

Turn Residues in β -Hairpin Peptides as
Points for Covalent Modification

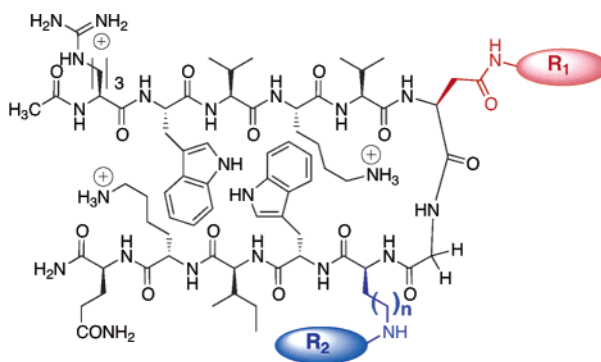
W. John Cooper and Marcey L. Waters*

Department of Chemistry, CB 3290, University of North Carolina,
Chapel Hill, North Carolina 27599

mlwaters@email.unc.edu

Received May 3, 2005

ABSTRACT



β -Turns are important sites for protein–protein and protein–peptide interactions, but little research has explored synthetic modifications of turn residue side-chains in a β -hairpin peptide. To this end, β -hairpin peptides were synthesized containing the type I' turn sequence Val-Asn-Gly-Lys with modifications at Asn and Lys. We found that these variations impose a small penalty, demonstrating that β -turns are capable of displaying a range of functionality, which may be exploited for biomolecular recognition and medicinal applications.

β -Turns have long been demonstrated to be important structural elements in biomolecular recognition, including protein–protein and protein–peptide interactions, such as somatostatin recognition, which is important to the regulation of growth hormone secretion in humans.¹ In addition, sites of antigenic recognition, phosphorylation, glycosylation, hydroxylation, and intein/exein splicing are frequently within turns.² For this reason, β -turn mimics have been an active area of synthetic research.³ One alternative to such peptidomimetics is the utilization of synthetically modified side chains within the context of a well-folded β -hairpin peptide.⁴ However, the current body of knowledge of β -turns is elementary with respect to other secondary structural elements. We report here the effect of side chain modification of residues within a favorable turn sequence on the structure

and stability of a β -hairpin. These model systems demonstrate that covalent amendment of side-chain functional groups in β -turns may occur with little loss in conformational stability in systems that are well-folded due to favorable strand–strand interactions.

β -Hairpins are the minimal unit of a β -sheet and hence are the secondary structure complement of an isolated α -helix. However, whereas the factors that contribute to α -helix formation have been investigated over the course of more than 30 years,⁵ the first structured β -hairpin was reported in 1993.⁶ Thus, the features that define a well-folded

(1) (a) Mattern, R.; Tran, T.; Goodman, M. *J. Med. Chem.* **1998**, *41*, 2686–2692. (b) Bauer, W.; Briner, U.; Doepfner, W.; Haller, R.; Huguenin, R.; Marbach, P.; Petcher, T. J.; Pless, J. *Life Sci.* **1982**, *31*, 1133–1140. (c) Melacini, G.; Zhu, Q.; Osapay, G.; Goodman, M. *J. Med. Chem.* **1997**, *40*, 2252–2258.

(2) Rose, G. D.; Gierasch, L. M.; Smith, J. A. *Adv. Protein Chem.* **1985**, *37*, 1–109.

(3) (a) Burgess, K. *Acc. Chem. Res.* **2001**, *34*, 826–835. (b) Loughlin, W. A.; Tyndall, J. D. A.; Glenn, M. P.; Fairlie, D. P. *Chem. Rev.* **2004**, *104*, 6085–6118. (c) Robinson, J. A. *Synlett* **1999**, 429–441. (d) Smith A. B.; Charnley, A. K.; Mesaros, E. F.; Kikuchi, O.; Wang, W. Y.; Benowitz A.; Chu, C. L.; Feng, J. J.; Chen, K. H.; Lin, A.; Cheng, F. C.; Taylor, L.; Hirschmann, R. *Org. Lett.* **2005**, *7*, 399–402. (e) Schneider, J. P.; Kelly, J. W. *Chem. Rev.* **1995**, *95*, 2169–2187.

