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E-Olefin Dipeptide Isostere Incorporation into a Polypeptide Backbone Enables Hydrogen Bond Perturbation: Probing the Requirements for Alzheimer's Amyloidogenesis

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Intermolecular backbone−backbone hydrogen bonding is observed in quaternary structures and is believed to be an important stabilizing force in protein−protein interactions. It is possible to eliminate both a backbone H-bond acceptor (C═O) and a donor (N−H) by replacing the amide functionality in a single dipeptide substructure within a protein with an *E*-alkene moiety (Figure 1). The idea of incorporating *E*-olefin dipeptide isosteres into polypeptides to perturb backbone H-bonding is not new. However, this strategy has been rarely realized in large part because of the difficulties involved in the chemical synthesis of *E*-alkene dipeptide isosteres and the challenge of incorporating these dipeptide isosteres into proteins.[1] Protein backbone H-bond perturbation has been realized using amide-to-ester backbone mutations.[2] Like ester perturbations, amide-to-*E*-alkene substitutions are ideal in that the conformational and geometrical preferences of the backbone are maintained (Figure 1). Moreover, unlike the situation in esters, where the non-carbonyl oxygen experiences an unfavorable electrostatic interaction with the amide carbonyl the NH once H-bonded to, *E*-olefin perturbations do not introduce unfavorable electrostatic interactions, making it easier to extract H-bond energies from $\Delta\Delta G$ measurements.

Herein, we describe a stereoselective *E*-olefin dipeptide isostere synthesis (Scheme 1) that can be used to make gram quantities of the amino acid required for eliminating a specific H-bond donor and acceptor in a protein while maintaining side-chain structures. The Phe−Phe *E*-olefin dipeptide isostere (**5**) was incorporated into the 40 residue Alzheimer's amyloid peptide, referred to as Aβ(1−40), in place of phenylalanines 19 and 20 utilizing a Boc/benzyl solid-phase synthesis strategy. The aim is to examine the role of intermolecular H-bonding in the process of amyloidogenesis thought to cause Alzheimer's disease. This amide-to-*E*-alkene backbone mutation precludes amyloid formation, but not spherical aggregate formation, providing insight into the structural requirements of amyloidogenesis.

Previously reported synthetic approaches to *E*-olefin isosteres often relied on S_N2' substitutions or rearrangements of allylic substrates[3] or generation of the double bond via 1,2 elimination.[4]

In the *E*-olefin dipeptide isostere synthesis described herein, the amino terminal C_α stereocenter comes from an amino aldehyde (derived from an amino acid), whereas the *C*-terminal stereocenter is produced via the diastereoselective (11:1) Evans alkylation of an oxazolidinone enolate (Scheme 1). The synthesis commenced with a Wittig reaction, followed by a hydroboration−oxidation, affording the *N*-Boc-Phe-Gly dipeptide *E*-olefin isostere using established procedures.[5] The acid within **2** was then condensed with Evans' chiral oxazolidinone[6] utilizing a mixed anhydride approach. Diastereoselective alkylation of the *C*-terminal C_α carbon in **3** is the key step in this approach. To avoid formation of N-alkylated or C_γ-alkylated side products and decomposition of the enolate, the reaction was conducted under kinetically controlled conditions, where **3** was added to 2 equiv of freshly prepared LDA at −78 °C. Addition of excess benzyl bromide afforded the desired product with a high diastereoselectivity ratio (11:1) and in good chemical yield (70%). Precursor **4** was treated with basic hydrogen peroxide to remove the oxazolidinone chiral auxiliary affording **5**, ready for incorporation into a polypeptide chain by solid-phase peptide synthesis. The methyl ester **6**, afforded by $TMSCHN_2$ treatment, was employed to confirm the structure of the Phe−Phe dipeptide *E*-olefin isostere by X-ray crystallography (Figure 1).

