***Supporting Information***

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# pH-Regulated anion transport activities of bis(iminourea) derivatives across the cell and vesicle membrane

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***Supporting Information***

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**Experimental procedures:**

**1. Ion recognition and transport activities:**

Anion binding analysis by $^1$H-NMR titrations[1, 2] ─ All synthesized compounds were dissolved in DMSO-$d_6$ and were used for the $^1$H-NMR titrations. The increasing concentrations of tetrabutylammonium chloride (TBACl) were used as $Cl^-$ ion source during the titration. The chemical shift (Δδ) of the both N-H proton of compounds were recorded and significant extents of shift (Δδ) of both N-H protons were observed for all the tested compounds. MestReNova software was used to stack all the titration spectra of $^1$H NMR. The changes in chemical shift *vs* concentration of $Cl^-$ ion were fitted using WinEQNMR2 program (1:1 binding model). All the dissociation constant ($K_d$) values were calculated by taking the reciprocal of binding constant ($K_a$; as mentioned in WinEQNMR2 program). The following mathematical equation (Eq.-1) was used for the calculation the binding constant.

$$\delta_{cal} = \sum_{m=1}^{m=i} \sum_{n=0}^{n=j} \frac{\delta_{mn}\beta_{mn} m[M]^m[L]^n}{[M]_{total}}$$

……Eq.-1

Where, *M* represents the free, uncomplexed receptor and *L* is the ligand; $\delta_{calc}$, is the weighted average of the chemical shifts of the various *M*-containing species present, $M_mL_n$, and i and j represent the maximum values of m and n respectively.[3]

Determination of anion binding stoichiometry by Job's plot[2] ─ A continuous variation method of Job's plot was adopted to determine the binding stoichiometry of the complex which was formed during the titration of compound 1c in the presence of $Cl^-$ ion. DMSO-$d_6$ solvent was used to prepare the solutions of host (compound) and the guest (TBACl). During the measurements total 10 separate NMR tubes containing the required amount of host and guest concentrations were prepared to keep the final concentration fixed to be at 5 (M). The chemical shift values were recorded and the changes of chemical shift of N-H proton at

different mole fraction of the $Cl^-$ ion were listed in the Table S1. The Job's plot of compound 1c indicates a 1:1 binding stoichiometry of the complex.

Ion transport activity studies using fluorescence based assay:

Preparation of EYPC/CHOL-LUV⊃HPTS[1, 2] ─ To prepare the large unilamellar vesicles (LUVs) of EYPC/CHOL-LUV⊃HPTS, first 50 μL of EYPC (100 mg/mL in deacidified $CHCl_3$) and 80 μL of cholesterol (25 mg/mL in deacidified $CHCl_3$) were taken in a clean and dry glass vial, so that the molar ratio of EYPC and cholesterol would be 6:4. Then, this solution was dried by continuous purging of nitrogen gas for 5-6 hours to obtain a thin film of lipids inside the glass vial. The dry film was then hydrated with 0.8 mL of 20 mM HEPES buffer, pH 7.2 containing 100 mM NaCl and 1 mM HPTS for 1 hours with 8-10 times occasional vortexing. The suspension was then passed through 14-15 cycles of freeze-thaw (freezing with liquid $N_2$ and thawing with lukewarm water, respectively) and vortexed for another 15 min. Next, the solution was extruded through a polycarbonate membrane (using a mini-extruder from Avanti Polar Lipids) having pore size of 200 nm for 21-times (as it must be an odd number), to give LUVs with a mean diameter of ~200 nm.[4] Finally, gel filtration (Sephadex G-50) column chromatography was performed with 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl as running solution to remove the untrapped HPTS present in the extravesicular solution. The HPTS encapsulated LUVs were collected and the final volume was adjusted to 0.8 mL using 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl. The final lipid concentration obtained was 25 mM (assuming 100% lipid regeneration).

Ion transport activity across EYPC/CHOL-LUV⊃HPTS[1, 2] ─ For the HPTS assay, first 2920 μL of 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl and 50 μL of the EYPC/CHOL-LUV⊃HPTS were taken in a 3 mL fluorescence cuvette and the cuvette was

placed in the fluorescence spectrophotometer (at 37 ºC), fluoromax-4 spectrofluorometer (Horiba Scientific, Singapore) under slow stirring condition. After that, compounds (10 μL of the stock solution in DMSO) were added to the buffer. The cuvette was then kept inside the fluorescence instrument under continuous stirring condition for 3 minutes to allow maximum incorporation of the compounds into the lipid bilayers. The HPTS fluorescence intensity was monitored (t = 0 sec) at 510 nm ($\lambda_{ex}$ = 450 nm). Subsequently, 20 μL of NaOH (0.75 M) solution was added into the cuvette after 50 sec to create a pH gradient (ΔpH = ~ 0.6) between the extra and intra-vesicular regions and to initiate the $Cl^-$ transport kinetics. After 450 sec the kinetic experiment was terminated by adding 20 μL of 20% Triton-X100 solution (to rupture the vesicular arrangements) into the cuvette and the fluorescent measurements were continued for another 50 sec (t = 500 sec).

Quantitative measurement of transport activity from HPTS assay[1, 2] ― The fluorescence emission intensities of the HPTS dye were normalized and the intensities appearing at t = 0 and t = 500 s were taken as 0 and 100 units, respectively. The normalized fluorescent intensities (FI) at t = 450 s (prior to the addition of Triton X-100) were considered to measure the transport activity of the compounds.

$$i.e.\ Transport\ activity,\ \ T_{HPTS} = \frac{F_t - F_0}{(F_\infty - F_0)} \times 100\% \qquad \text{..... Eq.-2}$$

Where, $F_t$ = fluorescence intensity at t = 450 s (prior to the addition of Triton X-100), $F_0$ = fluorescence intensity immediately before the addition of the NaOH (t = 0 s) and $F_\infty$ = fluorescence intensity after addition of Triton X-100 (i.e. at saturation after complete leakage at t = 500 s).

Ion transport activity studies using ion selective electrode based assay:

5.2.1. Chloride ion efflux studies using chloride ion selective electrode (chloride ISE)[5, 6] ─ The extent of chloride ion efflux from the liposomes was measured using a chloride selective electrode (chloride ISE from Thermo Scientific™ Orion™). Filling solution was also poured inside the electrode up to the mark prior to the calibration of the chloride ISE. The chloride selective electrode was calibrated prior to each experiment, using known concentrations of NaCl solution (1 ppm, 10 ppm and 100 ppm of aq. NaCl). Chloride concentration (ppm) appearing in the display of the ion meter was set in the continuous mode for the kinetic experiments.

Preparation of EYPC/CHOL-LUV[5, 6] ─ The LUVs were prepared according to the method mentioned in section 5.1.1. Briefly, 66 μL of EYPC (100 mg/mL in deacidified $CHCl_3$) and 40 μL of cholesterol (25 mg/mL in deacidified $CHCl_3$) were collected in a glass vial. The molar ratio of EYPC and cholesterol for LUVs was 8:2. Then, this solution was dried by continuous purging of nitrogen gas for 6 hours to obtain a thin film of lipids inside the glass vial. The dry film was then hydrated with 0.8 mL of 20 mM phosphate buffer, pH 7.2 containing 100 mM NaCl for 1 hours with 8-10 times occasional vortexing. The suspension was then passed through 14-15 cycles of freeze-thaw and vortexed for another 15 min. This suspension was allowed to sit for additional 30 min at room temperature. Next, the solution was extruded through a polycarbonate membrane having pore size of 200 nm for 21-times, to give LUVs with a mean diameter of ~200 nm. Finally, dialysis was performed with 20 mM phosphate buffer, pH 7.2, containing 100 mM $NaNO_3$ (iso-osmolar with the NaCl buffer) as external solution to remove NaCl present in the extra vesicular solution. The chloride-encapsulated LUVs were collected from the dialysis bag and the final volume was adjusted to

0.8 mL using 20 mM phosphate buffer, pH 7.2, containing 100 mM $NaNO_3$. The final lipid concentration obtained was 25 mM (assuming 100% lipid regeneration).

Chloride efflux study across EYPC/CHOL-LUV[5, 6] ─ In this experiment, 4.94 mL of 20 mM phosphate buffer, pH 7.2, containing 100 mM $NaNO_3$ and 50 µL of the EYPC/CHOL-LUV were taken in a glass vial. Then the chloride electrode was immersed into the vesicle solution under mild stirring condition (using external magnetic stirrer) for 3 min. The chloride efflux was monitored (t = 0 sec) using an ion meter. The compounds (10 µL of the stock solution in DMSO) were added (at t = 50 sec) into the vesicle solution to initiate the $Cl^-$ transport kinetics. The kinetic experiment was terminated after 500 sec by adding 20 µL of 20% Triton-X100 solution into the vesicle solution (to rupture the vesicular arrangements).

Quantitative measurement of transport activity from chloride ISE assay[5, 6] ─ The chloride efflux efficiency of the compounds were normalized and the intensities appearing at t = 0 and t = 700 s were taken as 0 and 100 units, respectively. The normalized chloride efflux efficiencies (EE) at t = 500 sec (prior to the addition of Triton X-100) were also considered for the measurement of transport efficacy of the compounds.

$$i.e.\ Chloride\ efflux\ efficiency,\ \ EE_{Chloride} = \frac{EE_t - EE_0}{(EE_\infty - EE_0)} \times 100\% \quad \text{.....}$$

Eq.-3

Where, $EE_t$ = chloride efflux efficiency at t = 500 s (prior to the addition of Triton X-100), $EE_0$ = chloride efflux efficiency immediately before the addition of the compound (t = 0 s) and $EE_\infty$ = chloride efflux efficiency after addition of Triton X-100 (i.e. at saturation after complete leakage at t = 700 s).

Ion selectivity studies:

Ion selectivity studies of the compounds were performed using HPTS assay with applied pH gradient as discussed in the section 5.1.2. The EYPC/CHOL-LUV⊃HPTS were prepared following a similar method as described in the previous section. Required amount of solid HEPES and the salts of either MCl or $Na_xA$ (LiCl, NaCl, KCl, $CaCl_2$, and $MgCl_2$, NaBr, NaI, $NaNO_3$, NaOAc) were dissolved in Milli-Q water to attain a final concentration of 20 mM and 100 mM, respectively. The pH of the solution was adjusted to 7.2 by drop wise addition of 1 M NaOH solution.

Cation selectivity studies[1, 2, 5] ─ In this assay, 2920 μL of 20 mM HEPES buffer of pH 7.2, containing 100 mM of MCl salt (where M = $Li^+$, $Na^+$, $K^+$, $Ca^{2+}$, and $Mg^{2+}$) and 50 μL of the EYPC/CHOL-LUV⊃HPTS were taken in a 3 mL fluorescence cuvette. To this solution 10 μL stock solution of the compound in DMSO was added. The cuvette was then kept inside the fluorescence instrument for 3 min under continuous stirring condition for equilibration. Then the HPTS fluoresce measurement ($\lambda_{em}$= 510 nm and $\lambda_{ex}$ = 450 nm) was started (t = 0 sec) and after 50 sec, 20 μL of MOH (0.75 M) solution was added into the cuvette to initiate the $Cl^-$ ion efflux kinetics and the fluorescence emission of HPTS dye was monitored. The kinetic experiment was terminated by the addition of 20 μL of Triton X-100 (20%) in the cuvette at t = 450 sec (to lyse the vesicles) and the fluorescence measurements were continued for another 50 sec (t = 500 sec). The fluorescence emission intensities (Y axis) of the HPTS dye at t = 0 and t = 500 s were normalized to 0 and 100 units, respectively.

Anion selectivity assay[1, 2, 5] ─ Anion selectivity assay was also performed using HPTS assay as described in the above section 5.1.2. In this assay, 100 mM of $Na_xA$ salt solution (where A

= $Cl^-$, $Br^-$, $I^-$, $NO_3^-$ and $OAc^-$; x = valency) was taken as external buffer. In all cases NaOH was used to initiate the transport kinetics.

Determination of the $pK_a$ values using UV-Vis Spectrophotometer[5, 7]:

The $pK_a$ values of compounds in aqueous solution were measured by monitoring the changes in absorbance of the compounds at different pH. The UV-Vis spectra of the compounds were recorded in a Perkin-Elmer Lambda 25 UV-Visible spectrophotometer and the pH measurements were performed using a HI 2210 pH meter (HANNA Instruments) at 298 K. The stock solutions of compounds were prepared in DMSO (5 mM) and appropriately diluted using 9:1 DMSO/$H_2O$ (v/v) solution containing 0.1 M NaCl to obtain a 0.5 mM solution of the compound. The pH of the solutions was varied from 4 to 10. The solutions were adjusted to acidic and basic pH using 0.1M HCl (in 0.1 M aq. NaCl) and 0.1M NaOH (in 0.1 M aq. NaCl) solutions, respectively. The absorbance values at the wavelength (nm) at which the maximum changes were observed were plotted against the log[pH] values. The $pK_a$ values were determined from the sigmoidal curves obtained from the plot of log[pH] *vs.* absorbance.

**Chloride efflux studies at different pH using ISE[5]:**

The chloride efflux efficiency of the potent compounds was measured at different pH using ISE. The EYPC/CHOL-LUVs were prepared following a similar method as described in the above section using 20 mM phosphate buffer containing 100 mM NaCl at different pH (pH 7.2 and pH 5.5). The LUVs were first dialyzed against 100 mM $NaNO_3$ (iso-osmolar with the NaCl buffer) in 20 mM phosphate buffer of appropriate pH. Then, the chloride efflux efficiency of the compounds was measured according to the above mentioned method for both the buffer solutions at different pH (same pH buffer was used in both extra and intra-vesicular solution).

Measurement of half maximal effective concentrations ($EC_{50}$) of the compounds at different pH from chloride efflux studies[5] ─ The chloride efflux efficiency at various concentration of compounds were measured at different pH (either pH 7.2 or pH 5.5) to determine the $EC_{50}$ values (i.e. half maximal effective concentration). The efflux efficiency (Y axis) obtained from the chloride ISE measurements were normalized [t = 0 to t = 700 s (X axis)]. The normalized efflux efficiency (EE) values at t = 500 s (prior to the addition of Triton X-100) were considered as the transport activity of the compounds. The efflux efficiency (EE) of a compound at a particular concentration was determined by using the equation Eq.-2. To get the effective concentration ($EC_{50}$) of the compound, the chloride efflux efficiency values were plotted against concentration (mol% with respect to lipid) and fitted in the sigmoidal equation.

$Chloride\ Efflux\ Efficiency,$

$$EE = EE_0 + \frac{EE_\infty - EE_0}{[1+10^{(logEC_{50}-logc)n}]} \quad \text{..... Eq.-4}$$

Here, $EE_0$ and $EE_\infty$ correspond to the efflux efficiency obtained at the lowest and at highest concentrations of the compound, respectively. The '*c*' represents concentration (mol% with respect to lipid) of the compounds. During calculations the ISE data were subtracted from the control experiment. The number of molecules required for the efflux and/or influx of a single ion is given by 'n' and it is the Hill coefficient for the compound.

Evidence for the HCl co-transport:

HPTS assay without applying any pH gradient[5] ─ First 2940 µL of 20 mM HEPES buffer, pH 7.2, containing 100 mM NaX, (where, $X^-$ = $Cl^-$, $Br^-$, $I^-$, $NO_3^-$ and $SO_4^{2-}$) and 50 µL of the EYPC/CHOL-LUV⊃HPTS were taken in a 3 mL fluorescence cuvette and the cuvette was placed inside the fluorescence spectrophotometer at 37 ºC under mild stirring condition. After

that the HPTS fluorescence intensity was monitored (from, t = 0 sec) at 510 nm ($\lambda_{ex}$ = 450 nm). The compounds (10 μL of the stock solution in DMSO) were added to the solution to initiate the $Cl^-$ transport kinetics. After 400 sec the kinetic experiment was terminated by adding 20 μL of 20% Triton-X100 solution (to rupture pH gradient completely) and the fluorescence measurements were continued for another 50 sec (t = 500 sec). Similar experiments were performed at pH 5.5 using citrate buffer containing 100 mM salt.

Ion transport activity in the presence of FCCP (FCCP assay)[2, 8] ─ The vesicles were prepared by following the same procedure as discussed in the section 5.1.1. The ion transport activity was measured in the absence and presence of FCCP ($H^+$ selective transporter). First 2920 μL of 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl and 50 μL of the EYPC/CHOL-LUV⊃HPTS were taken in a 3 mL fluorescence cuvette and the cuvette was placed in the fluorescence spectrophotometer at 37 ºC under mild stirring condition. After that, the compound (8 μL of the stock solution in DMSO) and 2 μL of FCCP solution in DMSO (4 μM) were added to the solution. The cuvette was then kept inside the fluorescence instrument under stirring condition for 3 minutes to allow maximum incorporation of the compounds into the lipid bilayers. After that the HPTS fluorescence intensity was monitored (t = 0 sec) at 510 nm ($\lambda_{ex}$ = 450 nm). Subsequently, 20 μL of NaOH (0.75 M) solution was added into the cuvette after 50 sec to create a pH gradient (ΔpH = ~ 0.6) between the extra and intra-vesicular regions and to initiate the $Cl^-$ transport kinetics. After 450 sec the kinetic experiment was terminated by adding 20 μL of 20% Triton-X100 solution (to rupture the vesicular arrangements) into the cuvette and the fluorescent measurements were continued for another 50 sec (t = 500 sec). The control experiment was performed in the absence of FCCP also.

Ion transport activity in presence of Valinomycin (Valinomycin assay)[2, 8] ─ The vesicles were prepared by following the exact same procedure as discussed in the above section. The ion transport activity was measured in the absence and presence of valinomycin. First 2920 μL of 20 mM HEPES buffer, pH 7.2, containing 100 mM KCl and 50 μL of the EYPC/CHOL-LUV⊃HPTS were taken in a 3 mL fluorescence cuvette and the cuvette was placed in the fluorescence spectrophotometer at 37 ºC under mild stirring condition. After that, the compound (8 μL of the stock solution in DMSO) and 2 μL of Valinomycin solution in DMSO (4 nM) were added to the solution. The cuvette was then kept inside the fluorescence instrument under stirring condition for 3 minutes to allow maximum incorporation of the compounds into the lipid bilayers. After that the HPTS fluorescence intensity was monitored (t = 0 sec) at 510 nm ($\lambda_{ex}$ = 450 nm). Subsequently, 20 μL of NaOH (0.75 M) solution was added into the cuvette after 50 sec to create a pH gradient (ΔpH = ~ 0.6) between the extra and intra-vesicular regions and to initiate the $Cl^-$ transport kinetics. After 450 sec the kinetic experiment was terminated by adding 20 μL of 20% Triton-X100 solution (to rupture the vesicular arrangements) into the cuvette and the fluorescent measurements were continued for another 50 sec (t = 500 sec).

U-Tube experiment for HCl cotransport[2, 8] ─ In the U-tube experiment $CHCl_3$ solvent, segregating the two aqueous phases was used to mimic the lipid bilayer. For the measurements of ion transport efficacy of the compounds, at first 0.2 mM solution of the compounds dissolved in $CHCl_3$ (20 mL) was placed in a U-tube (1.5 cm cone with 15 cm arm length). A tiny magnet was placed into the $CHCl_3$ solution to maintain the equilibrium in the organic phase solution. Then, the left arm of the U-tube was filled with 10 mL of 100 mM aqueous HCl solution (pH = 1.2) and the right arm was filled with 10 mL of 100 mM aqueous $NaNO_3$ solution. The changes in pH along with $Cl^-$ ion concentration in the right arm

aqueous solution of U-tube was measured time to time by employing pH meter and ISE, respectively (up to 70 hours). The changes of pH and $Cl^-$ concentrations at the right arm aqueous solution of the U-tube confirmed the transport of HCl from the left arm to the right arm.

Evidence for the carrier mechanism:

Preparation of DPPC-LUV⊃lucigenin[2] ─ For the preparation of DPPC-LUV⊃lucigenin, first 50 μL of DPPC (100 mg/mL stock in de-acidified $CHCl_3$) was taken in acid washed clean and dry glass vial and the organic solvent was removed under reduced pressure for 6 hours at room temperature. The dry thin film was then hydrated with 500 μL of 20 mM HEPES buffer, pH 7.2, containing 100 mM $NaNO_3$ and 1 mM lucigenin. The solution was then vortexed for 20 min at room temperature and sonicated for 30 min at 50 °C. After that, the solution was subjected to freeze-thaw cycle for 13-14 times. The vesicle solution was then extruded through a polycarbonate membrane having pore size of 200 nm for 21-times. Finally, gel filtration (Sephadex G-50) column chromatography was performed with 20 mM HEPES buffer, pH 7.2, containing 100 mM $NaNO_3$ as running solution to remove free lucigenin present in the extravesicular solution. The final volume of the collected vesicle solution was adjusted to 500 μL with 20 mM HEPES buffer pH 7.2 containing 100 mM $NaNO_3$. The final lipid concentration was 14 mM (assuming 100% lipid regeneration).

Transport activity across DPPC-LUV⊃lucigenin (DPPC-lucigenin assay)[2, 4] ─ The transport activity of the compounds using this DPPC-lucigenin assay was measured using fluorescence spectrophotometer. In this assay, 2890 μL of 20 mM HEPES buffer, pH 7.2, containing 100 mM $NaNO_3$ and 50 μL of the DPPC-LUV⊃lucigenin were taken in a 3 mL fluorescence cuvette. After that, 50 μL of NaCl solution (stock solution of 2 M) was added to the cuvette..

The kinetic experiment was started (at t = 0 s) and lucigenin fluorescence emission was monitored at 506 nm (excited at 455 nm). The cuvette was then kept under stirring condition and the chamber temperature was set to 25 ºC. After 50 sec, compound (10 µL of DMSO stock solution of the compound) was added to initiate the $Cl^-$ influx kinetics. Finally, to terminate the kinetic experiment, the vesicles were lysed by adding 20% Triton X-100 (20 µL) in the cuvette at t = 450 sec and fluorescent measurements were continued for another 50 sec (t = 500 sec). A similar measurement was performed to investigate the transport efficiency of the compound at 45 ºC. The time-dependent lucigenin fluorescence plot was fitted with 1$^{st}$ order exponential decay equation to calculate the half-life and initial rate at different temperatures.

Studies on the Stability of HPTS encapsulated vesicles in the presence of compounds:

Test for the leaching-out of the compounds from the membrane bilayer environment[2] ─ To examine whether the compounds get leached out during the transport activity measurement under the vesicular environment the leaching test was performed. It is hypothesized that if the transporters leached out from the membrane bilayer environment to the aqueous medium then transport efficiency of the compounds will be greatly affected (reduced) by the dilution of the vesicular solution. Whereas, dilution factor will not affect the transport efficiency if the transporters are localized inside the bilayer environment. In this assay, various concentration of EYPC/CHOL-LUV⊃HPTS vesicles in 20 mM HEPES buffer, pH 7.2 containing 100 mM $NaNO_3$ (final concentration of the vesicles were 300 µM, 400 µM, 500 µM and 600 µM) were taken in a 3 mL fluorescence cuvette. Consequently, 10 µL DMSO solution of the compound was added to the cuvette (to maintain a fixed anionophore/lipid ratio in all cases). The cuvette was then kept inside the fluorescence instrument under continuous stirring condition for 3 minutes for maximum incorporation of the compounds into the vesicles and

the HPTS assay was performed as mentioned earlier. The kinetic experiments showed no change of transport activity in four different lipid concentrations for compound, confirming the non-leaching property of the anionophores.

Calcein leakage assay[9] ─ The vesicles were prepared by following the same procedure as discussed in the section 5.1.1, where 50 mM calcein was used for the vesicle preparation instead of 1 mM HPTS. The phosphate and citrate buffers were used for the preparation of EYPC/CHOL-LUV⊃calcein at pH 7.2 and 5.5, respectively. Sodium nitrate solution (100 mM) was used as running solution. First, 2940 μL of 20 mM phosphate buffer, pH 7.2, containing 100 mM NaCl and 50 μL of the EYPC/CHOL-LUV⊃calcein were taken in a 3 mL fluorescence cuvette and the cuvette was placed in the fluorescence spectrophotometer at 37 ºC under slow stirring condition. The cuvette was then kept inside the fluorescence instrument under stirring condition for 3 minutes to allow maximum incorporation of the compounds into the lipid bilayers. The calcein fluorescence intensity was monitored (t = 0 sec) at 520 nm ($\lambda_{ex}$ = 490 nm). Subsequently, 10 μL of the compound (stock solution in DMSO) was added to the solution and the change in calcein fluorescence intensity was measured. After 450 sec the kinetic experiment was terminated by adding 20 μL of 20% Triton-X100 solution (to rupture the vesicle completely) into the cuvette and the fluorescent measurements were continued for another 50 sec (t = 500 sec).

Determination of the apparent pKa of the compounds within the membrane bilayer using ion selective electrode[5]:

The pH-dependent (pH from 4-9) chloride efflux efficiency of the compounds were measured using ISE. The EYPC/CHOL-LUVs were prepared following a similar method as described in the above section 5.2.2 using 20 mM phosphate buffer containing 100 mM NaCl at various

pH (9.0, 8.2, 7.2, 6.2, 5.5, 5.0 and 4.0), independently. The prepared LUVs were dialyzed against 100 mM $NaNO_3$ (iso-osmolar with the NaCl buffer) in 20 mM phosphate buffer of the appropriate pH. The chloride efflux efficiency measurements were performed according to the above mentioned method for all the pH solutions (same pH buffer was used in both extra and intra-vesicular solution).

