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β-Sheet mediated self-assembly of dipeptides of ω-amino acids and remarkable fibrillation in the solid state

Anita Dutt,[a] Michael G. B. Drew[b] and Animesh Pramanik*[a]

[a] *Department of Chemistry, University of Calcutta, 92, A. P. C. Road, Kolkata, 700 009, India. E-mail: animesh_in2001@yahoo.co.in*

[b] *School of Chemistry, The University of Reading, Whiteknights, Reading, UK RG6 6AD*

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Single crystal X-ray diffraction studies show that the extended structure of dipeptide **I** Boc–β-Ala–*m*-ABA–OMe (*m*-ABA: *meta*-aminobenzoic acid) self-assembles in the solid state by intermolecular hydrogen bonding to create an infinite parallel β-sheet structure. In dipeptide **II** Boc–γ-Abu–*m*-ABA–OMe (γ-Abu: γ-aminobutyric acid), two such parallel β-sheets are further cross-linked by intermolecular hydrogen bonding through *m*-aminobenzoic acid moieties. SEM (scanning electron microscopy) studies reveal that both the peptides **I** and **II** form amyloid-like fibrils in the solid state. The fibrils are also found to be stained readily by Congo red, a characteristic feature of the amyloid fiber whose accumulation causes several fatal diseases such as Alzheimer's, prion-protein *etc*.

Introduction

The design of peptides that self-assemble in supramolecular β-sheet structures through noncovalent interactions has attracted considerable attention because of their biological importance in various neurodegenerative diseases.[1–3] It has been established that the conformational changes of proteins are responsible for the formation of β-sheet rich fibrils through self-assembly, causing several neurodegenerative diseases including Alzheimer's and prion-protein diseases.[4–5] There is an increasing interest in studying the mechanism of self-assembly and the amyloid-fibrillation process with designed molecules. Few reports are found in the literature demonstrating β-sheet stabilization through only intramolecular hydrogen bonding.[6] Schrader and Kirsten first showed the nucleation of β-sheet structures by intermolecular hydrogen bonding on the basis of rigid molecular templates such as 3-aminopyrazole derivatives.[7] A series of model peptide derivatives were introduced by Yamada and coworkers to achieve amyloid-like parallel β-sheet assemblage in solution.[8] Kelly and coworkers have carried out various studies to understand the mechanism of β-sheet mediated self-assembly for amyloid fibril formation using 2,8-dibenzofuran-based peptidomimetics.[9] Recently, Banerjee *et al.* have reported several amyloid-like fibril formations in small peptides with coded and noncoded amino acids.[10] Since the mechanistic aspect of the process of fibrillation is not yet fully understood, studies with simple molecular systems of less structural complexities are necessary to gain more insights.

It is known that the amyloid-like fibrils are generally composed of a helical array of β-sheets, in which the β-strands are perpendicular and the cross-linking hydrogen bonds are parallel to the fiber axis.[11] We are interested in developing easily modifiable molecular systems that will self-assemble into a supramolecular β-sheet structure. Therefore, we chose two dipeptides, **I** Boc–β-Ala–*m*-ABA–OMe (*m*-ABA: *meta*-aminobenzoic acid) and **II** Boc–γ-Abu–*m*-ABA–OMe (γ-Abu: γ-aminobutyric acid), with noncoded ω-amino acids (Fig. 1) to study the self-assembly mechanism and the fibrillation process. The present model may decipher the importance of β-sheet cross-linking in the process of amyloid fibrillation. In both peptides the *N*-terminal ω-amino acids, such as β-alanine and γ-aminobutyric acid, will provide the extended conformation that is necessary for β-sheet formation and the *m*-aminobenzoic acid part at the *C*-termini may help in fabrication of inter-sheet cross-linking through intermolecular hydrogen bonding. Peptides **I** and **II** were synthesised by conventional solution phase methodology and their crystal structures were obtained by X-ray diffraction studies.† The morphological features of the fibrils generated from peptide **I** and **II** in the solid state were examined by scanning electron microscopy (SEM).

