DOI: 10.1002/cbic.200800053

Dehydrophenylalanine (ΔPhe) as a β Breaker: Extended Structure Terminated by a ΔPhe-Induced Turn in the Pentapeptide Boc-Phe1-Ala2-Ile3-ΔPhe4-Ala5-OMe

Madhvi Gupta,[a] Rudresh Acharya,[b] Aseem Mishra,[a] Suryanarayanarao Ramakumar,[b] Faizan Ahmed,[c] and Virander Singh Chauhan*[a]

Amyloid is a highly insoluble, aggregated state of certain polypeptide sequences associated with a range of debilitating diseases. A key step in amyloid formation is the transition of a protein from its native structure to a β-sheet arrangement; this suggests that the prevention of the ability of amyloidogenic proteins to adopt a β-sheet conformation would be useful as a way to impede the amyloid self-assembly process.[1] The use of β-breaker residues is one approach for the development of peptide-based fibrillization-inhibiting drugs. Soto et al. demonstrated that the incorporation of β-sheet-breaker elements into short peptides composed of the recognition sequence of the amyloidogenic proteins inhibited amyloid formation.[1c,d] In this context, β-sheet-breaker residues, such as proline and α-aminoisobutyric acid (Aib), which is an unnatural amino acid residue, have been found to inhibit amyloid fibril formation.[2a–d]

α,β-Dehydrophenylalanine (ΔPhe) is an analogue of phenylalanine, but it has a double bond between the Cα=Cβ atoms, which leads to an extended conjugation with the π electrons of the ring; this makes ΔPhe a planar residue. ΔPhe induces β turns in short peptides (even as short as tripeptides) and 3_{10} helices in long peptides.[3a–e] In this regard ΔPhe and Aib show similar conformational behavior.[2c,3d] The potential of Aib as a β-sheet-breaker residue is already known.[2a–c] Thus, the present study is an endeavor to explore the potential of ΔPhe as a β-sheet-breaker residue. Earlier studies from our laboratory have shown that the incorporation of ΔPhe along with protein-coded helix-forming residues induces 3_{10} helical structures.[3e] In this study, a peptide, Boc-Phe1-Ala2-Ile3-ΔPhe4-Ala5-OMe (**1**), which contains two β-strand-preferring amino acids (Phe1, Ile3),[4] was synthesized, and its molecular conformation was studied by using X-ray crystallography (Figure 1). Table S1 in the Supporting Information shows the crystallographic details of the peptide. The stretch, Phe1-Ala2-Ile3, has an extended (β strand) conformation, and has average ϕ,ψ values of (−125°, 114°). By assuming a helical conformation, however, ΔPhe4 terminated strand propagation. The important torsion angles for the peptide are shown in Table S2. The *tert*-butoxycarbonyl (Boc) group conformation is characterized by C1–O1–C5–N1 (θ^1) and O1–C5–N1–C1A (ω_o) torsion angles. It is generally noted that Boc groups with a *trans–trans* conformation permit the carbonyl oxygen O2 to act as an acceptor for the first 4→1 hydrogen bond in helical peptides.[5] In the present peptide, Boc has a *trans–trans* conformation ($\theta^1 = -176.1$ (3)°, $\omega_o = -166.9$ (3)°), and O2 is involved in a N–H···O intermolecular hydrogen bond with a symmetry-related parallel strand in the crystal lattice. All the peptide units are in the *trans* conformation (Table S2). As expected, the ΔPhe4 side chain is planar, as indicated by the angles $\chi^1 = -5°$ and $\chi^2 = -24°$ (Table S3). It is noteworthy that chain termination and turn formation occur together with the presence of C–H···O hydrogen bond between atoms Cγ2 of Ile3 and carbonyl oxygen of Ala5 (Table S3). In the crystal packing, β strands are arranged both in parallel and antiparallel orientations (Figure 2 A and B). The β strands extend approximately along the crystallographic *c* axis. Parallel β strands are related by translation symmetry along the crystallographic *a* axis and they define β sheets in the *a*–*c* plane, which are stabilized by a network of interstrand N–H···O and Cα–H···O hydrogen bonds (Figure 3). The pattern of interstrand hydrogen bonds is similar to that seen between parallel β strands in proteins.[6] Antiparallel strands are related to each other by a 2_1 screw axis along the *b* direction, and they are stabilized mainly through aromatic–aromatic and van der Waals interactions. (The results of a search carried out in the PDB database for proteins that contain side-chain to main-chain hydrogen bonds, similar to the arrangement seen in the

