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Conformationally Restricted Peptides: Solution Conformation of Tetra And Hepta Peptides Containing α,β-Dehydrophenylalanine Residues in Alternate Positions

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Abstract: Two model peptides containing dehydrophenylalanine, a tetrapeptide **1** (Ac- Δ^ZPhe-Pro-Δ^ZPhe-Ala-OMe) and a heptapeptide **2** (Boc-Gly-Δ^ZPhe-Val-Δ^ZPhe-Ala-Δ^ZPhe-Leu-OMe) have been synthesised and their solution conformations investigated by NMR and circular dichroism techniques. Assignment of amide protons and their involvement in intramolecular hydrogen bonding have been made by solvent and temperature dependence studies. These conformation studies indicate the presence of an incipient 3_{10}-helix in tetrapeptide **1**, with two consecutive β-turns and a right handed 3_{10}-helix in heptapeptide **2**. The results establish the potential of Δ^ZPhe residues to favour 3_{10}-helical conformations with Δ^ZPhe occupying alternate positions in the peptide. A comparison of solution conformation of analogous peptides containing Aib residue in place of Δ^ZPhe is also presented. These residues appear to induce similar conformation constraints in small peptides.

The α,β-dehydroamino acid residues are constituents of several microbial peptides and antibodies[1-4]. In addition, a few analogs with dehydro residues have been designed as a means of limiting both backbone and side chain flexibility at specific sites along the oligopeptide chain[3-6]. The presence of sp^2 hybridised C^α atom modulates the magnitude not only of the bonds and valence angles but also the usual conformational angles ϕ,ψ,ω according to the nature of the side chain of the dehydro residue incorporated[7].

Studies carried out both in solution[8-10] and in solid state[5,6,11-17] on model di-, tri- and tetrapeptides, containing Δ^ZPhe residues have indicated a strong tendency of dehydrophenylalanine to favour β-turn structures, accommodating itself at either corner positions (i+1 or i+2) of the turn. Theoretical conformation studies have also supported this view[14,18]. More recently, the potential of Δ^ZPhe residue in generating helices has been realised. Solution studies on peptides (5-8 residues) have shown the tendency of Δ^ZPhe residue to promote either 3_{10}-helical or α-helical conformation, depending on the length of the peptide chain[19-21]. However, the crystal structure studies have so far shown only the presence of 3_{10}-helices in peptides containing Δ^ZPhe residues (peptide length 5-9 residues)[22-24].

Extensive studies in solution and solid state on peptides containing an α,α dialkyl substituted amino acid, α-amino isobutyric acid (Aib), show the presence of either 3_{10} or α-helical conformation depending on the number of Aib residues and length of the peptide chain[25]. In some cases of Aib containing peptides, a mixture of $3_{10}/\alpha$ helical structures have also been observed[25] . Thus the credentials of Aib as a helix promoting residue are firmly established. In comparison the Δ^ZPhe has not been studied in much detail. Based on the limited number of studies reported so far, Δ^ZPhe seems to induce similar conformational constraints in peptide backbone as the Aib residue. However, a direct comparison of conformational preference of these two potentially highly useful residues in a given sequence has not been reported . As part of our continuing investigations on the conformational and spectroscopic properties of Δ^ZPhe containing peptides, this report describes solution conformational studies on a synthetic tetrapeptide Ac-Δ^ZPhe-Pro-Δ^ZPhe-Ala-OMe **1** and provides a direct comparison with the corresponding Aib peptide, Z-Aib-Pro-Aib-Ala-OMe[26]. Solution conformation of another synthetic peptide Boc-Gly-Δ^ZPhe-Val-Δ^ZPhe-Ala-Δ^ZPhe-Leu-OMe **2**, containing three Δ^ZPhe in alternate positions and its comparison with a similar peptide containing three Aib residues, in alternate positions, is also described (Boc-Val-Aib-Val-Aib-Val-Aib-Val-OMe)[27].

RESULTS AND DISCUSSION

Ac-Δ^ZPhe-Pro-Δ^ZPhe-Ala-OMe (1)

Assignment of Resonances. ^{1}H NMR spectra of the tetrapeptide in $CDCl_3$ and $(CD_3)_2SO$ is shown in Fig.1. Assignment of resonances other than amide and $C^\alpha H$ resonances was done on the basis of chemical shifts and splitting pattern. Assignments of individual NH groups and $N_iH<-->C_i^\alpha H<-->C_i^\beta H<-->C_i^\gamma H<-->C_i^\delta H$ coupling connectivities were established with the help of two dimensional COSY in $CDCl_3$ (spectrum not shown)[28]. The assignments were based on unambiguous recognition of chemical shift position of side chain protons such as $C^\beta H_2$ of Pro and $C^\beta H_3$ of Ala. Based on $N_iH<-->C_i^\alpha H$ connectivities, chemical shift value at 7.57δ was assigned to Ala(4) NH group . Due to the lack of corresponding $C^\alpha H$ protons, two Δ^ZPhe NH resonances could not be assigned by the COSY spectrum. However, the two Δ^ZPhe NH resonances were readily recognised as two broad singlets most downfield (7.65δ and 8.7δ) than all signals. Assignment of Δ^ZPhe NH groups were established with the aid of difference NOE spectra[29]. Irradiation of Δ^ZPhe at 8.7δ resulted in an enhancement of doublet at 7.57δ. Thus, the peak observed at 8.7δ was assigned to Δ^ZPhe(3)NH and the singlet at 7.65δ was of Δ^ZPhe(1) NH. The chemical shifts assigned to various NH groups are summarized in Table 1.

