Conformational Preferences of RNase A *C*-Peptide Derivatives Containing a Highly Constrained Analogue of Phenylalanine

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Abstract: Both enantiomers of a highly constrained derivative of phenylalanine, FiFi, were prepared in optically pure form. Studies were performed to elucidate the effects of substituting this amino acid for phenylalanine in RN-24, a derivative of the RNase A *C*-peptide. Thus RN-24, and the analogues **9** and **10**, in which Phe-8 was replaced by each of the enantiomers of FiFi, were prepared. Comparative circular dichroism (CD) experiments indicated relative tendencies to adopt helical structures, and variable-temperature CD studies showed the relative ease with which these conformations were lost at elevated temperatures. These observations were rationalized via computer-assisted molecular modeling, which showed that the phenyl groups of (*R,R*)-FiFi in the peptidomimetic **9** can be accommodated via distortion of the helical conformations and that such distorted conformations persist as the temperature is increased. Conversely, intolerable contacts occur in an analogous conformation of the (*S,S*)-FiFi peptidomimetic **10** and these preclude helicity. Consistent with these observations, molecular dynamics studies of these peptidomimetics at 276 K indicate that helical conformations of **9** and RN-24 are observable under conditions in which the analogous conformation of **10** is lost. Overall, these studies demonstrate that cyclopropane amino acids can be used to enforce elements of secondary structure (albeit distorted) or to preclude them altogether.

Introduction

Bovine pancreatic RNase A (Figure 1a) is a protein that mediates the hydrolysis of RNA. Fragments of RNase A are available via several enzymatic and chemical routes. Proteolytic degradation of RNase A with subtilisin, for instance, gives a C-terminal protein fragment, "the *S*-protein" (RNase A residues 21−124), and a N-terminal peptide called "the *S*-peptide" (RNase A residues 1−20).[1] The intermolecular complex formed between the *S*-protein and the *S*-peptide is "RNase S." A shorter peptide, "the *C*-peptide lactone," can also be formed from the *S*-peptide via cleavage with CNBr. Moreover, synthetic peptides based on the *C*-peptide lactone sequence have also been prepared and studied, notably the system named RN-24.[2]

$K^1E^2T^3A^4A^5A^6K^7F^8E^9R^{10}E^{11}H^{12}M^{13}D^{14}S^{15}S^{16}T^{17}S^{18}A^{19}A^{20}$

S-peptide

$K^1E^2T^3A^4A^5A^6K^7F^8E^9R^{10}E^{11}H^{12}$M-lactone^{13}

C-peptide lactone

succ-$A^1E^2T^3A^4A^5A^6K^7F^8L^9R^{10}A^{11}H^{12}A^{13}$-amide

RN-24

Several characteristics of RNase A derivatives make them prototypical models for studies in protein-folding[3] and for formation of helical secondary structures for small peptides in solution.[4] First, the *S*-peptide,[5] the *C*-peptide,[6] and RN-24[2] have significantly populated helical conformations at temperatures near 3 °C. At the time when these observations were originally made, it was unusual to find evidence of helicity in such small peptides, though subsequently these findings have inspired the design of other helical small-peptide systems.[7−10] Second, solid-state crystal structures have been determined for RNase A[11] and the *S*-protein/*S*-peptide complex;[12] for RN-24, a solution-state structure has been deduced from NMR.[13] Consequently, accurate physical models exist for structures of RNase A and its derivatives. For the *S*-peptide, these static models have been supported by computer-aided molecular simulations illustrating the dynamic conformational behavior of this system.[14] Finally, association complexes of the *S*-protein and the *S*-peptide have catalytic activities that are comparable with those of RNase A.[15] This is remarkable because the *S*-protein alone is inactive.

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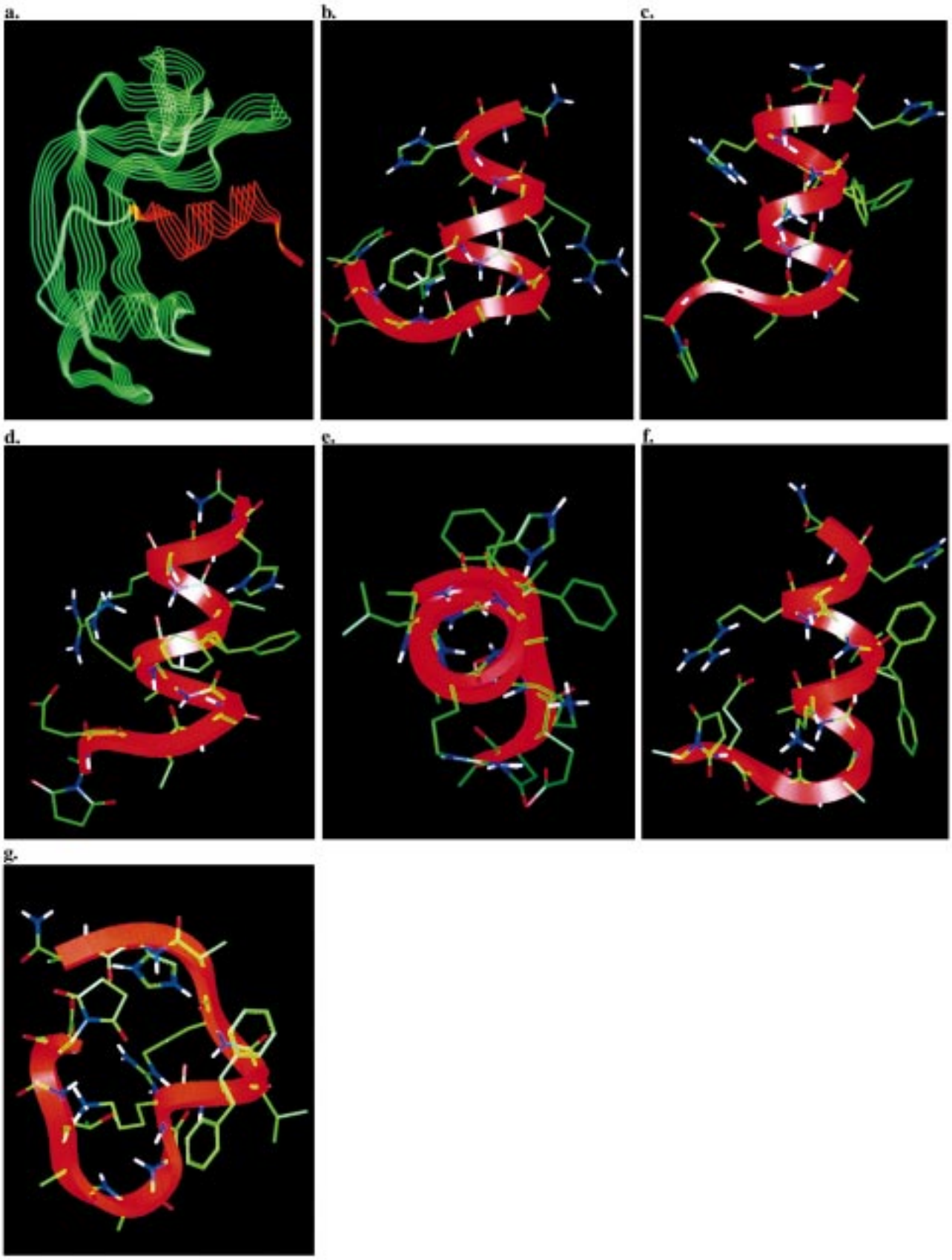


