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The Influence of Molecular Conformation upon the Self-Assembly of Cyclohexane Diamide Diacids

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Abstract—Background: Information regarding the self-association of small peptide motifs can be used in the design of peptide microstructures. Previous work in our laboratories illustrated the self-association of certain diamide diacids into microcapsules. In this report a series of cyclohexane diamide diacids are investigated. The cyclohexylene (R-C_6H_{10}-R) system (with its axial and equatorial requirements) provided an opportunity to study the influence of molecular conformation upon the self-aggregation process.

Results: Condensation of the respective *cis*- and *trans*-1,2-, 1,3-, and 1,4- cyclohexane dicarboxylic acid platforms with two equivalents of a L-Phe ester followed by deprotection gave the desired diamide diacids. Basic solutions of *cis*-1,2-, *trans*-1,3-, and *cis*-1,4-diamide diacids generated solid microspheres when acidified to pH 2.4. Molecular modeling revealed that 1,3-diaxial interactions favor a helical turn within these diamides.

Conclusions: Access to 'complementary' molecular geometries is needed to self-associate into microscopic architectures. © 1997 Elsevier Science Ltd.

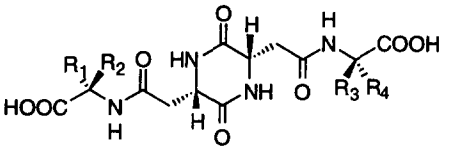
Introduction

In 1996 a helical peptide coil investigated by Lee et al.[1] was shown to autocatalyze its own synthesis from precursor peptides. Lee's discovery identified a new class of self-replicating structures beyond those predicated on Watson–Crick base pairing. Self-replicating peptides and artificial enzymes (such as molecular clefts) orient their functional groups in precise spatial arrangements to optimize substrate binding.[2–5] In the case of peptide molecular recognition both the template/enzyme and the substrate are peptides.

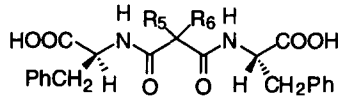
Peptide agglomeration also involves a degree of self-recognition.[6,7] The design of future templates and other peptide micro-structures should benefit from information regarding the self-association of small peptide motifs. Understanding which factors are responsible for the molecular ordering of model peptides is, therefore, important. Previous work in our laboratories illustrated the self-association of certain diamide diacids into microcapsules. These 'hybrid' peptides were comprised of two L-phenylalanine (L-Phe) groups appended via Nα amide bonds to an organic spacer group or platform. Each diamide was generated by the condensation of two equivalents of a protected L-Phe ester with a diacid platform (followed by ester deprotection) to give the respective diamide diacid. The bis-acid platforms investigated included malonic acid, maleic acid, and the diketopiperazine of L-aspartic acid. These platforms introduced both a structural tether and mobility constraints to the appended Phe ligands. These structural variations revealed that certain aromatic amino acid containing 'hybrid' peptides have a high affinity for self-organization (e.g., **1a–c**, **2a–c**, **3** in Fig. 1).[7]

In light of the current interest in how peptides recognize each other in aqueous environments,[1] we were interested in whether small self-agglomerating peptides exhibit conformational preferences prior to their self-assembly. In this report a series of cyclohexane diamide diacid frameworks were investigated. The cyclohexane platform allowed for the construction of isomeric diamide diacids with fixed distances between Phe ligands. In addition, the cyclohexane system (with its axial and equatorial requirements)

Key words: conformation, microspheres, molecular recognition, self-assembly.



1a: R_1, R_4 = CH_2Ph; R_2, R_3 = H
1b: R_1, R_4 = H; R_2, R_3 = CH_2Ph
1c: R_1, R_2, R_3, R_4 = either CH_2Ph or H;
where $R_1 \neq R_2$ and $R_3 \neq R_4$



2a: R_5, R_6 = H
2b: R_5, R_6 = CH_3
2c: R_5, R_6 = cyclo CH_2

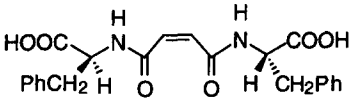


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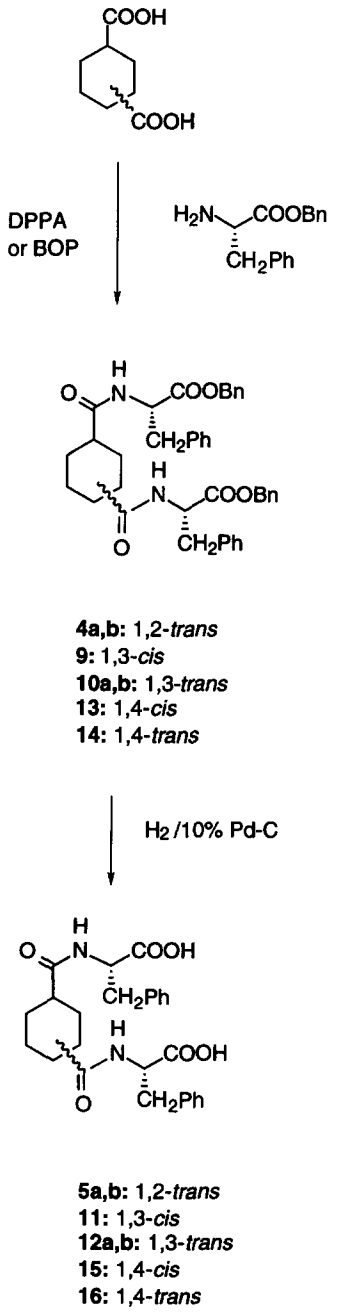
Figure 1. Diamide diacids which undergo aqueous self-assembly into microcapsules.

provided an opportunity to study the influence of molecular conformation upon the self-aggregation process. These studies were designed to test our earlier model[6] and to further define the conformational requirements of systems undergoing self-association into supramolecular arrays.

Results and Discussion

Synthesis

In order to access the various cyclohexyl substrates required for this study, we synthesized each bis-(L-Phe) diamide from its respective cyclohexanedicarboxylic acid platform. The diastereomeric *trans*-1,2-cyclohexane-bis-L-Phe conjugates were synthesized from the commercially available *trans*-1,2-cyclohexane diacid (Scheme 1). The diacid was condensed with two equivalents of L-Phe benzyl ester using the BOP reagent[8] to give the *trans*-1,2-diamide bis-benzyl ester as two diastereomers, **4a** and **4b** (47% yield). These diastereomers were separated and reduced to the respective *trans*-diacids **5a** and **5b** via hydrogenolysis over 10% Pd–C in MeOH in quantitative yield. The *cis*-1,2-cyclohexane derivative was generated from the stepwise condensation of L-Phe benzyl ester and *cis*-1,2-cyclohexanedicarboxylic acid anhydride (Scheme 2). This method initially gave the mono-amide acid **6** (78%), which was converted to the bisamide dibenzyl ester **7** (72%) after condensation with a second mole of L-Phe benzyl ester. This two step method utilized the acylation properties of the commercially available



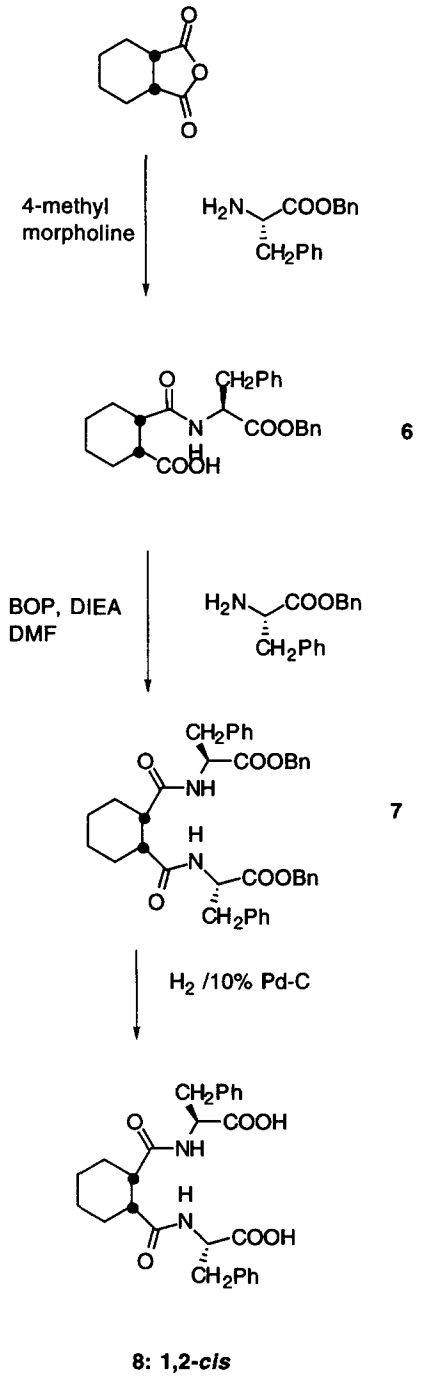
4a,b: 1,2-*trans*
9: 1,3-*cis*
10a,b: 1,3-*trans*
13: 1,4-*cis*
14: 1,4-*trans*

5a,b: 1,2-*trans*
11: 1,3-*cis*
12a,b: 1,3-*trans*
15: 1,4-*cis*
16: 1,4-*trans*

Scheme 1. Synthesis of bis-L-Phe cyclohexyl diamide diacids **5ab**, **11**, **12ab**, **15**, and **16**.

anhydride. The dibenzyl ester was subsequently cleaved (H_2, 10% Pd–C) to give the *cis*-1,2-cyclohexane diamide diacid **8** (100%).

