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De novo design, synthesis and solution conformational study of two didehydroundecapeptides: effect of nature and number of amino acids interspersed between ΔPhe residues

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***De novo* design of peptides and proteins has recently surfaced as an approach for investigating protein structure and function. This approach vitally tests our knowledge of protein folding and function, while also laying the groundwork for the fabrication of proteins with properties not precedented in nature. The success relies heavily on the ability to design relatively short peptides that can espouse stable secondary structures. To this end, substitution with α,β-didehydroamino acids, especially α,β-didehydrophenylalanine (Δ^{z}Phe), comes in use for spawning well-defined structural motifs. Introduction of ΔPhe induces β-bends in small and 3_{10}-helices in longer peptide sequences. The present work aims to investigate the effect of nature and the number of amino acids interspersed between two ΔPhe residues in two model undecapeptides, Ac-Gly-Ala-ΔPhe-Ile-Val-ΔPhe-Ile-Val-ΔPhe-Ala-Gly-NH_2 (I) and Boc-Val-ΔPhe-Phe-Ala-Phe-ΔPhe-Phe-Leu-Ala-ΔPhe-Gly-OMe (II). Peptide I was synthesized using solid-phase chemistry and characterized using circular dichroism spectroscopy. Peptide II was synthesized using solution-phase chemistry and characterized using circular dichroism and nuclear magnetic resonance spectroscopy. Peptide I was designed to examine the effect of incorporating β-strand-favoring residues like valine and isoleucine as spacers between two ΔPhe residues on the final conformation of the resulting peptide. Circular dichroism studies on this peptide have shown the existence of a 3_{10}-helical conformation. Peptide II possesses three amino acids as spacers between ΔPhe residues and has been reported to adopt a mixed $3_{10}/\alpha$-helical conformation using circular dichroism and nuclear magnetic resonance spectroscopy studies.**

Supporting information may be found in the online version of this article.

Keywords: α; β-didehydrophenylalanine (ΔPhe); β-branched residues; *de novo* design; nuclear magnetic resonance; circular dichroism; 3_{10}-helical conformation; α-helical conformation; simulated annealing

Introduction

De novo protein design endeavors to construct novel polypeptide sequences that fold into well-defined secondary and tertiary structures resembling those found in native proteins. Many *de novo* design strategies have relied on the known penchant of protein amino acids to espouse various secondary structures leading to several remarkable achievements [1–4]. Alternatively, the amalgamation of conformationally restricted, non-protein amino acids by chemical synthesis has led to triumphant designs of secondary and super-secondary structures that mimic proteins [5,6]. In this regard, α,β-didehydro residues, in particular α,β-didehydrophenylalanine (ΔPhe) [7], are being considered as one of the important inducers of conformational constraints in *de novo* design. α,β-Didehydro amino acids are characterized by a double bond between the α and β carbon atoms. This introduces strong steric effects in a didehydro residue, leading to significant alterations in the geometry, conformation and biochemical properties. The sp^2-hybridized carbon atom at both α and β positions, extended conjugation on account of the π-system, restricted mobility of the side chain due to the double bond between α and β carbon atoms and increased planarity of the residue as a whole are some of the special features that have made didehydro peptides attractive targets for conformational studies [8]. The presence of didehydro residues in peptides confers altered bioactivity as well as increased resistance to enzymatic degradation [9]. Thus, introduction of α,β-didehydroamino acid residues into bioactive peptide sequences has become a useful tool in structure–function relationship studies. The ability

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Abbreviations used: *Ac, acetyl; ACN, acetonitrile; Ac_2O, acetic anhydride; Boc, t-butoxycarbonyl; DCM, dichloromethane; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; Fmoc: 9-fluorenylmethoxycarbonyl; HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol; Rinkamide MBHA resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucylmethylbenzhydrylamine resin; MeOH, methanol; NaOAc, sodium acetate; OMe, methyl ester; RP-HPLC, reverse-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid; TFE, trifluoroethanol; TIS, triisopropylsilane; TLC, thin-layer chromatography.*

of ΔPhe to induce β-bends in small and 3_{10}-helices in longer peptide sequences has been well studied [10–19]. A novel, flat β-ribbon structure has been observed in a didehydro pentapeptide [20]. An α-helix in a pentapeptide with a ΔPhe residue in the second position has illustrated the context-dependent design rules for ΔPhe [21]. A notable number of consecutive ΔPhe-containing structures have been shown to adopt the 3_{10}-helix of both screw senses [22–24]. The potential of ΔPhe in achieving desired folding of super-secondary structural motifs including helix–turn–helix [5,25], helical bundle [26] and glycine zipper [27] has been amply demonstrated.

In continuation of our efforts in designing structural motifs using ΔPhe, we report here the design of two undecapeptides. The design strategy for the two peptides is as follows.

