On the Conformational Bias of F((2*R*,3*S*)-cyclo-M)RFa Induced by the *cis*-2,3-Methanomethionine Residue

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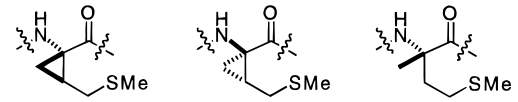
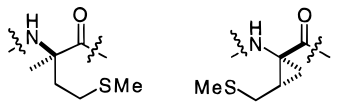
The objective of this work was to study conformational biases attributable to a *cis*-2,3-methanomethionine isomer substituted in a model sequence, FMRFa, and to compare them with previous studies of *trans*-2,3-methanomethionine stereoisomers in the same environment. Consequently, F((2*R*,3*S*)-cyclo-M)RFa was prepared via solid phase synthesis, and solutions of this material were examined by NMR and CD spectroscopies. These spectral studies were complemented by molecular simulations. These computational studies indicated γ- and β-turn structures were favored; however, the experimental data are consistent with only the γ-turn structure. Overall, this work and previous research indicates that both *cis*- and *trans*-2,3-methanomethionine stereoisomers tend to impart a conformational preference for γ-turns when substituted for methionine in FMRFa. It is proposed that this phenomenon is indirectly due to widening of the N−C_α−CO bond angle by the cyclopropane and might therefore be observed for 2,3-methanomethionine residues in other sequences.

The problem of finding small molecules that mimic pharmacological properties of biologically active peptides frequently arises in medicinal chemistry. α-Methyl amino and 2,3-methanoamino[1] acids can be valuable when addressing issues of this type. Peptidomimetics in which protein amino acids are substituted with these surrogates have very similar electrostatic and steric profiles, so their biological activities tend to resemble those of the parent peptide.[2] Moreover, they usually have enhanced proteolytic stabilities.[3−6] Perhaps the most interesting facet of conformationally constrained amino acids, however, is that the constraints that they impose can potentially enhance or suppress bioactivities that are shape dependent.[7−11] Consequently, peptidomimetics containing α-methyl amino or 2,3-methanoamino acids are potentially useful as "stepping stones" to facilitate the design of biologically active small molecules.

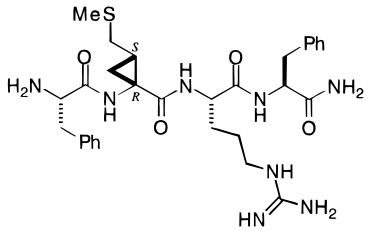
The literature on conformational constraints imposed by unusual amino acids can be difficult to interpret. Studies wherein different substitutions are made in different sequences, and are studied by different groups using different approaches, are difficult to assimilate. To achieve a degree of uniformity, members of this research group have been studying the conformational bias imposed by methionine surrogates when substituted for Met in the sequence Phe-Met-Arg-Phe-NH_2 (FMRFa), using similar experimental approaches.[7−11] Solution-phase NMR techniques supported by quenched molecular dynamics (QMD)[12] calculations have proved to be most efficacious. Other spectroscopic techniques such as IR and CD have proved to be relatively uninformative, and peptidomimetics of the sequence are slightly hygroscopic and not easily crystallized. Nevertheless, combinations of NMR and QMD studies have revealed evidence for various preferred conformations in the FMRFa[13] analogs in which the Met is substituted with (2*S*,3*S*)-cyclo-M,[10] (2*R*,3*R*)-cyclo-M,[11,14] (*S*)-αMe-M,[14] and (*R*)-αMe-M.[14] Both of the 2,3-methanomethionine derivatives studied so far have been *trans*-cyclopropanes (*trans* denotes the relative orientation of the amino and side-chain substituents). In this paper, we report the first studies of a *cis*-2,3-methanomethionine in the FMRFa sequence, *i.e.*, F((2*R*,3*S*)-cyclo-M)RFa.



