Multicyclic Polypeptide Model Compounds. 2. Synthesis and Conformational Properties of a Highly α-Helical Uncosapeptide Constrained by Three Side-Chain to Side-Chain Lactam Bridges

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Abstract: Further to our earlier report of the synthesis and conformational properties of cyclo(3-7,10-14,17-21)-H-[LysLeuLysGluLeuLysGlu]$_3$-OH (**1-1-1**) (Ösapay and Taylor, *J. Am. Chem. Soc.* **1990**, *112*, 6046–6051), two new amphiphilic α-helical peptides, cyclo(3-7,10-14,17-21)-H-[LysLeuLysGluLeuLysAsp]$_3$-OH (**2-2-2**) and its linear homologue H-[LysLeuLys(Ac)GluLeuLysLeuGln]$_3$-OH (**3-3-3**), have been synthesized in order to assess the relative helix stabilizing properties of multiple lactam bridges linking Lys^i,Glu^{i+4} and Lys^i,Asp^{i+4} residue pairs. These peptides were assembled using a combination of solid-phase peptide synthesis on the Kaiser-oxime resin and solution-phase segment condensations. During the preparation of the protected 7-residue building unit (**7**) for **2-2-2**, peptide cyclization with concomitant cleavage from the oxime resin yielded 54% product. Circular dichroism spectropolarimetry indicated that model peptide **2-2-2** was highly helical in aqueous buffer, pH 7.0, at 25 °C ($[\theta]_{222}$ = −23 800 deg·cm^2 dmol^{-1}). This bridged helical structure was resistant to thermal and chemical denaturation, being incompletely unfolded at 90 °C and, based on $[\theta]_{222}$, only 50% unfolded in 7.30 M guanidinium hydrochloride at 25 °C. In contrast, peptide **1-1-1** and the acyclic peptide **3-3-3** both displayed very little helical character in the aqueous buffer and were readily denaturated by guanidinium hydrochloride. However, in 50% trifluoroethanol or bound to hydrophobic coated quartz slides, the multicyclic peptides **1-1-1** and **2-2-2** gave almost identical CD spectra indicative of a highly α-helical conformation ($[\theta]_{222}$ = − 31 000 deg·cm^2 dmol^{-1}), whereas the linear peptide **3-3-3** was significantly less helical. From these results, we conclude that multiple lactam bridges linking the side chains of Lys^i,Asp^{i+4} residue pairs are strongly helix stabilizing, and those linking Lys^i,Glu^{i+4} residue pairs are weakly helix stabilizing.

Introduction

The biologically active conformations of receptor-bound linear polypeptides in the intermediate size range may often include secondary and even tertiary structures that are not readily identified in their unbound states in solution. One approach to identifying these receptor-bound conformations involves the synthesis and study of analogues that incorporate multiple conformational constraints, provided that a reasonable hypothesis exists as a guide in their design.[3] With this goal in mind, we have recently developed an efficient synthetic method for the preparation of peptides incorporating multiple lactam bridges.[4,5] This method involves the synthesis of fully protected cyclic intermediates via peptide assembly and cyclization on the Kaiser oxime resin[6] (PCOR), followed by the segment-condensation assembly of these peptides by solution- or solid-phase methods.

The α-helix is one of the most common structural elements in globular proteins, and there is significant evidence supporting a common functional role for this structure in bioactive peptides also. For example, Kaiser and Kēzdy and co-workers have demonstrated that the amphiphilic α-helical character of many bioactive peptides, including peptide hormones, is often more important than their specific amino acid sequences in determining function.[7] Other approaches to identifying functional roles for α-helical structures include the "scanning" of all single-residue substituted analogues for peptide segments that show the appropriate sinusoidal dependence of activity on the position of the modified residue in the peptide chain[8] and the correlation of helix stabilization in solution with biological activity, using structurally modified analogues.[9,10]

Helix stabilization also holds the promise of enhancing the potencies and/or specificities of a bioactive peptide[10] and of providing synthetically accessible model systems for studies of protein folding, and many recent efforts have been focused on this direction. Ion pair interactions between amino acid side chains that are adjacent on a helix surface have been investigated in considerable detail, and certain combinations can generate significant α-helical structure in short peptides in aqueous solution, even at room temperature.[11] Conformational restrictions involving

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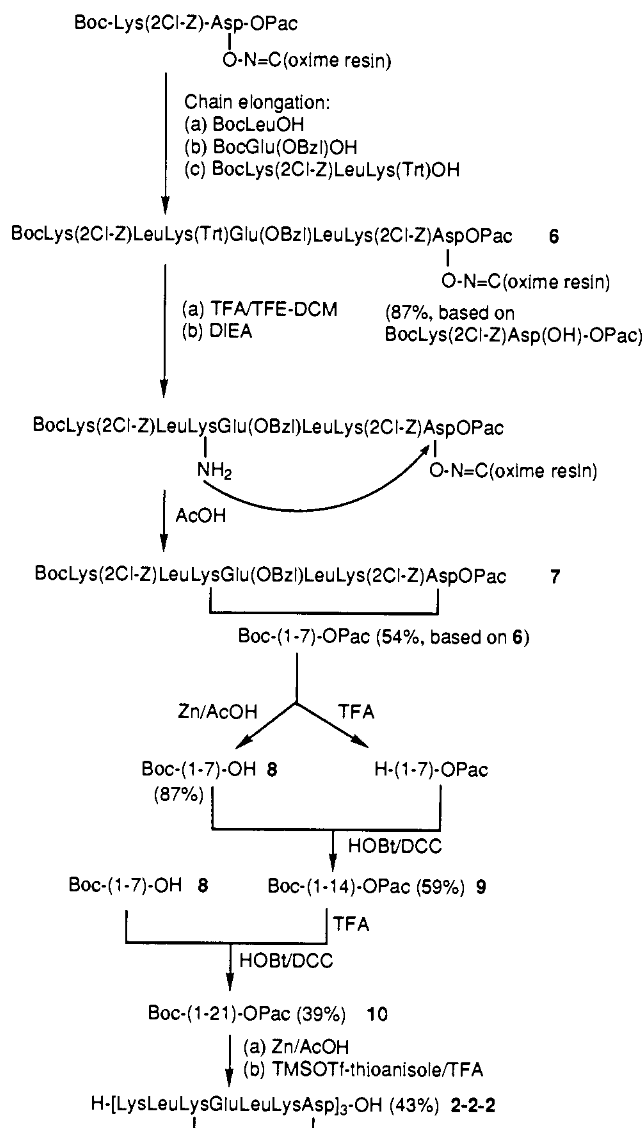
Scheme I



