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SUPPORTING INFORMATION

Quinoxaline derivatives disrupt the base stacking of hepatitis C virus-internal ribosome entry site RNA: reduce translation and replication

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Material and Methods

Dual Luciferase Assay

Huh 7 cells were seeded in 24 well plates using DMEM medium supplemented with 10 % Fetal Bovine Serum. The cells were kept overnight in CO_2 incubator at 37° C. Once the cells reach about 70-80 % confluency, they were transfected with bicistronic plasmid using Lipofectamine 2000 in OPTI-MEM medium following standard transfection protocol. Transfecting medium was removed and DMEM with 10 % FBS was added to the cell and kept overnight. Compounds were then added and cells were further kept in the incubator for 24 hours. The medium was removed; cells were washed with PBS and lysed using 1X Passive Lysis buffer from Promega. The Luciferase activity was checked using Dual Luciferase assay substrate from Promega as per protocol using a Glomax 20/20 Luminometer. Results were analyzed and plotted using Graphpad Prism 7.

Mutational Studies

The HCV IRES mutational experiment was done using QuikChange II Site-directed Mutagenesis kit from Agilent. The primer sequence used for the A57U mutation was sense primer: 5'-CCCCTGTGAGGAACTTCTGTCTTCACGCAGA-3' and antisense primer: 5'-TCTGCGTGAAGACAGAAGTTCCTCACAGGGG-3'. All mutations were verified by DNA sequencing prior to being used for dual luciferase assay.

HCV Replication Inhibition

Effect of compounds on HCV replication was measured using HCV-replicon cell line (Huh7 cells harboring HCV sub-genomic replicon RNA). These cells were treated with either DMSO control or test compounds at different concentrations for 5h. After 5h, fresh medium was supplemented, and the cells were then harvested 48 h post treatment. Total RNA was isolated from the harvested cells using Trizol Reagent. Changes in the HCV RNA were measured using RT-qPCR. GAPDH was used as an internal control.

Annealing of HCV subdomain IIa RNA

RNA oligo of sequence wild type (5'–GCGUGUCGUGCAGCCUCCGG– 3' and 5'–CGGAGGAACUACUGUCUUCACGCC–3') and A57U mutated (5'–CGGAGGAACUUCUGUCUUCACGCC–3') were purchased from IDT USA. Equal amounts of the oligo dissolved in HEPES buffer were mixed and heated at 91°C for 5 mins and cooled

gradually so that the RNA oligo is annealed to each other. The RNA was quantified using a spectrophotometer and the annealed oligonucleotide was stored at -80° C before use.

Circular Dichroism

The CD experiments were performed in a Jasco 814 CD Spectrometer in 10 mM NaP buffer supplemented with 1% DMSO and $MgCl_2$ for the titration experiments as per requirements. Annealed HCV IRES domain IIa RNA was mixed without and with increasing concentration of compounds and measured using a scan speed of 50 nm/min. Minimum of 2 accumulations were taken per sample concentrations and all data were done in triplicates. The data were analyzed using Graphpad Prism 7.

DNA Gel Shift assay

The pBR322 plasmid DNA used for the DNA gel shift assay was purchased from Thermo Fisher Scientific. 10 mM Tris Cl buffer (pH 7) and 10 mM Nacl was used with 40 µM of the pBR322 plasmid DNA and different concentrations of the compounds were added (calculated as compound: DNA base pair ratio) and incubated for 4 hours at 37 °C. Samples were run in 1 % Agarose gel at constant voltage of 50 V for 3 hours. Gels were stained with EtBr (1 µg/ml) at room temperature for 5 mins and developed in a Gel Doc imaging system from Bio-Rad and results were analyzed using Image Lab software.

Cytotoxicity assay

Huh 7 and HEK 293 cells were seeded in a 96 well plate in DMEM medium supplemented with 10 % FBS. Cells were incubated in a CO_2 incubator at 37° C overnight. Compounds were treated keeping a final concentration of DMSO at around 0.5 % for 24 hours in the incubator. MTT solution was added to each well and incubated for 3 hours in the CO_2 incubator. Media was discarded and equal amounts of DMSO were added to each well and shaked at room temperature for 20 mins in dark. The plates were analyzed using a multiplate reader and absorbance was measured at 595 nm.

Molecular Docking

The crystal structure of HCV IRES domain (PDB ID: 2NOK)[1] was retrieved from Protein Data Bank[2] and used as a receptor for docking. The receptor was prepared using protein preparation wizard of Schrodinger suite.[3] All the ligands were prepared using Ligprep wizard[4] of Schrodinger suite, which generate energy minimized structures with various

ionization states, stereo-chemistries, tautomer and ring conformations. The docking was performed using rDock [5,6] docking tool. The binding cavity was defined by using 3D coordinates of binding residues of known ligand benzimidazole. During docking, the receptor was kept rigid and ligands were treated as flexible to produce different docking solutions. Post-docking minimization was performed to improve the geometry of the docking pose. The docking poses were ranked on the basis of rDock Score and top most docking pose was selected.

Molecular dynamics simulations

The receptor-ligand complexes obtained from docking analysis were subjected to molecular dynamic simulation to study the ligand mediated structural change in HCV IRES domain. The molecular dynamic simulation was carried out using GROMACSv4.5.3 simulation package.[7] Coordinates and topology files of receptor molecule were generated with *Amberff99bsc0χOL3* force field.[8] The topology and coordinate files of ligands were generated using ACPYPE (Antechamber Python Parser interface) [9] and *Amberff99bsc0χOL3* force field.[8] The receptor-ligand complex was reconstructed by editing the topology and coordinate files of receptor and ligands. A cubic simulation box was defined and filled with TIP3P water molecules.[10] The simulation box was defined in such a way that the receptor-ligand complex was placed at least 1.0 nm from edge of the box. To neutralize the system, Na^+ was added. After building the solvated system, two-stage minimization of the system was performed using steepest-descent[11] and conjugate-gradient[12] minimization algorithms. Following minimization of the system, five equilibration steps (each for 100 ps) were performed to equilibrate the system. The system was equilibrated under NVT (constant number of particles, volume, and temperature) and NPT (constant number of particles, pressure, and temperature) conditions at a temperature of 300 K and 1 atm pressure. The temperature and pressure were kept constant by the Berendsen temperature coupling method[13] and Parrinello-Rahman barostat[14] methods, respectively. After equilibration step, final production run was performed under NPT condition for 30 ns at 300 K temperature and 1 atm pressure. During production run, leapfrog algorithm[15] was used for integrating Newton's equations of motion. The long-range electrostatics was calculated by the Particle-Mesh Ewald (PME) algorithm[16]. LINCS (LINear Constraint Solver) algorithm[17] was used to constrain the length of the bonds. The atom coordinates and trajectories were saved at every 0.002 ps.

After completion of MD simulation, the distance between terminal bases was calculated using *g_dist* utility of GROMACS package.

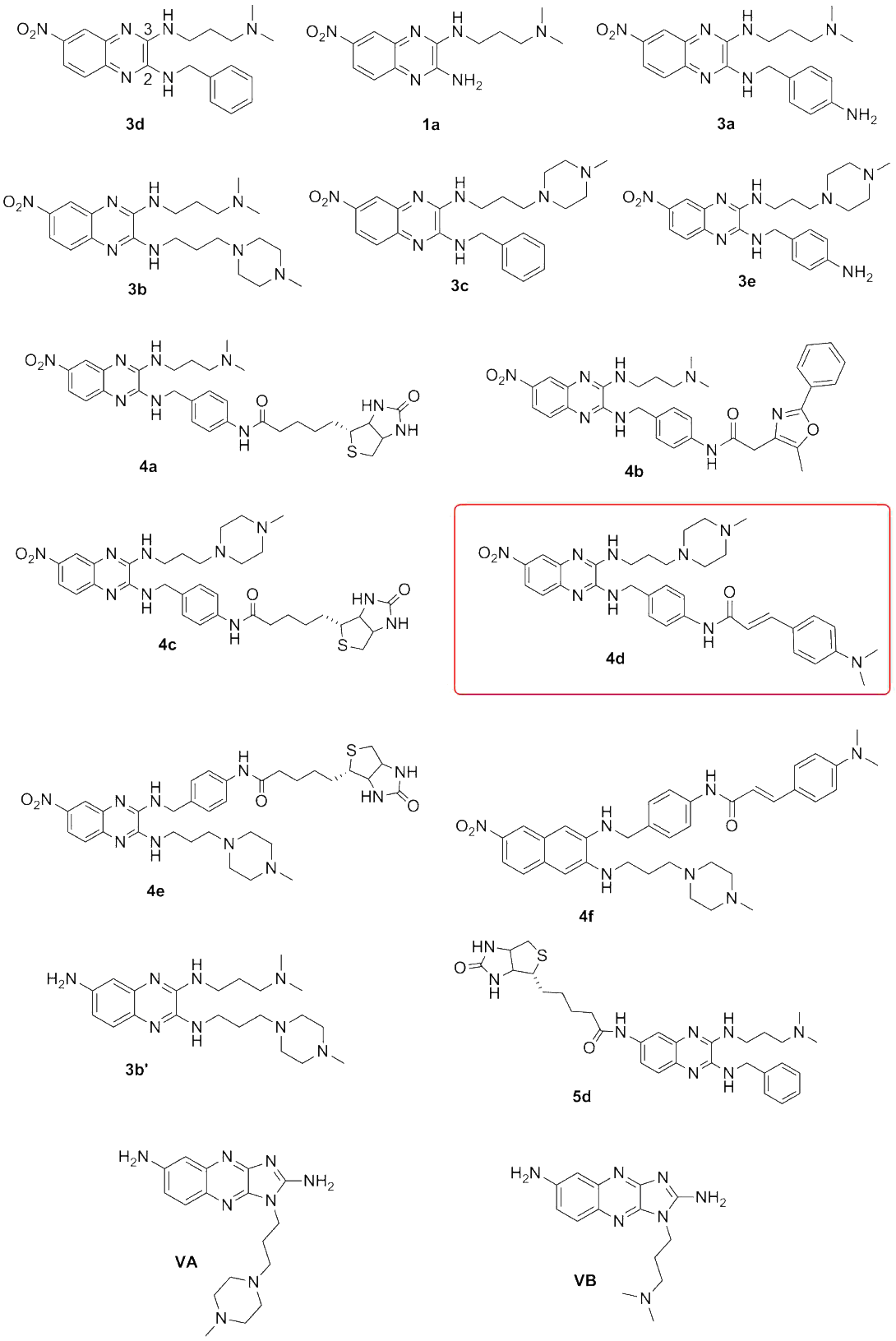


Figure S1: Structure of monoquinoxaline derivatives used in the study.

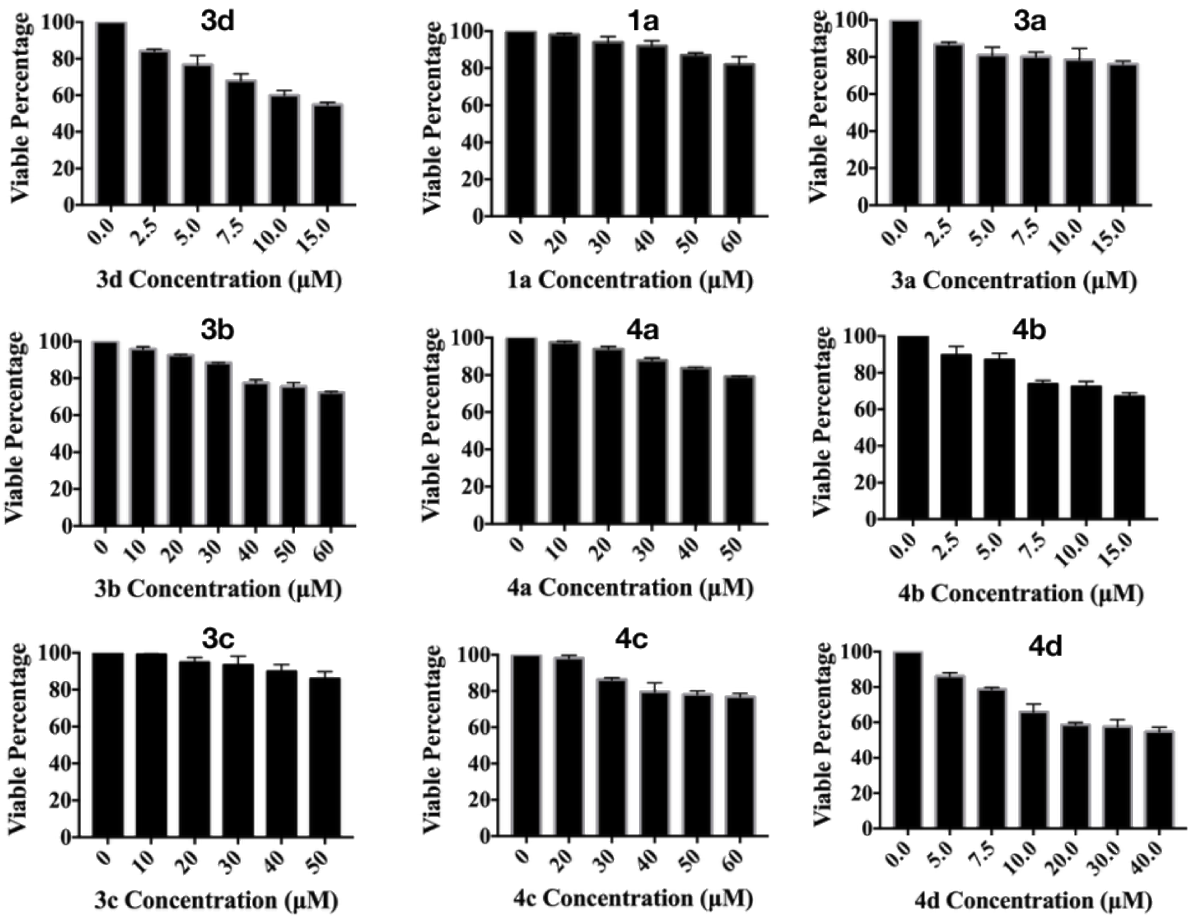


Figure S2a: Cytotoxic concentration evaluation for compounds **3d**, **1a**, **3a**, **3b**, **4a**, **4b**, **3c**, **4c** and **4d** by a colorimetric based assay (MTT assay) in Huh 7 cells. All data were done in triplicates and results were plot using Graphpad Prism 7. Error bars represent standard error calculated from three independent experiments.

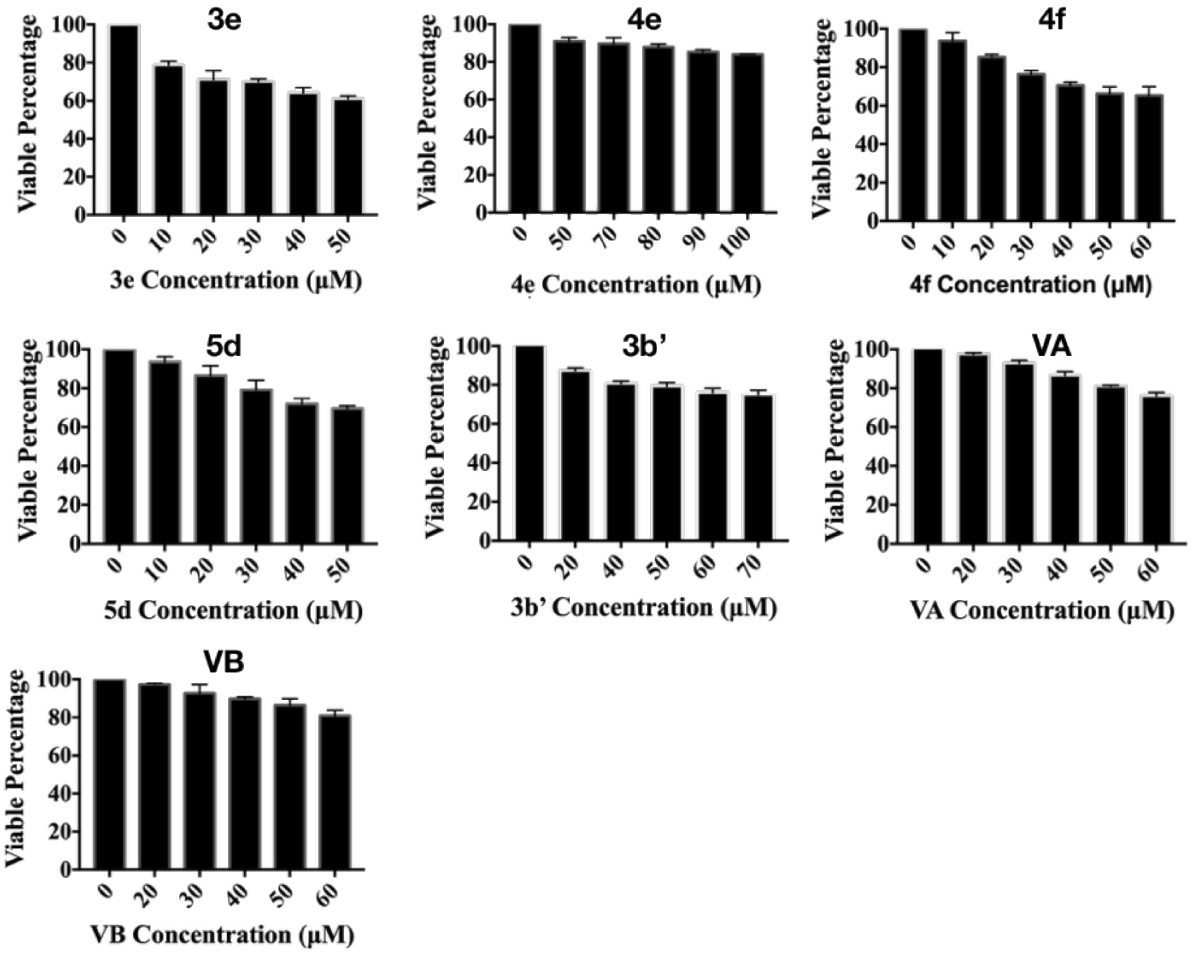


Figure S2b: Cytotoxic concentration evaluation for compounds **3e**, **4e**, **4f**, **5d**, **3b'**, **VA** and **VB** by a colorimetric based assay (MTT assay) in Huh 7 cells. All data were done in triplicates and results were plot using Graphpad Prism 7. Error bars represent standard error calculated from three independent experiments.

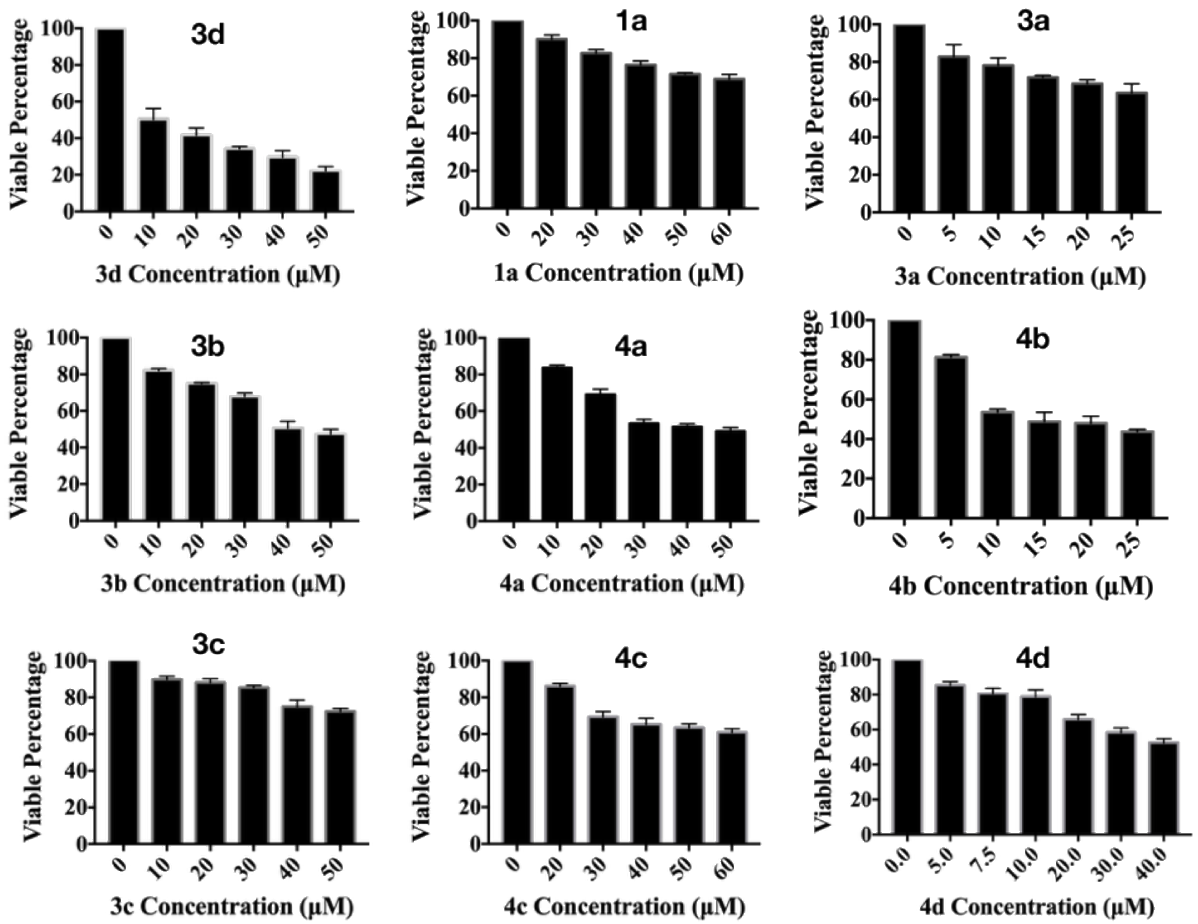


Figure S3a: Cytotoxic concentration evaluation for compounds **3d**, **1a**, **3a**, **3b**, **4a**, **4b**, **3c**, **4c** and **4d** by a colorimetric based assay (MTT assay) in HEK 293 cells. All data were done in triplicates and results were plot using Graphpad Prism 7. Error bars represent Standard error calculated from three independent experiments.