The 1,3-cyclohexane derivatives were prepared from a mixture of *cis*- and *trans*-1,3-cyclohexane dicarboxylic acids, which were coupled to L-Phe benzyl ester using diphenylphosphorylazide[9] (DPPA; 73% yield, Scheme 1). The resultant mixture of bis benzyl esters was separated by recrystallization and column chromatography to give the *cis*-1,3 benzyl ester **9** and a 1:1 mixture of the *trans*-1,3 diastereomers **10**. The *cis*-1,3 derivative was a single isomer, while its *trans* counter-



Scheme 2. Synthesis of bis L-Phe *cis*-1,2-cyclohexyl diamide diacid **8**.

part was a mixture of diastereomers: (1*R*, 3*R*) and (1*S*, 3*S*). The *cis*-1,3- and *trans*-1,3-isomers were assigned by their ^{1}H NMR spectra in $CDCl_3$. Since the *cis*-isomer is symmetrical in all respects save the two L-Phe chiral centers (which are not mirror images of each other), the observed multiplicity of each peak in the ^{1}H NMR spectrum should be less than that of the *trans*-isomeric mixture. The multiplicity differences are most marked for the amide NH peaks (*cis* isomer: broad singlet at 5.90 ppm; *trans*: two doublets centered at 5.90 ppm) and the CH_2Ph protons at 3.11 ppm (4H: *cis*: broad singlet; *trans*: multiplet). In addition to the multiplicities, there are significant chemical shift differences. The two cyclohexyl protons, which are α to the carbonyl of the amide, are both axial and shielded in the *cis*-isomer (broad triplet at 2.08 ppm) relative to the *trans*-isomers (multiplet at 2.41 ppm). This finding is in good agreement with the fact that cyclohexane equatorial hydrogens are consistently downfield of their axial counterparts due to the deshielding cone of the proximal σ bonds.[10] The benzyl esters (**9** and **10**) were converted to their respective *cis*-1,3- (**11**) and *trans*-1,3- (**12**) diamide diacids via hydrogenolysis (99% yield). We were not able to separate the *trans*-1,3 diastereomers of **12**, but did achieve partial enhancement of one diastereomer after several recrystallizations (albeit in much lower yield). All assembly experiments with **12** were done on the (60:40) mixture of *trans*-1,3 diastereomers.

In a similar synthesis the *cis*- and *trans*-1,4-cyclohexanedicarboxylic acids were individually coupled with L-Phe benzyl ester using DPPA[9] to give the *cis*-1,4- (**13**; 76%) and *trans*-1,4- (**14**, 75%) diamide bis-benzyl esters (Scheme 1). The bis benzyl esters were then debenzylated (H_2, 10% Pd–C) to the respective diamide diacids **15** (*cis*-1,4; 99%) and **16** (*trans*-1,4; 92%).

Assembly tests

Each of the synthesized diacids (**5**, **8**, **11**, **12**, **15**, and **16**) were tested for microcapsule formation using a modification of our previously described procedure.[7] The bis acid (0.01 mmol) is dissolved in 0.1 mL of aqueous Li_2CO_3 (0.1 M) to give a clear solution of the lithium salt in deionized water. Equal volumes of this 0.1 M peptide solution and 1 M aqueous citric acid were mixed and shaken. A positive response yielded a white suspension. Microscopic examination of the suspension revealed the formation of tiny spheres.

Scanning electron microscopy (SEM)

In order to discern whether hollow microcapsules or solid microspheres were being generated, the white suspensions were observed by SEM. The better resolution and higher magnification available in the scanning electron microscope allowed for a more descriptive evaluation of the assembly morphology. A typical procedure involved the generation of a white suspension by combining 50 μL of 500 mM citric acid and 50 μL of a 50 mM aqueous solution of the lithium salt of the peptide. The final concentration of the peptide was 25 mM (well above the concentration required for visible assembly). The aqueous suspension was deposited on polylysine-coated glass coverslips and fixed with 2% OsO_4 for 4 h. The sample was washed with distilled water, air dried, and sputter coated with gold. SEM photographs were then obtained. Examples of our findings (with peptide **15**) are shown in Figure 2. The aggregation phenomenon clearly results in the generation of solid microspheres with a distribution comprised of micron and submicron diameters.

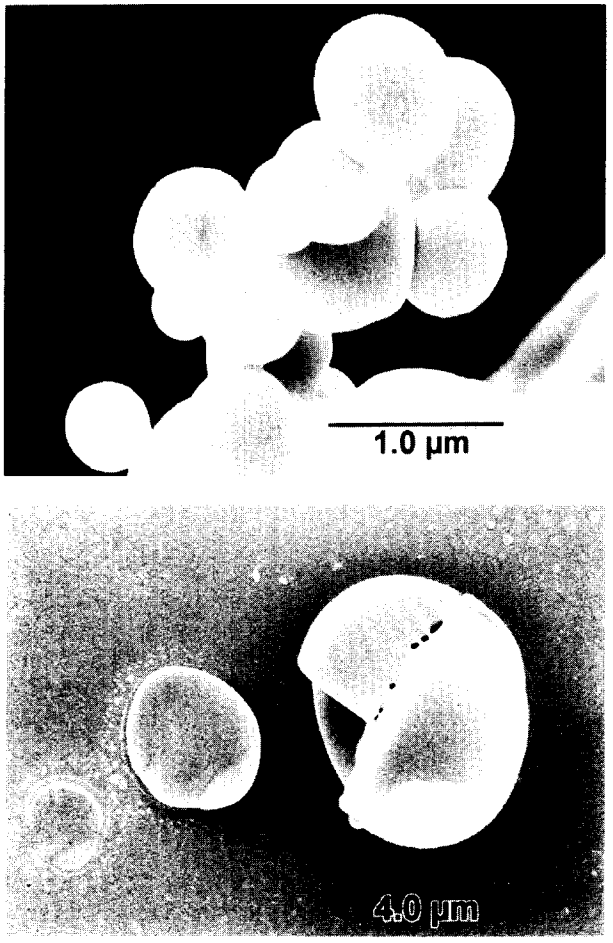


Figure 2. SEM pictures of microspheres generated from *cis*-1,4 derivative (**15**). (Top) Generated from a 25 mM **15** and 360 mM citric acid solution. (Bottom) Generated from a 50 mM **15** and 125 mM citric acid solution.

Surprisingly, the L-Phe diamides derived from **8**: *cis*-1,2-, **12**: *trans*-1,3-, and **15**: *cis*-1,4-cyclohexanedicarboxylic acids each generated solid microspheres and not the expected hollow microcapsules found with the previous systems **1a–c**, **2a–c**, and **3** (see Fig. 1). A control experiment with the microcapsule-forming peptide **2c** gave microcapsules under these conditions. The cyclohexylene amides are the first examples of microsphere formation in these diamide diacid systems. The results from the assembly and microscope studies are listed in Table 1. From this initial screen, peptides **8**, **12**, and **15** were selected for further study. Since the assembly properties of previous substrates **1a–c**, **2**, and **3** (Fig. 1) were shown to be sensitive to both substrate concentration and solution pH,[6,7] the concentration and pH dependence of peptides **8**, **12**, and **15** were determined.

Concentration and pH dependence studies

A stock solution containing the lithium salt of the diamide was prepared by stepwise addition of exactly two equivalents of a standardized solution of LiOH (stored under argon to prevent precipitation of lithium carbonate). The final concentration of the dilithium salt

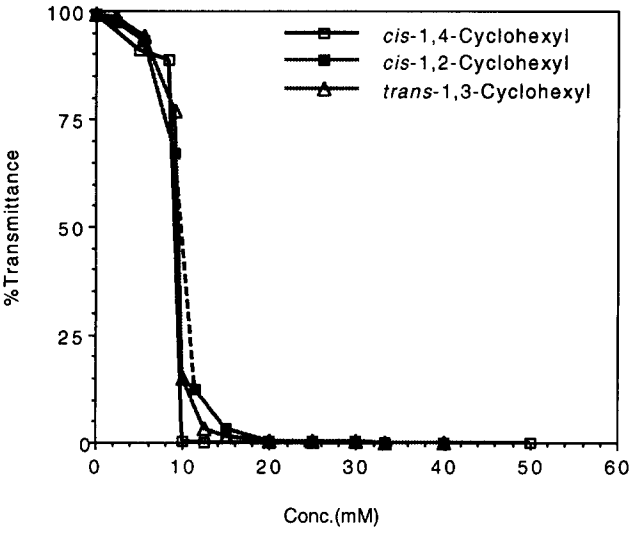


Figure 3. Percent transmittance (at 600 nm) vs. concn of peptide in 500 mM citric acid.

of the diamide was 100 mM, and the pH was always between 7.0 and 8.0. The solution was filtered through a 0.2 μm membrane prior to use. For concentration dependence studies, an appropriate amount of the 100 mM stock solution was diluted with deionized water to 500 μL. Microsphere formation was then initiated by addition of an equal volume of 1 M citric acid, so that the final concentration ranged from 0–50 mM dilithium diamide in 500 mM citric acid with the pH below 2.5. Turbidity was assessed over this range of concentrations by measuring percentage transmittance at 600 nm (Fig. 3). As seen in Figure 3, the concentration dependencies for **8**, **12**, and **15** were nearly identical (approximately 10 mM for 50% transmittance). This finding may simply reflect the intrinsic solubility of either the respective aggregates or the individual diamide diacids.

