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Solution Structure of the Antitumor Candidate Trunkamide A by 2D NMR and Restrained Simulated Annealing Methods

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Trunkamide A (**1**) is a cyclic heptapeptide extracted from the ascidian *Lissoclinum* sp. and has shown very promising cytotoxic activity. This compound incorporates several of the motifs commonly observed in the Patellin family, including dimethylallyl (Dma) Thr and Ser side chains and a thiazoline heterocycle. Given that little is known about the structures adopted by the cyclopeptides of the Patellin family, and with the aim of establishing structure−activity relationships, we have carried out the conformational analysis of trunkamide A by a combination of 2D NMR experiments and simulated annealing calculations. Our results show that the conformation of **1** is very rigid and is dominated by the volume of the dimethylallyl side chains and two trans-annular hydrogen bonds. We have also studied the conformation of **2**, the L-Phe diastereoisomer of **1**, the analysis of which provides a possible rationale for its epimerization to **1**, a process that is observed in solution. Finally, we show how a thorough NMR characterization can be used, in combination with simulated annealing methods, to confirm the configuration of a stereogenic center in the backbone of a rigid cyclic peptide such as trunkamide A (**1**).

Introduction

Marine organisms are proving to be a promising source of pharmaceutically interesting cyclic compounds.[1] Ascidians and tunicates[2] in particular produce several compounds that, due to their cytotoxic activity, have become candidate drugs for cancer therapy. For example, ecteinascidine-743[3] and aplidin,[4] both isolated from ascidians, are now in advanced phase III and phase II clinical trials, respectively. Another very relevant marine natural product with interesting cytotoxic activity is kahalalide F,[5] which was isolated from the Sacoglossan mollusk *Elysia rufescens* and the green alga *Bryopsis* sp. This compound is now in phase I clinical trials.

The ascidian *Lissoclinum patella* is of particular interest because various families of active cyclic peptides have been extracted from it. The Lissoclinamides,[6] which are a structural type of cyclic heptapeptide that contains one oxazoline and two thiazole (or thiazolines) units, have been the object of comprehensive synthetic, structural, and pharmacological research aimed at deriving very important structure−activity relationships.[7] Another very interesting structural type is represented by the Patellins,[8] whose most important feature is the presence of two dimethylallyl (Dma) Thr (or one Thr and one Ser) side chains and one thiazoline ring in the backbone. Among the moderately cytotoxic Patellins, trunkamide A (**1**) stands out due to its unique activity: it was initially selected by the National Cancer Institute for further testing due to a good COMPARE correlation analysis and specificity against the UO-31 renal cell line, which is a multidrug-resistant (MDR) line. Trunkamide A (**1**), like most members of the Patellin family, was isolated in 1996 by Bowden and co-workers,[8] but the structure initially suggested was incorrect in terms of the configuration of the Phe residue. The total synthesis performed by Wipf[9] showed that the correct configuration was D by comparison of the synthetic and natural products. Furthermore, a solid phase synthesis and preliminary characterization of trunkamide A[10] by our group confirmed the configuration proposed by Wipf. Cyclic peptides extracted from *Lissoclinum patella* tend to present very rigid conformations that have been shown to be essentially the same in solution and in the solid state.[11] The presence of between one and four conformationally restricted residues in the primary structure of the peptide is, especially in the Lissoclinamide family, the most important source of

