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Amino Acids that Specify Structure Through Hydrophobic Clustering and Histidine-Aromatic Interactions Lead to Biologically Active Peptidomimetics

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Abstract—Acyclic β-sheet structure can be nucleated in heptapeptides when the 4-(2-aminoethyl)-6-dibenzofuranpropanoic acid residue (**1**) is flanked in sequence by two His residues, a His residue and a hydrophobic residue or by two hydrophobic residues. Acyclic β-sheet peptidomimetics having an appropriate sequence have sufficient structural integrity to exhibit antimicrobial activity equivalent to that of gramicidin S.

Introduction

The concept of introducing a conformationally rigid molecule into a peptide to control its conformation was introduced by Hirschmann and his colleagues at Merck.[1] Since that time, the field of peptidomimetics has received considerable attention, particularly in the area of β-turn mimics.[2] The majority of the turn mimics are rigid skeletons designed to position peptide-like side chains in the proper orientation so as to be recognized by a given protein or receptor that typically recognizes a protein- or peptide-based β-turn. The Kemp laboratory[3] and our own laboratory[4] have set out to develop unnatural amino acids that nucleate secondary structure formation through short range non-covalent interactions. In the case of our amino acids, they are designed to replace a β-turn in only the simplest sense, i.e. they reverse the peptide chain direction.[4] We have not attempted to make these β-sheet nucleators look like an authentic β-turn with respect to the display of functional groups. Rather, we have concentrated on molecules that will reverse the peptide chain and in so doing facilitate tertiary interactions which will result in β-sheet nucleation. Some of these peptidomimetics have found applications in medicinal chemistry, and will likely prove useful in the design of protein mimics and for the development of self-assembling materials where small polypeptide sequences or peptide-like sequences are desirable.

The clustering of hydrophobic side chains during the early stages of a protein folding event is emerging as one of the common themes by which the primary amino acid sequence can specify a folding pathway(s).[5] These so called hydrophobic clusters, along with local conformational propensities, appear to limit the large number of possible folding pathways to a finite number so as to ultimately arrive at the proper protein fold. The work described within focuses on our efforts to evaluate the potential of amino acids that promote β-sheet folding through the intermediacy of hydrophobic clusters or related tertiary clusters to afford structurally well-defined biologically active peptides. We have previously reported that 4-(2-aminoethyl)-6-dibenzofuranpropionic acid (**1**) efficiently reverses the peptide chain direction and promotes antiparallel β-sheet formation when incorporated into an appropriate α-amino acid sequence.[4c-e] NMR and near-UV CD studies on related peptides containing residue **1** suggest that **1** needs to be flanked by two α-amino acid residues bearing relatively large hydrophobic side chains (Leu, Val, Phe, etc.) which allows the dibenzofuran skeleton and the side chains of the flanking α-amino acids to form a hydrophobic cluster which appears sufficient to nucleate β-sheet folding (Figure 1).[4e]

Typically, acyclic peptides that are composed of less than twenty amino acid residues do not undergo intramolecular folding into a unique conformation, rather they adopt an ensemble of conformations in aqueous solution.[6] However, a few exceptions exist.[7] Small cyclic peptides, on the other hand, can adopt well-defined conformations in aqueous buffers. An example is the cyclic decapeptide gramicidin S, which adopts an antiparallel β-sheet structure that is critical for its antimicrobial activity.[8] In an attempt to prepare small acyclic peptides with structural integrity, we synthesized peptides containing **1** which were designed to fold into an antiparallel β-sheet secondary structure.[4] NMR structural studies on these acyclic heptapeptide model systems indicate that they adopt a fluctuating β-sheet structure in aqueous solution,[4d] implying that these peptidomimetics may be active as antibacterial agents. In the work outlined here, we demonstrate that residue **1** can be utilized to create an acyclic peptidomimetic which has antimicrobial activity equivalent to that of the cyclic peptide gramicidin S.

In an effort to expand the utility of **1** as a β-sheet nucleator, we have begun to evaluate residues other than hydrophobic residues which can interact with **1** through space and in so doing nucleate β-sheet structure. Aware of the likely importance of π-cation-like interactions in aqueous solution, we set out to evaluate the potential of His as a flanking residue in acyclic heptapeptides. There are many examples in biological systems where His-aromatic interactions show what appear to be π-cation-like

