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Studies of β-turn opening with model peptides containing non-coded α-amino isobutyric acid

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Abstract—Single crystal X-ray diffraction studies show that among the three terminally protected model tripeptides **I**–**III**, Boc-Ile-Aib-Xx–OMe (Xx in peptide **I**: Val; **II**: Leu; **III**: Phe) with a centrally placed non-coded amino acid Aib (Aib: α-amino isobutyric acid), peptide **I** displays a conformational preference for β-turn, peptide **II** forms a hydrated β-turn representing the solvent mediated intermediate for the interconversion between β-turn and β-strand and peptide **III** adopts a completely unfolded β-strand like structure. By varying the steric bulk of the third residue, Xx(3), various conformations related to the structural interconversion between the β-turn and β-strand have been isolated. The peptide conformations in the solution phase have been probed by solvent dependent NMR titration and CD spectroscopy. Morphological studies with scanning electron microscopy (SEM) reveal that among the three peptides only peptide **III** can form filamentous fibrils in the solid state.

1. Introduction

It is believed that various hydrated reverse turns can be considered as folding intermediates that are trapped in the folding–unfolding process between an α-helix and a β-strand.[1] The surprisingly high stability of β-turns in several small linear peptides suggests that turns are to be expected in the polypeptide chains under folding conditions where they have a profound influence on protein folding pathways. The experimental observation of β-turns in linear peptides does not itself establish a role for these structures in the initiation of protein folding. Some studies do, however lend support to the notion that reverse turns play an important role in protein folding.[2] Although there are studies on the detection and isolation of α-helix,[3] hydrated helix,[4] reverse turns,[5] hydrated reverse turns[6] and β-strand,[7] no attempt has been made to isolate all the conformations related to a particular structural interconversion to gain a deeper insight into the mechanistic aspect of the process.

Therefore we are interested in designing small model peptides that exhibit β-turns, these peptides will have the potential to unfold under the influence of two important factors namely, solvent polarity and steric interactions. This creates the opportunity to isolate various conformations related to the structural interconversion between the β-turn and β-strand. In the present report, three terminally protected model tripeptides **I**–**III**, Boc-Ile-Aib-Xx–OMe (Xx in peptide **I**: Val; **II**: Leu; **III**: Phe) with a centrally placed non-coded amino acid Aib have been chosen where the first two residues Ile(1)–Aib(2) are kept unchanged (Fig. 1). By varying the steric bulk of the third residue, Xx(3), various conformations related to the structural interconversion between the β-turn and β-strand have been isolated. Generally conformationally restricted Aib is highly helicogenic and β-sheet breaker. Therefore tripeptides with a centrally positioned Aib(2) are found to adopt β-turn structure.[8] Interestingly the tripeptides **I**–**III** demonstrate conformational heterogeneity. This work has important implications in designing biologically active peptides.[9] All the peptides were synthesised using conventional solution phase methodology and their solid-state structures are determined by X-ray diffraction analysis.

2. Results and discussion

The crystal structure of peptide **I** reveals a type II β-turn structure with Ile(1) and Aib(2) occupying the *i*+1 and *i*+2 positions, stabilised by an intramolecular hydrogen bond (Fig. 2). The torsion angles at Ile(1) and Aib(2) were found

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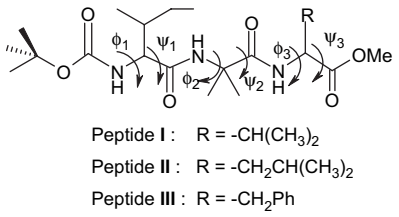

Figure 1. Schematic representation of peptides **I**–**III**.

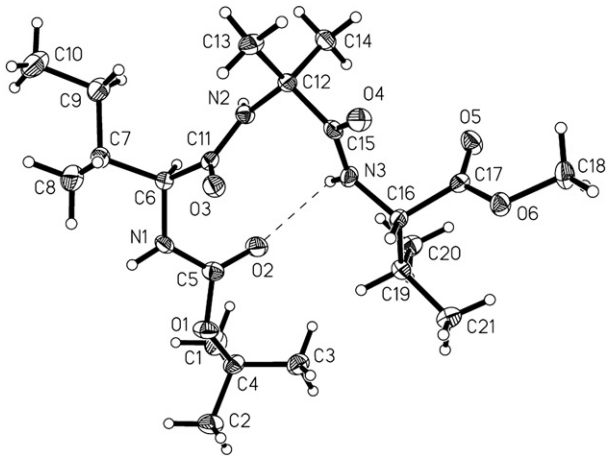

Figure 2. Crystal structure of peptide **I** with atom numbering scheme.

to be ϕ_1: −62.32, ψ_1: 134.96 and ϕ_2: 56.19, ψ_2: 34.73, respectively (Table 1), which are slightly deviated from the ideal values for a type II β-turn ϕ_1: −60, ψ_1: 120 and ϕ_2: 80, ψ_2: 0. As a result a weak intramolecular hydrogen bond between Boc–CO and Val(3)–NH is observed (H···O, 2.71 Å, Table 2). The steric interaction between the side chains of Ile(1) and Aib(2) is responsible for an unstable β-turn structure that is of interest to the present investigation. Although there are several examples of β-turns with centrally placed Aib in tripeptides,[8] we report the first use of Ile at the N-terminal position of the tripeptide.

