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α-Alkylated α-amino acids reduce the aggregation of unfolded peptides

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Aberrant aggregation complicates the development of bioactive peptides. Here, we generate new α-ethylated variants of proteinogenic amino acids and demonstrate that they reduce peptide aggregation through a mechanism distinct from their α-methylated counterparts. Whereas α-methylated residues reduce aggregation by favoring helix formation, α-ethylated residues prevent aggregation without promoting secondary structure.

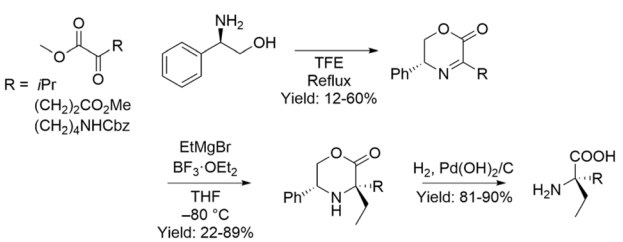
Introduction

Peptides hold great potential in applications across biotechnology and medicine, but they must generally adopt specific conformations in order to function. Failure to adopt the correct conformation not only reduces efficacy in the target activity/application, but in many cases, can lead to the gain of unwanted functions because unfolded or partially folded peptides and proteins are prone to aberrant aggregation into β-sheet-rich assemblies. These aggregates are often highly thermodynamically stable and can nucleate the aggregation of additional monomers, thereby propagating the misfolded state. Such aggregation not only diminishes functional performance but also leads to the formation of insoluble, heterogeneous species that hamper efforts to design, characterize, and formulate bioactive peptides. Moreover, these aggregation processes can be irreversible under physiological or experimental conditions, posing significant challenges for storage, delivery, and reproducibility. As a result, strategies that modulate peptide conformation and reduce aggregation propensity are critical for advancing the practical utility of peptide-based systems.[1,2] Backbone alkylation has emerged as a versatile strategy for modulating peptide secondary structure and aggregation.[3–5] For example, N-methylation disrupts amyloid β-hairpin formation and enables crystallization of oligomeric assemblies.[6–8] Alkylation at the α-carbon offers complementary control. The best-studied example, α-methylation, restricts backbone φ and ψ dihedral angles toward helical regions of the Ramachandran plot, potentially promoting α-helix formation and disfavoring extended β-sheets.[9–14]

Comparatively little is known about the structural consequences of α-ethylation. Diethylglycine (Deg), the most studied α-ethylated residue, adopts C5 geometry,[15–17] favors extended backbone conformations and disrupts both β-sheets (*e.g.*, TrpZip hairpins)[18] and helical structures (*e.g.*, oligo(Aib) host–guest foldamers).[19,20] Only limited structural studies on other α-ethylated amino acids have been reported; for example, hydrophobic oligomers of α-ethylated valine and leucine primarily favor the same extended conformations as Deg oligomers.[21,22] However, α-ethylated amino acids bearing functionalized side chains, such as α-Et-Glu and α-Et-Lys, have not previously been incorporated into peptides, and their effects on secondary structure and aggregation remain unknown. We were especially interested in the contribution of α-alkylation to the aggregation propensity of peptides, as these modifications could both bias backbone dihedral angles and introduce steric bulk that could reduce adoption of the aggregation-prone structure, though these effects could potentially be offset by increased hydrophobicity upon alkylation. We therefore sought to generate α-ethylated amino acids with functional side chains and evaluate their effects on peptide structure and aggregation.

Experimental

Inspired by prior syntheses of α-methylated amino acids,[23–25] we synthesized the functionalized α-ethylated amino acids stereoselectively using a morpholinone-based strategy (Scheme 1).[24,26] Condensation of α-keto ester derivatives with (2*S*)-phenylglycinol afforded the morpholinone scaffold, which positions the α-carbon for alkylation. A Lewis acid-promoted Grignard



Scheme 1 Synthesis of α-ethylated amino acids.

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reaction with EtMgBr installed the ethyl substituent, and subsequent hydrogenolysis liberated the free α-ethylated amino acid. Optical rotation measurements confirmed that stereoselectivity was consistent with precedent (Table S1).