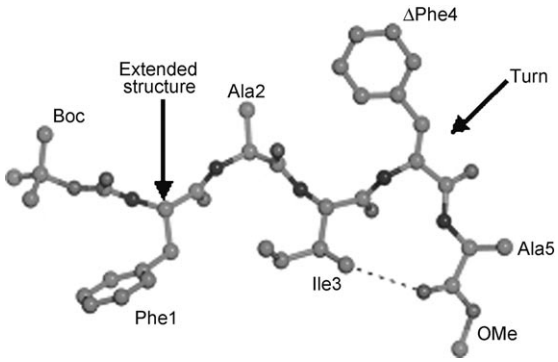

Figure 1. The molecular conformation of peptide **1** (Boc-FAIΔFA-OMe).

[a] *Dr. M. Gupta, A. Mishra, Prof. V. S. Chauhan*
International Center for Genetic Engineering and Biotechnology
ICGEB, New Delhi-67 (India)
Fax: (+91) 11-26102317
E-mail: virander@icgeb.res.in

[b] *Dr. R. Acharya, Prof. S. Ramakumar*
Department of Physics, Bioinformatics Center
Indian Institute of Science
Bangalore 560012 (India)

[c] *Prof. F. Ahmed*
Jamia Millia Islamia, National Islamic University
Biosciences Department
110025 New Delhi (India)

Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author.

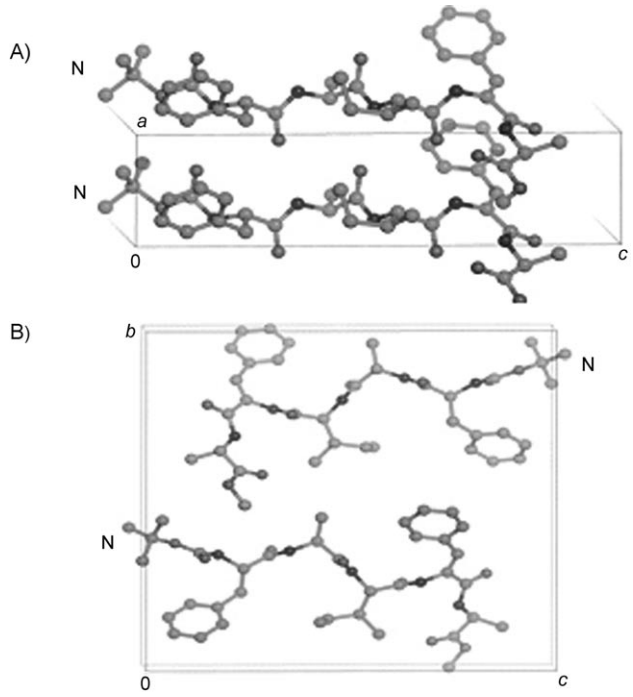

Figure 2. A) Parallel and B) antiparallel orientation of β strands in the crystal packing of peptide **1**.

