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Stepwise Self-assembly of a Tripeptide from Molecular Dimers to Supramolecular β-sheets in Crystals and Amyloid-like Fibrils in the Solid State

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Dedicated to the Memory of the Late Professor R.R. Rao

The terminally protected tripeptide Boc–Ala(1)–Leu(2)–Ala(3)–OMe 1 forms antiparallel hydrogen-bonded dimers of two different conformers in the asymmetric unit and the individual dimers then self-associate to form supramolecular β-sheet structures in crystals and amyloid-like fibrils in the solid state.

Keywords: Self-assembly; Molecular dimer; Peptide; Amyloid-like fibrils

INTRODUCTION

The construction of a supramolecular β-sheet architecture from molecular dimers through non-covalent interactions, including hydrogen bonding, is particularly important for their interdisciplinary aspects in supramolecular chemistry [1–5]. Organic moieties such as oligoamides [3,4] and pyridone dimers [5] form aggregated materials, exploiting their hydrogen-bonding functionalities. Highly specific and strong hydrogen-bonded dimer systems that have been designed so far are based on heterocycles [6–8], nucleobases [9–10], β-turn forming peptide strands containing non-coded amino acids [11] and urea functionalities [12]. The design of peptide-based systems that form β-hairpin structures has become a common method of constructing β-sheet structures [13–16]. Nowick and co-workers have designed a β-sheet forming dimer system that can be used as a model for studying β-sheet interactions in proteins [17,18]. However, examples of peptide dimer systems that can act as building units for supramolecular β-sheet structural motifs are relatively rare [11]. A peptide-based dimer system that forms a β-sheet is particularly important as it can act as a model system for studying the β-sheet interactions at the exposed edges of proteins. These types of dimer-based β-sheet aggregations also have clinical importance with regard to some fatal neurodegenerative diseases, particularly amyloid disease [19–22]. Several previous reports have suggested that a structural transition from a highly α-helical structure to a predominantly β-sheet conformation is a prerequisite for amyloid fibril formation [23] and many neurotoxic amyloid peptide sequences self-associate to form a quaternary β-sheet structure and ultimately fibrils by dimer formation [24,25]. However, atomic level characterization of the fibrillation process using single-crystal X-ray diffraction studies is still illusive because of the extremely low solubility and non-crystallinity of these amyloidogenic proteins and protein fragments. It is therefore important to study short model peptide systems that are capable of providing single crystals. We have reported previously that several short synthetic model monomeric peptides containing non-coded amino acids with extended backbone conformations self-assemble to form supramolecular β-sheet structures in crystals and amyloid-like fibrils in the solid state [26,27]. In comparison with our

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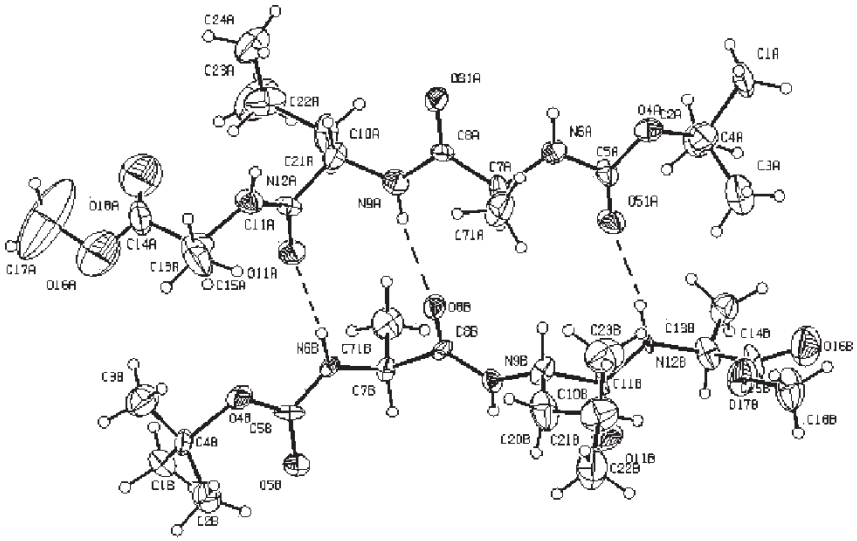

FIGURE 1 The molecular conformation of peptide **1** with atomic numbering scheme showing the formation of an intermolecular hydrogen-bonded dimer in the asymmetric unit. Ellipsoids at 20% probability. The intermolecular hydrogen bonds are shown as dotted lines.

