1-Aminocyclobutanecarboxylic Acid Derivatives as Novel Structural Elements in Bioactive Peptides: Application to Tuftsin Analogs

Eytan Gershonov,[†] Ruth Granoth,[†] Esther Tzehoval,[‡] Yehiel Gaoni,[†] and Mati Fridkin*,[†]

Departments of Organic Chemistry and Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

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Four novel 2,4-methano amino acids (MAAs, 1-aminocyclobutane-1-carboxylic acids) were synthesized. These include the basic MAA analogs of lysine (**16**), ornithine (**5**), and arginine (**6**) and the neutral methanovaline (**22**), related to proline. The above MAAs, as well as the MAA analog of homothreonine (**7**), were incorporated into the peptide chain of the immunomodulatory peptide tuftsin, Thr-Lys-Pro-Arg, known to enhance several biological activities mediated by phagocytic cells. The synthetic methano tuftsin analogs were assayed for their ability to stimulate interleukin-6 (IL-6) secretion by mouse peritoneal macrophages and for their stability in human serum toward enzymatic degradation. It was found that, at 2×10^{-7} M, [MThr[1]]tuftsin (**24**) and an isomer of [MVal[3]]tuftsin (**27a**) were considerably more active than the parent peptide in augmentation of cytokine release. [MOrn[2]]Tuftsin (**25**) was equally potent. The analogs [MThr[1]]tuftsin (**24**) and [MOrn[2]]tuftsin (**25**), both pertaining to the proteolytically sensitive Thr-Lys bond of tuftsin, exhibited high resistance to enzymatic hydrolysis as compared to tuftsin. Using specific rabbit anti-tuftsin antibodies in a competitive enzyme-linked immunosorbent assay (ELISA) revealed that none of the MAA analogs can cross-react with tuftsin. It may indicate that the peptides assume global structures different than that of tuftsin.

Introduction

The use of peptides in medically-oriented research, basic as well as applied, is constantly increasing. The major clinical potential of this family of compounds has, as yet, only slightly been realized. Hence, there is a growing demand for diversification of the arsenal of suitable building blocks whose incorporation into peptide chains may lead to augmentation of corresponding biopotency, selectivity and bioavailability. Efforts have been directed toward these goals through, notable among others, improving resistance to proteolysis[1,2] and stabilization of putative bioactive structures.[1,3] Methodologies such as peptide-bond modifications,[4] rational cyclization,[5−9] and introduction of bulky, motility-constraining elements into the peptide chain[7,10,11] have been investigated. 1-Aminocyclobutane-1-carboxylic acid (2,4-methano amino acid; MAA) derivatives belong to the latter category. Several of these amino acid analogs, bearing carboxyl or phosphoryl side chain moieties, have been studied in relation to excitatory receptors in the mammalian central nervous system.[12−14] The application of simple MAA derivatives, i.e. without side-chain functions, along with other $C^{\alpha,\alpha}$-symmetrically disubstituted cycloaliphatic glycine, has been limited, so far, to the synthesis of compounds related to L-aspartyl dipeptide sweeteners[15] and to basic conformational considerations.[16] Both of these latter studies have clearly pointed at the potentiality of MAA derivatives as beneficial structural components in bioactive peptide research. Aiming to test the feasibility of incorporating MAA, bearing "side-chain" moieties, into peptide chains and to evaluate its consequences we choose tuftsin as a model. Toward this goal we have synthesized four novel MAA derivatives.

Tuftsin, Thr-Lys-Pro-Arg, is a serum basic peptide, originating from immunoglobulin G through specific proteolysis, which has been found to exhibit a wide spectrum of biological activities associated with the reticuloendothelial and immune system function.[17] It is a potent immunomodulator whose capacity is manifested through and following binding to specific receptors present on virtually all phagocytic cells.[18,19] Tuftsin has a marked therapeutic potential, e.g. as an antimicrobial and anticancer agent,[17,20,21] and thus the development of potent and metabolically stable analogs is of major significance. The steric hindrance and conformational constrain induced on the tuftsin molecule by the cyclobutane ring of MAA may lead to realization of the above goals. Furthermore, all four amino acid residues in the tuftsin molecule are important, with certain allowed alterations, for manifestation of optimal biopotency.[17,22] Systematic substitution of amino acids by corresponding MAA derivatives may shed additional light on structure−function relationships and mode of action of tuftsin.[17] It may also lead to novel tuftsin-related drugs.

Chemistry

Four novel *trans*-2,4-methano amino acids derivatives were prepared in the present study and utilized in peptides synthesis. These compounds are analogs of three basic amino acids, i.e. ornithine (**5**; MOrn), arginine (**6**; MArg), and lysine (**16**; MLys), and of valine (**22**; MVal), used here as a substitute for proline. A previously reported[23,24] analog of homothreonine (**7**; MThr) was also synthesized (Scheme 1). The synthetic manipulations which led to compounds **5**−**7** and **16** are summarized in Schemes 1 and 2. They refer to and are

* Author to whom correspondence should be addressed. Tel: 972-8-9342505. Fax: 972-8-9344142.
[†] Department of Organic Chemistry.
[‡] Department of Immunology.
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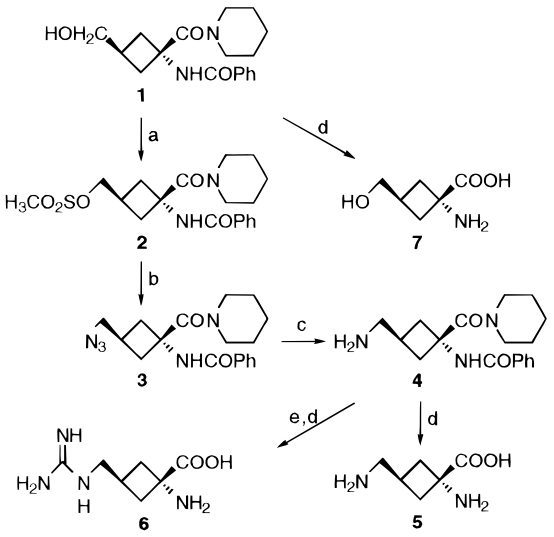
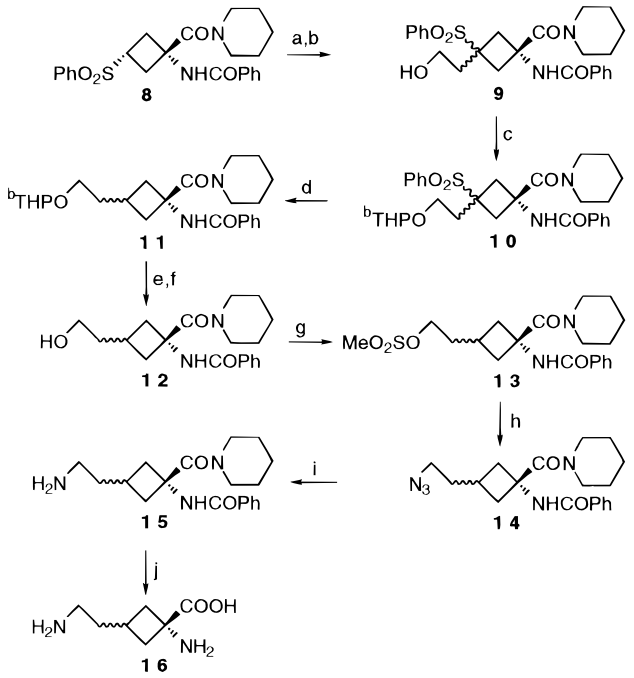
Scheme 1[a]



[a] (a) $MeSO_2Cl$, NEt_3, CH_2Cl_2; (b) TMGA, NMP; (c) H_2, 10% Pd/C, EtOH; (d) 6 N HCl; (e) DMPCN, NEt_3, DMF.

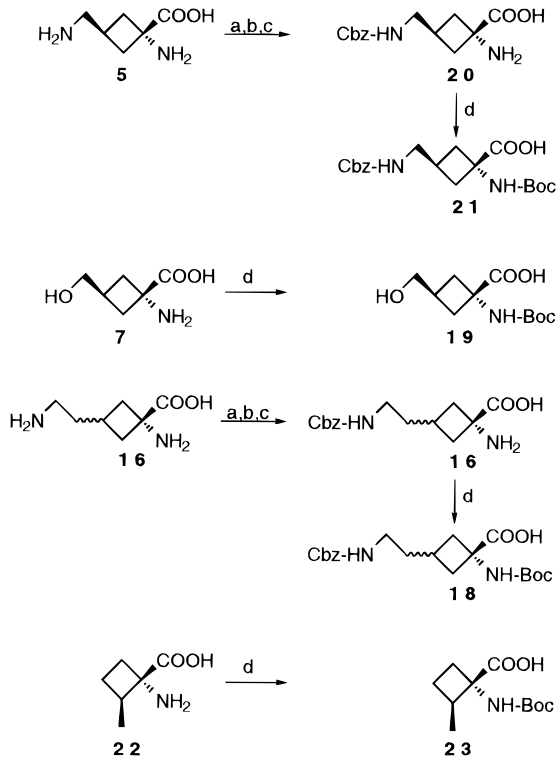
Scheme 2[a]



[a] (a) BuLi, THF; (b) oxirane; (c) DHP, PPTS, CH_2Cl_2; (d) Na/Hg 6%, MeOH:THF, 3:1; (e) unsuccessful isomers separation; (f) EtOH, PPTS; (g) $MeSO_2Cl$, NEt_3, CH_2Cl_2; (h) TMGA, NMP; (i) H_2, 10% Pd/C, EtOH; (j) 6 N HCl. [b] Tetrahydropyranyl ether.

based on the general scheme for the preparation of substituted cyclobutanes from 1-(phenylsulfonyl)bicyclo-[1.1.0]butane (**8**).[12,25] Thus, the high strain of the bicyclobutane ring, coupled with the olefinic π-bond nature of the central C−C bond and with the enhanced acidity of the bridgehead proton, make this system particularly prone to a large variety of chemical modifications.[26,27] As previously shown, the amino functions, at both α and side-chain positions of MAA derivatives, were introduced via reduction of corresponding azido groups of **3** and **14**. The guanidino side chain of the MAA analog of arginine (**6**) was introduced by reacting the free amino group of intermediate **4** with 3,5-dimethylpyrazole-1-carboxamidine nitrate (DMPCN).[28] The overall yields in the reactions leading to the MAA

Scheme 3[a]



[a] (a) Basic $CuCO_3$, H_2O; (b) $PhCH_2CO_2Cl$, MgO, H_2O; (c) Na_2S, 0.9 N HCl; (d) $((CH_3)_3COCO)_2O$, NEt_3, DMF/H_2O.

