2$)-Pro-Lys(Z)-Thr-Thr-Lys(Z)-Pro-Arg(NO$_2$)-OBzl (XXXIV). Peptide XXXII was prepared by the usual DCC method, followed by the usual hydrazine treatment. Peptide XXII, after TFA treatment, was further subjected to the coupling reaction with Z(OMe)-Lys(Z)-Pro-OH by the mixed anhydride procedure and then with Z-Thr-NHNH$_2$ by the azide procedure to give Z-Thr-Lys(Z)-Pro-Arg(NO$_2$)-Thr-Lys(Z)-Pro-Arg(NO$_2$)-Arg(NO$_2$)-Pro-Lys(Z)-Thr-OBzl (XLIII). Boc-Lys(Z)-Thr-OBzl[34] was treated with HCl/AcOEt, and the resulting N-deprotected dipeptide ester was condensed successively with Z(OMe)-Pro-OH and two Z(OMe)-Arg(NO$_2$)-OH residues by the DCC/HOBT procedure. Subsequently, Z(OMe)-Lys(Z)-Pro-OH and Z-Thr-NHNH$_2$ were introduced by the mixed anhydride procedure and the azide procedure, respectively, to afford XLIII.

Fully protected tuftsin analogues thus obtained were hydrogenated over a Pd catalyst for 7–9 h to remove all the protecting groups. The resulting deprotected peptides were purified by ion-exchange chromatography on carboxymethyl (CM)-Sephadex C-25, using a gradient elution

(33) Yajima, H.; Kitagawa, K.; Segawa, T. *Chem. Pharm. Bull.* **1973**, *21*, 2500.
(34) Yajima, H.; Kitagawa, K. *Chem. Pharm. Bull.* **1973**, *21*, 682.

Table II. Analytical Data of Unprotected Tuftsin Analogues

compd[a]	yield, %	$[\alpha]_D$ (temp, °C; concn, %, in 5% HOAc), deg	mobility (R_{f_3}; R_{f_4}; R_{Lys}[b])	formula	anal.
(III) H-Thr-Lys-β-Ala-Arg-OH	74	-3.83 (29; 0.79)	0.12; 0.10; 1.05	$C_{19}H_{38}O_6 \cdot 2CH_3COOH \cdot 0.5H_2O$	C, H, N
(VI) H-Thr-Lys-Pro-Cit-OH	48	-71.4 (29; 0.28)	0.18; 0.12; 0.77	$C_{21}H_{39}N_7O_7 \cdot CH_3COOH \cdot 0.5H_2O$	C, H, N
(X) H-Arg-Lys-Pro-Arg	83	-38.5 (28; 0.39)	0.08; 0.08; 1.25	$C_{23}H_{45}N_{11}O_5 \cdot 3CH_3COOH \cdot H_2O$	C, H, N
(XII) H-D-Thr-Lys-Pro-Arg-OH	61	-72.2 (28; 0.36)	0.11; 0.13; 1.05	$C_{21}H_{40}N_8O_6 \cdot 2CH_3COOH \cdot H_2O$	C, H, N
(XVI) H-Thr-Thr-Lys-Pro-Arg-OH	63	-71.1 (28; 0.38)	0.11; 0.13; 1.85	$C_{25}H_{47}N_9O_8 \cdot 2CH_3COOH \cdot 0.5H_2O$	C, H, N
(XVIII) H-Val-Thr-Lys-Pro-Arg-OH	74	-67.9 (28; 0.28)	0.13; 0.13; 0.84	$C_{26}H_{49}N_9O_7 \cdot 2CH_3COOH \cdot 1.5H_2O$	C, H, N
(XX) H-Lys-Thr-Lys-Pro-Arg-OH	67	-65.0 (28; 0.40)	0.07; 0.07; 1.10	$C_{27}H_{52}N_{10}O_7 \cdot 3CH_3COOH \cdot 2H_2O$	C, H, N
(XXIII) H-Arg-Thr-Lys-Pro-Arg-OH	40	-62.5 (29; 0.16)	0.10; 0.09; 1.12	$C_{27}H_{52}N_{12}O_7 \cdot 2CH_3COOH \cdot H_2O$	C, H, N
(XXVI) H-Thr-Lys-Lys-Pro-Arg-OH	57	-84.83 (28; 0.33)	0.06; 0.07; 1.04	$C_{27}H_{52}N_{10}O_7 \cdot 3CH_3COOH \cdot 0.5H_2O$	C, H, N
(XXX) H-Thr-Lys-Pro-Arg-Leu-OH	89	-183.9 (28; 0.31)	0.23; 0.25; 0.86	$C_{27}H_{15}N_9O_7 \cdot 2CH_3COOH \cdot H_2O$	C, H, N
(XXXV) H-Arg-Pro-Lys-Thr-Thr-Lys-Pro-Arg-OH	57	-100.0 (9; 0.21)	0.05; 0.11	$C_{42}H_{78}H_{16}O_{11} \cdot 4CH_3COOH \cdot H_2O$	C, H, N
(XXXVIII) H-Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg-OH	79	-103.6 (8; 0.28)	0.05; 0.12	$C_{42}H_{78}N_{16}O_{11} \cdot 4CH_3COOH \cdot 2H_2O$	C, H, N
(XLIV) H-Thr-Lys-Pro-Arg-Arg-Pro-Lys-Thr-OH	53	-103.8 (9; 0.26)	0.05; 0.12	$C_{42}H_{78}N_{16}O_{11} \cdot 4CH_3COOH \cdot 2H_2O$	C, H, N

[a] Prepared through catalytic hydrogenation of corresponding protected derivatives. [b] R_{Lys}, mobility assessed by high-voltage paper electrophoresis as compared to lysine·hydrochloride = 1.00.