previous study [26], the present study deals with a tripeptide, Boc–Ala(1)–Leu(2)–Ala(3)–OMe **1**, in which non-coded amino acids that were used in the previous study are replaced by coded amino acids to develop a more natural β-sheet mimicking sequence. We report here how the model synthetic tripeptide **1** forms a supramolecular β-sheet via intermolecularly hydrogen-bonded dimers in which the individual molecules in the dimer are in an antiparallel arrangement. This peptide forms amyloid-like fibrils upon further self-assembly, as is evident from Congo red binding studies and scanning electron microscopy (SEM) studies of these fibrils.

Preliminary information on the conformational preferences of peptide **1** was obtained from FT-IR studies. Concentration-dependent FT-IR studies indicated that peptide **1** is in a non-aggregated state at a concentration ranging from 1 to 14.6 mmol in $CHCl_3$ solution. The band corresponding to the NH stretching frequency at 3427 cm^{-1} suggests the occurrence of free NH groups [28]. In solution, a highly intense peak appears at 1674 cm^{-1} due to the C═O stretching of peptide **1**, indicating the presence of non-hydrogen-bonded C═O groups. However, in the solid state (KBr matrix), an intense band at 3304 cm^{-1} and a shoulder at 3277 cm^{-1} were observed for the reported peptide, indicating the presence of strongly hydrogen-bonded NH groups. No band has been observed at around 3400 cm^{-1}, indicating that all NH groups are involved in intermolecular hydrogen bonding [28]. The CO stretching band at 1639 cm^{-1} (amide I) and the NH bending peak at 1533 cm^{-1} (amide II) suggest the presence of a β-sheet conformation [29,30] for **1** in the solid state.

Preliminary conformational data were further supported by single-crystal X-ray diffraction studies. Single crystals of peptide **1** were grown from methanol–water solution. The molecular conformation of peptide **1** (Fig. 1) was established by X-ray crystallography. Figure 1 shows that there are two molecules (designated A and B) in the asymmetric unit of **1** and they are joined together by three intermolecular hydrogen bonds between amide C═O and NH functionalities to form a stable molecular dimer. Backbone torsion angles of each conformer (A and B) of the peptide **1** are mostly in the extended region of the Ramachandran diagram [31] (Table I). Thus, the peptide **1** provides an overall extended backbone structure for each conformer of the molecular dimer in the asymmetric unit. Each dimer then self-assembles via intermolecular hydrogen bonds and other non-covalent interactions to form an infinite antiparallel β-sheet assemblage in the crystal (Fig. 2) along the screw axis parallel to the crystallographic *c* direction. These individual β-sheet columns are themselves regularly stacked via van der Waals interactions to form a complex quaternary supramolecular sheet structure along the crystallographic *a* direction. The hydrogen-bonding

TABLE I Characteristics of peptide **1** in molecules A and B: selected torsional angles (°) for peptide **1**

Residue	Molecule	ϕ	φ	ω
Ala(1)	A	−141.5	138.2	171.5
	B	−128.8	137.5	−175.7
Leu(2)	A	−140.9	119.0	172.6
	B	−143.8	126.2	170.4
Ala(3)	A	−87.6	178.0	−178.8
	B	−101.6	21.0	178.1

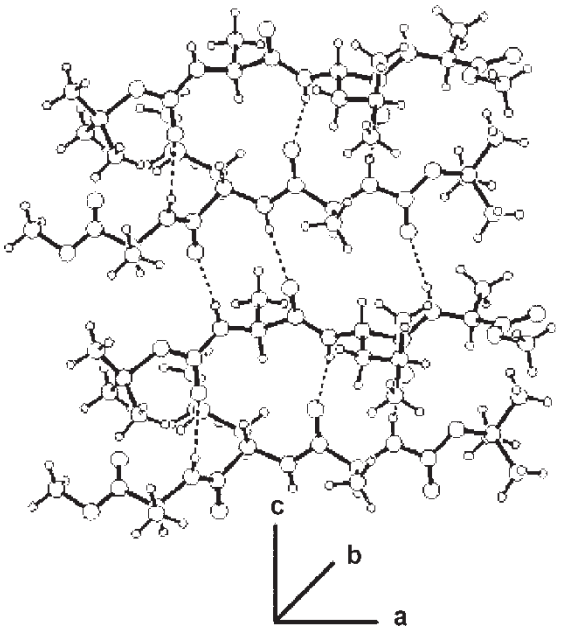

FIGURE 2 Packing of individual dimers of peptide **1** along the crystallographic *c* axis forming two-dimensional monolayer antiparallel β-sheet structures through intermolecular hydrogen bonds. Hierarchical self-assembly of these β-sheet monolayers along the *a* direction results in the formation of a highly ordered cross β-sheet structure.

