

Design and Synthesis of Small Molecule Chloride Receptors for Selective Transmembrane Transport Activity

A Thesis

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Doctor of Philosophy

BY

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Indian Institute of Science Education and Research (IISER), Pune

2019

Dedicated to...

My Parents



भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान, पुणे

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CERTIFICATE

Certified that the work incorporated in the thesis entitled “*Design and Synthesis of Small Molecule Chloride Receptors for Selective Transmembrane Transport Activity*” submitted by **Mr. Sopan Valiba Shinde** was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.

Date: 31 January 2019,

Place: Pune

Dr. Pinaki Talukdar

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I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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Table of Contents

Chapter 1:	Introduction of Membrane Transport	
1.1	Membrane Structure	01
1.2	Membrane Transport	02
1.3	Regulation of Ion Transport	03
1.4	Anions in Human Health	04
1.5	Chloride Channels in Cancer and Drug Resistance	05
1.6	Naturally Occurring Cation Transporters	06
1.7	Non-protein based Natural Occurring Cl^- Carriers and Their Synthetic Analogues	07
1.8	Techniques for Detecting and Quantifying Anion Transport	11
1.8.1	Fluorescence-based Assay	11
1.8.2	Ion Selective Electrode-based Assay	13
1.8.3	NMR based Assay	13
1.9	References	14
Chapter 2:	Triazole Linked Indole-2-Carboxamide as Anion Receptors for Transmembrane Transport Activity	
2.1	Introduction	18
2.2	Results and Discussions	19
2.2.1	Synthesis	19
2.2.2	Chloride Binding Study by ^1H NMR Titration	20
2.2.3	Ion Transport Activity	20
2.3	Geometry Optimized structures	26

2.3	Conclusion	27
2.4	Experimental Details	27
2.4.1	General Methods	27
2.4.2	Physical Measurements	28
2.4.3	Synthesis Procedures	28
2.4.4	Anion Binding Study by ^1H NMR Titration	31
2.3.5	Ion transport Experiments	32
2.5	Theoretical calculations	35
2.6	NMR Spectra	41
2.7	HRMS Spectra of Final compounds	51
2.8	References	55
Chapter 3:	Sulfonamide-based Anion Receptor for Transmembrane Proton-anion Co-transport Activity	
3.1	Introduction	57
3.2	Results and Discussions	60
3.2.1	Synthesis	60
3.2.2	Single Crystal X-Ray Analysis	61
3.2.3	Hirshfeld Surface Analysis	62
3.2.3	Anion Binding Study by ^1H NMR Titration	64
3.2.4	Ion Transport Activity	64
3.4	Conclusion	70
3.5	Experimental Section	70

3.5.1	General Methods	70
3.5.2	Physical Measurements	70
3.5.3	Synthesis Procedures	71
3.5.4	Single Crystal X-ray Crystallographic Study	75
3.5.5	Anion Binding Study by ^1H NMR Titration	78
3.5.6	Ion Transport Experiments	87
3.5	NMR Spectra	90
3.6	HRMS Spectra of Final compounds	99
3.7	References	102
 Chapter 4: Bis(amide)-substituted Imidazole Receptors for Transmembrane H^+/Cl^- Cotransport Activity		
4.1	Introduction	104
4.2	Results and Discussions	106
4.2.1	Synthesis	106
4.2.3	Anion Binding Study by ^1H NMR Titration	107
4.2.4	Ion Transport Activity	110
4.3	Conclusion	115
4.4	Experimental Section	115
4.4.1	General Methods	115
4.4.2	Physical Measurements	116
4.4.3	Synthesis Procedures	116
4.4.4	Anion Binding Study by ^1H NMR Titration Experiment	124

4.4.5	Ion transport Experiments	129
4.5	NMR Spectra	130
4.6	HRMS spectra of final compounds	142
4.7	References	146
 Chapter 5: A Dimeric Bis(melamine) Substituted Bispidine for Efficient Transmembrane H⁺/Cl⁻ Co-transport Activity		
5.1	Introduction	147
5.2	Results and Discussions	150
5.2.1	Synthesis	150
5.2.2	X-ray Crystal Structure Analysis	152
5.2.3	Ion Transport Study	152
5.3	Theoretical Support to protonation induced Conformational Changes	159
5.4	Conclusion	160
5.5	Experimental Section	161
5.5.1	General Methods	161
5.5.2	Physical Measurements	161
5.5.3	Synthesis Procedures	144
5.5.4	Single Crystal X-Ray Diffraction Studies	164
5.5.5	Ion Transport Experiments	165
5.5.6	U-Tube Experiment	167

5.5.7	pH-Dependent Cl ⁻ Efflux Assay	167
5.5	Theoretical Calculations	168
5.5.1	Theoretical Prediction of pK _a Values	168
5.5.2	Geometry Optimization	168
5.6	NMR Spectra	169
5.7	HRMS spectra of final compounds	172
5.8	References	173
	Overall Conclusion and Future Directions	175

Abbreviations and Symbols

A

α	Alpha
Å	Angstrom
A	Absorbance
Ar	Aromatic
Ac	Acetyl
ATP	Adenosine Tri-Phosphate

B

β	Beta
br	Broad singlet
BLM	Black lipid membrane
Boc	<i>tert</i> -Butoxycarbonyl
BF ₃ ·OEt ₂	Boron trifluoride diethyl etherate

C

<i>c</i>	Concentration
cm	Centimeter
CF	Carboxyfluorescein
Calc	Calculated
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CLC	Chloride Channel
CLCA	Calcium-activated Chloride Channel
CLIC	Chloride intracellular channel protein
CLNS1A	Chloride Nucleotide-Sensitive Channel 1A
CHCl ₃	Chloroform
CDCl ₃	Deuterated chloroform
CH ₃ CN	Acetonitrile
CsCl	Cesium chloride

CCDC Cambridge Crystallographic Data Centre

Cbz Carboxybenzyl

D

δ Delta (Chemical shift)

$^{\circ}\text{C}$ Degree Celsius

d Doublet

dd Doublet of doublet

dt Doublet of triplet

DPhPC 2-diphytanoyl-*sn*-glycero-3-phosphocholine

DMSO Dimethylsulfoxide

DMF Dimethylformamide

DCM Dichloromethane

D₂O Deuterated water

DMEM Dulbecco's Modified Eagle's Medium

DFT Density Functional Theory

DTT Dithiothreitol

E

EYPC L- α -phosphatidylcholine from egg-yolk

EC_{50} Effective concentration at half maximal activity

EtOAc Ethylacetate

EtOH Ethanol

Et₃N Triethylamine

ESI Electrospray ionization

EDC·HCl 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride

F

F_t Fluorescence intensity at time t

FCCP Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

G

G_{hyd} Free energy of hydration

H

Hz Hertz

h Hour

HPTS 8-Hydroxypyrene-1,3,6-trisulfonate trisodium salt

HOBt Hydroxybenzotriazole

HPF₆ Hexafluorophosphoric acid

HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HCl Hydrochloric acid

HPLC High performance liquid chromatography

HRMS High Resolution Mass Spectrometry

I

I_{F} Normalized Fluorescence Intensity

IR Infrared spectroscopy

ISE Ion selective electrode

ITC Isothermal titration calorimetry

J

J Coupling constant

Joule

K

k Rate constant

K Equilibrium constant

K_{a} Association constant

K₂CO₃ Potassium carbonate

KCl Potassium chloride

KBr Potassium bromide

KOH Potassium Hydroxide

L

λ Lambda

LUV Large unilamellar vesicle

logP Partition Coefficient

LiCl Lithium chloride

M

m Multiplet

M Molar

μ M Micromolar

μ L Microliter

M.P. Melting Point

MHz Mega hertz

min Minute(s)

max Maximum

mg Milligram(s)

mol Mole

mmol Millimole(s)

mM Millimolar

mL Milliliter

M. Wt. Molecular weight

MALDI Matrix Assisted Laser Desorption Ionization

MeOH Methanol

Mon Monensin

Me Methyl

N

n Hill coefficient

nm Nanometer

nM nanomolar

NMR Nuclear magnetic resonance

Na₂SO₄ Sodium sulfate

NaF Sodium fluoride

NaCl Sodium chloride

NaBr Sodium bromide

NaI Sodium iodide

NaNO₃ Sodium nitrate

NaClO₄ Sodium perchlorate

NaSCN Sodium thiocyanate

NaOAc Sodium acetate

NaOH Sodium hydroxide

O

obs Observed

ORTEP Oak Ridge Thermal Ellipsoid plot

P

Ph Phenyl

pM Picomolar

ppm Parts per million

R

R Ideal gas constant

ρ Resistivity

rt Room temperature

RbCl Rubidium chloride

S

s Second(s)

SCXRD Single crystal X-ray diffraction

T

t Triplet

td	Triplet of doublets
<i>t</i>	Time
Tx	Triton X-100
TLC	Thin Layer Chromatography
THF	Tetrahydrofuran
TFA	Trifluoroacetic acid
TBA	Tetrabutyl ammonium
TOF	Time of flight
V	
V_m	Membrane Potential
V or Vln	Valinomycin
VDAC	Voltage activated anion channel
Val	Valine
Y	
<i>Y</i>	Fractional fluorescence intensity

Synopsis

The thesis entitled as “**Design and synthesis of small molecule chloride receptors for transmembrane transport activity**” comprises of five chapters.

The objective of the thesis is to develop synthetic molecules capable of recognizing anions and transport them across the phospholipid membranes. In all genre of life, ion transporters play vital roles. Naturally occurring ion transporters have broad biological applications as antibiotics, antimalarial agents. The recent progress in the arena of ion transport research has opened up the use of natural and synthetic ion transporters as anticancer agents. The thesis work was performed to evaluate the behavior of certain small organic molecules as anion receptors by ^1H NMR titration method, their ion transport activity across the phospholipid membranes, and further the understanding of the mechanism by which these molecule transport ions across vesicular membranes.

Chapter 1: Introduction of membrane transport

To obtain the energy required to carry out the biochemical transformations, the cell has to acquire the dissolved solutes (e.g., cations, anions, small polar molecules, etc.) from the cell environment, and also need to exclude other dissolved solutes and the metabolic waste out from the cell across the membrane. Due to the large thermodynamic barrier created by the hydrophobic core of lipid membranes, these polar solutes cannot pass through on their own. However, the movement of ions across the lipid membranes is crucial for cell proliferation, control of osmotic pressure, cellular signaling, and pH regulation in different compartments of the cell. Hence, Nature has created a class of specialized membrane-embedded proteins that provide hydrophilic paths across the lipid membranes through which, ions can pass avoiding the hydrophobic lipid core. These membrane proteins are called transporters, which are further categorized as channels, carriers, and pumps. Natural ion transport proteins are large structures and adapt very complex mechanism for the transport of a specific type of ions. Chloride is the most abundant extracellular anion in the human body and plays an essential role in the healthy body functions. The selective transport of chloride, typically expedited by chloride selective channels, has to determine inference in diverse biochemical processes. Dysregulated chloride transport, mainly caused by mutations of the chloride transporters, results in some lives tethering diseases such as cystic fibrosis, Dent's disease, myotonia,

etc. Very recently, a new treatment modality called “channel replacement therapy” is introduced to restore the damaged ion transport by using engineered protein-based ion channels. Moreover, the dysregulated chloride transport is also known to induce the programmed cell death. The perturbation of healthy chloride transport process by using external transporting molecules has opened up the area of new drug therapy for cancer treatment.

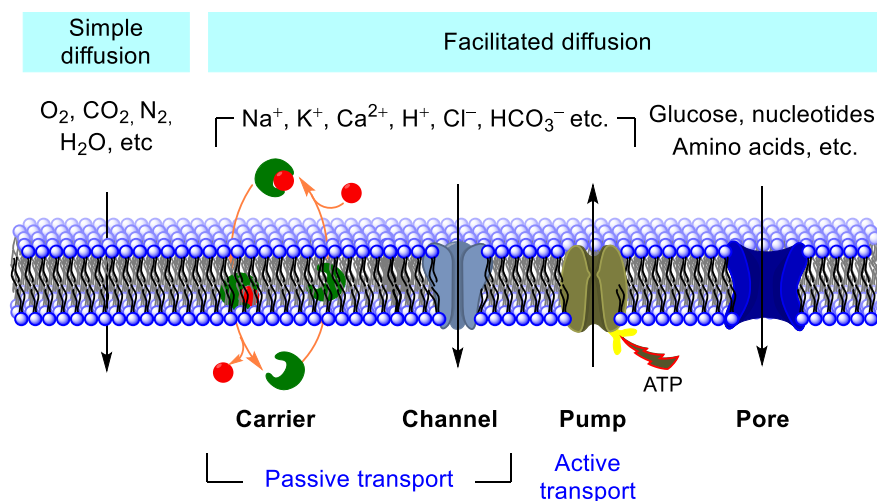


Figure 1 Schematic description of dissolved solute transport through the plasma membrane.

Chapter 2: Triazole linked indole-2-carboxamide as anion receptors for transmembrane transport activity

In this chapter, a series of triazole-appended indole-2-carboxamide-based anion receptors **1a-1i** were designed and synthesized. The lipophilic balance varied by changing the substitution (R group) on the aryl ring. ^1H NMR titration experiment of **1e** with tetrabutylammonium chloride were carried out to confirm the chloride recognition and involved interactions in the binding of the ion. The ion transport activity study was performed across large unilamellar vesicles with entrapped 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt as a fluorescent dye (i.e., EYPC-LUVs $\Rightarrow$ HPTS). The chloride selective ion transport was determined by a competitive HPTS assay. Monensin- and valinomycin-coupled assays confirmed the electrogenic transport mechanism.

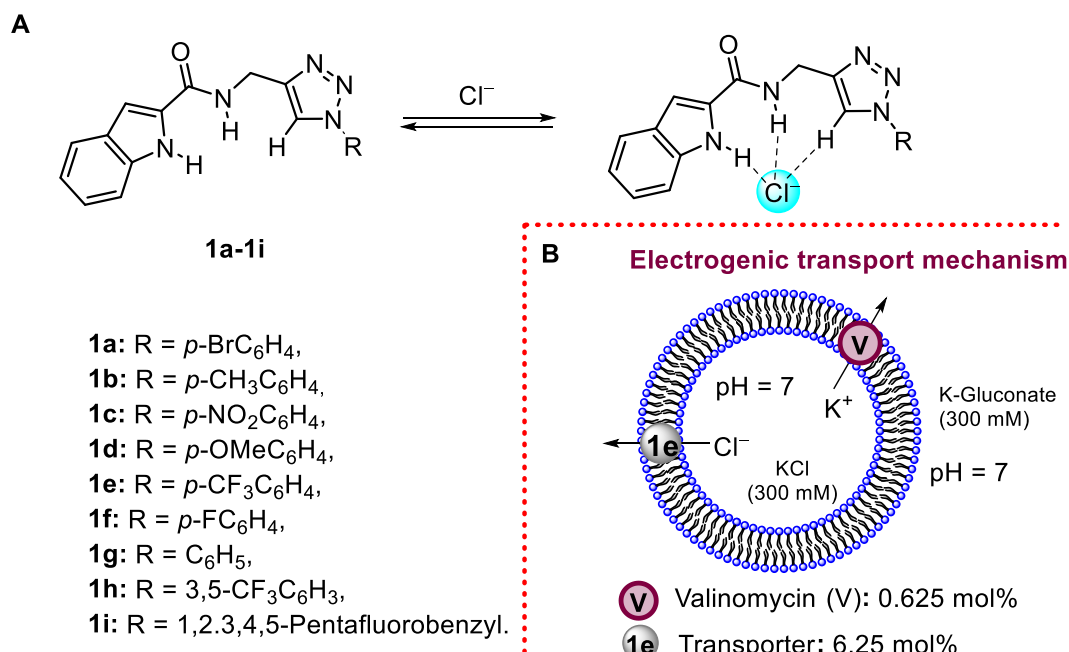


Figure 2 Structures of the receptors and receptor-chloride complex (A). The representative electrogenic chloride transport across phospholipid membrane.

Chapter 3: Sulfonamide-based anion receptor for transmembrane proton-anion cotransport activity

In this chapter, the design and synthesis of indole-linked amide-sulfonamide hybrids as synthetic anion receptors were reported. The anion recognition property was evaluated by X-ray single crystal structure analysis. The co-crystallization of **5e** with TBACl revealed the kosmotropic binding of two Cl[−] ions with two receptor molecules stabilized by hydrogen bond interactions with three water molecules. The anion recognition studies by ¹H NMR demonstrated a logical correlation between the acidity of sulfonamide N–H proton and binding strength. The comparison of anion recognition in solution state indicated the binding order Cl[−] >> Br[−] >> I[−]. The titration with basic anions (i.e., AcO[−] and F[−]) resulted in deprotonation of sulfonamide N–H proton. The ion transportability of these receptors across the phospholipids bilayer indicated that the receptors with sulfonamide N–H proton pK_a > 7 were able to transport anions more effectively than the receptors with sulfonamide N–H proton pK_a < 7. For the first time, it has been nicely depicted that instead of lipophilicity, the transport activity can be tuned by careful modification of the sulfonamide N–H acidity. The detailed insight into the ion

transport mechanism with the most efficient transporter revealed the proton-anion co-transport across lipid bilayer membrane.

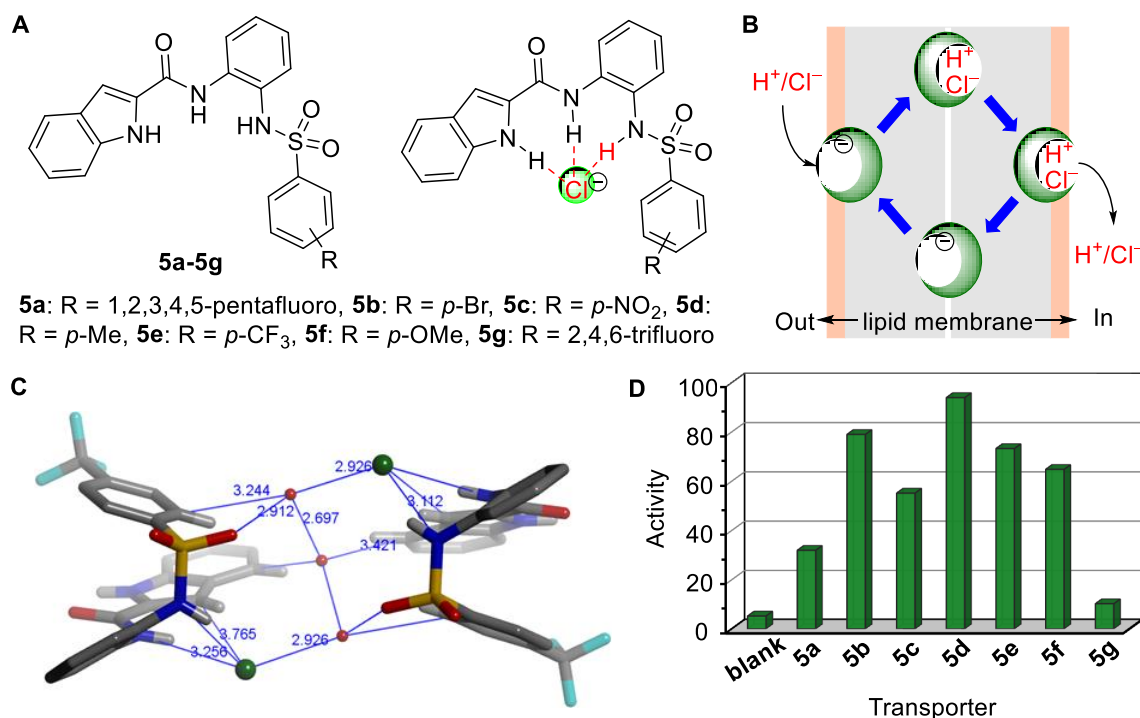


Figure 3 Structures of receptors **5a-5g** and representative Cl⁻ binding (A). Hypothesized H⁺/Cl⁻ transport across lipid vesicle (B). The X-ray crystal of kosmotropic **5e**-Cl⁻ complex (C), and relative transport activity across EYPC-LUVs (D).

Chapter 4: Bis(amide) substituted imidazole receptors for transmembrane H⁺/Cl⁻ cotransport activity

The design and synthesis of a series of imidazoles ($pK_a \sim 6.4 - 7.0$), decorated with correctly positioned bis(amide) groups, were reported. The anion recognition properties of these receptors were evaluated by a ¹H NMR titration method. The chloride recognition property of one of the receptors was compared with its mono-sulfonamide analog in their neutral as well as protonated states of the imidazole group. The investigation of binding strength with halide gave the trend Cl⁻ > Br⁻ > I⁻ suggesting a convenient chloride binding site. The comparison of ion transport activity indicated **6a** as the most active molecule. The coupling of the transport processes with FCCP (a proton transporter) and valinomycin (K⁺ transporter) suggested that the H⁺/Cl⁻ co-transport mechanism under the applied pH gradient across the vesicular bilayer membrane. The U-

tube experiments were performed to confirm the mobile carrier mechanism for such transport process.

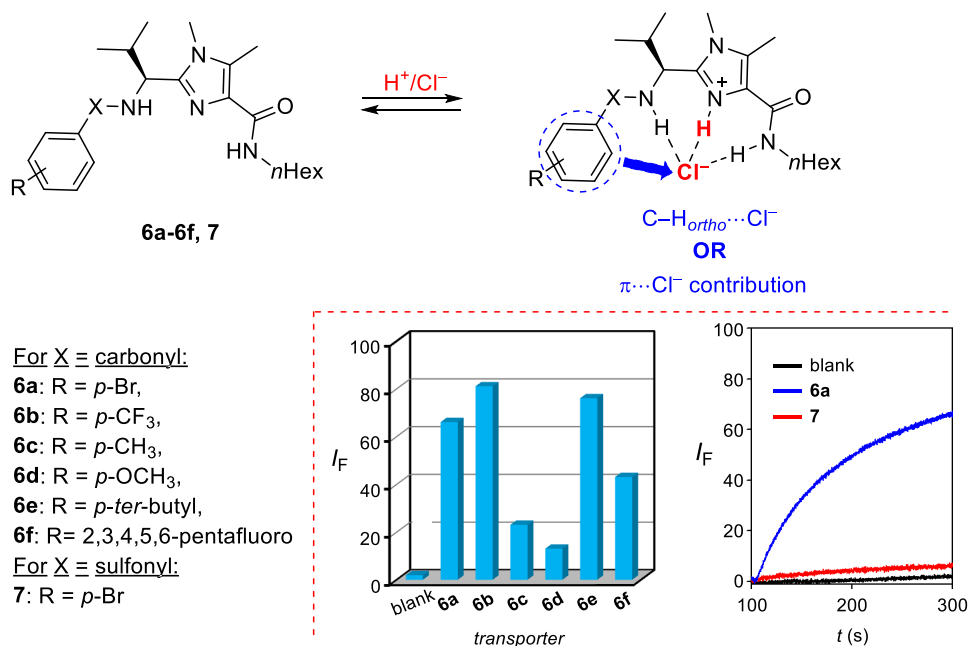


Figure 4 The design strategy for bis(amide) decorated imidazole as H^+/Cl^- receptors and the variation of ion transport activity.

Chapter 5: A dimeric bis(melamine) substituted bispidine for efficient transmembrane H^+/Cl^- cotransport

In this chapter, the design and synthesis of bis(2,4-dibutylamino-1,3,5-triazine) linked bispidine compound **3** was reported. The co-crystallization of **3** with HCl revealed that the V-shaped molecule forms a **3**:(HCl)₂ complex arranged in a *zig-zag* network through co-operative protonation of the triazine N-atom that intervenes two dibutylamino groups, and trifurcated cooperative N-H $\cdots$ Cl⁻ hydrogen bonding interactions between successive melamine units. The ion transport study demonstrated that in the lipid membrane, receptor **3** forms dimeric self-assembly involving multiple H^+ and Cl⁻ leads to the efficient co-transport of H^+/Cl^- . The pH-dependent Cl⁻ efflux observed for the compound was rationalized based on a gradual protonation model that confers an active transmembrane carrier at physiological pH.

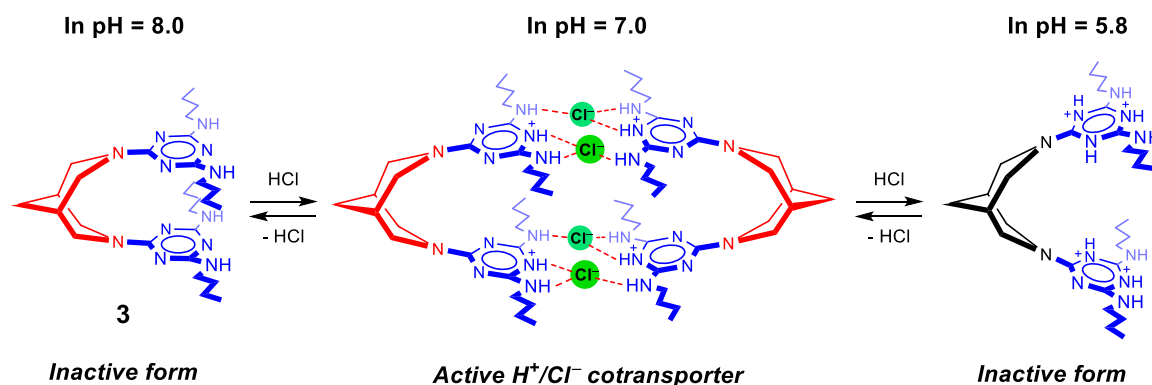


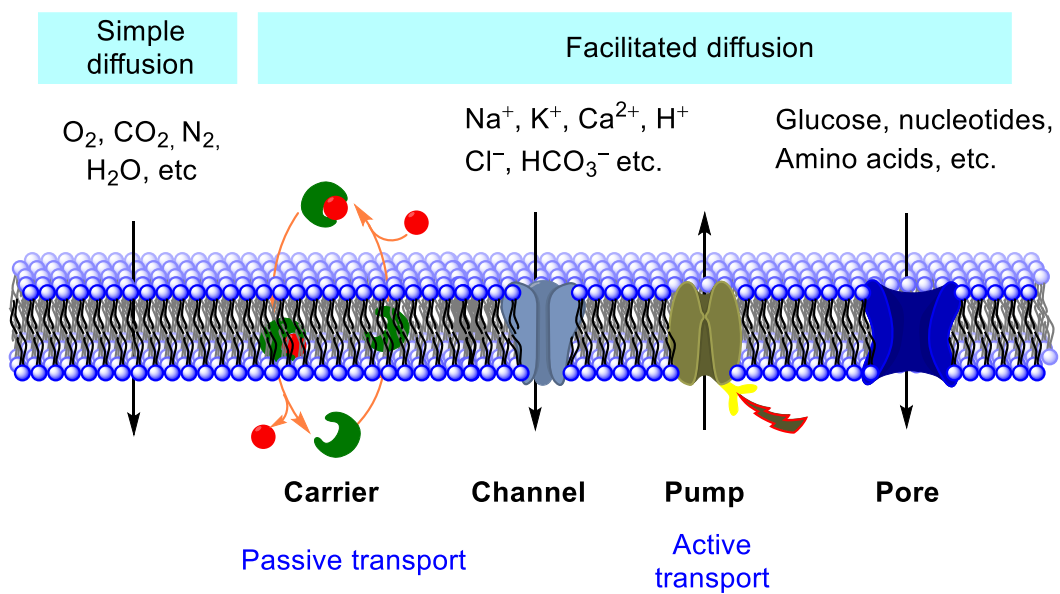
Figure 5 The illustrative change in conformations of bis(melamine) appended bispidine induced by protonation-deprotonation depending on pH.

List of publications:

1. Shinde, S. V.; Talukdar, P. A Dimeric Bis(melamine) Substituted Bispidine for Efficient Transmembrane H^+/Cl^- Cotransport. *Angew. Chem., Int. Ed.* **2017**, 56, 4238-4242.
2. Shinde, S. V.; Talukdar, P. Indole Based Receptor Employing Sulfonamide N-H Proton for Transmembrane Proton-Anion Symport. *Chem. Commun.* **2018**, 54, 10351-10354.
3. Shinde, S. V.; Talukdar, P. Bis(amide)-substituted Imidazole Receptors for Transmembrane H^+/Cl^- Cotransport Activity. (*Manuscript under preparation*).
4. Shinde, S. V.; Khan, S; Talukdar, P. Triazole Linked Indole Amide as a Transmembrane Chloride Transporter via an Antiport Mechanism. (*Manuscript under preparation*).

Chapter 1

Introduction of Membrane Transport



All the living organisms on earth included the tiniest bacterium or the big animals like whale originate from the one primitive or more cells. A cell is the smallest fundamental functional element which carries all the processes required to accomplish the complicated task of the life. Cell accomplishes the task of carrying out all the biochemical processes of the body, including production and storage of energy, transfer of genetic information, and making the molecules that have roles in metabolism. A cell is enclosed by the thin structures called plasma membranes which define the boundary of the cell separating the cytoplasm from that of exterior cell environment. Eukaryotic cells are the compartmentalized systems, i.e., cytosol is enriched with cellular machinery, and many structural elements also called cell organelles which perform the specific role in the overall task of the cell. The internal cell organelles are also separated from the cytoplasm by a unilamellar or multilamellar lipid bilayer.¹

1.1 Membrane Structure

Most of the plasma membranes are made mainly from the non-covalent assembly of phospholipid molecules – the most abundant class of lipid. Phospholipids are the amphipathic molecules having the polar, i.e., hydrophilic head group, and two nonpolar hydrocarbon chains, i.e., hydrophobic tail group. Thus in the aqueous environment of the cell, phospholipids spontaneously assemble into a compact, but fluidic bilayer core due to thermodynamically favored van der Waals interactions among the hydrocarbon tails as well as two leaflet of the bilayer and polar head groups face towards the watery environment of cell exterior and interior. (Figure 1.1) The most of the membranes have 3 – 4 nm thick hydrophobic cores. The hydrophobic interior of the membrane acts as a barrier between the cytosol and extracellular environment maintaining the significant differences in the interior and exterior ion concentrations.^{1,2}

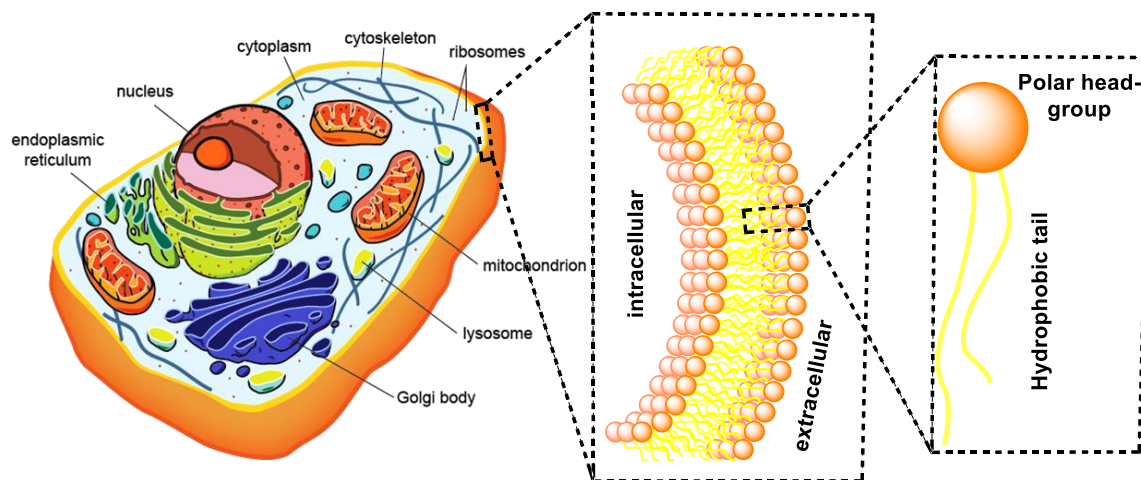


Figure 1.1 The representative structure of a eukaryotic cell and the plasma membrane. The image of the cell is accumulated from the link <https://www.thinglink.com/scene/507576839342915585>.

1.2 Membrane Transport

In order to obtain the energy required to carry out the biochemical transformation, the cell has to acquire the dissolved solutes from the cell environment and also need to exclude the metabolic waste out from the cell across the membrane. And so, it is crucial that the cell membrane should be selectively permeable. For example, the controlled movement of the ion across the membrane is essential in the regulation of pH, ion homeostasis osmotic balance, etc. Since the bilayer is a weak non-covalent assembly of lipids, it is semipermeable, and few apolar solutes like gases (e.g. N_2 , O_2 , CO_2 , etc.) and small polar molecules (e.g. H_2O , ethanol, urea) can readily slip through the membrane. The process is called simple diffusion. The diffusion rates highly depend on the concentration or electrochemical gradient, and the hydrophobicity of the diffusing molecule. The hydrophilic molecules (e.g. glucose and amino acids) and ions can not diffuse through the phospholipid membrane at substantial rates. These polar molecules and ions need to overcome the large thermodynamic energy barrier created by unfavorable interactions of ions with the hydrophobic interior of the bilayer membrane. Hence, the transport of such polar species is assisted by the specialized integral membrane-embedded ion transport proteins, which lower the energy barrier for the penetrating ions by providing a hydrophilic path through the membrane. The rigorous action of all this cellular protein machinery maintains the highly asymmetric concentrations of the different ions in the cytosol, cell organelles, and extracellular matrix.¹

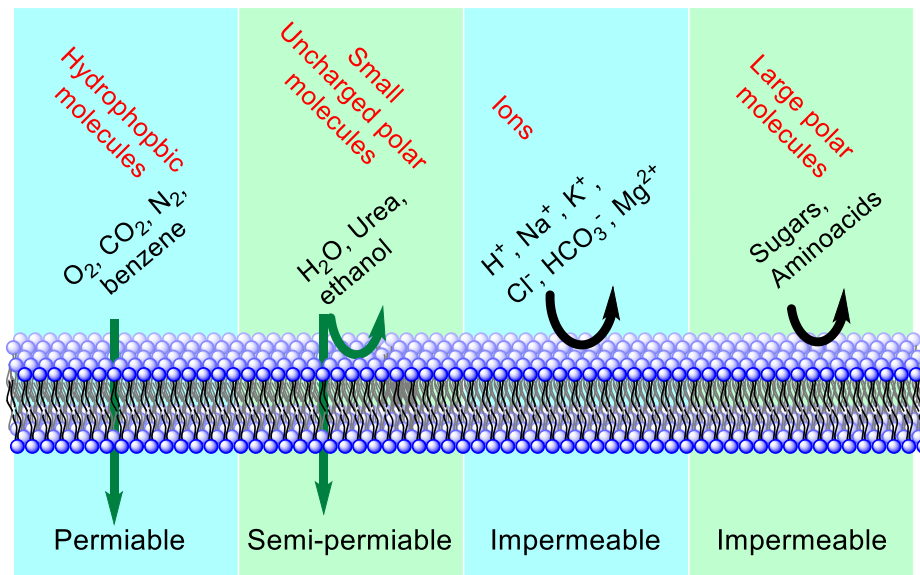


Figure 1.2 The relative permeability of small molecules and charged ions.

1.3 Regulation of Ion Transport

The transport of ions across the biological membranes and maintenance of ion gradient is essential for vital cellular processes. The electrochemical gradient of ions passively drives most of the ion transport processes. Electrochemical gradient is created by active membrane transporters (ion pumps) which utilize the ATP hydrolysis as an energy source. These processes are used to regulate fundamental tasks of sensory transduction, regulation of pH, osmotic stress, cell volume, nutrients transport, to produce electrical signals, cell proliferation, and also to regulate secretions across epithelial layers.³ Ion channels or carriers mainly facilitate the transmembrane ion transport.⁴ The natural channels are membrane-bound proteins provide the water field static path for the ions to move across the membrane. However, the carriers need not be necessarily associated with the bilayer membrane. Carriers are mobile molecules and shuttles along with an encased ion through the hydrophobic interior of the bilayer membrane.

Depending on the transport stoichiometry (number of solutes transported) and direction of transport, the transporters are categorized in uniporters – which transport a single species in a one direction (e.g. ion pumps), and cotransporters – which carry two or more species across the membrane. The cotransporters are further classified as symporters and antiporters. In symport, two species are transported in the same direction, however, in antiport, two species are carried in opposite directions of each other (Figure 1.3). The

natural ion proteins impose the required electrochemical gradient by selective uniport of the ions.

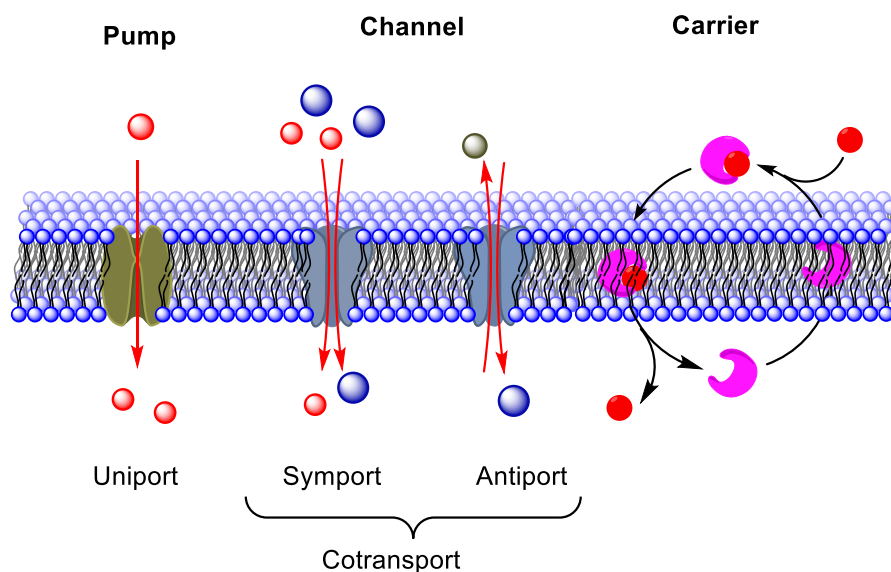


Figure 1.3 Transmembrane ion transports via a pump, channels, and carriers. Classification of ion transport processes based on the directions of ion transport.

1.4 Anions in Human Health

As previously stated, anions play a vital role in the maintenance of life, as they are involved in critical cellular functions such as regulating pH, cell volume, osmotic balance, and serve as cellular signals. The misregulated roles of natural anion transporters lead to the medical complications in humans so-called “channelopathies.”^{5,6} The chloride (Cl^-) is the most abundant extracellular anion in the human body and has a central role in physiology.⁷ For example, defective Cl^- ion channel has been interconnected with the pathology of diseases such as Bartter’s syndrome, osteopetrosis (bone disease), Dent’s disease, and cystic fibrosis (CF), etc.^{8,9} Similarly, CO_2 is the waste product of mitochondrial energy production and is transported in the form of HCO_3^- through the blood from respiring tissue to lungs. Therefore, despite the low abundance, the bicarbonate transport across membrane has a critical role in the acid/base secretion and affect the pH homeostasis. Also, the defects in HCO_3^- transport proteins have been associated with diseases of most organ systems including the brain, heart, and bones, as well as CF.¹⁰

Until early 1980’s, anion channels, typically the Cl^- transporters remained unexplored. Chloride transporters had gained lesser appreciation than that of cation (Na^+ ,

K^+ , and Ca^{+}) channels which are considerably focused from 1950's – 60's. The reason for this could be the rapid cellular excitability associated with cation channels. In 1984, Hille published the first edition of book “*Ionic Channels of Excitable Membranes*”³ describing ion channels, however, considering the chloride in electrochemical equilibrium across cell membranes, less than three pages were dedicated to the Cl^- channel in the book. Early in 1990's the involvement of chloride channels in stabilizing membrane potential was revealed. After a decade, in 2001, the disclosure of involvement of chloride channels (CFTR) in human disease, Cystic fibrosis acquired the attention of researchers. In the physiological system of organisms, Cl^- is most abundant than any other anion. Although chloride channels may also transport anions like NO_3^- , and I^- at a much faster rate than that of Cl^- , they are usually referred as the chloride channels, because Cl^- is principal permeating ion under a most set of circumstances. Cl^- transporters have a pivotal role in diverse physiological processes like controlling the ionic composition of cytosol, pH regulation in intracellular compartments, and regulation of cell volume. The naturally occurring Cl^- transporter includes cystic fibrosis transmembrane conductance regulator (CFTR), Chloride-conducting channels (CLC), chloride intracellular channel (CLIC), Chloride Nucleotide-Sensitive Channel 1A (CLNS1A), Calcium-Dependent Chloride Channel (CLCA), etc.

1.5 Chloride Channels in Cancer and Drug Resistance

There is cumulative evidence that the development of cancer, metastasis, and drug resistance have been associated with the dysregulated channel activity. To produce a daughter cell, the process of cell proliferation requires increasing the cell volume. However, the cell shrinkage is a characteristic of cell death. Volume-regulated anion channels (VRAC) has a significant role in maintaining constant cell volume.¹¹⁻¹³ VRAC in many cancer cells is encoded with CLC-3 as a major component.¹⁴ The CLC-3 is voltage-gated chloride channel affiliated to CLC superfamily. Cancer cells are characterized by abnormal proliferation of a different kind of cells in the body, and this is also linked to the extensive changes in cell volume.¹⁵ Unlike healthy cells, the elevated intracellular pH ($pH_i \geq 7.4$) than the extracellular pH ($pH_e = 6.7-7.1$) in a cancer cell is a perfect storm for cell proliferation by limiting the apoptosis.¹⁶ CLC-3, CLC-4, and CLC-5 act as a Cl^-/H^+ antiporter in intracellular membranes contributing to acidification of late endosomes and lysosomes. Also, the proton flux regulators, e.g. V-ATPase and Na^+/H^+ exchangers (NHE) regulate the unusual acidic pH in the microenvironment of tumor cells

and thereby acting as a hallmark to protect tumor cells from weak basic drugs.¹⁷ Acidic microenvironment induces the drug resistance by promoting the protonation of weak base drug molecules resulting in reduced effective concentrations of these drugs. Therefore the chemical agents which can modify the tumor cell acidity could be utilized for restoring the efficiency of such drug candidates.

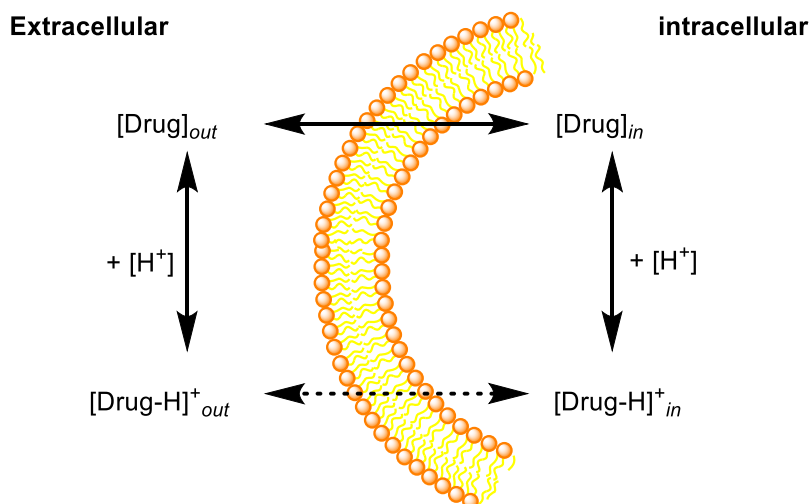


Figure 1.4 The effect of weak acidic microenvironment around tumor cell on weak base drug activity. The charged weak base drug $[\text{Drug-H}]^+$ tends to be excreted from the cell.

1.6 Naturally Occurring Cation Transporters

There are several examples of natural products mainly constructed from single or multiple proteins subunits, which have been found to mediate the transmembrane transport of cations, among these gramicidin A^{18,19} and valinomycin²⁰ (Figure 1.5) have been extensively used for the investigation of the transport mechanism of synthetic ion transporters. Gramicidin A is a monovalent cation channel which is a dimeric arrangement of two identical α -helical peptide units formed in biological membranes. On the other hand, valinomycin is a naturally occurring cyclic dodecadepsipeptide. It is a K^+ selective ionophore and usually used as a tool for many biochemical studies. The valinomycin is one of the best known K^+ carrier transporting about 104 ions per second. Valinomycin forms the six fold coordination complex with K^+ ion. The potassium-valinomycin complex is approximately 100,000 times more stable than that of the sodium-valinomycin complex.²¹

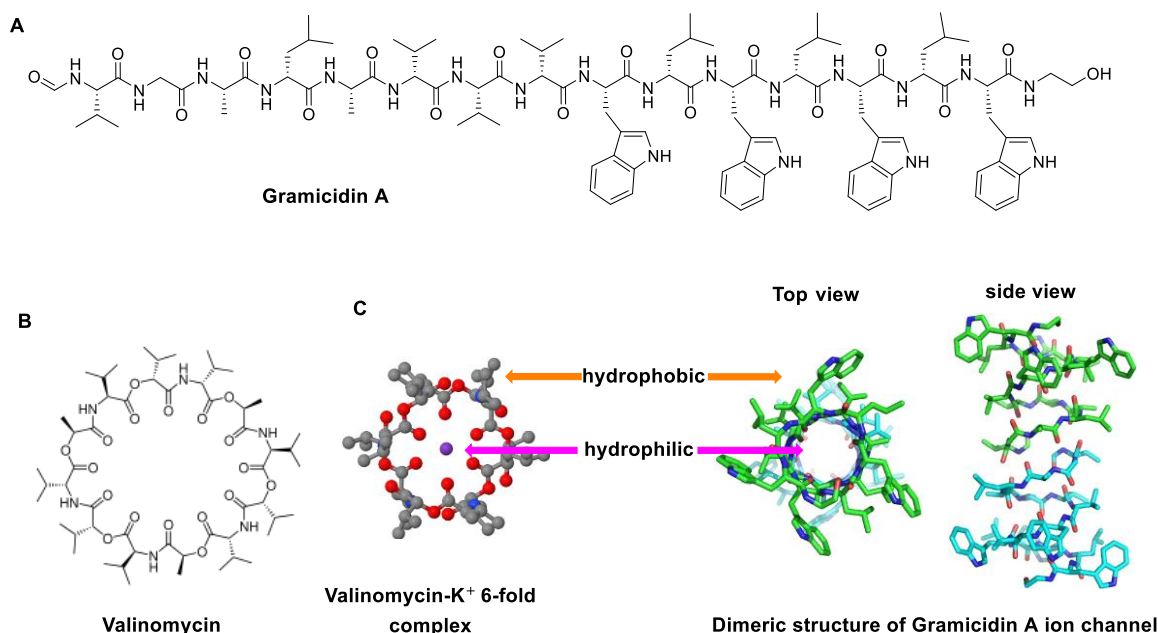


Figure 1.5 The structures of naturally occurring cation transporters, Gramicidin A (A), and valinomycin (B). The top view and side view of gramicidin A is shown on the right bottom corner, and the 6-fold complex of valinomycin with K^+ shown in the left bottom corner (C).

1.7 Non-protein based Natural Cl^- Carriers and Their Synthetic Analogues

Nature synthesizes the ion channels by genetically engineered biosynthesis. Due to the extremely complex structures and large sizes, synthetically creating and mimicking the natural peptide-based ion channels is very difficult. In natural channels, the induced mutations and sequencing of protein residues have been identified by crystallizing the ion channels. These discoveries provided the sufficient knowledge about conserved protein residues and their contributions to the channel gate and selectivity filter. This information is useful in the new designs of protein and non-protein based channels. However, this task of identifying the mutations and sequencing is eminently resource-intensive and challenging to execute. The small natural products or small synthetic molecules can offer a feasible, resource-superficial and easily adaptable alternative natural ion channels.

Prodigiosin **1** is a naturally occurring chloride complexing agent isolated from the gram-negative bacterium *Serratia marcescens*.²² It is a tripyrrolic compound with a sickle-shaped recognizing site. Prodigiosin received renewed attention due to its widespread biological activities including antifungal, antimalarial, immunosuppressive, and antibiotic activities.²³⁻²⁵ Perhaps, prodigiosin is best known to induce apoptosis of malignant cancer cells. Although prodigiosins have the marvelous biological significance,

the chemistry behind these bioactivities is complicated and not much explored. There are several hypotheses put forth to illuminate prodigiosin's biological activity. For example, the inhibition of phosphorylation and activation of JAK-3, a cytoplasmic tyrosine kinase by the prodigiosin's may bring about immunosuppressive properties.²⁶ Likewise, the prodigiosins are known to bind H^+/Cl^- by a combination of electrostatic interactions and hydrogen bonding interactions to form $1 \cdot HCl$ complex with 1:1 stoichiometry as shown in figure 1.6. Its ability to depolarize the acidic compartments within cells via H^+/Cl^- symport,²⁷⁻²⁹ or exchange of Cl^-/HCO_3^- across lipid bilayers,³⁰⁻³² has been attributed to the anti-cancer activity of prodigiosin.^{24,31,33} Another mechanism for anticancer activity of prodigiosin is proposed as a cleavage of double-stranded DNA mediated by the copper-complexed prodigiosenes.^{34,35} Prodigiosin **1** is currently in preclinical trials for the treatment of pancreatic cancer.^{23,36}

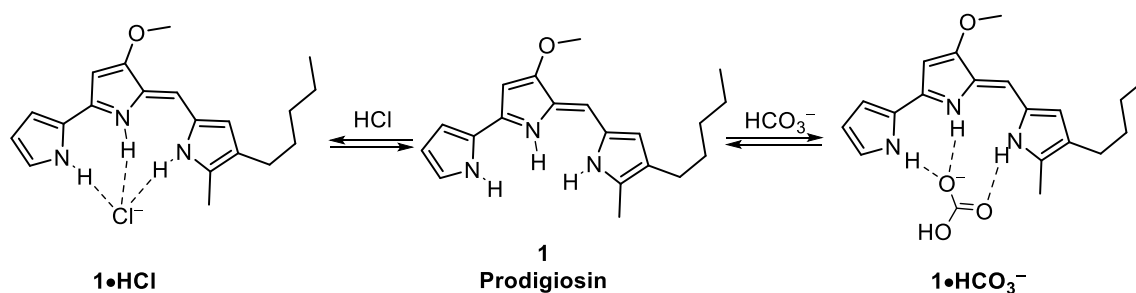


Figure 1.6 The structure of parent prodigiosin **1** and its complexes with HCl ($1 \cdot HCl$) and bicarbonate ($1 \cdot HCO_3^-$).

The therapeutic significance of parent prodigiosin has encouraged the structural modification of the molecule to develop new, less toxic but more effective analogs as a course of curing various anomalies.³⁷ In this progressive trailing, some derivatives have been selected for clinical and pre-clinical trials. For example, PNU-156804 **1b** (Figure 1.7) has been used as an anti-rejection candidate in organ transplants due to its promising immunosuppressive properties.^{38,39} The mesylate salt of obatoclax (GX15-070) **1c** (Figure 1.7) has undergone several phase II clinical trials for the treatment of various type of cancers such as leukemia, lymphoma, myelofibrosis, and mastocytosis.⁴⁰⁻⁴²

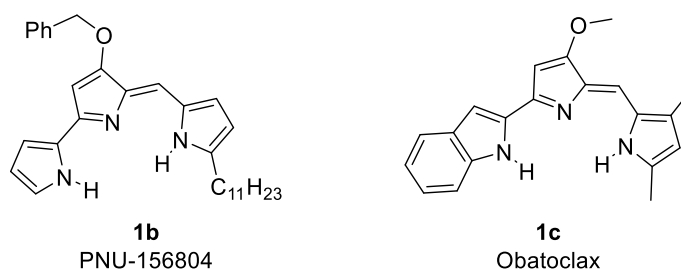


Figure 1.7 Structure of synthetic bioactive analogs of prodigiosin.

Tambjamines are another group of natural product structurally analogous to prodigiosin characterized by the 4-methoxy-2,2'-bipyrrolenamine structure (Figure 1.8).⁴³⁻⁴⁵ The pK_a value of tambjamine⁴⁶ is around 10 as disparate from the $pK_a \sim 7.2$ for prodigiosin.⁴⁷ Quesada and coworkers investigated the chloride binding and transport properties of these molecules **2a-2d** and synthetic analogs **2e-2f**. In ^1H NMR titration experiment of the protonated form of compound **2a-2f** with TBACl in $\text{DMSO}-d_6$, up to the addition of one equivalent of Cl^- resulted in significant changes in the chemical shifts of the NH protons involved in the binding event. This showed strong, 1:1 binding of protonated state of receptors with Cl^- . The X-ray crystal structures of compound **2f**·HCl showed that the bipyrrolenamine core interacts with chloride anion via hydrogen bonds (Figure 1.8). The high pK_a make these compounds weak H^+/Cl^- symporters. However, these compounds function as potent $\text{Cl}^-/\text{NO}_3^-$ and $\text{Cl}^-/\text{HCO}_3^-$ antiporters. These marine alkaloids (tamjamines) have shown interesting biological activities including anticancer and antimicrobial properties.

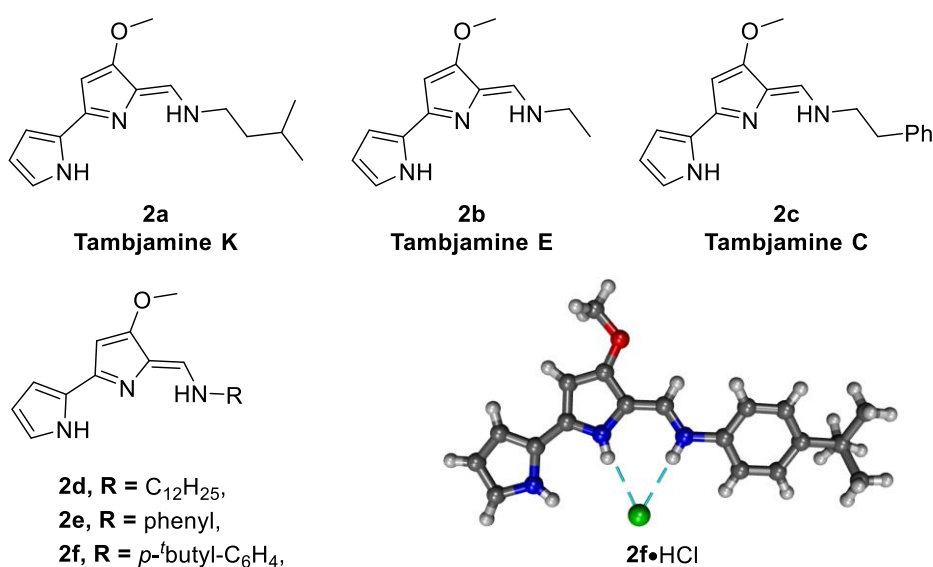


Figure 1.8 The Structures of tambjamine analogs and the crystal structure of **2f**·HCl.

A sphingolipid C₂- and C₁₆-Ceramides **3a** and **3b** respectively, are another class of naturally occurring anion transporters (Figure 1.9). It is hydrolysis product of sphingomyelin produced within the cells. It is a key component of intracellular stress response pathways and increased level of ceramide content (10 mol % of the total phospholipid) is implicated for the apoptotic cell death. In 2000, M. Colombini and co-workers investigated the ability of ceramides **3a** and **3b** to form channels in membranes using the planar membrane technique.^{48,49} The results obtained by M. Colombini and co-workers validated that both the C₂-, and C₁₆- ceramides forms a stable membrane spanning pores. Later in 2010, J. T. Davis and co-workers reinvestigated the ion transport activity of C₂-ceramide **3b**.⁵⁰ They observed that the further lowering the concentration of ceramide **3b** from 10 mol% to below 1 mol% (of total lipid can still facilitate anion transport. The C₂-ceramide **3b** was found to mediate antiport of Cl⁻/NO₃⁻ and Cl⁻/HCO₃⁻ in EYPC vesicle systems at a concentration of 1 mol%. Additionally, this concentration of **3b** was not sufficient to release the carboxyfluorescein (CF) dye from the vesicles. In contrast, the higher concentrations of **3b** were found to leak carboxyfluorescein (CF) from the vesicles. These observations suggest that the lower concentrations of **3b** function as a mobile carrier instead of forming the pores in lipid bilayer membranes. Davis group also demonstrated that naturally occurring monoacylglycerols **3a** and **3b** are even capable

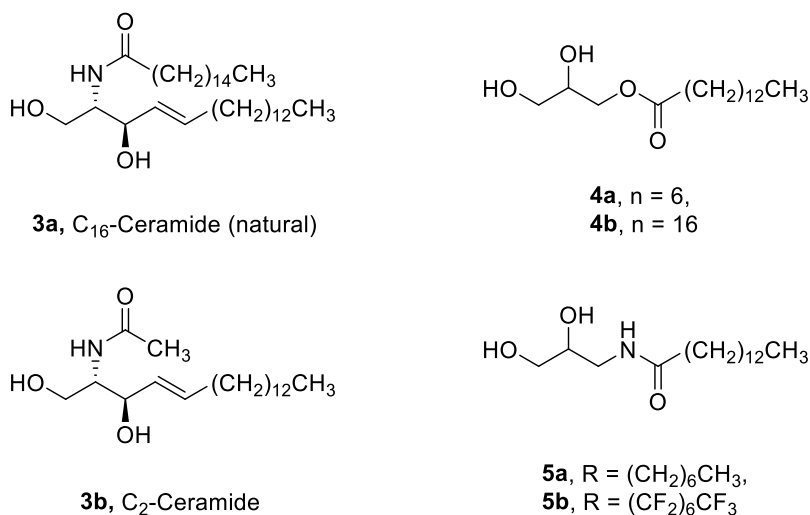


Figure 1.9 The structures of C₂- and C₁₆-ceramides and monoglycerol based natural (**4a**, and **4b**) and synthetic analogous (**5a**, and **5b**) anion transporters.

of mediating the through a chloride/nitrate antiport process across EYPC vesicles at the same carrier to lipid ratio. Both the ceramides and monoglycerol involves the OH...A⁻ interactions with an anionic guest during the transport process. The simple modification

of monoglycerol derivative **3a** and **3b** gave the synthetic analogs **4a** and **4b** (Figure 1.10). The ion transport activity of **4a**, and **4b** was significantly superior over the natural derivatives **3a** and **3b**. Similarly, the analogous short chain amide compound **5b** also showed the significant transport activity over the **5a** owing to the hydrophobicity of the perfluoro alkyl chain.” is added for citing these molecules. These examples suggest eminence of studying natural analogs and captivating creativeness from nature to develop new anionophores.⁵¹

1.8 Techniques for Detecting and Quantifying Anion Transport

The ion transporters change the concentration of solutes across the membrane. Hence it is crucial to detect and measure their transport activity accurately. The two most fundamental techniques used for measuring the transport activity of transporters include a conductance measurement across planar bilayer membranes often called as black lipid membrane (BLM) and the liposome-based assays. Former (i.e., BLM assay) measures the voltage directly and used to distinguish ion channel mechanism from carrier mechanism. To study the ion carriers, chemists use the liposome-based assays. Unilamellar phospholipid vesicles provide simple, reliable and reproducible models that can be used to mimic and study ion transport across cell membranes. Liposomes offer great versatility allowing careful control of the intra- and extra-vesicular solutions. The concentration gradients can be created across the vesicular membranes by replacing the extravesicular ions by either during size exclusion chromatography or dialysis technique. The ion transport enabled by the addition of a transporter compound can be quantified by fluorescence, ion-selective electrodes, NMR, etc.

1.8.1 Fluorescence-based Assay

In fluorescence-based assays, the transport activity is quantified by measuring the changes in fluorescence emission of particular encapsulated dye sensitive to change in pH, anion concentration, or electric potential over a period. Common fluorescent dyes used to measure ion transport in liposomal assays includes 8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (**HPTS**), *N,N'*-Dimethyl-9,9'-biacridinium dinitrate (**Lucigenin**), and Safranin O.

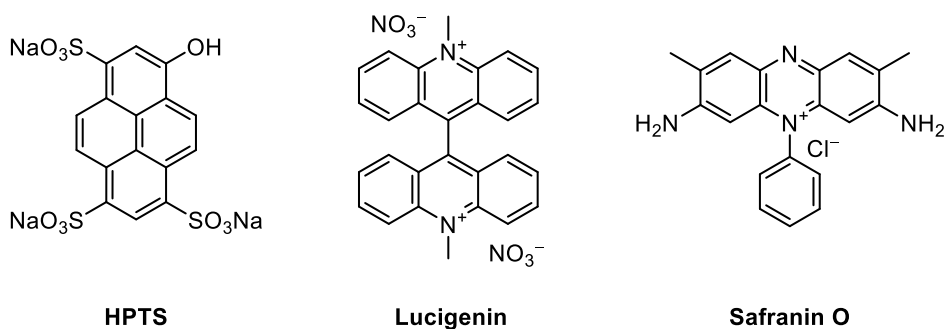


Figure 1.10 Commonly used fluorescent dyes in characterization of ion transport by vesicle-based assays.

HPTS is a highly water-soluble membrane-impermeable pH indicator with a pK_a of ~ 7.3 in aqueous buffers.⁵² The protonated and deprotonated forms of HPTS have two different excitation wavelengths, i.e., 403 nm in protonated form and 460 nm in deprotonated form. However, the excitation at both the wavelength gives the emission at the same wavelength (i.e., at 510 nm). In based assays, pH gradient is created across the HPTS entrapped vesicles, and the transport is characterized by monitoring the HPTS emission intensity after the addition of transporter molecule. The intravesicular pH change after addition of anion transporter indicates that the transport occurs either via H^+/A^- efflux, i.e., symport or OH^-/A^- exchange process (Figure 1.11A). This assay allows the measurement of intravesicular pH by determining the ratio of protonated and deprotonated dye.⁵²⁻⁵⁴ Lucigenin assays characterize the direct measurement of anion transport. Lucigenin fluorescence is selectively quenched in the presence of halide anions but is insensitive to oxoanions such as nitrate, or sulfate. The standard assay involves the vesicle entrapped with NO_3^- along with lucigenin dye and chloride gradient created by the addition of Cl^- to the extravesicular matrix. The transport process is characterized by monitoring the quenching of fluorescence after adding the transporter molecules. The NO_3^-/Cl^- exchange is followed during the transport (Figure 1.11B).⁵⁵ Similarly, the safranin O is sensitive to membrane potential used to detect the unidirectional ion transport. It is cationic dye associated with membrane and the development of membrane potential by adding the transporter lead to increase in fluorescence of safranin O.⁵⁶

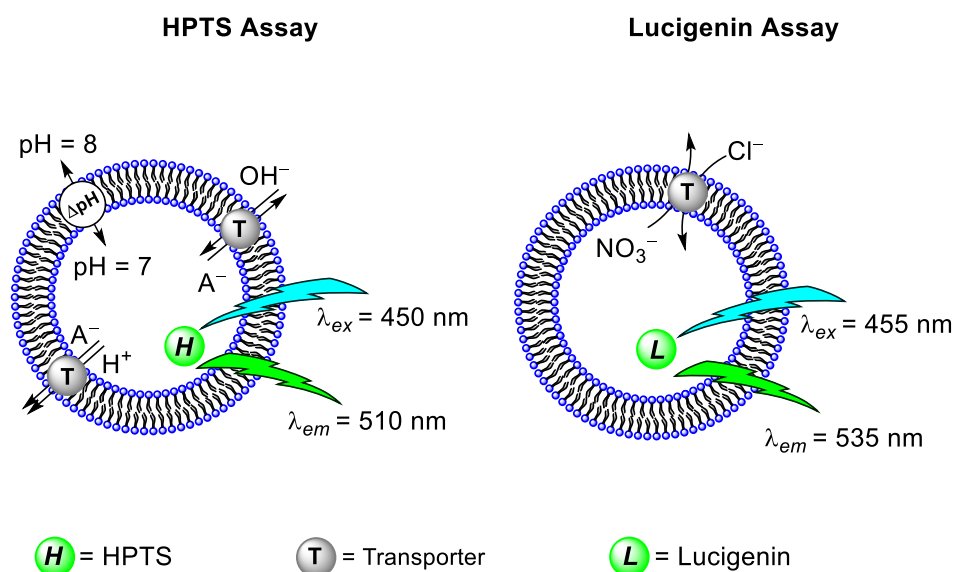


Figure 1.11 Representative fluorescent based assay in liposomes entrapped with the pH-sensitive HPTS (A), and lucigenin captured vesicles (B).

1.8.2 Ion Selective Electrode-based Assay

Similar to lucigenin based assay, use of ion selective electrode for detection of anion transport is direct and straightforward method. Both the statements are corrected as “The measure of the efflux of particular ions from vesicles can be measured by recording the conductivity of the extravesicular solution by using ion selective electrode.” and “In a typical chloride efflux assay, the liposomes entrapping relatively high concentration of MCl is added in isoosmolar MNO_3 or M_2SO_4 solution (where, M = monovalent alkali cations). Then the Cl^- efflux in the absence and presence of the transporter is measured by monitoring the conductivity of the extravesicular solution using a chloride selective electrode (Figure 1.12A).

1.8.3 NMR based Assay

The species being carried have to be necessarily NMR active (for example, ^{35}Cl , $H^{13}CO_3^-$, or $^{33}SO_4^{2-}$). In a typical experiment, unilamellar vesicles are prepared with NaA (where, $A^- = ^{35}Cl^-$, $H^{13}CO_3^-$, or $^{33}SO_4^{2-}$, and NMR inactive anion and paramagnetic shift reagents like Co^{2+} , Mn^{2+} are added to the extravesicular buffer. The paramagnetic shift reagent outside the liposome cause a significant difference in either the NMR chemical shift and/or the line-broadening of the peak for intra- and extravesicular anions (Figure 1.12B). Hence, in the presence of ion transporting agent, the line broadening or chemical

shift change of the peak for extravesicular NMR active anion can be monitored to check anion transport across the membrane.^{59,60}

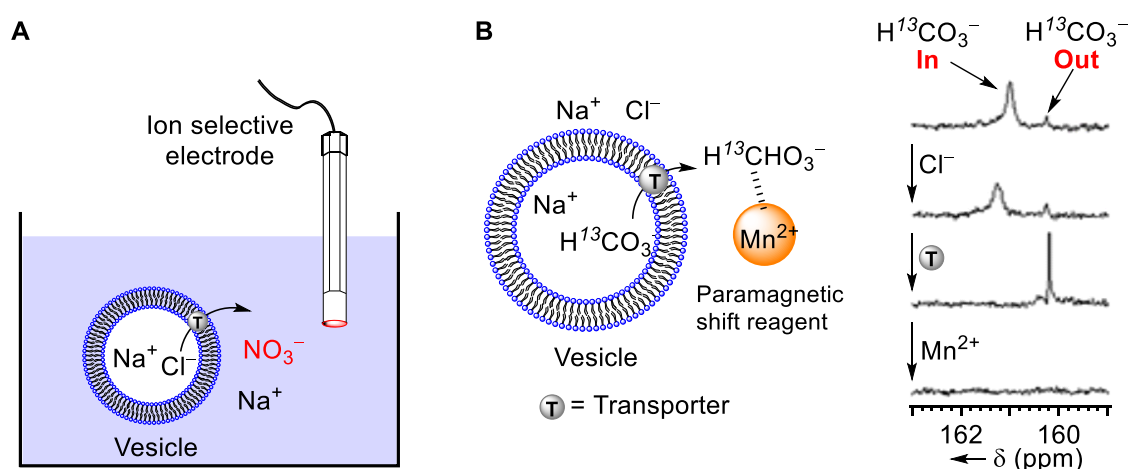


Figure 1.12 Schematic representation of ion transport study using ion selective electrode (A), and representative bicarbonate transport by ^{13}C NMR technique (B).

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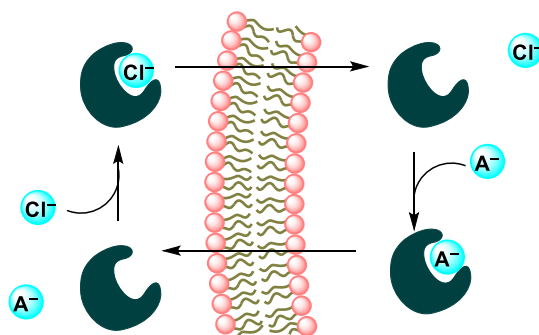
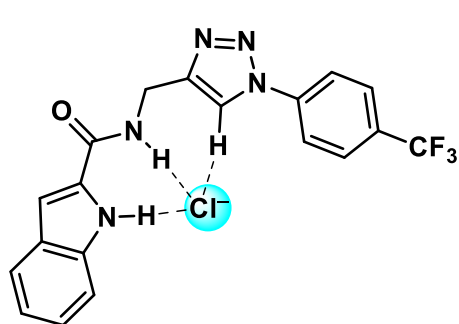
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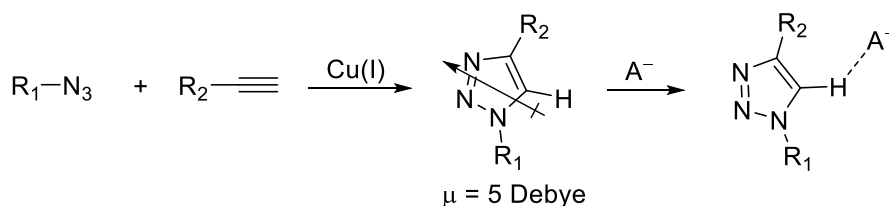
Chapter 2

Triazole Linked Indole-2-Carboxamide as Anion Receptor for Transmembrane Transport



2.1 Introduction

As discussed in chapter 1, the chloride ion flow regulates broad ranges of biological functions through the cell membranes. Additionally, the recent finding in the field of transmembrane chloride transporters demonstrates their future application in the cancer therapy.¹⁻⁵ Over the last decade, the development of structurally simple small molecules for selective anion recognition and their transport across phospholipid membrane have been focused intensively. As a tryptophan group, Indole is present in some of the natural anion receptors, *e.g.* nitrate binding protein NTrA,⁶ Sulfate binding protein, and bicarbonate binding protein CmpA.⁷ Inspired by nature, several synthetic indole-based receptors were designed and used as the transmembrane anion carriers.^{1,4,8,9} As discussed in the previous chapter, the anion recognition motifs in the majority of the receptors make use of strong hydrogen bond donor groups (*e.g.*, N–H and O–H). The strong to moderate hydrogen bonding affinity by such hydrogen bond donors are accrediting such moieties a selectivity and strong binding affinities. However, in such receptors, due to a strong binding affinity for anions the release of anions is a real issue. A design of truly reversible receptors with suitable binding affinity and selectivity can be obtained by taking the benefit from the treat fairly potential of the non-conventional hydrogen bonding interaction (*i.e.*, C–H $\cdots$ X interactions, where, X = anions or polar group). In this context, the triazole moiety is one of the well explored examples that revealed good potential for the design of various neutral anion receptors. Moreover, it is also known that due to the presence of three N-atoms, the functionalized 1,2,3-triazole derivatives have approximately 5 Debye dipole moment (μ). Therefore, the C–H bond in triazole is a polarized bond providing an anion binding site with weak C–H $\cdots$ X interactions.¹⁰ The acidity and hence the H-bonding ability of triazolic C–H could be controlled by substituents (R_1 and R_2) and thereby offer an additional advantage for making truly reversible systems. Additionally, the chemoselective high yielding click (chemoselective azide-alkyne cycloaddition) reaction makes the construction of triazole an easier task provides benefit for the design of triazole-based receptors for polar molecules and anions.



Scheme 2.1 Synthetic route to 1,2,3-triazole by copper(I)-catalyzed chemo selective azide-alkyne cycloaddition (CuAAC) reaction and representative $CH\cdots A^-$ hydrogen bond interactions.

In this chapter, we have designed and synthesized the indole-triazole based anion receptors by employing the advantage of $N-H\cdots Anion$ hydrogen bonding interactions from indole-2-amide and weak $C-H\cdots Anion$ hydrogen bond interactions from 1,2,3-triazoles. The lipophilicity of these receptors was modulated by varying the substituents on the aromatic ring connected to 1-position of triazole ring. The recognition of chloride ion by the receptor **1a** was evaluated by 1H NMR titration experiment. Then the transmembrane ion transport activity of **1a-1i** was evaluated by HPTS assay. The basic design of the indole-based transporters is shown in figure 2.1.

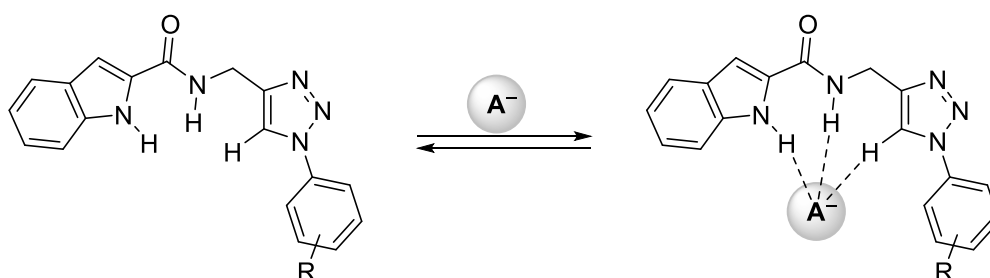
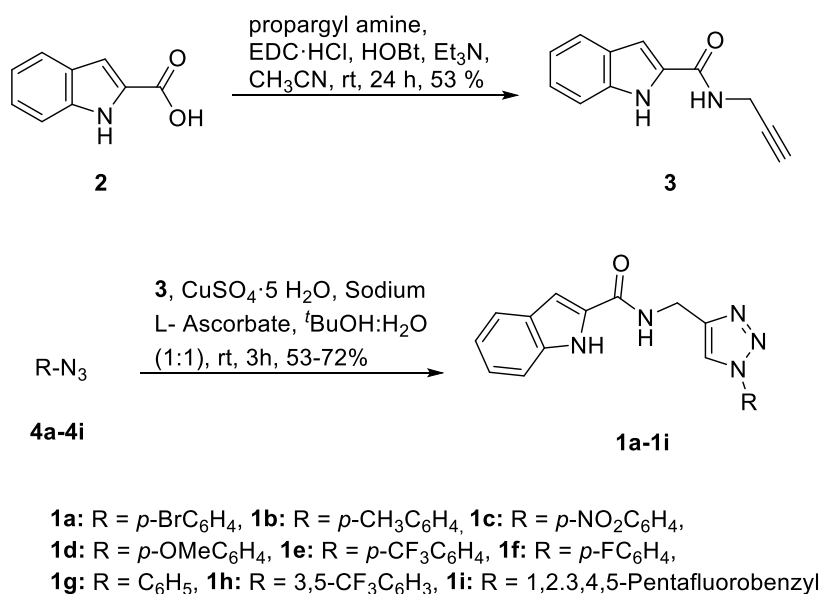


Figure 2.1 Design and proposed anion binding of receptors **1a-1i** with anions.

2.2 Results and Discussions

2.2.1 Synthesis

The synthesis of receptors **1a-1i** initiated from indole-2-carboxylic acid. Initially, the coupling of acid **2** with propargylamine **3** in the presence of EDC·HCl, and HOBt afforded the *N*-(prop-2-yn-1-yl)-1*H*-indole-2-carboxamide **3** in 53% yield. Simultaneously, following the known literature protocols, the azides **4a-4i** were prepared from the corresponding amine derivatives in quantitative yields. The click reaction of alkyne **3** with aryl azides **4a-4i** afforded the compounds **1a-1i** in 53–75% yield (Scheme 1).



Scheme 2.2 Synthesis of triazole linked anion receptors **1a-1i**.

2.2.2 Chloride Binding Study by ¹H NMR Titration

The chloride binding ability of receptor **1e** was evaluated with the help of ¹H NMR experiment in acetonitrile-*d*₃.¹¹ The stepwise addition of a TBACl solution (0 – 15 equivalents) in acetonitrile-*d*₃ resulted in a significant downfield shift of both indole and amide NH protons. Along with NH protons, the downfield shift in triazole CH ($\Delta\delta = 0.38$ ppm) indicate the presence of C–H $\cdots$ Cl[–] in the Cl[–] ion association (Figure 2.2A). The Job's plot analysis indicated the 1:1 stoichiometry of **1e**:Cl[–] (see inset in figure 2.2A). The association constant obtained by simultaneous fitting of the chemical shift of all three protons (i.e., H_a, H_b, H_c) in 1:1 binding model of Bindfit¹¹ indicated the binding constant of 468 M^{–1} (Figure 2.2B).

2.2.3 Ion Transport Activity

Ion transport behavior of molecules **1a-1i** was investigated fluorometrically by imposing pH gradient across the lipid bilayer of the large unilamellar vesicles made up of egg yolk L- α -phosphatidylcholine (EYPC). The suspension of EYPC large unilamellar vesicles (LUVs) charged with 8-hydroxypyrene-1,3,6 trisulfonate (HPTS) dye was added to the HEPES buffer.¹² To create the pH gradient across the lipid membrane, the NaOH

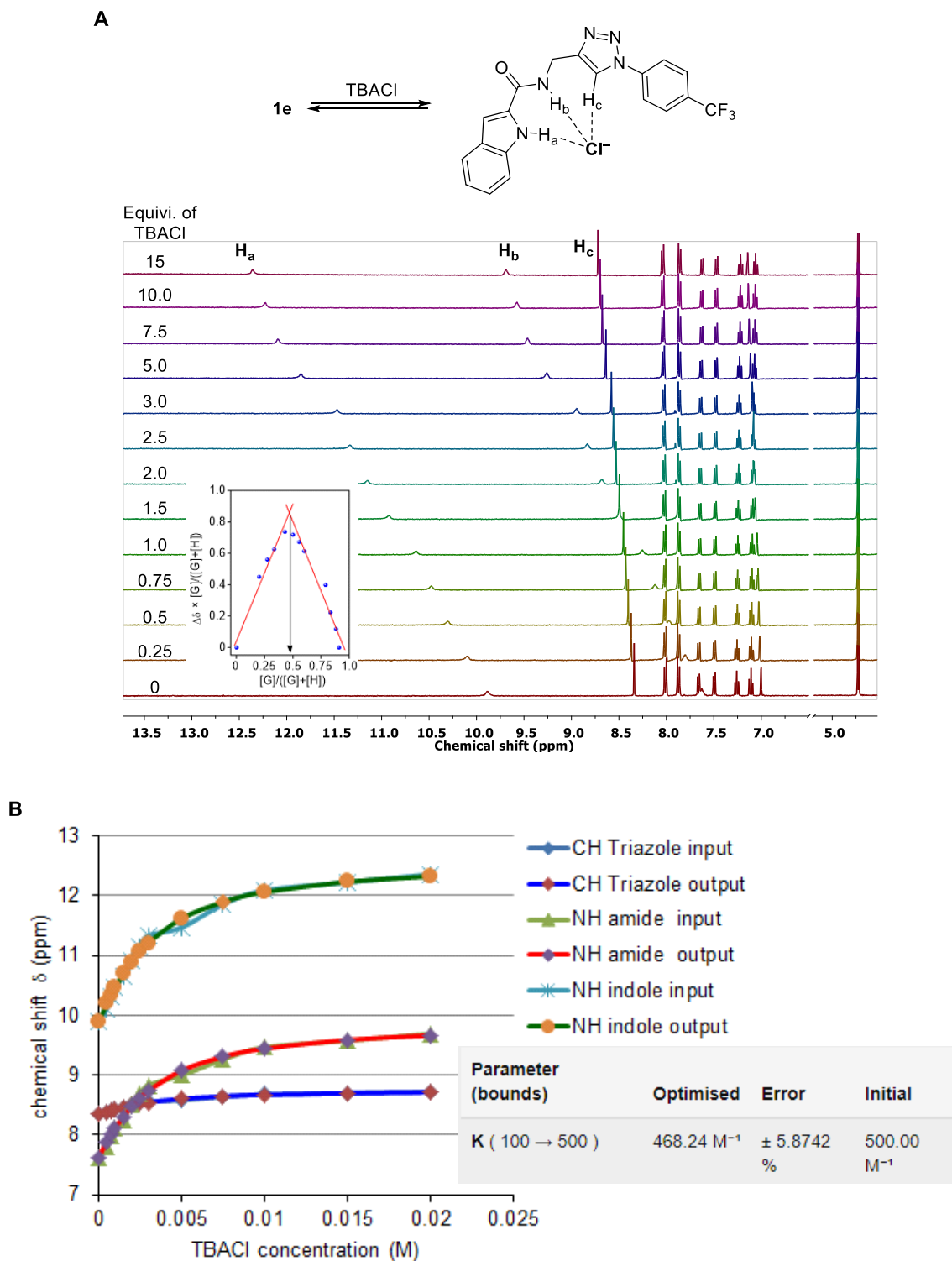


Figure 2.2 ^1H NMR titration of **1e** with TBACl in acetonitrile- d_3 . Equivalents of TBACl mentioned on stack spectra (A). Evaluation of association constant by fitting the chemical shift data for all three protons (H_a , H_b , H_c) from the 1:1 of Bindfit program (B). Inset in figure shows the Job's plot indicating 1:1 stoichiometry of **1e**: Cl^- .

pulse is applied to extravesicular buffer matrix ($\text{pH} = \sim 7.8$) followed by the addition of transporter molecule **1a-1i** ($10\ \mu\text{M}$). The ion transport activity of transporters **1a-1i** was assessed by monitoring the fluorescence intensity of entrapped HPTS dye ($\lambda_{\text{ex}} = 450\ \text{nm}$, and $\lambda_{\text{em}} = 510\ \text{nm}$) as a course of time. Finally, vesicles were lysed by the addition of 10 % Triton X-100 solution. The ion transport across the membrane was detected by an increase in intravesicular pH by either *via* Cl^-/OH^- antiport or H^+/Cl^- efflux which is accompanied by a rise in HPTS fluorescence intensity. In this experiment, we observed that the compounds **1a**, **1c**, and **1e** displayed 75%, 58%, and 72% enhancement in emission intensity of HPTS dye respectively after 200 seconds (Figure 2.3). However, compound **1b**, **1d**, **1f**, **1g**, **1h**, and **1i** displayed activity less than 35%. This behavior indicates that the compounds **1a**, **1d**, and **1e** can able to bind with ions more strongly and can easily penetrate through the lipid membrane.

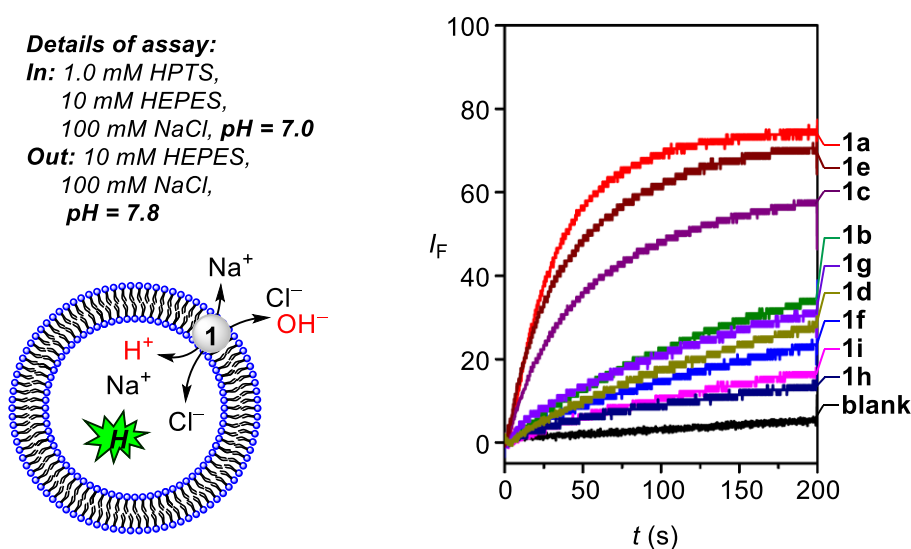


Figure 2.3 Comparison of ion transport activity of **1a-1i** ($10\ \mu\text{M}$) across EYPC-LUVs.

As compound **1a** and **1e** displayed the better relative transport activity as compared to other derivatives, we selected these two compounds for further study. The detailed examination of the ion transport activity of compound **1a** and **1e** was performed with increasing carrier concentration 0-14 μM (Figure 2.4A and 2.8A). The effective concentration required to reach 50% activity (EC_{50}) were calculated by fitting the plot of fractional activity at $t = 50$ seconds versus relative concentration with hill's equation (Figure 2.4B and 2.8B). From the Hill plot, the EC_{50} and the Hill coefficients were obtained. The EC_{50} values obtained for compound **1a** and **1e** was $6.16 \pm 0.54\ \mu\text{M}$ and

$5.14 \pm 0.47 \mu\text{M}$ respectively. The Hill coefficient (n) for both the transporter **1a** and **1e** were close to two indicating that three molecule cooperatively catalyzes the transport process of ions across phospholipids bilayer.

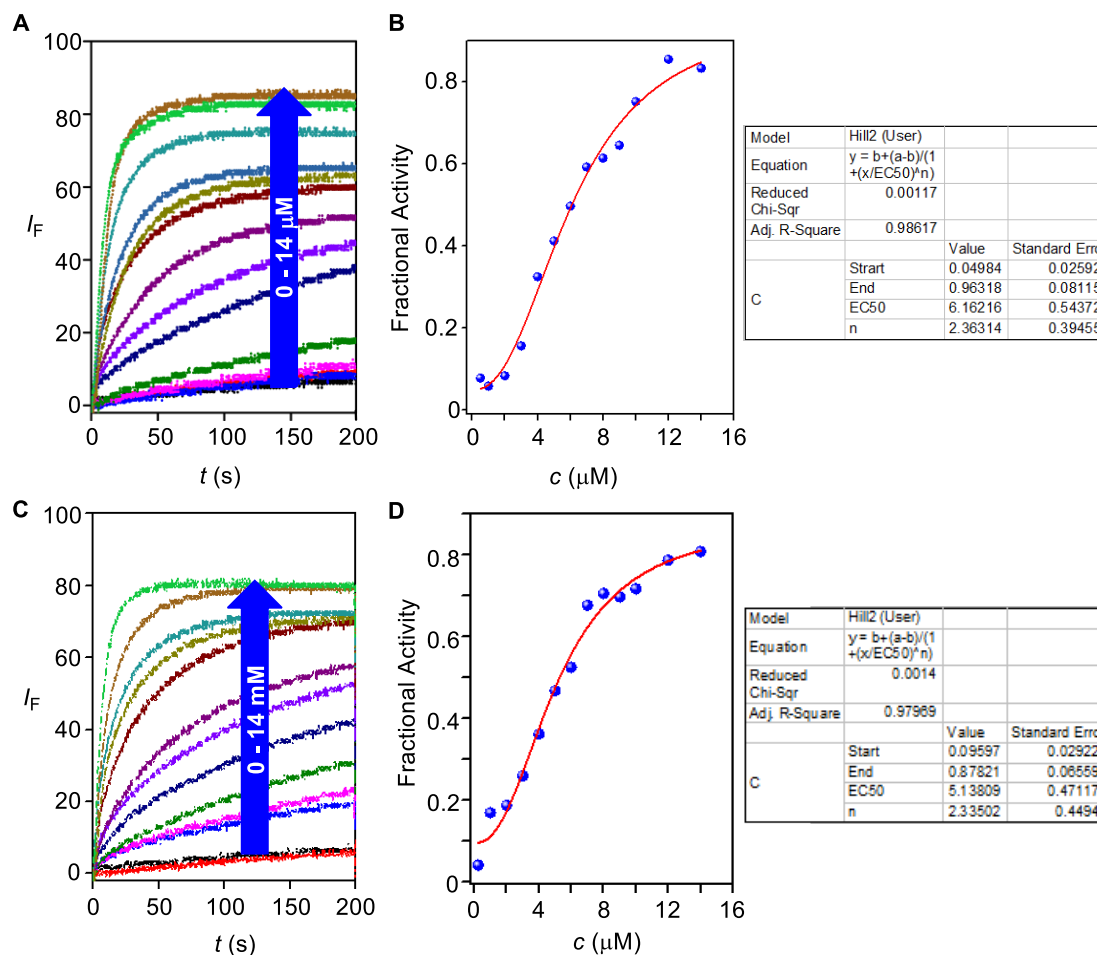


Figure 2.4 Dose-dependent ion transporting activity of transporter **1a** and **1e** across EYPC-LUV Δ HPTS, presented in the normalized emission intensity I_F as a function of time t (A and C). Hill plot of **1a** and **1e** generated from the fractional fluorescence intensity (Y) at $t = 150$ s versus concentration of **1a** or **1e** (B and C, respectively).

The effective transport rates for compound **1a** and **1e** encouraged us to investigate their ion selectivity sequences. For determining the ion selectivity sequence we have again employed the competitive HPTS assay.¹³ The EYPC-LUV charged with 1 mM HPTS dye and 100 mM NaCl in HEPES buffer were prepared. Initially, cation selectivity sequences were determined by adding the vesicle suspension in buffers containing isoosmolar different alkali metal chloride salts. (MCl, where $M^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+, \text{and Cs}^+$). In this experiment, we did not observe any characteristic change in transport

activities in the presence of different metal cations suggesting that compound **1e** and **1e** are not selective towards alkali cations (Figure 2.5A and 2.5B). In the next stage, we have determined the anion selectivity sequences by varying the extravesicular sodium salts (NaX, where $X^- = F^-, Cl^-, Br^-, I^-, SCN^-$ and OAc^-) isoosmolar with intravesicular NaCl. In this experiment, we observed that both the compound **1a** and **1e** displayed the difference in transport with the highest selectivity towards Cl^- ion (Figure 2.6A and 2.6B).

