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Bulky Dehydroamino Acids Enhance Proteolytic Stability and Folding in β-Hairpin Peptides

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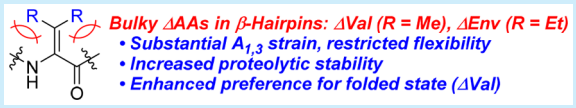
Supporting Information

ABSTRACT: The bulky dehydroamino acids dehydrovaline (ΔVal) and dehydroethylnorvaline (ΔEnv) can be inserted into the turn regions of β-hairpin peptides without altering their secondary structures. These residues increase proteolytic stability, with ΔVal at the (*i* + 1) position having the most substantial impact. Additionally, a bulky dehydroamino acid can be paired with a D-amino acid (i.e., D-Pro) to synergistically enhance resistance to proteolysis. A link between proteolytic stability and peptide structure is established by the finding that a stabilized ΔVal-containing β-hairpin is more highly folded than its Asn-containing congener.



Although peptides play crucial roles in medicine,[1] their use as drugs is hampered by rapid proteolytic degradation. Several methods for stabilizing peptides to proteolysis have been devised,[2−12] but additional strategies are needed if they are to realize their full potential as therapeutic agents. In seminal work, Stammer and co-workers demonstrated that incorporating α,β-dehydroamino acids (ΔAAs) such as ΔAla, *Z*-ΔPhe, and *Z*-ΔLeu into enkephalin mimics protected these peptides from proteolysis.[13] Presumably, the $A_{1,3}$ strain induced by the trisubstituted alkenes present in *Z*-ΔPhe and *Z*-ΔLeu rigidifies the backbones of peptides containing these residues, thereby conferring proteolytic stability by favoring folded states over more flexible random coil conformations.[14] We hypothesized that bulky ΔAAs containing tetrasubstituted alkenes (e.g., ΔVal and its homologue dehydroethylnorvaline, or ΔEnv) would be particularly effective at stabilizing peptides to proteolysis due to their elevated levels of $A_{1,3}$-strain caused by the presence of two allylic alkyl groups. We also reasoned that these residues would be less reactive Michael acceptors than ΔAAs possessing di- or trisubstituted alkenes, which are prone to attack by biologically relevant nucleophiles such as thiols.[15] To the best of our knowledge, the impact of bulky ΔAAs on the proteolysis of peptides that contain them has not been studied previously. Prompted by these considerations and inspired by the presence of ΔVal and ΔIle in bioactive peptides such as yaku'amides,[16] phomopsins,[17] and myxovalargin A,[18] we investigated the effect of bulky ΔAAs on proteolysis and peptide structure. Herein, we report that ΔVal and ΔEnv can impart substantial proteolytic stability to β-hairpin peptides. This result can be at least partially attributed to an increased thermodynamic preference for the folded states of the β-hairpins.

Reports describing the effect of ΔVal on peptide structure are scarce,[19] and we are unaware of published studies involving ΔEnv.[20] A dipeptide containing ΔVal exhibits a solution conformation that resembles a β-turn,[19a] and X-ray crystallography data show that ΔVal can adopt dihedral angles compatible with the (*i* + 1) position of a type II β-turn.[19b,e] Type II′ β-turns should be equally accessible given that this residue is achiral. Accordingly, we posited that β-hairpins containing ΔVal or ΔEnv in their turn regions would retain their secondary structure while possessing enhanced stability to proteolysis. We selected β-hairpins designed by Waters and co-workers[21] (**1** and **2**, Figure 1) to test this hypothesis. Close relatives of these peptides are well-folded and somewhat resistant to proteolysis, with the D-Pro-Gly-turn-containing peptide[22] analogous to **2** exhibiting the most stability.[21b] Thus, β-hairpins **1** and **2** presented a stringent test of the ability of bulky ΔAAs to impart rigidity and stability to peptides. Additionally, the amenability of **1** and **2** to be studied by NMR spectroscopy would yield structural insights that could help explain the increased proteolytic stability that we hoped to observe.

