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Synthesis and biological properties of β-turned $A\beta_{31-35}$ constrained analogues

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Abstract—A series of constrained pentapeptide analogues of the fragment $A\beta_{31-35}$ has been prepared using solid phase synthesis protocols. The results of conformational studies and surface plasmon resonance (SPR) experiments seem to indicate that the affinity of these constrained analogues for immobilized $A\beta_{25-35}$ peptide could be related to their ability to adopt a Leu34N-Ile31O β-turn-like folded conformation.

β-Amyloid peptide (Aβ) plays an important role in the neuropathology of Alzheimer disease (AD). Fibrillogenesis, the process of aggregation of Aβ into soluble fibers, and amyloidosis, the formation of insoluble amyloid plaques appear to be responsible for the triggering of the pathological process. Inhibition or reversion of formation of amyloid fibrils is thus an important target in AD therapy. Therefore, the use of compounds that specifically interfere in the Aβ–Aβ interaction and its aggregation, could prevent amyloid plaque formation, and decrease the toxicity associated to β-amyloid peptide and its aggregates.[1–3] In this context, peptide fibrillogenesis inhibitors have been described, not only for β-amyloid assembly, but also for other aggregating proteins and peptides.[4]

It has been reported that Aβ fragments such as, $A\beta_{16-22}$, $A\beta_{17-21}$, and $A\beta_{15-25}$, as well as some analogues modified at the N-terminus, bind to the amyloid peptide preventing the formation of $A\beta_{1-40}$ amyloid fibers, and in many cases inhibiting the cellular toxicity of the native peptide.[3]

Recently, special attention has been paid to fragment 31–35 of Aβ (Ile-Ile-Gly-Leu-Met, IIGLM) since this pentapeptide is able to induce cellular effects similar to those observed for $A\beta_{1-42}$, $A\beta_{1-40}$, and $A\beta_{25-35}$, suggesting a key role of residues 31–35 as a possible active site of the β-amyloid molecule.[5–8] Fragment $A\beta_{31-35}$ induces neurodegeneration in cultured cortical neurons promoting apoptosis and the corresponding changes in the expression of some related genes.[6,7] Moreover, and in a similar way to $A\beta_{1-40}$, this small peptide is able to incorporate spontaneously into lipid bilayers, generating channels permeable to different ions.[8]

X-ray diffraction shows that $A\beta_{31-35}$ in its associated form adopts a reverse turn structure with two intramolecular H-bonds at Leu34N-Ile32O and Met35N-Ile32O (Fig. 1). The peptide aggregates through the formation of intermolecular hydrogen bonds between Gly33N-Leu34O and Ile31N-Met35O (Fig. 1).[9] Additionally, hydrophobic residues Ile31, Ile32, Leu34, and Met35 have been suggested to be critical for oligomerization and toxicity.[10,11]

Keywords: β-Amyloid; Peptide derivatives; Turn inducers; Surface plasmon resonance.
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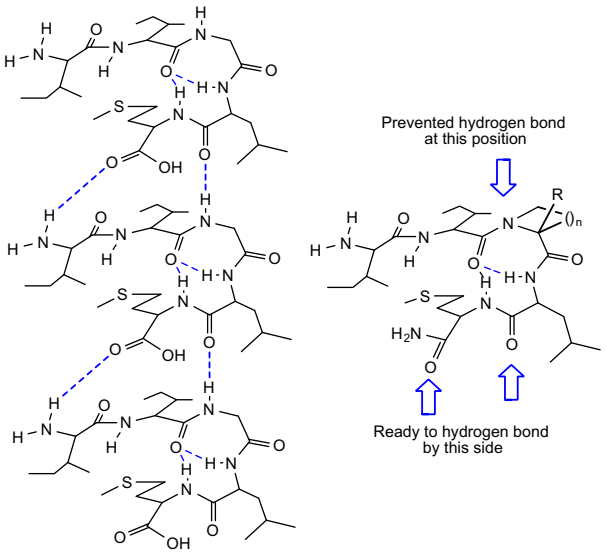


Figure 1. Hydrogen bond assembly of $A\beta_{31-35}$ and keys for the design of new analogues.

