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Fibril-forming model synthetic peptides containing 3-aminophenylacetic acid

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Abstract—The FT-IR, ^{1}H NMR, electrospray mass spectrometry studies of several dipeptides containing 3-APA (3-aminophenylacetic acid) and Aib/Val/Pro, revealed that all peptides share a common structural feature, an extended backbone conformation and they form the intermolecular hydrogen bonded supramolecular β-sheet structure in the solid state. The SEM images of all peptides exhibit amyloid-like fibrils, reminiscent of many neurodegenerative diseases like Alzheimer's, Prion-protein diseases.

1. Introduction

The design of small peptide-based molecules with extended β-strand conformations that self-assemble to form tubes, β-sheet tapes, fibrils is an active area of recent peptide research. Ghadiri and his co-workers have established that self-assembling cyclic peptides form hollow nanotubes, which can act as artificial ion channels and biosensors.[1] Recently, Zhang and his colleagues have shown that a self-assembling peptide scaffold can serve as a substrate for neurite outgrowth and synapse formation and this type of biologically compatible scaffold is also important for tissue repair and tissue engineering.[2] Higher order self-assembly of peptide molecules leads to the formation of fibrils and gels.[3] Recently, a nice theoretical model has been presented by Aggeli et al. describing the prediction of morphology and properties of self-assembled structures from peptide monomers and it has been verified by experimental studies.[4] The higher order molecular self-assembly of a peptide into a β-sheet structure is not only important for designing biomaterials, but also useful in studying pathogenesis of certain age-related disease causing fibrils where self-assembly of mis-folded proteins or protein fragments leads to the formation of the aggregated mass that is considered as amyloid fibrils.[5] The supramolecular β-sheet stabilization and consequent insoluble amyloid plaque formation are associated with several neurodegenerative diseases including Alzheimer's disease[6] and Prion-protein diseases.[7] The primary component of amyloid deposit varies from one disease to another: in Alzheimer's disease, it is the 42 residue Aβ peptide; in senile systemic amyloidosis it is the transthyretin protein; and in bovine spongiform encephalopathy it is the prion protein.[8] Despite substantial differences in sequences and lengths, these diverse proteins/protein-fragments self-assemble into amyloid fibrils that are resistant to enzymatic degradation and exhibit similar physicochemical properties (viz.: congophilicity, binds to thioflavin T). These amyloid fibrils consist of a cross β-sheet structure.[9] The therapeutic challenge in all forms of these fatal neurodegenerative diseases is to resist fibril formation. So, for designing the therapeutic agent against these diseases a detailed knowledge of the fibrillation process is required. Previously, many approaches have been pursued to establish the mechanism of fibrillogenesis of amyloid and human calcitonin fibrils.[10] However, many aspects of fibrillogenesis are still elusive.

The study of small, designed β-sheet forming peptide-based materials is important for their applications in biological and material sciences. In a recent report, we have demonstrated that a short peptide containing only non-coded amino acids has been self-assembled to form the amyloid-like fibril-forming supramolecular β-sheets in the solid state.[11] By utilizing non-coded amino acids, we have already established that in short model peptides the fibril-forming β-sheets are constructed via dimer formation and also via turn formation.[12]

The role of rigid templates for designing specific structural elements of proteins in de novo protein design is already well documented.[13] By utilizing stereochemically controlled unnatural amino acid residues (rigid/semi-rigid template), it is possible to make relatively short peptides with desired conformations such as α-helices,[13a,14] β-sheets.[13,15] Recently, we have demonstrated that a peptide and a pseudo-peptide containing 3-APA and N-substituted 3-APA, respectively, can adopt an extended backbone conformation which can self assemble to form supramolecular β-sheet structures in crystals.[16] Here we report a series of dipeptides containing 3-APA which form

Keywords: amyloid fibril; 3-aminophenylacetic acid; Aib; β-sheet.

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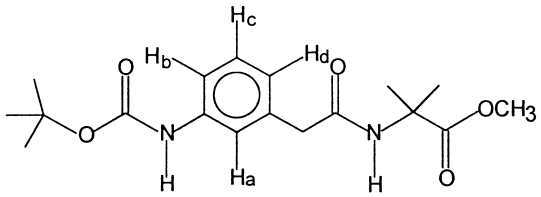
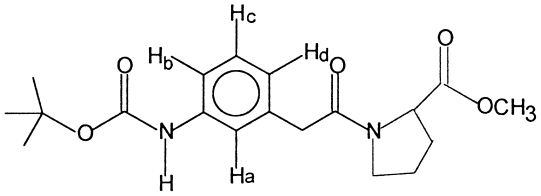
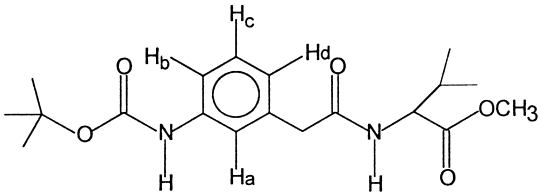