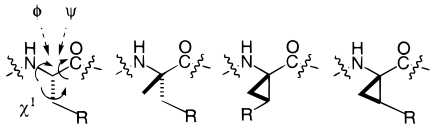
Figure 1. (a) RNase A; (b) RN-24 after 300-ps molecular dynamics simulation; (c) peptidomimetic **9** before molecular dynamics simulation; (d) side view of peptidomimetic **9** after molecular dynamics simulation; (e) top view of peptidomimetic **9** after molecular dynamics simulation; (f) peptidomimetic **10** before molecular dynamics simulation; (g) peptidomimetic **10** after molecular dynamics simulation.

However, the *S*-protein enhances the disposition of the *S*-peptide to adopt helical conformations,[16−18] so the inference of the catalytic activities is that RNase A and the *S*-protein have similar tertiary structures.

One of the consequences of research on the *S*-peptide, the *C*-peptide, and RN-24 is that the interactions that stabilize helical conformations in these systems are well understood. The most pertinent observation with respect to the work to be described here is that considerable evidence indicates a perpendicular π-interaction between Phe-8 and His^+-12.[19−22] Similar interactions have also been observed in some other systems.[23] Another crucial helix-stabilizing interaction in *C*-peptide analogues is a salt bridge between Glu-2 and Arg-10; the importance of this has been confirmed via experiments in media of variable pH.[19,24,25] Finally, the presence of a Glu^- residue near the N-terminus and a His^+ residue near the C-terminus has been postulated to be significant,[2,23,26−28] consistent with the generally accepted notion that helix dipoles are stabilized by negative charges at the N-terminus and positive charges at the *C*-terminus.[29]

Here we describe the first set of data on the effects of substituting cyclopropyl amino acids into an RN-24 analogue. This study has two goals: specifically, further elucidation of the effects of cyclopropane amino acids on secondary structures, and understanding how the secondary structure of *C*-peptide analogues may be deliberately and systematically perturbed. With respect to the first goal, RN-24 is significantly different from other model systems that have been used.[30−36] This is important because there is no ideal system for studying conformational biases of cyclopropane amino acids, so conclusions must be drawn from several different systems. Short linear peptides (e.g., 3−5 residues) yield useful data, but interpretation is complicated by ill-defined, random-coil conformational states that are hard to identify, even after substitution with a restricted amino acid.[30−35] Conversely, cyclic peptides tend to have better-defined conformational biases, but the effects of replacing a natural amino acid with a more-hindered one are obscured by the constraints of peptide-cyclization.[36] RN-24, a 13-mer, has characteristics that are midway between these extremes. It has more-pronounced conformational preferences than linear tetrapeptides, for instance, but it is more amenable to conformational perturbations than is a cyclic hexapeptide.

The specific approach described in this paper is to substitute the two enantiomers of the novel cyclopropane amino acid FiFi for Phe-8 in RN-24. The relevance of Phe-8 substitutions in RN-24 is evident from the postulated importance of the perpendicular π-stacking Phe-8/His^+-12 interaction, but the choice of the new cyclopropyl amino acid FiFi warrants explanation. Our rationale for this approach is as follows. The purpose of using sterically constrained amino acid analogues is that restricted rotation must enhance the probability that some conformations will be populated while others are less accessible. The nature of the critical interactions depends on the structures studied. For example, compared with amino acid **I**, the α-methyl substituent of **II** would be expected to exert influences of similar magnitudes on rotations about the ϕ, ψ, and χ^1 bonds. Conversely, a cis-substituent on a cyclopropane ring, as in **III**, locks χ^1, which has a very significant influence on ϕ but relatively minor effects on ψ. For the isomer **IV**, however, locking χ^1 impacts ψ most and ϕ least. A trans-disubstituted cyclopropane amino acid of type **V** imposes relatively severe constraints on ϕ and ψ simultaneously. Amino acids of this type, therefore, should have the most pronounced effects on secondary structure in the series and may provide clues to the possible influence of the monosubstituted derivatives **III** and **IV**.



I **II** **III** **IV**

V (*R*,*R*)-FiFi **ent-V** (*S*,*S*)-FiFi

To the best of our knowledge, there has been no previous synthesis of the structure we call FiFi, even as a racemate. Syntheses of dimethyl-substituted cyclopropane amino acids have been reported, via routes that do[37−41] and do not[42] involve elements of stereocontrol. An asymmetric synthesis of a *gem*-dimethyl cyclopropane amino acid has also been reported.[39,43]

Results and Discussion

Preparation of FiFi and Incorporation into Peptidomimetics via Solid-Phase Syntheses. Several synthetic routes to FiFi were explored before a satisfactory one was found. In a first attempt, rhodium-catalyzed cyclopropanation of *trans*-1,2-diphenylethene with an alkenyl diazoacetate was investigated, but the desired product was not formed under the conditions