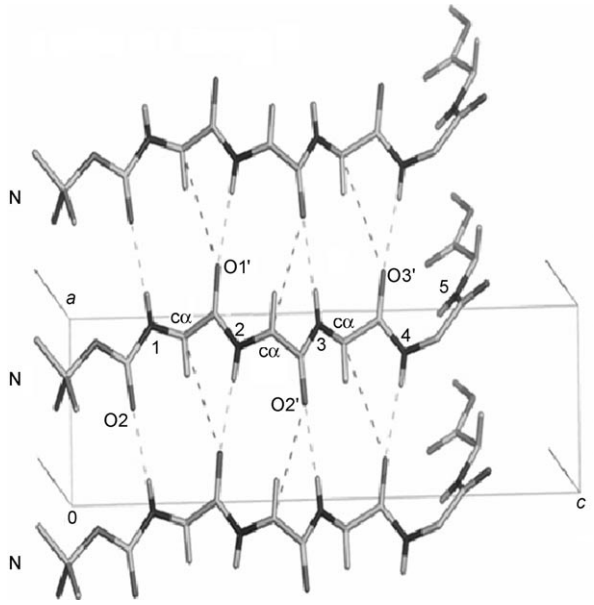

Figure 3. The network of N–H···O and Cα–H···O hydrogen bonds between parallel β strands in the crystal packing of peptide **1**.

present pentapeptide, is shown in Table S4.) The present peptide provides the first example of an extended structure that is terminated by a ΔPhe residue; this led us to design peptide-based inhibitors of amyloid-fibril formation.

Alzheimer's disease is the most common form of dementia and is associated with the deposition of senile plaques that are formed by the aggregation of β-amyloid polypeptide (39–42).[7a,b] Through peptide array technology, a central region ($A\beta_{16-20}$) that mediates intermolecular interactions between $A\beta_{1-40}$ (Aβ) monomers to form amyloid fibrils, has been identified.[1g] Short synthetic peptides that contain β-sheet-breaker residues have been shown to inhibit Aβ-induced fibrillization.[1c,f,7c] In this context, the specific role of ΔPhe as a β-sheet-breaker residue was investigated by incorporating it in the 16–20 segment of the Aβ sequence. Three peptides, $AcKLVFFNH_2$ (**2**; $A\beta_{16-20}$ with N- and C-terminal acetylation and amidation), $AcKL\Delta FVFNH_2$ (**3**), and $AcKLV\Delta FFNH_2$ (**4**), were synthesized by using SPPS chemistry. The synthetic peptides were purified by using RP-HPLC and characterized by using mass spectrometry and CD spectroscopy. Their amyloidogenic nature was determined by using transmission electron microscopy (TEM). We wanted to establish if the designed peptides would inhibit fibrillization of the Aβ peptide. We first examined whether the peptides (Aβ, **2**, **3**, and **4**) would form fibrils spontaneously by using TEM (Figure 4). As expected, Aβ demonstrated amyloid-like, long, unbranched fibrils whereas **2** demonstrated fibrils, but with a reduced density compared to Aβ alone.[8] Peptide **3** did not demonstrate any fibrillar material. In peptide **4**, although no fibrils were seen, a few ribbon-like structures were noticed. There was no change in the appearance of the electron micrographs of the peptides even after long periods of incubation (up to one month). The addition of peptides **2**, **3**, and **4** to Aβ had a dramatic effect on the morphology of Aβ fibrillar aggregates. Incubation of Aβ with **2** reduced the density of fibrils.[8] The fibrils were completely absent after incubation of Aβ with **3**. Incubation of preformed Aβ fibrils with peptide **3** led to complete disintegration of the fibril structure (data not shown). These results indicate that **3** can be a potent fibrillogenesis inhibitor, and might have a more potent antifibrillizing activity than the native peptide **2**. After incubation of Aβ with peptide **4**, the fibrils were less numerous and more thread-like; this suggests that **4** can also partially prevent fibrillization.

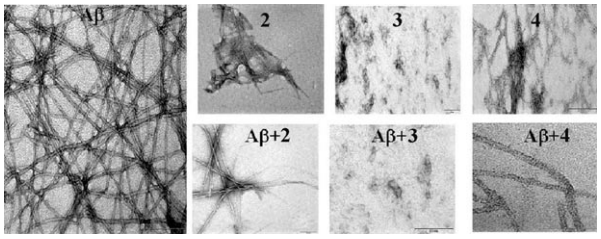

Figure 4. Electron micrographs of peptides Aβ, **2**, **3**, **4** alone, and Aβ incubated with peptides **2**, **3**, and **4** separately.