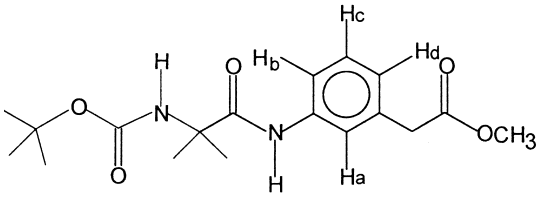
Peptide **1**



Peptide **2**



Peptide **3**



Peptide **4**

Figure 1. Schematic representation of peptides **1**–**4**.

supramolecular β-sheets and amyloid-like fibrils in the solid state.

2. Results and discussion

2.1. Solid state conformational analysis by FT-IR spectroscopy

The schematic representations of all dipeptides are shown in

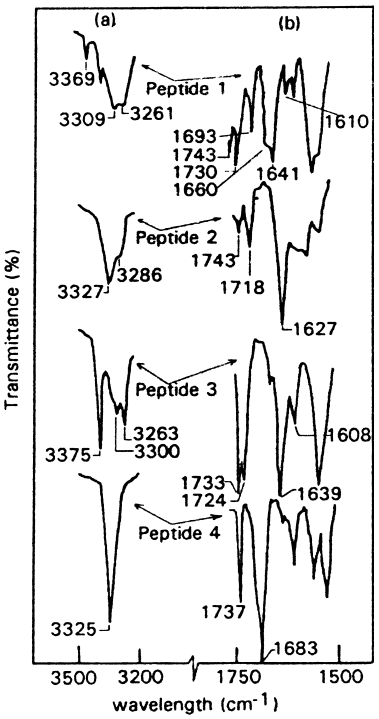


Figure 2. FT-IR bands at 3200–3500cm^{-1} (a) and 1500–1750 cm^{-1} (b) of peptides **1**–**4** in the solid state.

Fig. 1. Preliminary information on conformational preferences of all peptides were obtained from FT-IR studies.[17] The most informative frequency ranges are (i) 3500–3200 cm^{-1}, corresponding to the N–H stretching vibrations of the peptide and N-protecting urethane groups, and (ii) 1800–1600 cm^{-1}, corresponding to the C=O stretching vibrations of the peptide, urethane, and ester groups. The results in both solid-state and in solution are listed in Table 1. In the 3500–3200 cm^{-1} region (Fig. 2), no band is visible at a frequency >3430 cm^{-1} for all reported peptides in the solid state. However, an intense band corresponding to strongly hydrogen bonded NH groups has been observed at 3375–3275 cm^{-1} for each of the reported peptides. In the 1800–1600 cm^{-1} region, several bands have been observed (Fig. 2). The bands at 1783–1740 and 1737–1610 cm^{-1} (Fig. 2) are assigned to the stretching of the C=O groups of free methyl ester moieties and hydrogen-bonded urethane groups respectively. The observed strong and medium bands at 1733–1690 cm^{-1} are attributed to the stretching of the C=O groups of hydrogen bonded esters and/or free urethanes. The strong bands at 1627–1650 cm^{-1} are assigned to the C=O groups of hydrogen bonded peptide moieties. The bands observed at 1627–1650 and

Table 1. Infrared (IR) absorption frequencies (cm^{-1}) for all dipeptides in solid and in solution states

Peptide		NH stretch	C=O stretch
Boc-3-APA-Aib-OMe (peptide 1)	Solid state (KBr pellet)	3369 (w), 3309 (st), 3261 (m)	1743 (m), 1730 (st), 1693 (m), 1641–1660 (st), 1610 (v.w)
	Solution state ($CHCl_3$)	3436 (st), 3348 (sd)	1730 (st), 1670 (m), 1610 (w)
Boc-3-APA-Pro-OMe (peptide 2)	Solid state (KBr pellet)	3327 (st), 3286 (sd)	1743 (v.w), 1718 (v.w), 1627 (st)
	Solution state ($CHCl_3$)	3436 (st)	1726 (sd), 1647 (m), 1602 (s)
Boc-3-APA-Val-OMe (peptide 3)	Solid state (KBr pellet)	3375 (st), 3300 (w)	1762 (w), 1733 (st), 1724 (m), 1639 (v.s), 1608 (w)
	Solution state ($CHCl_3$)	3436 (st)	1730 (st), 1670 (st), 1610 (w)
Boc-Aib-3-APA-OMe (peptide 4)	Solid state (KBr pellet)	3325 (st)	1737 (m), 1783 (st)
	Solution state ($CHCl_3$)	3436 (m), 3415 (m)	1728 (st), 1697 (st), 1610 (w)

st=strong, w=weak, v.w=very weak, m=medium, sd=shoulder, v.s=very strong.