Peptide I

In most studies with model peptides containing -ΔPhe-X-X-ΔPhe- motif, the intervening amino acid residues have been those that generally favor helix formation [28]. The effect of incorporation of beta-strand-favoring residues in medium-sized peptides containing ΔPhe has not been investigated. Amino acids with branched β-carbon atom such as valine and isoleucine have strong preference for adopting beta-sheet conformation and are known to destabilize helical conformation. It would be interesting to study the effect of incorporation of these residues in medium-sized peptides containing ΔPhe. With this view in mind, an undecapeptide, Ac-Gly-Ala-ΔPhe-Ile-Val-ΔPhe-Ile-Val-ΔPhe-Ala-Gly-NH_2 (I), with 'Ile-Val' as a two-residue spacer between ΔPhe was designed. The peptide was synthesized using solid-phase peptide chemistry and studied in detail using CD spectroscopy.

Peptide II

Although a number of studies on peptides containing -ΔPhe-X-X-ΔPhe- that assume 3_{10}-helical conformation have been reported. Model peptides containing the -ΔPhe-X-X-X-ΔPhe- motif are relatively unexplored. Preliminary ^{1}H NMR studies on heptapeptides with a three-residue spacer between ΔPhe have suggested an α-helical conformation [29]. For further investigation, an undecapeptide, Boc-Val-ΔPhe-Phe-Ala-Phe-ΔPhe-Phe-Leu-Ala-ΔPhe-Gly-OMe (II), comprising of two -ΔPhe-X-X-X-ΔPhe- motifs was designed and synthesized by solution methods and its structure studied using CD and NMR spectroscopy.

Materials and Methods

Peptide Synthesis

Peptide I

Fmoc-protected amino acids for solid-phase peptide synthesis were obtained from Novabiochem ((Hohenbrunn, Merck, Germany). The undecapeptide was synthesized manually at a 0.5-mmol scale. Fmoc-Rinkamide MBHA resin (Novabiochem) was used to afford carboxyl-terminal primary amide. Couplings were performed by using diisopropylcarbodiimide. ΔPhe moiety was introduced as part of a dipeptide block, obtained through azlactonization and dehydration of Fmoc-AA-DL-threo-β-phenyl serine (AA = alanine or valine) using fused NaOAc and freshly distilled Ac_2O [17]. All reactions were monitored by TLC on precoated silica plates in 9 : 1 $CHCl_3$–MeOH system. Melting points were determined on a Fisher-Johns melting point apparatus (Thermo Fisher Scientific Inc., Waltham, MA, USA) and were uncorrected. Physical characterization of the dipeptide synthons was as follows: Fmoc-Ala-DL-Phe (β-OH)-OH, yield = 91.4%, m.p. = 72–74 °C, R_f = 0.40; Fmoc-Ala-ΔPhe-Azlactone, 93%, 102–104 °C, 0.95; Fmoc-Val-DL-Phe (β-OH)-OH, 90%, 112–115 °C, 0.3; Fmoc-Val-ΔPhe-Azlactone, 91%, 142–145 °C, 0.7. Fmoc deprotection was performed with piperidine (20% in DMF). After addition of the final residue, the amino terminus was Ac capped, and the resin was rinsed with DMF/DCM/MeOH and dried. The final peptide deprotection and cleavage from the resin was achieved with 10 ml of 95 : 2.5 : 2.5 TFA : H_2O : TIS for 2 h. Crude peptide was precipitated with cold ether, dried, taken up in acetic acid and lyophilized. Purification of the peptide was carried out by RP-HPLC using water–ACN gradient on Waters Delta-Pak C18 (19 mm × 300 mm) (Waters, Milford, MA, USA). A linear gradient of ACN from 10% to 70% over 60 min at a flow rate of 6 ml min^{-1} was employed.

Peptide identity was confirmed on a mass spectrometer (QSTAR XL MS/MS system (AB SCIEX, Darmstadt, Germany)). Physical characterization of peptide I with the molecular formula $C_{61}H_{82}N_{12}O_{12}$ was as follows: m.p. 155–160 °C, t_R 43 min, *m/z* calculated 1174 Da, observed 1197 Da (sodium salt).

Peptide II

Synthesis was carried out by conventional solution-phase procedures using Boc chemistry and fragment condensation method. ΔPhe moiety was introduced as part of a dipeptide block, obtained through azlactonization and dehydration of Boc-AA-DL-threo-β-phenyl serine (AA = Ala, Val, Phe) [17]. All reactions were monitored by TLC on precoated silica plates in the solvent system, chloroform/MeOH (9 : 1). Melting points were determined on a Fisher-Johns melting point apparatus and were uncorrected.

Boc-Ala-ΔPhe-Gly-OMe (1)

To a solution of Boc-Ala-ΔPhe-azlactone (10 g, 31.6 mM) in DCM (50 ml), glycine OMe hydrochloride (4 g, 31.6 mM) and triethylamine (31.6 mM) in DCM were added and stirred at room temperature for 48 h. After completion of the reaction, the solvent was removed *in vacuo*. The residue was dissolved in ethyl acetate, washed successively with 5% citric acid solution, water and saturated sodium bicarbonate and dried over anhydrous sodium sulfate. The solvent was finally evaporated to yield the tripeptide 1. Yield 9 g, 70.3%, m.p. = 115–117 °C; R_f = 0.61; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.78 (1H, s, NH ΔPhe), 7.48 (1H, s, CβH ΔPhe), 7.43 (1H, t, NH Gly), 7.35–7.26 (5H, m, aromatic protons), 5.12 (1H, d, NH Ala), 4.16 (1H, m, CαH Ala), 4.09 (2H, m, CαH_2 Gly), 3.73 (3H, s, OCH_3), 1.43 (3H, d, $C^{\beta}H_3$ Ala), 1.41 (9H, s, 3 × CH_3 Boc).

