Distance Distributions of End-Labeled Curved Bispeptide Oligomers by Electron Spin Resonance

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ABSTRACT We demonstrate the synthesis of a series of spin-labeled curved oligomers to determine their end-to-end lengths and distance distributions using electron spin resonance. We synthesize shape-persistent macromolecules from conformationally restricted, asymmetric monomers that are coupled through pairs of amide bonds to create water-soluble, spiro-ladder oligomers with well-defined three-dimensional structures. We synthesized seven different macromolecules, each containing eight monomers but differing in the sequence to create macromolecules with different curved shapes. The ends of the oligomers were labeled with nitroxide spin probes, and double electron—electron resonance (DEER) electron spin resonance (ESR) experiments were carried out to obtain quantitative information about the shapes and flexibility of the oligomers. The most probable end-to-end distance of the oligomers ranges from 23 to 36 Å, a range of length that we previously accessed by assembling rod-like homo-oligomers that contain 4—8 bisamino acid monomers. The relative distances measured for the oligomers confirm that, by varying the sequence of an oligomer, we are able to control its shape. The shapes of the ESR-derived population distributions allow us to compare the degree of shape persistence and flexibility of spiro-ladder oligomers to other well-studied nanoscale molecular structures such as *p*-phenylethynylenes.

KEYWORDS: bispeptides · nanostructures · EPR spectroscopy · curved nanostructures · organic nanostructures · spin probes · molecular flexibility

In this paper, we describe the construction of a series of constitutionally defined, nanometer-scale macromolecules with different programmed curved shapes and the use of DEER-ESR experiment to measure their end-to-end lengths and flexibility. The synthesis of ever larger and more complex molecules with designed shapes and properties is an overarching goal of synthetic chemistry. Oligomer synthesis is an efficient approach to macromolecules because it is modular and allows the rapid assembly of large structures from a collection of small monomers. Nature uses this approach in the construction of proteins, DNA, and RNA. Many groups have developed artificial monomers that are assembled through single bonds to form oligomers.[1,2] Some of these oligomers exhibit strong tendencies to form well-defined secondary structures through the influence of weak noncovalent interactions.[1,3−6] The development of a systematic approach to oligomers with designed tertiary structures is still elusive[7] because of the immense complexity involved in predicting the folded structure of molecules with large numbers of rotatable bonds.[8]

We have developed an alternative approach to shape-persistent macromolecules in which we use organic synthesis to create conformationally restrained, stereochemically pure, asymmetric monomers and couple them through pairs of amide bonds to create spiro-ladder oligomers that adopt well-defined three-dimensional structures defined by the monomer structure, stereochemistry, and their sequence within each oligomer.[9−13] In an application of this work, we have recently used bispeptide-based molecular rods as shape-persistent components within a metal-activated molecular actuator.[14] In future work, we intend to develop these actuators as molecular sensors and as active components of nanoscale valves. Toward this and other applications, we will need to create heterosequences of bispeptides that modeling suggests will form curved and crescent shaped structures and so we need to determine the degree of shape control that we have in the design of these molecules and the flexibility of these nanostructures.

This work builds on recent interest in macromolecules that form rod-like structures to act as structural elements and hold groups at defined distances relative to each other.[15,16] Constitutionally defined, conjugated rod-like molecules have been studied as mimics of their high molecular weight polymeric analogues as well as for their own

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unique electronic and photoelectronic properties.[17−19] Rod-like molecules have also been used as structural elements; for example, *p*-oligophenyl rigid-rod molecules have been used as scaffolding to create multifunctional pores in membranes to mimic biological channels.[20] While there are many examples of rod-like molecules, there are far fewer examples of curved and crescent shaped macromolecules. One example is the crescent oligoamides developed by Gong and co-workers.[21,22] These molecules consist of aromatic monomers connected through amide bonds, and the backbone is stabilized by three-center hydrogen bonding. Another example is the curved "molecular apple peels" of Huc and co-workers.[23] A third example are short *m*-phenylethynylenes which have a curved structure, and longer sequences fold to form helical structures displaying an 8 Å cavity in polar solvents.[24,25]

The DEER experiment provides a natural spectroscopic method to rapidly measure distance distributions in nanostructures. The DEER experiment has been used to measure distance distributions between pairs of spin labels in proteins,[26,27] peptides,[28] ribonucleic acids,[29] nanostructured materials,[30,31] and three-armed phenylethynylene compounds.[27] We have previously used the DEER experiment to measure the flexibility and lengths of rod-like bispeptide nanostructures containing different numbers of bisamino acid monomers.[13] We went on to use these data to develop a simplified approach to modeling rod-like bispeptides.[32]