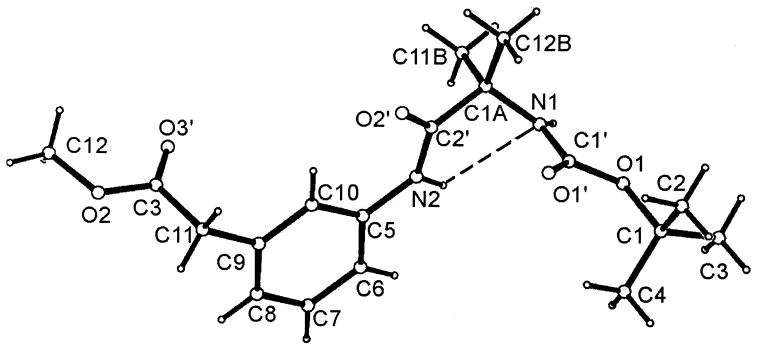


Figure 3. Molecular structure with atoms numbering scheme of the peptide **4** in the solid state showing 5-membered NH···N hydrogen bonded molecular scaffold.

3286–3227 cm^{-1} are typical of a fully developed β-sheet conformation.[17] For peptide **1**, a medium intensity band has been observed at 3261 and 1641–1660 (st) and 1610 cm^{-1} (v.w) bands are also found in solid state. All these data suggest an intermolecular β-sheet structure for peptide **1** in the solid state. Similarly, FT-IR data corresponding to peptides **3** and **4** (Table 1) are indicative of the β-sheet structure in the solid state.

2.2. X-Ray structure

From the single crystal X-ray diffraction studies of peptide **4**, it was evident that the peptide is in an extended backbone conformation with the formation of an intramolecular 5-membered NH···N hydrogen bond (N2···N1 2.85 Å, H2A···N1 2.48 Å, N2–H2A···N1 106.7°) between the 3-APA NH and N-atom of Aib amide[16a] (Fig. 3). Two intermolecular NH···O hydrogen bonds mediate the packing of the peptide to form a supramolecular parallel β-sheet structure (Fig. 4).

2.3. Solution conformational analysis

The concentration dependent FT-IR studies indicate that all peptides are in a non-aggregated state at the concentration range of 1–10 mmol in $CDCl_3$ solution. For peptides **2** and **3**, the bands corresponding to NH stretching appear at >3430 cm^{-1} (Fig. 5, Table 1) suggesting the occurrence of free NH groups.[18] The band at 3415 cm^{-1} of the peptide **4** is due to the presence of NH···N hydrogen bond in solution.[16a,19] For peptide **1** an intense band has been observed at 3436 cm^{-1} with a shoulder at 3386 cm^{-1} indicating the presence of mostly non-hydrogen bonded structure with a possibility of an extremely weak hydrogen bonded conformation. In solution, highly intense peaks appear at >1700 cm^{-1} due to the C═O stretch of all peptides indicating the presence of non-hydrogen bonded C═O groups (Fig. 5).

Further conformational characterization of peptides **1** and **3** has been performed using ^1H NMR studies in $CDCl_3$. Fig. 6

