

Development of Transmembrane Ion Transport Systems for Biomedical Applications

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

A thesis submitted in partial fulfillment of the requirements of the
degree of Doctor of Philosophy

द्वारा / By
नवीन जोसेफ रॉय / Naveen Joseph Roy

पंजीकरण सं. / Registration No.: 20173528

शोध प्रबंध पर्यवेक्षक / Thesis Supervisor: Prof. Pinaki Talukdar



भारतीय विज्ञान शिक्षा एवं अनुसंधान संस्थान पुणे
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Dedicated to...

Appa, Amma, Pappi, Appachan and Daisy



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CERTIFICATE

Certified that the work incorporated in the thesis entitled “**Development of Transmembrane Ion Transport Systems for Biomedical Applications**” submitted by Naveen Joseph Roy 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.

A handwritten signature in blue ink, reading 'Pinaki Talukdar'.

Date: 14 December 2023

Prof. Pinaki Talukdar

Place: Pune

(Supervisor)

Declaration

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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Place: Pune



Naveen Joseph Roy

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Symbols and Abbreviations

#

δ	Chemical shift (NMR)
λ	Wavelength (lambda)
μL	Microliter
μM	Micromolar
$\tilde{\nu}$	Wave number
$\text{\AA}$	Angstrom
$^{\circ}\text{C}$	Degrees Celsius

A

ACN	Acetonitrile
AcOH	Acetic acid
AO	Acridine orange
aq.	Aqueous medium
ATCC	American Type Culture Collection

B

B3LYP	Becke, 3-parameter, Lee–Yang–Parr (exchange-correlation functional)
BA	Benzoic acid
BLM	Bilayer lipid membrane (Black lipid membrane)
BSSE	Basis set superposition error

C

c	Concentration
CCD	Charge coupled device
CD ₃ CN	Acetonitrile- d_3
CDCl ₃	Chloroform-D
Ce(NH ₄) ₂ (NO ₃) ₆	Ceric ammonium nitrate
CHCl ₃	Chloroform
cm	Centimeter
CMOS	Complementary metal–oxide–semiconductor
<i>m</i> CPBA	<i>m</i> -Chloroperbenzoic acid
CsCl	Cesium chloride
Cu	Copper

D

D ₂ O	Deuterium oxide
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide
DCM	Dichloromethane
DEAD	Diethyl azodicarboxylate
DFT	Density functional theory
diPhyPC	Diphytanoylphosphatidylcholine
DLS	Dynamic light scattering

DMAP	4-Dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMF	<i>N,N</i> -Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTD	DT-diaphorase
DTi	Sodium dithionite

E

EC	Enzyme commission (number)
<i>EC</i> ₅₀	Half maximal effective concentration
EDC.HCl	<i>N</i> -Ethyl- <i>N'</i> -(3-dimethylaminopropyl)carbodiimide hydrochloride
ESI	Electron spray ionization
Et ₃ N	Triethylamine
EtOAc	Ethyl acetate
EtOH	Ethanol
EYPC	Egg yolk phosphatidylcholine

F

FAD	Flavin adenine dinucleotide
FBS	Fetal bovine serum
FCCP	Carbonyl cyanide- <i>p</i> -trifluoromethoxyphenylhydrazine

FESEM	Field emission SEM
FT-IR	Fourier transform infra-red spectroscopy

G

g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

H

H	Hour
H ₂ O	Water
H ₂ O ₂	Hydrogen peroxide
H ₂ SO ₄	Sulphuric acid
HCl	Hydrochloric acid
HEK293	Human embryonic kidney 293 (cell line)
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HF	Hartree-Fock
HOBt	Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HPTS	8-Hydroxypyrene-1,3,6-trisulfonic acid trisodium salt
HRMS	High resolution mass spectroscopy
Hz	Hertz

I

I_F	Normalized fluorescence intensity
IC_{50}	Half-maximal inhibitory concentration
ISE	Ion selective electrode
IR	Infra-red (spectroscopy)
IUPAC	International Union of Pure and Applied Chemistry

J

J	Joule
J	Coupling constant (NMR)

K

K	Kelvin
K_a	Association constant
kcal	Kilocalorie
KCl	Potassium chloride
kD	Kilodalton
KNO_3	Potassium nitrate
KOH	Potassium hydroxide

L

L-15	Leibovitz's L-15 medium
------	-------------------------

LED	Light emitting diode
LiCl	Lithium chloride
log <i>P</i>	Partition coefficient
LUVs	Large unilamellar vesicles

M

M	Molar (molarity)
MCF-7	Michigan Cancer Foundation-7 (cell line)
MD	Molecular dynamics
Me	Methyl
MEF	Mouse embryonic fibroblast (cell line)
MeOH	Methanol
mg	Milligram
MgCl ₂	Magnesium chloride
MHz	Megahertz
mL	Milliliter
mM	Millimolar
mmol	Millimole
MMP	Mitochondrial membrane potential
Mo	Molybdenum
Mol	Mole
Mon	Monensin

MP	Melting point
MsOH	Methanesulfonic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	Molecular weight
<i>m/z</i>	Mass to charge ratio
N	
N	Normal (normality)
<i>n</i>	Hill coefficient
N ₂	Nitrogen gas
Na ₂ CO ₃	Sodium carbonate
Na ₂ S ₂ O ₄	Sodium dithionite
Na ₂ SO ₄	Sodium sulphate
NaBH ₄	Sodium borohydride
NaBr	Sodium bromide
NAC	<i>N</i> -acetylcysteine
NaCl	Sodium chloride
NaClO ₄	Sodium perchlorate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO ₃	Sodium bicarbonate
NaNO ₃	Sodium nitrate

NaOH	Sodium hydroxide
NaI	Sodium iodide
NBS	<i>N</i> -Bromosuccinimide
nM	Nanomolar
nm	Nanometer
NMP	<i>N</i> -Methyl-2-pyrrolidone
NMR	Nuclear magnetic resonance (spectroscopy)
NQO1	NAD(P)H:Quinone acceptor oxidoreductase 1

O

ORTEP	Oak Ridge Thermal-Ellipsoid Plot Program
-------	--

P

PARP1	Poly(ADP-ribose) polymerase-1
PAGE	Polyacrylamide gel electrophoresis
PB	Phosphate buffer
PBS	Phosphate buffered saline
PCl ₃	Phosphorus trichloride
PET ether	Petroleum ether
pH	Potential of hydrogen
PI	Propidium iodide
PPh ₃	Triphenylphosphine

POCl ₃	Phosphoryl chloride (phosphorus oxychloride)
-------------------	--

ppm	Parts per million
-----	-------------------

PVDF	Polyvinylidene difluoride
------	---------------------------

Q

QM	Quinone methide
----	-----------------

R

RbCl	Rubidium chloride
------	-------------------

rcf	Relative centrifugal force
-----	----------------------------

RNA	Ribonucleic acid
-----	------------------

ROS	Reactive oxygen species
-----	-------------------------

rpm	Revolutions per minute
-----	------------------------

RT	Retention time
----	----------------

rt	room temperature
----	------------------

S

s	Second(s)
---	-----------

SCXRD	Single crystal X-ray diffraction
-------	----------------------------------

SDS	Sodium dodecyl sulphate
-----	-------------------------

SEM	Scanning electron microscope
-----	------------------------------

T

<i>t</i>	Time
TBA	Tetrabutyl ammonium
TBABr	Tetrabutyl ammonium bromide
TBACl	Tetrabutyl ammonium chloride
TBAI	Tetrabutyl ammonium iodide
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween® 20
TEM	Transmission electron microscope
THF	Tetrahydrofuran
TLC	Thin layer chromatography

U

UV	Ultraviolet
----	-------------

V

Val	Valinomycin
VMD	Visual Molecular Dynamics

Synopsis

The aim of this thesis is the advancement of transmembrane ion transport systems as prospective therapeutic agents for the treatment of cancer and bacterial infections by overcoming their limitation of non-specific activity. Different methods for selective activation of ion transport by formulation of the transporters into prodrug form or 'protransporters' are explored. This thesis deals with transporter and protransporter systems, starting from their synthesis and assessment of ion transport properties to in vitro studies in cancer cells. Protransporters were demonstrated to be successfully activated through enzyme action and light as stimuli. They were found to induce apoptotic cell death in cancer cells and inhibit growth in Gram-negative bacteria. This would be a significant step forward in the implementation of this novel therapeutic approach for various biomedical applications.

Chapter 1: Introduction

The outer envelopes of cells and subcellular compartments like organelles are comprised of lipid bilayers. The interior of these bilayer membranes comprises non-polar lipid tails facing each other, forming a hydrophobic layer. This core acts as a barrier to free solute movement, allowing for control of molecular exchanges with the environment, which enables diverse cellular functions. The movement of these solutes, including ions, across the membrane is controlled by a host of membrane proteins (carriers, channels, pores), which maintain tightly regulated solute gradients across the membranes in what is known as solute homeostasis.

Ion homeostasis in living systems is vital for a variety of essential processes, and homeostasis of chloride, which is the most abundant anion in mammal cells, is of particular importance as its regulation plays a crucial role in a variety of biological processes, including salt transport, cell volume regulation, cell cycle and apoptosis, and maintenance of potential gradients. The perturbation/disruption of this homeostasis is detrimental to the cell and can lead to cell death. Controlled, intentional disruption of ion homeostasis could hence find therapeutic use as it can be utilized for the destruction of diseased tissue like cancers or pathogens like bacteria. Artificial ion transporters are a class of small molecules capable of dissipating transmembrane ion gradients that have therapeutic potential as anticancer and antimicrobial agents in this regard. The non-target

mode of action of this approach can overcome the problem of cancer cell/bacterial resistance development that limits traditional treatment methods.

Various synthetic anion transporters have been proven to be capable of disruption of ion homeostasis in cancer cells and inducing apoptotic or programmed cell death. Despite the attractiveness of these systems, their utility as a viable therapeutic strategy is limited by their undesirable toxicity towards healthy non-malignant cells. To ensure selectivity of action, ion transporters need to be prepared either as ‘protransporters’, i.e., ion transporters formulated in a ‘prodrug’ form that gets activated in the target tissue, or through careful design of the active transporter such that it exhibits ion transport induced cytotoxicity only towards the target cell type. Protransporters are prepared by conjugation of the active transporter to a labile group that blocks its ion transport ability, which can then regain function in the presence of an internal or external stimulus, thereby enabling a high degree of spatiotemporal control to be achieved in targeted drug delivery. Both extrinsic and intrinsic stimuli, such as pH variations, enzyme action, light (photoisomerization and photocleavage), and differential expression of cellular metabolites, can be used to trigger targeted action from protransporters.