CD spectra of the peptides were acquired in trifluoroethanol (TFE; Figure 5). Peptide **2** displayed a relatively strong negative band near 200 nm that was accompanied by a weak negative band or shoulder at ~223 nm. This type of CD curve (class U spectrum) is generally characteristic of aperiodic conformers.[9] The CD spectrum of peptide **3** revealed a positive band at ~195 nm and two minima at ~228 and ~205 nm; the CD spectrum of peptide **4** revealed a positive band at ~195 nm and

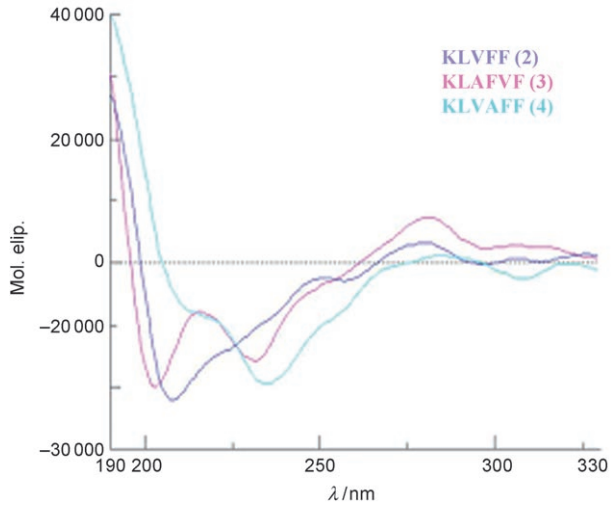

Figure 5. CD spectra of peptides **2**, **3**, and **4** in trifluoroethanol.

two minima at ~228 and ~210 nm. This type of spectrum is suggestive of 3_{10}-helical structure. The CD studies are suggestive of helical structures for peptides **3** and **4**. In this regard, Aib-containing helical peptides have been shown to not only disrupt β-sheet structures in a large number of peptides, but also inhibit fibrillization.[2a–c]

The work by Soto et al. has demonstrated the remarkable potential of pentapeptide, LPFFD, in inhibiting fibrils formed by Aβ(1–40) and (1–42); LPFFD exhibits high solubility, and low immunogenicity.[1c] However, one major drawback with the use of peptides as drugs in neurological diseases is their rapid metabolism by proteolytic enzymes.[10a,b] The ability of ΔPhe-containing peptides to resist enzymatic (chymotrypsin) degradation has been shown by using RP-HPLC, in which a single intact peak of similar intensity was seen before and after incubation of peptides **3** and **4** with the enzyme (data not shown). In this context, the stability of ΔPhe residue to enzymatic degradation could be an added advantage.[11] Moreover, the potential of ΔPhe residue to control not only Aβ amyloid aggregation but also inhibit amyloid formation by other polypeptides (hIAPP) has been observed (unpublished results). Taken together, these results suggest a new strategy for the development of amyloid-formation inhibitors by using ΔPhe as a β-breaker residue. This finding might be of relevance to chemists and biochemists in designing peptide-based antifibrillization drugs that are resistant to enzymatic degradation in vivo.

Experimental Section

Peptide synthesis: Pentapeptide **1** was synthesized by standard solution-phase procedures by using Boc chemistry. Amino acid couplings were performed by using the mixed-anhydride method. Trifluoroacetic acid (TFA) was used to remove the N-terminal Boc group in the peptide fragments. The dehydration of D,L-β-phenylserine was carried out by using fused NaOAc and Ac_2O to obtain the ΔPhe moiety. All reactions were monitored by thin layer chromatography (TLC) on precoated silica plates by using a $CHCl_3$/MeOH (9:1).

Boc-Ile-(β-OH)-Phe-OH: *N*-methylmorpholine (2.62 mL, 20 mmol) and IBCF (2.78 mL; 20 mmol) were added to a precooled solution (−20 °C) of Boc-Ile-OH (4.62 g, 20 mmol) in THF (30 mL). After being stirred for 30 min, a precooled solution of D,L-Phe-(β-OH) (3.982 g, 22 mmol), NaOH (0.88 g, 22 mmol), and H_2O (30 mL) was added, and the mixture was stirred at 0 °C for 1 h, and at room temperature, overnight. The organic solvent was removed under reduced pressure and the aqueous phase was acidified with citric acid to pH 3.0 and extracted with EtOAc (3×15 mL). The EtOAc layer was washed with H_2O, dried over anhydrous Na_2SO_4 and evaporated to yield a cream-colored, powdery compound; yield: 6.9 g (88%), R_f = 0.3, m.p. 85 °C.