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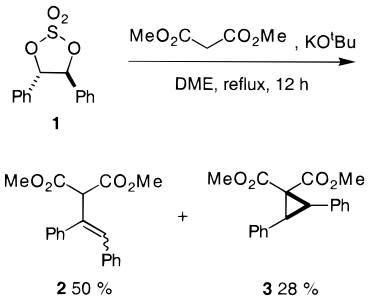
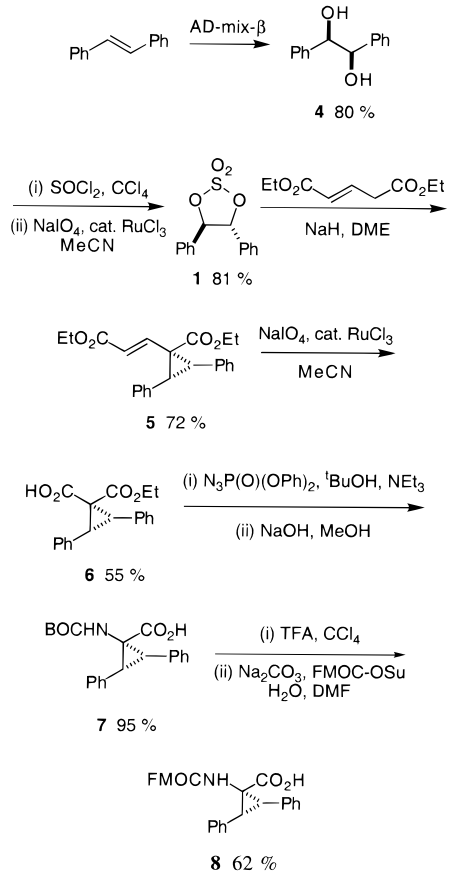
we used.[44] Later, other work from our group implied that the opening of cyclic sulfates with malonate derivatives would be appropriate, but these reactions proved unsuitable in this particular case.[45] For instance, the ring-opened product **2** predominated over cyclopropane **3** when dimethyl malonate was reacted with the cyclic sulfate **1**. Other nucleophiles such as 4-phenyl-3-butenoate and benzylidene glycinate[46] gave the desired material with no detectable elimination on a small scale, but failed to give the cyclopropane products cleanly when the reaction was scaled up to multigram amounts. Byproducts identified from these reactions included diphenyl acetylene and others derived from ester hydrolysis. Thus, the spectrum of chemical characteristics (e.g., nucleophilicity and basicity) that are tolerable for the nucleophilic component in the formation of cyclopropanes from cyclic sulfates seems to be limited.



Ultimately, a practical route to a usable cyclopropane was developed, involving reaction of diethyl glutaconate with **1**. Consequently, a preparation of (2*R*,3*R*)-3-phenyl-cyclo-Phe [*i.e.*, (*R*,*R*)-FiFi] from *trans*-1,2-diphenylethene was developed according to Scheme 1. Asymmetric dihydroxylation[47] gave diol **4** with an optical purity of >99% ee as assayed with a chiral aryl boronic acid.[48] This diol was converted first to the cyclic sulfate **1**,[49] then to cyclopropane **5** by a double displacement with diethyl glutaconate. Oxidation of alkene **5**[50] provided the acid ester **6**. Curtius rearrangement of this acid via treatment with diphenylphosphoryl azide (DPPA) in *tert*-butyl alcohol, followed by basic hydrolysis, afforded the *N*-butoxycarbonyl (BOC)-protected (2*R*,3*R*)-3-phenyl-cyclo-Phe. This protected form is appropriate for solid-phase syntheses of peptidomimetics. The enantiomeric product, *N*-BOC-protected (2*S*,3*S*)-3-phenyl-cyclo-Phe, was obtained in exactly the same way, except that AD-mix-α (Aldrich) was used in the first step.

For solid-phase syntheses of peptidomimetics from FiFi, we selected the fluorenyl methoxycarbonyl (FMOC) approach[51] to avoid problems that could arise because of the inconvenience and possible complications in HF cleavage procedures. FMOC-*N*-protected forms of the two FiFi enantiomers were therefore prepared via trifluoroacetic acid (TFA)-mediated removal of the *N*-BOC group, and reaction with FMOC−OSu (Su = succinimide). The FMOC-protected amino acids were purified via

Scheme 1. Asymmetric synthesis of FiFi



flash chromatography on silica gel. Yields of materials isolated after such purifications depend inversely on the residence time on the support since these (and incidentally some other hindered FMOC-protected amino acids that we have prepared) are not particularly robust with respect to this form of chromatography.

We synthesized a sample of RN-24 on Rink's amide resin,[52] using benzotriazoyloxy-tris(pyrrolidino)-phosphonium hexafluorophosphate/1-hydroxybenztriazole (PyBOP/HOBt)[53] for the couplings, performed for 1-h reaction times, and repeated once or twice as required for complete coupling (indicated by negative ninhydrin test results).[54] Peptidomimetics **9** and **10** containing (*R*,*R*)- and (*S*,*S*)-FiFi, respectively, were prepared via the same procedure except for the coupling to incorporate the FiFi residues, and for the coupling after that. For these couplings, acid fluorides were produced in situ with use of the reagent tetramethylfluoroformadinium hexafluorophosphate (TFFH)[55] with 1-hydroxy-7-azabenztriazole (HOAt)[56] as an activating agent. The coupling to incorporate the FiFi moieties required only 1 h, whereas the subsequent coupling was more difficult and was run for 12 h. In this work, the quantities of these relatively high-molecular-mass peptidomimetics obtained (∼6 mg) were sufficient for circular dichroism (CD) studies but not for NMR, although the route described above could be used to prepare enough for NMR studies if more time and resources were available.

CD Studies. Qualitative analysis of the CD spectra of the peptidomimetics in pH 5.2 buffer indicated that RN-24 itself

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succ-$A^1E^2T^3A^4A^5A^6K^7X^8L^9R^{10}A^{11}H^{12}A^{13}$-amide

X^8 = (*R*,*R*)-FiFi, **9**; X^8 = (*S*,*S*)-FiFi, **10**

and the (*R*,*R*)-FiFi derivative **9** had the same shape, indicative of helical character (Figure 2a). The RN-24 sample had molar ellipticity minima near 206 and 222 nm, and **9** had a very similar CD spectrum but with minima slightly shifted to ∼204 and 220 nm. However, the CD spectrum of the (*S*,*S*)-FiFi derivative **10** was an unusual shape that was reminiscent neither of a random-coil situation nor any other common secondary structure element. Because the compound gave no distinct minimum at 220 nm, this peptidomimetic does not appear to have any bias to a helical structure that is identifiable via this technique.

Comparisons of variable temperature CD data obtained for RN-24 with that for the (*R*,*R*)-FiFi derivative **9** were interesting and informative (Figures 2b and 2c). As RN-24 was warmed from 3 to 23 °C in 5 °C intervals, its molar ellipticity at 222 nm steadily decreased to 65% of its original value. However, the molar ellipticity for peptidomimetic **9** in an analogous experiment decreased to 90% of its original value; that is, the decrease was only 10%. Thus, the CD spectrum of this FiFi derivative, and therefore its secondary structure, are much less affected by a 20 °C increase in temperature than is RN-24. This appears to be a good example of stabilization of a secondary structure by substitution with a 2,3-methanoamino acid.

The secondary structure of peptidomimetic **9** was also less affected by pH than RN-24. Thus, whereas the molar ellipticity of RN-24 changes quite dramatically over a pH range of 7.3 units, the ellipticity of **9** is relatively insensitive to this perturbation (Figures 2d and 2e, respectively). Figure 2f shows the molar ellipticity at 222 nm as a function of pH for RN-24 and for **9**. The bell-shaped curve seen for RN-24 corresponds to the forming and breaking of the interactions between charged groups (i.e., Glu^--2 to Arg^+-10 and Phe-8 to His^+-12). However, the CD data show that those same factors cannot be as prevalent in stabilizing the presumed helical structure of **9**. For this peptidomimetic, the ellipticity increases in the pH range 2 to 4, corresponding to progressively more-deprotonated Glu-2, and decreases as Lys^+-7 is deprotonated, in the pH 9.5 to 10.5 region. The latter may be taken as evidence for formation of a Glu^--2 to Lys^+-7 salt bridge. This type of salt bridge was also observed in some of the molecular dynamics studies described below.