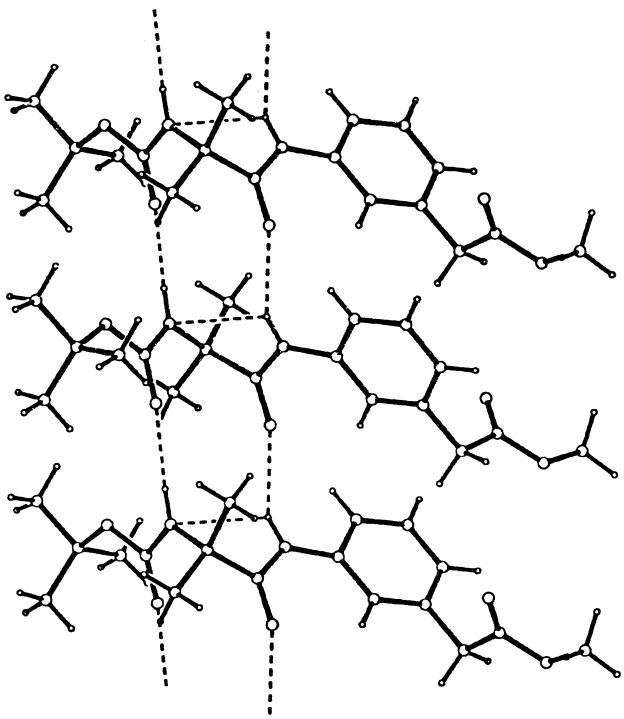


Figure 4. Packing diagram of the peptide **4** in crystal showing supramolecular parallel β-sheet formation in the solid state.

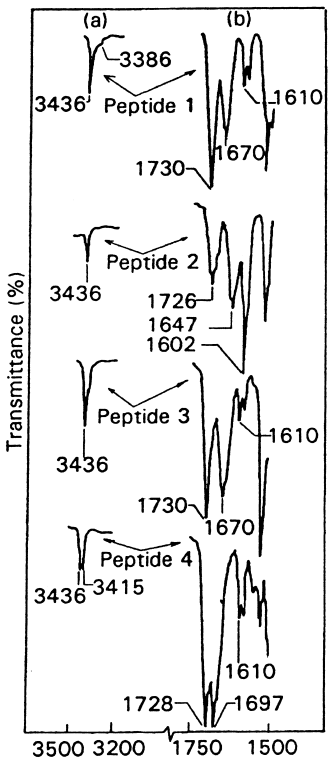


Figure 5. FT-IR spectrum at the region 3200–3500 cm^{-1} (a) and 1500–1750 cm^{-1} (b) of peptides **1**–**4** in solution.

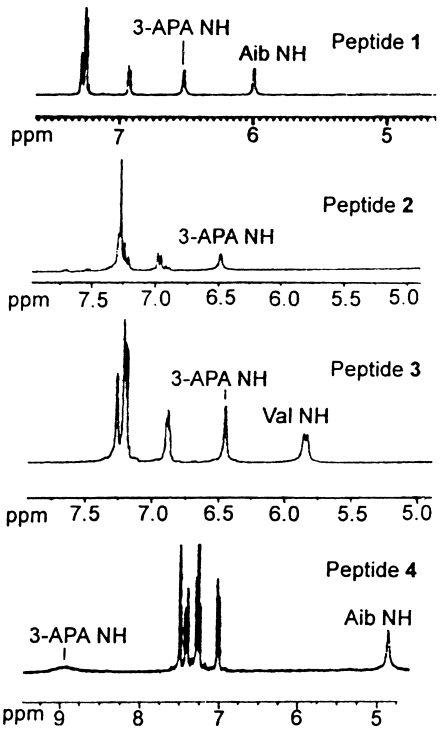


Figure 6. Comparative analysis ^{1}H NMR spectra at the range of δ 5–8 of peptides **1**–**4** in $CDCl_3$ solution.

represents the ^{1}H NMR spectrum of all dipeptides. Fig. 6 reveals that except for peptide **4**, all peptide NH resonances have similar δ values indicating their similar conformational feature in solution. The higher δ value of 3-APA NH in peptide **4** is due its involvement in an NH···N intramolecular hydrogen bond in solution. The effect of adding a hydrogen bond accepting solvent like $(CD_3)_2SO$ to $CDCl_3$ solutions of peptides **1** and **3** are represented in Figs. 7 and 8, respectively. Generally, addition of small amounts of $(CD_3)_2SO$ in $CDCl_3$ brings about monotonic downfield shifts of exposed NH groups in peptides, leaving solvent shielded NH groups largely unaffected. This allows identification of intramolecular hydrogen bonded NH groups in peptides using solvent titration experiments.[20] Figs. 7 and 8 show that, none of the amide NHs of both peptides are solvent shielded indicating the non-involvement in intramolecular hydrogen bond in solution. Unlike peptide **1** and **3**, the 3-APA NH of peptide **4**[16a] is solvent shielded upon the addition of $(CD_3)_2SO$ into $CDCl_3$ solution of peptide **4**, which clearly indicates that 3-APA NH is hydrogen bonded in solution. A similar feature is evident from IR studies.