Density functional theory (DFT) studies:

The density functional theory (DFT) is recognized as one of the most successful approaches in analyzing the structural and electronic properties of molecules and their interacting systems. Investigation of various non-covalent interactions like hydrogen bond and charge distribution of the molecules or atom are widely used. We performed DFT analysis (using the Gaussian 09 program) to explore the stability of compound-$Cl^-$ ion complex and interacting patterns of compound with the $Cl^-$ ion where the molecule 1d behave as host and the chloride ion behave as guest molecules in the complex system. All the considered electronic structure were fully optimized at B3LYP/6-31+G(d) and B3LYP/6-31++G(d,p) level of theories using Gaussian 09 program package.[2, 10, 11] The analysis showed that the $Cl^-$ ion interacts with neutral, monoprotonated and diprotonated compound. The interatomic bond distance [$r$ ($Cl^-$----H)] and the interaction energies were tabulated (Table S3). The interaction energies as well as the numbers of effective hydrogen bonds with the $Cl^-$ ion are increasing with increasing the protonation at the benzimidazole moiety which strongly support its higher ion recognition capability and also support the role of the additional proton in the benzimidazole moiety. These results strongly support the ion recognition and transport capabilities of these compounds both in neutral and acidic conditions. Similar calculations were also performed for compound 2 and the calculated bond distances as well as the interaction energy were

tabulated in Table S3. The DFT calculations with two different levels indicate that the level of the calculations does not affect the trend of the results as clear from Table S3.

Biological Activity Studies:

*MTT-based cytotoxicity assay* ─ BHK-21, MCF-7 and T-47D cells were seeded in a 96-well flat bottom tissue culture plates at a density of $10^5$ cells/well (per 100 µL). The cells were incubated at 37 °C and 5% $CO_2$ for 16 h. The media was discarded and each well was washed with PBS.[1, 2, 12] The compounds were added with media to each well in different concentrations and incubated for 24 h. After that incubation, 10 µL of MTT solution (5 mg MTT/mL of PBS) was added and cells were incubated for 4 h. MTT containing media was removed from each well and 100 µL of DMSO was added (in each well) to dissolve the formazan crystals. The absorbance was recorded in a microplate reader (Multiskan™ GO) at the wavelength of 570 nm. All experiments were performed in triplicate, and the relative cell viability (%) was expressed as a percentage relative to the untreated cells.

*Chloride mediated cell death studies* ─ MTT assay was performed using HBSS (Hank's balanced salt solution) buffer in the absence and presence of $Cl^-$ ion. T-47D cell line was selected for this study. Cells were maintained and prepared according to the mentioned method.[1, 2, 12] Cell culture media was replaced by HBSS buffer (either with $Cl^-$ or without $Cl^-$ ion) containing 10% FBS. Compound was added to each well in different concentration and incubated for 24 h. After that HBSS buffer solution was replaced by MTT-DMEM mixture and incubated for additional 4 h. MTT containing media was removed from each well and 100 µL of DMSO was added (in each well) to dissolve the formazan crystals. The absorbance was recorded in a microplate reader (Multiskan™ GO) at the wavelength of 570 nm. All experiments were performed in triplicate, and the relative cell viability (%) was expressed as

a percentage relative to the untreated cells. We also performed MTT assay in the absence and presence of compounds at pH 6.5 using HBSS buffer containing $Cl^-$ ion and10% FBS using MCF-7 cell line. The incubation time was 8 hours.

Hank's balanced salt solution with $Cl^-$ ion was prepared using 136.9 mM NaCl, 5.5 mM KCl, 0.34 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 0.81 mM $MgSO_4$, 1.25 mM $CaCl_2$, 5.5 mM D-glucose, 4.2 mM $NaHCO_3$ and 10 mM HEPES (pH 7.4).

Hank's balanced salt solution without $Cl^-$ ion was prepared using 136.9 mM Na-gluconate, 5.5 mM K-gluconate, 0.34 mM $Na_2HPO_4$, 0.44 mM $KH_2PO_4$, 0.81 mM $MgSO_4$, 1.25 mM Ca-gluconate, 5.5 mM D-glucose, 4.2 mM $NaHCO_3$ and 10 mM HEPES (pH 7.4).

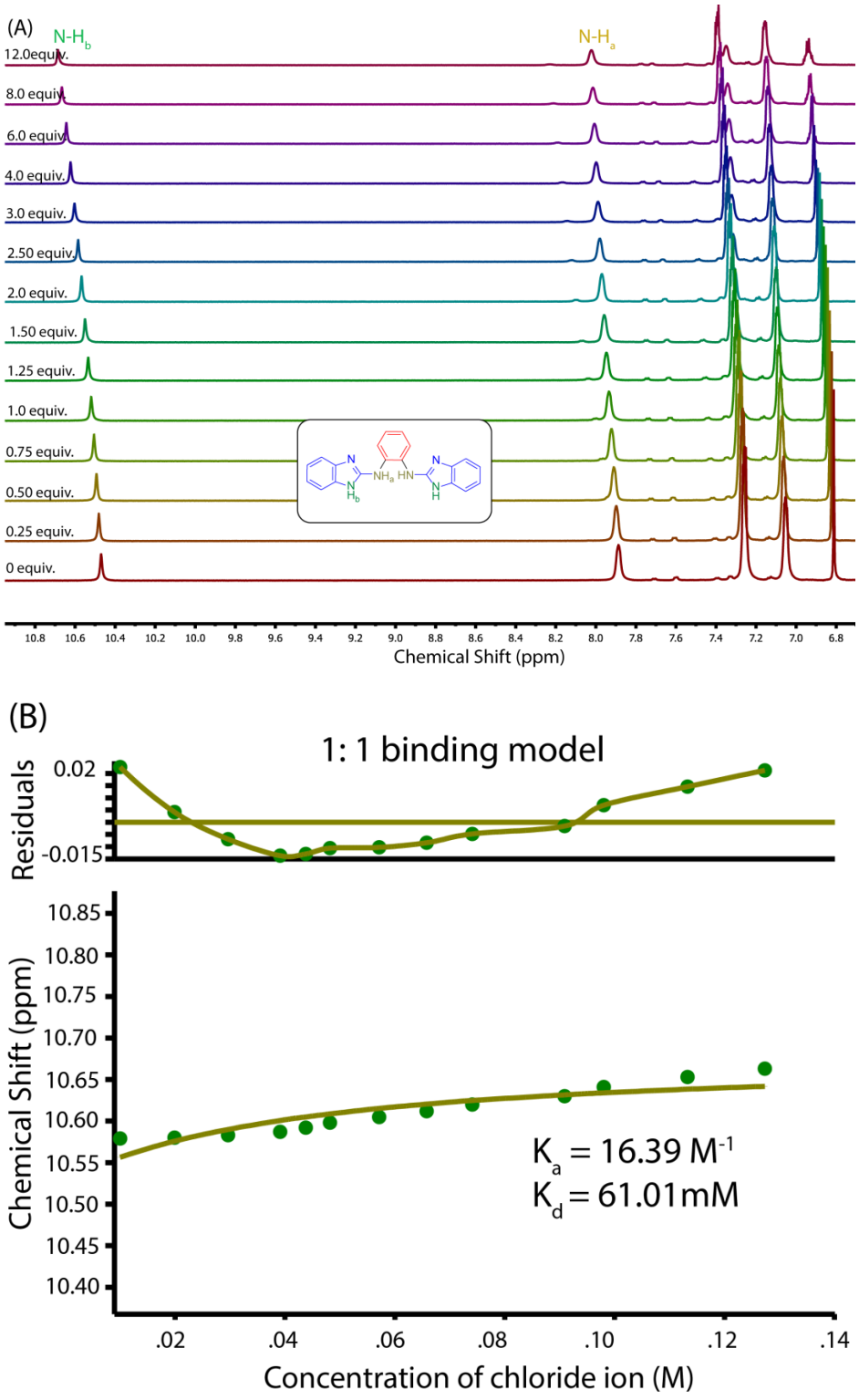



**Figure S1:** $^{1}$H NMR (600MHz) titration spectra for compound **1a** with sequential addition of TBACl in DMSO-$d_6$ solvent. The amounts of added TBACl are shown on the spectra (**A**). Plot of concentration of TBACl *vs* chemical shift of $^{1}$H signal, fitted to 1:1 binding model of WinEQNMR2 program (**B**).

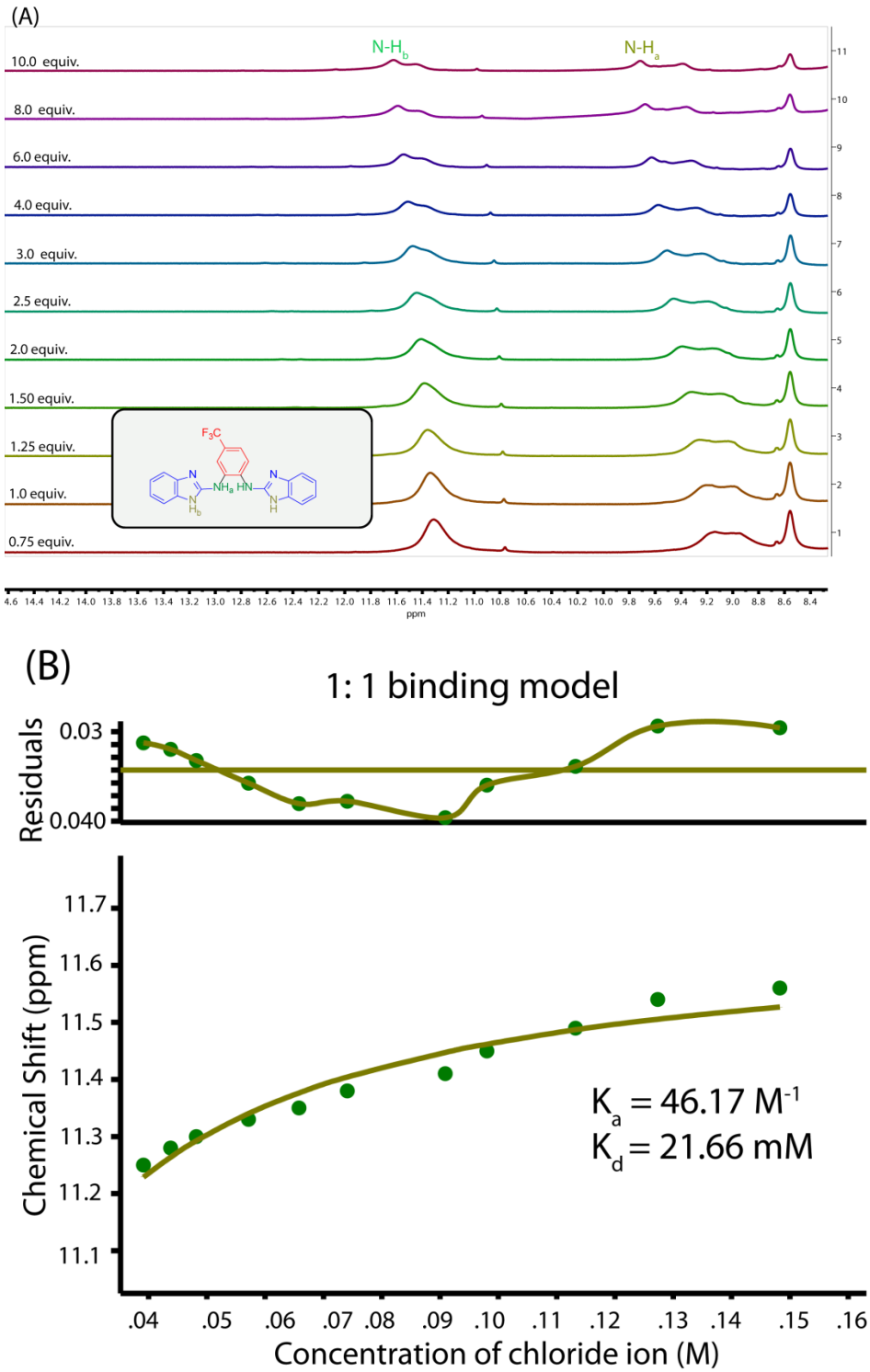
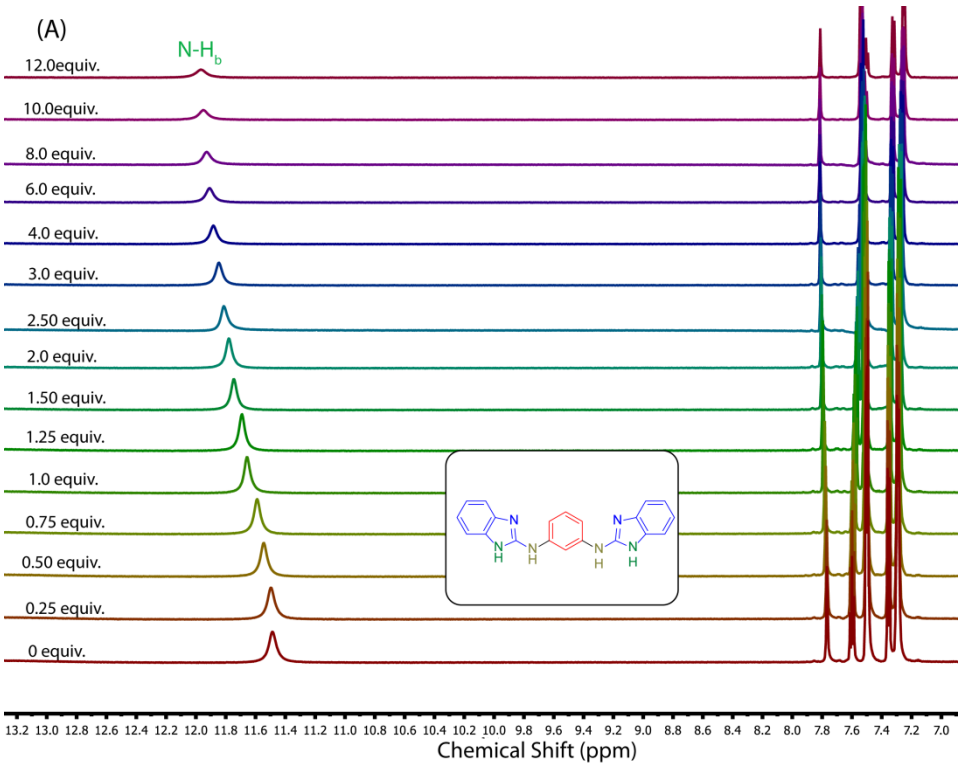



**Figure S2:** $^1$H NMR (600MHz) titration spectra for compound **1d** with sequential addition of TBACl in DMSO-$d_6$ solvent. The amounts of added TBACl are shown on the spectra (**A**). Plot of concentration of TBACl *vs* chemical shift of $^1$H signal, fitted to 1:1 binding model of WinEQNMR2 program (**B**).



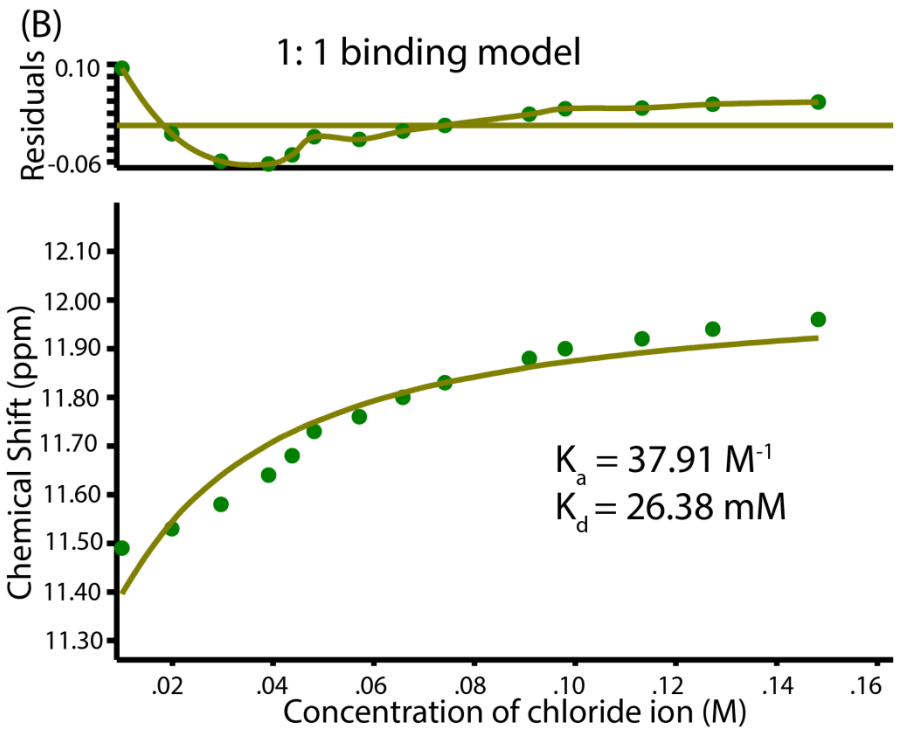




**Figure S3:** $^{1}$H NMR (600MHz) titration spectra for compound **2** with sequential addition of TBACl in DMSO-d$_6$ solvent. The amounts of added TBACl are shown on the spectra (**A**). Plot of concentration of TBACl *vs* chemical shift of $^{1}$H signal, fitted to 1:1 binding model of WinEQNMR2 program (**B**).

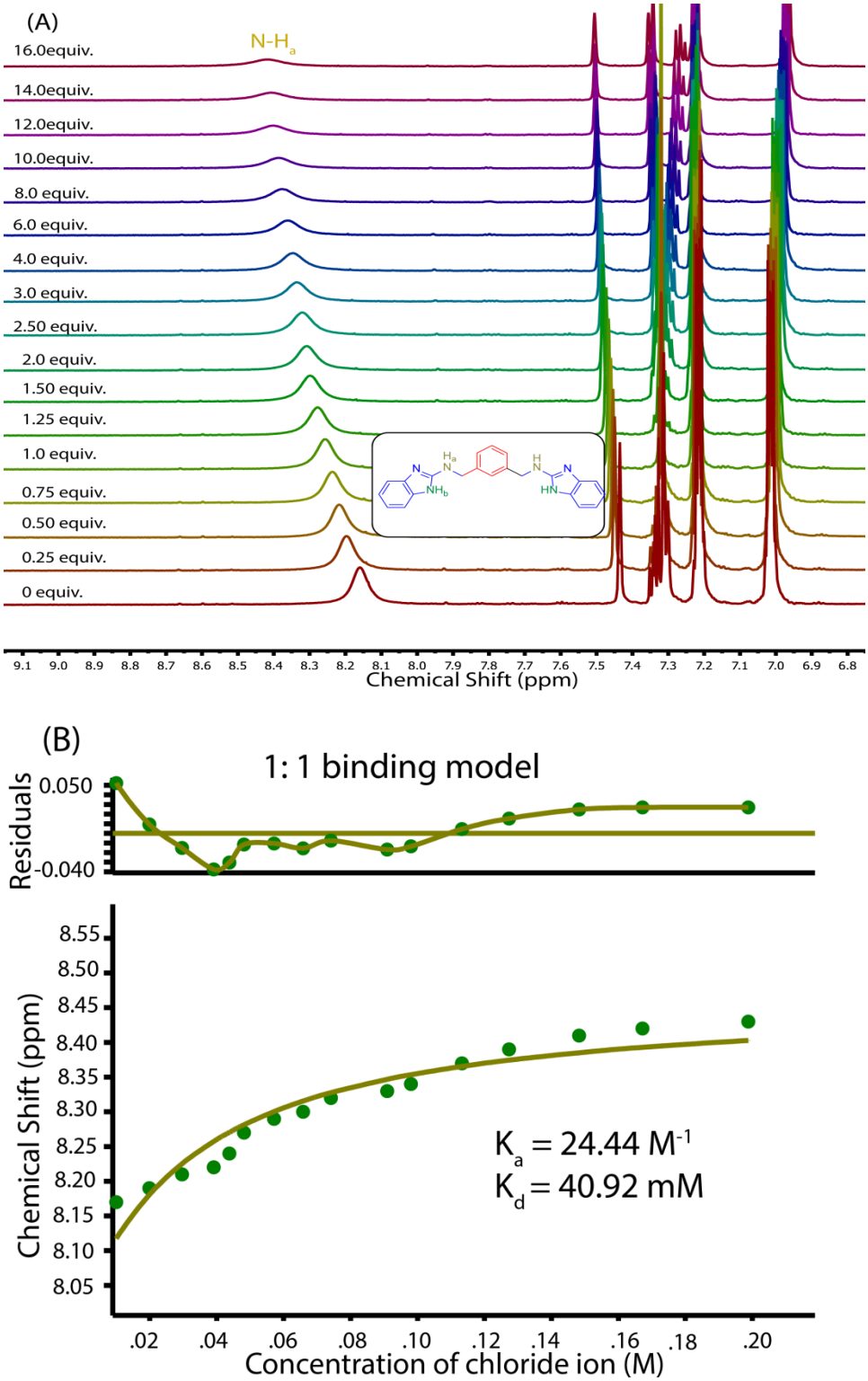



**Figure S4:** $^{1}$H NMR (600MHz) titration spectra for compound **3** with sequential addition of TBACl in DMSO-$d_6$ solvent. The amounts of added TBACl are shown on the spectra (**A**). Plot of concentration of TBACl *vs* chemical shift of $^{1}$H signal, fitted to 1:1 binding model of WinEQNMR2 program (**B**).

**Table S1:** Calculation and Result table for Job's plot analysis**.**

| Sample No. | Host conc. ([H], M) | Guest conc. ([G], M) | [H] + [G] (M) | [H]/ ([H]+[G]) | δ of proton | Δδ | {[H] / ( [H] +[G])}*Δδ |
|---|---|---|---|---|---|---|---|
| **JB-1-1d** | 0.5 | 4.5 | 5.0 | 0.1 | 11.33 | 0.22 | 0.022 |
| **JB-2-1d** | 1.0 | 4.0 | 5.0 | 0.2 | 11.31 | 0.2 | 0.04 |
| **JB-3-1d** | 1.5 | 3.5 | 5.0 | 0.3 | 11.27 | 0.16 | 0.048 |
| **JB-4-1d** | 2.0 | 3.0 | 5.0 | 0.4 | 11.24 | 0.13 | 0.052 |
| **JB-5-1d** | 2.5 | 2.5 | 5.0 | 0.5 | 11.22 | 0.11 | 0.055 |
| **JB-6-1d** | 3.0 | 2.0 | 5.0 | 0.6 | 11.19 | 0.08 | 0.048 |
| **JB-7-1d** | 3.5 | 1.5 | 5.0 | 0.7 | 11.17 | 0.06 | 0.042 |
| **JB-8-1d** | 4.0 | 1.0 | 5.0 | 0.8 | 11.14 | 0.03 | 0.024 |
| **JB-9-1d** | 4.5 | 0.5 | 5.0 | 0.9 | 11.13 | 0.02 | 0.018 |
| **JB-10-1d** | 5.0 | 0.0 | 5.0 | 1.0 | 11.11 | 0.00 | 0.00 |

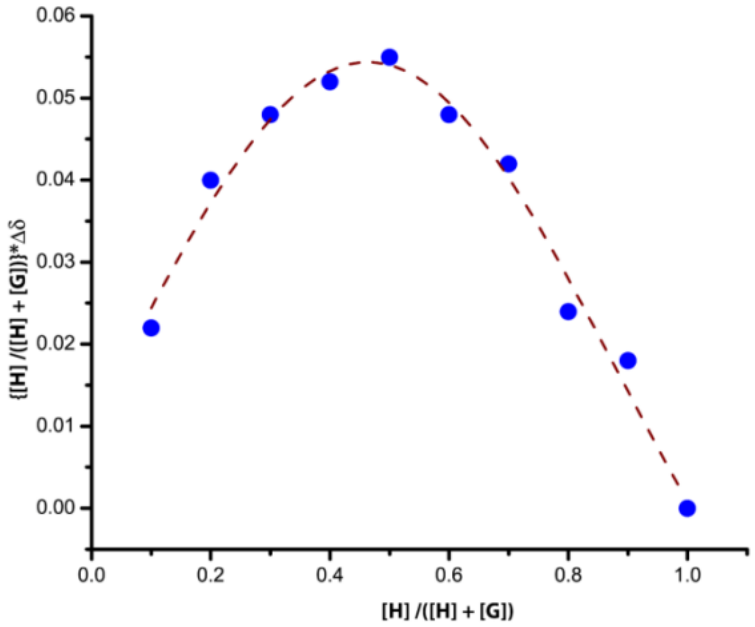



**Figure S5:** Job's plot for the compound **1c** (according to the Table S1).

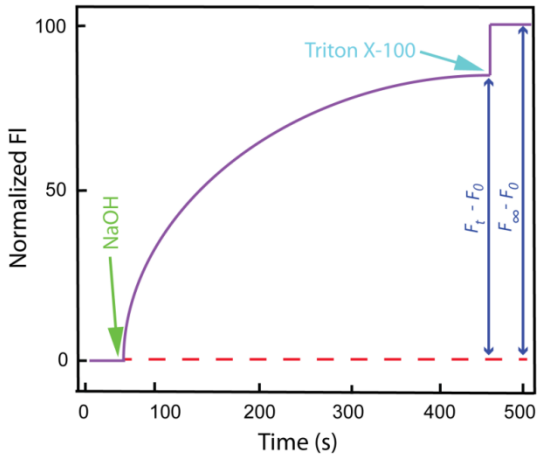



**Figure S6.** Representations of HPTS fluorescence based ion transport kinetics using EYPC/CHOL-LUV⊃HPTS.

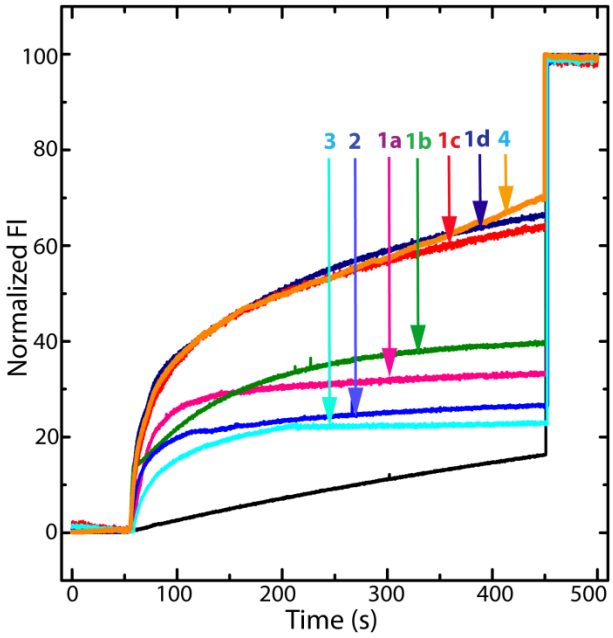



**Figure S7.** Transmembrane $Cl^-$ ion transport activity of the bis(benzimidazole) derivatives (**1-3**) as measured by HPTS assay at pH 7.2. Compound concentration = 5 mol% with respect to lipid.