The *N*-propanoyl derivative Pr-IIGL-NH_2 was the first analogue of $A\beta_{31-35}$ able to antagonize $A\beta_{1-42}$ mediated toxicity in glial cells.[12,13] However, in some in vitro experiments, Pr-IIGL-NH_2 proved to be neurotoxic, probably due to its ability to form fibrillar aggregates. A close pentapeptide analogue that did not form fibrils, RIIGL-NH_2, is an effective non-toxic inhibitor of both the aggregation and toxic effects of fibrillar Aβ.[14] On the other hand, it has been described that a series of turn-forming tripeptide derivatives self-assemble to form supramolecular β-sheet structures and amyloid-like fibrils, through intramolecular hydrogen bonds formed between the peptide linkages, and other non-covalent interactions.[15]

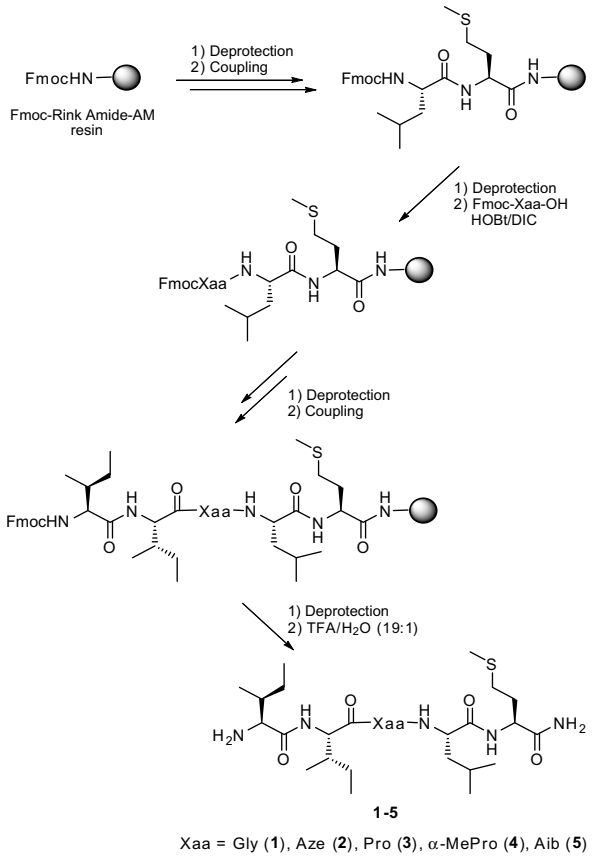
On the basis of these data, we have designed analogues of the fragment $A\beta_{31-35}$ with two ideas in mind. First, we wanted to know if the turn structure is related or not to the ability of these short peptide sequences to bind to Aβ fragments. For this purpose, replacing Gly33, the $i+1$ residue of the β-turn, by amino acids showing higher tendency to induce reverse turns could be instructive. Second, to interfere with the β-amyloid aggregation we have synthesized a series of compounds able to bind to Aβ peptides through only one face,[9] while they should be unable to interact by the other face by blocking the NH of Gly33 residue (Fig. 1). A similar approach, incorporating *N*-methyl amino acids, has been reported.[16,17]

With these aims, five pentapeptides have been prepared. Compound **1** corresponds to the amidated natural $A\beta_{31-35}$ sequence. Derivatives **2**, **3**, and **4** are analogues of **1** in which the Gly33 residue has been replaced by azetidine-2-carboxylate (Aze), Pro, and α-MePro, as constrained turn-promoting residues,[18,19] with no possibility of H-bond intermolecular interaction. Finally, an α-amino-isobutyric acid residue (Aib) has also been included to replace Gly33 (compound **5**). In this particular case, all functional groups involved in intra- and intermolecular H-bond formation are present in the molecule while, at the same time, the folded conformations are also favored.[15]

Compounds **1**–**5** were prepared as C-terminal carboxamides by solid phase methods. Parallel manual syntheses of $A\beta_{31-35}$-NH_2 (**1**) and its analogues were performed on a Rink amide AM polystyrene resin using a Fmoc/^{t}Bu strategy (Scheme 1). For analogues **2**–**5**, the Fmoc derivatives of Aze, Pro, αMePro, and Aib were used instead of Gly, respectively. Following chain assembly, peptides **1**–**5** were obtained by cleavage from the resin with TFA/H_2O (19:1, v/v). All analogues were isolated in good yields (>70%) and high purity (>80%).

