ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Acetyl-(D) Arginyl - Glycyl- (D) Aspartic Acid Is A Better Integrin Binding Analog Than Acetyl - Arginyl - Glycyl-Aspartic Acid In Membrane Mimicking Solvents.

Samir Kumar Nath[2], Ashish[2], and Veena Sharma[1] *

[1]Department of Bioscience and Biotechnology, Banasthali University, Banasthali, Rajasthan- 304022, India.
[2]CSIR-Institute of Microbial Technology, Sector-39A, Chandigarh- 160036, India.

ABSTRACT

Using molecular dynamics simulations and nuclear magnetic resonance techniques we explored the conformational preferences of linear and chiral analogs of acetyl-arginyl-glycyl-aspartic acid (Ac-RGD) and acetyl-arginyl-β-Alanyl-aspartic acid (Ac-RXD). Using tinker molecular modeling program and 5nano second molecular dynamics simulations in water/dimethyl sulphoxide to mimic the varying environment experienced by the peptide from water to membrane/dimethyl sulphoxide-d_6, in trans-cellular transport process. We validate the simulation results with the complementary nuclear magnetic resonance experiments. Results from both the techniques suggest that the Acetyl-(d)arginyl-glycyl-(d)aspartic acid is more susceptible for integrin binding than that of Ac-RGD. This is because more number of conformations was found to be fit with the pseudo-dihedral angle with $-45° \leq pdo \leq +45°$, an essential criterion for integrin binding and with valid interatomic distances in both the solvent conditions, laid down by various researchers. This peptide is stabilized by Arg-$N^{\varepsilon}H\cdots O=C^{\gamma}$-Asp hydrogen bonding interaction in dimethyl sulphoxide-d_6. Results from pseudo-dihedral angle and inter atomic distances of unusual β-Alanine analogs acetyl-arginyl-β-alanyl-aspartic acid suggest that the more number of conformers of this peptide is found to be fit in the pseudo-dihedral angle criteria than the other chiral analog except Ac-RGD and Ac-rGd. There is a good correlation between simulation and NMR experimental results.

Keywords: molecular dynamic simulations, NMR, peptide, conformation.

Corresponding author

ISSN: 0975-8585

INTRODUCTION

RGD peptide was identified as the fundamental sequence responsible for the recognizing number of cell surface proteins such as fibronectin, laminin, collagen, fibrinogen, osteopontin, thrombospondin, which play significant role in cell adhesion [1-6]. It is well studied that the peptide sequence containing RGD have high affinity towards integrin cell surface receptors and compete with the native proteins for binding to receptors [7]. Integrin binding behavior is sequence specific [8,9]. Various groups have studied the peptide/protein containing RGD sequence have shown to bind GPIIb/GPIIIa($\alpha_{IIb}\beta_3$) to inhibit platelet aggregation, as $\alpha v\beta 3$ integrin was reported to be implicated in human tumor angiogenesis and metastasis [10-12]. It is also well reported that disintegrins extracted from snake venoms were very potent inhibitors of fibrinogen binding to platelets and platelet aggregation [13].

Linear and cyclic RGD peptides have been extensively studied by NMR and MD simulations. A varying number of conformations including β-turns of types I, II or II' for Arg-Gly and/or Gly-Asp sequences have been suggested [14-18]. From these studies presence of Gly-Asp sequence as type II β-turn was a prerequisite for integrin binding, but the ligands selectiveness was correlated with the distances between either the C^{θ} atoms or the opposite charged centres of Arg and Asp residues [15, 17-21]. It was also not known that how the residues adjoining the RGD sequence influence its activity and selectivity. These flanking amino acid residues can be involved in binding to the receptor and also in inducing a distinct conformation of RGD sequence [22-25]. Structural analysis revealed that the β- turn is not a pre-condition for activity but a correlation between the pseudo-dihedral angle (*pdo*), which is formed by the (Arg)C^{ζ}-(Arg)C^{α}-(Asp)C^{α}-(Asp)C^{γ} and the activity was found having *pdo* -45° ≤ *pdo* ≤ +45° [26]. Stote *et. al* used *pdo* as an extra criterion for evaluation of structure–activity relationship of RGD sequence present in protein structures deposited in the Protein Data Bank [27]. In this study, the region of *pdo* was varied between −150° and 90°. The distances between C^{β} and C^{γ} atoms of Arg and Asp residues were distributed in the range of 5.5–9 Å and 7.8–11.5 Å, respectively. Bartels *et. al* carried out MD simulations on the RGDW peptide and established that the distance between the (Asp)C^{γ}-(Arg)C^{ζ} atom has an average value of 9.9Å and 11 Å was found to be the most populated distance [28]. The resultant distance was proposed to be 10–15 °A for decorsin, which is a potent antagonist of GPIIb/IIIa receptor having 39 amino acid residues [29,30].

Kang *et. al* work on simulations of Ac-RGD-NHMe in the nonhydrated and hydrated states interpreted that this tripeptide exists as an assembly of conformations rather than a single conformation [31]. The neutral nonhydrated tripeptide has a distorted type I β-turn stabilized predominantly by Asp-NH···O=C(NHMe) end group hydrogen bonding and also another two hydrogen bonds exits between Asp-N-H···$O^{\delta 1}$=C^{γ}(Asp) and Arg-N^{ε}H…O=C (NHMe). In hydrated state this tripeptide had also a distorted type I β-turn and stabilized by three intramolecular hydrogen bonds between Ac-C=O···H-$O^{\delta 2}$(Asp), Arg-C=O···HN-Asp and Gly C=O···HN(NHMe). In case of zwitterionic tripeptide, most probable conformation was found to be without a β-turn and is stabilized by four hydrogen bond between Arg^{+}-NH···O=C-Arg^{+}, Arg^{+}-C=O···HN-Asp^{-}, Arg^{+}-N^{ε}H···$O^{\delta 1}$=C-Asp^{-} and Arg^{+}-$N_{\eta 2}$-H···$O^{\delta 1}$=C-Asp^{-}. A detailed ^{1}H-NMR and molecular modeling analysis of Ac-RGD-NH_2 in DMSO-d_6 solution demonstrated that conformation of this peptide is stable in solution at room temperature and stabilized by a hydrogen bonded/ionic interaction between the Arg and Asp acid side chains with *pdo* values range between −70° ≤ *pdo* ≤ 0° [32]. This information revealed that the pseudo dihedral angle could be utilized as a measure for evaluating the structure–function relationship of RGD–peptides. Different criteria such as the occurrence of a β-turn, the distance between Arg and Asp C^{β} atoms, the distance between the oppositely charged centers and the presence of the ionic interaction could be predicted as dependent upon the relative orientation of the side chains, which further show a relationship between the activity and selectivity of an analogue.

The NMR experimental observations on RGDW, D-RGDW linear peptides and $c(RGDW)_2$ cyclic dipeptide, suggest that the linear peptides form a type II' β-turn with the Gly and Asp residues in positions 2 and 3 of the turn [33]. This observation is in accordance with the analysis of such turns in proteins [34-37]. Support for fast internal motions was provided for the linear tetrapeptide which undergoes a considerable flexibility in solution [38], so there is a prospective that not only turn conformations contribute to the average structure but also other conformation also plays a role. As only NMR experimental observations often cannot distinguish multiple conformations when exchange among them is fast on NMR time scale [39], the other conformations could not be identified. Therefore, it is important to supplement NMR data by simulations [40-42]. NMR experimental parameters and unconstrained MD simulation were used by Stote *et al* to probe the

structure and conformational dynamics of the short linear peptide RGDW [27]. The evaluation between the simulation results and the experimental observations demonstrated that the NMR data alone do not define a unique conformation for the RGDW peptide but that the NMR observation was well-suited with both turn and extended conformations in solution. It was proposed that a mixture of turn and extended conformations of the RGDW peptide, as suggested by the simulations, gives the best fit to the NMR data.

As the specificity of the RGD peptides are conformation dependent, any analogs containing unusual/D-amino acid may unravel a unique conformational feature which may be important for its biological activity. As of now there is no reported evidence of structure function relationship of chiral analogs of Ac-RGD peptides in membrane mimicking solvents. Keeping this in mind, in this paper we synthesized different chiral analogs, peptide **1**: Ac-RGD-OH, peptide **2**: Ac-rGD-OH, peptide **3**: Ac-RGd-OH, peptide **4**: Ac-rGd-OH, (where small letter alphabet represents D-isomer) and find out their conformational preferences in two different solvents by using MD simulation and complemented with NMR experimental results. We modified the peptide backbone by incorporating unusual amino acid β-Alanine (β-Ala), peptide **5**: Ac-RXD-OH, peptide **6**: Ac-rXd-OH (where as X=β-Alanine) to get some structural preferences by incorporating another main chain torsion angle μ between ϕ and ψ. The criteria of pseudo dihedral angles and inter atomic distances were calculated for all the analogs and compared with the native peptide **1**. From these results, we report for the first time that the Ac-rGd-OH analog is a better analog for integrin binding than all other analogs reported here.

MATERIALS AND METHODS

Fmoc-Amino acids Fmoc-Arg(Pbf)-OH, Fmoc-Gly-OH, Fmoc-Asp(OtBu)-OH, Fmoc- β-Ala-OH, 2-Chlorotritylchloride resin(100-200mesh) with 1% Divinylbenzene(DVB), O- (Benzotriazol-1-yl)-N,N,N′,N′-tetramethyluroniumhexafluorophosphate(HBTU), Diisopropyl ethylamine(DIPEA) and solvents like Dimethylformamide (DMF), Dichloromethane (DCM), methanol (MeOH), diethyl ether were purchased from Emerck India. 1-Hydroxybenzotriazole (HOBT) was purchased from GL Biochem China. Reagents for cleavage cocktails like Trifluoroacetic acid (TFA), Triisopropylsilane (TIS), Kaiser Test kit and Deuterated solvents like DMSO-d_6 and D_2O for NMR experiments were purchased from sigma Aldrich from Sigma Aldrich.