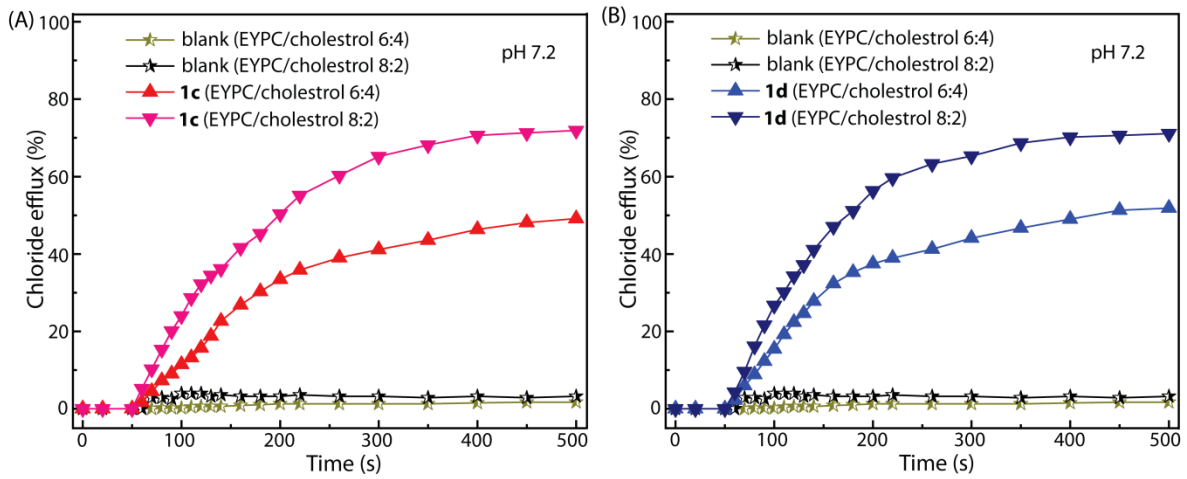



**Figure S8.** Transmembrane $Cl^-$ ion transport activity of the bis(benzimidazole) derivatives (**1c** and **1d**) as measured by HPTS assay at pH 7.2. Compound concentration = 5 mol% with respect to lipid.

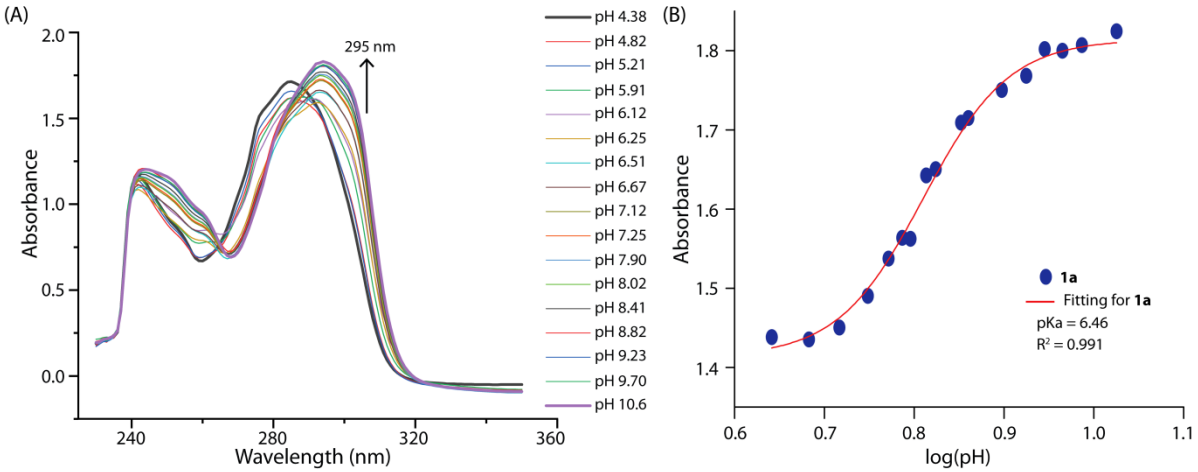



**Figure S9.** Absorbance spectra of compound **1a** (0.5 mM) at different pH in 9:1 DMSO/$H_2O$ (v/v) solution containing 0.1 M NaCl (A). Comparison plots of absorbance at 295 nm at different pH (B).

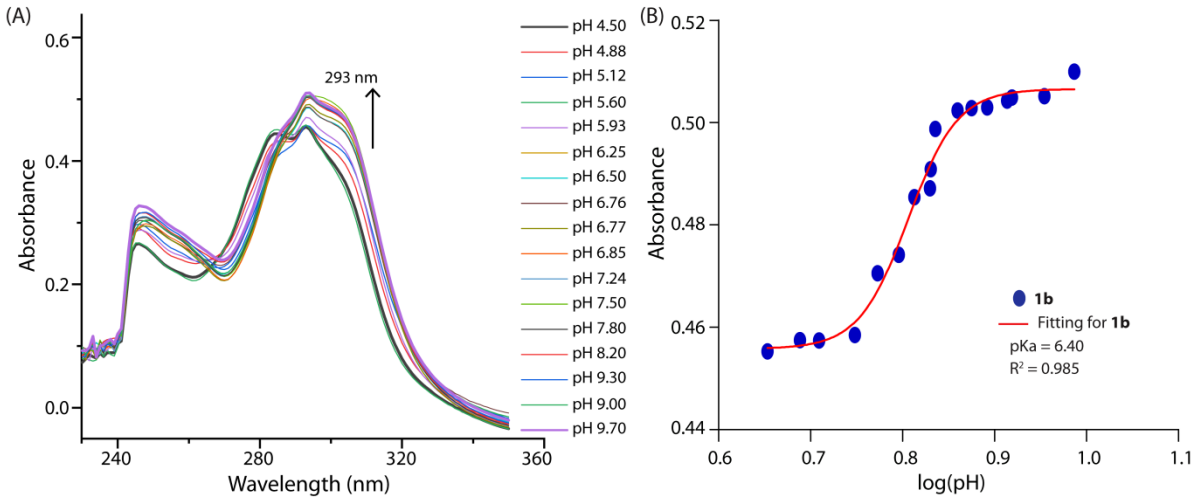



**Figure S10.** Absorbance spectra of compound **1b** (0.5 mM) at different pH in 9:1 DMSO/$H_2O$ (v/v) solution containing 0.1 M NaCl (A). Comparison plots of absorbance at 293 nm at different pH (B).

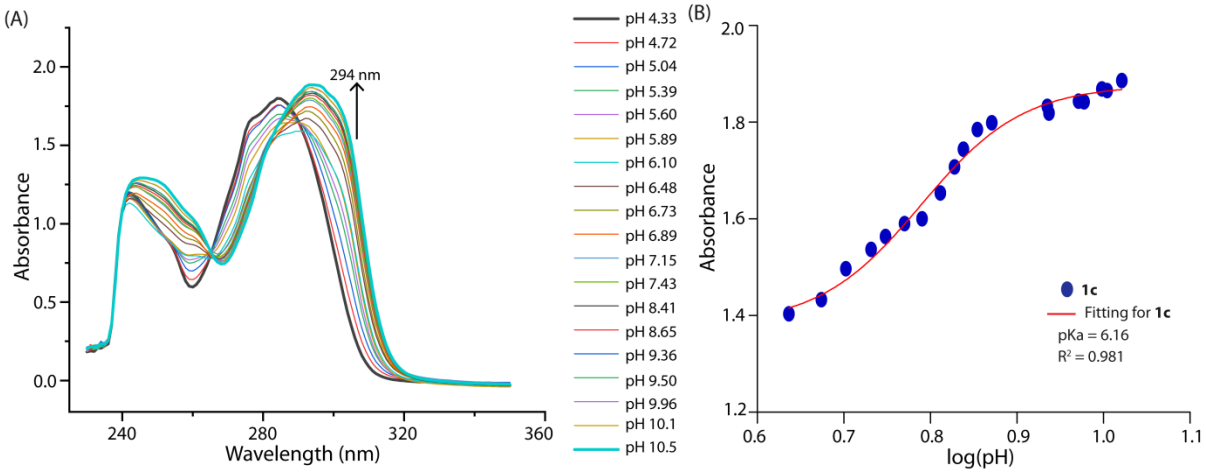



**Figure S11.** Absorbance spectra of compound **1c** (0.5 mM) at different pH in 9:1 DMSO/$H_2O$ (v/v) solution containing 0.1 M NaCl (A). Comparison plots of absorbance at 294 nm at different pH (B).

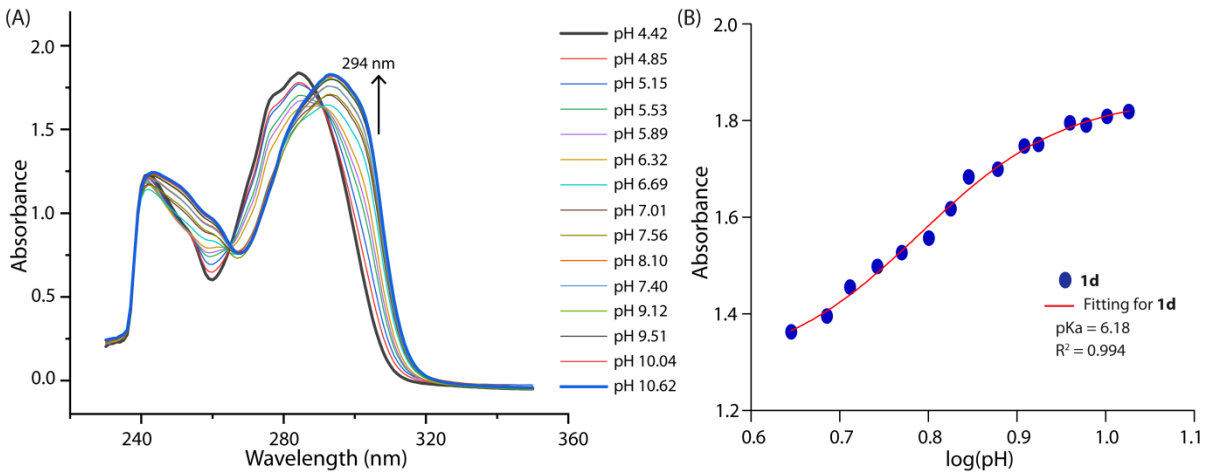



**Figure S12.** Absorbance spectra of compound **1d** (0.5 mM) at different pH in 9:1 DMSO/$H_2O$ (v/v) solution containing 0.1 M NaCl (A). Comparison plots of absorbance at 294 nm at different pH (B).

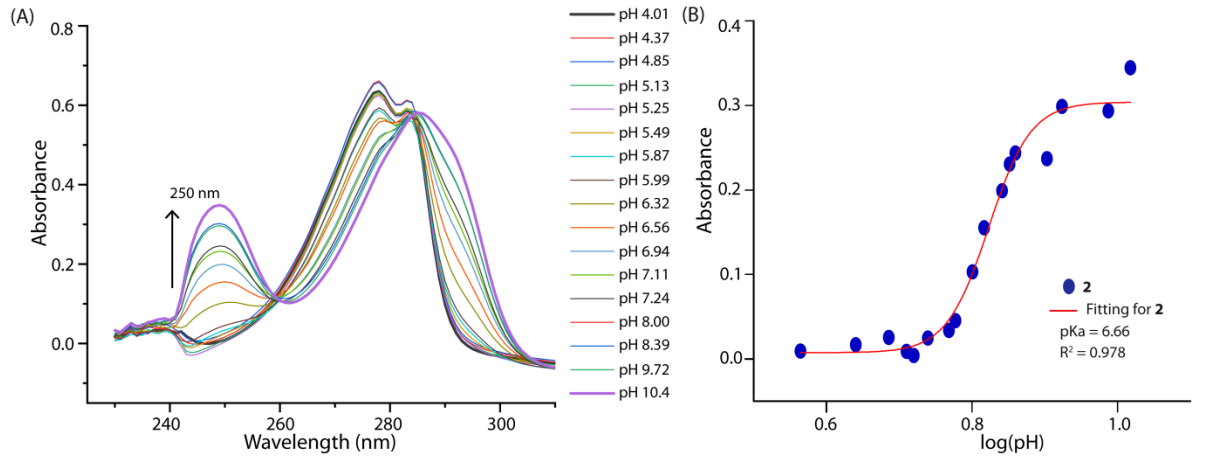



**Figure S13.** Absorbance spectra of compound **2** (0.5 mM) at different pH in 9:1 DMSO/$H_2O$ (v/v) solution containing 0.1 M NaCl (A). Comparison plots of absorbance at 250 nm at different pH (B).

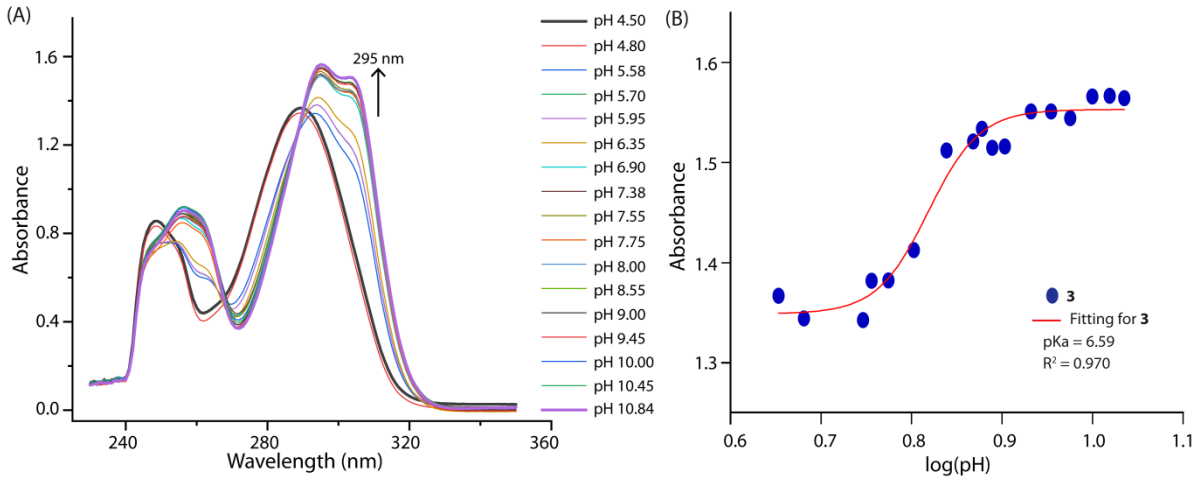



**Figure S14.** Absorbance spectra of compound **3** (0.5 mM) at different pH in 9:1 DMSO/$H_2O$ (v/v) solution containing 0.1 M NaCl (A). Comparison plots of absorbance at 295 nm at different pH (B).

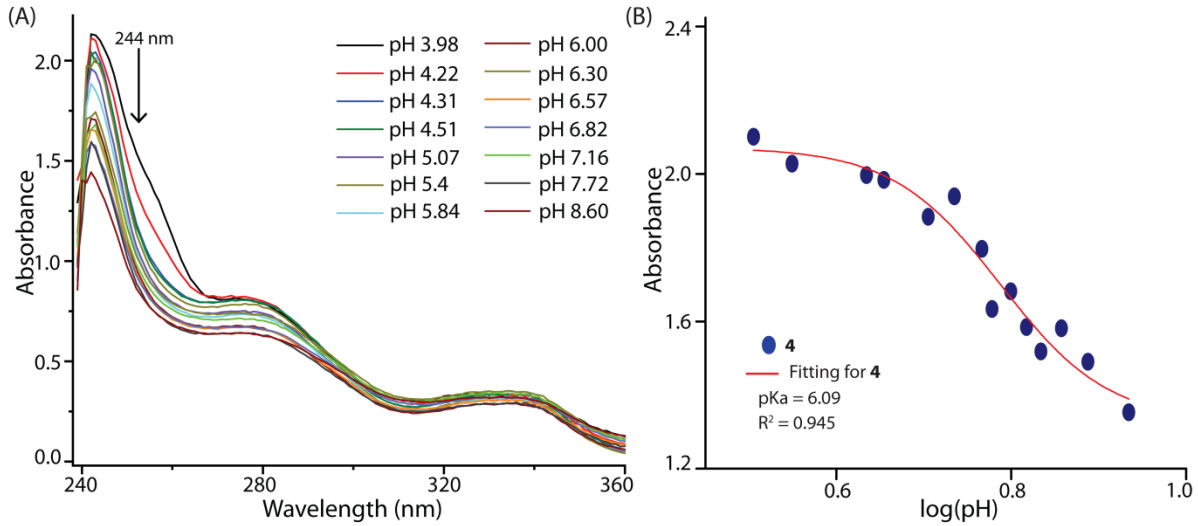



**Figure S15.** Absorbance spectra of compound **4** (0.5 mM) at different pH in 9:1 DMSO/$H_2O$ (v/v) solution containing 0.1 M NaCl (A). Comparison plots of absorbance at 244 nm at different pH (B).

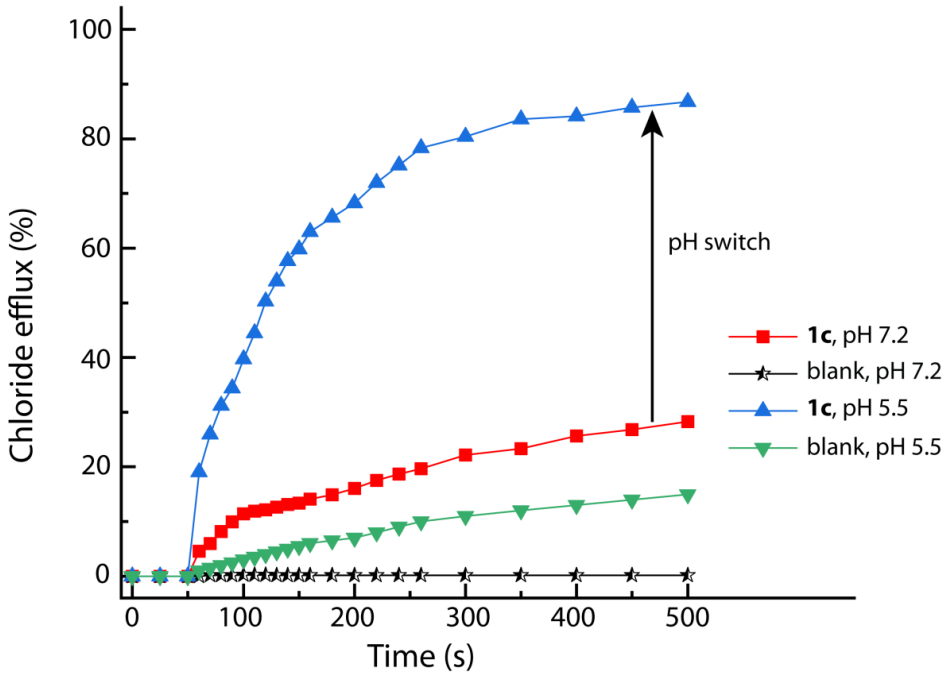



**Figure S16.** Chloride ion efflux capability of compound **1c** (0.5 mol% with respect to lipid) at pH 5.5 and 7.2.

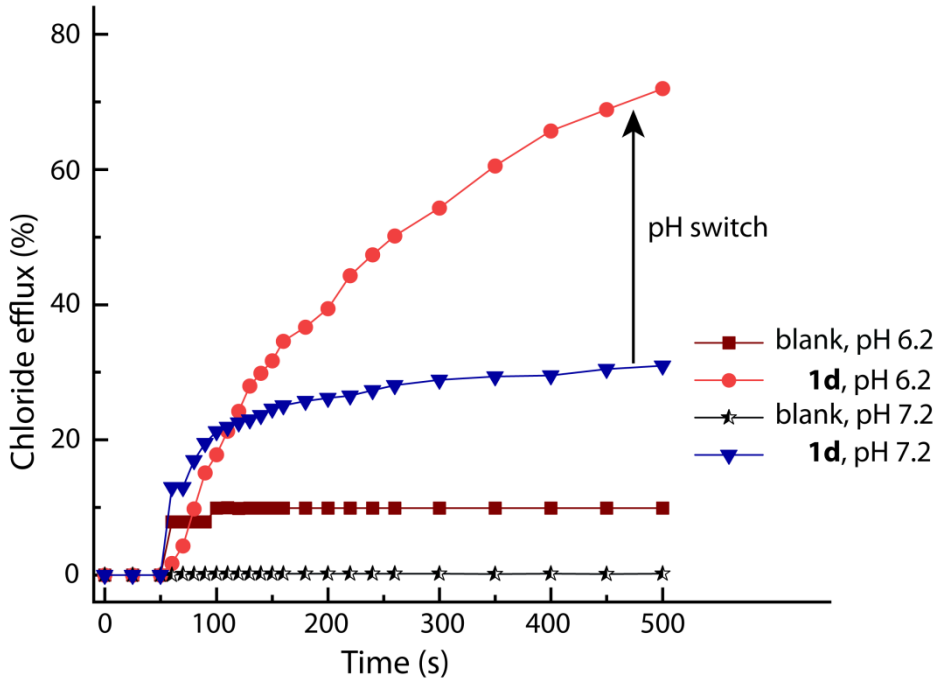



**Figure S17.** Chloride ion efflux capability of compound **1d** (0.5 mol% with respect to lipid) at pH 6.2 and 7.2.

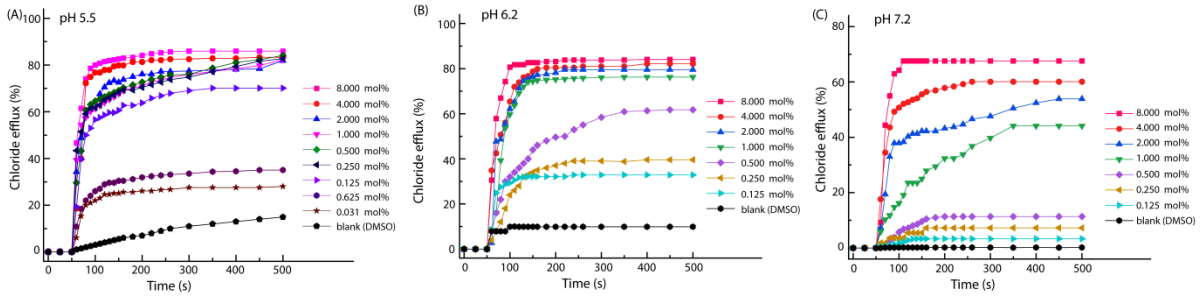



**Figure S18.** Chloride ion transport assays of compound **1a** by chloride ion selective electrode using EYPC/CHOL-LUVs at pH 5.5 (A), 6.2 (B) and 7.2 (C).

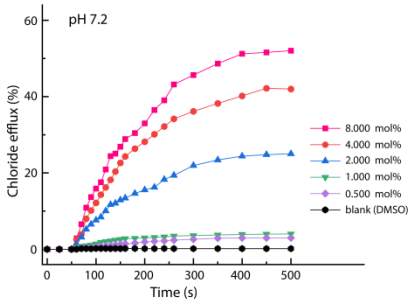



**Figure S19.** Chloride ion transport assays of compound **1b** by chloride ion selective electrode using EYPC/CHOL-LUVs at pH 7.2.