For pH dependence studies, 500 μL of the 100 mM dilithium diamide solution was mixed with an equal volume of one of a series of 1 M lithium citrate buffers containing 0–1 equiv of lithium hydroxide so that the final measured pH of the mixture ranged from ca. 2.4 to 4.0. Turbidity was assessed over this pH range by measuring percent transmittance at 600 nm (Fig. 4). The respective pK_as of **8**, **12**, and **15** were also determined and are listed in Table 2. As expected, the isomeric diacids gave similar pK_a values. The trend in pK_a ($pK_a1 \approx 3.67$) is reflected in the similar pH dependence (Fig. 4) found for each isomer (**8**, **12**, and **15**). Indeed, since the visible manifestation of the assembly process (i.e., a reduction in transmittance or increased turbidity) is related to the reprotonation propensity of each carboxylate substrate, it is tempting to assign the COOH group as a major factor in the self-aggregation process. This assessment is in keeping with our earlier premise that the COOH groups can form intermolecular hydrogen bonds to support the developing suprastructure.[7]

Table 1. Assembly test results

Compd	Diacid platform	Assembly observed
1a	Diketopiperazine of L-Asp	Microcapsules
2a	Malonic acid	Microcapsules
3	Maleic acid	Microcapsules
5a,b	*Trans*-1,2 cyclohexane	Crystalline ppt.
8	*Cis*-1,2 cyclohexane	Microspheres
11	*Cis*-1,3 cyclohexane	Amorphous ppt.
12a,b	*Trans*-1,3 cyclohexane	Microspheres
15	*Cis*-1,4 cyclohexane	Microspheres
16	*Trans*-1,4 cyclohexane	Amorphous ppt.

Molecular modeling

After our initial study involving the self-assembly of diketopiperazines of L-Asp,[6] we formulated a model which helped to explain how these compounds aggregate into supramolecular arrays. The model was predicated on the minimized geometries of the earlier peptides studied (i.e., compounds **1a–c**, **2a–c**, and **3**). The scaffolds of **1a–c**, **2a–c**, and **3** allow the respective peptides to complete the helical turn necessary for generation of a hydrophobic helix (with the terminal carboxylic acid groups oriented away from the core).[7] We speculated that acidification of the terminal carboxylates allows for intermolecular hydrogen bonding (between different helical subunits), thereby generating the observed larger arrays. This was consistent with both the observed pH dependence of these systems and our later observations that anionic species such as $R\text{-}COO^-$ (a non H bond donor) do not visibly aggregate and, in fact, disrupt the putative assembly process.[7]

Due to the similar behavior of the three assembling peptides (**8**, **12**, and **15**), the *cis*-1,4 derivative **15** was chosen as a representative for this family of peptides. Model building and molecular modeling of peptide **15** was carried out using *HyperChem* version 4.5 running on a computer workstation using the MM^+ forcefield.

Table 2. pK_a Data for peptides **8**, **12a,b**, and **15**

Peptide	pK_a1	pK_a2
Cis-1,2-(**8**)	3.71	4.83
Trans-1,3-(**12a,b**)	3.63	4.59
Cis-1,4-(**15**)	3.65	4.63

A search of the potential energy surface describing the conformational states of derivative **15** was performed by simultaneously changing (in a random manner) six dihedral angles (α1, α11, α2, α22, β1, and β2). The six angles are delineated in Figure 5. The six dihedral angles were altered 2000 times to generate a series of starting geometries. A conjugate-gradient method was then employed to minimize the respective energy of each conformer. Structures were minimized to an rms gradient of less than 0.10 kcal/mol-Å. Energies greater than 5 kcal/mol above the minimum energy were discarded.[11] From these 2000 structures, 254 conformers were obtained, each of which possessed an energy within the 5 kcal/mol window (−21.10 kcal/mol to −16.10 kcal/mol). Each of these structures exhibited the classical chair conformation.

The structures shown in Figure 6 represent the lowest energy structure (rank 1) and a representative higher energy structure (rank 23): (**A**, −21.1 and **B**, −18.4 kcal/mol, respectively). Boltzmann analysis indicates that structure **B** represents a 1% probability of occurrence. **B** is included with **A** to provide a 'flavor' of the conformational flexibility available to these systems.

In each of the two helical conformers (**A** and **B**), peptide **15** adopted the classic chair conformation and the amide groups are in the expected *Z* orientation. In the chair conformation, one ligand must be axial for each of the following disubstituted cyclohexanes: *cis*-1,2; *trans*-1,3; and *cis*-1,4. Of particular interest was the

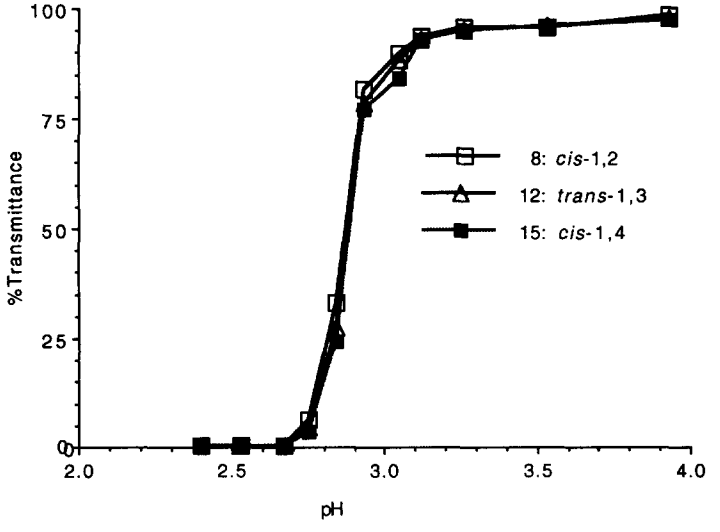


Figure 4. Percent transmittance (at 600 nm) vs. pH measurements with 50 mM peptide and 500 mM lithium citrate.

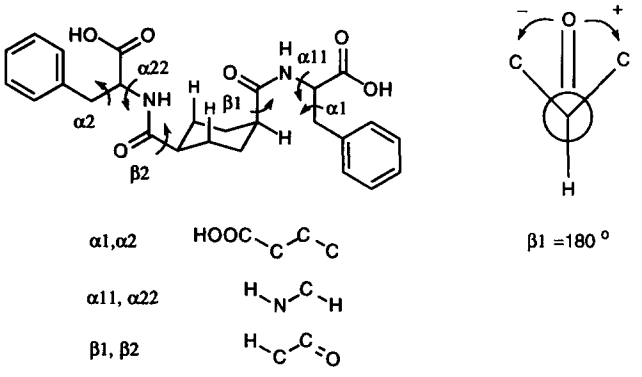


Figure 5. Drawing of derivative **15** (left) showing the dihedral angles of interest and their respective connectivities. Newman projection of β1 (right).

finding that precisely these isomers (**8**, **12**, and **15**, respectively) undergo the assembly process.

Using the *cis*-1,4 derivative (**15**) as an example, the axial amide pendant incurs two 1,3-diaxial interactions. It is possible that the *syn* hydrogens conformationally limit the free rotation of the axial pendant and direct the turn necessary for helix formation.

In an attempt to quantify this premise, we modeled the alteration of the H–C–C=O dihedral angle (β1) of the axial L-Phe substituent in **15**. The angle (β1) is defined as the dihedral angle for the axial substituent, which incorporates the cyclohexyl C–H, the axial C–C bond, and the C=O bond of the amide. Using a Newman projection and looking down the axial C–C bond (with the CH in the foreground and fixed), positive and negative values of β1 are defined as clockwise and counter-clockwise rotations of the carbonyl carbon, respectively (Fig. 5). To orient the reader, when β1=0° the axial amide NH bisects the *syn* 1,3 diaxial hydrogens and when β1=180°, the axial amide carbonyl bisects the *syn* 1,3 diaxial hydrogens. As shown in Figure 6, the β1 value is 115.5° for conformer **A** and −87.8° for conformer **B**. When β1 is varied in conformer **A** (while keeping the rest of the molecule conformationally fixed) single point energy calculations indicate that β1 values ranging from 115° to −140° define a deep potential well. Movement through this range allows the amide NH to move close to the *syn* 1,3 diaxial hydrogens, but not the amide C=O (e.g., β1=180°). Significantly, the energetically preferred value of β1 (i.e., 115.5°) represents a conformation which avoids bisection of the *syn* 1,3-diaxial hydrogens by either the amide NH or the amide C=O group.

These calculations support the premise that the *syn* (diaxial) hydrogens rotationally restrict the axial Phe pendant via their steric interactions. Furthermore, in developing a helical turn within these derivatives, an axial ring substituent and the proper dihedral angle (β) seem to be necessary and mutually inclusive (see Fig. 6).