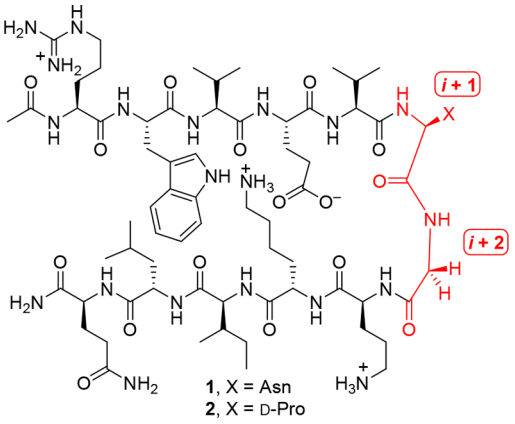


Figure 1. Model β-hairpins designed by Waters and co-workers.

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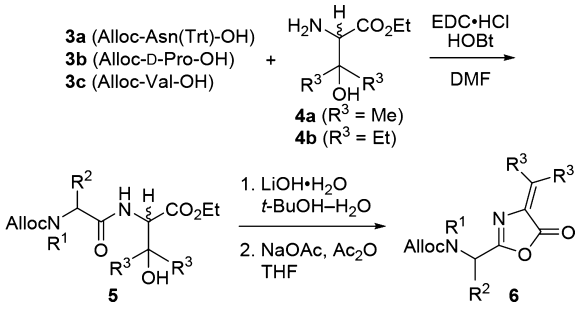
We used our recently published method for opening azlactones (i.e., oxazolones) with resin-bound amines[23] to construct the targeted bulky ΔAA-containing peptides. The requisite azlactone dipeptides were synthesized as shown in Table 1. Coupling of Alloc-protected amino acids **3** with racemic

Table 1. Synthesis of Azlactone Dipeptides

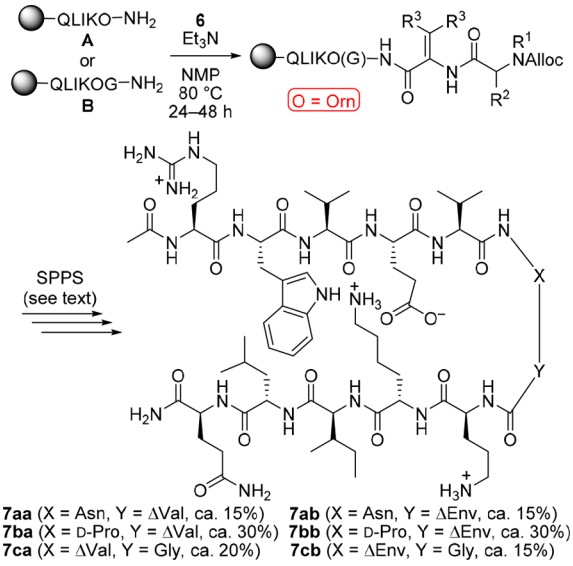


azlactone	yield of **5** (%)	yield of **6** (%)
6aa	77	70
6ab	71	64
6ba	81	72
6bb	79	73
6ca	84	80
6cb	78	74

β-OHVal-OEt (**4a**)[24] or racemic β-OHEnv-OEt (**4b**)[23] furnished dipeptides **5**. In contrast to our prior work, the use of rigorously purified samples of **4b** enabled EDC·HCl and HOBt to be employed in couplings instead of the more expensive COMU. Saponification was followed by a one-pot dehydration−cyclization sequence mediated by NaOAc and Ac_2O.[25] We originally employed Ac_2O as solvent,[23] but challenges in completely removing this high-boiling compound from **5** prior to the ring-opening reaction led us to devise a new procedure utilizing THF as solvent. The yields listed in Table 1 for azlactones **6** were affected by partial decomposition during SiO_2 chromatography. In practice, the purity of the crude azlactones was usually sufficient for use in the ring-opening reactions.