(4) For examples of turn sequence variations in the context of a stable β -hairpin, see: (a) Skelton, N. J.; Blandl, T.; Russell, S. J.; Starovasnik, M. A.; Cochran, A. G. *Spectroscopy* **2003**, *17*, 213–230. (b) Cochran, A. G.; Skelton, N. J.; Starovasnik, M. A. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 5578–5583. (c) Fesinmeyer, R. M.; Hudson, F. M.; Andersen, N. H. *J. Am. Chem. Soc.* **2004**, *126*, 7238–7243.

β -hairpin are still being established. Both the turn sequence and the strand residues of a β -hairpin have been shown to contribute to its stability.⁷

To date, only a small number of turn sequences have been shown to adequately induce β -hairpin formation, illustrating the role a turn sequence plays in determining peptide structure. As a consequence, the ability to display a wide range of turn sequences for biological applications has been severely limited. Most synthetic β -hairpins have utilized one of two two-residue turn sequences: an Asn-Gly sequence, which consists of the two most common residues found in a type I' turn sequence in the protein database,^{8,9} and the dPro-Gly sequence, which is the strongest two-residue turn sequence reported to date.¹⁰

The reason an Asn residue, in particular, is a good turn nucleator has been investigated but is still not well understood.^{7a} Its high left-handed α -helical propensity may allow reversal of the β -strand.¹¹ Carbonyl-carbonyl stacking interactions have also been proposed to influence its nucleating ability relative to other residues.¹² Even the modest replacement of Asn with Asp results in a measurable decrease in β -hairpin stability.^{9,11} More significant changes to the Asn side chain such as glycosylation have been shown to have a considerable impact on peptide secondary structure in peptide sequences excised from proteins and are credited with inducing type I turn formation.¹³

The residues flanking the turn have also been shown to be important to stabilizing the β -hairpin structure. A Val or Tyr residue N-terminal to the Asn has been shown to increase β -hairpin stability, and a Lys after the Gly is commonly utilized. Though its role in β -hairpin stability is not well established, Gellman and co-workers have shown that it may be substituted with ornithine at no penalty.¹⁴

As part of a study of the interactions between structured β -hairpins and oligonucleotides, we were interested in appending a fluorophore to a β -hairpin peptide at or near the β -turn. However, on the basis of the results of Danishefsky and Imperiali, it was not clear that these modifications would be tolerated.^{13,15} To investigate the impact of side-

chain-modified turn sequences on hairpin structure and stability, model peptides were examined in which Lys 8 was benzoylated or Asn 6 was alkylated (Figure 1). The previ-

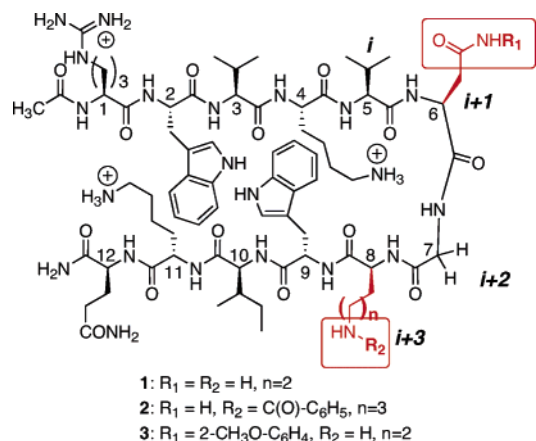
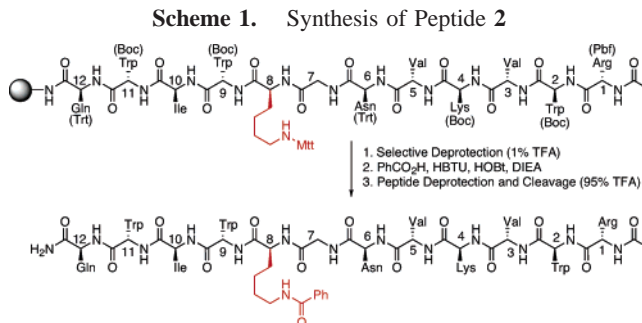


