

PhD Thesis

Development of Synthetic Anionophores with Potential Prospects in Therapeutic Applications

*A dissertation submitted to the
Indian Institute of Technology Guwahati
as partial fulfilment for the Degree of
Doctor of Philosophy in Chemistry*

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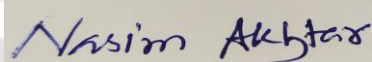


Declaration

9th September 2021

I hereby proclaim that the thesis entitled “Development of synthetic Anionophores with Potential Prospects in Therapeutic Application” is succinct research work. This thesis has been submitted by me to the Department of Chemistry, Indian Institute of Technology Guwahati, for the award of the degree of Doctor of Philosophy. In keeping with the general practice of reporting scientific observations, due acknowledgments have been made wherever the work described is based on the findings of other investigators.

I further declare that this work has not been submitted anywhere else for any degree, diploma, associateship, or membership, etc. of any Institute or University to the best of my knowledge.

A rectangular box containing a handwritten signature in blue ink that reads "Nasim Akhtar".

(Nasim Akhtar)





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To whom it may concern

This is to certify that the thesis entitled “**Development of synthetic Anionophores with Potential Prospects in Therapeutic Applications**” being submitted by Mr. Nasim Akhtar (Roll No. 166122036) for the award of the Ph.D. degree in Chemistry to the Indian Institute of Technology Guwahati, is a genuinely his own research work which was carried out by him during his PhD tenure. All the information and data reported by him are entirely his original findings. He has meticulously performed all the scientific investigations and also followed the guidelines of my laboratory. Neither this thesis nor any part of it has been submitted for awarding any degree/diploma anywhere before.

(Prof. Debasis Manna)



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Abstract

The thesis entitled “Development of Synthetic Anionophores with Potential Prospects in Therapeutic Applications” elucidates a succinct description of numerous new classes of anionophores with promising therapeutic potential. Within this thesis, the recognition, transmembrane transport, and the possible therapeutic prospects are reported. The thesis has been divided into five chapters based on the adept experimental results which were carried out during the entire research period.

Chapter 1 conveys a brief discussion on the importance of anion transportation to maintain normal physiological functions and how anionophores could be used as a therapeutic agent for cystic fibrosis, cancer, and the antibacterial field. Compact literature on anion transporting molecules and their numerous therapeutic potentiality have been also conversed.

Chapter 2 reports a series of bis-thiourea-based diphenylethylenediamine (DPEN) scaffolds with promising anion recognition as well as transport ability across the model membrane. The detailed transport mechanism and the correlations with apoptosis using a number of proficient experiments have also been pointed out.

Chapter 3 describes natural product-based anionophores, which were semi-synthetically derived from the well-known anti-malarial drug quinine. A comprehensive study on anion recognition, transportation at different pH has been discussed. In addition, a thorough investigation on anti-proliferative activity both *in vitro* and *in vivo* is also covered.

Chapter 4 illuminates glutathione (GSH) responsive proanionophore. A concise study on anion recognition, transport, and biological studies of sulphonium-based water-soluble derivatives is discussed.

Chapter 5 deals with a novel responsive strategy with nitroreductase (NTR) enzyme where transport, diagnosis, and possible bio imaging ability of fluorescent-based TPE molecules have been well conversed



List of Abbreviation

ATP	Adenosine triphosphate
MAPK	p38 mitogen-activated protein kinase
YFP	Yellow fluorescent protein
FRT	Fisher rat thyroid
VSOR	Volume-sensitive outwardly rectifying
RVD	Regulatory volume decrease
DPEN	1,2- diphenylethylenediamine
CHOL	Cholesterol
PARP	Poly(ADP-ribose) polymerase
HBSS	Hank's Balanced Salt Solution
DFT	Density Functional Theory
HPTS	8-Hydroxypyrene-1,3,6-trisulfonate
DPEN	1,2- diphenylethylenediamine
Lucigenin	<i>N,N'</i> -Dimethyl-9,9'-biacridinium dinitrate
PUMA	p53 upregulated modulator of apoptosis
EAC	Ehrlich ascites carcinoma
GSH	Glutathione
NTRs	Nitroreductase
GUVs	Giant unilamellar vesicles
Calcein	4',5'-Bis(<i>N,N</i> -bis(carboxymethyl)aminomethyl)fluorescein acetoxymethyl ester
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DMSO	Dimethyl sulfoxide
DMF	Dimethylformamide
DCM	Dichloromethane
DIEPA	<i>N,N</i> -Diisopropylethylamine
Et ₃ N	Triethyl amine
EtOH	Ethanol
FTIR	Fourier-transform infrared spectroscopy
H ₂ O	Water

IC ₅₀	The concentration of an inhibitor which causes 50% inhibition of a maximal activity
KCl	Potassium chloride
K _a	Association constant
K _d	Dissociation constant
LUV	Large unilamellar vesicles
KCl	Potassium chloride
LUV	Large unilamellar vesicles
m/z	Mass to charge ratio
M.P	Melting point
MeOH	Methanol
λ _{ex}	Excitation wavelength
λ _{em}	Emission wavelength
DFT	Density functional theory
NMDG-Cl	N-methyl-D-glucamine chloride
EAC	Ehrlich ascites carcinoma
H&E	Hematoxylin and eosin
PUMA	p53 upregulated modulator of apoptosis
DIAD	Diisopropyl azodicarboxylate
DPPA	Diphenylphosphoryl azide
DMEM	Dulbecco's Modified Eagle Medium
RPMI	Roswell Park Memorial Institute Medium
CPCSEA	Committee for Control and Supervision of Experimental Animals
DFT	Density functional theory
CLC	Chloride intracellular ion channel
NBD	Nitrobenzofurazan
TBACl	Tetrabutylammonium chloride
EYPC	Egg-yolk phosphatidylcholine
CHOL	Cholesterol
ISE	Ion selective electrode
FCCP	4-(trifluoromethoxy)phenylhydrazine
HPLC	High-performance liquid chromatography

PBS	Phosphate buffered saline
BSO	Buthionine sulfoximine
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization-Time Of Flight
CFTR	Cystic fibrosis transmembrane conductance regulator
MQAE	<i>N</i> -(Ethoxycarbonylmethyl)-6-methoxyquinolinium bromide
NTR	Nitroreductase
NADP	Nicotinamide adenine dinucleotide phosphate
TPE	Tetraphenylethylene
LUVs	Large unilamellar vesicles
AIE	Aggregate induced emission
AIBS	Aggregation-induced blue shift
LB	Luria Bertani
PMSF	Phenylmethanesulfonyl fluoride
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
DPPS	1,2-Dipalmitoyl-sn-glycero-3-phosphoserine
NADP	Nicotinamide adenine dinucleotide phosphate
AIE	Aggregate induced emission

For symbols/units

Å	Angstrom
atm	Atmosphere
β	Beta
C	Celsius
<i>J</i>	Coupling constant
°	Degree
δ	Delta
Hz	Hertz
K	Kelvin
MHz	Megahertz
<i>m</i>	Meta
μg	Micro gram
μM	Micro mole
mL	Millilitre
mV	Mili Volt
min	Minute
h	Hour
nM	Nano mole
<i>o</i>	Ortho
<i>p</i>	Para
%	Percent
s	Second







Chapter 1

Introduction of Anion Transportation and its Numerous Potential Therapeutic Applications

Maintenance of the exact adjustment of osmolality across the biological membrane is significant in controlling direct different physiological capacities. Generally, this balance of intra- or extracellular osmolality is harmonized by integral membrane transport proteins that accelerate the selective movement of ions across lipid bilayers.¹⁻² A network of noncovalent interaction originating from the backbone and side chains of these carriers or channel-forming proteins permit them to recognize these specific ions.³ The generation of electrochemical gradients across the membranes due to the transport of ions energies numerous biological processes such as electron transport chain, ion-homeostasis, signal transduction, cell volume regulation, migration, hormone secretion, blood pressure regulation, and induction of programmed cell death.⁴ However, mutation of the genes encoding the integral membrane transport proteins or their structural rearrangement could lead to innumerable channelopathies like cystic fibrosis, Bartter syndrome, generalized epilepsy, Isaacs' syndrome, QT syndrome, and others.⁵⁻⁷ Recent studies demonstrated that the homeostasis of several ions, including Cl^- , modulates cellular apoptosis.⁸ Increasing evidence also suggests that the dysregulated expression of chloride channel proteins in specific cancer cells leads to the efflux of Cl^- ions, which is considered as one of the reasons for the suppression of the apoptosis process.⁹

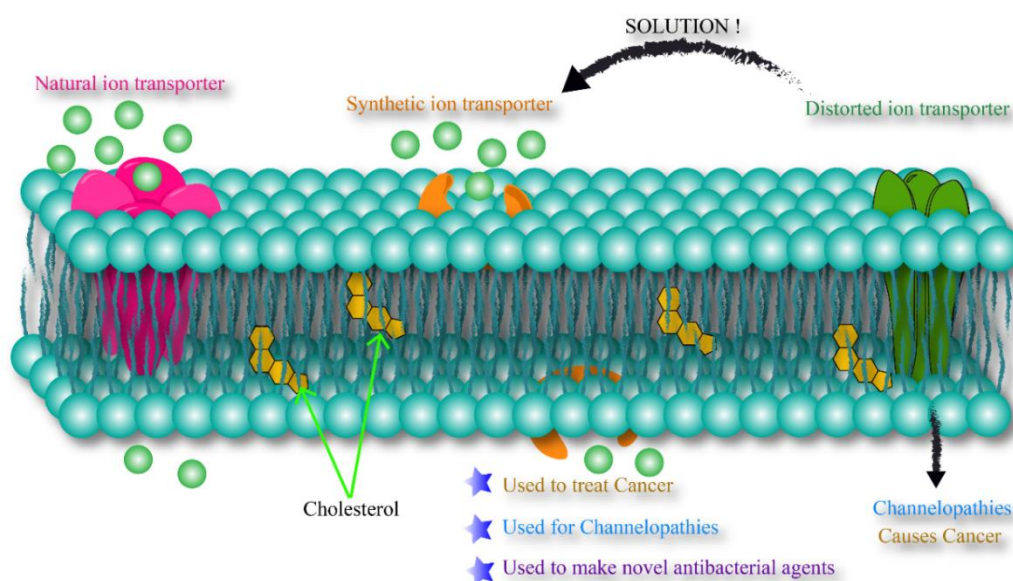


Figure 1.1. Schematic representation of numerous prospects of anionophores.

Unlike targeting the specific protein/enzyme or genetic material of the cancer cells, these ion transporters are located within the membrane bilayer and assist ions in passing through the hydrophobic layer of the membrane, leading to the induction of apoptosis, autophagy, and other cell death programs to eliminate cancer cells. The ion transporter facilitated regulation of ion homeostasis has the potential to deacidify the extracellular microenvironment and could have the potential to overcome the multidrug resistance-associated with the overexpression or mutation(s) of the proteins/enzymes or genetic materials.¹⁰⁻¹²

Further, the anionophores have also been explored against numerous channelopathies. Dysregulated transport of anion is the crucial reason for channelopathies like cystic fibrosis. Synthetic anionophores are used as one of the alternative approaches to restore anion transport in the cystic fibrosis transmembrane conductance regulator (CFTR) defective cells. Currently, few clinically approved drugs for cystic fibrosis disease are available that target the defective CFTR. However, a large number of mutations in the CFTR gene restricts the use of these drugs. So in this regard, the scientific fraternity developed numerous non-toxic anionophore to afford therapeutics for cystic fibrosis.¹³

The anionophores also have promising prospects for anti-microbial treatment. Antibacterial resistance is one of the foremost problems worldwide, which is endangering the efficacy of antibiotics. The centers for disease control and prevention (CDC) have already classified a number of bacteria as having severe threats to human civilization. Initially, the anion transport-related research was primarily concentrated on its development to treat cystic fibrosis and cancer. But recently, people have also been involved in developing anionophores having antibacterial activity. For this purpose, the potent anionophores should have low cytotoxicity against human cells, which is an indispensable parameter of an ideal antibacterial candidate. Several mechanistic studies confirmed that the membrane depolarization by these anionophores is the primary reason for its potent antibacterial properties. The membrane localization makes it challenging for bacteria to develop resistance against the anionophores. The anionophores should be active against drug-resistant bacteria.¹⁴

So overall, in the present thesis, we have focused on designing and synthesizing a plentiful potential anion transporting molecules where we have also explored its numerous

prospects in therapeutics, especially for the treatment of cancer through a set of well-known *in vitro* and *in vivo* experiments. The entire research work within this thesis is arranged into five chapters as given below. A different class of anionophore molecules has been discussed in each chapter, along with their possible therapeutic prospects. Also, the focus is given to minimize the limitations of known anionophores and provide an alternative approach to overcome those barriers.

Chapter 1 mainly discussed the numerous potential prospects of anionophore against a wide variety of pathological conditions and discussed their probable mode of action with an adept literature survey.

Chapter 2 focused on designing a new class of 1,2- diphenylethylenediamine (DPEN) derivatives as promising chloride ion transporter molecules and explored their anion transport mediated anticancer property through numerous detailed *in vitro* experiments.

Chapter 3 dealt with a new class of pH-responsive quinine-based anionophore molecules and explored their anticancer property, which is confirmed from both *in vitro* and *in vivo* studies. After thorough investigation, impressive improvement for the selectivity between the cancer cells and normal cells compared to our previous DPEN derivatives of chapter 2 were observed.

Chapter 4 mainly explored mainly glutathione responsive ionophore molecules with a new class of sulphonium-based water-soluble proanionophore and tried to solve the conventional hurdles associated with the classical anionophores such as poor deliverability, selectivity, and uptake issue which we had faced for all of our previous chapter's molecules.

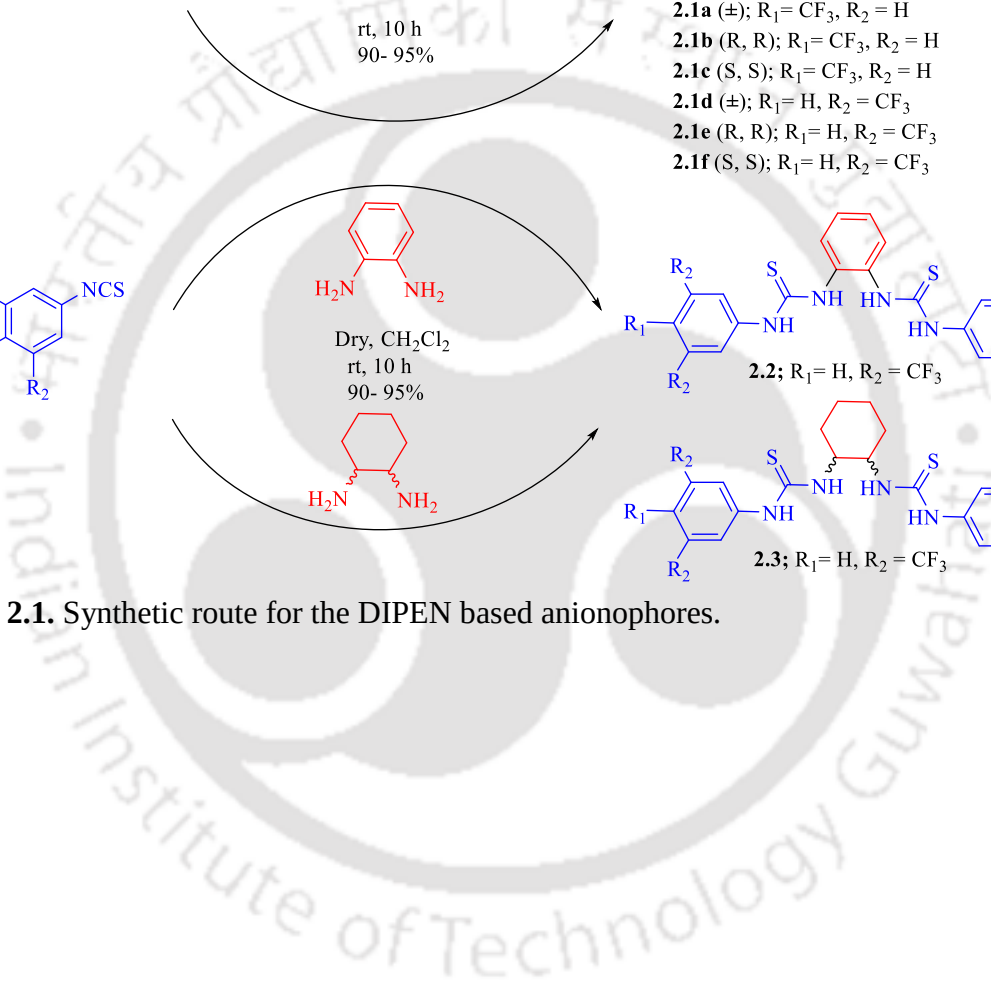
Chapter 5, the main emphasis was given to design a nitro reductase (NTRs) responsive TPE-based proanionophore with AIE active fluorescence property. To prove therapeutic potential, it was further explored as potent anionophores as well as diagnostic tools for the early detection of cancerous environments.

Chapter 2

Development of Diphenylethylenediamine-Based Potent Anionophores with Apoptosis-Inducing Activities

Inspired from the immense potential of anionophores, a new class of bis(thiourea)-containing anion transporter with conformationally controlled 1,2- diphenylethylenediamine (DPEN) was designed (**2.1a-f**) and synthesized where the thiourea derivatives of ortho-phenylenediamine (**2.1g**) and 1,2-diaminocyclohexane (**2.1h**) were also synthesized to understand the role of DPEN scaffold.¹⁵⁻¹⁶ In addition, the position and number of substituents on the aryl ring attached with the thiourea moiety were also altered to study the effect of substitution on the acidity of N-H proton as well as lipophilicity (Scheme 2.1). The initial anion recognition property of the DPEN derivatives was initially validated by the ¹H NMR titration and the single crystal analysis. This initial anion recognition ability prompted us to validate its anion transport efficiency under the model liposomal membrane containing (EYPC)/cholesterol (6:4). These anionophores induce measurable Cl⁻ transport efficiency in egg-yolk phosphatidylcholine (EYPC)/cholesterol (6:4) liposomes at a level down to transporter/lipid ratio of 1:750 000. Further, the potent thiourea derivatives (**2.1e** and **2.1f**) showed markedly higher selectivity for Cl⁻ ion in comparison with other tested biologically relevant anions such as Br⁻, I⁻, NO₃⁻, SO₄²⁻. Mechanistic studies revealed that potent compounds (**2.1e** and **2.1f**) follow Cl⁻/NO₃⁻ antiport pathway through the carrier mechanism. Further to check its anion transportation ability within the cellular environment, the MTT assay in the presence and absence of chloride ion using HBSS buffer was performed and found remarkable anti-proliferative activity under this environment (Figure 2.1E). Transportation of Cl⁻ ion by one of the potent compounds (**2.1e**) into the intracellular region of the cancer cells induces apoptosis through a caspase-dependent pathway. The anticancer property of compound **2.1e** (IC₅₀ value for the potent compound was 4.04 μM) was evaluated using a normal cell line BHK21 and two cancer lines such as HeLa and human tongue carcinoma SAS. Further, the detailed apoptosis induced by the potent anionophore was confirmed through numerous well-established assays using the HBSS, JC-1 DYE, DNA laddering experiments.¹⁷⁻¹⁹ The detailed western blot analysis also recommended the expression of cleaved Poly(ADP-ribose) polymerase (PARP) and other caspase enzymes, which confirmed the trigger in the apoptosis process *via* the intrinsic pathway of apoptosis.

So at the end of this chapter, the DPEN derivatives were successfully established as potential anionophore molecules with promising therapeutic prospects against cancer but failed to get the selectivity between the cancer cells and normal cells.



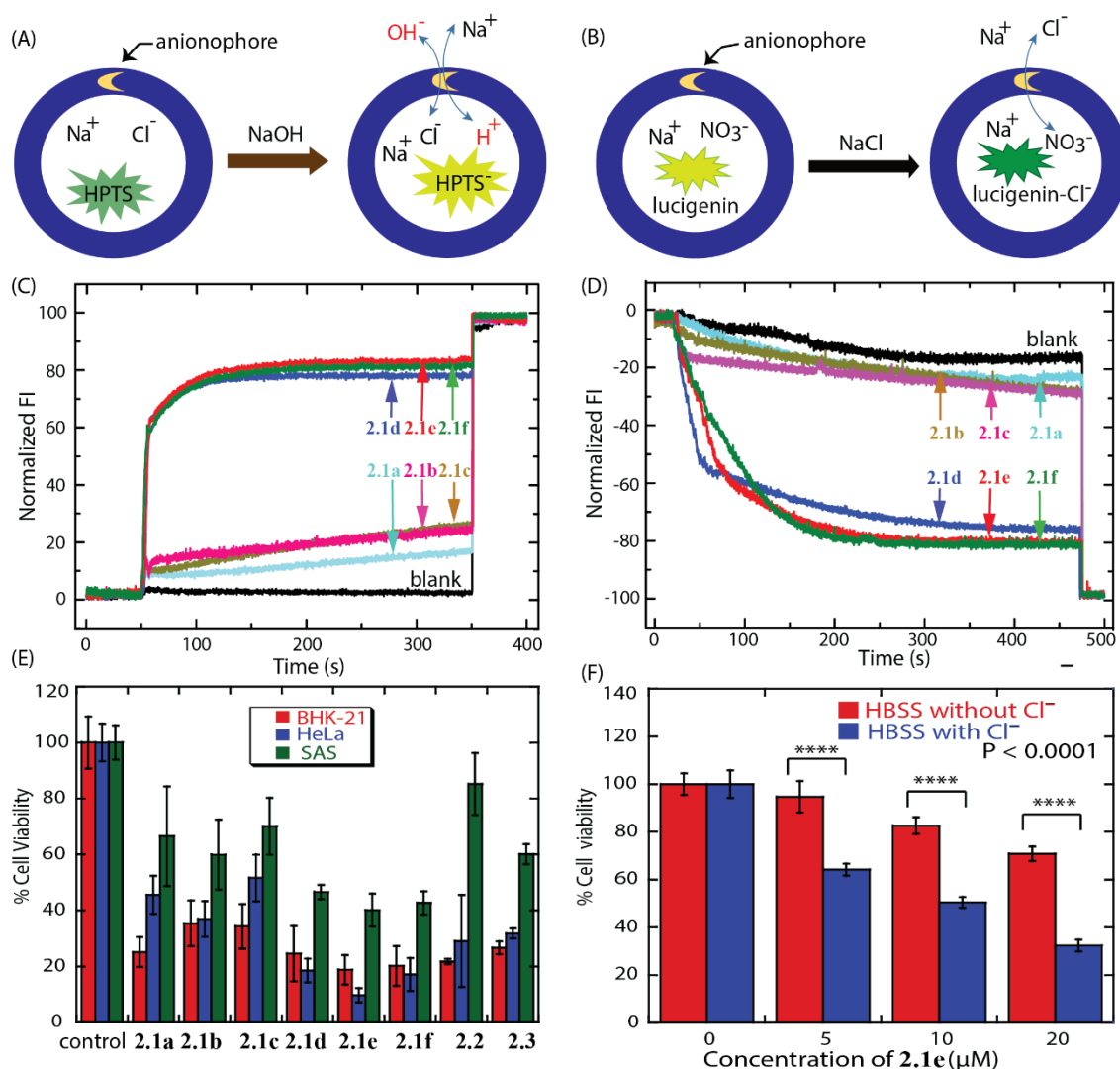


Figure 2.1. Schematic representation of fluorescence-based ion transport activity assay using EYPC/CHOL-LUVs $\supset$ HPTS (A) and EYPC/CHOL-LUVs $\supset$ lucigenin (B). Comparisons of ion transport activity of **2.1a-f** across EYPC/CHOL-LUVs $\supset$ HPTS (C) and comparisons of ion transport activity of **2.1a-f** across EYPC/CHOL-LUVs $\supset$ lucigenin (D). Cell viability of the compounds **2.1a-f**, **2.1g**, and **2.1h** was measured at a fixed concentration of 10 μ M. Cell viability was measured in BHK-21, HeLa, and SAS cells after 24 h of compound treatment (E). Viability of HeLa cells in the absence and presence of Cl^- ions in the HBSS buffer upon dose-dependent treatment of compound **2.1e** (0–20 μ M) after 24 h of incubation. Each bar represents the mean intensity of three independent experiments, and the differences in mean intensities are statistically significant ($P < 0.001$) according to two-way analysis of variance (ANOVA) (F).

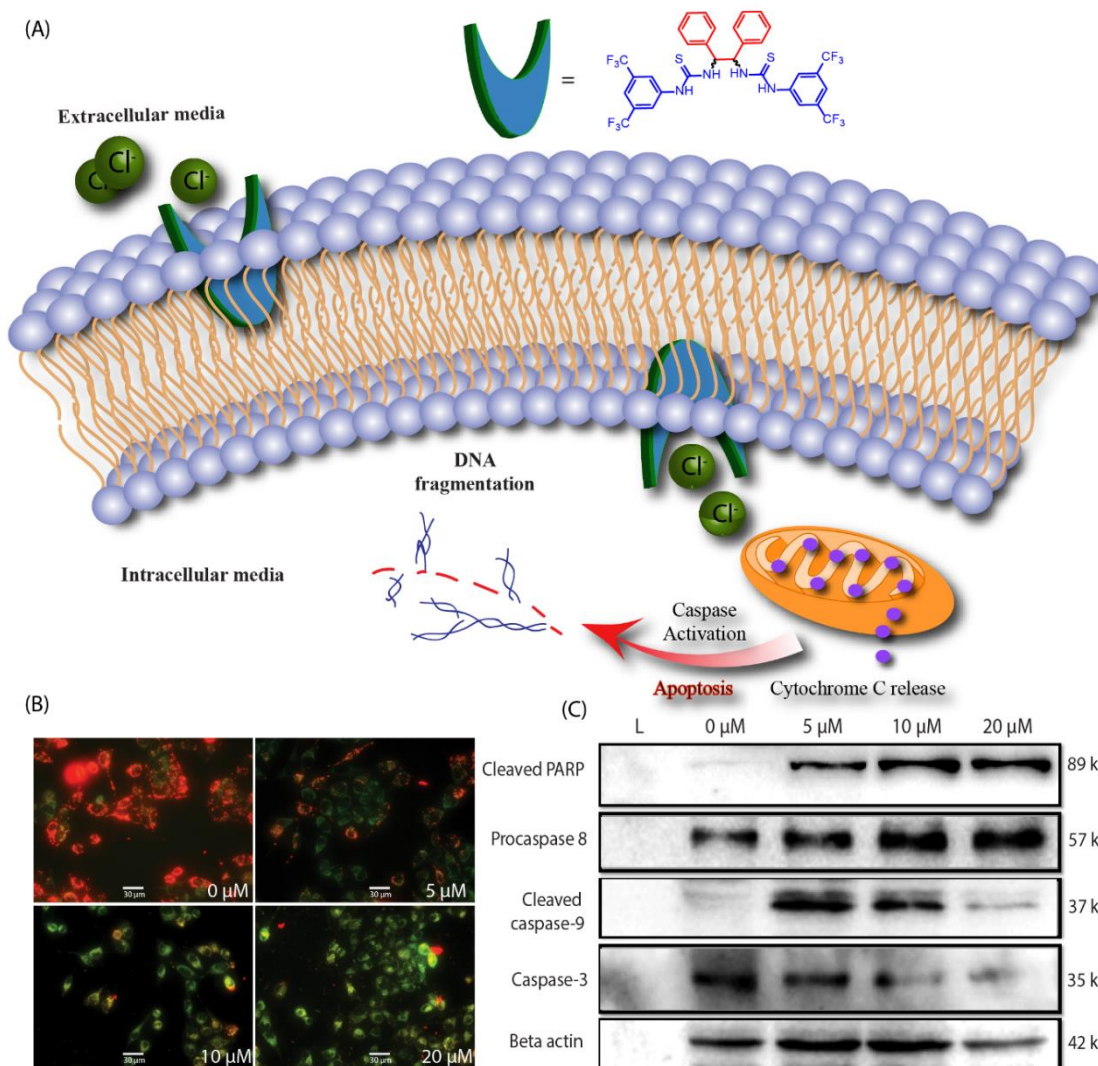
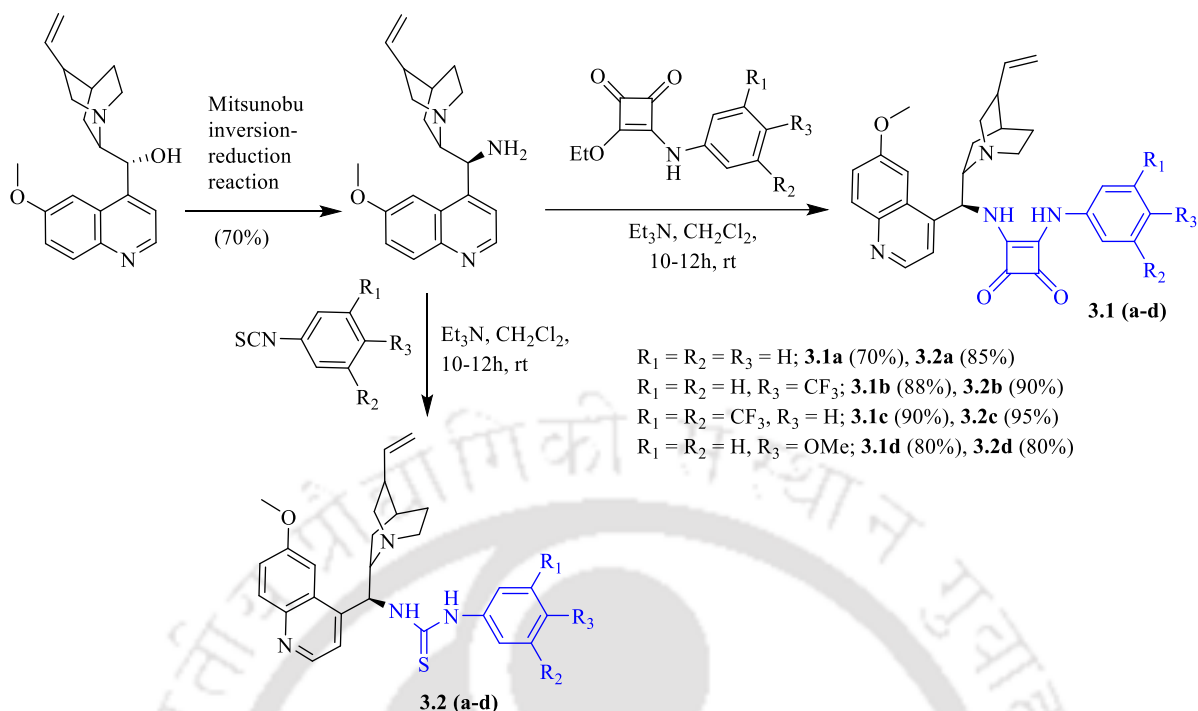


Figure 2.2. Schematic representation of diphenylethylenediamine based anionophores having chloride ion transportation and consequently triggers the apoptosis (A). Representative images of HeLa cells in the presence of 0, 5, 10, and 20 μM of compound **2.1e** after 24 hours of incubation. Cells were stained with JC-1 dye. Both red and green channels were merged in each image (B). Immunoblot analysis of HeLa cells in the presence of compound **2.1e** after 24 h of incubation with different concentrations (0, 5, 10 and 20 μM). L represents mock-infected HeLa cells. Antibody against poly(ADP-ribose) polymerase (PARP), Procaspase-8, pro-caspase-3, cleaved caspase-9, and beta-actin were used to develop the blot (C).

Chapter 3

Development of Quinine-based Semisynthetic Anionophores with Potential Anti-proliferative Activities

Though the aforementioned synthesized anionophore (**in chapter 2**) was found to be efficient to transport the ions but was not selective towards cancer cells over normal cells, which is the foremost obstacle to dictate its therapeutic potential, conversely, undesired toxicity limits the therapeutic potential of synthetic ion transporters to treat cancer. So a novel concept should be explored to reduce the toxicity of the anionophore in a normal cell. So in this chapter, importance was given to increase the selectivity between the normal and cancerous cell line. In this regard, some natural product-based derivatives were posited, which could have the potential to minimize the undesired side effects of the various ion transporters. Already it has been well accepted that despite tremendous progress in synthetic chemistry, natural products are still considered the best sources of drugs and drug leads. Currently, over 50% of clinically permitted drugs are either natural products or their derivatives because of their compatibility with the living system, as they have more “drug-likeness and biological friendliness” than synthetic molecules.²⁰ Motivated by the potential of the natural product-based molecules, natural product-based compound quinine was picked up, a century-old malaria drug. Initially, the quinine was semi-synthetically modified using the Mitsunobu inversion–reduction methodology, where the amine group was further modified with numerous squarate and thiourea moieties to provide an anion recognition motif (Scheme 3.1).²¹ Further, the role of the nitrogen atom of quinuclidine was also explored.



Scheme 3.1. Synthesis of squaramide and thiourea-based derivatives of quinine.

After confirming the anion recognition ability by the ¹H NMR and the DFT analysis, numerous fluorescence-based anion transport assay was performed using the Lucigenin, HPTS encapsulated dye across the model EYPC/CHOL LUVs. All these biophysical assays revealed that the potent compounds (**3.1b** and **3.2b**) could transport the chloride anions across the model membrane through the carrier mechanism (Figure 3.1). Additionally, a pH-dependent transportation ability was also noticed for these derivatives as quinuclidine nitrogen atom gets protonated at lower pH and contributes to anion binding. All these biophysical assays prompted us to explore its therapeutic potential at different cancer cell lines such as HCT-116 (a colon cancer cell line), OVCAR8 (an ovarian cancer cell line), MDA-MB-231 (a triple-negative breast cancer cell line), and MCF-7 (a luminal A breast cancer cell line). The concentration-dependent MTT assay showed that all other derivatives have concentration-dependent anti-proliferative activity (Figure 3.2). Analysis of the IC₅₀ values revealed that the potent compounds have the most potent anti-proliferative activity against different cancer cell lines. Interestingly, compound (**3.2b**) having the less EC₅₀ value at acidic environment has very low antiproliferative activity against the MCF-10A (normal breast epithelial cell line) compared to the other cancer cell line, which is really an impressive result (Figure 3.2). Subsequently, it's the detailed anti-proliferative activity was also confirmed using

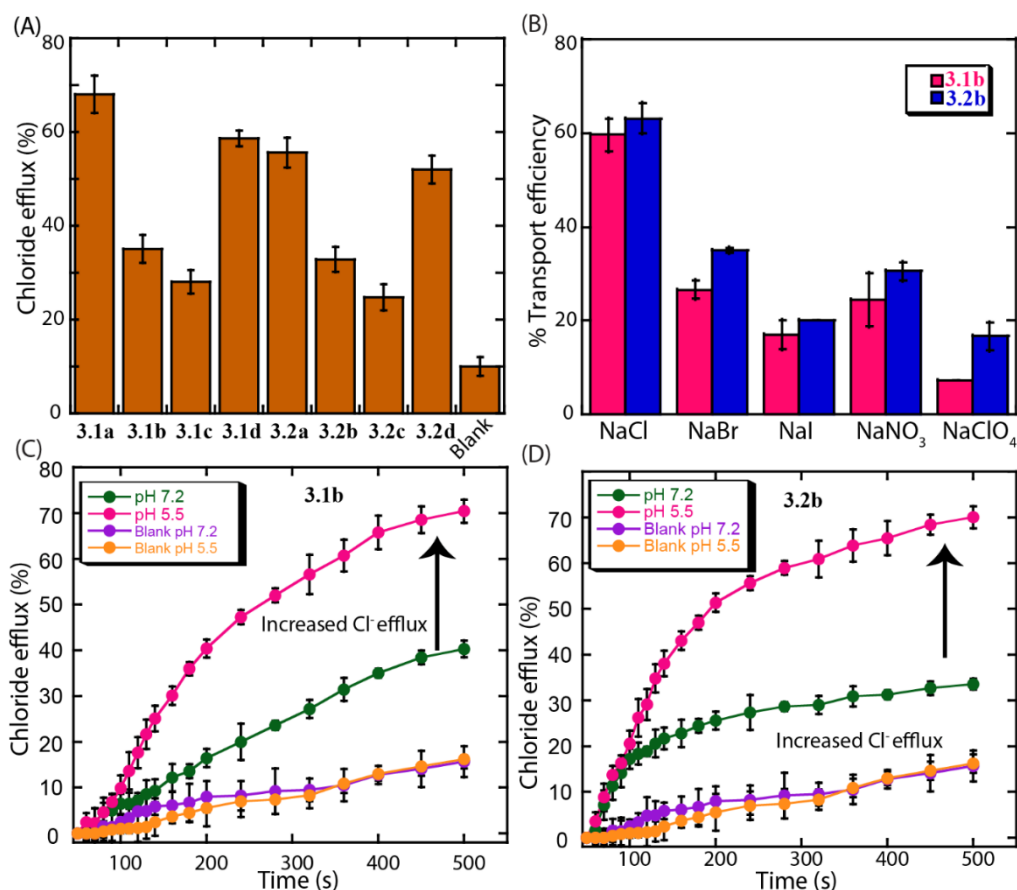


Figure 3.1. The Cl⁻ ion transport activities of the compounds, **3.1a–d** and **3.2a–d** (1.56 μ M = 0.5 mol % with respect to lipid) across the EYPC/CHOL-LUVs was measured by Ion Selective Electrode-based assay at pH 7.2 (A). Anion transport selectivity of compounds **3.1b** and **3.2b** was measured by a fluorescence-based assay using EYPC/CHOL-LUVs>HPTS in the presence of the base pulse in the extravesicular region (B) Chloride ion efflux capability of compound **3.1b** (0.56 μ M = 0.18 mol % with respect to the lipid) at pH 5.5 and 7.2 (C). The Cl⁻ ion efflux aptitudes of compound **3.2b** (2.29 μ M = 0.733 mol % with respect to lipid) across EYPC/CHOL-LUVs were measured by ISE-based assay at pH 5.5 and 7.2 (D). Compound concentrations were 0.56 μ M = 0.13 mol %, and 2.29 μ M = 0.55 mol % with respect to lipid for compound **3.1b** and **3.2b**, respectively. DMSO was used as blank. The efflux efficiency was measured after 450 sec of compound addition. All experiments were performed in triplicate.

numerous well known biological experiments such as MTT in the presence of HBSS buffer, clonogenic, and comet assay. The immunoblot analysis of the caspases and PUMA in the presence of the potent compound **3.2b** confirmed the mitochondria-mediated intrinsic pathway of apoptosis (Figure 3.3C). Further to investigate the applicability of

these ion transporters in therapeutics, *in vivo* studies were also executed in an established murine Ehrlich ascites carcinoma (EAC) solid tumor model (Figure 3.3D-F). The growth of the tumors was remarkably regressed in the presence of the potent compound (**3.1b** and **3.2b**). The treatment of potent compound **3.2b** also showed trivial toxicity to the kidney and spleen (Figure 3.3F). So overall, the *in vivo* studies also confirmed its potential role in the treatment of cancer.

In brief, at the end of this chapter, the selectivity between the cancer cells and the normal cells was successfully increased with the promising result both from *in vitro* and *in vivo* studies.²²

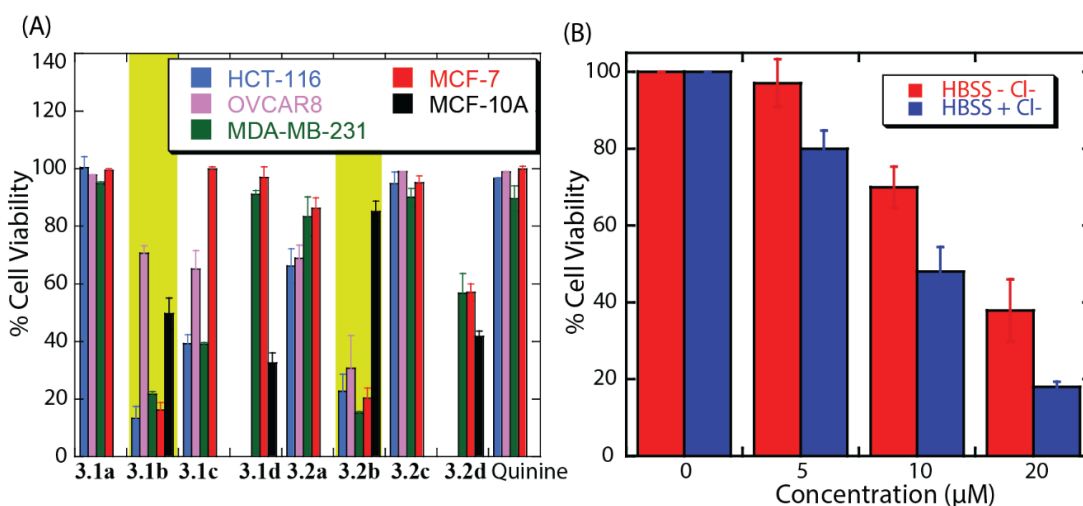


Figure 3.2. Cell viability of the compounds was measured (10 μM) in HCT-116 (a colon cancer cell line), OVCAR8 (an ovarian cancer cell line), MDA-MB-231 (a triple-negative breast cancer cell line), MCF-7 (a luminal A breast cancer cell line), and MCF-10A (a nontumorigenic breast epithelial cell line) (A). The MTT assay was performed after 48 h of compound treatment. Viability of MCF-7 cells in HBSS buffer with or without Cl⁻ ions at different concentrations of compound **3.2b** (0–20 μM). The MTT assay was performed after 24 h of incubation (B). All measurements were performed in triplicate.

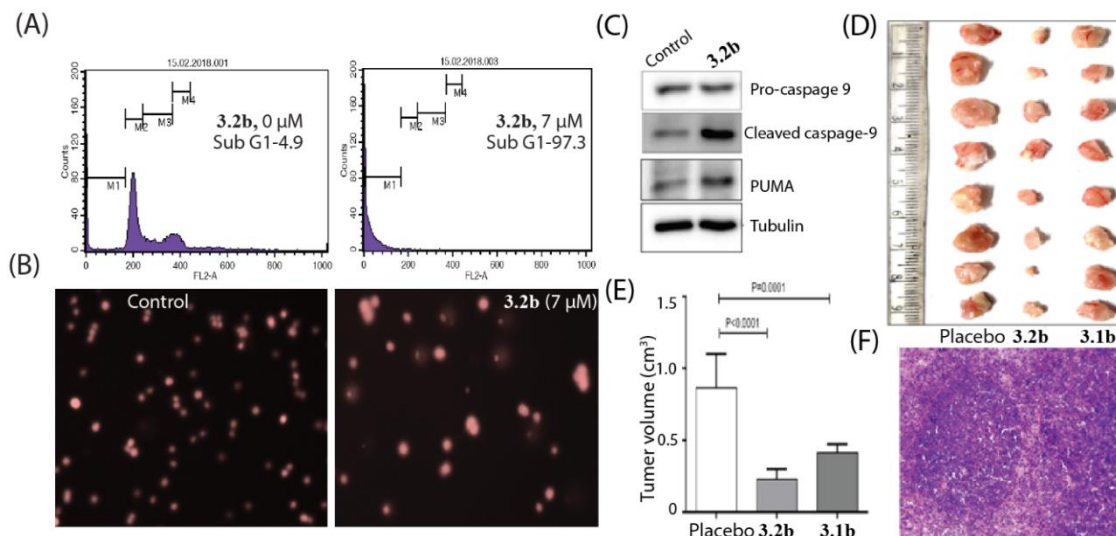


Figure 3.3. Fluorescent activated cell sorting (FACS) analysis of MCF-7 cells grown in the absence and presence of compound **3.2b**. The cell cycle distribution of the MCF-7 cell line was examined by staining the cells with propidium iodide (PI) in the absence and presence of the compounds. Cells were treated with the test compounds for 24 hours. Histograms were analyzed using Cell Quest Pro software (A). The compound induces DNA damage in MCF-7 cells. Comet assays for the determination of DNA damage in single cells following the treatment with compound **3.2b** (B). Whole-cell protein extracts of MCF-7 cells grown in the absence and presence of compounds (10 μM) for 24 h and immunoblotted for the indicated proteins (C). α -Tubulin was used as a loading control. The progression of a solid tumor in the female Swiss albino mice in the absence and presence of compounds **3.1b** and **3.2b** (D). The tumor was induced in Swiss albino mice by injecting EAC cells into the thigh muscle. The extent of tumor growth inhibition (E). Immunotoxicity assessment of spleen by hematoxylin and eosin staining after compound **3.2b** treatment (F) showed negligible toxicity.

Chapter 4

Development of Sulphonium Based Water Soluble Proanionophores with Potential Prospects in Therapeutic Applications

At the end of **chapter 3**, another inadequacy of anionophore was observed which is related to its uptake and cell deliverability. As the anionophore are generally lipophilic molecules, their cell deliverability is quite challenging where excess doses or additional delivery vehicles is required to deliver it to the targeted site. Hence, regulating the solubility of anionophores could be one of the strategies to increase their therapeutic

values. So the stimuli-responsive water-soluble proanionophore concept of hydrophobic anionophores was considered to augment the deliverability and generate the desired lipophilic transporter at specific tissues after the in situ cleavage of the hydrophilic appendages in the presence of certain stimuli, which is present at a higher amount in cancer cell than normal cells. In this regard, the glutathione (GSH)-responsive anion transport strategy was introduced to promote the controlled transport of chloride ions across lipid bilayers. As the concentration of the GSH in cancer cells is higher than the normal cells, it has immense efficiency towards the cancer cell line over the normal one.²³ An inspiration came from the information that the presence of the sulfonium moiety could enhance the cellular uptake of the target molecules.

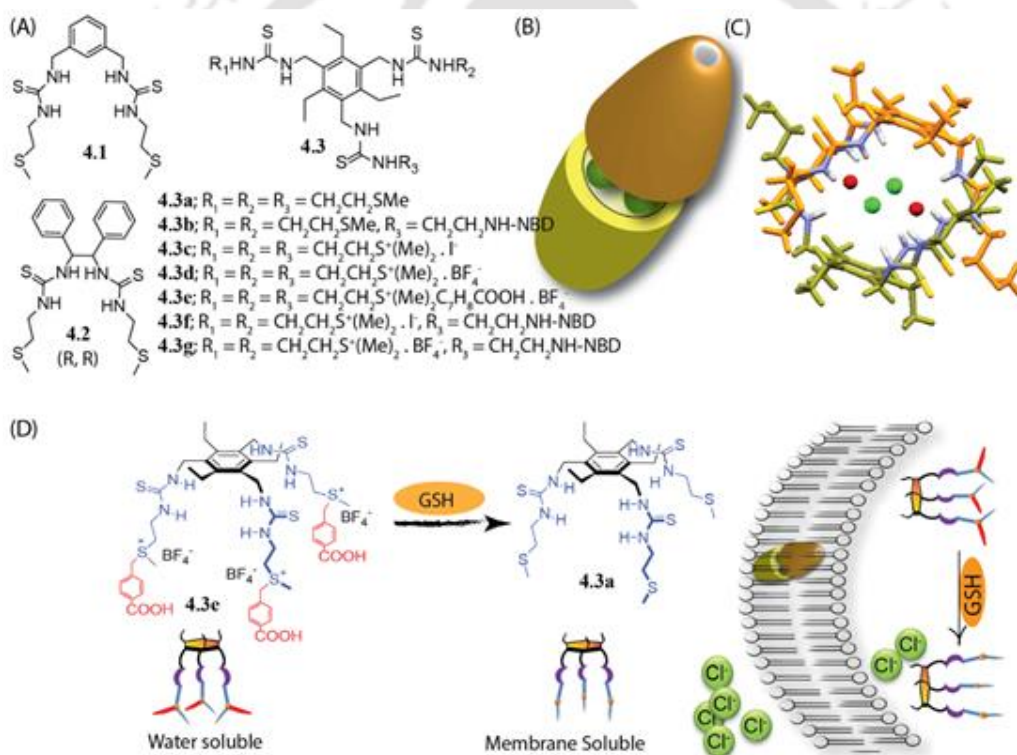


Figure 4.1. GSH responsive sulphonium-based anionophores and proanionophores (A). Schematic representation of Chloride ion encapsulation by capsule kind of arrangement (B). X-ray crystal structure of compound **4.3a** complexed with Cl^- ion and H_2O ((**4.3a**· Cl^- · H_2O)₂). The TBA⁺ cations present outside the cavity were omitted for clarity. The probable mode of transportation in the presence of GSH as stimuli (C). Probable mode of transportation across the membrane in the presence of GSH as stimuli (D).

Hence, the water-soluble sulfonium derivatives of hydrophobic anionophores could solve the conventional hurdles of solubility and deliverability.²⁴ So, in this regard, a tripodal-based water-soluble GSH responsive proanionophore was synthesized having the sulphonium moiety (Figure 4.1).

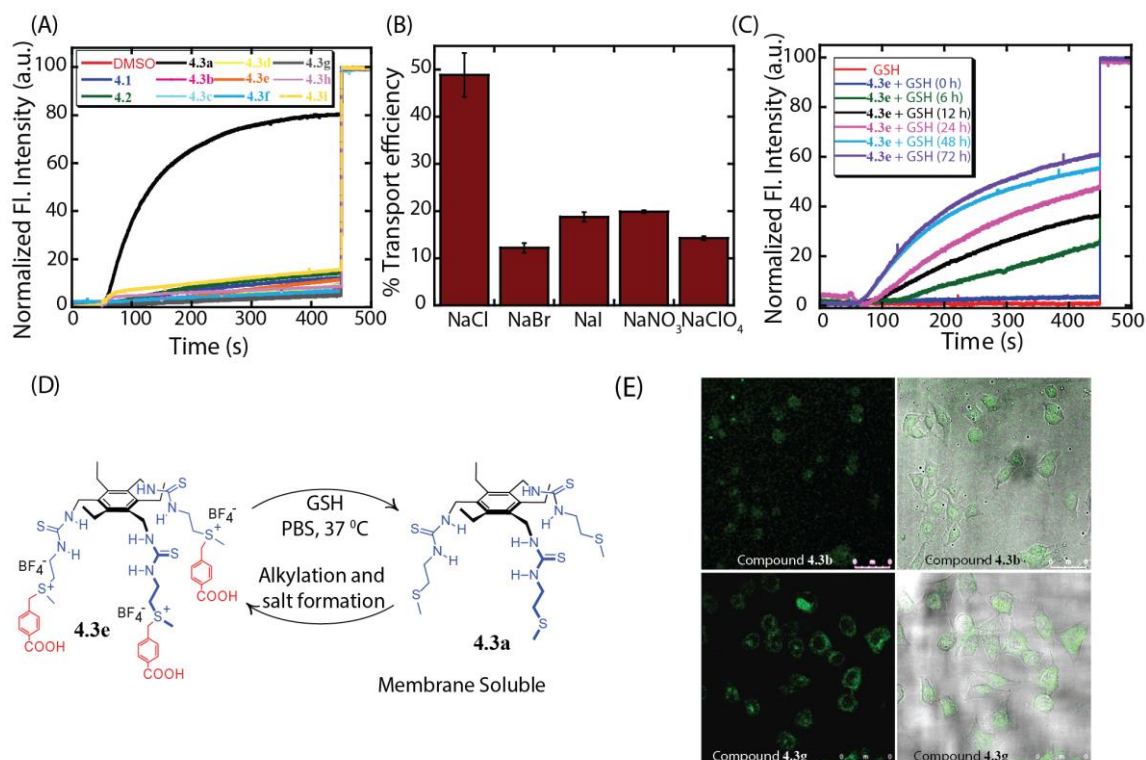


Figure 4.2. Comparisons of ion transport activities of the compounds (0.56 mol %) across the EYPC/CHOL-LUVs>HPTS (A). Anion selectivity of compound **3** at EC₅₀ = 0.14 mol % (B). Transport of Cl⁻ ion by the regenerated compound **4.3a** in the presence of GSH at different time intervals (C). Schematic representation of the alkylation/dealkylation strategy (D). Fluorescence microscopic images of the HeLa cells treated with NBD-labelled compounds **4.3b** and **4.3g**. Green channel illustrating compound uptake (E).

To monitor its uptake efficiency through fluorescence spectroscopy, we have modulated one hand of tripodal molecules with a fluorescence tagged NBD compound **4.3b**. Initial anion recognition by ¹H NMR and the crystal structure confirmed a capsule kind of arranging (Figure 4.1). Numerous bio-physical and *in vitro* assays suggested its potential chloride ion transport ability. The uptake of the NBD tagged proanionophore **4.3g**, and its corresponding active anionophore **4.3b** were performed in HeLa cells and found that the NBD tagged proanionophore **4.3g** has higher uptake from its corresponding

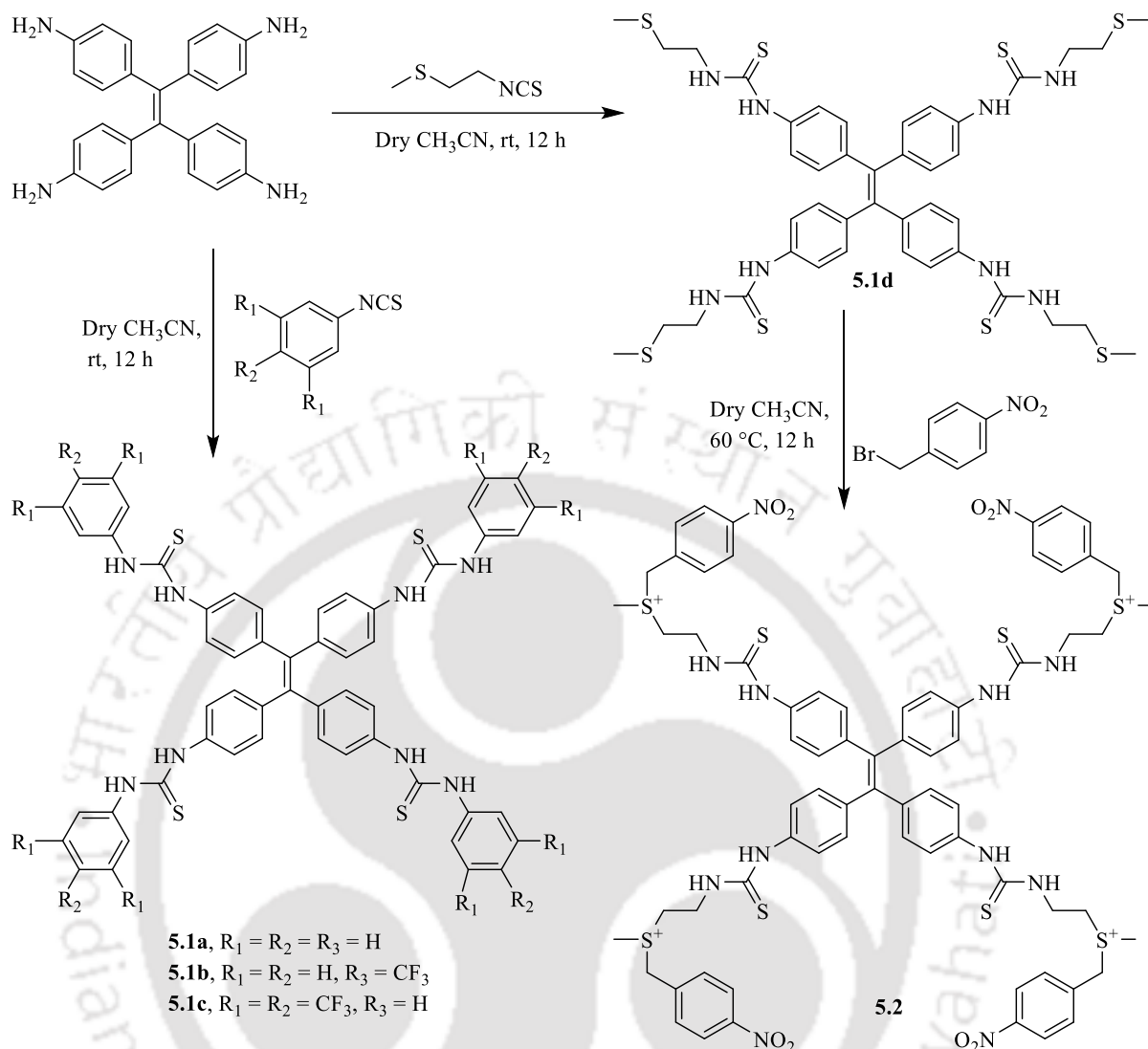
NBD tagged anionophore **4.3b**, which proved its potential applicability for numerous therapeutic applications (Figure 4.2E).

So at the end of this chapter, it has been concluded that this derivative could be used as a therapeutic agent for the treatment of cystic fibrosis due to its non-toxic nature, which is another highly demanded application of an anionophore. For cystic fibrosis, anion transporter molecules should have non-toxic behavior towards the normal cells, which is very rare to found. Fortunately, this sulphonium-based GSH responsive proanionophore has the criteria to be used as a therapeutic agent for the treatment of cystic fibrosis.

Chapter 5

Development of Nitroreductase Responsive Proanionophores with Potential Prospects in Therapy and Diagnosis

To find out the much more precise strategy for anti-cancer activity of the anionophore, another novel concept was looking to improve the efficacy selectively on cancer cells. The enzyme, light, and glutathione (GSH)-responsive proanionophores were successfully developed to improve the target-specific deliverability, uptake, and selectivity towards cancerous cells over healthy cells. However, the presence of significant esterase and glutathione levels in the healthy cell complicates their further biological applications. Hence, there is a need for precise stimuli-responsive proanionophores, which should have higher activity under the cancer environment. Besides solving these conventional obstacles associated with the anionophore related research, it is the right time to bolster the importance of the diagnostic tool for early detection of cancerous cells in the tumor microenvironment. For this diagnostic purpose, nowadays, scientists are developing numerous stimuli-responsive fluorescence molecules where the fluorescence property of the molecules will be enhanced in the presence of a certain biomarker present in the cancer environment. In this regard, nitroreductase responsive anionophores would potentially eliminate cancer cells under hypoxic conditions selectively. Nitroreductase (NTR) is a well-known flavin-containing oxidoreductases enzyme, the potential biomarker that has been upregulated in cells under hypoxic stress.²⁵ It is also well acknowledged that cells under the hypoxia environment finally transform into tumor cells, resistant to numerous therapy.



Scheme 5.1. Synthetic routes to TPE-based compounds.

So the selective NTR-responsive fluorescence molecules were designed that would be beneficial, both for the early detection of hypoxia-mediated tumor environment and the selective antiproliferative activity in cancer cells. Despite the remarkable progress in this field, fluorescent probes with superior properties such as emission at longer wavelengths (>500 nm), good water solubility, high selectivity, and sensitivity are still extremely valuable research for clinical diagnosis as well as biomedical applications.²⁶ Inspired by the immense potential of NTR enzyme, a novel strategy was adopted to develop anionophore that makes it much more efficient in cancer cells and has the potential to diminish the anionophores deliverability problems that arise due to their inherent lipophilic properties. Simultaneously its uptake efficiency could also be monitored through fluorescence microscopy due to the presence of the tetraphenylethylene (TPE) core unit within the anionophores. The aggregation-induced emission (AIE) property

provides a considerable advantage to these TPE-containing anionophores over the reported normal fluorophore-tagged anionophores. The AIE-based fluorophores have also been employed as novel probes for bioimaging and biosensing applications. The AIE probes possess many superior properties than the conventional fluorophores, including concentration-dependent enhancement of brightness, resistance to photo-bleaching that are useful for long-term tracking and imaging of cells. So keeping all these advantages in mind, TPE-based anionophores were synthesized where the chemistry of sulfonium salts was upgraded by using the p-nitrobenzyl moiety, which is susceptible to release the active anionophore in the presence of GSH and hypoxia environment (Scheme 5.1). The presence of both sulfonium and nitrobenzyl moieties improves the aqueous solubility. It provides a system where both GSH and NTR could act as dual stimuli to regenerate the active anionophore from its proanionophore. Within this chapter, a systematic investigation on regeneration and transportation of proanionophore **5.2** under the reductive environment of GSH, dithionite, and the presence of bacterial NTR enzymes were explored. Several biophysical studies suggested a carrier-mediated chloride ion transport ability of the synthesized compounds (Figure 5.2). Further, its transport ability was also explored using the liposome-based NMR assay (Figure 5.1). Besides, the investigation of the stimuli-mediated increase in TPE fluorescence was also carried out and proposed that it could be useful in detecting hypoxia-mediated cancer environments.

Overall, from this chapter, it was confirmed that these dual stimuli-responsive proanionophore **5.2** have the potential to regenerate the fluorescent active lipophilic compound **5.1d** that could transport the Cl^- ions across the lipid bilayers and useful for studying subcellular localization and early diagnosis of tumors.

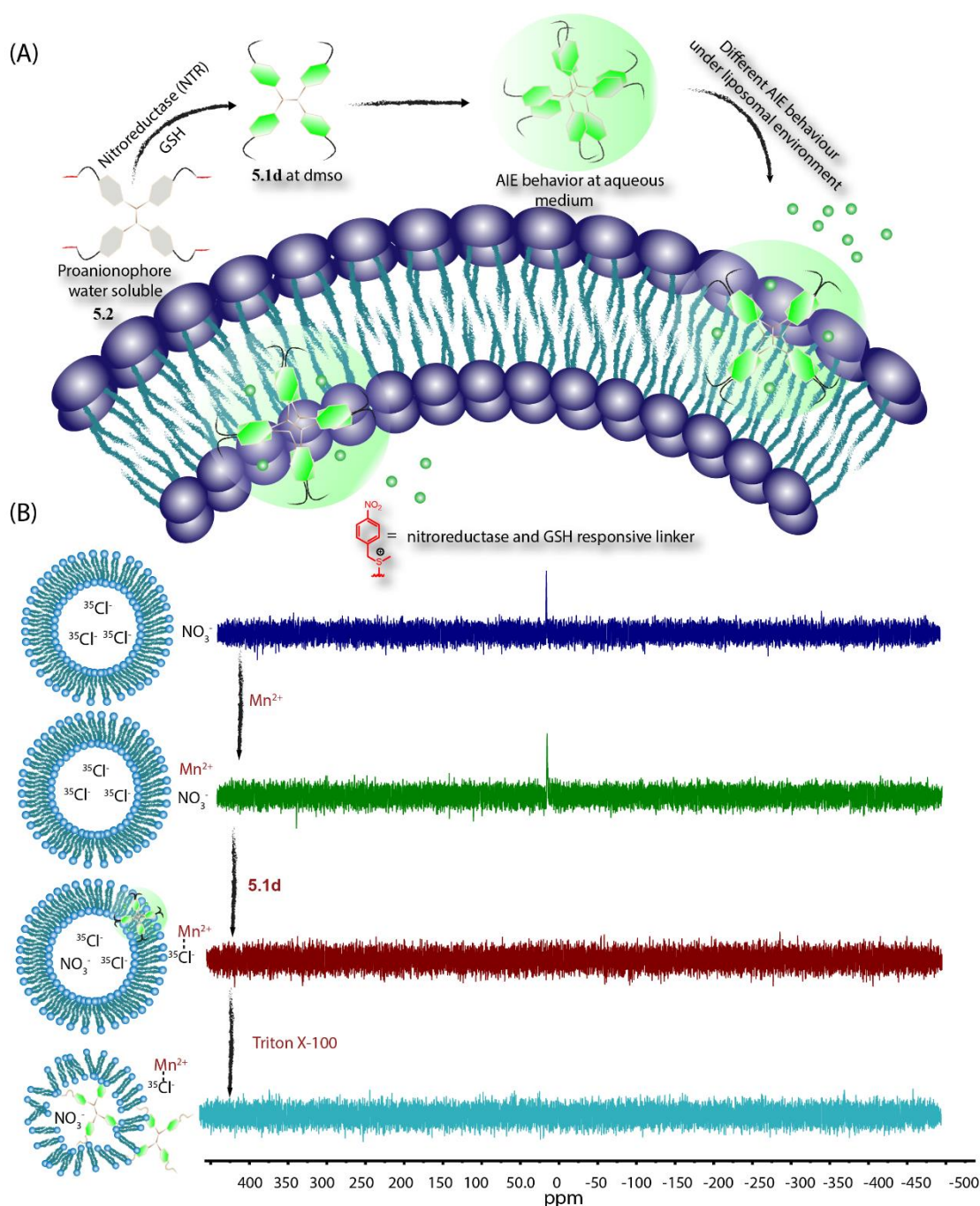


Figure 5.1. Schematic representation for the chloride ion transportation of TPE-based compounds (A). ^{35}Cl NMR experiment showed that **5.1d** (5 mM) is proficient in chloride ion transport via the $\text{Cl}^-/\text{NO}_3^-$ antiport pathway. The NMR experiment was performed in $\text{H}_2\text{O}:\text{D}_2\text{O}$ (9:1) solution. (B).

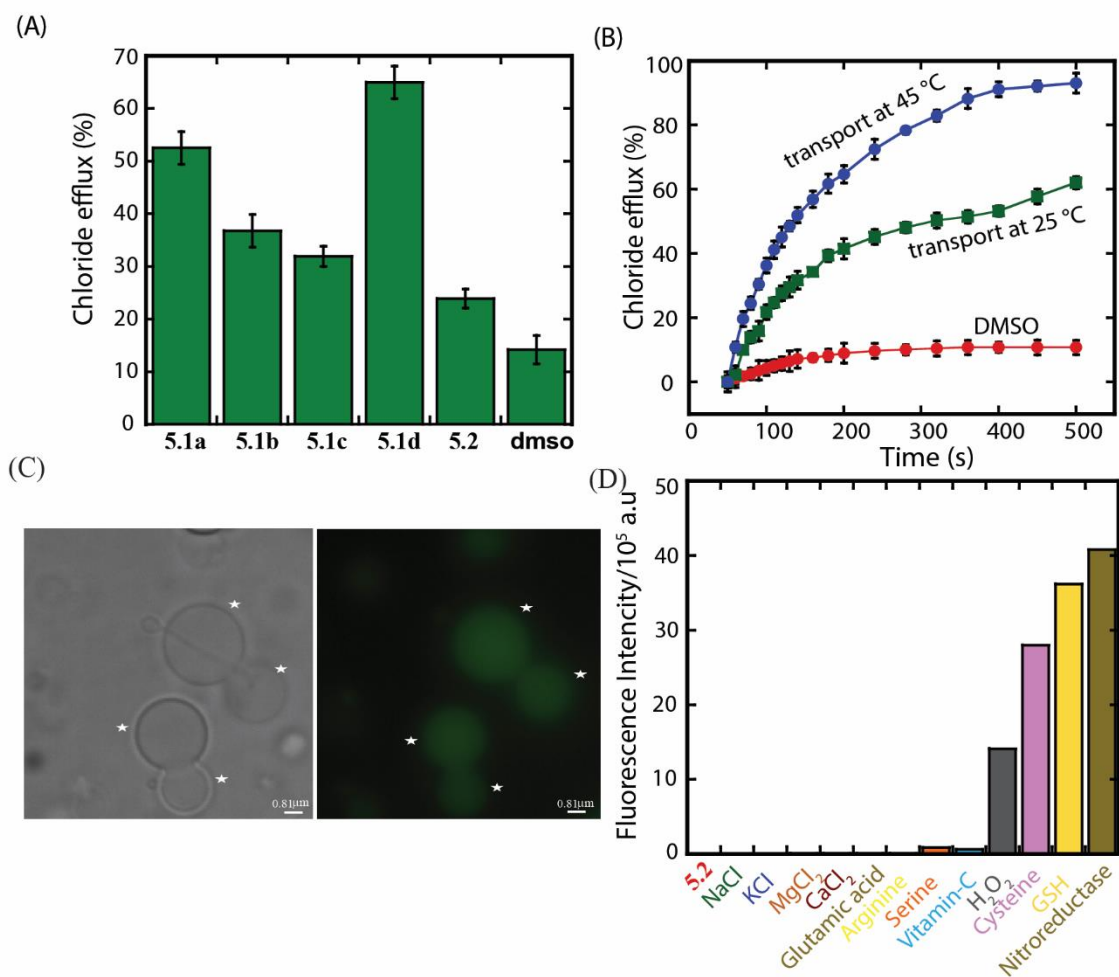


Figure 5.2. Chloride ion transport efficiency of the compounds (0.156 μM = 0.05 mol % with respect to lipid) across the EYPC/CHOL-LUVs (6:4 molar ratio) was measured by an ISE-based assay at pH 7.2 (A). Temperature-dependent transmembrane Cl⁻ ions transport (across DPPC-LUV) assay using chloride-ISE in the absence and presence of compound **5.1d** (B). Fluorescence microscopic images of the compound **5.1d** (10 μM) loaded GUVs under bright field and green channel. This study illustrates the potential use as a bio-imaging agent. The scale bar of the images was 0.81 μm (C). Regeneration of the active compound **5.1d** from the proanionophore **5.2** in the presence of the different substances. Proanionophore **5.2** (1 mM) was incubated with individual substances (10 mM), and fluorescence spectra were recorded. Regeneration efficacy in the presence of different analytes is represented in bar plot (D).

Conclusion

Within this thesis, numerous novel scaffolds were developed as a potential anionophore with numerous therapeutic prospects. Additionally, the significance of anionophore for several life-threatening diseases has also been described in **Chapter 1** with concise literatures. Next, the DPEN derivatives (in **Chapter 2**) were explored as a new class of anionophore with apoptosis-inducing activity. However, DPEN derivatives prove themselves as potential anionophore but fail to increase the gap of selectivity between the cancer cells and normal cells. To combat this limitation of DPEN derivatives, the natural product-based semi-synthetic quinine derivatives (In **Chapter 3**) were synthesized and found that these are able to increase the selectivity for cancer cells than normal cells up to 5 fold. In the next chapters (**Chapter 4** and **Chapter 5**) main emphasis was given to solve the hurdles associated with lipophilic anionophores such as cell deliverability, uptake, and selectivity using numerous novel stimuli-responsive approaches such as glutathione (GSH) and nitroreductase (NTRs). Overall, within this thesis, plenty of anionophores with a thorough investigation has been reported. Further, their detailed therapeutic prospects have also conversed with a set of well-known experiments or assay systems.

Future Perspectives

The anion transport-related research works are gaining significant advancement over the last 10-15 years because of their therapeutic potential against cancer, cystic fibrosis, and other channelopathies. The forecast of using anionophores to treat these diseases has been supported by a thorough understanding of the biological activities of anionophores.³ Although the development of classical anionophores has a considerable prospect in therapeutics, but still, this field is associated with a number of hurdles, such as poor deliverability due to lipophilic nature, cellular uptake, and selectivity between the cancer cells and normal cells. To overcome these hurdles, scientists across the globe are coming up with numerous new concepts like stimuli-responsive proanionophore. Using this concept, one may overcome these hurdles associated with an ionophore development. But this concept still needs to be explored more from the detailed biological point of view.

Mainly chloride anion transporting molecules are extensively explored for several therapeutic prospects. So the importance of designing and exploring the other ion transporting molecules is needed to be investigated. Besides that, the anion transport

facilitated by those molecules and their biological activities should be thoroughly investigated to open up numerous other therapeutic prospects from the conventional ones. It is also well documented that various monovalent anions have potential activity against multiple viruses. The antiviral property of the anionophores could have considerable potential, which could also be explored.²⁷ Recently, it has been revealed that chloride ion has an enormous efficient role in cardiac diseases. Anomalous functions of the chloride channel could lead to several pathological conditions like arrhythmogenesis in myocardial injury, cardiac ischemic preconditioning, and the adaptive remodeling of the heart during myocardial hypertrophy and heart failure.²⁸ So, in this scenario, the artificial chloride ion transporter molecules could have an immense perspective against these pathological conditions. Unregulated anion transport also plays an essential role in kidney diseases. The implications of the artificial anion transporter molecules could have an enormous role in controlling this anionic imbalance and preventing this chronic kidney disease. Mutation of the certain cation-chloride cotransporters (CCCs) channels like NKCC1 and KCC2 could have the potential to alter the intracellular chloride homeostasis, which eventually leads to numerous pathological conditions like ischemic, neonatal seizures, temporal lobe epilepsy, and neuropathic pain by hyperexcitability or hyperexcitability of neurons.²⁹ So, in this case, artificial anionophores could also come up with a solution against these pathologies. Hence, a search for the applications of anionophores against these unexplored areas is also required. Further, the anionophores could also be used alone or as a combination therapy with drug molecules that target anion transport-related diseases.

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Chapter 1

Introduction of Anion Transportation and its Numerous Potential Therapeutic Applications





1.1. Introductions

Maintenance of the precise balance of osmolality across the cell membrane is crucial in regulating various physiological functions. Usually, this balance of intra- or extracellular osmolality is harmonized by integral membrane transport proteins that expedite the selective movement of ions across lipid bilayers.¹⁻³ The generation of electrochemical gradients across the membranes due to the transport of ions drives biological processes such as ion-homeostasis, signal transduction, cell volume regulation, migration, hormone secretion, blood pressure regulation, and induction of programmed cell death.³ However, mutation of the genes encoding the integral membrane transport proteins or their structural rearrangement could result in various channelopathies like cystic fibrosis, Bartter syndrome, generalized epilepsy, Isaacs' syndrome, QT syndrome, and others.⁴⁻⁵ Recent studies demonstrated that the homeostasis of several ions, including Cl^- , regulates cellular apoptosis.⁶ Mutation of the membrane protein machinery is also associated with antibacterial resistances.⁷

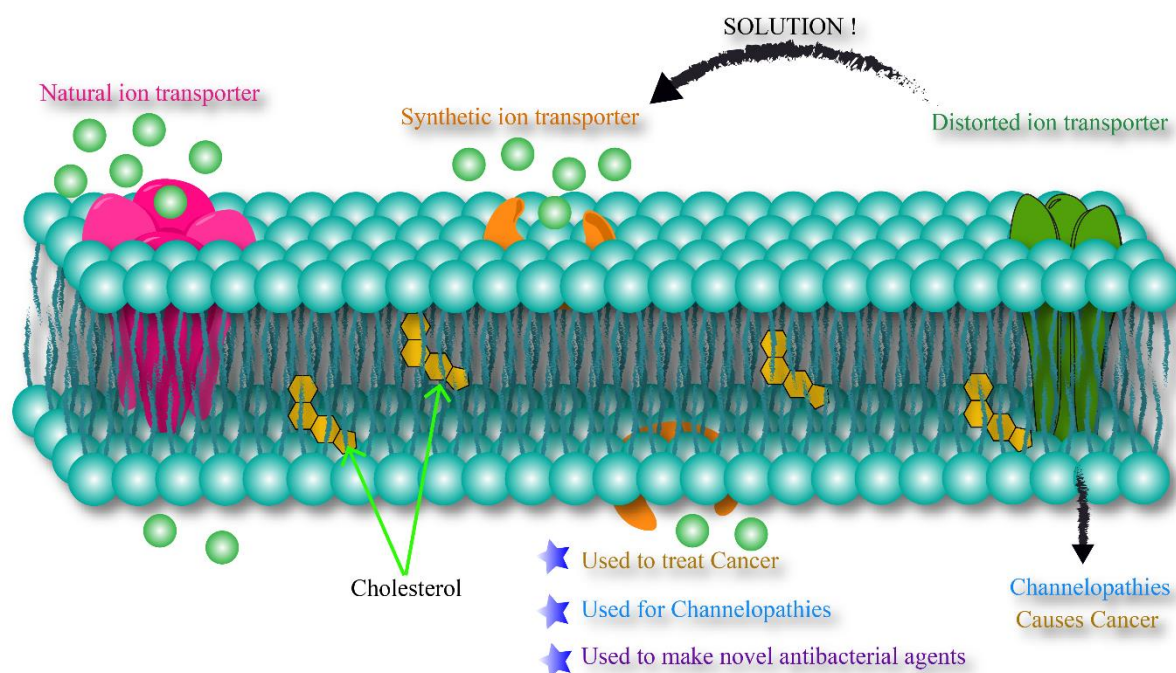


Figure 1.1. Numerous therapeutic prospects of anionophores.

To overcome all these pathological conditions which are generated from the abnormal ionic homeostasis, scientists across the globe have been devoted their spirit to

explore some unique ideas. Finally, they come up with an idea of artificial ion transporter molecules that will mimic the functions of the defected transport protein and solve all these aforementioned hurdles associated with it. So the development of simple organic molecules that could selectively bind with the anions and transport them across the membrane is really impressive and could lead to a revolutionary change in the healthcare arenas associated with numerous channelopathies, cancer, and anti-bacterial arenas. Within this chapter, the importance of anion transport and the recent advancement in the field of anion transportations with succinct literature are discussed in detail.

1.2. Transportation across the biological membrane

1.2.1. Different modes of transportations

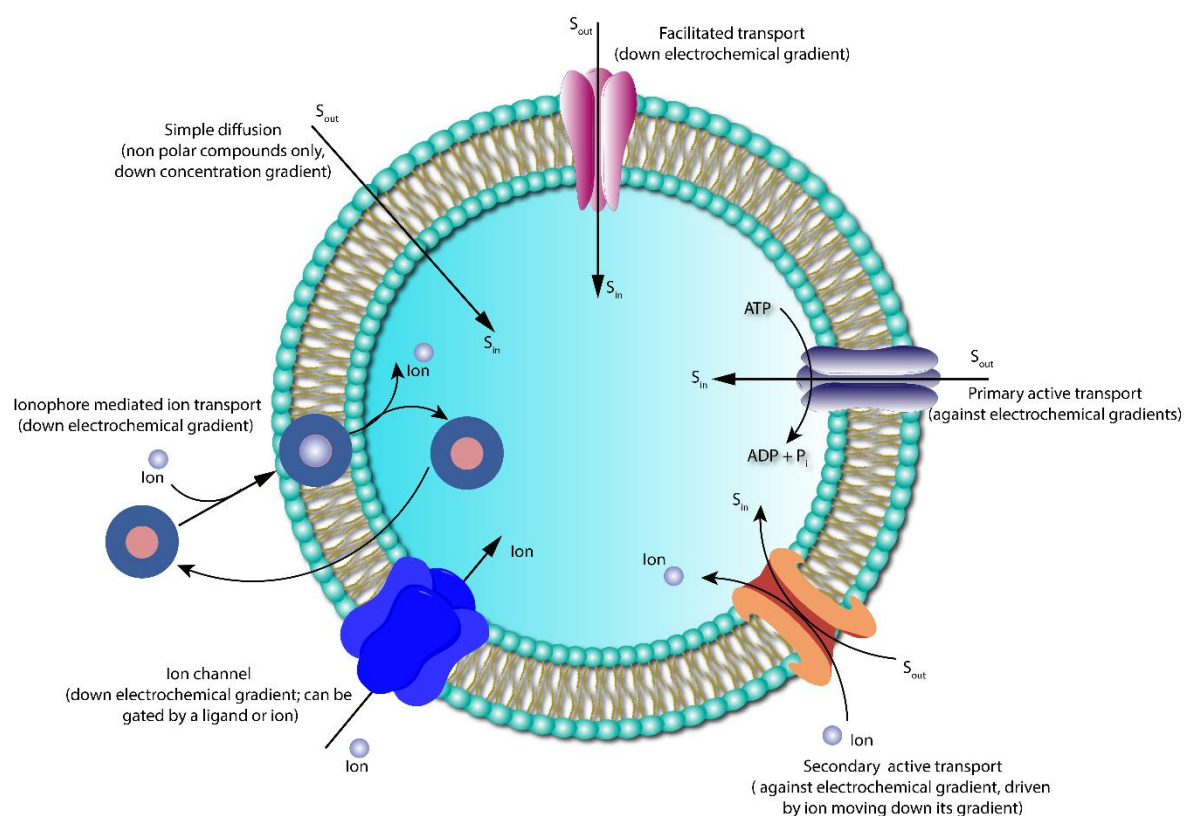


Figure 1.2. Different modes of transportations.

The cells require the raw materials from their surroundings for the desired biosynthesis and energy productions within the living organism. Simultaneously waste or by-products generated from these biosynthesis or energy productions should be released

to its environment to maintain a proper balance within the cells. The requirement of these raw materials is supplied by numerous elegant protein machinery which resites within the cell membrane. Although few non-polar ions could easily pass through the membrane for polar and charged species, a membrane protein is essential.⁸ These proteins are selective for particular ions and mainly transport these ions across the phospholipid cell membrane. In most cases, the membrane proteins facilitate the diffusion of the ions down its concentrations gradient. Still, transport often needs to be facilitated against the concentration gradient, electric charge, or both, in which cases the ions or solutes must be pumped in a sequential process that requires the energy which is coming from the hydrolysis of ATP or supplied in the form of the movement of another solute down its electrochemical gradient with sufficient energy to carry out another solutes or ions up its gradient.⁹ Ions could also move across the membrane *via* channels formed by specific proteins. The ionophore could also carry out small molecules that could mask the charge of the ions or solutes and facilitate them to diffuse through the membrane.¹⁰

1.2.2. Active and Passive Transport

The transportation of the ions or solutes could be assisted by two processes- the active process where the ions move from lower concentration to higher concentrations gradient with the help of energy in the form of ATP.⁹ On the other hand, passive transport deals with the movement of the ions from higher concentration gradient to lower concentrations gradient without any energy requirement.⁹ This passive transport is facilitated by numerous membrane proteins. To transport through the phospholipid membrane, the charged or polar solutes have to give up the interaction energy in their hydrated cells and then could diffuse the membrane where they are poorly soluble within it. During this transportation process, initially, the energy was peeled from its hydrated cells to move the water-soluble ions or solutes into the hydrophobic membrane. The numerous noncovalent interaction between the transporting molecules or proteins compensates this energy loss which is again regained as ions or solutes leave the membrane on the other side and is rehydrated again. Transmembrane passage of the ions or molecules is really a high energy state compared to the enzyme-catalyzed chemical reactions. For both cases, the activation barrier must be overcome to facilitate this process. The activation energy to translocate the polar solute across the membrane is so large that

the hydrophobic membrane is virtually impermeable to polar and charge species over time relevant to cell growth and division. The membrane proteins machinery lowers the activation energy by providing an alternative pathway to the ions through the bilayer. The charge and polar substrate are bound by numerous transporter with stereo-chemical specificity through multiple non-covalent interactions. The negative free energy change for these noncovalent interactions actually compensates for the loss of water from the hydrated cells, thereby lowering the free energy change for transmembrane passage. This magnitude of lowering the free energy change for channel-forming proteins is much more than the carrier kind of transporters. The channel offers alternative pathways for a specific substrate to transport across the bilayer without dissolve in the bilayer, which actually further lowers the free energy change for transmembrane diffusion.¹¹

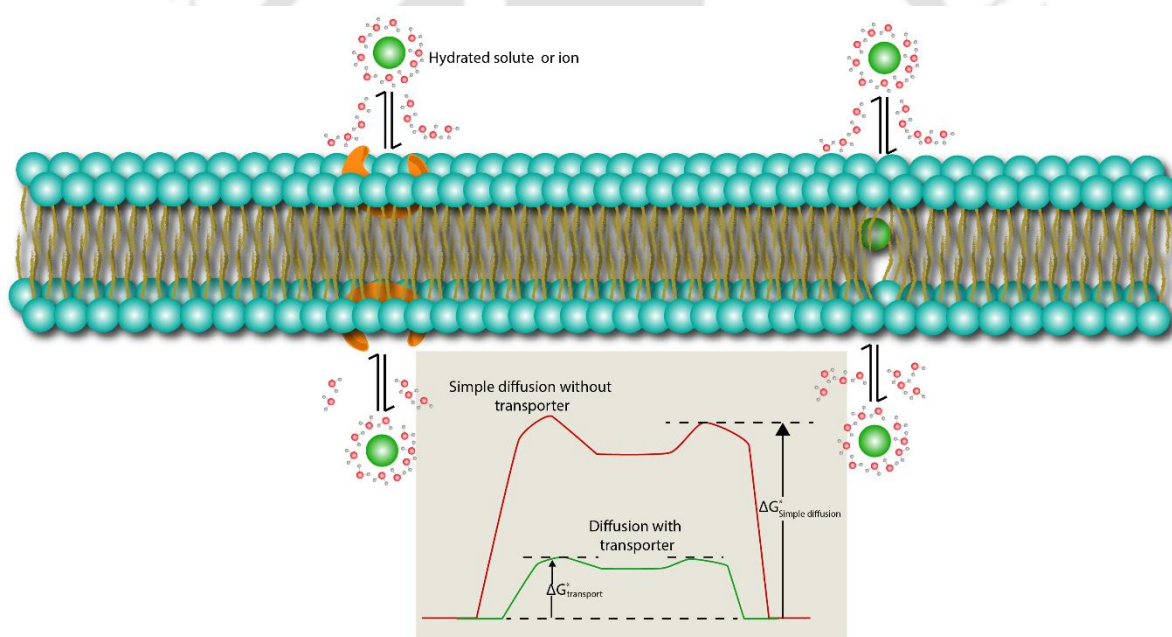


Figure 1.3. Energy changes during the passage of hydrophilic solute across the hydrophobic membrane.

1.2.3. Transport pathway and mechanism

The transmembrane transport assisted by the numerous transporter molecules generally follow the following pathways

- **Uniport:** The ions or solutes are transported in a single direction. Glucose transporter (GLUT) protein is a well-known example of a uniport mechanism that is found in erythrocytes.
- **Symport:** This is a co-transport pathway where the two different species of chemical entity are transported in the same direction. Na/glucose transporter proteins are examples of this kind of symport pathway.
- **Antiport:** This one is also a co-transport process where the two different chemical species are being transported in opposite directions. Na^+/H^+ antiporter proteins channels are a well-known example of this category.

All these processes could be active and passive depending upon the electrochemical gradient, which is already mentioned in detail. Most of the transporter molecules perform all these transportation either a channel mechanism or a carrier mechanism. Channel basically provides an alternative pathway by providing a hydrophilic pathway through which the transporter could avoid the hydrophobic lipid interaction. Whereas the mobile carrier is typically ionophore or small organic molecules that translocate within the membrane where it binds with the ions at the polar leaflet of the membrane and carry them across the hydrophobic membrane interior by simple diffusion and then release them on the opposite leaflet of the membrane in response to the electrochemical gradient.¹²

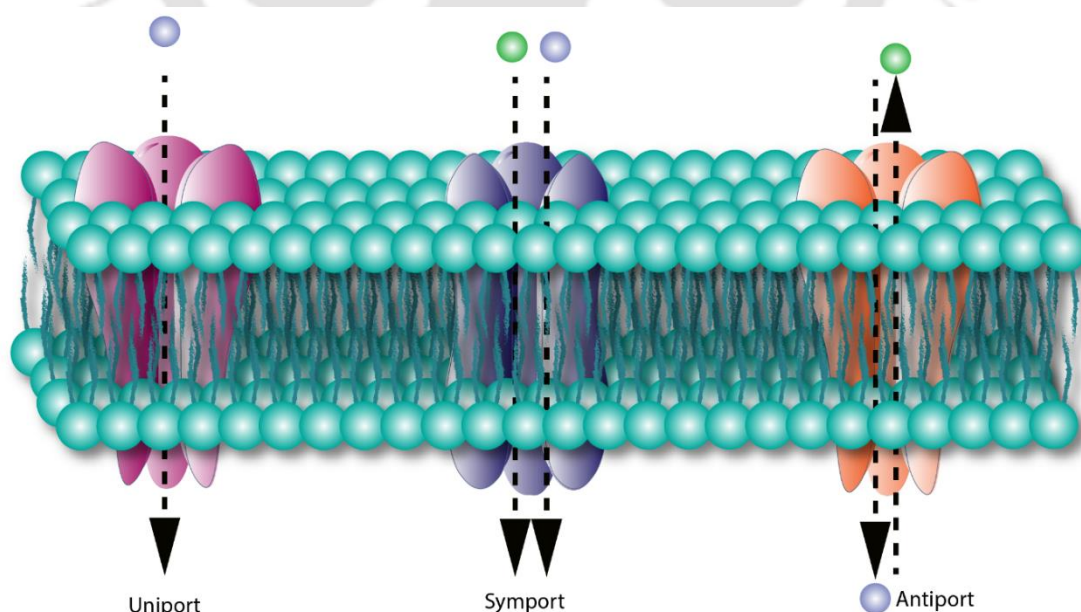


Figure 1.4. Different types of transportations pathway

1.3. Ion transport and cell death

It has been well accepted that natural ion transporters are associated with various cellular mechanisms responsible for cell proliferation and apoptosis; hence, their altered function is usually paralleled by apoptosis. Apoptosis is a distinctive programmed cell death process characterized by special morphological features like cell shrinkage, membrane blebbing, nuclear fragmentation, chromatin condensation, the formation of smaller apoptotic bodies with the scrambled cell membrane, and rapid engulfment by the surrounding cells. Apoptosis maintains the balance between cell proliferation and cell death by eliminating the potentially detrimental, damaged, senescent, or infected cells from the body. Any deregulation of this orderly mechanism could lead to excess un-dead cells, which could lead to several disease conditions like tumor growth, viral infections, and autoimmune diseases. One of the hallmarks of cancer is the attenuation of apoptosis of tumor cells, thereby allowing them to progress to the advanced stages of malignancy, activating invasion and metastasis and developing resistance towards any kind of therapy. The inhibition of apoptosis has been cited as one of the primary reasons for tumor growth, which imposes physiological restraints on apoptotic signaling pathways to escape from the immune response. Therefore, selective reactivation of apoptosis is considered a potential therapeutic approach to combat cancer. Apoptosis in cancer cells could be induced by targeting either the death receptor (extrinsic) pathway or the mitochondrial (intrinsic) apoptosis pathway.¹³ Apoptosis is generally marked by cell shrinkage, whereas an increase in cell volume parallels cell proliferation.¹⁴

The transmembrane transport of ions through ion channels plays a crucial role in regulating cell proliferation and apoptosis. Protein-based natural ion channels like Ca^{2+} channels, K^{+} channels, and Cl^{-} channels play a crucial role in various cellular processes. The regulation of transmembrane Ca^{2+} ion homeostasis by pro- and antiapoptotic proteins enables the Ca^{2+} -signaling process to regulate a wide variety of physiological events, especially apoptosis.¹⁵ Various Ca^{2+} -dependent apoptotic stimuli such as oxidative stress, ceramide, and arachidonic acid promote the release of Ca^{2+} ions from the endoplasmic reticulum, leading to the activation of apoptotic pathways, including the release of cytochrome *c* from the mitochondria.¹⁶ The flux of K^{+} , Na^{+} , and Cl^{-} ions is responsible for cell shrinkage, and it has been demonstrated in various studies that this ion flux is a requisite for apoptosis. Generally, the intracellular concentrations of Cl^{-} (5–40

mM) and Na^+ (10–30 mM) ions are much lower than their extracellular concentrations (120 and 142 mM, respectively), whereas the intracellular concentration of K^+ (140 mM) ions is much higher than their extracellular concentration (4 mM). Maintenance of the concentration gradients of these monovalent ions across the bilayers upholds the cell size and regulates the viability of the cells. K^+ channels are one of the key players of apoptosis, given the fact that the K^+ ions greatly influence the membrane potential and regulate cell volume in an isotonic environment, which are the hallmarks of apoptosis. Recent studies have reported that the efflux of K^+ ions and reduced intracellular K^+ ion concentration induces apoptosis. The activation of caspases and the apoptotic nuclease activity is also responsive to the K^+ ion concentration; however, once they are activated, the fluctuation of K^+ ion concentration could not control their activity.¹⁷ Other monovalent ions like Na^+ are also involved in the apoptotic volume decrease, resulting in the degradation of genetic material and, eventually, cell death. However, the efflux of K^+ ions does not solely guide the influx of Na^+ ions, suggesting the fact that separate ionic pathways do exist for the K^+ and Na^+ ions during apoptosis. The use of saxitoxin, a sodium ion channel blocker, prevents apoptotic cell death exemplifying the fact that the Na^+ ion not only assists the other monovalent ions in maintaining appropriate ion homeostasis and regulating the cell size, but its concentration also plays a pivotal role in initiating the signals for the apoptotic machinery. The Cl^- ion is also highly abundant in all organisms. K^+ efflux is inseparably related to apoptotic volume decrease (AVD), and it is also demonstrated that to drive the efflux of water, which leads to cell shrinkage, Cl^- ion usually follows K^+ ion efflux, thereby maintaining the charge neutrality of the process. In response to several apoptotic stimuli, various volume-regulated anion channels (VRACs) also get activated, which results in the efflux of Cl^- ions in the pre-apoptotic phase. The AVD under normotonic conditions is accompanied by regulatory volume decrease (RVD), which is accomplished with the help of both K^+ and Cl^- channels. Hence, quite expectedly, it was found that blocking the volume regulatory K^+ and Cl^- channels did not allow the cells to show any morphological features characteristic of apoptosis such as caspase activation, cytochrome *c* release, and DNA laddering, and hence the cells were rescued from cell death.¹⁸⁻¹⁹ The VRACs are also triggered by both cell swelling and increased production of reactive oxygen species (ROS).²⁰ During AVD, these VRACs get activated and instigate a variety of cellular processes, even in non-swollen cells. Pharmacological inhibition of

these channels leads to the prevention of AVD in many cell types. Several other channel-mediated Cl^- flux processes are also involved in apoptosis. The modulation of Cl^- flux is reported to damage the intrinsic pathways of apoptosis initiation, whereas it has a negligible effect on the extrinsic pathway of apoptosis. Volume-sensitive outwardly rectifying (VSOR) chloride channels are primarily involved in the transportation of Cl^- ions during apoptosis.²¹

Cells have primarily industrialized a defensive mechanism for maintaining cell volume and ion homeostasis across their membranes in response to the changes in ion concentration in the intra- and extracellular environments. Therefore, the deregulation of the natural ion transport system leads to various ion channel-related diseases, including cystic fibrosis and cancer. In this regard, synthetic ion channels and carriers have been developed to combat dysregulated ion transport-related diseases. Recently, several research groups have explored the ion transport properties of synthetic ionophores in the cellular environment.^{6-7, 22-23} Even a few studies have also demonstrated the activities of the synthetic ionophores under *in vivo* conditions. Although the investigation of ionophore arbitrated biological activities is in its early stage of development, it has tremendous therapeutic potential. The synthetic ionophores showed Cl^- ion transport efficiency across the membranes of epithelial cells with functionally faulty CFTR channels. The treatment with the synthetic ionophores also led to the induction of apoptosis in cancer cells. Hence, synthetic ionophores have the potential to fight against cancer, cystic fibrosis, and other ion transport-related diseases. In general, the ionophores are hydrophobic in nature and function within the lipid bilayer, and they do not bind to any bio-macromolecules like DNA, proteins, or enzymes but regulate ion transport to induce apoptosis. In this regard, the synthetic ionophores could overcome the perturbations due to the mutations and over-expression of genetic material and proteins/enzymes and established as an unorthodox approach for numerous pathological conditions. So based on this tremendous applicability to the healthcare system, scientists across the globe have been exploring numerous natural as well as artificial anion transporter molecules and trying to explore their numerous pharmacological efficacy. So within this chapter, several natural product-based anionophores and their biological applications as an anti-proliferative agent are summed up. Besides, numerous artificial anionophores having therapeutic prospects are also discussed.

1.4. Natural product-based anionophores

Prodigiosin is the first alkaloid-based transmembrane anion transporter (Figure 1.5), which shows the dissipation of the pH gradient in both liposomal and cellular environments.²⁴ The transport of the H^+ / Cl^- ion-pair by prodigiosin is driven by both electrostatic interaction and hydrogen bonding. Recent studies have revealed that the anion transport activities of prodigiosin are associated with its numerous biological properties, including immunosuppressive, antibacterial, antifungal, and anticancer properties. The cytotoxic effect of prodigiosin has led to the development of obatoclax, a synthetic analogue of prodigiosin. Ion-selective electrode (ISE)-based assays in the liposomal environment showed that obatoclax and its analogs transport anions *via* the Cl^-/HCO_3^- antiport pathway. Although obatoclax shows anion transport and potential anticancer activities, its mechanism of action is debatable.²⁴⁻²⁵ Quesada and co-workers developed a series of obatoclax derivatives (Figure 1.5).²⁶ Compounds **1.1c–1.1e** were synthesized to investigate their hydrogen bonding capabilities with anions. *N*-Boc protected compound **1.1d** was developed, assuming that the Boc protected indole moiety would not be available for interaction, and the bulkiness of the Boc group would also create hustle in the anion binding pocket. For the same reason, they also developed BODIPY derivative **1.1e**, wherein the hydrogen bond donor, the dipyrromethane motif, is not available for anion binding. Cellular activity studies showed that these potent compounds deacidify the acidic organelles to induce cell death in small-cell lung carcinoma cells (GLC-4). The concentration dependent cell viability assay showed that obatoclax was the most potent cytotoxic derivative against the GLC-4 cell line. The higher viabilities of the GLC-4 cells in the presence of compounds **1.1d** and **1.1e** were well supported by their reduced transmembrane Cl^- ion transport activities.²⁶ Meanwhile, marine alkaloids, tambjamines, showed anion transport arbitrated anticancer activities.²⁷ Structurally, the 2, 2' -bipyrrolic ring system present in tambjamines is closely related to prodigiosin. Its synthetic convenience led to the development of a series of tambjamine derivatives (Figure 1.6).²⁸ Bio-physical studies revealed that the tambjamines do not quickly get deprotonated under physiological conditions, and their transport mechanism follows the Cl^-/HCO_3^- exchange pathway. Cellular activity studies revealed that the 2, 2' -bipyrrole-based potential anion transporters deacidify the acidic organelles to induce cell death in GLC-4 cancer cells. The

reduced anion transport properties and anticancer activities of tambjamines (**1.2a-d**) could be due to their poor lipophilicity and weaker deacidifying ability.

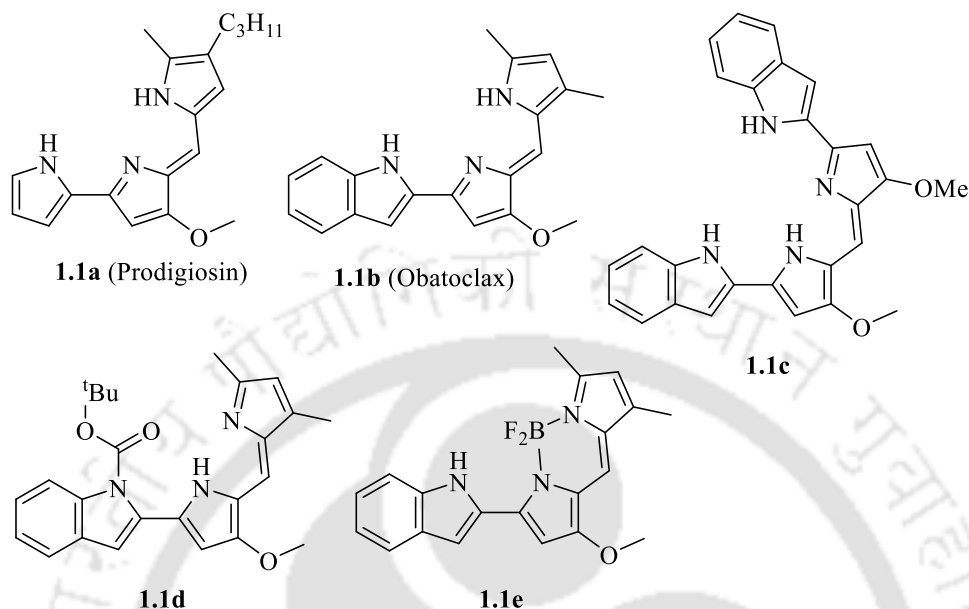


Figure 1.5. Prodigiosin-based anionophores.

Hence, the anionophoric activities of tambjamine derivatives are directly related to their capability of deacidifying the acidic cellular organelles.²⁸ Encouraged by the biological activities of tambjamines, Quesada and co-workers also developed tambjamine analogs **1.2e -1.2h** and explored their anion transport mediated anticancer activity (Figure 1.6).^{6, 28} The bipyrrrole-enamine ring containing synthetic tambjamines showed anion transport-dependent cytotoxic activities in various cancer cell lines. The potent synthetic tambjamines **1.2e** and **1.2g** showed higher anticancer activities with IC₅₀ values of 1.10 to 2.49 mM against human melanoma (A375) and mammary adenocarcinoma (MDA-MB-231) human cancer cell lines. Further biological studies by Soto-Cerrato and co-workers revealed that tambjamine analogue **1.2i** disturbs the anionic balance and alters the mitochondrial membrane potential (MMP), and induces mitochondrial autophagy in the human lung cancer cell line (A549).²⁹ The compound interceded mitochondrial autophagy was confirmed by the induced expression level of autophagy markers LC3II and p62. The cellular stress generated in the compound **1.2i** treated A549 cells also instigates the p38 mitogen-activated protein kinase (MAPK) pathways, which results in apoptotic cell death.

However, the addition of a universal caspase inhibitor in the compound **1.2i** treated A549 cells was not sufficient enough to recover the cell viability, suggesting that apoptosis may not be the only pathway of cell death for the compound **1.2i** treated cells. Necrosis may be one of the other possibilities of compound **1.1n** arbitrated cell death.

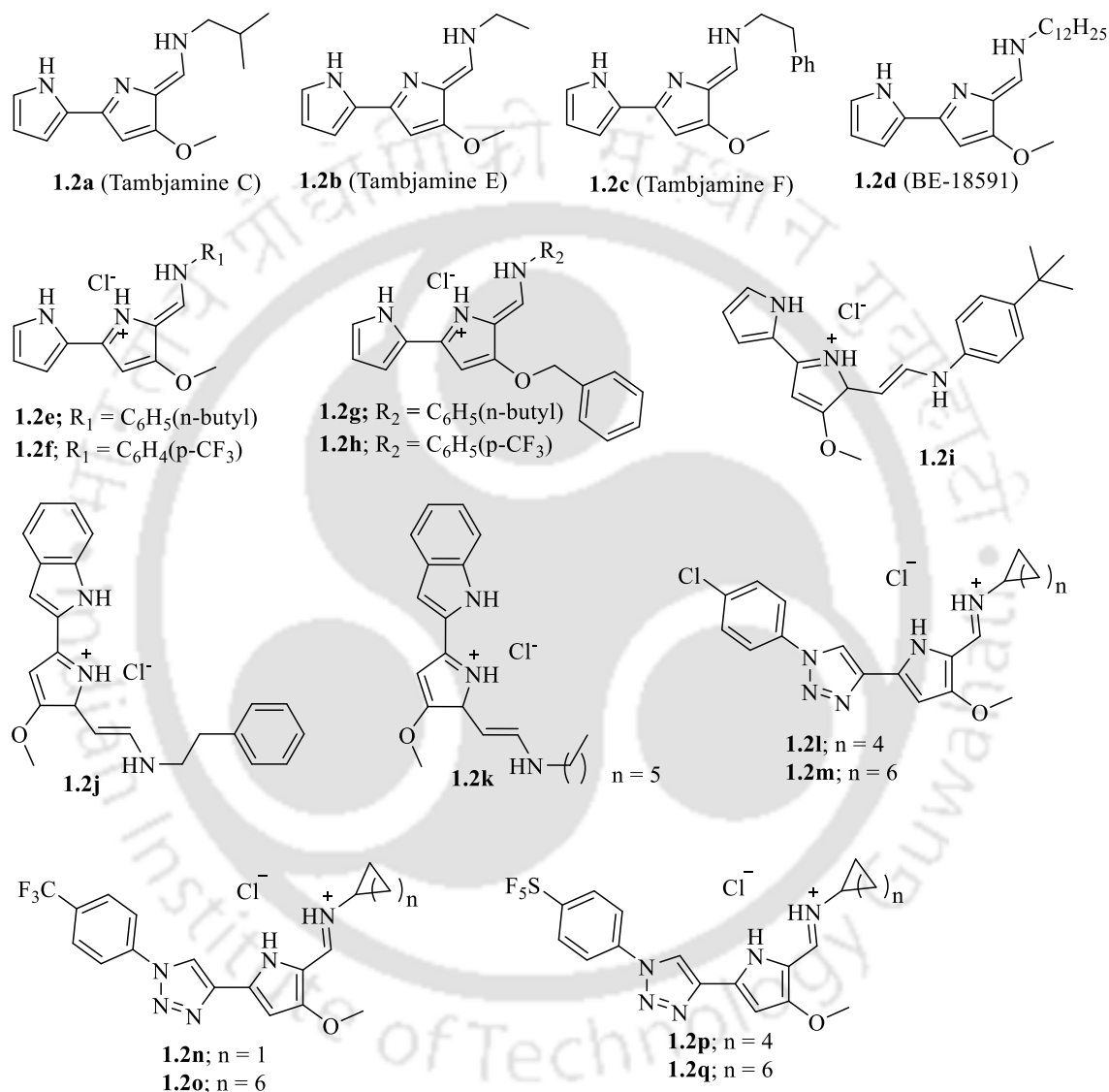


Figure 1.6. Tambjamine-based anionophores.

The other synthetic analogues of tambjamines **1.2j** and **1.2k** were successfuls in inducing the differentiation and elimination of highly active lung cancer stem cells (CSC).³⁰ Recently, Quesada and co-workers have reported a new class of tambjamine derivatives **1.2l–1.2q**, wherein the 4-methoxy-2, 20 -bipyrrole moiety of the natural tambjamine was

replaced by a 1,2,3-triazole ring (Figure 1.6).³¹ The anion transport activities of these compounds were investigated in the cellular environment. A variant of the iodide-sensitive yellow fluorescent protein (YFP) was transiently transfected in Fisher rat thyroid (FRT) cells. The YFP variants (YFP-H148Q/ I152L) are halide sensitive, and accordingly, chloride ion transport across the cells was detected in real-time using the fluorescence quenching of YFP. The investigation of the viability of human lung (A549) and breast (MCF7) adenocarcinoma cell lines, as well as the non-cancerous human mammary epithelial (MCF-10A) cell line, showed that these triazole containing tamjbamine derivatives induce anion transport mediated cell death.³¹ Inspired by the biological activities of the natural product-based ionophores, Gale and co-workers synthesized perenosins and investigated their ion transport mediated cell death-inducing properties. The naturally abundant pyrrole-based anion binding moiety is linked with the indole, benzimidazole, or indazole scaffold through the imine bond (Figure 1.7).³² Initial ISE and fluorescence-based studies revealed that the perenosins transport anions *via* the $\text{Cl}^-/\text{NO}_3^-$ antiport mechanistic pathway. Structure–activity relationship studies revealed that the acidity of the N–H proton and the lipophilicity of the molecules direct the anion transport behaviour of the perenosins. The benzimidazole and indazole containing perenosins displayed poor transmembrane Cl^- ion transport properties. However, pH-dependent anion transport studies revealed that the potent perenosins had higher Cl^- ion transport ability in the acidic environment (pH 6.2) than that under physiological conditions, which could be due to the augmented level of protonation. Additional mechanistic studies demonstrated that the perenosins function as HCl cotransporters in acidic medium and $\text{Cl}^-/\text{NO}_3^-$ antiporters under physiological conditions. Cell viability studies were performed using both normal and cancerous cell lines to investigate the anticancer activities of the perenosins. Indole-based perenosin molecules **1.3a–1.3e** showed higher viabilities in the MCF-10A cell line but lower viabilities in breast carcinoma model cell lines MDA-MB-231 (invasive) and MCF7 (non-invasive). Perenosin **1.3d** exhibited higher selectivity (~5.5 fold) for cancerous cell lines than the normal cell line. Benzimidazole derivative **1.3f** showed higher viabilities and lower selectivity against the tested cell lines. In 2017, Gale and co-workers again introduced a new family of perenosins motivated by the biological activities perenosin **1.3d**. They synthesized perenosins **1.3h–1.3l** and **1.3m–1.3n** using indole-pyrrole and bis-indole scaffolds (Figure 1.7).

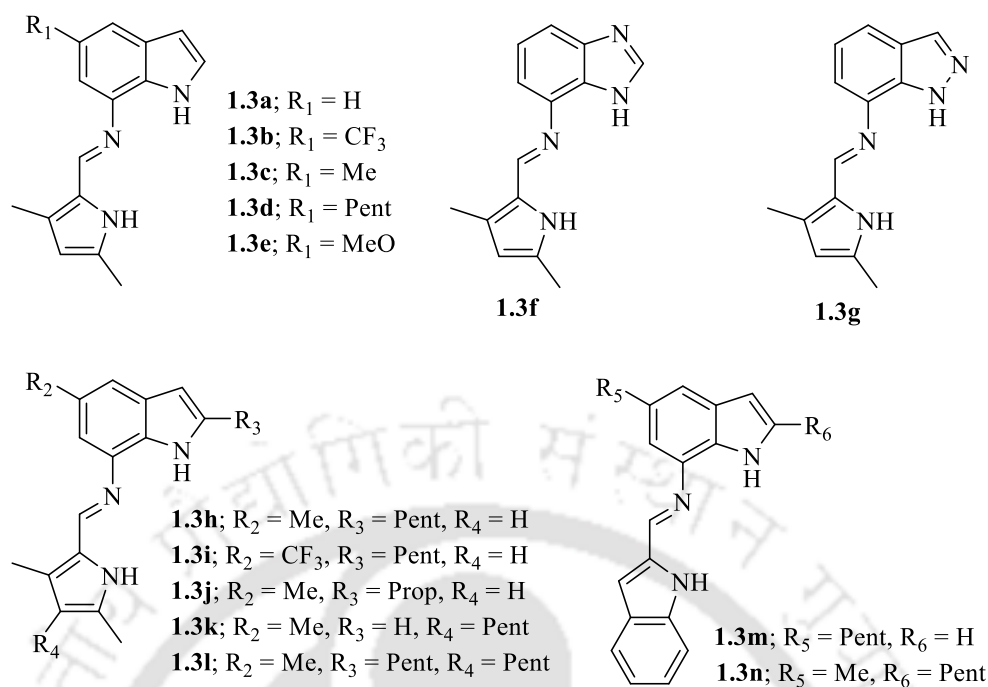


Figure 1.7. Perenosin-based anionophores.

The comprehensive ion transport studies of perenosins **1.3h–1.3n** confirm their HCl cotransport mechanism like prodigiosin. Initial screening showed that perenosins **1.3h–1.3n** promote cell death in five different cell lines (cancer cells MCF7, MDA-MB-231, A549, and SW620 and non-cancerous human cells MCF-10A). Time-dependent cell viability studies revealed that the second generation perenosins **1.3h–1.3n** exhibited an apparent structure-activity relationship for the onset of the cytotoxic effect. The compounds having alkyl substituents at R_3 (**1.3h–1.3j** and **1.3l**) and R_6 (**1.3n**) were shown to have a negligible effect on cell viability ($IC_{50} > 100$ mM). Interestingly a significant late-onset of the cytotoxic effect affecting the cell viability after 72 h was observed, with perenosin **1.3l** being the most potent compound to induce MDA-MB-231 cell death ($IC_{50} = 4.2$ mM after 72 hours). Perenosin **1.3k** having a pentyl substituent at R_3 showed cell viability effects similar to those of perenosin **1.3d**. The bis-indolebased perenosin **1.3m** with a pentyl substituent at R_1 showed a moderate degree of late-onset. Although the potent imine-based perenosins showed lower cell viabilities in breast cancer cells than that against normal breast cells, the imine moiety quickly gets hydrolyzed under physiological conditions. Hence, further studies are required to comprehend the stability and anion transport interceded biological activities of the imine-based perenosins.

1.5. Artificial Anionophore

1.5.1. Urea and thiourea based anionophores

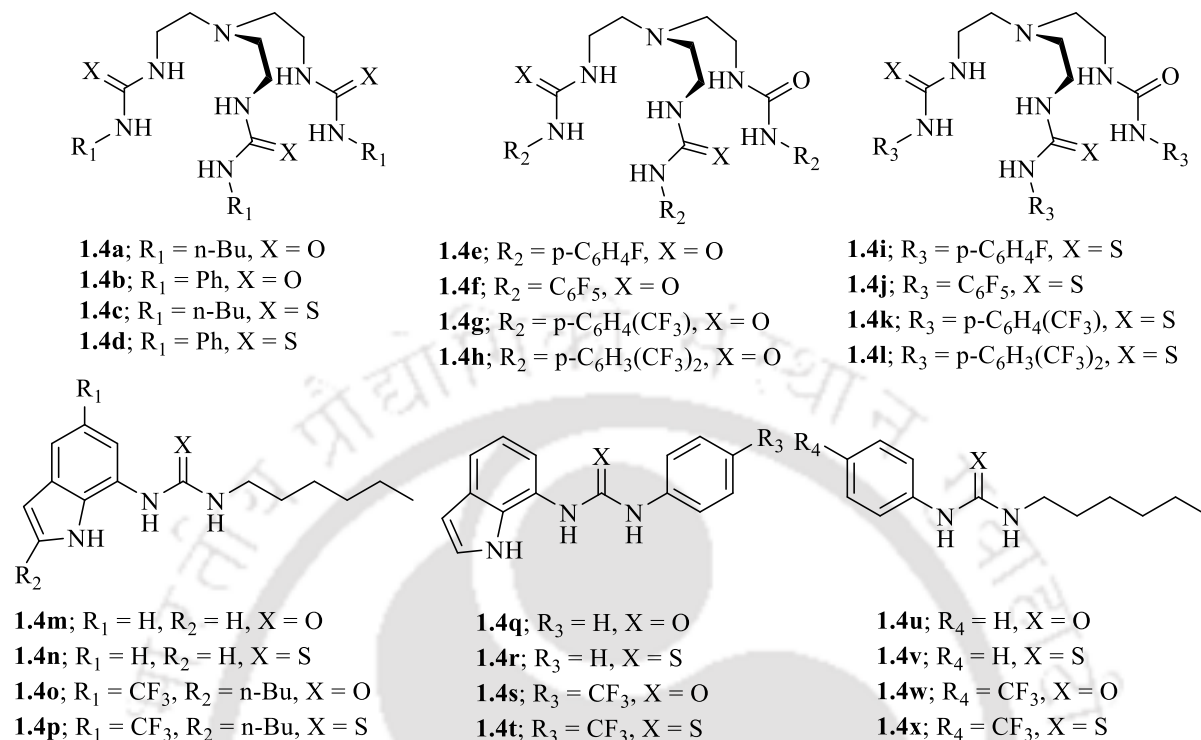


Figure 1.8. Tripodal and Indolyl containing urea/thiourea-based anionophores.

In 2010, Gale and co-workers developed simple TREN-based trisureas and thiourea as **1.4a-1.4d** and investigated their anion transport facilitated biological activities (Figure 1.8).³³ A similar class of compounds was already known to bind various anions both in solid and solution states.³³⁻³⁵ The fluorescence-based anion transport measurements confirmed that the TREN-based tris thioureas **1.4c** and **1.4d** were effective $\text{Cl}^-/\text{NO}_3^-$ and $\text{Cl}^-/\text{HCO}_3^-$ antiporters. In contrast, the urea analogues **1.4a** and **1.4b** had significantly reduced or no anion transport activity (Figure 1.8).³⁵ To improve the anion transport efficiencies, they also synthesized aryl-substituted TREN-based trisureas and thioureas (Figure 1.8).³⁵ The use of fluorine or trifluoromethyl substituents is considered as a potential approach in developing effective ionophores. The fluorine or trifluoromethyl substituents both increase the lipophilicity of the compounds and acidity of N-H proton of the urea or thiourea moieties.³⁶ The potent ionophores showed measurable anion transport abilities at transporter to lipid ratios of 1:250000. The biological activity studies showed that the potent TREN-based ionophores **1.4j** and **1.4l** induce cytotoxicity in various cancer

cell lines. The anion transport aptitude of the fluorinated tripodal carriers resulted in the alterations in the intracellular pH to induce apoptosis in cancer cells.³⁷ Inspired by the anticancer activities of tripodal trithioureas, Gale and co-workers developed indolylureas/thioureas **1.4m-1.4t** and structurally related ureas/thioureas **1.4u-1.4x** (Figure 1.8).³⁸⁻⁴⁰ The transmembrane anion transportability and biological activity studies were thoroughly investigated. The compound having the trifluoromethyl substituents exhibited higher anion transport activity than their non-fluorinated analogues, which could be due to the collective effect of lipophilicity and improved anion affinity. The anion transport data prompted them to probe into their behavior under the cellular environment. The viabilities of the compounds **1.4m-1.4x** were tested on various cancer cell lines such as GLC-4, A375, human colon adenocarcinoma (SW480), and human oral adenosquamous carcinoma CAL27 cell lines. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (single-dose screening) showed that the receptors **1.4o**, **1.4p**, **1.4s**, **1.4w**, and **1.4x** have significant cytotoxicity, especially in GLC4 and A375 cells. In contrast, no notable cytotoxic effect was observed after the treatment with other receptors. The dose-dependent MTT assay exhibited that the IC₅₀ values of the potent compounds were around 10 μ M in GLC-4 cells. Interestingly these compounds displayed a lower cytotoxic effect in MCF-10A cells in comparison with that of cancerous cells, signifying some cytotoxic selectivity of these receptors towards cancerous cells.

1.5.2 Orthophenyl/cyclohexyl based derivatives

The *ortho*-phenylenediamine scaffold has been successfully employed to recognize and transport anions across the membranes. To investigate the role of the anionophores in the anticancer activities, Gale, Davis, and co-workers synthesized a series of urea and thiourea-based derivatives (Figure 1.9).⁴¹ They also explored the effect of different substituents of the central aromatic moiety. Anion transport activities of the *ortho*-phenylenediamine derivatives were minutely investigated using the halide-sensitive fluorescent dye lucigenin under the liposomal environment. The *ortho*-phenylenediamine derivatives (**1.5a** and **1.5b**) were found to be active anion transporters and displayed great potential to reduce the viability of the cancer cell lines. The elaborate investigation also confirmed that it has a tendency to depolarize the acidic compartments, and as a consequence, it promotes apoptosis.⁴¹ In 2020, Gale and co-workers also prepared a series

of *ortho*-phenylene bisurea derivatives (Figure 1.9).⁴² The effects of fluorinated central aromatic moiety and thiophene or benzothiophene containing the urea motif on the anion transport activities were investigated (**1.5e-s**). The chloride-ion-selective-electrode was used to measure the anion transport activities of these compounds under the liposomal environment. The effective carriers in all biophysical assays were found to be compounds with fluorinated central aromatic moiety.

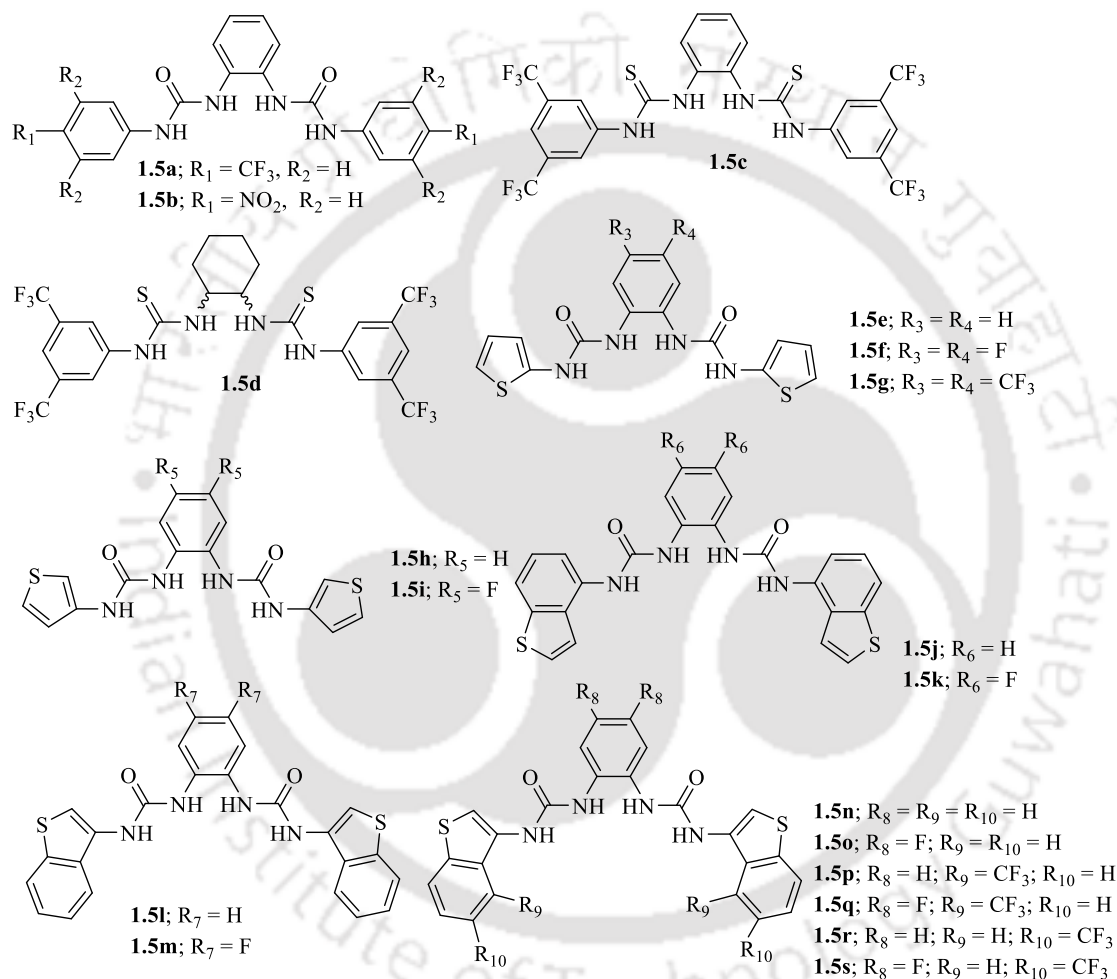


Figure 1.9. *Ortho*-phenylenediamine based anionophore.

The benzothiophene containing compounds **1.5k** ($\text{EC}_{50} = 0.0194 \text{ mol } \%$) and **1.5m** ($\text{EC}_{50} = 0.0089 \text{ mol } \%$) were identified to be the most active carrier of Cl^- ions. The interactions of the C–H proton of the benzothiophene moiety substantially contributed to the superior anion transport efficiency of compounds **1.5k** and **1.5m**. The pH-dependent 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) assay in the presence of base-pulse

demonstrated that the transport of H^+/Cl^- ion-pair is the primary operating anion transport pathway. The dose-dependent cell viability assay showed that the potent anion carriers have only moderate to low cytotoxicity towards cancer cell lines like MCF-7, A549, and HeLa and human CF bronchial epithelial cell CFBE.⁴² Compounds **1.5k** ($EC_{50} = 0.0194$ mol %) and **1.5m** ($EC_{50} = 0.0089$ mol %) were identified to be the most active carrier of Cl^- ion. The interactions of C-H proton of the benzothiophene moiety substantially contributed to the superior anion transport efficiency of the compounds **1.5k**

1.5.3. Benzimidazole based ion transporter

Chen and co-workers developed various benzimidazole-based anionophores and explored their anion transport-facilitated therapeutic implication through different *in vitro* experiments. Initially, they synthesized a series of 1,3-bis(benzimidazole-2-yl)benzene derivatives **1.6a-f** (Figure 1.10).⁴³⁻⁴⁴ Comprehensive biophysical studies, including HPTS and lucigenin assays, revealed that compound bearing benzimidazole moieties with either nitro or trifluoromethyl groups showed superior anion transport efficiency. To improve further the anion transport efficiency of the benzimidazole-based anionophores, they synthesized benzimidazole derivatives **1.6g-k** (Figure 1.10), where they incorporated electron-withdrawing nitro group at the 5-position of the central aromatic ring (**1.6g-1.6i**) or electron-donating 5-methoxy groups (**1.6j** and **1.6k**). Compound **1.6i** bearing nitro group in the central aromatic ring and benzimidazole moieties with a nitro group showed superior anion transport efficiency ($EC_{50} = 0.0027$ mol %) and followed the H^+/Cl^- synergistic pathway. The MQAE-based cellular assay showed that potent compounds also transport Cl^- ion under the cellular environment. The cell viability studies of compounds **1.6a-1.6k** against various cancer cell lines (HeLa and MCF-7) revealed that electron-withdrawing groups on the benzimidazole moieties do not always enhance the cytotoxicity, suggesting that the cytotoxic effect is not merely due to the anion transport behavior of the compounds. Whereas, compound **1.6h**, bearing the nitro group in the central aromatic ring and benzimidazole moieties with trifluoromethyl group, were most cytotoxic among the tested benzimidazoles. The cytotoxicity of compound **1.6h** was reduced in chloride-free buffer solution, supporting its anion transport mediated cytotoxic properties.

Supplementary cellular studies established that compound **1.6h** induces the release of cytochrome c from mitochondria, which results in apoptotic cell death. The same research group also synthesized benzimidazole containing compounds **1.6l-w** (Figure 1.10) to investigate the role of lipophilicity on the transportation of anions. The outcome of the transport measurements also revealed that the anion transport activity of these compounds is governed by the anion-binding affinity and its lipophilicity.⁴⁴ They observed that the installation of fluorine atoms at the 5-position of the central phenyl ring could significantly improve the anion transport activity and cytotoxicity. In contrast, the total number of fluorine atoms on the benzimidazole units has little effect on cytotoxicity. The potent compound **1.6w** also disrupts MMP and induces apoptosis in the cancer cell.

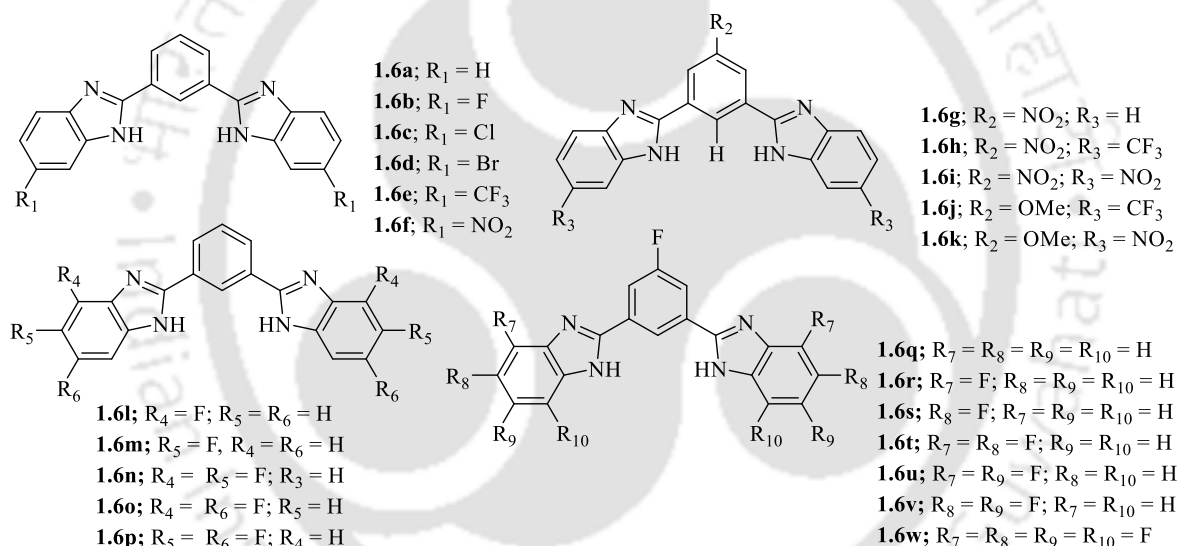


Figure 1.10. Benzimidazole based anionophores.

1.5.4. Squaramide based anionophores

Squaramide is considered as bioisostere of urea and thiourea moiety, and it has been extensively used in many applications of chemistry and related research areas, including supramolecular and medicinal chemistry. The higher affinity for hydrogen bonding of squaramides are governed by their concomitant increase in aromaticity.⁴⁵ It is already documented that squaramide-based compounds possess

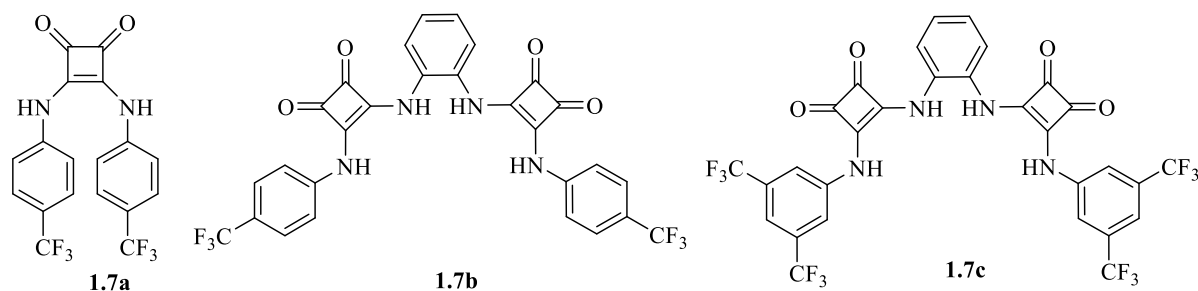


Figure 1.11. Squaramide based anion transporter molecules.

interesting anion-binding properties. Shin, Gale, Sessler, and co-workers extensively studied the anion transport mediated biological activities of the squaramide derivatives (Figure 11). Squaramide **1.7a** and bis squaramide derivatives of *ortho*-phenylenediamine **1.7b** and **1.7c** were validated to increase the transport of Cl^- ion under the liposomal environment.²² *In vitro* studies revealed that an increase in the transport of Cl^- ion by these anionophores and the concomitant influx of Na^+ ions through sodium channels results in the intensification of the intracellular concentrations of Cl^- and Na^+ ions in FRT cells. The dose-dependent viability studies showed that the squaramides **1.7a-c** induce cytotoxicity (IC_{50} values were 2-6 μM) in several cancer cell lines, including HeLa and A549 cells. The cytotoxicity of these compounds was substantially reduced in cancer cells incubated with Cl^- and Na^+ ions free buffer solutions. The use of sodium channel blockers like amiloride also reduced the cytotoxicity of these compounds, which could be due to the reduction of compound mediated transport of Cl^- ion to maintain the charge balance. Gale and co-workers also thoroughly investigated the biological activities of squaramide **1.7a**.^{22, 46} They observed that squaramide **1.7a** has the potential to increase the pH of lysosomes, which reduces the activities of cathepsin B and L and eventually affects the normal function of lysosomes resulting in the disruption of autophagy and induce apoptosis. The western blot analysis of the HeLa cell revealed the upregulation of LC3-II and p62 proteins levels, indicating that the squaramide **1.7a** interferes with autophagy. Several cellular activity studies also ascertained that the squaramides **1.7a-1.7c** promote the release of cytochrome c from mitochondria, which leads to caspase-dependent apoptotic cell death. Overall, the extensive experimental data support

that these ion transporters disrupt autophagy and induce apoptosis by perturbing cellular chloride concentrations.

1.5.5. Numerous other potent anionophores

The influence of anion transport mediated biological activities of the calixpyrroles were assayed by Gale and colleagues.⁴⁷ They hypothesized that the diamide functionality present in the pyridine strapped calix[4]pyrrole (C4P) would provide a useful ion-pair recognition site (Figure 1.12). The ion transport measurements under the liposomal environments revealed that the potent C4P derivative **1.8a** is an electrogenic transporter. However, the octafluorinated calix[4]P is both electrogenic transporter and H^+/Cl^- cotransporter. The transport studies in YFP expressed FRT cells displayed that the influx of Cl^- ion was more efficient for compound **1.8a**. The viability studies of the compounds in various cancer and normal cell lines (A549, pancreas adenocarcinoma cells (Capan-1), colorectal carcinoma cells (HCT116), HeLa, human fetal lung fibroblast cells (MRC-5), normal rat kidney cells (NRK), human fetal prostate epithelial cells (267B1)) showed moderate cytotoxicity with IC_{50} values in the range of 10-15 μM . The involvement of sodium ions was also confirmed using the sodium fluorescent probe sodium binding benzofuran isophthalate acetoxymethyl ester (SBFI-AM). Further, the detailed mechanistic studies demonstrated that compound **1.8a** mediates the transport of Cl^- and concomitantly the Na^+ influx in cellular milieus.⁴⁷ The potent compounds also induced anion transport mediated osmotic stress and ROS generation, which resulted in caspase-mediated apoptosis. Further studies revealed that the potent compounds **1.8b** also induced the cytosolic concentration of Ca^{2+} ions via the activation of calcium channel by inositol triphosphate (IP3R). The compound **1.8b** obstructed autophagy by persuading an escalation of lysosomal pH.

In 2016, Talukdar's group developed bis(sulfonamides)-based anionophores and thoroughly investigated the transmembrane transport mediated cellular activities (Figure 1.12).⁶ The HPTS-based fluorescence studies in the presence of pH-gradient proved that the transport of anion is greatly influenced by the lipophilicity of the receptor and follows the Cl^- /anions antiport process. Encouraged by the Cl^- ion transport activity of the potent bis(sulfonamide) derivatives, the authors measured the cytotoxicity of the compounds in MCF7,

mouse embryonic fibroblast cells (NIH3T3), human osteosarcoma cell line (U2OS), HeLa, and A549 cell lines. The MQAE assay in HeLa and MCF7 cells directly proved the Cl^- ion transport efficiency of compound **1.8c** (Figure 1.12). The MTT assay in chloride-free buffer solution showed that cytotoxicity of the compounds is directly related to their Cl^- ion transport activities. Further cellular studies revealed that the interference of cellular ionic homeostasis by the bis(sulfonamide) derivatives led to the disruption of MMP, which successively instigated the discharge of cytochrome *c*. The western blot analysis showed the bis(sulfonamide) derivative **1.8c** induces the expression of caspases and cleaved PARP-1, which resulted in mitochondria-dependent apoptosis (Figure 1.12).⁶

The development of artificial ion channels has also been motivated by the prospective application of these supramolecular arrangements for the treatment of ion transport-related diseases. Talukdar and colleagues recently demonstrated that bis-diol-based compounds self-assembled within the membrane bilayer (Figure 1.12).⁴⁸ The intermolecular hydrogen bonding among the hydroxyl groups and conformationally constrained 1,3-diethynylbenzene scaffold of the bis-diol **1.8d** could be the driving for the formation of the ion channel. The HPTS-based fluorescence assay in the presence of a pH gradient showed that the active compound **1.8d** transport anions via the Cl^-/OH^- exchange process. The measurement of electrical conductance across the planar lipid bilayer membrane proved that the bis-diol **1.8d** formed an ion channel within the lipid bilayer. The authors also performed thorough biological studies to understand the importance of anion transportation under cellular conditions. The viability studies showed that the bis-diol **1.8d** induces cytotoxicity in several cancer cell lines, including A549, HeLa, MCF7, NIH3T3, and U2OS. The cytotoxicity of compounds was significantly lowered in chloride-free buffer solution, which is in accordance with its anion transport activities (Figure 1.12). The immunoblot assay of the HeLa cell lysate showed the bis-diol **1.8d** induces apoptosis via a caspase-dependent pathway (Figure 1.12). Although there is no significant difference in the compound-mediated cytotoxicity between the normal and cancer cells, the generation of artificial ion channels could be a potential tool to fight against ion transport-related diseases. Their systematic studies showed that the transport of Cl^- ion by the anionophore

disrupted the MMP leading to the generation of ROS and induced the release of cytochrome c, which activates the caspase-dependent pathway of apoptosis (Figure 1.13).