Delineation of Hydrogen-bonded NH Groups. The involvement of NH groups in intramolecular H-bonding was investigated using temperature and solvent dependence of NH chemical shifts. The temperature coefficient values (dδ/dT) in $(CD_3)_2SO$ are listed in Table 1. The solvent titration curves are shown in Fig.2.

In tetrapeptide **1**, Ala(4)NH has low dδ/dT value ($<4x10^{-3}$ ppm$^oK^{-1}$) whereas Δ^ZPhe(3)NH resonance exhibits intermediate dδ/dT value of 0.004 ppm$^oK^{-1}$.

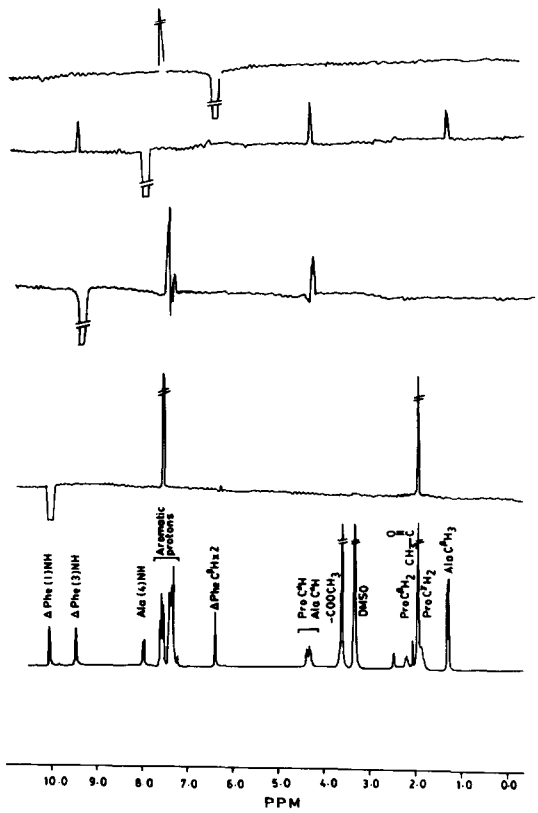

Fig.1 500 MHz ^{1}H NMR spectrum of **1** in $CDCl_3$ with traces of $(CD_3)_2SO$. Difference NOE spectra obtained on irradiation of various NH groups are shown.

These results show that Ala(4)NH is not accessible to the solvent and may be involved in hydrogen bonding , while Δ^ZPhe(3)NH is partially exposed to the solvent[30]. Both the resonances [Ala(4)NH & Δ^ZPhe(3)NH] also show significant lower chemical shift changes on increasing the composition of $(CD_3)_2SO$, a strong H-bonding solvent[31,32]. Whereas, the Δ^ZPhe(1)NH has high temperarure coefficient ($d\delta/dT > 4x10^{-3}$ ppm$^oK^{-1}$) and solvent dependence of chemical shifts characteristic of solvent exposed NH groups. The NMR data thus favours the conformation for the tetrapeptide **1** in which two NH groups are intramolecularly hydrogen bonded.

Nuclear Overhauser Effect. Difference NOE spectra recorded in $CDCl_3$ and $(CD_3)_2SO$ are shown in Fig.1. The NOEs observed on irradiation of various NH groups are summarised in Table 2. Irradiation of Δ^ZPhe(3)NH group resulted in the observation of interresidue NOEs Pro(2)C$^\alpha$H<-->Δ^ZPhe(3)NH and Δ^ZPhe(3)NH<-->Ala(4)NH. No NOE was

observed on irradiation of Δ^ZPhe(1)NH as expected. The observation of such $C^{\alpha}_{i+1}H$<-->$N_{i+2}H$ and $N_{i+2}H$<-->$N_{i+3}H$ NOEs is characteristic of type II β-turn conformation[8].