We have previously described the synthesis of two enantiomeric bisamino acid monomers **1** and **2**, their assembly into short bispeptide spiro-ladder oligomers, and the determination of the structures of these oligomers using NMR.[9,12] The NMR structures allowed us to determine the preferred conformations of the monomers and their relative orientations to one another within the context of heterosequences. This information was then incorporated into our software package CANDO (computer aided nanostructure design and optimization), for use in designing new sequences. CANDO constructs three-dimensional models of oligomers using a database of residue conformations and matrices that rotate and translate residues in three-dimensional space relative to each other in order to create chemically reasonable "strain-free" oligomer conformations. This approach to piece-wise modeling of large oligomers with complex sequences is imperfect because it builds on structural predictions from AMBER95,[33] a molecular mechanics force-field parametrized for proteins and nucleic acids. This approach does not account for the flexibility of the oligomers and small errors in the preferred conformation of each monomer accumulate and lead to larger errors in the end-to-end distances across long oligomers. We are, therefore, taking a combined experimental and theoretical approach to improve our ability to predict bispeptide structures. We experimentally determine

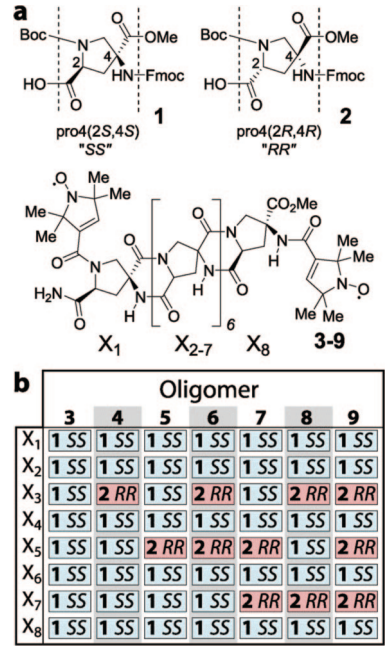


Figure 1. (a) Structure of the two residues pro4(2*S*,4*S*) "*SS*"[34] and pro4(2*R*,4*R*) "*RR*" in their fully protected monomer forms 1 and 2, respectively, and the chemical structures of the double spin probe labeled oligomers 3−9. (b) Sequences of monomers 1 *SS* and 2 *RR* used to assemble oligomers 3−9.

the preferred shapes of long bispeptide oligomers and use this information to develop new force-field parameters for modeling bispeptides.[13,32] Modeling of octameric oligomers assembled from different sequences of monomers **1** and **2** using CANDO led us to hypothesize that we could create a collection of shape-persistent macromolecules with different curved shapes. To test this hypothesis, we used CANDO to design a series of octamers with curved shapes that would hold their two ends at different distances with respect to each other. We then used the measurement of end-to-end distance by ESR to provide a simple assay of shape and design principles. These distance distributions will be used in the future to improve our modeling of curved bispeptide nanostructures.

RESULTS AND DISCUSSION

The Design of Curved Bispeptide Sequences. We had previously synthesized oligomer **3** (Figures 1b and 2), which is a homosequence consisting of eight pro4(2*S*,4*S*) ("*SS*") residues.[13] Modeling by CANDO suggests that oligomer **3** would form a left-handed helical molecular rod, the longest oligomer of the collection. When an *SS* residue follows another *SS* residue ("*SS-SS*"), each bicyclic diketopiperazine/pyrrolidine unit is rotated by approximately 90° with respect to the preceding unit due to being joined through a quaternary center; therefore, an unbroken chain of *SS* units creates a helix. Modeling suggested that the two-residue sequence "*SS-RR*" will introduce a kink into the structure (see Supporting Information for an illustration). On the basis of this mod-

