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Impact of Backbone Pattern and Residue Substitution on Helicity in $\alpha/\beta/\gamma$ -Peptides

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Abstract

We have evaluated the impact of changes in the chemical structure of peptidic oligomers containing α -, β - and γ -amino acid residues ($\alpha/\beta/\gamma$ -peptides) on the propensities of these oligomers to adopt helical conformations in aqueous and alcoholic solutions. These studies were inspired by our previous discovery that $\alpha/\beta/\gamma$ -peptides containing a regular $\alpha\gamma\alpha\alpha\beta\alpha$ hexad repeat adopt an α -helix-like conformation in which the β and γ residues are aligned in a stripe along one side and the remainder of the helix surface is defined by the α residues. This helix was found to be most stable when the β and γ residues were rigidified with specific cyclic constraints. Relaxation of the β residue constraints caused profound conformational destabilization, but relaxation of the γ residue constraints led to only a moderate drop in helicity. The new work more broadly characterizes the effect of γ residue substitution on helix stability, based on circular dichroism (CD) and two-dimensional nuclear magnetic resonance (2D NMR) measurements. We find that even a fully unsubstituted γ residue (derived from γ -amino butyric acid) supports a moderate helical propensity, which is surprising in light of the strong destabilizing effect of glycine residues on α -helix stability. Additional studies examine the effects of altering sequence in terms of amino acid type, by comparing a prototype with the $\alpha\gamma\alpha\alpha\beta\alpha$ hexad pattern to isomers with irregular arrangements of the α , β and γ residues along the backbone. The data indicate that the strong helix-forming propensity previously discovered for $\alpha/\beta/\gamma$ -peptide 12-mers is retained when sequence is varied, with small variations detected across diverse α - β - γ placements. These structural findings suggest that $\alpha/\beta/\gamma$ -peptide scaffolds represent versatile scaffolds for the design of peptidic foldamers that display specific functions.

Graphical Abstract

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Notes

The authors declare no competing financial interest.

Supporting Information. Experimental details, including polypeptide characterization, CD and NMR data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

The α -helix is a very common motif among proteins, and many protein-protein interactions depend upon the recognition of a helical segment from one partner by a complementary surface on the other.^{1,2} Considerable effort has been devoted to the design of unnatural oligomers that can mimic the surface displayed by a specific α -helix, because such agents can inhibit or augment interactions between natural proteins.^{3–12} Conventional peptides themselves are not ideal for this purpose since the conformational stability of isolated α -helices in aqueous solution is inherently low, and since such peptides are susceptible to rapid degradation by proteases *in vivo*. Properly designed unnatural oligomers can address both of these limitations.^{3–12} However, departure from the α -amino acid backbone usually causes a change in side chain presentation pattern relative to an authentic α -helix;¹² intrinsic structural mismatches become increasingly problematic with longer helices.

Previous α -helix mimicry efforts from our group have focused on oligomers in which α - and β -amino acid residues are intermixed, to generate “ α/β -peptides.”^{11–17} We discovered that α/β -peptides containing 25–33% β residues interspersed among α residues (backbone patterns $\alpha\alpha\beta$, $\alpha\alpha\alpha\beta$ and $\alpha\alpha\beta\alpha\alpha\beta$) can adopt helical conformations that are very similar to a canonical α -helix, despite the extra carbon atoms in the backbone relative to a conventional peptide.¹³ Side chain display is faithfully reproduced over as many as 10 helical turns. The even distribution of β residues, which limits the number of consecutive α residues in any region to two or three, can result in very substantial retardation of proteolytic cleavage.^{14–17} This type of α/β -peptide is readily generated from the prototype α -peptide sequence via periodic replacement of α residues with their β^3 homologues. In this scenario, the β residue maintains the original side chain but contains an “extra” backbone methylene unit. However, the resulting α/β^3 -peptides sometimes manifest lower affinity for the target than does the original α -peptide, an effect that may arise because of enhanced conformational freedom of the α/β^3 backbone relative to the α backbone. In such cases, α/β -peptide affinity can sometimes be regained if flexible β^3 residues are replaced with ring-constrained β residues.^{14,17,18}