In order to investigate the existence of intramolecular hydrogen bonding and peptide conformation in solution phase the solvent dependence of the NH chemical shifts was examined by NMR titration.[10] In this experiment a solution of the peptide in nonpolar $CDCl_3$ (10 mM in 0.5 ml) was gradually

Table 1. Selected backbone torsion angles of peptides

Torsion angles	Peptide **I**	Peptide **II**	Peptide **III**
ω_0[a]	172.81(14)	178.45(12)	−175.01(15)
ϕ_1	−62.32(19)	−85.04(15)	−58.30(2)
ψ_1	134.96(14)	126.87(12)	−44.50(2)
ω_1[b]	170.95(13)	171.42(11)	166.65(17)
ϕ_2	56.19(19)	54.74(16)	56.90(2)
ψ_2	34.73(19)	44.33(16)	50.90(2)
ω_2[c]	171.92(13)	173.18(12)	172.24(16)
ϕ_3	−64.24(18)	−73.96(17)	−48.3(2)
ψ_3	143.44(13)	−53.52(16)	141.50(19)

[a] Torsion angle around the amide C–N bond at the N-terminal of the peptides.
[b] Torsion angle around the peptide C–N bond between Ile(1) and Aib(2) of the peptides.
[c] Torsion angle around the peptide C–N bond between Aib(2) and Xx(3) of the peptides.

Table 2. Selected intra and intermolecular hydrogen bonding parameters

	D–H···A	H···A/Å	D···A/Å	D–H···A/°
Peptide **I**	N3–H3N···O2	2.71(2)	3.499(2)	152(2)
Peptide **II**	N3–H3N···O1O	2.22(2)	3.032(2)	176(2)
	O1O–H1O···O2	1.92(2)	2.759(2)	175(2)
	O1O–H1O···O5^{i}	1.96(2)	2.855(2)	173(2)
	N1–H1···O1O^{ii}	2.19(2)	3.002(2)	176(1)
Peptide **III**	N1–H1N···O3^{iii}	2.09(2)	2.959(2)	174(2)
	N2–H2N···O4^{iv}	2.15(2)	2.943(2)	164.5(19)

Symmetry codes: (i) 1+*y*, 1−*x*, 0.25+*z*; (ii) 1−*y*, *x*, *z*−0.25; (iii) 1+*x*, *y*, *z*; (iv) 1+*x*, *y*, *z*.

titrated against polar $(CD_3)_2SO$. The changes in the chemical shifts are presented in Figure 3. The assignment of NH resonances was quite straightforward. The Ile(1)–NH appears as doublet in the upfield region (δ=4.99 ppm in $CDCl_3$) among the three NH resonances resulting from the urethane functional group (Boc-group). The Aib(2)–NH appears at δ=6.53 ppm, as a singlet due to lack of an α-hydrogen atom. The only remaining doublet at δ=7.08 ppm was assigned to Val(3)–NH at the C-terminal of the peptide. The solvent titration shows that by increasing the percentage of $(CD_3)_2SO$ in $CDCl_3$ from 0 to 12% (v/v) the net changes in the chemical shift ($\Delta\delta$) values for Ile(1)–NH, Aib(2)–NH and Val(3)–NH are 0.55, 0.87 and 0.18 ppm, respectively (Fig. 3). The order of solvent exposure of the NH groups is Aib(2)–NH>Ile(1)–NH>Val(3)–NH. The $\Delta\delta$ values demonstrate that the Val(3)–NH is solvent shielded, and the other two NH groups are solvent exposed, which is a characteristic feature of a β-turn in a structure where the Val(3)–NH is involved in an intramolecular hydrogen bond to Boc–CO. The CD spectrum of the peptide at 25 °C in methanol reveals a clear positive ellipticity in the far-UV region with a distinct maximum at 203 nm, which indicates the presence of a β-turn structure (Fig. 4).[11]

It has been suggested that hydrated reverse turns may function as intermediates in unfolding of α-helices to give β-strands and β-sheets.[12] β-Sheet-driven amyloidogenesis is responsible for various neurodegenerative diseases.[13] Model studies may help to gain more insight into the mechanistic aspect of the structural interconversion between a β-turn and β-strand. Interestingly, the crystal structure of

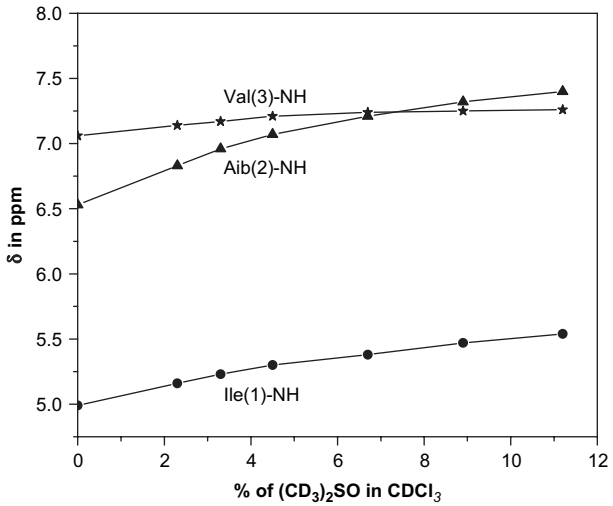

Figure 3. NMR solvent titration curve for NH protons in peptide **I**.