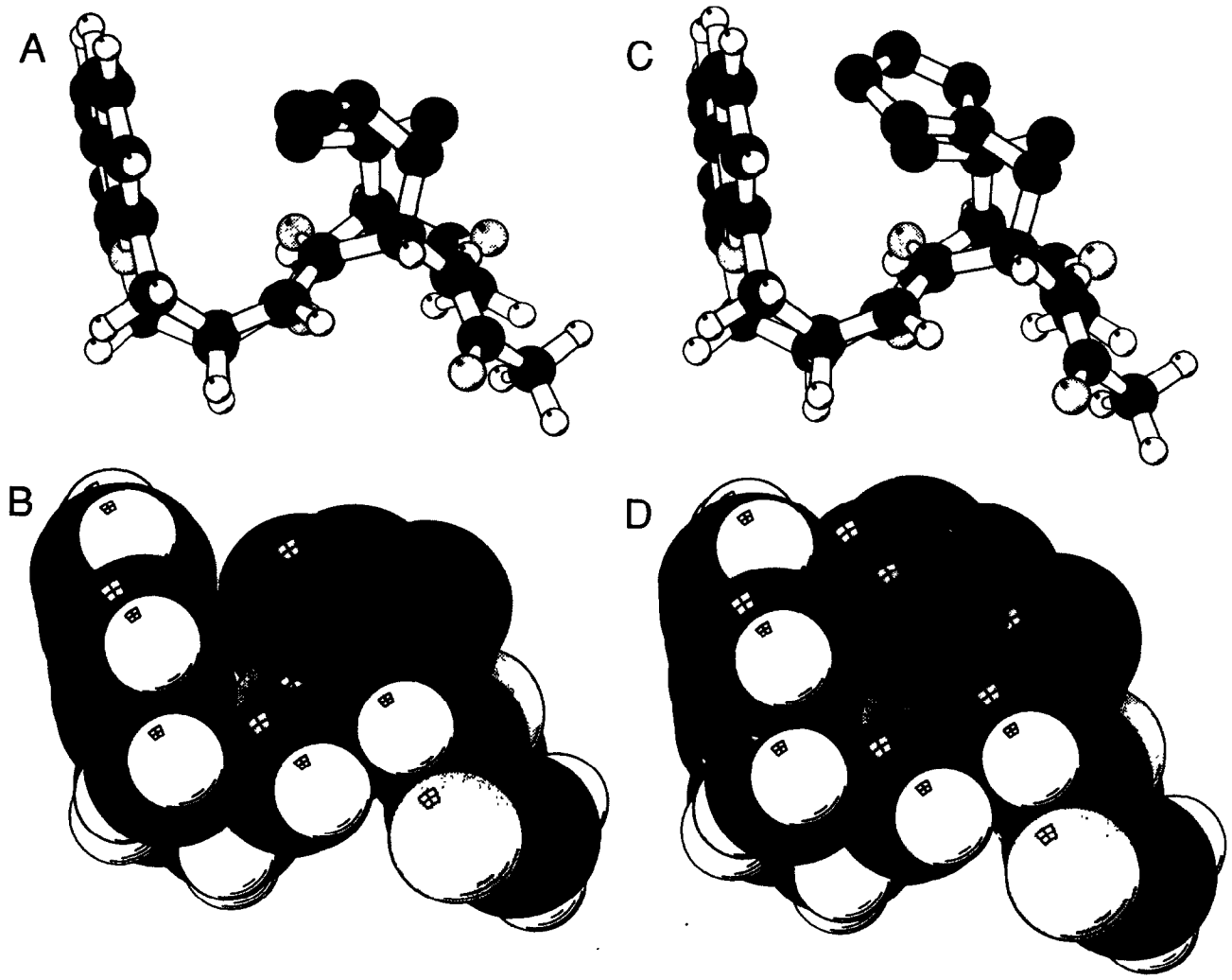


Figure 1. Molecular Graphics Depiction of Ac-Leu-1-Val-NH_2 (Structures A and B) and Ac-His-1-Val-NH_2 (Structures C and D) (see Figure 2 for a line drawing of residue 1). Structure A illustrates the hydrophobic cluster conformation adopted by 1 in aqueous solution (H's have been left off the α-amino acid side chains for clarity), which appears to be sufficient for the nucleation of β-sheet structure in the context of a larger peptide. In this view the dibenzofuran skeleton and the plane of the amides of the flanking hydrophobic amino acid residues are perpendicular to the plane of the page. The hydrophobic cluster conformation is stabilized by hydrophobic interactions between the dibenzofuran skeleton and the side chains of the flanking α-amino acid residues and potentially by intramolecular hydrogen bonding. A CPK representation of the hydrophobic cluster conformation (same view as in A) is shown in B. Note that the van der Waals surfaces of the dibenzofuran skeleton and the isobutyl group of the flanking Leu residue pack tightly to form a hydrophobic cluster or core (H's have been left off the α-amino acid side chains for clarity). Structure C illustrates the putative His-aromatic interaction (π-cation-like interaction) which stabilizes a conformation similar to that shown in A (H's have been left off the α-amino acid side chains for clarity), which appears to be sufficient for the nucleation of β-sheet structure in the context of a larger peptide. In this view the dibenzofuran skeleton and the plane of the amides of the flanking His and Val residues, which participate in intramolecular hydrogen bonding are perpendicular to the plane of the page. The nucleating cluster conformation is stabilized by hydrophobic interactions between the Val side chain and the dibenzofuran skeleton as well as a His^+ aromatic interaction between the imidazole NH^+ and the dibenzofuran skeleton, and potentially by intramolecular hydrogen bonding. A CPK representation of the His-aromatic cluster conformation (same view as in C) is shown in D. Note that the N^+ of the imidazole can interact nicely with the electron rich face of the dibenzofuran skeleton. These structures have not been minimized.