Boc-Ile-ΔPhe-azlactone: Boc-Ile-(β-OH)-Phe-OH (5.91 g; 15 mmol) was treated with anhydrous NaOAc (1.599 g, 19.5 mmol) and freshly distilled Ac_2O (30 mL) for 36 h at room temperature. The mixture was poured over crushed ice; the resultant precipitate was washed with cold H_2O and finally recrystallized from acetone/H_2O; yield: 6.3 g (88%), R_f = 0.6, m.p. 95 °C.

Fmoc-Val/Leu-ΔPhe-azlactone: This was synthesized by using a similar method as described above.

Fmoc-Val-(β-OH)-Phe-OH: Yield: 9.17 g (20 mmol; 91.4%), R_f = 0.30, m.p. 72–74 °C.

Fmoc-Val-ΔPhe-azlactone: Yield: 4.33 g (10 mmol; 93%), R_f = 0.95, m.p. 102–104 °C.

Fmoc-Leu-(β-OH)-Phe-OH: Yield: 9.8 g (20 mmol; 95%), R_f = 0.36, m.p. 82–84 °C.

Fmoc-Leu-ΔPhe-azlactone: Yield: 4.56 g (10 mmol; 95%), R_f = 0.82, m.p. 164–166 °C.

Synthesis of peptide 1: The tripeptide Boc-Ile-ΔPhe-Ala-OMe was first synthesized. Ala-OMe·HCl (2.196 g, 15.8 mmol) was added to a solution of Boc-Ile-ΔPhe-Azl (5.656 g, 15.8 mmol) in CH_2Cl_2 (20 mL), then Et_3N (2.15 mL, 15.8 mmol) was added, and the mixture was stirred at room temperature for 30 h. The solvent was removed under reduced pressure, and the aqueous phase was washed once with EtOAc and acidified with solid citric acid. The EtOAc layer was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated to yield the desired product. The tripeptide Boc-Ile-ΔPhe-Ala-OMe was deprotected at its N terminus by using a mixture of TFA in CH_2Cl_2 (1:1, *v/v*) at room temperature for 30 min. Excess acid was removed in vacuo, and the residue was triturated with dry Et_2O and filtered. Dicyclohexylcarbodiimide (DCC) (0.42 g, 2.1 mmol) and 1-hydroxybenzotriazole (HOBT) (0.33 g, 2.1 mmol) were added to a precooled solution of Boc-Ala-OH (0.397 g, 2.1 mmol) in DMF (20 mL), and the mixture stirred for 30 min. The trifluoroacetate salt of the tripeptide in DMF and Et_3N (0.28 mL) was added, and the mixture was stirred at room temperature for 16 h. The precipitated dicyclohexylurea was filtered off, and the solvent was removed in vacuo. The reaction was worked up as above to yield the tetrapeptide Boc-Ala-Ile-ΔPhe-Ala-OMe. The Boc group of the tetrapeptide was deprotected by using TFA in CH_2Cl_2, and the deprotected peptide was coupled with Boc-Phe-OH by using DCC and HOBT as above, to finally yield the cream-colored Boc-Phe-Ala-Ile-ΔPhe-Ala-OMe. The pentapeptide was purified by RP-HPLC with a Waters C18 column (300 mm×3.9 mm) by using a MeOH/H_2O gradient; yield: 700 mg (80%), R_f = 0.81 ($CHCl_3$/MeOH, 9:1), m.p. 165 °C (from MeOH). Peptide identity was confirmed with ES-MS; *m/z* calcd for $C_{36}H_{49}N_5O_8$: 679; found: 702 $[M+Na]^+$.