Modifying our published ring-opening procedure[23] by employing Et_3N instead of DMAP facilitated attachment of the bulky ΔAAs to solid-supported peptides (Scheme 1). Slow stirring was required to prevent degradation of the resin during the reaction. Pentapeptide **A** was used as the nucleophile to construct β-hairpins with bulky ΔAAs at the (*i* + 2) position (i.e., "Y" in Scheme 1), whereas hexapeptide **B** was enlisted to synthesize β-hairpins with Gly at (*i* + 2) and bulky ΔAAs at (*i* + 1). Subsequent acetylation capped any unreacted resin-bound amines. Alloc deprotection enabled attachment of the remaining residues via standard microwave-promoted solid-phase peptide synthesis (SPPS) protocols.[26] Acetylation, cleavage from the resin, and HPLC purification afforded the desired bulky ΔAA-containing peptides **7aa**−**7cb**. By considering the resin loadings, HPLC traces of the crude peptides, and concentrations of peptide solutions utilized in NMR and proteolysis studies, we estimated the overall yields of **7aa**−**7cb** that are given in Scheme 1. These approximations suggest that the ring-openings are fairly efficient, with the best results obtained from D-Pro-containing azlactones **6ba** and **6bb** and some attenuation in yield observed with the hindered Asn(Trt)-containing azlactones **6aa** and **6ab**.

Scheme 1. Synthesis of Bulky ΔAA-Containing Peptides



The proteolytic stabilities of **1** and its four bulky-ΔAA-containing analogues were determined by exposure to Pronase, an aggressive cocktail of nonspecific proteases from *Streptomyces griseus*.[21b] Monitoring of the proteolytic degradation by HPLC established that each analogue was substantially more stable than **1**. Peptides **7ca** and **7cb** containing ΔVal and ΔEnv, respectively, in place of the (*i* + 1) Asn were ca. 6−7 times more stable to proteolysis than **1** (Figure 2). Replacement of the (*i* +

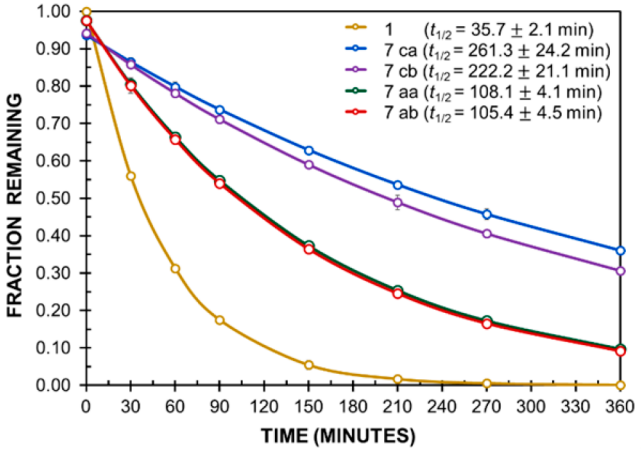


Figure 2. Proteolysis of **1** and analogues by Pronase.

2) Gly with either ΔVal (**7aa**) or ΔEnv (**7ab**) also improved stability, but was less beneficial than (*i* + 1) substitution, resulting in a ca. 3-fold increase in half-life. ΔVal exhibited a slightly stronger stabilizing effect at (*i* + 1) than did ΔEnv, and the impact of both bulky ΔAAs at (*i* + 2) was identical within experimental error.

