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Does Aluminium bind to Histidine? An NMR investigation of Amyloid β12 and Amyloid β16 fragments

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Running title : NMR study of Aβ fragments interaction with Aluminium.

Abstract:

Aluminium and zinc are known to be major triggering agents for aggregation of amyloid peptides leading to plaque formation in Alzheimer's disease. While zinc binding to histidine in Aβ (Amyloid β) fragments has been implicated as responsible for aggregation, not much information is available on the interaction of aluminium with histidine. In the NMR study of the N-terminal Aβ fragments, DAEFRHDSGYEV (Aβ12) and DAEFRHDSGYEVHHQK (Aβ16) presented here, the interactions of the fragments with aluminium have been investigated. Significant chemical shifts were observed for few residues near the C-terminus when aluminium chloride was titrated with Aβ12 and Aβ16 peptides. Surprisingly, it is non-histidine residues which seem to be involved in aluminium binding. Based on NMR constrained structure obtained by molecular modelling, aluminium binding pockets in Aβ12 were around charged residues such as Asp, Glu. The results are discussed in terms of native structure propagation and the relevance of histidine residues in

the sequences for metal binding interactions. We expect that the study of such short amyloid peptide fragments, will not only provide clues for plaque formation in aggregated conditions but also facilitate design of potential drugs for these targets.

Key Words: Aβ peptides, Aluminium interactions, NMR, Alzheimer's disease, metal binding, amyloid peptides.

Introduction:

Alzheimer's disease (AD) is characterized by memory impairment, disordered cognitive function, delusions and decline in language function (1) and the diseased brain is known to show atrophy (2). The disease leaves behind two major clues to the pathogenesis of AD (3). The neurofibrillary tangles which are a mass of paired helically wound filaments lie in the cytoplasm of neuronal processes (4) and the extracellular aggregates of neuritic plaques, the Amyloid beta (Aβ) (5). The Aβ known to be soluble (6) is produced by the proteolytic cleavage (7) of a larger protein, the Amyloid Precursor Protein (APP) (8) coded by the APP gene on the long arm of the human chromosome 21(9). The Aβ is a 39-43 amino acid residue peptide (10), with a hydrophobic C-terminal domain, assuming a beta-strand structure, and N-terminal region, which has the propensity to form different secondary structures (11). Two beta turns are predicted between residues 6 and 8 and 23 and 27 (12). The Aβ{1-42}, Aβ{1-39}, Aβ{1-28} and Aβ{29-42} are also known to form independent aggregates (13). The structure of Aβ{1-42} has been studied in aqueous hexafluoroisopropanol showing 2 helical regions connected by a type-I β turn (14). The structures of Aβ {1-40} (15) and Aβ{1-28}(16,17) have also been reported. Solid state NMR studies have been used to elucidate the structure of the full length Aβ (18,19). NMR studies of the Aβ {25-35} in various solvents and in a membrane mimicking environment have been carried out (20). The role of metals such as Zn^{2+}, Cu^{2+}, Al^{3+} and Fe^{2+} as a possible trigger for the onset of the disease has been studied and reviewed (21). Study of shorter fragments like Aβ16 and their interaction with Zn^{2+} have revealed the roles of specific amino acids like histidine (22). The role of histidines in zinc binding has been further established by solution NMR studies of truncated fragments of Aβ (23-27). Biophysical studies have revealed the formation of structured aggregates of the peptide on binding to Al^{3+} (28). The binding of Asp1, Glu3 to Fe^{2+} and Cu^{2+} has also been studied (29, 30). Metal chelators have been

shown to effectively reverse the aggregation of Aβ and silicates, borates and betaine are known to reverse the toxicity (31-33). Inhibitors of Amyloid deposits like platinum, curcumin, and nicotine have also been studied (34-36).