2.4. Morphology of peptides

The SEM images of peptides **1**, **2**, **3**, and **4** are shown in Figs. 9(a) and (b), 10(a) and (b), 11, and 12, respectively. From the SEM images of these peptides it is evident that they share a common morphological property despite the differences in sequences (of all reported peptides) and compositions (of peptides **1**–**3**). All these peptides give amyloid-like fibrils.[21] The morphology of peptide **3** is the most remarkable. Fig. 11(a) and (b) shows the SEM images of peptide **3**. Fig. 11(a) and (b) exhibits that the peptide **3** forms inter-twined helical filaments, a special characteristic of many neurodegenerative disease causing amyloid fibrils.[21b,22]

2.5. Self-association in the gas-phase

Electrospray mass spectrometry is one of the techniques to probe the association behavior of compounds in the gas-phase.[23] In order to determine whether self-association of the peptides persists in the gas phase, an electrospray mass spectrometric study has been performed for peptides **2** and **3.** From the electrospray mass spectrometric study an ion with m/z=$[2M+Na]^+$, presumably arising from a non-covalent dimer was observed for both peptides (peptides **2** and **3**). The monomeric form of the peptides **2** and **3** were also dominant in electrospray mass spectrometric study and this gives rise to the base peak in each spectrum.

3. Conclusion

The rigidity of the 3-APA residue provides an extended nature for all the peptide backbones under studies. Hence, these peptide models are excellent candidates for studying

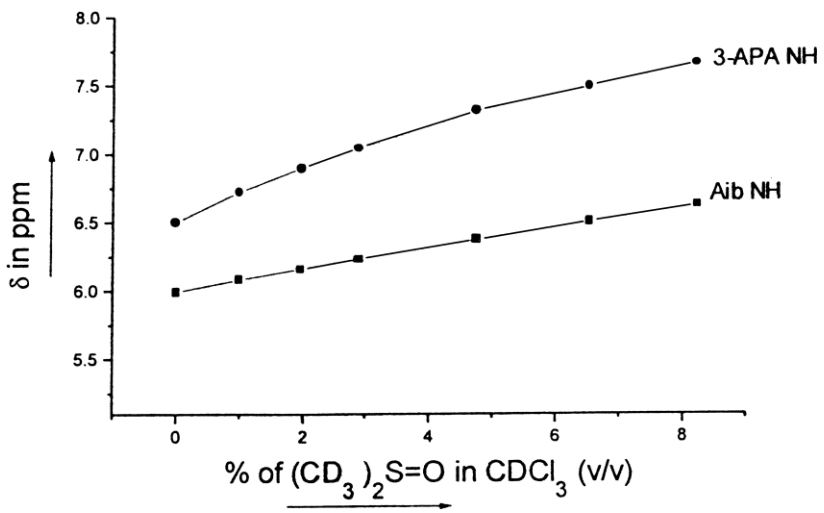


Figure 7. The plot of solvent dependence of NH chemical shifts of the peptide **1** at varying concentrations of $(CD_3)_2SO$ in $CDCl_3$.

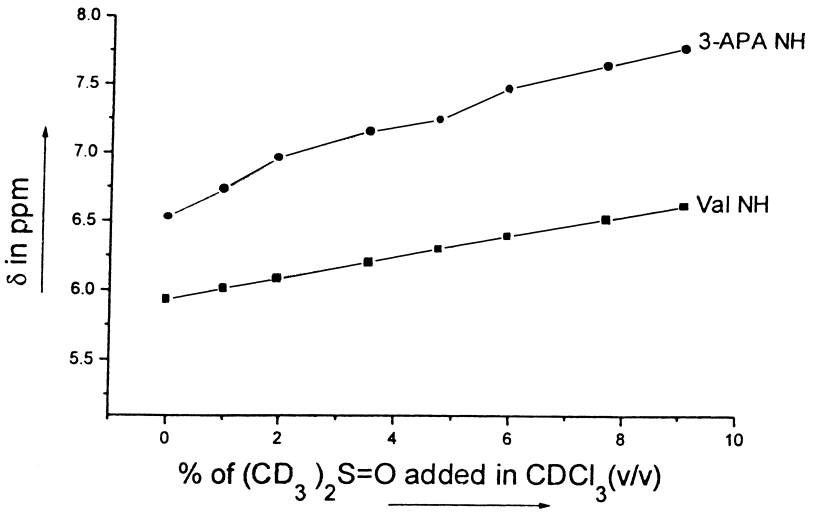


Figure 8. The plot of solvent dependence of NH chemical shifts of the peptide **3** at varying concentrations of $(CD_3)_2SO$ in $CDCl_3$.