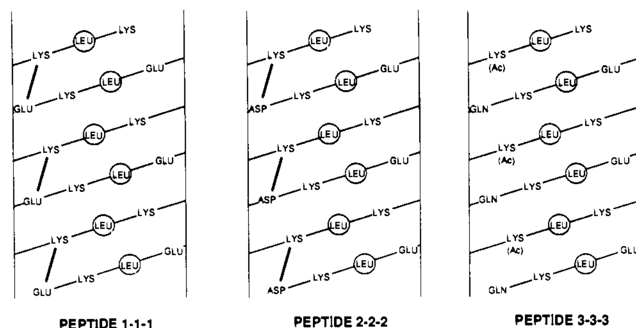
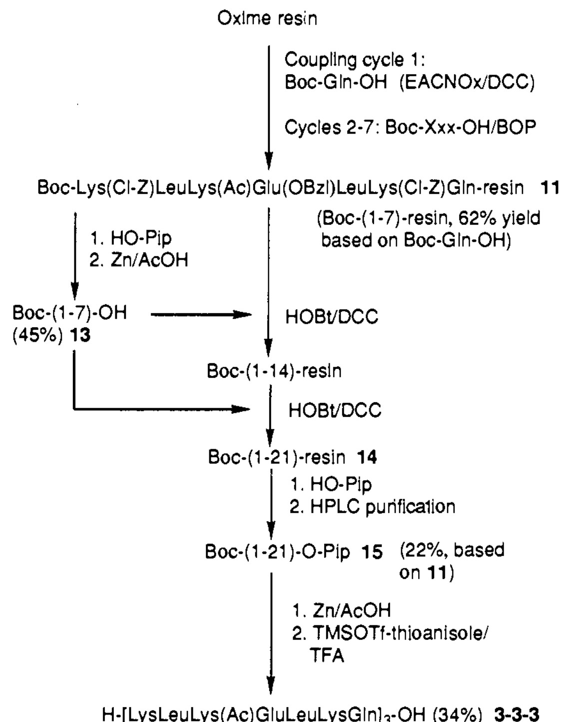


Figure 1. Helical net diagrams of the synthetic model amphiphilic α-helical peptides in this study.

Scheme II



the introduction of α,α-substituted amino acids such as α-aminoisobutyric acid[9a,12] or of metal-ion coordinated bridges linking the side chains of His^i,His^{i+4} residue pairs,[13a-c] covalent lactams formed by the side chains of Lys^i,Asp^{i+4} or Asp^i,Lys^{i+4} residue pairs,[9,10] or disulfide bridges linking 2-amino-6-mercaptohexanoic acid residues in positions *i* and *i*+7 of model peptide sequences[13d] have also proven effective in stabilizing the α-helical conformation.

In an earlier study,[4] we had designed and synthesized a 21-residue amphiphilic α-helical model peptide constrained by three side-chain bridged Lys^i,Glu^{i+4} residue pairs, cyclo(3-7,10-14,17-21)-H-[LysLeuLysGluLeuLysGlu]$_3$-OH (**1-1-1**). Given the helix-stabilizing effects demonstrated earlier for single lactam-bridged Asp^i,Lys^{i+4} residues,[9] we were surprised when **1-1-1** gave CD spectra indicative of mostly nonhelical, "disordered" conformations in aqueous solution. The Lys^i,Glu^{i+4} lactam bridges in **1-1-1** were, however, shown to be compatible with significant α-helical structure in this model peptide in 50% TFE or adsorbed onto siliconized quartz slides. In order to compare the helix-stabilizing effects of lactam bridges of the Lys^i,Glu^{i+4} and Lys^i,Asp^{i+4} types directly and assess the conformational effects of *multiple* lactam bridges more thoroughly, we have now synthesized a new multicyclic α-helical peptide analogue of **1-1-1** that is constrained by three Lys^i,Asp^{i+4} peptide bridges (**2-2-2**) as well as an acyclic analogue of these two peptides (**3-3-3**) that serves as a control for the inherent conformational preferences of the unbridged model peptide sequence (Figure 1). The syntheses of the two additional peptides by oxime-resin methods, and the conformational properties of the series of three peptides as compared by CD spectropolarimetry, are now described.

Results

Peptide Design. The design of the three peptides in this series is based on three repeats of a seven-residue peptide segment that gives rise to an amphiphilic structure in the α-helical conformation (Figure 1). Peptide **1-1-1** had previously been designed to have flexible constraints that were compatible with the α-helical conformation, consisting of lactam bridges linking Lys and Glu side chains in positions 3 and 7, respectively, in each repeat, corresponding to the hydrophilic face of the potential amphiphilic α helix. In contrast, the Lys^i,Asp^{i+4} lactam bridges incorporated into the same positions in **2-2-2** are one methylene group shorter. Model-building studies indicate that these bridges are the minimum length compatible with the α-helical conformation and would be highly constrained and closely packed against the helix surface. The acyclic analogue, **3-3-3**, incorporates N^ϵ-acetyl-Lys and Gln in positions 3 and 7, respectively, of the heptapeptide repeat. These residues provide side chains that mimic the lactam-bridged side chains but avoid introducing additional ionic

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interactions in aqueous solution at neutral pH that might affect helix stability.[11]

Peptide Synthesis. Peptides **2-2-2** and **3-3-3**, consisting of three repeats of amino acid sequences **2** and **3**, respectively, were assembled by repeat condensations of the corresponding protected seven-residue segments **7** and **13** that had been synthesized using the oxime resin method. However, whereas the condensations of **7** were performed in solution (Scheme I), condensations of **13** were performed by coupling on the oxime resin (Scheme II).

The synthesis of **7** was analogous to that described previously for the corresponding intermediate in the synthesis of **1-1-1**.[4] Dipeptide BocLys(2Cl-Z)Asp(OH)OPac (**5**) was prepared in solution phase as its DCHA salt.[14] Direct use of the DCHA salt for coupling to oxime resin with BOP resulted in only 29.2% yield.[15] However, the coupling of the dipeptide acid (**5**) with the use of EACNOx[16] and DCC gave 92% yield (substitution level 0.157 mmol/g). The next two amino acids (BocLeuOH and BocGlu(OBzl)OH) were introduced by the usual oxime resin synthesis protocol,[6] using their symmetrical anhydrides. The N-terminal tripeptide BocLys(2Cl-Z)LeuLys(Trt)OH[4] was then coupled to the tetrapeptide on the resin with HOBt/DIC in DMF. The overall yield of the three couplings was 86.8%, based on the substitution levels determined by amino acid analysis and on the picric acid titration method (0.12 mmol/g).

Selective cleavage of the trityl group used for Lys ϵ-amino protection was accomplished using 5% TFA in DCM–TFE (1/1, v/v). After neutralization, peptide cyclization (in the presence of AcOH as catalyst) then proceeded by internal aminolysis. The intrachain cyclization reaction gave **7** in 54% yield as the only major product cleaved from the resin. This result is consistent with our experiences with a variety of cyclization reactions carried out on the oxime resin.[4,5] The fully protected uncosapeptide (**10**) was then prepared by the repeated condensation of the cyclic heptapeptide derivatives in solution phase (yields 54% and 45%). Final deprotection was carried out in two steps, using Zn in aqueous AcOH and then TMSOTf/thioanisole/TFA.[17] Finally, the crude peptide product (**2-2-2**) was purified by gel filtration (Sephadex G-15) followed by RP-HPLC.

Peptide segment **13** was prepared starting from Boc-Gln-O-oxime resin (substitution level 0.21 mmol/kg) and using standard protocols to add the following amino acid derivatives: BocLys-(2Cl-Z)OH, BocLeuOH, BocGlu(OBzl)OH, BocLys(Ac)OH, BocLeuOH and BocLys(2Cl-Z)OH, by the BOP coupling procedure. The final substitution level (0.113 mmol/g based on the Leu content) indicated a 61% overall yield.