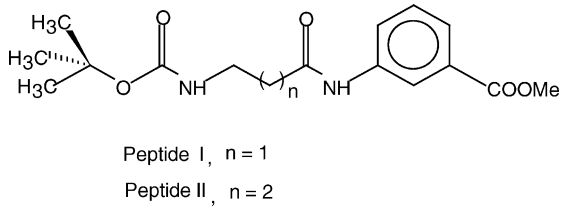

Fig. 1 Schematic representation of peptides **I** and **II**.

Results and discussion

Single crystal X-ray diffraction study

The ORTEP diagram of peptide **I** with the atom numbering scheme is presented in Fig. 2. The crystal structure of peptide **I** shows it adopts an infinite parallel β-sheet structure through intermolecular hydrogen bonding. The backbone torsion angles,

† CCDC reference numbers 263795 and 263796. See http://www.rsc.org/suppdata/ob/b5/b504112k/ for crystallographic data in CIF or other electronic format.

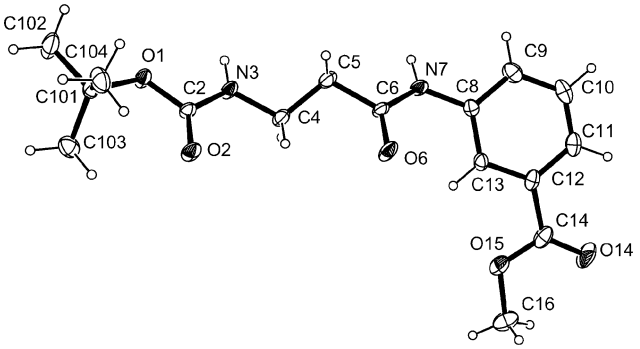

Fig. 2 The ORTEP diagram of peptide **I**, including the atom numbering scheme. Thermal ellipsoids are shown at the level of 25% probability.

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Table 1 Selected torsional angles (°) of peptides **I** and **II**

Peptide **I**			Peptide **II**		
C4–N3–C2–O1	−177.4(3)	ω_o	C16–N17–C18–O20	173.4(5)	ω_o
C2–N3–C4–C5	−139.2(3)	ϕ_1	C18–N17–C16–C15	155.8(5)	ϕ_1
N3–C4–C5–C6	173.1(3)	θ_1	N17–C16–C15–C13	70.1(6)	θ_1
C4–C5–C6–N7	142.8(3)	ψ_1	C16–C15–C13–C12	−177.8(5)	θ_2
			C15–C13–C12–N11	177.6(5)	ψ_1

that characterise an extended conformation, include C4–N3–C2–O1 (ω_o) = −177.4(3)°, C2–N3–C4–C5 (ϕ_1) = −139.2(3)°, N3–C4–C5–C6 (θ_1) = 173.1(3)° and C4–C5–C6–N7 (ψ_1) = 142.8(3)° (Table 1). The incorporation of the β-Ala residue in peptide **I** helps to attain a fully extended conformation, which is necessary for β-sheet formation. There are two intermolecular hydrogen bonds between N(3) and O(2) ($x-1, y, z$) and between N(7) and O(6) ($x-1, y, z$) with donor–acceptor distances of 2.963 and 2.947 Å, respectively (Table 2). These hydrogen bonds result in the formation of a 14-membered pseudo-ring that connects neighbouring molecules. A parallel β-pleated sheet along the direction of the *a*-axis is thus formed with the strands running in the same direction (Fig. 3). It is interesting that the spacegroup is P1 with just one molecule in the unit cell, which facilitates this packing formation. The molecules are also packed by π–π interactions between the phenyl rings. This arrangement is further stabilized by intermolecular hydrogen bonding. It is a well documented fact that π–π interactions have a significant role in amyloid aggregation.[12]

