Solution Conformation of a Potent Cyclic Analogue of Tuftsin: Low-Temperature Nuclear Magnetic Resonance Study in a Cryoprotective Mixture

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Tuftsin, a linear tetrapeptide (Thr-Lys-Pro-Arg), corresponding to the sequence 289−292 of the heavy chain of leukokinin, has been the object of intensive SAR studies during the past 30 years, owing to its numerous biological activities and to the possibility of generating a novel anticancer drug. A cyclic tuftsin analogue, c-[T-K-P-R-G], has biological activity 50 times higher than that of the parent linear peptide. Here we present a conformational study of c-[T-K-P-R-G] based on NMR data in a cryoprotective DMSO/water mixture. The preferred conformation is a type VIa turn centered on the K-P residues. The orientation of the side chains of the two basic residues (K and R) may represent the essential feature of the bioactive conformation of tuftsin. A possible role of tuftsin as a DNA binding motif is suggested by the similarity of the bioactive conformation of c-[T-K-P-R-G] and of the β-turn conformation proposed by Suzuki for the [T,S]-P-K-R motif.

Introduction

Tuftsin (Thr-Lys-Pro-Arg), a linear peptide corresponding to the sequence 289−292 of the heavy chain of leukokinin, a cytophilic γ-globulin,[1] is endowed with several remarkable biological activities. Complete release of this tetrapeptide from the parent protein occurs in two stages. The cleavage of the Arg^{292}−Glu^{293} peptide bond by a splenic enzyme, tuftsin endocarboxypeptidase, produces leukokinin-S containing the free carboxyl terminus of tuftsin. Second, after leukokinin-S is bound to the blood and tissue granulocyte, the Lys^{288}−Thr^{289} peptide bond is cleaved by the membrane enzyme leukokininase.

It is generally believed that tuftsin binds directly to specific tuftsin receptors on polymorphonucleates (PMN), monocyte-macrophages, and (natural killer) NK cells and modulates their biological activities,[2,3] but direct interaction with DNA has also been invoked to explain the biological activity of this peptide.[4] Tuftsin deficiency has been found in patients with some types of cancer, myelofibrosis, idiopathic trombocytopenic purpura, splenectomy, sickle cell disease, AIDS, and AIDS-related complex, in addition to tuftsin congenital abnormalities.[2,3,5,6] In animal and clinical studies, tuftsin displayed antitumor, antiinfection, and anti-AIDS activities with no detectable toxicity.[2,3,7]

Many attempts were made to produce active tuftsin analogues,[8] but these efforts were hampered by either loss of activity or formation of competitive inhibitors. Since tuftsin is degraded rapidly in serum with a half-life of several minutes,[9,10] preparation of peptidase-resistant tuftsin analogues would allow oral administration, thus facilitating clinical use. In addition, an ecto-enzyme on the membrane of PMNs, leucine aminopeptidase, inactivates tuftsin by cleaving the N-terminal threonine producing a competitive inhibitor,[11] Lys-Pro-Arg. As a result tuftsin analogues that are resistant to this peptidase attack would be expected to possess enhanced therapeutic potential.

The design of more active and resistant tuftsin analogues can greatly benefit from an accurate conformational study of tuftsin itself. A previous study on linear tuftsin in solution[12] performed in our laboratory identified two conformers (dubbed conformer II and conformer III, respectively) as relevant components of the mixture of solution conformations. Both structures, characterized by a trans Lys−Pro bond, are very compact, consistent with the observation that one NH proton is strongly solvent-shielded. The features of both conformations were strongly suggestive of possible cyclic analogues, an obvious choice when looking for analogues resistant to peptidase attack.

Cyclic analogues were in fact explicitly proposed by a computational study that appeared nearly simultaneously to our solution study.[13] These authors suggested the presence of a type IV β-turn for linear tuftsin. This conformer is not coincident with the absolute minimum energy conformation found by us but is fairly similar to the other less populated conformer of the quoted solution work.[12]

In addition to the simplest possible cycle, i.e., c-[T-K-P-R] (dubbed *ctuf*), O'Connor et al.[13] proposed three other analogues characterized by the insertion of an

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Table 1. Relevant Proton Chemical Shifts of the Cis Conformers of *ctuf2* and *ctufβ*[a]

ctuf2	*ctufβ*	NH	$C^{\alpha}H$	$C^{\beta}H$	$C^{\gamma}H$	$C^{\delta}H$	$C^{\epsilon}H$	NH_{sc}
Thr		7.28 (−2.5)	3.67	3.99	1.03			
	Thr	7.87 (−7.5)	4.07	3.83	1.10			
Lys		8.03 (−2.0)	4.08	1.59/1.63	1.11/1.28	1.46/1.48	2.72	7.68
	Lys	8.52 (−6.6)	4.13	1.59/1.62	1.20/1.40	1.49/1.52	2.77	7.77
Pro			4.44	2.02/2.21	1.67/1.86	3.38/3.62		
	Pro		4.52	1.98/2.20	1.53/1.87	3.38/3.43		
Arg		7.51 (−6.6)	4.32	1.56/1.61	1.34/1.38	3.06		7.48
	Arg	8.20 (−8.0)	4.23	1.60/1.77	1.35/1.40	3.05		7.49
Gly		9.05 (−3.3)	3.20/4.13					
	β-Ala	7.64 (−1.1)	3.47/3.23	2.14/2.46				

[a] All values were referenced to the residual DMSO resonance at 2.5 ppm. Temperature coefficients of NH resonances are reported in brackets.

appropriate spacer: glycine in c-[T-K-P-R-G] (dubbed *ctuf2*), lysine in c-[T-K-P-R-K] (dubbed *ctuf3*), and aspartic acid in c-[T-K-P-R-D] (dubbed *ctuf4*). In their calculations addition of either G or D in position 5 resulted in a backbone conformation similar to that they proposed for linear tuftsin, i.e., a type IV β-turn at the Lys-Pro position. Another computational study[14] reexamined the conformations accessible to linear tuftsin and *ctuf2*, treating explicitly the solvent (pure water and NaCl−water). The results of this study stressed the relevance of cis peptide bonds related to salt effects and prevalent in proline-containing peptides.

Ctuf2 and *ctuf4* were eventually synthesized and tested for biological activity.[15] One isomer of *ctuf4* proved nearly as active as linear tuftsin, whereas *ctuf2* was found to be 50 times more active than linear tuftsin.

Therefore we decided to examine the conformation of *ctuf2* in solution by means of a combination of NMR and molecular modeling. A new cyclic analogue, c-[Thr-Lys-Pro-Arg-β-Ala], dubbed *ctufβ*, was also studied for comparison, in particular to check whether a more flexible link might influence cis−trans isomerism.