Concerning the conformational preferences of the prepared derivatives, ^{1}H NMR variable temperature experiments were carried out in order to find information about the presence of intramolecular hydrogen bonds.[20,21] Spectra were recorded at 5 °C intervals within the 35–60 °C range in DMSO-d_6 and H_2O/D_2O (9:1). Temperature coefficients ($\Delta\delta/\Delta T$) were determined from the slopes of the linear regression lines obtained from chemical shift versus temperature plots.

It is assumed that, with DMSO as solvent, absolute values of $\Delta\delta/\Delta T > 4$ ppb/K are indicative of solvent accessibility of amide NH protons, and values lower than



Scheme 1.

3 ppb/K support the existence of a solvent-shielded NH.[20,21] In H_2O the $\Delta\delta/\Delta T$ values accepted for solvent accessibility and shielding are >5 and <4 ppb/K, respectively.[22] In small peptides, these data provide information about NH involvement in intramolecular hydrogen bonds. For compounds **1**–**3** in DMSO, temperature coefficients are consistent with all NH protons being solvent-accessible (Table 1). In contrast, $\Delta\delta/\Delta T$ values for the NH of the Met residue of compounds **4** and **5** the values found for the NH of the Met residue are 1.9 and 2.5 ppb/K (in absolute value), respectively, indicating that, in these derivatives, the Met NH is H-bonded. This suggests a certain degree of folding for these compounds, as expected from the presence of αMePro and Aib residues, and specifically would support a turn conformation stabilized by a Met35N-Ile32O intramolecular H-bond, similar to that reported for aggregated $A\beta_{31-35}$.[9] The other intramolecular H-bond observed in the solid associated state of $A\beta_{31-35}$, namely Leu34N-Ile32O, was not clearly observed for any derivative in the organic solvent.

A completely different conformational behavior was observed in aqueous solution, with all compounds exhibiting higher tendency to adopt folded conformations than in DMSO. Model compound, $A\beta_{31-35}$-NH_2 (**1**), showed three H-bonded amide protons, Gly, Leu, and Met NHs. The solvent shielding of Leu and Met amide protons could be explained through the double γ,β-turned conformation **A** (Fig. 2), equivalent to the spatial arrangement observed in solid state. The coexistence of an alternative conformation characterized by a γ′-turn or more plausibly a displaced double γ′-β′-turn (conformation **B**, Fig. 2) could explain the involvement of Gly NH in an intramolecular H-bond. Unfortunately, NOESY and ROESY experiments were not conclusive to clarify this point.[23]

Conformation **A** seems also to be adopted in aqueous solutions of compounds **2** and **3**, although the presence of the β′-turn (Leu34N-Ile31O) in conformation **B** cannot be discarded. In contrast, the incorporation of αMePro and Aib residues in **4** and **5** led to a preference for **B**-like conformations,[24] with Leu or Leu and Aib amide protons protected from the solvent. At the same time, the Met NH in these compounds showed an H/D interchange typical of a quite exposed amide proton, and high temperature absolute coefficient values (>5 ppb/K).

Surface plasmon resonance (SPR) experiments were carried out to evaluate the affinity of the new $A\beta_{31-35}$ pentapeptide derivatives for an immobilized Aβ peptide. It has been described that ligands exhibiting high affinity for β-amyloid peptides in SPR are effective at altering aggregation and at inhibiting cell toxicity.[25] The $A\beta_{25-35}$ fragment was selected in our case as the immobilized Aβ peptide, and attachment to the chip was performed following the procedure developed by Cairo.[25,26] Given the high affinity of Congo red for $A\beta_{10-35}$,[25] this dye was used as a control of a correct peptide immobilization and of the reliability of the ensuing results. A $K_D = 13\ \mu M$ was determined for Congo red against immobilized $A\beta_{25-35}$ fragment,[26,27] in good agreement with that reported for the longer sequence (38 μM).[25]