From the above studies we see that Aβ peptide fragment analysis has provided quite useful information. The aggregation of the peptide with metals has been studied using Circular Dichroism which revealed an alpha to beta sheet transition with the propensity in the order [Cu>Fe≥Al>Zn] (37). It has been well established that in the case of metal binding, the active part is the N-terminus containing the histidine residues and hence the studies are focussed on fragments such as Aβ28 (6), and Aβ16 (24,25). The current study was thus initiated to investigate aluminium interactions with Aβ16. Interestingly, we observed that in contrast to earlier studies involving zinc (26-27,38-39), histidine residues {His13 and His14} are not involved in the binding of Al^{3+}. Further NMR studies of Aβ12 fragment have thrown light on two aspects, (i) the idea of native structure progression as the size increases and (ii) the role of histidine residues {His13, and His14} in metal binding interaction, as these residues are truncated in Aβ12. Comparison of results obtained for Aβ12 and Aβ16 highlight the similarities and differences. Study of such shorter fragments may provide clues for plaque formation and could also be useful for design of potential drug targets.

Experimental Methods

Sample Preparation

The peptides for this study were custom synthesized from M/s USV peptides and purity of >95% was confirmed by HPLC. Aβ12 and Aβ16 peptide mass peaks (ESI-MS) (vide figure S1 in supplementary material) at 1424.97 and 1953.8 Da respectively agree well with the calculated mass. For NMR measurements, 2 mM concentrations of Aβ12 {DAEFRHDSGYEV} and Aβ16 {DAEFRHDSGYEVHHQK} in aqueous phosphate buffer at pH 6 containing 90% H_2O, and 10% D_2O were used. Generally, in case of short peptide sequences, aquatic media disrupt the structure; but in this case, the resonances were well dispersed both in 1D and 2D spectra indicating structural rigidity. The chemical shifts and $^3J_{NHC^{\alpha}H}$ were however close to the random coil values. A Circular Dichroism (CD) spectrum of Aβ12 also indicated a random coil structure (supplementary figure S2). Titration studies with aluminium were carried out using highest analytical grade of $AlCl_3$ dissolved in Mili Q water. Known volumes of $AlCl_3$ from 50 mM stock solution were added to NMR tube

containing peptides and NMR spectra were monitored at different peptide: metal stoichiometric ratios.

NMR Data Acquisition

All 1D and 2D NMR experiments were carried out either on a Bruker AV 700 MHz NMR spectrometer attached with a cryo-probe or on a Bruker AV 500 MHz NMR spectrometer. Spectra were recorded at 280K for Aβ12 and at 303K for Aβ16 to obtain a good dispersion of the amide resonances. Chemical shifts were referenced to external TSP Peak. Proton resonance assignments were carried out by recording TOCSY (40)and ROESY(41)spectra with mixing time of 100 and 250 ms respectively. $^{3}J_{NHC}{}^{\alpha}{}_{H}$ coupling were directly measured from the high resolution 1D spectra, from the splitting of the amide resonances. The amide proton temperature coefficients ($d\delta/dT_{HN}$) were determined from Proton 1D spectra recorded between 278K to 318K at 10 degree intervals. ^{1}H-^{13}C and ^{1}H – ^{15}N HSQC spectra were also recorded as additional confirmation of observations made using the TOCSY spectra. All data were offline processed using Bruker TOPSPIN software. Shifted sine bell window functions were used for processing 2D data.

Structure calculations

Structures of the peptides were calculated using the software CYANA (version no. 3.0) by including the constraints derived from NMR. The starting structure in all the cases was the extended form of the peptide sequence. The relative NOE cross peak volumes manually calculated from the ROESY spectrum were converted to approximate distance ranges and were provided as distance constraint input file (supplementary material tables ST2 and ST3). A set of structures are calculated by the software, based on torsion angle dynamics driven simulated annealing (42). If all experimental constraints are satisfied and all non bonded atom pairs are free of steric overlap, then a target function (f) is minimized which is indicative of quality of structure obtained. In our peptides we had a limited NOE distance and dihedral angle (ϕ) constraints which were obtained from NMR recordings. Though amide proton temperature coefficients were obtained, it could not be used to fix appropriate hydrogen bond distances because of lack of knowledge of the bonded partner. Twenty structures were calculated which resulted in no violations of any of the constraints. All the ϕ and ψ dihedral values were also within the allowed region in the Ramachandran map.