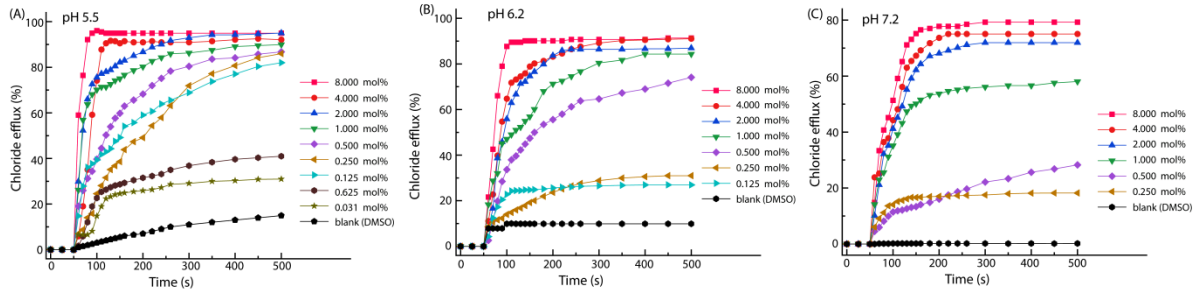



**Figure S20.** Chloride ion transport assays of compound **1c** by chloride ion selective electrode using EYPC/CHOL-LUVs at pH 5.5 (A), 6.2 (B) and 7.2 (C).

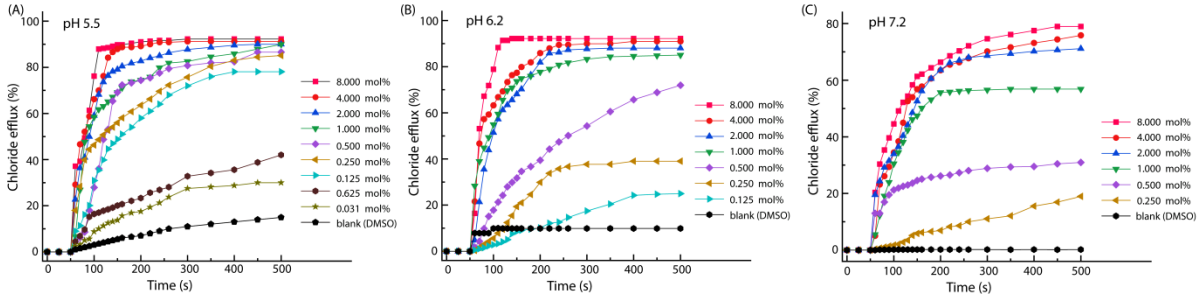



**Figure S21.** Chloride ion transport assays of compound **1d** by chloride ion selective electrode using EYPC/CHOL-LUVs at pH 5.5 (A), 6.2 (B) and 7.2 (C).

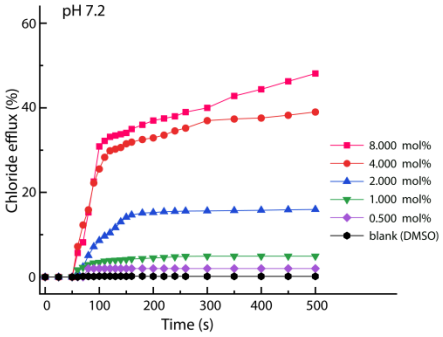



**Figure S22.** Chloride ion transport assays of compound **2** by chloride ion selective electrode using EYPC/CHOL-LUVs at pH 7.2.

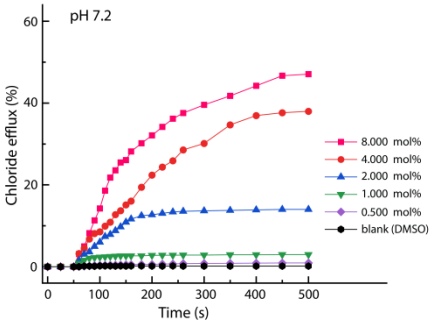



**Figure S23.** Chloride ion transport assays of compound **3** by chloride ion selective electrode using EYPC/CHOL-LUVs at pH 7.2.

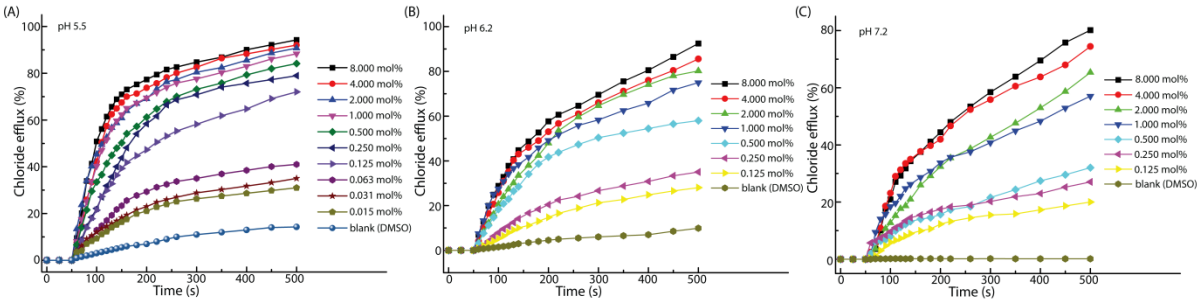



**Figure S24.** Chloride ion transport assays of compound **4** by chloride ion selective electrode using EYPC/CHOL-LUVs at pH 5.5 (A), 6.2 (B) and 7.2 (C).

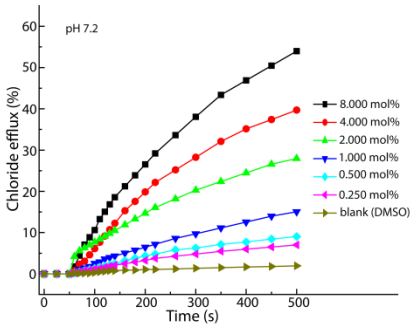



**Figure S25.** Chloride ion transport assays of compound **5** by chloride ion selective electrode using EYPC/CHOL-LUVs at pH 7.2.

The chloride influx-efflux process through membrane in the solution is an equilibrium process. Several experiments showed that synthesized compounds follow carrier mechanism. To get 100% chloride efflux, the liposome membrane supposed to be completely destroyed. Particularly, calcein assay shows that our compound does not destroy the membrane. However, the initial blast may be due to higher chloride efflux ability by the compounds at higher concentration.

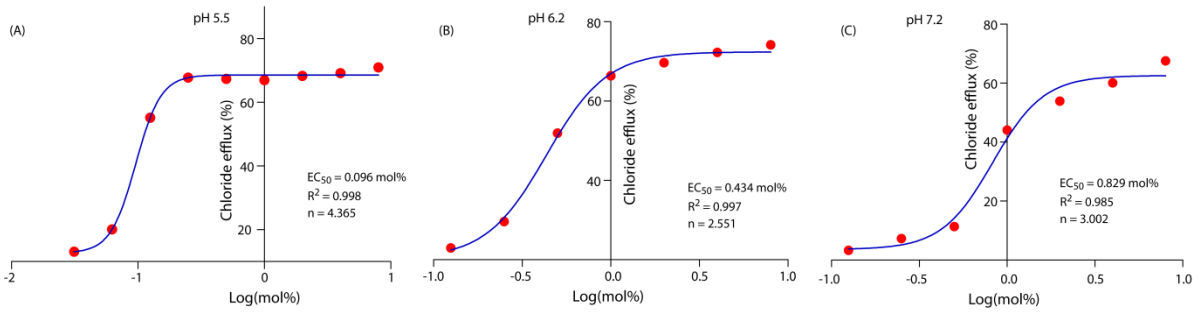



**Figure S26.** Concentration dependent $Cl^-$ ion transport activity of compound **1a** across EYPC/CHOL-LUVs at pH 5.5 (A), 6.2 (B) and 7.2 (C). The transport efficacy of the compound **1a** was measured using chloride ion selective electrode.

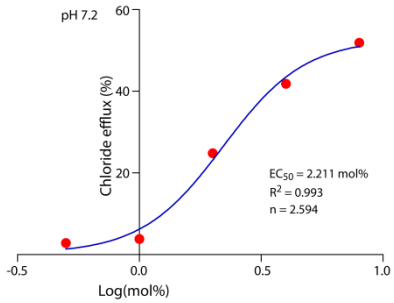



**Figure S27.** Concentration dependent $Cl^-$ ion transport activity of compound **1b** across EYPC/CHOL-LUVs at pH 7.2. The transport efficacy of the compound **1b** was measured using chloride ion selective electrode.

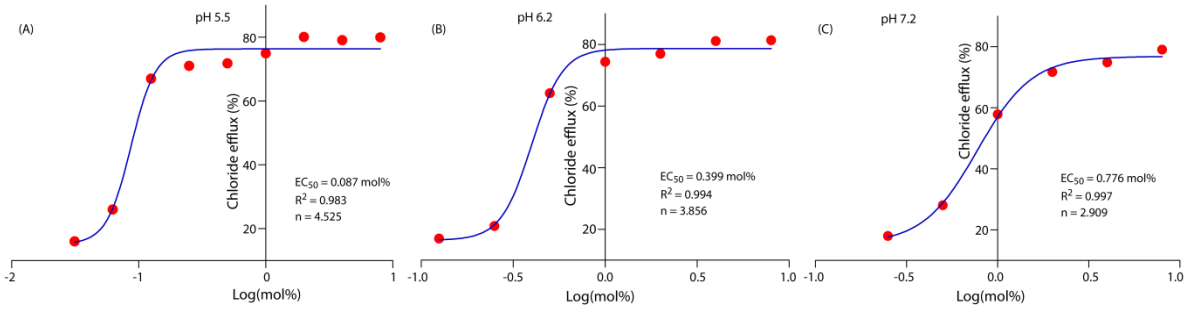



**Figure S28.** Concentration dependent $Cl^-$ ion transport activity of compound **1c** across EYPC/CHOL-LUVs at pH 5.5 (A), 6.2 (B) and 7.2 (C). The transport efficacy of the compound **1c** was measured using chloride ion selective electrode.

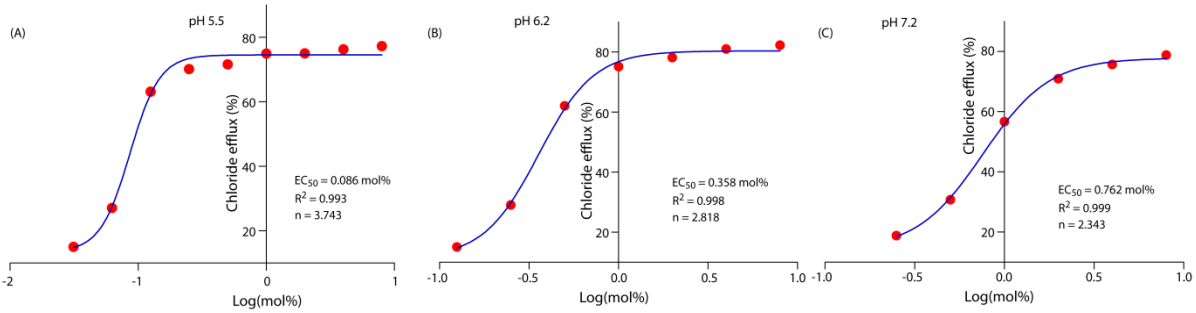



**Figure S29.** Concentration dependent $Cl^-$ ion transport activity of compound **1d** across EYPC/CHOL-LUVs at pH 5.5 (A), 6.2 (B) and 7.2 (C). The transport efficacy of the compound **1d** was measured using chloride ion selective electrode.

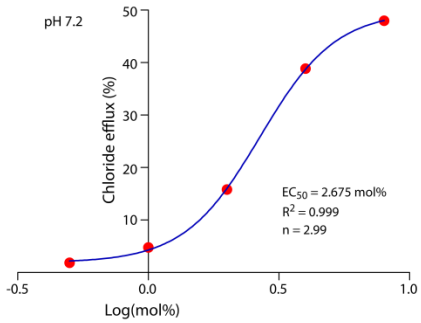



**Figure S30.** Concentration dependent $Cl^-$ ion transport activity of compound **2** across EYPC/CHOL-LUVs at pH 7.2. The transport efficacy of the compound **2** was measured using chloride ion selective electrode.

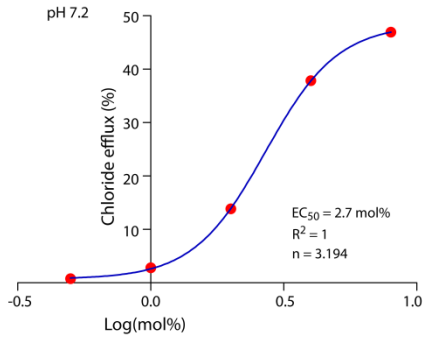



**Figure S31.** Concentration dependent $Cl^-$ ion transport activity of compound **3** across EYPC/CHOL-LUVs at pH 7.2. The transport efficacy of the compound **3** was measured using chloride ion selective electrode.

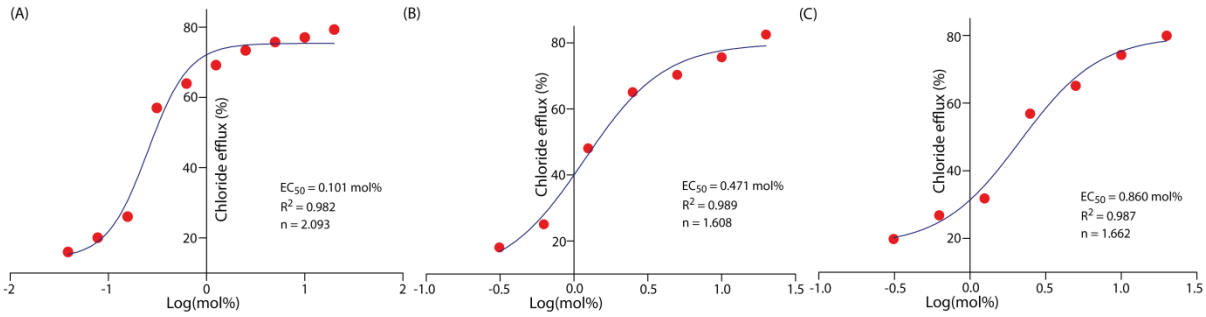



**Figure S32.** Concentration dependent $Cl^-$ ion transport activity of compound **4** across EYPC/CHOL-LUVs at pH 5.5 (A), 6.2 (B) and 7.2 (C). The transport efficacy of the compound **4** was measured using chloride ion selective electrode.

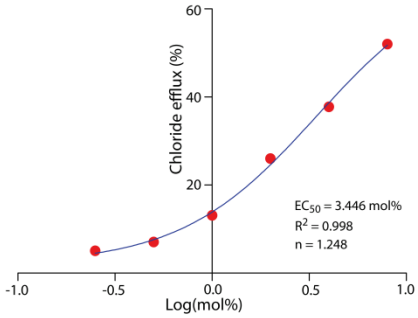



**Figure S33.** Concentration dependent $Cl^-$ ion transport activity of compound **5** across EYPC/CHOL-LUVs at pH 7.2. The transport efficacy of the compound **5** was measured using chloride ion selective electrode.

**Table S2.** The $Cl^-$ ion transport activities of the potent compounds under liposomal environment at various pH.

| | $EC_{50}$ (mol%)[a] | | |
|---|---|---|---|
| | pH 7.2 | pH 6.2 | pH 5.5 |
| **1a** | 0.829 | 0.433 | 0.096 |
| **1c** | 0.776 | 0.399 | 0.087 |
| **1d** | 0.762 | 0.360 | 0.086 |
| **4** | 0.860 | 0.471 | 0.101 |

[a]$EC_{50}$ of the compounds were calculated at 450 sec after the addition of the compounds.

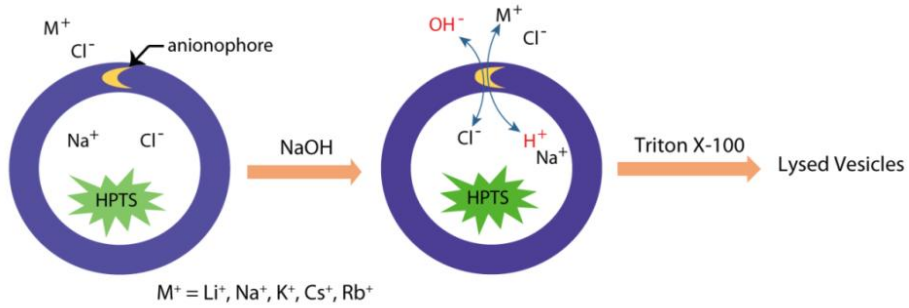



**Figure S34.** Schematic representation of fluorescence based cation transport activity assay using EYPC/CHOL-LUVs⊃HPTS.

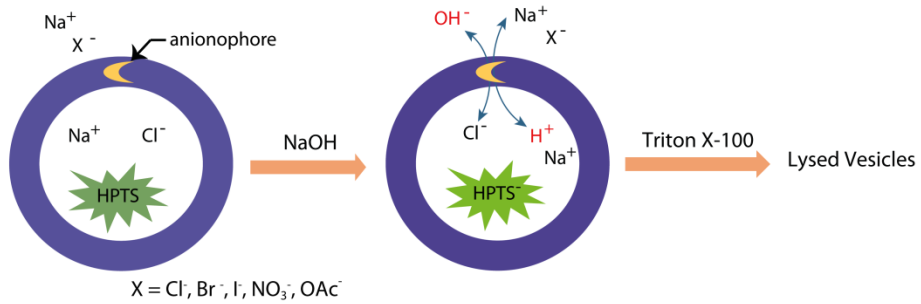



**Figure S35.** Schematic representation of fluorescence based anion transport activity assay using EYPC/CHOL-LUVs⊃HPTS.

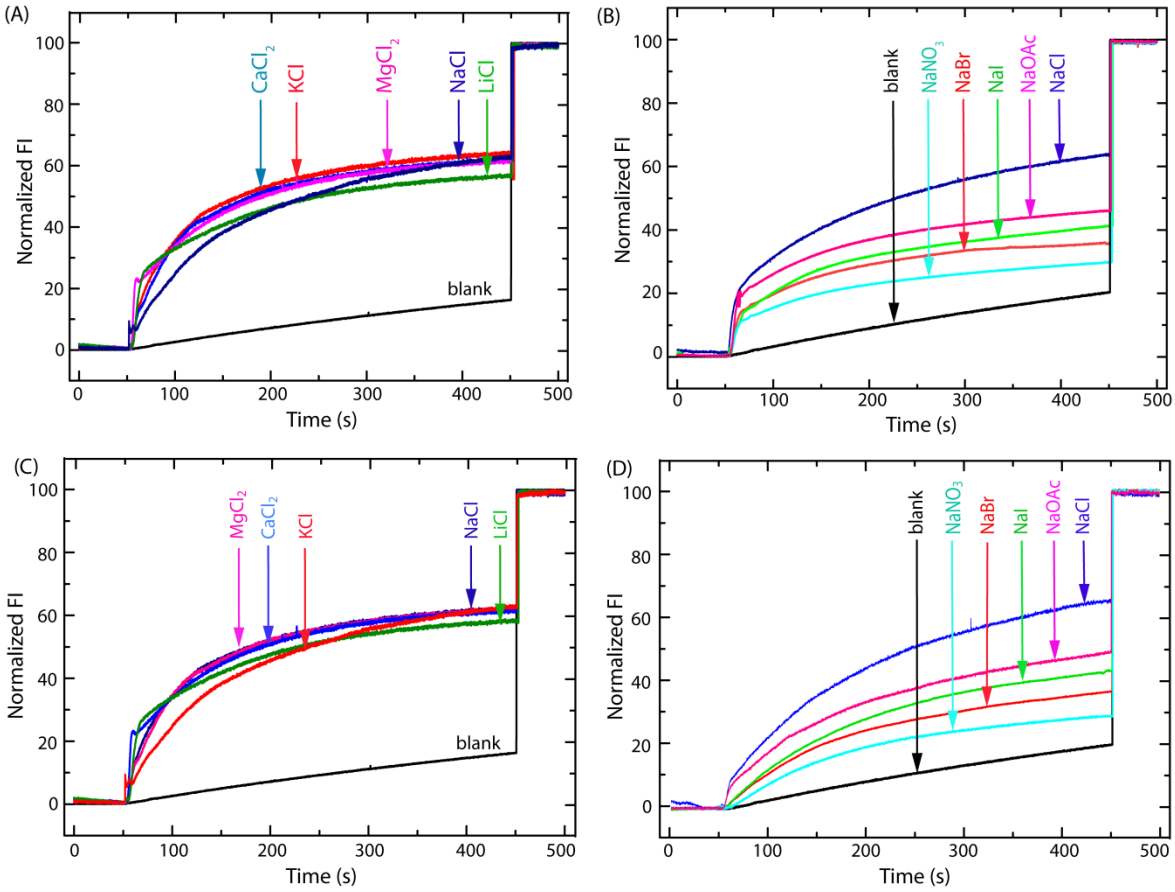



**Figure S36.** Cation (A and C) and anion (B and D) selectivity of compound **1c** and **1d,** respectively as measured by HPTS assay at pH 7.2.

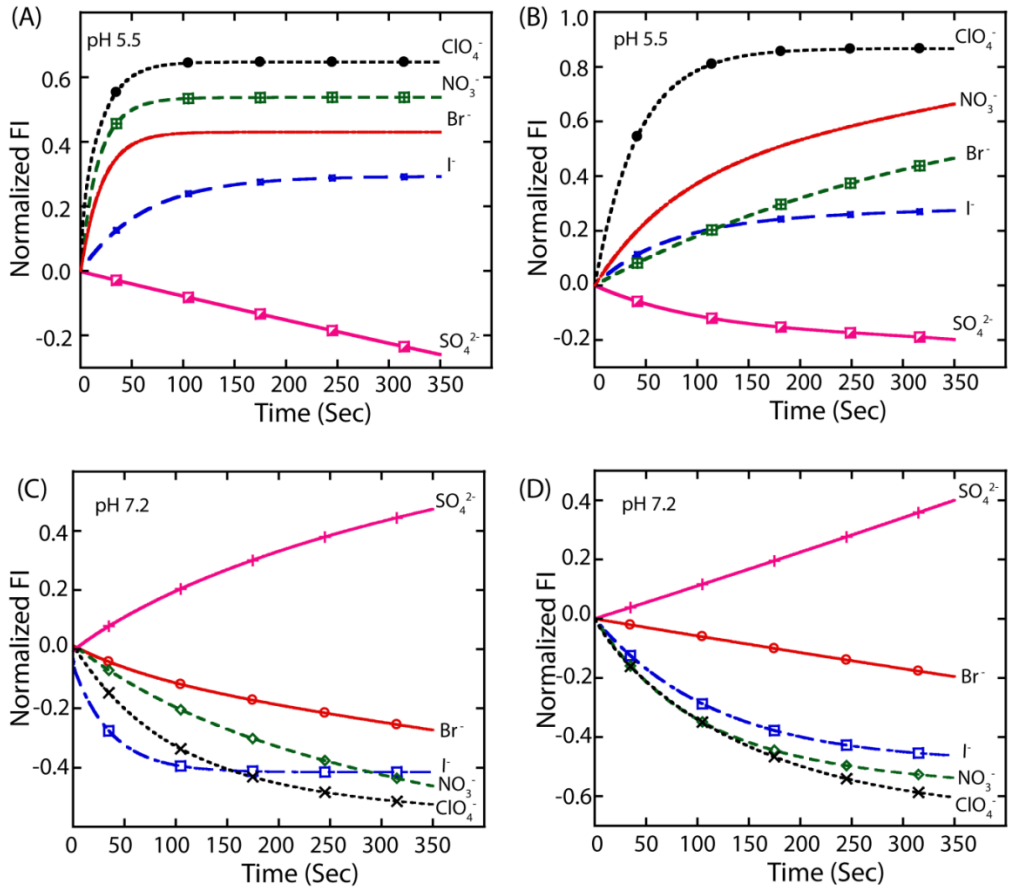



**Figure S37.** Anion selectivity of compound **1c** (A and C) and **1d** (B and D) at pH 5.5 and 7.2 (5 mol% with respect to lipid) was measured across EYPC/CHOL-LUV⊃HPTS without applying any pH gradient.