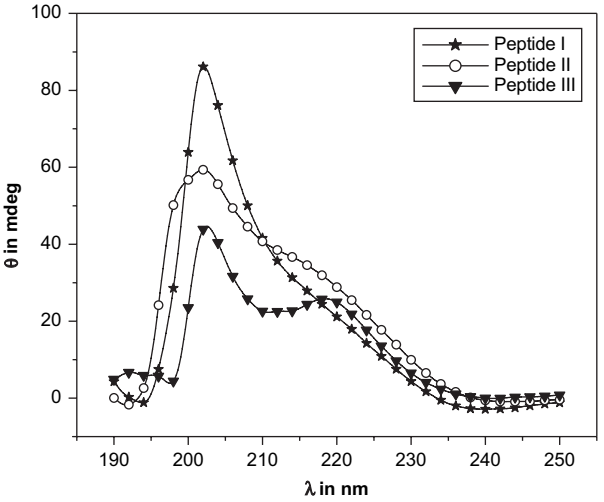

Figure 4. CD curves of peptides **I–III** in methanol (1.5 mM).

the peptide **II**, Boc-Ile-Aib-Leu–OMe, grown from acetone–water mixture shows a hydrated open β-turn structure (Fig. 5). A water mediated hydrogen bond is observed between Boc–CO and Leu(3)–NH with an open β-turn structure with Ile(1) and Aib(2) occupying the corner positions (Table 2). The torsion angles within the Ile(1) and Aib(2) moieties are ϕ_1: −85.04, ψ_1: 126.87 and ϕ_2: 54.74, ψ_2: 44.33, respectively (Table 1), which deviate significantly from the ideal values. These structural changes correspond to an open structure. The insertion of water molecules helps in opening the β-turn, which is further influenced by two neighbouring water molecules that are hydrogen bonded to Ile(1)–NH and Leu(3)–CO from that are located on the external side of the turn region (Fig. 6, Table 2). The interconversion between the β-turn and β-strand requires an aqueous environment for the formation of the hydrated β-turn intermediate.

The solvent dependence of the NH chemical shifts that was demonstrated in $CDCl_3$–$(CD_3)_2SO$ titration experiment indicates that all three –NH groups of peptide **II** are solvent exposed indicating an open structure (Fig. 7). The net change in the chemical shift ($\Delta\delta$) values for Ile(1)–NH, Aib(2)–NH and Leu(3)–NH are 0.43, 0.63 and 0.28 ppm, respectively. On the other hand the CD spectrum of peptide **II** in methanol displays a positive ellipticity in the far-UV region with distinct double maxima at 203 and 218 nm, suggesting the presence of a turn structure (Fig. 4). Thus in an aqueous methanolic environment the peptide **II** preferentially remains in the hydrated turn conformation. This finding is of particular biological relevance as it has been suggested that hydrated reverse turns promote helix–coil unfolding.[1]

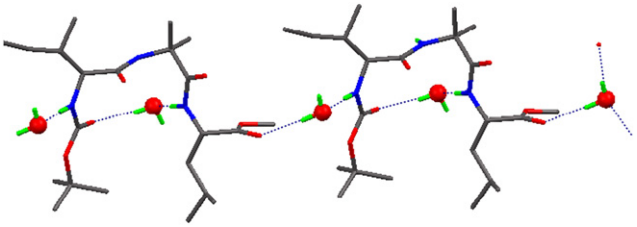

Figure 6. Solvent assisted β-turn opening of peptide **II** as observed in crystalline state.

Generally, short aromatic peptides are found to have the ability to form β-sheet mediated amyloid fibrils.[14] Therefore we have chosen peptide **III**, Boc-Ile-Aib-Phe–OMe, which incorporates a Phe(3) residue, to determine if this peptide forms a β-turn or β-strand structure. The peptide is found to adopt a β-strand structure in the solid state (Fig. 8). The torsion angles within Ile(1) are ϕ_1: −58.3 and ψ_1: −44.5, within Aib(2) they are ϕ_2: 56.9 and ψ_2: 50.9, and within Phe(3) they are ϕ_3: −48.3 and ψ_3: 141.5, indicating an open structure with three 'kinks' along the peptide backbone (Table 1). In the solid state the β-strand structure of peptide **III** self-assembles to supramolecular β-sheet through intermolecular hydrogen bonding along the crystallographic *a* axis (Fig. 9). There are two intermolecular hydrogen bonds N1–H1···O3 and N2–H2···O4 connecting individual peptide molecules to form a sheet like structure (Table 2). The C═O groups of Ile(1) and Aib(2) of one molecule are inter-linked with NH groups of Ile(1) and Aib(2) of another neighbouring molecule, respectively, through intermolecular hydrogen bonding. This type of supramolecular β-sheet mediated self-assembly of small peptide may create the possibility of