Results and discussions

To determine the effects of α-ethylation on peptide structure and aggregation, we incorporated three different α-ethylated building blocks (α-Et-Val, α-Et-Glu, and α-Et-Lys), or their α-methylated counterparts, into the fold-switching peptide VW19.[27–29] Previous studies have shown that VW19 can undergo secondary structure transitions between random coil, α-helix and β-sheet, depending on changes in buffer, pH, temperature or peptide concentration, as established by circular dichroism (CD) and electron microscopy (EM).[27–29] At low temperatures, WT VW19 forms an α-helical coiled coil, whereas at high temperatures, the structure transitions to β-sheet and aggregates irreversibly. The sequence of VW19 (Fig. 1B) is based on a 7-residue coiled-coil repeat (*i.e.*, *abcdefg*; Fig. 1C), where positions *a* and *d* mediate hydrophobic coiled-coil interactions and positions *e* and *g* provide stabilizing salt bridges. This model peptide therefore provides a convenient opportunity to evaluate the effects of α-alkylation of various amino acids on both α-helix and β-sheet formation, as well as peptide aggregation. In this way, we can simultaneously evaluate the structural propensities of these amino acids in the context of a simple sequence that contains only five different residues in a straightforward pattern, increasing the likelihood that the observations in this system might translate to others.

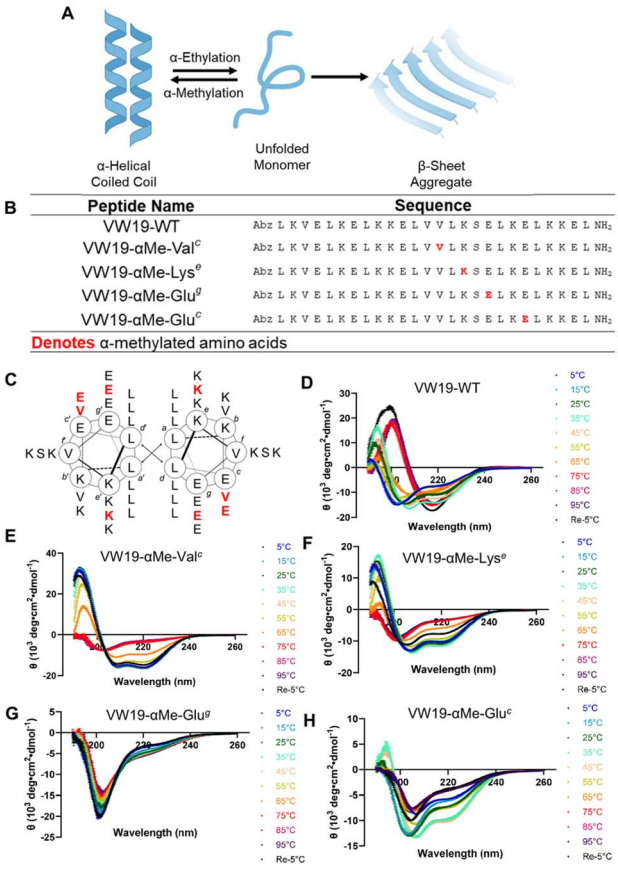

Fig. 1 (A) Schematic illustration showing that α-methylation promotes α-helical structure, whereas α-ethylation disrupts folding and prevents β-sheet aggregation. (B) VW19 peptide sequences. (C) Helical wheel of VW19 indicating substitution positions (red). CD spectra for 200 μM solutions of (D) VW19-WT, (E) VW19-αMe-Val^c, (F) VW19-αMe-Lys^e, (G) VW19-αMe-Glu^g, and (H) VW19-αMe-Glu^c recorded from 5 °C to 95 °C, followed by cooling to 5 °C.

To examine changes in VW19 structure as a function of backbone alkylation, we employed circular dichroism (CD) spectroscopy. CD is a spectroscopic technique that measures the differential absorption of circularly polarized light as a function of wavelength, which provides diagnostic fingerprints of major elements of secondary structure in peptides and proteins.[30] As expected, VW19-WT peptide at a concentration of 200 μM remained predominantly in a random coil state at temperatures below 15 °C (peptide concentration = 200 μM; buffer composition = 10 mM Tris/HCl buffer, pH 7.4). Upon increasing temperature, the peptide underwent a transition to an α-helix, followed by a return toward a random coil like state around 55 °C. With further heating, the secondary structure converted into a β-sheet state (Fig. 1D).[27,31] Upon cooling to 5 °C, the β-sheet structure was retained, establishing VW19 as a system in which loss of helical structure is coupled to irreversible β-sheet formation and aggregation. We then introduced α-Me or α-Et variants of glutamic acid, lysine, and valine at a variety of positions to assess how backbone alkylation affects secondary structure and aggregation.

