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De novo Design of Monomeric β-Hairpin and β-Sheet Peptides

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Summary

Since the first report in 1993 (JACS 115, 5887-5888) of a peptide able to form a monomeric β-hairpin structure in aqueous solution, the design of peptides forming either β-hairpins (two-stranded antiparallel β-sheets) or three-stranded antiparallel β-sheets has become a field of intense interest. These studies have yielded great insights into the principles governing the stability and folding of β-hairpins and antiparallel β-sheets. This chapter reviews briefly those principles and describes a protocol for the *de novo* design of β-sheet-forming peptides based on them. Criteria to select appropriate turn and strand residues and to avoid aggregation are provided. Because nuclear magnetic resonance is the most appropriate technique to check the success of new designs, the nuclear magnetic resonance parameters characteristic of β-hairpins and three-stranded antiparallel β-sheets are given.

Key Words: Antiparallel β-sheet; β-hairpin; NMR; peptide structure; β-sheet propensities; side chain–side chain interactions; solubility; β-turn prediction; β-turn propensities.

1. Introduction

Protein structures consist of a limited set of secondary structure elements, namely, helices, β-strands, and β-turns, which are organized in different numbers and orientations to produce an extraordinary diversity of protein tertiary structures. Therefore, a reasonable approach to understand protein folding and stability is the study of the conformational behavior of protein fragments and designed peptides. A large amount of information on α-helix folding and stability has been gathered since the early 1980s (*see* Chapter 1). In contrast, early efforts on studying β-sheet forming peptides in aqueous solution did not succeed, likely as a consequence of the strong tendency of sequences with high β-sheet propensity to self-associate. The first peptide able to adopt a monomeric

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β-hairpin in aqueous solution was reported in 1993 ***(1)***. A β-hairpin is the simplest antiparallel β-sheet motif, and antiparallel arrangements of β-strands are more easily studied in model peptides than parallel ones, since the latter requires a lengthy connector. In nature an α-helix acts as linker between most adjacent parallel β-strands. Peptides forming parallel β-sheets that incorporate unnatural templates ***(2,3)***, and the use of peptidomimetics at the β-turn or at the strands to induce β-hairpin structures ***(4,5)*** are beyond the scope of this chapter.

Since the report of the first β-hairpin forming peptide, the forces involved in the stability and folding of two- and three-stranded antiparallel β-sheets have been extensively investigated by several research groups (for reviews *see* **refs. *6–15***). Based on their conclusions, it is now possible to establish a general protocol for the design of new β-sheet–forming peptides that we will describe here (**Subheading 2.**). Previous to that description, the structural characteristics of β-hairpins and three-stranded antiparallel β-sheets that are relevant for the design will be illustrated (**Subheading 1.1.**). Next, the principles used in the design of the reported β-sheet peptides will be explained (**Subheading 1.2.**), and the main conclusions derived from the extensive studies on β-hairpin and β-sheet stability using peptide models will be summarized (**Subheading 1.3.**).

1.1. Characteristics of β-Hairpin and Three-Stranded Antiparallel β-Sheet Structures

A β-hairpin consists of two antiparallel hydrogen-bonded (H-bonded) β-strands linked by a loop region (**Figs. 1** and **2**). Characteristic average values for the ϕ and ψ angles of β-strand residues in antiparallel β-sheets are –139° and +135°, respectively ***(16)***. β-Hairpin motifs differ in the length and shape of the loop and are classified according to the number of residues in the turn and the number of interstrand hydrogen bonds between the residues flanking the turn (n – 1 and c + 1 in **Fig. 1**). This β-hairpin classification uses a X:Y nomenclature ***(17)***, with X being the number of residues in the turn region and either Y = X if the CO and NH groups of the two residues that precede and follow the turn form two hydrogen bonds (for example, in 2:2 and 4:4 β-hairpins; **Figs. 1A** and **1D**, respectively) or Y = X + 2 if these residues form only one hydrogen bond (as in 3:5 β-hairpins; **Fig. 1C**). In protein β-hairpins with short loops (2:2, 3:5, and 4:4), the loop conformation corresponds to β-turns with geometries adequate for the characteristic right-handed twist of antiparallel β-sheets. Thus, for 2:2 β-hairpins, the most frequent β-turn is type I', followed by type II', with type I more rarely found. This statistical occurrence is explained by the fact that type I' and II' β-turns have a right-handed twist suitable for the β-strand pairing, whereas type I and II β-turns are left-handed twisted, with the degree of twist being larger in the type I and I' β-turns than in the type II and II' turns. Type II β-turns are very uncommon in protein β-hairpins. A 3:5 β-hair-

