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Terminal Sidechain Packing of a Designed β -Hairpin Influences Terminal Conformation and Stability

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Abstract

While end capping in α -helices is well understood, the concept of capping a β -hairpin is a relatively recent development; to date, favorable Coulombic interactions are the only example of sidechains at the termini influencing the overall stability of a β -hairpin. While cross-strand hydrophobic residues generally provide hairpin stabilization, particular when flanking the turn region, those remote from this location appear to provide little stabilization. While probing for an optimal residue at a hydrogen bond position near the terminus of a designed β -hairpin a conservative, hydrophobic, V $\rightarrow$ I mutation was observed to not only result in a significant change in fold population but also effected major changes in the structuring shifts at numerous sites in the peptide. Mutational studies reveal that there is an interaction between the sidechain at this H-bonded site and the sidechain at the C-terminal non-H-bonded site of the hairpin. This interaction, which appears to be hydrophobic in character, requires a highly twisted hairpin structure. Modifications at the C-terminal site, for example an E $\rightarrow$ A mutation ($\Delta\Delta G_U = 6$ kJ/mol), have profound effects on fold structure and stability. The data suggests that this may be a case of hairpin end capping by the formation of a hydrophobic cluster.

Keywords

β -hairpin; capping interactions; hydrophobic cluster; chemical shift deviations

INTRODUCTION

The role of capping motifs in α -helices has been well documented and their stabilizing role is well understood^{1–6} For example acetylation (and to a lesser extent amidation) increase helicity, in part by removing charges that represent a repulsive interaction with the helix macrodipole. In the case of peptides that populate hairpin folds these same changes almost always decrease the fold population since they remove or decrease a potentially favorable columbic interaction between the ends of the hairpin. In fact this Coulombic interaction, which can provide 1–2 kJ/mole of hairpin stabilization, is perhaps the only end-capping effect known for hairpins.^{7–13} A recent data mining effort noted that Asn, Gly, and Pro are frequently found to be β -sheet (strand) terminators.¹⁴ The common Gly and Pro dihedral angles do correspond to disruption of β strands, however the frequency of Gly, Pro, and Asn at the end of β -strands is likely not an end-capping effect, rather a reflection of their β -turn propensity and the high frequency of β hairpin segments in β sheets. Little is known about the role of capping interactions and motifs in β -sheets outside of favorable Coulombic interactions and turn or loop initiators.

Both de novo design and studies of β -hairpins inspired by proteins have yielded a substantial amount of information on favorable turn sequences and cross-strand interactions.^{8,12,15–21} However, there are only a few instances of hydrophobic clusters placed at or near the termini increasing the stability of β -hairpins. Espinosa et al. noted that moving a hydrophobic cluster from near the turn nucleus nearer to the termini decreased terminal fraying of the β -hairpin.²² The Waters' group has reported a stabilizing cross-strand aromatic pair at the terminal nonhydrogen-bonded position.¹⁷ Recently they have described a stabilizing nonaromatic hydrophobic cluster at the termini of the excised N-terminal hairpin of ubiquitin²³; deleting the terminal interaction between Met1 and Val17 in the excised N-terminal hairpin of ubiquitin, which folds modestly in aqueous solution, was significantly destabilizing.

We were prompted to search for hairpin capping interactions by the observation of an unexpectedly large effect on hairpin stability observed for a conservative mutation in a truncated version of a GB1/Trpzip inspired peptide (Table I). The population increase for the T3V mutation (HP6V) had precedent,²⁵ however the T3I data was very surprising. The present study traces fold stability changes through a mutational analysis (see Figure 1) to an unusual interaction involving the C-terminal sidechain, which may represent a case of hairpin end capping by the formation of a hydrophobic cluster. This stands in contrast to prior studies that indicate that the more remote W/W and Y/V W/V pairs in trpzip2, HP5W, GB1p provide much less stabilization than the preserved turn flanking aryl/aryl interaction²⁰ in these species. In these prior systems, the hydrophobic cross-strand pairs near the termini appear to provide no further stabilization than that associated with the significant β -propensities of Trp, Tyr, and Val. The mutational analysis presented herein focuses on a diagonal cross-strand interaction at hydrogen-bonded positions remote from the turn (see Figure 1). Side-chains of residues at the S-3 and S+5 positions partake in a stabilizing hydrophobic interaction; there is no precedent for this type of interaction to provide stability to β -hairpins.