Consistent with prior reports,[12,32] incorporation of α-methylated residues potentially stabilized α-helical conformations and prevented aggregation. Both VW19-αMe-Val^c and VW19-αMe-Lys^e adopted stable α-helical structures at temperatures up to approximately 75 °C before unfolding to a random coil (Fig. 1E and F). Remarkably, upon cooling to 5 °C, the α-helical conformation was fully restored, which was not observed in the WT peptide. The effects of α-methylated glutamic acid, however, were position-dependent. VW19-αMe-Glu^g, with α-Me-Glu at the salt-bridge position *g*, remained in a random coil across all temperatures (Fig. 1G); we speculate that alkylation distorts the orientation of the side chain away from the conformation necessary for effective salt-bridge formation. In contrast, incorporating α-Me-Glu at the non-interacting position *c* (VW19-αMe-Glu^c) (Fig. 1H) restored helix formation and prevented the transition to β-sheet at high temperatures, similar to VW19-αMe-Val^c and VW19-αMe-Lys^e. This behavior is consistent with prior reports that α-methylation effects depend on residue identity and local structural context.[12,14] Collectively, these results demonstrate that α-methylation potentially biases the conformational landscape of VW19 toward helical states, thereby reducing access to aggregation-prone β-sheet conformations.

Incorporation of α-ethylated amino acids caused dramatically different effects compared to α-methylation. VW19-αEt-Val^c, VW19-αEt-Lys^e, and VW19-αEt-Glu^g each remained predominantly in a random coil state throughout the entire heating and cooling cycle (Fig. 2C, D and E). VW19-αEt-Lys^e

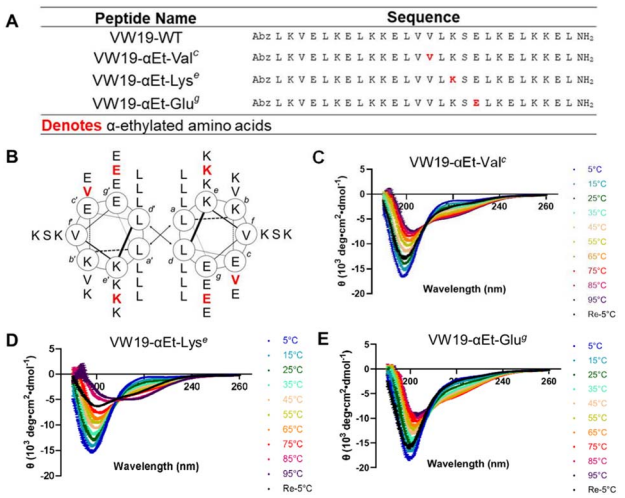

Fig. 2 (A) VW19 peptide sequences. (B) Helical wheel of VW19 indicating substitution positions (red). CD spectra for 200 μM solutions of (C) VW19-αEt-Val^c, (D) VW19-αEt-Lys^e, and (E) VW19-αEt-Glu^g recorded from 5 °C to 95 °C, followed by cooling to 5 °C.

showed weak helix-like spectral features above 85 °C, but no well-defined α-helical structure formed, as evidenced by the absence of characteristic negative bands at 208 and 222 nm (Fig. 2D). This disruption of ordered structure was independent of both the substituted residue and its position within the coiled-coil sequence; for example, Val14 is oriented away from the dimer interface of the coiled coil, so α-ethylation likely does not disrupt VW19 folding through direct disruption of the dimer interface. Instead, we attribute the disruption of secondary structure to the increased steric demands of the ethyl substituent, which can restrict backbone dihedral angles to conformations incompatible with both α-helical and β-sheet geometries, as has been seen previously for diethylglycine, and α-ethylated Val and Ala.[18,20,21] α-Ethylation is therefore sufficient to alter the secondary structure propensity of amino acids with functional side chains like Glu and Lys. Remarkably, the addition of even a single methylene at the α-position of a single residues in this assembly is sufficient to dramatically reshape folding (*i.e.*, from helical to extended/disordered conformations).

Strikingly, incorporation of any α-alkylated amino acid was sufficient to reduce aggregation of this aggregation-prone peptide. To assess the effects of α-alkylation on peptide aggregation, we measured turbidity of all peptide solutions after heating to 95 °C and cooling back to 5 °C (Fig. 3A). Upon aggregation, an increase in turbidity is expected due to the decrease in transmittance caused by suspended particles that scatter light.[33] Only VW19-WT showed detectable aggregation; the solution became visibly turbid after heating and cooling (Fig. 3B, statistically significant, $P < 0.0001$). In contrast, none of the α-methylated or α-ethylated variants exhibited turbidity greater than that of the unheated WT peptide, though we cannot eliminate the possibility that residual aggregates go undetected. The reduction in aggregation was consistent across peptide, regardless the side chain (*i.e.*, Val, Glu, or Lys), α-alkyl