Peptide synthesis

Linear peptides **1** to **6** were synthesized by solid phase peptide synthesis strategy using Fmoc (N-(9-fluronyl)-methoxycarbonyl) (Reference) chemistry in 0.12mmole scale. The chain elongation of the peptide was done on a Protein Technologies Inc, USA, PS-3 peptide synthesizer by using four equiv. of the protected Fmoc-amino acid with HBTU (2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) as a coupling reagent and HOBT (n-hydroxybenzotriazole) [44] as a recemisation suppressing agent. Synthesis strategy in synthesizer is depicted **Scheme 1**. For side chain protection pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) derivative of Fmoc-Arginine and tertbutyl ester (OtBu) derivative of Fmoc-Aspartic acid were used. For C-terminal activation 0.4M NMM in DMF was used and for N-terminal Fmoc-group deprotection 20% piperidine in DMF was used. The completion of the reaction was monitored by performing Kaiser Test. After final Fmoc-group removal, the peptide on resin was acetylated twice by treating with solution of DMF: acetic anhydride: DIPEA (8:1:1, v/v/v) for 2 hours each. Finally the resin was washed with DMF, DCM, 2-propanol, methanol and diisopropyl ether and, finally, dried. The peptide was cleaved from the resin by treating the resin with a cleavage cocktail Trifluroacetic acid/water/Triisopropylsilane (95:2.5:2.5) for 6 hours. Then it was filtered, dried under air pressure and subsequently on a vacuum to obtain 45-60mg crude peptides. Crude peptide was purified by Dionex Ultimate 3000 HPLC on a reverse phase C-18 (4.6x250mm) column using water/acetonitrile gradient containing 0.1% Trifluroacetic acid. Then from the pure eluting fraction, organic solvent was evaporated and then lyophilized to complete dryness yielded 25-32mg of the purified peptide in each case. The HPLC profile of the peptides were shown in supplementary material **Figure 1S**. Mass of the peptide confirmed by MALDI-TOF calculated 389.2, and obtained 389 for Ac-RGD-OH analogs and calculated 403.2 and obtained 402.9 for β-alanine analogs **Figure 2S**. The purified tripeptides were also fully characterized by ^{1}H NMR and found to be consistent with the covalent structure.

MD Simulations methods

Molecular Dynamics (MD) and energy minimization were carried out using the TINKER molecular modeling package v 4.2 [44,45], a freely available package for molecular mechanics based potential energy

calculations, geometry optimization, MD simulation, distance geometry and structural analysis. In order to map the available conformational space of peptides **1-6,** a fully extended ϕ = ψ= 180° was considered as a starting structure and all atom amber99 force field parameters were employed. We varied the dielectric constant ε = 80 for water and ε=45 for DMSO-d_6, during simulation runs in different explicit solvent conditions as different solvent environments are characterized primarily by their dielectric properties. Truncated Newton method was used to energy minimize the input structure having automated force field and automated precondition option with RMSD cut off of 0.001 kcal/mol/Å. Minimized coordinates were subjected to molecular dynamics for 5 ns at 310 K, with a snap shot saved at every 5 ps. The resultant 1000 structures for each peptide (under each dielectric) were analyzed for their conformational preferences. MD simulation results in different dielectric constants were tabulated in **Table-1**.

NMR experimental methods

Peptide solutions were prepared to a concentration of ~3.5mg in 0.6ml of water: D_2O (9:1) and DMSO-d_6. Both 1D and 2D ^{1}H NMR experiments were carried out in DMSO-d_6 at 25°C with 16 scans on Brucker advance II 400 NMR spectrometer available at SAIF, Panjab University, Chandigarh. Standard pulse sequences were applied for TOCSY and ROESY experiments. TOCSY experiments were recorded for a period of up to 1hour with 16 scans with mixing time 80ms and ROESY experiments were recorded for a period up to 3 hours with 16 scans using with mixing time 250ms with mlevph pulse program. The 1D ^{1}H NMR spectrum of Ac-RGD and its analogs were shown in supplementary material **Figure 3S**. From the 1D spectrum, the observed ^{1}H NMR parameters of NH resonances for peptides in water and DMSO-d_6 are summarized in **Table-2** and **Table-3** respectively. Resonance assignment of the peptides were done by standard procedure developed by Wüthrich et al [46]. Combined use of TOCSY and ROESY helped in the complete resonance assignment of the spin systems. TOCSY spectra in DMSO-d_6 is depicted in **Figure 4S**. Since peptides were synthesized with N-terminal acetyl, the first residue Arg_1 has ROESY cross peaks with N-terminal acetyl. The sidechain proton resonances were identified by TOCSY connectivities. The TOCSY spectrum shows proton–proton cross peaks related to correlations between NH, H^{α} and side chain protons. Arg is characterized by the $C^{\alpha}H$, $C^{\delta}H$, $C^{\beta}H$ and $C^{\gamma}H$ connectivities and here also $C^{\delta}H$ will have characteristic downfield shift value than the $C^{\beta}H$ and $C^{\gamma}H$ value. From NMR experiments all $3J_{NH\text{-}C\alpha H}$ coupling constants were measured for Ac-RGD analogs in water and DMSO-d_6 are summarized in **Table-2** and **Table-3** respectively**.** Calculated ϕ torsion angle by using Karplus equation [47] from the $^3J_{HN\alpha}$ values and observed torsion angle ϕ from NMR for all the residues found to be in good agreement with MD simulation data.

One dimensional variable temperature NMR studies

The variable temperature ^{1}H NMR studies were performed in DMSO-d_6 with 16 scans on JEOL ECX-300MHz NMR spectrometer available at Institute of Microbial Technology (IMTECH), Chandigarh. For all the peptides the temperature coefficients ($d\delta/dT$) of the backbone and Arg_1-$N^{\varepsilon}H$ were calculated by analyzing the chemical shift at five different temperatures (298, 308, 318, 328 and 338K) over the range of 298-338K. The FIDs were acquired after the desired temperature was stabilized for an additional 5 minutes. The variation in ^{1}H chemical shift values at different temperatures were measured from 1D spectrum depicted in **Figure 1** and tabulated in **Table-3**. The minimum energy conformers of Ac-RGD and Ac-RXD analogs were shown in **Figure 2**.

RESULTS AND DISCUSSIONS

Conformational preferences of Ac-RGD analogs in water

From MD simulation results, native peptide **1** conformation in water is characterized by averaged main chain torsion angle Arg ϕ_1 = -138.2° and ψ_1 = - 6.4° occupying the α_R region with semi-folded conformation where as Gly ϕ_2 = 53.7° and ψ_2 = - 1.0° occupying the α_L region and the terminal Asp has a semi-extended conformation with ϕ_3 = -137°.Overall, the peptide backbone experience a semi-folded turn like topology characterized by $N_iH^{\cdots}N_{i+2}$ interaction. Based on the preliminary condition of the bioactivity of the RGD peptides, *pdo* angle ~ (−45° ≤ *pdo* ≤ +45°)[26], we analyzed the 1000 simulated structure of the native peptide **1** in water and analyzed pdo_1 comprising $\phi(C^{\beta}(Arg)\text{-}C^{\alpha}(Arg)\text{-}C^{\alpha}(Asp)\text{-}C^{\beta}(Asp))$ and pdo_2 $\phi(C\zeta(Arg)\text{-}C^{\alpha}(Arg)\text{-}C^{\alpha}(Asp)\text{-}C^{\gamma}(Asp)$ atoms. The side chain of Arg (λ_1) prefers +gauche (g^+) ~60°(major) and trans (t) ±160° (minor) conformations. The terminal Asp (λ_3) residue side chain prefers –gauche (g^-) -60°(major) and +gauche

(g^+) ~60°(minor) conformations. Out of the 1000 ensembles, 657 and 600 conformations found fit in the above criteria for *pdo*$_1$ and *pdo*$_2$ which justify its activity. The average interatomic distances between Arg C^α- C^αAsp, Arg C^β- C^βAsp and Arg C^ζ- C^γAsp in this peptide found to be 6.6, 6.0, 6.6Å respectively and these observations are similar to that observed by Kostidis *et al*. [26] From NMR studies, semi-folded state of Arg is characterized by the $3J_N\alpha$ value 7.92Hz. The equal $3J_{N\alpha1}$ and $3J_{N\alpha2}$ of 5.88Hz of Gly indicate that Gly residue undergoes free rotation about N-C^α bond [48]. Proton resonance at 7.06ppm is indicative of non-hydrogen bonded state of $N_{\eta2}H_4$ [49]. The resonance at ~12.0ppm confirms the presence of protonated state of state of Asp-β-carboxylic acid in water. Peptide **2** in water adopts a similar conformation as that of Ac-RGD with opposite orientations and its main chain conformation is characterized by the Arg ϕ_1 =137.7°, ψ_1=-11.8°, Gly ϕ_2= -46.7°, ψ_2= 6.1° and Asp ϕ_3= -135.5° and side chains λ1 and λ3 favor major g^- conformations. This semi-folded conformation is stabilized by an intramolecular hydrogen bond between Arg $N^\varepsilon H\cdots O=C^\gamma$-Asp (distance 2.87Å) which also experience a similar semi-folded turn like topology as of the native peptide. For peptide **2** from 1000 ensembles only 130 and 170 conformations found fit in the above criteria for *pdo*$_1$ and *pdo*$_2$ which substantiate its inactivity, though the interatomic distances between Arg C^α- C^αAsp, Arg C^β- C^βAsp and Arg C^ζ- C^γAsp in this peptide found to be 7.1, 6.0, 6.3Å respectively. We also observed the similar folding behavior of Arg $3J_N\alpha$ 7.92Hz. The $3J_{N\alpha1}$ and $3J_{N\alpha2}$ 5.96 and 6.16Hz of Gly indicate that the free rotation slightly restricted about N-C^αbond than the native peptide.