2*S*,3*S*-cyclo-Met **2*R*,3*R*-cyclo-Met** ***S*-αMe-Met**



***R*-αMe-Met** **2*R*,3*S*-cyclo-Met**



F-{2*R*,3*S*-cyclo-M}RFa

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Table 1. Proton Chemical Shifts, Coupling Constants, and Temperature Coefficients for F((2*R*,3*S*)-cyclo-M)RFa in DMSO-d_6

amino acid	proton	δ (ppm)	J^3 N*H*-α (Hz)	temp coeff (ppb/K)
F[1]	N*H*	8.30	br s	
	α	4.04		
	β	3.03		
	β	2.94		
(2*R*,3*S*)-cyclo-M[2]	N*H*	8.66	s	−0.57
	β	1.54		
	β'_{cis}	0.27		
	β'_{trans}	1.50		
	γ	1.19		
	γ	2.35		
	ϵ	2.01		
R[3]	N*H*	7.46	8.0	−3.72
	α	4.33		
	β	1.46		
	β	1.63		
	γ	1.34		
	γ	1.41		
	δ	3.00		
	δ	3.00		
	*ϵ*N*H*	7.48		
F[4]	N*H*	8.02	8.5	−4.84
	α	4.35		
	β	2.77		
	β	2.94		

Results

NMR Studies. DMSO was used as solvent to be consistent with earlier studies in this series. Peak assignments were made via DQF-COSY spectra[15] in the usual way (Table 1).[16] ROESY spectra[17] were recorded to access close contacts, and the crosspeaks were classified according to their intensities (Table 2). Temperature coefficients were recorded for the N*H* protons (Table 1) to reveal those that are somehow insulated from the effect of temperature changes in the solvent.[18,19]

Several aspects of the data shown in Figure 1 and Table 1 are conspicuous. The chemical shift of the Arg[3]N*H* proton is shifted to higher field than the other backbone N*H* resonances (Table 1). One of the *γ*-CH_2 resonances for the 2,3-methanomethionine side chain is shifted upfield; this particular proton is observed at 1.19 ppm, whereas we have observed that the *γ*-CH_2 protons of cyclo-Met derivatives tend to occur in the range of 2.03−2.75 ppm (data from 10 different compounds containing cyclo-Met residues). A low-temperature coefficient was observed for the N*H* protons of the cyclo-Met[2] residue. Finally, the ROESY cross peaks around the cyclopropane are informative. A cyclo-MetN*H*−cyclo-MetC$H_{2\gamma}$ cross peak and the absence of a cyclo-MetN*H*−cyclo-MetC$H_{\beta' cis}$ contact are diagnostic of a preference for a positive *ϕ* angle (Figure 1).

Molecular Simulations. Molecular dynamics and molecular mechanics minimization were used to generate

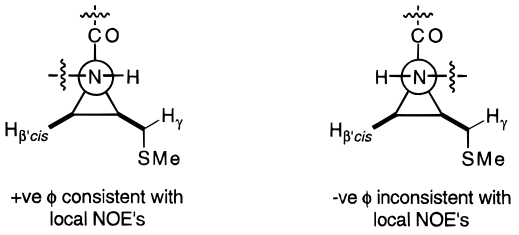

Figure 1. Local NOE's about the 2,3-methanomethionine residue in F((2*R*,3*S*)-cyclo-M)RFa.

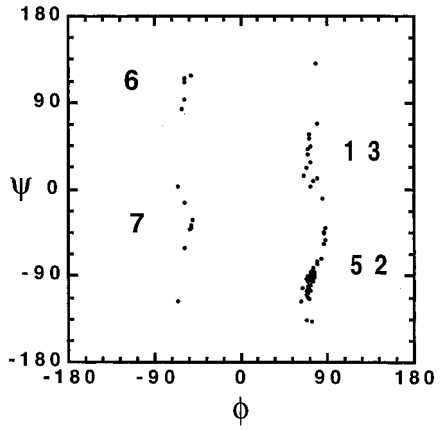

Figure 2. *ϕ*,*ψ* scatter plot for the cyclo-M[2] residue of F((2*R*,3*S*)-cyclo-M)RFa in the 78 lowest energy conformers generated in the QMD studies.

600 structures following a typical protocol for QMD. The energy distribution of these conformers relative to the overall lowest energy structure was surveyed. An energy cutoff was thereby selected such that 78 low energy conformers were set aside for further consideration.

Several observations can be made on the basis of the data generated from the molecular simulations alone. Figure 2 is *ϕ*,*ψ* dot plot showing the variation of *ϕ*,*ψ* angles for the cyclo-Met residue in the 78 low energy conformers. It indicates that the range of favorable *ϕ* values is small, but the spread of *ψ* values is large. Of the 78 structures, 52 are in a quite narrowly defined pocket in the (+,−) *ϕ*,*ψ* region. Finally, when the backbone atoms of the 78 lowest energy structures were systematically overlaid and grouped into families based upon root mean square deviations of the backbone atoms, eight families of structures were observed. The two most populated families are F2 and F4, and these contain the conformers with the second and fourth lowest energies.