Table III. Amino Acid Composition of Tuftsin Analogues

compd	Thr	Lys	Pro	Arg	Val	Leu	recovery, % Lys
	amino acid ratios						
X		1.00	1.14	2.00			91
XII	0.88	1.00	1.13	1.01			93
XVI	1.73	1.00	1.08	1.00			92
XVIII	0.94	1.02	1.06	0.92	1.00		91[a]
XX	0.91	2.00	1.17	1.01			89
XXIII	0.88	1.00	1.16	1.99			81
XXVI	0.94	2.00	1.27	1.05			84
XXX	0.92	0.98	1.06	0.90		1.00	94[b]
XXXV	1.90	2.00	2.21	1.81			90
XXXVIII	1.84	2.00	2.13	1.84			79
XLIV	1.88	2.00	2.09	1.76			86

[a] Recovery calculated as based on Val. [b] Recovery as based on Leu.

of ammonium acetate buffer followed by gel filtration on Sephadex G-10, and finally lyophilized to afford hygroscopic powders. Each of the tuftsin analogues thus obtained was found to be homogeneous on thin-layer chromatography (TLC), paper chromatography, and high-voltage paper electrophoresis. Amino acid and elemental analysis values also agreed well with theoretical values.

Biological Results

Evaluation of the stimulatory effect of tuftsin on both phagocytosis of ^{51}Cr-IgG-coated sheep red blood cells by mice peritoneal macrophages and on the immunogenic function of macrophages, i.e., on antigen presentation to lymphocytes, reveals a dose-dependent pattern. Thus, maximal potency was manifested at a peptide concentration of $\sim 5 \times 10^{-8}$ M, whereas at 1×10^{-7} M or at 1×10^{-8} M no activity was observed (Figure 1). These results are in close agreement with our previous finding that the calculated equilibrium dissociation constant, K_d, characterizing tuftsin–peritoneal macrophage association is 5×10^{-8} M.[17]

The activities of the various synthetic analogues were studied, at a concentration of 5×10^{-8} M, in comparison with that of tuftsin (Figure 2). Several conclusions can be derived from the data summarized in Figure 2: tuftsin is the most active peptide; some analogues, such as [Arg[1]]tuftsin, Arg-Pro-Lys-Thr-Lys-Pro-Arg (retro-tuftsinyltuftsin), Thr-Thr-Lys-Pro-Arg (threonyltuftsin), and [D-Thr[1]]tuftsin, exhibit considerable activity in both assay systems; several analogues, such as Lys-Thr-Lys-Pro-Arg (lysyltuftsin), Arg-Thr-Lys-Pro-Arg (arginyltuftsin), and [Cit[4]]tuftsin, do not stimulate phagocytosis and only slightly enhance immune response. Some derivatives, on the other hand, enhance markedly either

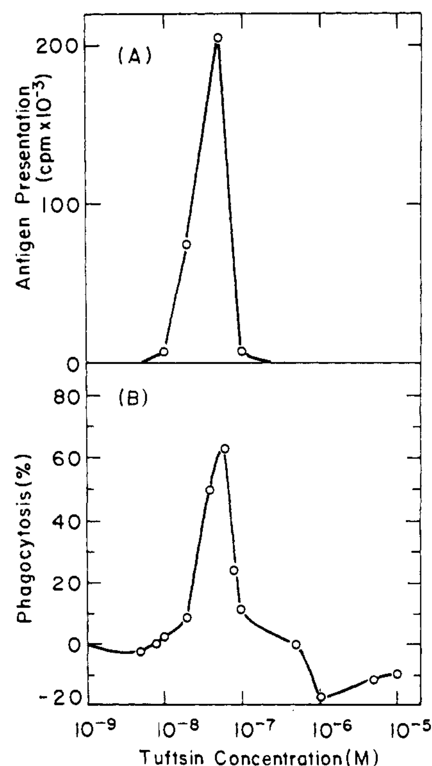

Figure 1. Effect of tuftsin on augmentation of macrophage activities: antigen presentation (A) and phagocytosis (B). In antigen presentation assay macrophages were fed with 50 μg of KLH/mL, and lymphocyte's response to 0.05 μg of KLH was measured. In phagocytosis assay, uptake of ^{51}Cr-IgG-SRBC by cells was measured. Zero stimulation refers to macrophages response without tuftsin.

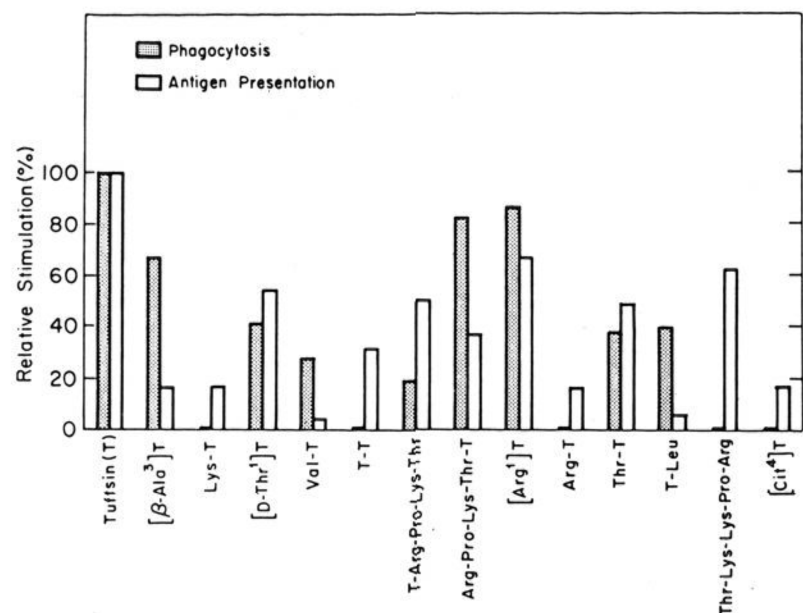

Figure 2. Effect of tuftsin's analogues on augmentation of macrophage activities. Analogues were applied at concentrations of 5×10^{-8} M; 100% stimulation refers to the augmentation of activities at a tuftsin concentration of 5×10^{-8} M.