MD simulation studies the peptide **3** conformation in water is characterized by Arg ϕ_1 = -138.2°, ψ_1 = 2.9°, Gly ϕ_2 = 53.2°, ψ_2 = -10.5° similar to that observed in peptide 1 with opposite Asp ϕ_3 = 136.6° because of the D-isomer and side chains λ_1 and λ_3 favor major g^+ and t conformations respectively. This peptide is stabilized by an intramolecular hydrogen bond between Gly $NH\cdots O=C^\gamma$-Asp (distance 2.72Å). For peptide **3** from 1000 ensembles only 99 and 112 structures are found to be fit in the above criteria for *pdo*$_1$ and *pdo*$_2$ which provide evidence that this peptide is inactive. The interatomic average distances between Arg C^α- C^αAsp, Arg C^β- C^βAsp and Arg C^ζ- C^γAsp in this peptide found to be 6.6, 6.0, 6.0Å respectively. NMR experiments revealed that this peptide folded Arg $3J_N\alpha$ 7.92Hz and freely rotated N-C^α bond of Gly having $3J_{N\alpha1}$ and $3J_{N\alpha2}$ 5.80 and 5.84Hz respectively. This peptide's inability to bind integrins may be attribute to its inability to fit into the pseudo-dihedral angle and occurrence of Gly $NH\cdots O=C^\gamma$-Asp intramolecular hydrogen bonding interaction. Peptide **4** in water also adopts a semi-folded conformation with Arg ϕ_1 = 139.2°, ψ_1 = 9.7°, Gly ϕ_2 = -71.1°, ψ_2 = 2.0°, Asp ϕ_3 = 136.8° and side chains λ_1 and λ_3 favor major g^- and g^+ conformations respectively. There is no such hydrogen bonding was observed in this case but surprisingly from 1000 MD simulated structure, 734 and 584 conformations found fit in the Kostidis, S. *et al*[26]criteria for *pdo*$_1$ and *pdo*$_2$ which corroborate for its better activity. The interatomic average distances between Arg C^α-C^αAsp, Arg C^β-C^βAsp and Arg C^ζ-C^γAsp in this peptide found to be 6.9, 6.0, 6.5Å respectively. NMR experiments revealed that this peptide folded Arg $3J_N\alpha$ 7.84Hz and freely rotated N-C^α bond of Gly having $3J_{N\alpha1}$ and $3J_{N\alpha2}$ 5.76 and 5.76Hz respectively similar to that of native peptide **1.**

In β-Ala containing peptides, the new torsion angle μ (C^β-C^α) is capable of adopting a compact folded *syn* μ ~0±20° to a tightly folded *gauche* μ ~±60±20° to a semi-extended *skew* ~±120±20° to a fully extended trans μ ~±180±20° orientation [50]. In this study, β-Ala analog peptide **5** in water experience similar main chain conformation as seen in native peptide **1** with main chain averaged torsion angle Arg ϕ_1 = -141.5° and ψ_1 = - 3.5° occupying the α_R region with semi-folded conformation whereas β-Ala occupied highly folded topology with ϕ_2 = 55.9°, *gauche* μ2 = ±60±10, ψ_2 = 23.0° and the terminal Asp has a semi-extended conformation with ϕ_3 = -134.2° and the side chain λ_1 is restricted only to g^+ and the terminal λ_3 occupying different orientation g^-, t and g^+. We also observed a weak intermolecular hydrogen bonding interaction between Arg $N^\varepsilon H\cdots O=C^\gamma$-Asp (distance 3.17Å). From 1000 structure, only 301 and 366 conformations found fit in the above criteria for *pdo*$_1$ and *pdo*$_2$ which may act better than the peptide **2** and **3**, The observed interatomic average distances between Arg C^α-C^αAsp, Arg C^β-C^βAsp and Arg C^ζ-C^γAsp in this peptide found to be 6.9, 7.2, 5.3Å respectively. From NMR parameters high value of Arg $3J_N\alpha$ 11.2Hz refers to extended ϕ = ±150±10°, β-Ala having $3J_N\alpha$ 6.84Hz and Asp $3J_N\alpha$ 7.80Hz justifies the conformation obtained from MD simulations. Peptide **6** in this environment shows averaged torsion angle Arg ϕ_1 = 139.3°, ψ_1 = 2.9°, β-Ala ϕ_2 = -57.9°, μ2 = *gauche* ±60±10°, ψ_2 = 17.6° Asp ϕ_3 = 134.9° and side chains λ_1 restricted to g^- and λ_3 g^+ and t conformations. From the simulated structures only 301 and 366 structures are found to in the fit in the above criteria for *pdo*$_1$ and *pdo*$_2$. The observed interatomic average distances between Arg C^α-C^αAsp, Arg C^β-C^βAsp and Arg C^ζ-C^γAsp in this

peptide found to be 6.6, 7.0, 5.5Å respectively. NMR parameter $3J_N\alpha$ of Arg, β-Ala and Asp residues 11.2, 6.80 and 7.84Hz respectively validate conformation obtained by MD simulation.

Conformational preferences of Ac-RGD analogs in DMSO-d_6

In this solvent peptide **1** conformation is characterized by averaged main chain torsion angle Arg ϕ_1 = -140.6° and ψ_1 = - 8.6° occupying the α_R region with semi-folded conformation where as Gly ϕ_2 = 81.7° and ψ_2 = - 1.3° occupying the α_L region and the terminal Asp has a semi-extended conformation with ϕ_3 = -135°. The semi-folded β-turn like conformation is stabilized by strong Arg-$N^{\varepsilon}H\cdots O=C^{\gamma}$-Asp hydrogen bonding (distance 2.67Å) interaction. This observation is also established from the NMR temperature coefficient experiment $d\delta/\delta T$ = -0.00260ppm/K for Arg-$N^{\varepsilon}H$ is characteristic of strong hydrogen bonding interaction (REF). Here also 716 and 554 conformations are and found to be fit for *pdo*$_1$ and *pdo*$_2$ respectively. The average interatomic distances between Arg C^{α}- C^{α}Asp, Arg C^{β}- C^{β}Asp and Arg C^{ζ}- C^{γ}Asp in this peptide found to be 6.6, 6.0, 6.5Å respectively.[26]. From NMR studies, semi-folded state of Arg is characterized by the $3J_N\alpha$ value 7.76Hz. In this solvent Gly residue also undergoes free rotation about N-C^{α} bond having 5.60Hz values for both$3J_{N\alpha 1}$ and $3J_{N\alpha 2}$[48]. Lower resonance at7.17Hz of Arg-$N^{\varepsilon}H$ than other analog also testifies the hydrogen bonded state. The resonance at ~12.0ppm confirms the presence of protonated state of state of Asp-β-carboxylic acid in DMSO-d_6. The side chain λ_1 prefers only g^+ conformations and terminal λ_3 side chain prefers g^+, g^-, t conformations. The observance of strong Arg-Gly (RG) and Gly-Asp (GD) N_iH-$N_{i+1}H$ and $N_{i+1}H$-$N_{i+2}H$ ROESY peaks **Figure 3(A)** clearly demonstrates the turn/folded conformation of this peptide.

Simulation results infers Peptide **2** adopt a folded turn like topology characterized by Arg ϕ_1 =135.8°, ψ_1= 4.0°, Gly ϕ_2= -35.2°, ψ_2= 10.2° and Asp ϕ_3= -129.5° and side chains λ1 and λ3 favor g^+, g^-, t and g^-, g^+,t conformation respectively. 152 and 118 conformations are and found to be fit for *pdo*$_1$ and *pdo*$_2$ respectively for this peptide. The average interatomic distances between Arg C^{α}- C^{α}Asp, Arg C^{β}- C^{β}Asp and Arg C^{ζ}- C^{γ}Asp in this peptide found to be 7.0, 6.0, 6.9Å respectively. Observed NMR parameters for this peptide correlates with the MD simulation results and the absence of hydrogen bonding interaction is proved from the temperature coefficient experiment **Table-3**. We also observed RG and GD ROESY medium peaks **Figure 3(B)** characterize the turn/semi-folded conformation of this peptide. Similarly Peptide **3** adopts similar folded conformation characterized by Arg ϕ_1 =-140.6°, ψ_1= -2.5°, Gly ϕ_2= 71.9°, ψ_2=-13.0° and Asp ϕ_3= 136.8° and side chains λ_1 favors only g^+ and λ_3 prefers g^-, g^+,t. This folded topology is stabilized by a Arg-$N^{\varepsilon}H\cdots O=C^{\gamma}$-Asp hydrogen bonding (distance 2.37Å) interaction and validated in NMR results $d\delta/\delta T$ = -0.0031ppm/K for Arg-$N^{\varepsilon}H$. In this case only 51 and 111 conformations are found to be fit for *pdo*$_1$ and *pdo*$_2$. The average interatomic distances between Arg C^{α}- C^{α}Asp, Arg C^{β}- C^{β}Asp and Arg C^{ζ}- C^{γ}Asp in this peptide found to be 6.6, 6.0, 5.8Å respectively. RG and GD ROESY medium peaks **Figure 3(C)** distinguish the turn/semi-folded conformation of this peptide. Simulation results for peptide **4** infers folded conformation with Arg ϕ_1 =139.7°, ψ_1= 9.9°, Gly ϕ_2= -85.8°, ψ_2=5.1° and Asp ϕ_3= 141.3° and side chains λ_1 favors g^-, g^+ and λ_3 prefers g^-, g^+,t exactly what was observed in water but in this solvent the peptide is stabilized by Arg-$N^{\varepsilon}H\cdots O=C^{\gamma}$-Asp hydrogen bonding interaction (distance 2.72 Å) with NMR validated an interaction $d\delta/\delta T$ = -0.00332ppm/K for Arg-$N^{\varepsilon}H$. Surprising results in water is also found at par in this solvent also with 729 and 644 conformations are found to be fit for *pdo*$_1$ and *pdo*$_2$ which confirmed that this peptide will have better efficacy than the native analog. The average interatomic distances between Arg C^{α}- C^{α}Asp, Arg C^{β}- C^{β}Asp and Arg C^{ζ}- C^{γ}Asp in this peptide found to be 7.0, 6.0, 6.44Å respectively. Here we also observed strong RG and GD ROESY cross peaks **Figure 3(D)** clearly established the turn/folded conformation of this peptide as that of native peptide **1**.