interactions, i.e. attractive electrostatic interactions between the positively charged imidazole-NH^+ and the face of an electron rich aromatic ring.[9] The His-aromatic or the π-cation interaction has been studied by several laboratories including those of Petsko, Dougherty, Fersht, and Baldwin.[9] We reasoned that substitution of one or both of the flanking hydrophobic residues by His could result in a favorable interaction between the protonated imidazole side chains and the dibenzofuran ring system affording a cluster similar to that produced by hydrophobic interactions (Figure 1).[4] Further knowledge regarding efficacious flanking residues would extend the utility of **1** as a nucleator and as a simple replacement residue for the i+1 and i+2 residues of a β-turn in a variety of applications.[4]

Results

Synthesis of peptides A–E

Acyclic peptidomimetics of gramicidin S were prepared conceptually by excising one of the two D-Phe–Pro dipeptide fragments from gramicidin S. The remaining D-Phe–Pro dipeptide fragment within the acyclic peptide was

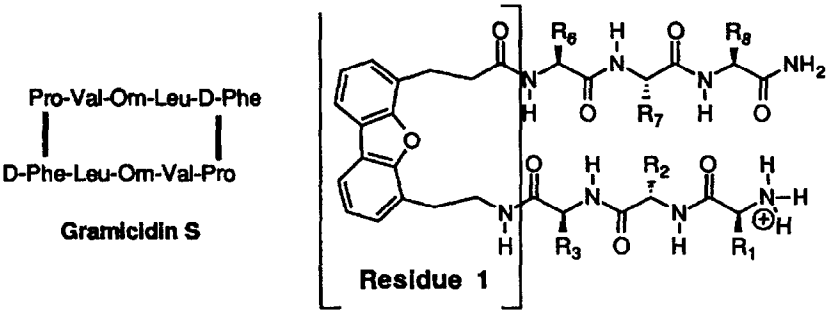
then replaced with residue **1** affording peptide **A**, (Figure 2). The D-Phe–Pro residues were chosen for replacement because these are the i+1 and i+2 residues of the β-turns in gramicidin S.[8] Residue **1** was designed to replace this portion of a β-turn and from previous work it appears to function quite well in this capacity.[4] A variety of reports from other laboratories have established that the antiparallel β-sheet conformation in gramicidin S is critical for its antibacterial activity.[8,10] Acyclic heptapeptides composed of residue **1** studied previously in our laboratory adopt a partial β-sheet structure as ascertained by CD and NMR spectroscopy, however these sheets are significantly less ordered when compared to sheet structure within a protein. The ability of heptapeptides incorporating **1** to populate a β-sheet structure suggests that similar peptides based on the sequence of gramicidin S may be biologically active.[4d,e] Peptide **A** was prepared, as were other peptides in this paper, using the benzhydrylamine resin employing standard *t*-Boc synthesis protocols and the BOP reagent for carboxyl activation of the α-amino acids.[4e,11] The β-sheet nucleator **1** was coupled to the growing peptide chain using the C-terminally activated pentafluorophenyl ester/*t*-Boc protected analog of **1** whose synthesis has been described previously.[4e] The peptides were cleaved from the resin and the side chain protecting groups were removed employing high-HF.[12] This route dictates that the C-terminus will be liberated from the resin as an amide.[12] The crude peptides were then purified by reverse-phase (C_{18}) HPLC. The primary structures of all peptides prepared in this study were confirmed by nominal resolution matrix assisted laser desorption mass spectrometry (MALDI-MS).[13]