Although the α/β -peptide approach for α -helix mimicry has been fruitful in several contexts, it is important to consider designs based on alternative backbones, i.e., backbones that are not limited to α - and β -amino acid residues. Many possibilities emerge from fundamental

studies of oligomers containing γ -amino acid residues pioneered by the groups of Schreiber,¹⁹ Hanessian²⁰ and Seebach²¹ and subsequently pursued in other laboratories.^{22–26} Peptidic oligomers containing α , β , and γ -residues ($\alpha/\beta/\gamma$ -peptides) have been investigated in a few cases.^{27–29}

Recently, we described an $\alpha/\beta/\gamma$ -peptide design that represents a new approach to α -helix mimicry.^{30,31} A six-residue segment with the backbone pattern $\alpha\gamma\alpha\alpha\beta\alpha$ is used to generate two helical turns, which in a true α -helix are formed by seven α residues. This approach involves an isoatomic backbone replacement, since $\alpha\gamma\alpha\alpha\beta\alpha$ and $\alpha\alpha\alpha\alpha\alpha\alpha$ segments have the same number of backbone atoms. In contrast, the α/β -peptide-based strategies mentioned above lead to mimics that contain more backbone atoms per turn than does an authentic α -helix. The $\alpha\gamma\alpha\alpha\beta\alpha$ hexad pattern was chosen based on the prediction that helix formation would cause the β and γ residues to be segregated on one side of the helix and the α residues to be segregated on the opposite side (Figure 1). Two-dimensional NMR and crystallographic data obtained for specific $\alpha/\beta/\gamma$ -peptides containing the $\alpha\gamma\alpha\alpha\beta\alpha$ hexad pattern were consistent with this prediction.³¹

Wilson, Aitken et al. have very recently described $\alpha/\beta/\gamma$ -peptides that function as specific inhibitors of the p53-*h*DM2 interaction.³² These workers used carefully designed hexamers with either a $\alpha\gamma\beta\gamma\beta\gamma$ or a $\alpha\gamma\beta\gamma\beta\alpha$ backbone to mimic the projection of three key side chains from an 8-mer α -helical segment in the N-terminal domain of tumor suppressor p53 (residues 19–26). 2D NMR studies conducted in chloroform provided clear evidence for adoption of a helical conformation involving C=O(*i*)–H–N(*i*+3) H-bonds; this H-bond pattern is observed also in helical conformations of the $\alpha/\beta/\gamma$ -peptides we have studied.³¹ The helical conformations of the Wilson-Aitken $\alpha/\beta/\gamma$ -peptides present a Phe-Trp-Leu side chain triad in an arrangement suitable for binding to the p53-recognition cleft on *h*DM2, as evidenced by the $\alpha/\beta/\gamma$ -peptides' ability to displace an authentic p53(15–31) peptide from this cleft.

Previous findings from our group^{30,31} raise fundamental questions regarding $\alpha/\beta/\gamma$ -peptide folding that are sharpened by the discoveries of Wilson, Aitken et al.³² Can all peptidic backbones containing a 4:1:1 $\alpha:\beta:\gamma$ residue ratio adopt a helical conformation, regardless of the backbone pattern? Do changes in the backbone arrangement of the residues lead to variations in helical propensity? The present study addresses these questions via comparisons involving several isomeric $\alpha/\beta/\gamma$ -peptide 12-mers with an invariant composition of eight α residues, two β residues and two γ residues. In addition, we evaluate the effect of γ residue substitution pattern on helical propensity. This portion of our study was motivated by the surprising observation that $\alpha/\beta/\gamma$ -peptide helicity was maintained when cyclically constrained γ residues were replaced with more conformationally flexible γ residues.³¹ The information from these basic studies provides a foundation for future application-oriented $\alpha/\beta/\gamma$ -peptide design efforts. In addition, these insights emerging from this work help to establish $\alpha/\beta/\gamma$ -peptides within a growing family of helical foldamers based on heterogeneous backbones (i.e., foldamers that contain more than one type of subunit).³³