Synthesis of peptides 2, 3, and 4: Fmoc-protected amino acids (Fmoc: 9-flourenylmethyoxycarbonyl) for solid-phase peptide syn-

thesis were obtained from Novabiochem (India). The three pentapeptides were synthesized manually on a 0.5 mmol scale. Fmoc-rinkamide *p*-methylbenzhydrylamine (MBHA) resin (0.5 mmol g^{-1}) was used to afford the C-terminal primary amides. Couplings were performed by using carbodiimide. Solution-phase methodology was used to introduce the ΔPhe residue in the pentapeptides as a dipeptide block by dehydration of Fmoc-aa-D,L-threo-β-phenylserine (where aa is either valine or leucine) by using fused NaOAc and freshly distilled Ac_2O as reported earlier. Fmoc deprotection was performed with piperidine (20% in DMF). After the addition of the final residue, the N terminus was acetyl capped, and the resin was rinsed with DMF/CH_2Cl_2/MeOH, and then dried. The final peptide deprotection and cleavage from the resin was achieved with TFA/H_2O/TIS 95:2.5:2.5 (10 mL) for 2 h. The resin was filtered and the filtrate was concentrated, after which the crude peptides were precipitated with cold diethyl ether. The supernatant was decanted, and the crude peptides were suspended in H_2O with a minimal amount of acetonitrile (ACN), then frozen and lyophilized to dryness. The crude peptides were purified by reverse-phase HPLC by using a TFA (0.1%) in H_2O/ACN on a Waters Deltapak C18 (19 mm × 300 mm). A linear gradient of ACN from 5 to 80% over 80 min at a flow rate of 6 mL min^{-1} was used. Physical characterization of the synthesized peptides was as follows: **2**: yield: 325 mg (94%), m.p. 170–175 °C, t_R 54 min, *m/z* 693.5 Da; **3**: yield: 304 mg (88%), m.p. 180–185 °C, t_R 57 min, *m/z* 691.2 Da; **4**: yield: 307 mg (89%), m.p. 189–194 °C, t_R 57 min, *m/z* 691.3 Da.

X-ray crystallography: The purified peptide was crystallized from MeOH by using the slow evaporation method over a period of three months. The X-ray diffraction data were collected by using a Bruker APEX CCD diffractometer equipped with Mo-K_α radiation. The structure was solved by using the direct method employed in the SHELXS 97 program. The refinement of the structure was carried out by using full-matrix least-squares refinement on F_o^2 implemented in SHELXL 97 software.

Transmission electron microscopy: The synthetic $A\beta_{1-40}$ was dissolved in H_2O prior to dissolution in PBS buffer (Aβ has low solubility in buffers) to produce a 0.5 mM stock solution. Lyophilized peptide ligands were dissolved in 100% hexafluoroisopropanol (HFIP) at a concentration of 100 mM. To avoid preaggregation, fresh stock solutions were prepared for each experiment. Peptide stock solutions were diluted with PBS to a final concentration of 10 mM. The Aβ peptide sample was incubated either with or without peptide ligands for 10 days at room temperature. A sample (10 mL) was placed on a 400-mesh copper grid covered with carbon-stabilized formvar film. After 1 min, the excess fluid was removed and the grid was negatively stained by treatment with 2% uranyl acetate in H_2O for 2 min.

Circular dichroism studies: The spectra were acquired between 190–330 nm (0.1 cm cell, peptide concentration ~100 mM) at 0.1 nm intervals with a time constant of 4 s and a scan speed of 200 nm min^{-1}, and averaged over six separate scans. The spectra were baseline corrected and smoothed. The peptide concentration was determined by using the molar extinction coefficient of ΔPhe (~19000 $M^{-1} cm^{-1}$).

Acknowledgements

Financial support from the Department of Science and Technology, India and WHO is acknowledged. We thank the Department of Biotechnology, India, for access to the facilities at the Bioinformatics and Interactive Graphics Facility (I.I.Sc., Bangalore). The CCD diffractometer facility is supported under the IRPHA program of the Department of Science and Technology.