Boc-Leu-Ala-ΔPhe-Gly-OMe (2)

The tripeptide 1 (6 g, 14.8 mM) was deprotected at its N-terminus using a mixture of TFA–DCM (1 : 1 v/v) to obtain TFA.H-Ala-ΔPhe-Gly-OMe. To a solution of Boc-Leu-OH (3.4 g, 14.8 mM) cooled to −10 °C in tetrahydrofuran (30 ml), *N*-methylmorpholine (1.6 ml, 14.8 mM) and isobutyl chloroformate (1.9 ml, 14.8 mmol) were gradually added. A precooled solution of TFA.H-Ala-ΔPhe-Gly-OMe (6 g, 14.8 mM) and triethylamine (2.1 ml, 14.8 mM) in tetrahydrofuran was then added to the reaction mixture. The reaction mixture was stirred for 2 h at 0 °C and overnight at room temperature. The solvent was evaporated *in vacuo* and the residue taken in ethyl acetate, washed with 5% citric acid solution, water and saturated $NaHCO_3$ solution, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to obtain the tetrapeptide 2. Yield 6.6 g, 85.5%, m.p. = 90–92 °C; R_f = 0.35; ^{1}H NMR (400 MHz, $CDCl_3$)

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δ (ppm): 8.19 (1H, s, NH ΔPhe), 7.52 (1H, s, CβH ΔPhe), 7.41 (1H, s, NH Gly), 7.25–7.42 (5H, m, aromatic protons), 6.82 (1H, d, NH Ala), 4.98 (1H, d, NH Leu), 4.37 (1H, m, CαH Ala), 4.12 (2H, d, $C\alpha H_2$ Gly), 4.03 (1H, m, CαH Leu), 3.74 (3H, s, OCH_3), 1.65 (1H, br, $C\beta H_2$ Leu), 1.51 (1H, d, CγH Leu), 1.47 (3H, d, $C\beta H_3$ Ala^3), 1.33 (9H, s, 3 × CH_3 Boc), 0.94–0.91 (6H, m, 2 × $C\delta H_3$ Leu).

Boc-Phe-Leu-Ala-ΔPhe-Gly-OMe (3)

The tetrapeptide 2 (5 g, 9.6 mM) was again deprotected at its N-terminus using a mixture of TFA–DCM (1 : 1 v/v) to yield TFA.H-Leu-Ala-ΔPhe-Gly-OMe. Boc-Phe-OH (2.6 g, 9.6 mM) was coupled to TFA.H-Leu-Ala-ΔPhe-Gly-OMe using the method described for 2. The reaction was worked up using the usual procedure to afford the pentapeptide 3, 5.5 g, 85.6%, m.p. = 180–182 °C; $R_f = 0.65$; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.34 (1H, s, NH ΔPhe), 7.62 (1H, s, CβH ΔPhe), 7.53 (1H, s, NH Ala), 7.26–7.35 (5H, m, aromatic protons), 6.31 (1H, d, NH Leu), 4.92 (1H, d, NH Phe), 4.45 (1H, t, CαH Ala), 4.23 (1H, m, CαH Leu), 4.15 (2H, t, $C\alpha H_2$ Gly), 4.07 (1H, br, CαH Phe), 3.73 (3H, s, OCH_3), 3.03 (1H, dd, CβH1 Phe), 2.8 (1H, m, CβH2 Phe), 1.7 (1H, br, $C\beta H_2$ Leu), 1.50 (1H, br, CγH Leu), 1.45 (3H, d, $C\beta H_3$ Ala), 1.44 (9H, s, 3 × CH_3 Boc), 0.94–0.88 (6H, m, 2 × $C\delta H_3$ Leu).

Boc-Phe-ΔPhe-Phe-Leu-Ala-ΔPhe-Gly-OMe (4)

The pentapeptide 3 (4 g, 6 mM) was deprotected at the N-terminus using TFA–DCM (1 : 1) and coupled to Boc-Phe-ΔPhe-Azl (2.2 g, 5.5 mM) using the procedure described for the synthesis of 1. Yield 3.3 g, 62.5%, m. p. = 118–120 °C; $R_f^1 = 0.63$; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.55 (1H, s, NH ΔPhe), 8.25 (1H, s, NH ΔPhe), 7.8 (1H, s, NH Leu), 7.45 (1H, s, NH Gly), 7.34 (1H, s, NH Ala), 7.22 (1H, s, NH Phe), 7.23–7.4 and 7.5–7.65 (21H, m, aromatic protons of ΔPhe and Phe residues and CβH of ΔPhe), 6.85 (1H, s, CβH ΔPhe), 4.95 (1H, d, NH Phe^1), 4.40–4.25 (4H, m, CαHs of Ala, Leu, Phe^1 and Phe^3), 4.10 (2H, m, CαH Gly), 3.68 (3H, s, OCH_3), 3.0–3.2 (4H, m, CβHs Phe^1 and CβHs Phe^2), 1.85 (2H, br, $C\beta H_2$ Leu), 1.65 (1H, br, CγH Leu), 1.45 (3H, d, $C\beta H_3$ Ala), 1.31 (9H, s, 3 × CH_3 Boc), 1.00–0.94 (6H, m, 2 × $C\delta H_3$ Leu).