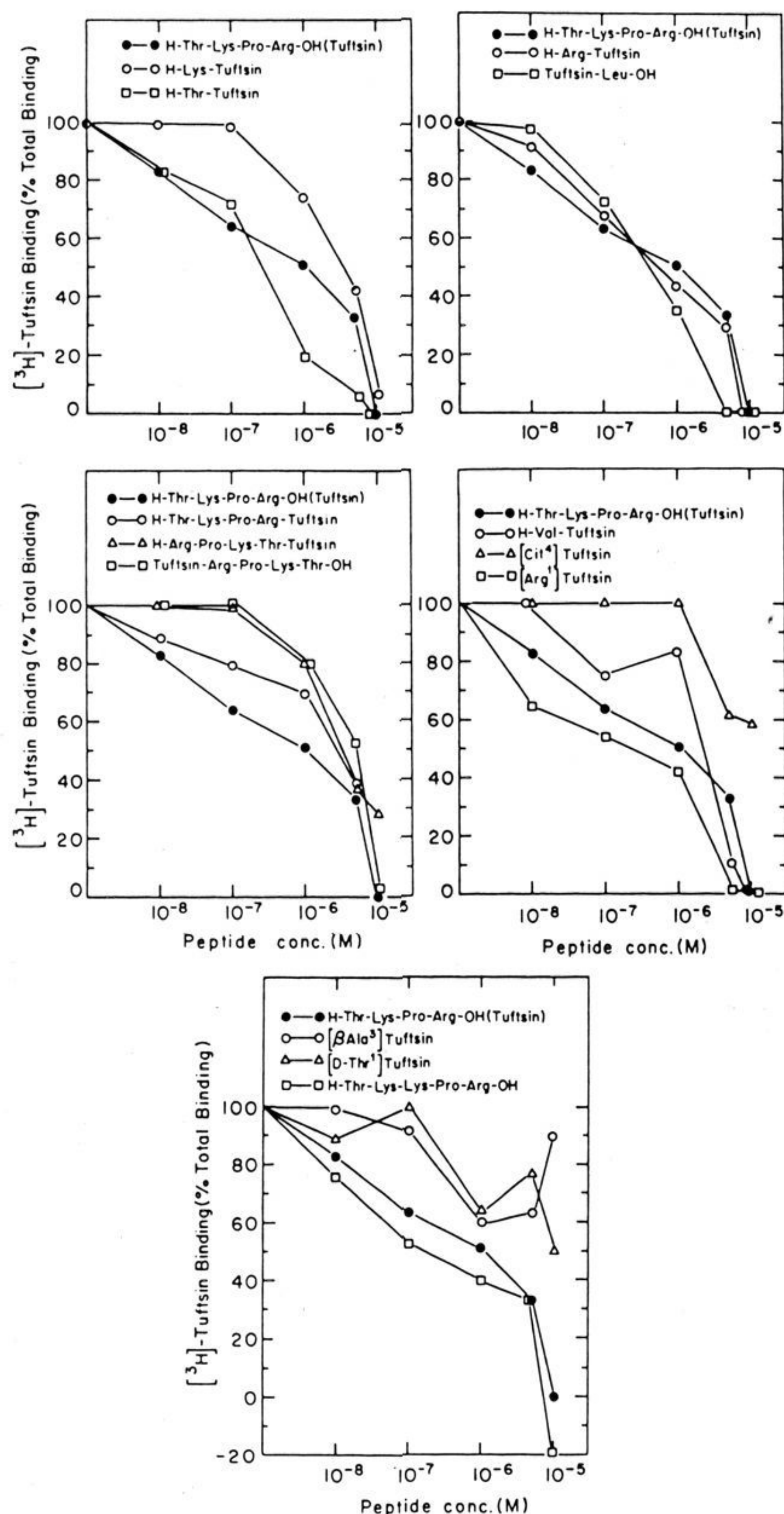

Figure 3. Displacement of [^{3}H-Arg^4]tuftsin binding to macrophages by different tuftsin analogues.

phagocytosis ([β-Ala^3]tuftsin) or the immune response (Thr-Lys-Lys-Pro-Arg). Similarly, we have previously

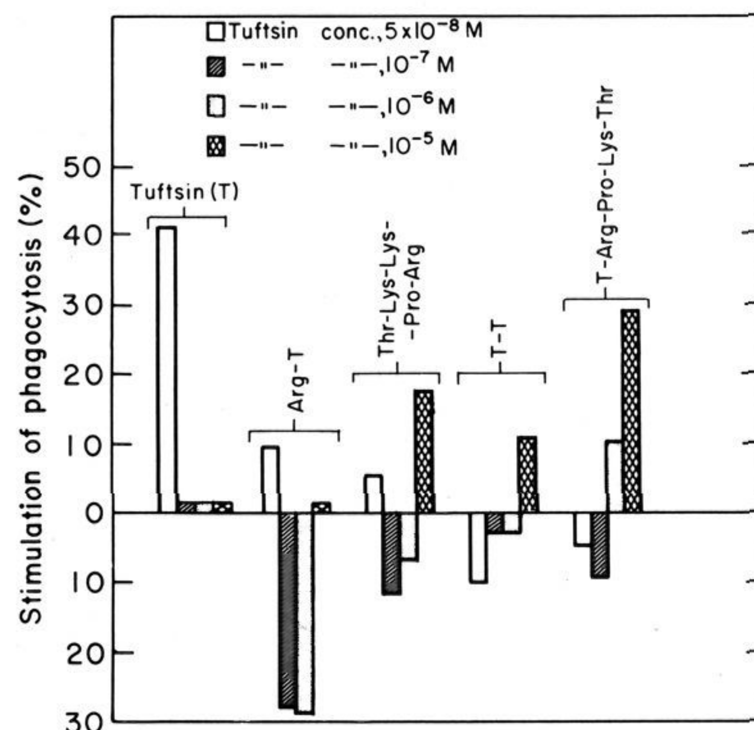

Figure 4. Inhibition of the phagocytic stimulation activity of tuftsin by tuftsin analogues. Peptides were applied to macrophages at a concentration of 1×10^{-7} M, together with tuftsin (at the indicated concentrations). Zero stimulation refers to cell's phagocytic response without tuftsin or peptides.