A major portion of peptidyl oxime resin **11** was treated with *N*-hydroxypiperidine, and the resulting HO-Pip ester was converted to the corresponding acid by reductive cleavage with Zn dust in acetic acid.[6c,d] This heptapeptide (**13**) was then repeatedly coupled to the remaining portion of the peptidyl oxime resin **11**. These solid-phase segment condensations were carried out using the DCC/HOBt method. The resultant 21-residue protected peptide was cleaved from the resin by treatment with *N*-hydroxypiperidine, and then the fully protected ester **15** was purified by RP-HPLC. The combined yield for the two solid-phase couplings and the cleavage from the resin was 22%. Ester **15** was converted to the free acid by reduction with Zn dust in 90% acetic acid, and final deprotection was carried out using TMSOTf/thioanisole/TFA.[17] After gel filtration on Sephadex G-25, **3-3-3** was obtained from the crude product by RP-HPLC.

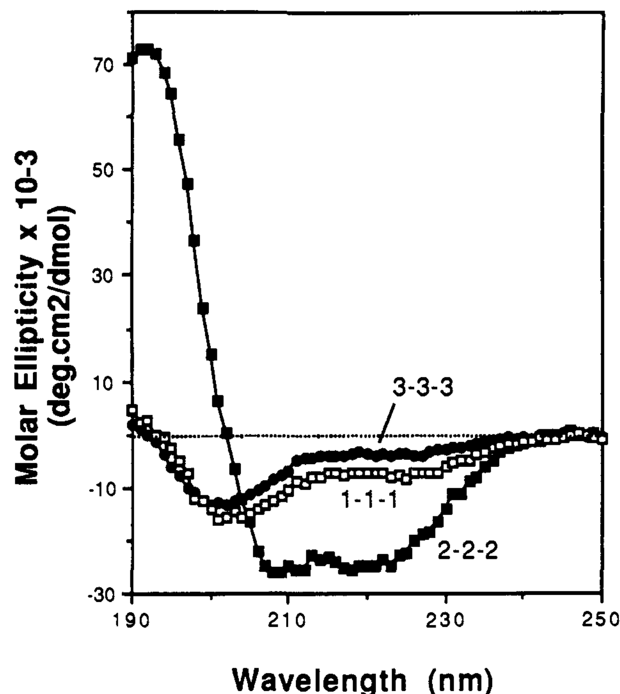

Figure 2. CD spectra of model peptides in aqueous 10 mM phosphate buffer, pH 7.0, at 25 °C: **1-1-1**, 40 μM (□); **2-2-2**, 30 μM (■); and **3-3-3**, 54 μM (●).

Optical Purity. For determination of racemization during the synthesis of peptides, assays with Marfay's reagent[18] were performed. The amounts of D-Asp, D-Glu, D-Leu, and D-Lys are expressed as percent of the total for each amino acid, as follows: **7** [(D-Asp+D-Glu)$_{<2.7}$, D-Leu$_{0.9}$, D-Lys$_{1.6}$], **2-2-2** [(D-Asp+D-Glu)$_{<9.7}$, D-Leu$_{<2.7}$, D-Lys$_{1.6}$], and **3-3-3** [D-Glx$_{4.2}$, D-Leu$_{1.7}$, D-Lys$_{2.0}$]. Within the limitations of this analysis, which arise mostly from racemization during peptide hydrolysis, these peptides appeared to be optically pure.

Circular Dichroism Studies. CD spectra of the three model peptides described in Figure 1 were first compared in aqueous 10 mM phosphate buffer, pH 7.0, at 25 °C. Each peptide gave a spectrum indicative of a mixture of α-helical and disordered conformations, but the CD spectrum of Lys^i,Asp^{i+4}-bridged peptide **2-2-2** was consistent with a very high α-helix content, whereas the spectra of **1-1-1** and **3-3-3** both indicated a low α-helix content (Figure 2).[19] Mean residue ellipticities, $[\theta]$ (deg cm^2 dmol^{-1}), were calculated per backbone peptide bond (n = 20), and the value measured at the 222-nm minimum that is characteristic of the α-helix n–π^* transition was used to calculate percent α helix, assuming that $[\theta]_{222}$ is proportional to percent helix and $[\theta]_{222}$ = −34 666 deg cm^2 dmol^{-1} corresponds to 100% α helix for a peptide of this length.[20] This calculation also assumes no contribution to $[\theta]_{222}$ arises from the lactam bridges in **1-1-1** and **2-2-2**. Calculated values of 69% α helix for **2-2-2** ($[\theta]_{222}$ = −23 800 deg cm^2 dmol^{-1}), compared to only 21% α helix for **1-1-1** ($[\theta]_{222}$ = −7300 deg cm^2 dmol^{-1}) and 13% α helix for **3-3-3** ($[\theta]_{222}$ = −4,400 deg cm^2 dmol^{-1}), were obtained.

The dependencies of the CD spectra on peptide concentration were also investigated under these conditions, in order to determine whether or not the amphiphilic character of the model peptides in the α-helical conformation favored self-association and con-

(14) Abbreviations used are those recommended by the IUPAC-IUB Joint Comission on Biochemical Nomenclature: BOP, benzotriazol-1-yloxytris-(dimethylamino)phosphonium hexafluorophosphate; CD, circular dichroism; 2Cl-Z, 2-chlorobenzyloxycarbonyl; DCC, *N,N'*-dicyclohexylcarbodiimide; DCM, dichloromethane; DIC, *N,N'*-diisopropylcarbodiimide; DIEA, *N,N*-diisopropylethylamine; EACNOx, ethyl 2(hydroxyimino)-2-cyanoacetate; HOBt, 1-hydroxybenztriazole; Pac, phenacyl; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol; TMSOTf, trimethylsilyl trifluoromethanesulfonate; Trt, trityl (=triphenylmethyl).

(15) BocLys(2Cl-Z)Asp(OH)OPac·DCHA (0.5 mmol) was coupled to oxime resin (0.75 g) in DCM with BOP (0.12 g, 0.55 mmol). After working up the reaction mixture the substitution level was 0.185 mmol/g, 0.146 mmol peptide, yield 29.2%.

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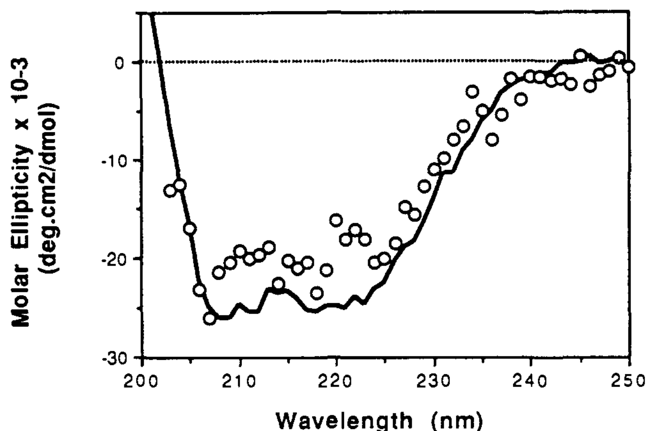

Figure 3. Concentration dependence of the CD spectra of **2-2-2**. The data points obtained for 45 nm **2-2-2** in aqueous 10 mM phosphate buffer, pH 7.0, at 25 °C in a 10-cm pathlength cell (O) are superimposed on the CD spectrum obtained for 30 μM **2-2-2** using a 1-mm pathlength cell (solid line).

sequent helix stabilization. In the concentration range between 0.5 and 100 μM, no concentration dependent changes in the CD spectra were observed for any of the three model peptides, indicating that the peptides do not change their aggregation states in this concentration range. For **2-2-2**, these studies were extended to extremely low concentrations (45 nM) using a 10-cm pathlength CD cell, since the dimerization of peptides consisting of somewhat similar coiled-coil sequence motifs has been observed in the nanomolar concentration range. Again, within the experimental errors arising mostly from peptide adsorption to the larger surface of the CD cell, no significant concentration-dependent changes in the CD spectrum were observed (Figure 3).

