Structure-activity Relationships of β-Hairpin Mimics as Modulators of Amyloid β-Peptide Aggregation

Nicolo Tonali,[a] Julia Kaffy,*[a] Jean-Louis Soulier,[a] Maria Luisa Gelmi,[b] Emanuela Erba,[b] Myriam Taverna,[c] Carine van Heijenoort,[d] Tap Ha-Duong,[a] and Sandrine Ongeri *[a]

[a] BioCIS, Univ. Paris-Sud, CNRS, Université Paris Saclay, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry Cedex, France.

[b] DISFARM-Sez. Chimica Generale e Organica "A. Marchesini", Università degli Studi di Milano, via Venezian 21, 20133 Milano, Italy

[c] Protéines et Nanotechnologies en Sciences Séparatives, Institut Galien Paris-Sud, Univ. Paris-Sud, CNRS, Université Paris Saclay, 5 rue Jean-Baptiste Clément, 92296 Châtenay-Malabry Cedex, France

[d] Equipe Biologie et Chimie Structurales, Dept Chimie et Biologie Structurales et Analytiques, CNRS, ICSN, Université Paris Saclay, 1 avenue de la terrasse, 91190 Gif sur Yvette FRANCE

Corresponding authors : E-mail: sandrine.ongeri@u-psud.fr; Tel : 0033 146835737.

E-mail: julia.kaffy@u-psud.fr; Tel : 0033 146835743.

ABSTRACT:

Aggregation of amyloid proteins is currently involved in more than 20 serious human diseases that are actually untreated, such as Alzheimer's disease (AD). Despite many efforts made to target the amyloid cascade in AD, finding an aggregation inhibiting compound and especially modulating early oligomerization remains a relevant and challenging strategy. We report herein the first examples of small and non-peptide mimics of acyclic beta-hairpins, showing an ability to delay the fibrillization of amyloid-β ($A\beta_{1-42}$) peptide and deeply modify its early oligomerization process. Modifications providing better druggability properties such as increased hydrophilicity and reduced peptidic character were performed. We also demonstrate that an appropriate balance between flexibility and stability of the β-hairpin must be reached to adapt to the different shape of the various aggregated forms of the amyloid peptide. This strategy can be investigated to target other challenging amyloid proteins.

KEYWORDS *amyloid, Alzheimer's disease, peptidomimetics, β-Hairpin, oligomerization, fibrillization.*

Introduction

Currently more than 30 human proteins are implicated in a range of degenerative disorders owing to their misfolding and misassembly into various aggregate structures and leading to at least 20 serious human diseases named amyloidosis.[1] The self-assembled insoluble aggregates are characterized by highly ordered cross-β structures.[1] In particular, Alzheimer's disease (AD) is associated with the aggregation of the amyloid-β ($A\beta_{1-42}$) peptide into senile plaques in the brain.[2] Approximately 47 million people worldwide are living with AD and this number is expected to reach 75 million by 2030, and 135.5 million by 2050.[3] Yet, despite significant means implemented, no causal treatment exists nor for AD nor for the other amyloid diseases. Many efforts have been made to design drugs targeting the amyloid cascade in AD but they have failed in clinical trials.[4] An ongoing promising strategy to find a therapy for AD involves the search for compounds that can inhibit $A\beta_{1-42}$ aggregation, to be used very early before the symptoms appear and before brain pathology (amyloid-plaques and tau tangles).[4c]

However, designing small molecular inhibitors that disrupt protein−protein interactions mediating the aggregation is still a challenge.[1] Indeed, protein−protein interactions occur over large surface areas that are difficult to characterize. Furthermore, many different forms of aggregates are produced while their structures and toxicity are far to be elucidated.[5] In particular, while the monomeric form of $A\beta_{1-42}$ is currently declared as non toxic, fibrils are able to generate damaging redox activity and promote the nucleation of toxic oligomers.[6] Recent studies demonstrate that soluble transient oligomers preceding fibril formation are highly toxic species constitute a major target to prevent the death of neurons.[7]
A large number of small molecules (e.g., tramiprosate, inositol, curcumin, epigallocatechin-3-gallate and resveratrol) have been reported as $A\beta_{1-42}$ inhibitors and have been studied in clinical trials.[5,8] However, the majority of these agents bind to $A\beta_{1-42}$ with low affinity, without selectivity and have no or unknown effect on the most toxic oligomeric species. Recently various monoclonal antibodies (such as Solanezumab) have been produced to inhibit $A\beta_{1-42}$ aggregation by targeting soluble species of the peptide. However they failed to show clinical efficacy in phase II or III clinical trials probably mainly because of their failure to cross the blood-brain barrier (BBB) due to their large size.[4a]
Peptide-based drug discovery could be a serious option for addressing new therapeutic challenging candidates. The number of approved peptides is dramatically increasing, in particular because they often offer greater efficacy, selectivity, specificity and a reduced risk of unforeseen side-reactions compared to small organic molecules.[9] Modified peptides and peptidomimetics can also be designed to decrease the susceptibility to proteases and to cross the BBB. A variety of small peptides or modified peptides that inhibit aggregation of Aβ and reduce its toxic effects have been already described.[10,4a] However, the use of pre-structured peptides, in particular small acyclic β-hairpins has been very rarely explored as β-sheet binders and inhibitors of aggregation.[11] We firmly believe that the greater flexibility of such compounds with respect to the cyclic peptides, allows them to adapt to the different shape of the various aggregated forms of the amyloid peptide. As a result, they can better bind to the sequences involved in the β-sheet structures of the amyloid peptide.

We have recently begun to demonstrate this concept by reporting a novel class of β-hairpin peptidomimetics, built on a piperidine-pyrrolidine semi-rigid β-turn inducer [12] and bearing two recognition pentapeptide sequences, designed on oligomeric and fibril structures of Aβ1-42 (compound 1, Figure 1).[13] This study was the first example of acyclic small β-hairpin mimics possessing such a highly efficient anti-aggregation activity.

We propose now to design more druggable compounds, particularly with a better hydrophobicity/ hydrophilicity balance and reduction of the peptidic character. To reach this target, starting from compound **1**, we planned three main modifications (compounds **3**-**7**, Figure 1): i) the substitution of the C-terminus peptide arm with a peptidomimetic, ii) the introduction of a di- or tripeptide peptide sequence at the *N*-terminus. In particular, according to our previous results with sugar based peptidomimetics (compound **2**, Figure 1),[14] the 5-amino-2-methoxybenzhydrazide (Amb) peptidomimetic strand linked to a Lys residue was selected for the *C*-terminal arm of the compounds and Val-Ala or Ala-Val-Ala was introduced at the *N*-terminus. Finally, the introduction of different substituents on the amino group at C-4 of the piperidine ring was also planned for assessing their effect on the activity.

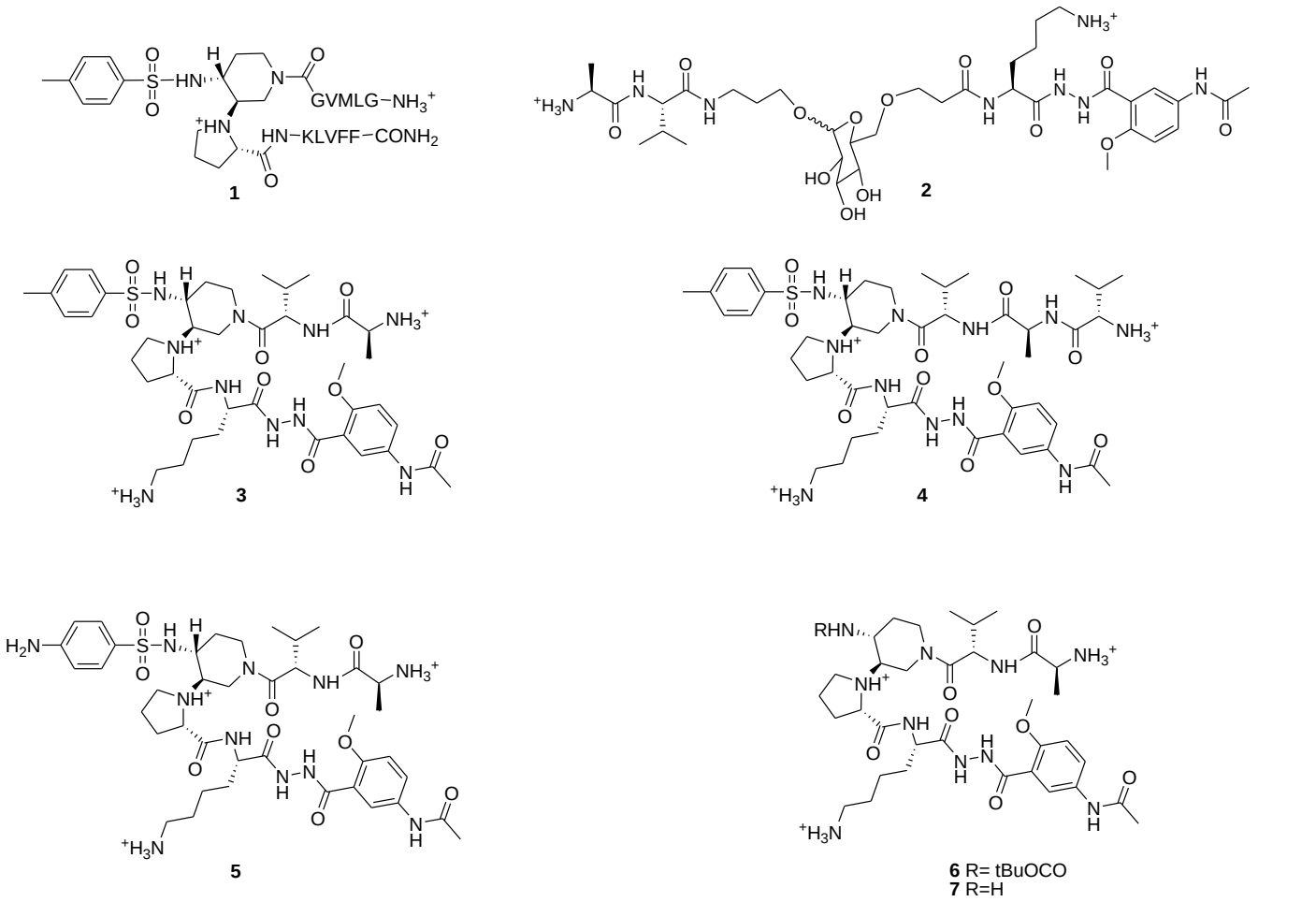


Figure 1. Structures of β-hairpin mimic **1** and glycopeptidomimetic **2** previously reported [13,14] and of the newly designed β-hairpin mimics **3**-**7**.

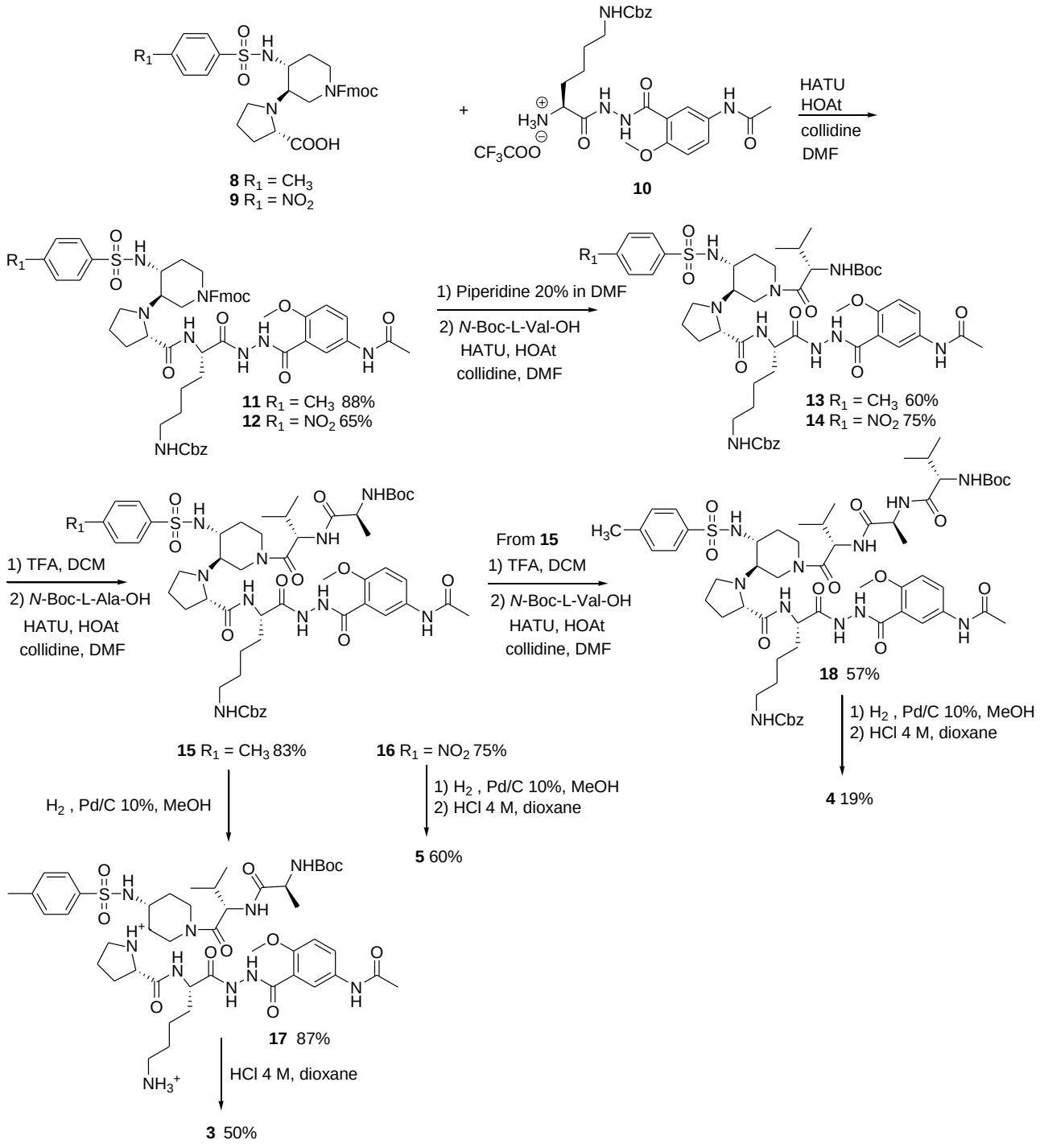
2. Results and discussion

2.1 Synthesis

The synthesis of the newly designed peptidomimetics 3-5 is described in scheme 1, starting from scaffolds 8 and 9. The tosyl scaffold 8 was prepared according to our published method, based on a key multicomponent reaction using *N*-benzylpiperidinone, tosyl azide and proline methyl ester.[12,13] We adapted this process to the synthesis of the nosyl scaffold 9, replacing tosyl azide by nosyl azide. This new synthetic protocol is fully described in the supporting information (Scheme 1S).