Apparently, compound **9** has less ellipticity at 222 nm and is therefore less helical than RN-24, though numerical differences between the two [θ]-values are hard to compare because of experimental errors. Accurate quantitative analyses of the CD data was not possible because of the problems with quantification that have arisen frequently in studies of the *C*-peptide.[21] Generally, the best method for measuring the concentration of RN-24 derivatives is to replace Phe-8 with a tyrosine residue; the molar UV absorbance at 280 nm is then relatively intense and easily calibrated.[21] This method is clearly not applicable in this work, however, where our purpose was to replace Phe-8 by another phenylalanine surrogate. We therefore used two other methods for concentration measurement in our studies: calibration of the UV-absorbance at 205 nm[57] and quantitative amino acid analyses. Reasonable agreement was obtained with both methods; however, we used the amino acid analysis results to obtain the molar ellipticities given in Figure 2. Calibration of concentrations by measuring the UV absorption at 205 nm was less accurate than amino acid analyses because of the slight absorbance of the FiFi residue at that wavelength, which is difficult to correct for. The overall consequence of these uncertainties in concentration determinations is that our comparisons between the molar ellipticities obtained for different samples are tenuous. Differences between the concentration data obtained from UV and amino acid analyses indicate that the percentage errors in the ellipticities are on the order of 10%.

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Molecular Modeling. The structure of RN-24 was built in CHARMm by using the coordinates of *S*-peptide as a starting point, minimized via a molecular mechanics routine, and then subjected to molecular dynamics studies at a simulated temperature of 276 K for 300 ps. This study parallels the analysis of an *S*-peptide previously performed by Jorgensen et al. and yielded very similar results (Figure 1b).[14] In the Jorgensen work, the helical structure of an *S*-peptide analogue was conserved throughout a 300-ps molecular dynamics run at a simulated temperature of 278 K. In our study, the simulated helical structure of RN-24 also persisted (276 K, 300 ps).

Peptidomimetic **9** was constructed by using modified coordinates from the crystal structure of the *S*-peptide as a starting point. A parameter set and topology file (see Supplementary Information) were constructed for the FiFi component by use of the approach previously outlined for other cyclopropane amino acids.[30,31] A simple molecular mechanics minimization gave the conformer shown in Figure 1c. This has seven hydrogen bonds from residues 3 to 13 that impart a helical character, but some of the key features that stabilize the RN-24 helix are not present. For instance, the Glu^--2 to Arg^+-10 salt bridge is disrupted, and the phenyl rings of the FiFi component are too far from His^+-12 for there to be a significant interaction (4.7 Å, compared with 3.9 Å in RN-24, for the distances between an NH proton of the His imidazole and the nearest phenyl ring carbon). However, when this structure was subjected to a molecular dynamics run, as outlined above, all the backbone hydrogen bonds were conserved and those factors that stabilize RN-24 were reinstated. Features of the simulation that led to that conclusion are as follows. First, conformers having the Glu^--2 to Arg^+-10 salt bridge predominated. Second, and most interesting, the protonated His-12 side chain tended to adopt conformations in which the imidazole interacted with both phenyl rings of the FiFi residue, one in a perpendicular edge-to-face orientation (3.5 Å separation) and the other in a parallel face-to-face arrangement (3.5 Å separation). The edge-to-face orientation is quite similar to that found in the *S*-peptide crystal structure. Overall, the conformations that prevail toward the end of the simulation (150−300 ps) are quite helical (Figures 1d and 1e), the phenyl rings of the FiFi residue being almost perpendicular to the helix axis. Finally, a Glu^-2 to Lys^+-7 electrostatic interaction (not present in RN-24) was also observed.

The protocol used to simulate the conformational biases of the (*S*,*S*)-FiFi isomer **10** was identical to that used for its diastereomer **9**, but the results were very different. After minimization, the helical conformation of this molecule is distorted (Figure 1f). There is no salt bridge between Glu^--2 and Arg^+-10 in the starting structure. One of the FiFi phenyl groups appears to interact with the protonated His-12 (2.6 Å separation), but the angle between these two groups is nonideal for a perpendicular or a face-to-face orientation. In fact, the FiFi phenyl groups are approximately aligned with the helical twist. This observation contrasts with the proposed conformational bias of peptidomimetic **9**, wherein the two phenyl rings are perpendicular to the helix. Overall, the region of the helix around the FiFi residue is crowded, implying that the molecule would relax away from this conformation if the energy were