Several attempts were made to confirm that the model peptides were monomeric in aqueous solution by measuring their apparent molecular weights by the sedimentation equilibrium method. However, we were unable to obtain significant concentration gradients by the tabletop airfuge method of Pollet et al.[21] for any of the peptides. We have previously used this method successfully to identify peptide aggregates with MW_{app} of around 8000 g mol^{-1} but have found that measurements of significantly lower MW_{app} values by this method are generally unreliable. Therefore, stable aggregation states involving three or more peptide monomers appear to be absent from the solution conformations of **1-1-1**, **2-2-2**, and **3-3-3**. In agreement with the CD studies, the lack of any observed sedimentation indicates that dimeric peptide structures are also unlikely, although they cannot absolutely be ruled out on the basis of these experiments alone.

Effects of Trifluoroethanol. Since the Lys,Glu-bridged peptide **1-1-1** and the acyclic control peptide **3-3-3** had similar low α-helix contents in aqueous solution, the effects of adding the helix-promoting solvent TFE were investigated in order to distinguish the helix stabilities of these peptides more clearly. Addition of TFE enhanced the helix contents of all three peptides, as indicated by the increased negative molar ellipticities in their CD spectra around 208 and 222 nm. From the dependence of $[\theta]_{222}$ on the TFE content of the solvent (Figure 4), it is clear that the helical conformation is favored by **1-1-1** compared to **3-3-3**. Lower TFE contents were required to stabilize α-helical structure in the Lys,Glu-bridged peptide compared to the acyclic model peptide, and the Lys,Glu-bridged peptide reached a higher maximum helix content ($[\theta]_{222}$ = −30 000 deg cm^2 $dmol^{-1}$), equal to that of the Lys,Asp-bridged peptide **2-2-2** ($[\theta]_{222}$ = −30 600 deg cm^2 $dmol^{-1}$), at high TFE contents than did the acyclic peptide ($[\theta]_{222}$ = −20 200 deg cm^2 $dmol^{-1}$).

Compared to the large changes in the CD spectra of **1-1-1** and **3-3-3** upon addition of TFE, a relatively small increase in negative molar ellipticity at 222 nm was observed for **2-2-2** (Figure 4).

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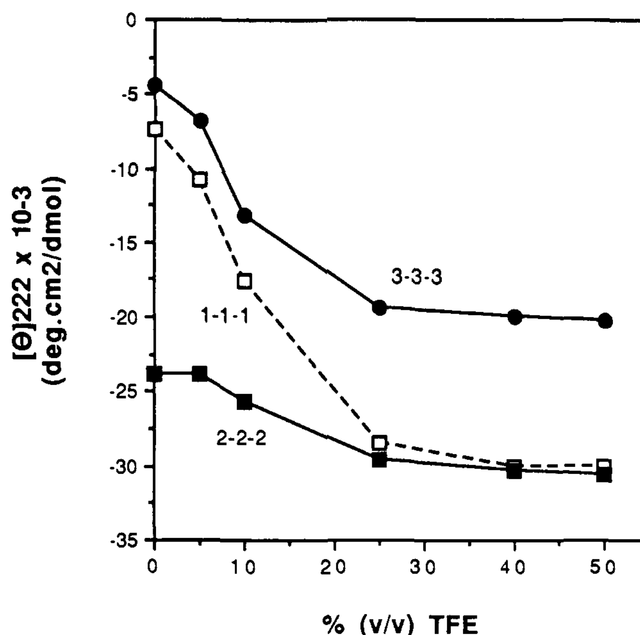

Figure 4. Effect of TFE on helix formation of synthetic peptides. Increasing proportions of TFE (%, v/v) were added to peptide solutions in aqueous 10 mM phosphate buffer, pH 7.0, at 25 °C and $[\theta]_{222}$ was monitored: **1-1-1**, 40 μM (□); **2-2-2**, 30 μM (■); **3-3-3**, 54 mM (●).

This suggests that the helical structure that is stabilized by Lys,Asp bridges in **2-2-2** in aqueous solution is already extensive, and a value of 88% α helix is calculated based on the limiting values described above. However, decreasing the temperature of a sample of **2-2-2** in 50% TFE to 5 °C resulted in a spectrum indicative of an even higher α-helix content corresponding to essentially 100% α helix ($[\theta]_{222}$ = −34 980 deg cm^2 $dmol^{-1}$).

Thermal Denaturation. The temperature dependencies of the conformations of the three model peptides were initially investigated by following $[\theta]_{222}$ as the temperature of the peptide solutions was increased stepwise from 1 to 90 °C. Equilibration of the sample at each new temperature was assessed by direct temperature measurement in the CD cell, and a 2.0-cm pathlength water-jacketed cell was used to minimize end effects. Equilibration of peptide conformation at each temperature was assessed by following $[\theta]_{222}$ using the spectropolarimeter in the kinetics mode for at least 20 min per measurement. Peptide **1-1-1** displayed an unusual temperature dependence in its CD spectra, which showed significantly increasing negative $[\theta]_{222}$ values as the sample temperature was increased from 1 °C to approximately 60 °C, followed by only a slight decrease in the negative $[\theta]_{222}$ values with further increases in temperature up to 90 °C (Figures 5 and 6A). In contrast, the acyclic peptide **3-3-3** showed essentially no temperature dependency. Thus, whereas **1-1-1** and **3-3-3** are both calculated to have 10% α helix at 1 °C, the values at 60 °C are 30% α helix for **1-1-1** and only 13% α-helix for **3-3-3**.

When frozen stock solutions of **2-2-2** were allowed to melt at room temperature and then diluted into cold buffer at 1 °C in the CD cell, the helix content was significantly increased compared to measurements at 25 °C, as evidenced by a $[\theta]_{222}$ value of −28 800 deg cm^2 $dmol^{-1}$. This corresponds to an estimated 83% α helix. Following $[\theta]_{222}$ with increasing temperature gave rise to a gently sloping, nonsigmoidal denaturation curve that appeared to be approaching the $[\theta]_{222}$ value observed for **1-1-1** at high temperatures but was still substantially more negative even at 90 °C (Figure 5). This behavior indicates that the thermal denaturation of **2-2-2** is incomplete even at 90 °C and is a noncooperative, multistep process, possibly involving unfolding of the helical structure around the central Lys^{10},Asp^{14} lactam bridge at higher temperatures than the helical structure around the other two bridges.