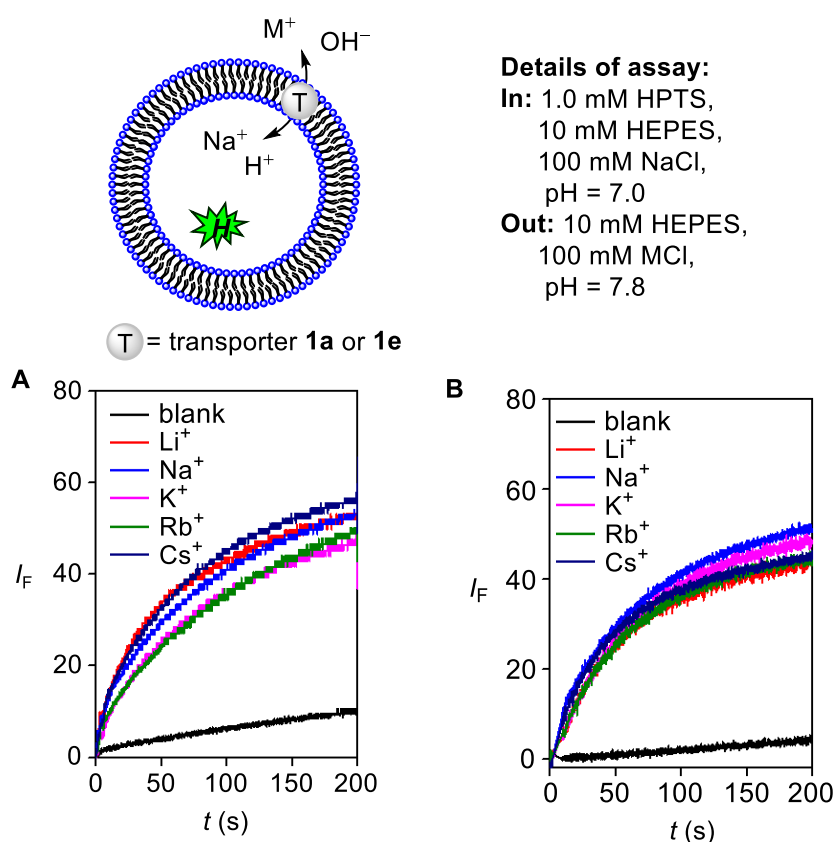


Figure 2.5 Cation selectivity experiment by compound **1a** (A), and **1e** (B), respectively across EYPC-LUVs entrapped with HPTS dye.

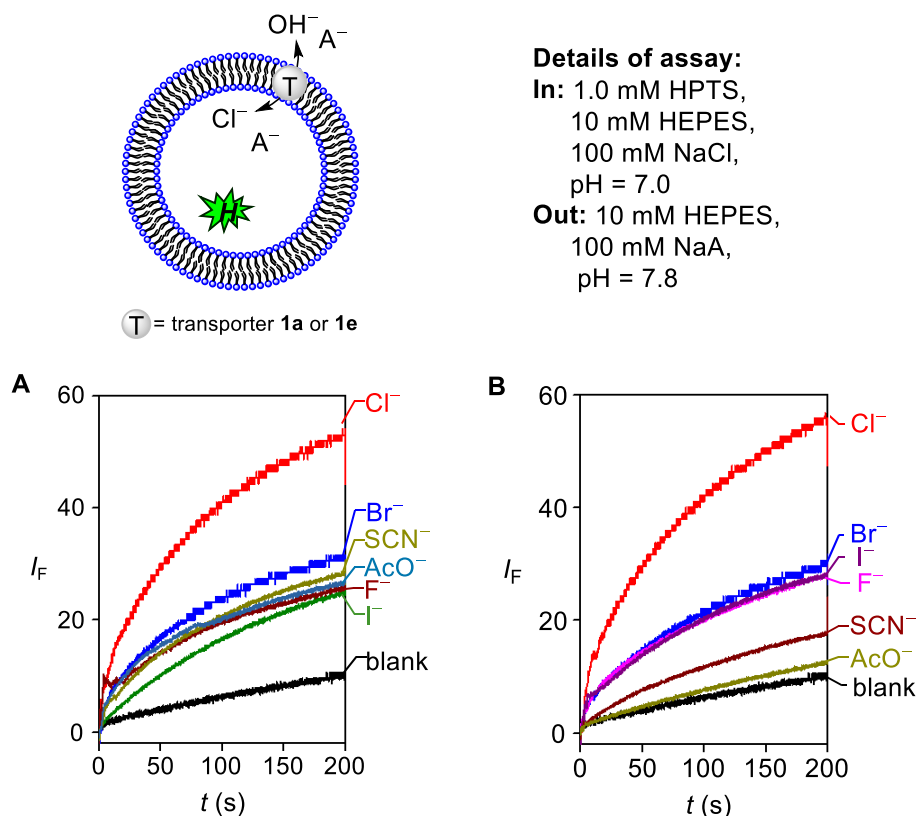


Figure 2.6 Anion selectivity experiment by compound **1a** (A), and **1e** (B), respectively across EYPC-LUVs entrapped with HPTS dye.

As the competitive HPTS assay displayed the selectivity Cl^- , next we investigated its transport mechanism by performing cooperative transport study using the valinomycin (an electrogenic K^+ carrier)¹⁴ and monensin (an electroneutral H^+/K^+ antiporter). The EYPC-LUVs entrapping KCl (300 mM, pH = 7.0) were suspended in the potassium gluconate (300 mM) solution. Formerly, **1e** induced efflux of Cl^- from KCl-loaded vesicles suspended in the potassium gluconate (K-Glu) solution was monitored in the presence and absence of monensin by chloride ion selective electrode (ISE). Monensin (Mon) transfers no net charge but builds up the pH gradient across the membrane. If the compound promotes H^+/Cl^- efflux, it should cooperate with monensin and prevent building up of the pH gradient (the net result of the assay is K^+/Cl^- efflux) resulting in increased Cl^- efflux rate. However, no net increase in transport rate was observed indicates transport occurs *via* an electrogenic transport mechanism. Later, the transport was assessed in the presence of 0.625 mol% valinomycin (Vln). In this assay, we observed that transport by compound **1e** coupled with valinomycin leading to increased

chloride efflux rate confirming the transport occurs via an electrogenic mechanism (Figure 2.7).

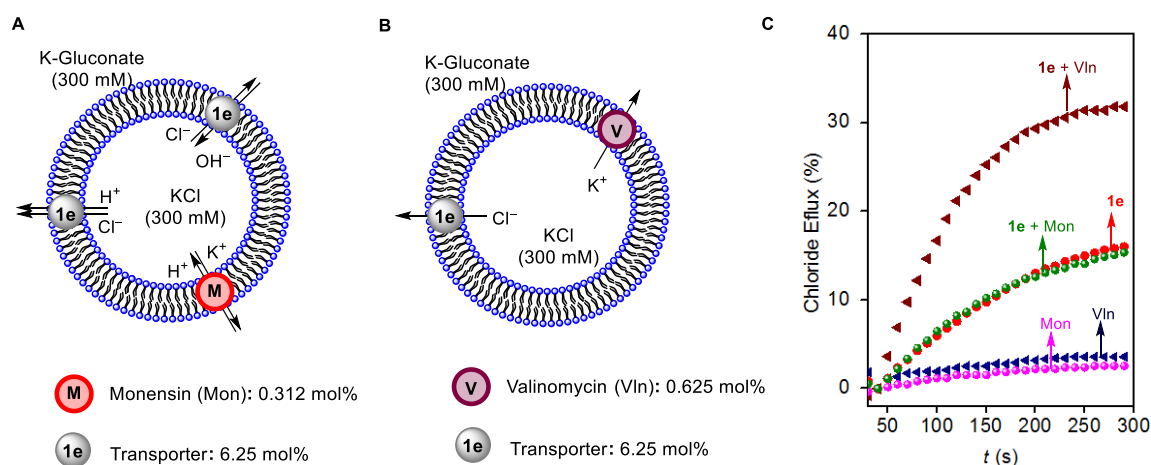


Figure 2.7 Cationophoric coupled chloride efflux assay. Cooperativity effect in chloride efflux observed in the presence of valinomycin and monensin. Representative scheme showing the net K^+/Cl^- efflux from coupled non-electrogenic transport processes (A), and coupled electrogenic transport processes, K^+ efflux by valinomycin and Cl^- efflux by transporter **1e** (B).

2.3 Geometry Optimized Structure

Based on the experimentally determined Hill coefficient value of ~ 2 , the geometry optimization studies were performed to identify the most probable active transporter structure. Initially, the most probable conformation of $[(\mathbf{1a})_2 + \text{Cl}^-]$ complex was obtained by using the CONFLEX 8 program (See experimental section Figure 2.10).^{15,16} and subsequently optimization was performed by using Gaussian 09 program¹⁷ using B3LYP functional and 6-311G++(d,p) basis set.¹⁸ Subsequently, the optimized structure of the $[(\mathbf{1d})_2 + \text{Cl}^-]$ was further modified by replacing the -Br atoms by $-\text{CF}_3$ groups and resulted $[(\mathbf{1e})_2 + \text{Cl}^-]$ complex was again optimized by Gaussian 09 program package using B3LYP functional and 6-311++G(d,p) basis set. The geometry optimized structures of complex $[(\mathbf{1a})_2 + \text{Cl}^-]$ and $[(\mathbf{1e})_2 + \text{Cl}^-]$ confirmed that each receptor participates in the anion recognition by $\text{N}_{\text{indole}}-\text{H}_a \cdots \text{Cl}^-$, as well as $\text{N}_{\text{amide}}-\text{H}_b \cdots \text{Cl}^-$ hydrogen bond interactions. In addition to these interactions, the $\text{C}_{\text{triazole}}-\text{H}_c \cdots \text{Cl}^-$ ($\text{C}_{\text{triazole}} \cdots \text{Cl}^- = 3.74 \text{ \AA}$) with a bond angle $\sim 144.4^\circ$ confirmed the presence of $\text{C}_{\text{triazole}}-\text{H}_c \cdots \text{Cl}^-$ interaction between receptors and Cl^- ion in the complex (Figure 2.8).

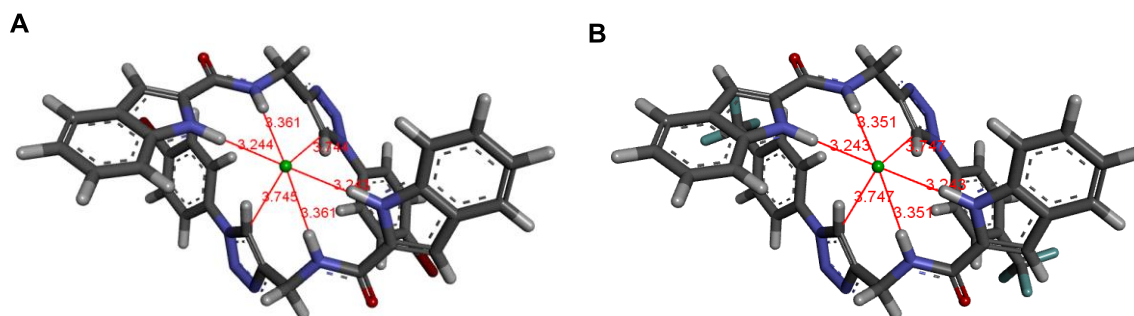


Figure 2.8 Geometry optimized structure of $[(\mathbf{1a})_2+\text{Cl}^-]$ complex (A) and $[(\mathbf{1e})_2+\text{Cl}^-]$ complex (B) showing the distances of hydrogen bond interactions.

2.4 Conclusion

In Summary, we have designed and synthesized indole-based chloride receptors. ^1H NMR titration experiment indicated both the NH proton and triazole C–H proton involved in chloride ion association. The transport study across EYPC-LUVs indicated the compound **1a**, **1c** and **1e** act as an active transporter. Based on the hill coefficient value the geometry optimized structure obtained for the active transporter structure showing the 2:1 The chloride ion selective transport was determined by competitive $1\text{a}:\text{Cl}^-$ interactions. HPTS assay. With the help of cooperative transport studies, the chloride transport shown to occurs via an electrogenic transport mechanism.

2.4 Experimental Details

2.4.1 General Methods

All reagents and solvents for synthesis were purchased commercially and used without further purification. Dichloromethane (CH_2Cl_2) was pre-dried over calcium hydride and then distilled. Column chromatographies were performed on Merck silica gel (100 – 200 mesh). Thin layer chromatographies (TLCs) were carried out with E. Merck silica gel 60-F254 plates. Egg yolk phosphatidylcholine (EYPC) obtained from Avanti Polar Lipids as a solution in CHCl_3 (25 mg/mL). HEPES buffer, HPTS dye, Triton X-100, NaOH and inorganic salts were purchased of molecular biology grade from Sigma. Large unilamellar vesicles (LUV) were prepared by using mini extruder, equipped with a polycarbonate membrane of 100 and 200 nm pore size, obtained from Avanti Polar Lipids.

2.4.2 Physical Measurements

All NMR spectra were recorded either on 400 MHz Bruker Ascend™ 400 spectrometer using either residual solvent signals as an internal reference the δ scale. The chemical shifts (δ) were reported in ppm and coupling constants (J) in Hz. The following abbreviations were used: m (multiplet), s (singlet), d (doublet), t (triplet) dd (doublet of doublet). High-resolution mass spectra were obtained on a MicroMass ESI-TOF MS spectrometer equipped with a Micromass Z-Spray electrospray ionization (ESI) source (Waters Co., Synapt G2, France). All melting points were measured in open glass capillary, and values are uncorrected. Fluoromax-4 from JobinYvon Edison-equipped with an injector port and a magnetic stirrer was used for recording the fluorescence spectra. For pH adjustment, Helmer pH meter was used. All data from fluorescence studies were processed by Origin 8.5 program.

2.4.3 Synthesis Procedures

Synthesis of *N*-(prop-2-yn-1-yl)-1*H*-indole-2-carboxamide (**3**):

To a suspension of 1*H*-indole-2-carboxylic acid (500 mg, 3.10 mmol) in dry acetonitrile (5 mL) was added EDC hydrochloride (514 mg, 3.10 mmol) and HOBT (419 mg, 3.10 mmol) in sequence. After 30 min, propargylamine (199 μ L, 3.10 mmol) and Et₃N (433 μ L, 3.10 mmol) were added in sequence. After stirring for 24 hours, the reaction mixture had thickened. The reaction solvent was removed in vacuum at 45 °C (bath temperature) and the residue was partitioned between EtOAc and saturated aqueous NaHCO₃. The organic layers were combined and dried over Na₂SO₄. The residue was obtained after concentration in vacuum and purified by column chromatography on SiO₂ (Eluent: 1% MeOH in CHCl₃) to obtain *N*-(prop-2-yn-1-yl)-1*H*-indole-2-carboxamide **3** as a white fluffy solid (435 mg, 53%).

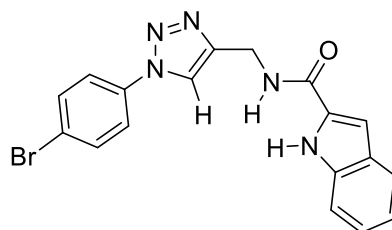
¹H NMR (400 MHz CDCl₃): δ = 9.45 (s, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.45 (d, J = 8.3 Hz, 1H), 7.65 (t, J = 7.6 Hz, 1H), 7.26 (s, 1H), 7.15 (t, J = 7.5 Hz, 1H), 6.9 (s, 1H), 6.36 (s, 1H), 4.31 (dd, J = 5.24 Hz & 2.36 Hz, 2H), 2.31 (t, J = 2.32 Hz); **¹³C NMR** (101 MHz DMSO-*d*₆): δ = 160.8, 136.5, 131.1, 127.0, 123.4, 121.6, 119.7, 112.3, 103.0, 81.3, 73.0, 28.0. **HRMS** (ESI): calcd. for C₁₂H₁₁N₂O: 199.0871 [M+H]⁺, found: 199.0869.

Synthesis of Azides 4a-4i: Azides **4a-4i** synthesized according to known literature protocols.

Synthesis of compounds 1a-1i:

***N*-((1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1a**):**

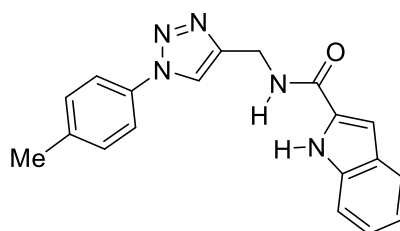
M.P. = 270–272 °C. ^1H NMR (400 MHz CDCl_3) δ = 11.01 (s, 1H), 9.06 (t, J = 5.6 Hz, 1H), 8.75 (s, 1H), 7.9 (d, J = 8.9 Hz, 2H), 7.8 (d, J = 8.9 Hz, 2H), 7.60 (d, J = 7.9 Hz, 1H), 7.4 (d, J = 8.2 Hz, 1H), 7.2 (t, J = 7.2 Hz, 2H), 7.0 (t, J = 7.4 Hz, 1H), 4.64 (d, J = 5.6 Hz, 2H);



^{13}C NMR (101 MHz $\text{DMSO}-d_6$) δ = 161.6, 146.9, 136.9, 136.3, 133.2 (2C), 131.9, 127.5, 123.8, 122.3 (2C), 122.0, 121.7, 121.6, 120.2, 112.8, 103.3, 34.8 ppm. **HRMS** (ESI): calcd. for $\text{C}_{18}\text{H}_{15}\text{BrN}_5\text{O}$: 396.0460 $[\text{M}+\text{H}]^+$, found: 396.0470.

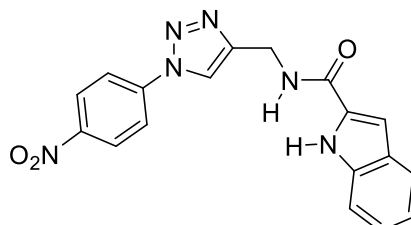
***N*-((1-(*p*-tolyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1b**):** **M.P.** =

258–261 °C. ^1H NMR (400 MHz $\text{DMSO}-d_6$) δ = 9.04 (t, J = 5.8 Hz, 1H), 8.6 (s, 1H), 7.78 (d, J = 8.5 Hz, 2H), 7.6 (d, J = 7.8 Hz, 1H), 7.4 (t, J = 7.4 Hz, 1H), 7.35 (d, J = 8 Hz, 2H), 7.17 (m, 2H), 7.02 (m, 2H), 4.63 (d, J = 5.7 Hz, 2H), 2.36 (s, 3H); ^{13}C NMR (101 MHz $\text{DMSO}-d_6$): δ = 161.1, 146.0, 138.1, 136.4, 134.4, 131.4, 130.2 (2C), 127.1, 123.3, 121.5, 121.1, 119.8 (2C), 119.7, 112.3, 102.8, 34.4, 20.5 ppm. **HRMS** (ESI): calcd. for $\text{C}_{19}\text{H}_{18}\text{N}_5\text{O}$: 332.1511 $[\text{M}+\text{H}]^+$, found: 332.1517.



***N*-((1-(4-nitrophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1c**):**

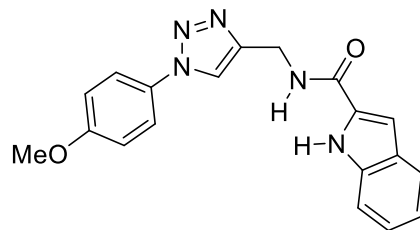
M.P.: 255 °C. ^1H NMR (400 MHz $\text{DMSO}-d_6$) δ = 11.6 (s, 1H), 9.1 (t, 1H, J = 5.54 Hz, 1H), 8.9 (s, 1H), 8.43 (d, J = 9 Hz, 2H), 8.25 (d, J = 9 Hz, 2H), 7.61 (d, J = 7.9 Hz, 1H), 7.4 (d, J = 8.2 Hz, 1H), 7.2 (t, J = 7.24 Hz, 2H), 7.0 (t, J = 7.3 Hz, 1H), 4.6 (d, J = 5.6



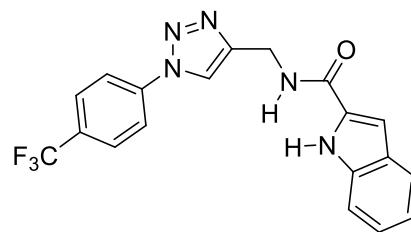
Hz, 2H); ^{13}C NMR (101 MHz $\text{DMSO}-d_6$) δ = 161.1, 146.9, 146.6, 140.9, 136.5, 131.4, 127.0, 125.5 (2C), 123.3, 121.6, 121.5, 120.4 (2C), 119.7, 112.3, 102.9, 34.3 ppm. **HRMS** (ESI): calcd. for $\text{C}_{18}\text{H}_{14}\text{N}_6\text{O}_3\text{Na}$: 363.1206 $[\text{M}+\text{H}]^+$, found: 385.1027.

***N*-((1-(4-methoxyphenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide**

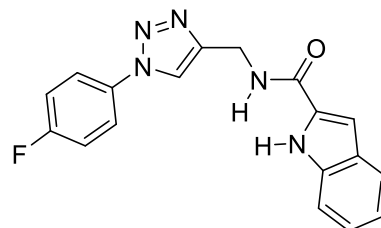
(1d): M.P. = 236–237 °C. ¹H NMR (400 MHz DMSO-*d*₆) δ = 11.6 (s, 1H), 9.04 (t, *J* = 6 Hz, 1H), 7.80 (d, *J* = 9 Hz, 2H), 7.60 (d, *J* = 8.8 Hz, 1H), 7.43 (d, *J* = 8.28 Hz, 1H), 7.18 (m, 2H), 7.1 (d, *J* = 9 Hz, 2H), 7.02 (t, *J* = 7.6 Hz, 1H), 4.63 (d, *J* = 5.64 Hz, 1H); ¹³C NMR (101 MHz DMSO-*d*₆) δ = 161.1, 159.2, 145.8, 136.4, 131.5, 130.1, 127.1, 123.3, 121.6 (2C), 121.5, 121.2, 119.7, 114.8 (2C), 112.3, 102.8, 55.5, 34.4 ppm. HRMS (ESI): calcd. for C₁₉H₁₈N₅O₂: 348.1460 [M+H]⁺, found: 348.1464.

***N*-((1-(4-(trifluoromethyl)phenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-**

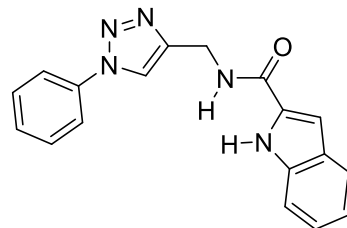
carboxamide (1e): M.P. = 255–256 °C. ¹H NMR (400 MHz DMSO-*d*₆) δ = 9.09 (t, *J* = 5.7 Hz, 1H), 8.9 (s, 1H), 8.2 (d, *J* = 8.4 Hz, 2H), 8.0 (d, *J* = 8.6 Hz, 2H), 7.6 (d, *J* = 8 Hz, 1H), 7.4 (d, *J* = 7.9 Hz, 1H), 7.19 (m, 2H), 7.03 (t, *J* = 7.5 Hz, 1H), 4.67 (d, *J* = 5.64 Hz, 2H); ¹³C NMR (101 MHz DMSO-*d*₆) δ = 161.2, 146.7, 139.4, 136.5, 131.4, 128.7, 127.2, 127.1, 125.2, 123.4 (2C), 121.54, 121.48, 120.4 (2C), 119.7, 112.3, 102.9, 34.4 ppm. HRMS (ESI): calcd. for C₁₉H₁₅F₃N₅O: 386.1229 [M+H]⁺, found: 386.1233.

***N*-((1-(4-fluorophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (1f):**

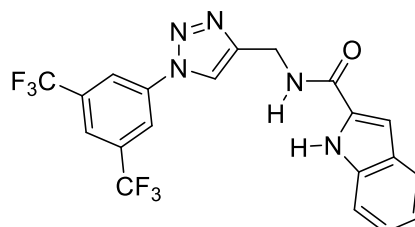
M.P. = 258–261 °C. ¹H NMR (400 MHz DMSO-*d*₆) δ = 11.6 (s, 1H), 9.06 (t, *J* = 4 Hz & 8 Hz, 1H), 8.7 (s, 1H), 7.9 (m, 2H), 7.61 (d, *J* = 8 Hz, 1H), 7.4 (t, *J* = 7.4 Hz, 3H), 7.2 (m, 2H), 7.03 (t, *J* = 8 Hz), 4.64 (d, *J* = 5.7 Hz, 2H); ¹³C NMR (101 MHz DMSO-*d*₆) δ = 162.2 (d, *J* = 245.5 Hz, 1C), 161.1, 146.2, 136.5, 133.3, 131.4, 127.1, 123.3, 122.3 (d, *J* = 8.8 Hz, 2C), 121.5, 121.4, 119.7, 116.7 (d, *J* = 23.2 Hz, 2C), 112.3, 102.9, 34.4 ppm. HRMS (ESI): calcd. for C₁₈H₁₅FN₅O: 336.1261 [M+H]⁺, found: 358.1085.



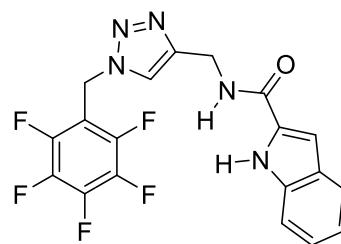
***N*-((1-phenyl-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1g**):** M.P. = 244–245 °C. ¹H NMR (400 MHz DMSO-*d*₆) δ = 11.6 (s, 1H), 9.06 (t, *J* = 5.4 Hz, 1H), 8.7 (s, 1H), 7.91 (d, *J* = 7.8 Hz, 2H), 7.6 (m, 3H), 7.46 (m, 2H), 7.18 (t, *J* = 7.2 Hz, 2H), 7.03 (t, *J* = 7.35 Hz, 1H), 4.63 (d, *J* = 5.6 Hz, 2H); ¹³C NMR (101 MHz DMSO-*d*₆) δ = 161.6, 146.7, 137.2, 137.0, 132.0, 130.4 (2C), 129.1, 127.6, 123.9, 122.1, 121.7, 120.5 (2C), 120.2, 112.8, 103.4, 34.9 ppm. **HRMS** (ESI): calcd. for C₁₈H₁₆N₅O: 318.1355 [M+H]⁺, found: 318.1359.



***N*-((1-(3,5-bis(trifluoromethyl)phenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1h**):** M.P. = 218–221 °C. ¹H NMR (400 MHz DMSO-*d*₆) δ = 9.2 (s, 1H), 8.2 (d, *J* = 6.3 Hz, 3H), 8.0 (s, 1H), 7.64 (d, *J* = 8 Hz, 1H), 7.43 (d, *J* = 8.2 Hz, 1H), 7.3 (t, *J* = 7.2 Hz, 1H), 7.0 (s, 1H), 6.9 (s, 1H), 4.9 (d, *J* = 5.9 Hz, 2H); ¹³C NMR (101 MHz DMSO-*d*₆) δ = 161.7, 147.6, 138.5, 137.0, 132.3 (q, *J* = 33.7 Hz, 2C), 131.9, 127.6, 123.9, 123.3 (q, *J* = 274.5 Hz, 2C), 122.4 122.1, 121.1 (2C), 120.3, 112.8, 103.4, 34.9 ppm. **HRMS** (ESI): calcd. for C₂₀H₁₄F₆N₅O: 454.1103 [M+H]⁺, found: 454.1107.



***N*-((1-((perfluorophenyl)methyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1i**):** M.P. = 258–261 °C. ¹H NMR (400 MHz DMSO-*d*₆) δ = 11.6 (s, 1H), 9.0 (t, *J* = 5.8 Hz, 1H), 8.1 (s, 1H), 7.6 (d, *J* = 7.8 Hz, 1H), 7.4 (dd, *J* = 0.76 Hz & 8.2 Hz, 1H), 7.03 (m, 1H), 7.14 (d, *J* = 1.1 Hz, 1H), 5.75 (s, 2H), 4.52 (d, *J* = 5.8 Hz, 2H); ¹³C NMR (101 MHz DMSO-*d*₆) δ = 161.1, 145.4, 145.0 (d, *J* = 245.3 Hz, 2C), 141.0 (d, *J* = 252.6 Hz, 1C), 137.11 (d, *J* = 251 Hz, 2C), 136.5, 131.4, 127.1, 123.5, 123.3, 121.5, 119.7, 112.3, 109.4 (m, 1C), 102.8, 40.3, 34.3 ppm. **HRMS** (ESI): calcd. for C₁₉H₁₃F₅N₅O: 422.1040 [M+H]⁺, found: 422.1041.



2.4.4 Anion Binding Study by ¹H NMR Titration

The stock solutions of **1e** (2 mM) and tetrabutylammonium chloride (200 mM) were prepared in acetonitrile-*d*₃. ¹H NMR spectra were recorded on a Bruker Ascent™ 400 spectrometer and calibrated with respect to residual solvent peak in Acetonitrile-*d*₃. ¹H

NMR titrations were performed by stepwise addition of aliquots of the tetrabutylammonium chloride (TBACl) in acetonitrile- d_3 . The change in the chemical shift of indole and amide NH and triazole CH was observed. The ^1H NMR stacked plots were processed using MestReNova 6 software. The association constant was obtained by fitting the data in 1:1 binding model of Bindfit.

2.4.5 Ion Transporting Activity Studies Across EYPC-LUV $\rhd$ HPTS

Buffer and Stock Solution Preparation: All buffer solutions were prepared at 25 °C temperature. HEPES buffer solutions were prepared by dissolving solid HEPES (10 mM) and appropriate salt *e.g.*, NaCl or KCl (100 mM) in Milli-Q water. The pH of the buffer was adjusted to 7.0 by addition of NaOH from 0.5 M stock solution. The stock solutions of all transporter molecules were prepared in HPLC grade DMSO.

Preparation of EYPC-LUVs Entrapped with HPTS Dye: In a clean dry 10 mL round bottom flask, 1 mL of egg-yolk phosphatidylcholine (25 mg/mL in chloroform) was transferred under a nitrogen atmosphere. The chloroform was removed by purging the nitrogen with continuous rotation of round bottom flask. The thin film of lipid was obtained further kept in high vacuum for four hours to remove traces of chloroform. The thin film of lipid was then hydrated by addition of buffer (HEPES 10 mM, NaCl 100 mM, HPTS dye 1 mM, pH = 7.0) and kept for 1 hour with occasional vortexing at 10 minute intervals. After 1 hour the suspension was subjected to 10 freeze–thaw cycles (hot water bath of 55 °C and liquid N_2). The suspension was then extruded through the polycarbonate membrane of 100 nm size (Avanti) for 19 or 21 times (must be an odd number). The extravesicular HPTS dye was removed by gel filtration using syphadex-G50 eluting with the buffer (HEPES 10 mM, NaCl 100 mM, pH = 7.0). The vesicle suspension was diluted to 6 mL to get EYPC-LUVs $\rhd$ HPTS: ~ 5.0 mM EYPC; *inside*: 1 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0, *outside*: 10 mM HEPES, 100 mM NaCl, pH = 7.0.

Ion Transport Assay: In a clean dry fluorescence cuvette, to a 1975 μL of buffer (HEPES 10 mM, NaCl 100 mM, pH = 7.0) solution 25 μL of above-prepared vesicles were added. The cuvette was placed in the fluorescence instrument with slowly stirring condition by a magnetic stirrer equipped in the instrument (at $t = 0$ s). The time course of

HPTS fluorescence emission intensity, I_t was observed at $\lambda_{em} = 510$ nm ($\lambda_{ex} = 450$ nm). The pH gradient ($pH_{in} = 7.0$, $pH_{out} = 7.8$) was created by addition of NaOH (20 μ L from 0.5M stock solution). Transporter molecules of different concentrations were added at $t = 100$ s, and finally 25 μ L of 10% Triton X-100 was added at $t = 300$ s to lyse vesicles resulting in complete destruction of the pH gradient. The emission intensity was monitored up to $t = 350$ s.

Final assay conditions: $c_{lipid} = 67$ μ M; *Inside the vesicles*: 1.0 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0; *Outside the vesicles*: 10 mM HEPES, 100 mM NaCl, pH = 7.8; $c_1 = 0$ –16 μ M of transporter for the concentration profile.

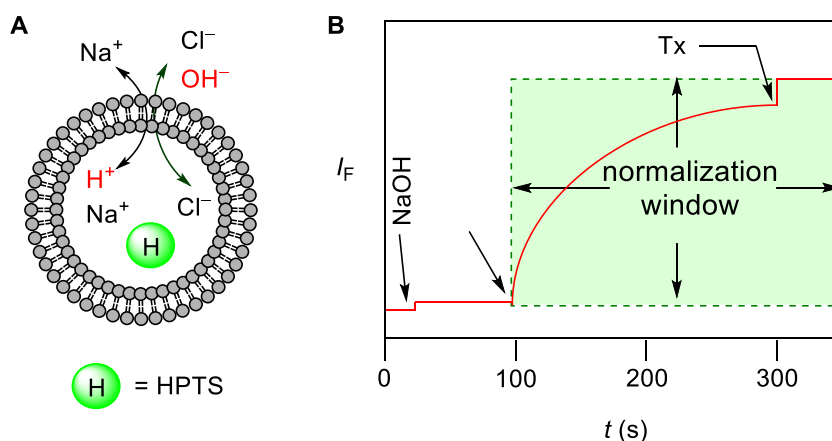


Figure 2.9 Representative ion transport assay across EYPC-LUVs containing HPTS (A), and illustration of ion transport kinetics showing normalization window (B).

For data analysis and comparison, the time axis (X-axis) was normalized to the point of transporter addition as $t = 0$ s (i.e., $t = 100$ s of the original data was normalized to $t = 0$ s). The emission intensities data were adjusted to normalized emission intensities I_F using Equation S1:

$$I_F = (I_t - I_0) / (I_\infty - I_0) \quad \text{Equation S1}$$

Where, $I_0 = I_t$ at transporter addition, $I_\infty = I_t$ at saturation after lysis with Triton X-100 (i.e., at $t = 100$ s after normalizing the X-axis).

Effective concentrations EC_{50} and Hill coefficients n were determined by plotting the fractional emission intensities Y as a function of the activator concentration c and fitting them to the Hill equation (Equation S2):

$$Y = Y_{\infty} + (Y_0 - Y_{\infty}) / [1 + (c / EC_{50})^n] \quad \text{Equation S2}$$

Where, Y_0 = fractional emission intensity without transporter, Y_{∞} = fractional emission intensity with excess transporter concentration, c = concentration of the transporter.

B. Ion Selectivity Studies Across EYPC-LUVs⊃HPTS

Preparation of EYPC-LUVs⊃HPTS: Vesicles were prepared according to the same procedure as stated above.

Anion Selectivity Assay: In a clean and dry fluorescence cuvette, 1975 μL of HEPES buffer (10 mM HEPES, 100 mM NaA, pH = 7.0; where, $A^- = \text{Cl}^-, \text{Br}^-, \text{I}^-, \text{and OAc}^-$). 25 μL of above prepared EYPC-LUVs⊃HPTS was added to this solution. The cuvette was kept in the fluorescence instrument equipped with a magnetic stirrer (at $t = 0$ s). The HPTS fluorescence emission intensity, I_F was observed at $\lambda_{\text{em}} = 510$ nm ($\lambda_{\text{ex}} = 450$ nm) with time. The pH gradient ($\Delta\text{pH} \sim 0.8$) between the intra and extra vesicular system was created by adding 20 μL of 0.5 M NaOH to the cuvette at $t = 20$ s. Transporter compounds were added at $t = 100$ s and of 10% Triton X-100 (25 μL) was added at $t = 300$ s to lyse vesicles for the complete destruction of pH gradient.

Cation Selectivity Assay: In a clean and dry fluorescence cuvette, 1975 μL of HEPES buffer (10 mM HEPES, 100 mM MCl, pH = 7.0; where, $M^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+$ and Cs^+). 25 μL of above prepared EYPC-LUVs⊃HPTS was added to this solution. The cuvette was kept in the fluorescence instrument equipped with a magnetic stirrer (at $t = 0$ s). The HPTS fluorescence emission intensity, I_F was observed at $\lambda_{\text{em}} = 510$ nm ($\lambda_{\text{ex}} = 450$ nm) with time. 20 μL of 0.5 M NaOH was added to the cuvette at $t = 20$ s to create the pH gradient ($\Delta\text{pH} = 0.8$) between the intra and extra vesicular system. Channel forming compounds were added at $t = 100$ s and 10% Triton X-100 (25 μL) was added at $t = 300$ s to lyse vesicles for complete destruction of the pH gradient.

C. Determination of Chloride Ion efflux by ion selective electrode (ISE):

Buffer solution prepared by adding 5 mM KH_2PO_4 (5 mM) and KCl (300 mM) or K-Gluconate (300 mM) to milli-Q water. pH was adjusted to 7.0 by adding the KOH.

Preparation of EYPC-LUVs for Chloride Efflux Assay: In a clean and dry small round-bottomed flask 1 mL of egg yolk phosphatidylcholine (EYPC, 25 mg/mL in CHCl_3) was dried by purging nitrogen gas with continuous rotation to form a transparent thin film of EYPC. The transparent film was kept under high vacuum for 4-5 h to remove all trace of CHCl_3 at room temperature. The resulting film was hydrated with 1 mL buffer (5 mM phosphate buffer, 300 mM KCl) solution for 1 hour with occasional vortexing at 10 minute intervals and subjected to freeze-thaw cycle (≥ 12 times). Extrusions were done 21 times (must be an odd number) by a Mini-extruder with a polycarbonate membrane of pore size 200 nm (Avanti). The extravesicular chloride ions were removed by dialysis (using MWCO 1 kD membrane) against K-Gluconate (300 mM) solution. The external dialysis solution was changed three times to ensure complete removal of Cl^- ions from extravesicular media.

Determination of Chloride Efflux by ISE: In a clean and dry glass tube, 1950 μL of K-Gluconate (300 mM) solution and 50 μL of vesicle suspension were taken and kept in slowly stirring condition by a magnetic stirrer (at $t = 0$ s). At 30 s, electrogenic K^+ carrier (Valinomycin) or a K^+/H^+ antiporter monensin was added. The chloride ion selective electrode was dipped into the vial and the concentration of chloride ion was monitored with time. Compound **1e** (in respective concentration) was added at $t = 50$ s and Triton X-100 (25 μL of 10% Tx-100) was added at 400 s to ensure the complete leakage of Cl^- ions (Fig. S2). The amount of extravesicular Cl^- before addition of compound **1e** has been normalized to 0% and after addition of Tx-100 has been normalized to 100%.

I. Theoretical Calculations.

To get an idea about the conformation of $[(\mathbf{1a})_2 + \text{Cl}^-]$, several initial geometries of the complex were generated using the CONFLEX 8^{15,16} software package using MMFF94s force field. The Boltzmann populations of five conformations obtained were **Conf-1** (Figure 2.10A) was most populated conformation i.e. 86.67% in addition to four minor conformations having the population of 4.59%, 3.99%, 2.68% and 1.84%, respectively (Figure 2.10B-E). Subsequently, **Conf-1** was optimized by Gaussian 09 program package using B3LYP functional and 6-311++G(d,p) basis set. The optimized structure of the $[(\mathbf{1a})_2 + \text{Cl}^-]$ was further modified by replacing the Br atoms by $-\text{CF}_3$ group and resulted

$[(1\mathbf{e})_2 + \text{Cl}^-]$ complex was again optimized by Gaussian 09 program package using B3LYP functional and 6-311++G(d,p) basis set.

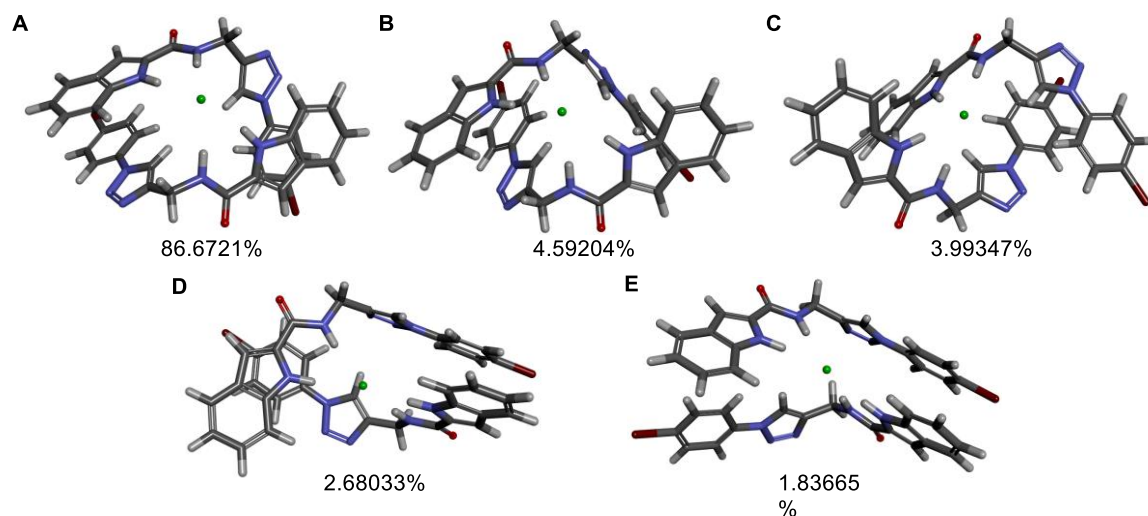


Figure 2.10 Initial geometries of **Conf-1** (A) and **Conf-2** (B) for $[(1\mathbf{a})_2 + \text{Cl}^-]$, optimized by CONFLEX 8 software using MMFF94s force field. The Boltzmann populations of conformations **Conf-1** (A) and **Conf-2** (B), **Conf-3** (C), **Conf-4** (D), and **Conf-5** (E) are also provided.

Table S1. Atomic coordinates of $[(1\mathbf{a})_2 + \text{Cl}^-]$ after geometry optimization by Gaussian 09 program using B3LYP functional and 6-311++G(d,p) basis set.

Charge = -1 Multiplicity = 1				
Atom #	Atom Type	x	y	z
1	C	4.987074	4.833534	-2.466963
2	C	3.884267	4.348267	-1.781395
3	C	4.121461	3.502327	-0.690754
4	C	5.43924	3.139382	-0.288147
5	C	6.536201	3.651075	-1.00607
6	C	6.302351	4.48916	-2.083569
7	N	3.236818	2.885806	0.156752
8	C	3.948253	2.134628	1.081471
9	C	5.297366	2.271665	0.835282
10	C	3.359087	1.324535	2.17709
11	O	4.087258	0.655816	2.909703
12	N	2.000713	1.359429	2.321157
13	C	1.338839	0.704028	3.442432
14	C	0.319576	-0.327579	3.04863
15	C	-0.659301	-0.301176	2.08219
16	N	-1.318104	-1.485197	2.22857
17	N	-0.770628	-2.202927	3.243007
18	N	0.209861	-1.50627	3.731258

19	C	-2.435918	-1.992776	1.508096
20	C	-3.223843	-2.990173	2.086667
21	C	-4.321835	-3.489015	1.395298
22	C	-4.631625	-2.977069	0.13828
23	C	-3.856619	-1.982655	-0.446729
24	C	-2.746523	-1.497352	0.242205
25	Br	-6.161255	-3.661176	-0.799586
26	H	4.83771	5.49106	-3.316541
27	H	2.874525	4.612156	-2.074037
28	H	7.549194	3.389017	-0.719191
29	H	7.138757	4.888984	-2.646612
30	H	2.220657	2.940643	0.066383
31	H	6.072852	1.795352	1.413041
32	H	1.425235	1.930208	1.706591
33	H	2.109622	0.216971	4.038242
34	H	0.863826	1.467148	4.071061
35	H	-0.918618	0.448678	1.353136
36	H	-2.969958	-3.3673	3.067607
37	H	-4.934834	-4.262911	1.838722
38	H	-4.101651	-1.580115	-1.421676
39	H	-2.129541	-0.740826	-0.224919
40	C	-4.985653	4.833748	2.467328
41	C	-3.88301	4.348414	1.781542
42	C	-4.120472	3.502558	0.690896
43	C	-5.438353	3.139765	0.28849
44	C	-6.535142	3.65152	1.006632
45	C	-6.301028	4.48952	2.08414
46	N	-3.236032	2.885989	-0.156788
47	C	-3.9477	2.134953	-1.081453
48	C	-5.296756	2.272126	-0.835034
49	C	-3.358826	1.324883	-2.177252
50	O	-4.087194	0.656145	-2.909655
51	N	-2.000491	1.359689	-2.321602
52	C	-1.338829	0.704043	-3.442856
53	C	-0.319712	-0.327689	-3.048987
54	C	0.65896	-0.301526	-2.082348
55	N	1.317753	-1.485539	-2.228837
56	N	0.770405	-2.203095	-3.243469
57	N	-0.209906	-1.506261	-3.731827
58	C	2.435402	-1.993293	-1.508226
59	C	3.223049	-2.991049	-2.086559
60	C	4.320877	-3.490059	-1.395052
61	C	4.630778	-2.977952	-0.138128
62	C	3.856046	-1.983189	0.446649
63	C	2.746125	-1.497689	-0.242432
64	Br	6.16016	-3.662343	0.799934
65	H	-4.83608	5.491215	3.316915
66	H	-2.873194	4.612196	2.074027
67	H	-7.548209	3.389583	0.719905

68	H	-7.137299	4.889394	2.647348
69	H	-2.219857	2.94073	-0.066608
70	H	-6.072387	1.795942	-1.412704
71	H	-1.424831	1.93037	-1.707115
72	H	-2.109734	0.217075	-4.03858
73	H	-0.863705	1.467003	-4.071594
74	H	0.918184	0.448236	-1.353163
75	H	2.969086	-3.368313	-3.067425
76	H	4.933661	-4.26423	-1.838296
77	H	4.101153	-1.580527	1.421529
78	H	2.129382	-0.740867	0.224523
79	Cl	0.000488	2.739995	-0.000521

Table S2. Atomic coordinates of [(**1e**)₂+Cl[−]] after geometry optimization by Gaussian 09 program using B3LYP functional and 6-311++G(d,p) basis set.

Charge = −1		Multiplicity = 1		
Atom #	Atom Type	x	y	z
1	C	-5.0338	4.7932	2.4296
2	C	-3.9191	4.3236	1.7526
3	C	-4.1359	3.4699	0.6637
4	C	-5.445	3.0836	0.255
5	C	-6.5547	3.5801	0.9641
6	C	-6.341	4.4259	2.0396
7	N	-3.2362	2.8654	-0.1767
8	C	-3.9299	2.0989	-1.1026
9	C	-5.2825	2.2143	-0.8644
10	C	-3.3217	1.2945	-2.1914
11	O	-4.0339	0.6054	-2.9208
12	N	-1.9637	1.3558	-2.3332
13	C	-1.2877	0.7057	-3.4483
14	C	-0.2697	-0.3247	-3.0477
15	C	0.6861	-0.3094	-2.0595
16	N	1.3575	-1.4871	-2.2124
17	N	0.8365	-2.1897	-3.2535
18	N	-0.1365	-1.4915	-3.749
19	C	2.461	-2.0035	-1.48
20	C	3.2265	-3.0315	-2.0399
21	C	4.3116	-3.5329	-1.3378
22	C	4.6475	-3.0079	-0.0869
23	C	3.8827	-1.9848	0.4693
24	C	2.7831	-1.4871	-0.2256
25	H	-4.9005	5.4563	3.2774
26	H	-2.9157	4.6056	2.0502
27	H	-7.5614	3.3004	0.6721
28	H	-7.1872	4.8142	2.596

29	H	-2.2217	2.9346	-0.0792
30	H	-6.047	1.7243	-1.4455
31	H	-1.4	1.938	-1.7184
32	H	-2.0502	0.218	-4.0545
33	H	-0.8073	1.4716	-4.0691
34	H	0.9229	0.4307	-1.3132
35	H	2.9628	-3.42	-3.0132
36	H	4.9095	-4.3249	-1.772
37	H	4.1361	-1.5629	1.4345
38	H	2.1852	-0.7048	0.2224
39	C	5.0331	4.7937	-2.4296
40	C	3.9185	4.324	-1.7525
41	C	4.1355	3.4703	-0.6637
42	C	5.4447	3.0841	-0.2551
43	C	6.5542	3.5808	-0.9643
44	C	6.3403	4.4266	-2.0397
45	N	3.2358	2.8656	0.1768
46	C	3.9297	2.0992	1.1025
47	C	5.2823	2.2147	0.8643
48	C	3.3217	1.2946	2.1914
49	O	4.034	0.6055	2.9206
50	N	1.9637	1.3559	2.3334
51	C	1.2879	0.7057	3.4485
52	C	0.2698	-0.3247	3.0478
53	C	-0.686	-0.3094	2.0597
54	N	-1.3573	-1.4871	2.2125
55	N	-0.8363	-2.1897	3.2535
56	N	0.1367	-1.4915	3.749
57	C	-2.4607	-2.0035	1.48
58	C	-3.2265	-3.0313	2.0401
59	C	-4.3115	-3.5327	1.3379
60	C	-4.6472	-3.0079	0.0868
61	C	-3.8822	-1.985	-0.4694
62	C	-2.7827	-1.4873	0.2254
63	H	4.8996	5.4569	-3.2774
64	H	2.915	4.6059	-2.05
65	H	7.561	3.3012	-0.6723
66	H	7.1864	4.8151	-2.5961
67	H	2.2214	2.9347	0.0794
68	H	6.0469	1.7248	1.4453
69	H	1.3999	1.9382	1.7187
70	H	2.0504	0.218	4.0545
71	H	0.8075	1.4715	4.0694
72	H	-0.9228	0.4308	1.3134
73	H	-2.963	-3.4196	3.0135
74	H	-4.9095	-4.3246	1.7721
75	H	-4.1354	-1.5633	-1.4348
76	H	-2.1846	-0.7052	-0.2225
77	Cl	-0.0002	2.7443	0.0001

78	C	-5.8111	-3.5909	-0.6613
79	C	5.8115	-3.5908	0.6612
80	F	-6.2631	-2.7858	-1.641
81	F	-6.8646	-3.8494	0.1594
82	F	-5.4979	-4.7803	-1.2463
83	F	6.2636	-2.7856	1.6407
84	F	6.8648	-3.8496	-0.1597
85	F	5.4982	-4.78	1.2465

2.6 NMR Spectra

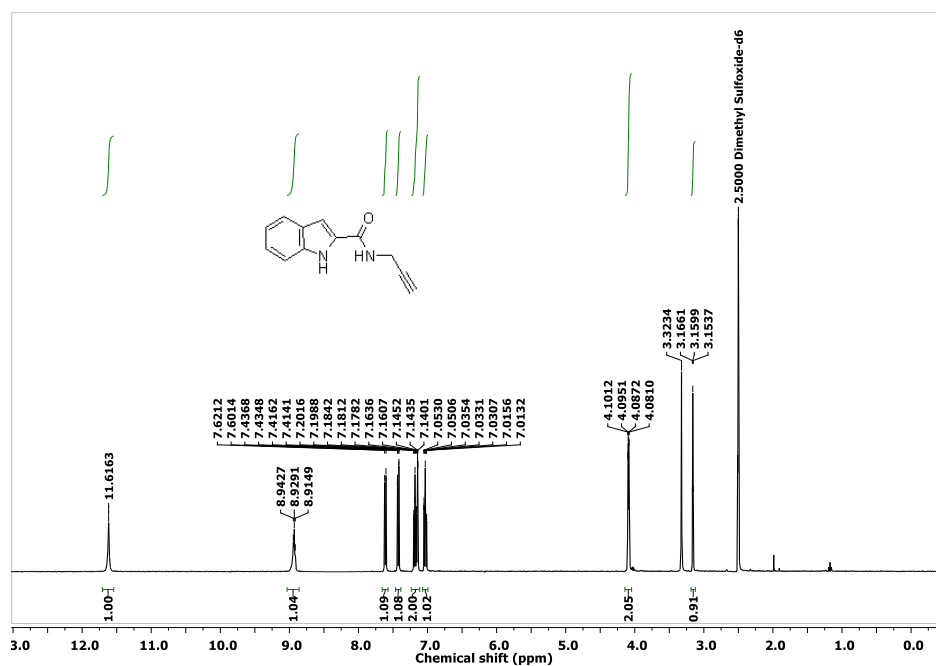


Figure 2.11 ¹H NMR spectrum of *N*-(prop-2-yn-1-yl)-1*H*-indole-2-carboxamide (3).

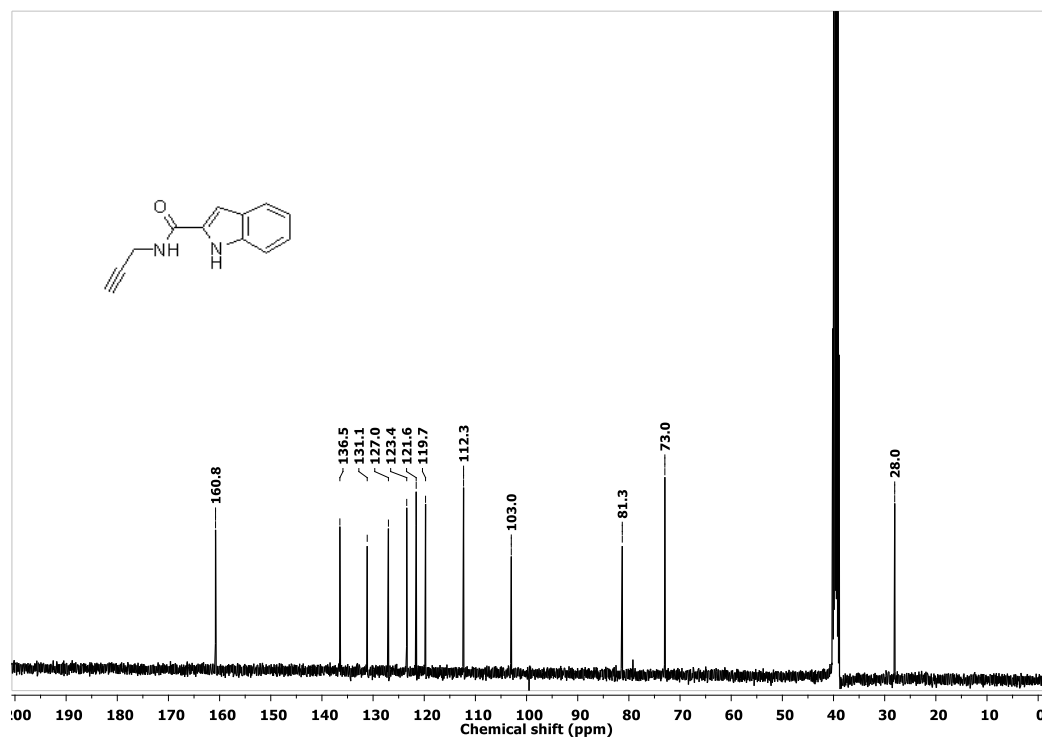


Figure 2.12 ¹³C NMR spectrum of *N*-(prop-2-yn-1-yl)-1*H*-indole-2-carboxamide (3).

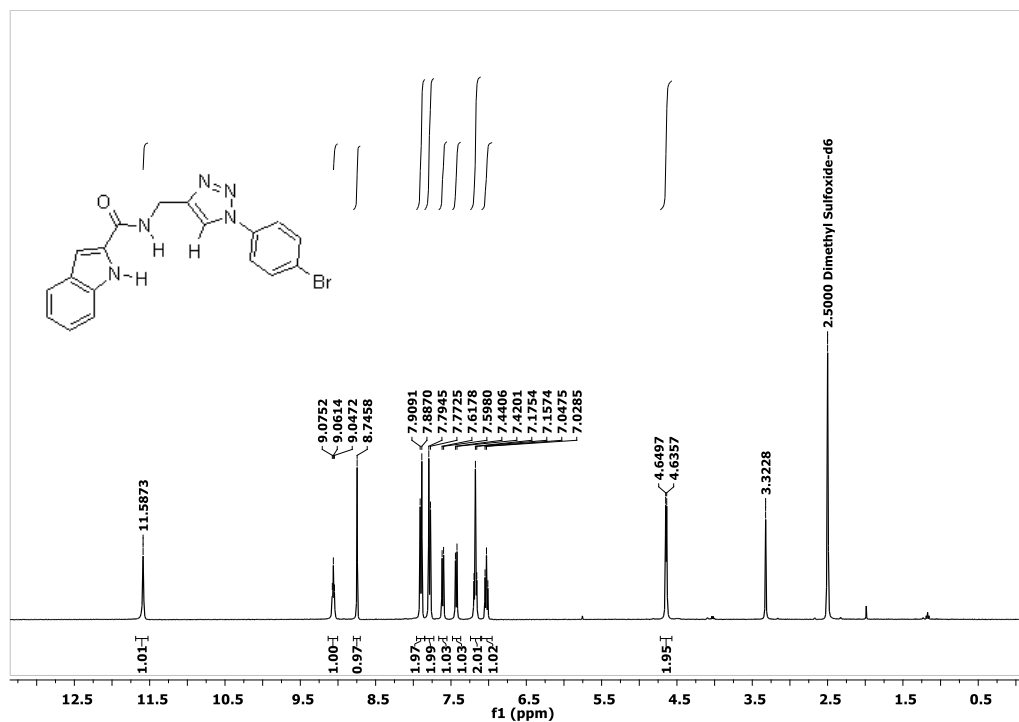


Figure 2.13 ¹H NMR spectrum of *N*-((1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1a**).

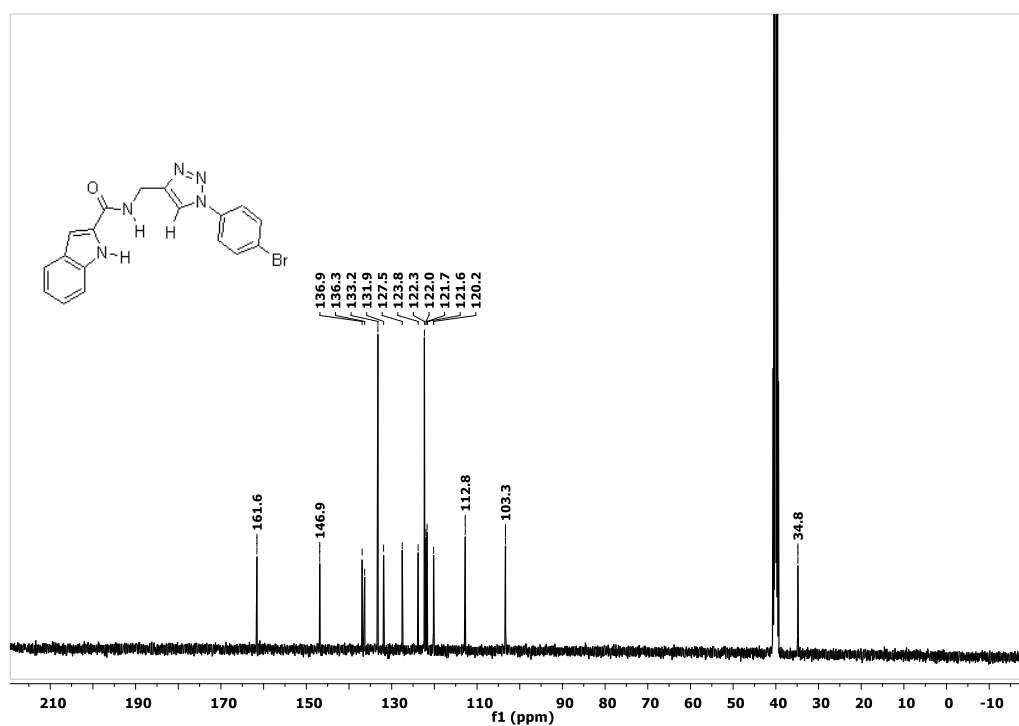


Figure 2.14 ¹³C NMR spectrum of *N*-((1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1a**).

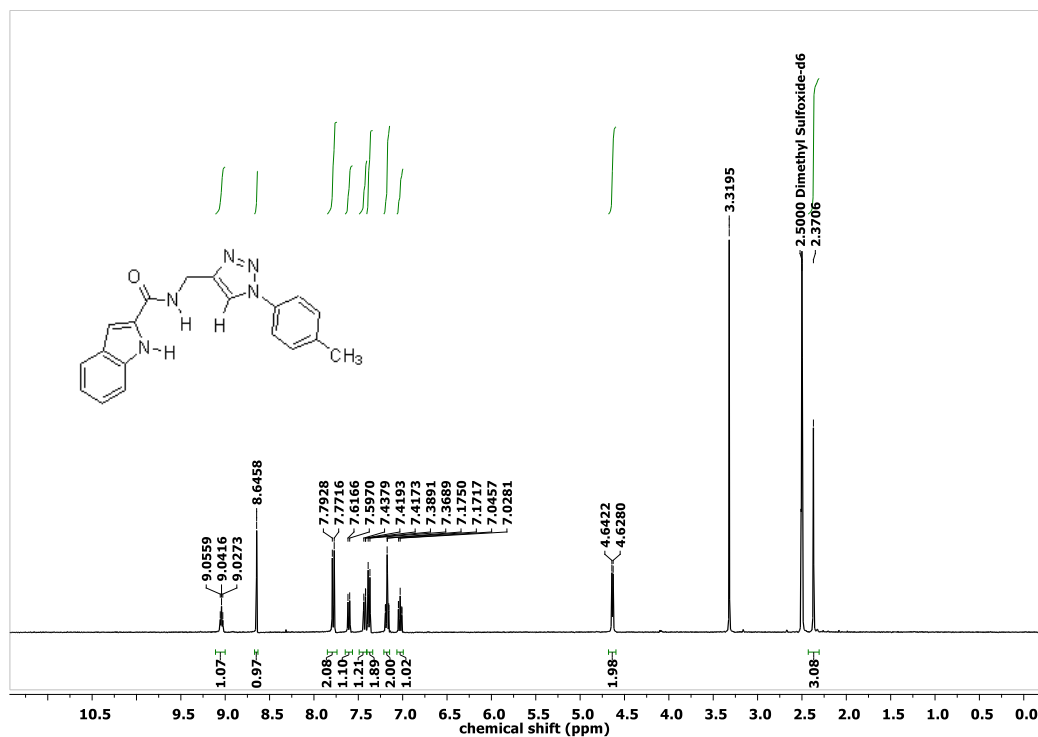


Figure 2.15 ¹H NMR spectrum of *N*-((1-(*p*-tolyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1b**).

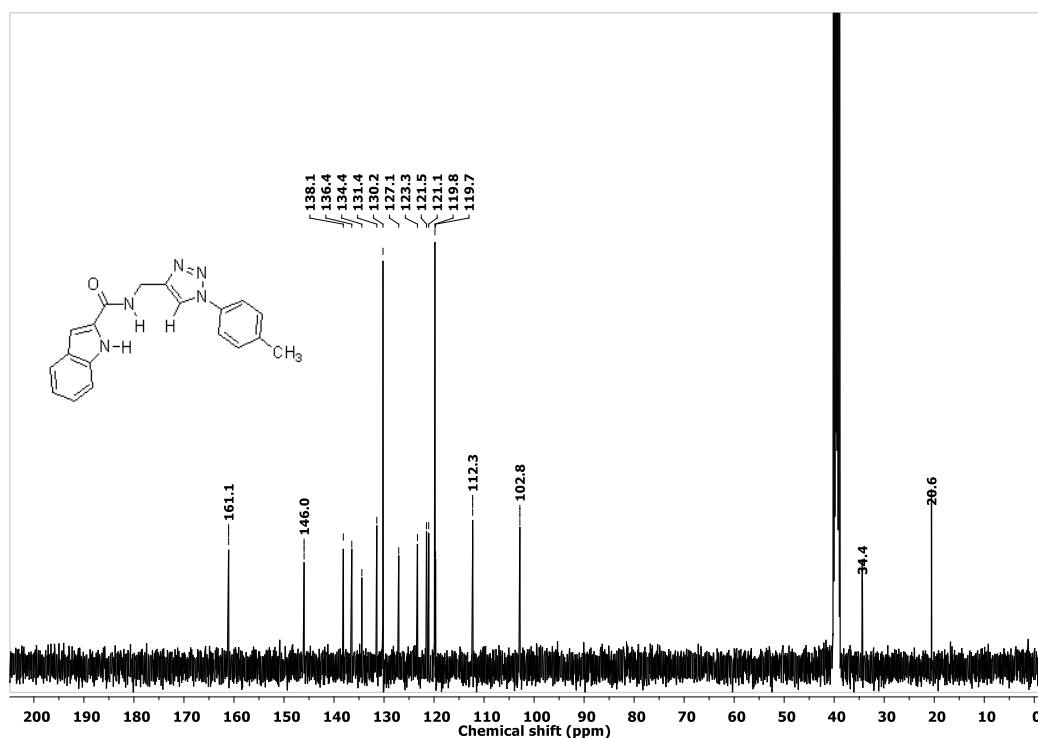


Figure 2.16 ¹³C NMR spectrum of *N*-((1-(*p*-tolyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1b**).

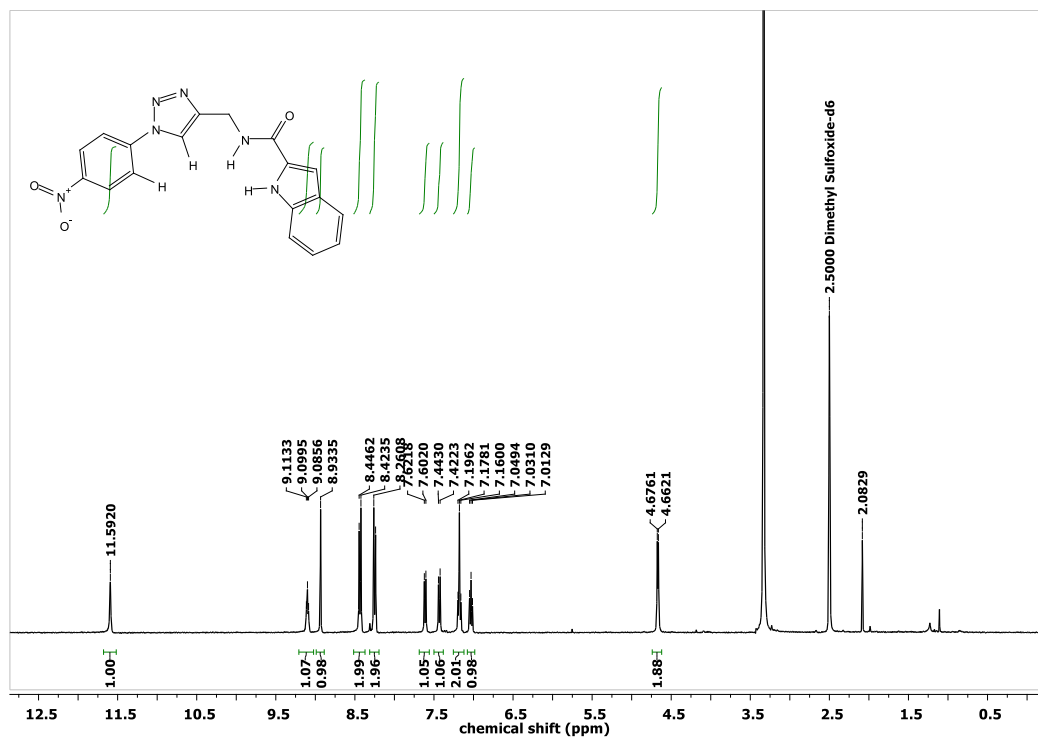


Figure 2.17 ¹H NMR spectrum of *N*-((1-(4-nitrophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1c**).

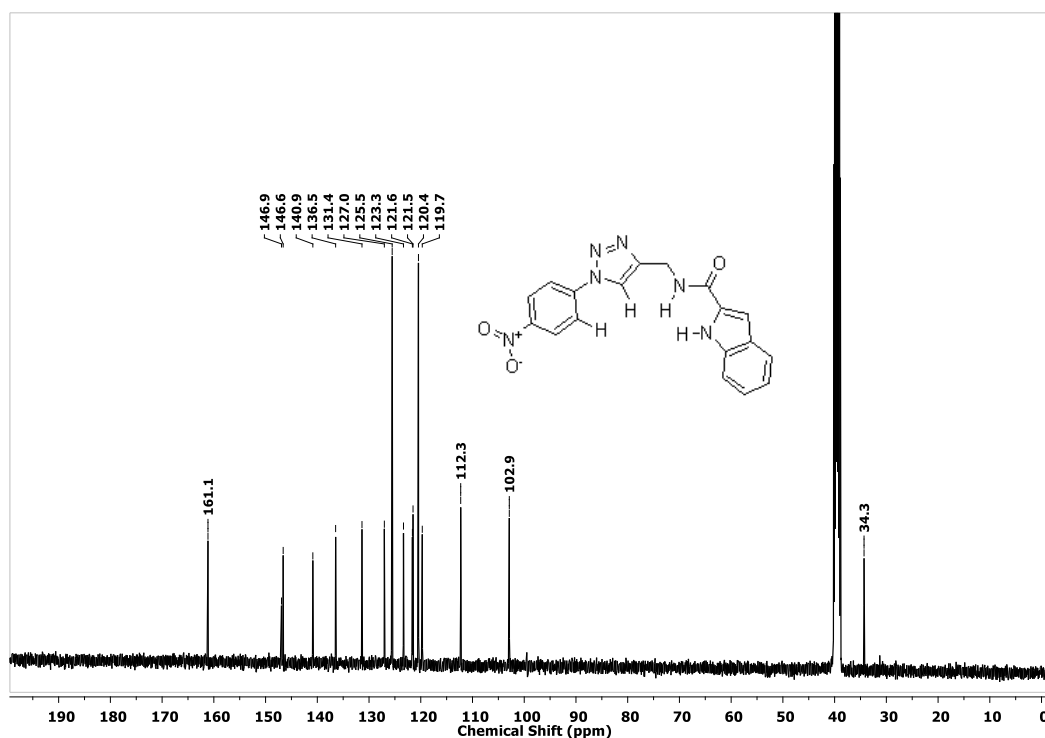


Figure 2.18 ¹³C NMR spectrum of *N*-((1-(4-nitrophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1c**).

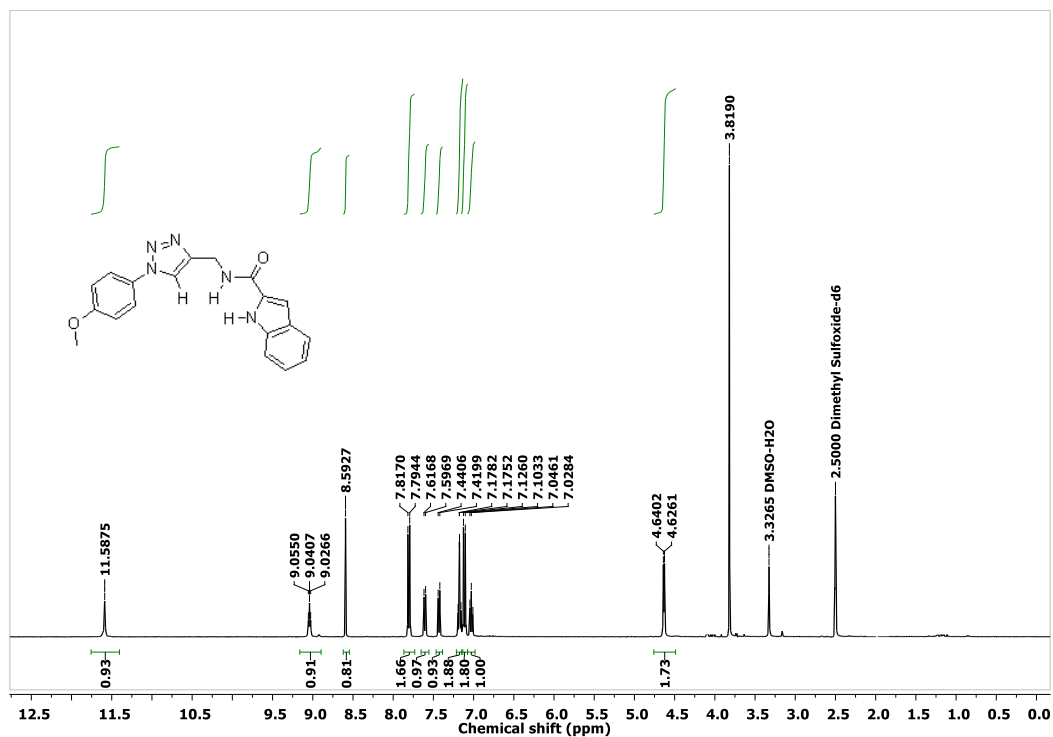


Figure 2.19 ^1H NMR spectrum of *N*-((1-(4-methoxyphenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1d**).

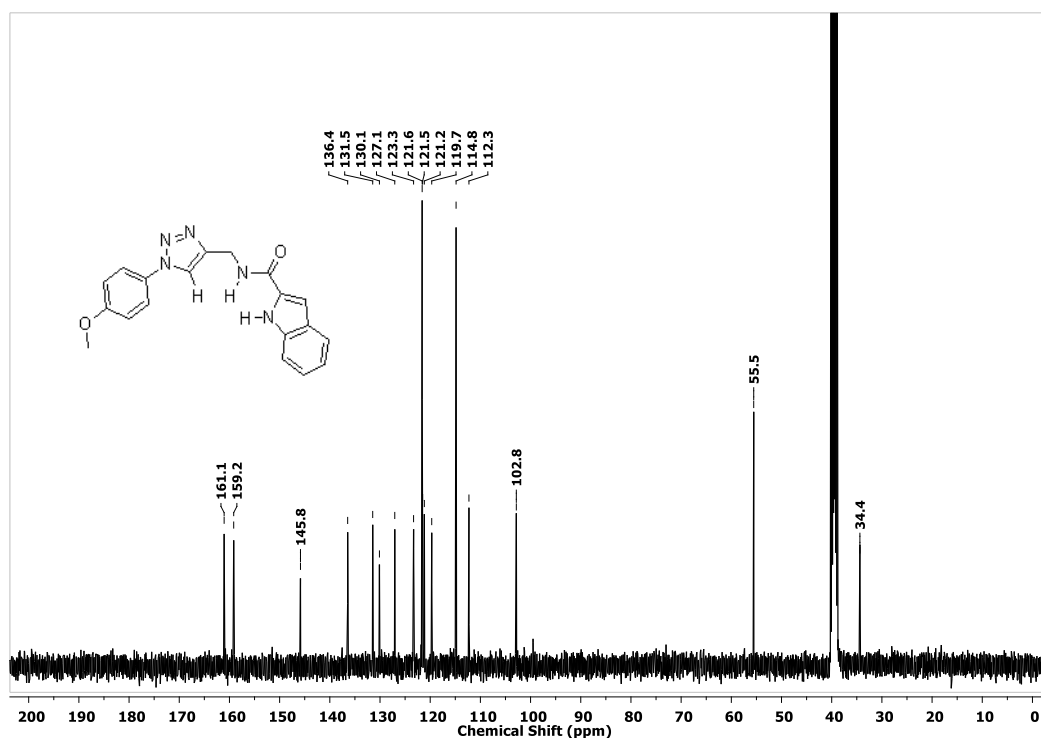


Figure 2.20 ^{13}C NMR spectrum of *N*-((1-(4-methoxyphenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1d**).

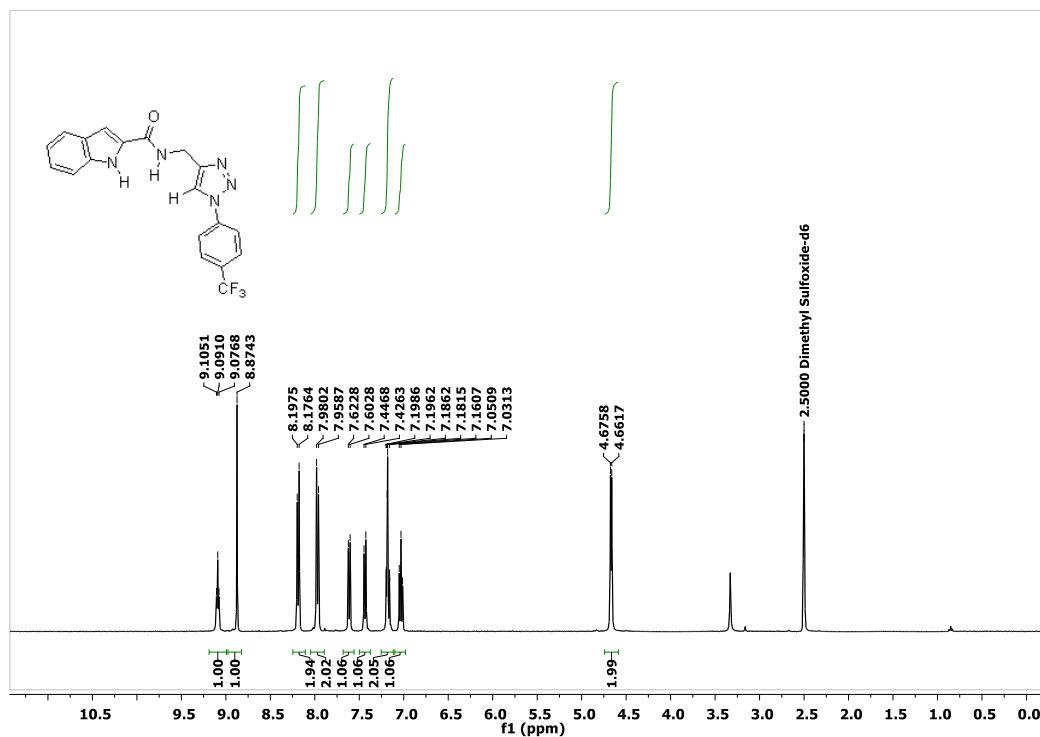


Figure 2.21 ¹H NMR spectrum of *N*-((1-(4-(trifluoromethyl)phenyl)-1H-1,2,3-triazol-4-yl)methyl)-1H-indole-2-carboxamide (**1e**).

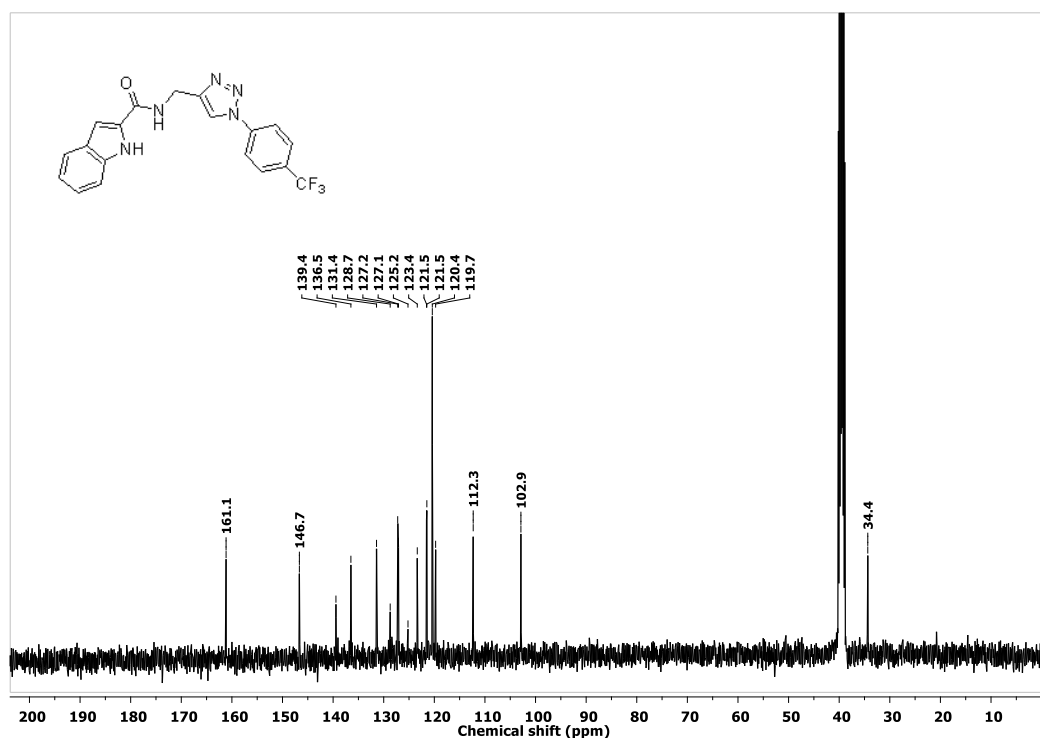


Figure 2.22 ¹³C NMR spectrum of *N*-((1-(4-(trifluoromethyl)phenyl)-1H-1,2,3-triazol-4-yl)methyl)-1H-indole-2-carboxamide (**1e**).

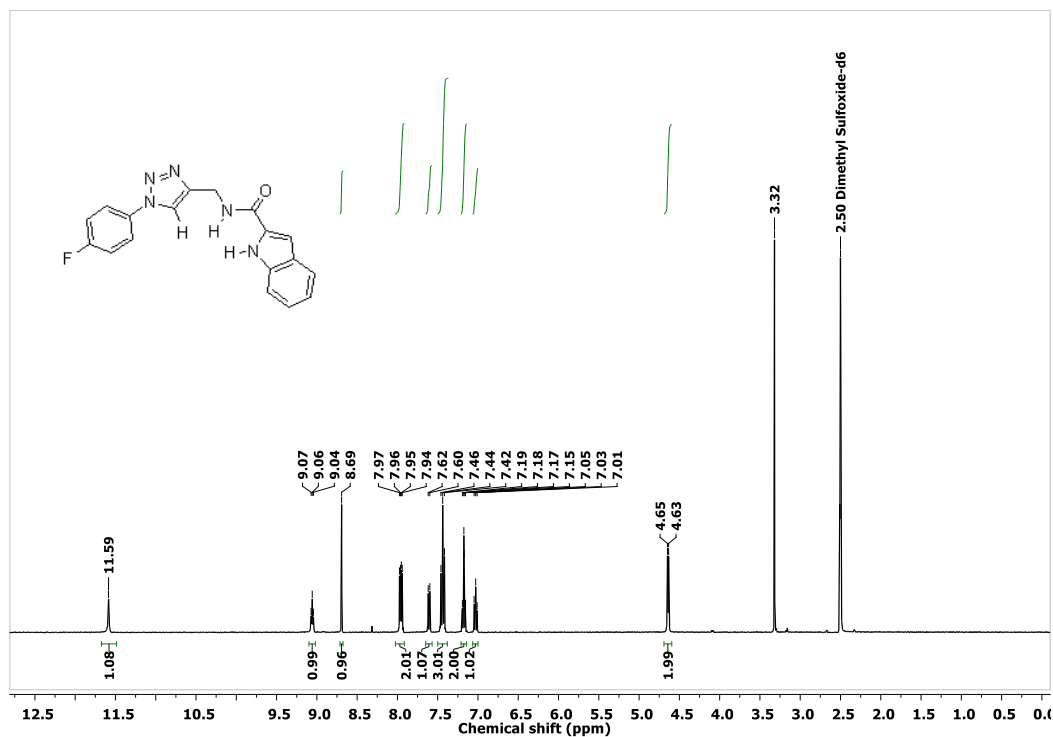


Figure 2.23 ¹H NMR spectrum of *N*-((1-(4-fluorophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1f**).

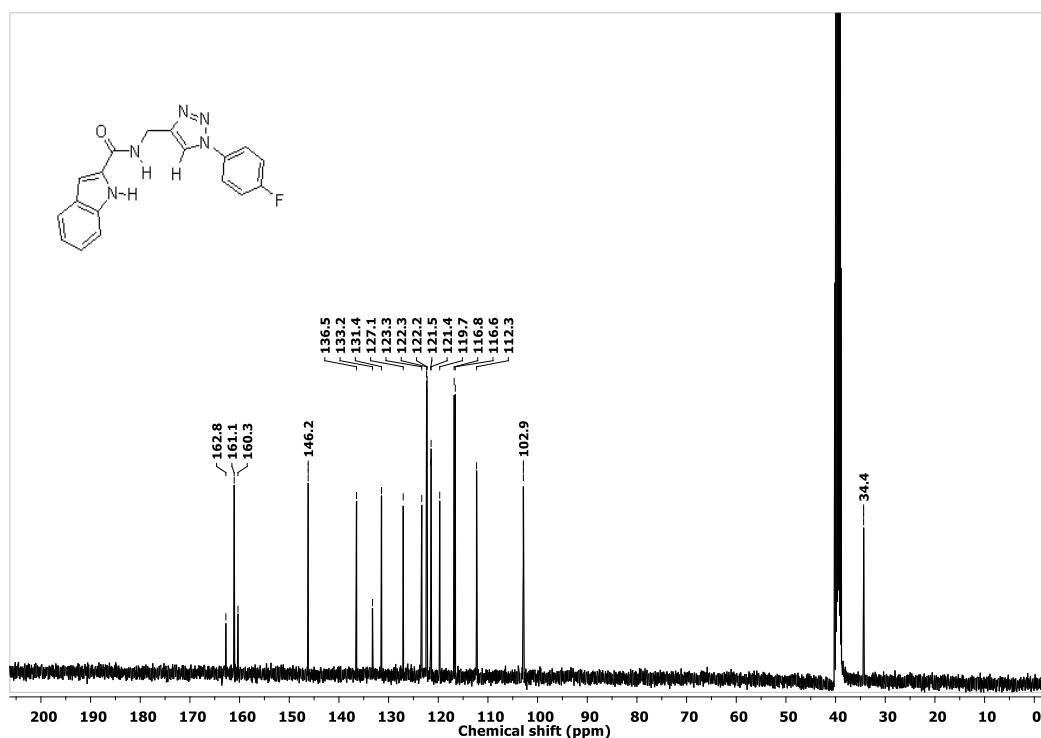


Figure 2.24 ¹³C NMR spectrum of *N*-((1-(4-fluorophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1f**).

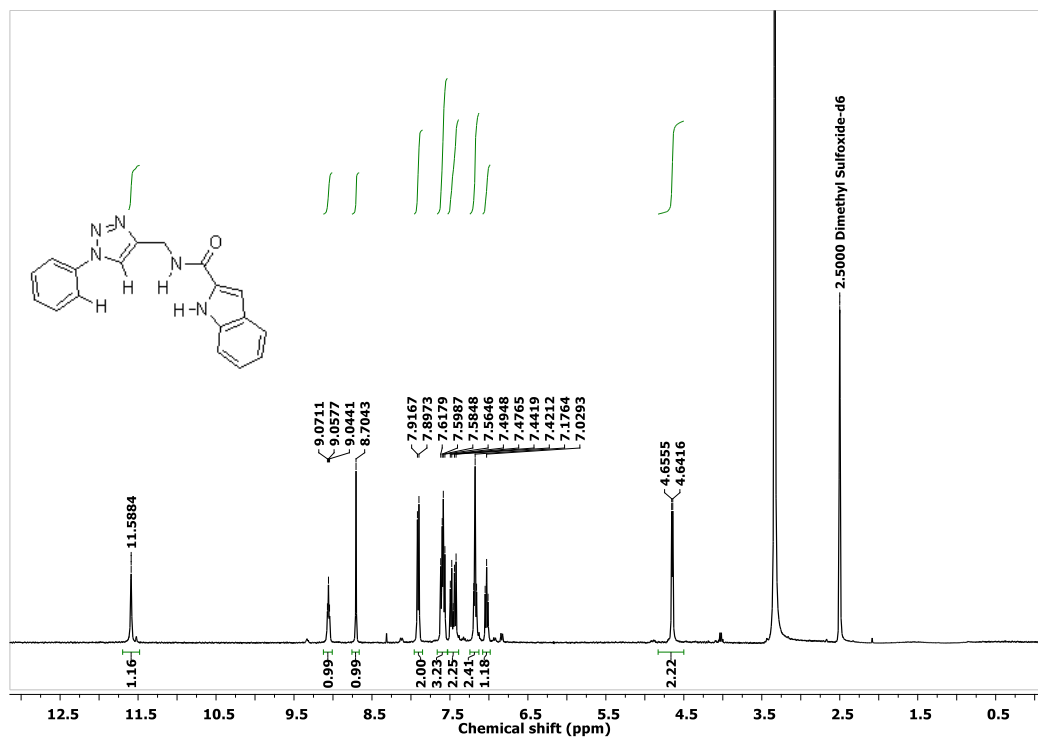


Figure 2.25 ¹H NMR spectrum of *N*-((1-phenyl-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (1g).