Chapter 2: NAD(P)H:Quinone Acceptor Oxidoreductase 1 (NQO1) Activable Salicylamide H^+/Cl^- Transporters

Cancer cells develop various pathways and overexpress many proteins that ensure their survival. These proteins can be used as targets for prodrug activation as they would ensure a high degree of selectivity of action towards cancerous cells over healthy cells. NAD(P)H:quinone acceptor oxidoreductase 1 (NQO1) is cell-protecting enzyme that is overexpressed 5-200 times in tumors that can catalyze the two electron reduction of quinones to hydroquinones. NQO1 has been considered an attractive candidate for prodrug activation and has been successfully applied in the case of DNA alkylating drugs such as Mitomycin C.

In this chapter, NQO1 activable protransporter systems were prepared by conjugating labile quinones to salicylamide-based ion transporters. Salicylamides are easily synthesizable small molecules that were hypothesized to be capable of transmembrane H^+/Cl^- symport. The ion transport activity of salicylamides can be caged by blocking the OH group with NQO1 activatable quinones via either ether or ester linkage. The ion-binding ability of salicylamides was proven through NMR-based titration assays and transport activity verified in liposomes. Various

liposomal studies were conducted to verify H^+/Cl^- symport mechanism of transport. The quinone-caged protransporters were found to have a minimal ion-binding ability, and the complete inhibition of transport activity was verified. The successful release of active transporters following both chemical and enzymatic (NQO1) reduction of quinone-caged ‘protransporters’ was verified through UV-Vis, NMR, HRMS, and HPLC analyses.

Both the transporters and protransporters were shown to exhibit significant toxicity towards the MCF-7 breast cancer line over the non-cancerous MEF cell line. Cytotoxicity was found to be mediated via ion homeostasis disruption triggered induction of oxidative stress, mitochondrial membrane depolarization, and lysosomal deacidification. Induction of cell death via intrinsic apoptotic pathway was also verified by monitoring PARP1 cleavage.

Chapter 3: Salicylanilide H^+/Cl^- Transporters for the Inhibition of Gram-negative Bacterial Proliferation with Application in Photorelease Antimicrobial Therapy

The advent of antibiotic resistance is a major global health crisis that is depleting the available antibiotic pool. The development of new antibiotics effective against Gram-negative bacteria is exceedingly difficult due to the protection offered by the bacterial outer membrane (OM). Treatment methods that disrupt this membrane are anticipated to have better chances of combating bacterial resistance.

In this chapter, salicylanilide H^+/Cl^- symporters were proposed as a system capable of destabilizing the Gram-negative OM, leading to inhibition of bacterial growth. A library of 14 monosubstituted salicylanilides was prepared, and transport properties were verified. Screening in the Gram-negative bacterium *Caulobacter crescentus* yielded five effective compounds from which structure-activity relationships were determined. Blocking of the salicylanilide OH via methylation led to the loss of both ion transport and antibacterial activities, confirming the role of ion transport in antibacterial action. The screened compounds were also found to be effective towards the free-living pathogenic Gram-negative strains: *Escherichia coli*, *Salmonella enterica* (serovar Typhimurium), and *Klebsiella pneumoniae*. Cell surface imaging of *E. coli* subjected to osmotic shock following salicylanilide treatment showed extensive cell damage, indicating transporter-facilitated H^+/Cl^- transport across the bacterial OM degrades its cell-protecting ability.

The scope of this system was extended by synthesizing a ‘protransporter’ by conjugating the salicylanilide to a photocleavable protecting group (PPG) that releases the active transporter on

red light activation. Recovery of ion transport and antimicrobial activities on irradiation under 655 nm light was verified. This system has the potential to be used for topical applications, where the acquisition of drug resistance due to unregulated use is a rampant problem, and the development of novel antimicrobial treatments has been largely overlooked.

Chapter 4: Photoactivable Bissalicylamide Transporters for Anticancer Applications

Light is attractive for prodrug activation due to its benign nature and the high degree of spatiotemporal control achievable during use. The use of light has been successfully demonstrated for protransporter activation in cancer cells, but these systems utilize UV light which is toxic to healthy tissue. Hence, for the practical anticancer application of light-activated protransporters, a shift to the visible region of the electromagnetic spectrum is necessary.

In this chapter, protransporters were prepared by conjugating *o*-nitrobenzyl photocleavable groups to an extended salicylamide/bissalicylamide ion transporter. Assessment of ion transport properties of the bissalicylamide system was performed across liposomes, following which their cytotoxicity was verified in the MCF-7 breast cancer cell line. Liposomal assays established the mechanism of ion transport to be anion antiport. Protransporters were prepared by doubly conjugating *o*-nitrobenzyl moieties to the free OH groups of the transporter. Both unsubstituted and dimethoxy-substituted *o*-nitrobenzyl groups were used. The caging of ion transport activity was verified in liposomes. The release of free transporter on irradiation of the protransporter with 405 nm light was verified through NMR analysis, and the subsequent recovery of ion transport activity was confirmed in liposomes. The protransporters by themselves were found to be non-toxic towards MCF-7 cells but, on activation under 405 nm light, were found to exhibit excellent cytotoxicity.

Chapter 5: Pyrrole-2-carboxamide System as a Step Toward Tunable, Targeted Ion Transport

The salicylamide and bissalicylamide systems though efficient ion transporters, are limited by the degree of structural tunability achievable for tweaking their ion transport activities. An analysis of new prospective designs, keeping the simplicity of the molecule as a core feature, yielded the pyrrole-2-carboxamide system, which has seen extensive application in anion receptor and sensor chemistry and is a component of many natural products. A large variety of substituted pyrrole-2-carboxamides are synthetically accessible, allowing for easy tuning of the transporter properties.

Despite its versatility, the pyrrole-2-carboxamide system has been little explored as a transmembrane ion transporter.

In this chapter, a series of eight pyrrole-2-carboxamides (pyrroleamides) were studied with four variations of the amide substituent and two variations on the pyrrole ring. Theoretical calculations yielded a wide range of $\log P$ and N-H pK_a values for the derivatives, indicating tunability of lipophilicity and ion binding ability are possible through variation of substitution on the pyrroleamide core. Dose-dependent studies of transport activity in liposomes yielded a wide range of ion transport activities and Hill coefficients ($n = 2-4$), indicating variation in the nature of ion transport complex with substitution. Mechanistic studies confirmed anion antiport as the dominant mechanism of ion transport. The ion binding ability of the system was verified through NMR titration experiments and anion co-crystallization. The structures of transmembrane ion transport complexes of derivatives with $n = 2$ and $n = 4$ were successfully predicted through theoretical calculations. The pyrrole-2-carboxamide system can be carried forward for testing in cancer cells and can be extended as a protransporter by attachment of a labile moiety to either the pyrrole or amide N-H groups.

Chapter 1

Introduction

Cells form the basic functional unit of life. Lipid membranes are an integral part of cells, forming both the outer envelope (cell membrane) and, in the case of eukaryotic cells, further compartmentalizing it into organelles. Cell membranes are protein-interspersed lipid bilayers where the polar lipid head groups are exposed to the intra/extracellular aqueous medium while the non-polar tails face each other to form a hydrophobic layer in between.^{1, 2} This hydrophobic core forms a barrier to free solute movement, which can only be permeabilized by small non-polar solutes (Figure 1). Polar molecules and ions are blocked by the membrane due to the high energy necessary for the movement of these solutes across through the hydrophobic medium. This allows cell membranes to function effectively as the outer border of the cell by controlling molecular exchanges with the environment and hence perform diverse functions such as signal transduction, osmotic control, and nutrient concentration in the cell.² A host of membrane proteins, namely channels, carriers, and pores, facilitate transmembrane solute movement by offering a shielded polar path for solute molecules that lowers the energy barrier posed by the interior hydrophobic environment of the lipid membrane. This lipid-protein “mosaic” allows for the maintenance of solute asymmetry across cell membranes and organelles.^{3, 4}

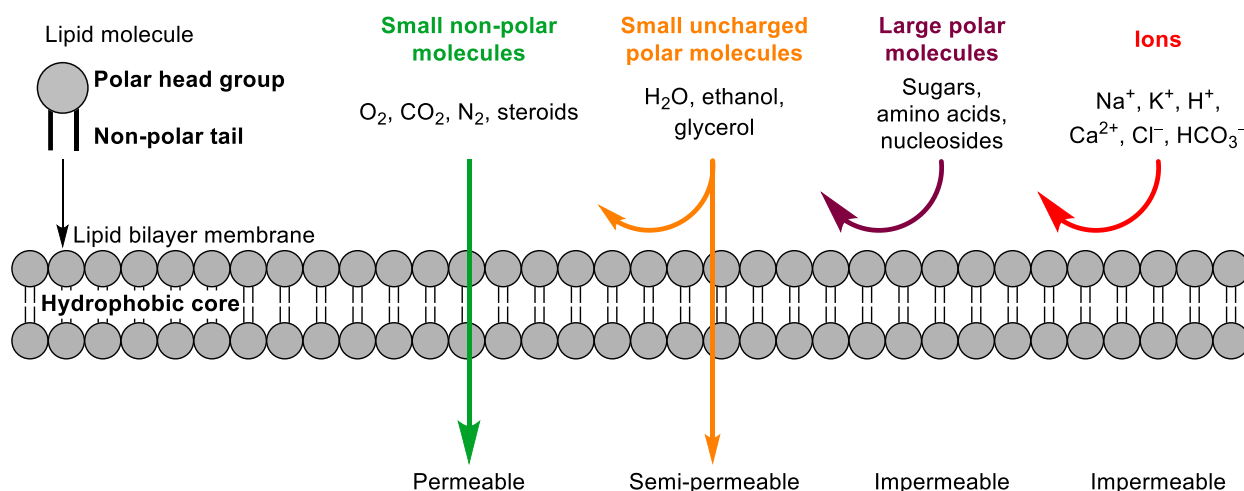


Figure 1: Schematic of lipid and lipid membrane structure and the relative membrane permeabilities of various solute species.