The "annealing" curve obtained by subsequent cooling of **2-2-2** solutions from 90 to 1 °C was nearly linear below approximately

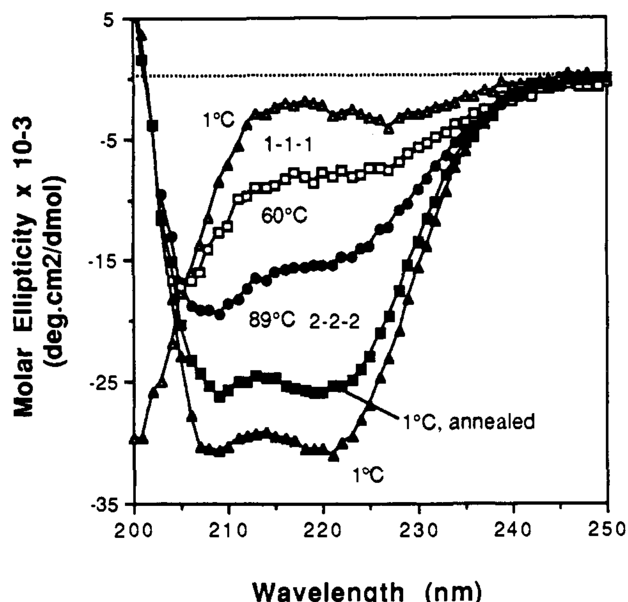

Figure 5. Effect of temperature on the CD spectra of peptides. Peptide **1-1-1** (3.7 μM, open symbols) after dilution into aqueous 10 mM phosphate buffer, pH 7.0, at 1 °C (△) and then heating to 60 °C (□); peptide **2-2-2** (4.0 μM, closed symbols), after dilution into the same buffer at 1 °C (▲), heating to 89 °C (●), and then slowly cooling down to 1 °C again (■).

80 °C and showed an increasing disparity with the denaturation curve as lower temperatures were reached. A spectrum that is significantly different from that initially obtained at 1 °C, having a $[\theta]_{222}$ value of only −25 150 deg cm^2 dmol^{-1}, was obtained upon returning the heated peptide solutions to 1 °C (Figure 5). In separate experiments, two of which are compared in Figure 6B, the difference between the heating and cooling curves was demonstrated to be independent of the rate of heating and cooling or the time that **2-2-2** was maintained at high temperature. Thus peptide losses due to degradation or precipitation are probably not responsible for the differences observed, suggesting that **2-2-2** may be present in aqueous solution at pH 7.0 in a mixture of two or more rigid helical conformations that have significantly different CD spectra and cannot readily equilibrate below about 70 °C.

Chemical Denaturation. Denaturation of the three model peptides by the chemical denaturant guanidinium hydrochloride (Gdn·HCl) was investigated by determining $[\theta]_{222}$ as a function of Gdn·HCl concentration in the 10 mM phosphate buffer, pH 7.0 at 25 °C. All three peptides showed increasing molar ellipticities at 222 nm at increasing Gdn·HCl concentrations, indicating unfolding of ordered secondary structure (Figure 7). Mixing peptide solutions with different Gdn·HCl concentrations indicated that equilibrations were rapid and that the denaturation process was reversible in each case.

Peptides **1-1-1** and **3-3-3** were readily denaturated by low concentrations of Gdn·HCl, and $[\theta]_{222}$ increased to similar constant values for each peptide (+870 deg cm^2 dmol^{-1} and +1330 deg cm^2 dmol^{-1}, respectively) at Gdn·HCl concentrations of at least 4 M. The conformational states in this plateau region are assumed to correspond to the completely unfolded states of these two peptides. In contrast, **2-2-2** retained most of its helical structure in Gdn·HCl concentrations up to 4 M and underwent a broad and incomplete unfolding transition in Gdn·HCl concentrations between 4 and 8 M (the solubility limit for Gdn·HCl). Assuming that complete denaturation of **2-2-2** would give the same plateau value of $[\theta]_{222}$ as for **1-1-1**, the estimated Gdn·HCl concentration required for 50% denaturation of **2-2-2** is 7.30 M.

Surface-Induced Conformations. The model peptides in this study were designed to be amphiphilic in an α-helical conformation so that helix induction at interfaces could be studied as a model for the receptor binding interactions of amphiphilic α-helical peptide hormones incorporating similar conformational constraints.[3] The conformations of the three model peptides that are

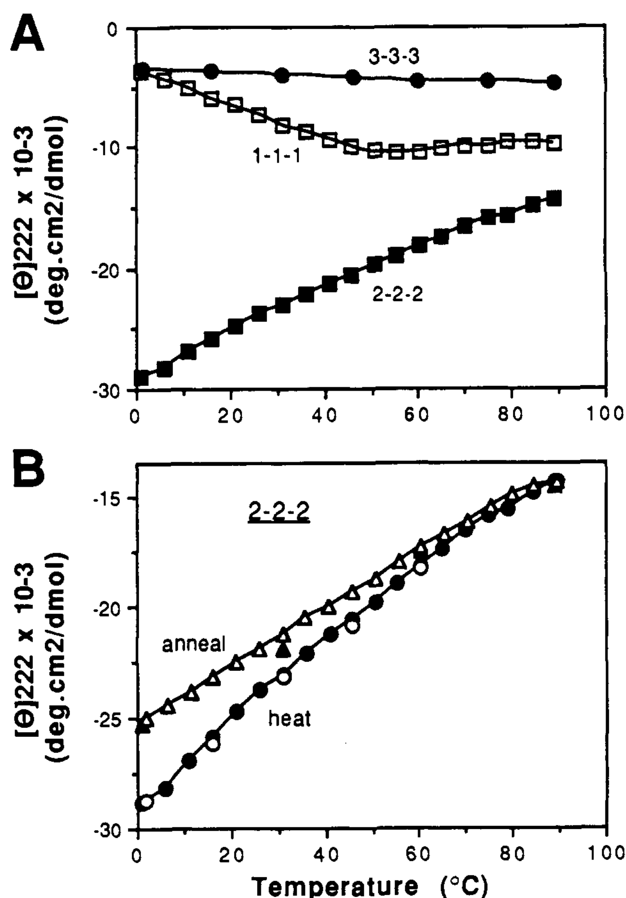

Figure 6. Thermal denaturation of peptides. (A) Ellipticity at 222 nm was followed for solutions of 3.7 μM **1-1-1** (□), 4.0 μM **2-2-2** (■), and 11 μM **3-3-3** (●) in aqueous 10 mM phosphate buffer, pH 7.0, as their temperatures were reequilibrated at increasing temperatures. (B) In two separate experiments, ellipticity at 222 nm was followed for **2-2-2** solutions in the phosphate buffer at 3.2 μM (open symbols) and 4.0 μM (filled symbols), as these solutions were reequilibrated at increasing temperatures (○, ●) and then decreasing temperatures (△, ▲).

