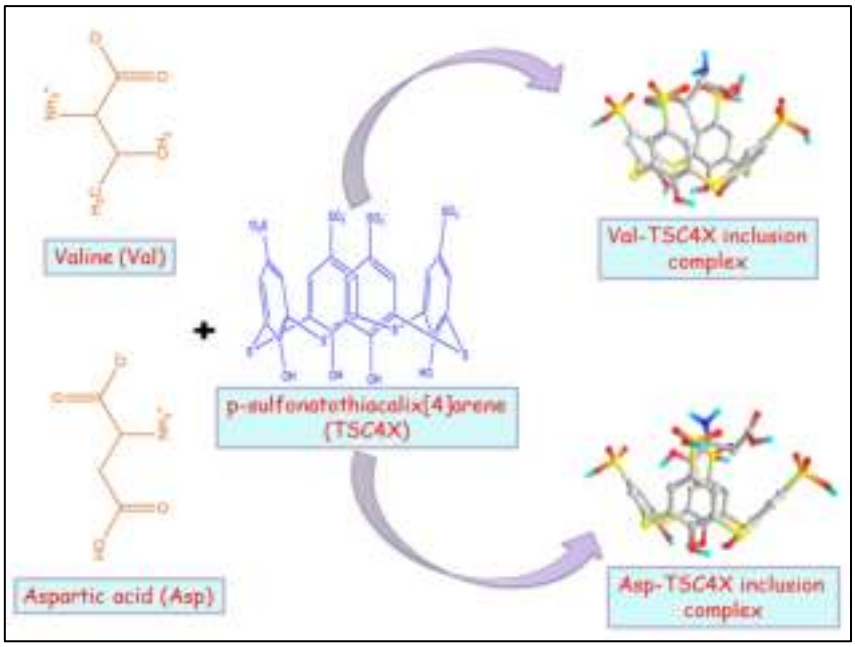
CHAPTER VII

Exploring Inclusion Complexes of Amino Acids with *p*-Sulfonatothiacalix[4]arene by Experimental and Computational Approach

Abstract : The supramolecular complexations of two natural amino acids, viz., L-Valine and L-Aspartic acid, with *p*-sulfonatothiacalix[4]arene have been investigated by means of DSC, SEM, PXRD, ESI-MS, ^{1}H NMR spectroscopy and molecular docking study. ^{1}H NMR spectral study suggested possible mode of molecular interactions, while ESI-MS analysis confirm that the inclusion complexes have been formed with 1:1 stoichiometry. Binding constants of the inclusion complexes were evaluated by NMR titrations, and the estimation of free energy of binding indicates the inclusion process to be thermodynamically feasible. PXRD and SEM image studies revealed the formation of inclusion complexes. DSC analysis showed that the thermal stability of amino acids has increased on encapsulation with *p*-sulfonatothiacalix[4]arene. Further, the molecular docking study presented the most stable binding orientation of L-Valine and L-Aspartic acid within the cavity of *p*-sulfonatothiacalix[4]arene.



Keywords : L-Valine ; L-Aspartic acid ; *p*-sulfonatothiacalix[4]arene ; Differential scanning calorimetry ; Powder X-ray diffraction ; Molecular docking.

VII.1. Introduction

Calixarenes, in supramolecular chemistry, are one of the most important classes of macrocyclic organic compounds.[1-3] They have received importance because of their flexible hydrophobic pockets and susceptibility to undergo facile rim modification to encapsulate specific targets.[4,5] This flexible behavior allows for efficient permeation of cell membrane without cell structure deformation.[6] In the calixarene family, sulphonated calixarenes are water-soluble, highly stable and less toxic supramolecules having widespread applications in the host-guest chemistry field.[7-10] Sulphonated calixarenes possess hydrophobic aromatic cavity and hydrophilic lower and upper rims.[11,12] They have achieved increasing attention over the last three decades owing to their inclusion properties with a wide range of guests.[13] Having high selectivity and affinity for various kinds of guests in an aqueous environment[14-17] and due to nontoxic character,[18,19] sulphonated calixarenes have been widely employed in the fields of smart materials,[20,21] chemical sensors,[22-24] supramolecular amphiphiles,[25] drug delivery,[26,27] protein surface recognition,[28] including molecular capsules synthesis in biocompatible environments.[29-31] Hence, the fundamental investigations involving the interactions of sulphonated calixarenes with different types of guests are important for their advanced applications.[32]

For our research, we have selected one of the sulfonated calixarenes, namely, *p*-sulfonatothiacalix[4]arene (TSC4X) [Scheme 1], as a unique macrocyclic host in which all four methylene bridges of the conventional calix[4]arenes are replaced by sulfur bridges. Being highly soluble in water, TSC4X have potential for application in biological systems. Therefore, it is important to develop systems that form and display stable associations as well as binding properties in aqueous solution.[33-36] To develop stable binary systems, we have chosen two biologically important amino acids, viz., L-Valine and L-Aspartic acid as the guests (Scheme 1) for the encapsulation into TSC4X. Both of these amino acids are used in biosynthesis of proteins. L-Aspartic acid (Asp) is the precursor of many essential amino acids and participates in gluconeogenesis process in mammals.[37] L-Valine (Val) is associated with insulin resistance and is essential for hematopoietic stem cell (HSC) self-renewal.[38,39] Encapsulation of amino acid guest molecules into TSC4X results in the modification of their some physicochemical properties such as solubility and stability after formation of inclusion complex. Changes

in the physicochemical properties have been used to characterize whether guest molecules are really included in the cavity of TSC4X. Hence, to explore the formation of inclusion complexes and their stability, the interaction study of amino acids with TSC4X has been investigated which may add a dimension to the modern field of science of controlled delivery of these two amino acids by using TSC4X.

Herein, we synthesized the inclusion complexes (ICs) of Asp and Val with TSC4X. The formation of ICs were investigated by ^{1}H NMR, PXRD, SEM study, mass spectroscopy and DSC analysis. Further, molecular docking was carried out to find out the possible binding mode of Asp and Val with TSC4X.

VII.2. Experimental section

VII.2.1. Materials

The two amino acids (viz., L-Valine, L-Aspartic acid) were purchased from Sigma-Aldrich, Germany and *p*-sulfonatothiacalix[4]arene (TSC4X) from TCI chemicals India Pvt. Ltd. The purity of the two amino acids and *p*-sulfonatothiacalix[4]arene were ≥ 98% and >98% respectively.

VII.2.2. Methods

Solution state ^{1}H NMR experiments were carried out on Bruker Avance 300 MHz NMR spectrometer using D_2O as a solvent at 298.15 K for acquiring the ^{1}H NMR spectra. The chemical shift (δ) values were reported in parts per million.

ESI-MS spectra of the prepared inclusion complexes were acquired on a high-resolution Q-TOF mass spectrometer with a positive-mode electrospray ionization.

All the DSC spectra were recorded using Perkin Elmer Pyris DSC 6. Heating of a solid sample was carried out in the temperature range of 30–300°C at a rate of 10°C/min under a N_2 gas flow of 40 mL/min.

Powder XRD analysis was performed on a Bruker SMART APEX diffractometer at 273 K using graphite-monochromated MoK_α (λ=0.71073 Å) radiation equipped with CCD area detector. All scans were carried out with scan rate 1^0/min and step size 0.042.

The surface morphologies of the samples was analysed using a JSM-6360 scanning electron microscope (SEM).