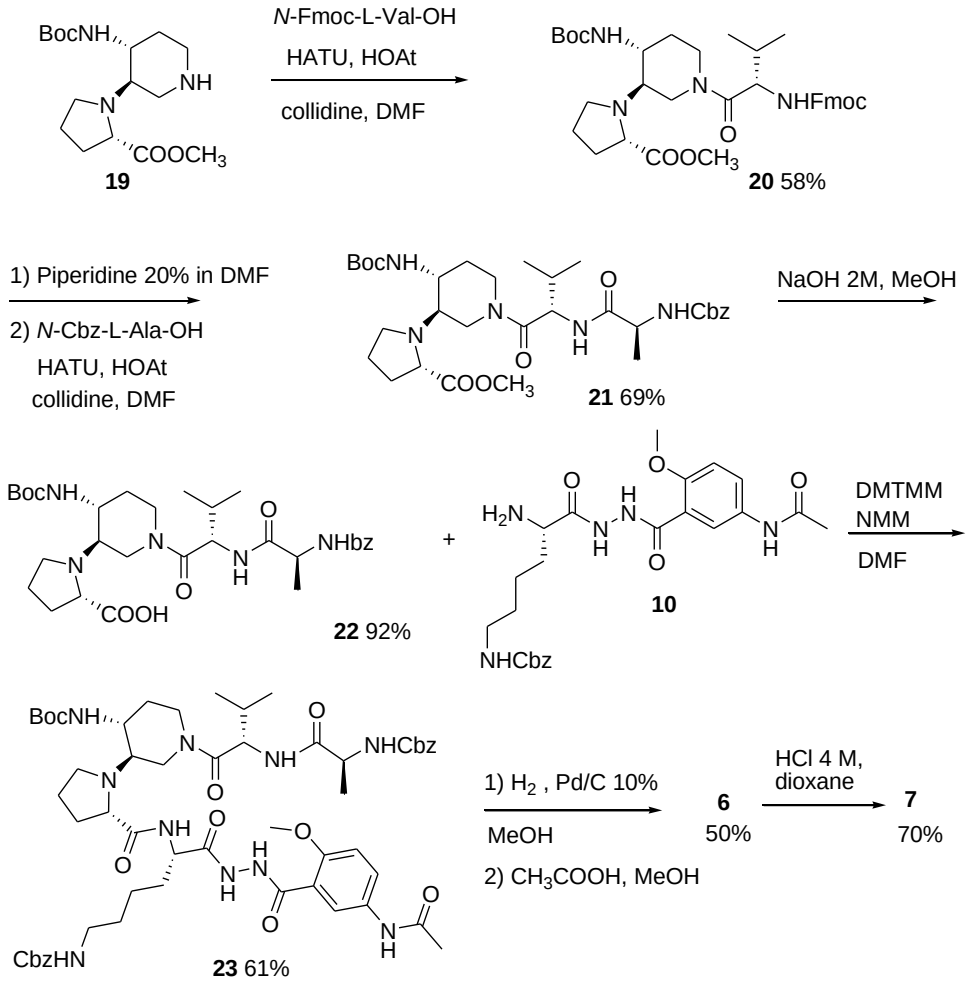
The coupling reaction between the peptidomimetic strand **10 [**14] and compounds **8** and **9** provided the desired products **11** and **12** in good yield (88 and 65%, respectively), using HATU and HOAt as coupling reagents, in the presence of collidine in DMF. Other coupling agents were also tested in parallel for the coupling of **10** with **8**, such as DMTMM in the presence of NMM, or EDC in the presence of HOBt and DIPEA but were less efficient (compound **11** was obtained in 18% and 43% of yield, respectively). After the Fmoc cleavage using 20% piperidine in DMF, the construction of the *N*-terminus arm was then performed using the Boc strategy. The coupling of *N*-Boc-L-Val-OH and compounds **11** or **12** was performed using HATU and HOAt, in the presence of collidine in DMF, to afford compounds **13** and **14** in 60 and 75% yields respectively. After the Boc cleavage, the second amino acid (*N*-Boc-L-Ala-OH) was coupled affording compounds **15** and **16** in 83 and 75% yield, respectively. The Cbz protecting group of **15** was then cleaved by hydrogenolysis

using hydrogen in the presence of 10% Pd/C in MeOH, and the resulting product **17** was finally Boc deprotected in the presence of 4M HCl in dioxane to afford the final compound **3**. The final compound **5** was obtained from **16**, in 60% yield, by following the same hydrogenation/hydrolysis procedures. On the other hand, the Boc cleavage of **15** followed by the coupling with *N*-Boc-L-Val-OH afforded compound **18** in 57% yield. Hydrogenolysis of the Cbz group followed by the Boc cleavage afforded the final compound **4**.



Scheme 1. Synthesis of compounds **3-5**

The synthesis of compounds **6-7** is described in scheme 2, starting from the Boc scaffold 19 whose synthesis is fully described in the supporting information (Scheme 2S, see supporting information). The direct cleavage of the nosyl group on the scaffold derivative S1 was only partially satisfactory, whatever the used conditions (thiophenol/K_2CO_3 in acetonitrile in the presence of DMSO, heating at 50°C or using ultrasounds or microwaves; thioglycolic acid/LiOH in DMF at room temperature or at 70°C). Thus, the nosylamide was converted first to the Boc-nosylimide S4 in order to activate the nosyl group towards the attack and cleavage by thiophenol.[15] Indeed, the cleavage of the nosyl group, giving S5, was then satisfactory and the Boc scaffold 19 was easily obtained after hydrogenolysis of S5. *N*-Fmoc-L-Val-OH and *N*-Cbz-L-Ala-OH were successively coupled, using HATU and HOAt as coupling reagents and collidine as the base to afford compound 21 (Scheme 2). Saponification of the methyl ester of 21, afforded product 22 (92% yield). The coupling reaction between 22 with the peptidomimetic strand 10, using HATU and HOAt, did not provide the desired product 23. Satisfactory results were obtained using the triazine-based reagent DMTMM (1.1 eq.) in the presence of NMM in DMF (61% yield). The two Cbz protecting groups of 23 were cleaved by hydrogenolysis to afford product 6 that was subjected to the Boc cleavage to afford the final compound 7 (70% yield).



Scheme 2. Synthesis of compounds **6-7**

2.2. Conformational studies

Conformational studies of compounds **3** and **4** were conducted by NMR in water, which is more challenging for intramolecular hydrogen bond formation in comparison with aprotic organic solvents such as chloroform, but that is closer to physiological conditions. The observed proton and carbon chemical shifts are displayed in Tables S1 and S2 for compound **3** and in table S7 for compound **4** (supporting information). Compounds **3** and **4** were present in solution as a mixture of two conformers in 3:1 and 4:1 *ratio,* respectively. The dynamic equilibrium between the two conformers was confirmed by the occurrence of splitting for some protons (see supporting information). The coalescence between the two forms could not be reached at 313 K. We next examined the temperature dependence of amide proton chemical shifts, as it can provide information on the network of hydrogen bonds and their relative stabilities.[16] None of the amide protons of the major isomer showed small negative temperature coefficient, except for the NH(b) proton ($\Delta\delta HN/\Delta T$ value of –4.47 ppb/K for **3** and –4 ppb/K for **4**, Tables S3 and S8, supporting information), presumably because of its hydrogen bonding with the methoxy group of the aromatic unit, as already reported by Nowick for the Hao motif.[17] In both conformers of **3**, the temperature coefficients of the Lys and Val amide proton fell within intermediate values ($\Delta\delta HN/\Delta T$ value of –6.33-7.33 ppb/K, Tables S3 and S4), suggesting their partial engagement in intramolecular hydrogen bonds. Similarly, the NH of Lys and Ala in compound **4** had intermediate $\Delta\delta HN/\Delta T$ values (Tables S8 and S9).

The vicinal $J_{NH\text{-}H\alpha}$ coupling constant also yields direct information on the main chain φ dihedral angle, through the Karplus relationship. In the two conformers of both compounds **3** and **4**, the coupling constants exhibited values between 7.4-8.7 Hz. As expected for extended conformation, positive difference between $J_{NH\text{-}H\alpha}$ values in the random coil and the values determined experimentally were found, reflecting φ angle values around -120° (Tables S5 and S6 for **3** and S10 for **4**, supporting information).[18] Chemical shift deviations (CSD) are defined as the difference between experimental chemical shifts and corresponding random coil values and are considered as good descriptors of backbone conformational space for each residue.[19] We calculated the CSD for the natural amino acids present in the molecule (Tables S5 and S6 for **3** and S10 for **4**, supporting information). In both conformers, the C_α and the carbonyl group were upfield shifted (negative CSD), indicating that an extended conformation for the peptide and peptidomimetic arms predominates. The downfield shifted H_α and NH (positive CSD, > 0.1 ppm) of Val and Lys indicated that they are more implicated in the β-conformation than the *N*-terminal Ala in **3**. Similarly, for compound **4**, Val-2, Ala and Lys CSD values indicated that they are more implicated in the β-conformation than the *N*-terminal Val-1. In the ROESY experiments performed at 278 K, strong sequential and medium intra-residual Hα-HN ROEs, which are characteristic of an extended backbone conformation, were detected for the dipeptide and tripeptide sequence in **3** and **4**, respectively. ROEs between the di- or tripeptide and the peptidomimetic arms in the major conformers of both **3** and **4** were observed (Figures 2A and 3A). ROEs between H2 of the 5-amino-2-methoxybenzhydrazide (Amb) motif and both Hβ and methyl protons of Val were observed (H2 Amb/Me_Val and H2 Amb/Hβ_Val in **3** and H2 Amb/Me_Val-2 and H2 Amb/Hβ_Val-2 in **4**). ROEs were also detected between the methoxy group of the benzhydrazide motif and Hα of Val (Hα_Val/OMe_Amb in **3** and Hα_Val-2/OMe_Amb in **4**). Furthermore, in compound **3** a ROE between H2 of the benzhydrazide motif and the methyl of Ala (H2 Amb/Me_Ala) was noticed. In both compounds **3** and **4**, we observed ROEs between the aromatic proton H2 of the tosyl group and both Hα and H4 of Pro, suggesting that the tosyl protecting group is stacked onto the proline ring. Three-dimensional structures of **3** and **4** were then generated using simulated annealings with restrained distances inferred from the six and five NMR ROEs previously described and reported in Figures 2A and 3A for compound **3** and **4**, respectively. As shown in Figures 2B and 3B, both compounds **3** and **4** exhibited a partially folded β-hairpin conformation in which the piperidine-pyrrolidine and lysine form the β-turn while Val in compound **3** or Val2 in compound **4** side chain make hydrophobic interactions with the benzhydrazide aromatic ring. However, the *N*-terminal residues (Ala in compound **3** or Val2-Ala in compound **4**) are not observed in contact with the *C*-terminal acetyl group, which would form a more extended β-hairpin. No particular « inter-arm» hydrogen bond was observed for stabilizing the β-hairpin. On the other hand, the carbonyl group of the alanine residue is close to and oriented toward the amine groups of both pyrrolidine, lysine and hydrazine, avoiding the formation of a fully extended β-hairpin. All these data suggest that compounds **3** and **4** adopt, a similar partial β-hairpin conformation.

A) B)

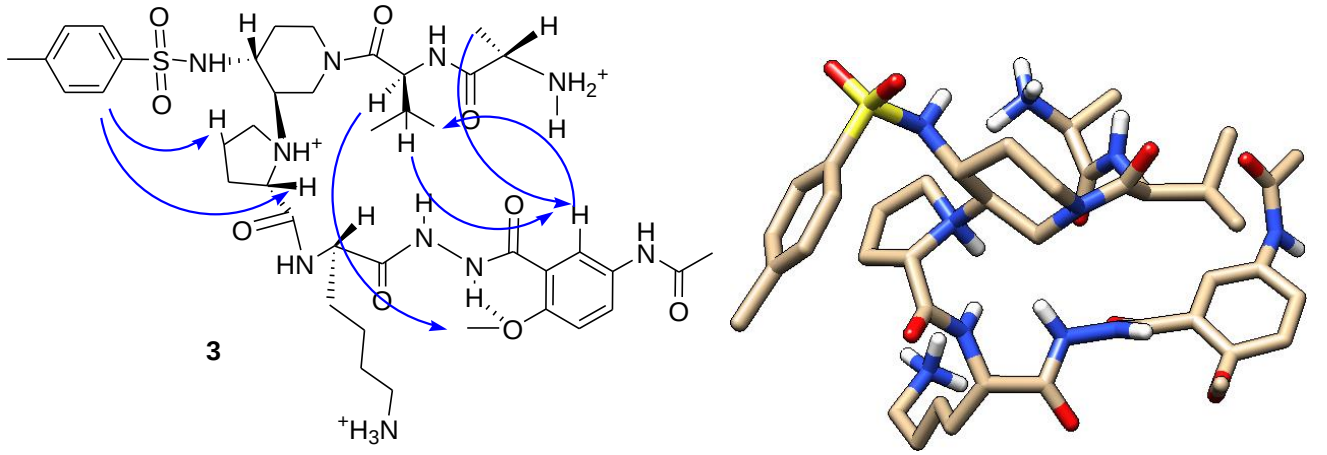


Figure 2. A) Structures of the major conformer of compound **3** showing the assigned ROEs by blue flashes; B) Structure of **3** generated by simulated annealing using restrained distances inferred from NMR ROEs. Sulfur, nitrogen, oxygen, atoms and polar hydrogens are coloured in yellow, blue, red, and white, respectively. The figure was prepared with UCSF Chimera.[25]

A) B)