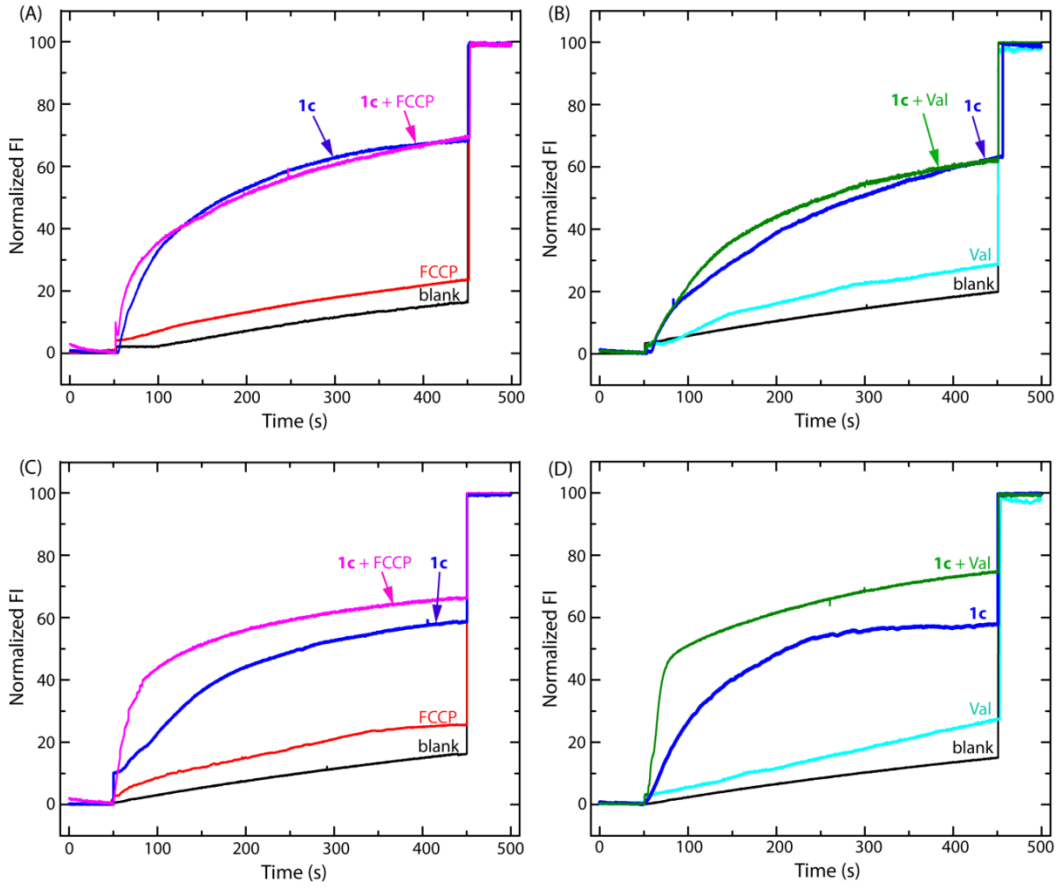



**Figure S38.** Comparison of ion transport activity of compound **1c** in the presence and absence of FCCP both at pH 5.5 (A) and 7.2 (C). Comparison of ion transport activity of compound **1d** in the presence and absence of valinomycin (Val) both at pH 5.5 (B) and 7.2 (D).

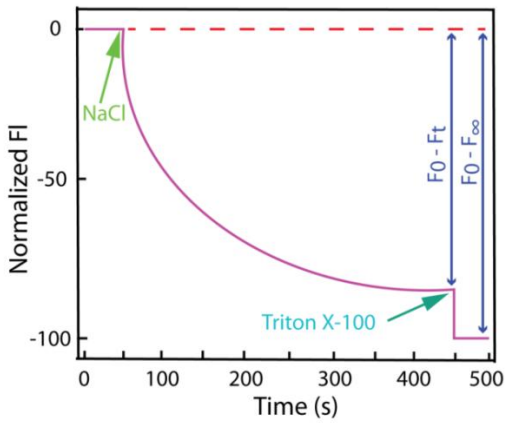



**Figure S39.** Representations of lucigenin fluorescence based ion transport kinetics using EYPC/CHOL-LUV⊃ lucigenin.

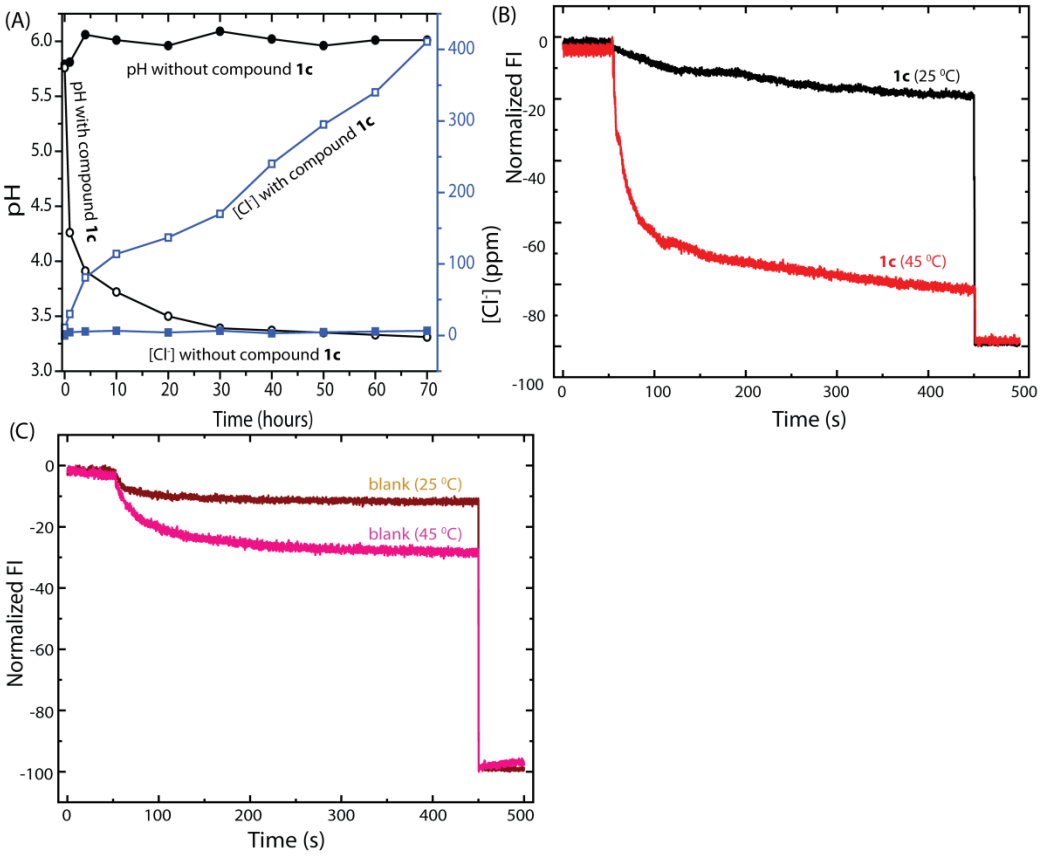



**Figure S40.** Measurement of the $H^+$/ $Cl^-$ transport efficacy by the compound **1c** (0.1 mM) using a chloride ion selective electrode across a U-tube in the presence of HCl gradient (A). Temperature dependent lucigenin assay to demonstrate the carrier-mechanistic pathway of $Cl^-$ ion transport activity by the compound **1c** (0.5 mol%) across DPPC-LUV⊃lucigenin (B). Control experiment with DPPC-LUV⊃lucigenin (C).

**Table S3.** Calculated half-life and initial rate of $Cl^-$ transport activity of compounds across DPPC-LUV⊃lucigenin at different temperatures.

| Entry | Half-life (s) | Initial rate ($s^{-1}$) |
|---|---|---|
| **1c** (25 °C) | 132.07 ± 1.12 | 0.0053 ± 0.0008 |
| **1c** (45 °C) | 23.24 ± 0.39 | 0.0298 ± 0.0054 |
| **1d** (25 °C) | 211.4 ± 1.53 | 0.0033 ± 0.0003 |
| **1d** (45 °C) | 25.77 ± 0.58 | 0.0269 ± 0.0029 |

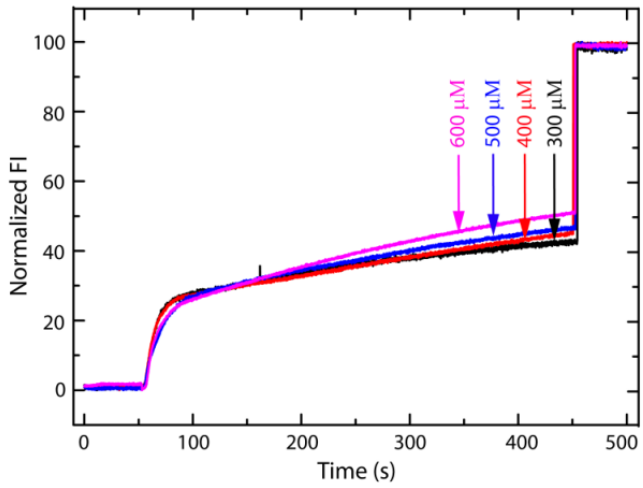



**Figure S41.** Leaching experiment for compound **1d** at transporter to lipid ratio of 0.001 mol%.

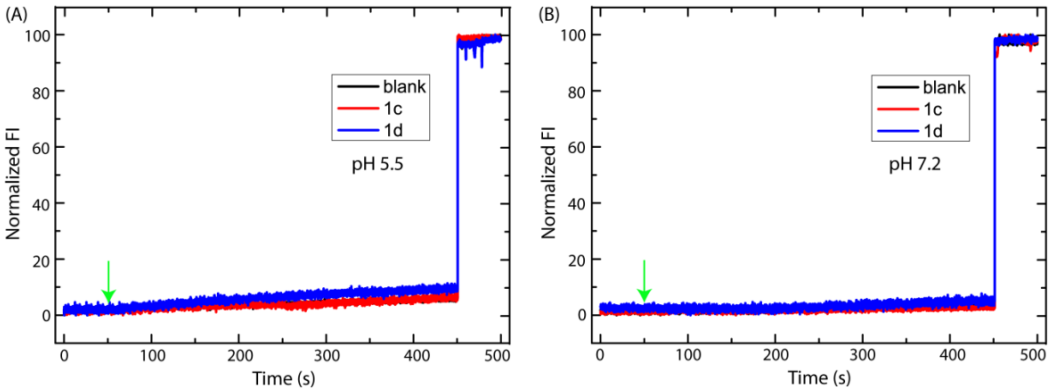



**Figure S42.** Extent of calcein leakage from the EYPC/CHOL-LUV⊃calcein with time in the presence of compounds (0.5 mol%) at pH 5.5 (A) and 7.2 (B). After time t = 50, DMSO solutions of compounds or DMSO (blank) was added, and the fluorescence intensity ($\lambda_{ex}$ = 490 nm, $\lambda_{em}$ = 520 nm) was recorded. After t= 450 sec the LUVs were lysed using triton-X100.

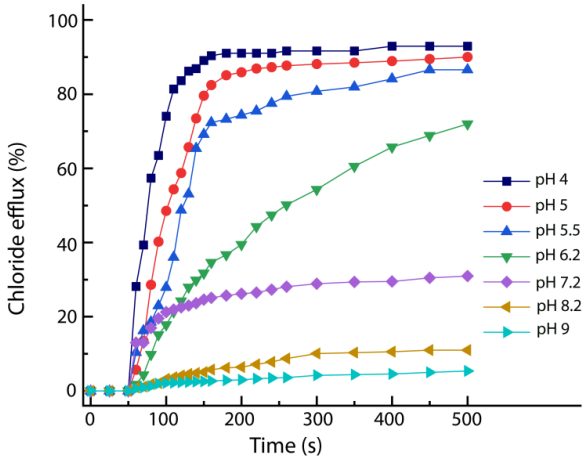
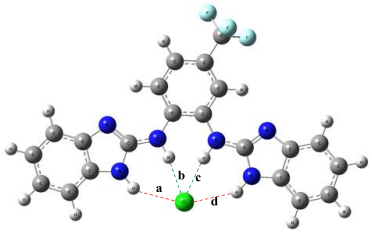
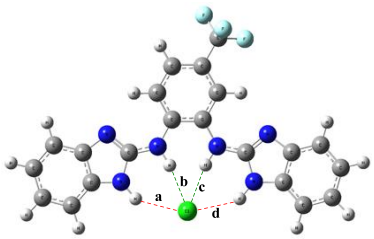
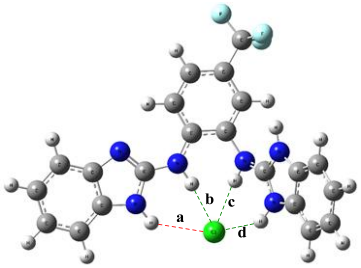
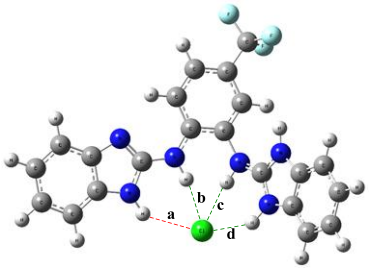
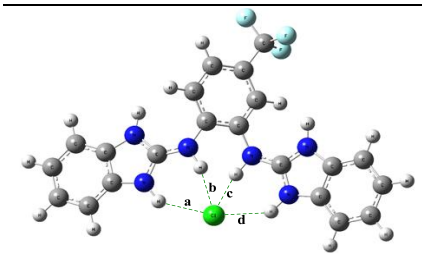
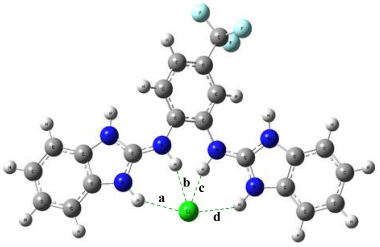
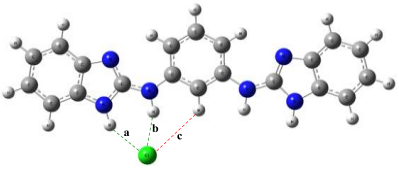
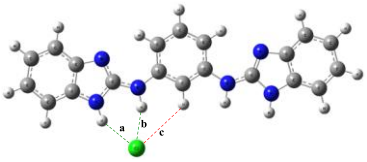
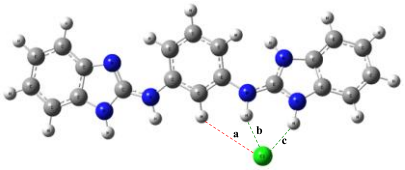
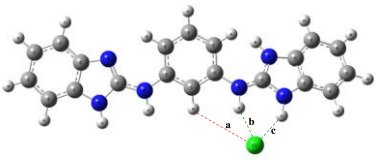
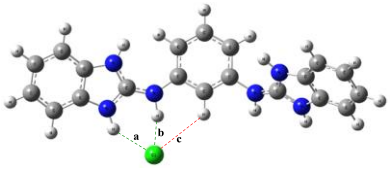
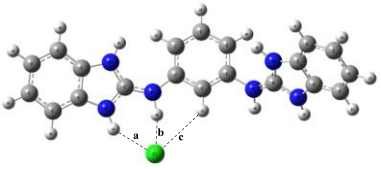



**Figure S43.** pH dependent $Cl^-$ ion transport ability of compound **1d** (5 mol%).

**Table S4:** Optimized structure of considered compounds at neutral, monoprotonated and deprotonated states at B3LYP/6-31+G(d) and B3LYP/6-31++G(d,p) level of theories.

| **B3LYP/6-31+G(d)** | ***IE* and *r* (Cl---- H)** | **B3LYP/6-31++G(d,p)** | ***IE* and *r* (Cl---- H)** |
|---|---|---|---|
|  **1d** (No Protonation) | *64.58*<br>a = 2.474<br>b = 2.259<br>c = 2.270<br>d = 2.497 |  **1d** (No Protonation) | *65.08*<br>a=2.474<br>b=2.259<br>c=2.270<br>d=2.497 |
|  **1d** (Mono-Protonation) | *129.16*<br>a =2.672<br>b =2.256<br>c =2.263<br>d =2.193 |  **1d** (Mono-Protonation) | *130.20*<br>a=2.669<br>b=2.256<br>c=2.256<br>d=2.192 |

| | | | | | |
|---|---|---|---|---|---|
|  **1d** (Di-Protonation) | *193.58* | a=2.342 | b=2.175 | c=2.187 | d=2.353 |
|  **1d** (Di-Protonation) | *194.88* | a=2.340 | b=2.176 | c=2.188 | d=2.351 |
|  **2** (No Protonation) | *41.72* | a=2.330 | b=2.168 | c=3.386 | |
|  **2** (No Protonation) | *42.16* | a=2.330 | b=2.167 | c=3.385 | |
|  **2** (Mono-Protonation) | *109.85* | a=3.729 | b=2.044 | c=2.098 | |
|  **2** (Mono-Protonation) | *111.07* | a=3.725 | b=2.043 | c=2.099 | |
|  **2** (Di-Protonation) | *161.18* | a=2.287 | b=1.859 | c=3.035 | |
|  **2** (Di-Protonation) | *162.36* | a=2.287 | b=1.858 | c=3.036 | |

Interaction Energy (IE) given in Italics. Dashed line indicates hydrogen bonding.

**Table S5**: XYZ Cartesian coordinate for compound **1d** (No Protonation).

| | B3LYP/6-31+G(d) | | | | B3LYP/6-31++G(d,p) | | |
|---|---|---|---|---|---|---|---|
| A | TE = -1897.7658907 | | #IF = 0 | A | TE = -1897.7985634 | | #IF = 0 |
| | x | y | z | | x | y | Z |
| C | -2.57237 | 3.969929 | -0.16654 | C | -2.5717 | 3.970135 | -0.17741 |
| C | -3.68181 | 3.083205 | -0.11933 | C | -3.68114 | 3.083803 | -0.12302 |
| C | -4.99782 | 3.525461 | -0.23961 | C | -4.9974 | 3.525571 | -0.24149 |
| C | -5.19548 | 4.900734 | -0.41213 | C | -5.19513 | 4.900099 | -0.41927 |
| C | -4.10848 | 5.793288 | -0.45924 | C | -4.1083 | 5.792045 | -0.47384 |
| C | -2.79067 | 5.3409 | -0.33738 | C | -2.79019 | 5.34027 | -0.35398 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | -1.75433 | 2.008631 | 0.104339 | C | -1.75386 | 2.009711 | 0.099979 |
| H | -5.83614 | 2.834604 | -0.20227 | H | -5.83495 | 2.835571 | -0.19863 |
| H | -6.20852 | 5.283637 | -0.5111 | H | -6.20742 | 5.282743 | -0.51674 |
| H | -4.29851 | 6.855869 | -0.59361 | H | -4.2987 | 6.853074 | -0.61226 |
| H | -1.94966 | 6.028332 | -0.37437 | H | -1.94983 | 6.026716 | -0.39659 |
| H | -3.57835 | 0.915911 | 0.083547 | H | -3.57598 | 0.917273 | 0.09393 |
| N | -1.38123 | 3.269719 | -0.02079 | N | -1.3804 | 3.27009 | -0.03231 |
| N | -3.12422 | 1.830478 | 0.061026 | N | -3.12402 | 1.8315 | 0.060913 |
| N | -0.98385 | 0.887516 | 0.271943 | N | -0.98436 | 0.889366 | 0.2709 |
| H | -1.56257 | 0.053909 | 0.402932 | H | -1.56553 | 0.057362 | 0.401341 |
| C | 0.405453 | 0.746683 | 0.254069 | C | 0.404552 | 0.748844 | 0.257405 |
| C | 0.959683 | -0.57117 | 0.250758 | C | 0.957307 | -0.56959 | 0.273659 |
| C | 1.27064 | 1.850911 | 0.241118 | C | 1.270061 | 1.852252 | 0.230163 |
| C | 2.34949 | -0.7294 | 0.271871 | C | 2.346841 | -0.72918 | 0.295306 |
| C | 2.65311 | 1.67745 | 0.250147 | C | 2.652384 | 1.677465 | 0.240853 |
| H | 0.837399 | 2.842781 | 0.226175 | H | 0.836195 | 2.843021 | 0.201818 |
| C | 3.191206 | 0.389114 | 0.271787 | C | 3.188764 | 0.389038 | 0.278455 |
| H | 2.753227 | -1.7336 | 0.282866 | H | 2.748993 | -1.73337 | 0.319619 |
| H | 3.301755 | 2.547733 | 0.241736 | H | 3.302323 | 2.545458 | 0.219946 |
| C | 4.67308 | 0.199485 | 0.354664 | C | 4.670465 | 0.198266 | 0.36196 |
| F | 5.09422 | -0.97797 | -0.17551 | F | 5.090452 | -0.97913 | -0.16975 |
| F | 5.365267 | 1.185089 | -0.28869 | F | 5.363713 | 1.183992 | -0.27971 |
| F | 5.138554 | 0.213982 | 1.644534 | F | 5.135301 | 0.210012 | 1.652046 |
| N | 0.086776 | -1.66567 | 0.217552 | N | 0.081783 | -1.66145 | 0.259183 |
| H | -0.90723 | -1.49852 | 0.047513 | H | -0.91553 | -1.49475 | 0.108309 |
| C | 1.167489 | -4.93404 | 0.812209 | C | 1.168548 | -4.93517 | 0.807833 |
| C | -0.23968 | -5.11386 | 0.727677 | C | -0.23949 | -5.11352 | 0.735204 |
| C | -0.84405 | -6.36043 | 0.877252 | C | -0.84307 | -6.36135 | 0.876661 |
| C | -0.00215 | -7.45312 | 1.118588 | C | 0.000849 | -7.45684 | 1.096827 |
| C | 1.393299 | -7.29316 | 1.202913 | C | 1.396881 | -7.29831 | 1.169519 |
| C | 1.991919 | -6.03811 | 1.051188 | C | 1.994792 | -6.04202 | 1.026374 |
| C | 0.342353 | -2.9922 | 0.442056 | C | 0.340012 | -2.98923 | 0.467714 |
| H | -1.92223 | -6.48215 | 0.811222 | H | -1.92096 | -6.48176 | 0.819941 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| H | -0.43732 | -8.44206 | 1.242558 | H | -0.43278 | -8.44632 | 1.213453 |
| H | 2.017998 | -8.16379 | 1.390135 | H | 2.022183 | -8.17067 | 1.34048 |
| H | 3.069292 | -5.91167 | 1.116978 | H | 3.071888 | -5.91668 | 1.082997 |
| H | -1.71315 | -3.53705 | 0.431256 | H | -1.71409 | -3.53277 | 0.46149 |
| N | 1.501215 | -3.5983 | 0.625294 | N | 1.500642 | -3.59735 | 0.633491 |
| N | -0.74245 | -3.8484 | 0.481729 | N | -0.74485 | -3.84546 | 0.509633 |
| Cl | -3.17175 | -1.52101 | 0.213225 | Cl | -3.15587 | -1.50909 | 0.249881 |

A=Atoms; TE = Total Energy in Hartree; #IF = Imaginary Frequency.