Transmembrane solute transport can be broadly classified into active and passive transport depending on the direction of solute movement with respect to the gradient. Active transport consists of the movement of solutes against their concentration gradient utilizing energy from ATP (e.g., sodium potassium pump, V-ATPase). Passive transport involves the movement of solute in

the direction of its concentration gradient, facilitated by either channels (e.g., ClC channel, potassium channel) and pores (e.g., nuclear pore complex, α -Hemolysin) that offer a continuous path through the membrane or carriers (e.g., valinomycin, ionomycin) that bind to solutes and move them across the membrane shielding their polar nature/charge (Figure 2). Depending on the number of solute molecules involved, the mode of transport can be classified into uniport- which concerns the unidirectional movement of a single solute species, symport – where two different solute species are moved in the same direction; and antiport – where two different solutes are moved in opposite directions across the membrane (Figure 3). In the case of ion transport, two ions of opposite charges are moved in the same direction during symport, and two ions of the same charge are moved in opposite directions during antiport, hence maintaining net charge neutrality during the transport process.

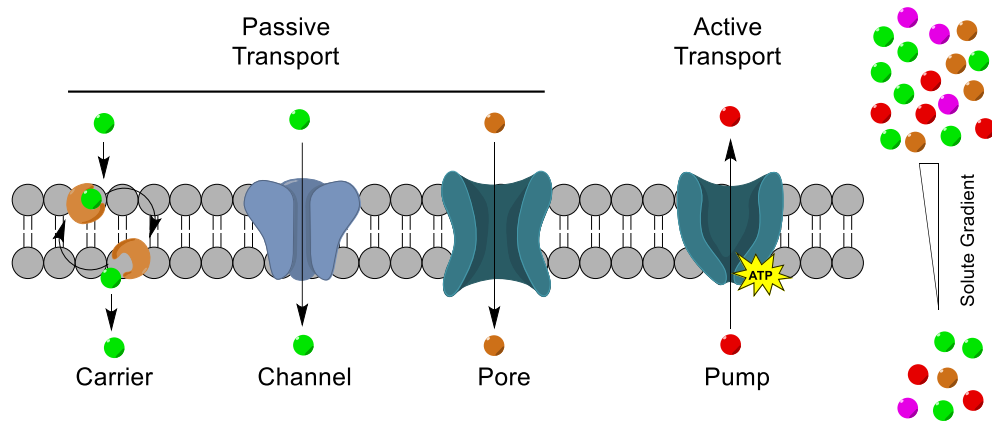


Figure 2: Schematic of types of natural transmembrane solute transport systems.

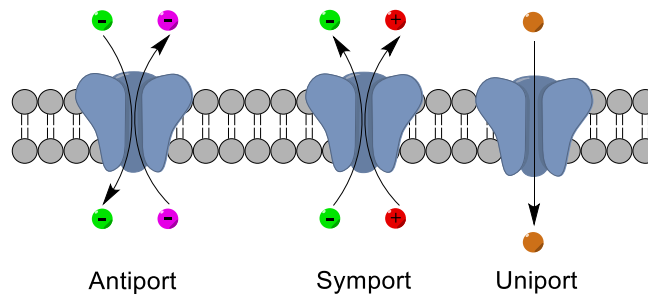


Figure 3: Schematic of modes of transmembrane solute transport.

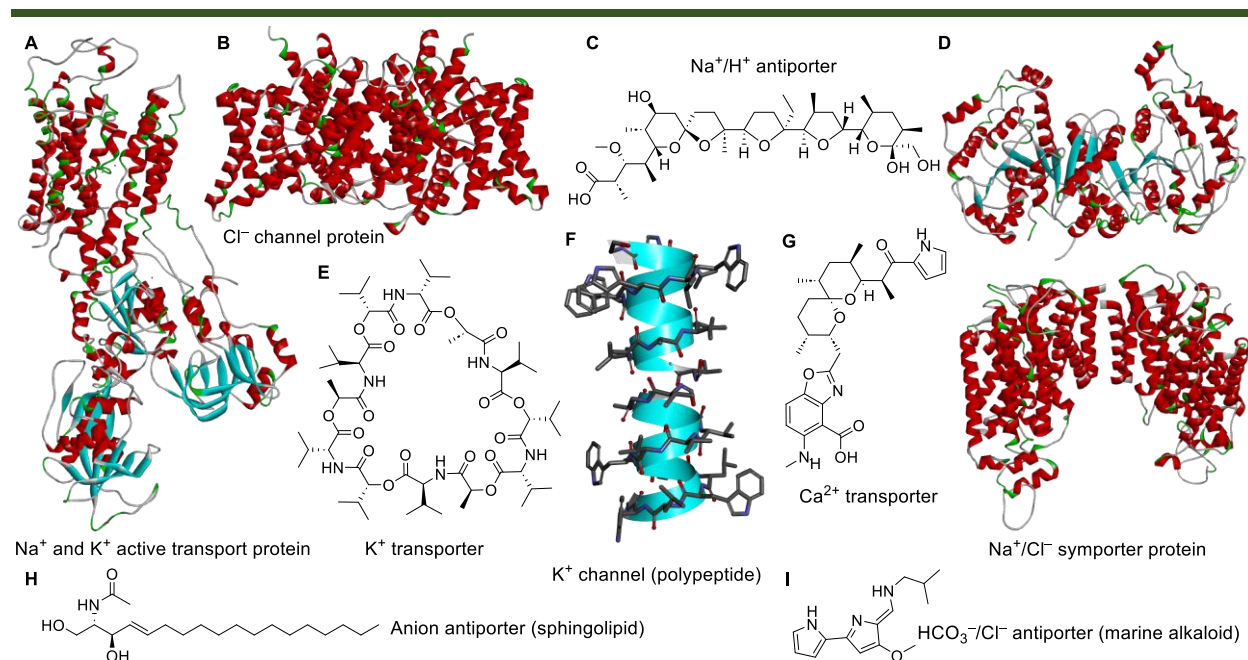


Figure 4: Examples of naturally occurring ion transport systems. (A) Na⁺/K⁺ ATPase (PDB: 3B8E), (B) ClC channel (PDB: 1OTS), (C) Monensin, (D) SLC12A3 (PDB: 7YG0), (E) Valinomycin, (F) Gramicidin A (PDB: 1GRM), (G) Calcimycin, (H) Ceramide, and (I) Tambjamine C.

The tight regulation of ion gradients across the cell, also known as ion homeostasis, is vital for a variety of essential life processes.⁴⁻⁷ Chloride being the most abundant anion in animal cells,⁸ is of particular importance, with its selective transport playing a key role in a variety of biological processes, including transepithelial salt transport, acidification of internal and extracellular compartments, cell volume regulation, cell cycle and apoptosis,^{7,9,10} and for maintaining potential gradient across the cell membrane.^{11,12} Malfunction of natural anion transport systems can lead to various maladies like cystic fibrosis, Bartter syndrome, Gitelman syndrome, Dent's diseases, renal tubular acidosis, deafness, etc.¹³⁻¹⁵

Being a vital component of cell function, the perturbation/disruption of the ion homeostasis is detrimental to the cell. It is known to correlate closely with the onset of programmed cell death or apoptosis in mammal cells.¹⁶ The intentional disruption of this homeostasis could find therapeutic use as it can be utilized for the destruction of diseased tissue like cancers or pathogens like bacteria. Both cancer cells^{17,18} and bacteria develop resistance to traditional treatments via the acquisition of various new pathways through mutations that render the treatment ineffective, leading to disease

relapse. In contrast, disruption of the ion homeostasis is a non-target approach against which can overcome these limitations, and molecules capable of this mode of action can find utility as novel anticancer and antimicrobial agents. Small molecule ion transporters can dissipate ion gradients across lipid membranes through transmembrane ion transport and hence have therapeutic potential in this regard (Figure 5). Various naturally occurring ion transporters are known for their biological activity, which is associated with their ion transport ability. The bacterial metabolite (isolated from *Serratia marcescens*) prodigiosin¹⁹ is capable of both H^+/Cl^- symport and $\text{HCO}_3^-/\text{Cl}^-$ antiport²⁰⁻²² and exhibits anticancer,²³ antibiotic,²⁴ antimalarial²⁵ and antifungal^{25, 26} properties. Marine alkaloids of the tambjamine family, analogues of prodigiosin, are anion antiporters with similar biological activities.²⁷ Cation transporters such as valinomycin (K^+ transporter),²⁸ monensin (Na^+/H^+ antiporter),²⁹ and nigericin (K^+/H^+ antiporter)³⁰ have antibiotic properties.



Figure 5: Therapeutic application of ion homeostasis disruption-induced cell death.

Small molecule ion transporters typically comprise of – (i) a polar pocket capable of binding to ions, and (ii) a non-polar outer shell that shields ionic charge from the hydrophobic core of the lipid membrane. These make the transport process energetically feasible. Inspired by natural anion transporters like prodigiosin, tambjamines, and ceramide, various ion transporters have been designed that incorporate suitably arranged polar NH (as part of amide, pyrrole, and indole moieties) and OH groups as part of the anion binding site, that are capable of binding to anions (A^-) via $\text{N-H}\cdots\text{A}^-$ and $\text{O-H}\cdots\text{A}^-$ hydrogen bonds. Hydrophobic groups, including aromatic rings and alkyl chains form the non-polar outer shell that shields the negative charge on the ion (Figure 6A).

These synthetic ion transport systems are receiving significant attention as possible anticancer agents due to their ability to perturb ion homeostasis across the lipid membrane.^{31, 32} Cancer cells develop resistance to cell death via disruption of the apoptotic process,^{17, 18, 33} which is necessary for maintaining healthy tissue homeostasis.³⁴⁻³⁶ The disruption of ion homeostasis in cancer cells

by artificial ion transport systems leads to loss of mitochondrial membrane potential, which in turn leads to cytochrome *c* release, inducing the caspase-dependent intrinsic pathway of apoptosis.³⁷⁻³⁹ Various synthetic anion transporters are known to have anticancer activity, including calix[4]pyrrole derivatives, urea/thioureas, bis(sulphonamides), and squaramides (Figure 6).³⁷⁻⁴⁵

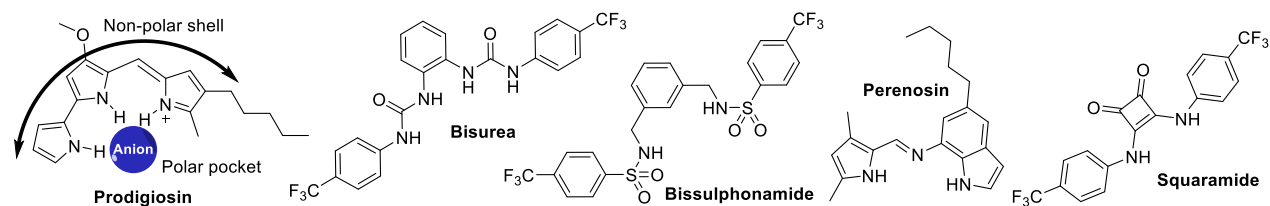


Figure 6: Basic design principle of anion transporters with prodigiosin as a reference and examples of transporter classes shown to have anticancer activity.