Table 1 NMR Parameters for peptide 1

(NH) Resonance Residue	$CDCl_3$ (ppm)	$(CD_3)_2SO$ (ppm)	Δ (ppm)	(dδ/dT) 10^{-3}ppm K^{-1}
Δ Phe (1)	7.65	10.03	2.38	5.1
Δ Phe (3)	8.69	9.45	0.76	4.0
Ala (4)	7.57	7.97	0.40	3.0

In peptide **1** Pro and Δ^ZPhe(3) occupy i+1 and i+2 positions respectively. Thus a β-turn is formed in which Ala(4)NH is intramolecularly hydrogen bonded with CO of Δ^ZPhe(1). Further, the vicinal coupling constant $J_{(NHC^{\alpha}H)}$ value for Ala(4)NH are 5.0Hz in

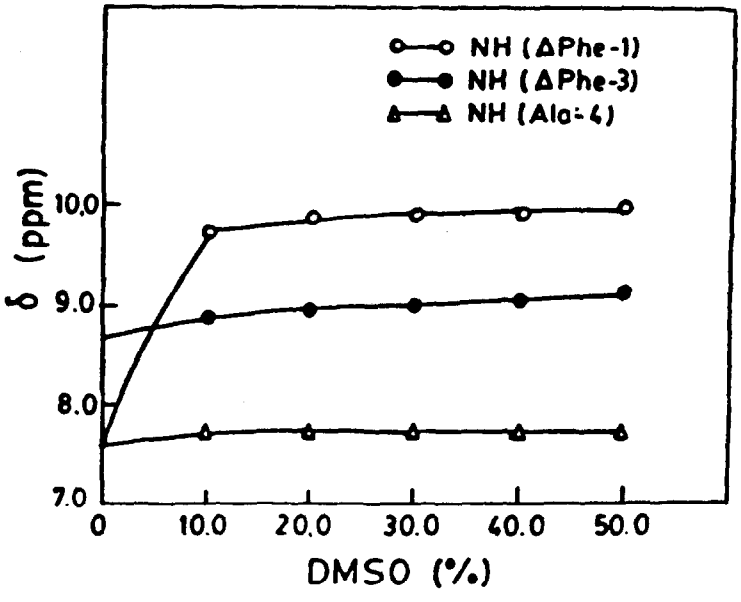

Fig.2 Solvent dependence of NH chemical shifts in tetrapeptide **1** in $CDCl_3$-$(CD_3)_2SO$ mixtures.

$CDCl_3$ and 5.1Hz in $(CD_3)_2SO$ respectively. These values are suggestive of ϕ Ala (4)NH in the range of -60 ± 10^0 which is compatible with a folded structure for **1**. In comparison, similar interresidue NOEs were observed in case of the Aib analogue, Z-Aib-Pro-Aib-Ala-OMe and $J_{NHC^{\alpha}H}$ values for Ala(4)NH was found to be 7.0Hz ($CDCl_3$) and 7.3Hz [$(CD_3)_2SO$][26].

Circular Dichroism Studies. CD spectra of the tetrapeptide were recorded in two solvents, acetonitrile and methylenechloride (Fig.3 a). In both the solvents, the spectra show an evident couplet of bands that can be associated with the dehydro chromophore[33]. The high elipticity values for the tetrapeptide suggest conformational rigidity in the molecule . Similar type of CD spectra were observed for Ac-Δ^ZPhe-Gly-Δ^ZPhe-Ala-OMe, which adopts an incipient 3_{10} helical conformation in solution[34] . The slight shift of maxima and minima observed in case of tetrapeptide **1** are perhaps due to chiral perturbation of the Δ^ZPhe choromophore by the proline residue[35]. However the positive band between 240nm to 320nm and a negative band between 210nm to 240nm are indicative of highly folded conformation for peptide **1**.

Table 2 NOEs observed in tetrapeptide 1

Resonance irradiated	$CDCl_3$		$(CD_3)_2SO$	
	Resonance Observed	% NOE	Resonance Observed	% NOE
Δ Phe (1) NH	-	-	Aromatic proton	1.63
Δ Phe (3) NH	Ala (4)NH Pro C^dH(2) Aromatic proton	1,56 1.11 1.1	Ala (4)NH Pro C^dH(2) Aromatic proton	1.04 2.30 1.37
Ala (4) NH	a	-	Ala $C^{\alpha}H$(4) Δ Phe (3) NH Ala $C^{\beta}H$	2.17 1.0 0.42

***Boc-Gly-Δ^ZPhe-Val-Δ^ZPhe-Ala-Δ^ZPhe-Leu-OMe* 2**

Assignment of Resonance. Fig.4 illustrates a two dimensional correlated spectrum ofheptapeptide **2** in $CDCl_3$. Derived assignments are listed in Table 3.