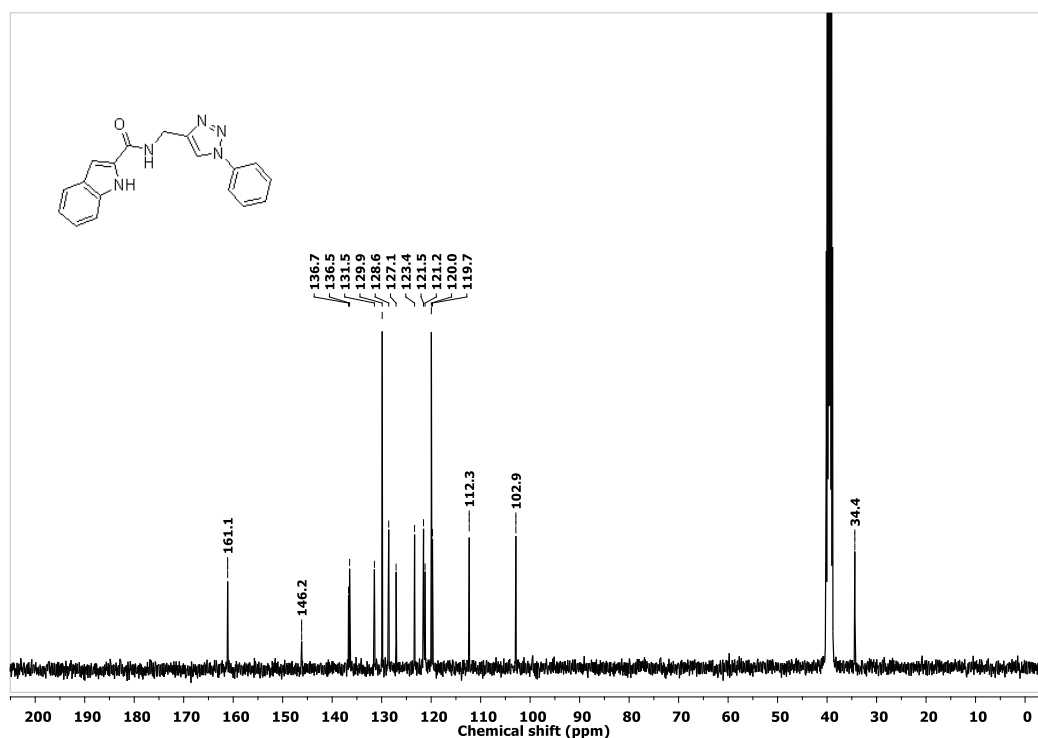


Figure 2.26 ¹³C NMR spectrum of *N*-((1-phenyl-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (1g).

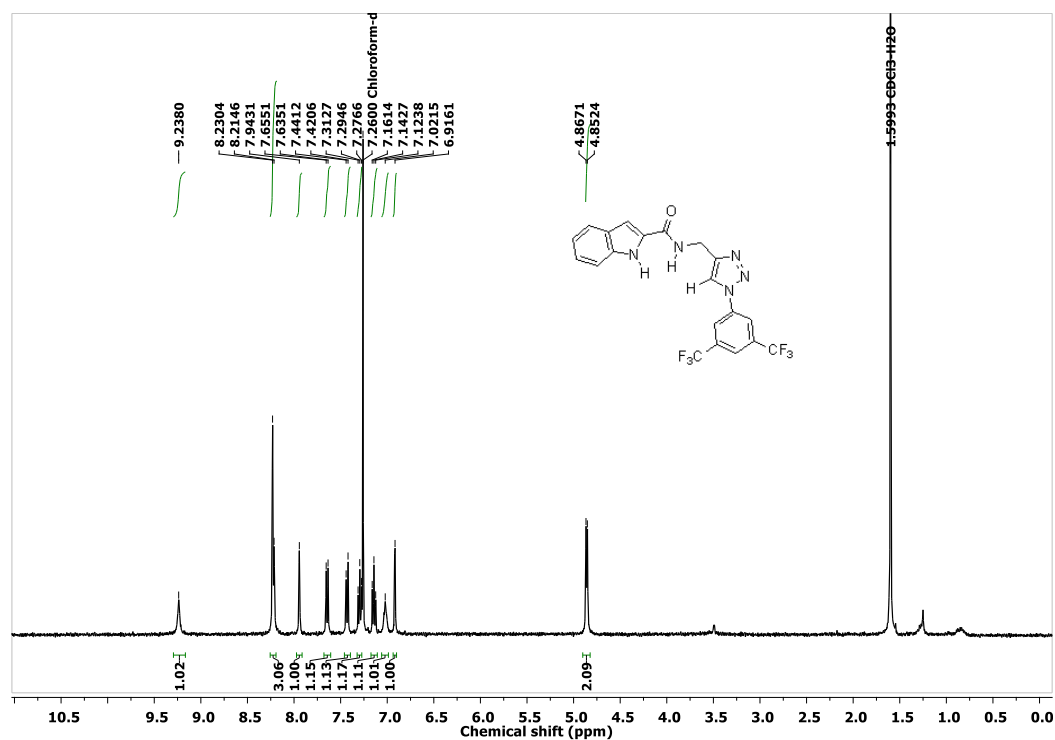


Figure 2.27 ¹H NMR spectrum of *N*-((1-(3,5-bis(trifluoromethyl)phenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1h**).

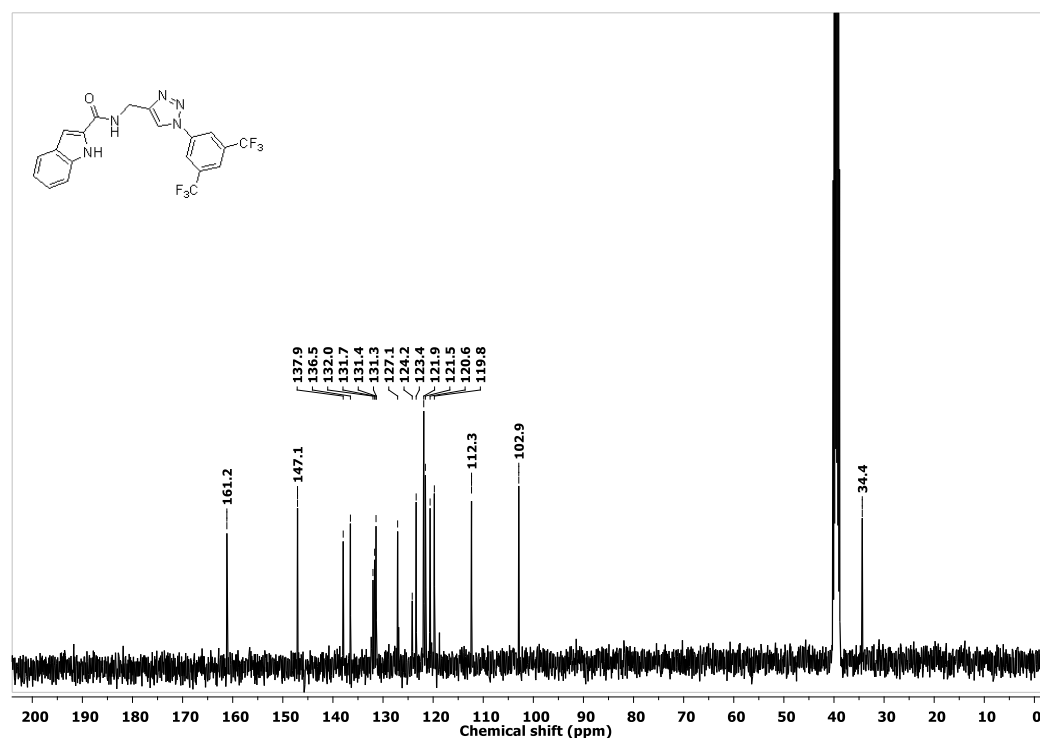


Figure 2.28 ¹³C NMR spectrum of *N*-((1-(3,5-bis(trifluoromethyl)phenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1h**).

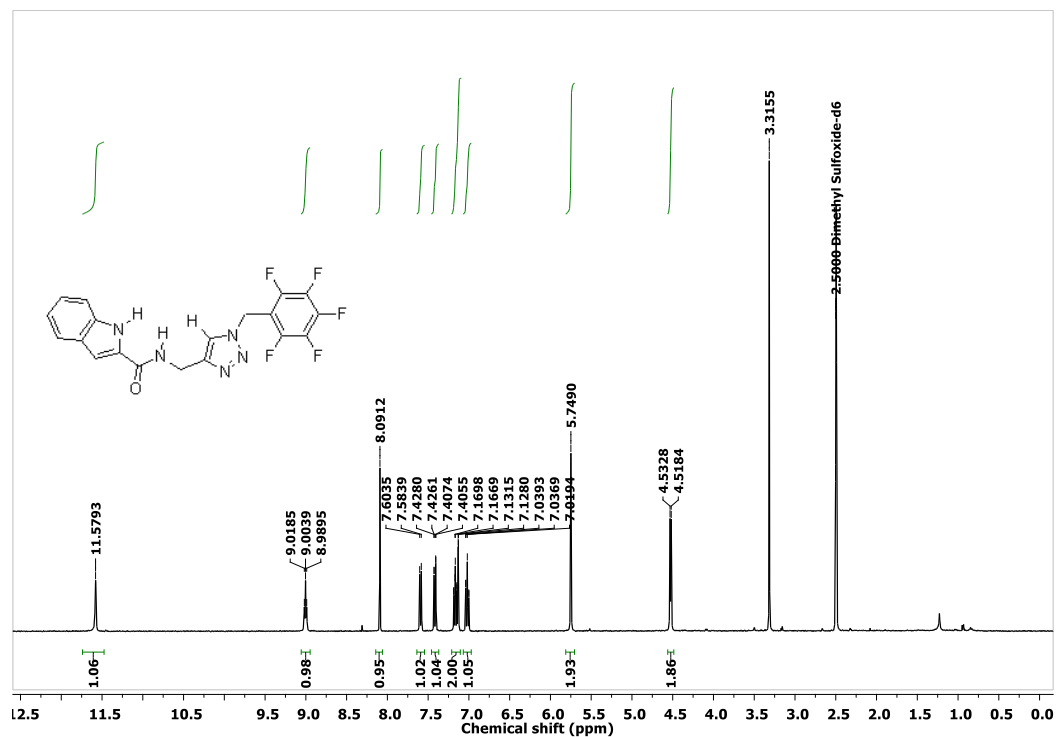


Figure 2.29 ¹H NMR spectrum of *N*-((1-((perfluorophenyl)methyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1i**).

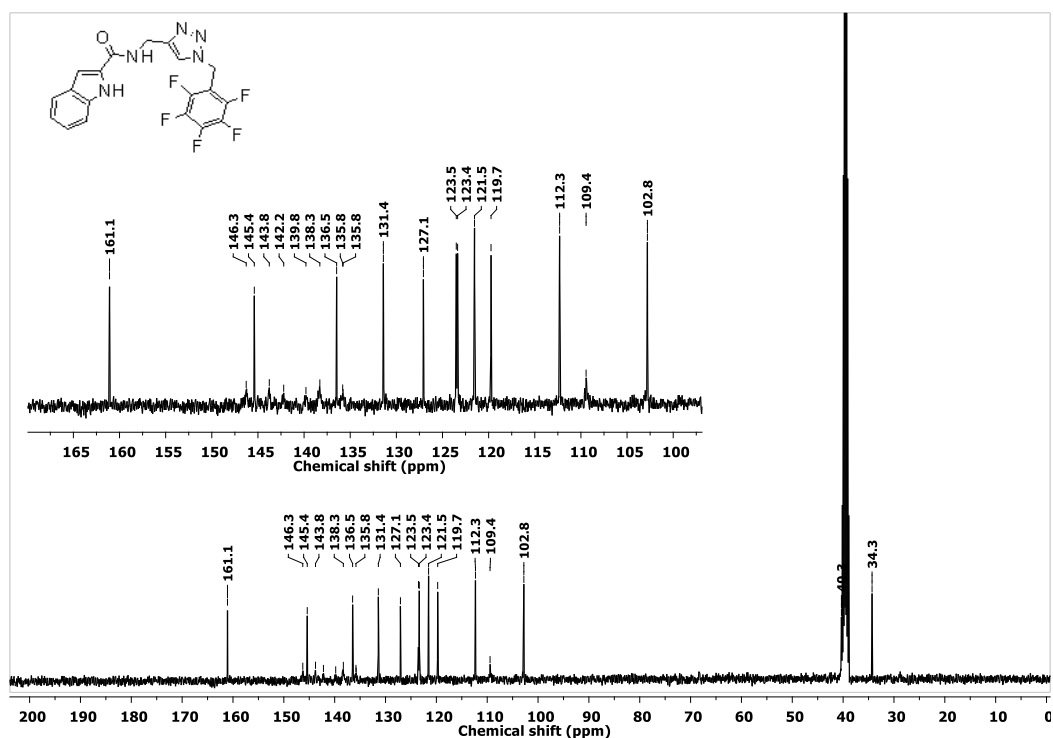


Figure 2.30 ¹³C spectrum of *N*-((1-((perfluorophenyl)methyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1i**).

2.7 HRMS Spectra of Final Compounds:

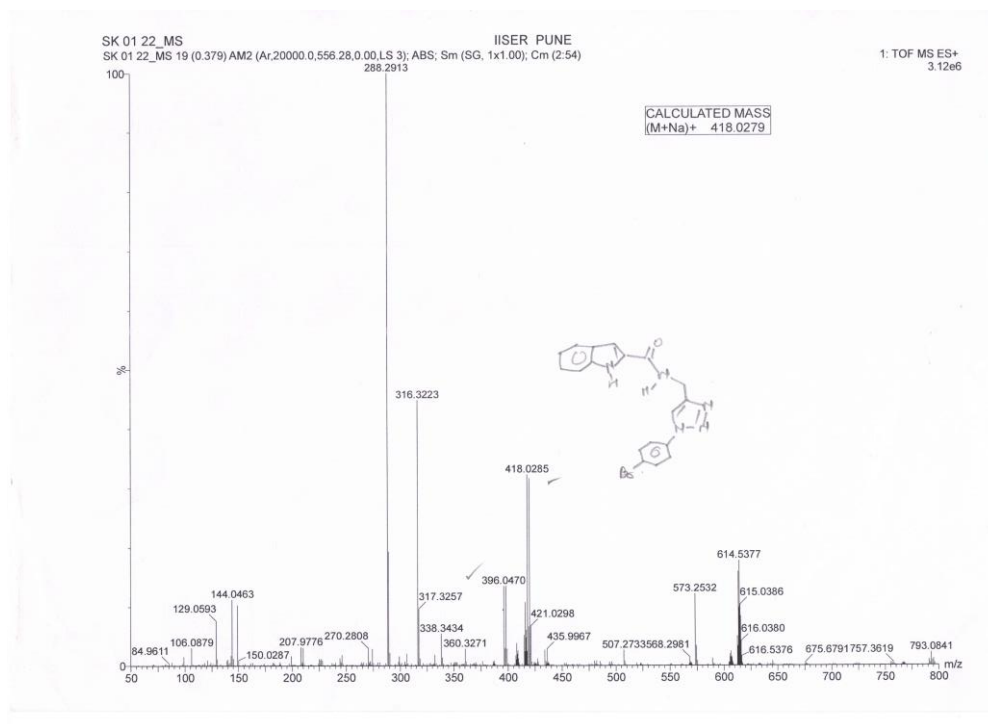


Figure 2.31 HRMS spectrum of *N*-((1-(4-bromophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1a**).

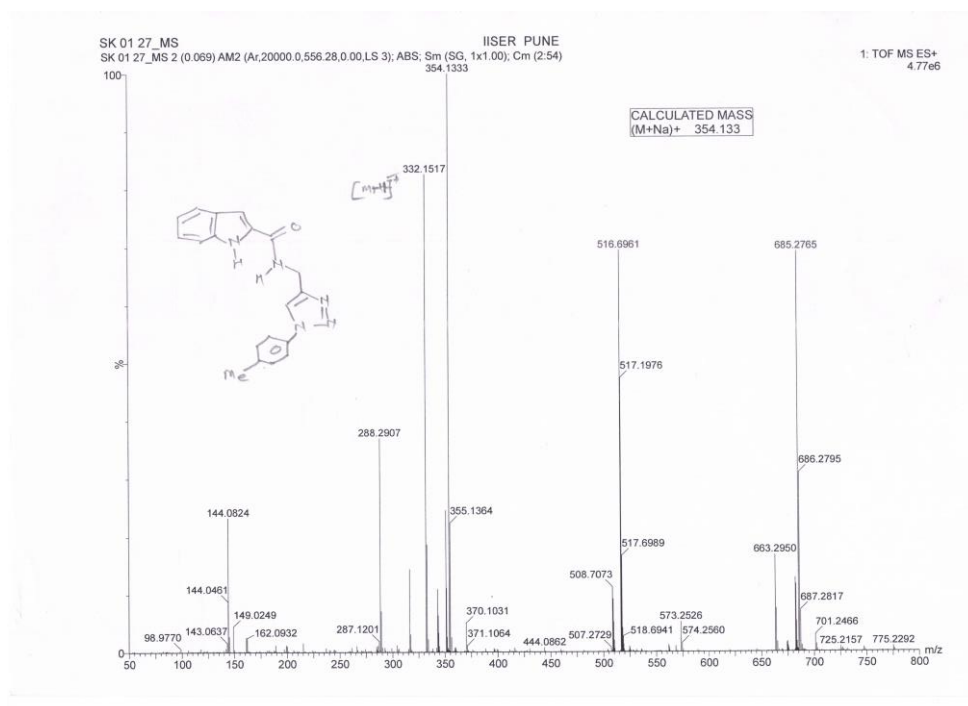


Figure 2.32 HRMS spectrum of *N*-((1-(*p*-tolyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1b**).

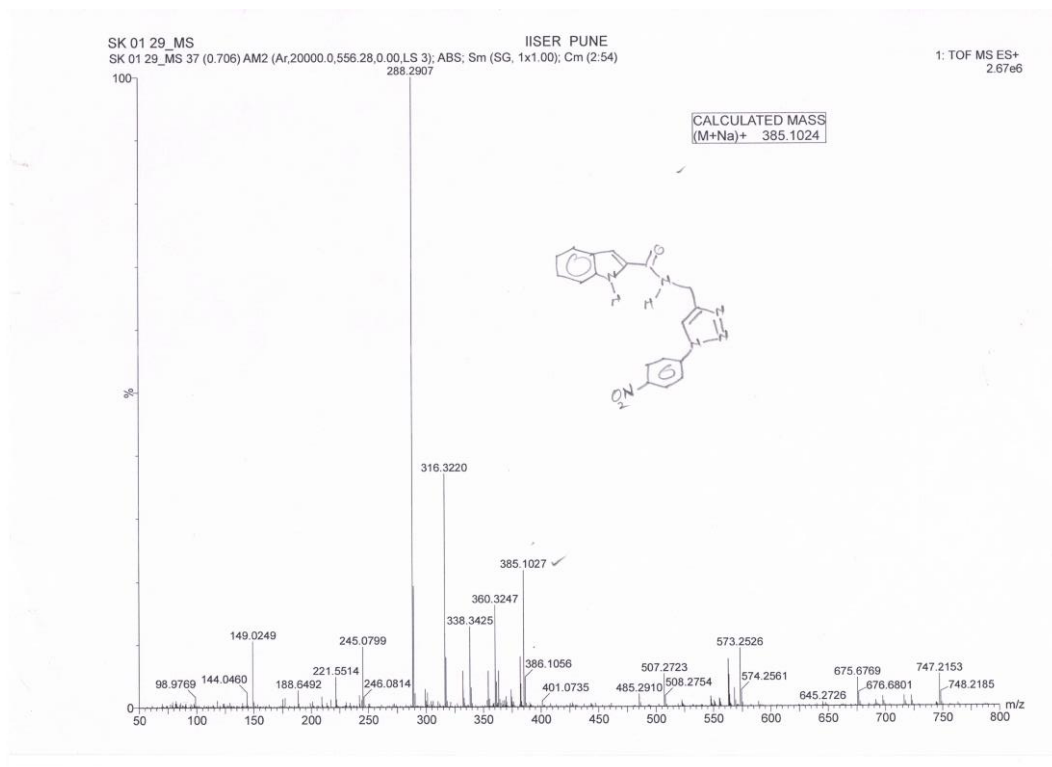


Figure 2.33 HRMS spectrum of *N*-((1-(4-nitrophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1c**).

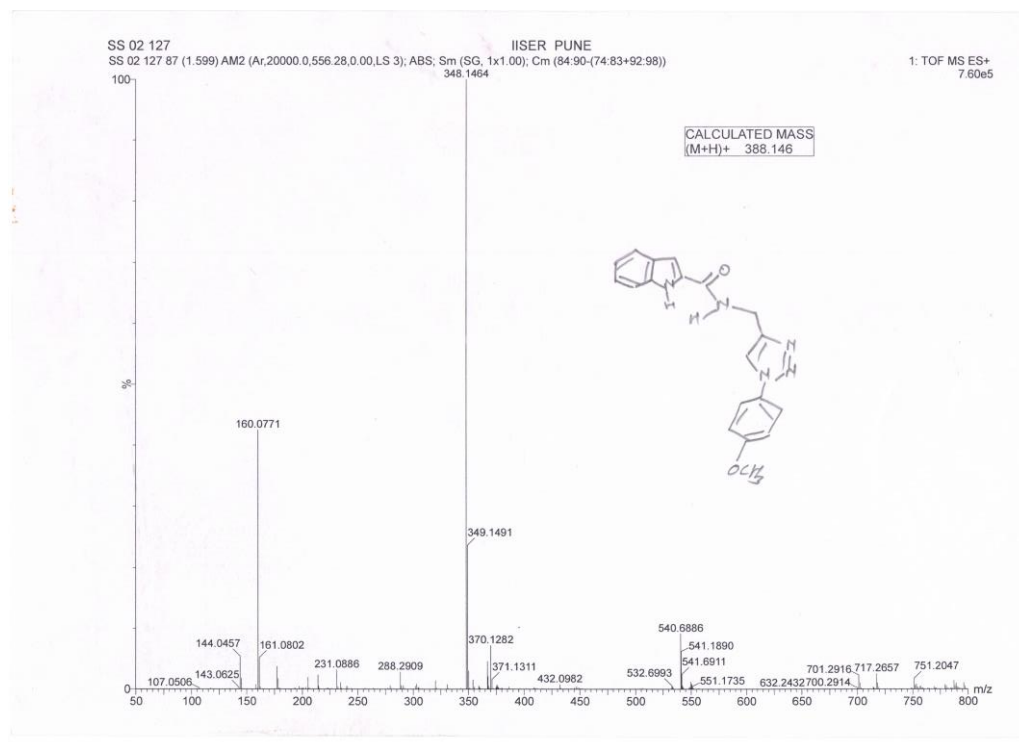


Figure 2.34 HRMS spectrum of *N*-((1-(4-methoxyphenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1d**).

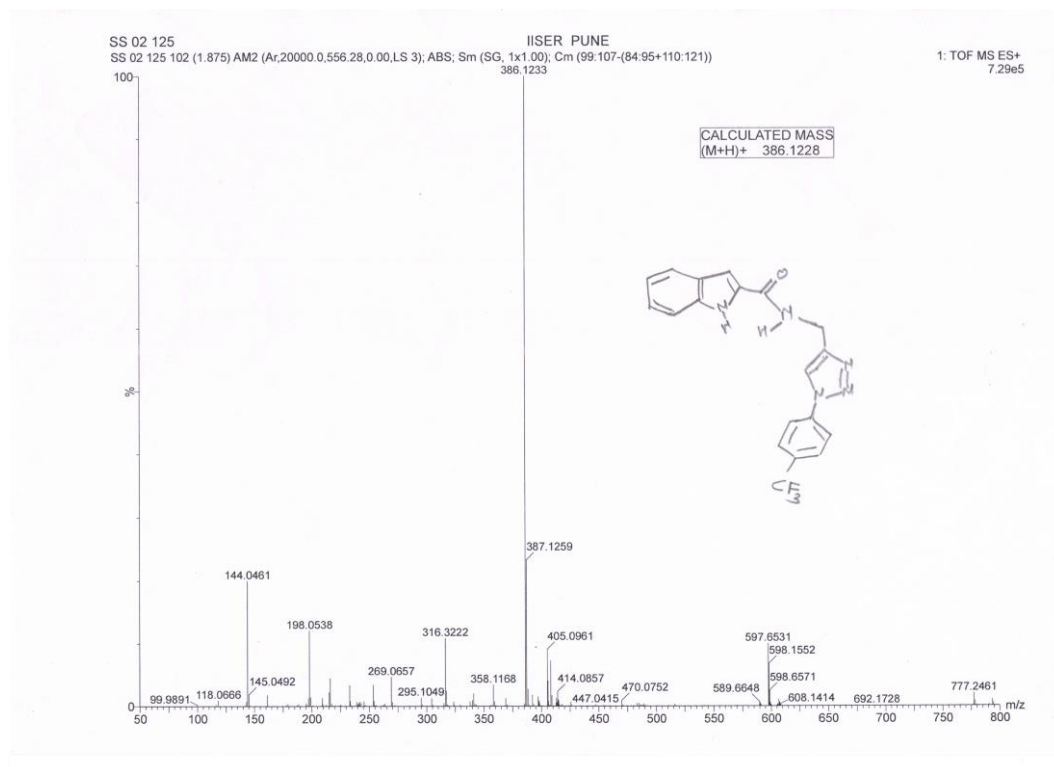


Figure 2.35 HRMS spectrum of *N*-((1-(4-(trifluoromethyl)phenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1e**).

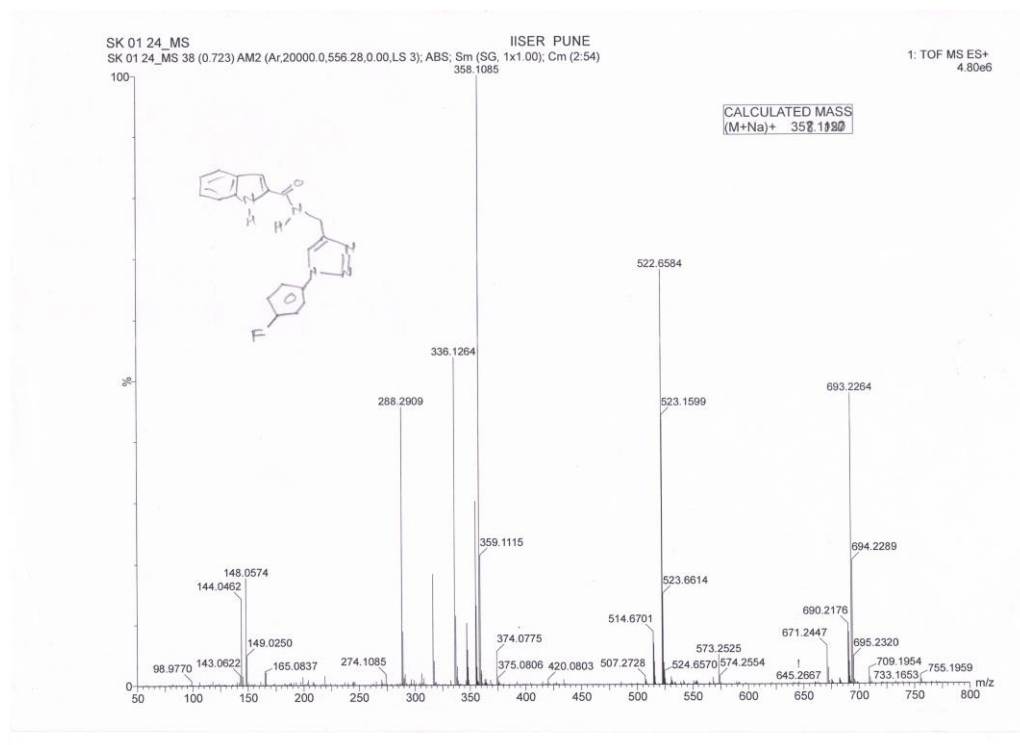


Figure 2.36 HRMS spectrum of *N*-((1-(4-fluorophenyl)-1*H*-1,2,3-triazol-4-yl)methyl)-1*H*-indole-2-carboxamide (**1f**).

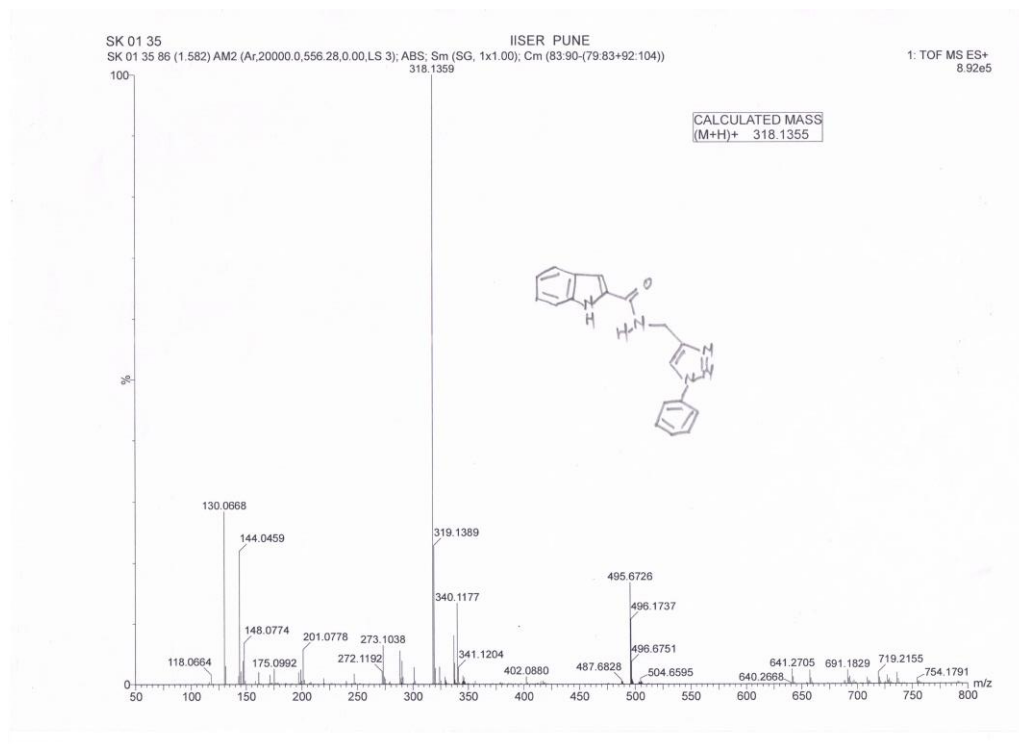


Figure 2.38 HRMS spectrum of *N*-((1-phenyl-1H-1,2,3-triazol-4-yl)methyl)-1H-indole-2-carboxamide (**1g**).

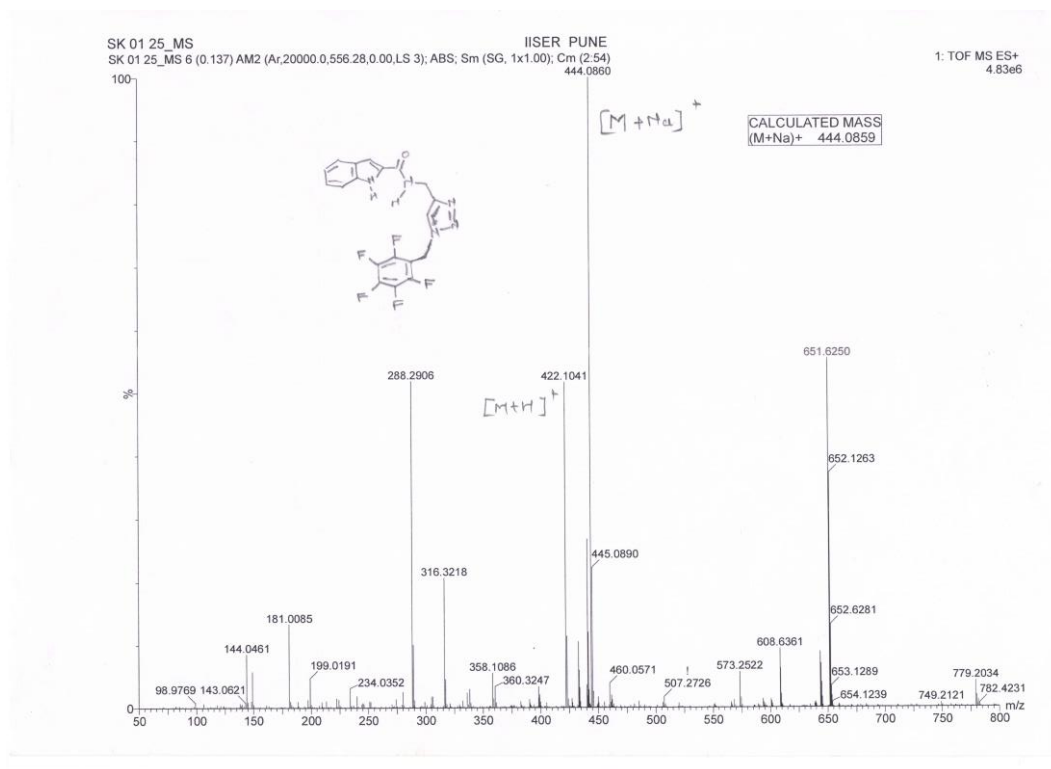


Figure 2.39 HRMS spectrum of *N*-((1-((perfluorophenyl)methyl)-1H-1,2,3-triazol-4-yl)methyl)-1H-indole-2-carboxamide (**1i**).

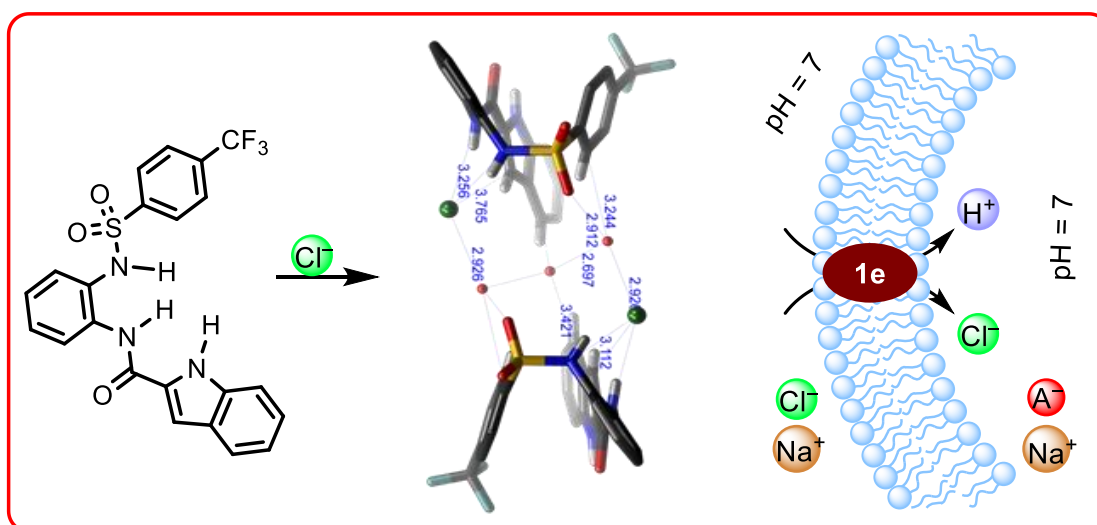
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Chapter 3

Sulfonamide-based Anion Receptors for Transmembrane Proton-Anion Co-transport Activity

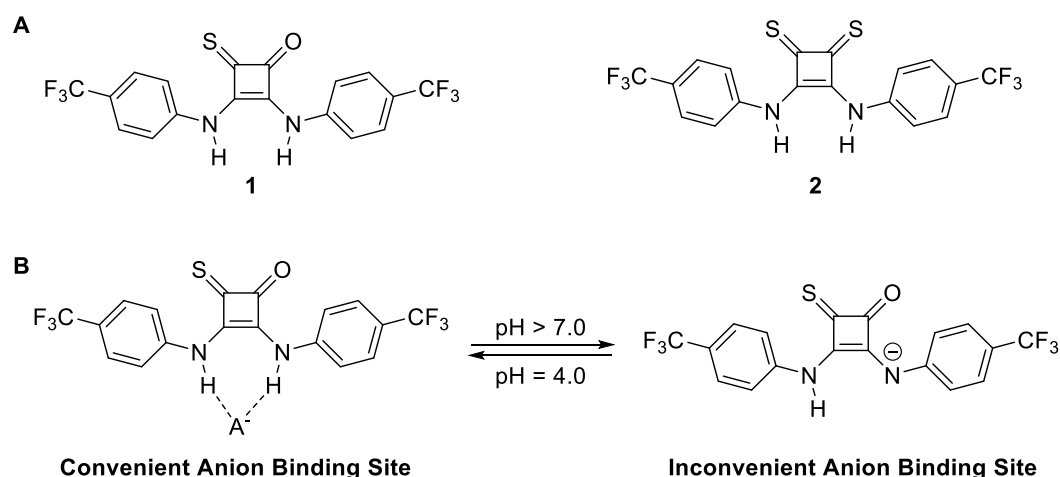


T H R E E

3.1 Introduction

Anions are essential to the maintenance of life of the cell. Most of the biochemical operations involve the recognition, transport of ions across bio-membranes, and ion transportation is involved in most of the biochemical performances. Due to the potential clinical, environmental and biological applications, biomimetic synthetic and semisynthetic receptors capable of selectively recognizing anionic species have attracted considerable interest in supramolecular chemistry.¹⁻³ To accommodate an anionic guest, a receptor molecule needs to be decorated with suitably positioned multiple noncovalent interactions, *e.g.* hydrogen bonding, anion- π , halogen bonding, electrostatic interactions, etc.⁴ Such recognition of anions has become a dominating topic in supramolecular chemistry. The organic and supramolecular chemists are aspired to identify the interactions involved in anion complexation, anion selectivity and binding strength. The techniques such as UV-Vis spectroscopy, NMR spectroscopy, and isothermal titration calorimetry (ITC) are used to quantify the binding strength. The NMR and X-ray crystal structure analysis can provide valuable insight about the geometry and particulars of interactions present in the complexation. It is, of course, the interactions can be evaluated from the association constants in solutions, but the actual verification of dynamic ion transport process is complicated.

Over a last two decades, immeasurable examples of anion complexing agents mimicking the anion transport behavior of specialized proteins have been developed.⁵ However, only a few of these synthetic molecules display gating/switching behavior that is seen in biological systems. For example, Gale and coworkers have reported the pH-switchable chloride transport activity by oxothiosquaramide and thiosquaramide analogs.^{6,7} The transmembrane Cl^- transport activity studies across vesicles indicated that the anion transport ability of thiooxasquaramide is switched ON at $\text{pH} < 7$ and turns OFF at $\text{pH} > 7$. However, lowering the pH from 7.0 to 4.0 resulted in reprotonation of the thiosquaramide nitrogen atom providing convenient anion binding site and turning ON the anion transport activity.

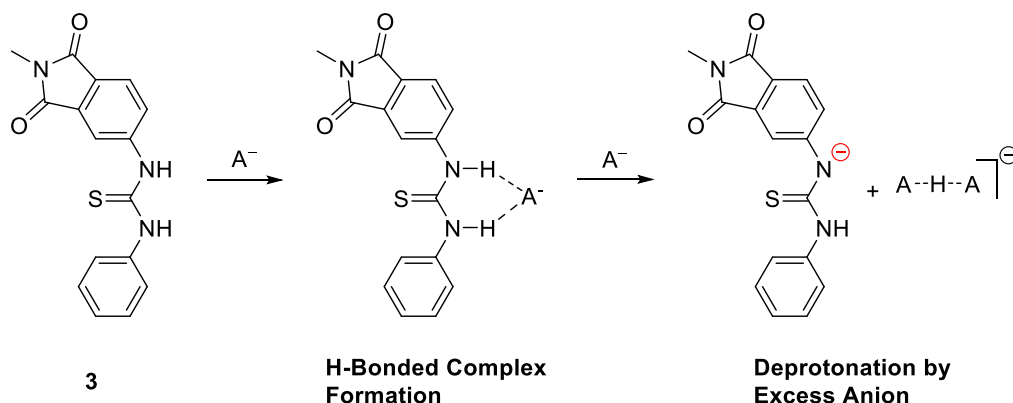


Scheme 3.1 Structures of oxothiosquaramide **1** and thiosquaramide **2** (A). pH-switchable anion binding by oxothiosquaramide **1** (B)

In chapter 2, we have shown that the triazole-linked indole-2-carboxamides selectively transports the chloride ions across the membrane. The detailed investigation of transport mechanism, indicated that when transport process was coupled with valinomycin (a electrogenic K⁺ carrier), the efflux of chloride by the triazole-linked indole-2-carboxamides was also electrogenic (the net effect of coupled assay as an electroneutral transport process). However, from the literature reports, it is already known that the influx of excessive chloride induces the hyperpolarization of the cell membrane activating the different cell death paths. Matsuyama et. al., reported that the modulation of cytosolic and intramitochondrial pH is an early events during the apoptotic cell death. Quesada and co-workers reported the indolic analogs of the tambjamines which induces both hyperpolarization and pH acidification in the cancer cells. Thus, the development of anionophores which can induce both the hyperpolarization and the pH acidification can generate the new strategies for potential therapeutics for the treatment of cancer treatment.

Prodigiosin has also inspired the development of several H⁺/Cl⁻ cotransporters, *e.g.*, perenosins,^{8,9} tren amides,¹⁰ imidazole-appended amidopyrrole,¹¹ tris-ureas and thioureas¹² and bis(melamine)-linked bispidine¹³ which have basic cores for protonation. Apart from simple anion recognition, the unanticipated competing processes can complicate a simple doubtless anion complexation processes in some cases. For example, over the last decade, deprotonation of the acidic hydrogen atom from the neutral donor group of the receptors by a basic anion (*e.g.*, fluoride and acetate) has been reported by several

research groups and such a cases are evident in thioureas (Scheme 3.2),¹⁴ pyrroles,¹⁵⁻¹⁷ amines,¹⁸ and amidoureas.¹⁹



Scheme 3.2 Thiourea containing receptor **3** deprotonates in the presence of basic anions A^- (where $A^- = F^-$ or AcO^-) due to the high stability of $[HA_2]^+$.

In general, sulfonamides have enhanced acidity than their isosteric amides. Hence, due to their propensity to make a stronger hydrogen bond with anions, the receptors based on sulfonamide appendages have become an important class of anion receptors and have been investigated by many research groups. For example, Gale and coworkers have reported the mixing the excess of tetrabutylammonium fluoride with receptors **4** containing aryl sulfonamide linkers, due to further increased conjugation from aryl groups lead to the enhanced acidity of sulfonamide NH proton, results in deprotonation of sulfonamide NH proton in both solution state and X-ray crystal structures (Figure 3.3B).²⁰

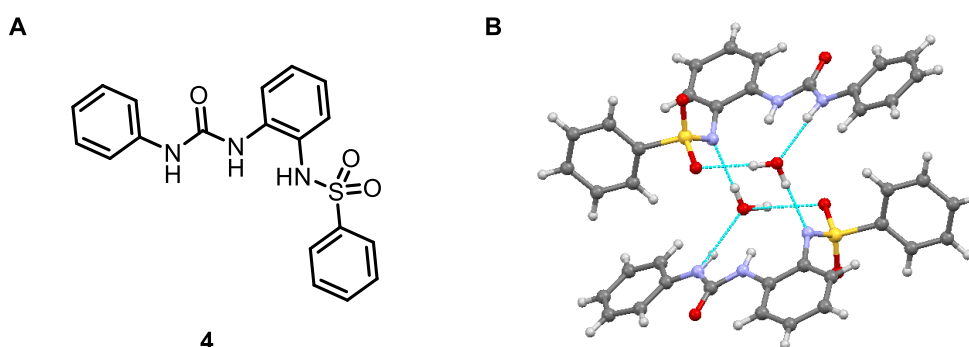


Figure 3.3 Sulfonamide **4** (A), and X-Ray crystal structure of deprotonated **4** complexed with water molecules.

However, no report is available taking advantage of enhanced acidity of sulfonamide NH proton for co-transport of proton as well as anion (i.e., symporter of

H^+/A^- , where A^- = anion) across the phospholipid membrane. In this chapter, we propose the design and synthesis of H^+/Cl^- cotransporter which does not contain any basic nitrogen centre. The indole-2-carboxamide was again taken as an chloride recognition site and connected to the aromatic sulfonamide groups has a pK_a in the range of 5.6 – 7.5 depending on its substitution. The structures of these molecules **5a-5g** are shown in Figure 3.4A. It was envisaged that such a receptor would bind to an anion through hydrogen bond interactions from three N-H moieties. The enhanced acidity of sulfonamide N-H proton results in the deprotonation under physiological conditions, with chloride transport across lipid bilayers. Nevertheless, Talukdar and co-workers have already reported that the pK_a of sulfonamide N-H protons dictates its deprotonation and this was reflected in the ion binding mode resulting in the pH-switchable cation vs anion transport in basic and in acidic pH respectively.^{21,22} Although, under acidic conditions, it can protonate to give a neutral form and bind with Cl^- and transport across the lipid bilayer driven by the pH gradient. However, in higher pH, it will deprotonate and cannot bind with Cl^- . The proposed model for the transport process is shown in Figure 3.4.

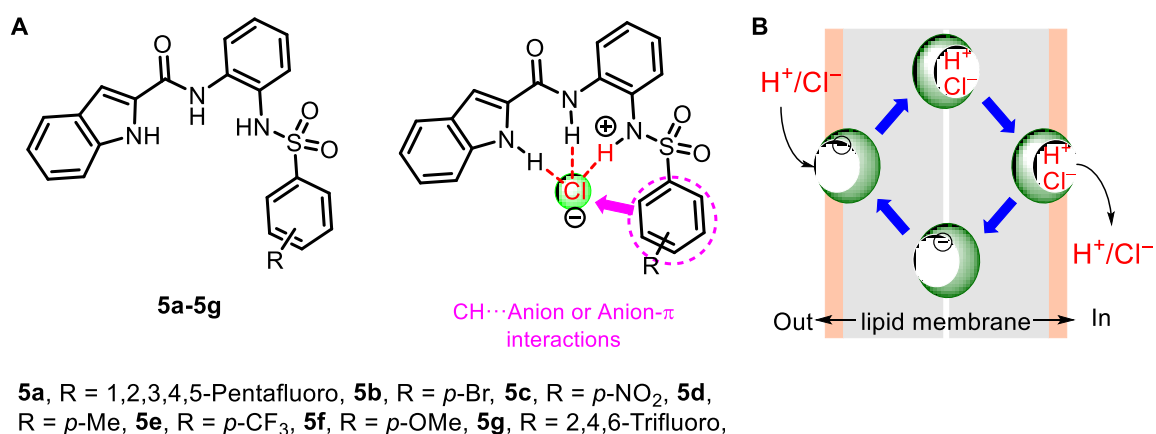


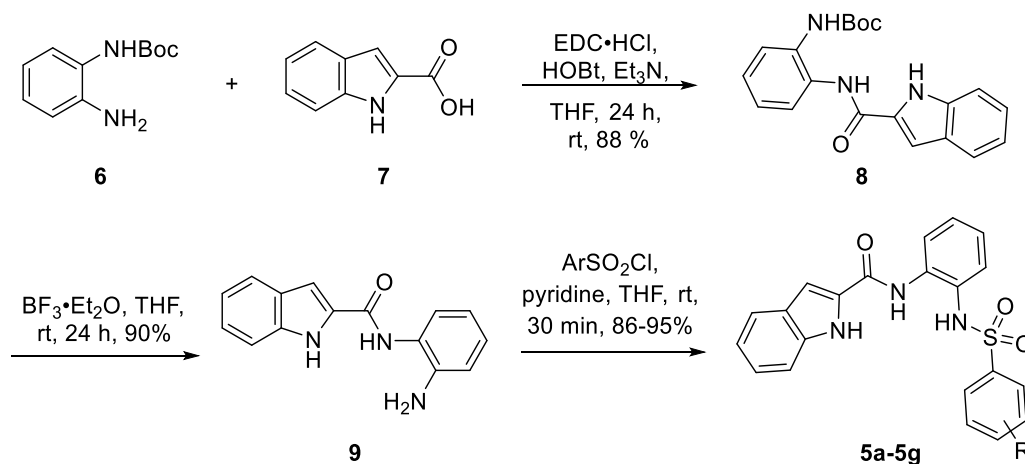
Figure 3.4 Structures of receptors **5a-5g** and representative Cl^- binding (A). Hypothesized H^+/Cl^- transport across lipid vesicle (B).

3.2 Results and Discussions

3.2.1. Synthesis

Synthesis of **5a-5g** was initiated from indole 2-carboxylic acid **7**. Initially, *N*-Boc-*o*-phenylenediamine **6** was synthesized using known literature procedure in 68% yield. The coupling of acid **7** with *N*-Boc-*o*-phenylenediamine **6** in the presence of EDC·HCl in THF afforded the amide **8** in 88% yield. The reaction of **8** with $BF_3 \cdot OEt_2$ resulted in the

deprotection of Boc group to obtain free amine **9** in 90% yield. Finally, the reaction of amine **9** with aryl sulfonyl chloride in pyridine-THF (1:3) mixture provided the receptor **5a – 5g** in 86-95% yields (Scheme 1).



Scheme 3.1 Synthesis of sulfonamide linked receptors **5a – 5g**.

3.2.2 Single Crystal X-Ray Analysis

The single crystals of compound **5c** and **5g**, suitable for X-ray analysis, were obtained by slow diffusion of atmospheric moisture into concentrated DMSO solutions of these compounds. The solid-state X-ray structure of both **5c** and **5g** showed hydrogen bonding interactions with DMSO through both the amide and sulfonamide N–H protons as well as indolic CH proton. The involvement of indolic NH in DMSO binding was not observed due to the cooperative hydrogen-bonded dimer formation in **5c** (Figure 3.5A) and interaction with additional DMSO molecule in **5g** (Figure 3.5B).

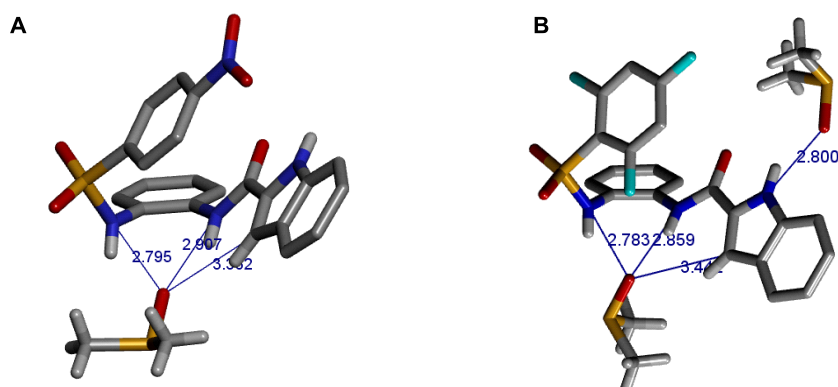


Figure 3.5 The single crystal X-ray crystal structure of **5c** (A), and **5g** (B) with dimethyl sulfoxide indicating NH···O and CH···O interaction with DMSO.

The co-crystal of **5e** with Cl^- was obtained by slow evaporation of acetonitrile solution of **5e** in the presence of excess tetrabutylammonium chloride (TBACl). The single crystal XRD structure showed the kosmotropic complex formation having 2:2 stoichiometry of **5e** and Cl^- , stabilized by three bridged water molecules (Figure 3.6, 3.15). The receptor **5e** forms two $\text{N}-\text{H}\cdots\text{Cl}^-$ hydrogen bonds ($\text{N}_{\text{sulfonamide}}\cdots\text{Cl}^-$ and $\text{N}_{\text{carboxamide}}\cdots\text{Cl}^-$ distances of 3.112(4) Å and 3.256(4) Å) with angles of 143.1° and 157.4, respectively. Each Cl^- further forms one $\text{C}-\text{H}\cdots\text{Cl}$ hydrogen bond with $\text{C}\cdots\text{Cl}$ distance of 3.765(4) Å and $\text{C}-\text{H}\cdots\text{Cl}$ angle of 148.7°, respectively. However, in the solid state, due to involvement in the cooperative hydrogen bonded homodimerization, Indolic NH has not involved the Cl^- binding. Each Cl^- also forms a hydrogen bond with one terminal water molecule ($\text{Cl}^-\cdots\text{OH}_2$ distance of 2.926(1) Å. At the same time, the complex is further stabilized by the additional hydrogen bond interactions of this water with one sulfonyl oxygen atom ($\text{H}_2\text{O}\cdots\text{O}_{\text{sulfonamide}}$ distance 2.912(1) Å), central water molecule ($\text{H}_2\text{O}\cdots\text{OH}_2$ distance 2.70(1) Å) and $\text{C}_{\text{ortho}}-\text{H}$ of the trifluoromethylphenyl ring 3.24(1) Å).

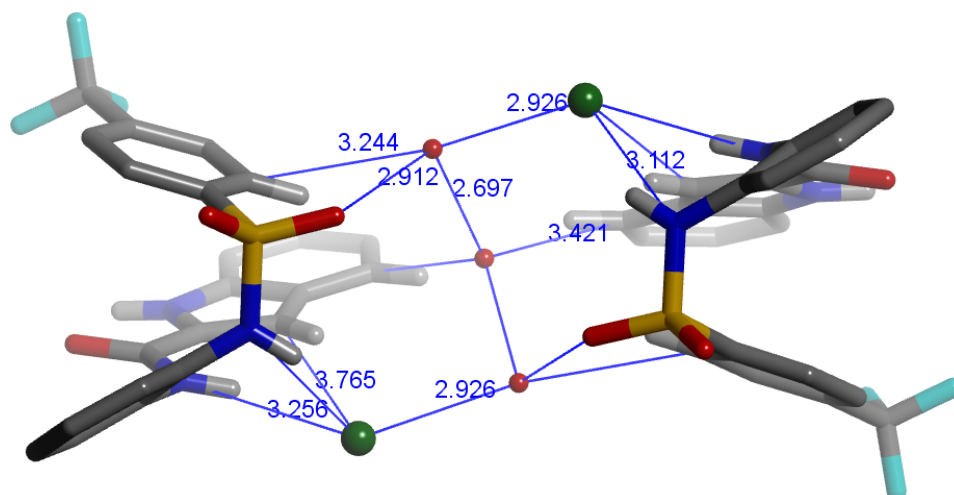


Figure 3.6 Front view of the X-ray crystal structure of $[\mathbf{5e}\cdot\text{Cl}^-][\text{Bu}_4\text{N}^+]$. Hydrogen bonds are shown by violet color lines. $[\text{Bu}_4\text{N}^+]$ and non-interacting H-atoms are omitted for clarity.

3.2.3 Hirshfeld Surface Analysis

The presence of hydrogen bonding interactions are also confirmed by the Hirshfeld surface analysis²³ of the **5d**: Cl^- complex. The Hirshfeld surface analysis is very useful tool to identify the intermolecular interactions in molecular crystals.²⁴ The tool has been widely used for analyzing the interactions in crystal structures. This tool also provide the $\text{C}-\text{H}\cdots\text{X}$ ($\text{X} = \text{N}, \text{O}, \text{Cl}$, etc.). The presences of hydrogen bonding interactions were visualized using the CrystalExplorer-17.5 software. The results of Hirshfeld surface

analysis shown in the Figure 3.7. The program was used to identify the $C(H)\cdots Cl^-$ and $C(H)\cdots O$ contacts as hydrogen bonds (see figure 3.7B). The electron density distribution on the receptor molecule in the co-crystal structure was identified using the CrystalExplorer-17.5 program. The Hirshfeld surface was also generated for the Cl^- ions in the same software (Figure 3.7C). The red, white, blue and color code indicates strong, medium and weak interactions respectively. The results obtained are analogous to the previously predicted results obtained using Mercury 3.0 software. We have also generated the electrostatic potential of the receptor using the crystal data. The electrostatic potential on receptor surface generated in crystalExplorer17 software using the default program “Tonto”, method “DFT” and basis set “6-31G(d)” (Figure 3.7D). In this case, the blue and red colors indicate the electron deficient and electron rich areas on a molecule. The

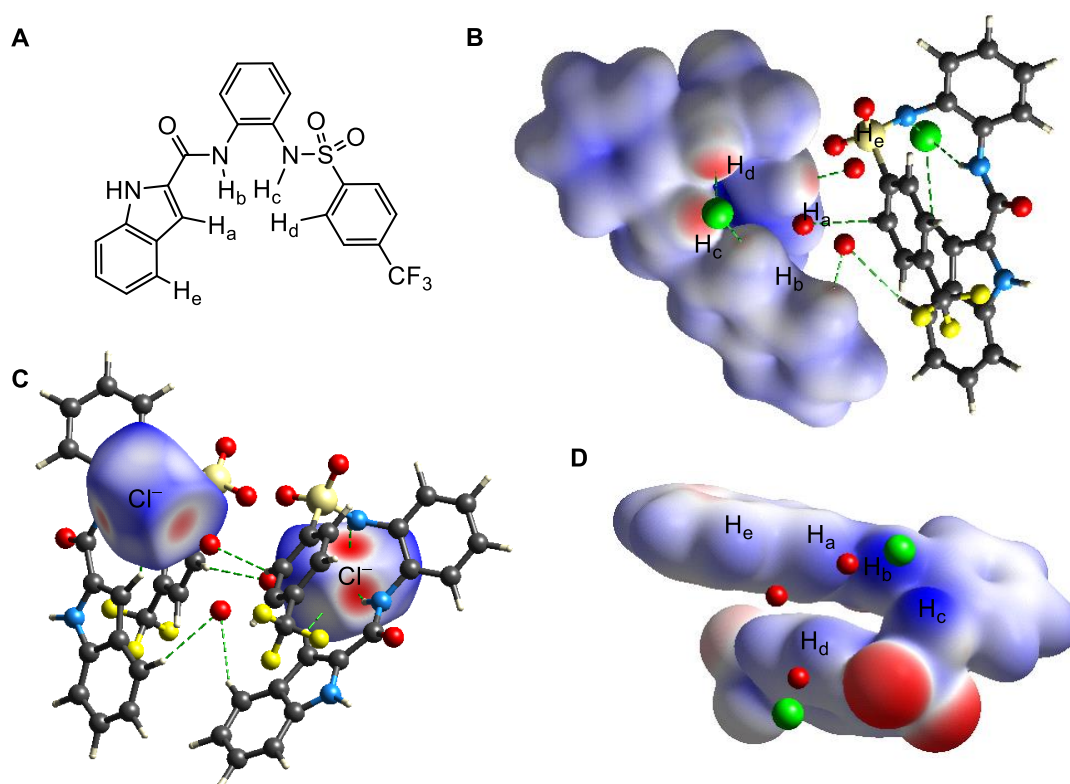


Figure 3.7 The ChemDraw structure of receptor **5e** (A). The d_{norm} mapped over the Hirshfeld surface using universal red, blue and white color code to show important interactions taking a neighbouring molecule. Hirshfeld surface generated for receptor (B) and Cl^- atoms (C). The red, white, blue and color codes on Hirshfeld surface indicate strong, medium and weak noncovalent interactions, respectively. The electrostatic potential on receptor surface generated in *crystalExplorer17* software using the default program “Tonto”, method “DFT” and basis set 6-31G(d) (D). In electrostatic potential surface the blue and red colors indicate the electron deficient and electron rich areas on a molecule.

analysis clearly indicates that the indole NH, carboxyamide NH and sulfonamide NH are strongly electron deficient while three CH protons (H_a , H_c and H_e) are moderately electron deficient. Therefore, these centers can participate in hydrogen bonds.

3.2.4 Anion Binding Study by ^1H NMR Titration

The anion binding abilities of receptors **5a-5g** in solution phase were evaluated by ^1H NMR titration²⁵ at 298 K in acetonitrile- d_3 by adding an increasing concentration of tetrabutylammonium (TBA) halides. For these receptors, titration data showed significant downfield shifts of all three NH signals, *e.g.*, $\Delta\delta = 1.72$, 2.57 and 1.75 ppm for H_a , H_b and H_c , respectively for **5d** (Figure 3.8A), indicating the hydrogen bond interactions of these protons with chloride ion. Job's plot analysis, based on the ^1H NMR titrations, showed a maximum at the 0.5 mole fraction, confirming 1:1 binding stoichiometry of **5d** with Cl^- (Figure 3.8B).

The binding constant (K_a) values were calculated by fitting the titration data of H_a proton in the WinEQNMR2 program according to the 1:1 binding model (Figure 3.8C, 3.14-3.21). For **5a** and **5d-5g**, K_a values were $> 10^4$, 3206.35 ± 88.3 , 4680.31 ± 146.8 , 1911.04 ± 140.3 and $8187.78 \pm 481.5 \text{ M}^{-1}$ (Table 1). For **5b** and **5c**, K_a values could not be determined due to their poor solubility in acetonitrile- d_3 . Overall, the trend of K_a values was dependent of the acidity of H_c , *i.e.*, stronger the electron withdrawing effect of the substituted benzene ring, next to the sulfonyl group, better is the binding with the Cl^- . For the receptor **5d**, a comparison of halide-binding provided the binding sequence of Cl^- ($K_a = 3206.35 \pm 88.3 \text{ M}^{-1}$) $>$ Br^- ($263.39 \pm 6.02 \text{ M}^{-1}$) $>$ I^- (K_a could not be determined due to the insignificant change of chemical shift) suggesting higher selectivity towards Cl^- (Figure 3.8, 3.23 - 3.24). The titration of **5d** with basic anions like AcO^- and F^- resulted in the deprotonation of sulfonamide N-H proton within the addition of 0.2 equivalent of analyte to the **5d** solution in acetonitrile- d_3 (Figure 3.25, 3.26).

3.2.5 Ion Transport Activity

The ion transport activity of **5a-5g** was investigated fluorometrically with the help of pH-sensitive 8-hydroxypyrene-1,3,6-trisulfonate dye (HPTS, $\text{p}K_a = 7.2$), entrapped into vesicles.²⁶ The large unilamellar vesicles were prepared at pH = 7.0 from egg yolk phosphatidylcholine (EYPC) lipid with entrapped HPTS to get EYPC-LUVs $\supset$ HPTS. Subsequently, the extravesicular pH was raised to 7.8 by adding NaOH. The collapse of

the pH gradient, upon addition of a transporter was monitored by measuring the fluorescence intensity of HPTS at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$). Finally, vesicles were lysed by adding Titron X-100 to get the complete collapse of the applied pH gradient.

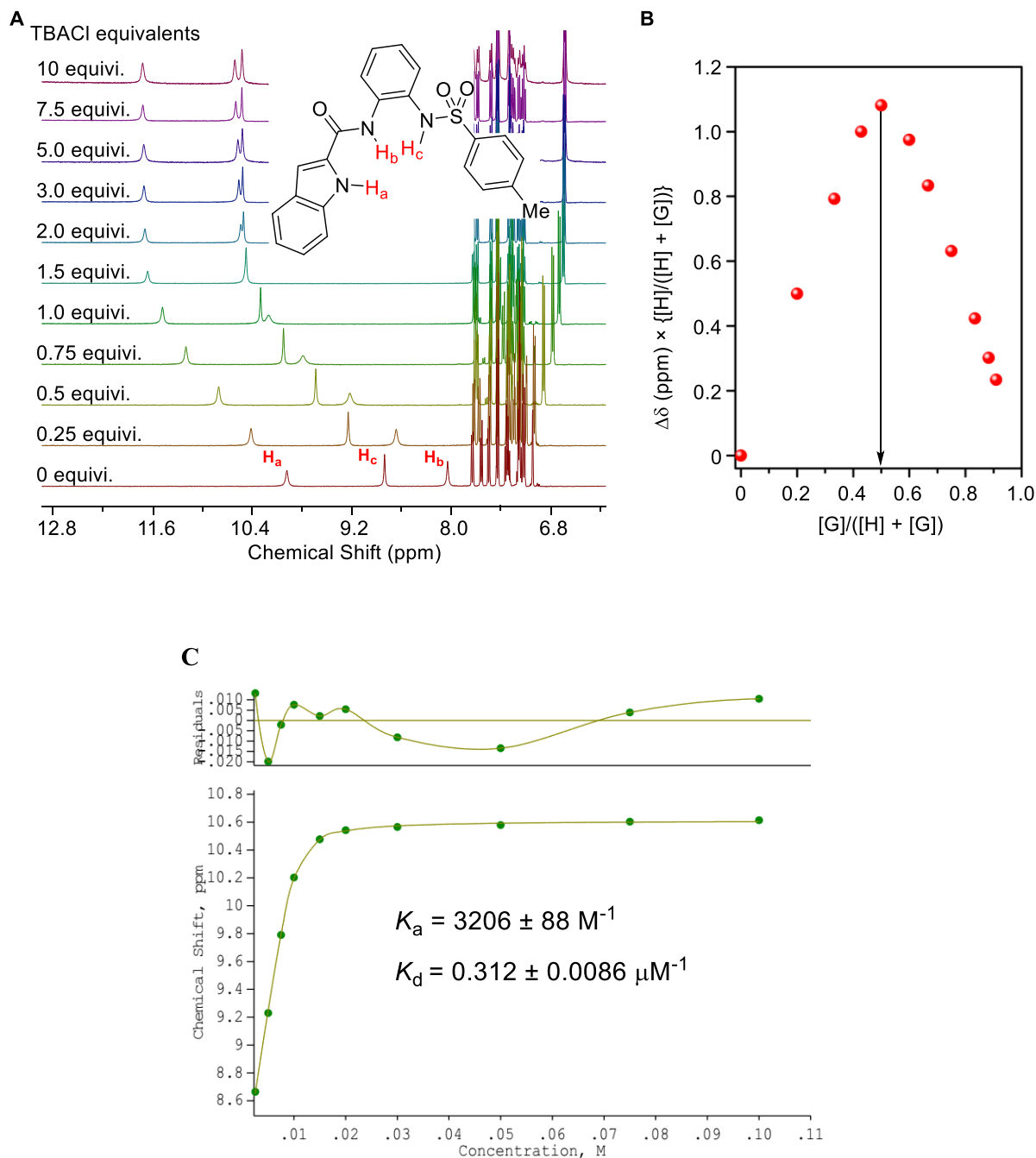


Figure 3.8 ^1H NMR titration spectra for **5d** with stepwise addition of TBACl in acetonitrile- d_3 at 25°C . The equivalents of added TBACl are shown on the stacked spectra (A). Job's plot generated from the same ^1H NMR titration indicating 1:1 binding of **5d**: Cl^- (B). The fit plot of chemical shift (δ ppm) of amide NH_a proton versus concentration of TBACl fitted to 1:1 binding model of WinEQNMR2 program (C).

Table 1 Summary of the chloride stability constants K_a (M^{-1}) of receptors **5a-5g** calculated at 298 K and pK_a values of receptors **5a-5g**.

Transporter	R	LogP	pK_a	K_a (M^{-1})	EC_{50} (μM)	n
5a	2,3,4,5,6-Pentafluoro	4.94	5.59	$>10^4$	ND	ND
5b	<i>p</i> -Br	4.99	7.28	ND	4.01	1.70
5c	<i>p</i> -NO ₂	4.16	6.8	ND	13.41	1.02
5d	<i>p</i> -CH ₃	4.73	7.44	3206 ± 88	2.33	2.18
5e	<i>p</i> -CF ₃	5.10	7.28	4680 ± 147	5.82	1.67
5f	<i>p</i> -OCH ₃	4.06	7.52	1911 ± 140	7.56	2.30
5g	2,4,6-Trifloro	4.65	5.75	8188 ± 482	ND	ND

^aCalculator plugin of the MarvinSketch program was used for logP of each molecule and pK_a calculation of each sulfonamide NH proton.²⁷ ^bBinding constant obtained by following amide NH proton. Data fitted to 1:1 binding model.

The synthetic receptors **5b** and **5d-5f** with a pK_a (of sulfonamide NH proton) greater than 7.0 exhibited significant increase in HPTS fluorescence intensity indicating the transport of ions across the EYPC-LUVs. Comparison of ion transport activity at identical concentration ($c = 10 \mu M$) provided the activity sequence: **5d** > **5b** > **5e** > **5f** > **5c** > **5a** > **5g** (Figure 3.9). Although **5a** has lower pK_a than **5f**, its higher ion transport activity could be attributed to its better membrane permeability ($\log P = 4.95$). The dose-response activity compound **5b-5f** also provided the EC_{50} and Hill coefficient n values (Table 1). The Hill coefficient n values, in general, suggested the involvement of two molecules in the active transporter formation. Subsequently, the most active compound **5d** was selected to investigate the ion selectivity and transport mechanism. The selectivity studies performed on compound **5d** by varying the extravesicular ions in HEPES buffer solution.²⁸ As expected, insignificant change in ion transport activity was observed upon varying metal chlorides (i.e., MCl,

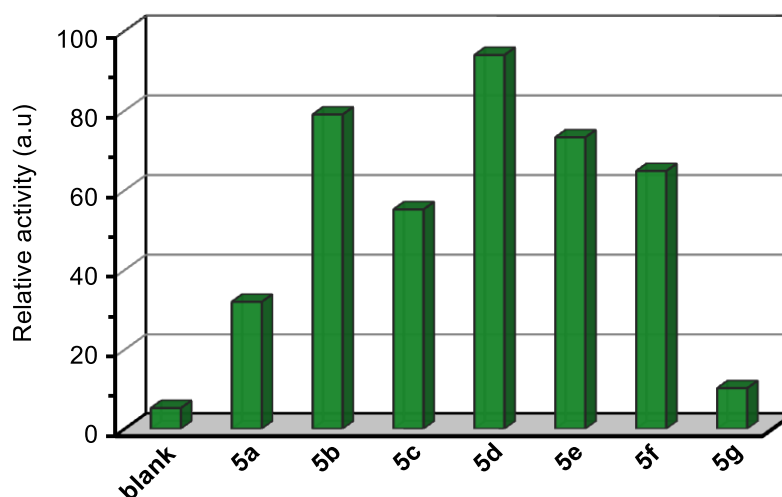


Figure 3.9 Comparison of ion transport activity of **5a-5g** across EYPC-LUVs $\Rightarrow$ HPTS. The fluorescence intensity values at 200 s after addition of transporters (10 μ M) were plotted.

where, $M^+ = Li^+, Na^+, K^+, Rb^+, Cs^+$) in extravesicular buffer solution. This data confirms that **5d** does not transport alkali cations (Figure 3.10A). However, the change of anions in the extravesicular buffer with sodium salts (i.e., NaA, where $A^- = F^-, Cl^-, Br^-, I^-, NO_3^-, ClO_4^-, SCN^-,$ and OAc^-) showed a significant difference in ion transport activity providing the activity sequence: $Cl^- > OAc^- \sim F^- \geq Br^- > SCN^- > NO_3^- > I^- > ClO_4^-$ (Figure 3.10B). The selectivity sequence neither fully concerted the Hofmeister series (where the dehydration energy limits transport) nor a complete dependence on the anion size. Also, among the halides, the dehydration dominated quasi-Hofmeister series (i.e., $Cl^- > Br^- > I^-$) was observed.²⁹ This indicates that both dehydration and carrier binding followed by membrane crossing are rate-limiting steps. The observation was also in agreement with the halide binding data of **5d** obtained from NMR studies. The chloride ion transport activity of **5d** across LUVs was further examined by monitoring the fluorescence intensity of lucigenin dye ($\lambda_{em} = 535$ nm, $\lambda_{ex} = 455$ nm) entrapped along with nitrate inside EYPC-LUVs.³⁰ The addition of NaCl (33 mM) followed by **5d** resulted in the quenching of intravesicular lucigenin fluorescence confirming the influx of Cl^- inside LUVs (Figure 3.10C).

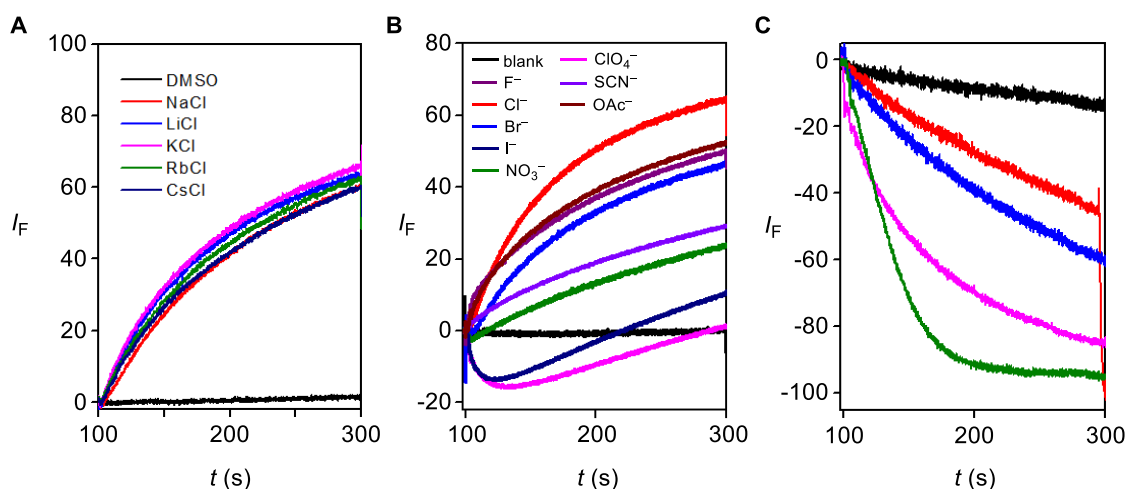


Figure 3.10 Cation selectivity (A), and Anion selectivity of **5d** (2.5 μ M) measured by varying the extravesicular cations and anions respectively, across EYPC-LUVs $\rightarrow$ HPTS (B). Chloride transport activity by lucigenin assay across EYPC-LUVs $\rightarrow$ Lucigenin.

Either of M^+/H^+ antiport, OH^-/A^- antiport, M^+/OH^- symport and H^+/A^- symport processes can achieve the collapse of the applied pH during the HPTS assay. The observed cation variation data ruled out the M^+/H^+ antiport and M^+/OH^- symport processes, and anion variation data are suggestive of either OH^-/A^- antiport or H^+/A^- symport by **5d**. The initial decrease of HPTS fluorescence, observed for $\text{A}^- = \text{I}^-$ and ClO_4^- (Figure 3.10B), is suggestive of H^+ influx via H^+/A^- symport as one possible mechanism. To further cross-examine, the transport activity of **5d** in the presence of either FCCP³¹ (a proton carrier) or valinomycin³² (a K^+ selective carrier), was implemented. In the FCCP coupled assay, the compound **5d** showed similar transport activity as in the absence of FCCP (Figure 3.11A). The outcome corroborated to the dissipation of pH gradient through the H^+ efflux by H^+/Cl^- cotransport mechanism, instead of the OH^- influx. The valinomycin assay performed by replacing the extravesicular Na^+ with K^+ ions. The valinomycin coupled HPTS assay (with intravesicular NaCl and extravesicular KCl) of **5d** also showed ion transport that was comparable to **5d** alone (Figure 3.11B). Therefore, no cooperative effect was seen between K^+ influx by valinomycin and ion transport by **5d**, and the outcome confirmed further the H^+/Cl^- symport mechanism of the indole derivative.

To get more insight about the mechanism, HPTS assays were carried out by varying the extravesicular anions and without applying the pH gradient (i.e., $\text{pH}_{\text{in}} = \text{pH}_{\text{out}} = 7.0$). The vesicles entrapped with NaCl were suspended in buffer containing either of

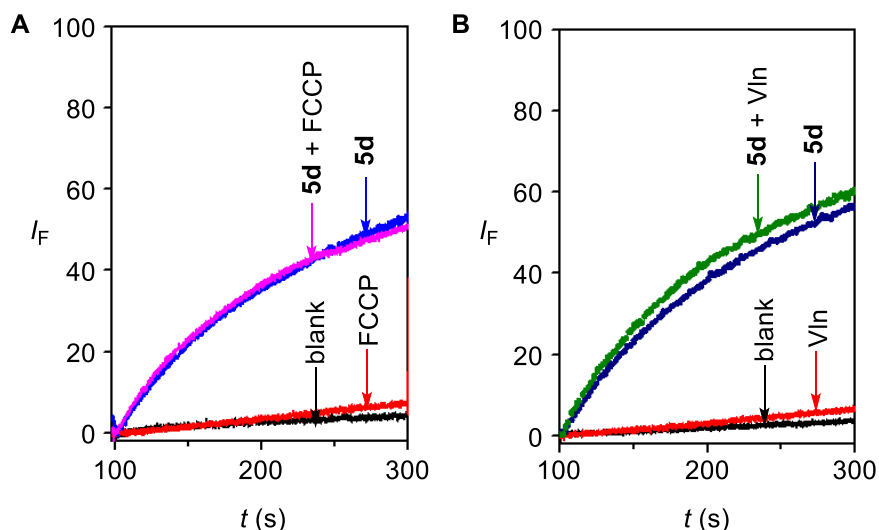


Figure 3.10 Comparison of ion transport activity of **5d** (2.5 μ M) in the presence and the absence of FCCP (0.8 μ M) (A). Comparison of ion transport activity of **5d** (2.5 μ M) in presence and absence of valinomycin (2.5 pM) (B).

NaCl, NaBr, NaI, NaClO₄ or Na₂SO₄ salts. In the presence of extravesicular NaCl, the addition of **5d** resulted in the initial influx of HCl (indicated by the initial decrease in HPTS fluorescence) followed by slow dissipation of the *in situ* generated pH gradient (indicated by the subsequent increase in HPTS fluorescence) (Figure 3.11). For extravesicular Br[−], I[−], and NO₃[−], similar trends were observed. In the presence of poor permeating external SO₄^{2−} (Hydration energy, $\Delta G_{\text{SO}_4^{2-}} = -1080 \text{ kJ mol}^{-1}$)³³ an immediate increase in HPTS fluorescence was observed which indicate the H⁺/Cl[−] coefflux.

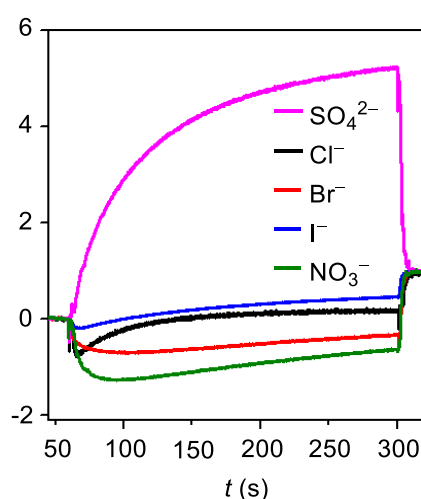


Figure 3.11 Anion selectivity of **5d** across EYPC-LUVs>HPTS without applying pH gradient (i.e., pH_{in} = pH_{out} = 7.0).

3.3 Conclusion

In summary, we have demonstrated the indole-based hybrid amide and sulfonamide appended synthetic chloride receptors. The receptors bind to chloride ion as revealed by the single crystal structure of **5b**·Cl[−] and solution state ¹H NMR titration experiments. Also, the receptor **5a-5g** found to be capable of transporting the anions across phospholipids bilayer. For the first time, it has been nicely depicted that instead of lipophilicity, the transport activity can be tuned by careful modification of the sulfonamide N–H acidity. The detailed mechanistic investigation demonstrated H⁺/Cl[−] symport mechanism under the pH gradient conditions. This approach may allow us to develop synthetic transporters sensitive to acidic pH medium. Without the use of any external stimuli, the approach provides a potential method for development of new synthetic transporter molecules capable of targeting acidic compartments in biological systems.

3.5 Experimental Section

3.5.1 General Methods

All chemicals, solvents and fluorescent dyes were purchased from commercial sources (Sigma-Aldrich, Alfa Aesar, Fisher Scientific, Spectrochem, and Avra). All commercial reagent grade chemicals were used as received without further purification unless otherwise specified. All deuterated solvents, HPTS dye, HEPES buffer, Triton X-100, sodium hydroxide and inorganic salts of molecular biology grade were obtained from Sigma-Aldrich. Egg-yolk phosphatidylcholine (EYPC) lipid as a solution in CHCl₃ (25 mg/mL), polycarbonate membrane (100 nm and 200 nm pore size), and mini-extruder used for vesicle preparation was obtained from Avanti Polar Lipids. All the Stock solutions were prepared in either HPLC grade DMSO, methanol or acetonitrile.

3.5.2 Physical Measurements

¹H-NMR spectra were recorded at 400 MHz using Bruker AscendTM 400 spectrometer, and ¹³C NMR spectra using Jeol ECS-400 at 101 MHz frequency. All the spectra were calibrated concerning residual solvent peaks. The following abbreviations were used to describe peak patterns wherever appropriate: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. All coupling constants (*J*) are reported in Hertz (Hz). High-resolution mass spectra (HRMS) were performed on an electron spray ionization time-of-

flight (ESI-TOF). Melting points were measured with a micro melting point apparatus. Single Crystal X-Ray Diffraction (SCXRD) measurements were done on a Bruker KAPPA APEX II CCD diffractometer. Fluorescence spectra were recorded from Fluoromax-4 from JobinYvon Edison-equipped with an injector port and a magnetic stirrer. All buffer solutions were adjusted to required pH using a Helmer pH meter. All data from fluorescence studies were processed by either KaleidaGraph 3.51 or OriginPro 8.5 software.

3.5.3 Synthesis procedures

Synthesis of *tert*-butyl (2-aminophenyl)carbamate ($C_{11}H_{16}N_2O_2$) **6**:

In a 100 mL round bottom flask, to a solution of *o*-phenylenediamine (1 g, 9.24 mmol) in CH_2Cl_2 (5 ml) was added Di-*tert*-butyl bicarbonate (2.01 g, 9.24 mmol) and triethylamine (1.28 ml, 9.24 mmol) at 0 °C with continuous stirring. The reaction mixture stirred for 4 hours at 0 °C under N_2 atm. The reaction was quenched with water and the aqueous layer extracted with EtOAc (2×50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated in *vacuo*. The solid residue was purified by column chromatography on SiO_2 (Eluent: Hexane: EtOAc from 91:9 to 88:12) to obtain **6** as a white solid (1.308 g, 67.94%).

1H NMR (400 MHz, $CDCl_3$): δ = 7.27 (d, J = 7.8 Hz, 1H), 7.00 (td, J = 7.6, 1.5 Hz, 1H), 6.79 (td, J = 7.6, 1.4 Hz, 1H) 6.77 (dd, J = 7.8, 1.2 Hz, 1H), 3.70 (s, 1H), 1.51 (s, 9H); HRMS (ESI $^+$) m/z : $[M + H]^+$ calcd. for $C_{11}H_{17}N_2O_2$: 209.1290, found: 209.1292.

Synthesis of *tert*-butyl (2-(1*H*-indole-2-carboxamido) phenyl) carbamate ($C_{20}H_{20}N_3NaO_3$) **6**:

In a 100 mL round-bottomed flask equipped with a magnetic stirrer, to a solution of Indole-2-carboxylic acid **7** (835 mg, 5.185 mmol) in dry THF (10 mL) was added EDC·HCl (994 mg, 5.185 mmol) and 1- hydroxy benzotriazole (HOBt, 700 mg, 5.185 mmol) at room temperature and the reaction mixture stirred for 10 minutes. After 10 minutes, *tert*-butyl (2-aminophenyl) carbamate **6** (900 mg, 4.321 mmol) and triethylamine (656 mg, 6.481 mmol) were added at room temperature and the reaction mixture stirred for 24 hours. After completion of the reaction mixture was concentrated to remove excess THF, The water (50 mL) was added and the mixture was extracted with EtOAc (3×30 mL), the organic layers were separated, washed with brine, dried over anhydrous Na_2SO_4 ,

filtered and concentrated under reduced pressure. The solid residue was purified by column chromatography on SiO₂ (*Eluent*: Hexane:EtOAc -91:9) to obtain **8** as a white solid (1.33 g, 87.8 %).

M.p.: 194 °C; **¹H NMR** (400 MHz, CDCl₃): δ = 9.36 (s, 1H), 7.83 (d, *J* = 8.0 Hz, 1H), 7.66 (d, *J* = 8 Hz, 1H) 7.44 (d, *J* = 8.3 Hz, 1H), 7.34 – 7.13 (m, 7H), 7.09 (s, 1H), 6.75 (s, 1H), 1.56 (s, 9H); **¹³C NMR** (101 MHz, CDCl₃): δ = 160.3, 154.8, 136.8, 130.9, 130.4, 130.0, 127.9, 126.2, 126.1, 125.9, 124.9, 124.5, 122.2, 120.8, 112.2, 103.6, 81.8, 28.5 (3C); **HRMS** (ESI⁺) *m/z*: [M + Na]⁺ calcd. for C₂₀H₂₁N₃NaO₃: 374.1481, found: 374.1481.

Synthesis of *N*-(2-aminophenyl)-1*H*-indole-2-carboxamide (C₁₅H₁₃N₃O) **9**:

In a 100 mL round bottom flask, to a solution of *tert*-butyl (2-(1*H*-indole-2-carboxamido) phenyl) carbamate **8** (740 mg) in THF (5 mL) was added BF₃·OEt₂ (98%, 6 mL) was added at room temperature. The reaction mixture was stirred for 24 hours (followed by TLC). After completion of the reaction, the THF was removed in *vacuo*. To the liquid residue, water (5 ml) was added. The excess of BF₃·OEt₂ was quenched with the dropwise addition of saturated NaHCO₃ until the effervescence of CO₂ stops. The mixture extracted with ethyl acetate (50 mL × 2), the organic layer separated, dried over Na₂SO₄, filtered and concentrated in *vacuo*. The residue was purified by column chromatography on SiO₂ (*Eluent*: 1% MeOH in CH₂Cl₂) to obtain **9** as a yellowish solid (510 mg, 96.5 %).

M.p.: 206 °C; **¹H NMR** (400 MHz, DMSO-*d*₆): δ = 11.69 (s, 1H), 9.68 (s, 1H), 7.65 (d, *J* = 8.0 Hz, 1H), 7.46 (d, *J* = 8.3 Hz, 1H), 7.36 (s, 1H), 7.21 (td, *J* = 6.2, 1.1 Hz, 2H), 7.06 (t, *J* = 7.5 Hz, 1H), 6.99 (t, 7.6 Hz, 1H), 6.81 (dd, *J* = 8.0, 1.2 Hz, 1H), 6.63 (td, *J* = 7.7, 1.3 Hz, 1H), 4.95 (s, 2H); **¹³C NMR** (101 MHz, DMSO-*d*₆): δ = 159.8, 143.2, 136.6, 131.5, 127.1, 126.7, 126.5, 123.5, 122.9, 121.6, 119.8, 116.3, 116.2, 112.3, 103.6; **HRMS** (ESI⁺) *m/z*: [M + H]⁺ calcd. for C₁₅H₁₄N₃O: 252.1137, found: 252.1144.

General Procedure for Synthesis of Transporter **5a** - **5g**:

In 10 mL round bottom flask, to a stirred suspension of *N*-(2-aminophenyl)-1*H*-indole-2-carboxamide **5** (50 mg, 0.199 mmol) and Aryl sulfonyl chloride (0.298 mmol) in dry THF (1 mL) was added pyridine (1 mL). The reaction mixture stirred for 30 minutes at room temperature. THF removed under vacuum, and the residue taken in 4M

HCl, extracted with ethyl acetate (10 mL $\times$ 2). The organic layer separated, dried over Na₂SO₄, filtered and concentrated in *vacuo*. The residue was purified by column chromatography (*Eluent*: 1% MeOH in CH₂Cl₂) to obtain product **5a–5g** in 86 – 95% yield.

***N*-(2-((perfluorophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide**

(C₂₁H₁₂F₅N₃O₃S) 5a: Beige color solid; **M.p.**: 286 °C; **¹H NMR** (400 MHz, DMSO-*d*₆): δ = 11.75 (s, 1H), 10.35 (s, 1H), 9.66 (s, 1H), 7.70 (d, *J* = 8.0 Hz, 1H), 7.56 (d, *J* = 7.88 Hz, 1H), 7.48 (d, *J* = 6.24 Hz, 1H), 7.44 – 7.37 (m, 1H), 7.31 (td, *J* = 7.34, 1.46 Hz, 1H), 7.25 (td, *J* = 7.58, 1.06 Hz, 1H), 7.20 (d, *J* = 1.5 Hz, 1H), 7.09 (t, *J* = 7.46 Hz, 1H); **¹³C NMR** (101 MHz, DMSO-*d*₆): δ = 159.7, 143.7 (dm, *J* = 242 Hz), 137.3 (dm, 252 Hz), 137.1, 132.8, 130.0, 129.0, 128.3, 127.6, 126.9, 126.3, 126.2, 124.2, 121.8, 121.1, 115.3 (t, 14 Hz), 112.5, 103.8; **HRMS** (ESI⁺) *m/z*: [M + H]⁺ calcd. for C₂₁H₁₃F₅N₃O₃S: 482.0598, found: 482.0595.