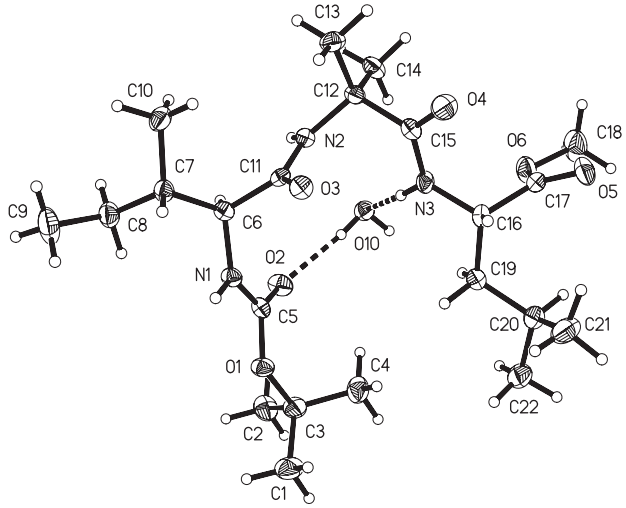

Figure 5. Crystal structure of peptide **II** with atom numbering scheme.

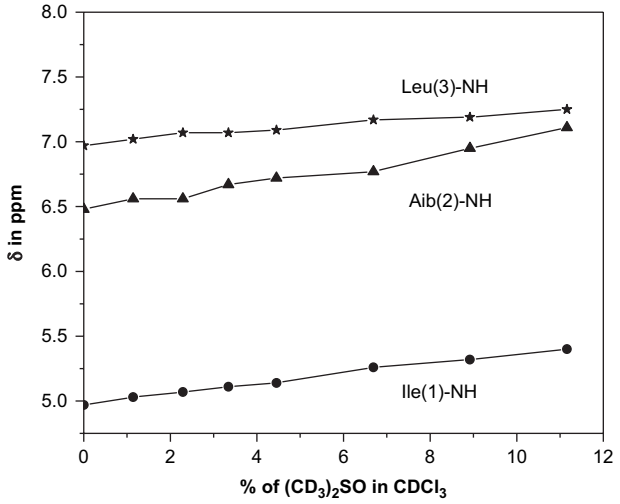

Figure 7. NMR solvent titration curve for NH protons in peptide **II**.

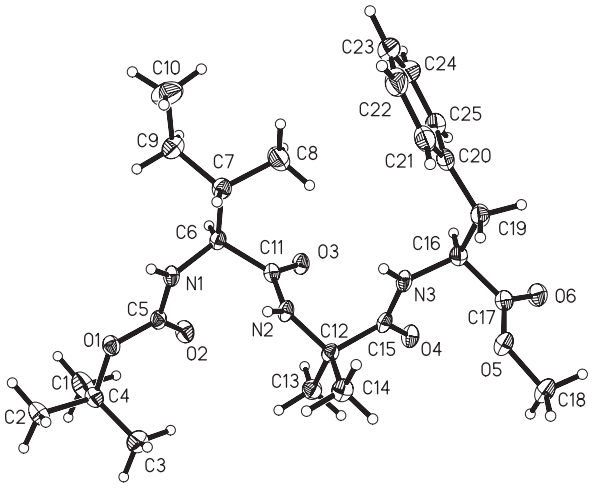

Figure 8. Crystal structure of peptide **III** with atom numbering scheme.

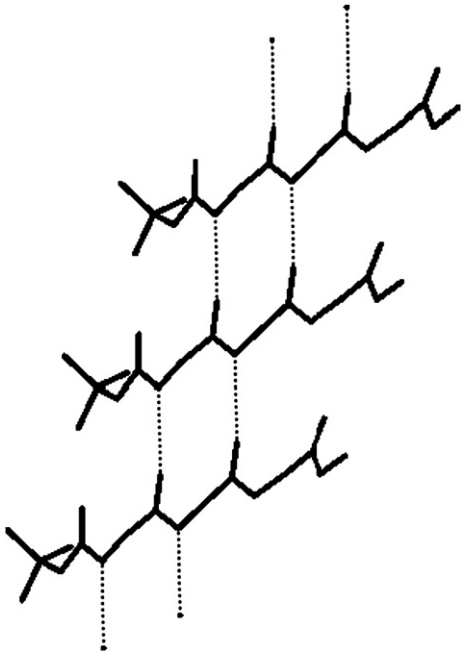

Figure 9. Packing diagram of peptide **III** along the crystallographic *a* direction showing intermolecular hydrogen bonding in the β-sheet structure (side chains and hydrogen atoms are omitted for clarity).

fibrillogenesis in the solid state. Interestingly the field emission scanning electron microscopic (FESEM) images of the dried fibrous materials of peptide **III** grown slowly from acetone clearly demonstrate that the aggregates in the solid state are bunches of filamentous fibrils, resembling the amyloid fibrils (Fig. 10). It is noteworthy that the other two peptides **I** and **II**, which adopt the turn structure, do not produce any such fibrous material under similar conditions.