shown that [Ala^1]tuftsin is a potent inhibitor of several tuftsin-mediated events: phagocytosis,[6] respiratory burst,[6] and chemotaxis,[35] while it is a powerful immunostimulator and even stronger than tuftsin.[9]

In an attempt to correlate biopotency or impotency of the tuftsin analogues with the nature of their association with macrophages, their ability to compete with specific binding of [^{3}H-Arg^4]tuftsin to cells, at 22 °C, was studied. It was previously reported by us that tuftsin is internalized into peritoneal macrophages through a receptor-mediated endocytosis process.[36] This event is marked and fast (~5–30 min) at 37 °C, but also occurs, though at a slower rate (≥30 min), at 22 °C. The experiments described in this study thus represent primarily data relevant to specific peptide–cell surface association. As shown in Figure 3, [Arg^1]tuftsin, Thr-Lys-Lys-Pro-Arg, Thr-Thr-Lys-Pro-Arg (Thr-tuftsin), and Thr-Lys-Pro-Arg-Leu are capable of competing with the binding of radiolabeled tuftsin as efficiently as unlabeled tuftsin, or even better. These peptides possess substantial biological activity in at least one of the assay systems. The analogues [β-Ala^3]tuftsin, [D-Thr^1]tuftsin, Thr-Lys-Pro-Arg-Arg-Pro-Lys-Thr (tuftsinylretrotuftsin), and Arg-Pro-Lys-Thr-Thr-Lys-Pro-Arg (retrotuftsinyltuftsin), on the other hand, exhibit rather considerable activities (Figure 2), but compete efficiently with tritiated tuftsin binding only at relatively high concentrations (>10^{-6} M) (Figure 3). The peptide analogues Lys-Thr-Lys-Pro-Arg (lysyltuftsin), Val-Thr-Lys-Pro-Arg (valyltuftsin), and [Cit^4]tuftsin, which possess considerably low activity (Figure 2), are also poor competitors of [^{3}H-Arg^4]tuftsin binding. As shown in Figure 3, Arg-Thr-Lys-Pro-Arg (arginyltuftsin), Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg (tuftsinyltuftsin), and Thr-Lys-Lys-Pro-Arg are inhibitors of tritiated tuftsin binding. The first peptide is practically biologically inactive, while the latter two

(35) Schnabel, E.; Kostermeyer, H.; Berndt, H. *Justus Liebigs Ann. Chem.* **1971**, *749*, 90.

(36) Gottlieb, P.; Stabinsky, Y.; Hiller, Y.; Beretz, A.; Hazum, E.; Tzehoval, E.; Feldman, M.; Segal, S.; Zakuth, V.; Spirer, Z.; Fridkin, M. In *Antineoplastic, Immunogenic and Other Effects of the Tetrapeptide Tuftsin: A Natural Macrophage Activator*; Najjar, V. A., Fridkin, M., Eds.; Annals of the New York Academy oc Sciences: New York, 1983; Vol. 419, p 93.

compounds are capable of stimulating antigen presentation by macrophages, but are incapable of stimulating phagocytosis by the cells. The three peptides do inhibit enhancement of tuftsin-mediated phagocytosis by macrophages (Figure 4). As shown, inhibition prevails even at tuftsin–peptide molar ratios greater than 20:1.

Discussion

Earlier, we referred to the multitude and diversity of tuftsin-mediated processes in phagocytic cells. The essential feature of tuftsin is seemingly phagocytic stimulation from which other cellular functions stem.[37] The present study reveals that while tuftsin is equally capable of augmenting both phagocytosis and immune response of macrophages, certain analogues of tuftsin can preferentially enhance only one activity. These findings, coupled with the data obtained in binding–competition assays of [^{3}H-Arg^4]tuftsin, suggest that, perhaps, the cellular receptors for tuftsin can accommodate a certain "core" peptidic sequence but are functionalized specifically only by particular alteration of that sequence. To date, none of the many analogues of tuftsin synthesized (>100) (with one exception, [Ala^1]tuftsin, in the antigen-presentation assay system[9]) has higher phagocyte stimulating activity than the parent peptide. Only very few derivatives have activity close to that of tuftsin. Hence, it is becoming evident that the biological activity of tuftsin is very specific, yet the exact physiological role of the peptide is not elucidated.

In general, a direct relation exists between the capacity of tuftsin derivatives to inhibit the specific binding of [^{3}H-Arg^4]tuftsin to macrophages and their ability to stimulate the cells, or to inhibit tuftsin-mediated stimulations. A direct binding of labeled analogue with cells will, however, give better information on the nature of macrophage–peptide interaction.

The study of analogues composed of double sequences of tuftsin and retrotuftsin is especially noteworthy. Tuftsinyltuftsin, H-Thr-Lys-Pro-Arg-Thr-Lys-Pro-Arg-OH, was previously synthesized and found to exert antitumor activity against B16/5B melanoma[22] and L1210 leukemia cells,[12,22] considerably more effectively than tuftsin. The octapeptide, however, was devoid of phagocytosis stimulating activity.[21] The use of peptidic tuftsin dimer was proposed for two reasons: (I) as a means to produce tuftsin in vivo, through enzymatic cleavage by serum trypsin-like peptidase at the -Arg-Thr- bond, at a slow steady rate, and at an effective therapeutical level; one molecule of dimer will yield two molecules of tuftsin; (II) to minimize accumulation of large amounts of H-Lys-Pro-Arg-OH.[12,38] This tripeptide is a potent tuftsin inhibitor[6,12,15] and may be generated from tuftsin by enzymatic splitting at the H-Thr-Lys peptide bond.[39,40] It should be emphasized, however, that the rationale which led to the study of tuftsin dimers is based, as yet, on pure speculation. Thus, the existence of serum-specific endopeptidase and the in vivo production of tripeptide inhibitor were still not validated. Both topics are under current research in our laboratory. Our experiments reveal that tuftsinyltuftsin does not stimulate the phagocytic capacity of mouse peritoneal macrophages and enhances slightly, but significantly, the cell's immune response (Figure 2). The peptide, however, competes rather efficiently with the specific binding of [^{3}H-Arg^4]tuftsin to macrophages (Figure 3). Moreover, it blocks the tuftsin-mediated stimulation of phagocytosis by the cells (Figure 4). These in vitro results indicate that the intact octapeptide may, perhaps, also be associated in vivo with macrophages and activate them in a certain specific manner. Therefore, the possibility that the antitumor effect of tuftsinyltuftsin is, at least, partially a direct one, rather than occurring through enzymatic breakdown to yield tuftsin, should not be excluded.