Despite their attractiveness, utilization of ion transporters as a viable therapeutic strategy is limited by their undesirable toxicity towards healthy non-malignant cells,^{37-39, 43, 45} a drawback they share with traditional broad-spectrum drugs.^{46, 47} To ensure their selectivity of action, ion transporters need to be prepared either as ‘protransporters’, i.e., ion transporters formulated in a ‘prodrug’ form that gets activated in the target tissue, or through careful design of the active transporter such that it exhibits ion transport induced cytotoxicity only towards the target cell type (Figure 7). Protransporters are prepared by conjugation of the active transporter to a labile group that blocks its ion transport ability, which can then regain function in the presence of an internal or external stimulus, thereby enabling a high degree of control to be achieved in targeted drug delivery.

Light is the preferred form of external stimulus due to its ease of use and the high degree of spatiotemporal control achievable.⁴⁸⁻⁵⁰ Light-activated ion transporters use one of two strategies, either photoisomerization⁵¹⁻⁵⁴ or photocleavage.^{55, 56} In the photoisomerization strategy, the ion transporter incorporates a photoswitchable group like azobenzene,⁵⁷ and the molecule can be switched between two conformers- the ‘ON’ state, which is capable of transmembrane ion transport and the ‘OFF’ state where the ion binding site is disrupted, leading to the loss of transport activity. The switching between these states can be controlled using light of different wavelengths. The photocleavage strategy involves blocking one or more of the H-bond donor groups in the ion binding cavity of the transporter with photocleavable groups (e.g., *o*-nitrobenzyl group),⁴⁸ thus ‘caging’ the ion transport activity to form the protransporter. These moieties undergo photolysis on irradiation with light in a specific wavelength range to release the transporter and regain activity.

Most photoactivable ion transport systems that have been reported utilize light in the ultraviolet (UV) region of the electromagnetic spectrum,^{52, 55, 58} which is both harmful for healthy cells and limited by its low tissue penetration ability.^{59, 60} There is a need for a shift in protransporter design to utilize light in the visible region, preferably the far red-infrared region of the electromagnetic spectrum, for this strategy to be useful for therapeutic applications.

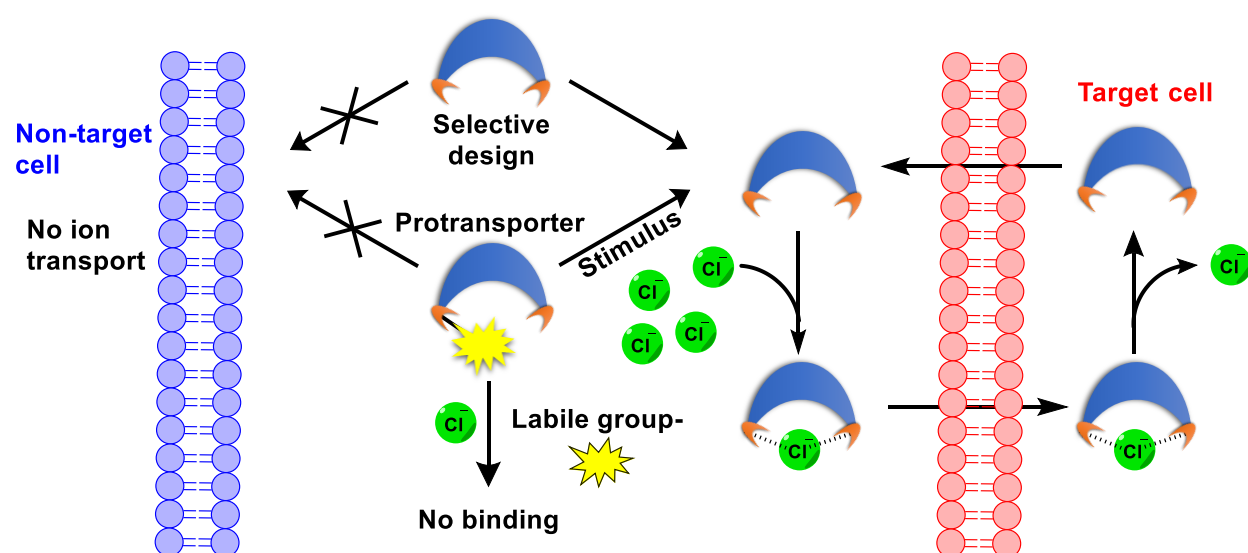


Figure 7: Selective anion transport in target cells, using protransporters or through selective transporter design.

Intrinsic stimuli are factors specific to the target cell or tissue microenvironment that distinguish them from the healthy tissue, which can be utilized for localized protransporter activation. These include overexpressed species, such as certain enzymes and metabolites,^{33, 61, 62} and conditions pertaining to the target cell microenvironment, such as pH.⁶³ The design of protransporters activatable by enzyme action,⁶⁴⁻⁶⁶ and differential expression of cellular metabolites^{67, 68} utilize an approach similar to that of the photocleavage strategy where the ion binding site is blocked with a labile group to prevent ion binding and inhibit transport activity. These labile groups undergo enzyme-catalyzed reactions/reactions with metabolites in the target cells, leading to cleavage from the binding site and the subsequent recovery of ion transport activity. Strategies using the pH of the target cell microenvironment (tumor tissue has a lower pH than healthy tissue)⁶³ as a trigger require careful transporter design such that pH-dependent protonation/deprotonation events at the transporter binding site can control the ion binding ability and, thus the ion transport.⁶⁹⁻⁷²

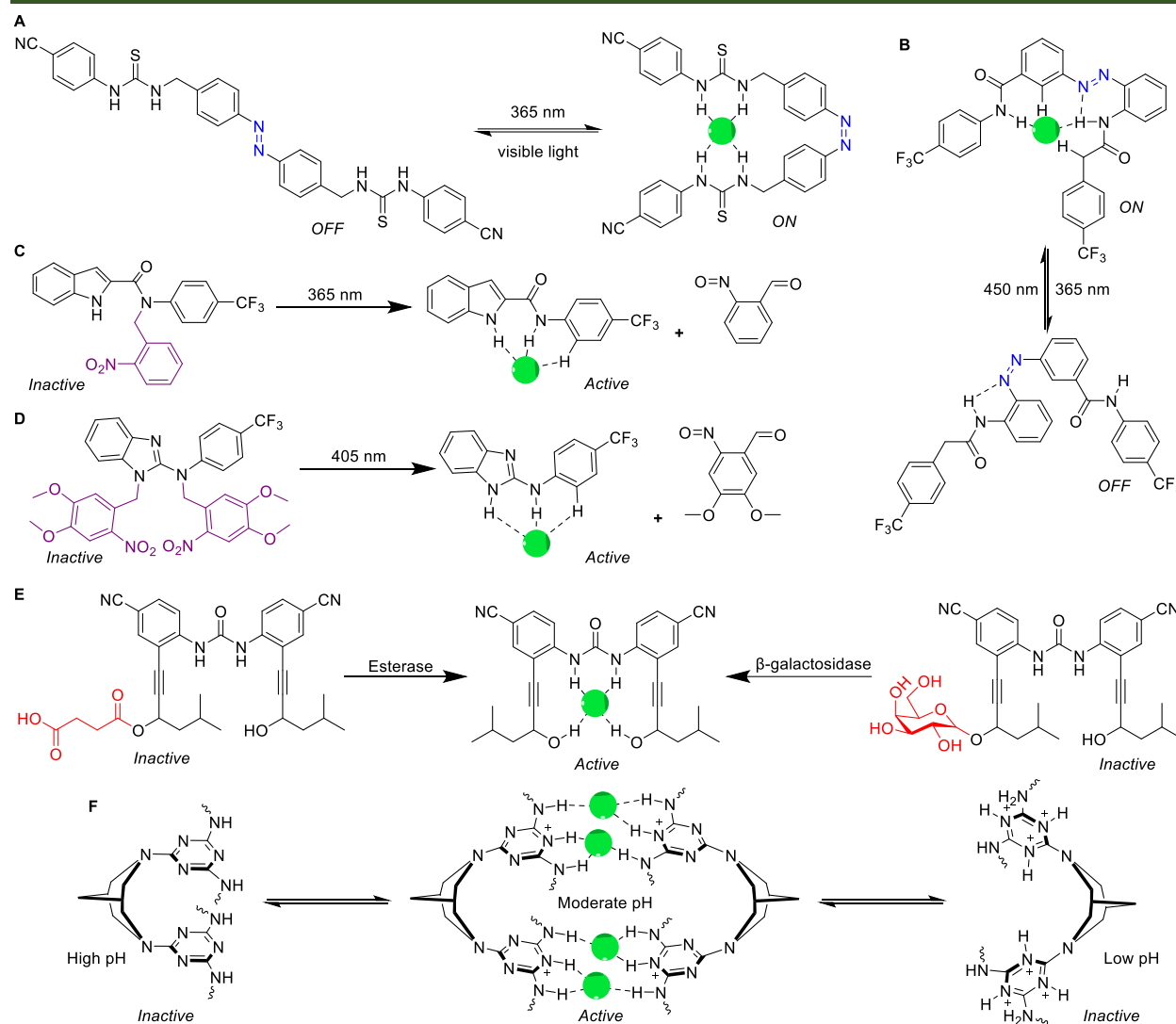


Figure 8: Examples of stimuli-responsive ion transporter systems. Photoactivable – (A), (B) photoisomerization strategy and, (C), (D) photocleavage strategy; (E) enzyme activation; and (F) pH variations.

The present work focuses on the development of methods for the implementation of selective ion transport in target cells as a step toward the implementation of ion transporter drugs for biomedical use. The simplicity of structure and ease of synthesis were among the core principles of design, as economic feasibility and scalability are major factors determining the commercial viability of a potential drug molecule. A total of three different transporter systems capable of activation under two different stimuli, one intrinsic (enzyme) and one extrinsic (light), were studied with the prospective application as both anticancer and antimicrobial agents.