These encouraging results caused us to wonder if bulky ΔAAs could work synergistically with other proteolytically stabilizing elements without negatively impacting secondary structure. Thus, analogues of **2** pairing the (*i* + 1) D-Pro with either ΔVal (**7ba**) or ΔEnv (**7bb**) at (*i* + 2) were subjected to the Pronase proteolysis assay (Figure 3). These assays were conducted with higher concentrations of the enzymes than those shown in Figure 2 in order to permit proteolysis of these more stable

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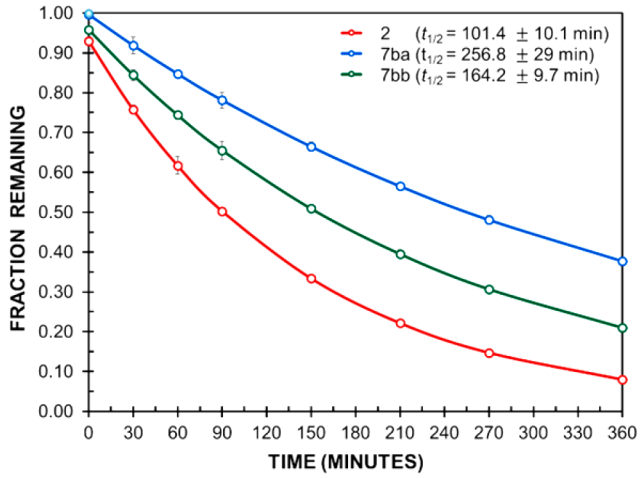

Figure 3. Proteolysis of **2** and analogues by Pronase.

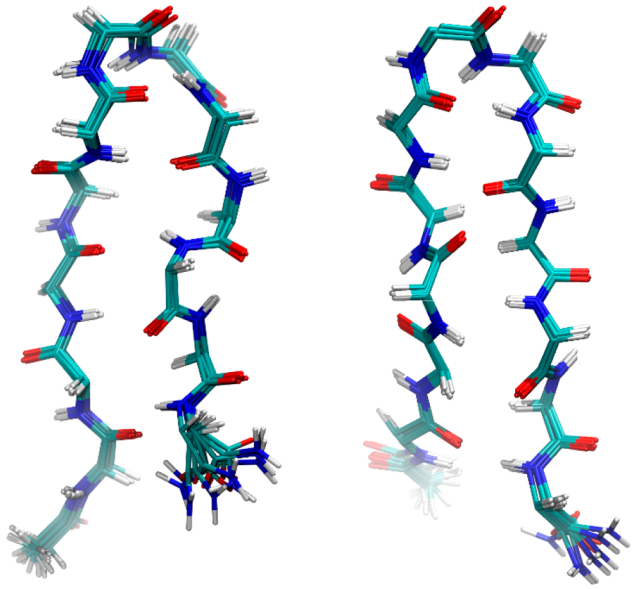

Figure 4. Solution-phase structures of **1** (left) and **7ca** (right) as calculated using NOE distance restraints. Side chains have been removed for clarity.

peptides to occur on a reasonable time scale. While this prevents direct comparison of the two sets of assays, examination of Figure 3 indicates that (1) bulky ΔAAs can impart additional proteolytic stability to peptides that contain a D-amino acid, and (2) in this case the stabilizing impact of ΔVal is more pronounced than that of ΔEnv.

NMR spectral data (1.0−3.5 mM of peptide in 10% v/v D_2O/H_2O buffered to pD 3.9) demonstrated that bulky ΔAA-containing peptides **7** retain the β-hairpin structures of **1** and **2**. For example, their Hα protons were shifted significantly downfield (i.e., ≥0.2 ppm) relative to random coil reference values,[27,28] with the exception of residues in the turn and *C*-terminal regions. The *C*-terminal regions are likely affected by fraying of the strand. Numerous cross-strand NOEs were also observed.[28] The ^{1}H NMR spectra of **1**, **2**, and most of the peptides **7** were characterized by one set of well-defined signals, which is indicative of either a single dominant low-energy conformation or rapid equilibration between multiple conformations. However, extra signals were present in the spectra of ΔEnv-containing peptides **7ab** and **7cb**. Since these compounds are homogeneous according to HPLC analysis, they presumably adopt two or more conformations that interconvert slowly relative to the NMR time scale. Finally, CD spectra of **1**, **2**, **7ca**, and **7ba** all showed the minimum at 215 nm that is characteristic of a β-sheet or a β-hairpin.