Typically, average RMSD values for all 20 structures (residues 3-12) were 0.37 +/- 0.11 Å for backbone atoms and 0.94 +/- 0.21 Å when all heavy atoms were considered. Best five structures were considered in each case for comparison.

Molecular modelling:

Molecular modelling to identify the metal binding sites was carried out using commercial Schrodinger software. NMR derived CYANA structures were exported to Macro model module of the software. Couple of potential aluminium binding site were identified near the charged residues such as Asp and Glu, which showed distinct chemical shift changes in the NMR spectra and are well suited for metal coordination through their carboxylate groups (43). Aluminium metal atom was placed in such positions and the whole bound structure was restrained energy minimized in vacuum. OPLS2005 force field and PRCG (Polak-Ribiere Conjugate Gradient) method for energy minimization were used.

Results and discussion:
Aβ12 structure:

A 700 MHz ^{1}H NMR spectrum of the 12 residue N-terminal fragment of Aβ peptide {DAEFRHDSGYEV}Aβ12 in phosphate buffer at 280K is shown in figure 1. It is seen in the spectrum that most of the amide resonances are well dispersed. Such well resolved sharp resonances imply a well folded peptide. Proton chemical shifts along with $^{3}J_{NHC^{\alpha}H}$ coupling obtained from the sepctrum are tabulated in Table 1. Resonance assignments were done with the help of the 2D-TOCSY and ROESY experiments. Figure 2a is a plot of partial region of the TOCSY spectrum showing connectivity of the amide protons to their coupled partners, providing residue specific assignments. Sequence specific assignment could also be done with the help of the ROESY spectrum. Figure 2b shows the finger print region along with the amide-amide connectivity of Aβ12 peptide. Presence of both and $d_{\alpha N}$ (i, i), and $d_{\alpha N}$(i,i+1) NOES with comparable intensities and also the presence of some d_{NN} NOEs suggests no particular preference for either the helical or the extended secondary structure. This is further corroborated by $^{3}J_{NHC\alpha H}$ values which are around 6-7 Hz (Table 1). Also most of the temperatures coefficients of amide protons are above -5ppb/K, implying no strong secondary structure hydrogen bonding pattern. CD spectrum shown in supplementary material (S2) is also indicative of a random coil structure which agrees with the NMR results mentioned

above. With the help of available limited NOEs and $^{3}J_{NHC\alpha H}$ values NMR structures were calculated using CYANA software. The first five low 'f' (target function) value structures out of 20 calculated are shown in figure 3. Though there is no definite secondary structure, they are all valid structures as all the ϕ, ψ values fall within the allowed region in the Ramachandran map.

Aβ16 structure:

Following the method described above, the structure of the 1 – 16 N-terminal fragment of Aβ peptide {DAEFRHDSGYEVHHQK, Aβ16} was also calculated. Aβ16 has four extra residues (HHQK) at C-terminus, in comparison to Aβ12. Relevent part of the TOCSY and ROESY spectra of Aβ16 are given in supplementary material (S3 and S4). The chemical shifts along with $^{3}J_{NHC}{}^{\alpha}{}_{H}$ values are provided in table 2. The NMR spectra recorded at 303K show a good dispersion in the amide region. Aβ16 structure is already available (26). However for the sake of comparison with Aβ12, we carried out the structure calculation of Aβ16 under conditions similar to those at which Aβ12 structure was obtained. Such a study would provide some understanding of structure propagation as the size increases. Another reason to study both peptides is to understand the metal interaction with and without histidine residues (H13 and H14) which are normally implicated in metal coordination. The NMR results for Aβ16 were similar to those obtained for Aβ12, indicating no definite secondary structure component.