Results and Discussion

The flexibility of small peptides, either linear or cyclic, may lead to inextricable mixtures of isoenergetic conformers and makes NOEs very difficult to detect, particularly in neat solvents of low viscosity such as water. A proper choice of the solvent medium can improve this situation. We have shown[16,17] that NMR problems linked to flexibility can be partially overcome by running NMR spectra at low temperature in a cryoprotective mixture. These experimental conditions imply fairly high viscosities that favor the building up of sizable NOEs.[16] In addition, the use of a viscous medium can affect the equilibrium among isoenergetic conformers, selecting the more ordered conformers.[18] Accordingly, we chose to rely on the best characterized among the cryoprotective mixtures, i.e., DMSO/water.[16−18] Such a mixture at 275 K, i.e., the temperature used for all NOESY experiments, has a viscosity of 6.7 cp.[19]

To check whether this cryoprotective mixture is compatible with the known conformational behavior of tuftsin in DMSO,[12] we examined also linear tuftsin in a 80:20 (v:v) DMSO-d_6/water cryomixture. A comparison of the ^{1}H NMR spectra of tuftsin in neat DMSO and in the 80:20 (v:v) DMSO-d_6/water cryomixture showed that the NMR data in the two solvent media are completely consistent, the only difference being that the cryomixture favors the detection of a larger number of NOEs.

Tuftsin analogues *ctuf2* (c-[Thr-Lys-Pro-Arg-Gly]) and *ctufβ* (c-[Thr-Lys-Pro-Arg-β-Ala]) were examined in a 80:20 (v:v) DMSO-d_6/water cryomixture at 275 K. Sequential assignment of all protons of *ctuf2* and *ctufβ* in the 80:20 (v:v) DMSO-d_6/water cryomixture was achieved by standard methods[20] via the usual systematic application of DQF-COSY,[21] TOCSY,[22] and NOESY[23] experiments.

1D protonic spectra of the two analogues show that several resonances are split in two signals corresponding to the two families of conformations expected from the cis−trans isomerism around the Lys−Pro bond. Trans conformers of linear tuftsin are largely predominant in water, neat DMSO, and the 80:20 (v:v) DMSO-d_6/water cryomixture. On the contrary, the cis conformers are more abundant in cyclic analogues, with a cis/trans ratio of 5:1 and 30:1 for *ctuf2* and *ctufβ*, respectively. Since *ctufβ* was designed primarily to check whether the introduction of a more flexible (and longer) linker was still consistent with the prevalence of a cis K−P bond, it is interesting to note that the cis/trans ratio in *ctufβ* is even higher than that in *ctuf2*. Another piece of interesting information that can be inferred from the 1D spectra in the temperature range 270−310 K is that some NH resonances have fairly low temperature coefficients.

Relevant proton chemical shift data are summarized in Table 1 along with temperature coefficients of NH resonances. The observation of small coefficients, −2.5 ppb/°C for T and −2.0 ppb/°C for K in *ctuf2* and −1.0 ppb/°C for β-Ala in *ctufβ*, hints at a high structural rigidity, but it is still not sufficient to indicate precise conformations nor even to guarantee that the corresponding hydrogens are involved in intramolecular hydrogen bonds. Amide hydrogens involved in intramolecular H-bonding or otherwise shielded from solvent can be identified by their relatively slow rate of H/D exchange. This information is commonplace in proteins but is generally very difficult to obtain in small peptides whose amide hydrogens normally exchange very fast. Nonetheless, owing to the peculiar behavior of the amide protons of tuftsin in DMSO,[12] we did perform these measurements for the two cyclic analogues. Slowly exchanging amide NH's of *ctuf2* were detected by dissolving the peptide in the DMSO-d_6/D_2O (80:20, v:v) cryomixture and monitoring signal intensities of residual amide NH resonances at 275 K. The NH's of Gly and Thr have half-lives respectively of 60 and 80 min, whereas the corresponding NH's of Arg and Lys have lower exchange rates, with half-lives respectively of 330

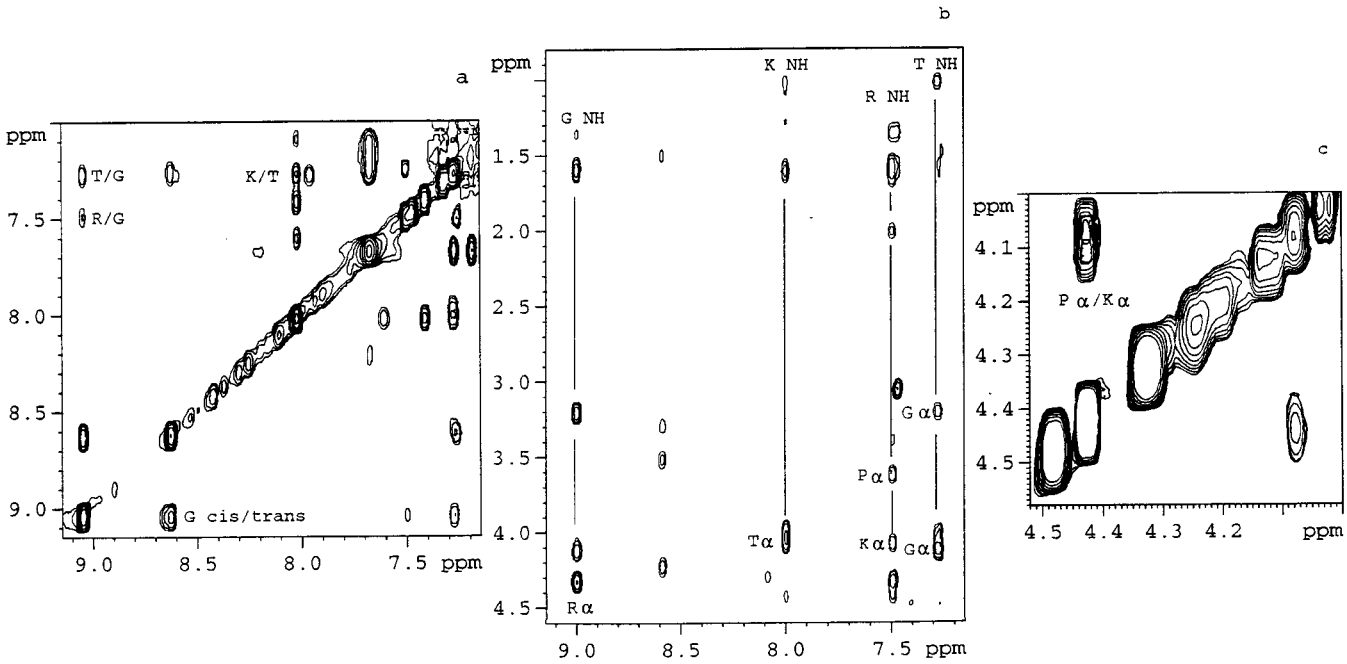

Figure 1. Low-field parts (a, b) of the 300-ms NOESY spectra of *ctuf2* in the 80:20 (v:v) DMSO-d_6/water cryomixture at 275 K. P/K diagnostic cross-peaks are shown in the expansion (c).