The NMR $CDCl_3$–$(CD_3)_2SO$ titration experiment implies that all three NH groups of peptide **III** are solvent exposed (Fig. 11), the $\Delta\delta$ values for Ile(1)–NH, Aib(2)–NH and Phe(3)–NH are 0.37, 0.56 and 0.38 ppm, respectively, indicating a β-strand like conformation. Interestingly, a positive ellipticity with two maxima at 205 and 220 nm is observed in the CD spectra of the peptide in methanol (Fig. 4), indicating a significant amount of turn structure. Actually in an aqueous environment peptide **III** exists as a mixture of hydrated turn and β-strand conformation. The gradual opening of β-turn structure from peptide **I** to **III** is quite evident from the changes in the pattern of CD spectra (Fig. 4). The increasing flattening of the CD curves indicates greater population of the open structure.

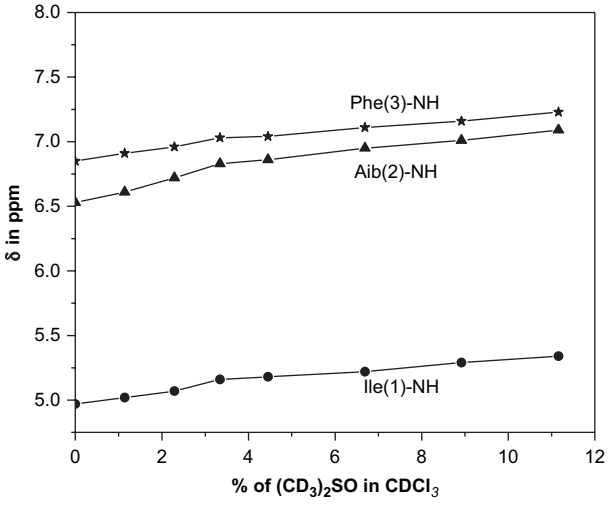

Figure 11. NMR solvent titration curve for NH protons in peptide **III**.

3. Conclusions

A comprehensive understanding of the molecular basis of β-turn formation and opening in small tripeptides is essential for designing small bioactive peptides. By selecting three

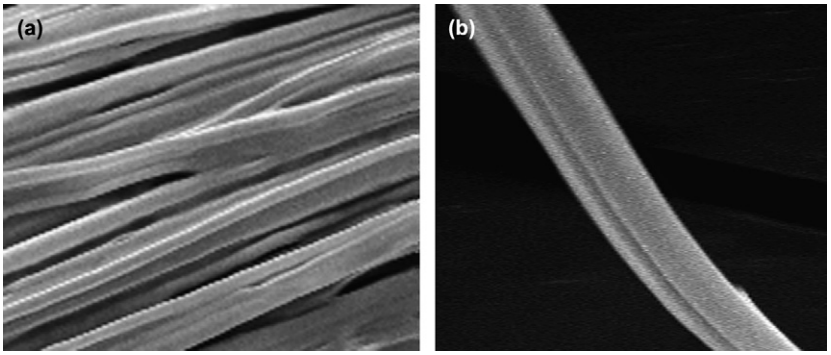

Figure 10. FESEM of peptide **III** (a) showing the formation of filamentous fibrils in the solid state; (b) the same at the higher resolution.

model peptides in which the first two residues are kept unchanged, and where the only perturbation introduced into the structure was variation in the steric bulk of the third residue, we are able to open up the β-turn structure. As a result, it was possible for various conformations related to the structural interconversion between β-turn and β-strand to be isolated. The crystal structure of the hydrated β-turn in peptide **II** provides an insight into the mechanism of interconversion between β-turns and β-strands, which could be a way of modulating β-sheet mediated amyloidogenesis. Generally, in nature, mutation within the protein causes misfolding by disrupting the local turn conformations.[15] The formation of β-strand like structure in peptide **III** where the third residue is a bulky Phe(3) group, suggests that steric factor plays an important role in regulating the transition between β-turn and β-strand structures. Therefore the results establish that even tripeptides containing a centrally positioned Aib(2) can display structural diversities in the backbone depending upon the co-operative steric interactions amongst the amino acid residues.

4. Experimental

4.1. Synthesis of peptides

The peptides **I–III** were synthesised by conventional solution phase procedures.[16] The *t*-butyloxycarbonyl and methyl ester groups were used for amino and carboxyl protections, respectively, and dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBT) as coupling agents. Methyl ester hydrochlorides of Aib, Val, Leu and Phe 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. All the final peptides were purified by column chromatography using silica gel (100–200 mesh) as the stationary phase and ethyl acetate and petroleum ether mixture as the eluent. The reported peptides **I–III** were fully characterised by X-ray crystallography, NMR and mass spectrometry.

4.1.1. Boc-Ile–OH. The amino acid isoleucine (5 g, 38.16 mmol) was suspended in a 1:1 dioxane–water mixture and Boc–N_3 (5.92 ml, 38.16 mmol) was added to it. The mixture was stirred at room temperature maintaining the pH in the alkaline range with 4 M NaOH. After 24 h, the solution was diluted and extracted with ether. The aqueous layer was cooled in an icebath, acidified with 2 M HCl and extracted with ethyl acetate. The organic layer was washed with excess of water and dried over anhydrous Na_2SO_4 and evaporated in vacuo producing a white solid.