CD Studies. A CD spectrum of the F((2*R*,3*S*)-cyclo-M)RFa in 65:35 MeOH:H_2O was recorded. This particular solvent was chosen since it has the same dielectric constant as DMSO (DMSO being unsuitable for CD studies). Figure 3 is an overlay of three CD spectra expressed in terms of mean residue ellipticity. The free cyclopropane amino acid has a relatively small negative ellipticity around 200 nm (Figure 3a), the parent sequence, FMRFa, has a positive ellipticity between 210 and 240 nm with a strong peak at 220 nm (Figure 3b), and the peptidomimetic has a broader and less intense positive ellipticities at approximately 200 and 220 nm (Figure 3c).

Discussion

Table 2 presents a comparison of the NOE intensities observed in the ROE studies and the interatomic dis-

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Table 2. Comparison of ROE Crosspeak Intensities with Interproton Distances for the Lowest Energy Conformer from Each of the Families Generated via QMD

contact	NOE intensity	contact distance from QMD (Å)							
		F1	F2	F3	F4	F5	F6	F7	F8
F^1NH-α	M	2.40	2.39	2.38	2.39	2.36	2.38	2.39	2.38
F^1α-(2*R*,3*S*)-M^2NH	S	2.09	1.99	3.57	1.99	***3.49***	1.98	1.98	*3.57*
R^3α-F^4NH	VS	*3.45*	2.04	2.21	*3.23*	*3.51*	*2.76*	2.06	*3.01*
F^4α-$CONH_2$	S	1.97	2.18	2.10	2.18	2.05	2.07	2.34	2.26
(2*R*,3*S*)-M^2NH-R^3NH	W	3.02	4.05	2.89	4.25	2.82	2.72	4.54	4.41
R^3NH-F^4NH		*2.21*	4.55	3.65	*2.46*	*2.18*	*2.46*	4.58	*2.23*
(2*R*,3*S*)-M^2NH-γH	VW	***2.74***	***2.49***	***2.19***	***2.42***	***2.47***	***2.34***	***2.78***	***2.18***
(2*R*,3*S*)-M^2NH-$\beta'_{cis}H$		*2.38*	3.34	3.26	3.27	3.20	3.23	*2.44*	3.30
F^1arom*H*-(2*R*,3*S*)-$M^2\delta H$[a]	W	*8.55*	2.56	***6.38***	2.58	4.02	2.59	*8.22*	*8.73*
F^1arom*H*-(2*R*,3*S*)-$M^2\beta'_{cis}H$	VW	4.81	2.87	***6.47***	2.80	*5.80*	2.88	*5.35*	*5.26*
F^1α-arom*H*	M	2.33	2.70	2.11	2.67	*4.04*	2.64	2.48	2.97
F^4α-arom*H*	M	2.53	2.61	2.66	2.45	2.54	2.59	*4.20*	*4.18*
ϕ,ψ of (2*R*,3*S*)-M^2		−53 −40	78 −77	72 3	72 −92	6 23	6 36	−5 112	7 −104
number in family[b]		3	12	3	21	6	2	9	9
energy (kcal/mol)		15.64	16.40	16.72	17.19	17.30	17.41	17.66	17.68

[a] This is the methyl group of the 2,3-methanomethionine side chain. [b] Families of conformers at higher energies than family 8 contained only two to three members each and were not considered further.

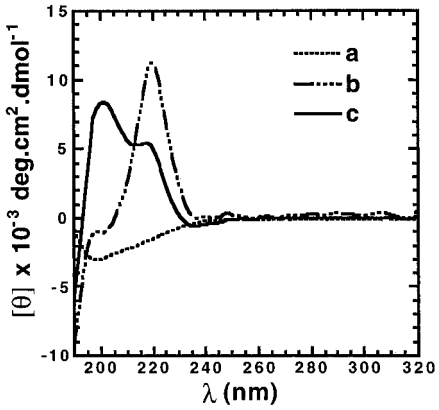

Figure 3. Mean residues ellipticities in 65:35 MeOH:H_2O for (a) H-((2*R*,3*S*)-cyclo-M)-OH; (b) FMRFa; and (c) F((2*R*,3*S*)-cyclo-M)RFa.

tances for the minimum energy conformer in each family. Serious deviations between the experimental and simulated values are shown in bold italics. There is only one discrepancy between the simulated proton separations and the observed NOE intensities for the lowest energy conformer in family 2. In general, differences between simulated distances and observed ROE's are more serious when the NMR cross peaks are greater than anticipated. Consequently, the sole discrepancy for family 2 is of the less serious kind. All the other families show greater divergence between the experimental and simulated parameters.