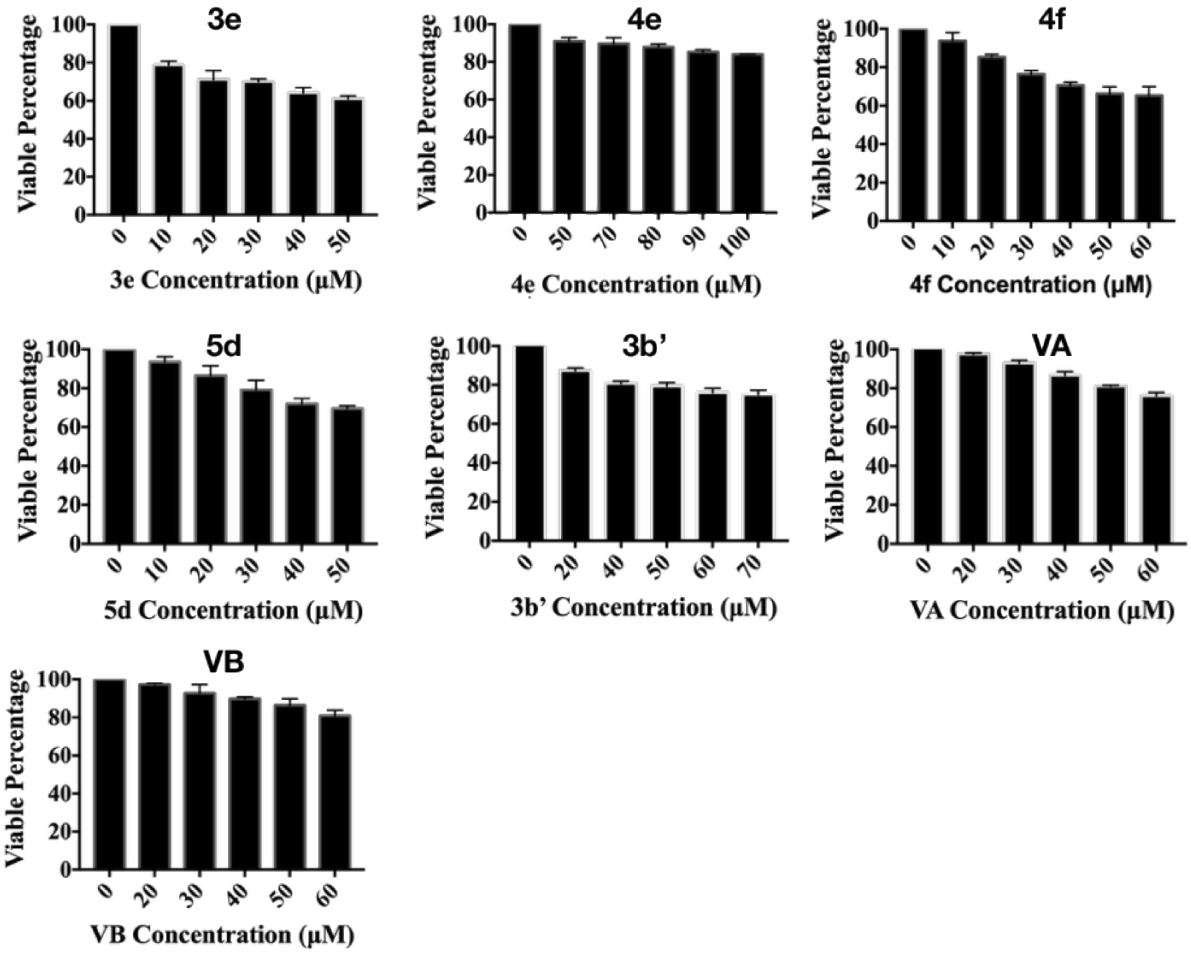


Figure S3b: Cytotoxic concentration evaluation for compounds **3e**, **4e**, **4f**, **5d**, **3b'**, **VA** and **VB** by a colorimetric based assay (MTT assay) in HEK 293 cells. All data were done in triplicates and results were plot using Graphpad Prism 7. Error bars represent standard error calculated from three independent experiments.

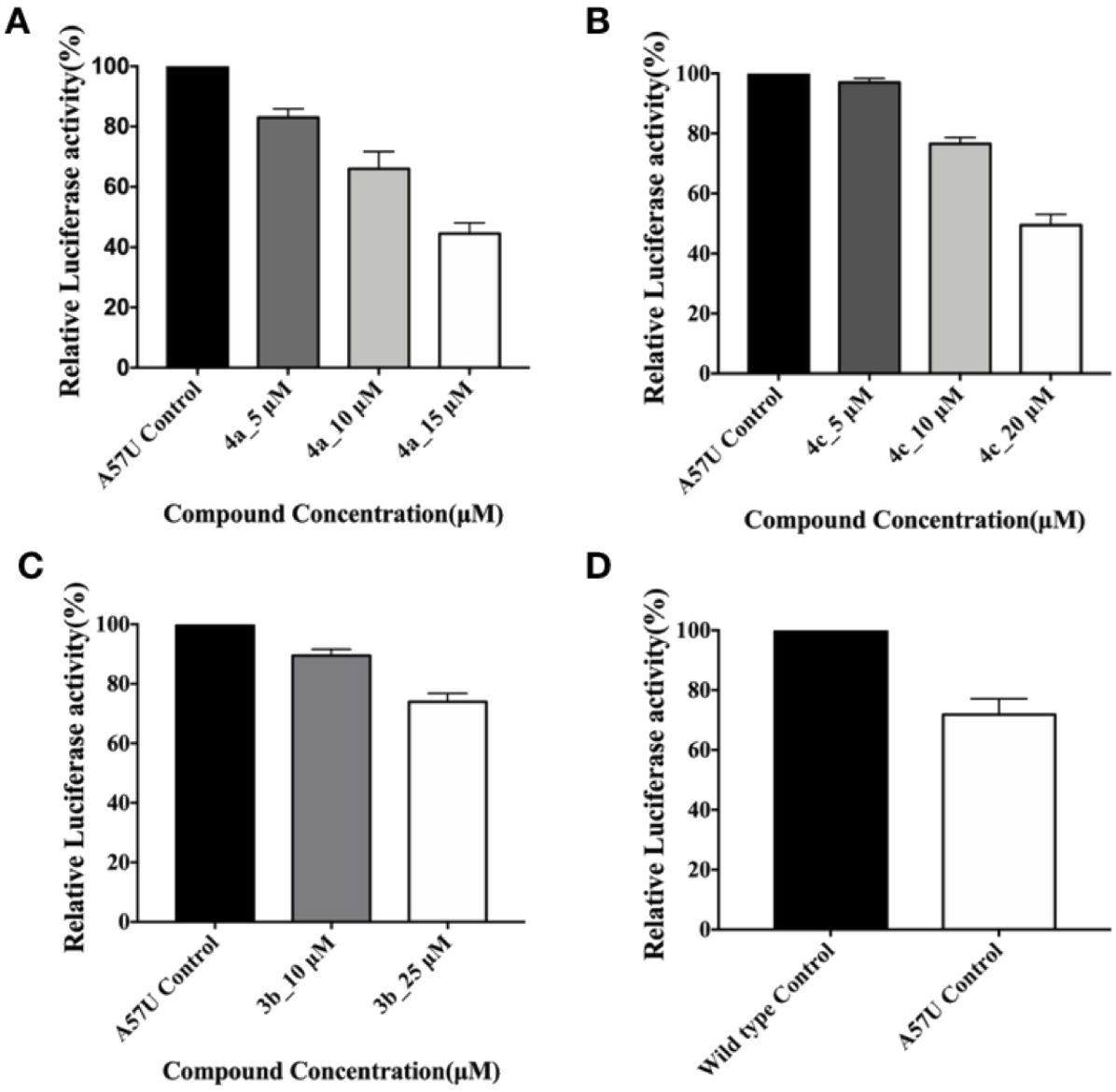


Figure S4: Dose dependent dual luciferase assay of A57U mutated HCV IRES containing bicistronic plasmid (pRL HCV 1b) with (A) **4a**, (B) **4c**, (C) **3b**. (D) Translational efficiency comparison of bicistronic plasmid carrying the wild type HCV IRES with the A57U mutant IRES. Although the absolute luciferase activity of A57U mutant was less than wild type IRES but for better representation of data, the relative luciferase activity for Figure A, B and C were plot using the luciferase activity of A57U mutated bicistronic plasmid (A57U control) as 100%. The relative luciferase activity for Figure D was plot using the luciferase activity of wild type bicistronic plasmid (Wild type Control) as 100%.

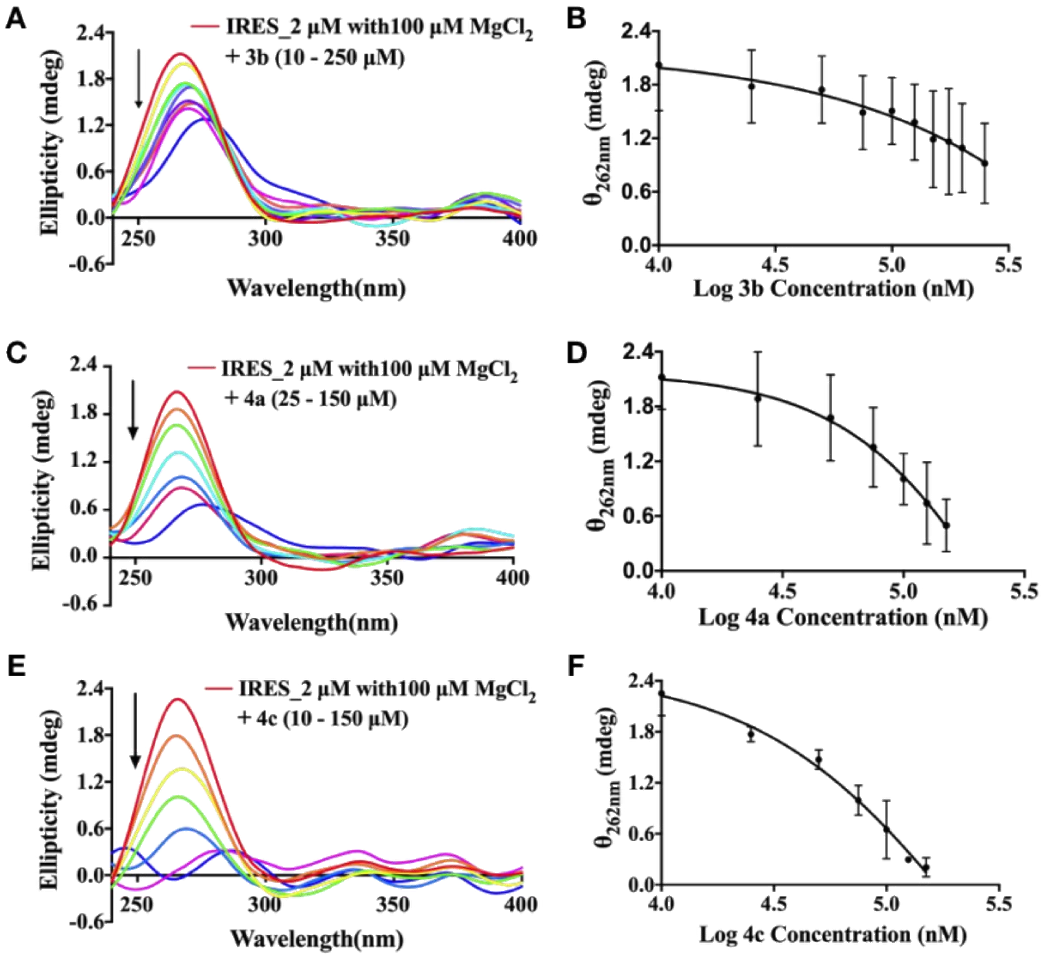


Figure S5: A) CD plot of HCV IRES subdomain IIa 2µM with **3b** (10 – 250 µM). B) Plot of CD ellipticity at 262 nm for HCV subdomain IIa 2µM with **3b** (10 – 250 µM). C) CD plot of HCV IRES subdomain IIa 2µM with **4a** (25 – 150 µM). D) Plot of CD ellipticity at 262 nm for HCV subdomain IIa 2µM with **4a** (25 – 150 µM). E) CD plot of HCV IRES subdomain IIa 2µM with **4c** (10 – 150 µM). F) Plot of CD ellipticity at 262 nm for HCV subdomain IIa 2µM with **4c** (10 – 150 µM). All plots were done using Graphpad Prism 7 and all data were done in triplicates.

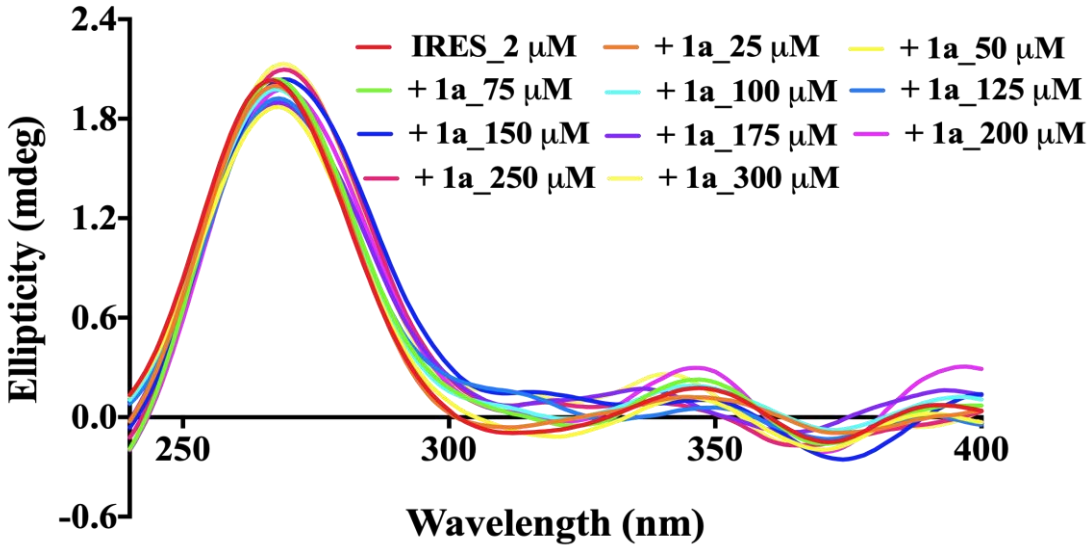


Figure S6: Circular dichroism plot of HCV IRES subdomain IIa (2 µM) with **1a** (25 – 300 µM) in the presence of 100 µM Mg^{2+} ion)

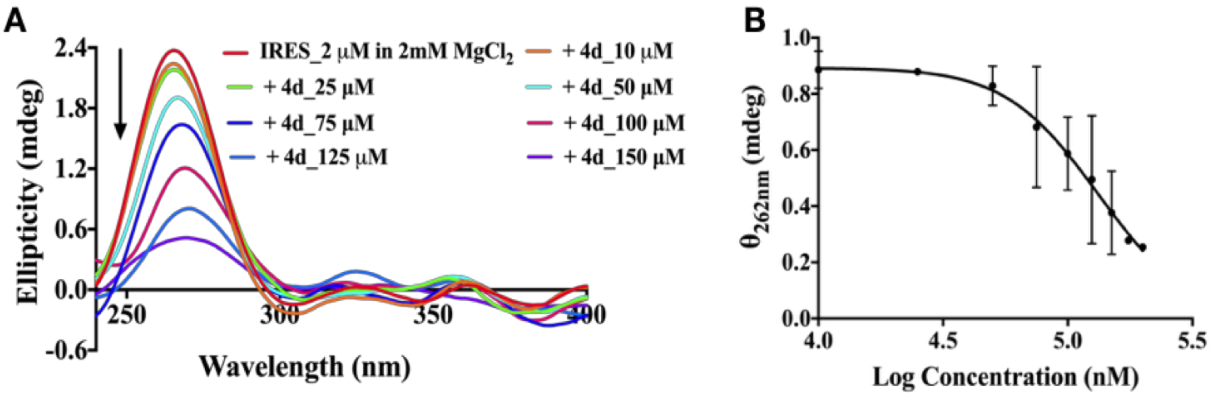


Figure S7: A) Circular dichroism plot of HCV IRES subdomain IIa (2 µM) with **4d** (10 – 200 µM) in the presence of 2 mM Mg^{2+} ion) B) CD ellipticity plot at 262 nm for HCV subdomain IIa (2µM) with **4d** (10 – 150 µM).

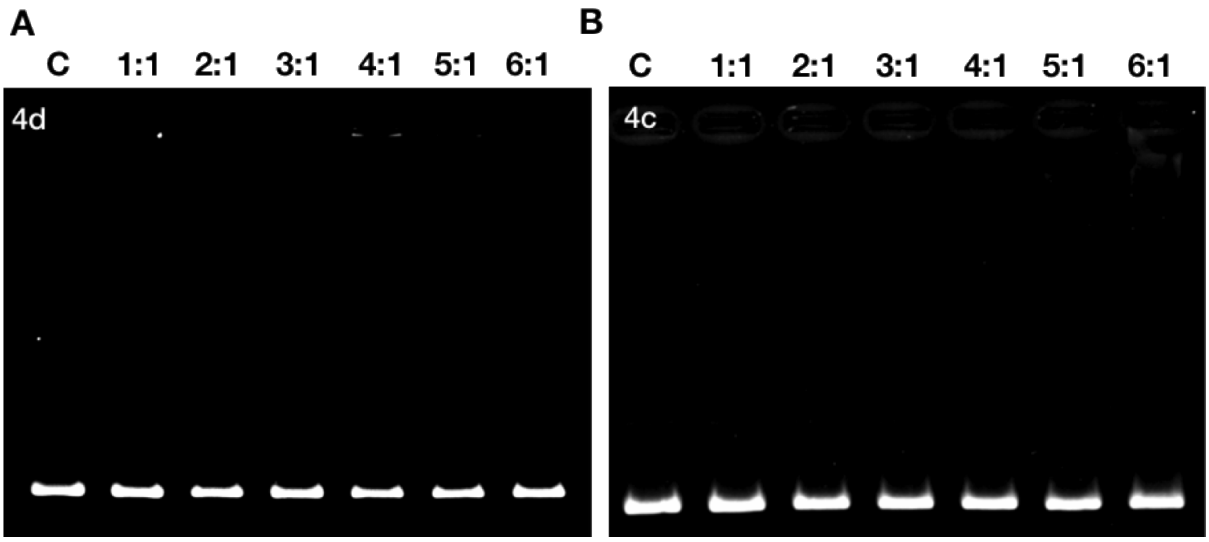


Figure S8: DNA gel shift assay of PBR 322 plasmid DNA with increasing concentration of the compound **4d** (Figure A) and **4c** (figure B). Each lane represents the compound: pBR 322 ratios. Figure was analyzed using Image lab^{TM} version 6. From the DNA gel shift studies it is quite evident that the two most potent molecule **4d** and **4c** donot intercalate double strand DNA.

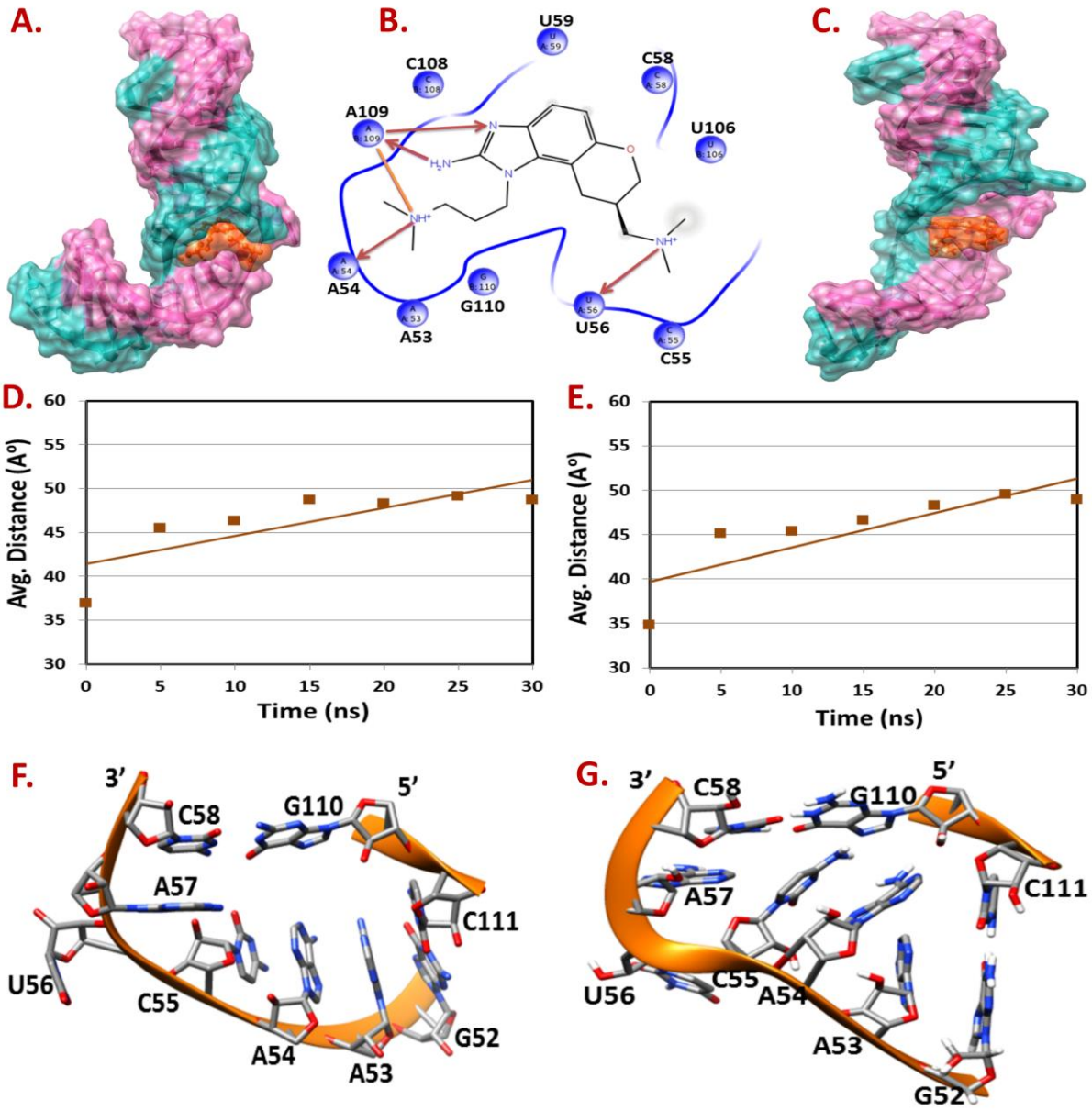


Figure S9: A) Benzimidazole docking pose, B) Ligand interaction diagram, C) Simulated pose of benzimidazole (orange) interacting with subdomain IIa. D) Distance between bases 47 and 70, E) Distance between bases 98 and 117, F) base stacking pattern before simulation, G) base stacking pattern after simulation with benzimidazole.