parameters of peptide **1** are listed in Table II. There are three intermolecular hydrogen bonds, namely N6B–H6B···O11A, N12B–H12B···O51A and N9A–H9A···O8B, that are responsible for connecting the individual molecules to form the dimer in the asymmetric unit of peptide **1**. Another three intermolecular hydrogen bonds, N12A–H12A···O5B, N6A–H6A···O11B and N9B–H9B···O81A, are involved in joining the individual dimers of peptide **1** to form the monolayer β-sheet structure. The side chains of the Ala and Leu residues also facilitate the self-association of each dimer through non-covalent interactions to form the β-sheet structure. Figure 3 is a schematic illustration of the stepwise self-assembly of peptide **1** into the supramolecular β-sheet architecture. The dimerization of peptide **1** is also evident from the MS (ESI) spectral analysis. A peak at 410.1 $(M + Na)^+$, corresponding to the monomeric peptide **1**, was

TABLE II Intermolecular hydrogen-bonding parameters for peptide **1**

D–H···A	H···A (Å)	D···A (Å)	D–H···A (°)
N6B–H6B···O11A	2.03	2.87	170
N9A–H9A···O8B	2.12	2.99	170
N12B–H12B···O51A	2.25	3.09	165
N12A–H12A···O5B*	2.27	3.06	158
N9B–H9B···O81A†	2.22	3.05	166
N6A–H6A···O11B*	2.10	2.86	146

Symmetry equivalent *$x, y, z + 1$, †$x, y, z - 1$.

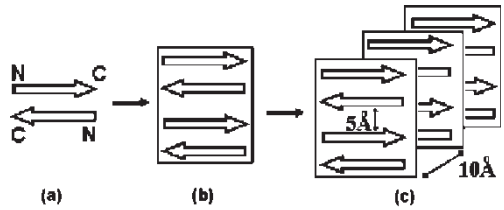

FIGURE 3 Schematic representation of the stepwise self-assembly of peptide **1** into supramolecular β-sheets: (a) the molecular dimer of peptide **1** in the asymmetric unit; (b) monolayer β-sheet formation through intermolecular hydrogen bonding between individual dimers; (c) higher order self-assembly of this monolayer β-sheet via van der Waals interactions to form the complex supramolecular β-sheet architecture.

observed, and a peak attributed to the dimeric form of peptide **1** was also found at 797.5 $(2M + Na)^+$.

SEM was used for the morphological studies of peptide **1**. The SEM picture (Fig. 4) of dried fibrous material obtained from methanol–water solution of this peptide shows amyloid-like fibrillar morphology [32–34]. Several disease-related proteins or protein fragments, including β-amyloid peptides, and even severely truncated variants of the disease-associated Aβ peptides self-assemble into fibrils [35]. To determine the similarity of these aggregated fibrils to Alzheimer's β-amyloid fibrils, fibrils obtained from peptide **1** were stained with Congo red and observed through cross-polarizers. Under cross-polarizer Congo red-bound fibrils of peptide **1** exhibited birefringence as shown in Fig. 5. These results are consistent with Congo red binding to an amyloid cross-β-sheet fibrillar structure [36].

In this report we have established the formation of hydrogen-bonded peptide dimers that form an antiparallel supramolecular β-sheet structure in crystals upon self-association. The peptide is composed of purely coded amino acids and a very important feature of this supramolecular structure is the amyloid-like fibril formation that is mediated by the dimerization of the peptide molecules. In this context, our present study may provide further understanding of the structure–function relationship of biologically important peptides that result in neurodegenerative disease causing amyloid fibrils.

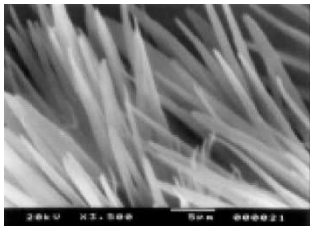

FIGURE 4 Scanning electron microscopy (SEM) image of peptide **1** exhibiting amyloid-like filamentous fibrillar morphology in the solid state.