The X-ray crystal structure of peptide **II** shows a different structural preferences from that of peptide **I**. Now the space-group is P-1 with two molecules in the unit cell. The ORTEP diagram of peptide **II**, including the atom numbering scheme, is shown in Fig. 4. The backbone torsions are mostly in fully extended and semi-extended conformations, a prerequisite for individual β-strand formation. The flexible γ-aminobutyric acid (γ-Abu) residue at the *N*-terminal induces an overall extended structure characterised by the backbone torsions C18–N17–C16–C15 (ϕ_1) = 155.8(5)°, N17–C16–C15–C13 (θ_1) = 70.1(6)°, C16–C15–C13–C12 (θ_2) = −177.8(5)° and C15–C13–C12–N11 (ψ_1) = 177.6(5)° (Table 1). Nearly similar conformational preferences are also observed in a γ-aminobutyric acid–choline ester diiodide and a Boc-derivative of 1,3-propylenediamines.[13] Each molecule of peptide **II** is connected with neighbouring molecules through an intermolecular hydrogen bond between N(17) and O(19) ($x-1, y, z$), with a donor–acceptor distance of 3.273 Å, to create an infinite parallel β-sheet structure (Table 2). Interestingly, one such parallel β-sheet is cross-linked

Table 2 Intermolecular hydrogen bonding parameters of peptides **I** and **II**

	D–H···A	H···A/Å	D···A/Å	D–H···A/°
Peptide **I**	N3–H3···O2[a]	2.13	2.963(6)	162
	N7–H7···O6[b]	2.13	2.947(6)	159
Peptide **II**	N11–H11···O34[c]	2.27	3.113(7)	167
	N17–H17···O19[d]	2.44	3.273(7)	162

[a] Symmetry equivalent $x-1, y, z$. [b] Symmetry equivalent $x-1, y, z$. [c] Symmetry equivalent $-x+2, -y+1, -z$. [d] Symmetry equivalent $x-1, y, z$.

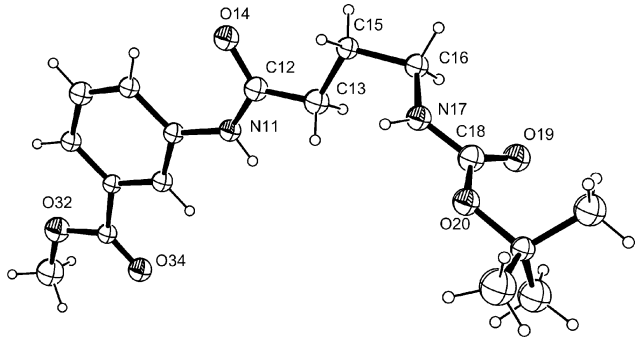

Fig. 4 The ORTEP diagram of peptide **II**, including the atom numbering scheme. Thermal ellipsoids are shown at the level of 25% probability.

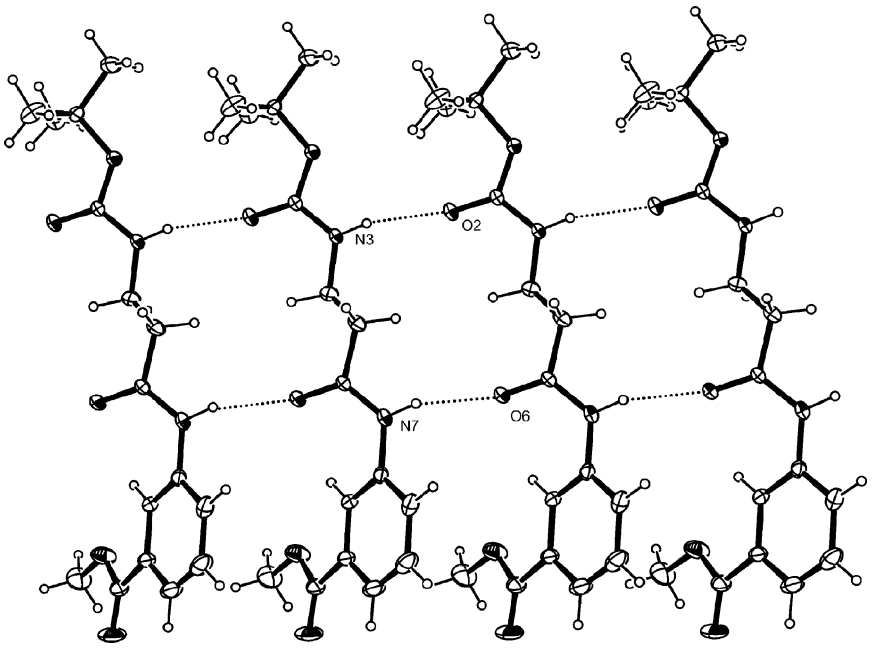

Fig. 3 Packing diagram of peptide **I** showing intermolecular hydrogen bonding in β-sheet structure.