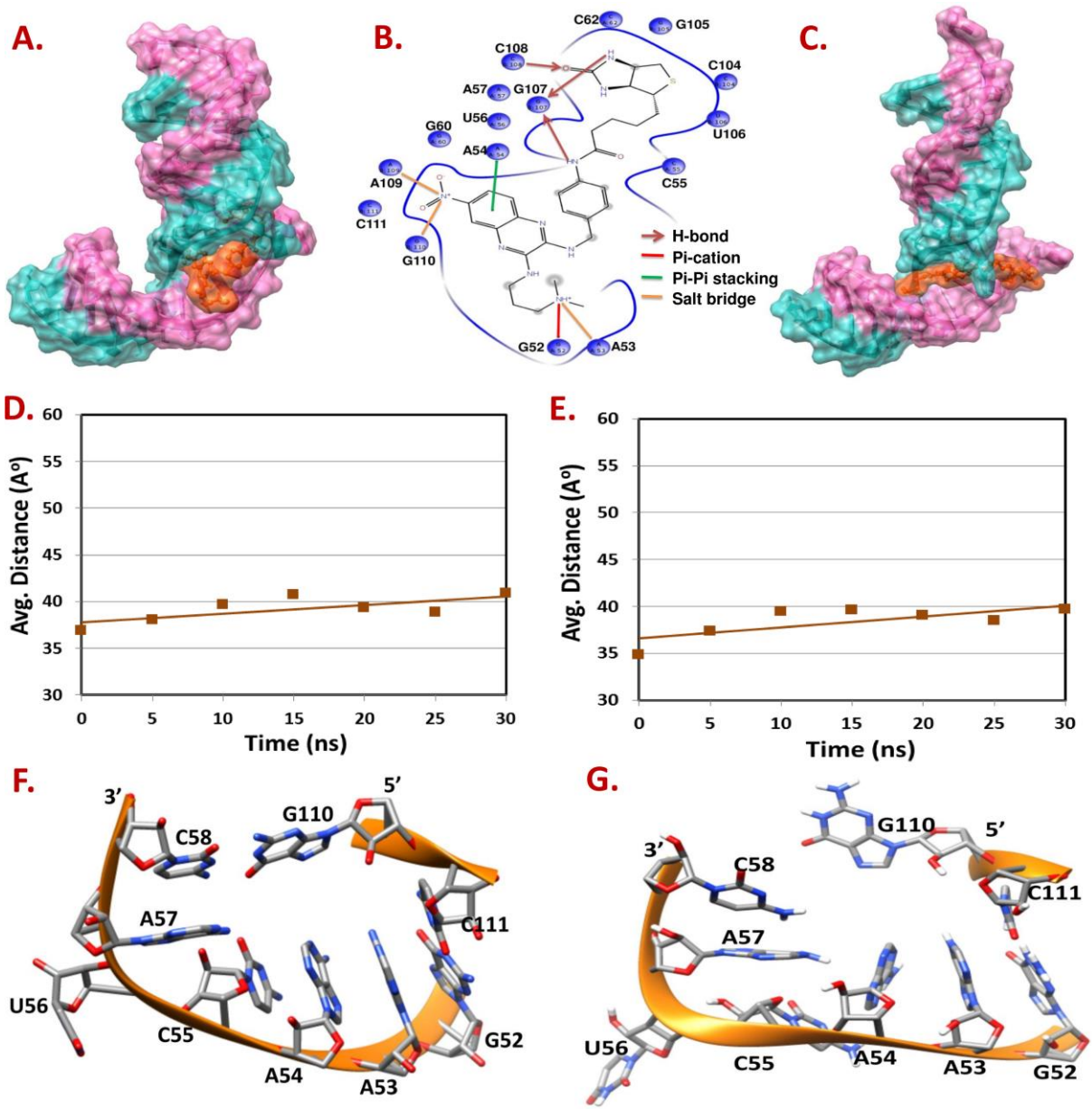


Figure S10: A) **4a** docking pose, B) Ligand interaction diagram, C) Simulated pose of **4a** (orange) interacting with subdomain IIa D) Distance between bases 47 and 70, E) Distance between bases 98 and 117, F) base stacking pattern before simulation, G) base stacking pattern after simulation with **4a**.

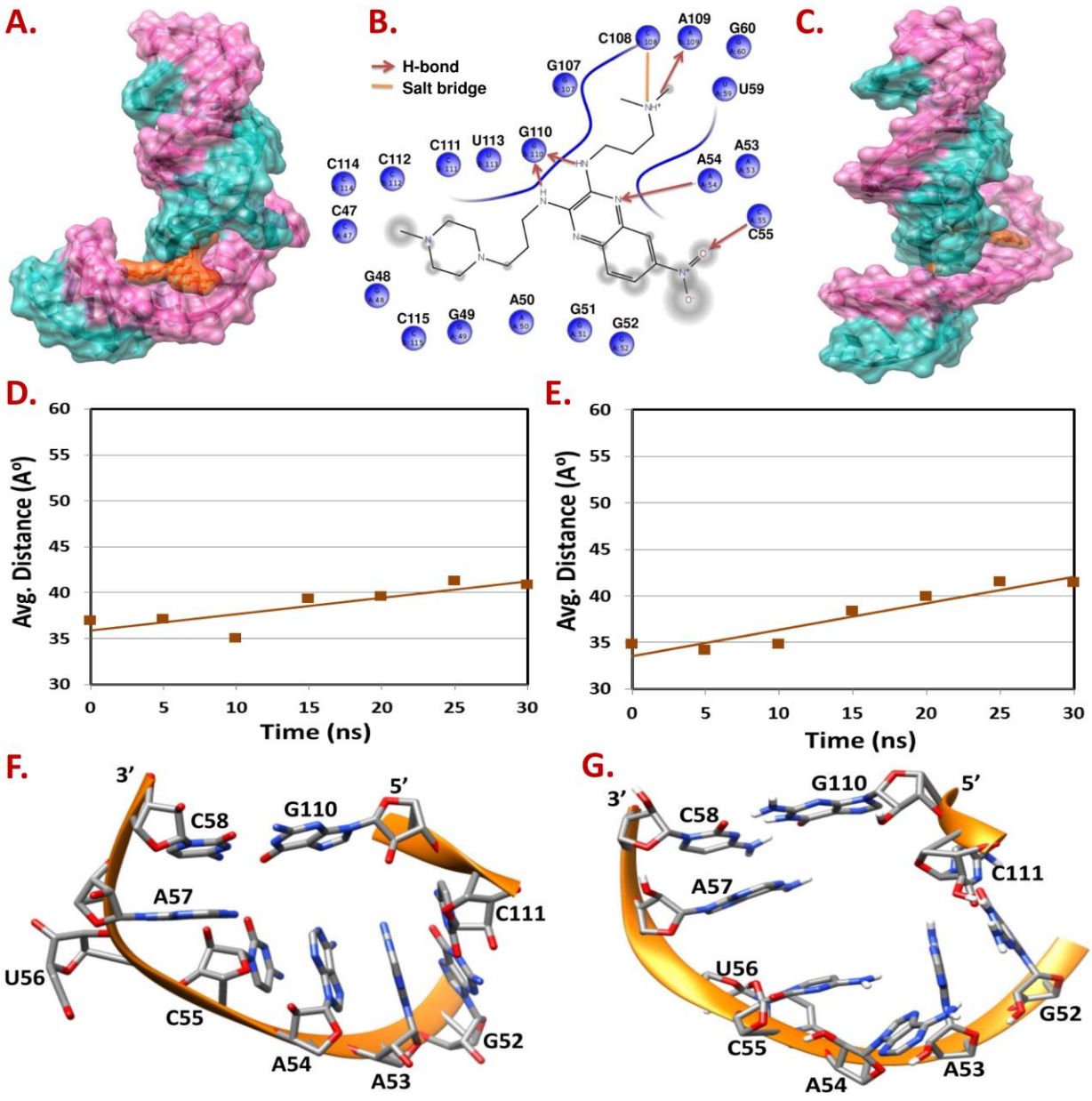


Figure S11: A) **3b** docking pose, B) Ligand interaction diagram, C) Simulated pose of **3b** (orange) interacting with subdomain IIa D) Distance between bases 47 and 70, E) Distance between bases 98 and 117, F) base stacking pattern before simulation, G) Base stacking pattern after simulation with **3b**.

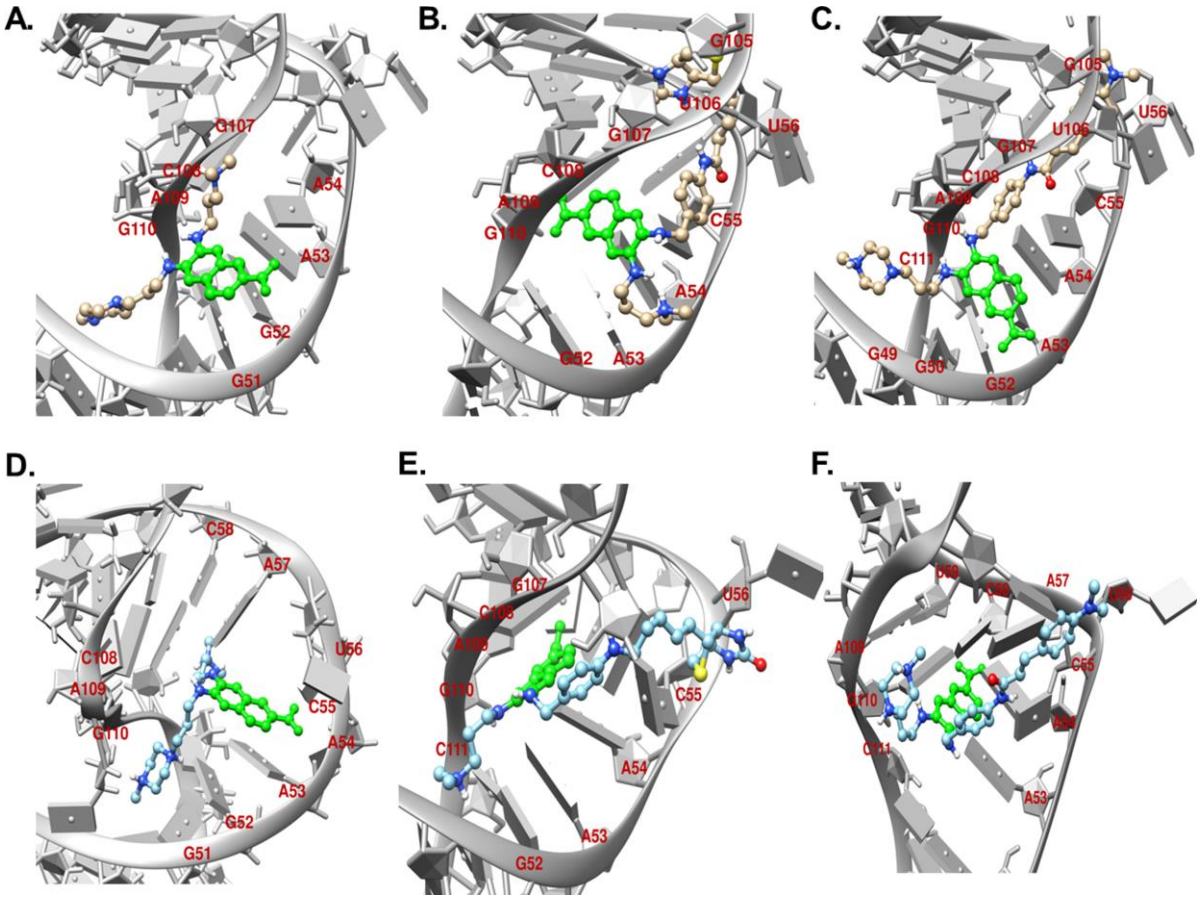


Figure S12: Representative docking poses of the molecules **3b** (Figure A), **4a** (Figure B), **4d** (Figure C) pre molecular dynamic simulation. Representative docking poses of the molecules **3b** (Figure D), **4a** (Figure E), **4d** (Figure F) post molecular dynamic simulations. The mono quinoxaline part of the molecules is marked in green. The docking orientation and region of docked monoquinoxaline part was found to be similar in case of **3b** and **4d** where the docked mono-quinoxaline parts were facing outside of the RNA groove (pre MD simulation). In the representative docking pose for **4a**, the monoquinoxaline part was docked in the subdomain IIa at regions similar to that of **4d** and **3b** but the orientation of NO_2 was pointing inside of the groove (pre MD simulation).

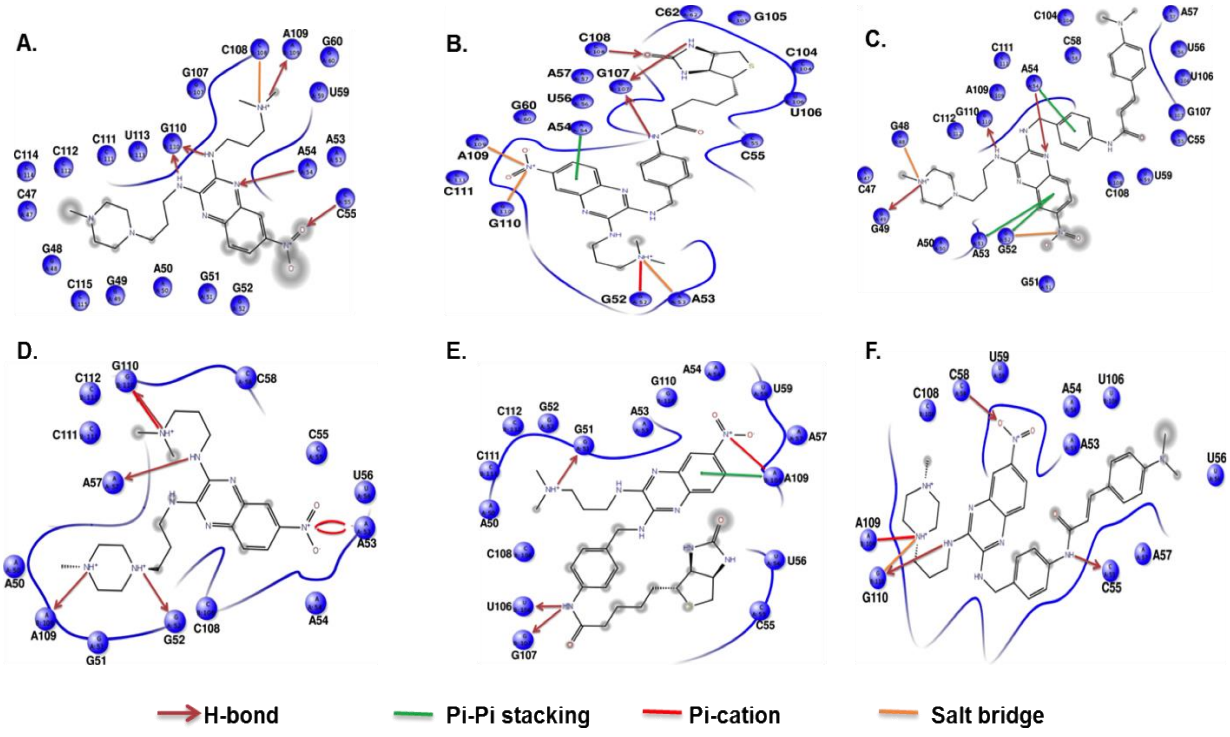


Figure S13: Probable interaction patterns of the molecules **3b** (Figure A), **4a** (Figure B) and **4d** (Figure C) extracted from the representative docking poses in pre MD simulation condition. Probable interaction patterns of the molecules **3b** (Figure D), **4a** (Figure E) and **4d** (Figure F) extracted from the representative docking poses in post MD simulation condition.

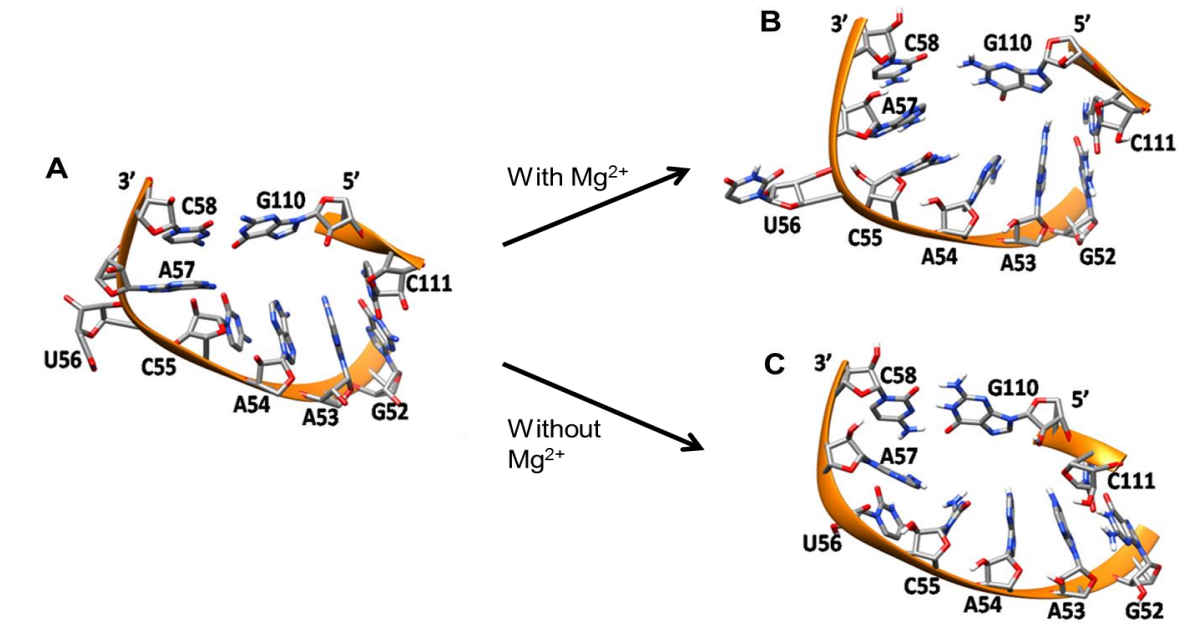


Figure S14: A) Base stacking pattern of the subdomain IIa in the absence of Mg^{2+} ions pre-simulation. B) Base stacking pattern of the subdomain IIa in the presnece of Mg^{2+} ions post-simulation (50 nano secs). C) Base stacking pattern of the subdomain IIa in the absence of Mg^{2+} ions post-simulation (50 nano secs).

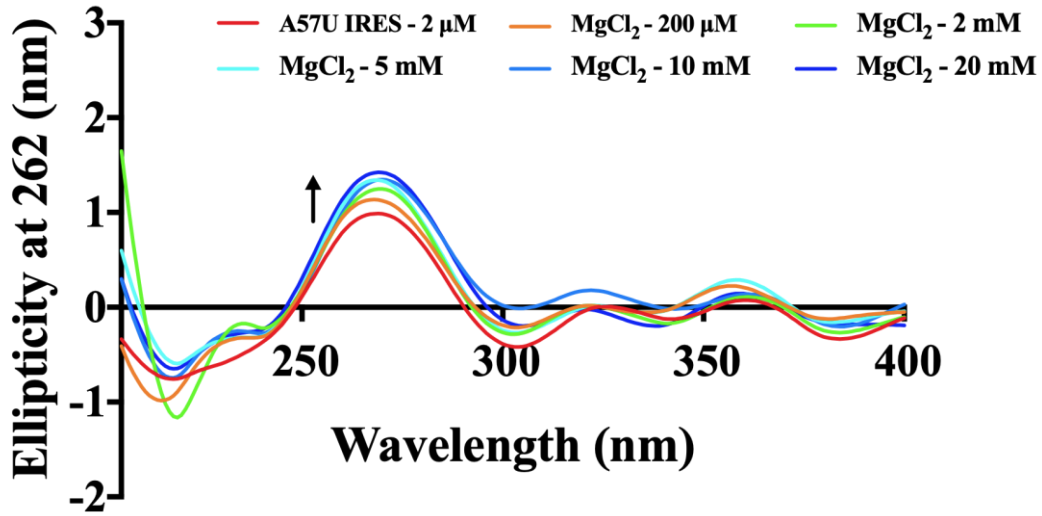
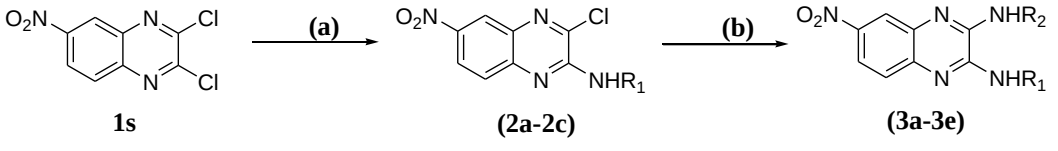


Figure S15: Circular Dichroism spectra of A57U mutated subdomain IIa(2 µM) titrated with increasing concentration of $MgCl_2$ (200 µM to 20 mM). Graph was plot using Graphpad Prism 7.