Initially, the salicylamide system was identified as a potential H^+/Cl^- transporter (Chapter 2) that could be easily conjugated to a labile group to yield potential protransporters. A few derivatives were synthesized and proven to be efficient transmembrane H^+/Cl^- symporters. The highly upregulated cancer cell protecting enzyme NAD(P)H: quinone acceptor oxidoreductase 1 (NQO1), which is known to catalyze quinone reduction, was chosen as the target stimulus dictating protransporter design. Protransporters were prepared by conjugation of NQO1 activable labile quinone moieties to the salicylamide, which effectively caged their transport activity. Enzymatic activation of the systems was verified, and they were proven to be capable of selectively disrupting ion homeostasis in cancer cells and inducing cell death.

Following this system's success, the salicylanilide core's utility as an antimicrobial agent capable of outer membrane depolarization in Gram-negative bacteria was also studied (Chapter 3). Screening of an extensive salicylanilide library yielded derivatives effective against various strains of Gram-negative bacteria, including free-living pathogens associated with nosocomial infections. The most promising transporters were conjugated to the pyrrolidinobenzoquinone photocleavable group to yield a red light activable protransporter. This system exhibited antibacterial activity on photoactivation under 655 nm light, making it suitable for controlled-release applications.

Next, the anticancer effects of visible light-activated ion transport were studied using an extension of the salicylamide system, namely bisalicylamide, doubly caged with *o*-nitrobenzyl photocleavable groups. Detailed mechanistic studies of the transport process were conducted along with verification of transport activity recovery on irradiation of protransporters with 405 nm light. The protransporters were found to efficiently kill MCF-7 breast cancer cells on photoactivation.

Though efficient systems, the salicylamide and bisalicylamide transporters lack synthetic tunability, which can be used to enhance ion transport activity. Hence, the substituted pyrrole-2-carboxamide system was explored, which offered a wide range of synthetically accessible structures due to the mature nature of pyrrole chemistry, but little studied in the way of ion transport despite being an efficient anion receptor. Two series of pyrrole-2-carboxamide transporters were synthesized and ion transport properties were assessed, which showed them to be capable of anion antiport, and small structural changes yielded a wide range of transport properties. These systems have the potential to be modified as protransporters for stimuli-responsive applications.

The ion transport properties of the described molecules were assessed across artificial unilamellar liposomes prepared from egg yolk phosphatidylcholine (EYPC), with sizes in the range of 100-200 nm, hence categorized as large unilamellar vesicles (LUVs). Following the induction of a pH or ion gradient across the liposomal membrane, transporters were added (as a solution in a water-miscible solvent), which would then dissipate the gradient via a transmembrane ion transport process (Figure 9). These changes in pH/ion concentrations are detected using either liposome encapsulated reporter dyes that provide a fluorescence readout or through direct measurement of extravesicular ion concentrations using ion selective electrodes (ISE). The reporter dyes used in this work are the pH-sensitive dye HPTS and the chloride-sensitive dye lucigenin.

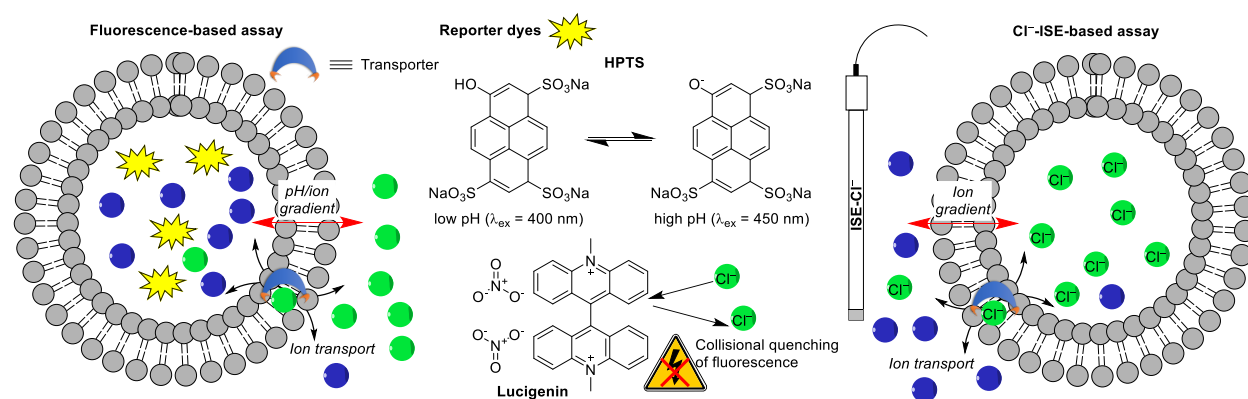


Figure 9: Schematic of liposomal assays for the study of transmembrane ion transport.

HPTS exists in two forms with different absorption maxima, a protonated (OH) form ($\lambda_{max} \sim 400$ nm) and a deprotonated form ($\lambda_{max} \sim 450$ nm), both of which have fluorescence emission maxima ~ 510 nm. The percentage of the deprotonated form increases with an increase in pH, and hence, fluorescence measurement at $\lambda_{ex}/\lambda_{em} = 450/510$ nm will indicate the change in intravesicular pH during the progress of ion transport. Lucigenin is a fluorescent dye ($\lambda_{ex}/\lambda_{em} = 455/535$ nm) that undergoes collisional quenching in the presence of Cl⁻. A quenching of the dye fluorescence is observed with increasing chloride concentration and can hence be utilized to detect transporter-facilitated Cl⁻ influx/efflux in liposomes. Lucigenin fluorescence is also quenched to varying degrees by other halides and anions like SCN⁻, which limits the range of conditions in which it can be used. In ISE-based assays, liposomes are prepared with entrapped Cl⁻ and having a non-chloride anion in the extravesicular buffer. Transporter-facilitated chloride efflux can be detected through current readings from a Cl⁻-ISE calibrated to known ion concentrations. These are mainly used for certain mechanistic studies and for activity assessment in cases where the optical

properties of the transporter interfere with the dye (HPTS/lucigenin) fluorescence, necessitating a dye-independent assay (seen in Chapters 2 and 3).

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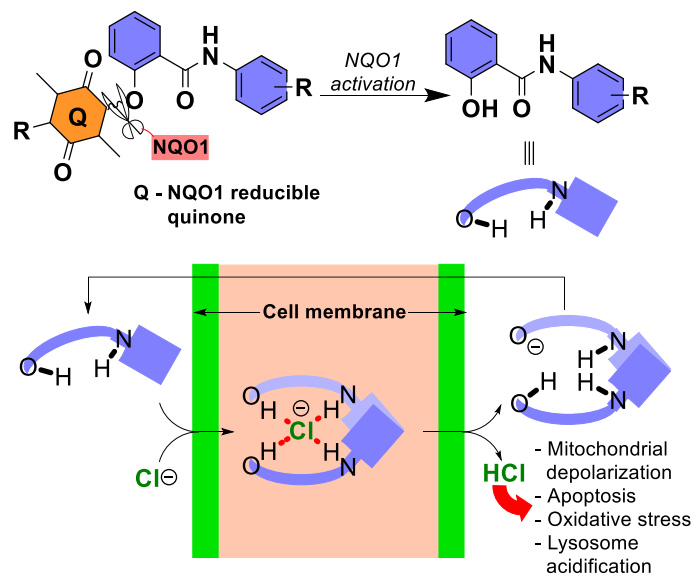
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Chapter 2

NAD(P)H:Quinone Acceptor Oxidoreductase 1 (NQO1) Activable Salicylamide H^+/Cl^- Transporters



2.1 Introduction

Cancer cells acquire various functional capabilities or hallmarks through extensive reprogramming of the cell circuits that allow for their survival and proliferation.¹⁻³ Hence, various proteins, including enzymes, necessary to support these capabilities are overexpressed/upregulated as part of the tumorigenic process. These dysregulated enzymes act as ideal targets for prodrug activation, and their heightened levels in cancer cells, relative to healthy cells, allow for very precise targeting and significantly lower non-target effects.⁴⁻⁶ Various enzymes from four IUPAC classes, namely, class 1- oxidoreductases, class 2- transferases, class 3- hydrolases, and class 4- lyases, have been found suitable for prodrug activation.⁴

One such target of interest is NAD(P)H: quinone acceptor oxidoreductase 1 or NQO1 (EC 1.6.5.2), also known as DT-diaphorase (DTD) or quinone reductase 1 (QR1), a dimeric flavin adenine dinucleotide (FAD) containing enzyme (~31 kDa per monomeric unit) (Figure 1A) found predominantly in the cytosol of mammal cells.⁷⁻⁹ It promotes the two-electron reduction of quinones to hydroquinones, utilizing NADH or NADPH as the electron donor substrate (Figure 1B).¹⁰ NQO1 acts as a Phase II detoxifying enzyme, protecting tissue against mutagens, carcinogens, and cytotoxics.^{8, 11, 12} It is also known to have an antioxidant function and to stabilize key regulatory proteins under stress.¹³ NQO1 plays a vital role in cellular defense during tumorigenesis due to its ability to detoxify a wide range of drugs¹¹ and in dealing with extreme oxidative stress conditions associated with elevated ROS levels.¹⁴ It is hence significantly overexpressed (~ 5-200 fold increase) in various solid tumor types, including colon,¹⁵ pancreatic,^{16, 17} lung,^{18, 19} liver,^{20, 21} and breast^{22, 23} cancers among others.²⁴⁻²⁶ This increased expression makes NQO1 a very attractive stimulus that can selectively activate quinone-based drug molecules in cancer tissue, resulting in higher levels of toxicity of these drugs toward cancerous tissue when compared to normal tissue due to the differential expression of the enzyme. The enzyme-catalyzed reduction of quinone-based drugs can show activity through either rearrangement of the resulting hydroquinone to produce a toxic alkylating product, like the clinical chemotherapeutic agent Mitomycin C (Figure 1C),²⁷ release of anticancer drug, or both.^{14, 28} As of the time of publication of this work, there exists no report of the use of NQO1 as a stimulus for the selective activation of protransporters.