^{1}H NMR and FTIR studies

The unique energetics and conformational preferences associated with the ring flip of cyclohexane ring systems have been thoroughly investigated by Jensen and Bushweller.[12] There is no innate preference as to which of the two L-Phe groups in **15** adopts the axial site. In fact, a dynamic equilibrium between the *ae* and *ea* conformers of **15** is expected. In order to observe this equilibration process and to obtain information regarding the intra- and intermolecular hydrogen bonding of **15**, FTIR and NMR studies were initiated. Because of the selection of **15** for modeling studies and its recrystallization properties from 8% MeOH/EtOAc,

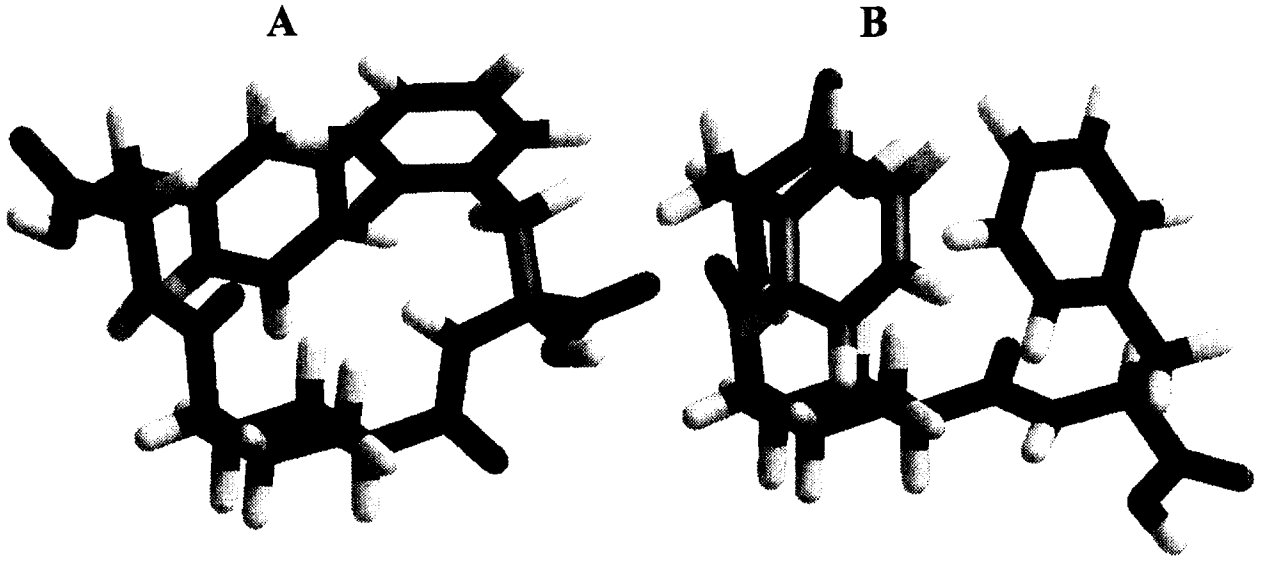


Figure 6. Calculated structures of the lowest energy conformer **A** (−21.1 kcal/mol) and a higher energy conformer **B** (−18.4 kcal/mol). The avoidance of unfavorable 1,3-diaxial interactions between the carbonyl oxygen of the axial phenylalanine pendant and the *syn*-hydrogens is governed by dihedral β1. The value of this dihedral angle for conformers **A** and **B** is 115.5° and −87.8°, respectively.

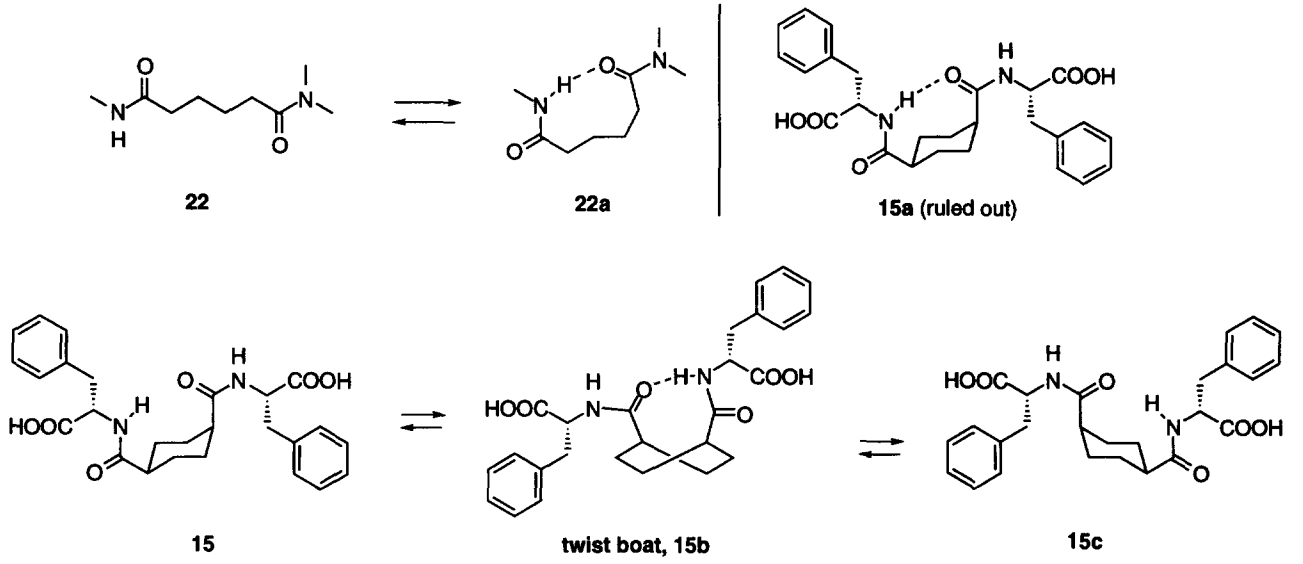


Figure 7. Proposed intramolecular hydrogen bonding patterns of adipamide **22** and cyclohexyl diamide diacid **15**.

the *cis*-1,4-cyclohexyl derivative **15** was chosen for further spectroscopic study.

Variable temperature-NMR (VTNMR) and FTIR studies aimed at elucidating hydrogen bonding patterns within peptides are typically carried out in non-hydrogen bonding solvents (e.g., CH_2Cl_2) according to the methods of Gellman and Kelly.[13–16] The use of VTNMR and FTIR in tandem is critical to properly decipher which hydrogen bonding states are being observed. Since the NMR time scale is relatively slow, it is possible that one may observe only the time-averaged NH bond environment instead of a particular NH bonding interaction. However, a FTIR study (with its faster time scale) allows for the direct observation of individual hydrogen bonded states.

In 1991 Gellman illustrated this tandem analytical method in his investigations of a homologous series of diamides, $(CH_3)_2NCO(CH_2)_nCONHCH_3$, where n = 1–6. Therein, he determined the unique energetics of a nine-membered intramolecular hydrogen bond associated with one member of the series, where n is equal to 4 (**22**, see Fig. 7). This substrate represents an acyclic model of the *cis*-1,4-cyclohexane derivative **15**. Gellman found a temperature dependence of the NH chemical shift ($\Delta\delta/\Delta T$) in CD_2Cl_2 (at 1 mM **22**) of −10 ppb/K using VTNMR methods.[13] A large absolute value in ppb/K (>6 ppb/K) implies that the hydrogen bond is undergoing a change in environment through the temperature range studied, while a small value (1–2 ppb/K) denotes a hydrogen bond which remains bonded throughout the temperature range.

In addition, Gellman demonstrated that the FTIR of **22** (1 mM at 235 K) in CH_2Cl_2 gave two bands at 3456 and 3310 cm^{-1}. The higher wavenumber was attributed to a solvent exposed NH and the 3310 cm^{-1} value attributed to an intramolecular hydrogen bond.[13] As shown in Figure 7, substrate **22** can adopt a nearly linear intramolecular H bond, N–H–O, which induces an anti torsional angle around the central methylenes of the butyl chain. Gellman concluded that the cyclic hydrogen bonded state **22a** (Fig. 6) is favored by the enthalpic gain associated with the hydrogen bond itself and not attributed to other 'conformational directing forces.'[13]

The question arose as to whether this phenomenon was present in the *cis*-1,4 system **15**, where conformational forces are clearly in play. Unfortunately, the proper VTNMR and FTIR experiments could not be run in CD_2Cl_2 due to the low solubility of peptide **15** imparting unexpected concentration constraints.

The diamide diacid **15** has many intramolecular hydrogen bonding patterns available (e.g., COOH to amide, amide to amide, etc.) as opposed to the singular hydrogen bond available to acyclic diamide **22** (i.e., **22a** in Fig. 7). It seemed likely that the intramolecular hydrogen bonding pattern seen in **22** might be observed with **15** in other solvent systems besides CD_2Cl_2. Therefore, diamide **15** was studied in four solvents with increasing dielectric constant: acetone, methanol, DMSO, and water. We recognized that polar solvents, which could act as either hydrogen bond donors or acceptors (or both), could disrupt the intramolecular association of **15** by also competing for hydrogen bonding sites within the molecule. However, this competitive scenario between solvent and substrate is more representative of the actual aqueous assembly process, wherein the monomeric subunits are likely to (a) seek out other solvated pre-assemblies and (b) bind together using non-covalent bonds into larger arrays.[17]

Table 3 lists the VTNMR studies of the amide NH of **15** in four solvents: 10% D_2O/H_2O, DMSO-d_6, MeOH (nondeuterated) and acetone-d_6. The temperature dependence ($\Delta\delta/\Delta T$) studies of **15** in DMSO-d_6, MeOH,

Table 3. Variable temperature ^{1}H NMR data for *cis*-1,4-diamide diacid **15**[a]

Solvent	(ε)[b]	ppb/K[c]	δ_{NH} (ppm)[c,d]
Water[e]	78.5	−7.4	7.38
DMSO-d_6	49	−6.5	7.82
MeOH	32.6	−9.6	7.61
Acetone-d_6	20.7	−5.9	6.93

[a]Dried just prior to use.
[b]Dielectric constants are from ref 18.
[c]Concentrations listed in text.
[d]At 300 K.
[e]10% D_2O/H_2O.