VII.2.3. Preparation of inclusion complex

The inclusion complexes of the mentioned amino acids with TSC4X were prepared in 1:1 molar ratio via co-precipitation method[40] with minor modifications. Briefly, weighted TSC4X was added to 50 ml deionized water taken in a 100 ml beaker at room temperature and stirred. The weighted amino acid was then slowly added to the aqueous TSC4X solution with continuous stirring at 55^0C for 6 hours using a magnetic stirrer. After that, the solution was kept overnight at 4^0C. The obtained cold precipitate of inclusion complexes was recovered by filtration and then stored at 50^0C for 24 hours to remove solvent. Finally, the complexes were kept in a dessicator at 25^0C for further analysis.

VII.2.4. ^{1}H NMR spectral titrations

Solutions for binding studies were made using D_2O as a solvent. A series of TSC4X solutions in the concentration range 1.0-5.0 mM (each 250 µl) was prepared. To each of these solutions, 250 µl of 1.0 mM amino acid solution was added.

VII.2.5. Molecular docking study

Molecular docking was performed to predict free energy of host-guest interaction and probable binding mode of guest molecule (Val and Asp) with host molecule (TSC4X) using PyRx software. Before executing docking, the host (TSC4X) and guest (Val and Asp) input files in the PDB format were converted into PDBQT format using inbuilt AutoDock software. During the docking process, Val and Asp were docked to TSC4X molecule using the following Cartesian coordinates as the binding site center : x = 8.3636 Å, y = 24.4146 Å and z = 1.2278 Å. A docking grid box of dimension 25 Å × 25 Å × 25 Å was constructed. To acquire best docking conformations, Lamarckian genetic algorithm was used with default values. Based on the docking score, the lowest binding energy conformer was chosen.

VII.3. Result and Discussion

VII.3.1. ^{1}H NMR analysis

^{1}H NMR spectroscopy is a useful technique that can be utilized to explore the possible binding mode of guest molecule with TSC4X by analyzing the changes in chemical shift values (Δδ) of guest protons.[41] In the present work, the inclusion of Val

and Asp into the cavity of TSC4X has been studied using ^{1}H NMR spectroscopy. The ^{1}H NMR spectra of pure Val, TSC4X and Val-TSC4X inclusion complex are shown in Figure 1 with their corresponding δ values in Table 1. After complex formation, H^d, H^e and H^f protons of Val were observed to undergo visible upfield shifts with Δδ -0.418, -0.358 and -0.134 respectively. These changes in chemical shift values is due to the anisotropic ring-current effects of the aromatic nuclei of TSC4X, which indicates the encapsulation of $CH(CH_3)_2$ and CH groups of Val into the TSC4X cavity [Scheme 2(a)]. Since H^d, H^e protons were found to undergo considerable upfield shifts than H^f proton, therefore, it could be suggested that $CH(CH_3)_2$ group is in close proximity to the aromatic nuclei of TSC4X compared to CH group.

Similarly, ^{1}H NMR spectra of pure Asp, TSC4X and Asp-TSC4X inclusion complex are shown in Figure 2 with their corresponding δ values in Table 1. It was observed that H^g and H^h protons of Asp undergo upfield shifts with Δδ -0.130 and -0.112 respectively due to the ring-current effects of the aromatic nuclei of TSC4X, indicating that CH and CH_2 groups of Asp are included into the TSC4X cavity [Scheme 2(b)]. Since H^g proton has higher upfield shift value than H^h proton, therefore, CH group could be possibly in the vicinity of the aromatic nuclei of TSC4X compared to CH_2 group.

VII.3.2. ^{1}H NMR titration

Encapsulation of a guest molecule into the TSC4X cavity results in the chemical shift changes (Δδ) of the guest protons in the ^{1}H NMR spectra.[42] Here, ^{1}H NMR titration of two amino acids (i.e., Val and Asp) with TSC4X was conducted by fixing the concentration of an amino acid and varying TSC4X concentration (Tables 2 and 3). The H^d, H^e, H^f protons of Val and H^g, H^h protons of Asp were found to undergo upfield shifts during titration, indicating the inclusion of the amino acid molecules into the cavity of TSC4X. For simplicity and to evaluate the binding affinity (K_a) using reliable Benesi-Hildebrand equation for 1:1 host-guest inclusion complexes,[43] we employed the Δδ of the H^d proton of Val and that of H^g proton of Asp (Tables 2 and 3).

$$\frac{1}{\Delta\delta} = \frac{1}{k'[\text{Amino acid}]K_a} \cdot \frac{1}{[\text{TSC4X}]} + \frac{1}{k'[\text{Amino acid}]} \qquad (1)$$

^{1}H NMR titration spectra of Val and Asp with TSC4X are shown in Figures 3 and 4 respectively. The binding affinity (K_a) values for both Val-TSC4X and Asp-TSC4X ICs

were evaluated from intercept/slope of double-reciprocal plot using equation 1 (Tables 2, 3 and Figure 5). The greater K_a value of Val-TSC4X IC (≃ 719 M^{-1}) compared to that of Asp-TSC4X IC (≃ 312 M^{-1}) reveals that the Val binds to TSC4X strongly than the Asp.

For the inclusion process between amino acids (i.e., Val and Asp) and TSC4X, the free energy of binding (ΔG) at 298.15 K can be derived using equation 2.

$$\Delta G = -RT \ln K_a \qquad (2)$$

The ΔG values for the Val-TSC4X and Asp-TSC4X complexes were found to be –3.9 kcal/mol and -3.4 kcal/mol respectively, which suggests that the inclusion process is spontaneous.

VII.3.3. ESI-MS studies

The synthesized Val-TSC4X and Asp-TSC4X inclusion complexes were analyzed by ESI-MS spectral technique.[44] The characteristic peak at m/z 1021.2722 (Figure 6(A)) corresponds to the molecular ion $[Val\text{-}TSC4X + 4Na]^+$ and that at m/z 949.8969 (Figure 6(B)) corresponds to the molecular ion $[Asp\text{-}TSC4X + 4H]^+$ (Table 4). This clearly confirms the formation of stable Val-TSC4X and Asp-TSC4X inclusion complexes with 1:1 stoichiometric ratio (Scheme 2).

VII.3.4. Thermal analysis

The differential scanning calorimetry (DSC) analysis was carried out on TSC4X, Val, Asp, Val-TSC4X IC and Asp-TSC4X IC to validate the thermal stability of Val and Asp after its encapsulation into the cavity of TSC4X (Figure 7). The DSC curve of TSC4X displayed a broader endothermal peak around 92°C, corresponding to the water loss. Val and Asp showed melting endothermic peaks at 315°C and 242°C respectively in their DSC thermograms. However, the disappearance of endothermic peaks at 315°C and 242°C corresponding to the pure Val and Asp, and the appearance of new endothermic peaks with reduced intensity at 328°C and 256°C for Val-TSC4X and Asp-TSC4X ICs respectively, were registered. Broader endothermal peaks were encountered around 99°C for Val-TSC4X IC and around 101°C for Asp-TSC4X IC, which corresponds to dehydration. These findings not only support the formation of inclusion complexes, but also indicate that the thermal stability of Val and Asp has enhanced on inclusion complexation.

VII.3.5. Powder XRD analysis

The PXRD analysis further presents insightful information regarding the formation of inclusion complexes.[44,45] The XRD patterns of TSC4X, Val, Asp, Val-TSC4X IC and Asp-TSC4X IC are shown in Figure 8. TSC4X exhibited broad diffraction peaks indicating amorphousness [Figure 8(a)]. Val and Asp displayed sharp and intense diffraction peaks confirming their crystalline nature [Figures 8(b) and 8(c)]. However, Val-TSC4X and Asp-TSC4X ICs showed diffraction profiles similar to the TSC4X, and the sharp crystalline peaks of Val and Asp were vanished [Figures 8(d) and 8(e)]. These observations confirms the complete complexation of Val and Asp with TSC4X.