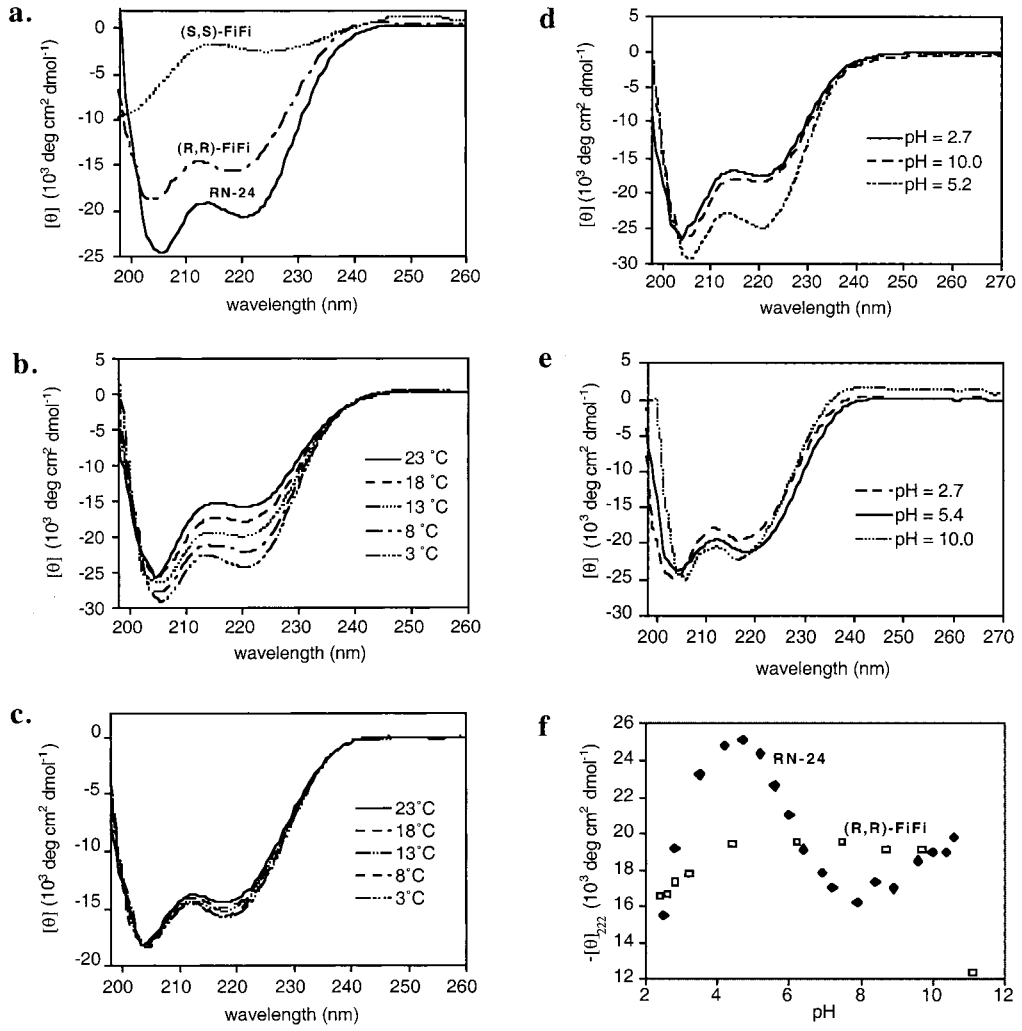

Figure 2. CD spectra of samples in pH 5.2 phosphate/citrate/borate buffer. (a) RNase A, **9**, and **10**; (b) RN-24 at various temperatures; (c) **9** at various temperatures; (d) RN-24 at various pH's; (e) **9** at various pH's; (f) the molar ellipticity of RN-24 and **9** at 222 nm as a function of pH.

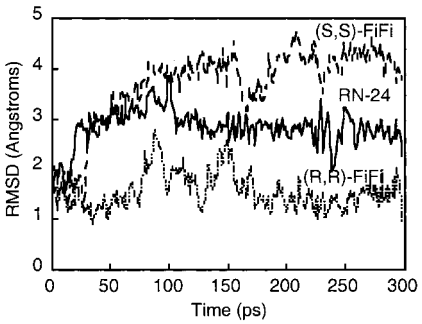

Figure 3. Deviation of the N, Cα, C, and O backbone atoms of conformers from their respective starting structures directly after the initial molecular mechanics minimization.

available to it. Indeed, upon molecular dynamics simulations (276 K for 300 ps), the helical conformation is lost in all the latter conformations sampled. The conformers sampled in the latter half of this simulation tended to have the protonated His^+-12 side chain and the FiFi phenyl rings on opposite sides of the structure (e.g., Figure 1g).

Figure 3 shows the root-mean-square (RMS) deviation of the backbone atoms (N, Cα, C, and O) of sampled conformers from their respective starting structures directly after the initial molecular mechanics minimization. Overall, peptidomimetic **9** shows less variance than RN-24 from its starting conformer, whereas compound **10** undergoes a more drastic relaxation.

Conclusions

Difficulties were encountered in our previous attempts to study conformational biases imposed by cyclopropane amino acids. Conformational ensembles rather than distinctly preferred conformers were generated when small linear tetrapeptides were used as model systems. These were difficult to characterize since so few long-range rotating-frame Overhauser enhancement (ROE) cross-peaks were observed in NMR studies. Conversely, incorporation of cyclopropane amino acids into model cyclic peptides gave more readily identified conformational biases, but these were clearly a compromise between the effects of the unusual amino acid and the cyclic structure. Neither approach was therefore ideal.

The model system described in this paper is large enough to have a recognizable secondary structure, but not so rigid that the effects of cyclopropane amino acid substitutions are negligible. It is a useful compromise between the short linear peptide and the cyclic system that have been studied previously in these labs. The synthesis and application of FiFi are also innovations, insofar as large steric influences on the ϕ and ψ bond vectors can be probed simultaneously in this disubstituted system. The CD data described indicates that the RN-24 derivative from (*R*,*R*)-FiFi, compound **9**, is helical; this conformation was prevalent in molecular simulations of that molecule, lending support to that hypothesis. Moreover, and perhaps most interesting of all, the stability of the helical

conformation for **9** is less perturbed by temperature increases and by pH changes than that of RN-24. We infer that this greater stability can be attributed to the enhanced conformational rigidity imparted by FiFi. Conversely, the effect of the (*S*,*S*)-isomer of FiFi was large enough to override the helical preference of the RN-24 sequence. We conclude this isomer is not conducive to right-handed helical secondary structures but would enhance populations of left-handed helices in an appropriate sequence (i.e., one formed predominantly from D-amino acids).

Experimental Section

General Procedures. Melting points were uncorrected. High-field NMR spectra were recorded on a Unity+ 300 spectrometer (^{1}H at 300 MHz, ^{13}C at 75 MHz); ^{1}H chemical shifts are reported in δ relative to C*H*Cl$_3$ (7.24 ppm) as internal standard, and ^{13}C chemical shifts are reported in ppm relative to C*D*Cl$_3$ (77.0 ppm) unless otherwise specified. Multiplicities in ^{1}H NMR are reported as (br) broad, (s) singlet, (d) doublet, (t) triplet, (q) quartet, and (m) multiplet. Thin-layer chromatography was performed on silica gel 60 F_{254} plates from Whatman. Flash chromatography was performed on SP silica gel 60 (230−600-mesh ASTM). *tert*-Butyl alcohol was distilled from CaH_2; dimethoxyethane (DME) was distilled from a sodium and benzophenone ketyl. Other chemicals were purchased from commercial suppliers and used as received.

(*1R*,*2R*)-1,2-Diphenyl-1,2-ethanediol (4). Prepared from *trans*-1,2-diphenylethene according to method of Sharpless et al.[47] the yield after one recrystallization was 80%, *ee* >99% [HPLC chiral column (*S*,*S*)-Whelk-O1, Regis Technologies]. Spectroscopic data for **1**:^{1}H NMR (200 MHz, $CDCl_3$) δ 2.91 (s, 2H), 4.69 (s, 2H), 7.08−7.25 (m, 10H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 79.09, 126.92, 127.92, 128.12, 139.81; mp 146−147 °C.

The (*S*,*S*)-enantiomer was prepared in an identical fashion; the yield after one recrystallization was 84%, *ee* >98%.