**Table S6**: XYZ Cartesian coordinate for compound **1d** (Mono-Protonation).

| | B3LYP/6-31+G(d) | | | | B3LYP/6-31++G(d,p) | | |
|---|---|---|---|---|---|---|---|
| A | TE = -1898.2509257 | | #IF = 0 | A | TE = -1898.2869576 | | #IF = 0 |
| | x | y | z | | x | y | Z |
| C | -4.56828 | -0.28198 | 0.65294 | C | -4.59837 | -0.26614 | 0.632899 |
| C | -4.41455 | -1.55403 | 0.046647 | C | -4.44141 | -1.54557 | 0.043466 |
| C | -5.43605 | -2.50284 | 0.02616 | C | -5.46449 | -2.49254 | 0.023845 |
| C | -6.64062 | -2.14585 | 0.637703 | C | -6.6741 | -2.12578 | 0.619242 |
| C | -6.80946 | -0.88688 | 1.246729 | C | -6.84627 | -0.85948 | 1.211282 |
| C | -5.78095 | 0.056611 | 1.262533 | C | -5.81622 | 0.082184 | 1.226276 |
| C | -2.57928 | -0.3289 | -0.13071 | C | -2.60147 | -0.32727 | -0.12978 |
| H | -5.30689 | -3.47408 | -0.44354 | H | -5.33276 | -3.4688 | -0.43243 |
| H | -7.46344 | -2.85576 | 0.642476 | H | -7.49793 | -2.83316 | 0.624529 |
| H | -7.76167 | -0.64714 | 1.712697 | H | -7.80187 | -0.61274 | 1.664417 |
| H | -5.90848 | 1.028396 | 1.730936 | H | -5.94629 | 1.059003 | 1.681255 |
| H | -2.64964 | -2.27124 | -0.99937 | H | -2.66696 | -2.27906 | -0.97387 |
| N | -3.39721 | 0.461958 | 0.526319 | N | -3.42407 | 0.473357 | 0.509408 |
| N | -3.11723 | -1.55707 | -0.44633 | N | -3.13943 | -1.55783 | -0.43691 |
| N | -1.27378 | -0.08403 | -0.51392 | N | -1.2923 | -0.09048 | -0.50281 |
| H | -0.85383 | -0.88052 | -1.00507 | H | -0.87072 | -0.89368 | -0.98023 |
| C | -0.57662 | 1.106494 | -0.49134 | C | -0.59022 | 1.097406 | -0.47903 |
| C | 0.74046 | 1.130265 | -1.03176 | C | 0.732104 | 1.112628 | -1.00671 |
| C | -1.0885 | 2.309319 | 0.037899 | C | -1.1031 | 2.304101 | 0.039402 |
| C | 1.484187 | 2.303052 | -1.06606 | C | 1.480113 | 2.282694 | -1.03976 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | -0.32188 | 3.470934 | 0.026459 | C | -0.33168 | 3.46252 | 0.031116 |
| H | -2.08579 | 2.30405 | 0.459622 | H | -2.10432 | 2.302716 | 0.450627 |
| C | 0.964434 | 3.483748 | -0.523 | C | 0.959541 | 3.467145 | -0.50565 |
| H | 2.461164 | 2.295565 | -1.54228 | H | 2.459768 | 2.271464 | -1.50865 |
| H | -0.73935 | 4.380716 | 0.445236 | H | -0.74792 | 4.375833 | 0.441401 |
| C | 1.816983 | 4.71613 | -0.51629 | C | 1.816049 | 4.696759 | -0.49709 |
| F | 2.403865 | 4.943494 | -1.72238 | F | 2.404116 | 4.923991 | -1.70278 |
| F | 1.126822 | 5.830955 | -0.18906 | F | 1.128992 | 5.813081 | -0.16877 |
| F | 2.847294 | 4.616934 | 0.380822 | F | 2.845694 | 4.592971 | 0.400175 |
| N | 1.303931 | -0.08555 | -1.54557 | N | 1.290544 | -0.10743 | -1.51253 |
| H | 0.72949 | -0.67719 | -2.16783 | H | 0.705435 | -0.71394 | -2.11167 |
| C | 3.82803 | -1.40425 | 0.576961 | C | 3.866307 | -1.39923 | 0.562492 |
| C | 3.437066 | -2.53412 | -0.16234 | C | 3.460504 | -2.53565 | -0.15846 |
| C | 4.019817 | -3.77903 | 0.053699 | C | 4.05113 | -3.77752 | 0.053635 |
| C | 5.010865 | -3.85202 | 1.037049 | C | 5.065154 | -3.84019 | 1.013817 |
| C | 5.404476 | -2.71982 | 1.769724 | C | 5.473105 | -2.70159 | 1.72804 |
| C | 4.817613 | -1.4692 | 1.55138 | C | 4.878315 | -1.45409 | 1.514095 |
| C | 2.207812 | -0.80021 | -0.85655 | C | 2.212759 | -0.81045 | -0.8373 |
| H | 3.714743 | -4.65233 | -0.51379 | H | 3.735099 | -4.65529 | -0.49919 |
| H | 5.487115 | -4.80712 | 1.237115 | H | 5.548339 | -4.79167 | 1.210052 |
| H | 6.180302 | -2.81495 | 2.52337 | H | 6.266142 | -2.7894 | 2.463265 |
| H | 5.119883 | -0.59482 | 2.119718 | H | 5.191489 | -0.57518 | 2.067711 |
| H | 1.731836 | -2.68051 | -1.5521 | H | 1.722034 | -2.69261 | -1.51196 |
| N | 3.039939 | -0.34057 | 0.108621 | N | 3.065596 | -0.34036 | 0.104691 |
| N | 2.443664 | -2.11318 | -1.05325 | N | 2.445223 | -2.12458 | -1.02859 |
| Cl | -0.33782 | -2.66989 | -2.27909 | Cl | -0.33062 | -2.6838 | -2.2207 |
| H | 3.045146 | 0.615812 | 0.439018 | H | 3.074575 | 0.617001 | 0.427937 |

A=Atoms; TE = Total Energy in Hartree; #IF = Imaginary Frequency.

**Table S7**: XYZ Cartesian coordinate for compound **1d** (Di-Protonation).

| | B3LYP/6-31+G(d) | | | | B3LYP/6-31++G(d,p) | | |
|---|---|---|---|---|---|---|---|
| A | TE = -1898.6381339 | | #IF = 0 | A | TE = -1898.6778235 | | #IF = 0 |
| | x | y | z | | x | y | z |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | -4.98907 | -0.02011 | 0.031737 | C | -4.99456 | -0.02184 | 0.032676 |
| C | -4.76536 | -1.3988 | 0.162687 | C | -4.76517 | -1.40019 | 0.156099 |
| C | -5.79521 | -2.32304 | 0.009623 | C | -5.79182 | -2.3276 | 0.001277 |
| C | -7.06025 | -1.80785 | -0.2805 | C | -7.05942 | -1.81549 | -0.28236 |
| C | -7.28307 | -0.42494 | -0.4074 | C | -7.2878 | -0.43308 | -0.40172 |
| C | -6.24777 | 0.499926 | -0.25186 | C | -6.25577 | 0.495024 | -0.24502 |
| C | -2.81066 | -0.34857 | 0.481044 | C | -2.81461 | -0.34413 | 0.473971 |
| H | -5.62328 | -3.38994 | 0.108398 | H | -5.61576 | -3.39352 | 0.094111 |
| H | -7.89201 | -2.49318 | -0.40989 | H | -7.88864 | -2.50252 | -0.41256 |
| H | -8.28254 | -0.06547 | -0.63105 | H | -8.2887 | -0.07695 | -0.62053 |
| H | -6.42212 | 1.56694 | -0.3501 | H | -6.43421 | 1.560977 | -0.33779 |
| H | -2.84563 | -2.41883 | 0.417522 | H | -2.83838 | -2.41251 | 0.401948 |
| N | -3.74361 | 0.601195 | 0.24785 | N | -3.75099 | 0.603615 | 0.247221 |
| N | -3.4041 | -1.55724 | 0.454346 | N | -3.40251 | -1.55529 | 0.442358 |
| N | -1.48797 | -0.19622 | 0.718556 | N | -1.49229 | -0.19025 | 0.707811 |
| H | -1.00594 | -1.11353 | 0.738689 | H | -1.0119 | -1.10916 | 0.724401 |
| C | -0.7147 | 0.958665 | 0.459257 | C | -0.7191 | 0.964972 | 0.453482 |
| C | 0.512824 | 0.821313 | -0.23987 | C | 0.516429 | 0.824342 | -0.23118 |
| C | -1.11322 | 2.227339 | 0.892672 | C | -1.12172 | 2.235273 | 0.878298 |
| C | 1.248528 | 1.963598 | -0.55862 | C | 1.255942 | 1.965333 | -0.54603 |
| C | -0.35197 | 3.360179 | 0.599894 | C | -0.35705 | 3.366534 | 0.589721 |
| H | -1.99194 | 2.326829 | 1.523442 | H | -2.00702 | 2.338164 | 1.497676 |
| C | 0.822728 | 3.227341 | -0.13815 | C | 0.825591 | 3.230195 | -0.13452 |
| H | 2.124374 | 1.878572 | -1.19485 | H | 2.13823 | 1.88007 | -1.1718 |
| H | -0.67278 | 4.334295 | 0.952332 | H | -0.68053 | 4.342076 | 0.933525 |
| C | 1.682094 | 4.428747 | -0.45624 | C | 1.688088 | 4.431017 | -0.44568 |
| F | 2.201341 | 4.350225 | -1.70404 | F | 2.237589 | 4.34016 | -1.67972 |
| F | 0.995708 | 5.583193 | -0.36405 | F | 0.994712 | 5.583434 | -0.38352 |
| F | 2.73418 | 4.510886 | 0.400626 | F | 2.718659 | 4.528055 | 0.435151 |
| N | 0.948908 | -0.46942 | -0.62427 | N | 0.951267 | -0.46864 | -0.6063 |
| H | 0.236943 | -1.21137 | -0.74316 | H | 0.240016 | -1.21376 | -0.71494 |
| C | 4.347114 | -1.31834 | 0.076431 | C | 4.352088 | -1.32104 | 0.070063 |
| C | 3.763072 | -2.56564 | -0.18969 | C | 3.76161 | -2.56805 | -0.18221 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | 4.50137 | -3.74451 | -0.13225 | C | 4.496344 | -3.74899 | -0.12287 |
| C | 5.851304 | -3.62128 | 0.203003 | C | 5.849425 | -3.62761 | 0.199538 |
| C | 6.435803 | -2.36917 | 0.465356 | C | 6.440219 | -2.37591 | 0.447944 |
| C | 5.692294 | -1.188 | 0.405912 | C | 5.700342 | -1.1927 | 0.387143 |
| C | 2.172851 | -0.99918 | -0.3985 | C | 2.175826 | -0.99806 | -0.3883 |
| H | 4.05064 | -4.71036 | -0.33613 | H | 4.041047 | -4.71401 | -0.31585 |
| H | 6.464235 | -4.5153 | 0.260618 | H | 6.459794 | -4.52239 | 0.257976 |
| H | 7.489716 | -2.31697 | 0.719778 | H | 7.495746 | -2.32591 | 0.69259 |
| H | 6.145227 | -0.22235 | 0.608082 | H | 6.157801 | -0.228 | 0.578759 |
| H | 1.646887 | -2.99827 | -0.53939 | H | 1.639266 | -2.99359 | -0.51239 |
| N | 3.321084 | -0.36504 | -0.07313 | N | 3.328239 | -0.36499 | -0.07638 |
| N | 2.416888 | -2.32047 | -0.49221 | N | 2.413645 | -2.32093 | -0.47434 |
| Cl | -0.66032 | -3.09591 | -0.0876 | Cl | -0.65776 | -3.07516 | -0.07641 |
| H | 3.405748 | 0.627297 | 0.099765 | H | 3.41789 | 0.627637 | 0.083625 |
| H | -3.55204 | 1.59001 | 0.164941 | H | -3.56378 | 1.592644 | 0.174655 |

A=Atoms; TE = Total Energy in Hartree; #IF = Imaginary Frequency.

**Table S8**: XYZ Cartesian coordinate for compound **2** (No-Protonation).

| | B3LYP/6-31+G(d) | | | | B3LYP/6-31++G(d,p) | | |
|---|---|---|---|---|---|---|---|
| A | TE = -1560.6809148 | | #IF = 0 | A | TE = -1560.714851 | | #IF = 0 |
| | x | y | z | | x | y | z |
| C | -0.76916 | 2.237827 | -2.12961 | C | -0.7726 | 2.234422 | -2.12663 |
| C | 0.218738 | 1.771314 | -3.01459 | C | 0.215235 | 1.767764 | -3.01166 |
| C | -1.0959 | 3.595059 | -2.06506 | C | -1.0983 | 3.591827 | -2.06279 |
| C | 0.883966 | 2.695168 | -3.8454 | C | 0.880931 | 2.691371 | -3.8426 |
| C | -0.44132 | 4.524523 | -2.88648 | C | -0.44351 | 4.521166 | -2.88406 |
| C | 0.538952 | 4.04403 | -3.76135 | C | 0.536542 | 4.040138 | -3.75872 |
| H | 1.645818 | 2.344228 | -4.52955 | H | 1.642038 | 2.338619 | -4.52579 |
| H | -0.69425 | 5.574769 | -2.83639 | H | -0.6968 | 5.570611 | -2.8332 |
| N | 0.464587 | 0.404686 | -2.99748 | N | 0.461126 | 0.401663 | -2.99521 |
| H | -0.09504 | -0.1658 | -2.33783 | H | -0.09681 | -0.17232 | -2.33677 |
| C | 2.845167 | -1.09421 | -5.06099 | C | 2.843588 | -1.09119 | -5.06018 |
| C | 2.336392 | -2.21644 | -4.34778 | C | 2.336169 | -2.21436 | -4.34743 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | 2.804194 | -3.51134 | -4.56442 | C | 2.805506 | -3.50859 | -4.5648 |
| C | 3.810584 | -3.67785 | -5.52475 | C | 3.811879 | -3.67313 | -5.52534 |
| C | 4.324659 | -2.57963 | -6.23864 | C | 4.324473 | -2.57406 | -6.23862 |
| C | 3.850135 | -1.28201 | -6.01594 | C | 3.848636 | -1.27712 | -6.01536 |
| C | 1.349284 | -0.33258 | -3.73055 | C | 1.346886 | -0.33326 | -3.72885 |
| H | 2.40527 | -4.35799 | -4.01151 | H | 2.407932 | -4.35519 | -4.01267 |
| H | 4.199678 | -4.67477 | -5.72002 | H | 4.202055 | -4.66847 | -5.72131 |
| H | 5.106161 | -2.74368 | -6.97783 | H | 5.105385 | -2.73695 | -6.97729 |
| H | 4.246203 | -0.43237 | -6.56664 | H | 4.243221 | -0.42737 | -6.56513 |
| H | 0.768336 | -2.15223 | -2.81422 | H | 0.765988 | -2.15018 | -2.81241 |
| N | 2.209885 | 0.070658 | -4.65473 | N | 2.206713 | 0.072396 | -4.65298 |
| N | 1.378396 | -1.69311 | -3.50267 | N | 1.377678 | -1.69373 | -3.50149 |
| Cl | -0.90521 | -1.83987 | -1.2228 | Cl | -0.88863 | -1.84556 | -1.23991 |
| C | -2.64906 | 5.153689 | -0.83036 | C | -2.64953 | 5.152526 | -0.83002 |
| C | -4.0164 | 6.555904 | 0.24978 | C | -4.01498 | 6.556979 | 0.248515 |
| H | -4.03079 | 4.446209 | 0.661047 | H | -4.0308 | 4.448669 | 0.661602 |
| C | -3.1923 | 7.227812 | -0.68862 | C | -3.19037 | 7.227331 | -0.69033 |
| C | -4.94933 | 7.217156 | 1.042638 | C | -4.9473 | 7.219375 | 1.041019 |
| C | -3.30911 | 8.613386 | -0.83615 | C | -3.3058 | 8.612813 | -0.83901 |
| C | -5.05185 | 8.605354 | 0.880398 | C | -5.04832 | 8.607444 | 0.877574 |
| H | -5.5742 | 6.68763 | 1.758017 | H | -5.57201 | 6.69127 | 1.756135 |
| C | -4.24365 | 9.289284 | -0.04455 | C | -4.23966 | 9.289826 | -0.0478 |
| H | -2.68408 | 9.140936 | -1.55122 | H | -2.6808 | 9.138754 | -1.55394 |
| H | -5.76891 | 9.159274 | 1.481079 | H | -5.7641 | 9.162389 | 1.477183 |
| H | -4.34816 | 10.36696 | -0.14592 | H | -4.34333 | 10.36661 | -0.14964 |
| N | -2.10753 | 3.94104 | -1.13382 | N | -2.10968 | 3.93915 | -1.13198 |
| N | -2.35597 | 6.332268 | -1.34205 | N | -2.35505 | 6.330172 | -1.34289 |
| N | -3.64647 | 5.220593 | 0.139112 | N | -3.64703 | 5.221071 | 0.139475 |
| H | -2.47596 | 3.151592 | -0.62024 | H | -2.47908 | 3.152114 | -0.61837 |
| H | 1.054319 | 4.755045 | -4.40411 | H | 1.051872 | 4.750254 | -4.40103 |
| H | -1.27708 | 1.515361 | -1.4921 | H | -1.28036 | 1.512633 | -1.48959 |

A=Atoms; TE = Total Energy in Hartree; #IF = Imaginary Frequency.

**Table S9**: XYZ Cartesian coordinate for compound **2** (Mono-Protonation).

| | B3LYP/6-31+G(d) | | | | B3LYP/6-31++G(d,p) | | |
|---|---|---|---|---|---|---|---|
| A | TE = -1561.1767972 | | #IF = 0 | A | TE = -1561.2147863 | | #IF = 0 |
| | x | y | z | | x | y | z |
| C | 0.097833 | 2.062774 | -1.11378 | C | 0.090554 | 2.062674 | -1.11615 |
| C | 1.206288 | 1.636241 | -1.84742 | C | 1.195576 | 1.636262 | -1.85508 |
| C | -0.12696 | 3.431471 | -0.89435 | C | -0.13347 | 3.431319 | -0.89639 |
| C | 2.114302 | 2.571184 | -2.36712 | C | 2.101293 | 2.572106 | -2.37775 |
| C | 0.757224 | 4.378568 | -1.44247 | C | 0.747877 | 4.379038 | -1.44758 |
| C | 1.863605 | 3.930669 | -2.16308 | C | 1.851217 | 3.931282 | -2.17252 |
| H | 3.03211 | 2.24943 | -2.8507 | H | 3.015696 | 2.251952 | -2.86653 |
| H | 0.575502 | 5.433348 | -1.28718 | H | 0.565677 | 5.432942 | -1.29042 |
| N | 1.382263 | 0.240223 | -1.99771 | N | 1.370215 | 0.240749 | -2.00533 |
| H | 1.01379 | -0.41973 | -1.26068 | H | 0.999776 | -0.42331 | -1.27117 |
| C | 2.414632 | -0.9501 | -5.18178 | C | 2.42083 | -0.95222 | -5.18122 |
| C | 2.216125 | -2.13313 | -4.44575 | C | 2.216822 | -2.13389 | -4.4446 |
| C | 2.404704 | -3.38427 | -5.02627 | C | 2.408579 | -3.38595 | -5.02218 |
| C | 2.797047 | -3.41094 | -6.3684 | C | 2.809694 | -3.41441 | -6.36151 |
| C | 2.996865 | -2.22852 | -7.0992 | C | 3.014817 | -2.23334 | -7.09262 |
| C | 2.810634 | -0.97082 | -6.51401 | C | 2.825074 | -0.9747 | -6.51089 |
| C | 1.775797 | -0.40678 | -3.0936 | C | 1.770097 | -0.40649 | -3.09849 |
| H | 2.249971 | -4.29659 | -4.45928 | H | 2.249805 | -4.29666 | -4.45532 |
| H | 2.950577 | -4.36966 | -6.85494 | H | 2.96613 | -4.37295 | -6.84561 |
| H | 3.302223 | -2.28781 | -8.13964 | H | 3.326771 | -2.29469 | -8.1301 |
| H | 2.965164 | -0.05645 | -7.07936 | H | 2.983278 | -0.06193 | -7.07607 |
| H | 1.477346 | -2.31244 | -2.34941 | H | 1.462939 | -2.30618 | -2.34752 |
| N | 2.145879 | 0.108181 | -4.29905 | N | 2.14592 | 0.107543 | -4.30258 |
| N | 1.84137 | -1.75401 | -3.15879 | N | 1.83376 | -1.75362 | -3.16056 |
| Cl | 0.500125 | -2.24424 | -0.4931 | Cl | 0.497747 | -2.23633 | -0.51643 |
| C | -1.72088 | 5.018831 | 0.217435 | C | -1.72155 | 5.019232 | 0.220631 |
| C | -3.07773 | 6.4748 | 1.219735 | C | -3.07703 | 6.476281 | 1.222059 |
| H | -3.32847 | 4.355018 | 1.481857 | H | -3.34035 | 4.357153 | 1.469529 |
| C | -2.07484 | 7.120862 | 0.459535 | C | -2.06972 | 7.121652 | 0.467353 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | -4.04487 | 7.172297 | 1.939168 | C | -4.04497 | 7.174124 | 1.939941 |
| C | -2.03317 | 8.517575 | 0.41183 | C | -2.02395 | 8.518277 | 0.423611 |
| C | -3.98699 | 8.568413 | 1.881577 | C | -3.98307 | 8.570097 | 1.885977 |
| H | -4.81054 | 6.664236 | 2.519682 | H | -4.81328 | 6.666924 | 2.515923 |
| C | -2.99756 | 9.228556 | 1.129313 | C | -2.98918 | 9.229535 | 1.139414 |
| H | -1.26861 | 9.02498 | -0.16922 | H | -1.25643 | 9.024767 | -0.15264 |
| H | -4.72186 | 9.152465 | 2.428798 | H | -4.71773 | 9.154474 | 2.431373 |
| H | -2.98532 | 10.31502 | 1.10796 | H | -2.97424 | 10.31513 | 1.121251 |
| N | -1.24876 | 3.772124 | -0.12731 | N | -1.25283 | 3.772231 | -0.12618 |
| N | -1.24587 | 6.184049 | -0.15376 | N | -1.24138 | 6.183948 | -0.14548 |
| N | -2.8296 | 5.11543 | 1.042055 | N | -2.8318 | 5.116668 | 1.043193 |
| H | -1.77374 | 2.986419 | 0.232197 | H | -1.77633 | 2.988483 | 0.235431 |
| H | 2.56223 | 4.663944 | -2.55742 | H | 2.547508 | 4.664042 | -2.56966 |
| H | -0.58549 | 1.31668 | -0.71415 | H | -0.59046 | 1.316991 | -0.71407 |
| H | 2.092488 | 1.090958 | -4.52942 | H | 2.104945 | 1.090036 | -4.53027 |

A=Atoms; TE = Total Energy in Hartree; #IF = Imaginary Frequency.

**Table S10**: XYZ Cartesian coordinate for compound **2** (Di-Protonation).

| | B3LYP/6-31+G(d) | | | | B3LYP/6-31++G(d,p) | | |
|---|---|---|---|---|---|---|---|
| A | TE = -1561.5599006 | | #IF = 0 | A | TE = -1561.60108 | | #IF = 0 |
| | x | y | z | | x | y | z |
| C | 0.488687 | 2.254828 | -0.13998 | C | 0.480155 | 2.242591 | -0.14043 |
| C | 1.595339 | 1.894024 | -0.93661 | C | 1.589146 | 1.880297 | -0.93315 |
| C | 0.458655 | 3.495596 | 0.481635 | C | 0.444685 | 3.487587 | 0.472244 |
| C | 2.645056 | 2.814794 | -1.10214 | C | 2.636071 | 2.804041 | -1.10273 |
| C | 1.4965 | 4.423861 | 0.321551 | C | 1.479641 | 4.418412 | 0.30803 |
| C | 2.579499 | 4.066038 | -0.48433 | C | 2.565117 | 4.058823 | -0.49362 |
| H | 3.54182 | 2.555287 | -1.65379 | H | 3.534047 | 2.544043 | -1.65053 |
| H | 1.48917 | 5.369752 | 0.855114 | H | 1.469084 | 5.366925 | 0.835125 |
| N | 1.536881 | 0.609256 | -1.4613 | N | 1.53803 | 0.592821 | -1.44944 |
| H | 0.767545 | -0.04116 | -1.05627 | H | 0.776147 | -0.06938 | -1.03949 |
| C | 3.64663 | -0.67089 | -4.04028 | C | 3.64339 | -0.66735 | -4.03979 |
| C | 2.885702 | -1.78333 | -3.64365 | C | 2.894983 | -1.78654 | -3.63847 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| C | 3.043403 | -3.0255 | -4.25366 | C | 3.060527 | -3.02728 | -4.24932 |
| C | 3.990201 | -3.10932 | -5.27683 | C | 4.001775 | -3.10219 | -5.27806 |
| C | 4.750188 | -1.99426 | -5.67182 | C | 4.749124 | -1.98047 | -5.6775 |
| C | 4.593376 | -0.74937 | -5.05617 | C | 4.584698 | -0.73701 | -5.06127 |
| C | 2.262958 | -0.03886 | -2.37812 | C | 2.264749 | -0.04909 | -2.36974 |
| H | 2.457001 | -3.88566 | -3.94737 | H | 2.484308 | -3.89208 | -3.93969 |
| H | 4.142472 | -4.05998 | -5.77844 | H | 4.159988 | -4.05064 | -5.78033 |
| H | 5.476477 | -2.10054 | -6.47159 | H | 5.470984 | -2.08051 | -6.48102 |
| H | 5.182955 | 0.109994 | -5.36114 | H | 5.163931 | 0.127055 | -5.3697 |
| H | 1.309826 | -1.85536 | -2.09585 | H | 1.324788 | -1.86787 | -2.07969 |
| N | 3.238885 | 0.397151 | -3.21827 | N | 3.230404 | 0.397218 | -3.21633 |
| N | 2.053324 | -1.35218 | -2.6113 | N | 2.064971 | -1.36387 | -2.60095 |
| Cl | -0.38256 | -1.43363 | -0.61521 | Cl | -0.33674 | -1.4742 | -0.61095 |
| C | -1.52706 | 4.794751 | 1.129225 | C | -1.53593 | 4.794 | 1.120822 |
| C | -3.28026 | 6.195112 | 1.359318 | C | -3.27981 | 6.203137 | 1.358487 |
| H | -2.88883 | 4.554644 | 2.711165 | H | -2.89553 | 4.556251 | 2.703326 |
| C | -2.54194 | 6.599142 | 0.238743 | C | -2.5418 | 6.60561 | 0.237397 |
| C | -4.4311 | 6.863757 | 1.761563 | C | -4.42564 | 6.877436 | 1.765306 |
| C | -2.92019 | 7.692056 | -0.53354 | C | -2.91434 | 7.703422 | -0.53049 |
| C | -4.81602 | 7.962932 | 0.989088 | C | -4.80487 | 7.981387 | 0.997118 |
| H | -5.00524 | 6.551563 | 2.628258 | H | -4.99929 | 6.566468 | 2.631737 |
| C | -4.0764 | 8.36829 | -0.13504 | C | -4.06527 | 8.385481 | -0.12717 |
| H | -2.35155 | 8.005885 | -1.4032 | H | -2.34596 | 8.016299 | -1.39963 |
| H | -5.70872 | 8.514798 | 1.265704 | H | -5.69277 | 8.537881 | 1.276917 |
| H | -4.40999 | 9.22645 | -0.70975 | H | -4.39452 | 9.247192 | -0.69763 |
| N | -0.66041 | 3.792644 | 1.339697 | N | -0.67661 | 3.785275 | 1.326479 |
| N | -1.46085 | 5.703738 | 0.134472 | N | -1.46611 | 5.704464 | 0.127965 |
| N | -2.61243 | 5.073173 | 1.887035 | N | -2.61785 | 5.075915 | 1.882084 |
| H | -0.92953 | 3.076083 | 2.005381 | H | -0.94696 | 3.072207 | 1.993342 |
| H | 3.407841 | 4.75833 | -0.60244 | H | 3.39069 | 4.752725 | -0.6147 |
| H | -0.32088 | 1.540043 | -0.01108 | H | -0.32663 | 1.526411 | -0.00832 |
| H | 3.509479 | 1.361586 | -3.33852 | H | 3.495535 | 1.36229 | -3.33203 |
| H | -0.75307 | 5.687834 | -0.58982 | H | -0.7575 | 5.688164 | -0.59373 |

A=Atoms; TE = Total Energy in Hartree; #IF = Imaginary Frequency.