VII.3.6. SEM analysis

The analysis of SEM image gives a supporting evidence for the inclusion complex formation by examining the surface morphology of solid substance.[46] The surface morphology of TSC4X, Val, physical mixture and solid Val-TSC4X complex are shown in Figure 9A. Pure TSC4X consisted of spherical flake-shaped particles [Figure 9A(a)], while Val appeared as polyhedral shaped crystals [Figure 9A(b)]. In physical mixture [Figure 9A(c)], a mixture of the particles of both TSC4X and Val were observed. However, Val-TSC4X complex was found to appear as triangular shaped crystal with a significant change in the morphological structure [Figure 9A(d)]. Similarly, Figure 9B shows the surface morphology of TSC4X, Asp, physical mixture and solid Asp-TSC4X complex. SEM images showed crystalline state Asp [Figure 9B(b)] and TSC4X as spherical flake-shaped particles [Figure 9B(a)]. Particles of both Asp and TSC4X were observed in case of physical mixture [Figure 9B(c)]. In contrast, solid Asp-TSC4X complex appeared as irregularly shaped crystal particles with a considerable alteration in the morphology [Figure 9B(d)]. These morphological variations indicates the encapsulation of amino acids, Val and Asp, into the cavity of TSC4X leading to the formation of Val-TSC4X and Asp-TSC4X inclusion complexes.

VII.3.7. Molecular docking studies

Molecular docking study has been performed to get a deeper insight on the binding orientation of Val and Asp into the TSC4X cavity.[47] Val and Asp were docked into the pocket of TSC4X using PyRx software.[48] The best docked conformation of Val-TSC4X and Asp-TSC4X complexes are shown in Figure 10. The lowest negative binding

energy (ΔG) value for the most stable docked conformation of Val-TSC4X and Asp-TSC4X complexes were found to be -3.7 kcal/mol and -3.4 kcal/mol respectively, which are close to the values (≃ -3.9 kcal/mol for Val-TSC4X and ≃ -3.4 kcal/mol for Asp-TSC4X) determined from ^{1}H NMR titration (Table 5), suggesting that the binding energy obtained from docking is in accordance with that deduced from ^{1}H NMR titration. Docking results demonstrated that in the docked conformation of Val-TSC4X complex [Figure 10(A)], $CH(CH_3)_2$ and CH groups are included into the hydrophobic aromatic cavity with amino (NH_2) and carboxylic (COOH) groups surrounded by polar sulphonate groups (SO_3^-) near the wider rim of TSC4X. However, in the docked conformation of Asp-TSC4X complex [Figure 10(B)], CH and CH_2 groups enters the hydrophobic aromatic cavity with amino (NH_2) and one of its carboxylic (COOH) groups surrounded by polar sulphonate groups (SO_3^-) near the wider rim of TSC4X. These docking interactions of Val and Asp with TSC4X agree well with the ^{1}H NMR results.

VII.4. Conclusion

The present work reports the formation of stable inclusion complexes of two naturally occurring amino acids, L-Valine (Val) and L-Aspartic acid (Asp), with TSC4X. The formation of inclusion complexes, Val-TSC4X and Asp-TSC4X, was confirmed by ^{1}H NMR study. ESI-MS study suggested the efficient 1:1 complexation of Val and Asp with TSC4X. ^{1}H NMR and molecular docking studies provided most stable binding orientation of Val and Asp within the cavity of TSC4X, such that $CH(CH_3)_2$, CH groups of Val and CH, CH_2 groups of Asp are included into the hydrophobic cavity of TSC4X. The greater binding affinity (K_a) value of Val-TSC4X IC (≃ 719 M^{-1}) compared to that of Asp-TSC4X IC (≃ 312 M^{-1}), obtained from ^{1}H NMR titration, revealed that the Val binds to TSC4X strongly than the Asp. ^{1}H NMR titration also accounts for the thermodynamic feasibility of the inclusion process, which was validated by the negative value of ΔG (≃ -3.9 kcal/mol for Val-TSC4X and ≃ -3.4 kcal/mol for Asp-TSC4X). The formation of Val-TSC4X and Asp-TSC4X complexes was further confirmed by PXRD study and SEM image analysis. Finally, the complexation with TSC4X improved the thermal stability of Val and Asp. Thus, the present work may add a dimension to the modern field of science of controlled delivery of these two amino acids by using TSC4X.

Tables

Table 1. ^{1}H NMR data of Val, Asp, TSC4X, Val-TSC4X complex and Asp-TSC4X complex in D_2O. ND : not detected

H protons	ppm (D_2O)			Δδ	H protons	ppm (D_2O)			Δδ
	δ_{Val}	δ_{TSC4X}	$\delta_{Val\text{-}TSC4X}$			δ_{Asp}	δ_{TSC4X}	$\delta_{Asp\text{-}TSC4X}$	
H^d	0.904	–	0.486	-0.418	H^g	4.041	–	3.911	-0.130
H^e	2.152	–	1.794	-0.358	H^h	2.980	–	2.868	-0.112
H^f	3.482	–	3.348	-0.134					
H^a	–	7.920	7.933	+0.013	H^a	–	7.920	7.906	-0.014
H^b	–	3.520	–	ND	H^b	–	3.520	3.530	+0.010

Table 2. Data for the Benesi-Hildebrand double reciprocal plot performed by ^{1}H NMR spectroscopy for aqueous Val-TSC4X system at 298.15 K[a]

Val (mM)	TSC4X (mM)	δ_o	δ	Δδ	1/[TSC4X] (M^{-1})	1/Δδ	Intercept	Slope	K_a (M^{-1})
0.5	0.5	0.904	0.801	0.103	2000	9.708738			
0.5	1.0	0.904	0.748	0.156	1000	6.410256			
0.5	1.5	0.904	0.711	0.193	666.67	5.181347	2.59746	0.00361	719
0.5	2.0	0.904	0.672	0.232	500	4.310345			
0.5	2.5	0.904	0.644	0.26	400	3.846154			

[a]Standard uncertainties in temperature u are: u(T) = ±0.01 K.

Table 3. Data for the Benesi-Hildebrand double reciprocal plot performed by ^{1}H NMR spectroscopy for aqueous Asp-TSC4X system at 298.15 K[a]

Asp (mM)	TSC4X (mM)	δ_o	δ	Δδ	1/[TSC4X] (M^{-1})	1/Δδ	Intercept	Slope	K_a (M^{-1})
0.5	0.5		4.011	0.03	2000	33.33333			
0.5	1		3.989	0.052	1000	19.23077			
0.5	1.5	4.041	3.969	0.072	666.67	13.88889	4.51151	0.01444	312
0.5	2		3.955	0.086	500	11.62791			
0.5	2.5		3.945	0.096	400	10.41667			

[a]Standard uncertainties in temperature u are: u(T) = ±0.01 K.