Biological activity

The antimicrobial activity of peptide **A** was evaluated using the Broth Dilution Method employing *Escherichia coli* strain LE 392 grown in Mueller–Hinton Broth at 37 °C for 24 h as described previously.[14] Briefly, peptide **A** or gramicidin S were added to Mueller–Hinton Broth that was inoculated with competent *E. coli* by serial dilution to discern the minimum concentration of drug (peptide) required to prevent growth of the organism. These so called minimum inhibitory concentrations of peptide **A** and gramicidin S are compared in Table 1. All experiments were carried out in triplicate. A difference of less than 4-fold is insignificant in this assay, therefore the activities of peptide **A** and gramicidin S are equivalent. The high activity of peptide **A** is consistent with its β-sheet structure, which presumably is its biologically active conformation. Interestingly peptide **B** (Figure 2), containing Lys instead of Orn, is not active as an antibiotic even though the backbone conformations of peptides **A** and **B** appear to be virtually identical by NMR and by CD. This result is supportive of previous studies which suggest that the Orn side chain is of critical importance for the biological activity of acyclic gramicidin S analogs.[16] The mechanism of action of gramicidin S is not established at the molecular level, but it is thought that gramicidin S damages the phospholipid bilayer of the prokaryotic cell membrane by absorption and prevents normal membrane function.[15] Further studies on the mechanism by which the peptides similar to peptide **A** act are underway.

Sequence specific His-nucleator (1) interactions

Previous studies have demonstrated that peptides containing residue **1** are capable of forming a hydrophobic cluster when **1** is flanked in sequence with hydrophobic α-amino acid residues.[4] The aromatic portion of residue **1** ends up being perpendicular to the plane of the β-sheet



PEPTIDE	R1	R2	R3	R4	R5	R6	R7	R8
Peptide A	Val	Orn	Leu		1	Val	Orn	Leu-NH_2
Peptide B	Val	Lys	Leu		1	Val	Lys	Leu-NH_2
Peptide C	Val	Lys	Leu		1	His	Lys	Leu-NH_2
Peptide D	Val	Lys	His		1	His	Lys	Leu-NH_2
Peptide E	Val	Lys	Ala		1	His	Lys	Leu-NH_2

Figure 2. Structural template for peptides **A**–**E** and gramicidin S. R_n refers to the amino acid residue at position n. Residue **1** is designed to replace the i+1 and i+2 residues of a β-turn, i.e. R_4 and R_5 in this case.

Table 1. Antibacterial activity of gramicidin S and its acyclic analogs

Peptide	Minimum Inhibitory Concentration μg / mL
Gramicidin S	**8**
Peptide A	**16**
Peptide B	**>128**

A difference of less than 4-fold in minimum inhibitory concentration is insignificant in this assay.

formed by the interacting peptide strands allowing the dibenzofuran skeleton to pack against the hydrophobic side chains of the flanking α-amino acid residues, Figure 1. The hydrophobic cluster conformation allows the α-amino acids flanking **1** to participate in intramolecular hydrogen bonding.[4b] The presence of this hydrogen bonded hydrophobic cluster appears to be sufficient to nucleate the folding of a β-sheet. Based on the published work of Petsko, Fersht, Dougherty and Baldwin,[9] it seemed likely that a similar conformation could be stabilized by the dibenzofuran skeleton interacting with the charged imidazole side chains from the His residues flanking **1** in sequence, Figure 1. Putative π-cation-like interactions of this type have been observed in proteins as discerned by their abnormally high pK_a's and inferred by analysis of X-ray crystal structures.[9] Because His is a hydrophilic residue it is unlikely that this residue would interact with the dibenzofuran skeleton owing to the hydrophobic effect. Therefore, it is expected that the ability of His to promote cluster formation, necessary for β-sheet nucleation, would be pH dependent since the π-cation-like interaction requires that the imidazole side chain be protonated.[9] Several analogs of peptide **A** have been prepared where one or both of the flanking α-amino acid residues are a His residue in order to determine if a π-cation-like interaction is capable of promoting a local nucleating competent conformation within heptapeptides composed of residue **1**.