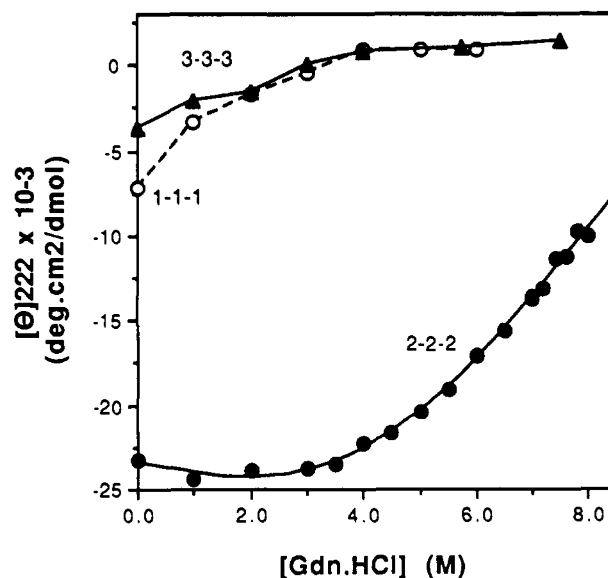

Figure 7. Chemical denaturation of peptides using Gdn·HCl. Ellipticity at 222 nm was determined for solutions of 40 μM **1-1-1** (○), 42 μM **2-2-2** (●), and 68 μM **3-3-3** (▲) equilibrated at 25 °C in aqueous 10 mM phosphate buffer, pH 7.0, containing various Gdn·HCl concentrations.

induced upon binding to a hydrophobic surface were determined by measuring the CD spectra of peptide films on siliconized quartz slides. Peptides were adsorbed from aqueous buffer at low con-

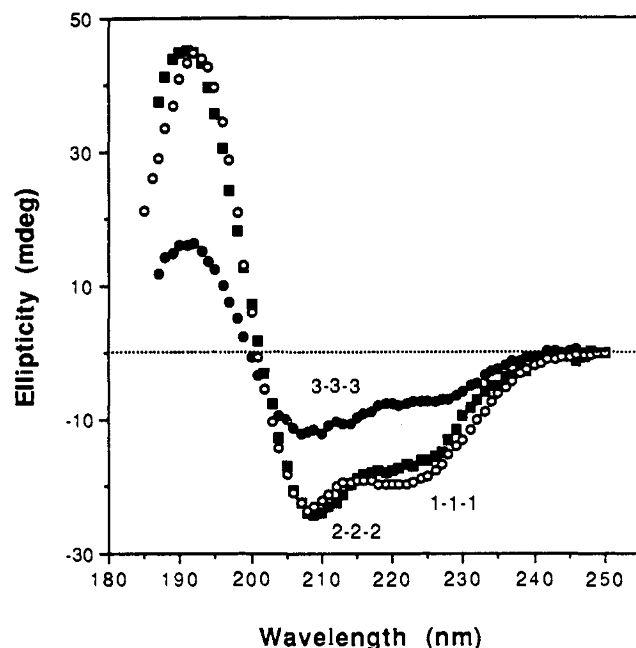

Figure 8. CD spectra of synthetic peptides adsorbed onto siliconized quartz slides. Peptides **1-1-1** (○), **2-2-2** (■), and **3-3-3** (●) were adsorbed from aqueous buffer containing approximately 10 μM peptide onto siliconized quartz slides. Sets of four slides for each peptide were then rinsed in H_2O and used to determine CD spectra for the adsorbed peptide films, as described previously.[26]

centrations onto the slides.[22] The slides were then rinsed in distilled water and CD spectra of sets of four of the slides were averaged for eight different orientations of the slides about the lightpath, in order to eliminate linear dichroism artefacts.[23] The shapes of the resultant spectra, shown in Figure 8, all indicated predominantly helical conformations, with the helix axis lying in the plane of the slide interface.[24] The spectra obtained for films of **1-1-1** and **2-2-2** were of similar intensities and were approximately twice the intensity of the spectrum obtained from **3-3-3**. Since the peptide content of the films could not be quantitated, this difference may indicate that **3-3-3** has a lower helix content, or that a smaller amount of **3-3-3** could be adsorbed onto the slides, or both. A higher fraction of disordered structure in the molecules of **3-3-3** bound to the slide surface would be expected to reduce the quantity of peptide bound, since the CD spectra of peptide films that are obtained by this method have generally been observed to correspond to monomolecular layers filling the available surface area.[3] A lower helix content for **3-3-3** in its surface-induced conformation would also be consistent with the lower degree of helix induction observed for this peptide in TFE solutions (Figure 4).

Discussion

Three model peptides have been designed to investigate the α-helix forming propensity of peptides incorporating multiple lactam bridges linking the side chains of lysine and glutamic acid residues (**1-1-1**) or lysine and aspartic acid residues (**2-2-2**) in positions *i* and *i*+4 of a peptide chain, compared to an appropriate acyclic analogue having the same amino acid sequence (**3-3-3**). The novel synthetic approach to multicyclic peptides previously reported for **1-1-1**[4] has now also been successfully applied to the synthesis of **2-2-2**. The key step in this synthetic approach, namely the cyclization/cleavage reaction on the oxime resin support, again proceeded in good yield (54%) and generated a protected peptide product of sufficiently high purity to be used in the subsequent solution-phase condensation steps after only minimal purification.

The acyclic analogue, **3-3-3**, was also prepared using the oxime resin as a solid support, but in this case the segment condensation strategy developed by Kaiser et al.[6] was employed. Solid-phase condensations of the repeated peptide segments were preferred over solution-phase reactions for optimal synthetic efficiency. No difficulties were encountered with this approach, since the size of the fully assembled peptide did not exceed the size range for which the oxime resin is useful.[25]

CD spectra of the model peptides in aqueous buffer at 25 °C and pH 7.0 (Figure 2), in TFE–water mixtures (Figure 4), and bound to siliconized quartz slides (Figure 8), indicate that α helix stability is ordered **2-2-2** ≫ **1-1-1** > **3-3-3**. Furthermore, **2-2-2** appears to be monomeric in aqueous buffer at 25 °C and neutral pH, so that its α-helical structure is not stabilized through self-association to form helix bundles or coiled coils, despite its amphiphilic character and surface-binding properties. Several structural features of **2-2-2** may inhibit aggregation. First, self-association in the helical conformation would place Lys residues at every position adjacent to the intermolecular interface formed by the buried Leu residues. Studies of the DNA binding proteins Fos and Jun have shown that dimerization of α helices in a parallel orientation to form a coiled-coil motif is strongly disfavored by such charge repulsion at the dimer interface.[26] Second, early studies of synthetic peptide models demonstrated that at least four of the seven-residue repeats that are characteristic of coiled-coil motifs, rather than the three repeats in our model peptides, are required for dimerization in the micromolar concentration range, even when favorable ionic interactions were designed into the dimer interface.[27] Finally, the arrangement of Leu residues in positions 2 and 5 of a seven-residue repeat is expected to generate an array of hydrophobic side chains on the helix surface that is unfavorable for self-association in an antiparallel orientation to form four-helix bundles.[28]

The maximum helix content that can be induced in the acyclic peptide **3-3-3** in TFE or upon binding to the hydrophobic coated slides (Figures 4 and 8) appears to be lower than that induced in the two multicyclic peptides, indicating the presence of residues for which the α-helical conformation is particularly unfavorable. NMR studies of model peptides have shown that certain peptide segments may remain in nonhelical conformations in TFE even when helical structure is fully induced in the adjacent peptide segments.[29] Presumably, even the residues corresponding to such nonhelical segments in **3-3-3** are effectively constrained in the α-helical conformation by the lactam bridges in **1-1-1** and **2-2-2**.