***N*-(2-((4-bromophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide**

(C₂₁H₁₆BrN₃O₃S) 5b: White solid; **M.p.**: 264 °C; **¹H NMR** (400 MHz, DMSO-*d*₆): δ = 11.78 (s, 1H), 9.80 (s, 1H), 9.58 (s, 1H), 7.75 (dd, *J* = 8.12, 1.12 Hz, 1H), 7.72 (d, *J* = 8.08 Hz, 1H), 7.60 – 7.44 (m, 5H), 7.34 – 7.21 (m, 2H), 7.20 – 7.07 (m, 4H); **¹³C NMR** (101 MHz, DMSO-*d*₆): δ = 159.6, 138.5, 137.0, 132.8, 132.3, 130.7, 128.5, 128.0, 127.5, 127.1, 126.9 (2C), 125.5, 124.7, 124.1, 121.9, 120.1, 112.5, 103.9; **HRMS** (ESI⁺) *m/z*: [M + H]⁺ calcd. for C₂₁H₁₇BrN₃O₃S: 470.0174, found: 470.0176.

***N*-(2-((4-nitrophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide (C₂₁H₁₆N₄O₅S)**

5c: Light brown color solid; **M.p.**: 295 °C; **¹H NMR** (400 MHz, DMSO-*d*₆): δ = 11.68 (s, 1H), 9.95 (s, 1H), 9.45 (s, 1H), 8.05 (d, *J* = 8.7 Hz, 2H), 7.79 (d, *J* = 8.7 Hz, 2H), 7.67 (dd, *J* = 16.5, 8.0 Hz, 2H), 7.45 (d, *J* = 8.3 Hz, 1H), 7.39 – 7.30 (m, 1H), 7.28 – 7.18 (m, 3H), 7.14 – 7.06 (m, 2H); **¹³C NMR** (101 MHz, DMSO-*d*₆): δ = 159.5, 149.4, 144.8, 136.9, 132.8, 130.5, 128.0 (2C), 127.9, 127.6, 126.8, 125.8, 125.4, 124.5, 124.5, 121.8, 120.1, 112.5, 104.0; **HRMS** (ESI⁺) *m/z*: [M + H]⁺ calcd. for C₂₁H₁₇N₄O₅S: 437.0919, found: 437.0915.

***N*-(2-((4-methylphenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide**

(C₂₂H₂₀N₃O₃S) 5d: White solid; **M.p.:** 230 °C; **¹H NMR** (400 MHz, DMSO-*d*₆): δ = 11.79 (s, 1H), 9.69 (s, 1H), 9.55 (s, 1H), 7.72 (d, *J* = 7.96 Hz, 1H), 7.72 (d, *J* = 8.04 Hz, 2H), 7.49 (m, 3H), 7.32 – 7.22 (m, 2H), 7.19 – 7.06 (m, 6H), 2.21 (s, 3H); **¹³C NMR** (101 MHz, DMSO-*d*₆): δ = 160.0, 143.8, 137.4, 137.0, 133.2, 131.4, 130.1, 128.6, 127.5, 127.4 (2C), 127.1, 125.7, 124.9, 124.5, 122.3, 120.6, 113.0, 104.2, 21.4; **HRMS** (ESI⁺) *m/z*: [M + H]⁺ calcd. for C₂₂H₂₁N₃O₃S: 406.1225, found: 406.1226.

***N*-(2-((4-(trifluoromethyl)phenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide**

(C₂₂H₁₆F₃N₃O₃S) 5e: White solid; **M.p.:** 234 °C; **¹H NMR** (400 MHz, DMSO-*d*₆): δ = 11.72 (s, 1H), 9.91 (s, 1H), 9.53 (s, 1H), 7.80 (d, *J* = 8.2 Hz, 2H), 7.76 – 7.66 (m, 4H), 7.47 (d, *J* = 8.3 Hz, 1H), 7.34 – 7.28 (m, 1H), 7.24 (t, *J* = 7.5 Hz, 1H), 7.21 – 7.15 (m, 2H), 7.14 – 7.06 (m, 2H); **¹³C NMR** (101 MHz, DMSO-*d*₆): δ = 159.6, 143.3, 137.0, 132.8, 132.2 (q, *J* = 33 Hz, CCF₃), 130.6, 127.9, 124.4, 127.4, 126.9, 126.5, 126.4, 125.6, 124.9, 124.1, 123.2 (q, *J* = 272 Hz, CF₃), 121.8, 120.1, 112.5, 103.9; **HRMS** (ESI⁺) *m/z*: [M + H]⁺ calcd. for C₂₂H₁₇F₃N₃O₃S: 460.0942, found: 460.0948.

***N*-(2-((4-methoxyphenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide**

(C₂₂H₁₉N₃O₄S) 5f: Pale yellow solid; **M.p.:** 231 °C; **¹H NMR** (400 MHz, DMSO-*d*₆) δ = 11.81 (s, 1H), 9.62 (s, 1H), 9.59 (s, 1H), 7.78 (d, *J* = 7.24 Hz, 1H), 7.72 (d, *J* = 7.96 Hz, 1H), 7.54 (d, *J* = 8.9 Hz, 2H), 7.49 (d, *J* = 8.3 Hz, 1H), 7.31 – 7.23 (m, 2H), 7.18 (d, *J* = 1.5 Hz, 1H), 7.16 – 7.05 (m, 3H), 6.86 (d, *J* = 8.9 Hz, 2H), 3.66 (s, 3H); **¹³C NMR** (101 MHz, DMSO-*d*₆) δ 162.4, 159.5, 136.9, 132.8, 130.9, 130.8, 128.8, 128.2, 127, 126.9 (2C), 125.2, 124.2, 124.0, 121.9, 120.1, 114.3, 112.5, 103.7, 55.5; **HRMS** (ESI⁺) *m/z*: [M + H]⁺ calcd. for C₂₂H₂₀N₃O₄S: 422.1174, found: 422.1178.

***N*-(2-((2,4,6-trifluorophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide**

(C₂₁H₁₄F₃N₃O₃S) 5g: Yellow–orange colored solid; **M.p.:** 238 – 239 °C; **¹H NMR** (400 MHz, DMSO-*d*₆): δ = 11.81 (s, 1H), 10.06 (s, 1H), 9.79 (s, 1H), 7.72 (d, *J* = 8.0 Hz, 1H), 7.64 (d, *J* = 8.1 Hz, 1H), 7.49 (d, *J* = 8.3 Hz, 1H), 7.35 – 7.30 (m, 2H), 7.28 – 7.21 (m, 3H), 7.15 – 7.05 (m, 3H); **¹³C NMR** (101 MHz, DMSO-*d*₆) δ 164.7 (dt, *J* = 256.4, 16.2

Hz), 156.2 (dm, $J = 259.3$ Hz, 2C) 137.0, 132.6, 130.5, 127.6, 127.4, 127.0, 125.9, 125.2, 124.2, 121.9, 120.1, 114.0 (dt, $J = 16.9$, 4.8 Hz), 112.6, 104.0, 102.5 (t, $J = 27.22$ Hz); HRMS (ESI⁺) m/z : $[M + H]^+$ calcd. for C₂₁H₁₅F₃N₃O₃S: 446.0786, found: 446.0785.

3.5.4 Single Crystal X-ray Crystallographic Study

Single crystals suitable for X-ray diffraction were obtained by slow diffusion of atmospheric moisture into DMSO solutions of compound **5c** and **5g**. The co-crystal of [**5e**·Cl[−]][TBA⁺] was obtained by mixing the **5e** and TBACl (10 equvi.) in acetonitrile at room temperature and slow evaporation of acetonitrile gave the uniform crystals.

Single crystal X-ray intensity data of compounds were collected on a Bruker-KAPPA APEX II CCD diffractometer with graphite-monochromatized (Mo-K α = 0.71073 Å) radiation. The X-ray generator was operated at 1500 W power, 50 kV and 30 mA. Data were collected with the scan width of 0.3° at different settings of ϕ (0°, 90° and 180°) keeping the sample to detector distance fixed at 40 mm and the detector position (2 θ) fixed at 24°. The X-ray data collection was monitored by SMART program (Bruker, 2003). The structure was solved by the direct method using SHELXTL and refined on F^2 by full-matrix least squares technique using the SHELXL-974 program package within the WINGX program. All non-hydrogen atoms were refined anisotropically. All the hydrogen atoms were placed in geometrically idealized position as riding over their parent atoms.

Details of Co-crystal of 5c·DMSO: CCDC 1840631, C₂₃H₂₂N₄O₆S₂, $M = 514.56$, colorless blocks, triclinic, space group: P $\bar{1}$, Cell: $a=7.567(2)$, $b=12.628(4)$, $c=12.890(4)$, $V = 1178.3(6)$ Å³, $Z = 2$, $T = 296$ K, $\theta_{\max} = 28.365^\circ$, $D_{\text{calc}} (\text{g cm}^{-3}) = 1.450$, $F(000) = 536.0$, $\mu (\text{mm}^{-1}) = 0.274$, 21482 reflections collected, 5854 unique reflections ($R_{\text{int}} = 0.0706$), $\text{GoF} = 0.980$, $R_1 = 0.0554$, $wR_2 = 0.1611$, R indices based on 5033 reflections with $I > 2s(I)$ (refinement on F^2). $\Delta\rho_{\max} = 0.792$, $\Delta\rho_{\min} = -0.752$ (eÅ^{−3}).

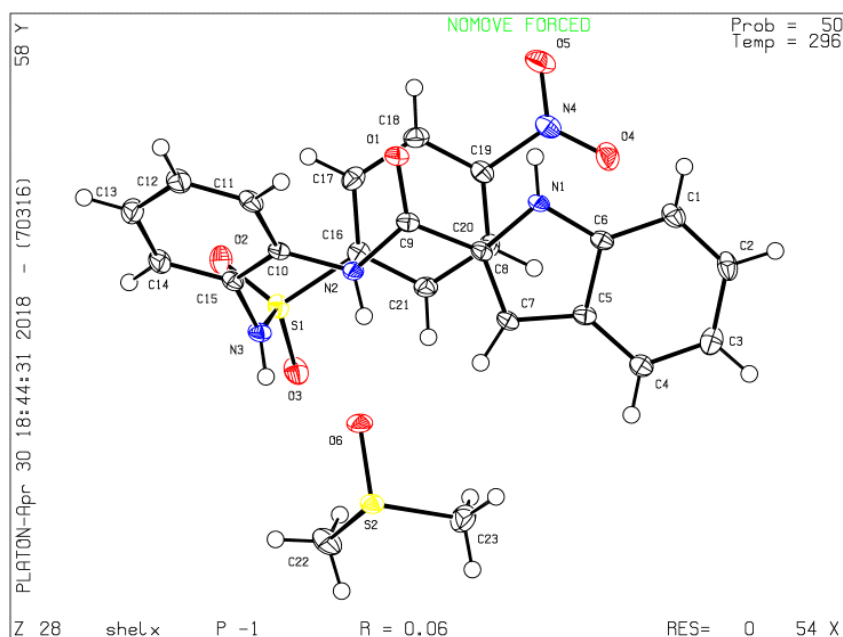


Figure 3.13 ORTEP diagram of X-ray crystal structure of [5c·DMSO].

Details of Co-crystal of 5g·DMSO: CCDC 1835297, $C_{25}H_{26}F_3N_3O_5S_3$, $M = 601.67$, colorless blocks, Monoclinic, space group $P 2_1/c$, Cell: $a = 19.508(2)$, $b = 8.2786(10)$, $c = 17.511(2)$ $V = 2713.5(5)$ $\text{\AA}^3$, $Z = 8$, $T = 296$ K, $\theta_{\max} = 28.363^\circ$, $D_{\text{calc}} (\text{g cm}^{-3}) = 1.473$, $F(000) = 1248$, $\mu (\text{mm}^{-1}) = 0.335$, 45772 reflections collected, 6778 unique reflections ($R_{\text{int}} = 0.0685$), $\text{GoF} = 0.905$, $R_1 = 0.0407$, $wR_2 = 0.1387$, R indices based on 5033 reflections with $I > 2s(I)$ (refinement on F^2). $\Delta\rho_{\max} = 0.544$, $\Delta\rho_{\min} = -0.507$ ($\text{e\AA}^{-3}$).

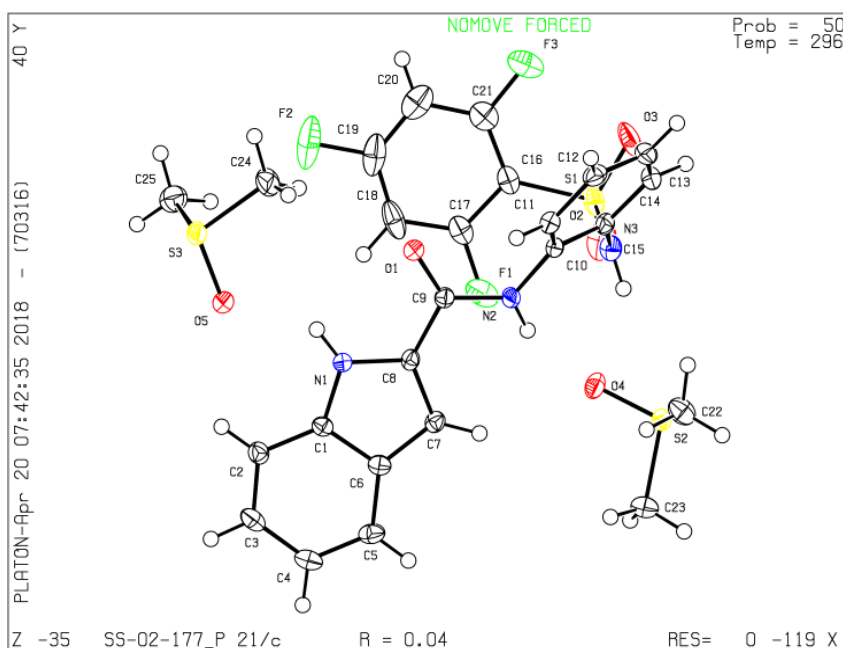


Figure 3.14 ORTEP diagram of X-ray crystal structure of [5g·DMSO].

Details of co-crystal of 5e with TBACl: CCDC 1835297, $C_{38}H_{52}ClF_3N_4O_{4.5}S$, $M = 761.34$, colorless blocks, Monoclinic, space group $C2/c$, Cell: $a = 20.234(4)$, $b = 13.246(2)$, $c = 30.365(6)$ $V = 7963(2)$ $\text{\AA}^3$, $Z = 8$, $T = 296$ K, $\theta_{\max} = 25.086^\circ$, $D_{\text{calc}} (\text{g cm}^{-3}) = 1.270$, $F(000) = 3232$, $\mu (\text{mm}^{-1}) = 0.207$, 38339 reflections collected, 7067 unique reflections ($R_{\text{int}} = 0.0625$), $\text{GoF} = 1.031$, $R_1 = 0.0888$, $wR_2 = 0.2830$, R indices based on 4668 reflections with $I > 2s(I)$ (refinement on F^2). $\Delta\rho_{\max} = 0.947$, $\Delta\rho_{\min} = -0.640$ ($\text{e\AA}^{-3}$).

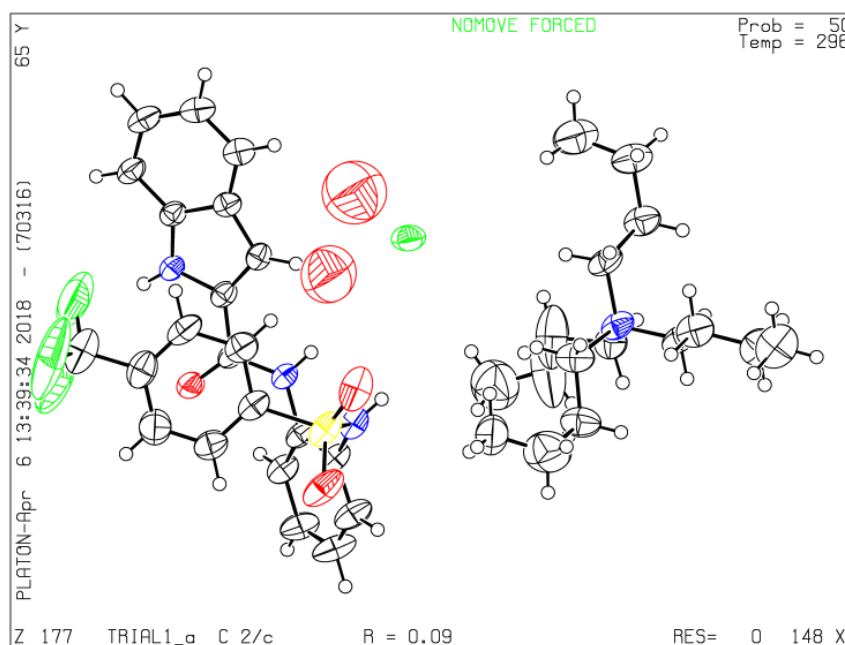


Figure 3.15 ORTEP diagram of **5e** co-crystalized with TBACl.

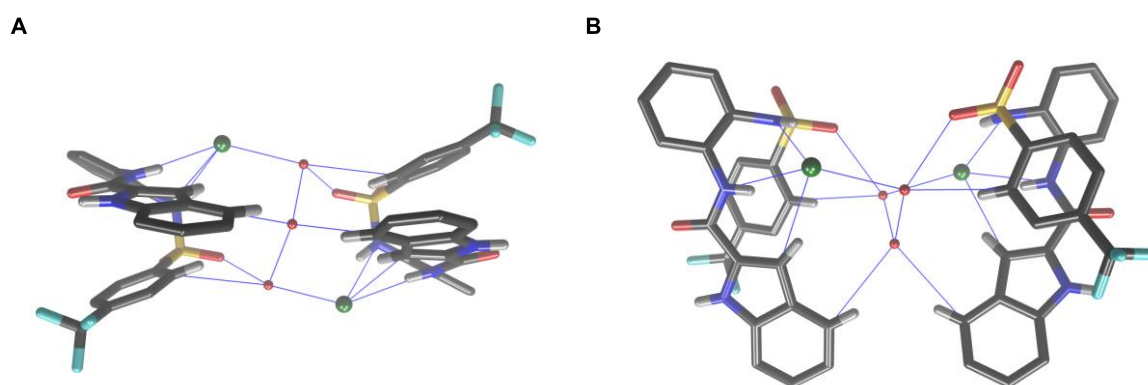


Figure 3.16 Top and back view of co-crystal of **5e·Cl⁻**.

3.5.5 Anion Binding Study by ^1H NMR Titration Experiment

The stock solutions of **5a-5g** (0.01M) and tetrabutylammonium chloride (1M) were prepared in either acetonitrile- d_3 or DMSO- d_6 . All the receptors and TBACl were dried in vacuum desiccator before use. ^1H NMR spectra were recorded on a Bruker AscentTM 400 spectrometer and calibrated with respect to residual protic solvent peak in Acetonitrile- d_6 ($\delta = 1.94$) or DMSO- d_6 ($\delta = 2.50$ ppm). NMR titrations were performed by successive addition of aliquots of the apparent anionic guest as the tetrabutylammonium (TBA) salt. In acetonitrile- d_6 , a change in the chemical shift of all three NH (Amide NH, indole NH, and Sulfonamide NH) was observed. However, in DMSO- d_6 , only the change in chemical shift of amide NH, and Sulfonamide NH protons were observed. Due to involvement of indolic NH in hydrogen bonding interactions with DMSO, it is not involved in Cl^- binding. The ^1H NMR stacked plots were processed using MestReNova 6 software. To obtain the binding constants data was curve fitted with 1:1 binding model of the WinEQNMR2 computer program.

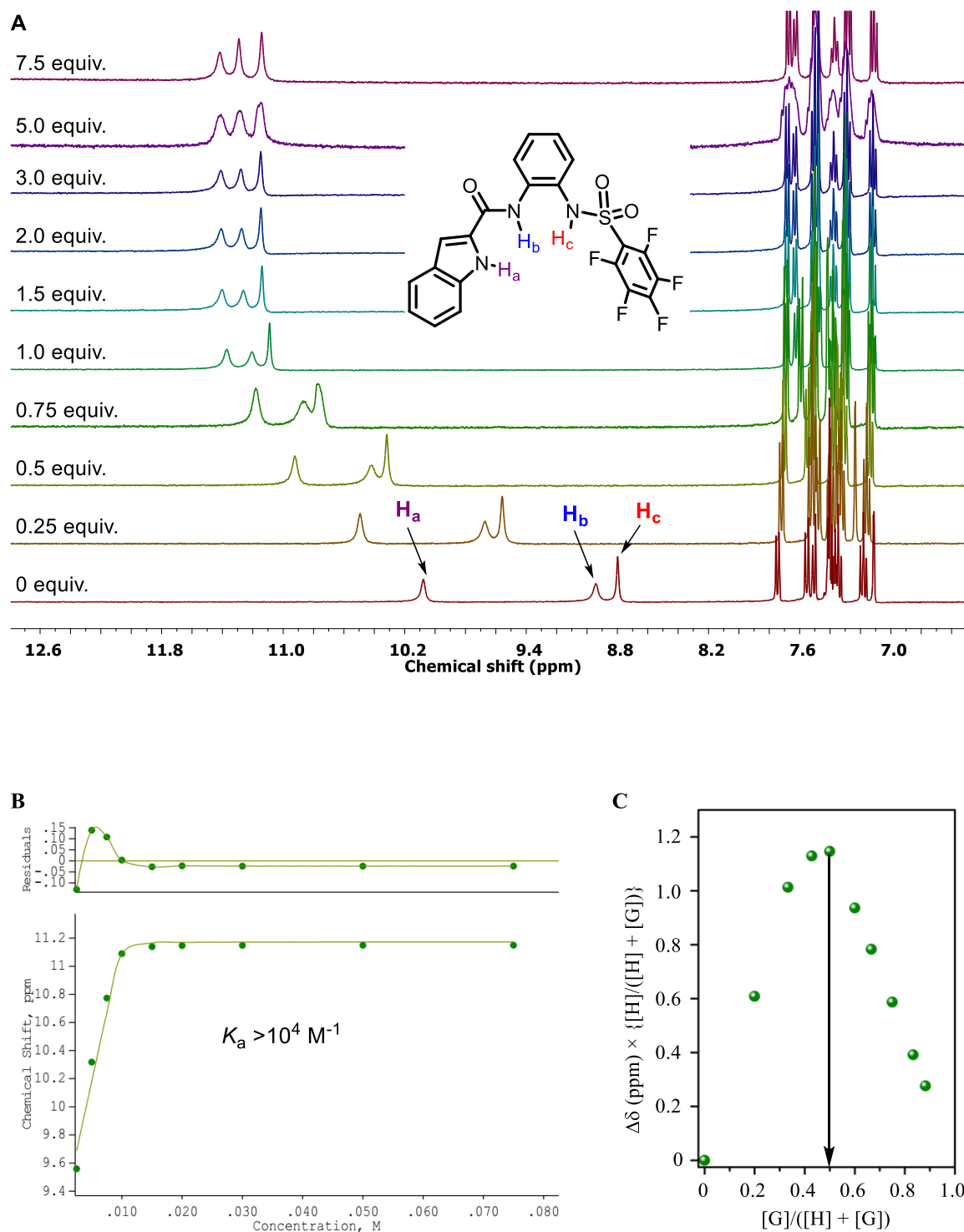


Figure 3.17 ^1H NMR titration spectra for **5a** with stepwise addition of TBACl in acetonitrile- d_3 at 25 °C. The equivalents of added TBACl are shown on the stacked spectra (A). Fit plot of chemical shift (δ ppm) of amide NH_a proton versus concentration of TBACl fitted to 1:1 binding model of WinEQNMR2 program (B). Job's plot generated from the same ^1H NMR titration indicating 1:1 binding of **5a**: Cl^- (C).

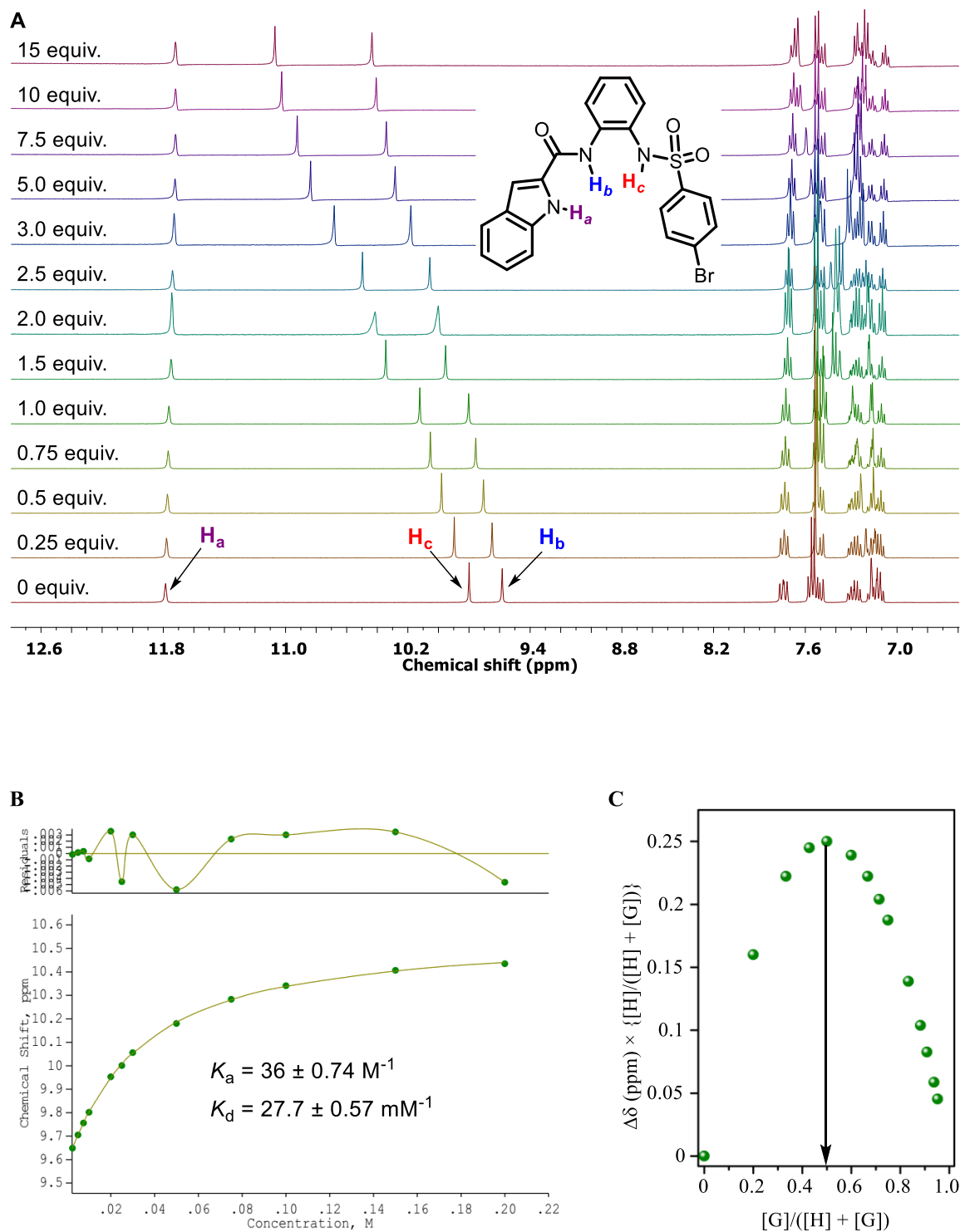


Figure 3.18 ^1H NMR titration spectra for **5b** with stepwise addition of TBACl in acetonitrile- d_3 at 25 °C. The equivalents of added TBACl are shown on the stacked spectra (A). Fit plot of chemical shift (δ ppm) of amide NH_a proton versus concentration of TBACl fitted to 1:1 binding model of WinEQNMR2 program (B). Job's plot generated from the same ^1H NMR titration indicating 1:1 binding of **5b**: Cl^- (C).

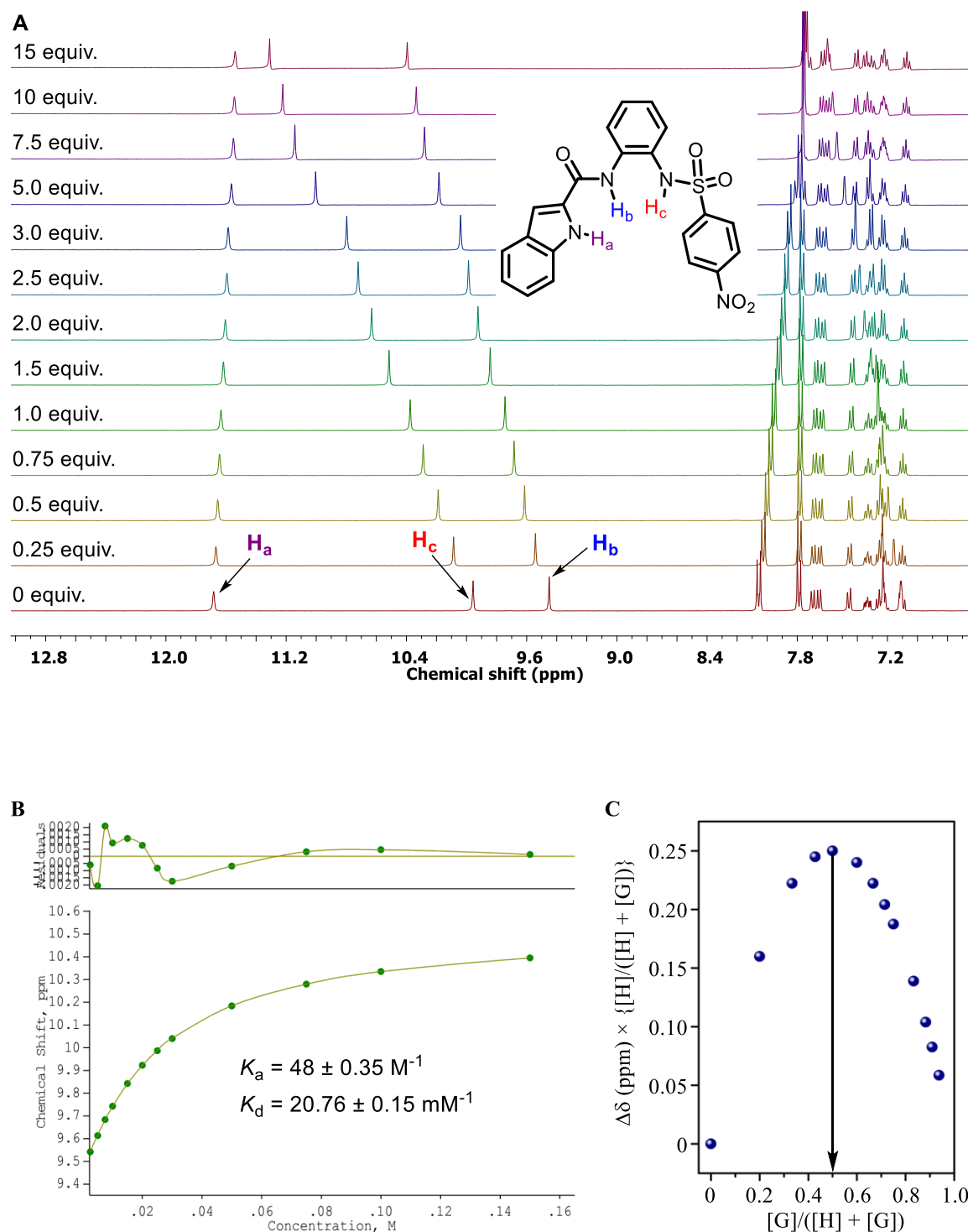


Figure 3.19 ^1H NMR titration spectra for **5c** with stepwise addition of TBACl in acetonitrile- d_3 at 25 °C. The equivalents of added TBACl are shown on the stacked spectra (A). Fit plot of chemical shift (δ ppm) of amide NH_a proton versus concentration of TBACl fitted to 1:1 binding model of WinEQNMR2 program (B). Job's plot generated from the same ^1H NMR titration indicating 1:1 binding of **5c**: Cl^- (C).

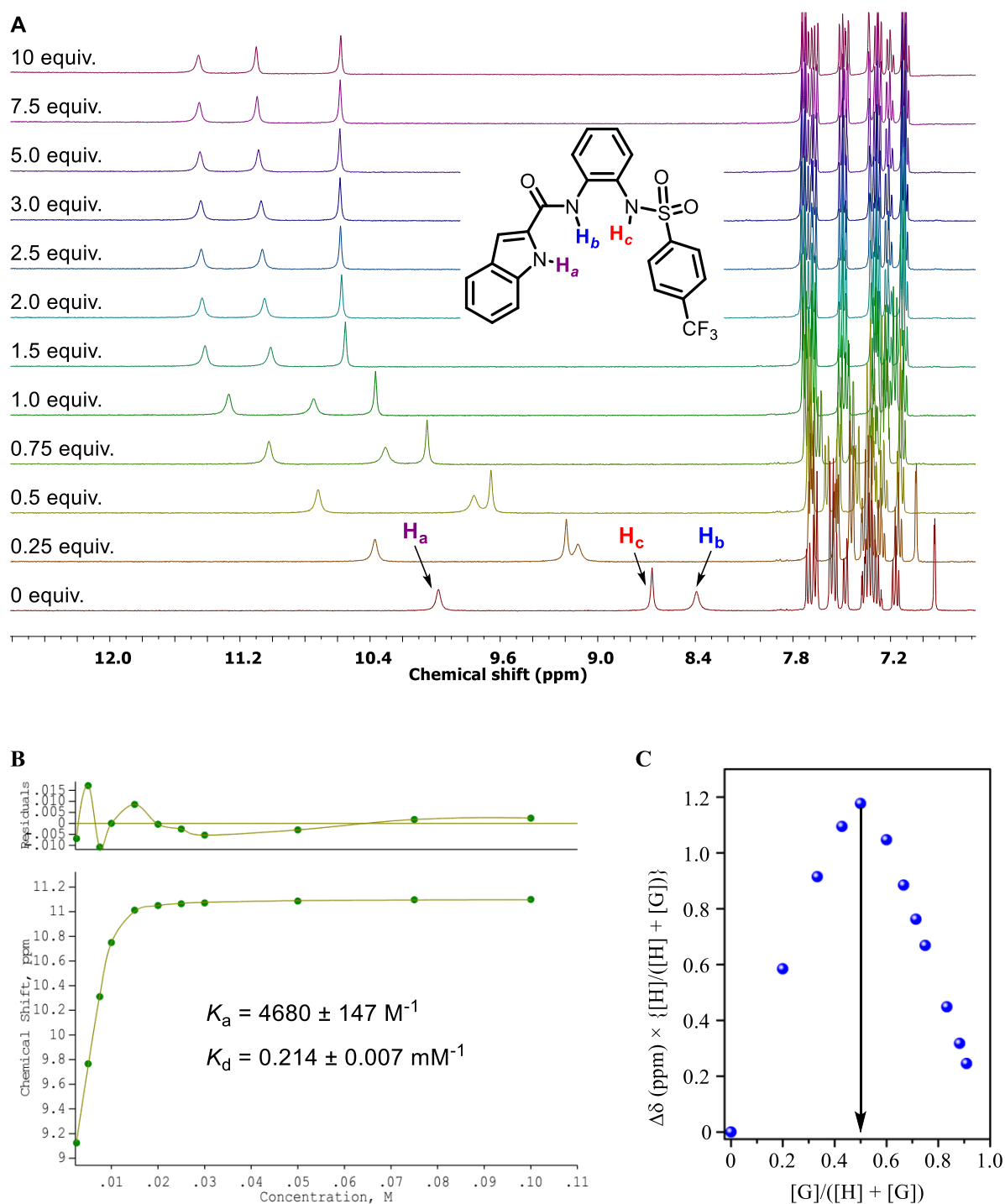


Figure 3.20 ^1H NMR titration spectra for **5e** with stepwise addition of TBACl in acetonitrile- d_3 at 25 °C. The equivalents of added TBACl are shown on the stacked spectra (A). Fit plot of chemical shift (δ ppm) of amide NH_a proton versus concentration of TBACl fitted to 1:1 binding model of WinEQNMR2 program (B). Job's plot generated from the same ^1H NMR titration indicating 1:1 binding of **5e**: Cl^- (C).

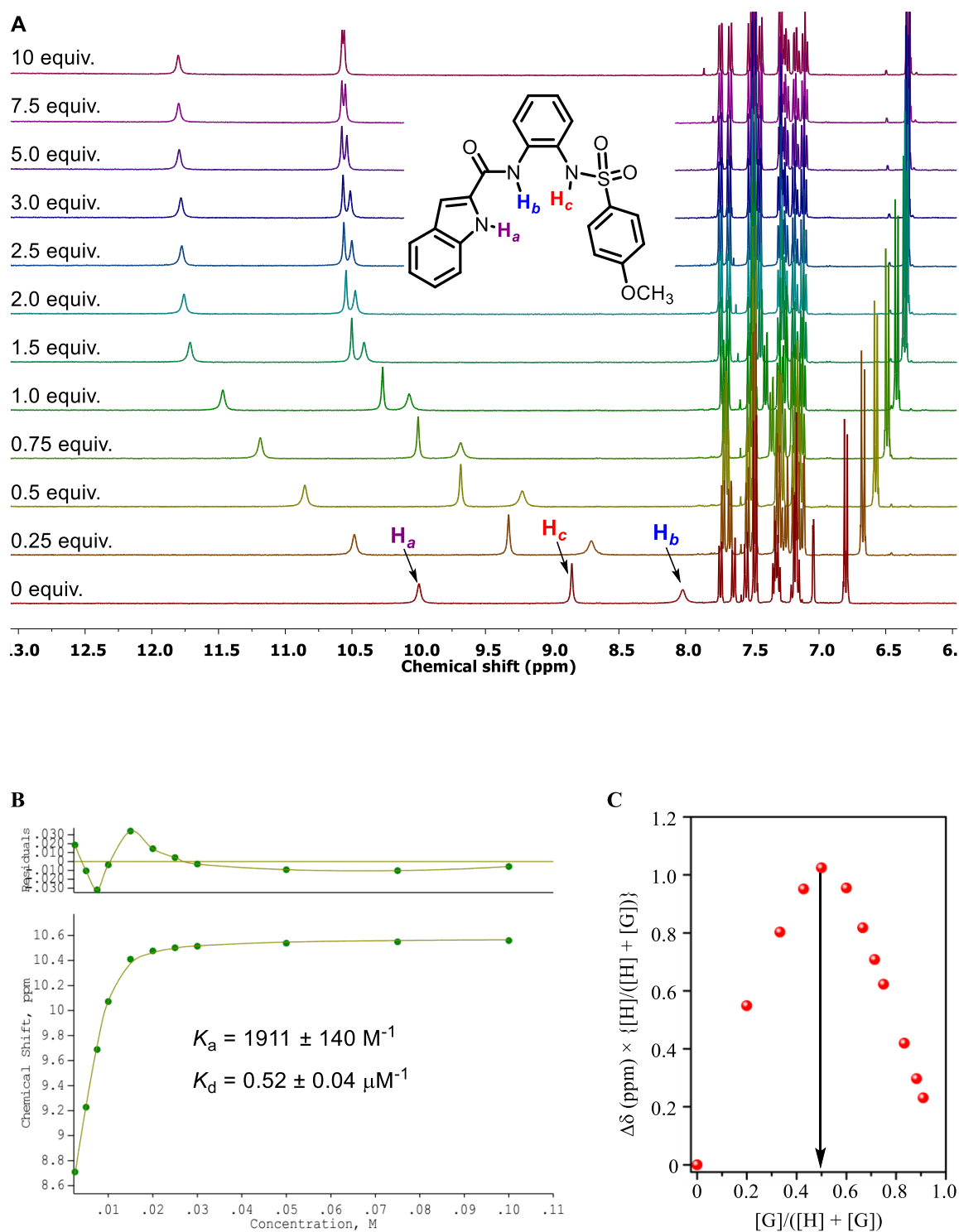


Figure 3.21 ^1H NMR titration spectra for **5f** with stepwise addition of TBACl in acetonitrile- d_3 at 25 °C. The equivalents of added TBACl are shown on the stacked spectra (A). Fit plot of chemical shift (δ ppm) of amide NH_a proton versus concentration of TBACl fitted to 1:1 binding model of WinEQNMR2 program (B). Job's plot generated from the same ^1H NMR titration indicating 1:1 binding of **5f**: Cl^- (C).

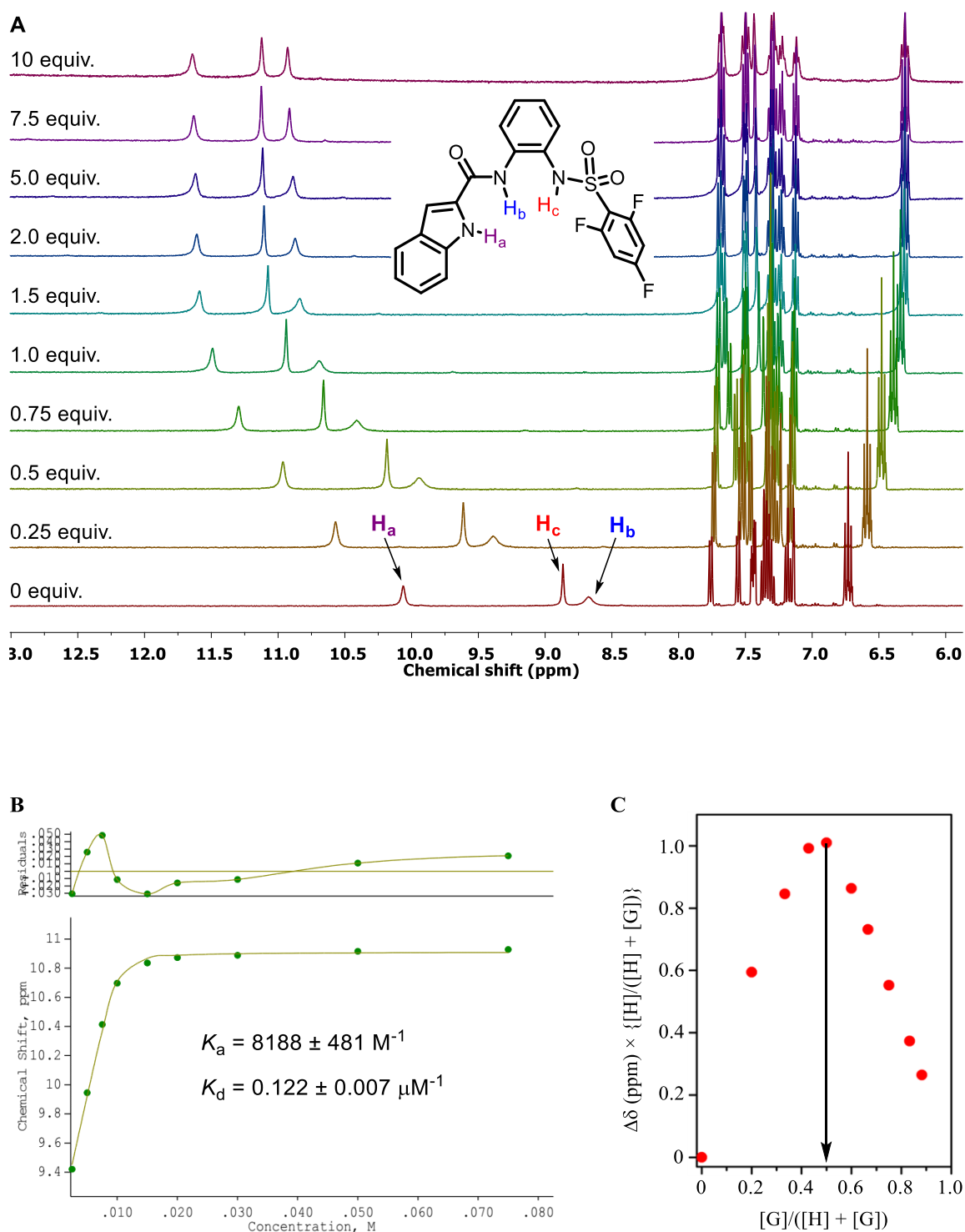


Figure 3.22 ^1H NMR titration spectra for **5g** with stepwise addition of TBACl in acetonitrile- d_3 at 25 °C. The equivalents of added TBACl are shown on the stacked spectra (A). Fit plot of chemical shift (δ ppm) of amide NH_a proton versus concentration of TBACl fitted to 1:1 binding model of WinEQNMR2 program (B). Job's plot generated from the same ^1H NMR titration indicating 1:1 binding of **5g**: Cl^- (C).

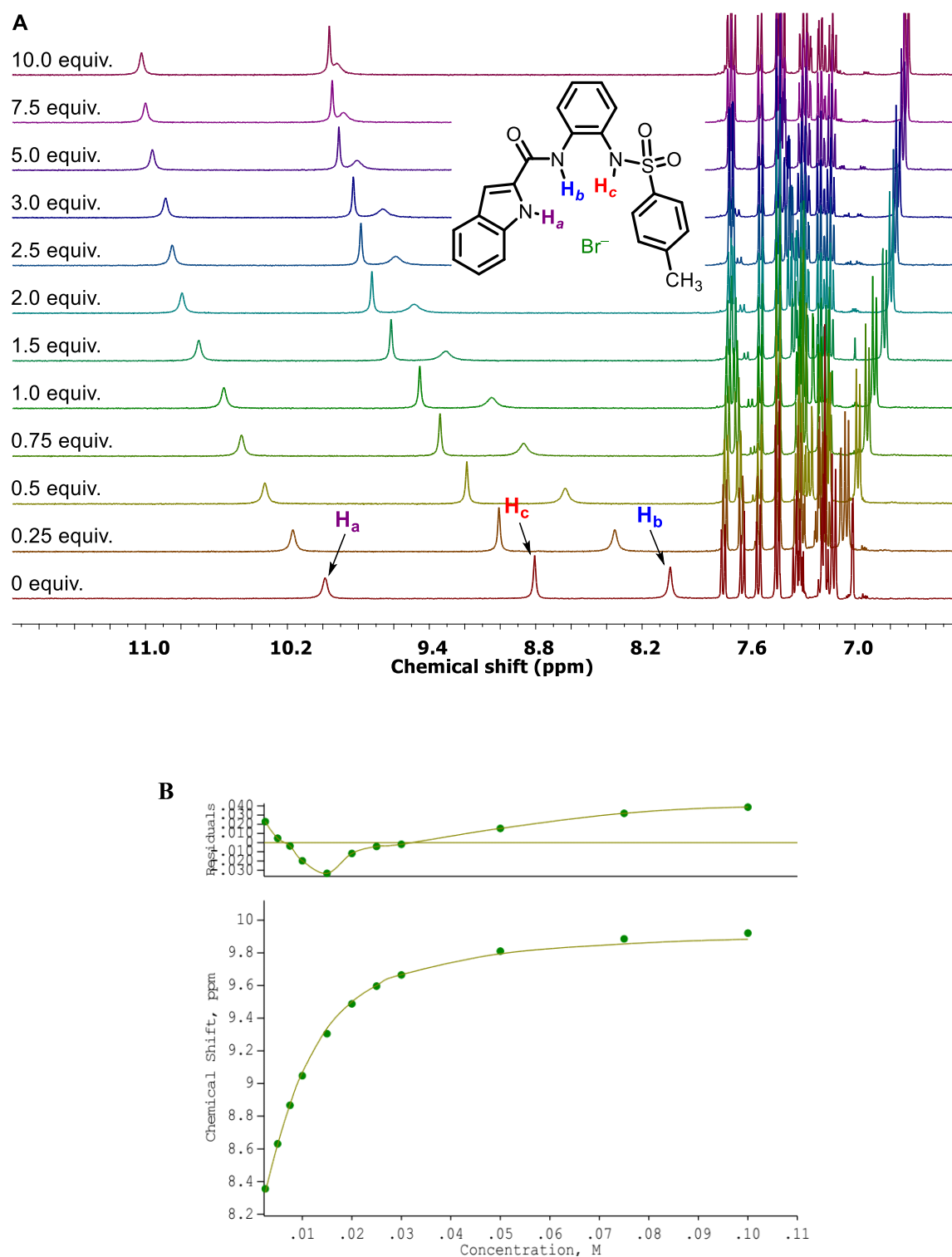


Figure 3.23 ^1H NMR titration spectra for **5d** with stepwise addition of TBABr in acetonitrile- d_3 at 25 °C. The equivalents of added TBABr are shown on the stacked spectra (A). Fit plot of chemical shift (δ ppm) of amide NH_a proton versus concentration of TBABr fitted to 1:1 binding model of WinEQNMR2 program (B)

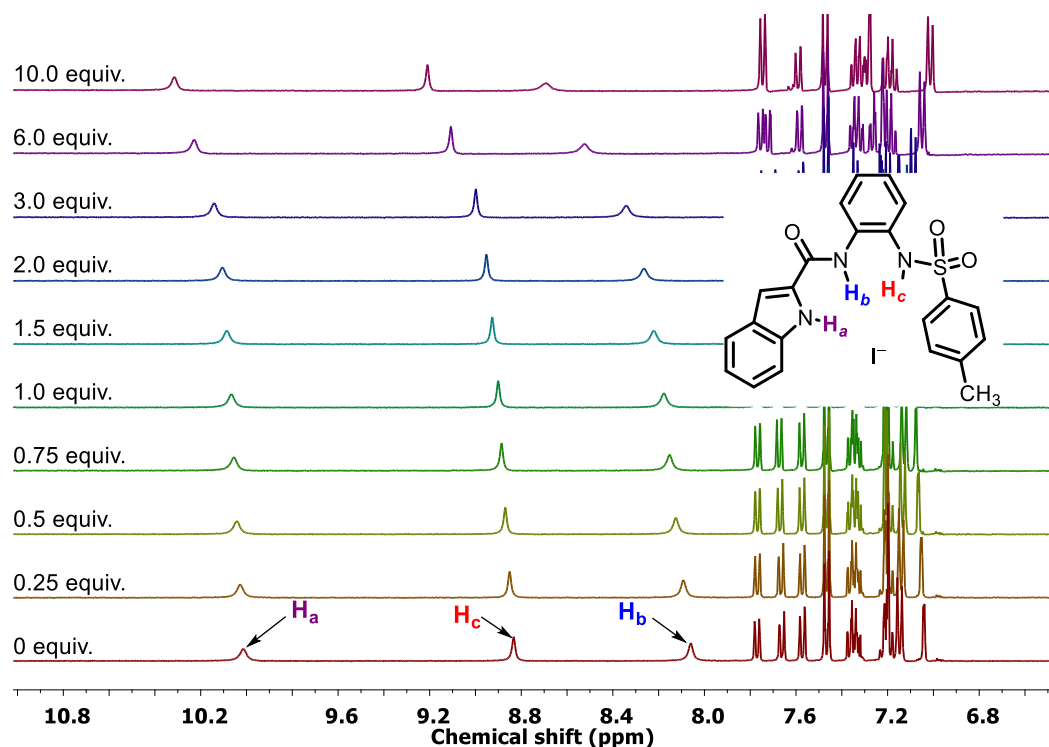


Figure 3.24 ^1H NMR titration spectra for **5d** with stepwise addition of TBAI in acetonitrile- d_3 at 25 °C. The equivalents of added TBAI are shown on the stacked spectra (A).

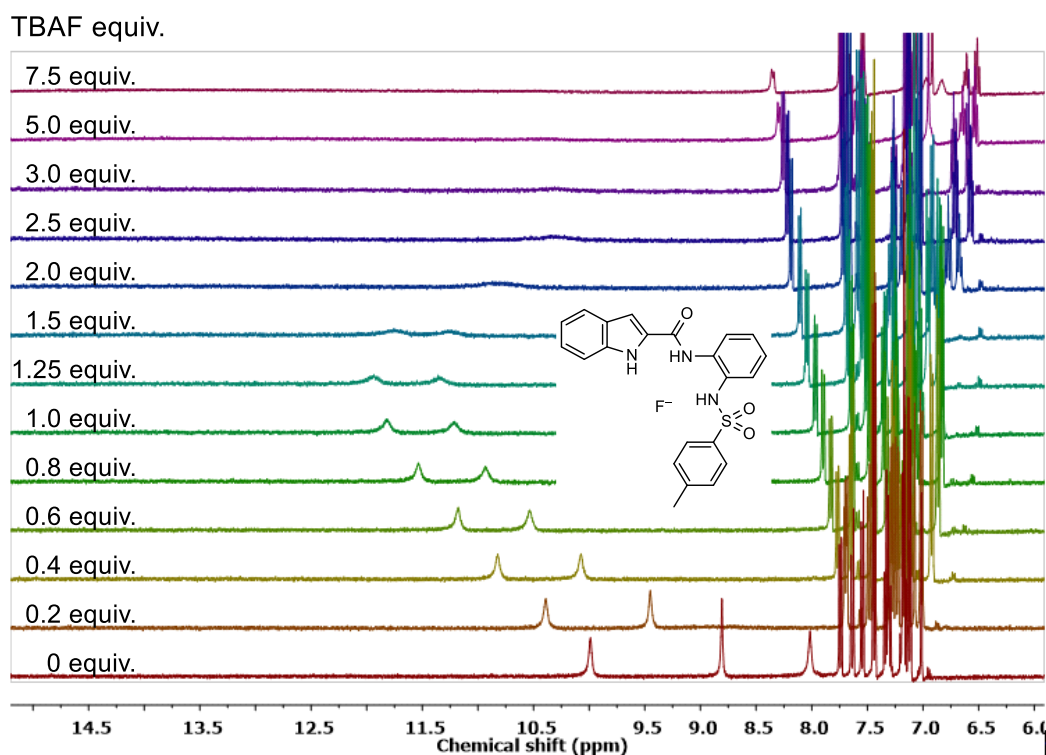


Figure 3.25 ^1H NMR titration spectra for **1d** with stepwise addition of TBAF in acetonitrile- d_3 at 25 °C. The equivalents of added TBAF are shown on the stacked spectra.

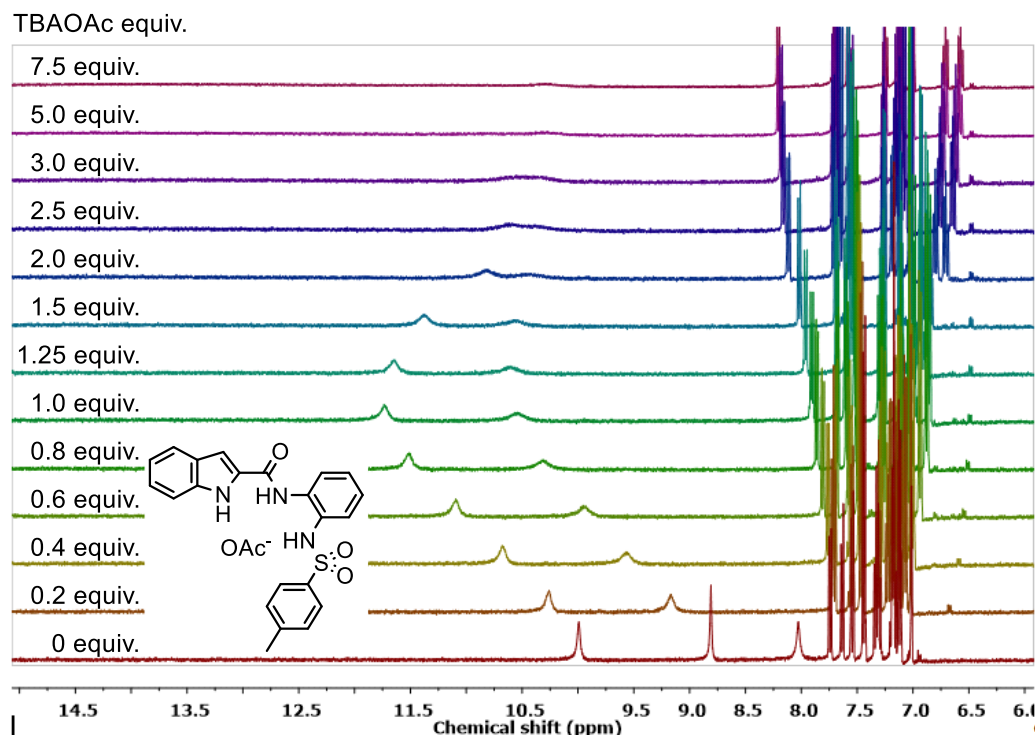


Figure 3.26 ^1H NMR titration spectra for **1d** with stepwise addition of TBAOAc (**B**) in acetonitrile- d_3 at 25 °C. The equivalents of added TBAOAc are shown on the stacked spectra.

3.5.6 Ion Transport Experiments

Ion Transport Activity and Selectivity

Ion transport activity and selectivity assays across EYPC-LUVs $\supset$ HPTS performed according to the similar methods as described in the chapter 2.

Preparation of EYPC-LUVs $\supset$ Lucigenin Vesicle

In a small clean and dry round bottom flask a transparent thin film of egg yolk phosphatidylcholine (EYPC) was prepared by drying a 1 mL of EYPC lipid (25 mg/ mL in CHCl_3) with continuous rotation and purging of nitrogen gas. The transparent film formed was kept in high vacuum for 4h to remove all the traces of CHCl_3 . The transparent lipid film was hydrated with 1 mL of buffer (Sodium phosphate 5mM, NaNO_3 200 mM, 1 mM lucigenin, pH 7) solution. The resulting suspension was vortexed at an interval of 10 minutes during 60 minutes and subjected to ≥ 10 freeze-thaw cycles (liq. N_2 , 55 °C water bath), and >19 times (must be an odd number) extruded through a polycarbonate membrane (pore size 200 nm). Extravesicular dye was removed by size

exclusion chromatography using Sephadex G-50 (Sigma Aldrich) eluting with aqueous NaNO_3 (200 mM) and diluted to 3 mL.

In a clean and dry fluorescence cuvette, 1975 μL of buffer (5 mM Na-phosphate, 200 mM NaNO_3 , pH 7.0) was added, followed by the addition of 25 μL EYPC-LUV $\Rightarrow$ Lucigenin. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The time-dependent change in fluorescence intensity at $\lambda_{\text{em}} = 510$ nm was monitored by excitation at wavelength $\lambda_{\text{ex}} = 455$ nm during the addition of NaCl (33 mM to create Cl^- gradient) at $t = 20$ s, (In valinomycin coupled assay, valinomycin added at 50 sec) transporter molecule in acetonitrile solution added at $t = 100$ s, and finally 10% Triton X-100 (25 μL .) at $t = 300$ s was added to lyse the vesicle resulting in complete destruction of chloride gradient. The collected data was then normalized to the percentage change in fluorescence intensity as a course of time using the equation 3.

$$I_F = (I_t - I_0) / (I_\infty - I_0) \times (-100) \quad \text{Equation 3.}$$

Where, I_0 is the initial intensity, I_t is the intensity at time t and I_∞ is the final intensity after addition of Triton X-100.

FCCP and Valinomycin Assay

FCCP Assay:

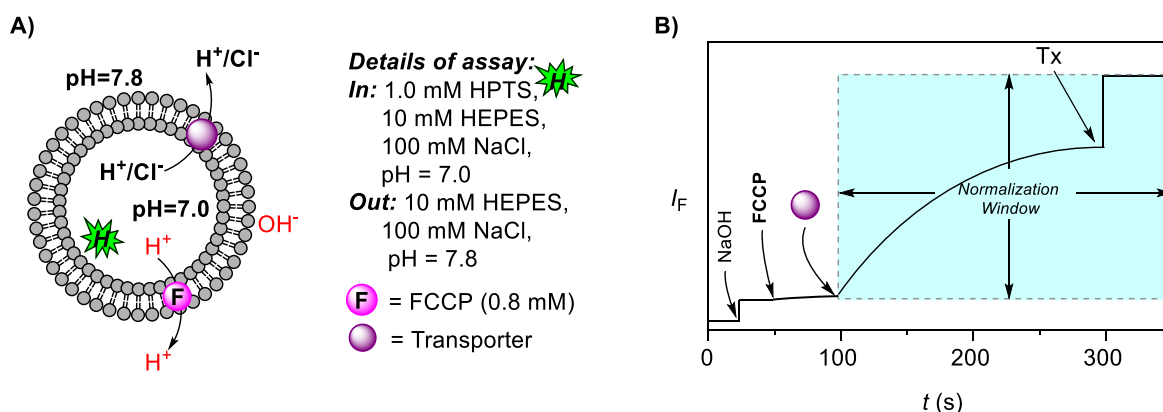


Figure 3.27 Representative fluorescence-based FCCP coupled assay using EYPC-LUV (A) and representation of ion transport kinetics showing normalization window (B).

The Vesicles were prepared by following the same procedure as mentioned above. The data were recorded with and without FCCP. In a clean and dry fluorescence cuvette, 1975 μL of buffer (10 mM HEPES, 100 mM NaCl, pH 7.0) was added, followed by the

addition of 25 μL EYPC-LUV $\rightarrow$ HPTS dye. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The time-dependent change in fluorescence intensity at $\lambda_{\text{em}} = 510 \text{ nm}$ was monitored by excitation at wavelength $\lambda_{\text{ex}} = 450 \text{ nm}$ during the addition of base (20 μL , 0.5 M NaOH to create pH gradient) at $t = 20 \text{ s}$, FCCP (0.8 μM) at 50 s, transporter molecule in DMSO solution at $t = 100 \text{ s}$, and finally 10% Triton X-100 (25 μL) at $t = 300 \text{ s}$ was added to lyse the vesicle resulting in complete destruction of pH gradient. The collected data was then normalized to the percentage change in fluorescence intensity as a course of time using the equation S1. It was observed that the presence of FCCP did not enhance transport rate, demonstrating that ion transport mechanism involves the symport process, probably H^+/Cl^- symport which delegates the charge balance.

Valinomycin Assay:

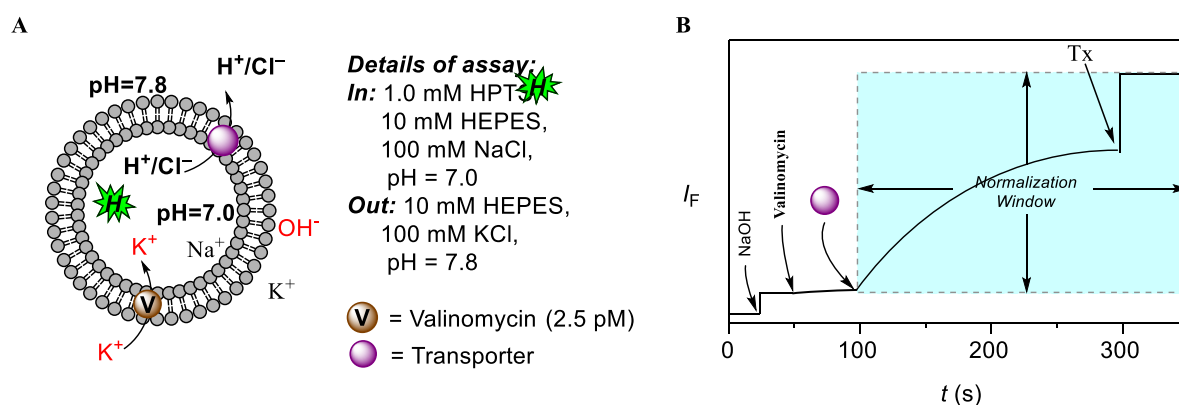


Figure 3.28 Representative fluorescence-based Valinomycin coupled assay using EYPC-LUV (A), and representation of ion transport kinetics showing normalization window (B).

The vesicles were prepared by following the same procedure as mentioned above. The data were recorded with and without the addition of valinomycin. In a clean and dry fluorescence cuvette, 1975 μL of buffer (10 mM HEPES, 100 mM KCl, pH 7.0) was added, followed by the addition of 25 μL EYPC-LUV $\rightarrow$ HPTS dye. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The time-dependent change in fluorescence intensity at $\lambda_{\text{em}} = 510 \text{ nm}$ was monitored by excitation at wavelength $\lambda_{\text{ex}} = 450 \text{ nm}$ during the addition of base (20 μL , 0.5 M NaOH to create pH gradient) at $t = 20 \text{ s}$, valinomycin (2.5 μM) at 50 s, transporter molecule in DMSO solution at $t = 100 \text{ s}$ and finally 10% Triton X-100 (25 μL) was added at $t = 300 \text{ s}$

to lyse the vesicle resulting in complete destruction of pH gradient. The collected data was then normalized to the percentage change in fluorescence intensity as a course of time using the equation S1.

3.5 NMR Spectra

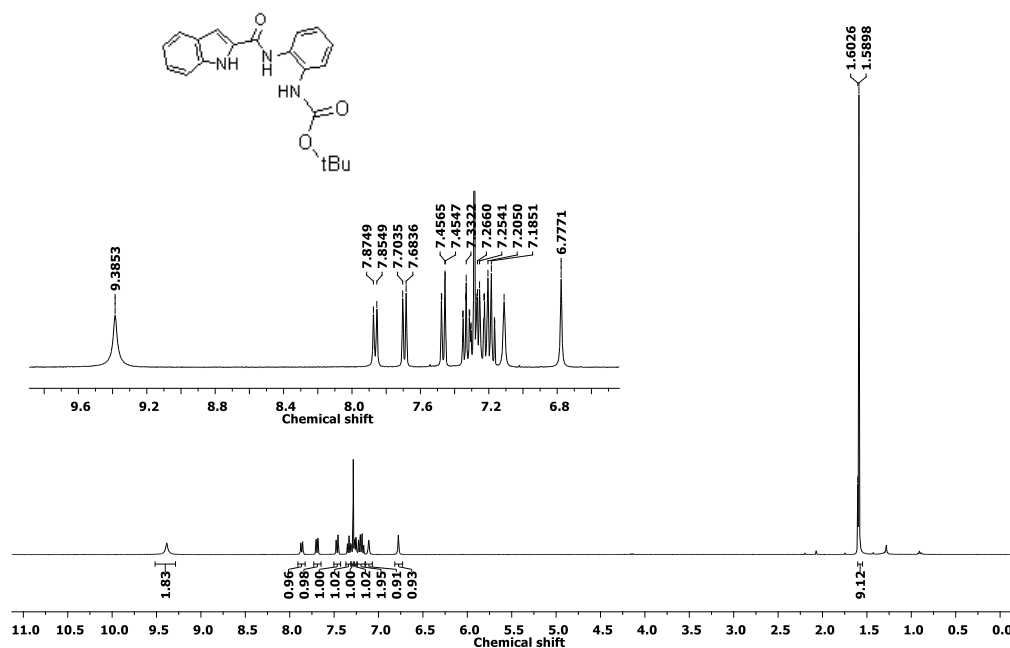


Figure 3.29 ¹H NMR spectrum of *tert*-butyl (2-(1*H*-indole-2-carboxamido)phenyl)carbamate **8**.

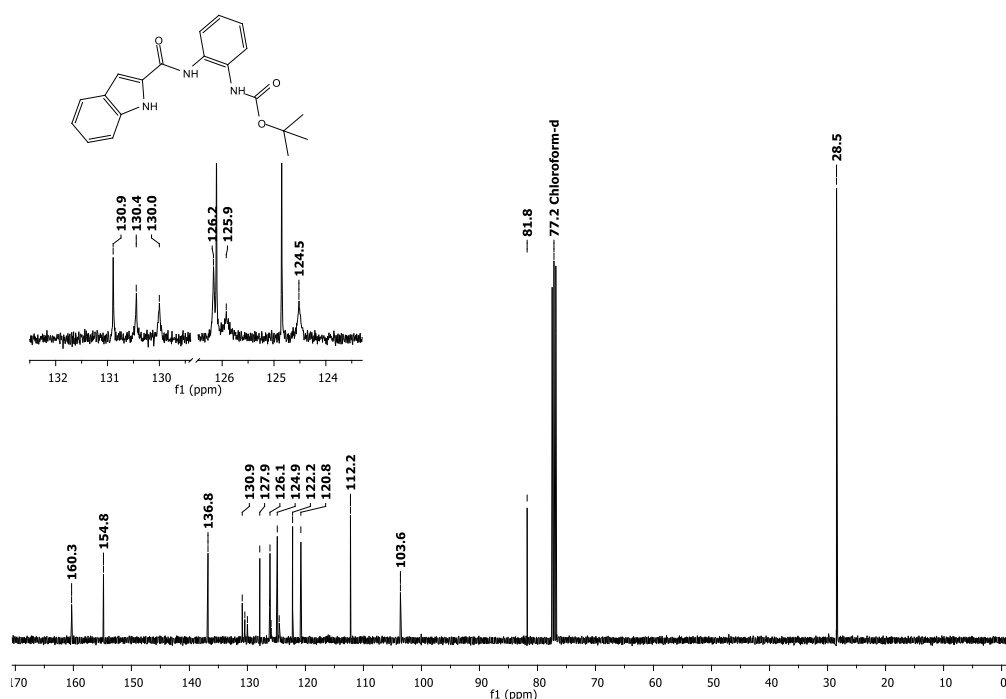


Figure 3.30 ¹³C NMR spectrum of *tert*-butyl (2-(1*H*-indole-2-carboxamido)phenyl)carbamate **8**.

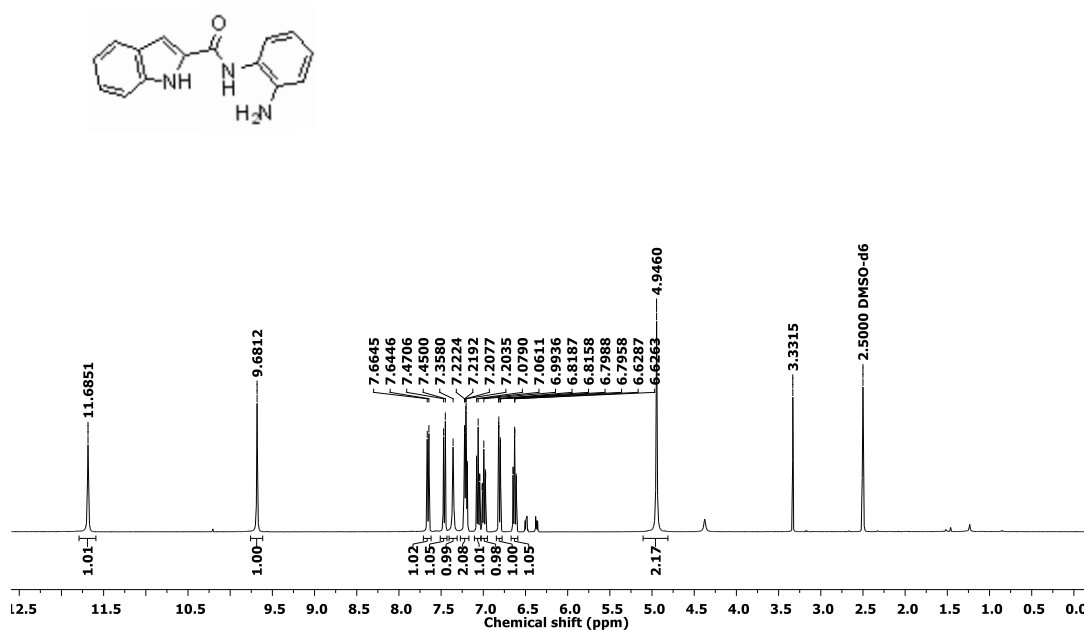


Figure 3.31 ^1H -NMR spectrum of *N*-(2-aminophenyl)-1*H*-indole-2-carboxamide **9**.

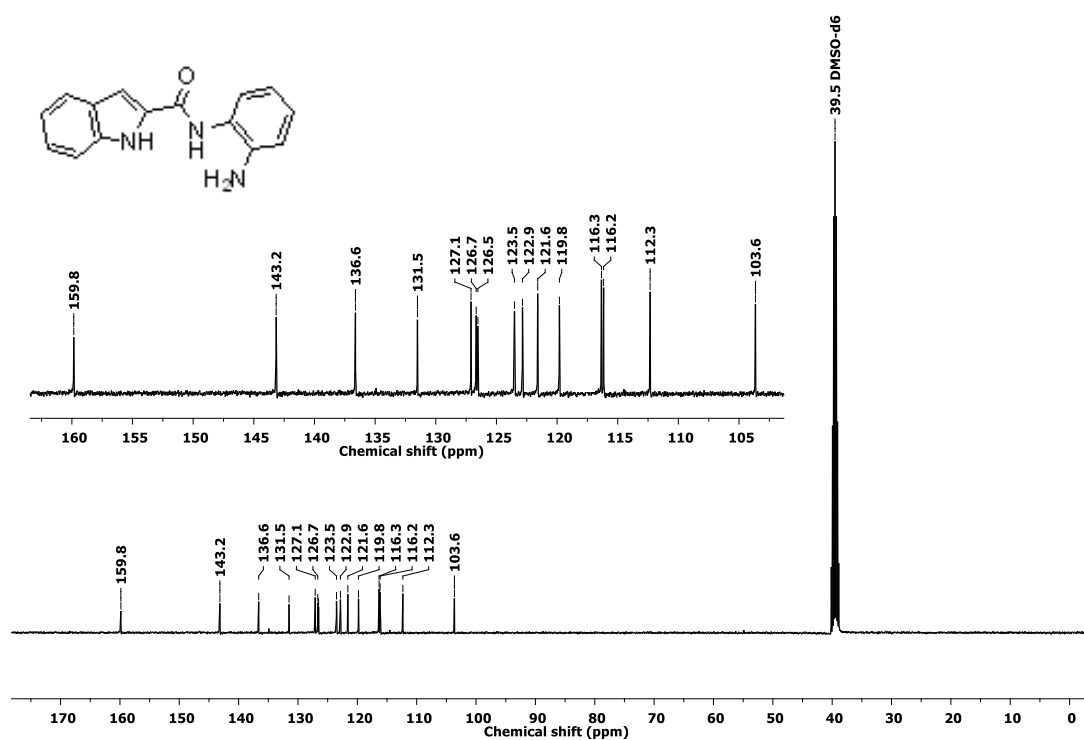


Figure 3.32 ^{13}C NMR spectrum of *N*-(2-aminophenyl)-1*H*-indole-2-carboxamide **9**.

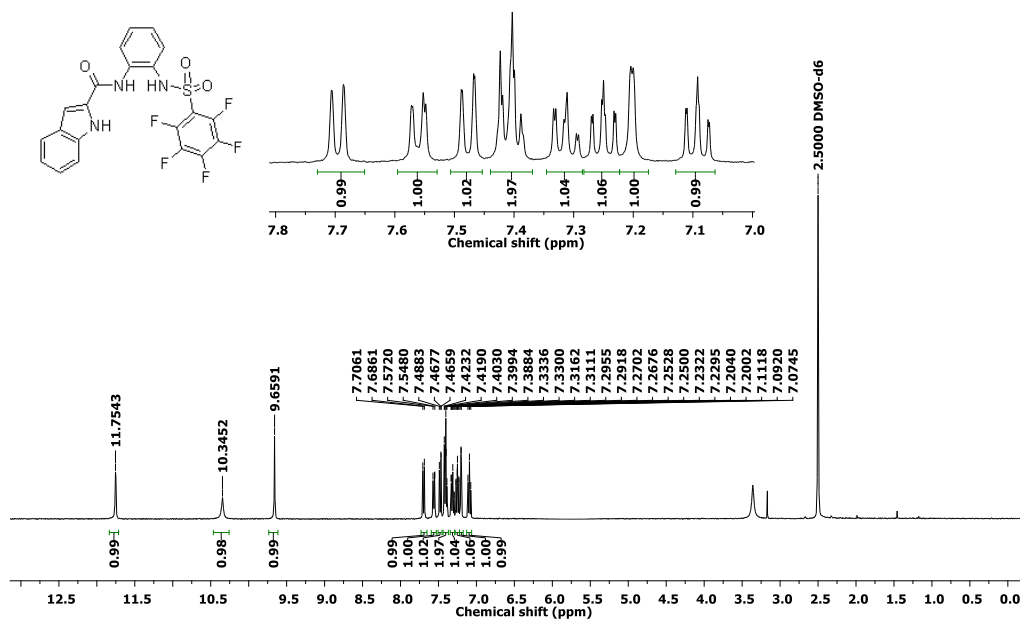


Figure 3.33 ¹H NMR spectrum of *N*-(2-((perfluorophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5a**.

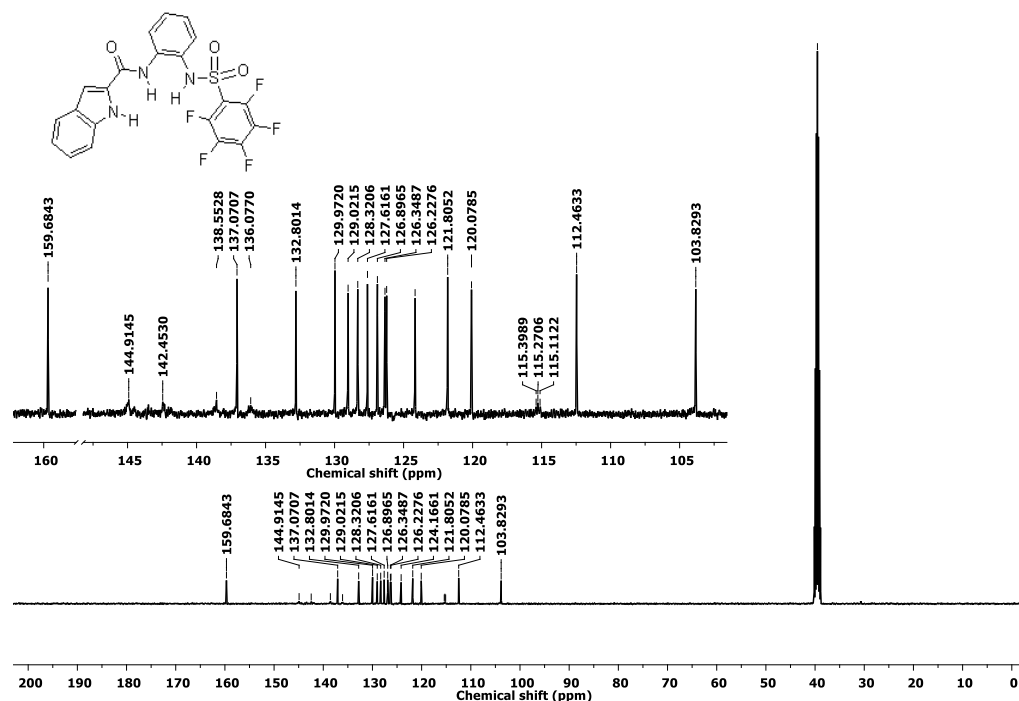


Figure 3.34 ¹³C NMR spectrum of *N*-(2-((perfluorophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5a**.

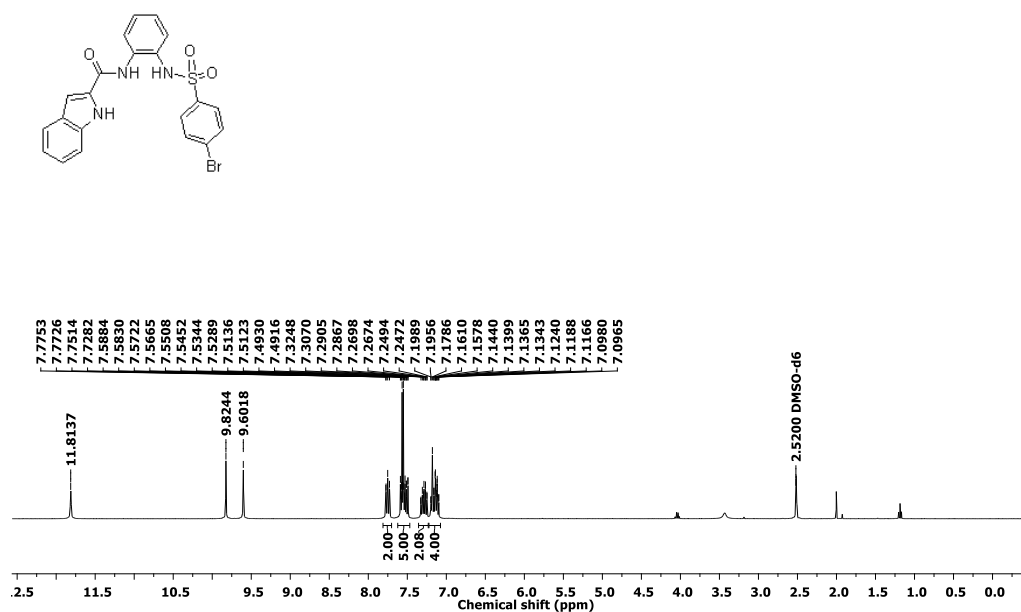


Figure 3.35 ¹H NMR spectrum of *N*-(2-((4-bromophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5b**.

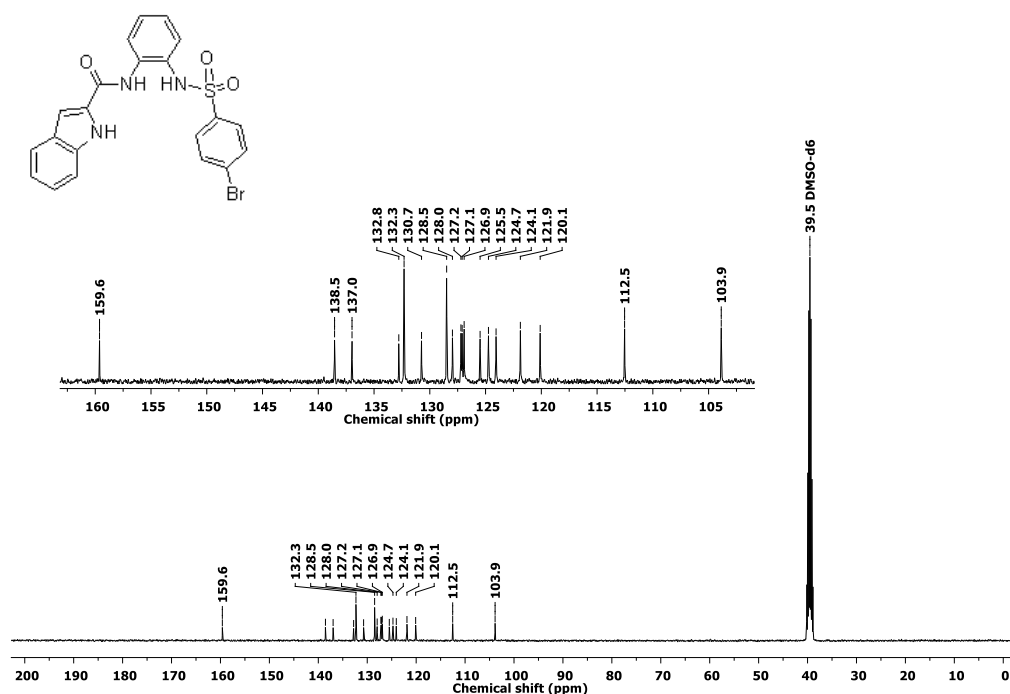


Figure 3.36 ¹³C NMR spectrum of *N*-(2-((4-bromophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5b**.

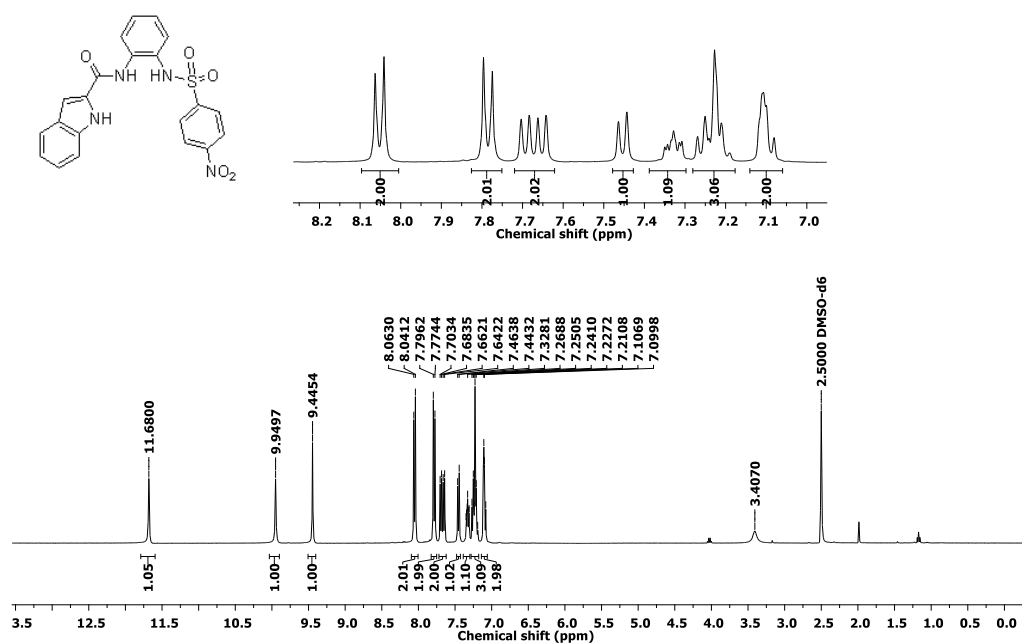


Figure 3.37 ¹H NMR spectrum of *N*-(2-((4-nitrophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5c**.

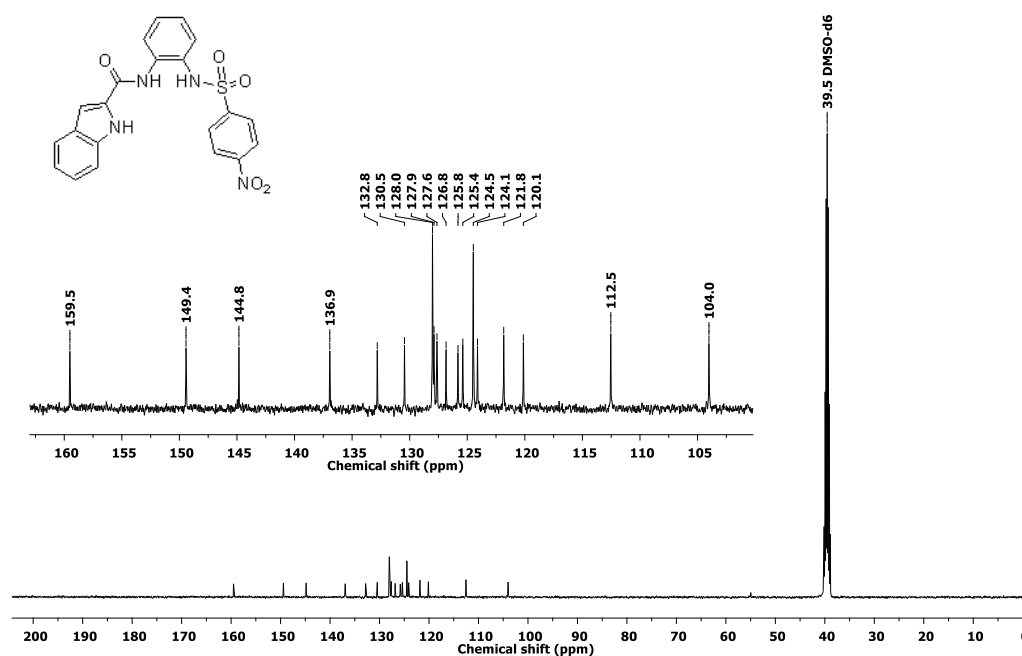


Figure 3.38 ¹³C NMR spectrum of *N*-(2-((4-nitrophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5c**.

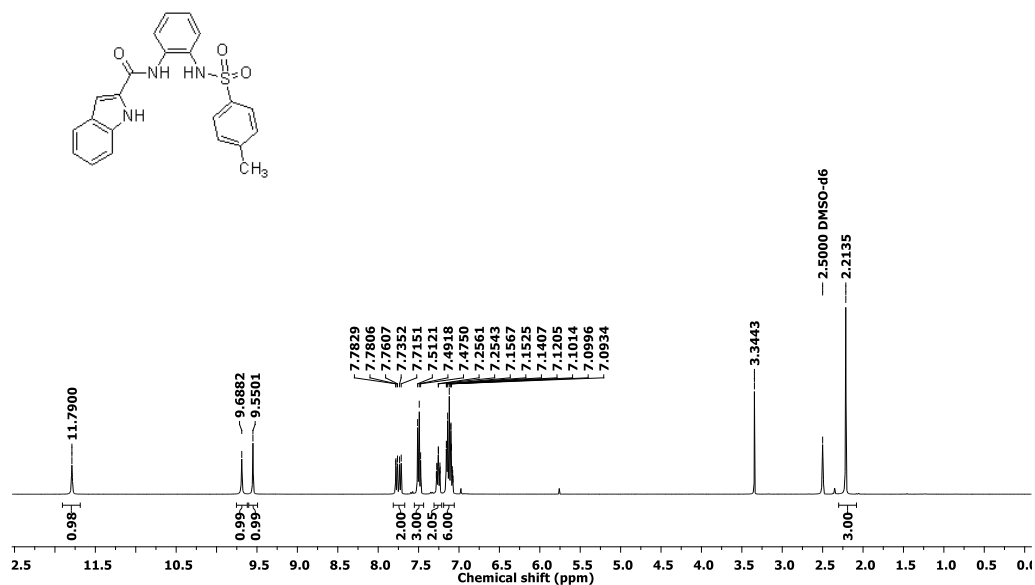


Figure 3.39 ¹H NMR spectrum of *N*-(2-((4-methylphenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5d**.