RESULTS

Quantifying Fold Populations of β -Hairpins With a W-loop-W Motif

The previously reported 12 residue peptides HP6 and HP6V exhibit the spectroscopic signatures of an edge-to-face (EtF) interaction of the indole rings for β -hairpins with a W-loop-W motif.^{20,26} These include an upfield chemical shift deviation of ~2.0 ppm for the H ϵ 3 proton of the edge tryptophan and an exciton couplet observed in the circular dichroism (CD) spectrum resulting from the chiral orientation of the Trp indole rings. These probes coupled with backbone chemical shift deviations (CSDs) are used to quantify hairpin fold population herein.

Mutations at S-3 of HP6V

Backbone CSD^{27,28} are well-established measures of β -hairpin folding and provide accurate measures of both turn and strand fold populations.^{29–35} Typically a β -hairpin would exhibit positive CSDs (downfield from random coil) for H α protons at the S $\pm$ 2 and S $\pm$ 4 positions and for H N protons at S $\pm$ 3, S $\pm$ 5, and S-1 positions.³⁵

The HP6 β -hairpin peptides differ somewhat from this pattern, particularly at the S-2 H α and the S+5 H N protons (see Figure 2). Ring current effects are the primary cause for these deviations in comparison with β -hairpins that do not contain aromatic residues. For example, a much larger negative CSD is seen for the S+1 H N , which has been noted for all the hairpins that employ a turn flanking Trp/Trp interaction whether an EtF or FtE geometry is observed.^{21,12,20,26} The exception is HP6I, which differs from HP6V by only one extra

methyl group on the sidechain at the S-3 positions; the S+1 Lys H_N CSD is -0.4 ppm, a value typical for hairpins lacking a W-loop-W motif and more than 1.0 ppm downfield from the corresponding proton of HP6V. Most β -hairpins display a positive (downfield) shift for the S-1 H_N, while HP6I exhibits an upfield CSD of -0.7 ppm. In addition the S-2 Trp H α CSD is even more negative and the diastereotopic shift difference at the turn glycine (Gly $\Delta\delta H\alpha$), 0.94 ppm at 280 K, is the largest value ever reported for an -NG- turn hairpin. 100% folded values from the literature for this probe are 0.43–0.75 ppm^{12,17–18,21,26,36,37}; 0.87 ppm is the 100% folded value for other hairpins of the HP6 series.

HP6I also shows significant deviations from HP6V in chemical shift for the aromatic protons of the edge Trp; in addition, the exciton couplet maximum is much larger than expected (see Figure 3) given the estimated fold population derived from NMR CSD data. We have previously found that changes in the exciton couplet maximum may not be an indication of changes in fold population but rather a change in the orientation of the interacting electronic transition dipoles.²⁶ It is also worth noting that a Thr at S-3 results in a less stable hairpin in comparison to HP6V, however the overall trend of CSDs remains the same, unlike the effect of Ile at the S-3 position. Given that the fold populations cannot be explained by the β -propensity of the respective residues, an alternative explanation is required for the fold stability and conformation changes evident in the data for HP6I.

Mutations at S+5 of HP6V

Having identified Val as optimal at the S-3 position, we turned to the NOEs for HP6V to decipher what other residues interact with the Val sidechain to aid in identifying which other residue would provide the most information from mutational analysis. Long range NOEs between the H β protons of Glu at S+5 and the H γ 's of Val at S-3 were observed; this was unexpected given that the two sidechains are at diagonal hydrogen bond positions (see Figure 1). The significance of the S+5 sidechain in the diagonal interaction was explored in a series of sequential mutations of the HP6V variant. NMR data (Figure 3A) indicates that an E12A mutation is fully tolerated, E12K and E12D mutations are somewhat destabilizing, only the E12G mutation resulted in a marked loss of thermodynamic stability. All folding measures imply that the E12 and A12 versions of HP6V are equally well folded at 280 K (Table II).