RESULTS AND DISCUSSION

Experimental design

$\alpha/\beta/\gamma$ -Peptide 12-mer **1** serves as a benchmark for the studies described here (Figure 1A). This $\alpha/\beta/\gamma$ -peptide contains two cyclic β residues and two acyclic γ residues in addition to eight α residues. $\alpha/\beta/\gamma$ -peptide **1** was previously shown to display $i,i+2$ and $i,i+3$ backbone NOEs in aqueous solution that are consistent with an α -helix-like conformation.³¹ The number of helix-characteristic medium-range NOEs increased when measurements were made in methanol, which is consistent with precedents that indicate that alcohol solvents enhance helicity relative to aqueous solution for peptides.³⁴ The crystal structure of a longer $\alpha/\beta/\gamma$ -peptide containing an $\alpha\gamma\alpha\alpha\beta\alpha$ hexad comparable to those in **1** confirmed that this backbone could adopt an α -helix-like conformation.³¹

Far-UV CD analysis of **1** in aqueous buffer, as previously reported,³¹ revealed a strong minimum at ~ 202 nm (Figure 2A), which we interpret as arising from at least partial population of an α -helix-like conformation, based on the 2D NMR and crystallographic data described above. Addition of 60 vol % 2,2,2-trifluoroethanol (TFE) causes a slight red-shift in the CD minimum for **1**, and a moderate increase in the intensity of this minimum (Figure 2B). These changes suggest that the TFE co-solvent enhances $\alpha/\beta/\gamma$ -peptide helicity relative to purely aqueous solution, as is well-established for other peptidic backbones.³⁴

The experiments described below involve peptides that are based on **1**. In the first set of studies, we vary substituents at carbon-4 of the γ residue while maintaining the $\alpha\gamma\alpha\alpha\beta\alpha$ hexad pattern. In the second set, we maintain residue identities but vary the arrangement of the α , β and γ residues. Additional studies explore the impact of cyclic vs. acyclic β or γ residues for selected backbone patterns. CD is used to make global comparisons among the $\alpha/\beta/\gamma$ -peptides, and selected examples are further characterized via 2D NMR.

Effects of varying γ residue substitution pattern

We compared $\alpha/\beta/\gamma$ -peptide **1** with analogues **1a** and **1b**, in which the identity of the γ residues is altered (Figure 1). In **1a**, the methyl side chains of the two γ residues have been replaced with H; in other words, the γ homologue of alanine (γ^4 -hAla) in **1** has been replaced with a γ homologue of glycine (γ -amino butyric acid (GABA) or γ -hGly) in **1a**. This change would be predicted to diminish helical folding propensity, by analogy to the diminution of α -helical propensity observed among conventional peptides when Ala is replaced with Gly.³⁵ In **1b**, a second methyl side chain is added to carbon-4 of the two γ residues; in other words, the γ^4 -hAla residues of **1** have been replaced with the γ^4 homologue of Aib (γ^4 -hAib) in **1b**. Aib itself promotes helicity among conventional peptides,³⁶ and an Aib homologue might be expected to promote helicity in the $\alpha/\beta/\gamma$ context. A further point of comparison involves $\alpha/\beta/\gamma$ -peptide **1c**, the analogue of **1** that contains γ^4 -hAla residues and in which the cyclic β residues have been replaced with acyclic β residues (β^3 -hGln at position 4 and β^3 -hPhe at position 10). As previously reported, the weak CD signal for **1c** in aqueous buffer (Figure 2A) suggests that the cyclic β -to- β^3 replacements cause a substantial decline in helicity.³¹ The data in Figure 2B suggest

that helicity increases for **1c** in aqueous TFE. $\alpha/\beta/\gamma$ -Peptide **1c** is included in the CD comparisons as a reference point for minimal helicity, particularly in aqueous buffer.