Figure 1. Peptides **1–3**: Ac-RWVKVN(R_1)GK(R_2)WIKQ-NH₂.

ously reported monomeric peptide **1** was used as an unmodified control.¹⁶

The synthesis of peptide **2** containing a benzoylated lysine (Scheme 1) was accomplished by standard Fmoc/tBu solid-



phase peptide synthesis protocols utilizing methyltrityl side chain protection of Lys 8, allowing for selective deprotection by treatment with 1% trifluoroacetic acid and subsequent coupling of the liberated amine with benzoic acid. The peptide was then wholly deprotected and cleaved from the resin with 95% trifluoroacetic acid.¹⁷ Following purification by RP-HPLC, the peptide sequence was confirmed by ESI-MS.

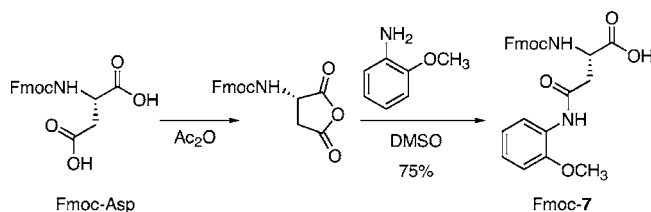
A similar approach to achieve an asparagine side-chain-modified β -hairpin **3** would invoke the incorporation of an aspartic acid-glycine dipeptide unit into the peptide followed by subsequent amidation. However, Asp-Gly sequences are

- (5) Scholtz, J. M.; Baldwin, R. L. *Annu. Rev. Biophys. Biomol. Struct.* **1992**, *21*, 95–118.
- (6) Blanco, F. J.; Jimenez, M. A.; Herranz, J.; Rico, M.; Santoro, J.; Nieto, J. L. *J. Am. Chem. Soc.* **1993**, *115*, 5887–5888.
- (7) (a) Searle, M. S. *J. Chem. Soc., Perkin Trans. 2* **2001**, 1011–1020.
- (b) Santiveri, C. M.; Santoro, J.; Rico, M.; Jimenez, M. A. *Protein Sci.* **2004**, *13*, 1134–1147. (c) De Alba, E.; Rico, M.; Jimenez, M. A. *Protein Sci.* **1999**, *8*, 2234–2244.
- (8) Hutchinson, E. G.; Thornton, J. M. *Protein Sci.* **1994**, *3*, 2207–2216.
- (9) Ramirez-Alvarado, M.; Blanco, F. J.; Niemann, H.; Serrano, L. J. *Mol. Biol.* **1997**, *273*, 898–912.
- (10) (a) Haque, T. S.; Gellman, S. H. *J. Am. Chem. Soc.* **1997**, *119*, 9, 22303–2304. (b) Stanger, H. E.; Gellman, S. H. *J. Am. Chem. Soc.* **1998**, *120*, 4236–4237.
- (11) Stotz, C. E.; Borchardt, R. T.; Siahaan, T. J.; Vander Velde, D.; Topp, E. M. *J. Peptide Res.* **2004**, *63*, 371–382.
- (12) Deane, C. M.; Allen, F. H.; Taylor, R.; Blundell, T. L. *Protein Eng.* **1999**, *12*, 1025–1028.
- (13) (a) Imperiali, B.; O'Connor, S. E.; Hendrickson, T.; Kellenberger, C. *Pure Appl. Chem.* **1999**, *71*, 777–787. (b) O'Connor, S. E.; Imperiali, B.; *Chem. Biol.* **1998**, *5*, 427–437. (c) Imperiali, B.; Rickert, K. W. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 97–101. (d) Imperiali, B.; O'Connor, S. E. *Pure Appl. Chem.* **1998**, *70*, 33–40.
- (14) Syud, F. A.; Espinosa, J. F.; Gellman, S. H. *J. Am. Chem. Soc.* **1999**, *121*, 11577–11578.