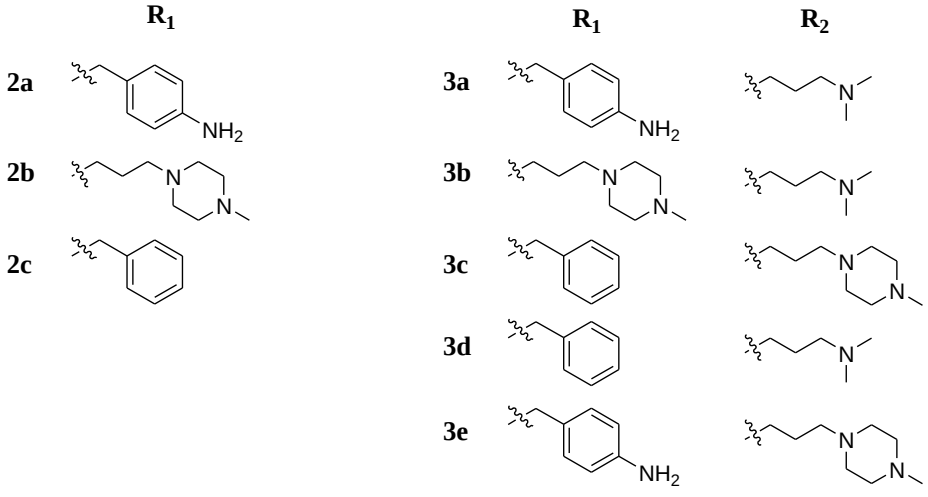
Section S1: Experimental Section

Materials and methods: All solvents and chemicals were purchased from common commercial vendors and used without further purification. Solvents were distilled prior to use. All reactions were carried out under nitrogenous atmosphere and anhydrous condition unless otherwise mentioned. ^{1}H NMR spectra were collected on a BRUKER-DPX 300 MHz and BRUKER AVANCE 600 MHz spectrometers. ^{1}H NMR data are reported as follows: chemical shift in parts per million (ppm) relative to tetramethylsilane, multiplicity (s = singlet; d = doublet; t = triplet; q = quartet; quin = quintet; m = multiplet; br = broadened), coupling constant (Hz), and integration. ^{13}C NMR spectra were recorded on BRUKER-AVANCE 600 (150 MHz) and BRUKER-DPX 300 (75 MHz) spectrometers. Mass spectra were performed using ESI and EI positive ionization mode. EI HRMS were collected using an EI mass spectrometer MS station Jms-700, Jeol, Japan and ESI HRMS were collected using Waters Q-TOF Micromass spectrometer. The compounds were purified by analytical HPLC on Shimadzu SCL-10A VP instrument. [21]

<u>Procedure A:</u> Synthesis of Quinoxaline derivatives (3a-3f): All the compounds (**3a**-**3f**) were synthesized by following the procedure as reported earlier for the synthesis of the reported compound **3d** (Scheme 1). [22, 23]



1s **(2a-2c)** **(3a-3e)**

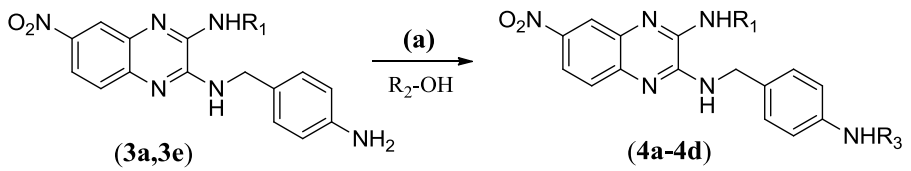


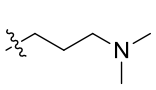
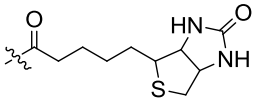
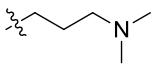
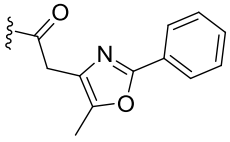
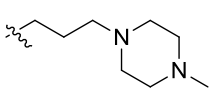
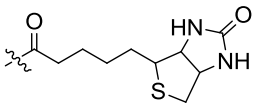
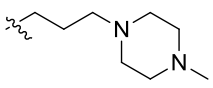
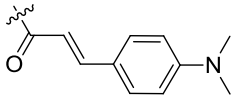
Scheme 1: Reagents and conditions: **(a)** $CaCO_3$ (3.442 mmol, 2.8 equiv), R_1NH_2 (1.475 mmol, 1.2 equiv), CH_2Cl_2, rt, 18 h. **(b)** X-Phos (0.025 mmol, 0.1 equiv), $Pd_2(dba)_3$ (0.0125 mmol, 0.05 equiv), Cs_2CO_3 (0.375 mmol, 1.5 equiv), R_2NH_2 (0.375 mmol, 1.5 equiv), dioxane, reflux at 110 ^{0}C, 6 hr.

<u>Procedure B:</u> Synthesis of quinoxaline derivatives (4a-4f):

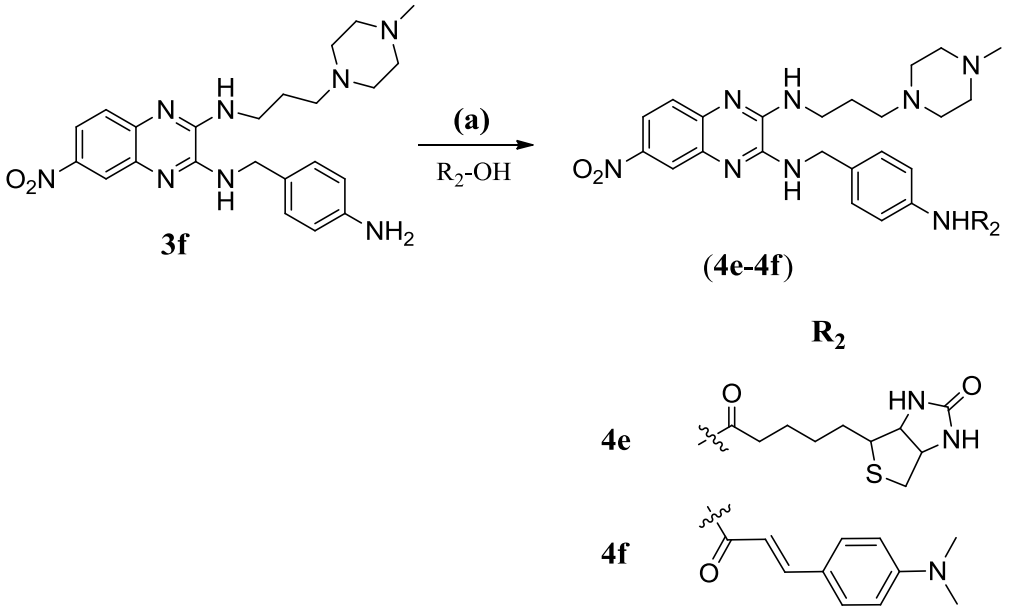
Compounds **4a-4f** (Scheme 2) were synthesized by using acid-amine coupling reaction. To a solution of carboxylic acid derivative (R_2-OH) (0.2 mmol, 1 equiv) in dry DMF (2 mL), a coupling reagent BOP (0.24 mmol, 1.2 equiv) and DIPEA (0.4 mmol, 2 equiv) were added on ice-bath which was then stirred for 15 minutes at room temperature. Then benzylamine derivative (**3a**, **3e**, **3f**) (0.24 mmol, 1.2 equiv) was added and stirred at room temperature for 18 hr. After the consumption of the starting material as indicated by TLC, work-up was done by using DCM and ice-cold water. Then the crude product was purified by using column chromatography with $CHCl_3$ and MeOH as eluents (with 60-80 % isolated yield).

Scheme 2a



	R_1	R_2
4a		
4b		
4c		
4d		

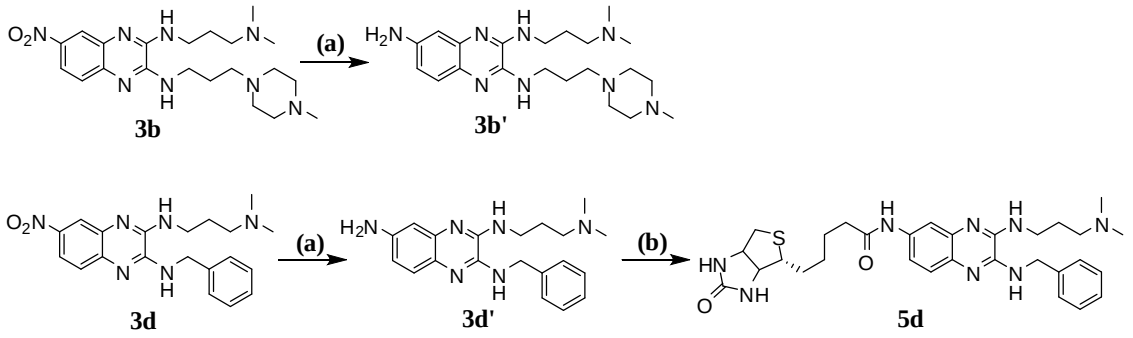
Scheme 2b



Scheme 2a and 2b: Reagents and conditions: **(a)** Acid (R_3-OH) (0.2 mmol, 1 equiv), BOP (0.24 mmol, 1.2 equiv), DIPEA (0.4 mmol, 2 equiv) in DMF, rt, 18 h.

<u>Procedure C:</u> Synthesis of quinoxaline derivatives (3b', 3d' and 5d):

Using **3b** or **3d** (0.2 mmol) as starting material, dissolved in dry EtOH (8 mL), followed by reductive hydrogenation with Pd/charcoal (0.02 mmol) at room temperature afforded 6-amino quinoxaline derivative (**3b**' or **3d'**) within 6 hr. After the consumption of the starting materials as indicated by TLC, it was diluted with ethanol (10 mL) and filtered through cellite bed. The filtrate was concentrated under reduced pressure, afforded the compound **3b'** or **3d'**. The resultant compound was used without further purification. In the next step, to a solution of D-biotin (0.1 mmol, 1 equiv) in dry DMF (1.5 mL), BOP (0.12 mmol, 1.2 equiv) and DIPEA (0.2 mmol, 2 equiv) were added on ice-bath and stirred for 15 minutes at room temperature. Upon addition of 6-aminoquinoxaline derivative **3d'** (0.12 mmol, 1.2 equiv) to the reaction mixture, stirred at room temperature for 18 hr. After completion of the reaction, crude was purified by column chromatography with $CHCl_3$ and MeOH as eluents (with 72 % isolated yield).



Scheme 3: Reagents and conditions: (a) Pd/C (0.02 mmol, 0.1 equiv), H_2 gas in dry ethanol (4 mL), rt, 6hr. (b) D-biotin (0.1 mmol, 1 equiv), BOP (0.12 mmol, 1.2 equiv), DIPEA (0.2 mmol, 2 equiv) in DMF (1.5 mL), rt, 18 hr.

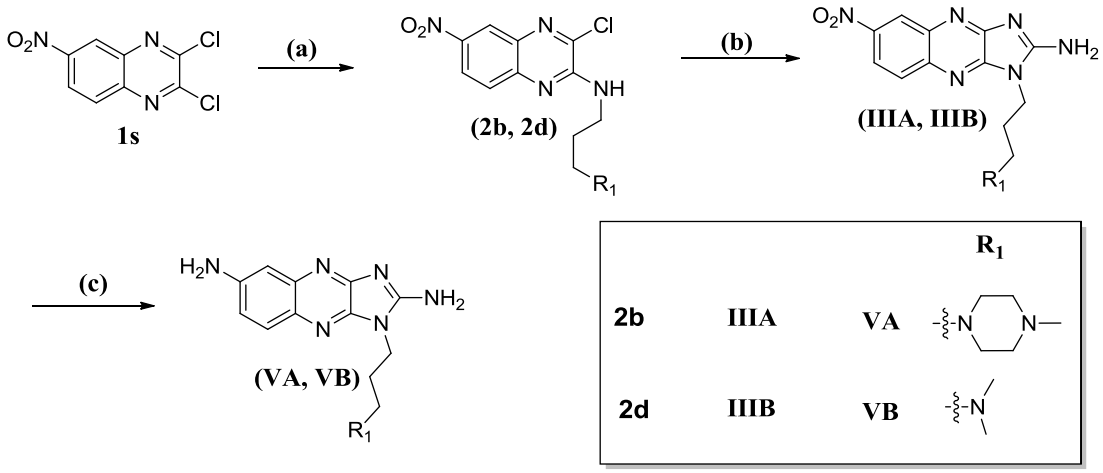
<u>Procedure D:</u> Synthesis of quinoxaline based derivatives VA and VB:

To a solution of 2,3-Dichloro-6-nitroquinoxaline (3 mmol, 1 equiv) in DCM (30 mL) , a propylamine derivative (3.6 mmol, 1.2 equiv) and $CaCO_3$ (9 mmol, 3 equiv) was added, which was then stirred for 18 h at rt. After the consumption of starting material, the crude was purified by using column chromatography with ethyl acetate and petroleum ether as eluents, isolating the intermediates **2b** and **2d** (with yield of 70 % and 55 % respectively).

Next to a solution of, **2b** or **2d** (2.23 mmol, 1 equiv) in DMF (10 mL), sodium hydrogen cyanamide (NaNHCN) (4.46 mmol, 2 equiv) was added slowly. Then the whole reaction mixture was stirred overnight under N_2 atmosphere. After the consumption of the starting materials as indicated by TLC, it was diluted with DCM (10 mL) and the reaction mixture was concentrated under vacuum, resulting dark red oil. Then the intermediates **IIIA** and **IIIB** were further purified by column chromatography (with isolated yield of 60 % and 52 % respectively) using methanol-chloroform solvent system and were used for the next step without further purification.

To a solution of **IIIA** or **IIIB** (1.21 mmol, 1 equiv) in ethanol (50 mL), Pd/Charcoal (0.12 mmol, 0.1 equiv) were added. Then the reaction mixture was stirred at rt for 6 hr under hydrogen atmosphere. After the consumption of the starting materials as indicated by TLC, it was diluted with ethanol (20mL) and filtered through celite bed. The filtrate was concentrated under reduced pressure which was further purified over neutral alumina in methanol-

chloroform affording pure products **VA** and **VB** (with isolated yield of 40 % and 55 % respectively).



Scheme 4: Reagents and conditions:(a) Amine, $CaCO_3$, Dichloromethane, r.t. , 18 h. (b) NaNHCN , DMF, r.t., 18 h. (c) H_2/ Pd (10%) , Ethanol: Ethylacetate (1:1), r.t., 6h.

N^2-(4-aminobenzyl)-N^3-(3-(dimethylamino)propyl)-6-nitroquinoxaline-2,3-diamine (3a): Compound **3a** was synthesized by following the procedure **A**. The crude compound was purified by column chromatography using silica gel (100-200) in methanol-chloroform (2-10 %) solvent system to afford the desired **3a**. Yellowish orange solid; yield ~ 70%.^{1}H-NMR (300 MHz, $CDCl_3$) δ(ppm): 8.45 (d, *J* = 2.4 Hz, 1H), 8.32 (br s, 1H), 8.07 (dd, *J* = 2.7, 9.0 Hz, 1H), 7.61 (d, *J* = 9.0 Hz, 1H), 7.23 (d, *J* = 8.4 Hz, 2H), 6.70 (d, *J* = 8.4 Hz, 2H), 5.26 (br s, 1H), 4.62 (d, *J* = 4.5 Hz, 2H), 3.72 (br s, 2H), 3.66 (t, *J* = 5.7 Hz, 2H), 2.61 (t, *J* = 5.4 Hz, 2H), 2.15 (s, 6H), 1.88 (m, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ (ppm): 146.3, 145.4,145.0,143.8, 141.9, 136.6, 129.9 (2C), 127.7, 125.6, 121.0, 118.4, 115.3 (2C), 59.6, 45.8, 44.8 (2C), 42.5, 23.7; HRMS (EI+): m/z calculated for $C_{20}H_{25}N_7O_2$ [M^+]: 395.2070; found 395.2069; HPLC purity:~ 100%.

N^2-benzyl-N^3-(3-(4-methylpiperazin-1-yl)propyl)-6-nitroquinoxaline-2,3-diamine (3c): Compound **3c** was synthesized by following the procedure **A**.The crude compound was purified by column chromatography using silica gel (100-200) eluting with 2- 8 % methanol in chloroform afforded the desired product.Orange solid;yield~ 50%. ^{1}H-NMR (600 MHz, DMSO-d_6) δ (ppm): 8.12 (s, 1H), 8.00 (brs, 1H), 7.93 (d, *J* = 8.4 Hz, 1H), 7.46 (d, *J* = 8.4 Hz, 1H), 7.41 (d, *J* = 7.8 Hz, 2H), 7.35 (t, *J* = 7.5 Hz, 2H), 7.27 (t, *J* = 6.9 Hz, 1H), 4.74 (d, *J* = 4.8 Hz, 2H), 3.49 (d, *J* = 5.4 Hz, 2H), 2.49 (s, 2H), 2.34 (m, 8H), 2.13 (s, 3H), 1.78 (m, 2H);^{13}C-NMR

(150 MHz, DMSO-d_6) δ (ppm): 145.6, 145.1, 143.0, 142.2, 139.0, 136.3, 128.9 (2C), 128.6 (2C), 127.7, 125.5, 120.1, 118.2, 56.0, 55.2 (2C), 53.1 (2C), 46.2, 44.9, 39.5, 25.9; HRMS (ESI^+): m/z calculated for $C_{23}H_{30}N_7O_2$+ H^+[M+H^+]: 436.2455; found 436.2463; HPLC purity:~ 100%.

N^2-(4-aminobenzyl)-N^3-(3-(4-methylpiperazin-1-yl)propyl)-6-nitroquinoxaline-2,3-diamine (3e): Compound **3e** was synthesized by following the procedure **A**.The crude product was purified by column chromatography using silica gel (100-200) eluting with 2-8% methanol in chloroform afforded the desired product.Yellow solid;yield~ 60%. ^{1}H-NMR (600 MHz, $CDCl_3$) δ (ppm): 8.46 (d, *J* = 2.4 Hz, 1H), 8.07 (dd, *J* = 2.4, 9.0 Hz,1H), 7.60 (d, *J* = 9.0 Hz, 1H), 7.31 (br s, 1H), 7.22 (d, *J* = 8.4 Hz, 2H), 6.66 (d, *J* = 8.4 Hz, 2H), 5.39 (br s, 1H), 4.74 (d, *J* = 5.4 Hz, 1H), 3.68 (br s, 2H), 3.65 (t, *J* = 6.0 Hz, 2H), 2.62 (t, *J* = 5.7 Hz, 2H), 2.56-2.34 (m, 4H), 2.19 (s, 3H), 2.16 (br s, 2H), 1.90 (m, 2H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ (ppm):146.1, 145.5, 145.1, 143.9, 141.9, 136.4, 129.7 (2C), 127.9, 125.7, 121.3, 118.7, 115.3 (2C), 58.4, 54.9 (2C), 53.3 (2C), 45.8, 45.2, 42.7, 23.3; HRMS (EI^+): m/z calculated for $C_{23}H_{30}N_8O_2$ [M^+]:450.2492; found 450.2497; HPLC purity:~ 100%.

N-(4-(((3-((3-(dimethylamino)propyl)amino)-6-nitroquinoxalin-2-yl)amino)methyl) phenyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (4a): The title compound **4a** was prepared according to the general procedure **B** from D-biotin and N^2-(4-aminobenzyl)-N^3-(3-(dimethylamino)propyl)-6-nitroquinoxaline-2,3-diamine (**3a**). The crude product was purified by column chromatography using silica gel(100-200), eluting with 2-10% methanol in chloroform afforded the desired product.Orange yellow solid;yield~ 60%; ^{1}H NMR (300 MHz, CD_3OD) δ (ppm): 8.33 (d, *J* = 2.4 Hz,1H), 8.03 (dd, *J* = 2.4, 9.0 Hz,1H), 7.56 (d, *J* = 8.7 Hz,3H), 7.41 (d, *J* = 8.4 Hz, 2H), 4.77 (s,1H), 4.50 (dd, *J* = 4.8, 7.8 Hz, 1H), 4.31 (dd, *J* = 4.5, 7.8 Hz, 1H), 3.66 (t, *J* = 6.6 Hz, 2H), 3.22 (m, 1H), 2.79 (m, 4H), 2.60 (s, 6H), 2.40 (t, *J* = 7.2 Hz, 2H), 2.04 (m, 2H), 1.77 (m, 4H), 1.52 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ(ppm): 171.5, 163.2, 145.5, 145.1, 143.0, 142.3, 138.9, 136.3, 133.4, 129.0 (2C), 125.5, 120.1, 119.6 (2C), 118.2, 79.6, 61.5, 59.7, 57.2, 55.9, 45.5, 44.5, 40.5, 39.6, 36.6, 28.7, 28.6, 26.5, 25.6; HRMS (ESI^+): m/z calculated for $C_{30}H_{40}N_9O_4S$ + H^+ [M+H^+]: 622.2918; found 622.2930; HPLC purity:~ 99%.

N-(4-(((3-((3-(dimethylamino)propyl)amino)-6-nitroquinoxalin-2-yl)amino)methyl) phenyl)-2-(5-methyl-2-phenyloxazol-4-yl) acetamide (4b): The title compound **4b** was

prepared according to the general procedure **B** from 2-(5-methyl-2-phenyloxazol-4-yl)acetic acid and N^2-(4-aminobenzyl)-N^3-(3-(dimethylamino)propyl)-6-nitroquinoxaline-2,3-diamine (**3a**). The crude product was purified by column chromatography using silica gel (100-200) eluting with 2-10% methanol in chloroform afforded the desired product. Yellow semi-solid; yield~ 60%; ^{1}H-NMR (600 MHz, $CDCl_3$) δ (ppm): 9.36 (s, 1H), 8.44 (s, 1H), 8.20 (br s, 1H), 8.03 (m,3H), 7.57 (m, 3H), 7.48 (s, 2H), 7.36 (d, *J* = 7.8 Hz, 2H), 5.20 (br s, 1H), 4.69 (d, *J* = 4.2 Hz, 2H), 3.63 (t, *J* = 4.8 Hz, 2H), 3.60 (s, 1H), 2.52 (t, *J* = 4.8 Hz, 2H), 2.39 (s, 3H), 2.10 (s, 6H), 1.83 (m, 2H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ (ppm): 167.5, 160.2, 145.5, 145.4, 145.0, 143.9, 141.7, 137.8, 136.7, 133.7, 130.6, 129.5, 129.2 (2C), 129.0 (2C), 127.0, 126.0 (2C), 125.6, 121.1, 120.1 (2C), 118.4, 59.6, 45.6, 45.0 (2C), 42.6, 34.4, 23.9, 10.2; HRMS (ESI^+): m/z calculated for $C_{32}H_{35}N_8O_4+H^+$ [$M+H^+$]: 595.2776; found 595.2778; HPLC purity: ~ 100%.

N-(4-(((3-((3-(4-methylpiperazin-1-yl)propyl)amino)-6-nitroquinoxalin-2-yl)amino) methyl)phenyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (4c): The title compound **4c** was prepared according to the general procedure **B** from D-Biotin and N^2-(4-aminobenzyl)-N^3-(3-(4-methylpiperazin-1-yl)propyl)-6-nitroquinoxaline-2,3-diamine (**3e**). The crude product was purified by column chromatography using silica gel (100-200) eluting with 2-10% methanol in chloroform afforded the desired product.Yellow semi- solid;yield~ 60%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ (ppm): 9.90 (s, 1H), 8.19 (br s, 1H), 8.11 (d, *J* = 1.8 Hz, 1H), 7.93 (dd, *J* = 2.7, 9.0 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 2H), 7.46 (d, *J* = 9.0 Hz, 1H), 7.32 (d, *J* = 7.8 Hz, 2H), 6.42 (s, 1H), 6.36 (s, 1H), 4.66 (d, *J* = 4.2 Hz, 2H), 4.29 (t, *J* = 6.3 Hz, 1H), 4.12 (t, *J* = 5.4 Hz, 1H), 3.49 (m, 4H), 3.10 (m, 1H), 2.80 (dd, *J* = 5.1, 12.3 Hz, 1H), 2.56 (d, *J* = 12.6 Hz, 1H), 2.36 (t, *J* = 6.6 Hz, 4H), 2.28 (t, *J* = 6.9 Hz, 4H), 2.13 (s, 3H), 1.78 (m, 2H), 1.60 (m, 3H), 1.47 (m, 1H), 1.35 (m, 2H), 1.20 (m, 2H). ^{13}C-NMR (150 MHz, DMSO-d_6) δ(ppm): 171.6, 163.2, 145.6, 145.2, 142.9, 142.3, 138.9, 136.3, 133.5, 128.9 (2C), 125.4, 120.0, 119.5 (2C), 118.2, 61.5, 59.7, 56.0, 55.9, 55.2 (2C), 53.1 (2C), 46.2, 44.4, 40.3, 36.6, 29.5, 28.7, 28.6, 25.9, 25.6; HRMS (ESI^+): m/z calculated for $C_{33}H_{44}N_{10}NaO_4S+Na^+$ [$M+Na^+$]: 699.3160; found 699.3160; HPLC purity: ~ 97.6%.