Two dimers retrotuftsinyltuftsin (H-Arg-Pro-Lys-Thr-Thr-Lys-Pro-Arg-OH) and tuftsinylretrotuftsin (H-Thr-Lys-Pro-Arg-Arg-Pro-Lys-Thr-OH) can block the specific association of [^{3}H-Arg^4]tuftsin with macrophages, but much less efficiently than tuftsin (Figure 3). The two peptides, and particularly the former, are rather effective mediators of macrophage activity in vitro (Figure 2). They may prove, as well, to be effective antitumor agents in vivo.

Experimental Section

Synthetic Procedures. Melting points were determined on a Yamato melting point apparatus, Model MP-21, and are uncorrected. Optical rotations were measured with a Union automatic polarimeter, Model P-101 (cell length, 1 cm). The hydrolyses of peptides for amino acid analyses were carried out in 6 N HCl at 110 °C for 24 h. Amino acid compositions of acid hydrolysates were determined with a Hitachi amino acid analyzer, Model 835-30. Concentration of solutions was carried out in a rotary evaporator under reduced pressure at a temperature of 40–50 °C. Thin-layer chromatography (TLC) was performed on silica gel (Kieselgel G 60, Merck). R_f values refer to the following solvent systems: R_{f_1}, $CHCl_3$–MeOH (9:1, v/v); R_{f_2}, $CHCl_3$–MeOH–H_2O (8:3:1, v/v, lower phase); and R_{f_3}, *n*-butanol–AcOH–pyridine–H_2O (15:3:10:12, v/v). Paper chromatography was performed on Whatman No. 3 filter paper using *n*-butanol–AcOH–pyridine–H_2O (15:3:10:12, v/v) as the developing solvent (R_{f_4}). High-voltage paper electrophoresis was performed on Whatman No. 3 paper for 60 min at 33.3 V/cm in 0.75 M formic acid (pH 1.9), using a Toyo high-voltage paper electrophoresis apparatus (Model HPE-V Toyo Roshi No. 51). Mobility of L-lysine hydrochloride was used as the standard (1.00). Mobility of synthetic tuftsin was Lys × 1.02. Peptide azides were prepared from corresponding hydrazides, using isoamyl nitrite.[24] Peptide mixed anhydrides were prepared employing isobutyl chloroformate.[29]

***N,N'*-Dicyclohexylcarbodiimide (DCC) Coupling Procedure.** The desired N^α-protected carboxylic free component (1 equiv) was coupled with the corresponding C-protected α-amino free component (1 equiv) using DCC (1 equiv) as an agent,[25] in an appropriate organic solvent (see below). After filtration of *N,N*-dicyclohexylurea, the solution was concentrated. The residue was dissolved in ethyl acetate (AcOEt), and the solution was extracted consecutively with 1 N HCl, 5% $NaHCO_3$, H_2O and aqueous saturated NaCl solution, dried over anhydrous Na_2SO_4, and the solvent was evaporated in vacuo. Products were purified from the resulting masses (see below).

The above procedure was employed for the synthesis of the following peptides (Table I): I, from Z-β-Ala-OH and H-Arg(NO_2)-OBzl; IV, from Z(OMe)-Pro-OH and H-Cit-OBzl; VII, from Z(OMe)-Pro-OH and H-Arg(NO_2)-OBzl; XXII, from Z(OMe)-Arg(NO_2)-OH and H-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl in presence of HOBT[32] (1 equiv); XXVII, from Z(OMe)-Arg(NO_2)-OH and H-Leu-OBzl in presence of HOBT; XXXI, from Z(OMe)-Lys(Z)-OH and H-Thr-OMe; XXXIX, from Z(OMe)-Pro-OH and H-Lys(Z)-Thr-OBzl in presence of HOBT; XL from Z(OMe)-Arg(NO_2)-OH and H-Pro-Lys(Z)-Thr-OBzl in presence of HOBT; and XLI, from Z(OMe)-Arg(NO_2)-OH and H-Arg(NO_2)-Pro-Lys(Z)-Thr-OBzl in presence of HOBT. Couplings were carried out in CH_2Cl_2 (I, XXXI), THF (IV, VII), or DMF (XXII, XXVII, XXXIX, XL, XLI). Products were recrystallized from EtOH (I,

(37) Beretz, A.; Hiller, Y.; Gottlieb, P.; Fridkin, M.; Zakuth, V.; Spirer, Z. In *Peptide Chemistry 1982*; Sakakibara, S., Ed.; Protein Research Foundation: Osaka, 1983; p 207.
(38) Bump, N. J.; Chaudhuri, M. K.; Munson, D.; Parkinson, D. R.; Najjar, V. A. *EOS-J. Immunol. Immunopharmacol.* **1985**, *5*, 8.
(39) Najjar, V. A.; Konopinska, D.; Chaudhuri, M. K.; Schmidt, D. E.; Linehan, L. *Mol. Cell. Biochem.* **1981**, *41*, 3.
(40) Florentin, I.; Bruley-Rosset, M.; Kiger, N.; Imbach, J. L.; Winternitz, F.; Mathe, G. *Cancer Immunol. Immunoether.* **1978**, *5*, 211.

IV), AcOEt (XXXI), and DMF–AcOEt (XXXIX) or purified through chromatography on silica gel utilizing $CHCl_3$–MeOH mixtures (VII, 50:1; XXII, 9:1; XL, 20:1; XLI, 15:1—v/v) or AcOEt (XXVII) as eluents.