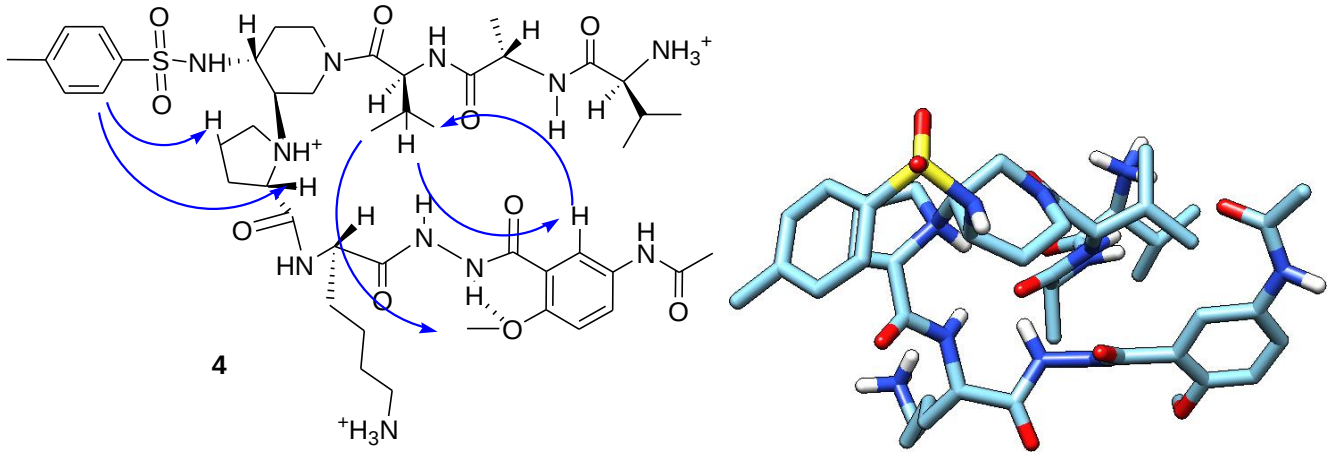


Figure 3. A) Structure of the major conformer of compound **4** showing the assigned ROEs by blue flashes. B) Structure of **4** generated by simulated annealing using restrained distances inferred from NMR ROEs. Sulfur, nitrogen, oxygen, atoms and polar hydrogens are coloured in yellow, blue, red, and white, respectively. The figure was prepared with UCSF Chimera.[25]

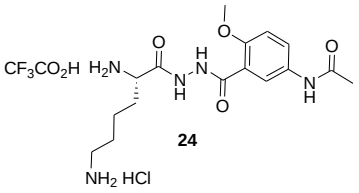
2.3 Evaluation of the inhibition of fibrillization

The anti-amyloidogenic activity of compounds **3-7** was investigated using the *in vitro* thioflavin-T (ThT) assay. ThT is a dye that fluoresces upon binding to β-sheet rich species and thus, is widely used to monitor the kinetics of aggregation of amyloid proteins.[20] For $A\beta_{1-42}$ alone (at a concentration of 10 μM), the fluorescence curve followed a typical sigmoidal shape with a lag phase around 4-6 h, an elongation phase and a final plateau reached after 12-20 h (Figure 4, purple curve). Two parameters were derived from the ThT curves: $t_{1/2}$, defined as the time at which the fluorescence has reached

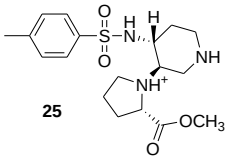
50% of its maximum, is a measure of the aggregation process rate; and *F*, the fluorescence value of the final plateau which is assumed to be dependent on the amount of fibers formed (Table 1).
Each compound was tested at a 10-fold excess (100 µM) and at a 1/1 ratio (10 µM). Compounds **3** and **4** were also tested at the substoichiometric ratio of 0.1/1 (compound/$A\beta_{1\text{-}42}$).
Significant differences in activity on the aggregation process were observed between all the evaluated compounds (Table 1, Figure 4 and Figure S6 supporting information). The most significant activity on the aggregation process of $A\beta_{1\text{-}42}$ was obtained with compound **3**, which is the analogue of the glycopeptidomimetic **2**,[14] composed of the same two arms. **3** displayed a comparable activity to **2** at the 1/1 ratio (extension of the $t_{1/2}$ by 36% and decreased of the fluorescence plateau by 38% for **3** compared to 48% and 29% for **2**). However, the activity of **3** was slightly lower than **2** at a 10-fold excess: delaying the rate of aggregation by almost 80% and decreasing the fluorescence plateau by almost 70% (*vs* no aggregation for **2**). Compound **17**, the analogue of **3**, protected by a terbutoxycarbonyl group on the *N*-terminal amine of Ala residue, was less active (extension of the $t_{1/2}$ by 59% and23% respectively for ratios 10/1 and 1/1 of **17**/$A\beta_{1\text{-}42}$ and no effect on the fluorescence plateau). The totally protected precursor **15** was inactive at the ratio 1/1 (**15**/$A\beta_{1\text{-}42}$) and self-aggregated at the 10-fold excess (100 µM). These results indicate that both free amines, either on the *N*-terminal position and on the side chain of both arms, are beneficial for the activity.[13,14]
The elongation of the *N*-terminus arm, by adding a third hydrophobic amino acid (Val), had more than halved the inhibitory activity of compound **4** with respect to **3**. Indeed, at the 10 fold excess concentration relative to $A\beta_{142}$, **4** increased the $t_{1/2\ \text{only}}$ by 30% and reduced the fluorescence plateau by 32%, compared to 76% and 66% for **3**. At the 1:1 ratio, **4** displayed no activity. A supplementary ThT fluorescence assay was performed by first, adding compound **3** after 3 hours or after 19h, when presumably oligomers are in formation or are already large species respectively. Finally, compound **3** was added after 44 hours, when presumably essentially fibrils are present (Figure S7 in supporting information). A similar activity was obtained with compound **3** added at the beginning of the kinetics or after 3 hours. However no effect was observed when compound **3** was added after 44 hours. When **3** was added after 19h, no effect was observed at the 1:1 ratio and the fluorescence observed at the 10:1 (**3**/$A\beta_{1\text{-}42}$) ratio after 19h, was maintained throughout the rest of the kinetics. This result indicates that **3** does not disaggregate large oligomers and preformed fibrils but rather prevent the early oligomerization process.
As these peptidomimetics were soluble in aqueous media, we verified that their activity on $A\beta_{1\text{–}42}$ aggregation was maintained when solubilized in water (without DMSO). Indeed, the most active compound **3**, dissolved in water (0.5% v/v), displayed a comparable or even slightly higher activity in the ThT fluorescence assay (at ratio 10/1 of **3**/$A\beta_{1\text{-}42}$) to that observed when solubilized in the same amount of DMSO ($t_{1/2}$ increased by 96% and F decreased by 81% in the case of a solubilization in water *vs* 76% and 66% in DMSO). The same result was obtained for the tripeptide analogue, **4**, the $t_{1/2}$ being increased by 58% and F decreased by 35% when the sample was solubilized in water compared to 30% and 32% in DMSO. This observation suggests that DMSO may perturb the secondary structure of **3**, reducing its affinity for $A\beta_{1\text{–}42}$.
Focusing on the pharmacomodulation induced by the substituent at C-4 of the piperidine ring, the aminophenyl-sulfonylamino derivative **5**, the terbutoxycarbonylamino compound **6** and the primary amine **7** were tested. Compound **5** retained some activity, however slightly less than that of the tosyl derivative **3**. For the Boc compound, **6**, the ability to inhibit the aggregation process was even lower ($t_{1/2}$ increased by 29% and F decreased by 43%, ratio 10/1 of **6**/$A\beta_{1\text{-}42}$). The activity of compound **7** almost disappeared (only F reduction of 18% at the same ratio). These results suggest that both the aromatic ring branched to the primary amine of the pyrrolidine of the scaffold through a sulfamide bond, and its para substitution by a methyl group rather than an amine are crucial for the inhibition of $A\beta_{1\text{-}42}$ aggregation. These data suggest that, the interaction of compounds **3** and **5** to $A\beta_{1\text{-}42}$ peptide is also probably driven by hydrophobic and/or aryl-aryl interactions involving the scaffold moiety.
Finally, to confirm that the entire molecule of β-hairpin was essential to interact and inhibit $A\beta_{1\text{-}42}$ aggregation, the scaffold **25** was also evaluated by the Tht-fluorescence assay (Table 1). It displayed a modest activity and only at the high 10/1 ratio (**25**/$A\beta_{1\text{-}42}$). F decreased by 23% and no effect was observed on the $t_{1/2}$. The activity of the peptidomimetic arm **24** has been already reported by us [14] and it is significantly lower than for the entire β-hairpin mimic ($t_{1/2}$ increased by only 7% and F decreased by 44% at the **24**/$A\beta_{1\text{-}42}$ ratio of 10/1 *vs* +76% and -66% in the case of **3**).
Transmission electron microscopy (TEM) analyses were performed on the most promising compounds **3** and **5** according to the Tht-fluorescence assay results. Images were recorded at 42 h of fibrillization kinetics with samples containing 100 µM of each compound corresponding to the compound/$A\beta_{1\text{-}42}$ ratio of 10/1 (Figure 5). Differences were observed in quantity of aggregates formed. At 42 h, a very dense network of fibers displaying a typical morphology was observed

for $A\beta_{1-42}$ alone (Figure **5a**). In the sample containing 3, the network of fibers, displaying the same morphology, was significantly less dense than in the control experiment after 42 h (Figure **5b**). The same trends were observed with compound 5, but to a lesser extent (Figure 5c). These results validated the ThT-fluorescence data, indicating that **3** significantly slowed down the aggregation of $A\beta_{1-42}$ and efficiently reduced the amount of typical amyloid fibrils, and that **5** is slightly less efficient.

Compounds (compound/Aβ ratio)[a]	$t_{1/2}$ extension [b]	F reduction [c]
1[13]10/1	NA[d]	−97±1 %
1[13] 1/1	NA	−90±2 %
2[14] 10/1	NA	−87±1 %
2 [14] 1/1	+48±12 %	−29±9 %
3 10/1	+76±16 %	−66±11 %
3 10/1 (water)	+96±10 %	−81±10 %
3 1/1	+36±12 %	−38±16 %
3 0.1/1	ne[e]	−38±10 %
4 10/1	+30±3 %	−32±9 %
4 10/1 (water)	+58±10 %	−35±10 %
4 1/1	ne	ne
4 0.1/1	ne	ne
5 10/1	+56±4 %	−54±4 %
5 1/1	+27±7 %	ne
6 10/1	+29±9 %	−43±7 %
6 1/1	+13±5 %	ne
7 10/1	ne	−18±13 %
7 1/1	ne	ne
15 10/1	SAT[f]	SAT[e]
15 1/1	ne	ne
17 10/1	+59±11 %	ne
17 1/1	+23±2 %	ne
24 10/1[14]	+7±1 %	−44±8 %
24 1/1[14]	ne	−16±9 %
25 10/1	ne	−23±14 %
25 1/1	ne	ne



24



25

[x] Parameters are expressed as mean ± SE, n=3-6. [a] Unless otherwise indicated, compounds were dissolved in DMSO with the final concentration kept constant at 0.5%. [b] See the experimental section for the calculation of the $t_{1/2}$ extension. [c] See the experimental section for the calculation of the *F* reduction. [d] NA = no aggregation. [e] ne = no effect. [f] SAT means that a saturation of the fluorescence signal is observed because **15** self-aggregates at 100 μM.

Table 1. Effects of compounds 3-7, 15, 23, 24 and 25 on $A\beta_{1-42}$ fibrillization assessed by ThT-fluorescence spectroscopy. The concentration of $A\beta_{1-42}$ is 10 μM. Compounds were tested at 10/1 and 1/1 compound/$A\beta_{1-42}$ ratios and compared to the values obtained for $A\beta_{1-42}$ alone ($t_{1/2}$ and *F*). Compounds 3 and 4 were also tested at the substoichiometric ratio of 0.1/1 (compound/$A\beta_{1-42}$). Data for compounds 1, 2 and 24 are extracted from references 13 and 14.

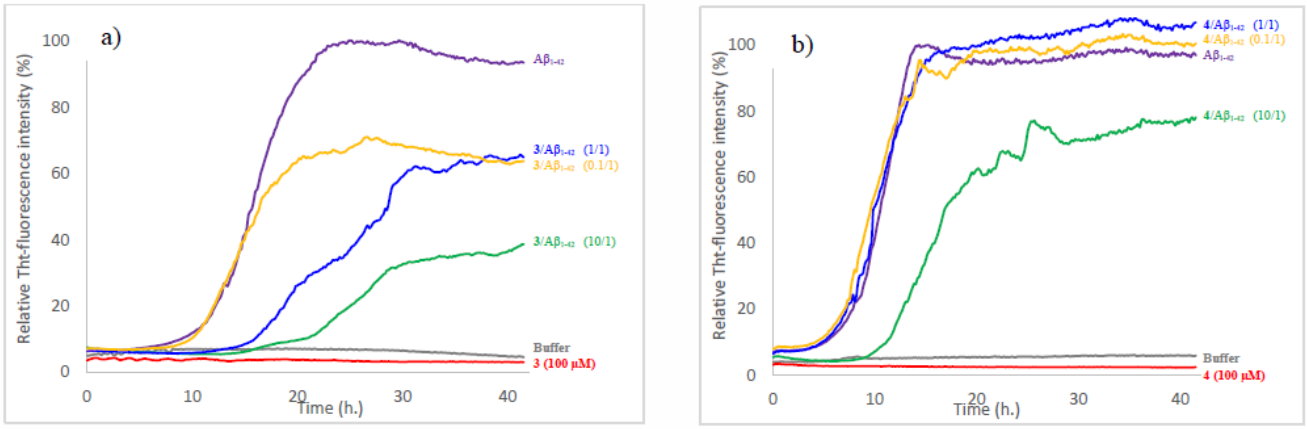


Figure 4. Representative curves of ThT fluorescence assays over time showing $A\beta_{1\text{-}42}$ (10 µM) aggregation in the absence (purple curve) and in the presence of compounds **3** (a) and **4** (b) at compound/$A\beta_{1\text{-}42}$ ratios of 10/1 (green curves), 1/1 (blue curves) and 0.1/1 (yellow curves). The control curves are represented in red lines and buffer in grey.

a) b) c)

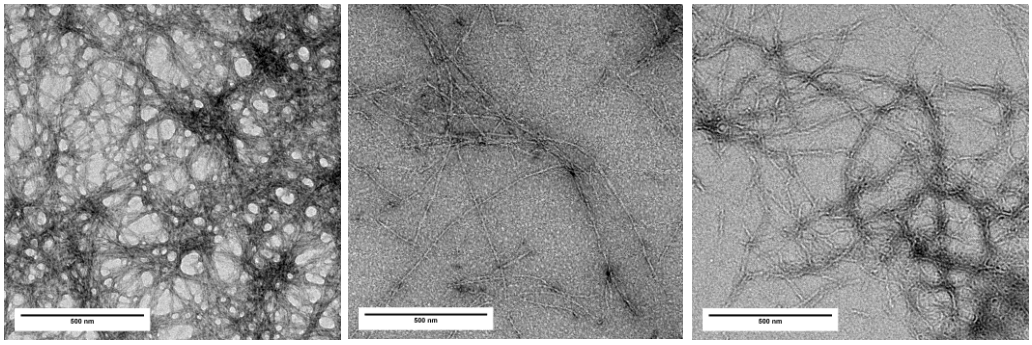


Figure 5. Fibril formation of **$A\beta_{1\text{-}42}$** visualized by TEM: negatively stained images recorded after 42 h of incubation of **$A\beta_{1\text{-}42}$** (10 µM in 10 mM Tris.HCl, 100 mM NaCl at pH = 7.4) alone (a) or in the presence of 100 µM of **3** (b) or **5** (d). Scale bars represent 500 nm.