Chen and colleagues reported iridium complexes **1.8e** and **1.8f** could transport anion primarily via the anion exchange mechanism (Figure 1.12).⁴⁹ These complexes have the potential to increase the pH from 4.6 to 6.8 of the lysozymes, and this increased in pH level hamper the proteolytic enzymes like cathepsin B. In addition, they also observed that the expression levels of LC3-II and p62 protein are also up-regulated after being treated with complex **1.8e** or **1.8f**. Additional cellular studies showed that the complexes persuade caspase-independent cell death

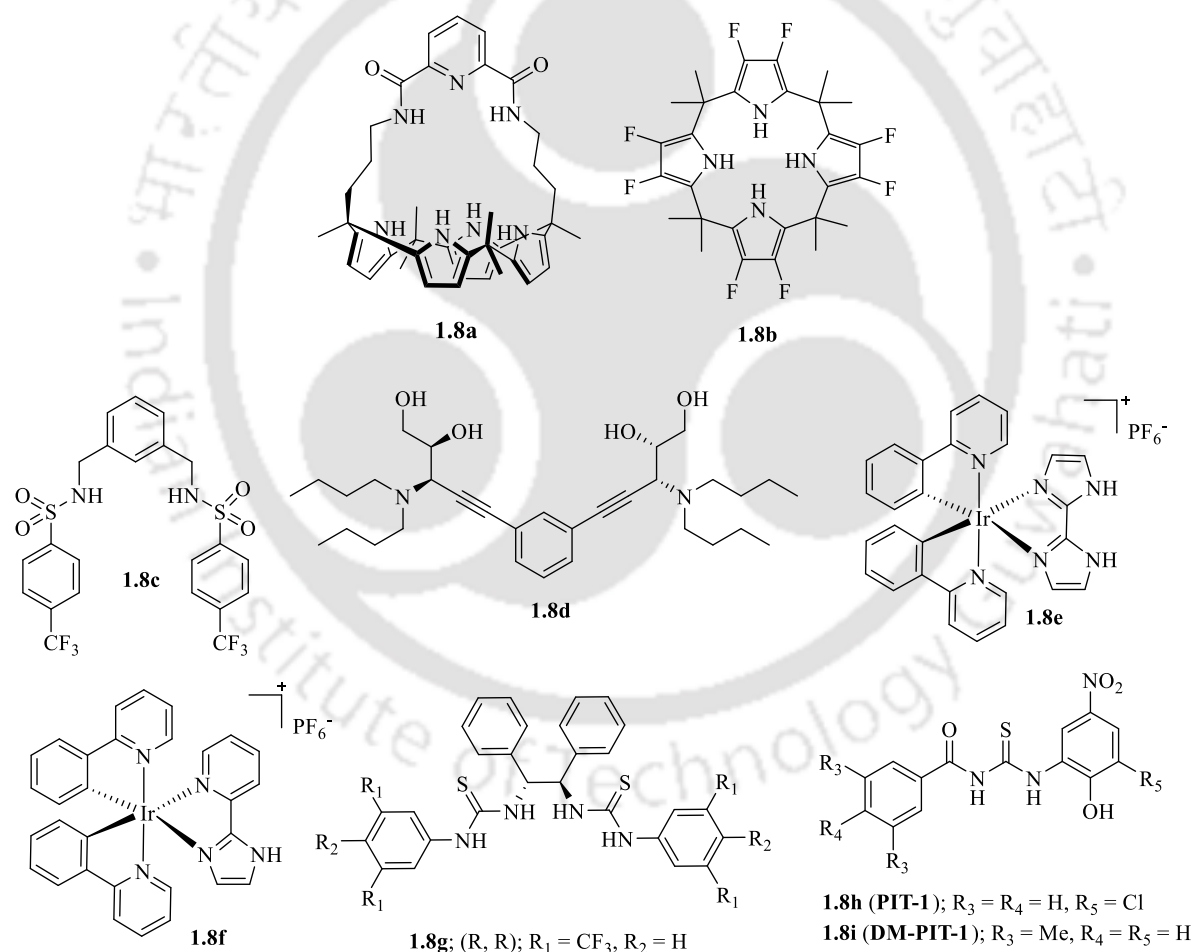


Figure 1.12. Numerous potent anionophores.

through ROS elevation pathway. Both the complexes also inhibit the autophagic flux due to their anion transport mediated lysosomal alkalinization property. The

transmission electron microscopy (TEM) images revealed that the complexes **1.8e** and **1.8f** treated cells show several vacuoles comprising of undegraded organelles or cytoplasmic substances, while the nuclei remain undamaged. These morphological changes are considered the characteristic property of autophagy. The *in vivo* studies revealed that the treatment of the complexes **1.8e** and **1.8f** remarkably suppressed the growth of the tumors in the female BALB/c-(nu/nu) nude mice. Additional hematoxylin and eosin (H&E) staining of major organs, including the heart, lung, liver, spleen, and kidney, after intratumoral administration of the complexes, revealed no significant abnormalities suggesting their low systematic toxicity.⁴⁹

To explore the role of anionophores in anticancer activities, Manna and co-workers also synthesized 1,2-diphenylethylenediamine-based bis(thiourea) derivative **1.8g** (Figure 1.12).⁵⁰ The HPTS and lucigenin-based fluorescence assays showed that the potent compound **1.8g** transport anions *via* the $\text{Cl}^-/\text{NO}_3^-$ antiport pathway. However, the conformations of the chiral centers present in the 1,2-diphenylethylenediamine scaffold did not play any significant role in their transmembrane anion transport activities. The viability studies using human cancer cells like cervical cancer (HeLa) and human tongue carcinoma SAS), and normal baby hamster kidney (BHK-21) cells showed that the potent compounds induce cytotoxicity with IC_{50} values 4-9 μM . Although the IC_{50} values of the potent compounds were promising, it has no significant selectivity towards the cancer cells over the normal cells. The viability studies with Cl^- and Na^+ ions free buffer solutions showed that the cytotoxicity of the compounds was considerably reduced in HeLa cells, suggesting that the transport of ions is one of the primary reasons for their cytotoxicity. Further cellular studies were performed to investigate the cell death mechanism of the potent compounds in HeLa cells. The microscopic studies using membrane potential sensitive JC-1 dye showed that the compounds disrupt MMP in HeLa cells. The presence of nuclear condensation, fragmentation, and apoptotic bodies in the compound and Hoechst 33342 dye treated HeLa cells suggested the apoptotic cell death pathway. The western blot analysis of the compound **1.8g** treated HeLa cells revealed the mitochondria-assisted caspase pathway of apoptosis. In 2020, Manna and colleagues reported that the well-known

anticancer agents, PITENINs, are also proficient transporters of Cl^- ion (Figure 1.12). A series of biophysical studies proved that the PITENINs interact with the Cl^- ion.⁵¹ The ion transport studies under the liposomal environment demonstrated that the presence of phenolic OH and acylthiourea moieties assist the lipophilic PITENINs in transporting the Cl^- ions across the lipid bilayers. The mechanistic studies revealed that the potent compounds PIT-1 (**1.8h**) and DM-PIT-1 (**1.8i**) prefer the H^+/Cl^- transport pathway. Due to the pH-switch ability, the compound DM-PIT-1 showed higher cytotoxicity in cancer cells over normal cells. In detail, cellular studies also showed that the potent compounds induce apoptosis *via* a caspase-dependent pathway.

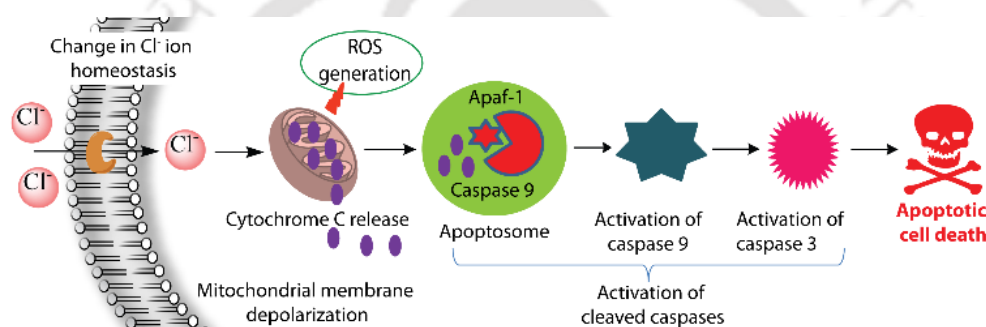


Figure 1.13. Schematic representation of the anionophore mediated intrinsic pathway of apoptosis.

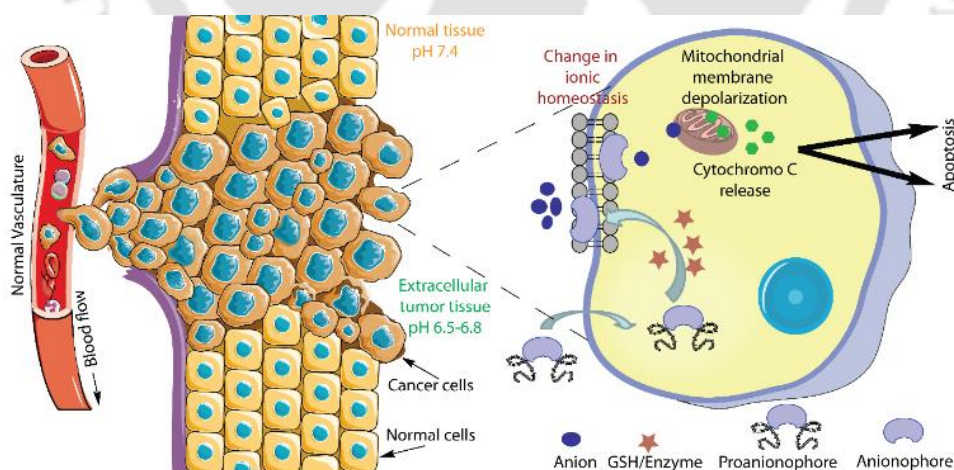


Figure 1.14. Schematic illustration of glutathione and enzyme-mediated regeneration of active anionophores from their proanionophores and its biological consequences on the tumor cells.

1.5.6. Stimuli-responsive anionophores

Although classical anionophores have a huge potential to be used in therapeutics, they also have certain limitations. As the anionophores are neutral and lipophilic, their deliverability into the cell is challenging. Selectivity between the normal cell and the cancerous cell is another major drawback of the classical anionophore. In general, in tumor cells, the pH value is lower, and reduction potential (due to the overexpression of glutathione and others) and enzyme activity (such as higher esterase activity) are higher. Therefore, pH, glutathione, enzyme, and others could act as endogeneous stimuli. Whereas near-infrared light, ultra sound, and others could act as exogeneous stimuli.⁵²⁻⁵⁴ In this regard, the design and synthesis of stimuli-responsive anionophores are regarded as one of the most favorable strategies that could selectively target cancer cells and reduce the side effects. Various researchers developed proanionophores, where the actual anionophore was masked with the various stimuli-responsive linker(s), which release the active anionophore depending on the cellular conditions. The abnormal metabolism allows the cancer cells to alter the pH of the intracellular and extracellular environments, enhance the level of glutathione (GSH) and enzyme, and others (Figure 1.14). These stimuli allow cancer cells to acquire their belligerent properties, including invasiveness, metastasizing ability, augmented motility, drug resistance capability, and others. The compounds that could amplify their anion transport activities under the acidic medium or in the presence of GSH are considered good targets for ion transport mediated anticancer activities. Therefore, ion transporters that could augment their ion transport properties under the acidic environment are considered as potential targets for selective anticancer activities. In 2019, Manna and co-workers synthesized 1,2-bis(benzimidazol-2-yl)benzene derivatives **1.9a-1.9d** and explored their ion transport mediated biological activities (Figure 1.15). The ISE-based studies showed that the iminourea-based potent compound **1.9d** supports pH-dependent transmembrane anion transport properties. The pK_a value of 6.2 of compound **1.9d** suggested that the compound gets easily protonated in a slightly acidic environment. It is well documented that normal healthy cells have lower internal pH ($pH_i \approx 7.2$) than the extracellular pH ($pH_e \approx 7.4$). However, in cancer cells, the pH_e is in the range of 6.2–6.9 and $pH_i \geq 7.4$. This reversal of pH gradient prompts cancer cells to evade apoptosis. Several biophysical

assays confirmed that compound **1.9d** could transport the H^+/Cl^- ion- pair across the lipid bilayers. The viability studies under different acidic mediums revealed that the potent compound has higher cytotoxicity at pH 6.5 compared to that at pH 7.4 in the MCF7 cells. The compound also showed Cl^- ion transport mediated cancer cell death. The cytotoxicity of the compound was more than 2-fold higher in cancer cells than that in normal cells. Alfonso and co-workers developed pseudopeptidic cage-like ion transporters that exhibit pH-dependent activity (Figure 1.15).⁵⁵ The ISE-based studies revealed that the potent pseudopeptidic cage **1.9g** transport anions *via* H^+/Cl^- pathway under the acidic environment. Thorough ion transport measurements revealed that the nature of the amino acid side chain plays a key role in their anion transport properties. The viability studies under different acidic mediums revealed that the potent compound has higher cytotoxicity at pH 6.2 in comparison with that at pH 7.6 in the A549 cells. The cytotoxicity of compounds was considerably reduced in chloride-free buffer solution, which is in good agreement with the potent compound's anion transport mediated biological activities.

In 2020, Manna and co-workers developed a series of thiourea and squaramide-based quinine derivatives. They thoroughly investigated anion transport mediated biological activities of the compounds both under *in vitro* and *in vivo* conditions (Figure 1.15).⁵⁶ The HPTS and ISE-based assays showed that the quinine derivatives showed superior anion transport efficiency under acidic medium due to the involvement of the bridge-head nitrogen of quinuclidine moiety. Mechanistic studies revealed that the potent compounds **1.9k** and **1.9o** transport the H^+/Cl^- ion-pair across the lipid bilayers under the acidic medium. The cell viability studies revealed that the cytotoxicity of compound **1.9o** is more than 5-fold selective for cancer cells (MCF7) than that in normal cells (MCF-10A). The detailed *In vitro* cell studies revealed the mitochondria-interceded caspase pathway of apoptosis. Further, the treatment with the potent compounds **1.9k** and **1.9o** remarkably reduced the growth of the solid tumors in the female Swiss albino mice. With negligible toxicity. Manna and co-workers designed and synthesized GSH-responsive sulfonium-based proanionophores (Figure 1.15).⁵⁷ The GSH-mediated cleavage of sulfonium moiety would detach the hydrophilic moieties and generate the

membrane soluble active anionophore preferentially within the cancer cells. The HPLC analysis showed the generation of membrane-active thiourea-based tripodal compound **1.9s** from its water-soluble proanionophore **1.9r**. The HPTS-based fluorescence assays in the presence of pH gradient displayed that the potent compound **1.9s** transport anions *via* the OH^-/Cl^- antiport pathway. The MALDI-TOF mass spectral analysis of a HeLa cell lysate also showed that regeneration of the active anionophore Talukdar and co-workers explored this GSH-mediated strategy to regulate transmembrane anion transport properties. They developed 2,4-nitrobenzenesulfonyl (DNS) protected 2-hydroxyisophthalamide derivatives that regenerate the membrane active 2-hydroxyisophthalamide derivative **1.9v** in the presence of GSH (Figure 1.15).⁵⁸ Systematic biophysical studies validated that the membrane active 2-hydroxyisophthalamide derivatives form channels within the membranes and transport ions *via* the M^+/Cl^- pathway. The ionic conductance measurement across the planar lipid bilayer membrane of diphytanoyl phosphatidylcholine (diPhyPC) lipid confirms the formation of ion channels. *In vitro* studies showed superior cellular uptake efficiency for the DNS protected compound in comparison to the 2-hydroxyisophthalamide derivative. The concentration-dependent cell viability studies showed that the active compounds **1.9v** had very high cytotoxicity (IC_{50} values 0.5-1 μM), which is linked with the augmented oxidative stress in MCF7 cells. The reduction of intracellular GSH levels, disruption of MMP, generation of ROS, the release of cytochrome c, and the activation of caspase cleavage confirmed the compound mediated apoptotic cell death in MCF7 cells. They also demonstrated that the active ionophore confines the proliferation and growth of spheroids of MCF7 cells. Additional cellular studies revealed that the active compound **1.9v** promote apoptosis *via* a caspase-dependent pathway and also disrupt autophagy due to alkylation of the lysosome. Jeong and co-workers first proposed the concept of procarrier to regulate the transport of anions across the cell membranes. They developed the procarriers **1.9w-1.9z** with hydrophilic appendages like polysaccharides or carboxylate groups, which are responsive towards specific enzymes like lipases, esterases, or glycosylase (Figure 1.15).⁵⁹ Bio-physical and cellular studies showed the regeneration of the active anionophore **1.9a1** from its water-soluble analogues. The transport studies in YFP

expressed FRT cells showed that the influx of Cl^- ion was more efficient for polysaccharides appended procarrier in the presence of β -galactosidase enzyme (from *Aspergillus oryzae*) under slightly acidic conditions (pH = 5.5–6). Hence, the attachment of polysaccharides could enhance the deliverability of the active anionophore in cancer cells, which could eventually display the anticancer activity.



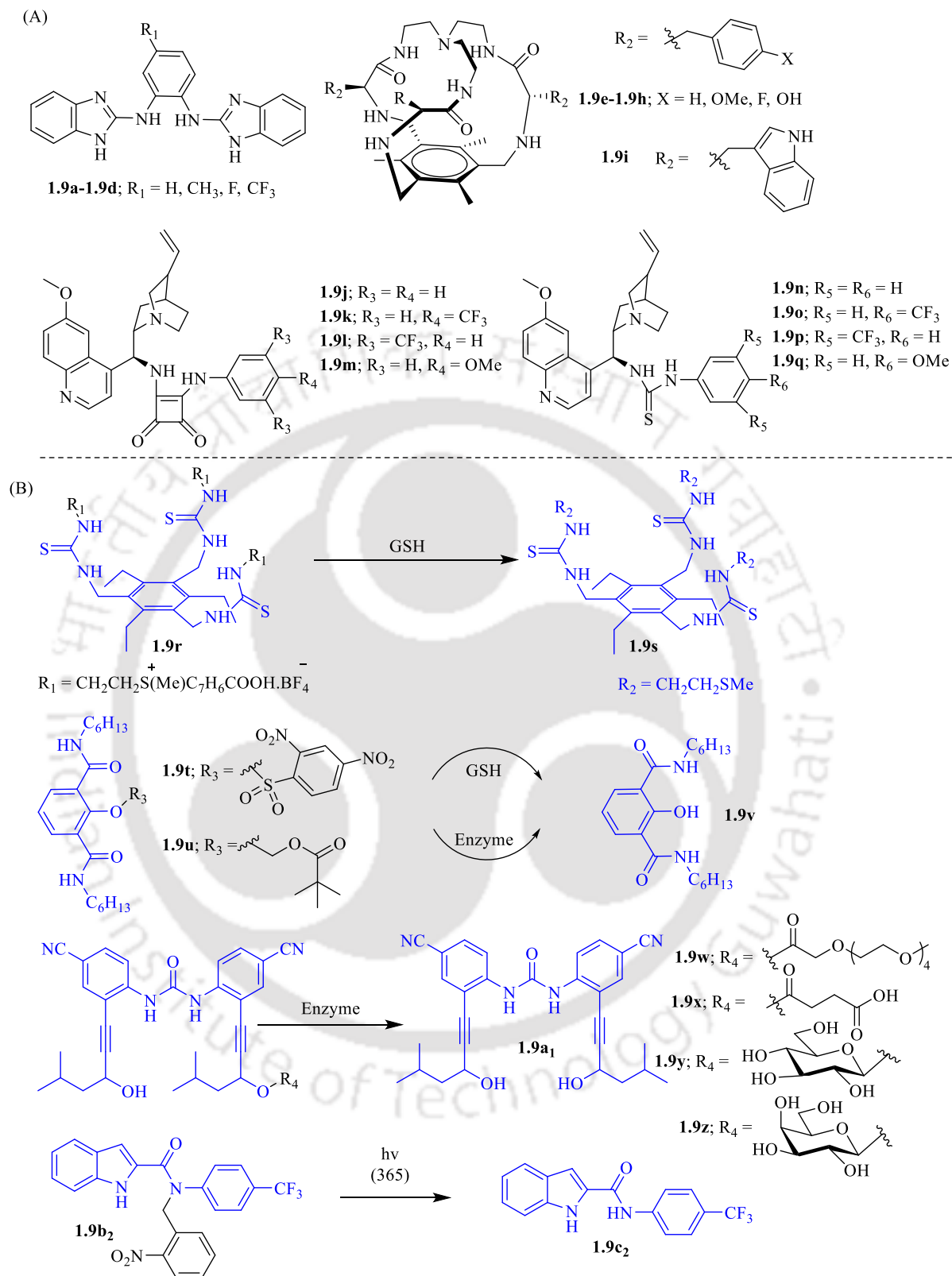


Figure 1.15. Stimuli responsive anionophores.

Talukdar and co-workers developed a photo-responsive proionophore, which was efficiently activated by UV-light to regenerate the active anionophore (Figure 1.15).⁶⁰ They synthesized indole-2-carboximide derivative **1.9b₂**, in which the photo-cleavable *ortho*-nitrobenzyl group protected the amide group. The ¹H NMR studies showed the generation of active ionophore from the proanionophore in the presence of 365 nm light. The HPTS-based fluorescence assay in the presence of base-pulse showed that the active ionophore **1.9c₂** transport anion *via* the OH⁻/Cl⁻ antiport pathway. The cell viability studies showed that the photo-release of the active ionophore induces moderate MCF7 cell death. Although the photo-activation with 365 nm light is not ideal for further biological studies, such a photo-responsive proionophore has potential use in photodynamic therapy.

1.6. Anionophores with non-toxic properties

Dysregulated transport of anion is the primary reason for channelopathies like cystic fibrosis. The use of synthetic anionophores is regarded as one of the alternative approaches to restore anion transport in the cystic fibrosis transmembrane conductance regulator (CFTR) defective cells. CFTR plays a fundamental role in the transport of water and salt across the epithelia. Currently, few clinically approved drugs for cystic fibrosis disease are available that target the defective CFTR. However, a large number of mutations in the CFTR gene restricts the use of these drugs (Figure 1.16). The synthetic anionophores avoid binding to any specific protein/enzyme or gene, and they function with the membrane to regulate the transport of ions. The synthetic anionophores could overawe the conflict linked to the mutations or over-expression of genes and proteins of CFTR. Hence, anionophores should be developed to afford therapeutics for cystic fibrosis. The detailed investigation of anionophores for cystic fibrosis disease is extremely rare, which should be explored. The anionophores for misregulated chloride channels like cystic fibrosis or Barter syndrome, in the first place, should have no or negligible cytotoxicity, and delivery of sufficient quantities of the anionophores to cells to produce significant effects of the same order of magnitude as endogenous anion channels should not produce any side effects. In many cases, it has been observed that when a proton is involved in the transport process, cell toxicity often

accompanies it. Hence anionophores capable of promoting $\text{Cl}^- > \text{H}^+/\text{OH}^-$ selectivity could preferably be used for channelopathies where we wish to achieve non-toxic behavior.

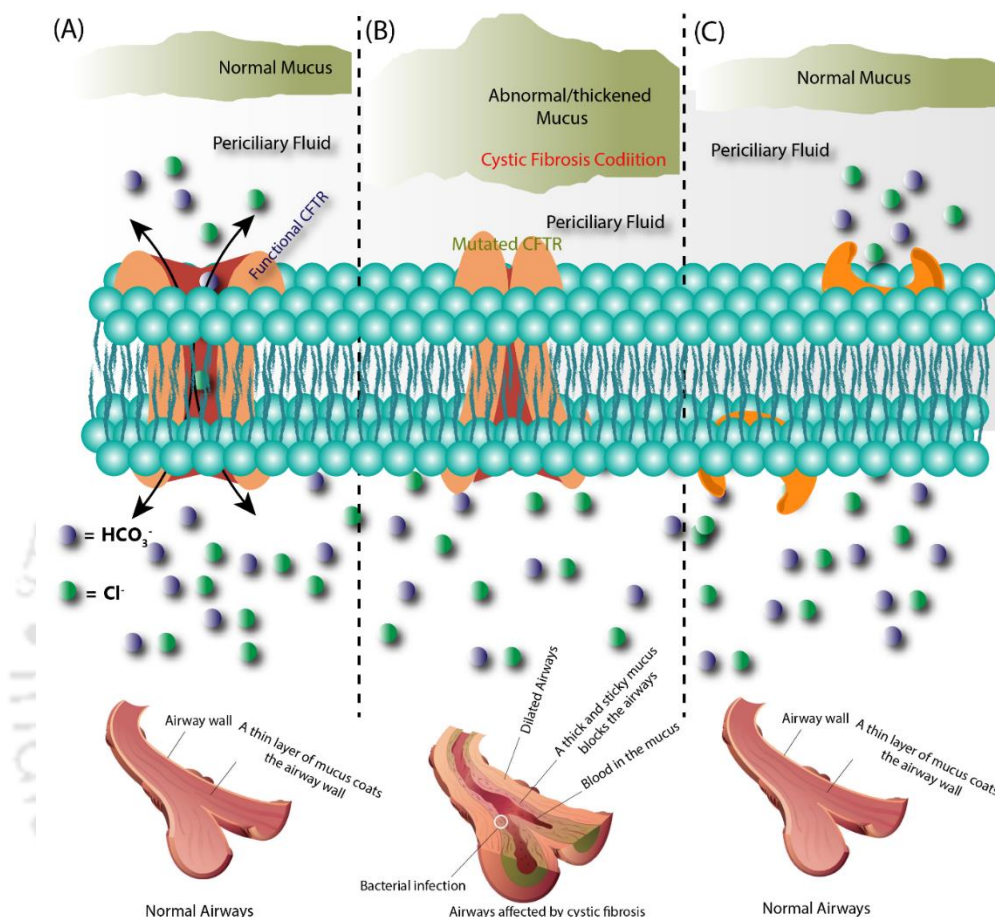
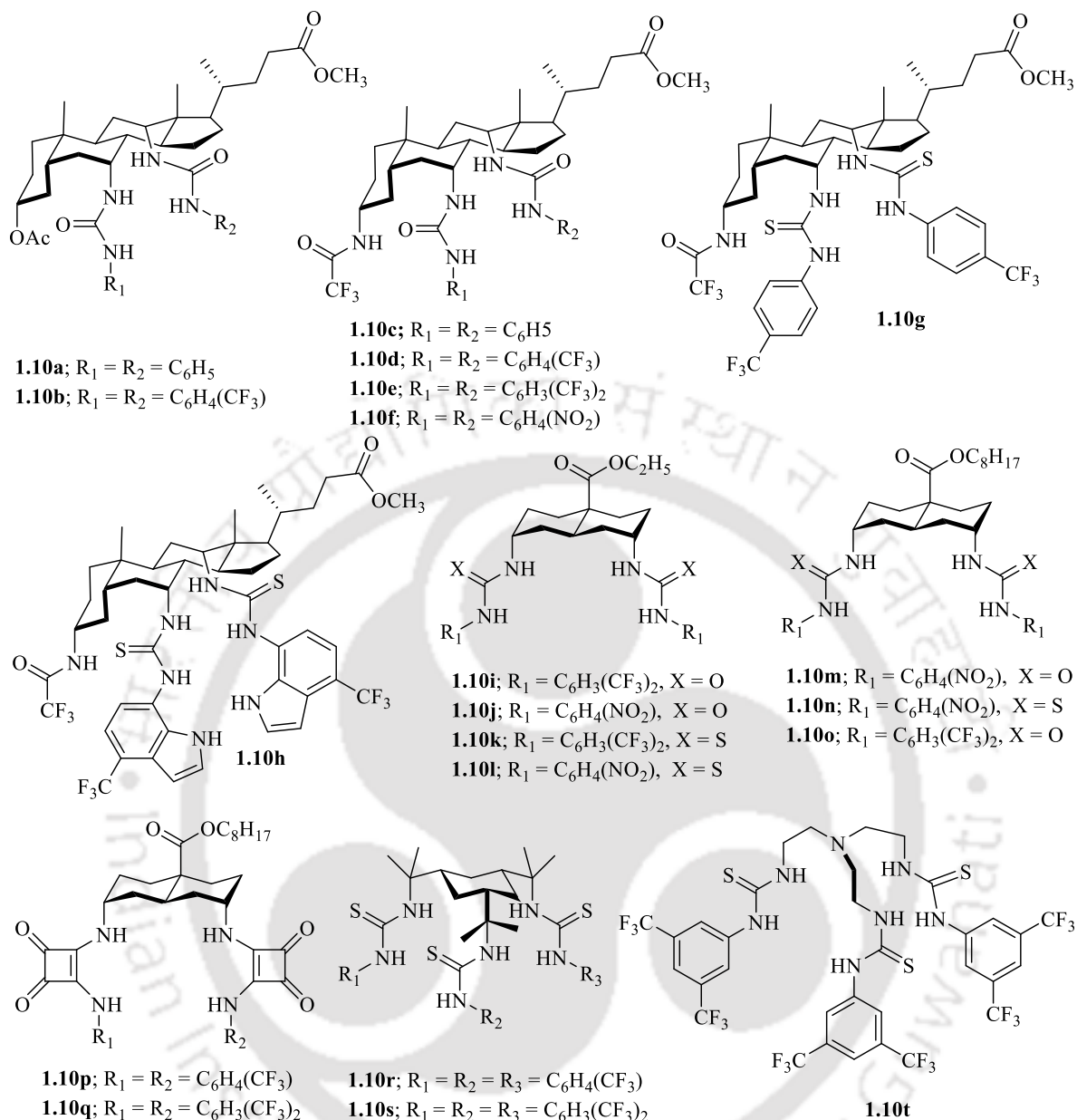


Figure 1.16. Transport of anions could address the lungs problems in cystic fibrosis.

However, in the end, the toxic property of an anionophore is compound-specific and could not be precisely tuned to our needs. Hence, though a wide variety of anionophores have been explored, the maximum number of molecules evinced their cytotoxic properties and was used for their anticancer properties. Consequently, in the search for a suitable anionophore for the treatment of cystic fibrosis, the Davis group did exhaustive investigations.⁶¹ They investigated molecules (**1.10a-1.10s**) having the scaffolds of steroids, trans-decalins, and substituted cyclohexanes (Figure 1.17). Testing of all the compounds to find out the most competent anionophore for CF using routine assays was a herculean task; hence they used the



Scheme 1.17. Anionophores with steroids, trans-decalins, and substituted cyclohexanes scaffolds.

fluorescence assay using the YFP expressed FRT cells. All the steroidal cholapods were unexpectedly inactive, which might be due to the ‘naturalness’ of the steroidal framework that might lead to specific inhibitory interactions with the biomolecules. Only compound **1.10e**, which is extremely active in LUVs, shows substantial activity in the YFP-FRT cells. They have observed a fascinating fact that the non-steroid compound **1.10h**, which is least active under the liposomal environment,

came out as the best candidate in the LUV deliverability test. Further, the evaluations confirmed that the bisureiododecalin **1.10h** could be readily delivered to cells without distinctive adjuvants. In continuation of the work, in 2019, the same group screened different classes of compounds to discover more potent or similarly potent compounds like compound **1.10h** (Figure 1.17).⁶² They have taken CF bronchial epithelial cell line (CFBE41o–), which was engineered to express YFP-H148Q/I152L (YFP-CFBE cells), and they had used this cell line as this has an advantage and have the ability to express the Ca^{2+} -activated Cl^- channel (CaCC), unlike YFP-FRT cells. The thorough investigation concluded that the anion transport by anionophores in YFP-CFBE cells is independent of CaCC activation, and for compound **1.10t**, it is additive to that of CaCC. They had examined overall toxicity and the induction of apoptosis in YFP-FRT cells using the live-cell analysis system. The cytotoxic data of anionophore **1.10i**, **1.10j**, and **1.10t** in YFP-CFBE cells showed some signs of cytotoxicity in YFP-CFBE cells, whereas **1.10e** showed no toxicity and gave a negative result in apoptosis. Overall these studies confirmed that the toxicity of these compounds is not merely associated with anion transport properties.

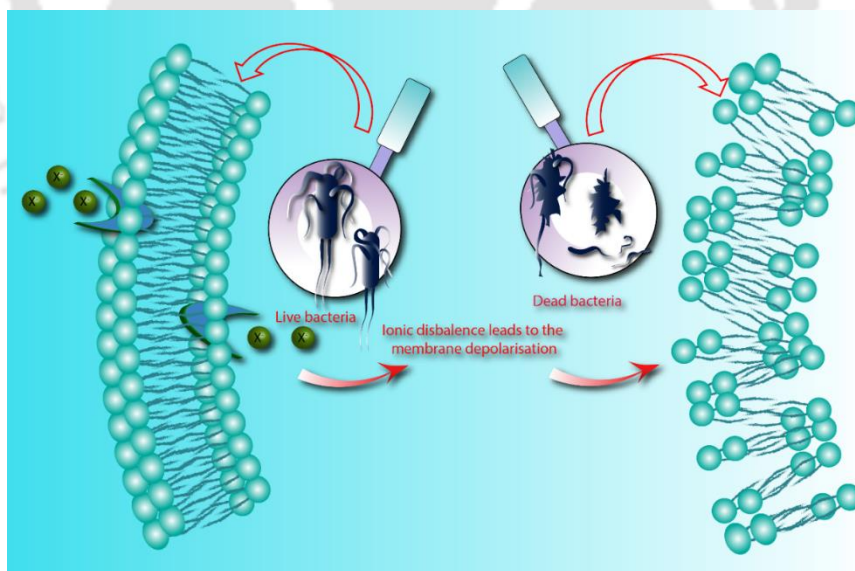
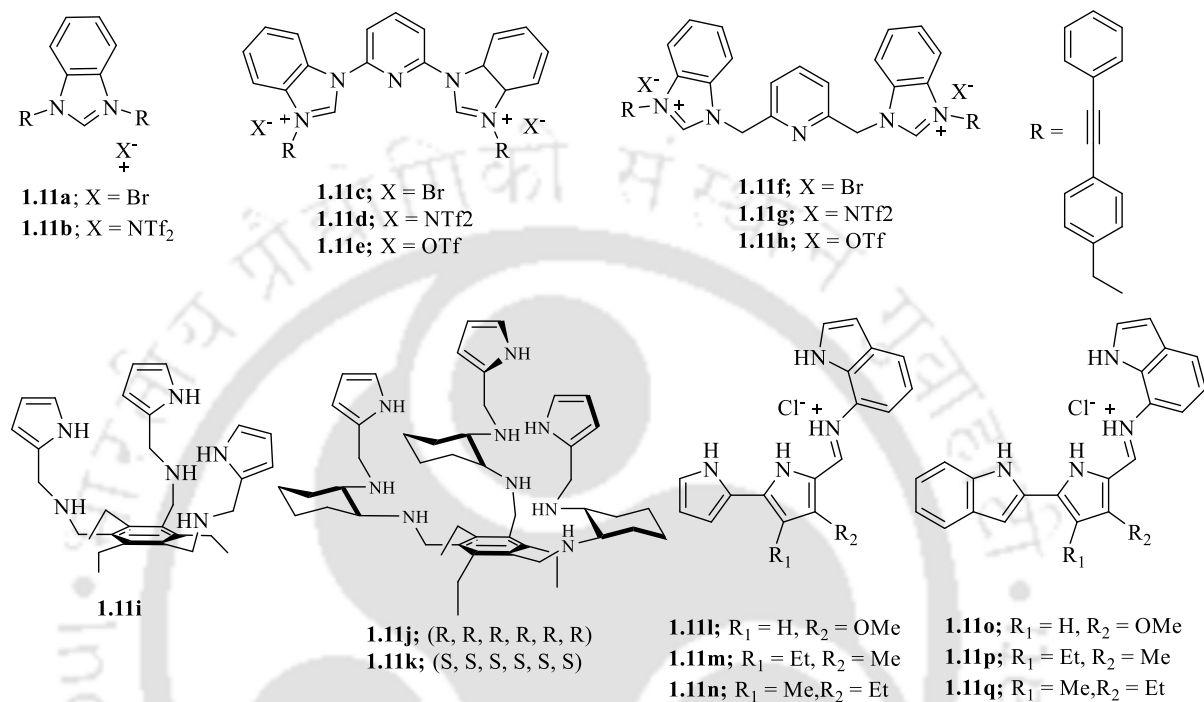


Figure 1.18. Schematic illustrations of membrane depolarization-mediated antibacterial properties of anionophores.

1.7. Anionophores with antibacterial activities

Antibacterial resistance is one of the leading problems worldwide, which is endangering the efficacy of antibiotics. The centers for disease control and prevention (CDC) have already classified a number of bacteria as having severe threats to humankind.² Initially, the anion transport-related research primarily focused on the development of ionophores for the treatment of cystic fibrosis and cancer. In 2015, Schmitzer and co-workers reported that the benzimidazolium salts-based anionophores (**1.11a-h**) possess antibacterial activity against both the gram-positive (*B. thuringiensis* strain) and gram-negative bacteria (*E. coli*) (Scheme 1.19).⁷ Subject to the types of the counter anion, the benzimidazolium salts-based anionophores transport anion *via* both channel and carrier pathways. The potent compounds have low cytotoxicity against human cells, which is also an essential parameter of an ideal antibacterial candidate. Various mechanistic studies confirmed that the membrane depolarization by these anionophores is the primary reason for its probable antibacterial property (Figure 1.18). The membrane localization makes it difficult for bacteria to develop resistance against the anionophores. By the same token, the anionophores should be active against drug-resistant bacteria. Sessler and colleagues reported that aminopyrrolic and TREN-based compounds promote anion transport mediated antibacterial activity against the *methicillin-resistant Staphylococcus aureus* (MRSA) strains, Mu50, and HP1173.⁶³ Comprehensive antibacterial studies confirmed that the compounds **1.11j-m** have noteworthy inhibitions both against the gram-positive (*S. aureus*) and gram-negative (*Pseudomonas aeruginosa*) bacterial strain (Figure 1.19). In the same study, they also investigated the antibacterial activities of the anionophores **1.4i-j**. These thiourea-based anionophores are quite active with MIC values in the range of 0.93-1.78 $\mu\text{g/mL}$ for the Mu50 and *S. aureus*, respectively. Although these anionophore showed antibacterial activities, there is no correlation between the anion transport and the antibacterial property. Moreover, careful assessment led to the conclusion that ion transport is not the only factor responsible for the antibiotic activity of these aminopyrrolic and TREN-based compounds. To explore the relationship between the anionophores and antibacterial activities, Quesada's group studied indolyl decorated tambjamine analogues (**1.11l-q**) bearing different substitutions on the pyrrole ring attached to the imine moiety (Scheme 1.19).⁶⁴ The anion transport activities of the compounds were investigated under the liposomal environment. The HPTS fluorescence-based studies revealed that the potent compounds transport anion ($\text{EC}_{50} = 16 \text{ nM}$) *via*

anion/ Cl^- exchange pathway. The potent compounds showed activities against both gram-positive bacteria such as *E. faecium* and *S. aureus*, and gram-negative bacteria like *A. baumannii* and *K. pneumonia* that are commonly present in hospital-acquired infections. The compounds also displayed decent haemo-compatibility.



Scheme 1.19. Anionophores with antibacterial activities.

1.8. Summary

Anion transport research in the past decade has been gained tremendous momentum, and it has made a pragmatic approach to combat various diseases. In terms of cancer, very selective approaches including, stimuli-responsive proanionophores have been recently developed to have control over the site of action of the anionophores. Regarding the channelopathies like cystic fibrosis, also several non-toxic anionophores showing negative toxicity results in the epithelial cells have been developed. Currently, the use of anionophores as antibacterial agents has also been well received. However, scientists across the globe successfully increase the gap between the selectivity of cancer cells and normal cells but are still not able to mute its effect on normal cells. Besides that improvement of deliverability, cellular uptake still needs more attention to overcome all conventional hurdles associated with anionophores. Besides all these conventional filed,

the anion transport field has huge prospects on angiogenesis, people with diabetes. Many new prospects are waiting in the arena of the anion transport field, which will be exciting to see in the near future.

1.9. References

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Chapter 2

Development of Diphenylethylenediamine-Based Potent Anionophores with Apoptosis-Inducing Activities





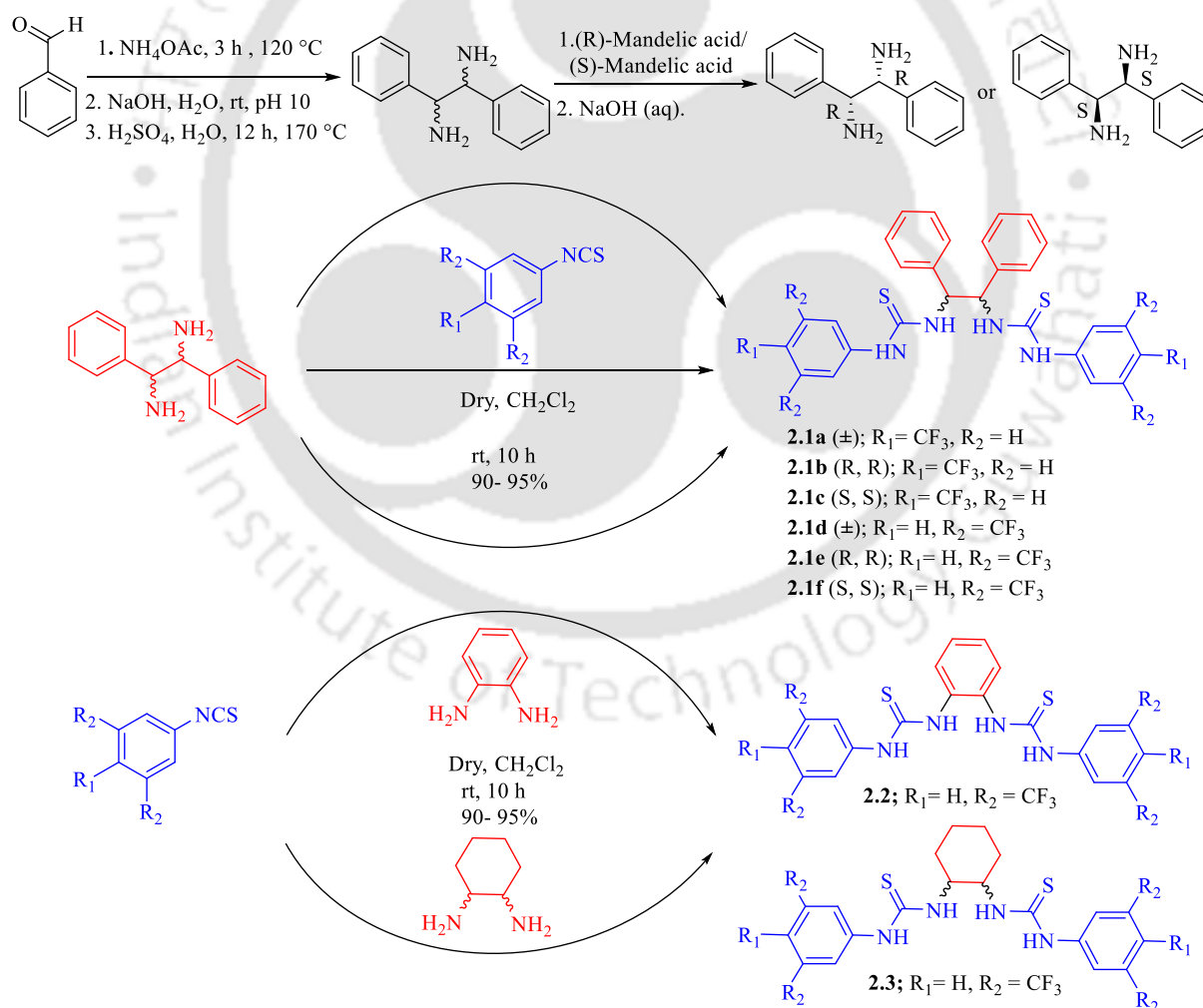


2.1. Background and objective of present work

The succinct literature of all bio-active anionophore in chapter 1 clearly demonstrates its potential against numerous pathological conditions. It is already well documented from the detailed literature survey that the transport of ions like Na^+ , K^+ , Ca^{2+} , Cl^- , and others across the membranes is essential in maintaining ion and pH-homeostasis, which regulates signaling pathways, proliferation, and other crucial cellular processes.¹⁻⁴ Chloride is one of the most abundant anions under normal physiological conditions. Selective conductance of Cl^- ions (potential gradient of 30–60 mV for eukaryotic cell membranes) is associated with various biological processes, including blood pressure regulation, chloride reabsorption, salt, and fluid secretion and electrical response generation.⁵⁻⁷ Malfunctioning of Cl^- ion transport proteins, primarily due to mutation(s), induces pathological conditions including myotonia, Barter's syndrome, epilepsy, cystic fibrosis, and cancer. To resolve all these pathological conditions, scientists across the globe thought about artificial ion transporting molecules as an alternative approach to deal with these hurdles.⁸⁻¹²

Motivated by its possible success, numerous synthetic anionophores were developed across the globe by the renewed scientists. Initially, it was started to deal with cystic fibrosis, but eventually, this technique applies to a broad spectrum of diseases. However, pertinent anion transporters have only recently been developed, and their biological activities beyond the “channel replacement therapy” have not been well explored. Recent studies revealed that Cl^- ions' transport is associated with the induction of apoptosis of the cancer cells by either disrupting the ionic homeostasis or altering the intracellular pH. It is also presumed that the synthetic ion transporter could prevent the drug resistance capability of the cancer cells, which is because of the reversal of pH and Cl^- ion concentration in the cytosolic and extracellular region of the cancer cells in comparison with that of the normal cells. The anticancer activity of natural product prodigiosin and its analogues are directly related to their Cl^- transport efficiency.¹³⁻¹⁴ The squaramide-based synthetic Cl^- ion transporters also showed disruption in autophagy and elevation of apoptosis by perturbing Cl^- ion concentration in cancer cells.¹⁵ In this regard, the development of synthetic Cl^- ion transporters adept at mimicking the cellular functions of natural Cl^- ion transporters is anticipated to be one of the most promising approaches to fight against diseases associated with the malfunctioning of natural Cl^- ion channel systems and others.

So encouraged by all these therapeutic prospects of anionophore, the focus was given to design some potent anion transporting molecules and within this chapter, a new class of bis(thiourea)-containing anion transporter with conformationally controlled 1,2-diphenylethylenediamine (DPEN) moiety were reported. To compare the potency of the DPEN moiety, thiourea derivatives of ortho-phenylenediamine and 1, 2-diaminocyclohexane have also been synthesized to understand the role of DPEN scaffold.¹⁶⁻¹⁷ In addition, the position and number of substituents on the aryl ring attached with the thiourea moiety were also altered to study the effect of substitution on the acidity of N-H proton as well as lipophilicity. Within this chapter, a thorough investigation of recognition, transport, and selectivity measurement was carried out through a set of biophysical experiments. Besides that, a number of *in vitro* assays were also performed to prove its apoptosis-inducing property.



Scheme 2.1. Synthetic route of anionophores.

Table 2.1. Structures and chemical properties of the compounds.

Compound	cLogP ^a	pK _{a1} (N-H) ^b	pK _{a2} (N-H) ^b	K _d (mM) ^c
2.1a (±), R ₁ = CF ₃ ; R ₂ = H	7.11	18.92	9.20	13.32
2.1b (R, R), R ₁ = CF ₃ ; R ₂ = H	7.11	18.92	9.20	9.43
2.1c (S, S), R ₁ = CF ₃ ; R ₂ = H	7.11	18.92	9.20	9.17
2.1d (±), R ₁ = H; R ₂ = CF ₃	8.80	18.94	8.97	6.62
2.1e (R, R), R ₁ = H; R ₂ = CF ₃	8.80	18.94	8.97	3.92
2.1f (S, S), R ₁ = H; R ₂ = CF ₃	8.80	18.94	8.97	3.87
2.2 , R ₁ = H; R ₂ = CF ₃	8.13	8.92	7.59	13.29
3.3 (±), R ₁ = H; R ₂ = CF ₃	7.43	19.18	8.96	22.37

^acLogP values of the compounds were calculated using DataWarrior program. ^bpK_{a1} and pK_{a2} were calculated using the MarvinSketch 5.3.1 program. ^cK_d values of the compounds were calculated using ¹H NMR titration

2.2. Results and discussions

2.2.1. Design and synthesis of the bis(thiourea) derivatives

A small series of bis(thiourea)-based compounds (**2.1a–f**) with stereo-chemical variations of the DPEN scaffold were synthesized. To understand the importance of DPEN scaffold on anion recognition and transport activities bis(thiourea), derivatives of ortho-phenylenediamine (**2.2**) and 1,2-diaminocyclohexane (**2.3**) scaffold were also synthesized.¹⁶⁻¹⁷ Most of the reported artificial ion transporters are either structurally complex or require multistep for their synthesis.^{12, 18-19} For prevalent use of these compounds, a single-step synthesis is evidently desirable. The compounds **2.1a–2.1f** were synthesized through the condensation of DPEN with corresponding isothiocyanates

(scheme 2.1).²⁰⁻²¹ A similar reaction method was followed for the synthesis of compounds **2.2** and **2.3** from orthophenylenediamine and 1, 2- diaminocyclohexane, respectively.²⁰⁻²² The characterizations, as well as the purity of the compounds, were scrutinized by recording the ^1H NMR, ^{13}C NMR, and HRMS spectroscopic data. The clog P values of the compounds were calculated (clog P = 7.11–8.80, 8.13, and 7.43 for compounds **2.1a–f**, **2.2**, and **2.3**, respectively) using DataWarrior program (Table 2.1).²³

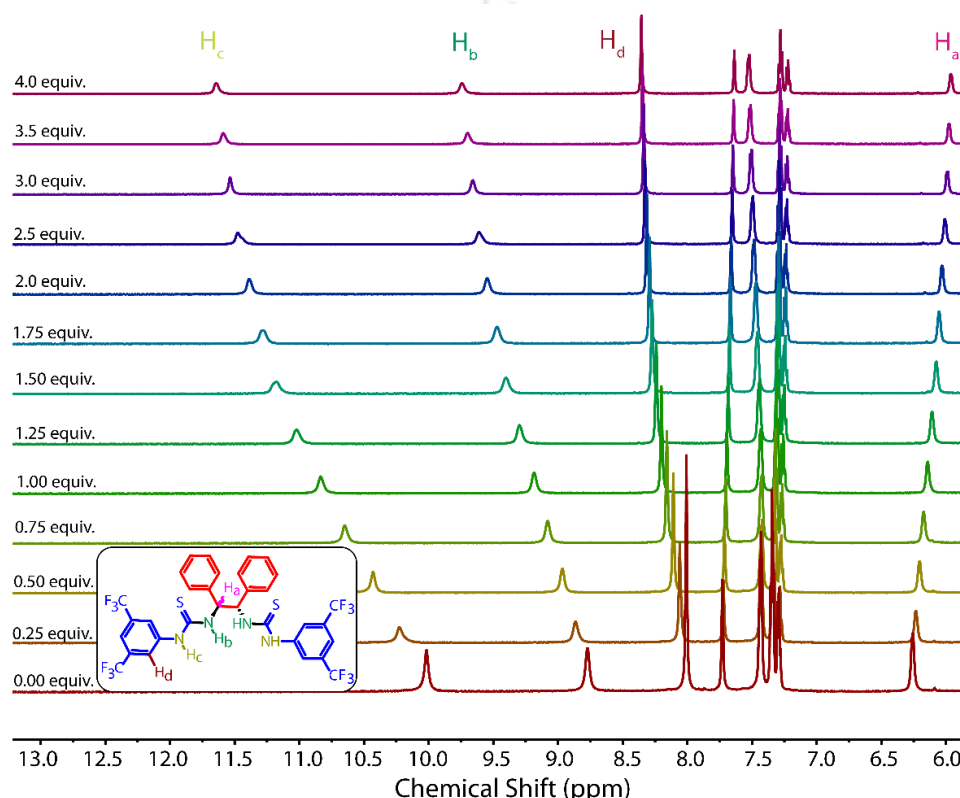


Figure 2.1. Representative ^1H -NMR (600 MHz) titration curve for compound **2.1f** (5.3 mM) with TBACl in $\text{DMSO}-d_6$ solvent. The amounts of added TBACl are shown on the spectra.

2.2.2. Anion binding studies of the bis(thiourea) derivatives

To investigate the strength and mode of interactions of the compound with the Cl^- ion, the ^1H NMR titration measurements were carried out in a $\text{DMSO}-d_6$ solvent. Tetrabutylammonium chloride (TBACl) was used as the source of Cl^- ion during these NMR titration measurements. Analysis of the ^1H NMR titration spectra in the presence of an increasing concentration of TBACl showed that the signals of H_b and H_c protons of the compounds shifted toward downfield (Figures 2.1 and Figure 2.21-2.26). These spectral

changes indicate the presence of $\text{N-H}_b \cdots \text{Cl}^-$ and $\text{N-H}_c \cdots \text{Cl}^-$ interactions between the compound and Cl^- ion. The chemical shifts ($\Delta\delta$) for N-H_c proton were used to calculate the binding constant and stoichiometry of binding. The $\Delta\delta$ for N-H_b protons were lower under similar experimental conditions, which could be because of weaker interaction with the N-H_b protons. However, the binding affinity pattern calculated using the N-H_b proton is similar to that calculated using the N-H_c proton (Table 2.1). In the solution state, the binding stoichiometry was determined by a continuous variation method of Job's plot, where the chemical shifts of N-H_c proton were used to calculate stoichiometry of binding (Table 2.3). This was done by varying the mole fractions (x) of the compounds. The chemical shift ($\Delta\delta$) of N-H_c proton was plotted against the mole fraction of compounds. The maximum interaction was observed at $x = 0.5$, which indicated a 1:1 binding (host/guest) stoichiometry of the selected compound (**2.1d**) with the Cl^- ion (Figure 2.28). Accordingly, the binding constants for the interaction of these compounds with Cl^- ion was calculated using WinEQNMR2 program, and the titration curves were fitted with an inbuilt 1:1 binding model. The values obtained for the dissociation constants (K_d) of the compounds were within the range of 3.87–22.37 mM. These dissociation constant values clearly suggest that the presence of trifluoromethyl group on the Ar-moieties was primarily responsible for the increasing acidity of the N-H proton as well as binding affinity for the Cl^- ion. It is well documented that the meso isomer of compound **2.3** showed poor Cl^- ion recognition capability over the R, R (**2.1e**), and S,S (**2.1f**) enantiomers.²² In this regard, the Cl^- ion binding ability of the meso isomers of compounds was not investigated. We hypothesized that the compounds would remain as monomers in the $\text{DMSO-}d_6$ solvent. The K_d values from the ^1H NMR titrations would not be significantly different among the enantiomers. Hence, the ^1H NMR titration measurements of the R, R enantiomers were not performed. Overall, the ion recognition abilities of these compounds also indicate the importance of the presence of a well-defined conformationally restrained environment of the thiourea derivatives. Additionally, Cl^- versus NO_3^- selectivity of the potent compound (**2.1f**) was also investigated using the ^1H NMR titration experiment (Figure 2.27).²⁴ The proportional change in $\Delta\delta$ values of $\text{N-H}_{b/c}$ protons confirmed negligible/nonspecific binding of NO_3^- ion (K_d could not be determined because of the unattainability of signal saturation even in the presence of >10 Equiv of TBANO_3 salt) with the compounds in comparison with the Cl^- ion. The higher charge density of Cl^- ion in comparison with NO_3^- ion could be the driving force for their stronger selectivity. Hence, these dissociation

constant values of the compounds indicate that compound **2.1f** has stronger binding affinity for Cl^- ion because of the presence of both DPEN and 1,3-bis(trifluoromethyl)phenyl moieties.

2.2.3. X-ray crystallographic analysis

The Cl^- ion recognition properties and their mode of interactions with these compounds were further corroborated from the X-ray crystal structure analysis. The crystallization was performed with compound **2.1d** ($\pm$) in the presence of excess TBACl salt as the source of Cl^- ion in the DMSO solvent system at room temperature. However, the structural analysis showed that in a single asymmetric unit, two Cl^- ions interact with three units of the meso-isomer of the compound **2.1d** and TBA^+ (Figure 2.29, Table 2.4). The observed coordination number of the Cl^- ion of this complex was found to be four. The space-filling model of the meso-compound **2.1d** also demonstrated that two symmetric equivalents of the molecule were responsible for the recognition of the Cl^- ion (Figure 2.2A-B). It is important to mention that the solid-state interaction pattern of the compound is quite different from that in the solution state (1:1 bind model). Further structural analysis revealed that the N–H groups of thiourea moiety were responsible for Cl^- ion recognition, and all of the measured bond distances were well within the distance of hydrogen bonding (all N–H–Cl bond distances were in the range of 2.370–2.390 Å). An interaction of Cl^- ion with the C–H bond of TBA^+ was also observed within the hydrogen bond distance (C–H–Cl distance 2.909 Å). This X-ray crystallographic analysis provides direct proof of Cl^- ion binding to the designed compounds.

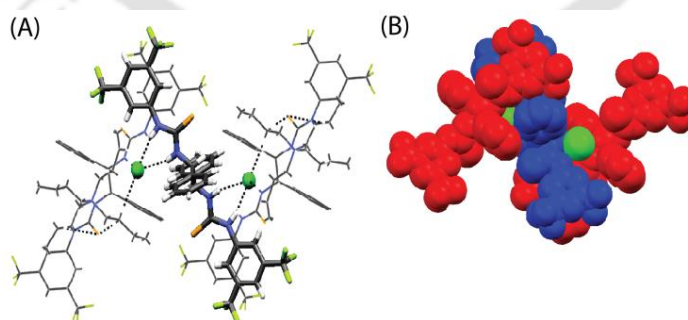


Figure 2.2. Wireframe view of chloride and TBA encapsulated complex of compound **2.1d** (A). Space-fill view of chloride ion (green ball) within the molecule-created cavity (B).

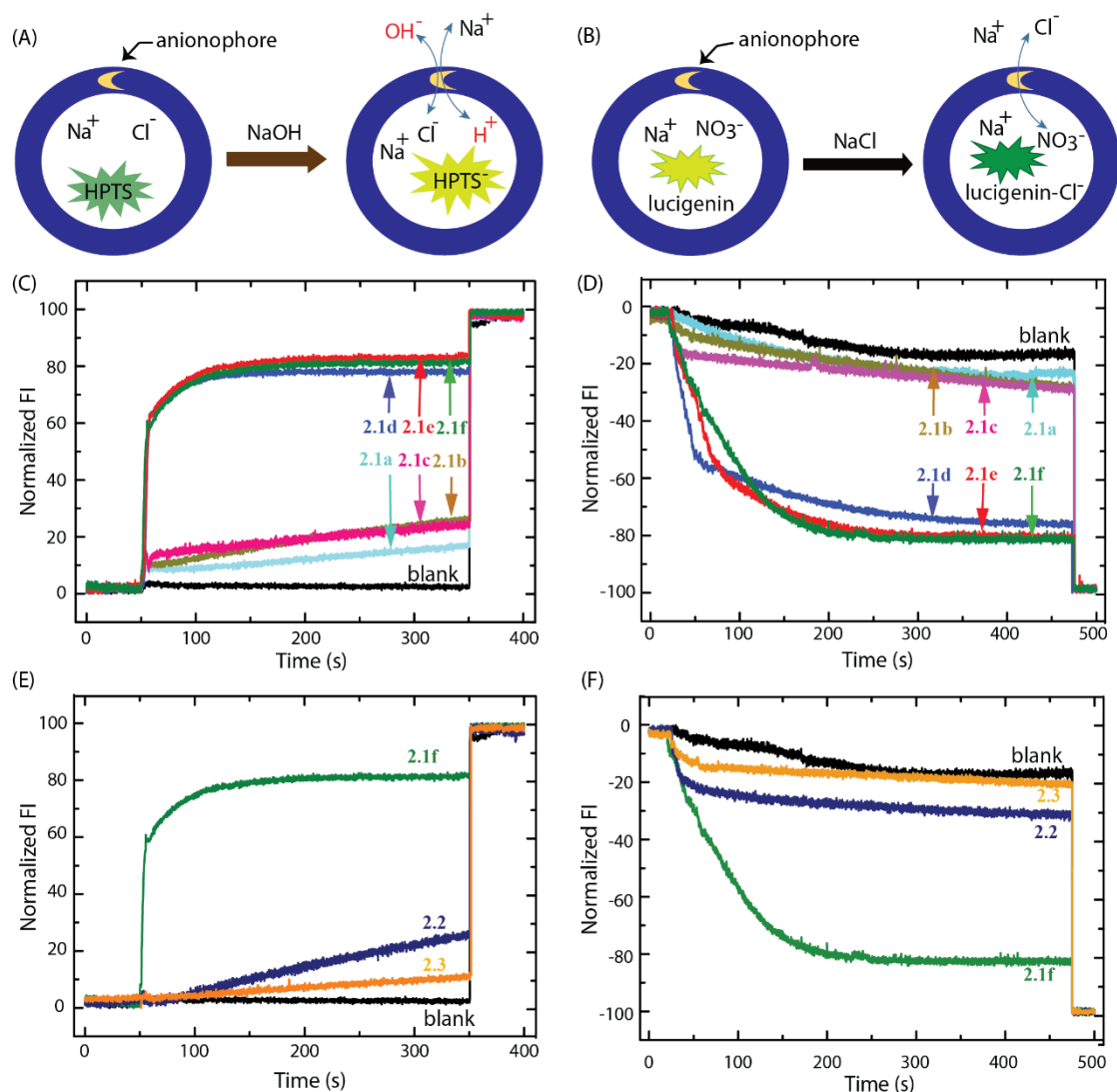


Figure 2.3. Schematic representation of fluorescence based ion transport activity assay using EYPC/CHOL-LUVs $\supset$ HPTS (A) and EYPC/CHOL-LUVs $\supset$ lucigenin (B). Comparisons of ion transport activity of **2.1a-2.1f** (C) and **2.1f**, **2.2**, and **2.3** (E) across EYPC/CHOL-LUVs $\supset$ HPTS. Comparisons of ion transport activity of **2.1a-f** (D) and **2.1f**, **2.2**, and **2.3** (F) across EYPC/CHOL-LUVs $\supset$ lucigenin. Compound/lipid ratio of 1:25000.

2.2.4. Chloride ion transport activities of the bis(thiourea) derivatives

The Cl^- ion binding properties and X-ray co-crystal structure of these compounds with TBACl prompted us to investigate their ion transport activity across the membrane bilayer of large unilamellar vesicles (LUVs). The transmembrane Cl^- ion transport efficacy of the compounds was evaluated using the well-established fluorescence-based assay.^{11-12, 25-27} The vesicles were prepared using EYPC and cholesterol (6:4 molar ratio) lipids with entrapped 8- hydroxypyrene-1,3,6-trisulfonate (HPTS, a pH-sensitive dye, pKa

= 7.2) dye in the presence of 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl to get EYPC/CHOLLUVs $\Rightarrow$ HPTS. The compounds were added in the extravesicular solution at compound/lipid ratios of 1:25 000 and allowed to equilibrate with the vesicles. For HPTS assay, the transport kinetics was initiated by generating a pH gradient ($\Delta\text{pH} = \sim 0.5$) using NaOH in the extravesicular solution and the increased fluorescence intensity of the deprotonated HPTS dye was monitored at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$). To get the maximum HPTS fluorescence intensity, the vesicular arrangements of the lipids were completely disrupted by using Triton X-100. The Cl^- influx/efflux affected by H^+ efflux or OH^- influx contributes to the enhancement of pH at the intravesicular environment of the vesicles, resulting in an increase of HPTS fluorescence intensity. Therefore, the higher HPTS fluorescence intensity is the measure of higher Cl^- transport efficacy (Figure 2.3A). Similarly, we performed a lucigenin assay to measure the Cl^- ion transport efficacy of the compounds (Figure 2.3B). The EYPC/CHOL-LUVs $\Rightarrow$ lucigenin were prepared by encapsulating the lucigenin (a halide sensitive dye) within the LUVs composed of EYPC and cholesterol (6:4 molar ratio) in the presence of 20 mM HEPES buffer, pH 7.2, containing 100 mM NaNO_3 .^{5, 27} For lucigenin assay, the chloride transport kinetics was initiated by addition of NaCl in the extravesicular solution, and the lucigenin fluorescence was monitored at $\lambda_{\text{em}} = 506 \text{ nm}$ ($\lambda_{\text{ex}} = 455 \text{ nm}$). The Cl^- influx resulted in the replacement of NO_3^- from the lucigenin and quenching of the fluorescence intensity of the dye. Maximum lucigenin fluorescence quenching was obtained by disrupting the vesicular arrangements of the lipids using Triton X-100. Hence, the higher lucigenin fluorescence quenching is the measure of higher Cl^- ion transport efficacy. All tested compounds showed significant fluorescence intensity change for both HPTS and lucigenin dyes, indicating their transport efficiency across the bilayer of the vesicles. The change in both HPTS and lucigenin fluorescence signals revealed that bis(trifluoromethyl)-substituted compounds have much higher Cl^- ion transport efficacies than the other tested compounds under similar experimental conditions. The higher transport efficacy of **2.1d–f** in comparison with the other tested compounds indicates the role of lipophilicity and acidity of N–H protons on Cl^- ion transport efficiency. The compounds **2.1d–f** are stereoisomers, and they showed very similar Cl^- ion binding and transport activities; hence, only **2.1f** was selected for further ion transport activity studies. It is important to mention that the potent compounds follow exponential decay kinetics for their transmembrane transportation of Cl^- ion (Figure 2.3C–F). However, a rapid initial increase/decrease in fluorescence

intensity followed by slower kinetics was repeatedly observed for both HPTS and lucigenin assay which could be due to the higher lipophilicity and stronger acidity of N–H protons (Table 2.1). It is well documented that anionic lipids are highly abundant both in the membranes of eukaryotic and prokaryotic cells.²⁸⁻³⁰ In this regard, additional Cl^- transport efficacy of the compound **2.1f** was investigated using EYPC/DPPS/CHOL-LUV $\supset$ lucigenin (EYPC/DPPS/cholesterol at a molar ratio of 4:2:4) to understand their ion transport efficacy under the physiological conditions (Figure 2.4). The kinetics parameters were calculated according to the reported method. The half-life and rate constants were calculated using first-order exponential decay kinetics. The initial rate constants were compared to investigate the efficiency of the Cl^- ion transport ability of the tested compounds. Analysis of the transport kinetics behavior in the presence of compound **2.1f** showed around a 1.7-fold decrease in the Cl^- ion transport rate when the anionic lipids were introduced in the liposomes (Table 2.5). These transport kinetic parameters of compound **2.1f** in the absence and presence of anionic lipids suggests that these compounds would have moderate Cl^- ion transport activity under physiological conditions. A similar Cl^- ion transport behavior was observed for compound **2.1e** in the presence of anionic lipids (Figure 2.4 and Table 2.5).

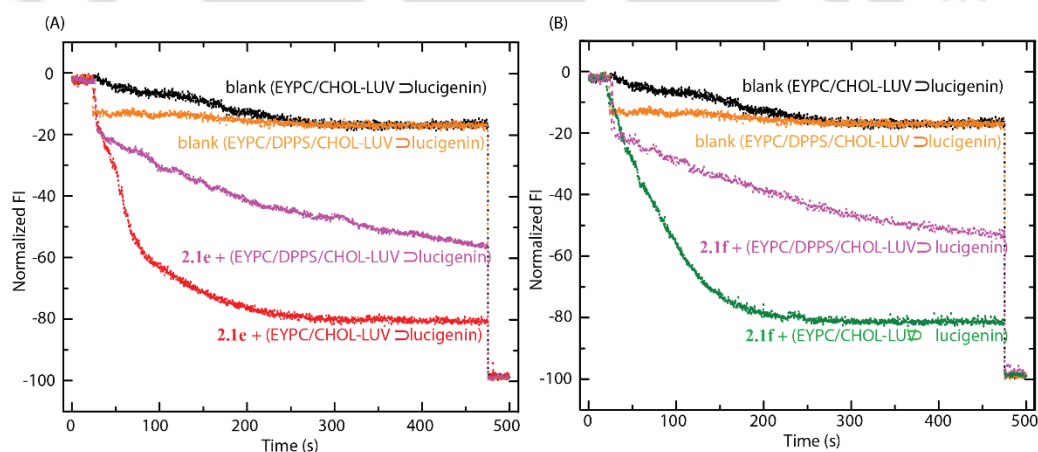


Figure 2.4. Comparisons of ion transport activity of compound **2.1e** (A) and **2.1f** (B) (Compound/lipid ratio of 1:25000) across EYPC/CHOL-LUVs $\supset$ lucigenin and EYPC/DPPS/CHOL-LUV $\supset$ lucigenin.

Dose-dependent HPTS fluorescence assay was used to calculate the EC_{50} value and Hill coefficient (n , number of compounds associated with a single ion) for the compounds

(Figure 31-38). The calculated EC_{50} values of the compounds were found to be very low and in the nanomolar range (Table 2.2). The Hill coefficients were found to be around 1, indicating the Cl^- ion recognition capabilities of the compounds (1:1 binding) in the solution phase (Table 2.2). The EC_{50} values of the compounds correlate well with the K_d and $clogP$ values of the compounds (Tables 2.1). The bis(trifluoromethyl)-substituted compounds (**2.1d-f**) with better Cl^- binding ability ($K_d = 3.87$ – 6.66 mM) and higher membrane permeability ($clog P = 8.80$) showed very strong Cl^- transport activity ($EC_{50} = 2.09$ – 4.15 nM). However, the ion transport activity of compound **2.3** ($EC_{50} = 98$ nM) was poor in comparison with **2.1d-f**. Interestingly, the compounds **2.1d-f** and **2.3** have identical Ar moieties and comparable permeability (Table 2.1) but weaker Cl^- binding affinity ($K_d = 22.37$ mM). The acidity of N–H protons of the thiourea moieties is lower for compounds **2.1d-f** in comparison with compounds **2.2** and **2.3**. The stability of LUVs in the absence and presence of compounds was confirmed by transmission electron microscopy (Figure 2.46).²⁸ Additional dynamic light scattering measurements also support the stability of LUVs (Figure 2.46).²⁸ The leaching test confirmed that the compounds exclusively reside in the lipophilic membrane-bilayer environment (Figure 2.45).³¹ Hence, the Cl^- ion transportation by these compounds indicates that the conformation control could be due to the presence of diphenyl rings in the diamine-scaffold is crucial for better Cl^- ion transport activity for compounds **2.1d-f**. For a similar reason, compounds **2.2** and **2.3** showed poor Cl^- ion transport activity than compounds **2.1d-f**.

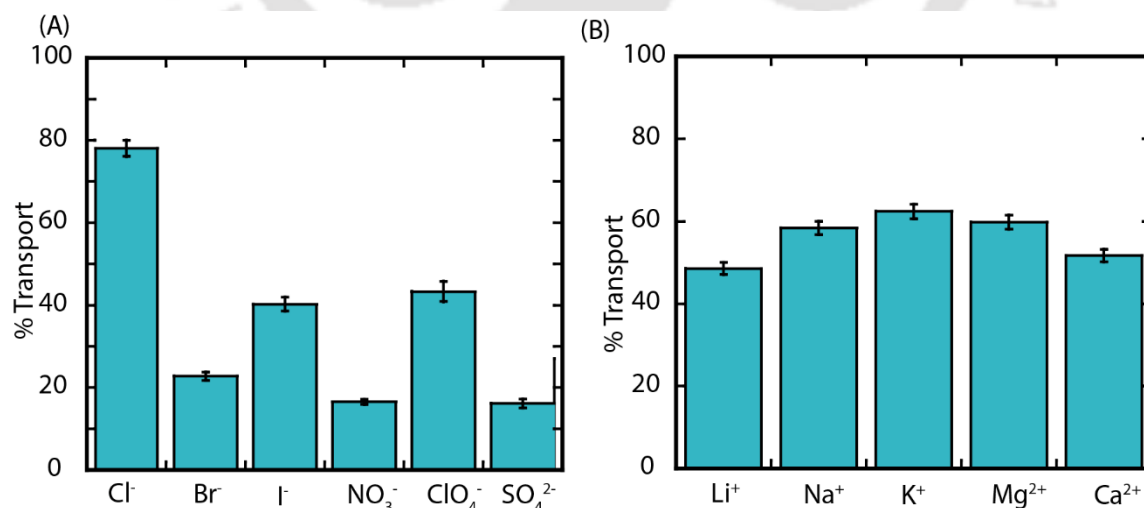


Figure 2.5. Anion and cation transport selectivity of compound **2.1f** across EYPC/CHOL-LUVs $\Rightarrow$ HPTS.

Table 2.2. Cl[−] Ion transport activities of the compounds under liposomal conditions.

Entry	% Cl [−] ion transport activity (lipid and compound ratio of 1 : 25,000) ^[a]		EC ₅₀ (nM)	Hill Co-efficient (n value)
	HPTS assay	Lucigenin assay		
2.1a	13.96 ± 1.23	7.03 ± 1.55	48.43 ± 7.9	1.675
2.1b	23.22 ± 1.23	12.01 ± 1.22	52.39 ± 3.6	0.931
2.1c	21.19 ± 2.21	12.49 ± 1.19	45.89 ± 4.1	0.977
2.1d	75.26 ± 4.89	59.86 ± 4.61	2.09 ± 0.2	1.347
2.1e	79.38 ± 2.31	63.86 ± 1.33	4.15 ± 0.5	0.761
2.1f	79.52 ± 1.58	65.08 ± 2.67	2.51 ± 0.2	1.394
2.2	22.43 ± 1.86	13.79 ± 2.44	36.62 ± 4.5	0.950
2.3	8.03 ± 0.31	3.53 ± 0.06	97.52 ± 11.7	0.851

^[a] % Cl[−] ion transport activities were compared at compound/lipid ratio of 1:25000

2.2.5. Relative cation and anion selectivity studies of the bis(thiourea) derivatives

The higher Cl[−] ion transport efficacy of the compounds **2.1d-f** inspired us to explore their Cl[−] ion selectivity in comparison with various other biologically relevant cations and anions. Anion selectivity was examined by monitoring anion transport activity across EYPC/CHOL-LUVs>HPTS bilayer with NaCl at the intravesicular region and iso-osmotic buffer solution of the sodium salt of various anions (Cl[−], Br[−], I[−], NO₃[−], ClO₄[−] and SO₄^{2−}) at the extravesicular region. Similarly, iso-osmotic solutions of the chloride salt of various cations (Li⁺, Na⁺, K⁺, Ca²⁺, and Mg²⁺) in the extravesicular region were used to investigate the selectivity of various cations. The relative change in the HPTS-fluorescence signal in the presence of compounds **2.1d-f** (at compound/ lipid ratios of 1:25,000) under similar experimental conditions indicates their ion recognition and transmembrane transport activity. These tested compounds **2.1d-f** follows an anion transport efficiency in the order of Cl[−] > ClO₄[−] > I[−] > Br[−] > NO₃[−] > SO₄^{2−} (Figure 2.5 and Figure 2.39-40). This Cl[−] ion transport selectivity of the compounds is in accordance with their respective ion binding affinity. We also observed that compound **2.1f** showed significant transport selectivity for Cl[−] ion over NO₃[−] ion. For Cl[−] ion transportation the calculated $r_i = 0.0726 \pm 0.0007 \text{ s}^{-1}$ and $t_{1/2} = 9.55 \pm 0.09 \text{ s}$, whereas for NO₃[−] ion

transportation the $r_i = 0.0213 \pm 0.0002 \text{ s}^{-1}$ and $t_{1/2} = 32.57 \pm 0.03 \text{ s}$ under the similar experimental conditions. This Cl^- vs. NO_3^- ion selectivity is in accordance with their binding affinities (measured from the ^1H -NMR titration measurements). The results also showed that in the presence of compounds, the monovalent (Li^+ , Na^+ , and K^+) and divalent (Ca^{2+} and Mg^{2+}) cations follow comparable transport rates regardless of the nature of the cation. This indicates no substantial role of these cations on Cl^- ion transportation. The moderate cations transport capabilities of these compounds suggest the possibility of nonspecific cations recognition through their alternative binding motif present in the molecular cavity.³² Anion ‘jump’ assay also displayed a similar order of anion selectivity of the compounds (Figure 2.42).

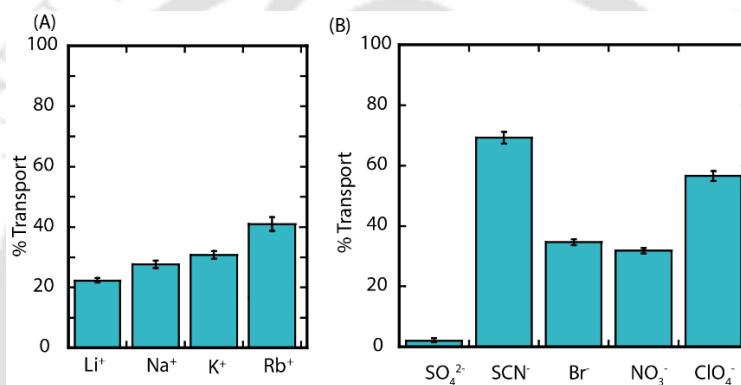


Figure 2.6. Influx of Cl^- ion across EYPC/CHOL-LUV $\supset$ lucigenin in the presence of compound 2.1f with intravesicular NaNO_3 and extravesicular MCl ($\text{M} = \text{Li}^+$, Na^+ , K^+ , and Rb^+) salt (A). Efflux of Cl^- ion across EYPC/CHOL-LUV $\supset$ lucigenin in the presence of compound 2.1f with intravesicular NaCl and extravesicular Na_xA ($\text{A}^- = \text{Br}^-$, SCN^- , SO_4^{2-} , ClO_4^- , NO_3^- ; $x = \text{valency}$) salt (B).

2.2.6. Mechanism of chloride ion transport activities of the bis(thiourea) derivatives

In the lucigenin-based Cl^- ion transport assay, a $\text{Cl}^-/\text{NO}_3^-$ gradient was constructed, and the influx of Cl^- ion into the vesicles was measured by monitoring the decrease of lucigenin fluorescence. This transmembrane Cl^- ion transportation into the NO_3^- containing vesicles incited us to investigate its transport mechanism. To retain the overall charge neutrality, the transportation of Cl^- ion may proceed through Cl^-/Na^+ symport or $\text{Cl}^-/\text{NO}_3^-$ antiport pathway. In this regard, chloride salt of various cations (LiCl , NaCl , KCl , and RbCl) and sodium salt of various anions (NaBr , NaSCN , Na_2SO_4 , NaClO_4 , and NaNO_3), were used in the extravesicular region to categorize the symport or

antiport mechanistic pathway (Figure 2.6 and Figure 2.43). Lucigenin assay of compound **2.1f** in the presence of these sodium salts of anions showed a substantial difference in the Cl^- ion transport rates. However, similar experiments in the presence of these chloride salts of cations revealed trivial differences in the Cl^- ion transportation rates. These results substantiate the possibility of the $\text{Cl}^-/\text{NO}_3^-$ antiport and rule out the prospect of Cl^-/Na^+ symport transport mechanistic pathway.

For further validation of this $\text{Cl}^-/\text{NO}_3^-$ antiport mechanistic pathway, we also performed lucigenin assay in the absence and presence of valinomycin (a K^+ selective transporter). EYPC/CHOL-LUV $\supset$ lucigenin in HEPES buffer containing NaNO_3 salt was used for this assay. The kinetic experiments were initiated with the addition of iso-osmolar KCl solution at the extravesicular region, and the ion transport rate of the compound **2.1f** was measured in the absence and presence of valinomycin. A significant increase in the anion transport rate in the presence of compound **2.1f** could be due to the synergistic effect of the compound and valinomycin under the vesicular environment (Figure 2.8A). Numerical analysis showed that in the presence of only compound **2.1f**, the $r_i = 0.00412 \pm 0.0012 \text{ s}^{-1}$ and $t_{1/2} = 168.17 \pm 0.09 \text{ s}$, whereas in the presence of both compound **2.1f** and valinomycin the $r_i = 0.04126 \pm 0.0003 \text{ s}^{-1}$ and $t_{1/2} = 16.80 \pm 0.11 \text{ s}$. This result showed ~10-fold increase in the Cl^- ion transport rate of compound **2.1f** in the presence of valinomycin, indicating their synergistic effect on Cl^- ion transportation across the bilayer (Figure 2.8B). This result clearly indicates the $\text{Cl}^-/\text{NO}_3^-$ antiport mechanistic pathway. We hypothesize that the other potent compounds **2.1d** and **2.1e** would also follow similar transport mechanisms.

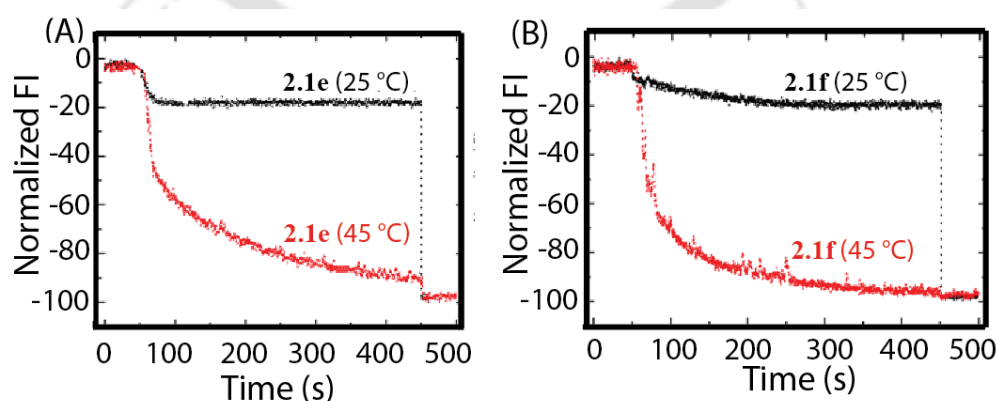


Figure 2.7. Temperature-dependent lucigenin assay to demonstrate the carrier-mechanistic pathway of Cl^- ion transport activity by the compound **2.1e** and **2.1f** across DPPC-LUV $\supset$ lucigenin.

2.2.7. Chloride ion transport pathway of the bis(thiourea) derivatives

Apart from the membrane lipids, most of the reported ion-binding small-molecules and supramolecules transport Cl^- ion across the biological membranes through mobile carrier or self-assembled channel pathway.^{14-15, 18, 26} To distinguish whether this bis(thiourea) derivative **2.1f** transport anion through carrier or channel mechanism, lucigenin assay was performed using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) lipid at different temperatures. Transitions from the solid gel to liquid crystal phase are regarded to be a crucial biophysical property of lipids to investigate the thermal stability and small molecule/ion release profile of the vesicles.^{18, 28} The randomness originated during phase transitions allow faster movement of the carriers, while the channels remain mostly unaffected. The phase transition temperature (T_m) of DPPC lipid is 41 °C, which indicates the increase in randomness of the lipid bilayer above this temperature. The transport activity was measured in the absence and presence of compounds using DPPC-LUV $\supset$ lucigenin in 20 mM HEPES buffer (at pH 7.2, containing NaNO_3) at 25 °C and 45 °C (temperature below and above phase transition, respectively). A sharp increase in ion transport activity of compound **2.1f** was observed when the temperature was raised from 25 to 45 °C (Figure 2.7). There is also a significant difference (5-6 fold) in the transport rates of the compound **2.1f** at the two different temperatures. Numerical analysis showed that the $r_i = 0.01179 \pm 0.0001 \text{ s}^{-1}$ and $t_{1/2} = 58.80 \pm 0.68 \text{ s}$ at 25 °C, whereas the $r_i = 0.0607 \pm 0.0008 \text{ s}^{-1}$ and $t_{1/2} = 11.42 \pm 0.16 \text{ s}$ at 45 °C (Table 2.6). A similar temperature-dependent Cl^- ion transport activity was observed for compounds **2.1e** (Figure 2.7). This strong correlation between Cl^- ion transport activity and membrane fluidity suggests its carrier-mechanistic pathway.³¹

2.2.8. Ion transport activities under cellular environment

2.2.8.1. MTT assay in presence and absence of chloride ions

Disruptions in Cl^- ion transportation could alter cellular pH and lead to the onset of apoptosis and related diseases. Recent studies showed that synthetic Cl^- ion transporters induce apoptosis-mediated activities in the cancer cells through the interference of ionic homeostasis of the cell.³³⁻³⁴ The strong Cl^- ion transportation properties of these bis(thiourea) derivatives prompted us to explore their activity under the cellular environment. The viability of normal (baby hamster kidney BHK-21) and model cancer (human cervical cancer HeLa and human tongue carcinoma SAS) cell lines in the presence

of these compounds correlate well with their Cl^- ion transport abilities. First, the viability of these cells was measured by MTT assay for compounds **2.1a-f**, **2.2**, and **2.3** at a fixed concentration (10 μM). For compounds, **2.1e** and **2.1f** maximum cell death was observed. (Figure 2.9A) The IC_{50} values of compounds **2.1e** and **2.1f** were in the range of 4.04–8.56 μM and 6.44–9.08 μM , respectively, for all these tested cell lines (Figure 2.47 and Table 2.7). The differences in IC_{50} values of the compounds **2.1e** and **2.1f** are very small. However, the IC_{50} values of compound **2.1e** is slightly lower than compound **2.1f**; hence it was selected for further cellular activity studies. The faster-growing cancer cells will presumably be more susceptible to these compounds than will be the kidney and other normal cells, which grow more slowly.

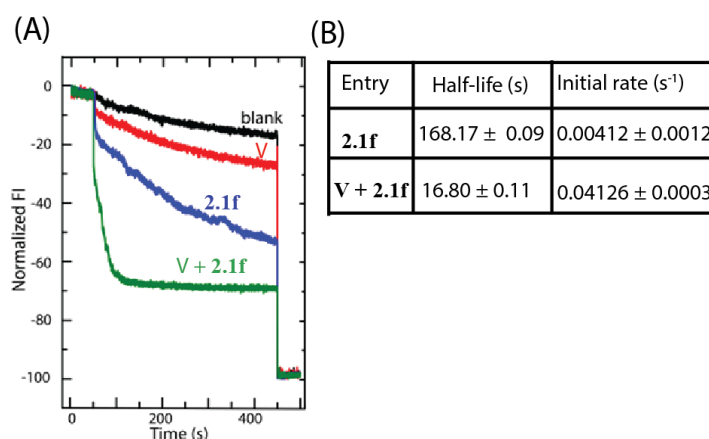


Figure 2.8. Comparison of Cl^- ion transport activity of compound **2.1f** in the absence and presence of valinomycin (V) (A). Calculated half-life and initial rate of Cl^- transport activity of compound **2.1f** (B).

To understand whether the cell viability of compound **2.1e** is due to the transportation of Cl^- ion into the cells and change in intracellular Cl^- ion concentration, MTT assay was performed using HBSS (Hank's balanced salt solution) buffer in the absence and presence of Cl^- ion. MTT assay showed that the extent of viability in the presence of compound **2.1e** is greater when the cells were suspended with Cl^- ion free HBSS buffer than the Cl^- ion-containing HBSS buffer (Figure 2.9B). This result suggests that intracellular transportation of Cl^- ion by compound **2.1e** is directly related to the extent of cell death.

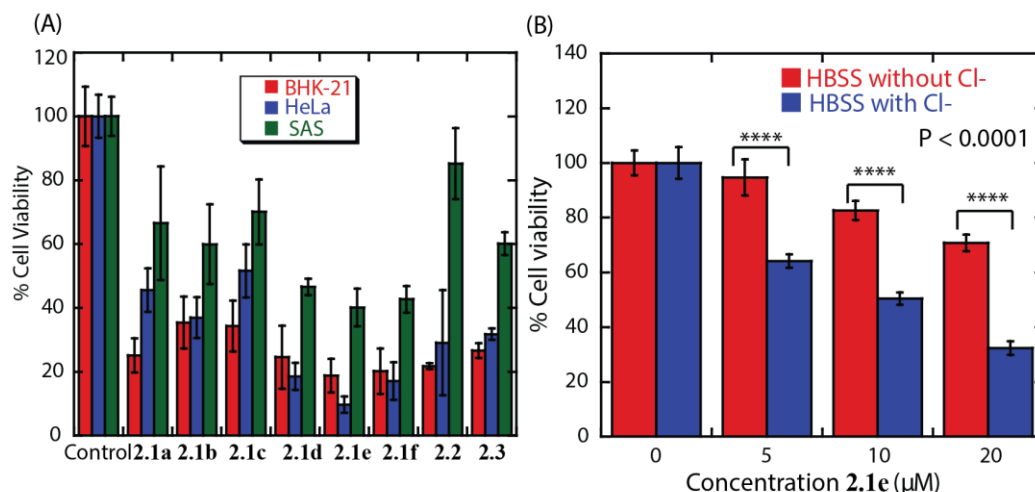


Figure 2.9. Cell viability of the compounds **2.1a-f**, **2.2**, and **2.3** was measured at a fixed concentration of 10 μM . Cell viability was measured in BHK-21, HeLa, and SAS cells after 24 hours of compound treatment. All experiments were performed in triplicate (A). Viability of HeLa cells in the absence and presence of Cl^- ions in HBSS buffer upon dose-dependent treatment of compound **2.1e** (0-20 μM) after 24 h of incubation. Each bar represents the mean intensity of three independent experiments, and the differences in mean intensities are statistically significant ($P < 0.001$ for according to two-way analysis of variance (ANOVA). (B).

2.2.8.2. Dye-based assay (JC 1, Hoechst 33342)

In general, Cl^- ion-assisted cell death is recognized to initiate the cellular apoptosis pathway.³⁴⁻³⁵ During apoptosis, a sequential change occurs in the cells, and this apoptotic process could be investigated by monitoring several cellular processes e.g., (1) mitochondrial membrane integrity, (b) phosphatidylserine redistribution and plasma membrane integrity, (c) expression level of the caspase family of proteins involved in caspase-dependent cascade pathways (d) nuclear fragmentation and DNA laddering of the cells.³⁴⁻³⁷ HeLa cells incubated with compound **2.1e** show morphological changes such as cell shrinkage and detachment from their neighboring cells. These observations indicate cell death through apoptosis.³⁸ Disruption of mitochondrial membrane potential (MMP) is considered as a pre-apoptotic indicator that could be monitored by using JC-1, a membrane potential sensitive dye.^{11-12, 33, 37} The decrease in red fluorescence signal of JC-1 dye due to the loss of its *J*-aggregates in the mitochondrial membrane indicates the disruption of MMP in the presence of compound **2.1e**. The depolarization of the mitochondrial membrane results in the diffusion of the JC-1 dye into the cytosol, resulting in green

fluorescence.^{11-12, 33} HeLa cells were incubated with compound **2.1e** (0-20 μ M) for 24 h, and the JC-1 dye was used to stain the cells. A stepwise reduction of the red fluorescence and enhancement of the green fluorescence signals of the JC-1 dye was observed in a concentration-dependent manner indicating the disruption of MMP in the presence of compound **2.1e** (Figure 2.10).

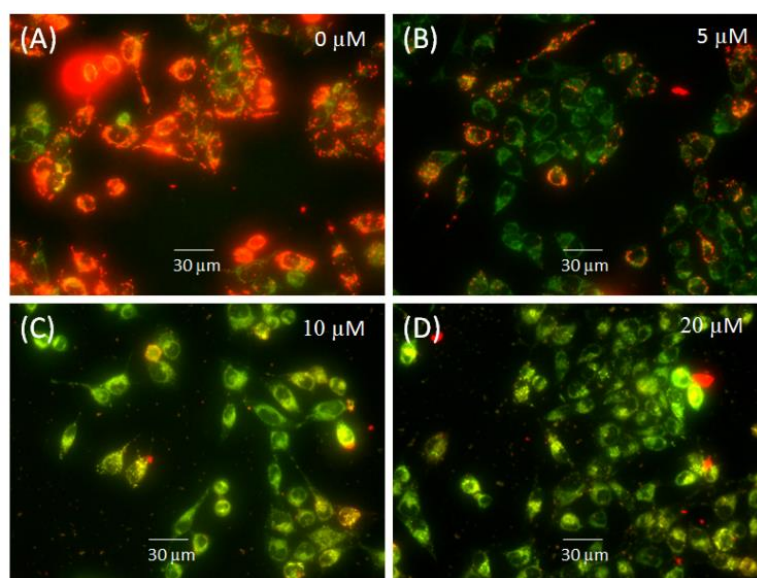


Figure 2.10. Representative images of HeLa cells in the presence of 0 (A), 5 (B), 10 (C) and 20 μ M (D) of compound **2.1e** after 24 hours of incubation. Cells were stained with JC-1 dye. Both red and green channels were merged in each image.

Nuclear condensation, fragmentation, and formation of apoptotic bodies are also considered as a hallmark of the late phase of apoptosis.³⁹⁻⁴⁰ The nuclear morphology of HeLa cells in the presence and absence of compound **2.1e** (10 μ M) shows significant changes that were investigated using Hoechst 33342 dye (Figure 2.11). The presence of nuclear condensation and fragmentation indicated the death of cells due to apoptosis.

2.2.8.3. Western blot and DNA laddering assay

This decrease in MMP instigates the release of cytochrome *c* to the cytoplasm where it binds to apaf-1 protein to form apoptosome.^{11-12, 36-37} The formation of apoptosome activates the caspase-9 protein that subsequently regulates different pathways, including caspase-3 dependent apoptosis through the intrinsic pathway of apoptosis.^{11-12, 36, 41-43} Apoptosis could also be initiated by a caspase-8 mediated extrinsic pathway.⁴³⁻⁴⁴ In this

regard, the expressions of the caspase enzymes in the HeLa cells were examined by immunoblot analysis for further understanding of the mitochondria-dependent pathway of apoptosis. The expression level of the initiator caspase-9, executor caspase-3, and caspase-8 were investigated in the presence of compound **2.1e** in a dose-dependent manner (0-20 μ M). The presence of cleaved caspase-9 and degradation of procaspase-3 indicates the caspase-dependent intrinsic pathway of apoptosis in the presence of compound **2.1e** (Figure 2.12). However, there is no activation of caspase-8 and no degradation of procaspase-8, which overlooks the prospect of the extrinsic pathway of apoptosis (Figure 2.12). In apoptotic cells, caspase-3 mediated proteolysis cleaves protein substrates into fragments. Poly(ADP-ribose) polymerase (PARP) family of proteins is involved in a number of cellular functions such as DNA repair, expression of essential genes during inflammation, and activation of chromatin remodeling enzymes, and apoptosis.⁴⁵ The presence of cleaved PARP is considered to be a hallmark of apoptosis in the presence of active caspase-3.⁴⁶ Presence of inactive PARP (89 kDa) in HeLa cells (Figure 2.12) supports the caspase-mediated apoptosis in the presence of compound **2.1e** (0-20 μ M).

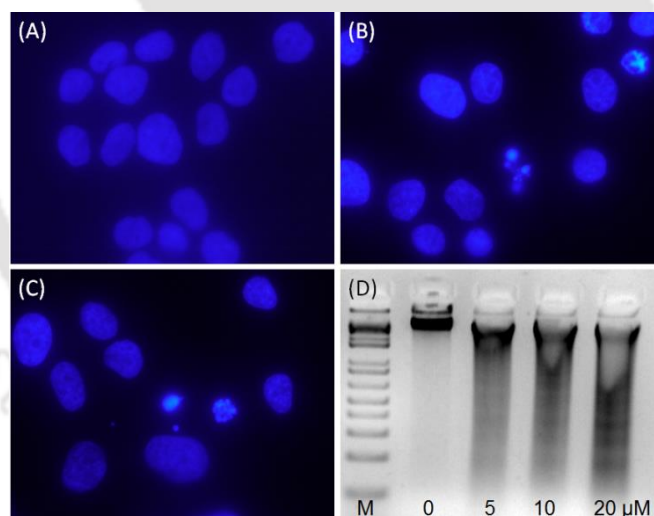


Figure 2.11. Compound **2.1e** induced apoptosis leads to nuclear fragmentation and release of the apoptotic body in HeLa cells. Nuclear fragmentation of HeLa cells in the presence of compound **2.1e** stained by Hoechst 33342 dye after 24 h incubation. The nucleus is intact in mock-infected cells (A), while it showed fragmentation following infection (B and C). The DNA laddering pattern in HeLa cells infected with compound **2.1e** (D).

Activation of caspase-3 also activates the cytosolic endonuclease CAD (or DFF40) by catalyzing the cleavage of its associated inhibitor ICAD (or DFF45). The active CAD enzyme enters into the nucleolus and degrades the DNA. Apoptotic DNA fragmentation is considered one of the key features of apoptosis, and a DNA laddering assay is being used to identify apoptotic cells.⁴⁷⁻⁴⁹ Activation of the endogenous nucleases of the apoptotic cells cleaved the DNA into internucleosomal fragments in the multiples of about 180-bp oligomers, which appears like a ladder on the agarose gel (Figure 2.11) demonstrates the classical DNA fragmentation in HeLa cells in the presence of compound **2.1e**. Phosphatidylserine is generally sequestered in the inner-plasma membrane but localizes to the outer plasma membrane during apoptosis and activates non-inflammatory phagocytic recognition of the apoptotic cell. Compound **2.1e** induced apoptotic property of HeLa cells was further reinforced by phosphatidylserine staining using fluorescein isothiocyanate-conjugated annexin-V under the flow cytometer. Compound **2.1e** (10 μ M) treated HeLa cells showed that 4.06% and 11.42% cells were present in early and late apoptotic stages, respectively. Whereas only 0.21% and 2.17% HeLa cells were present in the early and late apoptotic stages, respectively, in the absence of the compound **2.1e** (Figure 2.48). It delineates the apoptotic-like mode of death in HeLa cells following the treatment of compound **2.1e** (10 μ M) for 12 h.

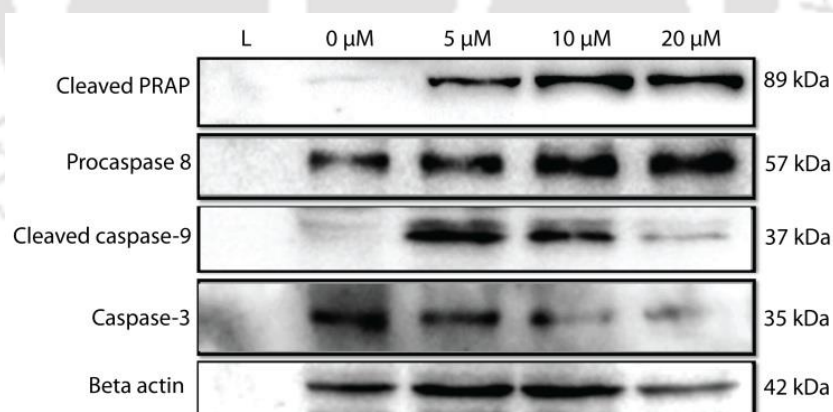


Figure 2.12. Immunoblot analysis of HeLa cells in the presence of compound **2.1e** after 24 h of incubation with different concentrations (0, 5, 10, and 20 μ M). L represents mock-infected HeLa cells. Antibodies against poly-ADP-ribose polymerase, Procaspase-8, caspase-3, cleaved caspase-9, and β -actin were used to develop the blot.

2.3. Summary

In this chapter, the bis(thiourea) derivatives of conformationally controlled 1,2-diphenylethylenediamin scaffolds are found to be promising anionophores with remarkable activities. The X-ray crystallographic study and ^1H NMR titrations confirmed their strong anion recognition capability. In solid-state, the Cl^- ion was found to have non-covalent interaction with two units of the anionophore (**2.1d**), whereas in the solution state, the interaction pattern was found to follow 1:1 binding model. The Cl^- ion transport properties of the potent compounds (**2.1d-f**) are very significant, with EC_{50} values in the range of 2.09-4.15 nM. The rapid and stronger Cl^- ion transport activity of these potent compounds could be due to their conformationally controlled structure and higher membrane permeability. The potent compounds are highly anion-selective and transported Cl^- ion across the lipid bilayer via $\text{Cl}^-/\text{NO}_3^-$ antiport mechanism. The dependence of high transport activity on membrane rigidity indicates the carrier pathway of Cl^- ion transport under the liposomal environment. The viability of different cell lines indicates that Cl^- transport activity is inversely related to their IC_{50} values. Additional MTT assay comparing the cell viability in the absence and presence of Cl^- ions in extracellular media confirmed the influx of Cl^- ion in the intracellular region of cells by the most active anionophore. The perturbation of Cl^- ion homeostasis of the cells altered the mitochondrial membrane potential indicating a pre-apoptotic pathway of cell death. Immunoblot analysis of the expressed family of caspases and cleaved PARP validated the mitochondria-dependent intrinsic apoptotic cell death pathway. The cellular expression level of caspase-9 and caspase-3 proteins indicates the intrinsic pathway of cell death in the most active molecule. Caspase-dependent nuclear fragmentation by this anionophore was also observed as the post-apoptosis process. Hence, these bis(thiourea) derivatives of the 1,2-diphenylethylenediamin scaffold are potent synthetic Cl^- ion transporters and act as apoptosis-inducing agents, which could be a useful tool in understanding the complex relationship between Cl^- ion transport and various disease associated with chloride dysfunction.

2.4. Experimental section

2.4.1. Synthesis of compounds

2.4.1.1. General information

Reagents and solvents were purchased from Sigma, Alfa Aesar and used directly without any further purification unless otherwise stated. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (0.25 mm). The column chromatography was performed with Merck silica gel of 120-200 mesh. ^1H NMR and ^{13}C NMR were recorded at 400 and 100 MHz, respectively, with a Bruker spectrometer. The chemical shifts were reported in parts per million (δ) using DMSO-d_6 as an internal solvent, coupling constants (J) were reported in hertz, and the abbreviation was reported as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiple), and br (broadened). High-resolution mass spectra (HRMS) were recorded at Agilent Q-TOF mass spectrometer with Z-spray source using built-in software for analysis of the recorded data. Egg yolk phosphatidylcholine (EYPC) and cholesterol were purchased from Sigma. Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylserine (DPPS) were purchased from Avanti Polar Lipids. HEPES buffer, HPTS, lucigenin, Triton X-100, NaOH, and inorganic salts were purchased of molecular biology grade from Sigma. Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers. Stock solutions of all the compounds were prepared in gas chromatographic grade DMSO (Sigma). Stock solutions of EYPC and cholesterol were prepared in spectroscopic grade $\text{CHCl}_3/\text{MeOH}$ at 8:2 ratios.

2.4.1.2. Synthesis of DPEN based anionophore

2.4.1.2.1. Synthesis of 1,2-diphenylethane-1,2-diamine moiety

To a stirring solution of ammonium acetate (84.9 mmol) in a 250 mL round bottom flask without any external solvent was added benzaldehyde (28.3 mmol) and the mixture was allowed to stir for 3 hours at 120 °C. Then the reaction mixture was allowed to cool down to room temperature, and the resulting yellow color cake was filtered and washed with water, 5% NaOH aqueous solution, and slightly hot alcohol simultaneously to get white color solid. This white solid was then allowed to suspend in concentrated sulphuric acid and heated at 180 °C for 3 hours. After that, the reaction mixture was cooled down to room temperature and diluted with ice water, and extracted with EtOAc (3 x 20 mL). The aqueous portion was basified with concentrated ammonium hydroxide solution until the white precipitation was observed. The aqueous portion was further extracted with EtOAc (3 x 20 mL). The resulting organic layer was washed with brine and dried over anhydrous

Na₂SO₄. Removal of organic solvent under reduced pressure produced a white solid, which was used further without any purification.⁵⁰

2.4.1.2.2. Synthesis of (1R, 2R)-1,2-diphenylethane-1,2-diamine / (1S, 2S)-1,2-diphenylethane-1,2 –diamine

First, hot EtOAc was added into a round bottom flask containing (R)-Mandelic or (S)-Mandelic acid. Then the solution was added slowly into the racemic mixture of DPEN compound, and the stirring was continued for 30 minutes. White precipitation was formed after refluxing the resulting solution for 45 minutes. Then the precipitate was filtered and washed with hot EtOAc to obtain the mandelate salt, further dissolved in 10 M NaOH solution. After that, the aqueous part was extracted using CH₂Cl₂ solvent (20 mL x 3). The organic layer was washed with brine and dried over anhydrous Na₂SO₄. Finally it was concentrated to obtain (1R, 2R)-1,2-diphenylethane-1,2-diamine or (1S, 2S)-1,2-diphenylethane-1,2-diamine as a colorless solid. Characterization of all these starting materials is in accordance with the literature.⁵¹⁻⁵²

2.4.1.2.3. General procedure for the synthesis of bis(thiourea) based anionophores

To a solution of respective diamine (0.471 mmol) in CH₂Cl₂ was added a solution of substituted isothiocyanate (0.942 mmol) in CH₂Cl₂, and the mixture was allowed to stir for 10 hours at room temperature. While precipitation was observed, which was filtered and washed with CH₂Cl₂ to get the pure product as white solid.^{21, 53-55}

2.4.1.3. Characterization of synthesized compounds

1,1'-(±1,2-diphenylethane-1,2-diyl)bis(3-(4-(trifluoromethyl)phenyl)thiourea (2.1a): ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 9.78 (s, 2H), 8.60 (brs, 2H), 7.55 (d, *J* = 8.5 Hz, 4H), 7.50 – 7.47 (m, 4H), 7.39 (t, *J* = 7.6 Hz, 4H), 7.33 – 7.29 (m, 2H), 7.27 (d, *J* = 8.4 Hz, 4H), 6.21 (brs, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ_{ppm} 179.2, 142.3, 138.7, 127.6, 127.4, 127.3, 127.1, 126.9, 126.6, 126.3, 125.1, 125.1, 125.0, 123.4, 123.0, 122.7, 122.4, 121.2, 60.7; HRMS (ESI) calcd. for C₃₀H₂₄F₆N₄S₂ (M⁺ H)⁺: 619.1419, found: 619.1419.

1,1'-((1R,2R)-1,2-diphenylethane-1,2-diyl)bis(3-(4-(trifluoromethyl)phenyl)thiourea (2.1b): ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 9.87 (s, 2H), 8.68 (brs, 2H), 7.55 (d, *J* = 8.5 Hz, 4H), 7.49 (d, *J* = 7.6 Hz, 4H), 7.38 (t, *J* = 7.5 Hz, 4H), 7.32 – 7.30 (m, 6H), 6.21 (brs,

2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ_{ppm} 179.2, 142.4, 138.7, 127.6, 127.4, 127.3, 127.1, 126.9, 126.5, 125.1, 125.0, 122.4, 121.2, 59.8; HRMS (ESI) calcd. for $\text{C}_{30}\text{H}_{24}\text{F}_6\text{N}_4\text{S}_2$ ($\text{M}^+ \text{H}^+$): 619.1419, found: 619.1420.

1,1'-((1S,2S)-1,2-diphenylethane-1,2-diyl)bis(3-(4-(trifluoromethyl)phenyl)thiourea

(2.1c): ^1H NMR (600 MHz, DMSO- d_6) δ_{ppm} 9.80 (s, 2H), 8.61 (brs, 2H), 7.55 (d, $J = 8.4$ Hz, 4H), 7.49 (d, $J = 7.6$ Hz, 4H), 7.38 (t, $J = 7.5$ Hz, 4H), 7.33 – 7.29 (m, 6H), 6.21 (brs, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ_{ppm} 179.2, 142.4, 138.4, 127.6, 127.4, 126.9, 125.1, 125.0, 125.0, 124.9, 123.3, 122.9, 122.7, 122.4, 121.1, 69.4; HRMS (ESI) calcd. for $\text{C}_{30}\text{H}_{24}\text{F}_6\text{N}_4\text{S}_2$ ($\text{M}^+ \text{H}^+$): 619.1419, found: 619.1417.

1,1'-±-1,2-diphenylethane-1,2-diyl)bis(3-(3,5-bis(trifluoromethyl)phenyl)thiourea

(2.1d): ^1H NMR (600 MHz, DMSO- d_6) δ_{ppm} 9.96 (s, 2H), 8.75 (brs, 2H), 7.99 (s, 4H), 7.74 (s, 2H), 7.45 (d, $J = 7.6$ Hz, 4H), 7.36 (t, $J = 7.3$ Hz, 4H), 7.34-7.28 (m, 2H), 6.26 (brs, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ_{ppm} 180.1, 141.3, 138.7, 130.6, 130.3, 129.4, 129.6, 128.0, 127.8, 127.5, 124.4, 122.2, 121.7, 116.4, 60.0; HRMS (ESI) calcd. for $\text{C}_{32}\text{H}_{22}\text{F}_{12}\text{N}_4\text{S}_2$ ($\text{M}^+ \text{H}^+$): 755.1167, found: 755.1167.

1,1'-((1R,2R)-1,2-diphenylethane-1,2-diyl)bis(3-(3,5-

bis(trifluoromethyl)phenyl)thiourea (2.1e): ^1H NMR (600 MHz, DMSO- d_6) δ_{ppm} 9.98 (s, 2H), 8.76 (s, 2H), 8.0 (s, 4H), 7.74 (s, 2H), 7.45 (d, $J = 7.5$ Hz, 4H), 7.36 (t, $J = 7.5$ Hz, 4H), 7.34-7.28 (m, 2H), 6.26 (brs, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ_{ppm} 180.1, 141.4, 138.7, 130.3, 129.9, 127.9, 127.8, 127.5, 124.5, 122.1, 121.7, 116.3, 61.0; HRMS (ESI) calcd. for $\text{C}_{32}\text{H}_{22}\text{F}_{12}\text{N}_4\text{S}_2$ ($\text{M}^+ \text{H}^+$): 755.1167, found: 755.1168.

1,1'-((1S,2S)-1,2-diphenylethane-1,2-diyl)bis(3-(3,5-

bis(trifluoromethyl)phenyl)thiourea (2.1f): ^1H NMR (600 MHz, DMSO- d_6) δ_{ppm} 10.02 (s, 2H), 8.78 (s, 2H), 8.01 (s, 4H), 7.74 (s, 2H), 7.45 (d, $J = 7.5$ Hz, 4H), 7.36 (t, $J = 7.5$ Hz, 4H), 7.31-129 (m, 2H), 6.25 (brs, 2H); ^{13}C NMR (100 MHz, DMSO- d_6) δ_{ppm} 180.1, 141.3, 138.7, 130.6, 130.3, 129.9, 129.6, 128.0, 127.8, 127.5, 122.2, 121.7, 60.0; HRMS (ESI) calcd. for $\text{C}_{32}\text{H}_{22}\text{F}_{12}\text{N}_4\text{S}_2$ ($\text{M}^+ \text{H}^+$): 755.1167, found: 755.1169.

1,2-phenylene)bis(3-(3,5-bis(trifluoromethyl)phenyl)thiourea (2.2): ^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ_{ppm} 10.12 (s, 2H), 9.69 (s, 2H), 8.14 (s, 4H), 7.56 (s, 2H), 7.52-7.49 (m, 2H), 7.33 (t, $J=6.0, 3.8\text{Hz}$, 2H); ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ_{ppm} 179.4, 140.9, 133.1, 129.7, 129.4, 129.1, 128.8, 127.9, 126.9, 126.6, 123.9, 123.0, 121.2, 1185.5 116.4; HRMS (ESI) calcd. for $\text{C}_{24}\text{H}_{14}\text{F}_{12}\text{N}_4\text{S}_2$ ($\text{M} + \text{H}$) $^+$: 651.0541, found: 651.0534.

1,1'-(cyclohexane-1,2-diyl)bis(3-(3,5-bis(trifluoromethyl)phenyl)thiourea (2.3): ^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ_{ppm} 10.14 (s, 2H), 8.18 – 8.15 (m, 6H), 7.70 (s, 2H), 4.35 (s, 2H), 2.20 (s, 2H), 1.73 – 1.72 (m, 2H), 1.30 (d, $J= 6\text{Hz}$, 4H); ^{13}C NMR (150 MHz, $\text{DMSO-}d_6$) δ_{ppm} 180.5, 142.1, 130.7, 130.5, 126.3, 124.5, 122.7, 122.5, 120.9, 116.6, 57.3, 40.5, 31.6, 24.7; HRMS (ESI) calcd. for $\text{C}_{24}\text{H}_{20}\text{F}_{12}\text{N}_4\text{S}_2$ ($\text{M} + \text{H}$) $^+$: 657.1011, found: 657.1007.

2.4.2. ^1H NMR titration experiments

2.4.2.1. Chloride ion binding through ^1H NMR titration

^1H -NMR titrations of the synthesized compounds (5.3 mM stock solution) in $\text{DMSO-}d_6$ were performed by the subsequent addition of tetrabutylammonium chloride (0- 20 equiv.) as a Cl^- ion source. The changes in the chemical shift of the N-H protons of the thiourea-moieties were analyzed. Significant extents of chemical shift ($\Delta\delta$) of both N-H protons were observed. All ^1H NMR spectra were stacked by using the MestReNova software. The changes in a chemical shift against the chloride ion concentration were fitted by the WinEQNMR2 program using a 1:1 binding model.⁵⁶ The dissociation constant (K_d) values were determined by taking the reciprocal of the binding constant (K_a ; as mentioned in WinEQNMR2 program). The following equation (Eq.-2.1) was used to calculate the binding constant-

$$\delta_{\text{cal}} = \sum_{m=1}^{m=i} \sum_{n=0}^{n=j} \frac{\delta_{mn} \beta_{mn} m [\text{M}]^m [\text{L}]^n}{[\text{M}]_{\text{total}}} \dots \dots \dots \text{Eq. 2.1}$$

Where, M represents the free, uncomplexed receptor and L is the ligand; δ_{calc} , is the weighted average of the chemical shifts of the various M -containing species present, $M_m L_n$, and i and j represent the maximum values of m and n respectively.

2.4.2.2. ^1H NMR titration with different anion

To investigate anion recognition capabilities of the DPEN derivatives, ^1H NMR titration of potent compound **2.1f** was also carried out with TBANO_3 using a similar experimental protocol as mentioned at section 2.4.2.1..²⁴

2.4.3. Job's plot

A continuous variation method of Job's plot was used to determine the binding stoichiometry of the complex which was formed during the titration of compound **2.1d** in the presence of Cl^- ion (TBACl). The main stock solution of the host (compound **2.1d**) and the guest (TBACl) were prepared in DMSO- d_6 solvent at room temperature. The concentration of the stock solution of both host and guest was fixed at 5.0 mM. During the measurements total of 10 separate NMR tubes containing the required host and guest, concentration were prepared to make the total final concentration fixed at 5.0 mM. The chemical shift values were recorded, and the change of chemical shift of N-H proton of the thiourea moiety at $\delta = 9.97$ ppm at a different mole fraction of the Cl^- ion was listed in Table 2.3. The Job's plot was shown in Figure 2.27, which indicated a 1:1 binding Stoichiometry of the complex.¹¹

2.4.4. Crystallographic Study

The crystallographic data were collected from the Bruker Nonius SMART APEX CCD diffractometer equipped with a graphite monochromator and Apex CCD camera using SMART software. All crystallographic data were refined using the SHELXL-2014/7 and or Olex2 1.2-alpha (Table S2). The ORTEP diagram was obtained with the help of ORTEP software (Figure S13). The tetrabutylammonium chloride salt was used as a chloride ion source for co-crystallization with the compound **2.1d** in the presence of DMSO solvent. In the co-crystal of compound **2.1d** with TBACl, the disorderliness of C-F bonds were refined by sharing the electron density with neighboring fluorine atom with equal sharing of 50% occupancies. All crystallographic parameters and refinement data were tabulated in Table S2. In the ORTEP presentation, all the H-atoms are omitted for clarity and listed in Figure 2.28.

2.4.5. Ion transport experiments

2.4.5.1. Ion transport activity studies using fluorescence-based assay

2.4.5.1.1. Preparation of HPTS encapsulated EYPC/CHOL-LUV $\Rightarrow$ HPTS

To prepare the large unilamellar vesicles (LUVs) of EYPC/CHOL-LUV $\Rightarrow$ HPTS, first 50 μ L of EYPC (100 mg/mL in 8:2 CHCl₃/MeOH) and 80 μ L of cholesterol (25 mg/mL in 8:2 CHCl₃/MeOH) 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 2-3 hours to obtain a thin film of lipids inside the glass vial. To remove any traces of CHCl₃ and MeOH solvent the transparent film was kept under reduced pressure for 4-5 hours at room temperature. The dry film was then hydrated with 800 μ L of 20 mM HEPES buffer, pH 7.2 containing 100 mM NaCl and 1 mM HPTS for 2 hours with occasional vortexing. Then, this suspension was sonicated (5 times, 30 sec of sonication followed by 30 sec of incubation in ice-water) to disrupt the aggregated vesicles. The suspension was further passed through 12-13 cycles of freeze-thaw (freezing with liquid N₂ and melting with lukewarm water, respectively) to break up the multilamellar vesicles. The vesicle solution was extruded through a polycarbonate membrane (using a mini-extruder from Avanti Polar Lipids) having a pore size of 200 nm (size of LUVs are > 200 nm) for 19/21-times (as it must be an odd number), to give LUVs with a mean diameter of $\sim$ 200 nm. Finally, gel filtration (Sephadex G-50) column chromatography was performed with 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl as a running solution to removed free/ uncapsulated HPTS. The HPTS encapsulated LUVs (without extra vesicular HPTS dye) were collected, and the final volume was adjusted to 800 μ L using 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl. The final lipid concentration obtained was 25 mM (assuming 100 % lipid regeneration).¹¹⁻¹²

2.4.5.1.2. Ion transport activity assay across EYPC/CHOL-LUV $\Rightarrow$ HPTS

For the HPTS assay, first 2925 μ L of 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl and 50 μ L of the EYPC/CHOL-LUV $\Rightarrow$ HPTS were taken in a 3 mL fluorescence cuvette, and the cuvette was placed in the fluorescence spectrophotometer under slow stirring condition (inbuilt magnetic stirrer in the instrument). After that, compounds (10 μ L from a 5 μ M stock solution in DMSO) were added to the solution to achieve a concentration ratio of 1: 25,000 for compound and lipids. The cuvette was then kept in the fluorescence instrument under stirring conditions 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 ($\lambda_{\text{ex}} = 450$ nm). Subsequently, 15 μ L of NaOH (0.5

M) solution was added into the cuvette after 50 sec to create a pH gradient ($\Delta\text{pH} = \sim 0.5$) between the extra and intra-vesicular regions and to initiate the Cl^- transport kinetics. After 350 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 (i.e., up to $t = 400$ sec).¹¹⁻¹²

2.4.5.1.3. Quantitative measurement of transport activity from HPTS

The fluorescence emission intensities (Y-axis) of the HPTS dye were normalized, and the intensities are appearing at $t = 0$ and $t = 400$ s were taken as 0, and 100 units, respectively, and the normalized fluorescent intensities (FI) at $t = 350$ s (prior to the addition of Triton X-100) were considered to measure the transport activity of the compounds.

$$\text{i.e. Transport activity, } T_{\text{HPTS}} = \frac{F_t - F_0}{(F_\infty - F_0)} \times 100 \% \dots\dots\dots \text{Eq. 2.2}$$

Where, F_t = fluorescence intensity at $t = 350$ 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_∞ = fluorescence intensity after addition of Triton X-100 (i.e., at saturation after complete leakage at $t = 400$ s).¹¹⁻¹²

2.4.5.1.4. Preparation of lucigenin encapsulated EYPC/CHOL-LUV $\rightarrow$ lucigenin

To prepare the LUVs of EYPC/CHOL-LUV $\rightarrow$ lucigenin, first 50 μL of EYPC (100 mg/mL in 8:2 $\text{CHCl}_3/\text{MeOH}$) and 80 μL of cholesterol (25 mg/mL in 8:2 $\text{CHCl}_3/\text{MeOH}$) 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 2-3 hours to obtain a thin film of lipids inside the glass vial. To remove any traces of CHCl_3 and MeOH solvent, the transparent film was kept under reduced pressure for 4-5 hours at room temperature. The dry film was then hydrated with 800 μL of 20 mM HEPES buffer, pH 7.2 containing 100 mM NaNO_3 and 1 mM lucigenin for 2 hours with occasional vortexing. Then, this suspension was sonicated (5 times, 30 sec of sonication followed by 30 sec of incubation in ice-water) to disrupt the aggregated vesicles. The suspension was further passed through 12-13 cycles of freeze-thaw (freezing with liquid N_2 and melting with lukewarm water, respectively) to break up the multilamellar vesicles. The vesicle

solution was extruded through a polycarbonate membrane with a pore size of 200 nm for 19/21-times to give LUVs a mean diameter of ~200 nm. Finally, gel filtration (Sephadex G-50) column chromatography was performed with 20 mM HEPES buffer, pH 7.2, containing 100 mM NaNO₃ as a running solution to removed free/ uncapsulated lucigenin. The lucigenin encapsulated LUVs (without extra vesicular lucigenin dye) were collected and the final volume was adjusted to 800 µL using 20 mM HEPES buffer pH 7.2, containing 100 mM NaNO₃. The final lipid concentration obtained was 25 mM (assuming 100 % lipid regeneration).¹¹⁻¹²

2.4.5.1.5. Ion transport activity assay across EYPC/CHOL-LUV $\rightarrow$ lucigenin

For the lucigenin assay, first 2890 µL of 20 mM HEPES buffer, pH 7.2, containing 100 mM NaNO₃ and 50 µL of the EYPC/CHOL-LUV $\rightarrow$ lucigenin were taken in a 3 mL fluorescence cuvette, and the cuvette was placed in the fluorescence spectrophotometer under slow stirring condition. After that, compounds (10 µL from a 5 µM stock solution in DMSO) were added to the solution to achieve a concentration ratio of 1: 25,000 for compound and lipids. The cuvette was then kept in the fluorescence instrument under stirring conditions for 3 minutes to allow maximum incorporation of the compounds into the lipid bilayers. The lucigenin fluorescence intensity was monitored (t = 0 sec) at 506 nm ($\lambda_{\text{ex}} = 455$ nm). Subsequently, 50 µL of NaCl (2.0 M) solution was added into the cuvette after 25 sec to initiate the Cl⁻ transport kinetics. After 475 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 25 sec (i.e., up to t = 500 sec).¹¹⁻¹²

2.4.5.1.6. Quantitative measurement of transport activity from lucigenin assay

The fluorescence emission intensities (Y-axis) of the lucigenin dye were normalized, and the intensities are appearing at t = 0 and t = 500 s were taken as 0, and 100 units, respectively, and the normalized fluorescent intensities (FI) at t = 475 s (prior to the addition of Triton X-100) were considered to measure the transport activity of the compounds.

$$\text{i.e. Transport activity, } T_{\text{lucigenin}} = \frac{F_0 - F_t}{(F_0 - F_{\infty})} \times 100 \% \dots\dots\dots \text{Eq. 2.3}$$

Where, F_0 = fluorescence intensity immediately before the addition of the NaCl (at $t = 0$ s), F_∞ = Fluorescence intensity after addition of Triton X-100 (i.e., at saturation, after complete leakage at $t = 500$ sec), F_t = Fluorescence intensity at $t = 475$ sec (prior to the addition of Triton X-100).¹¹⁻¹²

2.4.5.1.7. Half-life and initial transport rate calculation from lucigenin assay

The kinetic data obtained for quenching of fluorescence intensity of lucigenin were normalized for simplicity of analysis, and the Normalized fluorescence quenching curves (F/F_0) were fitted to a first-order exponential decay equation.

$$\frac{F}{F_0} = y + a \cdot e^{-[A]t} \quad \dots \dots \dots \text{Eq. 2.4}$$

At, $t = t_{1/2}$ the value of $\frac{F}{F_0} = y + \frac{a}{2}$

Introducing the Eq. 2.4 and we get

$$t_{1/2} = \frac{\ln(2)}{[A]} \quad \dots \dots \dots \text{Eq. 2.5}$$

The initial transport rates were determined from a second-order exponential decay equation

$$\frac{F}{F_0} = y + a \cdot e^{(-bt)} + c \cdot e^{(-dt)} \quad \dots \dots \dots \text{Eq. 2.6}$$

Differentiating according to t gives,

$$\frac{\partial y}{\partial t} = a \cdot b \cdot e^{(-bt)} + c \cdot d \cdot e^{(-dt)} \quad \dots \dots \dots \text{Eq. 2.7}$$

The initial transport rate r_i ($t = 0$) gives,

$$r_i = \frac{\partial y}{\partial t_{t=0}} = a \cdot b + c \cdot d \quad \dots \dots \dots \text{Eq. 2.8}$$

The initial transport rate was calculated from a second order exponential decay curve to obtain improved regression factor (R^2).¹¹⁻¹²

2.4.5.1.8. Measurement of half maximal effective concentrations (EC_{50}) of compound from HPTS assay

The fluorescence signals (Y-axis) from the HPTS- dye were normalized between 0 to 100 units [$t = 0$ to $t = 400$ s (X-axis)]. The normalized fluorescent intensity (FI) values at $t = 350$ s (prior to the addition of Triton X-100) were considered the compounds' transport activity. The transport activity (T) of a compound at a particular concentration was determined by using previous Eq.2.2. To get the effective concentration (EC_{50}) of the compound, the transport activity values (Y-axis) were plotted against concentration (X-axis) and fitted in the Hill equation (Eq.2.9).

$$T = T_{\infty} + \frac{T_0 - T_{\infty}}{\left[1 + \left(\frac{C}{EC_{50}}\right)^n\right]} \quad \dots \dots \dots \text{Eq. 2.9}$$

Here, T_0 and T_{∞} correspond to the transport activity obtained in the absence and at an excess concentration of the compound, respectively. C = concentration of the compound. EC_{50} value corresponds to the concentration of compound required to obtain half of the maximum transport activity. The number of molecules required for the transportation of a single ion is given by 'n', and it is the Hill coefficient for the transporter molecule.¹¹⁻¹²

2.4.6. Ion selectivity studies

2.4.6.1. Ion selectivity studies with base pulse using the fluorescence-based assay

2.4.6.1.1. Anion selectivity assay across EYPC/CHOL-LUV>HPTS

For anion selectivity assay, 2925 μ L of 20 mM HEPES buffer pH 7.2, containing 100 mM of Na_xA salt (where $A = Cl^-$, Br^- , I^- , NO_3^- , SO_4^{2-} and ClO_4^- ; x = valency) and 50 μ L of the EYPC/CHOL-LUV>HPTS were taken in a 3 mL fluorescence cuvette. To this buffer was added 10 μ L of 5 μ M stock solution of the compounds in DMSO (concentration ratio of compound and lipid was 1: 25,000). The cuvette was then kept inside the fluorescence instrument under continuous stirring conditions for 3 min to allow maximum incorporation of the compound into the vesicles. After that, 15 μ L of NaOH

(0.5 M) solution was added into the cuvette after 50 sec to initiate the Cl^- ion transport kinetics and the fluorescence emission of HPTS was monitored at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$). Finally, the kinetic experiment was terminated by the addition of 20 μL of Triton X-100 (20 %) in the cuvette at $t = 350 \text{ sec}$ (to lyse the vesicles), and fluorescent measurements were continued for another 50 sec (i.e., up to $t = 400 \text{ sec}$). The fluorescence emission intensities (Y-axis) of the HPTS⁻ dye were normalized, and the intensities at $t = 0$ and $t = 400 \text{ s}$ were normalized to 0 and 100 units, respectively.¹¹⁻¹²

2.4.6.1.2. Anion ‘jump’ assay

Anion jump assay was performed according to the mentioned assay conditions for anion selectivity assay. Only EYPC/CHOL-LUV $\Rightarrow$ HPTS was prepared using 20 mM HEPES buffer pH 7.2 (no ions added). No pH gradient was generated. The final external concentration of NaX was 33 mM.

2.4.6.1.3. Cation selectivity studies

Cation selectivity studies were also performed by the HPTS based assay as described above. HEPES Buffer and EYPC/CHOL-LUV $\Rightarrow$ HPTS were prepared following a similar method as described above, except 100 mM of MCl salt (where $\text{M} = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Ca}^{2+}$, and Mg^{2+}) was taken in the inner environment of the membrane instead of Na_xA salt (where $\text{A} = \text{Cl}^-, \text{Br}^-, \text{I}^-, \text{NO}_3^-, \text{SO}_4^{2-}$ and ClO_4^- ; $x = \text{valency}$). MOH was used to maintain the pH of the respective buffer.