Azide Coupling Method. A solution of desired N^{α}-amino free component (1 equiv, obtained by neutralization of the corresponding trifluoroacetate or hydrohalide salt with 1 equiv of TEA) in DMF was mixed with a solution of the N^{α}-protected azide component (1 equiv, prepared in situ from the corresponding hydrazide derivative using isoamyl nitrite[24]) and TEA (1 equiv) in DMF. The mixture thus obtained was stirred at 5 °C for 44 h. The solvent was evaporated in vacuo and the residue purified by extractions as described above for the DCC coupling procedure. Final purification was achieved as described below.

The above procedure was essentially utilized for the synthesis of the following peptides (Table I): II, from Z-Thr-Lys(Z)-$NHNH_2$[3] and H-β-Ala-Arg(NO_2)-OBzl; V, from Z-Thr-Lys(Z)-$NHNH_2$ and H-Pro-Cit-OBzl; XI, from Z-D-Thr-$NHNH_2$ and H-Lys(Z)-Arg-(NO_2)-OBzl; XIII, from Z-Thr-$NHNH_2$ and H-Thr-OMe; XV, from Z-Thr-Thr-$NHNH_2$ (XIV) and H-Lys(Z)-Pro-Arg(NO_2)-OBzl; XVII, from Z-Val-Thr-$NHNH_2$[30] and H-Lys(Z)-Pro-Arg(NO_2)-OBzl; XIX, from Z-Lys(Z)-Thr-$NHNH_2$[31] and H-Lys(Z)-Pro-Arg(NO_2)-OBzl; XXI, from Z(OMe)-Thr-$NHNH_2$ and H-Lys(Z)-Pro-Arg(NO_2)-OBzl; XXV, from Z-Thr-$NHNH_2$ and H-Lys(Z)-Lys(Z)-Pro-Arg(NO_2)-OBzl; XXXIII, from Z(OMe)-Lys(Z)-Thr-$NHNH_2$ (XXXII) and H-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl; XXXVII, from Z-Thr-$NHNH_2$ and H-Lys(Z)-Pro-Arg(NO_2)-Thr-Lys(Z)-Pro-(NO_2)-OBzl; and XLIII, from Z-Thr-$NHNH_2$ and H-Lys(Z)-Pro-Arg-(NO_2)-Arg(NO_2)-Pro-Lys(Z)-Thr-OBzl. Products were recrystallized from EtOH (II) or AcOEt–petroleum ether (40–60 °C) (XIII) or purified through chromatography on silica, employing $CHCl_3$–MeOH mixtures (XV, 10:1; XI, XXXVII, XLIII, 15:1; XVII, XIX, 20:1; V, XXXIII, 25:1; XXI, XXV, XXIX, 30:1—v/v) as eluents.

Mixed Anhydride (MA) Coupling Method. Mixed anhydride was prepared by reacting the desired N^{α}-protected derivative (1 equiv) with isobutyl chloroformate (1 equiv) in DMF in the presence of TEA (1 equiv).[29] It was then mixed with a solution of the corresponding C-protected derivative (1 equiv) in DMF, and the reaction mixture was stirred at room temperature for 20 h. the solution was concentrated in vacuo, and the residue was purified by the extraction procedure with AcOEt, as described above, following silica gel chromatography.

The procedure was employed to synthesize the following peptides (Table I): IX, from Z-Arg(NO_2)-OH and H-Lys(Z)-Pro-Arg(NO_2)-OBzl; XXVIII, from Z(OMe)-Lys(Z)-Pro-OH and H-Arg(NO_2)-Leu-OBzl; XXXVI, from Z(OMe)-Lys(Z)-Pro-OH and H-Arg(NO_2)-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl; and XLII, from Z(OMe)-Lys(Z)-Pro-OH and H-Arg(NO_2)-Arg-(NO_2)-Pro-Lys-(Z)-Thr-OBzl. Peptides were eluted from silica employing $CHCl_3$–MeOH mixtures (IX, XXVIII, 30:1; XXXVI, XLII, 15:1—v/v).

Active Ester Coupling Procedure: 5-Chloro-8-quinolyl (QCl) and Pentachlorophenyl (PCP) Esters. The appropriate N^{α}-protected C-terminal activated derivative (1 equiv) was allowed to react with the respective N^{α}-amino free derivative (1 equiv) in DMF in the presence of TEA (1 equiv). After 40 h at room temperature, the solution was concentrated in vacuo. The residue was purified by the extraction procedure with AcOEt, followed by silica gel chromatography.

The procedure was used to prepare the following peptides (Table I): VIII, from Z(OMe)-Lys(Z)-OQCl and H-Pro-Arg-(NO_2)-OBzl; XXIV, from Z(OMe)-Lys(Z)-OQCl and H-Lys(Z)-Pro-Arg(NO_2)-OBzl; and XXXIV, from Z-Arg(NO_2)-Pro-PCP and H-Lys(Z)-Thr-Thr-Lys(Z)-Pro-Arg(NO_2)-OBzl. Silica columns were eluted with AcOEt (VIII) or with $CHCl_3$–MeOH (XXIV, 40:1; XXXIV, 15:1—v/v).

Preparation of Hydrazides. Z-Thr-Thr-$NHNH_2$ (XIV) and Z(OMe)-Lys(Z)-Thr-$NHNH_2$ (XXXII) (Table I) were prepared by treating a methanolic solution of the corresponding methyl esters (1 equiv of XIII and XXXI, respectively; ~2 mmol/3 mL) with hydrazine hydrate (5 equiv) at room temperature for 24 h. The resulting precipitates obtained were collected by filtration and recrystallized from MeOH–H_2O.

Removal of N^{α}-*p*-Methoxybenzyloxycarbonyl [Z(OMe)] Protection. The corresponding N^{α}-protected peptide derivative was treated with a mixture of trifluoroacetic acid and anisole (~3:1, v/v; ~3 mL/mmol of peptide) in an ice bath for 60 min. Then, dry ether was added to precipitate the respective trifluoroacetate salt products. Powders were collected by filtration and dried over KOH pellets in vacuo, whereas oily products were washed with dry ether and similarly dried in vacuo.