Table 4. The peaks observed for Val-TSC4X and Asp-TSC4X inclusion complexes at different m/z with corresponding ions

Val-TSC4X inclusion complex		Asp-TSC4X inclusion complex	
m/z	Ion	m/z	Ion
1021.2722	$[Val\text{-}TSC4X + 4Na]^+$	949.8969	$[Asp\text{-}TSC4X + 4H]^+$

Table 5. Gibb's free energy of binding (ΔG) of Val and Asp with TSC4X obtained from Molecular Docking and ^1H NMR titration

Parameter	Ligand with receptor	Using Molecular Docking	Using ^1H NMR Titration
ΔG (kcal/mol)	Val-TSC4X	-3.7	-3.9
	Asp-TSC4X	-3.4	-3.4

Figures

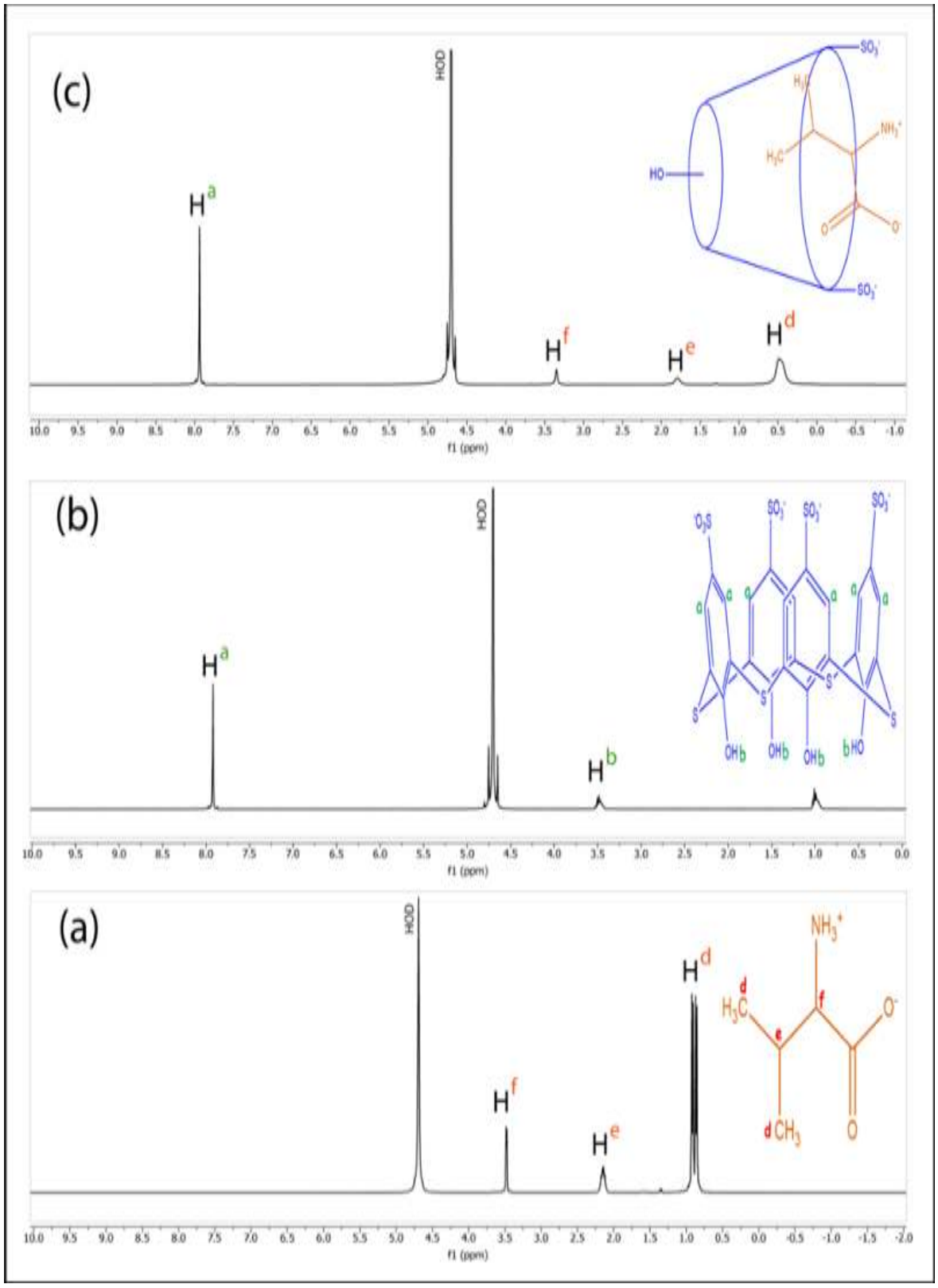


Figure 1. ^{1}H NMR spectra of **(a)** L-Valine (Val), **(b)** *p*-sulfonatothiacalix[4]arene (TSC4X) and **(c)** Val-TSC4X complex in D_2O at 298.15K.

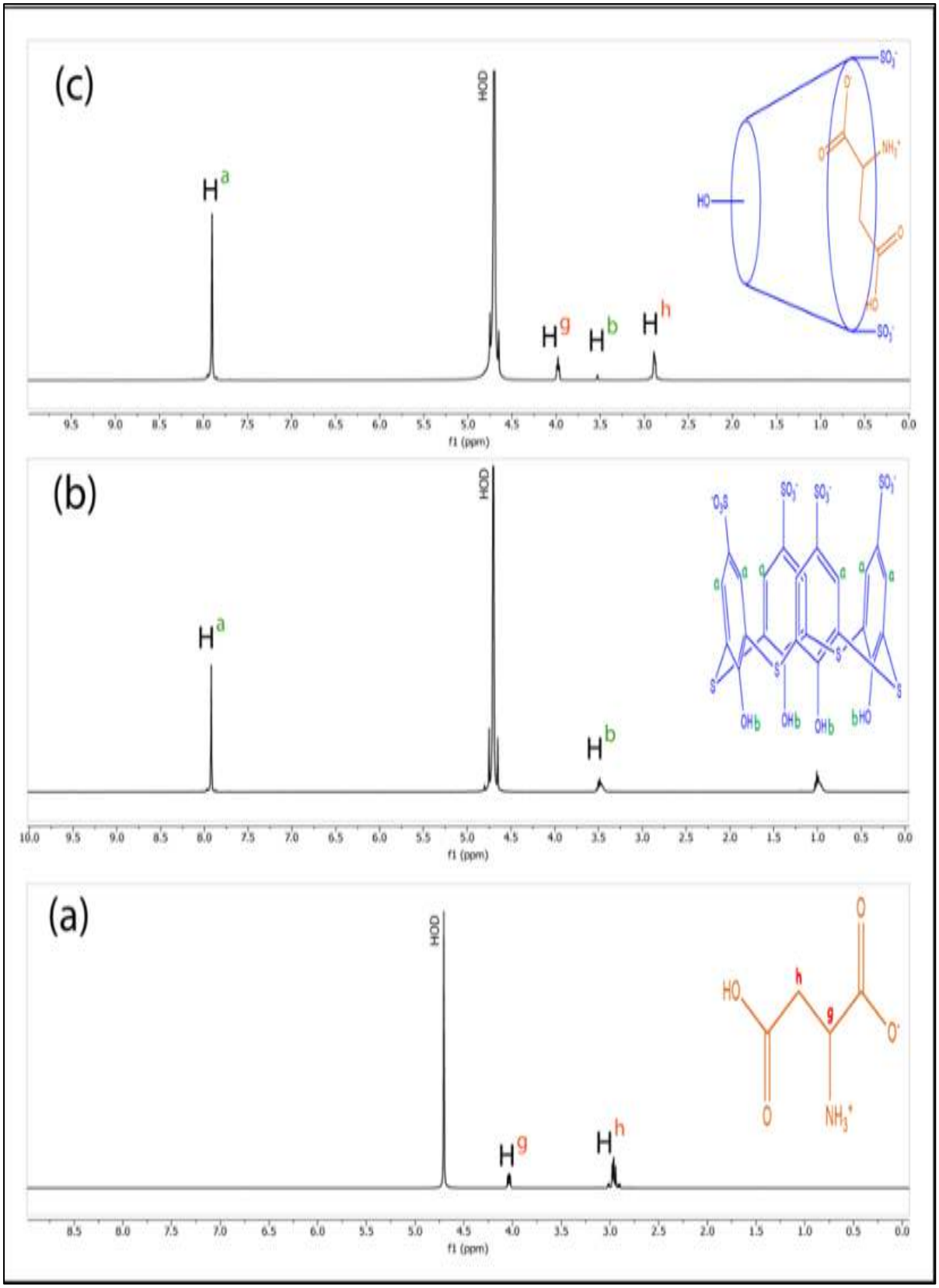


Figure 2. ^{1}H NMR spectra of **(a)** L-Aspartic acid (Asp), **(b)** *p*-sulfonatothiacalix[4]arene (TSC4X) and **(c)** Asp-TSC4X complex in D_2O at 298.15K.