The near identical stability of HP6V with Glu or Ala at the C-terminus was unexpected given that a potentially favorable Coulombic interaction was removed. A similar interaction has been shown to stabilizing in GB1p analogs.¹² This became more puzzling when the E12D mutant was shown to be destabilizing compared to the E12K mutant by NMR. A loss of stability for E12K was expected due to the introduction of an unfavorable interaction at the termini: the favorable Coulombic pairing (K1/D12) resulted in a less stable hairpin than the repulsive Coulombic pair (K1/K12) by NMR measures. The data for the E12K mutation demonstrates a distinctly diminished CD exciton couplet while retaining large ring current shifts for H ϵ 3 (see Figure 3) and requires some comment. Changes in angular orientation of the two Trps can occur without affecting the ring current shifts. Similarly diminished exciton couplets have been observed for MrH β -hairpins that employ a Trp/Trp pair when the EtF interaction geometry deviates from that of the trpzip-inspired HP peptides.²⁶

In the case of significantly less-stable HP6I system, the E12A mutation was stabilizing by both NMR and CD measures of folding (Figure 3 and Table II); (E12A)-HP6I actually displayed the greatest resistance to thermal melting (Figure 3A, and Table II). The rescue of the HP6I hairpin by this approach is discussed later.

Testing the β -Cap Function

In order to examine the potential of the V/E diagonal interaction at hydrogen bonded positions as a β -hairpin cap, we explored the effects of extension and truncation on HP6V. β -Strand truncation lead to a dramatic change in the indole/indole interaction orientation²⁶ and thus provided no quantitative information regarding the capping effect. Although the actual definition of a cap (particularly in the context of β -hairpins or sheets) is loosely established, two criteria should be met: the termini should not be frayed as a result of the interaction and the interaction should work only at the termini. For an effective β -cap, an extension of the β -strands past the capping unit should either decrease the fold population, or result in an increase that is less than expected for the addition of a hydrogen bonding pair.

The Gellman group has reported that β -strand extension increases the stability of the entire hairpin as a result of ‘length-wise cooperativity’.³⁸ In the case of HP6V, with the putative “diagonal capping interaction,” only a small increase in fold population is observed when each strand is extended by a (Thr)₂ unit (see Figure 4). In the case of HP6I, the (Thr)₂ additions, however, were significantly stabilizing and changed the CSD profiles along the sequence so that they resemble HP6V rather than HP6I.

The CD melt of HP6VTT is shown in Figure 5. The spectra differ from what is seen for other Trp/Trp peptides; the exciton couplet maximum is somewhat reduced versus that of HP6V even though all NMR measurements suggest that HP6VTT is more stable (Table II): the increased stability is confirmed by the CD melt. We postulate that the extension decreases the overall twist of the peptide and the orientation of the indole rings changes, although they are still close in space as indicated by NMR, the chiral interaction has been altered in a subtle manner.

DISCUSSION

In this study we have explored a case of apparent hairpin end capping by the formation of a hydrophobic cluster. A hydrophobic interaction between the S-3 and S+5 sidechains in KYX₃WSNGKWTVX₁₂ is suggested by fold population comparisons at 280 K that translate to the following $\Delta\Delta G_u$ values: 3.2 kJ/mol (V3/E12 vs T3/E12), 2.2 kJ/mol (V3/E12 vs V3/G12), 2.0 kJ/mol (V3/E12 vs V3/D12), and 1.7 kJ/mol (V3/E12 vs V3/K12). Hydrophobic interactions at or near the termini have historically provided little stabilization (1–2 kJ/mol) to β -hairpins systems; and, in general, cross-strand pairs near the termini, with few exceptions,²³ appear to provide no further stabilization than that associated with the β -propensities of the residues employed. If this interaction is indeed the result of cross-strand hydrophobic packing then a $\Delta\Delta G_u$ of 3.4 kJ/mol for V3/E12 vs I3/E12 and the fold rescuing effects seen for (E12A)-HP6I require explanation. These effects are particularly puzzling since the interacting side-chains would, in contrast to an S+5/S-3 interaction, be quite remote on a flat hairpin and there is no prior evidence of this type of interaction stabilizing β -sheets or hairpins.