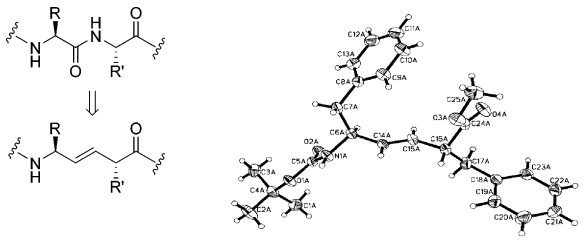
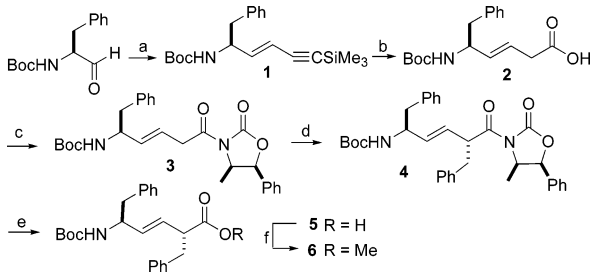


Figure 1. The backbone amide-to-*E*-olefin mutation eliminates both a H-bond acceptor (C═O) and a donor (N−H) (left). The X-ray structure of the Phe−Phe *E*-olefin dipeptide isostere synthesized herein (right).

Scheme 1. Synthesis of *E*-Olefin Isostere[a]



[a] Conditions: (a) $Ph_3PCH_2C{\equiv}CSiMe_3Br$, *n*-BuLi, *E*/*Z* 7:1, 99% ee, 70%. (b) (i) cyclohexene, BH_3; (ii) H_2O_2, 82% for two steps. (c) (i) *t*-BuCOCl, Et_3N; (ii) oxazolidinone, *n*-BuLi, 85% for two steps. (d) LDA, $PhCH_2Br$, 70%, dr 11:1. (e) LiOH, H_2O_2, 88%. (f) $TMS{-}CHN_2$, 92%.

The misassembly of Aβ, ultimately affording insoluble amyloid fibrils, is genetically linked to the pathogenesis of Alzheimer's disease.[7] Aβ(1−40) contains two hydrophobic subsequences which are underlined in one letter code below: DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV. The central hydrophobic core LVFFA (17−21) is essential for amyloidogenesis.[8] Since it has been argued that it is primarily the backbone that enables so many peptides to form cross-β-sheet-based amyloid fibrils,[9] it follows that intermolecular H-bonding should be essential. An amide-to-*E*-olefin substitution was therefore introduced into the key Phe19−Phe20 subsequence, removing both a hydrogen-bond donor and acceptor thought to make H-bonds with different neighboring Aβ molecules.[10]

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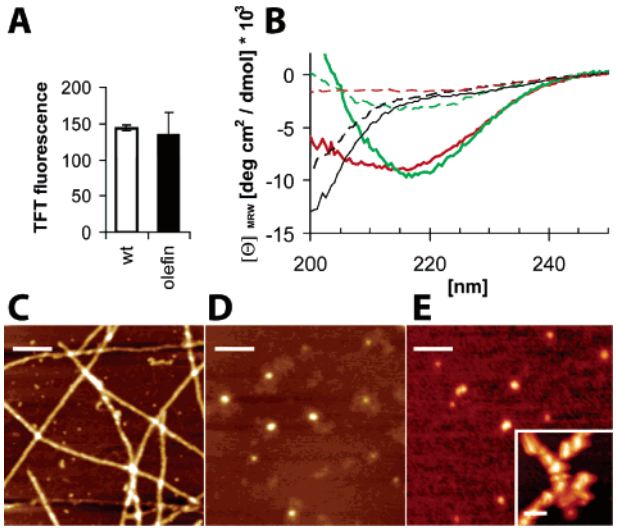

Figure 2. (A) TfT fluorescence of wt Aβ and the EOAβ analogue after agitated incubation at 37 °C for 25 h. (B) Mean residue ellipticity of Aβ(1−40) (solid lines) and EOAβ (dotted lines) before aggregation (black), after 25 h (red), and 6 weeks (green). AFM images (scale bars: 200 nm) of aggregated Aβ (C) and the EOAβ after 25 h (D) and 6 weeks (E).