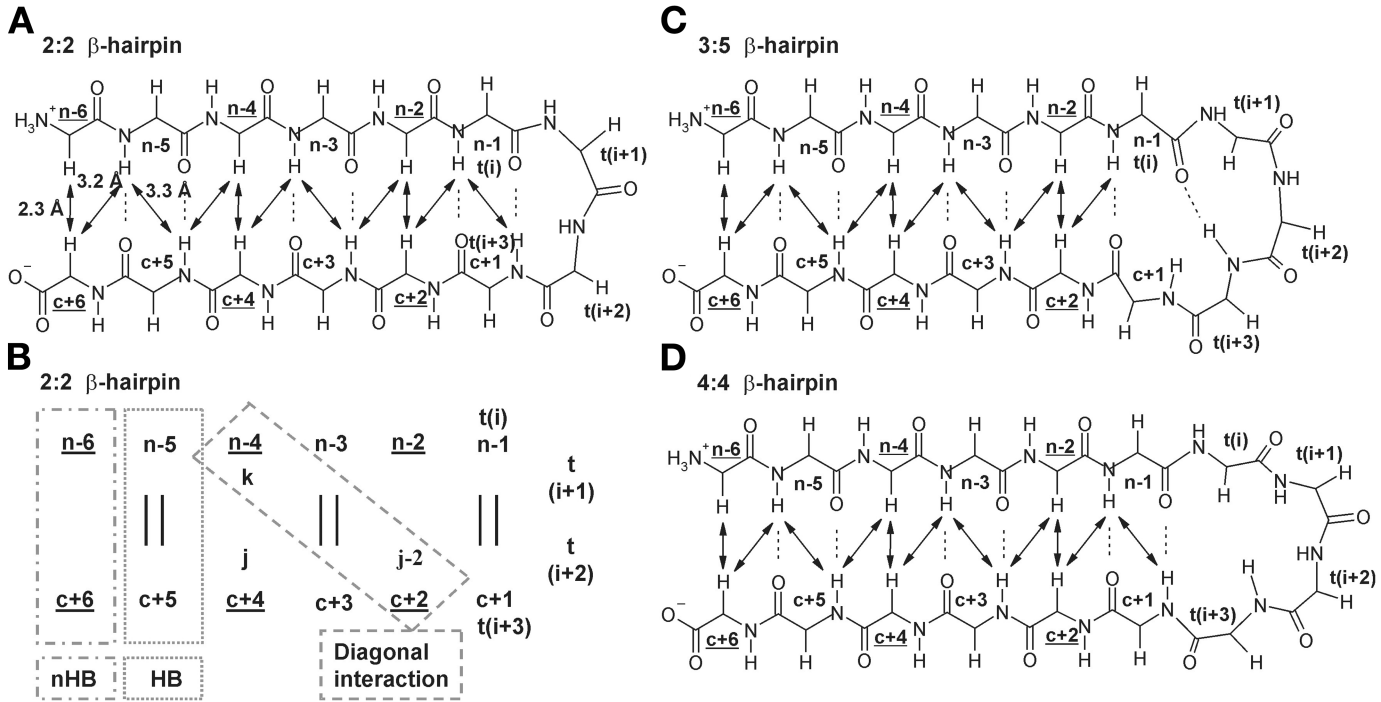

Fig. 1. Schematic representation of the peptide backbone conformation of 2:2 **(A,B)**, 3:5 **(C)**, and 4:4 **(D)** β-hairpins. Turn residues are labeled as t. Residues in the N-terminal and C-terminal strand are named as n and c, respectively. Side chains of underlined strand residues are pointing toward the same β-sheet face, and those not underlined toward the other. Dotted lines link the NH proton and the acceptor CO oxygen of the β-sheet hydrogen bonds. Black arrows indicate the observable long-range nuclear Overhauser enhancements (NOEs) involving H_α and NH backbone protons (**Subheading 2.7.**). The corresponding average distances in protein antiparallel β-sheets are shown in A. In B, two vertical lines connect H-bonded residues and rectangles indicate a pair of facing residues in a H-bonded site (HB) and in a non–H-bonded site (nHB), and a diagonal interaction between the side chains of residues located in non–H-bonded sites of adjacent strands (they are labeled k and j-2).

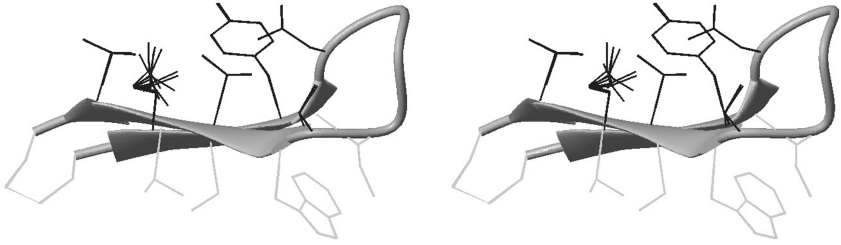

Fig. 2. β-Hairpin structure calculated for a designed 15-residue peptide (Santiveri and Jiménez, unpublished). Backbone atoms are displayed as a ribbon, side chains pointing upward in black and downward in gray.

pin normally exhibits a G1 bulge type I β-turn, a type I β-turn with a Gly residue at the i + 3 position forming a sort of bulge in the hairpin, whereas most of the 4:4 β-hairpins have a canonical type I β-turn. The ϕ and ψ dihedral angles characteristic of the i + 1 and i + 2 residues in types I, I', and II' β-turn are given in **Table 1**.

Two kinds of β-strand positions can be distinguished for facing residues according to whether they form hydrogen bonds or not, i.e., H-bonded sites and non–H-bonded sites (**Fig. 1**). In the β-hairpin, the side chains of consecutive residues in a strand point toward opposite sides of the β-sheet plane, whereas the side chains of facing residues corresponding to adjacent strands are on the same side of the β-sheet (**Figs. 1** and **2**). Because the average distances between the side chains of facing residues are 2.4 Å in non–H-bonded sites and 2.8 Å in H-bonded sites ***(18)***, the contribution of a given side chain–side chain interaction to β-hairpin stability depends on the site (**Subheading 1.3.2.2.**). As a consequence of the right-handed twist of β-sheets, the side chains of residues in two consecutive non–H-bonded sites (labeled as k and j-2 in **Fig. 1**) are also quite close (3.0 Å; ***[18]***). The interaction between these side chains, referred to as a diagonal interaction (**Fig. 1B**), also contributes to β-hairpin stability (**Subheading 1.3.2.3.**).

After β-hairpins, the next simplest kind of β-sheet motifs are three-stranded antiparallel β-sheets with topology β1-β2-β3. They can be regarded as being formed by two β-hairpins with a common β-strand (β2) that is the C-terminal strand of the hairpin 1 and the N-terminal strand of the hairpin 2 (**Fig. 3**).

1.2. Design of β-Sheet Peptides

Early β-sheet peptides were designed to understand protein β-sheet folding and stability ***(6–15)***. However, in the last several years some β-hairpin peptides have been designed with a biological functionality, such as, DNA-, ATP-, metal-, or flavin-binding properties in mind ***(11,19–21)***. According to design

Table 1
Residues With High Statistical Probabilities to Be in Each Position of Type I, Type I′, and Type II′ β-Turns Are Ordered From the Most Favorable to the Least *(22)*

Residue	Type I β-turn				Type I' β-turn		Type II' β-turn	
i	D>N>>H≈C≈S>P				Y>H>>I≈V		Y>V≈S≈H≈F	
i+1	P>>E≈S				N>H>>D>G		p>G	
ϕ, ψ angles	–60, –30				+60, +30		+60, –120	
i+2	D>N>>T>S≈W				G		N>S>D>H	
ϕ, ψ angles	–90, 0				90, 0		–80, 0	
i+3	G>>C≈T≈D≈R≈N				K>>N≈R≈E≈Q		T>G>N≈R≈F≈K	
Type of β-hairpin	3:5 G1 I		4:4 I		2:2 I'		2:2 II'	
Turn sequences in designed β-hairpins	**N**P**DG**	A**KDG**[a]	**AKAG**	**NSDG**[a]	V**NGK**	V**DGK**	V**pG**L	E**GNK**
	E**PDG**	A**PDG**[a]	**DATK**	**APDG**[a]	I**NGK**	I**DGK**	V**pG**O	N**GK**T
	N**SDG**[a]		**PATG**	**AKDG**[a]	Y**NGK**	E**NGK**	S**pG**K	E**pN**K
					V**NG**O		V**pG**K	S**pA**K
							I**pG**K	
							E**pG**K	

Average values of the ϕ and ψ dihedral angles are given for the i+1 and i+2 residues of each type of β-turn. Ornithine is indicated by "O" and DPro by a lower case "p."