Table 4. FTIR data for *cis*-1,4-diamide diacid **15**[a]

Solvent	Concn	IR Bands in cm^{-1} (area %)[b–e]
KBr pellet	3 wt%	3366 (100)
DMSO	10 mM	3469 (70), 3274 (30)
Acetone	10 mM	3612 (47), 3524 (20), 3372 (33)

[a]Dried just prior to use.
[b]After spectral subtraction of the appropriate solvent.
[c]Observed IR range was 3000–4000 cm^{-1} at 16 cm^{-1} resolution.
[d]At 300 K.
[e]Percentages were estimated from the length × the width (at half height) for each band reported in absorbance.

and acetone-d_6 were done at 10 mM. Due to solubility constraints, the water experiments were done at 1.1 mM **15**. It should be noted that this concentration is very near the saturation point of peptide **15** in water ($\approx$1.25 mM). In general the center of the NH amide doublet (δ_{NH} in ppm) seemed to mirror the dielectric constant (ε) of each solvent[18] with the exception of water (see Table 3). The NH resonance of **15** gave similar ppb/K values in each solvent. This was unexpected as the hydrogen bonding properties of each solvent vary dramatically. DMSO is considered a strong hydrogen bond acceptor, while acetone is a moderate acceptor. MeOH and water can each act as a hydrogen bond donor and acceptor. The observed value of ≤ -5.9 ppb/K in all of these solvents is consistent with a rapid equilibrium between hydrogen bonded states.

The δ_{NH} for **15** is concentration independent in DMSO-d_6 and in acetone from 1 to 10 mM. Therefore, **15** is likely to be monomeric throughout this concentration range in these solvents. FTIR studies (in DMSO at 10 mM **15**) revealed a strong peak at 3469 cm^{-1} and a shoulder at 3274 cm^{-1} in a 70:30 ratio (by area percent, estimated from the length × width at half height, see Table 4). The band at 3469 cm^{-1} was attributed to the solvated NH, and the band at 3271 cm^{-1} was tentatively assigned to an intramolecularly H bonded NH based on the prior studies by Gellman.[19] FTIR studies in acetone (10 mM **15**) revealed three populations centered at 3612, 3524, and 3372 cm^{-1}, respectively. The free NH stretching vibration of 2° amides in dilute solution usually occurs between 3500–3400 cm^{-1}.[10] The higher wavenumber populations are presumably due to solvent, while the signal at 3372 cm^{-1} is consistent with an intramolecular hydrogen bond based on Gellman's study of **22**.

Both the NMR (which showed the δ_{NH} to be concentration independent) and the FTIR [with N–H bands at 3372 (acetone) and 3274 cm^{-1} (DMSO)] studies are consistent with an intramolecular hydrogen bond in these solvents. A KBr pellet containing crystalline **15** gives a single band at 3366 cm^{-1}. Since this frequency is close to that observed in acetone (3372 cm^{-1}), it is possible that the NH is not involved in an intermolecular hydrogen bond in the crystal state. This postulate is in line with our earlier described model wherein the COOH groups were thought to play an important role in the intermolecular associations of the larger array.

During the NMR experiments in DMSO-d_6, MeOH-d_4, and acetone-d_6 (all at 10 mM **15** and 300 K), the chemical shifts of the equatorial cyclohexyl methylene hydrogens (both *cis* and *trans* to the Phe substituents) and the axial cyclohexyl methylene hydrogens (both *cis* and *trans* to the Phe substituents) were solvent dependent. The distinct axial and equatorial pairs of *cis* and *trans* diastereotopic methylene hydrogens were not resolved, but instead were observed as two multiplets in each of these three solvents. When assigning these two multiplets, the more downfield signal was assigned to both of the *cis* and *trans* equatorial pairs, while the corresponding axial pairs comprise the more upfield signal.[10] The ^{1}H NMR spectrum of **15** in each of these solvents is shown in Figure 8. These spectra are clearly different from the ^{1}H NMR spectrum of **15** (1.1 mM) in 10% D_2O/H_2O, which gave only one signal centered at 1.39 ppm for *all* of the cyclohexyl methylene hydrogens (see Fig. 9).

The cyclohexane ring flip process interconverts each equatorial position with its corresponding axial position and proceeds through a twist boat geometry.[20] It is likely that an intramolecular hydrogen bond could form between the amide groups as they pass in close proximity through this intermediary state (Fig. 7). A hydrogen bond of this type has been observed in *cis, cis, cis*-2,5-di-*t*-butyl-1,4-cyclohexanediols, which adopt twist boat geometries stabilized by a transannular H bond.[21,22] Structure **15a** (Fig. 7), which contains a transannular hydrogen bond spanning a chair conformation, was ruled out by the findings of Stolow.[21,22] The chemical shift (δ 1.39 ppm) observed in water is likely due to rapidly equilibrating chair conformers which appear to 'scramble' the pairs of equatorial and axial cyclohexyl ring protons on the NMR time scale. This scrambling process would be facilitated by the twist boat transannular H bond mentioned above. Moreover, there is precedent for water acting as a 'molecular lubricant' in aqueous studies of other globular proteins.[23,24] In water these equilibrating species should have more conformational mobility to adopt the

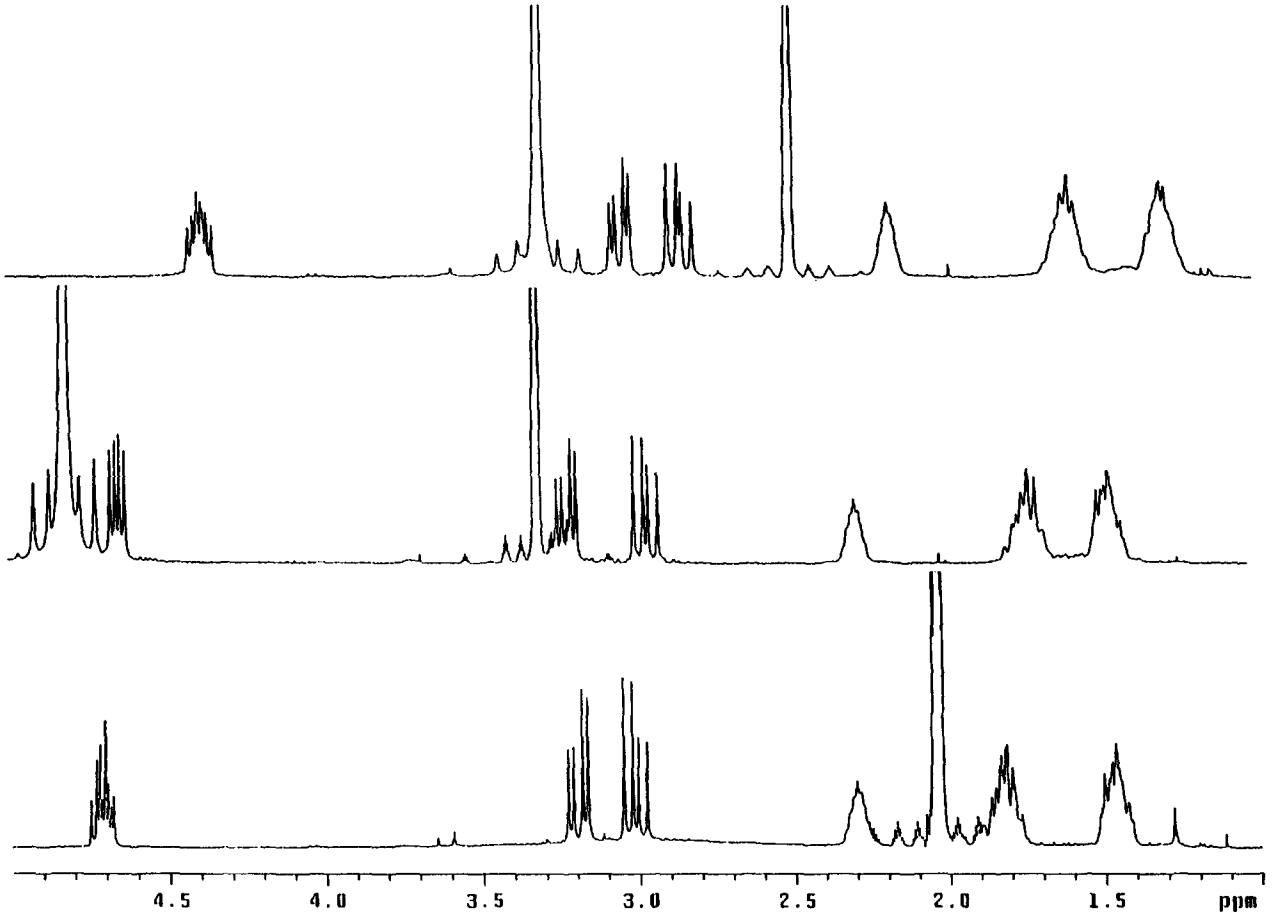


Figure 8. ^{1}H NMR spectra of **15** (10 mM) in DMSO-d_6 (top), MeOH-d_4 (middle), and acetone-d_6 (bottom) at 300 K.

penultimate structure(s) necessary for a densely-packed solid assembly. The rotational restrictions of β1, which direct a helical turn within the peptide, may also help in this dense packing process.