Keywords: Alzheimer's disease · circular dichroism · dehydrophenylalanine · protein structures · trifluoroethanol

[1] a) E. Gazit, *Curr. Med. Chem.* **2002**, *9*, 1725–1735; b) M. Stefani, C. M. Dobson, *J. Mol. Med.* **2003**, *81*, 678–699; c) C. Soto, E. M. Sigurdsson, L. Morelli, R. A. Kumar, E. M. Castano, B. Frangione, *Nat. Med.* **1998**, *4*, 822–826; d) C. Soto, *FEBS Lett.* **2001**, *498*, 204–207; e) L. A. Scrocchi, Y. Chen, S. Waschuk, F. Wang, S. Cheung, A. A. Darabie, J. McLaurin, P. E. Fraser, *J. Mol. Biol.* **2002**, *318*, 697–706; f) A. Kapurniotu, A. Schmauder, K. Tenidis, *J. Mol. Biol.* **2002**, *315*, 339–350; g) L. O. Tjernberg, J. Näslund, F. Lindqvist, J. Johansson, A. R. Karlström, J. Thyberg, L. Terenius, C. Nordstedt, *J. Biol. Chem.* **1996**, *271*, 8545–8548.

[2] a) S. Gilead, E. Gazit, *Angew. Chem.* **2004**, *116*, 4133–4136; *Angew. Chem., Int. Ed.* **2004**, *43*, 4041–4044 b) R. Kaul, P. Balaram, *Bioorg. Med. Chem.* **1999**, *7*, 105–117; c) I. L. Karle, P. Balaram, *Biochemistry* **1990**, *29*, 6747–6756; d) C. Soto, *Nat. Rev. Neurosci.* **2003**, *4*, 49–60.

[3] a) H. C. Patel, T. P. Singh, V. S. Chauhan, *Biopolymers* **1990**, *29*, 509–515; b) T. P. Singh, M. Haridas, V. S. Chauhan, A. Kumar, D. Viterbo, *Biopolymers* **1987**, *26*, 819–829; c) P. Mathur, S. Ramakumar, V. S. Chauhan, *Biopolymers* **2004**, *76*, 150–156; d) C. Toniolo, E. Benedetti, *Macromolecules* **1991**, *24*, 4004–4009; e) K. R. Rajashankar, S. Ramakumar, T. K. Mal, R. M. Jain, V. S. Chauhan, *Biopolymers* **1995**, *35*, 141–147.

[4] Y. Kallberg, M. Gustafsson, B. Persson, J. Thyberg, J. Johansson, *J. Biol. Chem.* **2001**, *276*, 12945–12950.

[5] E. Benedetti, C. Pedone, C. Toniolo, G. Nemethy, M. S. H. Pottle, A. Scheraga, *Int. J. Pept. Protein Res.* **1980**, *16*, 156–172.

[6] G. F. Fabiola, S. Krishanaswamy, V. Nagarajan, V. Pattabhi, *Acta. Crystallogr. D Biol. Crystallogr.* **1997**, *53*, 316–320.

[7] a) P. T. Lansbury, Jr., *Acc. Chem. Res.* **1996**, *29*, 317–321; b) F. E. Cohen, J. W. Kelly, *Nature* **2003**, *426*, 905–909; c) C. Soto, *Mol. Med. Today* **1999**, *5*, 343–350.

[8] D. J. Gordon, R. Tappe, S. C. Meredith, *J. Pept. Res.* **2002**, *60*, 37–55.

[9] M. Hollöasi, L. Ötvös, Jr., L. Ürge, J. Kajtár, A. Percel, I. Lackó, Z. S. Vadász, G. D. Fasman, *Biopolymers* **1993**, *33*, 497–510.

[10] a) J.-L. Fauchère, C. Thurieau, *Adv. Drug Res.* **1992**, *23*, 127–159; b) C. Adessi, C. Soto, *Curr. Med. Chem.* **2002**, *9*, 963–978.

[11] M. L. English, C. H. Stammer, *Biochem. Biophys. Res. Commun.* **1978**, *83*, 1464–1467.

Received: January 28, 2008
Published online on May 13, 2008