[a]The reported peptides with these turn sequences adopt a mixture of two β-hairpins, one a 3:5 with a G1 bulge type I β-turn and the other a 4:4 ***(54,100)***.

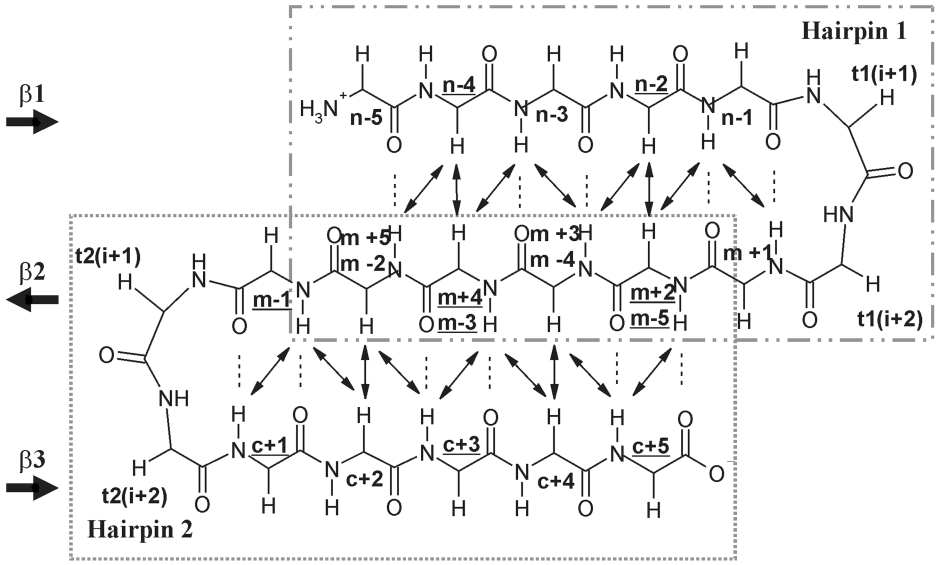

Fig. 3. Schematic representation of the peptide backbone conformation of a three-stranded antiparallel β-sheet motif. The β1-β2-β3 topology is indicated by black arrows (N to C direction) on the left site of the scheme. The two large rectangles surround the residues belonging to each of the 2:2 β-hairpins that compose this β-sheet motif. Turn residues of β-hairpin 1 and of β-hairpin 2 are named t1 and t2, respectively. Residues in the N-terminal strand are named as n, in the middle strand as m, and in the C-terminal strand as c. Side chains of underlined strand residues are pointing toward the same β-sheet face, and those not underlined toward the other. Dotted lines link the NH proton and the acceptor CO oxygen of the β-sheet hydrogen bonds. Double black arrows indicate the observable long-range NOEs involving backbone H_α and NH protons (**Subheading 2.7.**).

strategy, β-sheet forming peptides (*see* **Notes 1** and **2**) can be divided into two groups: (1) those derived from the sequence of native protein β-hairpins or β-sheets and (2) completely *de novo* sequences.

1.2.1. Peptides Derived From Protein β-Sheets

1.2.1.1. Peptides Designed by Modification of the Turn Sequence in Protein β-Hairpins

The earliest successful strategy in the design of β-hairpin peptides consisted in substituting the native turn sequence for those residues with the highest intrinsic probability to occupy the corresponding β-turn positions ***(22)***. Such strategy was used for a nonapeptide ***(1)*** and a 16-residue peptide ***(23)*** derived from residues 15–23 of Tendamistat and from the N-terminal hairpin of

ubiquitin, respectively. Both peptides adopt 3:5 β-hairpins with a G1 bulge type I β-turn. β-Hairpin formation had not been detected for the peptides encompassing any of the corresponding native protein sequences. Later, the same strategy was applied for the same ubiquitin hairpin by using a different turn sequence, one containing a DPro residue at position i + 1 of the turn, so that the resulting β-hairpin is a 2:2 with a type II' β-turn, instead of a 3:5 β-hairpin with a G1 bulge type I β-turn ***(24)***. Furthermore, a single residue substitution of the i + 1 β-turn residue (Thr by Asp) of that ubiquitin hairpin leads to a 3:5 β-hairpin ***(25)***.

1.2.1.2. Peptides Designed by Modification of Strand Residues in Protein β-Hairpins

Two β-hairpin peptides have been derived from the C-terminal hairpin of the B1 domain of protein G: a 16-residue peptide that adopts a 4:4 β-hairpin designed by substituting three strand residues by Trp to produce a cluster of four Trp residues in non–H-bonded sites of the β-strands ***(26)***, and a 10-residue 4:4 β-hairpin peptide designed using the consensus sequence found by statistical analysis of structurally aligned homologues for residues 41–56 of that domain ***(27)***.

1.2.1.3. Peptides Formed by Linking Two Nonsequential Antiparallel β-Strands by Means of a Short Loop

This strategy has only been used in the case of a designed DNA binding peptide that adopts a 2:2 β-hairpin ***(28)***. The DNA-binding motif of the met repressor protein dimer is formed by two antiparallel β-strands at the dimer interface. These two β-strands were linked by the Asn-Gly sequence which is the most favorable one for a type I' β-turn, appropriate for 2:2 β-hairpins (**Subheading 1.1.**; **Table 1**). In one of the two strands, an Ile residue was substituted by a Tyr to facilitate the nuclear magnetic resonance (NMR) assignment (**Subheading 2.7.2.**).