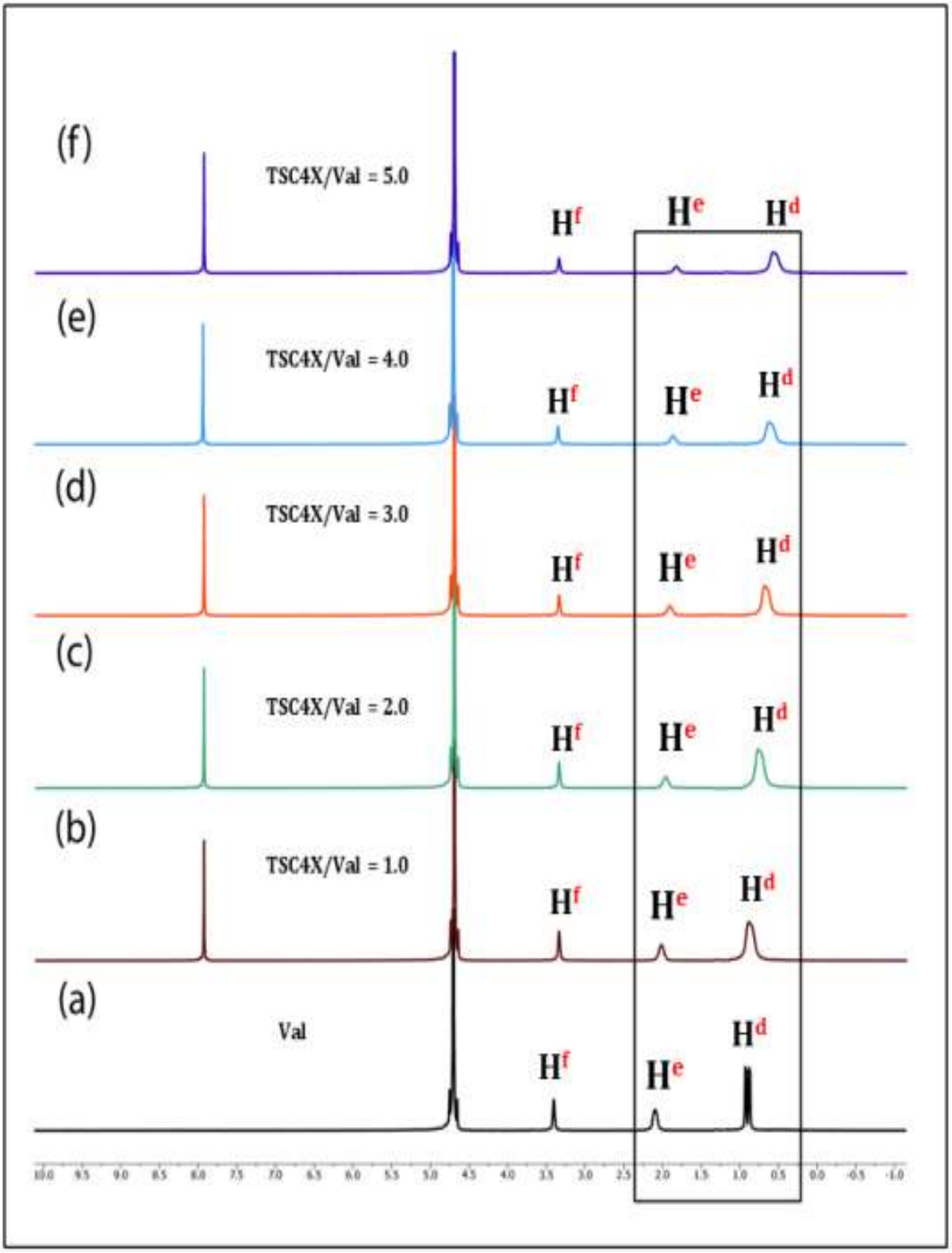


Figure 3. ^1H NMR titration spectra of Val (0.5 mM) in the presence of varying amount of TSC4X in D_2O at 298.15 K (0.5 - 2.5 mM).