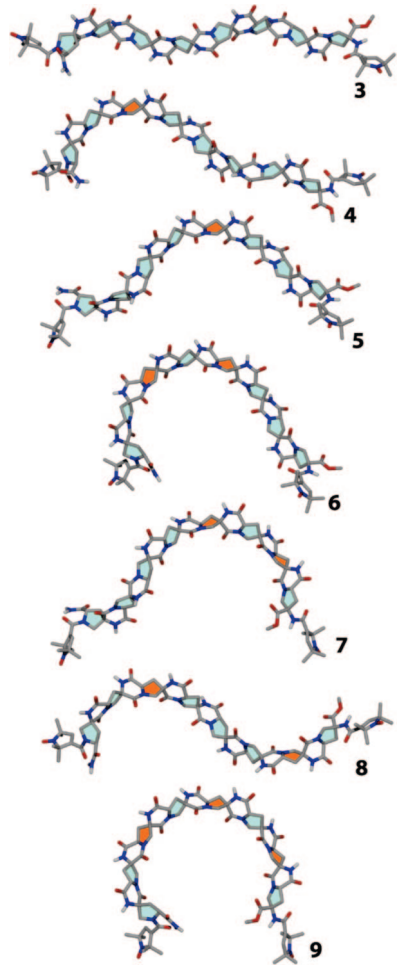


Figure 2. CANDO modeled shapes of bis-spin probe labeled oligomers 3−9. Hydrogens on carbon have been removed for clarity. The pro4(2*S*,4*S*) and pro4(2*R*,4*R*) pyrrolidine rings are filled with cyan and red, respectively.

eling, we designed oligomers **4** and **5**, hypothesizing that the inserted *SS-RR* sequence would bend the ends of the oligomer in toward each other and bring the spin probes closer together than in **3**. Modeling suggested that an "*SS-RR-SS-RR*" sequence would curve even more strongly, and so we designed oligomers **6** and **7**. We designed oligomer **8** to test if we could create an oligomer shaped like the letter "S". Oligomer **8** would have two *SS-RR* curves separated by an *SS-SS* sequence. The intervening *SS-SS* sequence would rotate the second *SS-RR* curve approximately 180° with respect to the first. These two curves would not curve in the same direction as in **6** and **7**, and so oligomer **8** should hold its two ends further apart than **6** and **7**. Finally, we designed oligomer **9** containing the sequence "*SS-RR-SS-RR-SS-RR*", which modeling suggested would have the most strongly curved structure and hold its two ends closer to each other than any of the other oligomers.

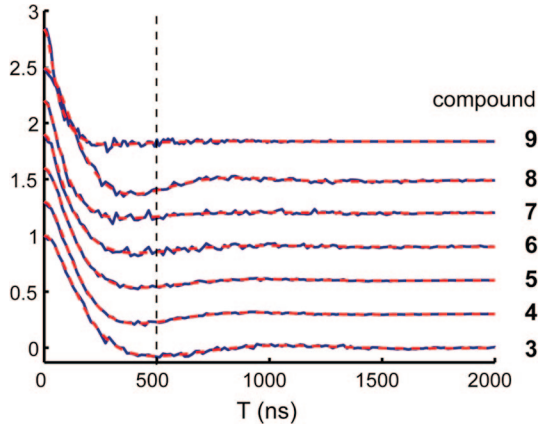
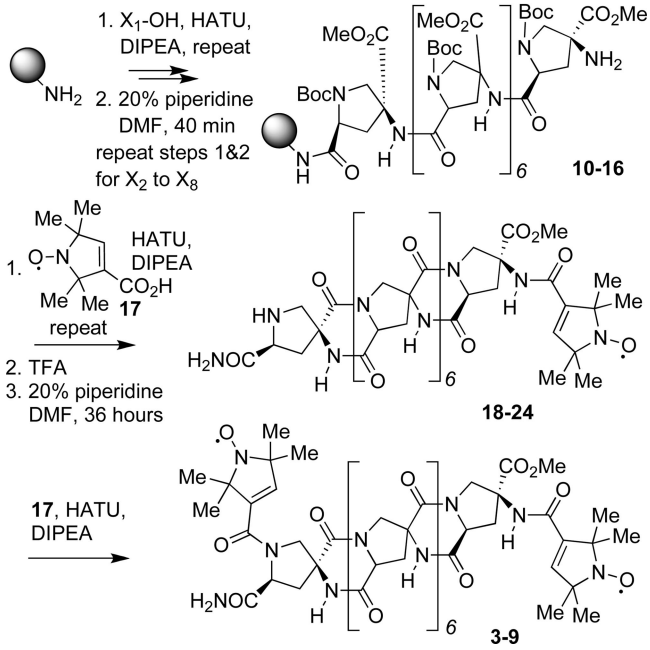


Figure 3. Time domain DEER experimental data (black) and data fitting (dashed red); the vertical dash line at 500 ns is for reference.