1.2.2. Completely de novo *Sequences*

1.2.2.1. β-Hairpins

The first reported *de novo*–designed β-hairpins were a decapeptide that adopts a 3:5 β-hairpin ***(29)*** and three dodecapeptides that form 2:2 β-hairpins ***(30,31)***. The 3:5 β-hairpin was designed exclusively by selecting strand residues with high intrinsic β-sheet propensities (**Subheading 2.3.**) and a turn sequence with high individual statistical probabilities for each position in a type I β-turn (NPDG as in the 9-residue β-hairpin derived from Tendamistat ***(1)***; **Subheading 1.2.1.1.**). The three 2:2 β-hairpins contain either the Asn-Gly turn sequence ***(30,31)***, which is the most optimal sequence for a type I' β-turn or the DPro-Gly ***(30)*** that leads to a type II' β-turn, with both types of β-turn

being appropriate for 2:2 β-hairpins (**Subheading 1.1.**). Strand residues in the 12-residue peptide designed by Ramírez-Alvarado et al. ***(30)*** are those statistically favorable in the corresponding β-strand positions according to the examination of the structure protein database included in the WHAT IF program ***(32)***. To prevent aggregation and to increase solubility, this peptide contains two positively charged Arg residues at the peptide ends separated from the residues involved in the β-hairpin structure by one Gly residue (**Subheading 2.4.**).

A recently *de novo*–designed β-hairpin system is a series of disulfide-cyclized 10-residue peptides ***(33,34)***, being the Cys residues placed in a non–H-bonded site. By examination of disulfide-bonded Cys residues that connect adjacent antiparallel strands in proteins, side chains of either Leu or aromatic residues were those providing better packing with the disulfide bond; therefore, this type of residues were selected. The turn sequences are Gly-Asn, DPro-Asn, and DPro-Gly for 2:2 β-hairpins with type II' β-turns and Asn-Gly for 2:2 β-hairpins with type I' β-turns. Very stable monomeric β-hairpins were derived from these peptides by removing the Cys residues, conserving the turn sequences and incorporating a cluster of four Trp residues ***(26)***.

Finally, an ATP-binding β-hairpin peptide ***(19)*** has been designed by incorporating a Trp-Trp diagonal interaction (**Fig. 1B**) into a 12-residue β-hairpin peptide previously designed by Gellman's group ***(31)***. This Trp-Trp diagonal interaction should allow nucleobase intercalation because in one β-hairpin peptide designed by Cochran et al. ***(26)*** two Trp diagonal residues seem to form a cleft. Lys residues were incorporated on the same face as the Trp residues to afford electrostatic interactions with nucleotide phosphates. The peptide has a net charge of +4 to increase solubility.

1.2.2.2. Three-Stranded β-Sheets

After the success in designing β-hairpin peptides, several research groups addressed the design of three-stranded antiparallel β-sheets (*see* **Note 3**), the next step up in motif complexity. Almost simultaneously, four different peptides that adopt monomeric three-stranded β-sheets in aqueous solution were reported ***(35–38)***. The two β-hairpins that compose the β-sheet motif in the four peptides are type 2:2 (**Fig. 3**). Taking into account the crucial role of the turn in β-hairpin folding and stability (**Subheading 1.3.1.**), the incorporation of sequences optimal to form either type I' β-turns or type II' β-turns was a key factor for successful designs. Although the design strategies differ in the procedure followed to select the strand residues, all of them took into account β-sheet propensities ***(39–44)*** and statistical preferences for interstrand residue pairs ***(18,45–47)***. The four β-sheet models also differ in the number of residues per strand. Criteria to prevent aggregation and to aid solubility were also important and consequently all of them incorporate from two to five positively

charged residues with their side chains pointing toward different sides of the β-sheet plane (*see* **Note 4**). In this way, positive charge is distributed over both faces of the β-sheet and self-association is minimized. Apart from the principles described here, the sequence of the 24-residue β-sheet ***(38)*** was chosen by statistically analyzing the Protein Data Bank (PDB) ***(48)*** as was previously done for a β-hairpin peptide (***30***; *see* **Subheading 1.2.2.1.**). The sequence of a 20-residue β-sheet ***(36)*** was selected by evaluating the van der Waals energies of several sequences using a template backbone structure derived from two proteins with antiparallel β-sheets (a dehydrogenase fragment and a WW domain). Later, the β-sheet stability was improved in two of these peptides, in one of them ***(36)*** by using a protein engineering rotamer library algorithm to evaluate the effect of amino acid substitutions on the parent β-sheet peptide ***(49)***, and in the other ***(35)*** by including a DPro residue at the β-turn ***(50)***; *see* **Subheading 1.3.1.**

Another β-sheet design consists in extending the sequence of a β-hairpin peptide by adding a type I' β-turn, Asn-Gly sequence and a third strand to its C-terminus. This third strand contains Phe and Trp residues in non–H-bonded sites which face Tyr and Val residues in the second strand giving rise to a stabilizing hydrophobic cluster ***(51)***.

1.3. Main Conclusions About Contributions to β-Sheet Folding and Stability

Analysis of the conformational behavior of peptides able to adopt β-sheet motifs in solution has provided information about their formation and stability ***(6–15,52,53)***. These conclusions are summarized here with emphasis on their applicability as design rules rather than on the physical-chemical basis of β-hairpin and β-sheet stability (*see* **Note 5**).

1.3.1. Essential Role of the Turn Sequence

The peptides derived from Tendamistat ***(1)*** and ubiquitin ***(23)*** by incorporating the NPDG turn sequence (favorable for a type I β-turn; **Table 1**) adopt β-hairpins whose strand registers surprisingly differ from the one in the corresponding protein. These results were the first evidence indicating the importance of the turn region in β-hairpin formation. Later, the essential role played by the turn in directing β-hairpin formation ***(24,54,55)*** and also in its final stability has been demonstrated in several β-hairpin peptide systems (*see* reviews, **refs. *6–15***). A strong correlation between β-hairpin population and the statistical occurrence of the residues at the position i+1 of type I' β-turns in protein 2:2 β-hairpins has been found (***56***; *see* **Fig. 1A**). As a general rule, a good β-turn sequence is a necessary, but not sufficient condition for a sequence to adopt a β-hairpin structure ***(55)***. The crucial role of the β-turn sequence in dictating the β-strand register has also been confirmed in three-stranded antiparallel β-sheet systems ***(57)*** using the β-sheet model designed by Gellman's

group *(37)*. The incorporation of a DPro as i + 1 turn residue greatly stabilizes β-hairpins as well as multistranded β-sheets while substitution of DPro by LPro prevents the β-sheet formation *(31,37,49,50,57–59)*.