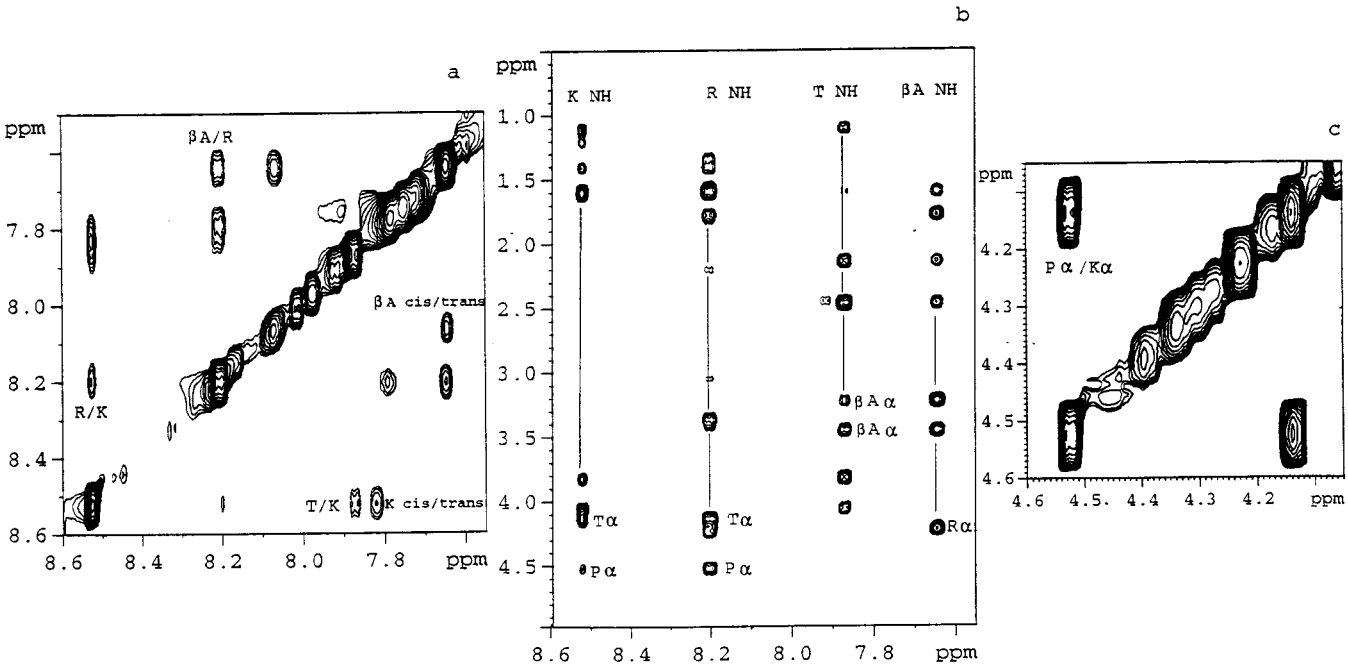

Figure 2. Low-field parts (a, b) of the 300-ms NOESY spectra of *ctufβ* in the 80:20 (v:v) DMSO-d_6/water cryomixture at 275 K. P/K diagnostic cross-peaks are shown in the expansion (c).

and 450 min, thus confirming that the NH of Lys is indeed involved in hydrogen bonding.

Figure 1 shows the low-field parts of the NOESY spectra of *ctuf2* (Figure 1a,b) in the 80:20 (v:v) DMSO-d_6/water cryomixture at 275 K and a mixing time of 300 ms, together with the expansion of the α-CH region that shows the P/K correlations crucial for the sequential assignment of the resonances to the cis isomer (Figure 1c). The corresponding spectra for *ctufβ* at the same experimental conditions are shown in Figure 2a−c, respectively. The resonances can be grouped in two sets of different intensity corresponding to molecules containing a cis or trans Lys−Pro bond, respectively. Assignment of the two components to cis and trans isomers is crucial for the subsequent structure interpretation; it is well-known that the two isomers are best discriminated on the basis of α−α X−P or α−δ X−P cross-peaks.

The quoted conformational analysis of linear tuftsin in DMSO[12] has shown that the most populated conformational family for tuftsin is characterized by a trans Lys−Pro bond. The two preferred conformations (conformer II and conformer III in the original paper[12]), consistent with all NOE−derived constraints, are rigid, folded structures, characterized by an approximate inverse γ-turn centered on Pro. The findings that the prevailing family of conformers in the cyclic analogues contains a cis Lys−Pro bond and that *ctuf2* is 50 times

more active than the linear parent peptide imply that the conformers previously identified in the DMSO solution of tuftsin may not be representative of the bioactive conformation. Accordingly, it is not possible to use them as starting conformers for the interpretation of the NMR data of the cyclic analogues.

It is possible, in principle, to run an exhaustive conformational search on the cyclic analogues, similar to those previously performed on tuftsin[12,24] since the size of both peptides is rather small. However, the main conformational preferences of tuftsin and *ctuf2* are well-known from the quoted conformational studies,[12−14,24] and an improvement over these analyses would require a much finer mesh in the search with a substantial scaling up of computing times. Accordingly, we did not attempt to map out the conformational space of the two analogues but chose to rely on a combination of known tuftsin structures and NMR data to find the prevailing conformers of *ctuf2* in solution. The data of *ctufβ* are so similar to those of *ctuf2* that the structure determination of this analogue can be tailored after that of *ctuf2*. The features of the prevailing conformer of *ctuf2* were used as an internal check, i.e., to examine whether the stability of conformers, of *ctuf2* and *ctufβ*, containing a K−P cis bond is influenced by the flexibility of the whole cycle.

The strategy adopted to find the prevailing conformers of *ctuf2* in solution was based on two methods: (a) a simple check of canonical conformations, containing a cis Lys−Pro peptide bond, to test their consistency with experimental NMR data and (b) the use of the structure of the parent protein[25] to generate starting conformations in procedures of EM and MD.

Alternatively one might resort to more sophisticated approaches, e.g., ensemble calculations,[26] but the number of the constraints that can be derived from the NMR data in our case does not warrant this kind of approach.