Conclusions

In summary, certain isomeric forms of bis L-Phe cyclohexane diamide diacids [*cis*-1,2-(**8**), *trans*-1,3-(**12**),

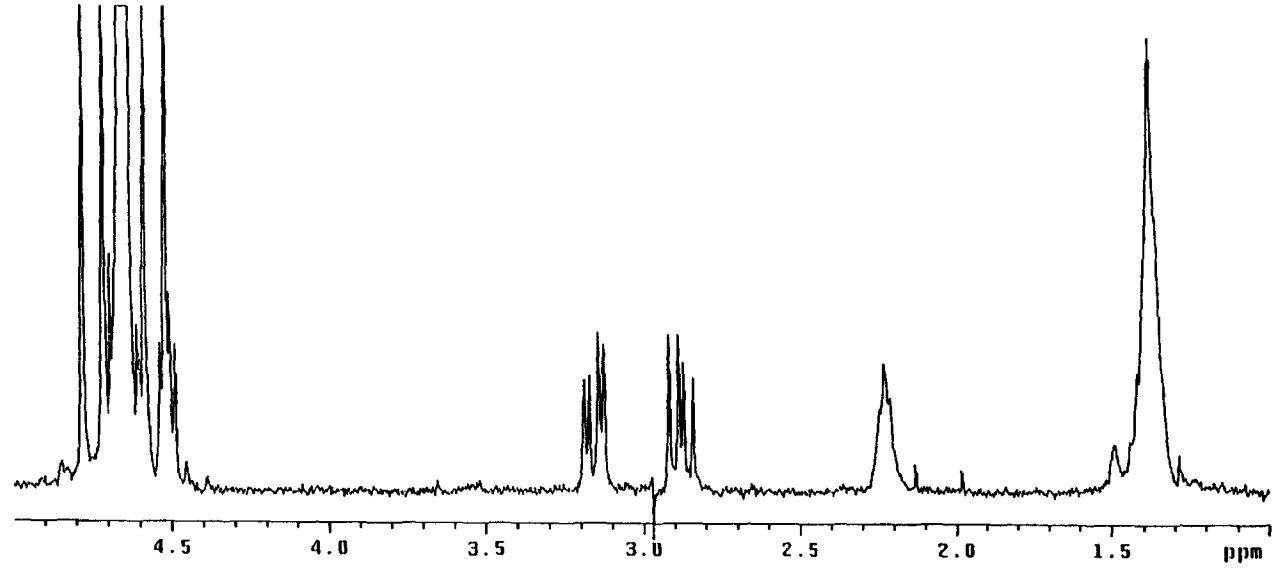


Figure 9. ^{1}H NMR spectrum of **15** (1.1 mM) in 10% D_2O/H_2O at 300 K.

and *cis*-1,4-(**15**)] were shown to form microspheres upon a pH adjustment. Structure–activity studies clearly demonstrate that a balance exists between the platforms' conformation and the ability of its conjugates with L-Phe to self-assemble into solid architectures. These microstructures were confirmed by scanning electron microscopy (SEM). In contrast, the isomeric L-Phe diamides of *trans*-1,2-(**5a** and **5b**), *cis*-1,3-(**11**), and *trans*-1,4-(**14**) cyclohexane dicarboxylic acid generated neither microcapsules nor microspheres.

To date, we have demonstrated the aqueous self-assembly of four different diamide diacid families (compounds **1–3**, **8**, **12**, and **15**) each predicated upon a distinct dicarboxylic acid platform (i.e., the diketopiperazine of L-aspartic acid,[6] malonic acid,[7] maleic acid,[7] and di-substituted cyclohexanedicarboxylic acids). Previous diamide diacid systems generated hollow geometries (i.e., microcapsules). In this report we have demonstrated conformational control of a self-assembly process to give a solid array (i.e., microspheres).

^{1}H NMR and FTIR studies hinted at how **15** may assemble into these densely packed solid motifs. The NMR and FTIR data indicate that derivative **15** (in its monomeric state in acetone) contains an intramolecularly hydrogen bonded NH. In an aqueous environment this bond would certainly be solvated. However, access to this transannular hydrogen bond may facilitate the ring flip process for derivatives containing axial pendants (even in water). Furthermore, NMR studies in 10% D_2O/H_2O confirmed the rapid ring flip of the cyclohexyl ring system in aqueous environments. We believe that the dynamic ring flip process, when coupled with the rotational restriction around β1 (which creates a 'packable' helix), allows these unique peptides to pack closely together into a solid array. Perhaps most compelling is the observation that only those derivatives which contained axial Phe ligands underwent this form of self-assembly. These results support the hypothesis that access to 'complementary' molecular geometries is needed to self-associate into solid microscopic architectures.

Experimental

General methods

All reagents were purchased from the Aldrich and Sigma Chemical Companies and were used without further purification. Silica gel 40 mm, obtained from J. T. Baker, was used for flash column chromatography. NMR spectra are listed with chemical shifts given in parts per million downfield from an internal tetramethylsilane (TMS) or 3-(trimethylsilyl)propionic 2,2,3,3-d_4 acid, sodium salt (TSP) when indicated. Mass spectra were carried out on a Kratos MS 80RFA or a Finnigan 4516 MS instrument. Optical rotations were run at 589 nm (the Na D-line) on a Perkin–Elmer 241 polarimeter, with *c* expressed as g of compound per 100 mL. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. Melting points were uncorrected. Light microscopy was performed on a camera mounted-Zeiss light microscope. SEM pictures were obtained on a Hitachi 4000 Scanning Electron Microscope.

Assembly test protocol

The diacid (0.01 mmol) was dissolved in 0.1 mL of aq Li_2CO_3 (1 M) to give a clear solution of the lithium salt in deionized water. An amount of 50 μL of this 0.1 M solution was mixed with 50 μL of 1 M citric acid and shaken. A white suspension was generated. Microscopic examination of the suspension revealed the formation of tiny spheres which moved randomly throughout the field of inspection. A wide size distribution was observed qualitatively (ranging from 10 μm to submicron diameters).

Scanning electron microscopy (SEM)

A typical procedure involved the generation of a white suspension by combining 50 μL of 500 mM citric acid and 50 μL of a 50 mM aqueous solution of the lithium salt of the peptide. The aqueous suspension was deposited on polylysine-coated glass coverslips and fixed with 2% OsO_4 for 4 h. The sample was washed with distilled water, air dried, and sputter coated with gold. SEM photographs were then obtained.

NMR experiments in water

The NMR experiments with **15** (1.1 mM) in 10% D_2O/H_2O were done both in the presence of 3-(trimethylsilyl)propionic 2,2,3,3-d_4 acid, sodium salt (TSP) and in the absence of TSP. The temperature dependence of the NH was the same in each instance (−7.4 ppb/K). However, the chemical shift of the NH was sensitive to TSP. Therefore, δ_{NH} reported in Table 3 (i.e., 7.38 ppm) was determined using TSP as an external standard. Samples studied at lower concentrations of **15** (0.5 mM) in water gave similar $\Delta\delta/\Delta T$ values.

***Trans*-1,2-bis(*N*α-L-phenylalanine benzyl ester)cyclohexane dicarboxylate (4a and 4b).** To a well-stirred solution of (+)-*trans*-1,2-cyclohexanedicarboxylic acid (0.86 g, 5.0 mmol) and L-phenylalanine benzyl ester *p*-toluenesulfonate salt (4.28 g, 10.0 mmol) in DMF (50 mL) was added benzotriazolyl-N-oxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP, 4.42 g, 10 mmol) at 0°C. The mixture was stirred for 20 min at 0°C and diisopropylethylamine (DIEA, 4.86 g, 22.0 mmol) was added dropwise over a 5-min period. The solution was allowed to warm slowly to room temperature and stirred overnight. The volatiles were removed under reduced pressure, and the residue was dissolved in 100 mL of CH_2Cl_2. The organic layer was washed successively with 50 mL aliquots of H_2O, 10% aqueous citric acid, 10% $NaHCO_3$, and H_2O. The organic layer was separated, dried over anhydrous

$MgSO_4$, filtered, and concentrated to half volume. Upon standing a white solid precipitated from the solution. This solid was filtered off to give **4a** (0.7 g). The filtrate was evaporated to dryness and the resulting solid chromatographed (10% EtOAc/hexane) to afford **4b** (0.8 g), the diastereomer of **4a**. Total yield of **4a** and **4b** was 1.50 g (47%). Recrystallization of the above products from 15% EtOAc/hexane provided analytical samples. **4a**: mp 158–160 °C. ^{1}H NMR ($CDCl_3$) δ 7.26 (m, 16H), 6.96 (m, 4H), 6.21 (d, 2H), 5.15 (q, 4H), 4.78 (m, 2H), 3.06 (m, 4H), 2.38 (m, 2H), 1.74 (m, 4H), 1.30 (m, 2H), 1.23 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 174.4, 171.0, 135.8, 135.0, 129.3, 128.4, 126.8, 66.8, 53.1, 46.4, 37.6, 29.0, 24.7. Optical rotation $[\alpha]^{28}_D$ 22° (*c* 1; $CHCl_3$). Anal. calcd for $C_{40}H_{42}N_2O_6$: C, 74.28; H, 6.55; N, 4.33; found C, 74.01; H, 6.51; N, 4.34. **4b**: mp 186–188 °C. ^{1}H NMR ($CDCl_3$) δ 7.26 (m, 16H), 7.03 (m, 4H), 6.10 (d, 2H), 5.08 (q, 4H), 4.86 (m, 2H), 3.00 (m, 4H), 2.46 (m, 2H), 1.80 (m, 4H), 1.35 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 174.4, 171.1 135.4, 135.0, 129.3, 128.4, 126.9, 67.0, 53.0, 46.5, 37.9, 29.4, 24.9. Optical rotation $[\alpha]^{28}_D$ 25° (*c* 1; $CHCl_3$). Anal. calcd for $C_{40}H_{42}N_2O_6$: C, 74.27; H, 6.55; N, 4.33; found C, 74.13; H, 6.57; N, 4.34.