Four expected connectivities between NH and $C^{\alpha}H$ resonances [Gly (1), Val(3), Ala(5) and Leu(7)] are clearly identified. Urethane NH of Gly(1) at 5.5δ and its coupling to $C^{\alpha}H$ (3.89δ) is readily recognized. By virtue of $C^{\beta}H_3$ (1.4δ)<-->$C^{\alpha}H$ (4.36δ) connectivities,

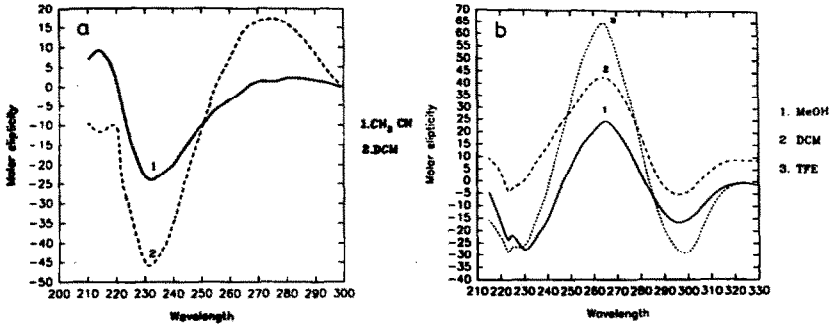

Fig.3 (a) CD spectra of tetrapeptide **1** in various solvents.
(b) CD spectra of heptapeptide **2** in various solvents.

Ala (5) NH is identified at (7.85δ). Similarly Val(3)NH (8.1δ) and Leu(7)NH (7.5δ) were recognised by bond to bond connectivities[28]. Two of the Δ^ZPhe NHs appear as a singlet at

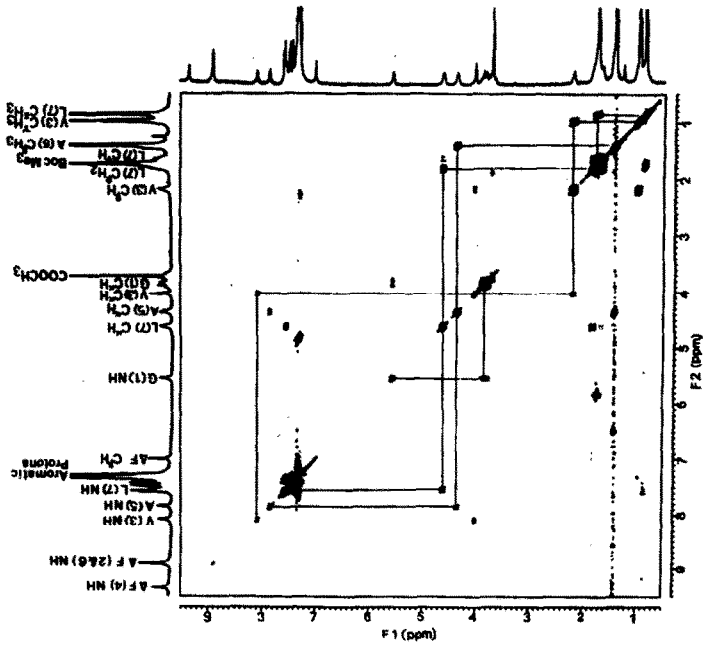

Fig.4 400 MHz COSY spectrum of heptapeptide **2** in $CDCl_3$.

8.88δ [integration of the peak is for two protons & the peaks get resolved in to two peaks in 10% $(CD_3)_2SO$] and the singlet at 9.3δ is assigned to the third Δ^ZPhe NH group. The specific assignment of NHs of Δ^ZPhe residues were not possible by COSY spectrum due to lack of the
corresponding $C^\alpha H$ protons. However, the NH resonances of Δ^ZPhe(2), Δ^ZPhe(4) and Δ^ZPhe(6) were assigned unambiguously on the basis of diagnostic NOEs recorded in $CDCl_3$:$(CD_3)_2SO$ mixture. Δ^ZPhe(2) NH (9.3δ which observes downfield shift in 10 % $(CD_3)_2SO$) gives a sequential NOE (in ROESY spectrum) with $C^\alpha H$ of the Gly(1) residue. Δ^ZPhe(4) NH (9.22δ) yields NOE with $C^\alpha H$ of the Val(3) residue. Subsequently the third singlet at 8.88δ was assigned to be Δ^ZPhe(6)NH, which also gives a NOE with Ala(5)$C^\alpha H$ and Leu(7)NH.

Delineation of H-bonded NH Groups. The dδ/dT values for various groups in $(CD_3)_2SO$ and the solvent dependence of chemical shifts are summarized in Table 3 and Fig.5 respectively.