5−**7** and **16** from **8** were 11%, 10%, 22%, and 7%, respectively. Racemic *trans*-1-amino-2-methylcyclobutane-1-carboxylic acid (MVal; **22**) was synthesized by the same procedures described for the preparation of intermediate **8** using crotyl bromide instead of allyl chloride.[29] Protection of the side-chain amino group and of the α-amino group of the MAA is described in Scheme 3. The purity of MAA, as well as that of en-route intermediates, was ascertained by NMR, by mass spectrometry, and by homogeneity in amino acid analysis. This latter technique was further employed for the quantitative determination of MAA content in the corresponding peptides.

Four analogs of tuftsin, [MThr[1]]tuftsin (**24**), [MOrn[2]]-tuftsin (**25**), [MLys[2]]tuftsin (**26**), and [MVal[3]]tuftsin (**27**) (Figure 1), were synthesized following solid phase methodology,[30] on a chloromethylated polystyrene carrier, utilizing N^{α}-*t*-Boc strategy. An equimolar combination of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBT) was employed as coupling agent. The various stages of peptide chain extensions were monitored by qualitative ninhydrin test. As a rule, double couplings were performed throughout syntheses. Respective incorporation of MAA derivatives did not create difficulties related to incomplete couplings, as might perhaps been anticipated due to steric considerations. The methanoarginine analog of tuftsin, [MArg[4]]tuftsin (**28**) (Figure 4), was prepared in solution by coupling of unprotected MArg (**6**) with the tripeptide active ester Boc-Thr-Lys(Boc)-Pro-OSu[17] following a corresponding deprotection. All crude tuftsin analogs were purified to homogeneity by semipreparative HPLC on silica RP-18 column and their correct structures ascertained by amino acid analysis and mass spectrometry.

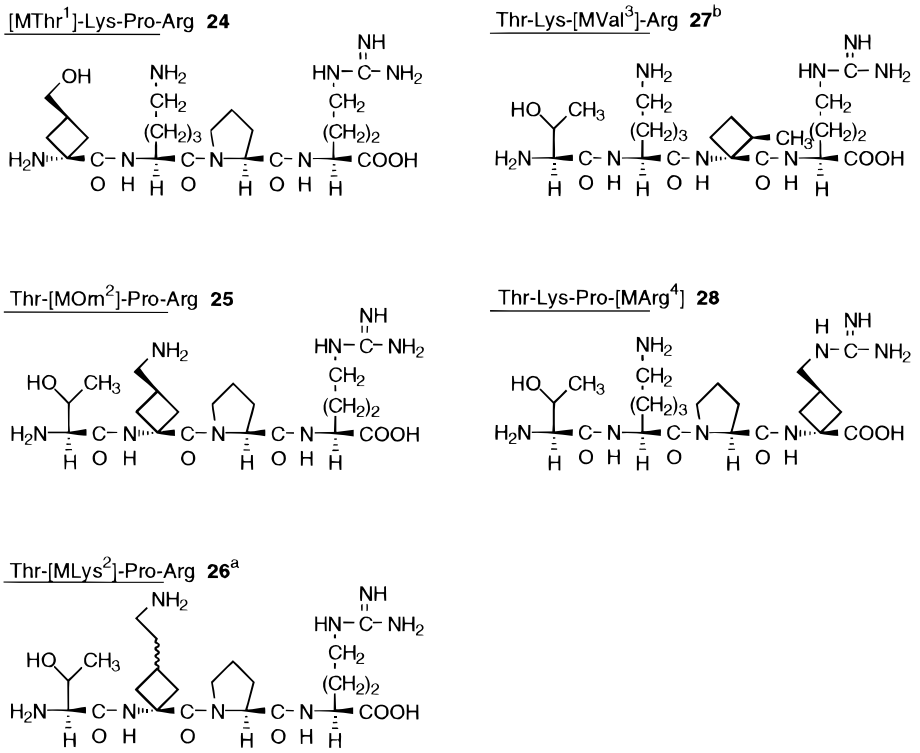


Figure 1. Structures of methano tuftsin analogs. [a]Tuftsin analog **26** was obtained as a mixture of both cis and trans isomers of methanolysine and was separated by HPLC. They will be referred to as **26a** and **26b**. [b]Tuftsin analog **27** was obtained as a mixture of two diastereomers (due to racemic MVal) and was separated by HPLC. They will be referred to as **27a** and **27b**.

Circular Dichroism (CD) Measurements

The possibility that tuftsin may assume a preferred conformation in solution was investigated by many groups, using a variety of physical techniques. However, rather conflicting conclusions have been drawn. Considerations based on X-ray, NMR, and IR data[31] as well as on IR studies[32] suggested that tuftsin has a certain tendency to form a 4 → 1 hydrogen-bonded β-turn of type 3. It has also been suggested that this structure is further stabilized by ionic interaction between the carboxyl terminal of Arg[4] and the α-amino terminal of Thr[1]. This was further supported by CD spectra of tuftsin and its analogs.[33] Conformational calculations based on energy minimization[34] revealed that the peptide may assume numerous low-energy conformations which share a general characteristic described as a "hairpin with two split ends", where each of the splits is composed of one terminus of the backbone and a Lys or Arg side chain. Other conformational studies based on calculations[35] and CD spectra[36] proposed that the tuftsin chain is folded to form a quasi-cyclic structure, stabilized by ionic interaction between the carboxylic group of Arg[4] and the ϵ-amino group of Lys[2]. Further ^{1}H NMR and ^{13}C NMR studies[37] suggested that tuftsin in aqueous solution exists in a random conformation, while in DMSO a slightly ordered structure is obtained. More recently it was postulated, on the basis of results of high-temperature quenched molecular dynamics calculations of tuftsin in solution, that a type 4 β-turn characterize the Lys[2]-Pro[3] position.[38] In view of the above it is evident that there is no consensus as to the structure of tuftsin in aqueous media. It can perhaps be assumed that tuftsin has a rather random structure in aqueous solution. This notion is further supported by extensive structure–function studies on tuftsin analogs.[22] The high specificity of its biological action as well as its binding to specific receptors are therefore indicative of the possibility that tuftsin assumes an "active conformation" only upon adhering to its target cells.

Our present CD studies of tuftsin in water (pH 5) reveal, in agreement with previous studies,[36] a maximal negative ellipticity at 198 nm characteristic of an ensemble of random conformations.[39] The positive maximum observed at 221 nm[36] appears, however, now as a positive shoulder at 226 nm (Figure 2A). The CD spectrum of tuftsin in trifluoroethanol (TFE) is similar in shape to that obtained in water but with a marked decrease in intensity of the negative maximum at 198 nm. This effect was considered,[36] on the ground of pH dependence of CD spectra, to be indicative of an interaction of the carboxylic Arg[4] group with the amino group Thr[1] contributing to the stabilization of the tuftsin's conformation. However, this change was also interpreted as the result of an increasing population of β-turns conformers in solution. This suggestion is further supported by the appearance of the small negative maximum at 225–226 nm.

The CD curves of [MArg[4]]tuftsin **28** in water is similar to that of tuftsin but with a slight decrease of maximum negative intensity in the region 198–200 nm. No changes were observed between the CD spectra of **28** in water and in TFE, suggesting that this analog had no apparent secondary structure in solution (not shown). The CD curves of [MVal[3]]tuftsin (**27a**) (Figure 2A), [MOrn[2]]tuftsin (**25**) (Figure 2B), and [MLys[2]]tuftsin (**26b**) (not shown) are completely different from the spectrum of tuftsin. There is a marked decrease in intensity of ellipticity and a red concomitant shift of the negative maximum, which becomes broader. The CD spectra obtained for [MVal[3]]tuftsin (**27a**) in TFE and for [MOrn[2]]tuftsin (**25**) in water display a negative