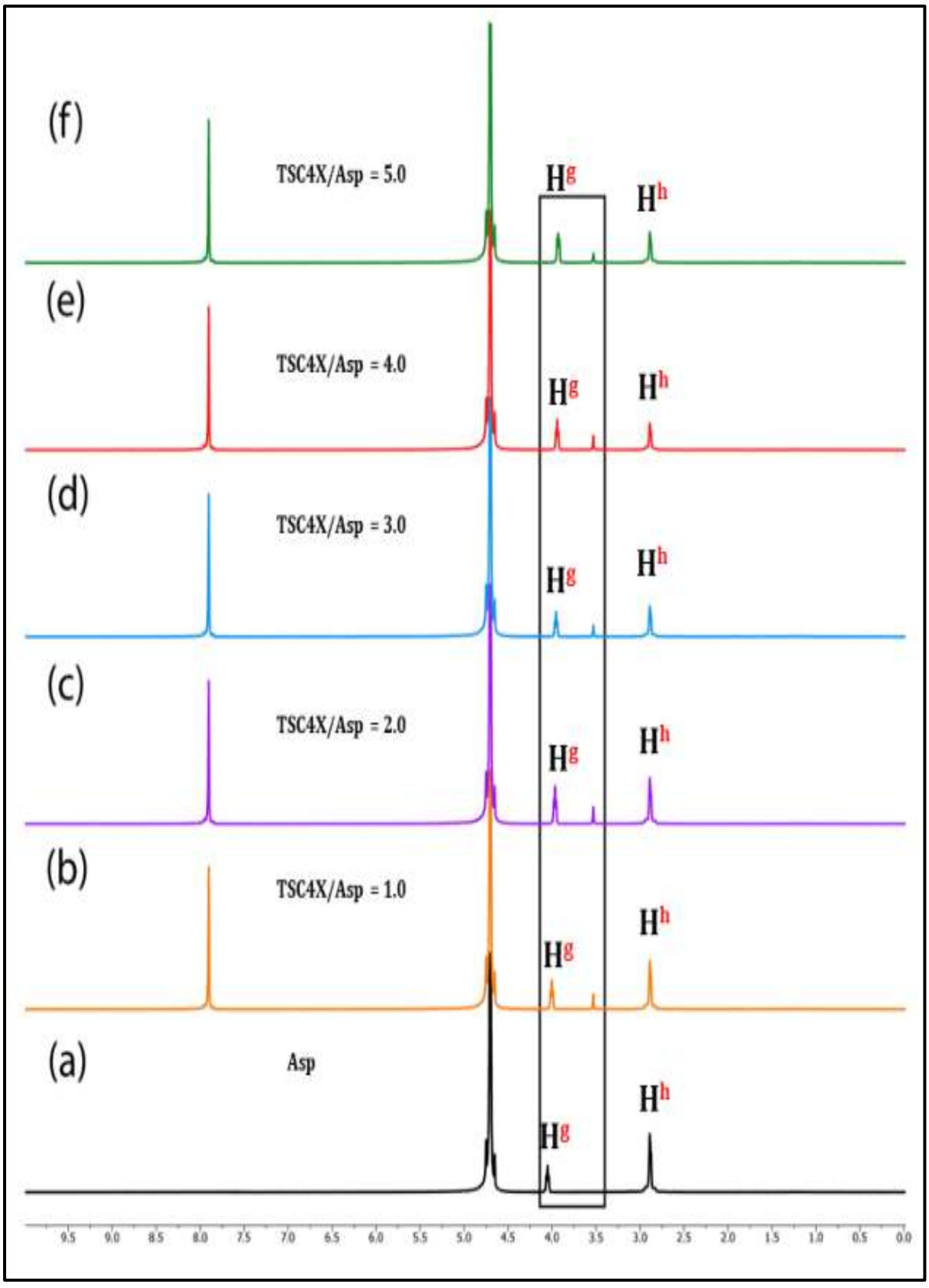


Figure 4. ^{1}H NMR titration spectra of Asp (0.5 mM) in the presence of varying amount of TSC4X in D_2O at 298.15 K (0.5 - 2.5 mM).

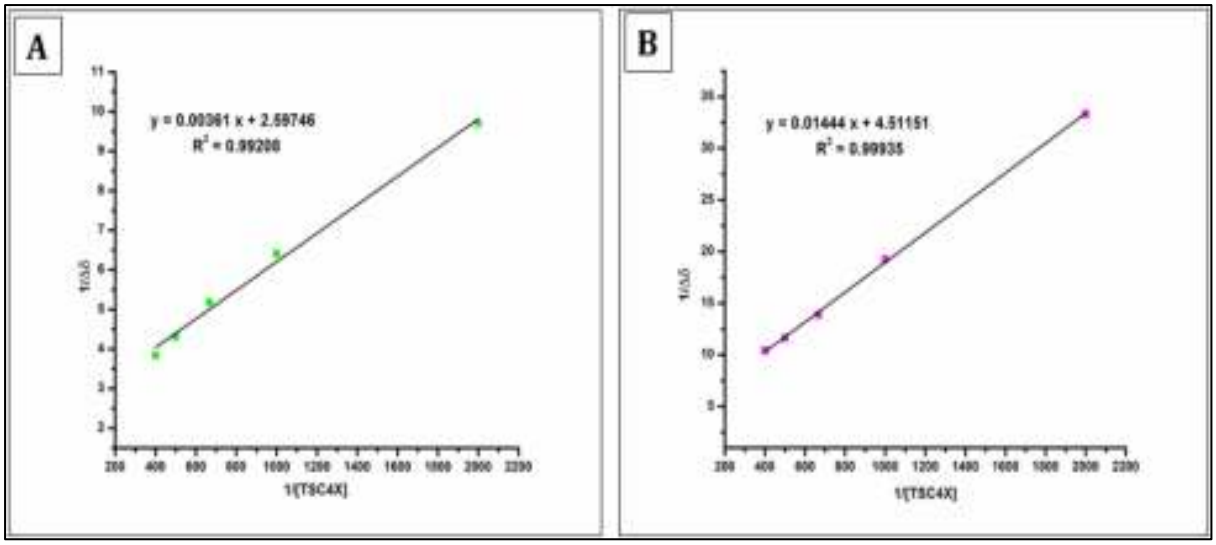


Figure 5. Double reciprocal Benesi-Hildebrand plots of $1/\Delta\delta$ versus $1/[TSC4X]$ for **(A)** Val-TSC4X system and **(B)** Asp-TSC4X system in D_2O at 298.15 K.

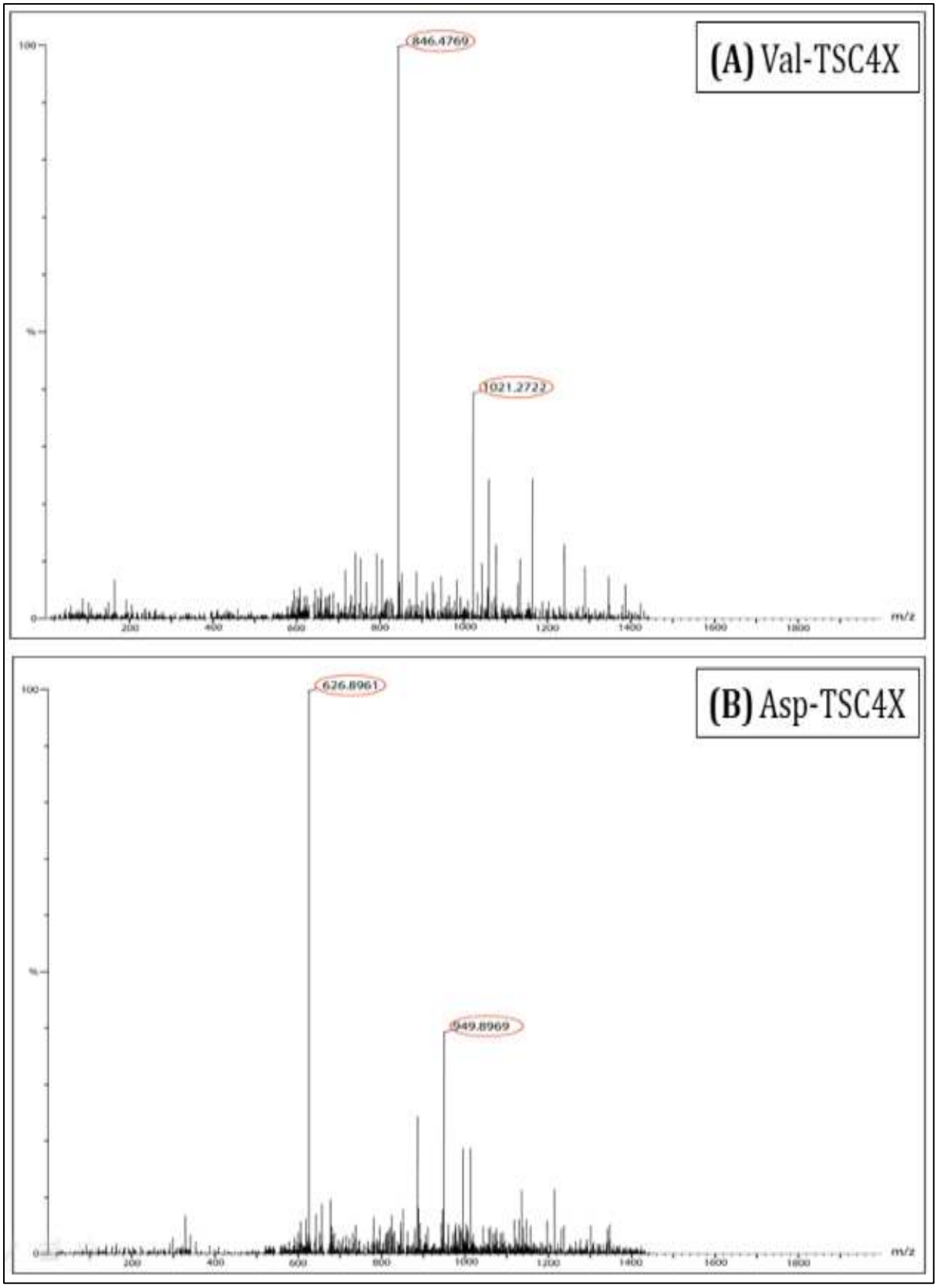


Figure 6. ESI-MS mass spectra of **(A)** Val-TSC4X inclusion complex and **(B)** Asp-TSC4X inclusion complex.