β-Ala analog Peptide **5** in DMSO-d_6 behave similar way as observed in water characterized by Arg ϕ_1 = -134.3°, ψ_1 = 8.2°, β-Ala ϕ_2 = 41.2°, μ_2 = *gauche* ±60±10°, ψ_2 = 7.4° Asp ϕ_3 = -136.3° and side chains λ_1 restricted to g^- ,t and λ_3 g^+ g^-,t conformations. In this case 408 and 477 conformations are found to be fit for *pdo*$_1$ and *pdo*$_2$. The observed interatomic average distances between Arg C^{α}-C^{α}Asp, Arg C^{β}-C^{β}Asp and Arg C^{ζ}-C^{γ}Asp in this peptide found to be 6.9, 6.7, 5.8Å respectively. NMR experimental results Arg $3J_N\alpha$ 8.36Hz refers to extended ϕ = ±140±10°, β-Ala having $3J_N\alpha$ 5.0Hz justify the *gauche* and helical orientation and Asp $3J_N\alpha$ 8.16Hz validate ϕ_3 = -136.3° conformation obtained from MD simulations. This conformation is stabilized by a weak hydrogen bonding interaction between Arg-$N^{\varepsilon}H\cdots O=C^{\gamma}$-Asp (distance 3.03 Å) and corroborate with the NMR observation of $d\delta/\delta T$ = -0.00317ppm/K for Arg-$N^{\varepsilon}H$. But the absence of Arg-β-Ala (RX) and β-Ala-Asp(XD) ROESY peaks confirmed that the overall main chain conformation of the peptide is not folded. Peptide **6** in this

ISSN: 0975-8585

solvent behaves differently than that observed in water and distinguished by Arg ϕ_1 = 134.5°, ψ_1 = -5.2°, β-Ala ϕ_2 = -44.6°, μ_2 = *gauche* ±60±10°, ψ_2 = -56.0° Asp ϕ_3 = 133.4° and side chains λ_1 restricted to g^-, g^+, t and λ_3 g^+,t conformations. In this case 442 and 405 conformations are found to be fit for pdo_1 and pdo_2. The observed interatomic average distances between Arg C^{α}-C^{α}Asp, Arg C^{β}-C^{β}Asp and Arg C^{ζ}-C^{γ}Asp in this peptide found to be 6.6, 7.4, 5.6Å respectively. NMR results substantiate the MD results with the observation of Arg $3J_N\alpha$ 8.44Hz, β-Ala $3J_N\alpha$ 4.6Hz and Asp $3J_N\alpha$ 7.88Hz. This conformation is stabilized by a hydrogen bonding interaction between Arg-$N^{\varepsilon}H\cdots O=C^{\gamma}$-Asp (distance 2.56 Å) and support the NMR observation of $d\delta/\delta T$ = -0.0031ppm/K for Arg-$N^{\varepsilon}H$. Absence of RX and XD ROESY peaks confirmed that the overall main chain conformation of the peptide is not folded.

Table 1: Average torsion angles preference (°) from Molecular Dynamics simulation for Ac-RGD-OH and its analogs in different dielectric constants

Dielectric Constant	Peptide*	ϕ_1	ψ_1	λ_1	ϕ_2	μ_2	ψ_2	ϕ_3	λ_3
80	**1**	-138.2	-6.4	+g, -g, t	53.7	--	-1.0	-137.0	-g, t, +g
	2	137.7	-11.8	-g, t, +g	-46.7	--	6.1	-135.5	-g, +g, t
	3	-138.2	2.9	+g, t, -g	53.2	--	-10.5	136.6	t, +g, -g
	4	139.2	9.7	-g	-71.1	--	2.0	136.8	+g, t, -g
	5	-141.5	-3.5	+g	55.9	±60.0±10	23.0	-134.2	-g, t, +g
	6	139.3	2.9	-g	-57.9	±60.0±10	17.6	134.9	+g, t
45	**1**	-140.6	-8.6	+g	81.7	--	-1.3	-135.0	+g, -g, t
	2	135.8	4.0	+g, -g, t	-35.2	--	10.2	-129.5	-g, +g, t
	3	-140.6	-2.5	+g	71.9	--	-13.0	136.8	-g, +g, t
	4	139.7	9.9	-g, +g	-85.8	--	5.1	141.3	-g, +g, t
	5	-134.3	8.2	-g, t	41.2	±60.0±10	7.4	-136.3	+g, -g, t
	6	134.5	-5.2	-g, +g, t	-44.6	±60.0±10	-56.0	133.4	+g, t

*1: Ac-RGD-OH, 2. Ac-rGD-OH, 3.Ac-RGd-OH, 4.Ac-rGd-OH, 5.Ac-RXD-OH, 6.AC-rXd-OH

Table 2: ^{1}H NMR Chemical shifts (δ) of Ac-RGD and its analogs in H_2O:D_2O (90:10)

Peptide	Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	3J (Hz)	Others
1	Arg	8.12	4.13	1.72 1.61	7.92	$C^{\gamma}H$ 1.51, $C^{\delta}H$ 3.06, $N^{\varepsilon}H$ 7.06
	Gly	8.41	3.81		5.88, 5.88	
	Asp	8.25	4.61	2.80	6.44	
2	d-Arg	8.11	4.14	1.71 1.61	7.92	$C^{\gamma}H$ 1.51, $C^{\delta}H$ 3.06, $N^{\varepsilon}H$ 7.06
	Gly	8.39	3.81		6.16, 5.96	
	Asp	8.25	4.59	2.80	3.64, 2.6	
3	Arg	7.91	4.15	1.73, 1.62	7.92	$C^{\gamma}H$ 1.43, $C^{\delta}H$ 3.07, $N^{\varepsilon}H$ 6.90
	Gly	8.22	3.82		5.80, 5.84	
	d-Asp	8.09	4.60	2.80	6.52	
4	d-Arg	8.09	4.16	1.74, 1.64	7.84	$C^{\gamma}H$ 1.57, $C^{\delta}H$ 3.08, $N^{\varepsilon}H$ 7.06
	Gly	8.41	3.83		5.76, 5.76	
	d-Asp	8.25	4.59	2.80	6.36	
5	Arg	8.02	4.07	1.67, 1.60	11.2	$C^{\gamma}H$ 1.52, $C^{\delta}H$ 3.06, $N^{\varepsilon}H$ 7.07
	β-Ala	8.14	3.34		6.84	
	Asp	8.31	4.58	2.80	7.80	
6	d-Arg	8.04	4.07	1.65, 1.57	11.2	$C^{\gamma}H$ 1.50, $C^{\delta}H$ 3.06, $N^{\varepsilon}H$ 7.09
	β-Ala	8.14	3.34		6.80	
	d-Asp	8.24	4.54	2.77	7.84	

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Table 3: Observed Chemical shift and temperature coefficients for Ac-RGD and its analogs in DMSO-d_6.

Peptide	Residue	NH	$C^{\alpha}H$	$C^{\beta}H$	Others	3J	dδ/dT (ppm/K)
Ac-RGD-OH	Arg	8.10	4.22	1.74	$C^{\gamma}H$ 1.49, $C^{\delta}H$ 3.08, $N^{\varepsilon}H$ 7.17	7.76	-0.00413 - 0.00260
	Gly	8.34	3.78,3.65			5.60, 5.60	-0.00473
	Asp	8.11	4.43	2.58,2.57		7.28	-0.00416
Ac-rGD-OH	d-Arg	8.10	4.23	1.75	$C^{\gamma}H$ 1.50, $C^{\delta}H$ 3.09, $N^{\varepsilon}H$ 7.21	7.6	-0.00468 - 0.00341
	Gly	8.35 8.29	3.75,3.68			5.64, 5.80	-0.00506 - 0.00536
	Asp	8.11	4.42	2.58,2.56		7.72	-0.00474
Ac-RGd-OH	Arg	8.07	4.24	1.74	$C^{\gamma}H$ 1.50, $C^{\delta}H$ 3.09, $N^{\varepsilon}H$ 7.20	7.56	-0.00454 - 0.0031
	Gly	8.32	3.77,3.65			5.76, 5.92	-0.00505
	d-Asp	8.11	4.39	2.57, 2.55		7.76	-0.00486
Ac-rGd-OH	d-Arg	8.08	4.23	1.75	$C^{\gamma}H$ 1.50, $C^{\delta}H$ 3.07, $N^{\varepsilon}H$ 7.21	7.68	-0.00477 - 0.00332
	Gly	8.36	3.79,3.63			5.84, 5.84	-0.00534
	d-Asp	8.12	4.41	2.57,2.55		7.60	-0.00483
Ac-RXD-OH	Arg	8.00	4.26	1.67	$C^{\gamma}H$ 1.44, $C^{\delta}H$ 3.09, $N^{\varepsilon}H$ 7.22	8.36	-0.00518 - 0.00317
	β-Ala	8.05	3.19	2.25		5.0	-0.00524
	Asp	8.19	4.46	2.70, 2.67		8.16	-0.00603
Ac-rXd-OH	d-Arg	8.00	4.27	1.68	$C^{\gamma}H$ 1.46, $C^{\delta}H$ 3.08, $N^{\varepsilon}H$ 7.24	8.44	-0.00506 - 0.00310
	β-Ala	8.06	3.19	2.24		4.96, 5.40	-0.00524
	d-Asp	8.19	4.47	2.71, 2.65		7.88	-0.00582

Scheme-1 : Schematic representation of solid phase synthesis protocol for Ac-RGD-OH and its analogs.