The method was employed to remove the Z(OMe) group from the following peptides (Table I): IV, VII, VIII, XXII, XXIV, XXVII, XXVIII, XXXIII, XXXVI, XXXIX, XL, XLI, and XLII. The Z(OMe) group of XXI was removed by treatment with 2 N HCl-AcOEt-anisole (5:1, v/v) for 60 min at room temperature. The Z group of I was cleaved by 25% HBr-AcOH-anisole (6:1, v/v) for 60 min at room temperature.

Removal of Protecting Groups by Catalytic Hydrogenation. The corresponding protected peptide derivative was dissolved in a mixture of MeOH–H_2O–AcOH (1:1:1, v/v; ~0.1 mmol/10 mL), Pd/C catalyst added, and hydrogenation carried out for 8 h at room temperature. The catalyst was removed by filtration, and the filtrate was concentrated in vacuo. The residue was dissolved in water (~0.1 mmol/50 mL) and applied to a CM-Sephadex C-25 column (2.8 × 2.5 cm), which was generally eluted with a linear gradient of ammonium acetate buffer (pH ~7.0; salt molarity increasing up to 0.25). The peptide's elution was monitored by Sakaguchi and ninhydrin test. Fractions containing the desired compound were pooled and concentrated in vacuo. The residue dissolved in a small amount of 0.1 M HOAc, loaded on a Sephadex G-10 column (1.8 × 65 cm), and eluted with 0.1 M HOAc. The desired fractions were pooled and lyophilized to give a hygroscopic powder.

The procedure was essentially used for the preparation of the following peptides (Table II): III from II; VI from V; X from IX; XII from XI; XVI from XV; XVIII from XVII; XX from XIX; XXIII from XXII; XXVI from XXV; XXX from XXIX; XXXV from XXXIV (purification by three gel filtrations on Sephadex G-10 using 0.1 AcOH as eluent); XXXVIII from XXXVII; and XLIV from XLIII.

Bioactivity Assays. Macrophages. Peritoneal exudate cells were aseptically collected from thioglycolate-stimulated (Balb/c × C_3H)F1-strain female mice (6–8-weeks old). The stimulating agent, thioglycolate broth (2.98 g/100 mL, Difco Laboratories, Detroit, MI), was injected intraperitonealy (3 mL/mouse) 4 days prior to cell harvest. Cells were collected by washing the peritoneum with cold phosphate-buffered saline (PBS, Gibco, pH 7.4), centrifuged, and resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco) at a final concentration of 1×10^6 cells/mL. Cell preparations consistently contained >90% macrophages of which more than 87% were viable cells.

IgG-Coated Sheep Red Blood Cells (SRBC). Erythrocytes (10^9 cells suspended in 3 mL of PBS) were sensitized with rabbit anti-SRBC antibodies (7-S IgG, Cordis Laboratories) (3 mL of 1:150 dilution in PBS) for 45 min at 37 °C. Following three washes in PBS, cells were resuspended in PBS (3 mL) and ^{51}Cr (150 μCi, Amersham) was added. After incubation for 2 h at 37 °C, cells were washed (3×) in PBS and resuspended in PBS (60 mL).

Phagocytosis Assay. Macrophages, suspended in DMEM (1×10^6 cells/mL) were layered on tissue culture trays (Costar, 24 wells/tray, 0.5 mL/well). After incubation for 2 h at 37 °C in a humidifed incubator (5% CO_2, in air), nonadherent cells were removed by washing (3×) with PBS and cultures were supplemented with 1 mL of DMEM-5% fetal calf serum (GIBCO, Grand Island, NY). Subsequent to cultivation for 24 h, cells were washed in PBS and preincubated with tuftsin or its analogues for 15 min at 37 °C. ^{51}Cr-IgG-coated SRBC (0.5 mL) were then added, and the macrophage monolayers were further incubated for 45 min at 37 °C. The cells were then washed once with PBS, treated with 0.83% NH_4Cl solution to remove free and surface-bound SRBC, washed again with PBS (3×), and finally dissolved in 0.2% sodium dodecyl sulfate (SDS, 0.5 mL). The solution obtained was collected and radioactivity was measured in a Hewlett-Packard γ counter. Results given are from triplicate wells whose SEM is less than 5%.

Immune Response Assay. Antigen Presentation of Macrophages. Macrophages, suspended in DMEM (2×10^6 cells/mL) were layered on 100-mm plastic tissue culture petri dishes (Falcon No. 3003), 10 mL/dish. The dishes were incubated overnight at 37 °C in a humidified atmosphere of 5% CO_2 in air

to provide maximum adherence of macrophages as a monolayer. The cells were then washed 3 times with PBS to remove nonadherent cells. Five milliliters of DMEM, containing 50 μg of KLH (Keyhole limpet hemocyanine)/mL was added to each petri dish. In the experimental groups, the medium also contained tuftsin or its tested analogue at various concentrations. After 4 h at 37 °C, excess free antigen was washed out with three successive washes with an excess of PBS. Ten milliliters of spleen cell suspension (10^7 cells/mL) was added on top of the antigen-fed monolayers, and incubation proceeded for 18 h at 37 °C, in an atmosphere of 5% CO_2 in air. The nonadherent cells were then collected gently, reseeded on another set of petri dishes, and incubated for 90 min to adsorb residual adherent cells. The spleen cells were then irradiated with 1000 rad, using a Dermavolt X-ray machine (Siemens X-ray tube, 56 kV, 20 mA, 0.5 Al filter, 750 rad/min). The suspension was collected, centrifuged, and washed twice with PBS. The cells were resuspended in DMEM (10^8 cells/mL). Fifty microliters of this suspension was injected in the hind foot pads of syngeneic mice. On the seventh day, the popliteal lymph nodes were removed and a cell suspension was placed in culture medium (RPMI 1640, Gibco, supplemented with 0.5% syngeneic mouse serum and 50 μM 2-mercaptoethanol) adjusted to a concentration of 5×10^6 cells/mL. Samples (0.1 mL) of these cells were cultured in tissue microtiter plates (Costar 96 wells/tray) in the presence of antigen or control reagents at 37 °C in a humidified atmosphere of 5% CO_2 in air. After 72 h, 1 μCi of tritiated thymidine (Israel AEC, Negev, Israel) was added and the cells were incubated for 16 h. Cells were then collected by a Titertek cell harvester (Skatronas, Liebyen, Norway) on glass filter, washed twice with saline, dried, and placed in a Bray's scintillation fluid. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Competitive Binding Assay. All binding studies were performed in PBS at 22 °C for 30 min at a final volume of 0.5 mL. Macrophage suspensions were incubated with gentle agitation in plastic tubes (NUNC 70 × 12 mm; 1×10^6 cells/tube), with tritiated tuftsin([^{3}H-Arg^4]tuftsin; specific activity, 20.8 Ci/mmol; 5×10^{-8} M) and varying concentrations of unlabeled tuftsin or the tested peptide analogue. Binding was terminated by dilution with PBS (3 mL), followed by centrifugation and removal of the supernatant by aspiration. Cells were subsequently washed once more with PBS and similarly isolated. The cell pellets were then dissolved in 0.3 mL of SDS (0.2%), and the solutions obtained were collected and added to vials, each containing 5 mL of Triton-toluene mixture. Radioactivity was measured in a Beckman 7500 liquid scintillation spectrophotometer. Each point on the resulting binding curves derives from duplicate tubes, and the standard error of the mean (SEM) did not exceed 5%. Nonspecific binding was defined as the amount of tritiated tuftsin not inhibited by 10 nM tuftsin.