Our assumptions in interpreting the CD spectra as described above are that the lactam bridge amides do not contribute significantly to the molar ellipticities observed at 222 nm in either the disordered or the α-helical conformations and that the α-helical conformation is not significantly distorted by the bridges, since this is also expected to affect the intensity of the CD signal at 222 nm.[30] The reasonable agreement reported for the α-helix contents of lactam-bridged GRF analogues calculated from CD spectra and from NOE-constrained molecular dynamics and energy minimization[10a] indicates that these assumptions are approximately valid. In our experiments, the CD signal at 222 nm for Gdn·HCl-denatured Lys,Glu-bridged **1-1-1** and Gdn·HCl-denatured acyclic **3-3-3** are essentially identical and near to zero (Figure 7), as is generally observed for linear Gdn·HCl-denatured peptides of this size.[31] This confirms that the lactam bridges do

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not contribute significantly to the CD signal of the nonhelical (disordered) state. Similar comparisons may be applied to the fully helical state. The molar ellipticity at 222 nm measured at 25 °C for **2-2-2** reaches a plateau value of −31 600 cm^2 dmol^{-1} upon incremental additions of TFE up to 50% v/v. Since this represents only a 1.3-fold increase over the value in purely aqueous solution, compared to the 4.1- and 4.6-fold increases observed for **1-1-1** and **3-3-3**, respectively, it seems reasonable to assume that the molar ellipticity observed for **2-2-2** in 50% TFE represents close to 100% α-helical conformation. This molar ellipticity is only enhanced a further 1.1-fold to −34 980 deg cm^2 dmol^{-1} by reducing the temperature to 5 °C. Molar ellipticity values in this range agree well with the value of −34 666 deg cm^2 dmol^{-1} estimated for a linear 21-residue peptide that is 100% α-helical.[20] Despite this approximate agreement, however, our thermal denaturation studies of **2-2-2** suggest that interpreting the CD signal at 222 nm in terms of fractional helix contents may not be straightforward (see below).

Thermal denaturation studies (Figures 5–7) demonstrate that the helical conformation of **2-2-2** that is stabilized by multiple Lys^i,Asp^{i+4} side-chain lactam bridges is remarkably resistant to denaturation. There is very little indication that $[\theta]_{222}$ is approaching a plateau that might be representative of the heat-denatured state of **2-2-2**, even at 90 °C. If one assumes that this plateau value would be the same for **2-2-2** as for the Lys^i,Glu^{i+4} bridged **1-1-1**, then the α-helix content of fully denatured **2-2-2** would be reduced to 28%, but at 90 °C a calculated 41% α-helix still remains.

The resistance of the helical structure in **2-2-2** to Gdn·HCl denaturation (Figure 7) appears equally remarkable, when it is compared to natural globular proteins,[32,33] a four-helix bundle protein of de novo design,[34] or the helical dimerization domain of transcription factor LFB1 (a "leucine zipper" coiled coil).[35] However, even compared to the LFB1 peptide,[35] the denaturation curve determined for **2-2-2** is unusually shallow at its assumed midpoint. This is a characteristic expected for Gdn·HCl denaturation of a monomeric helical peptide, which would have virtually no hydrophobic core consisting of the buried hydrophobic side chains and backbone structural elements that normally contribute to the cooperativity of the denaturation process in globular proteins.[32] Thus, **2-2-2** owes its extreme Gdn·HCl resistance primarily to the fact that Gdn·HCl is a poor denaturant for an isolated helical structure.

The lack of any significant change in $[\theta]_{222}$ as the Gdn·HCl concentration is increased from 0 to 3.5 M suggests that **2-2-2** in aqueous solution at 25 °C consists predominantly of a small number of discrete Gdn·HCl-resistant conformations in which up to 69% of the residues are in an α-helical conformation almost 100% of the time. (At the other extreme, a very flexible structure might have a large population of different conformations having widely different helix contents such that each residue is α-helical only 69% of the time.) The high thermal stability of the α-helical conformation of **2-2-2** and the lack of correspondence between the heat denaturation and annealing curves (Figure 6) also indicate that a small number (two or more) of rigid α-helical structures may be contributing to the CD spectrum and that these structures do not interconvert at a measurable (by CD) rate in aqueous solution at room temperature. Examination of Corey–Pauling–Koltun molecular models, or space filling models built by running Quanta software (Polygen) on a Stellar GS 1000 Supermini computer, shows that a Lys^i,Asp^{i+4} lactam bridge would be tightly packed against the surface of a regular α helix, and that there appear to be two distinct sterically allowed bridge conformations. These conformations are determined primarily by the orientation on the helix surface of the amide moiety, which is assumed to be in the trans configuration and nearly planar, and they allow for very little additional flexibility in the rest of the structure. The sterically allowed Lys^i,Asp^{i+4} lactam bridge conformations on the surface of an α helix also include unfavorable eclipsed conformations in the lysine side chain. This suggests that different distortions in the α-helical conformation of **2-2-2** might result from the two major bridge conformations available and give rise to a CD signals of different magnitudes.[30] A high energy barrier to interconversion between these bridge conformations, which requires uncoiling of the helix, may prevent the central Lys^{10},Asp^{14} bridge in **2-2-2** in particular from reaching thermodynamic equilibrium without heat denaturation and annealing. However, high energy barriers for interconversion with alternative, nonhelical conformations may also explain the present data and more detailed conformational studies using NMR techniques are required.

Thermal denaturation of **3-3-3** generated a nonzero plateau value for $[\theta]_{222}$ close to −5000 deg cm^2 dmol^{-1} (−4640 deg cm^2 dmol^{-1}) (Figure 6). A nonzero plateau value of $[\theta]_{222}$ is a common feature of the thermal denaturation of many linear peptides as well as globular proteins[31] and indicates that thermal denaturation is less complete than Gdn·HCl denaturation. However, the increasing negative ellipticity at 222 nm with increasing temperature and high negative $[\theta]_{222}$ plateau value observed for **1-1-1** are both very unusual and suggest that the formation of α-helical structure in **1-1-1** is an entropy-driven process. Both the increased flexibility of the Lys^i,Glu^{i+4} lactam bridges in the helical structures of **1-1-1**, compared to the shorter Lys^i,Asp^{i+4} bridges in **2-2-2**, and the increased rigidity in the nonhelical conformations of **1-1-1** that contribute to the "disordered" component of the CD spectrum, relative to linear peptides such as **3-3-3**, might contribute to this effect.