By analogy, we use CANDO to produce "turn-by-turn driving instructions" in three dimensions. From a common starting point (the first spin probe of each oligomer), each monomer moves forward a distance X_i angstroms, rotates around the direction of movement by Y_i degrees, turns right by Z_i degrees, and then repeats this process for seven additional monomers with different X_i, Y_i, and Z_i values for each monomer. The initial CANDO values for X_i, Y_i, and Z_i from modeling are approximate, but they suggest that the other end of each oligomer will end up at different distances from the starting point. We then synthesize each oligomer end-labeled with two POAC spin labels and measure the end-to-end distances and population distributions of the oligomers. We will then use these data to develop better X'_i, Y'_i, and Z'_i values to improve bispeptide modeling.

Bispeptide Synthesis. We synthesized oligomers **3**−**9** with the monomer sequences shown (Figure 1b) and labeled each end of each oligomer with nitroxides.[13] The monomers **1** and **2** were synthesized according to previously described procedures, and their absolute stereochemical purity was confirmed using optical rotation and by derivatizing each enantiomer with (*S*)- and (*R*)-methylbenzylamine to produce diastereomers that are separable by C_{18} reverse phase high performance liquid chromatography (RP-HPLC).[9,12] Each of the syntheses of compounds **3**−**9** were carried out on solid support following previously described procedures on 5 mg of Rink amide resin (Scheme 1). While still on solid support, the terminal free amine of each intermediate **10**−**16** was acylated with the spin probe 2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid **17** activated with *O*-(7-azabenzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU). The flexible oligomers were then cleaved from the resin, and the Boc group was removed using trifluoroacetic acid (TFA). Intramolecular aminolysis between the secondary amine of each monomer and the methyl ester of the monomer preceding it was catalyzed using 20% piperidine in dimethylformamide at room temperature for 36 h to form the spin-labeled spiro-ladder oligomers **18**−**24**. Finally, a second spin label **17** was coupled to the remaining free secondary amine in solution, and the



Scheme 1. Synthesis of bispeptides 3−9.

products **3**−**9** were purified using C_{18} RP-HPLC, and their masses (all identical, 1485.6 Daltons) were confirmed by mass spectrometry. The mass spectrometry suggested that some disproportionation of the nitroxides to hydroxylamines and oxoammonium cations had occurred,[35] but this does not effect our results because the DEER experiment selects the molecules with two intact nitroxides.

ESR-DEER Experiments. For ESR experiments, ~0.2 mM of the double labeled molecules were prepared in 70% buffer (50 mM phosphate buffer, pH 7.4, 200 mM NaCl, 3 mM NaN_3, 1 mM EDTA) and 30% glycerol. The four-pulse double electron−electron resonance (DEER) ESR experiment[36] was carried out on 10 μL samples of the oligomers flash-frozen in liquid ethane at 80 K. The DEER data, shown in Figures 3 and 4, yield characteristic ESR line shapes that directly reveal the magnetic dipolar interaction between the POAC spins. The magnetic dipolar interaction is proportional to r^{-3}, where r is the distance between the electron spins. The DEER data were inverted to

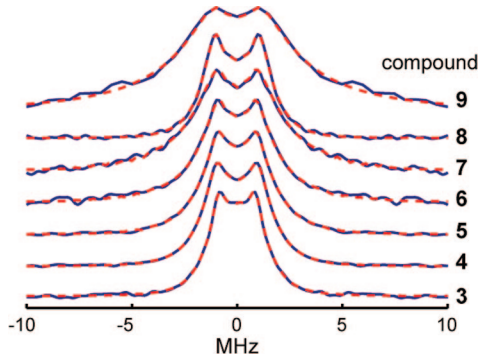


Figure 4. Fourier transformed DEER spectra for compounds 3−9, experimental data (black), and data fitting (dashed red).