(E)-3-(4-(dimethylamino)phenyl)-N-(4-(((3-((3-(4-methylpiperazin-1-yl)propyl)amino)-6-nitroquinoxalin-2-yl)amino)methyl)phenyl)acrylamide (4d): The title compound **4d** was prepared according to the general procedure **B** from (E)-3-(4-(dimethylamino)phenyl)acrylic

acid and N^2-(4-aminobenzyl)-N^3-(3-(4-methylpiperazin-1-yl)propyl)-6-nitroquinoxaline-2,3-diamine (**3e**). The crude product was purified by column chromatography using silica gel (100-200), eluting with 2-10% methanol in chloroform afforded the desired product. Yellow semi-solid; yield~ 60%; ^{1}H-NMR (600 MHz, $CDCl_3$) δ (ppm): 8.44 (d, *J* = 2.4 Hz, 1H), 8.04 (dd, *J*= 2.4, 9.0 Hz, 1H), 7.80 (brs, 1H), 7.59 (d, *J* = 15.6 Hz, 1H), 7.56 (d, *J* = 9.0 Hz, 1H), 7.46 (d, *J* = 7.8 Hz, 2H), 7.37 (d, *J* = 8.4 Hz, 2H), 7.28 (d, *J* = 8.4 Hz, 2H), 6.62 (d, *J* = 8.4 Hz, 2H), 6.30 (d, *J* = 15.6 Hz , 1H), 6.22 (br s, 1H), 4.76 (d, *J* = 4.8 Hz, 2H), 3.63 (t, *J* = 6.0 Hz, 2H), 2.99 (s, 6H), 2.55 (t, *J* = 6.0 Hz, 2H), 2.51 (br s, 4H), 2.36 (br s, 4H), 2.19 (s, 3H), 1.88 (t, *J* = 6.3 Hz, 2H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ(ppm): 165.7, 151.7, 145.4, 145.1, 143.8, 143.1, 141.8, 137.6, 136.5, 134.1, 129.7 (2C), 129.1 (2C), 125.5 (2C), 122.0, 121.1, 120.6, 118.5, 114.8, 111.8 (2C), 57.5, 54.9 (2C), 53.1 (2C), 45.8, 45.1, 41.6, 40.1 (2C), 24.3; HRMS (ESI^+): m/z calculated for $C_{34}H_{42}N_9O_3$+ H^+ [$M+H^+$]: 624.3405; found 624.3398; HPLC purity: ~ 100%.

N-(4-(((3-((3-(4-methylpiperazin-1-yl)propyl)amino)-7-nitroquinoxalin-2-yl)amino) methyl)phenyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (4e): The title compound **4e** was prepared according to the general procedure **B** from D-biotin and N^2-(4-aminobenzyl)-N^3-(3-(4-methylpiperazin-1-yl)propyl)-6-nitroquinoxaline-2,3-diamine (**3f**). The crude product was purified by column chromatography using silica gel (100-200), eluting with 2-10% methanol in chloroform afforded the desired product. Yellow semi- solid; yield~ 60%; ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 8.21 (d, *J* = 3.0 Hz, 1H), 7.92 (dd, *J* = 2.4, 9.0 Hz, 1H), 7.54 (d, *J* = 8.4, 2H), 7.41 (d, *J* = 8.4, 1H), 7.38 (d, *J* = 9.0, 2H), 4.67 (s, 2H), 4.45 (dd, *J* = 4.8, 7.8 Hz, 1H), 4.26 (dd, *J* = 4.8, 7.8 Hz,1H), 3.56 (t, *J* = 7.2 Hz, 2H), 3.31 (m, 2H), 3.17 (m, 1H), 2.89 (dd, *J* = 4.8, 13.2 Hz, 1H), 2.68 (d, *J* = 13.2 Hz, 3H), 2.49 (t, *J* = 7.2 Hz, 4H), 2.37 (t, *J* = 7.2 Hz, 4H), 2.28 (s, 3H), 1.89 (m, 2H), 1.72 (m,3H),1.60 (m,1H), 1.47 (m,2H); ^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 173.0, 164.7, 145.4, 144.5, 143.1, 141.9, 137.7, 135.50, 134.2, 128.6 (2C), 124.6, 120.0, 119.9 (2C), 117.8, 61.9, 60.2, 55.7, 55.6, 54.1 (2C), 52.1 (2C), 44.5, 44.4, 39.7, 39.5, 36.2, 28.4, 28.1, 25.4, 25.3; HRMS (ESI^+): m/z calculated for $C_{33}H_{45}N_{10}O_4S$+H^+ [$M+H^+$]: 677.3340; found 677.3335; HPLC purity: ~ 100%.

(E)-3-(4-(dimethylamino)phenyl)-N-(4-(((3-((3-(4-methylpiperazin-1-yl)propyl)amino)-7-nitroquinoxalin-2-yl)amino)methyl)phenyl)acrylamide (4f): The title compound **4f** was

prepared according to the general procedure **B** from (E)-3-(4-(dimethylamino)phenyl)acrylic acid and N^3-(4-aminobenzyl)-N^2-(3-(4-methylpiperazin-1-yl)propyl)-6-nitroquinoxaline-2,3-diamine (**3f**). The crude product was purified by column chromatography using silica gel (100-200), eluting with 2- 10 % methanol in chloroform afforded the desired product. Yellow semi-solid;yield~ 60%; ^{1}H-NMR (600 MHz, CDCl3) δ (ppm): 8.44 (d, *J* = 2.4 Hz, 1H), 8.05 (dd, *J* = 2.4, 9.0 Hz, 1H), 7.90 (br s,1H), 7.61 (d, *J* = 15.5 Hz, 1H), 7.53 (d, *J* = 9.0 Hz, 1H), 7.50 (d, *J* = 6.6 Hz, 2H), 7.38 (d, *J* = 9.0 Hz, 2H), 7.36 (d, *J* = 8.4 Hz, 2H), 6.62 (d, *J* = 8.4 Hz, 2H), 6.44 (br s, 1H), 6.36 (d, *J* = 15.0 Hz, 1H), 4.77 (d, *J* = 4.8 Hz, 2H), 3.66 (s, 2H), 2.99 (s, 6H), 2.66 (t, *J* = 6.0 Hz, 2H), 2.48 (br s, 4H), 2.26 (s, 3H), 2.16 (br s, 4H), 1.91 (t, *J* = 6 Hz, 2H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ (ppm): 165.5, 151.7, 145.7, 144.7, 143.5, 143.0, 142.3, 137.6, 135.9, 129.7 (2C), 129.1 (2C), 125.2 (2C), 124.9, 122.1, 121.4, 120.4, 118.8, 115.1, 111.8 (2C), 57.0, 54.2 (2C), 52.5 (2C), 45.5, 45.0, 41.1, 40.2 (2C), 23.8; HRMS (ESI$^+$): m/z calculated for $C_{33}H_{45}N_{10}O_4S$ + H^+ [M+H^+]: 677.3340; found 677.3335; HPLC purity: ~ 98%.

N^3-(3-(dimethylamino)propyl)-N^2-(3-(4-methylpiperazin-1-yl)propyl)quinoxaline-2,3,6-triamine (3b'): Compound **3b**' was synthesized by following the procedure **C**.The crude product was purified by column chromatography using neutral alumina eluting with 2- 15 % methanol in chloroform afforded the desired product. Light yellow solid; yield~ 45%. ^{1}H-NMR (300 MHz, D_2O) δ (ppm): 7.32 (d, *J* = 8.7 Hz, 1H), 6.88 (d, *J* = 2.4 Hz, 1H), 6.76 (dd, *J* = 2.4, 8.7 Hz, 1H), 3.54 (dd, *J* = 7.2, 15.6 Hz, 4H), 3.33 (m, 2H), 2.52 (dd, *J* = 7.5, 15.0 Hz, 10H), 2.32 (s, 6H), 2.30 (s, 3H), 1.92 (m, 4H); ^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 144.4, 144.0, 142.3, 137.3, 129.9, 124.8, 114.5, 108.6, 56.9, 56.0, 54.2 (2C), 52.3 (2C), 44.6, 43.9 (2C), 39.5, 39.2, 26.4, 25.6; HRMS (ESI$^+$): m/z calculated for $C_{21}H_{36}N_8$ + H^+ [M+H^+]: 401.3141; found 401.3141; HPLC purity: ~ 99.6%.

N-(2-(benzylamino)-3-(3-(dimethylamino)propylamino)quinoxalin-6-yl)-5-(2-oxo-hexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (5d): The title compound **5d** was prepared according to the general procedure **C** from D-biotin and N^2-benzyl-N^3-(3-(dimethylamino)propyl)quinoxaline-2,3,6-triamine (**3d**'). The crude product was purified by column chromatography usingneutral alumina in methanol-chloroform (2- 10 %)solvent system afforded the desired product. light greenish semisolid;yield~ 55%; ^{1}H-NMR (600

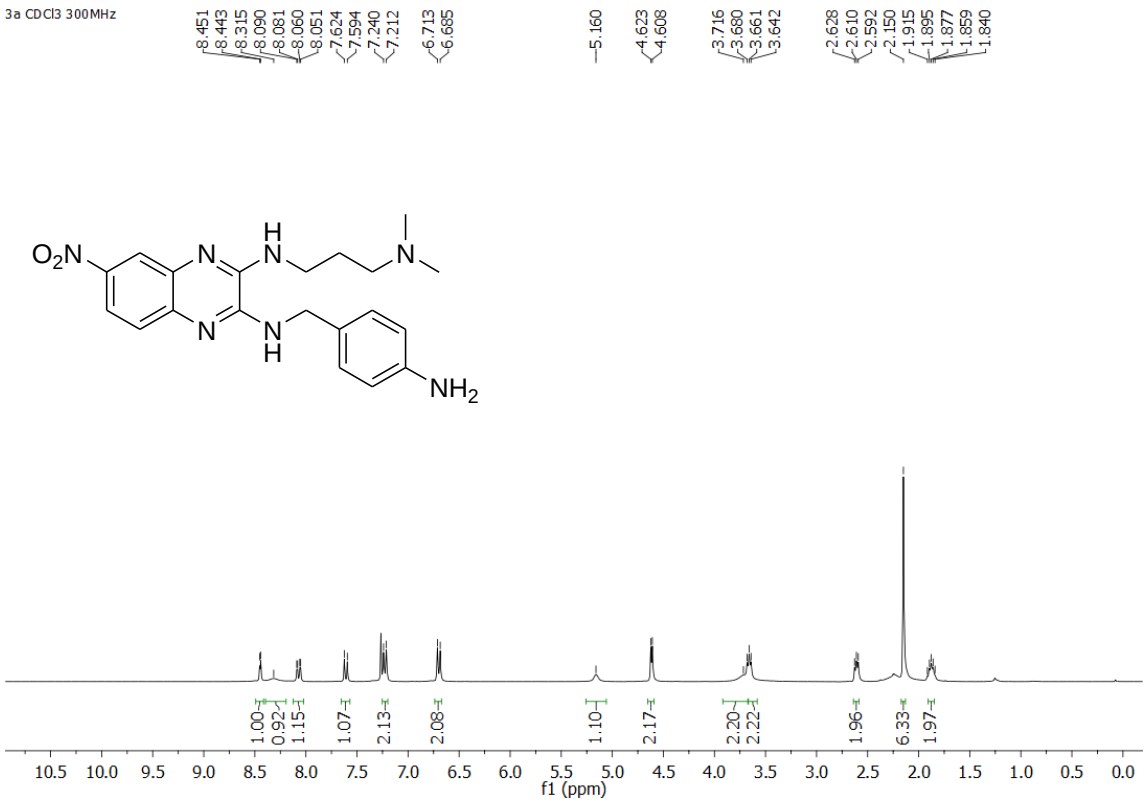
MHz, CD_3OD) δ (ppm): 7.87 (s, 1H), 7.45 (d, *J* = 8.4 Hz, 1H), 7.38 (d, *J* = 7.8 Hz, 2H), 7.33 (dd, *J* = 1.8, 8.4 Hz, 1H), 7.30 (t, *J* = 7.2 Hz, 2H), 7.22 (t, *J* = 7.2 Hz, 1H), 4.69 (s, 2H), 4.38(m, 1H), 4.18 (dd, *J* = 4.2, 7.2 Hz, 1H), 3.51 (t, *J* = 7.2 Hz, 2H), 3.07 (m, 1H), 2.83 (dd, *J* = 4.8, 12.6 Hz, 1H), 2.64 (d, 12.6 Hz, 1H), 2.38 (m, 4H), 2.21 (s, 6H), 1.84 (m, 2H), 1.69 (m, 3H), 1.57 (m, 1H), 1.44 (m, 2H); ^{13}C-NMR (150 MHz, CD_3OD)δ (ppm): 172.9, 164.7, 144.4, 143.4, 139.0, 136.8, 134.7, 133.3, 128.2 (2C), 127.9 (2C), 126.9, 124.5, 117.1, 115.3, 61.9, 60.2, 57.0, 55.6, 45.0, 44.1 (2C), 39.7, 39.4, 36.4, 28.5, 28.1, 26.3, 25.5; HRMS (ESI^+): m/z calculated for $C_{30}H_{41}N_8O_2S + H^+$ [$M+H^+$]: 577.3068; found 577.3062; HPLC purity: ~ 98.1%.

1-(3-(4-methylpiperazin-1-yl)propyl)-1H-imidazo[4,5-b]quinoxaline-2,6-diamine (VA): The title compound **VA** was prepared according to the general procedure **D** by the reductionof1-(3-(4-methylpiperazin-1-yl)propyl)-6-nitro-1H-imidazo[4,5-b]quinoxalin-2-amine (**IIIA**). The crude product was purified by column chromatography using neutral alumina in methanol-chloroform (2- 10 %) solvent system afforded the desired product. Light yellow solid;yield~ 40%; ^{1}H-NMR (600 MHz, CD_3OD) δ (ppm): 7.64 (d, *J* = 9.0 Hz, 1H), 7.07 (d, *J* = 1.8 Hz, 1H), 7.03 (dd, *J* = 1.8, 9.0 Hz, 1H), 4.14 (t, *J* = 6.0 Hz, 2H), 2.43 (m, 10H), 2.27 (s, 3H), 2.03 (t, *J* = 6.6 Hz, 2H); ^{13}C-NMR (150 MHz, CD_3OD) δ (ppm): 162.2, 151.5, 147.2, 141.4, 141.0, 131.1, 127.2, 117.3, 107.5, 54.1 (2C), 53.8, 51.7 (2C), 44.4, 38.4, 24.5; HRMS (ESI^+): m/z calculated for $C_{17}H_{25}N_8 + H^+$ [$M+H^+$]:341.2197; found 341.2186; HPLC purity: ~ 98.5%.

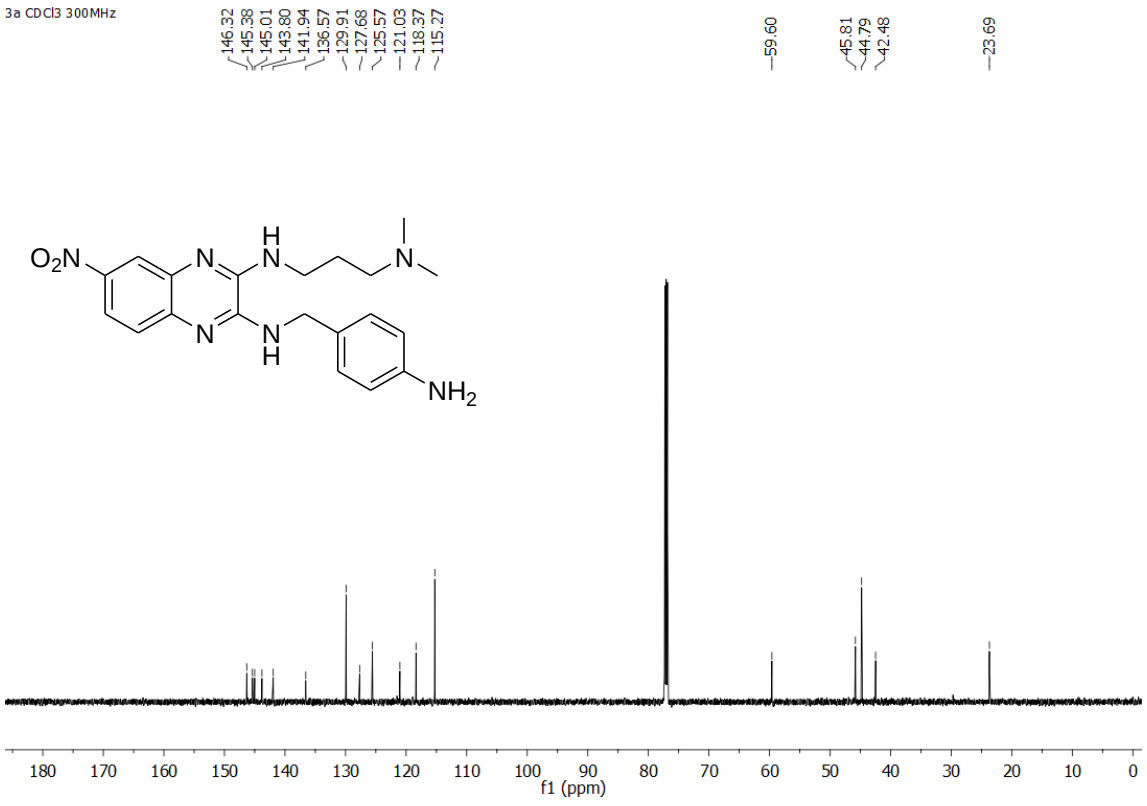
1-(3-(dimethylamino)propyl)-1H-imidazo[4,5-b]quinoxaline-2,6-diamine(VB): The title compound **VB** was prepared according to the general procedure **D** by the reduction of 1-(3-(dimethylamino)propyl)-6-nitro-1H-imidazo[4,5-b]quinoxalin-2-amine (**IIIB**).The crude product was purified by column chromatography using neutral alumina in methanol-chloroform (2- 5 %) solvent system afforded the desired product. greenish semisolid;yield~ 50%; ^{1}H-NMR (600 MHz, DMSO-d_6) δ (ppm): 7.87(br s, 2H), 7.52 (d, *J* = 9.6 Hz, 1H), 6.86 (m, 2H), 5.31 (s, 2H), 4.00 (t, *J* = 6.6 Hz, 2H), 2.22 (t, *J* = 6.6 Hz, 2H), 2.12(s, 6H), 1.83 (m, 2H); ^{13}C-NMR (150 MHz, DMSO-d_6) δ (ppm): 162.0, 152.7, 147.5, 142.6, 141.0, 130.4, 127.7, 116.7, 107.4, 55.8, 45.3 (2C) , 38.6, 26.5; HRMS (EI^+): m/z calculated for $C_{14}H_{19}N_7$ [M^+]:285.1702; found 285.1712; HPLC purity: ~ 100%.

Section S2: NMR Spectra (^{1}H and ^{13}C)

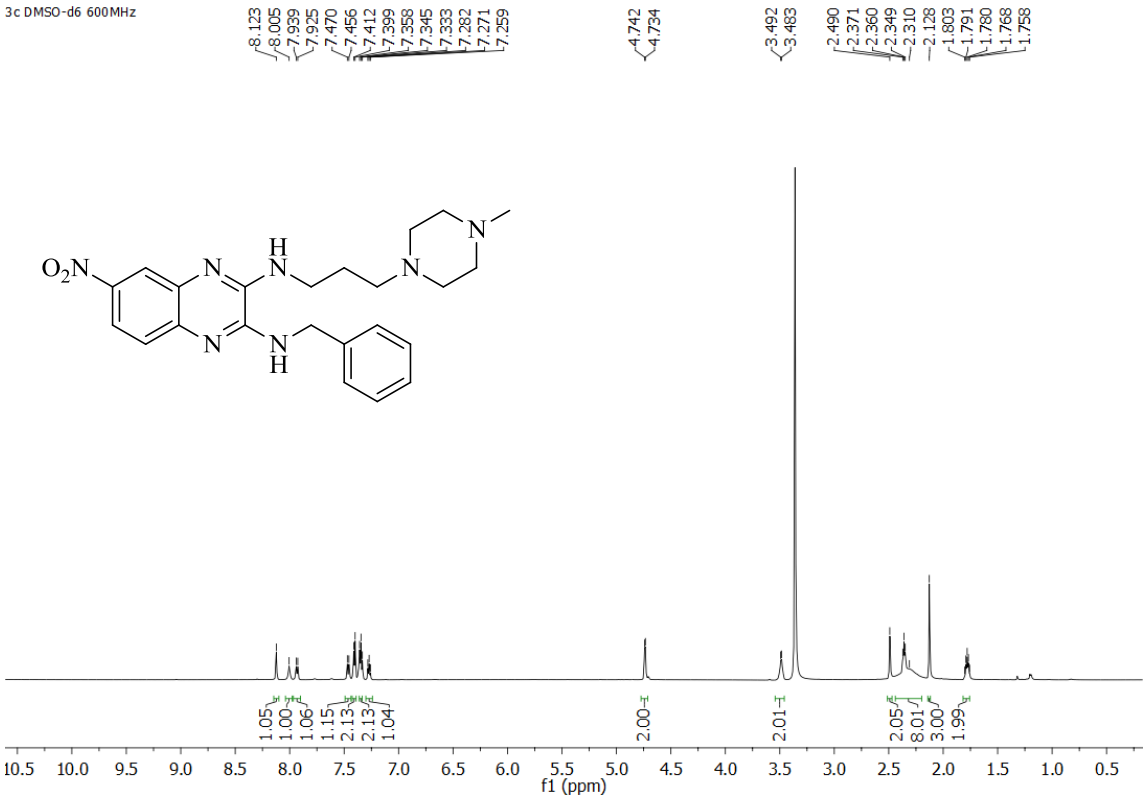
^{1}H NMR of **3a** ($CDCl_3$, 300 MHz):-