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(1) Wipf, P. *Chem. Rev.* **1995**, *95*, 2115−2134.
(2) Davidson, B. S. *Chem. Rev.* **1993**, *93*, 1771−1791.
(3) (a) Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Stroh, J. G.; Keifer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. *J. Org. Chem.* **1990**, *55*, 4512−4515. (b) Rinehart, K. L.; Holt, T. G.; Fregeau, N. L.; Stroh, J. G.; Keifer, P. A.; Sun, F.; Li, L. H.; Martin, D. G. *J. Org. Chem.* **1990**, *55*, 4512−4515.
(4) Schmitz, F. J.; Yasumumoto, T. *J. Nat. Prod.* **1991**, *54*, 1469−1490.
(5) López-Macià, A.; Jiménez, J. C.; Royo, M.; Giralt, E.; Albericio, F. *J. Am. Chem. Soc.* **2001**, *123*, 11398−11404.
(6) Wipf, P.; Fritch, P. C. *J. Am. Chem. Soc.* **1996**, *118*, 12358−12367.
(7) Wipf, P.; Fritch, P. C.; Geib, S. J.; Sefler, A. M. *J. Am. Chem. Soc.* **1998**, *120*, 4105−4112.
(8) Carroll, A. R.; Coll, J. C.; Bourne, D. J.; MacLead, J. K.; Zahriskie, T. M.; Ireland, C. M.; Bowden, B. F. *Aust. J. Chem.* **1996**, *49*, 659−667.
(9) Wipf, P.; Uto, Y. *J. Org. Chem.* **2000**, *65*, 1037−1049.
(10) Caba, J. M.; Rodríguez, I. M.; Manzanares, I.; Giralt, E.; Albericio, F. *J. Org. Chem.* **2001**, *66*, 7568−7574.

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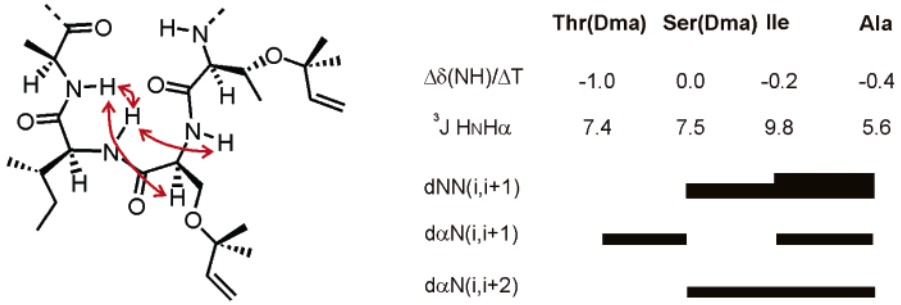
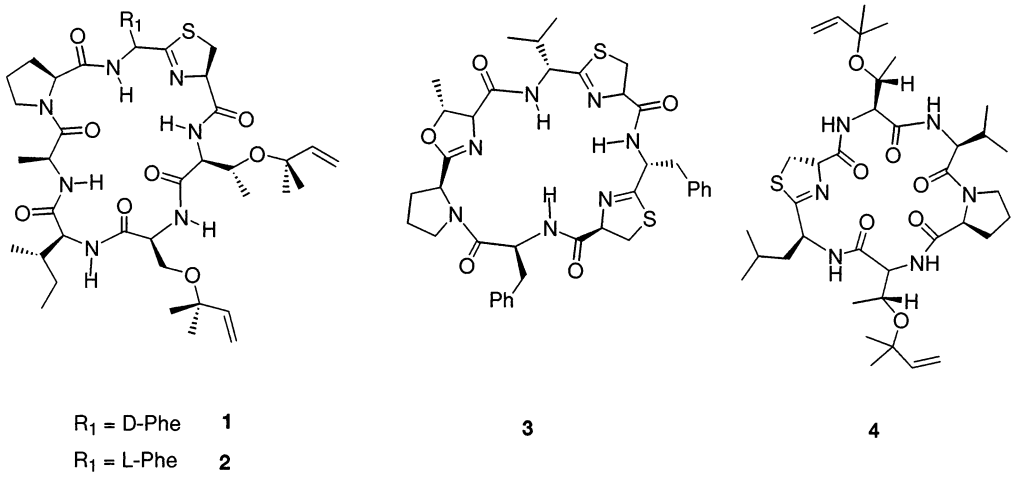


FIGURE 1. Summary of the NMR evidence for the formation of a β-turn in trunkamide A. The width of the bars is proportional to the volume of the cross-peaks in the NOESY spectrum. Temperature coefficients are expressed in ppb/K and coupling constants in Hz.