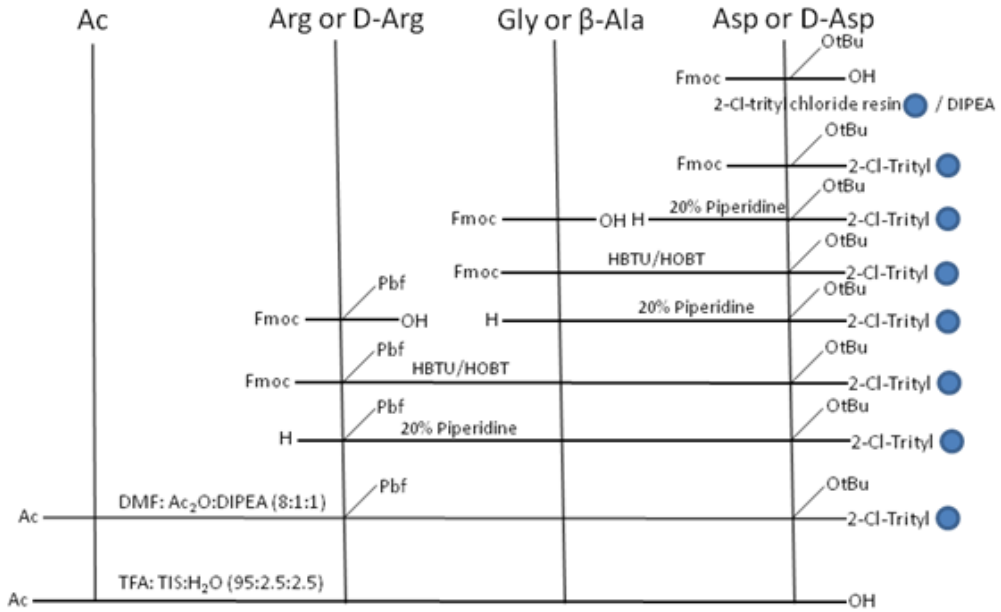

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Figure 1: Temperature coefficient experiments of (A) Ac-RGD-OH (B) Ac-rGD-OH, (C) Ac-RGd-OH, (D) Ac-rGd-OH, (E) Ac-RXD-OH, (E) Ac-rXd-OH in DMSO-d_6.

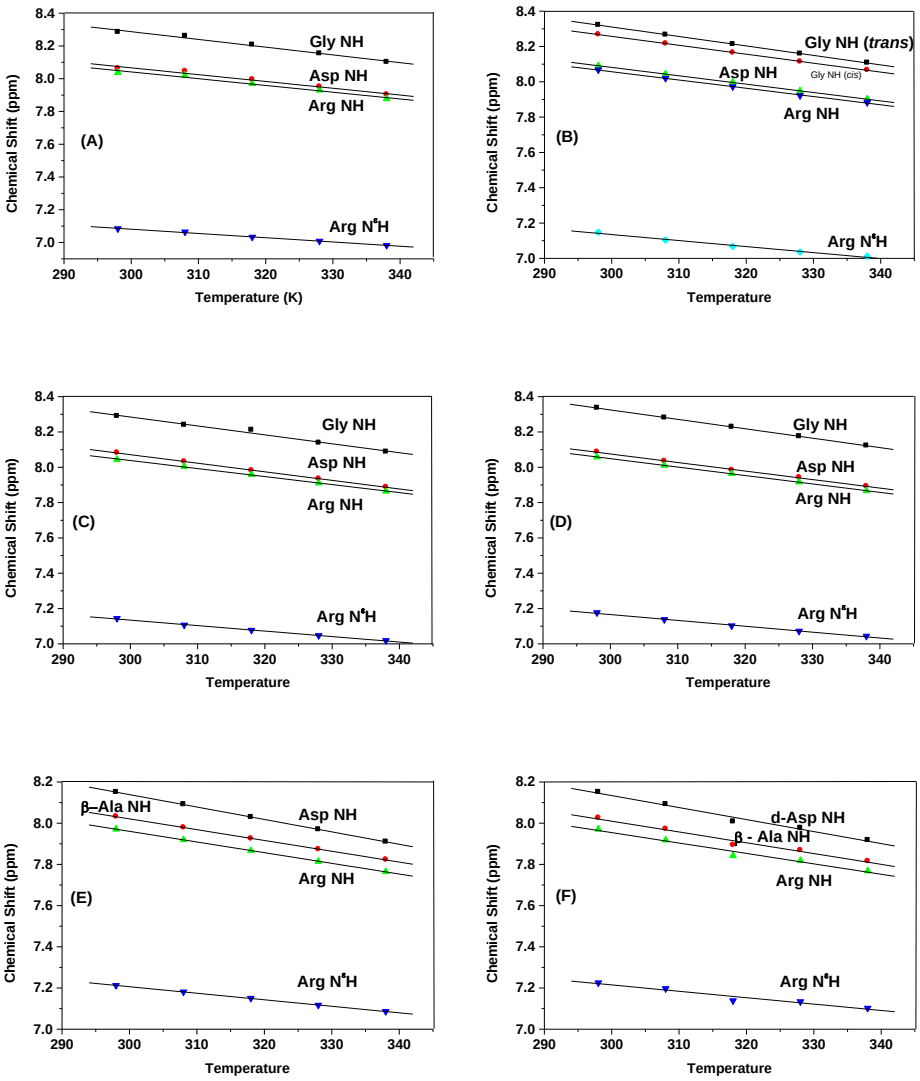
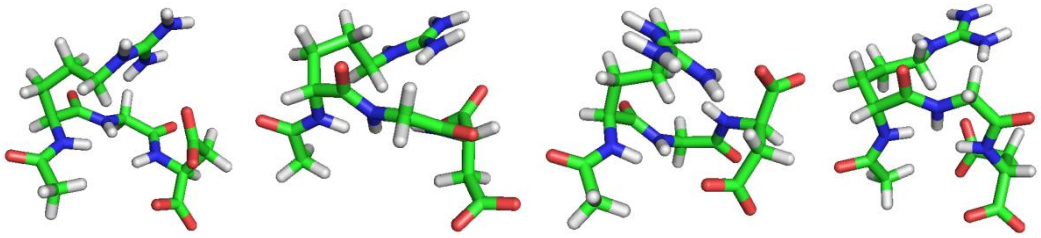


Figure 2: Minimum energy conformations of the Ac-RGD and its analogs (a)Ac-RGD-OH (b) Ac-rGD-OH, (c) Ac-RGd-OH, (d) Ac-rGd-OH in water (e) Ac-RGD-OH (f) Ac-rGD-OH, (g) Ac-RGd-OH, (h) Ac-rGd-OH in DMSO-d_6 (i) AC-RXD-OH, (j) Ac-rXD-OH, (k) Ac-RXd-OH,(l) Ac-rXd-OH in water and m)Ac-RXD-OH (n) Ac-rXD-OH, (o) Ac-RXd-OH, (p) Ac-rXd-OH in DMSO-d_6.



(a) (b) (c) (d)