Far- and near-UV circular dichroism of His containing peptides

Circular dichroism studies of the His containing peptides **C–E** (Figure 2) have been carried out to probe the scope and limitations of the π-cation-like interaction for the creation of a nucleation cluster. The residues flanking **1** in peptide **B** were replaced by one or two His to discern if these peptides were capable of folding. Peptide **B** has been studied extensively[4a,b] and serves as the paradigm for β-sheet nucleation via hydrophobic cluster formation. Peptide **C** is an analog of peptide **B** in which the Val-6 residue has been replaced by His. The pH dependent far-UV CD spectra of peptide **C** are shown in Figure 3. As expected, this peptide adopts a fluctuating β-sheet structure at low pH similar to that exhibited by peptides **A** and **B**. However, as the pH is increased from 4.5 to 8.5 the amount of β-sheet structure (indicated by the 213 nm minimum) decreases at the expense of random coil (indicated by the 197 nm minimum). This can be rationalized on the basis of the His-6 pK_a (6.8) in peptide **C**. At low pH the imidazole is protonated and as such is expected to interact with the dibenzofuran ring system via a π-cation-like interaction. As the pH is increased, the fraction of free imidazole increases. The extent of π-cation-like interactions in a given population of peptide **C** molecules would be expected to directly correlate with the fraction of the total protonated imidazole groups. While the extent of β-sheet structure in peptide **C** relative to random coil decreases with increasing pH, it does not disappear due to the fact that the Leu–dibenzofuran hydrophobic interaction is sufficient to maintain a partial hydrophobic cluster which retains partial β-sheet structure. Further evidence that the imidazole–dibenzofuran interaction is severed at high pH comes from a comparison of the CD spectra of peptide **C** and the peptide Val-Lys-Leu-**1**-Ala-Lys-Leu-NH_2 where it is established that only the Leu and not the Ala interacts with the dibenzofuran skeleton.[4c] The far-UV CD spectra of these peptides are very similar, strongly supporting the lack of the imidazole–dibenzofuran interaction at high pH. Moreover, if the β-sheet band (213 nm) of peptide **C** is compared at low and high pH, a difference of approximately 5000 mean residue ellipticity units is observed. Significantly, this is the same difference that is observed when one compares peptide **B** to the peptide Val-Lys-Leu-**1**-Ala-Lys-Leu-NH_2, previously studied.[4c]

In order to further evaluate the utility of the conjugate acid of His as a flanking residue in peptides containing residue **1**, peptide **D** was prepared where both of the flanking residues in peptide **B** (Leu-3 and Val-6) are replaced by His. As expected the structure of this peptide as discerned by far-UV CD exhibits a marked pH dependence, Figure 4. At low pH, peptide **D** adopts a dynamic β-sheet structure which is very similar to that adopted by peptides **A**, **B** and **C**. However, as the pH is increased the peptide loses β-sheet structure at the expense of random coil. This observation is consonant with the conversion of the conjugate acid of imidazole to the free base which is incapable of supporting a π-cation-like interaction. When peptide **D** is subjected to alkaline pH (> 7.5) the peptide appears to lose all of its β-sheet structure consistent with the measured pK_a's of the His residues (6.5 and 6.2). The free imidazole form of peptide **D** (high pH) cannot adopt a β-sheet structure, consistent with the idea that there is no interaction between the dibenzofuran moiety in **1** and the non-protonated imidazole rings of the flanking His residues. Contrarily, when the imidazole groups of the flanking His residues are charged (at low pH) the π-cation-like interaction facilitates an interaction between the dibenzofuran skeleton and the flanking His residues which is apparently sufficient to nucleate and stabilize a β-sheet structure. The near-UV CD spectrum of peptide **D** is significantly attenuated as one increases the pH, which is in agreement with the idea that the dibenzofuran chromophore is spending less time in an asymmetrical

environment (on the same face of the β-sheet as the imidazole side chains), Figure 5. The changes in the near-UV CD spectrum of peptide **D** are twice those observed in the near-UV CD spectrum of peptide **C** (data not shown), consistent with the idea that the nucleating conformation in peptide **C** is partly controlled by hydrophobic interactions (Leu-3) and partly by π-cation like interactions (His-6).

In order to determine whether a single π-cation-like interaction with **1** is of sufficient strength to nucleate a β-sheet structure in aqueous solution, we prepared peptide **E** which has Ala and His in the flanking positions. From independent studies[4e] we know that Ala, which has a methyl side chain, is too small to allow for hydrophobic interaction with residue **1** as discerned from the observation that peptide Val-Lys-Ala-**1**-Ala-Lys-Leu-NH_2 is a random coil over a wide pH range.[4e] Peptide **E** is also incapable of adopting a β-sheet structure over a wide pH range suggesting that a single π-cation-like interaction is not of sufficient strength to nucleate a β-sheet structure in this context (data not shown). In contrast, a single hydrophobic residue in the flanking position does nucleate a dynamic β-sheet structure under identical conditions in the peptide Val-Lys-Leu-**1**-Ala-Lys-Leu-NH_2, studied previously.[4e]