CD comparisons in both aqueous buffer (Figure 2A) and aqueous TFE (Figure 2B) suggest that the two new $\alpha/\beta/\gamma$ -peptides, **1a** and **1b**, display partial helicity, since there is a minimum in the 200–210 nm region in both cases. This helicity seems to be promoted by introduction of TFE, as indicated by the greater intensity of the minimum in TFE-containing solvent relative to aqueous buffer for each of the oligomers. In addition, the minimum is shifted slightly to higher wavelength for each oligomer in the presence of TFE, relative to aqueous buffer. $\alpha/\beta/\gamma$ -Peptides **1a** and **1b** appear to have lower helix populations than does **1**, since the intensities of the characteristic minima are smaller for **1a** and **1b** than for **1**. Both of the new $\alpha/\beta/\gamma$ -peptides appear to display a greater helical population in aqueous buffer than does **1c**. These results suggest that the *gem*-dimethyl substitution of the γ residues in **1b** decreases helical propensity relative to the single methyl substitution of the γ residues in **1**, which contrasts with the trend among conventional α -peptides.³⁶ The decline in helicity observed for **1a** relative to **1** is not surprising, given that Ala→Gly changes among α -peptides cause a decline in α -helicity;³⁵ however, we were surprised that helix formation remained detectable in **1a**.

To gain higher-resolution information on the conformational behavior of **1a**, we conducted 2D NMR studies of this flexible $\alpha/\beta/\gamma$ -peptide in methanol, a solvent that is more favorable for helix formation relative to water. Several NOEs between backbone protons were observed (Figure 2C), all with *i,i*+2 or *i,i*+3 patterns that are characteristic of the $\alpha/\beta/\gamma$ -peptide helix previously documented via NMR and crystallography.^{30,31} These data support the conclusion that significant helical propensity can be manifested by a medium-length $\alpha/\beta/\gamma$ -peptide even if completely unsubstituted (and therefore maximally flexible) γ residues are employed.

Effects of varying $\alpha/\beta/\gamma$ residue arrangements

Figure 3 shows the sequences of six $\alpha/\beta/\gamma$ -peptide 12-mers, **2–7**, that were prepared in order to evaluate the effects of changing subunit pattern on helical propensity. Each of the new 12-mers is an isomer of **1**, but none has the $\alpha\gamma\alpha\alpha\beta\alpha$ hexad pattern. Some of these compounds retain a more-or-less even distribution of β and γ residues along the backbone (**2** and **3**), but others feature an irregular backbone arrangement. Collectively, this set of new $\alpha/\beta/\gamma$ -peptides allows us to determine whether helical propensity is strongly influenced by subunit placement among oligomers with a 4:1:1 $\alpha:\beta:\gamma$ residue ratio.

Figure 4 shows far-UV CD comparisons of the six new $\alpha/\beta/\gamma$ -peptides with prototype **1**, in both aqueous buffer and aqueous TFE. All six compounds seem to display roughly comparable extents of helix formation, given the similarities in intensity of the characteristic minima in the 200–205 nm region. The largest differences are observed in aqueous TFE,

with $\alpha/\beta/\gamma$ -peptides **4**, **5** and **6** manifesting slightly more intense minima than **1**, while **2**, **3** and **7** display somewhat less intense minima relative to **1**. However, we note that CD measurements provide structural information that is inherently of low resolution, and that differences in the sequences of α , β and γ residues among **1**–**7** might give rise to differences in CD signatures that are independent of extent of helicity. Therefore, interpretation of the differences among the CD data in Figure 4 must be regarded as provisional.