with another neighbouring β-sheet by intermolecular hydrogen bonds between two closely placed *m*-aminobenzoic acid moieties (Fig. 5a and b). Two *m*-aminobenzoic acid units recognize each other through intermolecular hydrogen bonds between N(11) and O(34) ($-x + 2$, $-y + 1$, $-z$), with a donor–acceptor distance of 3.113 Å. The results clearly show that the array of β-strands are perpendicular to the cross-linking hydrogen bonds; a common morphological feature observed in amyloid fibrils, where a compact and highly ordered structure is formed by a cross-β-sheet organization.[11] Therefore, peptide **II** shows a remarkable cross-linking property to generate a cross-β-sheet structure which is rarely found in small peptides (Fig. 5a and b).

Morphological study

Some recent reports in the literature show that β-sheet forming small peptides promote amyloid-like fibrillation in the solid state.[10] Therefore, we became interested in exploring the possibility of fibril formation in the present peptides. The morphological similarity of the assemblages grown from peptides **I** and **II** with the amyloid fibrils have been examined using SEM.[9,10] The SEM images of the dried fibrous material (grown slowly from ethylacetate) of both the peptides show the formation of amyloid-like fibrils in the solid state (Fig. 6a and b). The filaments generated from peptide **II** are found to be much longer and more well structured than those of peptide **I** (Fig. 6c), which may be due to inter-sheet cross-linking present in peptide **II** (Fig. 5a and b).

The morphological resemblance of these peptide fibrils with amyloid plaques have been studied further by Congo red staining. It has been reported that Congo red binds to amyloid fibrils, which are responsible for several fatal diseases including Alzheimer's, prion-protein *etc.*[14,15] Interestingly, the aggregated fibrils originating from peptides **I** and **II** are found to be stained