Table 3 NMR parameters for NH resonances in peptide 2

Residue	$CDCl_3$ (ppm)	$(CD_3)_2SO$ (ppm)	Δ (ppm)	(dδ/dT) 10^{-3} ppm K^{-1}
Gly (1)	5.5	7.16	1.66	6.7
Δ Phe (2)	8.88	9.66	0.78	5.1
Val (3)	8.1	8.22	0.12	5.0
Δ Phe (4)	9.3	9.8	0.5	4.0
Ala (5)	7.85	8.0	0.15	2.25
Δ Phe (6)	8.88	9.66	0.78	3.0
Leu (7)	7.5	7.93	0.43	0.8

The dδ/dT values (<0.004 ppm$^oK^{-1}$) in $(CD_3)_2SO$ provide evidence for the involvement of 4 NH groups [Δ^ZPhe(4)NH, Ala(5)NH, Δ^ZPhe(6)NH and Leu(7)NH] in intramolecular hydrogen bonding. While the remaining 3 NH [(Gly(1)NH, Δ^ZPhe(2)NH & Val(3)NH] groups have high temperature coefficient values (>4x10^{-3}ppm$^oK^{-1}$) indicative of their exposure to the solvent. However, the solvent titration experiments suggest that five NH groups [Val(3), Δ^ZPhe(4), Ala(5), Δ^ZPhe(6) & Leu(7)] in the heptapeptide **2** are relatively insensitive to change in solvent composition, whereas Gly(1)NH and Δ^ZPhe(2)NH show significant

downfield shifts on increasing the percentage of $(CD_3)_2SO$. These results suggest that five NHs may be involved in intramolecular hydrogen bonding in $CDCl_3$ while in $(CD_3)_2SO$ only 4-bond structure may be favoured. Further, the vicinal coupling constants ($J_{NHC^{\alpha}H}$) for Val, Ala and Leu are observed to be between 5-7Hz. This suggest a conformationally averaged value for ϕVal,ϕAla and ϕLeu to be between -50^{o} to -70^{o}. These values are consistent with consecutive 4-->1 intramolecular bonding in heptapeptide **2**[26].

Nuclear Overhauser Effect. NOE dipolar correlated 2D spectra were obtained by using ROESY experiment in $CDCl_3$ with traces of $(CD_3)_2SO$. Fig. 6 illustrates a magnitude mode of ROESY spectrum of the heptapeptide.

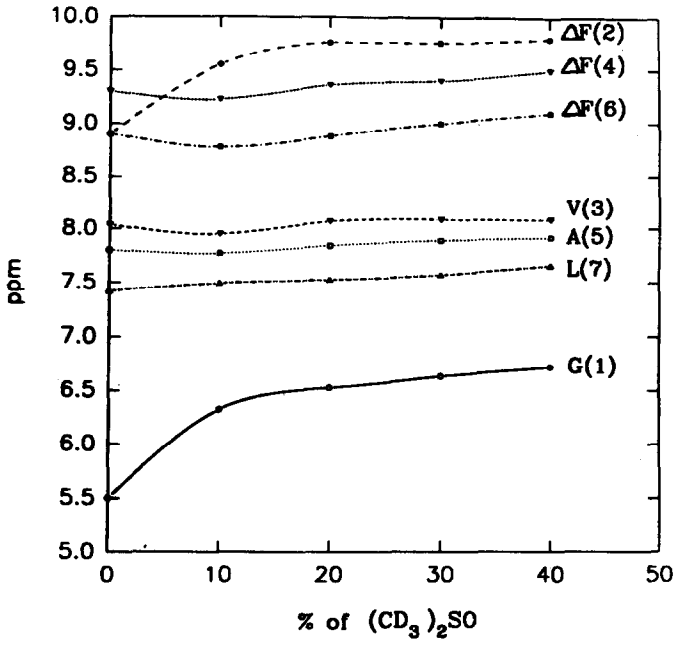

Fig.5 Solvent dependence of NH chemical shifts in heptapeptide **2** in $CDCl_3$-$(CD_3)_2SO$.

Along with sequential NOEs, appreciable $N_iH<-->N_{i+1}H$ ROE cross peaks are observed between the following pairs of protons; Gly(1)NH<-->Δ^{Z}Phe(2)NH; Δ^{Z}Phe(2)NH<-->Val(3)NH; Val(3)NH<-->Δ^{Z}Phe(4)NH; Δ^{Z}Phe(4)NH<-->Ala(5)NH; Ala(5)NH<->Δ^{Z}Phe(6)NH and Δ^{Z}Phe(6)NH<-->Leu(7)NH. Such ROE cross peaks are characteristic of helical conformation[28] with $N_iH<-->N_{i+1}H$ interproton distance $2.6A^{o}$ (α-helix) or $2.8A^{o}$ (3_{10}-helix) and backbone torsional angles ϕ~-50, ψ~-50. In addition, cross peaks due to $d_{\alpha N}$ (i,i+2) and $d_{\alpha N}$ (i,i+3) are also noticed in the ROESY spectrum (cross peaks 11 & 10 respectively in Fig.6). Such NOEs are observed for short (< 4 A^{o}) $C_i^{\alpha}H<-->N_{i+2}H$ and

$C_i^{\alpha}H<-->N_{i+3}H$ interproton distances as seen in 3_{10}- or α-helical peptide conformation[28]. ROE cross peaks due to $d_{\alpha N}(i,i+4)$, which are characteristic of α-helical conformation[36] are not observed in the spectrum. These results are consistent with a 3_{10}-helical conformation for peptide **2**.