Yield: 8.37 g (95%); ^{1}H NMR 300 MHz ($CDCl_3$, δ ppm): 5.17 (Ile NH, 1H, d, J=8.4 Hz); 4.27–4.31 (C^{α}H of Ile, 1H, m); 1.91 (C^{β}H of Ile, 1H, m); 1.45 (Boc–CH_3s, 9H, s); 1.21–1.23 (C^{γ}Hs of Ile, 3H, m); 0.91–0.97 (C^{γ}Hs and C^{δ}Hs of Ile, 5H, m). Anal. Calcd for $C_{11}H_{21}N_1O_4$ (231.28): C, 57.12; H, 9.15; N, 6.05. Found: C, 57.25; H, 9.21; N, 6.14.

4.1.2. Boc-Ile-Aib–OMe (1). Boc-Ile–OH (5.8 g, 25.10 mmol) was dissolved in dimethylformamide (DMF; 8 ml). Aib–OMe (2.94 g, 25.10 mmol) obtained from its hydrochloride was added, followed by DCC (5.17 g, 25.10 mmol). The reaction mixture was stirred at room temperature for 3 days. The precipitated dicyclohexylurea (DCU) was filtered and diluted with ethyl acetate. The organic layer was washed with excess of water, 1 M HCl (3×50 ml), 1 M Na_2CO_3 solution (3×50 ml) and again with water. The solvent was then dried over anhydrous Na_2SO_4 and evaporated in vacuo, giving a light yellow solid.

Yield: 7.45 g (90.0%). IR (KBr): 3307, 1656 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$, δ ppm): 6.62 (Aib NH, 1H, s); 5.07 (Ile NH, 1H, d, J=7.2 Hz); 3.84–3.89 (C^{α}H of Ile, 1H, m); 3.68 (OCH_3, 3H, s); 1.91 (C^{β}H of Ile, 1H, m); 1.49 (C^{β}Hs of Aib, 6H, s); 1.45 (Boc–CH_3s, 9H, s); 1.21–1.23 (C^{γ}Hs of Ile, 3H, m); 0.91–0.97 (C^{γ}Hs and C^{δ}Hs of Ile, 5H, m). Anal. Calcd for $C_{16}H_{30}N_2O_5$ (330.42): C, 58.15; H, 9.15; N, 8.48. Found: C, 58.22; H, 9.20; N, 8.39.

4.1.3. Boc-Ile-Aib–OH (2). Compound **1** (6.0 g, 18.18 mmol) was dissolved in methanol (15 ml) and 2 M NaOH (10 ml) was added. The reaction mixture was stirred at room temperature for 2 days. The progress of the reaction was monitored by TLC. After completion of the reaction the methanol was evaporated. The residue obtained was diluted with water and washed with diethylether. The aqueous layer was cooled in ice and neutralised by 2 M HCl and extracted with ethyl acetate. The solvent was evaporated in vacuo to give a light yellow waxy solid.

Yield: 4.57 g (80.0%). IR (KBr): 3314, 1666 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$, δ ppm): 7.19 (Aib NH, 1H, s); 5.57 (Ile NH, 1H, br s); 4.31 (C^{α}H of Ile, 1H, m); 1.60–1.80 (C^{β}H of Ile, 1H, m); 1.57 (C^{β}Hs of Aib, 6H, s); 1.45 (Boc–CH_3s, 9H, s); 1.21–1.40 (C^{γ}Hs of Ile, 3H, m); 0.92–0.98 (C^{γ}Hs and C^{δ}Hs of Ile, 5H, m). Anal. Calcd for $C_{15}H_{28}N_2O_5$ (316.39): C, 56.93; H, 8.92; N, 8.86. Found: C, 56.80; H, 8.84; N, 8.75.

4.1.4. Boc-Ile-Aib-Val–OMe (peptide I). Compound **2** (1.0 g, 3.16 mmol) was dissolved in DMF (3 ml). Val–OMe (0.42 g, 3.16 mmol) obtained from its hydrochloride was added followed by DCC (0.65 g, 3.16 mmol). The reaction mixture was stirred at room temperature for 5 days. The precipitated dicyclohexylurea (DCU) was filtered and diluted with ethyl acetate. The organic layer was washed with excess of water, 1 M 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 light yellow solid. Purification was done using silica gel as stationary phase and ethyl acetate–petroleum ether mixture as the eluent. Single crystals were grown from acetone–water mixture (90:10) by slow evaporation and were stable at room temperature.