A qualitative interpretation of the NMR data suggests a well-defined family of structures characterized by a cis Lys−Pro bond. In particular the two adjacent short NH−NH distances (K-T and T-G), concomitant low-temperature coefficients, and slow H/D exchange rates are consistent with the features of canonical (cis) type VI β-turns.[27] Using families of type VI β-turns (VIa and VIb) as starting conformers in unrestrained EM runs, it was clear that the best agreement with the NMR data can be obtained with turns of the VIa type. Most conformational features of the backbone of this structure (G1) are in fair agreement with the NOE-derived distances reported in Table 2. The relative arrangement of the side chains was optimized by MD runs in which the backbone conformation was kept fixed at the values reported in Table 3. Subsequent restrained EM runs yielded a structure (G2) that is in good agreement with the experimental data. In particular, there are small violations of observed NOE-derived constraints but also no significant violation of absent distance constraints (ADC in the terminology of Brüschweiler et al.[28]). Significant internal rotation angles of G1 and G2 are reported in Table 3. The differences between overall geometrical features are small: such a behavior is mirrored by the fact that the "restrained structure" relaxes back, to some extent, to the "unrestrained structure" when the NMR-derived restraints are relieved. The molecular model of G2 is shown in Figure 3.

Table 2. Comparison of Experimental Interproton Distances Derived from NOEs (d_{exp}) and Calculated Distances (d_{calc}) of Models of *ctuf2* Calculated from Canonical Turns (G1, G2) or the Conformation of the Corresponding Tuftsin Sequence (TKPR) in the Parent Protein (G3, G4)

		d_{exp}, Å	d_{calc}, Å			
residue	NOE	NMR	G1	G2	G3	G4
Thr-1 NH						
	NH Gly-5	3.64	3.14	3.79	3.57	4.28
	NH Lys-2	3.22	3.45	3.55	3.79	3.76
	CHα1 Gly-5	2.74	2.27	2.28	3.70	3.75
	CHα2 Gly-5	3.79	3.43	3.40	4.07	4.11
Lys-2 NH						
	HN Thr-1	3.22	3.55	3.08	2.5	3.11
	HN Arg-4	4.27	5.64	4.80	3.24	2.99
	CHα Pro-3	4.49	5.12	5.25	2.54	3.16
	CHα Thr-1	3.08	2.16	2.15	2.20	2.24
	CHγ THr-1	4.41	3.81	3.66	4.62	5.34
Pro-3 CHα						
	CHα Lys-2	3.34	2.44	2.58	4.03	4.01
	*CHγ1Lys-2	3.70	3.15	4.04	5.34	5.52
	CHβ Lys-2	4.05	4.11	4.22	4.08	4.97
Arg-4 NH						
	HN Lys-2	4.27	5.64	4.80	3.24	2.99
	CHα Pro-3	3.89	3.57	3.52	2.25	3.45
	CHα Lys-2	3.43	3.70	3.11	5.71	4.28
	*CHβ Pro-3	3.67	3.32	3.57	3.42	3.81
	*CHδ Pro-3	3.41	2.68	3.22	5.66	3.54
Gly-5 NH						
	HN Arg-4	4.39	2.89	3.73	3.80	3.48
	HN Thr-1	3.64	3.14	3.79	3.57	4.28
	CHα Arg-4	2.80	3.56	3.33	3.61	3.64
	*CHβ Arg-4	3.33	3.85	2.19	3.06	2.92
	*CHγ Arg-4	4.51	3.30	4.52	3.64	4.65

Table 3. Significant Internal Rotation Angles of Models of *ctuf2* Calculated from Canonical Turns (G1, G2) or the Conformation of the Corresponding Tuftsin Sequence (TKPR) in the Parent Protein (G3, G4) Calculations

	dihedral	G1	G2	G3	G4
E, kcal mol^{-1}		29	32	34	32
Thr-1	ϕ	94.24	98.94	−103.9	−104.44
	ψ	108.41	111.70	88.10	78.56
Lys-2	ϕ	−58.05	−51.32	−115.02	−92.05
	ψ	127.81	126.23	−46.38	−35.15
Pro-3	ϕ	−74.22	−67.64	−75.76	−72.57
	ψ	−45.06	−26.53	159.56	−9.50
Arg-4	ϕ	−78.33	−113.69	114.81	−50.90
	ψ	−50.42	−112.22	−80.29	−58.72
Gly-5	ϕ	−164.42	−82.58	−161.30	−162.86
	ψ	74.69	78.54	85.43	75.47

Alternatively, we tried to use the structure of the tuftsin fragment in the parent protein as a starting point. We are aware, of course, of the fact that the K−P peptide bond in the protein is trans and that the conformation of the tuftsin fragment in the protein may bear no relationship with the bioactive conformation of tuftsin or even with the solution conformation of tuftsin. However, it seemed interesting to verify whether using the "protein conformation" as a starting point we might "naturally" end up with a correct cyclic tuftsin conformation. In human Igg tuftsin residues are part of a β-sheet strand in the Fc fragment. Tuftsin is actually produced, in vivo, as a cleavage fragment of this leukokinin (residues 289−292). We 'extracted' the four residues with the conformation they have in the protein[25] and tried to use this 'protein conformer' to produce

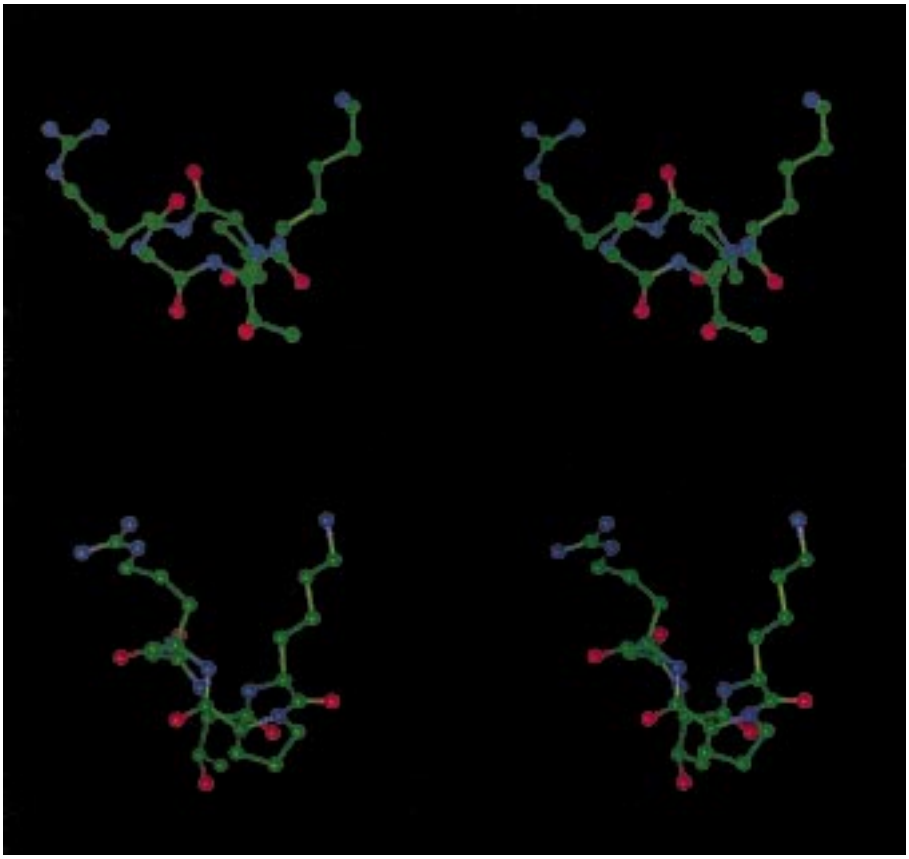

Figure 3. Stereoviews of the molecular models of the conformers of *ctuf2* derived from a starting canonical VIa turn centered on the K−P bond (G2, top) and from the conformation of the sequence T-K-P-R in the parent protein (G4, bottom).