Circular Dichroism Studies. Heptapeptide **2** shows significant features in the CD spectra which also supports the NMR data (Fig.3 b). In methanol, methylene chloride and

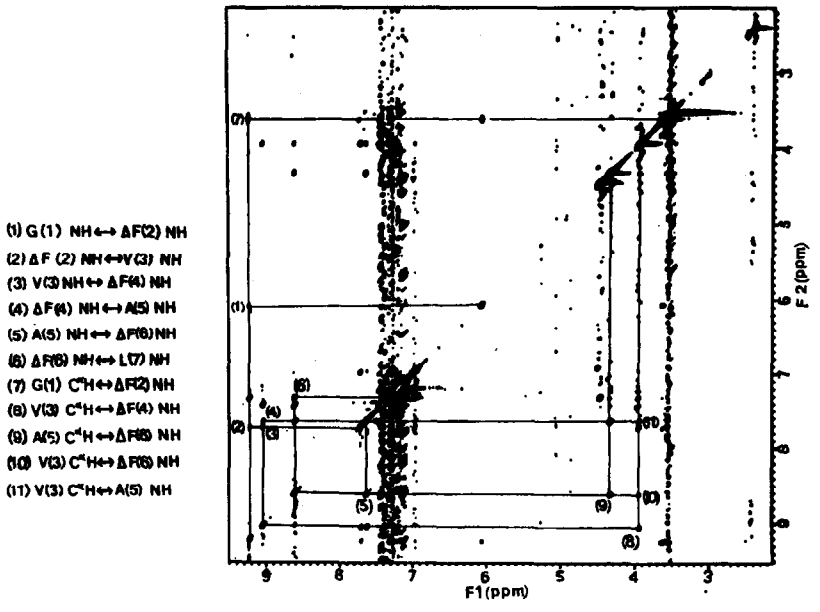

Fig.6 500 MHz ROESY spectrum of heptapeptide **2** in $CDCl_3$ with traces of $(CD_3)_2SO$.

triflouroethanol, the spectrum displayed characteristic couplet of intense bands with opposite signs at ~295nm and 265nm and a crossover at ~280nm, in correspondence with absorption maximum of the dehydrochromophore[33]. The observed CD pattern is typical exciton couplet of bands due to splitting of 280nm transition which may originate from the rigid and fixed disposition of the three dehydrophenylalanine residues within the molecule and provides a strong proof that the peptide assumes a unique folded structure in solution. The CD spectra are similar to the spectra reported by Pieroni and coworkers on a series of 3_{10}-helical peptides containing two Δ^ZPhe residues[34]. The CD spectra of another didehydrophenylalanine pentapeptide, Boc-Ala-Δ^ZPhe-Gly-Δ^ZPhe-Ala-OMe matches well

with the above peptide. A right handed 3_{10}-helical conformation for this pentapeptide has already been established in both solution[34] and solid state[22].

Conformation of 1 and 2 and their comparison with the corresponding Aib Peptides

The ^{1}H NMR and CD results described above support the following conclusions:

I. In relatively apolar solvent like $CDCl_3$, tetrapeptide Ac-Δ^ZPhe-Pro-Δ^ZPhe-Ala-OMe **1** favours folded conformation in solution stabilized by two intramolecular H-bonds involving Δ^ZPhe(3)NH and Ala(4)NH in two consecutive β-turns. The CD spectra in acetonitrile and methylene chloride confirm the presence of an incipient 3_{10}-helical confomation in solution for peptide **1**. The NMR data of **1**, in $CDCl_3$ and $(CD_3)_2SO$ compares well with Z-Aib-Ala-Aib-Ala-OMe, for which an incipient 3_{10}-helical structure in solution and in solid state has already been established[26]. A further point of similarity is noticed in relatively higher dδ/dT value of the NH of the third residue in these two analogous peptides. The observed dδ/dT values of 4.0×10^{-3}ppm$^oK^{-1}$ and 4.9×10^{-3}ppm$^oK^{-1}$ for Δ^ZPhe and Aib respectively in the two peptides may be indicative of this NH to be partially exposed in highly polar solvent. This may suggest some degree of flexibility in both the peptides.