2.4.7. Evidence of antiport mechanistic pathway from the lucigenin assay

2.4.7.1. Contribution of cations in the chloride transport process

The contribution of cations in the chloride transport process was investigated using the lucigenin assay. The EYPC/CHOL-LUV $\Rightarrow$ lucigenin were prepared following a similar method as described above. In this assay, 2890 μL of 20 mM HEPES buffer pH 7.2, containing 100 mM NaNO_3 and 50 μL of 25 mM EYPC/CHOL-LUV $\Rightarrow$ lucigenin (with EYPC: cholesterol ratio of 6:4) were taken in a 3 mL fluorescence cuvette. To this, 10 μL solution of 5 μM compound dissolved in DMSO was added. The cuvette was then kept inside the fluorescence instrument under continuous stirring conditions for 3 min. The kinetic measurements were started after 3 minutes, and the fluorescence emission of lucigenin was monitored at $\lambda_{\text{em}} = 506 \text{ nm}$ ($\lambda_{\text{ex}} = 455 \text{ nm}$). After 50 sec, 50 μL of 2 M MCl

($M = Li^+$, Na^+ , K^+ , and Rb^+) was added to initiate the Cl^- influx kinetics. Finally, the kinetic experiment was terminated by the addition of 20 μL of Triton X-100 (20 %) in the cuvette at $t = 350$ sec (to lyse the vesicles), and fluorescent measurements were continued for another 50 sec (i.e., up to $t = 400$ sec). The fluorescence emission intensities (Y axis) of the lucigenin dye were normalized, and the intensities at $t = 0$ and $t = 400$ s were normalized to 0 and 100 units, respectively. (Here, compound and lipid concentration ratio of 1: 25,000).¹¹⁻¹²

2.4.7.2. Contribution of anions in the chloride transport process

The contribution of anions in the chloride transport process was also assessed using the modified lucigenin assay. EYPC/CHOL-LUV $\supset$ lucigenin were prepared following a similar method as described above (except, 100 mM NaCl was used in place of $NaNO_3$ during the preparation of the LUVs). For this assay, 20 mM HEPES buffer, pH 7.2 was prepared using sodium salts of various anions keeping the concentration fixed at 100 mM Na_xA ($A^- = Br^-$, I^- , NO_3^- , ClO_4^- , SO_4^{2-} ; $x =$ valency). In this assay, 2890 μL of this solution was taken in a 3 mL fluorescence cuvette, and 50 μL of 25 mM EYPC/CHOL-LUV $\supset$ lucigenin was added. The cuvette was then kept inside the fluorescence instrument under continuous stirring conditions for 3 minutes. Lucigenin emission kinetics was started (at $t = 0$ s) and monitored at $\lambda_{em} = 506$ nm ($\lambda_{ex} = 455$ nm). After 50 sec of the start of the experiment, 10 μL of DMSO solution of the compound was added to initiate the Cl^- influx kinetics. Finally, the kinetic experiment was terminated by the addition of 20 μL of Triton X-100 (20 %) in the cuvette at $t = 350$ sec (to lyse the vesicles), and fluorescent measurements were continued for another 50 sec (i.e., up to $t = 400$ sec). The fluorescence emission intensities (Y-axis) of the lucigenin dye were normalized, and the intensities at $t = 0$ and $t = 400$ s were normalized to 0 and 100 units, respectively. (Here, compound and lipid concentration ratio of 1: 25,000).¹¹

2.4.7.3. Preferential transportation mechanism across EYPC/CHOL-LUV $\supset$ lucigenin (in the presence of valinomycin)

To confirm the preferential Cl^- ion transport mechanism, lucigenin assay was performed in the presence and absence of valinomycin. 20 mM HEPES buffer pH 7.2, containing 100 mM $NaNO_3$ and EYPC/CHOL-LUV $\supset$ lucigenin were used for this experiment. In this assay, 2890 μL of 20 mM HEPES buffer pH 7.2, containing 100 mM

NaNO₃ and 50 μ L of EYPC/CHOL-LUV $\supset$ lucigenin (stock solution of vesicles, 25 mM) were taken in a 3 mL fluorescence cuvette. After that, valinomycin (5 μ L of 2.5 μ M DMSO stock solution) or 5 μ L only DMSO (in the absence of valinomycin) and compound (5 μ L of 2.5 μ M DMSO stock solution) were added into the cuvette (to achieve a concentration ratio of 1: 25,000 for compound and lipids). The cuvette was then kept inside the fluorescence instrument under continuous stirring conditions for 3 minutes for maximum incorporation of the compounds into the vesicles. Lucigenin emission kinetics was started (at $t = 0$ sec) and monitored at $\lambda_{em} = 506$ nm ($\lambda_{ex} = 455$ nm). After 50 sec of the start of the experiment, KCl (50 μ L of 2 M solution) was added to initiate the Cl⁻ influx kinetics. Finally, the kinetic experiment was terminated by the addition of 20 μ L of Triton X-100 (20 %) in the cuvette at $t = 450$ sec (to lyse the vesicles), and fluorescent measurements were continued for another 50 sec (i.e., up to $t = 500$ sec). The lucigenin dye's fluorescence emission intensities (Y-axis) were normalized, and the intensities at $t = 0$ and $t = 500$ s were normalized to 0 and 100 units, respectively. The half-life of the Cl⁻ ion transport efficacy in the absence and the presence of the valinomycin were determined by applying equations, Eq.2.5 and Eq.2.8, respectively.¹¹

2.4.8. Evidence for the carrier mechanism

2.4.8.1. Preparation of lucigenin encapsulated 1,2-dipalmitoylphosphatidylcholine (DPPC) LUVs

Lucigenin assay using 1,2-dipalmitoylphosphatidylcholine (DPPC) was employed to determine the transport mechanism of the compounds. DPPC-LUV $\supset$ lucigenin were prepared in 20 mM HEPES buffer pH 7.2, containing 100 mM NaNO₃. For the preparation of DPPC-LUV $\supset$ lucigenin first 50 μ L of DPPC (100 mg/mL stock in de-acidified CHCl₃) was taken in a clean and dry glass vial, and the organic solvent was removed under reduced pressure (for 5-6 hours) at room temperature. The dry, thin film was then hydrated with 500 μ L of 20 mM HEPES buffer, pH 7.2, containing 1 mM lucigenin and 100 mM NaNO₃. The solution was then sonicated for 30 min at 50 °C and was vortexed occasionally for 15-20 min. After that, the solution was subjected to a freeze-thaw cycle 12-13 times and again was sonicated 10 times (40-sec sonication followed by 20-sec incubation in ice-water). The LUVs were prepared by extrusion using Avanti Mini-Extruder (Avanti Polar Lipids, Alabaster, AL) through 200 nm pore size polycarbonate membranes according to the manufacturer's protocol. The unencapsulated dye was removed using gel filtration

(Sephadex G-50) column with 20 mM HEPES buffer, pH 7.2 containing 100 mM NaNO₃. The final volume of the collected vesicle solution was adjusted to 500 μ L with 20 mM HEPES buffer pH 7.2 containing 100 mM NaNO₃. The final lipid concentration was 13.62 mM (assuming 100 % lipid regeneration). For the transport activity assay, first 2890 μ L of 20 mM HEPES buffer, pH 7.2, containing 100 mM NaNO₃ and 50 μ L of the DPPC-LUV $\Rightarrow$ lucigenin (concentration of stock solution = 13.62 mM) were taken in a 3 mL fluorescence cuvette and the cuvette was placed in the fluorescence spectrophotometer under slow stirring condition. After that, compounds (10 μ L from a 5 μ M stock solution in DMSO) were added to the solution to achieve a concentration ratio of 1: 25,000 for compound and lipids. The lucigenin fluorescence-based kinetic measurements were performed as mentioned above.¹⁸

2.4.8.2. Transport activity across DPPC-LUV $\Rightarrow$ lucigenin (DPPC Assay)

DPPC assay was performed using fluoromax-4 spectrofluorometer (Horiba Scientific, Singapore) connected with a refrigerated system for temperature control (where the temperature was regulated using a Peltier temperature controller). In this assay, 2890 μ L of 20 mM HEPES buffer, pH 7.2 containing 100 mM NaNO₃ and 50 μ L of the DPPC-LUV $\Rightarrow$ lucigenin were taken in a 3 mL fluorescence cuvette. 10 μ L of DMSO stock solution of the compounds was added to the cuvette (to make the anionophore and lipid ratio of 1: 25,000). The kinetic experiment was started (at $t = 0$ s) and lucigenin fluorescence emission was monitored as mentioned above. The cuvette was then kept under stirring condition, and the chamber temperature was set to 25 °C. After 50 sec, NaCl (50 μ L, 2 M) 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 μ L) in the cuvette at $t = 450$ sec, and fluorescent measurements were continued for another 50 sec (i.e., up to $t = 500$ sec). A similar experiment was performed for measurement of the transport rate of the compound at 45 °C.¹⁸

2.4.8.3. Cholesterol Assay

It is well documented that the fluidity of the EYPC/CHOL membrane gets affected by the amount of cholesterol present in the bilayer, which directly affects the ion carrier in the transmembrane transportation process.⁵⁷ Thus, lucigenin assay using different concentrations of cholesterol in the EYPC/CHOL-LUV $\Rightarrow$ lucigenin was performed to

further confirm the transportation mechanism of the compounds. The EYPC/CHOL-LUV $\rightarrow$ lucigenin was prepared following a similar method as described above. In this assay, 2890 μ L of 20 mM HEPES buffer pH 7.2, containing 100 mM NaNO₃ and 50 μ L of 25 mM EYPC/CHOL-LUV $\rightarrow$ lucigenin (here amount of cholesterol was varied 10% to 50%) were taken in a 3 mL fluorescence cuvette. To this, 10 μ L DMSO solution of compound **2.1f** was added (at a fixed concentration of 5 nM). The cuvette was then kept inside the fluorescence instrument under continuous stirring conditions for 3 minutes. The kinetic measurements were started after 3 minutes, and the fluorescence emission of lucigenin was monitored. After 50 sec, 50 μ L of 2 M NaCl was added to initiate the Cl⁻ influx kinetics. Finally, the kinetic experiment was terminated by the addition of 20 μ L of Triton X-100 (20 %) in the cuvette at $t = 450$ sec (to lyse the vesicles), and fluorescent measurements were continued for another 50 sec (i.e., up to $t = 500$ sec). The fluorescence emission intensities (Y-axis) of the lucigenin dye were normalized, and the intensities at $t = 0$ and $t = 500$ s were normalized to 0 and 100 units, respectively.

2.4.9. Test for the leaching-out of the compounds from the membrane bilayer environment

To examine whether the compounds get leached out during the transport activity measurement under the vesicular environment, we performed a lucigenin-based leaching test. It is hypothesized that if the transporters leached out from the membrane bilayer environment to the aqueous medium, then the transport rate of the compounds will be greatly affected (reduced) by the dilution of the vesicular solution. In comparison, the dilution factor will not affect the transport rate if the transporters are localized inside the bilayer environment. In this assay, the various concentration of EYPC/CHOL-LUV $\rightarrow$ lucigenin vesicles in 20 mM HEPES buffer, pH 7.2 containing 100 mM NaNO₃ (final concentration of the vesicles were 300 μ M, 400 μ M, 500 μ M, and 600 μ M) were taken in a 3 mL fluorescence cuvette. Consequently, the compound **2.1f** (10 μ L of DMSO stock solution) was added to the cuvette (in all cases fixed anionophore/lipid ratio). The cuvette was then kept inside the fluorescence instrument under continuous stirring conditions for 3 minutes for maximum incorporation of the compounds into the vesicles, and the lucigenin assay was performed as mentioned earlier. Half-Life and chloride transport activities were determined by applying equation Eq.-S5 and equation Eq.-S8, respectively. The kinetic experiments showed no change of transport activity in four

different lipid concentrations for compound **2.1f** was observed (for fixed anionophore/lipid ratio in all cases), confirming the non-leaching property of the anionophores.

2.4.10. Studies on the stability of lucigenin encapsulated vesicles in the presence of the compounds

The stability of lucigenin encapsulated LUVs in the presence of anionophore was investigated using dynamic light scattering (DLS) and transmission electron microscopy (TEM) measurements. For these assays, 25 mM EYPC/CHOL-LUV $\Rightarrow$ lucigenin (having EYPC: cholesterol ratio 6:4) and 20 mM HEPES buffer pH 7.2, containing 100 mM NaNO₃, were used. The stock solution of the compounds was prepared in DMSO solvent (ionophore and lipid concentration ratio of 1: 25,000 was maintained). This solution was mixed well, allowed to stand for 10 min, and was used for DLS and TEM measurements.

2.4.10.1. Dynamic light scattering (DLS) study

For the DLS study, the above-prepared solution was taken in a 3 mL quartz cuvette, and the mean particle size of the vesicles was measured using Zetasizer Nano ZS90 (Malvern) instrument. Particle sizes were measured in triplicate and presented as mean particle diameter (d_H). Standard deviations were calculated from several readings. The corresponding polydispersity indexes (PDI) were within the range of 0.2-0.5.

2.4.10.2. Transmission electron microscopy (TEM) analysis

For transmission electron microscopic (TEM) imaging, 10 μ L of the vesicles was drop-coated onto a carbon-coated copper grid and allowed to stand for 1 minute. Then, the grid was carefully blotted with filter paper and was allowed to dry for 10 min at 30 °C. Next, the grid was coated with 5-10 μ L aqueous solution of uranyl acetate (2%) and allowed to dry for another 1 minute. The excess uranyl acetate solution was wiped off, and the grid was dried overnight at 30 °C. The images were collected using a JEOL JEM 2100 transmission electron microscope (operated at a maximum accelerating voltage of 200 kV).

2.4.11. Biological Activity Studies

2.4.11.1. MTT-based cytotoxicity assay

HeLa, BHK-21, and MG-63 cells were seeded in 96-well flat-bottom tissue culture plates at a density of 10^5 cells/well (per 100 μ L) in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). The cells were incubated at 37 °C and 5% CO₂ for 16 h. The media was discarded, and each well was washed with PBS. The compounds were added with media to each well in different concentrations and incubated for 24 h. After that incubation, 10 μ L 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 μ L 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.¹¹⁻¹²

2.4.11.2. 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. HeLa cell line was selected for this study. Cells were maintained and prepared according to the mentioned method. Cell culture media was replaced by HBSS buffer (either with Cl⁻ or without Cl⁻ ion) containing 10% FBS. Compound **2.1e** was added to each well in different concentrations 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 μ L 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. Hank's balanced salt solution with Cl⁻ ion was prepared using 136.9 mM NaCl, 5.5 mM KCl, 0.34 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 0.81 mM MgSO₄, 1.25 mM CaCl₂, 5.5 mM D-glucose, 4.2 mM NaHCO₃ 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 Na₂HPO₄, 0.44 mM KH₂PO₄, 0.81 mM MgSO₄, 1.25 mM Ca-gluconate, 5.5 mM D-glucose, 4.2 mM NaHCO₃ and 10 mM HEPES (pH 7.4).¹¹⁻¹²

2.4.11.3. Measurement of intracellular Cl⁻ ion transportation by MQAE dye

HeLa cells were seeded in 96-well flat-bottom tissue culture-treated plates at a density of 10^5 cells/well and incubated at 37 °C and 5% CO₂ for 16 h. After that, the fluorescent dye *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE) was added to each well (final concentration of 5 mM) and incubated for 3.5 h. Each well was washed with PBS to remove extracellular MQAE dye, and compound **2.1e** was added at various concentrations (final concentrations of 5, 10, and 20 μ M). After 24 h of incubation, the MQAE fluorescence was measured by using a fluorescence plate-reader (λ_{ex} = 350 nm and λ_{em} = 460 nm).¹¹⁻¹²

2.4.11.4. Immunoblot analysis

Cells were seeded in a 6-well plate at a density of 6×10^5 cells per well and incubated at 37 °C for 16 h. Then compound **2.1e** (dissolved in DMSO) with different concentrations (5, 10, and 20 μ M) was directly added to the culture medium, and cells were incubated for 24 h. An equal volume of DMSO was added to the control cells. After 24 h of incubation, media was removed, and the cells were washed with PBS. Cells were lysed by using Lysis-buffer containing 1(M) Tris (pH 6.8), 20% glycerol, 4% sodium dodecyl sulphate (SDS), 400 mM dithiothreitol (DTT), and 0.004% bromophenol blue and stored at -20 °C for further analysis.

The proteins of cell lysates were separated by 12% SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were blocked with 5% skimmed milk and probed with specific primary and secondary antibodies. Enhanced chemiluminescent HRP substrate (Bio-Rad Laboratories, Hercules, CA, USA) were used to visualize the specific proteins. The following were used as a primary antibody: rabbit polyclonal Anti-poly-ADP-ribose polymerase (PARP) (214/215) cleavage-specific, mouse anti-caspase 3, 9, 8, and mouse monoclonal β -actin (all from Life Technologies, USA).^{11-12, 37}

2.4.11.5. Nuclear fragmentation by cell imaging

The HeLa cells were seeded on coverslip at the concentration of 1×10^5 cells per wells. Cells were incubated with compound **2.1e** (20 μ M) for 24 h. Cells were then washed with PBS and fixed by using 4% formaldehyde at room temperature for 10 min. After that, cells were washed thoroughly and incubated with 100 μ g ml⁻¹ Hoechst 33342 dye

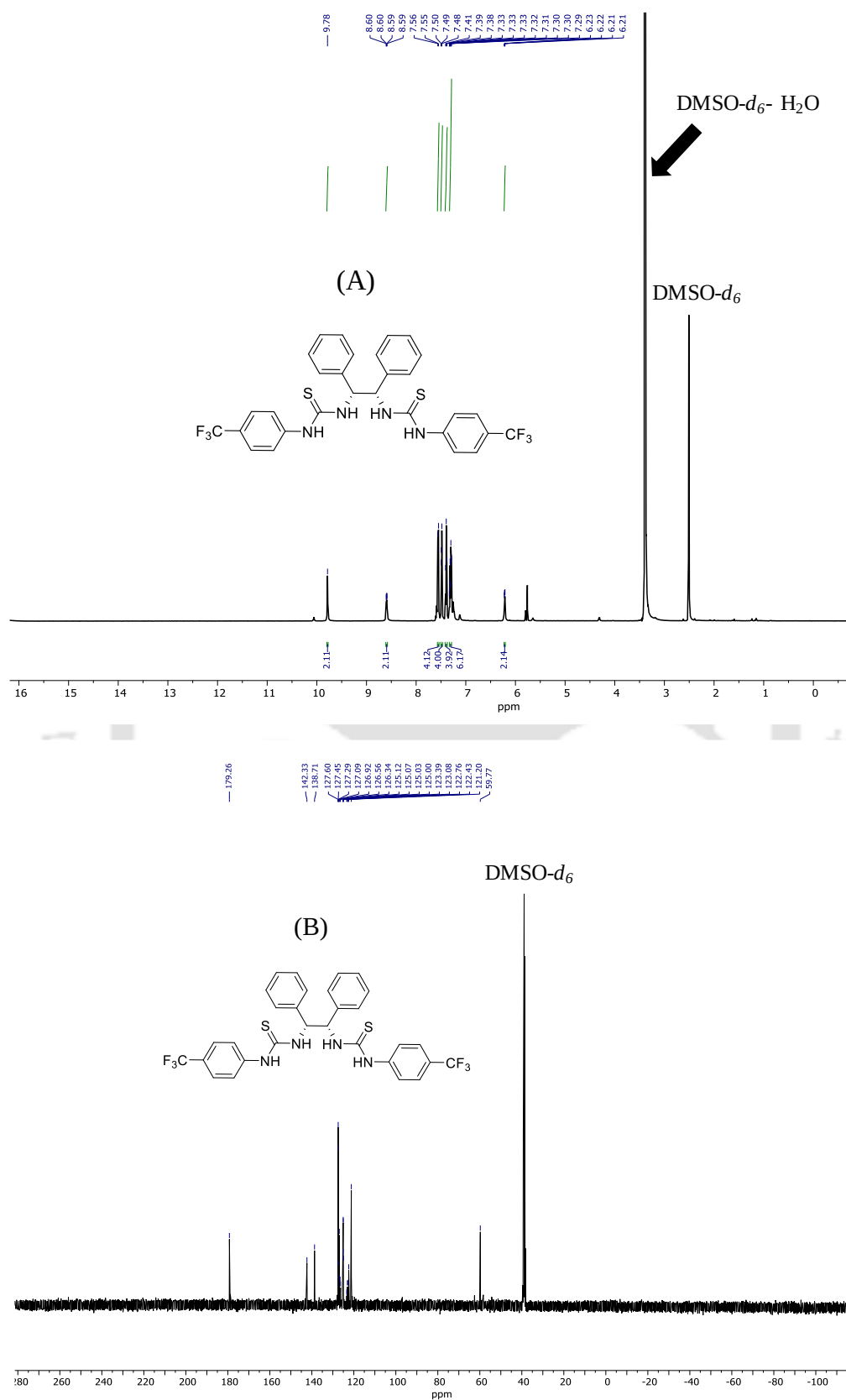
(Invitrogen) for 15 min in the dark. Further, cells were washed with PBS, and fluorescence microscopic images were recorded.¹¹⁻¹²

2.4.11.6. DNA laddering

The HeLa cells were seeded in 6 wells plate as described above and treated with compound **2.1e** for 24 h at different concentrations (0, 5, 10, and 20 μM). The cells were harvested and lysed in lysis buffer (1% NP-40 in 20mM EDTA, 50mM Tris-HCl, pH 7.5) for 20 s, followed by centrifugation, and the supernatant was collected. Furthermore, the supernatant was treated with 10% SDS and 50 mg ml^{-1} RNase A (final concentration of 5 $\mu\text{g } \mu\text{l}^{-1}$) and incubated for 2 h at 56 °C. The supernatant was then treated with protease k for 2 h at 37°C, and 10 M ammonium acetate was added. The DNA content of the supernatant was precipitated with isopropanol and analyzed on 2% agarose gel.¹¹⁻¹²

2.4.11.7. Study on the mode of death of cells using FACS analysis

To understand the mode of HeLa cells death in the presence of compound **2.1e**, the cells were treated with FITC Annexin V/Dead Cell Apoptosis Kit with FITC Annexin-V and Propidium Iodide (PI) for flow cytometry (FACS Calibur, San Jose, CA, USA) following the manufacturer's protocol (Invitrogen, USA).³⁷

2.4.12. ^1H and ^{13}C NMR spectra of synthesised molecules.Figure 2.13. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 2.1a.

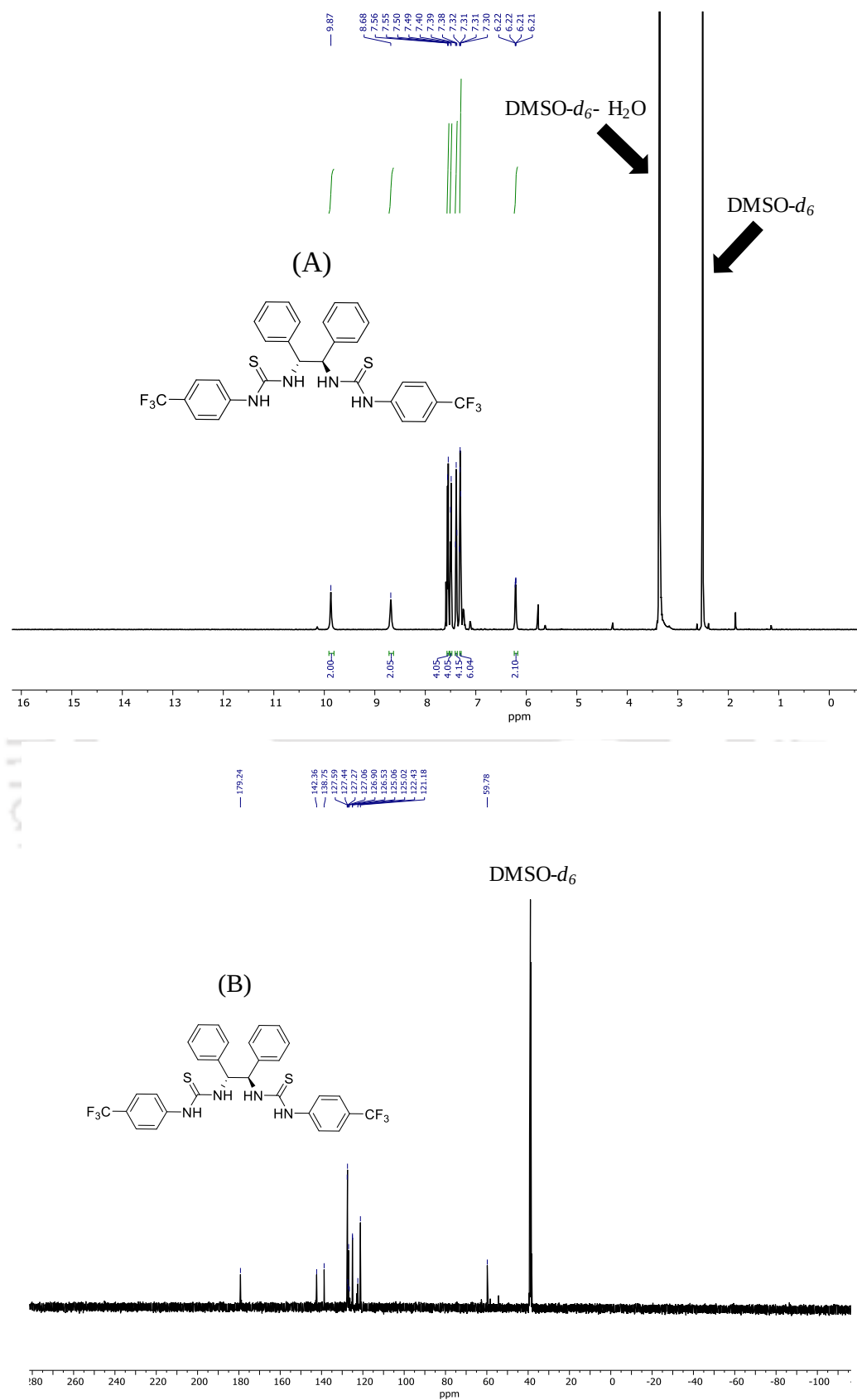


Figure 2.14. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 2.1b.

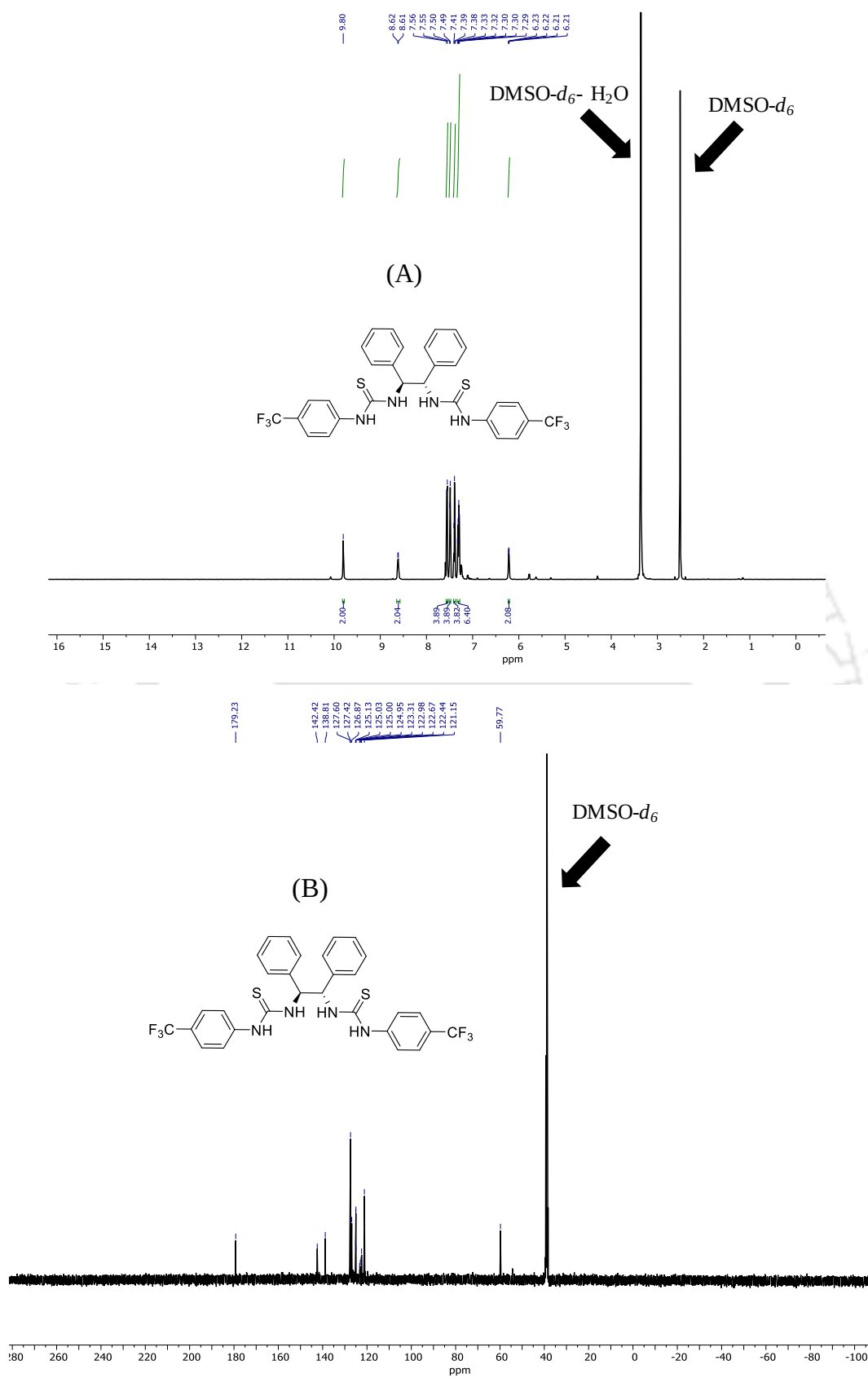


Figure 2.15. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 2.1c.

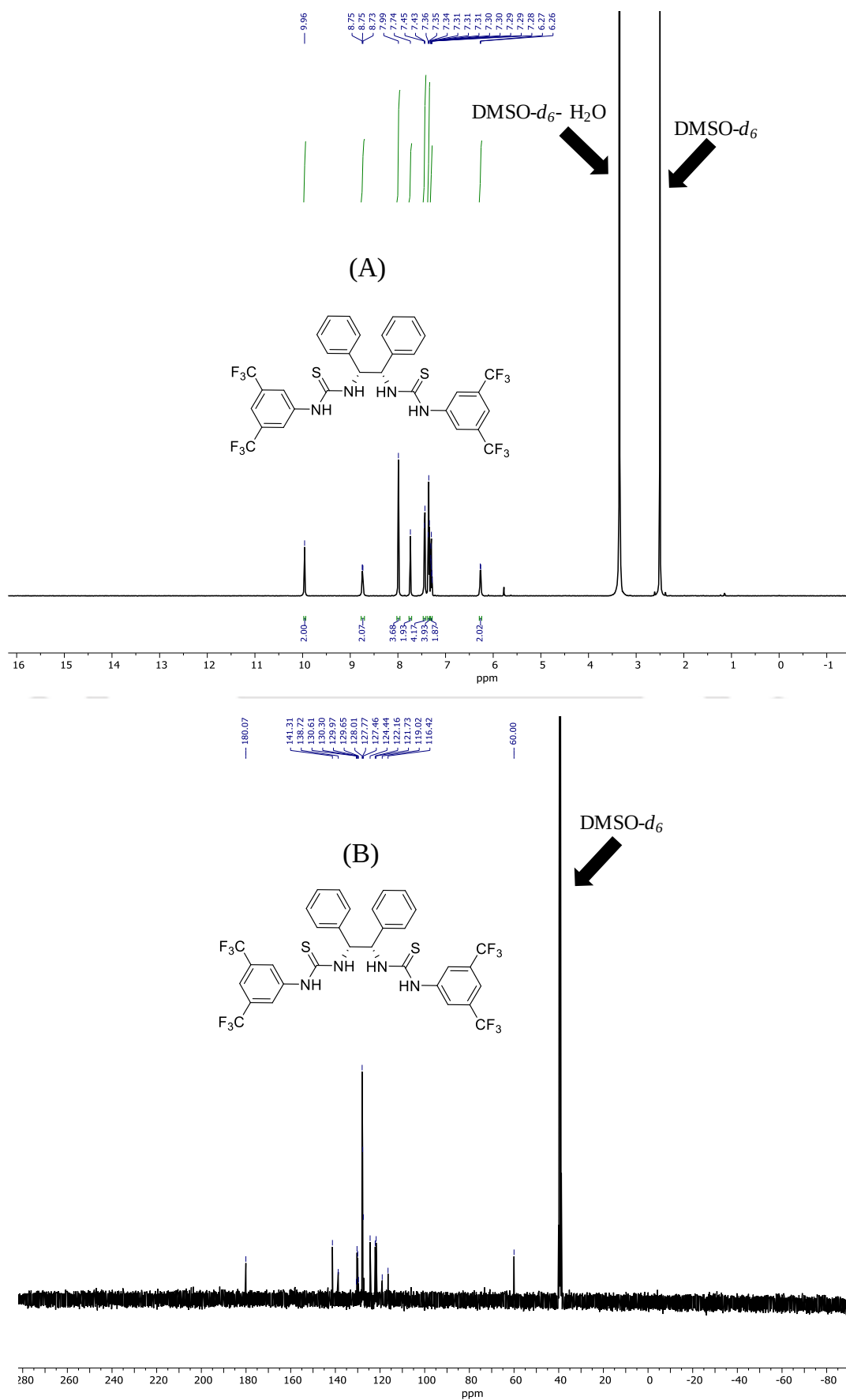


Figure 2.16. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 2.1d.

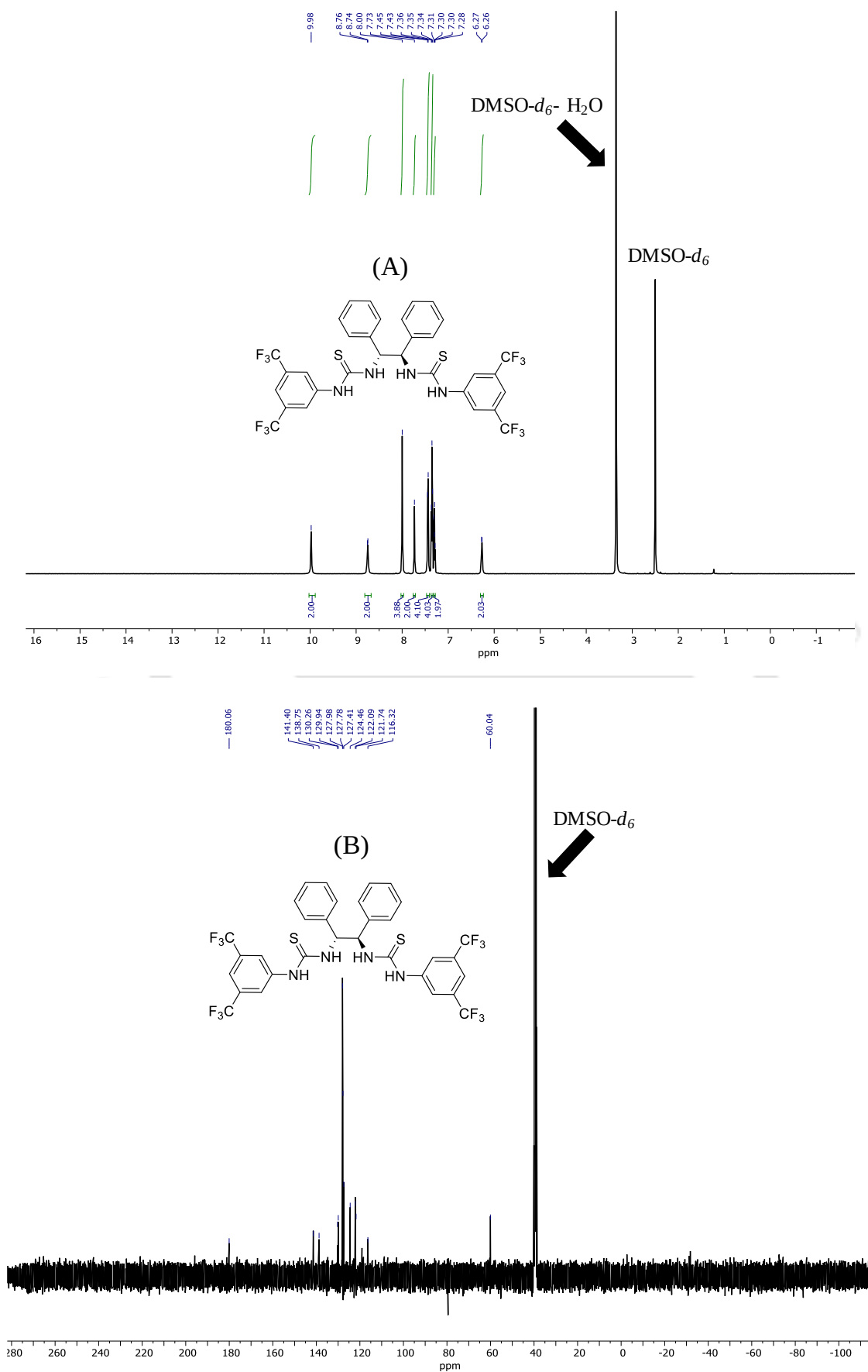


Figure 2.17. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **2.1e**.

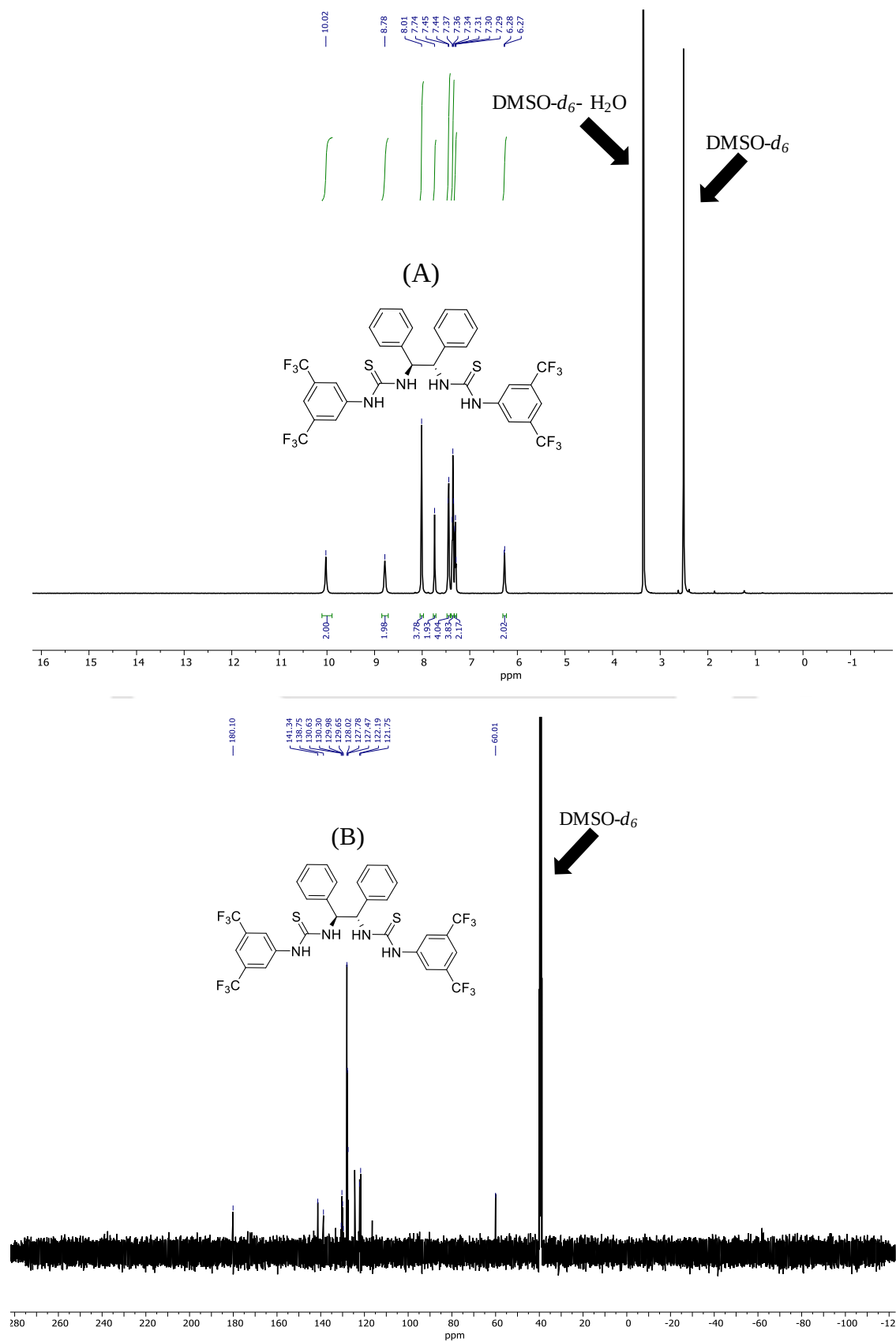


Figure 2.18. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 2.1f.

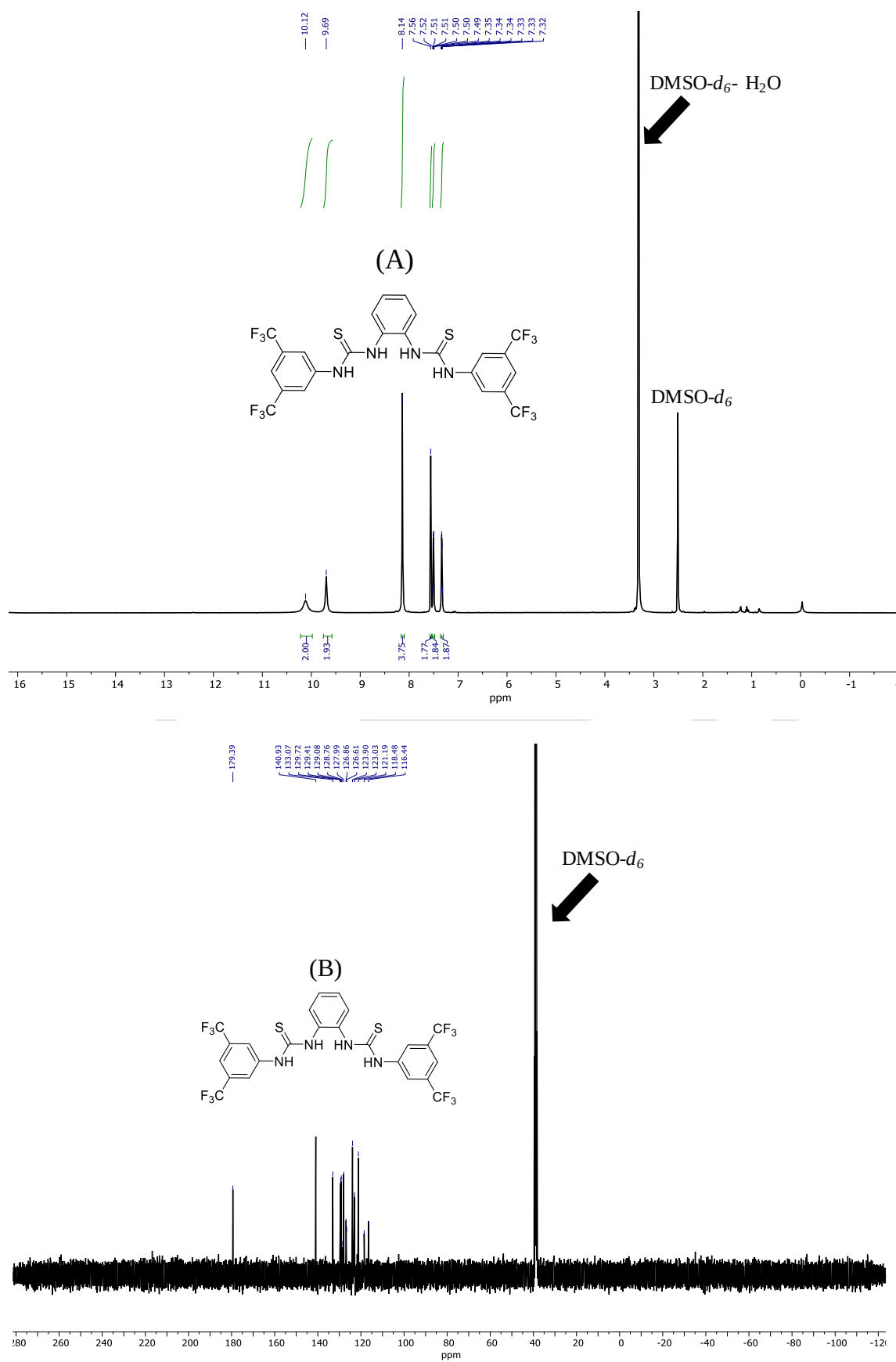


Figure 2.19. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 2.2.

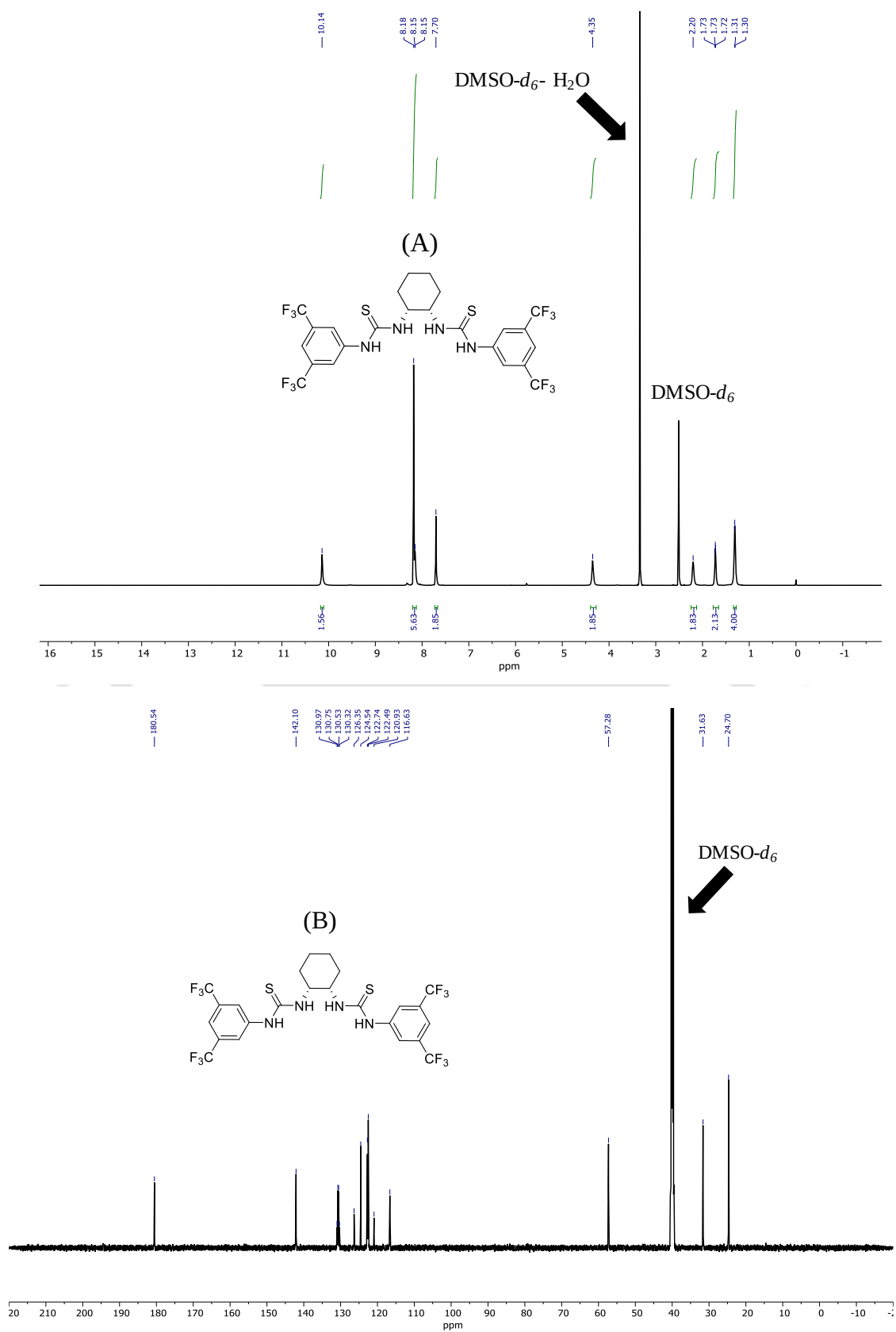


Figure 2.20. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 2.3.

2.4.13. ^1H NMR titration plots

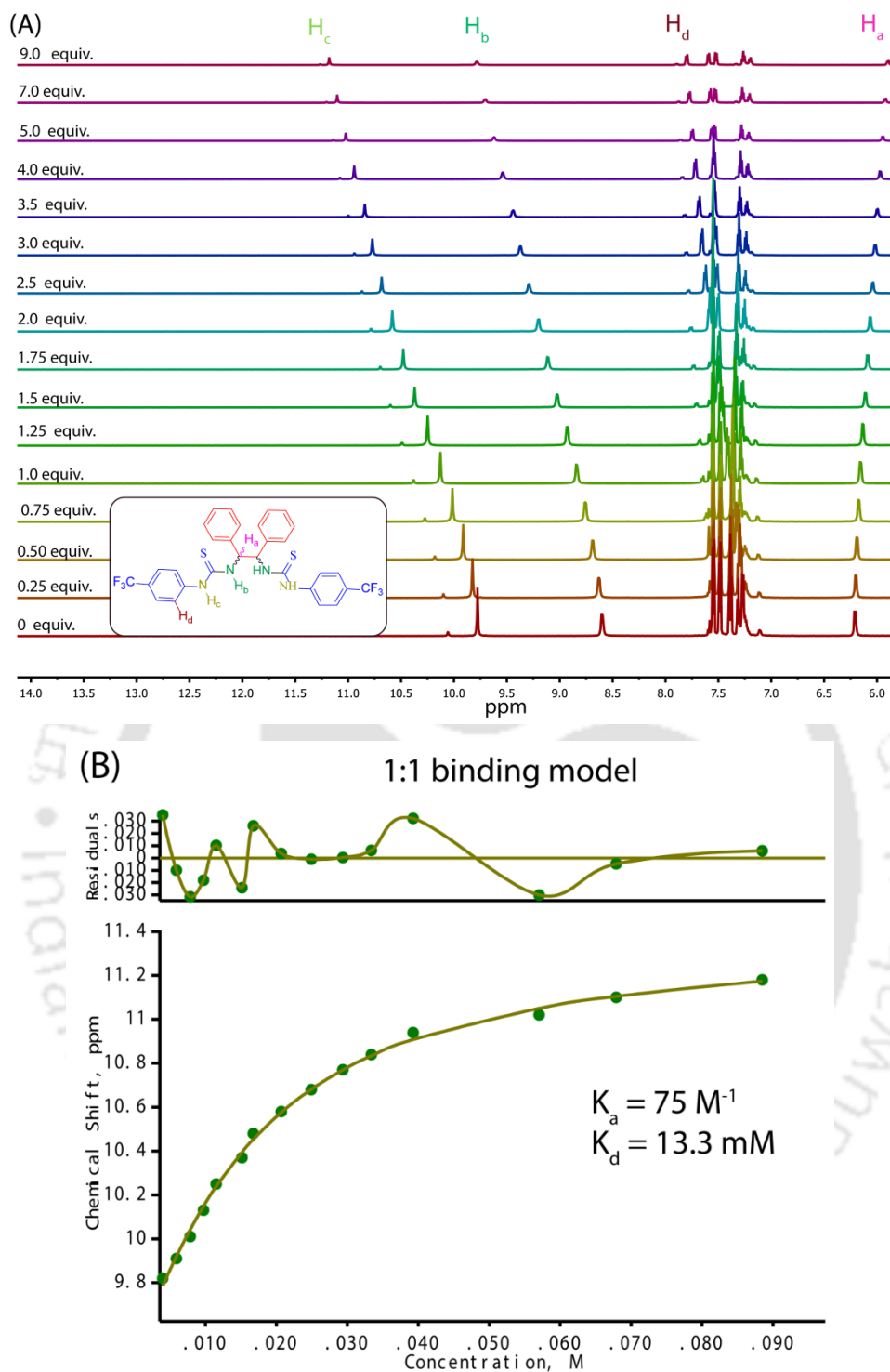


Figure 2.21. ^1H -NMR (600MHz) titration spectra for compound **2.1a** with sequential addition of TBACl in $\text{DMSO}-d_6$ solvent. The amounts of added TBACl are shown on the spectra (A). Plot of concentration of TBACl versus chemical shift of ^1H signal, fitted to 1:1 binding model of WinEQNMR2 program (B).

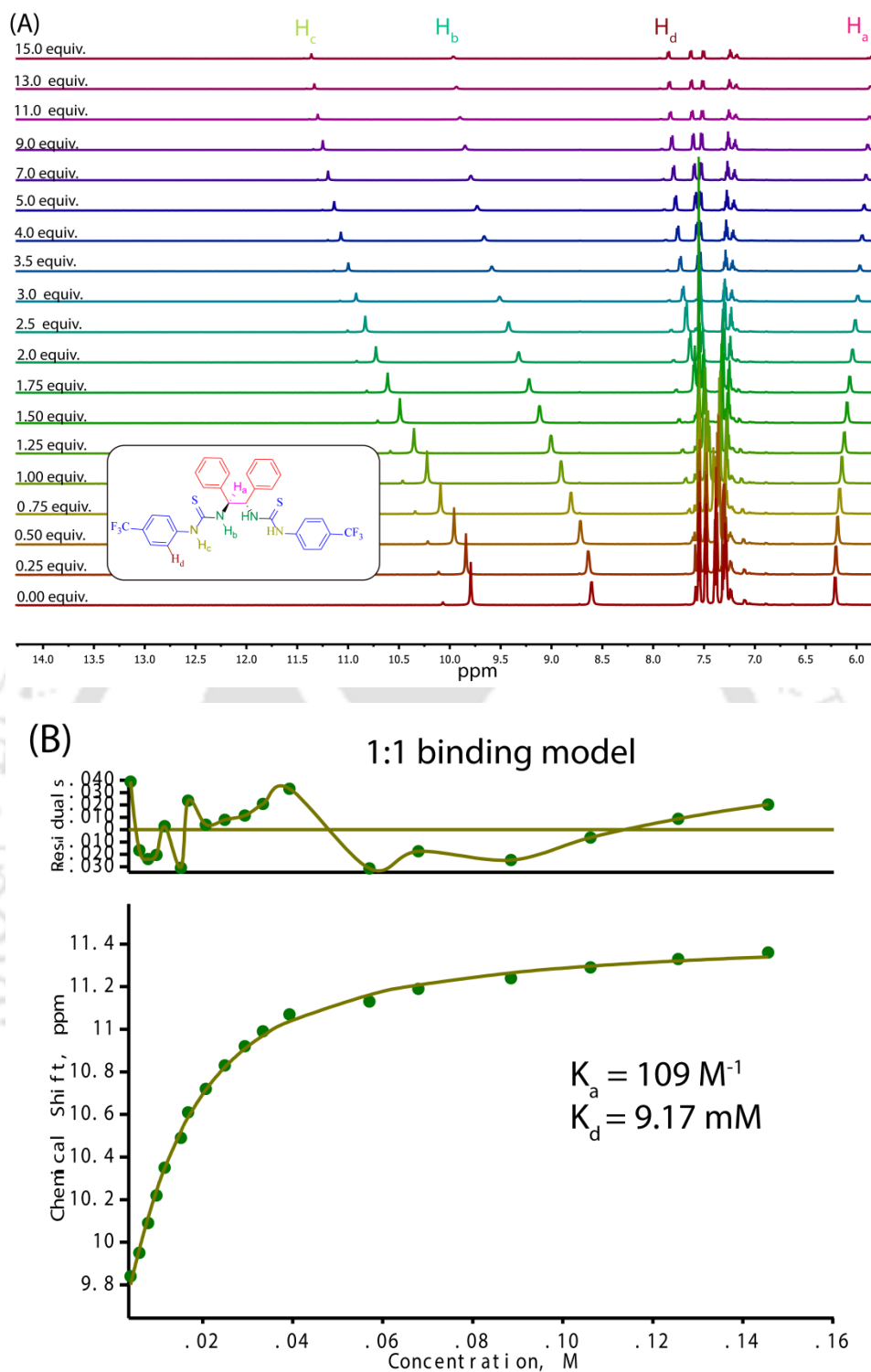


Figure 2.22. ^1H -NMR (600MHz) titration spectra for compound **2.1c** with sequential addition of TBACl in $\text{DMSO}-d_6$ solvent. The amounts of added TBACl are shown on the spectra (A). Plot of concentration of TBACl versus chemical shift of ^1H signal, fitted to 1:1 binding model of WinEQNMR2 program (B).

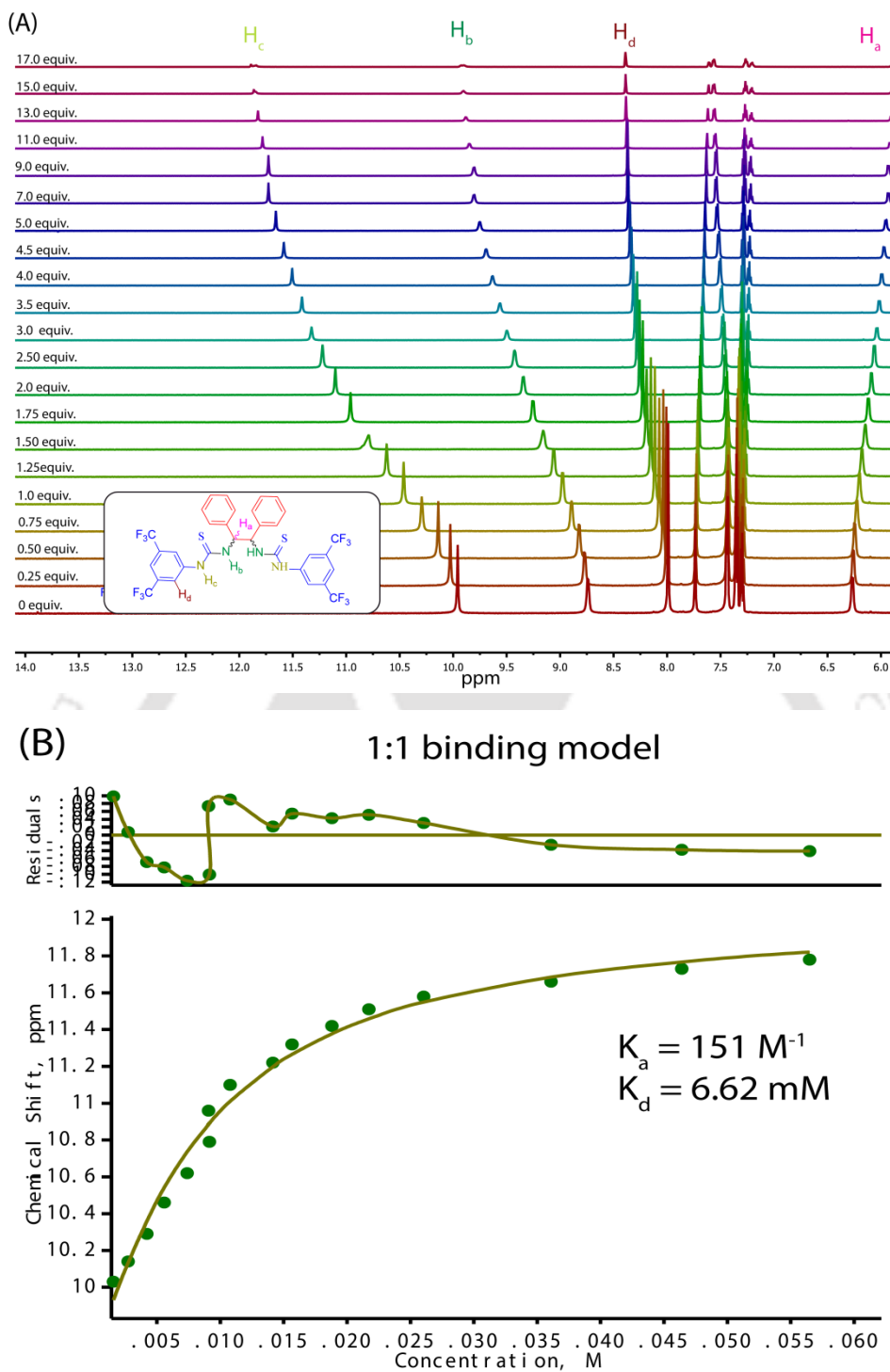


Figure 2.23. ^1H -NMR (600MHz) titration spectra for compound **2.1d** with sequential addition of TBACl in $\text{DMSO}-d_6$ solvent. The amounts of added TBACl are shown on the spectra (A). Plot of concentration of TBACl versus chemical shift of ^1H signal, fitted to 1:1 binding model of WinEQNMR2 program (B).

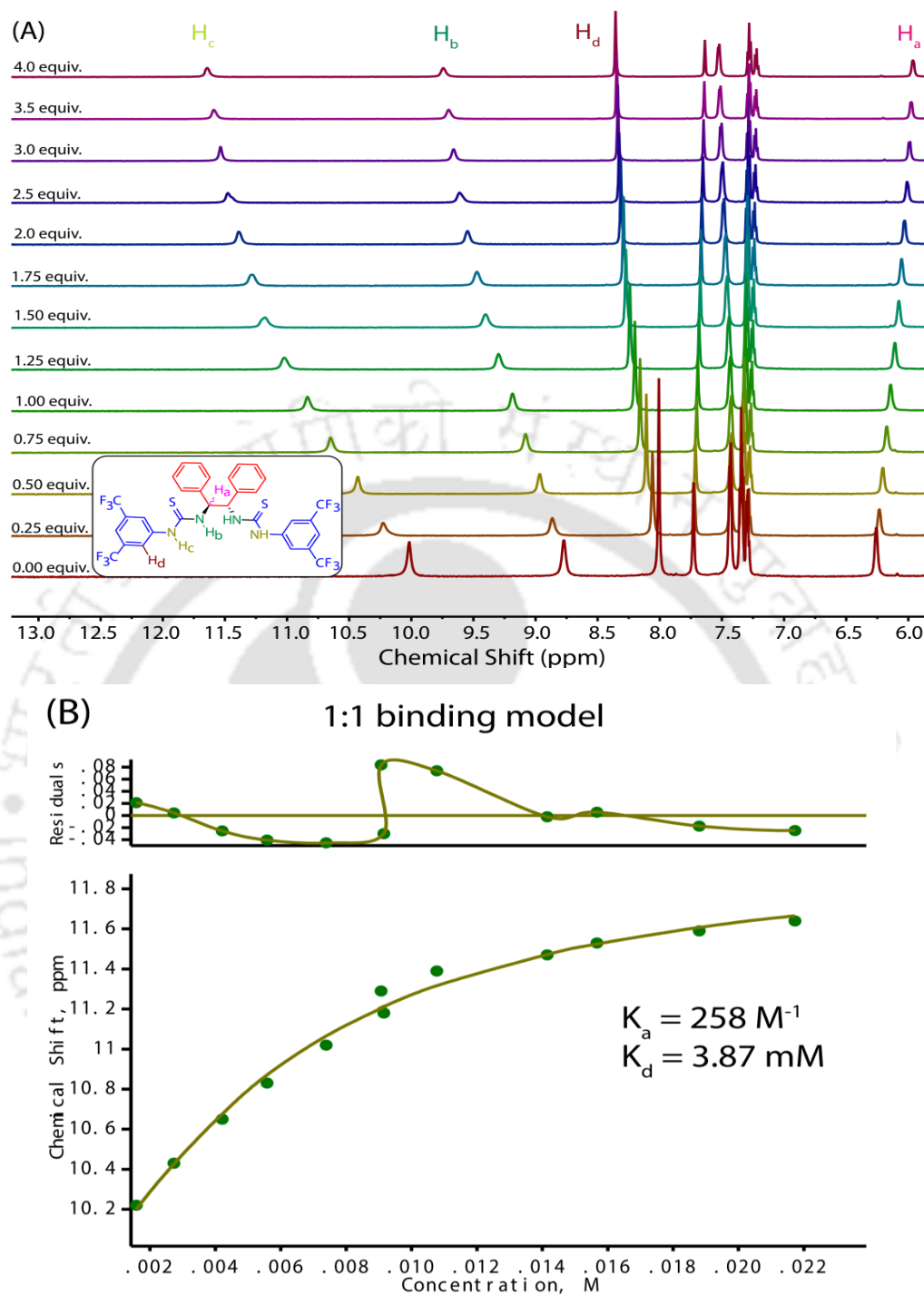


Figure 2.24. ^1H -NMR (600MHz) titration spectra for compound **2.1f** with sequential addition of TBACl in $\text{DMSO-}d_6$ solvent. The amounts of added TBACl are shown on the spectra (A). Plot of concentration of TBACl versus chemical shift of ^1H signal, fitted to 1:1 binding model of WinEQNMR2 program (B).

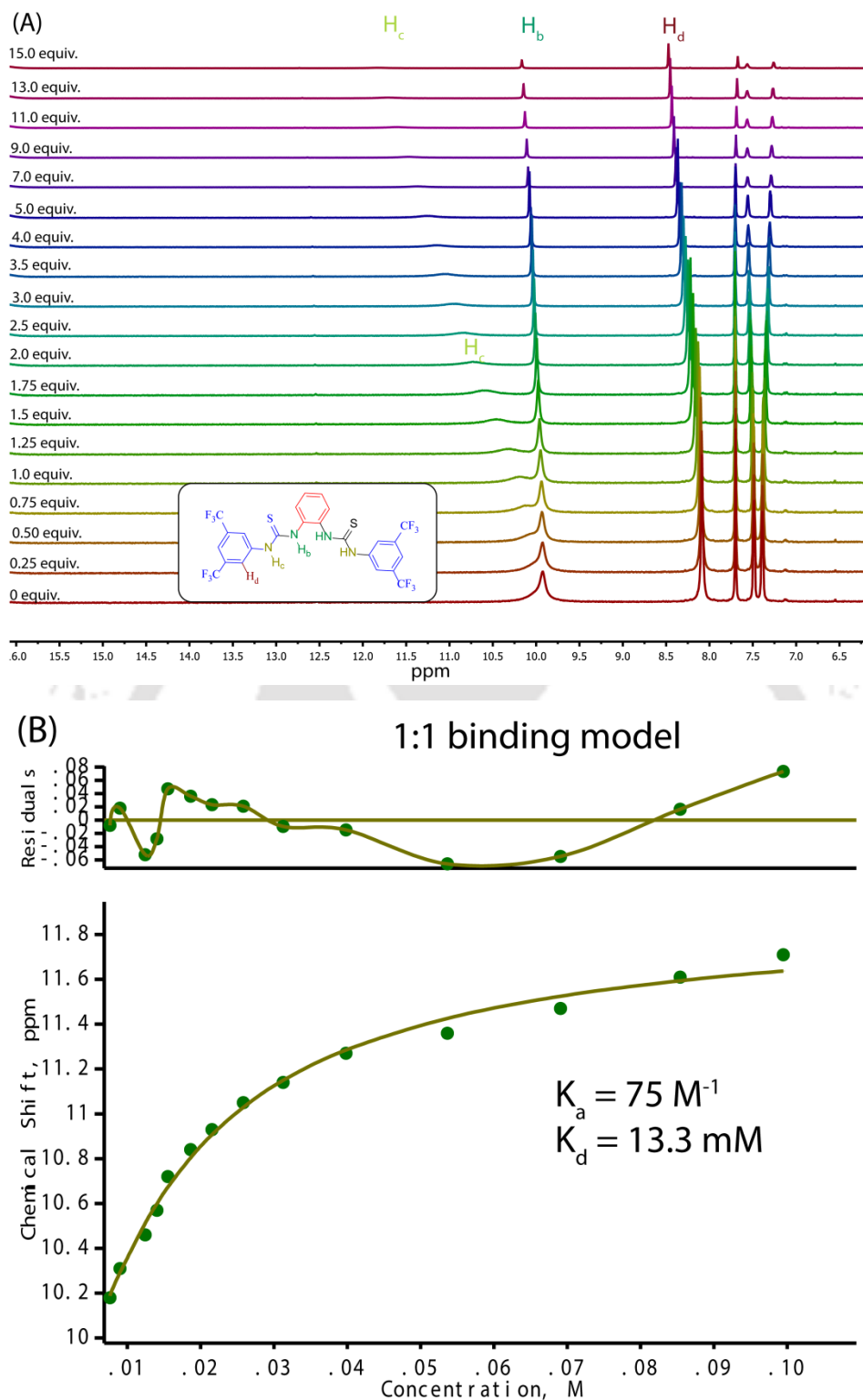


Figure 2.25. ^1H -NMR (600MHz) titration spectra for compound **2.2** with sequential addition of TBACl in $\text{DMSO-}d_6$ solvent. The amounts of added TBACl are shown on the spectra (A). Plot of concentration of TBACl versus chemical shift of ^1H signal, fitted to 1:1 binding model of WinEQNMR2 program (B).

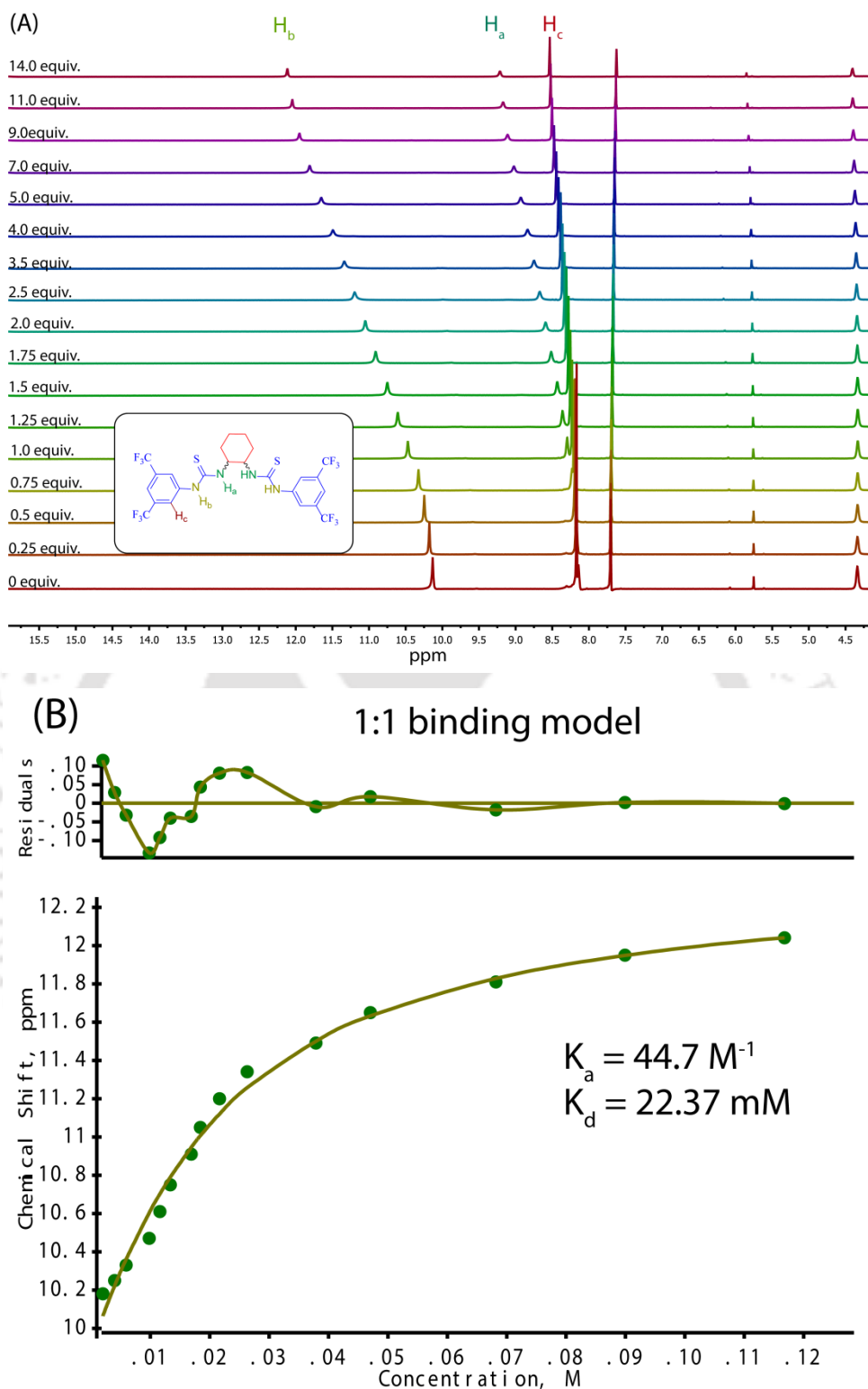


Figure 2.26. ^1H -NMR (600MHz) titration spectra for compound **2.3** with sequential addition of TBACl in $\text{DMSO}-d_6$ solvent. The amounts of added TBACl are shown on the spectra (A). Plot of concentration of TBACl versus chemical shift of ^1H signal, fitted to 1:1 binding model of WinEQNMR2 program (B).

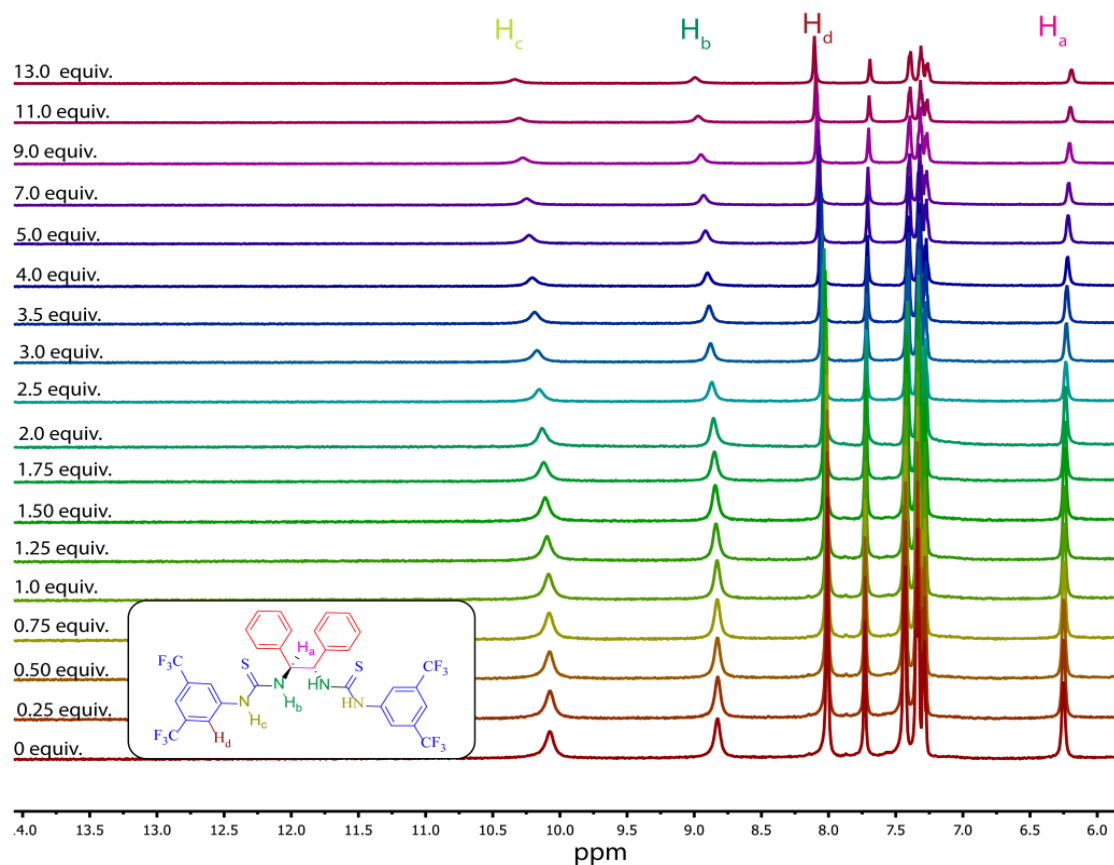


Figure 2.27. ^1H -NMR (600MHz) titration spectra for compound **2.1f** with sequential addition of TBANO_3 in $\text{DMSO}-d_6$ solvent. The amounts of added TBANO_3 are shown on the spectra.

2.4.14. Calculation and spectra of job's plot

Table 2.3. Calculation and Result table for Job's plot analysis.

Sample No.	Host conc. ([H], mM)	Guest conc. ([G], mM)	[H] + [G] (mM)	[H]/([H]+[G])	δ of proton	$\Delta\delta$	$\{[H]/([H]+[G])\} * \Delta\delta$
JB-1- 2.1d	0.5	4.5	5.0	0.1	10.49	0.52	0.052
JB-2- 2.1d	1.0	4.0	5.0	0.2	10.45	0.48	0.096
JB-3- 2.1d	1.5	3.5	5.0	0.3	10.38	0.41	0.123
JB-4- 2.1d	2.0	3.0	5.0	0.4	10.33	0.36	0.144
JB-5- 2.1d	2.5	2.5	5.0	0.5	10.28	0.31	0.155
JB-6- 2.1d	3.0	2.0	5.0	0.6	10.21	0.24	0.144
JB-7- 2.1d	3.5	1.5	5.0	0.7	10.15	0.18	0.126
JB-8- 2.1d	4.0	1.0	5.0	0.8	10.09	0.12	0.096
JB-9- 2.1d	4.5	0.5	5.0	0.9	10.03	0.06	0.054
JB-10- 2.1d	5.0	0.0	5.0	1.0	09.97	0.00	0.000

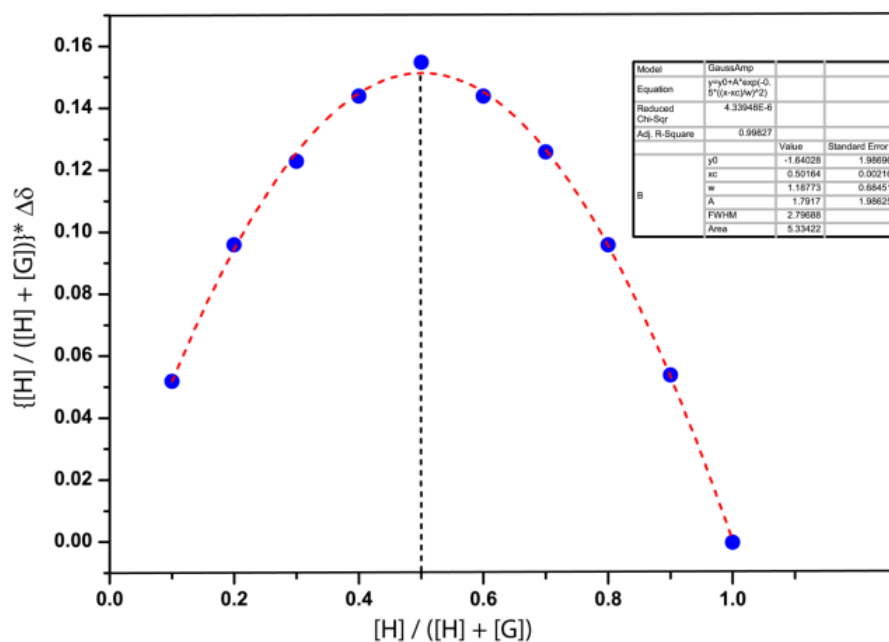


Figure 2.28. ^1H -NMR titration spectra for **2.1d** with TBACl in $\text{DMSO}-d_6$ solvent by varying mole fraction of **2.1d** (according to Table 2.3) (A). Job's plot of the same ^1H -NMR titration (B).

2.4.15. ORTEP diagram of crystal structure

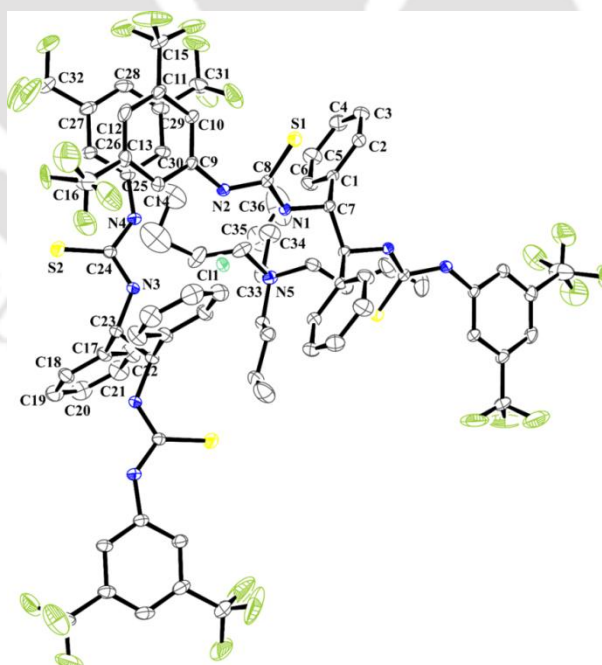


Figure 2.29. ORTEP diagram of complex **2.1d** (15% thermal ellipsoid plot, H-atoms are omitted for clarity)

Table 2.4. Crystal parameters and refinement data of the anion complex.

Parameters	Compound 2.1d
Formula	C ₉₆ H ₁₁₆ Cl ₂ F ₂₄ N ₁₀ S ₄
Fw	2065.13
Crystal system	triclinic
Space group	'P -1'
a/Å	12.1101(10)
b/Å	14.4753(11)
c/Å	15.7433(13)
$\alpha/^\circ$	94.946(3)
$\beta/^\circ$	96.866(3)
$\gamma/^\circ$	100.918(3)
V/Å ³	2673.5(4)
Z	1
Dc/g cm ⁻³	1.283
μ Mo K α mm ⁻¹	0.228
F000	1076
T/K	296(2)
θ max.	25.05
Total no. of reflections	38391
Independent reflections	9472
Observed reflections	9472
Parameters refined	626
R1, I > 2 σ (I)	0.0926
wR2, I > 2 σ (I)	0.2059
GOF (F2)	0.991
CCDC	1820120

2.4.16. Ion transport plot

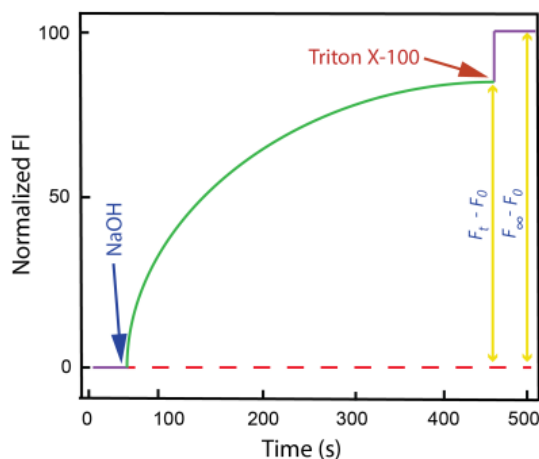


Figure 2.30. Representations of HPTS fluorescence based ion transport kinetics using EYPC/CHOL-LUV $\supset$ HPTS.

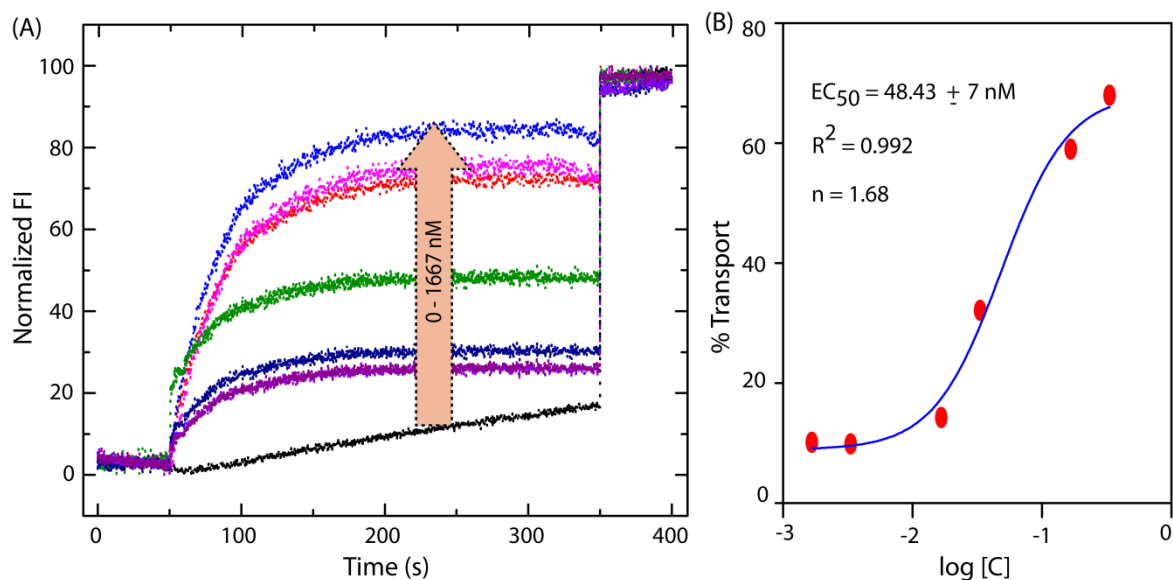


Figure 2.31. Concentration dependent activity of compound **2.1a** across EYPC/CHOL-LUV $\supset$ HPTS (A). Dose-response plot of compound **2.1a** at 350 sec, after the addition of NaOH (B).

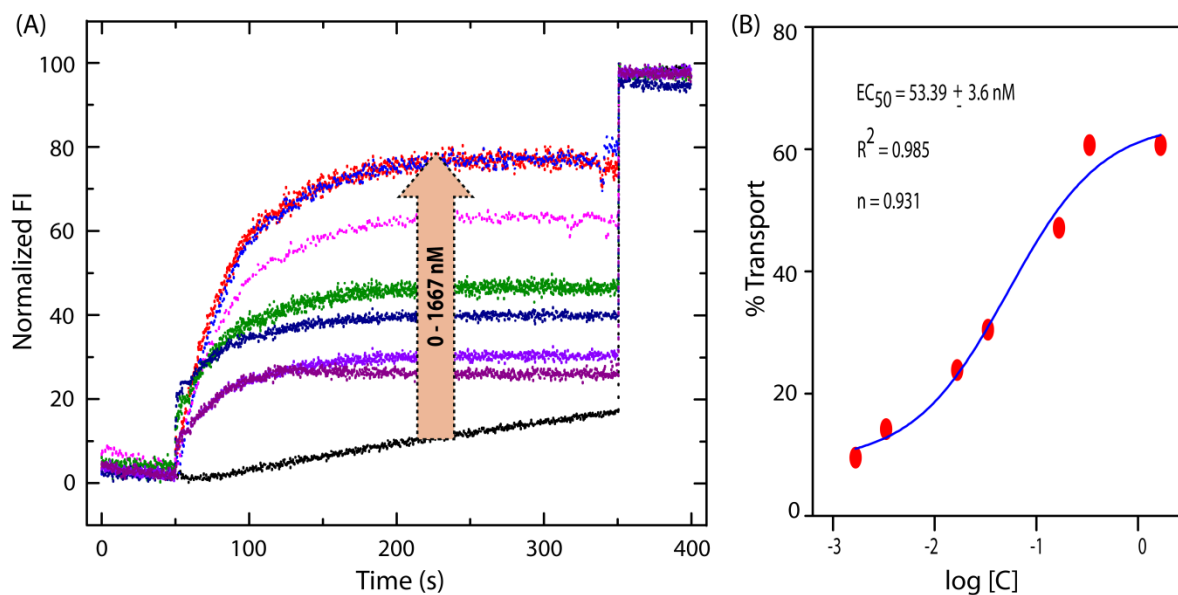


Figure 2.32. Concentration dependent activity of compound **2.1b** across EYPC/CHOL-LUV $\rightarrow$ HPTS (A). Dose-response plot of compound **2.1b** at 350 sec, after the addition of NaOH (B).

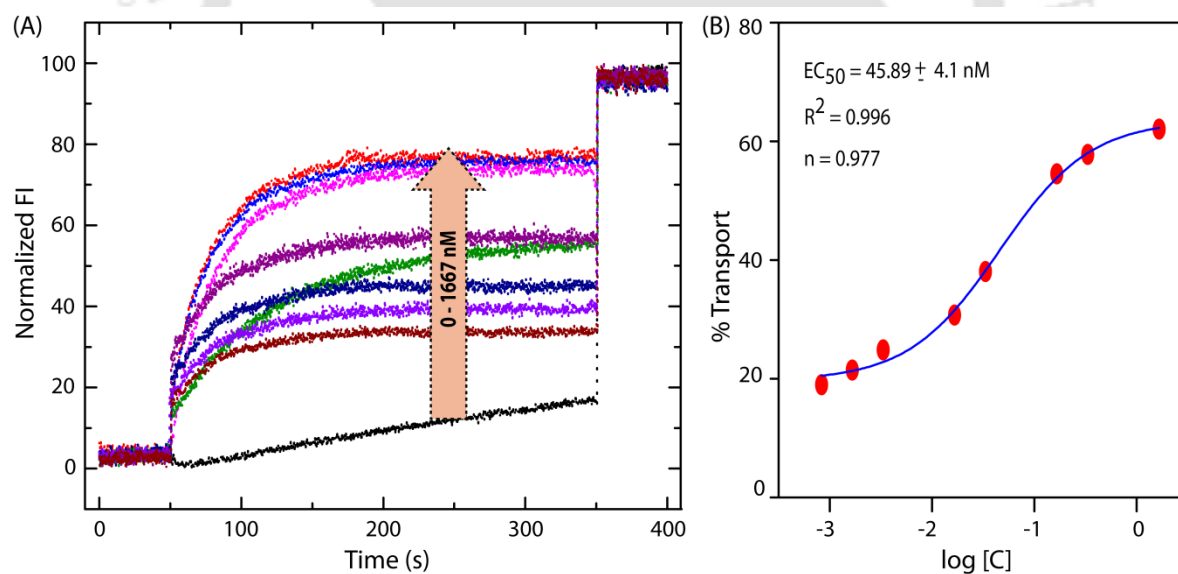


Figure 2.33. Concentration dependent activity of compound **2.1c** across EYPC/CHOL-LUV $\rightarrow$ HPTS (A). Dose-response plot of compound **2.1c** at 350 sec, after the addition of NaOH (B).

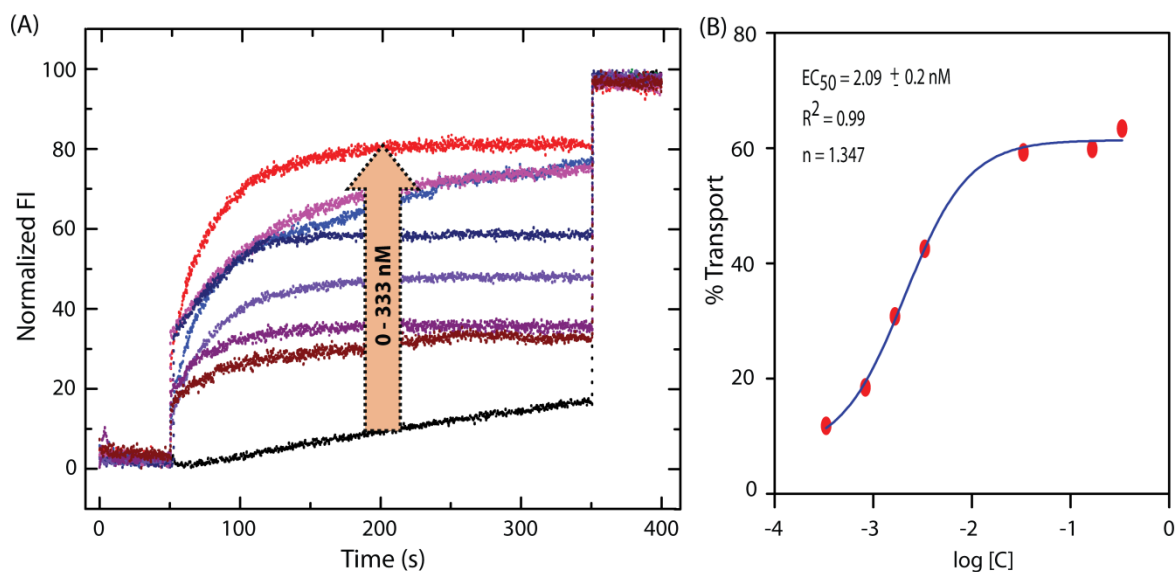


Figure 2.34. Concentration dependent activity of compound **2.1d** across EYPC/CHOL-LUV>HPTS (A). Dose-response plot of compound **2.1d** at 350 sec, after the addition of NaOH (B).

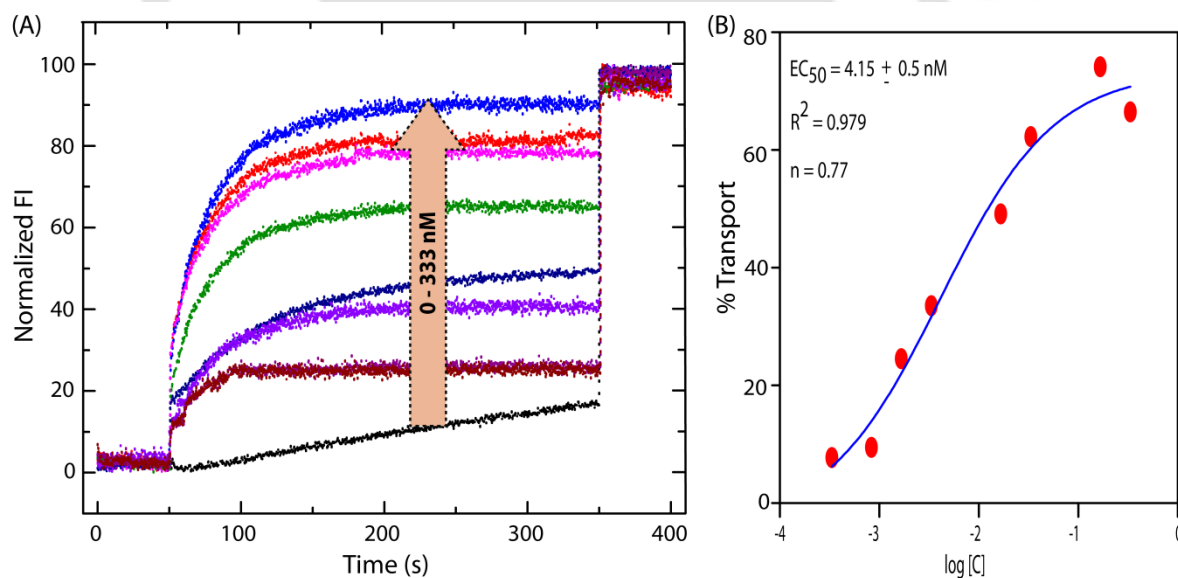


Figure 2.35. Concentration dependent activity of compound **2.1e** across EYPC/CHOL-LUV>HPTS (A). Dose-response plot of compound **2.1e** at 350 sec, after the addition of NaOH (B).

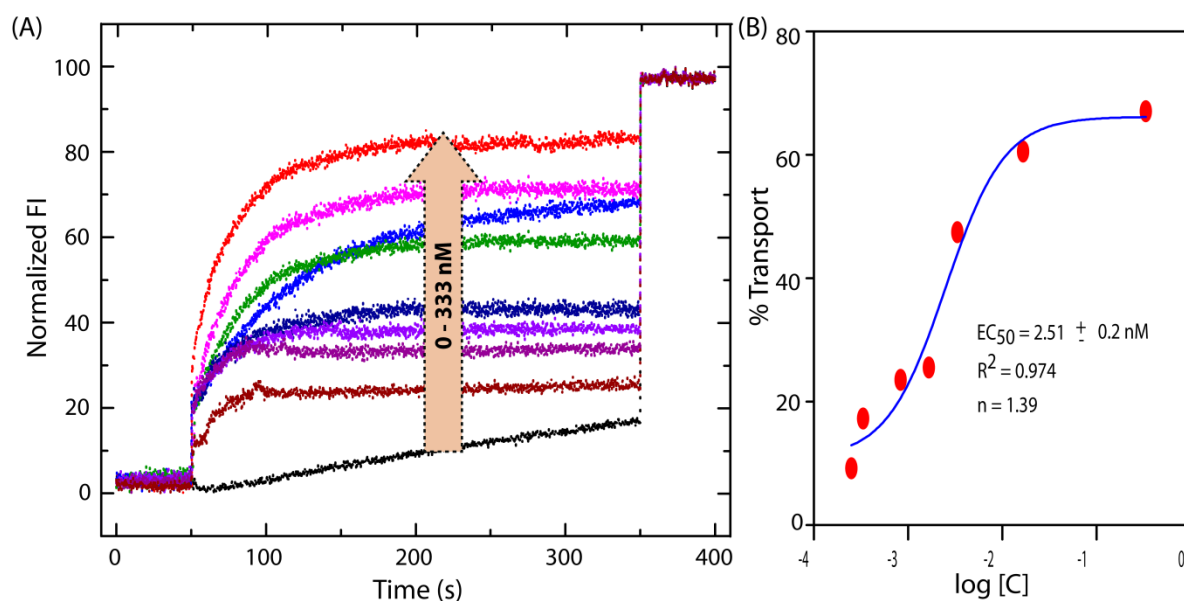


Figure 2.36. Concentration dependent activity of compound **2.1f** across EYPC/CHOL-LUV $\rightarrow$ HPTS (A). Dose-response plot of compound **2.1f** at 350 sec, after the addition of NaOH (B).

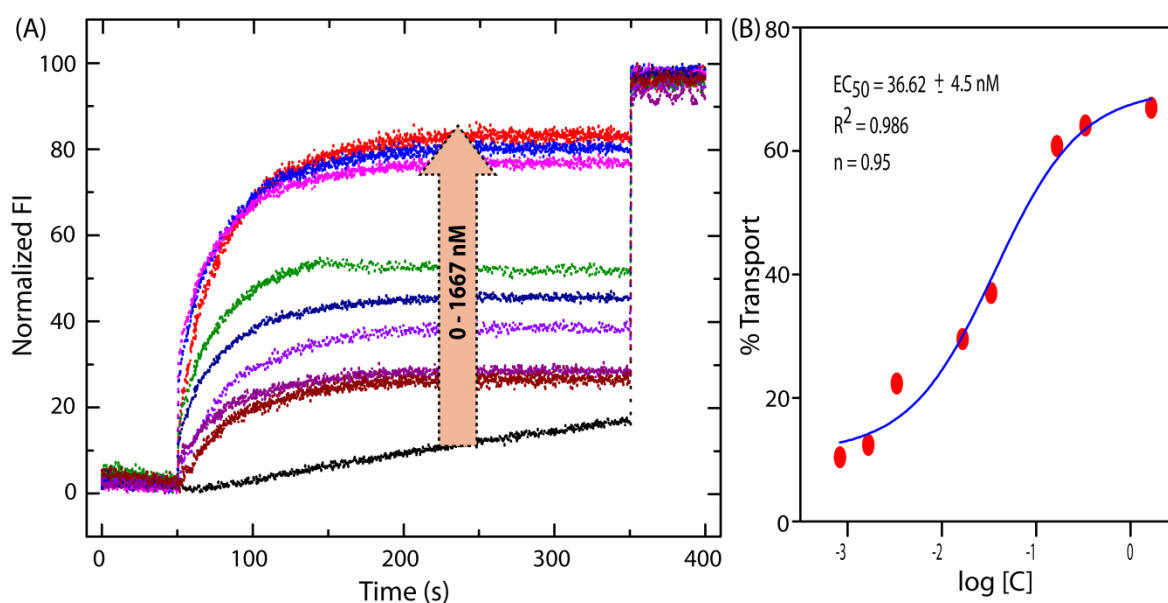


Figure 2.37. Concentration dependent activity of compound **2.2** across EYPC/CHOL-LUV $\rightarrow$ HPTS (A). Dose-response plot of compound **2.2** at 350 sec, after the addition of NaOH (B).

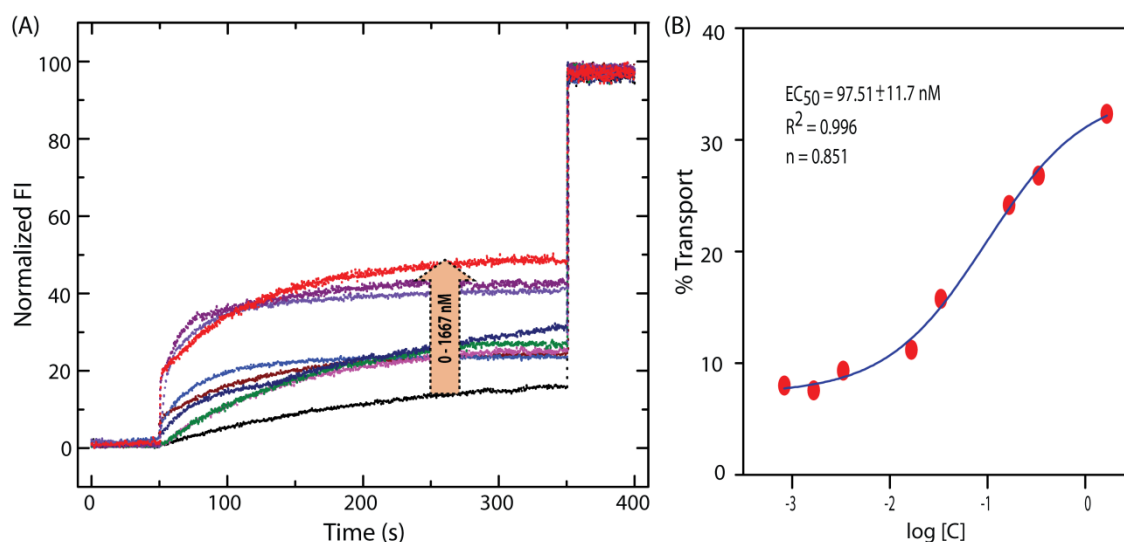


Figure 2.38. Concentration dependent activity of compound **2.3** across EYPC/CHOL-LUV-HPTS (A). Dose-response plot of compound **2.3** at 350 sec, after the addition of NaOH (B).

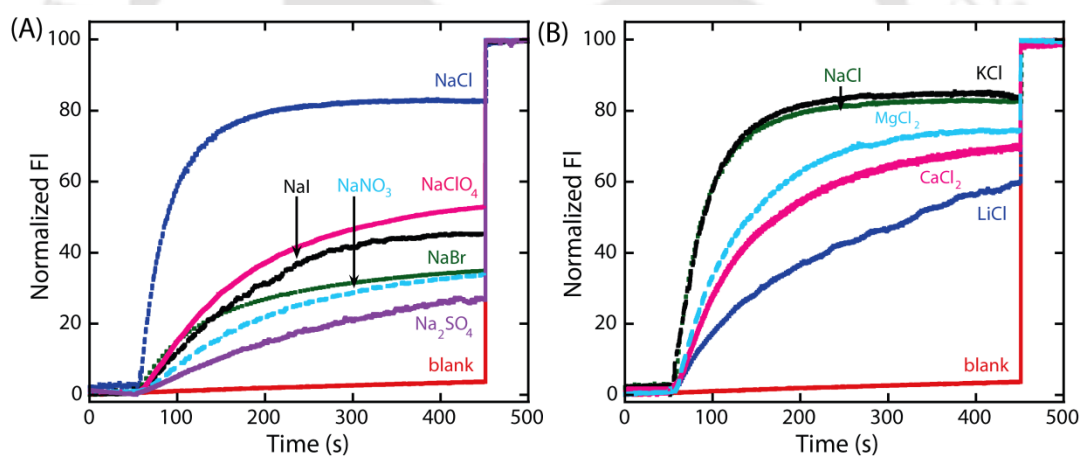


Figure 2.39. Measurement of anion (A) and cation (B) transport selectivity of compound **2.1e** across EYPC/CHOL-LUV-HPTS.

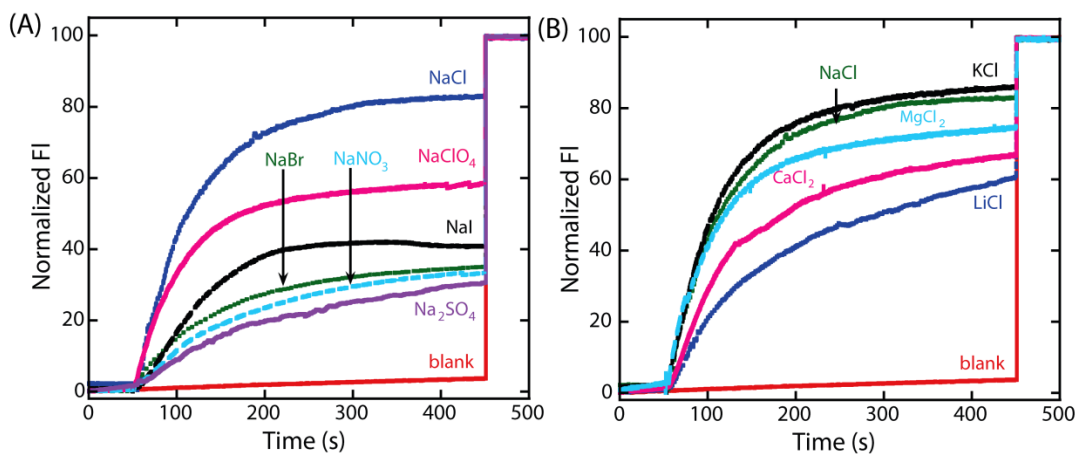


Figure 2.40. Measurement of anion (A) and cation (B) transport selectivity of compound **2.1f** across EYPC/CHOL-LUV $\Rightarrow$ HPTS.

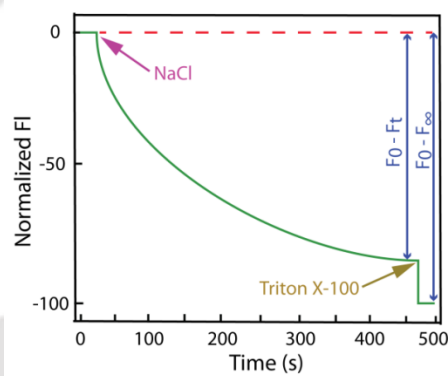


Figure 2.41. Representations of lucigenin fluorescence-based ion transport kinetics using EYPC/CHOL-LUV $\square$ lucigenin.

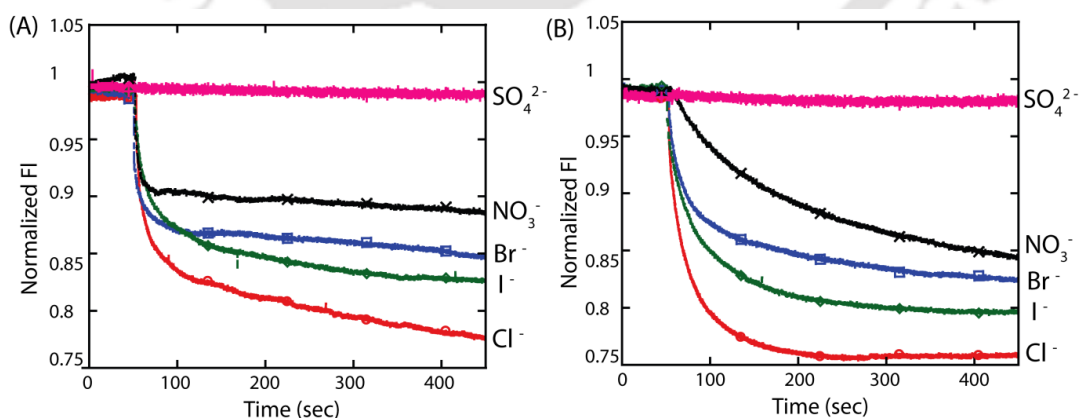


Figure 2.42. Anion 'jump' assay in the presence of compound **2.1e** (A) and **2.1f** (B) across EYPC/CHOL-LUV $\Rightarrow$ HPTS.

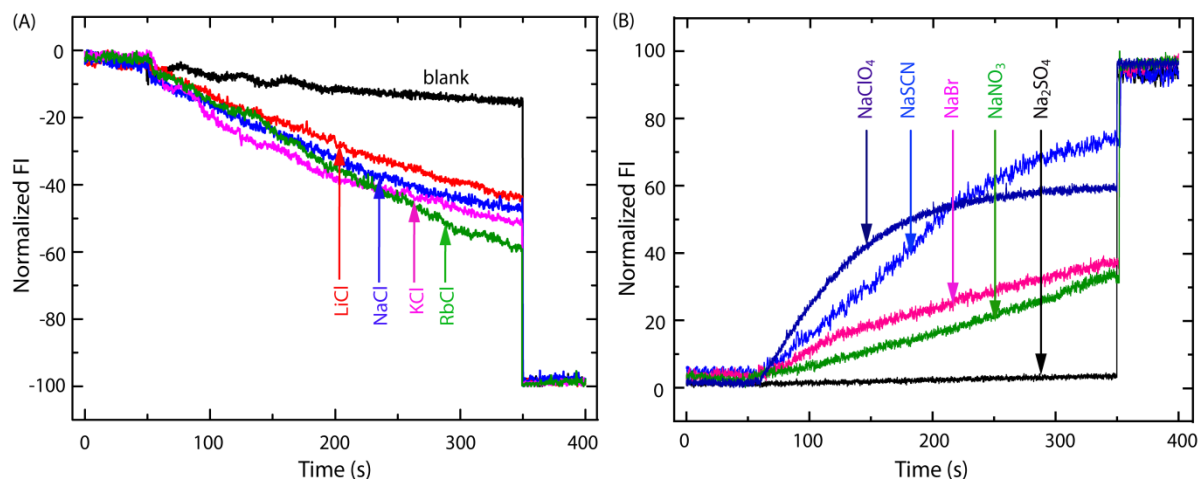


Figure 2.43. The influx of Cl^- ion across EYPC/CHOL-LUV ∇ lucigenin in the presence of compound **2.1f** with intravesicular NaNO_3 and extravesicular MCl ($\text{M} = \text{Li}^+$, Na^+ , K^+ , and Rb^+) salt (A). Efflux of Cl^- ion across EYPC/CHOL-LUV ∇ lucigenin in the presence of compound **2.1f** with intravesicular NaCl and extravesicular Na_xA ($\text{A}^- = \text{Br}^-$, I^- , NO_3^- , ClO_4^- , SO_4^{2-} ; $x = \text{valency}$) salt (B).

Table 2.5. Calculated half-life and initial rate of Cl^- transport activity of compound **2.1e** and **2.1f**.

Entry	Half-life (s)	Initial rate (s^{-1})
2.1e + EYPC/CHOL-LUV ∇ lucigenin	121.0433 ± 46.6730	0.0064 ± 0.0027
2.1e + EYPC/DPPS/CHOL-LUV ∇ lucigenin	192.9623 ± 23.2162	0.0036 ± 0.0003
2.1f + EYPC/CHOL-LUV ∇ lucigenin	90.7712 ± 08.0467	0.0077 ± 0.0007
2.1f + EYPC/DPPS/CHOL-LUV ∇ lucigenin	169.6167 ± 15.6586	0.0041 ± 0.0004

Table 2.6. Calculated half-life and initial rate of Cl^- transport activity of compound **2.1e** and **2.1f** across DPPC-LUV ∇ lucigenin at different temperatures.

Entry	Half-life (s)	Initial rate (s^{-1})
2.1e (25 °C)	60.18 ± 2.1	0.012 ± 0.0063
2.1e (45 °C)	26.21 ± 0.12	0.026 ± 0.0011
2.1f (25 °C)	41.36 ± 0.68	0.091 ± 0.0048
2.1f (45 °C)	17.42 ± 0.16	0.024 ± 0.0013

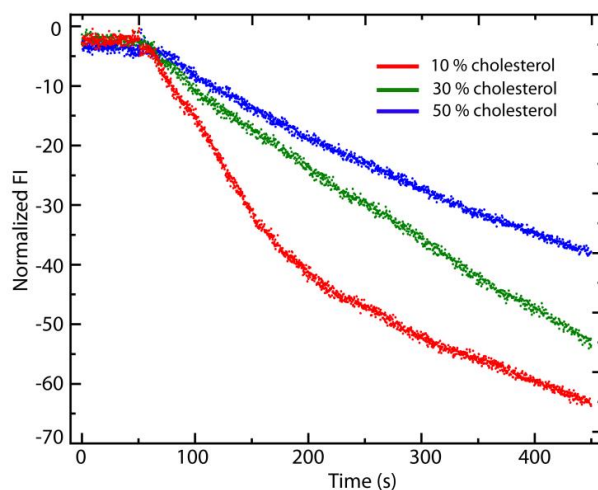


Figure 2.44. Effect of the concentration of cholesterol in the EYPC/cholesterol membrane on transport by compound **2.1f**.

2.4.17. Spectra corresponds to stability test

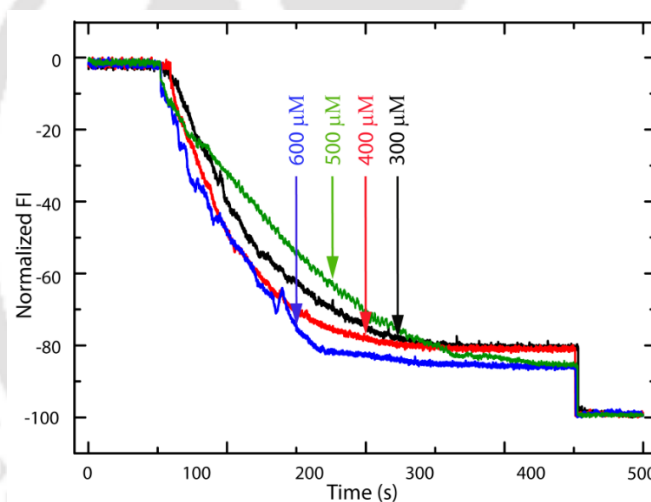


Figure 2.45. Leaching experiment for compound **2.1f** at transporter to lipid ratio of 1:25,000.

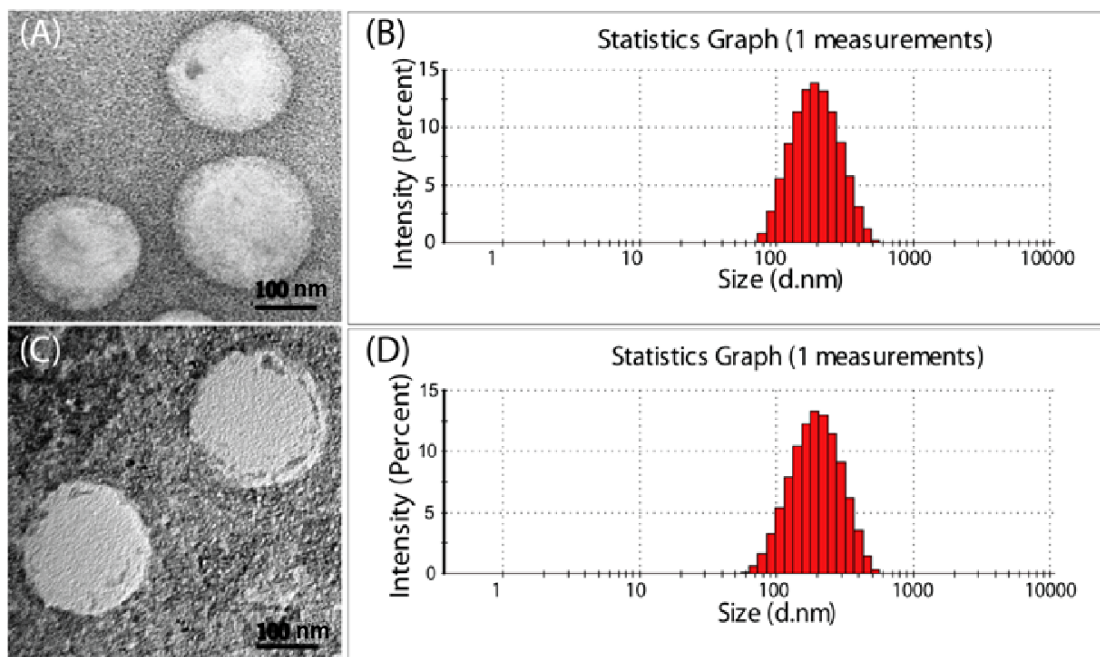


Figure 2.46. TEM image and DLS analysis of the EYPC/CHOL-LUV $\supset$ lucigenin in the absence of anionophore (A-B). TEM image and DLS analysis of the EYPC/CHOL-LUV $\supset$ lucigenin in the presence of anionophore **2.1f** (C-D).

2.4.18. *In vitro* data and plot

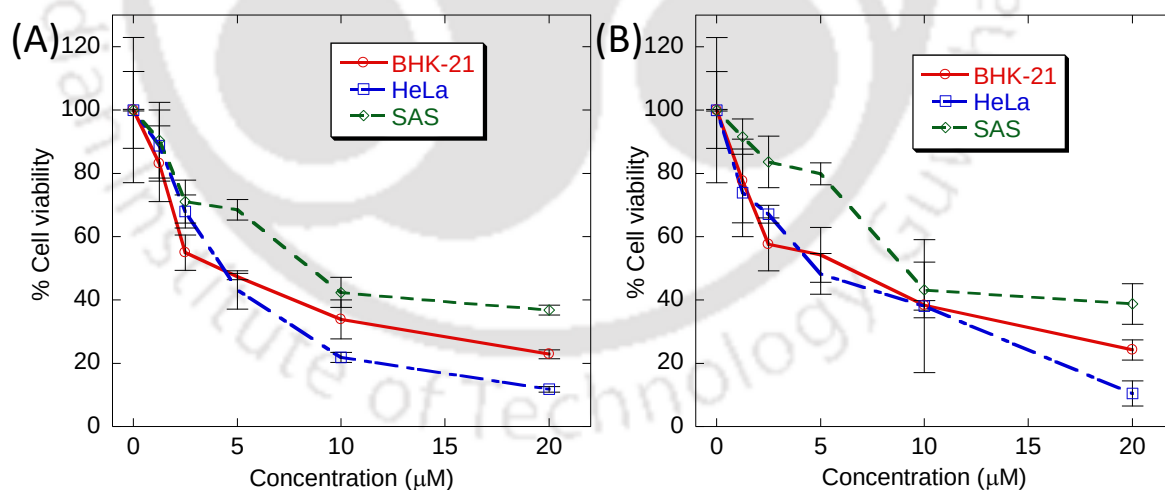
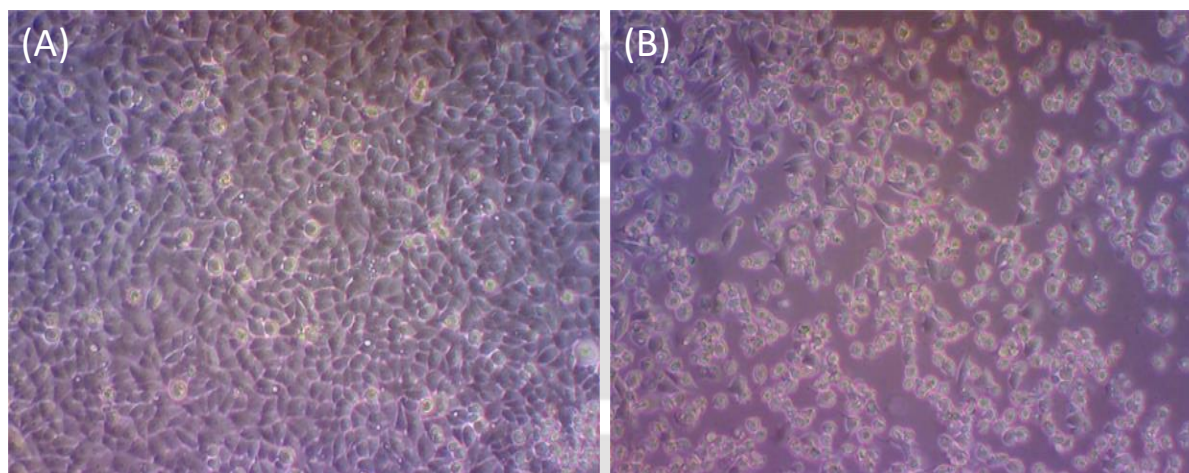
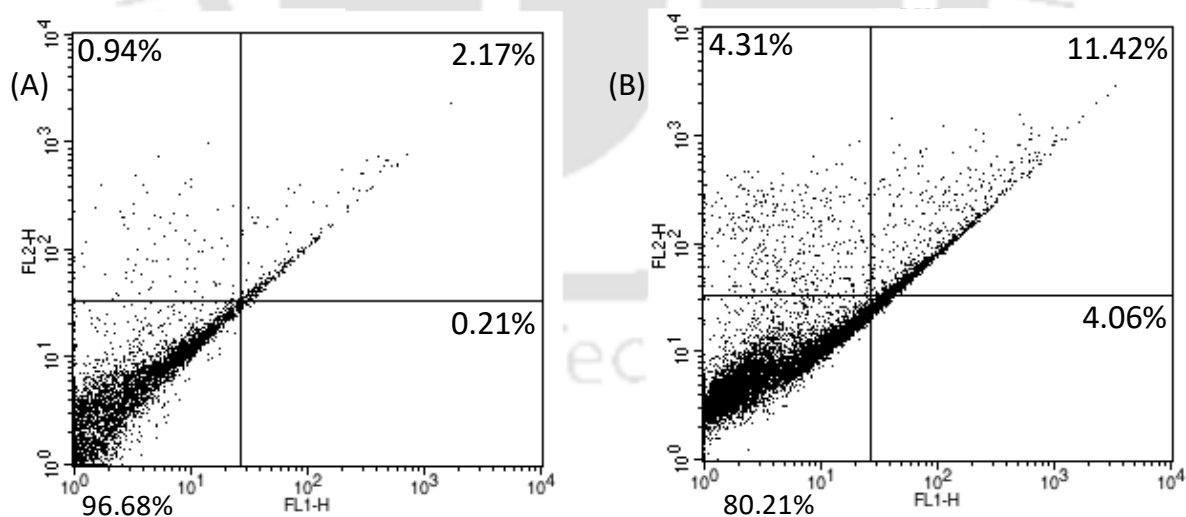


Figure 2.47. Viability of BHK-21, HeLa, and SAS cells treated with varying concentrations of compounds **2.1e** (A) and compound **2.1f** (B) after 24 hours of incubation.

Table 2.7. Calculated IC₅₀ values of the compounds.

Cell Line	Compound 2.1e	Compound 2.1f
BHK-21	4.04 μ M	6.44 μ M
HeLa	4.35 μ M	4.78 μ M
SAS	8.56 μ M	9.08 μ M

**Figure 2.48.** Comparison of cell morphology of HeLa cells in the absence (A) and presence (B) of compound **2.1e** (10 μ M) after 24 h of incubation.**Figure 2.49.** The phosphatidylserine redistribution in HeLa cells treated with compound **2.1e** (10 μ M) was analyzed after 12 h post-infection by staining the cells with fluorescein isothiocyanate-conjugated annexin-V and propidium-iodide double staining under flow cytometer. The quadrants (-1, -1) of each dot plot represent live cells in which Annexin-

V binding was absent on the surface and have an intact membrane. The quadrants (1, - 1) contain the apoptotic cells, which are Annexin-V positive and propidium-iodide negative. The necrotic cells reside in quadrants (-1, 1) which are propidium iodide positive and annexin-V negative. The cell control showed 0.21% and 2.17% cells in the early apoptotic stage and late apoptotic stage, respectively (A), while compound **2.1e** treated cells showed 4.06% and 11.42% cells (B).

2.5. References

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Chapter 3

Development of Quinine-Based Semisynthetic Ion Transporters with Potential Anti-proliferative Activities



3.1. Background and objective of present work

Unlike targeting the specific protein/enzyme or genetic material of the cancer cells, the ion transporters reside within the membrane bilayer and assist ions in crossing the hydrophobic barrier of the lipid bilayer, leading to the induction of apoptosis autophagy and other cell death programs to eliminate cancer cells. The ion transporter-mediated regulation of ion homeostasis has the potential to de-acidify the extracellular microenvironment and overcome the multidrug resistance associated with the overexpression or mutation(s) of the proteins/enzymes or genetic materials. However, several synthetic ion transporters induce apoptosis in normal cells at a much higher or very similar rate in comparison with the cancerous cells (Figure 3.1).¹⁻⁵ This undesired toxicity limits the therapeutic potential of the synthetic ion transporters in human cancer. Recently, imine-based prodigiosin analogue perenosins were reported to show higher toxicity to the breast cancer cells in comparison with normal breast cells. However, due to the presence of imine moiety, the perenosins readily get hydrolyzed under physiological conditions.⁵⁻⁶ Undecylprodigiosin was identified as a selective promoter of apoptosis in breast cancer cell lines.⁷ The triazole derivatives of prodigiosin showed lower toxicity than the prodigiosin.⁸ Therefore, there is a need to develop stable synthetic ion transporters that could selectively induce cancer cell death. The development of stimuli-responsive systems is considered one of the most promising approaches that could be selectively applied against cancer cells. The tumor cells produce or alter various stimuli including, redox (glutathione), pH, and enzymes, which are capable of bestowing cancer cells with belligerent comportment that is categorized by invasiveness, propensity to metastasize, increased motility, drug resistance, and others.⁹ The pH-dependent selective transport of ion by small synthetic molecules has already been applied to explore the benefit of the acidic extracellular microenvironment of the tumor cells in comparison with that of healthy cells. However, the biological activities of these pH-sensitive synthetic Cl⁻ ion transporters were not thoroughly investigated.¹⁰⁻¹⁴ Within this chapter, we thought about the natural product derivatives, which could be one of the feasible alternatives and have the potential to minimize the undesired side effects of the various ion transporters. Despite tremendous progress in synthetic chemistry, natural products are still considered the best sources of drugs and drug leads. Currently, over 50% of clinically permitted drugs are either natural products or their derivatives. The natural products and their semi-synthetic derivatives are more compatible with the living system, as they have more “drug-likeness

and biological friendliness” than synthetic molecules.¹⁵⁻¹⁷ In this regard, natural product-based ion transporters could be developed to induce cancer cell death selectively.

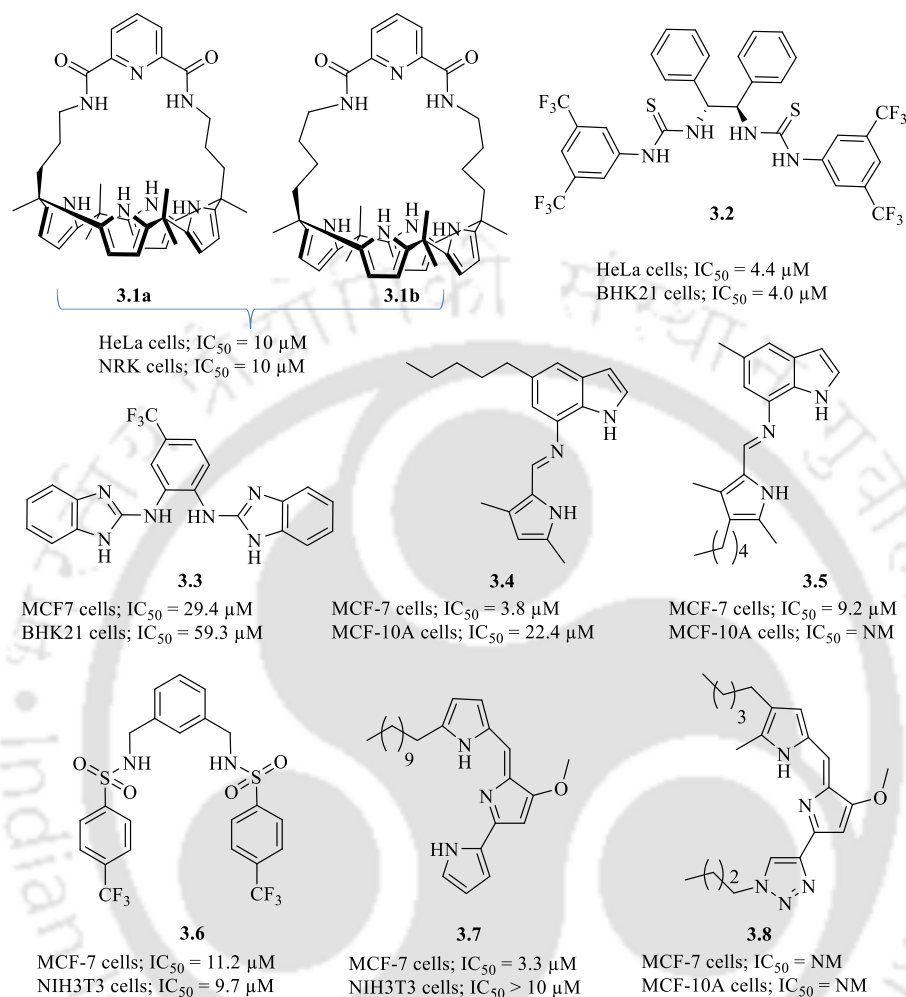


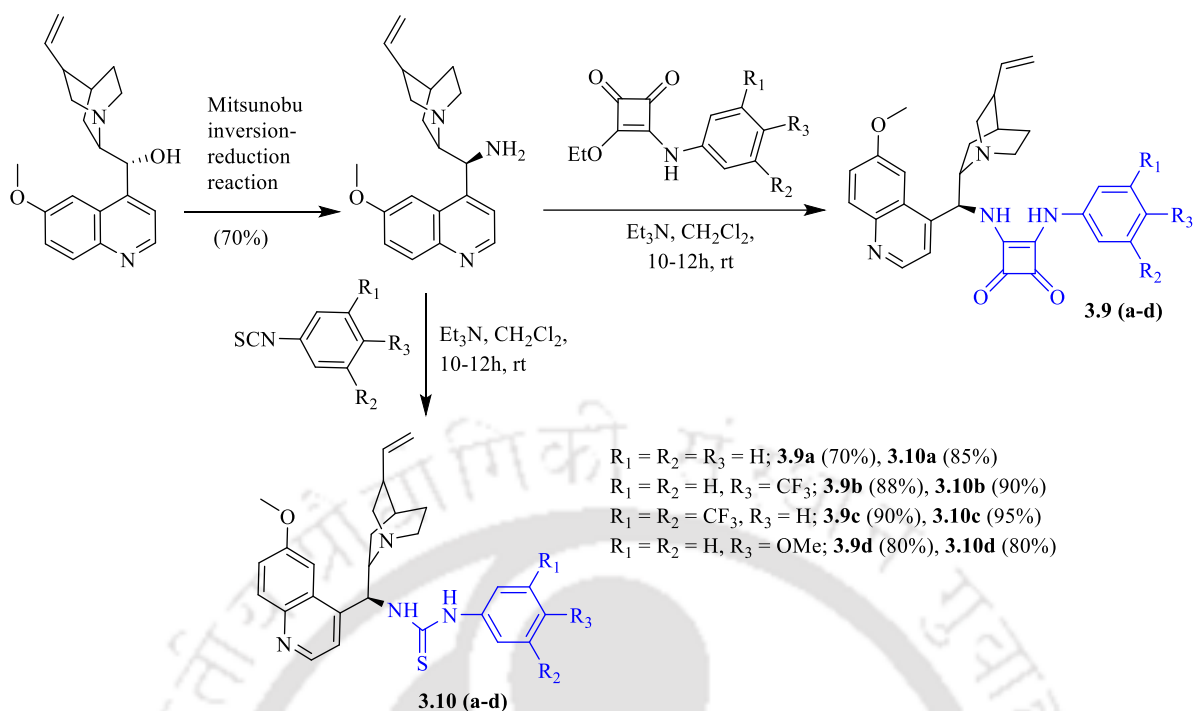
Figure 3.1. Antiproliferative activity of the known potent ion transporters in cancer and normal cell lines. NM = IC_{50} values were not measured, but point screening was performed.

The present study describes the design and synthesis of quinine derivatives as a new class of natural product-based pH-sensitive Cl^- ion transporters. The current study thoroughly investigated the relationship between physicochemical properties, ion recognition, ion transport, and biological activities. Additionally, the detailed transmembrane transport of Cl^- ion by the potent compounds also demonstrate apoptotic cell death through a caspase-dependent pathway. The *in vivo* studies also revealed that the potent ion transporters could reduce the tumor volume with minimum immunotoxicity.

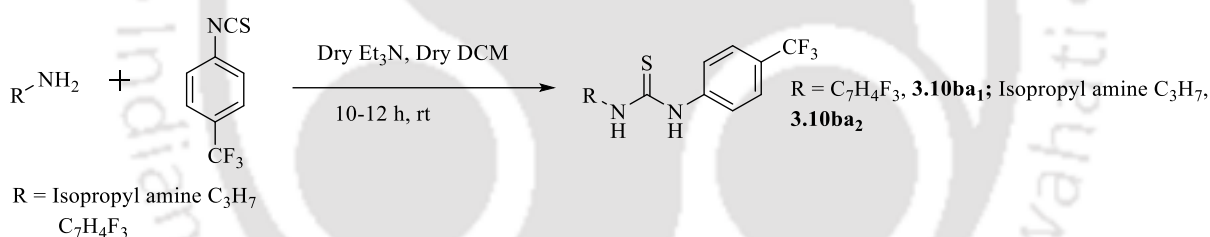
3.2. Results and discussions

3.2.1. Design and synthesis of quinine based anionophores

The cinchona alkaloid quinine is a century-old drug for malaria.¹⁸⁻¹⁹ Interestingly, the quinine and its derivatives are extensively used as organocatalysts for the asymmetric synthesis of numerous compounds through C-C/O/X bond formations, decarboxylation, hydrogenation, desymmetrization, and other reactions over the last forty years.²⁰⁻²² A detailed structural analysis revealed that quinine is a lipophilic molecule with an interesting membrane orientation pattern because of the presence of quinoline and quinuclidine rings. The quinoline moiety is linked with the quinuclidine ring through a secondary alcohol group. The quinine itself provides a preorganized structure, and the secondary alcohol group could be easily modified to create an ion binding cavity. We hypothesized that the installation of the ion-binding motif would allow the quinine derivatives to transport ions across the membrane. The protonation at the quinuclidine moiety could also play a significant role in better ion recognition, and their binding affinity could be tuned upon the variation of the pH of the surrounding medium.²³ The squaramide and thiourea are common Cl^- ion recognition and transport moieties. It is reported that squaramide and thiourea moieties are good hydrogen bond donors for anions.¹³ The N-H protons of the squaramide moiety have lower pK_a values, but the squaramide moiety provides discontinuous lipophilic surface. Whereas the hydrophobicity of the thiourea moiety provides a continuous shield of the hydrophobic surface to the compounds within the interior of the membrane, which assist the compounds for higher ion transport activities. Utilizing the quinine moiety as a core unit, a small series of squaramide and thiourea-based compounds were synthesized (Scheme 3.1), where the quinine was first converted to 9(S)-amino-9-deoxyepiquinine using one-pot Mitsunobu inversion–reduction methodology.²³ Condensation of 9(S)-amino-9-deoxyepiquinine with substituted isothiocyanates and 3-ethoxy-4-(phenylamino)cyclobut-3-ene-1,2-diones produced the thiourea and squaramide derivatives of 9(S)-amino-9-deoxyepiquinine, respectively (Scheme 3.1). The substituents of the phenyl ring of the isothiocyanates and 3-ethoxy-4-(phenylamino)cyclobut-3-ene-1,2-diones were varied to investigate their effect on the acidity of the N-H proton and hydrophobicity of the compounds. To investigate the effect of stereochemistry on anion transportation, the compounds **3.9bi** and **3.10bi** were also synthesized using the reported literature procedure. Besides these parent anionophores, few control compounds were also synthesized to compare the role of the quinine moiety using a simple condensation reaction (scheme 3.2).



Scheme 3.1. Synthesis of squaramide and thiourea derivatives of quinine.



Scheme 3.2. Synthesis of the control compounds.

3.2.2. Anion binding studies of the quinine derivatives

The ¹H NMR titration experiments were performed to explore the pattern of interactions of the quinine derivatives with the Cl[−] ion. The substantial downfield shift of both N-H_a and the N-H_b protons of the squaramide (**3.9a-d**) and thiourea (**3.10a-d**) moieties support their interaction with Cl[−] ion through hydrogen bond formation (Figure 2A and Figure 3.23-30). The Job's plot analysis confirmed 1:1 binding stoichiometry of the compounds with the Cl[−] ion (Figure 3.44-45 and Table 3.6-7). The strength of interactions was also investigated by calculating the binding constant (*K*) from the chemical shifts ($\Delta\delta$) values for both the N-H signals, using the BindFit v0.5 program.²⁴⁻²⁵

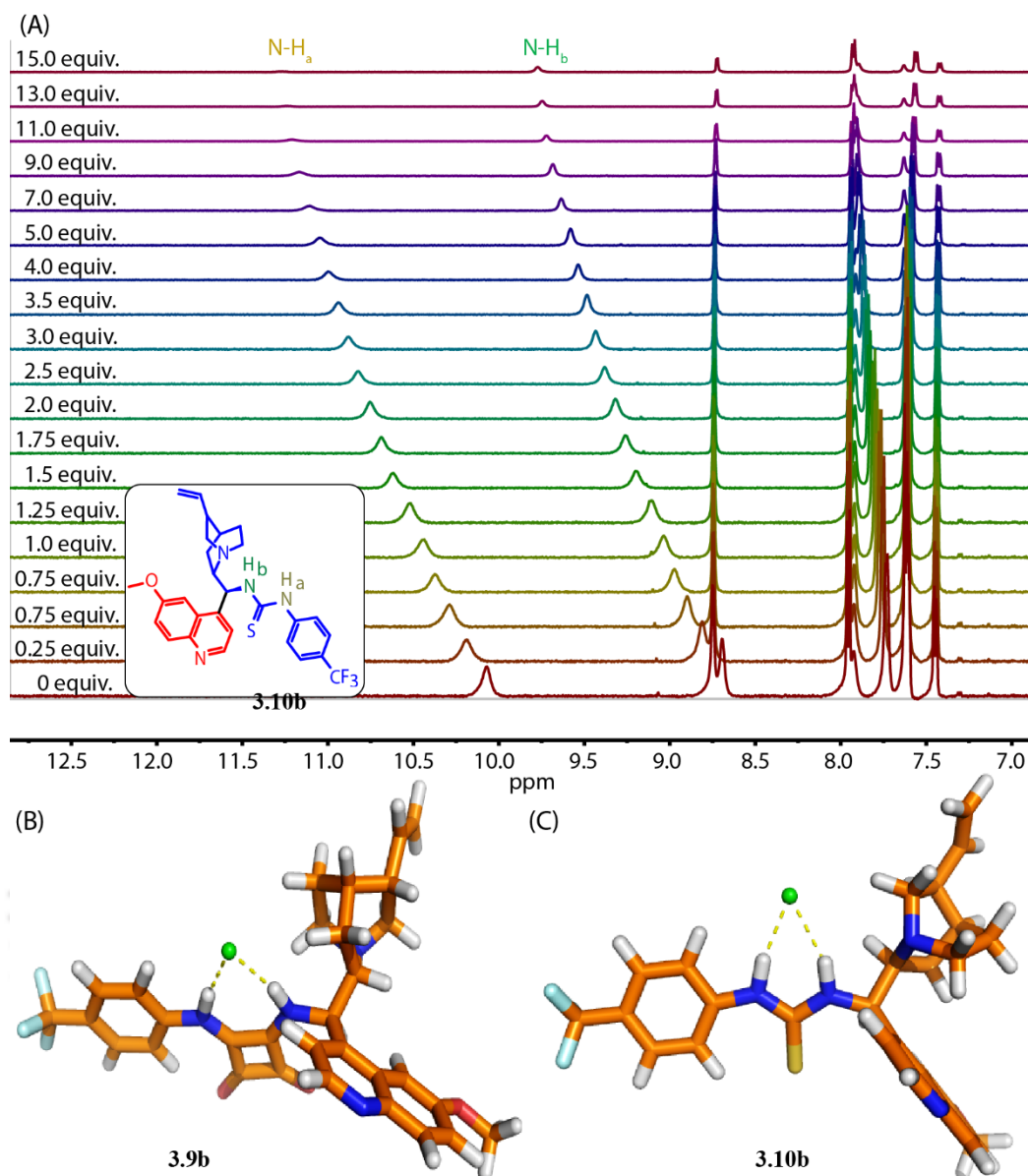


Figure 3.2. Representative ¹H NMR titration spectra for compound **3.10b** (8.4 mM) with TBACl in DMSO-*d*₆ solvent (A). The quantities of TBACl are shown on the spectra. The geometry optimized structures of [3.9b + Cl⁻] (B) and [3.10b + Cl⁻] (C) complexes.