stable structures of the two cyclic analogues. If one uses this 'protein conformer' in EM runs that do not overemphasize the role of electrostatic interactions, i.e., by using neutral end groups and/or employing a distance-dependent dielectric constant, the predictable result is a structure still characterized by a trans Lys−Pro bond. However, when one performs an EM calculation with fully charged N- and C-terminal groups and a low value of the dielectric constant, as already noted by Valdeavella et al.,[14] overestimation of electrostatic interactions leads to a "spontaneous cyclization", i.e., to a structure rearrangement characterized by a cis Lys−Pro bond and a quasi-cyclic backbone shape.

The gap between N- and C-terminal regions can be easily filled by a glycine residue. After cyclization with glycine another unrestrained EM run yielded a stable structure of *ctuf2* containing a cis Lys−Pro amide bond (G3). Its relevant distances are reported in Table 2. As already described for conformer G1, the relative arrangement of the side chains of G3 was optimized by MD runs in which the backbone conformation was kept fixed at the values reported in Table 3. After constrained EM calculations using NOE-derived distances, the resulting structure (G4) shows a better agreement with experimental NOEs with respect to the starting conformer (G3). Relevant conformational parameters are reported in Table 3. A final EM calculation was performed to allow relaxation of the structure derived by the constrained application of NOE distances and to check whether it represents a true energy minimum conformational state. The molecular model of G4 is shown in Figure 3.

A comparison of calculated distances of the two final conformers (G4 and G2) derived from the two methods described above with experimental data (Table 2) shows that, although the agreement of G2 is fairly good, neither structure is fully consistent with the NOE-derived distances. However, it can be seen that nearly in all cases where there is a violation, the calculated distances can be interpolated between the two structures to give a satisfactory agreement with experimental data. An interesting feature of these structures is the fact that, despite differences in the backbone torsion angles, they have similar energies and a very similar topology of the side chains, which implies a very similar surface of interaction. It is interesting to note that while refinement of the starting VIa conformer does not alter the backbone topology too much, i.e., conformer G2 is still a VIa turn, restrained minimization alters the backbone conformation of G3, as indicated by the rmsd value between G3 and G4 (0.96), leading to significant transitions of ψ_3 and ϕ_4 torsion angles.

A crucial point in this structure determination is the stability of conformations containing a cis K−P peptide bond. It is surprising that in going from linear tuftsin to *ctuf2* there is a drastic change in the relative stability of conformers containing a trans or cis K−P peptide bond. Although it can be expected that cyclization imposes some sterical strain on the K−P peptide bond, it must be taken into account that a cyclic pentapeptide has considerable conformational flexibility. An indirect way to check the intrinsic stability of the conformers of *ctuf2* containing a cis K−P peptide bond is to modify the constitution of the peptide by inserting a residue

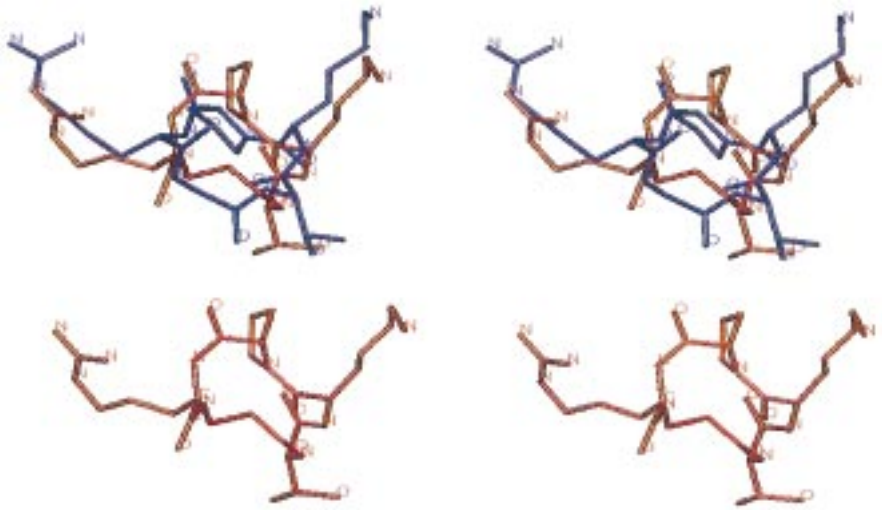

Figure 4. Comparison of the molecular models of the minimum energy conformer of *ctufβ*, B2 (red), and of the corresponding *ctuf2*, G2 (blue), displayed using capped sticks renderings. Heteroatoms are labeled.

even more flexible than Gly. We have examined the solution conformation of *ctufβ* and found that both the NMR data and energy calculations similar to those performed on *ctuf2* lead to the same results, i.e., to isomers containing a cis K−P peptide bond and characterized by backbone torsion angles very similar to those of *ctuf2*. Even in this case, using families of type VI β-turns (VIa and VIb) as starting conformers in unrestrained EM runs, it was clear that the best agreement with the NMR data can be obtained with turns of the VIa type. The relative arrangement of the side chains was optimized by MD runs in which the backbone conformation was kept fixed at the values of the starting VIa conformation. Subsequent restrained EM runs yielded a final structure that is in good agreement with the experimental data. Figure 4 shows a comparison of this model of *ctufβ* with the corresponding model of *ctuf2* (G2).