2.4 Evaluation of the inhibition of oligomerization

We recently developed a capillary electrophoresis (CE) method to monitor the early steps of the oligomerization process over time.[21] This method allows to follow the formation of small soluble oligomers and to evaluate the impact of small molecules on three kinds of species, (i) the monomer of $A\beta_{1\text{-}42}$ (peak ES), (ii) different small metastable oligomers grouped under peak ES' and (iii) transient later species corresponding to oligomers larger than dodecamers (peak LS).[13,14,21] The control kinetics ($A\beta_{1\text{-}42}$, 100 µM) showed that overtime, the peak of monomer ES decreases in favor of the peaks of LS, and that soluble species are no longer visible after 6 h (Figure 6A). Moreover, ES' species are hardly visible while spikes, corresponding likely to insoluble and larger oligomers, are clearly visible as soon as 2 h.
The electrophoretic profiles obtained in the presence of compound **3** indicate that **3** significantly modified the aggregation process. **3** was able to maintain the non-toxic monomer ES overtime. Indeed, ES is still present after 12 h (Figure 6B): 49% and 31% of the monomer are still present after 6 h and 12 h respectively in the presence of **3**, while it was no longer detected in the control sample after 6 h (Figure 6C). Furthermore, LS migrated slower and higher number of spikes were

observed from the beginning of the kinetics (0-2 h) and on each electrophoretic profile. These two features suggest another pathway of $A\beta_{1-42}$ aggregation in the presence of **3**.

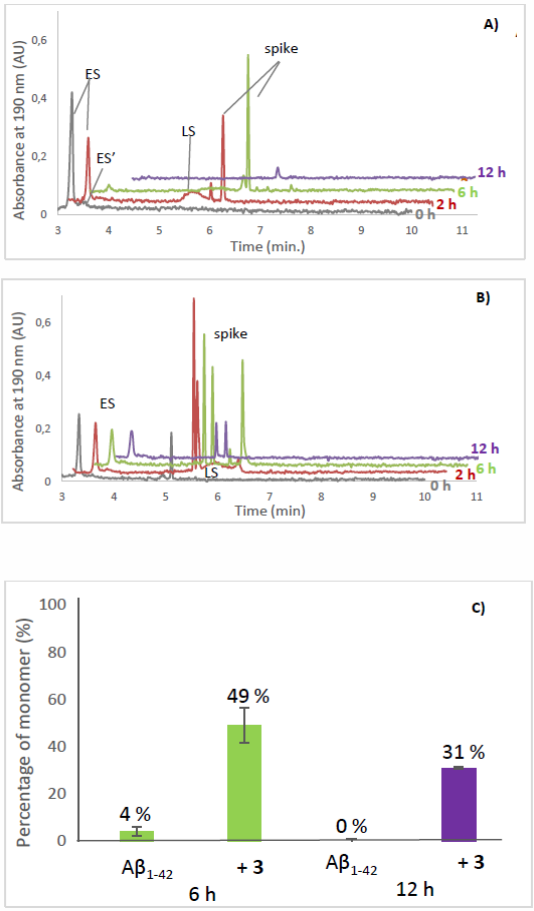


Figure 6. Electrophoretic profile obtained immediately (0 h), 2 h, 6 h and 12 h after sample reconstitution (t_0) of $A\beta_{1-42}$ peptide (100 µM) alone (A), in the presence of compound **3** at **3**/$A\beta_{1-42}$ ratio of 10/1 (B). Peak area of the monomer (ES) related to its peak area in the sample of $A\beta_{1-42}$ alone at 0 h, 6 h and 12 h (C).

Conclusion

We described the design and the synthesis of five new acyclic peptidomimetics based on a piperidine-pyrrolidine β-turn inducer bearing a di- or a tri- peptide *N*-terminus arm and a peptidomimetic arm containing a 5-amino-2-methoxybenzhydrazide unit at the *C*-terminus. Compound **3** is the most active compound of this series. It is able to greatly delay the fibrillization process of $A\beta_{1-42}$, as demonstrated by Tht fluorescence spectroscopy and TEM images. It is also able to modify the early oligomerization steps and to maintain the presence of the non toxic monomer of $A\beta_{1-42}$. The results obtained in this study allowed us to establish some informative structure-activity relations. 1) With respect to compound **1**, the reduction of the peptidic character by both the shortening from pentapeptide arms to a di- or tripeptide, and the substitution of the *C*-terminus peptide arm by a peptidomimetic arm, maintained a satisfactory activity of $A\beta_{1-42}$ aggregation inhibition. Moreover, compounds **3** and **4** were hydrosoluble. 2) The length of the peptide arm dramatically influences the aggregation inhibition activity, a dipeptide being more advantageous than a tripeptide sequence. 3) Concerning the substituent on the amino group at C-4 of the piperidine ring of the β-turn mimic, the tosyl residue is crucial for an inhibitory activity, as the Boc compound **6** and the free amine derivative **7** are almost inactive. The more polar aminophenyl-sulfonylamino derivative **5** is much less active than **3**. We hypothesize that the tosyl moiety of **3** interacts with

aromatic residues of $A\beta_{1-42}$ through hydrophobic and aryl-aryl interactions. 4) Compounds **15** and **23**, being the di- and mono-*N*-protected analogues of **3**, exhibited a strong decrease of activity. This result supports our previous hypothesis on the importance of establishing ionic interactions between the amino groups and acidic residues of $A\beta_{1-42}$.[13,14] The whole β-hairpin construct is necessary to strongly delay the $A\beta_{1-42}$ aggregation kinetics, as the isolated scaffold **25** and the peptidomimetic arm **24** displayed only very modest activity. 6) The conformational studies allowed us to hypothesize that a fully extended β-hairpin structure is more convenient and can further inhibit the early oligomerization and fibrillization process. In fact, we observed that compound **3** exhibits a partially folded β-hairpin conformation while the more active reference compound **1** was able to adopt a more stable β-hairpin conformation.

Thus, this work demonstrates that a compromise between flexibility of the β-hairpin, hydrosolubility and reduction of the peptidic character can be reached. These peptidomimetics are the first examples of small and non-peptide analogues of acyclic beta-hairpins, which have preserved anti-aggregation activity after modifications that provide better druggability properties. This strategy can now be envisaged to design inhibitors of other types of amyloid-forming proteins.

Experimental

Chemistry

General Experimental Methods. Solvents were purchased from commercial sources and dried and distilled by standard procedures. Protected amino acids, ***N***-[(Dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide (HATU), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM(Cl^-)), and 1-Hydroxy-7-azabenzotriazole (HOAt) were purchased from commercial sources. Compounds **1**,[13] **2** [14] **8** [13]**, 24** [14] and **25** [13] were prepared according to published methods. The syntheses of compounds **9** and **18** are described in the supporting information. Pure products were obtained after liquid chromatography using Merck silica gel 60 (40-63 µm). TLC analyses were performed on silica gel 60 F_{250} (0.26 mm thickness) plates. The plates were visualized with UV light (λ = 254 nm) or revealed with a 4% solution of phosphomolybdic acid in EtOH or with a solution of ninhydrin in EtOH. Melting points were determined on a Kofler melting point apparatus. NMR spectra were recorded on an ultrafield Bruker AVANCE 300 (^{1}H, 300 MHz, ^{13}C, 75 MHz), a Bruker AVANCE 400 (^{1}H, 400 MHz, ^{13}C, 100 MHz), a Bruker AVANCE I 600MHz (^{1}H, 600 MHz, ^{13}C, 150 MHz) or a Bruker AVANCE III 800MHz (^{1}H, 800 MHz, ^{13}C, 200 MHz) spectrometers, the last two equipped with a z-gradient TCI cryprobe. Chemical shifts δ are in ppm and the following abbreviations are used: singlet (s), doublet (d), doublet of doublet (dd), doublet of triplet (dt), triplet (t), triplet of doublet (td), quintuplet (qt), multiplet (m), broad multiplet (bm), and broad singlet (bs). Mass spectra were obtained using a Bruker Esquire electrospray ionization apparatus. HRMS were obtained using a TOF LCT Premier apparatus (Waters), with an electrospray ionization source. The purity of compounds **3**-**7** was determined by HPLC using the 1260 Infinity system (Agilent Technologies) and a column SUNFIRE (C18, 3.5 µm, 150 mm X 2.1 mm); mobile phase: H_2O + 0.1% formic acid/CAN gradient from 5 to 100% in 20 min. or from 1 to 60% in 20 min.; detection at 230, 254, 310 nm; flow rate 0.25 mL/min.

(2*S*)-*N*-[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-amino-pentyl]-1-[(3*R*,4*R*)-1-[(2*S*)-2-[[(2*S*)-2-aminopropanoyl]amino]-3-methyl-butanoyl]-4-(p-tolylsulfonylamino)-3-piperidyl]pyrrolidine-2-carboxamide trihydrochloride 3. Compound **15** (64.0 mg, 0.058 mmol, 1.0 eq.) was dissolved in MeOH (4.0 mL) and 10% Pd/C (10 mg) was added. The reaction was kept 2 hours under stirring and under hydrogen atmosphere at room temperature. The catalyst was then removed over a celite pad and the solvent was evaporated under vacuum. The resulting product was dissolved in dioxane (2.0 mL) and a 4 N solution of HCl in dioxane (0.293 mL, 30.0 eq.) was added. The reaction was kept 2 hours under stirring at room temperature. Diethyl ether was then added to allow precipitation of product **3** that was isolated as the hydrochloride salt after filtration. The crude was purified by recrystallization in MeOH and diethyl ether to afford the pure compound **3** as a white powder (19.0 mg, 0.019 mmol, 50%). mp = 221–223 °C; 1H NMR (H_2O/ D_2O, 600 MHz): δ = 10.42 (1H, bp), 10.31 (1H, bs), 9.79 (1H, s,), 9.09 (1H, d, *J* = 7.5 Hz), 8.52 (1H, d, *J* = 7.5 Hz), 8.06 (3H, bs), 7.95 (1H, bs), 7.88-7.84 (3H, m), 7.57-7.56 (4H, m), 7.47 (2H, m), 7.22 (1H, d, *J* = 9.4 Hz), 4.60 (1H, m), 4.52 (1H, m),4.23 (1H, m), 4.22 (1H, m), 4.11 (1H, m), 3.96 (3H, s), 3.93 (1H, m), 3.72 (1H, m), 3.48 (1H, m), 3.15 (1H, m), 3.04 (2H, m), 3.01 (1H, m), 2.85 (1H, m), 2.60 (1H, m), 2.50 (1H, m), 2.44 (3H, s), 2.18 (3H, s), 2.11

(1H, m), 2.06 (1H, m),1.98 (2H, m), 1.94 (1H, m), 1.90 (1H, m), 1.76 (2H, m), 1.63 (1H, m), 1.53 (2H, m),1.48 (1H, m), 1.46 (3H, d, *J* = 7.5 Hz), 0.82-0.83 (6H, m);^{13}C NMR (H_2O/ D_2O, 133 MHz): δ = 173.1, 171.9, 171.3, 170.7, 166.5, 164.9, 155.1, 145.6, 135.6, 130.2, 130.2, 128.4, 126.7, 124.7, 118.7, 112.9, 56.3, 55.1, 52.2, 52.1, 48.8, 44.2, 44.1, 41.4, 39.3, 37.0, 31.4, 30.3, 30.2, 30.1, 29.7, 26.4 , 24.1, 22.5, 21.8, 20.6, 18.5, 16.7, 16.5; IR (neat): ν_{max} = 3194, 2926, 1652, 1546, 1493, 1457, 1252, 1157; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{41}H_{63}N_{10}O_9S$ 871.4500, found: 871.4497,m/z $[M + Na]^+$ calcd for $C_{41}H_{62}N_{10}O_9NaS$, 893.4320, found: 893.4316; HPLC purity (XBridge C18, 3.5 µm, H_2O + 0:2% form. ac./ACN, gradient 5–100% in 20 min): TR = 10:08 min, 100%.

(2*S*)-*N*-[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-amino-pentyl]-1-[(3*R*,4*R*)-1-[(2*S*)-2-[[(2*S*)-2-[[(2*S*)-2-amino-3-methyl-butanoyl]amino]propanoyl]amino]-3-methyl-butanoyl]-4-(p-tolylsulfonylamino)-3-piperidyl]pyrrolidine-2-carboxamide trihydrochloride 4. The same procedure was used as that described for the synthesis of compound **3:** from **17** (50.0 mg, 0.042 mmol, 1.0 eq.) to afford, after recrystallization in MeOH and diethyl ether, the pure compound **4** as a white powder (9.0 mg, 0.008 mmol, 19%). mp = 229–231 °C; ^{1}H NMR (H_2O/ D_2O, 800 MHz): δ = 10.27 (1H, bs), 10.09 (1H, bs), 9.59 (1H, s), 8.89 (1H, d, *J* = 7.4 Hz), 8.46 (1H, d, *J* = 6.2 Hz), 8.18 (1H, d, *J* = 8.1 Hz), 7.82 (3H, bs), 7.79 (1H, bs),7.59-7.54 (3H, m), 7.33 (3H, bs,), 7.27-7.18 (3H, m), 6.92 (1H, d, *J* = 10.2 Hz), 4.34 (1H, m), 4.25 (1H, m), 4.24 (1H, m), 4.12 (1H, m), 3.92 (1H, m), 3.83 (1H, m), 3.69 (3H, s), 3.64 (1H, m), 3.49-3.47(2H, m), 2.84-2.76 (5H, m), 2.29 (1H, m), 2.26 (1H, m), 2.15 (3H, s), 1.91 (1H, m), 1.88 (3H, s), 1.81 (1H, m), 1.71 (1H, m), 1.70 (2H, m), 1.67 (1H, m), 1.65 (1H, m), 1.49 (2H, m), 1.30 (1H, m), 1.28 (2H, m), 1.19 (1H, m), 1.05 (3H, d, *J* = 7.5 Hz), 0.73-0.71 (6H, m), 0.57-0.54 (6H, m); ^{13}C NMR (H_2O/ D_2O, 200 MHz): δ = 174.1, 172.9, 171.8, 171.6, 170.8, 169.0, 166.3, 155.1, 145.3, 135.3, 130.1, 129.8, 128.4, 126.6, 124.7, 118.4, 112.7, 58.3, 56.0, 54.6, 52.4, 51.7, 49.3, 48.9, 46.8, 43.6, 40.9, 39.2, 39.1, 30.1, 30.1, 29.9, 29.9, 29.7, 26.3, 23.8, 22.4, 21.9, 20.6, 18.4, 16.9, 17.5, 16.6, 16.3; IR (neat): ν_{max}= 3194, 2966, 1652, 1539, 1494, 1456, 1250, 1161 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{46}H_{72}N_{11}O_{10}S$ 970.5184, found: 970.5181; HPLC purity (XBridge C18, 3.5 µm, H_2O + 0:1% form. ac./ACN, gradient 5–100% in 20 min): TR = 10:47 min, 92%