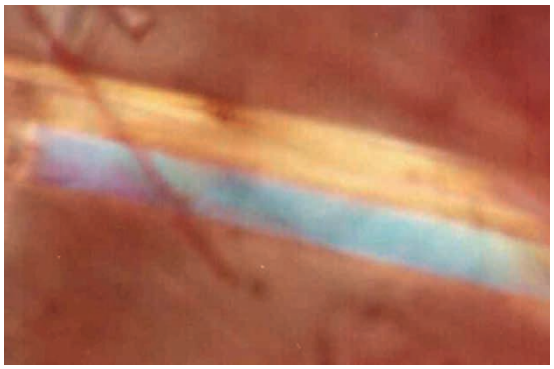

FIGURE 5 Congo red-stained peptide **1** fibrils observed through crossed polarizers showing birefringence, a characteristic of amyloid fibrils.

EXPERIMENTAL

Synthesis of Peptide 1

The peptide Boc–Ala(1)–Leu(2)–Ala(3)–OMe **1** was synthesized by conventional solution-phase methodology [37] and characterized by ^{1}H NMR spectroscopy and mass spectrometry. ^{1}H NMR (300 MHz; $CDCl_3$; Me_4Si) δ 6.64 (d, 1H, J 5.43 Hz), 6.58 (d, 1H, J 7.53 Hz), 4.94 (d, 1H, J 6.25 Hz), 4.51–4.56 (m, 1H), 4.42–4.46 (m, 1H), 4.15 (m, 1H), 3.74 (s, 3H), 1.62–1.72 (m, 3H), 1.44 (s, 9H), 1.4 (d, 3H), 1.36 (d, 3H), 0.93 (m, 6H). IR (KBr): 3304, 3277, 2958, 1742, 1697, 1676, 1639, 1533 cm^{-1}; IR ($CHCl_3$): 3427, 1742, 1674, 1499 cm^{-1}. $[\alpha]_D^{27.4} = -62.58$ (C 2.0, $CHCl_3$). Anal. Calcd for peptide **1** ($C_{18}H_{33}N_3O_6$): C, 55.85; N, 10.8; H, 8.5. Found: C, 55.81; N, 10.85; H, 8.53%. MS (ESI) *m/z* 410.1 $(M + Na)^+$, *m/z* 797.5 $(2M + Na)^+$.

FT-IR Spectroscopy

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

X-ray Structural Analysis

Single crystals of tripeptide **1** suitable for X-ray crystallographic analysis were grown from methanol–water solution by slow evaporation. Intensity data were collected at 293 K with Mo Kα radiation using the MARresearch Image Plate System. The absolute structure could not be determined from the diffraction data but was determined by the known chirality of the peptide. The crystal was positioned at 70 mm from the image plate. One hundred frames were measured at 2° intervals with a counting time of 5 min to give 1352 independent reflections. Data analysis was carried out with the XDS program [38]. Crystal data are as follows: $C_{18}H_{33}N_3O_6$, $M = 387.47$, monoclinic, $P2_1$, $a = 10.044(11)$, $b = 23.089(27)$, $c = 9.711(10)$ Å, $\beta = 104.93(1)$, $U = 2176.01$ Å^3, $Z = 4$, $d_{calc} = 1.183$ gm/cm^{-3}. The structure was solved using direct methods with the SHELX86 program [39]. The non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atoms were included in geometric positions and given thermal parameters equivalent to 1.2 times those of the atom to which they were attached. The structure was refined on F^2 using SHELXL [40]. The final R values were $R1 = 0.0641$ and $wR2 = 0.1597$ for 1352 data with $I > 2\sigma(I)$ for peptide **1**. The largest peak and hole in the final difference Fourier were 0.111 and -0.131 e Å^{-3}. The crystallographic data have been deposited at the Cambridge Crystallographic Data Centre with reference number CCDC 221587.

Morphological Study

The morphology of peptide **1** was investigated by SEM. For this study, fibrous materials (grown slowly from methanol–water mixtures) were dried and gold coated and then the micrographs were taken using a Hitachi S-415A SEM instrument.

Congo Red Binding Study

An alkaline saturated Congo red solution was prepared. The peptide fibrils were stained with 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 in vacuum at room temperature for 24 h, and then visualized at 500 × magnification and birefringence was observed between crossed polarizers.

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