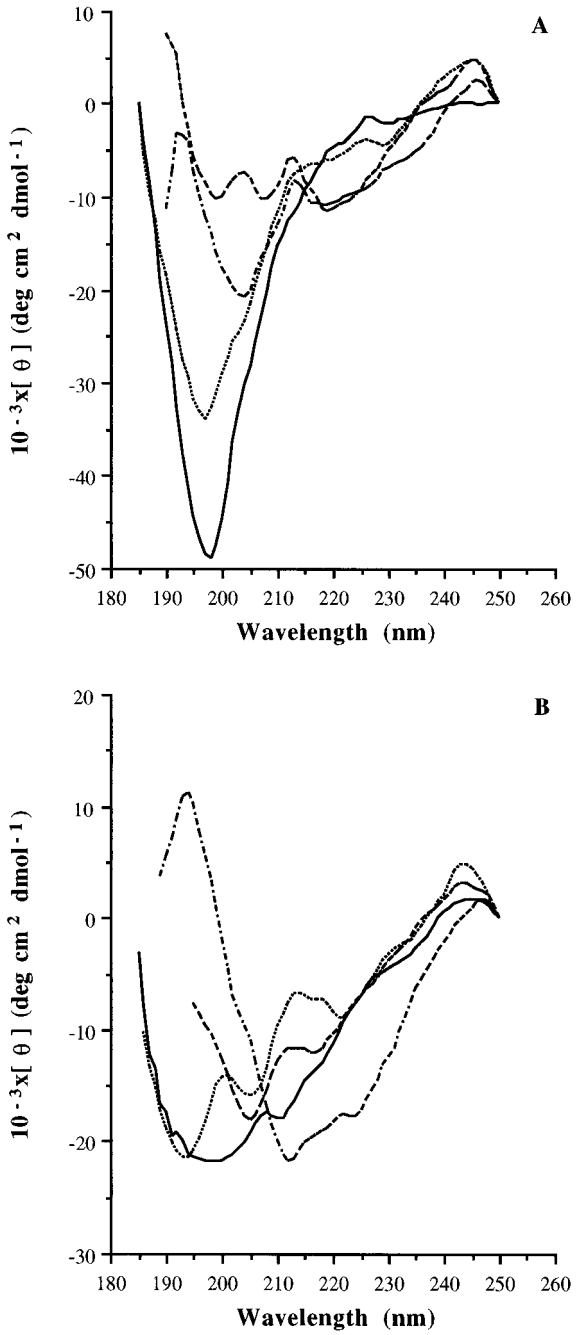


Figure 2. CD spectra of aqueous and TFE solutions of tuftsin and methano tuftsin analogs. Symbols correspond to the following peptides: (A) (—) tuftsin (T) in H_2O, (···) tuftsin in TFE, (- - -) [MVal^3]T **27a** in H_2O, (- · -) [MVal^3]T **27a** in TFE. (B) (—) [MThr^1]T **24** in H_2O, (···) [MThr^1]T **24** in TFE, (- - -) [MOrn^2]T **25** in H_2O, (- · -) [MOrn^2]T **25** in TFE.

maximum at 204 nm and a positive maximum at 192−194 nm with a shoulder at about 214 nm. This curve shape is very similar to that observed in the CD spectrum of gramicidin S and of [DLys^2]tuftsin, where the type 2′ β-turn is present.[33] The CD curves of [MOrn^2]tuftsin (**25**) in TFE (Figure 2B) and of [MLys^2]-tuftsin (**26b**) (not shown) in water and in TFE display a broader negative maximum in the region 212−218 nm and a positive maximum at 190−195 nm, suggesting the presence of different types of β-turns.[40] The intensity of the positive maximum is smaller than expected which may indicate the presence of another population of conformations or some distortion of the β-turn. [MVal^3]Tuftsin (**27a**) in water (Figure 2A), [MThr^1]-tuftsin (**24**), both in water and TFE (Figure 2B), and [MVal^3]tuftsin (**27b**) (not shown) gave a spectrum which did not correspond to any of the characteristic common patterns of well-defined structures.

In contrast to the above, major alterations in CD spectra were obtained with [MVal^3]tuftsin (**27a**), [MOrn^2]-tuftsin (**25**), and [MLys^2]tuftsin (**26b**), as compared to tuftsin, indicating perhaps the presence of certain preferred conformers, probably of the β-turn types. The [MArg^4]tuftsin (**28**) spectrum was, however, similar to that of tuftsin, suggesting the existence of random conformations. [MVal^3]Tuftsin (**27b**) and [MThr^1]-tuftsin (**24**), as already mentioned, were not indicative of any known structure. A further conclusion that can be drawn from our CD data is that more pronounced effects on the peptide conformation are afforded by a change of configuration in residues 2 or 3 of the peptide chain. It is also very tempting to relate the information obtained from CD to the biological activity of the analogs, tested for their effect on augmentation of IL-6 secretion by macrophages. [MVal^3]Tuftsin (**27a**), which showed a certain ordered structure, was very active as compared to tuftsin, indicating that this structure might be closer to the "bioactive conformation". This suggestion is further supported by the inactivity of [MVal^3]-tuftsin (**27b**), the optical isomer of **27a**, which showed unordered structure and diminished bioactivity.

Biological Evaluations

Stability in Serum. In order to apply tuftsin as a drug, its stability toward enzymatic degradation would be of utmost importance. It has been reported[41] that the biological activity of tuftsin is destroyed by leucine aminopeptidase, carboxypeptidase B, pronase, or subtilisin but not by trypsin, chymotrypsin, papain, or pepsin. Leucine aminopeptidase located on the plasma membrane of human neutrophils plays, presumably, a role in the degradation of tuftsin.[42] This enzyme removes the N-terminal threonine to yield Lys-Pro-Arg, an inhibitor of tuftsin activity.[43] The blood obviously contains a battery of various nonspecific peptidases. It was not surprising to find, using an assay of phagocytosis, that indeed tuftsin is degraded efficiently by blood enzymes.[44]

Various approaches were carried out to develop more stable, potent long-acting analogs of tuftsin and to avoid generation of the Lys-Pro-Arg inhibitor. These studies included substitution of the N-terminal threonyl residue[10] or its cyclization,[45,46] substitution of L-amino acids by D-amino acids,[47] elongation of the tetrapeptide chain by the addition of further amino acid residues to the N-terminus,[17] sequential coupling of two molecules of tuftsin to create the dimer tuftsinyltuftsin,[48] and introduction of an isopeptide bond into the molecule, taking advantage of lysine's ϵ-amino group.[2] Although some of these approaches increased metabolic stability of tuftsin, it was usually accompanied by a marked decrease or even an abolishment of biological activity. Tuftsinyltuftsin, as an exception, was found to exert antitumor activity but did not stimulate the phagocytic capacity of macrophages.[49] Loss of activity might stem from major receptor-related structural alterations or be due to blocking of functional groups essential for activity, caused by the above modifications.[22]

Methanotuftsin analogs were tested, as compared to tuftsin, for their stability in human serum toward

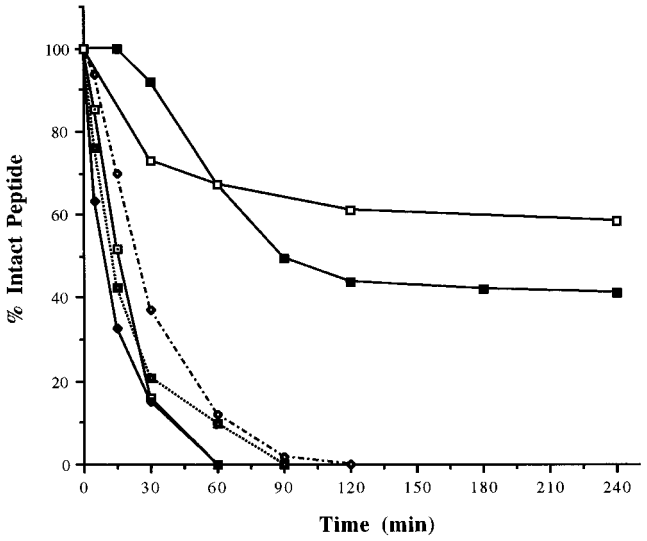


Figure 3. Stability toward enzymatic degradation by human serum of tuftsin and methano tuftsin analogs. Symbols correspond to the following peptides: (⊡) tuftsin (T), (◆) [MArg^4]T **28**, (··■··) [MLys^2]T **26a**, (◇) [MVal^3]T **27a**, (− ■ −) [MThr^1]T **24**, (□) [MOrn^2]T **25**.

enzymatic degradation. Thus, tuftsin and the synthetic analogs were incubated with human serum at 37 °C, aliquots were taken at various time intervals, and the amount of intact peptides was evaluated by analytical HPLC. From the results obtained (Figure 3), it is clear that tuftsin was totally degraded after 60 min with a half-life ($t_{1/2}$) of 16 min. [MThr^1]Tuftsin (**24**) and [MOrn^2]tuftsin (**25**) underwent comparatively very slow degradation with $t_{1/2}$ of 90 and 420 min, respectively. [MVal^3]Tuftsin (**27a**) was degraded with $t_{1/2}$ of 25 min, while [MVal^3]tuftsin (**27b**) (not shown) underwent degradation similar to tuftsin with $t_{1/2}$ of 15 min and total degradation after 60 min. Total degradation was revealed after 90 min with the two [MLys^2]tuftsin isomers **26a** and **26b**, with $t_{1/2}$ of 13 min for both of them. [MArg^4]Tuftsin (**28**) was totally degraded, like tuftsin, after 60 min with $t_{1/2}$ of 11 min. These results clearly show that only analogs **24** and **25**, which possess one methano amino acid at the Thr^1-Lys^2 bond of tuftsin, are much more resistant to enzymatic hydrolysis as compared to the parent peptide. This further indicates that it is this bond which is indeed more prone to the enzymatic cleavage of tuftsin to produce the tripeptide inhibitor. It is not clear, as yet, why analogs **26a** and **26b**, which contain methanolysine (at the sensitive Thr-Lys bond), show insignificant enhanced stability toward enzymatic degradation. The analog [MArg^4]-tuftsin (**28**) was degraded exactly as tuftsin, indicating that substitution by methano amino acid in this position does not contribute to stability.

IL-6 Assay. On the basis of previous findings that the immunomodulatory capacity of tuftsin apparently lies in mediation of interleukin production,[17,50] the relative potencies of tuftsin and its methano analogs were evaluated, *in vitro*, in stimulating mouse peritoneal macrophages to secrete interleukin-6 (IL-6). IL-6 plays a central role in host-defense mechanisms relevant to the action of tuftsin[51] (also R. Granoth, manuscript in preparation), thus for example, it promotes the differentiation of B cells into antibodies producing cells and induces synthesis of acute phase proteins by hepatocytes.