SPR experiments were performed at 10 and 30 μl/min and referenced to an Ahx-Cys peptide surface. Data treatment for kinetic parameter determination included subtraction of a blank injection (buffer) from all curves. Affinities of the constrained $A\beta_{31-35}$ analogues for the $A\beta_{25-35}$ fragment, measured in a Biacore 3000 instrument, turned out to be much lower than that of Congo red in all cases. To establish the significance of these low affinity values, pentapeptide MIGLI-NH_2, a scrambled version of **1**, was synthesized and tested as an additional control. The pentapeptide showed a K_D value of 34.8 mM, with steady-state fitting suggesting basically non-specific interaction with $A\beta_{25-35}$ (see Supplementary data). Slightly better (10- to 20-fold) affinities were found for compounds **4** and **5** in this series (Table 1 and Fig. 3). According to the ^1H NMR data, the latter compounds were the ones exhibiting a differential conformational behavior in H_2O/D_2O with respect to model compound **1**. Since the SPR experiments were also

Table 1. Synthesized compounds (Ile-Ile-Xaa-Leu-Met-NH_2), temperature coefficients: $\Delta\delta/\Delta T$ (ppb/K), and dissociation constants (SPR)

Compound	Xaa	Amide NH $\Delta\delta/\Delta T$ (ppb/K)										K_D (μM)
		DMSO					H_2O/D_2O, 9:1					
		Ile2	Xaa	Leu	Met	$CONH_2$	Ile2	Xaa	Leu	Met	$CONH_2$	
1 ($A\beta_{31-35}$)	Gly	−3.9	−5.1	−4.2	−5.3	−4.2 −4.4	—[a]	**−2.7**	**−2.7**	**−2.7**	−5.6 −5.5	ND[b]
2	Aze	−4.1	—	−3.9	−4.8	−4.2 −4.2	—[a]	—	**−1.9**	**−2.4**	−5.2 −5.4	4600
3	Pro	−4.4	—	−4.6	−4.2	−4.4 −4.8	−4.7	—	**−1.5**	**−2.4**	−5.4 −5.7	4490
4	αMePro	−5.0	—	−7.2	**−1.9**	−6.3 **−1.2**	—[a]	—	**−2.2**	−5.5	−6.3 −4.8	1530
5	Aib	−3.5	−6.2	−3.7	**−2.5**	−4.8 −3.1	—[a]	**−2.8**	**−3.0**	−5.2	−5.8 −4.4	2560
Congo red	—											13

Amide protons inaccessible to solvent are indicated in bold.
[a] Not observed due to rapid H/D interchange.
[b] Not determined due to insolubility.

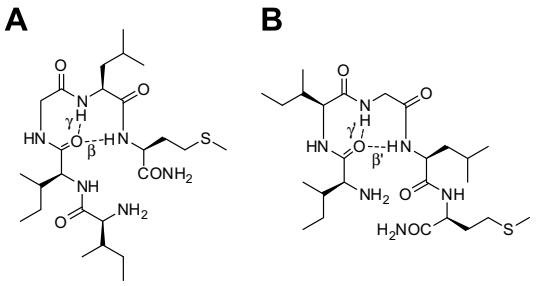


Figure 2. Proposed alternative conformations for compound **1** in H_2O/D_2O.

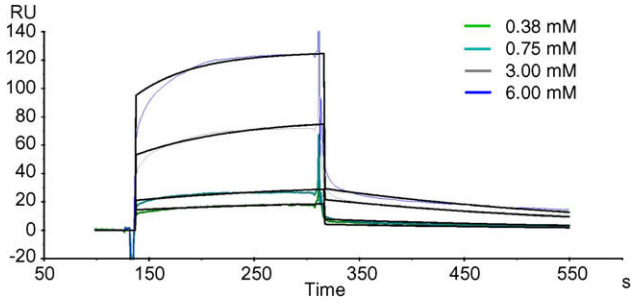


Figure 3. Representative set of binding data for compound **4** in SPR experiments. A similar graphic was obtained for compound **5**.

carried out in aqueous solution, it is fair to assume that the particular folding (**B** conformation, Fig. 2) favored by constrained analogues **4** and **5** might be related to their ability to bind the $A\beta_{25-35}$ peptide, while conformation **A** would be responsible for the amyloid aggregation process observed in solid state.