- (15) Live, D. H.; Kumar, R. A.; Beebe, X.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12759–12761.
- (16) Butterfield, S. M.; Waters, M. L. *J. Am. Chem. Soc.* **2003**, *125*, 9580–9581.
- (17) Chan, W. C.; White, P. D. *Fmoc Solid-Phase Peptide Synthesis: A Practical Approach*; Oxford University Press: New York, 2000.

of notorious synthetic difficulty due to side reactions occurring during synthesis.^{17,18} Hence, following the method of Keillor et al.,¹⁹ a side-chain-modified Asn was prepared beginning with *N*-Fmoc *L*-aspartic acid, which was dehydrated by heating in acetic anhydride and then treated with 2-anisidine in DMSO to give the desired product with good regioselectivity (Scheme 2). Incorporation of this modified

Scheme 2. Synthesis of Fmoc-7



Asn residue proceeded by standard peptide synthesis procedures to give peptide **3**.

A β -hairpin structure was demonstrated for each peptide by cross-strand NOEs and the downfield shifting of the α -protons (H_α) of the peptides relative to random coil reference sequences.²⁰ NOEs indicate that the peptides are folded into a β -hairpin with correct register (Figure 2a). The H_α downfield shifting indicates that the folded state is well pop-

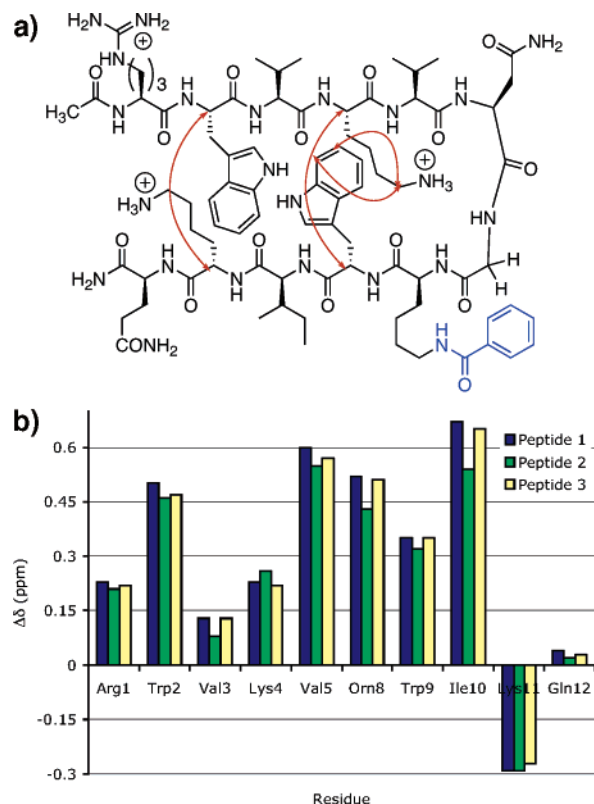


Figure 2. (a) Representative cross-strand NOEs observed for peptide **2**. (b) Observed downfield shifting of residue H_α relative to random coil values (Asn 6 and Gly 7 are not shown).

ulated, as the shifts are ≥ 0.1 ppm for all strand residues, except the N- and C-termini, which are frayed (Figure 2b). The extent of folding of each hairpin was quantified by comparison of H_α chemical shifts and the degree of glycine H_α splitting relative to random coil and fully folded values.²¹ The fully folded state was derived from a cyclized β -hairpin as had been reported previously (See Supporting Information).¹⁶

Characterization of peptide **3** by NMR revealed that alkylation of Asn 6 resulted in little change in H_α chemical shifts relative to peptide **1** (Figure 2b). The effect was quantified by averaging the fraction folded value for residues in hydrogen-bonded sites with that determined from glycine splitting (See Supporting Information).²² A minor decrease from 99% folded for the parent sequence **1** to 96% folded for peptide **3** was measured by this method (Table 1). The

Table 1. Comparison of β -Hairpin Stabilities with Modified Turn Sequences: Ac-RWVKV(NR₁)G(KR₂)XIKQ-NH₂ at 298 K

peptide	R ₁	R ₂	X	average fraction folded ^a
1	H	H ^b	Trp	0.99 (0.95)
2	H		Trp	0.93 (0.93)
3		H ^b	Trp	0.96 (0.91)
5	H	H ^b	Phe	0.69 (0.67)
6	H		Phe	0.54 (0.56)
7		H ^b	Phe	0.57 (0.57)

^a Fraction folded was determined from an average of the H_α chemical shift of residues 3, 5, and 10 and the Gly splitting. The value in parentheses was determined from the Gly splitting alone. ^b Orn was used in place of Lys.

small change in the population fraction in the folded state is surprising since Asn 6 is directly involved in reversing the peptide chain.