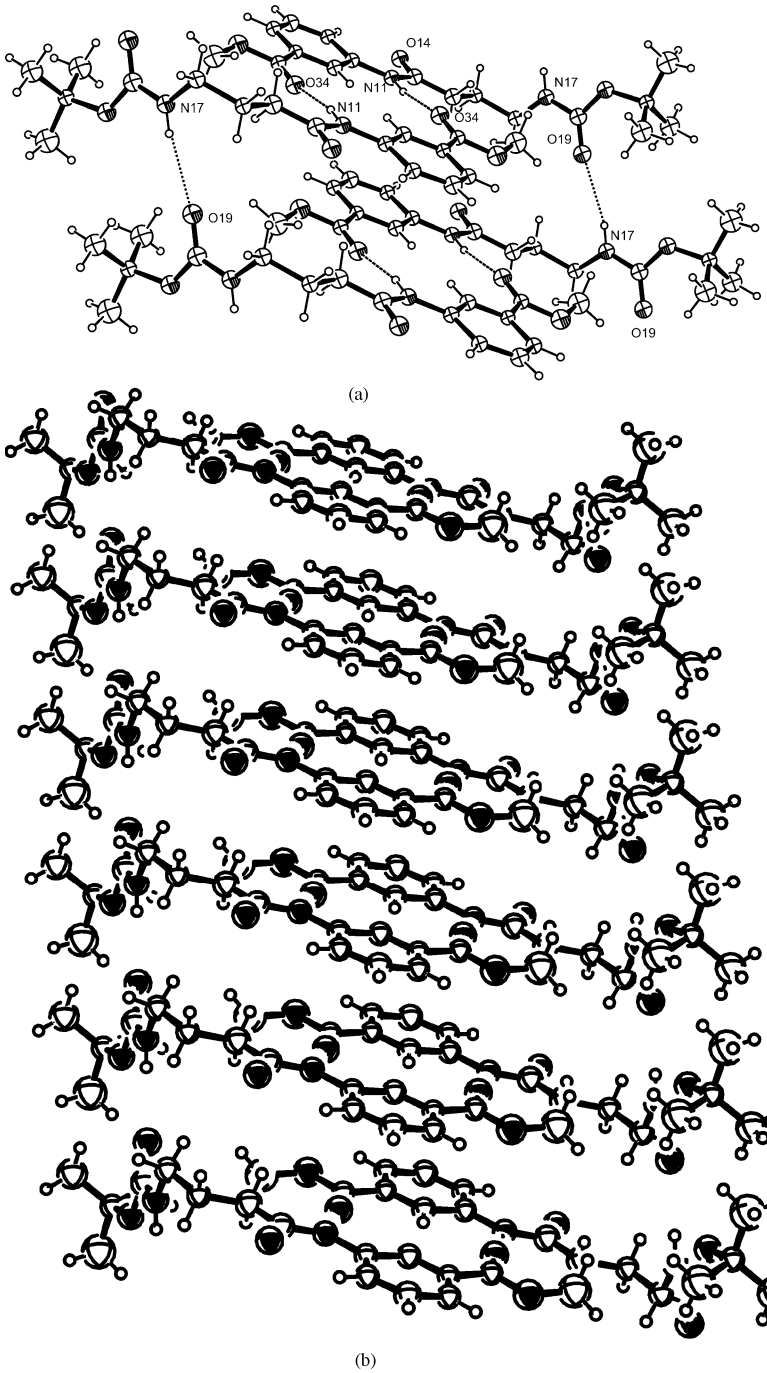

Fig. 5 (a) Packing diagram of peptide **II** showing intermolecular hydrogen bonding and β-sheet cross-linking. (b) Packing diagram of peptide **II** showing the self-assembly of cross-β-sheets.

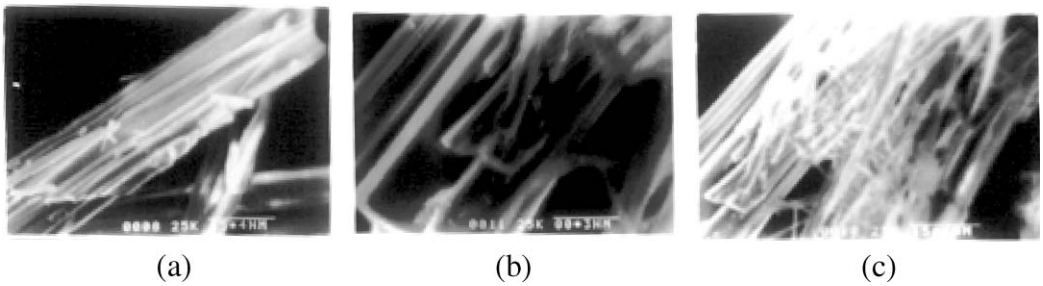

Fig. 6 SEM images of (a) peptide **I** and (b) peptide **II** showing amyloid-like fibrillar morphology in the solid state. (c) The higher resolution SEM image of peptide **II**.

readily by Congo red (Fig. 7a and b). Therefore, these *de novo* designed supramolecular β-sheet forming peptides share some characteristic properties of amyloidogenic proteins.

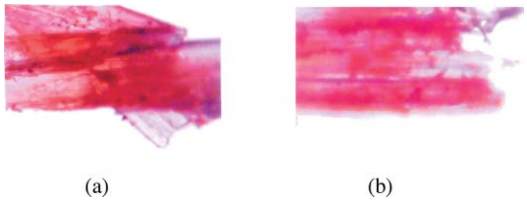

Fig. 7 Light microscope images of Congo red stained fibrils of peptide **I** (a) and peptide **II** (b) observed at × 25 and × 100 magnification, respectively; a characteristic feature of amyloid fibrils.