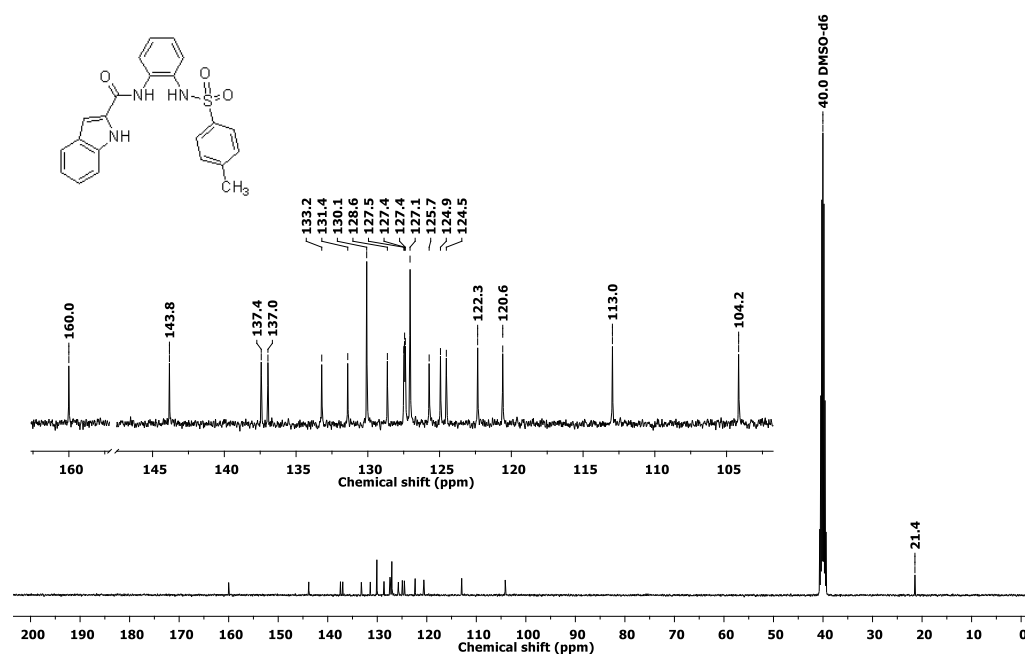


Figure 3.40 ¹³C NMR spectrum of *N*-(2-((4-methylphenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5d**.

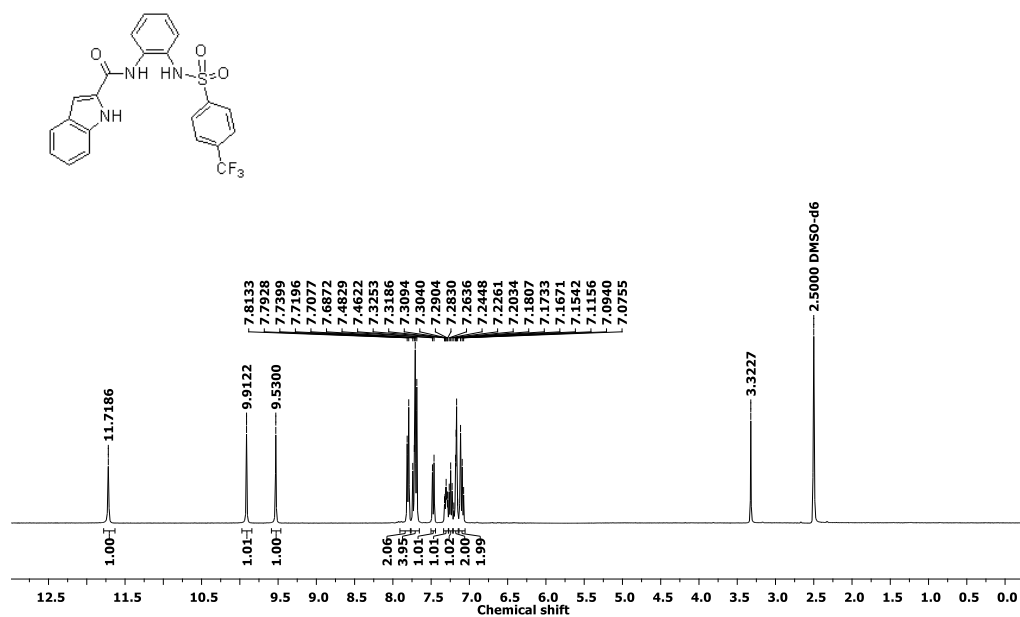


Figure 3.41 ¹H NMR spectrum of *N*-(2-((4-(trifluoromethyl)phenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide 5e.

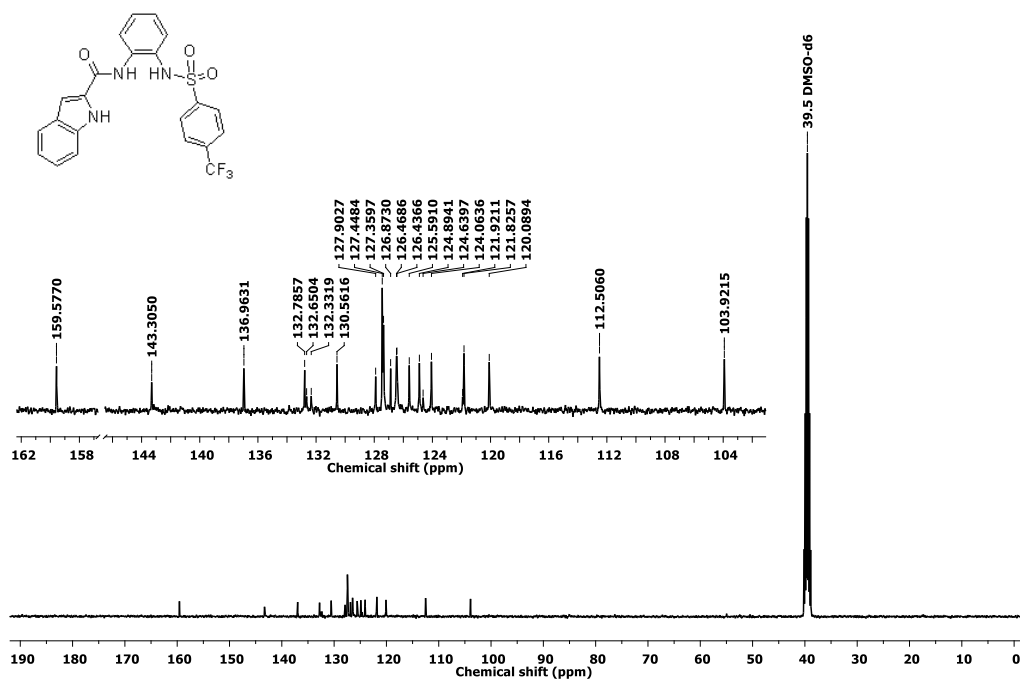


Figure 3.42 ¹³C NMR spectrum of *N*-(2-((4-(trifluoromethyl)phenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide 5e.

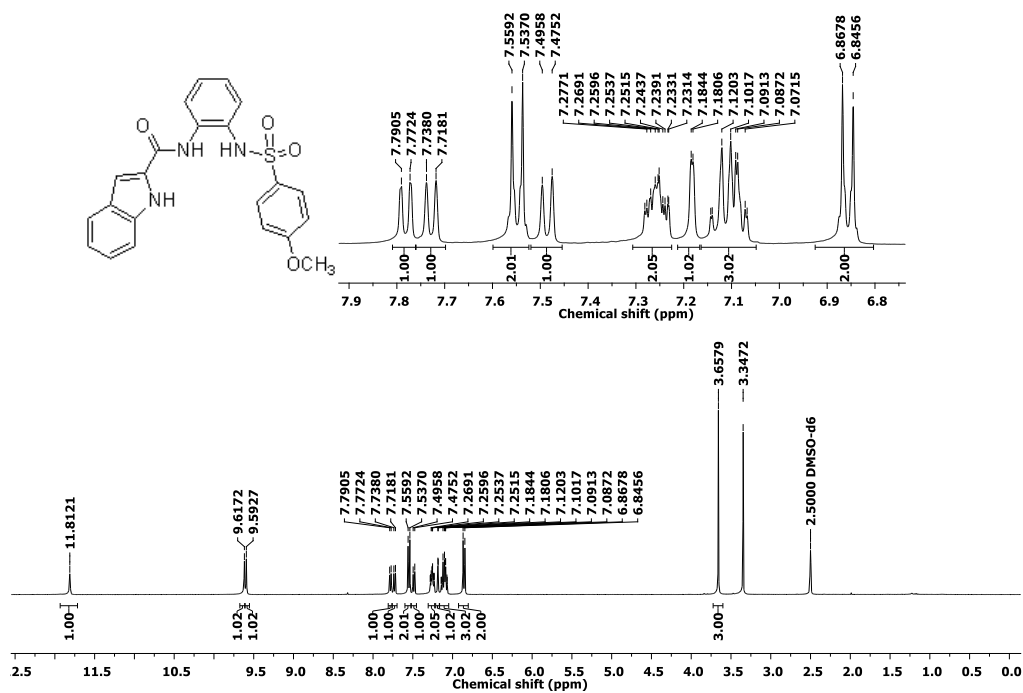


Figure 3.43 ¹H NMR spectrum of *N*-(2-((4-methoxyphenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5f**.

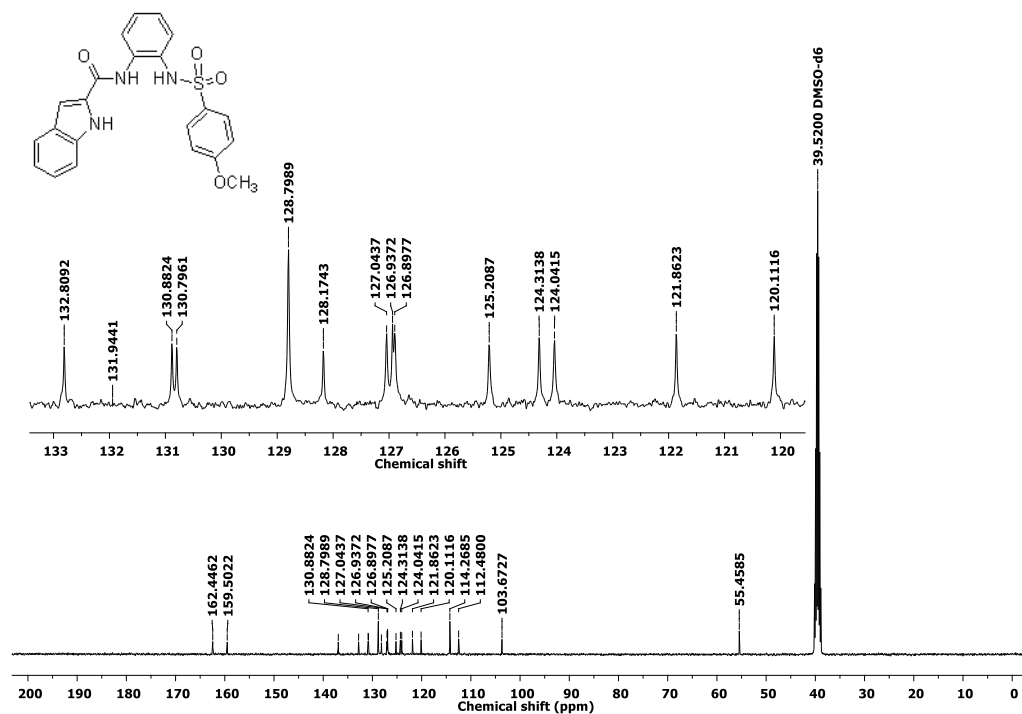


Figure 3.44 ¹³C NMR spectrum of *N*-(2-((4-methoxyphenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5f**.

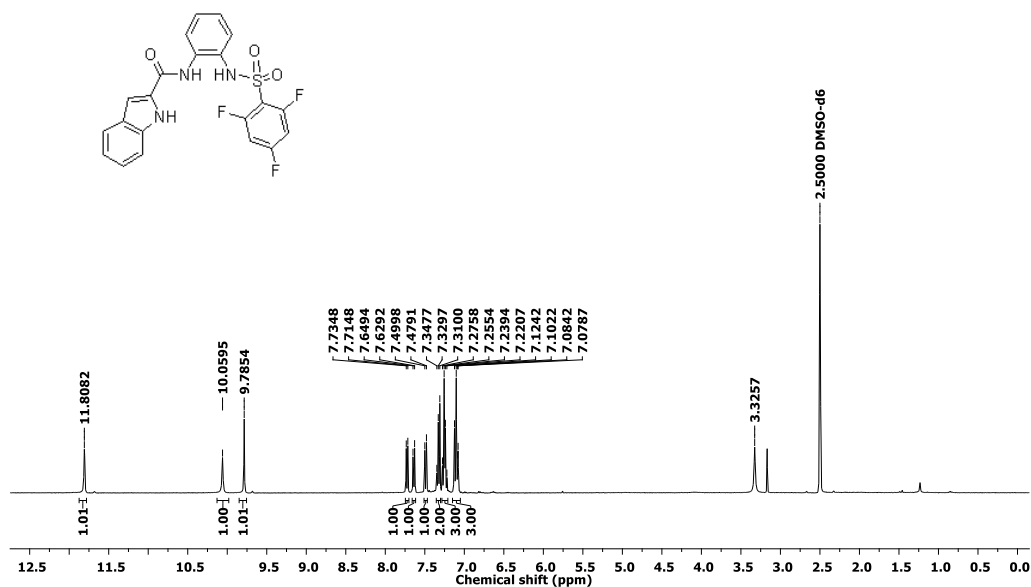


Figure 3.45 ^1H NMR spectrum of *N*-(2-((2,4,6-trifluorophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5g**.

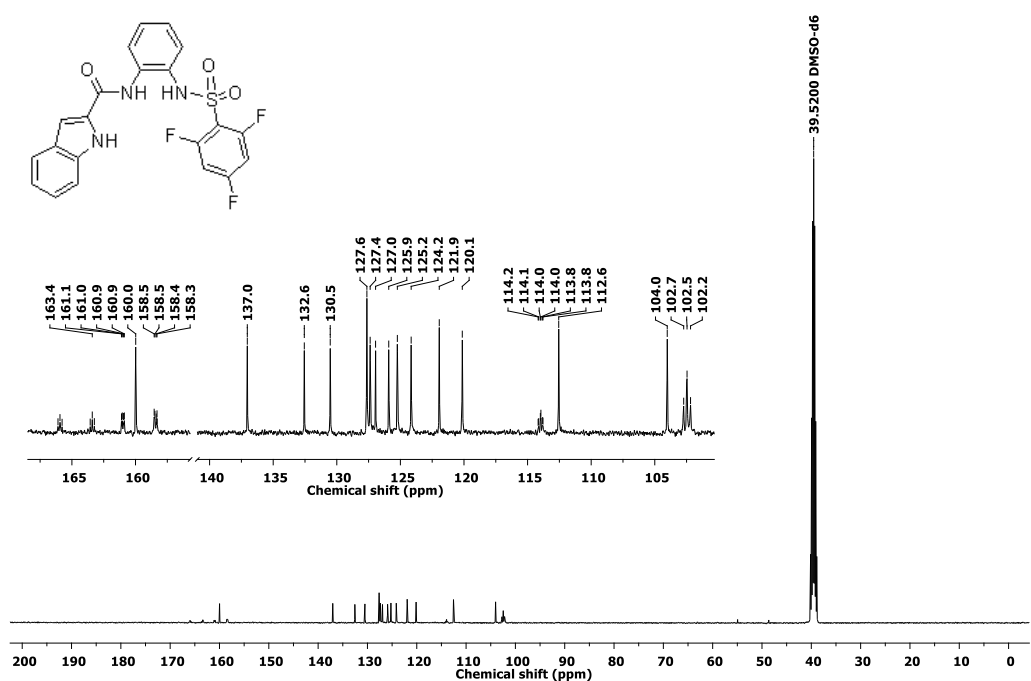


Figure 3.46 ^{13}C NMR spectrum of *N*-(2-((2,4,6-trifluorophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5g**.

3.6 HRMS Spectra of final compounds:

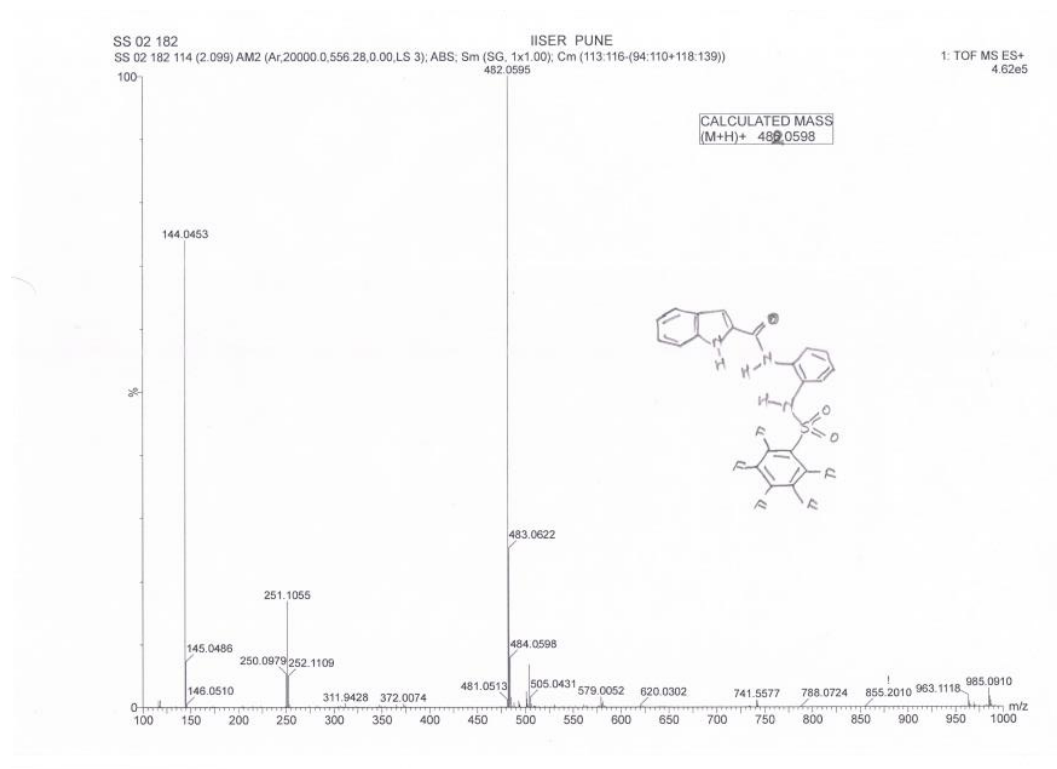


Figure 3.47 HRMS spectrum of *N*-(2-((perfluorophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5a**.

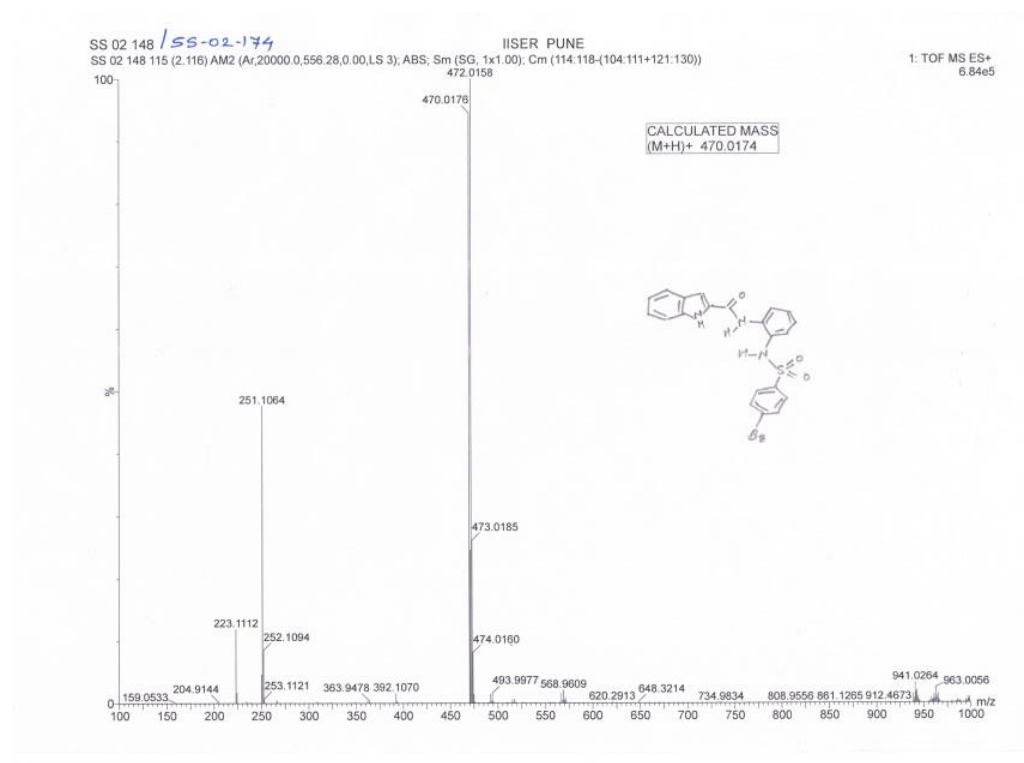


Figure 3.48 HRMS spectrum of *N*-(2-((4-bromophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5b**.

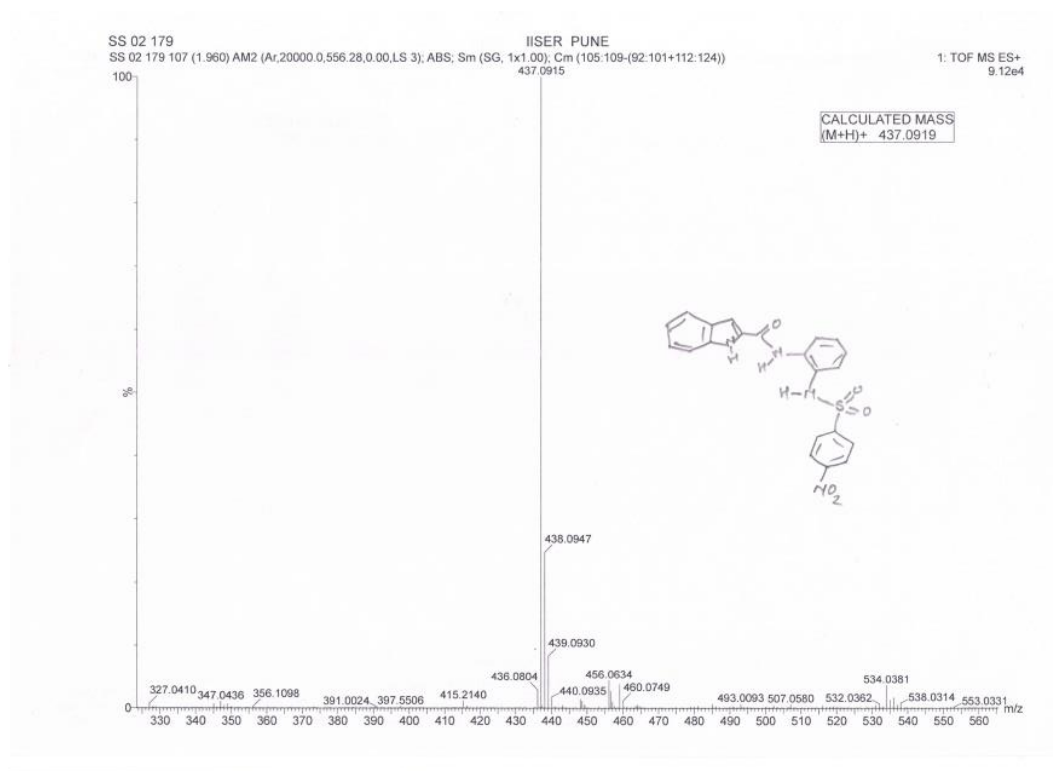


Figure 3.49 HRMS spectrum of *N*-(2-((4-nitrophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5c**.

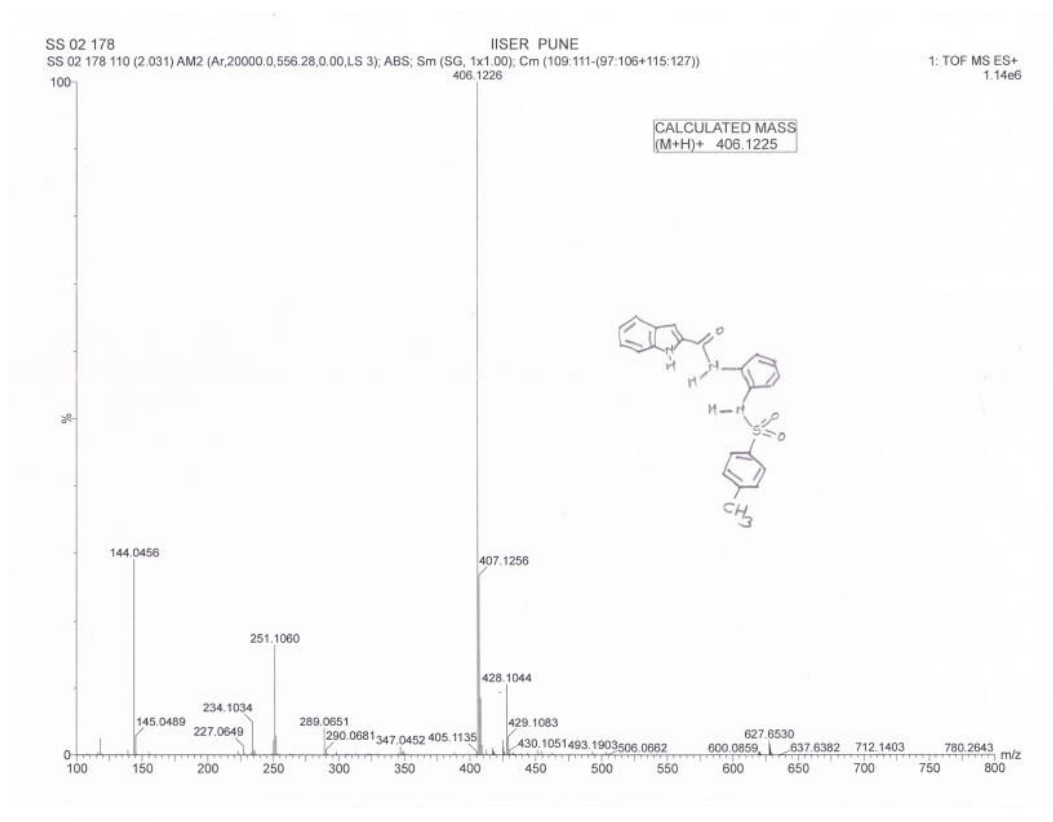


Figure 3.50 HRMS (ESI) spectrum of *N*-(2-((4-methylphenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5d**.

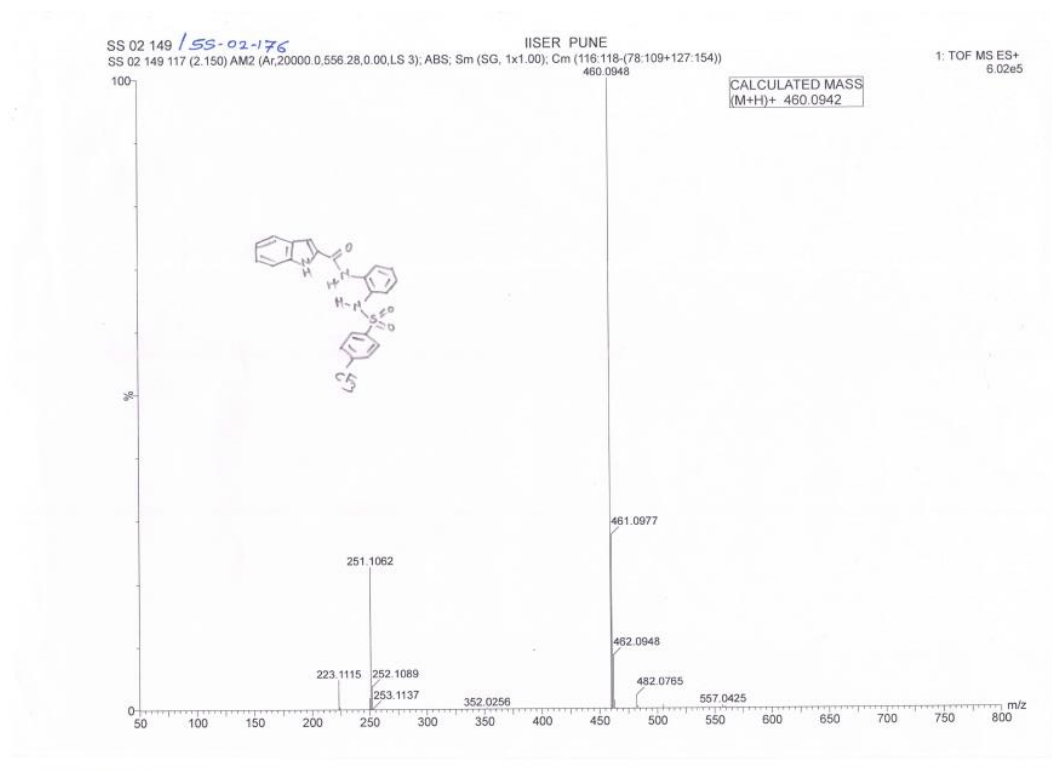


Figure 3.51 HRMS spectrum of *N*-(2-((4-(trifluoromethyl)phenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5e**.

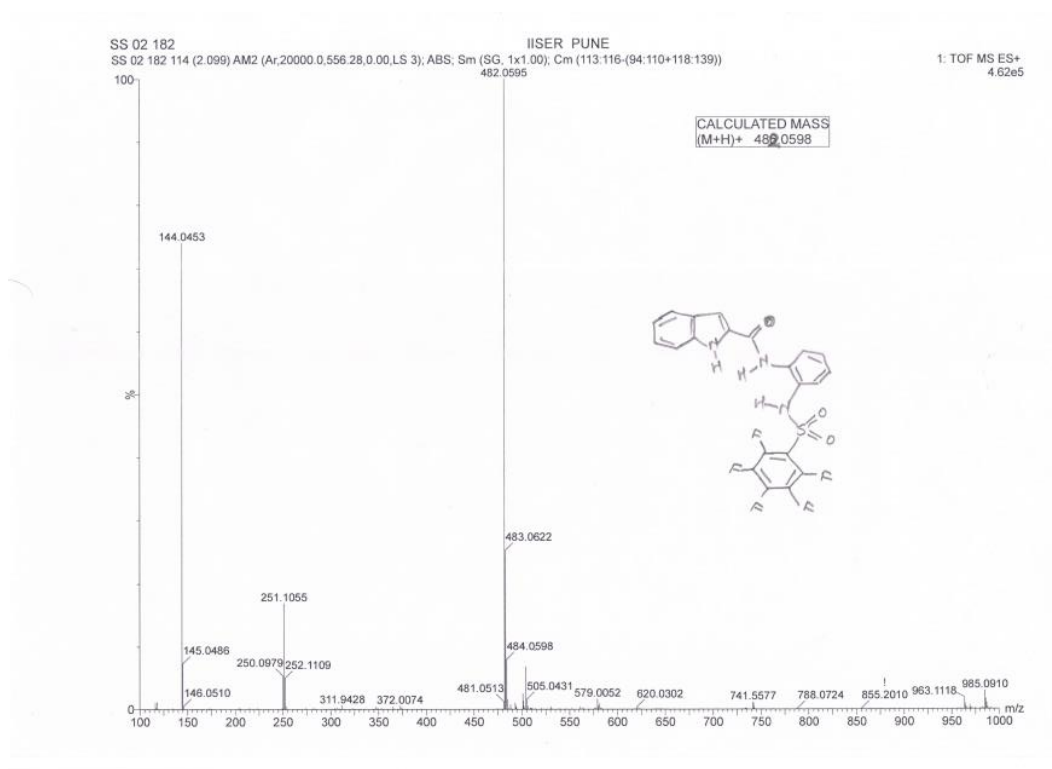


Figure 3.52 HRMS (ESI) spectrum of *N*-(2-((2,4,6-trifluorophenyl)sulfonamido)phenyl)-1*H*-indole-2-carboxamide **5g**.

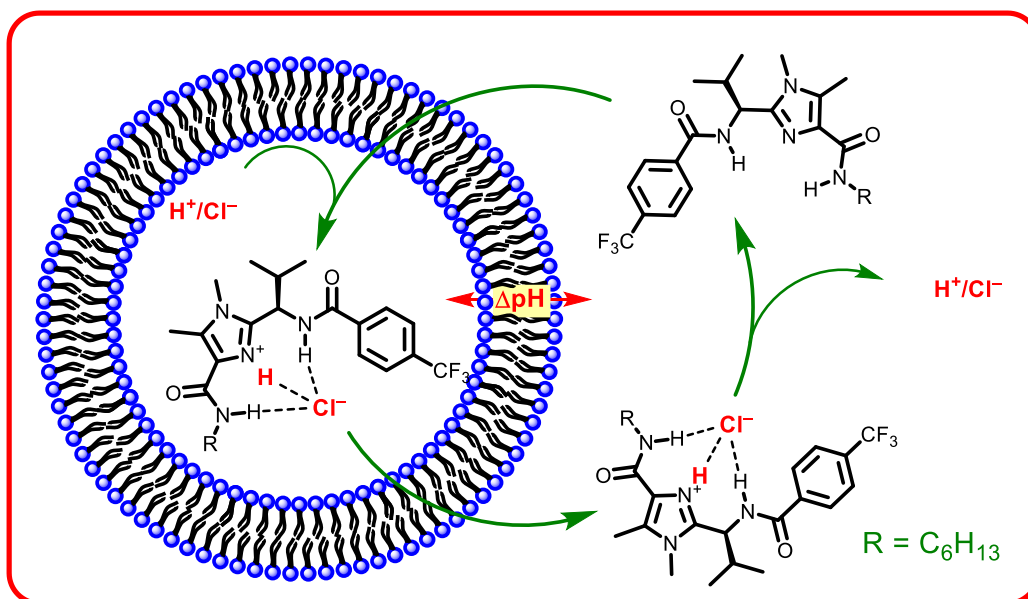
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Chapter 4

Bis(amide)-substituted Imidazole Receptors for Transmembrane H^+/Cl^- Co- Transport Activity



4.1 Introduction

Transmembrane anion transport across biological membranes is an essential process in conserving the cellular pH and ion homeostasis. Over the past decades, encouragement towards the development of biomimetic synthetic chloride transporters materialized through the realization of their involvement in a disparate set of influence in normal physiology and acquired diseases.¹ For designing the small molecule synthetic ion transporters two primary constituents have to take into account, a) the ion recognition territory to emphasize the ion selectivity, and b) the hydrophobic periphery to enhance the membrane insertion. Such a design can also be inspired by the small molecules natural products. However, there are only few small-molecule natural products are reported for mediating transmembrane anion transport. Among such natural compounds, prodigiosin is a remarkable and most studied example of naturally occurring anionophore known for its anticancer activity.^{2,3} The structure of prodigiosin has a hydrophobic core and the sickle-shaped hydrogen bonding domain for anion recognition. Unlike healthy cells, the elevated intracellular pH ($\text{pH}_i \geq 7.4$) than the extracellular pH ($\text{pH}_e = 6.7\text{--}7.1$) in cancer cells is a perfect storm for cell proliferation by limiting the apoptosis.^{4,5} Prodigiosin facilitates the chloride transport in both antiport ($\text{Cl}^-/\text{HCO}_3^-$) and symport (H^+/Cl^-) manner is altering the intracellular pH (i.e., pH_i)⁶⁻⁹ and Cl^- concentration. Cycloprodigiosin, an analog of the natural product, is reported to induce apoptosis in human colon cancer cell lines.^{10,11} As a result, the natural product-inspired design of ion transporters has gained immense attraction. For example, Quesada and co-workers have reported that indole analogs of the natural product tambjamine induce intracellular pH (i.e., pH_i) acidification and hyperpolarization of the cellular membrane which triggers cancer stem cell death.¹²

Many peptides and proteins consist of the imidazole moiety as a side-arm of histidine residue and this plays enzymatic functions to lead critical biological functions. Imidazole also has a significant role in conformational acidity and thereby modulation of the activity of many proteins. Imidazole in the side chain of proteins can turn into either

charged or uncharged species by small alteration in environmental pH. Taking the inspiration from the prodigiosin family, Gale and coworkers have developed the relatively modest H^+/Cl^- receptors **1** – **3** containing an imidazole moiety as proton acceptor (Figure 4.1). The H^+/Cl^- transport activity of imidazole analog **2** (where, imidazole is a proton acceptor) across the phospholipid bilayer was compared with the pyridyl analog **3** (where, pyridine moiety is a proton acceptor). Their result indicated that the compound **2** is able to bind with the H^+/Cl^- as indicated by the **1**·HCl cocrystal. The H^+/Cl^- co-transport activity by the imidazole analog **2** was much more superior over the pyridyl analog **3**.

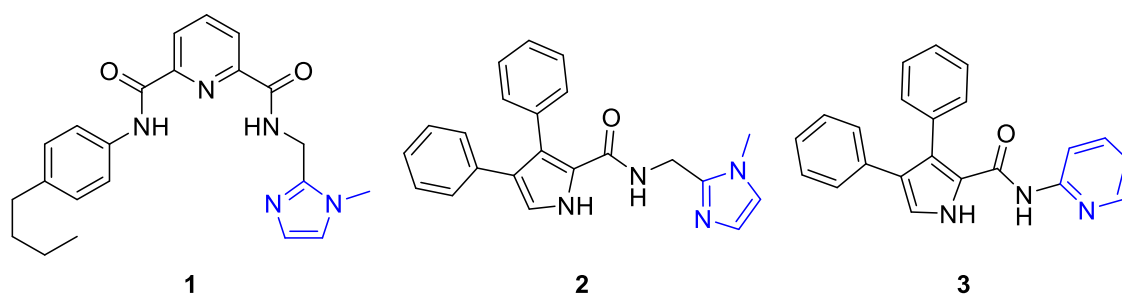


Figure 4.1 Structures of imidazole appended HCl receptors **1**, **2** and **3**.

In 2003, Haberhauer and co-workers have reported the synthesis of cyclopeptide **5**, which is an imidazole analog of marine alkaloid westellamide **4** (Figure 4.2).¹³ Both the westellamide and its imidazole analog contain the sickle-shaped trivalent hydrogen bonding motif and also a proton acceptor moieties (i.e., oxazole and imidazole) similar to that of prodigiosin and tambjamine family. Although both of the analogs include the azole as a proton acceptor, the ability of imidazole ($\text{pK}_a \sim 7$) to accept the proton near physiological condition is acceptable than the oxazole analog ($\text{pK}_a \sim 0.8$).

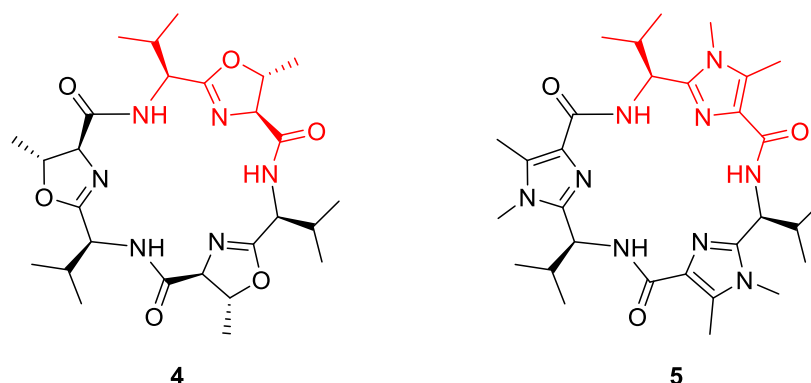


Figure 4.2 Structures of natural product westellamide **6**, and its synthetic imidazole analog **7**.

In chapter 3, we have shown that the neutral sulfonamide connected indole amide was used as proton-anion symporter across the phospholipid bilayer membrane of EYPC-vesicles. The transport activity of these receptors has been tuned by tuning the acidity of sulfonamide N–H protons. In this chapter, we are reporting the H^+/Cl^- transporters whose transport activity can be tuned by the lipophilic control on receptor molecules. In order to change the lipophilic characters of the receptors while retaining the H^+/Cl^- ability of the receptors, the fragment on which the lipophilicity need to be control has to be attached on the chemical entity which will not abandon the pK_a values of the proton binding site in receptors. To design such receptors, the imidazole was chosen as the basic core because it can be protonated similarly to the histidine side-arm (pK_a of histidine imidazole ~ 6.4 – 7.0).^{14,15} The design of transmembrane H^+/Cl^- cotransporters **6a–6f** is based on the one-third fragment of imidazole analog **5** of westellamide **4** (Figure 4.3). Two amide functionalities were correctly positioned around the imidazole core to favor cooperative hydrogen bonding interactions with an anion. *n*-Hexyl and isopropyl groups were introduced to improve the lipophilicity. Substituted phenyl rings were selected to vary lipophilicity as well as pK_a of the neighboring amide NH. A sulfonamide compound **7** was also designed to identify the effect of binding unit and angle (angle of Ar–CO–NH and Ar–SO₂–NH) on the anion binding and transport activity.

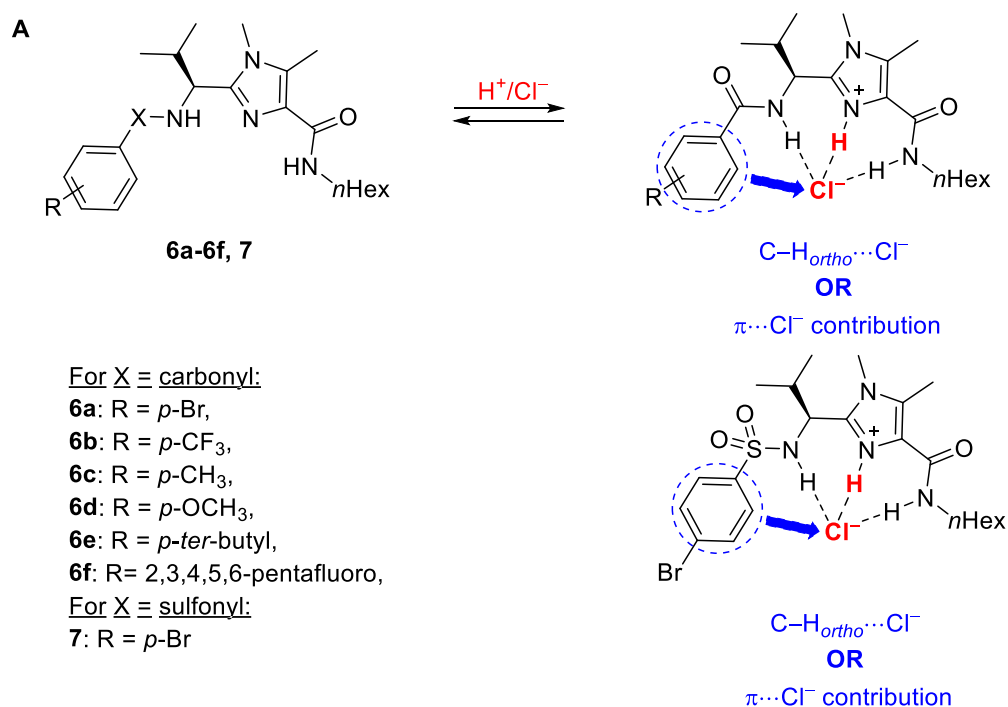
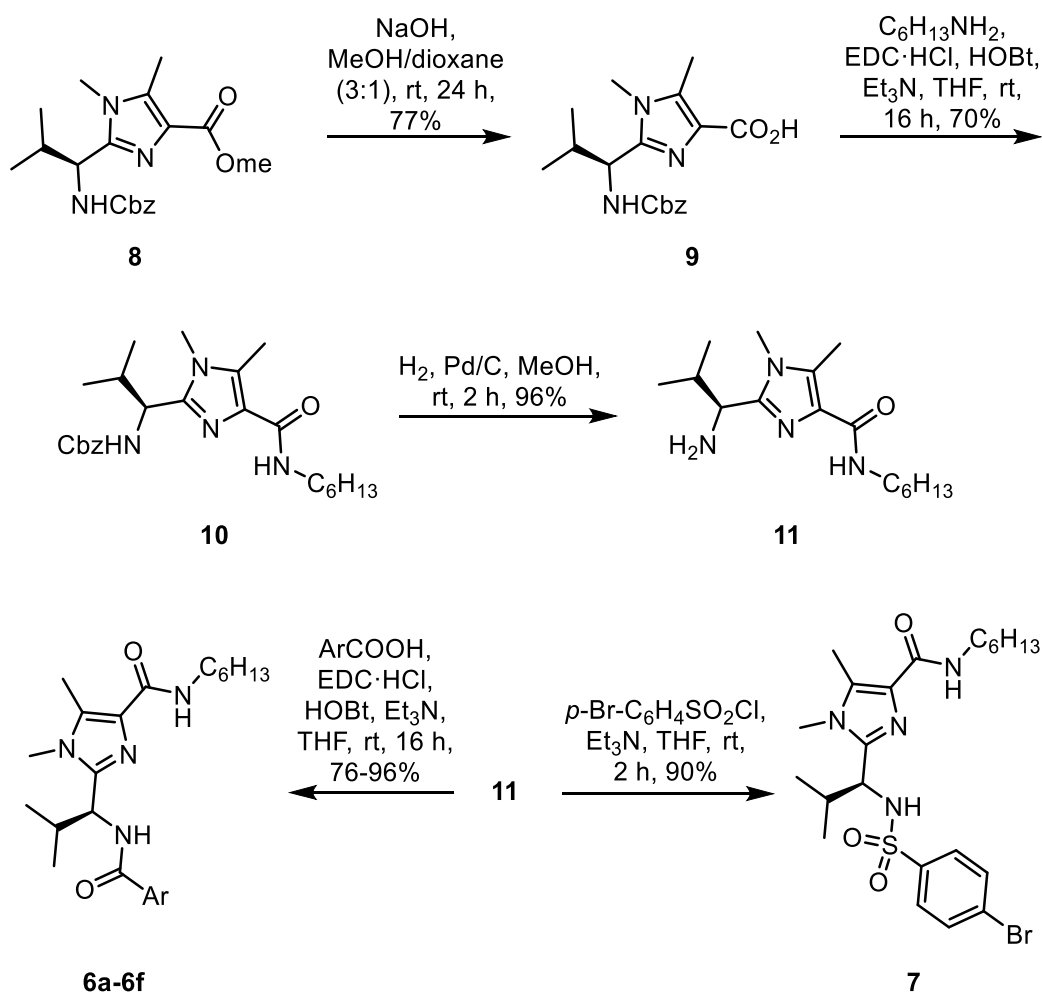


Figure 4.3 The structures and representative H^+/Cl^- binding by the bis(amide) appended imidazole **6a–6f** and its sulfonamide analog **7**.

4.2 Results and Discussions

4.2.1. Synthesis

Synthesis of the receptor **6a-6f** and **7** is described in Scheme 4.1. The synthesis of Methyl 2-amino-3-oxobutanoate hydrochloride **13** performed by modified literature protocol (See experimental section).¹⁶ The coupling of **13** with Cbz-L-Val-OH **14** furnished the amido ketone **15** in 77% yield (see experimental section), which on condensation with methylamine resulted in methyl ester **8**.¹³ The hydrolysis of ester **8** with NaOH in methanol/dioxane mixture provided the acid **9** in 77% yield. The coupling of acid **9** with *n*-hexylamine furnished the amide **10** in 70% yield. The hydrogenolysis of **10** resulted in the formation of free amine **11**. The coupling of the amine **11** with ArCO₂H in the presence of EDC·HCl afforded **6a-6f** in 76-96% yields. Similarly, the reaction of amine **11** with *p*-bromobenzenesulfonyl chloride gave **7** in 90% yield.



Scheme 4.1 Synthesis of imidazole-based receptors **6a-6f** and **7**.

4.2.2 Anion Binding Study by ^1H NMR Titration

At first, the chloride ion binding ability of neutral molecules **6a** and **7** was examined by ^1H NMR titration in acetonitrile- d_3 . ^1H NMR titration of bis(amide) **6a** with tetrabutylammonium chloride (TBACl) resulted in only 0.097 and 0.021 ppm shifts of the N-H_b and $\text{C}_{\text{Ar-H}_a}$ protons up to 1.0 equivalents of Cl^- indicating poor binding of the receptor with the ion (Figure 4.4). Likewise the titration of neutral sulfonamide analog **7** with 1.0 equivalents of TBACl showed only 0.24 ppm shift of N-H_b proton and imperceptible shift in $\text{C}_{\text{Ar-H}_a}$ protons up to 1 equivalent of TBACl addition (Figure 4.5). It is conclusive that, for both the compounds **6a** and **7**, the insignificant change in N-H_c proton chemical shift during titration indicates very poor binding of these receptors with the chloride. Therefore, the hexafluorophosphate salts of bis(amide) **6a** and sulfonamide analog **7** were prepared and then chloride binding strengths were determined using ^1H NMR by titrating with tetrabutylammonium chloride (TBACl) in acetonitrile- d_3 . Upon addition of TBACl, the replacement of weak coordinating PF_6^- by Cl^- was indicated by the significant downfield chemical shift of N-H protons (See figure 4.15, 4.16A, 4.17, 4.18A). In addition to N-H protons, a shift in $\text{C}_{\text{Ar-H}}$ was also observed. For amide derivative **6a**, the downfield shift of $\text{C}_{\text{ortho-H}}$ proton indicates the $\text{C}_{\text{ortho-H}}\cdots\text{Cl}^-$ contribution in chloride binding (Figure 4.6A). However, the upfield shift of all $\text{C}_{\text{Ar-H}}$ protons, observed for sulfonamide **7**, suggests the $\pi\cdots\text{Cl}^-$ influence from the aromatic ring (Figure 4.6B).¹⁷ The Job's plot analysis denoted the 1:1 binding stoichiometry in each case (Figure 4.16B). The association constants^{18,19} K_a with chloride were calculated to be $8041 \pm 55 \text{ M}^{-1}$ and $7215 \pm 265 \text{ M}^{-1}$ for **6a**·HPF₆ and **7**·HPF₆, respectively. Further halide binding studies of **6a**·HPF₆ with tetrabutylammonium bromide (TBABr) and tetrabutylammonium iodide (TBAI) provided the binding sequence of Cl^- ($K_a = 8041 \pm 55 \text{ M}^{-1}$) > Br^- ($K_a = 7215 \pm 256 \text{ M}^{-1}$) > I^- ($K_a = 2030 \pm 188 \text{ M}^{-1}$) which clearly indicates better Cl^- recognition by protonated receptor **6a** (Figure 4.6C, and 4.15, 4.16A, 4.19-4.22).

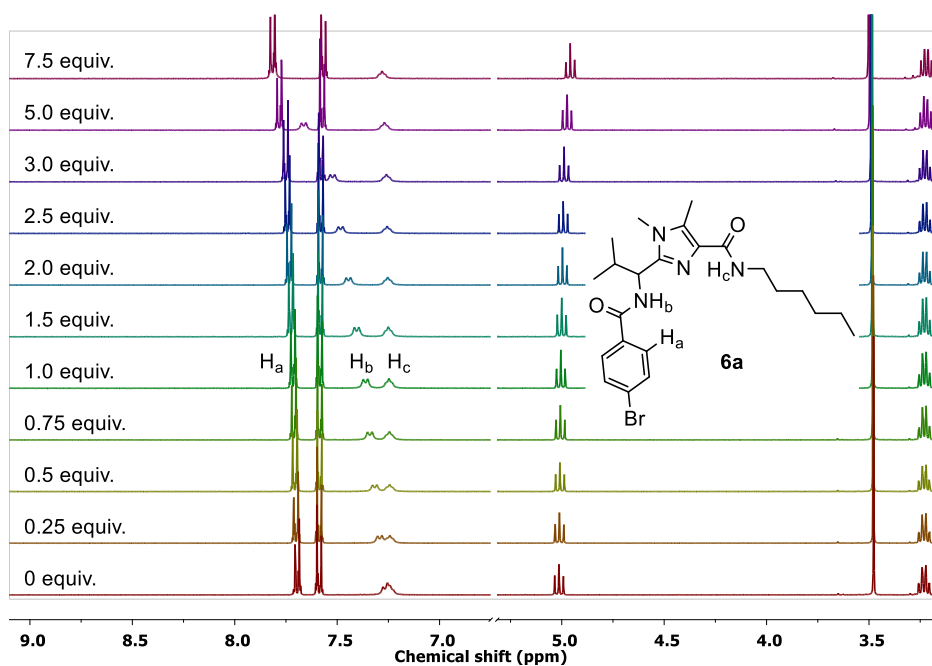


Figure 4.4 ^1H NMR titration spectra of neutral receptors **6a** with tetrabutylammonium chloride (TBACl) in acetonitrile- d_3 . The equivalents of TBACl shown on stack spectra.

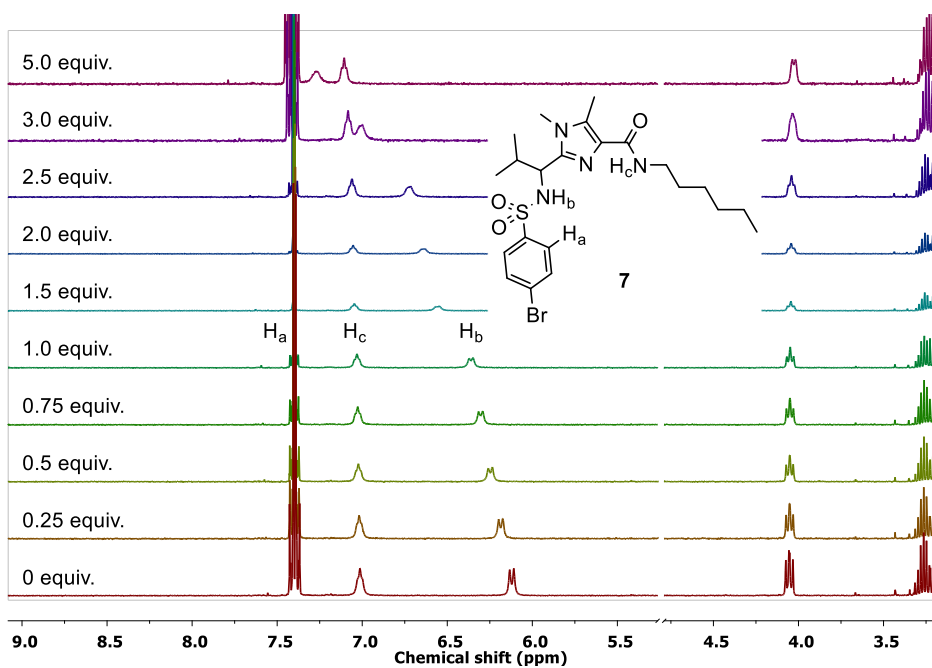


Figure 4.5 ^1H NMR titration spectra of neutral receptors **7** with tetrabutylammonium chloride (TBACl) in acetonitrile- d_3 . The equivalents of TBACl shown on stack spectra.

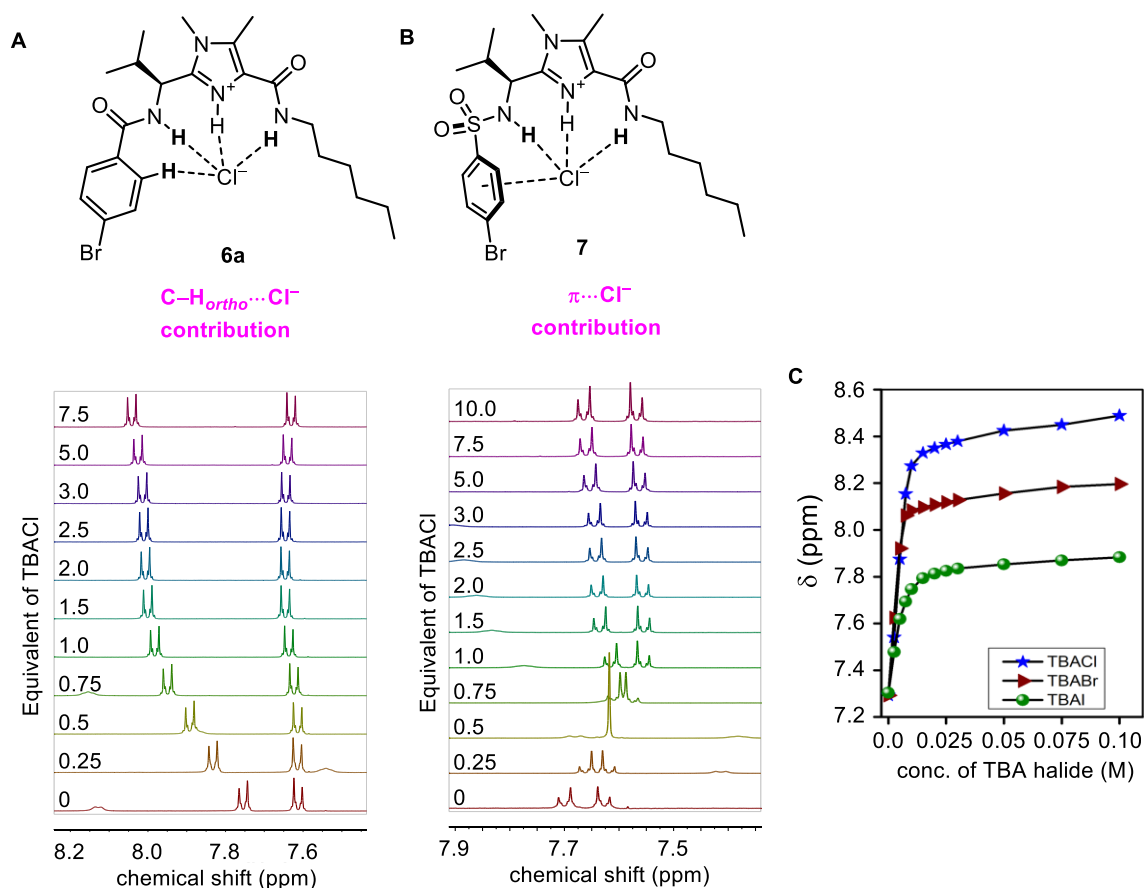


Figure 4.6 Chemical shift change in $C_{Ar}\text{-H}$ of amide **6a**·HPF₆ (A), and sulfonamide **7**·HPF₆ (B) upon titration with TBACl. ¹H NMR titration plot showing changes in the NH (CH₂NHCO) chemical shifts of **6a**·HPF₆ (0.01 M) with an increasing concentration of different halides in acetonitrile-*d*₃ (C).

4.2.3 Ion Transport Activity

The ion transport activities of **6a–6f** and **7** were evaluated by monitoring the collapse of pH gradient ($\text{pH}_{\text{in}} = 7.0$ and $\text{pH}_{\text{out}} = 7.8$) applied across large unilamellar vesicles (LUVs) prepared from egg yolk phosphatidylcholine (EYPC) lipid entrapping 8-hydroxypyrene 1,3,6-trisulfonate (HPTS).²⁰ Upon addition of **6a–6f** (10 μM), the collapse of pH gradient after 200 s were 66%, 81%, 23%, 13%, 76%, and 43%, respectively providing the activity sequence: **6d** < **6c** < **6f** < **6a** < **6e** < **6b** (Figure 4.7A).

The dose-response activity of compounds **6a**, **6b**, **6e**, and **6f** having logP values 5.05, 5.16, 5.82, and 3.99 respectively, (Figure 4.7B) provided the EC_{50} (i.e., concentration required to reach 50% activity) values of 6.14, 5.85, 6.92, 9.97 μM , respectively (Figure 4.8A, 4.8B). The EC_{50} for **6c** and **6d** (with logP of 4.79 and 4.12) could not be determined unambiguously because of its poor activity. For these

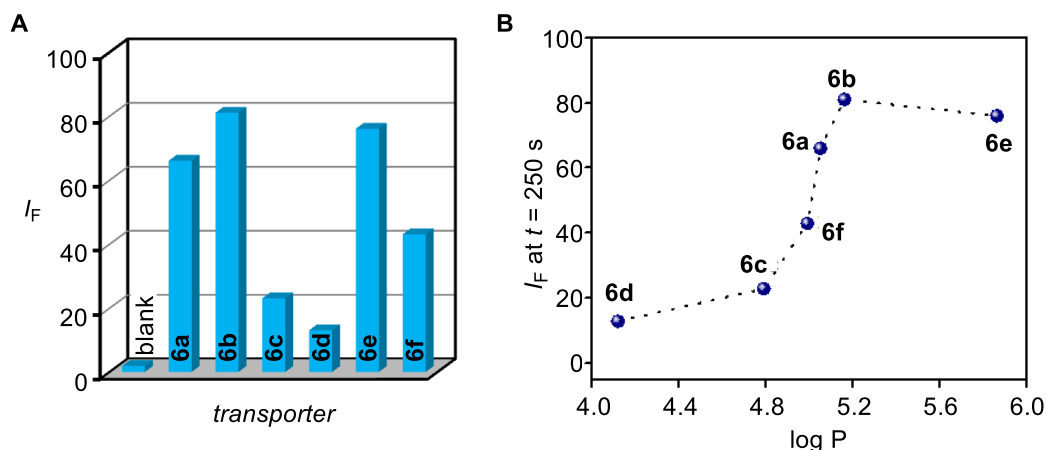


Figure 4.7 Comparison of ion transport activity of **6a-6f** at an identical concentration of 10 μM (A). The plot of relation between ion transport activity and $\log P$ (B).

compounds, the Hill coefficient i.e., n values of 2.83, 3.33, 3.45, and 2.89 indicate the involvement of three molecules in the active transporter formation. Such an outcome is different than that obtained from the NMR based binding model in solution, however, not surprising considering the environments (i.e., acetonitrile- d_3 for NMR and lipid for ion transport). Despite the identical aromatic side-arm, $\log P$ values (i.e., 5.03 for **6a** and 4.80 for **7**)²¹ and comparable Cl^- binding strength, ion transport activity of compound **6a** was far superior to that of **7** (Figure 4.8C). This may be ascribed to the tetrahedral geometry of the sulfur atom resulting in the inadequate complexation with the Cl^- in the lipid membrane.

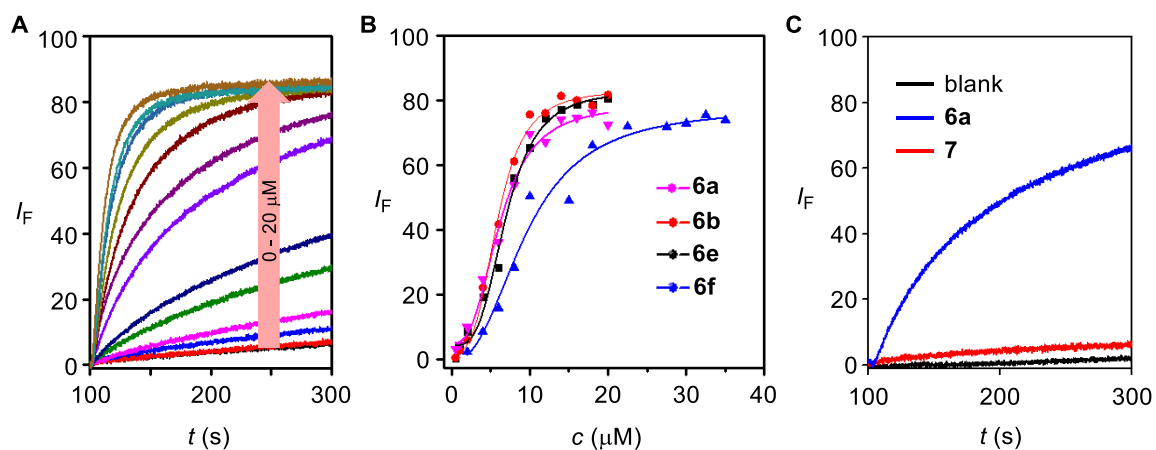


Figure 4.8 Dose-dependent enhancements in transport activity by **6b** across the EYPC-LUVs $\supset$ HPTS (A), and comparison of concentration-dependent transport activity of **6a**, **6b**, **6e**, and **6f** (B). Comparison of ion transport activity of bis(amide) **6a** with sulfonamide analog **7** at the identical concentration of 10 μM (C).

Aforestated transport activity encouraged us to determine the ion selectivity of the most active compound **6b**. Initially, the cation transport activity of **6b** was screened by varying the external alkali metal cations (i.e., MCl, where $M^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+, \text{and } \text{Cs}^+$) of the vesicles trapped with HPTS and isoosmolar NaCl.²² However, such variation of cations did not provide any significant change in transport activity, which rules out the involvement of alkali metal cations in the transport process (Figure 4.9A). Instead, the change in anions of external sodium salts (i.e., NaA, where $A^- = \text{F}^-, \text{Cl}^-, \text{Br}^-, \text{I}^-, \text{NO}_3^-, \text{ClO}_4^-$, and AcO^-) resulted in a notable trend in transport activity with the selectivity sequence: $\text{Cl}^- > \text{F}^- \approx \text{AcO}^- > \text{SCN}^- > \text{I}^- \approx \text{Br}^- > \text{ClO}_4^- > \text{NO}_3^-$. In the presence of an extravesicular NO_3^- ion, we observed an initial decrease in intravesicular pH as the result of competitive H^+/NO_3^- influx by **6b** (Figure 4.9B). Then the chloride transport is also established with the help of lucigenin assay.²³ The EYPC-LUVs (with a mean diameter of 200 nm) enclosed with NaNO_3 and lucigenin were prepared and suspended in pH 7 buffer containing isoosmolar NaNO_3 . The chloride gradient was created by adding 33 mM NaCl in the extravesicular matrix. The chloride influx was assessed by monitoring the decay in lucigenin fluorescence. The transporter **6b** displayed the concentration-dependent change in the chloride transport activity (Figure 4.9C).

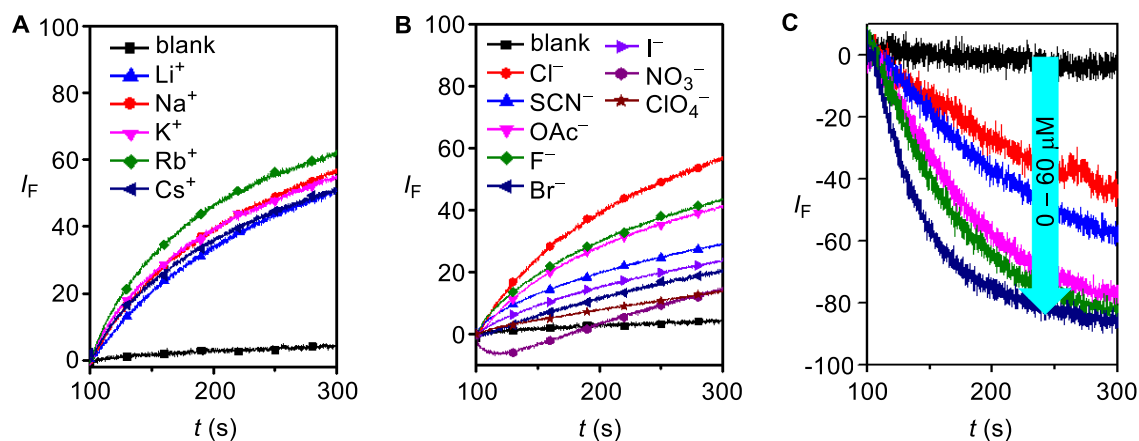


Figure 4.9 Ion selectivity of **6b** (5.5 μM). Cation selectivity (A) and, (B) Anion selectivity (B) across EYPC-LUVs $\supset$ HPTS measured in presence of applied pH gradient ($\Delta\text{pH} = 0.8$). Chloride transport activity by **6b** across EYPC-LUVs $\supset$ lucigenin (C).

From the ion selectivity assay, two possible mechanisms H^+/A^- symport and Cl^-/A^- antiport are possible. To get the unblemished idea about the contribution of these mechanisms, we determined the ion transport activity of **6b** in the presence of FCCP (F) and valinomycin assay, after applying the pH gradient.^{22,24-26} Formerly, H^+/Cl^- selectivity

was examined in the presence and absence of FCCP.²⁷ Under applied pH gradient FCCP allows the transport of H^+ ion from the intravesicular pool to the extravesicular pool. For a Cl^-/A^- antiport mechanism, it is expected that transport rate should increase in the presence of FCCP due to the cooperative effect two transporters.²⁸ However, we did not observe any change in the transport rate indicating that transport mechanism occurs preferentially via the symport mechanism (Figure 4.10A). In similar way, the effect of valinomycin (V), a K^+ ion carrier,²⁹ on the ion transport rate of **6a** was investigated. In this case, the study was carried out in the presence of extravesicular KCl instead of NaCl. However, in this assay, we did not observe any difference in transport activity of **6b** and that of the same compound in the presence of valinomycin. The outcome confirmed that the transport process by **6b** involves the H^+/Cl^- symport instead of Cl^-/A^- antiport (Figure 4.10B).

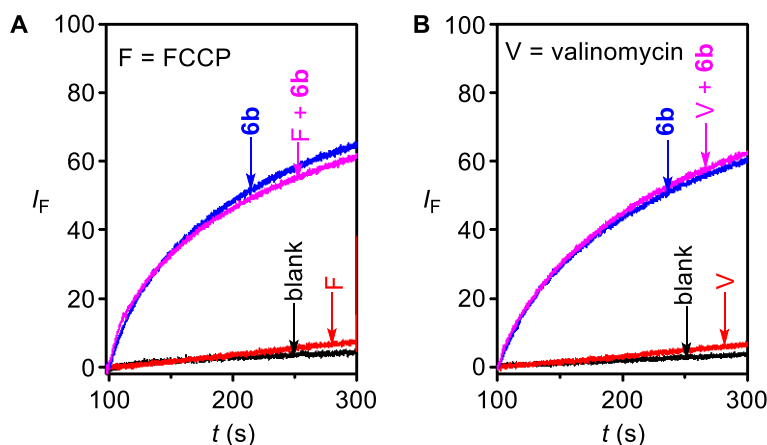


Figure 4.10 Comparison of ion transport activity of **6b** (5.5 μ M) in the presence and the absence of FCCP (0.8 μ M) (A). Comparison of ion transport activity of **6b** (5.5 μ M) in the presence and the absence of valinomycin (2.5 pM) (B).

To investigate the prodigiosin like behaviour in symmetrical pH solution (i.e., exchange of anion across the membrane under symmetrical intra- and extravesicular pH conditions),³⁶ the valinomycin coupled lucigenin assay was performed by adding 33 mM KCl to the extravesicular buffer. The chloride influx by compound **6b** alone and in the presence of valinomycin was determined. It was observed that the chloride influx efficiency by **6b** (25 μ M) in the presence of an excess of valinomycin (5 μ M) was more than that of compound **6b** alone supporting the exchange of anions under the symmetrical pH conditions (Figure 4.11).

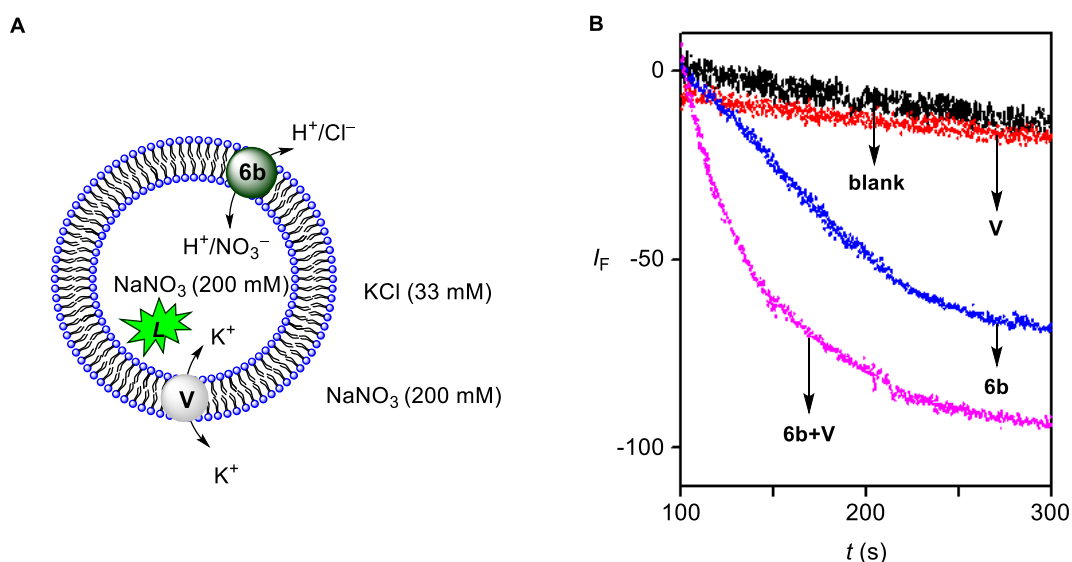


Figure 4.11 Valinomycin coupled anion exchange experiment across lucigenin entrapped vesicle (A). Data showing the enhancement in transport activity in coupled assay condition (B).

Both classes of transporters (i.e., channels and carriers) are functional in phospholipid bilayers. The channel including natural channel proteins are not longer than the 60 Å. However, the path length of the hydrophobic layer of U-tube is comparatively several thousand times longer than thickness of phospholipid. Therefore, only the mobile carrier molecules can show the transport activity across such long organic layers in U-tube experiment. Therefore, the U-tube experiment to explore the possibility of mobile carrier formation by the transporter **6e** was carried out. In this experiment, a solution of the compound **6e** (2 mM) in chloroform was placed at the bottom of the U-tube. One arm of U-tube was filled with aqueous NaNO_3 (500 mM) while the other arm was filled with 100 mM HCl and 400 mM NaCl. The chloride concentration and pH change were measured in the NaNO_3 side (receiver arm) with chloride selective electrode and pH meter. No change in chloride concentration was observed in the absence of **6e**. A significant increase in the chloride concentration and decrease in the pH was seen in the receiver arm when compound **6e** was studied. The results suggested a carrier-mediated H^+/Cl^- co-transport mechanism (Figure 4.12B).

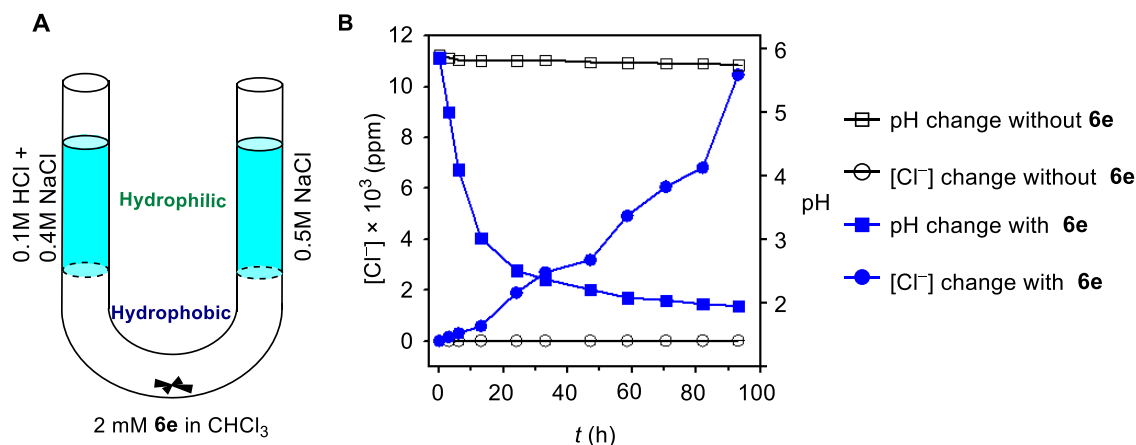


Figure 4.12 Representative U-tube experiment (A). Change in pH and Cl⁻ ion concentration in aqueous phase of receiver arm of U-tube in the presence and absence of compound (B).

4.3 Conclusion

In summary, we have developed bis(amide)-based imidazole receptors **6a-6f** as the transmembrane cotransporter of H⁺/Cl⁻. The ¹H NMR titration study corroborated to the inconvenient chloride binding by the neutral form of the compounds **6a** and **7**. However, the strong Cl⁻ binding by the protonated form of **6a** and **7** at imidazole core nitrogen was observed. The investigation of binding strength with halide gave the trend Cl⁻ > Br⁻ > I⁻ suggesting convenient chloride binding site. The comparison of ion transport activity indicated **6a** as the most active molecule. The elucidation of the transport activity with cooperative FCCP and valinomycin assays suggested that chloride transport occurs via the H⁺/Cl⁻ symport mechanism. The U-tube experiment further confirmed the mobile carrier mechanism for such transport process.

4.4 Experimental Section

4.4.1 General Methods

All reagents and solvents for synthesis were purchased commercially and used without further purification. Dichloromethane (CH₂Cl₂) was pre-dried over calcium hydride and then distilled. Column chromatographies were performed on Merck silica gel (100 – 200 mesh). Thin layer chromatographies (TLCs) were carried out with E. Merck silica gel 60-F254 plates. Egg yolk phosphatidylcholine (EYPC) was obtained from Avanti Polar Lipids as a solution in CHCl₃ (25 mg/mL). HEPES buffer, HPTS, lucigenin, Triton X-100, NaOH and inorganic salts were purchased of molecular biology grade from

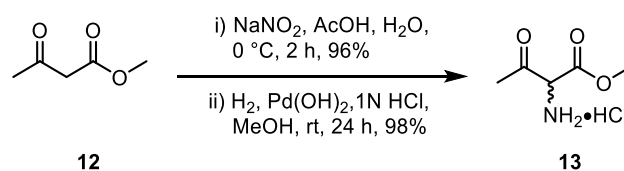
Sigma. Large unilamellar vesicles (LUVs) were prepared by using mini extruder, equipped with a polycarbonate membrane of 100 nm pore size, obtained from Avanti Polar Lipids.

4.4.2 Physical Measurements

All NMR spectra were recorded either on 400 MHz Bruker, or 400 MHz Bruker Ascend™ 400 spectrometer using residual solvent signals as an internal reference on the δ scale. The chemical shifts (δ) were reported in ppm and coupling constants (J) in Hz. The following abbreviations were used: s (singlet), br s (broad singlet), d (doublet), t (triplet), dd (doublet of doublet), m (multiplet). High-resolution mass spectra were obtained on a MicroMass ESI-TOF MS spectrometer equipped with a Micromass Z-Spray electrospray ionization (ESI) source (Waters Co., Synapt G2, France). FT-IR spectra were obtained using NICOLET 6700 FT-IR spectrophotometer and reported in ν (cm^{-1}). Melting points were measured using a VEEGO Melting point apparatus. All melting points were measured in open glass capillary, and values are uncorrected. Fluorescence spectra were recorded from Fluoromax-4 from JobinYvon Edison-equipped with an injector port and a magnetic stirrer. Measurements of pH were done using a Helmer pH meter. All data from fluorescence studies were processed either by KaleidaGraph 3.51 or Origin 8.5 program. ChemBio Draw 16 Ultra software was used for drawing structures and processing figures.

4.4.3 Synthesis procedures

Synthesis of ($\pm$)-2-Amino-3-oxo-butyric acid methyl ester hydrochloride (**9**).^{30,16}



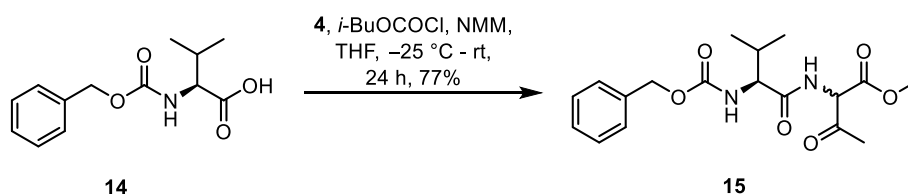
Scheme 4.2 Synthesis of amine **13** from methyl acetoacetate **12**.

In a 250 mL round bottom flask, to an ice-cold solution of methyl acetoacetate **12** (10.2 g, 77 mmol) in glacial acetic acid (20 mL), the solution of NaNO_2 (7.0 g, 100 mmol) in 8 mL water was added dropwise at such a rate that the temperature was not allowed to rise above 5 °C. After complete addition, the reaction mixture stirred at room

temperature for 2 hours. Water (50 mL) was added and stirred for additional 30 minutes. The product was extracted with diethyl ether (100 mL $\times$ 2). The organic layer washed with saturated NaHCO_3 (100 mL), dried over Na_2SO_4 and filtered. The solvent was evaporated under vacuum to obtain methyl-2-(hydroxyimino)-3-oxobutanoates (12.2 g, 96%) as a pale yellow liquid.

To a degassed solution of methyl 2-(hydroxyimino)-3-oxobutanoate 3.5 g in methanol (35 mL) was added conc. HCl (3 mL) and stirred for 15 minutes. 20% (w/w) $\text{Pd}(\text{OH})_2$ (200 mg) was added, and the reaction mixture was hydrogenated for 30 hours (bladder pressure). The reaction mixture was filtered through the small bed of celite and washed with methanol. The filtrate was concentrated to obtain the yellowish residue. The residue was washed with dichloromethane to obtain ($\pm$)-2-Amino-3-oxo-butyric acid methyl ester hydrochloride **13** as a white solid (3.99 g, 98%).

Methyl 2-((S)-2-(((benzyloxy)carbonyl)amino)-3-methylbutanamido)-3-oxobutanoate (15).

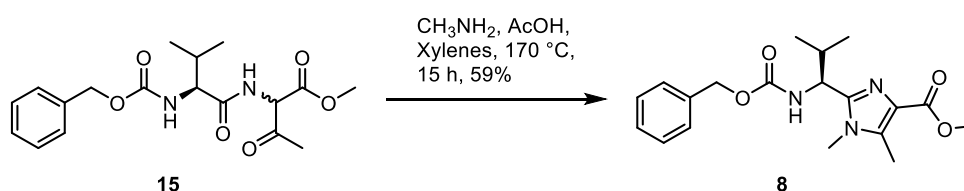


Scheme 4.3 Synthesis of amidoketone **15** from Cbz-Val-OH **14**.

In a 100 mL round bottom flask, to a solution of ((benzyloxy)carbonyl)-*L*-valine (1.0 g, 3.98 mmol) in tetrahydrofuran (12 mL) was added *N*-methyl morpholine (806 mg, 3.98 mmol) and stirred for 5 minute. After cooling the reaction mixture to $-25\text{ }^\circ\text{C}$, isobutyl chloroformate was added in one portion, while reaction mixture maintained at $-25\text{ }^\circ\text{C}$ for 45 minutes. After 45 minute, aminoketone (667 mg, 3.98 mmol) and second equivalent of NMM (806 mg, 3.98 mmol) was added at $-25\text{ }^\circ\text{C}$. The reaction mixture was allowed warming to room temperature and the stirring was continued for additional 22 hours. The THF was evaporated under vacuum. The residue was dissolved in dichloromethane and washed with water and brine. The organic layer was dried over Na_2SO_4 , filtered and concentrated. The residue was purified by column chromatography with silica gel (*Eluent*: 30-35% EtOAc/petroleum ether) to obtain diastereomeric amidoketone **15** as white solid (1.07 g, 73%). **M.p.**: $132\text{ }^\circ\text{C}$; **IR (neat, cm^{-1})**: 3415, 3297,

3064, 3036, 2962, 2874, 1754, 1725, 1689, 1651, 1536, 1454, 1438, 1366, 1289, 1249, 1160, 1042, 700; ^1H NMR (400 MHz, CDCl_3): δ = 7.38-7.28 (m, 10H; $\text{C}_{\text{Ar}}\text{H}$), 7.09-6.93 (m, 2H; CONH), 5.30 (m, 2H; NHCO_2), 5.23 (m, 2H; CHCO_2Me), 5.12 (s, 2H; $\text{C}_{\text{Ar}}\text{CH}_2$), 5.11 (s, 2H; $\text{C}_{\text{Ar}}\text{CH}_2$), 4.22-4.12 (m, 2H; CHCONH), 3.81 (s, 3H; CO_2CH_3), 3.80 (s, 3H; CO_2CH_3), 2.38 (s, 3H; COMe), 2.37 (s, 3H; COCH_3), 2.26-2.10 (m, 2H; CHMe_2), 1.02-0.90 (m, 12H; $\text{C}(\text{CH}_3)_2$); ^{13}C NMR (101 MHz, CDCl_3): δ = 197.92, 197.78, 171.05, 166.21, 166.18, 156.35, 136.18, 136.15, 128.56, 128.55, 128.22, 128.20, 128.09, 128.08, 67.19, 67.14, 63.02, 62.88, 59.94, 59.91, 53.40, 53.34, 31.23, 31.07, 28.10, 27.93, 19.14, 19.03, 17.55, 17.38; HRMS (ESI): Calcd. for $\text{C}_{18}\text{H}_{25}\text{N}_2\text{O}_6$: 365.1712 $[\text{M}+\text{H}]^+$, found: 365.1727.

Methyl (S)-2-(1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxylate (8).¹³



Scheme 4.4 Synthesis of imidazole **8** from amidoketone **15**.

In a 100 mL round bottom flask, to a solution of amidoketone **15** (1.28 g, 3.51 mmol) in mixture of xylenes (60 mL), acetic acid (4 mL) and methyl amine (40% v/v in water, 1.36 mL, 17.55 mmol) was added. The reaction mixture was stirred at 170 °C temperature with azeotropic removal of water for 5 hours and then cooled down to room temperature. Methyl amine (40% v/v in water, 1.36 mL, 17.55 mmol) and acetic acid (4 mL) was added and stirred at 170 °C with azeotropic removal of water for 10 hours. The solution was cooled and concentrated in vacuum. Residue was purified by column chromatography with silica gel (*Eluent*: 30% EtOAc/ petroleum ether) to obtain product **8** as a white solid (710 mg, 56%). **M.p.**: 123 °C; **IR** (neat, cm^{-1}): 3334, 3056, 2965, 1702, 1575, 1512, 1444, 1378, 1345, 1307, 1267, 1223, 1170, 1077, 1026, 983, 911, 870, 780, 729, 699, 650, 580; ^1H NMR (400 MHz, CDCl_3): δ = 7.38 – 7.27 (m, 5H), 5.59 (d, J = 9.7 Hz, 1H), 5.1 (d, J = 12.4, 1H), 5.03 (d, J = 12.4 Hz, 1H), 4.57 (t, J = 9.3 Hz, 1H), 3.87 (s, 3H), 3.57 (s, 3H), 2.52 (s, 3H), 2.27 (m, 1H), 1.03 (d, J = 6.7 Hz, 3H), 0.83 (d, J = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, CDCl_3): δ = 164.3, 156.4, 148.3, 136.4, 136.1, 128.5,

128.1, 127.9, 127.8, 66.9, 53.0, 51.6, 33.2, 30.4, 19.7, 18.8, 10.3; **HRMS (ESI):** Calcd. for $C_{19}H_{26}N_3O_4$: 360.1923 $[M+H]^+$, found: 360.1927.

(S)-2-(1-(((Benzyloxy)carbonyl)amino)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxylic acid (9).

In a 25 mL round bottom flask, to a solution of methyl (S)-2-(1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxylate (500 mg, 1.39 mmol) in methanol (8 mL) and dioxane (5 mL), NaOH (4.7 M, 3 mL, 13.91 mmol) was added dropwise at 0 °C. The reaction mixture stirred for 16 hours. After completion of reaction (followed by TLC), solvent was evaporated and residue was dissolved in ice cold water/ CH_2Cl_2 mixture and acidified with 6M aqueous HCl solution to pH = 1. The aqueous layer saturated with addition of solid NaCl and extracted multiple times with CH_2Cl_2 . The combined organic layer dried over Na_2SO_4 and concentrated. Residue was purified by column chromatography with silica gel (Eluent: 2% MeOH/ CH_2Cl_2) to obtain acid as white solid (350 mg, 77%). **M.p.:** 65 °C; **IR (neat, cm^{-1}):** 3261, 3054, 2966, 1707, 1672, 1585, 1513, 1455, 1374, 1310, 1268, 1234, 1113, 1078, 1027, 986, 912, 867, 730, 697, 568; **1H NMR (400 MHz, $CDCl_3$):** δ = 8.30 (br s, 1H), 7.33-7.18 (m, 5H), 5.09 (d, J = 12.6 Hz, 1H), 4.96 (d, J = 12.6 Hz, 1H), 4.50 (t, J = 9.7 Hz, 1H), 3.62 (s, 3H), 2.48 (s, 3H), 2.47-2.30 (m, 1H), 1.07 (d, J = 6.4 Hz, 3H), 0.71 (d, J = 6.1 Hz, 3H); **^{13}C NMR (101 MHz, $CDCl_3$):** δ = 165.9, 157.3, 149.0, 136.9, 135.8, 128.4, 127.9, 127.8, 127.6, 66.6, 23.4, 32.5, 30.7, 19.9, 19.6, 10.2; **HRMS (ESI):** Calcd. for $C_{18}H_{24}N_3O_4$: 346.1767 $[M+H]^+$, found: 346.1766.