Boc-Val-ΔPhe-Phe-Ala-Phe-ΔPhe-Phe-Leu-Ala-ΔPhe-Gly-OMe (Peptide II)

The heptapeptide 4 (2 g, 2.1 mM) was deprotected using TFA–DCM (1 : 1) to yield TFA.H-Phe-ΔPhe-Phe-Leu-Ala-ΔPhe-Gly-OMe. Boc-Val-ΔPhe-Phe-Ala-OH (1.2 g, 2.1 mM) was coupled to the TFA salt of the heptapeptide 4 using the procedure reported above for 2. After evaporation of the solvent, the residue was taken up in ethyl acetate and washed with 5% citric acid, $NaHCO_3$ and water to yield the undecapeptide II. Yield 1.8 g, 60%, m. p. = 120–122 °C; $R_f = 0.55$; ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 8.55 (1H, s, NH ΔPhe), 8.25(1H, s, NH ΔPhe), 7.8 (1H, s, NH Leu), 7.45 (1H, s, NH Gly), 7.34 (1H, s, NH Ala), 7.22 (1H, s, NH Phe), 7.23–7.4 and 7.5–7.65 (21H, m, aromatic protons of ΔPhe and Phe residues and CβH of ΔPhe), 6.85 (1H, s, CβH ΔPhe), 4.95 (1H, d, NH Phe^1), 4.40–4.25 (4H, m, CαHs of Ala, Leu, Phe^1 and Phe^3), 4.10 (2H, m, CαH Gly), 3.68 (3H, s, OCH_3), 3.0–3.2 (4H, m, CβHs Phe^1 and CβHs Phe^2), 1.85 (2H, br, $C\beta H_2$ Leu), 1.65 (1H, br, CγH Leu), 1.45 (3H, d, $C\beta H_3$ Ala), 1.31 (9H, s, 3 × CH_3 Boc), 1.00–0.94 (6H, m, 2 × $C\delta H_3$ Leu).

The crude peptide was purified by RP-HPLC using water–MeOH gradient on Waters Delta-Pak C18 (19 mm × 300 mm). A linear gradient of MeOH from 60% to 95% over 40 min at a flow rate of 6 ml min^{-1} was employed (t_R: 36.4 min).

FT-IR Spectroscopy

FT-IR spectra of peptide I were collected on a PerkinElmer Spectrum BX-II FT-IR (Seer Green, UK) spectrometer. The peptide was dissolved in chloroform-*d* at a concentration of 20 mg ml^{-1}. A thin film of this peptide solution was dried over a freshly made KBr pellet. Each spectrum was an average of 1000 collections at a resolution of 4 cm^{-1} in the spectral range of 1300 to 1900 cm^{-1}. The FT-IR spectra were smoothed with a smoothing length of 20. The spectral processing was performed with a spectrum supplied by Perkin Elmer (Waltham, MA, USA).

CD Spectroscopy

CD spectra were recorded on a JASCO J-810 CD spectropolarimeter (JASCO International Company Limited, Tokyo, Japan). The spectra were acquired between 240 and 330 nm (0.1-cm cell, peptide concentration ~100 μM) at 0.1-nm intervals with a time constant of 4 s and a scan speed of 100 nm min^{-1} and averaged over six separate scans. The spectra obtained were baseline corrected and smoothened. Peptide concentration was determined using the molar extinction coefficient of ΔPhe (~19,000 M^{-1} cm^{-1}). CD spectra of I were recorded in chloroform, MeOH, TFE, ACN, HFIP, dioxane and DMSO. $CHCl_3$–MeOH titration was carried out for peptide I. CD spectra of II were recorded in chloroform, MeOH, TFE, ACN and DMSO.