The observation that turns and strand–strand interactions appear to make independent and additive contributions to β-hairpin stability *(34)* provides a solid basis for the design protocol described below in which β-turn and β-strand residues are selected independently (**Subheading 2.**).

1.3.2. Contributions to Stability of β-Strand Residues

1.3.2.1. β-Sheet Propensities

Strand residues with high intrinsic β-sheet propensities help to stabilize the β-hairpins and three-stranded β-sheets, whereas those with low intrinsic β-sheet propensities destabilize them. For example, the incorporation of a Gly residue either in an edge strand or in the central strand led to a large destabilization of a three-stranded antiparallel β-sheet peptide *(60)*. That residues with high intrinsic β-sheet propensities (**Subheading 2.3.**) are generally hydrophobic accounts for the high tendency of β-sheet peptides to aggregate.

1.3.2.2. Cross-Strand Side Chain–Side Chain Interactions of Facing Residues

Side chain–side chain interactions of cross-strand facing residues contribute to β-hairpin and β-sheet stability (**Fig. 1**). Some of these stabilizing interactions, either hydrophobic or electrostatic, have been identified and quantified *(6–8,10–12,26,33,34,61–71)* (**Subheading 2.3.** and **Table 2**).

As occurs in α-helices, ionic interactions were demonstrated to be stabilizing in several β-hairpin peptides *(10,11)*. The contributions of Glu-Lys salt bridges placed in non–H-bonded sites are pH dependent decreasing at low pH, where the Glu residue is neutral *(63)*. Two Glu-Lys salt bridges produce a much larger overall contribution to β-hairpin stability than the sum of the two individual Glu-Lys interactions, indicating that co-operativity plays an important role in determining the energetics *(63)*. A Glu-Lys ion pair located at the β-hairpin ends turned out to be more stabilizing than a Lys-Glu pair at the same position *(62)*. The interaction between the N-terminal positively charged amino group and the negatively charged carboxylate group at the C-terminus also contributes to β-hairpin stability *(72)*. Furthermore, a 2:2 β-hairpin peptide is stabilized by a salt bridge interaction between the N-terminal positively charged Lys residue and the C-terminal carboxylate *(10)*.

A series of cross-strand interactions involving mainly hydrophobic and aromatic residues in either a non–H-bonded site *(33,34)* or in a H-bonded site *(61)* has been investigated using a 10-residue disulfide-cyclized β-hairpin system and a method to evaluate β-hairpin population based on the rate of disulfide bond formation (**Subheading 2.7.4.**). Contributions to β-hairpin stability of favorable cross-strand facing interactions (Ile-Trp at a non–H-bonded site and

Table 2
Favorable Cross-Strand Side Chain–Side Chain Interactions Between Facing and Diagonal β-Strand Residues (Fig. 1)

	Facing residues Non–H-bonded sites	H-bonded site
Statistical data	**C-C**>>**E-K**>D-H>N-N>**W-W**>C-W≈ D-G≈D-R>K-N≈N-S>H-P≈Q-R ***(47)***	C-C>E-K≈E-R>H-H≈Q-R≈ D-N≈F-F≈C-H≈S-S≈D-K≈ K-Q≈**N-T** ***(47)***
Experimental[a]	**W-W**>>W-F>W-Y>W-L>W-M>W-I> W-V>>Y-L>M-L>F-L>L-L>I-L≈V-L ***(33,34)***	V-V>H-V≈V-H ***(61)*** S-T, T-T ***(64)*** **N-T** ***(54)***
	C-C ***(33,34)***; Y-W ***(64)***; I-W ***(65)*** F-F ***(66)***; Y-F ***(69)***; Y-L ***(70)*** **K-E** ***(62,63)***	S-T ***(65)***
	Diagonal interactions	
Statistical data	W-Y, K-E, E-R, R-E ***(18)***	
Experimental[a]	Y-K ***(70)*** W-K, W-R, F-K, F-R ***(67)***	

Pair-wise interactions found to be favorable statistically and experimentally are shown in bold.
[a]As deduced from experimental studies on β-hairpin peptides (**Subheading 1.2.**).

Ser-Thr in a H-bonded site ***[65]***) as well as a hydrophobic cluster (Trp/Val-Tyr/Phe in non–H-bonded sites ***[7]***) have been shown to be dependent on their proximity to the turn region. They make larger contributions when they are closer to the turn.

Aromatic interactions have also been investigated by using the 12-residue peptide designed by Gellman's group ***(31,74)***. The Phe-Phe cross-strand interaction in a non–H-bonded site was found to have edge-face geometry ***(66–68)***. This implies that the interaction is not driven by the hydrophobic effect, which would favour the maximum burial of surface area. Edge-face interactions are proposed to be driven by electronic or van der Waals forces or a combination of both between the partially positive hydrogen on one aromatic ring and the π-cloud of the other ring. In the same peptide model, the Phe-Phe pair interaction in a non–H-bonded site has been shown to be more stabilizing than the Glu-Lys interaction ***(75)***.

1.3.2.3. Diagonal Side Chain–Side Chain Interactions

Diagonal side chain–side chain interactions also contribute to β-hairpin stability (**Fig. 1B**). Evidence for the contribution to stability of a favorable Tyr-Lys diagonal interaction in a 12-residue β-hairpin peptide was found ***(70)***. Afterwards, the diagonal cation-π interactions between Phe or Trp with either Lys or Arg were investigated ***(67)***. The Lys residue was found to interact

through the polarized C_ε carbon, whereas the Arg guanidinium moiety is stacked against the aromatic ring of Phe or Trp.

1.3.2.4. Hydrophobic Clusters

Hydrophobic clusters stabilise β-hairpin structures. These stabilizing effects have been demonstrated by incorporating the Trp/Val-Tyr/Phe hydrophobic cluster taken from the C-terminal β-hairpin of protein G B1 domain into non–H-bonded sites of two designed 12-residue β-hairpin peptides ***(71)***. A remarkable high stability has also been reported for designed β-hairpins containing a cluster of four Trp residues in non–H-bonded sites ***(26)***.