(2*S*)-*N*-[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-amino-pentyl]-1-[(3*R*,4*R*)-4-[(4-aminophenyl)sulfonylamino]-1-[(2*S*)-2-[[(2*S*)-2-aminopropanoyl]amino]-3-methyl-butanoyl]-3-piperidyl]pyrrolidine-2-carboxamide trihydrochloride 5 .The same procedure was used as that described for the synthesis of compound **3:** from **16** (25.0 mg, 0.022 mmol, 1.0 eq.) in MeOH (4.0 mL) to afford, after recrystallization in MeOH and diethyl ether, the pure compound **5** as a pale yellow powder (13.0 mg, 0.013 mmol, 60%). mp = 233-235 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.50 (1H, bs), 10.20 (1H, bs), 9.96 (1H, s), 9.22 (1H, bs), 8.69 (1H, bs), 8.30 (3H, bs), 8.22 (3H, bs), 8.02 (1H, bs), 8.00 (1H, s), 7.75 (1H, m), 7.61 (2H, m), 7.10 (1H, d, *J* = 8.8 Hz), 6.86 (2H, m), 4.59 (1H, m), 4.48 (1H, m), 4.46 (1H, m), 4.44 (1H, m), 4.19 (2H, m), 3.97-3.86 (2H, m), 3.84 (3H, s), 3.66 (1H, m), 3.15-3.09 (3H, m), 2.79 (2H, m), 2.49 (1H, m), 2.10 (1H, m), 2.06-2.05 (2H, m), 2.02 (3H, s), 1.89-1.63 (6H, m), 1.47-1.20 (8H, m), 0.87-0.82 (6H, m);^{13}C NMR (DMSO-d_6, 100 MHz): δ = 169.9, 169.5, 169.6, 169.4, 168.3, 163.6, 152.8, 149.9, 132.7, 128.6, 128.2, 123.6, 121.4, 120.9, 115.1, 112.4, 64.8, 61.5, 56.3, 53.8, 51.9, 50.5, 47.5, 42.6, 38.3, 38.1, 31.0, 29.7, 29.6, 29.4, 29.3, 26.1, 23.8, 23.0, 22.0, 19.5, 18.1, 17.3; IR (neat): ν_{max}= 3220, 2970, 1636, 1546, 1494, 1304, 1254 cm^1; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{40}H_{62}N_{11}O_9S$ 872.4453, found 872.4440; HPLC purity (Sunfire C18, 3.5 µm, H_2O + 0.1% form. ac./ACN – gradient 5-100% in 20 min): TR = 7.31 min, 97%.

acetic acid; tert-butyl N-[(3*R*,4*R*)-3-[(2*S*)-2-[[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-amino-pentyl]carbamoyl]pyrrolidin-1-yl]-1-[(2*S*)-2-[[(2*S*)-2-aminopropanoyl]amino]-3-methyl-butanoyl]-4-piperidyl]carbamate 6. Compound **22** (63.0 mg, 0.06 mmol, 1.0 eq.) was dissolved in MeOH (4.0 mL) and 10% Pd/C (10 mg) was added. The reaction was kept 2 hours under stirring and under a hydrogen atmosphere at room temperature. The catalyst was then removed over a celite pad and the solvent was evaporated under vacuum. The crude was diluted in a minimum amount of MeOH and 2.0 eq. of acetic acid were added. The amine was isolated as an acetate salt by precipitation in diethyl ether. The crude product was purified by recrystallization in MeOH and diethyl ether to afford the pure compound **6** (25.0 mg, 0.03 mmol, 50%) as a white powder. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.03 (1H, s), 8.25 (1H, bs), 8.03 (1H, s), 7.85-7.61 (2H, bs), 7.10 (1H, m), 6.94 (3H, bs); 4.47-4.32 (7H, m), 3.93-3.84 (5H, m), 3.33-2.64

(4H, m), 2.73 (2H, m), 2.24 (1H, m), 2.06 (1H, m), 2.02 (3H, s), 1.83-1-56 (8H, m), 1.43-1.23 (6H, m), 1.34 (9H, s), 1.09 (3H, s), 0.75, 0.67 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 174.2, 173.1, 172.5, 169.4, 168.0, 155.1, 152.8, 142.6, 138.9, 132.7, 123.4 121.6, 112.4, 78.1, 63.0, 58.7, 56.2, 53.4, 52.8, 50.5, 49.8, 43.4, 39.0, 38.6, 32.2, 31.6, 30.5, 28.6, 28.2, 27.5, 24.2, 23.8, 22.9, 22.4, 19.4, 17.9, 17.7; IR. HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{39}H_{65}N_{10}O_9$ 817.4936, found 817.4939; HPLC purity (X Bridge C18, 3.5 µm, H_2O + 0.1% formic acid /ACN – gradient 5-100% in 20 min): TR = 9.13 min, 86%.

(2*S*)-*N*-[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-amino-pentyl]-1-[(3*R*,4*R*)-4-amino-1-[(2*S*)-2-[[(2*S*)-2-aminopropanoyl]amino]-3-methyl-butanoyl]-3-piperidyl]pyrrolidine-2-carboxamide tetrahydrochloride 7. Compound **6** (20.0 mg, 0.02 mmol, 1.0 eq.) was dissolved in dioxane (2.0 mL) and a 4 N solution of HCl in dioxane (0.15 mL, 30.0 eq.) was added. The reaction was kept 2 hours under stirring at room temperature. Diethyl ether was then added to allow precipitation of product **7** that was isolated as the hydrochloride salt after filtration.. The crude product was purified by recrystallization in MeOH and diethyl ether to afford the pure compound **7** (12.0 mg, 0.014 mmol, 70%) as a white powder. mp = 226-228 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.60 (1H, s), 10.20 (1H, s), 10.00 (1H, s), 8.97 (3H, bs), 8.51 (1H, bs), 8.30 (1H, bs), 8.25 (3H, bs), 8.05 (3H, bs), 8.04 (1H, s), 7.71 (1H, m),7.11 (1H, d, *J* = 8.8 Hz), 4.52-4.44 (3H, m), 4.08 (1H, m), 3.89 (1H, m), 3.86 (3H, s), 3.39 (1H, m), 3.37 (1H, m), 3.12 (1H, m), 3.06 (1H, m), 2.73 (2H, m), 2.70 (1H, m), 2.69 (1H, m), 2.48 (1H, m), 2.09 (1H, m), 2.03 (3H, m), 1.90-1.59 (8H, m), 1.36-1.22 (7H, m), 0.84-0.78 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 171.3, 171.1, 169.7, 168.5, 168.0, 163.8, 153.2, 133.1, 124.2, 121.9, 120.9, 112.9, 65.2, 58.4, 56.7, 54.1, 50.5, 50.4, 48.2, 44.3, 42.5, 38.3, 37.9, 30.8, 30.5, 29.4, 28.4, 26.0, 24.2, 23.4, 22.0, 19.1, 17.6, 16.8; IR (neat): ν_{max}= 3235, 2967, 1634, 1547, 1494, 1252 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{34}H_{57}N_{10}O_7$ 717.4412, found 717.4409; HPLC purity (X Bridge C18, 3.5 µm, H_2O + 0.1% formic acid /ACN – gradient 1-60% in 20 min): TR = 7.75 min, 85%.

Procedure A for coupling reactions using HATU and HOAt: To a solution of the carboxylic acid (1.2 eq.) in dry DMF (10 mL) at 0°C under nitrogen atmosphere, were successively added collidine (6.0 eq.), HATU (2 eq.) and HOAt (2 eq.). The solution was let stirring at 0 °C for 1h. and then the solution of the amine (free or as a salt, 1 eq.) in DMF (5 mL) was added to the previous solution. The reaction mixture was stirred at room temperature overnight. The solvent was evaporated under vacuum and the residue was taken up with EtOAc or CH_2Cl_2. The organic phase was successively washed with water, saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, filtered and concentrated under vacuum to afford the crude product which was purified by column chromatography on silica gel.
Procedure B for coupling reactions using DMTMM: To a solution of the carboxylic acid (1 eq.) in dry DMF under nitrogen atmosphere and at 0ºC, were added DMTMM(Cl^-) (1,1 eq.) and NMM (4 eq.). After 30 min, a solution of the free amine or its salt (1 eq.), in DMF was added and the reaction mixture was stirred at 0°C for 1 h and at room temperature overnight. The solvent was evaporated under vacuum and the residue was taken up with EtOAc. The organic phase was successively washed with water, saturated $NaHCO_3$ and brine, dried over Na_2SO_4, filtered and concentrated under vacuum to afford the crude product which was purified by column chromatography column on silica gel using EtOAc 100% to EtOAc/MeOH 95:5 as eluent.

(3*R*,4*R*)-(9H-fluoren-9-yl)methyl 3-((*S*)-2-((((*S*)-1-(2-(5-acetamido-2-methoxybenzoyl)hydrazinyl)-6-(((benzyloxy)carbonyl)amino)-1-oxohexan-2-yl)oxy)carbonyl)pyrrolidin-1-yl)-4-(4-methylphenylsulfonamido)piperidine-1-carboxylate 11. Compound **11** was synthesized according to the general procedure **A** from compound **8** (452 mg, 0.93 mmol, 1.2 eq.) and compound **10** (454 mg, 0.77 mmol, 1.0 eq.) to give, after purification by column chromatography on silica gel using EtOAc 100% to EtOAc/MeOH 95:5 as eluent, compound **11** (713 mg, 0.67 mmol, 88%) as a white solid. mp = 113-115 °C; R_f = 0.60 (EtOAc/MeOH 90:10); ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.56 (1H, bs), 10.08 (1H, bs), 9.95 (1H, s), 8.19 (1H, bs), 8.01 (1H, s), 7.84 (2H, m),7.77 (1H, m), 7.72 (2H, d, *J* = 7.9 Hz), 7.56 (3H, m), 7.37 (2H, m), 7.35 (2H, m), 7.34 (5H, m), 7.30 (2H, m),7.20 (1H, bs), 7.11 (1H, m), 5.00 (2H, s), 4.54 (1H, m), 4.36 (2H, m), 4.24 (1H, m), 3.86 (1H, m), 3.84 (3H, s), 3.83 (1H, m), 3.57 (1H, m), 3.21 (1H, m), 3.00 (2H, m), 2.79 (1H, m), 2.48 (1H, m), 2.36 (3H, s), 2.23 (1H, m), 2.03 (3H, s), 2.01 (1H, m), 1.83 (1H, m),1.73 (2H, m), 1.72 (1H, m), 1.69 (1H, m H_{11}), 1.64 (1H, m), 1.57 (1H, m), 1.45 (2H, m H_{24}), 1.43 (1H, m), 1.35 (2H, m), 1.06 (1H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 170.8, 168.4, 163.7, 156.5, 154.5, 153.2, 152.8, 143.8, 142.6, 140.8, 138.7, 137.3, 132.7, 129.6, 128.3,

128.2, 127.1, 126.5, 124.8, 123.7, 121.4, 120.7, 120.1, 112.5, 66.9, 65.1, 58.9, 56.5, 56.1, 53.4, 50.3, 47.1, 44.9, 41.9, 40.1, 32.8, 32.3, 32.0, 30.9, 29.2, 24.7, 23.8, 23.2, 20.9; IR (neat): ν_{max}= 3294, 2943, 1656, 1610, 1521, 1450 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + Na]^+$ calcd for $C_{56}H_{64}N_8O_{11}NaS$ 1079.4313, found 1079. 4354.

(3*R*,4*R*)-(9H-fluoren-9-yl)methyl 3-((*S*)-2-(((*S*)-1-(2-(5-acetamido-2-methoxybenzoyl)hydrazinyl)-6-(((benzyloxy)carbonyl)amino)-1-oxohexan-2-yl)carbamoyl)pyrrolidin-1-yl)-4-(4-nitrophenylsulfonamido)piperidine-1-carboxylate 12. Compound **12** was synthesized following the procedure described according to method **A** from compound **9** (246 mg, 0.41 mmol, 1.2 eq) in DMF (5 mL) and compound **10** (235 mg, 0.34 mmol, 1.0 eq) in (10 mL) to give after purification by column chromatography, compound **12** (239 mg, 0.22 mmol, 65%) as a pale yellow solid. mp = 142-144 °C: R_f = 0.50 (EtOAc/MeOH 95:5); ^{1}H NMR (DMSO-d_6, 400 MHz): δ = (1H, bs), 10.08 (1H, bs), 9.95 (1H, s),8.36 (2H, d, *J* = 8.8 Hz), 8.26 (1H, bs), 8.09 (2H, d, *J* = 8.7 Hz), 8.01 (1H, s), 7.88 (2H, m), 7.83 (2H, m), 7.73 (1H, m), 7.42 (2H, m), 7.36-7.34 (7H, m), 7.24 (1H, bs), 7.13 (1H, m), 6.28 (1H, bs),5.00 (2H, s), 4.54 (1H, m), 4.35 (2H, m), 4.21 (1H, m), 3.85 (3H, s), 3.38 (1H, m), 3.20 (1H, m), 3.01(2H, m), 2.94(1H, m), 2.70 (1H, m), 2.46 (1H, m),2.37 (1H, m), 2.33 (1H, m), 2.02 (3H, s), 2.00 (1H, m),1.85-1.65 (6H, m), 1.59 (1H, m),1.44-1.23 (2H, m),1.42 (1H, m),1.32 (2H, m), 1.22 (1H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 174.6, 171.1, 168.1, 165.7, 163.4, 156.2, 152.8, 149.4, 147.6, 139.7,137.8, 137.3, 132.7, 129.2, 127.9, 127.9, 127.7, 127.3, 124.4, 123.5, 121.5, 121.3,120.4, 120.1, 112.6, 69.4, 66.8, 63.2, 59.6, 59.6, 54.5, 50.7, 47.0, 44.7, 44.3, 40.1, 34.6, 32.4, 32.0, 30.5, 29.0, 24.5, 23.8, 22.8; IR (neat): ν_{max} =3298, 2934, 1693, 1528, 1477, 1449, 1347 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{55}H_{62}N_9O_{13}S$ 1088.4188, found 1088.4180; m/z $[M + Na]^+$ calcd for $C_{55}H_{61}N_9O_{13}NaS$ 1110.4007, found 1110.4021.