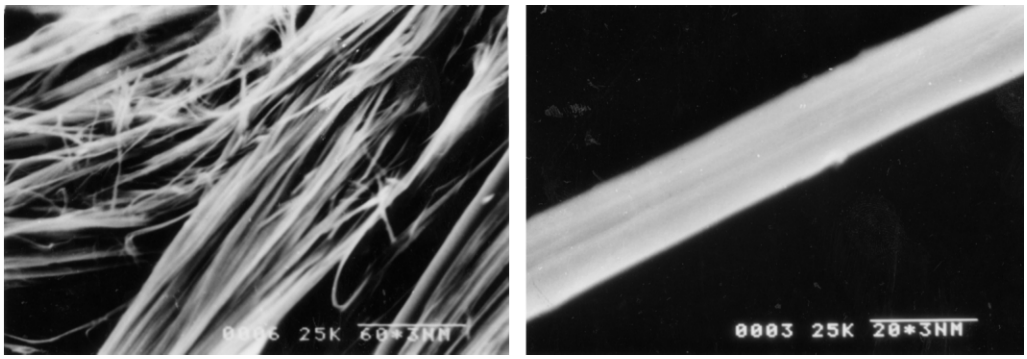


Figure 9. (a) Typical SEM image of peptide **1**. (b) The SEM image of peptide **1** showing rod-like morphology.

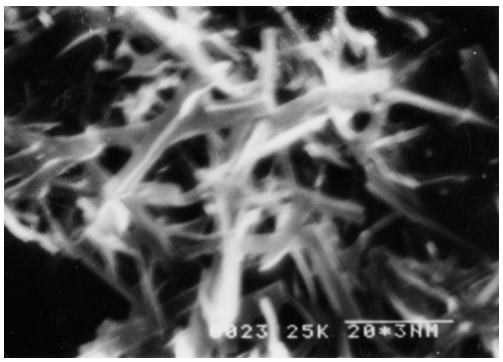


Figure 10. Typical SEM image of peptide **2**.

supramolecular β-sheet assembly. Moreover, all model peptides preferentially adopt extended backbone conformations in gas and solution phases. The FT-IR studies of all peptides **1**–**4** reveals that all reported peptides form supramolecular β-sheet structure in the solid state. Peptides **2**–**4** have different compositions and the sequences of all peptides are also different. However, their structural behaviors are strikingly similar: all peptides possess amyloid-like fibril forming supramolecular β-sheet structure. Fibril formation of these peptides does not depend upon sequence and composition like amyloid fibrillogenesis.[6–8,21,22]

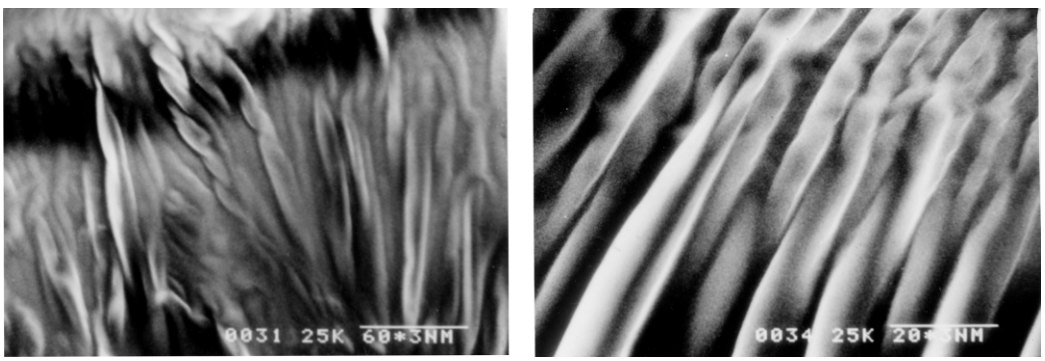


Figure 11. (a) Typical SEM image of peptide **3** showing intertwined helical filaments. (b) Typical SEM image of peptide **3** showing intertwined helical filaments with higher magnification.

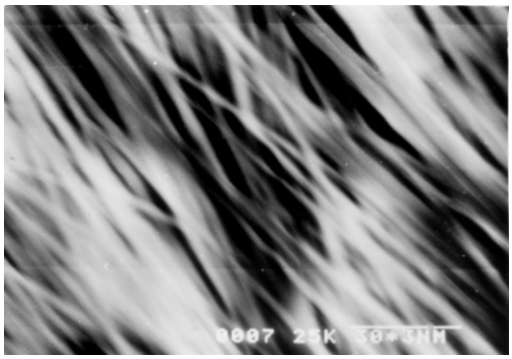


Figure 12. Typical SEM image of peptide **4**.