We turned to 2D NMR to test the hypothesis that $\alpha/\beta/\gamma$ -peptides with varying sequences of α , β and γ residues adopt helical conformations in aqueous buffer and/or aqueous-TFE mixtures. $\alpha/\beta/\gamma$ -Peptide **4** was selected for initial studies because the CD minimum for this oligomer is among the most intense in each solvent among **1**–**7**. In addition, we were interested in the contrast between the irregular placement of β and γ residues within **4** and the regular placement in **1**. This effort was stymied, however, because of poor dispersion in the ^1H NMR spectrum of **4**. We therefore prepared **8**, an analogue of **4** that shares the $\alpha\gamma\beta\alpha\alpha\alpha\gamma\alpha\alpha\alpha\beta\alpha$ backbone and contains two α residue side chain modifications, Ala5 $\rightarrow$ Gln and Ala8 $\rightarrow$ norleucine (Figure 5, Figure S1). The ^1H NMR spectrum of **8** in 60 vol % aqueous TFE displayed sufficient dispersion to allow assignment of a set of $i,i+2$ and $i,i+3$ NOEs that is entirely consistent with the expected α -helix-like conformation (Figure 5A). When comparable NMR studies of **8** were conducted in purely aqueous solution, however, NOEs could be detected between only sequentially adjacent residues. Our inability to detect medium-range NOEs for **8** in aqueous solution suggests that this $\alpha/\beta/\gamma$ -peptide is not highly folded in this solvent, although the pronounced minimum near 200 nm in the far-UV CD spectrum of **8** in aqueous buffer raises the possibility of partial helicity under these conditions (Figure 6).

$\alpha/\beta/\gamma$ -Peptide **8-cyc** is the analogue of **8** in which the flexible γ^4 -hAla residues have been replaced with constrained γ residues that are trisubstituted and in which the $\text{C}_\beta\text{-C}_\gamma$ bond is incorporated into a *cis*-cyclohexyl unit. Our initial studies with **1** featured a similar comparison, involving the analogue of **1** containing the same cyclic γ residues in place of the two γ^4 -hAla residues in **1**.³¹ This previous comparison indicated that the *cis*-cyclohexyl-constrained γ residue can participate in the α -helix-like conformation available to $\alpha/\beta/\gamma$ -peptides. However, we were surprised to find that replacing this constrained residue with a more flexible γ^4 -hAla residue caused only a small decline in helicity, according to CD data in aqueous solution. The analogous comparison of **8-cyc** vs. **8** by CD (Figure 6) indicates similar behavior: once again, replacing the constrained γ residues with γ^4 -hAla causes relatively little change in the intensity of the CD signature, in aqueous buffer or in 60 vol % aqueous TFE.

2D NMR analysis of **8-cyc** in 60 vol % aqueous TFE revealed a set of $i,i+2$ and $i,i+3$ NOEs comparable to those observed for **8** in this solvent (Figure 5B). Several of these medium-range NOEs could be detected for **8-cyc** in aqueous solution as well, which contrasted with the lack of medium-range NOE detection for **8** in aqueous solution. Overall, these data suggest that a strong tendency for helical folding is retained when the regular $\alpha\gamma\alpha\beta\alpha$ repeat of **1** is replaced with the irregular but isomeric backbone sequence of **4** and **8**. CD comparison of **8** vs. **8-cyc** suggests that these two $\alpha/\beta/\gamma$ -peptides both manifest some degree

of helicity in water and in aqueous TFE; however, comparison of **8** vs. **8-cyc** via 2D NMR indicates that replacing flexible γ^4 residues with the *cis*-cyclohexyl-constrained γ residues enhances helix propensity.

In $\alpha/\beta/\gamma$ -peptides **5–7**, the β and γ residues are grouped toward the N-terminus, with maximal clustering in **5** ($\beta\gamma\beta\gamma$ segment), one α residue between two $\beta\gamma$ dyads in **6**, and one α residue between each pair of non- α residues in **7**. CD comparisons (Figure 4) suggest that **5** and **6** exhibit similar extents of helicity relative to **1** in aqueous buffer and modestly enhanced helicity relative to **1** in aqueous TFE. These interpretations must be made with caution because of backbone differences: **5** and **6** have substantial stretches of consecutive α residues (seven and six, respectively), while **1** has no more than two consecutive α residues. The backbone variations among **1**, **5**, **6** and **7** could be significant since the α -helical conformation of a pure α backbone gives rise to two CD minima in the far-UV region (at 208 and 222 nm), while helical conformations of backbones containing mixtures of α and β residues^{12–14} or α , β and γ residues^{30,31} give rise to a single minimum in the 205–210 region. Nevertheless, the CD data in Figures 4C and 4D, which are normalized for the number of residues, strongly suggest that **5** and **6** have helical propensities at least as pronounced as that of **1**. $\alpha/\beta/\gamma$ -Peptide **7** may have a slightly smaller helical propensity relative to **1**.