tert-butyl *N*-[(1*S*)-1-[(3*R*,4*R*)-3-[(2*S*)-2-[[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-(benzyloxycarbonylamino)pentyl]carbamoyl]pyrrolidin-1-yl]-4-(p-tolylsulfonylamino)piperidine-1-carbonyl]-2-methyl-propyl]carbamate 13. The *N*-Fmoc protected compound **11** (682 mg, 0.64 mmol, 1.0 eq.) was dissolved in a 20% solution of piperidine in DMF (10 mL) and let under stirring at room temperature for 2 h. After evaporation of DMF and piperidine in excess, the free amine was coupled with Boc-NH-Val-OH (278 mg, 1.28 mmol, 2.0 eq.) in DMF (10.0 mL) according to the procedure **A** to give, after purification by column chromatography on silica gel using EtOAc 100%, then EtOAc/MeOH 95:5 as eluent, compound **13** (400 mg, 0.39 mmol, 60%) as a white solid. mp = 151-153 °C; R_f = 0.55 (EtOAc/MeOH 90:10); ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.55 (1H, bs), 10.04 (1H, bs), 9.95 (1H, s), 8.21 (1H, bs), 8.00 (1H, s), 7.77 (1H, m), 7.73 (2H, d, *J* = 7.8 Hz), 7.57 (1H, bs), 7.38 (2H, m), 7.36-7.34 (5H, m), 7.21 (1H, bs), 7.12 (1H, m), 6.70 (1H, s), 5.01 (2H, s), 4.52 (1H, m), 4.32 (1H, m), 4.11 (1H, m), 3.88 (1H, m), 3.86 (3H, s), 3.37 (1H, m), 3.29 (1H, m), 3.00 (2H, m), 2.95 (1H, m), 2.64 (1H, m), 2.36 (3H, s), 2.21 (1H, m), 2.05 (1H, m), 2.02 (3H, s), 1.85 (3H, m), 1.82-1.58 (5H, m), 1.45 (2H, m), 1.40 (1H, m),1.35 (2H, m),1.33 (9H, s), 1.25 (1H, m), 0.79-0.72 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 175.7, 169.9, 168.0, 163.1, 162.1, 156.5, 156.1, 152.8, 142.6, 138.4, 137.3, 132.7, 129.6, 128.3, 127.7, 126.6, 126.0, 123.3, 121.5, 120.9, 112.5, 77.1, 65.1, 62.6, 58.0, 55.8, 54.7, 52.8, 50.0, 42.4, 39.9, 39.2, 32.8, 31.8, 31.6, 29.8, 29.5, 28.5, 28.1, 23.6, 23.8, 22.3, 20.9,19.1, 17.8; IR (neat): ν_{max}= 3280, 2938, 2870, 1652, 1520, 1454 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{51}H_{72}N_9O_{12}S$ 1034.5021, found 1034.5016.

[4-[[(3*R*,4*R*)-3-[(2*S*)-2-[[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5(benzyloxycarbonylamino)pentyl]carbamoyl]pyrrolidin-1-yl]-1-[(2*S*)-2-(tert-butoxycarbonylamino)-3-methyl-butanoyl]-4-piperidyl]sulfamoyl]phenyl]azinic acid 14. The Fmoc protected compound **12** (209 mg, 0.19 mmol, 1.0 eq.) was dissolved in a 20% solution of piperidine in DMF (10 mL) and the reaction mixture was let stirring at room temperature for 2 h. After concentration, the free amine in DMF (5 mL) was coupled with Boc-NH-Val-OH (83 mg, 0.38 mmol, 2.0 eq.) was dissolved in DMF (10.0 mL) following the procedure described according to method **A** to give after purification by column chromatography, compound **14** (152 mg, 0.14 mmol, 75%) as a pale yellow solid. mp = 146-148 °C; R_f = 0.60 (EtOAc/MeOH 90:10); ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.54 (1H, bs), 10.06 (1H, bs),9.95 (1H, s), 8.38 (2H, d, *J* = 8.7 Hz), 8.25 (1H, bp), 8.12 (2H, d, *J* = 8.8 Hz), 8.03 (1H, s), 7.72 (1H, m), 7.36-7.34 (5H, m),7.21 (1H, bs), 7.12 (1H, m), 6.72 (1H, s), 6.28 (1H, bs),4.99 (2H, s), 4.51 (1H, m), 4.32 (1H, m),4.09 (1H, m), 3.85 (4H, m), 3.39 (2H, m), 2.99 (3H, m), 2.66 (1H, m), 2.27 (1H, m), 2.05 (1H, m), 2.02 (3H, s), 1.90 (1H, m),1.82-1.60 (7H, m), 1.44-1.40 (3H, m), 1.32 (11H, m), 1.28 (1H, m), 0.79-0.72 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 173.6, 172.1, 170.1, 168.0, 164.0, 156.8, 156.1, 153.4, 150.0, 147.6, 137.4, 133.3, 127.7, 128.3, 127.7,126.6, 124.5, 123.9, 123.3, 121.1, 112.0, 78.0, 65.1,

62.7, 58.6, 56.2, 55.0, 53.5, 50.1, 43.3, 40.1, 39.8, 33.0, 31.9, 31.7, 30.4, 28.7, 28.1, 24.2, 23.8, 22.7, 21.1, 19.4, 18.0; IR (neat): ν_{max} =3296, 2939, 1684, 1527, 1477, 1450, 1348 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{50}H_{69}N_{10}O_{14}S$ 1065.4715, found 1065.4714; m/z $[M + Na]^+$ calcd for $C_{50}H_{68}N_{10}O_{14}NaS$ 1087.4535, found 1087.4546.

tert-butyl *N*-[(1*S*)-2-[[(1*S*)-1-[(3*R*,4*R*)-3-[(2*S*)-2-[[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-(benzyloxycarbonylamino)pentyl]carbamoyl]pyrrolidin-1-yl]-4-(p-tolylsulfonylamino)piperidine-1-carbonyl]-2-methyl-propyl]amino]-1-methyl-2-oxo-ethyl]carbamate 15. To a solution of the N-Boc protected compound **13** (256 mg, 0.25 mmol, 1.0 eq.) in DCM (5.0 mL) was added TFA (0.56 mL, 7.5 mmol, 30.0 eq.) and the reaction mixture was let under stirring at room temperature for 2 h. The solvent was evaporated, toluene (2 x 10 mL) was added followed by evaporation, and then ether was added and evaporated to afford the corresponding TFA salt. This TFA salt in solution in DMF (5.0 mL) in the presence of collidine (0.13 mL, 1.0 mmol, 4.0 eq.) was coupled with Boc-NH-Ala-OH (95 mg, 0.5 mmol, 2.0 eq.) following the procedure **A** to give after purification by column on silica gel using EtOAc 100% then EtOAc/MeOH 95:5 as eluent, compound **15** (228 mg, 0.21 mmol, 83%) as a white solid. mp = 149-151 °C: R_f = 0.25 (EtOAc/MeOH 95:5); ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.58 (1H, bs); 10.06 (1H, bs), 9.95 (1H, s), 8.22 (1H, bs,), 8.01 (1H, s), 7.76 (1H, m), 7.74 (2H, m), 7.63 (1H, bs), 7.58 (1H, bs),7.37 (2H, m), 7.34-7.29 (5H, m), 7.21 (1H, bs), 7.12 (1H, m), 6.95 (1H, bs), 5.00 (2H, s), 4.52 (1H, m), 4.48 (1H, m), 4.33 (1H, m), 3.95 (1H, m), 3.86 (1H, m), 3.85 (3H, s), 3.37 (1H, m) 3.29, (1H, m), 3.00 (2H, m), 2.98 (1H, m), 2.65 (1H, m), 2.36 (3H, s), 2.24 (1H, m), 2.04 (1H, m), 2.03 (3H, s),1.85 (3H, m), 1.81-1.71 (2H, m), 1.67 (1H, m), 1.59 (1H, m), 1.44 (2H, m), 1.43 (1H, m),1.34 (2H, m), 1.35 (9H, s), 1.28 (1H, m),1.26 (1H, m),1.12 (3H, s), 0.74-0.72 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 174.5, 172.5, 170.5, 169.3, 168.0, 163.1, 156.1, 155.0, 152.8, 142.7, 138.5, 137.3, 132.7, 129.6, 128.3, 127.7, 126.6, 126.9, 123.7, 121.5, 120.6, 112.5, 78.1, 65.1, 62.9, 58.4, 56.2, 53.2, 52.8, 50.4, 49.8, 43.8, 40.3, 39.9, 33.7, 33.6, 31.8, 30.7, 30.2, 29.9, 28.2, 24.5, 23.8, 22.7, 20.9, 19.4, 17.5, 17.9; IR (neat): ν_{max} = 3298, 2932, 1652, 1626, 1518, 1455 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{54}H_{77}N_{10}O_{13}S$ 1105.5392, found 1105.5410; m/z $[M + Na]^+$ calcd for $C_{54}H_{76}N_{10}O_{13}NaS$ 1127.5212, found 1127.5217; HPLC purity (Sunfire C18, 3.5 µm, H_2O + 0.2% form. ac./ACN – gradient 5-100% in 20 min): TR = 17.83 min, 99%.

[4-[[(3*R*,4*R*)-3-[(2*S*)-2-[[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-(benzyloxycarbonylamino)pentyl]carbamoyl]pyrrolidin-1-yl]-1-[(2*S*)-2-[[(2*S*)-2-(tert-butoxycarbonylamino)propanoyl]amino]-3-methyl-butanoyl]-4-piperidyl]sulfamoyl]phenyl]azinic acid 16. To a solution of the N-Boc protected compound **14** (125 mg, 0.12 mmol, 1.0 eq.) in DCM (8.0 mL) was added TFA (0.45 mL, 6.0 mmol, 50.0 eq.) and the reaction mixture was let stirring at room temperature for 2 h. The solvent was evaporated, then toluene (2 x 10 mL)) was added followed by evaporation, and then ether was added and evaporated to afford the corresponding TFA salt. The solution of the previous TFA salt and collidine (0.10 mL, 0.72 mmol, 6.0 eq.) in DMF (5.0 mL) was coupled with Boc-NH-Ala-OH (46 mg, 0.24 mmol, 2.0 eq.) following the procedure described according to method **A** to give after purification by column chromatography, compound **16** (100 mg, 0.09 mmol, 75%) as a pale yellow solid. mp = 148-150 °C; R_f = 0.55 (EtOAc/MeOH 95:5; ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.54 (1H, bs), 10.05 (1H, bs), 9.94 (1H, s), 8.40 (2H, d, J = 8.5 Hz), 8.24 (1H, bs), 8.11 (2H, d, J = 8.5 Hz), 8.09 (1H, bp), 8.04 (1H, s), 7.71 (1H, m),7.63 (1H, bp), 7.34 (5H, m), 7.22 (1H, bs), 7.12 (1H, m), 6.95 (1H, bs),5.00 (2H, s), 4.51 (1H, m), 4.46 (1H, m), 4.32 (1H, m), 3.95 (1H, m), 3.85 (4H, m), 3.69 (1H, m), 3.39 (1H, m), 3.38 (1H, m), 3.01 (2H, m), 2.96 (1H, m), 2.30 (1H, m), 2.04 (1H, m), 2.02 (3H, s), 1.90-1.62 (8H, m), 1.43-1.42 (3H, m), 1.36 (9H, s), 1.33 (2H, m), 1.29 (1H, m), 1.10 (3H, s), 0.77-0.72 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 174.6, 172.7, 172.3, 170.7, 169.6, 168.3, 163.8, 156.1, 153.1, 149.5, 147.4, 137.3, 132.7, 127.9, 127.8, 124.5, 123.4, 121.4, 120.6, 112.5, 78.4, 65.1, 63.1, 58.5, 56.2, 53.4, 52.6, 50.2, 49.6, 43.3, 40.1, 39.5, 33.0, 31.9, 31.6, 30.3, 30.2, 28.9, 28.1, 24.2, 23.8, 22.7, 19.2, 17.5, 17.8; IR (neat): ν_{max} = 3297, 2933, 1653, 1527, 1494, 1454, 1348 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{53}H_{74}N_{11}O_{15}S$ 1136.5087, found 1136.5095; Anal. calcd for $C_{53}H_{73}N_{11}O_{15}S.1.5\ H_2O$: C 54.72, H 6.60, N 13.25; found C 54.70, H 6.25, N 12.41; HPLC purity (Sunfire C18, 3.5 µm, H_2O + 0.2% form. ac./ACN – gradient 5-100% in 20 min): TR = 17.68 min, 100%.

(*S*)-6-(2-(5-acetamido-2-methoxybenzoyl)hydrazinyl)-5-((*S*)-1-((3*R*,4*R*)-1-((*S*)--2-((*S*)-2-((tert-butoxycarbonyl)amino)propanamido)-3-methylbutanoyl)-4-(4-methylphenylsulfonamido)piperidin-3-yl)pyrrolidine-2-carboxamido)-6-oxohexan-1-aminium acetate 17.
Compound **15** (64.0 mg, 0.058 mmol, 1.0 eq.) was dissolved in MeOH (4.0 mL) and 10% Pd/C (13 mg) was added. The reaction was kept 2 hours under stirring in hydrogen atmosphere at room temperature. The catalyst was then removed over a celite pad and the solvent evaporated under vacuum. The crude was diluted in a minimum amount of MeOH and 1.0 eq. of acetic acid were added. Product **17** was isolated as the acetate salt by precipitation in diethyl ether. The crude product was purified by crystallization in MeOH and diethyl ether to afford the pure compound **17** as a white powder (52.0 mg, 0.050 mmol, Yield 87%). ^{1}H NMR (DMSO-*d6*, 400 MHz): δ = 10.00 (1H, bs), 8.27-8.18 (2H, bs), 8.05 (1H, s), 7.74 (3H, m), 7.65 (1H, bs), 7.35 (2H, m), 7.11 (1H, m), 6.95 (1H, bs), , 4.48-4.33 (3H, m), 3.94-3.63 (5H, m), 3.34-3.30 (2H, m), 2.95 (1H, m), 2.74 (2H, m), 2.64 (1H, m), 2.36-2.23 (4H, m), 2.04-2.02 (4H, m), 1.83-1.56 (9H, m); 1.431.27 (13H, m), 1.10-1.07 (4H, m), 0.80-0.70 (6H, m); ^{13}C NMR (DMSO-*d6*, 400 MHz): : δ = 174.2, 173.0, 172.5, 169.4, 168.0, 162.3, 155.1, 152.8, 142.6, 138.9, 132.7, 129.6, 126.5, 123.4, 121.6, , 112.4, 111.6, 78.1, 62.9, 58.5, 56.2; 53.2, 52.8, 50.5, 49.8, 43.4, 39.3, 38.9, 33.2, 32.2, 31.6, 30.4, 30.3, 28.2, 27.5, 24.2, 23.8, 22.4, 20.9, 19.4, 17.9, 17.7; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ Calcd. for C46H70N10O11S 971.5025, found: 971.5021; calcd for $[M + Na]^+$ 993.4844, found: 993.4814; HPLC purity (SUNFIRE C18, 3:5 µm, H2O + 0:2% form. ac./ACN, gradient 5–100% in 20 min): TR = 11:12 min, 82%.