Role of the C-Terminal Carboxylate in Angiotensin II Activity: Alcohol, Ketone, and Ester Analogues of Angiotensin II[†]

Kun-hwa Hsieh*[‡] and Garland R. Marshall[§]

Department of Pharmacal Sciences, School of Pharmacy, Auburn University, Auburn University, Alabama 36849, and Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110.
Received August 26, 1985

[Ac-Asn^1,Val^5]angiotensin II analogues containing a C-terminal alcohol (Phe-ol), methyl ketone (Pmk), methyl ester (Phe-OMe), or α-methyl methyl ester (Phe(αMe)-OMe) were prepared in order to examine the relative importance of COOH-mediated ionic vs. hydrogen bonding interactions in angiotensin activities. Based on the observation that only [Ac-Asn^1,Phe-OMe^8]AII (AII, angiotensin II) had significant activities (20% oxytocic and 13% pressor) in the rat, with all other analogues having negligible agonistic and antagonistic effects, it is concluded that ionic interaction of the C-terminal carboxylate with the receptor is necessary for angiotensin binding and that hydrogen bonding has little effect. Thus, the different potencies observed for the AII methyl ester and for various C-terminal analogues previously reported may simply reflect their relative abilities to generate the active carboxylate species in situ.

The COOH-terminus of biologically active peptides is frequently a target for drug design, in that the COOH group predisposes its parent peptides to metabolic breakdown by carboxypeptidase and converting enzyme/dipeptidase. In addition, peptides with a carboxylate group are often poorly absorbed through gastrointestinal membranes due to their ionic nature at physiological pH. Consequently, conversion of the COOH group to a proteolytic-resistant and nonionic alcohol in enkephalin or to a monoester in enalapril, a converting enzyme inhibitor, significantly improved their oral effectiveness.[1,2] Since the COOH group acts primarily through ionic and hydrogen bondings, hormone-receptor and enzyme-substrate interactions involving the peptide COOH-terminus would require a counterion or nucleophile on the enzyme/receptor. This concept has been utilized in the design of selective and irreversible affinity labels, such as peptide chloromethyl ketone[3,4] and aldehyde.[5]

In angiotensin II (Asp-Arg-Val-Tyr-Val-His-Pro-Phe, AII), C-terminal esterification or amidation significantly reduces its biological activities,[6-12] suggesting that a free

[†]The abbreviations used to denote amino acids and peptides are those recommended by the IUPAC Commission on Nomenclature: *Biochemistry* **1975**, *14*, 449, and *Biochemistry* **1967**, *6*, 362. Other abbreviations are as follows: AI, angiotensin I (Asp-Arg-Val-Tyr-Val-His-Pro-Phe-His-Leu); AII, angiotensin II (Asp-Arg-Val-Tyr-Val-His-Pro-Phe); Phe-ol, phenylalaninol; Pmk, phenylalanine methyl ketone or 4-phenyl-L-3-aminobutan-2-one; Phe(αMe), α-methylphenylalanine; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole monohydrate; PMSF, phenylmethylsulfonyl fluoride.

[‡]Auburn University.

[§]Washington University School of Medicine.

(1) Roemer, D.; Buescher, H. H.; Hill, R. C.; Pless, J.; Bauer, W.; Cardinaux, F.; Closse, A.; Hauser, D.; Huguenin, R. *Nature (London)* 1977, *268*, 547.
(2) Sweet, C. S. *Fed. Proc.* 1983, *42*, 167.
(3) Kettner, C.; Shaw, E. *Biochemistry* 1978, *17*, 4778.
(4) Kettner, C.; Shaw, E. *Thrombosis Res.* 1979, *14*, 969.
(5) Thompson, R. C. *Biochemistry* 1973, *12*, 47.
(6) Needleman, P.; Marshall, G. R.; Rivier, J. *J. Med. Chem.* 1973, *16*, 968.
(7) Schwyzer, R. *Helv. Chim. Acta* 1961, *44*, 667.
(8) Rioux, F.; Park, W. K.; Regoli, D. *Can. J. Physiol. Pharmacol.* 1975, *53*, 383.
(9) Guttmann, St. *Helv. Chim. Acta* 1961, *44*, 744.
(10) Bumpus, F. M.; Khairallah, P. A.; Arakawa, K.; Page, I. H.; Smeby, R. R. *Biochim. Biophys. Acta* 1961, *46*, 38.
(11) Regoli, D.; Park, W. K.; Rioux, F. *Pharmacol. Rev.* 1974, *26*, 69.

0022-2623/86/1829-1968$01.50/0 © 1986 American Chemical Society