II. Together with the known stereochemical preferences of Δ^ZPhe residue, the NMR and CD data suggest that heptapeptide, Boc-Gly-Δ^ZPhe-Val-Δ^ZPhe-Ala-Δ^ZPhe-Leu-OMe **2** exclusively favours 3_{10}-helical conformation. Such a folded conformation is stabilised by five 4-->1 H-bonds . Circular dichroism studies also confirm a highly folded structure for this peptide. The presence of negative couplet at 295nm is an indication that the peptide **2** assumes a right handed 3_{10}-helical conformation[34]. A folded structure with five intramolecular H-bonds[27] was reported in $CDCl_3$ for heptapeptide Boc-X-(Aib-X)$_3$-OMe (where X =Val or Ala or Pro). Also similar to the Aib analogue , peptide **2** shows evidence of conformational changes on going from a relatively non-polar solvent ($CDCl_3$) to a highly polar, H-bonding solvent $(CD_3)_2SO$[27]. Observation of high dδ/dT value for Val(3)NH (>4×10^{-3} ppm K^{-1}) suggest loosening of one of the 4-->1 H-bonds in both the peptides containing Aib and Δ^ZPhe residues. Thus conformational constraints induced by Δ^ZPhe are found to be similar to those induced by the Aib residue, in both the peptide sequences discussed above.

Spectroscopic studies on two Δ^ZPhe containing peptides suggest that peptides upto seven residues containing –Δ^ZPhe-X-Δ^ZPhe- or -Δ^ZPhe-X-Δ^ZPhe-X-Δ^ZPhe- motifs tend to stabilize 3_{10}-helix. We have earlier shown that in a hexapeptide containing -Δ^ZPhe-X-X-Δ^ZPhe- motif also a 3_{10}-helical conformation is preferred[19]. In peptides, containing -Δ^ZPhe-X-X-X-Δ^ZPhe- and -Δ^ZPhe-X-X-X-X-Δ^ZPhe- motifs an α-helical structure was found compatible with the NMR data[20,21]. The length of the peptide and specific positioning and the number of Δ^ZPhe residues in a peptide sequence appear to be important factors in determining the conformation of the peptide. Clearly, many more peptides of different lengths with different Δ^ZPhe contents will have to be studied to understand the exact conformational preferences of Δ^ZPhe containing peptides. There is little doubt though that together with Aib, Δ^ZPhe has great potential in designing helical peptides.

EXPERIMENTAL

Peptides **1** and **2** were synthesised by conventional procedure and fully characterized by 500 MHz ^{1}H NMR. The homogeneity of the peptides was monitored by thin layer chromatography (TLC) in three solvent systems namely, A) $CHCl_3$:MeOH(9:1), B) nBuOH:CH_3COOH:H_2O(4:1:1) and C) nBuOH: CH_3COOH:Pyridine:H2O(4:1:1:2). Purification of the peptides was carried out by reverse-phase high performance liquid chromatography (HPLC) on Water's Deltapak C_{18} column (3.9 mm x 30cm) with gradient elution (70-90% MeOH in H_2O in 40 minutes, flow rate 1.5 ml min^{-1}, detection 280nm) on a water's HPLC system. ^{1}H NMR spectra were recorded on a Bruker 500MHz FT-NMR spectrometer at Tata Institute of Fundamental Research, Bombay and at Sophisticated Instrument Facility, Bangalore. CD spectra were recorded by using a JASCO J-500 at Indian Institute of Science, Bangalore.

Peptide Synthesis. Amino acid couplings were performed by either mixed anhydride or dicyclohexyl carbodiimide. Boc-Gly-Δ^ZPhe-OH, Boc-Val-Δ^ZPhe-OH and Boc-Ala-Δ^ZPhe-OH were obtained on mM scale from corresponding azlactones using already reported procedure[21].

Boc-Ala-Δ^ZPhe-Leu-OCH_3 **3**. To a precooled (0^{o}C) solution of Boc-Ala-Δ^ZPhe-OH (5g,15.0mmol) in dimethylformamide (DMF) (20ml), was added dicyclohexylcarbodiimide (DCC) (3.1g,15.0mmol) and hydroxybenzatriazole (HOBT) (2.03g,15.0mmol) and the mixture stirred for 30 min. Leucine methyl ester hydrochloride (3.3g,18.0mmol) and triethylamine (TEA) (18.0mmol) in DMF (10ml) were added and the mixture was stirred for 4hrs at 0^{o}C and overnight at room temperature. For workup, the precipitated dicyclohexylurea(DCU) was filtered off and the solvent was removed in vacuo. The residue dissolved in ethyl acetate (50ml), was washed successively with saturated $NaHCO_3$ solution, water, 5% citric acid solution, dried over anhydrous Na_2SO_4 and finally evaporated to yield the tripeptide **3**. Yield, 4.6g (67%); m.p., 136-138^{o}C; R_f(A), 0.79; R_f(B), 0.82; R_f(C), 0.79. ^{1}HNMR, δ,ppm($CDCl_3$,60MHz):7.9 (1H, s, NH Δ^ZPhe), 7.4-7.1 (6H, m, aromatic protons & C$^\beta$H Δ^ZPhe), 5.2 (1H, d, NH Ala), 4.8 (1H, m, C$^\alpha$H Leu),4.2 (1H, m, C$^\alpha$H Ala), 3.8 (3H, s, OMe), 1.9-1.5 (3H, m, C$^\beta H_2$ Leu & C$^\gamma$H Leu), 1.4 (9H, s, $BocMe_3$), 1.35 (3H, d, C$^\beta H_3$ Ala), 0.9 (6H, d, 2xC$^\delta H_3$ Leu).