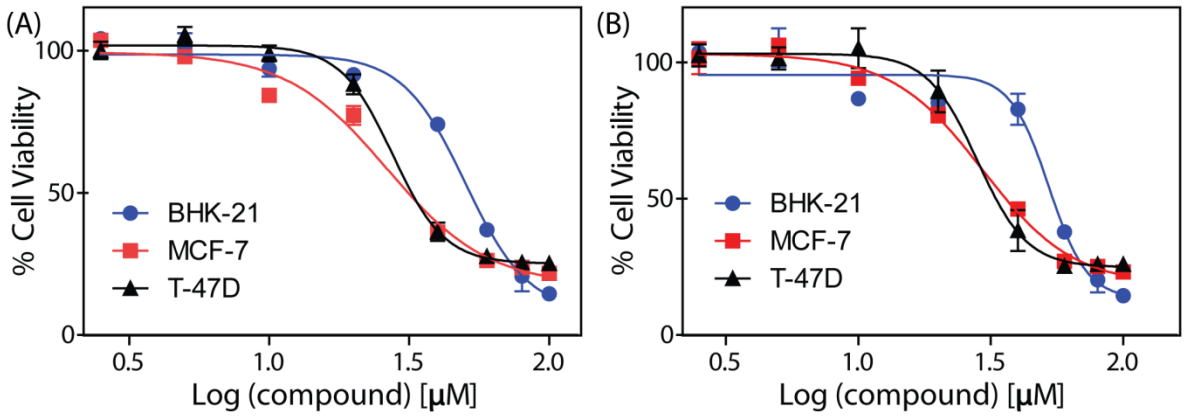



**Figure S44.** The viabilities of BHK-21, MCF-7 and T-47D cell lines in the presence of compounds **1c** (A) and **1d** (B) were measured at different concentrations (after 24 hours of incubation).

**Table S11.** Calculated $IC_{50}$ values of the compounds.

| Cell Line | $IC_{50}$ values (μM) | |
|---|---|---|
| | Compound **1c** | Compound **1d** |
| BHK-21 | 50.22 | 52.37 |
| MCF-7 | 26.39 | 29.39 |
| T-47D | 27.60 | 28.12 |

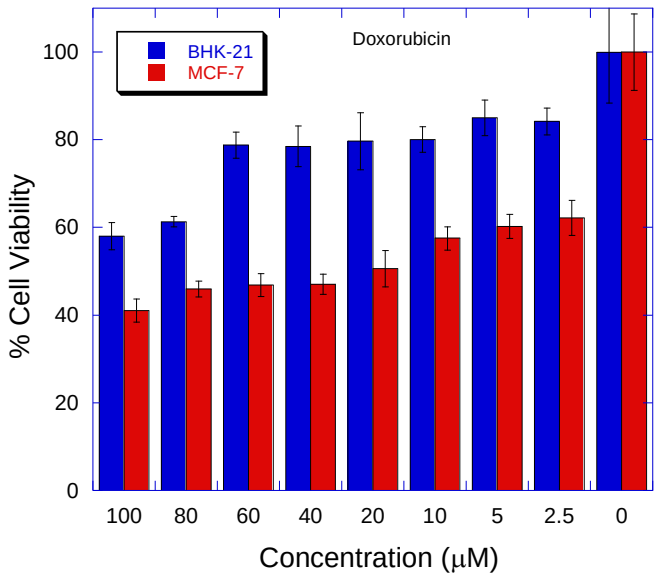



**Figure S45.** The viabilities of BHK-21 and MCF-7 cell lines in the presence of doxorubicin were measured at different concentrations (after 24 hours of incubation).

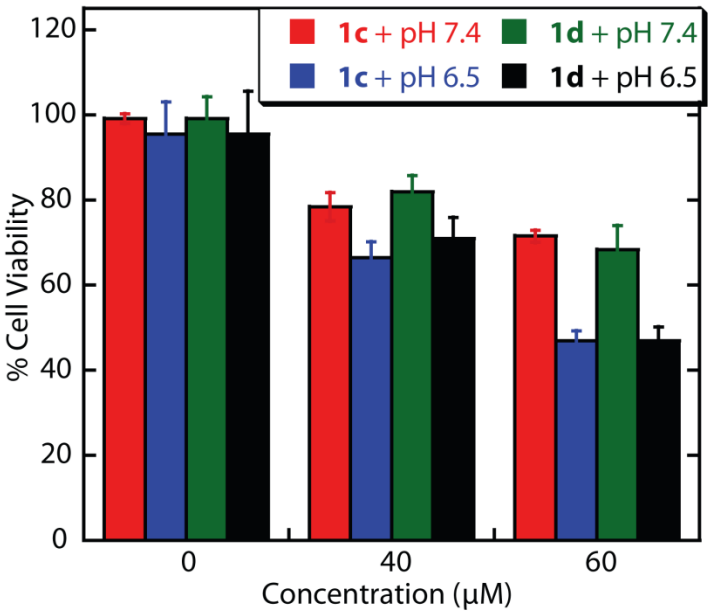



**Figure S46.** The viabilities of MCF-7 cells at pH 6.5 and 7.4 in the absence and presence of compounds (after 12 hours of incubation).

**Note**: **Few solvent impurities (including high boiling impurity from solvent, water and others which cannot be removed during drying process even after several attempts) were taken under consideration during the calculation of the yield of the final compounds.**