Yield: 1.22 g (90.0%). Mp=109–110 °C; IR (KBr): 3423, 3367, 3301, 1732, 1703, 1658 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$, δ ppm): 7.09 (Val NH, 1H, d, J=8.4 Hz); 6.58 (Aib NH, 1H, s); 5.03 (Ile NH, 1H, d, J=7.8 Hz); 4.48–4.53 (C^{α}H of Val, 1H, m); 3.90–3.91 (C^{α}H of Ile, 1H, m); 3.73 (–OCH_3, 3H, s); 2.12–2.24 (C^{β}H of Val, 2H, m); 1.87–1.92 (C^{β}H of Ile, 1H, m); 1.56 (C^{β}Hs of Aib, 6H, s); 1.45 (Boc–CH_3s, 9H, s); 1.07–1.21 (C^{γ}Hs of Ile, 5H, m); 0.90–0.96 (C^{γ}Hs of Val and C^{δ}Hs of Ile, 9H, m);

^{13}C NMR 75 MHz ($CDCl_3$, δ ppm): 172.99, 172.27, 171.53, 155.88, 80.15, 59.83, 57.60, 57.44, 51.99, 36.94, 31.14, 30.86, 28.26, 25.64, 24.79, 18.95, 17.72, 15.57, 11.35. Anal. Calcd for $C_{22}H_{41}N_3O_6$ (443.57): C, 59.57; H, 9.32; N, 9.48. Found: C, 59.52; H, 9.28; N, 9.52; HR-MS (M^+Na^+)=451.86, M_{calcd} (M^+Na)$^+$=452.54.

4.1.5. Boc-Ile-Aib-Leu–OMe (peptide II). Compound **2** (1.0 g, 3.16 mmol) was dissolved in DMF (3 ml). Leu–OMe (0.46 g, 3.16 mmol) obtained from its hydrochloride was added followed by DCC (0.65 g, 3.16 mmol). The reaction mixture was stirred at room temperature for 5 days. The precipitated dicyclohexylurea (DCU) was filtered and diluted with ethyl acetate. The organic layer was washed with excess of water, 1 M 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 light yellow solid. Purification was done using silica gel as stationary phase and ethyl acetate–petroleum ether mixture as the eluent. Single crystals were grown from acetone–water mixture (90:10) by slow evaporation and were stable at room temperature.

Yield: 1.27 g (90.0%). Mp=123–124 °C; IR (KBr): 3390, 3314, 1715, 1666 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$, δ ppm): 7.05 (Leu NH, 1H, d, J=7.8 Hz); 6.61 (Aib NH, 1H, s); 5.05 (Ile NH, 1H, d, J=7.2 Hz); 4.53–4.60 (C$^{\alpha}$H of Leu, 1H, m); 3.87–3.90 (C$^{\alpha}$H of Ile, 1H, m); 3.72 (–OCH_3, 3H, s); 1.87–1.89 (C$^{\beta}$Hs of Ile and Leu, 3H, m); 1.55 (C$^{\beta}$Hs of Aib, 6H, s); 1.45 (Boc–CH_3s, 9H, s); 1.12–1.16 (C$^{\gamma}$Hs of Ile, 2H, m); 0.92–0.93 (C$^{\delta}$Hs of Ile and Leu, 9H, m); ^{13}C NMR 75 MHz ($CDCl_3$, δ ppm): 173.93, 173.23, 171.37, 155.88, 80.16, 59.81, 57.40, 52.09, 50.97, 41.28, 36.91, 28.25, 25.40, 24.81, 24.78, 24.75, 22.76, 21.83, 15.50, 11.33. Anal. Calcd for $C_{22}H_{41}N_3O_6$ (443.57): C, 59.57; H, 9.32; N, 9.48. Found: C, 59.53; H, 9.29; N, 9.53; HR-MS (M^+Na^+)=466.06, M_{calcd} (M^+Na)$^+$=466.57.

4.1.6. Boc-Ile-Aib-Phe–OMe (peptide III). Compound **2** (1.0 g, 3.16 mmol) was dissolved in DMF (3 ml). Phe–OMe (0.57 g, 3.16 mmol) obtained from its hydrochloride was added followed by DCC (0.66 g, 3.16 mmol). The reaction mixture was stirred at room temperature for 5 days. The precipitated dicyclohexylurea (DCU) was filtered and diluted with ethyl acetate. The organic layer was washed with excess of water, 1 M 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 light yellow solid. Purification was done using silica gel as stationary phase and ethyl acetate–petroleum ether mixture as the eluent. Single crystals were grown from acetone–water mixture (90:10) by slow evaporation and were stable at room temperature.