1.3.3. Co-Operativity

The question of whether the folding of β-hairpin and β-sheet peptides is co-operative or not still remains open. Evidence for co-operativity has been reported for some β-hairpin and β-sheet peptides ***(37,38,51,76)***, but not found for others ***(35)***. Two types of co-operativity can be distinguished in antiparallel β-sheet peptides, the longitudinal or parallel to the strand axis, and the perpendicular to the strand direction. The increase of β-hairpin stability observed upon strands lengthening indicates the existence of longitudinal co-operativity ***(77)***. Regarding to perpendicular co-operativity, it has been shown that adding a fourth strand to a three-stranded β-sheet peptide leads to further β-sheet stability ***(59)***.

1.3.4. β-Sheet Twist

The right-handed twist characteristic of β-sheets (**Subheading 1.1.**) seems to be related to β-sheet stability, being generally the most twisted β-hairpins the most stable ones. This is the case for the 3:5 β-hairpins, which are more twisted and also more stable than the 4:4 β-hairpins ***(78)***. The existence of a correlation between the degree of twist and the buried hydrophobic surface has been found in a three-stranded β-sheet ***(50)***. Thus, β-sheet twist appears to contribute to β-sheet stability by increasing hydrophobic surface burial.

1.3.5. Hydrogen Bonds

As occurs in proteins, contribution of hydrogen bonds to β-hairpin stability is not clear. The infrared spectrum of a 16-residue β-hairpin peptide showed no features in the amide I region to suggest a significant contribution from interstrand hydrogen bonds ***(79)***, whereas in another 16-residue β-hairpin peptide, an amide I band at approx 1617 cm^{-1} was identified and attributed to hydrogen-bonding across the strand ***(80)***. That the rare protein 2:2 β-hairpins with the unsuitable type I β-turn usually exhibit longer strands than those with the appropriate type I' β-turns might be explained by the additional hydrogen bonds compensating for the unfavorable conformation of the type I β-turn ***(45)***.

1.3.6. Disulfide Bonds

In general, disulfide bonds stabilize the β-hairpin structures because the unfolded state becomes more rigid and a decrease in the loss of entropy on folding is observed. Some natural antimicrobial peptides are β-hairpins stabilized by disulfide bonds ***(81)***. Disulfide cyclization of designed peptides, as well as backbone cyclization, have been used as references for the folded state ***(74)***. In addition, one of the peptide systems used to investigate β-hairpin stability is a series of disulfide-cyclized decapeptides ***(33,34)***.

2. Methods

The protocol proposed here for the *de novo* design of monomeric β-sheet peptides consists of the following steps.

1. Selection of goal β-sheet and peptide length (**Subheading 2.1.**).
2. Selection of β-turn sequence (**Subheading 2.2.**).
3. Selection of β-strand residues. Solubility criteria (**Subheadings 2.3.** and **2.4.**).
4. *In silico* validation of the sequence resulting from **steps 1–3** (**Subheading 2.6.**).
5. Peptide preparation.
6. Experimental validation of the designed β-sheet (**Subheading 2.7.**).

The two structurally different regions that constitute a β-hairpin, the β-turn (**step 2**), and the two antiparallel β-strands (**step 3**) are considered independently. In most reported β-sheet forming peptides, **step 4** was not performed. Methods to carry out peptide preparation (**step 5**) either by chemical synthesis or by biotechnology are beyond the scope of the current review. From the design perspective, the procedure used to obtain the designed peptide is important only regarding to the possibilities for the protection of peptide ends or the incorporation of non-natural amino acids. Concerning **step 6**, the method used for confirming that the designed peptide adopts the goal structure has also to be considered. Thus, NMR assignment is facilitated by nonrepetitive sequences.

2.1. Selection of Goal β-Sheet Structure and Peptide Length

The design of a protein or peptide structure consists in finding a sequence able to form a selected goal structure. The choice of this structure is the starting point in any design project. The principles described on the following sections are applicable for β-hairpins with short loops and can be extended to three-stranded antiparallel β-sheets. If the aim of the project is to re-design a protein domain or fragment of known β-sheet structure (for example, to improve certain structural characteristics of a natural biologically active sequence), the rules given in **Subheadings 2.2.–2.4.** can be applied by maintaining the residues important for the biological activity of the molecule, and substituting some other residues to improve its stability. Sequence alignment procedures are very useful in these redesign cases (**Subheading 2.5.**).

The length of a β-hairpin peptide is n + c + t, where n and c are the number of residues at N- and C-terminal strands, respectively, and t the number of residues in the turn. This last value depends on the type of β-hairpin, being two in a 2:2 β-hairpin, three in a 3:5 β-hairpin, and four in a 4:4 β-hairpin (**Subheading 1.1.**; **Fig. 1**). If n and c are different, residues at the end of the longest strand are not paired. Strand length in the reported β-hairpin peptides ranges from two to nine, most of them having three to seven strand residues. In terms of stability, the strand length increases stability up to seven residues long, but no stability increment is observed with further strand lengthening (*77*; *see* **Subheading 1.3.3.**).

To design a three-stranded antiparallel β-sheet, the procedure followed for β-hairpins (**Subheadings 2.2.–2.6.**) is applied twice, once for the N-terminal hairpin and then again for the C-terminal hairpin (hairpins 1 and 2 in **Fig. 3**). These two β-hairpins can be of the same type and thus have the same number of turn residues, or they can be of a different type. It is necessary to bear in mind that the sequences of the C-terminal strand of hairpin 1 and the N-terminal strand of hairpin 2 are the same and that every residue at this middle strand that is located in an H-bonded site in hairpin 1 is in a non–H-bonded site in hairpin 2 and vice versa (**Fig. 3**). Designed three-stranded β-sheets have three to seven residues per strand.

2.2. Selection of β-Turn Residues

Because the turn region plays an essential role in determining β-hairpin conformation and its stability (**Subheading 1.3.**), the selection of an adequate β-turn sequence is crucial to ensure that the designed peptide will adopt the target β-hairpin. **Table 1** has been built using β-turn positional potentials statistically derived from protein structures *(22)*. **Table 1** is very useful in selecting the β-turn residues by taking into account which is the most appropriate turn for the desired β-hairpin, namely type I for 3:5 and 4:4 β-hairpins, and type I' or II' for 2:2 β-hairpins. The final designed β-hairpin not only must have an optimal β-turn sequence but also it should not contain any sequence likely to form an alternative β-turn.