(*4R*,*5R*)-4,5-Diphenyl-(1,3,2)-dioxathiolane-(2,2)-dioxide (1). A 250-mL two-neck round-bottom flask was fitted with a reflux condenser carrying a drying tube with an HCl trap, and the sidearm was stoppered. 1,2-Diphenyl-1,2-ethanediol **4** (23.3 mmol, 5 g, 1 equiv) and CCl_4 (25 mL) were placed in the flask, then thionyl chloride (1.2 equiv, 2.1 mL) was added via syringe over ∼10 min. The resulting mixture was refluxed for 30 min. The solution was cooled in an ice−water bath and then diluted with acetonitrile (25 mL). Solid $NaIO_4$ (7.5 g, 1.5 equiv) and $RuCl_3$·H_2O (4 mg, 0.001 equiv) were added, followed by H_2O (35 mL), and the resulting biphasic mixture was rapidly stirred at room temperature for 60 min. The mixture was then diluted with diethyl ether (Et_2O) (200 mL), and the phases were separated. After the organic layer was washed with H_2O (10 mL), saturated aqueous $NaHCO_3$ (2 × 10 mL), and brine (10 mL) and was dried over $MgSO_4$, the solution was filtered through a small pad of silica to remove the dark color, concentrated, and dried under a high vacuum to give 5.2 g (81%) of **1** as white crystals. This product decomposes at room temperature but is stable for several months in the freezer. The crude product[49] was used without further purification: ^{1}H NMR (200 MHz, $CDCl_3$) δ 5.76 (s, 2H), 7.29−7.34 (m, 5H), 7.41−7.51 (m, 5H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 89.72. 127.15, 129.19, 130.60.

The enantiomer of this product, (*S*,*S*)-4,5-diphenyl-(1,3,2)-dioxathiolane-(2,2)-dioxide (ent-1), was prepared by an identical procedure, starting with the enantiomeric diol to give 11 g of the product (81%).

(2′*R*,3′*R*)-[1-(2′,3′-Diphenyl)-cyclopropyl] diethyl glutaconate (5). To a 250-mL two-neck round-bottom flask equipped with a reflux condenser, N_2 inlet, and a magnetic stirrer was added NaH (2.5 equiv, 26.7 mmol, 0.304 g). The sidearm was stoppered and freshly distilled DME was added by syringe. Diethyl glutaconate (1.1 equiv, 10.7 mmol, 2.19 g) was added dropwise to the NaH suspension. (4*R*,5*R*)-4,5-diphenyl-(1,3,2)-dioxathiolane-(2,2)-dioxide, **1** (1 equiv, 2.95 g), was dissolved in 50 mL of DME and added dropwise to the anion mixture. The resulting orange solution was stirred at room temperature for 2 h. The solution was diluted with saturated aqueous NH_4Cl (60 mL) and extracted with ethyl acetate (EtOAc) (2 × 200 mL). The combined organics were dried over Na_2SO_4, filtered, evaporated, and dried. The crude material was purified by silica gel chromatography using 10% EtOAc/hexanes as eluent, R_f = 0.60, collected, and dried to give 2.8 g (72%) of **5** as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.92 (t, 3H, J = 7.2 Hz), 1.22 (t, 3H, J = 7.2 Hz), 3.36 (d, 1H, J = 8.4 Hz), 3.92 (m, 3H), 4.06 (m, 2H), 5.87 (d, 1H, J = 15.9 Hz), 6.77 (d, 1H, J = 15.9 Hz), 7.28−7.35 (bm, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 13.77, 14.15, 37.23, 39.50, 40.79, 60.23, 61.23, 121.38, 130.80, 127.39, 127.42, 128.22, 128.54, 128.90, 129.33, 134.89, 135.10, 143.91, 166.18, 168.48. IR (CH_2Cl_2) 1684, 1602, 1228 cm^{-1}. HRFABH+: calcd 365.1753, found 365.1738. $[\alpha]_D$ +69.7, (c =1.4, $CHCl_3$). Anal. Calcd for $C_{23}H_{24}O_4$: C, 75.79; H, 6.64; O, 17.57. Found: C, 74.93; H, 6.53.

(2′*S*,3′*S*)-[1-(2′,3′-Diphenyl)-cyclopropyl] diethyl glutaconate was prepared in the same way as **5**, affording 3.3 g of a yellow oil (62%). $[\alpha]_D$ = −78.7, (c = 1.2 $CHCl_3$).

(2*R*,3*R*)-1-Carboxy-1-(ethyloxycarbonyl)-2,3-diphenyl cyclopropane (6). To a rapidly stirred mixture of compound **5** (7.72 mmol, 1 equiv, 2.80 g) and $NaIO_4$ (61.7 mmol, 8 equiv) in acetonitrile and CCl_4 (15 mL each) and H_2O (23 mL) was added $RuCl_3$·H_2O (0.154 mmol, 0.02 equiv). The resulting slurry was stirred at room temperature for 15 h. The mixture was filtered, and the filtrate was washed with H_2O and CH_2Cl_2. The aqueous layer was extracted with CH_2Cl_2 (2 × 150 mL). The combined organics were dried ($MgSO_4$), filtered, and evaporated to give **6**, 1.6 g (70%), as a brown oil. The crude acid was used in the next step without further purification. A small amount of **6** was purified by silica gel chromatography (25−40% EtOAc/hexanes) to give the following analytical data: ^{1}H NMR (300 MHz, D_6-acetone) δ 0.93 (t, 3H, J = 7.2 Hz), 3.77 (q, 2H, J = 6.3, 8.4 Hz), 3.91−4.00 (m, 2H), 7.31−7.44 (m, 10H); ^{13}C NMR (75 MHz, D_6-acetone) δ 13.21, 34.10, 34.21, 60.82, 127.12, 121.16, 128.12, 128.69, 128.77, 135.139, 166.66; IR (CH_2Cl_2) 1700, 1265 cm^{-1}. HRFABH+: calcd 311.1283, found 311.1302. (c = 1.04, $CHCl_3$). Anal. Calcd for $C_{19}H_{18}O_4$: C, 73.52; H, 5.85; O, 20.63. Found: C, 72.71; H, 5.92.

(2*S*,3*S*)-1-Carboxy-1-(ethyloxycarbonyl)-2,3-diphenyl cyclopropane was prepared the same way as **6**, providing 5.1 g of a brown oil (67%).