Yield: 1.43 g (95.0%). Mp=114–115 °C; IR (KBr): 3394, 3319, 1716, 1664 cm^{-1}; ^{1}H NMR 300 MHz ($CDCl_3$, δ ppm): 7.13–7.26 (phenyl ring protons, 5H, m); 6.88 (Phe NH, 1H, d, J=7.2 Hz); 6.55 (Aib NH, 1H, s); 4.99 (Ile NH, 1H, d, J=7.2 Hz); 4.80–4.87 (C$^{\alpha}$H of Phe, 1H, m); 3.84–3.89 (C$^{\alpha}$H of Ile, 1H, m); 3.72 (–OCH_3, 3H, s); 3.06–3.20 (C$^{\beta}$Hs of Phe, 2H, m); 1.86–1.90 (C$^{\beta}$Hs of Ile, 1H, m); 1.52 (C$^{\beta}$Hs of Aib, 6H, s); 1.46 (Boc–CH_3s, 9H, s); 1.05–1.35 (C$^{\gamma}$Hs of Ile, 5H, m); 0.90–0.94 (C$^{\delta}$Hs of Ile, 3H, m); ^{13}C NMR 75 MHz ($CDCl_3$, δ ppm): 173.75, 171.85, 171.18, 155.89,135.96, 129.28, 128.46, 127.02, 80.14, 59.64, 57.27, 53.50, 52.09, 37.87, 36.90, 28.27, 25.08, 24.89, 24.79, 15.52, 11.37. Anal. Calcd for $C_{25}H_{39}N_3O_6$ (477.56): C, 62.86; H, 8.23; N, 8.80. Found: C, 62.83; H, 9.19; N, 8.75; HR-MS (M^+Na^+)=499.99, M_{calcd} (M^+Na)$^+$=500.56.

4.2. FTIR spectroscopy

IR spectra were examined in Perkin–Elmer-782 model spectrophotometer. The solid-state FTIR measurements were performed using the KBr disk technique.

4.3. NMR experiments

All ^{1}H NMR and ^{13}C NMR studies were recorded on a Bruker Avance 300 model spectrometer operating at 300, 75 MHz, respectively. The peptide concentrations were in the range 1–10 mM in $CDCl_3$ for ^{1}H NMR and 30–40 mM in $CDCl_3$ for ^{13}C NMR. Solvent titration experiments were carried out at a concentration of 10 mM in $CDCl_3$ with gradual addition of d_6-DMSO from 0 to 12% v/v approximately.

4.4. Mass spectrometry

Mass spectra were recorded on a HEWLETT PACKARD Series 1100MSD and Micromass Qtof Micro YA263 mass spectrometers by positive mode electrospray ionisation.

4.5. Circular dichroism spectroscopy

Methanolic solution of peptides **I–III** (1.5 mM as final concentration) was used for obtaining the spectra. Far-UV CD measurements were recorded at 25 °C with 0.5 s averaging time, a scan speed of 50 nm/min, using a JASCO spectropolarimeter (J 720 model) equipped with a 0.1 cm pathlength cuvette. The measurements were taken at 0.2 nm wavelength intervals, 2.0 nm spectral bandwidth and five sequential scans were recorded for each sample.

4.6. Field emission scanning electron microscopic study

Morphology of peptide **III** was investigated using field emission scanning electron microscope (FESEM). For the study, fibrous materials (slowly grown from acetone) were dried and gold coated. The micrograph was taken in a FESEM apparatus (JEOL JSM-6700F).

4.7. Single crystal X-ray diffraction study

All data were collected with graphite monochromated X-radiation (λ=0.71073 Å) on a Bruker SMART diffractometer. Data processing was performed with standard Bruker software.[17] Structure solutions were by direct methods. Refinement was on F^2 using full-matrix least-squares techniques. All data were corrected for absorption with SADABS.[18] All hydrogen atoms are placed at calculated positions, and have been refined with a riding model. The exception being the hydrogen atoms bound to nitrogen atoms, and the hydrogen atoms of the water molecule in the model of peptide **II**, which were all located in the difference Fourier maps and

Table 3. Crystallographic refinement details for peptides **I–III**

	Peptide **I**	Peptide **II**	Peptide **III**
Crystal size (mm^3)	0.28×0.20×0.12	0.51×0.39×0.12	0.28×0.27×0.03
Crystal colour	Colourless	Colourless	Colourless
Crystal habit	Block	Block	Plate
Chemical formula	$C_{21}H_{39}N_3O_6$	$C_{22}H_{41}N_3O_6 \cdot H_2O$	$C_{25}H_{39}N_3O_6$
Formula weight (g)	429.55	461.59	477.59
Crystal system	Monoclinic	Tetragonal	Triclinic
Space group	$P2_1$	$P4_3$	$P1$
Z	2	4	1
A (Å)	6.0564(2)	12.0348(5)	6.087(1)
B (Å)	21.34406(1)	12.0348(5)	9.968(2)
C (Å)	9.8971(2)	18.412(1)	11.413(2)
α (°)	90.000	90.000	92.977(4)
β (°)	107.339(1)	90.000	100.634(4)
γ (°)	90.000	90.000	102.505(4)
V ($Å^3$)	1221.24(6)	2666.8(2)	661.4(2)
Temperature (K)	120(2)	120(2)	120(2)
μ (Mo Kα) (mm^{-1})	0.085	0.085	0.086
Collected reflections	7726	10,735	6060
Unique reflections	4601	5089	4866
No. parameters	293	319	327
R(int)	0.0215	0.0209	0.0211
GoF	1.047	1.037	1.055
R_1 [$I>2\sigma(I)$]	0.0329	0.0293	0.0364
wR_2 [$I>2\sigma(I)$]	0.0736	0.0645	0.0731

refined freely. All non-hydrogen atoms were refined anisotropically. Additional crystallographic information can be found in Table 3.

Cambridge Crystallographic Data Center references CCDC 630721–630723 for peptides **I–III**, respectively.

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