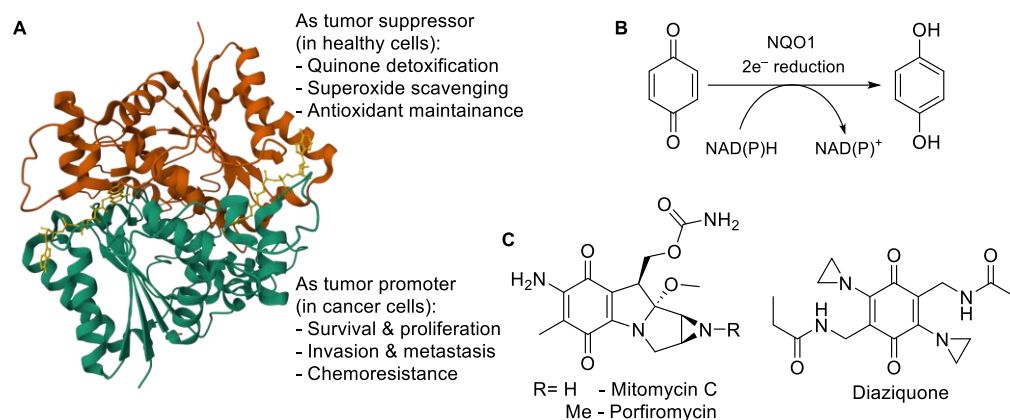


Figure 1: (A) Structure of NQO1 enzyme (PDB ID- 8C9J)²⁹ showing linked FAD groups (in yellow). Functions of the enzyme in tumor suppressor and promoter roles are listed. (B) NQO1 catalyzed reduction of quinone to hydroquinone. (C) Structures of NQO1 activatable DNA alkylating prodrugs Mitomycin C, porfiromycin, and diaziquinone.

For the creation of NQO1 enzyme activable protransporters, the salicylamide system was chosen as the transporter component whose ion transport activity would be caged by the attachment of an NQO1 reducible quinone. The salicylamide core has been proposed as a proton transporter/protonophore, but no evidence was provided in the form of ion transport experiments to support this hypothesis.³⁰ Instead, we envisaged them to be capable of H⁺/Cl⁻ symport across the lipid membrane due to the presence of both polar phenolic OH and amide NH groups capable of anion binding,³¹ and the OH group pK_a being close to physiological pH,³² facilitating protonation-deprotonation cycles (Figure 2). They are also easy-to-synthesize small molecules. The blocking of the OH group with a quinone moiety can inactivate the transporter, and the subsequent enzymatic reduction to hydroquinone triggers a rearrangement cascade that would release the free transporter (Figure 2).

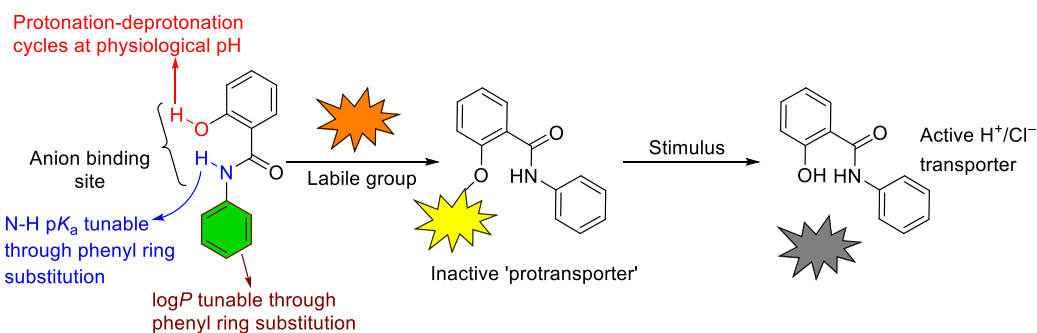


Figure 2: Description of salicylamide transporter and its transport activity caging and uncaging with stimulus-responsive labile group.

Two different quinone-based systems were chosen for study, namely a pyrrolidinobenzoquinone (**PT-1**)³³ and a trimethyl lock quinone (**PT-2**)³⁴, each with a different mode of reduction-induced release of the transporter (Figure 3). The pyrrolidinobenzoquinone system **PT-1** is capable of releasing a toxic quinone methide **QM**, an electrophilic species capable of reacting with DNA and a variety of nucleophiles,^{35, 36} along with the active transporter **T** (Figure 3, Strategy 1), hence could exhibit a synergistic effect. The trimethyl lock quinone **PT-2**, when “triggered” by NQO1, releases the active agent **T** (i.e., the ion transporter) along with an inactive metabolite **L**,³⁷ and should showcase the effect of the transporter alone in cancer tissue (Figure 3, Strategy 2). A series of six ion transporters (**1a-1f**) were designed along with one pyrrolidinobenzoquinone-based protransporter (**2**) and one trimethyl lock quinone protransporter (**3**).

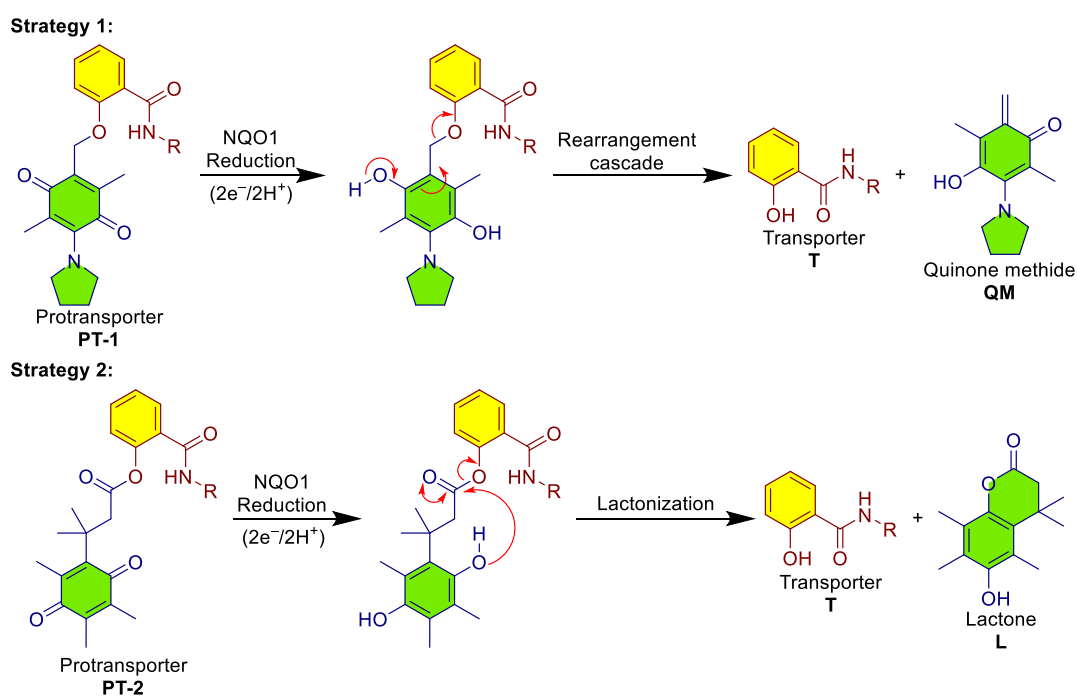


Figure 3: Structures of proposed protransporters (**PT-1**, **PT-2**) and their mechanism of NQO1 ‘triggered’ release of active ion transporter (**T**) in cancer cells.

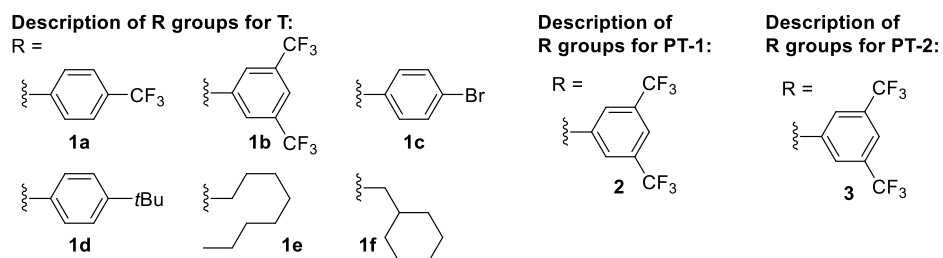
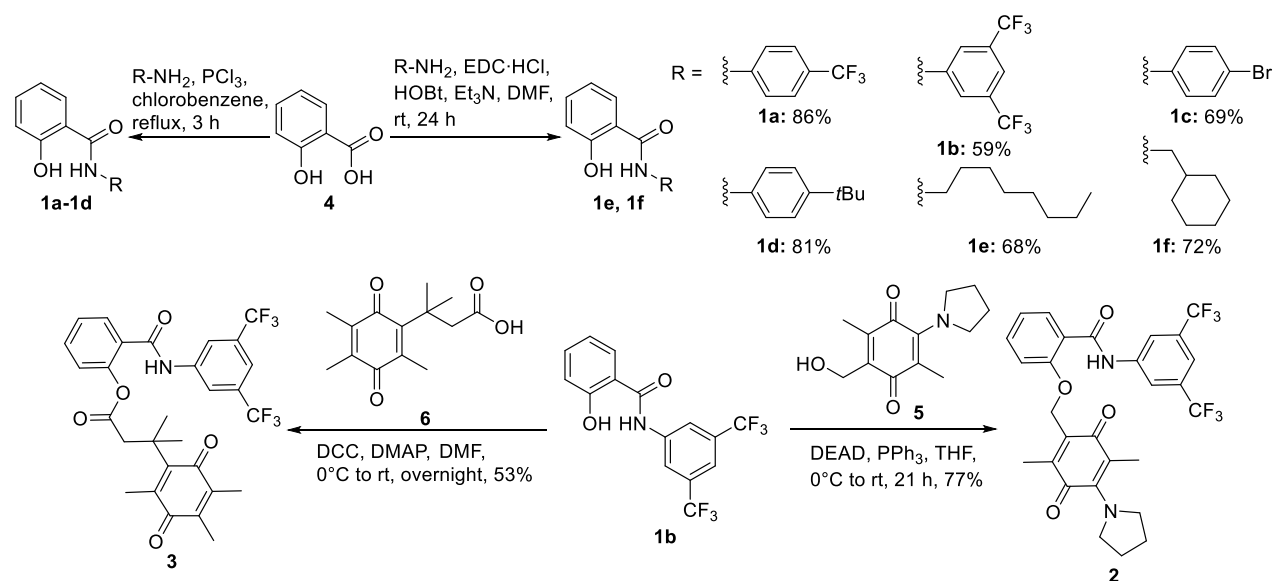


Figure 4: Structure of R groups of transporters and protransporters.