NMR Spectroscopy and Structure Calculation

Because of aggregation observed for peptide I, it could not be studied by NMR. NMR spectra for peptide II (4 mM) were recorded in *d*-chloroform ($CDCl_3$) on a Bruker DRX 500-MHz spectrometer. TMS was used as internal reference. Two-dimensional experiments such as double quantum filtered COSY [30] and TOCSY [31] (mixing time = 73 ms) were used to assign the peptide spectra. ROESY [32] experiments were performed to determine spatial proximity at various mixing times: 100, 200 and 300 ms. Five hundred twelve t_1 values were recorded with 2048 data points using a spectral width of 6009.6 Hz in both dimensions. 2D was performed in phase-sensitive mode with time-proportional phase incrementation. NMR data were processed using the software FELIX v. 97.2 (Accelrys, San Diego, CA, USA) on a Silicon Graphics Indigo2 Workstation (Silicon Graphics, Mountain View, CA, USA). A shifted (π/2) sine-squared window function was applied prior to FT in both dimensions, and zero filling to 2*k* points was applied in F1. To estimate distances from the ROESY spectra for calculation of the ordered structures, we broadly classified all cross-peaks as strong, medium and weak on the basis of the intensity of the peak. Well-resolved ROESY peaks between geminal protons of side chain methylene groups of Phe^3 and Phe^7 $C\beta H_2$ were used as reference peaks for the undecapeptide II, as the distance (1.75 Å) between geminal protons is independent of conformation. Final NOE distance restraints were created by assigning the strong, medium and weak intensities to the distance ranges of 1.0–2.5, 2.5–3.5 and 3.5–5.0 Å, respectively. Pseudoatom corrections were used for methyl, methylene and aromatic ring protons where stereospecific assignments were not available. Peptide II was minimized before being subjected to simulated annealing calculations with the DISCOVER (Accelrys Inc., San Diego,

CA, USA) program using the consistent-valence force field. The simulated annealing started with an extended conformation of the peptide backbone. A total of 71 NOEs for the undecapeptide II were used as distance restraints. The molecule was heated up to a temperature of 1000 K and slowly cooled in steps of 100–300 K. At the end of the simulated annealing, all the structures were energy minimized until a gradient of 0.01 kcal mol^{-1} $Å^{-1}$ or lower was achieved. A total of 50 structures were generated for the peptide.

Results and Discussion

CD Studies of Peptide I

Peptide I has three ΔPhe residues interspersed by two amino acid residues. The CD spectra displayed a negative couplet (−, +) in HFIP, MeOH, ACN, chloroform dioxane and TFE. A negative band was observed at about 295 nm and an intense positive band at about 265 nm, with a crossover point at ~280 nm (Figure 1). This CD pattern corresponds to the absorption maximum at 270–280 nm and arises from the dipole–dipole interactions between the charge transfer electronic moments of the two dehydroamino acid chromophores placed in a mutual fixed disposition within the molecule. This pattern, as reported earlier, is typical of a right-handed 3_{10}-helix [33,34]. The FT-IR spectrum of I further validated the structure. An intense peak at around 1676 cm^{-1} revealed a 3_{10}-helix-like conformation, and the absence of amide I absorption at 1630 cm^{-1} excluded the presence of beta-sheet (Figure 2). The varying intensity of bands in different solvents suggested different contents of the 3_{10}-helical conformer. The $CHCl_3$–MeOH titrations showed exciton couplet bands of higher intensity in both 50 : 50 (chloroform : MeOH) and MeOH alone (Figure S1, supplementary information). The peptide was completely insoluble in water, but it was soluble in different percentages of TFE/water. Following the above observation, the experiments were performed in aqueous TFE that acts as a lipomimetic solvent. CD

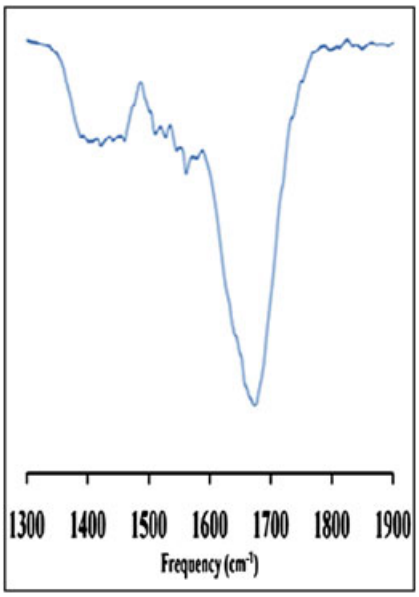

Figure 2. FT-IR spectra of peptide I (dissolved in chloroform-*d* at a concentration of 20 mM) dried over a freshly made KBr pellet.

spectra of the undecapeptide in TFE/water solution showed intense exciton-coupled band, a characteristic of a right-handed 3_{10}-helical conformer (Figure S2, supplementary information). Thus, the peptide was found to attain more stability in a membrane mimetic environment at relatively low percentage, suggesting the propensity of the peptide to exist in an ordered 3_{10}helical conformation in a hydrophobic environment and depicting stabilization achieved by molecular association [35].

Interestingly, the phenomenon of self-association was not an impediment on the stability of the right-handed 3_{10}-helical conformation. The thermal denaturation of the peptide in ACN displayed overall stability of the peptide to variation in temperature (Figure 3). Despite some melting of the structure at higher temperature, the overall conformation of the peptide was maintained. The 3_{10}-helical conformation in both far-UV and near-UV region was retained on heating up to 50 °C, suggesting enhanced stability and order for the 3_{10}-helical conformer.