SCHEME 1. Representative Structures of Peptides Extracted from *Lissoclinum patella*: Trunkamide A (1), Compound 2 (the diastereoisomer of 1 where D-Phe is replaced by L-Phe and which was described by Bowden as trunkamide A), Lissoclinamide 7[7] [from the Lissoclinamide family] (3), and Patellin 2[10] (4, the only member of the Patellin family whose three-dimensional structure is known)



rigidity.[12] However, the effect that bulky or β-branched residues such as Thr, Val, or Ile can have on the overall conformation of the macrocyclic backbone cannot be overlooked, especially in the light of the high frequency with which these types of side chains are encountered in these compounds. Trunkamide A, as a member of the Patellin family, contains only one thiazoline cycle and would therefore be expected to have a rather flexible conformation. In the context of our efforts to obtain structure−activity relationships within the Patellin family we present here the three-dimensional structure of trunkamide A (**1**) and that of its L-Phe diastereoisomer (**2**), as obtained by restrained simulated annealing calculations. The results of this study highlight the power of these techniques both for understanding the properties of these closely related compounds and for the confirmation of the stereochemistry in natural rigid cyclic peptides.

Results and Discussion

A single set of resonances was obtained for trunkamide A despite the presence of a Pro residue in the sequence of this peptide, which indicates that there is no *cis*−*trans* isomerization around the Ala−Pro peptide bond and suggests that the macrocyclic ring is relatively rigid compared to the other member of the Patellin family for which the structure is known, i.e., patellin 2.[11] The presence of strong NOEs between the δ protons of Pro and the α and β protons of Ala, together with the absence of an NOE between the α protons of those residues, shows that, as expected, the Ala−Pro amide bond is *trans*, like the rest of the amide bonds of trunkamide A.

The most relevant backbone-to-backbone NOEs in the Thr(Dma)-Ser(Dma)-Ile-Ala segment of trunkamide A are shown in Figure 1. The NOE pattern observed for these residues clearly indicates the formation of a β-turn, which is confirmed by the presence of NOE contacts between the γ-methyl of Thr and all the amide protons of this segment (Figure 2).[13] The other segment of the macrocycle is less well-defined in terms of number of NOEs: the fact that two of the residues in this segment of the macrocycle are N-alkyl residues severely diminishes the number of interesting NOEs that can be observed and, at the same time, severely restricts the number of possible conformations available to the backbone. An indication of the rigidity of this part of the cycle comes from the observation that the side-chain protons of the

(11) Abbenante, G.; Fairlie, D. P.; Gahan, L. R.; Hanson, G. R.; Pierens, G. K.; van den Brenk, A. L. *J. Am. Chem. Soc.* **1996**, *118*, 4−10388.

(12) Zabriskie, T. M.; Foster, M. P.; Stout, T.; Clardy, J.; Ireland, C. M. *J. Am. Chem. Soc.* **1990**, *112*, 8080−8084.

(13) Wüthrich, K. *NMR of Proteins and Nucleic Acids*; Wiley: New York, 1986.

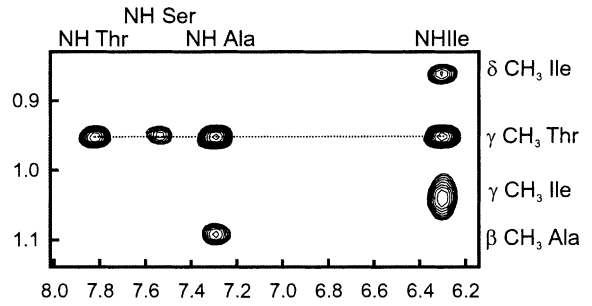


FIGURE 2. Long-range NOEs between the γ-CH_3 of Thr and the amide protons of Ser, Ala, and Ile.