Macrophages were incubated in the presence of antigen keyhole limpet hemocyanin (KLH), with various concentrations of tuftsin or methanotuftsin analogs. Twenty hours later the supernatants were collected and measured for IL-6, employing cytokine-dependent cell (B9) assay.[51] The experimental data are compared to KLH alone. The results, average of three experiments (Figure 4), indicate that tuftsin augments secretion of IL-6 by macrophages by 78% at 2×10^{-7} M, by 42% at 2×10^{-8} M and by 32% at 2×10^{-9} M. [MVal^3]Tuftsin (**27a**) and [MThr^1]tuftsin (**24**) significantly augment secretion of IL-6 (141% and 125%, respectively) at 2×10^{-7} M and to a lesser extent at 2×10^{-8} M (95% and 29%, respectively). [MOrn^2]Tuftsin (**25**) augments secretion of IL-6 by 68% at 2×10^{-7} M and by 54% at 2×10^{-8} M. [MVal^3]Tuftsin (**27b**) and the diastereomers mixture **27** hardly augment IL-6 secretion (30%) at 2×10^{-7} M, do not affect the macrophages at 2×10^{-8} M, and show inhibitory effect at 2×10^{-9} M. [MLys^2]-Tuftsin isomers **26a** and **26b**, as well as [MArg^4]tuftsin (**28**), have no effect on the secretion of IL-6 at any concentration and even show inhibitory effect at 2×10^{-9} M (not shown). All the peptides (except for **26b**) show a dose-dependent pattern with maximal potency at 2×10^{-7} M. Peptides alone (without KLH) did not show any stimulatory effect at the above concentrations. According to the results, [MVal^3]tuftsin (**27a**) and [MThr^1]tuftsin (**24**) were more effective as compared to tuftsin. [MOrn^2]Tuftsin **25** was as effective as tuftsin, while all the other analogs were less effective and even showed some inhibitory effect in diluted concentration. The higher capacity of [MThr^1]tuftsin (**24**) as compared to tuftsin, as well as that of [MOrn^2]tuftsin (**25**), which was similar to that of tuftsin, can be explained, perhaps, by the lower sensitivity of the MThr-Lys and Thr-MOrn bonds to enzymatic cleavage, thus a lower production rate of the inhibitory fragment Lys-Pro-Arg. The higher effectiveness of [MVal^3]tuftsin (**27a**), however, can be explained both by the lower sensitivity of the "proline"-centered bonds toward endo-peptidases as well as to conformational constraints which may lead to a facilitated and more fruitful binding to the tuftsin's receptor. Moreover, enhanced affinity of the analog toward tuftsin's specific receptor should not be excluded. The inactivity of all other analogs can be explained either by metabolic instability or by structural constraints that prevent the peptide from attaining the bioactive conformation.

Enzyme-Linked Immunosorbent Assay (ELISA). Anti-tuftsin antibodies, raised in rabbits against a conjugate of (*p*-aminophenylacetyl)tuftsin and tetanus toxoid, were employed in competitive ELISA assays with the MAA−tuftsin analogs. It is evident from the results (not shown) that none of the peptides can compete with tuftsin, indicating further structural dissimilarity.

Conclusions

We have synthesized a series of 2,4-methano amino acid analogs of lysine, ornithine, and arginine and of a methano analog of valine related to proline and incorporated them into the peptide chain of tuftsin. The results of these experiments confirm our working hypothesis that substitution of amino acid residues in tuftsin by methano amino acids, in positions which are more susceptible to degradation, may lead to resistance toward enzymatic degradation and thus to enhanced metabolic stability. Consequently, biological potency

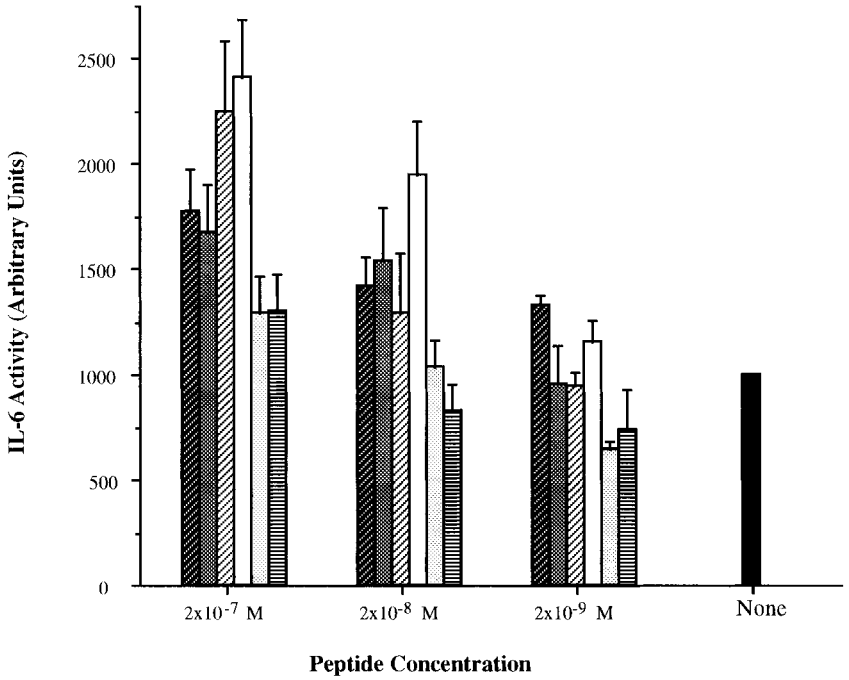


Figure 4. Augmentation of IL-6 secretion from macrophages by tuftsin and methano tuftsin analogs. From left to right, the bars correspond to the following peptides: tuftsin (T), [MOrn[2]]T **25**, [MThr[1]]T **24**, [MVal[3]]T **27a**, [MVal[3]]T **27b**, **27a**+**27b**. A solid black bar is used for KLH alone.

may be retained and even augmented. The hypothesis that restriction of bond rotations, imposed by the cyclobutane ring, may affect global conformation and biopotency was realized in the case of methanovaline, i.e. "proline-related" analog of tuftsin, which possesses a conformation different from that of tuftsin and is highly active. Introduction of more then one methano amino acid into tuftsin might be beneficial and is currently under investigation.

The basic amino acids lysine and arginine, as well as the cyclic species proline, constitute the "active site" of many and versatile bioactive peptides, e.g. neurotensin, adrenocorticotropin hormone, and bradykinin. Thus, incorporation of methano-analogs into these peptides may lead to stable and long-acting potent analogs.

Experimental Section

Chemistry. General Procedures. Melting points were taken on a Fisher-Johns apparatus and were not corrected. Proton NMR spectra were measured on a Bruker 270 or Bruker AMX 400 spectrometer. Chemical shifts are reported in δ units downfield from internal Me_4Si when measurements were taken in deuterated chloroform, from DHO (δ 4.80) when measurements were taken in deuterium oxide, and from CHD_2OH (δ 3.30) when measurements were taken in deuterated methanol. All J values are given in hertz. Mass spectra were determined with a Finnigan 4500 or Finnigan TSQ-70B triple stage quadropole spectrometer. Chemical ionization was performed with isobutane as reagent gas. Fast atom bombardment (FAB) was determined, unless indicated otherwise, in magic bullet matrix (dithiothreitol−dithioerythritol, 5:1, v/v) and with Xenon as the bombarding atom. TLC was done on Merck Kieselgel 60-F254, or on Riedel-de Haën DC-Karten CE F cellulose, precoated aluminum plates, using the following solvent systems: (A) dichloromethane (DCM)−ethyl acetate−ether (2:1:1); (B) DCM−methanol (9:1); (C) 1-butanol−pyridine−H_2O−AcOH (15:10:12:3); (D) $CHCl_3$−methanol−AcOH (5:3:1); (E) 1-butanol−AcOH−H_2O (2:1:1); (F) $CHCl_3$−methanol−AcOH (90:8:2); (G) DCM−methanol (19:1); (H) CH_3CN−H_2O (9:1). The products were detected on the TLC plates by one of the following methods or by a combination of them: Ultraviolet light, iodine vapor, ninhydrin, and Sakaguchi's test (for detection of guanidine function of Arg). The silica gel for column chromatography was Merck Kieselgel 60 (70−230 mesh). Tetrahydrofuran (THF) was dried by distillation from sodium diphenyl ketyl. Commercial BuLi in hexane was titrated with diphenylacetic acid to determine its concentration. All reactions with BuLi were carried out under argon atmosphere by using syringe and septum techniques for handling of air-sensitive reagents. Preparative high-voltage paper electrophoresis was performed on a Whatman No. 3 paper for 60 min at 33.3 V/cm in 1.6 M formic acid (pH 1.65). Products were extracted from paper by 10% solution of acetic acid. Methano amino acids were analyzed with a Dionex automatic amino acid analyzer.

Synthesis of 2,4-Methano Amino Acids. *trans*-1-(Benzoylamino)-3-[[(methylsulfonyl)oxy]methyl]cyclobutane-*N*,*N*-pentamethylenecarboxamide (2). To solution of alcohol **1** (1.14 g, 3.6 mmol; prepared according to published procedure[12]) in dichloromethane (DCM, 36 mL) under Ar, at 0 °C, was added triethylamine (365 mg, 3.6 mmol). This was followed by dropwise addition of methanesulfonyl chloride (412 mg, 3.6 mmol). The solution was then slightly warmed to initialize the reaction and set aside at room temperature for 1 h. An extractive workup with acidified water yielded a crude product which was further purified by recrystallization from chloroform−hexane to give 1.0 g (70%) of mesylate **2**: TLC system A, R_f 0.25; mp 193−194 °C; NMR (400 MHz, $CDCl_3$) δ 1.52 and 1.60 (two br, 4 H and 2 H, piperidine protons), 2.71−2.73 (d, J = 7.45, 4 H, cyclobutane (CB) protons), 2.90−2.92 (m, 1 H, CB proton), 3.03 (s, 3 H, $MsCH_3$), 3.50 (br, 4 H, piperidine protons), 4.23−4.25 (d, J = 6.8, 2 H, CH_2OMs), 7.43−7.77 (m, 5 H, aromatic protons). The product was not characterized further and was used as such for the next step.