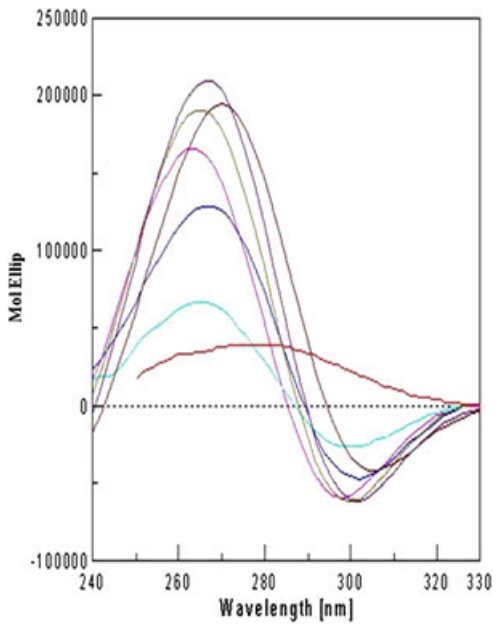

Figure 1. CD spectra of peptide I (concentration 100 μM) in different solvents.

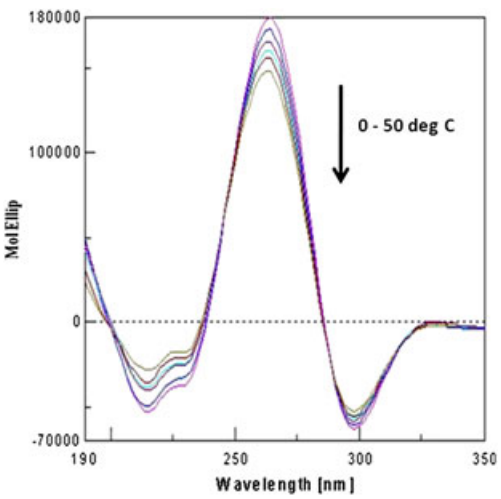

Figure 3. Variable-temperature CD spectrum of peptide I (concentration 100 μM) in ACN.

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CD Studies of Peptide II

Peptide II has three ΔPhe residues interspersed by three saturated amino acids. The CD spectra of the peptide in different solvents (Figure 4) clearly suggested conformational heterogeneity in solution. In chloroform, the CD spectrum showed an intense positive band at about 280 nm. This intensity decreased in other solvents. A negative band of varying intensity was observed at 235 nm in solvents like MeOH, TFE and ACN. On the basis of a previously documented octapeptide containing two ΔPhe interspersed by four saturated amino acids [36], α-helix and 3_{10}-helix or helical structures with mixed 3_{10}/α-helical turns could be proposed for peptide II. However, we do not claim that the obtained CD spectra would be diagnostic for the stated conformation. Further evidence would be required to establish the exact conformational situation. At the same time, it would be interesting to note that peptides with Aib residues, which behave quite similarly to peptides containing dehydro residues, display either the 3_{10}-helical or α-helical pattern, and the solvent could play an important role near a critical chain length [37–40]. The higher intensity of CD band at 280 nm in chloroform for peptide II might be an indicator of extended hydrogen bonded pattern, thus stabilizing the helical conformer. However, no exciton splitting was observed in all the solvents used. This could be possible when the styryl side chains of ΔPhe residues were placed on the opposite sides of the helix: so the rotational strength of exciton splitting was 0. An inspection of the 3_{10}-helical wheel projection of peptide II revealed that the side chains of ΔPhe were indeed placed on different sides of the helix. The positive band at 280 nm arose from the contributions of the non-interacting but chirally perturbed chromophores. The negative band at 235 nm observed in solvents like MeOH, TFE and ACN could be attributed to π–π^* electronic transition of the didehydro chromophore and might suggest the presence of type III β-bends [36]. In DMSO, however, intramolecular hydrogen bonds were broken, and a highly reduced CD band was seen.

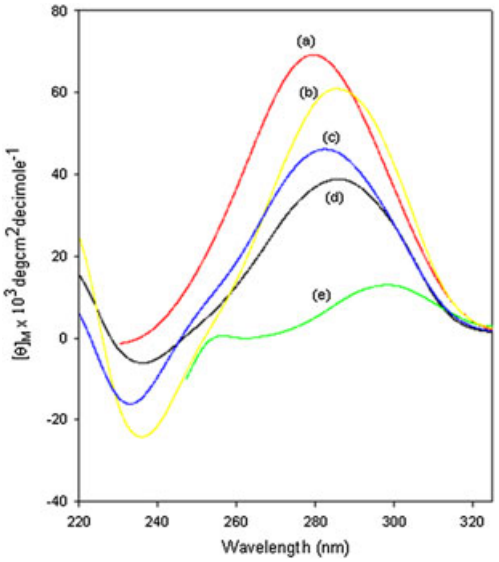

Figure 4. CD spectra of peptide II in (a) $CHCl_3$, (b) MeOH, (c) TFE, (d) ACN and (e) DMSO.