It can be noted that all models of Figures 3 and 4, despite differences in constitution and/or backbone conformation, are characterized by similar orientations of the two basic side chains. It is difficult to say whether this feature is characteristic of a bioactive conformation interacting with a specific membrane receptor, but it is interesting that this orientation is shared also by one of the conformers (III) of linear tuftsin in DMSO solution,[12] by the native structure of the sequence in the parent protein,[25] and even by the type I turn proposed by Suzuki for the S-P-X-X peptide corresponding to the [S,T]-P-X-X motif that the same author proposed as a general motif in gene regulatory proteins,[29] if one substitutes K and R for the two X. This observation, in turn, prompted a more in-depth check of the hypothesis that the main biological action of tuftsin is a direct interaction with DNA.[4]

In fact the sequence of tuftsin is reminiscent of the [S,T]-P-X-X motif that is involved in specific binding of AT-rich sequences along the minor groove of DNA.[29] The similarity is even higher with other peptides that bind AT-rich DNA such as the so-called BD peptide, a consensus peptide from HMGI[30] whose sequence contains a proline flanked by two basic residues: TPKRPRGRPKK. It is interesting to note that the frequency of occurrence in the data banks of the pattern [T,S]-[K,R]-P-[K,R], corresponding to tuftsin and its closest analogues, is comparable to that of the Suzuki motif, [T,S]-P-[K,R]-[K,R]. For instance, in the SwissProt data bank, the pattern [T,S]-[K,R]-P-[K,R] was found in 1 737 instances in 1 664 proteins out of a total of 70 423 entries, whereas that of the Suzuki motif was found 2 290 times in 2 012 proteins. Thus the sequence permutation showed by tuftsin and its analogues with respect to the [S,T]-P-[K,R]-[K,R] motif, i.e., T-[K,R]-P-[K,R], may not prevent interaction with DNA, particularly if tuftsin acts as an inhibitor with respect to DNA binding proteins. As noted above it is interesting that the two basic side chains of *ctuf2* in the G2 and G4 models are oriented in way very similar to that of the turn proposed by Suzuki.[29] Figure 5 shows the comparison of all mentioned models: i.e., G2 (yellow) and G4 (pink) of *ctuf2*, *ctufβ* (orange), model III of linear tuftsin (green), type I β-turn of the Suzuki peptide (gray), and parent protein TKPR sequence (blue). The similarity of the orientation of the K and R side chains in all structures is remarkable, especially if one takes into account the differences in constitution.

We can conclude by saying that the prevailing conformer of *ctuf2* is characterized by a cis K−P bond and has a type VIa turn conformation. This structure appears a likely candidate for the 'bioactive conformation' of tuftsin and has an outer shape strongly suggestive of a possible interaction with DNA.

Experimental Section

Peptides. Tuftsin was purchased from Bachem and used without further purification. c-[Thr-Lys-Pro-Arg-Gly] and c-[Thr-Lys-Pro-Arg-β-Ala] were synthesized by standard solution methods according to the following procedure.

Boc(*tert*-butyloxycarbonyl)-Lys(2-Cl-Z)(2-chlorobenzyloxycarbonyl)-Pro-OBg(*N*-benzhydryl-glycolamide ester) (1). To a stirred solution of 3.56 g of Boc-Lys(2-Cl-Z)-OH (8.6 mmol) in 100 mL of CH_2Cl_2 were added at −10 °C 1.16 g of iBCCl (isobutyl chloroformate) (8.6 mmol) and 1.16 g of NMM (*N*-methylmorpholine) (11.4 mmol). After 10 min a precooled solution of 2.9 g of H-Pro-OBg (8.6 mmol) in 30 mL of CH_2Cl_2 was added; the reaction mixture was stirred at room temperature for 2 h, extracted with aqueous 0.5 M $KHSO_4$, a saturated $NaHCO_3$ solution, and water, dried with Na_2SO_4, and evaporated in vacuo, to give 5.9 g (95% yield) of title

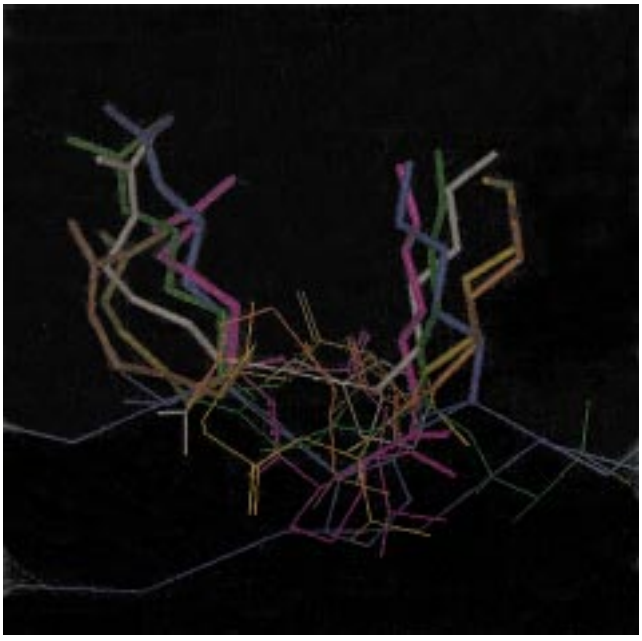

Figure 5. Comparison of the molecular models G2 (yellow), G4 (pink) of *ctuf2* with the models of *ctufβ* (orange), linear tuftsin (green), type I β-turn of the Suzuki peptide (gray), and parent protein TKPR sequence (blue). The K and R side chains are represented as capped stick (thick sticks) models, whereas all other atoms of the peptides and of the parent protein are represented as lines.

compound as a TLC (thin-layer chromatography) pure oil (R_f = 0.7 in ethyl acetate−diethyl ether, 8:2).

Boc-Lys(2-Cl-Z)-Pro-OH (2). To a solution of 5.9 g of **1** (8 mmol) in 20 mL of DMF (dimethylformamide) was added a solution of 2.26 g of K_2CO_3 (16 mmol) in 20 mL of water. After stirring overnight at room temperature, the reaction solution was diluted with water and extracted with 200 mL of diethyl ether. The aqueous phase, acidified with 0.5 M $KHSO_4$, was extracted with ethyl acetate. The organic phase, washed with water, dried with Na_2SO_4, and evaporated in vacuo, afforded 4 g (100% yield) of **2** as a TLC pure foam (R_f = 0.3 in ethyl acetate−acetic acid, 95:5).

Boc-Lys(2-Cl-Z)-Pro-Arg(NO_2)-OBg (3). To a solution of 4 g of **2** (8 mmol) in 60 mL of CH_2Cl_2 were added at −10 °C under stirring 1.08 g of iBCCl (8 mmol) and 1.08 g of NMM (10.68 mmol). After 10 min a solution of 3.54 g of H-Arg(NO_2)-OBg (8 mmol) in a mixture of 60 mL of CH_2Cl_2−THF (tetrahydrofuran) (1:1) was added. After 3 h stirring at room temperature, the reaction mixture, worked up as previously described, gave 7.2 g of a residue which was chromatographed on a silica gel column (150 cm × 2.5 cm) in chloroform−methanol (92:8) as eluent, obtaining 5.6 g (75% yield) of **3** (R_f = 0.4 in chloroform−methanol 9:1).