NOE-restrained structural calculations using CYANA[29] revealed further insights regarding the solution structures of peptides **1**, **2**, and **7**. Superimposing the 10 lowest-energy conformations of each peptide that fit the NOE data indicated that β-hairpins were formed in all cases.[28] Comparison of the structural ensembles for peptides **1** and **7ca** (Figure 4) illustrates the conformational similarities between these compounds. Qualitative examination of the simulated structures suggested that peptides containing ΔVal are more rigid than those containing ΔEnv. This observation was supported by RMSD calculations[28] and is consistent with the multiple conformations seen in the NMR spectra of **7ab** and **7cb**. Since each ΔVal-containing peptide is either more stable to Pronase exposure than its ΔEnv-containing congener or equivalently stable, it appears that proteolytic stability and rigidity of the peptide backbone are correlated for this series of β-hairpins.

Fraction folded values for **1** and its most proteolytically stable analogue **7ca** were determined using ^{1}H NMR spectroscopy.[21,30,31] Specifically, the Hα chemical shifts of Val3, Val5, Orn8, and Ile10, each of which are engaged in cross-strand hydrogen bonding, were compared to chemical shift values obtained from fully folded (i.e., cyclic) and random coil control peptides.[21] This method showed **1** and **7ca** to be 77% and 90% folded, respectively. Similar fraction folded values of 73% for **1** and 94% for **7ca** were calculated by measuring the amount of chemical shift separation between the diastereotopic Gly7 α-hydrogens of these peptides.[21] Conversion of these values into differences in free energy suggests that insertion of ΔVal at the $(i + 1)$ position of a β-turn can increase the preference for the folded state by ca. 0.6−1.0 kcal/mol. Thus, the substantially improved resistance to proteolysis of **7ca** relative to **1** can at least partially be attributed to the impact of the bulky ΔAAs on the equilibrium between the folded and unfolded states. The observed shift of the equilibrium toward the folded state could be accomplished by either stabilizing the folded state or by destabilizing the random coil conformations. Although further study is required to determine which scenario is operative, we suggest that the bulky ΔAAs are destabilizing many of the random coil conformations of β-hairpins **7** through their inherently high levels of $A_{1,3}$ strain.

In conclusion, we have found that the bulky dehydroamino acids dehydrovaline and dehydroethylnorvaline can be inserted into the turn regions of β-hairpins without perturbing secondary structure. These residues enhance proteolytic stability, with more pronounced effects seen at the $(i + 1)$ versus the $(i + 2)$ position and with ΔVal versus ΔEnv. A bulky ΔAA can also be paired with a D-amino acid (i.e., D-Pro) to synergistically increase resistance to proteolysis. In one case, the greater proteolytic stability of a ΔVal-containing β-hairpin (**7ca**) relative to its parent peptide (**1**) was correlated with a stronger thermodynamic preference for the folded state versus unfolded states. This work adds to the growing number of uses for dehydroamino acids.[32] Studies of the impact of bulky ΔAAs on other secondary structures and on the proteolytic stability and efficacy of bioactive peptides are in progress.

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■ ASSOCIATED CONTENT

Supporting Information

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Experimental procedures, compound characterization data, simulated structures, NMR spectra, and HPLC traces (PDF)

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Notes

The authors declare no competing financial interest.

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(31) The multiple conformations in the NMR spectra of **7ab** and **7cb** precluded calculation of fraction folded values for these peptides. Additionally, the absence of Gly in **7aa**, **7ba**, and **7bb** eliminates one of the two methods of obtaining fraction folded data for these compounds. Accordingly, **7ca** and its parent **1** were the only peptides in this study for which the fraction folded could be reliably determined.

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