Comparison of Aβ12 and Aβ16 structures:

The structures of Aβ12 and Aβ16 were overlayed and are shown in figure 4. The reasonable overlap of structures suggests extended propagation of Aβ12 structure in Aβ16 inspite of the addition of 4 more C-terminal residues (-H-H-Q-K). A comparative Chemical Shift Index {CSI}(44) plot shown at the bottom of figure 4 show quite similar $\Delta\delta$ values suggesting similar structures. Based on NMR structures couple of metal binding sites could be identified in Aβ12 which are discussed in the following sections. As Aβ16 structure is similar to Aβ12, the same binding site may also be invoked here; in addition there could be additional binding pocket at the C-terminus involving histidine (H13, H14) residues which have been implicated in metal binding studies involving zinc and copper (24,25).

Surprisingly, in the current study with aluminium, it is observed that both these H13 and H14 residues do not show any significant changes in their chemical shift implying non involvement of these residues in binding. A natural abundance $^{1}H - ^{15}N$ correlation spectrum (HSQC) of Aβ16 is shown in Figure 5, where the movement of amide resonances is monitored in the presence of aluminium. Clearly it can be observed that there is no significant movement of H13 and H14 amide peaks. The residues which showed significant changes are similar to those of Aβ12 peptide. A more detailed analysis of Aβ12 with aluminium interaction is discussed in the following section.

Interaction of Aβ12 with Aluminium:

Aluminium metal interactions with the Aβ12 peptide were studied by titrating known amounts $AlCl_3$ into the NMR tube containing Aβ12 peptide solution at 280K. Figure 6a shows the changes in the amide region of the NMR spectrum of Aβ12. Amide protons of E3, F4, H6, G9, E11 and V12, which show significant changes, are marked in the figure. There were also some changes in the aliphatic region of the C-terminal V12 residue. As there is no terminus protection for the Aβ12 peptide, the C-terminus acid group (COOH) interaction probably causes the shifts and hence the terminal residue shifts are not taken into consideration. Figure 6b and 6c bar chart show the relative changes in the Aβ12 chemical shifts respectively of amide and C^{α} protons with and without metal. Overlap of the relevant TOCSY spectral regions shown in Figure 7 (with and without metal), clearly identify the movement of various resonances. The same figure also has ROESY spectra overlapped which show that the NOEs are similar between free and bound form. Few exceptions being small intensity changes, especially with the tyrosine (Y10) ring protons and valine (V12) protons (data not shown). Similar shifts in proton-carbon and proton-nitrogen hetero-correlations can also be seen as shown in supplementary material (S5 and S6).

The procedure used for the structure calculation of metal free Aβ12, were also applied to the metal bound Aβ12. Though there were significant chemical shifts changes, other NMR parameters like $^{3}J_{NHC^{\alpha}H}$ coupling and NOES, amide temperature coefficients were similar (table 1). Couple of NOEs were different near C-terminus. Structure calculation using CYANA yielded the structure of the metal bound peptide which was similar to the metal free peptide with some differences such as small reorientations near C-terminus and quite

significant movement in arginine side chain. Figure 8 is a comparative chemical shift index (CSI) plot of C^{α} protons of Aβ12 with and without metal. The chemical shift difference (Δδ), though small throughout the sequence, is indicative of similar structures. Figure 9 shows an overlap of the best CYANA structures of Aβ12 peptide with and without metal interaction. Wide distribution of ϕ, ψ dihedral values in the allowed region of Ramachandran map for both free and bound form, (supplementary figure S7) can be attributed to some structure with no definite secondary structure component.

To identify the possible sites of aluminium interaction with the available CYANA structure, molecular modelling using commercially available Schrodinger software was used. Overlapping the Aβ12 structure with and without metal bound as shown in figure 9 indicated many of the N-terminal residues has not changed, whereas there were some deviations towards the C-terminus. We could locate two potential sites for placing Al^{3+} metal, one around the centre of the molecule near the aspartic acid (D7) residue, and the other towards the C-terminus centred around residues Gly (G9), Tyr (Y10) and Glu (E11). At both these sites carboxylate functional groups of charged residues provide a good geometry for aluminium metal coordination (43). Energy minimization was carried out with the Metal at these positions restraining the N-terminal residues. The C-terminus metal position shows relatively lower energy and also the Metal-oxygen coordination distances were shorter compared to the other site. The results also match with the NMR chemical shift changes of these C-terminal residues.