Figure 4, parts a and b, shows overlays of the conformers in the two most populated families, 2 and 4, respectively. Family 2 (Figure 4a) shows a γ-turn conformation about the cyclo-M residue wherein the F^1CO-R^3NH distance is 2.28 Å. The temperature coefficient for the R^3NH residue was marginally over −3 ppb/K; this value neither confirms or eliminates H-bonding for this proton. A hydrogen bond to this proton is inherent in the C^7 conformation depicted by family 2. However, hydrogen bonding of the R^3NH resonance is consistent with the observed upfield shift of this proton. Backbone coordinates were used to sort the conformers into families; less deviation in the backbone conformation than the side-chain conformation is therefore to be expected, and this

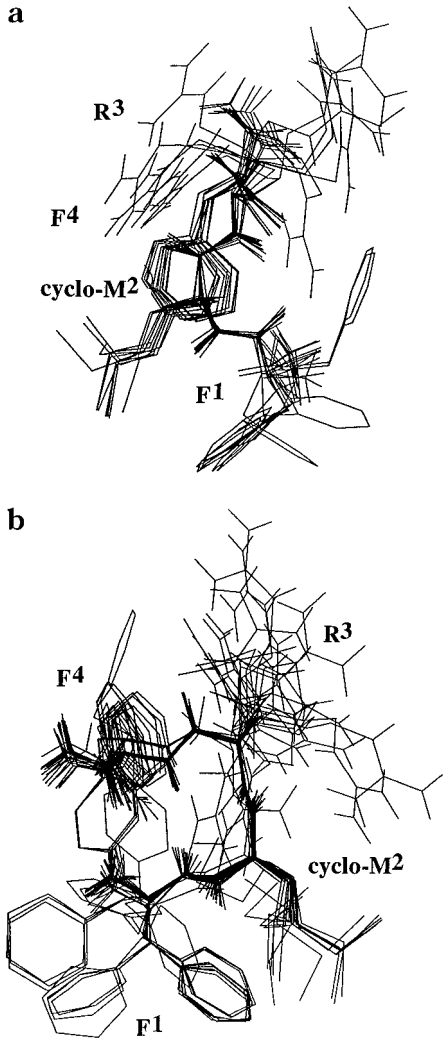

Figure 4. Overlaid conformers in (a) family 2 showing a γ-turn preference, and (b) family 4 showing a β-turn preference. In part a the F^4 residue is behind the R^3 residue.

is what is observed in Figure 4. Despite the variations of side chain orientations, it is evident that the F^4 phenyl group tends to shield the cyclo-M^2NH, and this could account for the very low temperature coefficient observed

for this proton (−0.57 ppb/K, Table 1). Moreover, the proximity of the phenyl side chain to the γ-C*H* resonances for the 2,3-methanomethionine side-chain could account for the abnormally low chemical shift observed for this proton.

The CD studies also lend support to the hypothesis that the F((2*R*,3*S*)-cyclo-M)RFa has a tendency to fold into a γ-turn. Subtraction of the mean residue ellipticities of H-((2*R*,3*S*)-cyclo-M)-OH and FMRFa from that of the peptidomimetic F((2*R*,3*S*)-cyclo-M)RFa gives a negative absorbance at around 220 nm. Other workers have associated negative ellipticities in this region with C^7 conformations.[20]

The discussion above illustrates the harmony between the experimental and simulated data for family 2, but there is an equally clear divergence between the two sets of data for family 4. Figure 4b shows that the conformers in the latter family are all of a certain β-turn type. If this were the predominant conformation there should be a relatively weak NOE for the $R^3\alpha-F^4NH$ contact and a more intense NOE for transfer between R^3NH and F^4NH. In fact, the corresponding NOE's for these contacts were very strong and not observed, respectively. Moreover, the temperature coefficient for the F^4NH was the highest of all the backbone amide protons indicative of lack of involvement of this proton in a β-turn hydrogen bond.