Examination of the H_α chemical shifts of peptide **2** illustrates a slightly larger effect that benzylation of the Lys

(18) Wade, J. D.; Mathieu, M. N.; Macris, M.; Tregear, G. W. *Lett. Pept. Sci.* **2000**, 7, 107–112.

(19) Huang, X.; Luo, X.; Roupioz, Y.; Keillor, J. W. *J. Org. Chem.* **1997**, 62, 8821–8825.

(20) (a) Wishart, D. S.; Sykes, B. D.; Richards, F. M. *Biochemistry* **1992**, 31, 1647–1651. (b) Wishart, D. S.; Sykes, B. D.; Richards, F. M. *J. Mol. Biol.* **1991**, 222, 311–333. (c) Maynard, A. J.; Sharman, G. J.; Searle, M. S. *J. Am. Chem. Soc.* **1998**, 120, 1996–2007.

(21) Griffith-Jones, S. R.; Maynard, A. J.; Searle, M. S. *J. Mol. Biol.* **1999**, 292, 1051–1069.

(22) Syud, F. A.; Stanger, H. E.; Gellman, S. H. *J. Am. Chem. Soc.* **2001**, 123, 8667–8677.

8 side chain has on stability (Figure 2b). Nonetheless, with an overall fraction folded value of 0.93, this peptide represents a well-folded system despite the conversion of a charged polar side chain to an uncharged polar side chain.

We also compared the magnitude of these synthetic turn modifications to the naturally occurring Gly-Gly turn sequence (peptide **4**), which has been shown to be a weaker turn nucleator than Asn-Gly due to a larger allowed region on a Ramachandran plot.⁹ Replacement of Asn-Gly with Gly-Gly results in a decrease of the folded population to 92%, similar to that observed in the case of peptide **2**.²³

To further investigate the cost in stability of the modification of a turn residue side chain, this approach was applied to a more moderately folded system where larger changes in the fraction folded are observed for equivalent energetic changes, resulting in smaller errors in measurement. A previous study indicated that mutating Trp 2 to Phe to give peptide **5** results in a decrease in the fraction folded by about one-third.²⁴ On the basis of the average fraction folded determined from the H_α chemical shifts and glycine H_α splitting, the unmodified peptide **5** is 69% folded, and when the lysine proximal to the turn is benzoylated (peptide **6**), the fraction folded decreases to 54%. As in peptides **1–3**,

the alkylation of Asn again has a slightly smaller effect than benzoylation of Lys, as demonstrated by peptide **7**, which is 57% folded. Thus, in moderately folded systems, these side chain variations result in a loss of about 0.3 to 0.4 kcal/mol at 298 K.

These findings demonstrate the ability to incorporate synthetically modified turn sequences in well-folded β -hairpins with only a small effect on the extent of folding. This is contrary to the observed effect of Asn glycosylation on peptide structure in some sequences and demonstrates the importance of strong strand–strand interactions. We expect that the use of β -turn residues as sites for covalent modification in the context of well-folded systems will provide a new avenue for exploring protein–peptide and receptor–ligand interactions.

Acknowledgment. W.J.C. gratefully acknowledges a Burroughs–Wellcome fellowship. This research was supported in part by an NSF Career Award (CHE-0094068) and an Alfred P. Sloan fellowship to M.L.W. and a grant from the NIH NIGMS (GM072691).

Supporting Information Available: Peptide NMR assignments and experimental procedures. This material is available free of charge via the Internet at <http://pubs.acs.org>.

OL0510116

(23) NMR concentration studies indicate that peptide **4** is monomeric between 200 μ M and 2 mM.

(24) Butterfield, S. M.; Sweeney, M. M.; Waters, M. L. *J. Org. Chem.* **2005**, *70*, 1105–1114.