(2*R*,3*R*)-1-[*N*-[(*tert*-Butyloxycarbonyl)]amino]2,3-diphenyl-cyclopropane-1-carboxylic Acid (7). Compound **6** (4.19 mmol, 1 equiv), triethylamine (5.03 mmol, 1.2 equiv), and dry *tert*-butyl alcohol (60 mL) were added to a 200-mL two-neck round-bottom flask with reflux condenser and a N_2 inlet. Diphenylphosphoryl azide (9.22 mmol, 2.2 equiv) was added, and the resulting mixture was refluxed for 15 h. The solution was concentrated and the crude material was extracted with EtOAc (2 × 200 mL), washed with H_2O, brine (1 × 20 mL each), and dried over Na_2SO_4. After evaporation of the solvent, the crude residue was purified via flash chromatography (10−25% EtOAc/hexanes to give 0.89 g of white crystals (55%): R_f = 0.55 (20% EtOAc/hexanes); ^{1}H NMR (30 MHz, D_6-acetone) δ 0.90 (t, 3H, J = 7.0 Hz), 1.33 (s, 9H), 3.39 (d, 1H, J = 8.7 Hz), 3.74 (d, 1H, J = 8.7 Hz), 3.84 (dq, 2H, J = 1.8, 5.1 Hz), 6.68 (s, 1H), 7.27−7.52 (m, 10H); ^{13}C NMR (75 MHz, D_6-acetone) δ 14.20, 14.43, 27.96, 28.36, 35.41, 35.81, 37.78, 38.04, 48.01, 61.05, 61.22, 79.01, 79.29, 127.29, 127.44, 127.61, 128.61, 128.70, 129.50, 129.87, 130.02, 136.59, 136.90, 156.46, 169.51. IR (CH_2Cl_2) 3425, 1724, 1269, cm^{-1}. HRFABH+ calcd: 382.2018, found: 382.2026. $[\alpha]_D$ = +56.41 (c = 1.01, $CHCl_3$). Mp = 88−89 °C. Anal. Calcd for $C_{23}H_{27}NO_4$: C, 72.4; H, 7.14; N, 3.67; O, 16.78. Found: C, 72.5; H, 7.13; N, 3.71.

The enantiomeric product, (2*S*,3*S*)-ethyl-1-[*N*-[(*tert*-butyloxycarbonyl)]amino]-2,3-diphenyl cyclopropane-1-carboxylate, was prepared by a procedure identical to that described above, with ent-6 as substrate (50%). $[\alpha]_D$ = −60.2, (c = 1.28, $CHCl_3$).

In a 150-mL round-bottom flask equipped with a reflux condenser and stir bar, (2*R*,3*R*)-ethyl-1-[*N*-[(*tert*-butyloxycarbonyl)]amino]-2,3-diphenyl cyclopropane-1-carboxylate (1.83 mmol, 1 equiv) was mixed with NaOH (5.49 mmol, 3 equiv) and aqueous methanol (50 mL) and refluxed for 48 h. The methanol was evaporated and the residue was extracted with Et_2O, 100 mL. The aqueous was acidified to pH∼2 with 1M HCl and extracted with EtOAc (3 × 150 mL). The combined organic layers were washed with H_2O and brine (1 × 20 mL, each) and dried (Na_2SO_4). After evaporation of the solvent, the crude residue was purified via flash chromatography (20−40% EtOAc/hexane, R_f =

0.23), collected, and dried to give 0.6 g (98%) of BOC−FiFi **7** as a white solid: ^{1}H NMR (300 MHz) δ 1.18 (s, 3H), 1.31 (s, 6H), 3.38−3.33 (m, 1H), 3.72 (d,1H, J = 8.7 Hz), 6.67 (s, 1H), 7.22−7.53 (bm, 10H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 27.74, 28.15, 35.32, 35.60, 37.80, 38.07, 47.76, 78.77, 79.14, 127.01, 127.17, 127.31, 128.39, 128.43, 129.23, 129.74. 129.82, 136.51, 136.51, 136.66, 155.29, 156.29, 170.47. IR (CH_2Cl_2) 3426, 1696, 1264 cm^{-1}. HRFABH+ calcd: 354.1705, found: 354.1696. $[\alpha]_D = +70.1$ (c = 1.03, $CHCl_3$). Anal. Calcd for $C_{21}H_{23}NO_4$: C, 71.4; H, 6.56; N, 3.97. Found: C, 71.3; H, 6.58 N, 3.93.

(2*S*,3*S*)1-[*N*-[(*tert*-Butyloxycarbonyl)]amino]2,3-diphenyl-cyclopropane-1-carboxylic acid was prepared by a procedure identical to that as described above but using the (*S*,*S*)-BOC amino ester as substrate (95%). $[\alpha]_D = -66.8$ (c = 1.28, $CHCl_3$).

(2*R*,3*R*)-1-[*N*-[(9-Fluorenylmethoxy)carbonyl]amino]-2,3-diphenyl-cyclopropane-1-carboxylic Acid (8). A 1:1 mixture of TFA/CCl_4 at 0 °C was added dropwise to 0.5 g of **7**. This mixture was stirred at 25 °C for 30 min, concentrated, neutralized with 2 M NaOH, and lyophilized to dryness. The crude amine was then dissolved in a mixture of 1 M Na_2CO_3 (5.1 mL), *N*,*N*-dimethylformamide (DMF) (7 mL), and H_2O (1.8 mL) and cooled to 0 °C. FMOC−OSu was added in one portion and the mixture was stirred for 8 h at 25 °C. The mixture was then diluted with H_2O (80 mL) and washed with Et_2O (40 mL 5). The aqueous layer was acidified to pH ∼2 with citric acid (4.5 g) and extracted with EtOAc (150 mL). The organic layer was washed with H_2O and brine and then was dried over $MgSO_4$. After evaporation of the solvent, the crude residue was purified via flash chromatography with 10−40% EtOAc/hexanes eluent to obtain 0.42 g (62%) of the target molecule **8** as a white solid: R_f = 0.29 (40% EtOAc/hexanes); ^{1}H NMR (300 MHz) δ 3.45 (d, 1H, J = 8.7 Hz), 3.73 (d, 1H, J = 8.4 Hz), 4.00−4.31 (3H, m), 7.14−7.84 (m, 18 H), 7.99 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 35.04, 37.73, 47.05, 66.15, 78.32, 119.85, 125.29, 125.37, 126.81, 127.02, 127.57, 127.62, 127.88, 128.00, 128.69, 129.02, 129.32, 135.56, 135.94, 141.13, 143.90, 144.28, 156.50, 169.29, 169.73. IR (CH_2Cl_2) 3416, 1736, 1268 cm^{-1}. HRFABH+ calcd: 476.1862, found: 476.1869. $[\alpha]_D = +47.1$, (c = 0.73, $CHCl_3$).

(2*S*,3*S*)1-[*N*-[(9-Fluorenylmethoxy)carbonyl]amino]-2,3-diphenyl-cyclopropane-1-carboxylic acid was prepared in an identical fashion to **8** but with ent-7 as substrate and yielded 0.63 g (48%) of product as a white solid. $[\alpha]_D = -46.4$, (c = 0.74, $CHCl_3$).