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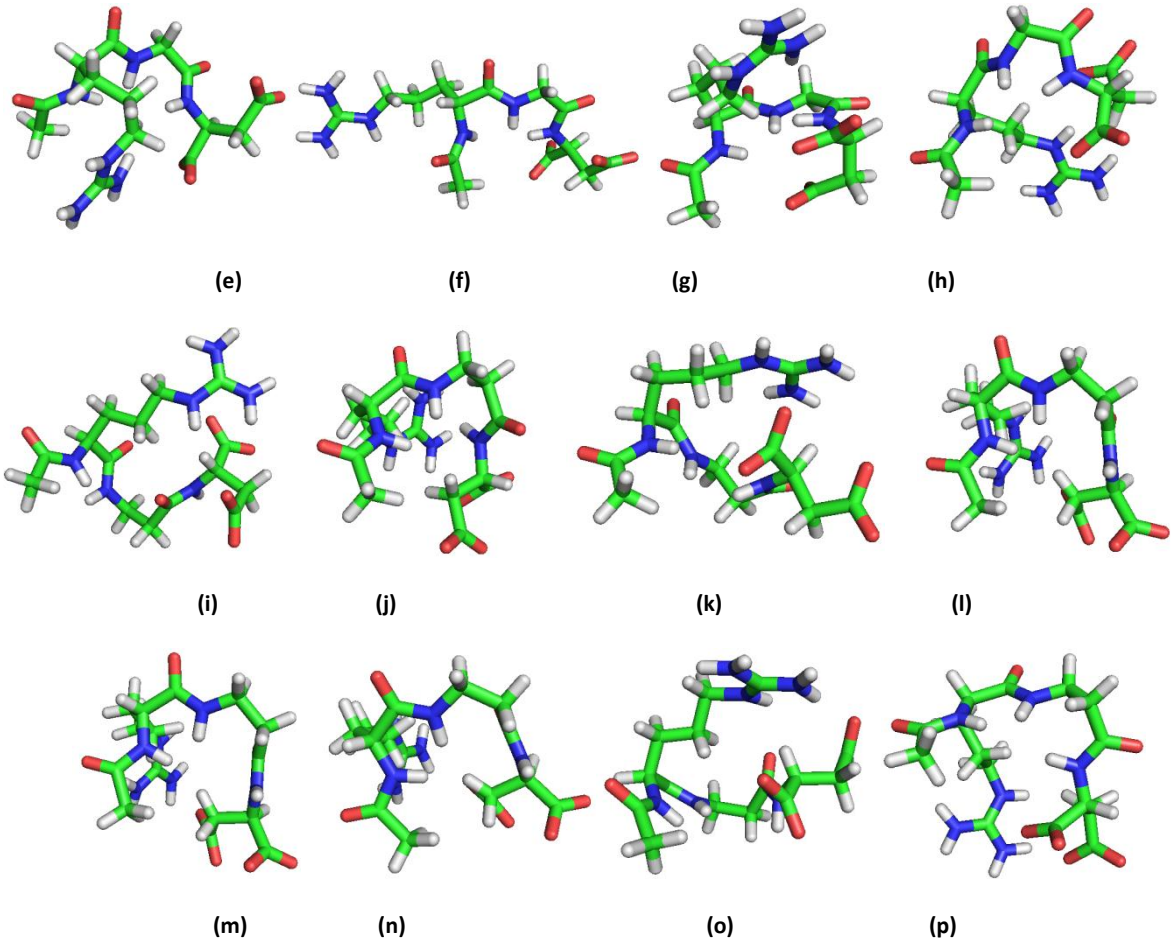
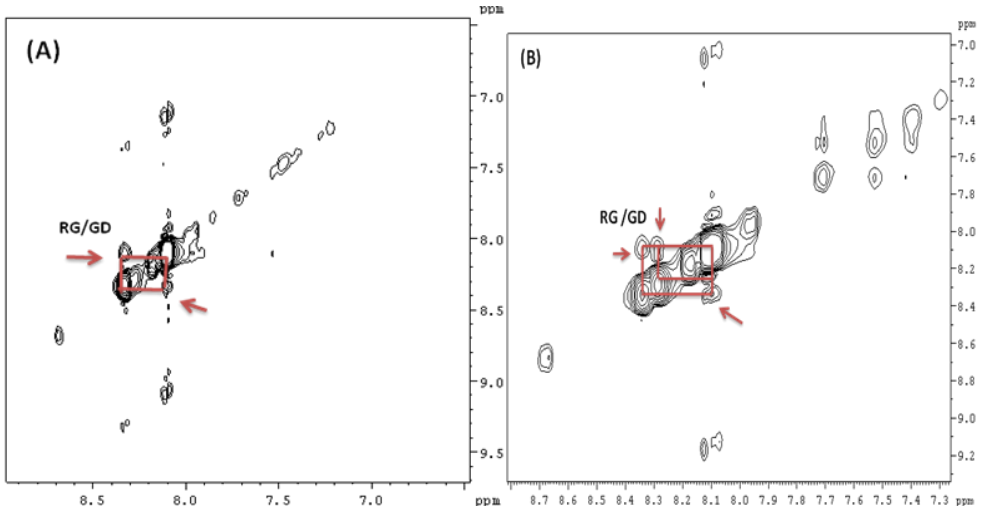


Figure 3: NMR ROESY spectrum NH region of (A) Ac-RGD (B) (C) Ac-RGd and (D) Ac-rGd (E) Ac-RXD and (F) Ac-rXd in DMSO-d_6 showing the Arg-Gly(RG) and Gly-Asp (GD) ROESY cross peaks in A, B, C and D characteristics of folding conformation and Arg-β-Ala/ β-Ala-Asp (RX/XD) ROESY cross peak is absent in E&F.



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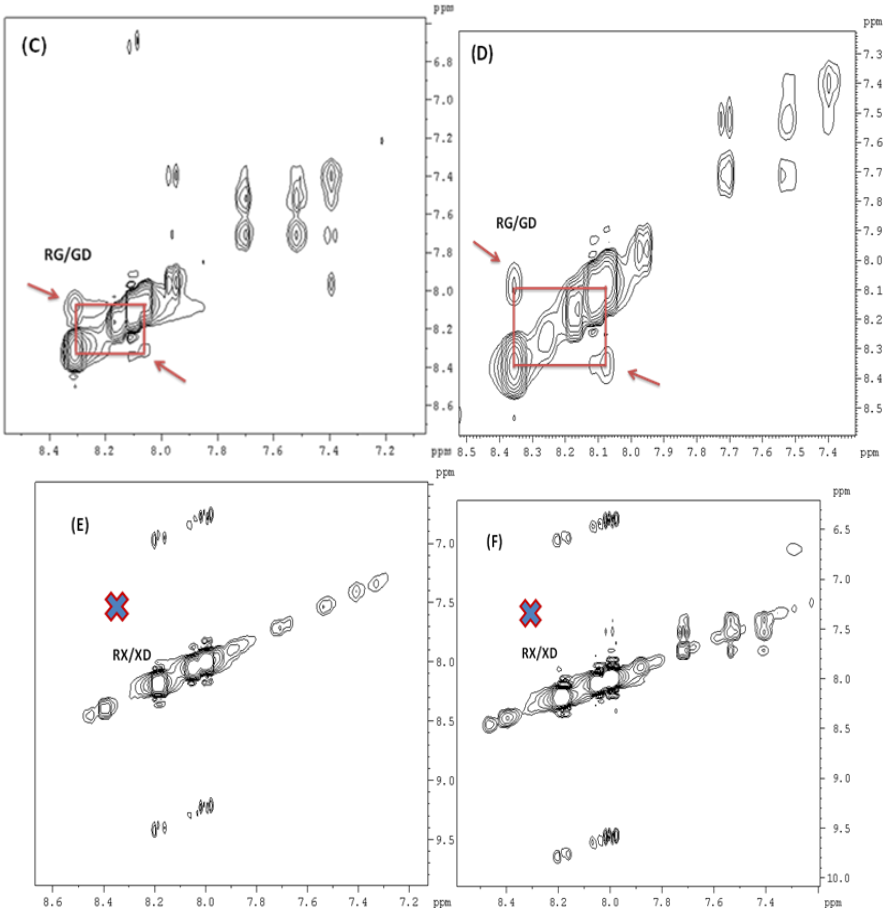

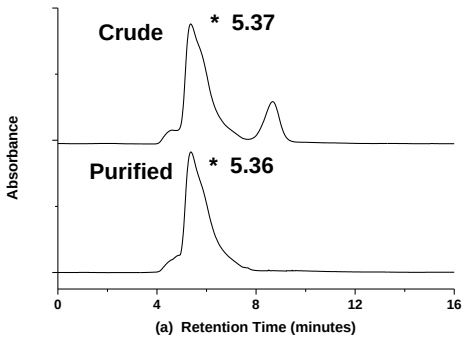

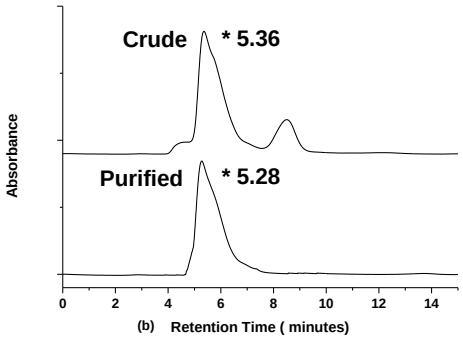

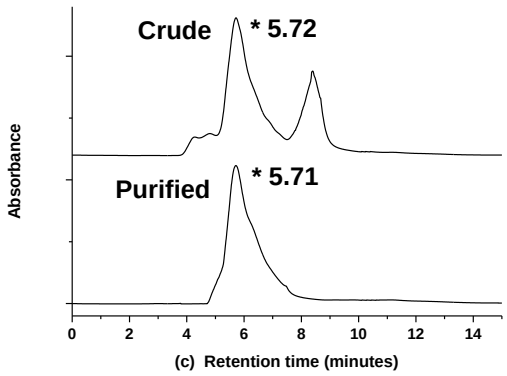

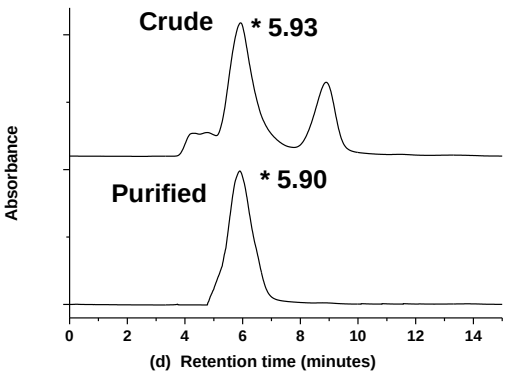

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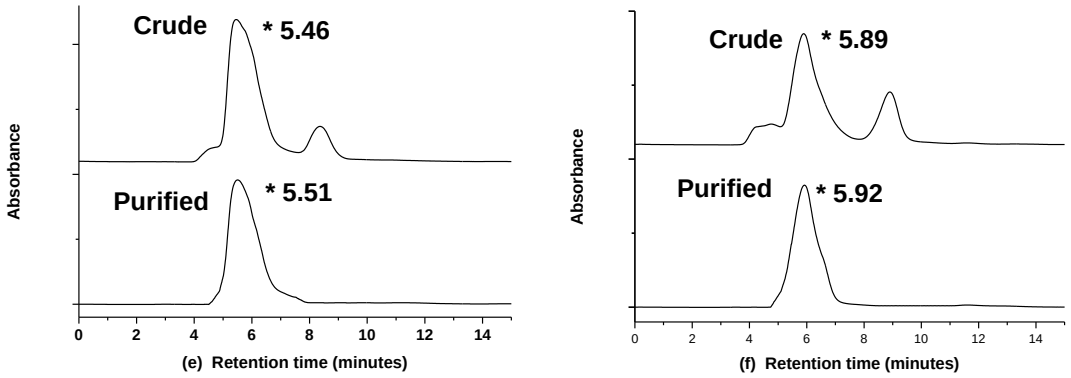

Figure 1S: HPLC profile of the (a) Ac-RGD, (b)Ac-rGD, (c) Ac-RGd, (d) Ac-rGd, (e) Ac-RXD and (f) Ac-rXd