Histidine pK_a determinations by NMR

Exchange deuterated peptides **C**–**E** were dissolved in D_2O and spectra were recorded every 0.2–0.3 pH units to afford a titration curve derived from the chemical shifts of the 2- and 4-protons on the imidazole ring. The pK_a's reported are obtained by averaging the value obtained from the 2- and 4-protons. These values have not been corrected for the isotope effect, which can be approximated by subtracting 0.2 units from each value. The pK_a's determined for peptides **C** (6.8), **D** (6.5 and 6.6) and **E** (6.8) did not exhibit the elevated pK_a that might have been expected based on that observed in Barnase.[9c] However, it may be that other effects in addition to the π-cation-like interaction

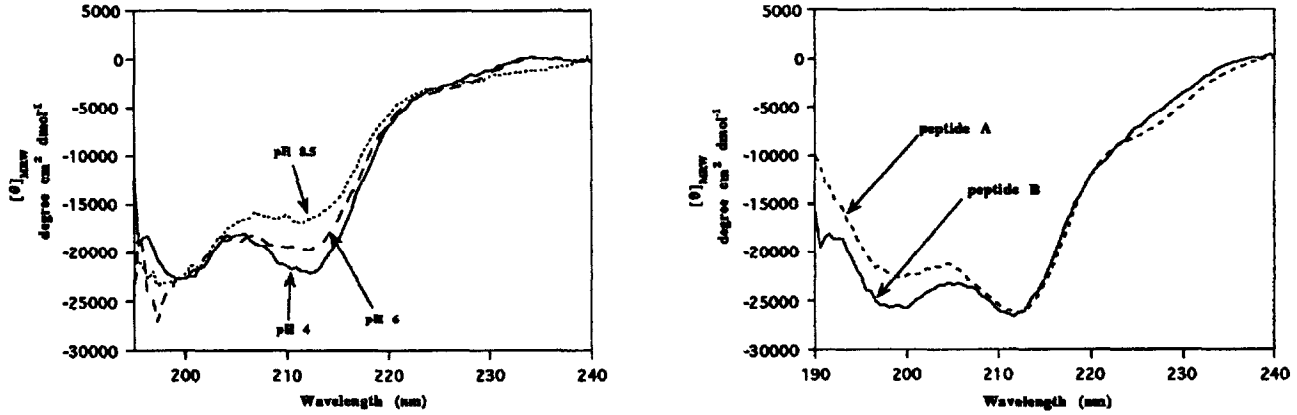


Figure 3. Far-UV CD spectra of peptides **A**–**C**. The CD data were collected on a Jasco J-600 spectropolarimeter at 25 °C using a 1 mm quartz cell. The spectra for peptide **C** (0.1 mM) are presented in the left panel as a function of pH and were recorded in either 10 mM acetate or borate buffer. The spectra of peptides **A** and **B** are shown in the right panel in 10 mM acetate buffer at pH 4.9. All spectra were corrected for buffer contributions and are reported in units of mean residue ellipticity.[16]

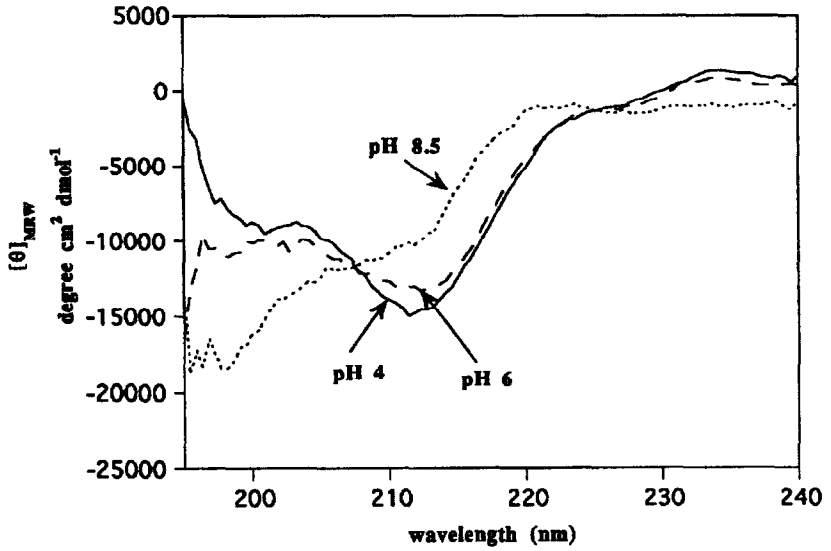


Figure 4. Far-UV CD spectra of peptide **D** (0.1 mM) as a function of pH. The CD data were collected on a Jasco J-600 spectropolarimeter at 25 °C using a 1 mm quartz cell. The spectra were recorded in either 10 mM acetate or borate buffer, corrected for buffer contributions and are reported in units of mean residue ellipticity.[16] The data for pH's 5, 7.5 and 8 were left out of the figure for clarity.