***Trans*-1,2-bis(*N*α-L-phenylalanine)cyclohexane dicarboxylate (5a).** The dibenzyl ester **4a** (0.37 g, 0.57 mmol) and 10% Pd–C (0.05 g) were suspended in absolute MeOH (100 mL). The suspension was degassed three times, and hydrogen gas was introduced. The absorption of hydrogen ceased in 2 h. TLC (15% EtOAc/hexane) showed no starting material remaining after 15 min. The black suspension was filtered, and the filtrate was concentrated to give **5a** as a white solid (0.27 g, 100%); mp 202–203 °C. ^{1}H NMR (CD_3OD) δ 7.26 (m, 10H), 4.62 (m, 2H), 3.13 (m, 4H), 2.44 (m, 2H), 1.71 (m, 4H), 1.25 (m, 4H); ^{13}C NMR (CD_3OD) δ 176.6, 174.1 138.5, 130.5, 129.3, 127.6, 54.5, 47.1, 38.6, 30.8, 26.2. Optical rotation $[\alpha]^{28}_D$ 65° (c 1.0; MeOH). Anal. calcd for $C_{26}H_{30}N_2O_6$: C, 66.94; H, 6.48; N, 6.01; found C, 66.78; H, 6.51; N, 5.99.

***Trans*-1,2-bis(*N*α-L-phenylalanine)cyclohexane dicarboxylate (5b).** The dibenzyl ester **4b** (0.50 g, 0.77 mmol) was combined with 10% Pd–C (0.05 g) in degassed CH_3OH (100 mL), and H_2 gas was introduced. After 1 h the black suspension was filtered, and the filtrate was concentrated to give a white crystalline product **5b** (0.36 g, 100%); mp 233–235 °C. ^{1}H NMR (CD_3OD) δ 7.06 (m, 10H), 4.39 (m, 2H), 2.77 (m, 4H), 2.39 (m, 2H), 1.70 (m, 4H), 1.19 (m, 4H); ^{13}C NMR (CD_3OD) δ 177.2, 174.6, 138.1, 130.4, 129.3, 127.7, 55.0, 47.3, 38.4, 30.7, 26.3. Optical rotation $[\alpha]^{28}_D$ 29° (*c* 1.0; MeOH). Anal. calcd for $C_{26}H_{30}N_2O_6$: C, 66.94; H, 6.48; N, 6.01; found C, 66.76; H, 6.46; N, 6.07.

***Cis*-1-carboxy-2-(Nα-L-phenylalanine benzyl ester)-cyclohexane carboxylate (6).** To a well-stirred solution of *cis*-1,2-cyclohexane dicarboxylic anhydride (1.54 g, 10.0 mmol) and L-phenylalanine benzyl ester-*p*-toluenesulfonate salt (4.28 g, 10.0 mmol) in DMF (40 mL) was added 4-methylmorpholine (2.5 mL) dropwise at 0 °C. The mixture was stirred and warmed to room temperature overnight. Evaporation in vacuum provided a white solid, which was dissolved in CH_2Cl_2 (100 mL), washed with H_2O (4 × 40 mL), dried over anhydrous $MgSO_4$, and filtered. Removal of the solvent from the filtrate gave a white powder. Column chromatography (40% EtOAc/hexane) gave mono amide acid **6** (3.2 g, 78%); mp 107–108 °C. ^{1}H NMR ($CDCl_3$) δ 7.16 (m, 10H), 6.23 (m, 1H), 5.10 (dd, 2H), 4.92 (m, 1H), 3.10 (d, 2H), 2.81 (m, 2H), 2.00 (m, 2H), 2.60 (m, 6H); ^{13}C NMR ($CDCl_3$) δ 177.8, 173.4, 171.0, 135.1, 128.8, 128.1, 126.5, 66.8, 52.6, 43.3, 42.1, 37.3, 26.4, 23.0. $[\alpha]^{24}_D$ 20° (*c* 1.0; $CHCl_3$). Anal. calcd for $C_{24}H_{26}N_1O_5$: C, 70.57; H, 6.42; N, 3.43; found C, 70.42; H, 6.48; N, 3.49.

***Cis*-1,2-bis(*N*α-L-phenylalanine benzyl ester)cyclohexane carboxylate (7).** BOP (1.33 g, 3.0 mmol) was added to a solution of **6** (1.22 g, 3.0 mmol), L-phenylalanine benzyl ester *p*-toluenesulfonate salt (1.28 g, 3.0 mmol) and DMF (50 ml) at 0 °C. The mixture was stirred for 20 min. Diisopropylethylamine (DIEA, 2.71 g, 21 mmol) was added dropwise. The resulting mixture was stirred and warmed to room temperature overnight. The volatiles were removed under pressure. The remaining oil was dissolved in 100 mL of CH_2Cl_2; washed with 50 mL of H_2O, 10% aq citric acid, 10% aq $NaHCO_3$ and H_2O; dried over anhydrous $MgSO_4$; and filtered. Evaporation of the filtrate followed by flash chromatography (40% EtOAc/hexane) gave the diamide dibenzyl ester **7** as a white solid (1.4 g, 72%); mp 107–108 °C. ^{1}H NMR ($CDCl_3$) δ 7.18 (m, 20H), 6.41 (m, 2H), 5.11 (m, 4H), 3.06 (dd, 4H), 2.62 (m, 2H), 1.99 (m, 2H), 1.62 (m, 4H), 1.32 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 171.8, 171.5, 169.3, 133.9, 133.1, 127.3, 126.5, 124.9, 65.0, 51.1, 42.3, 41.7, 35.7, 25.0, 21.4. Optical rotation $[\alpha]^{24}_D$ 20°, (*c* 1; $CHCl_3$). Anal. calcd for $C_{40}H_{42}N_2O_6$: C, 74.28; H, 6.55; N, 4.33; found C, 74.40; H, 6.53; N, 4.35.

***Cis*-1,2-bis(*N*α-amido-L-phenylalanine)cyclohexane diamide (8).** The dibenzyl ester 7 (0.97 g, 1.5 mmol) and 10% Pd–C (0.15 g) were suspended in degassed CH_3OH (100 mL). Hydrogen gas was introduced. TLC (40% EtOAc/hexane) was used to monitor the reaction. After 2 h the black suspension was filtered and the filtrate concentrated to give the diamide diacid **8** as a white crystalline solid (0.70 g, 100%); mp 88–90°C. ^{1}H NMR (DMSO-d_6) δ 7.82 (m, 2H), 7.18 (m, 10 H), 4.40 (m, 2H), 2.92 (m, 4H), 1.90 (m, 2H), 1.42 (m, 4H), 1.21 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 173.6, 173.1, 172.0, 137.6, 129.1, 128.0, 126.4, 53.3, 42.8, 36.8, 27.1, 26.5, 23.2. Optical rotation $[\alpha]^{24}_D$ 33°, (*c* 1; $MeOH_3$). Anal. calcd for $C_{26}H_{30}N_2O_6$: C, 66.94; H, 6.48; N, 6.01; found C, 66.72; H, 6.53; N, 6.05.

***Cis*- and *trans*-1,3-bis(*N*α-L-phenylalanine benzyl ester) cyclohexane dicarboxylate (9 and 10a,b).** The

1,3-cyclohexane derivatives were prepared in a similar manner as their 1,4 counterparts (i.e., compounds **13** and **14**). Since the starting 1,3-cyclohexane dicarboxylic acid (2.0 g, 11.6 mmol) was a mixture of *cis* and *trans* isomers, three isomers were generated in the product mixture of diamides (5.5 g, 73%). An analytical sample of the *cis*-isomer **9** was isolated by recrystallization from $CHCl_3$. A sample of the *trans*-diastereomeric mixture **10a,b** was isolated by column chromatography using a trisolvent system (30% EtOAc/40% hexane/30% $CHCl_3$, R_f =0.31) on silica gel. *Cis*-1,3 (**9**): mp 192–194 °C. ^1H NMR ($CDCl_3$) δ 7.37 (m, 10H), 7.21 (m, 6H), 6.97 (m, 4H), 5.90 (broad s, 2H, NH), 5.15 (m, 4H), 4.90 (m, 2H), 3.11 (broad s, 4H), 2.08 (br t, 2H), 1.95 (d, 1H), 1.80 (m, 3H), 1.55 (q, 1H), 1.29 (m, 3H). Optical rotation $[\alpha]^{28}_D$ 30° (*c* 0.32; $CHCl_3$). Anal. calcd for $C_{40}H_{42}N_2O_6$: C, 74.28; H, 6.55; N, 4.33; found C, 74.39; H, 6.62; N, 4.38. Diastereomeric mixture of *trans*-1,3 isomers (**10a,b** mixture): mp 85–88 °C. ^1H NMR ($CDCl_3$) δ 7.37 (m, 16H), 6.97 (m, 4H), 5.90 (dd, 2H, NH), 5.16 (m, 4H), 4.87 (m, 2H), 3.10 (m, 4H), 2.41 (m, 2H), 1.79 (d, 2H), 1.55 (m, 4H), 1.42 (m, 2H). Optical rotation $[\alpha]^{25}_D$ 9° (*c* 0.5; $CHCl_3$). High-resolution mass spectrum for $C_{40}H_{42}N_2O_6$: theory 646.3043, found 646.3161.