4. Experimental

4.1. Peptide synthesis

The synthesis and characterization of peptide **4** (Boc-Aib-3-APA-OMe) have already been reported previously.[16a] The other peptides were synthesized by conventional solution phase methods using racemization free fragment condensation strategy.[24] The Boc group was used for N-terminal protection and the C terminus was protected as a methyl ester. Couplings were mediated by dicyclohexylcarbodiimide-1-hydroxybenzotriazole (DCC/HOBt). All intermediates have been characterized by ^{1}H NMR (300 and 500 MHz) and thin layer chromatography (TLC) on silica gel and used without further purification. The final products were purified by column chromatography using silica (100–200 mesh size) gel as stationary phase and ethyl acetate–toluene mixture as eluent. The purified final compounds have been fully characterized by 500 and 300 MHz ^{1}H NMR spectroscopy.

4.2. Synthesis of peptide 1

4.2.1. Boc-3-APA-OH. A solution of 3-aminophenylacetic acid (3.02 g, 20 mmol) in a mixture of dioxan (40 mL), water (20 mL), and 1N NaOH (20 mL) was stirred and cooled in an ice-water bath. Di-tertiarybutylpyrocarbonate (4.8 g, 22 mmol) was added, and stirring was continued at room temperature for 6 h. Then, the solution was concentrated under vacuo to about 20–30 mL, cooled in an ice water bath, covered with a layer of ethyl acetate (about 20 mL), and acidified with a dilute solution of $KHSO_4$ to pH 2–3 (congo red). The aqueous phase was extracted with ethyl acetate, and this operation was done repeatedly. The ethyl acetate extracts were pooled, washed with water, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The pure material was obtained, 4.81 g, 19.16 mmol, 95.8%); [Found: C, 66.34; H, 7.18; N 6.01. $C_{13}H_{17}NO_3$ requires C, 66.38; H, 7.23; N, 5.95%].

4.2.2. Boc-3-APA-Aib-OMe. A 0.50 g (2 mmol) sample of Boc-3-APA-OH was dissolved in a mixture of 10 mL of dichloromethane (DCM) in an ice-water bath. H-Aib-OMe was isolated from 0.61 g (4 mmol) of the corresponding methyl ester hydrochloride by neutralization, subsequent extraction with ethyl acetate, and concentration (5 mL), and this was added to the reaction mixture, followed immediately by 0.41 g (2 mmol) of di-cyclohexaylcarbodiimide (DCC). The reaction mixture was allowed to come to room temperature and stirred for 24 h. DCM was evaporated, and the residue was taken in ethyl acetate (20 mL); dicyclohexylurea (DCU) was filtered off. The organic layer was washed with 2N HCl (3×20 mL), brine, 1 M sodium carbonate (3×20 mL), and brine (2×20 mL), dried over anhydrous sodium sulfate, and evaporated under vacuo to yield 0.52 g (1.48 mmol, 74.28%); [Found: C, 61.56; H, 7.35; N, 8.25. $C_{18}H_{26}N_2O_5$ requires C, 61.71; H, 7.43; N, 8.0%]; δ_H (500 MHz, $CDCl_3$) 7.36 [Ha (ph), 1H, s]; 7.30–7.29 [Hb and Hc (ph), 2H, d, J=11.0 Hz]; 6.94–6.95 [Hd (ph), 1H, s]; 6.54 [3-APA NH, 6.10 [Aib NH, 1H, s]; 1H, s]; 3.71 [$-OCH_3$, 3H, s]; 3.50 [benzyl protons (ph), 2H, s]; 1.51 [Boc-CH_3s, 9H, s]; 1.49 [Aib C$^\beta$Hs, 6H, s].

4.3. Synthesis of peptide 2

4.3.1. Boc-3-APA-Pro-OMe. A 0.50 g (2 mmol) sample of Boc-3-APA-OH was dissolved in a mixture of 10 mL of dichloromethane (DCM) in an ice-water bath. H-Pro-OMe was isolated from 0.66 g (4 mmol) of the corresponding methyl ester hydrochloride by neutralization, subsequent extraction with ethyl acetate, and concentration (5 mL), and this was added to the reaction mixture, followed immediately by 0.41 g (2 mmol) of di-cyclohexaylcarbodiimide (DCC). The reaction mixture was allowed to come to room temperature and stirred for 24 h. DCM was evaporated, and the residue was taken in ethylacetate (20 mL); dicyclohexylurea (DCU) was filtered off. The organic layer was washed with 2N HCl (3×20 mL), brine, 1 M sodium carbonate (3×20 mL), and brine (2×20 mL), dried over anhydrous sodium sulfate, and evaporated under vacuo to yield 0.48 g (1.32 mmol, 66%); [Found: C, 62.68; H, 7.35; N, 7.25. $C_{19}H_{26}N_2O_5$ requires C, 62.98; H, 7.73; N, 7.18%]; δ_H (300 MHz, $CDCl_3$) 7.20–7.27 [Ha, Hb, Hc (ph), 3H]; 6.95–6.97 [Hd (ph), 1H, d, J=6 Hz]; 6.47 [3APA NH, 1H, s]; 4.50 [Pro C$^\alpha$H, 1H, m]; 3.72 [$-OCH_3$, 3H, s]; 3.61 [benzyl CH_2 (ph), 2H, s]; 3.49 [Pro C$^\delta$H, 2H, m]; 1.97 [C$^\beta$H Pro, 2H, m]; 1.73 [C$^\gamma$H Pro, 2H, m]; 1.49 [Boc-CH_3s, 9H, s]; mass spectral data M+Na$^+$=385.2, $C_{19}H_{26}N_2O_5$ requires=362.