Phe residue have restricted rotation: the $^3J\alpha\beta$ coupling constants and the intraresidue NOEs to the α and NH protons are very different for the two β protons of the Phe side chain. The rest of the coupling constants confirm the qualitative assignment of the secondary structure. The $^3J\alpha$N values for the residues involved in the β-turn show that the β-turn is of type I, albeit slightlty distorted, as evidenced by the presence of NOE contacts between the Me at γ of Thr and the NH (Ile). The value of the $^3J\alpha$N coupling for Ile (9.8 Hz) is in agreement with the presence of a type I β-turn, whereas a value of 7.5 Hz for Ser, the residue in the $i + 1$ position of the β-turn, is almost 3 Hz larger than expected. Strikingly, all amide protons in trunkamide A have very low temperature coefficients[14] (e.g., lower or equal to 1 ppb/K in absolute value) except for the amide proton of the Phe residue. This trend, which is exceptional in cyclic peptides of this size, indicates that trunkamide A has a very rigid conformation that is stabilized by hydrogen bonds, a situation in agreement with the NOE and ^3J data.

A quantitative treatment of the NMR data presented here has allowed the determination of the structure of trunkamide A in solution. A total of 48 restraints were implemented in a simulated annealing protocol, which yielded 50 structures. The 14 structures of lowest restraint violation are displayed in Figure 3A. A detailed analysis of the 14 structures shows that two families of conformations fulfill the restraints, but that they have the same fold and only differ in the conformation of the thiazoline ring, a feature that is already evident in the solid state structure of patellin 2.[11]

The most representative structure of the ensemble, shown in Figure 3B, displays the secondary structure elements qualitatively inferred from the NMR data. A type I distorted β-turn is present in the Thr-Ser-Ile-Ala segment and the γ-CH_3 of Thr points toward the center of the β-turn. The nature of the side chains of the residues in positions $(i + 1)$ and $(i + 2)$ determines to a large extent the type of β-turn that can be formed by four residues in proteins and cyclic peptides.[15] The L configuration and the absence of Gly residues in these positions typically leads to the formation of the most common type I β-turn, where the side chains of the residues at $(i + 1)$ and $(i + 2)$ lie in a "pseudoaxial" arrangement on the same side of the plane defined by the turn. Inspection of the 3D structure reveals that the distortion of the turn is caused by the large volume of the Ser(Dma) and Thr-

(14) Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1982**, *21*, 512−523.
(15) Wilmot, C. M.; Thornton, J. *Protein Eng.* **1990**, *3*, 479−493.

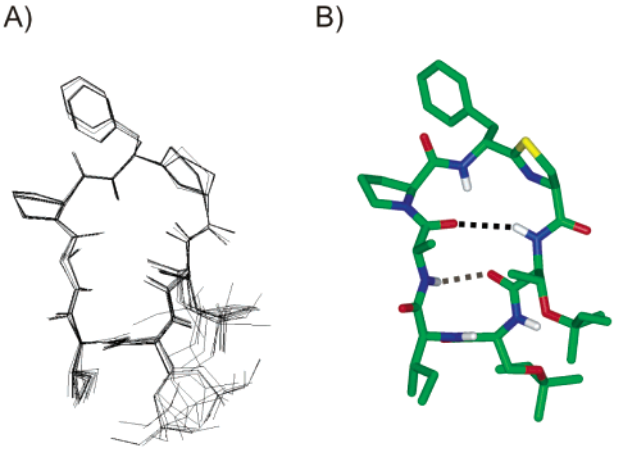


FIGURE 3. (A) Superimposition of the 14 most representative structures of trunkamide A obtained by restrained simulated annealing. Only heavy atoms and amide hydrogens are displayed for clarity. (B) Most respresentative structure of the ensemble.

(Dma) side chains. Although the configuration of the Ser and Ile residues of trunkamide A suggests the formation of a canonical type I β-turn, the large size of the dimethylallyl Ser and β branched Ile side chains precludes the pseudoaxial arrangement and distorts the turn. The low-temperature coefficients of the amide protons of Ala and Thr are caused by the formation of two trans-annular hydrogen bonds, which help to stabilize the structure of trunkamide A. The low values for Ser and Ile are more difficult to interpret but are very probably due to the formation of hydrogen bonds between these amide protons and the oxygen atoms in the Thr and Ser side chains, respectively.