The calculated *K* values of the compounds were within the range of 23.7-1032.6 M⁻¹ (Table 3.1), suggesting their Cl⁻ ion recognition abilities in the solution phase. The Cl⁻ ion binding properties of squaramide-based compounds **3.9a-d** were relatively stronger than the thiourea-based compounds **3.10a-d** (Table 3.1). The *pK_a* values of squaramides are generally lower than the corresponding thioureas, which results in their stronger hydrogen bond formation capabilities.²⁶ However, the binding constants for compounds **3.9b** and **3.10b** with Br⁻, I⁻, and NO₃⁻ could not be calculated accurately due to small

shifts of the N-H_a and N-H_b protons (Figure 3.31-34). Hence, the ¹H NMR titration experiments confirmed superior Cl⁻ ion recognition capabilities of compounds.

Table 3.1. Lipophilicity and anion recognition properties of the compounds.

Compound	LogP ^a	pK _{a1} , and pK _{a2}	Binding constant (K; M ⁻¹) ^b
3.9a	4.0	-	279.6 ± 1.0
3.9b	4.9	4.7, 6.8	282.9 ± 2.4
3.9c	5.8	-	1032.6 ± 8.1
3.9d	3.9	-	265.6 ± 2.7
3.10a	5.0	-	30.7 ± 0.2
3.10b	5.9	4.4, 6.8	68.9 ± 1.2
3.10c	6.8	-	80.1 ± 1.0
3.10d	4.9	-	23.7 ± 0.8

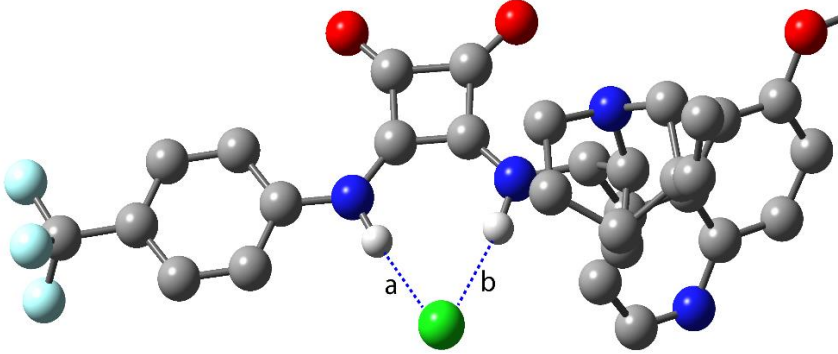
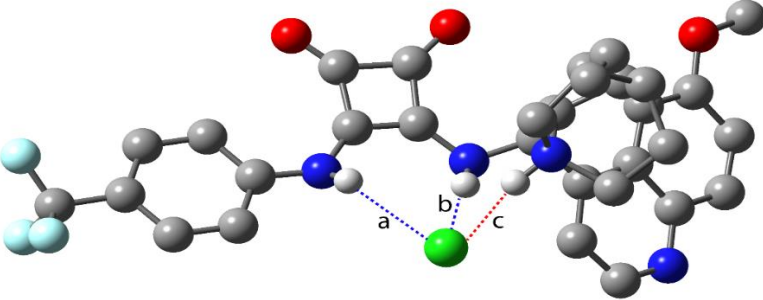
^aLog P values of the compounds were calculated using Marvin Sketch 17.27 program. The pK_{a1} (quinoline) and pK_{a2} (quinuclidine) values of the compounds were calculated by ¹H NMR titration in D₂O/ DMSO-*d*₆ solvent mixture (1:20, v/v) at different pH. ^bBinding constant (K) values were calculated by monitoring the changes in chemical shifts of both N-H_a and N-H_b protons using the BindFit v0.5 program.

3.2.3. Theoretical anion binding studies

To gain atomic insight into the binding mode of the quinine derivatives with the Cl⁻ ion, the density functional theory (DFT) calculations were performed.²⁷⁻²⁸ The optimized structures with the relevant bonded parameters of chloride-bound molecules are shown in Figure 3.2B-C. The structure of all possible conformations of compounds **3.9b** and **3.10b** showed that the N-H protons of the squaramide and thiourea moieties are mainly accountable for their interactions with the Cl⁻ ion (Figure 3.2B-C and Table 3.8-9). The DFT analysis also showed that the distance for the interactions of compounds **3.9b** and **3.10b** with Cl⁻ ion is within the hydrogen-bond distance (2.13-2.95 Å). It should be noted that the calculated pK_a values of the conjugate acid of the quinuclidine moiety are 6.8 (Table 3.1 and Figure 3.35-43), suggesting that the extent of protonation of this nitrogen atom at a lower pH range (4-6.5) is much greater in comparison with that at physiological

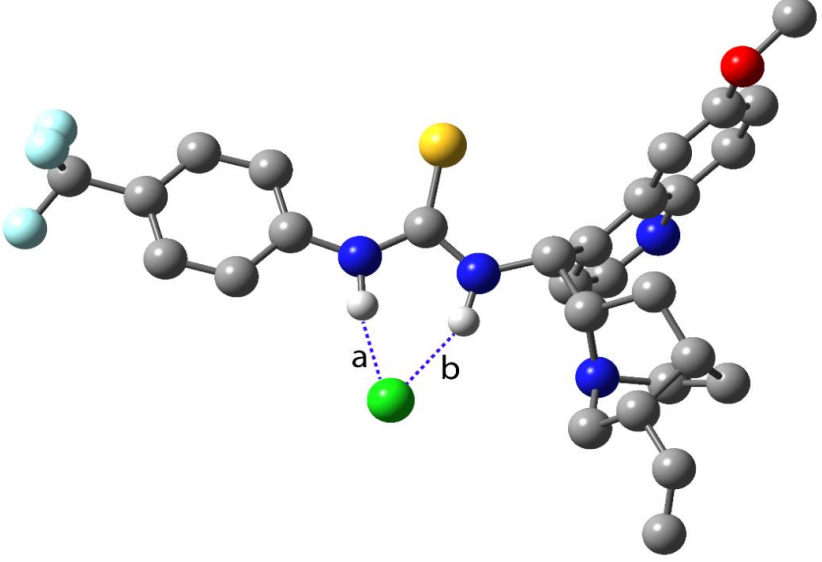
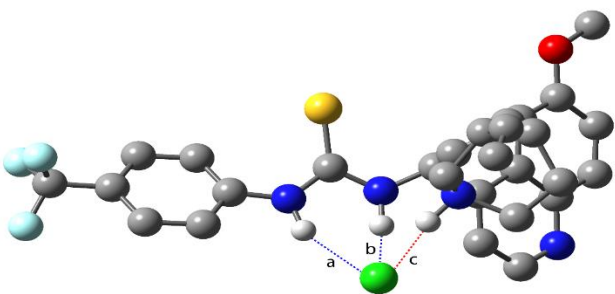
conditions.²⁹⁻³⁰ In this regard, the DFT analysis was also performed with the protonated form of compounds **3.9b** and **3.10b**, which showed tridentate chloride coordination

Table 3.2. Optimized structure of ‘**3.9b**---Cl[−] complex’ where the compound is at neutral and protonated states at B3LYP/6-31+g* level of theories.

3.9b---Cl[−] complex (3.9b in the neutral state)	
	Interaction Energy (Kcal/mol) 60.051
	Cl[−]---H Bond Distance (Å) a = 2.163 b = 2.158
3.9b-H⁺---Cl[−] complex (3.9b in the protonated state)	
	Interaction Energy (Kcal/mol) 123.842
	Cl[−]---H Bond Distance (Å) a = 2.590 b = 2.192 c = 2.068

Only interacting N-H protons are shown here. The dashed line indicates hydrogen bonding. The effect of the additional proton is designated by a red color dashed line.

Table 3.3. Optimized structure of ‘**3.10b**---Cl⁻ complex’ where the compound is at neutral and protonated states at B3LYP/6-31+g* level of theories.

3.10b---Cl⁻ complex (3.10b in the neutral state)	
	Interaction Energy (Kcal/mol) 44.252
	Cl⁻---H Bond Distance (Å) a = 2.129 b = 2.295
3.10b-H⁺---Cl⁻ complex (3.10b in the protonated state)	
	Interaction Energy (Kcal/mol) 122.351
	Cl⁻---H Bond Distance (Å) a = 2.465 b = 2.261 c = 1.973

Only interacting N-H protons are shown here. The dashed line indicates hydrogen bonding. The effect of the additional proton is designated by a red color dashed line.

involving squaramide or thiourea and protonated quinuclidine moieties. The distance for the interactions of the protonated form of compounds **3.9b** and **3.10b** with Cl⁻ ion are also

within the hydrogen-bond distance (1.97-2.59 Å). Noticeable elongation of quinuclidine N–H bond length from 1.03 Å to 1.07 Å (Table 3.2-3) in response to chloride binding with short $\text{Cl}^- \cdots \text{H}$ distance of $\sim 1.98 \text{ Å}$ indicate stronger interaction between chloride and quinine moiety. Interestingly, the distance between protonated quinuclidine moiety and Cl^- ion (1.97-2.06 Å) is shorter than the distances between Cl^- ion and squaramide/thiourea moiety (2.19-2.59 Å), which indicate stronger interaction between chloride and quinuclidine moiety due to electrostatic reasons (Table 3.2-3). The interaction energy for the Cl^- ion with the protonated compounds (122.351 to 123.842 kcal mol^{-1}) is much higher in comparison with that of the neutral form (44.252 to 60.051 kcal mol^{-1}). The outcome of the DFT calculations supports the quinuclidine-mediated Cl^- ion binding aptitudes of the compounds.

3.2.4. Ion transport activities of the quinine derivatives

The ion recognition abilities of these lipophilic quinine derivatives encouraged us to explore their ion transport activity across the bilayer of large unilamellar vesicles (LUVs). The Cl^- ion transport activities of all the compounds were investigated by the classical ion-selective electrode (ISE)-based assay.⁴ The phosphatidylcholine (EYPC) and cholesterol (CHOL) lipids (6:4 molar ratios) were used to prepare the LUVs with intravesicular NaCl solution (5 mM phosphate buffer at pH 7.2 containing 100 mM NaCl) and extravesicular NaNO_3 solution (5 mM phosphate buffer at pH 7.2 containing 100 mM NaNO_3). The compounds were added in the extravesicular solution, and the extent of Cl^- ion efflux was monitored using the chloride ion-selective electrode in the absence of any pH gradient. A continuous increase in the Cl^- ion concentration in the extravesicular solution suggests the Cl^- ion transport activities of the compounds. The initial screening revealed that all quinine derivatives (1.56 μM = 0.5 mol% with respect to lipid) efficiently increase the Cl^- ion efflux rate, demonstrating their transport efficiencies across the lipid bilayers at pH 7.2. As it is already mentioned that the protonation of the quinuclidine ring could significantly affect the anion binding properties of the compounds (Figure 3.46).²³ Hence, to investigate the effect of protonation of the quinuclidine ring on Cl^- ion transport activity, the ISE-based assays were also performed at pH 5.5 (Figure 3.3C-D and Figure 3.46). The dose-dependent ISE assay was used to calculate the EC_{50} values for the quinine derivatives both at pH 7.2 and 5.5 (Figure 3.49-58). The outcome of the dose-dependent ISE assay demonstrated that in acidic medium (pH 5.5), these compounds have much

stronger Cl^- ion efflux aptitude than that of under physiological conditions. The EC_{50} values of the compounds were within 1.6-26.3 and 0.9-8.9 μM at pH 7.2 and 5.5, respectively (Table 3.4). The compounds **3.9b** and **3.10b** showed 5.3 and 8.9-folds increase in Cl^- ion transport abilities, respectively, at pH 5.5 in comparison with that at pH 7.2, suggesting their pH-dependent Cl^- ion transport activities (Table 3.4).

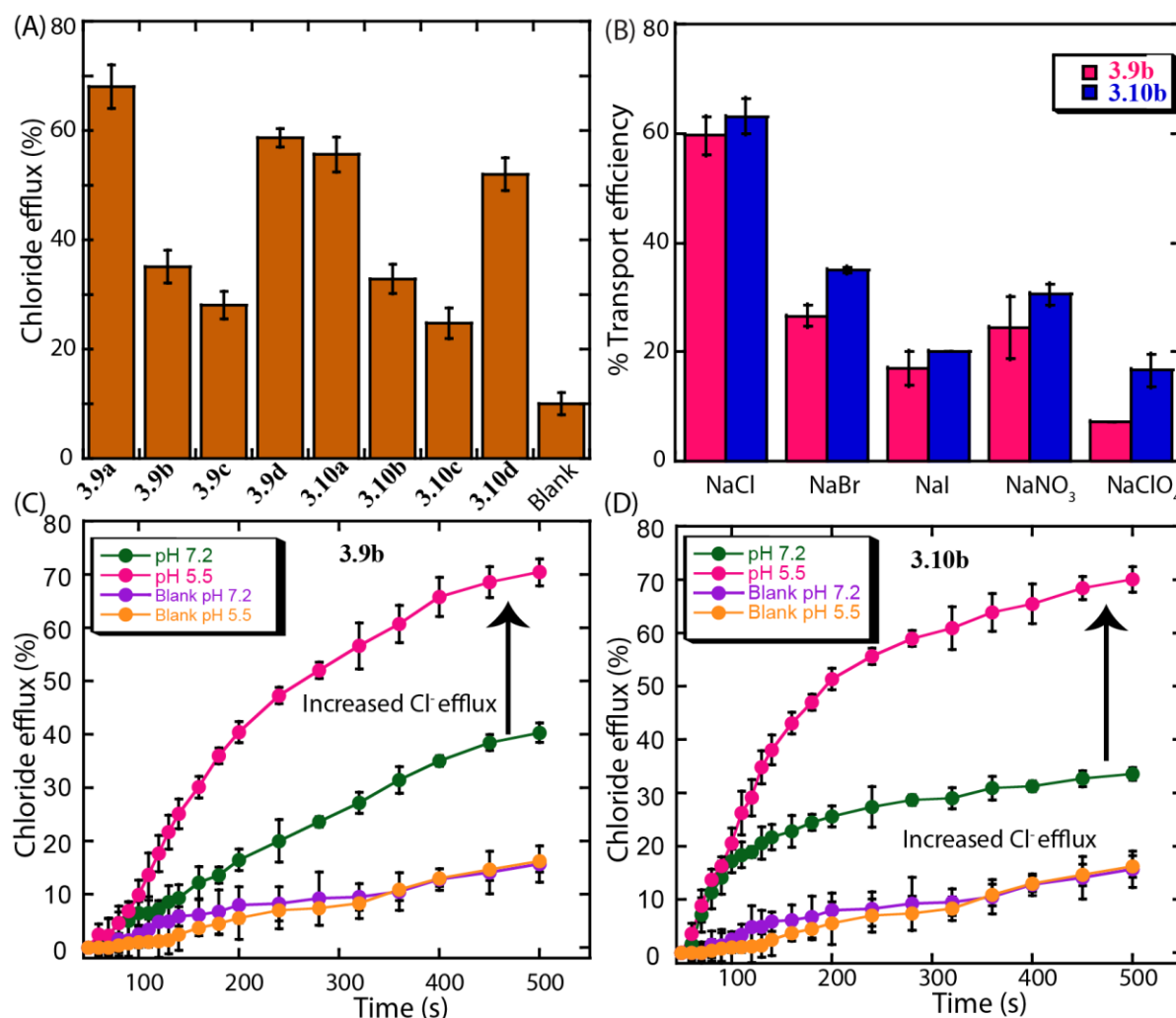


Figure 3.3. Representations of chloride ion transport activity across EYPC/CHOL-LUVs>HPTS (A). Demonstration of ion transport kinetics displaying normalization plot (B). The Cl^- ion transport activities of the compounds, **3.9a–d** and **3.10a–d** (1.56 μM = 0.5 mol % with respect to lipid) across the EYPC/CHOL-LUVs was measured by ISE-based assay at pH 7.2 (C). Anion transport selectivity of compounds **3.9b** and **3.10b** was measured by a fluorescence-based assay using EYPC/CHOL-LUVs>HPTS in the presence of the base pulse in the extravesicular region (D) Chloride ion efflux capability of compound **3.9b** (0.56 μM = 0.18 mol % with respect to the lipid) at pH 5.5 and 7.2

(E). The Cl^- ion efflux aptitudes of compound **3.10b** ($2.29 \mu\text{M} = 0.733 \text{ mol } \%$ with respect to lipid) across EYPC/CHOL-LUVs were measured by ISE-based assay at pH 5.5 and 7.2 (F). Compound concentrations were $0.56 \mu\text{M} = 0.13 \text{ mol } \%$, and $2.29 \mu\text{M} = 0.55 \text{ mol } \%$ with respect to lipid for compound **3.9b** and **3.10b**, respectively. DMSO was used as blank. The efflux efficiency was measured after 450 sec of compound addition. All experiments were performed in triplicate.

Table 3.4. Transmembrane transport efficiencies of the compounds at different pH.

Compound	EC ₅₀ (μM)		EC ₅₀ fold change (pH 7.2/pH 5.5)
	pH 7.2 (n ^a)	pH 5.5 (n ^a)	
3.9a	1.6 ± 0.2 (1.0)	0.9 ± 0.1 (0.8)	1.8
3.9b	5.8 ± 0.3 (1.1)	1.1 ± 0.1 (0.9)	5.3
3.9c	13.6 ± 1.0 (0.8)	4.4 ± 0.4 (0.8)	3.1
3.9d	3.6 ± 0.2 (0.7)	1.7 ± 0.1 (0.8)	2.1
3.10a	3.9 ± 0.3 (1.1)	1.2 ± 0.1 (1.0)	3.3
3.10b	10.7 ± 0.4 (0.8)	1.2 ± 0.2 (0.8)	8.9
3.10c	26.3 ± 1.6 (0.8)	8.9 ± 1.1 (0.7)	3.0
3.10d	7.2 ± 0.5 (0.7)	2.1 ± 0.3 (0.7)	3.4

^an = Hill coefficient

Besides, the pH-dependent Cl^- ion transport properties of the potent compounds were also performed to understand the effect of protonation on Cl^- ion transport efficiency. The quinoline moiety is at the distant site from the Cl^- ion binding thiourea or squaramide moiety. Hence, quinoline moiety does not have a considerable impact on the Cl^- ion transport properties of the compounds. The pH-dependent Cl^- ion transport activities of the potent compounds showed that the 'apparent pK_a' values of the quinuclidine moiety of compounds **3.9b** and **3.10b** were found to be at 6.99 and 6.96, respectively, under the liposomal environment (Figure 3.59). Hence, the extent of protonation of the quinuclidine moiety at a lower pH range is much greater than that of under physiological conditions, resulting in superior Cl^- ion transport activities of these compounds. Overall, the compounds with CF_3 substituted aromatic moieties showed lower Cl^- ion transport

activities. In general, the CF_3 substituted compounds are active ion transports. Hence, to investigate the possibility of the formation of intramolecular hydrogen bonding between the quinuclidine-nitrogen and the opposite NH group, the DFT analysis was performed. The outcome of the DFT analysis revealed that the thiourea moiety forms an intramolecular hydrogen bond with the quinuclidine-nitrogen, which could be one of the reasons for the less protonation ability of compound **3.10b** at physiological conditions and more significant change in EC_{50} values at acidic pH. The strength of such intramolecular hydrogen in compound **3.10c** was much stronger than that of compound **3.10b**, suggesting its weaker quinuclidine-nitrogen protonation ability and lower Cl^- ion transport activities even in acidic pH (Figure 3.47). A similar intramolecular hydrogen-bonding pattern was observed for compounds **3.9b** and **3.9c** (Figure 3.47). Hence, compounds **3.9b** and **3.10b** were selected for further studies.

3.2.5. Relative ion selectivity studies of the potent compounds

The effects of extravesicular anions or cations on their Cl^- ion transport activities of the compounds were also investigated by HPTS assay in the presence of pH-gradient ($\text{pH}_{\text{out}} = 7.8$ and $\text{pH}_{\text{in}} = 7.2$). Considerable differences in the anion transport activities for the compounds were observed when NaCl was used as the intravesicular solution and isosmotic buffer solution of different NaX salts (F^- , Cl^- , Br^- , I^- , NO_3^- , ClO_4^-) were used in the extravesicular solution, suggesting the involvement of anions in the transport process. The compounds **3.9b** and **3.10b** follows an anion transport efficiency in the order of $\text{Cl}^- \gg \text{Br}^- > \text{NO}_3^- > \text{I}^- > \text{ClO}_4^-$ (Figure 3.3B and Figure 3.4). The strong binding of the ion supports this Cl^- ion selectivity of compounds **3.9b** and **3.10b** in the cavity of the squaramide and thiourea moieties. However, lower transport activities for the other tested anions indicate their poor binding abilities due to the disparity of their sizes with the binding pocket of the compounds. Similarly, iso-osmotic solutions of the chloride salt of various monovalent metals (Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+) in the extravesicular solution were used to examine the role of different cations on the Cl^- ion transport efficiency of the compounds. Figure 3.4 showed that there might be a small effect of the cation on the kinetics of ion transport for compound **3.10b**, although the final value before the addition of detergent is similar. The ion selectivity studies were also performed in the presence of vesicles prepared with the salt under investigation present in both intra- and extravesicular

media. The outcome of this selectivity study follows a similar pattern as described by the other method in this section (Figure 3.5).

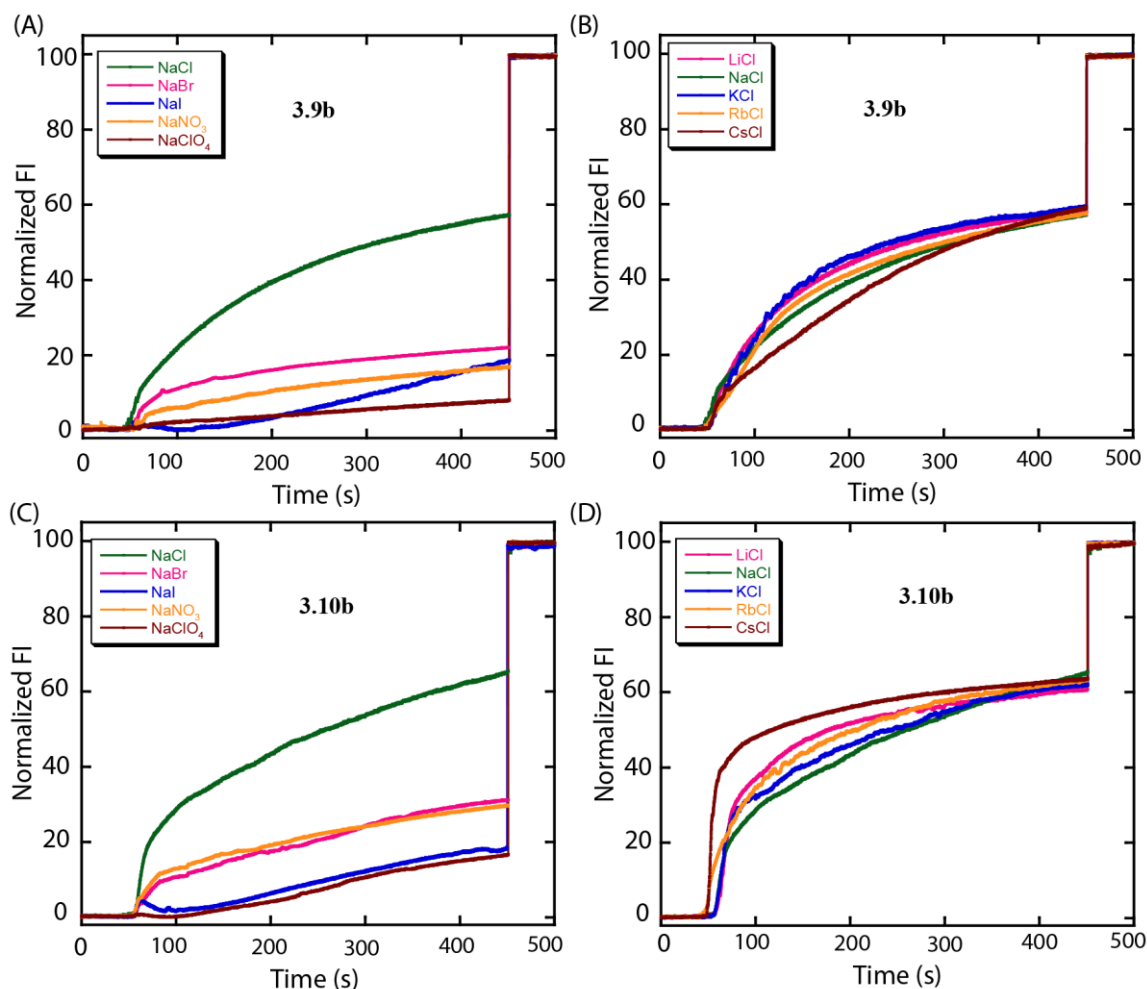


Figure 3.4. Representative plots of the anion (A and C), cation (B and D) transport selectivity of compounds **3.9b** ($0.56 \mu\text{M} = 0.13 \text{ mol } \%$ with respect to the lipid), and **3.10b** ($2.29 \mu\text{M} = 0.55 \text{ mol } \%$ with respect to the lipid) across EYPC/CHOL-LUVs $\Rightarrow$ HPTS in the presence of the base pulse. All experiments were performed in triplicate.

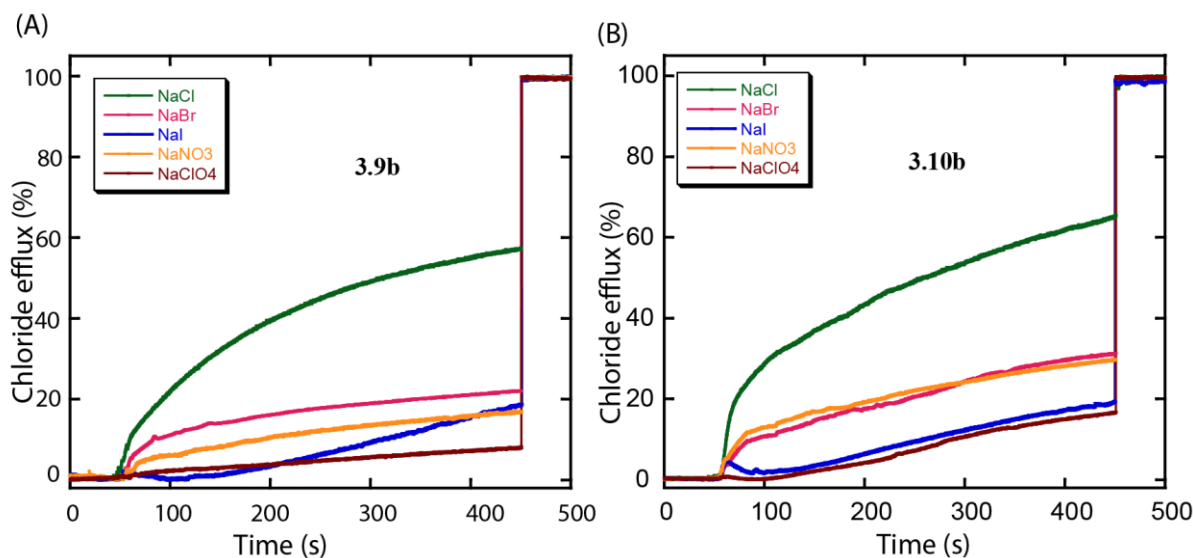


Figure 3.5. Representative plots of the anion transport selectivity of compounds **3.9b** (A; 0.56 μM = 0.13 mol % with respect to the lipid), and **3.10b** (B; 2.29 μM = 0.55 mol % with respect to the lipid) across EYPC/CHOL-LUVs $\rightarrow$ HPTS in the presence of the base pulse. The ion selectivity studies were also performed in the presence of vesicles prepared with the salt under study present in both intra- and extravesicular media. All experiments were performed in triplicate.

3.2.6. Mechanism for transport of chloride ion of the potent compounds

The transport of Cl^- ion by the compounds **3.9b** and **3.10b** may follow either antiport (OH^-/A^-) or symport (H^+/A^-) mechanism.⁴ In this regard, the transport activity was first measured with intravesicular NaCl and an isotonic solution of NaNO_3 , NaI, NaBr, and NaClO_4 in the extravesicular region by HPTS assay in the absence of pH gradient (i.e., $\text{pH}_{\text{in}} = \text{pH}_{\text{out}} = 7.2$).⁴ Even though no pH gradient was applied still a notable change in HPTS fluorescence intensity in the presence of compounds suggests the possibility of an influx of H^+ across the membrane bilayer. It is also expected that for the charge neutrality, the influx of X^- ion would be accompanied. However, in the presence of SO_4^{2-} ion, an increase in the HPTS fluorescence intensity was observed. The higher hydration energy restricts the SO_4^{2-} ion (hydration energy = 1080 kJ mol^{-1}) to cross the lipid bilayer, but the enhancement in the HPTS fluorescence signal indicates the efflux of the H^+/Cl^- ion pair as symport mechanism (Figure 3.6A-B).

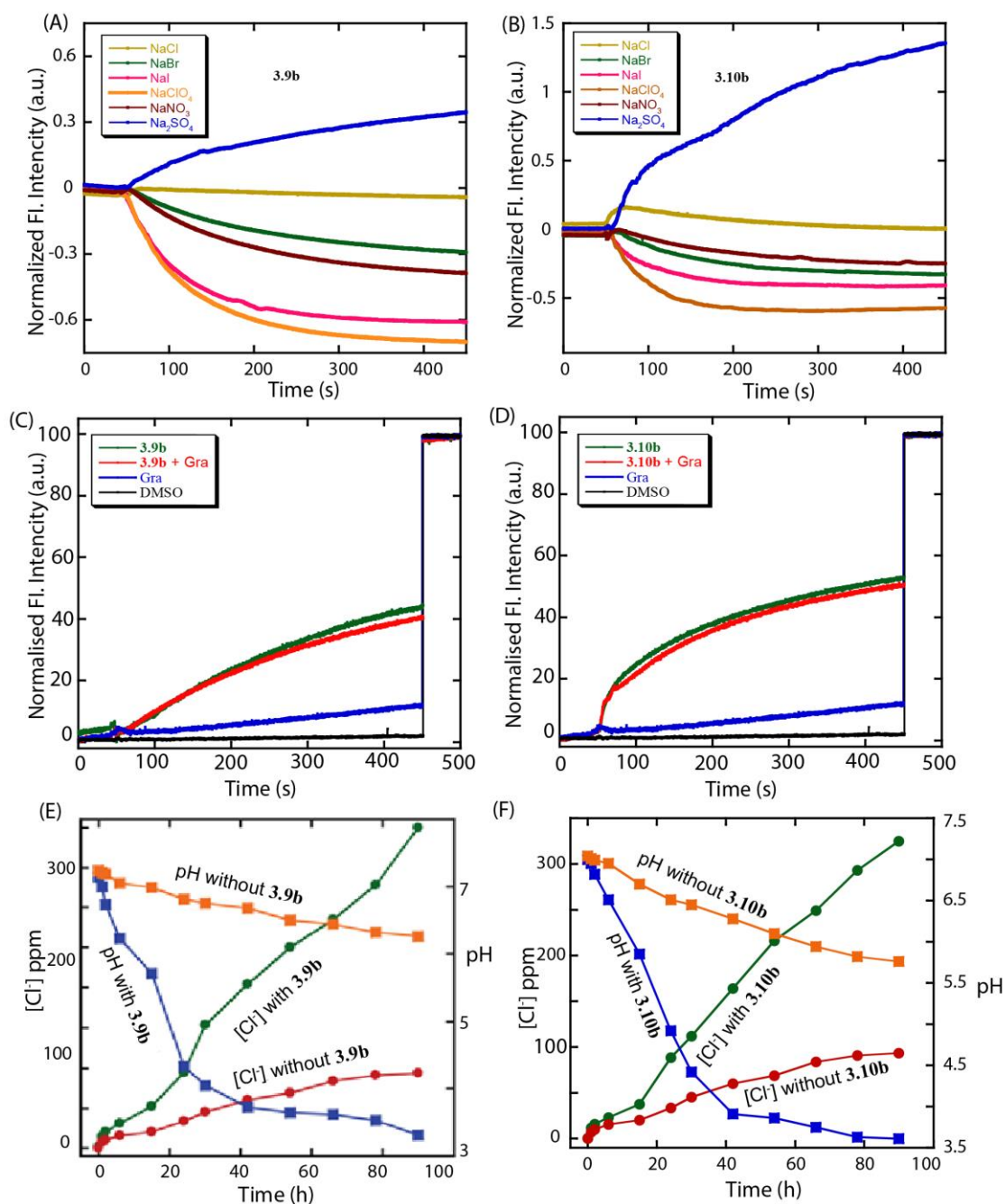


Figure 3.6. Anion selectivity of compounds **3.9b** (A) and **3.10b** (B) was measured across EYPC/CHOL-LUV Δ HPTS by varying the extravesicular anions without applying pH gradient. NMDG-Cl assay for compound **3.9b** (C) and **3.10b** (D) in the absence and presence of proton channel gramicidin D (Gra). The H⁺/Cl⁻ transport efficacy of compound **3.9b** (E) **3.10b** (F) was measured across a U-tube by applying the HCl gradient, using chloride ion-selective electrode and pH meter. The concentration of compound **3.9b** and **3.10b** were 0.56 μ M = 0.13 mol %, and 2.29 μ M = 0.55 mol % with respect to the lipid, respectively.

The HPTS assay was performed in the absence and presence of 4-(trifluoromethoxy)phenylhydrazone (FCCP; a protonophore) in 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl for further investigation of the Cl^- ion transport properties of compounds. The compounds showed a similar increase in the Cl^- ion transport activity both in the absence and presence of the FCCP (Figure 3.7A and 3.7C). The rate of dissipation of the pH gradient in the FCCP experiment is not affected by the protonophore, which suggests that the involvement of Cl^- ion in the rate-limiting step of the H^+/Cl^- symport transportation. We also performed the HPTS assay in the absence and presence of valinomycin (a potassium ionophore) in 20 mM HEPES buffer, pH 7.2, containing 100 mM KCl. Comparable enhancement in the Cl^- ion transport rate in the absence and presence of the valinomycin suggests preferential transport of Cl^- ion over OH^- ion (Figure 3.7B and 3.7D). As a consequence, the cotransport of H^+/Cl^- by the ionophore was the operating mechanism for the transport of Cl^- ion. Besides, the NMDG-Cl (*N*-methyl-D-glucamine chloride) assay was performed in the absence and presence of gramicidin-D in 100 mM NMDG-Cl buffer, pH 7.0. The NMDG (5 mM) was used in the *extravesicular region* to create the pH gradient. The compound exhibited a comparable change in the Cl^- ion transport activity both in the absence and presence of the gramicidin-D, suggesting that Cl^- transport is the rate-limiting step (Figure 3.6C-D). Hence, these HPTS-based assays propose that the transport rate of H^+/OH^- is faster than Cl^- ion, and H^+/Cl^- symport is the operating mechanism.

To confirm the H^+/Cl^- co-transport mechanism, we performed the classical U-tube assay. The left-arm of the U-tube was filled with the aqueous HCl (100 mM) solution (pH 1.05), while the right-arm was filled with aqueous NaNO_3 (100 mM) solution, and these two arms were separated by the chloroform solution, which mimics the membrane environment. A steady decrease in the pH and a sharp increase in Cl^- ion concentration directly confirmed its H^+/Cl^- co-transport mechanism (Figure 3.6E-F). The U-tube assay was also performed in the presence of bromothymol-blue (a pH-sensitive dye) in the right-arm of the U-tube along with the NaNO_3 solution. The time-dependent change in the color of bromothymol-blue visually confirmed the difference in the pH of the solution in the presence of the compound (Figure 3.65).

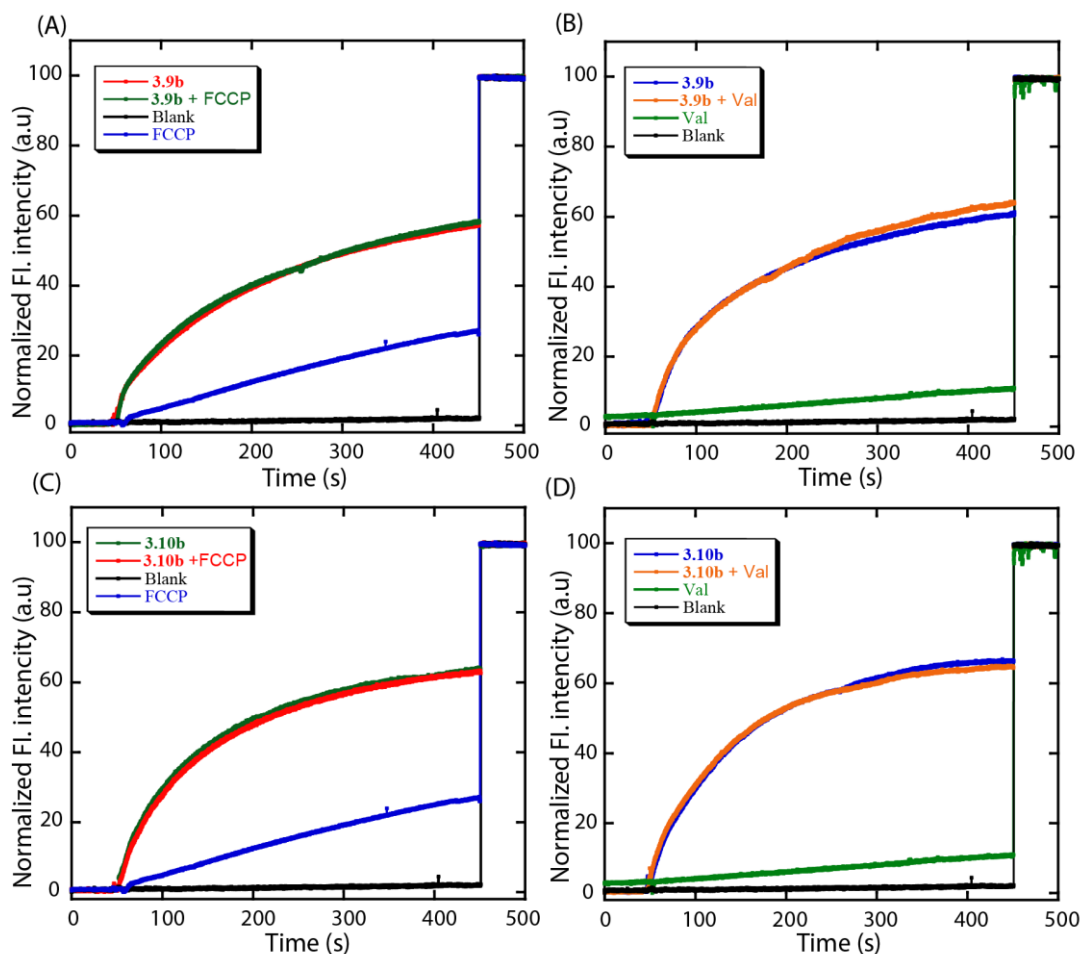


Figure 3.7. Assessment of Cl^- ion transport properties of compound **3.9b** and **3.10b** (2.29 μM = 0.55 mol% with respect to lipid) in the absence and presence of FCCP (A, C) or valinomycin (B, D) in 20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl/KCl.

To investigate whether the compound-mediated transport of Cl^- ion follows carrier or channel mechanism, the cholesterol concentration dependency assay was performed (Figure 3.8). The outcome of the experiment revealed that the transport of Cl^- ion depends on the concentration of the cholesterol present in the membrane, confirming the carrier-mediated Cl^- ion transport pathway of the compounds. The classical U-tube assay also confirmed that the potent compounds **3.9b** and **3.10b** transport Cl^- ion through carrier mechanism. Meanwhile, the

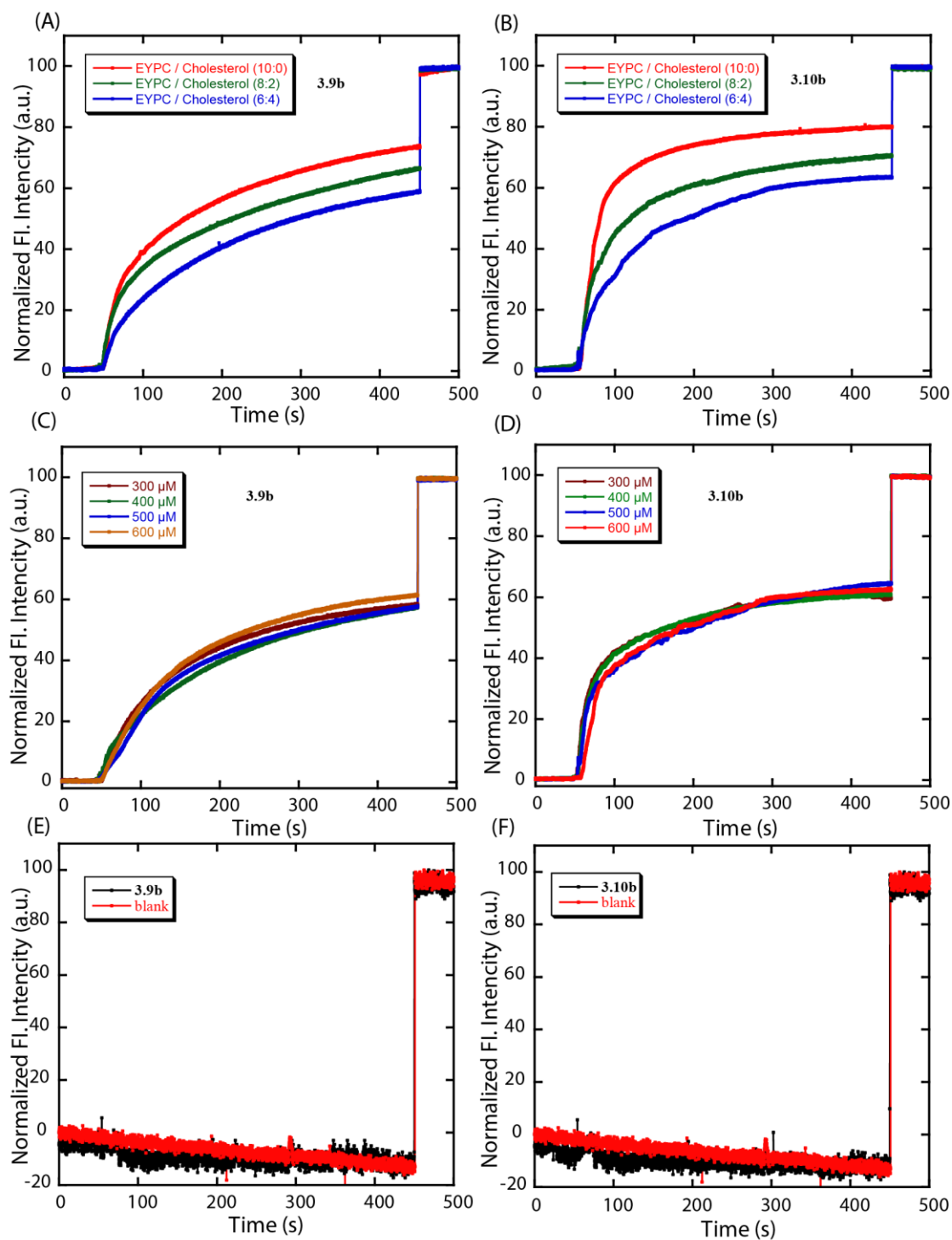


Figure 3.8. Effect of the concentration of cholesterol in the EYPC/CHOL membrane on Cl^- ion transport activity of compound **3.9a** (A) and **3.10b** (B), respectively. Leaching experiment of compounds **3.9b** (C) and **3.10b** (D). The extent of calcein leakage from the EYPC/CHOL-LUV $\Rightarrow$ calcein in the presence of compounds **3.9b** (E) and **3.10b** (F). The concentration of compound **3.9b** and **3.10b** were $0.56 \mu\text{M} = 0.13 \text{ mol\%}$, and $2.29 \mu\text{M} = 0.55 \text{ mol\%}$ with respect to the lipid, respectively.

leaching test demonstrated the non-leaching behaviors of the compounds from the lipid bilayers (Figure 3.8C-D). The non-leaking ability of calcein from the LUVs in the presence of compound also supports carrier mechanism and dismisses the probability of pores or channels formations (Figure 3.8E-F). Further, the stability of the potent compounds in different buffer solutions was also performed (pH 5.5-8.0) after 72 hours of incubation. The HPLC analyses clearly showed that the structural integrity of the compounds **3.9b** and **3.10b** are intact (Figure 3.66).

3.2.7. Ion transportation under cellular environment

3.2.7.1. MTT assay in presence and absence of chloride ions

The excellent Cl^- ion transport activities of the selected quinine derivatives encouraged us to investigate their biological applicability.^{1, 4, 30} Recent studies showed that the perturbation of Cl^- ion homeostasis, modification of the extracellular or intracellular pH level is associated with anticancer activities. Various studies also demonstrated that the synthetic Cl^- ion transporters promote apoptosis-mediated cell death through the influx of Cl^- ion into cells.^{1, 31} The antiproliferative activity of the compounds was investigated in HCT-116 (colon cancer cell line), OVCAR8 (ovarian cancer cell line), MDA-MB-231 (triple-negative breast cancer cell line), MCF-7 (Luminal A breast cancer cell line), and MCF-10A (a non-tumorigenic breast epithelial cell line) by MTT assay.³²⁻³⁴ Initially, the viability of these cells in the presence of these compounds was investigated at a fixed concentration of 10 μM after 48 hours of the treatment (Figure 3.9A). The outcome of this initial screening showed that maximum cancer cell death was observed in the presence of compounds **3.9b** and **3.10b**. The concentration-dependent MTT assay showed that except for quinine, all other compounds have dose-dependent antiproliferative activity (Figure 3.67). Analysis of the IC_{50} values revealed that compounds **3.9b** and **3.10b** have the most potent antiproliferative activity against different cancer cell lines (Table 3.5). Interestingly, compound **3.10b** has very low antiproliferative activity against the normal breast epithelial cell line, MCF10A (growth inhibition was around 30% in the presence of 50 μM of the compound after 48 hours of treatment) as compared to the cancer cell counterpart. Whereas compound **3.10b** has an almost similar antiproliferative activity for both normal and cancer cells (Figure 3.67F). It is well documented that the internal pH ($\text{pH}_i \approx 7.2$) of normal healthy cells is lower than the extracellular pH ($\text{pH}_e \approx 7.4$). Conversely, the pH_e is in the range of 6.2–6.9 and $\text{pH}_i \geq 7.4$ in cancer cells due to the

anaerobic excess metabolism of glucose. This acidic microenvironment of tumor tissues has been exploited to target the cancer cell, reduce the unwanted toxicity, and anticancer drug resistance capability.^{4, 35} The calculated ion transport activity (EC_{50}) is not directly correlated with cytotoxicity (IC_{50}) of the compounds (Table 3.4 and Table 3.5). However, the viability difference between normal and cancerous cells in the presence of compounds **3.9b** and **3.10b** is directly related to their pH-dependent Cl^- ion transport abilities (5.3 and 8.9 folds, respectively). The other tested compounds proficiently transport Cl^- ion but are not as effective as compounds **3.9b** and **3.10b** in inducing selective cancer cell death and also have poor pH-dependent Cl^- ion transport properties. It is important to mention that all the tested compounds (except **3.9c** and **3.10c**) have low EC_{50} values similar to that of compounds **3.9b** and **3.10b** at lower pH. However, the viabilities of these compounds are comparatively higher in cancerous cells. In particular, compound **3.9a**, with low EC_{50} values at both pH seems to be almost inactive against the tested cell lines. These observations clearly suggest that only the anion transportability is not always sufficient enough to promote cell death. Therefore, both the structure of the individual compound and its anion transport efficiency could be the driving force for their anion transport mediated selective cancer cell death. There are also several reported compounds that are potent anion transporter but not active enough to promote selective cancer cell death.^{6, 36-37} Hence, further detailed biological investigations are required to unravel the difference between the EC_{50} and IC_{50} values of several anionophores and to investigate whether these compounds have any specific drug target, which is beyond the scope of this study. In the meantime, we also performed the MTT assay of compounds **3.9ci** and **3.10ci**, which are the squaramide and thiourea derivative of 9(R)-amino-9-deoxyepiquinine (Figure 3.67). The compounds **3.9ci** and **3.10ci** contain the inversed chiral center as that of compounds **3.9c** and **3.10c**. However, the MTT assay revealed that the alternation in the stereochemical configuration does not affect the cell viabilities of the compounds. Earlier, we also showed that stereoisomers do not have any significant effect on ion transport mediated cellular activities. The other control compounds without the quinine moiety (**3.10ba1** and **3.10ba2**; Figure 3.69) showed pH-independent Cl^- ion transport activities but failed to show selective antiproliferative activity in the cancer cell (Figure 3.69). Hence, the MTT results demonstrate that the promising antiproliferative activity of compound **3.10b** could be attributed to the quinine structure.

Table 3.5. The half-maximal inhibitory concentration (IC₅₀) of the compounds in different cell lines.

Compounds	IC ₅₀ (μM)				
	HCT-116	OVCAR8	MDA-MB-231	MCF-7	MCF-10A
Quinine	>50	>50	>50	>50	--
3.9a	17.2 ± 5.3	44.3 ± 0.1	30.2 ± 1.2	18.5 ± 0.5	--
3.9b	6.1 ± 0.1	9.2 ± 3.3	6.0 ± 1.3	6.0 ± 0.1	10.3 ± 3.3
3.9c	8.5 ± 1.5	11.5 ± 0.5	8.4 ± 3.5	16.5 ± 3.5	--
3.9ci	14.2 ± 6.7	10.5 ± 2.2	8.2 ± 2.3	14.5 ± 3.5	--
3.9d	--	--	19.2 ± 1.1	21.5 ± 2.1	8.5 ± 0.5
3.10a	12.5 ± 0.5	11.5 ± 5.5	16.2 ± 3.4	16.5 ± 0.5	--
3.10b	6.1 ± 1.0	10.5 ± 3.5	6.0 ± 2.6	7.1 ± 1.2	>50
3.10c	18.2 ± 1.1	38.3 ± 4.2	38.2 ± 5.3	19.2 ± 1.1	--
3.10ci	13.5 ± 0.5	13.1 ± 1.3	12.2 ± 5.3	21.1 ± 8.1	--
3.10d	--	--	11.5 ± 1.9	12.5 ± 1.5	8.5 ± 0.5

Similarly, MTT assay was performed using Hank's balanced salt solution (HBSS) in the absence and presence of Cl⁻ ion in the extracellular matrix (Figure 3.9B) to investigate whether the higher cancer cell death is due to the transmembrane Cl⁻ ion transportability of the compounds.¹ The MCF-7 cells were treated with compound **3.10b** in a dose-dependent manner in the HBSS buffer with or without Cl⁻ ions and the cell viability was investigated by MTT assay after 24 hours of treatment. The result clearly described that the MCF-7 cells underwent more cell death when suspended in Cl⁻ ion-containing HBSS buffer in comparison with that of Cl⁻ ion-free HBSS buffer (Figure 3.9B). The gradual decrease in cell viability confirms that the compound **3.10b** mediated intracellular transport of Cl⁻ ion is directly associated with the extent of cell death. Nevertheless, a significant effect of the transporter is also observed in the absence of Cl⁻ ion, which could be due to the presence of KCl and NaCl salts in the cell culture media that could not be obliterated. Complete removal of these salts also may affect the cell morphology and viability as they are of the important nutrients of the cells. The compounds also could transport other ions that may also dissipate the pH gradients.

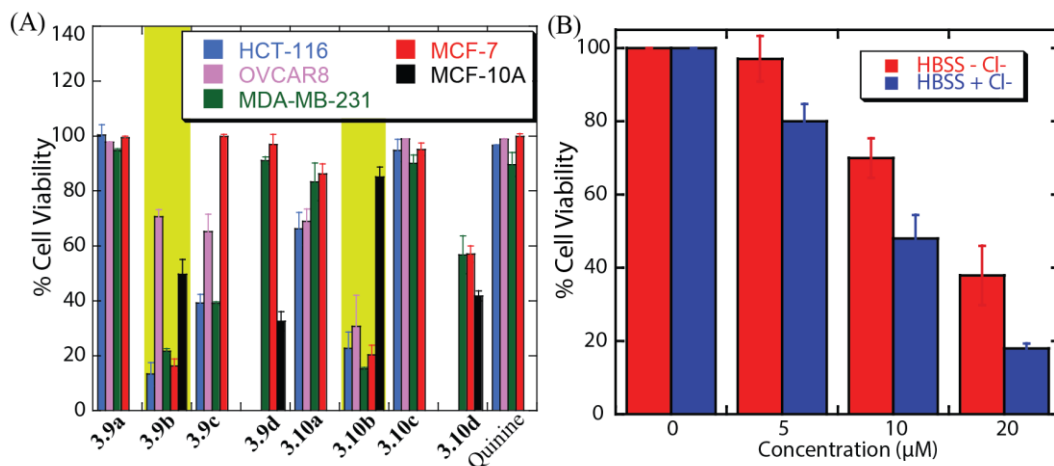


Figure 3.9. Cell viability of the compounds was measured (10 μM) in HCT-116 (a colon cancer cell line), OVCAR8 (an ovarian cancer cell line), MDA-MB-231 (a triple-negative breast cancer cell line), MCF-7 (a luminal A breast cancer cell line), and MCF-10A (a nontumorigenic breast epithelial cell line) (A). The MTT assay was performed after 48 h of compound treatment. Viability of MCF-7 cells in HBSS buffer with or without Cl⁻ ions at different concentrations of compound **3.10b** (0–20 μM). The MTT assay was performed after 24 h of incubation (B). All measurements were performed in triplicate.

3.2.7.2. Clonogenic assay

To authenticate the effect of potent compounds on the colony-forming ability of the cancer cells, we performed a clonogenic survival assay. The cancer cells were seeded and allowed to form colonies for four days. The colonies were then grown in the absence and presence of compounds **3.9b** and **3.10b** for only 24 hours. The next day, compound-containing media was replaced with complete fresh media and allowed to grow for another six days. Finally, colonies were stained with crystal violet solution. The results showed that both compounds **3.9b** and **3.10b** significantly inhibited the formation of colonies in a dose-dependent manner as compared to the untreated control (Figure 3.10). However, compound **3.10b** generates more resistance against the colony-forming ability of the cancer cells. Overall, the MTT and colony formation assays demonstrated that compound **3.10b** strongly induces cancer cell death.

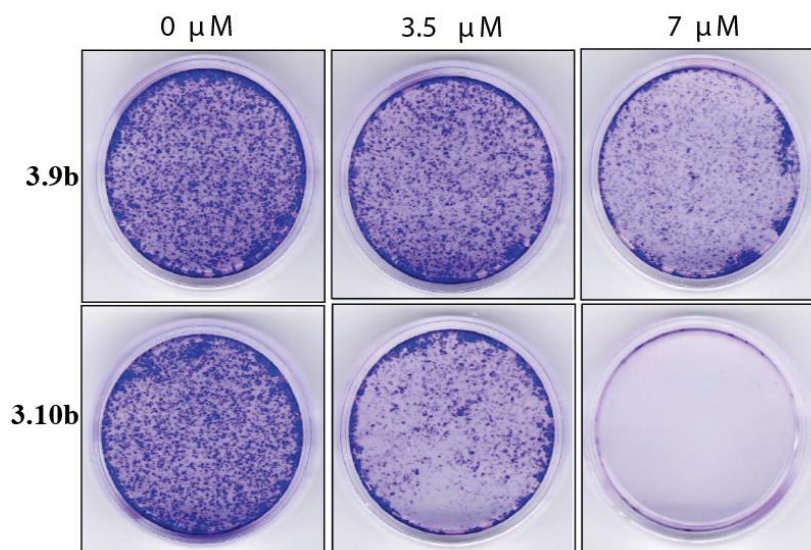


Figure 3.10. Representative clonogenic analysis of MCF-7 cells after treatment with compounds **3.9b** and **3.10b**.

3.2.7.3 FACS, comet, and immunoblot assay

Further, a series of experiments were performed to investigate whether the compound-mediated reduction in the growth of cancer cells is due to the induction of apoptosis. It is important to mention that Cl^- ion-mediated cell death generally promotes the apoptotic pathways in live cells.^{1, 31} The Cl^- ion transport-mediated apoptosis is known to disrupt mitochondrial membrane potential, which stimulates the release of cytochrome-*c* from mitochondria.^{1, 31} The association of released cytochrome *c* with *Apaf-1* activates caspase-9, which promotes caspase-3 mediated apoptosis.^{1, 38-39} The apoptotic process could also be monitored by monitoring the nuclear fragmentation of the cell.^{1, 31} In this regard, the cell cycle profiles of MCF7 cells in the presence of the compounds were examined by using a flow cytometer.⁴⁰ The fluorescence-activated cell sorting (FACS) analysis showed that compound **3.10b** caused severe cell death of MCF7 cells in comparison with that of compound **3.9b**. The FACS analysis also revealed that the sub-G1 (cells with $< 2N$ DNA content) population was significantly increased, indicating that cells are undergoing apoptosis in the presence of the compounds (Figure 3.11A and Figure 3.70). Increased level of Sub-G1 population upon the treatment of compounds also suggests the possibility of induction of apoptosis through DNA fragmentation.⁴¹ Hence, to explore the possibility of DNA fragmentation in the presence of compounds, the comet assay was performed. The cells with genotoxic damage could repair their genomic DNA,

whereas the apoptotic cells could not reverse DNA fragmentation. A significant increase in the DNA in the comet tail was observed following the treatment of compounds, which confirms DNA fragmentation due to apoptosis (Figure 3.11B and Figure 3.71). It is well documented that the mitochondria-mediated apoptotic pathway activates caspase-9 enzymes.

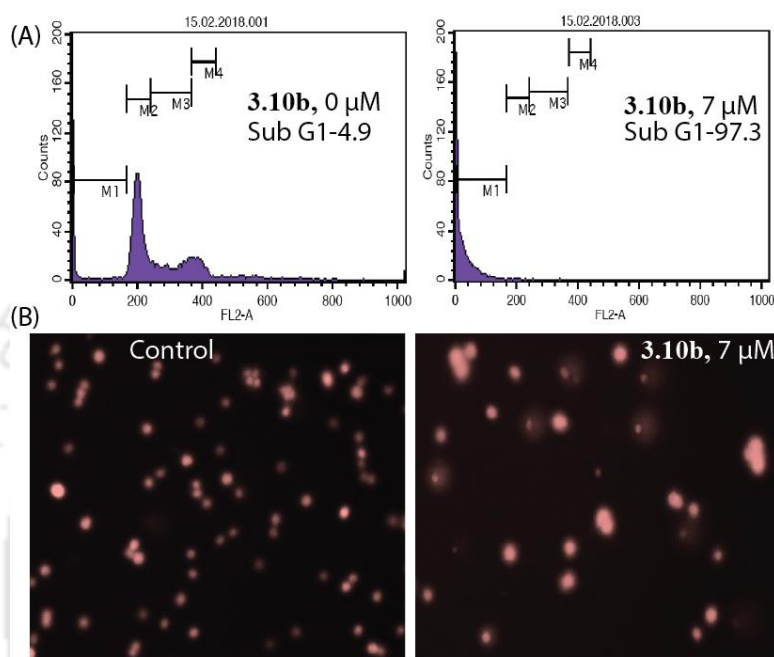


Figure 3.11. Fluorescent activated cell sorting (FACS) analysis of MCF-7 cells grown in the absence and presence of compound **3.10b**. The cell cycle distribution of the MCF-7 cell line was examined by staining the cells with propidium iodide (PI) in the absence and presence of the compounds. Cells were treated with the test compounds for 24 hours. Histograms were analyzed using Cell Quest Pro software (A). The compound induces DNA damage in MCF-7 cells. Comet assays for the determination of DNA damage in single cells following the treatment with compound **3.10b** (B).

Hence, we examined the activation of initiator caspase-9 in the absence and presence of the compounds. We also investigated the expression level of other known markers of apoptosis upon treatment with the compounds.⁴² The immunoblot analysis was performed using MCF-7 cells upon treatment with compounds (10 μ M) for 24 hours by using suitable antibodies (Figure 3.12A and Figure 3.72). The enhancement of the extent of cleaved caspase-9 expression level in the presence of the compounds suggests the caspase-dependent intrinsic pathway of apoptosis. The apoptosis is also known to proceed via

activation of the p53-mediated pathway. The p53 upregulated modulator of apoptosis (PUMA) is a pro-apoptotic protein, and the transcription factors regulate its expression. The activated PUMA interacts with anti-apoptotic Bcl-2 proteins and provides a signal of apoptosis to the mitochondria.⁴² Elevated levels of pro-apoptotic PUMA was also observed in the presence of the compounds, suggesting their mitochondria-mediated caspase pathway of apoptosis.

3.2.7.4. *In vivo* activities of the potent compounds

To investigate the applicability of these ion transporters in therapeutics, the *in vivo* studies were performed in an established murine Ehrlich ascites carcinoma (EAC) solid tumor model (n = 8 for each group).⁴³ Post-treatment of the potent compounds **3.9b** and **3.10b**, the growth of the tumors was remarkably regressed (Figure 3.12B-C). The hematoxylin and eosin (H&E) staining was executed to investigate any commotion in the tissue architecture of systemic organs after treatment with compounds **3.9b** and **3.10b**. The spleen is a major secondary lymphoid organ and has immense importance in tumor progression. Interestingly, in the only tumor-bearing mice, massive splenic inflammation and disruption of the regular arrangement of the splenocytes were observed. The compound **3.10b** treated spleen group showed no splenic inflammation (Figure 3.12D and Figure 3.73). The treatment of compound **3.9b** also showed a reduced inflammatory profile in the spleen, but not as effective as that of compound **3.10b**. The kidneys are another vital organ of our body that has shown to be affected adversely post-treatment with a series of drugs. Hence we investigated the effect of compounds **3.9b** and **3.10b** on kidneys. While examining the architecture of kidneys in all four experiment groups signifying, there is limited renal toxicity of compound **3.10b** treatment, but the higher inflammatory profile was observed for the compound **3.9b** treated group (Figure 3.73). Hence, the H&E staining revealed no significant abnormalities in the stained organ slices, suggesting that compound **3.10b** as a potent anti-tumor drug with no visible side effects on associated organs like kidneys and spleen. Therefore, both cellular and *in vivo* studies indicate that suitable quinine-based Cl⁻ ion carrier selectively induces apoptosis in cancerous cells. However, further studies are required to thoroughly investigate the undesired side toxicity of these compounds to normal cells.

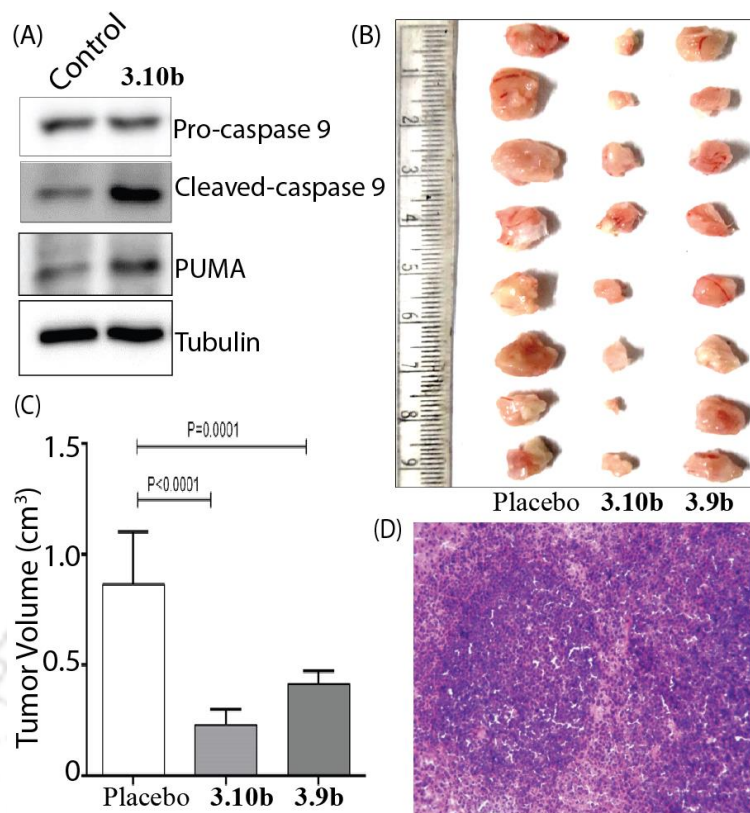


Figure 3.12. Whole-cell protein extracts of MCF-7 cells grown in the absence and presence of compounds (10 μ M) for 24 h and immunoblotted for the indicated proteins (A). α -Tubulin was used as a loading control. The progression of a solid tumor in the female Swiss albino mice in the absence and presence of compounds **3.9b** and **3.10b** (B). The tumor was induced in Swiss albino mice by injecting EAC cells into the thigh muscle. The extent of tumor growth inhibition (C). Immunotoxicity assessment of spleen by hematoxylin and eosin staining after compound **3.10b** treatment (D).

3.3. Summary

The present study demonstrated that potent quinine derivatives selectively transport Cl^- ion across the lipid bilayer *via* a carrier pathway. Further, the NMR studies corroborated with the Cl^- ion recognition properties of the quinine derivatives. The pH-dependent assay was also performed where transmembrane transport of Cl^- ion by the compounds significantly increases under the acidic environment. The results also showed that the quinuclidine ring-mediated cooperative interactions of H^+ and Cl^- ions with the thiourea or squaramide moiety of the quinine derivatives result in proficient transport of H^+/Cl^- ion pair across the lipid bilayer. Interestingly, in the presence of the potent quinine derivative, the growth inhibition in non-cancerous cells is much lower than in the cancer

cells. The Cl^- ion concentration-dependent cytotoxicity assays suggest that the compound-mediated transport of Cl^- ion plays a crucial role in cell death. The western blot analysis of the caspases and PUMA in the presence of the potent compounds confirmed the mitochondria-mediated intrinsic pathway of apoptosis. Therefore, the quinine derivatives are potent synthetic Cl^- ion carriers and promote caspase-mediated apoptosis in cancer cells. The *in vivo* investigations also emphasized the therapeutic potential of the quinine-based ionophores. So we hypothesize that the natural product-based synthetic ion transporter could be a valuable tool in exploring the ion transport mediated activities against cancer and other diseases related to the malfunction of ion transporters.

3.4. Experimental section

3.4.1. Synthesis of compounds

3.4.1.1. General information

Described in section 2.5.1.1.

3.4.1.2. Synthesis of quinine based anionophores

3.4.1.2.1. Synthesis of (1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methanamine

To a stirring solution of quinine (6.2 mmol, 1.0 equiv.) in dry THF was (10 mL) added triphenylphosphine (7.4 mmol, 1.2 equiv.) solution in dry THF under N_2 atmosphere. After that, the solution was cooled down to 0 °C and diisopropyl azodicarboxylate (DIAD) (7.4 mmol, 1.2 equiv.), followed by diphenylphosphoryl azide $(\text{PhO})_2\text{P}(\text{O})\text{N}_3$ (7.4 mmol, 1.2 equiv.) in dry THF was added slowly (dropwise). Then, the reaction mixture was allowed to stir at room temperature for 24 hours. Additional stirring for 3 hours was also continued at 60 °C. The solution of triphenylphosphine (7.4 mmol, 1.2 equiv.) in dry THF was added slowly. After that, the reaction mixture was stirred at 50 °C for 3 hours and then cooled down to room temperature, and 700 μL of distilled H_2O was added to quench the reaction. Finally, the reaction mixture was stirred for another 18 hours. In the meantime, the progress of the reactions in every-steps was monitored by TLC. The solvent was removed under reduced pressure. Then, CH_2Cl_2 (30 mL) and HCl (10%, 30 mL) were added to the crude reaction mixture. The organic layer was separated, and the aqueous phase was extracted with CH_2Cl_2 (3×30 mL). After that, the aqueous layer was basified with NH_4OH (30-33%) until the pH of the solution was 12 and

subsequently extracted with CH_2Cl_2 (3×30 mL). The combined organic solution was then dried over dry Na_2SO_4 , filtered, and the solvent was removed under reduced pressure. The crude reaction mixture was purified through the column chromatography with a solvent gradient system of EtOAc/MeOH to give a light yellow liquid compound with a 70% yield.⁴⁴

3.4.1.2.2. Synthesis of the sqaramide-based aninophores

To a stirring solution of (1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methanamine (1 equiv.) and triethylamine (2 equiv.) in CH_2Cl_2 was added a solution of sqaramide based ligand (1.1 equiv.) and the reaction mixture was allowed to stir for 10-12 hours at room temperature under N_2 atmosphere. For compounds **3.9a** and **3.9b**, white precipitation was observed, which was filtered and washed with CH_2Cl_2 and diethyl ether to get the pure product as a white solid. While for the other compounds, there was not any precipitation. Hence, the reaction mixture was diluted with water and extracted with EtOAc (3×30 mL). The separated organic layer was washed with brine solution and dried over anhydrous Na_2SO_4 . The organic solvent was then removed under reduced pressure, and the purification was done through column chromatography with a solvent gradient system of MeOH/DCM to get the pure product as a crystalline solid.⁴⁴

3.4.1.2.3. Synthesis of the thiourea-based aninophores

To a stirring solution of (1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methanamine (1 equiv.) and triethylamine (2 equiv.) in CH_2Cl_2 was added a solution of substituted aryl isothiocyanates (1.1 equiv.) in CH_2Cl_2 and the reaction mixture was allowed to stir for 10-12 hours at room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was diluted with water and extracted with EtOAc (3×30 mL). The separated organic layer was washed with brine solution and dried over anhydrous Na_2SO_4 . The organic solvent was then removed under reduced pressure, and the purification was done through column chromatography with a solvent gradient system of MeOH/DCM to get the pure product as a crystalline solid.²³

3.4.1.2.4. Synthesis of the control compounds:

To a stirring solution of propan-2-amine or 4-(trifluoromethyl)aniline (1 equiv.) and triethylamine (2 equiv.) in CH_2Cl_2 was added a solution of substituted aryl 1-isothiocyanato-4-(trifluoromethyl)benzene (1.1 equiv.) in CH_2Cl_2 and the reaction mixture was allowed to stir for 10-12 hours at room temperature. The progress of the reaction was monitored by TLC. After completion of the reaction, the mixture was diluted with water and extracted with EtOAc ($3 \times 30 \text{ mL}$). The separated organic layer was washed with brine solution and dried over anhydrous Na_2SO_4 . The organic solvent was then removed under reduced pressure, and the purification was done through column chromatography with a solvent gradient system of MeOH/DCM to get the pure product as a crystalline solid.

3.4.1.3. Characterization of the synthesized quinine derivatives

3-(((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)amino)-4-(phenylamino)cyclobut-3-ene-1,2-dione (3.9a): ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ ppm 9.11 (s, 1H), 8.70 (d, $J = 6.0 \text{ Hz}$, 1H), 7.91 (d, $J = 9.1 \text{ Hz}$, 1H), 7.74 (s, 1H), 7.41 – 7.40 (m, 1H), 7.32 – 7.28 (m, 4H), 7.19 (t, $J = 7.9 \text{ Hz}$, 2H), 6.91 (t, $J = 7.4 \text{ Hz}$, 1H), 6.09 (s, 1H), 5.80-5.75 (m, 1H), 4.97 – 4.93 (m, 2H), 3.94 (s, 3H), 3.27-3.20 (m, 3H), 2.71 – 2.63 (m, 2H), 2.26 (brs, 1H), 1.58 – 1.46 (m, 4H), 0.68 (brs, 1H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 184.4, 164.4, 158.7, 148.4, 147.5, 145.1, 141.7, 136.7, 129.6, 123.2, 118.4, 114.8, 56.6, 56.3, 41.0, 39.64, 27.9, 27.6, 26.7. HRMS (ESI) calcd. for $\text{C}_{30}\text{H}_{30}\text{N}_4\text{O}_3$ ($\text{M} + \text{H}$) $^+$: 495.2391, found: 495.2398.

3-(((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)amino)-4-((4-(trifluoromethyl)phenyl)amino)cyclobut-3-ene-1,2-dione (3.9b): ^1H NMR (600 MHz, $\text{DMSO}-d_6$): δ ppm 9.28 (s, 1H), 8.71 (d, $J = 6.0 \text{ Hz}$, 1H), 7.92 (d, $J = 9.3 \text{ Hz}$, 1H), 7.71 (s, 1H), 7.41 (d, $J = 7.3 \text{ Hz}$, 4H), 7.33-7.30 (m, 3H), 6.09 (s, 1H), 5.78 – 5.74 (m, 1H), 4.98-4.94 (m, 2H), 3.94 (s, 3H), 3.35 – 3.20 (m, 3H), 2.70 (s, 2H), , 2.26 (s, 1H), 1.62-1.52 (m, 4H), 0.68 (s, 1H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$): δ ppm 185.1, 180.2, 168.9, 163.6, 158.4, 148.3, 144.8, 143.5, 142.8, 142.7, 132.0, 127.9, 127.6, 127.1, 125.8, 124.0, 123.01 (q, $J_{\text{C-F}} = 258 \text{ Hz}$), 122.41, 122.2, 119.8, 118.6, 114.8, 101.9, 59.3, 56.2, 56.1, 53.7, 40.58, 40.51, 27.8, 26.5. HRMS (ESI) calcd. for $\text{C}_{31}\text{H}_{29}\text{F}_3\text{N}_4\text{O}_3$ ($\text{M} + \text{H}$) $^+$: 563.2265, found: 563.2270.

3-((3,5-bis(trifluoromethyl)phenyl)amino)-4-(((1S)-(6-methoxyquinolin-4-**yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)amino)cyclobut-3-ene-1,2-dione**

(3.9c): ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ ppm 10.67 (s, 1H), 8.82 (brs 1H), 8.02 – 7.98 (m, 3H), 7.76-7.64 (m, 3H), 7.47-7.45 (m, 1H), 6.07-5.97 (m, 1H), 5.06-4.98 (m, 2H), 3.96 (s, 3H), 3.59 – 3.47 (m, 3H), 3.21-3.17 (m, 1H), 2.72 – 2.62 (m, 2H), 2.28 (brs, 1H), 1.90 (s, 1H), 1.60 – 1.58 (m, 1H), 1.55-1.48 (m, 4H), 0.91 – 0.77 (m, 1H), 0.64 (s, 1H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 183.9, 181.2, 169.5, 163.1, 158.7, 147.4, 144.6, 140.2 (d, $J = 56.7$ Hz), 132.5 (q, $J = 32.9$ Hz), 131.6, 125.6, 123.7, 121.9, 120.1, 118.3, 116.3, 115.2, 101.2, 55.8, 55.6, 40.8, 38.9, 29.7, 27.2, 25.7. HRMS (ESI) calcd. for $\text{C}_{32}\text{H}_{28}\text{F}_6\text{N}_4\text{O}_3$ ($\text{M}^+ \text{H}^+$): 631.2138, found: 631.2139.

3-((4-methoxyphenyl)amino)-4-(((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-

vinylquinuclidin-2-yl)methyl)amino)cyclobut-3-ene-1,2-dione (3.9d): ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ ppm 9.54 (brs, 1H), 8.83 (brs, 1H), 8.09 (brs, 1H), 8.00-7.97 (m, 1H), 7.79 (s, 1H), 7.67-7.66 (m, 1H), 7.47-7.45 (m, 1H), 7.31-7.30 (m, 2H), 6.90-6.89 (m, 2H), 6.02-5.98 (m, 2H), 5.07-4.99 (m, 2H), 3.94 (s, 3H), 3.71 (s, 3H), 3.33-3.29 (m, 1H), 2.73-2.62 (m, 3H), 2.30 (brs, 1H), 1.60-1.53 (m, 4H), 1.26-1.23 (m, 1H), 0.64 (brs, 1H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 181.9, 169.3, 162.7, 158.7, 156.5, 147.7, 144.8, 143.6, 141.6, 131.8, 130.5, 128.1, 122.6, 121.2, 119.2, 114.6, 114.1, 101.3, 59.4, 55.9, 55.8, 55.1, 53.7, 40.8, 39.7, 31.9, 29.7, 27.7, 26.3, 22.7, 14.2. HRMS (ESI) calcd. for $\text{C}_{31}\text{H}_{32}\text{N}_4\text{O}_4$ ($\text{M}^+ \text{H}^+$): 525.2496, found: 525.2498.

1-(((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)-3-

phenylthiourea(3.10a): ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ ppm 9.71 (s, 1H), 8.71 (d, $J = 6.0$ Hz, 1H), 8.34 (brs, 1H), 7.93-7.91 (m, 2H), 7.56-7.55 (m, 1H), 7.42 – 7.40 (m, 3H), 7.25 (t, $J = 7.7$ Hz, 2H), 7.03 (t, $J = 7.3$ Hz, 1H), 5.99 (s, 1H), 5.83-5.74 (m, 1H), 4.99-4.89 (m, 2H), 3.92 (s, 3H), 3.22-3.16 (m, 2H), 2.69-2.66 (m, 2H), 2.27 (s, 1H), 1.57 (s, 3H), 1.20 (s, 2H), 0.82 (s, 1H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ ppm 180.2, 170.8, 157.5, 148.3, 144.6, 142.3, 139.9, 131.7, 129.0, 128.5, 124.3, 122.8, 121.7, 114.7, 103.6, 66.8, 60.2, 59.8, 56.1, 55.5, 41.4, 27.8, 27.5, 25.9, 21.2, 14.5. HRMS (ESI) calcd. for $\text{C}_{27}\text{H}_{30}\text{N}_4\text{OS}$ ($\text{M}^+ \text{H}^+$): 459.2213, found: 459.2217.

1-((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)-3-(4-(trifluoromethyl)phenyl)thiourea (3.10b): ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ ppm 10.08 (s, 1H), 8.75 (d, $J = 6.0$, 1H), 8.71 (brs, 1H), 7.96 (d, $J = 9.2$ Hz, 1H), 7.93 (s, 1H), 7.75 (d, $J = 8.3$ Hz, 2H), 7.66 – 7.60 (m, 3H), 7.45–7.43 (m, 1H), 6.02 (s, 1H), 5.86–5.80 (m, 1H), 5.02–4.93 (m, 2H), 3.97 (s, 3H), 3.25–3.21 (m, 2H), 2.73–2.71 (m, 2H), 2.28 (brs, 1H), 1.63 – 1.55 (m, 3H), 1.23 (s, 2H), 0.85 (brs, 1H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ ppm 180.0, 157.5, 148.1, 144.6, 143.9, 142.3, 131.7, 129.2 (d, $J = 12.1$ Hz), 128.4, 127.6, 126.1 (d, $J = 3.8$ Hz), 125.77, 124.3 – 122.9 (m), 122.2, 60.2, 56.1, 55.5, 41.4, 29.5, 27.8, 27.5, 25.8, 22.5, 21.2, 14.5, 14.4. HRMS (ESI) calcd. for $\text{C}_{26}\text{H}_{29}\text{F}_3\text{N}_4\text{OS}$ ($\text{M}^+ \text{H}^+$): 527.2087, found: 527.2093.

1-(3,5-bis(trifluoromethyl)phenyl)-3-((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)thiourea (3.10c): ^1H NMR (600 MHz, $\text{DMSO}-d_6$) δ ppm 8.94 (s, 1H), 8.71 (d, $J = 6.0$ Hz, 1H), 8.12 (s, 2H), 7.94 – 7.91 (m, 2H), 7.67 (s, 1H), 7.58 (d, $J = 4.6$ Hz, 1H), 7.43–7.40 (m, 1H), 6.04 (s, 1H), 5.84–5.76 (m, 1H), 5.00–4.90 (m, 2H), 3.94 (s, 3H), 3.23–3.16 (m, 2H), 2.70 (s, 2H), 2.27 (s, 1H), 1.57 (s, 3H), 1.20 (s, 2H), 0.81 (brs, 1H). ^{13}C NMR (150 MHz, $\text{DMSO}-d_6$) δ ppm 179.9, 157.6, 148.1, 144.5, 142.2, 131.7, 130.8 (q, $J = 32.8$ Hz), 129.21, 28.5, 126.4, 124.6, 122.8, 121.8, 120.9, 116.3, 114.8, 103.5, 59.8, 56.1, 55.5, 41.5, 27.4, 25.7. HRMS (ESI) calcd. for $\text{C}_{29}\text{H}_{28}\text{F}_6\text{N}_4\text{OS}$ ($\text{M}^+ \text{H}^+$): 595.1961, found: 595.1969.

1-(4-methoxyphenyl)-3-((1S)-(6-methoxyquinolin-4-yl)((1S,2S,4S)-5-vinylquinuclidin-2-yl)methyl)thiourea (3.10d): ^1H NMR (600 MHz, CDCl_3) δ ppm 8.43 (s, 1H), 7.97 (d, $J = 9.2$ Hz, 1H), 7.82 (s, 1H), 7.36–7.34 (m, 1H), 7.16 – 7.09 (m, 3H), 6.90–6.89 (m, 3H), 5.95 (s, 1H), 5.67–5.62 (m, 1H), 4.95 – 4.90 (m, 2H), 4.14–4.09 (m, 1H), 3.95 (s, 3H), 3.83 (s, 3H), 3.35 (s, 1H), 3.11–3.07 (m, 1H), 2.69 – 2.58 (m, 2H), 2.27 – 2.23 (m, 1H), 2.05 (s, 1H), 1.68 – 1.56 (m, 3H), 1.28 – 1.25 (m, 1H), 0.95 – 0.91 (m, 1H). ^{13}C NMR (150 MHz, CDCl_3) δ ppm 180.9, 171.2, 158.3, 157.7, 147.5, 144.6, 140.9, 131.5, 130.3, 128.3, 121.9, 114.7, 114.6, 102.4, 60.4, 55.7, 55.5, 55.2, 41.5, 39.3, 29.7, 27.7, 27.3, 25.7, 22.7, 21.1, 14.2. HRMS (ESI) calcd. for $\text{C}_{28}\text{H}_{32}\text{N}_4\text{O}_2\text{S}$ ($\text{M}^+ \text{H}^+$): 489.2319, found: 489.2328.

1,3-bis(4-(trifluoromethyl)phenyl)thiourea (3.10ba₁): ¹H NMR (600 MHz, DMSO-*d*₆) δppm 10.39 (s, 2H), 7.75-7.66 (m, 8H). ¹³C NMR (150 MHz, CDCl₃) δppm 179.5, 140.0, 128.8 (d, *J* = 32.9 Hz), 126.9 (q, *J* = 3.7 Hz), 124.4, 122.7. HRMS (ESI) calcd. for C₁₅H₁₀F₆N₂S (M+ H)⁺: 365.0542 found: 365.0547.

1-isopropyl-3-(4-(trifluoromethyl)phenyl)thiourea (3.10ba₂): ¹H NMR (600 MHz, DMSO-*d*₆) δppm 9.66 (s, 1H), 7.95 (s, 1H), 7.71 (d, *J* = 6, 2H), 7.64 (d, *J* = 8.4 Hz, 2H), 4.39 (s, 1H), 1.18 (d, *J* = 6.6 Hz, 6H). ¹³C NMR (150 MHz, DMSO-*d*₆) δppm 179.5, 143.9, 127.6, 126.1, 125.8, 124.0, 121.9, 45.9, 22.2. HRMS (ESI) calcd. for C₁₁H₁₃F₃N₂S (M+ H)⁺: 263.0842, found: 263.0848.

3.4.2. ¹H NMR titration experiments

3.4.2.1. Chloride ion binding through ¹H NMR titration

The process is already described in section 2.4.2.1 with minor modifications. For this case, all the titration data were fitted with the BindFit v0.5 program, which is available online.²⁴⁻²⁵

3.4.2.2. ¹H NMR titration with different anions

To investigate the anion recognition capabilities of the squaramide and thiourea derivatives of the quinine, we also performed ¹H NMR titration of representative potent compounds **3.9b** and **3.10b** with other anions (Br⁻, I⁻, NO₃⁻), using the similar experimental protocol as mentioned earlier. The TBABr, TBAI, and TBANO₃ salts were taken as the source of Br⁻, I⁻, NO₃⁻ respectively.

3.4.2.3. Determination of pK_a values through the ¹H NMR titration

Before starting the experiment, we arranged the following items like NMR tubes (~17), pH meter and calibration solutions (pH 4, pH 7), pipettes, compound **3.9b**, and **3.10b**, HCl (1 M), KOH, (1 M), HCl (0.33 M) and KOH (0.33 M). Next, 10 mM stock solution was prepared of the compound **3.9b** and **3.10b** in DMSO-*d*₆. Similarly, the HCl and KOH solution were also prepared in the D₂O solvent. The small aliquot of HCl or KOH solutions was added into each NMR tube containing the compound solution and determined each pH of the solution.⁴⁵ The pH of the solution was adjusted by HCl or KOH solution before recording the ¹H NMR spectra. In each tube, the compound was dissolved

in 500 μL of $\text{DMSO-}d_6$, and the maximum volume of D_2O solution of HCl or KOH was around 25 μL . In each NMR spectra, the peak at 2.5 corresponds to the $\text{DMSO-}d_6$ reference peak, and we recorded the chemical shift value from this standard $\text{DMSO-}d_6$ peak. After recording all the ^1H NMR spectra, all spectra were stacked by the Mestronovo software. For both the potent compounds, the A-type and C-type peaks were broadened after few pH values; hence B-type proton shifts were used for the calculation of the pK_a value of the quinuclidine nitrogen. The chemical shifts of the D-type proton at different pH were used to calculate the pK_a value of the quinoline ring. For compound **3.9b**, the pK_a values of the quinuclidine and the quinoline rings are 6.75 and 4.66, respectively. For compound **3.10b**, the pK_a values of the quinuclidine and the quinolone rings are 6.77 and 4.35, respectively.

3.4.3. Determination of anion binding stoichiometry by job's Plot

Described in section 2.4.3.

3.4.4. Density functional theory (DFT) studies

The density functional theory (DFT) calculations were performed using the Gaussian 09 program to explore the binding mode, stability of compound- Cl^- ion complex, and mode of their interactions. All the considered electronic structures were fully optimized at B3LYP/6-31+g* level of theories using the Gaussian 09 program.²⁸ The input geometries were optimized in a lower basis set (3-21g*) first and subjected to further optimization in 6-31+g* basis set.⁴⁶ In order to ensure true local minimum geometry, we performed a normal mode analysis of the compounds and complexes. Several possible geometries by rotating around the single bonds and positioning chloride at different distances were generated and subjected to energy minimization. The most stable geometries were reported here. The analysis showed that the Cl^- ion interacts with neutral and protonated forms of the compound. The interatomic bond distance ($\text{Cl}^- \cdots \text{H}$) and the interaction energies were tabulated. The interaction energies, as well as the numbers of effective hydrogen bonds with the Cl^- ion, were higher for the protonated quinuclidine moiety, which strongly support its higher ion recognition capability and also support the role of the additional proton. These results also strongly support the ion recognition capabilities of these compounds both in neutral and acidic conditions.

3.4.5. Ion transport activity studies

3.4.5.1. Ion transport activity studies using ion-selective electrode based assay

3.4.5.1.1. Chloride ion efflux studies using chloride ion-selective electrode (chloride ISE)

Ion transport activities of the synthesized compound were investigated by monitoring the efflux of chloride ions from the EYPC/CHOL-LUV using a chloride ion-selective electrode (ISE; Thermo Scientific™ Orion™). Before each experiment, the ISE was calibrated using solutions of 1 ppm, 10 ppm, and 100 ppm of standard chloride solution with an ionic strength adjuster solution. A filling solution was poured inside the electrode up to the mark before each experimental session. Chloride concentration (ppm) appearing in the display of the ion meter was set in continuous mode for the time-dependent measurements.

3.4.5.1.2. Preparation of EYPC/CHOL-LUV

The LUVs were prepared according to the reported procedure. Briefly, egg yolk phosphatidylcholine (EYPC, 50 mg/mL in deacidified CHCl_3) and cholesterol (25 mg/mL in de-acidified CHCl_3) were taken in a clean glass vial (with a molar ratio of 6:4). Then, the solution was dried under vacuum for at least 5 hours to form a thin film of lipid. The dry film was then hydrated with 500 μL of 5 mM phosphate buffer, pH 7.2, or 5.5 containing 100 mM NaCl. This solution was kept for 1 hour with occasional vortexing (6-7 times). The suspension was further passed through 12-13 cycles of freeze-thaw (freezing with liquid N_2 and melting with lukewarm water, respectively) to break up the multilamellar vesicles. The vesicle solution was extruded through a polycarbonate membrane (using a mini-extruder from Avanti Polar Lipids) having a pore size of 200 nm (size of LUVs are > 200 nm) for 19/21-times (as it must be an odd number), to give LUVs with a mean diameter of ~ 200 nm. The resulting unilamellar vesicles were dialyzed with respective 5 mM phosphate buffer, pH 7.2 or pH 5.5 containing 100 mM NaNO_3 to remove the extravesicular NaCl from the solution. Finally, the LUVs were collected, and volume was adjusted to 500 μL using 5 mM phosphate buffer, pH 7.2 or pH 5.5 containing 100 mM NaNO_3 . The final lipid concentration was 25 mM (assuming 100 % lipid regeneration).

3.4.5.1.3. Chloride efflux study across EYPC/CHOL-LUV

To measure the extent of efflux of Cl^- ion in the absence and presence of the compound, the EYPC/CHOL-LUVs (50 μL) and 5 mM phosphate buffer, pH 7.2, containing 100 mM NaNO_3 (3940 μL) were taken in a clean and dry glass vial and kept under mild stirring condition. The glass electrode was immersed in the solution under mild stirring conditions. To initiate the Cl^- ion transport kinetics at $t = 50$ s, 10 μL of the respective compound (from DMSO stock solution) was added into the stirring solution, and the readings were noted from the ion meter. After 5 minutes, the vesicles were lysed using 50 μL of 20% Triton X-100 solution. The total Cl^- ion efflux reading was taken at 7 minutes (allowing complete disruption of the LUVs). The initial reading was considered 0% Cl^- ion efflux, and the final reading at 7 min was considered 100% Cl^- ion efflux.

3.4.5.1.4. Quantitative measurement of transport activity from chloride ISE assay

To find out the EC_{50} value of all the compounds, ion-selective electrode based $\text{Cl}^-/\text{NO}_3^-$ exchange assay was performed at varying concentrations. From these experiments the chloride efflux (%) at 500 s was plotted as a function of the carrier concentration (μM). The data points were then fitted to the modified Hill equation using the Origin program.⁶

$$y = a + (b - a) * \left(\frac{x^n}{k^n + x^n} \right) = V_{\max} \frac{x^n}{k^n + x^n} = 100 * \left(\frac{x^n}{\text{EC}_{50}^n + x^n} \right) \dots \dots \text{Eq. 3.1}$$

In this equation, we have fixed the $a = 0$, $b = 100$, y is the chloride efflux at 500 s (%), and x is the carrier concentration (μM). The $V_{\max}$, k , and n are the parameters to be fitted. $V_{\max}$ is the maximum efflux possible (usually fixed to 100 % as this is the maximum chloride efflux possible), n is the Hill coefficient, and k is the carrier concentration needed to reach $V_{\max}/2$ (when $V_{\max}$ is fixed to 100%, then k is the EC_{50}). EC_{50} values at 500 s could be obtained directly from the Hill plot.

3.4.5.1.5. Measurement of half-maximal effective concentrations (EC_{50}) of the compounds at different pH from chloride ISE assay

The chloride ion efflux efficiency at various concentrations of the compounds were measured at different pH (pH 7.2 and pH 5.5) to determine the half-maximal effective concentration (EC_{50} values). The efflux efficiency obtained from the ISE measurements

were normalized [$t = 0$ to $t = 700$ s]. The normalized efflux efficiency (EE) values at $t = 500$ s (before the addition of Triton X-100 solution) were considered as the transport activity of the compounds. To get the effective concentration (EC_{50}) of the compound, the chloride ion efflux efficiency values were plotted against concentration and fitted in the Hill equation.

3.4.6. Ion selectivity studies

3.4.6.1. Ion selectivity studies with base pulse using the fluorescence-based assay

3.4.6.1.1. Buffer and stock solution preparation

The required buffer solution was prepared by dissolving HEPES and MCl or Na_xA salt (LiCl, NaCl, KCl, RbCl, CsCl and NaCl, NaBr, NaI, $NaNO_3$, $NaClO_4$) in Milli-Q water to obtain a buffer composition of 20 mM HEPES buffer, pH 7.2, containing 100 mM of the respective salt (MCl or Na_xA).

3.4.6.1.2. Preparation of EYPC/CHOL-LUVs $\Rightarrow$ HPTS

Described in section 2.4.5.1.1.

3.4.6.1.3. Ion transport activity across EYPC/CHOL-LUV $\Rightarrow$ HPTS

Described in section 2.4.5.1.2.

3.4.6.1.4. Quantitative measurement of transport activity from HPTS assay

Described in section 2.4.5.1.3.

3.4.6.1.5. Measurement of half-maximal effective concentrations (EC_{50}) of the compounds across EYPC/CHOL-LUV $\Rightarrow$ HPTS

The Cl^- ion transport activity at various concentrations of the compounds was measured at pH 7.2 to determine the half-maximal effective concentration (EC_{50} values). The transport activity obtained from the fluorescence-based HPTS assay were normalized [$t = 0$ to $t = 700$ s]. The normalized chloride ion transport activity values at $t = 500$ s (before the addition of Triton X-100 solution) were considered as the transport activity of the compounds. To get the compound's effective concentration (EC_{50}), the chloride ion efflux efficiency values were plotted against concentration and fitted in the Hill1 equation.

3.4.6.1.6. Anion selectivity studies across EYPC/CHOL-LUVs>HPTS

Described in section 2.4.6.1.1.

3.4.6.1.7. Cation selectivity studies across EYPC/CHOL-LUVs>HPTS

Described in 2.4.6.1.3.

3.4.6.2. Ion selectivity studies without base pulse using the fluorescence-based assay**3.4.6.2.1. Buffer and stock solution preparation**

The required buffer solution was prepared by dissolving HEPES and Na_xA salt (NaCl, NaBr, NaI, NaNO₃, NaClO₄, and Na₂SO₄) in Milli-Q water to attain a final concentration of 20 mM HEPES buffer, pH 7.2, containing 100 mM of the respective salt.

3.4.6.2.2. Preparation of EYPC-LUVs>HPTS

Described in section 2.4.5.1.1.

3.4.6.2.3. Anion selectivity studies across EYPC/CHOL-LUVs>HPTS

The anion selectivity studies were performed according to the reported procedure. Briefly, 2940 μ L of 20 mM HEPES buffer, pH 7.2, containing 100 mM of the respective salt and 50 μ L of EYPC/CHOL-LUVs>HPTS were taken in a fluorescence cuvette. Then, it was placed in the fluorescence spectrophotometer at room temperature under slow stirring conditions. The HPTS fluorescence intensity of the solution was monitored. After 50 s, 10 μ L of the respective compound (from DMSO stock) was added to initiate the anion transport kinetics. At 450 s, the vesicles were lysed by adding 20 μ L of 20% Triton X-100 solution, and the fluorescence measurement was continued up to $t = 500$ s.

3.4.6.2.4. Quantitative measurement of transport activity from HPTS assay

The fluorescence emission intensity of the HPTS dye (at $t = 50$ to $t = 450$ s) was converted to start all the kinetics spectra at 0. The converted fluorescent intensity (FI) at $t = 450$ s (before the addition of Triton X-100 solution) was considered as the transport activity of the compounds.

3.4.7. Evidence for the mechanistic pathway for chloride ion transportation**3.4.7.1. Preferential ion transport activity in the presence of FCCP (FCCP assay)**

For the FCCP assay, the EYPC/CHOL-LUV-HPTS was prepared by following a similar procedure as mentioned in section 2.5.4.1. The ion transport activity was measured in the absence and presence of FCCP at a fixed compound concentration. For this assay, 2890 μL of buffer solution (20 mM HEPES buffer, pH 7.2, containing 100 mM NaCl), 50 μL of 25 mM EYPC/CHOL-LUV-HPTS and 50 μL of 0.5 M NaOH (to generate a pH gradient of ~ 0.6 between the extra and intravesicular regions) were taken in a clean and dry fluorescence cuvette, and the cuvette was placed in the fluorescence spectrophotometer under slow stirring condition for approximately 3 minutes. The HPTS fluorescence intensity of the solution was monitored. After 50 s, the solution of 10 μL of the respective compound (from DMSO stock) and/or FCCP (1.5 μM) was added to initiate the anion transport kinetics. At $t = 450$ s, the vesicles were lysed by adding 20 μL of 20% Triton X-100 solution, and the fluorescence measurement was continued up to $t = 500$ s. The control experiment was carried out in the absence of FCCP (2 μL of DMSO was added in place of FCCP). The fluorescence emission intensity of the HPTS dye was normalized at $t = 50$ and $t = 500$ s and transfer as 0 and 100 units, respectively.

3.4.7.2. Preferential ion transport activity in the presence of valinomycin (valinomycin assay)

For the valinomycin assay, the EYPC/CHOL-LUV-HPTS was prepared by following a similar procedure as mentioned in section 2.4.5.1.1. The ion transport activity was performed as following the similar for FCCP assay, which is described in section 2.4.7.3

3.4.7.3. NMDG-Cl assay

This experiment is intended to evaluate the chloride transport activity and $\text{Cl}^- > \text{H}^+ / \text{OH}^-$ selectivity of test anionophores. In this experiment, the pH gradient after the addition of NMDG (5 mM) could be dissipated by H^+ / Cl^- symport or functionally equivalent $\text{Cl}^- / \text{OH}^-$ antiport. The effect of the proton channel gramicidin D (0.1 mol %) on the rate of pH gradient dissipation prompted by the ionophore is monitored. If H^+ / OH^- transport is the rate-limiting process in H^+ / Cl^- symport or $\text{Cl}^- / \text{OH}^-$ antiport, i.e., the anionophore shows $\text{Cl}^- > \text{H}^+ / \text{OH}^-$ selectivity, the HPTS response will be accelerated by gramicidin which facilitates electrogenic proton transport. Conversely, if H^+ / OH^- transport is faster than Cl^- transport, the rate of HPTS response will be unaffected by gramicidin.

Conditions: Internal and external solutions (excluding HPTS): 100 mM NMDG-Cl, buffered at pH 7.0 with 10 mM HEPES; base pulse: 5 mM NMDG.

3.4.8. Evidence for mobile carrier mechanism

3.4.8.1. Cholesterol dependency assay

The EYPC/CHOL-LUVs/HPTS were prepared similarly, as mentioned in section 2.4.5.1.1., with minor modifications using the HPTS dye. The transport activity was performed similarly (only one exception is there, the experiments are performed in the presence of HPTS encapsulated LUVs) as mentioned in section 2.4.8.3, with these respective vesicles.

3.4.8.2. U-tube experiment

According to the reported procedure, the classical U-tube experiment was performed to confirm the compounds' mechanistic pathway for the Cl^- ion transport. The lipid bilayer was mimicked by using chloroform (12 mL) as the organic layer. The compound (0.4 mM) in chloroform was placed at the bottom of the U-tube with mild stirring conditions. The left-arm of the tube was filled with 0.1 M aqueous HCl solution (10 mL), and the right one was filled with 0.1 M aqueous NaNO_3 solution (10 mL). The Cl^- ion concentration of the receiver end was monitored using a chloride ion-selective electrode. In the meantime, the pH of the receiver end was monitored using a pH meter. H^+/Cl^- co-transport by the compound was observed by monitoring the right-arm of the tube using both pH meter and ISE.

3.4.8.3. U-Tube experiment in the presence of the bromothymol blue indicator

To confirm the H^+/Cl^- co-transport mechanism of potent compound **3.10b**, we performed the classical U-tube assay where we have slightly modified the assay system. We incorporated the Bromothymol blue (1%) and aqueous 0.1 M aqueous NaNO_3 solution (10 mL) at the right-arm, and the left-arm of the U-tube was filled with 0.1 M aqueous HCl solution (10 mL). These two arms were separated by a compound (0.4 mM) containing chloroform solution with mild stirring conditions. Here the chloroform layer mimics the biological membrane environment. As we know, the bromothymol blue is a pH-dependent indicator that exhibited the yellow color at lower pH (2.0-6.0) from the green color at higher pH (7-7.4). A steady decrease in the pH with the different pH-

dependent color of bromothymol blue at right-arm directly confirmed its H^+/Cl^- co-transport mechanism.

3.4.9. Test for the leaching-out of the compounds from the membrane bilayer environment

To confirm that the compounds were present within the lipid bilayer during the ion transport process, the leaching test was performed according to the reported procedure. It is hypothesized that if the compounds leached out from the membrane bilayer environment to the aqueous medium, then the transport rate of the compounds will be greatly affected (reduced) by the dilution of the vesicular solution. Whereas the dilution factor will not affect the transport rate if the compounds are localized inside the bilayer environment. Briefly, various concentrations of EYPC/CHOL-LUV-HPTS in the buffer (20 mM HEPES, pH 7.2, containing 100 mM NaCl) were taken in fluorescence cuvette (final concentration of the vesicles were 300 μM , 400 μM , 500 μM , and 600 μM) followed by addition of NaOH. The cuvette was then placed in the fluorescence spectrophotometer at room temperature under slow stirring conditions. The transport kinetics was started after the addition of the compound (a fixed anionophore/lipid ratio in all cases) at $t = 50$ s. Finally, at $t = 450$ s, the vesicles were lysed by adding 20 μL of 20% Triton X-100 solution. The fluorescence intensity measurement was continued for further 50 s.

3.4.10. Evidence of vesicle stability in the presence of the compounds (calcein leakage assay)

The vesicles were prepared according to the method described in section 2.5.4.1. instead of 1 mM HPTS, 50 mM calcein was used for the vesicle preparation. The assay was performed according to the described procedure in section 3.4.6.2.4 with minor modification. The calcein fluorescence intensity was monitored at 520 nm ($\lambda_{\text{ex}} = 490$ nm).

3.4.11. Stability test for the potent compounds

To check the pH-dependent stability of the potent compounds, first, the compounds **3.9b** and **3.10b** (50 μM) were dissolved in 10 mM phosphate buffer at different pH (5.5, 6.5, 7.2, and 8.0), and the solution was incubated in 37 $^{\circ}\text{C}$ for 72 h. After that, the aliquot was injected (1 mL) in HPLC (UltiMate 3000) with Luna® 5 μm C18(2) 100 Å columns using a UV-Vis detector at 254 nm to monitor the stability of the compounds.

Methanol/water gradient (95% methanol and 5% water) was used as the mobile phase at a flow rate of 10 mL/minute for 15 minutes run time. The HPLC traces showed that our potent compounds are stable in these conditions.

3.4.12. Biological activity studies

3.4.12.1. Cell cultures

MCF7 breast cancer cells were grown in DMEM, whereas MDA-MB-231 (breast cancer cells), OVCAR8 (ovarian cancer cells), and HCT116 (colon cancer cells) were grown in RPMI medium as a monolayer. The media was supplemented with 10% FBS, 100 µg/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in a 5% CO₂ atmosphere at 37°C in a humid condition. Normal breast epithelial cells MCF10A were grown in DMEM/F-12 supplemented with 10% horse serum.

3.4.12.2. MTT-based cytotoxicity assay

Described in section 2.4.11.1.

3.4.12.3. Chloride mediated cell death studies

Described in section 2.4.11.2.

3.4.12.4. Colony formation assay

MCF7 breast cancer cells (10,000 cells per 35 mm dish) were grown for 4 days. Cells were then treated with the compounds **3.9b** and **3.10b** (0, 3.5, and 7 µM) for 24 h. The next day, a compound containing media was replaced with complete fresh media (without compound) and allowed to grow for another 6 days. The media was replaced every alternative day. Finally, cells were stained with 0.1 % crystal violet solution for 30 minutes at room temperature. The extra solution was removed and washed with PBS for 3 times.⁴⁷

3.4.12.5. Flow cytometry

MCF7 cells were grown for 24 h. The next day, cells were treated with either vehicle (DMSO) or the indicated compounds for 24 h. Then, the cells were trypsinized, washed with PBS buffer, and fixed with 95 % ethanol. On the day of FACS acquisition, fixed cells were washed with PBS, stained with propidium iodide (50 µg/ml PI, 50 µg/ml

RNase A, and 2 mM MgCl₂) and acquired using BD FACS Calibur. The data were analyzed using the Cell Quest Pro software.⁴⁰

3.4.12.6. Comet assay

MCF7 cells were grown for 24 h and treated with the indicated compounds for 24 h. Then, cells were trypsinized, washed with PBS buffer, embedded in 0.8 % agarose gel, and lysed in lysis buffer (2.5 M NaCl, 100 mM EDTA, and 10 mM Trizma base) overnight at 4°C. The next day, the cells embedded in the gel were incubated with the running buffer (300 mM NaOH and 1mM EDTA) for 30 min at 4°C followed by running at 300 mA for 30 min. The cells were neutralized in 0.4 M Tris (pH 7.5), washed in ice-cold water, and stained in EtBr (8 µg/ml). The images were taken at 532 nm (TRITC) using OLYMPUS 1X71.

3.4.12.7. Immunoblotting

MCF7 cells were grown in the absence or presence of 10 µM either compound **3.9b** or 10 µM compound **3.10b** for 24 h. Cells were then harvested and lysed in lysis buffer (50 mM Tris-Cl, 5 mM EDTA, 250 mM NaCl, 50 mM NaF, 0.5 mM sodium vanadate, and 0.05% Triton X-100 containing protease inhibitors) as described previously. The whole-cell lysates were resolved on SDS-PAGE and immunoblotted for the indicated antibodies. The blots are developed in Amersham Imager 600 using chemiluminescence substrates. Anti-PUMA (Cat# SC-374223) antibody and anti-Caspase 9 (Cat# SC-56073) antibody were purchased from Santa Cruz, and anti- α -Tubulin (Cat# T5168) was purchased from Sigma.^{1, 42}

3.4.13. In vivo activity studies

3.4.13.1. Animals and ethical statement

Swiss albino mice of 20 gms weight were selected for the experiments. Animals were housed in an animal facility room, Department of Zoology, the University of Calcutta at 25 ± 2° C, the relative humidity of nearly 45 ± 5 % with alternative 12 hours day/ night cycle with the access to food and water. All the experiments were carried out as per the guideline of the Committee for Control and Supervision of Experimental Animals (CPCSEA), Government of India (Registration No: 885/GO/Re/S/05/CPCSEA), and approved by the Institutional animal ethical committee (IAEC), University of Calcutta.

3.4.13.2. Tumor cell preparation and transplantation

Ehrlich ascites carcinoma (EAC) cells were maintained in the ascetic form in Swiss albino mice by intraperitoneal transplantation every 10th day on each mice. The ascitic fluid was drawn on the 7th day of implantation according to the previously described protocol. Isolated cells were diluted in sterile PBS. Then, 2×10^6 cells were then implanted on the thigh muscle of all the animals selected for the experiment.⁴³

3.4.13.3. Experiment design

Swiss albino mice were divided into four groups (n = 8 each group). Post inoculation of the cells in thigh muscle on day 0 mice in their respective group was allowed to treat for 5 days on every alternate day at the treatment of 5 mg/ kg body weight described below. All the mice were sacrificed on day 10. The tumor cells post-treatment remarkably regressed tumor growth.

A. Placebo control group (5% DMSO at 10 mL/kg)

B. Compound **3.10b** group

C. Compound **3.9b** group

3.4.13.4. Tissue preparation and hematoxylin and eosin staining

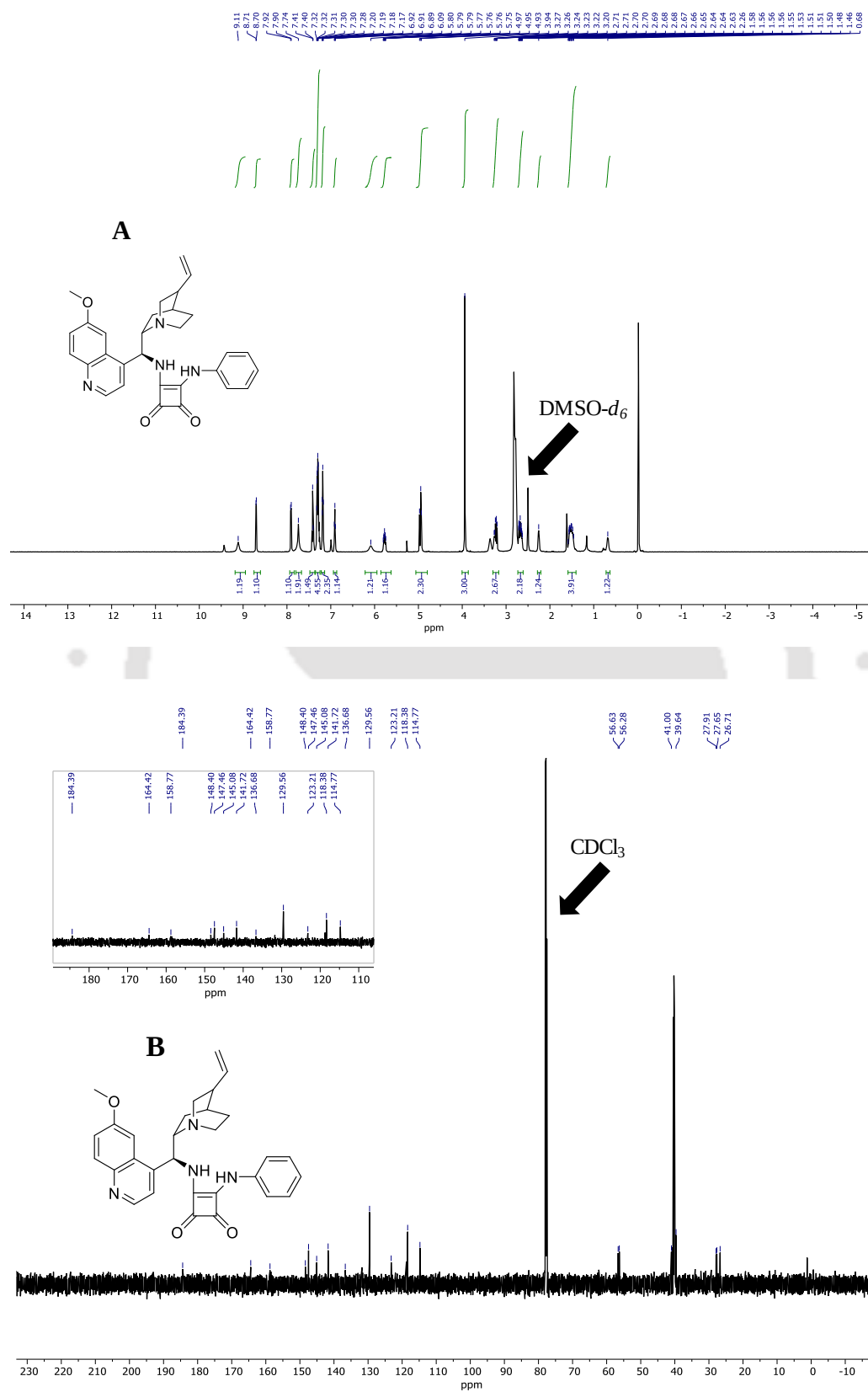
Spleen and kidney were harvested post-treatment with compounds **3.9b** and **3.10b** for tissue processing and sectioning. 5 μ m sections of each organ of all the three groups were nuclear stained with hematoxylin and cytosol stained with eosin. Posts processing the tissues were thoroughly examined to understand cellular toxicity in different tissues. In the alone tumor-bearing mice, we found huge splenic inflammation and disruption of the regular arrangement of the splenocytes. Whereas compounds **3.9b** and **3.10b** treated spleen group resembles nearly with the control spleen architecture. While examining the architecture of kidneys in all the three experimental groups, we observed no significant change in any of the groups signifying there is limited renal toxicity of compounds **3.9b** and **3.10b** treatment. The P-value for tumors:

By T-test:

Control vs compound **3.10b**: 0.0004

Control vs compound **3.9b**: 0.0027

By ANOVA: <0.0001

3.4.14. ^1H NMR and ^{13}C NMR spectra of synthesized compoundsFigure 3.13. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 3.9a.

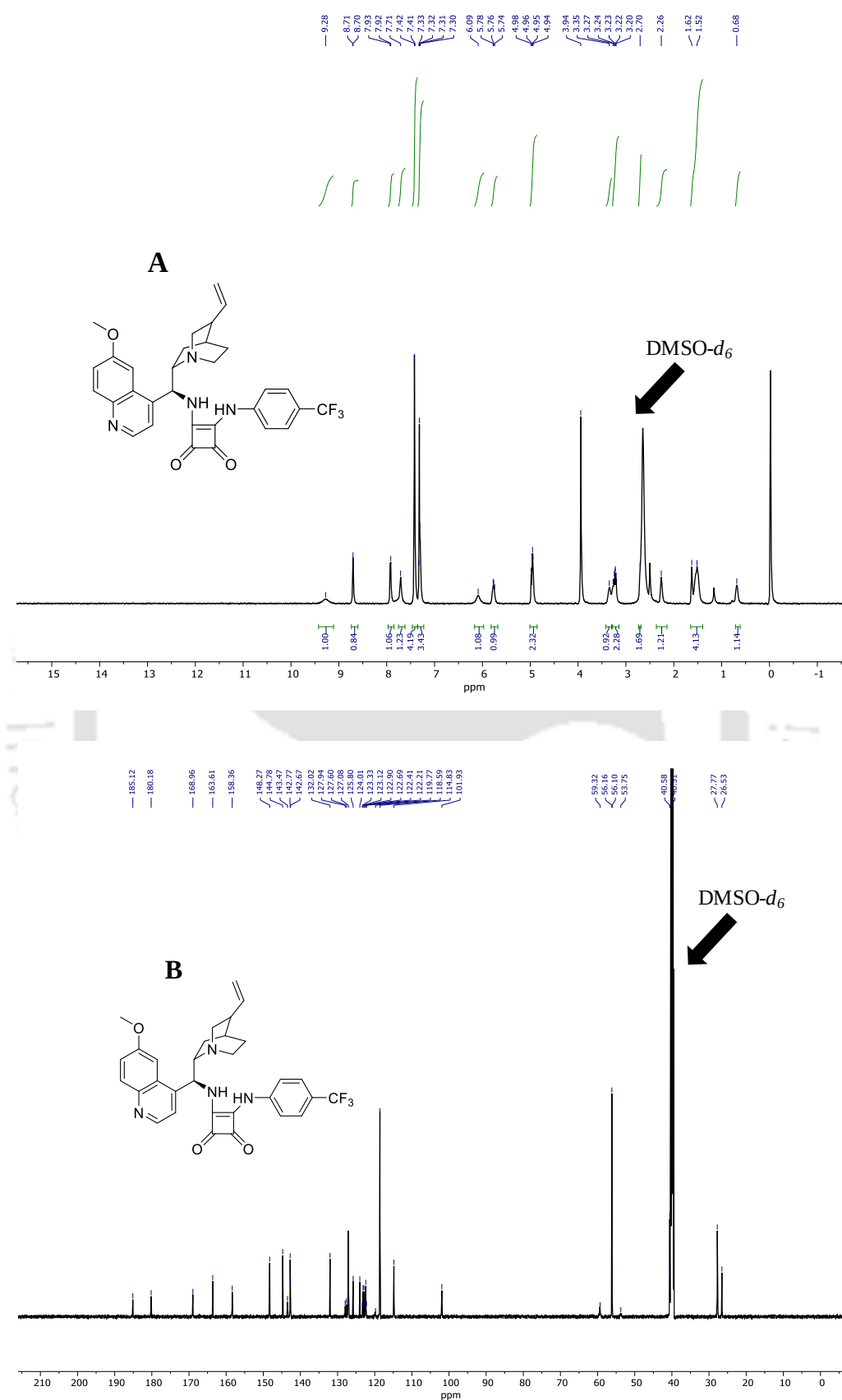


Figure 3.14. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **3.9b**.

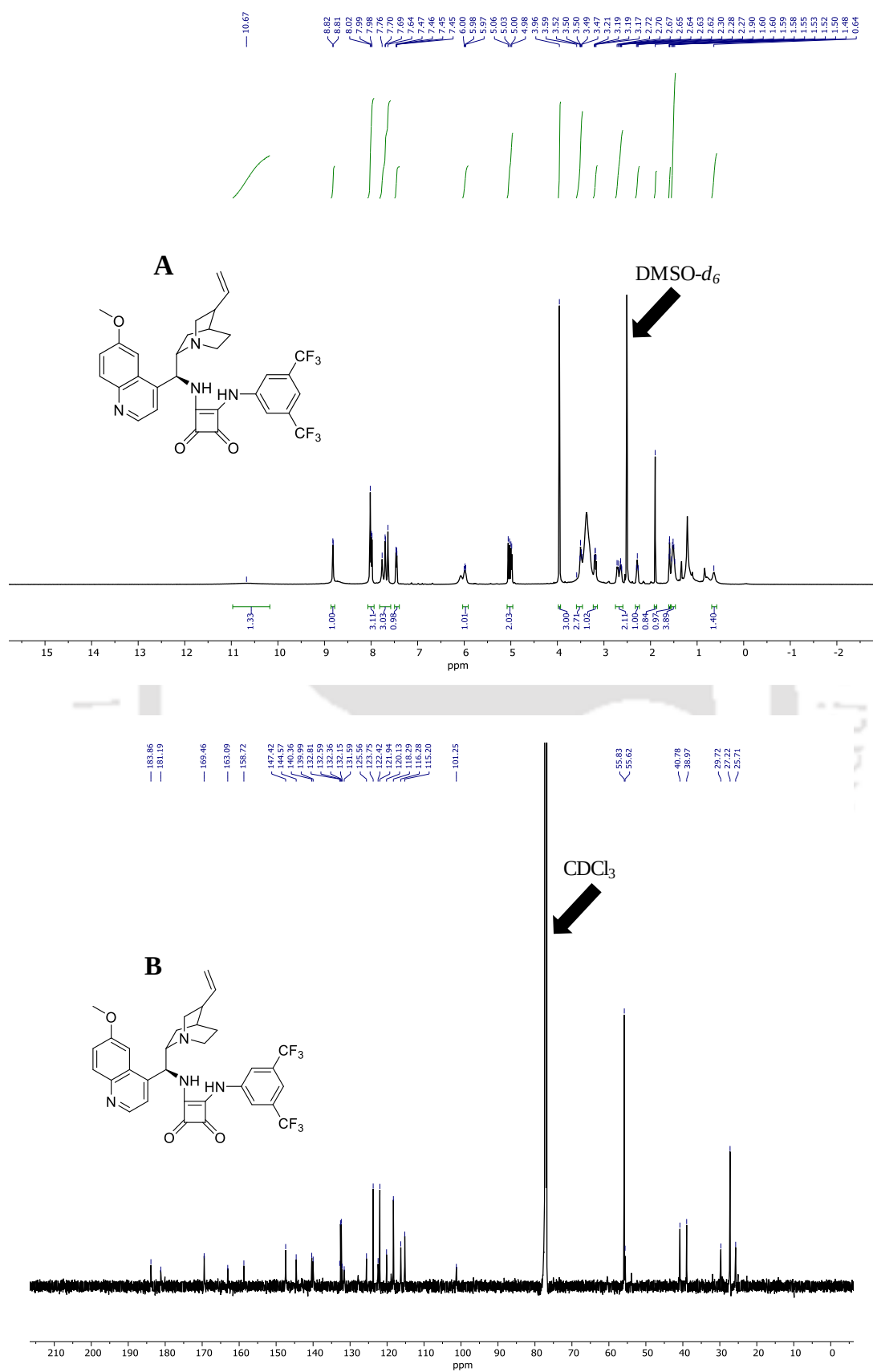


Figure 3.15. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **3.9c**.

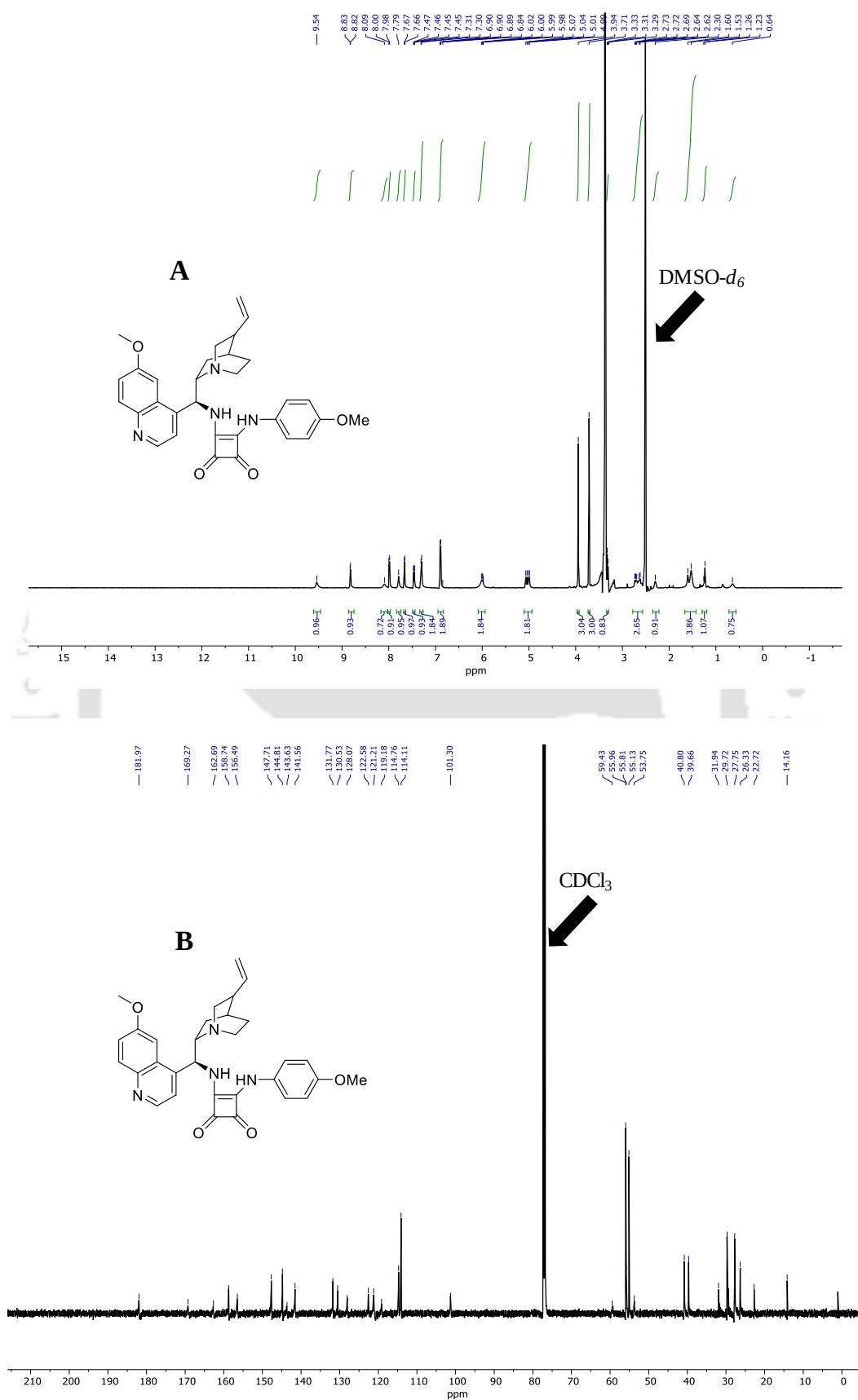


Figure 3.16. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **3.9d**.

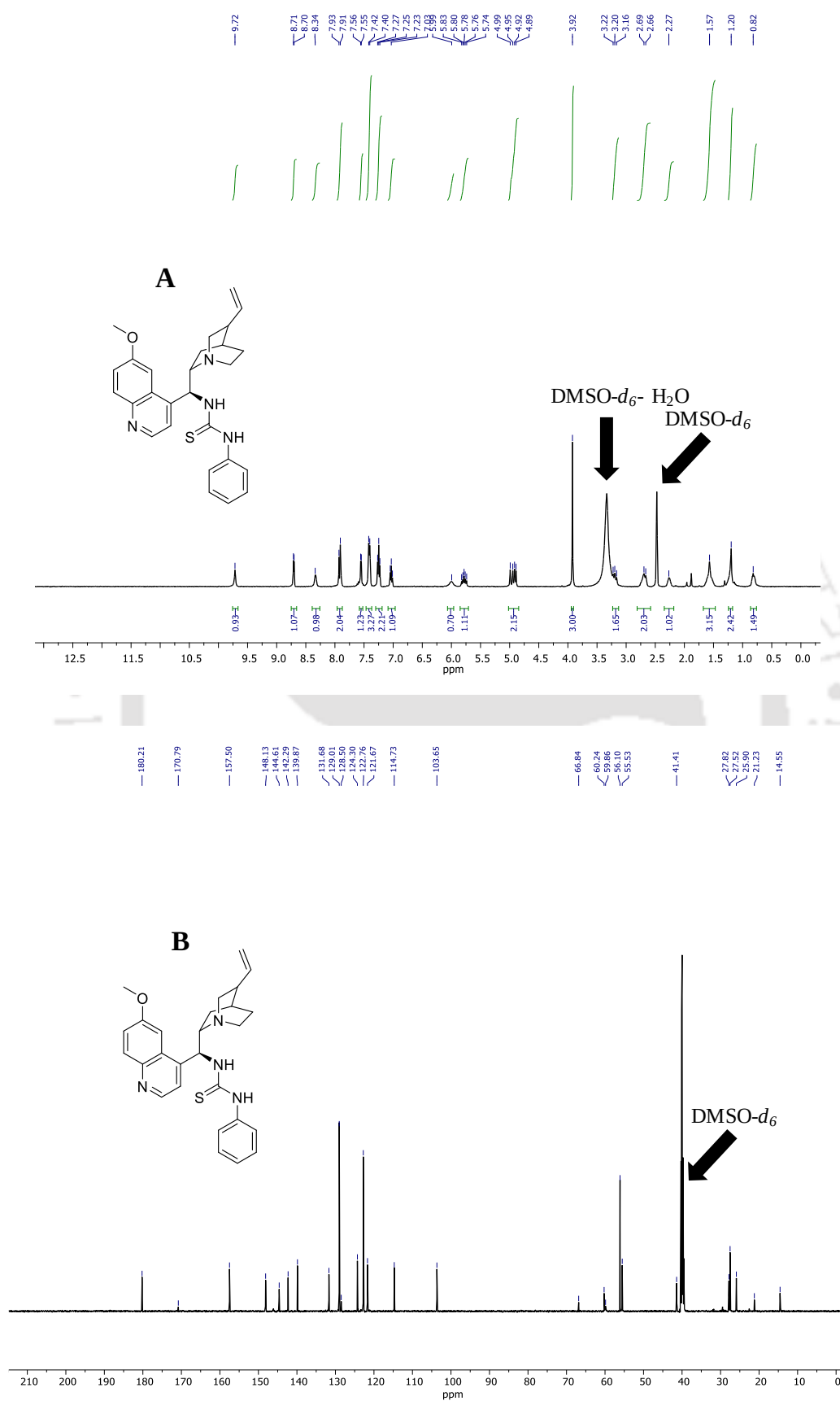


Figure 3.17. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **3.10a**.

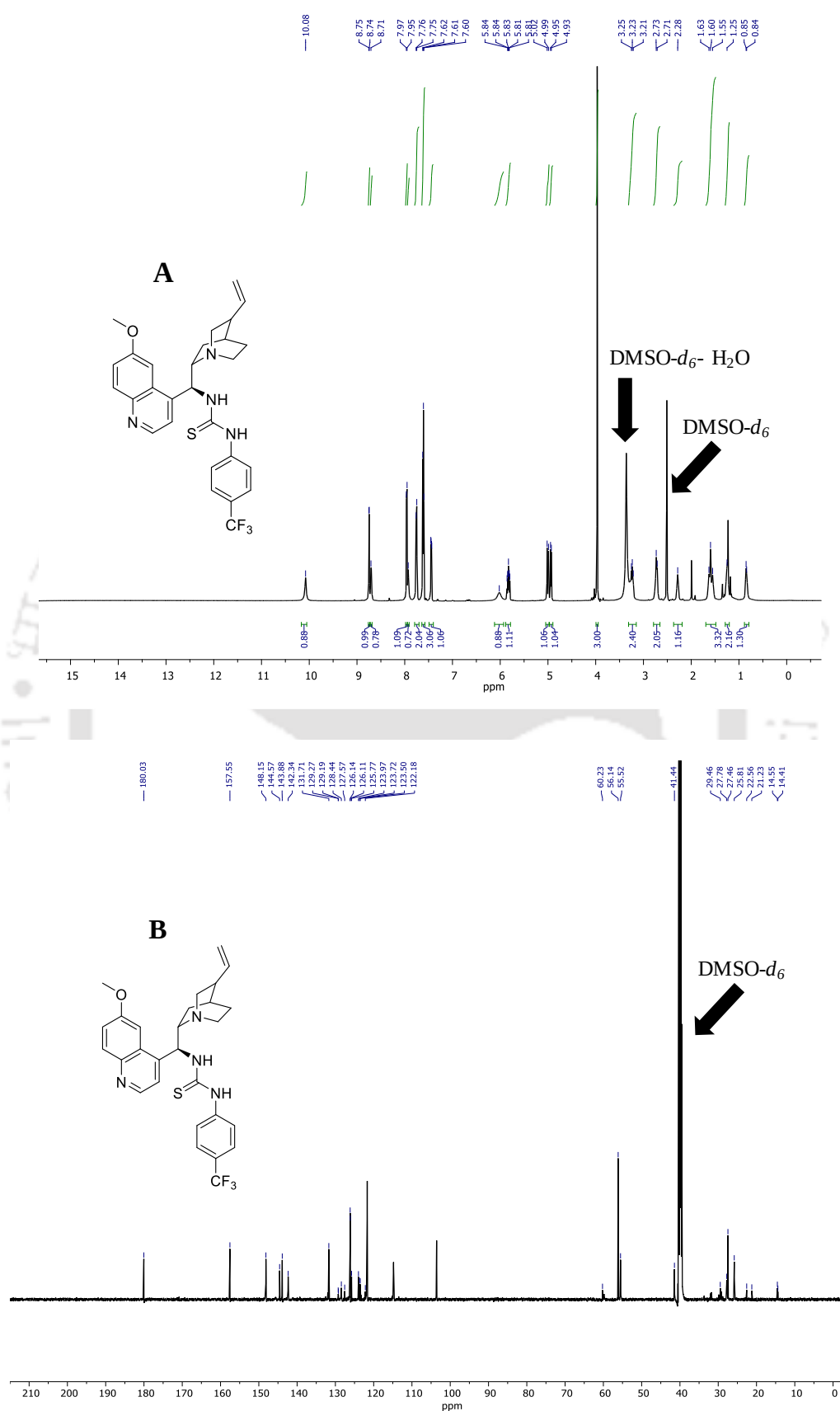


Figure 3.18. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **3.10b**.