## 2. NMR spectra of the synthesized compounds:

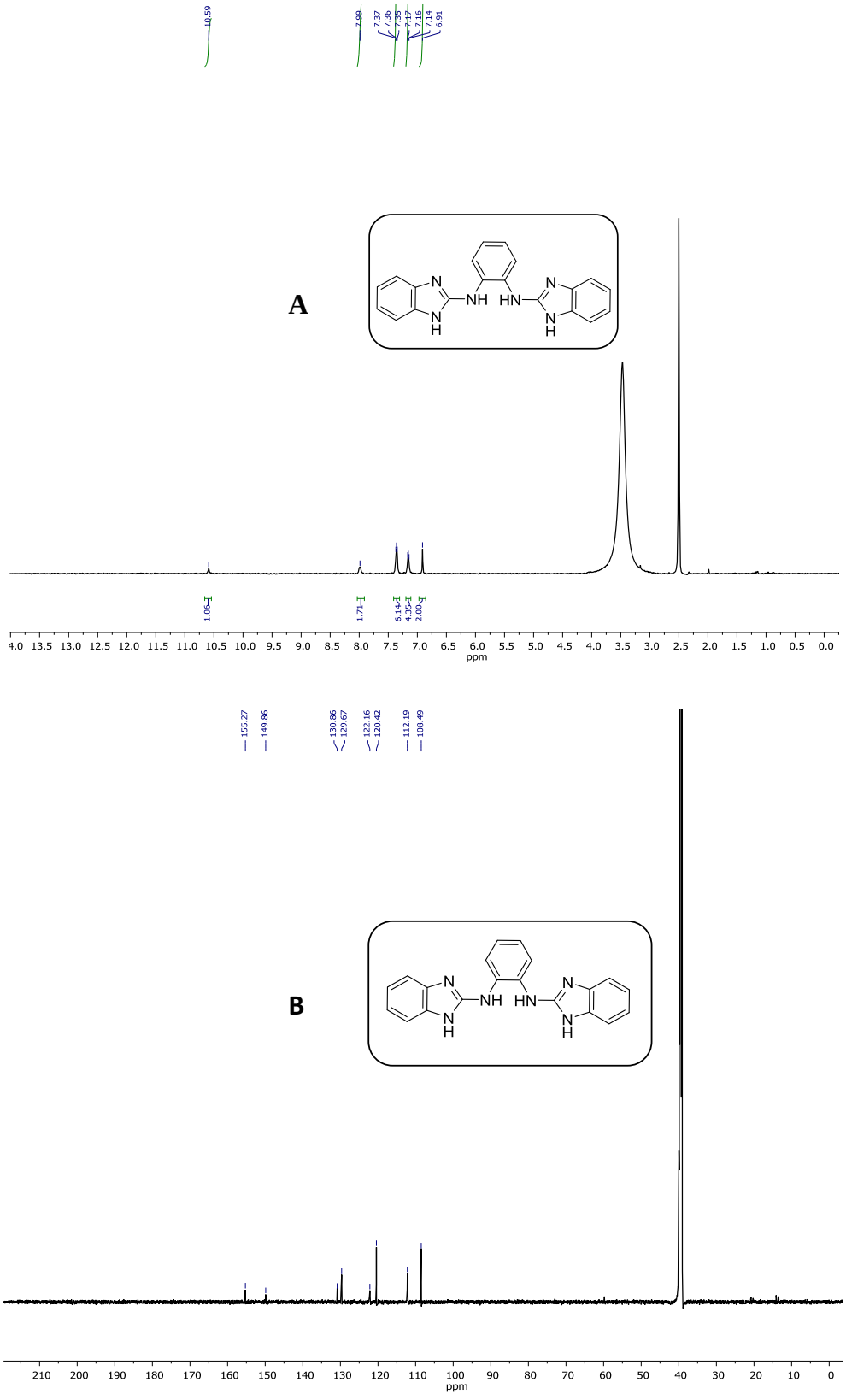



**Figure S47:** $^{1}$HNMR (A) and $^{13}$CNMR (B) of compound **1a.**

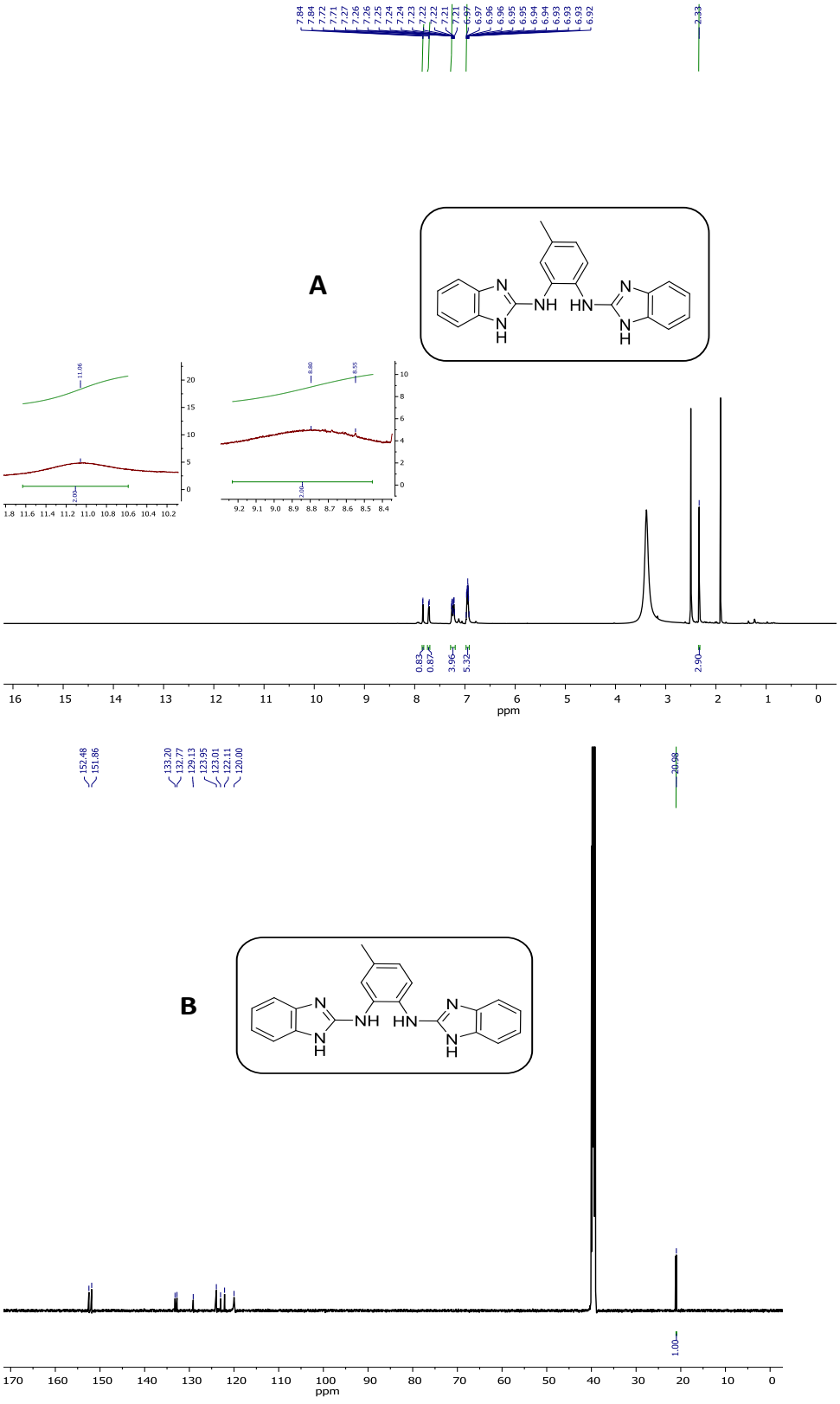



**Figure S48:** $^{1}$HNMR (A) and $^{13}$CNMR (B) of compound **1b.**

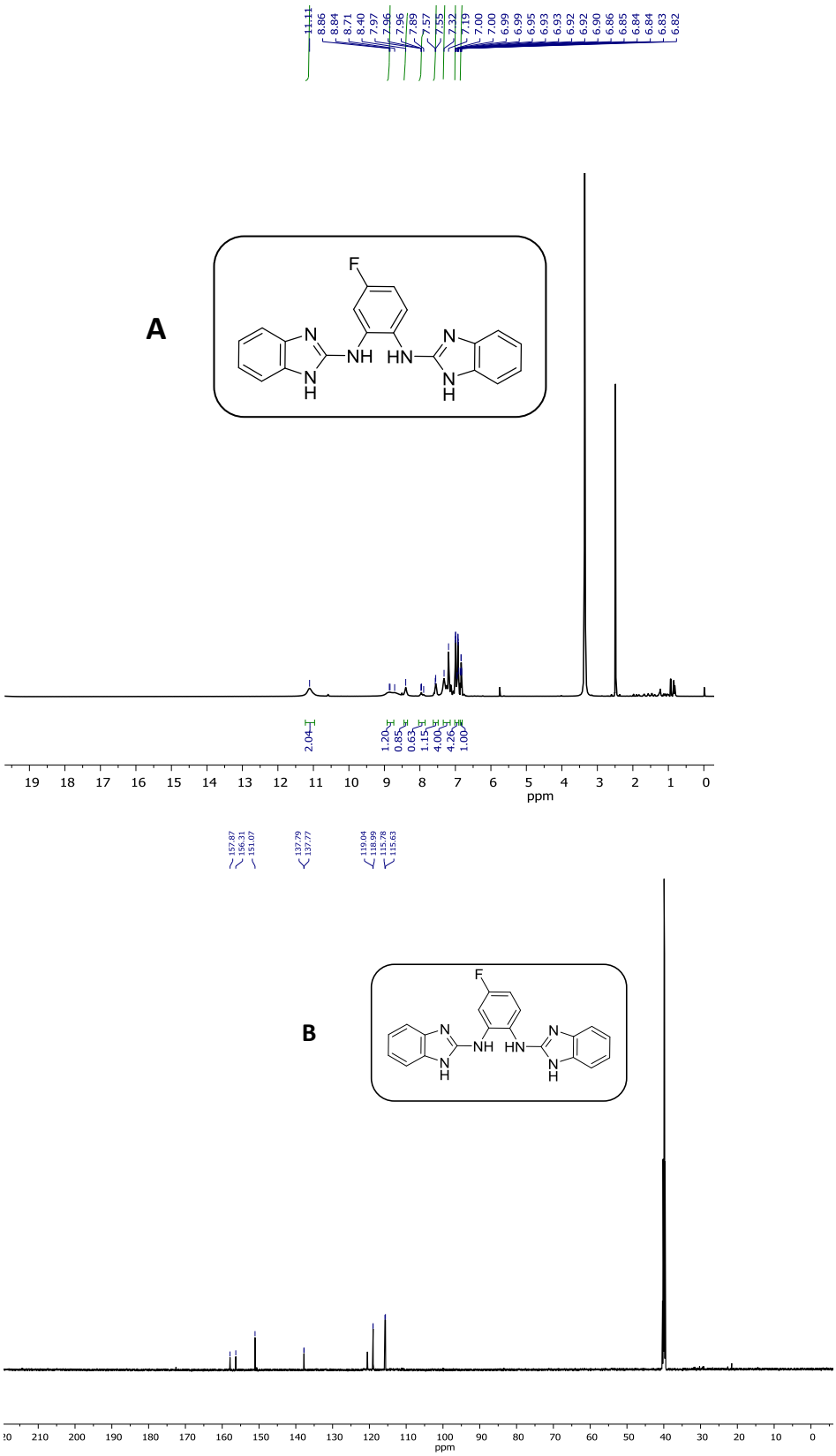



**Figure S49:** $^{1}$HNMR (A) and $^{13}$CNMR (B) of compound **1c.**

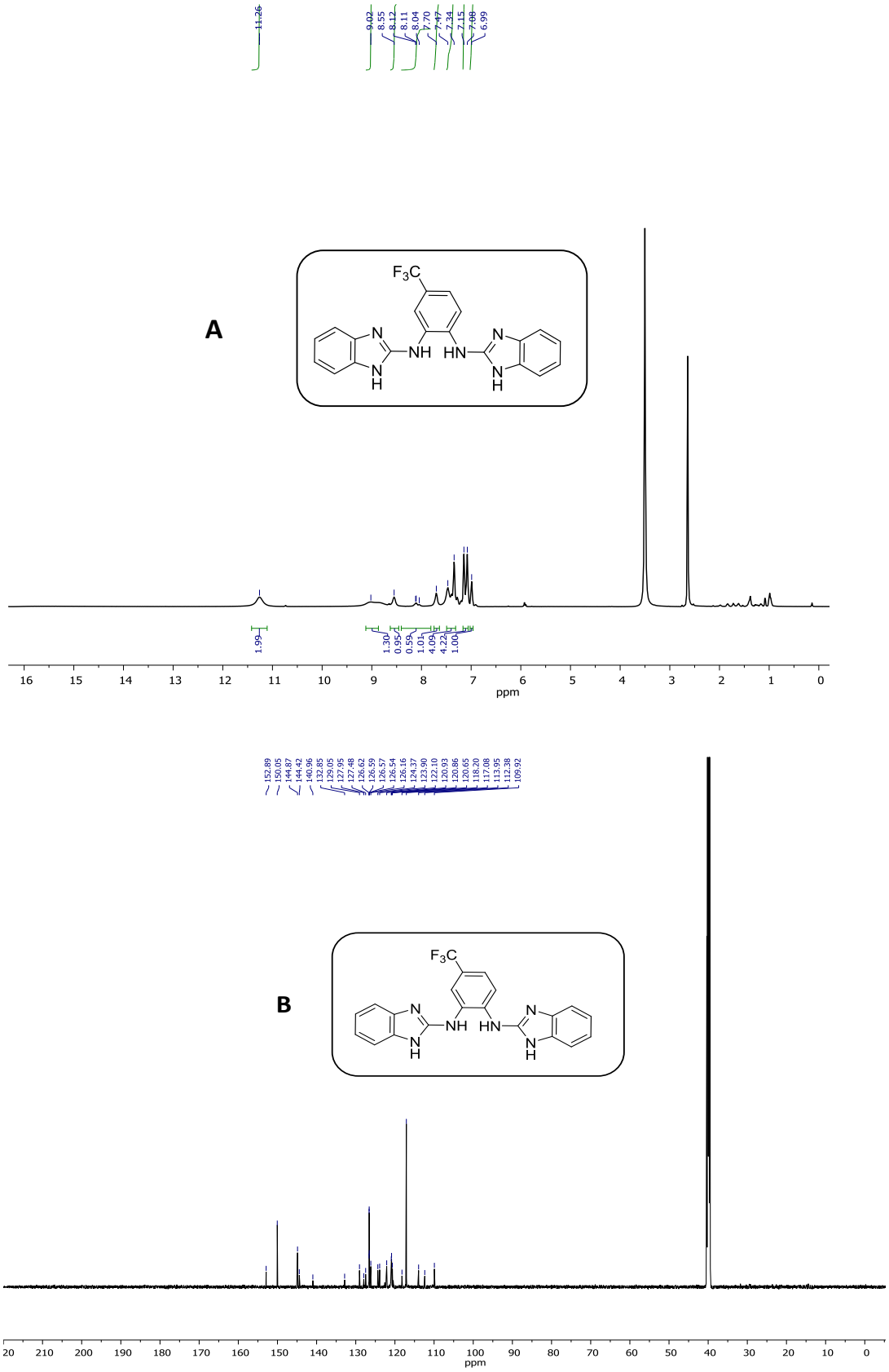



**Figure S50:** $^{1}$HNMR (A) and $^{13}$CNMR (B) of compound **1d.**

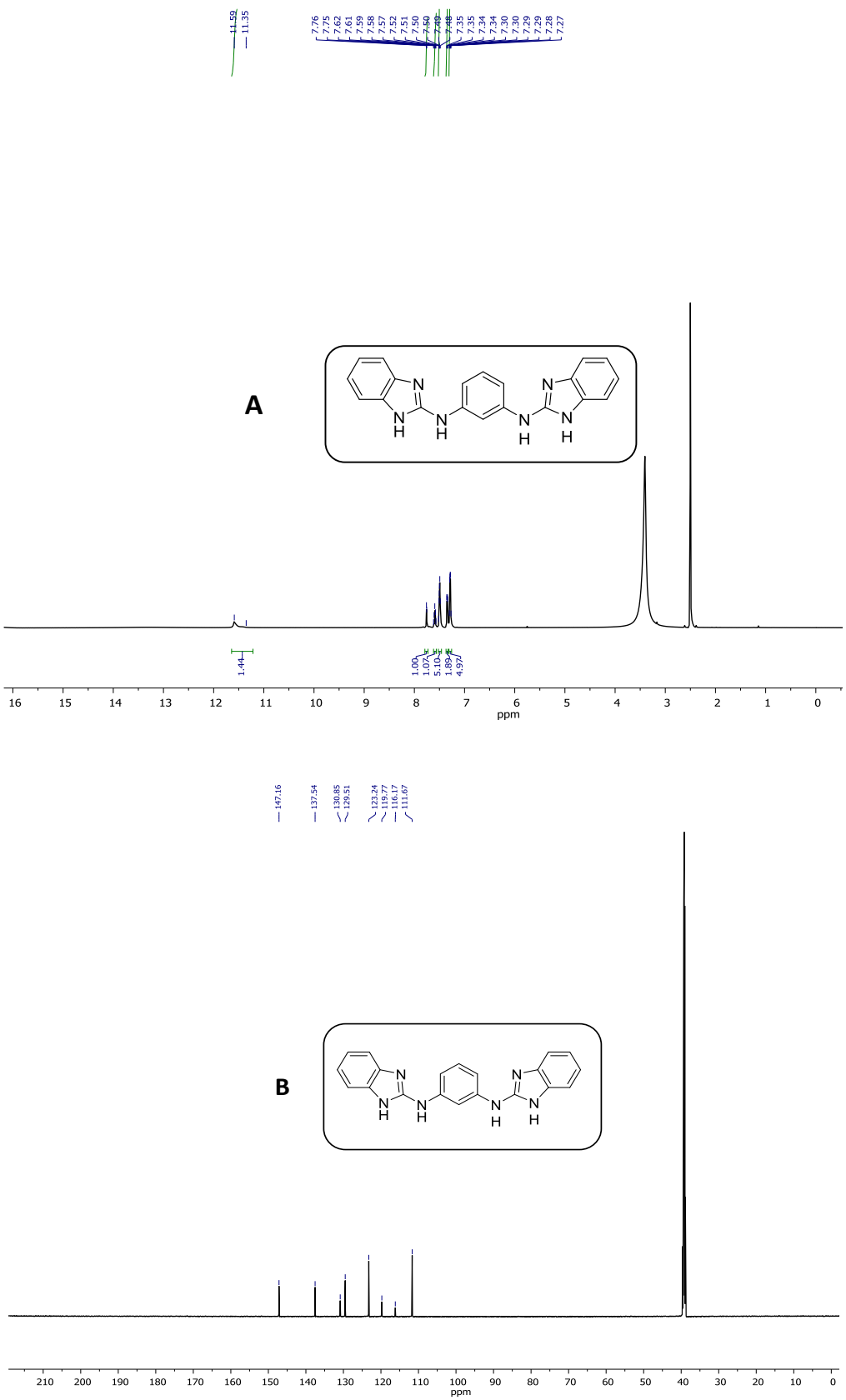



**Figure S51:** $^{1}$HNMR (A) and $^{13}$CNMR (B) of compound **2.**

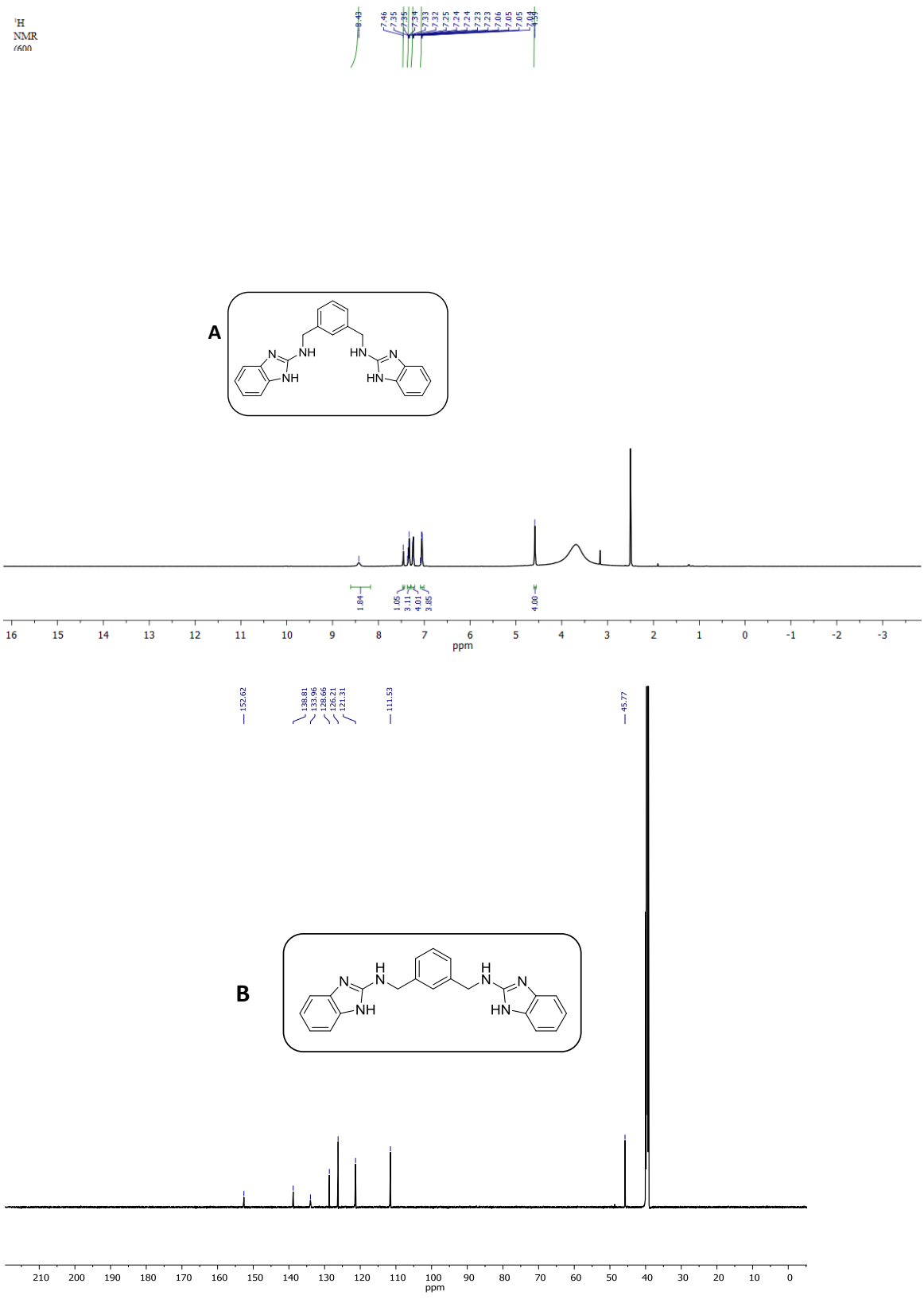



**Figure S52:** $^{1}$HNMR (A) and $^{13}$CNMR (B) of compound **3.**

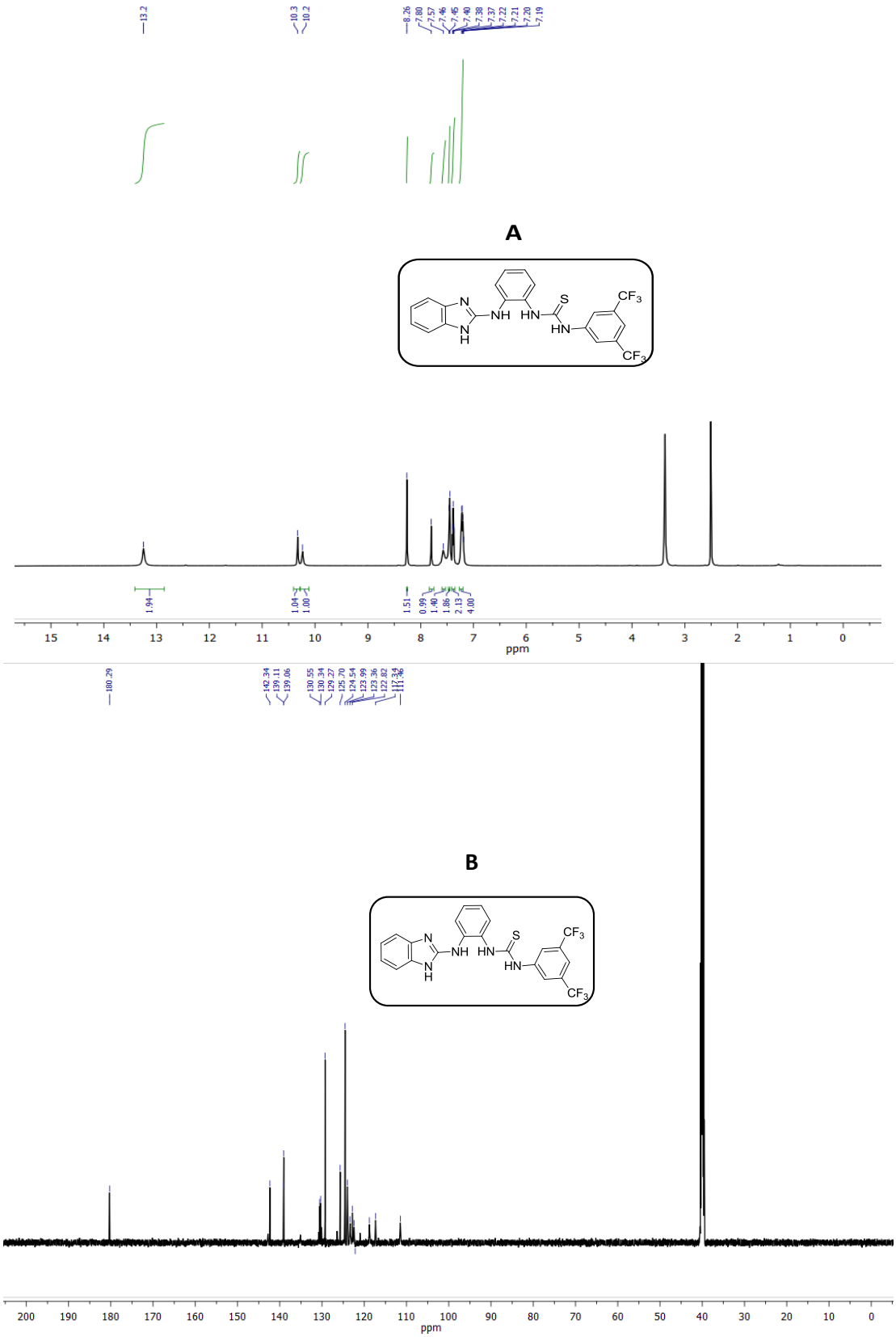



**Figure S53:** $^{1}$H NMR (A) and $^{13}$C NMR (B) of compound **4.**

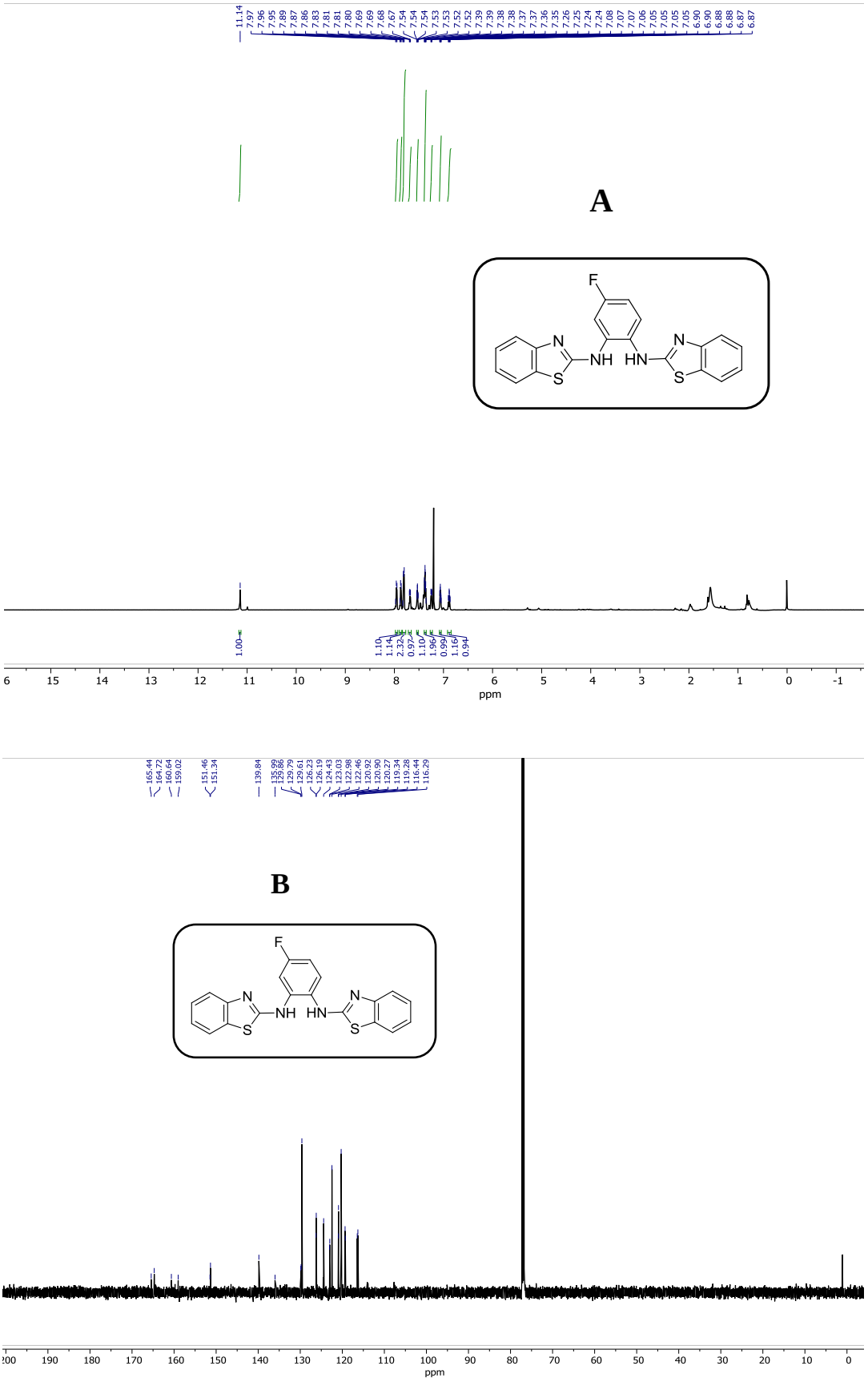



**Figure S54:** $^{1}$H NMR (A) and $^{13}$C NMR (B) of compound **5.**

## 3. HRMS spectra of compounds:

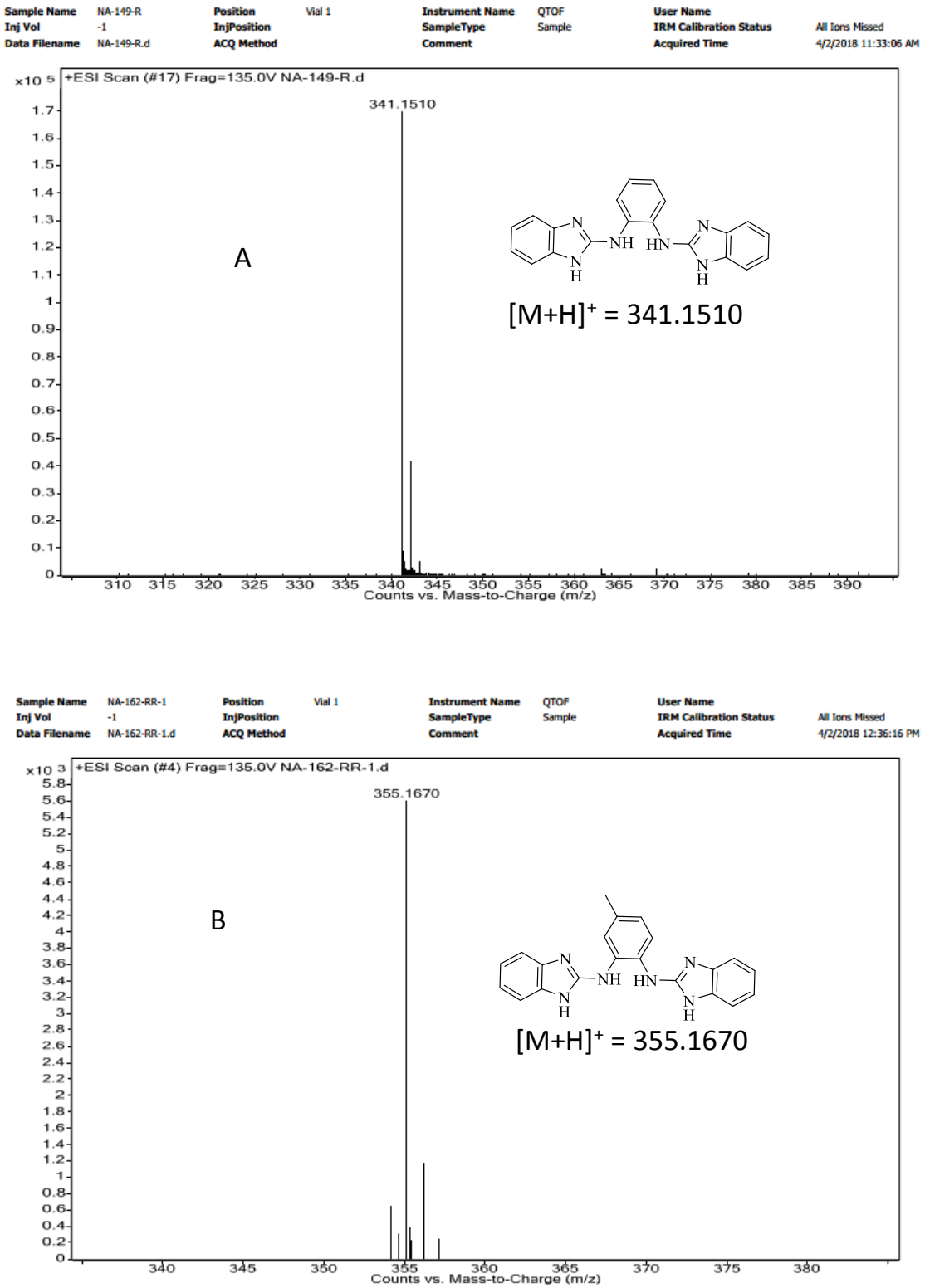



**Figure S55:** HRMS spectra of compound **1a (A)** and for compound **1b** (B).

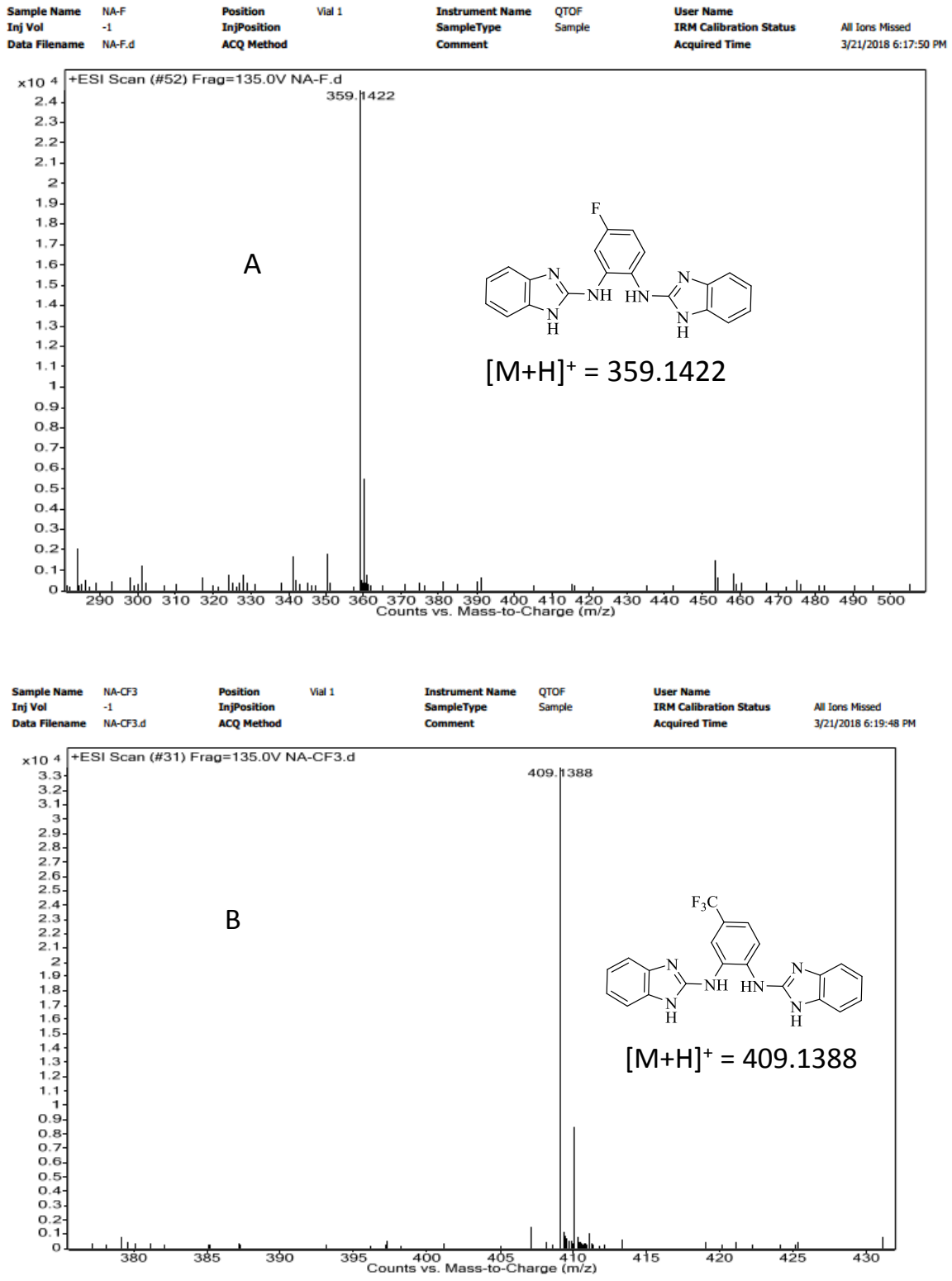
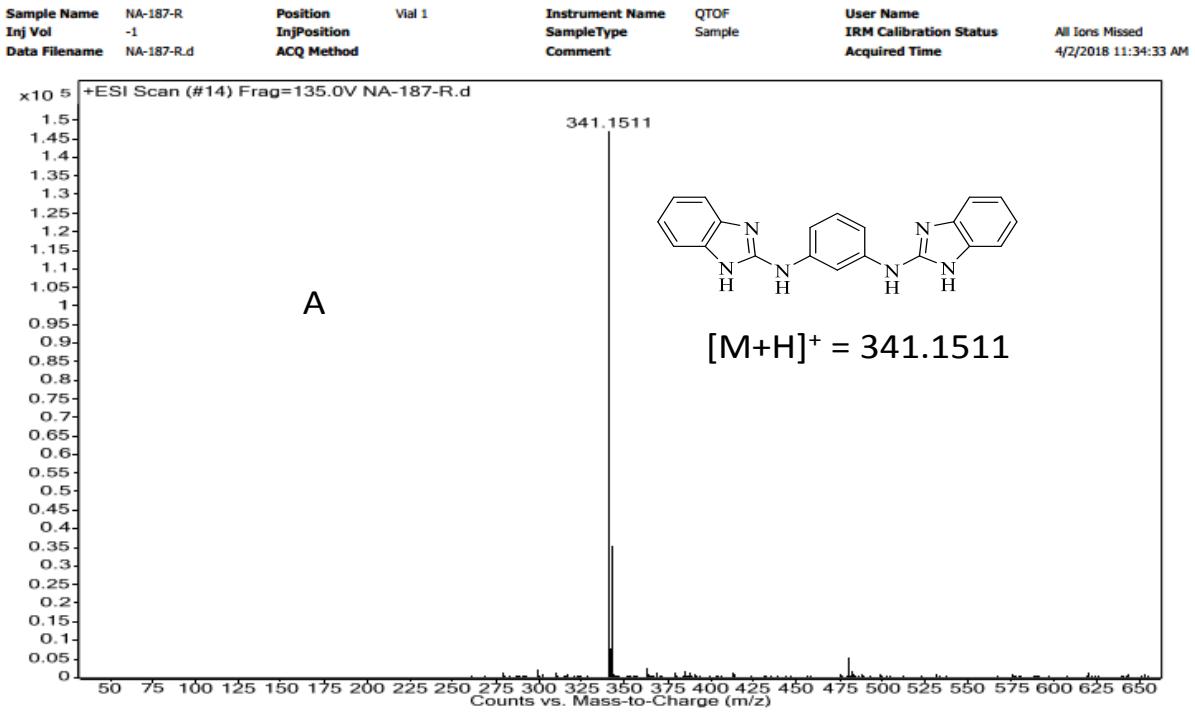



**Figure S56:** HRMS spectra of compound **1c (A)** and for compound **1d** (B).



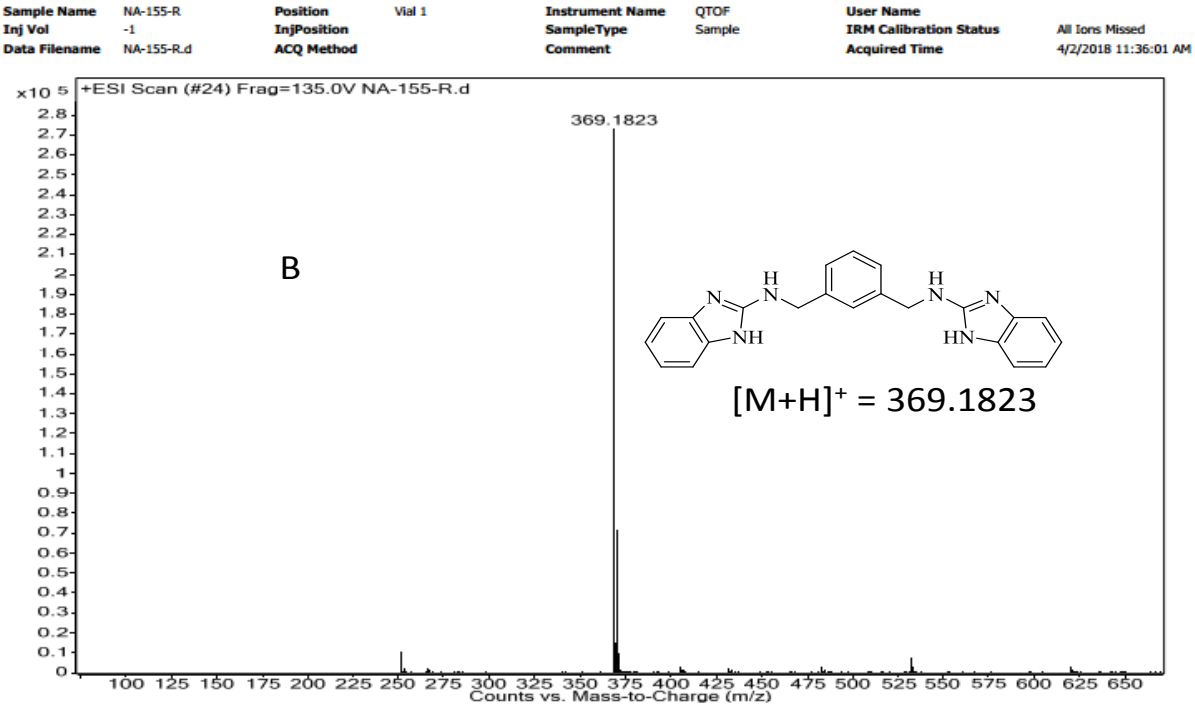




**Figure S57:** HRMS spectra of compound **2 (A)** and for compound **3** (B).

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