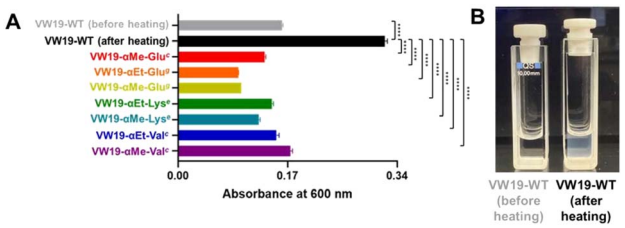

Fig. 3 (A) Turbidity (absorbance at 600 nm) of VW19-WT before heating and all peptide variants after heating and cooling. (B) VW19-WT solution before and after heating, showing visible aggregation. ****: $P < 0.0001$.

substituent (*i.e.*, methyl or ethyl), or position in the protein (*i.e.*, *c*, *e*, or *g* positions in the heptad repeat), despite the increased hydrophobicity imparted by α-alkylation. These results suggest that α-alkylation could be a minimal strategy for promoting solubility of even highly aggregation-prone peptides like VW19. Future studies are needed to evaluate this propensity in other molecular contexts.

The reduction of aggregation by α-methylation can be explained in part by its effects on secondary structure; α-methylation increased the helicity of VW19 (Fig. 2), thereby reducing the propensity to unfold and subsequently transition to β-sheet. However, α-alkylation must impose additional barriers to aggregation because neither VW19-αMe-Glu^g nor any of the α-ethylated variants were helical at any temperature. Though determining the conformation of residues in unfolded peptides remains a general challenge, one that is exacerbated by the lack of an α-proton, we hypothesize based on prior reports[18] that α-alkylation reduces aggregation by disrupting β-sheet formation.

To test this hypothesis, we incorporated α-alkylated amino acids into the tryptophan zipper (TrpZip),[34] a well-characterized β-hairpin peptide[35–38] whose structural features have been thoroughly investigated by CD, IR, and NMR spectroscopy.[34,37] Previous studies have shown that α-ethylated residues with hydrophobic side chains disrupt β-sheet formation,[18] but it was unclear whether α-ethylated amino acids bearing polar and/or charged side chains behave similarly. We therefore

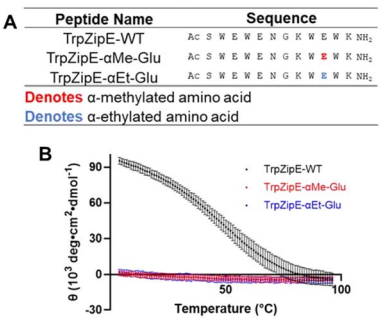

Fig. 4 (A) TrpZipE peptide sequences. (B) Thermal denaturation of TrpZipE peptides monitored by molar ellipticity at 228 nm, demonstrating that α-alkylated glutamic acid substitutions abolish β-sheet folding.

substituted the glutamic acid in TrpZipE with α-Me-Glu or α-Et-Glu. Consistent with our hypothesis both substitutions abolished β-sheet folding, as assessed by thermal denaturation monitored by CD (Fig. 4; peptide concentration = 20 μM; buffer composition = 20 mM potassium phosphate, pH 7.0). These results suggest a propensity of α-alkylated amino acids, including α-ethylated variants, to disrupt β-sheet formation.[13,20] This destabilization likely results from a combination of altered backbone dihedral angles[9–20] and nascent steric clashes between the α-alkyl substituent and the backbone of partner β-strands.[39]

Conclusion

In summary, α-methylation and α-ethylation both reduce aggregation of the aggregation-prone peptide VW19, but through partially divergent mechanisms. Both α-methylated and α-ethylated amino acids prevent β-sheet formation, while α-methylation stabilizes α-helical conformations that resist unfolding, whereas α-ethylation disrupts both α-helix and β-sheet formation, favoring more disordered or extended states. These results demonstrate that even small backbone modifications can significantly reshape the conformational landscape of peptides and influence their aggregation behavior. Taken together, our findings suggest that α-ethylation represents a complementary strategy to α-methylation for preventing peptide aggregation, particularly in systems where maintaining an extended or disordered conformation is desirable. More broadly, α-ethylated residues might serve as useful tools for promoting non-aggregating states in synthetic peptides, which are otherwise prone to deleterious aggregation.[40,41] This approach could be especially applicable to the design of synthetic intrinsically disordered peptides, which are often enriched in charged residues, like those we report herein.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information: synthetic and analytical methods, characterization data (PDF). See DOI: https://doi.org/10.1039/d6ra03257e.

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