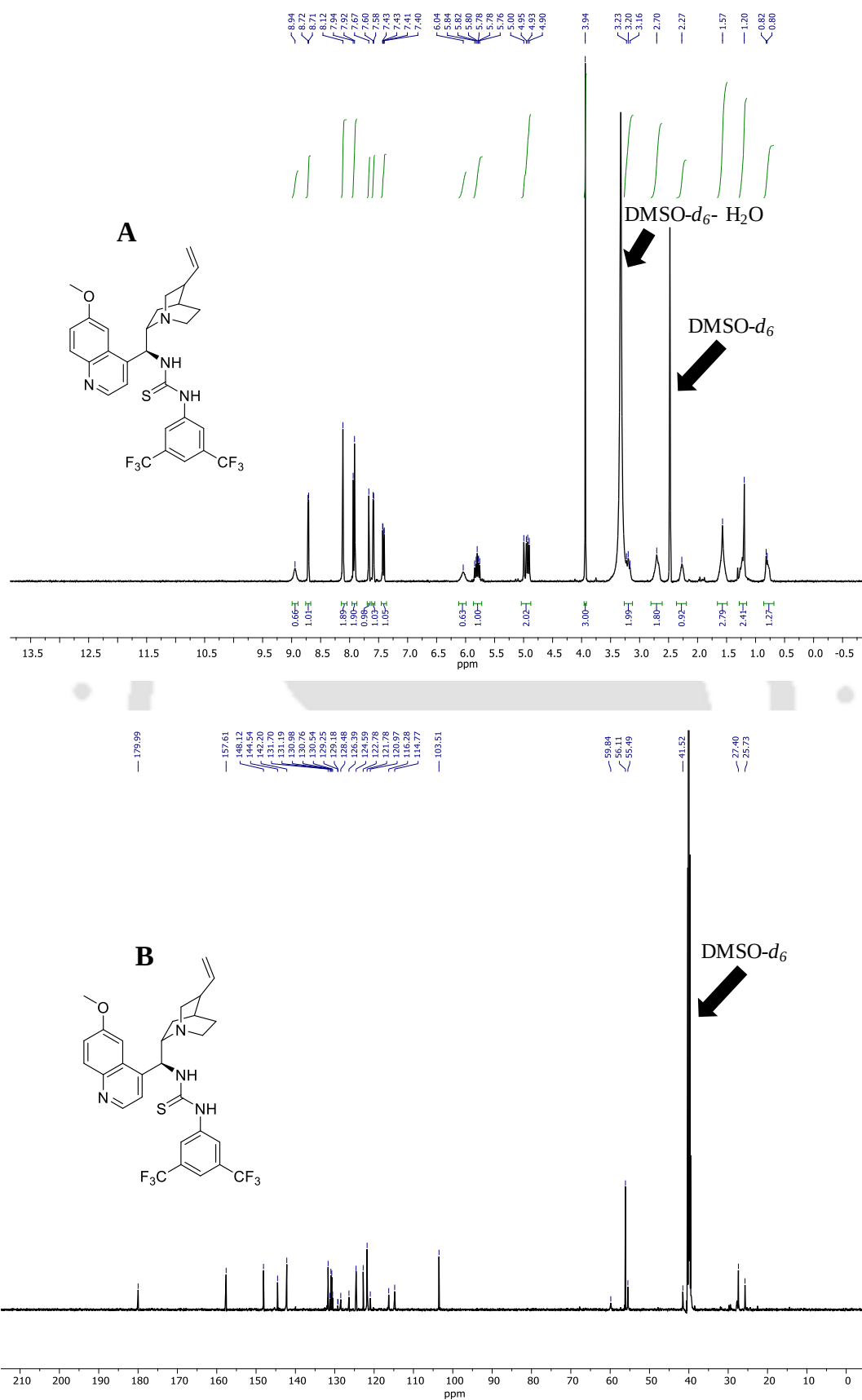


Figure 3.19. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 3.10c.

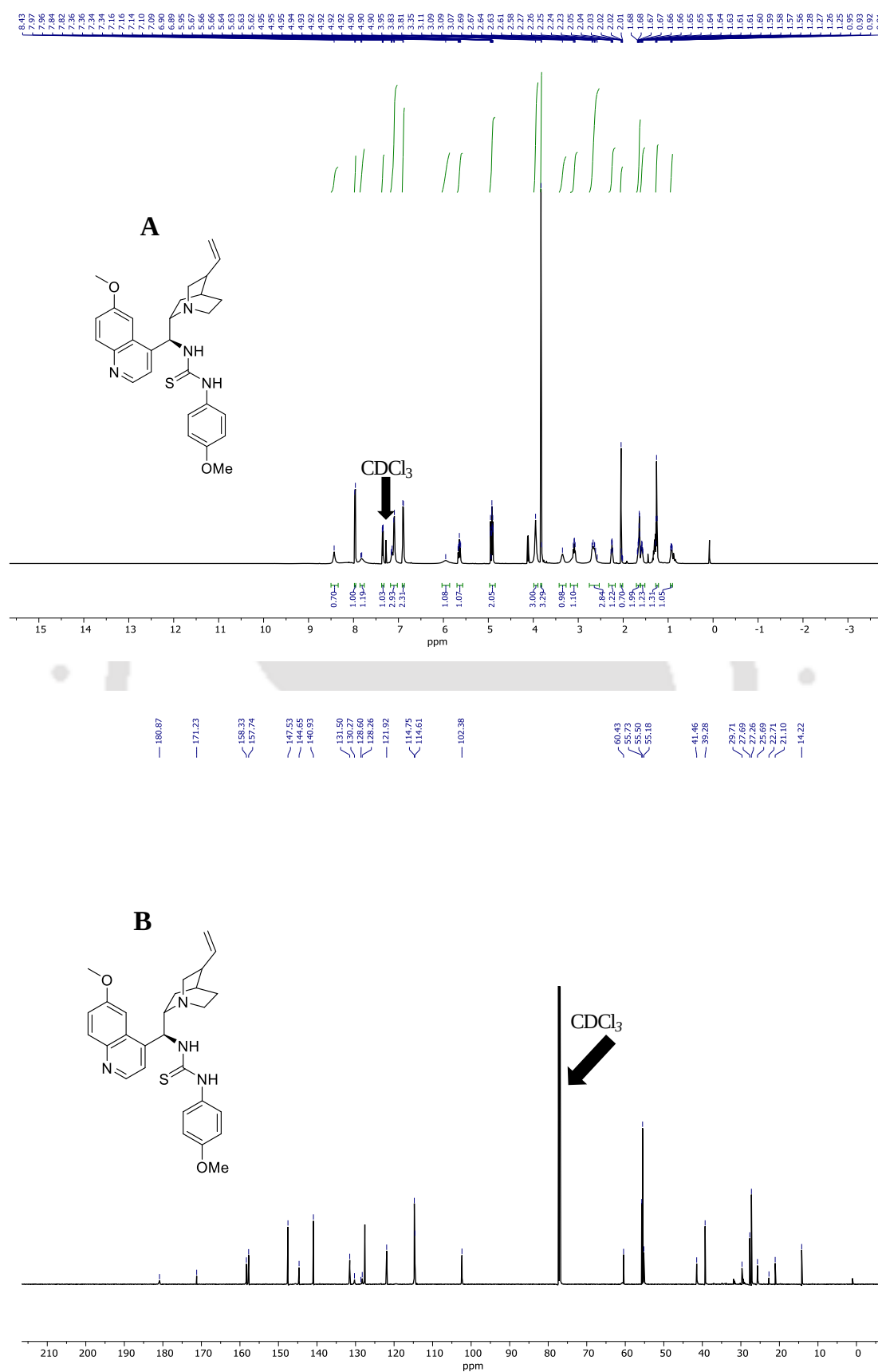


Figure 3.20. ¹H NMR (A) and ¹³C NMR (B) spectra of compound **3.10d**.

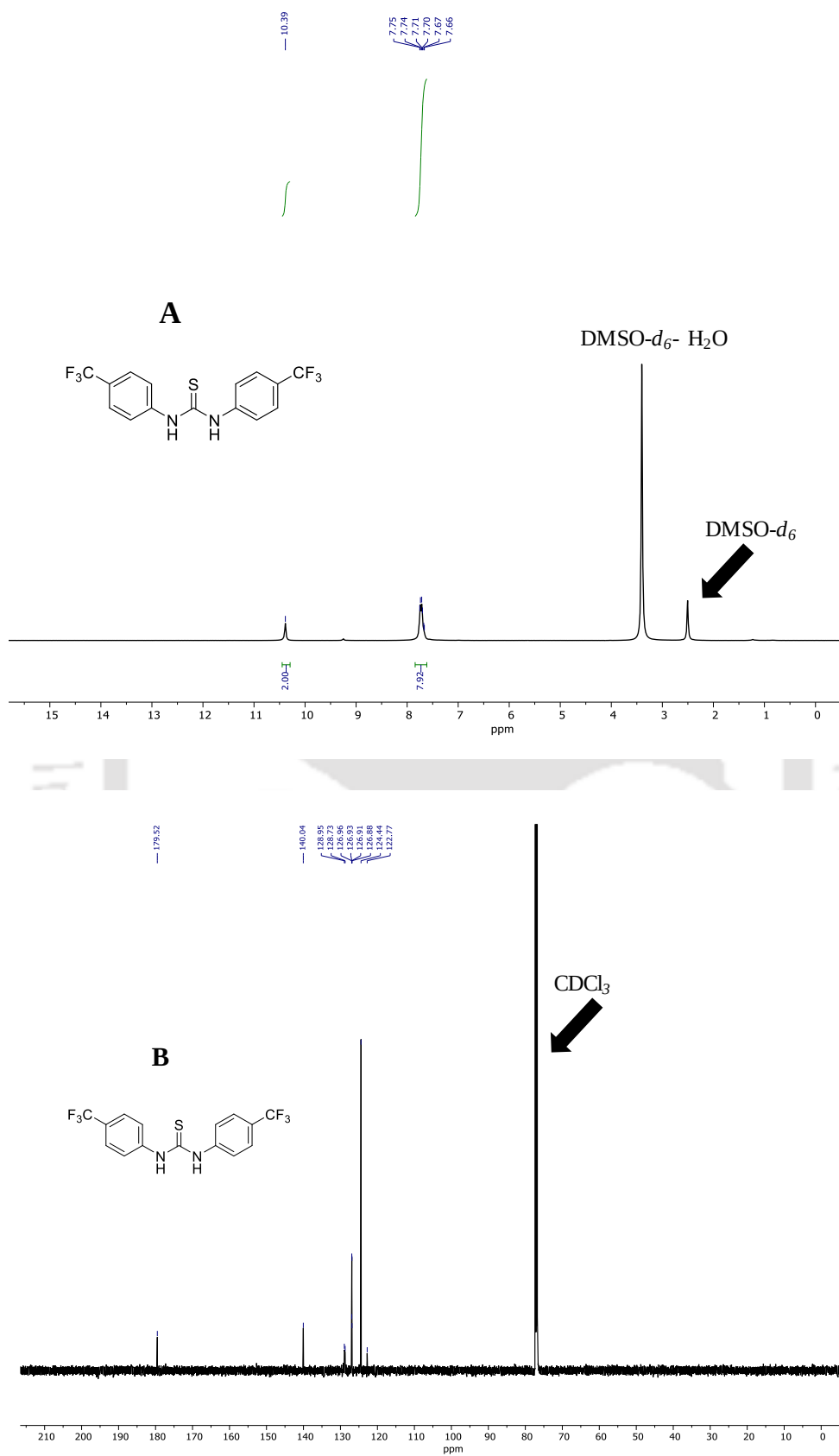


Figure 3.21. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **3.10ba1**.

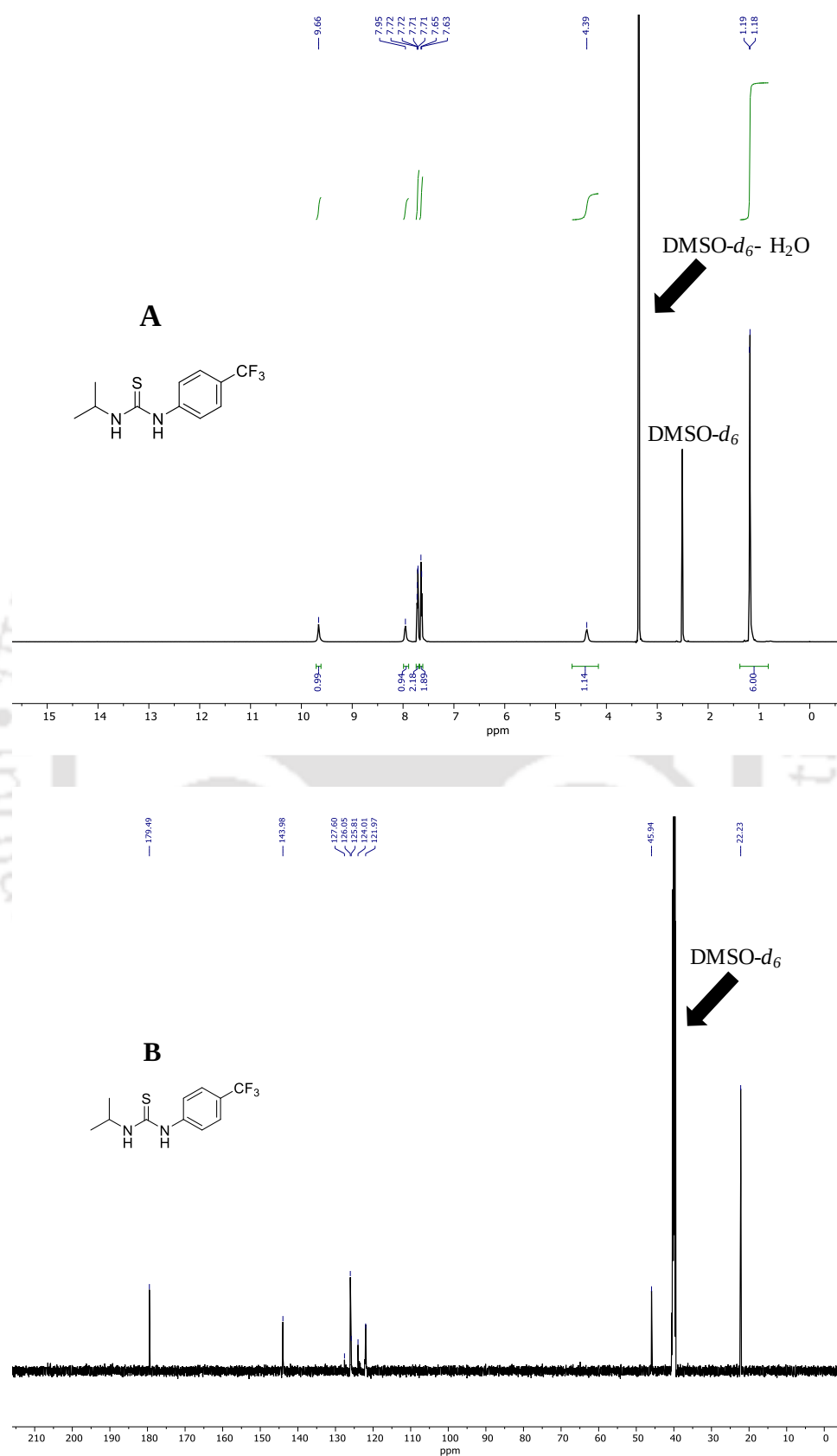


Figure 3.22. ¹H NMR (A) and ¹³C NMR (B) spectra of compound **3.10ba2**.

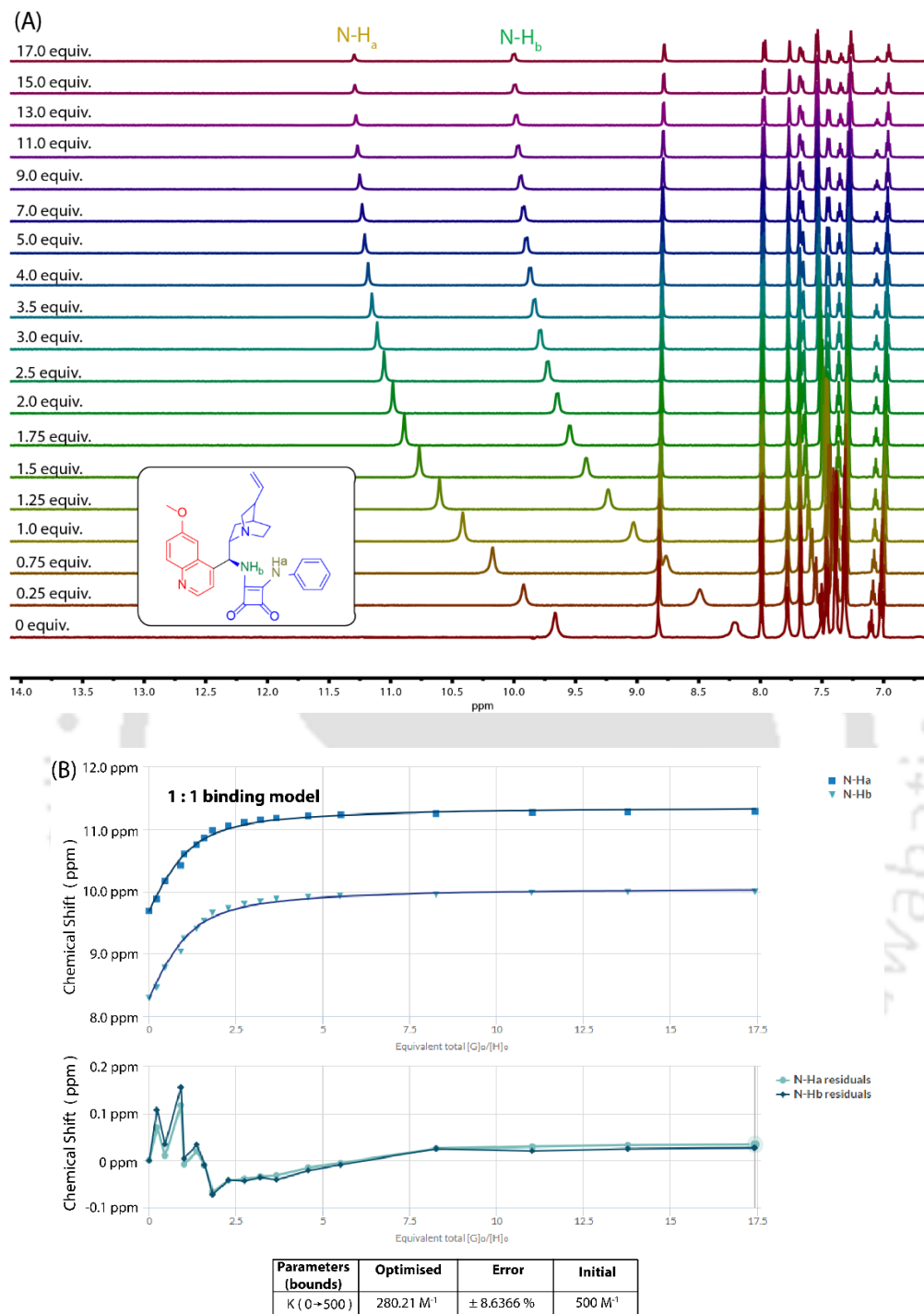
3.4.15. ^1H NMR titration plots

Figure 3.23. Representative ^1H -NMR (600MHz) titration spectra for compound **3.9a** with the sequential addition of TBACl in DMSO- d_6 solvent. The amounts of added TBACl are shown on the spectra (A). Representative plot of chemical shift (δ) of N-Ha and N-Hb protons vs. equivalent total ($[G]/[H]_0$) added, fitted to 1:1 binding model of BindFit v0.5 program (B). H = host = **3.9a** and G = guest = TBACl.

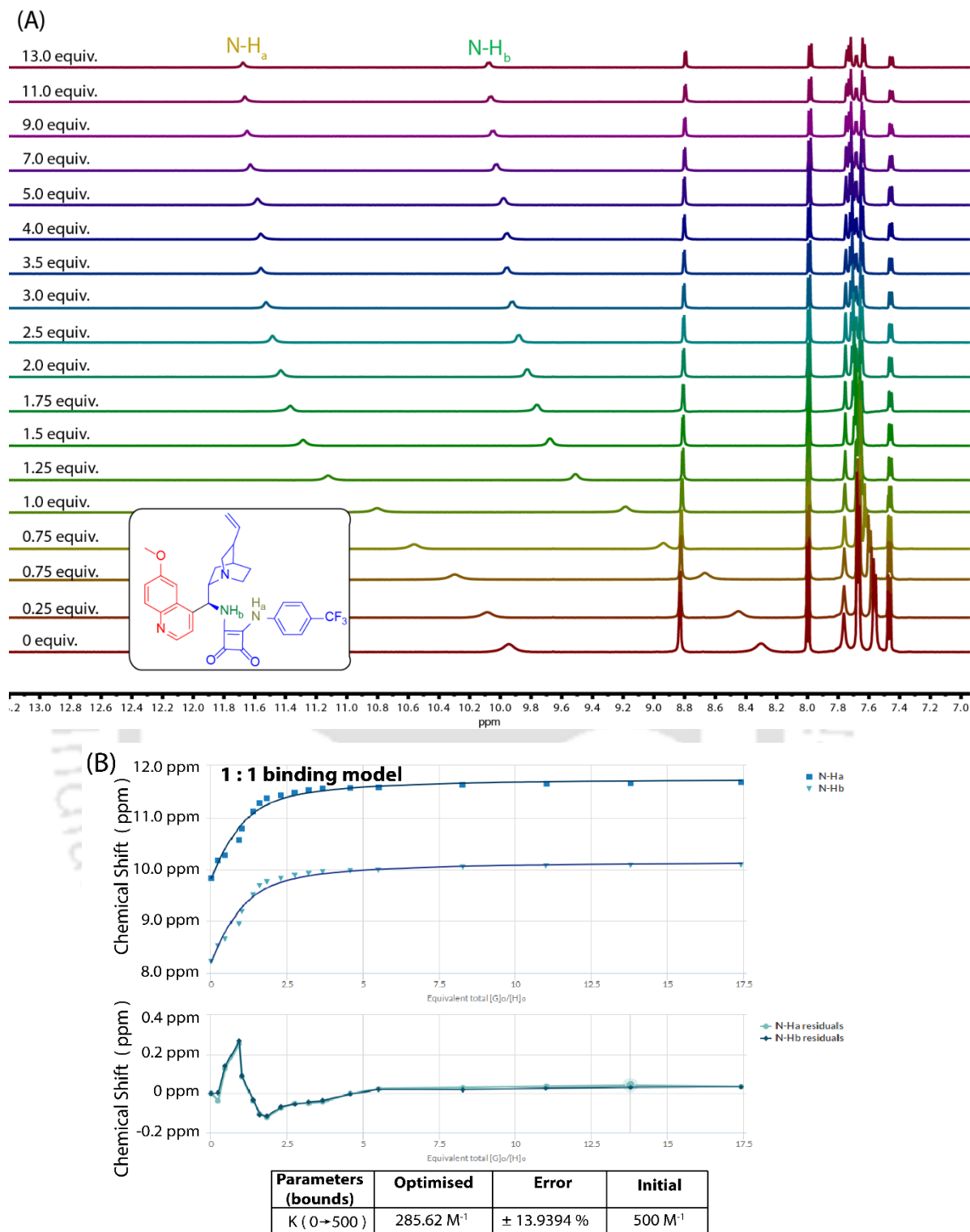


Figure 3.24. Representative ¹H-NMR (600MHz) titration spectra for compound **3.9b** with the sequential addition of TBACl in DMSO-*d*₆ solvent. The amounts of added TBACl are shown on the spectra (A). Representative plot of chemical shift (δ) of N-H_a and N-H_b protons vs. equivalent total ($[G]_0/[H]_0$) added, fitted to 1:1 binding model of BindFit v0.5 program (B). H = host = **3.9b** and G = guest = TBACl

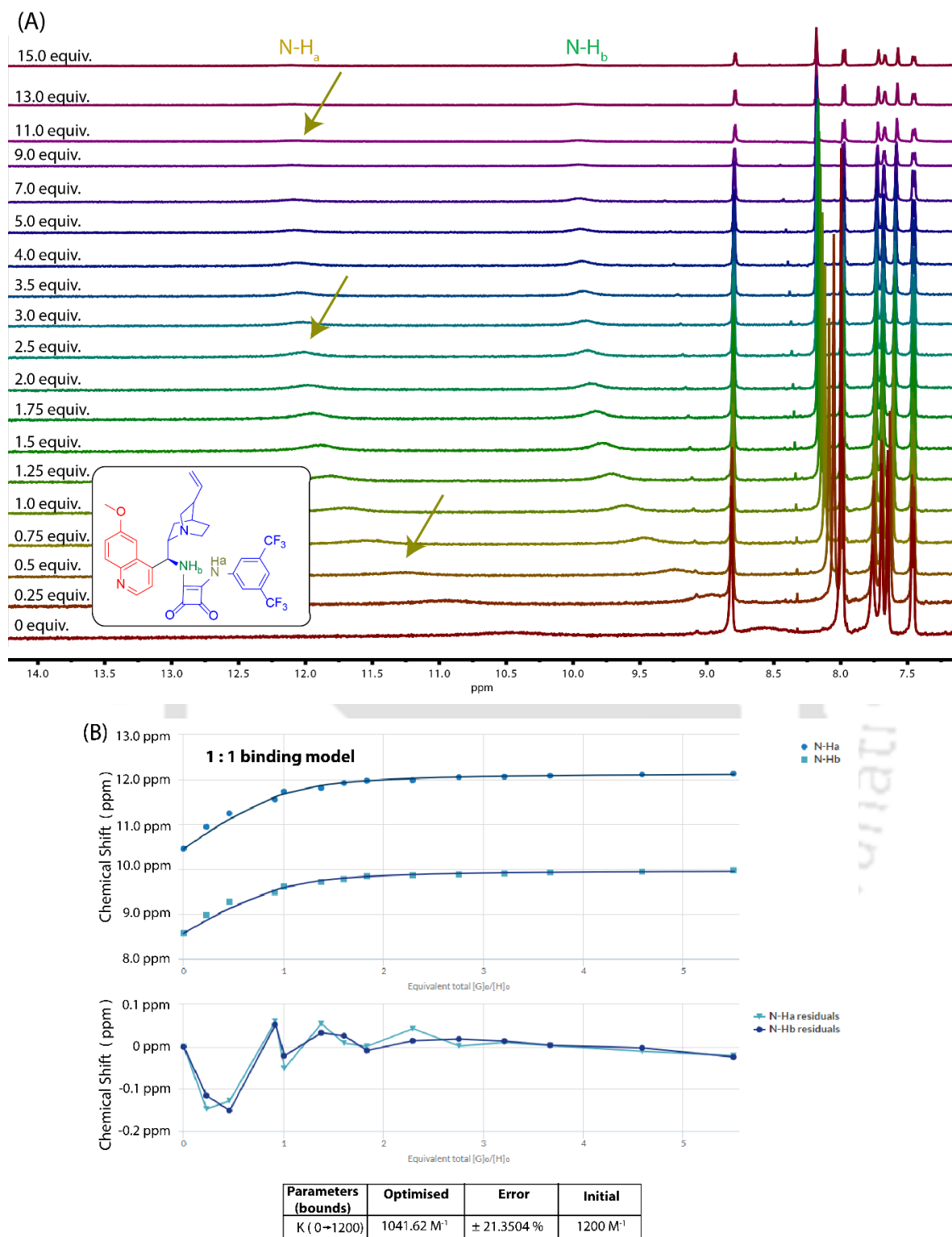


Figure 3.25. Representative ¹H-NMR (600MHz) titration spectra for compound **3.9c** with the sequential addition of TBACl in DMSO-*d*₆ solvent. The amounts of added TBACl are shown on the spectra (A). Representative plot of chemical shift (δ) of N-H_a and N-H_b protons vs equivalent total ($[G]_0/[H]_0$) added, fitted to 1:1 binding model of BindFit v0.5 program (B). H = host = **3.9c** and G = guest = TBACl.

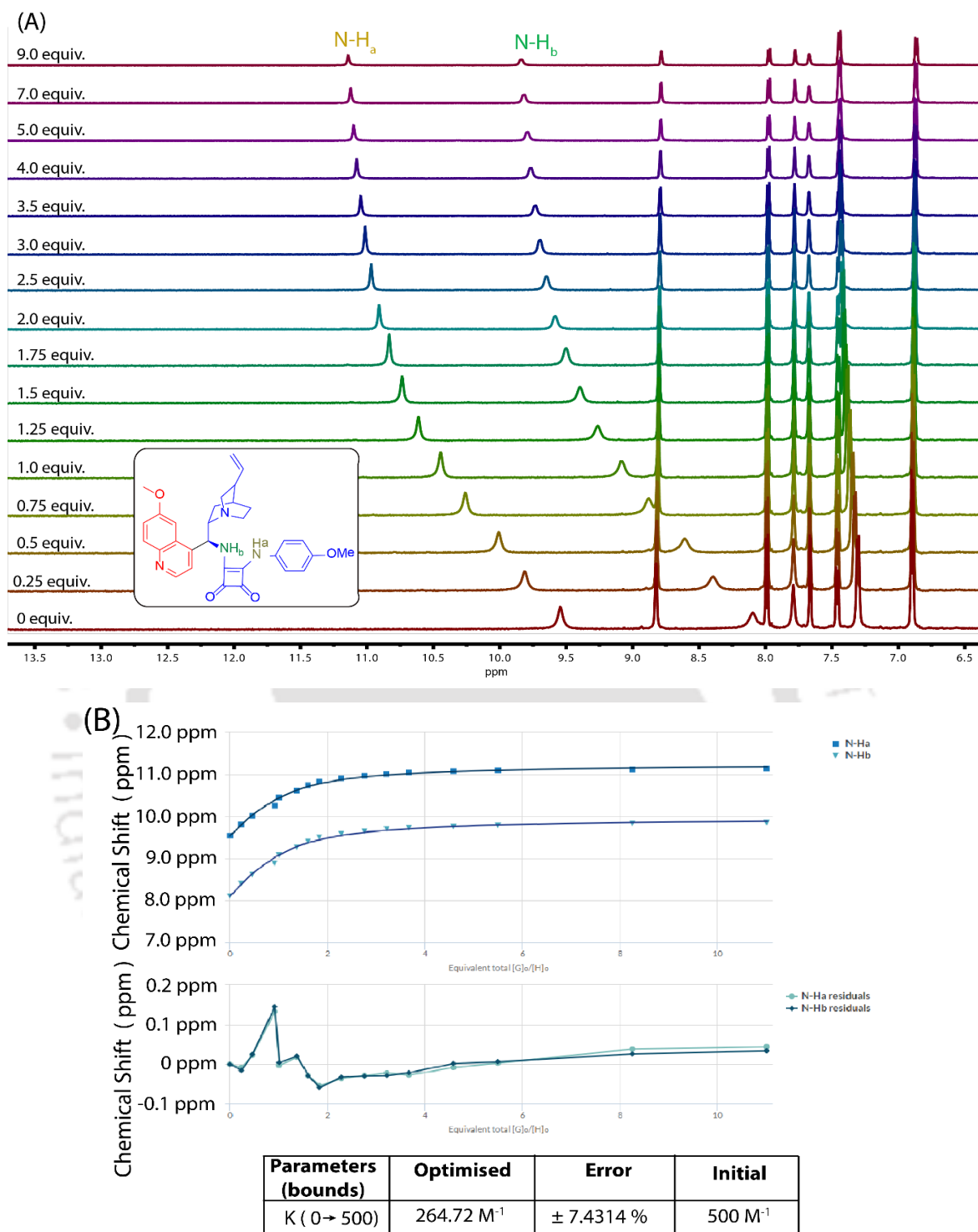


Figure 3.26. Representative ¹H-NMR (600MHz) titration spectra for compound **3.9d** with the sequential addition of TBACl in DMSO-*d*₆ solvent. The amounts of added TBACl are shown on the spectra (A). Representative plot of chemical shift (δ) of N-H_a and N-H_b protons vs equivalent total ($[G]_0/[H]_0$) added, fitted to 1:1 binding model of BindFit v0.5 program (B). H = host = **3.9d** and G = guest = TBACl.

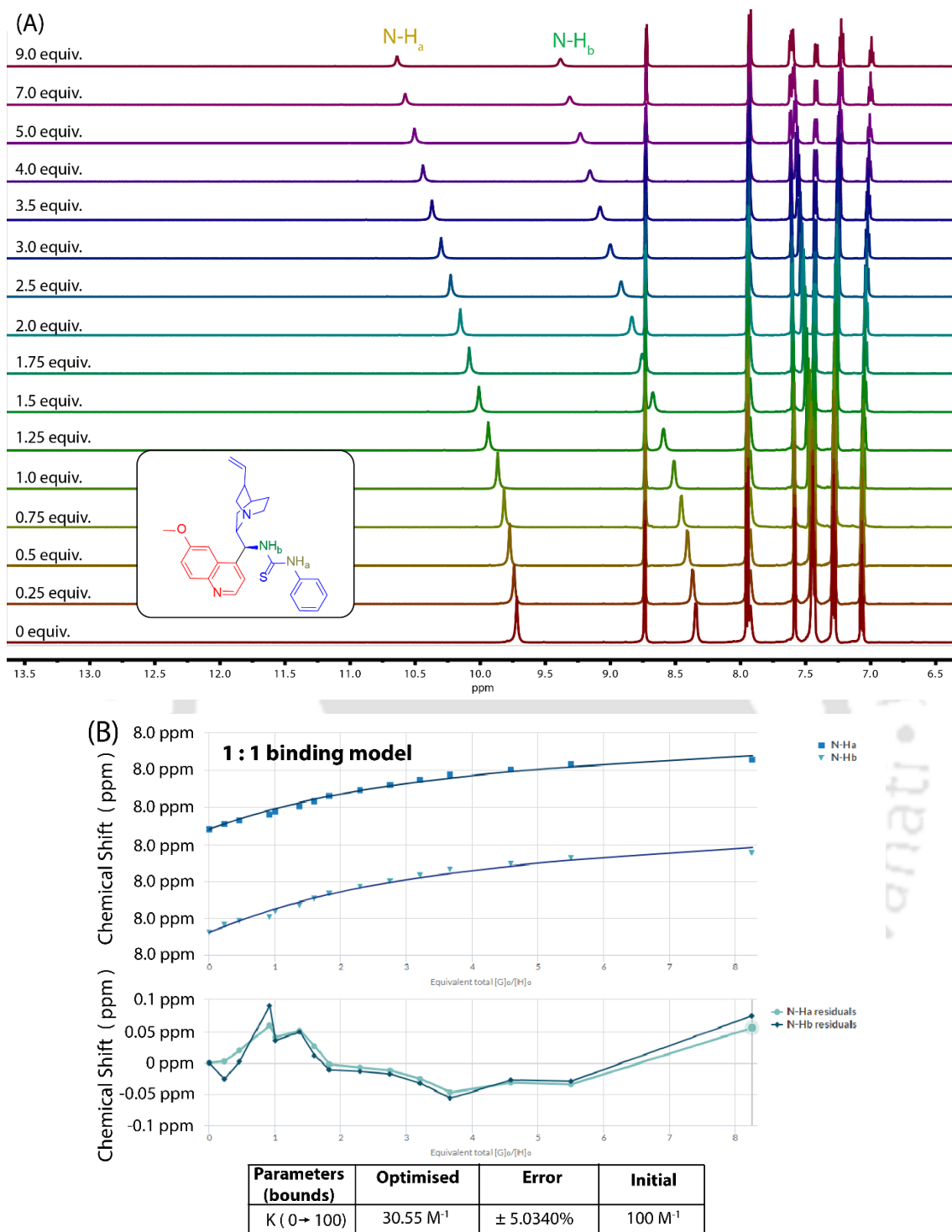


Figure 3.27. Representative ¹H-NMR (600MHz) titration spectra for compound **3.10a** with the sequential addition of TBACl in DMSO-*d*₆ solvent. The amounts of added TBACl are shown on the spectra (A). Representative plot of chemical shift (δ) of N-H_a and N-H_b protons vs equivalent total ($[G]_0/[H]_0$) added, fitted to 1:1 binding model of BindFit v0.5 program (B). H = host = **3.10a** and G = guest = TBACl.

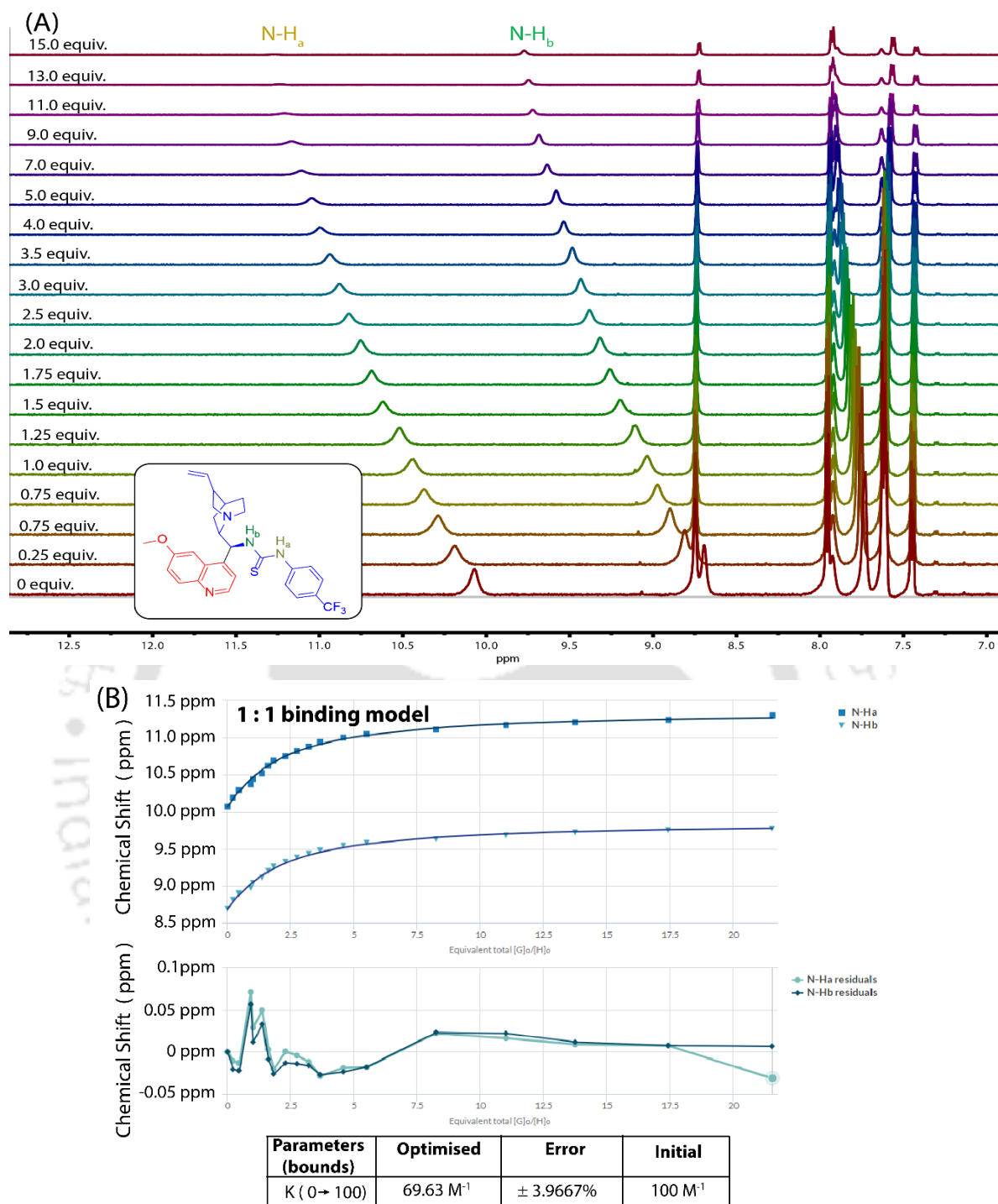


Figure 3.28. Representative ¹H-NMR (600MHz) titration spectra for compound **3.10b** with the sequential addition of TBACl in DMSO-*d*₆ solvent. The amounts of added TBACl are shown on the spectra (A). Representative plot of chemical shift (δ) of N-H_a and N-H_b protons vs equivalent total ($[G]_0/[H]_0$) added, fitted to 1:1 binding model of BindFit v0.5 program (B). H = host = **3.10b** and G = guest = TBACl.

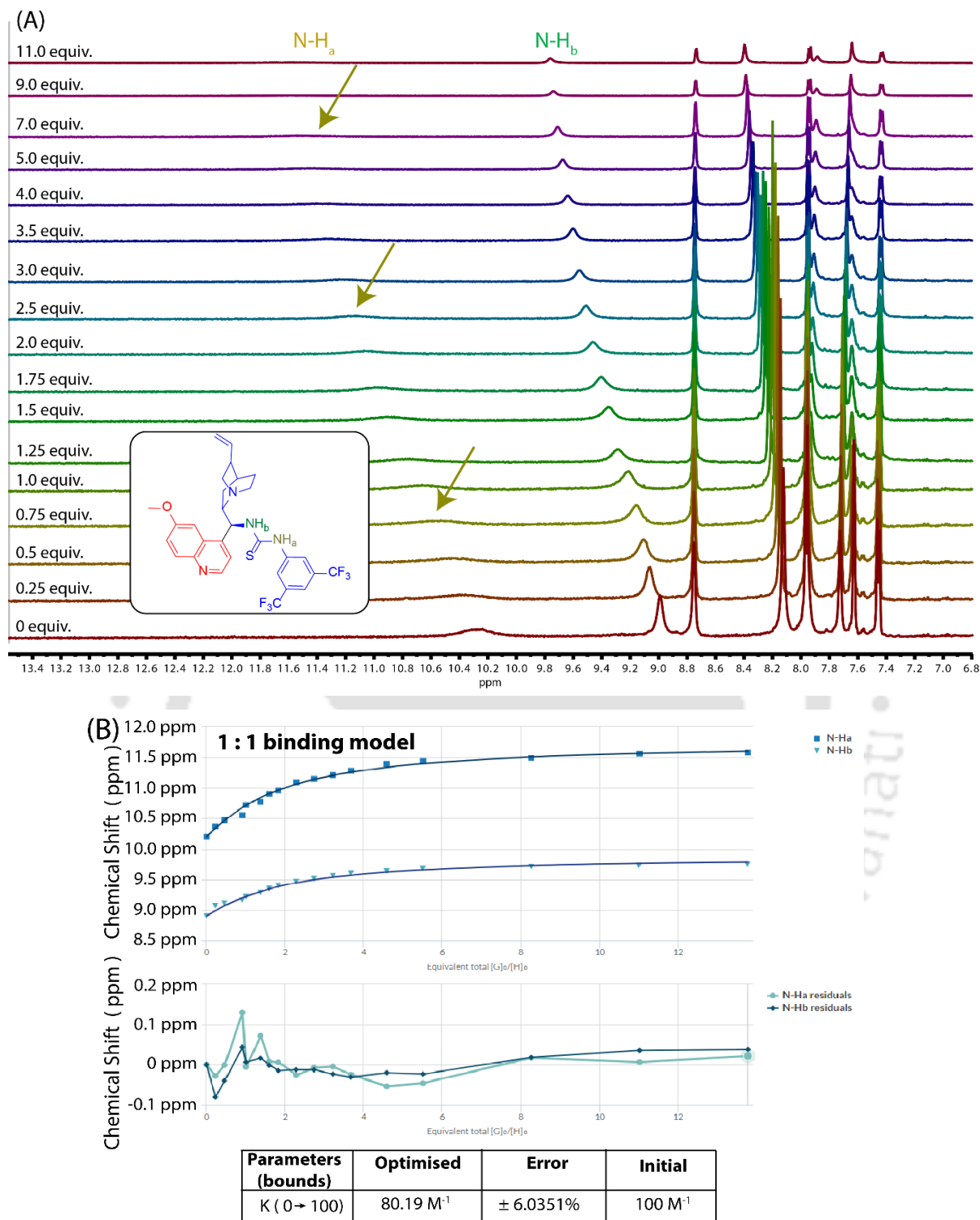


Figure 3.29. Representative ¹H-NMR (600MHz) titration spectra for compound **3.10c** with the sequential addition of TBACl in DMSO-*d*₆ solvent. The amounts of added TBACl are shown on the spectra (A). Representative plot of chemical shift (δ) of N-H_a and N-H_b protons vs equivalent total ($[G]_0/[H]_0$) added, fitted to 1:1 binding model of BindFit v0.5 program (B). H = host = **3.10c** and G = guest = TBACl.

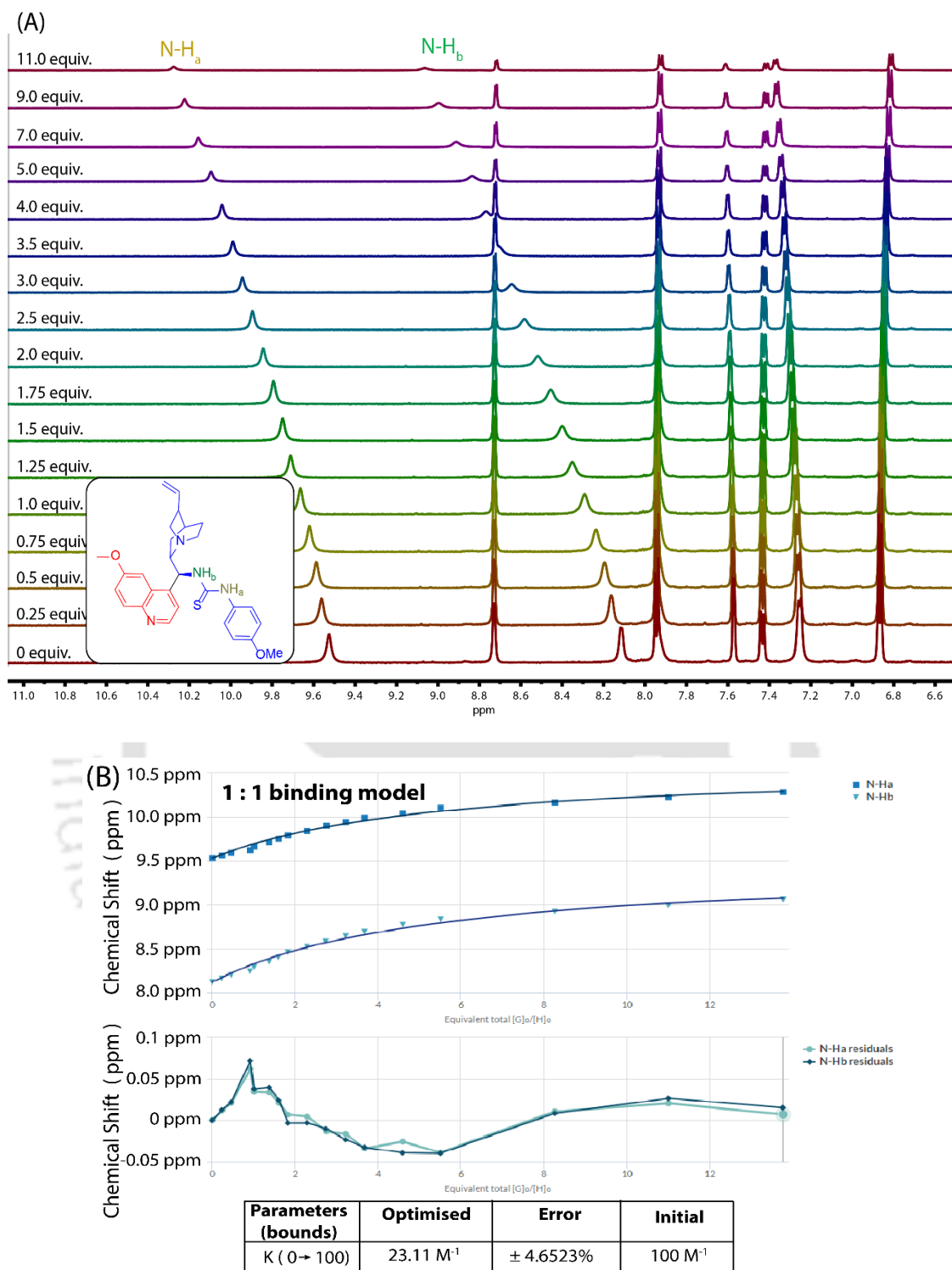


Figure 3.30. Representative ¹H-NMR (600MHz) titration spectra for compound **3.10d** with the sequential addition of TBACl in DMSO-*d*₆ solvent. The amounts of added TBACl are shown on the spectra (A). Representative plot of chemical shift (δ) of N-H_a and N-H_b protons vs equivalent total ([G]₀/[H]₀) added, fitted to 1:1 binding model of BindFit v0.5 program (B). H = host = **3.10d** and G = guest = TBACl.

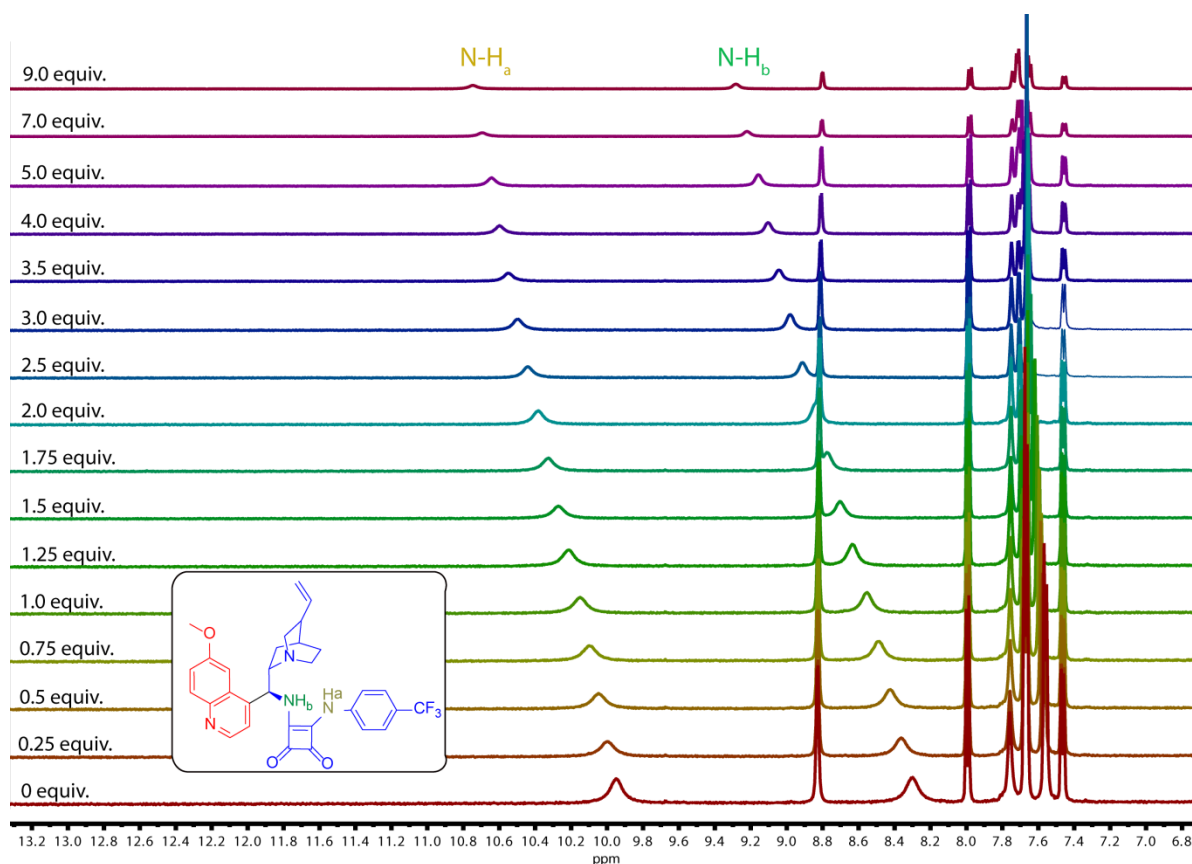
^1H NMR Titration plots with different anions

Figure 3.31. Representative ^1H -NMR (600MHz) titration spectra for compound **3.9b** with the sequential addition of TBABr in $\text{DMSO}-d_6$ solvent. The amounts of added TBABr are shown on the spectra.

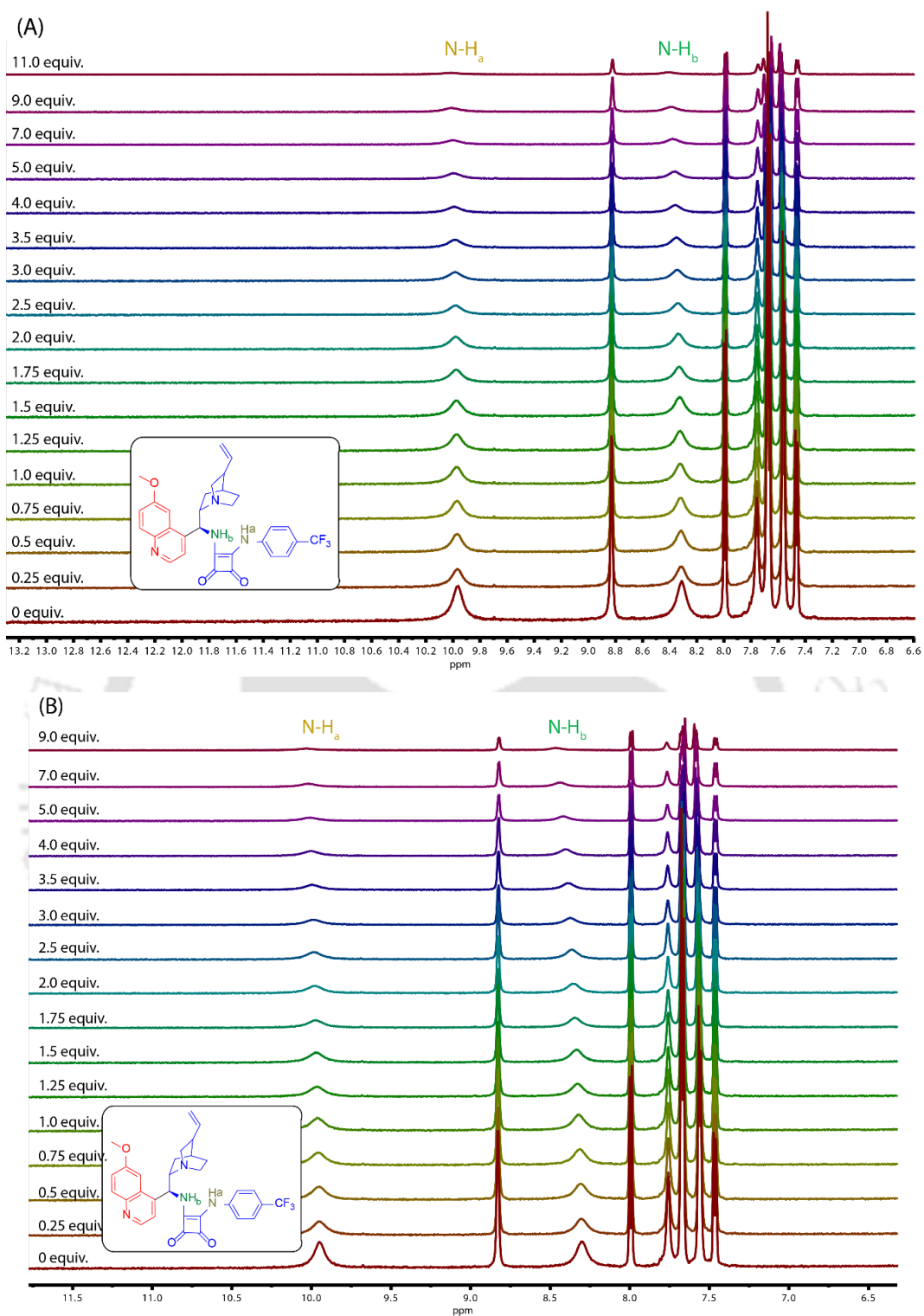


Figure 3.32. Representative ^1H -NMR (600MHz) titration spectra for compound **3.9b** with the sequential addition of TBAI (A) and TBANO_3 (B) in $\text{DMSO}-d_6$ solvent.

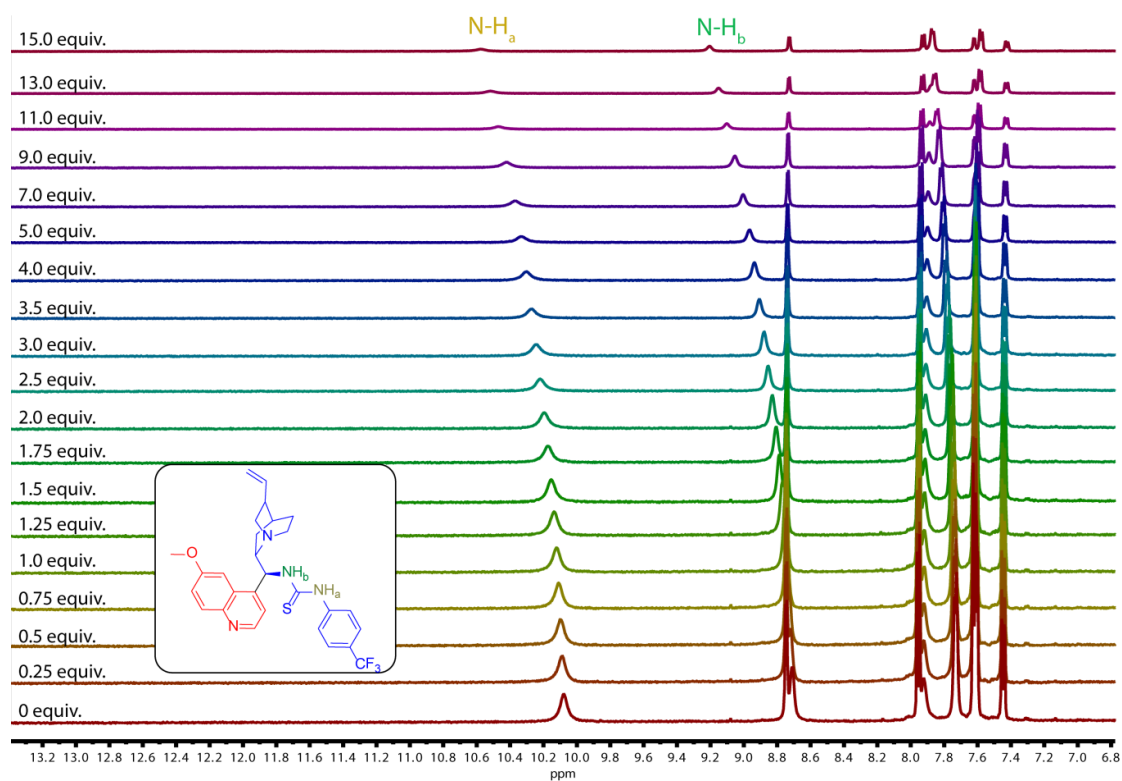


Figure 3.33. Representative ^1H -NMR (600MHz) titration spectra for compound **3.10b** with the sequential addition of TBACl in $\text{DMSO}-d_6$ solvent. The amounts of added TBACl are shown in the spectra.

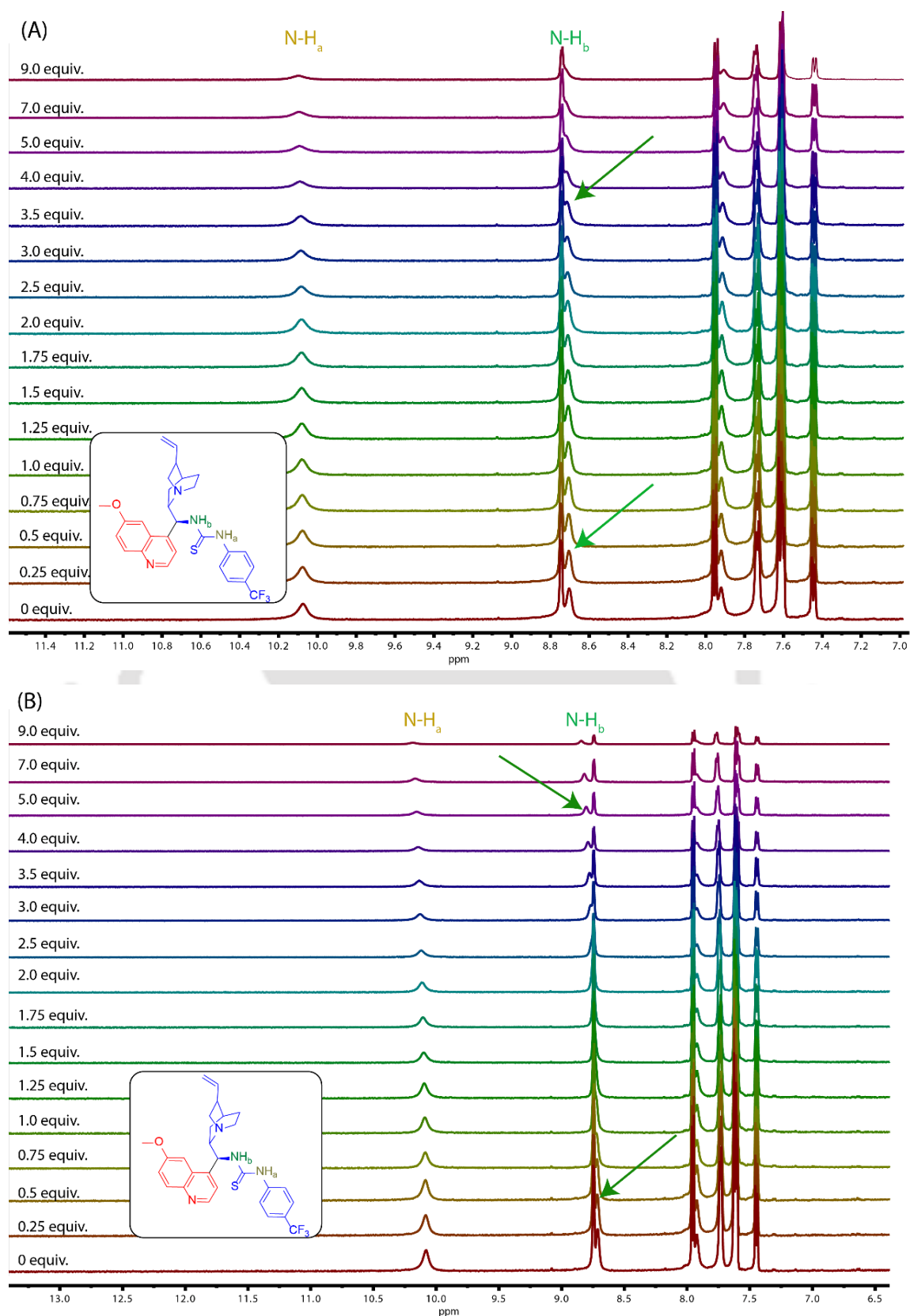


Figure 3.34. Representative ^1H -NMR (600MHz) titration spectra for compound **3.10b** with the sequential addition of TBAI (A) and TBANO_3 (B) in $\text{DMSO}-d_6$ solvent.

3.4.16. ^1H NMR titration and the corresponding fitting spectra for pKa determination

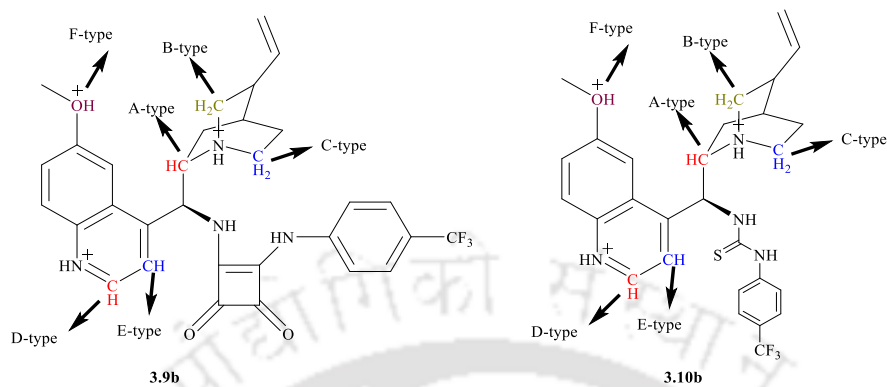


Figure 3.35. Structures of compounds **3.9b** and **3.10b** in the acidic medium.

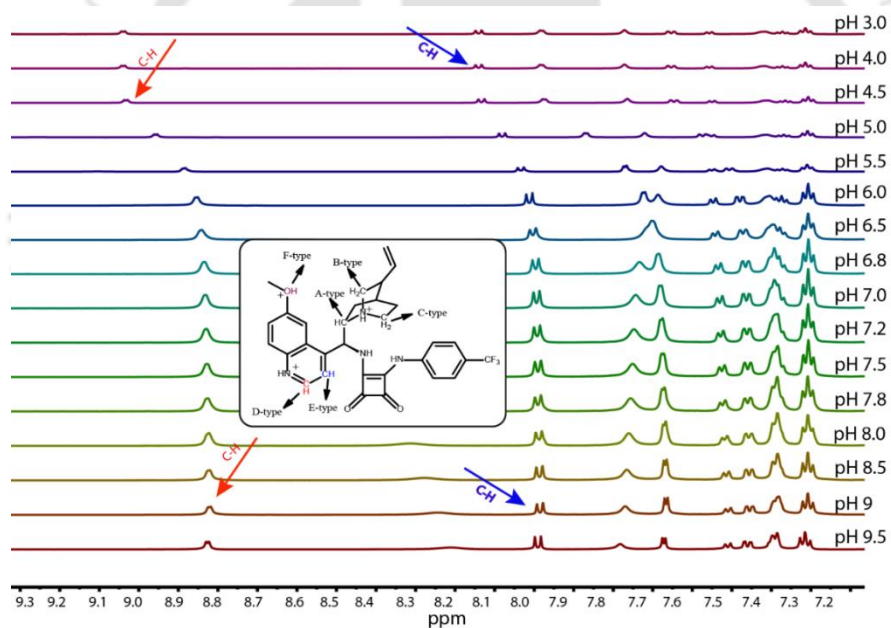


Figure 3.36. Partial ^1H NMR spectra of the compound **3.9b** at different pH. The chemical shifts of the quinoline moiety upon changing the pH of the medium are shown here.

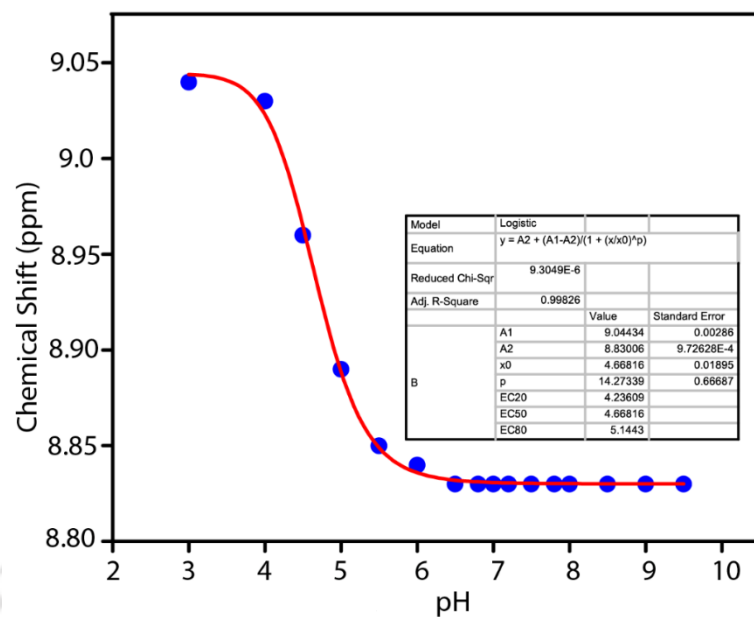


Figure 3.37. The change in chemical shifts of compound **3.9b** D-type proton peak near the quinoline nitrogen) plotted against pH of the medium. The calculated pKa1 value was 4.66.

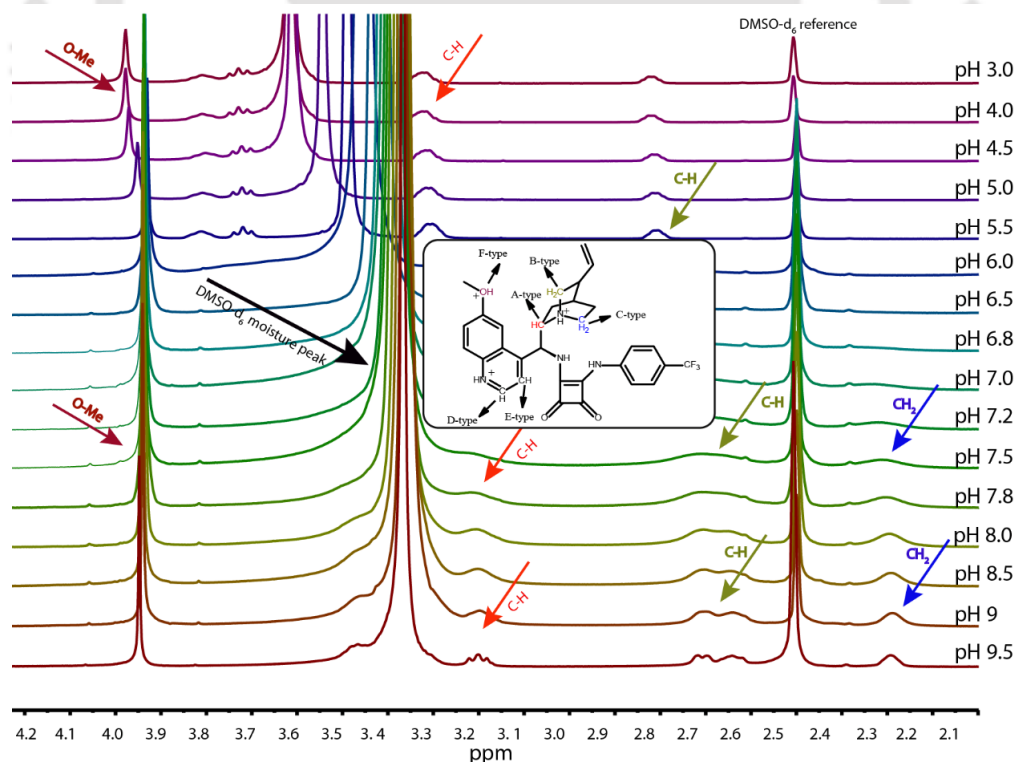


Figure 3.38. Chemical shift upon the changing the pH of the medium near the quinuclidine nitrogen atom.

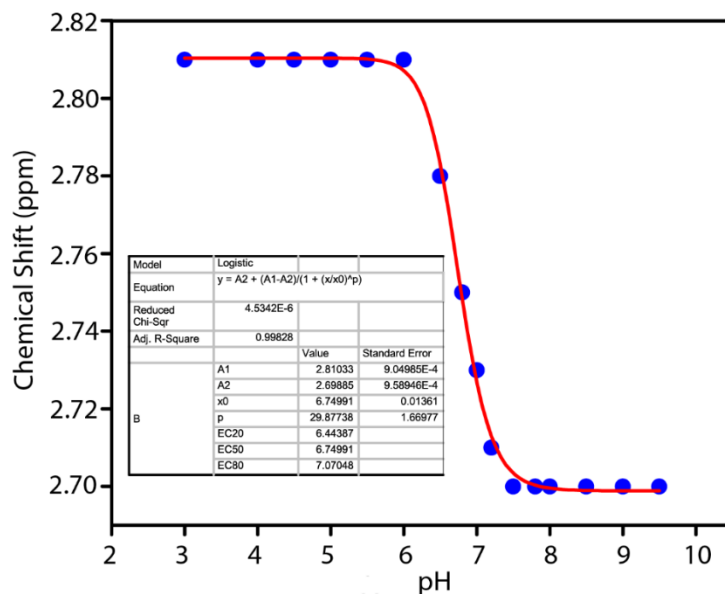


Figure 3.39. Fitting plot of the chemical shift Vs pH of the B-type proton peak near the quinuclidine nitrogen atom. The pKa came around 6.75.

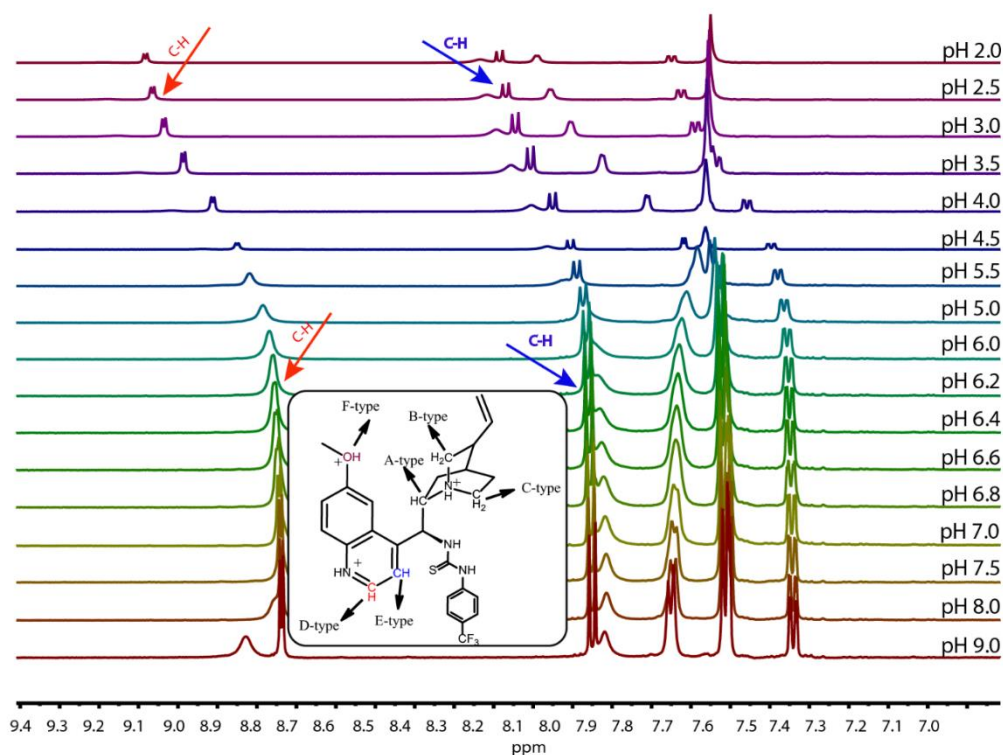


Figure 3.40. Partial ^1H NMR spectra of the compound **3.10b** at different pH. The chemical shifts of the quinoline moiety upon changing the pH of the medium are shown here.

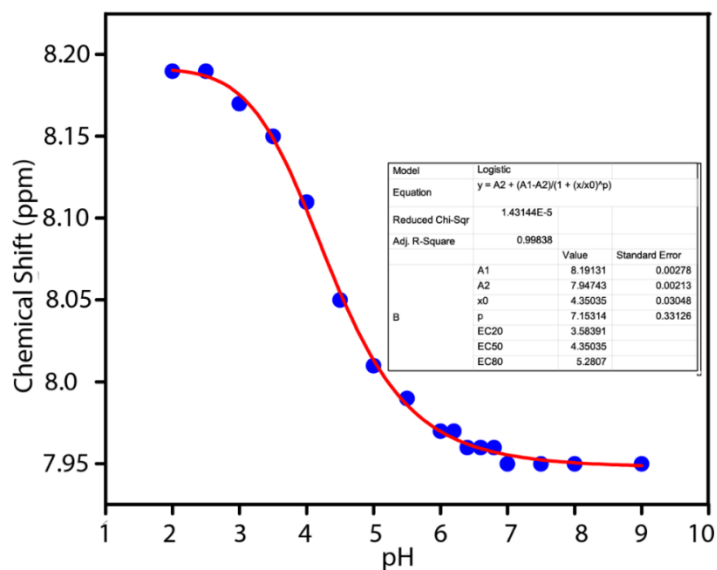


Figure 3.41. Fitting plot of the chemical shift Vs pH of the D-type proton peak near the quinoline nitrogen atom. The pKa came around 4.35.

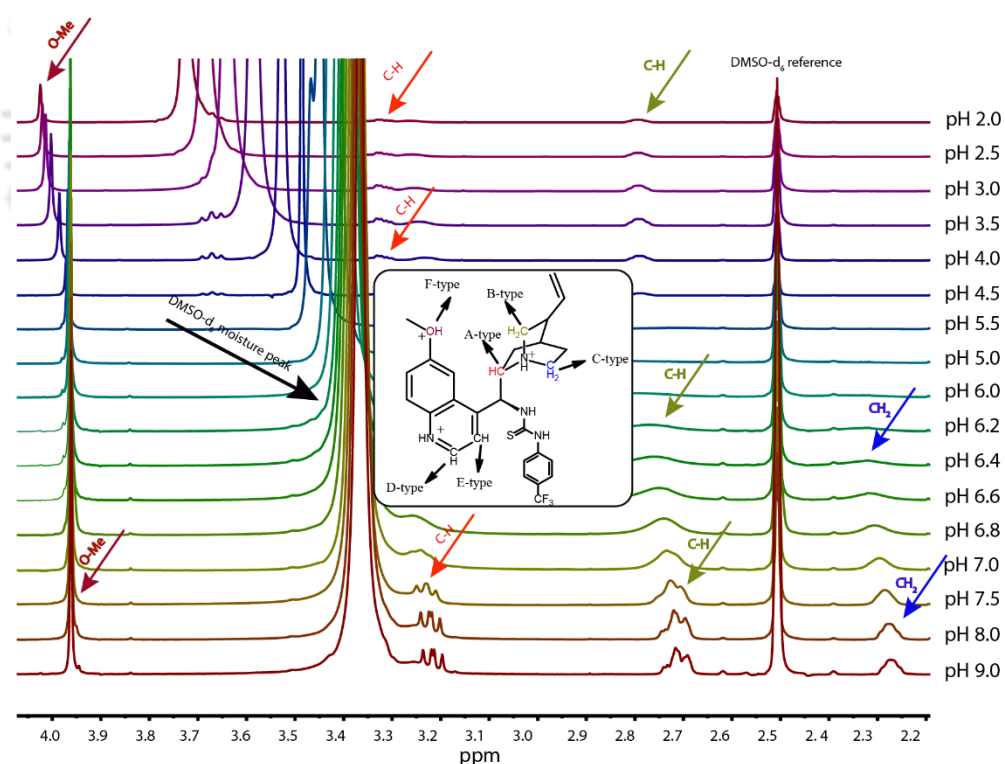


Figure 3.42. Partial ^1H NMR spectra of the compound **3.10b** at different pH. The chemical shifts near the quinuclidine nitrogen atom upon changing the pH of the medium are shown here.

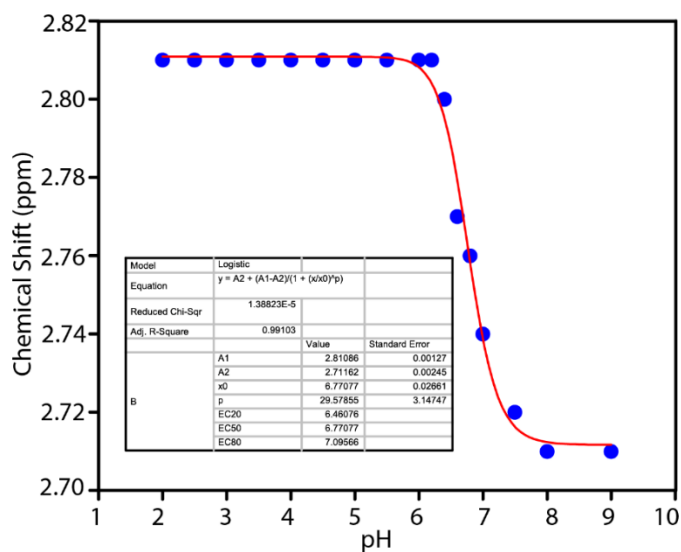


Figure 3.43. Fitting plot of the chemical shift Vs pH of the B-type proton peak near the quinuclidine nitrogen atom. The pKa came around 6.77.

3.4.17. Calculation and spectra of Job's plot

Table 3.6. Calculation and result table for Job's plot analysis of compound **3.9b**.

Sample No.	Host conc. ([H], mM)	Guest conc. ([G], mM)	[H] + [G] (mM)	[H]/([H]+[G])	δ of proton	$\Delta\delta$	$\{[H] / ([H] + [G])\}^* \Delta\delta$
JB-1- 3.9b	1	9	10	0.1	10.98	1.03	0.103
JB-2- 3.9b	2	8	10	0.2	10.92	0.97	0.194
JB-3- 3.9b	3	7	10	0.3	10.79	0.84	0.252
JB-4- 3.9b	4	6	10	0.4	10.69	0.74	0.296
JB-5- 3.9b	5	5	10	0.5	10.57	0.62	0.31
JB-6- 3.9b	6	4	10	0.6	10.45	0.5	0.3
JB-7- 3.9b	7	3	10	0.7	10.31	0.36	0.252
JB-8- 3.9b	8	2	10	0.8	10.18	0.23	0.184
JB-9- 3.9b	9	1	10	0.9	10.06	0.11	0.099
JB-10- 3.9b	10	0	10	1	9.95	0	0

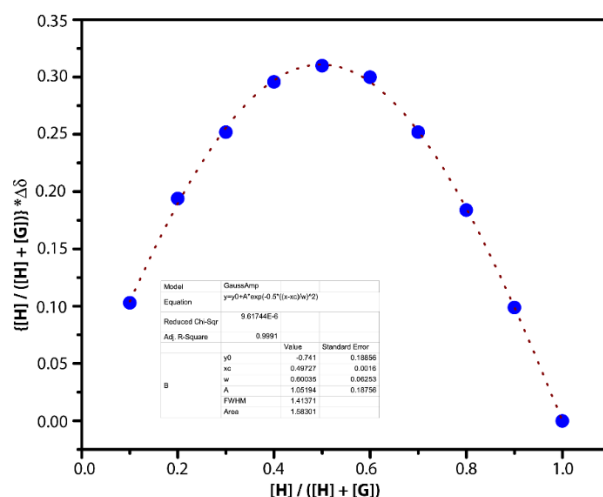


Figure 3.44. Job's plot of compound **3.9b** (according to Table 3.6).

Table 3.7. Calculation and result table for Job's plot analysis of compound **3.10b**.

Sample No.	Host conc. ([H], mM)	Guest conc. ([G], mM)	[H] + [G] (mM)	[H]/ ([H]+[G])	δ of proto n	$\Delta\delta$	$\{[H] /$ $([H] + [G])\} * \Delta\delta$
JB-1- 3.10b	1	9	10	0.1	10.25	0.18	0.018
JB-2- 3.10b	2	8	10	0.2	10.23	0.16	0.032
JB-3- 3.10b	3	7	10	0.3	10.22	0.15	0.045
JB-4- 3.10b	4	6	10	0.4	10.2	0.13	0.052
JB-5- 3.10b	5	5	10	0.5	10.18	0.11	0.055
JB-6- 3.10b	6	4	10	0.6	10.16	0.09	0.054
JB-7- 3.10b	7	3	10	0.7	10.14	0.07	0.049
JB-8- 3.10b	8	2	10	0.8	10.12	0.05	0.04
JB-9- 3.10b	9	1	10	0.9	10.1	0.03	0.027
JB-10- 3.10b	10	0	10	1	10.07	0	0

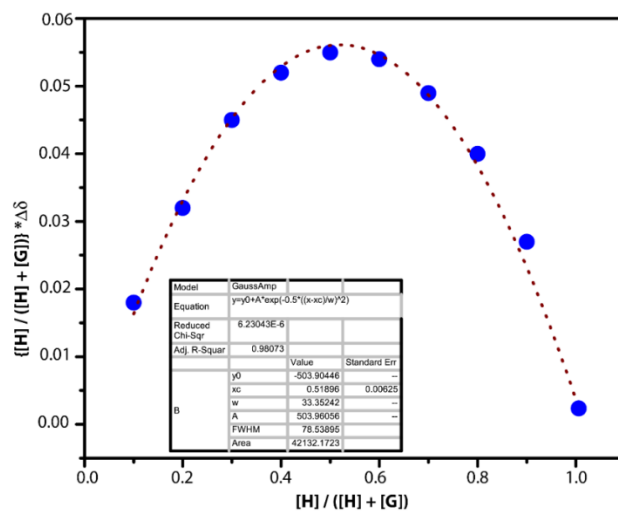


Figure 3.45 Job's plot of the compound **3.10b** (according to Table 3.7).

3.4.18. Plots and cartesian coordinates of potent compounds in DFT

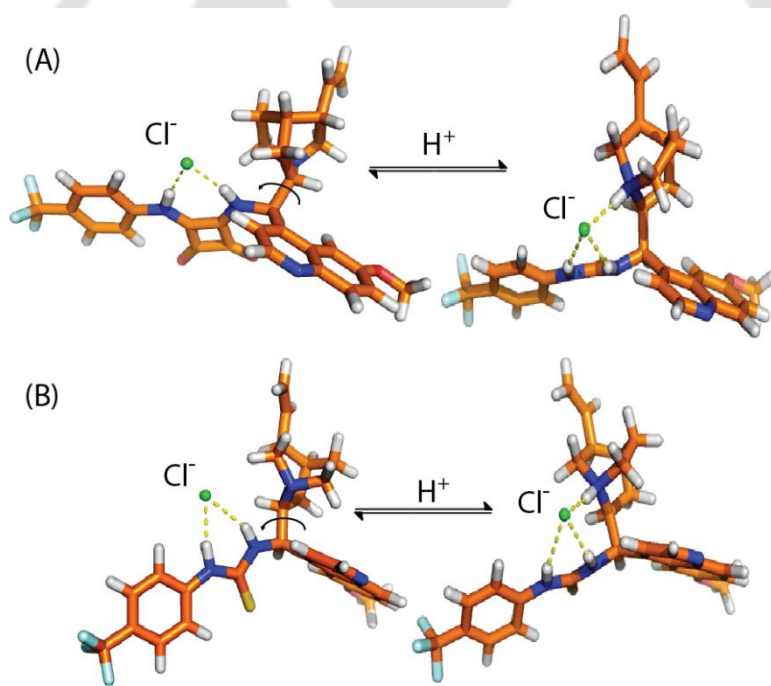


Figure 3.46. Probable mode of interaction of compound **3.9b** (A) and **3.10b** (B) with the Cl^- ion in the neutral and protonated form

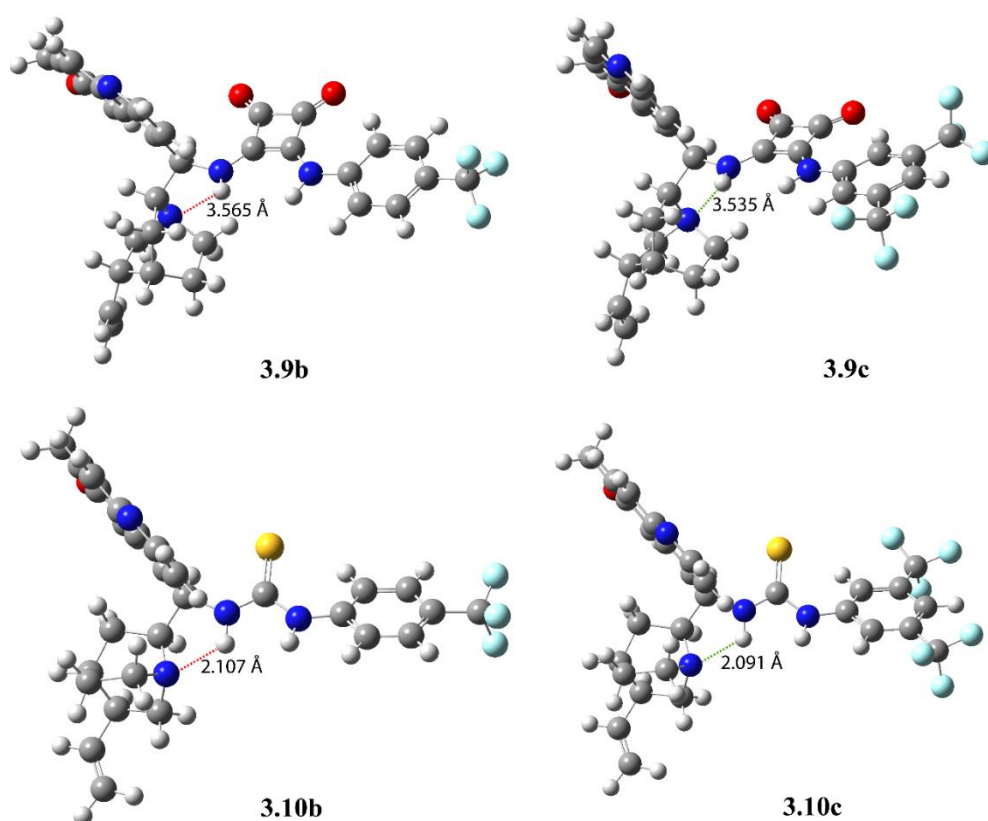


Figure 3.47. Probable mode of intramolecular hydrogen-bonding between the quinuclidine nitrogen and the opposite NH group of compounds. All the structures were analyzed by DFT analysis

Table 3.8. XYZ Cartesian coordinate for compound ‘**3.9b---Cl⁻ complex**’ using B3LYP/6-31+g* basic sets.

3.9b---Cl⁻ (3.9b in the neutral state)				3.9b-H⁺---Cl⁻ (3.9b in the protonated state)			
Total Energy (a.u.) = -2403.230712				Total Energy (a.u.) = -2403.734003			
Imaginary Frequency = 0				Imaginary Frequency = 0			
Atom	x	y	z	Atom	x	y	Z
N	0.409436	0.045655	0.007686	N	0.42753	-0.75233	0.330988
N	-2.75441	-0.19646	-0.02042	N	-2.75878	-0.56399	0.159503
C	1.860196	-0.11255	-0.07837	C	1.814002	-0.42175	-0.00576
C	-6.24991	-0.95578	-1.03631	C	-6.27604	-1.17237	-0.89366
O	0.710516	-1.84597	-2.6572	O	0.682581	-1.59416	-2.78977

C	-0.42557	-0.60845	-0.80546	C	-0.45375	-1.01359	-0.65944
O	-2.56659	-2.12013	-2.83198	O	-2.62093	-1.8103	-3.01651
C	-4.8587	-0.94169	-1.07297	C	-4.88535	-1.18156	-0.93472
C	-4.144	-0.27063	-0.06496	C	-4.16231	-0.5904	0.112395
C	-1.83057	-0.72231	-0.8639	C	-1.85422	-0.99094	-0.74978
C	-6.95171	-0.31342	-0.01283	C	-6.96109	-0.58412	0.173257
C	-6.23603	0.353044	0.992357	C	-6.23604	0.006977	1.212529
C	-4.85114	0.377126	0.971491	C	-4.84783	0.006911	1.184464
C	-0.24695	-1.44525	-2.01646	C	-0.28219	-1.44209	-2.0565
C	-1.77108	-1.56933	-2.08582	C	-1.81503	-1.49821	-2.1657
H	-0.00887	0.537176	0.816734	H	0.036941	-0.31779	1.172066
H	-2.36465	0.328742	0.784972	H	-2.36248	-0.06448	0.961906
C	2.392114	-1.07616	0.986226	C	2.765558	-0.79895	1.125206
C	2.634633	1.235483		C	1.889774	1.037455	-0.56081
H	-4.29172	0.891948	1.748198	H	-4.28521	0.47485	1.988156
C	-8.44424	-0.31787	0.037511	C	-8.45879	-0.63842	0.23449
H	-4.3259	-1.44934	-1.87197	H	-4.36871	-1.62353	-1.78031
C	3.611944	-1.80567	0.775086	C	4.04558	-1.38896	0.847489
C	4.071702	-2.6517	1.839623	C	4.87218	-1.71998	1.977174
N	3.410998	-2.79595	3.027374	N	4.498849	-1.51055	3.271621
C	2.292811	-2.11635	3.170436	C	3.308581	-0.9911	3.481168
C	1.744089	-1.2524	2.189957	C	2.410205	-0.6254	2.448627
C	5.271002	-3.3831	1.661379	C	6.142271	-2.30644	1.749683
H	1.769166	-2.23742	4.118676	H	3.015708	-0.84058	4.519223
H	0.814795	-0.73331	2.410096	H	1.447534	-0.20449	2.72931
N	2.475678	2.066707	-1.21649	N	1.369157	2.122755	0.377204
C	1.226512	2.861135	-1.18696	C	2.428234	2.646642	1.320067
C	1.213033	3.873503	-0.00165	C	3.479552	3.432132	0.506965
C	2.43959	3.57281	0.880234	C	3.298808	3.071647	-0.97937
C	3.741789	3.8231	0.064248	C	1.95065	3.673379	-1.47937
H	2.430505	4.205889	1.776408	H	4.126979	3.471454	-1.57287
C	3.605096	3.011878	-1.2645	C	0.85738	3.267499	-0.4612

H	3.423782	3.678667	-2.11405	H	0.613817	4.06582	0.241731
H	4.525846	2.455889	-1.47856	H	-0.07033	2.939112	-0.93418
C	2.38612	2.08525	1.28658	C	3.24717	1.532196	-1.10431
H	3.689116	0.935802	-0.007	H	1.164766	1.036302	-1.37963
H	1.412117	1.880535	1.746009	H	4.07995	1.088259	-0.54937
H	3.149456	1.858746	2.041376	H	3.346738	1.211198	-2.14634
C	4.058711	5.291819	-0.07833	C	2.046693	5.156506	-1.74982
H	4.56704	3.39297	0.65643	H	1.725234	3.193389	-2.44325
C	4.379352	5.971894	-1.18138	C	1.29303	6.140749	-1.25771
H	4.440221	5.510849	-2.16349	H	0.471011	5.981336	-0.56521
H	4.601236	7.035459	-1.13927	H	1.466428	7.171487	-1.55264
H	4.032737	5.84072	0.866748	H	2.834664	5.415418	-2.45845
H	1.150348	3.377069	-2.15154	H	1.901932	3.253883	2.05984
H	0.384889	2.172291	-1.12262	H	2.851495	1.784637	1.832029
H	1.252762	4.908742	-0.36308	H	3.360791	4.510903	0.650026
H	0.298583	3.76113	0.590933	H	4.484157	3.164928	0.849463
C	4.373793	-1.74266	-0.41703	C	4.532784	-1.67756	-0.45063
C	5.54261	-2.47053	-0.55722	C	5.777743	-2.25701	-0.63747
C	6.000729	-3.30097	0.495578	C	6.595864	-2.57218	0.478518
O	6.199858	-2.32833	-1.75181	O	6.138825	-2.48678	-1.9298
C	7.370281	-3.09013	-1.97476	C	7.37328	-3.13939	-2.19502
H	5.594701	-4.01552	2.482567	H	6.742537	-2.54442	2.62196
H	4.055536	-1.13615	-1.25725	H	3.947614	-1.47647	-1.34072
H	6.914638	-3.8752	0.391347	H	7.570154	-3.02591	0.338814
H	7.702808	-2.83857	-2.98429	H	7.42616	-3.23952	-3.28024
H	7.172876	-4.16972	-1.9206	H	7.40922	-4.13626	-1.73788
H	8.16758	-2.83699	-1.26156	H	8.228404	-2.54673	-1.84504
H	2.056842	-0.5313	-1.06809	H	2.05364	-1.02803	-0.88284
Cl	-1.28269	1.339161	2.362199	H	0.549384	1.787453	0.976601
F	-9.0042	-0.99468	-0.99355	Cl	-0.93173	1.398424	2.23893
F	-8.96569	0.938907	0.00863	F	-8.97779	0.42606	0.891086
F	-8.92186	-0.882	1.180137	F	-8.90333	-1.74397	0.882782

H	-6.79511	-1.47527	-1.81753	F	-9.01652	-0.66425	-0.998
H	-6.76941	0.855538	1.794663	H	-6.83353	-1.62257	-1.70858
				H	-6.75803	0.475304	2.040497

Table 3.9. XYZ Cartesian coordinate for compound '**3.10b**---Cl⁻ complex' using B3LYP/6-31+g* basic sets.

3.10b---Cl⁻ (3.10b in the neutral state)				3.10b-H⁺---Cl⁻ (3.10b in the protonated state)			
Total Energy (a.u.) = -2403.23071174 a.u.				Total Energy (a.u.) = -2403.73400307 a.u.			
Imaginary Frequency = 0				Imaginary Frequency = 0			
Atom	x	y	z	Atom	x	y	z
N	-0.1154	0.391923	0.182921	N	0.00911	-0.52779	0.66876
N	-2.36592	0.386225	0.06282	N	2.24848	-0.45343	0.62071
C	1.246302	0.001997	-0.1643	C	1.33771	-0.37471	0.09517
C	-5.63912	-1.40389	-0.16613	C	5.47819	-1.74139	-0.74211
C	-1.21804	-0.34599	-0.11686	C	1.11400	-0.75435	-0.08525
C	-4.25927	-1.22827	-0.1202	C	4.10548	-1.62656	-0.54005
C	-3.71942	0.067582	-0.01712	C	3.61032	-0.62887	0.31201
C	-6.51151	-0.31418	-0.10498	C	6.37300	-0.88557	-0.09662
C	-5.97748	0.976796	0.004909	C	5.88069	0.09804	0.76679

C	-4.60598	1.167309	0.050114	C	4.51321	0.22653	0.96871
H	-0.26766	1.383275	0.423029	H	0.15232		
H	-2.18971	1.40069	0.213462	H	7	-0.05001	1.55899
C	1.864049	-0.89179	0.9093	H	2.08725	0.13922	1.43980
C	2.01747	1.321488	-0.48912	H	3	5	7
H	-4.18977	2.167672	0.140445	C	2.42158		1.08290
C	-7.98718	-0.50977	-0.22125	C	1	-0.7997	9
H	-3.59458	-2.07886	-0.16956	C	1.46075	1.03442	-0.56342
C	2.774568	-1.95903	0.605009	H		0.99314	1.63876
C	3.332403	-2.69003	1.709337	H	4.13207	7	1
N	3.032036	-2.43114	3.016616	C	7.84681		
C	2.166367	-1.46432	3.244485	C	9	-0.97895	-0.36223
C	1.560769	-0.67861	2.237558	H	3.42413		
C	4.240002	-3.74469	1.443151	H	1	-2.30204	-1.03749
H	1.921863	-1.27065	4.289348	C	3.57391		0.64081
H	0.862348	0.104453	2.510224	C	4	-1.53631	2
N	2.364973	2.146767	0.690192	C	4.54023	-1.90592	1.63969
				N	4.41134		2.96285
				C	3	-1.60228	3
				C	3.33252		
				C	8	-0.94435	3.32775
				C	2.31655	-0.52635	2.43306
				C	5.69029		1.24582
				H	3	-2.63451	4
				H			4.38793
				H	3.23381	-0.71542	8
				H	1.46728		2.84055
				H	6	0.01692	9
				C	2.46836	2.67322	1.08555
				C	4	1	5

C	3.704791	1.819213	1.222526	C	3.43783	3.29357	0.05641
C	4.832359	2.188937	0.212616	C	7	4	2
C	4.150637	2.421705	-1.147	C	2.96881	2.87946	-1.35058
C	3.27078	3.706431	-1.04113	C	5	7	-1.35058
H	4.8971	2.53904	-1.94332	C	1.62067	3.59861	-1.65887
C	2.385529	3.554899	0.2403	H	9	1	-1.65887
H	2.790425	4.148598	1.067057	C	3.71781	3.15644	-2.09935
H	1.354585	3.880101	0.069121	C	0.68279	3.36278	-0.44958
C	3.246008	1.198286	-1.42911	C	9	5	-0.44958
H	1.265358	1.907025	-1.02748	C	2.74433	1.35101	-1.35942
H	3.816577	0.279102	-1.24836	H	6	1	-0.92735
H	2.917527	1.172994	-2.47637	H	3.61547	0.84768	-0.92735
C	4.098761	4.964206	-1.13639	H	2.62728	0.97158	-2.38013
H	2.609828	3.712843	-1.92351	C	8	6	-2.38013
C	4.073194	6.0329	-0.337	C	1.81658	5.04747	-2.03879
H	3.414086	6.108997	0.523126	H	9	2	-2.03879
				H	1.18605	3.10117	-2.5386
				C	4	3	-2.5386
				C	1.25106	6.13071	-1.50432
				H	4	1	-1.50432
				H	0.53974	6.09539	-0.68377
				H	2	5	-0.68377
				H	1.47422	7.12057	-1.89157
				H	4	7	-1.89157
				H	2.50158	5.18471	-2.87684
				H	9	5	-2.87684
				H	3.45469	4.38492	0.14111
				H	8	2	8
				H		2.93422	0.24459
				H	4.45446	5	9

H	4.711241	6.892145	-0.52996	C	3.80991		
H	4.781325	4.983966	-1.99079	C	5	-1.92854	-0.69931
H	3.82621	2.361346	2.167629	C	4.94341		
H	3.734715	0.755788	1.461784	C	4	-2.64371	-1.0504
H	5.369238	3.093593	0.523638	C	5.89932		
H	5.571391	1.38068	0.139496	C	3	-2.99924	-0.06414
C	3.138399	-2.34476	-0.70852	O	5.06149		
C	4.022461	-3.38429	-0.93278	C	8	-2.96243	-2.36994
C	4.58849	-4.092	0.156598	C	6.16729		
O	4.298572	-3.66703	-2.24747	C	5	-3.74936	-2.79081
C	5.139345	-4.76571	-2.53831	H	6.40041		2.02277
H	4.648179	-4.2759	2.29773	H	8	-2.89896	8
H	2.712834	-1.8521	-1.57385	H	3.10839		
H	5.28305	-4.90761	-0.01175	H	9	-1.69869	-1.49274
H	5.190039	-4.82386	-3.62803	H	6.78734		
H	4.731952	-5.70841	-2.14727	H	5	-3.55999	-0.33163
				H	6.03221		
				H	2	-3.89815	-3.86337
				H	6.18248		
				H	3	-4.72555	-2.28976
				H	7.12143		
				H	9	-3.23554	-2.61547
				H	1.35520		
				H	9	-1.05204	-0.76265
				S	1.04511		
				S	4	-1.29473	-1.68493
				Cl	0.76527	1.83904	2.63865
				Cl	7	6	4
				N	1.21857	2.21674	0.37017
				N	9	4	5
				H	0.60653		
				H	9	1.05916	-1.24614

H	6.154536	-4.62441	-2.14058	H	0.33757	3.10783	
H	1.182323	-0.5712	-1.09487	H	6	5	-0.74264
S	-1.10839	-1.94793	-0.6661	H	0.63704	4.21862	0.22620
Cl	-1.4067	3.374792	0.366717	H	7	1	7
H	-6.0411	-2.4098	-0.24155	H	2.13885	3.37404	1.85581
H	-6.64075	1.834655	0.06219	H	3	3	2
F	-8.38469	-1.73638	0.20293	H	2.88659	1.80000	1.58261
F	-8.43632	-0.39357	-1.50215	H	5	6	5
F	-8.68805	0.403787	0.499216	H	0.48528	2.00306	1.12185
				H	3	7	1
				H	5.85517		
				H	6	-2.51813	-1.39908
				H	6.56702	0.76057	1.28419
				F	8.21420		
				F	9	-2.21549	-0.76893
				F	8.24483	-0.11695	-1.33106
				F	8.57723		0.73823
				F	5	-0.68101	5

3.4.19. EC₅₀ plot

3.4.19.1 Ion selective electrode (ISE) based EC₅₀ plots

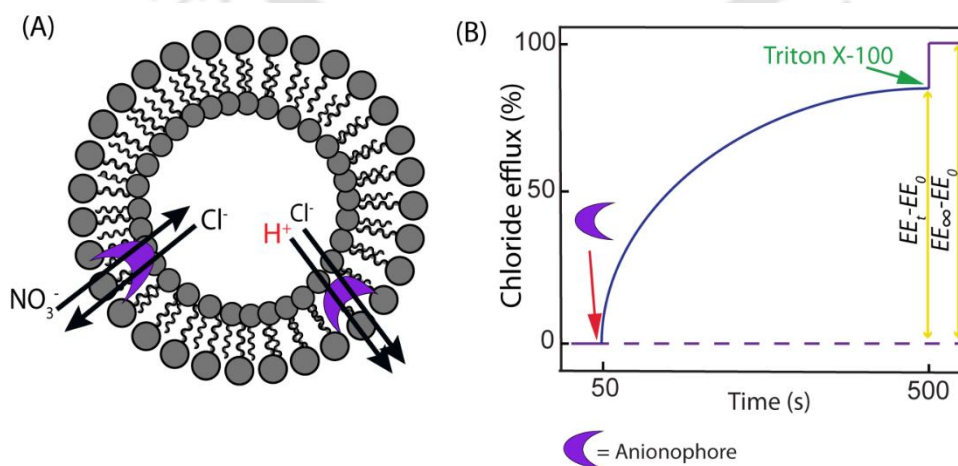


Figure 3.48. Representations of chloride efflux study across EYPC/CHOL-LUV (A). Demonstration of ion transport kinetics displaying normalization plot (B).

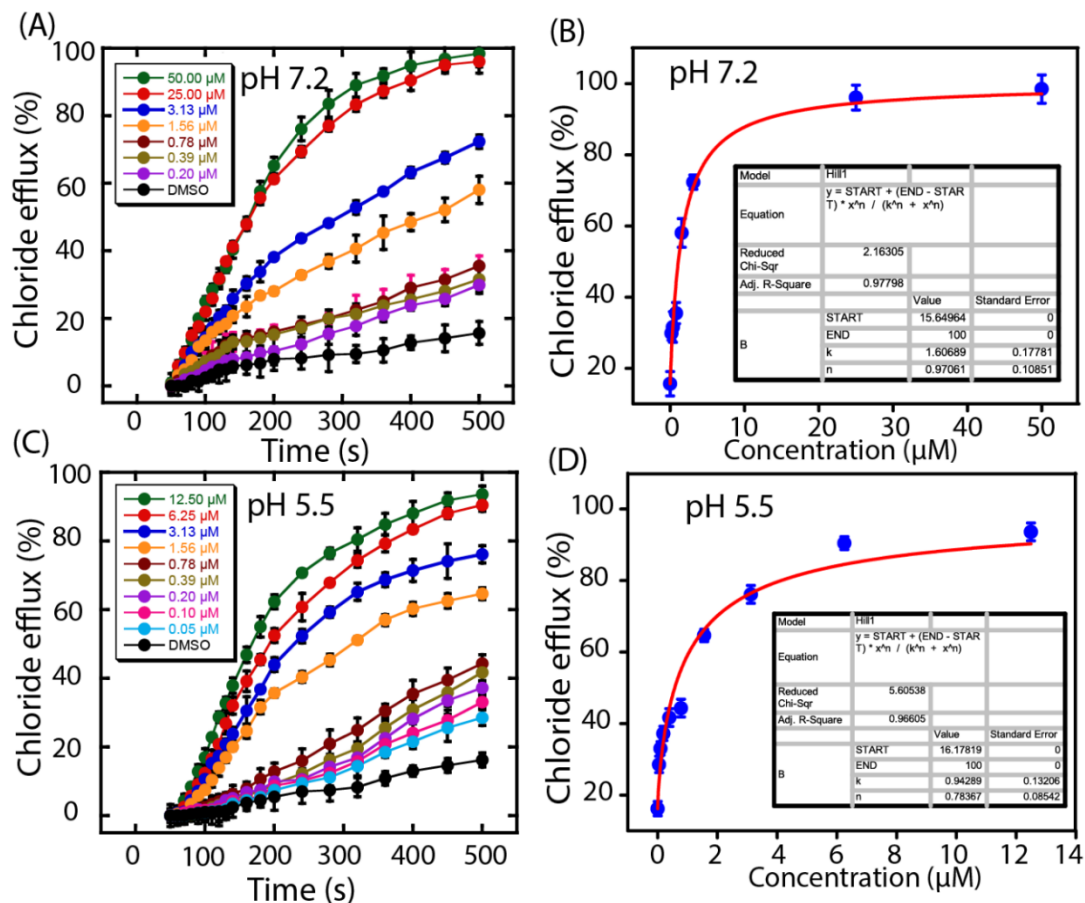


Figure 3.49. Concentration-dependent Cl^- ion transport activity of compound **3.9a** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentration of the compounds were varied from 0-50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0-12.5 μM (4 mol% with respect to the lipid).

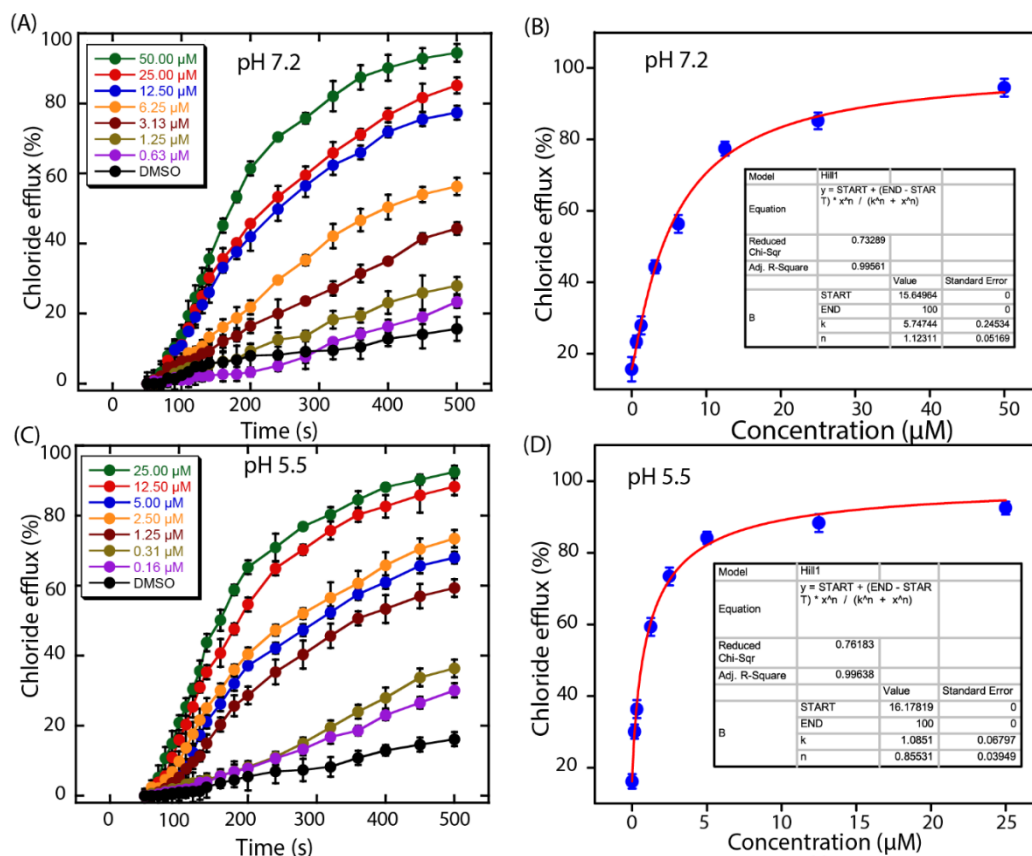


Figure 3.50. Concentration-dependent Cl[−] ion transport activity of compound **3.9b** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentration of the compounds was varied from 0–50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0–25 μM (8 mol% with respect to the lipid).

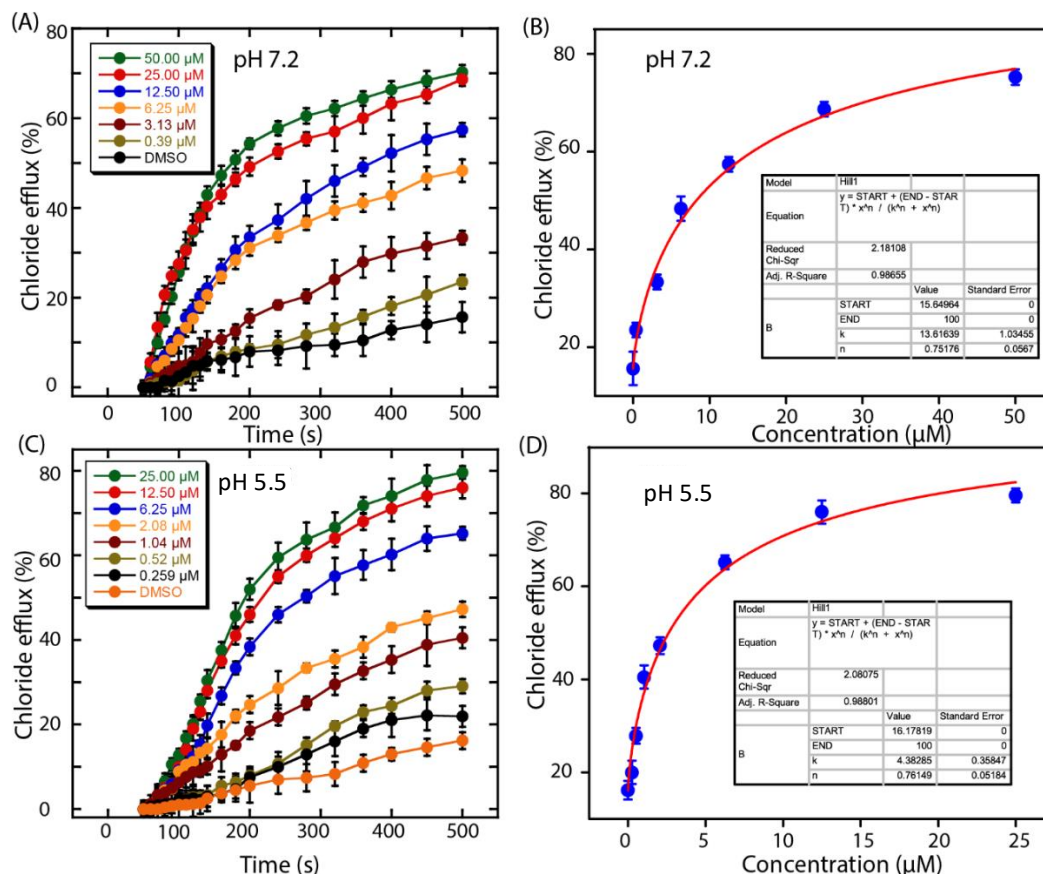


Figure 3.51. Concentration-dependent Cl^- ion transport activity of compound **3.9c** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentration of the compounds was varied from 0-50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0-25 μM (8 mol% with respect to the lipid).

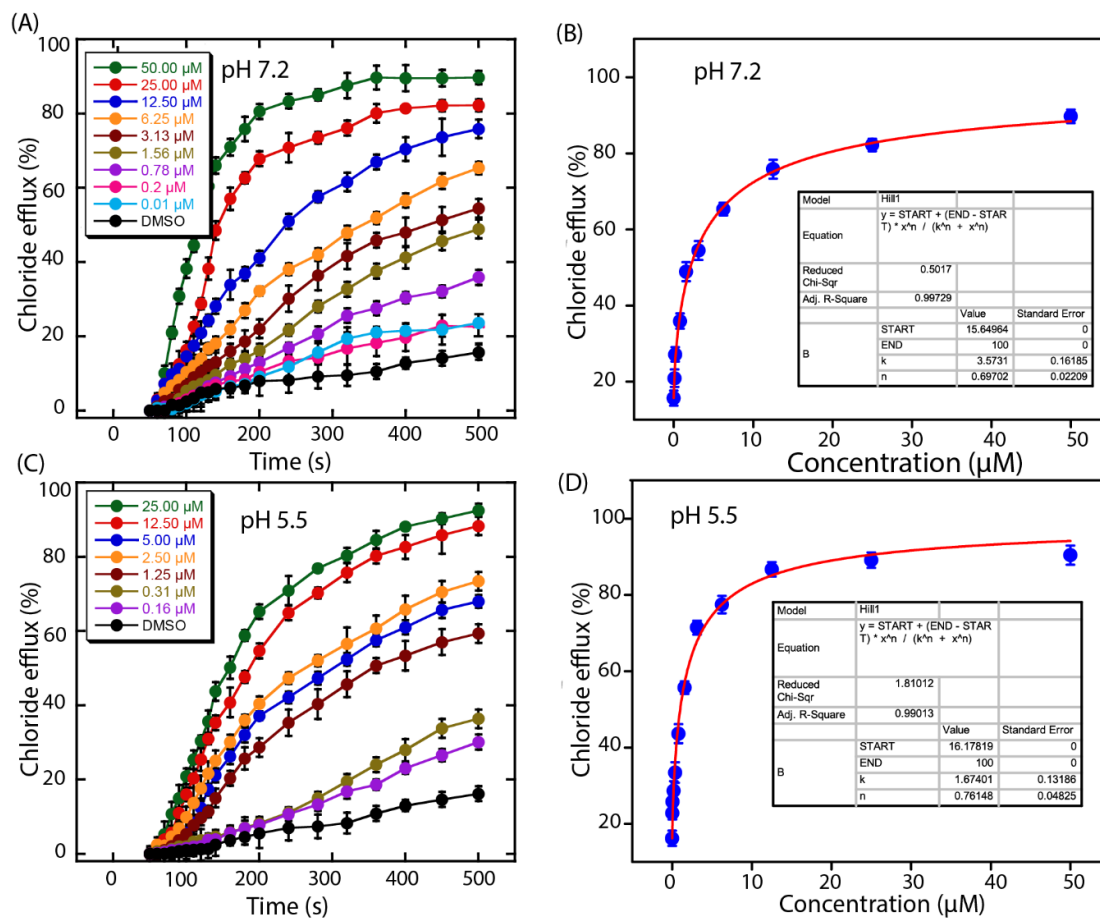


Figure 3.52. Concentration-dependent Cl^- ion transport activity of compound **3.9d** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentration of the compounds was varied from 0-50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0-50 μM (16 mol% with respect to the lipid).

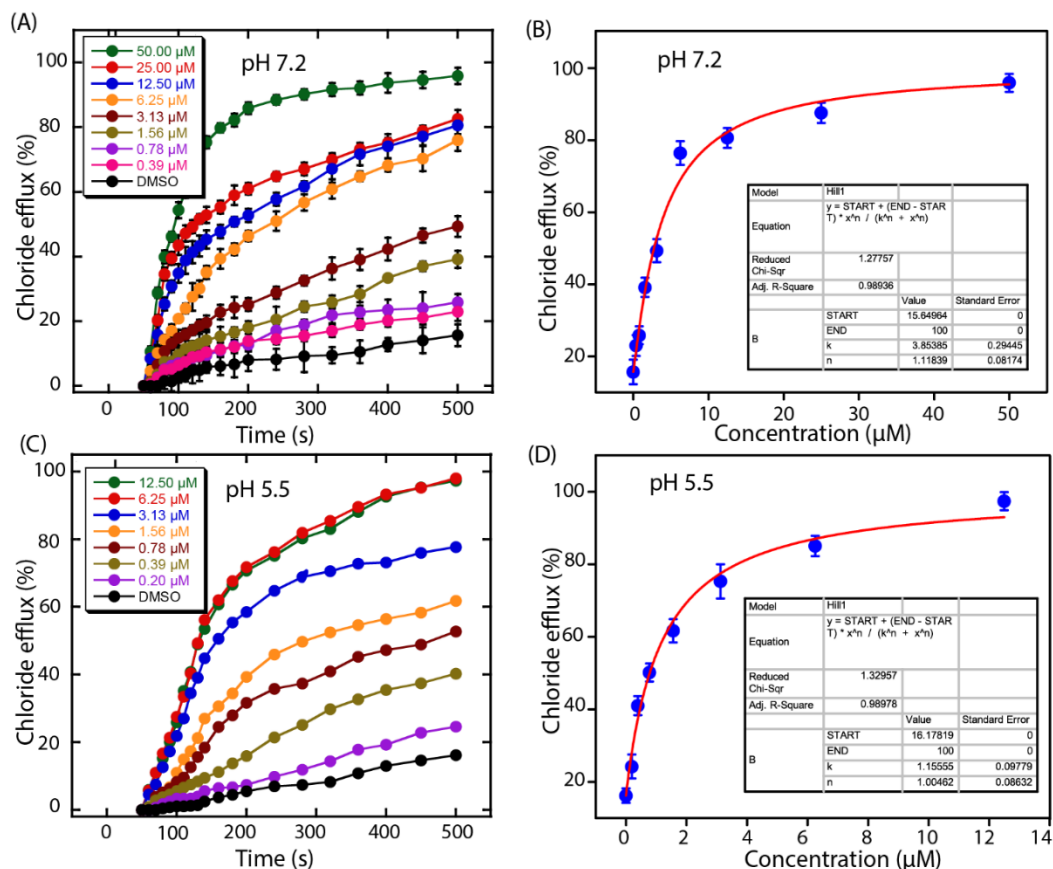


Figure 3.53. Concentration-dependent Cl^- ion transport activity of compound **3.10a** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentrations of the compound were varied from 0-50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0-12.5 μM (4 mol% with respect to the lipid).

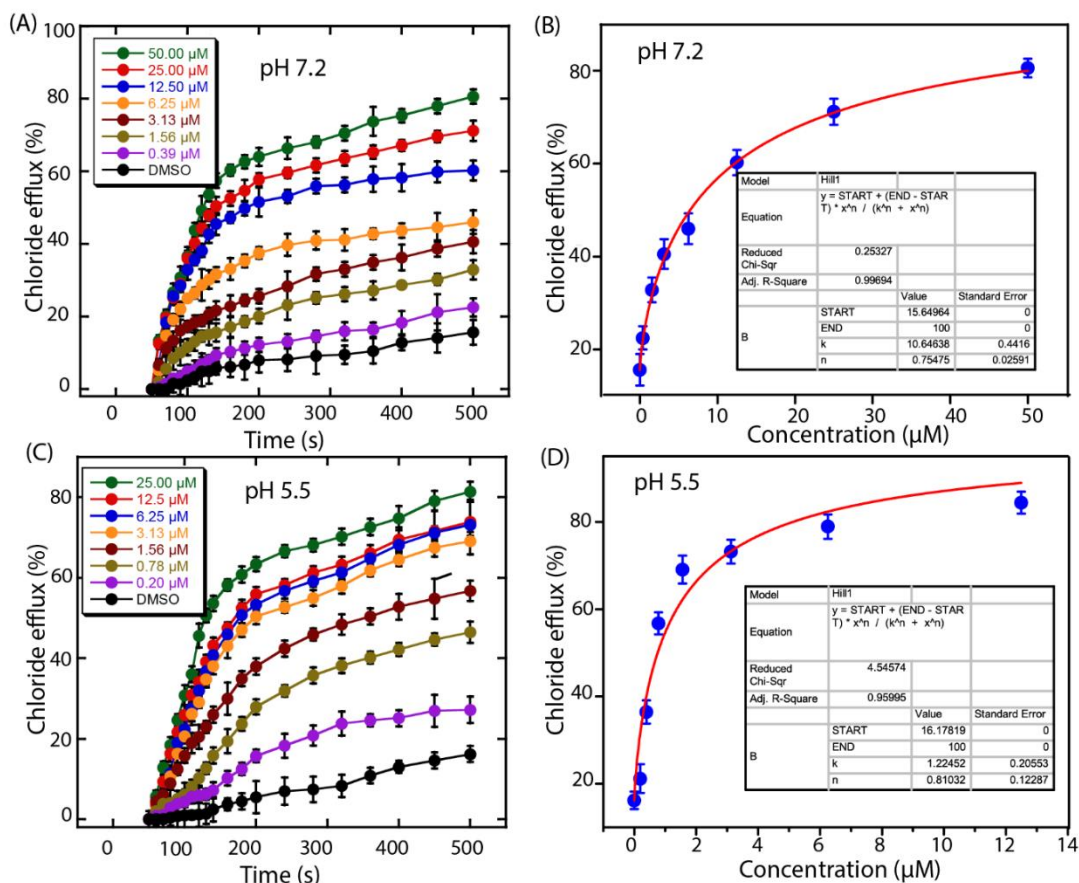


Figure 3.54. Concentration-dependent Cl^- ion transport activity of compound **3.10b** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentrations of the compound were varied from 0-50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0-12.5 μM (4 mol% with respect to the lipid).

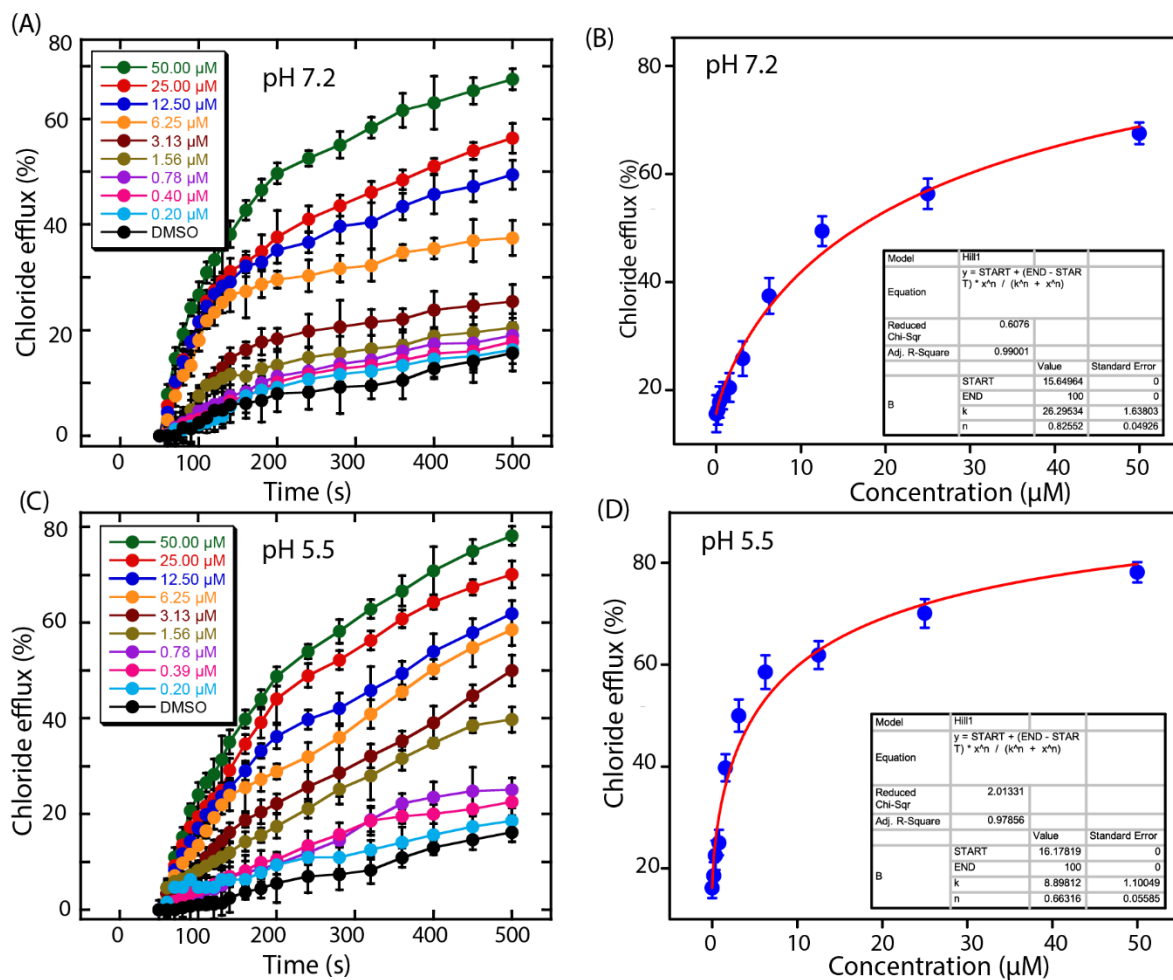


Figure 3.55. Concentration-dependent Cl^- ion transport activity of compound **3.10c** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentrations of the compound were varied from 0-50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0-50 μM (16 mol% with respect to the lipid).

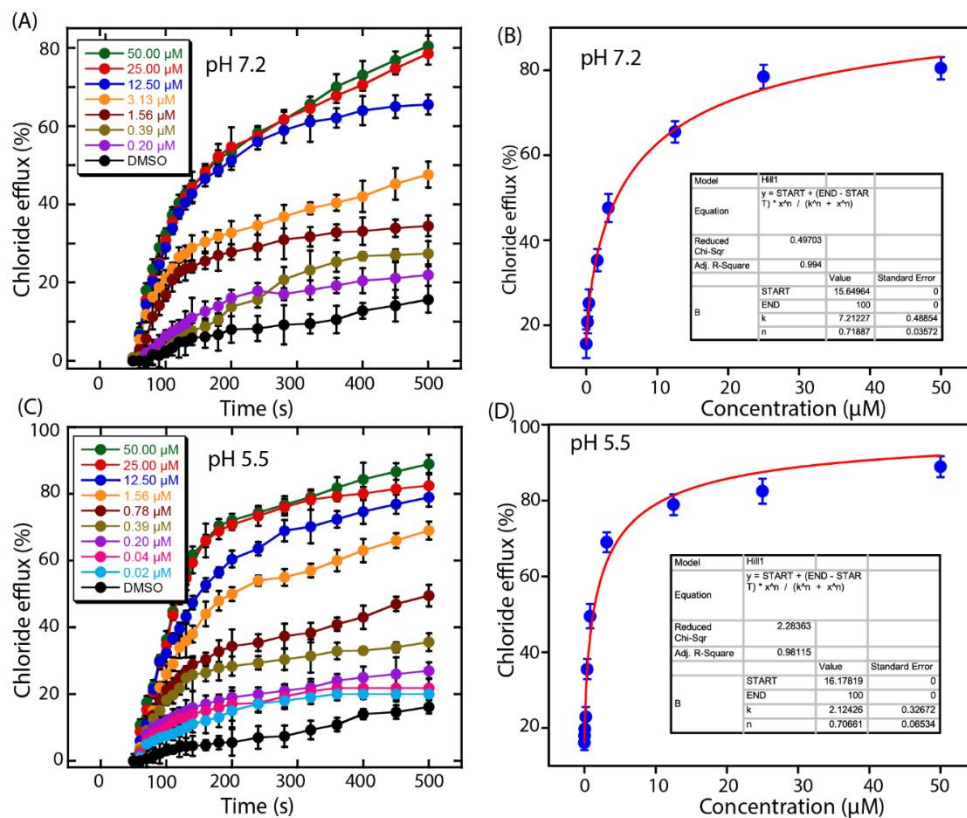


Figure 3.56. Concentration-dependent Cl^- ion transport activity of compound **3.10d** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentration of the compounds were varied from 0-50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0-50 μM (16 mol% with respect to the lipid).

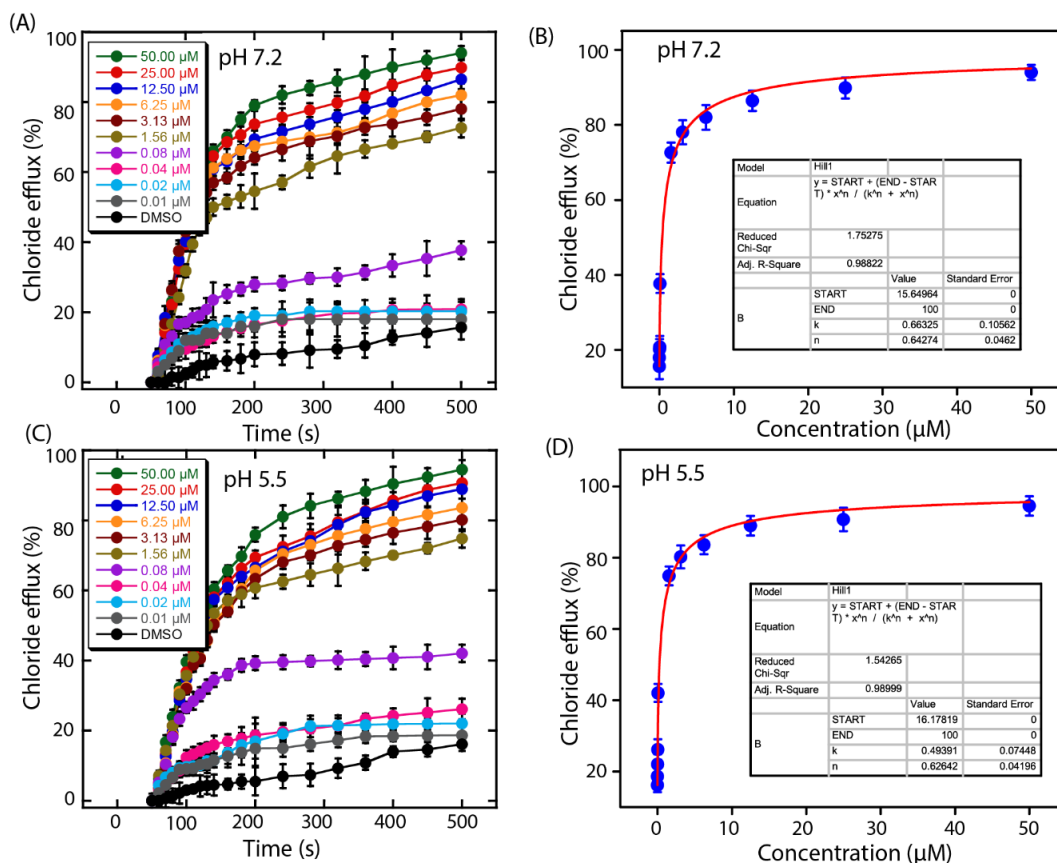


Figure 3.57. Concentration-dependent Cl^- ion transport activity of compound **3.10ba1** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentrations of the compound were varied from 0-50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0-50 μM (16 mol% with respect to the lipid).

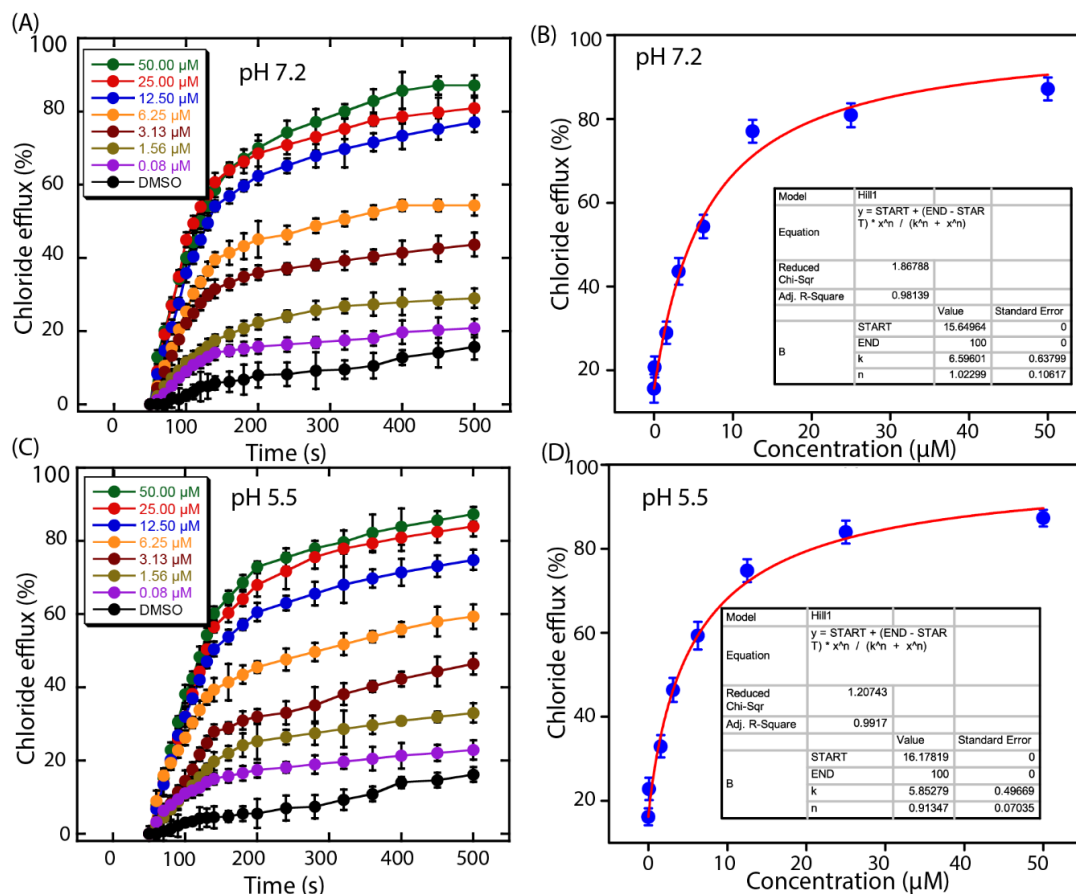


Figure 3.58. Concentration-dependent Cl^- ion transport activity of compound **3.10ba2** was measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2 (A and B) and pH 5.5 (C and D). The concentrations of the compound were varied from 0-50 μM (16 mol% with respect to the lipid) at pH 7.2 and 0-50 μM (16 mol% with respect to the lipid).