Benzyl (S)-2-(1-(4-(hexylcarbamoyl)-1,5-dimethyl-1H-imidazol-2-yl)-2-methylpropyl) carbamate (10).

In a 25 mL round bottom flask, a solution of acid (400 mg, 1.16 mmol), EDC·HCl (244 mg, 1.27 mmol) and HOBt (172 mg, 1.27 mmol) was stirred for 15 minutes. *n*-Hexylamine (129 mg, 1.27 mmol) and triethyl amine (194 μ L, 1.40 mmol) was added. The reaction mixture was stirred at room temperature for 16 hours. The solvent was evaporated under vacuum. The residue was dissolved in CH_2Cl_2 and extracted with water and brine. The organic layer was dried over Na_2SO_4 , Filtered and concentrated. Residue was purified by column chromatography with silica gel (eluent: 30% EtOAc/ petroleum ether to provide amide (343 mg, 69%) as white solid. **M.p.:** 86 – 87 °C; **IR (neat, cm^{-1}):** 3413, 3052, 2930, 2866, 1714, 1646, 1594, 1515, 1460, 1373, 1307, 1265, 1228, 1108,

1027, 983, 911, 730, 645, 576; **¹H NMR (400 MHz, CDCl₃):** δ = 7.43–7.28 (m, 5H), 7.06 (t, J = 5.4 Hz, 1H), 5.44 (d, J = 9.1 Hz, 1H), 5.12 (d, J = 12.2 Hz, 1H), 5.06 (d, J = 12.2 Hz, 1H), 4.62 (dd, J = 9.1 Hz & 7.8 Hz, 1H), 3.44–2.27 (m, 2H), 2.55 (s, 3H), 2.22–2.05 (m, 1H), 1.58 (quint, J = 7.2 Hz, 2H), 1.42–1.26 (m, 6H), 0.98 (d, J = 6.8 Hz, 3H), 0.92–0.84 (m, 6H); **¹³C NMR (101 MHz, CDCl₃):** δ = 164.0, 156.3, 146.1, 136.4, 132.2, 130.0, 128.7, 128.3, 128.2, 67.1, 52.8, 38.9, 33.4, 31.7, 30.2, 30.0, 26.9, 22.7, 19.6, 18.4, 14.2, 9.9; **HRMS (ESI):** Calcd. for C₂₄H₃₇N₄O₃: 429.2865 [M+H]⁺, found: 429.2860.

(S)-2-(1-Amino-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (11).

In a 25 mL round bottom flask, to a degassed solution of Cbz-protected amine **10** (650 mg, 1.38 mmol) in methanol, Pd/C (10 % w/w) was added and stirred under hydrogen atmosphere for 3 hours. The reaction mixture filtered through small bed of celite. The filtrate was concentrated to obtain amine (407 mg, 96%) as colorless oil. **IR (neat, cm⁻¹):** 3401, 2926, 2864, 1642, 1593, 1516, 1462, 1370, 1321, 1271, 1230, 1126, 1051, 977, 937, 902, 733, 579; **¹H NMR (400 MHz, CDCl₃):** δ = 7.16 (br s, 1H), 3.67 (d, J = 6.8 Hz, 1H), 3.49 (s, 3H), 3.40–3.30 (m, 2H), 2.55 (s, 3H), 2.01 (sext, J = 6.8 Hz, 1H), 1.58 (quint, J = 7.3 Hz, 2H), 1.41–1.26 (m, 6H), 0.98 (d, J = 6.7 Hz, 3H), 0.91–0.84 (m, 6H); **¹³C NMR (101 MHz, CDCl₃):** δ = 164.3, 148.8, 132.3, 129.7, 54.6, 38.9, 34.1, 31.7, 30.2, 30.0, 26.9, 22.7, 20.0, 18.2, 14.2, 9.8; **HRMS (ESI):** Calcd. for C₁₆H₃₁N₄O: 295.2498 [M+H]⁺, found: 295.2498.

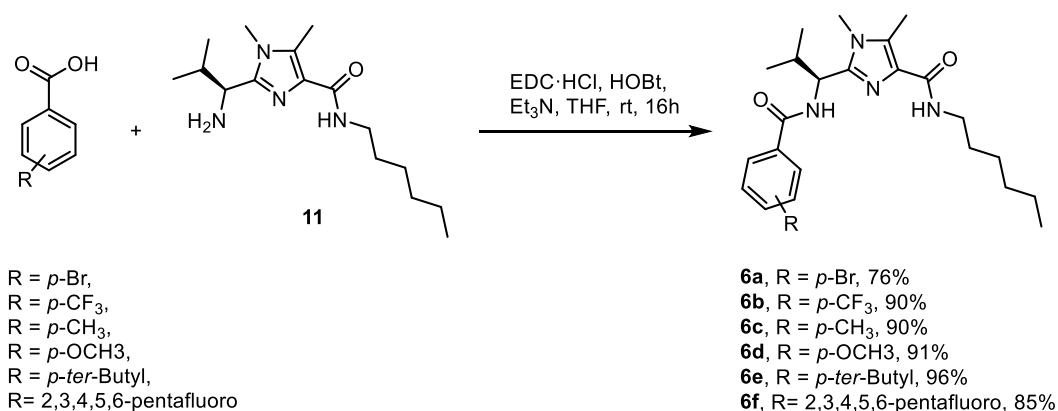
(S)-2-(1-((4-Bromophenyl)sulfonamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (7).

In a 10 mL round bottom flask, to the solution of amine (105 mg, 0.356 mmol) and 4-bromobenzenesulfonyl chloride (100 mg, 0.392 mmol) in dry THF (2 mL) was added the triethylamine (75 μ L, 0.534 mmol) and stirred for 2 hours at room temperature. After completion of reaction THF was evaporated under vacuum. CH₂Cl₂ was added and extracted with saturated NaHCO₃. The organic layer was dried over Na₂SO₄, filtered and concentrated. The residue was purified by column chromatography with silica gel (*eluent*: 30% EtOAc/ petroleum ether) to obtain **7** as a colorless liquid. **IR (neat, cm⁻¹):** 3392, 2928, 2864, 1644, 1592, 1519, 1465, 1392, 1332, 1268, 1232, 1162, 1062, 1012, 978, 914, 822, 733, 610, 557; **¹H NMR (400 MHz, CDCl₃):** δ = 7.42 (d, J = 8.9 Hz, 2H), 7.41

(d, $J = 8.9$ Hz, 2H), 5.69 (m, $J = 6.8$ Hz, 1H), 3.95 (dd, $J = 9.6$ & 7.2 Hz, 1H), 3.45-3.28 (m, 2H), 3.12 (s, 3H), 2.40 (s, 3H), 2.01 (m, 1H), 1.60 (quint, $J = 7.3$ Hz, 2H), 1.43-1.28 (m, 6H), 0.99 (d, $J = 6.7$ Hz, 3H), 0.90 (t, $J = 7$ Hz, 3H), 0.84 (d, $J = 6.7$ Hz, 3H); ^{13}C NMR (101 MHz, CDCl_3): $\delta = 163.5, 145.0, 138.7, 131.8, 130.1, 128.5, 127.5, 55.6, 38.9, 34.1, 31.7, 30.1, 29.9, 26.9, 22.7, 19.1, 18.4, 14.2, 9.6$; HRMS (ESI): Calcd. for $\text{C}_{22}\text{H}_{34}\text{BrN}_4\text{O}_3\text{S}$: 513.1535 $[\text{M}+\text{H}]^+$, found: 513.1528.

General procedure for the synthesis of amides 6a-6f.

In a 10 mL round bottom flask, to a solution of carboxylic acid (1 equiv.) in dry THF (1 mL), EDC·HCl (1.1 equiv.), HOBT (1.1 equiv.) were added and stirred for 15 minutes. Amine (1.1 equiv.) in THF (1 mL) was added followed by the addition of Et_3N (1.2 equiv.). The reaction mixture stirred for 15 hours. The THF was evaporated under vacuum. The residue was dissolved in CH_2Cl_2 and extracted with water and brine. The organic layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated and purified by column chromatography with silica gel eluting with 30-40% EtOAc/petroleum ether to obtain the amide **6a-6f** in good to excellent yields.



Scheme 4.5 Synthesis of compound **6a-6f**.

(S)-2-(1-(4-Bromobenzamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (**6a**).

M.p.: 145 °C; **IR** (neat, cm^{-1}): 3404, 3282, 3061, 2957, 2926, 2863, 1639, 1592, 1521, 1474, 1372, 1322, 1233, 1144, 1070, 1011, 980, 913, 844, 733, 559; ^1H NMR (400 MHz, CDCl_3): $\delta = 7.67$ (d, $J = 8.7$ Hz, 2H), 7.57 (d, $J = 8.6$ Hz, 2H), 7.02 (m, $J = 5.7$ Hz, 1H), 6.78 (d, $J = 9.2$ Hz, 1H), 5.12 (dd, $J = 9.0$ & 8.2 Hz, 1H), 3.57 (s, 3H), 3.44-3.27 (m, 2H), 2.55 (s, 3H), 2.36-2.25 (m, 1H), 1.59 (quint, $J = 7.3$ Hz, 2H), 1.42-1.27 (m, 6H), 1.04 (d,

$J = 6.7$ Hz, 3H), 0.93 (d, $J = 6.7$ Hz, 3H), 0.88 (t, $J = 7$ Hz, 3H); ^{13}C NMR (101 MHz, CDCl_3): $\delta = 166.0, 163.9, 146.0, 133.0, 132.4, 131.9, 130.1, 128.8, 126.5, 50.9, 38.9, 33.4, 31.6, 30.3, 30.0, 26.8, 22.7, 19.6, 18.7, 14.1, 9.8$; HRMS (ESI): Calcd. for $\text{C}_{23}\text{H}_{34}\text{BrN}_4\text{O}_2$: 477.1865 $[\text{M}+\text{H}]^+$, found: 477.1858.

(S)-N-Hexyl-1,5-dimethyl-2-(2-methyl-1-(4-(trifluoromethyl)benzamido)propyl)-1H-imidazole-4-carboxamide (6b).

M.p.: 140 °C; **IR (neat, cm^{-1}):** 3418, 3276, 2960, 2865, 1639, 1592, 1521, 1463, 1409, 1372, 1324, 1231, 1166, 1124, 1067, 1018, 984, 912, 857, 771, 730, 691, 644, 596, 552; ^1H NMR (400 MHz, CDCl_3): $\delta = 7.92$ (d, $J = 8.1$ Hz, 2H), 7.70 (d, $J = 8.2$ Hz, 2H), 7.08-6.93 (m, 2H), 5.14 (t, $J = 8.6$ Hz, 1H), 3.57 (s, 3H), 3.44-3.27 (m, 2H), 2.54 (s, 3H), 2.41-2.26 (m, 1H), 1.58 (quint, $J = 7.3$ Hz, 2H), 1.42-1.26 (m, 6H), 1.06 (d, $J = 6.7$ Hz, 3H), 0.94 (d, $J = 6.7$ Hz, 3H), 0.87 (t, $J = 7$ Hz, 3H); ^{13}C NMR (101 MHz, CDCl_3): $\delta = 165.7, 163.8, 145.8, 137.4, 133.4$ (q, $J = 35$ Hz), 132.4, 130.1, 127.7, 125.8, 123.6 (q, $J = 283$ Hz), 50.9, 38.9, 33.4, 31.6, 30.3, 30.0, 26.8, 22.7, 19.6, 18.7, 14.1, 9.8; HRMS (ESI): Calcd. for $\text{C}_{24}\text{H}_{34}\text{F}_3\text{N}_4\text{O}_2$: 467.2634 $[\text{M}+\text{H}]^+$, found: 467.2625.

(S)-N-Hexyl-1,5-dimethyl-2-(2-methyl-1-(4-methylbenzamido)propyl)-1H-imidazole-4-carboxamide (6c).

M.p.: 160 °C; **IR (neat, cm^{-1}):** 3413, 3273, 2927, 2865, 1639, 1593, 1507, 1463, 1372, 1321, 1231, 1189, 1117, 1047, 983, 913, 838, 727, 643, 588, 553; ^1H NMR (400 MHz, CDCl_3): $\delta = 7.69$ (d, $J = 8.1$ Hz, 2H), 7.22 (d, $J = 8.1$ Hz, 2H), 7.03 (t, $J = 5.7$ Hz, 1H), 6.78 (d, $J = 9.0$ Hz, 1H), 5.13 (t, $J = 8.7$ Hz, 1H), 3.55 (s, 3H), 3.34 (td, $J = 13.5$ & 7.3 Hz, 2H), 2.53 (s, 3H), 2.38 (s, 3H), 2.36-2.25 (m, 1H), 1.57 (quint, $J = 7.3$ Hz, 2H), 1.41-1.24 (m, 6H), 1.05 (d, $J = 6.7$ Hz, 3H), 0.94 (d, $J = 6.8$ Hz, 3H), 0.87 (t, $J = 6.7$ Hz, 3H); ^{13}C NMR (101 MHz, CDCl_3): $\delta = 166.9, 164.0, 146.2, 142.3, 132.3, 131.3, 130.0, 129.3, 127.1, 50.8, 38.8, 33.3, 31.6, 30.3, 30.0, 26.8, 22.7, 19.7, 18.7, 14.1, 9.8$; HRMS (ESI): Calcd. for $\text{C}_{24}\text{H}_{37}\text{N}_4\text{O}_2$: 413.2916 $[\text{M}+\text{H}]^+$, found: 413.2937.

(S)-N-Hexyl-2-(1-(4-methoxybenzamido)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxamide (6d).

M.p.: 151-153 °C; **IR (neat, cm^{-1}):** 3403, 2957, 2928, 2864, 1638, 1600, 1503, 1462, 1372, 1311, 1252, 1178, 1114, 1031, 983, 914, 845, 770, 729, 683, 641, 592, 555; ^1H

NMR (400 MHz, CDCl₃): δ = 7.76 (d, J = 8.9 Hz, 2H), 7.04 (t, J = 5.8 Hz, 1H), 6.91 (d, J = 8.8, 2.4 Hz, 2H), 6.73 (d, J = 8.9 Hz, 1H), 5.13 (t, J = 8.7 Hz, 1H), 3.83 (s, 3H), 3.45-3.27 (m, 2H), 2.53 (s, 3H), 2.41-2.24 (m, 1H), 1.57 (quint, J = 7.3 Hz, 2H), 1.42-1.26 (m, 6H), 1.05 (d, J = 6.7 Hz, 3H), 0.93 (d, J = 6.7 Hz, 3H), 0.87 (t, J = 6.6 Hz, 3H); **¹³C NMR (101 MHz, CDCl₃):** δ = 166.5, 164.0, 162.5, 146.3, 132.4, 130.1, 129.0, 126.4, 113.9, 55.5, 50.8, 38.9, 33.3, 31.7, 30.3, 30.0, 26.9, 22.7, 19.8, 18.8, 14.2, 9.9; **HRMS (ESI):** Calcd. for C₂₄H₃₇N₄O₃: 429.2865 [M+H]⁺, found: 429.2857.

(S)-2-(1-(4-(*Tert*-butyl)benzamido)-2-methylpropyl)-*N*-hexyl-1,5-dimethyl-1*H*-imidazole-4-carboxamide (6e).

M.p.: 140 °C; **IR (neat, cm⁻¹):** 3411, 2958, 2927, 2865, 1640, 1594, 1511, 1463, 1368, 1322, 1274, 1232, 1150, 1049, 983, 917, 851, 774, 731, 643, 568; **¹H NMR (400 MHz, CDCl₃):** δ = 7.74 (d, J = 8.5 Hz, 2H), 7.46 (d, J = 8.5 Hz, 2H), 7.05 (t, J = 5.9 Hz, 1H), 6.77 (d, J = 9.1 Hz, 1H), 5.14 (t, J = 8.7 Hz, 1H), 3.56 (s, 3H), 3.45 – 3.27 (m, 2H), 2.55 (s, 3H), 2.42 – 2.25 (m, 1H), 1.58 (quint, J = 7.3 Hz, 2H), 1.43-1.27 (m, 15H), 1.05 (d, J = 6.8 Hz, 3H), 0.94 (d, J = 6.7 Hz, 3H), 0.88 (t, J = 6.9 Hz, 3H); **¹³C NMR (101 MHz, CDCl₃):** δ = 166.9, 164.0, 155.4, 146.2, 132.4, 131.3, 130.1, 127.0, 125.7, 50.8, 38.9, 35.1, 33.4, 31.7, 30.3, 30.1, 26.9, 22.7, 19.8, 18.8, 14.2, 9.9; **HRMS (ESI):** Calcd. for C₂₇H₄₃N₄O₂: 455.3386 [M+H]⁺, found: 455.3391.

(S)-*N*-Hexyl-1,5-dimethyl-2-(2-methyl-1-(perfluorobenzamido)propyl)-1*H*-imidazole-4-carboxamide (6f).

M.p.: 126-127 °C; **IR (neat, cm⁻¹):** 3419, 3270, 2960, 2928, 2865, 1652, 1593, 1496, 1414, 1373, 1340, 1260, 1230, 1163, 1111, 1049, 989, 915, 861, 823, 730, 682, 645, 611, 575; **¹H NMR (400 MHz, CDCl₃):** δ = 7.18 (d, J = 8.1 Hz, 1H), 7.0 (t, J = 5.8 Hz, 1H), 5.09 (t, J = 8.5 Hz, 1H), 3.56 (s, 3H), 3.40 – 3.21 (m, 2H), 2.45 (s, 3H), 2.41 – 2.27 (m, 1H), 1.55 (quint, J = 7.3 Hz, 2H), 1.39-1.24 (m, 6H), 1.07 (d, J = 6.8 Hz, 3H), 0.96 (d, J = 6.7 Hz, 3H), 0.87 (t, J = 6.8 Hz, 3H); **¹³C NMR (101 MHz, CDCl₃):** δ = 163.8, 157.2, 144.8 (dm, J = 242.4 Hz), 132.5 (dm, J = 252.5 Hz), 130.0, 111.7 (t, 20 Hz), 51.5, 38.9, 32.9, 31.7, 30.3, 30.0, 26.8, 22.7, 19.7, 18.4, 14.1, 9.7; **HRMS (ESI):** Calcd. for C₂₃H₃₀F₅N₄O₂: 489.2289 [M+H]⁺, found: 489.2265.

4.4.4 Anion Binding Study by ^1H NMR Titration Experiment

Initially the imidazolium hexafluorophosphate salts **6a**·HPF₆ and **7**·HPF₆ were prepared quantitatively by treatment of dichloromethane solutions of neutral **6a** and **7** (30 mg in 15 mL) with aqueous HPF₆ 1M (3 x 10 mL). The comparative ^1H NMR spectra of **6a** with **6a**·HPF₆ and **7** with **7**·HPF₆ in acetonitrile-*d*₃ are provided below. All the receptors and TBACl were dried in vacuum desiccator before use.

The stock solutions of **6a**·HPF₆ and **7**·HPF₆ (0.01M) and tetrabutylammonium chloride (1M) were prepared in either acetonitrile-*d*₃. ^1H NMR spectra were recorded on a Bruker Ascent™ 400 spectrometer and calibrated with respect to residual protic solvent peak in Acetonitrile-*d*₃ (δ = 1.94 ppm). NMR titrations were performed by successive addition of aliquots of the apparent anionic guest as the tetrabutylammonium (TBA) salt. In acetonitrile-*d*₃, a change in the chemical shift of NH and aromatic CH was observed. The ^1H NMR stacked plots were processed using MestReNova 6 software. To obtain the binding constants data was curve fitted with 1:1 binding model of the WinEQNMR2 computer program.¹⁹

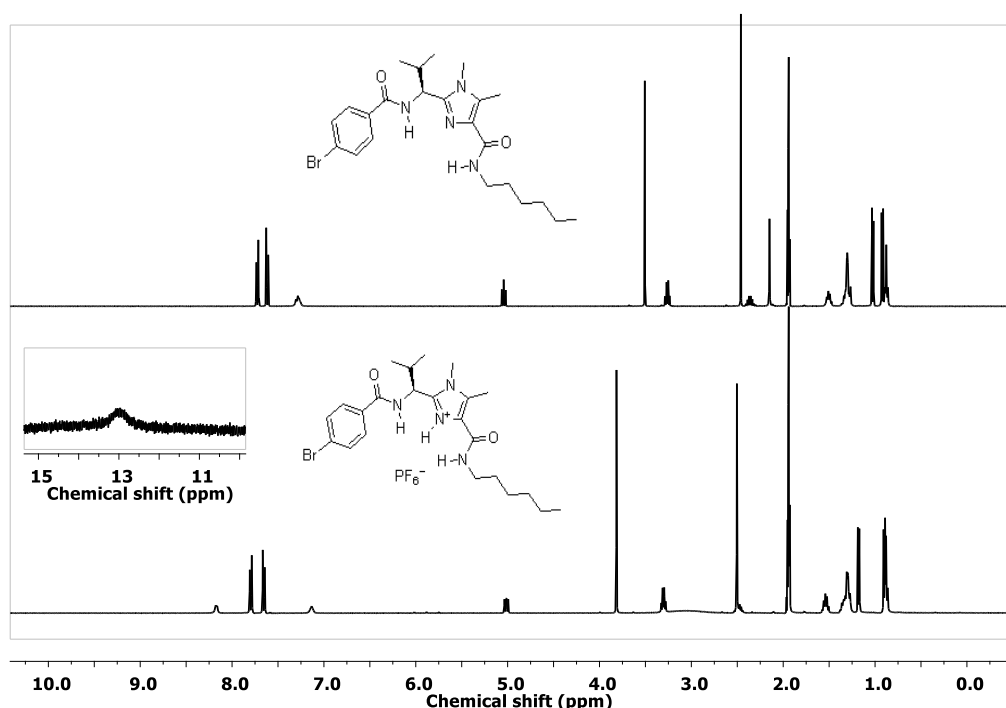


Figure 4.13 Comparative ^1H NMR spectra of **6a** and **6a**·HPF₆.

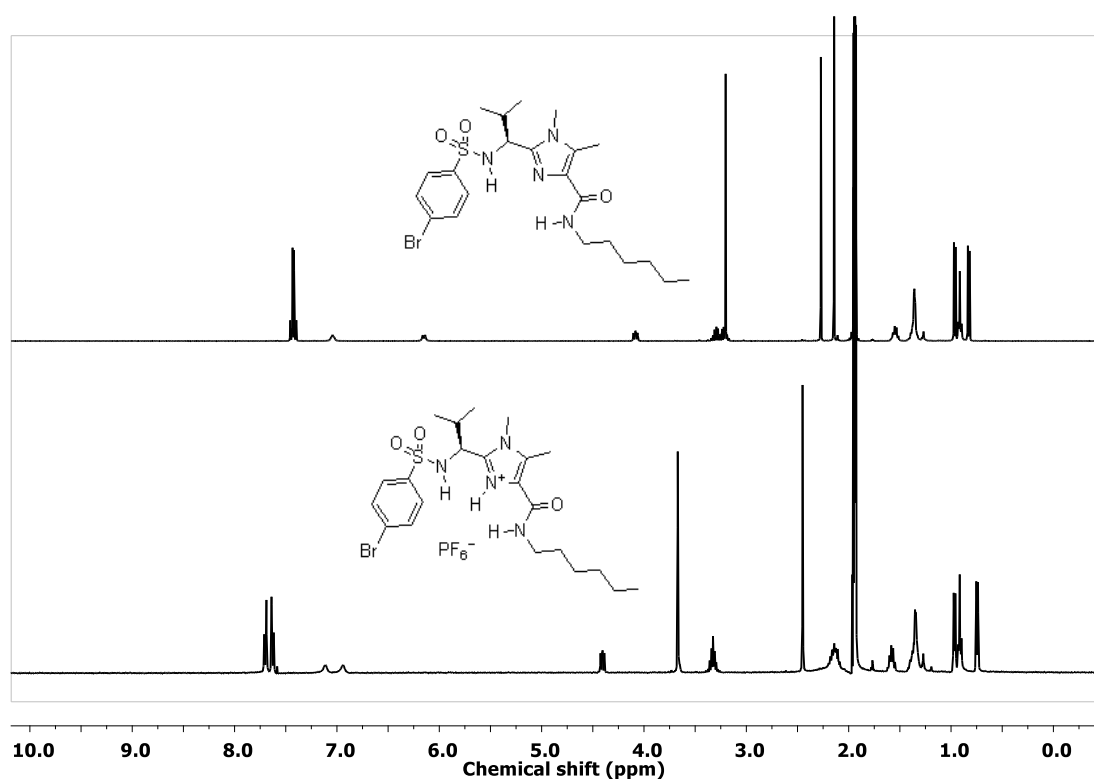


Figure 4.14 Comparative ^1H NMR spectra of **7** and **7·HPF₆**.

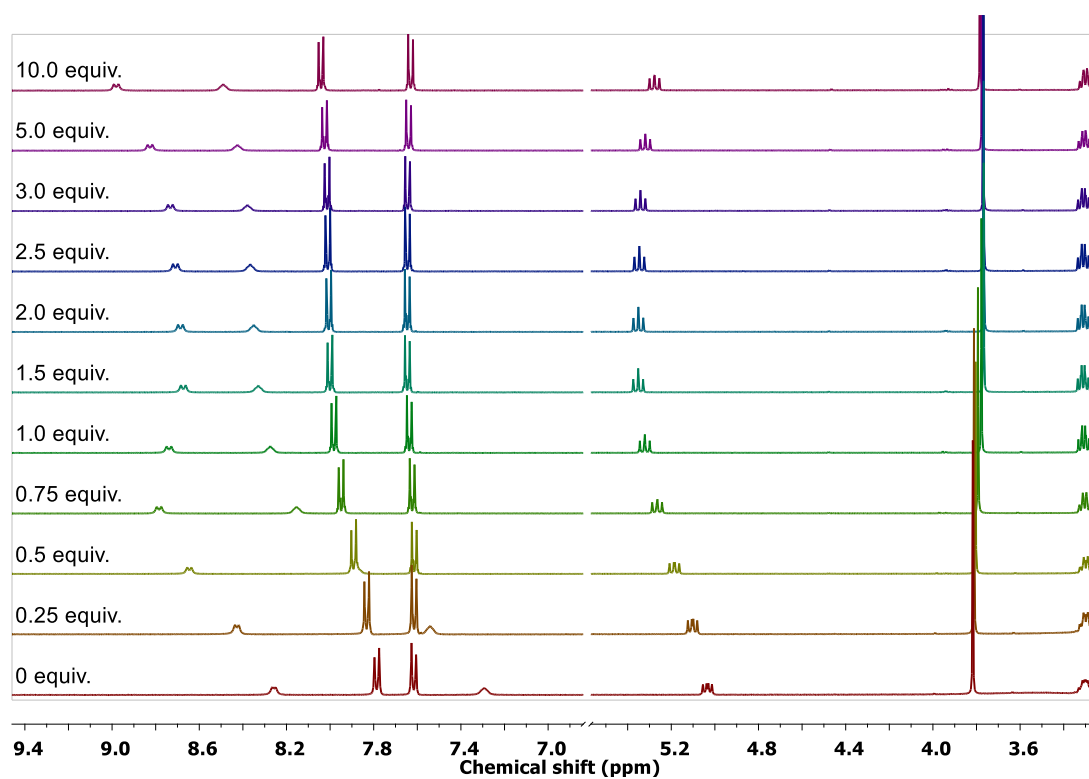


Figure 4.15 Stack plot of ^1H NMR titration of **6a·HPF₆** with TBACl in acetonitrile- d_3 . Equivalent of TBACl is given on stacked spectra.

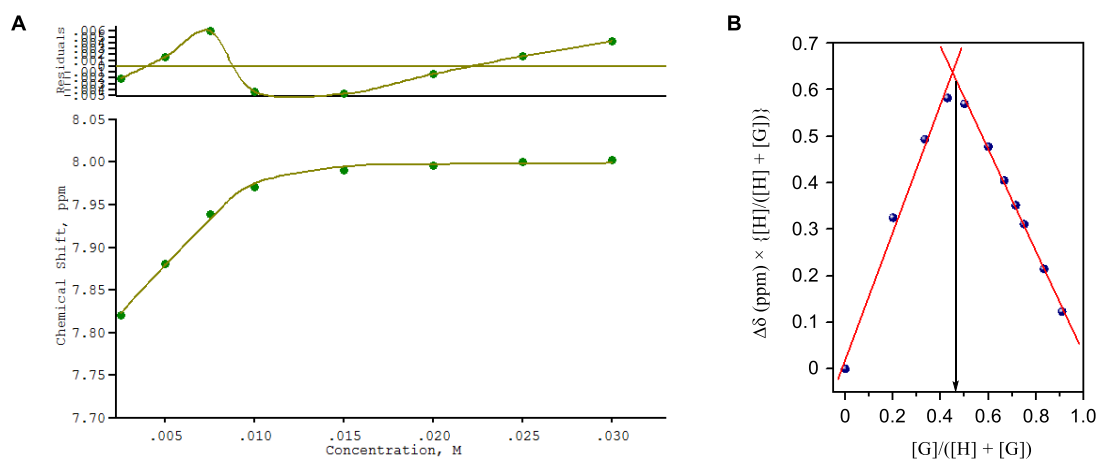


Figure 4.16 Fit plot of chemical shift (δ ppm) of amide CH_2NH proton of $6a \cdot HPF_6$ versus concentration of TBACl fitted to 1:1 binding model of WinEQNMR2 program (A). Job's plot generated from the same 1H NMR titration indicating 1:1 binding of $6a \cdot H:Cl^-$ (B).

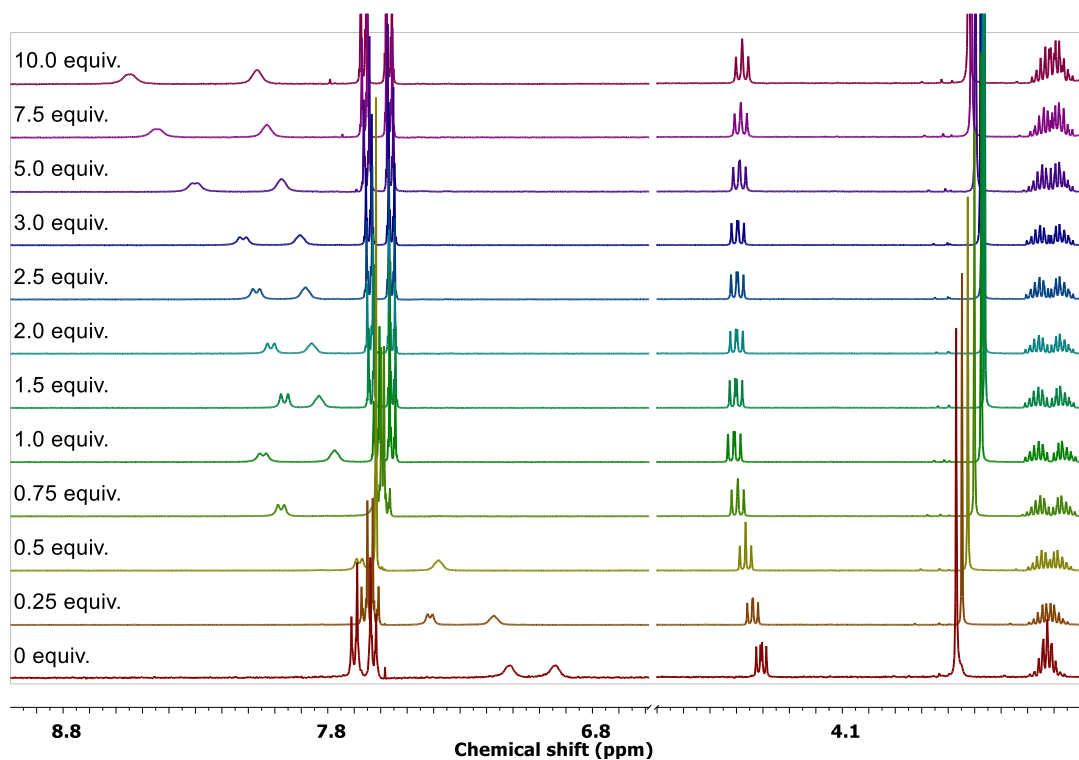


Figure 4.17 Stack plot of 1H NMR titration of $7 \cdot HPF_6$ with TBACl in $acetonitrile-d_3$. Equivalent of TBACl is given on stacked spectra.

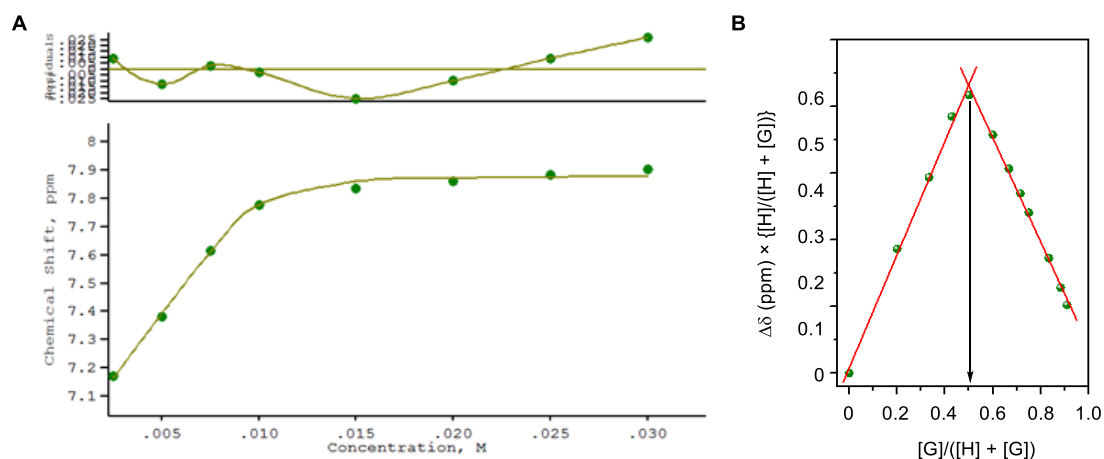


Figure 4.18 Fit plot of chemical shift (δ ppm) of amide CH_2NH proton of $7\cdot\text{HPF}_6$ versus concentration of TBACl fitted to 1:1 binding model of WinEQNMR2 program (A). Job's plot generated from the same ^1H NMR titration indicating 1:1 binding of $7\cdot\text{H}:\text{Cl}^-$ (B).

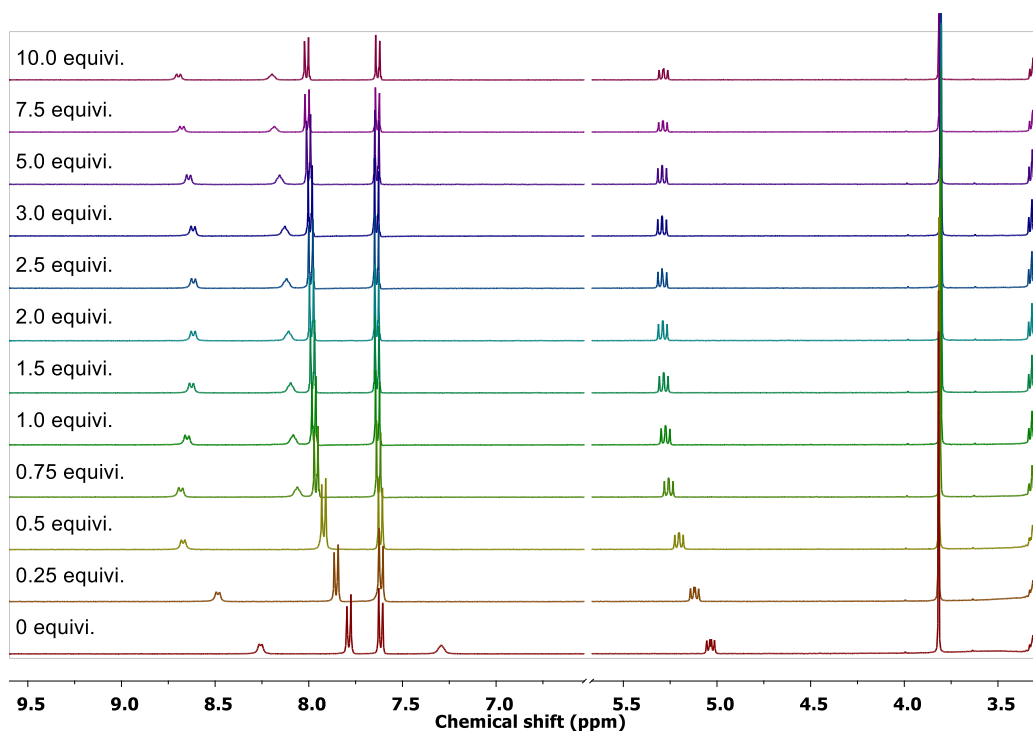


Figure 4.19 Stack plot of ^1H NMR titration of $6\text{a}\cdot\text{HPF}_6$ with TBABr in $\text{acetonitrile-}d_3$. Equivalent of TBACl is given on stacked spectra.

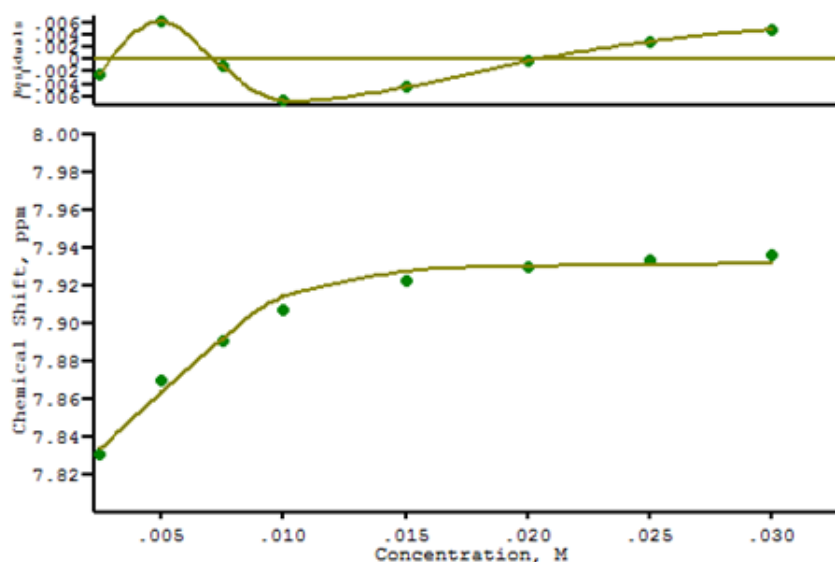


Figure 4.20 Fit plot of chemical shift (δ ppm) of amide CH_2NH proton of $6\mathbf{a}\cdot\text{HPF}_6$ versus concentration of TBABr fitted to 1:1 binding model of WinEQNMR2 program.

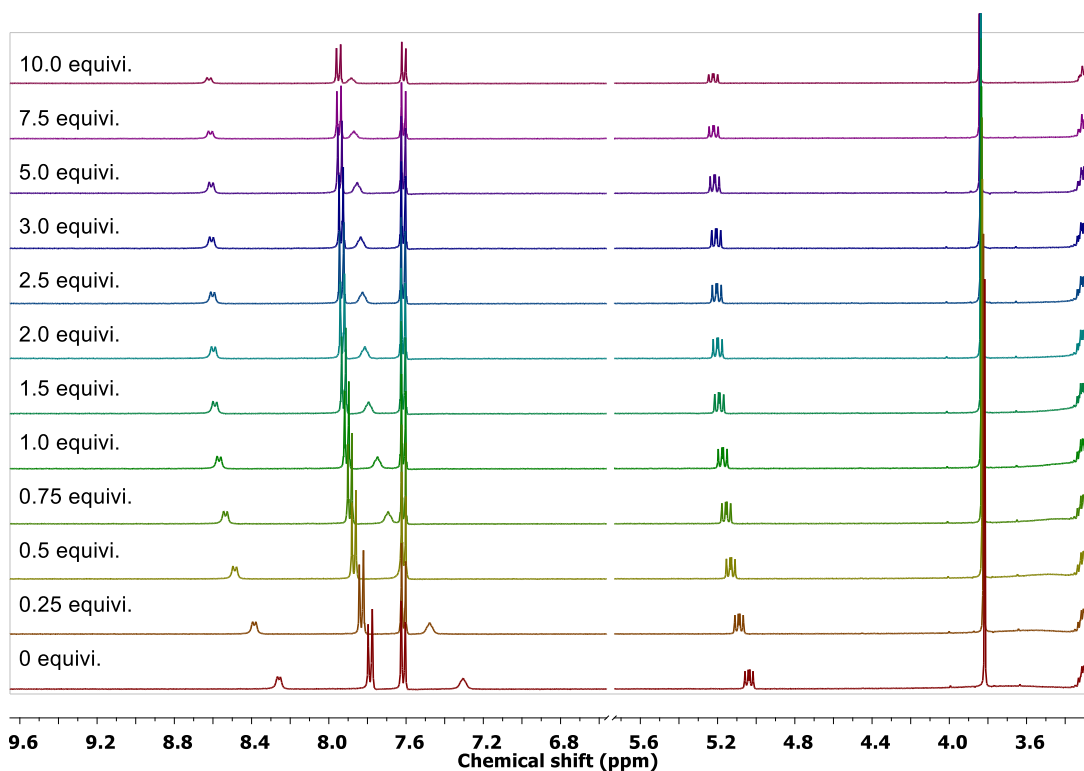


Figure 4.21 Stack plot of ^1H NMR titration of $6\mathbf{a}\cdot\text{HPF}_6$ with TBABr in acetonitrile- d_3 . Equivalent of TBAI is given on stacked spectra.

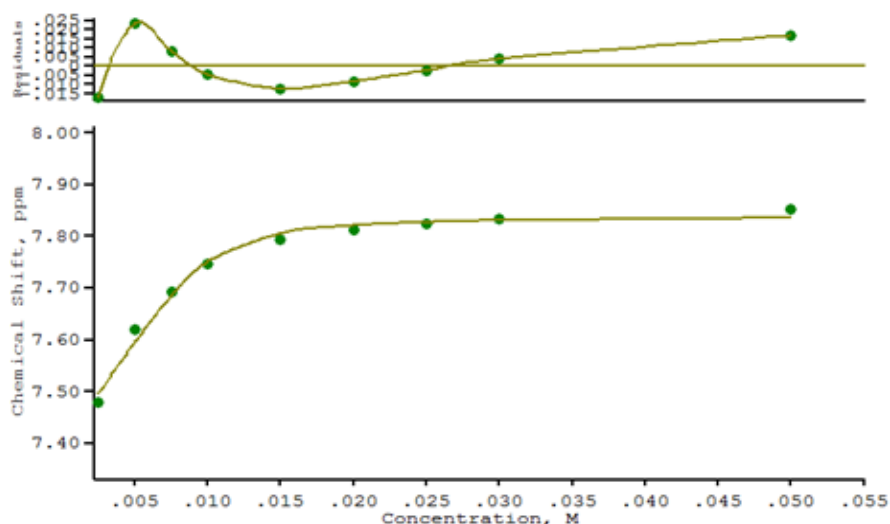


Figure 4.22 Fit plot of chemical shift (δ ppm) of amide CH_2NH proton of **6a**· HPF_6 versus concentration of TBAI fitted to 1:1 binding model of WinEQNMR2 program.

4.4.5 Ion transport Experiments

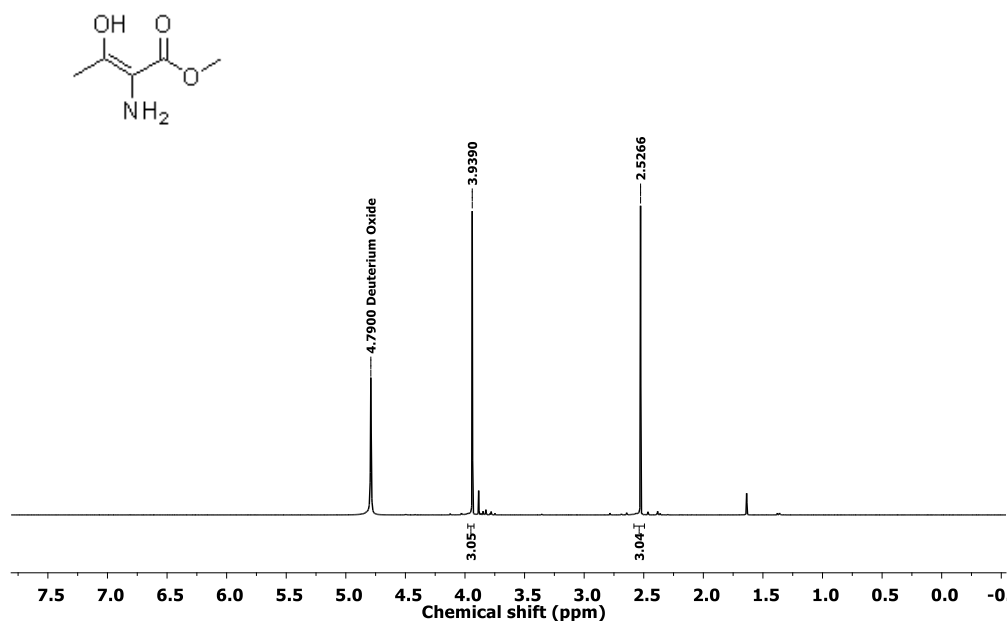
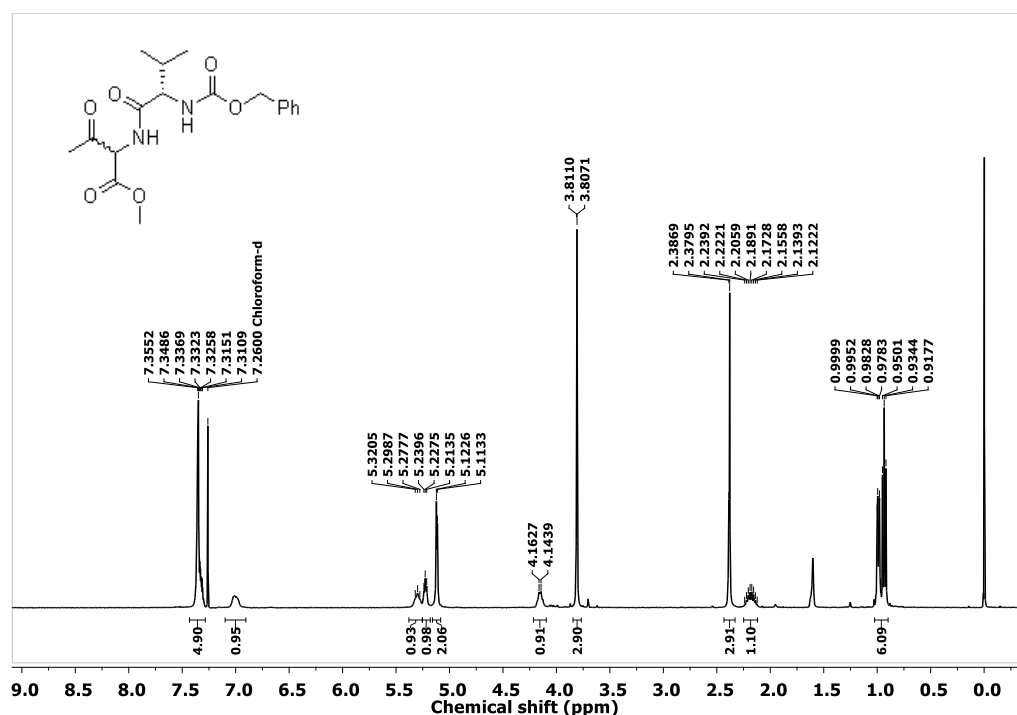
Ion Transport Activity and Selectivity

Ion transport activity and selectivity assays across EYPC-LUVs $\supset$ HPTS and EYPC-LUVs $\supset$ Lucigenin screened according to the similar methods as described in the chapter 2 and Chapter 3.

FCCP and Valinomycin and Lucigenin Assays

Ion transport activity and selectivity assays across EYPC-LUVs $\supset$ HPTS and EYPC-LUVs $\supset$ Lucigenin screened according to the similar methods as described in chapter 3.

4.5 NMR Spectra

Figure 4.23 ¹H NMR spectrum of amine 13.Figure 4.24 ¹H NMR spectrum of methyl 2-((S)-2-(((benzyloxy)carbonyl)amino)-3-methylbutanamido)-3-oxobutanoate (15).

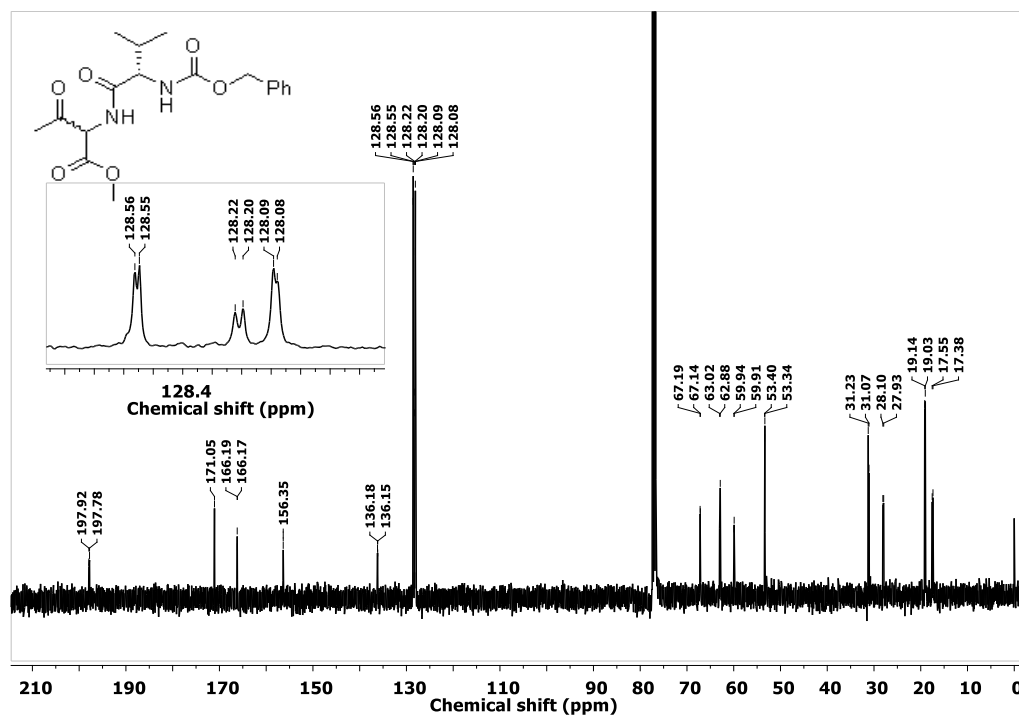


Figure 4.25 ¹³C NMR spectrum of methyl 2-((S)-2-(((benzyloxy)carbonyl)amino)-3-methylbutanamido)-3-oxobutanoate (15).

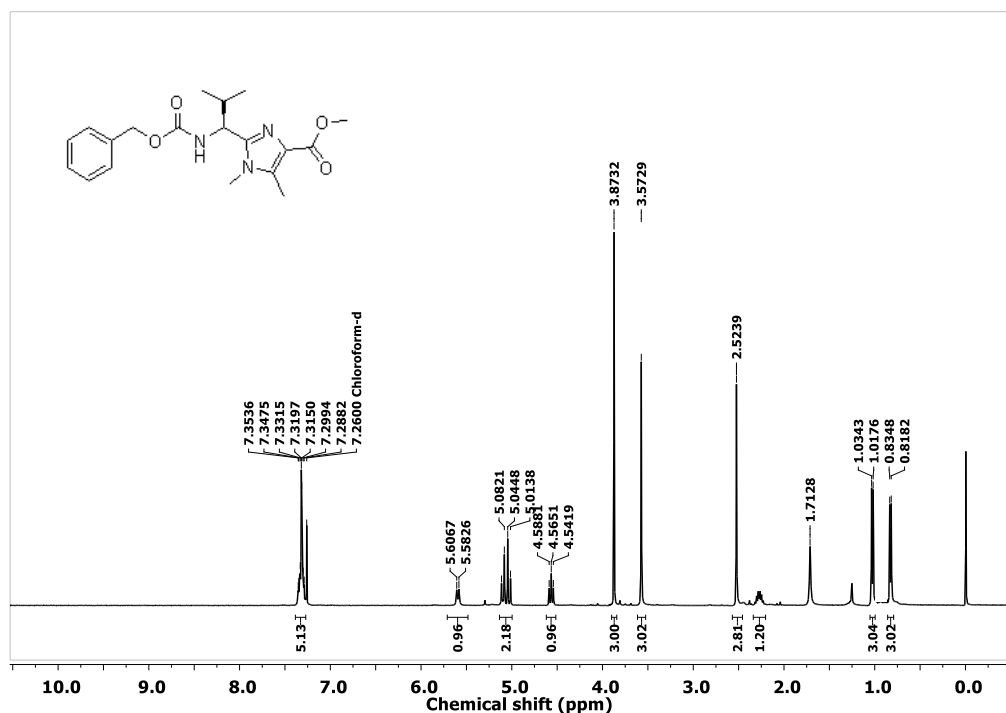


Figure 4.26 ¹H NMR spectrum of methyl (S)-2-(1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxylate (8).

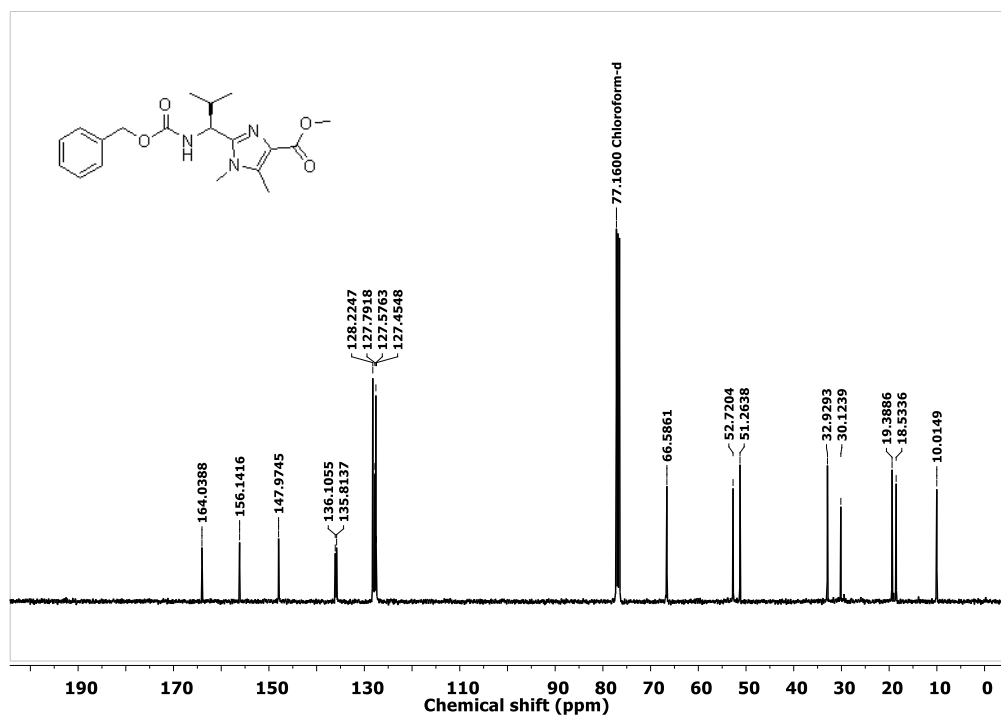


Figure 4.27 ^{13}C NMR spectrum of methyl (S)-2-(1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxylate (8).

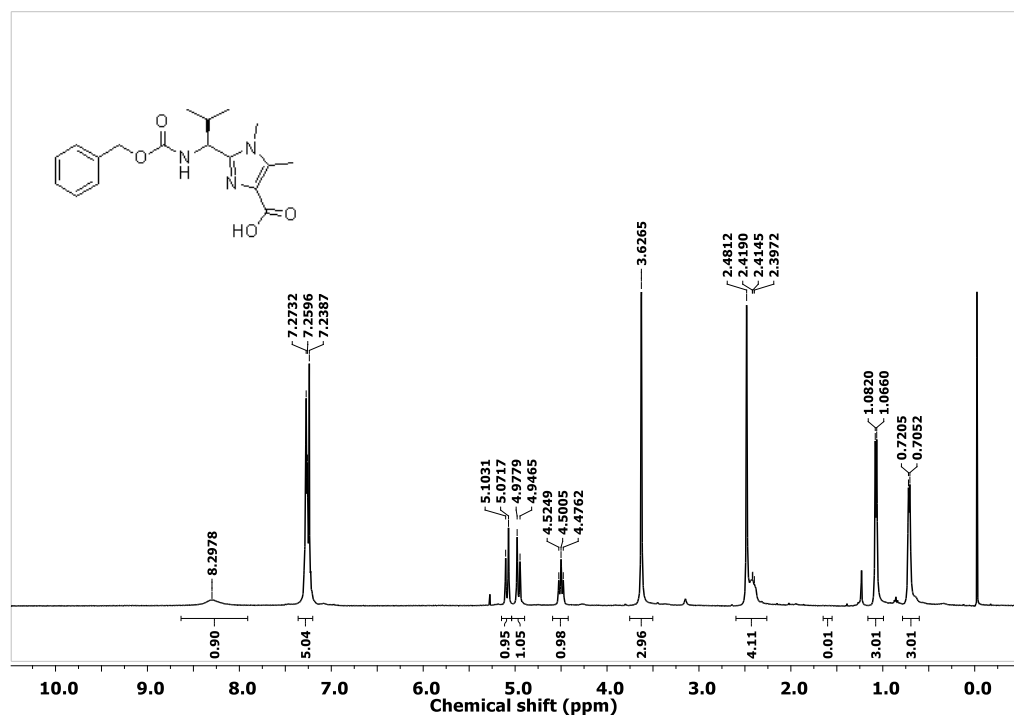


Figure 4.28 ^1H NMR spectrum of (S)-2-(1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxylic acid (9).

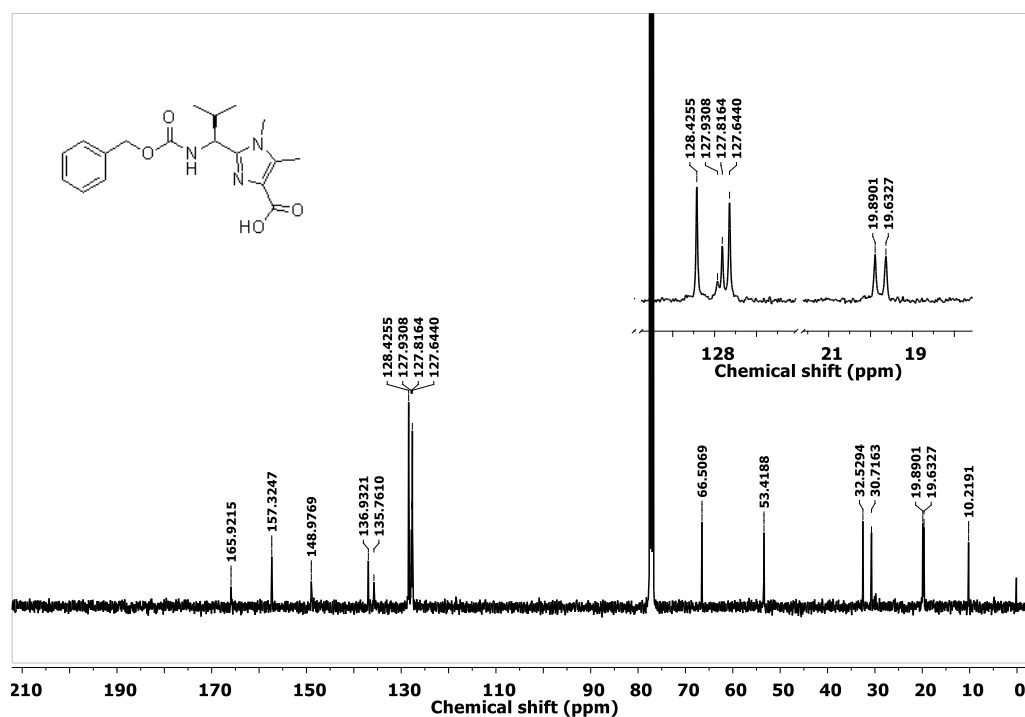


Figure 4.29 ^{13}C NMR spectrum of (S)-2-(1-(((benzyloxy)carbonyl)amino)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxylic acid (9).

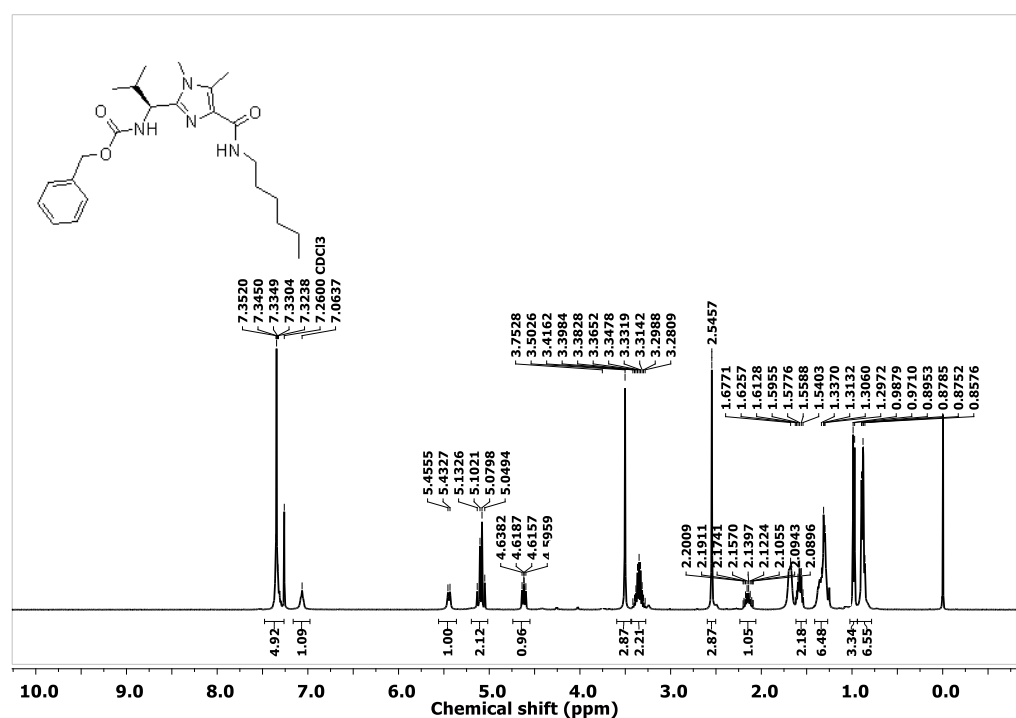


Figure 4.30 ^1H NMR spectrum of benzyl (S)-1-(4-(hexylcarbamoyl)-1,5-dimethyl-1H-imidazol-2-yl)-2-methylpropylcarbamate (10).

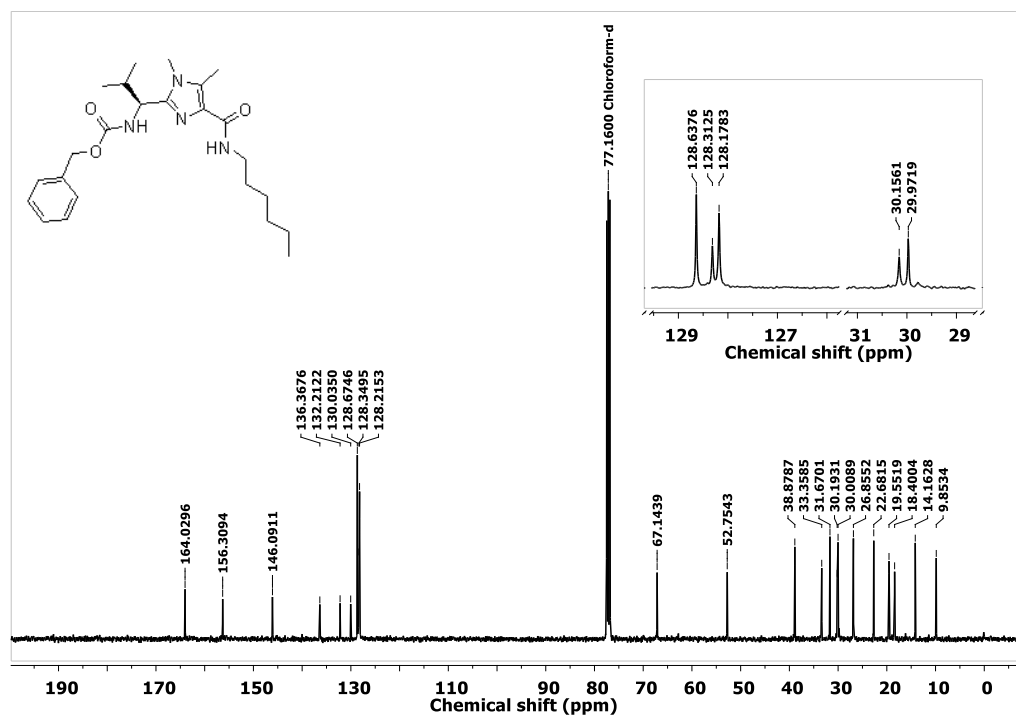


Figure 4.31 ¹³C NMR spectrum of benzyl (S)-1-(4-(hexylcarbamoyl)-1,5-dimethyl-1H-imidazol-2-yl)-2-methylpropylcarbamate (**10**).

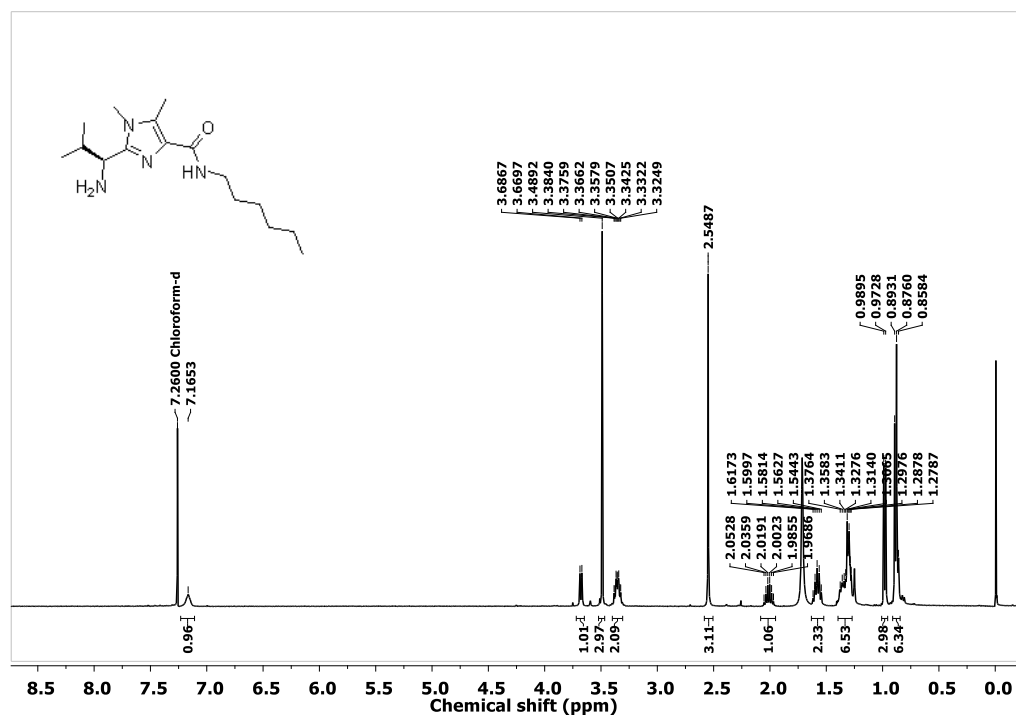


Figure 4.32 ¹H NMR spectrum of (S)-2-(1-amino-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (**11**).

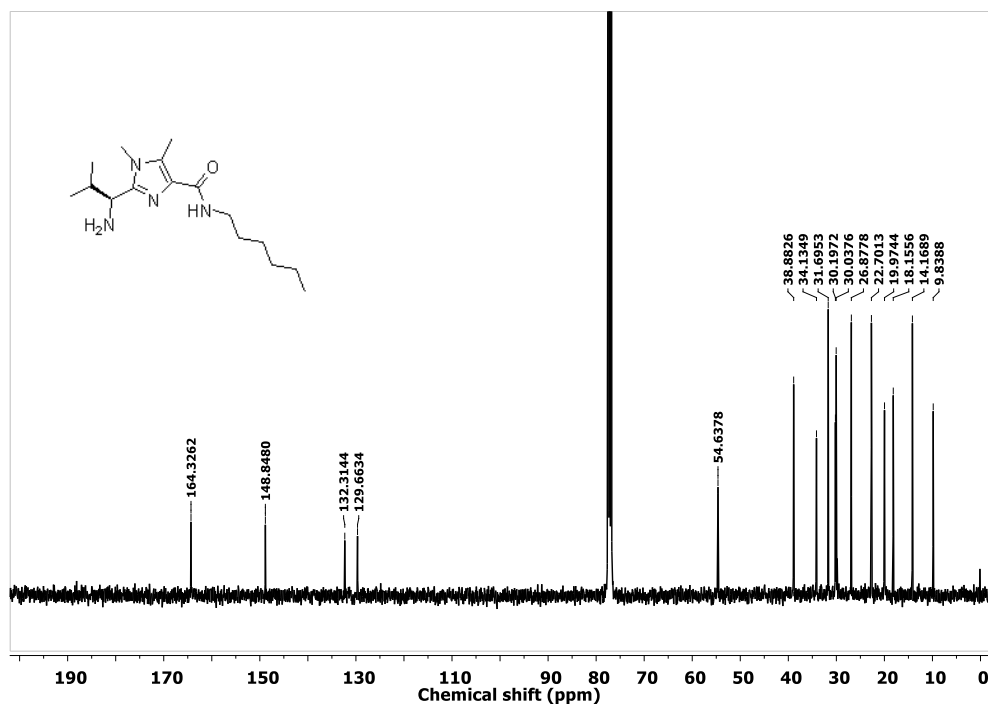


Figure 4.33 ¹³C NMR spectrum of (S)-2-(1-amino-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (11).

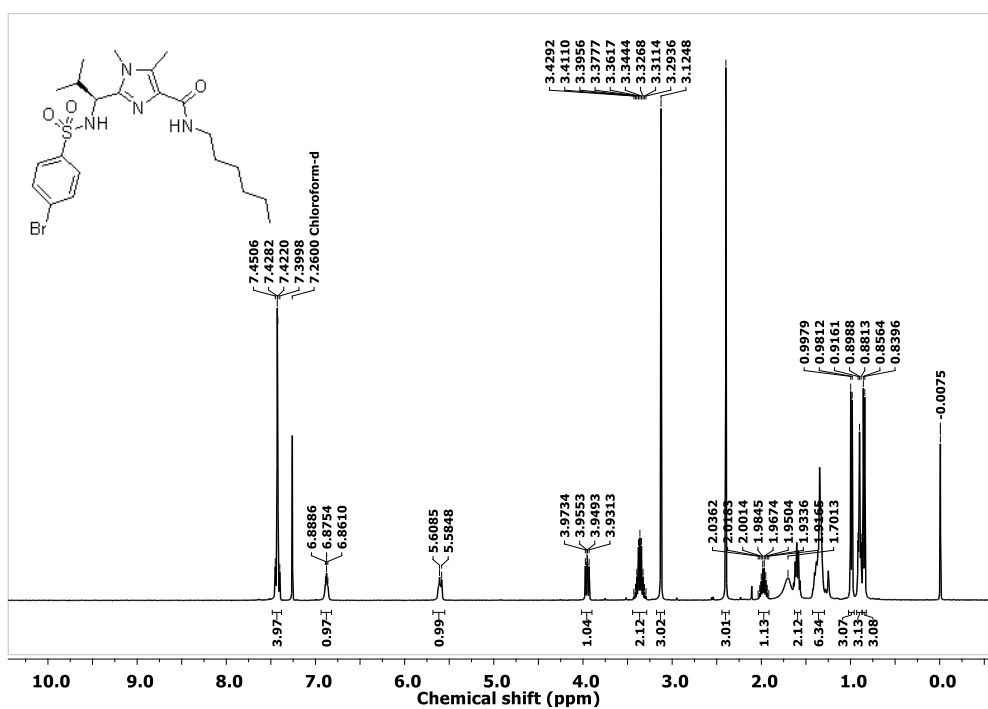


Figure 4.34 ¹H NMR spectrum of (S)-2-(1-((4-bromophenyl)sulfonamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (7).

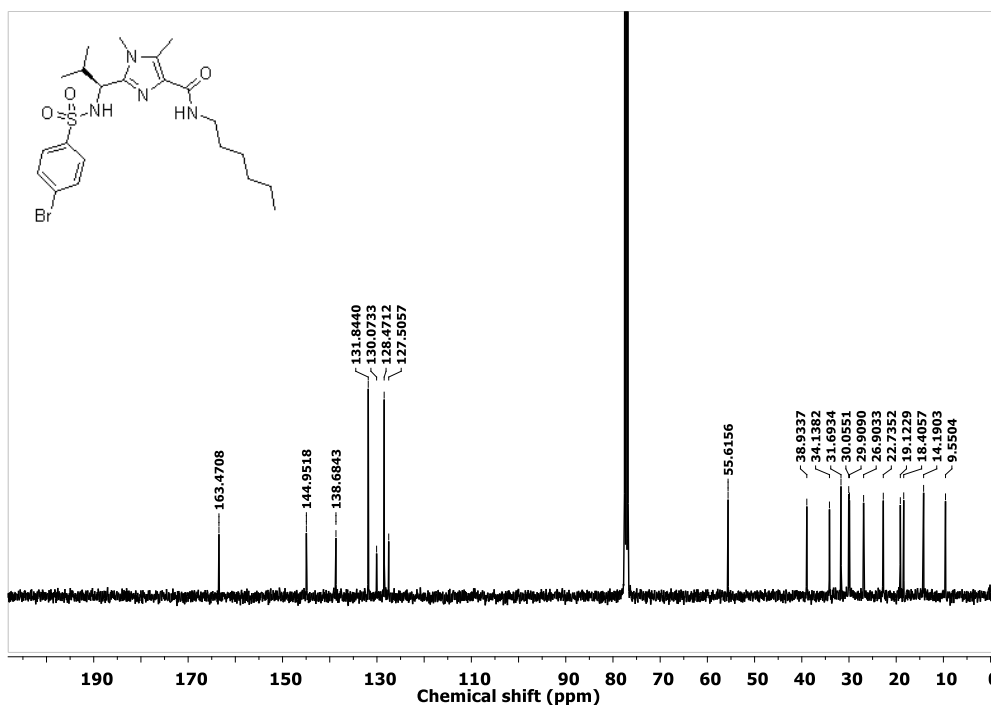


Figure 4.35 ^{13}C NMR spectrum of (S)-2-(1-((4-bromophenyl)sulfonamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (7).

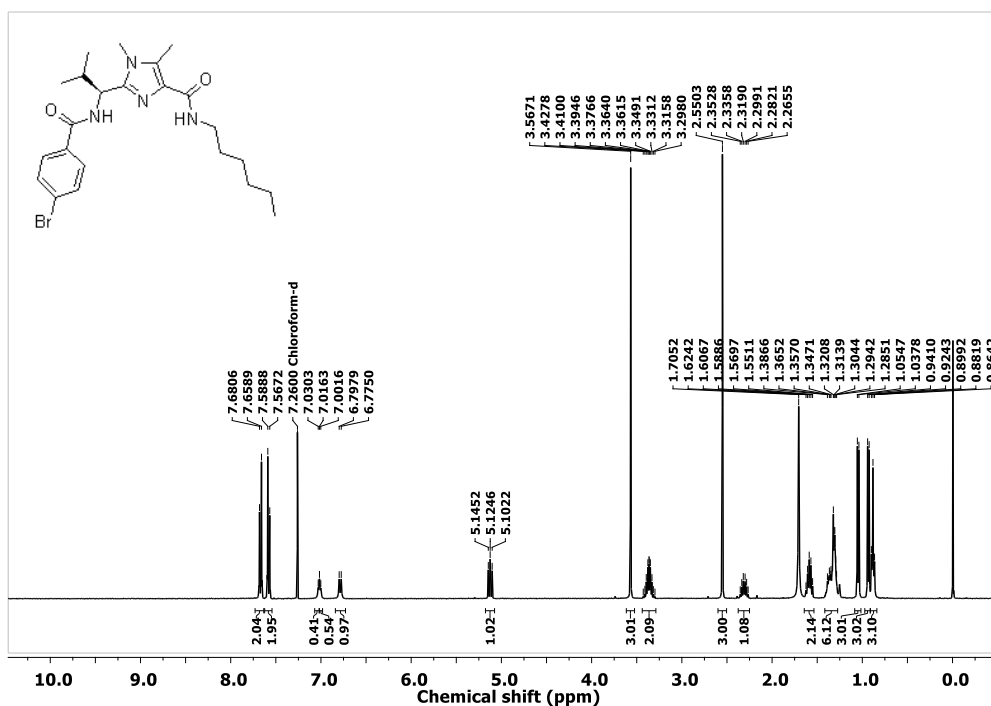


Figure 4.36 ^1H NMR spectrum of (S)-2-(1-((4-bromophenyl)sulfonamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (6a).

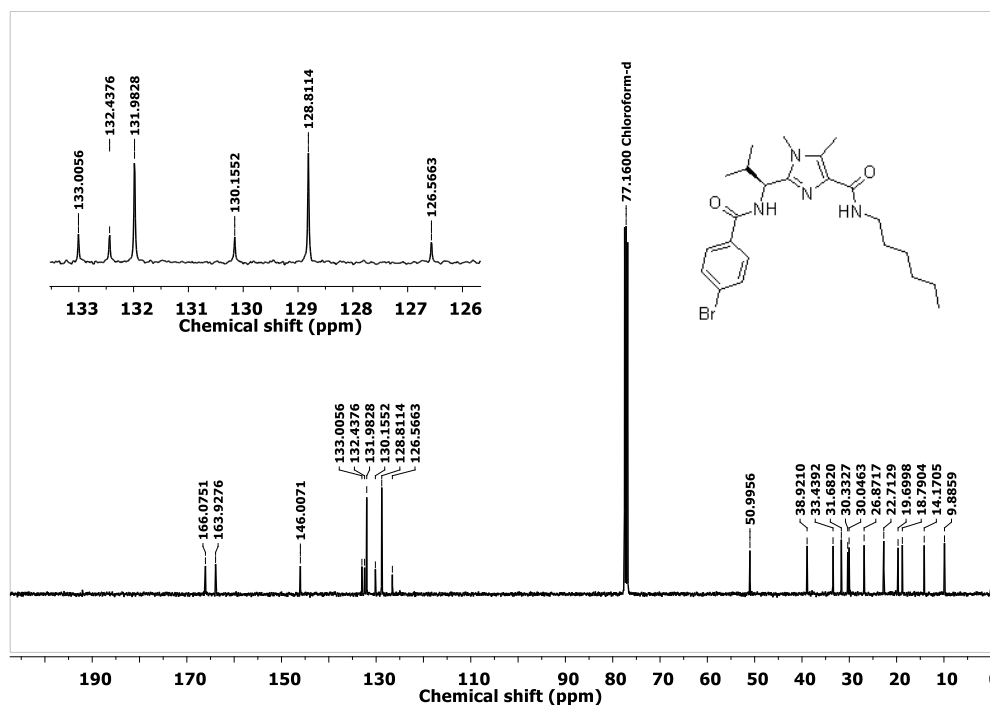


Figure 4.37 ¹³C NMR spectrum of (S)-2-(1-(4-bromobenzamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (6a).

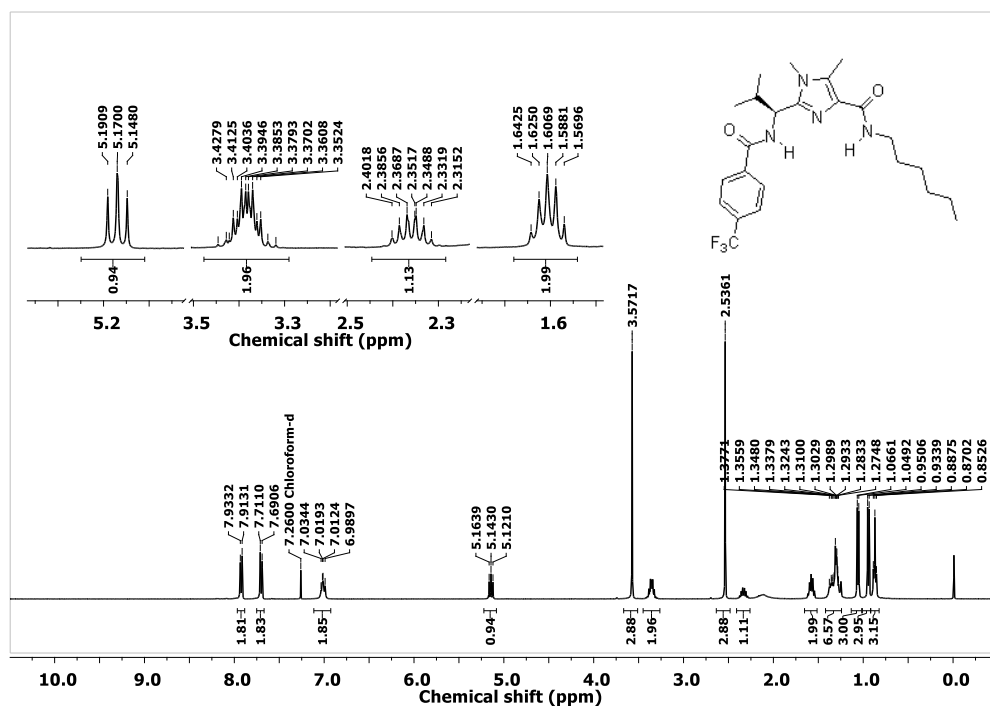


Figure 4.38 ¹H NMR spectrum of (S)-N-hexyl-1,5-dimethyl-2-(2-methyl-1-(4-(trifluoromethyl)benzamido)propyl)-1H-imidazole-4-carboxamide (6b).

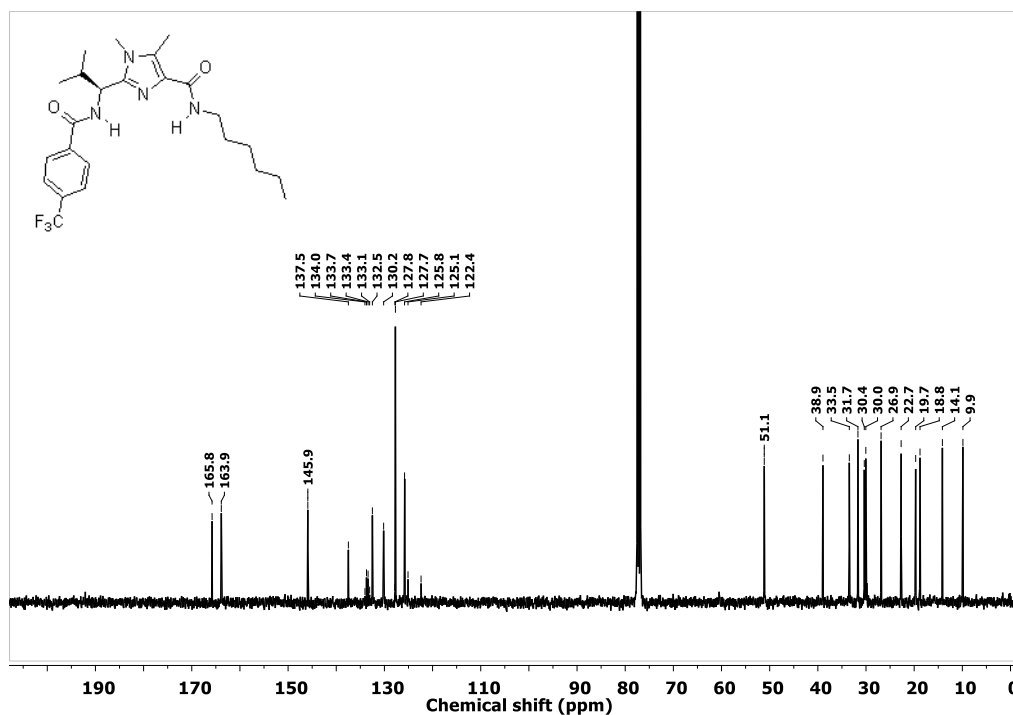


Figure 4.39 ^{13}C NMR spectrum of (S)-N-hexyl-1,5-dimethyl-2-(2-methyl-1-(4-(trifluoromethyl)benzamido)propyl)-1H-imidazole-4-carboxamide (**6b**).

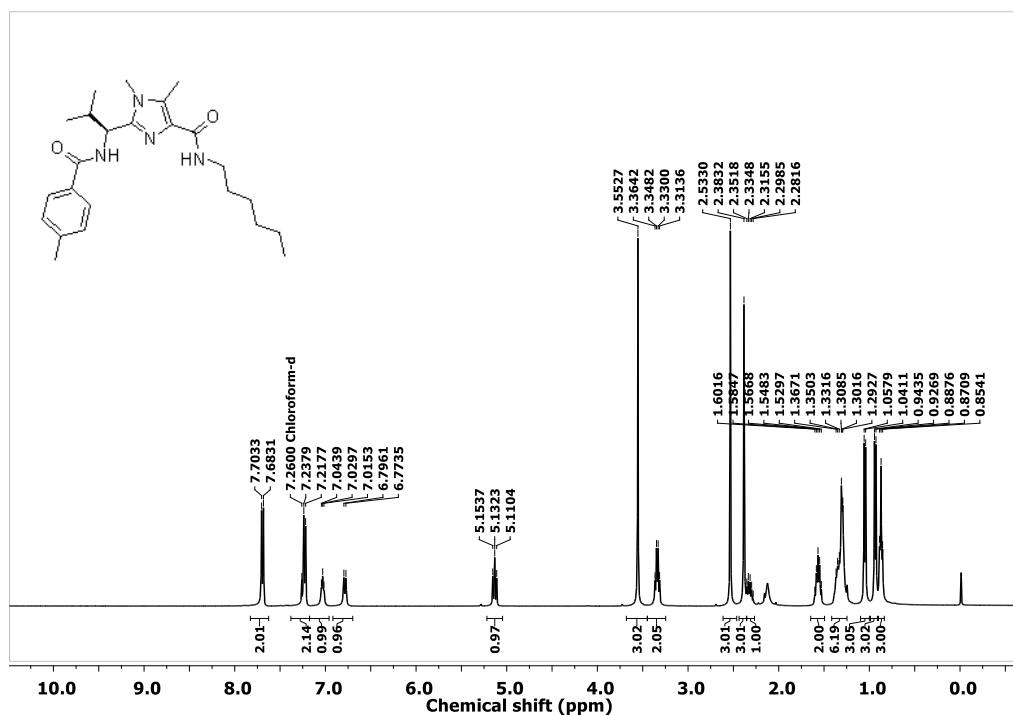


Figure 4.40 ^1H NMR spectrum of (S)-N-hexyl-1,5-dimethyl-2-(2-methyl-1-(4-methylbenzamido)propyl)-1H-imidazole-4-carboxamide (**6c**).

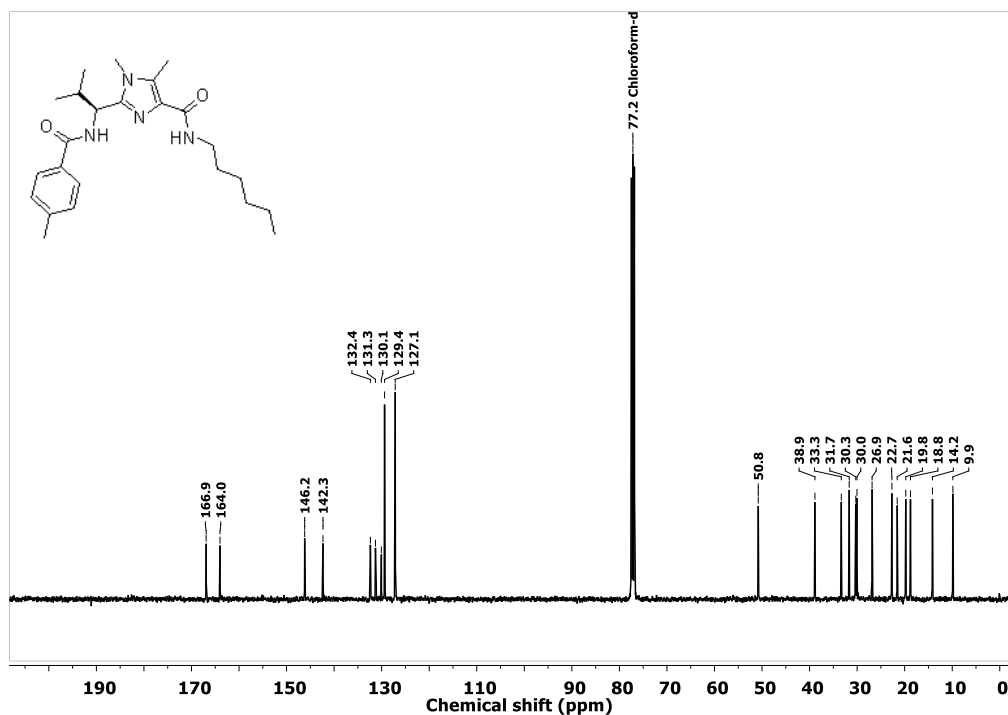


Figure 4.41 ¹³C NMR spectrum of (S)-N-hexyl-1,5-dimethyl-2-(2-methyl-1-(4-methylbenzamido)propyl)-1H-imidazole-4-carboxamide (6c).