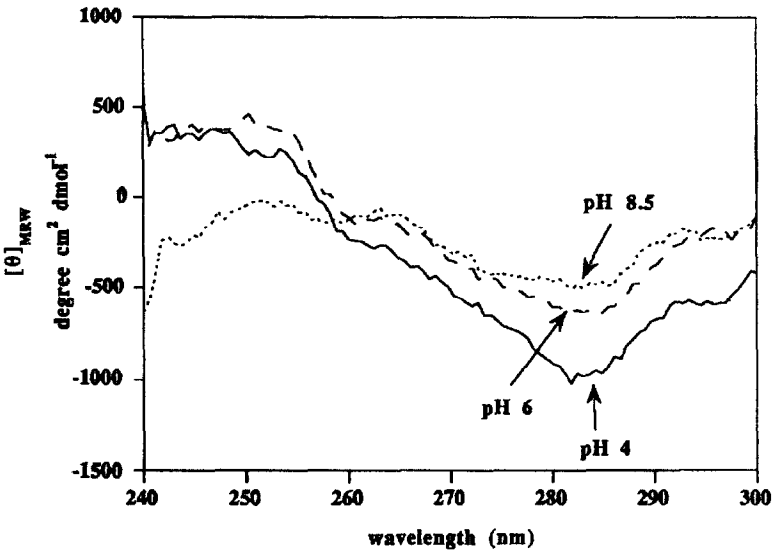


Figure 5. Near-UV CD spectra of peptide **D** (0.05 mM)as a function of pH. The CD data were collected on a Jasco J-600 spectropolarimeter at 25 °C using a 5 mm quartz cell. The spectra were recorded in 10 mM acetate or borate buffer, corrected for buffer contributions and are reported in units of mean residue ellipticity.[16]

are also influencing His-18's pK_a in Barnase. For example, it is established that the His-12–Phe-8 interaction stabilizes the C-peptide helix of ribonuclease A, yet the pK_a of His-12 is normal (6.3).[9e] It appears that the elevation of His pK_a is not a reliable probe for analyzing a His-aromatic interaction, particularly in the case of peptides.

Discussion

The dibenzofuran-based amino acid residue **1**, which controls the conformation of peptides within which it is incorporated, should prove to be important in medicinal chemistry, bioorganic chemistry, and materials applications. Previous structural studies on heptapeptides composed of a single 4-(2-aminoethyl)-6-dibenzofuranpropanoic acid residue (**1**) have shown that the resulting monomeric β-sheets in these peptides are well-defined proximal to the nucleator (**1**) and become less ordered towards the end of each peptide strand.[4] Since the known structural preferences of these peptides met the requirements for antimicrobial activity (gramicidin S) we carried out a structure–function study on peptide **A** as described in detail within. The excellent antibacterial activity of peptide **A** argues that our understanding of its structure must be very close to reality and that the β-sheet structure is critical for biological activity. Ongoing high field NMR studies should help us better understand the conformation in solution, particularly with regard to the dynamics of the strands. Future experiments will also address whether the mode of action of peptide **A** and gramicidin S are the same.

In order to extend the utility of **1** as a β-sheet nucleator, we have demonstrated that it is possible to stabilize a nucleation cluster employing π-cation-like interactions instead of, or in addition to, hydrophobic interactions. Studies on peptides **C–E** demonstrate that **1** is functional as a β-sheet nucleator when flanked by two charged His or one charged His and a hydrophobic residue. The efficacy of His as a flanking residue is also useful from the standpoint that a charged flanking residue enhances solubility, which is an important issue when very hydrophobic β-sheets are desired. In summary, His-aromatic interactions and/or hydrophobic interactions with the dibenzofuran skeleton in **1** appear sufficient to stabilize a nucleation cluster competent for β-sheet nucleation.

Experimental

General methods

NMR titrations were performed on a Varian XL-400 spectrometer. A matrix assisted laser desorption ionization (MALDI) mass spectrometer constructed by Dave Russell and coworkers was used to obtain nominal masses of peptides described within to corroborate their structure. Far- and near-UV CD spectra were recorded on a Jasco J-600 spectrometer. The data from the Jasco spectrometer were imported into the Macintosh version of KaleidaGraph and processed. Preparative HPLC was carried out on a dual pump system equipped with Altex 110A pumps and a 420 gradient programmer. The column employed was a Waters RCM Delta Pak C_{18} (15 μm, 300 Å, 25 × 100 mm) attached to a Knauer 86 variable wavelength detector set at 254 nm. Solvent A was composed of 95 % water, 5 % acetonitrile (Fisher, Optima grade), and 0.2 % TFA. Solvent B was composed of 5 % water, 95 % acetonitrile, and 0.2 % TFA.