***trans*-1-(Benzoylamino)-3-(azidomethyl)cyclobutane-*N*,*N*-pentamethylenecarboxamide (3).** To a solution of mesylate **2** (1.0 g, 2.5 mmol) in 1-methyl-2-pyrrolidone (NMP; 12 mL), was added *N*,*N*,*N*′,*N*′-tetramethylguanidinium azide (TMGA; 0.6 g, 3.8 mmol). The solution was then warmed to 80 °C for 2 h. Acidified water (120 mL) was added, and the solution was repeatedly extracted with ethyl acetate. The combined ethyl acetate extracts were washed three times with water to remove NMP. After evaporation of the solvent, the crude material was triturated with ether to remove excess NMP, yielding 0.6 g (70%) of azide **3**. TLC system A, R_f 0.35; system B, R_f 0.75; mp 194−195 °C; NMR (400 MHz, $CDCl_3$) δ

1.53 and 1.60 (two br, 4 H and 2 H, piperidine protons), 2.61−2.80 (two m, 1 H and 4 H, cyclobutane (CB) protons), 3.36 (d, $J = 6.8$, 2 H, CH_2N$_3$), 3.52 (br, 4 H, piperidine protons), 6.64 (s, 1 H, N*H*), 7.43−7.79 (m, 5 H, aromatic protons); mass spectrum (CI, positive mode) *m*/*z* 342 (100, MH$^+$), 299 (9, MH$^+$ − HN$_3$).

***trans*-1-(Benzoylamino)-3-(aminomethyl)cyclobutane-*N*,*N*-pentamethylenecarboxamide (4).** Azide **3** (510 mg, 1.5 mmol) was stirred in ethanol (25 mL) with 10% Pd/C catalyst (100 mg) under hydrogen, at atmospheric pressure and room temperature, for 20 h (extra catalyst and fresh hydrogen might be added if the starting material is still detected by TLC: system B; the amine product is more polar then the azide). After filtration of the catalyst through Celite, the solvent was evaporated and the residue was recrystallized from DCM−hexane to yield 440 mg (93%) of impure amine **4**. The compound was further purified by dissolving in diluted HCl, extraction of aqueous solution with DCM, addition of base to pH ∼10 and re-extraction of the amine with DCM: TLC system C, R_f 0.67; system D, R_f 0.65; mp 180−182 °C; NMR (400 MHz, CDCl$_3$) δ 1.50 and 1.57 (two br, 6 H, piperidine protons), 2.52−2.68 (m, 5 H, CB protons), 2.74 (d, $J = 4.9$, 2 H, CH_2NH$_2$), 3.50 (br, 4 H, piperidine protons), 7.27−7.79 (m, 5 H, aromatic protons); mass spectrum (CI, positive mode) *m*/*z* 316 (100, MH$^+$), 231 (41, MH$^+$ − piperidine).

***trans*-1-Amino-3-(aminomethyl)cyclobutane-1-carboxylic Acid (*trans*-Methanoornithine, 5).** (Aminomethyl)-cyclobutane **4** (450 mg, 1.43 mmol) was dissolved in 6 N HCl (20 mL), and the solution was heated at 130 °C for 20 h. Water was added to the reaction mixture, and the solution was extracted with ether. The aqueous layer was evaporated, and the residue was dissolved in water and evaporated, repeating this process for three times. The residue was then dissolved in ethanol, treated with propylene oxide, and triturated to furnish 200 mg (97%) of pure *trans*-methanoornithine **5**: TLC system C (cellulose), R_f 0.18; system E, R_f 0.25; NMR (400 MHz, D$_2$O) δ 2.51 (d, $J = 8.5$, 4 H, CB protons), 2.88 (m, 1 H, CB proton), 3.23 (d, $J = 7.3$, 2 H, CH_2NH$_2$); mass spectrum (CI, positive mode) *m*/*z* 145 (100, MH$^+$), 127 (77, MH$^+$ − H$_2$O); amino acid analysis, t_R 36.62 min.

***trans*-1-Amino-3-(guanidinomethyl)cyclobutane-1-carboxylic Acid (*trans*-Methanoarginine, 6).** To a solution of amine **4** (200 mg, 0.63 mmol) and triethylamine (667 mg, 6.6 mmol) in dried DMF (10 mL) was added 3,5-dimethylpyrazole-1-carboxamidine nitrate (1.27 g, 6.3 mmol), and the reaction mixture was stirred, protected from moisture, at room temperature for 24 h. TLC (system H, Sakaguchi and ninhydrin detection) showed disappearance of starting material. The DMF was removed entirely in vacuo, and the crude residue was hydrolyzed, in the same procedure used for methanoornithine **5**, without any purification. The resultant product was dissolved in water and passed through strong acidic cation exchange column (Dowex 50W×8). The eluent was concentrated and freeze-dried to give still impure product. Preparative purification by high-voltage paper electrophoresis was then performed, providing 94 mg (80%) of *trans*-methanoarginine **6**: TLC system C (cellulose), R_f 0.27; system E, R_f 0.31; NMR (400 MHz, D$_2$O) δ 2.45 (m, 4 H), 2.84 (m, 1 H), 3.40 (d, $J = 7.1$, 2 H, CH_2NH); mass spectrum (CI, positive mode) *m*/*z* 187 (20, MH$^+$), 169 (15.4, MH$^+$ − H$_2$O), 145 (48, MH$^+$ − (NHC═NH)), 127 (100, M − guanidine$^+$); mass spectrum (CI, negative mode) *m*/*z* 185 (100, [M − H]$^-$), 143 (70, [M − H − (NHC═NH)]$^-$; mass spectrum (FAB, glycerol matrix, positive mode) *m*/*z* 187 (100, MH$^+$); amino acid analysis, t_R 46.32 min.

***trans*-1-Amino-3-(hydroxymethyl)cyclobutane-1-carboxylic Acid (*trans*-Methanothreonine, 7).** Alcohol **1** (475 mg, 1.5 mmol) was hydrolyzed in the same procedure used for methanoornithine **5**, providing 190 mg (87%) of the free amino acid **7**: TLC system C (cellulose), R_f 0.36; system E, R_f 0.45; mp 250 °C dec (lit. mp 255 °C dec) with similar ^{1}H NMR spectra to that of an authentic sample;[23] NMR (400 MHz, D$_2$O) δ 2.35−2.41 and 2.45−2.50 (two symmetrical m, 2 H each), 2.74−2.80 (m, 1 H), 3.73 (d, $J = 7.1$, 2 H, CH_2OH); mass spectrum (FAB, positive mode) *m*/*z* 146 (8.6, MH$^+$); amino acid analysis, t_R, 17.28 min.

1-(Benzoylamino)-3-(phenylsulfonyl)-3-(2-hydroxyethyl)cyclobutane-*N*,*N*-pentamethylenecarboxamide (9). To a stirred solution of sulfone **8** (5 g, 11.7 mmol; prepared according to published procedure[12]) in THF (120 mL) at −50 °C was added a solution of BuLi in hexane (2 equiv). The mixture was allowed to warm to −20 °C for about 45 min before ethylene oxide was added in excess. Stirring was continued for 1 h before the reaction was quenched with water. The THF was evaporated, and the aqueous solution was extracted with DCM. During extraction, part of the product precipitated and was collected and triturated with ether to give 1.8 g (33%) of the cis alcohol **9**. The DCM layer was evaporated, and the resulting crude was chromatographed on silica gel (120 g, elution: DCM−ethyl acetate−ether, 1:1:1) to give 0.9 g of the starting material and 0.8 g (14%) of mixture of cis and trans alcohol **9**: TLC system B, R_f 0.60; system F, R_f 0.42; NMR (270 MHz, CDCl$_3$ + TFA) of the cis isomer δ 1.57 (br, 6 H, piperidine protons), 2.22 (t, $J = 7.1$, 2 H, CH_2-CH$_2$OH), 2.94 and 3.74 (AB$_q$, $J = 14.1$, 4 H, CB protons), 3.48 and 3.58 (two br, 2 H each, piperidine protons), 4.07 (t, $J = 7.1$, 2 H, CH$_2$CH_2OH), 7.36−7.89 (m, 10 H, aromatic protons); mass spectrum (CI, positive mode) *m*/*z* 471 (29, MH$^+$), 427 (100, MH$^+$ − C$_2$H$_3$OH), 143 (89, PhSO$_2$H$_2^+$).

1-(Benzoylamino)-3-(phenylsulfonyl)-3-[2-(tetrahydropyranyloxy)ethyl]cyclobutane-*N*,*N*-pentamethylenecarboxamide (10). Alcohol **9** (4 g, 8.5 mmol), 3,4-dihydro-2*H*-pyran (1.5 g, 17.8 mmol), and pyridinium tosylate (PPTS; 220 mg, 0.88 mmol) were dissolved in DCM (60 mL) and stirred at room temperature for 3 days (alcohol **9** was not completely soluble in DCM and dissolved during the reaction). An additional 1 equiv of dihydropyran and 0.5 equiv of PPTS were added after 2 days for the completion of the reaction (TLC monitoring, system G). The crude mixture was then washed with water and brine, dried, and evaporated. The residue was triturated with ether to give 5.2 g of crude solid **10**, which was utilized in the following step without further purification: TLC system G, R_f 0.73; the NMR (270 MHz, CDCl$_3$) spectrum was a superposition of the two isomers and was too complexes for interpretation. At δ 4.55 and 4.47, two br were observed (ratio 9:1) related to OC*H*(CH$_2$)O proton of the cis and trans isomers.