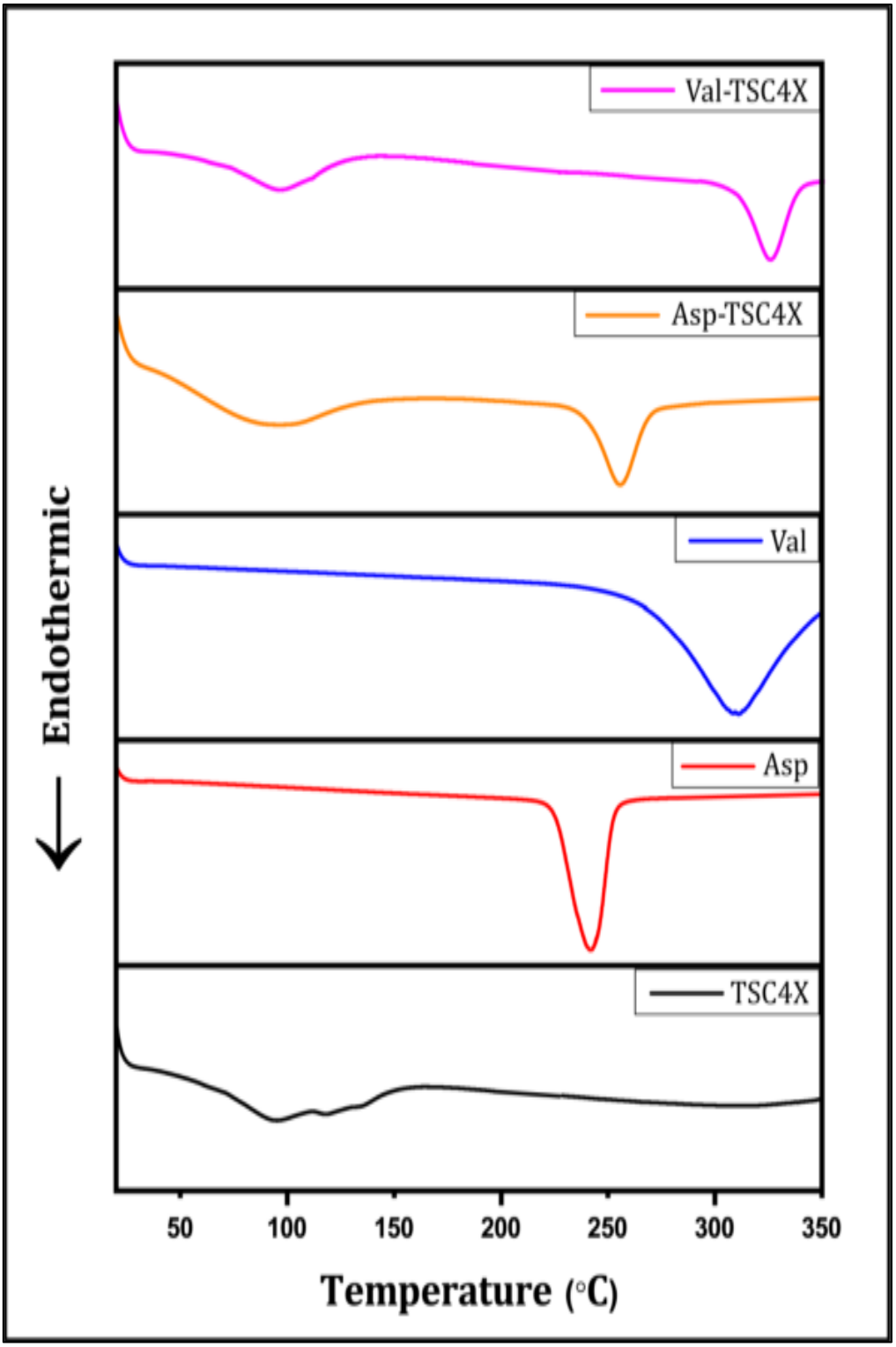


Figure 7. DSC thermograms of TSC4X, Asp, Val, Asp-TSC4X inclusion complex and Val-TSC4X inclusion complex.

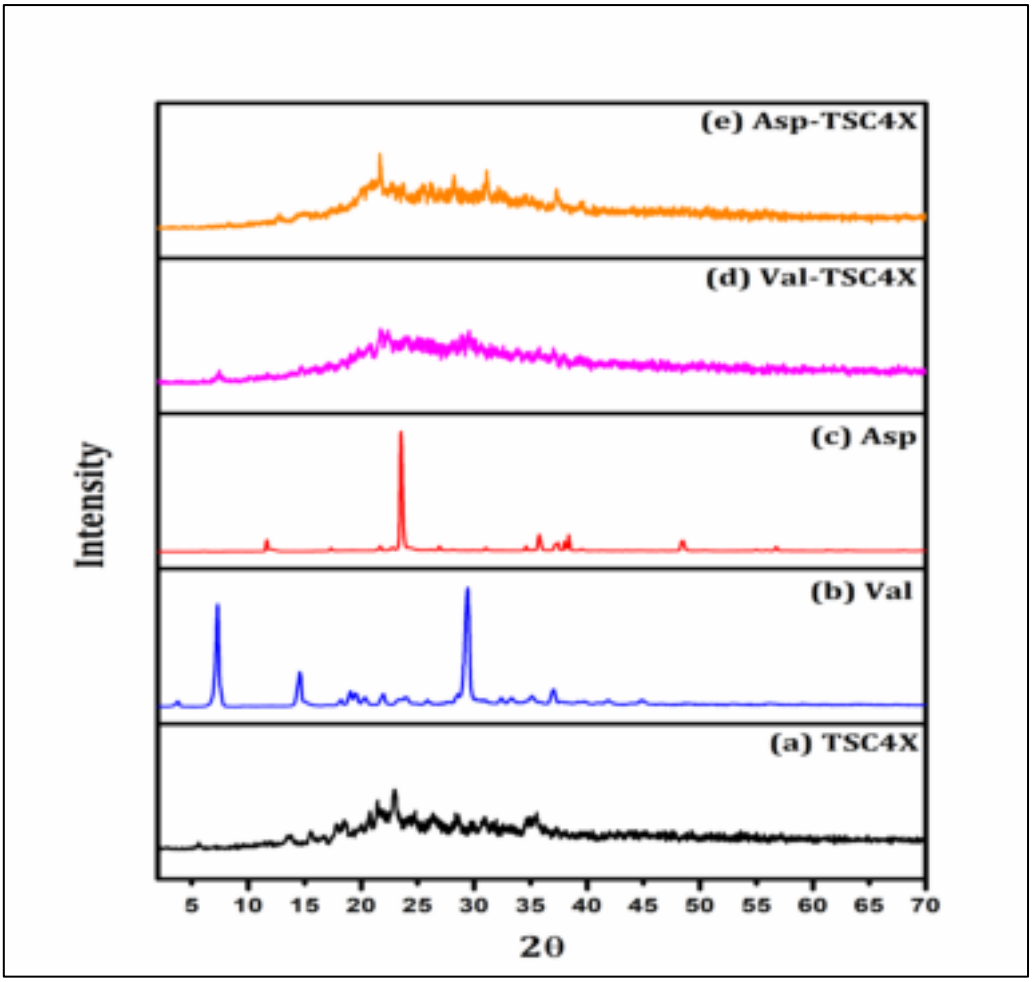


Figure 8. PXRD profiles of **(a)** TSC4X, **(b)** Val, **(c)** Asp, **(d)** Val-TSC4X inclusion complex and **(e)** Asp-TSC4X inclusion complex.