We turned to an examination of the NMR structure ensemble of the most similar sequence available, (S5I)-HP6V²⁶ (see Figure 6) for further insights. NOE data suggests that HP6V and its S5I mutant are nearly identical in structure; the only difference are long range NOEs between the Val S-3 H γ protons and the Ser S-1 H β protons observed for HP6V, while NOEs between the sidechains of Val at S-3 and Ile at S-1 are not observed for (S5I)-HP6V. A lack of agreement between NMR and CD measures of melting behavior between the two hairpins²⁶ suggests a slight difference in geometry of the indole rings in the EtF interaction; the bulk of the Ile sidechain at S-1 is likely responsible for this spectroscopic difference.

(S5I)-HP6V displays analogous NOEs to HP6V between the H β protons of Glu at S+5 and the H γ of Val S-3. Diastereotopic splitting of the H β methylene protons indicates that the Glu sidechain does not visit all χ_1/χ_2 values, which is unexpected in a part of the hairpin that has a substantial amount of conformational freedom and particularly for a long sidechain. In the dominant conformer one of the Glu H β s fits directly between the Val methyls. Given that the two sidechains, although on the same face of the hairpin, are at diagonal hydrogen bonded positions, only a high degree of twist, as is present in (S5I)-HP6V, would result in such a conformation and sidechain interaction. The twist is the most likely cause of intolerance for the Ile at the S-3 site. Consideration of the NMR structure poses two possibilities as to why the twist causes this intolerance. One is that there is simply not enough space for the extra methyl of Ile, which disrupts the packing of the hairpin, or the extra methyl is forced into vicinity of the glutamate carboxylate which requires solvation (resulting in a solvation penalty), see panel C of Figure 6.

In order to clarify whether the V3/X12 interaction is made possible because of the high degree of twist, a (Thr)₂ extension was applied to both strands of HP6V and HP6I. Given the CD data for HP6VTT (in comparison to NMR probes) we hypothesized that HP6ITT would be “untwisted” enough to provide space for the Ile sidechain; space that is not present in HP6I. The backbone CSDs support this hypothesis (see Figure 4), HP6ITT is nearly as folded as HP6VTT, which would not have been possible if the highly twisted conformation persisted in HP6ITT.

The NMR structure of (S5I)-HP6V also provides a rationale for the observation that V3/D12 is less stable than both V3/E12 and V3/K12. Shortening of the sidechain (from E $\rightarrow$ D) brings the polar part of the molecule closer to the Val3 methyls, inhibiting the solvation of the sidechain carboxylate function. The reduced fold population for the V3T mutation may also reflect decreased favorable van der Waals contacts of a hydrophobic cluster (methyl vs hydroxyl). In the case of the E12G mutant, reduced fold stability might be expected based on greater configurational entropy; however in the extreme C-terminal position, β -propensity and terminal fraying effects may not be as hairpin-destabilizing as they would be in a central β strand site. Lacking a sidechain, G12 cannot participate in the hydrophobic interaction proposed.

The data suggests that hydrophobic sidechain packing plays an important role in a diagonal interaction at the termini of the HP6 peptides. When S-3 is occupied by Val, the corresponding diagonal sidechain can either be a methyl or $-(CH_2)_2-X$. When S-3 is occupied by Ile, only Ala accommodates the extra spatial needs for the Ile methyl while still supplying an aliphatic chain contacts to satisfy the hydrophobic cluster requirement. The “rescue” of the HP6I hairpin validates the conclusion that sidechain packing must be optimized in this system.

The most remarkable finding of this study is that a single mutation (E $\rightarrow$ A) at the terminus of a β -hairpin results in an increase in stability of ~ 6 kJ/mole ($\Delta\Delta G_0$); the rescue of HP6I highlights the importance of sidechain packing at the termini of HP6 peptides, and the ability of hydrophobic non-aromatic interactions to provide structural stability at the end of β -hairpins. The lack of fraying as seen in the structure ensemble of (S5I)-HP6V demonstrates that one criteria of a β -cap is fulfilled. However, the extent of stabilization caused by the (Thr)₂ extension of HP6V, prevents us from claiming this interaction is a specific β -cap.

In previous studies, we have observed²⁶ that the geometry of the Trp/Trp pair can be modulated by interactions at the termini, such as a π -cation interaction, or deletion of cross-strand pairs. These effects have not involved nonaromatic hydrophobic sidechains. The

convergence of the terminal backbone of (S5I)-HP6V is highly unusual for a relatively short yet well-structured β -hairpin; we have demonstrated that this is a result of optimal hydrophobic packing of hydrogen bonded diagonal cross-strand sidechains, which requires an unusual extent of hairpin twist.