Conclusions

The present study clearly demonstrates that even small peptides of ω-amino acids can self-assemble into parallel β-sheet structures through intermolecular hydrogen bonding. SEM and Congo red binding studies reveal that both the peptides form amyloid-like fibrils in the solid state. In the case of peptide **II**, the fibrillation is more effective, which may be due to β-sheet cross-linking. Importantly, crystallographic evidence of β-sheet cross-linking in small peptides is presented here, which may help to understand the structure and function of various abnormal peptides such as prion and the Alzheimer's amyloid.

Experimental

Synthesis of peptides

The dipeptides were synthesised by a conventional solution phase fragment condensation strategy. The *t*-butyloxycarbonyl and methyl ester groups were used for amino and carboxyl protections and dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBT) as coupling agents. Methyl ester hydrochlorides of *m*-ABA were prepared by the thionyl chloride–methanol procedure. All the intermediates obtained were checked for purity by thin layer chromatography (TLC) on silica gel and used without further purification. The final peptides were purified by column chromatography using silica gel (100–200 mesh) as the stationary phase and an ethyl acetate and petroleum ether mixture as the eluent.

Methyl *m*-aminobenzoate. A mixture of absolute methanol (40 mL) and *m*-amino benzoic acid 1.37 g (10 mmol) was cooled in an ice–salt bath for 30 min. Then, thionyl chloride (30 mL) was dropwise added to the reaction mixture with constant stirring and it was slowly allowed to attain rt. After stirring the clear mixture for 15 h. the excess methanol and thionyl chloride were removed and the residue was treated with ether. The solid methyl ester hydrochloride obtained (1.03 g) was dissolved in water, neutralised with sodium bicarbonate solution and extracted with ethyl acetate. Finally, the liquid ester was obtained by removal of solvent and it was used without further purification.

Methyl *m*-aminobenzoate hydrochloride. Mp = 186–188 °C; IR (KBr): 2838, 1721, 1279, 1210, 1091, 755 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$, δ ppm): 3.64 (2H, s, $–NH_2$), 3.91 (3H, s, $–OCH_3$), 3.96 (1H, s, $–^+NH_2–H$), 7.44 (1H, t, J = 7.8 Hz, Hc (*m*-ABA)), 7.70 (1H, d, J = 7.4 Hz, Hd (*m*-ABA)), 7.95 (1H, d, J = 7.7 Hz, Hb (*m*-ABA)), 8.09 (1H, s, Ha (*m*-ABA)).

Methyl *m*-aminobenzoate. IR (KBr): 1711 cm^{-1}. ^1H NMR (300 MHz, $CDCl_3$, δ ppm): 3.73 (2H, s, $–NH_2$), 3.91 (3H, s, $–OCH_3$), 6.79, 7.14, 7.32, 7.37 (4 Ar–Hs).

Boc–β-Ala–*m*-ABA–OMe (I). Boc–β-Ala–OH (0.95 g, 5 mmol) was dissolved in dimethylformamide (DMF; 3 mL) and 0.76 g (5 mmol) of *m*-ABA–OMe obtained from its hydrochloride was added, followed by DCC (1.0 g, 5 mmol). The reaction mixture was stirred at rt for 3 days. The precipitated dicyclohexylurea (DCU) was filtered and diluted with ethyl acetate (80 mL). The organic layer was washed with an excess of water, 1 N HCl (3 × 30 mL), 1 M Na_2CO_3 solution (3 × 30 mL) and again with water. The solvent was then dried over anhydrous Na_2SO_4 and evaporated *in vacuo*, giving a brown solid. Yield: 1.46 g (90%). Single crystals were grown from a chloroform–toluene mixture by slow evaporation and were stable at rt. Mp = 120–122 °C; (found: C, 59.72; N, 8.82; H, 6.99%. $C_{16}H_{22}N_2O_5$ requires: C, 59.61; N, 8.69; H, 6.89%); IR(KBr): 3345, 2936, 1684, 1530 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$, δ ppm): 1.44 (Boc–CH_3s, 9H, s), 2.62–2.65 (C^{β}Hs of β-Ala, 2H, m), 3.49–3.53 (C^{α}Hs of β-Ala, 2H, m), 3.91 ($–OCH_3$, 3H, s), 5.2 (β-Ala–NH, 1H, m), 7.40 (Hc (*m*-ABA), 1H, t, J = 7.9 Hz), 7.79 (Hd (*m*-ABA), 1H, d, J = 7.7 Hz), 7.87 (Hb (*m*-ABA), 1H, d, J = 7.9 Hz), 7.98 (*m*-ABA NH, 1H, br. s), 8.11 (Ha (*m*-ABA), 1H, s).