2.3. Selection of β-Strand Residues

The following principles must be considered to select β-strand residues:

2.3.1. Intrinsic β-Sheet Propensities

As a general rule, residues in the strands should have high intrinsic β-sheet propensities. Because intrinsic β-sheet propensities seem to be context dependent *(41)*, the reported scales of β-sheet propensities show differences. Nevertheless, they are useful as a guide because of their concordance with respect to

which residues are good β-sheet formers. The differences are in the rank order of the residues. Thus, according to statistical data ***(39)***, the residues with high intrinsic β-sheet propensities are V>I>T>Y>W>F>L, whereas C>M>Q>S>R are more or less neutral to adopt β-sheet conformations. Among experimental scales ***(40–44)***, the β-sheet favorable residues are Y>T>I>F>W>V and the neutral ones are S>M>C>L>R, for example ***(43)***.

2.3.2. Residues Adjacent to the Turn

Apart from having high intrinsic β-sheet propensities, the best residues to be in positions preceding the β-turn in 2:2 and 3:5 β-hairpins (n – 1 in **Figs. 1A** and **1C**) should also have high intrinsic probability to be at the position i of the corresponding type of β-turn (**Table 1**), and the best one to follow the β-turn in 2:2 β-hairpins (c + 1 in **Fig. 1C**) should have high intrinsic probability to be at the position i + 3 of type I' or type II' β-turns (**Table 1**).

2.3.3. Pair-Wise Cross-Strand Interactions

Facing residues should correspond to pairs with favorable side chain–side chain interactions. **Table 2** that lists the most favorable pair-wise interactions according to statistical data ***(47)*** is useful as a guide to select them, though the statistical data on cross-strand pair-wise interactions does not always coincide with the experimental results regarding to β-sheet stability and statistical analysis by different authors show some discrepancies. Other statistical data on pair-wise interactions, which are not included in **Table 2**, might also be used ***(18,45,46)***. The cross-strand side chain–side chain interactions found to be stabilizing in model β-hairpin peptides are also given in **Table 2**.

2.3.4. Diagonal Interactions

In addition to pair-wise cross-strand interactions between facing residues, favorable diagonal interactions and hydrophobic clusters will contribute to β-hairpin stability (**Subheading 1.3.2.3.**). **Table 2** lists preferred diagonal interactions according to statistical data ***(18)*** as well as those found experimentally and servicable as aids for selecting favorable diagonal interactions.

2.3.5. Peptide Ends

Peptide ends can be nonprotected or protected by acetylation of the N-terminal and by amidation of the C-terminal. If they are not protected and the two terminal amino acids are facing residues, the interaction between the positively charged amino group and the negatively charged carboxylate makes a favorable contribution to β-hairpin stability ***(72)***. To increase solubility, it may be advisable to protect one of the peptide ends (**Subheading 2.4.**). The method to be used for obtaining the peptide can also determine the possibilities for pep-

tide ends; for example, protection by acetylation and amidation is easy and convenient if the peptide is going to be prepared by chemical synthesis. Some residues at the N-terminus will be added in the case of cloning and expression of the peptide.

2.4. Solubility Criteria (see Note 6)

Because aggregation and solubility problems in peptides and proteins seem to be higher close to the isoelectric point, a peptide with either a net positive charge or a net negative charge will be likely more soluble. Incorporation of an electrostatic interaction resulted in peptide aggregation in one case *(78)*. Thus, it can be advisable to protect the N-termini in Asp/Glu-containing peptides and the C-termini in those containing positively charged residues (Lys, Arg, Orn). The distribution of the charged and polar residues also plays an important role in solubility. Because amphipathic sheets with a hydrophilic face and a hydrophobic one are more prone to aggregate than the nonamphipathic ones, the charged and polar side chains should be pointing toward both faces of the β-sheet. Other strategy to avoid aggregation used in designed β-hairpin peptides consists in the incorporation of charged residues at the peptide ends separated from the hairpin by spacing linkers consisting of Gly residues *(30)*.

2.5. Alignment to Sequences That Adopt the Target β-Sheet Motif

Naturally occurring peptides or protein domains having the desired β-sheet motif or designed peptides previously reported to adopt the target structure can be used as the starting point for the design. In this case the alignment of all the known peptides or protein domains that have the same structure or function that the target one can be used to get a consensus sequence. A 10-residue β-hairpin peptide *(27)* has been designed using this strategy (**Subheading 1.2.2.**).

2.6. In silico *Validation of Designed β-Sheet Sequences (see Note 7)*

The probability of the sequence designed according to the principles in **Subheadings 2.1.–2.5.** to adopt the goal β-sheet structure can be examined by applying a β-hairpin prediction program developed in our laboratory (*see* **Note 8**). The program contains two principal subroutines: one predicts the β-turn residues and the other deals with the β-strand residues. In a first step, a normalized version of the β-turn positional potentials published by Hutchinson and Thornton *(22)* is employed to predict the residues most likely to form a β-turn and the type of turn they will adopt. In a second independent step, the prediction of most favorable residues in β-sheet conformation is determined by a linear combination of terms derived from intrinsic β-sheet propensities *(43)*, cross-strand pair interactions *(47)*, and number of hydrogen bonds formed. Finally, the prediction of the β-hairpin type adopted by the amino acid sequence is a

combination of both results, which are ranked numerically together with rules based on the most favorable type of protein β-hairpins.

2.7. Checking the Success of the Design

Monomeric state of the β-hairpin peptides is usually confirmed by analytical ultracentrifugation and by dilution experiments monitored by circular dichroism (CD) or NMR.

2.7.1. CD Spectroscopy

Characteristic far-ultraviolet CD spectra for β-sheets exhibit one minimum at approx 216 nm and one maximum at approx 195 nm ***(82)***. Thus, CD spectroscopy provides a quick way to confirm whether or not a designed peptide adopts a β-sheet structure. However, no information about strand register can be gathered from a CD spectrum.

2.7.2. NMR Spectroscopy

NMR is the best technique to demonstrate that a particular peptide adopts its target β-sheet structure. The observation of NOEs between backbone protons of residues in adjacent strands provides unambiguous evidence about the strand register, and thus the type of β-hairpin. Type of β-turn can also be determined by NMR.

2.7.2.1. NOEs

In antiparallel β-sheets, the backbone protons that are close enough to give rise to NOEs are (1) the H_α protons of residues facing each other in a non–H-bonded site (2.3 Å; **Fig. 1A**), (2) the NH amide protons of facing residues in H-bonded sites (3.3 Å; **Fig. 1A**), and (3) the H_α protons in a non–H-bonded site and NH protons in an H-bonded site of residues in adjacent strands, when these two sites are consecutive (3.2 Å; **Fig. 1A**). Because some H_α signals may be obscured by that of water, to observe H_α–H_α NOEs is convenient to dissolve the peptide in D_2O. Further information on the β-hairpin adopted can be gathered from the NOEs between protons of side chains located at the same β-sheet face.