Differences in quantitative measurements of the EtF interaction (the exciton couplet by CD and the edge residue H ϵ 3 NMR structuring shift), indicate these measurements should be used in conjunction with backbone CSDs to obtain a complete understanding of mutational effects and for obtaining fold population estimates. In addition, the placement of the Trp indole rings relative to the strand or turn can be assessed from the backbone CSDs; due to the large effect on chemical shift by ring currents, the extent of the effect can be correlated with its proximity to a given proton. HP6I for example has a much larger CSD for the T2 Gly H α than the S+1 Lys H N , in contrast with HP6V. This suggests that the EtF interaction is in closer proximity to the T2 position.

Whether caused by specific ring current effects or amide bond diamagnetic anisotropy and H-bonding, resonances with a large CSD (1.0 ppm or higher) continue to provide reliable measures of fold population. CD measurements provide insightful information on the nature of the Trp/Trp interaction but should be used cautiously for fold population quantification of Trp containing peptides due to the greater geometric sensitivity of the exciton couplet magnitude.

This study represents one of the first explorations of C-terminal sidechain effects on a β -hairpin construct. We have shown that the sidechain at the extreme terminus of a β -hairpin can have a substantial effect on overall stability. In the present case, a high degree of twist is needed to facilitate the interaction. β -Hairpins continue to provide useful information and further our understanding of β -sheet folding in proteins, and de novo peptide and protein design. As these constructs continued to be used and provide insight for material and biological applications,^{39–41} understanding the interactions that modulate their stability and overall structure is essential.

MATERIALS AND METHODS

All peptides were synthesized using fast Fmoc chemistry on an ABI 433A peptide synthesizer, and were cleaved from the resin by shaking in excess trifluoroacetic acid (TFA) with 2.5% water and 2.5% triisopropylsilane for 1.5 h. Peptides were purified by reverse-phase HPLC (C18 column) with a water (0.1% TFA)/acetonitrile (0.085% TFA) gradient. The identity and purity of the peptides were verified by positive ion mass spectrometry on a Bruker Esquire ion trap spectrometer. NMR established purity and proper connectivities by NOESY or ROESY spectra.

CD Spectroscopy

CD stock solutions were made by dissolving weighed amounts of peptide in 20 mM pH 6 potassium phosphate buffer, and the concentrations of the stock solutions were measured by the combined expectation UV absorption of Trp ($\epsilon = 5580 \text{ M}^{-1} \text{ cm}^{-1}$ per Trp) and Tyr ($\epsilon = 1420 \text{ M}^{-1} \text{ cm}^{-1}$ per Tyr) at 280 nm. Stock solutions were diluted with buffer to yield 30 μM solutions of peptide. Spectra were recorded on a Jasco J715 spectropolarimeter using 0.10 cm path length cells. CD data are reported in molar units ($\text{deg cm}^2 \text{ dmol}^{-1}$), shown as degrees molar ellipticity throughout, rather than residue-molar ellipticity units ($\text{deg cm}^2 \text{ res-dmol}^{-1}$) because the exciton couplet, which dominates the CD spectra for most peptides, is due to a single chromophore and not the backbone amide units. The exciton couplet has a maximum at 227–229 nm, and this maximum value was used (after subtraction of the coil

control value of $-11,000^\circ$ for each Trp at 228 nm) to provide a folding measure that should approach zero when the fold population of each peptide approaches zero.

NMR Spectroscopy

NMR spectra were recorded on 500 and 750 MHz NMR instruments with Bruker DRX and AV consoles respectively. Solvent suppression was accomplished in all cases by a WATERGATE⁴² pulse train. TOCSY spectra employed a 60 ms MLEV-17 spinlock,⁴³ and NOESY spectra had a 150 ms mixing time. Aqueous samples were comprised of 1–2 mM peptide in 20–50 mM sodium phosphate buffer, pH 6–7, with 10% D₂O and sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal standard. Complete resonance assignments appear in the Supporting Information.