All structures of the ensemble display the same overall fold in the Pro-Phe-Tzn segment of the macrocycle despite the relatively low number of distance restraints that were imposed. This situation should be regarded as a consequence of the rigidity imparted by the Pro and thiazoline cycles on the backbone. As a result of its D-configuration, the side chain of Phe faces the side-chain-poor face of the macrocycle but adopts what can be considered as a "pseudoequatorial" position in which NOEs cannot be observed between the protons of the side chain and the other protons of the peptide. Furthermore, and in contrast with the other amide protons of the backbone, the amide proton in Phe has a very high temperature coefficient, which indicates that it is hydrogen bonded to the solvent. Overall, the structure of trunkamide A is pseudoplanar and is very rigid for a heptapeptide of this size. The structure is dominated by the size of the dimethylallyl side chains of Ser and Thr and by the formation of two trans-annular hydrogen bonds.

As mentioned before, when trunkamide A was first isolated in 1996, the configuration of the Phe unit was incorrectly assigned.[8] The total synthesis of trunkamide A by Wipf and Uto[9] and by our group[10] showed, by comparison with a natural sample, that the correct configuration of the Phe residue was D. To study the effect of changing the configuration of this chiral center, the L-Phe diastereoisomer (**2**) of trunkamide A (**1**) was prepared using a solid phase approach totally analogous to the one used in our recent solid phase synthesis of

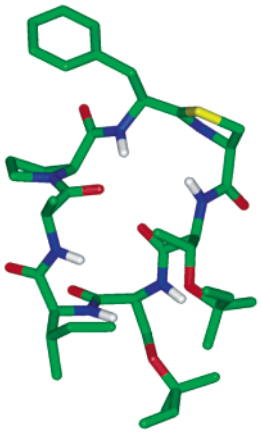


FIGURE 4. Most representative structure of the ensemble of 14 structures calculated for **2** (the usual color scheme is used).

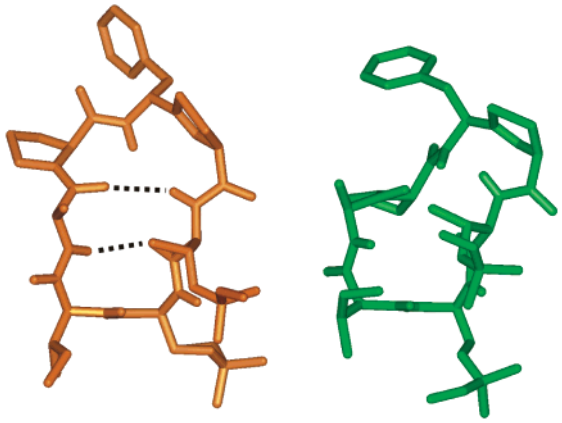


FIGURE 5. Comparison of the structures of trunkamide A (**1**) (brown) and its L-Phe diastereoisomer (**2**) (green). The presence of hydrogen bonds is indicated by a black dotted line.

trunkamide A.[10] As commonly observed in strongly structured cyclic peptides with rigid conformations, the L-Phe diastereoisomer (**2**)[7] epimerized to the natural diastereoisomer (**1**) within days and, as a consequence, we were able to carry out only one NOESY experiment with **2**. This experiment, however, yielded enough distance restraints to calculate its solution structure.

Inspection of the NOE data for **2** (data not shown) reveals that the β-turn is essentially preserved in the L-epimer but that major rearrangements must take place on the other end of the macrocycle, which is where the Phe unit is located. The most striking observation is the number of NOEs displayed by the amide proton of Phe, which in the natural peptide fails to show any long-range contact except for a weak NOE with the amide proton of Thr. Not only is the intensity of this NOE higher in **2** but the amide proton of Phe has contacts with the amide and β methyl of Ala and γ methyl of Thr. These contacts clearly indicate that the L-Phe diastereoisomer (**2**) displays a very different conformation in this part of the macrocycle compared to the natural product.