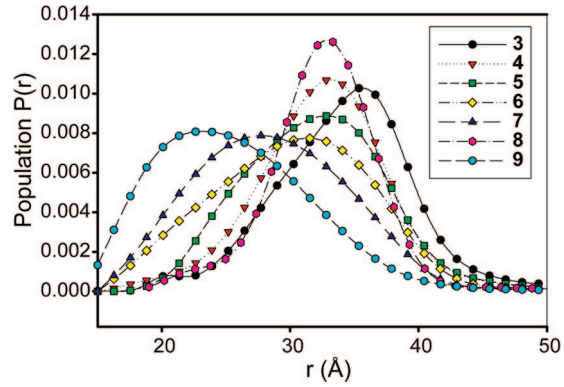


Figure 5. Population distribution functions $P(r)$ for 3−9, derived from the time domain DEER experimental data (Figure 3).

obtain the distance distribution functions, $P(r)$, using Tikhonov regularization method by DEERAnalysis 2006 program (Figure 5).[37,38] The most probable distance (r_{prob}), the mean distance $<r>$, and the standard deviations σ were calculated using a moment analysis and are shown below (Table 1). Model dependent simulations that assume a Gaussian distribution in distances did not adequately fit the DEER data. The asymmetric shapes of $P(r)$ in Figure 5 provide a better fit to the data, and this is in accordance with the $P(r)$ shapes of shorter oligomers with 4−8 *SS* monomers.[13]

The End-to-End Distances of Curved Bispeptides. The population distributions for oligomers **3**−**9** show distributions that are offset relative to one another (Figure 5). While the half-height width of the distance distributions is on the order of 10 Å (see below), the average and most probable distances are determined to within approximately 1−2 Å.[13] The experimentally determined most probable distances of these oligomers range from 36 Å for **3**, the longest, to 23 Å for **9**, the most curved. This is similar to the range of distances spanned by a series of helical molecular rods composed of 4−8 *SS* monomers that we characterized previously.[13] A comparison of the CANDO modeled distances and the DEER determined most probable distances is shown in Figure 6. The trends of the experimental data and the modeled distances are the same with the exception of oligomers **6** and

TABLE 1. Most Probable Distance, r_{prob}, Average Distance, and Standard Deviation Were Obtained from the Population Distributions Shown in Figure 5

compound	r_{prob} (Å)	$<r>$ (Å)	S (Å)	modeled distance (Å)[a]
3	36	33.3	5.3	40
4	33	32.2	5.2	37
5	33	31.6	5.4	32
6	31	29.9	6.1	23
7	28	28.5	5.9	29
8	33	32.4	4.2	38
9	23	25.6	5.9	17

[a]CANDO modeled distances.

7. All of the CANDO modeled distances are within the experimentally determined minimum and maximum distances at half-height of the population distributions (Figure 6). CANDO overestimates the distances of the longer oligomers and underestimates the distances of the shorter ones. We observe small changes in distance in the less curved compounds **3**, **4**, **5**, and **8** because they can only deviate a little from being fully extended. We observe the largest changes in end-to-end distance in the more curved oligomers **6**, **7**, and **9** because small changes in curvature cause the ends to lever large distances.

The experimental data are in reasonable accord with the shapes and design principles deduced from CANDO. The differences likely come about because of small uncertainties in the modeling of the monomers in CANDO's database and that get amplified when eight of them are assembled by CANDO into models of the octamers. We have previously shown that molecular dynamics calculations based on the AMBER95 force field used by CANDO overestimate the lengths of long rod-like homo-oligomers.[13] In addition, while we modeled the orientations of the POAC spin probes on each oligomer in a consistent way, if they differ from oligomer to oligomer, then together they can shift the measured distance distribution in either direction by up to 14 Å, depending on their orientation. We have recently described a simplified parametrized model that allows us to optimize the parameters to fit ESR determined distance distributions for homo-oligomers of bispeptides, and this model uses molecular dynamics simulations to model the dynamic behavior of the spin probes.[32] The use of this approach to model hetero-oligomers such as those described here is underdetermined and is a problem that we are continuing to work on.