Peptide synthesis

Peptide synthesis was accomplished using procedures described in detail previously.[4e,11] Briefly, manual solid phase peptide synthesis was carried out by employing the benzhydrylamine resin (0.66 meq./g).[11] The first Boc-protected amino acid was attached to the resin employing DIC. After TFA deprotection and neutralization of the

resin-bound amino terminus, the subsequent residues were added to the resin-bound peptide employing BOP activation.[11] A final N-terminal deprotection followed by high-HF cleavage afforded the desired peptide which was purified by HPLC.[12]

Summary of purification and characterization of peptides A–E

Peptide A ($C_{49}H_{78}N_{10}O_8$). HPLC Purification by preparative (C_{18}) HPLC employing a linear gradient from 35 % to 50 % solvent B over 30 min; overall yield 34 %; MALDI-TOFMS (MH^+) calcd 935.6, obsd 935.6; AAA: Leu 2.1 (2), Orn 1.3 (2), Val 2.0 (2).

Peptide B. (See reference 4e for analytical data).

Peptide C ($C_{52}H_{80}N_{12}O_8$). HPLC Purification by preparative (C_{18}) HPLC employing a linear gradient from 15 % to 30 % solvent B over 20 min; overall yield 30 %; MALDI-TOFMS (MH^+) calcd 1001.6, obsd 1001.6.

Peptide D ($C_{52}H_{76}N_{14}O_8$). HPLC Purification by preparative (C_{18}) HPLC employing a linear gradient from 15 % to 50 % solvent B over 25 min; overall yield 35 %; MALDI-TOFMS (MH^+) calcd 1025.6, obsd 1023.5.

Peptide E ($C_{49}H_{74}N_{12}O_8$). HPLC Purification by preparative (C_{18}) HPLC employing a linear gradient from 22 % to 32 % solvent B over 10 min; overall yield 11 %; MALDI-TOFMS (MH^+) calcd 959.6, obsd 959.9.

Circular dichroism studies

Far-UV CD spectra were collected on a Jasco J-600 spectropolarimeter using a 1 mm quartz cell. The samples were prepared as stock solutions in water and diluted to afford 0.1 mM solutions in 10 mM acetate buffer (pH 4–6) or borate buffer (pH 7.5–8.5), 75 mM NaCl. The concentrations of the peptides **A**–**E** were determined by UV spectroscopy at 282 nm (ε = 17,797/cm/M). The samples were allowed to equilibrate at room temperature over 24 h and degassed prior to performing the CD experiments. CD data were collected at 25 °C using a scan speed of 20 nm/min, a time constant of 0.5 s, a band width of 1 nm and a sensitivity of 50 mdeg. Near-UV CD spectra were recorded using 0.05 mM peptide concentrations in a 5 mm quartz cell. All other conditions were identical with the exception that a sensitivity of 10 mdeg was used. All spectra were corrected for buffer contributions and are presented in units of mean residue ellipticity.[16]

His titrations by NMR

The method followed was that adapted from Fersht.[9c] The peptide of interest was lyophilized from D_2O three times and redissolved in 1.5 mL of D_2O to afford a 2 mM peptide solution (TSP was used as an internal standard). The sample was divided into two equal volumes and titrated with either DCl or NaOD in D_2O solution. Spectra were recorded every 0.2–0.3 pH units. Two singlets were observed in the downfield region between 6.5 and 8.5 ppm corresponding to the imidazole H-4 and H-2 signals. For peptide **D**, two sets of singlets were observed, but residue specific assignments were not made. Resonances which overlapped with the dibenzofuran protons were not used. The chemical shift of H-4 and H-2 were plotted separately as a function of pH. The curves were fit to the following equation[18] to extract the pK_a's: $\delta_i = \delta_{His} + (\delta_{His+} - \delta_{His})\{10^{n(pK_a - pH)}$; where δ_i is the chemical shift observed, δ_{His} is the chemical shift of the unprotonated imidazole, δ_{His+} is the chemical shift of protonated imidazole, and n = Hill coefficient. The pK_a's reported are the average of those discerned from three separate data sets on H-4 and H-2. The pK_a values reported were not corrected for the isotope effect which usually decreases the pK_a by approximately 0.2 pK_a units.[9c]

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