NMR Study of II

NMR spectra of peptide II were acquired in chloroform-*d* (4 mM) and assigned using TOCSY and ROESY experiments. On the basis of TOCSY and the identification of the 'sequential walks' through the αH-NH, βH-NH and NH-NH regions, the undecapeptide was completely assigned. The CαH and NH protons were well dispersed. A summary of all NOEs observed in the undecapeptide has been shown (Figure 5). Continuous d_{NN} and $d_{\beta N}$ cross-peaks were observed throughout the entire length of the peptide from NH Val[1] to NH Gly[11], a characteristic of a helical conformation (Figure S3, supplementary information). Cross-peaks of medium intensity of the type $d_{\alpha N}$ (i,$i+3$) were also observed between Val1/Ala4, Phe3/ΔPhe6, Ala4/Phe7, Phe5/Leu8, Phe7/ΔPhe10 and Leu8/Gly11 (Figure S4, supplementary information), which provided compelling evidence for a helical backbone [41]. All possible medium-intensity $d_{\alpha N}$ (i,$i+2$) peaks were also observed, which suggested the predominance of a 3_{10}-helix [42,43]. However, the absence of a cross-peak between CαH Phe[7] and NH Ala[9] and the presence of medium-intensity peaks of the type $d_{\alpha\beta}$ (i,$i+3$) between CαH Phe[5] and Cβ1H/Cβ2H Leu[8] pointed to the existence of an α-helical turn. Such weak $d_{\alpha\beta}$ (i,$i+3$) peaks were also observed between Val1/Ala4 and Ala4/Phe7. The

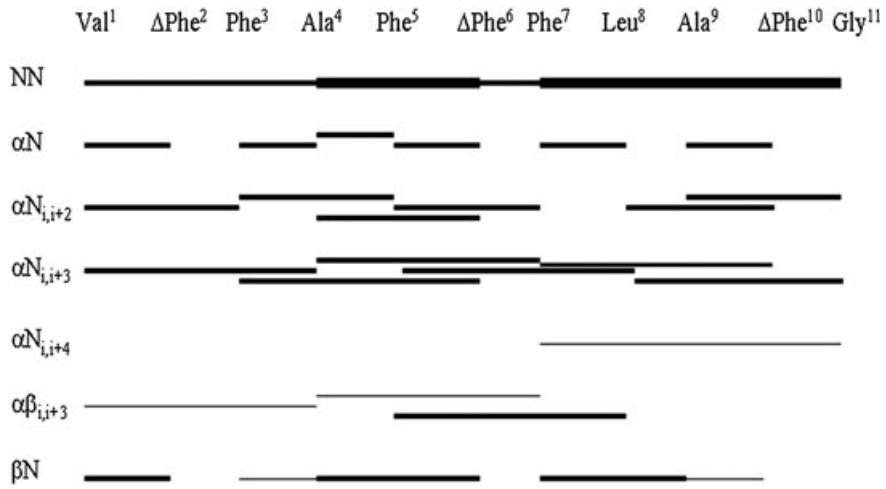

Figure 5. Summary of NOEs observed in peptide II. Thick, medium and thin bars indicate strong, medium and weak NOEs, respectively.

distance $d_{\alpha N}$ $(i,i+4)$ is above 4 Å in α-helical conformations and thus gives rise to very weak NOE intensity. Such a weak-intensity peak was observed between CαHPhe[7] and NHGly[11]. Thus, the ^{1}H NMR spectra of peptide II revealed NOEs consistent with a mixture of 3_{10}-helix and α-helix. A total of 71 NOE restraints were calculated and used for structure calculations. Out of these, 55 were inter-residue and 16 were intra-residue. Dihedral restraints were not used because the coupling constants were found to be averaged because of conformational heterogeneity. A summary of statistics associated with the final simulated annealing structures and parameters used for restrained molecular dynamics simulations is given in Table 1. Figure 6 shows six best-converged structures along with hydrogen bonds. Three 1←5 hydrogen bonds and three 1←4 hydrogen bonds were stabilized. NH Ala[9] and NH Phe[5] form 1←5 hydrogen bonds with Phe[5]C=O and Val[1]C=O, respectively. However, NH ΔPhe[6] and NH Phe[7] form hydrogen bonds with Phe[3]C=O and Ala[4]C=O, respectively. At the C-terminus, a larger conformational heterogeneity was observed. NH Gly[11] was involved in both 1←5 and 1←4 hydrogen bonds with Phe[7]C=O and Leu[8]C=O, respectively, thus indicating the simultaneous presence of a 3_{10}-helix and an α-helix.

Earlier studies have shown that incorporation of β-branched residues in tripeptides such as Boc-Val-ΔPhe-Val-OCH3 [44] and Boc-Val-ΔPhe-Ile-OCH3 [45] resulted in complete loss of structure. Remarkably, the presence of these β-branched residues in peptide I did not disrupt the structure, and the 3_{10}-helical structure was maintained, as evidenced by using CD spectroscopy and FT-IR spectroscopy. CD studies showed folding of I into a right-handed 3_{10}-helix and self-association-mediated conformational integrity. The aggregation of the peptide was however a major impediment in carrying out either crystal or NMR structural studies. Detailed structural studies of other peptides containing ΔPhe at two-residue spacers including, Boc-Phe-ΔPhe-Val-Phe-ΔPhe-Val-OMe [46], Boc-Val-ΔPhe-Leu-Ala-ΔPhe-Ala-OMe [47], helical hairpin, helical bundle and glycine zipper have also shown a preferential 3_{10}-helical conformation.