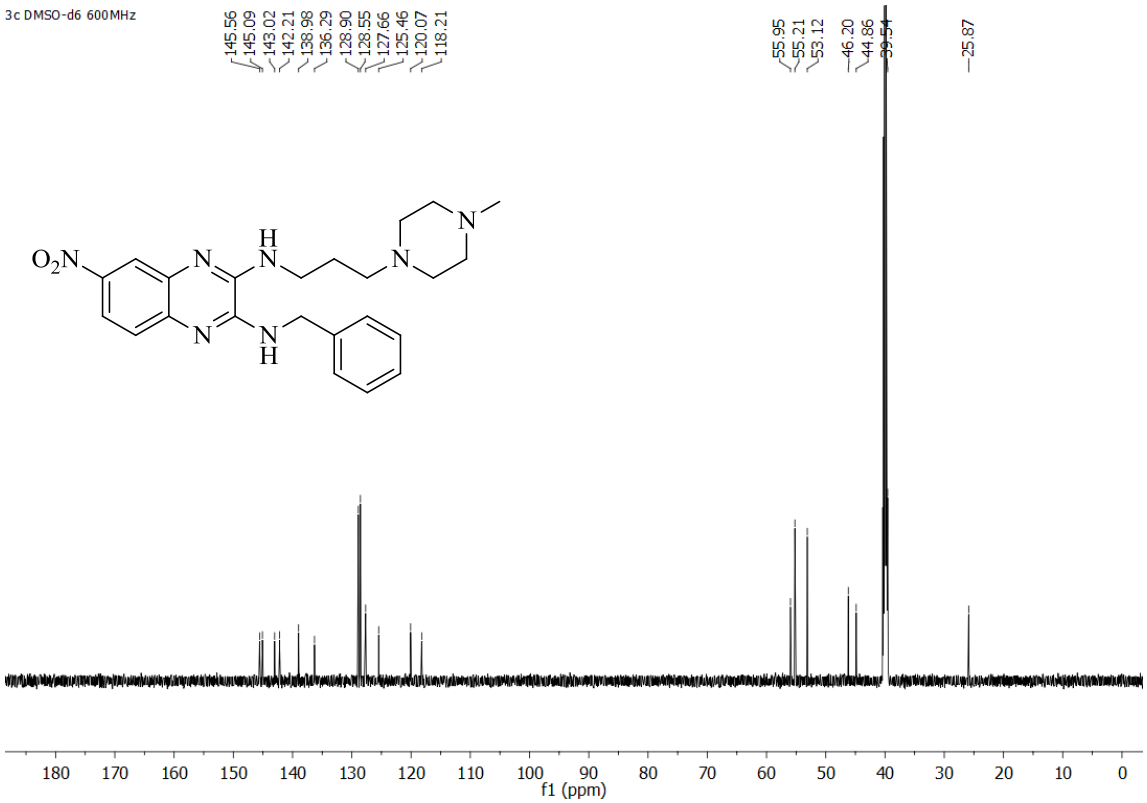
^{13}C NMR of 3a ($CDCl_3$, 75 MHz):-



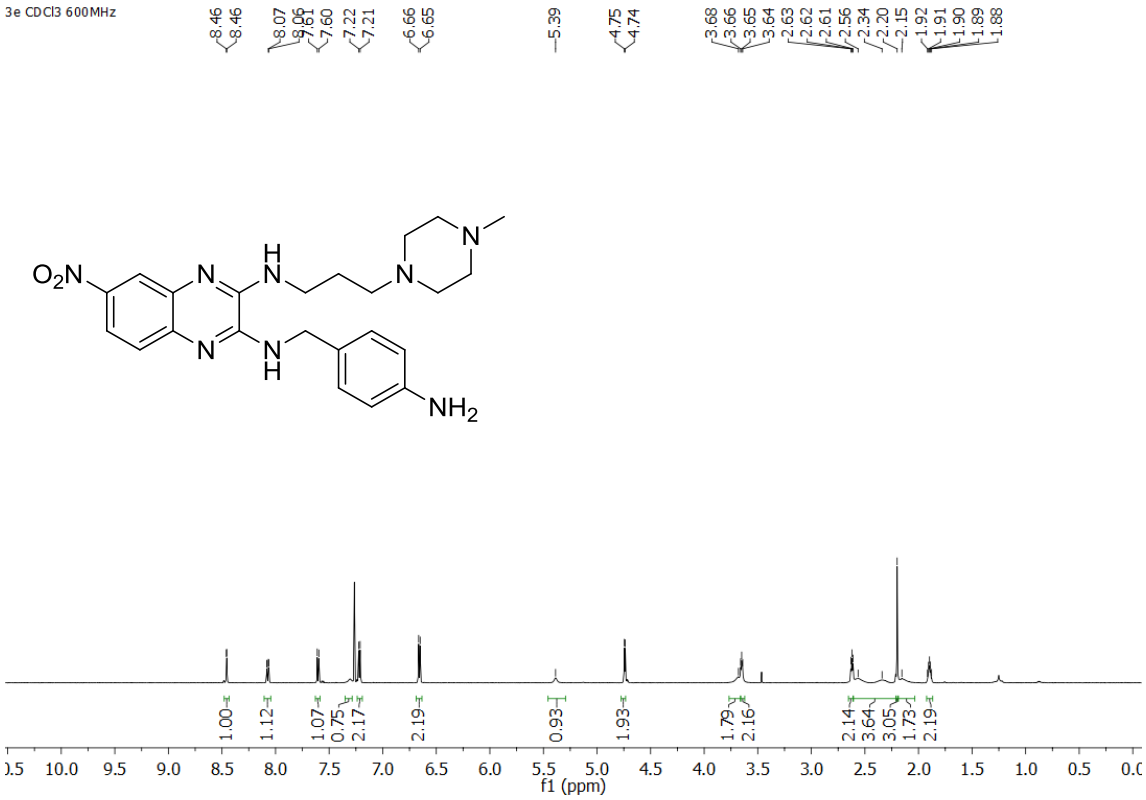
^{1}H NMR of **3c** (DMSO-d_6, 600 MHz):-



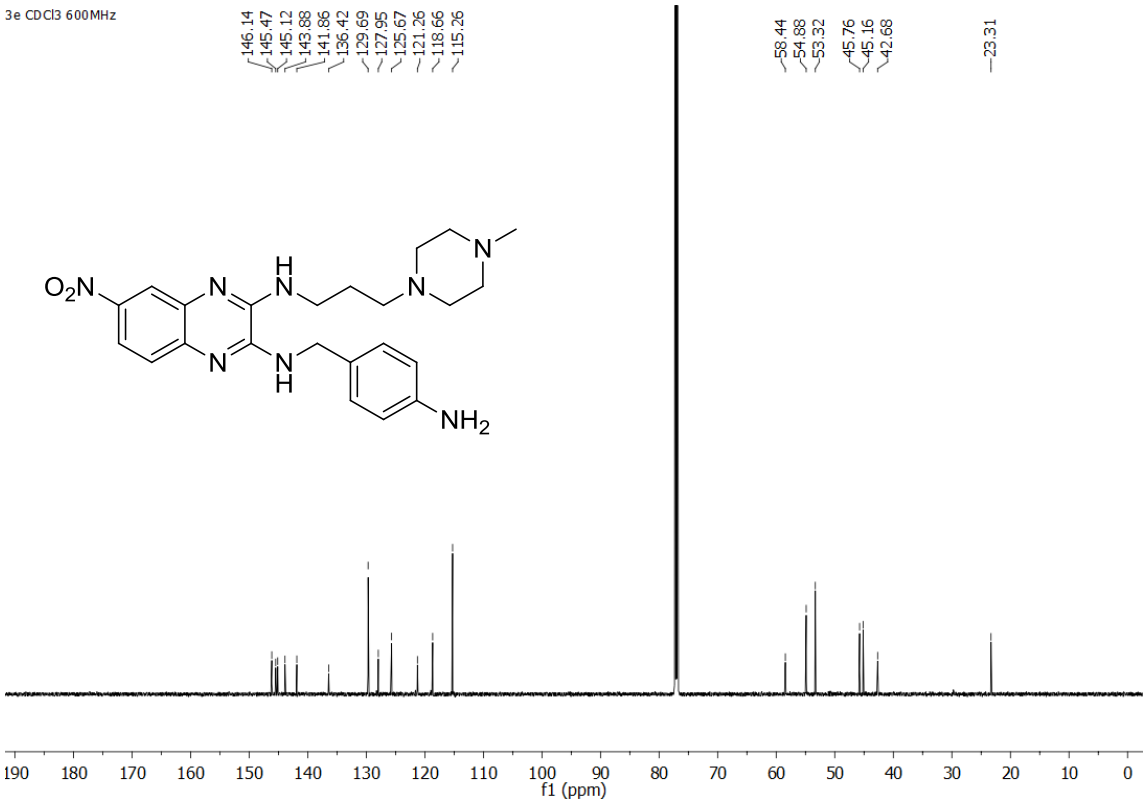
^{13}C NMR of **3c** (DMSO-d_6, 150 MHz):-



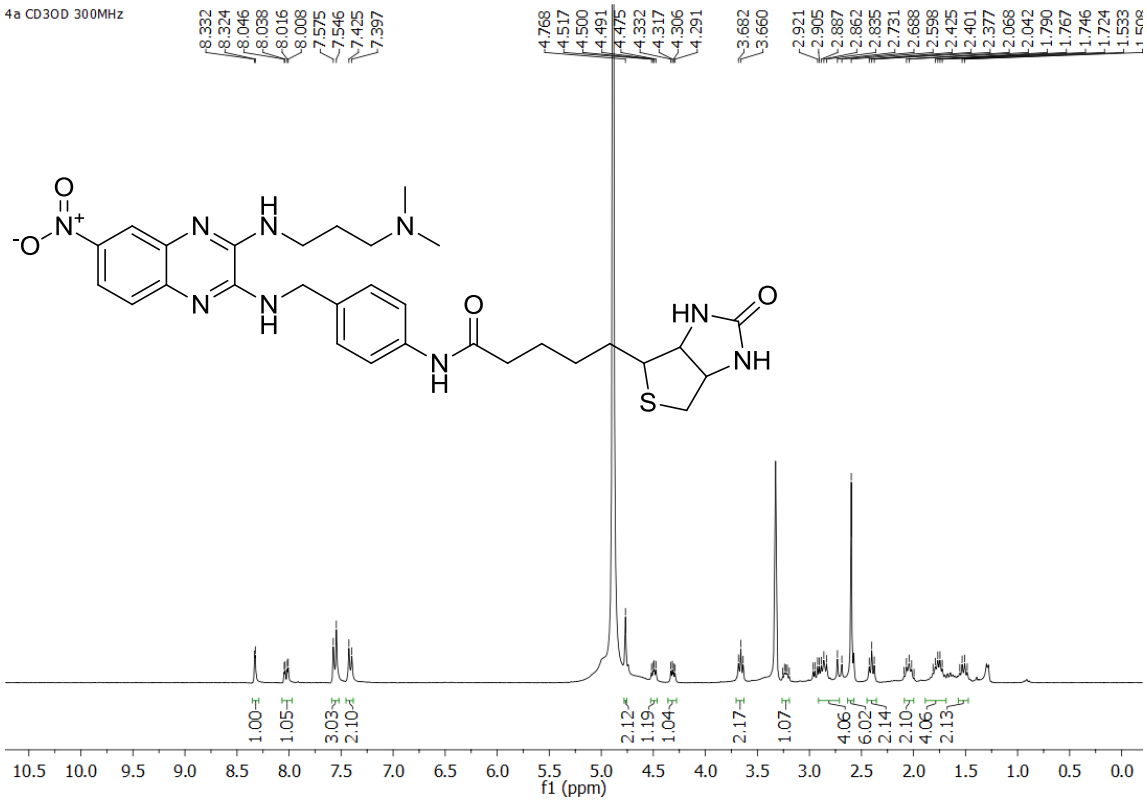
^{1}H NMR of **3e** ($CDCl_3$, 600 MHz):-



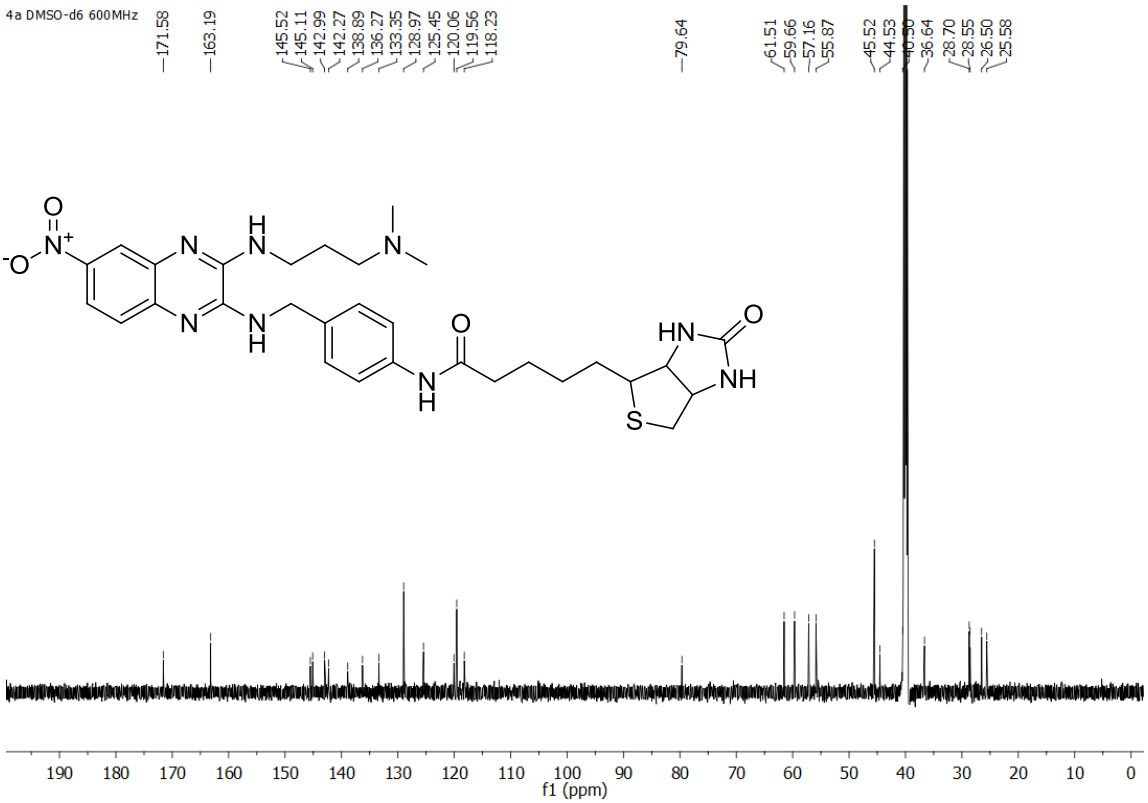
^{13}C NMR of **3e** ($CDCl_3$, 150 MHz):-



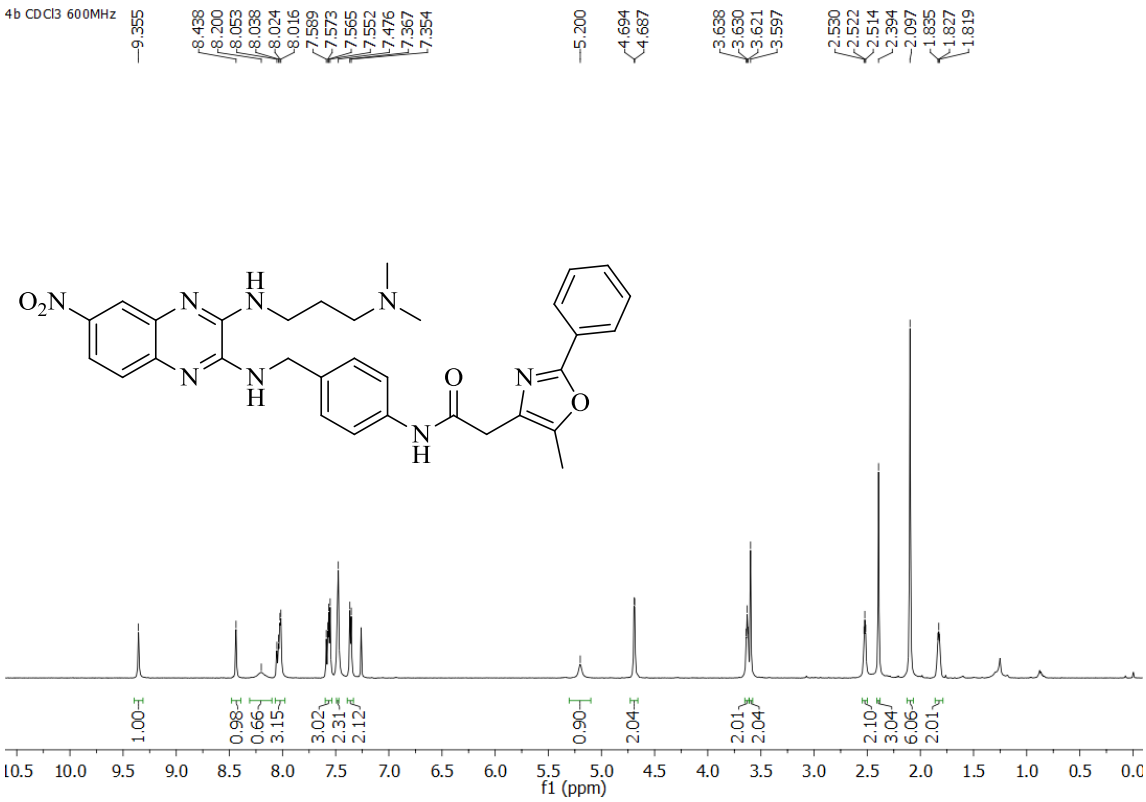
^{1}H NMR of **4a** (CD_3OD, 300 MHz):-



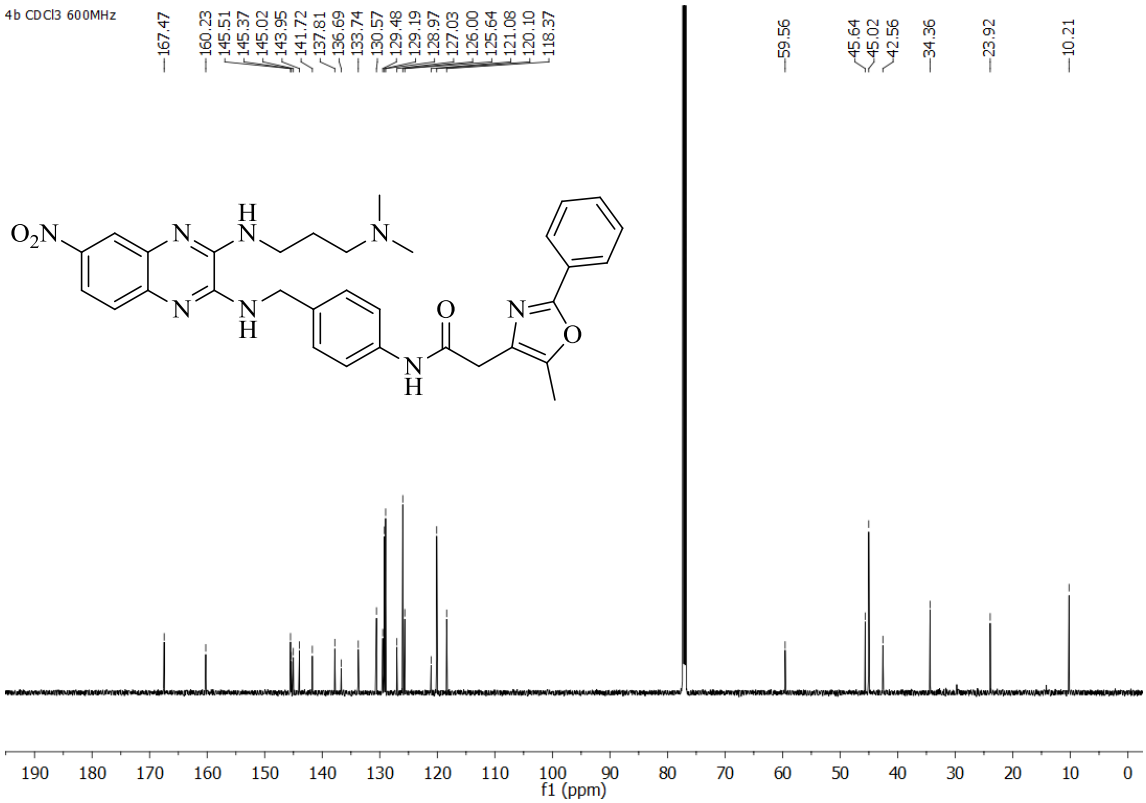
^{13}C NMR of **4a** (DMSO-d_6, 150 MHz):-



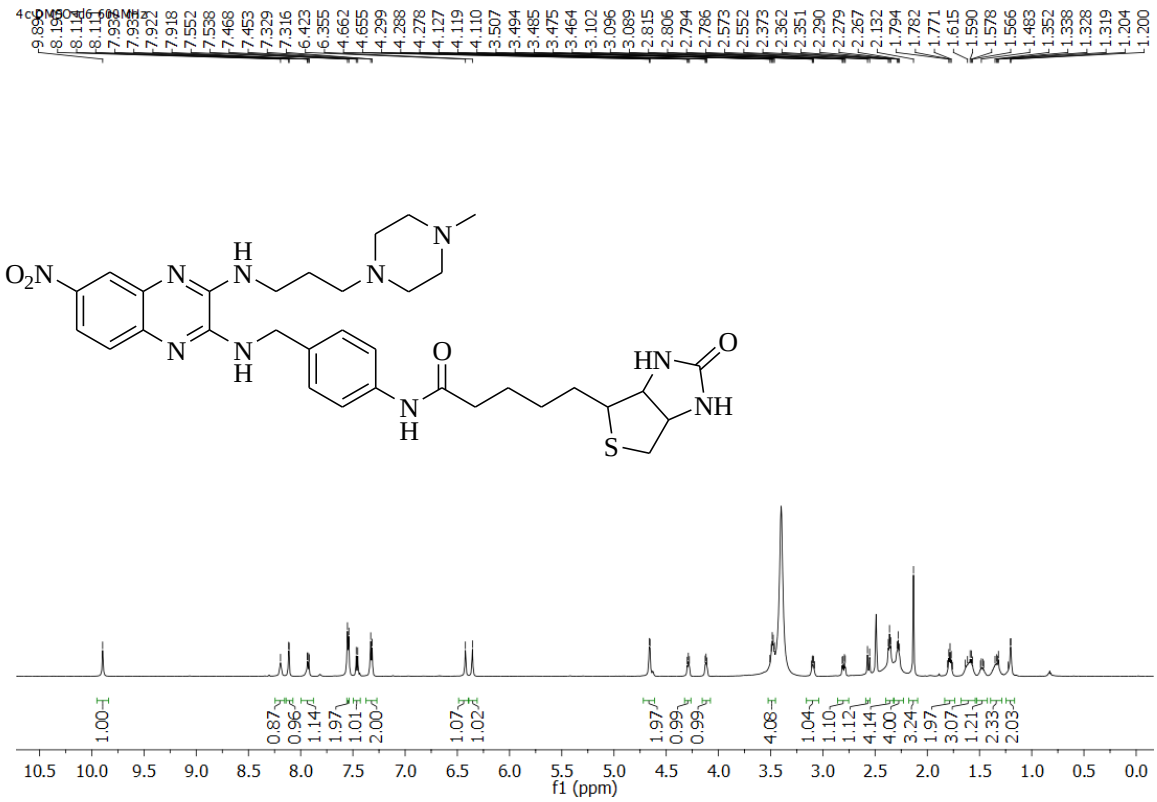
^{1}H NMR of **4b** ($CDCl_3$, 600 MHz):-