4.4. Synthesis of peptide 3

4.4.1. Boc-3-APA-Val-OMe. A 0.50 g (2 mmol) sample of Boc-3-APA-OH was dissolved in a mixture of 10 mL of dichloromethane (DCM) in an ice-water bath. H-Val-OMe was isolated from 0.67 g (4 mmol) of the corresponding methyl ester hydrochloride by neutralization, subsequent extraction with ethyl acetate, and concentration (5 mL), and this was added to the reaction mixture, followed immediately by 0.41 g (2 mmol) of di-cyclohexaylcarbodiimide (DCC). The reaction mixture was allowed to come to room temperature and stirred for 24 h. DCM was evaporated, and the residue was taken in ethyl acetate (20 mL); dicyclohexylurea (DCU) was filtered off. The organic layer was washed with 2N HCl (3×20 mL), brine, 1 M sodium carbonate (3×20 mL), and brine (2×20 mL), dried over anhydrous sodium sulfate, and evaporated under vacuo to yield 0.62 g (1.7 mmol, 85%); [Found: C, 62.48; H, 7.45; N, 7.72. $C_{18}H_{26}N_2O_5$ requires C, 62.64; H, 7.69; N, 7.69%]; δ_H (300 MHz, $CDCl_3$) 7.26 [Ha (ph), 1H, s]; 7.19–7.21 [H_b and H_c (Ph), 2H, d, J=8.1 Hz]; 6.89–6.87 [Hd (ph), 1H, d,

J=6.6 Hz]; 6.45 [3APA NH, 1H, s]; 5.92 [Val NH, 1H, d, J=8.4 Hz]; 4.01 [Val $C^{\alpha}H$, 1H, m]; 3.67 [$-OCH_3$, 3H, s]; 3.50 [benzyl CH_2 (ph), 2H, s]; 1.84 [Val $C^{\beta}H$, 1H, m]; 1.44 [Boc-CH_3s, 9H, s]; 0.7–0.8 [Val $C^{\gamma}Hs$, 6H, m]; mass spectral data $M+Na^+$=387.2, $C_{18}H_{26}N_2O_5$ requires=364.

4.5. NMR experiments

All NMR studies were carried out on a Brüker DRX 500 MHz and DPX 300 MHz spectrometer at 300 K. Peptide concentrations were in the range 1–10 mmol in $CDCl_3$.

4.6. FT-IR spectroscopy

The FT-IR spectra were taken using shimadzu (Japan) model FT-IR spectrophotometer with sample-shuttle device, averaging over 40 scans. Solvent (chloroform) spectra were obtained under the same condition using a cuvette with 1 mm path length. For the solid-state measurements the KBr disk technique was used.

4.7. Scanning electron microscopic studies

The morphology of all reported compounds was investigated using scanning electron microscopy (SEM). The scanning electron microscopic studies of the peptides **2** and **3** were done using a small amount of the $CHCl_3$ solution (10 mmol) of the corresponding compounds on microscopic slides and drying in air at room temperature. These dried samples were gold coated and observed under the microscope. The SEM Studies of other peptides (peptides **1** and **4**) were taken from fibrous materials (growing from ethyl acetate solution by slow evaporation) after gold coating. SEM pictures were taken in a SEM apparatus (Hitachi S-415 A).

4.8. X-Ray diffraction studies

The single crystal X-ray diffraction structure of Boc-Aib-3-APA-OMe (peptide **4**) has already been reported previously.[16a]

4.9. Mass spectrometry

Mass spectra were recorded on a HEWLETT PACKARD Series 1100MSD mass spectrometer by positive mode electrospray ionization.

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