Conclusions

NMR data collected for F((2*R*,3*S*)-cyclo-M)RFa is consistent with a γ-turn structure as depicted by one family of conformers (family 2) generated in a QMD study. Conversely, the NMR data do not fit so well with β-turn conformers like those in family 4 of the QMD study.

Overall, when (2*R*,3*S*)-cyclo-M is substituted into our model FMRFa sequence it illustrates a preference for a C^7-turn conformation, specifically γ-turn structures. Other work from these laboratories suggests that *trans*-cyclo-M stereoisomers tend to exert a conformational bias in favor of γ- or inverse γ-turn structures.[9−11,14] Therefore, the initial indications are that both *cis*- and *trans*-cyclo-M residues tend to bend peptidomimetics into C^7 turn orientations. However, α-methylmethionine in the same sequence tends to impart partial helical structures. We suggest that there are two reasons why C^7 turns are favored for the cyclo-M stereoisomers. First, the NH−C_α−CO bond angle is wider for the 2,3-methanoamino acids than for the corresponding amino acids (approximately 118° as compared with 112° from the parameters we collected from crystal structures in the literature).[21,22] Second, the side chain substituent on the cyclopropane is locked. The relatively open bond angle of cyclopropane amino acids increases steric repulsions between both the *N*-and the *C*-termini and the cyclopropane, forcing the *N*-and *C*-termini to point away from the cyclopropane. The rigidly constrained side chain of these compounds accentuates this effect (Figure 5).

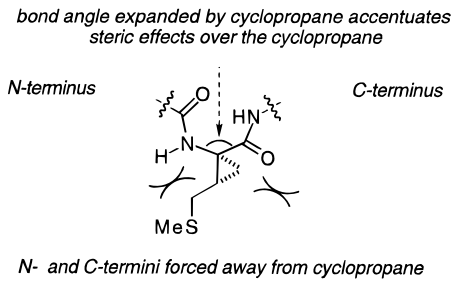

Figure 5. Diagram to illustrate the steric interactions that may favor C^7 turn conformations for 2,3-methanoamino acids.

Almost all of our conformational studies so far have focused on 2,3-methanomethionine stereoisomers in the FMRFa sequence. However, the conclusions outlined here are likely to be applicable to other 2,3-methanoamino acids in other sequences. For these reasons we predict that conformational biases in favor of C^7 turns conformers centered about 2,3-methanoamino acids may be the norm rather than an exception. It will be interesting to see if future studies confirm or disprove this prediction.

Experimental Section

Solid-Phase Synthesis of F((2*R*,3*S*)-cyclo-M)RFa. The peptide was prepared via stepwise couplings of 9-fluorenylmethoxycarbonyl (FMOC) amino acids derivatives on 4-((2′,4′-dimethoxyphenyl)(FMOC-amino)methyl)phenoxy resin (Rink amide resin).[23] The 4-methoxy-2,3,6-trimethylbenzenesulfonyl (Mtr)[24] group was used for side chain protection for the Arg residue. (Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) and *N*-hydroxybenzotriazole (HOBt) were used as coupling reagents, and DMF was used as solvent.[25] Manual peptide synthesis was carried out in a 20 mL vessel fitted with a coarse glass frit by using a manual wrist action shaker (Burrel, Model 75), and the reagents were added manually. All reactions were carried at 25 °C unless otherwise specified. A 1 min DMF washing cycle (10 × *ca.* 10 mL) was performed after each coupling and deprotection. FMOC-(2*R*,3*S*)-cyclo-Met was synthesized as previously described.[26] Other chemicals were purchased from commercial suppliers. DMF was stored over 4 Å molecular sieves.