Conclusion

We have demonstrated that multicyclic peptides may be assembled in routine fashion using an oxime resin assembly and cyclization procedure. CD studies of multicyclic model peptides **1-1-1** and **2-2-2**, in comparison to their acyclic analogue **3-3-3**, indicate that linking the side chains of three Lys^i,Asp^{i+4} or Lys^i,Glu^{i+4} pairs to form multiple lactam bridges stabilizes the α-helical conformation in aqueous solution in a 21-residue peptide at neutral pH in the order Lys^i,Asp^{i+4} bridges $\gg$ Lys^i,Glu^{i+4} bridges > no bridges. Both types of bridge also favor helix formation upon surface binding by the model peptides in this study, which have the potential to form amphiphilic α helices. Introduction of three Lys^i,Asp^{i+4} bridges into the hydrophilic face of an amphiphilic α-helical motif in a biologically active peptide in place of nonessential residues should, therefore, significantly enhance the biological activities that are associated with the helical structure and may also result in substantially prolonged biological halflives. However, thermal and chemical denaturation studies of **2-2-2** suggest that these multiple bridges will also introduce significant rigidity and/or distortions into the helical structure, and these changes might be incompatible with a given biological function. In such cases, the more flexible Lys^i,Glu^{i+4} bridges may be more useful, although the potential for potency enhancement would be lower.

Experimental Section

Peptide Synthesis. Detailed procedures for the peptide syntheses described in Schemes I and II are provided in the supplementary material supporting this work. Only the assembly of **6** on oxime resin and its deprotection and cyclization to give **7** are described below.

General Methods for Peptide Synthesis. All amino acids were of the L configuration. Materials were purchased and purified as described previously.[4] *p*-Nitrobenzophenone oxime resin,[6] *N*-hydroxypiperidine,[36] and EACNOx[16] were prepared according to the literature. Melting points are the uncorrected values measured in a capillary melting point apparatus. Analytical investigations (HPLC, TLC, carboxyl end-group titration, optical rotation measurements, amino acid analysis, and optical purity) were performed as described previously.[4] Crude and purified peptides were analyzed on precoated silica gel F-254, 0.25 mm plates (Aldrich) using (B) $CHCl_3$/dioxane/EtOH/AcOH, 11/2/0.8/0.2 and (D) $CHCl_3$/MeOH/AcOH, 18/1/1.

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Mass spectrometric analyses were performed under the direction of Dr. Brian Chait in the Rockefeller University Mass Spectrometry Laboratory, using the ^{252}Cf fission fragment method.[37]

Boc-Lys(2Cl-Z)LeuLys(Trt)Glu(OBzl)LeuLys(2Cl-Z)Asp(O-resin)-OPac (6). Boc-Lys(2Cl-Z)Asp(OH)-OPac, **5** (3.07 g, 4.62 mmol), was attached to *p*-nitrobenzophenone oxime resin (25 g) with EACNOx (1.31 g, 9.24 mmol) and DCC (1.03 g, 5 mmol) in DCM (300 mL) at −10 °C for 30 min and at 20 °C overnight. Acetylation of excess oxime groups and coupling of the next two amino acids [BocLeuOH, BocGlu(OBzl)-OH] and the N-terminal BocLys(2Cl-Z)LeuLys(Trt)OH were then performed exactly as described for the synthesis of **1-1-1**:[4] yield 3.79 mmol peptide (86.9%); substitution level 0.12 mmol/g, based on Leu; amino acid analysis $Asp_{1.20}Glu_{1.15}Leu_{2.00}Lys_{2.86}$.

Cyclization Reaction: Cyclo(1-5)-Boc-Lys(2Cl-Z)LeuLysGlu(OBzl)-LeuLys(2Cl-Z)Asp-OPac. Boc-(1-7)-OPac (7). Peptidyl resin **6** (10 g, 1.2 mmol peptide on resin) was swollen in DCM (100 mL) in a solid-phase peptide synthesis vessel. The Trt group was removed from the Lys ε-amino group with 5% TFA in DCM–TFE 1/1 (3 × 100 mL), shaking the reaction vessel at room temperature for 4 h. The peptidyl resin was then drained, washed, and neutralized according to the standard oxime resin protocol.[6] The cyclization reaction was then carried out by shaking the peptidyl resin in DCM (14 mL) in the presence of 10 equiv of AcOH at room temperature for 72 h. The cyclic peptide product was collected from the reaction vessel by draining and then washing the resin with DCM (3×). These solutions were combined, evaporated to a reduced volume, and then washed at 0 °C with water, 0.1 N HCl, 5% $NaHCO_3$, and brine. The solvent was then evaporated, and the crude product was purified by silica gel flash chromatography (2 × 20 cm, $CHCl_3$/MeOH 10/1). The appropriate fractions were pooled, and the solvent was evaporated. The pure solidified product was recrystallized from methanol/ether: yield 970 mg (54%); mp 92–95 °C; R_f(B) 0.47; R_f(D) 0.65; $[\alpha]_D$ −7.3 ± 0.2 (*c* 1.7, $CHCl_3$); amino acid analysis $Asp_{0.83}Glu_{1.16}Leu_{2.00}Lys_{3.14}$; MS (^{252}Cf fission fragment) m/e = 1500.5 (theoretical 1500.5 for $(M + Na)^+$, $\Delta = 0.0$); racemization assasy <2.7% D-Asp + D-Glu, 0.97% D-Leu, 1.6% D-Lys.

Circular Dichroism Studies. CD measurements were performed on an Aviv Model 62ds spectropolarimeter fitted with a fused silica modulator. Stock peptide concentrations were determined in triplicate by amino acid analyses after hydrolysis in 6 N HCl at 110 °C for 24 h, using crystalline alanine (Sigma Chemical Co., St. Louis, MO) as an internal standard. Spectra were determined using 10 mM KH_2PO_4 titrated to pH 7.0 at 25 °C with NaOH as buffer and 1.0-mm, 5.0-mm, 2.0-cm or 10-cm pathlength cells, as appropriate for the peptide concentration under study. Data were typically collected using a time constant of 2.0 s and averaging the data from five scans. Data collected at dynode voltages greater than 450 V were discarded. The CD spectra of peptides adsorbed onto siliconized circular quartz slides (22 × 1 mm, Hellma Cells Inc., Jamaica, NY) from the same phosphate buffer were determined using the method described previously.[4,22] These spectra represent the sum of spectra collected for eight different orientations of a set of four slides about the lightpath.

Characterization of Model Peptides by Equilibrium Sedimentation. The method described by R. Pollet et al.[21] was used. The peptide samples were dissolved in 100 μL of buffer (0.02 M Na_2HPO_4 buffer, pH 7.4, containing 0.16 M KCl and 5 mg/mL Dextran T 40). Peptide solutions were centrifuged at 95 000–100 000 rpm in a Beckman Spinco Airfuge at room temperature. After 20-h centrifugation, the peptide content of 10-μL fractions was determined by fluorescence after derivatization with *o*-phthalaldehyde in sodium borate buffer at pH 9.0. The correct determination of MW_{app} for hemoglobin in experiments carried out at lower rotor speeds immediately prior to the peptide analyses ensured that these experiments were correctly executed.

Acknowledgment. This research was supported by U.S.P.H.S. Grants HL-18577 and DA-04197. We are grateful for the amino acid analyses and racemization assays performed by Mr. Adam A. Profit and the mass spectrometric analyses performed in the laboratory of Dr. Brian Chait. We thank Dr. Klara Ösapay for help with molecular graphics studies using Quanta.

Supplementary Material Available: Experimental data for **4–11**, **13**, **15**, **2-2-2**, and **3-3-3** (10 pages). Ordering information is given on any current masthead page.

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