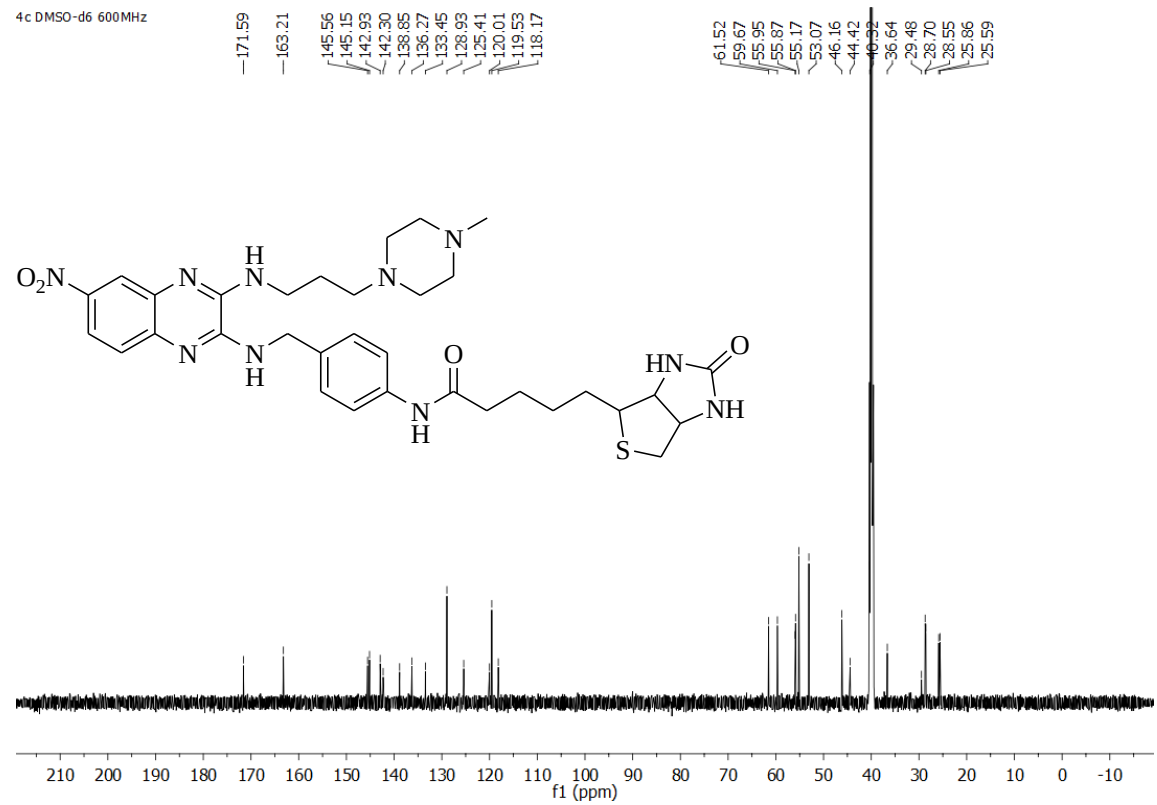
^{13}C NMR of **4b** ($CDCl_3$, 150 MHz):-



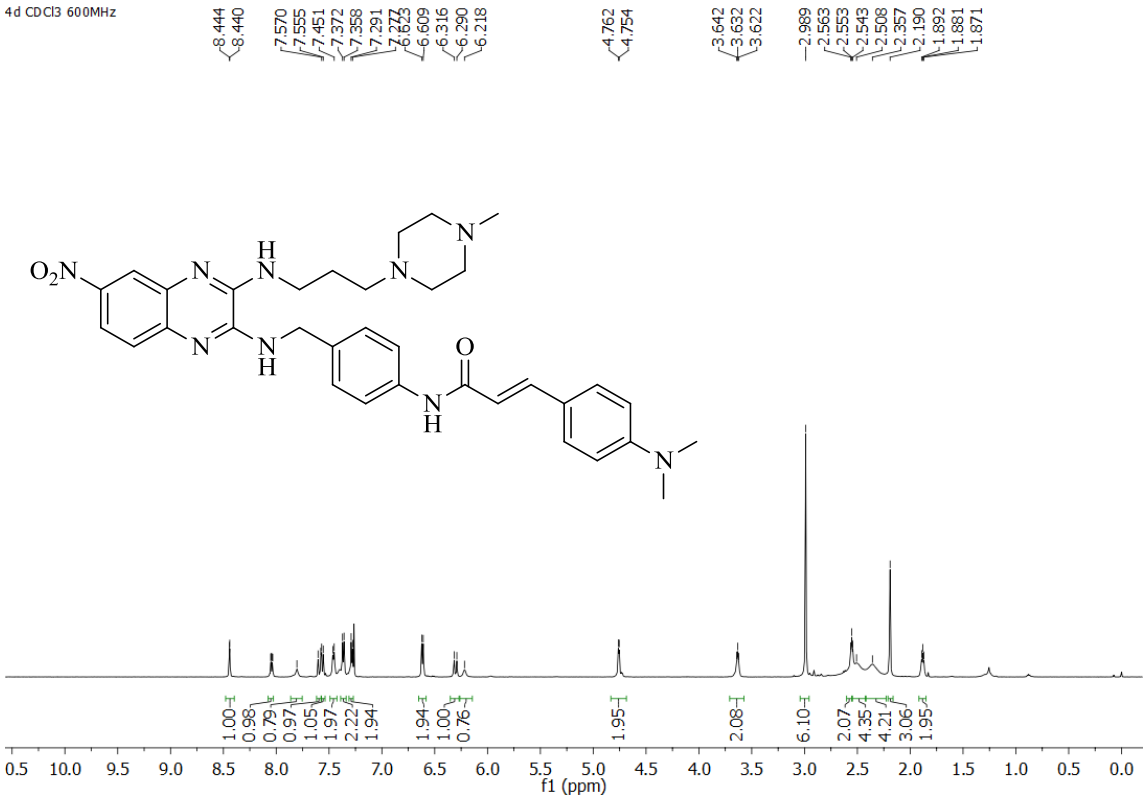
^{1}H NMR of **4c** (DMSO-d_6, 600 MHz):-



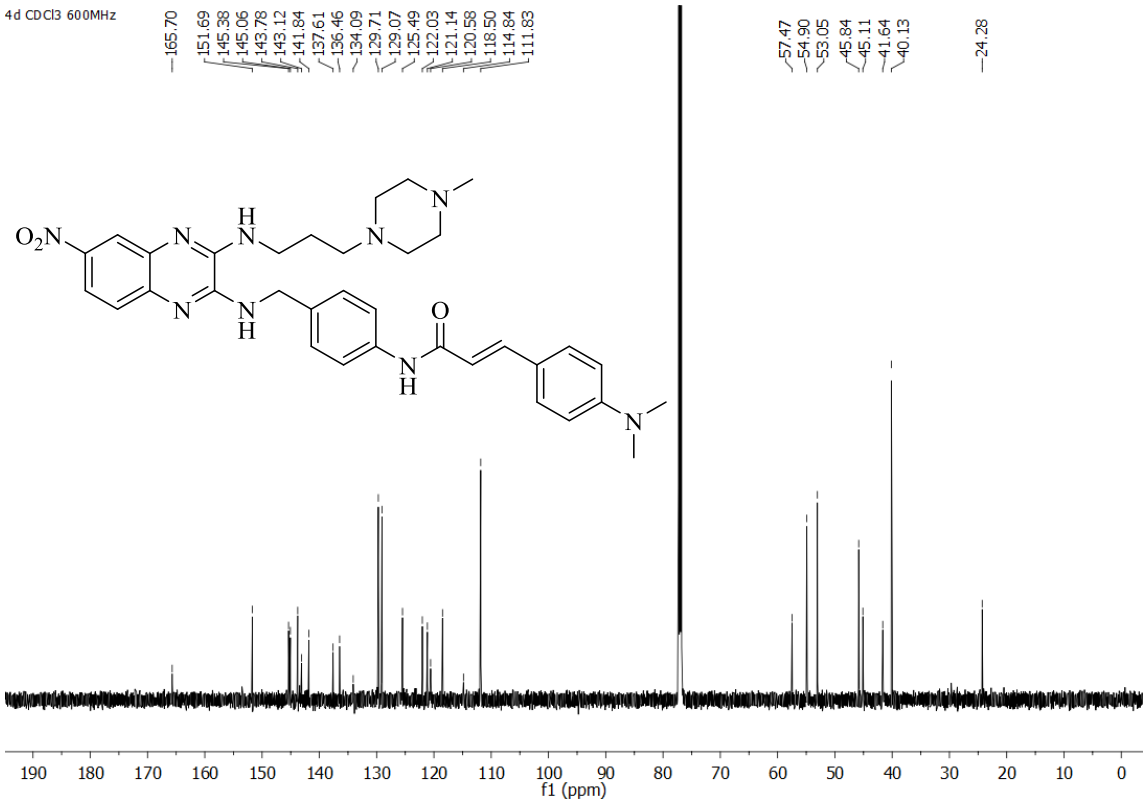
^{13}C NMR of **4c** (DMSO-d_6, 150 MHz):-



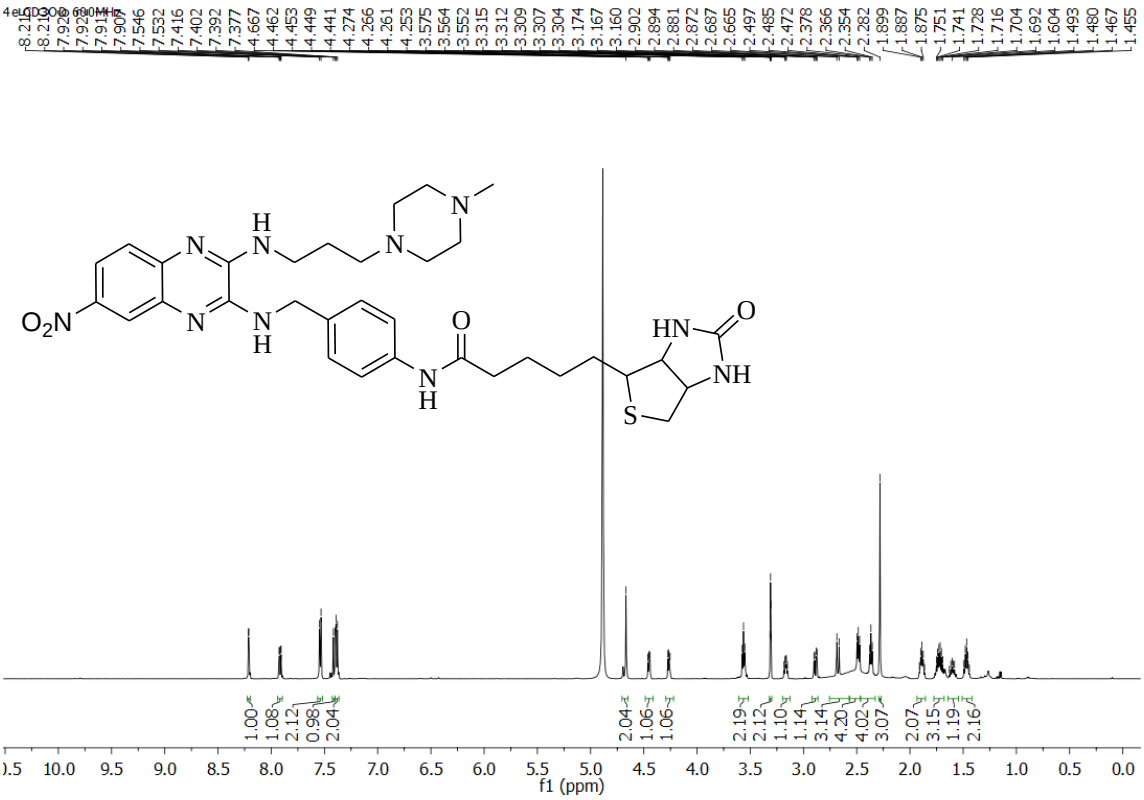
^{1}H NMR of **4d** ($CDCl_3$, 600 MHz):-



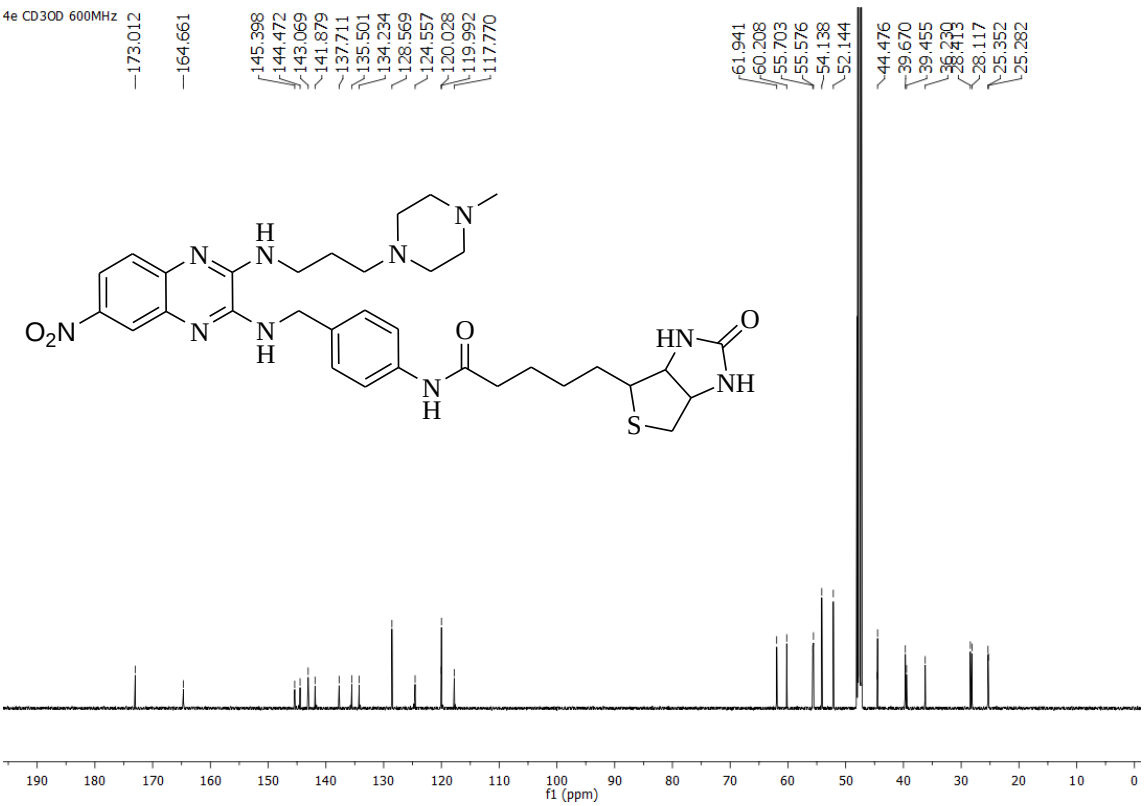
^{13}C NMR of **4d** ($CDCl_3$, 150 MHz):-



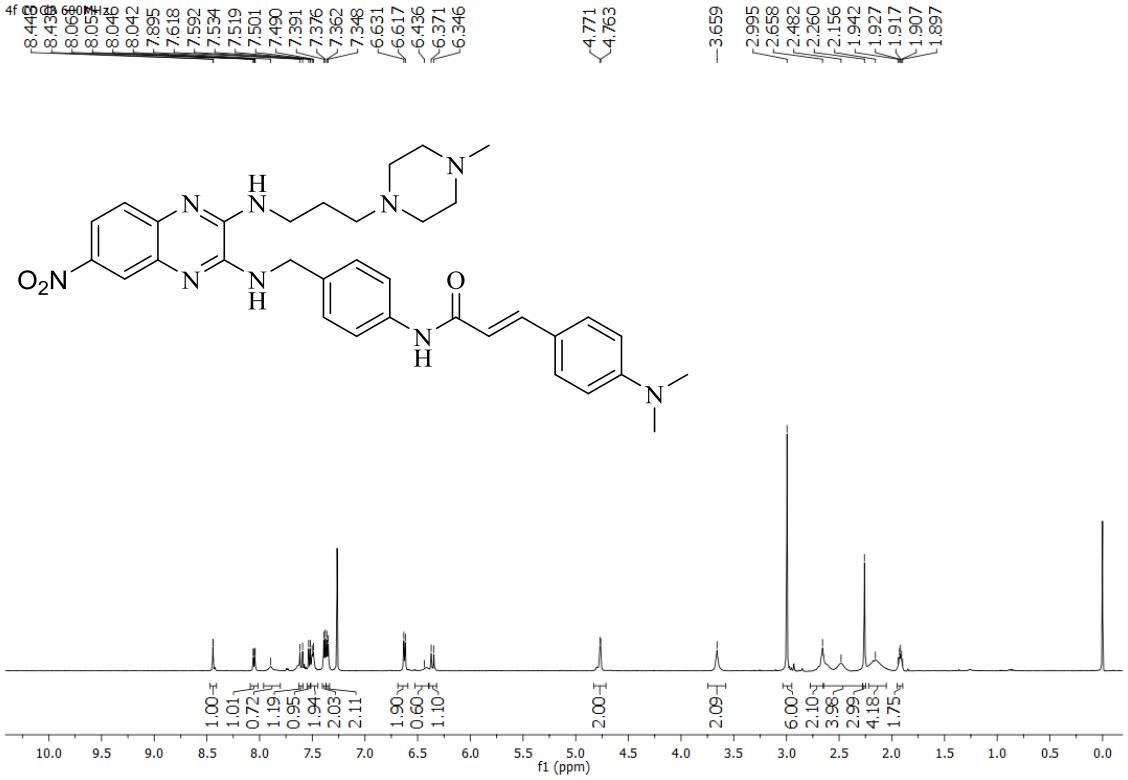
^{1}H NMR of **4e** (CD_3OD, 600 MHz):-



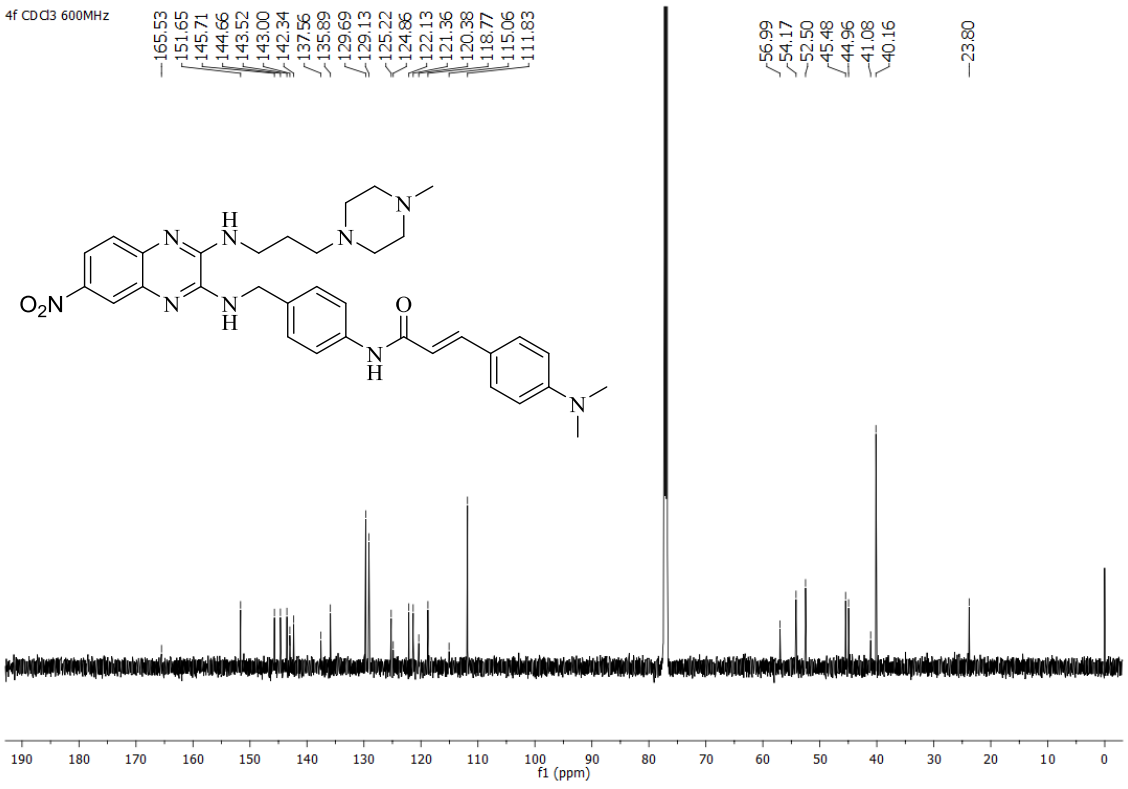
^{13}C NMR of **4e** (CD_3OD, 150 MHz):-