Comparing the Flexibility of Bispeptides to Other Macromolecules. The half-height widths of the distance distributions provide a measure of the flexibility of bispeptide oligomers,[13] and they range from 8.3 Å for oligomer **8** to 15.7 Å for oligomer **6**. These values can be compared to the half-height widths of distance distributions determined for phenylethynylenes. Jeschke and co-workers measured the distance distribution for a double spin-labeled, rod-like *para*-phenylethynylene molecule that was 36−39 Å long, similar in length to oligomer **3**; it had a half-height width of 1.1 Å (see Supporting Information).[27] Oligomer **3** is more flexible than Jeschke's extremely stiff, rod-like *para*-phenylethynylene oligomer. This can be understood in terms of the different construction of these very different molecules. When rod-like *para*-phenyleneethynylene oligomers are bent, stiff bond angles are distorted, which is energetically costly and it requires a considerable amount of bend to bring the spin probes closer to each other. Bending of bispeptides causes distortion of dihedral angles, and these are easier to bend then bond angles. This is supported by comparison of population distributions derived from molecular dynamics simulations that have been carried out on the rod-like *para*-phenylethynylene oligomer[27] and the rod-like bispeptide **3**.[13] In the same work, Jeschke measured the distance distribution across the arms of a three-armed phenylethynylene triradical that spanned ~36.5 Å; its distance distribution had a half-height width of 7.2 Å (see Supporting Information).[27] This value is larger than the linear phenylethynylene because, in the triradical, small amounts of bending lead to large changes of distance between the spin probes. The triradical phenylethynylene is more stiff than the bispeptide curved oligomers such as **6**, **7**, and **9**, which have half-height widths of 16, 16, and 15 Å, respectively.

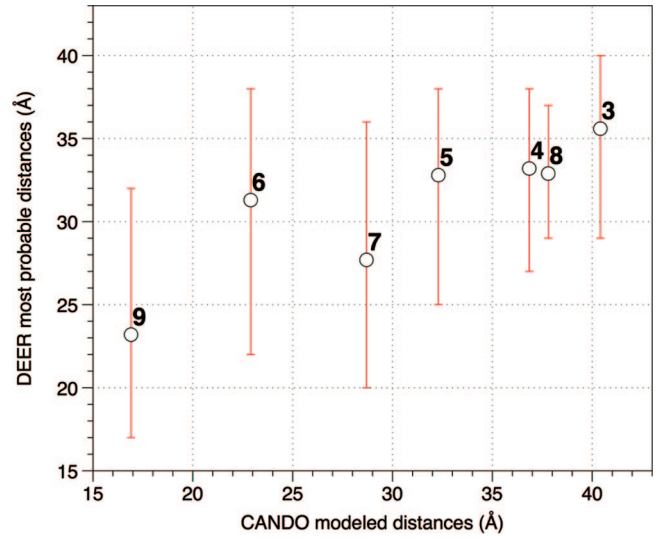


Figure 6. Comparison of the end-to-end modeled distances calculated using CANDO and the DEER determined r_{prob} values. The vertical bars span the minimum distance at half-height and the maximum distance at half-height of the population distributions in Figure 5. The experimental error in the determination of the most probable distances is about 1−2 Å.

CONCLUSIONS

We have demonstrated the ability to design, synthesize, and characterize bispeptide-based macromolecules with different shapes. We synthesized seven double spin probe labeled oligomers and measured their end-to-end distances using the DEER-ESR experiment. We will use these experimentally determined distances and population distributions in the future to develop better bispeptide modeling tools. This work validates the approach of coupling rigid bisamino acid monomers through pairs of amide bonds to create shape-persistent curved macromolecules. As we create oligomers using other building blocks,[10,11] we will be able to create macromolecules with more divers

e shapes, and as we become more proficient at decorating them with functional groups, we will be able to construct macromolecules with new functions not found in nature.

METHODS

General Methods: Dichloromethane was distilled from CaH_2. All other reagents were used as received, unless stated otherwise. Solid phase synthesis was performed in a 1.5 mL disposable polypropylene reaction column, connected to a three-way valve equipped with vacuum and argon for mixing. Dichloromethane (DCM) used in coupling reactions was distilled over calcium hydride. Dry grade dimethylformamide (DMF) from Aldrich was used for coupling. *N*,*N*-Diisopropylethylamine (DIPEA) was distilled under nitrogen sequentially from ninhydrin and potassium hydroxide and stored over molecular sieves. *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) was obtained from Acros. All solid phase reactions were mixed by bubbling argon up through the reactor, allowing for mixing and an inert atmosphere over the reaction. HPLC-MS analysis and preparative purification were performed on a Hewlett-Packard Series 1050 instrument equipped with a Waters Xterra MS C_{18} column (3.5 μm packing, 4.6 mm × 150 mm) and a diode-array detector, while the MSD-ESI was Series 1100. All reverse phase C_{18} HPLC was carried out using H_2O/MeCN with 0.1% formic acid.