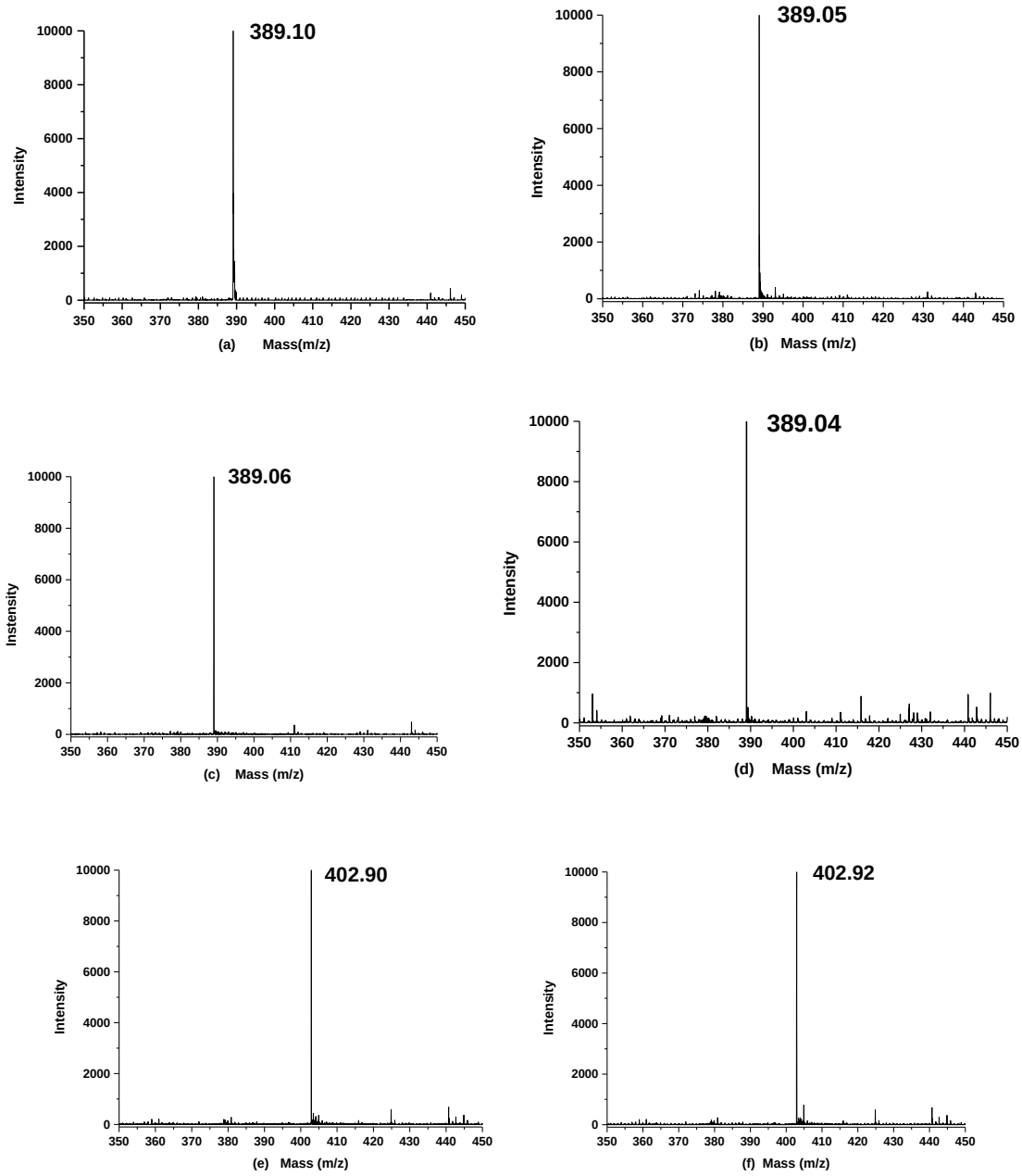

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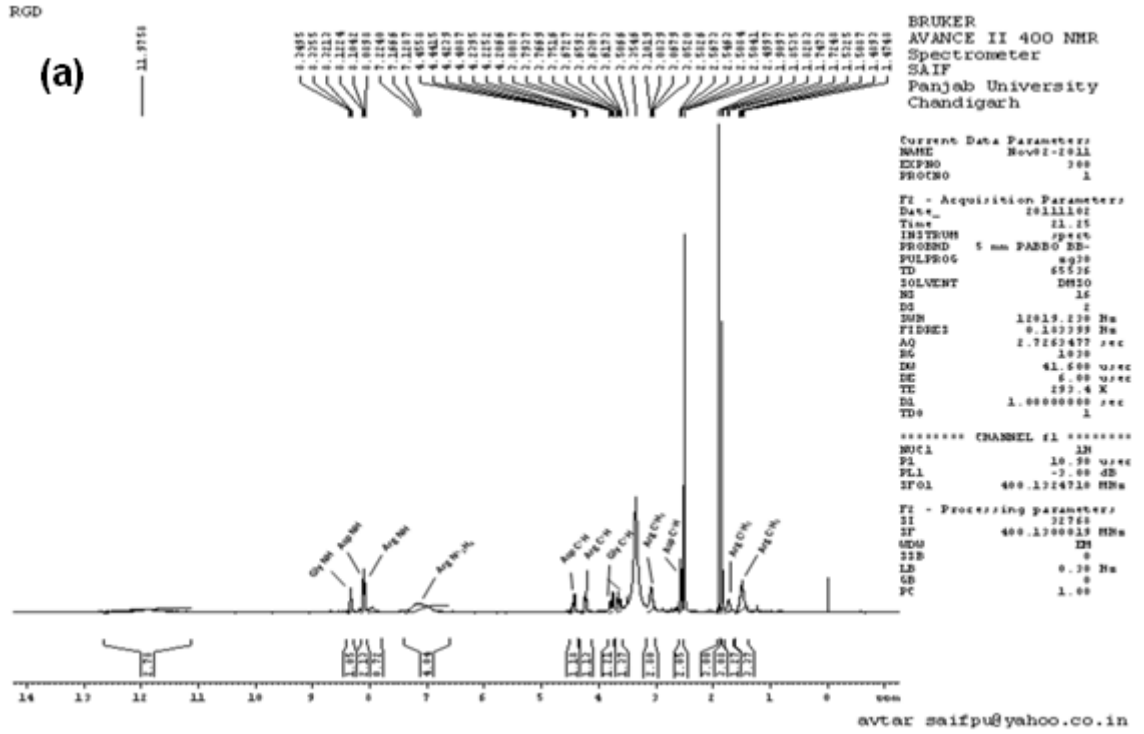
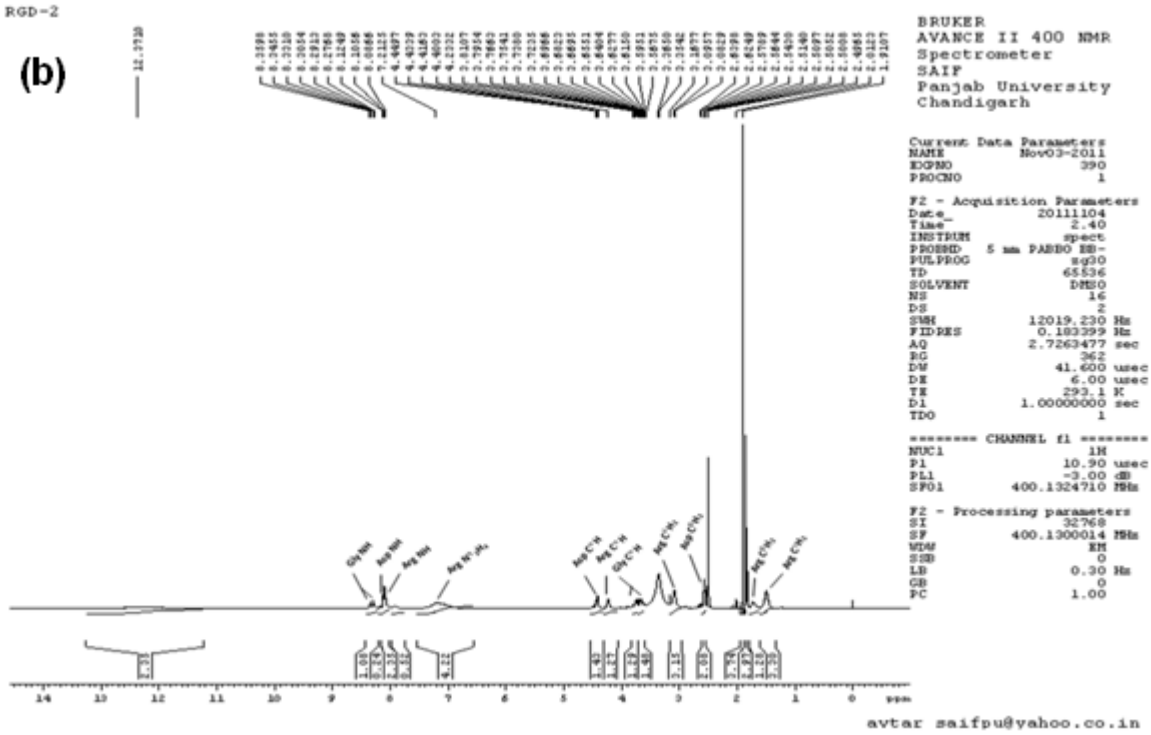
Figure 2S: MALDI-TOF mass spectra of the (a) Ac-RGD, (b)Ac-rGD, (c) Ac-RGd, (d) Ac-rGd, (e) Ac-RXD and (f) Ac-rXd