All resonances displayed sharp signals: line widths consistent with the tumbling times of monomeric peptides. NMR fold population measures are corroborated by CD fold population measures taken at 20 μ M, additional support for monomeric behavior of all peptides discussed. The algorithms for CSD calculations are automated and a recently updated version¹² is used throughout, and is publicly available at (<http://andersenlab.chem.washington.edu/CSDb>). The program calculates and graphs H α and H $_N$ CSDs across a sequence and includes corrections for near neighbor effects in the coil state. For H $_N$'s, alternative temperature gradients are used depending on the degree of solvent sequestration present in the folded state and the degree of unfolding observed over the temperature range employed in a melting study. Generation of the structure of HP6V and ensemble statistics have previously been reported.²⁶

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Schematic of β -hairpin HP6V with the turn (T) and Strand ($S^\pm$) positions indicated (A). This nomenclature is used throughout the text however residue numbers (1–12) are employed when discussing specific mutations at site X_3 and X_{12} . The S-3 and S+5 positions are hydrogen-bonded sites and are diagonal in relation to each other. Names and sequences of peptides used in this study (B).

FIGURE 2. H_α and H_N CSDs of β -hairpins at 300 K with mutations at residue 3: X = V (light purple), T (pink), and I (red).

FIGURE 3.

Melting curves derived from the upfield H ϵ 3 values (A) and the CD exciton couplet maximum at 228 nm (B).

FIGURE 4.

$H\alpha$ and H_N CSDs of the most stable constructs, and HP6I for comparison. Data at pH 3.0 is shown for both (Thr)₂-extended peptides; at pH 6, HP6ITT aggregated at NMR concentrations. HP6VTT is stabilized slightly by changing the pH from 6 to 3. The divergent shifts of HP6I (green bars) disappear upon hairpin extension.

FIGURE 5.
CD melt of HP6VTT from 5–75°C.

FIGURE 6.

NMR structure ensemble of (S5I)-HP6V with side chains of Val3 and Glu12 highlighted (A). Lowest energy conformation of (S5I)-HP6V (B). Space-filling model of the lowest energy conformation of (S5I)-HP6V (C). The close packing of the Val3 and Glu12 (CH₂)₂ unit is shown. The pink arrows highlight the Val3 H_γ's, the bottom arrow points to the γ₂ methyl and the top arrow points to the γ₁ methyl. If Val3 was replaced with Ile, the methyl would be extended from γ₂.

Table I

Previously Reported GB1/Trpzip Analogs That Inspired this Study

Name	Sequence	T _m (°C)
Trpzip2 ^a	SWTW-ENGK-WTWK	72
HP6 ^b	KYTW-SNGK-WTVE	45
HP6V ^b	KYVW-SNGK-WTVE	59
HP6I ^c	KYIW-SNGK-WTVE	31
GB1p ^d	GEWTW-DDATKT-FTVTE	1–11 ^{a,b}
HP5W ^e	KKYTW-NPATGK-WTVQE	66
HP7 ^b	KTW-NPATGK-WTE	66

^aReported by Cochran et al.²¹^bReported by Andersen et al.²⁰^cReported herein.^dReported by Kobayashi et al.²⁴^eReported by Fesinmeyer et al.¹²

Table II

Percent Fold Values for HP6 and Mutants at 280 K

Peptide	CD	H α 3	Strand ^a	Turn Gly $\Delta\delta H\alpha$	Turn Lys H _N
HP6	71	78 (65)	72	86	76
HP6V	91	91 (82)	91	91	91
HP6I	79	68 (53)	70	na ^b	23
HP6V E12G	74	82 (65)	80	82	82
HP6V E12A	84	91 (82)	91	91	94
HP6V E12D	77	85 (72)	81	82	82
HP6V E12K	52 ^c	86 (74)	83	82	88
HP6I E12A	84	93 (85)	94	90	90
HP6V TT	84	93 (84)	94	91	95
HP6I TT	94	92 (88)	92	89	91

Values in parentheses are at 300 K. 100% fold measures from NMR CSDs employ extrapolated 100% folded values based on the HP6V CSD values at 280 K (as 91% folded) for each method.

^a Calculated from backbone CSDs of H α at Y2, T/V/I3, W9, and T10 and H_N at T/V/I3, W4, and T10.

^b Fold population exceeds 100% based on the 100% fold value (0.87 ppm) applied.

^c Although the CD measure is aberrant from NMR probes, this is not without precedent as the exciton couplet is extremely sensitive to the orientation of the tryptophans.