Synthesis of Oligomer 3: This was synthesized and characterized as described previously.[13]

General Fmoc Deprotection Procedure A: The terminal Fmoc-protected amine was deprotected by adding 0.5 mL of 20% piperidine/DMF to the resin and bubbling with argon for 40 min at room temperature. By measuring the absorbance at 301 nm of a few microliters of the deprotection solution diluted into 100 volumes of DMF, the number of moles of Fmoc removed was calculated by using $\epsilon = 7800\ M^{-1}\ cm^{-1}$.

General Coupling Procedure B: Coupling solutions were prepared by dissolving 17.1 mg (31.5 μmol) of **1** (Fmoc-(Boc)-pro4(2*S*,4*S*)) or **2** (Fmoc-(Boc)-pro4(2*R*,4*R*))[12] and 12.0 mg of HATU (31.5 μmol) in 160 μL of 20% DCM/DMF. These solutions were mixed at rt using a micropipette, after which 11.0 μL of DIPEA (63.0 μmol) was added to form the active ester. After 10 min activation time, the solutions were added to each resin and allowed to mix with the resin by argon bubbling for 30 min at room temperature.

Synthesis of Oligomers 4−8: To five 1.5 mL polypropylene solid phase peptide synthesis (SPPS) reaction vessels was added Rink amide AM Resin (Novabiochem) (5 mg, 3.15 μmol loading). The resin was swollen for 1 h in dimethylformamide (DMF). The terminal Fmoc-protected amine was deprotected using Fmoc deprotection procedure A. The resin was washed with DMF, isopropanol, DMF, isopropanol, and DMF for 2 min each.

In a 5 × 1.5 mL microcentrifuge tube, coupling procedure B was carried out using monomer **1** or **2** depending on the sequence. The resins were then washed 3 × 2 min with 1 mL of DMF and then coupling procedure B was carried out again using the same monomer to affect a double coupling. The resins were then washed using 3 × 2 min with 1 mL of DMF. Fmoc deprotection procedure A was carried out to remove the N-teminal Fmoc group. This process of coupling/deprotection was repeated seven additional times for each monomer in the sequence as needed to make the desired octamer.

After the final building block was coupled and Fmoc group removed, the first spin label was attached. A solution of 29.0 mg (158 μmol) of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid and 60.0 mg of HATU (158 μmol) in 800 μL of 20% DCM/DMF was mixed using a micropipette, after which 55.0 μL of DIPEA (315 μmol) was added to prepare the active ester. After 10 min activation time, the solution was aliquoted into five portions and each portion added to one reactor and allowed to react for 30 min. Each resin was then washed 3 × 2 min with 1 mL of DMF. A second coupling was carried out using the same quantities as described above. After the second coupling, the resin was prepared for cleavage by washing with 1 mL volumes of DMF, isopropanol, DMF, isopropanol, DCM, methanol, DCM, methanol, and DCM for 2 min each. The reactors were then put in a vacuum tube and dried *in vacuo* overnight.

The resins were cleaved in 1 mL of 2.5% water, 2.5% triisopropylsilane, in trifluoroacetic acid (TFA), with stirring at room temperature for 2 h. The solution was filtered away from the resin beads, and an additional 2 × 1 mL of TFA was used to wash the beads. The solutions were pooled, and the TFA was removed under a stream of dry nitrogen. Residual solvent was removed *in vacuo* for 1 h, yielding a colorless residue.

The cleaved products were dissolved in 125 μL of 20% piperidine/*N*-methylpyrrolidinone (NMP) and allowed to sit at room temperature for 48 h. After 48 h at room temperature, the products were precipitated by dripping into 2 mL of ether stirring in a 2.2 mL polypropylene microcentrifuge tube. The precipitates were collected by centrifugation at 10 000*g*, 4 °C, for 5 min. The pellets were washed with 2 mL of fresh ether, sonicated, and the centrifugation was repeated. The ether was removed, and the pellets were allowed to dry. A solution of 29.0 mg (158 μmol) of 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid (POAC-OH) and 60.0 mg of HATU (158 μmol) in 800 μL of 20% DCM/DMF was mixed using a micropipette, after which 55.0 μL of DIPEA (315 μmol) was added to prepare the active ester. The pellets were dissolved in this active ester solution of the spin probe. This reaction was allowed to proceed for 30 min. The final, crude bis-spin-labeled oligomers were precipitated by dripping into 2 mL of ether stirring in a 2.2 mL polypropylene microcentrifuge tube. The precipitates were collected by centrifugation at 10 000*g*, 4 °C, for 5 min. The pellets were washed with 2 mL of fresh ether, sonicated, and the centrifugation was repeated. The ether was removed, and the pellets were allowed to dry. The precipitates were dissolved in 30 μL of 30% acetonitrile in water with 0.1% trifluoroacetic acid. This crude material was purified by analytical HPLC as described in the General Methods section using a 5−50% MeCN in H_2O gradient. A small aliquot of each final product was reinjected on the LC-MS and confirmed by mass analysis. In all cases, the desired product was clean, with the only impurity being a small amount of HOAt in some cases.