To gain further insight on the folding of **5–7**, we conducted 2D NMR studies in aqueous solution and in aqueous TFE; results are summarized in Figure 7. In each solvent and for each of the three $\alpha/\beta/\gamma$ -peptides, one or two medium range NOEs is evident between pairs of backbone protons from residues that are not sequentially adjacent. Each of these NOEs is consistent with α -helix-like conformations. It is noteworthy that all of the medium-range NOEs involve a γ^4 hAla residue, which raises the possibility that γ^4 residues have an intrinsically high folding propensity.

We used $\alpha/\beta/\gamma$ -peptides **5** and **6** to evaluate the generality of a trend previously observed among analogues of prototype $\alpha/\beta/\gamma$ -peptide **1**: replacement of cyclic β residues with acyclic β residue (i.e., β^3 residues) causes a substantial decline in helicity (Figure 2). The CD comparisons in Figure 8 indicate that this trend obtains also among peptides that have arrangements of α , β and γ residues very different from the repeating $\alpha\gamma\alpha\alpha\beta\alpha$ pattern in **1**. In **5-acyc** and **6-acyc**, the two cyclic β residues found in **5** and **6** have been replaced with β^3 -hAla residues. In both aqueous buffer and 60 vol % aqueous TFE, **5-acyc** and **6-acyc** are significantly less helical than are **5** and **6**, respectively.

CONCLUSIONS

The present study offers an expanded perspective on the folding behavior of $\alpha/\beta/\gamma$ -peptides that contain a 4:1:1 $\alpha:\beta:\gamma$ residue ratio. This ratio originally drew our interest because of the hypothesis that a $\alpha\gamma\alpha\alpha\beta\alpha$ hexad pattern could provide a basis for α -helix-mimetic oligomers in which the unnatural β and γ residues are aligned along one side of the helical conformation.³⁰ Testing this hypothesis led us to discover that β and γ residues preorganized with small-ring constraints could generate very stable helical conformations. Subsequent

work revealed that a high folding propensity does not require constrained γ residues,³¹ which enhances the synthetic accessibility of helixprone $\alpha/\beta/\gamma$ -peptides.

Here we have explored the impact of varying the pattern of α , β and γ residues among a set of $\alpha/\beta/\gamma$ -peptide isomers with the 4:1:1 $\alpha:\beta:\gamma$ ratio and constant residue composition. Qualitative comparisons of folding in aqueous solution and aqueous TFE solution suggest that a substantial helical propensity is manifested regardless of the pattern among α , β and γ residues; however, altering the residue pattern can exert modest effects on helicity. It is noteworthy that an analogous conventional peptide containing 14 α residues, which has a backbone containing the same number of atoms as the backbones of the $\alpha/\beta/\gamma$ -peptides discussed here (8 α residues, 2 β residues and 2 γ residues), shows little or no evidence for α -helix formation in aqueous buffer, according to CD.³⁰ The experimental results we have provided for $\alpha/\beta/\gamma$ -peptides with a 4:1:1 $\alpha:\beta:\gamma$ ratio and varying degrees of β and γ residue preorganization provide benchmarks that should be useful for computational studies designed to elucidate differences in helical propensity between pure α and $\alpha/\beta/\gamma$ backbones.