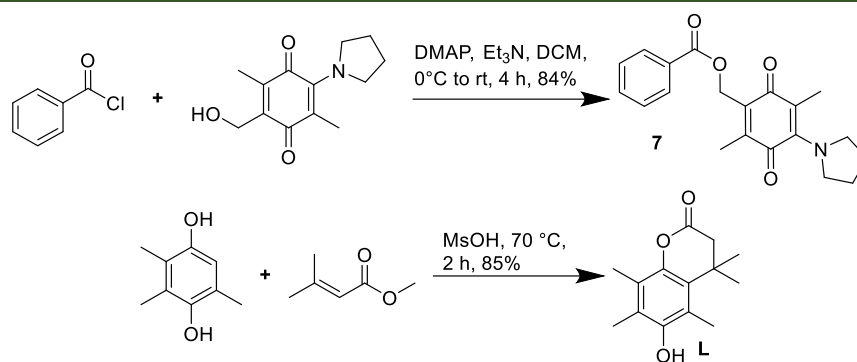
2.2 Results and Discussions

2.2.1 Synthesis

The salicylamide ion transporters (**1a-1f**) were synthesized by coupling salicylic acid (**4**) to the respective commercially available aryl/alkylamines. Synthesis of aryl-substituted salicylamides (**1a-1d**) required refluxing **4** and suitably substituted anilines with PCl₃ in chlorobenzene (Scheme 1).³⁸ The *N*-alkyl amide derivatives (**1e, 1f**) were synthesized by coupling **4** with the alkylamines **4a-4b** in the presence of EDC·HCl and HOBt (Scheme 1). Compound **1f** was prepared exclusively for the study of crystal structure.³⁹ The protransporter **2** was synthesized by blocking the OH group on transporter **1b** with the quinone **5**³³ via ether linkage under Mitsunobu conditions (Scheme 1).⁴⁰ To evaluate the effect of the quinone methide (**QM**), a reactive transient species generated after transporter release, compound **7** was synthesized by coupling non-cytotoxic benzoic acid (**BA**) to **5** (Scheme 2). The resulting rearrangement cascade after the reduction of **7** by NQO1 would release **BA** along with the quinone methide. Protransporter **3** was synthesized by coupling quinone **6**⁴¹ to **1b** via ester linkage using DCC and DMAP (Scheme 1). The lactone **L**, synthesized as the precursor for quinone **6**, was purified for use in the evaluation of cytotoxicity of the enzymatic cleavage byproducts of protransporter **3** (Scheme 2). Synthesis of a protransporter control for Strategy-2, that would release **L** (along with an inert compound) *in situ* on enzymatic reduction in the cell, was attempted by DCC-DMAP coupling of 4-methoxyphenol to quinone **6**. Due to low yields and issues with the stability of the resulting product, lactone **L** was used directly as the control.



Scheme 1: Synthesis of salicylamide ion transporters **1a-1f**, and protransporters **2** and **3**



Scheme 2: Synthesis of control compounds **7** and **L**.

2.2.2 log*P* and p*K*_a Calculations

The p*K*_a values of amide N-H protons of the *N*-salicylamides were estimated using calculator plugins of MarvinSketch program⁴² (Table 1). The p*K*_a values of O-H protons were comparable across the series and were estimated to be 7.93 for **1a-1d** and 8.19 for **1e**. The variation in p*K*_a values indicates higher acidity of the N-H proton when stronger electron withdrawing groups were used and hence could influence the ion transport rates. The log*P* values were also predicted using MarvinSketch and were found to be in the optimal range for an ion transporter (Table 1). The log*P* values for the protransporters were predicted to be 6.18 and 7.41 for **2** and **3**, respectively, indicating good membrane permeability of these compounds (Table 1).

Table 1: Estimated log*P*, and N-H, O-H p*K*_a values of compounds **1a-1e**, **2**, and **3**. Values were estimated using calculator plugins of MarvinSketch.

Compound	log <i>P</i>	N-H p <i>K</i> _a	O-H p <i>K</i> _a
1a	3.64	12.15	7.93
1b	4.52	11.02	7.93
1c	3.53	12.19	7.93
1d	4.31	12.61	7.93
1e	4.50	15.90	8.19
2	6.18	10.22	-
3	7.41	10.05	-

2.2.3 Anion Binding Studies

The chloride ion binding properties of **1a-1e** were investigated through ¹H NMR titration experiments in CD₃CN. On stepwise addition of chloride ion in the form of tetrabutylammonium chloride (TBACl) to **1a-1e** (5 mM), downfield shifts were observed for H_a, H_b, H_c, and H_d, (Figures 5-11), indicating the presence of hydrogen bonding interactions of the corresponding bonds (O-H_a, N-H_b, C-H_c, and C-H_d) with Cl⁻. The binding constants calculated from ¹H NMR titration data using BindFit program,⁴³ assuming 1:1 transporter-ion binding, were $K_a(1:1) = 164.65 \pm 1.63 \text{ M}^{-1}$, $261.80 \pm 9.01 \text{ M}^{-1}$, $167.17 \pm 1.07 \text{ M}^{-1}$, $82.08 \pm 0.41 \text{ M}^{-1}$, and $32.35 \pm 0.12 \text{ M}^{-1}$ for **1a-1e**, respectively (Figures 5-9, Table 2). The Br⁻ and I⁻ binding properties of **1b** were also analyzed through ¹H NMR titration experiment using TBABr ($K_a(1:1) = 51.77 \pm 0.71 \text{ M}^{-1}$) and TBAI ($K_a(1:1) = 8.83 \pm 0.09 \text{ M}^{-1}$), respectively (Figures 10-11, Table 2).

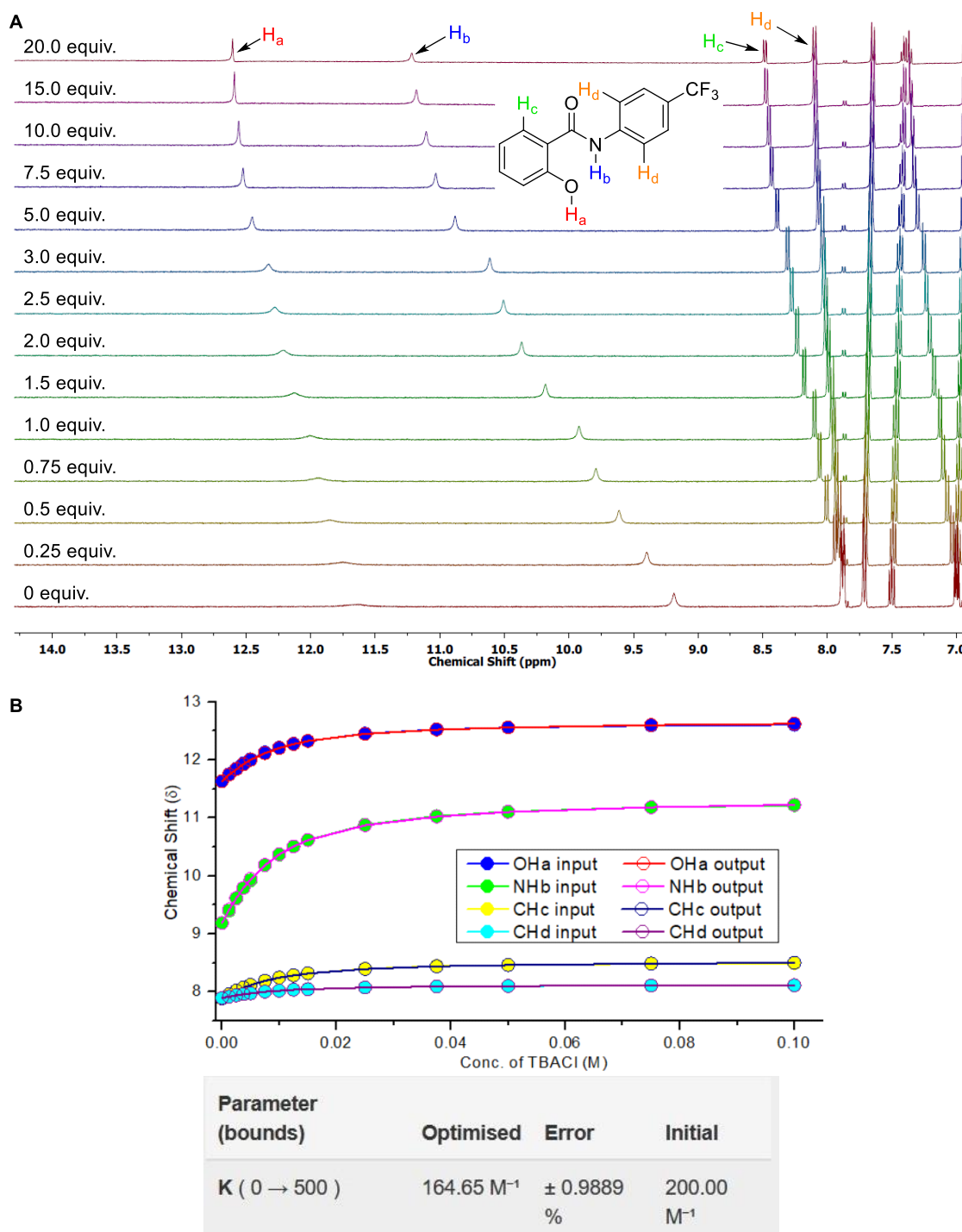


Figure 5: (A) ¹H NMR titration spectra of **1a** (5 mM) with stepwise addition of TBACl in CD₃CN. The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) The plot of chemical shifts (δ) of protons H_a, H_b, H_c, and H_d vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