Boc-Lys(2-Cl-Z)-Pro-Arg(NO_2)-OH (4). To a stirred solution of 5.6 g of **3** (6 mmol) in 30 mL of DMF was added a solution of 1.65 g of K_2CO_3 (12 mmol) in 30 mL of water. After stirring 1.5 h at room temperature, the reaction mixture was worked up as described for the analogous deprotection reaction, obtaining 4.8 g (100% yield) of **4** (R_f = 0.3 in ethyl acetate−acetic acid, 9:1).

Boc-Lys(2-Cl-Z)-Pro-Arg(NO_2)-Gly-OBg (5). To a MA (mixed anhydride solution), prepared as already described, from 2.26 g of **4** (3.10 mmol), 0.42 g of iBCCl (3.10 mmol), and 0.42 g of NMM (4.14 mmol) in CH_2Cl_2 was added 0.92 g of H-Gly-OBg (3.10 mmol) in 20 mL of CH_2Cl_2. After the usual workup, 3.2 g of the obtained residue was purified by chromatography on a silica gel column (100 cm × 2.5 cm) in chloroform−methanol (92:8) as eluent obtaining 1.8 g (79% yield) of **5** as a TLC pure foam (R_f = 0.4 in chloroform−methanol, 9:1).

Boc-Thr-Lys(2-Cl-Z)-Pro-Arg(NO_2)-Gly-OBg (6). For removal of Boc, 1.8 g of Boc-tetrapeptide **5** (1.8 mmol) was kept in 20 mL of TFA for 1 h at room temperature. The solution was evaporated to dryness and repeatedly evaporated after addition of ether. The TFA salt was dissolved in 40 mL of acetonitrile and added to a solution of 0.394 g of Boc-Thr-OH (1.8 mmol), 0.795 g of BOP (benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate) (1.8 mmol), and 0.528 mL of TEA (triethylamine) (3.76 mmol) in 20 mL of acetonitrile. After stirring overnight at room temperature, the reaction mixture was evaporated to dryness, and the residue, kept in ethyl acetate, was washed with aqueous 0.5 M $KHSO_4$, saturated $NaHCO_3$ solution, and water. The organic phase, dried with Na_2SO_4 and evaporated in vacuo, gave 1.9 g of a residue which was chromatographed on a silica gel column (100 cm × 2.5 cm) in chloroform−methanol (85:15) as eluent. Fractions (30 mL) 20−45 afforded 1.52 g (85% yield) of TLC pure **6** (R_f = 0.6 in chloroform−methanol, 85:15).

Boc-Lys(2-Cl-Z)-Pro-Arg($N0_2$)-β-Ala-OBg (7). The protected tetrapeptide **7** was obtained by reacting a MA prepared from 2.26 g of **4** (3.10 mmol) with 0.96 g of H-β-Ala-OBg (3.10 mmol) following the procedures described in the previous paragraphs. The crude reaction residue was purified on a silica gel column (100 cm × 2.5 cm) in chloroform−methanol (9:1) as eluent obtaining 1.84 g (59% yield) of **7** as aTLC pure foam (R_f = 0.55 in chloroform−methanol, 9:1).

Boc-Thr-Lys(2-Cl-Z)-Pro-Arg(NO_2)-β-Ala-OBg (8). A total of 1.5 g of Boc-tetrapeptide **7** (1.5 mmol) was deprotected from Boc by TFA (trifluoroacetic acid) treatment as previously reported. The obtained TFA salt, dissolved in 20 mL of acetonitrile, was added at room temperature to a solution of 0.463 g of Boc-Thr-OH (1.5 mmol), 0.660 g of BOP (1.5 mmol), and 0.41 mL of TEA (5.6 mmol) in 20 mL of acetonitrile. The reaction mixture was left under stirring overnight and worked up as previously described obtaining a residue which was chromatographed on a silica gel column (100 cm × 2.5 cm) in chloroform−methanol (85:15) as eluent. Fractions (30 mL) 29−35 afforded 1.35 g (81% yield) of the title compound (R_f = 0.6 in chloroform−methanol, 85:15).

TFA·Thr-Lys(2-Cl-Z)-Pro-Arg(NO_2)-Gly-OH (9). For OBg deprotection, 1.56 g of protected pentapeptide **8** (1.42 mmol) was dissolved in 20 mL of DMF and treated with a solution of 0.39 g of K_2CO_3 (2.84 mmol) in 20 mL of water. After stirring 1 h at room temperature, the reaction mixture, diluted with water, was extracted with 100 mL of ether. The aqueous phase was acidified with $KHSO_4$ and extracted with ethyl acetate. The organic layer, washed with water and dried with Na_2SO_4, was evaporated in vacuo, and the obtained residue was kept for 1 h at room temperature in 10 mL of TFA for Boc removal. The TFA was evaporated in vacuo, and the residue, triturated with ether, gave a white solid which was filtered and repeatedly washed with ether obtaining 1.08 g (77% yield) of **9** (R_f = 0.3 in *n*-buthanol−acetic acid−water, 4:1:1).

TFA·Thr-Lys(2-Cl-Z)-Pro-Arg(NO_2)-β-Ala-OH (10). In an analogous way, as described in the preceding paragraph, 1 g of title compound (1.1 mmol, 88% yield) was obtained from 1.35 g (1.22 mmol) of protected pentapeptide **8**.

Cyclo[Thr-Lys(2-Cl-Z)-Pro-Arg(NO_2)-Gly] (11). To the stirred solution of 0.89 g (1 mmol) of **9** in 120 mL DMF and 80 mL of THF was added at −15 °C 0.136 g of iBCCl (1 mmol). After 10 min stirring, the precooled solution of 0.2 mL of NMM in 340 mL of DMF and 160 mL of THF was added. After stirring 1 h at 0 °C and overnight at room temperature, the reaction mixture was evaporated in vacuo and the residue chromatographed on a Sephadex LH 20 column (250 cm × 2.5 cm) in methanol as eluent. The residue obtained from fractions (27 mL) 54−59 was further chromatographed on a silica gel column (30 cm × 2.5 cm) in chloroform−methanol−water (65:25:4) as eluent. Fractions (20 mL) 6−7 gave 276 mg (37% yield) of title compound (R_f = 0.4 in chloroform−methanol−water, 65:25:4).

Cyclo(Thr-Lys-Pro-Arg-Gly) (12). A total of 276 mg of **11** dissolved in 40 mL of acetic acid−water (1:1) was hydrogenated over 40 mg of Pd black. After 1 day further 40 mg of catalyst was added; total deprotection occurred in 3 days. After removal of catalyst by filtration, the filtrate was evaporated in vacuo

and the residue chromatographed on a Sephadex LH 20 column (100 cm × 2.5 cm) in water as eluent. Fractions (20 mL) 49−50 afforded 90 mg of cyclic title compound which was further purified by preparative HPLC using a C_{18} RP column eluted by linear gradient H_2O/0.1% TFA and CH_3CN/0.1% TFA obtaining 70 mg (36% yield) of pure **12**. Fast atom bombardment mass value for $(M + H)^+$ was 539 as expected.