Calculation of the 3D structure of the L-epimer of trunkamide A was carried out using exactly the same protocol as developed for the determination of the structure of the natural product **1** with the exception that in this case only 20 NOE-derived distance restraints were used, and a representative structure of the family of 14 conformations of **2** is shown in Figure 3. Inspection of the NMR structure of trunkamide A (**1**) (Figure 3) shows that, due to its configuration, the side chain of Phe does not share the same side of the macrocycle as the L side chains of the peptide. The change of configuration, however, reverses this situation: all the side chains of peptide **2** are now on the same side of the macrocycle, and the backbone adopts a saddle structure that is reminiscent of the solid state structure adopted by patellin 2.[12] The curvature of the saddle allows the amide proton of Phe to approach the side chains with which it shows NOE contacts. As can be seen in Figure 4, the amide proton of Phe, which in the natural compound is in the plane of the macrocycle, in the structure of **2** is pointing toward the center of the saddle, where it contacts the side chains of Ala and Thr. Interestingly, as can readily be seen in Figure 5, the most important difference between the two solution structures presented here is the absence of trans-annular hydrogen bonds in the nonnatural diastereoisomer. This result offers a rationale for the epimerization of **2** observed in solution. Indeed, the formation of these two trans-annular hydrogen bonds possibly acts as a driving force for the epimerization reaction, which yields a more planar, less congested, and more stable solution structure.

The complete characterization of marine cyclic peptides is a challenge for the natural products chemist. These compounds are rich in nonnatural amino acids for which the configuration is often very difficult to determine using the traditional degradation and derivatization techniques. The synthesis of compounds of this type is also particularly challenging,[1] a feature that can be related to the rigidity shown by most of the compounds that have be subjected to a thorough conformational analysis, and render the characterization of the configuration by the synthesis of the putative structure particularly costly. A combination of NMR experiments and simulated annealing calculations can, however, take advantage of this rigidity and be used as a technique for assigning the configuration of stereogenic centers in the backbone of rigid cyclic peptides. In these cases, after a comprehensive NMR characterization of the natural product, it is useful to carry out restrained simulated annealing calculations with floating chirality in the stereogenic center of unknown configuration. A statistical and energetical analysis of the ensemble of conformations can then provide an answer to the stereochemical problem.[16] Another possibility is to carry out the restrained simulated annealing calculation without floating chirality, but in this case two calculations need to be carried out, one for each of the two putative structures. Assuming that the peptide is relatively rigid, the set of experimental restraints should yield only a single correct ensemble, with no significant restraint violation and reasonable total energy, when the proposed structure matches that of the natural compound.

Given the initial uncertainty regarding the configuration of the Phe residue in trunkamide A, we decided to take advantage of our thorough NMR characterization

(16) Constantine, K. L.; Mueller, L.; Huang, S.; Abid, S.; Lam, K. S.; Li, W.; Leet, J. E. *J. Am. Chem. Soc.* **2002**, *124*, 7284−7285.

of this product to test whether the second approach could have been used in this case. We applied the set of restraints derived from the NMR experiments of the natural product in a simulated annealing calculation in which the model of trunkamide A had the configuration of the Phe incorrectly set to L. The results of the structure calculation are far less satisfactory than those obtained for the correct configuration (1.23 kcal·mol^{-1} average restraint violation with the incorrect configuration vs 0.23 kcal·mol^{-1} with the correct one and a total energy difference of 5.65 kcal·mol^{-1}). If the same methodology is applied to the determination of the configuration of the L-epimer, a similar result is obtained, although the energy difference is lower in this case due to the lack of dihedral restraints (total energy difference of 3.11 kcal·mol^{-1}). The conclusion that can be drawn from these energy comparisons is that forcing a peptide with the incorrect configuration to fulfill the restraints of its diastereoisomer causes an increase of its total energy (nonrestraint-derived) as expressed by the force field applied herein. From these results it appears that a comprehensive NMR characterization of the natural product, like the one carried out here with trunkamide A and its L-Phe diastereoisomer, can be used to confirm the configuration of stereogenic centers in the backbone of rigid cyclic peptides of marine origin.