tert-butyl *N*-[(1*S*)-1-[[(1*S*)-2-[[(1*S*)-1-[(3*R*,4*R*)-3-[(2*S*)-2-[[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-(benzyloxycarbonylamino)pentyl]carbamoyl]pyrrolidin-1-yl]-4-(p-tolylsulfonylamino)piperidine-1-carbonyl]-2-methyl-propyl]amino]-1-methyl-2-oxo-ethyl]carbamoyl]-2-methyl-propyl]carbamate 18. To a solution of the *N*-Boc protected compound **15** (228 mg, 0.21 mmol, 1.0 eq.) in DCM (5.0 mL) was added TFA (0.46 mL, 6.19 mmol, 30.0 eq.) and the reaction mixture was let stirring at room temperature for 2 h. The solvent was evaporated, toluene (2 x 10 mL) was added followed by evaporation, and then ether was added and evaporated to afford the corresponding TFA salt. The solution of the previous TFA salt and collidine (0.11 mL, 0.84 mmol, 4.0 eq.) in DMF (5.0 mL) was coupled with Boc-NH-Val-OH (91 mg, 0.42 mmol, 2.0 eq.) following the procedure described according to method **A** to give after purification by column chromatography, compound **18** (144 mg, 0.12 mmol, 57%) as a white solid. mp = 153-155 °C; R_f = 0.40 (EtOAc/MeOH 95:5); ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.56 (1H, bs), 10.05 (1H, bs), 9.95 (1H, s), 8.23 (1H, bs), 8.00 (1H, s), 7.87 (1H, bs), 7.86 (1H, bs), 7.76 (1H, m), 7.74 (2H, m), 7.68 (1H, bs), 7.37 (2H, m), 7.36-7.16 (6H, m), 7.12 (1H, m), 6.67 (1H, bs), 5.01 (2H, s), 4.52 (1H, m), 4.44 (1H, m), 4.30 (1H, m), 4.34 (1H, m), 3.87 (1H, m),3.85 (3H, s), 3.79 (1H, m), 3.38 (1H, m),3.29 (1H, m), 3.00 (2H, m), 2.96 (1H, m), 2.65 (1H, m), 2.36 (3H, s), 2.24 (1H, m), 2.05 (1H, m),2.03 (3H, s), 1.91 (2H, m), 1.84 (2H, m), 1.69 (2H, m),1.65 (1H, m),1.59 (1H, m), 1.43-1.23 (16H, m), 1.13 (3H, s), 0.83-0.73 (12H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 174.2, 171.8, 170.9, 170.5, 169.4, 168.0, 163.3, 156.1, 155.4, 152.8, 142.6, 138.6, 137.3, 132.7, 129.6, 128.3, 127.7, 126.5, 126.6, 123.7, 121.5, 120.6, 112.5, 78.0, 65.1, 63.3, 59.4, 58.9, 56.2, 53.6, 53.2, 50.3, 47.9, 43.8, 40.5, 40.0, 33.7, 32.1 30.8, 30.4, 30.0, 29.0, 28.1, 24.7, 23.8, 23.2, 20.9, 19.4, 17.9, 19.2, 17.7, 18.3; IR HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{59}H_{86}N_{11}O_{14}S$ 1204.6076, found 1204.6078; m/z $[M + Na]^+$ calcd for $C_{59}H_{85}N_{11}O_{14}NaS$ 1226.5896, found 1226.5890; HPLC purity (XBridge C18, 3.5 µm, H_2O + 0.2% form. ac./ACN – gradient 5-100% in 20 min): TR = 17.60 min, 90%.

(*S*)-methyl 1-((3*R*,4*R*)-1-((*S*)-2-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-3-methylbutanoyl)-4-((tert-butoxycarbonyl)amino)piperidin-3-yl)pyrrolidine-2-carboxylate 20. Compound **20** was synthesized following the procedure described according to method **A** from **19** (264 mg, 0.8 mmol, 1.0 eq.) in DMF (5 mL) and Fmoc-NH-Val-OH (543 mg, 1.6 mmol, 2.0 eq.) in DMF (5.0 mL) to give compound **20** (296 mg, 0.46 mmol, 58%) as a white solid. In this case purification by column chromatography on silica gel used c-Hex/EtOAc 6:4 as eluent. mp = 104-106 °C; R_f = 0.40 (c-Hex/EtOAc 6:4); ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 7.88 (2H, m); 7.74 (2H, m); 7.51 (1H, bs,); 7.41 (2H, m); 7.32 (2H, m); 6.45 (1H, bs); 4.31-4.21 (5H, m), 4.07 (1H, m), 3.81 (1H, m), 3.64 (4H, m), 3.07 (1H, m),2.96 (1H, m), 2.78 (1H, m), 2.57 (1H, m), 2.47 (1H, m), 2.04 (2H, m), 1.98 (1H, m),1.78 (2H, m), 1.65 (2H, m), 1.38 (9H, s), 0.88, 0.83 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 175.1, 169.9, 156.1, 155.2, 143.8, 140.7, 127.6, 127.0, 125.3, 120.0, 77.5, 65.7, 60.1, 59.7, 59.3, 55.4, 51.5, 46.7, 47.1, 44.2, 39.9, 31.1 29.8, 29.1, 28.2, 23.7, 19.5, 18.1; mp = 104-106 °C;

R_f = 0.40 (c-Hex/EtOAc 6:4); IR (neat): ν_{max} = 3324, 2967, 1707, 1506, 1219, 1166 cm^{-1}; ; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{36}H_{49}N_4O_7$ 649.3601, found 649.3597.

(*S*)-methyl 1-((3*R*,4*R*)-1-((*S*)-2-((*S*)-2-(((benzyloxy)carbonyl)amino)propanamido)-3-methylbutanoyl)-4-((tert-butoxycarbonyl)amino)piperidin-3-yl)pyrrolidine-2-carboxylate 21.The *N*-protected compound **20** (253 mg, 0.39 mmol, 1.0 eq.) was dissolved in a 20% solution of piperidine in DMF (10 mL) and the reaction mixture was let stirring at room temperature for 2 h. After concentration, the free amine in DMF (5 mL) was coupled with CbZ-NH-Ala-OH (174 mg, 0.78 mmol, 2.0 eq.) in DMF (5.0 mL) following the procedure described according to method **A** to give **21** (170 mg, 0.27 mmol, 69%) as a white solid. In this case purification by column chromatography on silica gel used c-Hex/EtOAc 6:4 as eluent. mp = 97-99 °C; R_f = 0.50 (c-Hex/EtOAc 6:4); ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 7.82 (1H, bs), 7.42 (1H, bs),7.34-7.30 (5H, m), 6.49 (1H, bs), 5.01 (2H, s), 4.54 (1H, m), 4.28 (1H, m), 4.09 (1H, m), 3.83 (2H, m), 3.65 (1H, m), 3.60 (3H, s), 3.11 (1H, m), 2.95 (1H, m), 2.78 (1H, m), 2.57 (1H, m), 2.45 (1H, m), 2.01 (2H, m), 1.93 (1H, m), 1.80 (1H, m), 1.67 (2H, m), 1.38 (9H, s), 1.29 (1H, m), 1.18 (3H, d, *J* = 7.1 Hz), 0.82, 0.81 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 175.5, 172.2, 169.3, 156.7, 155.5, 137.0, 128.3, 128.0, 127.8, 77.7, 65.4, 60.2, 59.9, 59.0, 53.0, 51.5, 50.1, 46.8, 42.7, 39.9, 30.8, 30.3, 29.1, 28.2, 23.5, 18.1, 17.7, 18.0; IR (neat): ν_{max} =3306, 2970, 1708, 1624, 1504, 1453, 1221, 1167 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{32}H_{50}N_5O_8$ 632.3659, found 632.3663.

(*S*)-1-((3*R*,4*R*)-1-((*S*)-2-((*S*)-2-(((benzyloxy)carbonyl)amino)propanamido)-3-methylbutanoyl)-4-((tert-butoxycarbonyl)amino)piperidin-3-yl)pyrrolidine-2-carboxylic acid 22. Compound **21** (150 mg, 0.24 mmol, 1.0 eq.) was dissolved in MeOH (8.0 mL) and NaOH 2 M (0.6 mL, 1.2 mmol, 5.0 eq.) was added dropwise to the solution. The reaction was stirred at 60° C for 2 h. The solvent was evaporated and the solid obtained was solubilized in water. The mixture was acidified with 10% solution of $KHSO_4$ until pH = 2-3. A part of the product was extracted from the water phase with EtOAc and another part of the desired compound was recuperated by solving the solid residue of the water phase with MeOH. The two combined organic phases were dried over Na_2SO_4 and concentrated under vacuum to yield compound **22** (137 mg, 0.22 mmol, 92%) as a white solid. mp = 129-131 °C; ^{1}H NMR (DMSO-d_6, 30 MHz): δ = 8.31 (1H, bs), 7.87 (1H, bs), 7.44 (1H, bs), 7.35-7.22 (5H, m), 6.80 (1H, bs), 5.02 (2H, s), 4.55 (1H, m), 4.28 (1H, m), 4.09 (1H, m), 3.84-3.81 (2H, m), 3.57 (1H, m), 3.13 (1H, m), 3.04 (1H, m), 2.82 (1H, m), 2.56 (1H, m), 2.49 (1H, m), 2.06 (1H, m), 1.91 (1H, m), 1.83 (1H, m), 1.71 (1H, m), 1.62 (1H, m), 1.38 (9H, s), 1.24 (2H, m), 1.18 (3H, d, *J* = 7.1 Hz), 0.82- 0.76 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 178.7, (C_6); 172.1, 169.4, 156.5, 155.5, 136.9, 127.8, 127.4, 126.4, 77.7, 65.0, 61.4, 61.0, 59.6, 52.7, 49.6, 46.9, 42.8, 39.8, 29.8, 29.6, 27.9, 28.6, 23.8, 19.2, 17.4, 17.9; IR (neat): ν_{max} =1702, 1627, 1547, 1453, 1225, 1160, cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{31}H_{48}N_5O_8$ 618.3503, found 618.3514; m/z $[M + Na]^+$ calcd for $[M + Na]^+$ $C_{31}H_{47}N_5O_8Na$ 640.3322, found 640.3322.

***tert*-butyl *N*-[(3*R*,4*R*)-3-[(2*S*)-2-[[(1*S*)-1-[[(5-acetamido-2-methoxy-benzoyl)amino]carbamoyl]-5-(benzyloxycarbonylamino)pentyl]carbamoyl]pyrrolidin-1-yl]-1-[(2*S*)-2-[[(2*S*)-2-(benzyloxycarbonylamino)propanoyl]amino]-3-methyl-butanoyl]-4-piperidyl]carbamate 23.**

Compound **23** was synthesized following the procedure described according to method **B** from compound **22** (132 mg, 0.22 mmol, 1.2 eq.) solved in DMF (5.0 mL) and compound **10** (108 mg, 0.18 mmol, 1.0 eq.) in DMF (5.0 mL) to give after purification by column chromatography, compound **23** (114 mg, 0.11 mmol, 61%) as a white solid. mp = 130-132 °C; R_f = 0.50 (EtOAc/MeOH 9:1); ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 10.64 (1H, bs); 10.03 (1H, bs), 9.96 (1H, s), 8.01 (1H, s), 7.95-7.69 (3H, m), 7.41 (1H, d, *J* = 7.7 Hz), 7.30-7.34 (10H, m), 7.21 (1H, bs), 7.11 (1H, t, *J* = 8.9 Hz), 6.84 (1H, bs), 5.00 (4H, s), 4.55-4.38 (3H, m), 4.10 (1H, m), 3.97 (1H, m); 3.84 (3H, s), 3.78 (1H, m), 3.63 (1H, m), 3.31 (1H, m), 3.09 (2H, m); 2.99 (2H, m), 2.79-2.63 (2H, m), 2.07 (1H, s); 2.02 (3H, s); 1.95-1.66 (6H, m); 1.53-1.44 (3H, m), 1.37-1.33 (11H, s), 1.22 (1H, m); 1.14 (3H, d, *J* = 7.2 Hz), 0.75- 0.66 (6H, m); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 172.4, 172.2, 171.9, 169.4, 162.5, 156.2, 155.7, 155.3, 152.9, 137.3, 137.1, 132.8, 128.4, 127.7, 127.3, 123.9, 121.6, 120.4, 112.6, 78.4, 65.4, 62.4, 61.2, 55.8, 53.2, 52.8, 50.1, 49.3, 49.6, 45.1, 43.7, 40.3, 39.1, 32.6, 32.0, 30.0, 29.7, 28.7, 23.4, 27.7, 24.3, 22.7, 19.1, 17.1, 17.8; IR (neat): ν_{max} = 3297, 2935, 2870, 1658, 1625, 1520, 1455, 1245 cm^{-1}; HRMS (TOF ESI, ion polarity positive): m/z $[M + H]^+$ calcd for $C_{55}H_{77}N_{10}O_{13}$ 1085.5672, found 1085.5688; HPLC purity (X Bridge C18, 3.5 µm, H_2O + 0.1% formic acid /ACN – gradient 5-100% in 20 min): TR = 16.38 min, 94%.

NMR Conformational studies

Proton NMR spectra were recorded on Bruker spectrometers operating at 600 MHz for **3** and at 800 MHz for **4**, equipped with a TCI cryoprobe (ICSN, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette). ^{1}H and ^{13}C resonances were completely assigned using 1D ^{1}H WATERGATE, 2D ^{1}H-^{1}H TOCSY (mixing time set to 30, 60 or 100 ms), 2D ^{1}H-^{1}H ROESY (200 ms mixing time), 2D ^{1}H-^{13}C HSQC and 2D ^{1}H-^{13}C HMBC spectra, recorded at 278 K and 298 K. ^{1}H and ^{13}C chemical shifts were calibrated using the solvent residual peak (H_2O/D_2O, δ ^{1}H 4.70 ppm). The chemical shifts deviations were calculated as the differences between observed chemical shifts and random coil values reported in water reported by Wishart *et al.* (^{1}H, ^{13}C and ^{15}N random coil NMR chemical shifts of the common amino acids).[19e] Vicinal coupling constants were extracted from 1D ^{1}H spectrum and from 2D ^{1}H-^{1}H COSY at 278 K and 298 K.

Restrained molecular simulations

Simulated annealing were performed using the GROMACS package (version 5.0.2) [22], with the Generalized Amber Force Field [23]. The aqueous solvent was implicitly modelled by a continuum medium with a dielectric constant of 80. Compound topologies and parameters were generated for GROMACS using the ACPYPE tool [24]. Simulated annealings consisted in running molecular dynamics (MD) simulations at a temperature linearly decreasing from 900 to 300 K over a period of 10 ns. Then the compounds were simulated at constant temperature (300 K) using a standard MD protocol over an additional period of 10 ns for analysis.