1-(Benzoylamino)-3-[2-(tetrahydropyranyloxy)ethyl]-cyclobutane-*N*,*N*-pentamethylenecarboxamide (11) was prepared from **10** by treatment with 6% sodium amalgam, as described previously.[12] After evaporation of the solvents, the residue was dissolved in ethyl acetate, washed with water, dried, and evaporated. The resulting crude mixture was chromatographed on silica gel (200 g, elution DCM−ether−ethyl acetate, 16:8:3), but separation of the cis and trans isomers could not be achieved. The overall yield from alcohol **9** was 72%: TLC system A, R_f 0.44; system B, R_f 0.69; the NMR (270 MHz, CDCl$_3$) spectrum is complex, δ 1.35−1.62 (m, 12 H, piperidine + THP protons), 1.63−1.83 (m, superimposed of two dt, 2 H, CHCH_2CH$_2$O), 2.37−2.67 (m, 2 H, CB trans protons) and 2.10−2.32 and 3.05−3.13 (two m, 3 H, CB cis protons), 3.40−3.58 (br, 6 H, piperidine + THP protons), 3.33 and 3.70 (two dt, $J_{AB} = 9.8$, $J_{AX} = 6.3$, 1 H each, ABX of CH$_2$CH_AH$_B$O), 4.53 (br, 1 H, OC*H*(CH$_2$)O), 7.38−7.80 (m, 5 H, aromatic protons); mass spectrum (CI, positive mode) *m*/*z* 415 (49, MH$^+$), 330 (62, MH$^+$ − THP), 287 (100, MH$^+$ − C$_2$H$_3$-OTHP).

1-(Benzoylamino)-3-(2-hydroxyethyl)cyclobutane-*N*,*N*-pentamethylenecarboxamide (12). Pyranyl ether **11** (2.3 g, 5.5 mmol) and PPTS (138 mg, 0.55 mmol) were dissolved in ethanol (44 mL), and the mixture was stirred at 55 °C for 20 h. The mixture was evaporated under reduced pressure and chromatographed on silica gel (30 g, elution: DCM−methanol, 19:1), providing 1.53 g (84%) of alcohol **12**. TLC system G, R_f 0.45; NMR (270 MHz, CDCl$_3$ + CD$_3$OD) revealed some impurities δ 1.53 (br, 6 H, piperidine protons), 1.66 (dt, $J_1 =$ ∼6.3, $J_2 =$ ∼10.2, 2 H, CH_2CH$_2$OH), 2.06−2.28 and 2.99−3.06 (two m, 3 H, CB cis protons), and 2.42−2.55 (m, 2 H, CB trans protons), 3.48 (br, 4 H, piperidine protons), 3.54 (t, $J = 6.3$, 2 H, CH$_2$CH_2OH), 7.39−7.83 (m, 5 H, aromatic protons). The product was not characterized further and was used as such for the next step.

1-(Benzoylamino)-3-[2-[(methylsulfonyl)oxy]ethyl]cyclobutane-*N,N*-pentamethylenecarboxamide (13). Mesylation of **12** (1.53 g, 4.6 mmol) was carried out as described before for **2**, but since the reaction was not homogeneous, the mixture was stirred for 20 h. The crude product was triturated with ether, providing 1.56 g (83%) of a solid, further purified by recrystallization from DCM−hexane to give pure **13**: TLC system B, R_f 0.69; system F, R_f 0.44; mp 138−140 °C; NMR (400 MHz, $CDCl_3$) δ 1.51−1.59 (3 br, 6 H, piperidine protons), 1.90 and 1.98 (2q, J = 6.2 and 6.4, respectively, 2 H, CH_2CH_2-OMs of cis and trans isomers), 2.23−2.36 (m, 2 H, CB trans protons) and 2.48−2.67 and 3.05−3.10 (2m, 3 H, CB cis protons), 3.00 (s, 3 H, $MsCH_3$), 3.60 (br, 4 H, piperidine protons), 4.20 and 4.21 (m, superimposed of 2t, J = 6.1 and 6.3 respectively, 2 H, CH_2CH_2OMs of cis and trans isomers), 7.38−7.81 (m, aromatic protons); mass spectrum (CI, positive mode) m/z 409 (28.3, MH^+), 324 (100, M − piperidine$^+$), 319 (39.1, M − OMs^+).

1-(Benzoylamino)-3-(2-azidoethyl)cyclobutane-*N,N*-pentamethylenecarboxamide (14) was prepared from **13** (1.54 g, 3.8 mmol) as described for **3**. The crude product (800 mg) was chromatographed on silica gel (20 g, elution; DCM−methanol, 19:1), providing 450 mg (34%) of azide **14**: TLC system B, R_f 0.73; system F, R_f 0.51; mp 165−167 °C dec; NMR (400 MHz, $CDCl_3$) δ 1.47 and 1.56 (2 br, 6 H, piperidine protons), 1.69 and 1.76 (2q, J = 6.7 and 6.8, respectively, 2 H, $CH_2CH_2N_3$ of cis and trans isomers), 2.17−2.24 (m, 2 H, CB trans protons) and 2.45−2.65 and 3.03−3.07 (2m, 3 H, CB cis protons), 3.19 and 3.20 (m, superimposed of 2t, J = 6.9 and 6.7 respectively, 2 H, $CH_2CH_2N_3$ of cis and trans isomers), 3.48 (br, 4 H, piperidine protons), 7.35−7.80 (m, aromatic protons); mass spectrum (CI, positive mode) m/z 356 (100, MH^+), 328 (14.7, $MH^+ - N_2$), 271 (73, M − piperidine$^+$), 105 (43.5, $COPh^+$).

1-(Benzoylamino)-3-(2-aminoethyl)cyclobutane-*N,N*-pentamethylenecarboxamide (15) was prepared from **14** (420 mg, 1.2 mmol) as described for **4**. The crude product was recrystallized from DCM−hexane to give 370 mg (94%) of the amine **15** and was not further purified: TLC system D, R_f 0.66; system E, R_f 0.68; mp 170−175 °C (not sharp); NMR (400 MHz, CD_3OD) δ 1.42 and 1.51 and 1.60 (3 br, 6 H, piperidine protons), 1.76−1.86 (m, 2 H, $CH_2CH_2NH_2$ of cis and trans isomers), 2.05−2.22 and 3.02−3.07 (2m, 3 H, CB cis protons) and 2.48−2.60 (m, 2 H, CB trans protons), 2.84−2.90 (m, 2 H, $CH_2CH_2NH_2$ of cis and trans isomers), 3.44 and 3.54 (2br, 4 H, piperidine protons), 7.45−7.87 (m, aromatic protons); mass spectrum (CI, positive mode) m/z 330 (100, MH^+), 245 (43.9, M − piperidine$^+$).

1-Amino-3-(2-aminoethyl)cyclobutane-1-carboxylic acid (methanolysine, 16) was prepared from **15** (320 mg, 0.97 mmol) as described for **5**. The product did not crystallize when treated with propylene oxide. After evaporation of the solvent, the residue was triturated with ether, collected, dissolved in water, and freeze-dried to give 142 mg (90%) of the free amino acid **16**: TLC system C (cellulose), R_f 0.12; system E, R_f 0.26; mp 210−213 °C dec; NMR (400 MHz, D_2O) δ 1.84 and 1.92 (2q, J = 7.6 and 7.8, respectively, 2 H, $CH_2CH_2NH_2$ of cis and trans isomers), 2.02−2.08 and 2.52−2.68 (2m, 3 H, CB cis protons) and 2.36−2.42 (m, 2 H, CB trans protons), 2.89−2.95 (q, J = ∼7.8, 2H, ABX spectrum of $CH_2CH_AH_BNH_2$); mass spectrum (CI, positive mode) m/z 159 (100, MH^+), 142 (21.3, M − NH_2^+), 141 (12, $MH^+ - H_2O$); mass spectrum (EI) showed typical fragmentation, 159 (11.3, MH^+), 87 (92.1, $CH_2{=}C(COOH)NH_2^+$), 71 (61.3, $CH_2{=}CHCH_2CH_2NH_2^+$), 72 (100, $CH_2{=}CHCH_2CH_2NH_3^+$); amino acid analysis, one peak with retention time of 38.95 min.

***N*$^\epsilon$-Carbobenzoxymethanolysine (17)** was prepared from **16** (137 mg, 0.87 mmol) as described below for **20**, but the product did not crystallize from the final water solution and was isolated by freeze-drying. The crude product was dissolved in absolute ethanol and filtered to remove most of the salts, providing 250 mg of **17**, which was utilized without further purification: TLC system E, R_f 0.80; NMR (400 MHz, CD_3OD) δ 1.73−1.78 (m, 2 H, CH_2CH_2NH), 2.12 and 2.69 (2m, 3 H, CB cis protons) and 2.44 (m, 2 H, CB trans protons), 3.09 (br, 2 H, CH_2CH_2NH), 5.14 (br, 2 H, CH_2OCO), 7.31−7.35 (m, 5 H, aromatic protons); mass spectrum (FAB, positive mode) m/z 293 (100, MH^+), 585 (44.6, $[2M + H]^+$).

***N*$^\alpha$-Boc-*N*$^\epsilon$-carbobenzoxymethanolysine (18).** *N*$^\epsilon$-CBZ-methanolysine (**17**; 250 mg, 0.86 mmol), sodium hydroxide (420 mg, 10.5 mmol), and di-*tert*-butyl dicarbonate (2.81 g, 12.9 mmol) were dissolved in *tert*-butyl alcohol and water (30 mL each) and stirred at room temperature for 24 h. The *tert*-butyl alcohol was removed in vacuo, and the aqueous solution was extracted with ether. The aqueous layer was then cooled and acidified to pH ∼2 and repeatedly extracted with ethyl acetate. The combined ethyl acetate extracts were evaporated to give a mixture of the starting material **17** and the Boc derivative **18** (TLC monitoring, system E). The mixture was, therefore, reacted again with di-*tert*-butyl dicarbonate as described below for **19**, using only DMF as the solvent and stirring only for 2 h. The resulting crude was triturated with petroleum ether to give 135 mg (40% yield from **16**) of a sticky product: TLC system E, R_f 0.92; system B, R_f 0.94; NMR (400 MHz, $CDCl_3$) δ 1.42 (s, 9 H, *t*-BOC), 1.62−1.69 (m, 2 H, CH_2CH_2NH), 2.00−2.88 (3m, 5 H, CB protons of both isomers), 3.10−3.15 (m, 2 H, CH_2CH_2NH), 5.08 (s, 2 H, CH_2OCO), 7.34 (br, 5 H, aromatic protons); NMR also indicated the presence of some impurities; mass spectrum (FAB, positive mode) m/z 392 (41.5, M^+), 293 (100, MH^+ − *t*-BOC); mass spectrum (FAB, negative mode) m/z 391 (86.1, $[M - H]^-$), 317 (100, $[M - H - CO(CH_3)_3]^-$).