The Phe19−Phe20 *E*-olefin dipeptide isostere was incorporated into Aβ by manual solid-phase peptide synthesis using HATU activation. The reverse phase HPLC purified Phe19−Phe20 *E*-olefin analogue of Aβ(1−40), hereafter referred to as EOAβ, was subjected to high pH treatment and membrane filtration to monomerize the sample.[11] TfT is an environmentally sensitive fluor that binds and fluoresces when bound to spherical and cross-β-sheet aggregates of Aβ.[11] Incubation of EOAβ (50 μM) with agitation on a rocker (30/min) in the buffer (150 mM NaCl, 50 mM phosphate, pH 7.4, 37 °C) that typically leads to the aggregation of Aβ(1−40) resulted in a strong thioflavin T signal after 25 h, comparable to that exhibited by wt Aβ (Figure 2A), indicating that EOAβ was able to self-assemble. TfT fluorescence remained essentially constant for both peptides over the course of 6 weeks.

To examine whether aggregated EOAβ adopts a β-sheet rich structure analogous to that exhibited by wt Aβ, far-UV circular dichroism (CD) was employed. The CD spectra of unaggregated Aβ peptides display strong minima around 200 nm, whereas aggregated wt Aβ exhibits a strong minimum at 217 nm, characteristic of cross-β-sheet structure. The amplitude at 217 nm was very weak if present at all in EOAβ (25 h), but increased very slowly over 6 weeks, long after the TfT signal had plateaued (Figure 2B). Absorbance at 280 nm revealed that the EOAβ concentrations remained very similar to those of wt Aβ.

Since the differences in secondary structure suggested differences in aggregate morphology, atomic force microscopy (AFM) was employed to examine the wt Aβ and EOAβ assembled structures. Fibrillar aggregates were observed with wt Aβ (Figure 2C), but not with EOAβ, which aggregated predominantly, if not exclusively, into spherical aggregates (Figure 2D) that partially assembled into larger amorphous aggregates after 6 weeks (Figure 2E, inset). In each case, ∼50% of the sample could be pelleted at 100 000*g* after 4 days of aggregation. No fibril formation was observed with EOAβ even after 6 weeks of incubation. Electron micrographs of these samples revealed analogous results, suggesting that it is unlikely that these results are biased by the mica- or carbon-coated Cu surfaces used for AFM and EM, respectively. AFM measurements determined the height of the spherical aggregates to be ∼5 nm, EM measurements reveal a ∼10 nm diameter, similar to the dimensions seen in diffusible oligomer aggregation intermediates ("ADDLs")[12] and in cholesterol metabolite induced spherical aggregates.[11] There is substantial evidence that Aβ aggregation proceeds first by forming spherical aggregates which transform into protofilaments and then into fibrils having a cross-β-sheet structure.[7b] It is striking that removal of 1 of the 39 amide bonds in Aβ, and thus one H-bond donor and one acceptor, could prevent the typical progression of spherical aggregates into protofilaments and fibrils. The results herein indicate that the 19−20 amide bond is critical for protofilament and fibril formation, but not for spherical aggregate formation. Ongoing studies to discern whether replacement of other amides will influence amyloidogenesis will be reported in due course. The discovery of an Aβ analogue that can form spherical aggregate morphologies but cannot progress to protofilaments or fibrils is very valuable for delineating the structure, toxicity, and antigenicity of spherical Aβ aggregates.

In summary, we report a synthesis of *E*-olefin isosteres that can be prepared on a scale that enables their incorporation into polypeptide sequences to examine the role of individual hydrogen bonds in protein structure acquisition.

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Supporting Information Available: Experimental details and characterization data for the synthesis of **6**; crystal structure of **6**; procedures for peptide assembly. This material is available free of charge via the Internet at http://pubs.acs.org.

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