ESI-MS spectra as shown in figure 10, corroborate aluminium metal interaction as proposed above. Intense mass spectral lines corresponding to both free Aβ12 peptide with mass 1424 Da. and aluminium bound Aβ12 peptide with mass 1448 Da (1424 – 3H + 27) could be observed. A closer look as ESI-MS also show a diminished peak corresponding to a species with mass 1472 Da, which corresponds to two aluminium bound to the peptide (1424 – 6H +27+27). The corresponding M/2 peaks of all three species could also be identified at mass of 713, 725 and 737 Da. respectively on expansion of ESI-MS spectrum (data not shown). These results thus confirm the conclusions arrived on the basis of the NMR studies.

Interaction of Aβ16 with metal:

Titration of $AlCl_3$ with Aβ16 peptide showed changes in the NMR spectrum similar to those observed with Aβ12 peptide. Amide proton chemical shifts (figure 5) as well as other parameters like $^3J_{NHC}{}^{\alpha}{}_{H}$, NOEs and amide temperature coefficients (dδ/dT) remained almost unchanged. As in the previous case, the unprotected C-terminal residue K16 with its acid (COOH) interaction showed a large chemical shift change for its amide proton. Thus as in the case of Aβ12, the Aβ16 structure with metal binding remains essentially the same, except for small local perturbations where the metal could possibly interact as explained in Aβ12. As mentioned elsewhere, it would be interesting to see whether the extension of Aβ12 with four residues (-H_{13}-H_{14}-Q_{15}-K_{16}) would provide additional binding pockets for aluminium at the C-terminus. Surprisingly, it is observed that both the histidine H13 and H14 residues do not show any significant changes in their chemical shift implying non involvement of these residues in binding. The residues which showed significant changes are the same as that of Aβ12 peptide.

Discussion

In literature there are several reports on the interaction of metals like zinc, copper, etc. (23-27, 37) with Aβ peptides. But in spite of aluminium being implicated as a trigger for the onset of Alzheimer's disease (22), very limited NMR studies involving aluminium have been reported. In a bioinformatics analysis carried out by us involving the residues of the Aβ12 fragment in various metals bound protein structures in the protein data bank (45), we find that there are very few aluminium bound structures reported as shown in ST1 (supplementary table 1). Of the reported structures, none are NMR structures.

It is well known that one of the reasons for the plaque formation is the metal induced aggregation of Amyloid peptide Aβ42. It is also known that the C-terminus residues being hydrophobic are generally found inside the membrane, and hence the exposed region of 1-28 residues (Aβ28) is responsible for aggregation leading to plaque formation (6). Further the focus of several studies have been to 1-16 residues as it was shown that His13, and His14 are implicated in metal binding (39).

Conformational changes in peptides and proteins induced by aluminium(46) have been considered as neurotoxic(47).and recent evidences have shown that the smaller fragments of Aβ are produced in presence of elastase (48). Hence the present work on fragments 1-12 (Aβ12) and 1-16 (Aβ16) becomes significant to understand possible structural changes and the relevance, if any, of His13 and His14 in metal binding. The NMR structures of these fragments presented here were found to be very similar, but for the extension at the C-terminus as shown in figure 4, and Al^{3+} titrations with Aβ16 (which contained His13 and His14) showed no interactions with the histidine residues. On the other hand, the study reveals additional plausible metal interaction sites specific to aluminium, namely Gly9, Tyr10 and Glu11.

Conclusions:

Metal interactions with Aβ peptides are strongly implicated in fibril formation. This study was carried out as there are limited information available on aluminium interactions with Aβ peptides. Such study becomes important as aluminium entry into brain along with zinc and other metals has been clearly identified. aluminium in food packing and cooking vessels are common sources for aluminium entry into the human system. Here we show that aluminium does bind to Aβ fragments, but not at the generally expected histidine residue site. The binding does not bring about any major structural changes but causes small local perturbations. Studies also show that there are no major structural changes when the 16 residue fragment Aβ16 is reduced to 12 residues Aβ12, where the two important metal binding histidine residues (His13 and His14) along with the remaining two Gln15 and Lys16 are deleted. In both Aβ12 and Aβ16, the same set of resonances is disturbed implying the interactions with aluminium are similar.