Synthesis of Oligomer 9: Initial attempts to synthesize **9** using the above procedure failed because the diketopiperazine formation reaction would not go to completion in 20% piperidine/DMF. To deal with this, we developed the following bispeptide synthesis route with an on-resin diketopiperazine formation reaction. Synthesis of this most curved scaffold **9** was identical to that described above except for the three following changes. We used regular Rink amide resin (NOT AM). After the last bisamino acid monomer was attached, the Boc groups were removed on resin using TMSOTf-lutidine, as follows: The Boc groups on the building blocks were removed on resin using a procedure from the Burgess laboratory.[39] The beads were washed with DCM for 5 min. A solution of 5 mL of 1 M trimethylsilyl triflate (Aldrich) and 1.5 M 2,6-lutidine in DCM was prepared. This solution was added to the resin 3 × 0.75 mL × 5 min, with gentle argon bubbling at room temperature; between TMSOTf solution additions, we washed with 5 mL of DCM for 5 min. After deprotection, the resin was washed vigorously with DCM, followed by MeOH/DCM washes, ending up with swelling in DMF for 5 min. The diketopiperazines were then closed on the resin using a stirred solution of 0.5 mL of 20% piperidine in NMP for 20 h at 35−40 °C. After 20 h, the resin was washed extensively with DMF and isopropanol. Spin labels were attached to both amines simultaneously, while the oligomer still attached to the resin. A solution of 63 μmol of HATU, 63 μmol 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid, and 126 μmol DIPEA was combined with DMF for a final concentration of 0.2 M POAC-OH in DMF. This solution was added to the resin and mixed by gentle argon bubbling for 30 min. The resin was washed with 3 × 2 min with 2 mL of DMF. A second solution of 63 μmol of HATU, 63 μmol 2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl-3-carboxylic acid, and

126 μmol DIPEA was combined with DMF for a final concentration of 0.2 M POAC-OH in DMF. This solution was added to the resin and mixed by gentle argon bubbling for 30 min. After the second coupling, the resin was prepared for cleavage by washing with 1 mL volumes of DMF, isopropanol, DMF, isopropanol, DCM, methanol, DCM, methanol, and DCM for 2 min each. The reactors were then put in a vacuum tube and dried *in vacuo* overnight. The resins were cleaved in 1 mL of 2.5% water, 2.5% triisopropylsilane, in trifluoroacetic acid (TFA), with stirring at room temperature for 2 h. The solution was filtered away from the resin beads, and an additional 2 × 1 mL TFA was used to wash the beads. The solutions were pooled, and the TFA was removed under a stream of dry nitrogen. Residual solvent was removed *in vacuo* for 1 h. RP-HPLC purification was carried out using a 5−50% MeCN in H_2O gradient as described above.

Electron Spin Resonance. The ESR experiments were performed at 80 K on a Bruker EleXsys E580 CW/FT X-band ESR spectrometer equipped with a Bruker X-band ER 4118X-MS2 split ring resonator. A $\pi/2_{\nu a} - \pi_1 - \pi_{\nu a} - (\tau_1 + T) - \pi_{\nu b} - (\tau_2 - T) - \pi_{\nu a}$ pulse sequence was used for the DEER experiment. The observer frequency, ν_A, was set at the central field of the spin label peak, which is around 9.5−9.6 GHz, and the pump frequency, ν_B, was set 70 MHz higher. The length of the $\pi/2$ and π pulses was 16 and 32, respectively. The delay τ_1 was 200 ns, and τ_2 was 2200 ns for compounds **3**−**8** and 1500 ns for compound **9**. The increment of time T after the second pulse was 16 ns for 128 points for compounds **3**−**8** and 10 ns for compound **9**. For each step of the phase cycle, 500 averages were collected at a repetition rate of 1 KHz. The acquisition time used for each sample was roughly 24 h.

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Supporting Information Available: Purification of all compounds and estimation of half-height peak widths for distance distributions in phenylethynylenes. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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