Succ-Ala-Glu-Thr-Ala-Ala-Ala-Lys-Phe-Leu-Arg-Ala-His-Ala-NH$_2$, (Peptide RN-24). Stepwise couplings of FMOC-amino acid derivatives on Rink's amide resin (Advanced Chemtech) were used to prepare the 13-residue peptide. Manual peptide synthesis was carried out in a 30-mL vessel fitted with a coarse glass frit and using a wrist-action shaker (Burrel, model 75). The reagents were added manually. All reactions were carried out at 25 °C unless otherwise specified. FMOC deprotection was performed by shaking the resin twice with 20% piperidine in DMF (5 mL for 3 min and 5 mL for 7 min); DMF washing cycles (10 × 1 min, ∼10 mL) were performed after each coupling and deprotection. The coupling conditions were as follows. Each FMOC-amino acid coupling was accomplished by premixing the amino acid (2 equiv) with 4-methylmorpholine (NMM) (3 equiv), HOBt (2 equiv), and PyBOP (2 equiv) in DMF (7 mL); this solution was added to the resin (200 mg, 1 equiv, 0.6 mmol/g loading) and the mixture was shaken for 1 h. A negative ninhydrin test result was observed after this stage. Deprotection of the side chains, cleavage from the resin, and purification of the peptide were performed as follows. A mixture of phenol (1 mL), 1,2-ethanedithiol (0.5 mL), thioanisole (1 mL), deionized water (1 mL), and TFA (16.5 mL) was cooled to 0 °C and added to the crude peptide. The reaction mixture was stirred for 12 h at 25 °C and then concentrated to dryness. Et_2O (30 mL) was added to precipitate the product, after which the ethereal solution was decanted away from the solid residue. The crude peptide was further purified by preparative reversed-phase HPLC (Vydac C_{18} column, 22 mm × 25 cm, 10 mm) with a linear gradient obtained by mixing solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile). The gradient was programmed to increase from 5% to 20% B over 60 min with a flow rate of 6 mL min^{-1}. The desired peptide RN-24 was obtained as a white powder (13 mg, 7%). MALDI-TOF [M + H_2O] calcd: 1455.73, found: 1455.91. Amino acid analysis revealed Ala:Thr of 19.716:3.310 (6:1).

Succ-Ala-Glu-Thr-Ala-Ala-Ala-Lys-[(*R*,*R*)FiFi]-Leu-Arg-Ala-His-Ala-NH$_2$ (Peptidomimetic 9). This peptide was synthesized by a protocol similar to the one used for RN-24 except that FMOC−(*R*,*R*)-FiFi-OH was substituted for FMOC−Phe in the sequence. Additionally, to incorporate this hindered amino acid, the coupling conditions for this and the next residue were slightly modified. The acid fluoride of FiFi was prepared in situ by mixing FMOC−FiFi (1.1 equiv) with TFFH (2 equiv) EtN^iPr_2 (2 equiv), HOAt (2 equiv), and DMF for 7 min, then adding to the resin. The coupling was complete in 1 h (negative ninhydrin test result). The next residue, Lys-7, was also coupled by using TFFH, diisopropylethylamine (DIEA), and HOAt (1:2:2:2 equiv) for 12 h at 25 °C. The remainder of the peptide was prepared by using the standard conditions outlined above. The desired peptide was collected and dried to give **9** (6 mg, 3%) as a white powder. MALDI-TOF [M + H_2O] calcd: 1543.76, found: 1543.90. Amino acid analysis revealed Ala:Thr of 38.946:6.124 (6:1).

Succ-Ala-Glu-Thr-Ala-Ala-Ala-Lys-[(*S*,*S*)FiFi]-Leu-Arg-Ala-His-Ala-NH$_2$, (Peptidomimetic 10). Synthesis of this analogue was performed by a protocol identical to that for **9** except that FMOC−(*S*,*S*)-FiFi-OH (1.1 equiv) was used for the coupling at the eighth position. The desired peptide was collected and dried to give **8** as a white powder (5 mg, 3%). MALDI-TOF [M + H_2O] calcd: 1543.76 found: 1543.90. Amino acid analysis revealed Ala:Thr of 7.433:1.058 (7:1).

Molecular Modeling. A Silicon Graphics IRIX−O_2 workstation was used for the molecular simulations performed in this work. All calculations were performed with QUANTA97/CHARMm version 23.2 software (Molecular Simulations Inc.) with extended representations of the nonpolar hydrogen atoms.

Since CHARMm does not have parameters for the cyclopropane amino acids, additional atom types were assigned and a parameter set was built, based on crystallographic data and CHARMm default parameters, and then was appended to the CHARMm standard parameter file. The residue topology files (RTFs) of the two FiFi amino acids were built according to the standard geometry of these two unnatural amino acids and then appended to the CHARMm standard RTF. The combined CHARMm parameters and RTFs were imported to QUANTA/CHARMm for the following calculations.

Initial coordinates for RN-24 and the FiFi derivatives were obtained from the X-ray structures of ribonuclease A (Protein Data Bank entry 1RTB.[11] The first 13 residues were selected and then modified to form the structure of RN-24 and the (*R*,*R*)-FiFi and (*S*,*S*)-FiFi peptidomimetics. These initial structures were first minimized by 500 steepest decent cycles followed by conjugate gradient minimization until the RMS energy deviation was <0.01 kcal mol^{-1} Å^{-1}. The resulting conformations were used as the starting structures in the following molecular dynamics simulations.

Free dynamics simulations were performed on the three peptidomimetics at 276 K. The Verlet algorithm was used during the dynamics, with a time step of 1 fs and SHAKE to constrain all bond lengths containing polar hydrogens. A dielectric continuum with ϵ = 80 was used to model the aqueous environment. A nonbonded pair list was generated by using a 12-Å cutoff distance and was updated every 10 steps. For each peptide, the system was heated from 150 to 276 K over 5 ps; assignment of the starting random velocities was according to a Gaussian distribution. The resulting structure was equilibrated at 276 K for an additional 5 ps. The molecular dynamics calculations were then performed for a total time of 300 ps, with coordinates, velocities, and energies saved every 1 ps for further analysis.

CD Studies. CD measurements were obtained on an Aviv (model 62DS) spectrometer. The peptide/peptidomimetics were dissolved in a buffer solution formed from 1 mM each of sodium phosphate, sodium citrate, and boric acid. The pH of the samples was adjusted at room temperature with HCl or NaOH. Peptide concentrations were determined by UV absorption assays[57] and by quantitative amino acid analysis. The concentration of peptide/peptidomimetics studied was in the range of 2−13 mM. The data presented represent an average of 4 scans per sample with a time constant of 2 s, bandwidth of 1 nm, and sampling every 0.5 nm from 260 to 195 nm. For temperature-

dependence studies, the samples were equilibrated for at least 10 min before data acquisition. Likewise, for the pH-dependence experiments conducted at 3 °C, the samples were allowed to equilibrate for at least 10 min prior to measurement of θ_{222}.

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Supporting Information Available: Comparison of peptide concentration determination by UV and AAA and parameter sets and topology files for FiFi (7 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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