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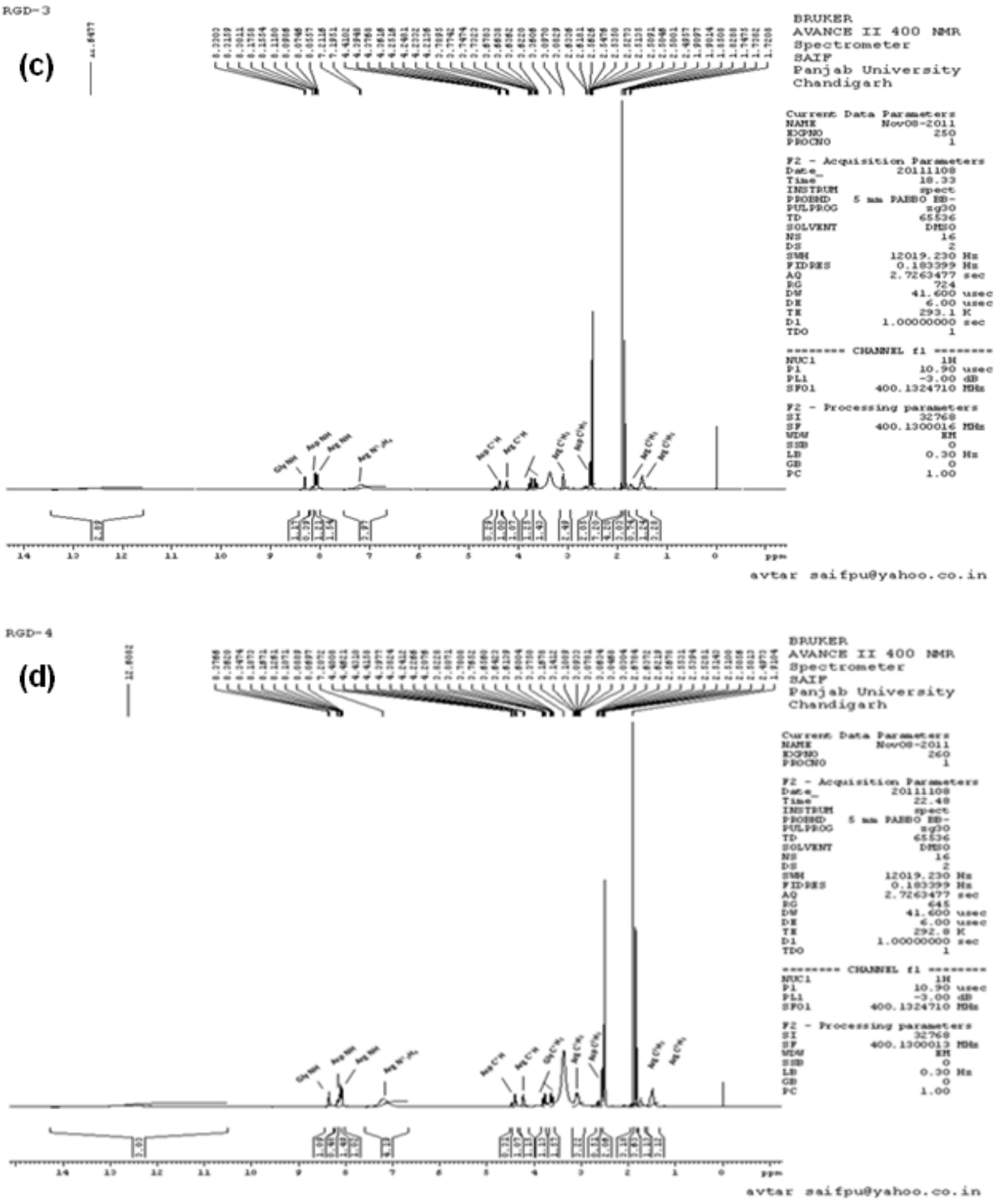

Figure 3S: Fully characterized ^{1}H NMR spectrum of (a) Ac-RGD, (b)Ac-rGD, (c) Ac-RGd, (d) Ac-rGd, (e) Ac-RXD and (f) Ac-rXd in DMSO-d_6

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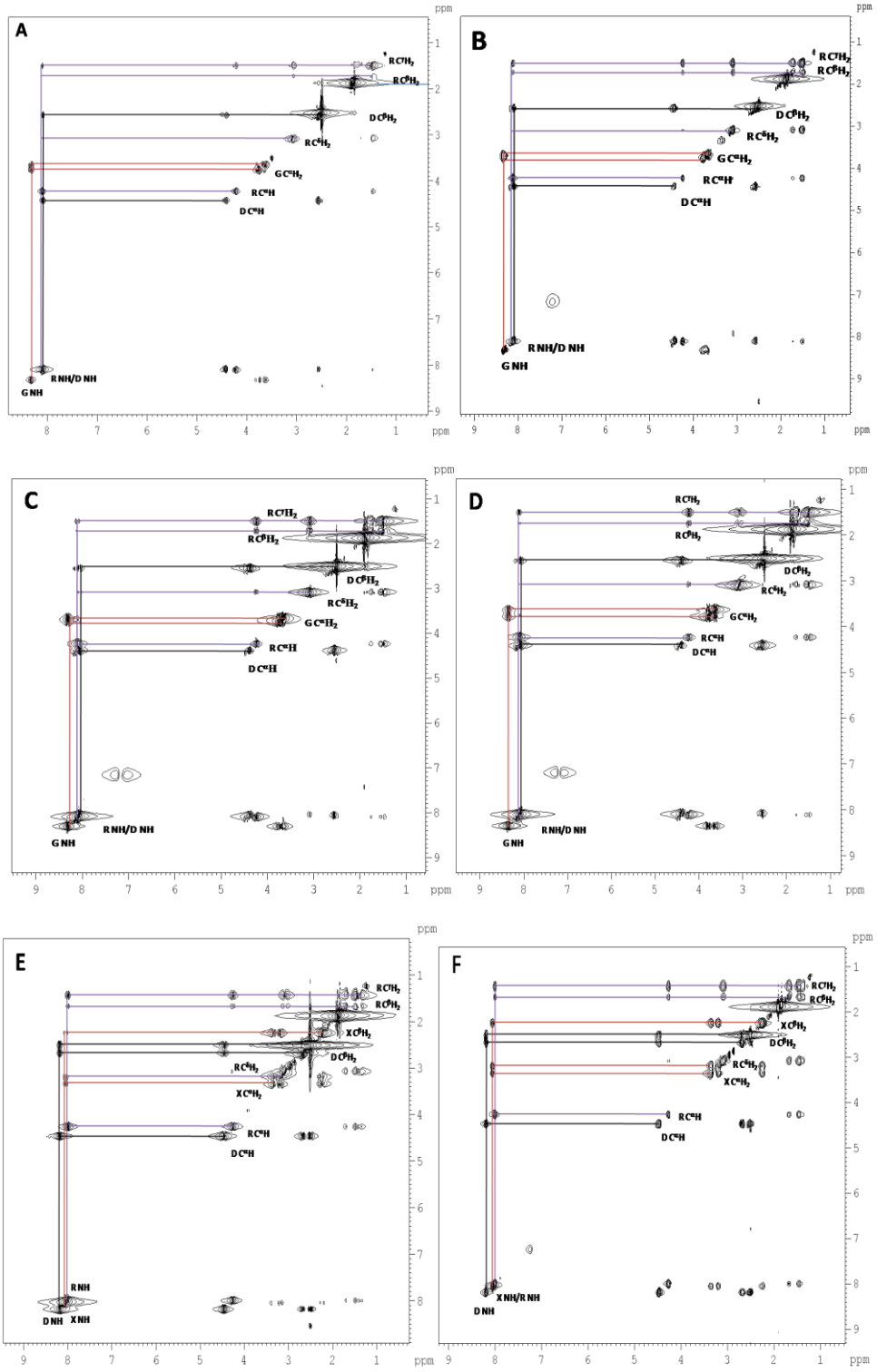

Figure 4S: Fully characterized TOCSY spectrum of (A) Ac-RGD OH (B) Ac-rGD-OH (C) Ac-RGd-OH (D) Ac-rGd-OH (E) Ac-RXD-OH (F) Ac-rXd-OH in DMSO-d_6

CONCLUSION

In this paper we described and compared the conformational preferences of Ac-RGD and Ac-RXD peptides in membrane mimicking solvents. MD simulations and experimental NMR results provide evidence for the unique conformational features of Ac-rGd, than the other analogs. This peptide experience a folded conformation in both the solvents and stabilized by Arg-$N^{\varepsilon}H\cdots O{=}C^{\gamma}$-Asp hydrogen bonding interaction in DMSO-

d_6. The prevalence of hydrogen bonding interactions were validated from the NMR temperature coefficients experiments. To enhance our understanding of the structure and function of RGD analogs we introduced another torsion angle μ between ϕ and ψ by incorporating β-Ala residue in between Arg and Gly, both MD simulation and NMR results suggest that though we were able to enhance the activity than the Ac-rGD and Ac-RGd, it could not achieve the same conformational features as that of Ac-rgD and Ac-RGD. From these results we conclude that Ac-rGd is a better integrin binding analog than the native peptide Ac-RGD by changing the chirality of both chiral residues and we also infer another conclusion that introduction of β-Ala in various bioactive compounds instead of Gly or any structural similar compound may unravel unique conformational features and thereby its activity.

ACKNOWLEDGEMENTS

Authors are grateful for the consistent encouragement and support from Director, IMTECH, faculty/staff of IMTECH and required financial support. Authors are also grateful to the Vice-Chancellor, faculty and staff of Banasthali University, Banasthali, Rajasthan for their support during the research work. Authors acknowledge the help from Mr Avtar Singh, SAIF, Panjab University, Chandigarh, India for carrying out NMR experiments. Authors also acknowledge the help of Mr. Chander Parkash Midha for temperature coefficient NMR experiments at IMTECH.

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