Despite the low affinity of $A\beta_{31-35}$ pentapeptides for $A\beta_{25-35}$, their activity in neuronal cell cultures was also evaluated.[28,29] First, the effect of compounds **1**–**5** in cell viability was investigated to assess their possible toxicity, as found for the natural fragment $A\beta_{31-35}$.[6,7] Neuronal viability was measured at both 10 and 100 μM, and only the C-terminal amide of $A\beta_{31-35}$, IIGLM-NH_2 (**1**), was found to be slightly neurotoxic. At both concentrations, **1** induced a 10% mortality, lower than that observed for the C-terminal carboxylate derivative IIGLM-OH (21% mortality in the same assay). The protective effect of compounds **1**–**5** against neurotoxicity induced by soluble oligomeric forms of Aβ-peptide was then measured.[29] In this assay, only compound **5**, at a 100 μM concentration, was somewhat effective at preventing the cellular toxicity of Aβ (~24% protection). Unfortunately, compound **4**, showing a similar conformational behavior than **5**, could not be assayed due to solubility problems.

In short, the constrained $A\beta_{31-35}$ analogues showed weak affinity for immobilized Aβ peptides and low protective effects against Aβ induced toxicity. However, the possible relationship between some particular conformations and affinity, inferred from our study, opens the way toward new constrained analogues. The preparation of derivatives with frozen **A** or **B** conformations through covalently linked peptides is now being considered.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.01.092. General procedures for the solid phase synthesis of compounds **1**–**5**, characterization of peptide derivatives **1**–**5** (HPLC, ^{1}H NMR, ESI MS), SPR data, and molecular modeling results.

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23. Although the existence of aggregated species, like in the solid state, could explain the solvent shielding of the Gly NH, this was discarded since no signs of aggregation were observed by ^{1}H NMR in a twofold concentrated solution than the used for temperature coefficient measurements.
24. A preliminary molecular dynamic study carried out with compound **4** supports its high tendency to adopt the Leu34N-Ile31O β′-turn of conformation **B** within the low energy conformers (see Supporting information).
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26. Immobilization (for details, see Ref. 22) was performed through the thiol group of a Cys residue, linked to C-terminal part of $A\beta_{25-35}$ through a 6-aminohexanoic acid (Ahx) spacer. The sequence GSNKGAIIGLM-Ahx-C was made by Fmoc solid phase synthesis, purified by RP-HPLC, and characterized by MALDI-TOF MS. Upon attachment to the chip, unusually high immobilization levels (RU) were initially observed, most likely due to aggregation between immobilized and free peptide. Consistent responses were obtained when the channel was repeatedly washed with regeneration buffer before use.
27. Congo red (1.5, 3, 6.25, 12.5, 25, 50, and 100 μM) and peptide (0.38, 0.75, 1.5, 3, 6, and 12 mM) samples were prepared in HBS-EP buffer. Compounds **4** and **5** were only soluble up to 6 mM, while IIGLM-NH_2 (**1**) formed a suspension that could not be dissolved.
28. Hippocampal neuronal cultures were prepared from 18-day-old Wistar rat embryos and maintained in Neurobasal medium supplemented with B27 for 11 days and then used for these studies. Cell viability was determined by measuring the mitochondrial dehydrogenase activity, that cleaves the tetrazolium salt MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2*H*-tetrazolium bromide) to the formazan product, in a colorimetric assay. The effect of compounds on cell viability was assayed after incubation of neurons at 10 and 100 μM for 24 h.
29. For Aβ soluble oligomers (amyloid-derived diffusible ligands, ADDLs) preparation, $A\beta_{1-42}$ peptide was initially dissolved to 1 mM in hexafluoro-2-propanol (HFIP) and separated into aliquots. After removing the solvent under vacuum, the peptide film was stored desiccated at −80 °C until its use. For the oligomeric aggregation, the peptide was first resuspended in dry DMSO to make a 5-mM solution. The DMSO stock was immediately diluted into cold Ham's F-12 (phenol red-free) medium to make a 100 μM Aβ solution. After incubation for 24 h at 4 °C, Aβ solution was centrifuged at 15,000*g* for 10 min at 4 °C and the supernatant, containing soluble oligomeric Aβ, was collected. Each ADDL preparation was evaluated for the presence of soluble oligomers by Western blotting using 6E10 monoclonal antibody. The cytotoxicity of Aβ oligomers was determined under different experimental conditions and the appropriate concentration and time of exposure of cultures were selected in order to induce a 50% of mortality. For evaluating the potential neuroprotective effect of $A\beta_{31-35}$ analogues, cultures were pretreated with the compounds (10 and 100 μM) for 30 min and then synthetic ADDLs were added at a final concentration of 10 μM. After 24 h, cell viability was determined as described above.