Fluorescence-detected Thioflavin-T binding assay

Thioflavin T was obtained from Sigma. $A\beta_{1-42}$ was purchased from American Peptide. The peptide was dissolved in an aqueous 1% ammonia solution to a concentration of 1 mM and then, just prior to use, was diluted to 0.2 mM with 10 mM Tris-HCl, 100 mM NaCl buffer (pH 7.4). Stock solutions of β-hairpin compounds and fragments were dissolved in DMSO with the final concentration kept constant at 0.5% (v/v). Thioflavin T fluorescence was measured to evaluate the development of $A\beta_{1-42}$ fibrils over time using a fluorescence plate reader (Fluostar Optima, BMG labtech) with standard 96-well black microtiter plates. Experiments were started by adding the peptide (final $A\beta_{1-42}$ concentration equal to 10 µM) into a mixture containing 40 µM Thioflavin T in 10 mM Tris-HCl, 100 mM NaCl buffer (pH 7.4) with and without the tested compounds at different concentrations (100, 10 and 1 µM for the compounds active at 10 µM) at room temperature. The ThT fluorescence intensity of each sample (performed in triplicate) was recorded with 440/480 nm excitation/emission filters set for 42 hours performing a double orbital shaking of 10 s. before the first cycle. The fluorescence assays were performed between 2 and 4 times on different days, with the same batch of peptide. The ability of compounds to inhibit $A\beta_{1-42}$ aggregation was assessed considering both the time of the half-life of aggregation ($t_{1/2}$) and the intensity of the experimental fluorescence plateau (F). The $t_{1/2}$ extension is defined as the experimental $t_{1/2}$ in the presence of the tested compound relative to the one obtained without the compound and is evaluated as the following percentage: $t_{1/2}$ ($A\beta_{1-42}$+compound) – $t_{1/2}$($A\beta_{1-42}$) / $t_{1/2}$($A\beta_{1-42}$) X 100. The F reduction is defined as the intensity of experimental fluorescence plateau observed with the tested compound relative to the value obtained without the compound and is evaluated as the following percentage : ($FA\beta_{1-42}$+compound – $FA\beta_{1-42}$) / $FA\beta_{1-42}$ X 100.

Transmission electron microscopy

Samples were prepared under the same conditions as in the ThT-fluorescence assay. Aliquots of $A\beta_{1-42}$ (10 µM in 10 mM Tris-HCl, 100 mM NaCl, pH 7.4 in the presence and absence of the tested compound) were adsorbed onto 300-mesh carbon grids for 2 min, washed and dried. The samples were negatively stained for 45 s. on 2% uranyl acetate in water. After draining off the excess of staining solution and drying, the grids were observed using a JEOL 2100HC TEM operating at 200 kV with a LaB6 filament. Images were recorded in zero-loss mode with a Gif Tridiem energy-filtered-CCD camera equipped with a 2k x 2k pixel-sized chip (Gatan inc., Warrendale, PA). Acquisition was accomplished with the Digital Micrograph software (versions 1.83.842, Gatan inc., Warrendale, PA).

Capillary electrophoresis

Sample preparation: the commercial $A\beta_{1-42}$ was dissolved upon reception in 0.16% NH_4OH (at 2 mg/mL) for 10 minutes at 20°C, followed by an immediate lyophilization. The dried sample was then stored at -20°C until use.
CE experiments were carried out with a PA800 ProteomeLab instrument (Beckman Coulter Inc., Brea, CA, USA) equipped with a photodiode array detector. UV Detection was performed at 190 nm. The prepared sample (as previously described) was reconstituted by dissolution in 20 mM phosphate buffer pH 7.4 containing DMSO (control or stock solutions of the evaluated compound dissolved in DMSO). A constant DMSO/phosphate buffer ratio of 2.5% (v/v) was used for each sample. The final peptide concentration was set at 100 µM regardless the peptide/compound ratio.
For the CE separation of Aβ oligomers, fused silica capillary 80 cm (10.2 cm to the detector) ´ 50 mm I.D. were used. The background electrolyte was a 80 mM phosphate buffer, pH 7.4.
The separation was carried out under -30 kV at 20°C. The sample was injected from the outlet by hydrodynamic injection at 3.44 KPa for 10 s. After each run, the capillary was rinsed for 5 min with water, 1 min with SDS 50 mM, 5 min with NaOH 1 M and equilibrated with running buffer for 5 min.

Acknowledgment

The Ministère de l'Enseignement Supérieur et de la Recherche (MESR) is thanked for financial support for N. Tonali. Marianna Munafo (BioCIS, UMR 8076, Erasmus student from the Università degli Studi di Milano) is thanked for her help with the synthesis. Ghislaine Frébourg (Institut de Biologie Paris Seine (IBPS)/FR 3631, Service de Microscopie Electronique, Université Pierre et Marie Curie (UPMC), France) is thanked for her contribution to cryo-electron microscopy. Camille Dejean (BioCIS, UMR 8076) and Jean-François Gallard (ICSN, UPR2301) are thanked for their help with the NMR experiments. The Laboratory BioCIS is a member of the Laboratory of Excellence LERMIT supported by a Grant from ANR (ANR-10-LABX-33).

References

1 a) M. Stefani, C. M. Dobson, *J. Mol. Med*., 2003, *81*, 678–699; b) F. Chiti, C. M. Dobson, *Annu. Rev. Biochem*., 2006, *75*, 333–366; c) A. Aguzzi, T. O'Connor, *Rev. Drug Discov*., 2010, *9*, 237-248; d) Y. S. Eisele, C. Monteiro, C. Fearns, S. E. Encalada, R. L. Wiseman, E. T. Powers, J. W. Kelly, *Nat. Rev.*, 2015, *14*, 759-780; e) P. C. Ke, M.-A. Sani, F. Ding, A. Kakinen, I. Javed, F. Separovic, T. P. Davis,R. Mezzenga, *Chem. Soc. Rev*., 2017, *46*, 6492-6531.
2 a) M. Goedert, M. G. A. Spillantini, *Science*, 2006, *314*, 777-780; b) C. Haas, D. J. Selkoe, *Nat. Rev. Mol. Cell. Biol.*, 2007, *8*, 101-112.
3 https://www.alz.co.uk/research/WorldAlzheimerReport2016.pdf
4 a) D. Goyal, S. Shuaib, S. Mann, B. Goyal, *ACS Comb. Sci.* 2017, *19*, 55−80; b) H. Coman, B. Nemes, *Int. J. Gerontol.*, 2017, *11* , 2-6; c) E. McDade, R. J. Bateman, *Nature*, 2017, *457*, 153-155.
5 a) F. Belluti, A. Rampa, S. Gobbi, *Exp. Opin. Ther. Pat.*, 2013, *23*, 581-596; b) T. Härd, C. Lendel, *J. Mol. Biol*., 2012, *421*, 441–465; c) A. J. Doig, P. Derreumaux, *Curr. Opin. Struct. Biol.*, 2015, *30*, 50–56.
6 a) J. Mayes, C. Tinker-Mill, O. Kolosov, H. Zhang, B. J. Tabner, D. Allsop, *J. Biol. Chem.*, 2014, *289*, 12052-12062; b) S. I. A. Cohen, S. Linse, L. M. Luheshi, E. Hellstrand, D. A. White, L. Rajah, D. E. Otzen, M. Vendruscolo, C. M. Dobson, T. P. J. Knowles, *Proc. Natl. Acad. Sci. U S A.* 2013, *110*, 9758-9763; c) M. J. Guerrero-Muñoza, D. L. Castillo-Carranza, R. Kayed, *Biochem. Pharm*., 2014, *88*, 468–478.
7 a) C. Haas, D.J. Selkoe, *Nat. Rev. Mol. Cell Biol*., 2007, 101-112; (b) K. Ono, M. M. Condron, D.B. Teplow, *Proc. Natl. Acad. Sci. U S A.*, 2009, *106*, 14745-14750; (c) P. Prangkio, E. C. Yusko, D. Sept, J. Yang, M. Mayer, *PLoS One*, 2012, *7*, e47261; (d) P. Cizas, R. Budvytytec, R. Morkuniene, R. Moldovan, M. Broccio, M. Lösche, G Niaura, G. Valincius, V. Borutait *Arch. Biochem. Biophys.*, 2010, *496*, 84-92; e) I. Benilova, E. Karran, B. De Strooper *Nat. Neurosci.*, 2012, *15*, 349-357.
8 Q. Nie,, X.Du, M. Geng, *Acta Pharmacol. Sin.*, 2011, *32*, 545−551.
9 a) P. Vlieghe, V. Lisowski, J. Martinez, M. Khrestchatisky, *Drug Discov. Today.*, 2010, *15*, 40-56; (b) F. Albericio, H.G. Kruger, *Future Med. Chem.*, 2012, 4, 1527-1531; c) T. Uhlig, T. Kyprianou, F. G. Martinelli, C. A. Oppici, D.

Heiligers, D. Hills, X. R. Calvo, P. Verhaert, *EuPA Open Proteom.*, 2014, 4, 58-69; d) Keld Fosgerau, and Torsten Hoffmann, *Drug Discov. Today.*, 2015, *20*, 122-128.
10 a) S. Aileen Funke, D. Willbold, *Curr Pharm Des*. 2012, *18*, 755–767; b) M. Chemerovski-Glikman, M. Richman, S. Rahimipour in *Top. Heterocycl. Chem.*, 2016, Publisher: Springer Berlin Heidelberg, 1-32**; c**) C. I. Stains, K. Mondal, I. Ghosh, *ChemMedChem*, 2007, *2*, 1674-1692; c) T. Takahashi, H. Mihara, *Acc. Chem. Res.*, 2008, *41*, 1309-1318; d) J. Kumar, R. Namsechi, V. L. Sim, *PLoS One*, 2015, *10*, e0129087; e) V. Parthsarathy, P.L. McClean, C. Hölscher, M. Taylor, C. Tinker, G. Jones, O. Kolosov, E. Salvati, M. Gregori, M. Masserini, D. Allsop, *PLoS One*, 2013; *8*, e54769; f) P.-N. Cheng, C. Liu, M. Zhao, D. Eisenberg, J. S. Nowick, *Nat. Chem*., 2012, *4*, 927-933; g) S. Brogi, S. Butini, S. Maramai, R. Colombo, L. Verga, C. Lanni, E. De Lorenzi, S. Lamponi, M. Andreassi, M. Bartolini, V. Andrisano, E. Novellino, G. Campiani, M. Brindisi, S. Gemma *CNS Neurosci Ther*. 2014, 20, 624-32.
11 a) G. Hopping, J. Kellock, B. Caughey, V. Daggett, *ACS Med. Chem. Lett*., 2013, *4*, 824−828; b) K. N. L. Huggins, M. Bisaglia, L. Bubacco, M. Tatarek-Nossol, A. Kapurniotu, N. H. Andersen, *Biochemistry*, 2011, *50*, 8202−8212; c) Y. Hamada, N. Miyamoto, Y Kiso, *Bioorg. Med. Chem. Lett.*, 2015, *25*, 1572–1576; d) L. Vahdati, D. Brinet, G. Bernadat, I. Correia, S. Panzeri, R. Fanelli, O. Lequin, M. Taverna, S. Ongeri, U. Piarulli, *Eur. J. Org. Chem*. 2017, 2971–2980.
12 S. Pellegrino, A. Contini, M. L. Gelmi, L. Lo Presti, R. Soave, E. Erba, *J. Org. Chem.*, 2014, *79*, 3094-3102.
13 S. Pellegrino, N. Tonali, E. Erba, J. Kaffy, M. Taverna, A. Contini, M. Taylor, D. Allsop, M. L. Gelmi, S. Ongeri *Chem. Sci.*, 2017, *8*, 1295 - 1302.
14 J. Kaffy, D. Brinet, J-L Soulier, I. Correia, N. Tonali, K. F. Fera, Y. Iacone, A. R. F. Hoffmann, L. Khemtemourian, B. Crousse, M. Taylor, D. Allsop, M. Taverna, O. Lequin, S. Ongeri, *J. Med. Chem.* 2016, *59*, 2025−2040.
15 P. E. Maligres, M. M. See, D. Askin, P. J. Reider, *Tet. Lett.*, 1997, *38*, 1586-1587.
16 T. Cierpicki and J. Otlewski, *J. Biomol. NMR.*, 2001, *21*, 249-261.
17 J. S. Nowick, K. S. Lam, T. V. Khasanova, W. E. Kemnitzer, S. Maitra, H. T. Mee, R. Liu, *J. Am. Chem. Soc.*, 2002, *124*, 4972–4973.
18 a) G.W. Vuister and A. Bax, *J. Am. Chem. Soc.* 1993, *115*, 7772-7777; b) F. Avbelj, S. G. Grdadolnik, J. Grdadolnik and R. L. Baldwin, *Proc. Natl. Acad. Sci. USA.*, 2006, *103*, 1272-1277.
19 a) L. Szilagyi and O. Jardetzky, *J. Magn. Reson*., 1989, 83, 441–449; b) S. Spera and A. Bax, *J. Am. Chem. Soc.*, 1991, *113*, 5490–5492; c) D. S. Wishart and B. D. Sykes, *Methods Enzymol.*, 1994, *239*, 363-392; d) D. S. Wishart and B. D. Sykes, *J. Biomol. NMR.*, 1994, *4*, 171–180; e) D. S. Wishart, G. C. Bigam, A. Holm, R. S. Hodges, B. D. Sykes., *J. Biomol. NMR.*, 1995, *5*, 67-81; f) L. J. Smith, K. M. Fiebig, H. Schwalbe, C.M. Dobson, *Fold. Des*., 1996, *1*, R95–R106.
20 H. LeVine, *Methods Enzymol.*, 1999, *309*, 274-284.
21 D. Brinet, J. Kaffy, F. Oukacine, S. Glumm, S. Ongeri, M. Taverna, *Electrophoresis*, 2014, *35*, 3302–3309.
22 M. J. Abraham, T. Murtola, R. Schulz, S. Páll, J.C. Smith, B. Hess, E. Lindahl, *SoftwareX*, 2015, *1-2*, 19–23.
23 J. Wang, R.M. Wolf, J. W. Caldwell, P. A. Kollman, D. A Case, *J. Comput. Chem.*, 2004, *25*, 1157-1174.
24 A. W. Sousa da Silva, W. F. Vranken, *BMC Research Notes*, 2012, *5*, 367.
25 E. F. Pettersen, T.D. Goddard, C. C. Huang, G. S. Couch, D. M. Greenblatt, E. C. Meng, T. E. Ferrin, *J. Comput. Chem.* 2004, *25*, 1605-1612.