Table 1. Summary of experimental restraints for structure calculation of the undecapeptide and results of simulated annealing calculations

Parameter	Value
Distance restraints	
All	71
Intra-residue	16
Inter-residue	55
Sequential	30
Medium range	25
$i,i+2$	10
$i,i+3$	14
$i,i+4$	1
Long range	0
Results of structure calculations	
Average fractional violation/constraint	0.0017
Average number of violations/structure	4.0
RMSDs with average structure backbone atoms (Å)	
Maximum	1.39
Minimum	1.24
Average	1.32
Average pair wise RMSD (Å)	1.67 ± 0.08

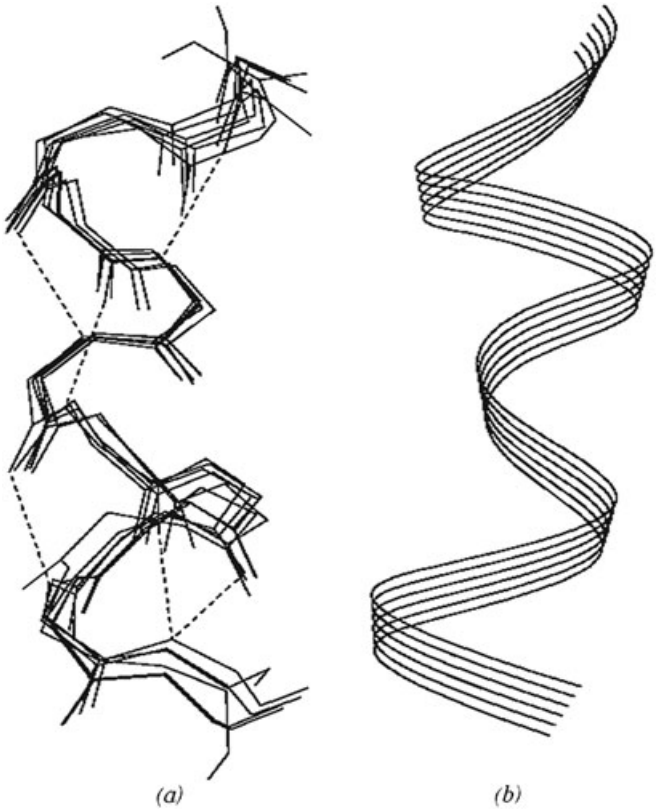

Figure 6. (a) Overlay of six structures for peptide II from the simulated annealing run. (b) Ribbon diagram of the same.

The present structure therefore cemented the claim that a peptide containing a -ΔPhe-X-X-ΔPhe- motif would stabilize a 3_{10}-helix.

Peptide II, Boc-Val-ΔPhe-Phe-Ala-Phe-ΔPhe-Phe-Leu-Ala-ΔPhe-Gly-OMe, stabilized a helical structure with a mixed $3_{10}/\alpha$-helical conformation. This could be attributed to the presence of a three-residue spacer between two ΔPhe, the length of the peptide or both. Further studies are warranted to establish this hypothesis. Previous ^{1}H NMR studies on heptapeptides with a three-residue spacer between ΔPhe have suggested an α-helical conformation. However, this interpretation was based only on the hydrogen bonding pattern obtained from solvent titration experiments [29]. Several examples of Cα-substituted amino-acid-containing peptides, depicting $3_{10}/\alpha$-helical conformations, have been reported [48,49]. In peptides containing Aib residues, a 3_{10}-helix is preferred for shorter peptides ($n \sim 8$ or less) and an α-helix for longer peptides ($n = 9$–20) [38,50]. Factors governing the type of helical structure (3_{10} vs α), namely, main chain length, sequence dependence (Aib content), inherent relative stabilities of the two helices, the additional intramolecular C=O···H–N hydrogen bond in a 3_{10}-helix for the same number of residues, environmental factors like solvent polarity and temperature, helix macro dipoles, have been investigated and discussed [51–54]. Molecular dynamics simulations and free energy calculations suggest that energetically the two helical forms are very close [55–57]. The 3_{10}-helix is suggested to be an intermediate conformation in the thermodynamic pathway of formation of an α-helix from the nascent helix [41,58,59]. The backbone torsion angles for a right-handed 3_{10}-helix ($\sim\phi = -60°$, $\psi = -30°$) are within the same region of conformational map as those of an α-helix ($\sim\phi = -55°$, $\psi = -45°$). As there is no disallowed region of the

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conformational space completely separating these two regularly folded secondary structures, the α-helix may be gradually transformed into a 3_{10}-helix (and vice versa), maintaining a near-helical conformation in the chain throughout.

Conclusion

The present solution conformational study on two undecapeptides illustrated the following: (1) in spite of the presence of β-sheet-favoring amino acids that acted as two-residue spacers between ΔPhe, peptide I adopted a 3_{10}-helical conformation and (2) the presence of a three-residue spacer between ΔPhe in peptide II stabilized a mixed $3_{10}/\alpha$-helical conformation. This is an important contribution to the rule book of peptide design involving didehydrophenylalanine. These examples reflected on the possibilities of fine tuning peptide design. The results extracted from the present structures could be incorporated during the design of longer peptides of desired fold.

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Supporting information

Supporting information may be found in the online version of this article.

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