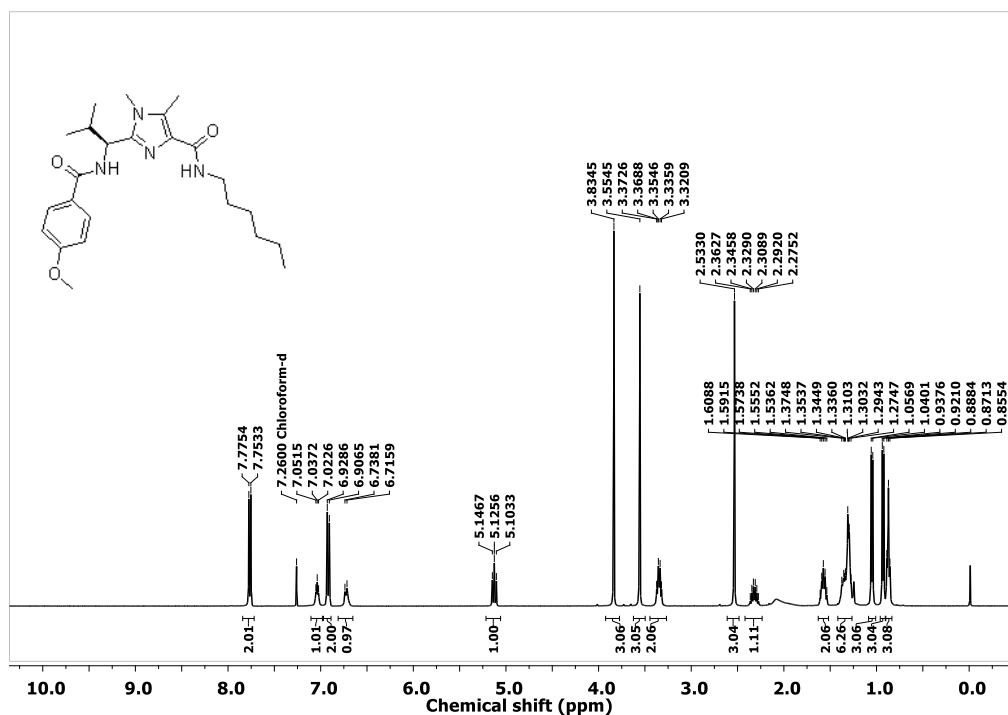


Figure 4.42 ¹H NMR spectrum of (S)-N-hexyl-2-(1-(4-methoxybenzamido)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxamide (6d).

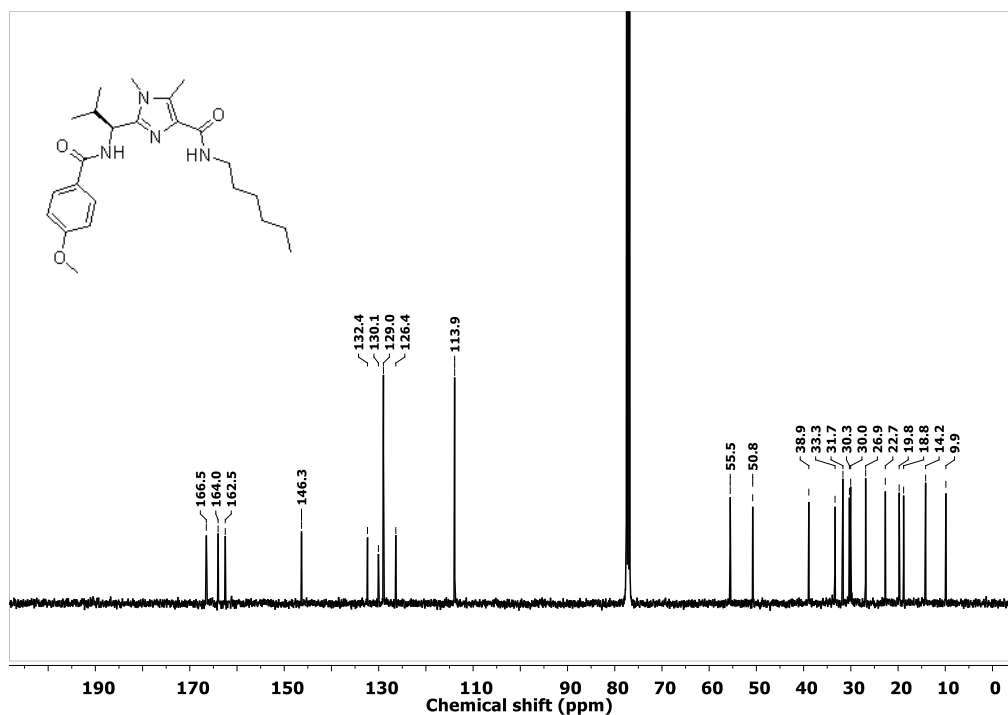


Figure 4.43 ¹³C NMR spectrum of (*S*)-*N*-hexyl-2-(1-(4-methoxybenzamido)-2-methylpropyl)-1,5-dimethyl-1*H*-imidazole-4-carboxamide (**6d**).

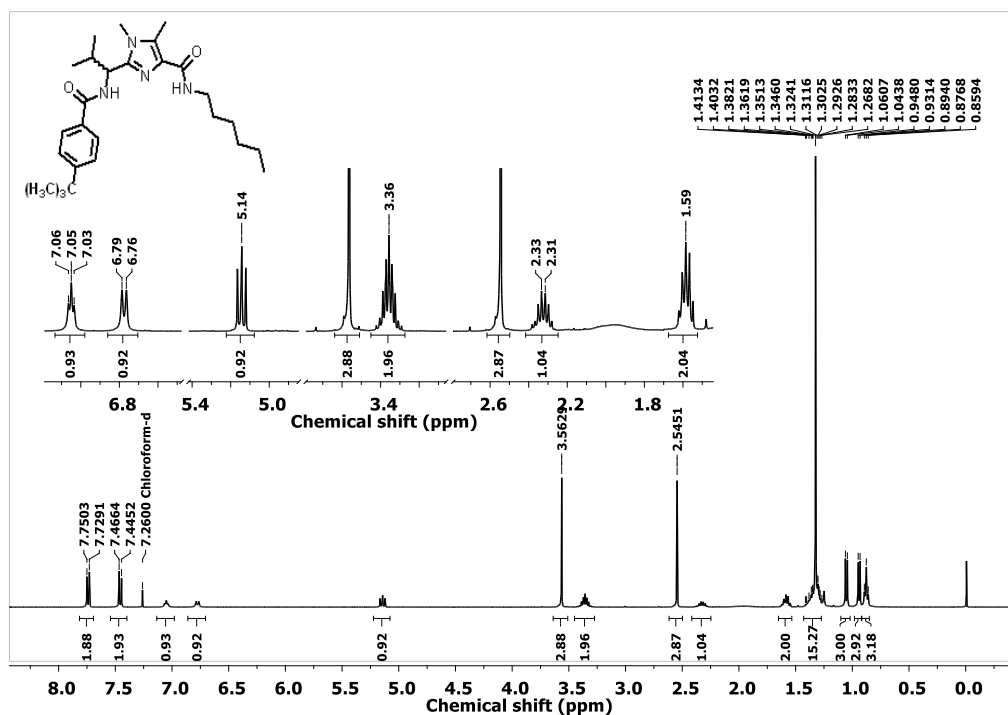


Figure 4.44 ¹H NMR spectrum of (*S*)-2-(1-(4-(*tert*-butyl)benzamido)-2-methylpropyl)-*N*-hexyl-1,5-dimethyl-1*H*-imidazole-4-carboxamide (**6e**).

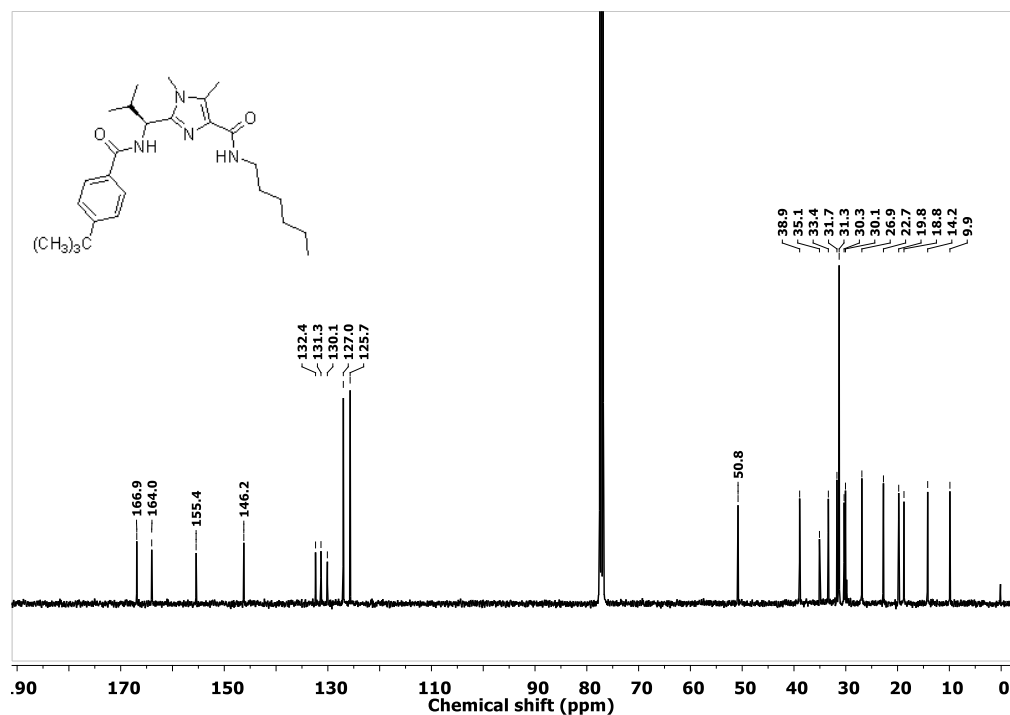


Figure 4.45 ¹³C NMR spectrum of (S)-2-(1-(4-(tert-butyl)benzamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (6e).

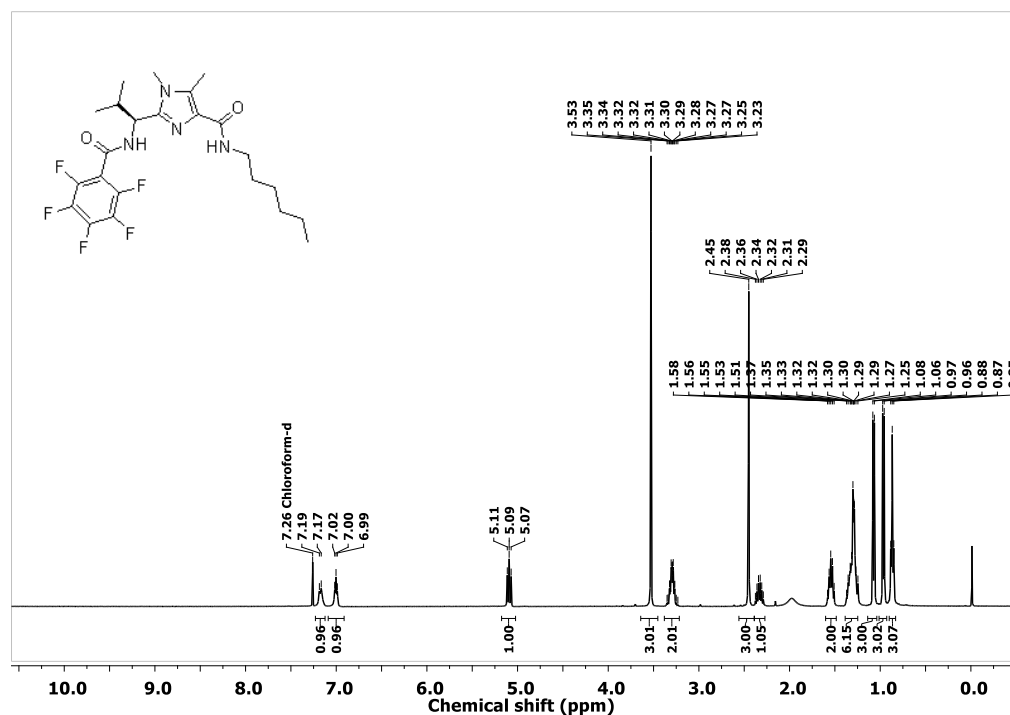


Figure 4.46 ¹H NMR spectrum of (S)-N-hexyl-1,5-dimethyl-2-(2-methyl-1-(perfluorobenzamido)propyl)-1H-imidazole-4-carboxamide (6f).

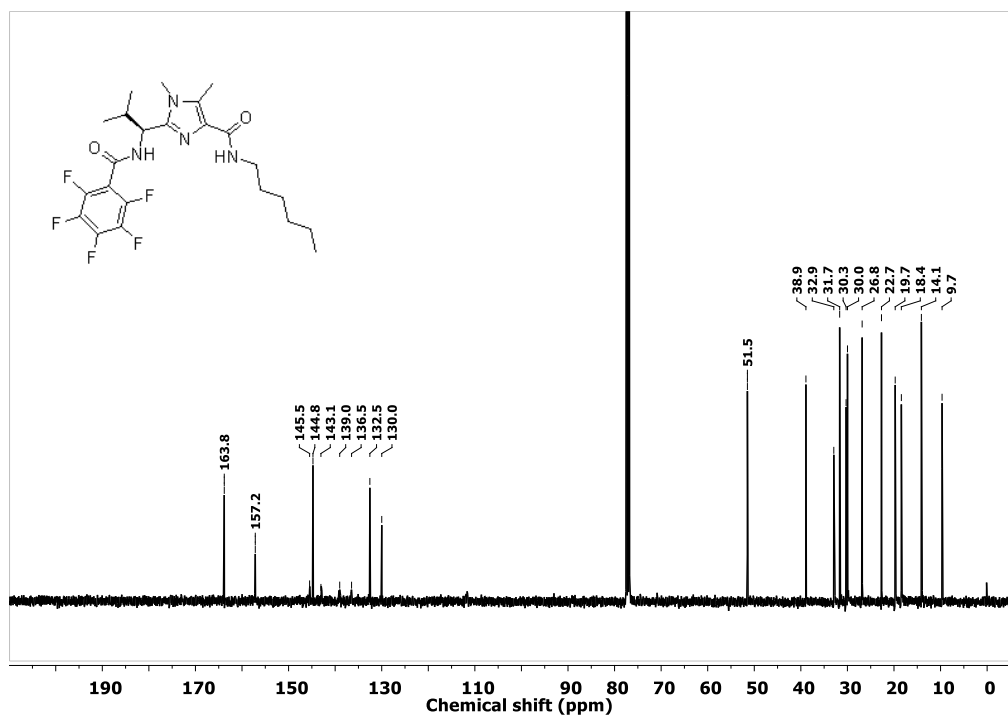


Figure 4.47 ^{13}C NMR spectrum of (S)-N-hexyl-1,5-dimethyl-2-(2-methyl-1-(perfluorobenzamido)propyl)-1H-imidazole-4-carboxamide (6f).

4.6 HRMS spectra of final compounds:

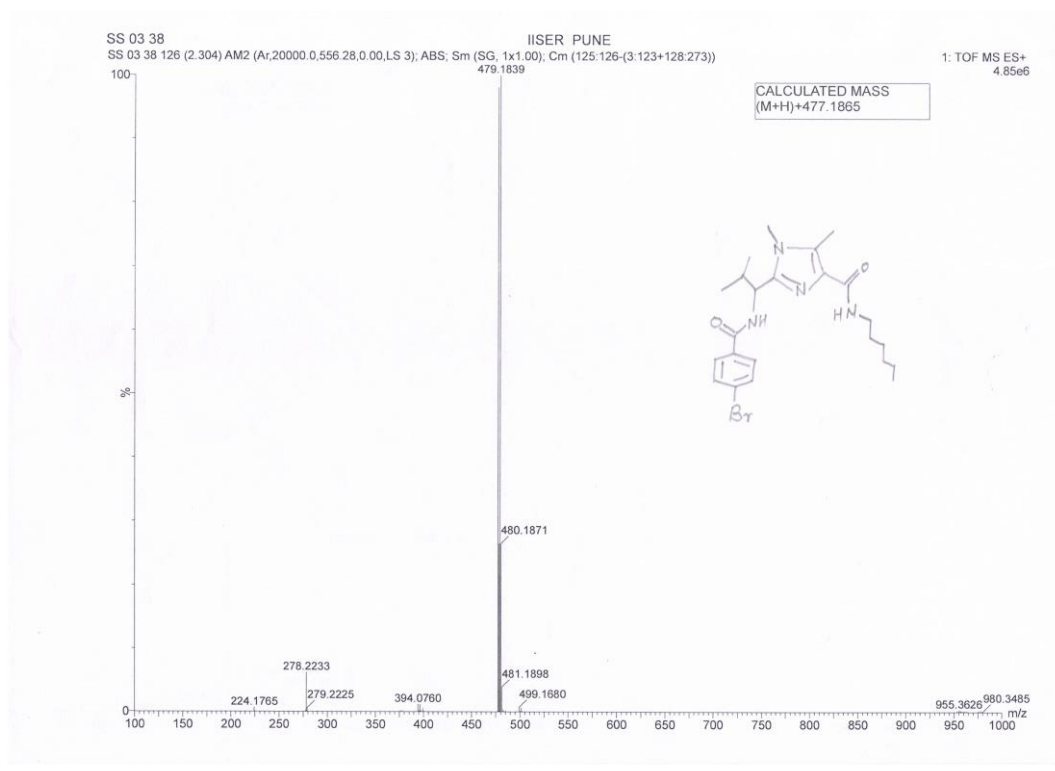


Figure 4.48 HRMS spectrum of (S)-2-(1-(4-bromobenzamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (6a).

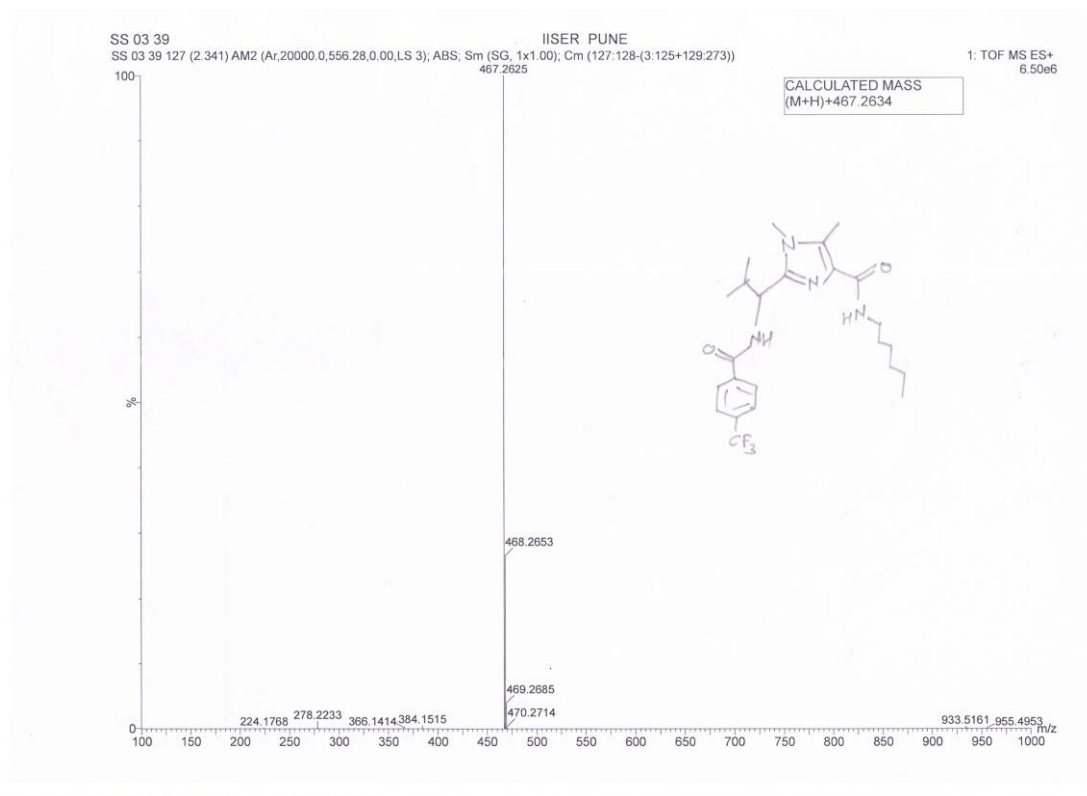


Figure 4.49 HRMS spectrum of (S)-N-hexyl-1,5-dimethyl-2-(2-methyl-1-(4-(trifluoromethyl)benzamido)propyl)-1H-imidazole-4-carboxamide (**6b**).

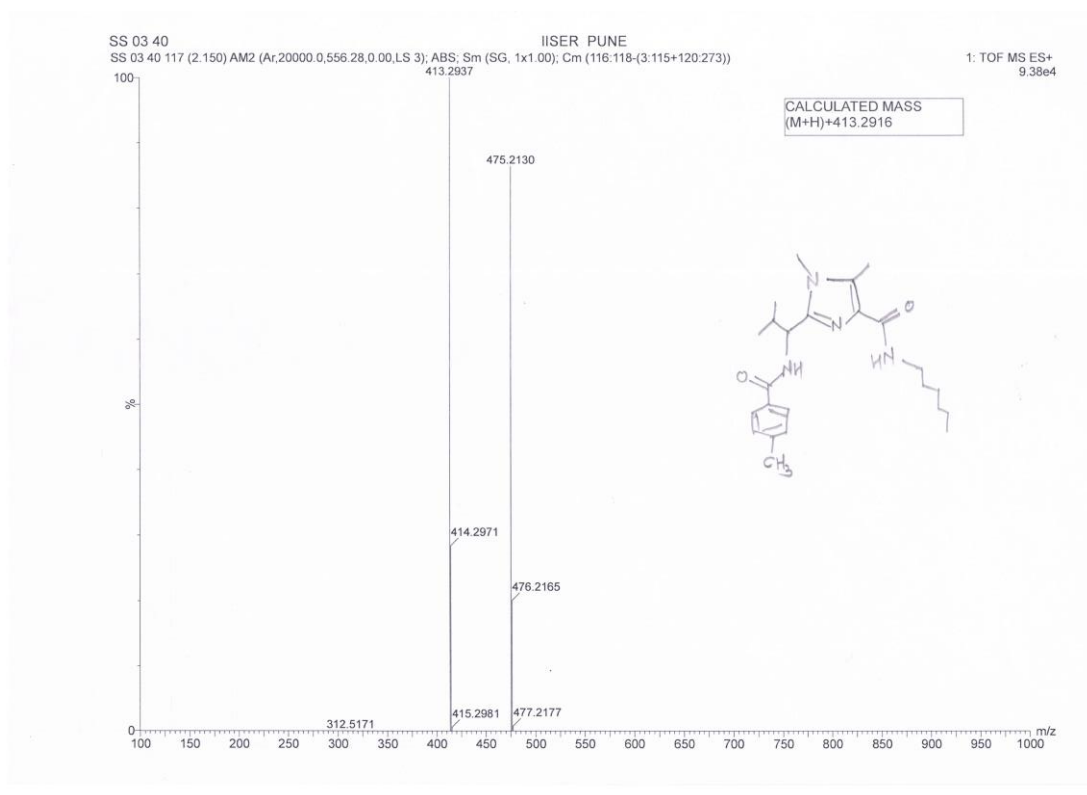


Figure 4.50 HRMS spectrum of (S)-N-hexyl-1,5-dimethyl-2-(2-methyl-1-(4-methylbenzamido)propyl)-1H-imidazole-4-carboxamide (**6c**).

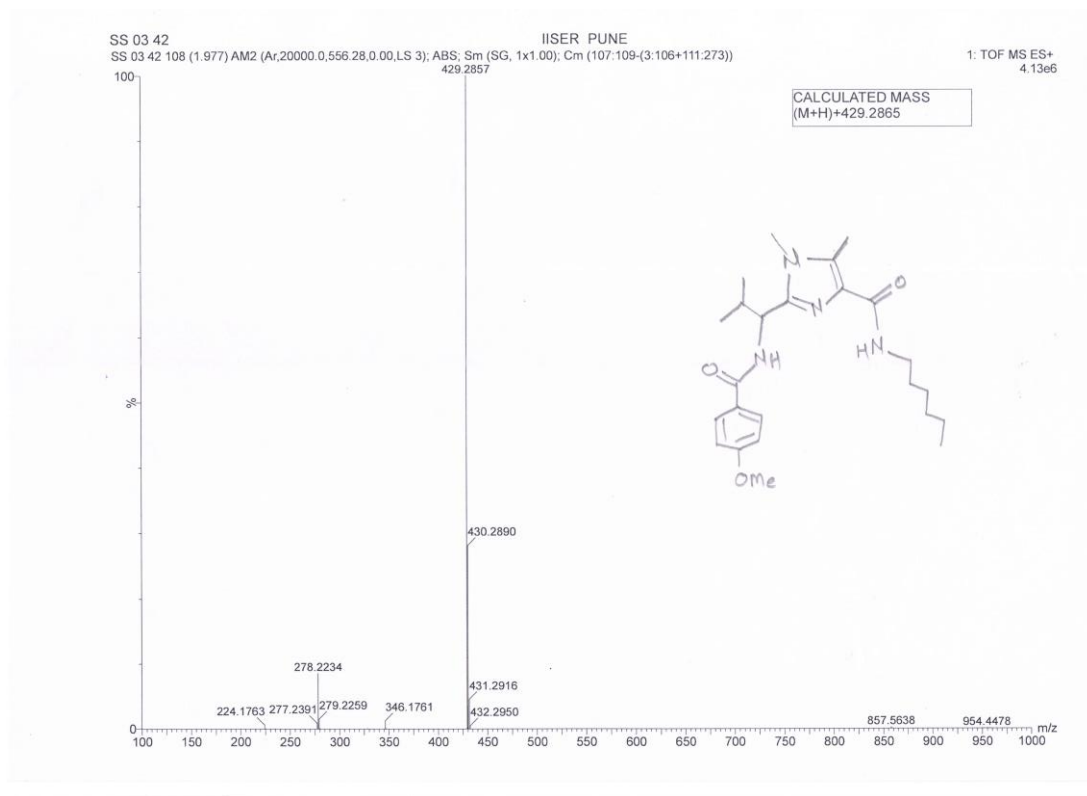


Figure 4.51 HRMS spectrum of (S)-N-hexyl-2-(1-(4-methoxybenzamido)-2-methylpropyl)-1,5-dimethyl-1H-imidazole-4-carboxamide (**6d**).

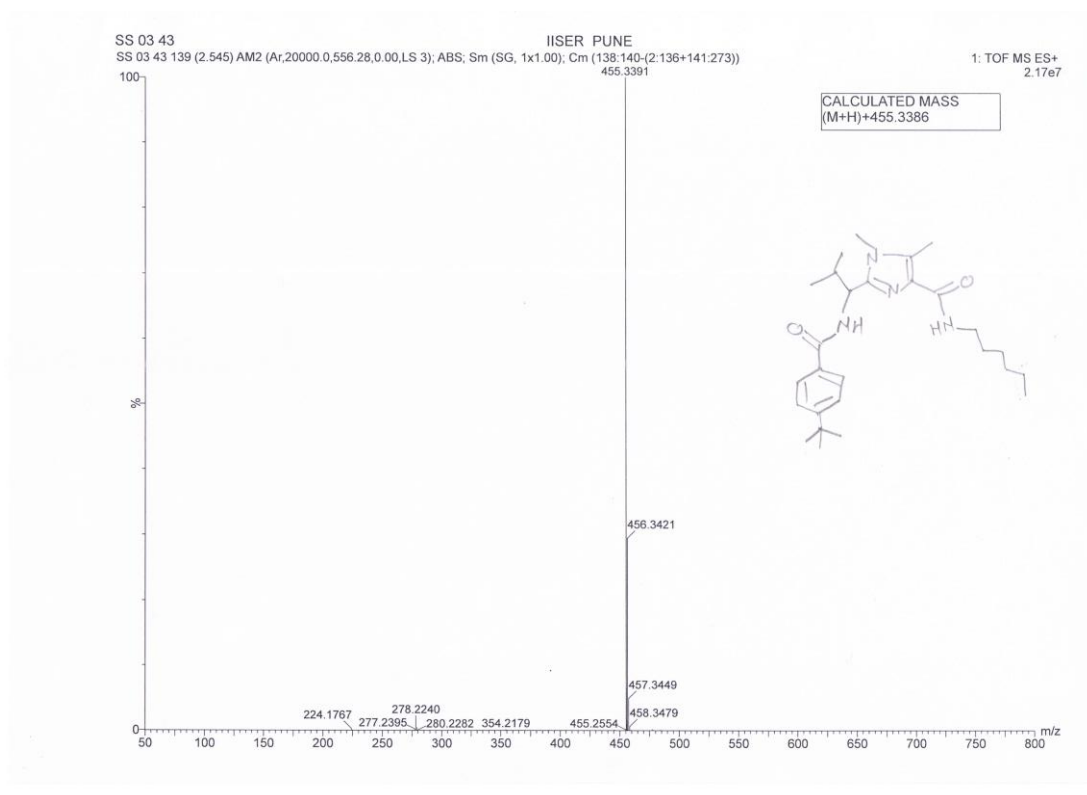


Figure 4.52 HRMS spectrum of (S)-2-(1-(4-(tert-butyl)benzamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (**6e**).

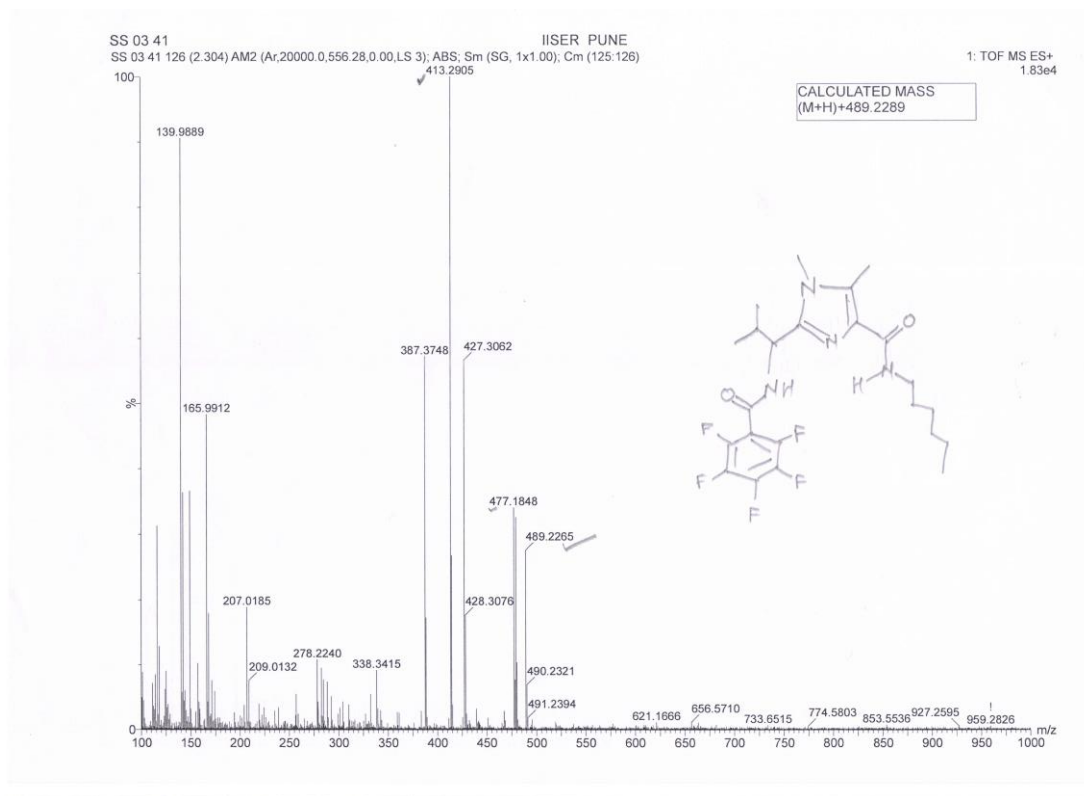


Figure 4.53 HRMS spectrum of (S)-N-hexyl-1,5-dimethyl-2-(2-methyl-1-(perfluorobenzamido)-propyl)-1H-imidazole-4-carboxamide (6f).

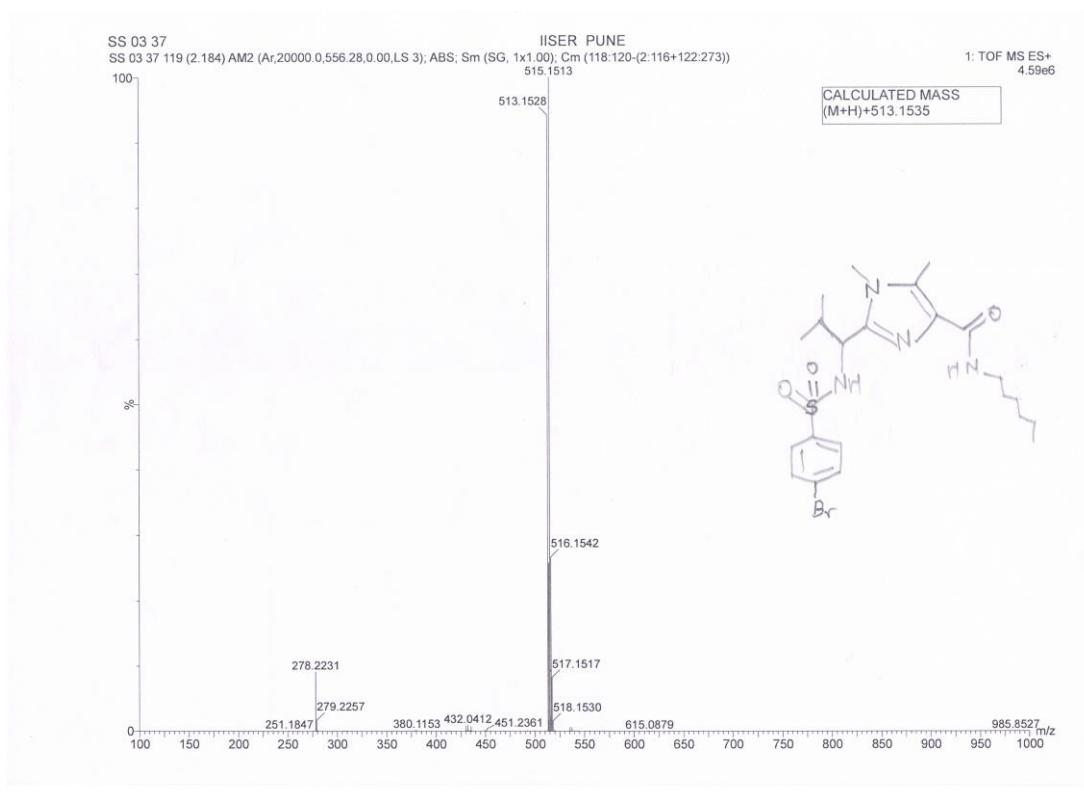


Figure 54 HRMS spectrum of (S)-2-(1-((4-bromophenyl)sulfonamido)-2-methylpropyl)-N-hexyl-1,5-dimethyl-1H-imidazole-4-carboxamide (7).

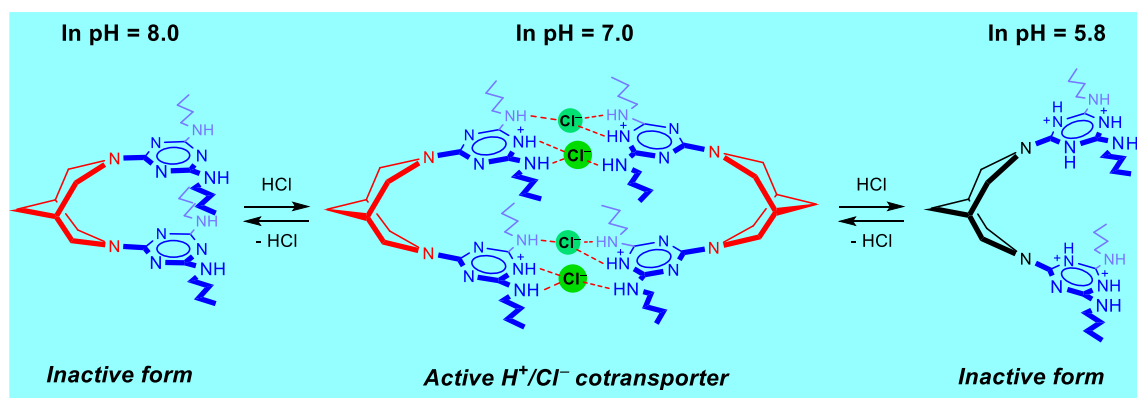
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Chapter 5

A Dimeric Bis(melamine)-Substituted Bispidine for Efficient Transmembrane H^+/Cl^- Cotransport Activity



5.1 Introduction

In nature, high-affinity recognition of a particular anionic species is achieved by the sophisticated protein hosts that allow the passage of the ions across the cellular membranes, overcoming the hydrophobic barrier of the lipid bilayer. This distinctive feature of anion-binding protein is the outcome of multiple non-covalent interactions originating from the protein backbone and its amino acid side-chains. Inspired by biological processes of anion recognition and transportation numerous preorganized artificial receptors, capable of recognizing anionic guests have been investigated. In most of such systems, the complementary induced fit conformation is used to bind an anionic guest in a specific hydrophobic recognition environment.

The microenvironment around tumor cells is unusually acidic compared to healthy cells, and this low pH environment acts as a defense shield for cancerous cells, generating drug resistance. Therefore, chemical agents capable of deacidifying the intracellular organelles can restore the drug-sensitivity.^{1,2} For example, the red pigment prodigiosin, isolated from microorganisms such as *Serratia* and *Streptomyces*,³ is an anticancer agent, and this activity is perceived based on its H^+/Cl^- cotransport and Cl^- transport properties across lipid membranes. Beyond prodigiosin⁴ and its analogs,⁵⁻⁸ calix[4]arene amide from Davis group,⁹ imidazole-linked pyrrole amide by Gale group,¹⁰ perenosins,¹¹ tren-amide by D. K. Smith et al.¹² and tris-ureas, tris thioureas¹³ are instances of synthetic receptors which allow the H^+/Cl^- cotransport across lipid membranes. The point to be noted here is that all these receptors are capable of recognizing the single HCl molecule at a time. However, the developments of chemical entities capable of recognizing multiple anions at the time are of high importance. Such receptors could be capable of delivering the multiple anions simultaneously across the membranes in a single mobile carrier cycle.

The natural protein channels show the inherent properties of gated ion transport activity. Gated ion transport by protein channels causes due to changes in stimulus across biological membranes. However, mimicking such a gated transport activity in synthetic carrier molecules is a challenging task. Gale and coworkers have reported the pH-

switchable anion transport activity by an oxothiosquaramides, thiosquaramides, and phenylthiosemicarbazones mimicking the function of acid-sensitive ion channels. The pH-induced switch ON/OFF transport activity of the squaramides and thiosquaramides was already discussed in chapter 3. Both of these derivatives showed deprotonation in the neutral and the basic pH conditions providing the inconvenient anion binding site. However, switching from pH 7.2 to pH 4 leads to the re-protonation of N-centers gave suitable anion binding site thereby resulting in the switch-ON anion transport activity. The phenylthiosemicarbazones **1** are inactive at neutral pH conditions. However, the decreasing the pH from 7.2 to 4.0 triggers the concomitant conformational switch from *anti* to *syn* as a result of protonation of the imine nitrogen atom to give $[1\cdot H]^+$ as it was indicated by NMR titration of **1** with $\text{HBF}_4\cdot(\text{CH}_3\text{CH}_2)_2\text{O}$ in acetone- d_6 solution. Then the protonated form $[1\cdot H]^+$ in its *syn* conformation binds to a Cl^- to give $1\cdot\text{HCl}$. The detailed investigation of the transport mechanism by valinomycin and monensin ionophores with this receptor confirmed the non-electrogenic H^+/Cl^- transport mechanism similar to that of natural product prodigiosin.

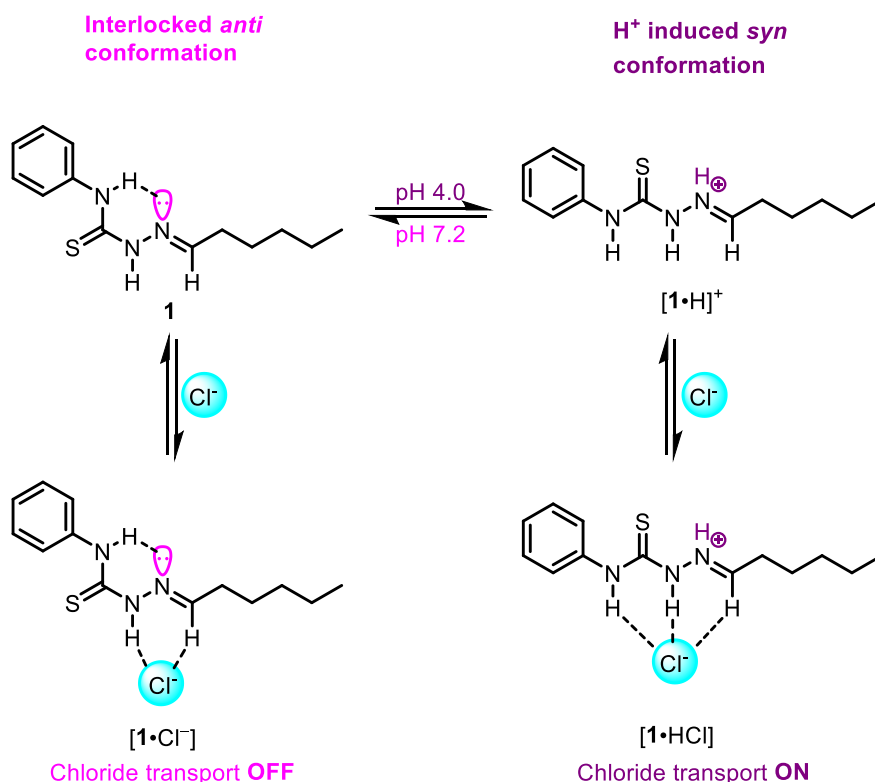


Figure 5.1 pH-gated ON/OFF chloride transport mechanism of thiosemicarbazones **1**. Transport was switched by pH-induced conformational changes (as *syn* and *anti* conformations) in the phenylthiosemicarbazones **1**.

In chapter 1-4, the small organic structures capable of recognizing the single chloride ion or HCl molecules were discussed. In order to design a new receptor capable of transporting the multiple H^+/Cl^- molecules, we thought to couple two recognizing unit in a single molecule. While doing the literature survey, we found that in 2012, Prof. Xi Zhang and coworkers reported the formation of 2D organic micro sheets from the controlled growth of suppressed self-association of protonated melamine derivatives **2**. In an organic solvent, the melamine and its dialkylated derivatives on treatment with halogen acid protonates and self-assemble through the counter ion bridged multiple hydrogen bonds to form 2D and 3D assemblies, respectively (Figure 5.2). In the HCl co-crystal structure, the hydrogen bond interactions of chloride with two melamine moieties were observed.

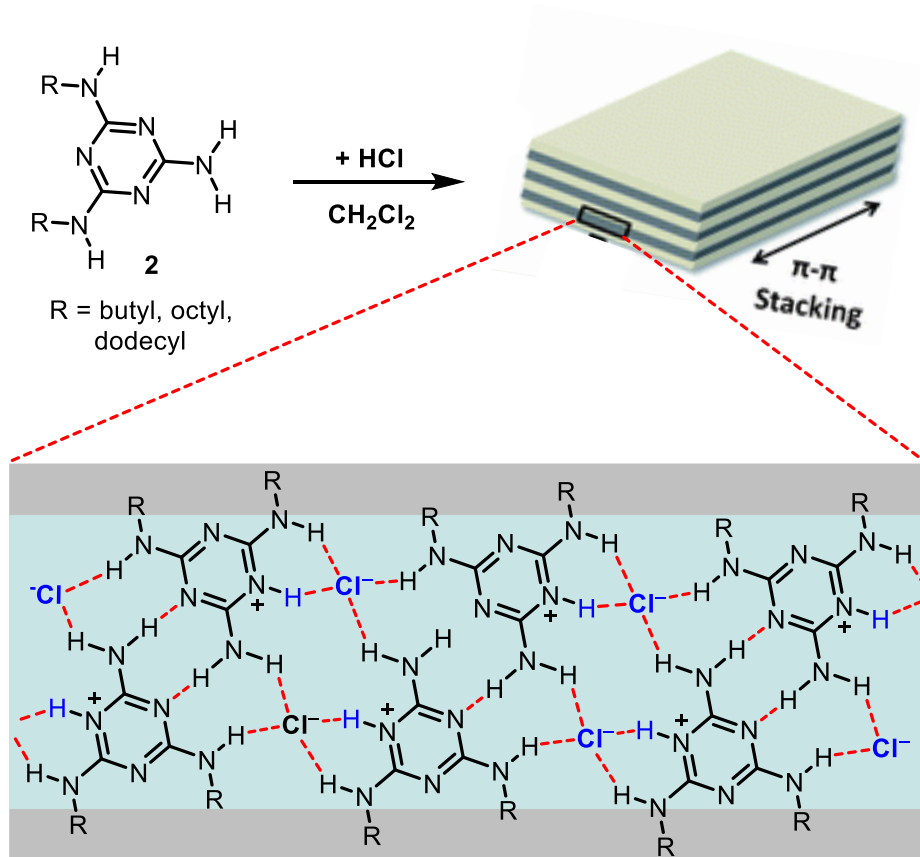


Figure 5.2 Schematic illustration of the 2D network produced from self-assembly of protonated dialkylated melamine derivatives through multiple counter ion bridged hydrogen bonds and π - π stacking interactions

In this chapter, by taking the advantage from above report, we report a melamine-substituted bicyclic receptor **3** that forms a homodimeric liplock taking advantage of

cooperative interactions involving multiple H^+ and Cl^- , and thereby functions as transmembrane H^+/Cl^- co-transporter (Figure 5.3B). The 3,7-diazabicyclo[3.3.1]nonone (or bispidine) moiety was selected as the jaw component of the receptor (i.e., locked stable *chair-chair* conformation)¹⁴ due to its rigidity and hydrophobic outer surface (Figure 5.3A). Two butylamino groups, each at C-2 and C-4 positions of the 1,3,5-triazine ring were attached to generate hydrogen bond donor sites as well as for providing the hydrophobic cover for the hydrophilic binding site of melamine to make it more lipophilic. A control molecule **2** that lacks the hydrogen bond donor sites was designed by connecting *n*-butoxy groups at C-2 and C-4 positions of the 1,3,5-triazine ring (Figure 5.3B).

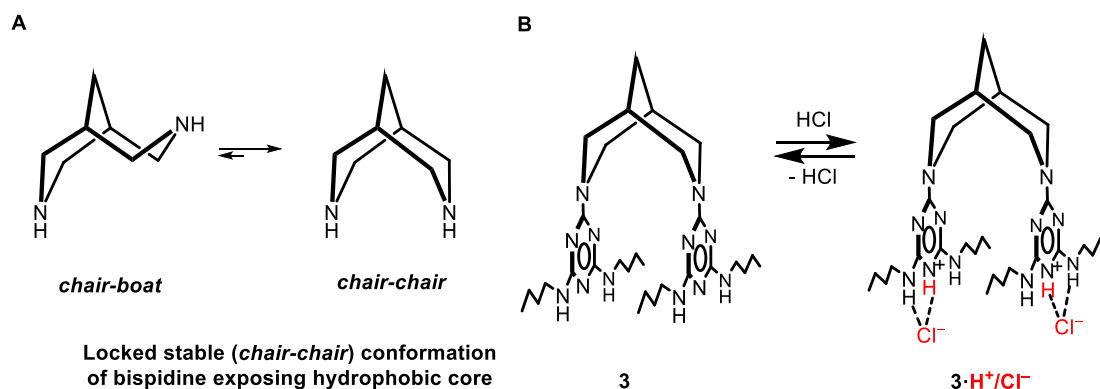
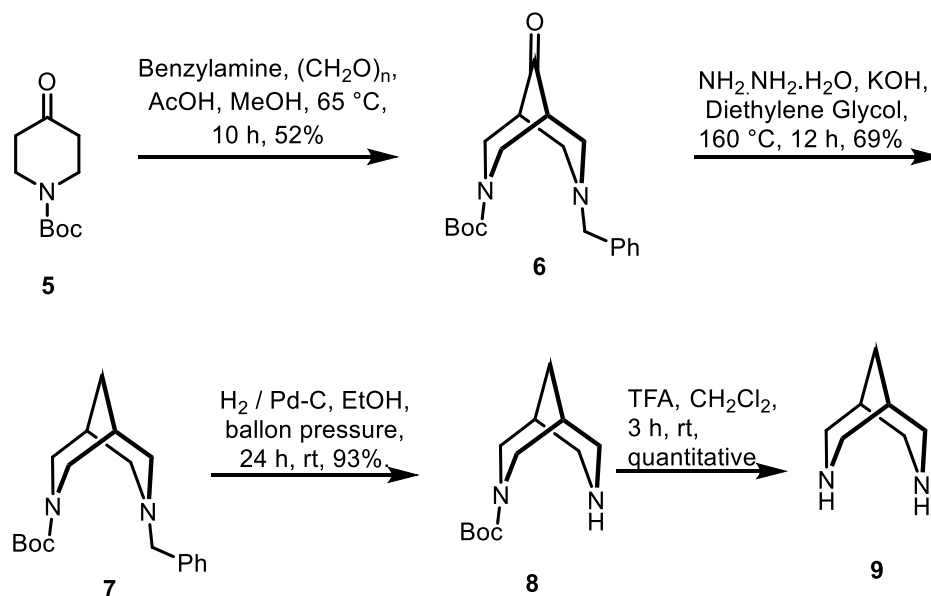


Figure 5.3 Chair-boat and locked *chair-char* conformation of bispidine (A). Illustrative H^+/Cl^- binding with receptor **3** (B).

5.2 Results and Discussions

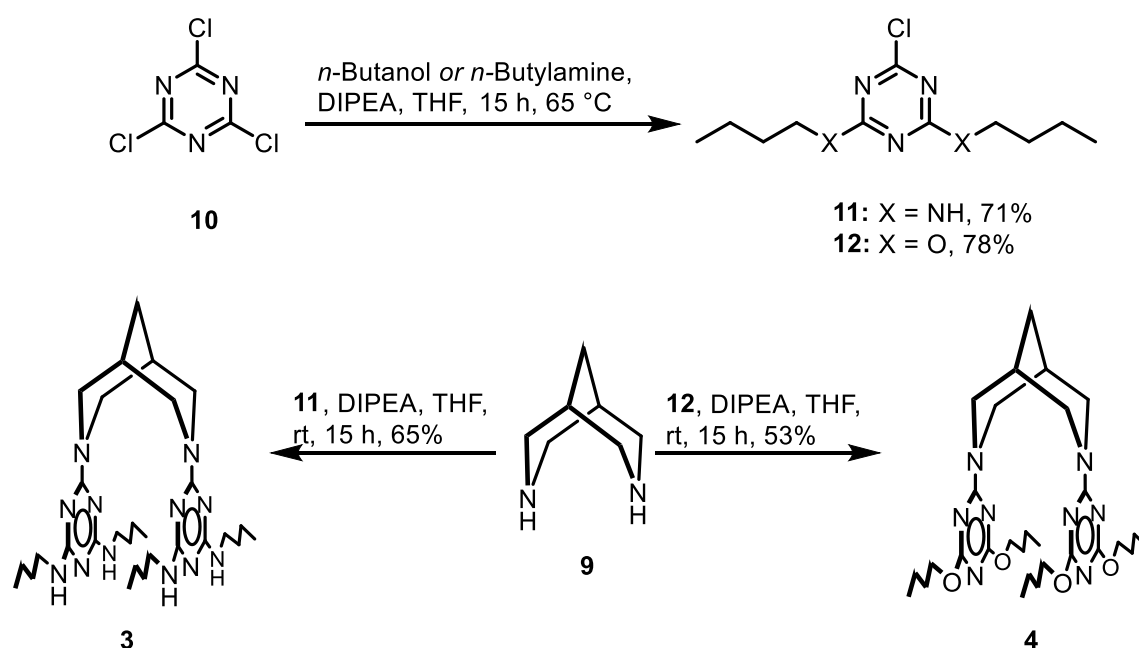
5.2.1 Synthesis

The synthesis of bispidine **9** was achieved in four steps by following the known literature protocol.¹⁵ Initially, The double Mannich reaction of 4-boc-piperidone **5** with benzylamine and paraformaldehyde in the presence of 5% acetic acid afforded the ketone **6** in 52% yield. The ketone is then subjected to Wolf–Kishner reduction condition resulted in the formation of compound **7** in 69% yield. The hydrogenolysis of compound **7** by Pd-C (10% w/w) under balloon pressure afforded the Boc protected amine **8** in 93% yield which further on treatment with trifluoroacetic acid in dichloromethane gave bispidine **9** in quantitative yield (Scheme 5.1).



Scheme 5.1 Synthesis of 3,7-diazabicyclo[3.3.1]nonane (or bispidine) derivative 7.

Simultaneously, the compound **10** was converted first into the dibutylamino substituted derivative **11** following a reported protocol. Subsequent reaction of **11** with bispidine **9** in the presence of DIPEA provided the bicyclic compound **3** in 53% yield. The reaction of **10** with *n*-butanol under basic conditions gave the dibutoxy substituted derivative **12** in 78% yield. Further reaction of **12** with **9** provided the second bicyclic derivative **4** in 67% yield.



Scheme 5.2 Synthesis of transporter **3** and control compound **4**.

5.2.2 X-Ray Crystal Structure Analysis

Compound **3** was co-crystallized with HCl by purging dry HCl gas to a solution of the compound **3** in methanol and crystals were isolated by slow evaporation of the solvent. The crystal structure, as shown in Figure 5.4A, revealed the formation of a zig-zag chain of molecule **3** in which the triazine rings of two adjacent molecules are connected edge-to-edge with the help of two HCl molecules. It is also interesting that each Cl^- was held via accepting trifurcated hydrogen bonds with three N-H bonds at hydrogen bond distances of 3.163 Å, 3.092 Å and 3.156 Å, respectively (Figure 5.4A). Therefore, the protonation of **3** at the N-3 position over either N-1 or N-5 position is a perfect consequence of positive cooperativity¹⁶⁻¹⁸ and microenvironment¹⁹ between two melamine units of two neighboring molecules although, the theoretical prediction²⁰ did not indicate any significant preference for the protonation of N-3 atom of a triazine ring (Figure 5.4A). Based on the crystal structure information, an ion transporter model, consisting of two units of **3** and four HCl molecules, was proposed (Figure 5.4B). However, other possible stoichiometries involving $3_2 \cdot (\text{HCl})_2$ and $3_2 \cdot (\text{HCl})_3$ may also play a role in the H^+/Cl^- cotransport process.

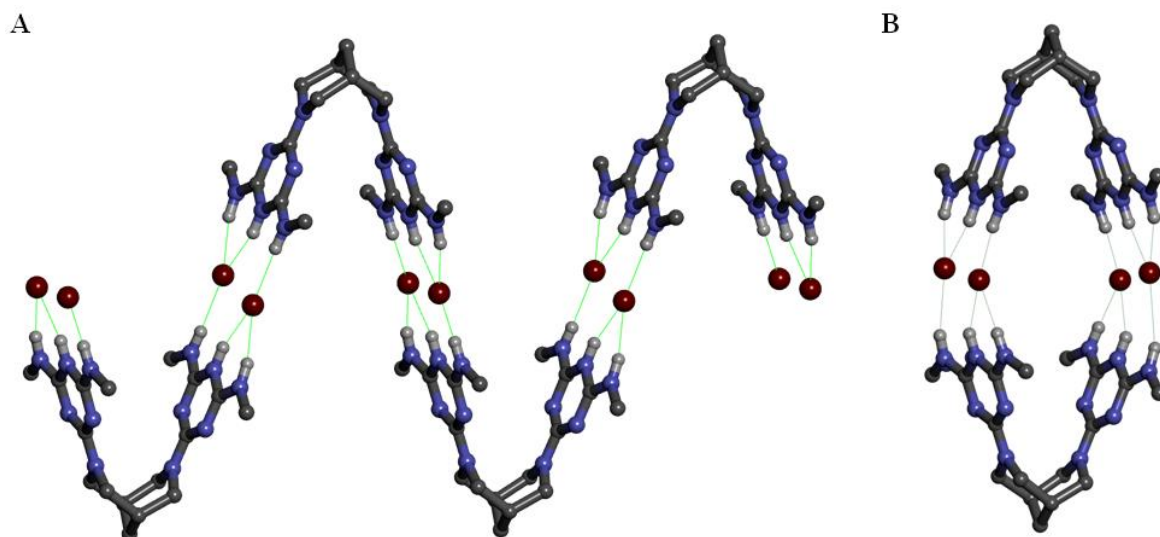


Figure 5.4 X-ray crystal structure molecule **3** complexed with HCl (A). Hypothesized homodimeric model of the active transporter (B).

5.2.3 Ion Transport Study

The ion transport properties of compounds **3** and **4** were studied across large unilamellar vesicles (LUVs), prepared from egg yolk phosphatidylcholine (EYPC) lipid

by entrapping 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) dye²¹ and NaCl, and after applying a $\Delta\text{pH} \cong 0.8$ ($\text{pH}_{\text{out}} = 7.8$ and $\text{pH}_{\text{in}} = 7.0$).^{22,23} Faster equilibration of the applied pH gradient by **3** (100 nM) compared to **4** (100 nM) indicated that the dibutylamino substituted derivative is a superior ion transporter (Figure 5.5A). Concentration-dependent ion transport activity of **3** (0 – 140 nM) (Figure 5.5B) followed by Hill analysis provide the $\text{EC}_{50} = 30.1$ nM (i.e., 0.045 mol % with respect to the lipid) confirming the compound as an efficient transmembrane transporter. The Hill coefficient, $n = 1.91$ signifies that the two molecules of **3** were involved in the formation of the active transporter (Figure 5.5C).

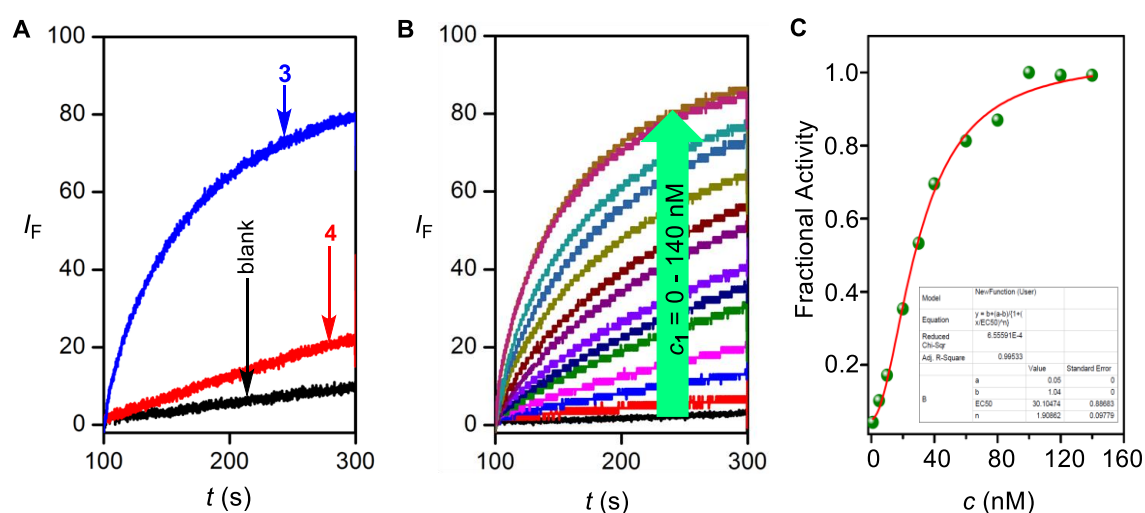


Figure 5.5 Comparison of ion transport activity of **3** (100 nM) and **4** (100 nM) (A), and dose-response activity of compound **3** (0 – 140 nM) across EYPC-LUVs, presented as normalized emission intensity I_F with respect to time, t (B). The hill analysis obtained from the dose- response activity data.

An effective transport rate obtained from HPTS assay encouraged us to investigate its ion transport mechanism and ion selectivity sequence for compound **3**. The ion transport assay under applied pH gradient can occur by multiple possibilities such as, OH^-/A^- antiport, H^+/A^- cotransport, H^+/M^+ antiport and M^+/OH^- cotransport are possible for the preceding transport data of **3**. Therefore, to scrutinize further, ion transport properties of **3** in the presence of FCCP (a H^+ selective transporter) was studied using same experimental protocol. Compound **3** showed similar ion transport behavior when studied in the absence and in the presence of FCCP (Figure 5.7A). The outcome suggests that the H^+ efflux rather than the OH^- influx by **3** was responsible for the equilibration

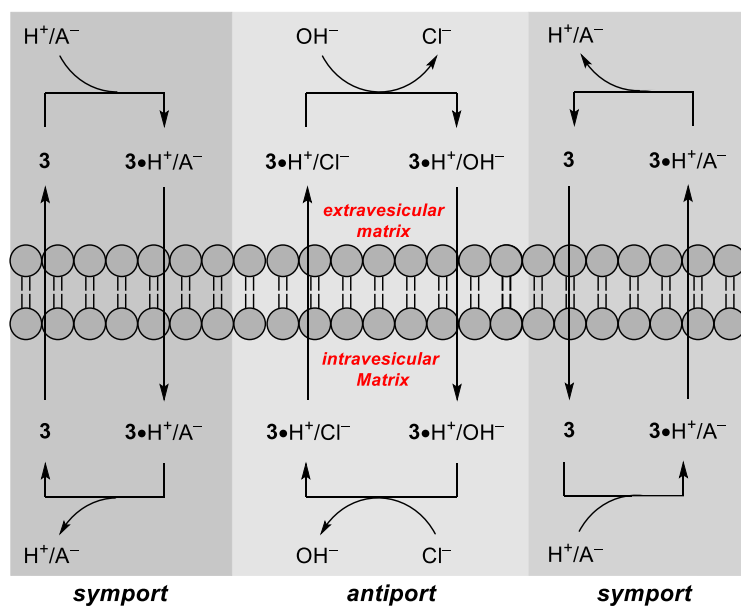


Figure 5.6 The proposed mechanism of ion transport showing an initial influx of H^+/A^- (left) followed by antiport (middle) or symport (right) mechanism.

of the pH gradient. The study excluded the possibilities of OH^-/A^- antiport and M^+/OH^- cotransport by the bicyclic compound. Replacement of extravesicular Na^+ with K^+ , and evaluation of ion transport by **3** in the presence of valinomycin (a K^+ ion selective carrier) resulted in the comparable HPTS fluorescence intensity enhancement (Figure 5.7B). The

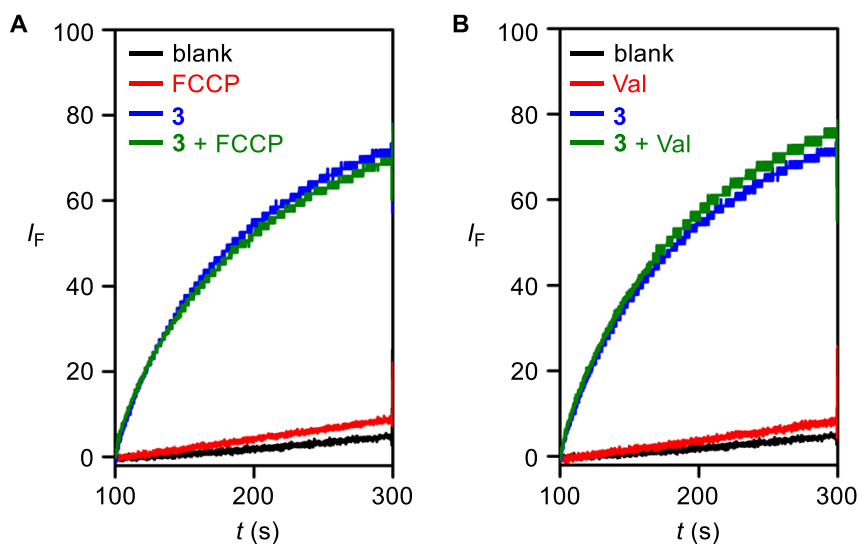


Figure 5.7 Comparison of ion transport activity of **3** (40 nM) in the presence and the absence of FCCCP (2.5 μM) (A). Comparison of ion transport activity of **3** (40 nM) in the presence and the absence of valinomycin (5 pM) (B).

lack of any cooperative effect between the H^+ efflux by **3**, and K^+ influx by valinomycin corroborates to an efflux of Cl^- by **3**. Therefore, the cotransport of H^+/Cl^- by the designed bicyclic molecule was confirmed from these studies.

Insignificant differences in ion transport activity, observed by varying the metal ion in the extravesicular chloride salt solution (i.e., MCl , $M^+ = Li^+, Na^+, K^+, Rb^+, Cs^+$) confirmed that the compound **3** was not selective towards any metal cation (Figure 5.8A). The nonparticipation of cations in transport process also ruled out the M^+/H^+ antiport and M^+/A^- symport mechanisms. The change of anion in the extravesicular sodium salt solution (NaA , $A^- = F^-, Cl^-, Br^-, I^-, OAc^-, ClO_4^-, NO_3^-, SCN^-$) showed considerable differences in the ion transport activity of **3** (Figure 5.8B). For $A^- = F^-, Cl^-, Br^-, OAc^-$, ClO_4^- and SCN^- , the pH equilibration process was marked by an enhancement in HPTS fluorescence upholding the strong influence of anions, however, with the Cl^- selectivity. In the absence of any OH^- transport by **3**, the increase in HPTS fluorescence corroborates to the H^+ coefflux process. When the anions in the extravesicular buffer were changed to NO_3^- , ClO_4^- , Br^- and I^- , an initial quenching followed by enhancement of HPTS fluorescence were observed for extravesicular. These results suggested a primary H^+ influx process against the applied pH gradient, and the process is accompanied by A^- co-influx.

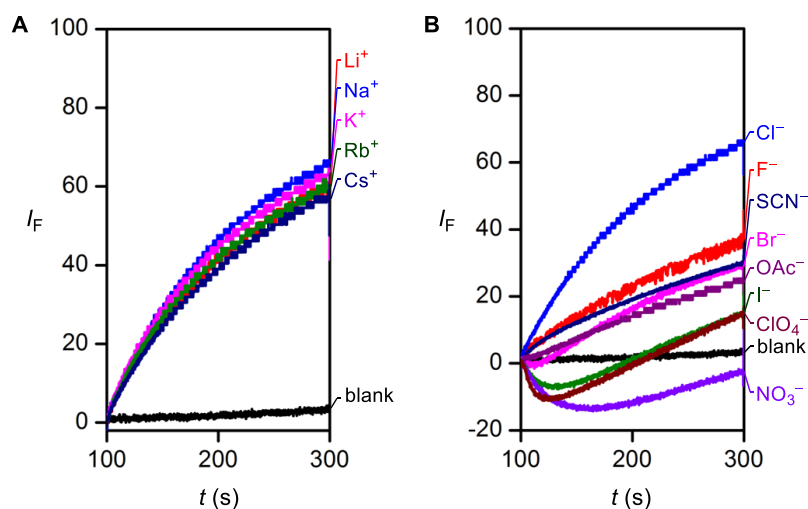


Figure 5.8 Ion selectivity of **3** (40 nM) across EYPC-LUVs $\Rightarrow$ HPTS measured by varying extravesicular ions. Cation selectivity (A) measured by varying extravesicular alkali cations, and Anion selectivity (B) measured by varying the extravesicular anions.

The H^+/A^- cotransport process was further proved by measuring the ion transport across EYPC-LUVs with intravesicular Cl^- and extravesicular I^- , Br^- , ClO_4^- , and NO_3^- ions, however without applying the pH gradient (that is, $pH_{in} = pH_{out} = 7.0$) (Figure 5.9A). The addition of **3** resulted in the decrease in intravesicular HPTS fluorescence (Figure 5.9B). The observation was corroborated the H^+ influx, accompanied by A^- for the charge balance. When SO_4^{2-} , a strongly hydrated anion (hydration energy $\Delta G = 1080 \text{ kJmol}^{-1}$)²⁴ was used in the extravesicular buffer, a sharp enhancement observed for the HPTS fluorescence indicates the efflux of the H^+/Cl^- ion pair, further confirming the symport mechanism.

The direct evidence of the H^+/Cl^- cotransport was obtained from the U-tube experiment. In this experiment, the lipid bilayer was substituted by $CHCl_3$ segregating the two aqueous phases. The left arm of the U-tube was filled with aqueous HCl (100 mM) solution (pH 1.05) while the right arm was filled with aqueous $NaNO_3^-$ (100 mM) (Figure 5.10A). Subsequently, 1.1 mM solution of **3** was placed at the bottom of the U-tube, and the changes in pH as well as Cl^- concentration were monitored in the right arm by a pH and Cl^- selective electrodes, respectively. The steady decrease in pH and increase in Cl^- concentration in the right arm of U-tube confirmed the H^+/Cl^- cotransport by **3** (Figure 5.10B). The study also confirmed a via mobile carrier mechanism during the ion transport process.

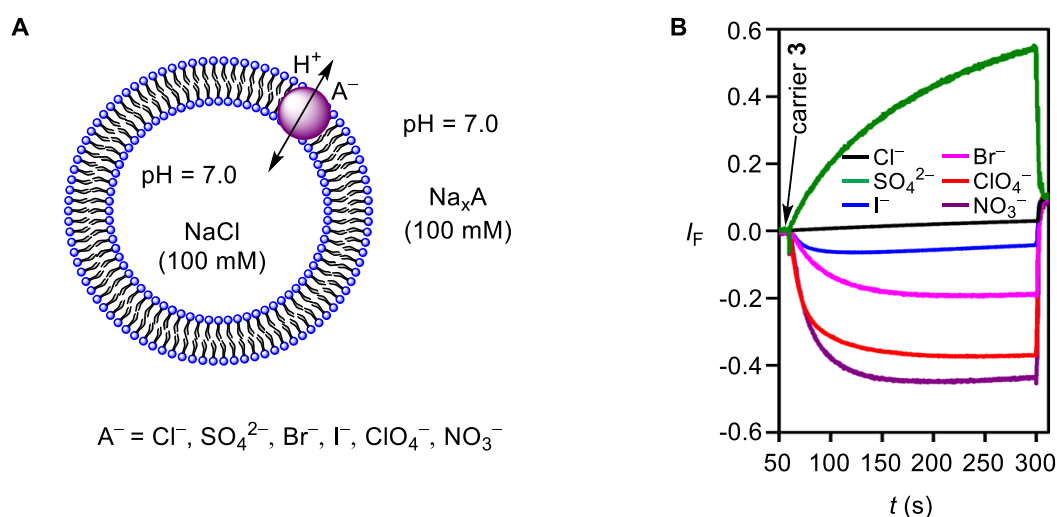


Figure 5.9 Anion selectivity of **3** (40 nM), measured across EYPC-LUVs by varying the extravesicular anions without applying pH gradient. Representative assay condition (A), and change in intravesicular pH in the presence of different extravesicular anion upon adding transporter **3** ($c = 40 \text{ nM}$) (B).

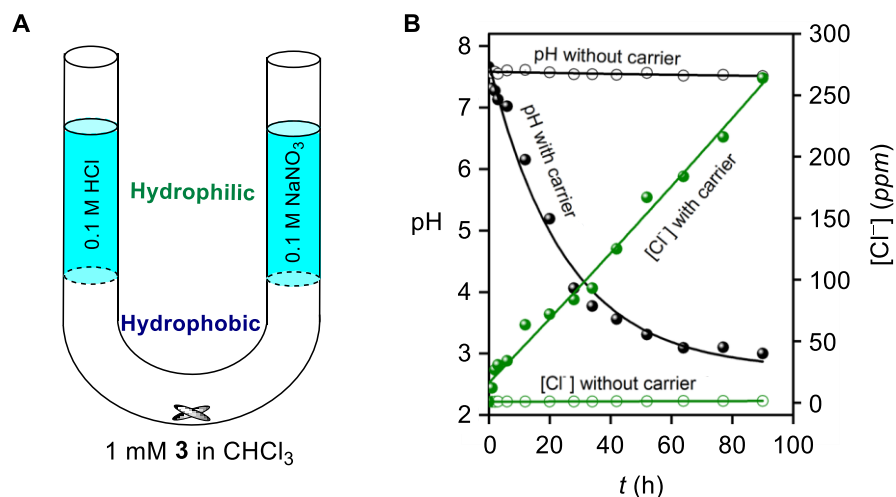


Figure 5.10 Representative U-tube experiment (A). Change in pH and Cl⁻ ion concentration in the aqueous phase of receiver arm of U-tube (B).

The Cl⁻ ion transport activity of **3** across LUVs was investigated further by monitoring the fluorescence of lucigenin dye²⁵ which was encapsulated along with NaNO₃ (200 mM) within the EYPC-LUVs. After adding NaCl (33 mM) in the extravesicular media, the influx of Cl⁻ ions was monitored in the presence of **3**. A sharp quenching of lucigenin fluorescence was observed indicating the influx of Cl⁻ ion via an exchange NO₃⁻ (Figure 5.11A). Study of the concentration profile followed by Hill analysis provided $n = 1.86$ (Figure 5.11B) which further confirms the active carrier formation involved two molecules of **3**.

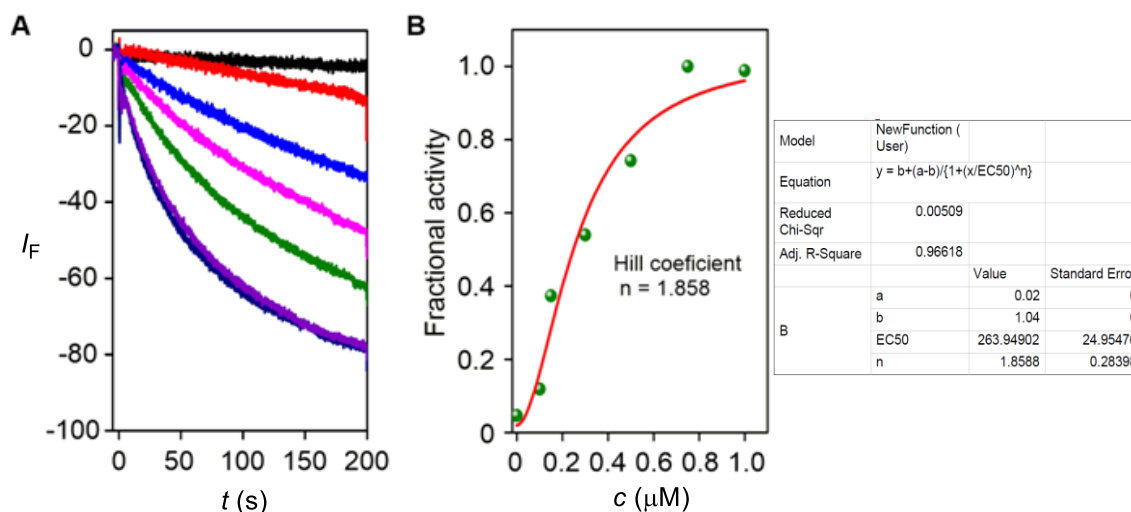


Figure 5.11 Cl⁻ ion transport activity across EYPC-LUVs with lucigenin with increasing concentration of **3** (0 – 1 μM) (A), and Hill's plot (B).

Finally, to validate the proton-induced change in transport activity, the effect of pH on Cl^- efflux process across EYPC-LUVs was monitored at pH 5.8, 7.0 and 8.0. The vesicles were prepared by entrapping the NaCl and suspended in extravesicular NaNO_3 solution in phosphate buffer medium of pH 5.8, 7.0, or 8.0 respectively. The efflux of Cl^- upon addition of the carrier **3** ($2\ \mu\text{M}$) was monitored by a chloride ion selective electrode (ISE). Interestingly, the most prominent Cl^- efflux was noticed at pH 7.0 (Figure 5.12A). On the other hand, the Cl^- efflux efficiency decreased when the pH was either increased (at pH 8.0) or decreased (at pH 5.8). The plausible explanation for such pH-dependent activity variation can be provided based on the conformational changes in molecule **3** due to protonation of the triazine ring N-atoms as shown in Figure 5.12B. At higher pH, no protonation occurs on the triazine ring. As a result, the receptor molecules do not offer any anion binding site although, intermolecular two-arm H-bonding between two melamine units is possible. Lowering of pH increases the probability of a cooperative cascade process involving the protonation at the N-5 atom of each triazine ring, recognition of Cl^- ions, and a liplock dimer formation. The H^+/Cl^- bound dimer of **3** favors the transport of the ion pair. Further lowering of pH allows protonation, also at

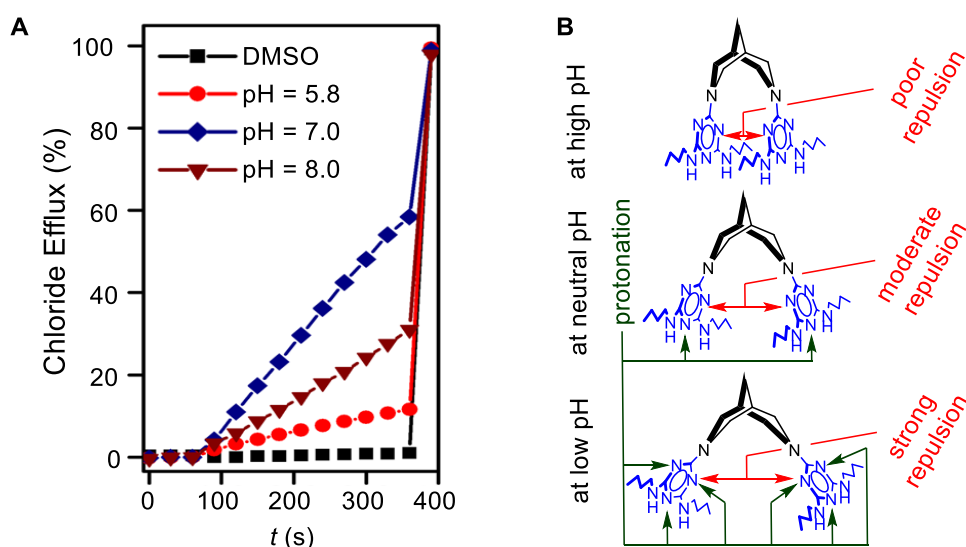


Figure 5.12 Effect of pH on Chloride efflux from EYPC-LUVs (200 nm). EYPC vesicles were loaded with a 500 mM NaCl solution buffered to pH 5.8, 7.0, 8.0 with 5 mM phosphate salts, and were suspended in a 500 mM NaNO_3 solution buffered to pH 5.8, or 7.0, or 8.0, respectively with 5 mM phosphate salts. At the end of the experiment ($t = 360\ \text{s}$), Triton X-100 was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. DMSO was used as a control before each measurement (A). Probable pH-induced conformational change in the compound **3**.

other N-atoms of each triazine ring. As a result, charge repulsion between melamine units of **3** is increased, leading to an opening of molecular jaw and distortion of the dimeric H^+/Cl^- bound complex. The structural distortion weakens the cooperative Cl^- ion binding and therefore, transport activity of **3** is decreased. The computed pK_a values (in the range of 5.9–4.2) were not correlating with the pH and HCl uncoupling activity (Figure 5.13). However, it is known that the pK_a values in lipid bilayer can differ significantly.^{26,27}

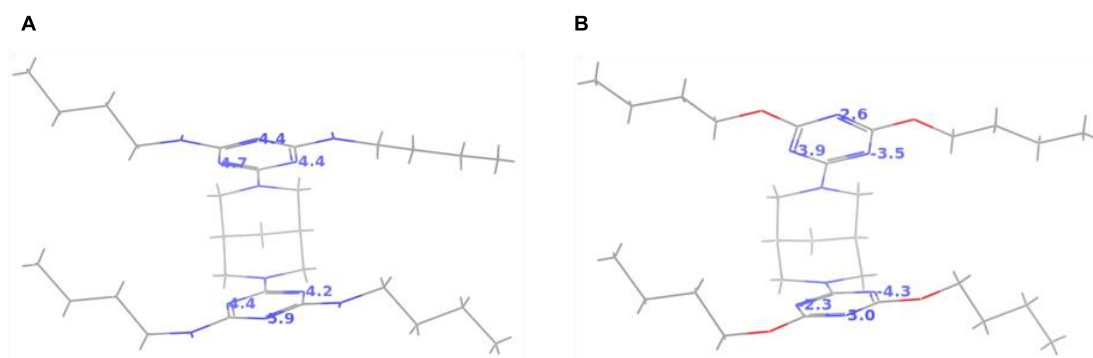


Figure 5.13 Theoretical prediction of pK_a values of compound **3** (A), and **4** (B).

5.3 Theoretical Support to protonation induced Conformational Changes

The protonation-dependent opening of V-Shaped structures was also in agreement with theoretical calculations performed using CS Chem Office 2015 molecular modeling software (Figure 5.14). The calculations were done by replacing the butyl chains by methyl groups (see structure **13**, Figure 5.14A). The results indicated that the neutral compound **13** adapts a V-shape conformation (5.14B). However, with increasing the protonation on triazine ring, more distraction from the V-shape along with rotation of melamine ring from its original plane was observed as shown in figure 5.14C-E.

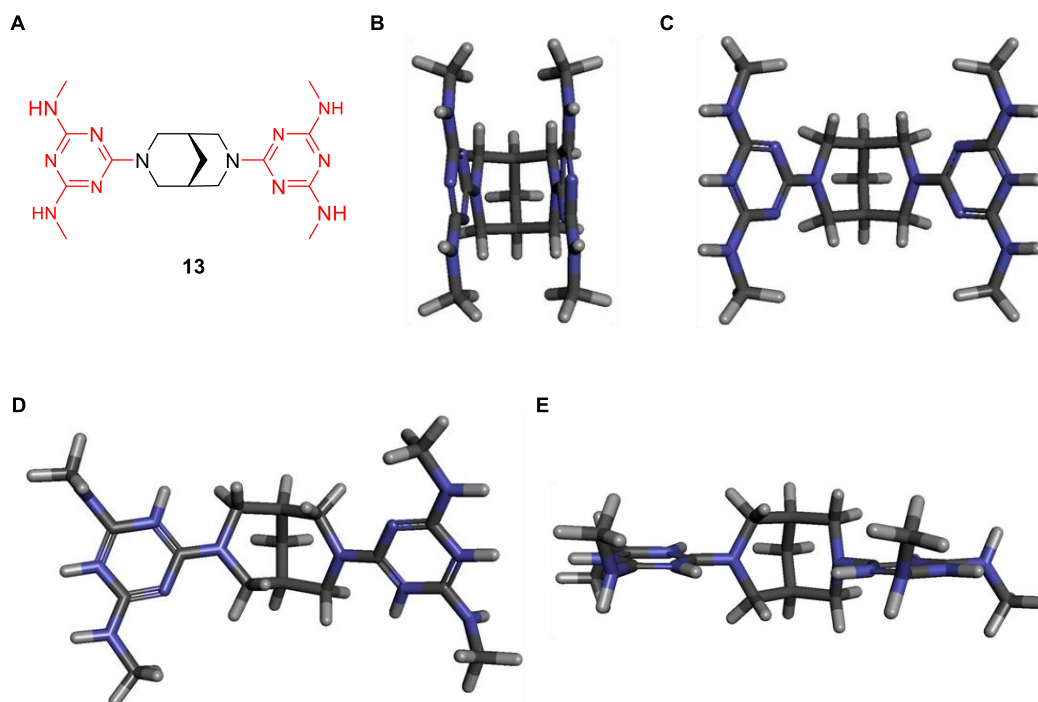


Figure 5.14 Structure of molecule **13** (A). Geometry optimized structures of **13** (B), [**13** + (2 × H)]²⁺ (C), [**13** + (4 × H)]⁴⁺ (D) and [**13** + (6 × H)]⁶⁺ (E).

5.4 Conclusion

In nut shell, we have developed a bis(2,4-dibutylamino- 1,3,5-triazine) appended bispidine compound **3** as an efficient HCl co-transporter across bilayer lipid membranes. The X-ray crystallographic study revealed that the V-shaped molecule forms a **3**·(HCl)₂ complex arranged in a continuous *zig-zag* network. In this assembly, protonation of the triazine N-atom that intervenes two dibutylamino groups, and trifurcated N–H···Cl[−] interactions were evident as the motif for the binding of two H⁺ and two Cl[−] between successive melamine units. This HCl recognition was rationalized based on cooperativity and micro environmental effects because theoretical prediction suggested comparable basicities of all N-atoms of a triazine ring. Compound **3** displayed an excellent ion transport activity which corroborated to a dimeric locking and delivers multiple H⁺ and Cl[−], simultaneously. The H⁺/Cl[−] co-transport mechanism was also established. Cl[−] efflux characteristics of **3** indicates maximal activity at pH 7, and this was explained based on a protonation-dependent widening of the mouth of the V-shaped molecule. The mechanism suggests that the dimeric locking involving multiple H⁺ and Cl[−] is stable at pH 7.

5.5 Experimental Section

5.5.1 General Methods

Reagents for synthesis were purchased from commercial sources (Sigma Aldrich, and Spectrochem) and used as it is without further purification. HPTS, HEPES buffer, Triton X-100, NaOH and inorganic salts were of molecular biology grade obtained from Sigma. Egg-yolk phosphatidylcholine (EYPC) lipid as a solution in CHCl₃ (25 mg/mL), polycarbonate membrane (100 nm and 200 nm pore size), and mini-extruder used for vesicle preparation was obtained from Avanti Polar Lipids. All the Stock solutions were prepared in either HPLC grade DMSO or Methanol.

5.5.2 Physical Measurements

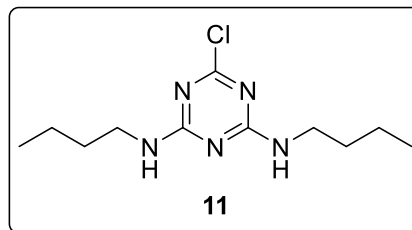
¹H NMR spectra were recorded at 400 MHz, and ¹³C NMR spectra at 101 MHz. All the spectra were calibrated with respect to residual solvent peak. The following abbreviations were used to describe peak patterns wherever appropriate: br, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. Coupling constants are reported in hertz (Hz). High-resolution mass spectra (HRMS) were performed on an electron spray ionization time-of-flight (ESI-TOF). Melting points were measured with a micro melting point apparatus. Single Crystal X-Ray Diffraction (SCXRD) measurements were done on a Bruker-KAPPA APEX II CCD diffractometer. Fluorescence spectra were recorded from Fluoromax-4 from JobinYvon Edison equipped with an injector port and a magnetic stirrer. Measurements of pH were done using a Helmer pH meter. All data from fluorescence studies were processed either by either KaleidaGraph or Origin 8.0 software. The theoretical predictions of pK_a values were done using the Jaguar model of Schrödinger Suite 2015 program.