There are attempts to reverse the effect of metal interaction by use of metal chelators (49). Curcumin and Betaine have also been tested for such reversal, wherein they compete with the metal binding sites (31-35). Further investigations are under way in this direction.

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Figure captions:

Figure 1: 700 MHz Proton spectrum of Aβ12 in phosphate buffer pH 6 at 280K. The expanded regions are (a) amide and aromatic protons, (b) C^{α} Protons, and (c) remaining side chain protons.

Figure 2: (a) Partial region of (a) TOCSY and (b) ROESY spectra of Aβ12 in phosphate buffer at 280K. Projection on either side is the corresponding 1D plot of Aβ12. ROESY spectra are split into NH-NH (bottom) and NH-C^{α}H regions (top).

Figure 3: Stereo view of best five structure out of 20 structures calculated using CYANA software by using only limited NOE distant and ϕ dihedral constraints. Hydrogen bond constraints could not be used due to lack of identification of partner Carbonyl oxygen to amide protons.

Figure 4: Backbone atom overlap of Aβ12 (green) with Aβ16 (blue) CYANA structures. A comparative CSI plot of C^{α} proton chemical shifts difference with respect to standard random coil values (Δδ) show no major changes providing evidence for similarity of structures (blue: Aβ12, red: Aβ16).

Figure 5: Overlap of Aβ16 free (blue) and bound (red) natural abundance ^{1}H-^{15}N HSQC spectra. Significant movement are observed for circled peaks. Note: H13 and H14 do not show any major differences.

Figure 6: (a) Amide region plots of titration experiments of Aβ12 with $AlCl_3$ at 298K. Bar charts (Blue: free peptide and Red: metal bound) showing the chemical shifts differences of (b) amide, and (c) C^{α} protons.

Figure 7: (Left panel) Partial regions of the TOCSY spectra showing the amide correlations with other regions of Aβ12 in phosphate buffer at 280K with (Blue) and without (black) metal interaction. (Right panel) Partial region of ROESY spectra with (Red) and without (black) metal interaction.

Figure 8: Comparative chemical shift index (CSI) plot of Aβ12 with (Red) and without (Blue) aluminium metal. Most of the residues show small negative Δδ values indicate random coil, bordering α_R region of Ramachandran map.

Figure 9: Overlap of Aβ12 structures with (green) and without metal (orange). Proposed binding sites of aluminium metal are also shown. C-terminus binding site has relatively lower energy, and also compatible with NMR studies.

Figure 10: ESI-MS recording of Aβ12 with aluminium metal. Free peptide peak is observed at 1424 Da, whereas aluminum metal bound peptide peak appear with highest intensity at 1448 Da. Peptide bound to two aluminium can also be identified as a low intensity peat at 1472Da.

Table 1: NMR parameter of free Aβ12 along with Al^{3+} metal bound peptide (in brackets)