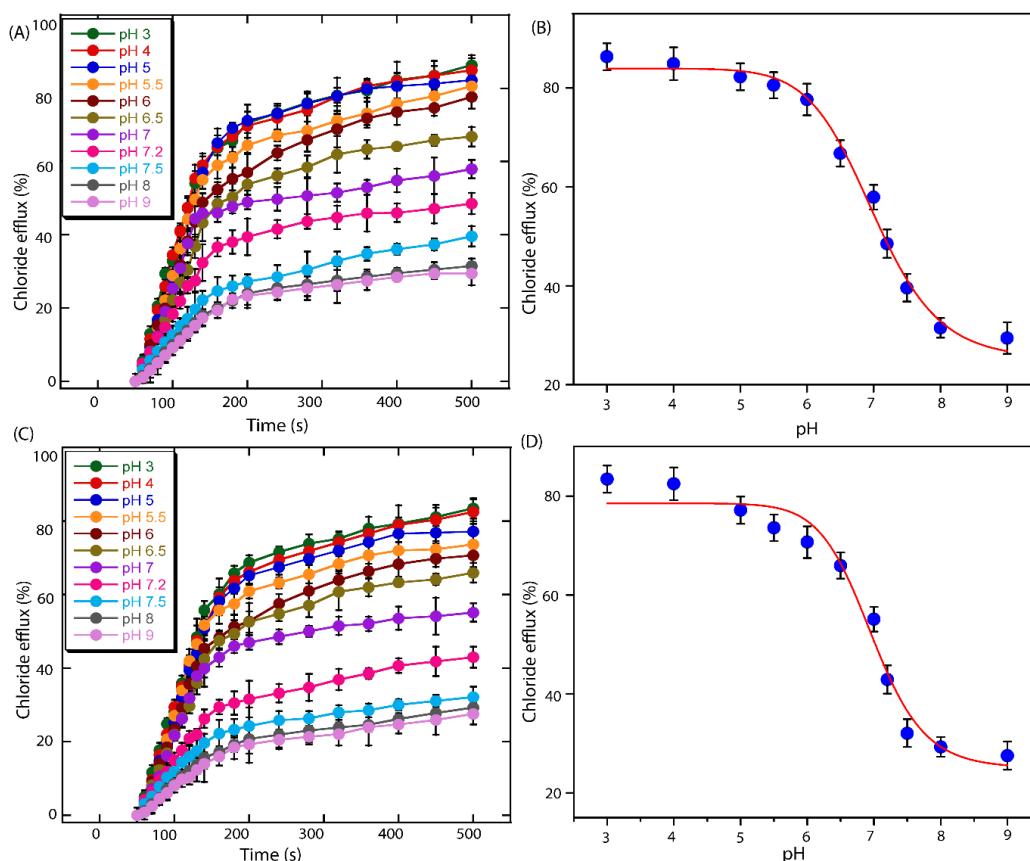


Figure 3.59. pH-dependent Cl^- ion transportability of the compound **3.9b** (A and B) and **3.10b** (C and D) within the membrane environment. The concentration of **3.9b** and **3.10b** were $0.56 \mu\text{M} = 0.18 \text{ mol}\%$ and $2.29 \mu\text{M} = 0.73 \text{ mol}\%$ with respect to the lipid.

3.4.19.2. Fluoresce based ion transport plots

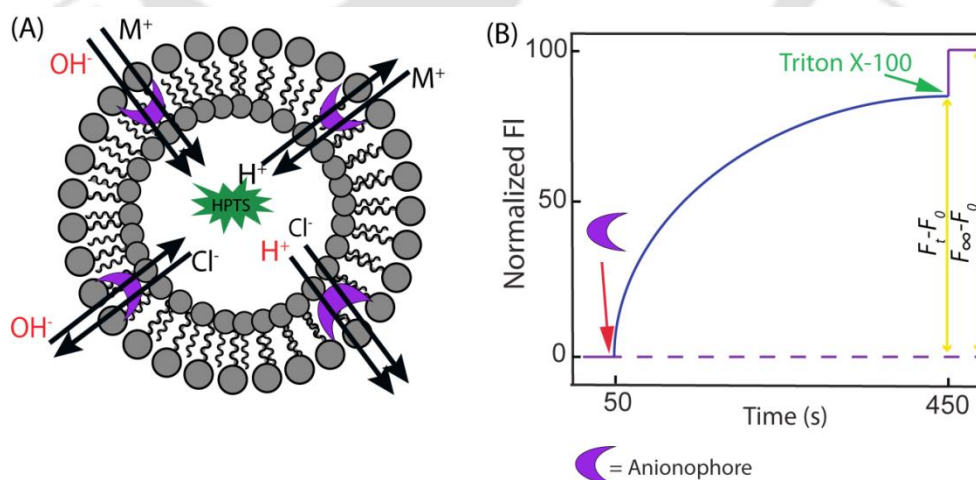


Figure 3.60. Representations of chloride ion transport activity across EYPC/CHOL-LUV $\rightarrow$ HPTS (A). Demonstration of ion transport kinetics displaying normalization plot (B).

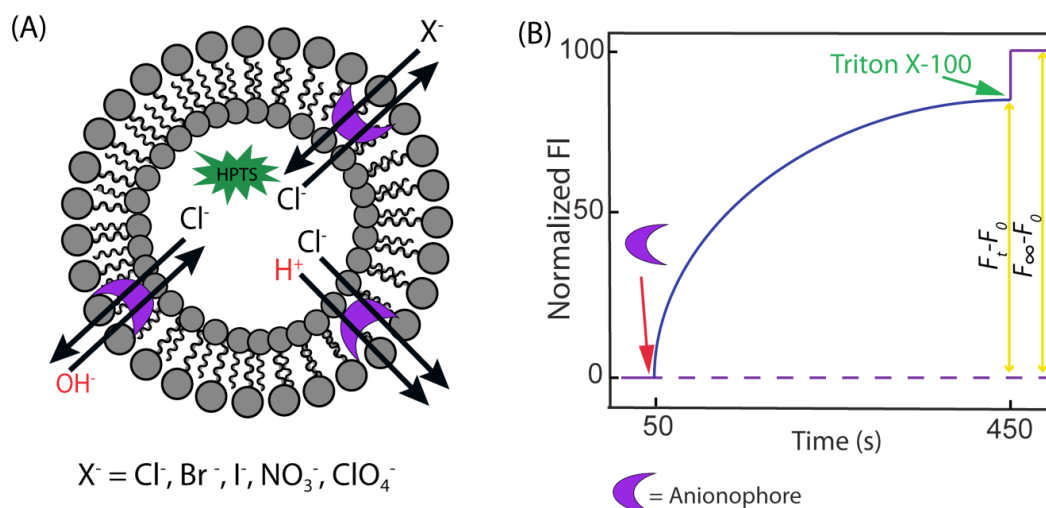


Figure 3.61. Representations of anion selectivity studies across EYPC/CHOL-LUV-HPTS (A). Demonstration of ion transport kinetics displaying normalization plot (B).

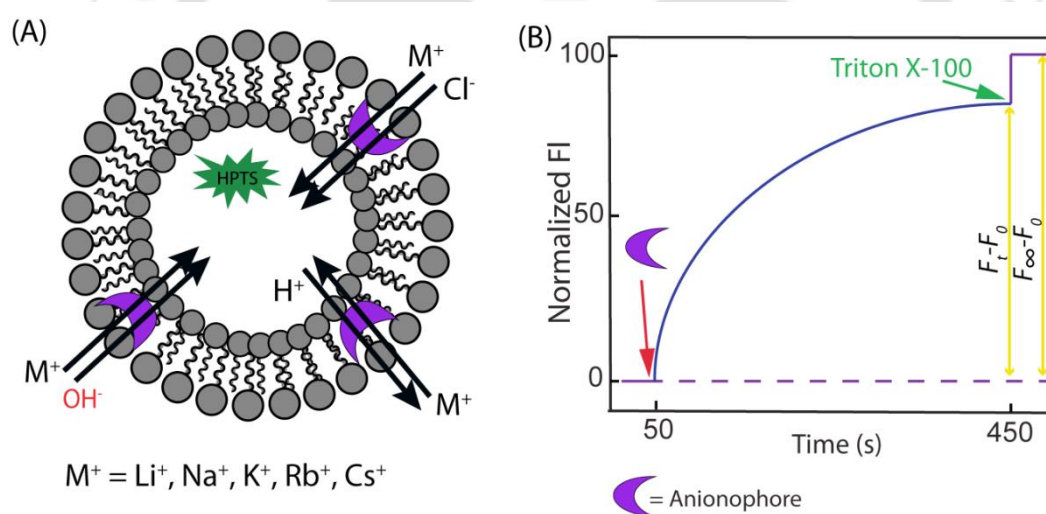


Figure 3.62. Representations of cation selectivity studies across EYPC/CHOL-LUV-HPTS (A). Demonstration of ion transport kinetics displaying normalization plot (B).

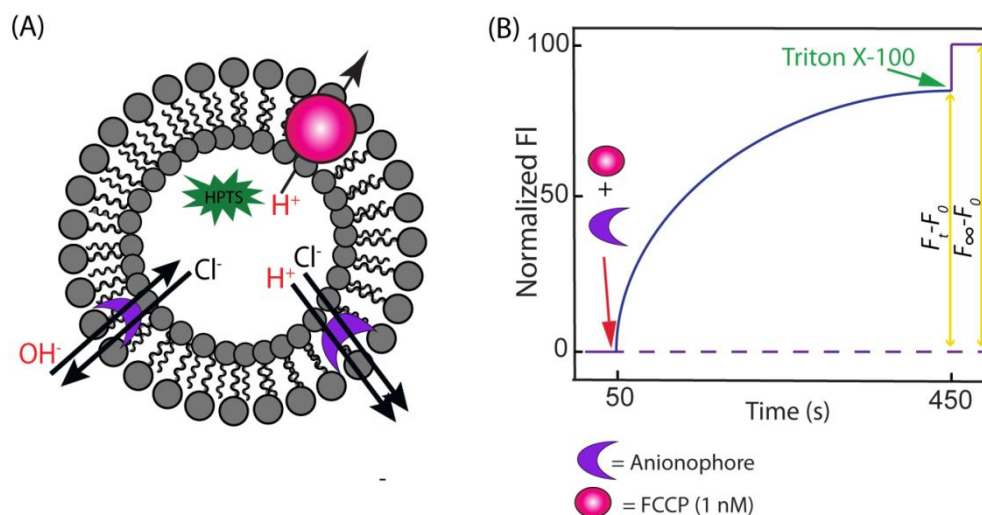


Figure 3.63. Representations of preferential ion transport activity in the presence of FCCP across EYPC/CHOL-LUV-HPTS (A). Demonstration of ion transport kinetics displaying normalization plot (B).

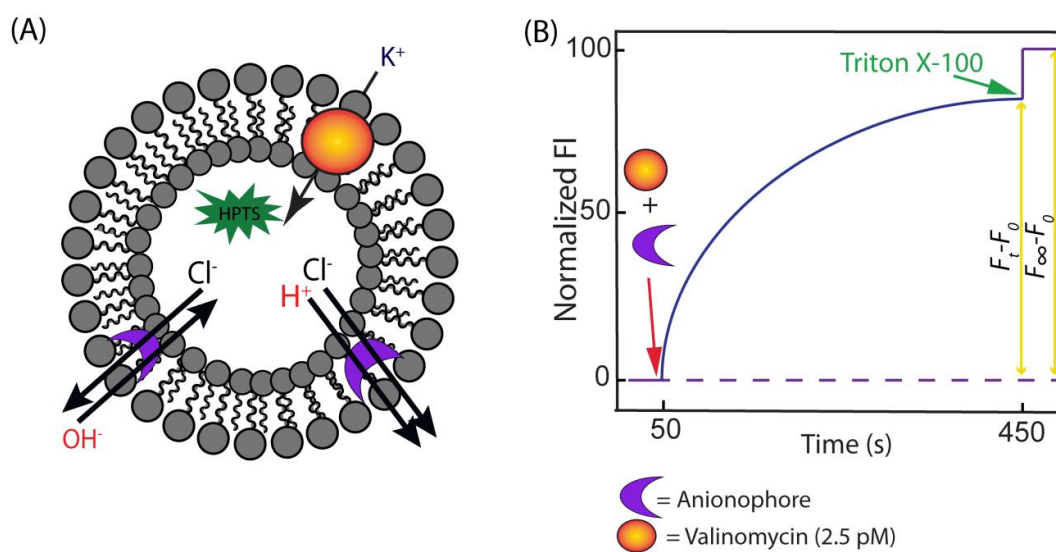


Figure 3.64. Representations of preferential ion transport activity in the presence of valinomycin across EYPC/CHOL-LUV-HPTS (A). Demonstration of ion transport kinetics displaying normalization plot (B).

3.4.20. U tube plot using bromothymol blue



Figure 3.65. Three representative color changes during the U-tube experiment in the presence of the bromothymol blue indicator.

3.4.21. Spectrum of stability test under different pH

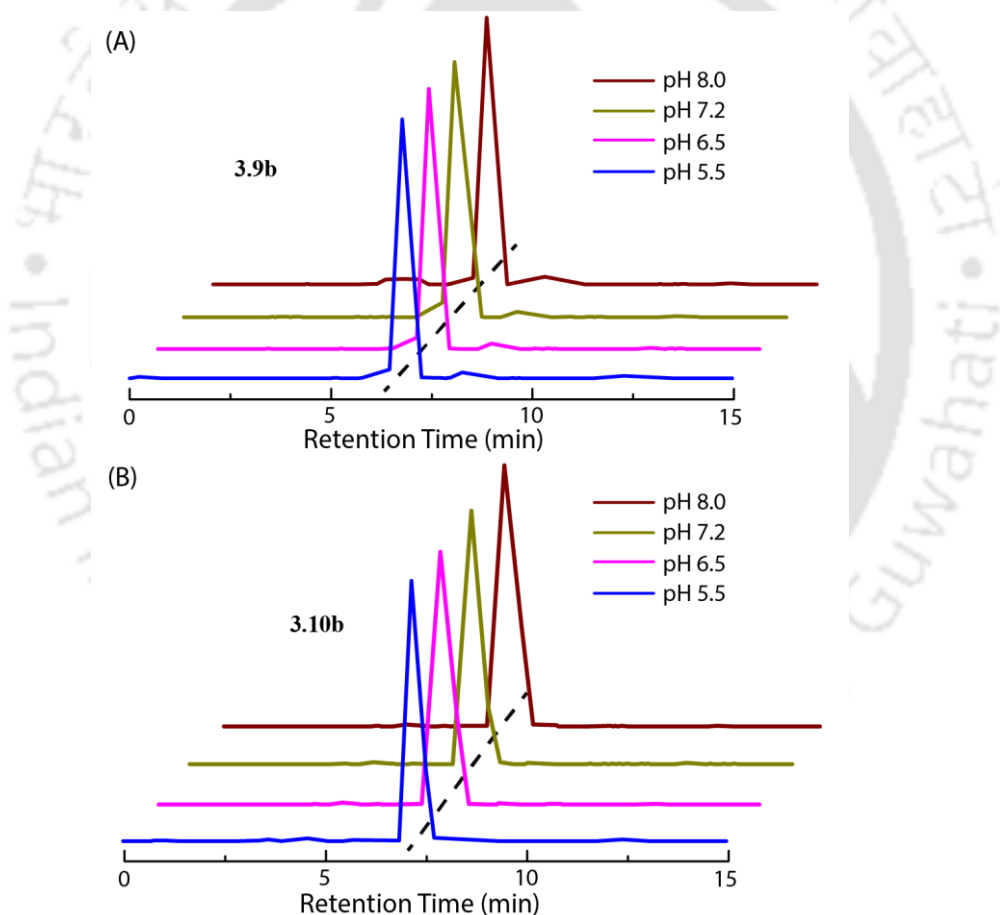


Figure 3.66. HPLC analysis of the compounds **3.9b** (A) and **3.10b** (B) incubated in 10 mM phosphate buffer at different pH (5.5, 6.5, 7.2, and 8.0). The HPLC analyses were performed by using a UV-Vis detector at 254 nm. Compound concentrations were 1 mM.

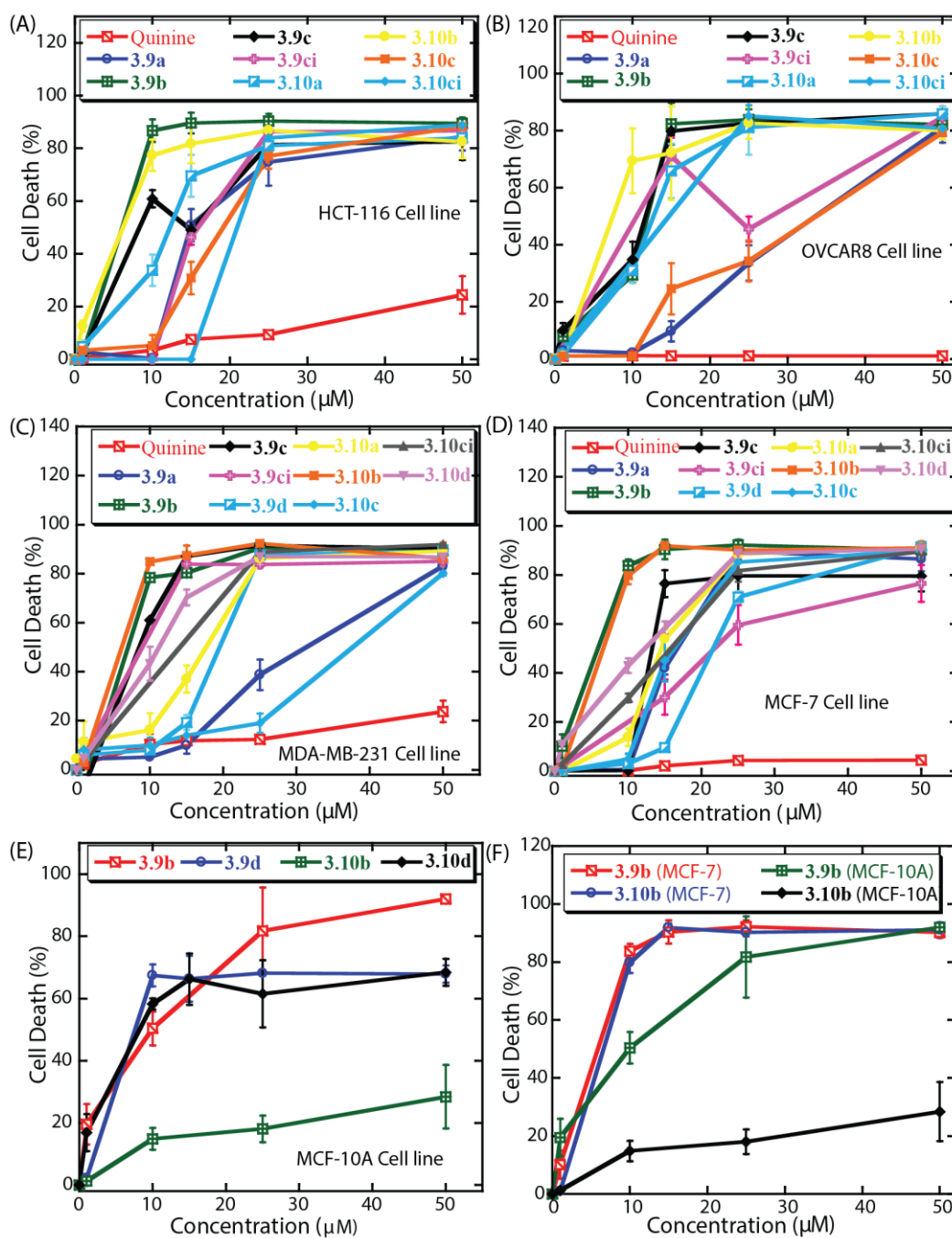
3.4.22. *In vitro* data and plot

Figure 3.67. Cell viability of the compounds was measured in HCT116 (A), OVCAR8 (B), MDA-MB-231 (C), MCF-7 (D), and MCF-10A (E). Cells were grown in the presence of vehicle (DMSO) or different concentrations of the compounds for 48 hours, and then MTT assay was performed. All experiments were performed in triplicate.

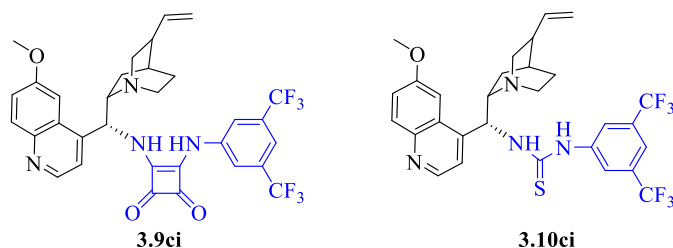


Figure 3.68. Chemical structures of compounds **3.9ci** and **3.10ci**.

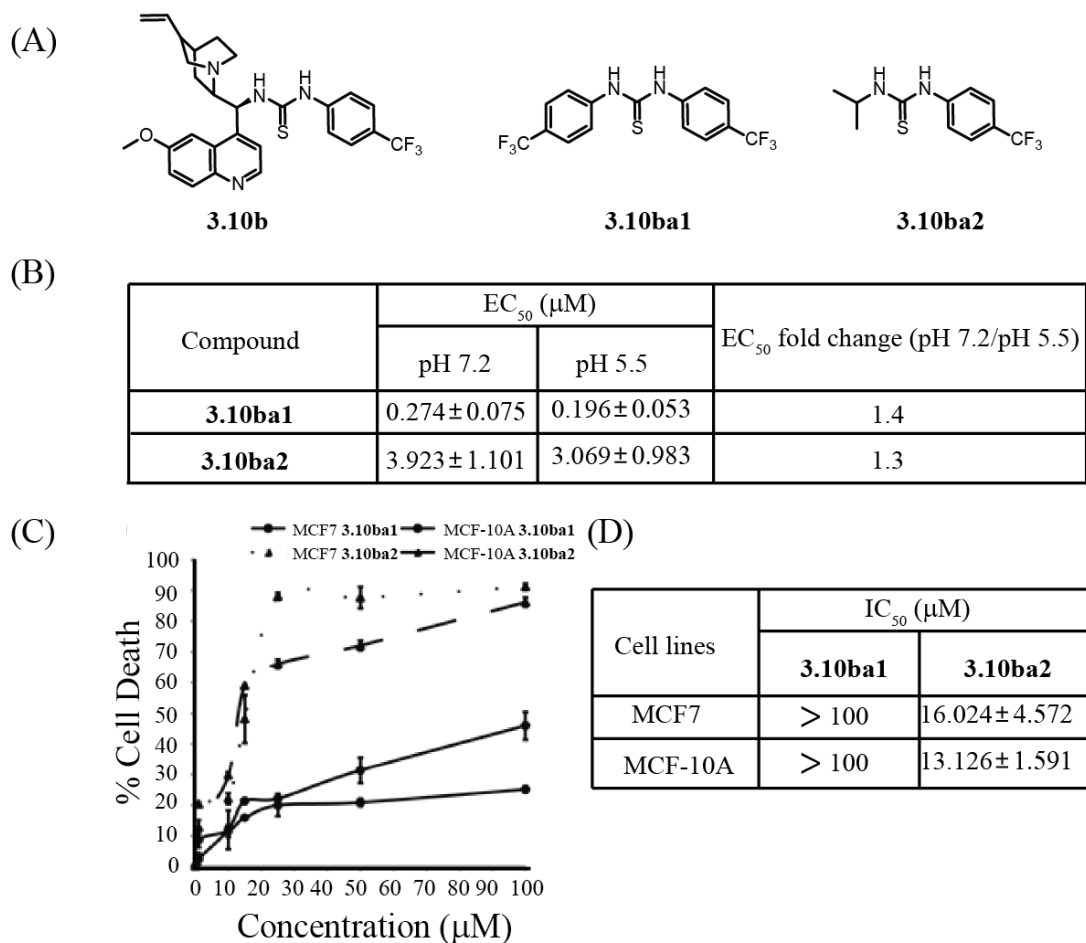


Figure 3.69. Structure of the potent quinone derivative and control compounds without the quinone moiety (A). Transport efficiency of the control compounds (B). Cell viability of the control compounds was measured in MCF-7 and MCF-10A cell lines (C). Cells were grown in the presence of vehicle (DMSO) or different concentrations of the compounds for 48 hours, and then MTT assay was performed. All experiments were performed in triplicate. The half-maximal inhibitory concentration (IC₅₀) of the control compounds in MCF-7 and MCF-10A cell lines (D).

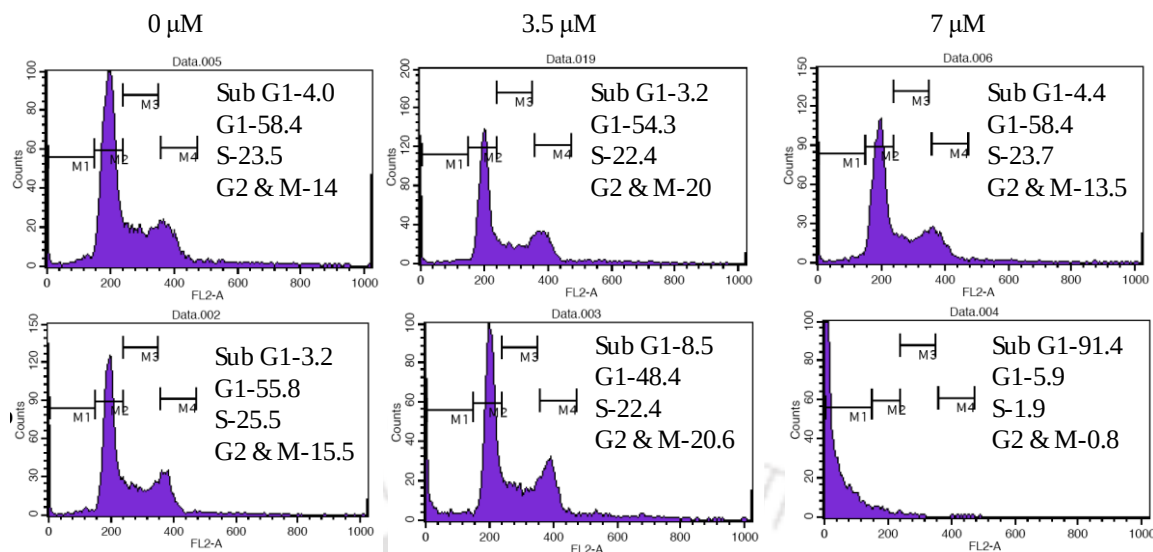


Figure 3.70. Fluorescent activated cell sorting (FACS) analysis of compounds **3.9b** and **3.10b**. The cell cycle distribution of MCF-7 cells grown in the absence and presence of indicated compounds was examined by staining the cells with propidium iodide (PI). Cells were treated with the test compounds for 24 hours. Histograms were analyzed using Cell Quest Pro software.

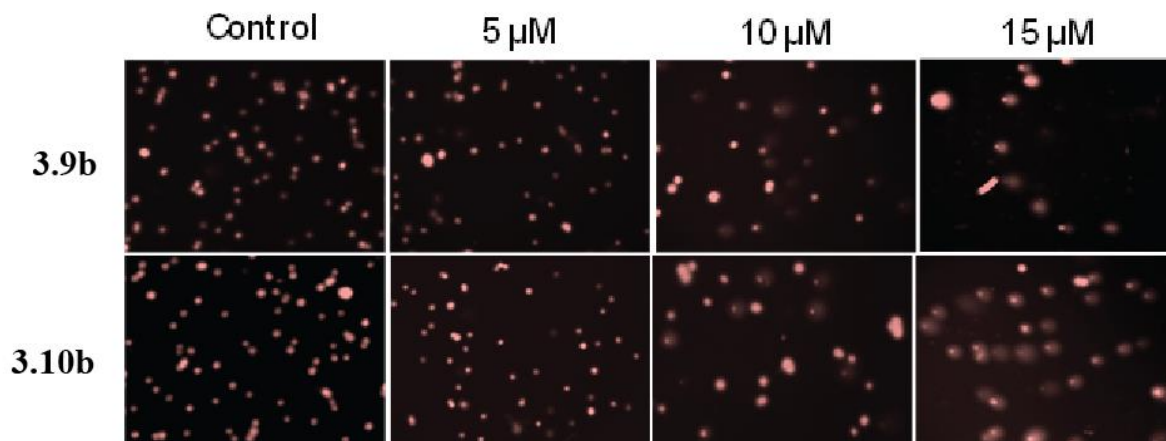


Figure 3.71. The compound induces DNA damage in MCF-7 cells. Comet assays for determination of DNA damage in single cells following the treatment with compound **3.9b** and **3.10b**.

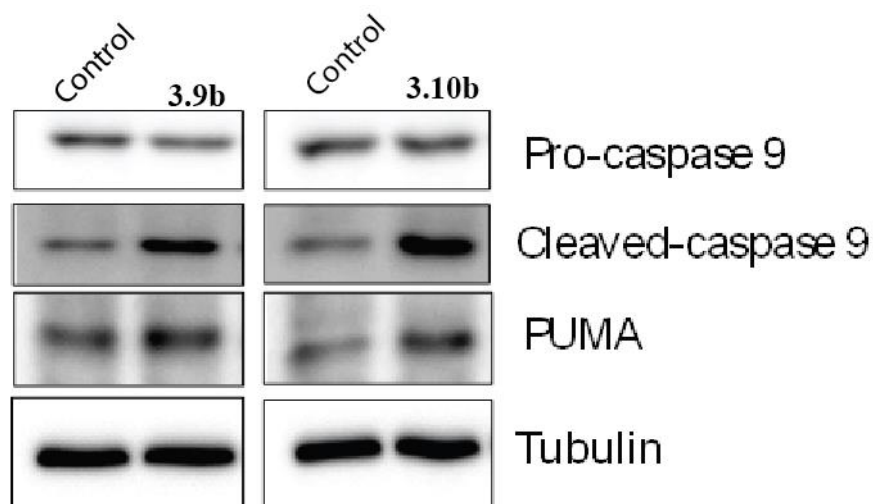


Figure 3.72. Whole-cell protein extracts of MCF-7 cells grown in the absence and presence of compounds (10 μ M) for 24 h and immunoblotted for the indicated proteins. α -Tubulin was used as a loading control.

3.4.23. *In vivo* plot

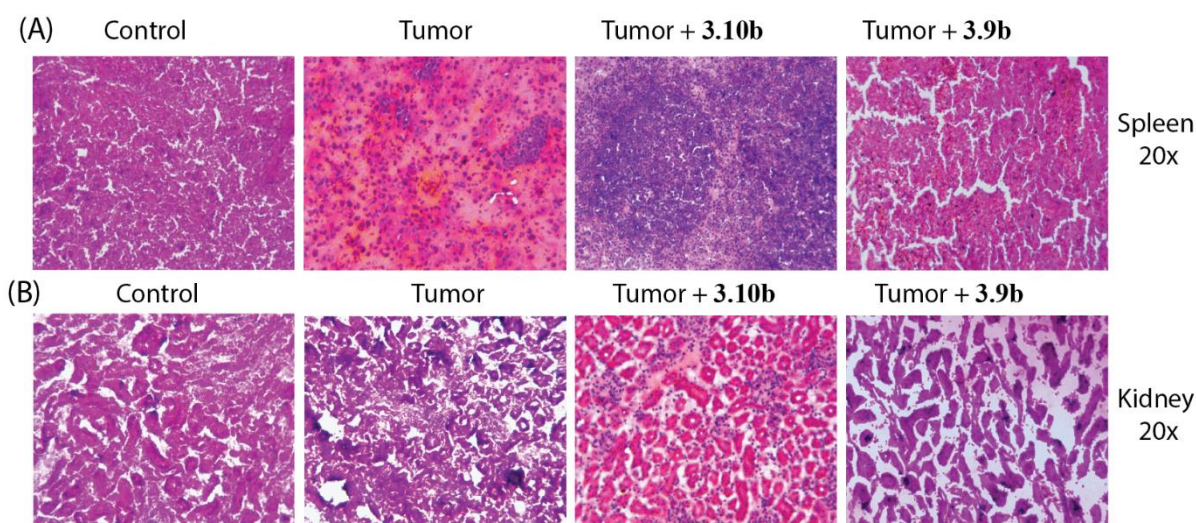


Figure 3.73. Immunotoxicity assessment of different organs by hematoxylin and eosin staining method. Massive inflammation was noted in the spleen of tumor-bearing mice that reduced significantly after compound **3.10b** treatment, but inflammation was still observed in compound **3.9b** treated experimental group (A). No significant change in renal architecture was observed in the compound **3.10b** treated experimental group, but inflammatory cells were observed in the compound **3.9b** treated experimental group (B). All the images were acquired at 20 $\times$ magnification.

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Chapter 4

Development of Sulphonium Based Water Soluble Proanionophores with Promising Prospects in Therapeutic Applications



4.1. Background and objective of present work

A variety of synthetic anionophores have been developed to mimic the Cl^- ion transport behavior of chloride intracellular ion channel (CLC) forming proteins, and few of them have shown exciting capabilities to act as alternative therapeutic agents against cancer and other diseases.¹⁻³ However, their cell deliverability seems to be quite challenging.² It is important to note that the general structure of most of the synthetic Cl^- ion transporters follow two basic design elements — hydrophobic motif(s) for their membrane localization and ion-recognition motif(s) for better selectivity and transport efficiency. Therefore, the anionophores are generally lipophilic molecules.²⁻⁴ The lower water solubility of the anionophores precluded the *in vivo* applications. Either additional doses or the assistance of an additional delivery vehicle is required to have an effect. The synthetic anionophore, obatoclax failed to clear its phase I/II clinical trials due to its poor water solubility.⁵

In this regard, the regulation of the solubility could be one of the strategies to increase the therapeutic values of the anionophores. Stimuli-responsive water-soluble derivatives of hydrophobic anionophores would augment the deliverability and generate the desired lipophilic transporter at specific tissues after the *in situ* cleavage of the hydrophilic appendages. Recently, enzyme and photo were used as stimuli to generate active anionophores from proanionophores. Unfortunately, both stimuli have non-specific cellular activity and showed burst regeneration of the active carrier.⁶⁻⁷

In this chapter, we have discussed the glutathione (GSH)-responsive anion transport strategy that used proanionophores to promote controlled transport of Cl^- ion across the lipid bilayers. The GSH responsive molecules have been widely used in drug delivery, biomolecular sensing, but the GSH-responsive synthetic proanionophore has not been reported so far.⁸⁻¹⁰ The GSH-dependent reductive cleavage of disulfide bonds is well exploited in various applications. However, the poor uptake efficiency of the disulfide-containing molecules reduces its applicability. Recently, GSH-responsive sulfonium-based molecules were developed, which not only increased the water solubility but also undergo facile cleavage under physiological conditions.¹⁰ The presence of sulfonium moiety enhanced the cellular uptake of the target molecules. So within this chapter, sulfonium derivatives of the hydrophobic anionophores were synthesized, and after that, their thorough investigation on anion recognition, transport, regeneration, and cellular uptake was carefully investigated.

4.2. Results and discussions

4.2.1. Design and synthesis of the anionophores and proanionophores

These successful GSH-responsive strategies inspired to design of thiourea-based anion transporters and their respective water-soluble sulfonium derivatives. The synthesis of these thiourea derivatives (**4.1-3**) was highly modular, where S-methylcysteamine was readily appended onto the preorganized scaffolds (Figure 4.1). The fluorescent derivative **4.3b** was synthesized by installing the nitrobenzofurazan (NBD) moiety. The requisite iodosulfonium derivatives **4.3c** and **4.3f** and tetrafluoroboratesulfonium derivatives **4.3d**, **4.3e**, and **4.3g** were synthesized from compounds **4.3a** and **4.3b** to enhance the water solubility.¹¹ Although various tripodal

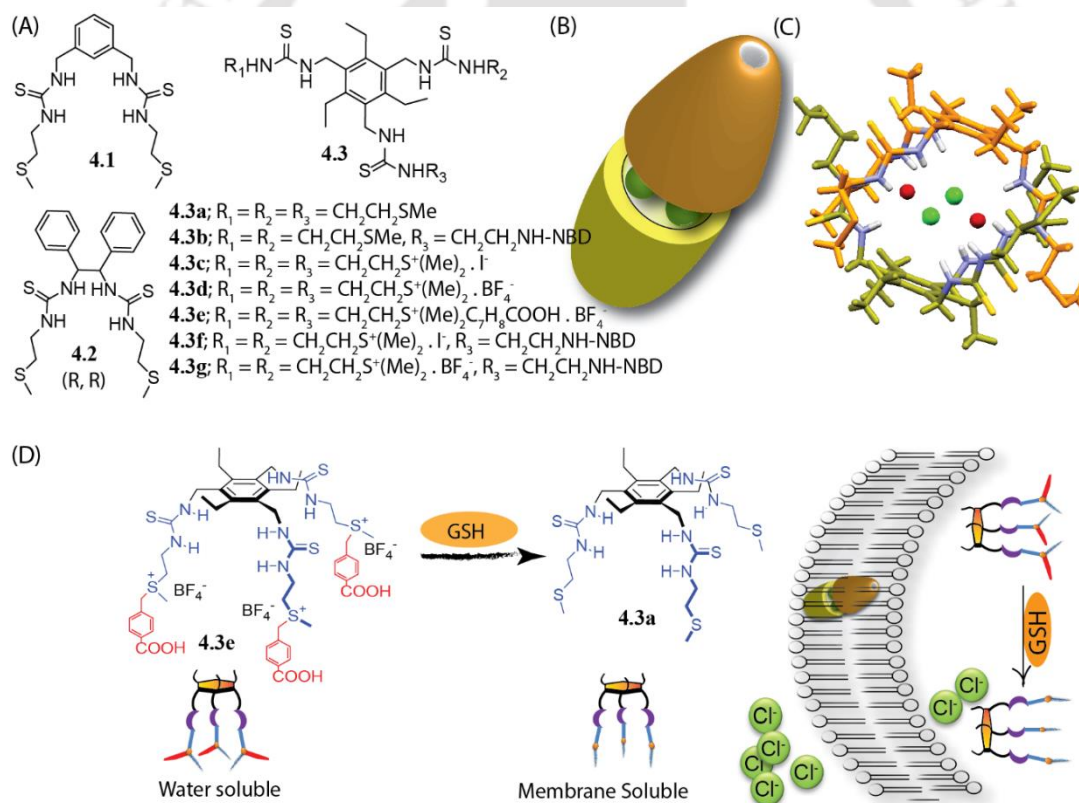


Figure 4.1. GSH responsive sulfonium based anionophores and proanionophores (A). Schematic representation of Chloride ion encapsulation by capsule kind of arrangement (B). X-ray crystal structure of compound **4.3a** complexed with Cl^- ion and H_2O ($(\mathbf{4.3a} \cdot \text{Cl}^- \cdot \text{H}_2\text{O})_2$). The TBA⁺ cations present outside the cavity were omitted for clarity. Probable mode of transportation in presence of GSH as a stimuli ((D).

anionophores were already reported but, these synthesized tripodal compounds contain methylthioethyl moieties allowing them to form water-soluble sulfonium derivatives, which could *in situ* generate the active ionophore via GSH mediated dealkylation process.¹²

4.2.2. Anion binding studies of the bis(thiourea) derivatives

The Cl^- ion binding abilities of the compounds were investigated in the solution phase by ^1H NMR titration experiments using tetrabutylammonium chloride (TBACl) as the Cl^- ion source. The titration plots showed considerable downfield shifts of all N-H signals, indicating the interaction with Cl^- ion through hydrogen bond formation (Figure 4.23-24). The Job's plot analysis based on ^1H NMR titrations revealed that the maximum interaction of compound **3** with Cl^- ion was observed at $x = 0.5$, indicating 1:1 binding stoichiometry (Figure 4.25). The association constants (K_a) values of the compounds were within the range of $12.22\text{--}20.99\text{ M}^{-1}$, suggesting their moderate Cl^- ion recognition abilities in the solution phase. The single crystal of compound **4.3a** displayed an interesting binding pattern, where two molecules of the receptor were found to form a capsular structure. Two Cl^- ions as well as the two water molecules ($(\text{Cl}^- \cdots \text{H}_2\text{O})_2$) were found to be encapsulated within this molecular capsule (Figure 4.1B-C and Figure 26-27). This solid-state Cl^- ion binding pattern is in good agreement with that in the solution phase (1:1 binding model). Further structural analysis revealed that the N-H proton of two different units of the same molecules was actually responsible for the Cl^- ion recognition, and the measured bond lengths were within the hydrogen bond distance (N-H $\cdots$ Cl^- bond distances were 2.336-2.642 Å). This crystallographic information also suggests a dimeric species might be involved in the transport of Cl^- ion.

4.2.3. Chloride ion transport activities of the synthesized derivatives

The transmembrane Cl^- ion transport activities were measured by fluorescence assay using 8-hydroxypyrene-1,3,6-trisulfonate (HPTS, $\text{p}K_a \sim 7.3$) encapsulated large unilamellar vesicles (LUVs) composed of egg-yolk phosphatidylcholine (EYPC) and cholesterol (CHOL) lipids. The ion transport activities of the compounds across the bilayer were examined in the presence of a pH gradient (Figure 4.2A). The lower Cl^- ion transport efficiency of the

iodosulfonium and tetrafluoroborate sulfonium salts indicate their poor membrane permeability.¹¹

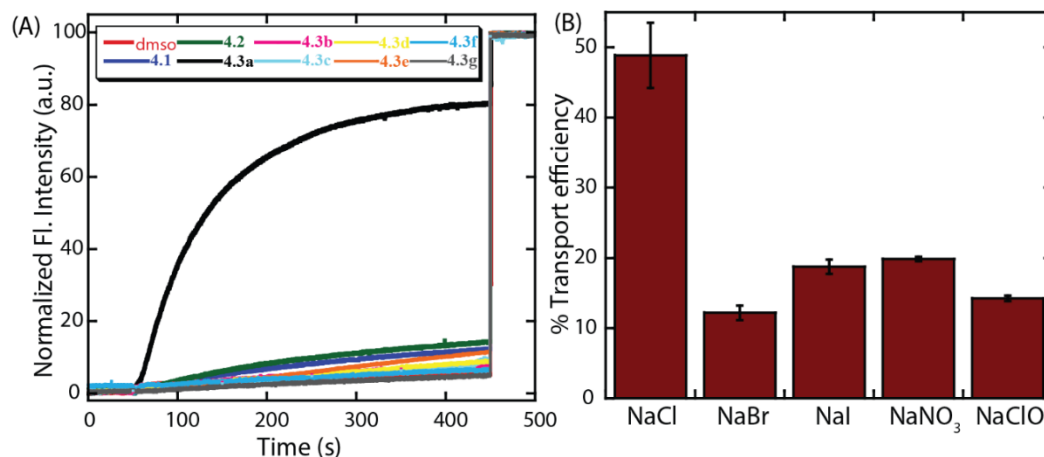


Figure 4.2. Comparisons of the ion transport activities of the compounds (0.56 mol %) across the EYPC/CHOL-LUVs>HPTS (A). Anion selectivity of compound **4.3a** at $EC_{50} = 0.14$ mol % (B). These measurements were performed in the presence of a pH gradient ($\Delta pH = 0.6$). Appropriate control experiments were carried out to exclude the effect of DMSO on the anion transport efficacies of compound **4.3a**.

The ion-selective electrode (ISE) based assay also demonstrated the Cl^- ion transport activities of the compounds in the absence of any pH gradient (Figure 4.29). Both HPTS and ISE-based assays revealed that compound **4.3a** had higher Cl^- ion transport activity. The dose-dependent HPTS assay also showed that the EC_{50} value of the compound **4.3a** was 0.14 mol % (Figure 4.28 and Figure 4.30). Whereas the EC_{50} values of ditopic compounds **4.1** and **4.2** could not be measured properly due to the formation aggregation at higher concentrations.

4.2.4. Ion selectivity studies

To explore the effect of extravesicular anions or cations on the Cl^- ion transport activity of the compound **4.3a**, the HPTS assay was performed by applying a pH gradient. A considerable difference in the anion transport activities for compound **4.3a** was observed when different NaX salts (Cl^- , Br^- , I^- , NO_3^- and ClO_4^-) were used in the extravesicular solution and iso-osmotic buffer solution of NaCl was used as the intravesicular solution at pH 7.2. The outcome clearly

suggests the higher selectivity of Cl^- ion over the other tested anions (Figure 4.2B), and the outcome is in good agreement with its Cl^- ion binding properties. Whereas the use of various chloride salts of metal ions ($\text{M}^+ = \text{Li}^+, \text{K}^+, \text{Na}^+, \text{Ca}^{2+}$, and Mg^{2+}) in the extravesicular solution showed very similar Cl^- ion transport activity (Figure 4.3). The results suggest that the presence of pH-gradient cations does not take part in the rate-limiting step of the ion transport process by compound **4.3a**.

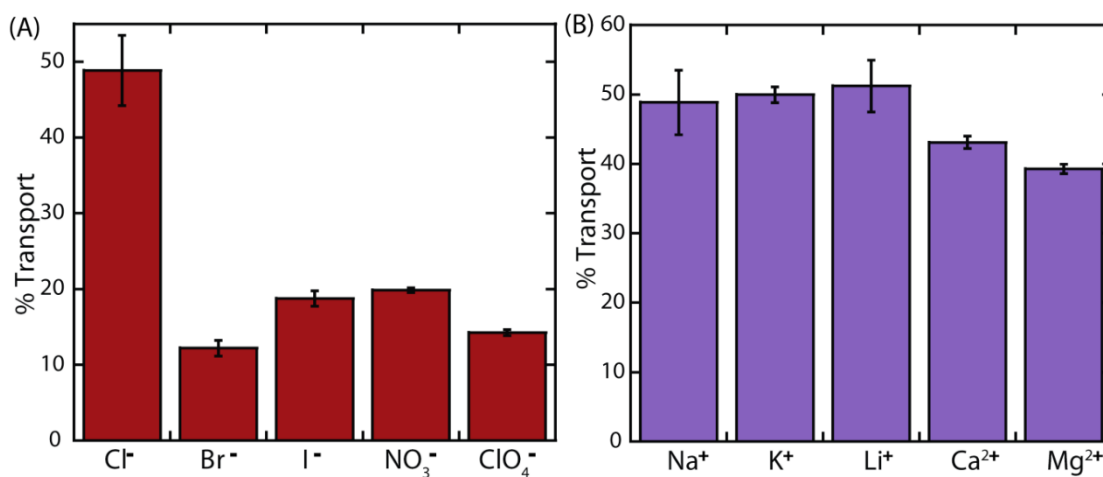


Figure 4.3. Anion (A) and cation (B) selectivity of compound **4.3a** as measured by HPTS-based fluorescence assay in the presence of a pH gradient of ~ 0.6 .

4.2.5. Mechanism of transportation

This transmembrane transport of Cl^- ion by compound **4.3a** may follow either antiport (H^+/M^+ and OH^-/A^-) or symport (H^+/A^- and M^+/OH^-) mechanism in the presence of a base pulse. Meanwhile, the cation selectivity studies eliminate any considerable role of the cations on the Cl^- ion transport activity. The variable anion selectivity data suggest the probability of OH^-/A^- antiport or H^+/A^- symport mechanism. For further investigation, the Cl^- ion transport properties of compound **4.3a** in the presence of 4-(trifluoromethoxy)phenylhydrazone (FCCP) were investigated by HPTS assay. The compound **4.3a** showed an enhancement in the Cl^- ion transport activity in the presence of the FCCP (Figure 4.4A). The result proposes a cooperative effect between the compound and FCCP, which eliminates the probability of H^+ ion transport. The HPTS assay was also performed in the absence and presence of valinomycin. The influx of K^+ ion by valinomycin could promote the influx of OH^- ion and/or efflux of Cl^- ion for the equilibration of the pH gradient. A similar enhancement in the Cl^- ion transport rates in

the absence and presence of valinomycin suggests that the OH^-/Cl^- antiport mechanism is operative with superior transport of Cl^- over OH^- ion for compound **4.3a** (Figure 4.4B).

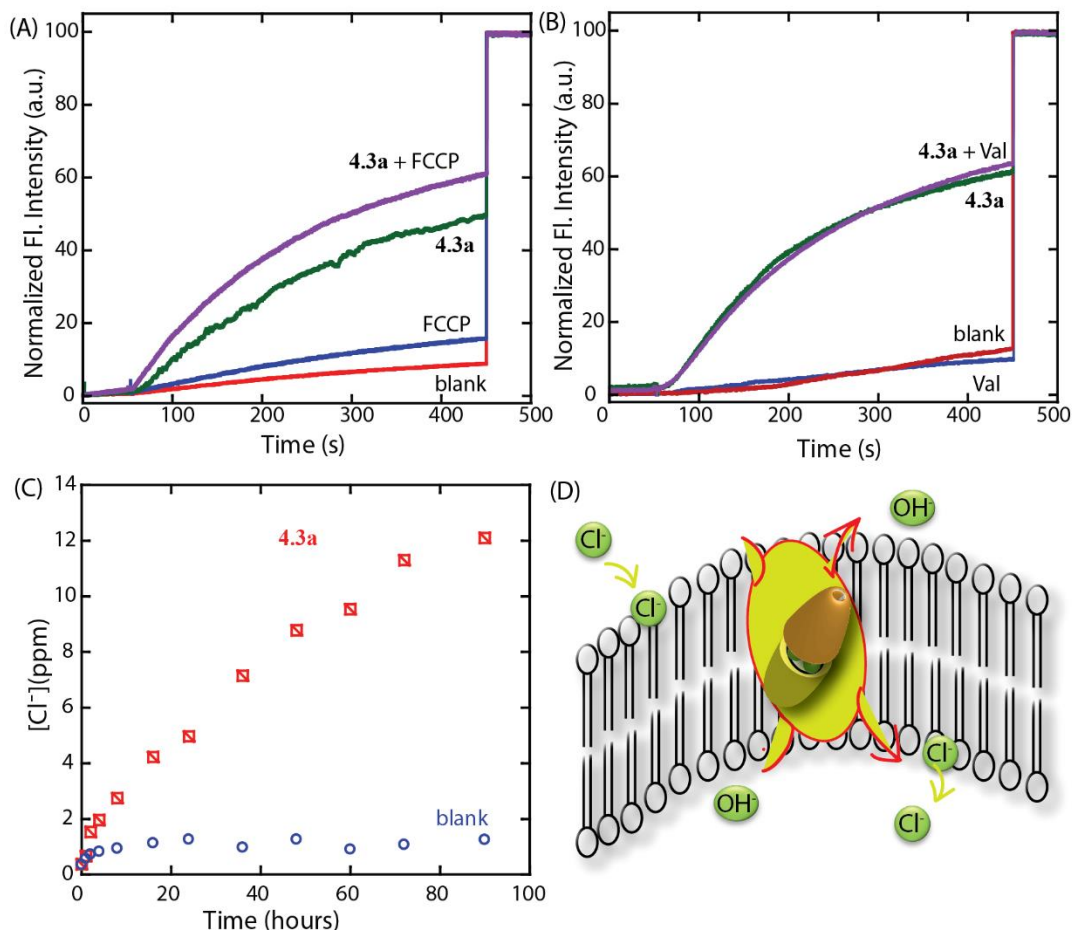


Figure 4.4. Ion transport activity of compound **4.3a** (at $\text{EC}_{50} = 0.14$ mol %) in the absence and presence of FCCP (4 nM) (A) and valinomycin (2.5 nM) (B). Measurement of the Cl^- ion transport efficacy by U-tube assay in the presence of compound **4.3a**, using a chloride ISE and NaCl gradient (C). Schematic representation to illustrate the transport of Cl^- ion *via* carrier mechanism (D).

4.2.6. Pathway of transportation

The cholesterol-dependent ISE assay suggests the carrier pathway for the transport of Cl^- ion in the presence of compound **4.3a** (Figure 4.5A).¹³ The classical U-tube assay confirmed the transport of Cl^- ion *via* carrier pathway for compound **4.3a** (Figure 4.4C).¹⁴ In the meantime, the dose-dependent Cl^- ion transport activity of compound **4.3a** showed that the Hill coefficient (n) value was around 2, indicating the involvement of two molecules in the formation of active transporters under the liposomal environment. This

observation was in accordance with the crystallographic results. The ^1H NMR titration experiments showed that compound **4.3a** have weak binding affinity for Cl^- ion, but the HPTS assay showed its strong transmembrane Cl^- ion transport activity, which also suggests the possibility of the formation of a self-assembled carrier. To investigate whether a dimeric species is really involved in the transportation of Cl^- ion, the initial rates were calculated at different concentrations of compound **4.3a**. An upward-curvature in the transport activity vs concentration profile clearly suggests the possibility of Cl^- ion transport by dimeric capsule *via* carrier pathway (Figure 4.31).¹⁵

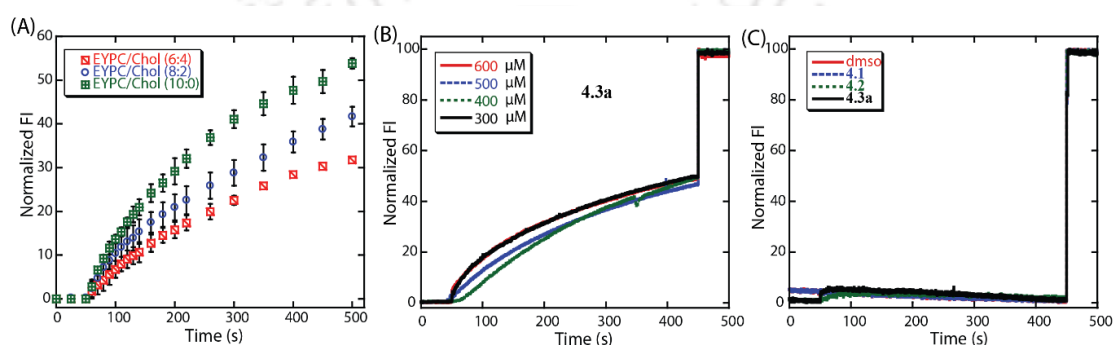


Figure 4.5. Effect of the concentration of cholesterol in the EYPC/cholesterol membrane on Cl^- ion transport activity of compound **4.3a** (A). Leaching experiment of compound **4.3a** (at compound to lipid ratio of 0.142 mol %) (B). Extent of calcein leakage from the EYPC/CHOL-LUVs in the presence of compounds. After time $t = 50$ sec., DMSO solutions of compound or DMSO (blank) was added and the fluorescence intensity ($\lambda_{\text{ex}} = 490$ nm, $\lambda_{\text{em}} = 520$ nm) was recorded. After $t = 450$ sec the LUVs were lysed using triton X-100 (C)

To confirm that these synthetic compounds reside in the EYPC/CHOL membrane bilayer and do not come out in the aqueous solution over the period of time, leaching test was performed (Figure 4.5B). It is hypothesized that if the compounds leached out from the membrane bilayer environment to the aqueous medium then transport rate of the compounds will be greatly affected (reduced) by the dilution of the vesicular solution. Whereas, dilution factor will not affect the transport rate if the compounds are localized inside the bilayer environment. To confirm, this experiment was carried out and it was observed that there was no effect of transportation on dilution effect which clearly suggested its non-leaching behaviour. Additionally, the calcein experiment was also

performed to confirm that whether a molecule had any adverse effect on membrane integrity or not. Fortunately, we have not observed the fluorescence intensity enhancement of calcein on dye during transportation which clearly suggested that membrane integrity remains unchanged (Figure 4.5C).¹

4.2.7. Regeneration of active anionophore

4.2.7.1. Regeneration studies through biophysical experiment

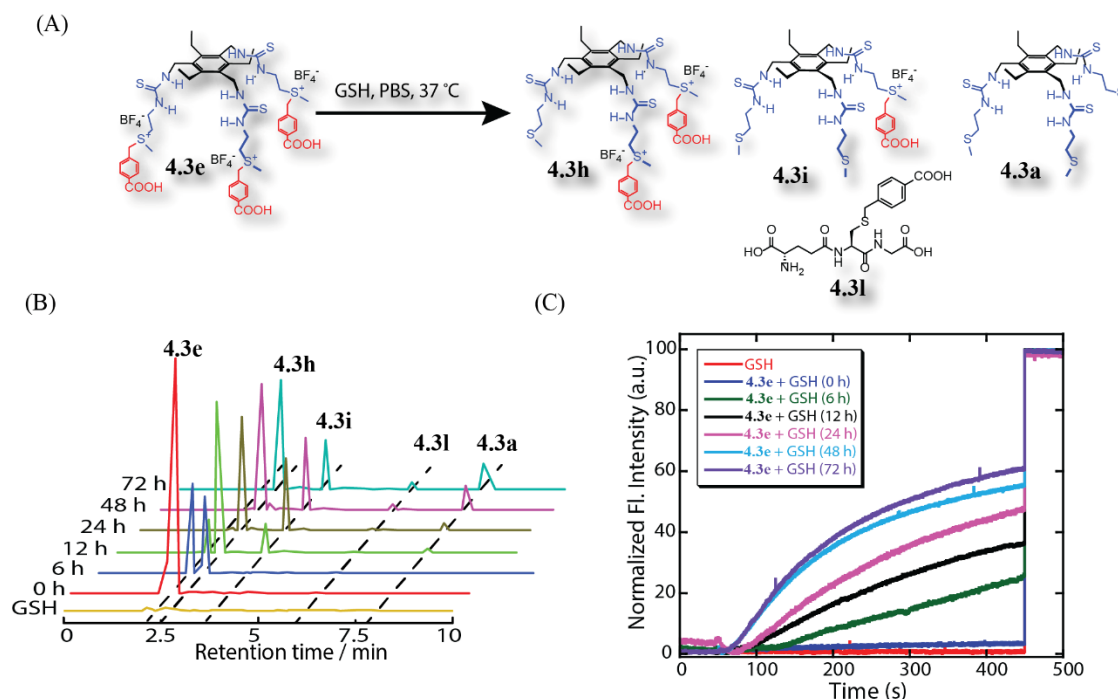


Figure 4.6. Schematic representation of regeneration strategy (A) Dealkylation of compound **4.3e** (1 mmol) in the presence of GSH (10 mmol) in PBS (pH = 7.4) at 37 °C. HPLC traces for the dealkylation of compound **4.3e** and the regeneration of active compound **4.3a** along with different other possible intermediate such as **4.3h**, **4.3i** and **4.3l** at different time intervals (B) Transport of Cl⁻ ion by the regenerated compound **4.3a** in the presence of GSH at different time intervals (C)

Overall, these studies confirmed the Cl⁻ ion recognition and transport properties of compound **4.3**. However, hydrophobicity could limit its deliverability and bio-applicability.¹⁶ Therefore, to regulate the solubility of anionophores, trialkylsulfonium salts of compound **4.3a** and **4.3b** were investigated. The tetrafluoroboratesulfonium salts of compound **4.3d**, **4.3e**, and **4.3g** showed better water solubility than iodosulfonium salts **4.3c** and **4.3f** (Table 4.3).¹¹ To confirm the dealkylation of water-soluble analogue **4.3e** and the

regeneration of the active carrier **4.3e** (Figure 4.6A-B), the HPLC analysis was performed. In most normal cells and cancer cells the GSH concentrations are within 1-2 and 7-10 mM, respectively. However, the GSH present at a very low concentration of 2-20 μ M in blood serum.¹⁷ Hence, dealkylation of compound **4.3a** (1 mmol) was performed in the presence of GSH (10 mmol) in PBS (pH 7.4) at 37 °C. The outcome of the HPLC analysis confirmed the time-dependent dealkylation of compound **4.3e** and regeneration of the parental hydrophobic carrier **4.3a** along with mono- and di-dealkylated intermediates in the presence of GSH (Figure 4.6A-B and Figure 4.32-34). The Cl^- ion transport ability of the regenerated compound **4.3a** was also investigated by the HPTS assay using the GSH containing reaction mixture. The time-dependent enhancement of Cl^- ion transport ability also confirmed the regeneration of the active carrier **4.3a** in the presence of GSH (Figure 4.6C). Although, the GSH (10 mM) mediated rate of dealkylation was quite slow, it did produce the active compound **4.3a** over time, which is highly beneficial for their bio-applicability. The step-wise cleavage of compound **4.3e** generates active carrier **4.3a**, mono- and di-dealkylated intermediates, which could be the primary reason for the slow transport of Cl^- ion under the experimental conditions. The cleavage of compound **4.3e** was very slow in the presence of a low concentration of GSH (< 2 mM). Interestingly, in the absence of GSH, the structural integrity of compound **4.3e** was intact (Figure 4.35), and it failed to transport Cl^- ion (Figure 4.37).

4.2.7.2. Regeneration studies under cellular environment

The GSH-mediated ionophore regeneration strategy under cellular environments was investigated where HeLa cells were treated with compound **4.3e** in the presence and absence of GSH inhibitor buthionine sulfoximine (BSO).¹⁸ The MALDI-TOF analysis of the cell lysate confirmed the formation of active anionophore **4.3a** with mono- and di-dealkylated intermediates (Figure 4.7B and Figure 4.39-4.41). In BSO treated cells proanionophore **4.3e** and a small amount of mono cleaved intermediate **4.3h** were observed. The formation of intermediate **4.3i** and the anionophore **4.3e** were not detected, which clearly suggests that in the presence of the GSH inhibitor, the dealkylation rate was very slow (Figure 4.42). The extent of dealkylations was higher when external GSH was used, confirming the operability of this strategy under the cellular environment. Cystic fibrosis is a genetic disease that mostly affects the lungs.¹⁹ It is anticipated that these nontoxic

anionophores or proanionophores could be easily delivered to the lungs of cystic fibrosis patients. The water-soluble proanionophore could be easily delivered to the affected patients by the oral delivery method.¹⁹⁻²⁰ However, the GSH content in the apical fluid in cystic fibrosis patients, is low due to the abnormal transport of GSH by the defective CFTR proteins. Hence, additional GSH as external stimuli might be necessary to generate the active anionophore from proanionophore for both pulmonary and oral drug delivery methods.

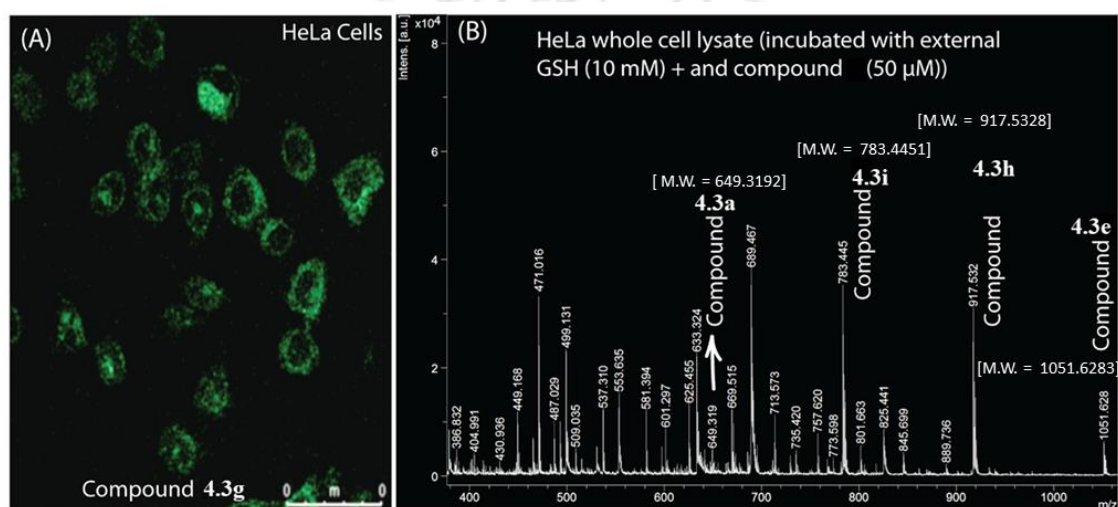


Figure 4.7. CLSM images of HeLa cells treated with NBD-labelled tetrafluoroboratesulfonium derivative **4.3g** (A). The green channel illustrates the uptake of the NBD-labelled compound. Scale bar 50 mm. MALDI-TOF mass spectral analysis of a HeLa cell lysate (B). The HeLa cells were incubated with external GSH (10 mM) and compound **4.3e** (50 mM).

4.2.8. Cell-based experiment

4.2.8.1. MTT assay

To investigate the bio-applicability of the compounds, the cytotoxicity was measured in BHK-21, HeLa, and MDCK cell lines because of their lack of ability to express cystic fibrosis transmembrane conductance regulator (CFTR) protein endogenously.²⁰⁻²¹ The MTT assay revealed that the compounds **4.3a** and **4.3e** have low toxicity in all three tested cell lines (Figure 4.8A-F). The MQAE assay showed a concentration-dependent quenching of the MQAE fluorescence signal, suggesting an intracellular transport of Cl^- ion is facilitated by the compound **4.3a** (Figure

4.9).¹ In addition, HPTS quenching assay was also performed using giant unilamellar vesicles (GUVs). The result showed that the presence of compound **4.3a** significantly induces the influx of Cl^- ion (Figure 4.10).²²

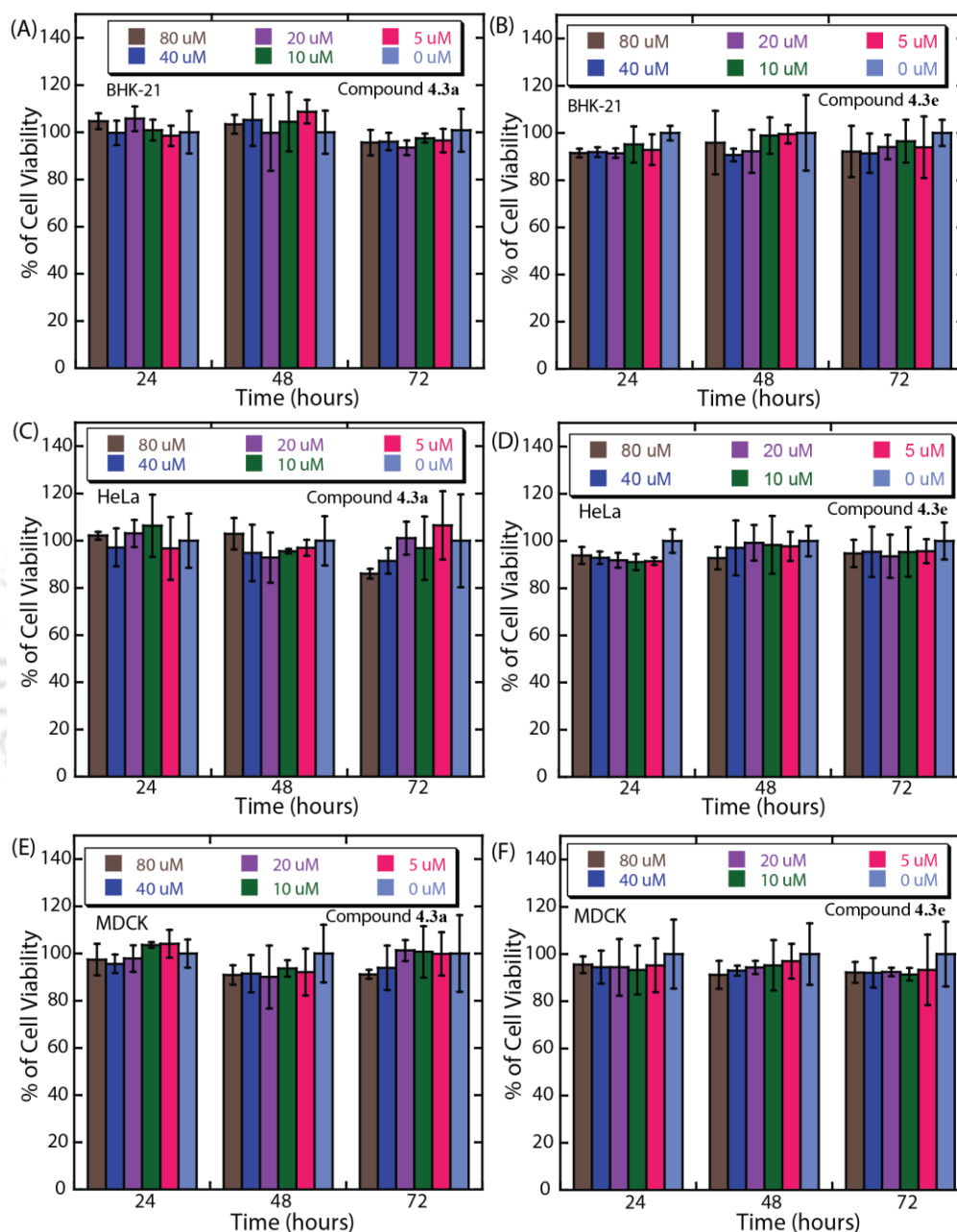


Figure 4.8. Cell viability of the compounds **4.3a** and **4.3e** was measured at different concentrations of 0-80 μM . Cell viability was measured in BHK-21, HeLa, and MDCK cells after 24, 48, and 72 hours of compound treatment. All experiments were performed in triplicates.

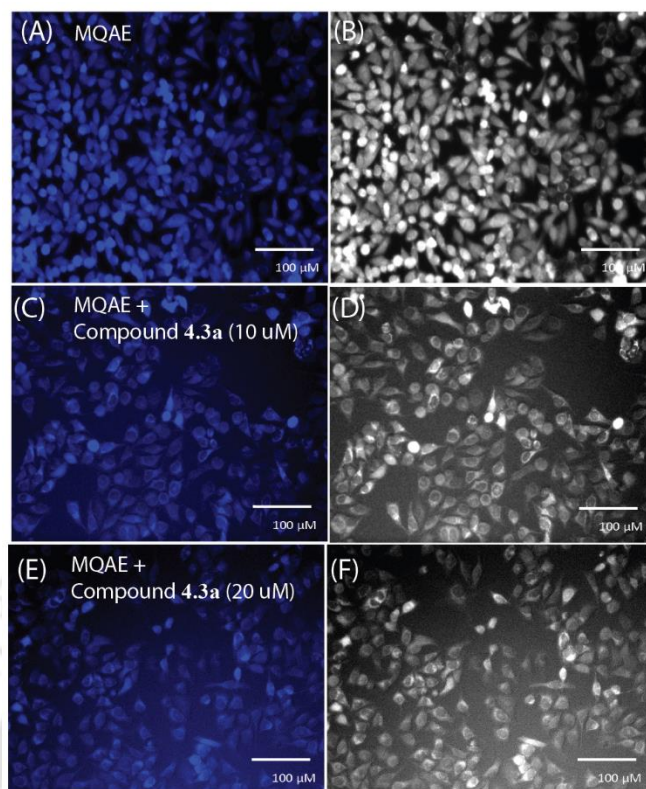


Figure 4.9. Fluorescence microscopic images of the MQAE dye ($\lambda_{\text{ex}} = 350 \text{ nm}$ and $\lambda_{\text{em}} = 460 \text{ nm}$) labeled HeLa cells in the absence and presence of the compound **4.3a**. Both blue channel (A, C, and E) and grey channel (B, D and F) illustrate the fluorescence intensity of the MQAE dye in the absence and presence of the compound **4.3a**.

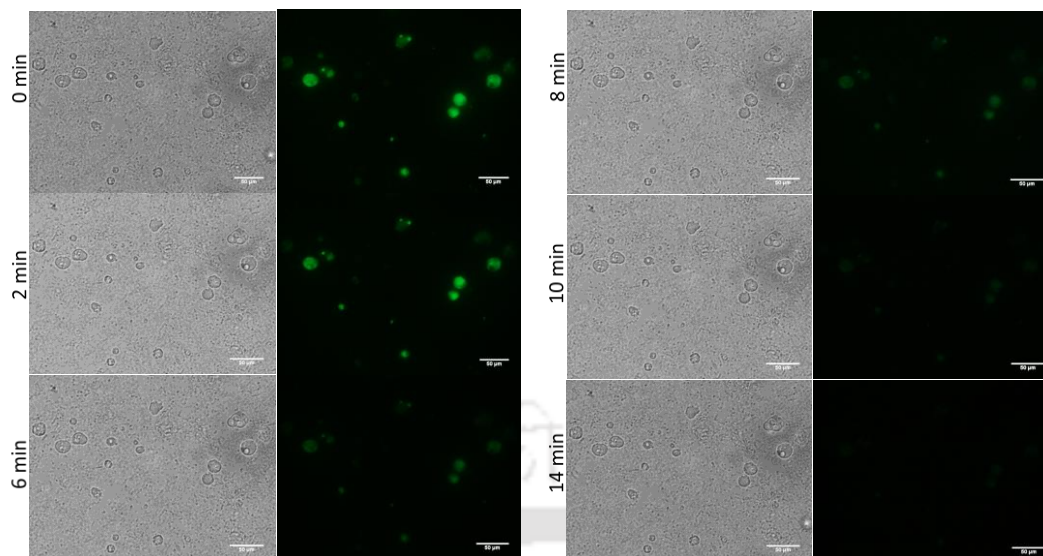


Figure 4.10. Fluorescence microscopic images of the HPTS loaded GUVs were recorded at different time intervals (0, 2, 10, 20, and 30 minutes), after the addition of compound **4.3a** and NaCl (0.1 M). The change in the integrity of the GUVs and encapsulated HPTS fluorescence intensity were monitored using bright-field and green channels, respectively.

4.2.8.2. Cell-based microscopic analysis

The microscopic analysis revealed that the sulfonium derivative **4.3g** (Figure 4.11) has better cellular uptake than neutral hydrophobic derivative **4.3b**, which could be due to the positive charge of the sulfonium moieties.²³ It was hypothesized that a similar cellular uptake pattern would be observed for compounds **4.3a** and **4.3e**. The outcomes of MTT and MQAE assays suggest that the toxicity of the anionophores depends on the structure of the parent compound and only transport of anion is inadequate to promote cell death. It is hypothesized that the non-toxic nature of compounds **4.3a** and **4.3e** would find a suitable application for cystic fibrosis treatment.

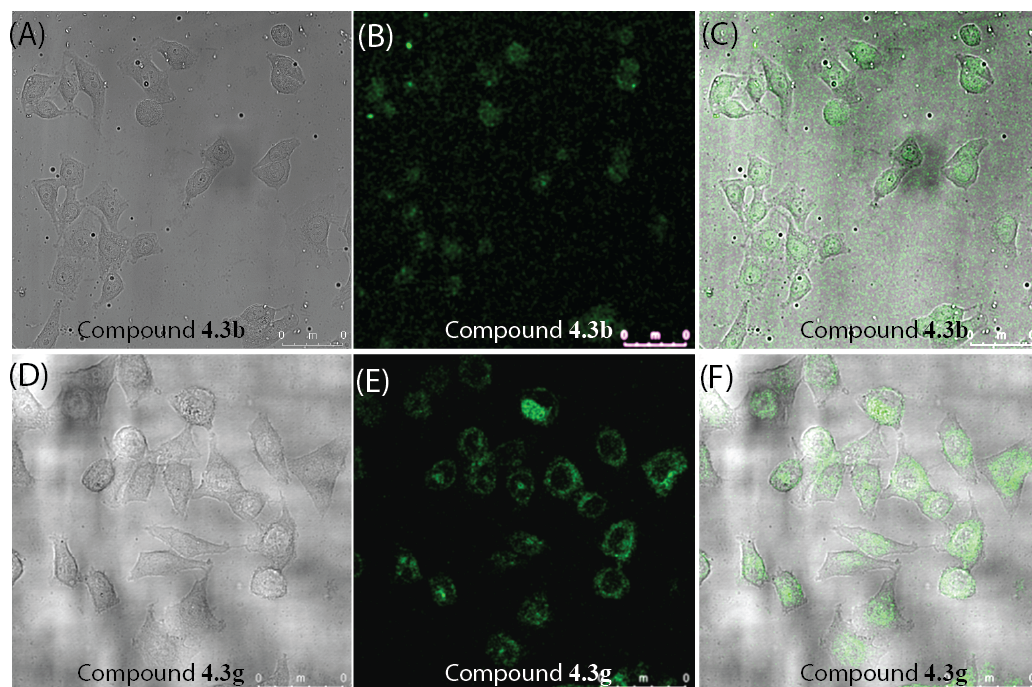


Figure 4.11. Confocal microscopic images of the HeLa cells treated with NBD-labelled compounds **4.3b** and **4.3g**. Green channel illustrating compound uptake, bright field illustrating the cell morphology, merge of green and bright field channels illustrating cell morphology in the presence of NBD-labelled compounds.

4.3. Summary

Within this chapter, the tripodal compound was found to be an efficient transporter of Cl^- ion. The compound could form a molecular capsule within the membranes to transport the Cl^- ion *via* the carrier pathway. Modification of each arm with sulfonium moiety allowed forming the targeted water-soluble stimuli-responsive proanionophore. The GSH was successfully applied to achieve a controlled regeneration of the hydrophobic active anionophore and transport of Cl^- ion. Several biophysical analyses confirmed the regeneration of the active anionophore under a cellular environment. The microscopic analysis revealed higher cellular uptake of the sulfonium-based proanionophore, suggesting its better deliverability. The lower toxicity level of the potent compound suggests that the GSH-responsive anion transport strategy has realistic potential for the treatments of ion transport-related diseases including, cystic fibrosis.

4.4. Experimental section

4.4.1. Synthesis of compounds

4.4.1.1. General information

Described in section 2.4.5.1.1.

4.4.1.2. General procedure for the synthesis of targeted compounds

4.4.1.2.1. Synthesis of 1-isothiocyanato-2-(methylthio)ethane

To a stirring solution of 2-(methylthio)ethan-1-amine (830 mg, 7.90 mmol) in chloroform (10 mL) and water (10 mL) at 0 °C was added a solution of thiophosgene (0.9 ml, 11.86 mmol) and NaOH (948 mg, 23.7 mmol) in chloroform (10 mL). After 10 minutes, the reaction mixture was allowed to warm up to room temperature, and the stirring was continued for 1-1.5 hours. The progress of the reaction was monitored by TLC analysis. After completion of the reaction, the organic layer was extracted with chloroform (2 × 50 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The crude reaction mixture was purified through the column chromatography with a solvent gradient system using methanol/chloroform to give a light yellow liquid compound with 70% yield.²⁴

4.4.1.2.2. Synthesis of 1, 3, 5-triethyl-2, 4, 6-tris(isothiocyanatomethyl)benzene

To a stirring solution of 1,3,5-tris(bromomethyl)-2,4,6-triethylbenzene (200 mg, 0.45 mmol) in dry DMF solvent, were added tetrabutylammonium bromide (585 mg, 1.81 mmol), potassium thiocyanate (308 mg, 3.17 mmol) and sodium iodide (55 mg, 0.36 mmol) at room temperature under N₂ atmosphere. Then the reaction mixture was continued to stir for 2-3 hours at 80 °C temperature. The progress of the reaction was monitored by TLC analysis. After completion of the reaction, the unwanted salts were filtered through the filter paper. The filtrate was diluted with water (50 mL), and the organic layer was extracted with ethyl acetate (2 × 50 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The crude reaction mixture was purified through the column chromatography with a solvent gradient system using ethyl acetate/hexane to give a white solid compound with 60% yield.^{12, 25}

4.4.1.2.3. Synthesis of N-(2-aminoethyl)-7-nitrobenzo[c][1,2,5]oxadiazol-4-amine

To a stirring solution of NBD-Cl (250 mg, 1.252 mmol) in CH₂Cl₂ was added N-Boc-1,2-diaminoethane (221 mg, 1.378 mmol) and triethylamine (2 mL, 1.378 mmol) in CH₂Cl₂ (10 mL). The resulting dark-brown color solution was continued to be stirred until the disappearance of the starting materials. The progress of the reaction was monitored by the TLC analysis. After completion of the reaction, the reaction mixture was diluted with CH₂Cl₂ (10 mL), and the organic layer was washed with NaHCO₃ solution in water (2 × 10 mL). The collected organic layers were dried over the anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. Then, the crude reaction mixture was dissolved in CH₂Cl₂ (5 mL), and 30% trifluoroacetic acid solution in CH₂Cl₂ (2 mL, 1:9 (v/v)) was slowly added at room temperature. After 2 hours, diethyl ether (30 mL) was added, and the precipitated product was collected through filtration, washed with cold diethyl ether to give the brown color product with 70% yield.²⁶

4.4.1.2.4. Synthesis of bis(thiourea) derivatives

To a stirring solution of 1, 3-phenylenedimethanamine (100 mg, 0.734 mmol) or 1, 2-diphenylethane-1, 2-diamine (100 mg, 0.4710 mmol) in CH₂Cl₂ (15 mL) was added a solution of 1-isothiocyanato-2-(methylthio)ethane (1.615 mmol for compound **4.1** and 1.036 mmol for **4.2**) in CH₂Cl₂ and the whole reaction mixture was allowed to stir for 4-5 hours at room temperature. The progress of the reaction was monitored by TLC analysis. After completion of the reaction, the mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with brine solution and dried over anhydrous Na₂SO₄. The organic solvent was removed under reduced pressure. The crude reaction mixture was purified through the column chromatography with a solvent gradient system using MeOH/ CH₂Cl₂ to give the targeted compounds **4.1** and **4.2** with 70 and 75% yield, respectively.

4.4.1.2.5. Synthesis of tris(thiourea) derivatives

To a stirring solution of *N*¹-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)ethane-1,2-diamine (62 mg, 0.266 mmol) in CH₃CN and/or 2-(methylthio)ethan-1-amine (97 mg, 1.065 mmol for compound **4.3a** or 46 mg, 0.5054 mmol for compound **4.3b**) was added a solution of 1,3,5-triethyl-2,4,6-tris(isothiocyanatomethyl)benzene (100 mg, 0.266 mmol for both compounds **4.3a** and **4.3b**) in CH₃CN. The reaction mixture was then allowed to

stir for 4-5 hours at room temperature. The progress of the reaction was monitored by the TLC analysis. White precipitation was observed (for the compound **4.3a**) and the precipitate was filtered and washed with CH_2Cl_2 and CH_3CN to get the pure compound **4.3a** as a white solid with 90% yield. However, there was no precipitation for compound **4.3b**. After completion of the reaction, the reaction mixture was diluted with water and extracted with ethyl acetate. The organic layer was washed with brine solution and dried over anhydrous Na_2SO_4 . The organic solvent was removed under reduced pressure, and the purification was done by column chromatography using a gradient solvent system of $\text{MeOH}/\text{CH}_2\text{Cl}_2$. The targeted compound **4.3b** was obtained as a white solid with 50% yield.

4.4.1.2.6. Synthesis of sulfonium derivatives

To a stirring solution of 1,1',1''-((2,4,6-triethylbenzene-1,3,5-triyl)tris(methylene))tris(3-(2-(methylthio)ethyl)thiourea) (compound **4.3a**; 1 equiv.) in CH_3CN was added a solution of iodomethane (3.2 equiv. for compound **4.3c** and **4.3d**) or 4-(bromomethyl)benzoic acid (3.2 equiv. for compound **7**) in CH_3CN . To this reaction mixture, a trace amount of dry DMF was added, and the mixture was heated (at 40-50 °C) to solubilize the whole reaction mixture and was allowed to stir for 24 hours at room temperature. For the synthesis of the compound **4.3d** and **7**, 20 mol% of AgBF_4 were also added to prepare their respective tetrafluoroborate salts for making them as better water-soluble compounds. The progress of the reaction was monitored by TLC analysis. The precipitation due to the silver salt was filtered, and the filtrate part was washed with diethyl ether, CH_2Cl_2 , and CH_3CN to obtain the pure product **4.3d** and **4.3e** as sticky liquid with 60% and 70% yields, respectively. As AgBF_4 was not used for the preparation of compound **4.3c**, so there was not any precipitation under the experimental conditions and after the completion of the reaction, the reaction mixture was simply washed with the diethyl ether, CH_2Cl_2 and CH_3CN to get the pure product as sticky liquid with 90% yield.¹¹

4.4.1.2.7. Synthesis of NBD-labeled sulfonium derivatives

To a stirring solution of compound **4.3b** in CH_3CN (10 mL), a solution of iodomethane was added in CH_3CN , and the whole reaction mixture was allowed to stir for 24 hours at room temperature. After completion of the reaction, the reaction mixture was washed with diethyl ether, CH_2Cl_2 , and CH_3CN to obtain a sticky compound **4.3f** with

90% yield. For the synthesis of the compound **4.3g**, 20 mol% of AgBF₄ was added to the solution of compound **4.3b** in CH₃CN. The precipitate due to the presence of excess silver salt was filtered off, and the filtrate part was washed with diethyl ether, CH₂Cl₂, and CH₃CN to get the pure product **4.3g** as sticky compounds with 50% yield.¹¹

4.4.1.3. Characterization of synthesized compounds

1,3,5-triethyl-2,4,6-tris(isothiocyanatomethyl)benzene: ¹H NMR (600 MHz, CDCl₃) δ_{ppm} 4.68 (s, 6H), 2.79 (q, $J = 7.7$ Hz, 6H), 1.21 (t, $J = 7.7$ Hz, 9H). ¹³C NMR (150 MHz, CDCl₃) δ_{ppm} 144.1, 132.1, 129.9, 42.8, 23.1, 15.7. ES-MS (ESI+) m/z : 376.098. HRMS (ESI) calcd. for C₁₈H₂₁N₃S₃ (M+ H)⁺: 376.0970, found: 376.0981.

*N*¹-(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)ethane-1,2-diamine

Characterization data were matched with the reported literature.

1,1'-(1,3-phenylenebis(methylene))bis(3-(2-(methylthio)ethyl)thiourea) (4.1):

¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 7.97 (s, 2H), 7.60 (s, 2H), 7.29 (t, $J = 7.5$ Hz, 1H), 7.19 – 7.16 (m, 3H), 4.64 (s, 4H), 3.62 (s, 4H), 2.63 (t, $J = 7.0$ Hz, 4H), 2.08 (s, 6H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 183.6, 139.8, 128.7, 126.7, 126.3, 47.5, 43.3, 32.9, 15.1. ES-MS (ESI+) m/z : 403.1119. HRMS (ESI) calcd. for C₁₆H₂₆N₄S₄ (M+ H)⁺: 403.1113, found: 403.1119.

1,1'-(1,2-diphenylethane-1,2-diyl)bis(3-(2-(methylthio)ethyl)thiourea) (4.2):

¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 7.91 (brs, 2H), 7.61 (brs, 2H), 7.29-7.27 (m, 6H), 7.24-7.22 (m, 4H), 6.03 (brs, 2H), 3.51 (brs, 4H), 2.01 (s, 6H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 182.9, 157.6, 140.4, 128.3, 128.0, 127.5, 61.1, 43.2, 32.7, 15.0. ES-MS (ESI+) m/z : 479.1429. HRMS (ESI) calcd. for C₂₂H₃₀N₄S₄ (M+ H)⁺: 479.1426, found: 479.1429.

1,1',1''-((2,4,6-triethylbenzene-1,3,5-triyl)tris(methylene))tris(3-(2-

(methylthio)ethyl)thiourea) (4.3a): ¹H NMR (600 MHz, DMSO-*d*₆) δ_{ppm} 7.40 (brs, 6H), 4.61 (s, 6H), 3.64-3.61 (s, 6H), 2.67-2.61 (m, 12H), 2.08 (s, 9H), 1.11 (t, $J = 7.4$ Hz, 9H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_{ppm} 182.6, 143.9, 132.7, 43.0, 42.5, 32.9, 23.2, 16.8,

14.9. ES-MS (ESI+) m/z: 649.2336. HRMS (ESI) calcd. for $C_{27}H_{48}N_6S_6$ ($M+H$)⁺: 649.2337, found: 649.2336.

Compound 4.3b: ¹H NMR (600 MHz, DMSO-*d*₆): δppm 8.44 (s, 1H), 8.36 (brs, 1H), 7.57-7.49 (m, 4H), 6.44 (brs, 2H), 6.35 (s, 1H), 4.57 (s, 6H), 3.05 (s, 4H), 2.79 (s, 2H), 2.60 (s, 6H), 2.06 (s, 6H), 1.88 (s, 6H), 1.29-1.07 (m, 9H). ¹³C NMR (150 MHz, DMSO-*d*₆) δppm 173.0, 152.9, 147.6, 144.8, 143.9, 136.9, 100.7, 45.1, 32.9, 23.2, 22.6, 22.0, 16.8, 15.0. ES-MS (ESI+) m/z: ($M+3H$)⁺: 783.2679 ($M+4H$)⁺ 784.2703, ($M+5H$)⁺ 785.2652.

Compound 4.3c: ¹H NMR (600 MHz, DMSO-*d*₆) δppm 9.53-9.43 (m, 1H), 9.22-8.75 (m, 3H), 7.57-7.32 (m, 2H), 4.70-4.48 (m, 6H), 4.04-3.99 (m, 1H), 3.77 (s, 2H), 3.63-3.61 (m, 3H), 2.83-2.76 (m, 4H), 2.69-2.63 (m, 8H), 2.18 (s, 8H), 2.15 (s, 4H), 2.09-2.05 (m, 6H), 1.13-1.11 (m, 9H). ¹³C NMR (150 MHz, DMSO-*d*₆) δppm 168.9, 146.7, 129.5, 128.8, 62.8, 55.5, 49.1, 44.7, 42.6, 26.7, 25.5, 25.3, 24.0, 16.7, 15.5. ES-MS (ESI+) m/z: [$M - 2I + H$]⁺ 821.2031.

Compound 4.3d: ¹H NMR (600 MHz, DMSO-*d*₆): δppm 9.52 -9.49 (m, 1H), 9.14-8.98 (m, 2H), 8.79-8.75 (m, 1H), 7.48-7.31 (brs, 2H), 4.64-4.51 (m, 6H), 4.04-3.98 (m, 1H), 3.77 (brs, 2H), 3.62-3.60 (m, 3H), 2.98-2.96 (m, 1H), 2.82-2.81 (m, 3H), 2.68-2.62 (m, 8H), 2.18 (s, 8H), 2.15 (s, 4H), 2.08-2.04 (m, 6H), 1.12-1.10 (m, 9H). ¹³C NMR (150 MHz, DMSO-*d*₆) δppm 168.9, 146.6, 129.4, 128.7, 62.7, 55.4, 49.0, 44.6, 42.5, 26.6, 25.2, 23.9, 16.6, 15.5. ES-MS (ESI+) m/z: [$M/3 + NH_4^+$] 337.1539. MALDI-TOF m/z: [$M/2 + 1H$]⁺ 478.5.

Compound 4.3e: ¹H NMR (600 MHz, DMSO-*d*₆) δppm 13.07 (brs, 3H), 9.81-9.21 (m, 4H), 7.99-7.97 (m, 1H), 7.93-7.90 (m, 4H), 7.63 (s, 1H), 7.61 (m, 2H), 7.58-7.42 (m, 4H), 4.76 (s, 1H), 4.61-4.45 (m, 11H), 3.76 (s, 3H), 3.63 (s, 3H), 2.72 (s, 4H), 2.62 (s, 8H), 2.08 (s, 9H), 1.12-1.06 (m, 9H). ¹³C NMR (150 MHz, DMSO-*d*₆) δppm 183.1, 167.6, 165.3, 144.5, 144.3, 143.3, 140.7, 131.0, 130.3, 130.1, 129.9, 129.8, 129.7, 129.4, 44.9, 43.3, 42.6, 37.2, 33.8, 33.0, 23.5, 23.4, 23.2, 16.9, 16.8, 16.6, 15.3, 15.0, 14.8. ES-MS (ESI+) m/z: [$M - 2BF_4^- + NH_4^+$] 1159.7907, [$M - 2BF_4^- - 2Me + H$]⁺ 1111.7909. MALDI-TOF m/z: [$(M - BF_4)/3 + 1H$]⁺ 410.1.

Compound 4.3f: ^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ_{ppm} 8.44 (s, 1H), 8.35-8.34 (m, 1H), 7.61-7.49 (m, 4H), 6.50 (s, 2H), 6.45-6.34 (m, 1H), 4.57 (s, 6H), 3.84 (s, 2H), 3.05 (s, 2H), 2.79 (s, 4H), 2.59 (s, 4H), 2.06 (s, 6H), 1.88 (s, 12H), 1.22 (s, 3H), 1.08-1.07 (m, 6H). ^{13}C NMR (150 MHz, $\text{DMSO-}d_6$) δ_{ppm} 173.0, 145.5, 144.8, 143.9, 137.0, 100.7, 100.4, 63.5, 44.8, 43.2, 32.9, 31.7, 30.3, 29.5, 23.2, 22.6, 21.9, 16.8, 15.0, 14.4. ES-MS (ESI $^{+}$) m/z : $[\text{M} + \text{Br}^{-} + \text{H}]^{+}$ 1145.5355, $[\text{M} + \text{Br}^{-} + 2\text{H}]^{+}$ 1146.5326, $[\text{M} + \text{Br}^{-} + 2\text{H}]^{+}$ 1147.5347. MALDI-TOF m/z : $[\text{M}/2 + 2\text{H}]^{+}$ 534.6, $[\text{M}/2 + 3\text{H}]^{+}$ 535.3, $[\text{M}/2 + 4\text{H}]^{+}$ 536.6.

Compound 4.3g: ^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ_{ppm} 8.45-8.42 (m, 1H), 8.34-8.33 (m, 1H), 7.75-7.49 (m, 4H), 6.50-6.43 (m, 2H), 6.35-6.33 (m, 1H), 4.57 (s, 6H), 3.83 (s, 2H), 3.04 (s, 2H), 2.78 (m, 4H), 2.59 (s, 4H), 2.06 (s, 6H), 1.87 (s, 12H), 1.22 (s, 3H), 1.09-1.06 (m, 6H). ^{13}C NMR (150 MHz, $\text{DMSO-}d_6$) δ_{ppm} 173.2, 145.6, 144.9, 144.1, 137.1, 100.8, 100.5, 63.6, 44.9, 43.3, 33.1, 31.9, 30.4, 29.6, 23.3, 22.7, 22.1, 16.9, 15.1, 14.5. ES-MS (ESI $^{+}$) m/z : $[\text{M} + \text{Br}^{-}]$ 1063.5571. MALDI-TOF m/z : $[\text{M-2BF}_4]$ 810.5, $[\text{M-2BF}_4 + 2\text{H}]^{+}$ 812.5, $[\text{M-2BF}_4 + 4\text{H}]^{+}$ 814.5.

4.4.2. Anion binding analysis by ^1H -NMR titrations

Described at section 2.4.2.1.

4.4.3. Crystallographic Study

The crystal was obtained by the crystallization of compound **4.3a** in the presence of $\text{Me}_4\text{N}^{+}\text{Cl}^{-}$ in DMSO solvent at room temperature. The crystallographic data were recorded using SMART software by the Bruker Nonius SMART APEX CCD diffractometer equipped with a graphite monochromator and Apex CCD camera. All crystallographic data were refined using the software SHELXL-2014/7 and or Olex2 1.2-alpha. The ORTEP diagram was obtained with the help of ORTEP software with 50% thermal ellipsoid. The tetrabutylammonium chloride salt was used as a chloride ion source for co-crystallization with the compound **4.3a** in the presence of DMSO solvent. The crystallographic parameters and refinement data were listed in Table 4.2. All H-atoms are omitted from the ORTEP diagram for clarity.

4.4.4. Job's plot experiment

Described in section 2.4.3.

4.4.5. Ion transport studies

4.4.5.1. Ion transport experiments through fluorescence spectroscopy

4.4.5.1.1. Preparation of EYPC/CHOL-LUVs

Described in section 2.4.5.1.1

4.4.5.1.2. Ion transport activity (HPTS) assay

Described in section 2.4.5.1.2.

4.4.5.1.3. Quantitative measurement of transport activity from HPTS

Described in section 2.4.5.1.3.

4.4.5.1.4. Measurement of half-maximal effective concentrations (EC₅₀) of the compound from HPTS assay

Described in section 2.4.5.1.8.

4.4.5.2. Ion Transport Experiments through ion-selective electrode

4.4.5.2.1. Chloride ion efflux studies using chloride ion-selective electrode (chloride-ISE)

Described in section 3.4.5.1.1.

4.4.5.2.2. Preparation of EYPC/CHOL-LUV

Described in section 3.4.5.1.2.

4.4.5.2.3. Chloride efflux study across EYPC/CHOL-LUVs

Described in section 3.4.5.1.3.

4.4.5.2.4. Quantitative measurement of transport activity from chloride-ISE assay

The Cl⁻ ion efflux efficiency of the compounds was normalized, and the Cl⁻ ion efflux 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 s (prior to the addition of Triton X-100 solution) were considered for the measurement of chloride transport efficiency of the compounds-

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

Eq.4.1

Where, EE_t = Cl^- ion efflux efficiency at $t = 500$ s (prior to the addition of Triton X-100 solution), EE_0 = Cl^- ion efflux efficiency immediately before the addition of the compounds ($t = 0$ s) and EE_∞ = Cl^- ion efflux efficiency after addition of Triton X-100 solution (i.e., at saturation after complete leakage at $t = 700$ s).

The differences in the Cl^- ion transport activities of the compounds between the HPTS and ISE-based assays could be due to the concentration differences of the compounds used for both the assay.

4.4.6. Ion Selectivity Studies

4.4.6.1. Anion selectivity assay

Described in section 2.4.6.1.1

4.4.6.2. Cation selectivity assay

Described in section 2.4.6.1.3

4.4.7. Evidence for the mechanistic pathway for chloride ion transport

4.4.7.1. Preferential ion selectivity assay using FCCP

Described in section 3.4.7.1

4.4.7.2. Preferential ion selectivity assay using valinomycin

Described in section 3.4.7.2

4.4.8. Evidence for the carrier mechanism

4.4.8.1. Cholesterol dependency assay

The LUVs were prepared according to the procedure mentioned in section 2.4.5.1.1. by varying the molar ratios of EYPC and cholesterol as 6:4, 8:2, and 10:0 to delve into the fact whether increasing the cholesterol concentration in preparing the vesicles (cholesterol is known to increase the rigidity of the lipid bilayer) has any effect on the Cl^- ion transport activity of the compound. The chloride transport activity of the compound was measured using ISE. In case of a mobile carrier the transport activity was

anticipated to decrease on increasing the molar ratio of cholesterol (increasing cholesterol slows the diffusion process) while for an ion channel mechanistic pathway, no difference in transport activity should be there. This similar assay procedure, as described in section 2.4.8.3. was followed using three compositions of liposomes. No significant change was observed in the chloride ion transport activity, indicating the mobile carrier mechanism being followed by the compound.

4.4.8.2. U-tube experiment

The 'U-tube' experiment was performed to confirm the mechanistic pathway for the Cl^- ion transport. The lipid bilayer was mimicked here by using nitrobenzene as the organic layer. First, compound (0.2 mM) and $\text{TBA}^+\text{PF}_6^-$ (2 mM) were dissolved in nitrobenzene (16 mL) and placed in a U-tube (1.5 cm cone with 15 cm arm length). The $\text{TBA}^+\text{PF}_6^-$ (2 mM) was used to provide a counter anion for the transportation of Cl^- ion. In the source end, 8 mL of 5 mM phosphate buffer at pH 7.2 containing 489 mM NaCl was used. In the receiver end, 10 mL of 5 mM phosphate buffer at pH 7.2 containing 489 mM NaNO_3 was used. The organic phase was stirred gently using a magnetic stirrer, and the Cl^- ion concentration of the receiver end was monitored using a chloride ion-selective electrode.

4.4.9. Test for the leaching-out of the compounds from the membrane bilayer environment.

Described in section 3.4.9.

4.4.10. Evidence of vesicle stability in the presence of the compounds using calcein assay

Described in section 3.4.11.

4.4.11. Regeneration of the active transporter by dealkylation of the proanionophores:

The tetrafluoroboratesulfonium salt **4.3e** (1 mM) was dissolved in 10 mM PBS, pH 7.4 containing 10 mM reduced glutathione (GSH), and the mixture was incubated at 37 °C. At different time points, an aliquot of the reaction solution was removed and monitored by HPLC analysis. The aliquot was dissolved in methanol before its injection into the

analytical HPLC (Waters 600E HPLC) system. The Hypersil GOLD™ C18 Selectivity LC Column and a UV-detector (214 nm) were used for the analysis. Methanol/water gradient (70% methanol and 30% water) was used as the mobile phase at a 1.0 mL/minute flow rate for 20 minutes run time. Regeneration of lead compound and reduction of sulfonium compound was analyzed by inbuilt software and confirmed by ES-MS (ESI+).

4.4.12. Biological Activities of the Compounds

4.4.12.1. MTT-based cytotoxicity assay

HeLa, BHK-21, and MDCK cells were seeded in 96-well flat-bottom tissue culture plates at a density of 10^4 cells/well (per 100 μ L) in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS). The detailed experimental procedure is similar which is As described in section 2.4.11.1

4.4.12.2. Measurement Cl^- transport in the presence of the compounds

Transport of Cl^- ion in the presence of compound into the HeLa cell was measured by using chloride selective cell-permeable fluorescent dye *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE). The detailed experimental procedure is similar which is As described in section 2.4.11.3

4.4.12.3. Cellular uptake of the compounds

The uptake of NBD-tagged compounds was also investigated under the cellular environment. The HeLa cells were seeded on a cover slip and incubate for 16 hours at 37 °C and 5% CO_2 . After the incubation period, the media was removed and washed three times with PBS. The NBD-tagged compounds were added at 50 and 100 μ M concentrations and incubated for additional 4 hours. The cells were then washed three times with PBS, and fixation was performed with 4% formaldehyde at room temperature for 10 minutes. After that, cells were washed 3-4 times, and the slides were prepared for microscopic (both confocal and fluorescence) analyses

4.4.13. Transport of Chloride Ion across Giant Unilamellar Vesicles (GUVs):

4.4.13.1. Preparation of GUVs

In a clean and dry glass vial EYPC, cholesterol, and DPPS (from the respective stock solution) were taken in the molar ratio of 6:3.5:0.5 and dried under vacuum for 6

hours. To that lipid film, 200 μL of light liquid paraffin oil was added, and the mixture was sonicated for 30 minutes; until the lipid film gets fully dissolved in the paraffin oil. After that, 20 μL of the upper buffer (100 mM HEPES, 200 mM sucrose, 1 mM 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) in H_2O , $\text{pH} = 7.4$) was added, and the solution was mixed thoroughly to form an emulsion. The emulsion was then carefully added to 500 μL of lower buffer (100 mM HEPES, 200 mM glucose in H_2O , $\text{pH} = 7.4$) in a centrifuge tube, and the whole mixture was pipetted up and down thoroughly to mix everything. The emulsion was centrifuged for 15 minutes at 10000 rpm to remove the oil as well as the extravehicular free dye. This process was repeated 4-5 times to remove the maximum amount of the paraffin oil from the solution. The final pellet (dense solution containing the GUVs) was mixed with 100 μL of lower buffer (final vesicle conc. of 15 mM).

4.4.13.2. Ion transport measurements using GUVs coated on the glass surface

The coated glass surface was prepared by following the reported method with minor modifications. In an acid-washed glass vial, *N*-(3,4-dihydroxyphenethyl)palmitamide and DPPS solutions were taken in the molar ratio of 1:4 and dried under reduced pressure for 6 hours to remove any traces of organic solvents. The lipid mixture was then dissolved in 0.1 mL of THF/water (9:1 v/v), and the solution was added to 0.9 mL of 5 mM PBS buffer, $\text{pH} 5.6$ (final conc. of 0.2 mM). The solution was then sonicated for 5 minutes. The prepared vesicle solution was directly used for the glass coating. The vesicle solution was drop casted on the surface of a glass-bottom disk and incubated overnight at 37 $^{\circ}\text{C}$. Finally, the substrate was washed with HEPES buffer thoroughly.

The prepared GUV solution (100 μL) and compound **4.3a** were added to the hydrophobic coated glass-bottom dish. The GUVs were allowed to settle down for 5 hours. After that, NaCl solution (0.1 M) was added. The images were recorded (in a Nikon ECLIPSE Ts2R fluorescence microscope) before and after the addition of NaCl in the green as well as in the bright field channel to ensure the integrity of the GUVs and quenching of HPTS fluorescence intensity. Here the decay of the HPTS fluorescence intensity is due to Cl^- influx and consequently OH^- efflux (as compound follows OH^-/Cl^- antiport mechanism for its chloride transport activity).

4.4.14. Regeneration of anionophore from proanionophore under cellular environments

HeLa cells were cultured in T25 culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) along with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, and 1% pyruvate (Gibco), maintaining at 37 °C in a humidified atmosphere with 5% CO₂. Cells were re-cultured and maintained for further study. The cells are counted using trypan blue stain using Countess II FL Automated Cell Counter (Thermo Fisher Scientific). In order to examine the effect of GSH and GSH inhibitors, 5×10^6 cells/well were seeded onto a six-well plate and maintained in DMEM media containing 10% FBS. After 12 h the cells were washed twice with PBS and treated with external GSH (10 mM) and GSH inhibitor (1 mM) in serum-free media for 12 h; after the incubation period, GSH and GSH inhibitor were aspirated and replaced with media containing 2%FBS. After 72 h the media was aspirated and washed twice with PBS. The cells were harvested by centrifugation at 480g for 10 min. The cell pellet was washed twice with ice-cold PBS and kept on ice. The pellets were re-suspended in 1 mL of the lysis buffer (150 mM NaCl + 50mM Tris-Cl (pH 7.4) and incubated in ice for 15 min. The lysate was centrifuged at 20,000g for 10 min at 4 °C. The supernatant was removed carefully in a fresh tube, and the samples were used for further experiments.

Later, matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (Autoflex speed, Bruker, Germany) analysis was carried out to confirm the formation of anionophore and intermediates. The HeLa cell lysates were mixed with matrix (Sinapinic acid) as 1:1 ratio for the MALDI-TOF analysis.

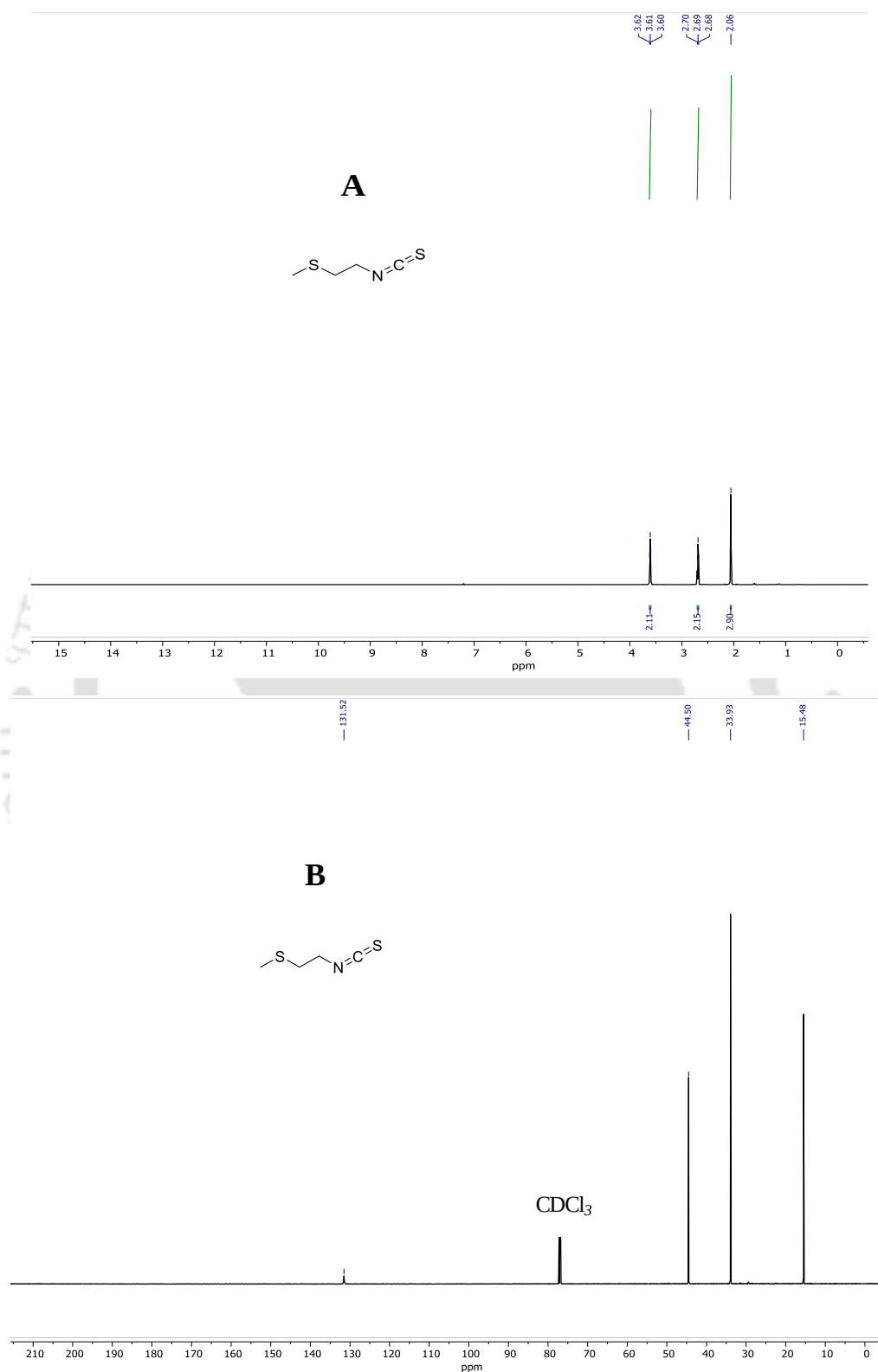
4.4.15. ^1H and ^{13}C spectra of synthesized compounds

Figure 4.12. ^1H NMR (A) and ^{13}C NMR (B) spectra of (2-isothiocyanatoethyl)(methyl)sulfane.

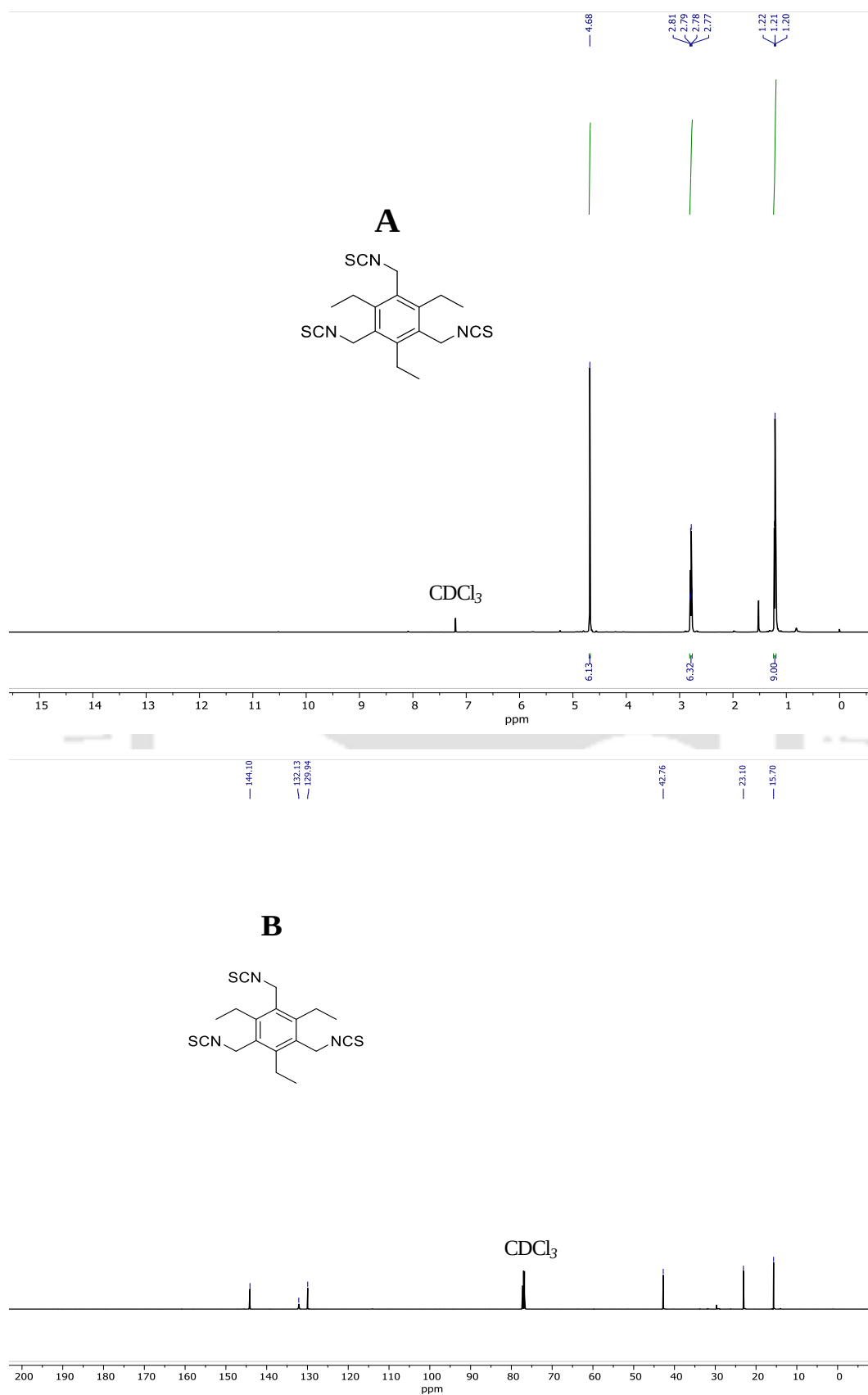


Figure 4.13. ^1H NMR (A) and ^{13}C NMR (B) spectra of 1,3,5-triethyl-2,4,6-tris(isothiocyatomethyl)benzene.

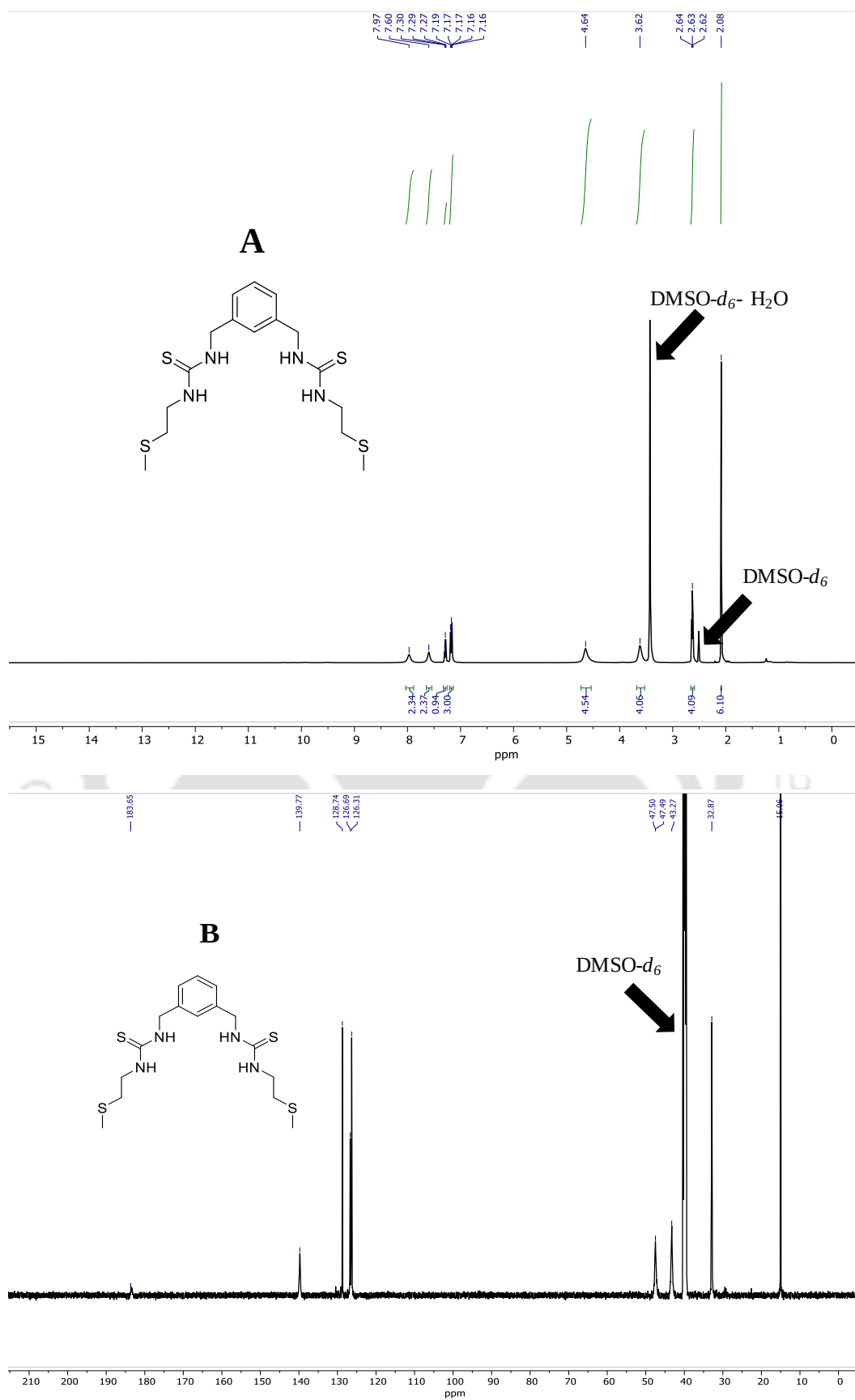


Figure 4.14. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 4.1.

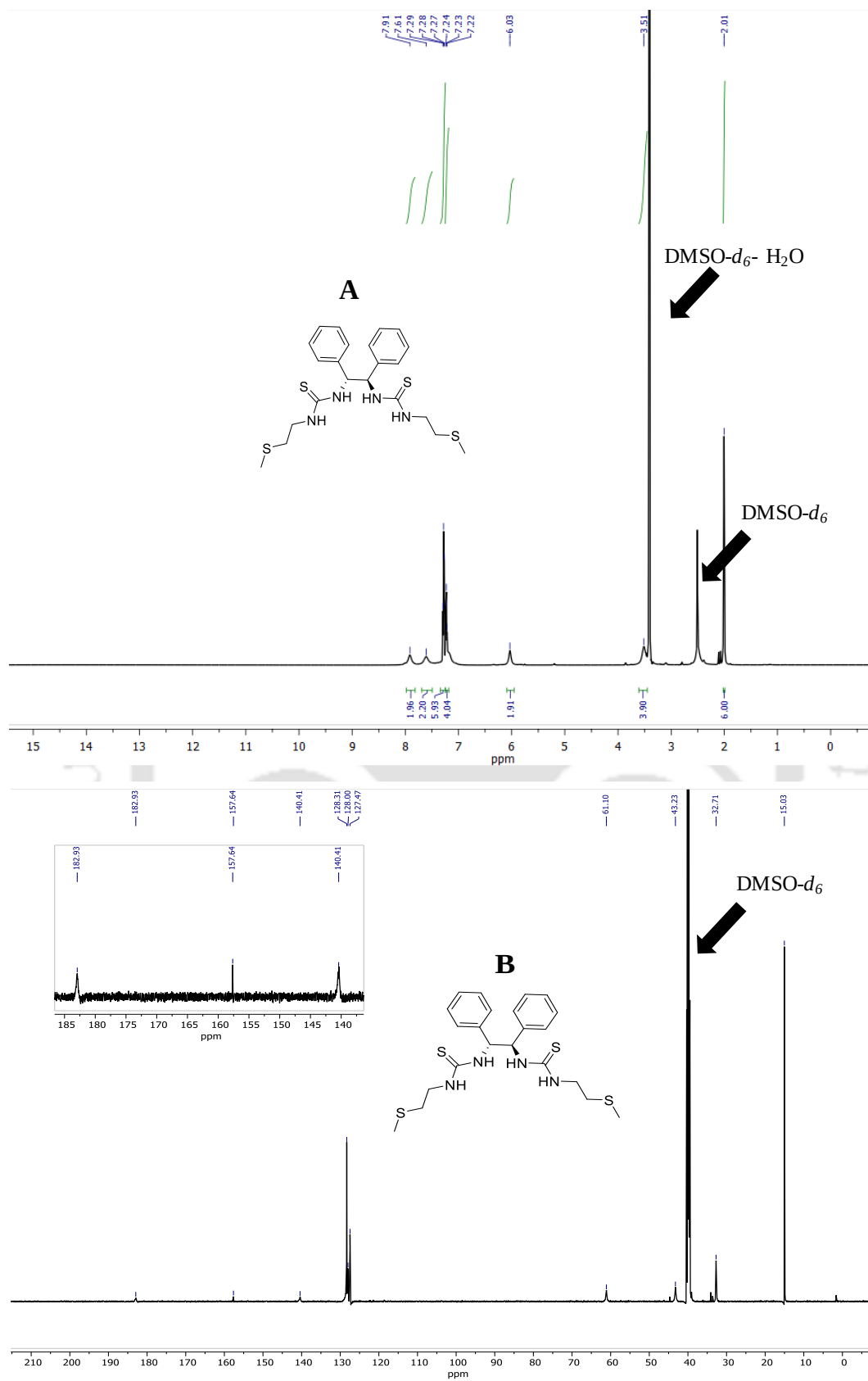


Figure 4.15. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 4.2.

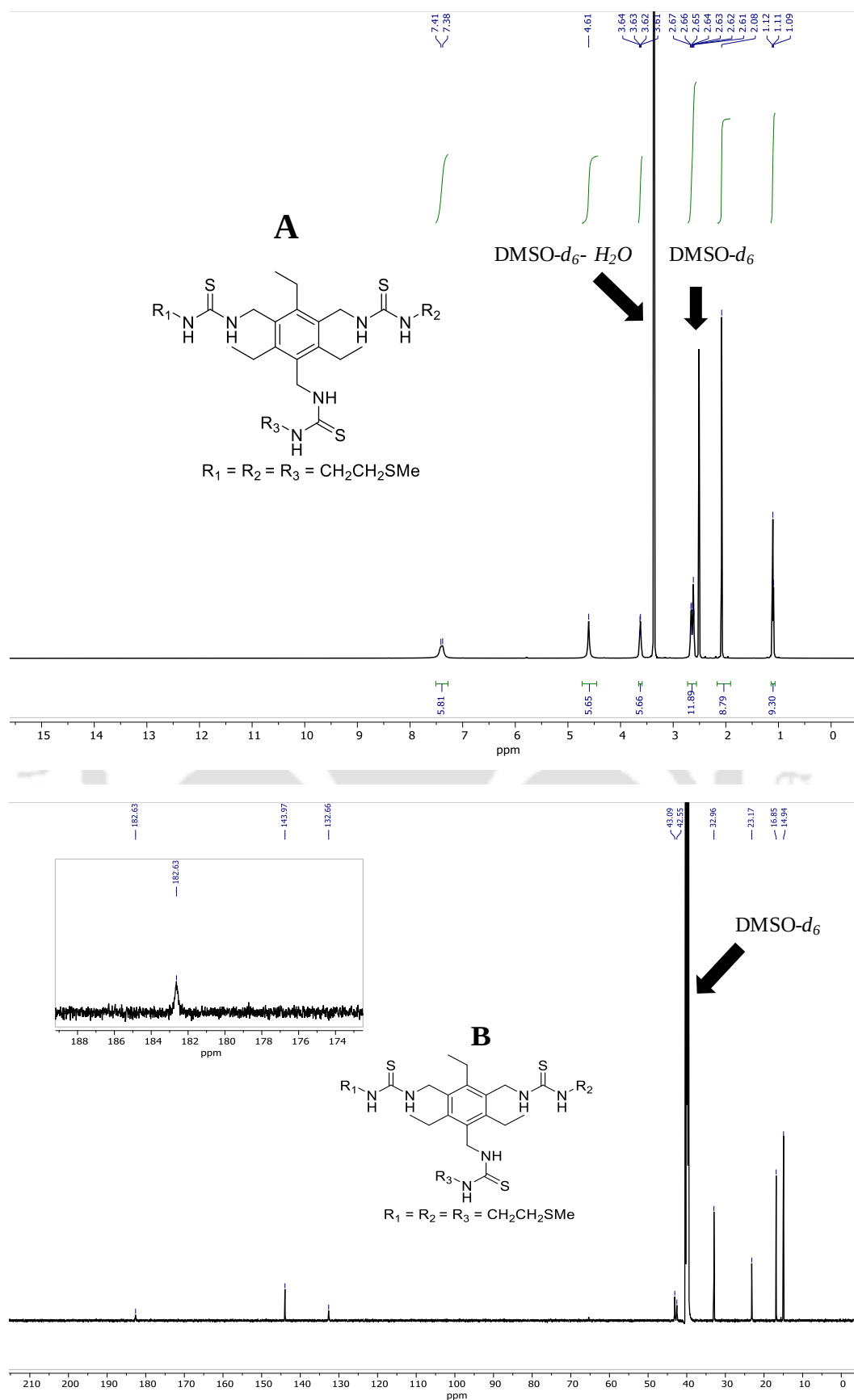


Figure 4.16. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 4.3a.

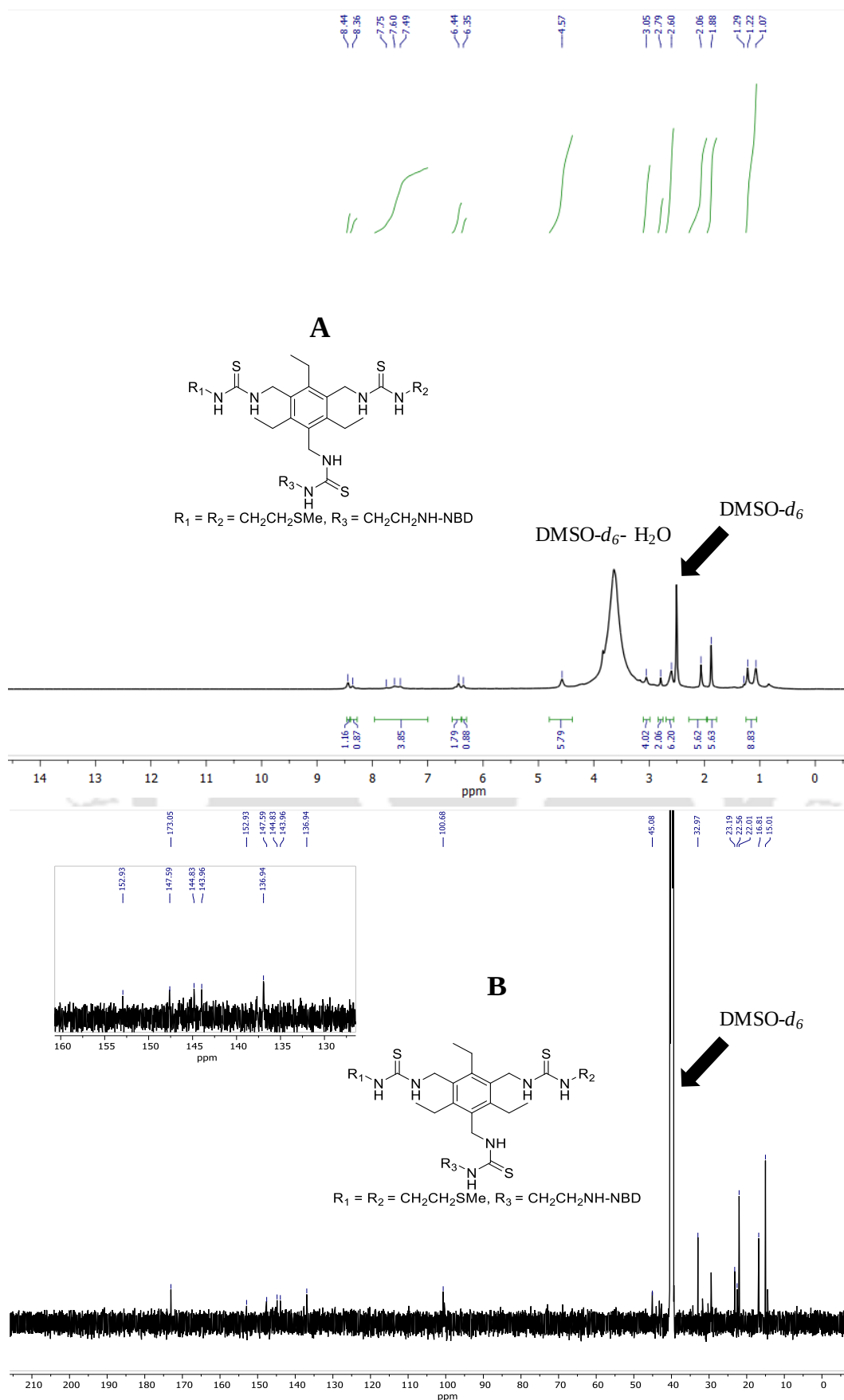


Figure 4.17. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.3b**.

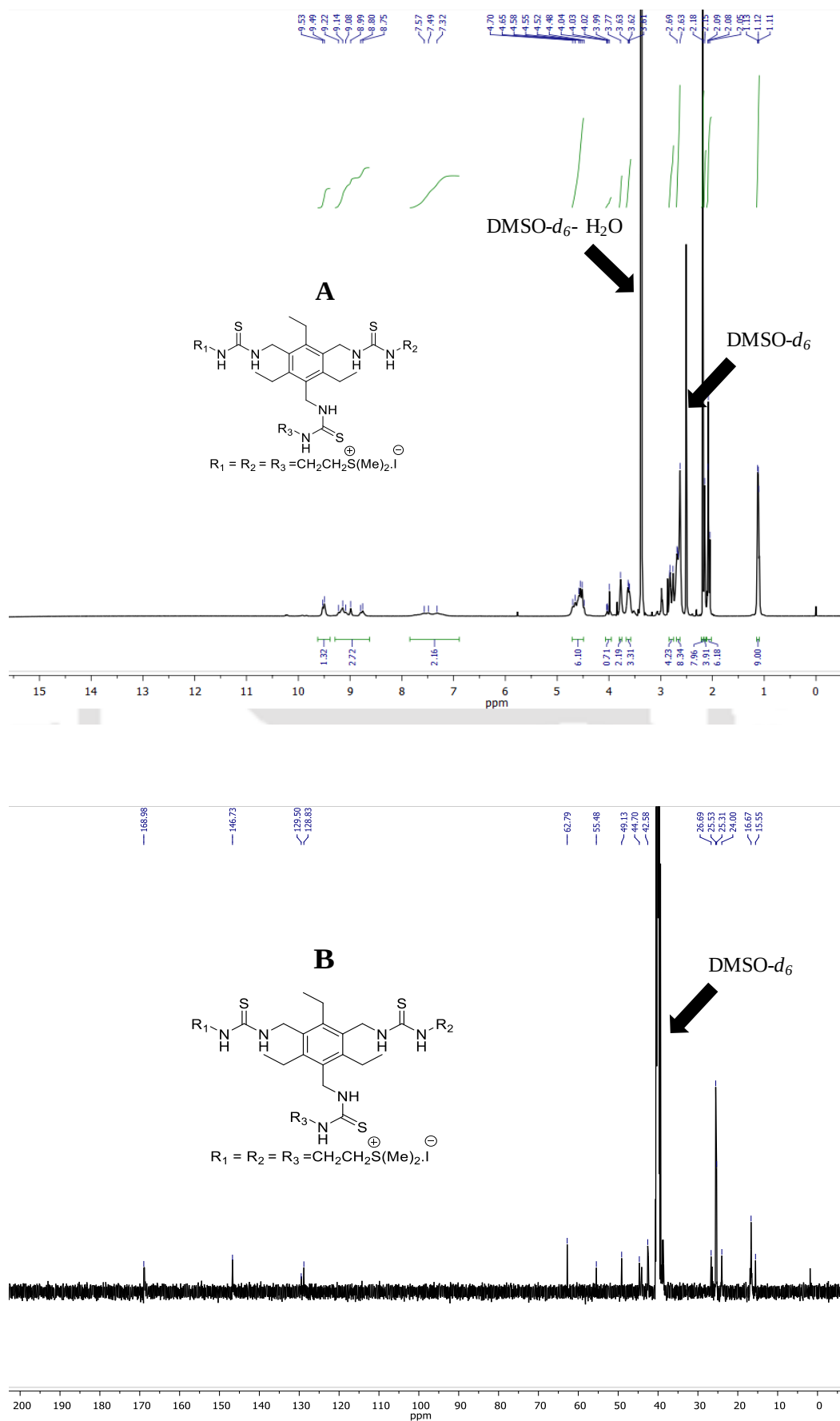


Figure 4.18. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.3c**.