Rink amide resin (0.161 g of 0.62 mmol g^{-1} capacity, 0.1 mmol) was swelled in DMF (ca. 10 mL) for 45 min, and the FMOC protecting group was removed by shaking the resin with 20% piperidine in DMF (twice, 3 and 7 min). Coupling of FMOC-Phe (0.116 g, 0.2 mmol, 2 equiv relative to resin) was performed by premixing the amino acids with *N*-methylmorpholine (NMM; 0.030 g, 0.3 mmol, 3 equiv), HOBt (0.027 g, 0.2 mmol, 2 equiv), and BOP (0.088 g, 0.2 mmol, 2 equiv) in DMF (6 mL), and then the mixture was added to the resin, shaken for 105 min, and washed with DMF (10 × 1 min, ca. 10 mL). A negative ninhydrin test[27] was observed. The FMOC protecting group was removed as described above. The same coupling cycle was repeated for FMOC-Arg(Mtr) (0.134 g, 0.2 mmol, 2 equiv) and FMOC-(2*R*,3*S*)-cyclo-Met (0.077 g, 0.2 mmol, 2 equiv). FMOC-Phe-fluoride (0.156 g, 0.4 mmol, 4 equiv)[28,29] was coupled using DIEA (diisopropylethylamine)

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(0.052 g, 0.4 mmol, 4 equiv) in DMF (5 mL) for 35 min. Washing cycles and the final deprotection were performed as described above. The resin was then washed with CH_2Cl_2 (10 × 1 min) and stored under vacuum overnight.

Cleavage of the peptidomimetic from the resin was performed using 0.5 mL of 82.5% TFA, 5% phenol, 5% water, 5% thioanisole, and 2.5% ethanedithiol. The reaction was stirred at 25 °C for 15 h and filtered, and the residual material on the resin was collected by washing the resin with *ca*. 3 mL of water. The organic materials were extracted away with Et_2O (3 × 10 mL), and the aqueous layer was lyophilized to obtain the crude product as colorless solid. This was purified by preparative HPLC (Vydac C18 column, 22 mm × 25 cm, 10 μm) with a linear gradient obtained by mixing solvent A (0.05% TFA in water) and solvent B (0.05% TFA in acetonitrile). The gradient was increased from 5 to 60% B over 30 min with a flow rate of 6 mL min^{-1}. The peak with a retention time of 26.84 min was collected and lyophilized to produce 40 mg of the pure peptidomimetic (TFA salt) as a colorless powder: HR-FABMS (NBA/MeOH) *m*/*z* calcd for $C_{30}H_{43}N_8O_4S$ ($M + H^+$) 611.3128, found 611.3096.

Molecular Simulations. The CHARMm 22 modeling package was used for the molecular simulations. The procedure used was the same as that previously reported for similar studies.[10,11] Structures within 3.5 kcal mol^{-1} were selected for further analyses.

NMR Studies. NMR experiments were performed on a Varian XL-400 (400 MHz) or a Varian Unity Plus 500 (500 MHz) spectrometer. The sample was prepared in DMSO-d_6 at 4.36 mM concentration. One-dimensional ^{1}H NMR spectra were recorded using 8000.0 Hz spectral width and 80 000 data points with a temperature range of 20−40 °C in 2.5 °C increments to determine the temperature dependence of the amide chemical shifts. The phase-sensitive double-quantum-filtered correlation spectrum (DQF-COSY) was recorded using 2 s relaxation delay, 2048 data points, 8000.0 Hz spectral width, 512 t_1 increments, 16 scans per t_1 increment, and 2 K data points at t_2. The rotating frame nuclear Overhauser enhancement spectroscopy (ROESY)[17] was recorded using similar parameters but with a mixing time of 300 ms and 32 scans per t_1 increment for ROESY. This mixing time and transmitter offset for the ROESY spectra was that previously determined to be optimal for an isomeric compound.[10,11] The NMR spectra were processed using Vnmr version 5.1 software operating on a Sun workstation. Two-dimensional spectra were zero-filled to 2 K × 2 K data set and multiplied by a Gaussian function prior to Fourier transformation. Distance constraints were derived from the intensities of the ROESY crosspeaks. The intensities of the ROESY crosspeaks were classified as VS (very strong), S (strong), M (medium), W (weak), and VW (very weak) corresponding to the number of contours of crosspeaks.

CD Studies. CD spectra were recorded at 25 °C on an Aviv CD spectrometer Model 62DS. The solution of the peptidomimetic (0.1939 mM) was made up in spectral grade MeOH and H_2O that had been degassed immediately before use. A mixture of 65:35 MeOH:H_2O was used since it has the same dielectric constant as DMSO (the latter being unsuitable for CD). All spectra were monitored from 320 to 190 nm at a scan of every 0.5 nm, a time constant of 4 s, and a band width of 1.0 nm. A quartz cell with a path length of 0.1 cm was used. CD spectra recorded at seven different concentrations gave almost identical spectra.

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