Boc–γ-Abu–*m*-ABA–OMe (II). Peptide **II** was synthesised following a similar procedure to that of peptide **I**. Yield: 1.51 g (90%). Single crystals were grown from a methanol–water mixture by slow evaporation and were stable at rt. Mp = 104–106 °C; (found: C, 60.61; N, 8.45; H, 7.28%. $C_{17}H_{24}N_2O_5$ requires: C, 60.70; N, 8.33; H, 7.19%); IR(KBr): 3363, 2975, 1691, 1543 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$, δ ppm); 1.59 (Boc–CH_3s, 9H, s), 1.86–1.91(C^{γ}Hs of γ-Abu, 2H, m), 2.40–2.42 (C^{β}Hs of γ-Abu, 2H, m), 3.26–3.28 (C^{α}Hs of γ-Abu, 2H, m), 3.91 ($–OCH_3$, 3H, s), 4.81 (γ-Abu NH, 1H, m), 7.40 (Hc (*m*-ABA), 1H, t, J = 7.9 Hz), 7.77 (Hd (*m*-ABA), 1H, d, J = 7.7 Hz), 7.92 (Hb (*m*-ABA), 1H, d, J = 7.9 Hz), 8.21 (Ha (*m*-ABA), 1H, s), 9.03 (*m*-ABA NH, 1H, s).

Crystal data

Peptide I. $C_{16}H_{22}N_2O_5$, M = 322.36, triclinic, spacegroup P1, a = 5.108(7) Å, b = 5.710(7) Å, c = 14.903(16) Å, α = 79.66(1)°, β = 81.13(10)°, γ = 89.62(1)°, U = 422.4 $Å^3$, d_{calc} = 1.267 g cm^{-3}, 2247 independent reflections.

Peptide II. $C_{17}H_{24}N_2O_5$, M = 336.38, triclinic, spacegroup P-1, a = 5.448(6) Å, b = 11.674(12) Å, c = 14.810(14) Å, α = 79.94(1)°, β = 79.89(1)°, γ = 85.35(1)°, U = 911.8 $Å^3$, d_{calc} = 1.225 g cm^{-3}, 3040 independent reflections.

Data were measured with MoKα radiation using the MAR research Image Plate System. The crystals were positioned at 70 mm from the image plate. 100 Frames were measured at 2° intervals with a counting time of 2 min. Data analysis was carried out with the XDS program.[16] The structures were solved using direct methods with the Shelx86 program.[17] Non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atoms bonded to carbon were included in geometric positions and given thermal parameters equivalent to 1.2 times those of the atom to which they were attached. The structures were refined on F^2 using Shelxl[18] to $R1$, $wR2$ 0.0520, 0.1146 for **I** and 0.0931, 0.2465 for peptide **II** with 1886, 1305 reflections with $I > 2\sigma(I)$. The data have been deposited at the Cambridge Crystallographic Data Center.†

Congo red binding study [14,15]

The fibrils generated from peptides **I** and **II** were stained by alkaline Congo red solution (80% methanol–20% glass distilled water containing 10 μL of 1% NaOH) for 2 min and then the excess stain (Congo red) was removed by rinsing the stained fibrils with 80% methanol–20% glass distilled water solution several times. The stained fibrils were dried under a vacuum at rt for 24 h and then visualized under a light microscope at × 25 and × 100 magnification for peptide **I** and **II**, respectively.

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