The high folding propensity conferred by interspersing a relatively small number of β and γ residues among α residues, and the tolerance of the resulting helical conformation to different arrangements of the α , β and γ residues should make this 4:1:1 $\alpha/\beta/\gamma$ -peptide family attractive for the design of foldamers that manifest specific activities. Such functional studies will reveal whether the isoatomic replacement of α residue heptads with $\alpha/\beta/\gamma$ hexads leads to more effective α -helix mimicry than has already been achieved with α/β -peptide backbones.¹² The results presented here enable the $\alpha/\beta/\gamma$ system to take its place among other helix-forming foldamer families that have been generated by combining different types of subunits.^{11,12,33}

EXPERIMENTAL METHODS

Materials

Fmoc α -amino acids and resin for solid phase peptide synthesis were purchased from Novabiochem or Chem-Impex. Coupling reagents and additives, including O-benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), O-(7-azabenzotriazol-1-yl)-N,N,N,N'-tetramethyluronium hexafluorophosphate (HATU), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), hydroxybenzotriazole (HOBt), and 1-hydroxy-7-azabenzotriazole (HOAt), were purchased from Chem-Impex. Fmoc β -amino acids, including Fmoc-(1*S*,2*S*)-2-aminocyclopentane carboxylic acid and Fmoc β^3 -amino acids, were purchased from Chem-Impex. Acyclic γ -amino acids were purchased from Polypeptide group. The ring-constrained γ -amino acid, EtACHA, was synthesized according as previously described.³⁷ Other reagents and solvents were purchased from Aldrich.

Synthesis of $\alpha/\beta/\gamma$ -peptides

All of the $\alpha/\beta/\gamma$ -peptides described here were synthesized on Nova PEG rink amide resin (25–50 μ mol scale) by microwave-assisted solid-phase reactions using a CEM MARS microwave reactor.^{30,31,38} NovaPEG Rink Amide resin was swelled in DMF for 30 minutes

before sequential amino acid coupling reactions. For the α - and β -amino acids coupling reactions, 4 equiv of Fmoc-protected α - or β -amino acids, 3.95 equiv of HBTU, 8 equiv of diisopropylethylamine (DIEA), and 4 equiv of HOBT (0.1 M) were dissolved in DMF (1 mL DMF per 25 μ mol resin scale) in a separate vial 1–2 minutes prior to the coupling reaction to pre-activate the amino acids. The amino acid solution was added to resin in a fritted syringe, which was then subjected to microwave irradiation: 2 minutes ramp to 70 °C, 4 minutes or 12 minutes hold at 70 °C for α - or β -amino acids, respectively. For acyclic γ -amino acid coupling reactions, the conditions used for β -amino acid coupling were employed, but DIEA was added just before the start of microwave irradiation in order to avoid the cyclization side-reaction of the γ -amino acid. Cyclic γ -amino acid coupling reactions were performed using 4 equiv of Fmoc-amino acid, 4 equiv of EDCI, 8 equiv of DIEA and 4 equiv of HOAt (0.1 M at final) in DMF (1 mL DMF per 25 μ mol resin). The amino acid solution was added to the resin and allowed to nutate for 14 hours at room temperature. Fmoc deprotection reactions were carried out using 20 % (v/v) piperidine in DMF under microwave irradiation (2 min ramp to 80 °C, 2 min hold at 80 °C). The resin was washed with 3–5 resin volumes of DMF after each coupling and Fmoc deprotection reactions. The N-terminal amino group of the final residue was acetylated by stirring the resin in 8:2:1 (e.g., 1.6 mL:0.4 mL:0.2 mL for 25–50 μ mol resin scale) DMF:DIEA:acetic anhydride solution for 10 minutes at room temperature.

Cleavage, Deprotection, HPLC Purification, and Characterization

Peptides were globally deprotected and cleaved from the resin by suspending the resin in cleavage cocktail (95% trifluoroacetic acid (TFA), 2.5 % water, and 2.5% triisopropylsilane) for 4–5 hours at room temperature. After the filtration, the crude peptide in TFA filtrate was concentrated under a stream of nitrogen, precipitated by addition of cold diethyl ether, then centrifuged. The crude peptide was purified by reverse-phase HPLC using a C18 column. Peptide identity was confirmed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrometry. Peptide purity was evaluated by analytical reverse-phase HPLC.

Circular dichroism measurements

CD spectra (260 nm – 190 nm) were recorded in a 1 mm quartz cell with an averaging time of 6 sec for each step (1 nm step size) in pH 7.5 PBS buffer or in 60% TFE/water mixture at 20 °C (AVIV circular dichroism spectrometer model 420). Variable-temperature measurements (10 °C – 90 °C) were conducted with 10 °C intervals and an equilibration time of 10 min at each new temperature.