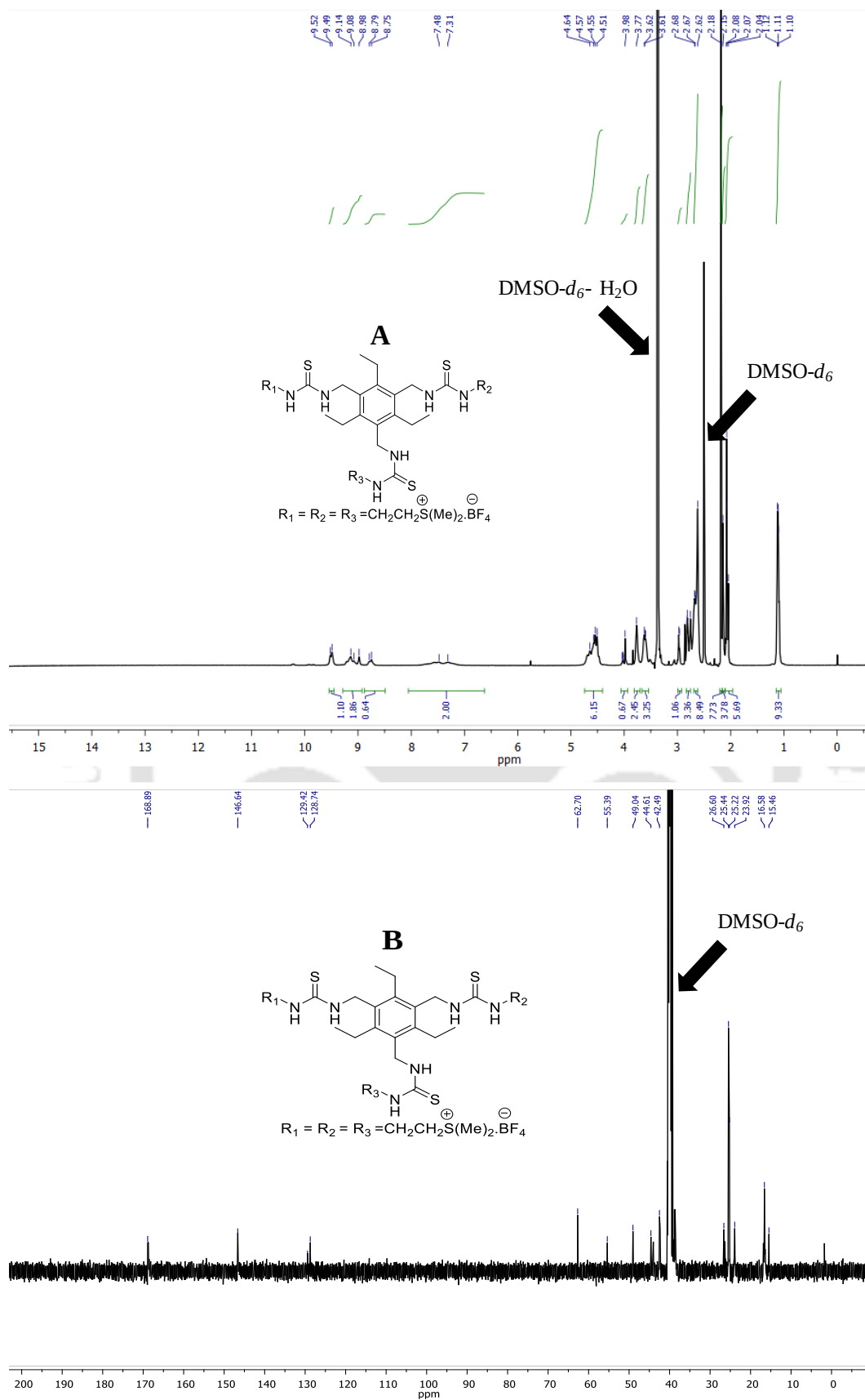


Figure 4.19. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.3d**.

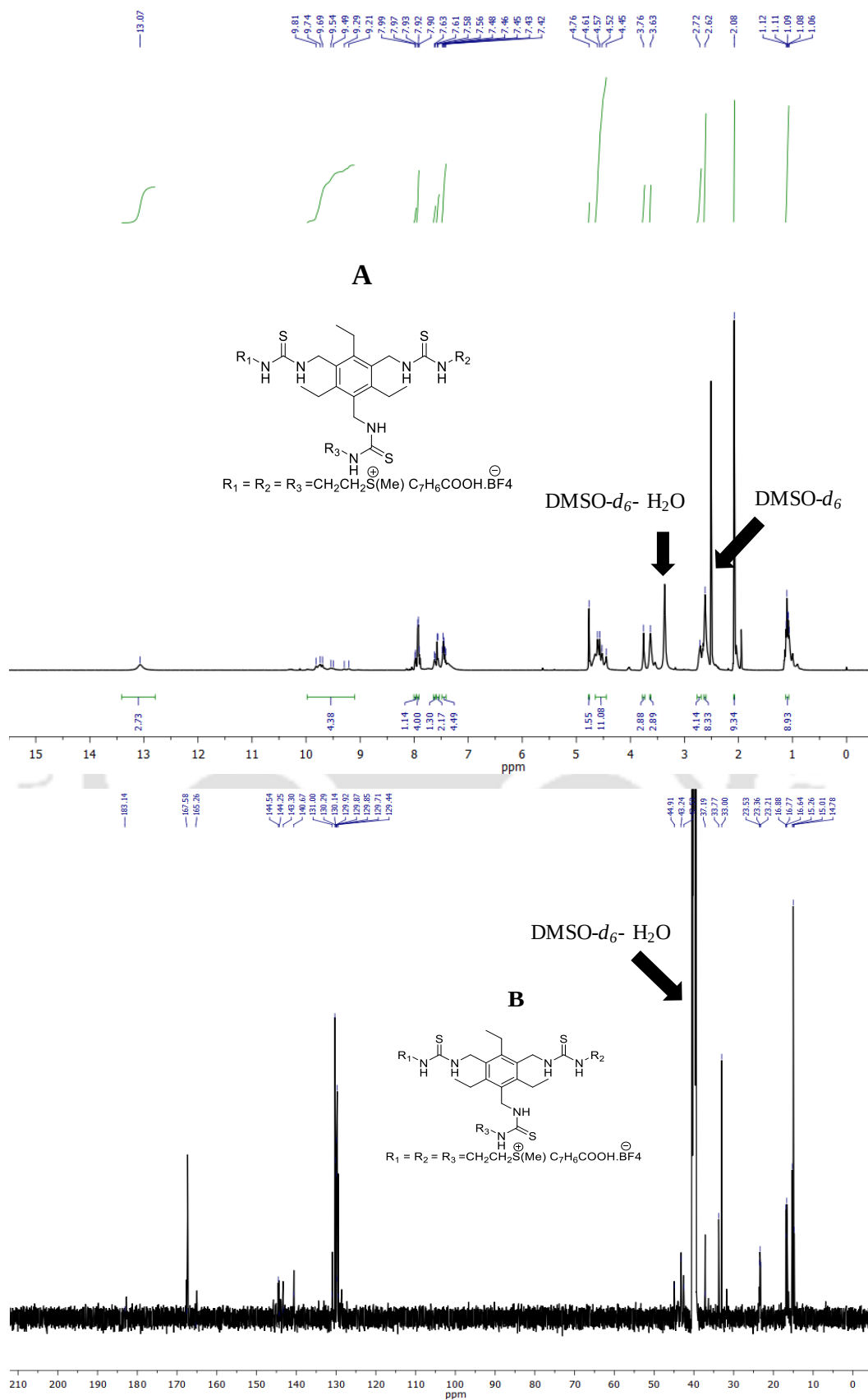


Figure 4.20. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **4.3e**.

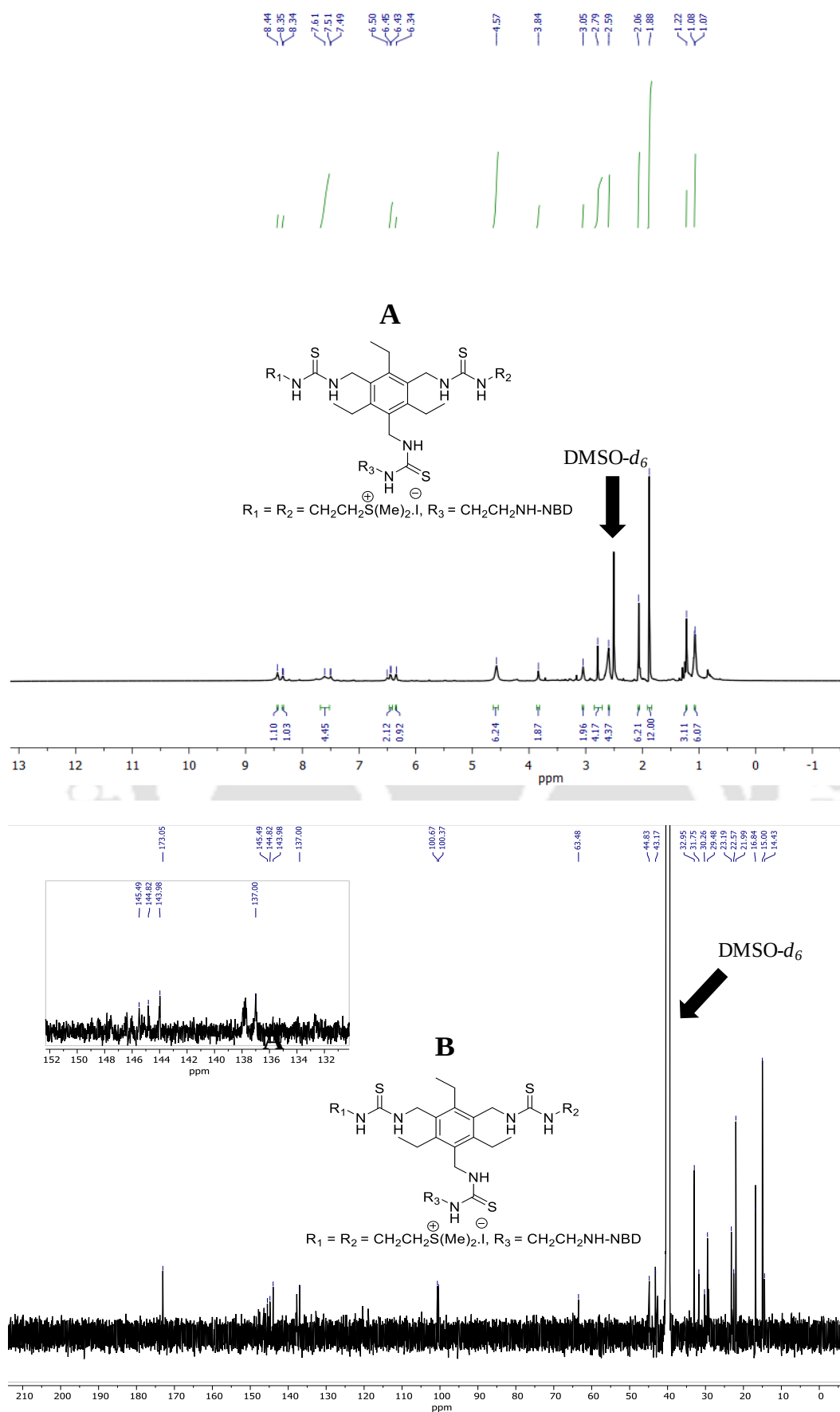


Figure 4.21. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 4.3f.

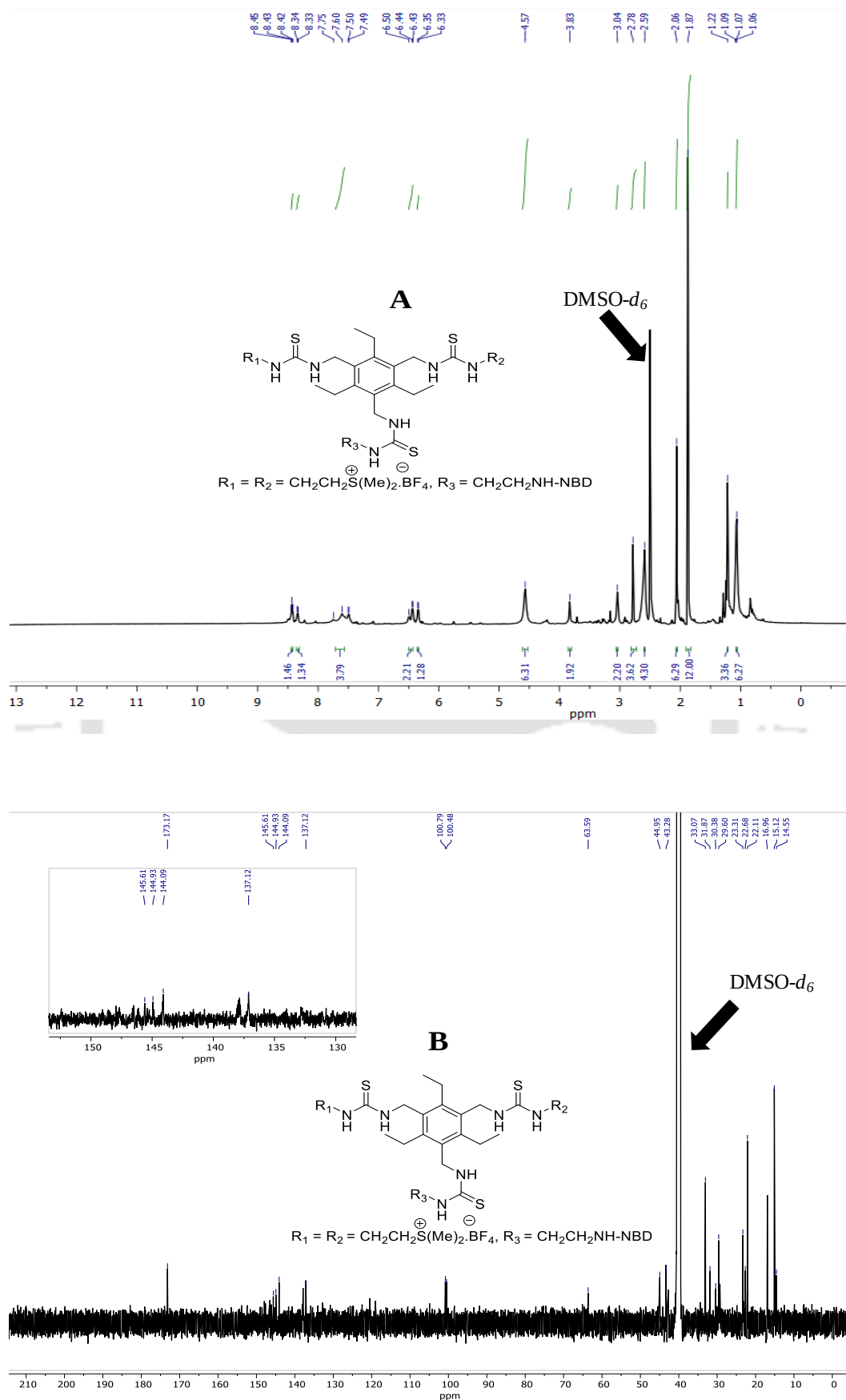


Figure 4.22. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 4.3g.

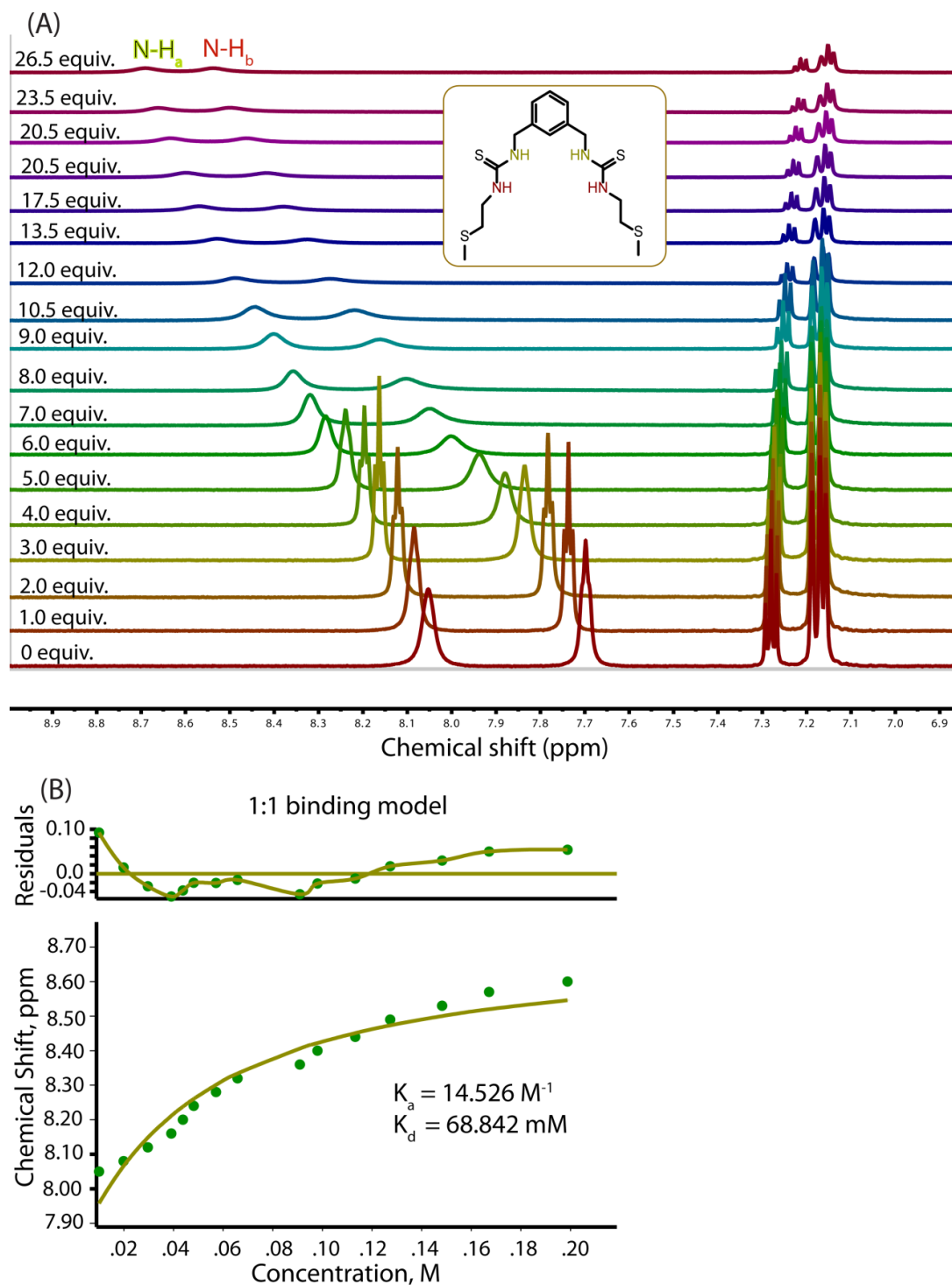
4.4.16. ^1H NMR titration plots

Figure 4.23. ^1H -NMR (600MHz) titration spectra for compound **4.1** with the sequential addition of TBACl in $\text{DMSO}-d_6$ solvent. The amounts of added TBACl are shown on the spectra (A). The plot of concentration of TBACl versus chemical shift of ^1H signal fitted to 1:1 binding model of WinEQNMR2 program (B).

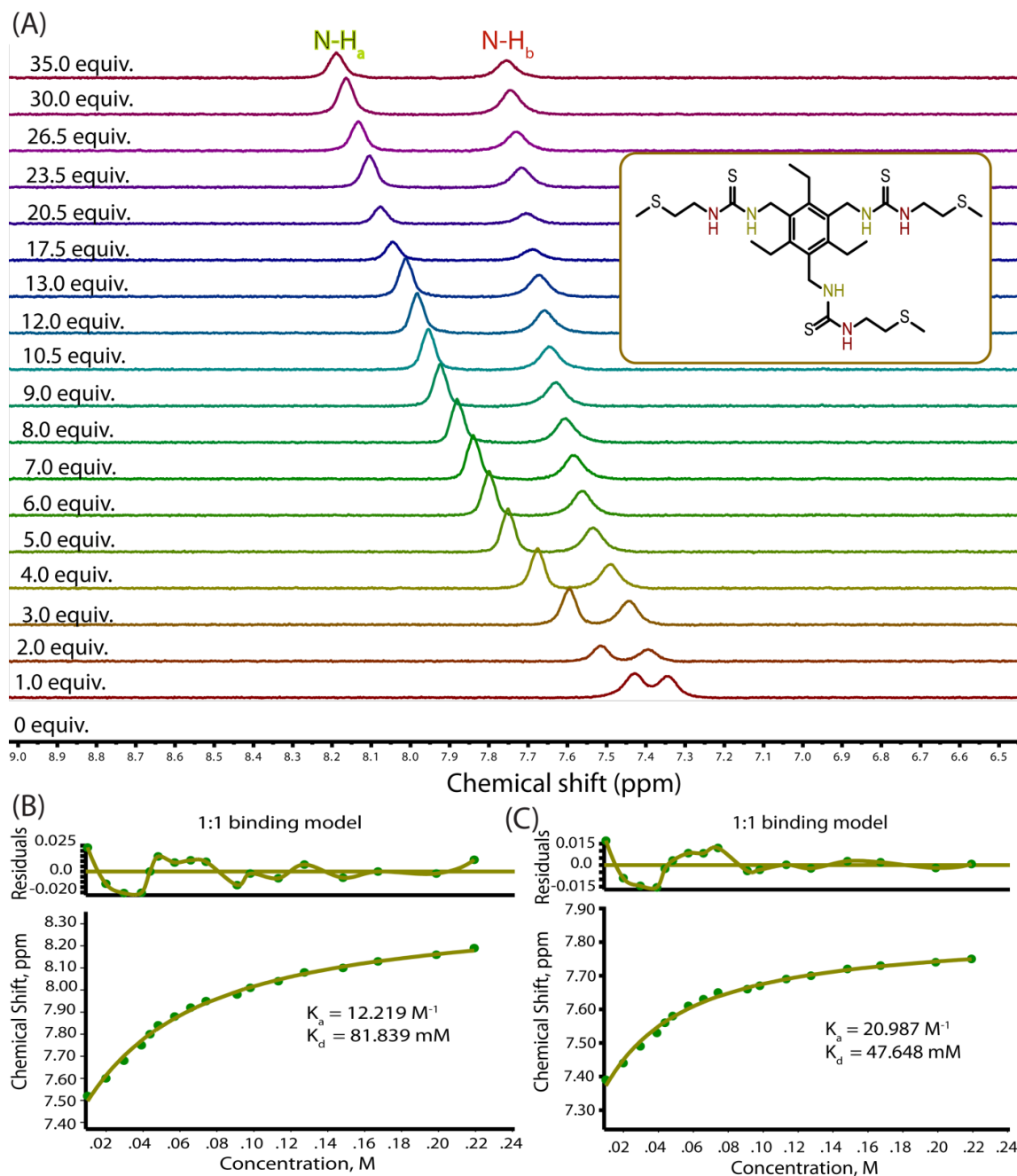
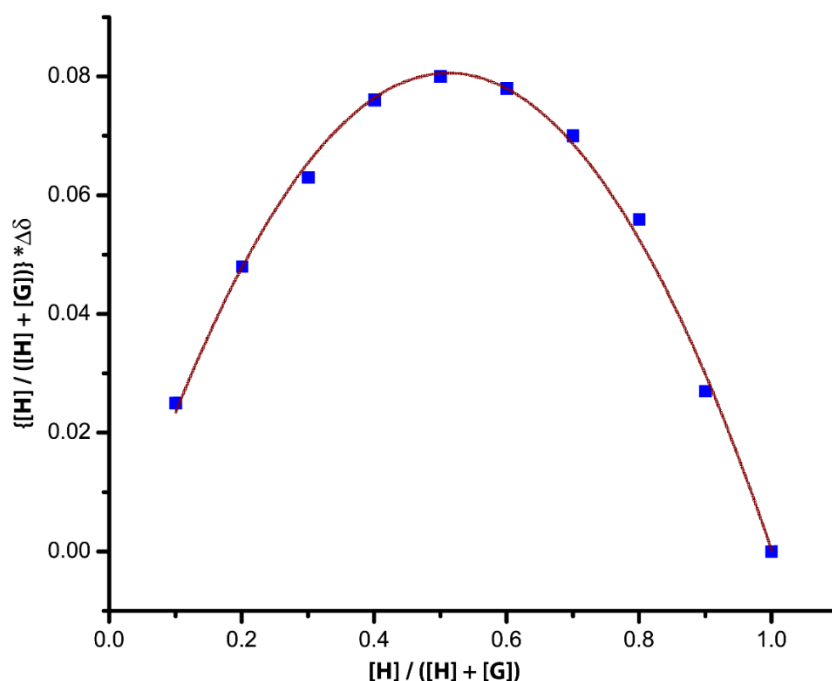


Figure 4.24. ^1H -NMR (600MHz) titration spectra for compound **4.3a** with the sequential addition of TBACl in $\text{DMSO}-d_6$ solvent. The amounts of added TBACl are shown on the spectra (A). The plot of concentration of TBACl versus chemical shift of ^1H signal (for N-H_a), fitted to 1:1 binding model of WinEQNMR2 program (B). The plot of concentration of TBACl versus chemical shift of ^1H signal (for N-H_b), fitted to 1:1 binding model of WinEQNMR2 program (C).

4.4.17. Calculations and plots for job's plot

Table 4.1. Calculation and result table for the Job's plot analysis.

Sample No.	Host conc. ([H], mM)	Guest conc. ([G], mM)	[H] [G] (mM)	+ [H]/ ([H]+[G])	δ of proton	$\Delta\delta$	$\{[H] /$ ([H] +[G]) $\} * \Delta\delta$
JB-1-4.3a	1.0	9.0	10	0.1	7.66	0.25	0.025
JB-2-4.3a	2.0	8.0	10	0.2	7.65	0.24	0.048
JB-3-4.3a	3.0	7.0	10	0.3	7.62	0.21	0.063
JB-4-4.3a	4.0	6.0	10	0.4	7.6	0.19	0.076
JB-5-4.3a	5.0	5.0	10	0.5	7.57	0.16	0.08
JB-6-4.3a	6.0	4.0	10	0.6	7.54	0.13	0.078
JB-7-4.3a	7.0	3.0	10	0.7	7.51	0.1	0.07
JB-8-4.3a	8.0	2.0	10	0.8	7.48	0.07	0.056
JB-9-4.3a	9.0	1.0	10	0.9	7.44	0.03	0.027
JB-10-4.3a	10	0.0	10	1	7.41	0	0

**Figure 4.25.** Job's plot for the compound 4.3a (according to Table 4.1).

4.4.18. Data and plots of crystal structure

Table 4.2. Crystal parameters and refinement data of the anion complex.

Parameters	Compound 4.3a
Formula	C ₂₇ H ₄₈ N ₆ S ₆ , C ₁₆ H ₃₆ N, Cl, O
Fw	942.98
Crystal system	triclinic
Space group	'P -1'
a/Å	12.8015(8)
b/Å	13.8204(8)
c/Å	16.5396(7)
$\alpha/^\circ$	67.517(5)
$\beta/^\circ$	87.324(4)
$\gamma/^\circ$	85.158(5)
V/Å ³	2693.8(3)
Z	2
Dc/g cm ⁻³	1.163
μ Mo K α mm ⁻¹	0.341
F000	1024.0
T/K	293(2)
θ max.	25.00
Total no. of reflections	18456
Independent reflections	9485
Parameters refined	557
R1, I > 2 σ (I)	0.0739
wR2, I > 2 σ (I)	0.1708
GOF (F2)	1.020
CCDC	1900065

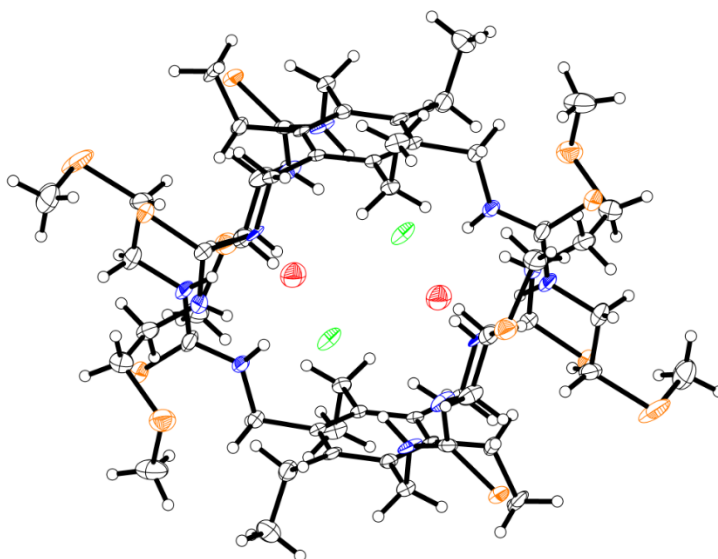


Figure 4.26. ORTEP diagram of complex **4.3a** (50% thermal ellipsoid plot, H-atoms are omitted for clarity). The TBA⁺ cations outside the cavity were omitted for clarity.

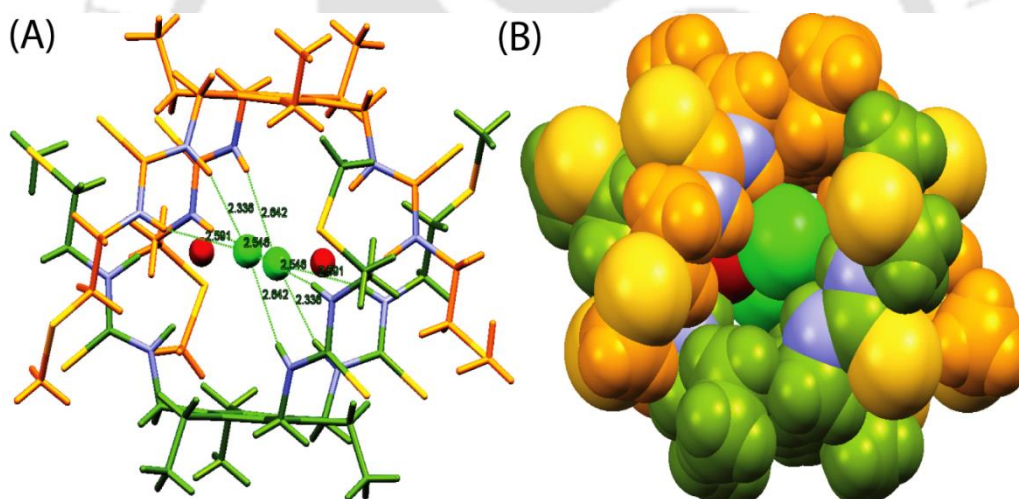


Figure 4.27. X-ray crystal structure depicting the non-covalent interactions involved in 2:2 Cl[−] ion encapsulation (capped sticks and space-fill model) by compound **4.3a** ((**4.3a**·Cl[−]·H₂O)₂), where the green ball represents the chloride ion, the red ball represents the water molecules, the purple color represents the nitrogen atom, and yellow color represents a sulphur atom. One molecule of compound **4.3a** is colored orange, and the other is colored grey. The TBA⁺ cations outside the cavity were omitted for clarity.

4.4.19. Ion transport related plots

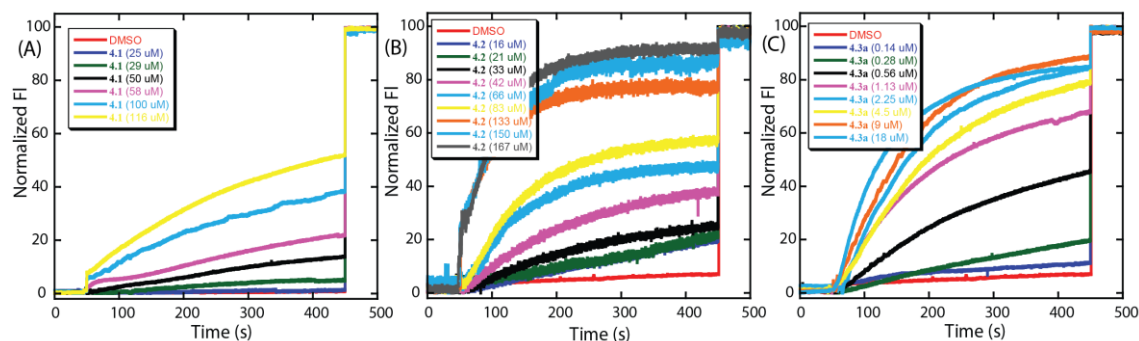


Figure 4.28. Concentration-dependent curves for the compounds **4.1** (A), **4.2** (B) and **4.3a** (C). The Cl^- ion transport activity of the compounds were measured across the EYPC/CHOL-LUV $\rightarrow$ HPTS.

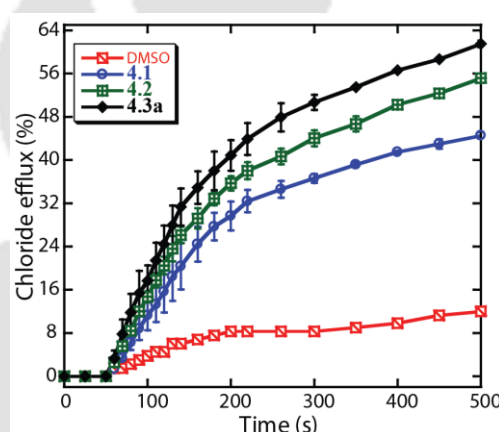


Figure 4.29. Transmembrane Cl^- ion transport activity of the compounds (**4.1-4.3a**, Compound concentration = 18 μM) across the EYPC/CHOL-LUVs as measured by chloride-ion selective electrode at pH 7.2.

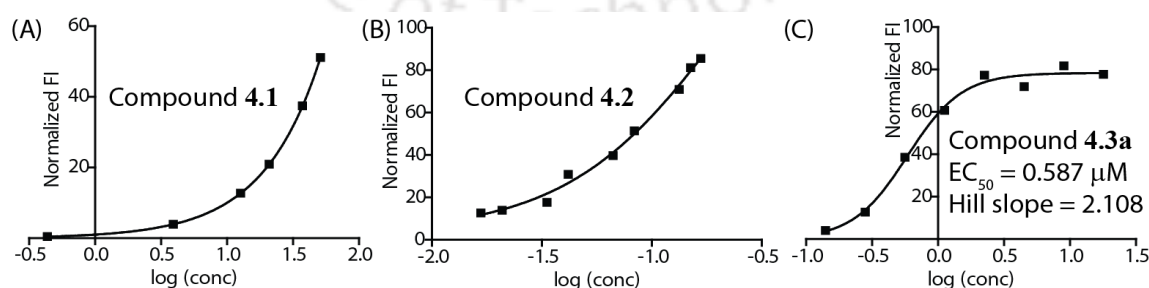


Figure 4.30. Concentration dependent Cl^- ion transport activity of the compounds across the EYPC/CHOL-LUV $\rightarrow$ HPTS. Dose-response plot of Cl^- ion transport activity of the compounds at 450 sec (after the addition of the compounds). The effect solvent on the

transport activities was corrected. The EC₅₀ values of compounds **4.1** and **4.2** could not be measured because of the aggregation formation at higher concentrations.

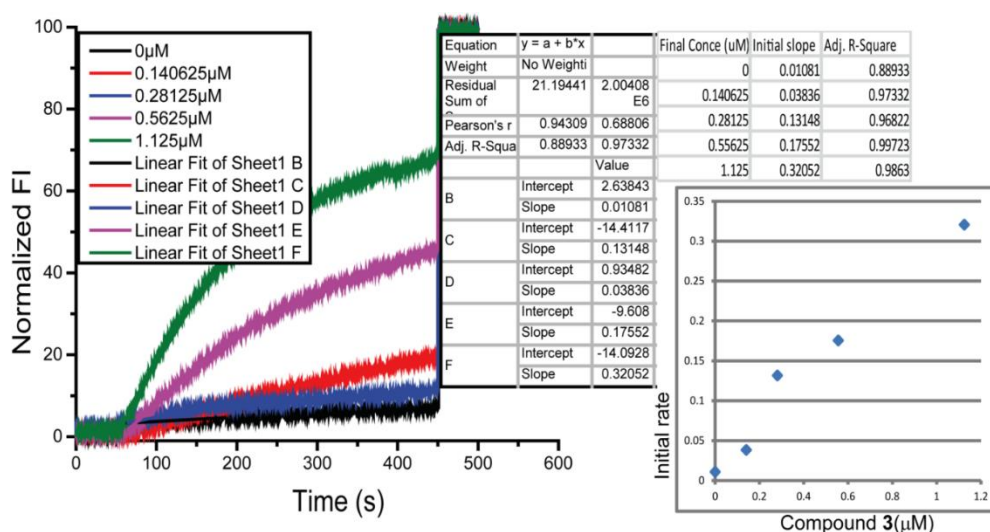


Figure 4.31. Calculation of the initial slope from the concentration dependent transport activities of compound **4.3a**. Plot of the initial rates of Cl⁻ ion transport activities at different concentrations of compound **4.3a**.

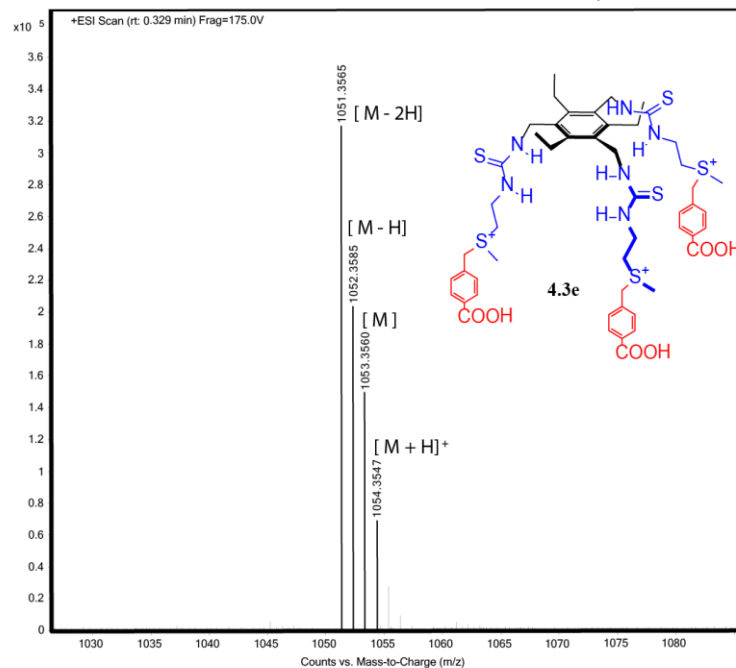
Table 4.3. Aqueous solubility of the selected compounds.

Compound Code	Aqueous solubility (mg/mL)
4.3a	insoluble ^a
4.3b	2.0
4.3c	3.3
4.3d	4.0
4.3e	2.5
4.3g	3.3

^ainsoluble in 1 mL water.

4.4.20. Regeneration confirmation by mass data and plots

Sample Name	SAMPLE	Position	P1-D11	Instrument Name	Instrument 1
User Name		Inj Vol	20	InjPosition	
Sample Type	Sample	IRM Calibration Status	Success	Data Filename	DI-ADUCT-HPLC.d
ACQ Method	ESI ALS 200-1500.m	Comment		Acquired Time	17-05-2019 12:54:29 (UTC+05:30)



Sample Name	SAMPLE 11	Position	P2-A6	Instrument Name	Instrument 1
User Name		Inj Vol	20	InjPosition	
Sample Type	Sample	IRM Calibration Status	Success	Data Filename	EXP-M-ADD-HPLC.d
ACQ Method	ESI ALS 100-1500.m	Comment		Acquired Time	22-05-2019 16:10:55 (UTC+05:30)

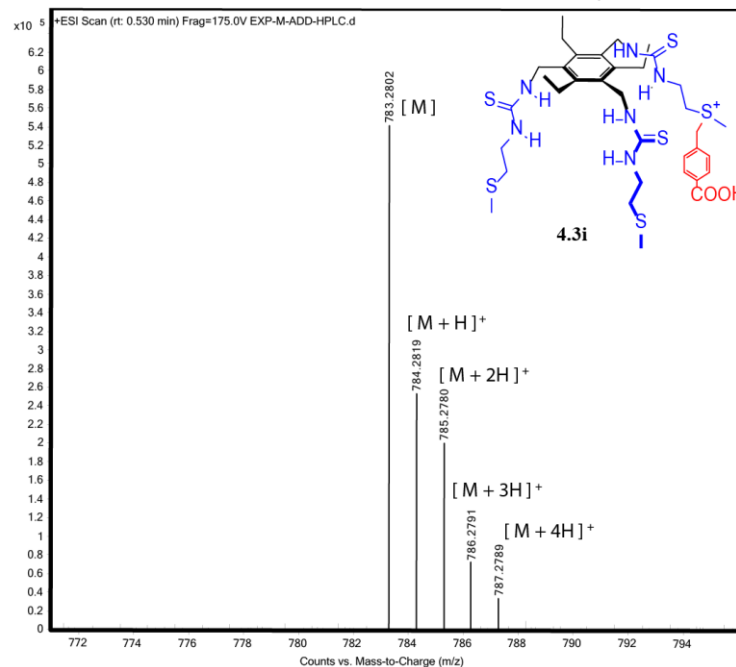
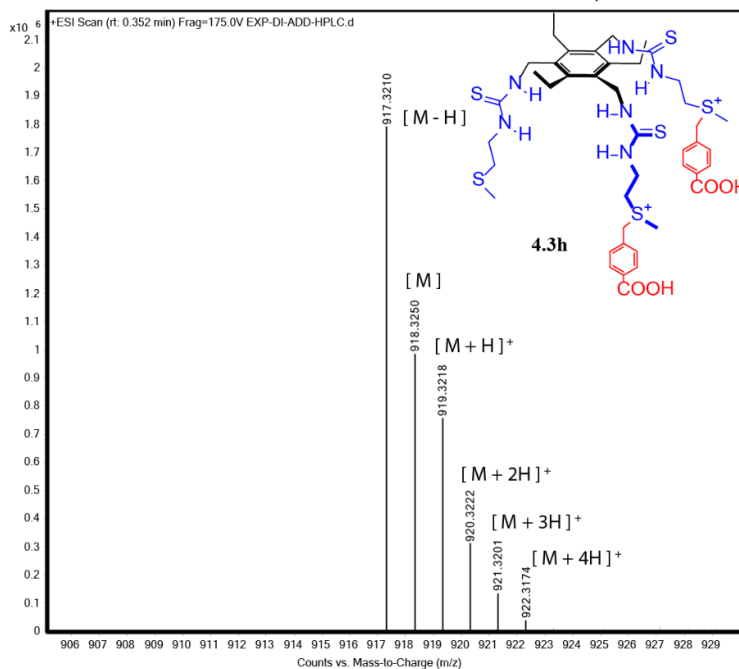


Figure 4.32. Mass spectral signals of compounds **4.3e** and **4.3i** (in water) after separation by HPLC analyses.

Sample Name	SAMPLE 10	Position	P2-A5	Instrument Name	Instrument 1
User Name		Inj Vol	20	InjPosition	
Sample Type	Sample	IRM Calibration Status	Success	Data Filename	EXP-DI-ADD-HPLC.d
ACQ Method	ESI ALS 100-1500.m	Comment		Acquired Time	22-05-2019 16:09:01 (UTC+05:30)



Sample Name	SAMPLE 13	Position	P2-A8	Instrument Name	Instrument 1
User Name		Inj Vol	20	InjPosition	
Sample Type	Sample	IRM Calibration Status	Success	Data Filename	EXP-MAIN-HPLC.d
ACQ Method	ESI ALS 100-1500.m	Comment		Acquired Time	22-05-2019 16:14:41 (UTC+05:30)

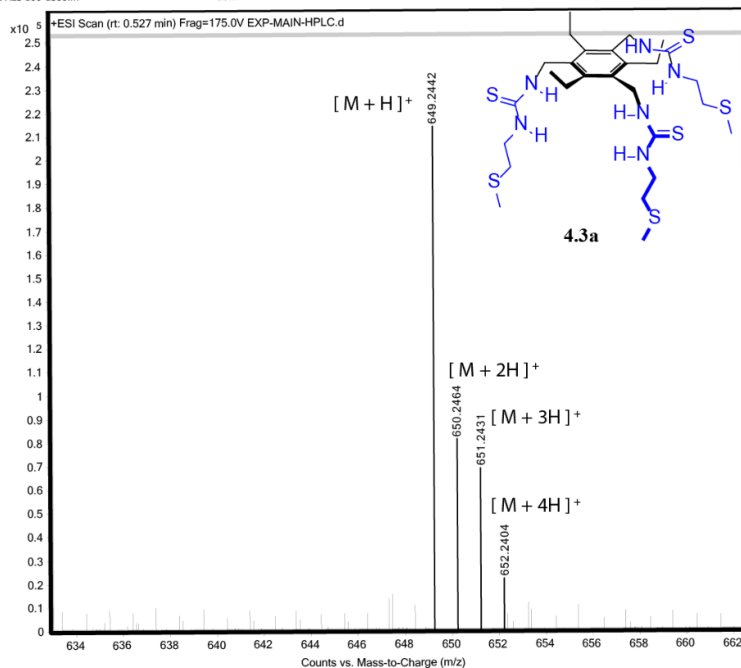


Figure 4.33. Mass spectral signals of compounds **4.3h** and **4.3a** (in water) after separation by HPLC analyses.

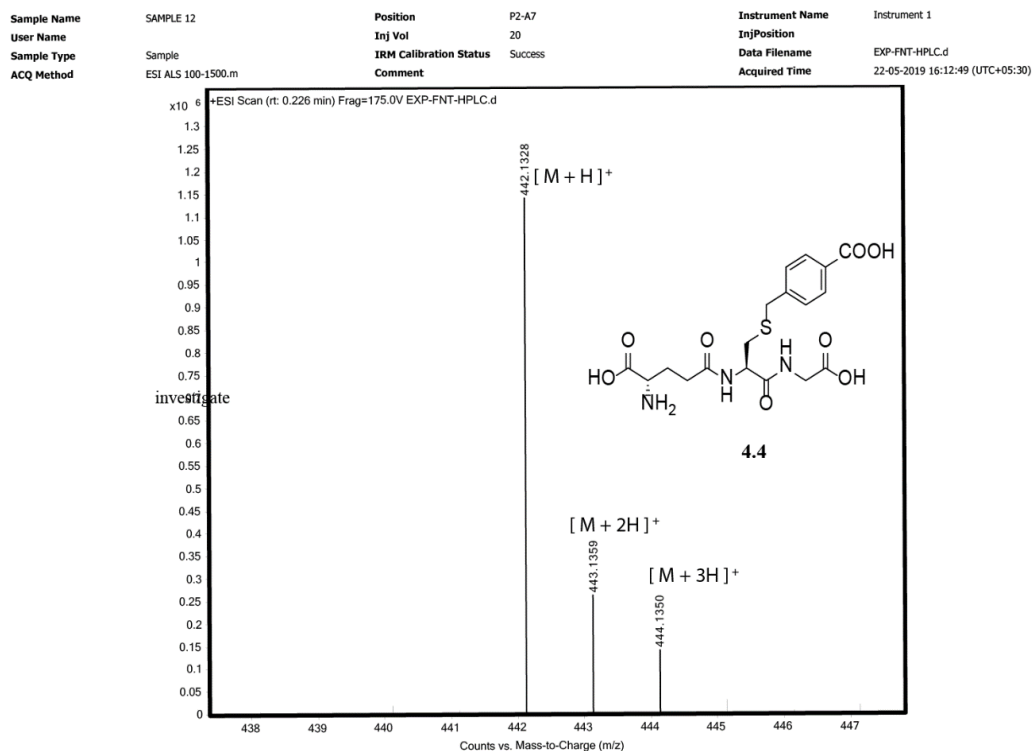


Figure 4.34. Mass spectral signals of GSH derivative, **4.4** in water (from a reaction mixture).

4.4.21. Regeneration confirmation by HPLC and Fluorescence and their corresponding plots

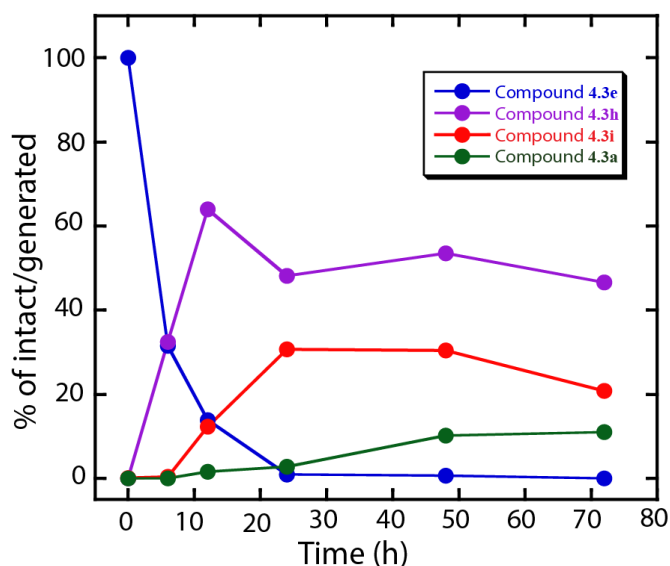


Figure 4.35. Plot of dealkylated compounds at different time intervals.

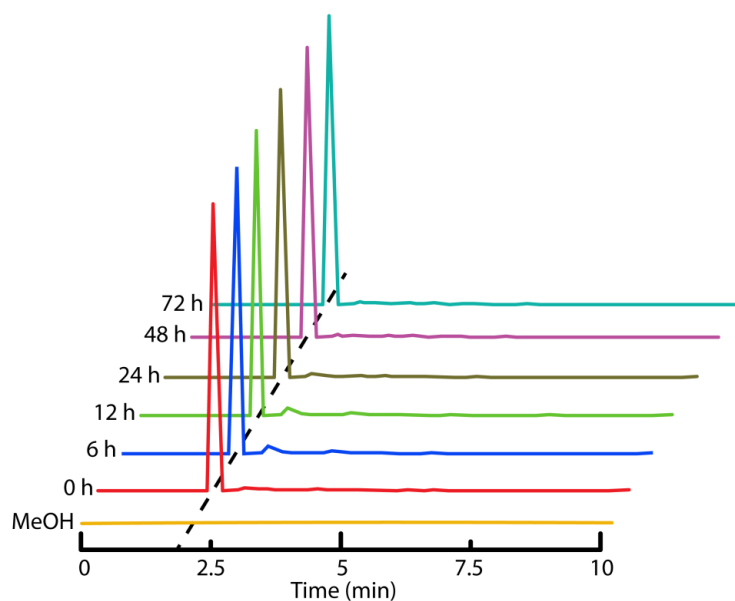


Figure 4.36. HPLC traces of compound **4.3e** at different time intervals in the absence of GSH.

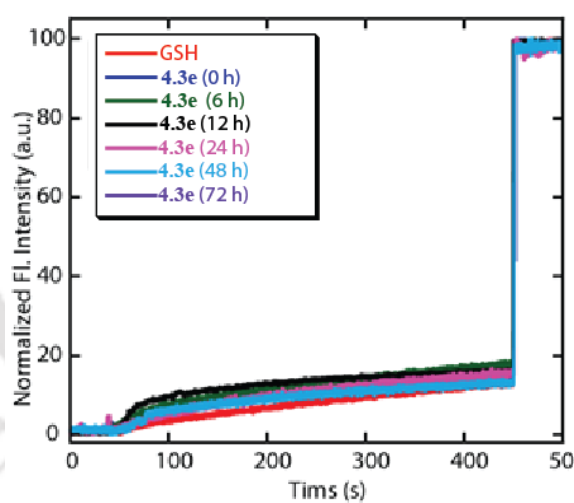


Figure 4.37. Transport of Cl⁻ ion by the compound **4.3e** in the absence of GSH at different time intervals (B).

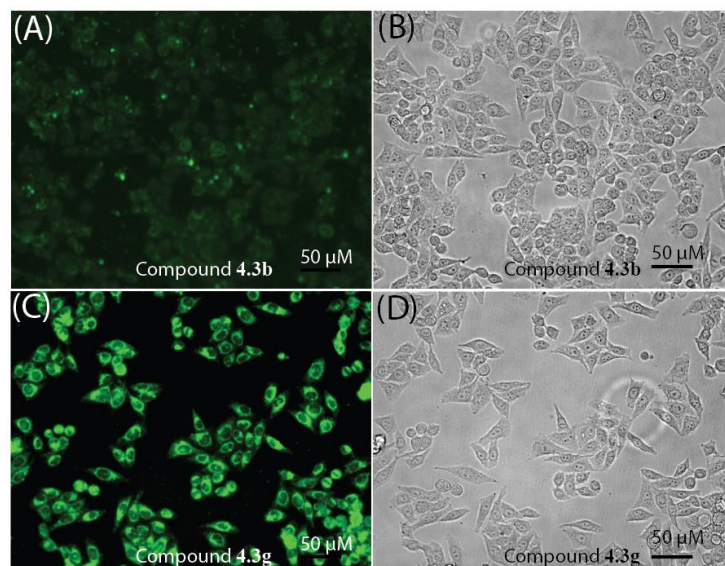


Figure 4.38. Fluorescence microscopic images of the HeLa cells treated with NBD-labelled compounds **4.3b** and **4.3g**. Green channel illustrating compound uptake, bright field illustrating the cell morphology.

4.4.22. Regeneration under cellular environments and corresponding plots

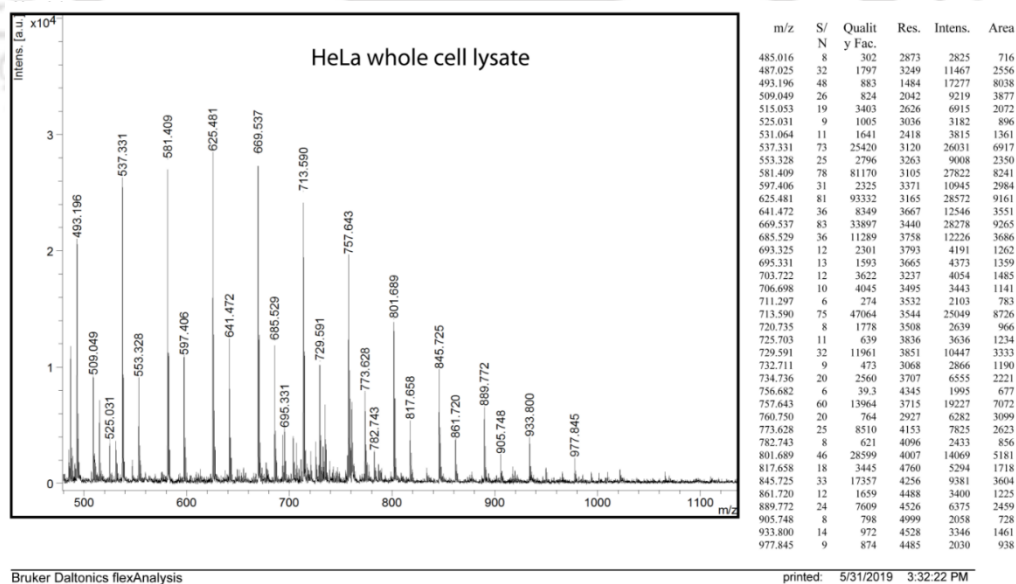


Figure 4.39. MALDI-TOF mass spectral analysis of HeLa whole cell lysate.

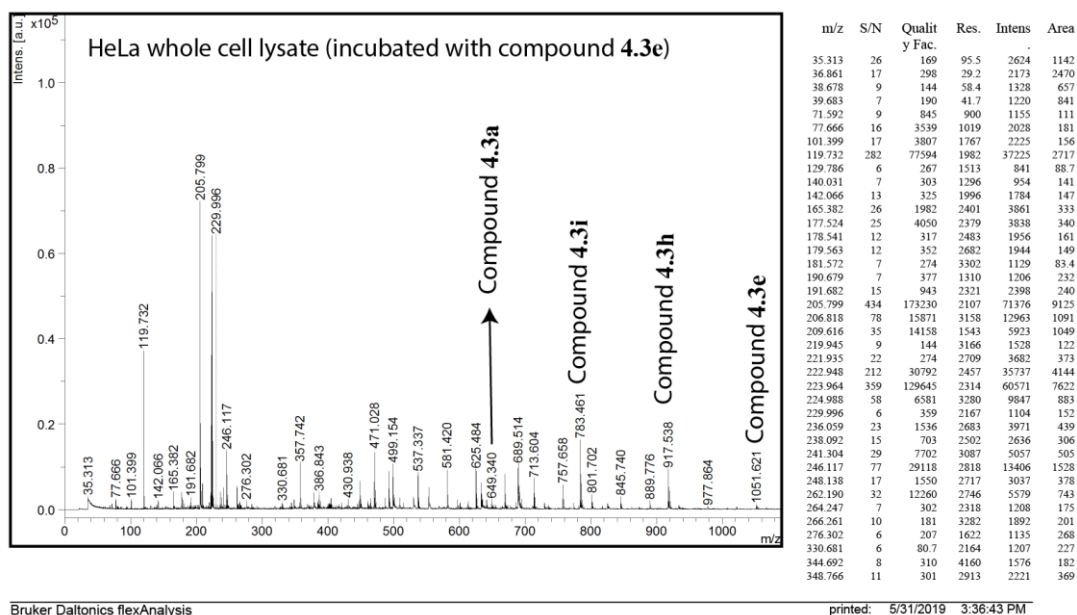


Figure 4.40. MALDI-TOF mass spectral analysis of HeLa whole cell lysate. The HeLa cells were incubated with compound **4.3e** (50 μ M).

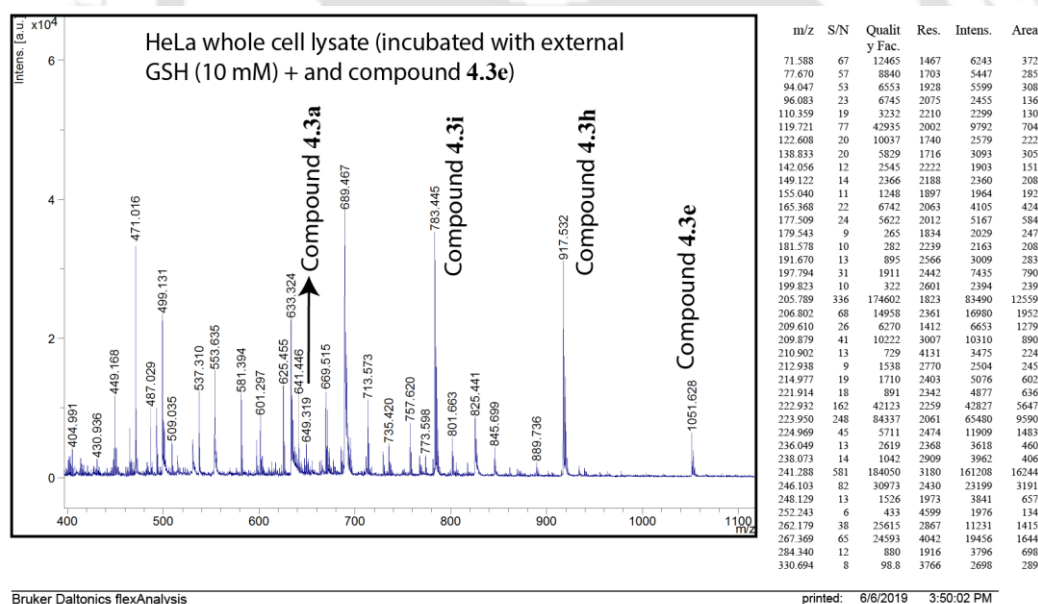


Figure 4.41. MALDI-TOF mass spectral analysis of HeLa whole cell lysate. The HeLa cells were incubated with external GSH (10 mM) and compound **4.3e** (50 μ M).

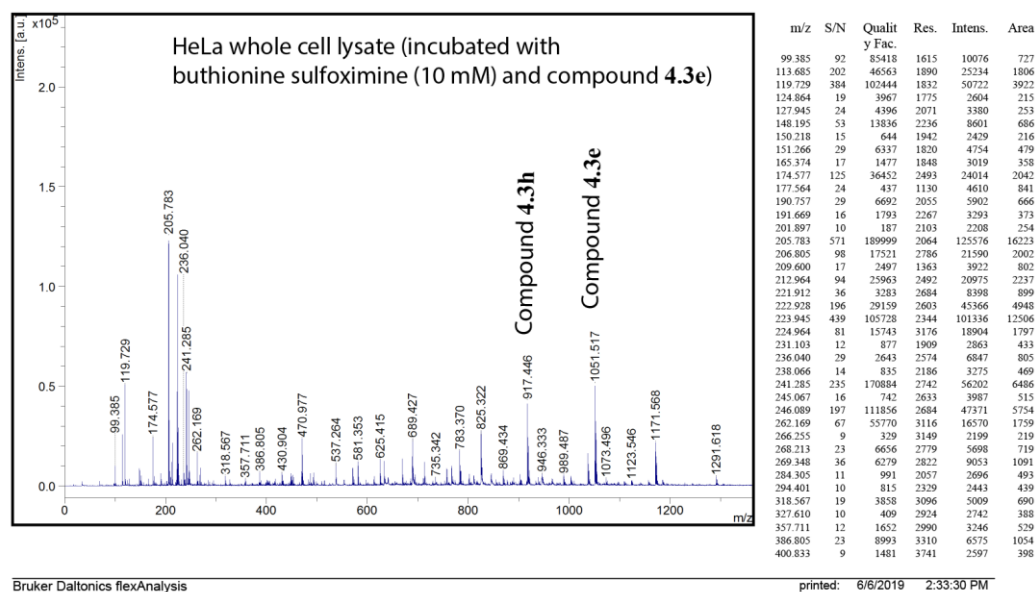


Figure 4.42. MALDI-TOF mass spectral analysis of HeLa whole cell lysate. The HeLa cells were incubated with buthionine sulfoximine (BSO; 1 mM) and compound **4.3e** (50 μ M).

4.5. References

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Chapter 5

Development of Nitroreductase Responsive Proanionophores with Potential Prospects in Therapy and Diagnosis



5.1. Background and objective of present work

Within the previous chapter, the focus was given to solve the conventional hurdle associated with anionophore, such as poor deliverability, cellular uptake, and selectivity. Although selectivity between the cancer cells and normal cells was increased in a significant ratio in chapter 3, still complete toxicity on normal cells was still not eliminated. Further, within chapter 4, the problems associated with deliverability and cellular uptake were carefully investigated and provided an alternative concept of water-soluble proanionophore. Therefore, there is a need for precise stimuli-responsive proanionophores, which should have higher activity under the cancer environment. Besides solving these conventional obstacles associated with the anionophore related research, now is the time to bolster the importance of the diagnostic tool for early detection of cancerous cells in the tumor microenvironment. For this diagnostic purpose, nowadays, scientists are developing various stimuli-responsive fluorescence molecules where the fluorescence property of the molecules will be enhanced in the presence of a particular biomarker present in the cancer environment. Thus, nitroreductase (NTR) responsive anionophores would potentially eliminate cancer cells under hypoxic conditions selectively. NTR is a well-known flavin-containing oxidoreductase enzyme, the potential biomarkers that have been upregulated in cells under hypoxic stress. NTR catalyzes the reduction of nitroaryl to arylamines using NADP as an electron donor. It is also well acknowledged that cells under the hypoxia environment finally transform into tumor cells, which is resistant to numerous therapy. The NTR-responsive strategy already received momentum due to its overexpression in hypoxic conditions.¹⁻⁴ Hence, developing the selective NTR-responsive fluorescence molecules would be beneficial for early detection of hypoxia-mediated tumor environment and selective antiproliferative activity in cancer cells. Despite the remarkable progress in this field, fluorescent probes with superior properties such as emission at longer wavelengths (>500 nm), good water solubility, high selectivity, and sensitivity are still extremely valuable research for clinical diagnosis as biomedical applications.⁵

Inspired by the immense potential of the NTR enzyme, a novel strategy was adopted to develop anionophore that could make it more efficient in the cancer cell and reduce the anionophores deliverability problems that arise due to their inherent lipophilic properties. Simultaneously its uptake efficiency could also be monitored through fluorescence microscopy due to the presence of the tetraphenylethylene (TPE) core unit within the anionophores. The aggregation-induced emission (AIE) property provides a considerable

advantage to these TPE-containing anionophores over the reported normal fluorophore-tagged anionophores. The AIE-based fluorophores have been employed as novel probes for bio-imaging and bio-sensing applications. The AIE probes possess many superior properties than the conventional fluorophores, including concentration-dependent enhancement of brightness, resistance to photo-bleaching that are useful for long-term tracking and imaging of cells. The conventional fluorophores show strong emission in the diluted solutions, but their fluorescence signals get quenched or reduced in the concentrated solutions or aggregated state, which creates a substantial barrier for their theranostic applications.^{3, 6-9}

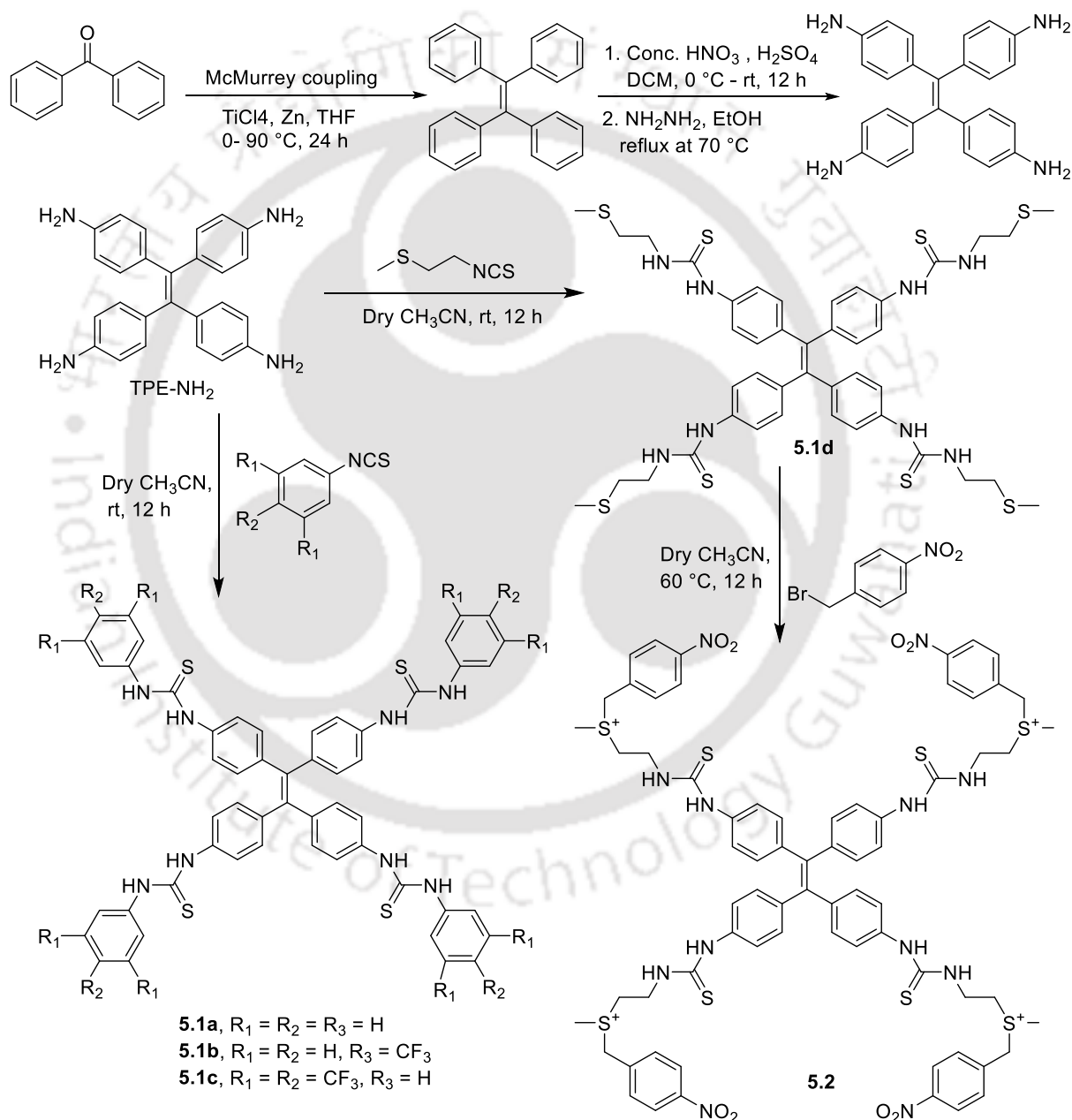
Within the present chapter, the chemistry of sulfonium salts was upgraded to incorporate *p*-nitrobenzyl moieties to develop GSH and hypoxia-responsive compounds. The presence of both sulfonium and nitrobenzyl moieties improves the aqueous solubility. It provides a system where both GSH and NTR could act as dual stimuli to regenerate the active anionophore from its proanionophore. In the current study, a systematic investigation on the ion transport properties and numerous theranostic prospects of TPE-based molecules was carried out. The regeneration of membrane-active anionophores under the reductive environment of GSH and dithionite and the presence of bacterial NTR enzymes were also explored. The stimuli-mediated increase in TPE fluorescence could be helpful in detecting hypoxia-mediated cancer environments. Therefore, these dual stimuli-responsive proanionophores have the potential to regenerate the fluorescence active lipophilic compounds that could transport the Cl⁻ ions across the lipid bilayers and are helpful in studying subcellular localization and early diagnosis of tumors.

5.2. Results and discussions

5.2.1. Design and synthesis of the anionophores and proanionophores

Inspired by the biological applications of NTR enzyme-mediated AIE-based fluorescent probes, a small series of TPE containing thiourea-based compounds were synthesized (Scheme 5.1). The TPE core-unit was synthesized from commercially available benzophenone using the well-known McMurry reaction.¹⁰ The nitration and its subsequent reduction resulted in the formation of a tetraamino-TPE (TPE-NH₂) derivative. Further reaction of TPE-NH₂ with various aryl isothiocyanates or (2-isothiocyanatoethyl)(methyl)sulfane resulted in the desired

TPE containing thiourea-based compounds with excellent yields. To synthesize NTR-responsive compounds, the *p*-nitrobenzyl group was installed to the methylsulfane-substituted TPE derivative using a simple nucleophilic substitution reaction between the anionophore and the *p*-nitrobenzyl bromide. This also resulted in the synthesis of a water-soluble *p*-nitrobenzyl based sulfonium-containing compound.



Scheme 5.1. Synthetic route for TPE based anionophore and proanionophore.

5.2.2. Anion binding studies of the TPE derivatives

The initial anion binding propensities of the TPE derivatives were investigated via ^1H NMR titration experiment in $\text{DMSO-}d_6$ solvent system, where the concentrations of

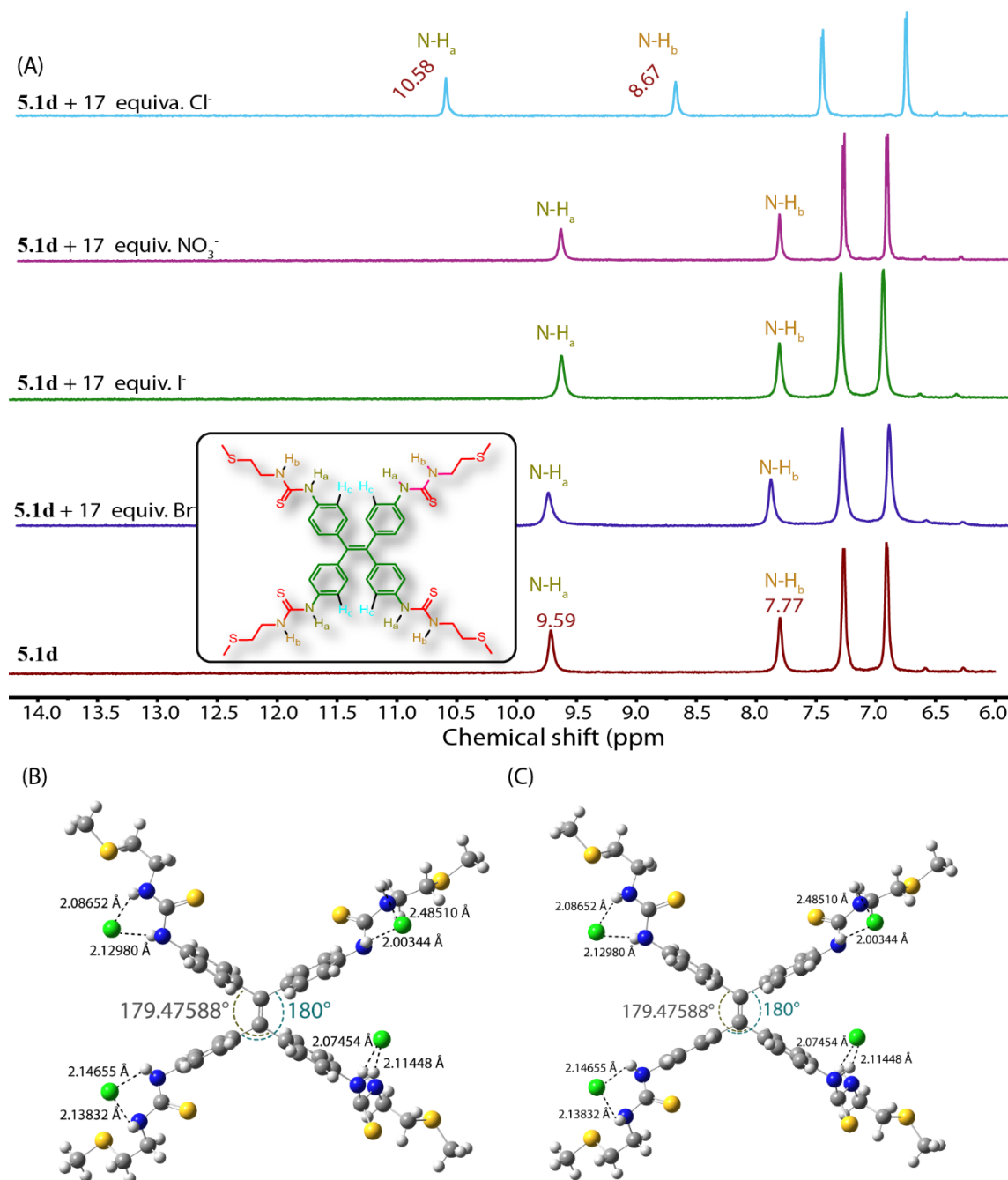


Figure 5.1. The ^1H NMR titration spectra of compound **5.1d** (3.45 mM) with fixed concentrations of different anions (58.65 mM) of TBA salt in $\text{DMSO-}d_6$ solvent at room temperature (A). The geometrically optimized structures of the $[\mathbf{5.1d} + \text{Cl}^-]$ complex were obtained using the B3LYP/6-31+G (d) (B) and B3LYP/6-31++G(d,p) (C) basic sets.

tetra-n-butylammonium (TBA) salts of the respective anions were fixed (17 equivalents with respect to the compound concentration). The outcome of this ^1H NMR titration experiments showed the higher Cl^- ion recognition selectivity of the TPE derivatives compared to the other tested anions such as NO_3^- , Br^- and I^- . The difference in chemical shift ($\Delta\delta$) value for the Cl^- ions for aryl thioureas having the electron-withdrawing group ($\Delta\delta$ values for compound **5.1a** and **5.1c** are 1.16 and 1.18, respectively) have the higher chemical shift values compared to that of alkyl thiourea ($\Delta\delta$ values of compound **5.1d** is $\Delta\delta$ 0.99) (Figure 5.1A and Figure 5.18-19). Further to find out the binding stoichiometry of the compound **5.1d** with the Cl^- ions, we performed the Job's plot analysis using the continuous variation method of ^1H NMR titration. The outcome of Job's plot analysis revealed maximum interaction at a mole fraction of 0.43 (Figure 5.27), which clearly suggested the complex binding stoichiometry. The concentration-dependent ^1H NMR titration spectra of compound **5.1d** with TBACl ($\text{DMSO}-d_6$) also showed a significant downfield chemical shift of the thiourea N-H proton signal (Figure 5.21). The shift in N-H proton signals for compounds could be fitted with the winEqNMR2 program using 1:1 or a mixture of 1:1 and 1:2 host/guest stoichiometries.¹¹ The analysis revealed that the concentration-dependent chemical shift values could be fitted with either 1:1 or a mixture of 1:1 and 1:2 models (Figure 5.22-5.24 and Table 5.1). The calculated binding affinities revealed that compounds with aryl thioureas having the electron-withdrawing group (compounds **5.1a** and **5.1c**) have higher affinities for Cl^- ions than that of alkyl thiourea (compound **5.1d**). In contrast, the concentration-dependent ^1H NMR titration of compound **5.1d** with other tested anions (NO_3^- , Br^- and I^-) showed no significant shift of the thiourea N-H proton signal (Figure 5.25-5.26), which indicates its Cl^- ion recognition selectivity in the $\text{DMSO}-d_6$ solvent. Further theoretical analysis was also carried out using the density functional theory (DFT) in the gas phase with the help of B3LYP/ 6-31+G(d) and B3LYP/ 6-31++G(d,p) basic sets where the optimized structure of the potent compound **5.1d** revealed that the N-H protons of the thiourea moiety are primarily responsible for their interactions with the Cl^- ions. The DFT analysis also demonstrated that the interacting distances between compound **5.1d** and Cl^- ions were within the hydrogen-bond distance (2.003–2.485 Å) (Figure 5.1B-C and Table 5.2-3). Although the DFT analysis revealed the interactions of four Cl^- ions with one molecule of **5.1d**, the true recognition pattern during transportation could be different and observed in Job's plot analysis.

5.2.3. Anion transportation across the lipid bilayers

Initial anion recognition properties of the compounds prompted us to investigate their transmembrane anion transport activities. The Cl^- ion transport activities of the TPE-based compounds across the bilayers of large unilamellar vesicles (LUVs) of

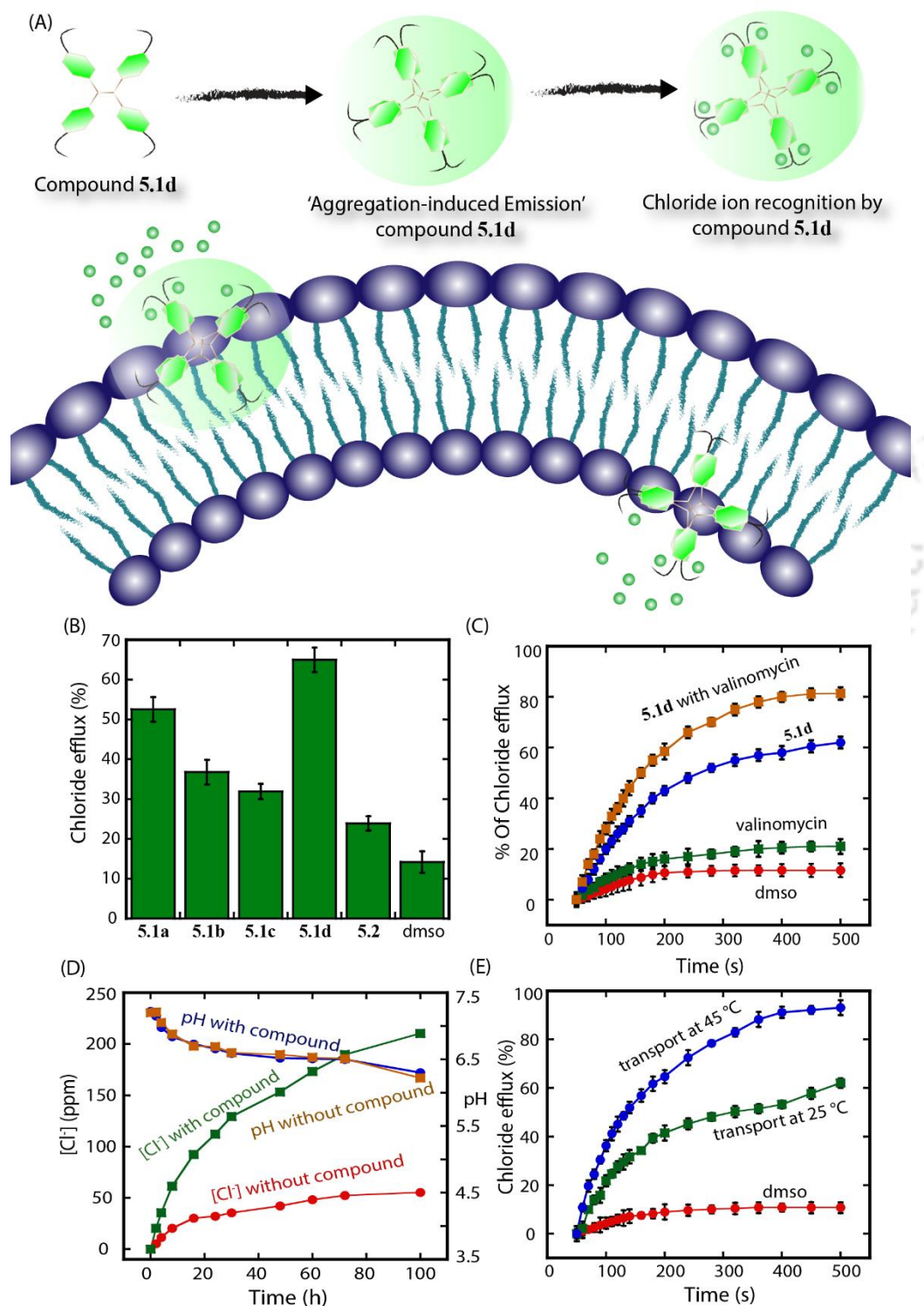


Figure 5.2. Schematic representation of the chloride ion transportation of the TPE-based compounds (A). The chloride ion transport efficiency of the compounds ($0.156\ \mu\text{M} = 0.05\ \text{mol \%}$ with respect to lipid) across the EYPC/CHOL-LUVs (6:4 molar ratio) was measured by an ISE-based assay at pH 7.2 (B). The percentage of the Cl^- ion transport activity of compound **5.1d** in the absence and presence of valinomycin (C). Eliminating the probability of H^+/Cl^- transport efficacy of compound **5.1d** by the U-tube assay in the presence of HCl gradient, using a pH meter and a Cl-ISE (D). Temperature-dependent transmembrane Cl^- ion transport (across DPPC-LUV) assay using Cl-ISE in the absence and presence of compound **5.1d** (E).

model membranes (Figure 5.2A) were investigated using the ion-selective electrode (ISE)-based assay. The commercially available model lipids, phosphatidylcholine (EYPC), and cholesterol (CHOL) lipids (6:4 molar ratio) were utilized to prepare the LUVs. The NaCl solution (5 mM phosphate buffer at pH 7.2 containing 100 mM NaCl) and NaNO_3 solution (5 mM phosphate buffer at pH 7.2 containing 100 mM NaNO_3) were used as the intravesicular and extravesicular solutions of the LUVs, respectively (Figure 5.28). The extent of Cl^- ions efflux from the LUVs was recorded by the Cl-ISE-based assay (without applying any pH gradient) after the addition of the compound to the extravesicular buffer solution. The persistent increase in the Cl^- ion concentration in the extravesicular buffer solution recommends the compound ($0.156\ \mu\text{M} = 0.05\ \text{mol \%}$ with respect to lipid) mediated transmembrane transport of the Cl^- ions (Figure 5.2 and Figure 5.29). In contrast, the water-soluble *p*-nitrobenzyl-based sulfonium-containing compound showed a very similar Cl^- ion transportation behavior to that of only DMSO solution (control), which could be due to its lower membrane-permeable ability. The dose-dependent ISE-based assay revealed that compound **5.1d** (LogP 10.42) had higher transmembrane Cl^- ions transport efficiency ($\text{EC}_{50} = 0.03\ \text{mol \%}$) among the other tested compounds (Figure 5.29 and Figure 5.31). The dose-dependent transport behaviors of compounds **5.1b** and **5.1c** could not be adequately measured due to their precipitation in the buffer medium at higher concentrations, which could be due to their higher hydrophobicity (LogP for **5.1a**, **5.1b**, and **5.1c** are 15.32, 18.83, and 22.34 respectively). Although the binding affinities of compounds **5.1a** and **5.1c**

for Cl^- ions were higher than that of compound **5.1d**, their transportation behavior under the liposomal environment for compound **5.1d** is higher than that for compounds **5.1a** and **5.1c**. These results suggest that both the recognition and release of ions are crucial for the transmembrane transportation of anions. The lipophilicity of these compounds also plays a significant role in their transport properties. A balance of these properties is crucial for optimized transport properties of the anionophores.

5.2.4. Transport mechanism and pathway

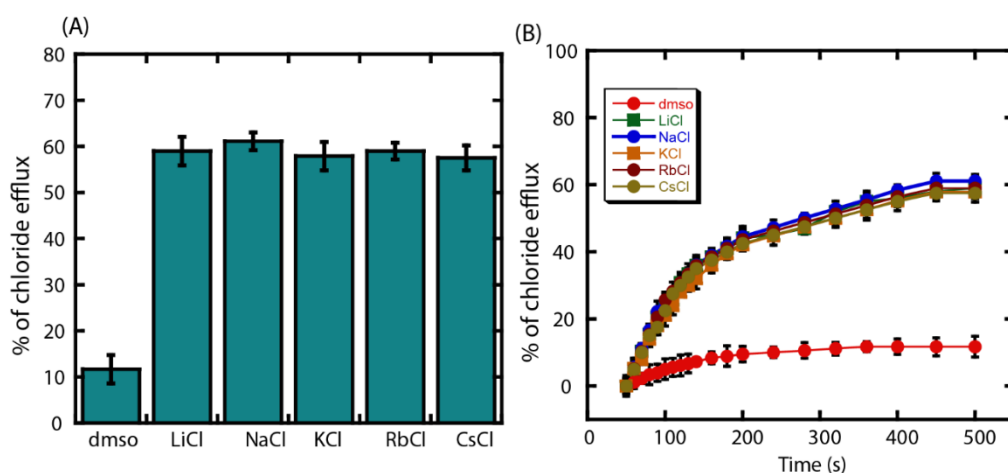


Figure 5.3. Chloride ion transport efficiency of the compound **5.1d** ($0.156 \mu\text{M} = 0.05 \text{ mol } \%$ with respect to lipid) across the EYPC/CHOL-LUVs (6:4 molar ratio) was measured by an ISE-based assay at pH 7.2 in the presence of different metal ions to rule out the possibility of metal ion dependency where figure A represents the bar plot and figure B corresponding plot.

For the ISE-based assay, a $\text{Cl}^-/\text{NO}_3^-$ gradient was constructed, and the efflux of Cl^- ion was measured by monitoring the chloride ion concentration. To retain the overall charge neutrality, the transportation of Cl^- ions may proceed through Cl^-/M^+ symport, H^+/Cl^- cotransport, and a $\text{Cl}^-/\text{NO}_3^-$ antiport pathway. In this regard, chloride salt of various cations (Li, Na, K, Cs, and Rb) was used intravesicular and nitrate in the extravesicular solution to categorize the symport or antiport mechanistic pathway (Figure 5.3). However, the outcome of this study is suggested that no significant difference in the Cl^- ion efflux rates in the presence of different metal ions were there, which ruled out the possibility of a metal-dependent transport

(Cl⁻/M⁺) pathway. Further, we performed a U-tube assay using 100 mM aqueous HCl solution (pH 1.2) in the left-arm and isotonic NaNO₃ solution in the right-arm of the U-tubes (Figure 5.2D). Although a significant increase in Cl⁻ ions concentration was observed, almost no change in the pH of the solution in the right-arm of the U-tube was observed, which ruled out the possibility of H⁺/Cl⁻ cotransport. To confirm the operational mechanism (an antiport (Cl⁻/NO₃⁻)) of the transmembrane transport of Cl⁻ ions by compound **5.1d**, we have performed chloride-ISE assay in the absence and presence of valinomycin (selective K⁺ ion transporter) and classical U-tube assay. For this particular chloride-ISE assay, the extravesicular solution was filled with the potassium nitrate solution, and the intravesicular solution was filled with KCl solution. The extent of Cl⁻ ions efflux efficiency was measured after the addition of the compound to the extravesicular solution. The Cl⁻ ion transport measurement was done in the presence of compound **5.1d** along with the presence and absence of valinomycin. The valinomycin-mediated transports of K⁺ ions from extravesicular solution to intravesicular solution could create an imbalance of this ionic homeostasis. A significant increment in the Cl⁻ ions transport rate in the presence of valinomycin could be due to the restoration of the ionic homeostasis *via* compound **5.1d** promoted efflux of Cl⁻ ions. The outcome of this assay suggested the involvement of Cl⁻ and NO₃⁻ ions during transportation (Figure 5.2C). Another U-tube assay was performed where the left-arm was filled with NaCl solution, and the right-arm was filled with the NaNO₃ buffer. These two aqueous solutions were separated by chloroform (organic phase; a mimic of the lipid bilayer). The concentration of Cl⁻ ions in the right-arm of the U-tube was measured by chloride-ISE. The time-dependent increase in the Cl⁻ ion concentrations in the right-arm of the U-tube suggests the Cl⁻/NO₃⁻ antiport mechanism as the operational mechanism of transmembrane transportation of Cl⁻ ions (Figure 5.32A).¹²

To investigate whether the compound follows a carrier or channel pathway for Cl⁻ ions transportation, we performed the chloride-ISE-based assay using a different molar ratio of EYPC and CHOL lipids. A reduction of Cl⁻ ions transport activity of compound **5.1d** was observed when a higher CHOL concentration with respect to the EYPC lipids was used for the liposome preparation (Figure 5.32B). The outcome of this study suggests that the transport of Cl⁻ ions by compound **5.1d**

depends on the fluidity of the model membrane. Hence, compound **5.1d** follows the carrier pathway for the transmembrane transport of Cl^- ions. Additionally, we performed the DPPC based transport assay where the Cl^- ions transport activities were recorded by chloride-ISE at 25 °C and 45 °C temperatures (Figure 5.2E). A considerable difference in the Cl^- ions transportation rate clearly suggested the carrier pathway. The classical U-tube assay also supports the carrier pathway for the transportation of Cl^- ions. Similar Cl^- ion transport properties were also observed for compound **5.1a** (Figure 5.31).

5.2.5. Regeneration of active anionophore

It is hypothesized that active Cl^- ion transporter (compound **5.1d**) could be regenerated from proanionophore **5.2** under the reductive environment. Henceforth, we monitored the regeneration aptitude of the active anionophore using fluorescence spectrophotometry in the presence of sodium dithionite, NTR enzyme, and GSH (Figure 5.4). It is important to mention that the fluorescence property of proanionophore **5.2** is insignificant compared to the active anionophore **5.1d**. First, the proanionophore **5.2** was incubated with sodium dithionite in PBS buffer, and the aliquot from the solution was collected at a different time interval, and fluorescence spectra were recorded. The time-dependent increase in fluorescence signal suggests the generation of active anionophore **5.1d** from the nonfluorescent proanionophore **5.2** (Figure 5.33-40). The anionophore regeneration aptitude was also investigated in the presence of bacterial cellular lysate, which contains the NTR enzyme at a higher concentration. The time-dependent increase in fluorescence signal also suggests the generation of active anionophore **5.1d**. During measurements, the whole reaction mixtures were incubated at 37 °C under constant stirring conditions. The conversion of proanionophore **5.2** to active compound **5.1d** and the generation of intermediates in the presence of both sodium dithionite¹³ and NTR enzyme were also measured by mass spectrophotometric analysis, which supports the proposed regeneration pathway (Figure 5.4A). The NTR enzyme (from bacterial cell lysate) mediated regeneration of the active anionophore **5.1d** suggests that similar studies could also be performed under hypoxic cellular environments (Figure 5.4B and Figure 5.37-40).

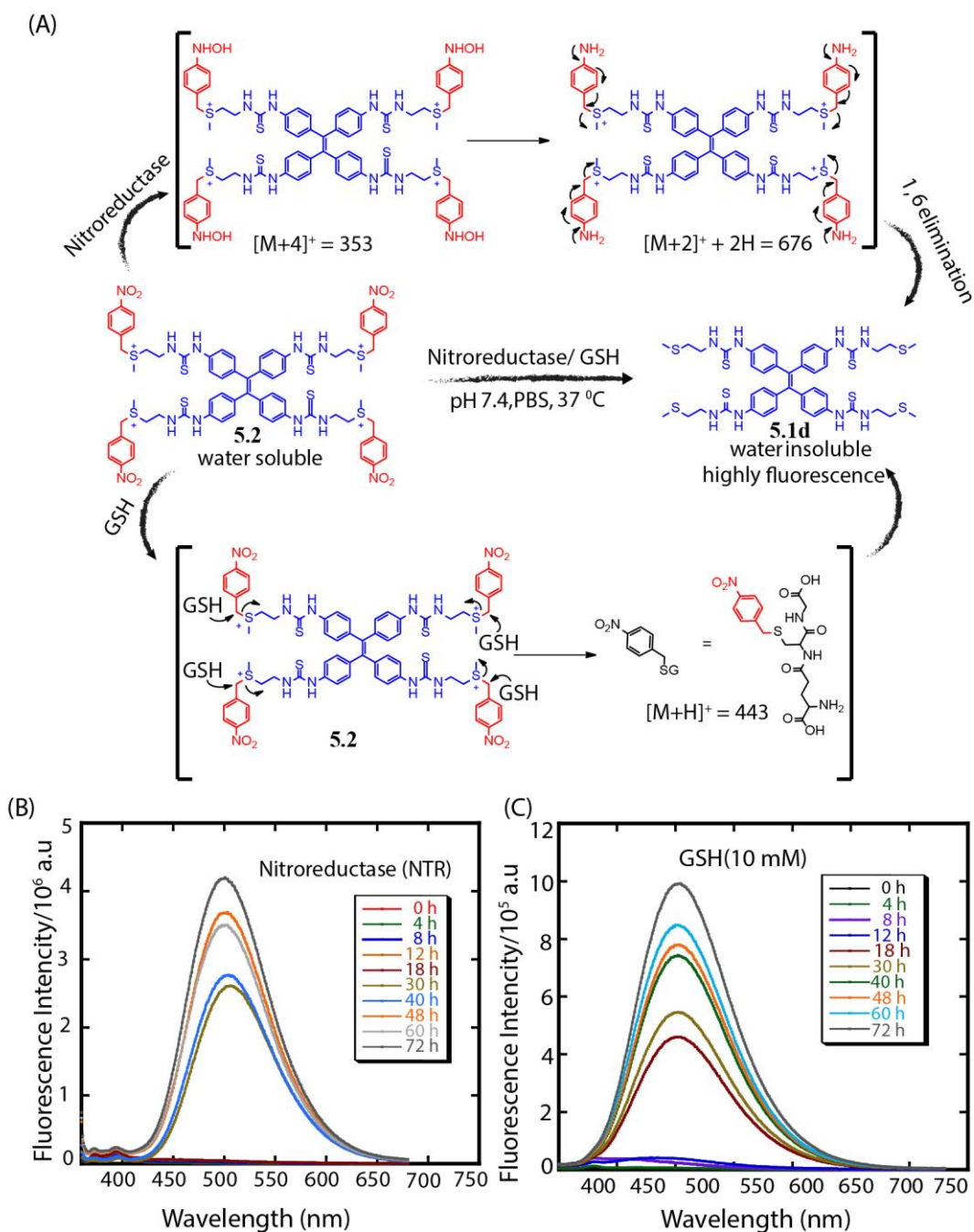


Figure 5.4. The proposed regeneration pathway for the active anionophore from the proanionophore (A). Regeneration of the active anionophore **5.1d** from its non-fluorescence proanionophore **5.2** in the presence of bacterial NTR enzyme (B) and GSH (C)

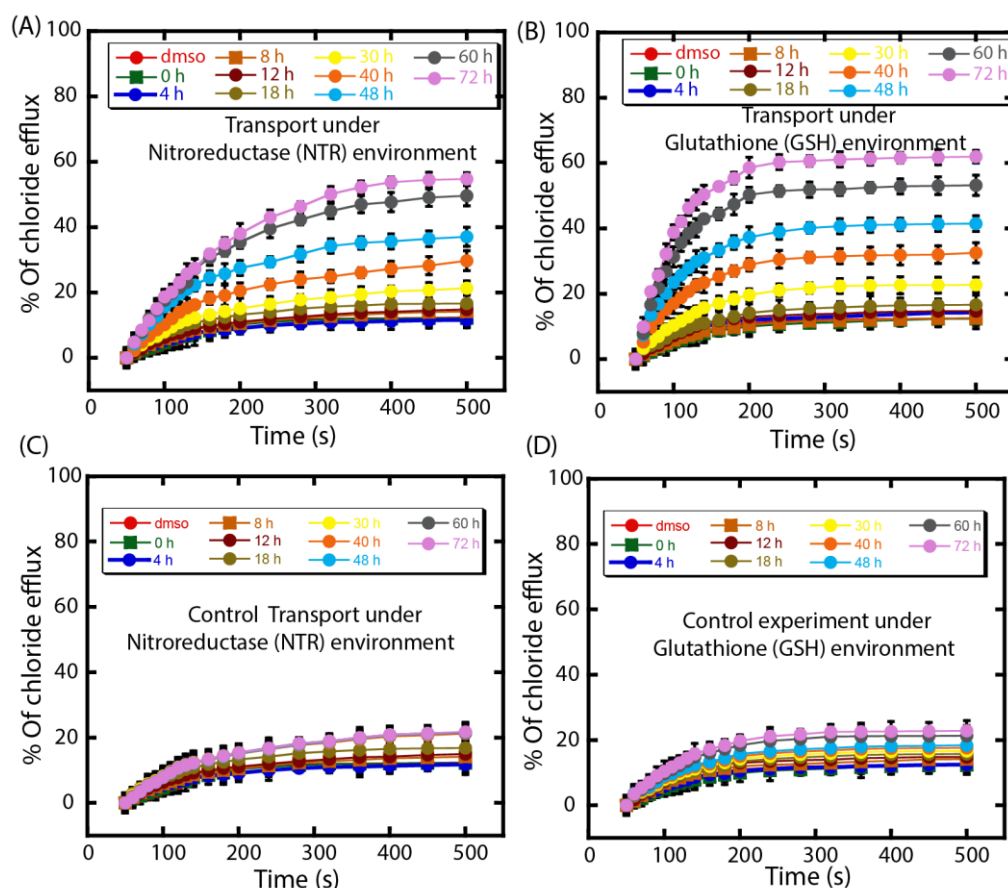


Figure 5.5. Cl^- ion transport activities of regenerated anionophore **5.1d** from the proanionophore **5.2** in the presence of NTR enzyme (A, C) and GSH (B, D). The concentration of the proanionophore **5.2** was 1 mM.

Due to the presence of sulfonium moieties, we also investigated the possibility of GSH mediated regeneration of the active anionophore. To validate this, we have similarly incubated compound **5.2** in the presence of GSH and found a similar kind of fluorescence increment, suggesting the GSH responsive nature of the proanionophore **5.2** (Figure 5.4C and Figure 5.41-43). The GSH mediated cleavage was also supported by the ES-MS analysis of the reaction mixture. The Cl^- ion transportability of the regenerated compound **5.1d** was also scrutinized by the Cl^- ISE-based assay using the GSH and NTR containing reaction mixture. The time-dependent enhancement of the Cl^- ion transportability of these reaction mixtures also confirmed the regeneration of the active carrier **5.1d** from its corresponding proionophore **5.2** in the presence of GSH and NTR. Both these stimuli (GSH, NTR enzyme) are present at higher concentrations in cancer cells (hypoxia-mediated)

than the healthy cells. Therefore, we hypothesized that this dual responsive strategy would improve the Cl^- ion mediated biological activities.

5.2.6. Transportation of chloride ion using the giant unilamellar vesicles (GUVs) and ^{35}Cl NMR

To confirm the transport efficiency of the TPE-based potent compound, we performed the lucigenin quenching assay using giant unilamellar vesicles (GUVs).¹⁴⁻¹⁵ The initial fluorescence microscopic image revealed the encapsulation of lucigenin within the core of the GUVs. However, after adding the NaCl and compound **5.1d** (1 μM), the fluorescence intensity of lucigenin was quenched (Figure 5.6). The result of the lucigenin quenching assay revealed that the presence of compound **5.1d** significantly induces the influx of Cl^- ions. Therefore, these microscopic images suggest the influx of Cl^- ions in the presence of compound **5.1d**. The U-tube assay showed no significant change in the pH of the solution in the right-arm of the U-tube (Figure 5.2D), which ruled out the possibility of the H^+/Cl^- symport process. The valinomycin assay recommends the involvement of Cl^- and NO_3^- ions during the transportation process. Hence, the quenching of lucigenin fluorescence could be only due to the transport of Cl^- ions and suggest $\text{Cl}^-/\text{NO}_3^-$ antiport mechanism (Figure 5.6). Besides, we designed and performed the ^{35}Cl NMR study to investigate the transmembrane transport of the Cl^- ions by the compound. The higher isotopic abundance of ^{35}Cl (chloride abundance around 75.77%) prompted us to perform the ^{35}Cl NMR-based Cl^- ions transport assay in the presence of vesicles (EYPC/CHOL; molar ratio of 6:4). The intravesicular solution of the vesicles was filled with the NaCl solution, and the extravesicular solution was filled with the NaNO_3 solution. The ^{35}Cl NMR experiment was designed using a paramagnetic Mn^{2+} ion, which interacts with the chloride ion and quenches the chloride signal of the ions. Initially, the ^{35}Cl NMR spectra showed a distinct signal for the Cl^- ions at 4.79 ppm. The ^{35}Cl signal peak for the Cl^- ions remains at the same position even after the addition

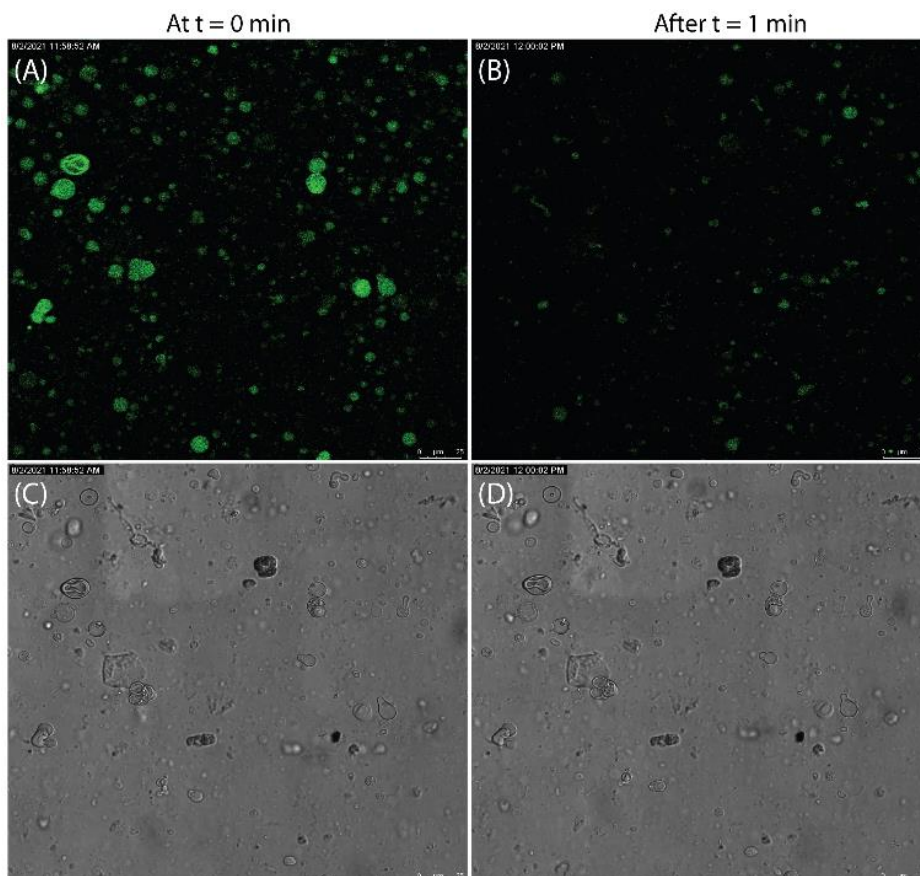


Figure 5.6. The fluorescence microscopy images of the lucigenin encapsulated GUVs were recorded at different time intervals ($t = 0$ and 1 minutes) in the presence of compound **5.1d** ($1 \mu\text{M}$) and NaCl (0.1 M). The fluorescence intensity of lucigenin was monitored using green (A and B) and bright-field (C and D) channels. The scale bar of the images was $25 \mu\text{m}$.

of the paramagnetic Mn^{2+} ions in the extravesicular solution. We did not observe the disappearance of the Cl^- ions peak as there were no Cl^- ions present in the extravesicular medium. Next, we stirred the whole liposome solution in the presence of compound **5.1d** for few minutes and recorded the ^{35}Cl spectra, and observed the complete disappearance of the Cl^- ion peak in the spectra. A control experiment was also performed in the absence of the compound where the ^{35}Cl peak was not disappeared, which confirmed the compound **5.1d** mediated Cl^- ion transportation across the lipid bilayers (Figure 5.7 and Figure 5.45).

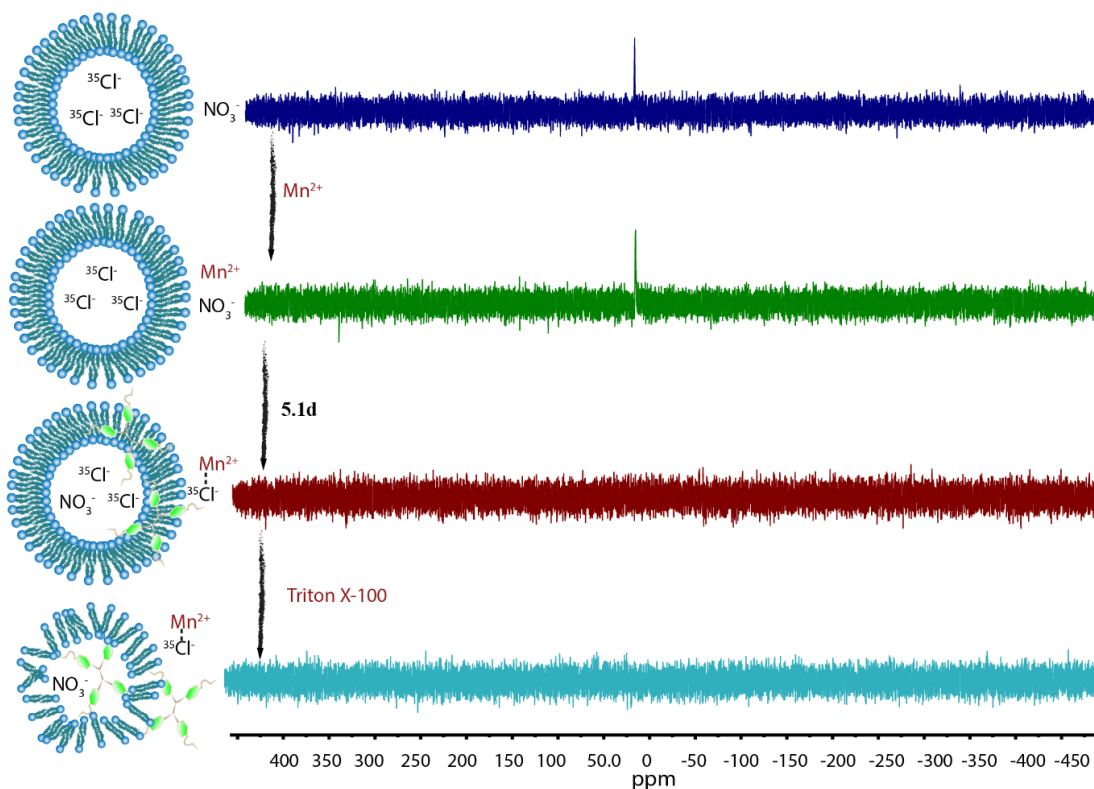


Figure 5.7. The ^{35}Cl NMR spectra experiment showed that **5.1d** (5 mM) is proficient in chloride ion transport *via* the $\text{Cl}^-/\text{NO}_3^-$ antiport pathway. The NMR experiment was performed in $\text{H}_2\text{O}:\text{D}_2\text{O}$ (9: 1) solution

5.2.7. Aggregate induced emission property

The TPE is a well-characterized molecule with AIE property. Herein we used TPE moiety to develop AIE sensitive anionophores. Regular organic fluorophore-linked anionophores face several limitations, including fluorescence quenching at higher concentrations, which prompted the researchers to use a very dilute solution of the fluorophore-linked anionophores. However, photobleaching significantly affects their prospect of obtaining quantitative data. The AIE-active molecules have been successfully utilized in overcoming these limitations for bioimaging. To investigate the AIE properties of the synthesized compounds, we recorded their fluorescence emission properties in a mixture of DMSO and H_2O solutions (Figure 5.8). The outcome of the

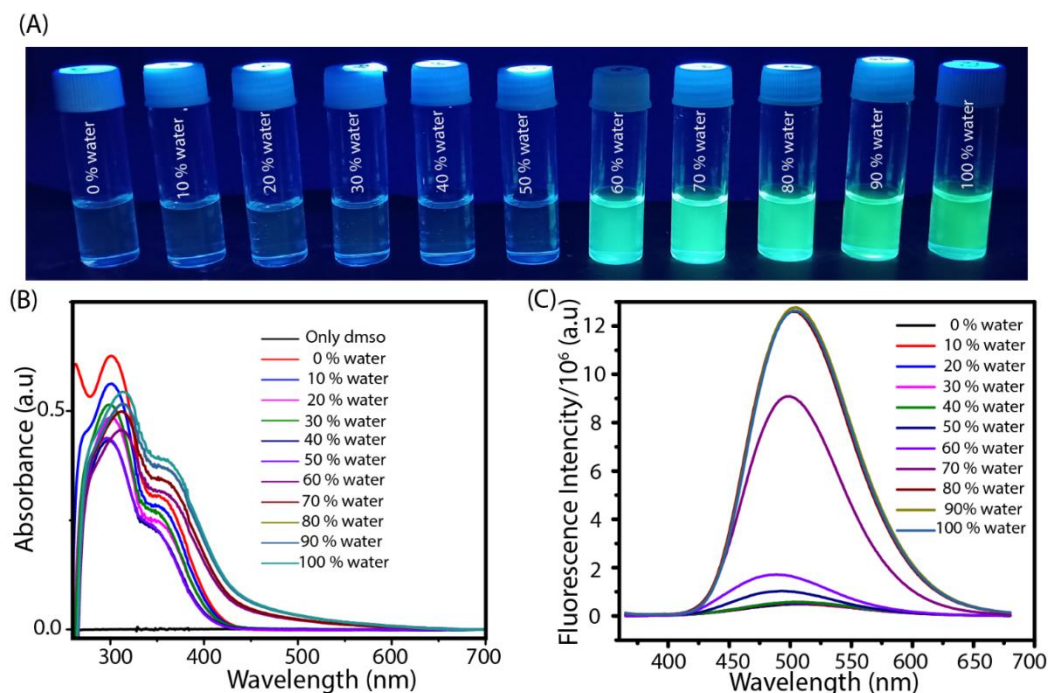


Figure 5.8. The fluorescent photographs of compound **5.1d** at different fractions of DMSO/H₂O mixture under UV irradiation (365 nm) at a concentration of 2 μ M (A). The UV-vis spectra of the solution containing different DMSO/H₂O mixture fractions at a concentration of 2 μ M. A clear peak shift was observed after 60% of the DMSO/H₂O fraction (B). Abnormal increase in the fluorescence intensity after 60% of water (C).

studies showed that with an increase in water fraction, the fluorescence intensity of the molecules increases abnormally. The presence of 80-90% H₂O in the solution showed > 14-folds increase in their fluorescence signal compared to that of only DMSO solvent (Figure 5.8). This fluorescence enhancement could even be visualized in the naked eye, where the compound is almost non-emissive when dissolved in only DMSO solvent. It became emissive after the 50% addition of water, which clearly supports their efficient AIE activity.

To investigate the AIE property under the liposomal environment (EYPC/CHOL-LUVs), we monitored the emission spectra of compound **5.1d**. We recorded the initial emission spectra of compound **5.1d** (0.03 μ M) in the presence of 100% water (fully AIE-active state). Then liposomal solutions were sequentially added, and the emission spectra of compound **5.1d** were recorded. The analysis revealed a considerable enhancement of the emission intensity with a large blue-shift (504 to 450 nm), which could be due to the

effect of the liposomal (hydrophobic) environment or its different aggregation pattern from that in aqueous solution (Figure 5.46). It is already documented that the aggregation-induced blue shift (AIBS) phenomenon is because of its rotation restrain ability due to low temperature, high viscosity, or high aggregation state. Herein liposomal environment might behave like a viscous environment which could promote aggregation of TPE.¹⁶ Further to explore the potential of the synthesized TPE derivatives as bio-imaging agents, we performed giant unilamellar vesicles (GUVs) staining measurements. The microscopic images showed that the TPE derivative **5.1d** localized within the lipid bilayers of the liposomes (Figure 5.47).

Hypoxia, a condition where tissue or an organ is deprived of enough oxygen levels, is crucial in many diseases, including inflammatory diseases, cardiac ischemia, and solid tumors.¹⁷⁻²¹ Hypoxic regions in solid tumors indicate tumor progression with malignant phenotype, metastatic ability, and unresponsiveness to the treatment. Tumor hypoxia is a prospective therapeutic problem because it creates more drug-resistant tumors.^{4, 22} For that purpose, tumor hypoxia is considered a potential target for diagnosis and therapy. As a result, the development of a novel strategy for hypoxia detection has become a great biomedical tool for the early detection of tumors. The elevated level of the NTR enzyme is a biomarker of a hypoxia environment.

On the other hand, the overexpression of the GSH is also observed in different cancer cell lines. Hence detection of the NTR enzyme is highly anticipated for its immense therapeutic value. In earlier sections, we already demonstrated that the proanionophore **5.2** is responsive towards the GSH and NTR. Further to prove their selectivity over the other potentially interfering bioactive molecules, we also performed the fluorescence analysis for the conversion of proanionophore **5.2** to active anionophore **5.1d** in the presence of various inorganic salts (CaCl₂, MgCl₂, KCl, and NaCl), cysteine, glutathione, glutamic acid, arginine, serine, vitamin C and reactive oxygen species (H₂O₂).³ The outcome of this study revealed that NTR and GSH showed maximum regeneration of the active anionophore (Figure 5.9). However, a significant amount of compound **5.1d** regeneration was also observed in the presence of cysteine and H₂O₂, which could be due to the cysteine and OH⁻ (active species generated from H₂O₂)

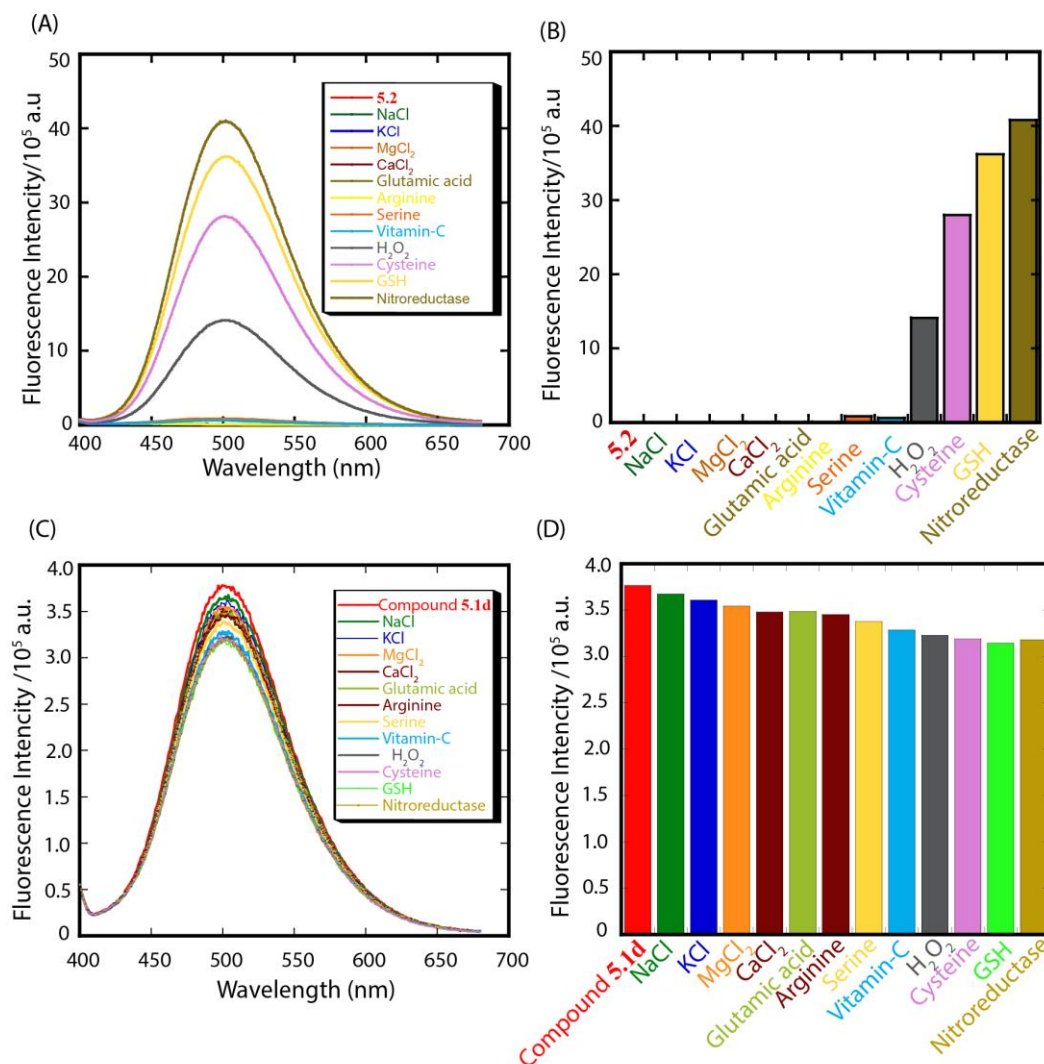


Figure 5.9. Regeneration of the active compound **5.1d** from the proanionophore **5.2** in the presence of the different substances. Proanionophore **5.2** and active compound **5.1d** was (1 mM) were incubated with individual substances (10 mM), and fluorescence spectra were recorded after 72 h of incubation. Fluorescence spectra of proanionophore **5.2** (A) compound **5.1d** (C) and bar plot diagram of proanionophore **5.2** (B) compound **5.1d** (D).

mediated cleavage of the sulfonium moieties. A control experiment was carried out to nullify any interfering effect of these species with the AIE property of the regenerated active anionophore **5.1d**. The compound **5.1d** was incubated with these similar species and recorded their spectra where there was no evidence of any interference in AIE property by these species (Figure 5.9D). We hypothesize that the higher fluorescence behavior of the proanionophore **5.2** in the presence of the NTR enzyme could also be explored as a diagnostic strategy for detecting a hypoxia environment. Overall, the AIE data suggested

the aggregation of **5.1d** within the membrane bilayers. However, the transmembrane Cl^- ion transport studies revealed that the Hill coefficients (n values) were around 1. Hence, to identify the active species in the transport process, we calculated the initial transportation rate at different initial concentrations of compound **5.1d** (Figure 5.48). An upward-curvature (nonlinear) of the transport properties against the concentration of the compound **5.1d** clearly suggests the possibility of Cl^- ion transport by the dimeric/oligomeric compounds *via* a carrier pathway.

5.3. Summary

So within this chapter, the potent TPE derivatives were found to be selective for Cl^- ions transportation across the bilayers of the model membrane *via* carrier mechanistic pathways. The ^1H NMR titration and DFT analysis confirmed the anion recognition ability of the synthesized compounds in the solution phase. The Cl-ISE-based transport assay and ^{35}Cl NMR titration experiments revealed its potential Cl^- ions transportation properties. The HPTS-based fluorescence quenching assay also demonstrated the compound-mediated transportation of Cl^- ions across the lipid bilayers of GUVs. The regeneration ability of the highly fluorescent active lipophilic anionophore from its nonfluorescent water-soluble proanionophore was also investigated in the presence of bacterial NTR enzyme, sodium dithionite, and GSH, which represents its potential applications in cancer and other diseases as well as could also be explored as a diagnostic tool for detection of the hypoxia environment. The AIE of the active anionophore could be useful for bioimaging, particularly under the hypoxic cancer environment.

5.4. Experimental section

5.4.1. Synthesis of compounds

5.4.1.1. General information

Described in section 2.4.1.1

5.4.1.2. General procedure for the synthesis of targeted compounds

5.4.1.2.1. Synthesis of 1,1,2,2-tetraphenylethene

To a stirring solution of zinc powder (2.6 g, 40 mmol) in anhydrous THF (50 mL) at 0 °C under N_2 atmosphere was added TiCl_4 (2.2 mL, 20 mmol). After complete addition,

the ice-salt bath was removed, and the reaction mixture was refluxed for two hours. Next, the benzophenone solution (1.82 g, 10 mmol) in dry THF (10 mL) was slowly added to the reaction mixture and refluxed overnight. The progress of the reaction was monitored using thin-layer chromatography (TLC). After maximum consumption of the starting material, the reaction mixture was quenched using a saturated aqueous NaCl solution and extracted with CH₂Cl₂ (3 × 50 mL). The combined organic layers were then dried over anhydrous Na₂SO₄, filtered, and the solvent was removed under reduced pressure. The crude reaction mixture was then purified through column chromatography with 100% hexane, which resulted in a white solid compound with 70% yield. The product was characterized by ¹H NMR, ¹³C NMR, and HRMS that matched the reported literature.²³

5.4.1.2.2. Synthesis of 1,1,2,2-tetrakis(4-nitrophenyl)ethene

To a stirring solution of concentrated sulphuric acid (4 mL) and concentrated nitric acid. (15 mL) at -10 °C, a solution of tetraphenylethylene (2g, 6.01 mmol) in CH₂Cl₂ was added dropwise for a period of 30 minutes. The whole mixture was then stirred at -5 to -10 °C temperature overnight. After maximum consumption of the starting material, water was added, and the reaction mixture was extracted with CH₂Cl₂ (3 × 50 mL). The organic layer was then washed with a saturated solution of NaCl and dried over anhydrous Na₂SO₄, and the solvent was removed under reduced pressure. The obtained crude product was purified by column chromatography with a gradient solvent system of EtOAc/hexane (0-10%) to get a light yellow solid compound with 80% yield. Further, the product was scrutinized by ¹H NMR, ¹³C NMR, and HRMS analyses, which matched the reported literature.²⁴

5.4.1.2.3. Synthesis of 4,4',4'',4'''-(ethene-1,1,2,2-tetrayl)tetraanilin

This compound was synthesized using the previously reported procedure. To a stirring solution of tetrakis(4-nitrophenyl)ethylene (2.50 g, 4.34 mmol) in dry THF were added palladium on carbon (wt 10 % Pd, 500 mg) and NH₂NH₂·H₂O (25 mL, 516 mmol) and the whole reaction mixture was refluxed for 48 h. After maximum consumption of the starting material, the reaction mixture was cooled down to room temperature, and the insoluble residues were filtered off. Finally, the solvent was removed under reduced pressure to afford a brown solid of the targeted compound with 70 % yield. The product

was characterized by ^1H NMR, ^{13}C NMR, and HRMS analyses which matched with the reported literature.²⁴

5.4.1.2.4. Synthesis of 1,1',1'',1'''-(ethene-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl))tetrakis(3-phenylthiourea)

To a stirring solution of TPE-NH₂ (200 mg, 0.508 mmol) in dry CH₃CN (50 mL) was added a solution of phenyl isothiocyanate (0.3 mL, 2.54 mmol) in dry CH₃CN, and then the reaction mixture was stirred at 60 °C temperature for 12 h. Greenish grey precipitation was observed, which was further washed with solvents like CH₂Cl₂ (3 × 50 mL), CH₃CN (3 × 50 mL) to get the pure product as the greenish solid with 75 % yield. The product was characterized by ^1H NMR, ^{13}C NMR, and HRMS analysis.

5.4.1.2.5. Synthesis of 1,1',1'',1'''-(ethene-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl))tetrakis(3-(4-(trifluoromethyl)phenyl)thiourea)

To a stirring solution of TPE-NH₂ (200 mg, 0.508 mmol) in dry CH₃CN (50 mL) was added a solution of 1-isothiocyanato-4-(trifluoromethyl)benzene (516 mg, 2.54 mmol) in dry CH₃CN, and then the reaction mixture was stirred at 60 °C temperature for 12 h. Greenish grey precipitation was observed, which was further washed with solvents like CH₂Cl₂ (3 × 50 mL), CH₃CN (3 × 50 mL) to get the pure product as the greenish solid with 80 % yield. The product was scrutinized by ^1H NMR, ^{13}C NMR, and HRMS analysis.

5.4.1.2.6. Synthesis of 1,1',1'',1'''-(ethene-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl))tetrakis(3-(3,5-bis(trifluoromethyl)phenyl)thiourea)

To a stirring solution of TPE-NH₂ (200 mg, 0.508 mmol) in dry CH₃CN (50 mL) was added a solution of 1-isothiocyanato-3,5-bis(trifluoromethyl)benzene (0.46 mL, 2.54 mmol) in dry CH₃CN, and then the reaction mixture was stirred at 60 °C temperature for 12 h. Greenish grey precipitation was observed, which was further washed with solvents like CH₂Cl₂ (3 × 50 mL), CH₃CN (3 × 50 mL) to get the pure product as the greenish solid with 80 % yield.

5.4.1.2.7. Synthesis of 1,1',1'',1'''-(ethene-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl))tetrakis(3-(2-(methylthio)ethyl)thiourea)

To a stirring solution of TPE-NH₂ (200 mg, 0.508 mmol) in dry CH₃CN (50 mL) was added a solution of (2-isothiocyanatoethyl)(methyl)sulfane (338 mg, 2.54 mmol) in dry CH₃CN, and then the reaction mixture was stirred at 60 °C temperature for 12 h. Greenish grey precipitation was observed, which was further washed with solvents like CH₂Cl₂ (3 × 50 mL), CH₃CN (3 × 50 mL) to get the pure product as the greenish solid with 80 % yield.

5.4.1.2.8. Synthesis of (((((ethene-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl))tetrakis(azanediyl))tetrakis(carbonothioyl))tetrakis(azanediyl))tetrakis(ethane-2,1-diyl))tetrakis(benzyl(methyl) sulfonium)

To a stirring solution of compound **5.1d** (200 mg, 0.216 mmol) in dry CH₃CN (50 mL) was added a solution of 1-(bromomethyl)-4-nitrobenzene (231 mg, 1.08 mmol) in CH₃CN. To this reaction mixture, a small amount of dry DMF (0.2 mL) was added, and the mixture was heated (at 40-50 °C) to solubilize the whole reaction mixture. The whole reaction mixture was allowed to stir for 24 hours at this temperature. The progress of the reaction was monitored by TLC analysis. After maximum consumption of the starting material, precipitation was observed, which was then washed with diethyl ether, CH₂Cl₂, and CH₃CN to obtain the pure compound **5.2** with 70% yields.

5.4.1.3. Characterization of synthesized compounds

1,1,2,2-tetraphenylethene (TPE): ¹H NMR (600 MHz, CDCl₃) δppm 7.15-7.13 (m, 12H), 7.08 – 7.07 (m, 8H). ¹³C NMR (150 MHz, CDCl₃) δppm 143.7, 141.0, 131.4, 127.7, 126.4. HRMS (ESI) calcd. for C₂₆H₂₀ (M+ H)⁺: 333.1565, found: 333.1560.

1,1,2,2-tetrakis(4-nitrophenyl)ethane: ¹H NMR (600 MHz, CDCl₃) δppm 8.10 (d, *J* = 8.8 Hz, 8H), 7.21 (d, *J* = 8.8 Hz, 8H). ¹³C NMR (150 MHz, CDCl₃) δppm 147.4, 147.1, 141.6, 131.8, 124.0. HRMS (ESI) calcd. for C₂₆H₁₆N₄O₈ (M+ H)⁺: 513.0968, found: 513.0976

4,4',4'',4'''-(ethene-1,1,2,2-tetrayl)tetraanilin : ¹H NMR (600 MHz, DMSO-*d*₆) δppm 6.57 (d, *J* = 8.3 Hz, 8H), 6.26 (d, *J* = 8.3 Hz, 8H), 4.87 (s, 8H). ¹³C NMR (150 MHz, DMSO-*d*₆) δppm 146.5, 137.1, 133.4, 132.2, 113.6. HRMS (ESI) calcd. for C₂₆H₂₄N₄ (M+ H)⁺: 393.2001, found: 393.2010.

1,1',1'',1'''-(ethene-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl))tetrakis(3-phenylthiourea) (5.1a): ^1H NMR (600 MHz, DMSO- d_6) δ ppm 9.79 (s, 8H), 7.36-7.31 (m, 16H), 7.12 (t, $J = 7.4$ Hz, 4H), 6.93 (d, $J = 8.5$ Hz, 8H). ^{13}C NMR (150 MHz, DMSO- d_6) δ ppm 179.5, 139.8, 138.2, 131.3, 128.9, 124.9, 124.2, 124.1, 122.5. HRMS (ESI) calcd. for $\text{C}_{54}\text{H}_{44}\text{N}_8\text{S}_4$ ($\text{M} + \text{H}$) $^+$: 933.2572, found: 933.2595.

1,1',1'',1'''-(ethene-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl))tetrakis(3-(4-(trifluoromethyl)phenyl)thiourea) (5.1b): ^1H NMR (600 MHz, DMSO- d_6) δ ppm 10.39-10.29 (m, 4H), 7.76 (s, 4H), 7.69 (s, 4H), 7.39 (s, 8H), 7.24-7.19 (m, 6H), 6.97-6.89 (m, 14H). ^{13}C NMR (150 MHz, DMSO- d_6) δ ppm 179.8, 162.8, 156.4, 142.7, 142.1, 140.1, 139.9, 137.6, 131.5, 130.8, 130.6, 130.4, 130.2, 126.4, 124.6, 123.8, 123.0, 122.7, 120.7, 120.9, 117.3, 117.0. HRMS (ESI) calcd. for $\text{C}_{58}\text{H}_{40}\text{F}_{12}\text{N}_8\text{S}_4$ ($\text{M} + \text{H}$) $^+$: 1205.2140, found: 1205.2151.

1,1',1'',1'''-(ethene-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl))tetrakis(3-(3,5-bis(trifluoromethyl)phenyl)thiourea) (5.1c): ^1H NMR (600 MHz, DMSO- d_6) δ ppm 10.26 (br 8H), 8.22 (s, 10H), 7.79 (s, 4H), 7.34 (d, $J = 8.2$ Hz, 7H), 7.01 (d, $J = 8.3$ Hz, 7H). ^{13}C NMR (150 MHz, DMSO- d_6) δ ppm 179.8, 162.8, 156.4, 142.7, 142.2, 140.1, 140.0, 137.7, 131.5, 130.52 (d, $J = 32.8$ Hz), 126.4, 124.6, 123.8, 123.0, 122.8, 121.0, 117.3, 117.0. HRMS (ESI) calcd. for $\text{C}_{62}\text{H}_{36}\text{F}_{24}\text{N}_8\text{S}_4$ ($\text{M} + \text{H}$) $^+$: 1477.1635, found: 1477.1637.

1,1',1'',1'''-(ethene-1,1,2,2-tetrayltetrakis(benzene-4,1-diyl))tetrakis(3-(2-(methylthio)ethyl)thiourea) (5.1d): ^1H NMR (600 MHz, DMSO- d_6) δ ppm 9.63 (s, 4H), 7.80 (s, 4H), 7.27 (d, $J = 8.2$ Hz, 8H), 6.93 (d, $J = 8.2$ Hz, 8H), 3.66 (s, 8H), 2.67 (t, $J = 6.8$ Hz, 8H), 2.09 (s, 12H). ^{13}C NMR (150 MHz, DMSO- d_6) δ ppm 180.2, 139.6, 139.3, 138.0, 131.5, 121.8, 43.2, 40.5, 32.4, 15.0. HRMS (ESI) calcd. for $\text{C}_{42}\text{H}_{52}\text{N}_8\text{S}_8$ ($\text{M} + \text{H}$) $^+$: 925.2153, found: 925.2159.

(((((ethene-1,1,2,2-tetraoltetrakis(benzene-4,1-diyl))tetrakis(azanediyl))tetrakis(carbonothioyl))tetrakis

(azanediyl))tetrakis(ethane-2,1-diyl))tetrakis(benzyl(methyl) sulfonium) (5.2):

^1H NMR (600 MHz, $\text{DMSO-}d_6$) δ ppm 9.67-9.65 (m, 4H), 8.23-8.21 (m, 4H), 7.85-7.82 (m, 4H), 7.74 (d, $J = 8.5$ Hz, 1H), 7.56 (s, 1H), 7.32-7.27 (m, 8H), 7.08-7.0 (m, 7H), 6.93-9.62 (m, 8H), 4.43 (s, 2H), 3.66 (s, 8H), 3.56 (s, 6H), 2.68-2.63 (m, 8H), 2.09-2.07 (m, 9H), 2.04-2.03 (m, 3H). ^{13}C NMR (150 MHz, $\text{DMSO-}d_6$) δ ppm 180.2, 147.4, 139.6, 139.3, 138.9, 138.3, 138.0, 131.5, 130.5, 124.3, 121.8, 44.9, 43.2, 43.0, 40.5, 32.4, 15.0. HRMS (ESI) calcd. for $\text{C}_{70}\text{H}_{76}\text{N}_{12}\text{O}_8\text{S}_8$ ($\text{M}^+ \text{H}^+$): 1469.3747 found: 1469.3741.

5.4.2. Anion binding analysis by ^1H -NMR titrations

Described in 2.4.2.1.

5.4.3. Job's plot experiment

Described in 2.4.3.

5.4.4. Density functional theory studies

Described in 3.4.4.

5.4.5. Ion transport studies

5.4.5.1. Ion transport activity studies using ion-selective electrode based assay:

5.4.5.1.1. Chloride ion efflux studies using chloride ion-selective electrode (chloride-ISE)

Described in section 3.4.5.1.1.

5.4.5.1.2. Preparation of EYPC/CHOL-LUVs

Described in section 3.4.5.1.2.

5.4.5.1.3. Chloride efflux study across EYPC/CHOL-LUVs

Described in section 3.4.5.1.3.

5.4.5.1.4. Quantitative analysis of transport activity from chloride-ISE assay

Described in section 3.4.5.1.4.

5.4.5.1.5. Measurement of half-maximal effective concentrations (EC_{50}) of the compounds from chloride-ISE assay

Described in section 3.4.5.1.5.