***trans*-*N*$^\alpha$-Boc-methanothreonine (19).** To an ice-cooled, stirred solution of methanothreonine **7** (180 mg, 1.24 mmol) and triethylamine (250 mg, 2.48 mmol) in DMF (10 mL) and H_2O (10 mL) was added di-*tert*-butyl dicarbonate (810 mg, 3.71 mmol). The reaction was allowed to warm to room temperature and stirred for 24−30 h, until no starting material was observed by TLC. The DMF was removed in vacuo, and the aqueous solution was extracted with ether. The aqueous layer was then cooled to 0 °C, acidified to pH ∼2, and repeatedly extracted with ethyl acetate. The combined ethyl acetate extracts were dried ($MgSO_4$) and concentrated, and the product was crystallized with petroleum ether to give 240 mg (79%) of solid **19**: TLC system B, R_f 0.20; system H, R_f 0.35; NMR (400 MHz, D_2O) δ 1.40 (s, 9 H, *t*-Boc), 2.17−2.23 and 2.34−2.39 (two symmetrical m, 2 H each, CB protons), 2.60−2.68 (m, 1 H, CB proton), 3.60 (d, J = 6.8, 2 H, CH_2OH); mass spectrum (FAB, positive mode) m/z 246 (11.5, MH^+); mass spectrum (FAB, negative mode) m/z 244, (100, $[M - H]^-$).

***trans*-*N*$^\delta$-Carbobenzoxymethanoornithine (20).** Excess basic $CuCO_3$ (∼5 equiv) was added gradually to a boiling solution of methanoornithine **5** (210 mg, 1.46 mmol) in water (20 mL). After being cooled to room temperature, the mixture was filtered. The resulting deep-blue solution was stirred at 0 °C and treated portionwise during 0.5 h with benzyl chloroformate (683 mg, 4 mmol) in the presence of magnesium oxide (300 mg, 7.5 mmol). Stirring was continued for 2 h at room temperature, and the precipitated copper complex was collected and washed on the filter, first with water and then with ethanol. The air-dried precipitate was suspended in 0.9 N HCl and decomposed with excess sodium sulfide. The mixture was then boiled for a few minutes and rapidly filtered from the CuS which was further washed with boiling water. On cooling, the combined filtrate and washings were brought to pH 4−5 with 3 N HCl, and the product (152 mg, 37.5%) crystallized out and was collected. The mother liquors yielded on concentration in vacuum a mixture of **20** with salts (∼1 g): TLC system D, R_f 0.66; system E, R_f 0.70; mp 232−235 °C dec; NMR (400 MHz, $CDCl_3$) δ 2.52−2.63 (m, 4 H, CB protons) 2.84−2.89 (m, 1 H, CB proton) 3.39 (d, J = 6.6, 2 H, CH_2NH) 5.10 (br, 2 H, CH_2OCO) 7.33 (br, 5 H, aromatic protons); mass spectrum (CI, positive mode) m/z 279 (100, MH^+), 171 (4.5, M − $PhCH_2O^+$); mass spectrum (CI, negative mode) m/z 277 (100, $[M - H]^-$).

***trans*-*N*$^\alpha$-Boc-*N*$^\delta$-carbobenzoxymethanoornithine (21)** was prepared from **20** as described for **19**, except for using only DMF as the solvent: yield 87%; TLC system B, R_f 0.55; system E, R_f 0.94; mass spectrum (CI, positive mode) m/z 379 (8.5, MH^+), 323 (100, $MH^+ - C(CH_3)_3$); mass spectrum (CI, negative mode) m/z 377 (100, $[M - H]^-$), 243 (13.1, M − CBZ^+). The second batch of **20**, which contained salts (see above), was dissolved in absolute ethanol and filtered to

remove most of the salts. It was then treated as described for **19**, adding (dimethylamino)pyridine (0.3 equiv) for the completion of the reaction. The product (yellow oil) contained impurities and did not crystallize. It was used later in tuftsin analog synthesis only in the second coupling of methanoornithine.

***rac-trans*-1-(N^{α}-Boc-amino)-2-methylcyclobutane-1-carboxylic acid (23)** was prepared from **22**[29] (340 mg, 2.63 mmol) as described for **19**, but using only DMF as the solvent, to give 400 mg (66%). Product **23**: TLC system E, R_f 0.92; system H, R_f 0.83; mp 157−160 °C; NMR (400 MHz, CD_3OD) δ 1.01 (d, J = 7.00, 3 H, Me), 1.41 (s, 9 H, *t*-Boc) 1.63−1.68 and 2.59−2.62 (2m, 1 H each, ring-CH_2CH_2CHMe), 1.90−2.04 (m, 2 H, ring-CH_2CH_2CHMe), 2.69−2.75 (m, 1 H, ring-CH_2CHMe); mass spectrum (FAB, positive mode) m/z 230 (100, MH^+), 459 (63.8, $[2M + H]^+$; mass spectrum (FAB, negative mode) m/z 228 (90, $[M - H]^-$).

Synthesis of Methanotuftsin Analogs. All protected amino acids derivatives were obtained from Bachem, CA. The α-amino functional groups were protected by *tert*-butyloxycarbonyl (*t*-Boc). Side-chain protecting groups were as follows: Lys, N^{ϵ}-benzyloxycarbonyl; Arg, N^{γ}-tosyl; Thr, *O*-benzyl. All protected methano amino acids were synthesized as described previously. Chloromethylated polystyrene, Merrifield resin, with 0.4 mequiv of active chlorine per 1 g was purchased from Nova, Switzerland. Tuftsin was synthesized in our laboratory using conventional methods.[17] The methanoarginine (MArg) analog of tuftsin was synthesized by liquid phase coupling, while all other tuftsin analogs were synthesized by a solid phase method using a manual procedure.[30] Coupling of the first amino acid Arg to the resin was achieved through its cesium salt. All coupling stages were performed in NMP with a 2-fold excess of protected amino acids derivatives using *N,N'*-dicyclohexylcarbodimide (DCC) as a coupling agent in the presence of 1-hydroxybenzotriazole (HOBT). Usually the coupling stage was repeated twice. Coupling stages employing methano amino acids were performed in the same manner. After the first coupling stage, the protected methano amino acids were recovered from the reaction solution and used for the repeated coupling stage. All stages of coupling and of α-amino deprotection were monitored with ninhydrin and/or isatin for the presence or absence of free amino groups. After synthesis, the peptides were cleaved from the resin and deprotected simultaneously by treatment with anhydrous HF containing 10% anisole (used as scavenger) for 1 h at 0 °C. Evaporation of HF followed by addition of dried ether yielded solid residue. The crude peptide was filtered, washed with dried ether, and extracted with 50% acetic acid solution. The solution was concentrated, diluted with water, and lyophilized to give the crude peptides. The peptides were then purified by preparative HPLC. Reversed phase HPLC was performed on a Spectra-Physics SP8800 liquid chromatography system equipped with an Applied Biosystems 757 variable-wavelength absorbance detector. The column effluents were monitored by UV absorbance at 220 nm, and chromatograms were recorded on a Chrom-Jet integrator. HPLC prepacked columns used are (1) Merck, LiChroCART 250−10 mm containing LiChrosorb RP-8 (7 μm); (2) Merck, LiChrospher 100 RP-8 (5 μm), 250 × 4 mm (3); Merck, LiChrospher 100 RP-18 (5 μm), 250 × 4 mm. HPLC purification was achieved using a gradient formed from 0.1% triflouroacetic acid (TFA) in H_2O (solution A) and 0.1% TFA in acetonitrile−H_2O, 75:25 (solution B), run at the following times: t_{min} = 0, A = 100%, B = 0%; t_{min} = 5, A = 100%, B = 0%; t_{min} = 30, A = 75%, B = 25%. The desired fraction was then concentrated and lyophilized to give pure peptide. For amino acid composition analysis, peptides were hydrolyzed in 6 N HCl at 110 °C for 24 h under vacuum and analyzed with a Dionex automatic amino acid analyzer. Methano amino acids were evaluated separately. Optical rotations were measured with a Perkin-Elmer model 141 polarimeter and were taken in 5% acetic acid.

Synthesis of Thr-Lys-Pro-[MArg^4] (28). Boc-Thr-Lys-(Boc)-Pro-OSu was synthesized in our laboratory via a solution procedure.[17] To a stirred solution of Boc-Thr-Lys(Boc)-Pro-OSu (3 equiv) in dioxane (3 mL) was added at room temperature a solution of MArg (1 equiv) and $KHCO_3$ (2 equiv) in H_2O (1 mL). The reaction mixture was stirred until all starting material was consumed (∼3−4 h, TLC monitoring in system E). Water (15 mL) was then added, and the solution was acidified (pH ∼2) with 0.5 N H_2SO_4 at 0 °C before extracting three times with ethyl acetate. The aqueous layer was then cooled, brought to pH ∼5 with 0.5 N NaOH, and evaporated under reduced pressure. Water was removed by dissolving the residue in absolute ethanol and evaporation, following four similar repetitions. The residue was then dissolved in TFA, for deprotection, and allowed to stand at room temperature for 15 min. After TFA was evaporated, the peptide was dissolved in water, lyophilized, and purified by HPLC: amino acid analysis, Thr (1.00), Lys (0.96), Pro (0.94), MArg (1.04); mass spectrum (FAB, positive mode) m/z 513 (100, MH^+), 339 (23, MH^+ − Thr − (Guanidine-CH_2)); mass spectrum (FAB, negative mode) m/z 511 (73, $[M - H]^-$), 409 (100, $[M - H -$ Guanidine $- CH_2CH_2NH_2]^-$); TLC system C, R_f 0.17; system E, R_f 0.15; HPLC (isocratic, 1% solution B, RP-8) t_R 18.2 min; $[\alpha]^{23}_D$ −34 (c 0.1).