2D-NMR Analysis

Two-dimensional NMR studies were performed on a Varian INOVA 600 MHz spectrometer equipped with a Varian 5 mm or 3 mm $^1\text{H}/^{13}\text{C}/^{15}\text{N}$, 3-axis PFG probe in 5 mm Shigemitsu tubes or in 3 mm tubes at 10 °C or at 4 °C. Peptide NMR samples were prepared in (v/v) 90% $\text{H}_2\text{O}/10\%$ D_2O , in CD_3OH , or in (v/v/v) 60% $\text{CF}_3\text{CD}_2\text{OH}/30\%$ $\text{H}_2\text{O}/10\%$ D_2O with 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) sodium salt as internal standard. gCOSY, wgTOCSY, and wgROESY (watergate suppression) data were processed and analyzed using

Varian VNMR 6.1 software and Sparky³⁹, respectively. Peak assignments are summarized in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Sequence of $\alpha/\beta/\gamma$ -peptide **1** and structures of cyclic β - and acyclic γ -residues. (B) Helical wheel diagrams for the $\alpha\alpha\alpha\alpha\alpha\alpha\alpha$ heptad and the $\alpha\gamma\alpha\alpha\beta\alpha$ hexad. (C) Structures of $\alpha/\beta/\gamma$ peptides **1a**, **1b** and γ amino acids incorporated in them; GABA and $\gamma^4\text{hAib}$.

Figure 2.

(A) Circular dichroism data for α/β/γ-peptides **1**, **1a**, **1b** and **1c** (0.1 mM) measured in PBS buffer pH 7.5 at 20°C. (B) CD comparison in aqueous TFE. (C) NOEs observed between non-adjacent residues for α/β/γ-peptide **1a** (8 mM) in CD₃OH at 10 °C. Medium intensity NOEs are shown as curved arrows on the peptide structure and on the helical wheel diagram.

Figure 3.

(A) Different $\alpha/\beta/\gamma$ -peptide patterns used in this study. (B) Sequences of $\alpha/\beta/\gamma$ -peptides **1–7** based on patterns shown in (A).

Figure 4.

Circular dichroism data from (A) peptides **1–4** in PBS buffer pH 7.5 (B) peptides **1–4** in 60% TFE (C) peptides **1** and **5–7** in PBS buffer pH 7.5 (D) peptides **1** and **5–7** in 60% TFE at 20°C. All peptide concentrations 0.1 mM.

Figure 5.

(A) NOEs observed between non-adjacent residues for (A) $\alpha/\beta/\gamma$ -peptide **8** (1.5 mM) and (B) $\alpha/\beta/\gamma$ -peptide **8-cyc** (8 mM) in 60% CF₃CD₂OH/30% H₂O/10% D₂O mixture (shown in pink) or in water (shown in blue) at 4 °C. NOEs are shown as curved arrows on the peptide structures and on the helical wheel diagrams.

Figure 6.

(A) Circular dichroism data for peptides **8-cyc** (black) and **8** (red) in PBS buffer (solid line) and in 60% TFE/water mixture (dashed line) at pH 7.5 at 20 °C. (B) Circular dichroism data for **8-cyc** measured in 50% MeOH/50% H₂O at varied temperature from 10 °C to 90 °C. All peptide concentrations 0.1 mM.

Figure 7.

NOEs from non-adjacent residues for peptides **5**, **6** and **7** measured in 60% CF₃CD₂OH/30% H₂O/10% D₂O (shown in pink) or in 90% H₂O/10% D₂O (shown in blue) at 4 °C (peptide concentration 5–8 mM).

Figure 8.

Circular dichroism data from (A) peptides **5** (red) and **5-acyc** (blue) and (B) peptides **6** (red) and **6-acyc** (blue) in PBS buffer (solid line) and in 60% TFE/water mixture (dashed line) at pH 7.5 at 20 °C. All peptides 0.1 mM.