Cyclo[Thr-Lys(2-Cl-Z)-Pro-Arg(NO_2)-β-Ala] (13). A total of 0.9 g (1 mmol) of **10** was reacted with 0.136 g of iBCCl adopting the identical cyclization conditions used for the synthesis of cyclopentapeptide **11** as previously described. After purification of the crude reaction material by subsequent chromatographic procedures on Sephadex LH 20 in methanol as eluent and on silica gel eluting with chloroform−methanol−water (65:25:4), 195 mg (25% yield) of cyclic title compound was recovered (R_f = 0.45 in chloroform−methanol−water, 65:25:4).

Cyclo(Thr-Lys-Pro-Arg-β-Ala) (14). A total of 195 mg of cyclopentapeptide **13** in 40 mL of acetic acid−water (1:1) were hydrogenated over 20 mg of Pd black following the same procedure used for obtaining cyclopeptide **12**. The residue was purified by chromatography on Sephadex LH 20 in water as eluent followed by a preparative HPLC purification on a C_{18} RP column eluted by a linear gradient H_2O/0.1% TFA and CH_3CN/0.1% TFA obtaining 55 mg (40% yield) of pure **14**. FAB $(M + H)^+$ was 553 as expected.

NMR. NMR samples were prepared by dissolving appropriate amounts of tuftsin, *ctuf2,* and *ctufβ* in a DMSO-d_6/water (80:20, v:v) cryomixture to obtain 1 mM solutions. NMR spectra were run at 600 MHz on a Bruker DRX-600 instrument. The samples for amide proton exchange were prepared dissolving the peptides in a DMSO-d_6/D2O water (80:20, v:v) cryomixture to obtain 1 mM solutions.

1D NMR spectra were recorded in the Fourier mode, with quadrature detection, and the water signal was suppressed by a low-power selective irradiation in the homogated mode. DQF-COSY,[21] TOCSY,[22] and NOESY[23] experiments were run in the phase-sensitive mode using quadrature detection in ω_1 by time-proportional phase incrementation of initial pulse.[30] Data block sizes were 2048 addresses in t_2 and 512 equidistant t_1 values. Before Fourier transformation, the time domain data matrices were multiplied by shifted $\sin^2$ functions in both dimensions. A mixing time of 70 ms was employed for the TOCSY experiment. NOESY experiments were run at 275 K with mixing times in the range 100−300 ms. Cross-peak volumes were quantitated in NOESY spectra with 75, 150, 225, and 300 ms mixing times, using the AURELIA software (Bruker). Distances were calibrated using $d(\delta,\delta')$ Pro^3 distances of 0.180 nm and calculating other distances according to the r^{-6} relationship, fitting the build-up curve to a second-order polynomial function. The necessary pseudoatom corrections were applied for nonstereospecifically assigned protons at prochiral centers and for Me group of Thr. The resulting values were used as input upper distance restraints, considering a percent error limit of 10%.

Energy Calculations. Energy calculations were based on the all-atom parametrization of the CVFF force field[32] as provided by the MSI InsightII package. Every atom parameter was assigned explicitly since the CVFF force field is especially parametrized to reproduce peptide and protein properties. EM calculations have been performed in vacuo with no distance cutoff for nonbonded interactions; charges were computed as provided by the CVFF force field.

X-ray coordinates of human immunoglobulin G[25] were extracted from the Brookhaven Protein Data Bank. Model building was performed according to two different methods (vide infra). In method (a) starting models for *ctuf2* and *ctufβ* were built from standard geometries based on canonical peptide structures containing a cis K−P peptide bond.[27] All molecules were subjected to extensive preliminary EM and then compared to NMR data. Introduction of NMR-based restraints led to a good agreement only in the case of the VIa model, which was thus chosen for subsequent calculations. In method (b) linear tuftsin, used as a guideline to generate starting structures of cyclic analogues, was modeled starting from the conformation adopted by the corresponding residues (T-K-P-R) in human IgG. Peptide coordinates were transferred directly from the protein X-ray structure using InsightII Homology module. The obtained molecule was subjected to an energy minimization, in its zwitterionic form. To allow a gradual structural relaxation, first a steepest descents minimization using a larger convergence criterion (until maximum rms derivative was less than 0.5 kcal mol^{-1} $Å^{-1}$) was applied; second the geometry optimization proceeded using conjugate gradient until the maximum rms derivative was less than 0.001 kcal mol^{-1} $Å^{-1}$. This protocol was adopted in each EM run.

Using InsightII Biopolymer module, tuftsin has been cyclized with glycine by automatic addition of the new amino acid to tuftsin NH terminus. In the case of β-Ala, it proved necessary to introduce this nonstandard residue manually. The cyclic models, obtained by these two methods, were subjected to an unrestrained EM calculation to let the peptide conformation reach its local energy minimum and then to a restrained EM calculation by using distance restraints from NOESY spectra. All restrained calculations were made using a range of distances according to the percent error calculated for NOE-derived distances.

Restraints were applied by a "flat-bottomed" potential function with a force constant value of 1000 kcal mol^{-1} $Å^{-1}$. This function applies a further energy penalty on the atoms which exceeds the limits of the distance restraints ranges. Regarding this latter parameter, two different energy penalty values were assigned, to force more and less strongly the conformation to fit NOE distances. These values were set to 1 and 100 kcal mol^{-1} $Å^{-1}$, respectively, leading to two different sets of structures defined as 'less restrained' and 'more restrained'. The obtained structures were relaxed by steepest descents minimization until maximum rms derivative was less than 0.5 kcal mol^{-1} $Å^{-1}$ to let them reach the nearest local minimum. The observed and absent NOEs were fully checked. The conformation with better respected NOEs distances has been chosen as starting point for restrained molecular dynamics calculations.

A molecular dynamics procedure was performed on the peptide side chains while keeping the backbone geometry fixed. Molecular dynamics simulations were run at high temperature for 300 000 ps so that thermal energy sufficient to cross energy barriers was added. Local minima conformations were then equilibrated by running dynamics at 310 K for 600 000 ps to allow the system to escape from high-energy states.

An average conformation was extracted from molecular dynamics runs. The whole molecule was then relaxed using a steepest descents minimization until maximum rms derivative was less than 0.5 kcal mol^{-1} $Å^{-1}$ to let it reach the nearest local minimum. Observed and absent NOEs were fully checked.

The conformation of "Suzuki's peptide", T-P-K-R, was built using canonical values of the type I β-turn for the internal rotation angles as suggested in the original paper[29] and subsequently performing a molecular dynamics procedure on the peptide side chains while keeping the backbone geometry fixed.

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