***Cis*-1,3-(bis-*N*α-amido-L-phenylalanine)cyclohexane dicarboxylate (11).** Hydrogenation of **9** (0.69 g, 1.07 mmol) over 10% Pd–C (0.10 g) in 100 mL MeOH gave the *cis*-1,3-diamide diacid **11** as a white solid (0.52 g, 100%); mp 192–193 °C. ^1H NMR (CD_3OD) δ 7.23 (m, 10H), 4.65 (m, 2H), 3.18 (m, 2H), 2.93 (m, 2H), 2.18 (t, 2H), 1.70 (m, 4H), 1.30 (m, 4H); ^{13}C NMR (CD_3OD) δ 177.93, 177.86, 174.86, 138.53, 138.48, 130.34, 130.33, 129.45, 129.40, 127.82, 127.77, 54.73, 54.68, 45.41, 45.31, 38.45, 38.43, 33.08, 29.92, 29.61, 25.96. Optical rotation $[\alpha]^{23}_D$ 15° (*c* 1; MeOH). Anal. calcd. for $C_{26}H_{30}N_2O_6$: C, 66.94; H, 6.48; N, 6.01; found C, 66.65; H, 6.50; N, 5.90.

***Trans*-1,3-(bis-*N*α-amido-L-phenylalanine)cyclohexane dicarboxylate (12a,b).** Hydrogenation of mixture **10a,b** (0.32 g, 0.5 mmol) over 10% Pd–C in EtOH gave the diamide diacid **12** as an oil (0.23 g, 99%). Recrystallization from 50% EtOAc/hexane gave the enhanced 60:40 mixture **12** as a white solid. ^1H NMR (CD_3OD) δ 7.20 (m, 10H), 4.65 (m, 2H), 3.20 (m, 2H), 2.95 (m, 2H), 2.50 (m, 2H), 1.72 (t, 1H), 1.58 (m, 6H), 1.41 (m, 1H); ^{13}C NMR revealed the enhancement of one diastereomer after recrystallization. The 60:*40* mixture was used in all assembly experiments. The minor isomer is in italics. ^{13}C NMR (CD_3OD) δ 178.15, *178.13*, *175.02*, 174.94, *138.67*, 138.65, 130.31, 129.45, *129.42*, 127.79, *127.75*, 58.86, *54.73*, *40.64*, 40.59, 38.41, 31.22, *30.90*, *29.45*, 29.32, 22.64, *22.53*. Optical rotation $[\alpha]^{24}_D$ 5° (*c* 1; MeOH). High-resolution mass spectrum for M+H ($C_{26}H_{31}N_2O_6$): theory 467.2182, found 467.2156.

***Cis*-1,4-bis(*N*α-amido-L-phenylalanine benzyl ester)-cyclohexane dicarboxylate (13).** The *cis*-isomer was prepared in a similar manner as its *trans*-counterpart **14**. Column chromatography (5% acetone/$CHCl_3$, R_f = 0.14) gave pure **13** (76%); mp 102–104 °C. ^1H NMR ($CDCl_3$) δ 7.35 (m, 10H), 7.20 (m, 6H), 7.00 (m, 4H), 5.95 (d, 2H), 5.20 (q, 4H), 4.98 (dt, 2H), 3.19 (m, 4H), 2.13 (m, 2H), 1.82 (m, 4H), 1.60 (m, 4H). Optical rotation $[\alpha]^{22}_D$ 23° (*c* 0.5; $CHCl_3$). Anal. calcd for $C_{40}H_{42}N_2O_6$: C, 74.28; H, 6.55; N, 4.33; found C, 74.00; H, 6.50; N, 4.57.

***Trans*-1,4-bis(*N*α-amido-L-phenylalanine benzyl ester)cyclohexane dicarboxylate (14).** Diphenylphosphoryl azide (DPPA, 6.71 g, 24.4 mmol) was added dropwise to a solution of DMF (75 mL), *trans*-cyclohexane dicarboxylic acid (2.0 g, 11.6 mmol), and L-phenylalanine benzyl ester *p*-toluenesulfonate salt (10.44 g, 24.4 mmol) cooled to 0 °C. The mixture was stirred for 15 min at 0 °C and DIEA (6.60 g, 51.1 mmol) was added dropwise over a 5 min period. The solution was allowed to warm slowly to room temperature and stirred overnight. The volatiles were removed under reduced pressure; the residue was dissolved in 150 mL CH_2Cl_2. The cloudy solution was filtered to give a white solid and a yellow filtrate. The solid was washed with EtOAc and dried to give 1.43 g of **14**. The filtrate was washed with 1 N HCl, water, 10% $NaHCO_3$, and water. The organic layer was separated, dried, filtered, and concentrated to give a yellow solid. Flash column chromatography (3% EtOH/$CHCl_3$) gave additional *trans*-diamide **14** (4.22 g). Total yield of **14** was 5.65 g (75%); mp 211–213 °C. ^1H NMR (DMSO-d_6/$CDCl_3$, 3:1) δ 8.10 (d, 2H, NH), 7.26 (m, 20H), 5.08 (s, 4H), 4.55 (m, 2H), 3.00 (m, 4H), 2.10 (m, 2H), 1.65 (m, 4H), 1.30 (m, 4H). Optical rotation $[\alpha]^{21}_D$ 30° (*c* 1; $CHCl_3$). Anal. calcd for $C_{40}H_{42}N_2O_6$: C, 74.28; H, 6.55; N, 4.33; found C, 74.12; H, 6.54; N, 4.30.

***Cis*-1,4-bis(*N*α-L-phenylalanine)cyclohexane dicarboxylate (15).** The *cis*-isomer was prepared in a similar manner as its *trans*-counterpart, compound **16.** The reduction was carried out in degassed MeOH and stirred under H_2 for 4 h. TLC (10% EtOH/$CHCl_3$) was used to monitor the reaction. The suspension was filtered and concentrated to give **15** as a white solid (2.7 g, 99%). An analytical sample was obtained by recrystallization from 8% MeOH/EtOAc; mp 185–186 °C. ^1H NMR (DMSO-d_6) δ 7.82 (d, 2H), 7.20 (m, 10H), 4.35 (m, 2H), 3.05 (m, 2H), 2.82 (m, 2H), 2.18 (m, 2H), 1.60 (m, 4H), 1.25 (m, 4H); ^{13}C NMR (DMSO-d_6) δ 174.42, 173.10, 137.72, 128.92, 127.93, 126.16, 52.98, 40.19, 36.57, 26.04, 25.65. FTIR (KBr pellet) 3366, 2953, 1738, 1622, 1539 cm^{-1}. High-resolution mass spectrum: ($C_{26}H_{31}N_2O_6$, M+H) theory 467.2182, found 467.2156. Optical rotation $[\alpha]^{21}_D$ 26° (*c* 1; MeOH). Anal. calcd for $C_{26}H_{30}N_2O_6$: C, 66.94; H, 6.48; N, 6.01; found C, 66.79; H, 6.51; N, 5.94.

***Trans*-1,4-bis(*N*α-L-phenylalanine)cyclohexane dicarboxylate (16).** The dibenzyl ester **14** (1.33 g, 2.06 mmol) and 10% Pd–C (0.1 g) were suspended in

absolute EtOH (200 mL). The suspension was degassed three times, and hydrogen gas was introduced. TLC (4% EtOH/$CHCl_3$) showed no starting material remaining after 90 min at rt. The black suspension was filtered, and the filtrate was concentrated to give **16** as a white solid (0.88 g, 92%); mp 249–251 °C. ^1H NMR (CD_3OD) δ 7.21 (m, 10H), 4.64 (dd, 2H, J^3_{H-H} = 9.3 Hz, J^3_{H-H} = 4.9 Hz), 3.21 (dd, 2H, J^2= 13.7 Hz, J^3= 9.5 Hz), 2.13 (m, 2H), 1.80 (d, 2H, J^3= 7.55 Hz), 1.56 (d, 2H, J^3= 7.3 Hz), 1.38 (m, 2H), 1.35 (m, 2H). Optical rotation $[\alpha]^{21}_D$ 3° (*c* 0.5; MeOH). Anal. calcd for $C_{26}H_{30}N_2O_6$: C, 66.94; H, 6.48; N, 6.01; found C, 66.65; H, 6.55; N, 5.98.

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