The following tuftsin analogs were prepared by solid phase peptide synthesis following the general procedure described previously.

[MThr^1]Lys-Pro-Arg (24): amino acid analysis, MThr (1.26), Lys (1.37), Pro (1.17), Arg (1.00); mass spectrum (FAB, positive mode) m/z 527 (15, MH^+), 491 (18, $M^+ - H_2O - NH_3$), 481 (21, M^+ − COOH); TLC system C, R_f 0.16; system E, R_f 0.22; HPLC (isocratic, 1% solution B, RP-8) t_R 17.8 min; $[\alpha]^{23}_D$ −5.5 (c 0.2).

Thr-[MOrn^2]Pro-Arg (25): amino acid analysis, Thr (1.04), MOrn (0.99), Pro (0.89), Arg (1.00); mass spectrum (FAB, positive mode) m/z 499 (55, MH^+), 309 (100, $M^+ - NH_2$ − Arg); TLC system C, R_f 0.14; system E, R_f 0.10; HPLC (isocratic, 1% solution B, RP-18) t_R 14.0 min; $[\alpha]^{23}_D$ −57 (c 0.1).

Thr-[MLys^2]Pro-Arg (26). Isomers **26a** and **26b** were obtained in a 10:7 ratio from **26** by HPLC separation, and their configurations at C_3 of MLys have not been established yet. **26a**: amino acid analysis, Thr (1.03), MLys (0.99), Pro (1.14), Arg (1.00); mass spectrum (FAB, positive mode) m/z 513 (26, MH^+), 481 (50, $MH^+ - 2NH_2$), 373 (100, $M^+ - H_2O - NH_2 -$ OH − C(NH)NH_2 − Thr side chain), 395 (36, M^+ − Thr − NH); TLC system C, R_f 0.25; system E, R_f 0.20; HPLC (isocratic, 4% solution B, RP-18) t_R 9.3 min; $[\alpha]^{23}_D$ −62 (c 0.1). **26b**: amino acid analysis, Thr (1.32), MLys (0.94), Pro (0.96), Arg (1.00); mass spectrum (FAB, positive mode) m/z 513 (42, MH^+), 481 (50, $MH^+ - 2NH_2$), 427 (45, MH^+ − Arg side chain), 373 (63, $M^+ - H_2O - NH_2 -$ OH − C(NH)NH_2 − Thr side chain), 339 (73, M − Arg^+); TLC system C, R_f 0.31; system E, R_f 0.33; HPLC (isocratic, 4% solution B, RP-18) t_R 18.5 min; $[\alpha]^{23}_D$ −62 (c 0.1).

Thr-Lys-[MVal^3]Arg (27). Diastereomers **27a** and **27b** were obtained in 3:2 ratio from **27** by HPLC separation, using analytical column RP-8. Their configurations at C_1 of MVal have not been established as yet. **27a**: amino acid analysis, Thr (0.96), Lys (1.05), MVal (0.98), Arg (1.00); mass spectrum (FAB, positive mode) m/z 515 (100, MH^+), 481 (30, $MH^+ - NH_2 - H_2O$), 453 (47, $M^+ - NH_2$ − Thr side chain), 411 (75, M^+ − Thr side chain − guanidine), 369 (87, M^+ − Thr and Arg side chains); mass spectrum (FAB, negative mode) m/z 513 (45, $[M - H]^-$), 419 (100, $[M - H - H_2O - NH_2 -$ OH − C(NH)$NH_2]^-$); TLC system C, R_f 0.21; system E, R_f 0.18; HPLC (isocratic, 8% solution B, RP-18) t_R 13.2 min; $[\alpha]^{23}_D$ −39 (c 0.1). **27b**: amino acid analysis, Thr (0.94), Lys (1.01), MVal (0.96), Arg (1.00); mass spectrum (FAB, positive mode) m/z 515 (100, MH^+), 469 (11, M^+ − Thr side chain); TLC system C, R_f 0.23; system E, R_f 0.20; HPLC (isocratic, 8% solution B, RP-18) t_R 16.4 min; $[\alpha]^{23}_D$ −110 (c 0.05).

Circular Dichroism (CD). CD were recorded on a Jasco J-500C spectropolarimeter equipped with a Jasco DP-500N data processor. A path length of 0.1 mm was used. Each spectrum represents the average of four scans. The dried, weighed peptides were dissolved in double-distilled water or in 98% 2,2,2-trifluoroethanol (TFE), in concentration of 1 mg/mL. The pH of the solutions was ∼5.0. The data are presented as molar ellipticity, $[\theta]$.

Biological assays. Stability in Serum. Blood from human donors was incubated at room temperature. The serum was separated by centrifugation at 2500 rpm for 10 min. Tuftsin (0.5 mg) and 0.5 mg of each of the tested tuftsin analogs were added to 0.5 mL of serum, and the samples were then incubated at 37 °C. Aliquots of 50 μL were taken at the following times: 0, 5, 15, 30, 60, 90, 120, 180, and 240 min. The samples were denatured, in tightly closed microtubes, by boiling at 90−95 °C for 5 min. H_2O (950 μL) was then added, and the mixture was thoroughly stirred and then centrifuged twice. Recovery of tuftsin, following these manipulations, is rather quantitative.[17] Supernatants were analyzed by HPLC. Each sample (200 μL, containing approximately 10 μg of peptide at time 0) was eluted at isocratic conditions at flow rate of 1 mL/min. The area corresponding to the peptide peak (in percent, calculated by the integrator) was always referred to an internal peak of the serum (eluted after about 5−7 min, depending on the above conditions). The peptide's peak area at time 0 was always assigned as 100%.

IL-6 Production Assay. The methods were described in detail elsewhere.[21] In brief, (BALB/c x C3H.eb)F1 mice were bred in our rodent facilities and used at the age of 6−8 weeks. The parents, BALB/c and C3H.eb, were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were injected intraperitoneally (3 mL/mouse) with thioglycollate broth (Difco Laboratories, Detroit, MI). Four days later the macrophages were aspirated in phosphate buffer saline (PBS, pH 7.4, Gibco, Grand Island, NY) supplemented with 5 IU/mL heparin, centrifuged and resuspended in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY) containing 40 μg/mL gentamycin. Cell preparation consistently contained >90% macrophages, of which more than 90% were viable cells. Macrophages (10^6/mL) were cultured in 1 mL of DMEM (Nunclon, 24-well plates) at 37 °C in a humidifier atmosphere of 5% CO_2 in air for 48 h prior to the assay. After 24 and 48 h the cells were washed twice with warm medium. The cultures were then incubated in triplicate in the presence of KLH (Calbiochem, CA) alone, KLH + tuftsin or tuftsin analogs at concentrations range of 2×10^{-7} to 2×10^{-9} M, and tuftsin or tuftsin analogs alone for 20 h. The supernatants were then collected, and triplicates were combined and centrifuged at 900*g* for 10 min. Supernatants were then assayed for IL-6. The IL-6 dependent murine hybridoma cell line, B9, was used to determine IL-6 levels.[51] B9 cells were cultured in RPMI containing 5% fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 40 μg/mL gentamycin, and 10 IU/mL human rIL-6 (Genzyme, Boston, MA). The B9 cells were starved in IL-6-free medium 24 h prior to the assay. B9 cells (5000/200 μL) were cultured in 96-well plates (flat-bottom, Falcon 3072, NJ) in triplicate wells in the presence of samples to be tested in eight sequential 2-fold dilutions. Proliferation was measured by a [^{3}H]thymidine pulse (1 μCi/well) from 48 h for 16 h. All results are expressed as arbitrary units. Supernatants obtained from macrophages cultured in presence of KLH alone were assigned to 1000 units. All samples were calculated based on corresponding cpm at different dilutions and the slope of the obtained curve.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was performed using the conventional procedure.[52] Briefly, 96 wells of microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μL of ovalbumin (Sigma, USA)−tuftsin conjugate in PBS (0.1 mg/mL) at 4 °C for 20 h and then washed three times with PBS containing 0.05% Tween 20 (Sigma, USA). The wells were incubated for an additional 1 h at 37 °C with 200 μL of 1% bovine serum albumin (BSA, Sigma, USA) in PBS to saturate free sites (in order to prevent non specific binding). Following three more washes, 100 μL of preincubated (20 h, 4 °C) tuftsin and tuftsin analogs at different concentrations with tuftsin antisera (1:1000) in PBS were applied to duplicate wells and incubated for 2 h at 37 °C. After three washes, 100 μL samples of affinity-purified enzyme-labeled (Horse-reddish peroxidase) goat anti-rabbit IgG (H+L, Biomakor, Israel) at a dilution of 1:1000 in PBS were added, and the samples were incubated for another 2 h at 37 °C. Finally, the plates were washed three times, 100 μL of substrate (1 mg/mL, ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)), Sigma, USA) solution (dissolved in 0.1 M citric acid, 28 mL; 0.2 M Na_2HPO_4, 22 mL; H_2O, 50 mL; H_2O_2, 6 μL) was added, and the samples were then incubated at room temperature. After 15 min the reaction was stopped with 100 μL of 0.2 M citric acid. Optical density (OD) was recorded at 630 nm using Microplate autoreader instrument (Bio-tek, Vermont).

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