5.5.3 Synthesis Procedures

3,7-Diazabicyclo[3.3.1]nonane (9): Compound 3,7-diazabicyclo[3.3.1]nonane **9** was synthesized in four steps starting from *N*-Boc-4-piperidone according to known literature protocol.²⁸

Synthesis of 2,4-bis(butylamino)-6-chloro-1,3,5-triazine (11):¹⁵ In a 250 mL round bottom flask, to a solution of *n*-butylamine (6.30 g, 68.36 mmol) in THF (100 mL) was added dropwise at 0 °C the solution of cyanuric chloride (6.3 g, 34.18 mmol) and DIPEA

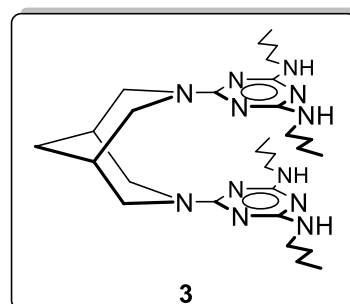
(15 mL) in THF (100 mL). After 1 hour the reaction mixture was allowed to warm to room temperature and stirred for additional 15 hour. The solvent was removed under vacuum on a rotatory evaporator and the obtained white residue was washed with methanol-



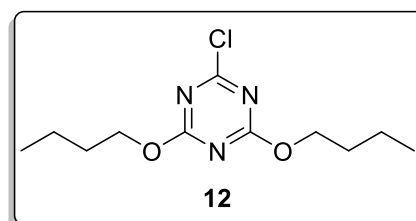
water (2:1) to afford a pure **11** (6.3 g, 74%) as a white solid. ^1H NMR (400 MHz, DMSO- d_6) 7.78 (s, br, 1H), 7.66 (s, br, 1H), 3.20 (m, 4H), 1.46 (m, 4H), 1.23 (m, 4H), 0.87 (t, $J = 7.4$ Hz, 6H).

Synthesis of 6,6'-(3,7-diazabicyclo[3.3.1]nonane-3,7-diyl)bis(N^2,N^4 -dibutyl-1,3,5-triazine-2,4-diamine) (3**):**

In a 25 mL round bottom flask 3,7-diazabicyclo[3,3,1]nonane **9** (100 mg, 0.79 mmol) and 2,4-bis(butylamino)-6-chloro-1,3,5-triazine **11** (510 mg, 1.98 mmol) were dissolved in anhydrous THF (3 mL). To the reaction mixture a solution of DIPEA (306 mg, 2.37 mmol) in THF (2 mL) was added drop wise at room temperature. The resulting reaction mixture was then allowed to reflux for 15 hour under nitrogen atmosphere. After completion of the reaction (followed by TLC) THF was removed under vacuum on a rotatory evaporator. The residue was extracted in ethyl acetate, dried over Na_2SO_4 , and concentrated under reduced pressure to obtain the thick oil which was further purified by column chromatography on silica gel (Eluent: 2% MeOH in CHCl_3) to obtain **3** (425 mg, 53%) as a white solid. **M.P.**: 181 °C; **IR** (ν cm^{-1}): 3213, 2933, 2869, 1682, 1637, 1537, 1442, 1353, 1269, 1190, 1136, 991, 947, 835, 752, 722 cm^{-1} ; ^1H NMR (400 MHz, Methanol- d_4): δ 4.9-5.12 (d, br, $J = 13.08$, 4H), 3.55 (s, br, 4H), 3.16 (m, br, 8H), 2.04 (s, br, 4H), 1.47-1.61 (m, 8H), 1.36 (sext, $J = 6.8$ Hz, 8H), 0.94 (t, $J = 7.3\text{Hz}$, 12H); ^{13}C NMR (101 MHz, Methanol- d_4): δ 165.61 (4C), 165.16 (2C), 47.68 (4C), 40.56 (4C), 32.09 (4C), 31.99, 28.68 (2C), 20.31 (4C), 14.02 (4C); **HRMS** (ESI): calcd. 569.4516 $[\text{M}+\text{H}]^+$; found 569.4516 $[\text{M}+\text{H}]^+$.



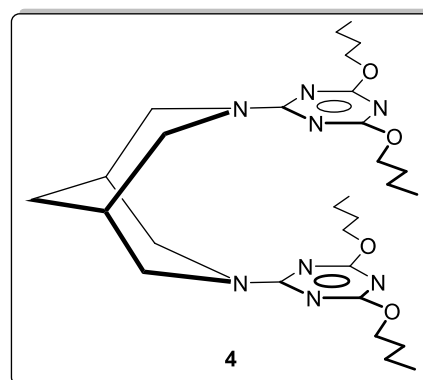
2,4-dibutoxy-6-chloro-1,3,5-triazine (12): To a solution of cyanuric chloride **3** (836 mg, 4.53 mmol, 1 equiv.) in THF (3 mL) placed in a 25 mL round bottom flask was added dropwise at 0 °C the solution of *n*-butanol (840 mg, 11.33 mmol) and DIPEA (1.97 mL, 11.31 mmol) in THF (2 mL).



After 1 h the reaction mixture was allowed to warm to room temperature and stirred for additional 12 h. The solvent was removed under vacuum and the reaction mixture was extracted with ethyl acetate. The organic layer was dried with Na₂SO₄, concentrated *in vacuo* on a rotatory evaporator and subjected to column chromatography on silica gel (*Eluent*: 2% EtOAc in petroleum ether) to afford **12** as a colorless liquid (928 mg, 78%). **IR** (ν cm⁻¹): 2962, 2874, 1538, 1500, 1448, 1356, 1298, 1248, 1173, 1038, 930, 848, 804, 758 cm⁻¹; **¹H NMR** (400 MHz, CDCl₃): δ 4.42 (t, *J* = 6.66 Hz, 4H), 1.80 (q, *J* = 7.1 Hz, 4H), 1.49 (sext, *J* = 7.46 Hz, 4H), 0.96 (t, *J* = 7.46 Hz, 6H); **¹³C NMR** (101 MHz, CDCl₃): δ 172.56 (2C), 171.16, 70.53(2C), 30.38 (2C), 18.97(2C), 13.71 (2C). **HRMS (ESI)**: calcd. 260.1166 [M+H]⁺; found 260.1167 [M+H]⁺.

Synthesis of 3,7-bis(4,6-dibutoxy-1,3,5-triazin-2-yl)-3,7-diazabicyclo[3.3.1]nonane

(4): In a 25 mL round bottom flask 3,7-diazabicyclo[3,3,1]nonane **9** (50 mg, 0.396 mmol) and 2,4-dibutoxy-6-chloro-1,3,5-triazine **12** (258 mg, 0.99 mmol) were dissolved in anhydrous THF (3 mL). To the reaction mixture was added dropwise at room temperature the solution of DIPEA (153 mg, 1.188 mmol) in THF (2 mL). The resulting reaction mixture was then allowed to reflux for 15 h under nitrogen



atmosphere. After completion of the reaction (followed by TLC) the solvent was removed under vacuum on a rotatory evaporator. The residue was extracted with ethyl acetate and dried over Na₂SO₄, solvent was removed under reduced pressure to obtain the thick oil which was further purified by column chromatography on silica gel (*Eluent*: 0.5% MeOH in CHCl₃) to obtain **4** (172 mg, 67%) as colorless liquid. **IR** (ν cm⁻¹): 2959, 2927, 2867, 1565, 1500, 1424, 1344, 1289, 1210, 1124, 1068, 1031, 984, 802; **¹H NMR** (400 MHz, CDCl₃): δ 4.92 (d, *J* = 13.2 Hz, 4H), 4.23 (t, *J* = 6.9 Hz, 8H), 3.08 (dd, *J* = 13.2 & 2.6 Hz, 8H), 2.02 (s, br, 2H), 1.96 (s, br, 2H), 1.71 (quin, *J* = 7.26 Hz, 8H), 1.42 (sext, *J* = 7.47 Hz, 8H), 0.94 (t, *J* = 7.38 Hz, 12H); **¹³C NMR** (101 MHz, CDCl₃): δ 171.52 (4C), 166.99 (2C), 67.22 (4C), 48.09 (4C), 32.07 (2C), 30.93, 28.37 (4C), 19.21(4C), 13.96 (4C); **HRMS (ESI)**: calcd. 573.3877 [M+H]⁺; found 573.3878 [M+H]⁺.

5.5.4 Single Crystal X-Ray Diffraction Studies

To a solution of compound **3** in methanol dry HCl gas (generated by dropwise addition of 98% H₂SO₄ on solid NaCl) was purged for 5 min and the solution was left undisturbed in the closed vial at ambient temperature in the dark for four days and then cap of vial was removed and kept for three days to allow slow evaporation of methanol. The suitable colorless rectangular crystals were obtained was analyzed in single crystal XRD.

Single crystal X-ray crystallographic analysis of **3 co-crystallized with HCl:** Single crystal X-ray intensity data of compound **1** co-crystallized with HCl was collected at 298K on a Bruker KAPPA APEX II CCD diffractometer with graphite-monochromatized (Mo-K α = 0.71073 Å) radiation. The X-ray generator was operated at 1500 W power, 50 kV and 30 mA. Data were collected with the scan width of 0.3° at different settings of ϕ (0°, 90° and 180°) keeping the sample to detector distance fixed at 40 mm and the detector position (2θ) fixed at 24°. The X-ray data collection was monitored by SMART program (Bruker, 2003). The structure was obtained by the direct method using SHELXTL and refined on F^2 by full-matrix least squares techniques using the SHELXL-974 program package within the WINGX program. All non-hydrogen atoms were refined anisotropically. All the hydrogen atoms were placed in geometrically idealized position as riding over their parent atoms.

Details of co-crystal of **3 with HCl:** CCDC 1528648, C₂₉H₅₄Cl₂N₁₂, M = 641.74, colorless blocks, Monoclinic, space group C₂/c, Cell: a = 26.804(7), b = 29.655(7), c = 15.103(4), V = 10534(5), Z = 4, , T = 296 K, $\theta_{\max}$ = 28.470°, D_{calc} (g cm⁻³) = 1.224, $F(000)$ = 744, $\mu(\text{mm}^{-1})$ = 0.091, 7410 reflections collected, 3101 unique reflections (R_{int} = 0.0389), multi-scan absorption correction, $T_{\min}$ = 0.948, $T_{\max}$ = 0.956, number of parameters = 217, number of restraints = 1, GoF = 1.068, R_1 = 0.0680, wR_2 = 0.1836, R indices based on 2043 reflections with $I > 2s(I)$ (refinement on F^2). S_{pmax} = 0.437, S_{pmin} = -0.360 (eÅ⁻³).

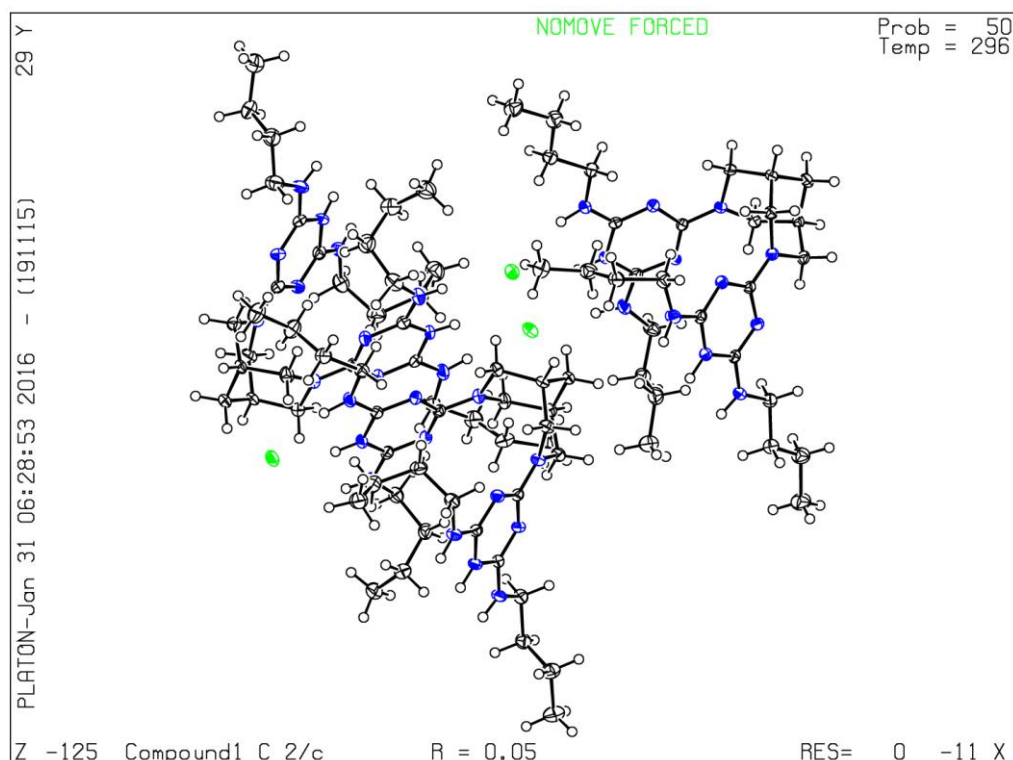


Figure 5.15 ORTEP diagram of **3** co-crystallized with HCl.

5.5.5 Ion Transport Experiments

Preparation of EYPC-LUVs $\supset$ HPTS in NaCl: Vesicles prepared according to the same protocol as discussed in Chapter 2.

Ion transport activity by HPTS assay:²³ Same protocol as discussed in Chapter 2. The collected data was then normalized to the percentage change in fluorescence intensity as a course of time using the equation 1:

$$I_F = [(I_t - I_0)/(I_\infty - I_0)] \times 100 \quad (\text{Equation 1})$$

Where, I_0 is the initial intensity, I_t is the intensity at time t , and I_∞ is the final intensity after addition of Triton X-100.

Ion transport activities of **3** with were measured at different concentrations (0-140 nm). The I_F values at 150 s after addition of **3** were plotted against respect concentration values. Subsequently, the plot was fitted with the Hill equation (Equation 2):

$$Y = Y_\infty + (Y_0 - Y_\infty) / [1 + (c / EC_{50})^n] \quad (\text{Equation 2})$$

Where, Y_0 is the Fluorescence intensity just before the transporter addition (at $t = 0$ s), Y_∞ is the Fluorescence intensity with excess transporter concentration, c = Concentration of transporter compound, and EC_{50} = Effective concentration required to reach 50% activity.

Ion selectivity studies by HPTS assay with applied pH gradient:

The EYPC-LUVs $\rightarrow$ HPTS for ion selectivity were prepared following the same protocol as discussed in Chapter 2.

Anion selectivity assay: The anion selectivity data in the presence of eight different anion as NaA salts, where, $X^- = F^-, Cl^-, Br^-, I^-, OAc^-, NO_3^-, SCN^-$ and ClO_4^- .

Cation selectivity assay: In the similar way, cation selectivity was investigated by varying extravesicular chloride salts (MCl) of different cation; where, $M^+ = Li^+, Na^+, K^+, Rb^+$, and Cs^+ . Under the applied experimental conditions, no difference in transport activity was observed for different cations.

Ion selectivity studies by HPTS assay without applying pH gradient: In a clean and dry fluorescence cuvette 1975 μ L of buffer (10 mM HEPES, 100 mM NaX, pH 7.0; where, $X^- = Cl^-, Br^-, I^-, NO_3^-,$ and ClO_4^-) was added followed by the addition of 25 μ L EYPC-LUVs $\rightarrow$ HPTS vesicles. The resulting solution was slowly stirred with the magnetic stirrer equipped with the fluorescence instrument. The time-dependent change in fluorescence intensity at $\lambda_{em} = 510$ nm was monitored at excitation wavelength $\lambda_{ex} = 450$ nm during the addition of compound **3** (30 nM as DMSO solution, at $t = 60$ s) to start the ion transport, and finally 10% Trion X-100 (25 μ L) at $t = 300$ s was added to lyse the vesicles so that the complete destruction of pH gradient is achieved. The collected data was then normalized to the percentage change in fluorescence intensity as a course of time using the Equation 1 ($I_F = [(I_t - I_0)/(I_\infty - I_0)] \times 100$), where, I_0 is the initial intensity, I_t is the intensity at time t , and I_∞ is the final intensity after addition of Triton X-100).

Ion transport mechanism by HPTS assay:

Ion transport activity in presence of FCCP and Valinomycin Assay: Both the FCCP and valinomycin assays were performed as discussed in Chapter 3.

Preparation of EYPC-LUVs $\rightarrow$ lucigenin vesicles: In a 10 mL clean and dry round bottom flask a transparent thin film of egg yolk phosphatidylcholine (EYPC) was

prepared by drying a 1 mL of EYPC lipid (25 mg / mL in CHCl_3) with continuous rotation and purging of nitrogen gas. The transparent film formed was kept in high vacuum for 6 hours to remove all the traces of CHCl_3 . The transparent lipid film was rehydrated with 1 mL of aqueous NaNO_3 (200 mM, 1 mM lucigenin) solution and resulting suspension was vortexed at an interval of 10 min during period of 60 min. The suspension was subjected to ≥ 10 freeze-thaw cycles (liquid N_2 , 55 °C water bath), and >19 times (must be an odd number) extruded through a polycarbonate membrane (pore size 200 nm). Extravesicular dyes were removed by size exclusion chromatography using Sephadex G-50 (Sigma-Aldrich) eluting with aqueous NaNO_3 (200 mM) and diluted to 3 mL. Final conditions: ~ 5 mM EYPC; *inside*: 200 mM NaNO_3 , 1 mM lucigenin, pH 7.0; *outside*: 200 mM NaNO_3 .

5.5.6 U-Tube Experiment for H^+/Cl^- Symport:

In this experiment, a thin lipid bilayer was substituted by the bulk amount of CHCl_3 segregating the two aqueous phases. In this experiment, the 1 mM solution of receptor **3** in CHCl_3 (12 mL) was placed at the bottom of U-tube. Left arm of U-tube was filled with 6 mL of 0.1 M aqueous HCl solution (pH = 1.05) and right arm was filled with 6 mL aqueous NaNO_3 solution (100 mM). The HCl transport was monitored by the change in pH as well as chloride ion concentration in the right arm of U-tube over a period of 90 hours.

5.5.7 pH-Dependent Cl^- Efflux Assay

Chloride efflux during transport experiments were measured using chloride-selective electrode (*Accumet*). The electrode was calibrated against sodium chloride solutions of known concentrations prior to each experiment.

A thin film of egg yolk phosphatidylcholine (EYPC) was formed from a chloroform solution (25mg/mL) with purging nitrogen and dried under vacuum for at least 6 hours. The lipid film was rehydrated by vortexing with a metal chloride (NaCl) salt solution buffered to either pH 5.8, 7.0 or 8.0 with sodium phosphate salts. The lipid suspension was then subjected to 10 freeze-thaw cycles, where the suspension was alternately allowed to freeze in a liquid nitrogen bath, followed by thawing in a water bath (55 °C). The lipid suspension was allowed to age for 30 min at room temperature and was subsequently extruded 19 times through a 200 nm polycarbonate membrane. The

resulting unilamellar vesicles were dialyzed (using membrane MWCO 1 kD) against NaNO_3 (500 mM) in sodium phosphate buffer of corresponding pH to remove encapsulated Cl^- ions. Internal and external solution pH varied from experiment to experiment.

Unilamellar EYPC vesicles containing NaCl, prepared as described above were suspended in the external medium consisting of NaNO_3 solution buffered to pH 5.8 or 7.0 or 8.0. The lipid concentration in the cuvette was 1.0 mM. The chloride efflux was monitored using a chloride selective electrode. A DMSO solution of the carrier molecule was added at 1 min after starting the measurements and at 5 min, the vesicles were lysed with 25 μL of triton X-100 (10% solution in water) and a total chloride reading was taken at 6 min. The value at 60 second was set at 0% chloride efflux and the final chloride reading (at 7 minutes) was set as 100% chloride efflux.

5.6 Theoretical Calculations

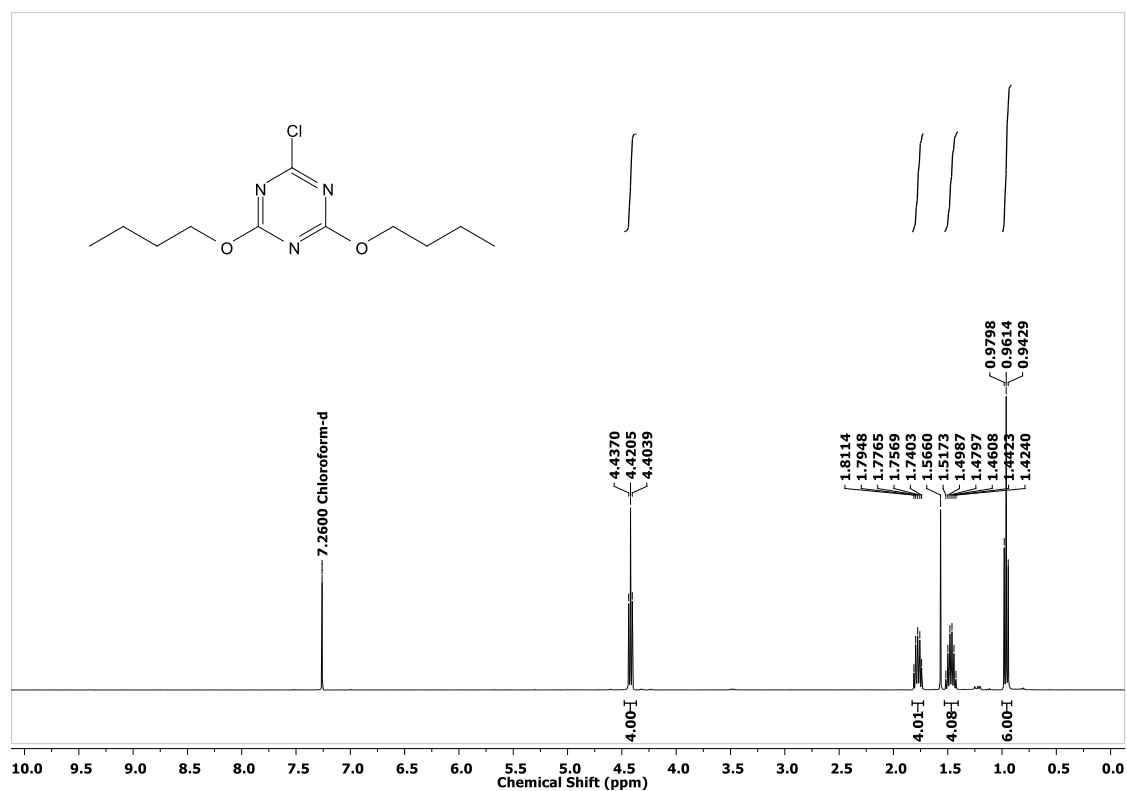
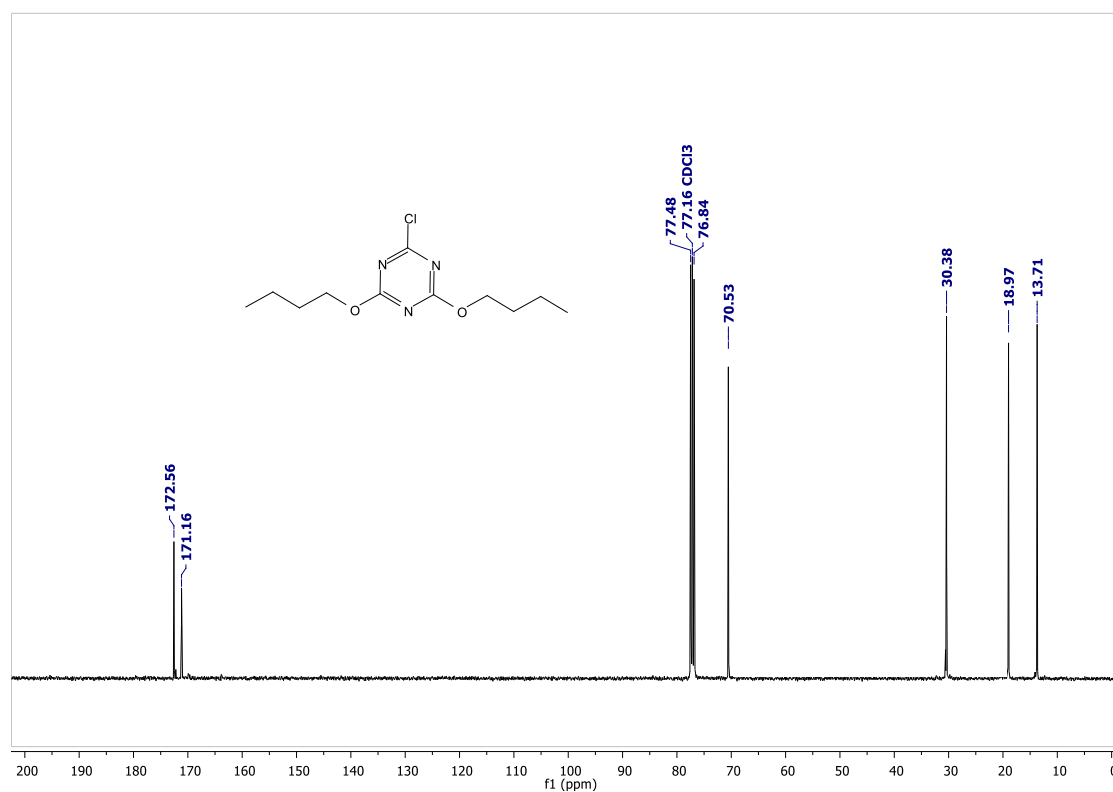
5.6.1 Theoretical Prediction of pK_a Values

Jaguar model²⁰ of Schrödinger Suite 2015 was used for calculating pK_a values of side arm of triazine ring nitrogen atoms of compound **3** and **4** using all default parameters except, selecting water as solvent and Macromodel for conformational search.

5.6.2 Geometry optimization of protonated form of compound 6,6'-((1s,5s)-3,7-diazabicyclo[3.3.1]nonane-3,7-diyl)bis(N^2,N^4 -dimethyl-1,3,5-triazine-2,4-diamine) (**13**):

The compound 6,6'-((1s,5s)-3,7-diazabicyclo[3.3.1]nonane-3,7-diyl)bis(N^2,N^4 -dimethyl-1,3,5-triazine-2,4-diamine) **13** (Figure 5.15A) was used to understand the protonation dependent opening of its V-shape. The butylamine groups of compound **3** were replaced by methylamino groups to get the simpler structure **13**, which was further used for theoretical calculations. The structures of **7** (neutral form), $[\mathbf{13} + (2 \times \text{H})]^{2+}$ (di-protonated form), $[\mathbf{13} + (4 \times \text{H})]^{4+}$ (tetra-protonated form) and $[\mathbf{13} + (6 \times \text{H})]^{6+}$ (hexa-protonated form) were generated using the CS Chem Office 2015 molecular modeling software. The Geometries of generated 3D structures (i.e., **13**, $[\mathbf{13} + (2 \times \text{H})]^{2+}$, $[\mathbf{13} + (4 \times \text{H})]^{4+}$ and $[\mathbf{13} + (6 \times \text{H})]^{6+}$) were optimized using molecular mechanics method (MM2)²⁹ implemented within the Chem 3D module of CS ChemOffice 2015. All default parameters of the program were used for the calculations.

5.7 NMR Spectra:

Figure 5.16 ¹H NMR spectrum of compound 12.Figure 5.17 ¹³C NMR spectrum of compound 12.

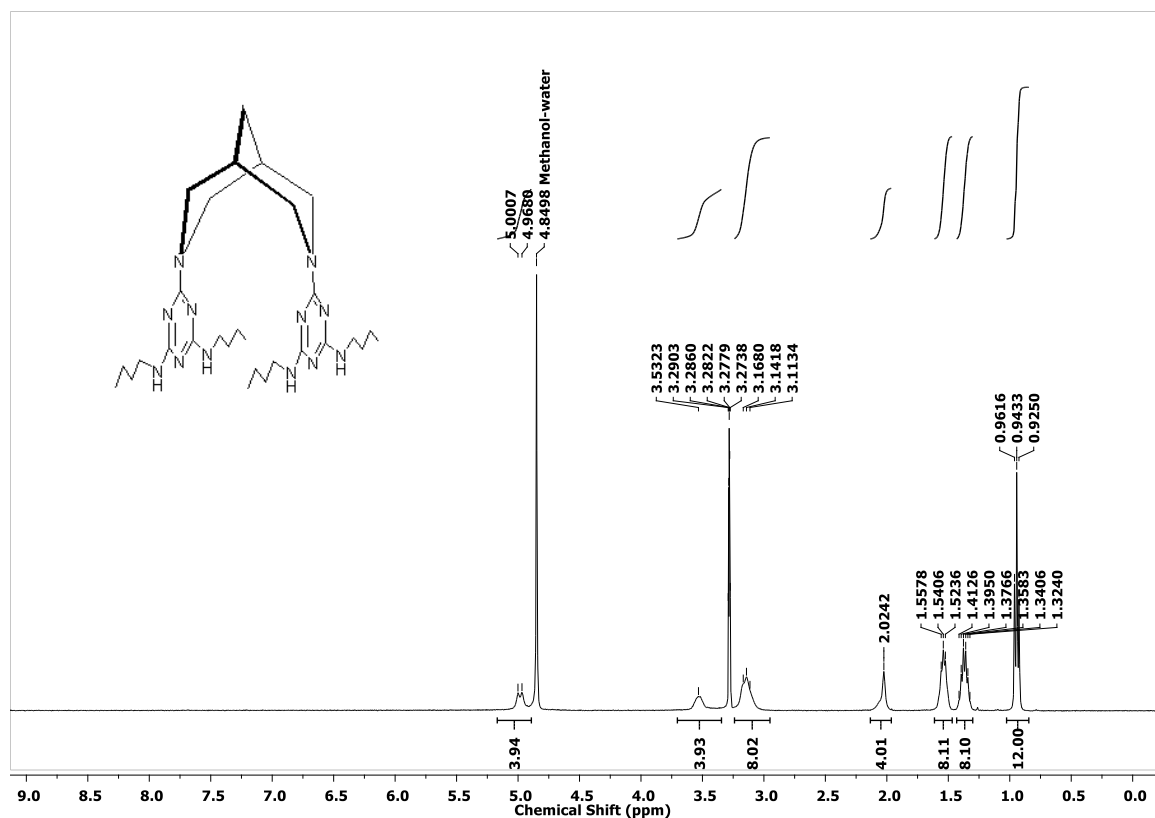


Figure 5.18 ^1H NMR spectrum of compound 3.

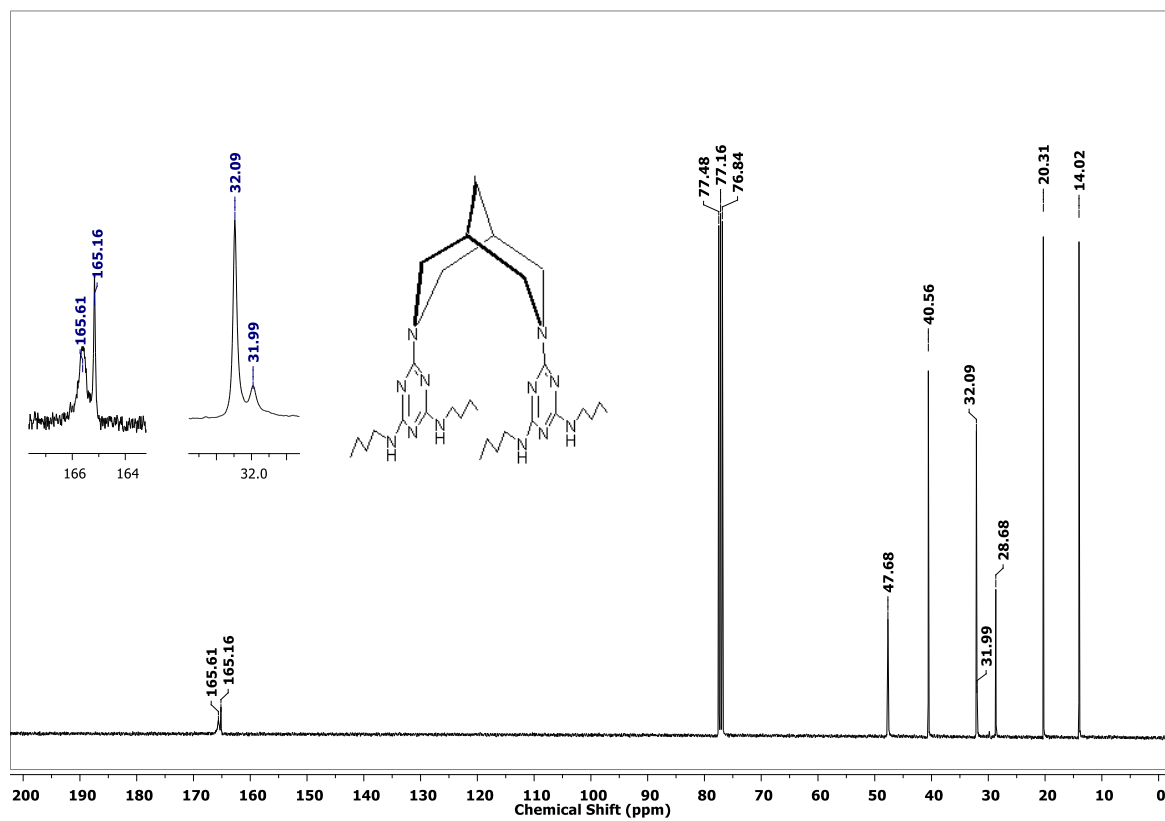


Figure 5.19 ^{13}C NMR spectrum of compound 3.

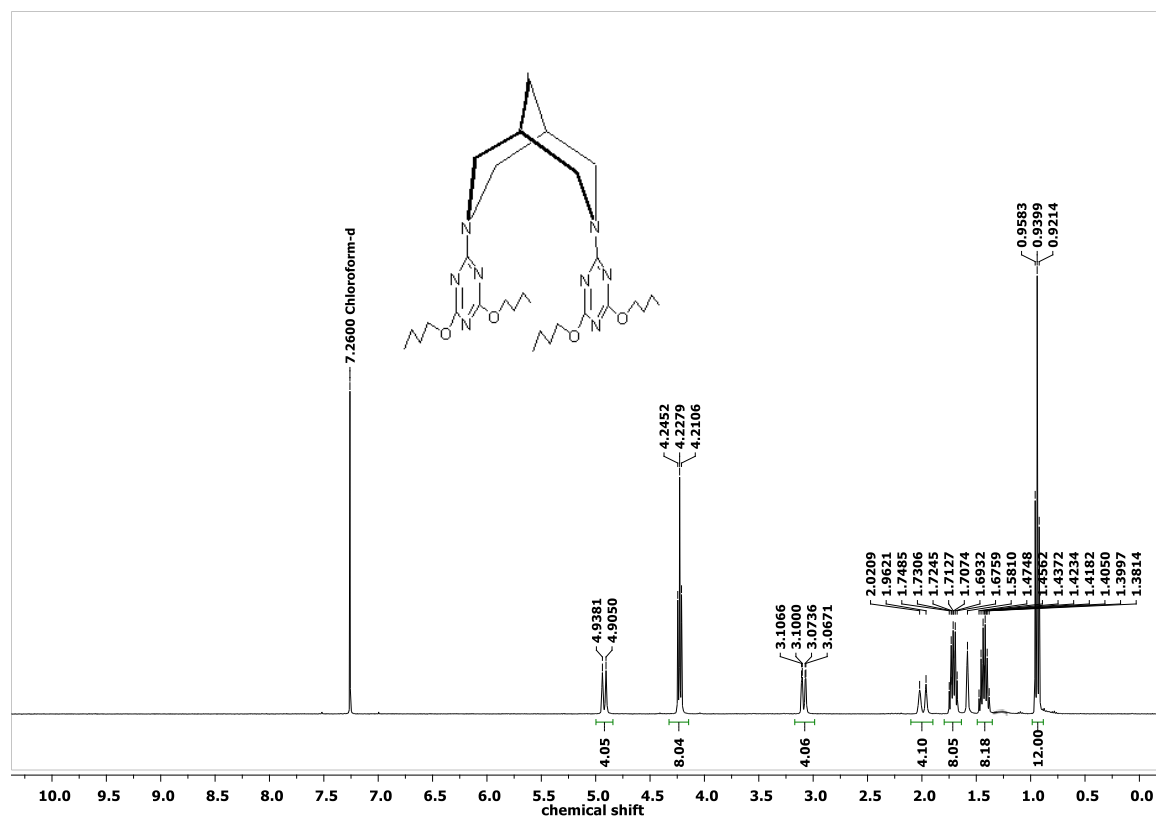


Figure 5.20 ¹H NMR spectrum of compound 4.

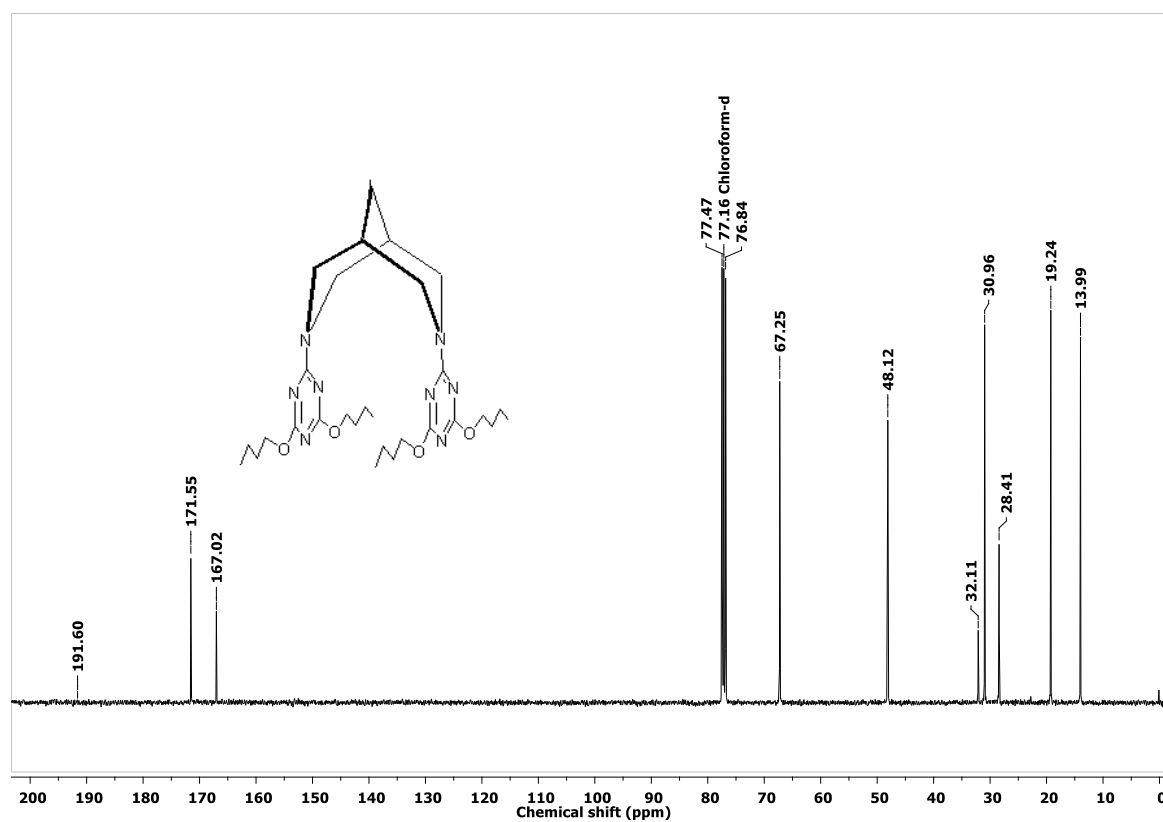


Figure 5.21 ¹³C NMR spectrum of compound 4.

5.7 HRMS Spectra of final compounds

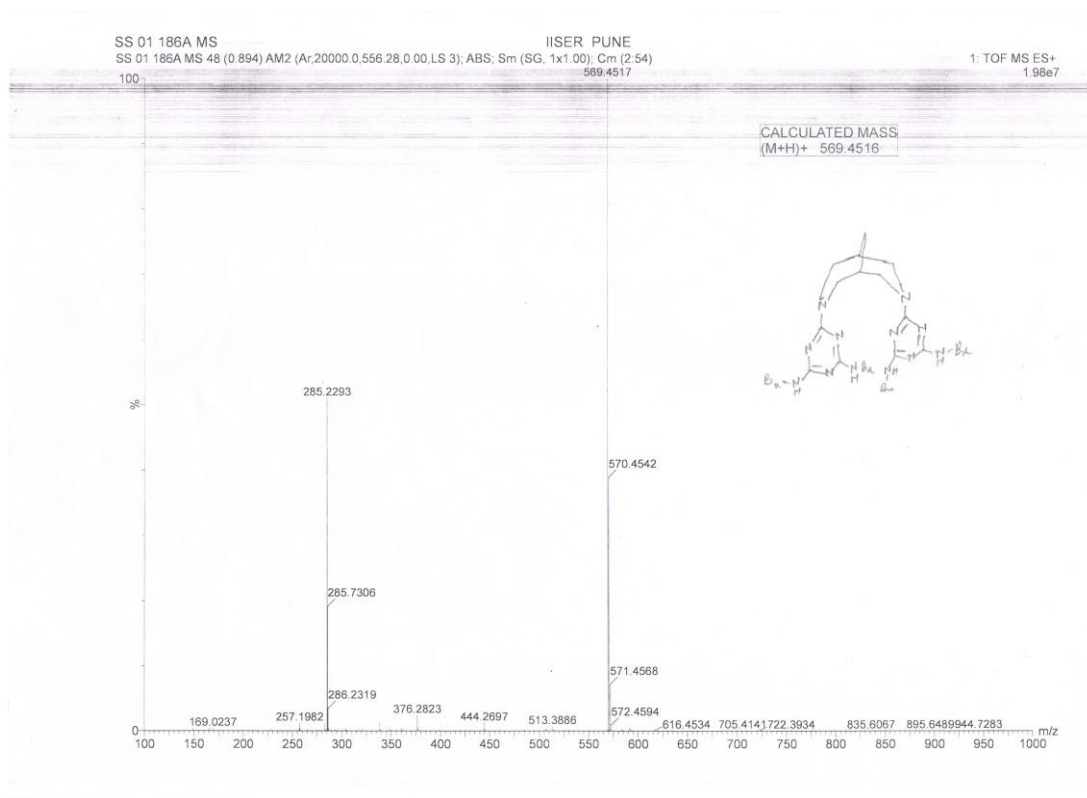


Figure 5.22 HRMS spectrum of compound 3.

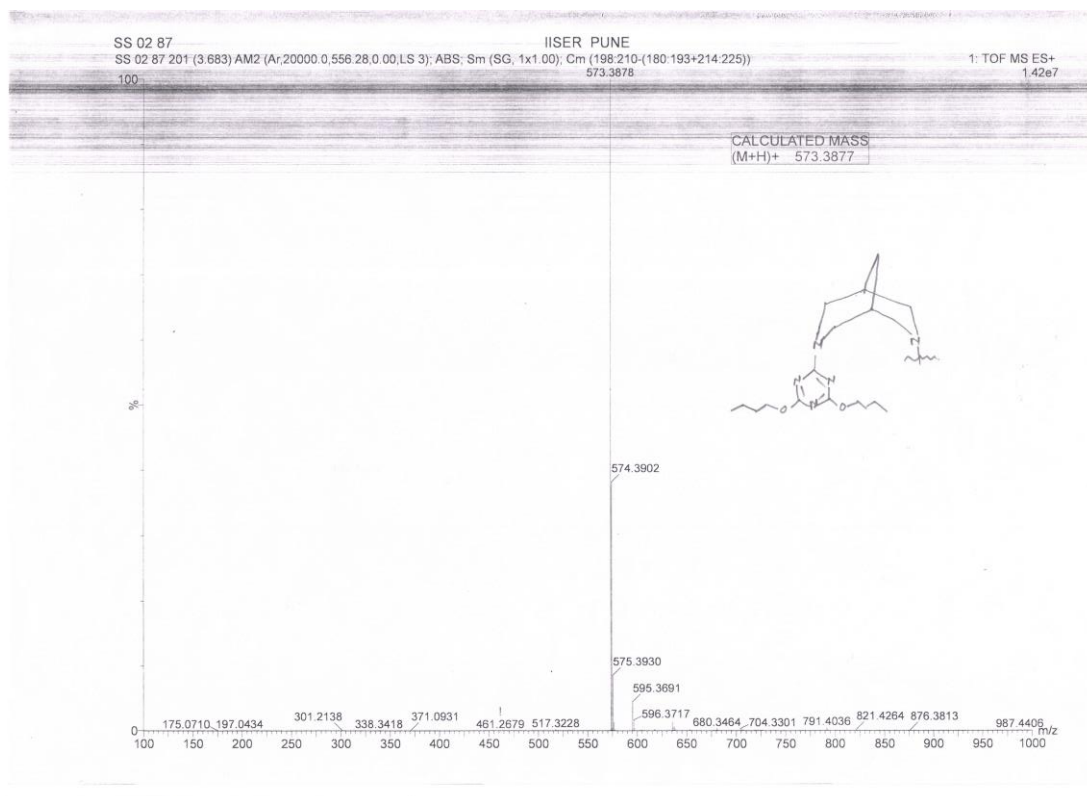


Figure 5.23 HRMS spectrum of compound 4.

5.8 References

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❖ Overall Conclusion and Future Directions

In the overall conclusion, I have achieved the fundamental objectives of my doctoral research study by introducing the synthetic transmembrane anion transport systems. The thesis is focused on the design, synthesis, and characterization of small organic molecules as transmembrane chloride carriers. The study included the investigation of lipophilic and pK_a balance on ion transport activity, ion selectivity, and the transport mechanism across the membrane. In chapter 2, I have introduced the small molecule as an electrogenic chloride transporter while in chapter 3-5, I have designed and synthesized chloride receptors which recognize the chloride selectively in different modes and transport across the membrane as an H^+/Cl^- symport process. In chapter 5, I have shown that the receptors capable of recognizing the multiple HCl molecules lead to efficient transport of chloride across the membrane

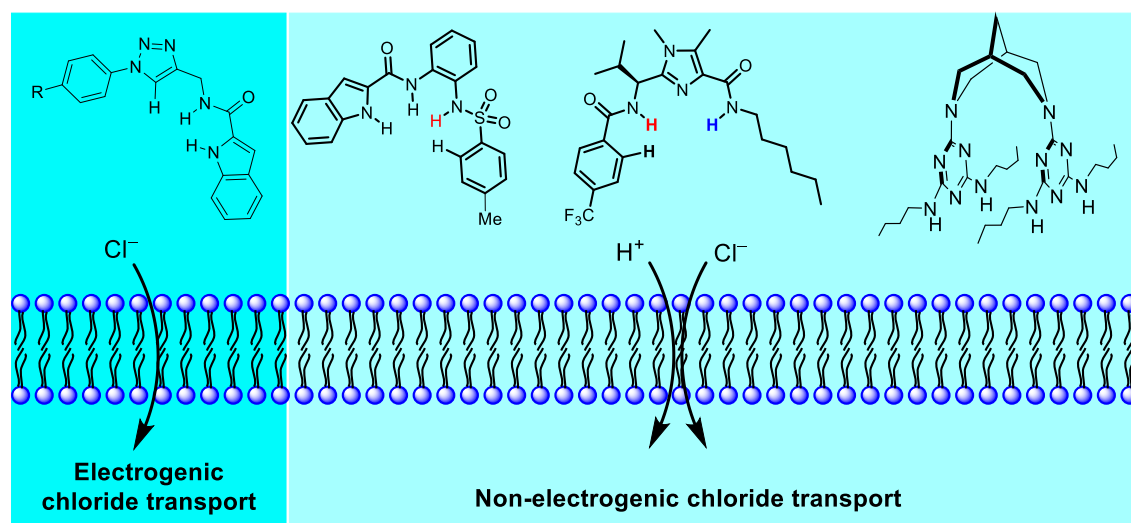


Figure: Schematic illustration of chloride transporters and mechanisms of ion transport discussed in the thesis.

As most of the transporters developed during my Ph.D. work transport both the proton and anion across the membrane, they can show the remarkable biological applications in the line of future therapeutic tools for cancer treatment. Therefore, our next plan is to utilize these proton-anion transporters for deacidifying the microenvironment of tumor cells and simultaneously delivers Cl^- ions into cell cytosol

to induce hyperpolarization followed by apoptosis. Based on the outcome, the active molecules will be further taken forward for their application in targeting cancer cells.

End of the Thesis

Ion Transport

International Edition: DOI: 10.1002/anie.201700803
German Edition: DOI: 10.1002/ange.201700803A Dimeric Bis(melamine)-Substituted Bispidine for Efficient Transmembrane H^+/Cl^- Cotransport

Sopan Valiba Shinde and Pinaki Talukdar*

Abstract: A 3,7-diazabicyclo[3.3.1]nonane linking to two melamines is a unique transmembrane H^+/Cl^- carrier. In the solid state, the V-shaped compound forms a HCl-bound zig-zag network through cooperative protonation and hydrogen bond interactions. In the lipid membrane, the receptor forms a dimeric self-assembly involving multiple H^+ and Cl^- leading to the efficient transport of the acid. The pH-dependent Cl^- efflux observed for the compound was rationalized based on a gradual protonation model that confers an active transmembrane carrier at physiological pH.

Ion transport systems in nature are sophisticated protein machinery that allows the passage of ions across the cellular membranes, overcoming the hydrophobic barrier of the lipid bilayer.^[1] Each transmembrane ion transport system is selective towards a specific type of ions. This distinctive feature of ion channels is the outcome of selective ion recognition by multiple noncovalent interactions originating from the protein backbone and side-chains.^[2] However, dysfunction of these transport proteins, mainly by mutations, results in a number of diseases, known as “channelopathies”.^[3] For example, Bartter’s syndrome, Best disease, and cystic fibrosis are common clinical syndromes arising from the dysfunction of chloride channel proteins.^[4] The introduction of synthetic channel-forming peptides as a novel therapy for fighting chronic infections in cystic fibrosis has promoted the applications of artificial ion transport systems in medical research.^[5] Therefore, the development of synthetic systems capable of mimicking the functions of natural ion transporters is focused extensively because these synthetic congeners have the potential to either complement or replace current therapies for ion channel related diseases. Beyond the so-called channel replacement therapy, synthetic ion transporters are also applicable as antibacterial and anticancer agents. For example, multiple chloride selective ion transporters are reported for their apoptosis-inducing activity in cells,^[6] demonstrating a plausible approach for anticancer drug development.

The microenvironment around tumor cells is unusually acidic compared to healthy cells, and this low pH environment acts as a defense shield for cancerous cells, generating drug

resistance. Therefore, chemical agents capable of deacidifying the intracellular organelles can restore the drug sensitivity.^[7] For example, the red pigment prodigiosin, isolated from microorganisms such as *Serratia* and *streptomyces*,^[8] is an anticancer agent, and this activity is perceived based on its H^+/Cl^- cotransport and Cl^- transport properties across lipid membranes. Beyond prodigiosin^[9] and its analogues,^[10] calix-[4]arene amide from the Davis group,^[11] imidazole-linked pyrrole amide^[12] and perenosins^[13] by the Gale group, trenamide by Smith et al.,^[14] and tris-ureas and tris-thioureas^[15] are instances of synthetic receptors which allow the H^+/Cl^- cotransport across lipid membranes.

Herein we report a melamine-substituted bicyclic receptor **1** that forms a homodimeric liplock taking advantage of cooperative interactions involving multiple H^+ and Cl^- , and thereby functions as transmembrane H^+/Cl^- cotransporter (Figure 1 B). The 3,7-diazabicyclo[3.3.1]nonane (or bispidine)

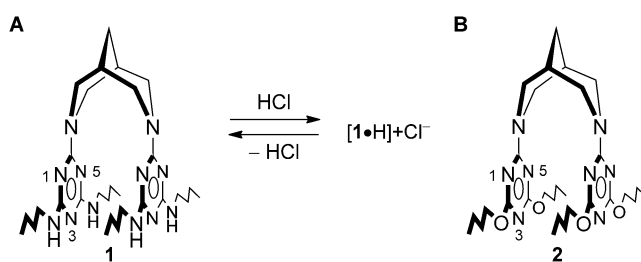


Figure 1. A) The structure of the molecule **1** as a HCl transporter. B) The structure of the control molecule **2**.

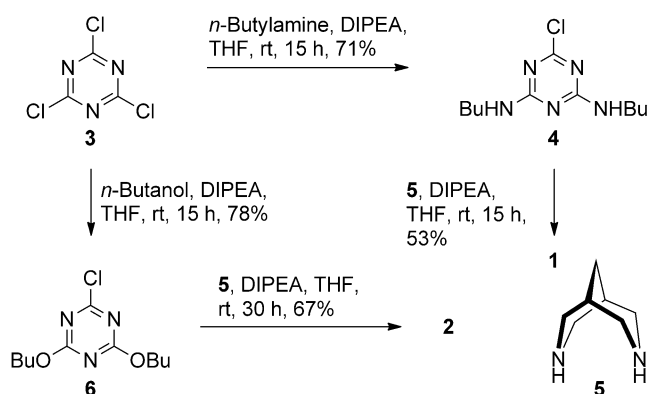
moiety was selected as the jaw component of the receptor due to its rigidity and hydrophobic outer surface. Two butylamino groups, each at C-2 and C-4 positions of the 1,3,5-triazine ring, were attached to generate hydrogen bond donor sites as well as for controlling the amphiphilicity of the V-shaped molecule. A control molecule **2** that lacks the hydrogen bond donor sites was designed by connecting n-butoxy groups at C-2 and C-4 positions of the 1,3,5-triazine ring (Figure 1 B).

The melamine substituted bicyclic molecules **1** and **2** were synthesized from cyanuric chloride **3** (Scheme 1). The compound **3** was converted first into the dibutylamino substituted derivative **4** following a reported protocol.^[16] Subsequent reaction of **4** with bispidine **5** in the presence of DIPEA provided the bicyclic compound **1** in 53 % yield. The reaction of **3** with n-butanol under basic conditions gave the dibutoxy substituted derivative **6** in 78 % yield. Further reaction of **6** with **5** provided the second bicyclic derivative **2** in 67 % yield.

Crystals of compound **1**, complexed with HCl, were obtained by purging dry HCl gas to a solution of the

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Scheme 1. Synthesis of bispidine derivatives **1** and **2**.

compound in MeOH followed by slow evaporation of the solvent. The crystal structure, as shown in Figure 2A, revealed the formation of a zig-zag chain of molecule **1** in which the triazine rings of two adjacent molecules are connected edge-to-edge with the help of two H^+ and two Cl^- . It is also interesting that each Cl^- was held via accepting

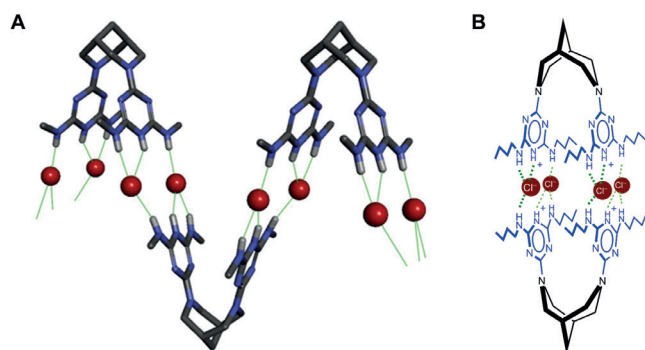


Figure 2. A) X-ray crystal structure molecule **1** complexed with H^+ and Cl^- . Terminal propyl groups on each molecule and noninteracting hydrogens were removed for clarity. B) Proposed homodimeric model of the active transporter.

trifurcated hydrogen bonds with three N–H bonds at hydrogen bond distances of 3.163, 3.092, and 3.156 Å (Figure 2A; Supporting Information, Figure S1). Therefore, the protonation of **1** at the N-3 position over either N-1 or N-5 position is a perfect consequence of positive cooperativity^[17] and micro-environment^[18] between two melamine units of two neighboring molecules, although the theoretical prediction^[19] did not indicate any significant preference for the protonation of N-3 atom of a triazine ring (Supporting Information, Figure S10A). Based on the crystal structure information, an ion transporter model, consisting of two units of **1** and four units of HCl, was proposed (Figure 2B). However, other possible stoichiometries involving $1_2 \cdot (HCl)_2$ and $1_2 \cdot (HCl)_3$ may also play a role in the H^+/Cl^- cotransport process.

The ion transport properties of compounds **1** and **2** were studied across large unilamellar vesicles (LUVs), prepared from egg yolk phosphatidylcholine (EYPC) lipid by entrapping 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) dye^[20] and NaCl, and after applying a ΔpH 0.8 ($pH_{out} = 7.8$ and $pH_{in} = 7.0$).^[21] Faster equilibration of the applied pH gradient by **1** (100 nM) compared to **2** (100 nM) indicated that the dibutylamino substituted derivative is a superior ion transporter (Figure 3A). Concentration-dependent ion transport activity of **1** (0–140 nM; Supporting Information, Figure S3A) followed by Hill analysis provide the $EC_{50} = 30.1$ nM (i.e. 0.045 mol% with respect to the lipid) confirming the compound as an efficient transmembrane transporter. The Hill coefficient, $n = 1.91$ signifies that the two molecules of **1** were involved in the formation of the active transporter (Supporting Information, Figure S3B).

Multiple possibilities such as OH^-/A^- antiport, H^+/M^+ antiport, H^+/A^- cotransport, and M^+/OH^- cotransport are possible for the preceding transport data of **1**. Therefore, to scrutinize further, ion transport properties of **1** in the presence of FCCP (a H^+ selective transporter) was studied using same experimental protocol. Compound **1** showed similar ion transport behavior when studied in the absence and in the presence of FCCP (Figure 3B). The outcome suggests that the H^+ efflux rather than the OH^- influx by **1** was responsible

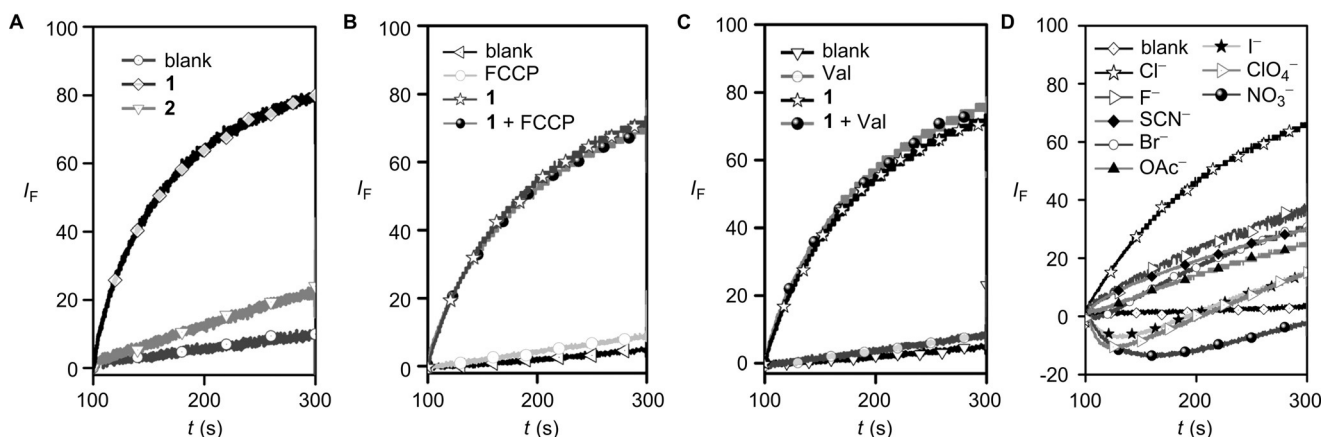


Figure 3. A) Comparison of ion transport activity of **1** (100 nM) and **2** (100 nM) across EYPC-LUVs, presented as normalized emission intensity I_F with respect to time, t . B) Comparison of ion transport activity of **1** (40 nM) in the presence and the absence of FCCP (2.5 μM). C) Comparison of ion transport activity of **1** (40 nM) in the presence and the absence of valinomycin (5 μM). D) Anion selectivity of **1** (40 nM) measured by varying the extravesicular anions.

for the equilibration of the pH gradient. The study excluded the possibilities of OH^-/A^- antiport and M^+/OH^- cotransport by the bicyclic compound. Replacement of extravesicular Na^+ with K^+ , and evaluation of ion transport by **1** in the presence of valinomycin (a K^+ ion selective carrier) resulted in the comparable HPTS fluorescence intensity enhancement (Figure 3C). The lack of any cooperative effect between the H^+ efflux by **1**, and K^+ influx by valinomycin corroborates to an efflux of Cl^- by **1**. Therefore, the cotransport of H^+/Cl^- by the designed bicyclic molecule was confirmed from these studies.

Insignificant differences in ion transport activity, observed by varying the metal ion in the extravesicular chloride salt solution (that is, MCl , $\text{M}^+ = \text{Li}^+$, Na^+ , K^+ , Rb^+ , Cs^+), confirmed that the compound **1** was not selective towards any metal cation (Supporting Information, Figure S4B, S5). Change of anion in the extravesicular sodium salt solution (NaA , $\text{A}^- = \text{F}^-$, Cl^- , Br^- , I^- , OAc^- , ClO_4^- , NO_3^- , SCN^-) showed considerable differences in the ion transport activity of **1** (Figure 3D). For $\text{A}^- = \text{F}^-$, Cl^- , Br^- , OAc^- , ClO_4^- and SCN^- , the pH equilibration process was marked by an enhancement in HPTS fluorescence upholding the strong influence of anions, however, with the Cl^- selectivity. In the absence of any OH^- transport by **1**, the increase in HPTS fluorescence corroborates to the H^+ coefflux process. When the anions in the extravesicular buffer were changed to NO_3^- , ClO_4^- , Br^- and I^- , an initial quenching followed by enhancement of HPTS fluorescence were observed for extravesicular. These results suggested a primary H^+ influx process against the applied pH gradient, and the process is accompanied by A^- coinflux.

The H^+/A^- cotransport process was further proved by measuring the ion transport across EYPC-LUVs with intravesicular Cl^- and extravesicular I^- , Br^- , ClO_4^- , and NO_3^- ions, however without applying the pH gradient (that is, $\text{pH}_{\text{in}} = \text{pH}_{\text{out}} = 7.0$). The addition of **1** resulted in the decrease in intravesicular HPTS fluorescence (Figure 4A). The observation was corroborated the H^+ influx, accompanied by A^- for the charge balance. When SO_4^{2-} , a strongly hydrated anion (hydration energy $\Delta G = -1080 \text{ kJ mol}^{-1}$)^[22] was used in

the extravesicular buffer, a sharp enhancement observed for the HPTS fluorescence indicates the efflux of the H^+/Cl^- ion pair, further confirming the symport mechanism.

The direct evidence of the H^+/Cl^- cotransport was obtained from the U-tube experiment. In this experiment, the lipid bilayer was substituted by CHCl_3 segregating the two aqueous phases. The left arm of the U-tube was filled with aqueous HCl (100 mM) solution (pH 1.05) while the right arm was filled with aqueous NaNO_3 (100 mM). Subsequently, 1.1 mM solution of **1** was placed at the bottom of the U-tube, and the changes in pH as well as Cl^- concentration were monitored in the right arm by a pH and Cl^- selective electrodes, respectively. The steady decrease in pH and increase in Cl^- concentration in the right arm of U-tube confirmed the H^+/Cl^- cotransport by **1** (Figure 4B). The study also confirmed a via mobile carrier mechanism during the ion transport process.

The Cl^- ion transport activity of **1** across LUVs was investigated further by monitoring the fluorescence of lucigenin dye^[23] which was encapsulated along with NaNO_3 (200 mM) within the EYPC-LUVs. After adding NaCl (33 mM) in the extravesicular media, the influx of Cl^- ions was monitored in presence of **1**. A sharp quenching of lucigenin fluorescence was observed indicating the influx of Cl^- ion via an NO_3^- exchange (Supporting Information, Figure S8A). Study of the concentration profile followed by Hill analysis provided $n = 1.86$ (Supporting Information, Figure S8B) which further confirms the active carrier formation involved two molecules of **1**.

Finally, the effect of pH on Cl^- efflux process across EYPC-LUVs was monitored under intravesicular NaCl and extravesicular NaNO_3 at pH 5.8, 7.0 and 8.0. The efflux of Cl^- upon addition of the carrier **1** ($2 \mu\text{M}$) was monitored by a chloride ion selective electrode (ISE). Interestingly, the most effective Cl^- efflux was observed at pH 7.0 (Figure 5A).

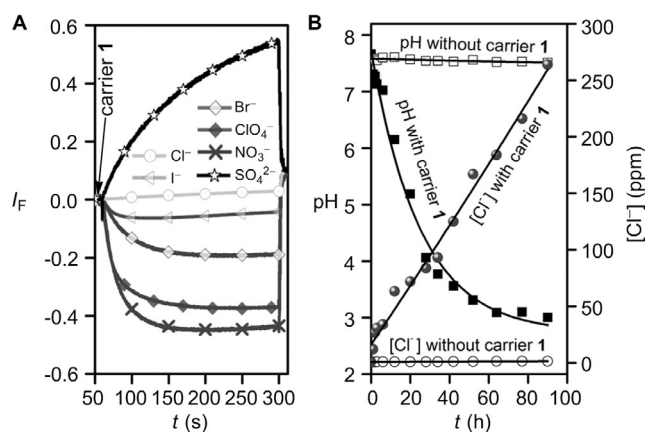


Figure 4. A) Anion selectivity of **1** (40 nM), measured across EYPC-LUVs by varying the extravesicular anions without applying pH gradient. B) Transport of H^+/Cl^- by **1**, measured across a U-tube by applying a HCl gradient.

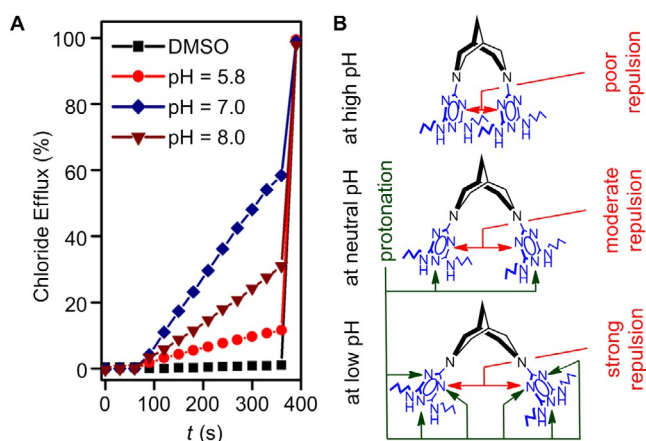


Figure 5. Effect of pH on chloride efflux from EYPC-LUVs (200 nm). EYPC vesicles were loaded with a 500 mM NaCl solution buffered to pH 5.8, 7.0, 8.0 with 5 mM phosphate salts, and were suspended in a 500 mM NaNO_3 solution buffered to pH 5.8, or 7.0, or 8.0, respectively with 5 mM phosphate salts. At the end of the experiment ($t = 360 \text{ s}$), Triton X-100 was added to lyse the vesicles and calibrate the ISE to 100% chloride efflux. DMSO was used as a control before each measurement.

The Cl^- efflux efficiency decreased when the pH was either increased (at pH 8.0) or decreased (at pH 5.8). The plausible explanation for such pH-dependent activity variation can be provided based on the conformational changes in molecule **1** due to protonation of the triazine ring N-atoms as shown in Figure 5B. At higher pH, no protonation occurs on the triazine ring. As a result, the receptor molecules do not offer any anion binding site although, intermolecular two-arm H-bonding between two melamine units is possible. Lowering of pH increases the probability of a cooperative cascade process involving the protonation at the N-5 atom of each triazine ring, recognition of Cl^- ions, and a liplock dimer formation. The H^+/Cl^- bound dimer of **1** favors the transport of the ion pair. Further lowering of pH allows protonation, also at other N-atoms of each triazine ring. As a result, charge repulsion between melamine units of **1** is increased, leading to an opening of molecular jaw and distortion of the dimeric H^+/Cl^- bound complex. The structural distortion weakens the cooperative Cl^- ion binding and therefore, transport activity of **1** is decreased. The computed pK_a values (in the range of 5.9–4.2) were not correlating with the pH and HCl uncoupling activity. However, it is known that the pK_a values in lipid bilayer can differ significantly.^[24] The protonation-dependent opening of V-Shaped structures was also in agreement with theoretical calculations performed using CS Chem Office 2015 molecular modeling software (Supporting Information, Figure S11).

In summary, we have developed a bis(2,4-dibutylamino-1,3,5-triazine) linked bispidine compound **1** as an efficient HCl transporter across bilayer lipid membranes. The X-ray crystallographic study revealed that the V-shaped molecule forms a $1:(\text{HCl})_2$ complex arranged in a zig-zag network. In this assembly, protonation of the triazine N-atom that intervenes two dibutylamino groups, and trifurcated $\text{N}-\text{H}\cdots\text{Cl}^-$ interactions were evident as the motif for the binding of two H^+ and two Cl^- between successive melamine units. This HCl recognition was rationalized based on cooperativity and microenvironmental effects because theoretical prediction suggested comparable basicities of all N-atoms of a triazine ring. Compound **1** displayed an excellent ion transport activity which corroborated to a dimeric locking and delivers multiple H^+ and Cl^- , simultaneously. The H^+/Cl^- cotransport mechanism was also established. Cl^- efflux characteristics of **1** indicates maximal activity at $\text{pH} \approx 7$, and this was explained based on a protonation-dependent widening of the mouth of the V-shaped molecule. The mechanism suggests that the dimeric locking involving multiple H^+ and Cl^- is stable at pH 7.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: cooperativity · ion transport · pH-dependent activity · protonation · symport mechanism

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An anion receptor that facilitates transmembrane proton–anion symport by deprotonating its sulfonamide N–H proton†

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Indole-based amide-sulfonamide derivatives were synthesized. The X-ray crystal structure and chloride binding studies in solution showed a 1 : 1 stoichiometry. The ion transport efficiency directly correlated to the pK_a of the sulfonamide N–H protons. The mechanistic study indicated proton–anion symport across the lipid bilayer membrane.

Anions play a significant role in the survival of cells by regulating many vital processes, such as cellular pH, signal transduction, osmotic balance, *etc.*¹ Naturally, anion transport across the phospholipid bilayer is facilitated by membrane protein machinery which assists in anion transport as either a mobile carrier or static channel.² However, the malfunctioning of these proteins leads to a number of medical complications (*i.e.* channelopathies) such as cystic fibrosis, kidney disorders, epilepsy and myotonia.³ Many synthetic small molecules have emerged mimicking the anion transport behavior of specialized proteins. However, only a few of these synthetic molecules display gating/switching behavior that is seen in biological systems. The proton flux regulators, *e.g.* V-ATPase and Na^+/H^+ exchangers (NHE), regulate the unusual acidic pH in the microenvironment of tumor cells, thereby acting as a hallmark to protect tumor cells from weak basic drugs.⁴ The activity of such weak basic drugs can be restored by using a chemical agent, which is capable of deacidifying the microenvironment of tumor cells.⁵ One of the most studied carriers is prodigiosin,⁶ a red pigment isolated from the Gram-positive bacteria *Serratia* and *Streptomyces*.⁷ Prodigiosin and its synthetic analogs are well documented for their anticancer^{6,8} and immunosuppressive activity, and toxicity against fungi⁹ and malaria parasites.¹⁰ These molecules take advantage of the basic nature of one ring nitrogen and bind to proton–chloride through electrostatic and hydrogen bond interactions, and facilitate

the cotransport of the inorganic acid across cell membranes thereby triggering apoptotic cell death.¹¹

Prodigiosin also inspired the development of several proton–chloride cotransporters, *e.g.* perenosins,¹² tren-amides,¹³ imidazole-appended amidopyrroles,¹⁴ tris-ureas and thioureas¹⁵ and bis(melamine)-linked bispidines¹⁶ which have basic cores for protonation. Herein, we propose the design and development of a sulfonamide-based receptor that takes advantage of its sulfonamide N–H proton acidity to cotransport proton–anions across the lipid bilayer membrane.

The structures of these molecules **1a–1g** are shown in Fig. 1A. In this design, *o*-phenylenediamine was connected to an indole moiety at one end *via* carboxamide linkages and a range of substituted phenyl rings at the other end *via* sulfonamide linkages. It was envisaged that such a receptor would bind to an anion through hydrogen bond interactions of three N–H moieties (Fig. 1B). Upon transporting the anion across the lipid bilayer membrane, the receptor may also give up its sulfonamide N–H proton depending on the acidity of the proton and pH of the environment. Such an outcome is feasible because Talukdar and co-workers have already reported that the pK_a of sulfonamide N–H protons dictates their deprotonation, which gets reflected in the ion binding mode.¹⁷ The theoretically estimated

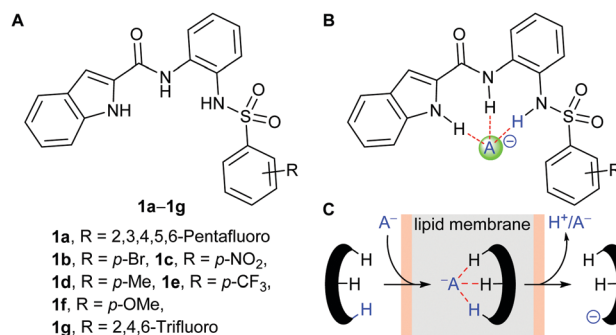


Fig. 1 Structures of receptors **1a–1g** (A). Representative anion (A^-) binding (B) and transport across lipid vesicles (C).

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Table 1 Summary of the chloride stability constants K_a (M^{-1}) of receptors **1a–1g** calculated at 298 K and the pK_a values of receptors **1a–1g**

Transporter	R	log P	pK_a of sulfonamide NH^a	K_a^b (M^{-1})	EC_{50} (μM)	n
1a	2,3,4,5,6-Pentafluoro	4.94	5.59	$> 10^4$	ND	ND
1b	<i>p</i> -Br	4.99	7.28	ND	4.01	1.70
1c	<i>p</i> -NO ₂	4.16	6.8	ND	13.41	1.02
1d	<i>p</i> -CH ₃	4.73	7.44	3206.35 ± 88.3	2.33	2.18
1e	<i>p</i> -CF ₃	5.10	7.28	4680.31 ± 146.8	5.82	1.67
1f	<i>p</i> -OCH ₃	4.06	7.52	1911.04 ± 140.3	7.56	2.30
1g	2,4,6-Trifloro	4.65	5.75	8187.78 ± 481.5	ND	ND

^a Calculator plugin of the MarvinSketch program was used for calculating log P and pK_a . ^b Binding constants were obtained based on the 1 : 1 binding model for sulfonamide NH protons.

pK_a values of the sulfonamide N–H proton for molecules **1a–1g** are 5.59, 7.28, 6.8, 7.44, 7.28, 7.52 and 5.75, respectively (Table 1).¹⁸ Under acidic conditions, these receptors were expected to transport anions. However, in some cases, the deprotonation of the sulfonamide N–H proton was expected resulting in a proton–anion symport process (Fig. 1C).

Synthesis of **1a–1g** was initiated from indole 2-carboxylic acid **3**. Initially, acid **3** was coupled with *N*-Boc-*o*-phenylenediamine **2** to get the compound **4** in 88% yield. The reaction of **4** with $BF_3 \cdot OEt_2$ led to the deprotection of the Boc group to obtain free amine **5** in 90% yield. Finally, the reaction of amine **5** with aryl sulfonyl chloride **6a–6g** in a pyridine–THF (1 : 3) mixture provided the receptors **1a–1g** in 86–95% yields (Schemes S1–S4, ESI†).

The crystal structure of **1e** with Cl^- was obtained by the slow evaporation of an acetonitrile solution of **1e** in the presence of excess tetrabutylammonium chloride (TBACl). The single crystal XRD structure showed the 2 : 2 kosmotropic complexation of **1e** and Cl^- , stabilized by three bridged water molecules (Fig. 2A and Fig. S22, ESI†). The receptor **1e** forms two $N-H \cdots Cl^-$ hydrogen bonds ($N_{sulfonamide} \cdots Cl^-$ and $N_{carboxamide} \cdots Cl^-$ distances of 3.112(4) Å and 3.256(4) Å with angles of 143.1° and 157.4°, respectively. Each Cl^- ion further forms one $C-H \cdots Cl^-$ hydrogen bond with the $C \cdots Cl$ distance of 3.765(4) Å and the $C-H \cdots Cl^-$ angle of 148.7°, respectively. Each Cl^- ion also forms a hydrogen bond with one terminal water molecule ($Cl^- \cdots OH_2$ distance of 2.926(1) Å. At the same time, the complex is further stabilized by the additional hydrogen bond interactions of this water with one

sulfonyl oxygen atom ($H_2O \cdots O_{sulfonamide}$ distance 2.912(1) Å), the central water molecule ($H_2O \cdots OH_2$ distance 2.70(1) Å) and the C_{ortho} -H of the trifluoromethylphenyl ring 3.24(1) Å). Additionally, the Hirshfeld surface analysis¹⁹ also confirmed these hydrogen bonding interactions (Fig. S23, ESI†). The indolic NH was engaged in the homodimerization with another molecule of **1e** through the $NH \cdots O=C$ hydrogen bond making it unavailable for anion binding.

The solution phase anion binding abilities of receptors **1a–1g** were evaluated by 1H NMR titration²⁰ at 298 K in acetonitrile- d_3 by titrating tetrabutylammonium (TBA) halides. Titration data showed significant downfield shifts of all three NH signals, e.g., $\Delta\delta = 1.72$, 2.57 and 1.75 ppm for H_a , H_b and H_c , respectively for **1d** (Fig. 2B), indicating the hydrogen bond interactions of these protons with chloride ions. Job's plot analysis, based on the 1H NMR titrations, showed a maximum at the 0.5 mole fraction, confirming a 1 : 1 binding stoichiometry of **1** with Cl^- (Fig. S24C, ESI†). Mass spectrometric analysis also supported a 1 : 1 binding of **1d** : Cl^- (Fig. S47, ESI†). The binding constant K_a values were calculated by fitting the titration data of the H_a proton in the WinEQNMR2 program according to the 1 : 1 binding model. For **1a** and **1d–1g**, the K_a values were $> 10^4$, 3206.35 ± 88.3 , 4680.31 ± 146.8 , 1911.04 ± 140.3 and $8187.78 \pm 481.5 M^{-1}$, respectively (Table 1). For **1b** and **1c**, the K_a values could not be determined due to their poor solubility in acetonitrile- d_3 . Overall, the trend of the K_a values was dependent of the acidity of H_c , i.e. the stronger the electron withdrawing effect of the substituted benzene ring next to the sulfonyl group, the better the binding with the Cl^- . For receptor **1d**, a comparison of halide-binding provided the binding sequence of Cl^- ($K_a = 3206.35 \pm 88.3 M^{-1}$) $>$ Br^- ($263.39 \pm 6.02 M^{-1}$) $>$ I^- (K_a could not be determined due to the insignificant change of chemical shift) suggesting a higher selectivity towards Cl^- (Fig. S29 and S30, ESI†). However, the titration of **1d** with the basic anions F^- and AcO^- as TBA salts in acetonitrile- d_3 resulted in deprotonation of the receptor (Fig. S31 and S32, ESI†).

The ion transport activity of **1a–1g** was investigated fluorometrically with the help of a pH-sensitive 8-hydroxypyrene-1,3,6-trisulfonate dye (HPTS, $pK_a \sim 7.3$), entrapped into vesicles.²¹ The large unilamellar vesicles were prepared at pH = 7.0 from the egg yolk phosphatidylcholine (EYPC) lipid with entrapped HPTS to get EYPC-LUVs $\supset$ HPTS. Subsequently, the extravesicular pH was raised to 7.8 by adding NaOH. The collapse of the pH gradient,

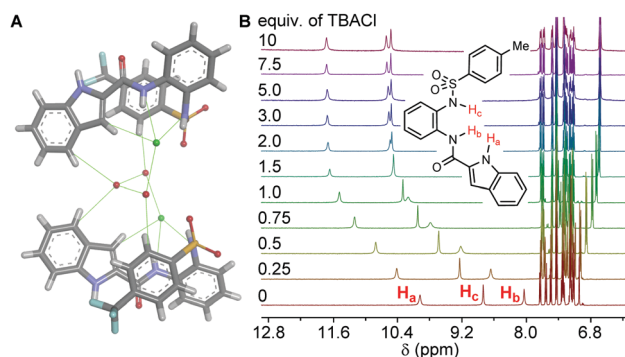


Fig. 2 Side view of the X-ray crystal structure of **[1e·Cl[−]][Bu₄N⁺]** (A). Chemical shift change in NH protons of **1d** upon titration with TBACl (0–10 equiv.) (B).

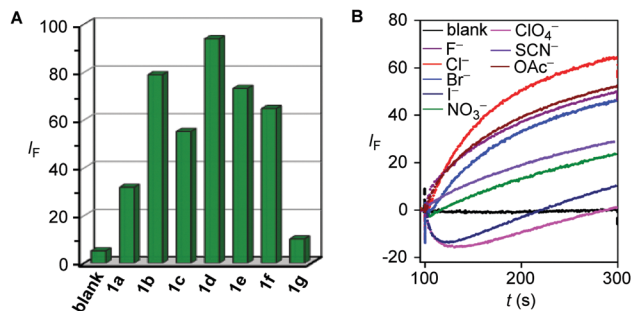


Fig. 3 Comparison of the ion transport activities of **1a–1g** (10 μM) across EYPC-LUVs Δ HPTS recorded at 200 s after sample addition (A). Anion selectivity of **1d** across EYPC-LUVs Δ HPTS measured in the presence of an applied pH gradient ($\Delta\text{pH} = 0.8$) (B).

upon addition of a transporter was monitored by measuring the fluorescence intensity of HPTS at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$). Finally, the vesicles were lysed by adding Titron X-100 to achieve the complete collapse of the applied pH gradient. The synthetic receptors **1b** and **1d–1f** with a pK_a value (of sulfonamide NH protons) greater than 7.0 exhibited a significant fluorescence intensity enhancement indicating the transport of ions across the EYPC-LUVs. Comparison of the ion transport activities at an identical concentration ($c = 10 \mu\text{M}$) provided the activity sequence: **1d** > **1b** > **1e** > **1f** > **1c** > **1a** > **1g** (Fig. 3A). Although **1a** has a lower pK_a value than **1f**, its higher ion transport activity could be attributed to its better membrane permeability ($\log P = 4.95$). The dose-response activity of compounds **1b–1f** also provided the EC_{50} (the concentration required to reach 50% activity) and Hill coefficient n values (Table 1). The Hill coefficient n values, in general, suggested the involvement of two molecules in the formation of active transporters. For compound **1d** (40 μM), no leakage of the ANTS dye from EYPC-LUVs Δ ANTS/DPX was observed, confirming the vesicle integrity in the presence of **1d** (Fig. S40, ESI[†]).

Subsequently, the most active compound **1d** was selected to investigate the ion selectivity and transport mechanism. The selectivity studies were performed on compound **1d** by varying the extravesicular ions in the HEPES buffer solution.²² As expected, an insignificant change in the ion transport activity was observed upon varying the metal chloride (*i.e.* MCl, where, $\text{M}^+ = \text{Li}^+, \text{Na}^+, \text{K}^+, \text{Rb}^+, \text{and } \text{Cs}^+$) in the extravesicular buffer solution (Fig. S41B and C, ESI[†]). These data confirm that the cations are not involved in the rate limiting steps of the ion transport process by **1d**. However, the change of the anions in the extravesicular buffer with sodium salts (*i.e.* NaA, where, $\text{A}^- = \text{F}^-, \text{Cl}^-, \text{Br}^-, \text{I}^-, \text{NO}_3^-, \text{ClO}_4^-, \text{SCN}^-, \text{and } \text{OAc}^-$) showed a significant difference in the ion transport activity providing the activity sequence: $\text{Cl}^- > \text{OAc}^- \sim \text{F}^- \geq \text{Br}^- > \text{SCN}^- > \text{NO}_3^- > \text{I}^- > \text{ClO}_4^-$ (Fig. 3B). The selectivity sequence indicated the involvement of extravesicular anions in the ion transport process. The initial drop in the intravesicular pH in the case of I^- and ClO_4^- indicated the possibility of an initial H^+/A^- influx or A^-/OH^- antiport. The selectivity sequence neither fully concerted the Hofmeister series (where the dehydration energy limits transport) nor showed a complete dependence on

the anion size. Also, among the halides, the dehydration dominated quasi-Hofmeister series (*i.e.* $\text{Cl}^- > \text{Br}^- > \text{I}^-$) was observed.²³ This indicates that both dehydration and carrier binding followed by membrane crossing are rate-limiting steps. The observation was also in agreement with the halide binding data of **1d** obtained from NMR studies. The chloride ion transport activity of **1d** across LUVs was further examined by monitoring the fluorescence intensity of lucigenin dye ($\lambda_{\text{em}} = 535 \text{ nm}$, $\lambda_{\text{ex}} = 455 \text{ nm}$) entrapped along with nitrate inside EYPC-LUVs.²⁴ The addition of NaCl (33 mM) followed by **1d** resulted in the quenching of the intravesicular lucigenin fluorescence confirming the influx of Cl^- (Fig. S43, ESI[†]).

The collapse of the applied pH during the HPTS assay can be achieved by either M^+/H^+ antiport, OH^-/A^- antiport, M^+/OH^- symport, or H^+/A^- symport processes. The observed cation variation data ruled out the M^+/H^+ antiport and M^+/OH^- symport processes, and anion variation data are suggestive of either OH^-/A^- antiport or H^+/A^- symport by **1d**. The initial decrease of the HPTS fluorescence, observed for $\text{A}^- = \text{I}^-$ and ClO_4^- (Fig. 3B), is suggestive of H^+ influx *via* H^+/A^- symport as one possible mechanism. To further cross-examine, the transport activity of **1d**, in the presence of either FCCP (a proton carrier) or valinomycin (a K^+ carrier), was implemented.²⁵ In the FCCP coupled assay, the compound **1d** showed similar transport activity as in the absence of FCCP (Fig. 4A). This outcome corroborated the dissipation of the pH gradient through the H^+ efflux by the H^+/Cl^- cotransport mechanism, instead of OH^- influx. Valinomycin assay^{25b} performed by replacing the extravesicular Na^+ with K^+ . The valinomycin coupled HPTS assay (with intravesicular NaCl and extravesicular KCl) of **1d** also showed ion transport that was comparable to **1d** alone (Fig. 4B). Therefore, no cooperative effect was seen between the K^+ influx by valinomycin and the ion transport by **1d**, and the outcome further confirmed the H^+/Cl^- symport mechanism of the amide–sulfonamide derivative.

To get more insight about the mechanism, HPTS assays were carried out by varying the extravesicular anions and without applying a pH gradient (*i.e.* $\text{pH}_{\text{in}} = \text{pH}_{\text{out}} = 7.0$). Vesicles entrapped with NaCl were suspended in buffer containing either NaCl, NaBr, NaI, NaClO_4 or Na_2SO_4 salts. In the presence of extravesicular NaCl, the addition of **1d** resulted in the initial

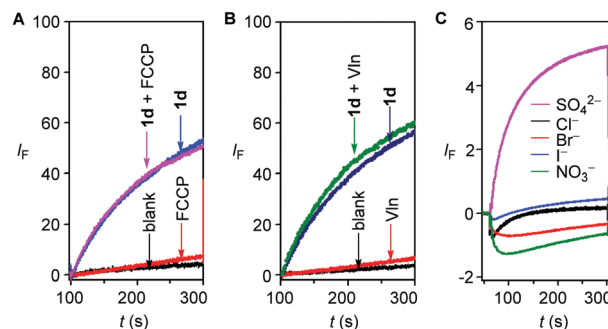


Fig. 4 Ion transport activity of **1d** (2.5 μM) in the presence and absence of FCCP (0.8 μM) (A), and valinomycin (2.5 pM) (B). Anion selectivity without a pH gradient (C).

influx of HCl followed by slow dissipation of the *in situ* generated pH gradient (Fig. 4C). In the presence of poor permeating external SO_4^{2-} (hydration energy, $\Delta G_{\text{SO}_4^{2-}} = -1080 \text{ kJ mol}^{-1}$)²⁶ an immediate increase in the HPTS fluorescence was observed which indicates the H^+/Cl^- coefflux. For extravesicular Br^- , I^- , and NO_3^- , quenching of the fluorescence was observed suggesting the influx of H^+/A^- . The anion symport mechanism is further confirmed by checking the transport across EYPC-LUVs (inside 1 mM HPTS, 100 mM NaNO_3 , HEPES 10 mM, pH 7.0). These vesicles were suspended in the buffer (100 mM NaCl, 10 mM HEPES, pH = 7.0) and the transport is monitored after the addition of **1d** resulting in the H^+/NO_3^- efflux confirming the symport mechanism (Fig. S46, ESI†).

In summary, we have synthesized 1*H*-indole-2-carboxamide-based anion receptors by further linking the moiety to a sulfonamide with varied aryl groups. This class of molecules was envisaged to bind anions, thereby transporting the anion along with a proton by deprotonating its sulfonamide group. The co-crystallization of **1e** with TBACl revealed the binding of two Cl^- ions by two receptor molecules with the help of multiple water molecules. The ^1H -NMR titration experiments also confirmed anion binding. All receptors showed ion transport activity in the order: **1d** > **1b** > **1e** > **1f** > **1c** > **1a** > **1g**. The detailed mechanistic investigation with the most efficient transporter demonstrated the proposed proton-anion symport process. This class of molecules may be useful in targeting solid tumors by promoting intracellular acidification.

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Conflicts of interest

There are no conflicts to declare.

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