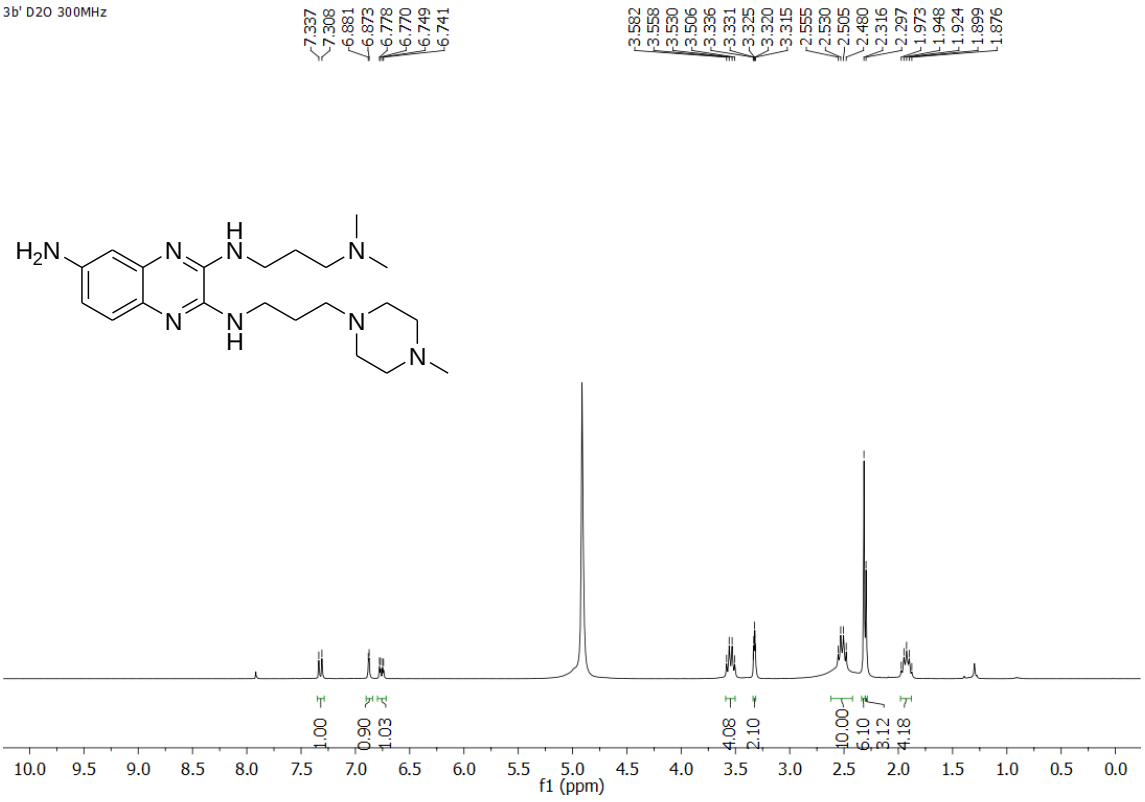
^{1}H NMR of **4f** ($CDCl_3$, 600 MHz):-



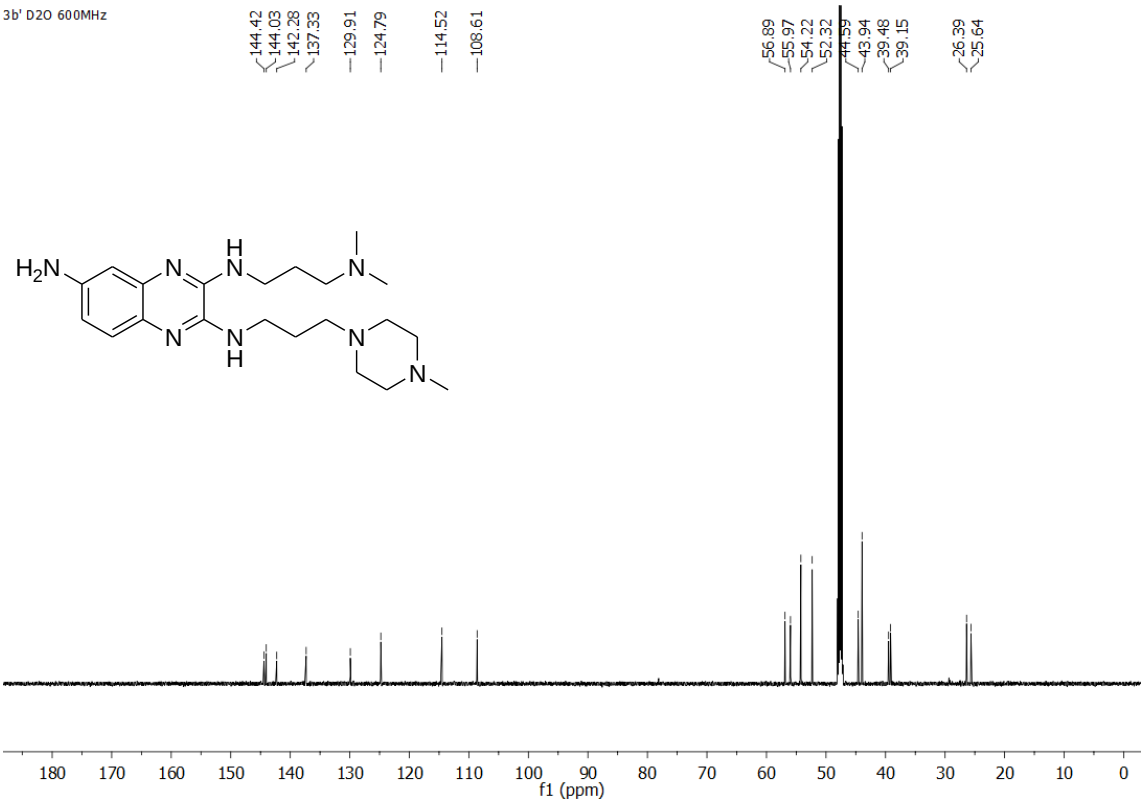
^{13}C NMR of **4f** ($CDCl_3$, 150 MHz):-



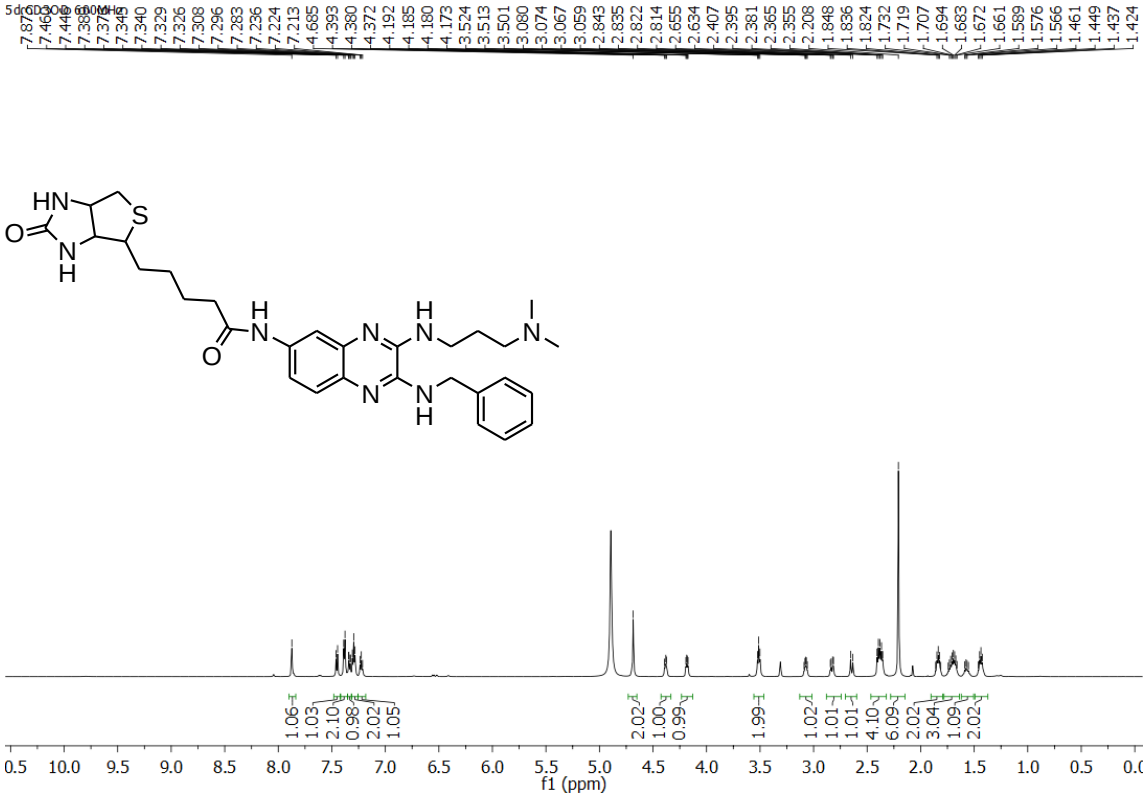
^{1}H NMR of **3b'** (D_2O, 300 MHz):-



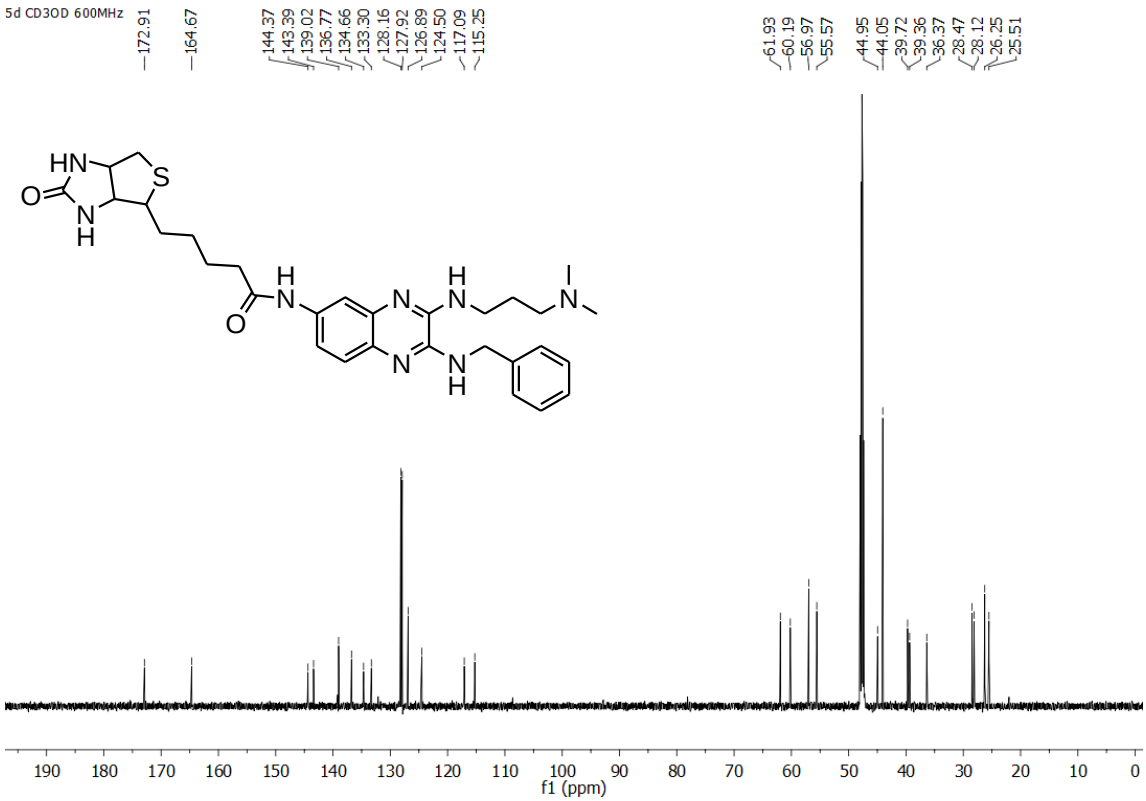
^{13}C NMR of **3b'** (D_2O, 150 MHz):-



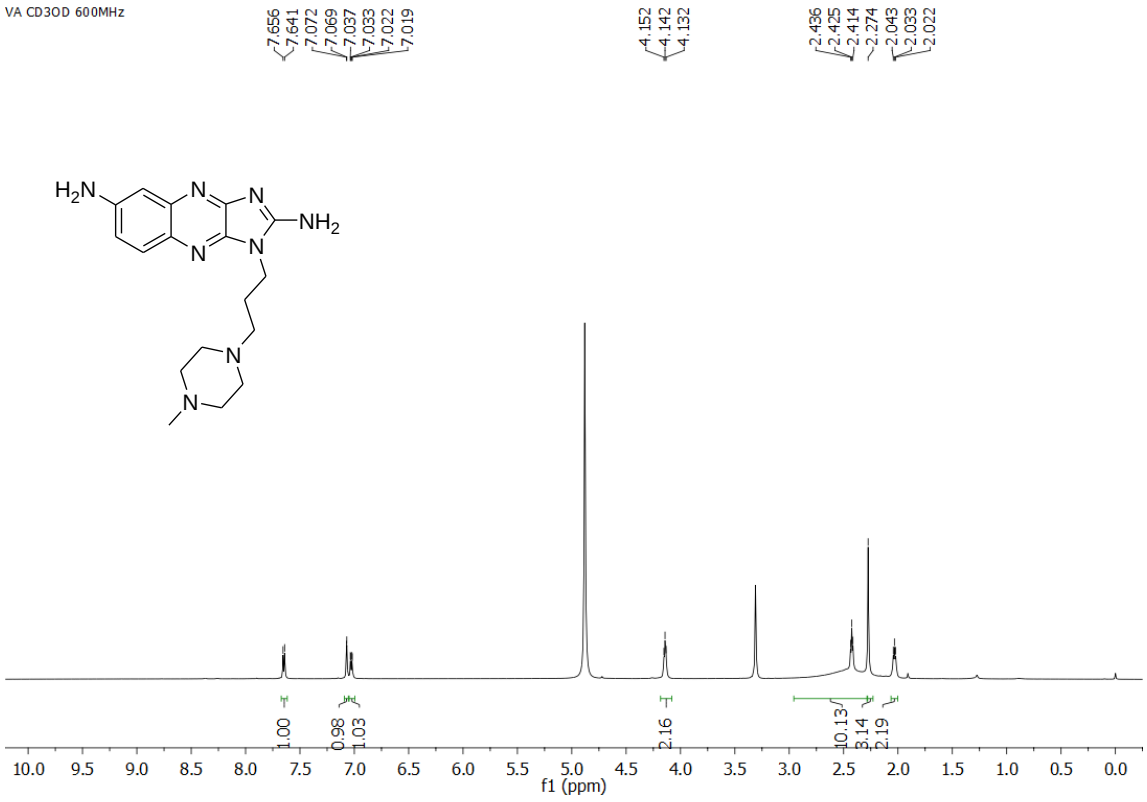
^{1}H NMR of **5d** (CD_3OD, 300 MHz):-



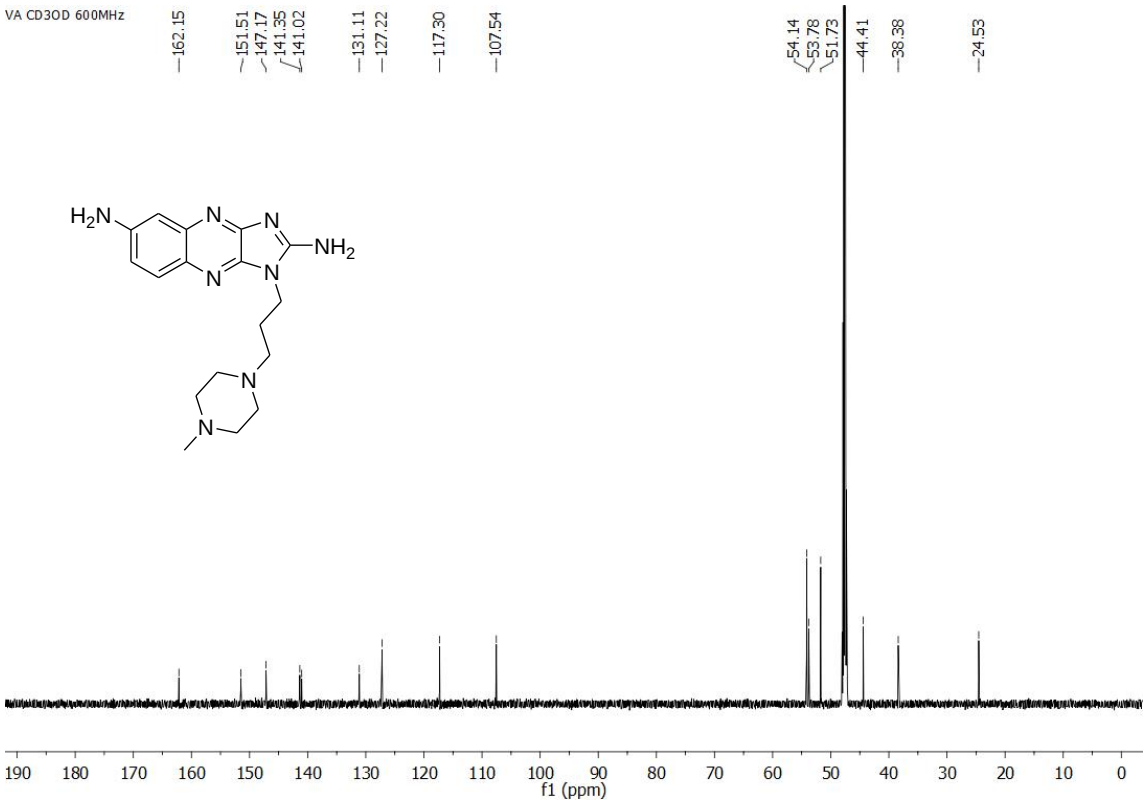
^{13}C NMR of **5d** (CD_3OD, 150 MHz):-



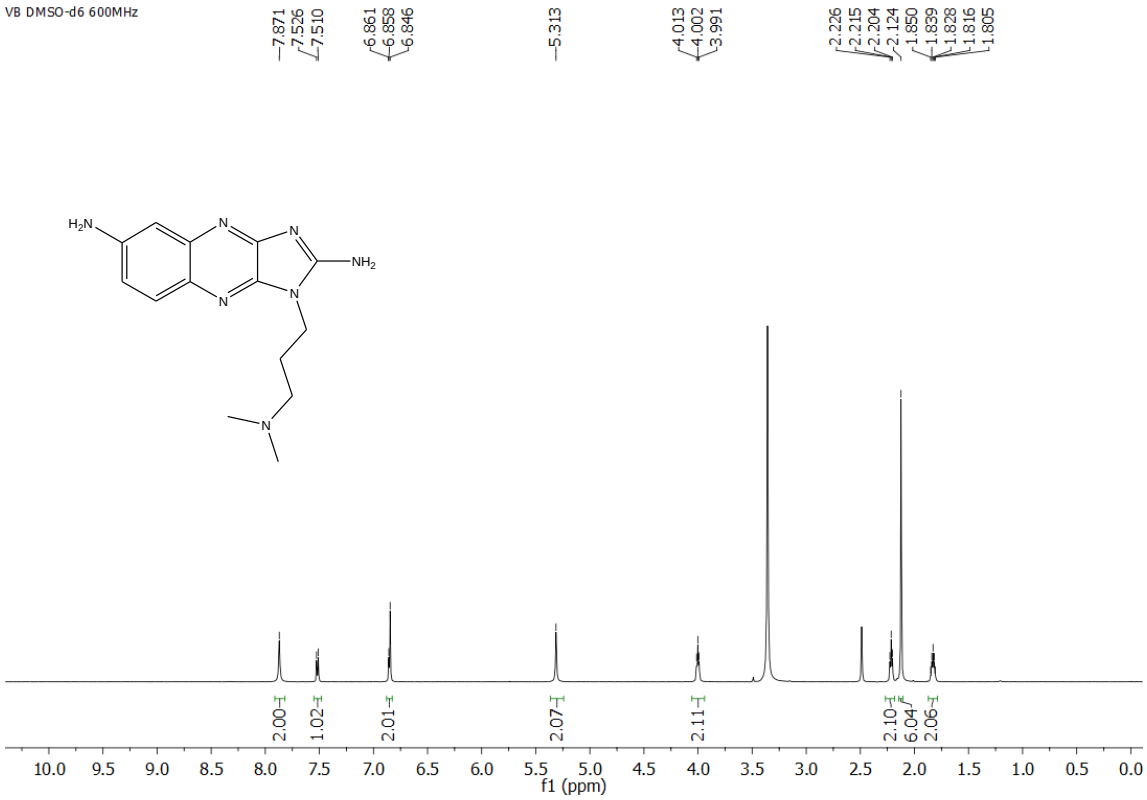
^{1}H NMR of **VA** (CD_3OD, 600 MHz):-



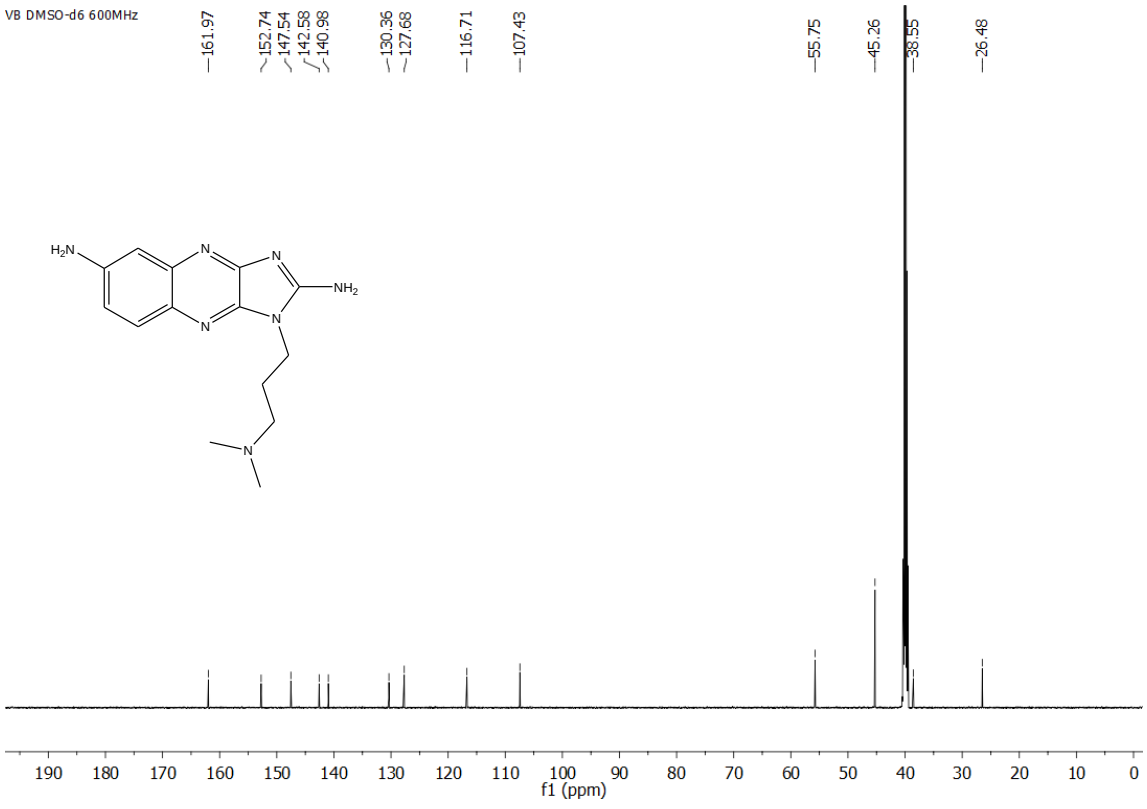
^{13}C NMR of **VA** (CD_3OD, 150 MHz):-



^{1}H NMR of **VB** (DMSO-d_6, 600 MHz):-



^{13}C NMR of **VB** (DMSO-d_6, 150 MHz):-



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