In three-stranded β-sheets, the set of NOEs involving backbone protons is also compatible with the independent formation of β-hairpin 1 and β-hairpin 2 (**Fig. 3**). Only the observation of at least one long-range NOE involving side chain protons of residues at the N-terminal strand and at the C-terminal one demonstrates the formation of the three-stranded β-sheet motif (***50***; *see* **Fig. 3**).

2.7.2.2. Conformational Shifts

The patterns of $^1H_\alpha$, $^{13}C_\alpha$, and $^{13}C_\beta$ conformational shifts ($\Delta\delta = \delta^{observed} - \delta^{random\ coil}$, ppm; *see* **Note 9**) are also useful for identifying β-hairpins and three-

stranded β-sheets. β-Strands can be delineated by the stretches of at least two consecutive residues having positive $\Delta\delta_{H\alpha}$ and $\Delta\delta_{C\beta}$ values and negative $\Delta\delta_{C\alpha}$ values. Two of such stretches are observed in β-hairpins and three in three-stranded β-sheets. These stretches are separated by two to four residues with $\Delta\delta_{H\alpha}$ negative or very small in absolute value, with at least one of them having a positive $\Delta\delta_{C\alpha}$ value and a negative $\Delta\delta_{C\beta}$ value ***(83)***. A more thorough analysis of the $\Delta\delta_{C\alpha}$ and $\Delta\delta_{C\beta}$ profiles at the turn region allows us to identify the particular type of β-hairpin and β-turn (for details *see* **ref. *83***).

2.7.3. Quantification of β-Hairpin and β-Sheet Populations

In contrast to helical peptides in which CD spectroscopy provides a method for quantifying helix population, there is not a well-established method to quantify β-sheet populations. Assuming a two-state behavior for β-hairpin or β-sheet formation, populations can be evaluated from different NMR parameters, such as H_α–H_α NOEs and $^1H_\alpha$, $^{13}C_\alpha$, and $^{13}C_\beta$ chemical shifts. Apart from the validity of the two-state assumption, the absence of accurate reference values for the completely folded and random coil states limits the precision and accuracy of the quantification of β-sheet populations (for details on this question *see* **refs. *6–8*,*10*,*74*,*83***).

2.7.4. Method Based on Disulfide Bond Formation

A recently reported nonspectroscopic method to measure β-hairpin stability is based on thiol-disulfide equilibrium in cystine-cyclized peptides ***(33,34)***. β-Hairpin stabilities in disulfide-cyclized decapeptides were compared on the basis of the changes in the thiol-disulfide equilibrium constant upon residue mutation.

3. Notes

1. Two protein fragments that adopt their native β-hairpin structures have been reported ***(84,85)***.
2. Non-water-soluble peptides that adopt β-hairpin structures in chloroform, benzene, and alcoholic solvents have also been designed ***(86)***. As in many water-soluble β-hairpins, their β-turn sequences are either DPro-Gly or Asn-Gly. Some of these short and very hydrophobic β-hairpin peptides have been crystallized ***(87)***.
3. A designed three-stranded antiparallel β-sheet with a β2-β1-β3 topology instead of the β1-β2-β3 of the meander β-sheets reviewed here has been reported ***(88)***. Several antiparallel β-sheets with more than three strands have also been designed: two four-stranded antiparallel β-sheet peptides, a 26-residue peptide that adopts the β-sheet in either pure methanol or in water-methanol solutions ***(89,90)***, and a 50-residue molecule composed of two BPTI-derived β-hairpin modules that are connected by a cross-link between two Lys residues in the inner strands ***(91)***; a 34-residue peptide that forms a five-stranded β-sheet and contains a metal binding site ***(92)***; and an eight-stranded antiparallel β-sheet formed by connecting two four-stranded β-sheets with a disulfide bond ***(93)***. Dimeric and tetrameric β-

sheets ***(94)*** and a trimer composed of three β-hairpin modules ***(95)*** have also been designed.

4. N-methyl amino acids were incorporated in a designed three-stranded β-sheet to prevent aggregation ***(96)***. A non-water-soluble three-stranded antiparallel β-sheet containing DPro-Gly sequences in the two turns has also been designed ***(89)***.
5. β-Hairpins are stabilized by alcohol cosolvents, trifluoroethanol, and methanol. For deeper discussion on the physicochemical origin of the contributions to β-hairpin and β-sheet stability, see reviews ***(6–15)*** and references therein and here.
6. A recently published program that predicts the tendency of a given sequence to aggregate may be used to screen out sequences with the strongest tendency to self-associate ***(97)***.
7. Methods developed for predicting β-turns in a protein from its amino acid sequence could also be used to validate the designed β-sheet sequences. However, so far the use of such methods has not been reported in the case of any designed β-hairpin or β-sheet peptide. A web server that predicts β-turns from sequence by implementing several reported statistical algorithms is available (http://imtech.res.in/raghava/betapred/home.html; ***[98]***). A method reported for the recognition of β-hairpins in proteins that combines secondary structure predictions and threading methods by using a database search and a neural network approach could also be used ***(99)***. Another possibility is to construct a model structure and evaluate its stability by rotamer library algorithms, as done in the case of a 20-residue three-stranded β-sheet (***49***; *see* **Subheading 1.2.2.2.**).
8. BEHAIRPRED is accessible from http://bionmr.cipf.es/software/behairpred.htm (Pantoja-Uceda and Jiménez, unpublished). It accepts the non-natural residues Orn and DPro as input. In addition, it is able to identify type II β-turns, even though they are quite uncommon in β-hairpins.
9. $^{1}H_{\alpha}$, $^{13}C_{\alpha}$, and $^{13}C_{\beta}$ conformational shifts ($\Delta\delta_{H\alpha}$, $\Delta\delta_{C\alpha}$, and $\Delta\delta_{C\beta}$, respectively) are defined as the deviation of the experimentally measured chemical shift ($\delta^{observed}$, ppm) from reference δ values for the random coil state ($\delta^{random\ coil}$, ppm; ***[83]***).

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