Resi-due	**Chemical Shift (ppm)**						**$^3J_{NHc^aH}$ (Hz)**
	NH	**$C^{\alpha}H$**	**$C^{\beta}H$**	**$C^{\gamma}H$**	**$C^{\delta}H$**	**$C^{\varepsilon}H$ / Others**	
D(1)	-	4.17 (4.19)	2.79, 2.82 (2.74, 2.83)	-	-	-	-
A(2)	8.72 (8.73)	4.23 (4.24)	1.29 (1.29)				5.5 (5.4)
E(3)	8.40 (8.36)	4.15 (4.17)	1.89 (1.89)	2.12,2.21 (2.16, 2.30)			6.5 (6.5)
F(4)	8.31 (8.29)	4.52 (4.52)	2.94, 3.00 (2.95, 3.01)			7.14 (7.19)	7.0 (6.8)
R(5)	8.25 (8.25)	4.14 (4.14)	1.59,1.68 (1.59,1.67)	1.44,1.39 (1.42)	3.03 (3.06)	7.32 (7.26)	6.9 -
H(6)	8.65 (8.62)	4.57 (4.57)	3.07, 3.15 (3.08, 3.15)		7.19 (7.17)	8.51 (8.52)	7.3 (7.2)
D(7)	8.54 (8.57)	4.58 (4.61)	2.64, 2.71 (2.77)				6.8 (6.8)
S(8)	8.47 (8.47)	4.33 (4.33)	3.80 (3.80)				6.1 (5.6)
G(9)	8.50 (8.48)	3.86 (3.82)					- (5.9)
Y(10)	7.98 (7.98)	4.48 (4.48)	2.88, 2.93 (2.90)		6.99 (6.99)	6.71 (6.71)	6.9 -
E(11)	8.29 (8.24)	4.26 (4.28)	1.83 (1.82,1.97)	1.98,2.26 (2.28)			7.4 (6.7)
V(12)	7.87 (8.00)	3.94 (3.98)	2.05 (2.04)	0.85 (0.86)			8.1 (7.7)

Table 2: NMR parameter of free Aβ16 along with Al^{3+} metal bound peptide (in brackets)

Resi-due	Chemical Shift (ppm)						$^3J_{NHc^\alpha H}$ (Hz)
	NH	$C^\alpha H$	$C^\beta H$	$C^\gamma H$	$C^\delta H$	$C^\varepsilon H$ / Others	
D(1)	- (-)	4.25 (4.25)	2.73, 2.81 (2.82)				-
A(2)	8.61 (8.62)	4.27 (4.29)	1.30 (1.30)				5.8 (5.8)
E(3)	8.27 (8.21)	4.18 (4.22)	1.89 (1.89)	2.15, 2.24 (2.22,2.31)			6.7 (6.7)
F(4)	8.15 (8.13)	4.55 (4.55)	2.94, 3.03 (2.95, 3.05)		7.16 (7.17)	7.26 (7.27)	7.1 (7.1)
R(5)	8.11 (8.11)	4.20 (4.20)	1.64, 1.68 (1.62, 1.68)	1.46 (1.46)	3.08	7.17 (7.18)	7.1 (-)
H(6)	8.49 (8.48)	4.61 (4.60)	3.06, 3.19 (3.09, 3.19)		7.24 (7.24)	8.55 (8.56)	7.2 (7.6)
D(7)	8.39 (8.44)	4.67 (4.66)	2.69 (2.77)				7.1 (7.1)
S(8)	8.37 (8.35)	4.36 (4.37)	3.83 (3.83)				6.3 (6.3)
G(9)	8.44 (8.38)	3.86 (3.87)					- (-)
Y(10)	7.91 (7.92)	4.45 (4.46)	2.91 (2.91)		7.02 (7.02)	6.73 (6.73)	6.5 (6.3)
E(11)	8.18 (8.11)	4.20 (4.20)	1.85, 1.91 (1.84, 1.92)	2.18 (2.26)			6.8 (-)
V(12)	7.93 (7.92)	3.85 (3.85)	1.88 (1.87)	0.76, 0.81 (0.73,0.82)			7.2 (7.0)
H(13)	8.42 (8.43)	4.70 (4.70)	3.04,3.17 (3.07, 3.17)		7.22 (7.22)	8.54 (8.55)	7.9 (7.8)
H(14)	8.48 (8.50)	4.66 (4.66)	3.09, 3.19 (3.06, 3.21)		7.20 (7.21)	8.52 (8.54)	7.7 (7.6)
Q(15)	8.47 (8.49)	4.30 (4.30)	1.96, 2.07 (1.95, 2.06)	2.34 (2.35)			- (6.9)
K(16)	8.08 (8.26)	4.12 (4.12)	1.70,1.76 (1.74, 1.80)	1.38 (1.39)			7.3 (7.2)