5.4.6. Evidence for the mechanistic pathway for chloride ion transportation:**5.4.6.1. Cation selectivity**

Cation selectivity studies were also performed by the ISE based assay as described above using EYPC/CHOL-LUVs where 5 mM phosphate buffer, pH 7.2 containing 100 mM MCl salt (where $M = Li^+$, Na^+ , K^+ , Rb^+ , and Cs^+) were used as an intravesicular medium, and extravesicular medium was filled with 5 mM phosphate buffer, pH 7.2, containing 100 mM $NaNO_3$.

5.4.6.2. Preferential ion transport activity in the presence of valinomycin (valinomycin assay)

For the valinomycin assay, the EYPC/CHOL-LUVs were prepared by following a similar procedure as mentioned earlier. For this particular chloride-ISE assay, the extravesicular solution was filled with the potassium nitrate solution, and the intravesicular was filled with KCl solution. The chloride efflux activity was performed in the presence and absence of the compound to determine the effect of the transportation rate. Here at 50 s, the solution of 10 μ L of the respective compound (from DMSO stock) and/or 2 μ L valinomycin (2.0 pM) was added to initiate the anion transport kinetics.

5.4.7. Evidence for mobile carrier mechanism**5.4.7.1. Cholesterol dependency assay**

Described in section 4.4.8.1

5.4.7.2. DPPC assay

To measure the magnitude of chloride efflux of the compound **5.1d** at different temperatures (25 and 45 $^{\circ}C$) in the presence of DPPC-LUVs (50 μ L) and

5 mM phosphate buffer, pH 7.2, containing 100 mM NaNO_3 (3940 μL) were taken in a clean and dry glass vial and kept under gentle stirring condition. The Cl-ISE was then immersed into the solution under mild stirring conditions. To initiate the Cl^- ion transport kinetics at $t = 50$ s, 10 μL of the respective compound (from DMSO stock solution) was added into the stirring solution, and the corresponding readings were recorded from the ISE instrument. After 5 minutes, the vesicles were completely lysed using 50 μL of 20% Triton X-100 solution. The total Cl^- ion efflux reading was taken at 7 minutes (permitting complete disruption of the LUVs). The initial reading was considered 0% Cl^- ion efflux, and the final reading at 7 min was considered 100% Cl^- ion efflux to calculate the percentage of chloride ion efflux.

5.4.7.3. U-tube experiment

The U-tube experiment was performed according to the reported procedure to confirm the mechanistic pathway for the Cl^- ion transport by the potent compound **5.1d**. The lipid bilayer was mimicked by using chloroform (12 mL) as the organic layer. The compound (0.3 mM) in chloroform was placed at the bottom of the U-tube with mild stirring conditions. The left-arm of the tube was filled with 0.1 M aqueous HCl solution (10 mL), and the right one was filled with 0.1 M aqueous NaNO_3 solution (10 mL). The Cl^- ion concentration of the receiver end was monitored using a Cl-ISE. In the meantime, the pH of the receiver end was monitored using a pH meter. We did not observe any pH changed by the compound **1d**, which ruled out the possibility of H^+/Cl^- co-transport. We also performed a similar kind of U-tube with minor modifications. This time the left-arm was filled with NaCl solution, and the right-arm was filled with the NaNO_3 buffer, and a steady increase in chloride ion concentration was observed at the right-arm, which clearly suggested its $\text{Cl}^-/\text{NO}_3^-$ antiport mechanism.

5.4.8. Regeneration of the active compound from its proanionophore

5.4.8.1. Regeneration in the presence of the sodium dithionite

The proanionophore **5.2** (1 mM) was dissolved in 10 mM PBS, pH 7.4 containing 10 mM sodium dithionite, and the mixture was incubated at 37 °C. At

different time points, an aliquot of the reaction solution was removed and monitored through fluorescence spectroscopic analysis. The fluorescence property of the proanionophore **5.2** was insignificant, whereas the active compound **5.1d** showed prominent fluorescence property in the aqueous medium. Utilizing this difference in fluorescence property, we recorded the spectra of the aliquot, which were collected at a different time interval. We observed a clear increment of the fluorescence spectra after a certain time interval which clearly confirmed the regeneration of the active compound **5.1d** in the presence of the reductive environment, which was created using the sodium dithionite, and it is already well documented that in the presence of the water the sodium dithionite generates the two free electrons which are mainly responsible for the reduction of the nitro group to its corresponding NHOH /NH₂ conversion and eventually released the active anionophore using the 1, 6 elimination. To support this regeneration pathway, we recorded the mass of the intermediates.

5.4.8.2. Regeneration anionophore 5.1d in the presence of nitroreductase enzyme

As we proposed that active compound **5.1d** could be regenerated under the reductive environment. To validate the proposed mechanism, we also monitored the regeneration study under the cellular medium where bacterial lysate was taken as the source of NTR, which is highly abundant in bacterial. In this regard, *E. Coli* strain (DE3) was inoculated in 10 mL Luria Bertani (LB) media till the mid-logarithm phase, and after that, the bacterial cells were centrifuged at 4,000 rpm for 10 min to get the bacterial pellet. Further, the resulting bacterial pellet was washed twice with PBS and re-suspended in fresh PBS (pH 7.4, 10 mM). Bacterial cells were then incubated with 300 mM NaCl, 10 mM MgCl₂, and 30 μM phenylmethanesulfonyl fluoride (PMSF) at mild shaking. Bacterial cells were then lysed using a probe sonicator for 30 min. under ice-cold conditions and followed by the centrifugation of bacterial lysate at 10000 rpm for 15 min. Next, 1 mL of this supernatant was taken into a washed vial and treated with compound **2** (25 μM). The whole reaction mixture was incubated at 37 °C with constant stirring. Aliquots were taken at certain time intervals like 0, 4, 8, 12, 18, 30, 40, 48, 60, and 72 hours. Using these aliquots, we observed a fluorescence enhancement which confirmed

the regeneration of the fluorescence active anionophore **5.1d** from its nonfluorescent proanionophore **5.2**.

5.4.8.3. Regeneration in the presence of the GSH

The proanionophore **5.2** (1 mM) was dissolved in 10 mM PBS, pH 7.4 containing 10 mM GSH, and the mixture was incubated at 37 °C. At different time points, an aliquot of the solution was removed and monitored through fluorescence spectroscopic analysis. The fluorescence property of the proanionophore **5.2** was insignificant, whereas the active compound **5.1d** showed prominent fluorescence properties in the aqueous medium. Utilizing this difference in fluorescence property, we recorded the spectra of the aliquot, which were collected at a different time interval. We observed an increase in the fluorescence signal after a certain time interval which clearly confirmed the regeneration of the active compound **5.1d** in the presence of GSH, and it is already well documented that GSH has the ability to attack the benzyl position through its SH group and could release back the potent anionophore from its inactive analog. To support this cleavage pathway, we recorded the ES-MS of the reaction mixture and observed the ES-MS of the intermediate, which clearly supported its regeneration process.

5.4.5.4. Transport studies of regenerated compound

The proanionophore **5.2** (1 mM) was dissolved in 10 mM PBS, pH 7.4, containing 10 mM GSH/ NTR enzyme. After that, the whole reaction mixture was incubated at 37 °C and stirred up to 72 h. At different time points, an aliquot of the solution was collected. Using these aliquots, the transport behavior of this reaction mixture was carried out using the Cl-ISE and observed significant transport after 18 h of incubation which clearly supports the regeneration of our active anionophore **5.1d** from the inactive proanionophore **5.2** under these conditions.

5.4.9. Transport of chloride ions across the giant unilamellar vesicles (GUVs): 5.4.9.1. Preparation of GUVs

Described in section 4.4.13.1.

5.4.9.2. Ion transport measurements using GUVs coated on the glass surface

As described in section 4.4.13.2., with minor modification. Here, the lucigenin dye was used instead of the HPTS dye.

5.4.10. Chloride ion transport by ^{35}Cl NMR measurements

The EYPC/CHOL-LUVs were prepared according to the method described earlier. The dry, thin film of EYPC and CHOL was rehydrated with HEPES buffer (500 μL of 20 mM HEPES containing 50 mM NaCl, pH 7.2). The resultant suspension was then vortexed 6-7 times for 1 hour. This suspension was then subjected to 17-19 times freeze-thaw cycles followed by 15 minutes of thorough vortexing. To obtain a uniform liposome size of 200 nm, the lipid suspension was then extruded using a mini extruder (a polycarbonate membrane from Avanti Polar Lipids having 200 nm pore size) for a minimum of 19-21 times (must be an odd number). Finally, the dialysis was performed overnight using 20 mM HEPES containing isoosmotic NaNO_3 , pH 7.2. The ^{35}Cl NMR studies were performed using 59 MHz Bruker NMR (ASCEND 600). First, the vesicle solution (450 μL) was taken in an NMR tube, and D_2O (50 μL) was added to it (final H_2O : D_2O mixture of 9:1), and the first spectra were taken (clearly showing the ^{35}Cl NMR peak) which was come around 4.79 ppm. Next, Mn^{2+} solution (10 μL) from its stock solution of 115 mM was added to the vesicle solution (5 mol % Mn^{2+} : Cl), and the solution was shaken for 2-3 minutes followed by recording the NMR spectra. After that, the solution was incubated for 1 hour with 5 mM of the transporter **5.1d** or DMSO, and consequently, another spectrum was recorded displaying the disappearance of the ^{35}Cl NMR signal, which represents Cl^- ions transportation across the lipid bilayers. Finally, the vesicles were completely lysed by adding 20 μL of 20% Triton X-100 (prepared in 9:1 H_2O : D_2O mixture), leading to all chloride ions coming outside and getting quenched due to the presence of Mn^{2+} (in the NMR spectra no ^{35}Cl NMR signal could be seen).

5.4.11. Validation of AIE property:

5.4.11.1. Under an aqueous environment

To confirm the AIE properties of potent compound **5.1d**, the fluorescence enhancement property of compound **5.1d** in the presence of the different fractions

of water was investigated. The emission characteristics of compound **5.1d** (2 μM) with different water fractions (0 to 100%) were conducted. During this experiment, the compound was excited at 350 nm.

5.4.11.2. AIE behavior under liposomal environment

The AIE property of compound **5.1d** under the liposomal environment was also validated in the presence of the EYPC/CHOL-LUVs, where initially we recorded the emission spectra of compound **5.1d** (0.03 μM) in 100% water environment (fully AIE state). After that, the liposomal solution was added sequentially to the fluorescence cuvette and recorded the emission spectra. The emission spectra displayed a blue shift (at around 450 nm) with significant fluorescence enhancement upon the addition of the liposomal solution, which could be due to the hydrophobic environment of the lipid bilayer or a different kind of AIE pattern from its aqueous solution pattern. A similar experiment was performed where we added the same amount of water (which was earlier liposomal solution) and observed a decrease in emission spectra which is because of the dilution effect. This blank experiment confirmed its different kind of AIE property under the liposomal environment. For both experiments, initially, we took compound **5.2** within 800 μL of water in the fluorescence cuvette and sequentially added the liposomal solution/water solution (2 μL each time) to the cuvette.

5.4.12. Staining of GUVs

To explore the potential of the synthesized TPE derivatives as bioimaging agents, we performed GUV staining measurements. The GUVs were prepared as mentioned in the earlier section with minor modifications. After preparation, the GUVs (EYPC/ CHOL/ DPPS in the molar ratio of 6:3.5:0.5) were incubated with the compound **5.1d** (10 μM ; higher concentration was used for better visibility) and kept for 7-8 h under mild shaking conditions. After that, we centrifuged the whole solution at 1000 rpm for 10 minutes to remove the excess compound **5.1d**, and this process was repeated another 5-times using a buffer solution (100 mM HEPES, 200 mM glucose in H_2O , pH = 7.4). After each centrifugation, we observed a clear pellet at the bottom of the microcentrifuge tube. The final pellet (dense solution containing the GUVs with compound **5.1d**) was mixed with 100 μL of lower buffer (final

vesicle concentration of 15 mM) and used for the microscopic visualization. For the microscopic images, we used the coated glass coverslip, which was described in the earlier section

5.4.13. Selectivity of the stimuli

The selectivity of compound **5.2** for NTR and GSH was examined by monitoring the fluorescence analysis of proanionophore **5.2**, after incubation with NADH and various potentially interfering species, such as inorganic salts (CaCl₂, MgCl₂, KCl, and NaCl), biothiols (cysteine), GSH, glutamic acid, arginine, serine, vitamin C and reactive oxygen species (H₂O₂) under the similar experimental conditions as that for NTR and GSH. As shown in Figure 5.9, fluorescence enhancement was not detected for active compound **5.1d** after incubation with each of these potentially interfering species. Only under the GSH and NTR, we observed the AIE property of the active compound **5.1d**, which confirmed its selectivity towards the GSH and the NTR. A significant amount of fluorescence was also observed in the presence of the cysteine, H₂O₂, which may be due to the presence of the thiol and reactive OH⁻ group that could attack the benzyl position of the proanionophore and subsequently could release the active molecules from its proanionophore. To nullify any intrusive effect of these species with AIE property of regenerated **5.1d**, a control experiment was carried out where the **5.1d** compound was incubated with these similar species and recorded their spectra where there was no evidence of any interference in AIE property observed.

5.4.14. ^1H and ^{13}C NMR spectra of TPE-based anionophores and proanionophores

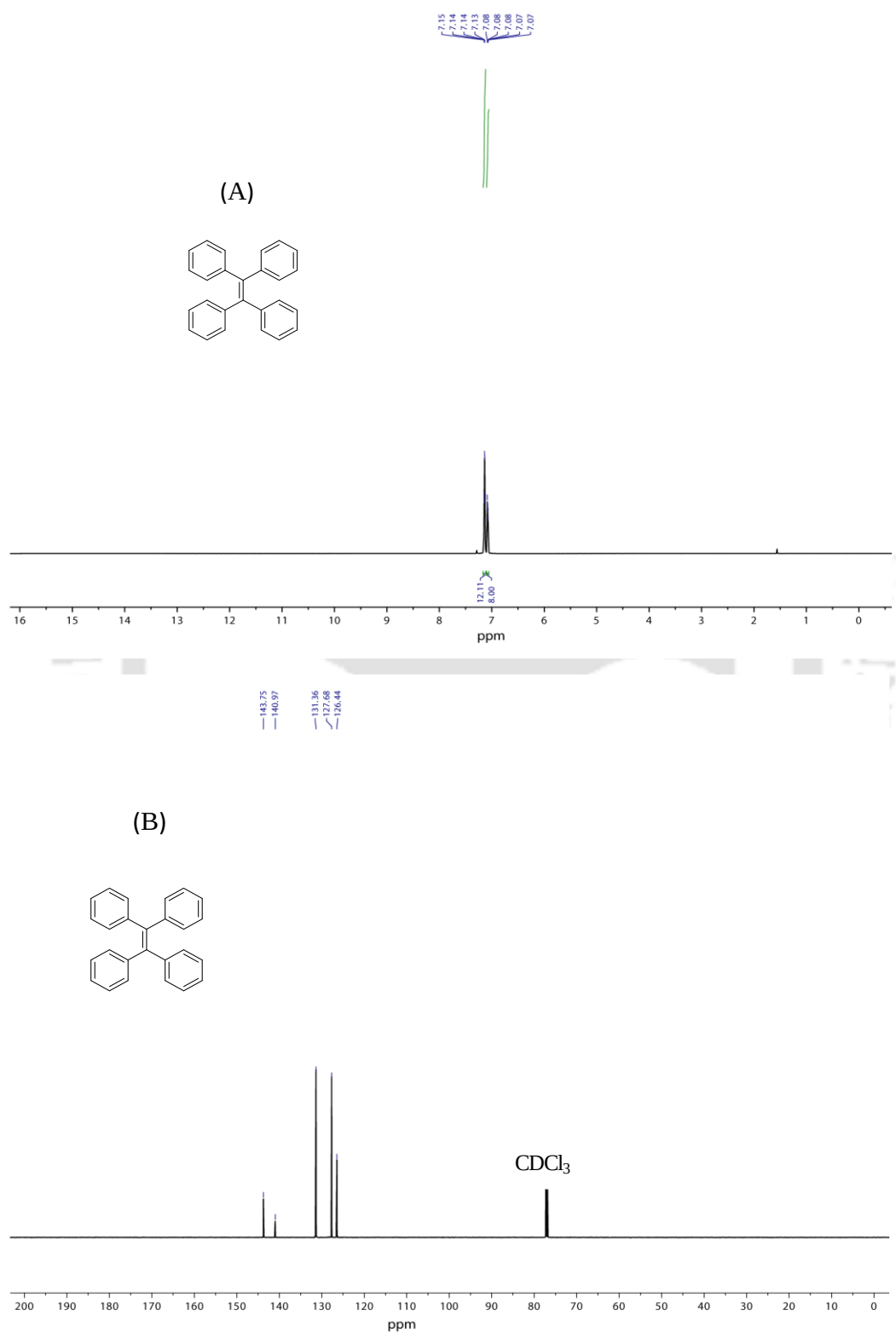


Figure 5.10. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound TPE.

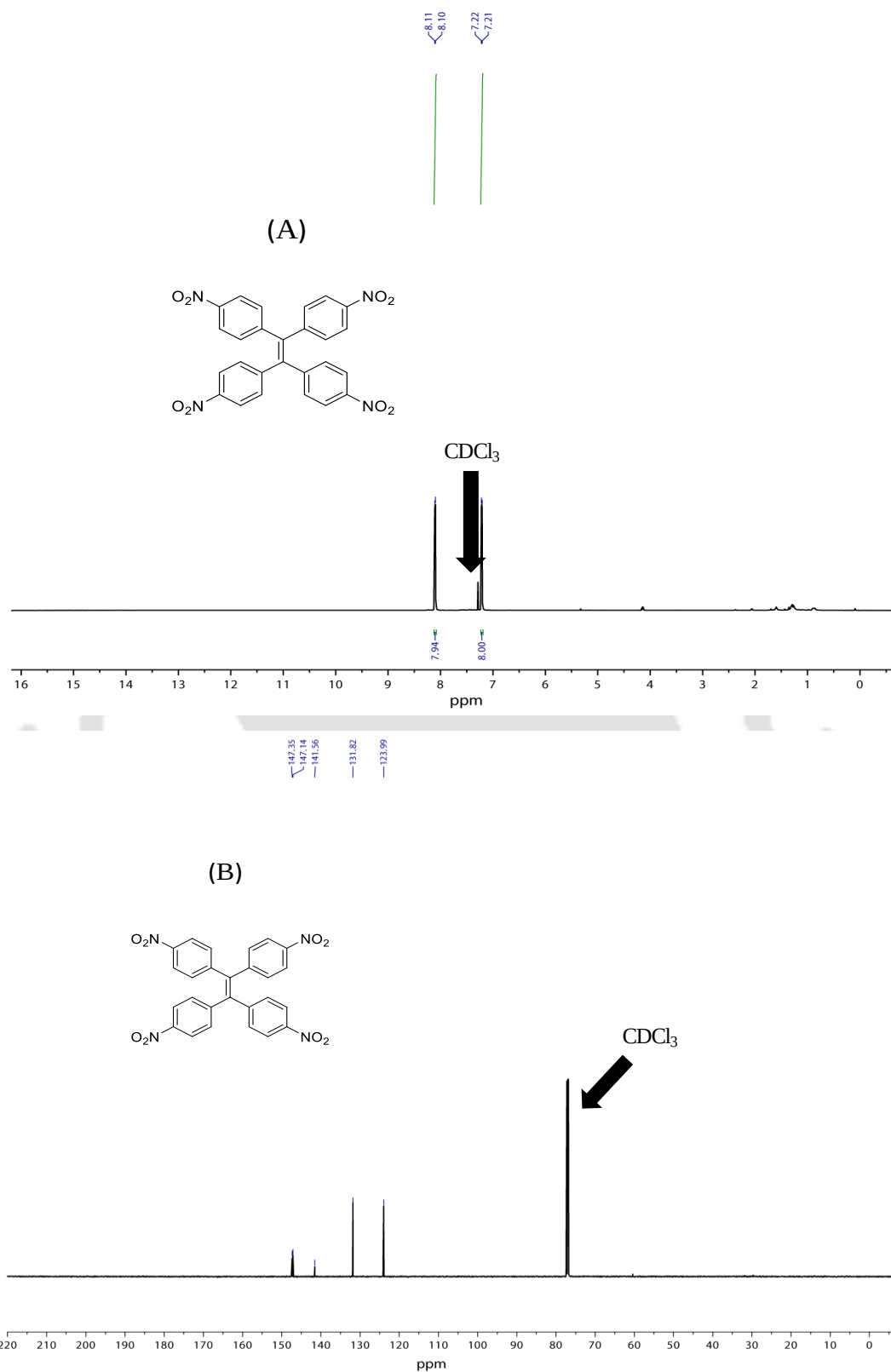


Figure 5.11. ¹H NMR (A) and ¹³C NMR (B) spectra of compound TPE-NO₂.

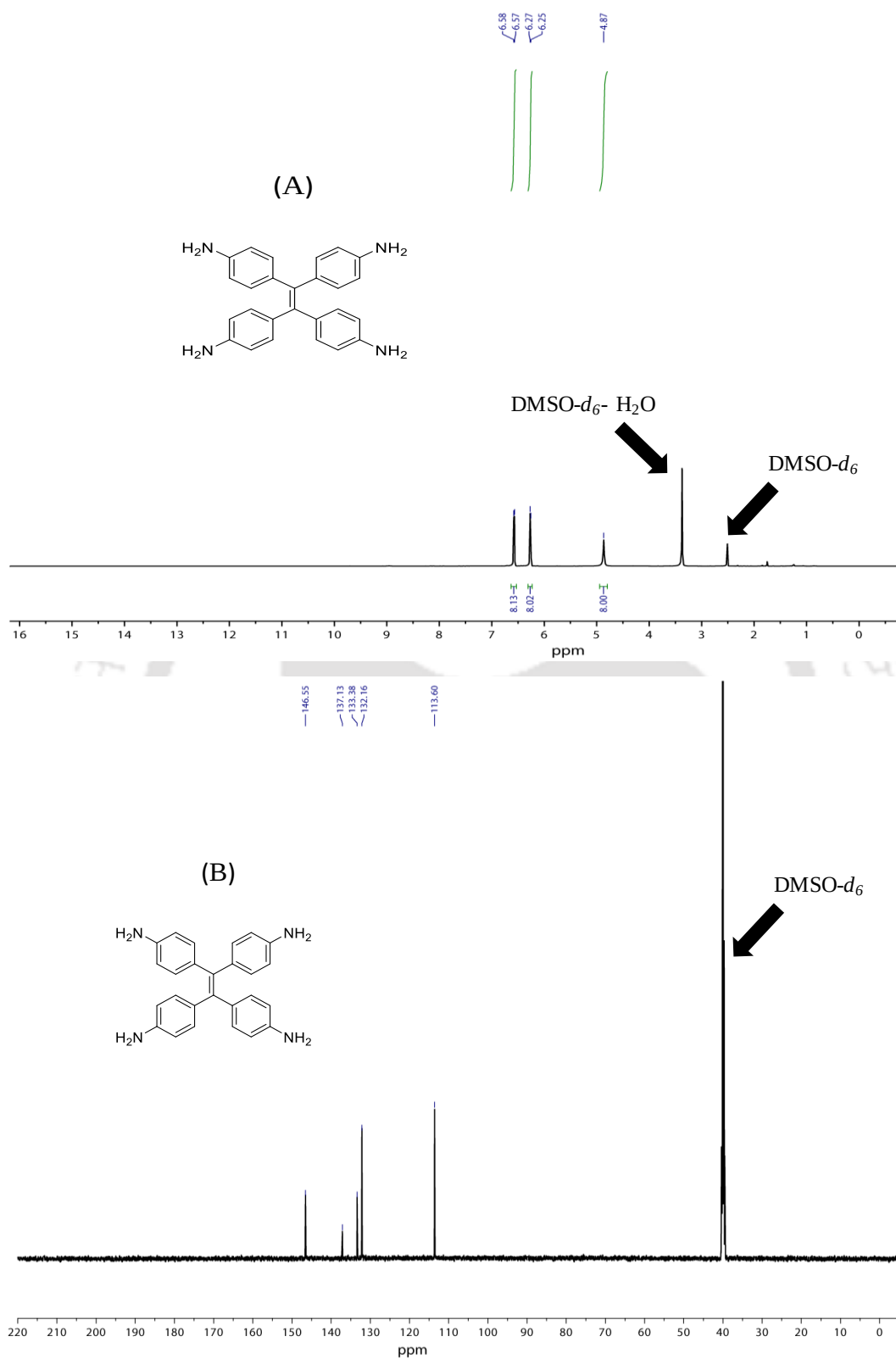


Figure 5.12. ¹H NMR (A) and ¹³C NMR (B) spectra of compound TPE-NH₂.

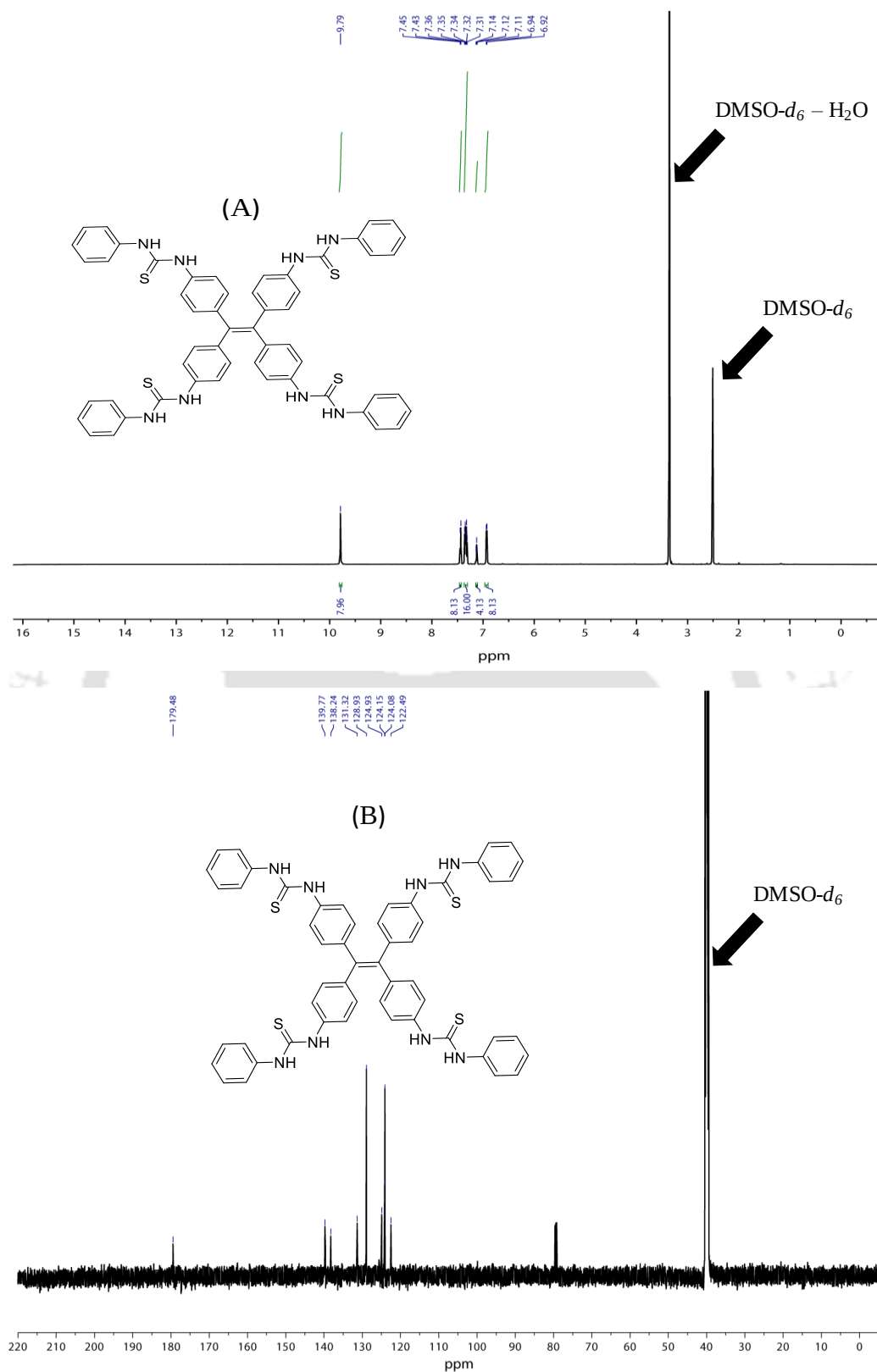


Figure 5.13. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 5.1a.

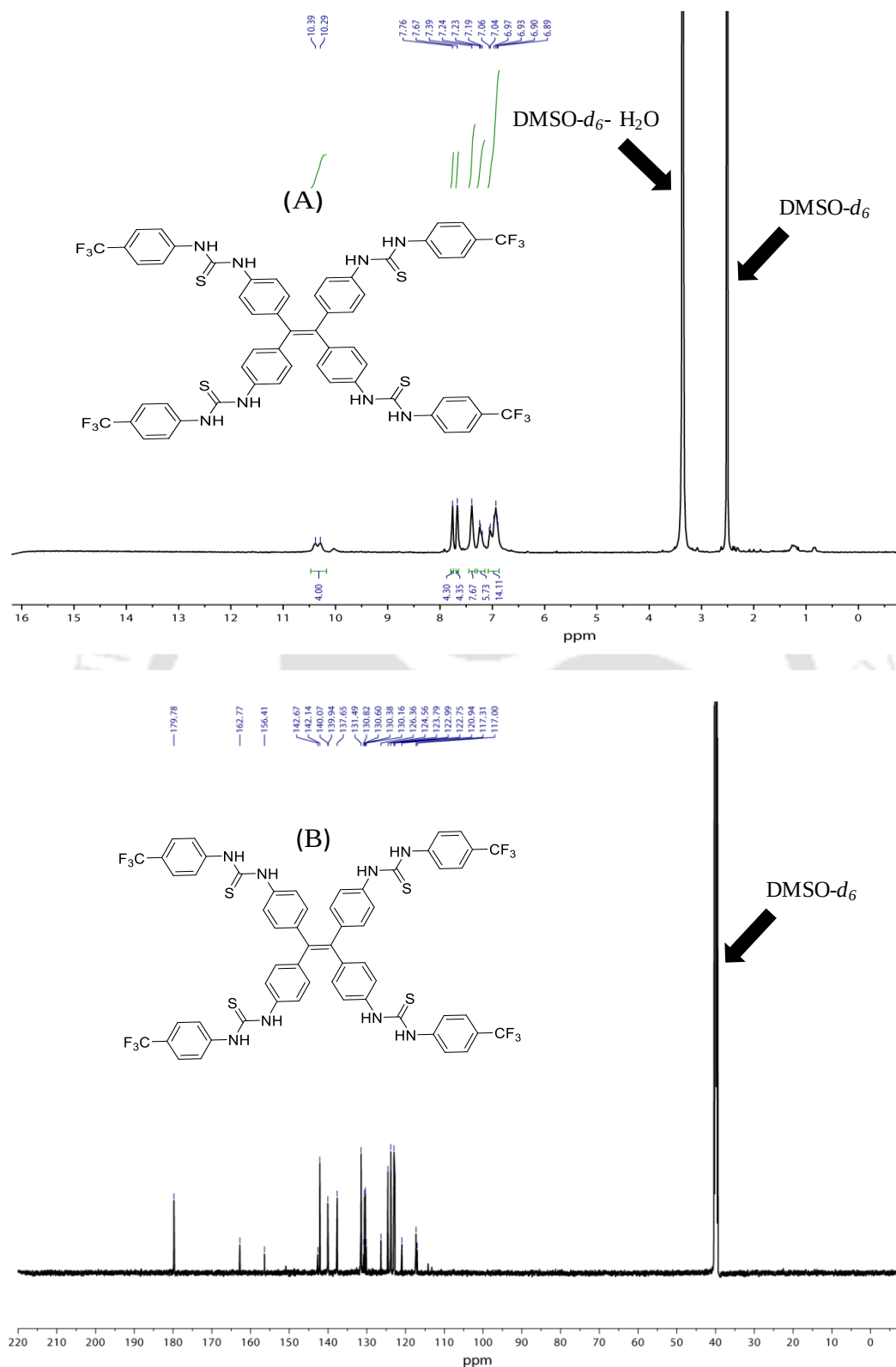


Figure 5.14. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound **5.1b**.

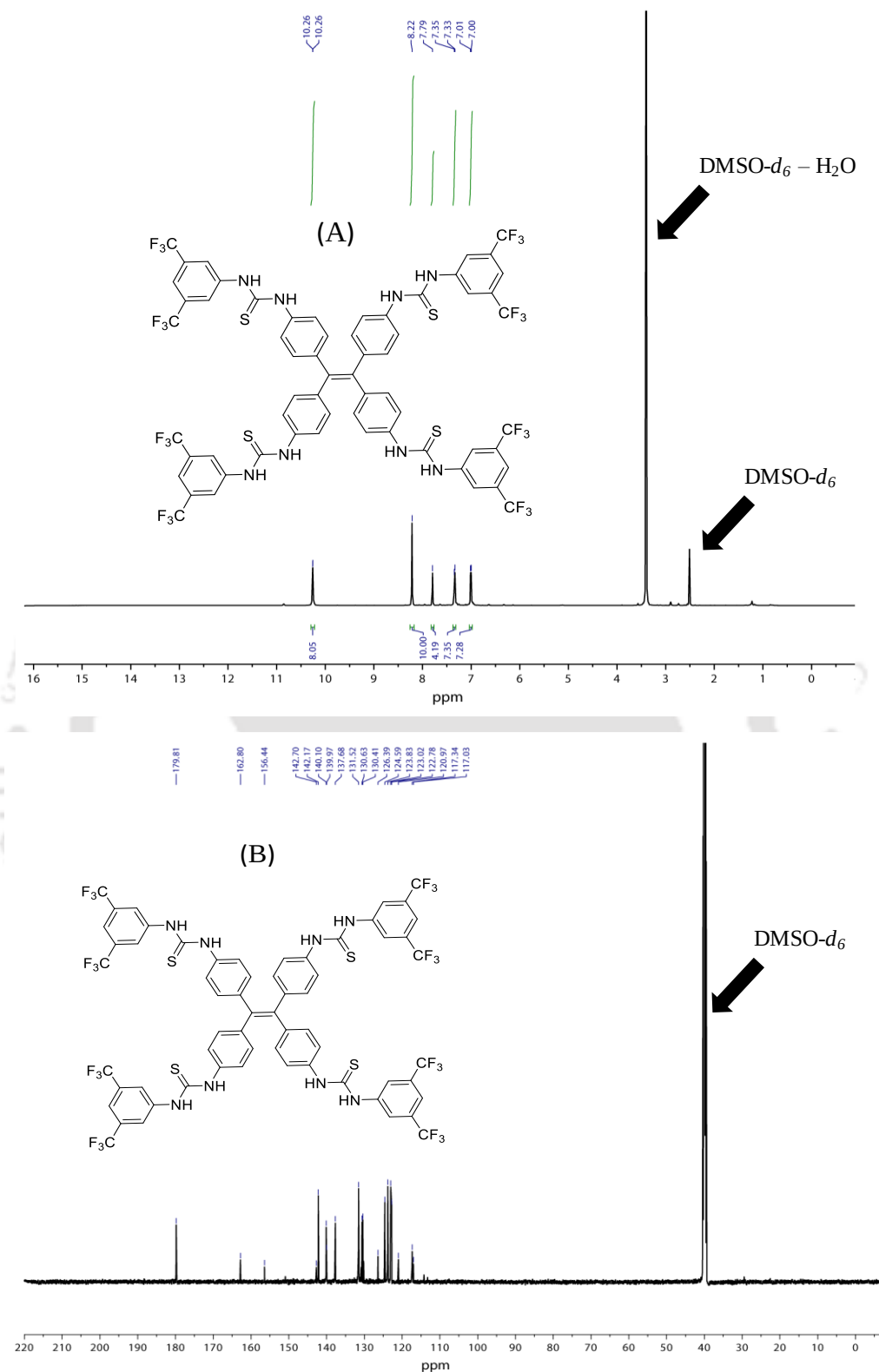


Figure 5.15. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 5.1c.

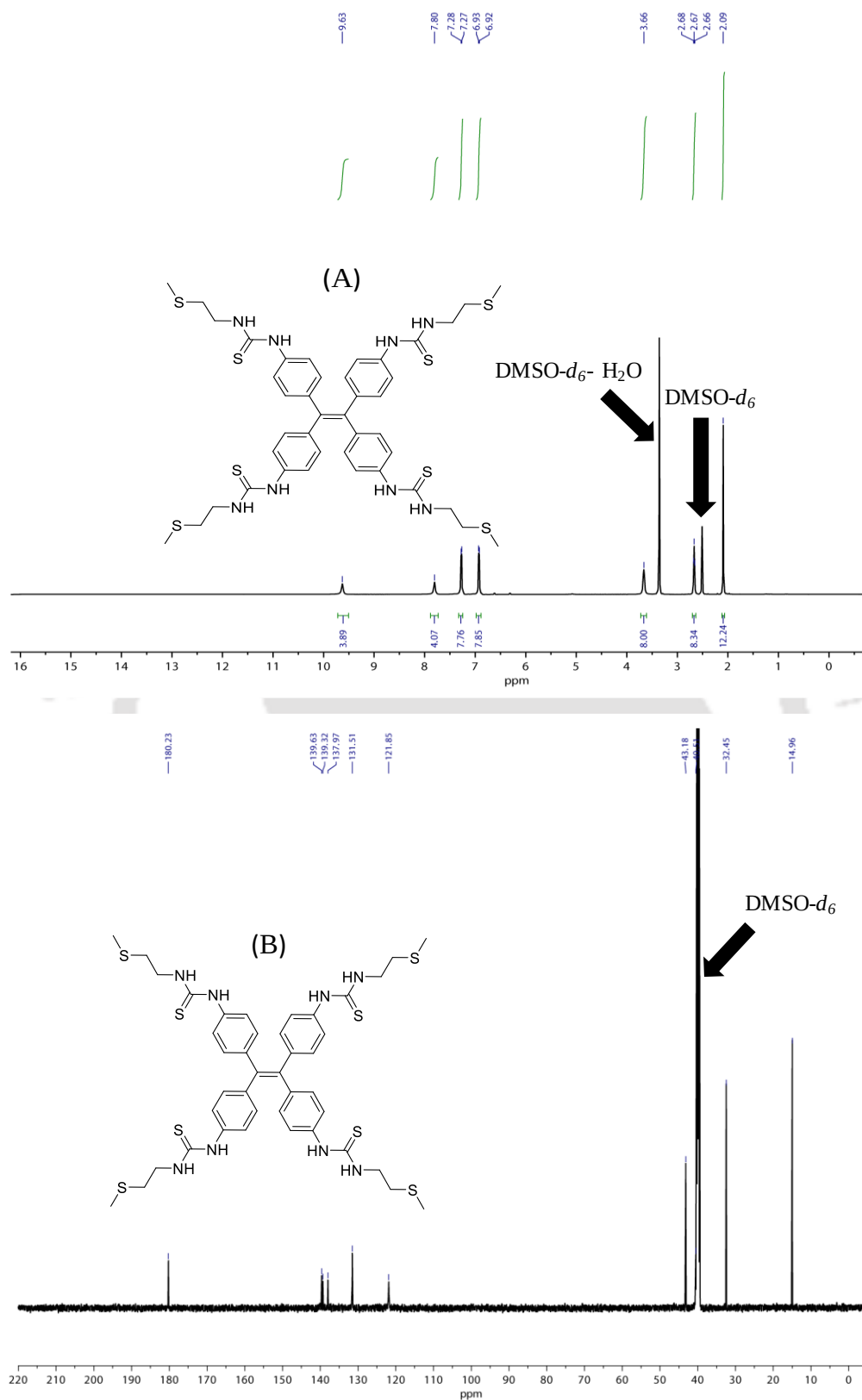


Figure 5.16. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 5.1d.

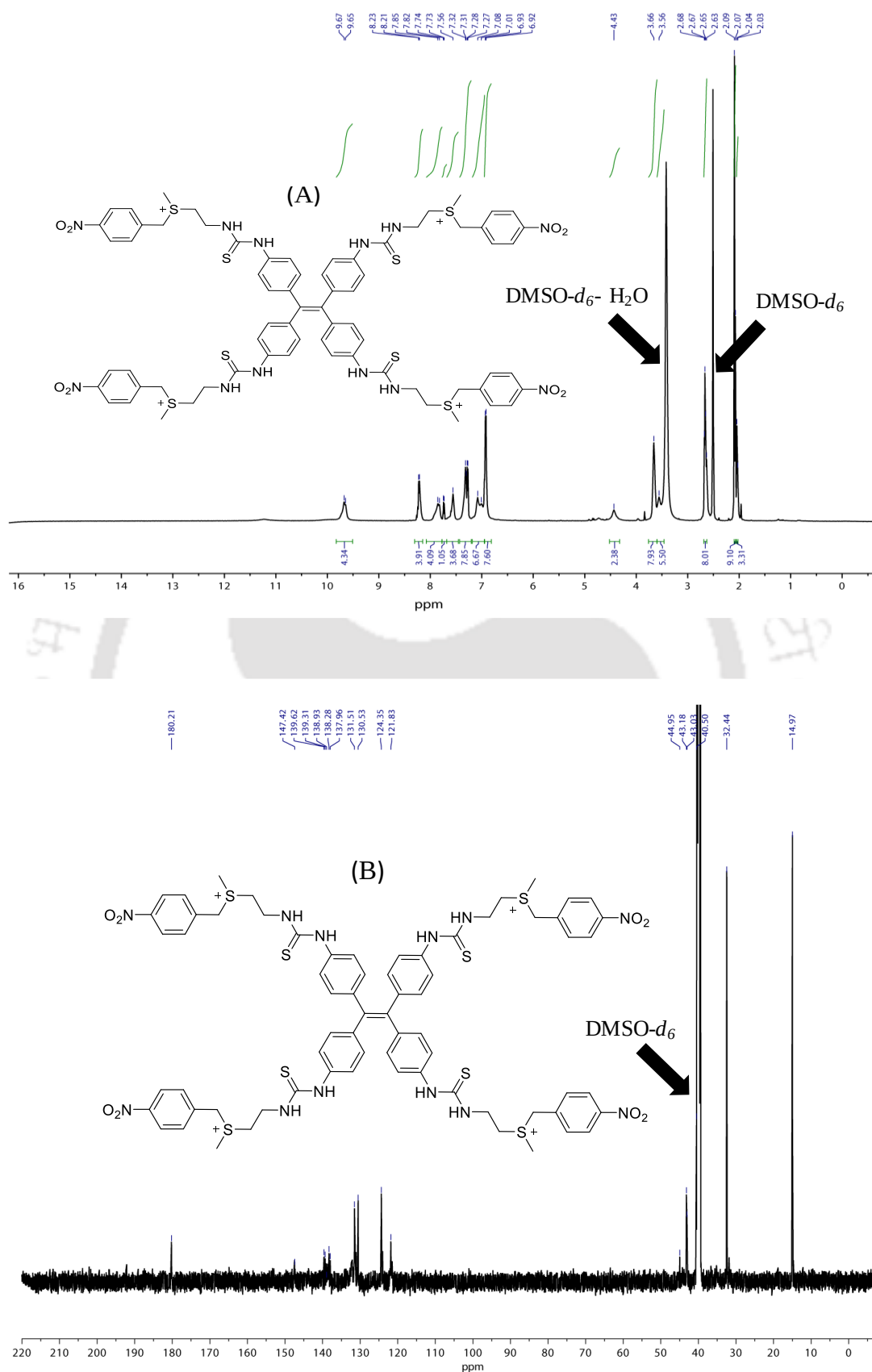


Figure 5.17. ^1H NMR (A) and ^{13}C NMR (B) spectra of compound 5.2.

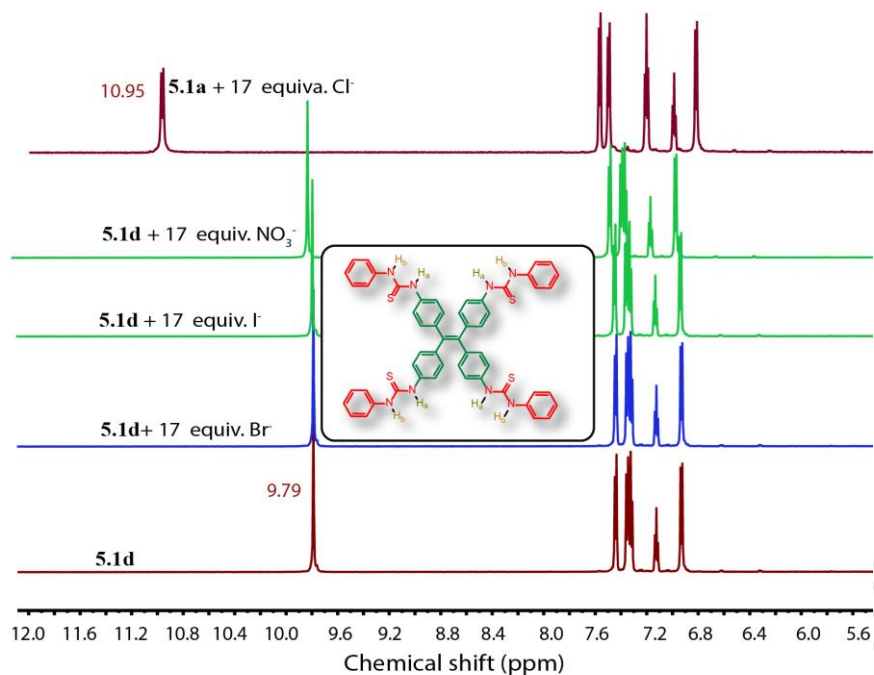
5.4.15. Spectra of anion recognition through ^1H NMR titration

Figure 5.18. The ^1H NMR titration spectra for compound **5.1a** (3.45 mM) with a fixed concentration of numerous anions salt of TBA (58.65 mM) in $\text{DMSO}-d_6$ solvent at room temperature.

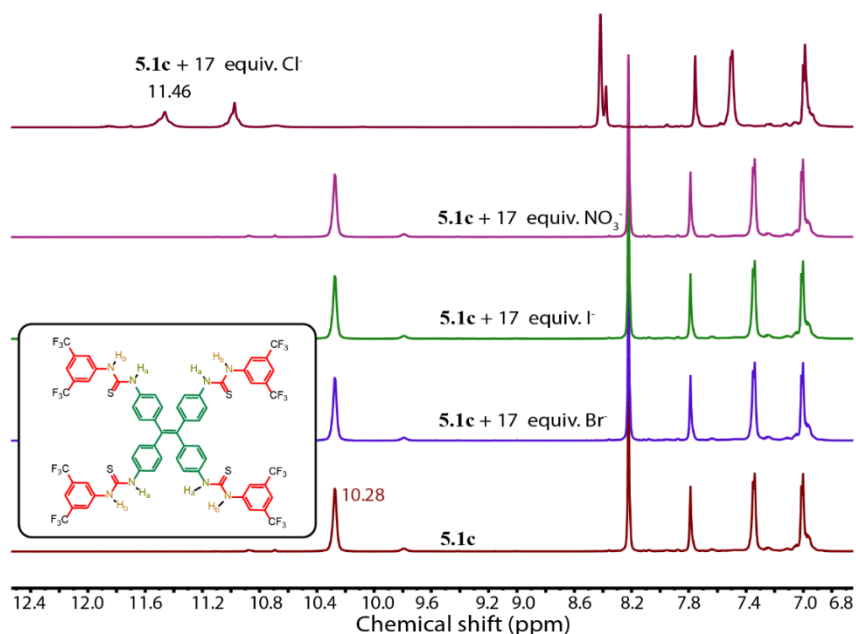


Figure 5.19. The ^1H NMR titration spectra for compound **5.1c** (3.45 mM) with a fixed concentration of numerous anions salt of TBA (58.65 mM) in $\text{DMSO}-d_6$ solvent at room temperature.

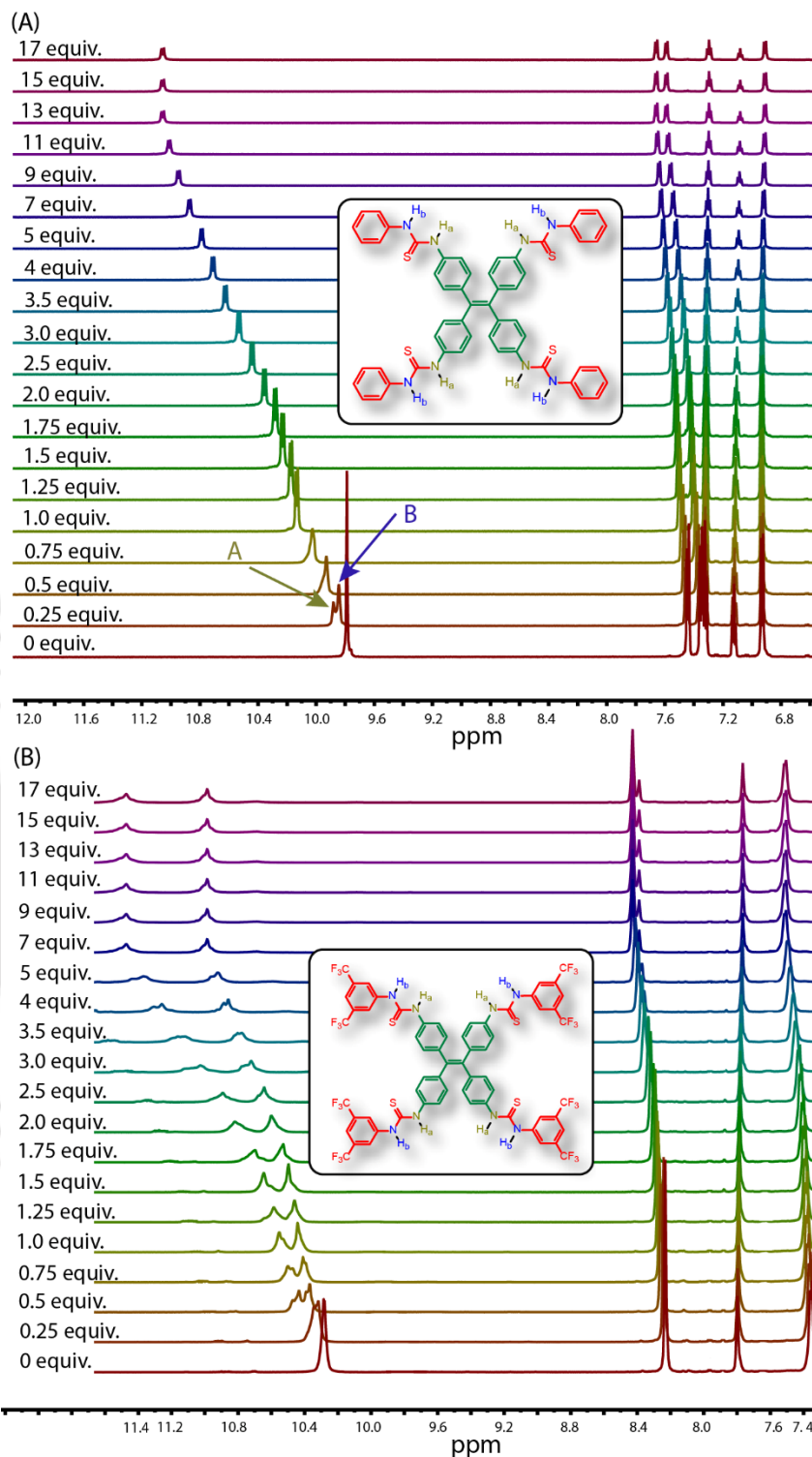


Figure 5.20. ^1H -NMR (600MHz) titration spectra for compound 5.1a (A) and 5.1c (B) with the chronological addition of TBACl in DMSO- d_6 solvent, respectively. The equivalent amounts of added TBACl were shown on the spectra.

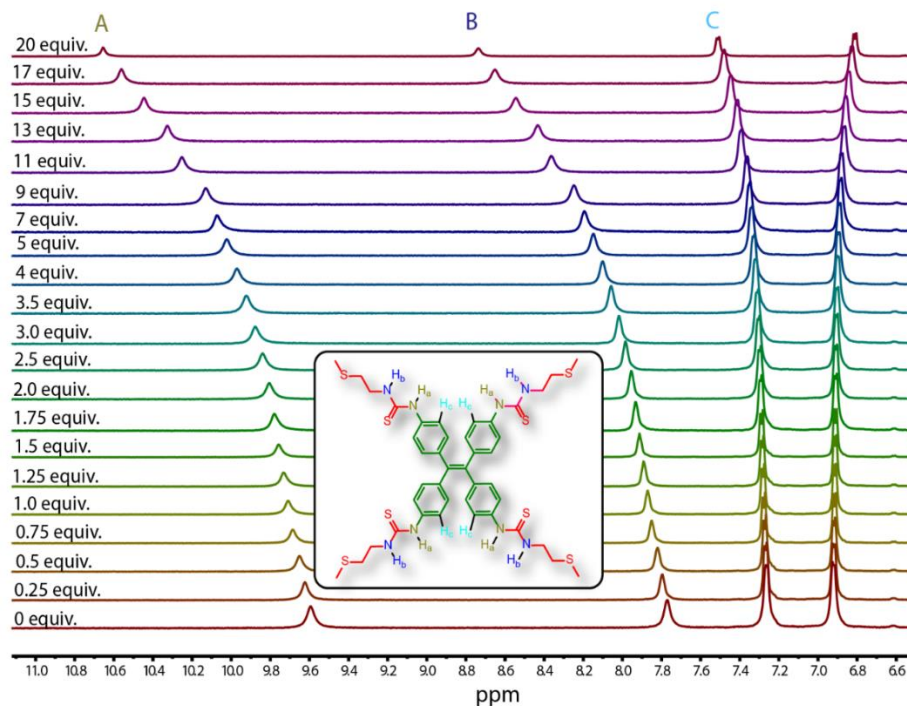


Figure 5.21. ^1H -NMR (600MHz) titration spectra for compound **5.1d** with the chronological addition of TBACl in $\text{DMSO-}d_6$ solvent. The amounts of added TBACl were shown on the spectra.

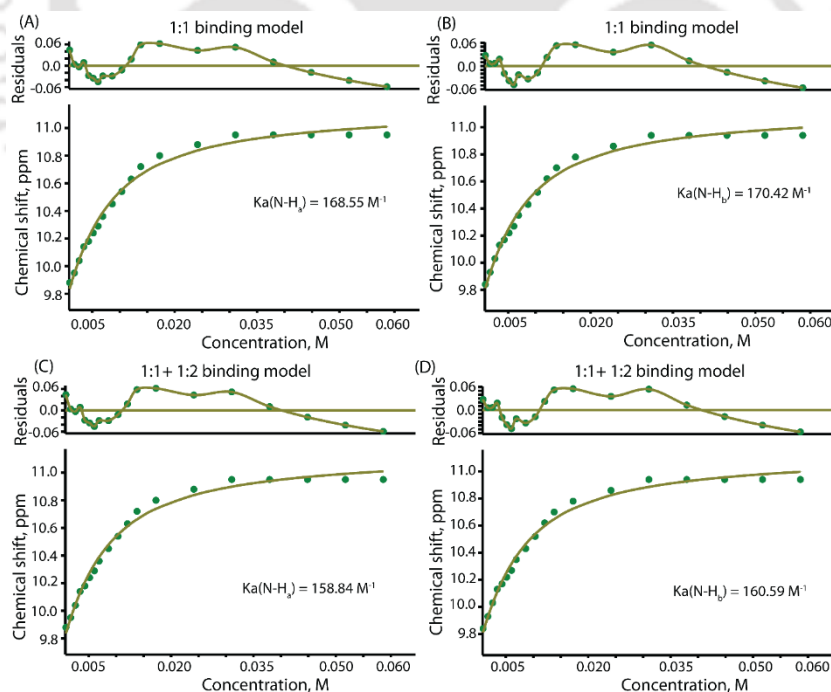


Figure 5.22. Plots of non-linear fit using WinEqNMR and experimental points (symbols) against the concentration of TBACl for **5.1a** in $\text{DMSO-}d_6$ using either N-H_a (A, C), N-H_b (B, D) with 1:1 or a mixture of 1:1 and 1:2 host/guest stoichiometries.

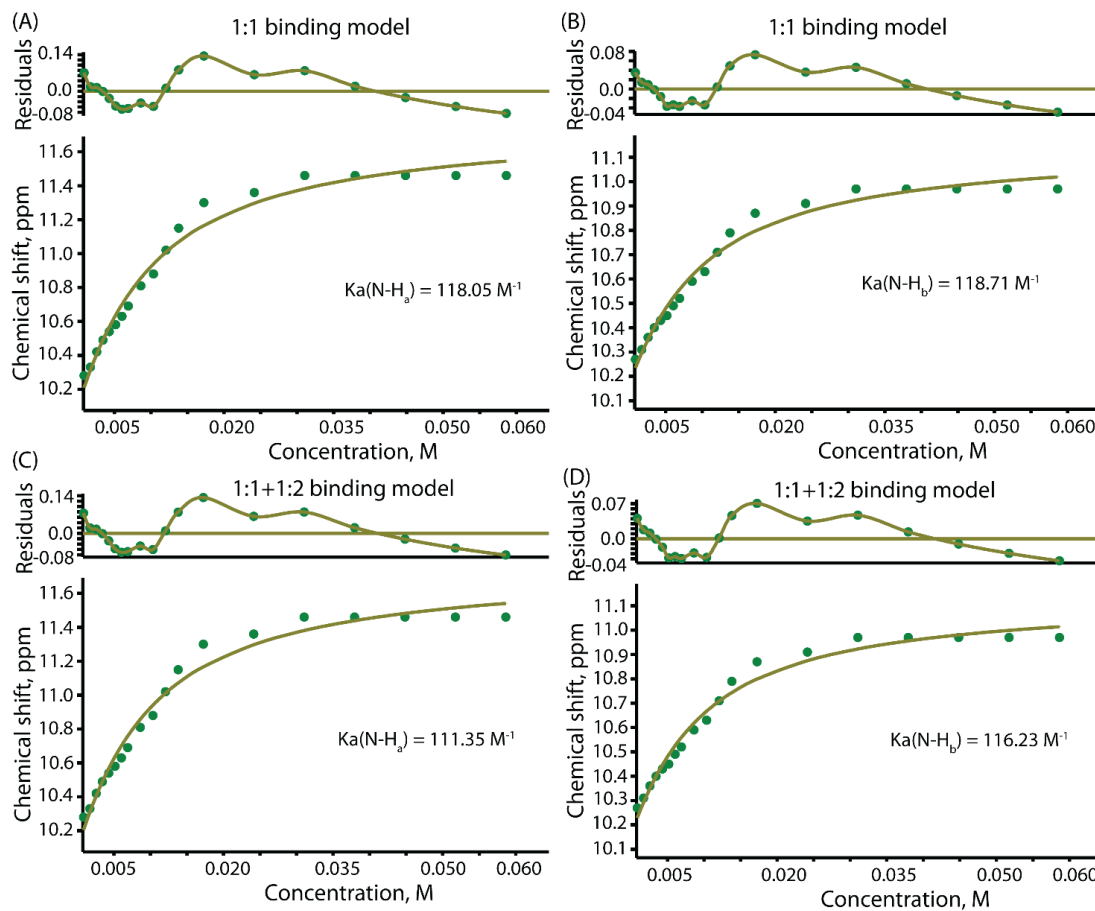


Figure 5.23. Plots of non-linear fit using WinEqNMR and experimental points (symbols) against the concentration of TBACl for **5.1c** in DMSO- d_6 using either N-H_a (A, C), N-H_b (B, D) with 1:1 or a mixture of 1:1 and 1:2 host/guest stoichiometries.

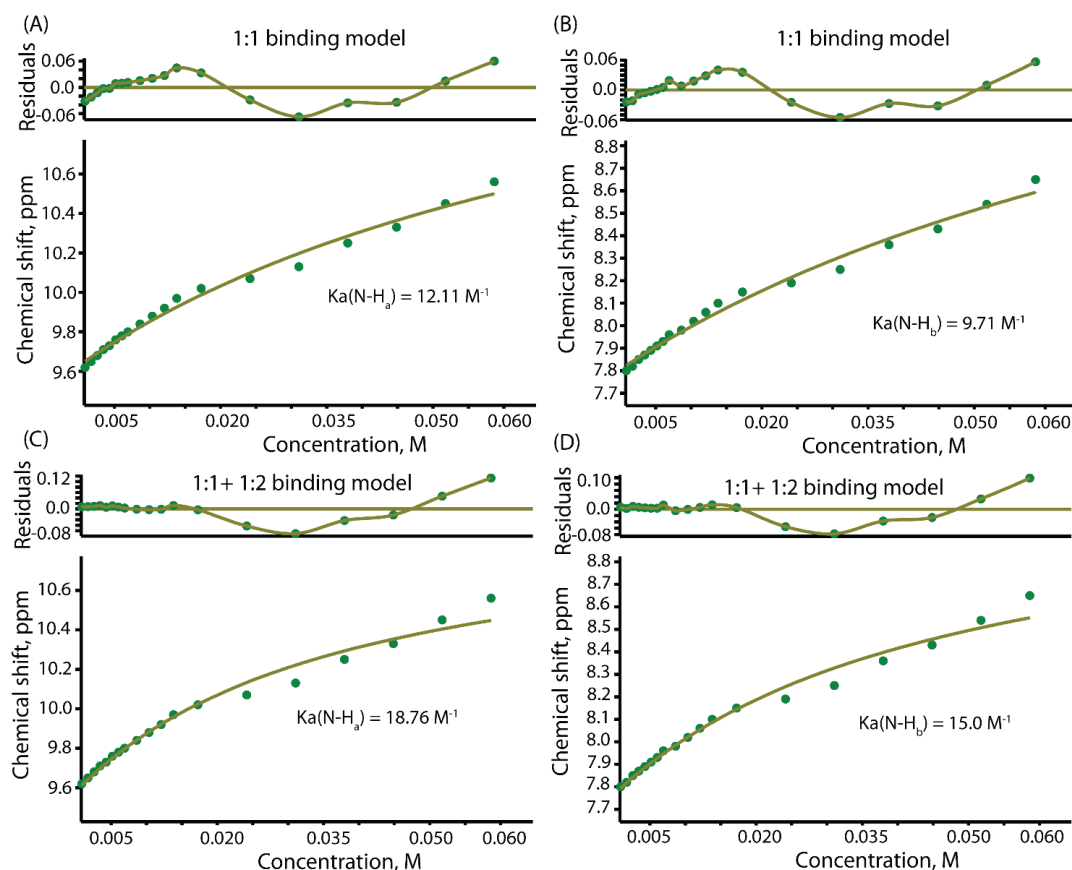


Figure 5.24. Plots of non-linear fit using WinEqNMR and experimental points (symbols) against the concentration of TBACl for **5.1d** in DMSO- d_6 using either N-H_a (A, C), N-H_b (B, D) with 1:1 or a mixture of 1:1 and 1:2 host/guest stoichiometries.

Table 5.1. Binding affinities of the compounds.

1:1 binding model		
Compound Code	K for N-H _a	K for N-H _b
5.1a	168.55	170.42
5.1c	118.05	118.71
5.1d	12.11	9.71
1:1 + 1:2 binding model		
5.1a	158.84	160.59
5.1c	111.35	116.23
5.1d	18.76	15.0

N.B. Due to the broadening of N-H peaks, binding affinities from the ^1H NMR could not be measured for compound **5.1b** compound.

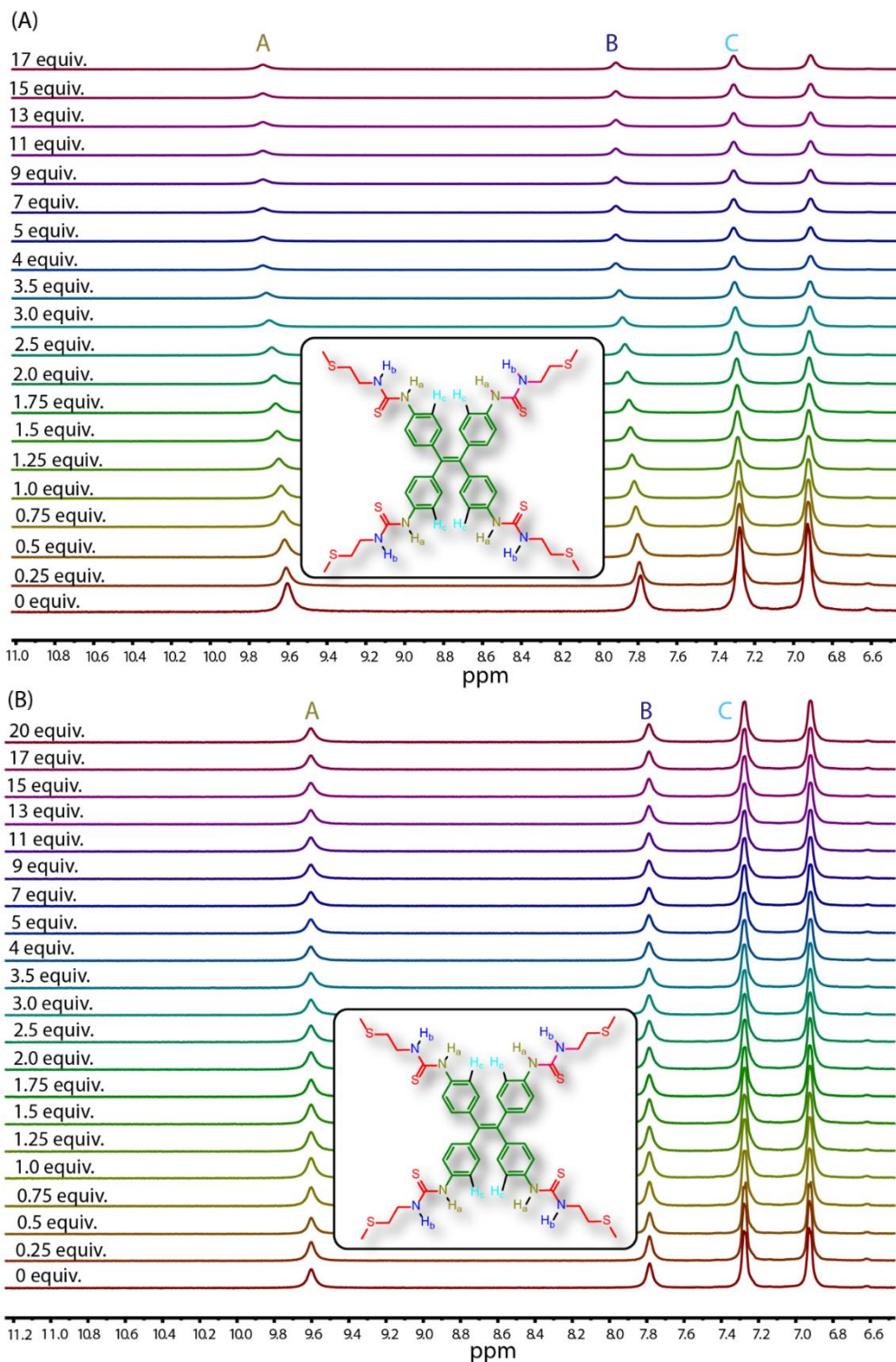


Figure 5.25. ^1H -NMR (600MHz) titration spectra for compound **5.1d** with the chronological addition of TBABr (A) and TBAI (B) in $\text{DMSO}-d_6$ solvent, respectively. The amounts of added TBABr and TBAI were shown on the spectra. An insignificant extent of the chemical shift was observed.

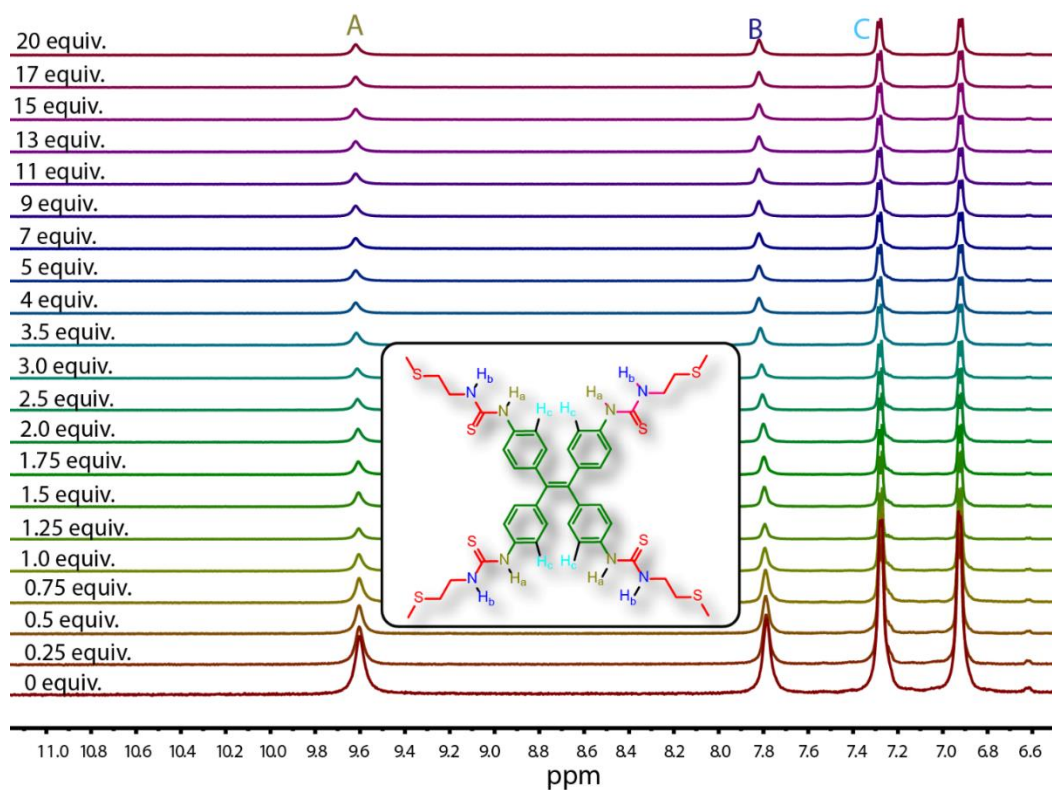


Figure 5.26. ^1H -NMR (600MHz) titration spectra for compound **5.1d** with the chronological addition of TBANO_3 (A). The amounts of added TBANO_3 were shown on the spectra. An insignificant extent of the chemical shift was observed.

5.4.16. Job's plot spectra

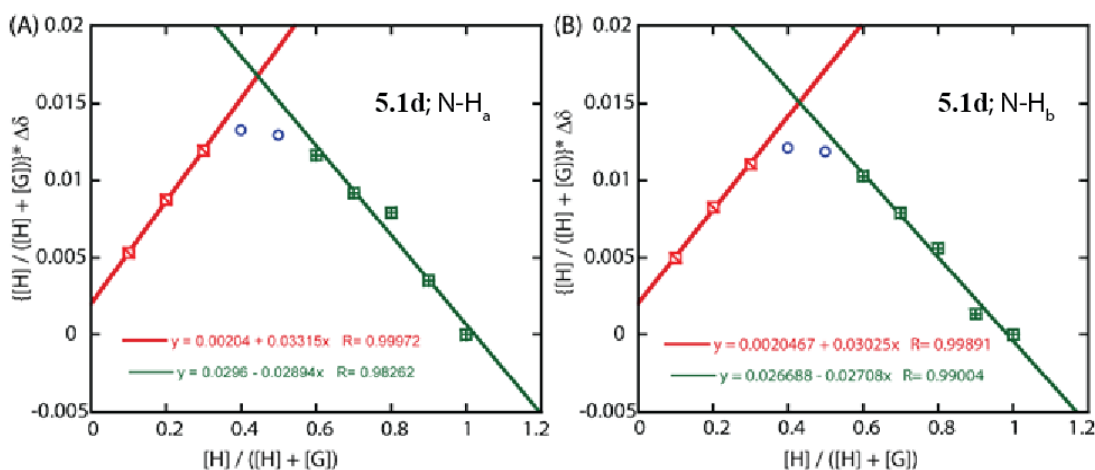


Figure 5.27. Job's plot analysis for **5.1d** with TBACl in $\text{DMSO-}d_6$ solvent by varying mole fraction of **5.1d** against the chemical shift of N-H_a (A) and N-H_b (B).

5.4.17. DFT spectra and corresponding co-ordinate:

Table 5.2. Optimized structure of compound **5.1d** using the using B3LYP/ 6-31+G (d) and B3LYP/ 6-31++G (d,p) basic sets.

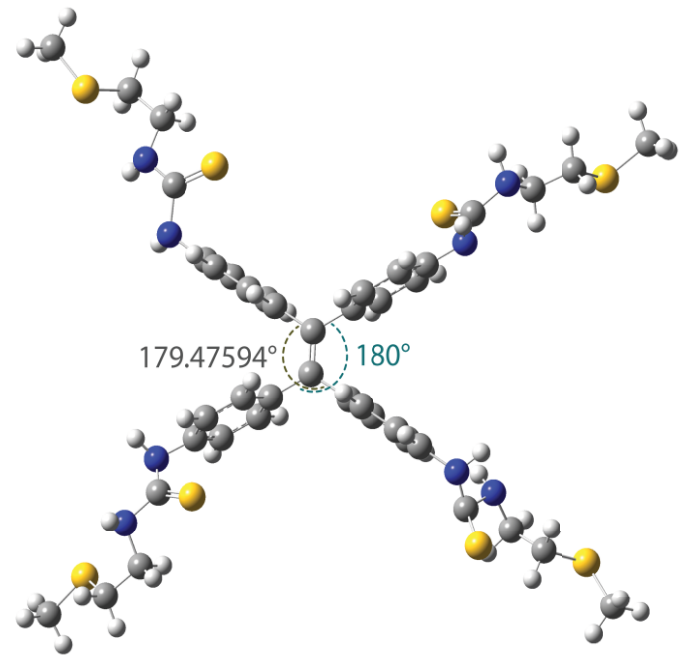
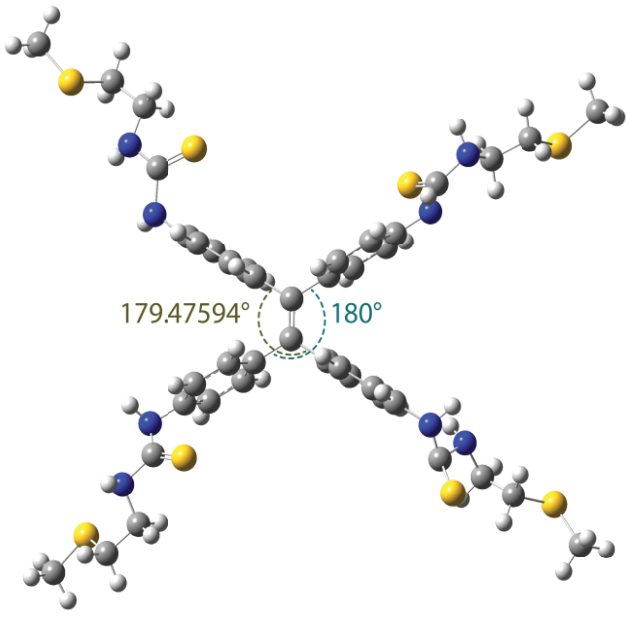
5.1d; B3LYP/ 6-31+G (d)	
	Optimized Energy (a.u) -5254.85514260
5.1d; B3LYP/ 6-31++G (d,p)	
	Optimized Energy (a.u) -5254.94950766

Table 5.3. XYZ Cartesian coordinate for compound '5.1d--- Cl⁻ complex' using B3LYP/ 6-31+G (d) and B3LYP/ 6-31++G (d,p) basic sets.

5.1d---Cl ⁻ { (B3LYP/ 6-31+G (d) basic set}				5.1d--- Cl ⁻ { (B3LYP/ 6-31++G (d,p) basic set}			
Total Energy = -7095.460848 Hartree Imaginary Frequency = 0				Total Energy = -7095.560816 Hartree Imaginary Frequency = 0			
Atom	X	Y	Z	Atom	X	Y	Z
C	-0.280605	0.472282	0.506758	C	-0.28061	0.472282	0.506758
C	-0.274936	-0.86771	0.50743	C	-0.27494	-0.86771	0.50743
C	-1.596381	1.236604	0.269807	C	-1.59638	1.236604	0.269807
C	-2.421745	1.582429	1.312236	C	-2.42175	1.582429	1.312236
C	-1.949324	1.583617	-1.05731	C	-1.94932	1.583617	-1.05731
C	-3.588658	2.263758	1.066673	C	-3.58866	2.263758	1.066673
H	-2.139887	1.309255	2.339405	H	-2.13989	1.309255	2.339405
C	-3.137006	2.275676	-1.29396	C	-3.13701	2.275676	-1.29396
H	-1.294576	1.311183	-1.89788	H	-1.29458	1.311183	-1.89788
C	-3.976151	2.625019	-0.21521	C	-3.97615	2.625019	-0.21521
H	-4.230318	2.52675	1.920108	H	-4.23032	2.52675	1.920108
H	-3.419464	2.54873	-2.32107	H	-3.41946	2.54873	-2.32107
C	1.04295	-1.63203	0.732365	C	1.04295	-1.63203	0.732365
C	1.540279	-1.9827	1.895773	C	1.540279	-1.9827	1.895773
C	1.776663	-1.97415	-0.5088	C	1.776663	-1.97415	-0.5088
C	2.74581	-2.67532	2.011694	C	2.74581	-2.67532	2.011694
H	0.972045	-1.71336	2.797833	H	0.972045	-1.71336	2.797833
C	2.982148	-2.66619	-0.39296	C	2.982148	-2.66619	-0.39296
H	1.394259	-1.69788	-1.50224	H	1.394259	-1.69788	-1.50224
C	3.467017	-3.01656	0.867736	C	3.467017	-3.01656	0.867736
H	3.12784	-2.95097	3.005331	H	3.12784	-2.95097	3.005331
H	3.550939	-2.9354	-1.29479	H	3.550939	-2.9354	-1.29479
C	1.021672	1.247935	0.778858	C	1.021672	1.247935	0.778858
C	1.827778	1.640118	-0.30354	C	1.827778	1.640118	-0.30354

C	1.401941	1.55883	2.073428	C	1.401941	1.55883	2.073428
C	3.009625	2.343013	-0.06765	C	3.009625	2.343013	-0.06765
H	1.52988	1.39541	-1.33336	H	1.52988	1.39541	-1.33336
C	2.583732	2.261139	2.309372	C	2.583732	2.261139	2.309372
H	0.767877	1.249843	2.917208	H	0.767877	1.249843	2.917208
C	3.387984	2.653027	1.238474	C	3.387984	2.653027	1.238474
H	3.643392	2.651385	-0.91174	H	3.643392	2.651385	-0.91174
H	2.882229	2.505739	3.339112	H	2.882229	2.505739	3.339112
C	-1.577212	-1.64336	0.23533	C	-1.57721	-1.64336	0.23533
C	-2.399684	-1.84642	1.504057	C	-2.39968	-1.84642	1.504057
C	-1.998474	-2.14256	-0.86011	C	-1.99847	-2.14256	-0.86011
C	-3.599473	-2.54897	1.388322	C	-3.59947	-2.54897	1.388322
H	-2.085219	-1.45272	2.48143	H	-2.08522	-1.45272	2.48143
C	-3.197732	-2.84537	-0.9758	C	-3.19773	-2.84537	-0.9758
H	-1.367328	-1.98197	-1.7463	H	-1.36733	-1.98197	-1.7463
C	-3.998285	-3.04892	0.148876	C	-3.99829	-3.04892	0.148876
H	-4.229957	-2.70954	2.27486	H	-4.22996	-2.70954	2.27486
H	-3.512416	-3.2396	-1.95296	H	-3.51242	-3.2396	-1.95296
N	4.739687	-3.77084	0.990829	N	4.739687	-3.77084	0.990829
H	5.514413	-3.13347	1.016642	H	5.514413	-3.13347	1.016642
C	5.070263	-4.83802	0.360004	C	5.070263	-4.83802	0.360004
S	5.59424	-6.07846	1.147632	S	5.59424	-6.07846	1.147632
N	5.89716	-4.1809	-0.5109	N	5.89716	-4.1809	-0.5109
H	5.348401	-3.5598	-1.07677	H	5.348401	-3.5598	-1.07677
C	6.491695	-5.21613	-1.36865	C	6.491695	-5.21613	-1.36865
H	6.863222	-4.76611	-2.26551	H	6.863222	-4.76611	-2.26551
H	5.747924	-5.94429	-1.61661	H	5.747924	-5.94429	-1.61661
N	-5.249959	3.395685	-0.42651	N	-5.24996	3.395685	-0.42651
H	-5.620373	3.219883	-1.34248	H	-5.62037	3.219883	-1.34248
C	-5.148814	4.925089	-0.20873	C	-5.14881	4.925089	-0.20873
S	-3.809923	5.517047	0.330301	S	-3.80992	5.517047	0.330301
N	-6.165809	5.696477	-0.38384	N	-6.16581	5.696477	-0.38384

H	-6.67892	5.383281	-1.18742	H	-6.67892	5.383281	-1.18742
C	-5.691704	7.071241	-0.59866	C	-5.6917	7.071241	-0.59866
H	-5.325848	7.470636	0.324129	H	-5.32585	7.470636	0.324129
H	-4.904155	7.067839	-1.32299	H	-4.90416	7.067839	-1.32299
C	-6.855061	7.941538	-1.10933	C	-6.85506	7.941538	-1.10933
H	-6.547339	8.965869	-1.14024	H	-6.54734	8.965869	-1.14024
H	-7.134037	7.623126	-2.09202	H	-7.13404	7.623126	-2.09202
S	-8.247537	7.773138	-0.0134	S	-8.24754	7.773138	-0.0134
C	-9.423791	9.067565	-0.34398	C	-9.42379	9.067565	-0.34398
H	10.285729	8.933006	0.27558	H	-10.2857	8.933006	0.27558
H	-8.977814	10.0171	-0.13328	H	-8.97781	10.0171	-0.13328
H	-9.714903	9.030705	-1.37296	H	-9.7149	9.030705	-1.37296
N	-5.252187	-3.7418	0.055707	N	-5.25219	-3.7418	0.055707
H	-5.940861	-3.20059	0.545531	H	-5.94086	-3.20059	0.545531
C	-5.092158	-4.86259	0.662335	C	-5.09216	-4.86259	0.662335
S	-3.900077	-5.06382	1.648262	S	-3.90008	-5.06382	1.648262
N	-6.16081	-5.86801	0.586896	N	-6.16081	-5.86801	0.586896
H	-6.591614	-5.96588	1.487974	H	-6.59161	-5.96588	1.487974
C	-5.590483	-7.15831	0.173691	C	-5.59048	-7.15831	0.173691
H	-5.09541	-7.61251	1.006463	H	-5.09541	-7.61251	1.006463
H	-4.886845	-7.00049	-0.61681	H	-4.88685	-7.00049	-0.61681
C	-6.718247	-8.0835	-0.32004	C	-6.71825	-8.0835	-0.32004
H	-6.306146	-9.03086	-0.5986	H	-6.30615	-9.03086	-0.5986
H	-7.434184	-8.22233	0.462948	H	-7.43418	-8.22233	0.462948
S	-7.516316	-7.34598	-1.72984	S	-7.51632	-7.34598	-1.72984
C	-8.681603	-8.49892	-2.42354	C	-8.6816	-8.49892	-2.42354
H	-9.166267	-8.05249	-3.26657	H	-9.16627	-8.05249	-3.26657
H	-8.165945	-9.38305	-2.7355	H	-8.16595	-9.38305	-2.7355
H	-9.413078	-8.7543	-1.68555	H	-9.41308	-8.7543	-1.68555
C	7.650295	-5.90032	-0.61957	C	7.650295	-5.90032	-0.61957
H	7.340262	-6.87113	-0.29351	H	7.340262	-6.87113	-0.29351
H	7.924317	-5.30957	0.229442	H	7.924317	-5.30957	0.229442

S	9.049359	-6.05891	-1.70855	S	9.049359	-6.05891	-1.70855
C	10.020478	-7.47064	-1.22652	C	10.02048	-7.47064	-1.22652
H	9.437927	-8.36065	-1.3424	H	9.437927	-8.36065	-1.3424
H	10.891945	-7.53183	-1.84434	H	10.89195	-7.53183	-1.84434
H	10.315325	-7.36808	-0.20308	H	10.31533	-7.36808	-0.20308
N	4.633887	3.392542	1.486954	N	4.633887	3.392542	1.486954
H	4.599328	3.809169	2.399281	H	4.599328	3.809169	2.399281
C	4.789265	4.445475	0.473007	C	4.789265	4.445475	0.473007
N	5.883664	5.420959	0.580814	N	5.883664	5.420959	0.580814
H	5.50265	6.333815	0.750038	H	5.50265	6.333815	0.750038
S	3.78522	4.528287	-0.72643	S	3.78522	4.528287	-0.72643
C	6.65239	5.435392	-0.67208	C	6.65239	5.435392	-0.67208
H	6.112721	5.984221	-1.41535	H	6.112721	5.984221	-1.41535
H	6.803215	4.431276	-1.00958	H	6.803215	4.431276	-1.00958
C	8.017366	6.105783	-0.42915	C	8.017366	6.105783	-0.42915
H	7.867277	7.121185	-0.12694	H	7.867277	7.121185	-0.12694
H	8.541821	5.578496	0.340144	H	8.541821	5.578496	0.340144
S	8.972286	6.068646	-1.93087	S	8.972286	6.068646	-1.93087
C	10.319456	7.225459	-1.80711	C	10.31946	7.225459	-1.80711
H	10.936666	7.148635	-2.67777	H	10.93667	7.148635	-2.67777
H	9.930355	8.219394	-1.73232	H	9.930355	8.219394	-1.73232
H	10.901164	7.003737	-0.93685	H	10.90116	7.003737	-0.93685
Cl	-7.753512	-4.33365	0.740831	Cl	-7.75351	-4.33365	0.740831
Cl	-7.714784	3.572063	-1.18294	Cl	-7.71478	3.572063	-1.18294
Cl	6.099755	-1.72971	-0.45231	Cl	6.099755	-1.72971	-0.45231
Cl	6.386077	4.715264	2.416044	Cl	6.386077	4.715264	2.416044

5.4.18. Spectra for ion transport

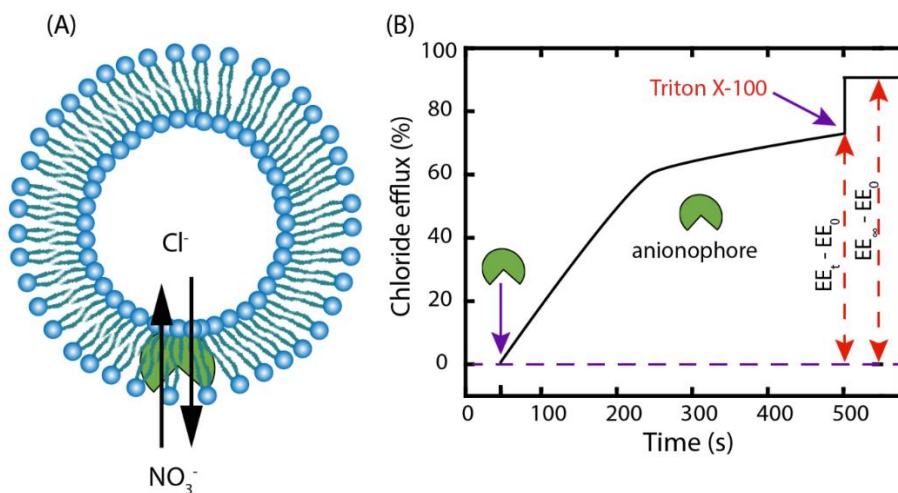


Figure 5.28. Representations of chloride efflux study across the EYPC/CHOL-LUVs (A and B).

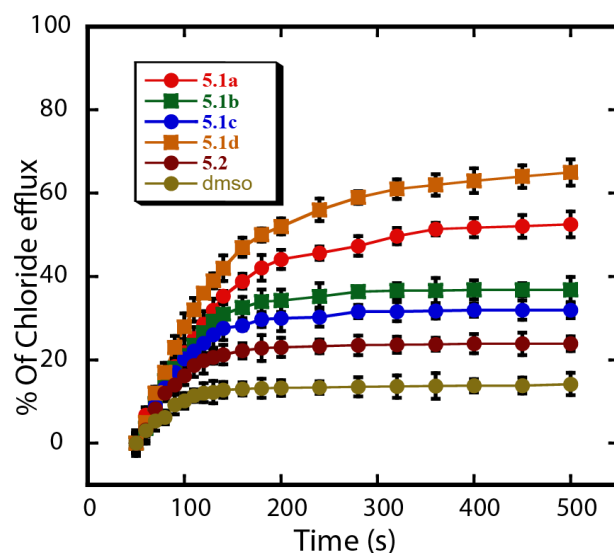


Figure 5.29. Initial screening for the compound-mediated transmembrane transport of Cl^- ions (at concentration $0.156 \mu\text{M}$).

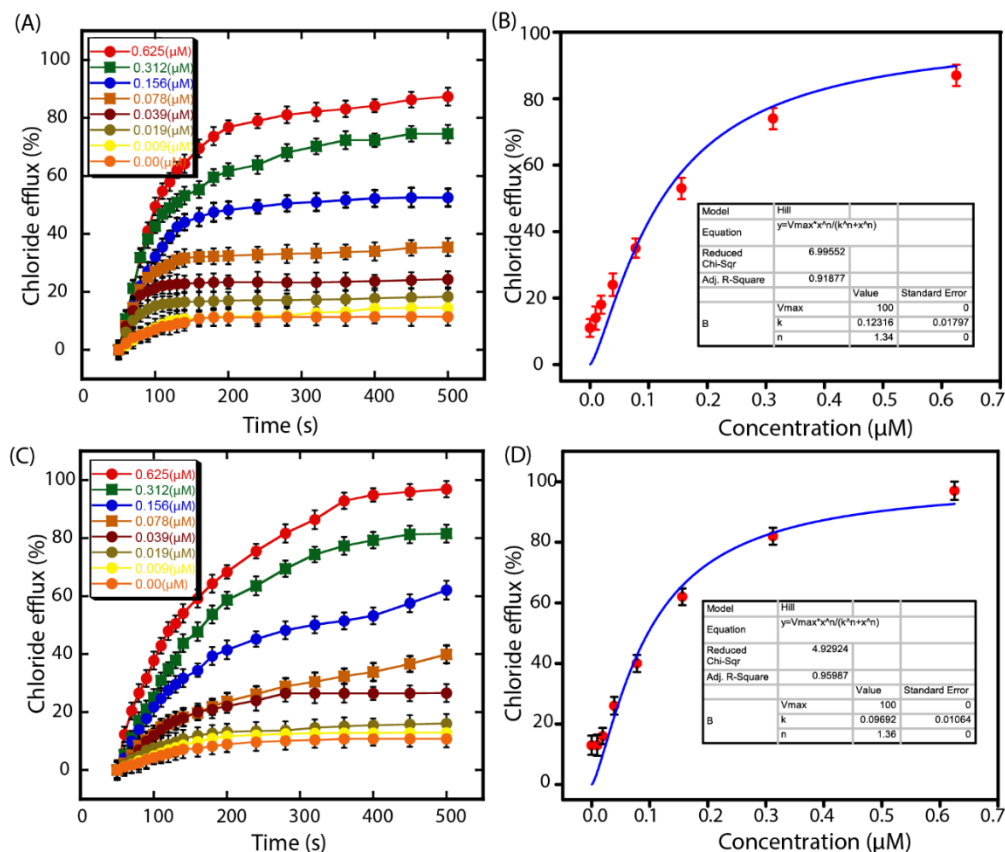


Figure 5.30. Concentration-dependent Cl^- ion transport activity of compounds 5.1a (A and B) and 5.1d (C and D) were measured by chloride ion-selective electrode using EYPC/CHOL-LUVs at pH 7.2.

Table 5.4. Cl^- ion transport properties of the compounds.

Compound Code	EC ₅₀ values (μM)	EC ₅₀ values (mol %)	Hill coefficient (n)
5.1a	0.123 ± 0.018	0.039 ± 0.006	1.34
5.1b	-	-	-
5.1c	-	-	-
5.1d	0.097 ± 0.011	0.031 ± 0.003	1.36

N.B. EC₅₀ values of compounds 5.1b and 5.1c could not be done due to the precipitation at higher concentrations.

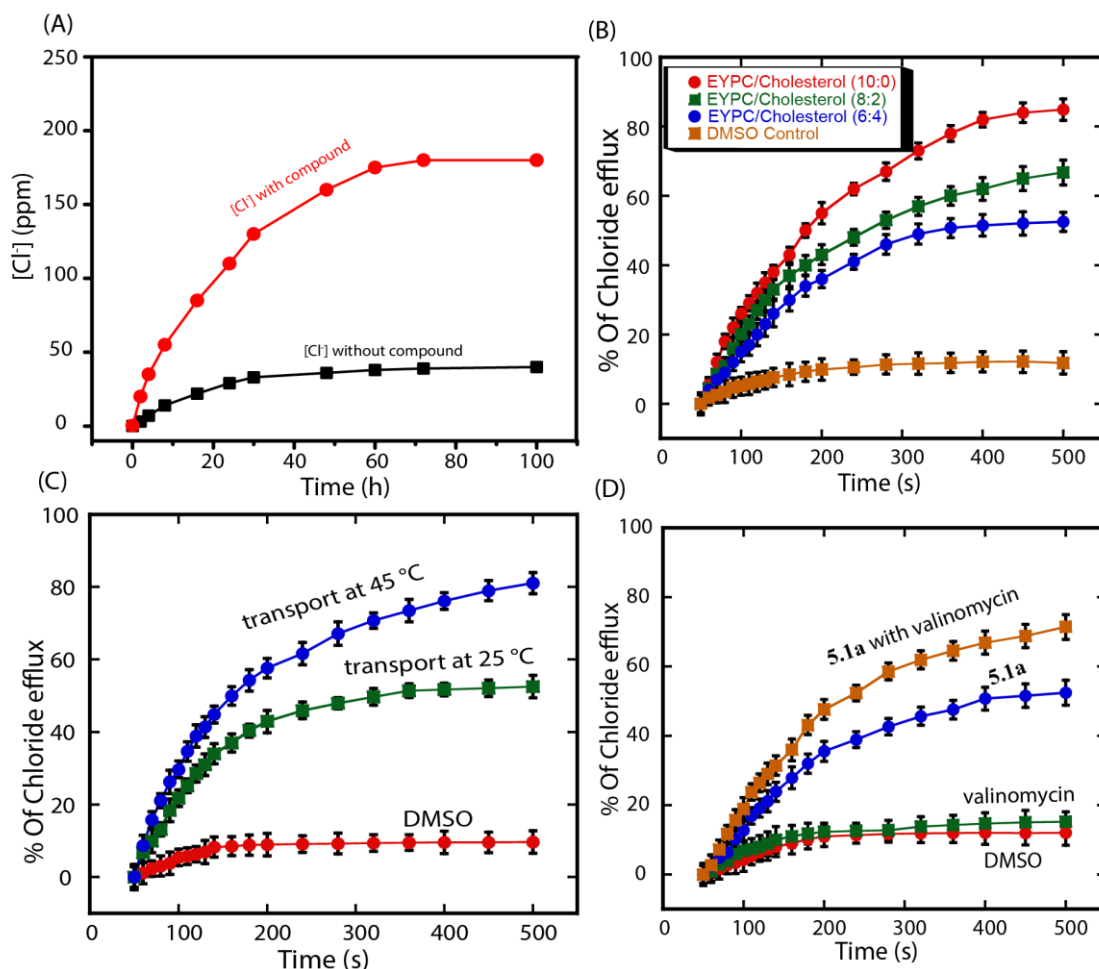


Figure 5.31. The probability of Cl^-/NO_3^- antiport mechanistic pathway of compound **5.1a** by the U-tube assay in NaCl presence at the left-arm and $NaNO_3$ at right-arm (A). Cholesterol-dependent chloride efflux assay using ISE to validate the carrier-mechanistic pathway of Cl^- ion transport activity by the compound **5.1a** across the different ratios of cholesterol encapsulated EYPC-LUVs (B). Cholesterol-dependent chloride efflux assay using ISE to validate the carrier-mechanistic pathway of Cl^- ion transport activity by the compound **5.1a** across the different ratios of cholesterol encapsulated EYPC-LUVs (C). Experiment was performed at $0.156 \mu M = 0.05 \text{ mol } \%$ with respect to lipid. Percentage of Cl^- ion transport activity of compound **5.1a** in the absence and presence of valinomycin (D).

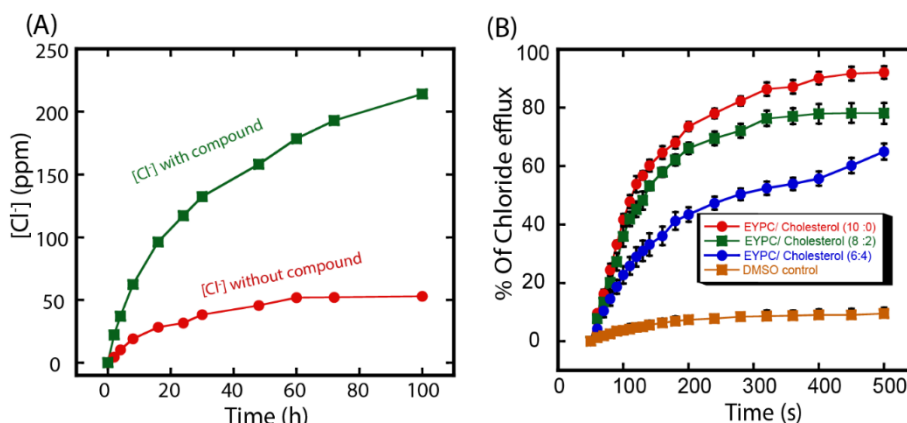


Figure 5.32. The probability of $\text{Cl}^-/\text{NO}_3^-$ antiport mechanistic pathway of compound **5.1d** by the U-tube assay in the presence of NaCl at left-arm and NaNO_3 at right-leftarm (A). Cholesterol-dependent chloride efflux assay using ISE to validate the carrier-mechanistic pathway of Cl^- ion transport activity by the compound **5.1d** across the different ratios of cholesterol encapsulated EYPC-LUVs (B). Experiment was performed at $0.156 \mu\text{M} = 0.05 \text{ mol } \%$ with respect to lipid.

5.4.19. Regeneration of the active compound **5.1d** from its proanionophore **5.2**

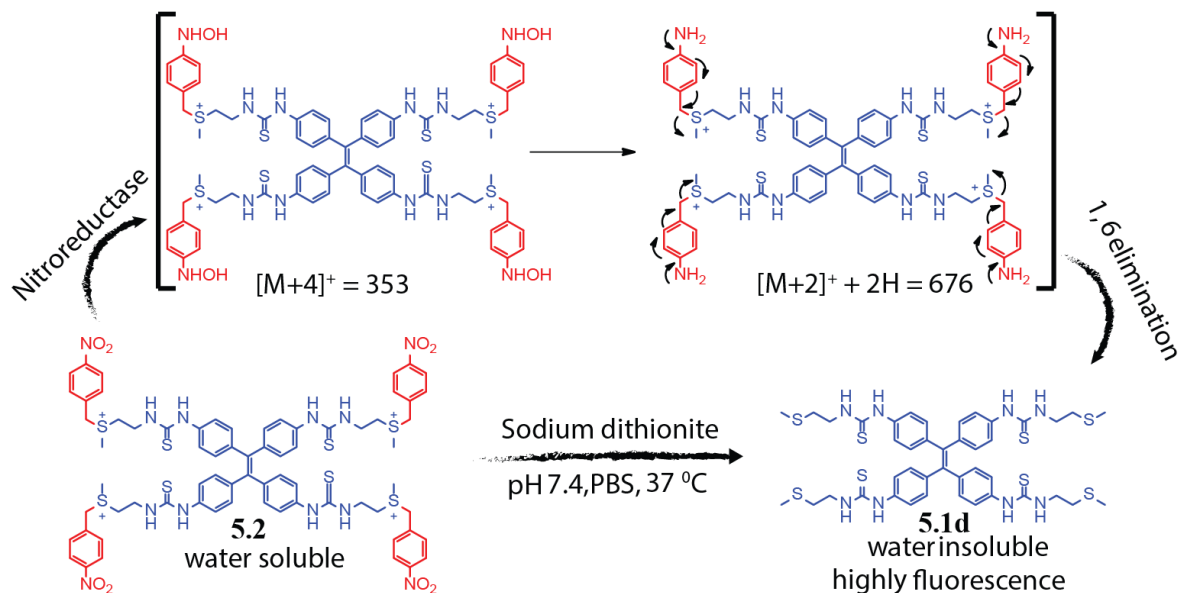


Figure 5.33. Regeneration of the active compound in the presence of sodium dithionite.

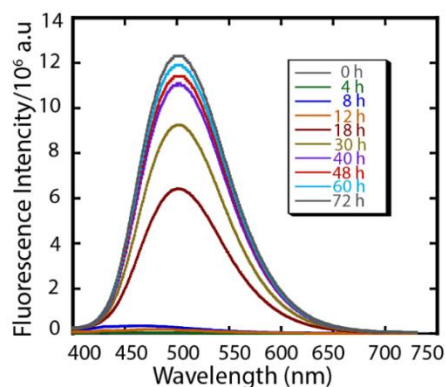


Figure 5.34. Regeneration of the active compound **5.1d** from the proanionophore **5.2** in the presence of sodium dithionite. The initial concentration of the proanionophore **5.2** was 1 mM.

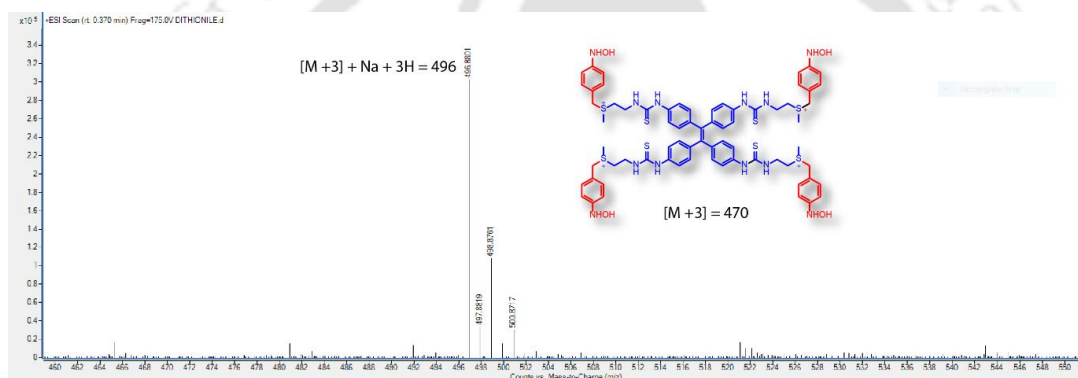


Figure 5.35. The reaction of proanionophore **5.2** in the presence of sodium dithionite in PBS at 37 °C led to the generation of intermediate compound, which was also confirmed by ES-MS analysis.

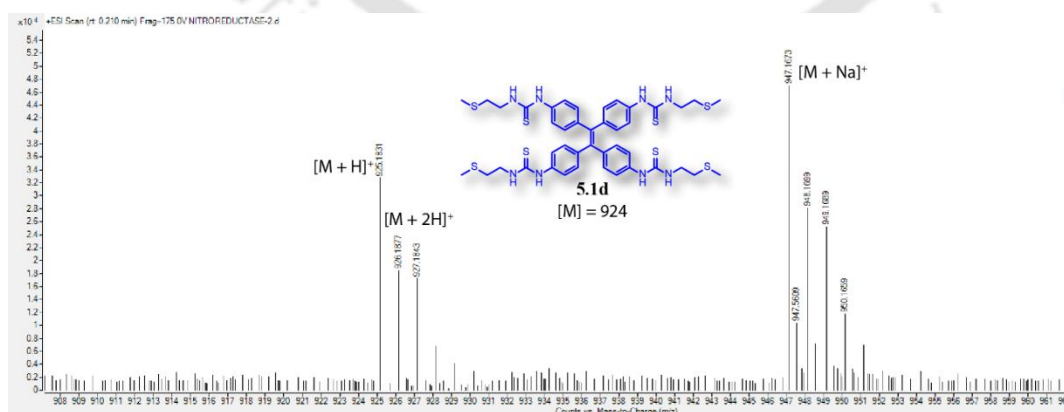


Figure 5.36. The reaction of proanionophore **5.2** in the presence of sodium dithionite in PBS at 37 °C led to the regeneration of active compound **5.1d**, which was also confirmed by ES-MS analysis.

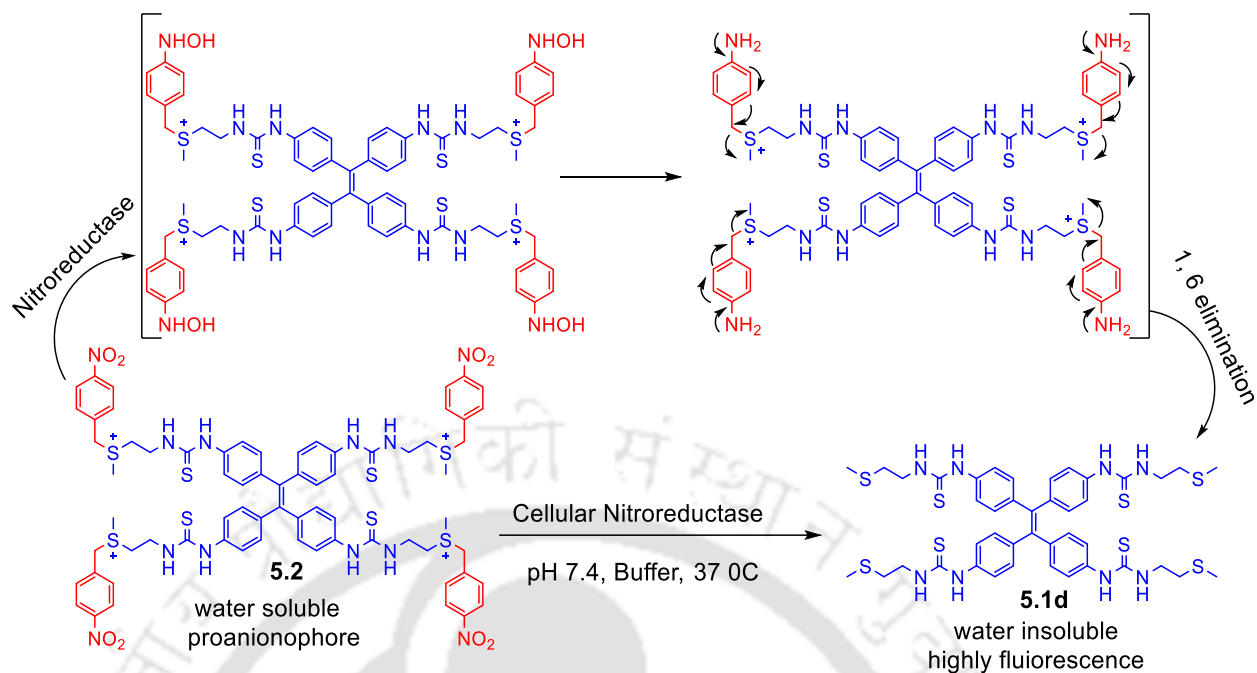


Figure 5.37. Regeneration of the active compound in the presence of nitroreductase enzyme (bacterial cellular extract).

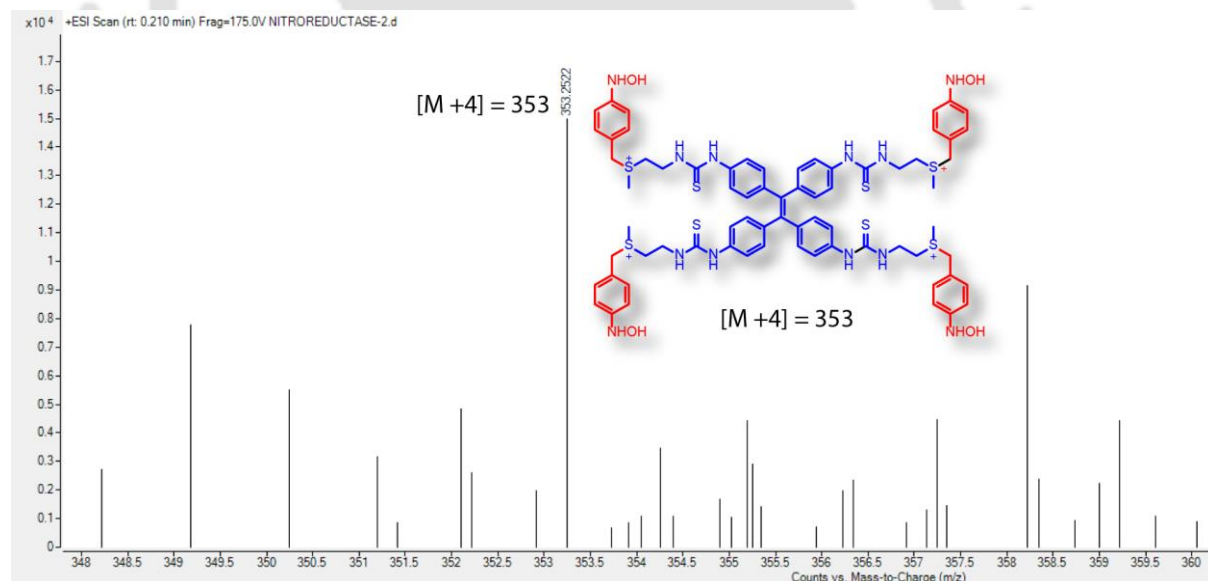


Figure 5.38. The reaction of proanionophore **5.2** in the presence of bacterial cell lysate containing nitroreductase in PBS at 37 °C led to the generation of intermediate compound, which was also confirmed by ES-MS analysis.

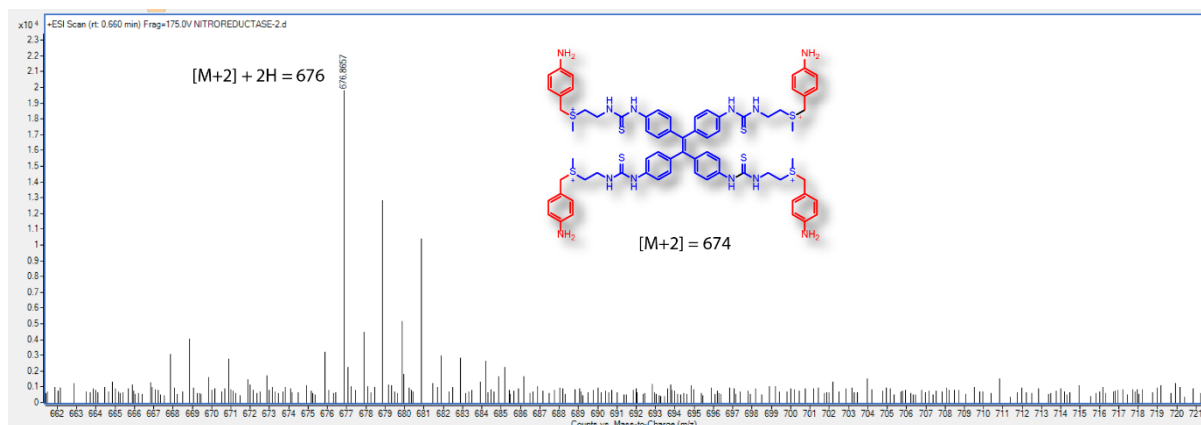


Figure 5.39. The reaction of proanionophore **5.2** in the presence of bacterial cell lysate containing nitroreductase in PBS at 37 °C led to the generation of intermediate compound **5.1c**, which was also confirmed by ES-MS analysis.

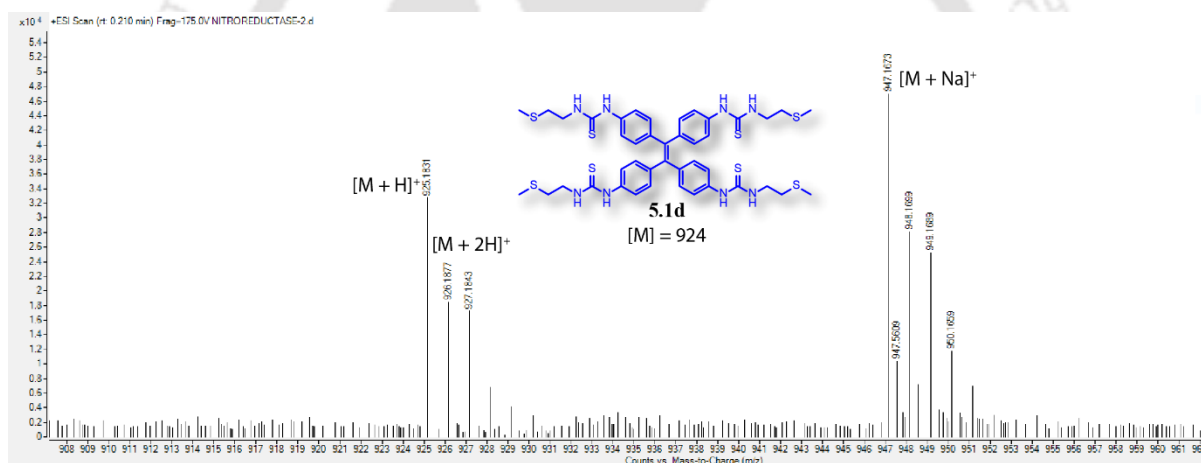


Figure 5.40. The reaction of proanionophore **5.2** in the presence of bacterial cell lysate containing nitroreductase in PBS at 37 °C led to the regeneration of active anionophore **5.1d**, which was also confirmed by ES-MS analysis.

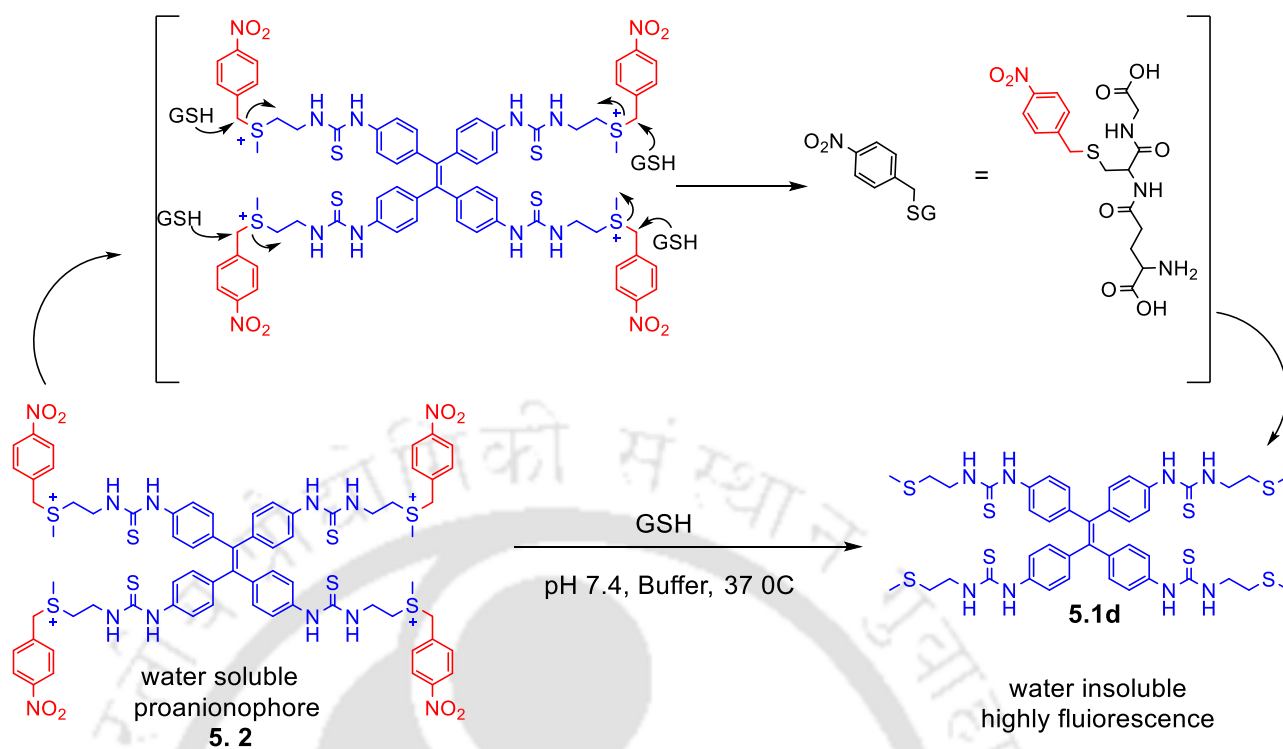


Figure 5.41. Regeneration of the active compound in the presence of the GSH.

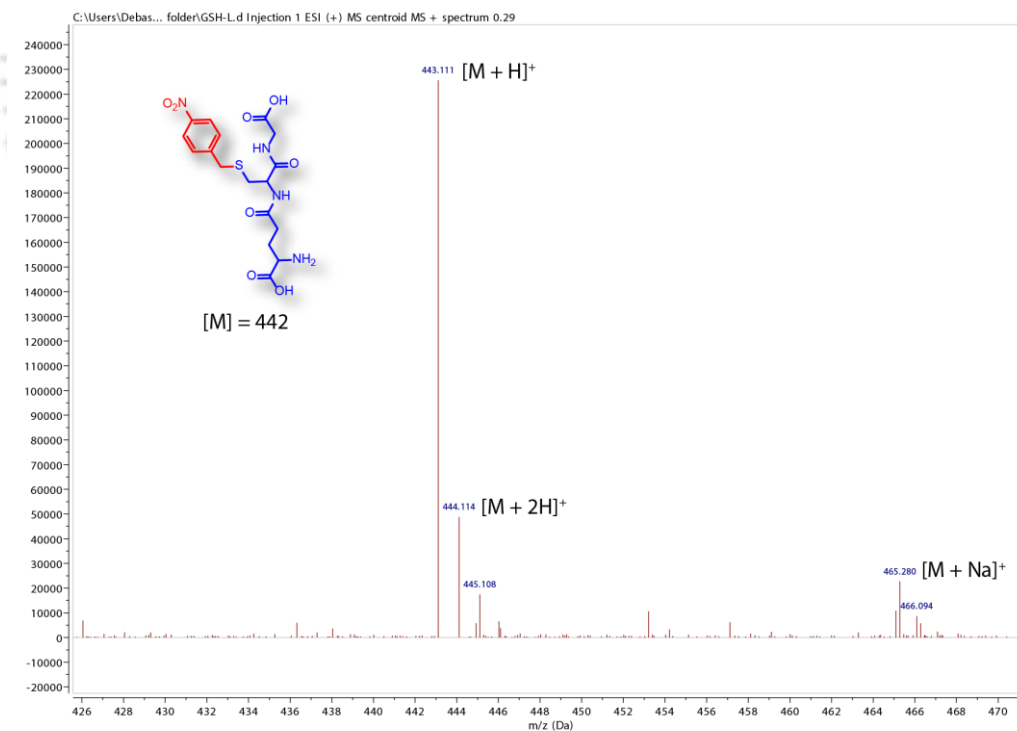


Figure 5.42. The reaction of proanionophore **5.2** in the presence of GSH in PBS at 37 °C led to the generation of intermediate compound, which was also confirmed by ES-MS analysis.

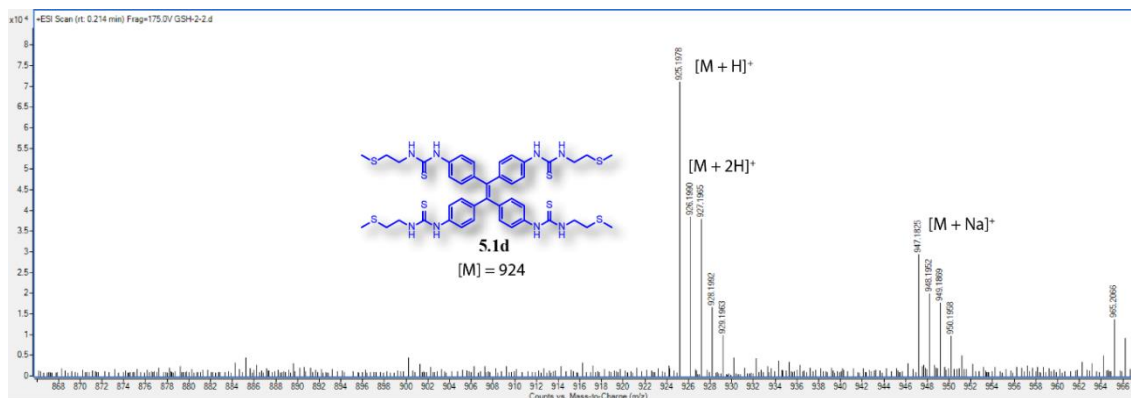


Figure 5.43. The reaction of proanionophore **5.2** in the presence of GSH in PBS at 37 °C led to the regeneration of active anionophore **5.1d**, which was also confirmed by ES-MS analysis.

5.4.20. Transport of chloride ions across the giant unilamellar vesicles:

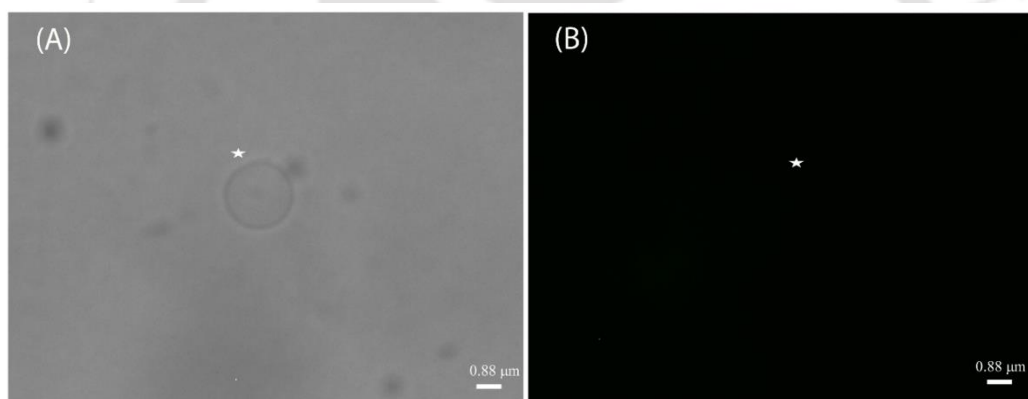


Figure 5.44. Fluorescence microscopic images of the compound **5.1d** (1 μ M) loaded GUVs. Bright field (A) and green channel (B). Microscopic images showed that at this concentration of compound **5.1d**, the fluorescence intensity in the green channel is not detectable, which ruled out the interference of the fluorescence of the compound during lucigenin encapsulated GUVs assay.

5.4.21. Chloride transport by ^{35}Cl NMR measurements:

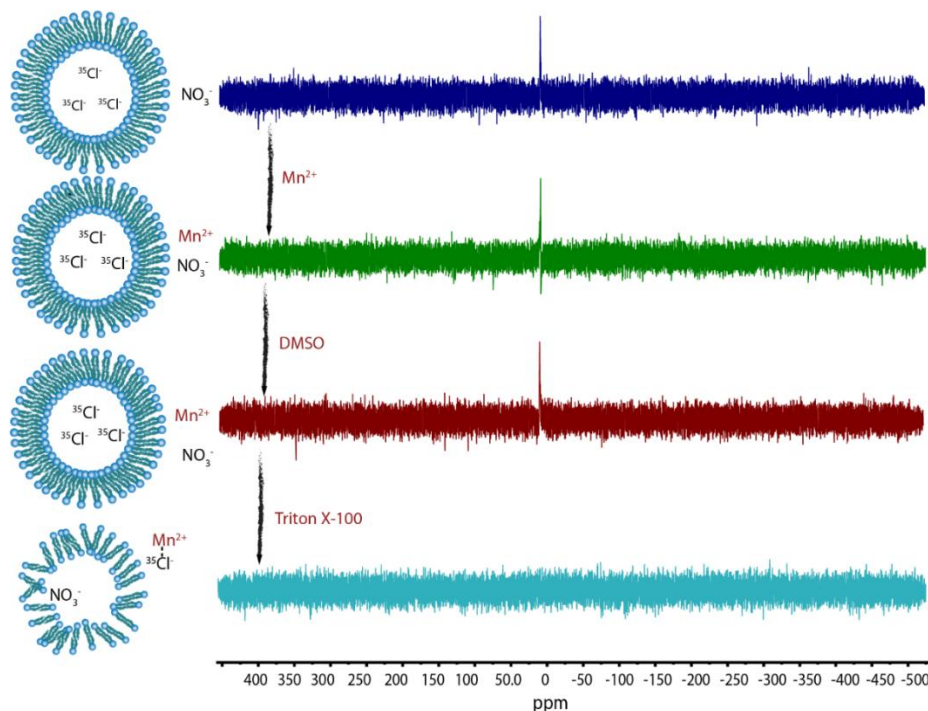


Figure 5.45. The ^{35}Cl NMR control experiment was performed in the absence of a compound. The NMR experiment was performed in $\text{H}_2\text{O}:\text{D}_2\text{O}$ (9:1) solution.

5.4.22. Validation of AIE property:

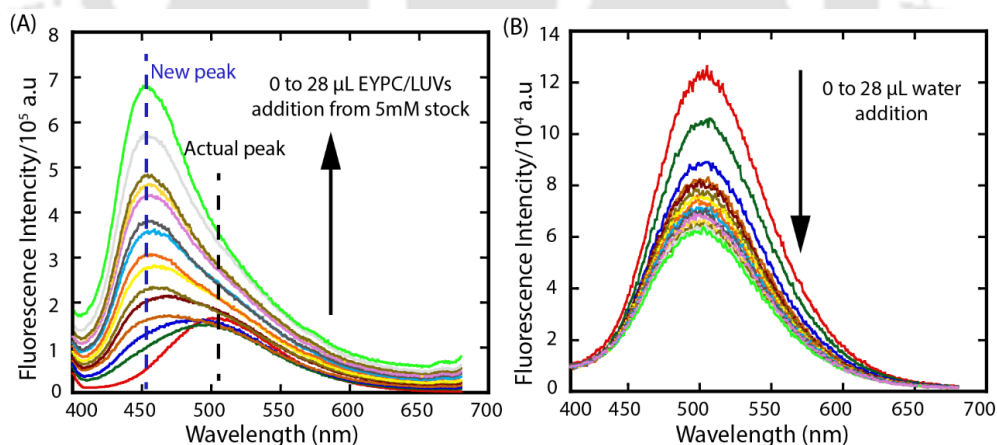


Figure 5.46. Fluorescence emission spectra of compound **5.1d** ($0.03\ \mu\text{M}$) in the presence of the liposomal solution (0 - $28\ \mu\text{L}$ addition of EYPC-LUVs from $5\ \text{mM}$ stock), where a clear blue shift with significant fluorescence enhancement was observed (A). The control experiment in the presence of the same amount (0 - $28\ \mu\text{L}$) of water (B). For both cases, the compound was excited at $350\ \text{nm}$.

5.4.23. Staining of GUVs:

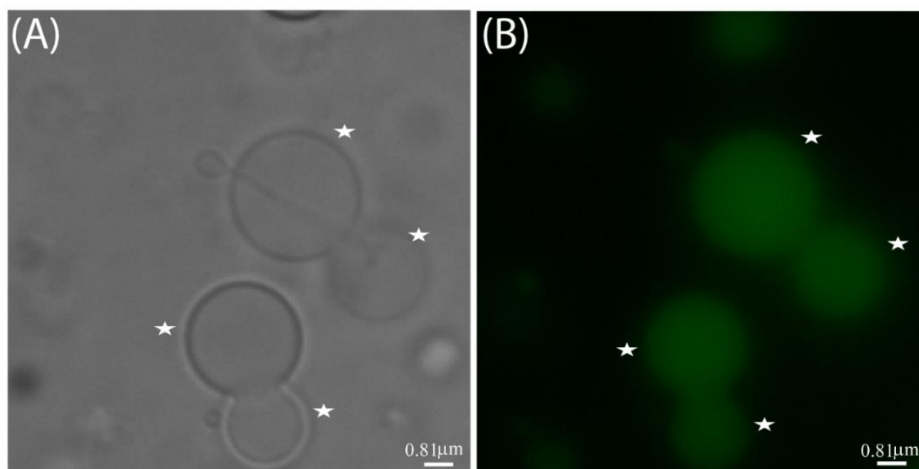


Figure 5.47. Fluorescence microscopic images of the compound **5.1d** (10 μM) loaded GUVs. Bright field (A) and green channel (B) illustrates the compound **5.1d** encapsulation efficiency of the GUVs. The scale bar of the images was 0.81 μm .

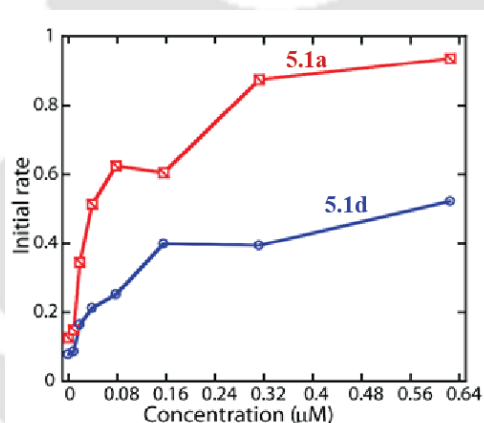


Figure 5.48. A plot of the initial rates of Cl^- ion transport activities at different concentrations of compounds.

5.5. References

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6. Thesis Conclusion

In this thesis, we have successfully described numerous prospects of anionophore research. Additionally, concise literature on the biologically active anionophores has also been documented in **Chapter 1**. Inspired by the numerous prospects of anionophore, DPEN based anionophores derivatives (in **Chapter 2**) have been explored and showed promising anion transport ability with potential apoptosis-inducing activities. However, DPEN derivatives have been found to be active but failed to get the selectivity difference between the cancer cells line and normal cell line. To overcome the limitation of chapter 1, quinine-based semi-synthetic anionophores molecules (in **Chapter 2**) have been developed. The detailed *in vitro* and *in vivo* studies were also carried out to prove the potential therapeutic agent. The selectivity problem is a little bit improved as 4-5 fold selectivity has been observed between the cancer cells and normal cells, which is quite satisfactory. But still, the classical anionophores are having the problems such as deliverability and cellular uptake due to the lipophilic nature of the anionophore. So in this regard, in **chapter 4**, the main focus has been given to overcome all these conventional hurdles associated with anionophore and come up with sulphonium-based proanionophore concepts where the sulphonium-based proanionophore molecules have been developed and explored their transport, deliverability, and cellular uptake studies. Within **chapter 5**, the focus has been given to merging the concept of therapy and diagnosis, and in this regard, NTRs responsive sulphonium molecules have been designed and explored their possible therapy and diagnostic applicability. So overall, within this thesis, the focus has been given to develop numerous novel anionophores molecules and studied them for the numerous therapeutic prospects.

7. Future Prospects

Anion transport-related research works have been gaining renewed attention since 1990, and significant advancement has been made in this regard. In particular, over the last 10–15 years, the transmembrane transport of anions has been getting more attention because of its therapeutic potential against cancer, cystic fibrosis, and other channelopathies. So motivated by all these therapeutic prospects, a diverse range of novel anionophores molecules have been developed throughout this thesis. To date, most of the developed anion transporters have failed to show selective induction of apoptosis in cancer cells over normal cells. Their poor deliverability and selectivity between the normal and

the cancerous cells are significant obstacles. Recent interest in the proanionophore concept has been gaining momentum since last year. The proanionophores generate the anionophores in the presence of internal or external stimuli (such as GSH, enzymes, and light), which could selectively disrupt the ion homeostasis in the tumor cells and trigger cell death. Hence this concept needs to be more exploited to provide the highest possible selectivity for cancer cells against normal cells. The use of targeted drug delivery systems could be a practical approach for therapeutic purposes. Anionophores should also be explored against the unexplored field. The antiviral properties of the anionophores could have considerable potential, which could also be explored. Recently it has been shown that the chloride ion has an enormous functional role in cardiac diseases. Abnormal functions of the chloride channel could lead to various pathological conditions like arrhythmogenesis in myocardial injury, cardiac ischemic preconditioning, and the adaptive remodeling of the heart during myocardial hypertrophy and heart failure. So, in this scenario, artificial chloride ion transporter molecules could have colossal potential against these pathological conditions. Unregulated anion transport also plays an essential role in kidney diseases. Artificial anion transporter molecules could have an immense role in controlling this anionic imbalance and preventing chronic kidney diseases.⁷¹ Mutation of certain cation–chloride cotransporters (CCC) channels like NKCC1 and KCC2 could alter the intracellular chloride homeostasis, which invites numerous pathological conditions like neonatal ischemic seizures, temporal lobe epilepsy, and neuropathic pain caused by the hyperexcitability of neurons. So, in this case, artificial anionophores could also come up with a solution against these pathologies. Hence, a search for the applications of anionophores in these unexplored areas is also required. Anionophores might be used alone or as part of combination therapy with drug molecules that target anion transport-related diseases. Nevertheless, the development of new anion transporting scaffolds and systematic biological activity studies will be useful for future research and therapeutic applications of anionophores.

Biological applications of synthetic anion transporters

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Diphenylethylenediamine-Based Potent Anionophores: Transmembrane Chloride Ion Transport and Apoptosis Inducing Activities

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Publisher: American Chemical Society

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Quinine-Based Semisynthetic Ion Transporters with Potential Antiproliferative Activities

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Tuning the solubility of ionophores: glutathione-mediated transport of chloride ions across hydrophobic membranes

N. Akhtar, N. Pradhan, A. Saha, V. Kumar, O. Biswas, S. Dey, M. Shah, S. Kumar and D. Manna, *Chem. Commun.*, 2019, **55**, 8482 DOI: 10.1039/C9CC04518J

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Stimuli-responsive transmembrane anion transport by AIE-active fluorescent probes

N. Akhtar, O. Biswas and D. Manna, *Org. Biomol. Chem.*, 2021, Advance Article, DOI: 10.1039/D1OB00584G

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