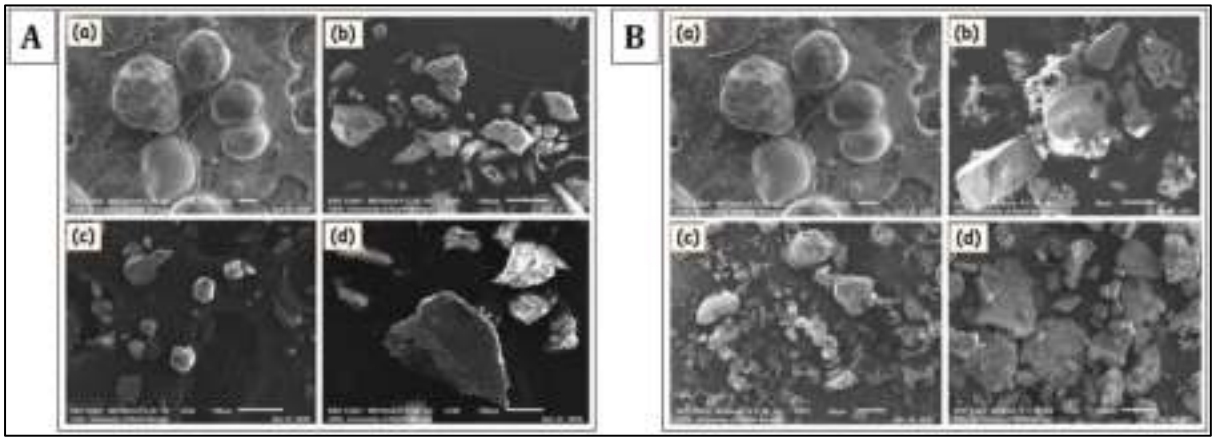


Figure 9. (A) SEM images of (a) TSC4X, (b) Val, (c) physical mixture of Val and TSC4X, (d) Val-TSC4X inclusion complex ; **(B)** SEM images of (a) TSC4X, (b) Asp, (c) physical mixture of Asp and TSC4X, (d) Asp-TSC4X inclusion complex.

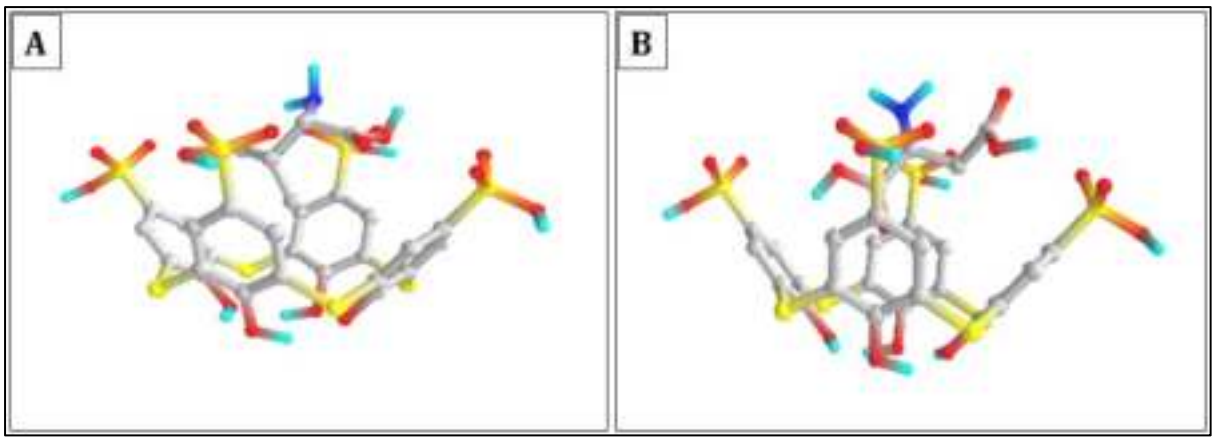
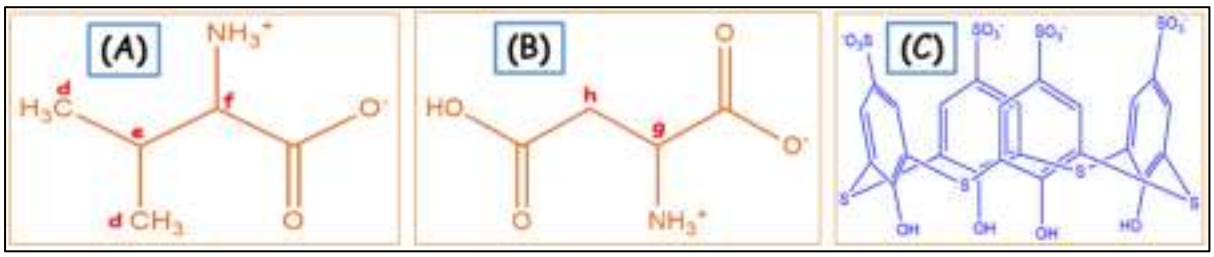
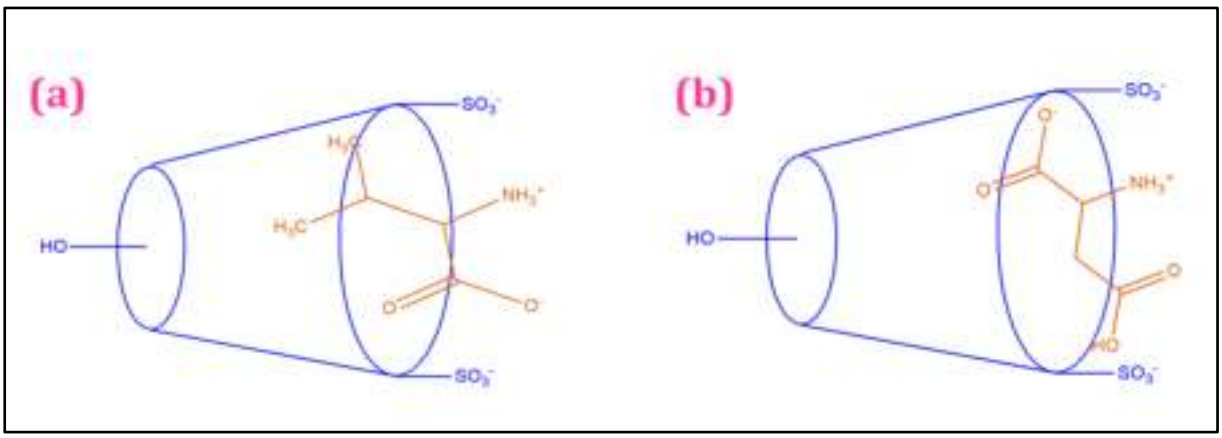


Figure 10. Docked conformation of **(A)** Val-TSC4X inclusion complex and **(B)** Asp-TSC4X inclusion complex.

Schemes



Scheme 1. Molecular Structures of **(A)** L-Valine (Val), **(B)** L-Aspartic acid (Asp) and **(C)** *p*-sulfonatothiacalix[4]arene (TSC4X).



Scheme 2. Illustration of the complexation of **(a)** L-Valine (Val) and **(b)** L-Aspartic acid (Asp) with TSC4X forming 1:1 inclusion complex.