Boc-Val-Δ^ZPhe-Ala-Δ^ZPhe-Leu-OCH_3 **4**. Tripeptide Boc-Ala-Δ^ZPhe-Leu **3** (4.5g,9.4mmol) was deprotected at its N-terminal using a mixture of trifluoroacetic acid in dichloromethane (TFA:DCM; 1:1 v/v) using the procedure described elsewhere[20]. To a solution of Boc-Val-Δ^ZPhe-OH (3.8g,10.4mmol) in DMF (20ml), cooled to 0^{o}C, were added HOBT(1.4g, 10.4mmol) and DCC (2.1g, 10.4mmol). After 30min , a precooled solution of TFA salt of **3** and TEA (9.4mmol) in DMF (10ml) was added to the above solution. The reaction mixture was stirred at room temperature overnight and worked up using the same procedure as for tripeptide **3**. Yield, 3.6g (54%); m.p., 155-158^{o}C; R_f(A), 0.87; R_f(B), 0.82; R_f(C), 0.85. ^{1}HNMR, δ, ppm($CDCl_3$, 90MHz): 8.8 (1H, s, NH Δ^ZPhe), 8.6 (1H, s, NH Δ^ZPhe), 7.75 (1H, d, NH Ala), 7.5-7.2 (11H, m, aromatic protons & NH Leu), 6.85 (1H, s,

$C^{\beta}H$ Δ^{Z}Phe), 6.75 (1H, s, $C^{\beta}H$ Δ^{Z}Phe), 5.1 (1H, d, NH Val), 4.2 (1H, m, $C^{\alpha}H$ Leu), 4.05 (1H, m, $C^{\alpha}H$ Ala), 4.0 (1H, m, $C^{\alpha}H$ Val), 3.8 (3H, s, COOMe), 2.2 (1H, m, $C^{\beta}H$ Val), 1.85 (2H, m, $C^{\beta}H$ Leu), 1.45 (9H,s, Boc Me_3), 1.25(1H, m, $C^{\gamma}H$ Leu), 1.1 (3H, $C^{\beta}H$ Ala), 0.95-0.9 (12H, $C^{\gamma}H$ Val & $C^{\delta}H$ Leu).

Boc-Gly-Δ^{Z}Phe-Val-Δ^{Z}Phe-Ala-Δ^{Z}Phe-Leu-OCH_3 **2**. Pentapeptide **4** (3.0g, 4.2mmol) was deprotected using the same procedure as above. To a precooled solution (-10°C) of Boc-Gly-Δ^{Z}Phe-OH (1.47g, 4.6mmol) in tetrahydrofuran (THF) (20ml) was added N-methylmorpholine (0.5ml, 4.6mmol) and isobutylchloroformate (0.6ml, 4.6ml). After 10min of stirring a solution of TFA salt of pentapeptide **4** and TEA (4.2ml) in THF (10ml) was added. The reaction mixture was stirred at 0°C for 2hrs and overnight at room temperature. Workup of reaction (same as for peptide **4**) afforded the crude heptapeptide as pale yellow solid. Yield, 1.3g (34%); m.p.,138-140°C; R_f(A), 0.87; R_f(B), 0.95; R_f(C), 0.85. The heptapeptide was purified by HPLC using gradient of methanol and water (Retention time 21.7min). ^{1}HNMR of **2** in $CDCl_3$ is shown in Fig.4.

Ac-Δ^{Z}Phe-Pro-Δ^{Z}Phe-Ala-OCH_3 **1**. Boc-Pro-Δ^{Z}Phe-Ala-OCH_3 was obtained from Boc-Pro-Δ^{Z}Phe-Azlactone and alanine methyl ester hydrochoride using the procedure reported earlier[16]. The tetrapeptide **1** was synthesised by coupling of Ac-Δ^{Z}Phe-OH[37] and Boc-Pro-Δ^{Z}Phe-Ala-OMe using the same procedure as described above for peptide **2**. The peptide **1** was obtained as a white solid. It was crystallized from ethyl acetate/pet. ether. Yield, 0.7g (62.5%); m.p., 185-187°C; R_f(A), 0.66; R_f(B), 0.60; R_f(C), 0.89; $[\alpha]_D^{19}$-74.49 (c, 0.7 MeOH). ^{1}HNMR in $CDCl_3$ is shown in Fig.1.

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