Conclusions

A combination of NMR spectroscopy and simulated annealing calculations has been used for the determination of the solution structure of trunkamide A. The cyclic heptapeptide appears as a pseudoplanar molecule that is stabilized by the formation of two trans-annular hydrogen bonds. The structure of trunkamide is very rigid and optimized, and a change in the configuration in the Phe unit from D to L causes epimerization in solution. NMR experiments carried out on its L-Phe diastereoisomer have allowed the determination of its solution structure, which shows no formation of intramolecular hydrogen bonds and provides a rationale for the experimentally observed epimerization. Finally, we have shown how NMR can, in combination with simulated annealing calculations, help in the determination of the configuration of stereogenic centers in rigid cyclic peptides.

Experimental Section

NMR Spectroscopy. Samples were 2 mM in **1** (or **2**) in 30% DMSO-d_6/70% $CDCl_3$. Traces of acid were removed from $CDCl_3$ by passing it through an alumina column. Spectra were acquired at 600 MHz on a NMR spectrometer equipped with pulsed field gradients. The following experiments were carried out (all at 300 K): TOCSY[17] (mixing time 70 ms), NOESY[18] (mixing times 100, 200, and 300 ms), and E-COSY.[19] In some NOESY experiments presaturation was used during the mixing time to remove the residual H_2O signal. All spectra were processed using nmrPipe and analyzed using NMRVIEW (4.1.2). TOCSY and NOESY experiments were acquired as 1024 × 256 (real points) data matrixes, processed using 90° squared sine bells in both dimensions and zero filled to yield 2048 × 512 spectra. The E-COSY experiment was acquired as a 2048 × 600 (real points) data matrix, processed using a 35° sine bell in both dimensions and zero filled to yield a 4098 × 1024 spectrum. Amide hydrogen temperature coefficients were measured from 1D experiments carried out at 278, 283, 288, 293, and 298 K.

Restrained Simulated Annealing Calculations. Distance restraints used for structure calculations were derived from the volume of cross-peaks in the NOESY spectrum acquired with a mixing time of 300 ms. Upper limit restraints were sorted as strong (d < 2.5 Å), medium (d < 3.5 Å), weak (d < 4.0 Å), and very weak (d < 5.0 Å). The usual methodology for dealing with methyl and nonstereospecifically assigned methylene protons was used.[20] Torsion angle restraints were calculated from three-bond coupling constants measured from the fine structure of the E-COSY cross-peaks. No hydrogen bond restraints were used. Trunkamide was built in InsightII (Accelrys Inc.) using the cvff force field. Partial charges and initial geometries for the nonpeptidic functional groups were calculated using semiempiric methods (MOPAC, Hamiltonian AM1). All structure calculations were carried in vacuo using a distance dependent dielectric ($\epsilon = 4r$) constant and a 9.5 Å cutoff for nonbonding interactions in Discover 3 (Accelrys Inc.) on an Octane2 Silicon Graphics Workstation. A 50 ps unrestrained molecular dynamics carried out at 300 K was used to generate the initial structure: the lowest energy frame of the trajectory was energy minimized to ensure correct local geometry. Distance restraints were harmonic (only upper-bound limits were used) while torsion angle restraints were cosine centered on all possible values. Calculation of the structure was performed using 50 steps of simulated annealing at 700 K in which the force constant for the restraints and the nonbonding interactions were scaled down with temperature. The 50 structures were then checked for restraint violation and clustered in families according to backbone conformation.

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Supporting Information Available: 2D TOCSY and NOESY spectra of trunkamide A (**1**) and of its L-Phe diastereoisomer (**2**), lists of chemical shift assignments (^{1}H), and two tables containing a summary of the statistical and energetical analysis of the structure calculations. This material is available free of charge via the Internet at http://pubs.acs.org.

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(17) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521−528.

(18) Jeener, J.; Meier, B. H.; Buchman, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546−4553.

(19) Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1990**, *107*, 6394−6396.

(20) Neuhaus, D.; Williamson, M. P. *The Nuclear Overhauser Effect in Structural and Conformational Analysis*, 2nd ed.; John Wiley and Sons: New York, 2000.