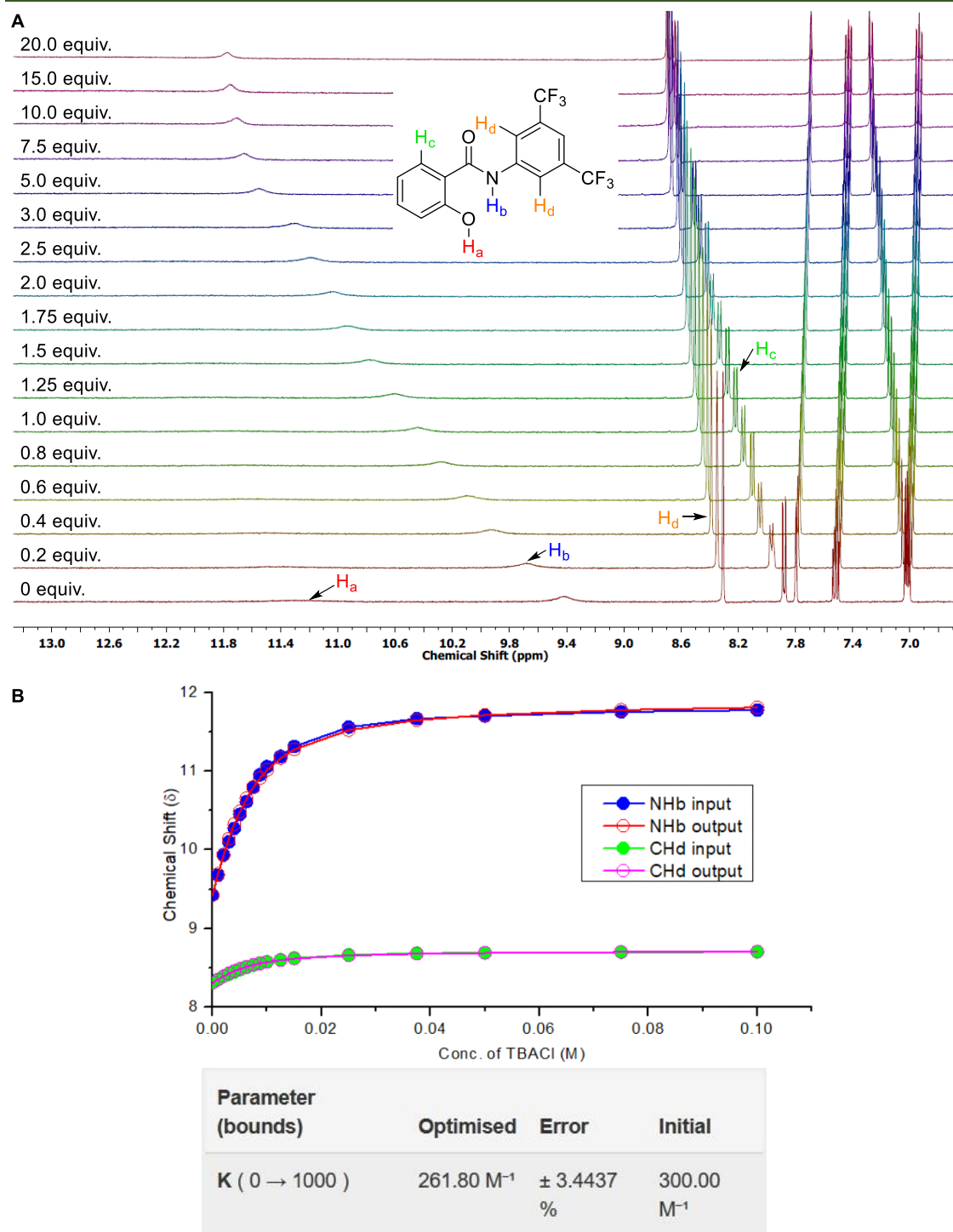


Figure 6: (A) ¹H NMR titration spectra of **1b** (5 mM) with stepwise addition of TBACl in CD₃CN. The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) Plot of chemical shifts (δ) of protons H_b and H_d vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

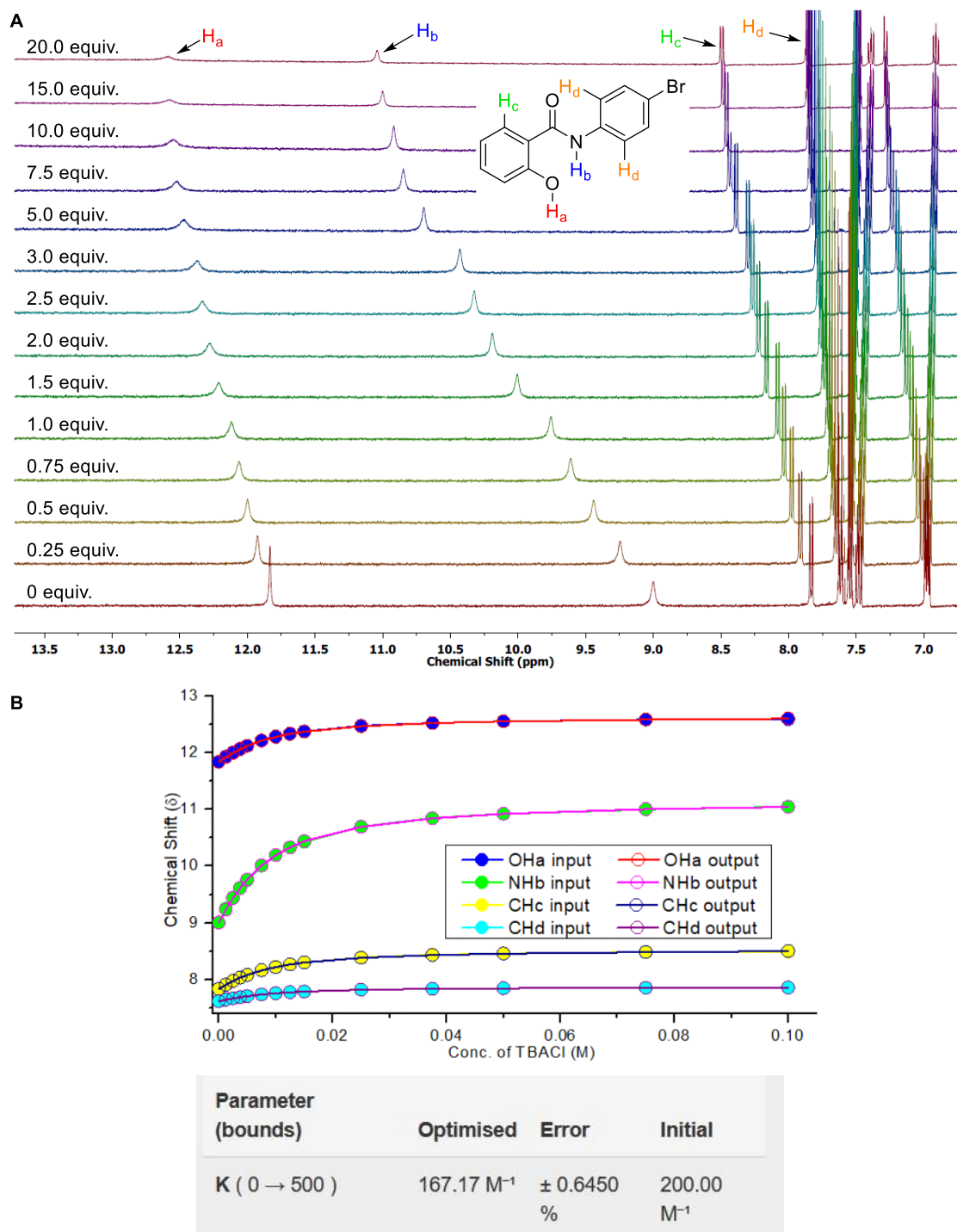


Figure 7: (A) ¹H NMR titration spectra of **1c** (5 mM) with stepwise addition of TBACl in CD₃CN. The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) Plot of chemical shifts (δ) of protons H_a, H_b, H_c, and H_d vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

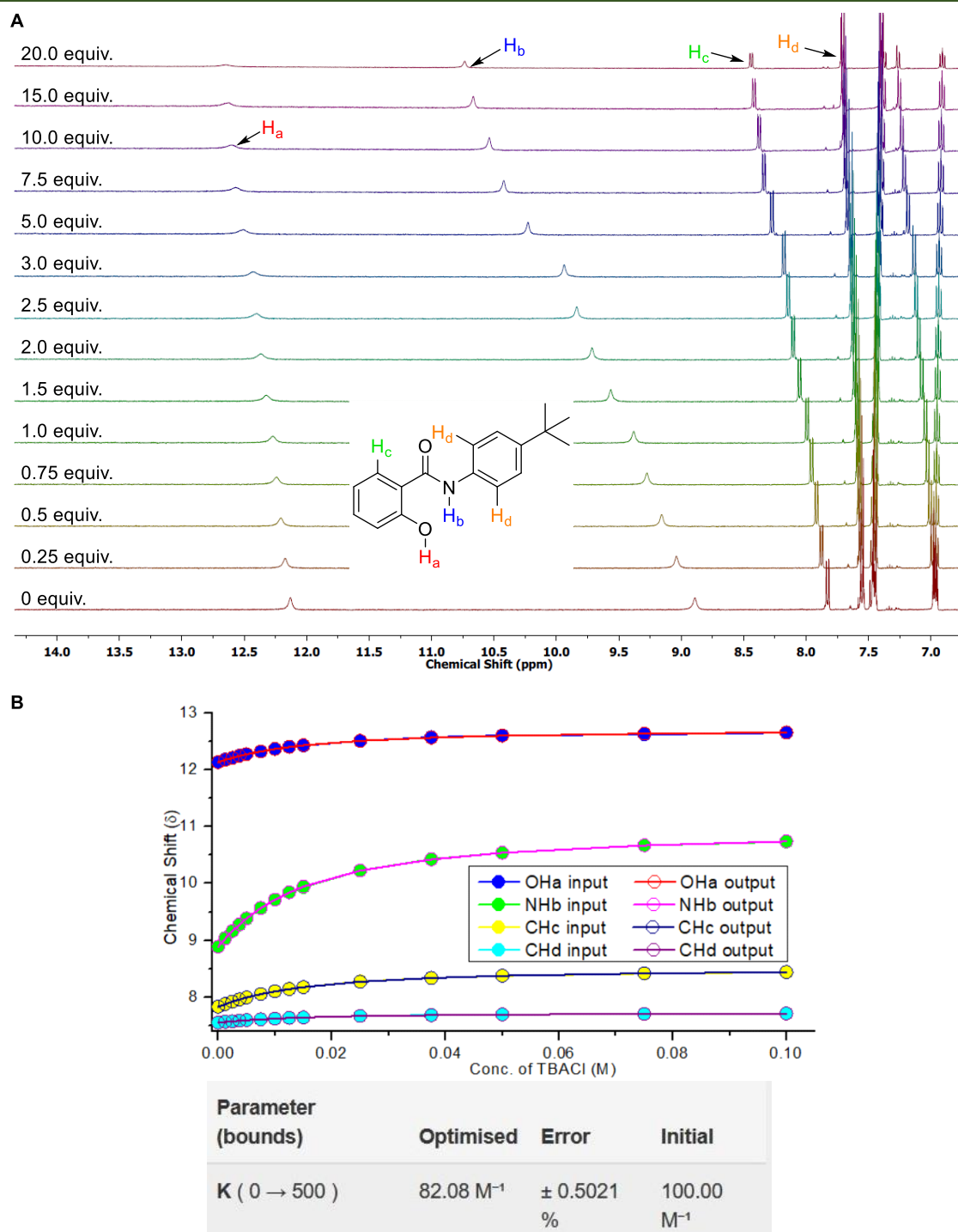


Figure 8: (A) ¹H NMR titration spectra of **1d** (5 mM) with stepwise addition of TBACl in CD₃CN. The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) Plot of chemical shifts (δ) of protons H_a, H_b, H_c, and H_d vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

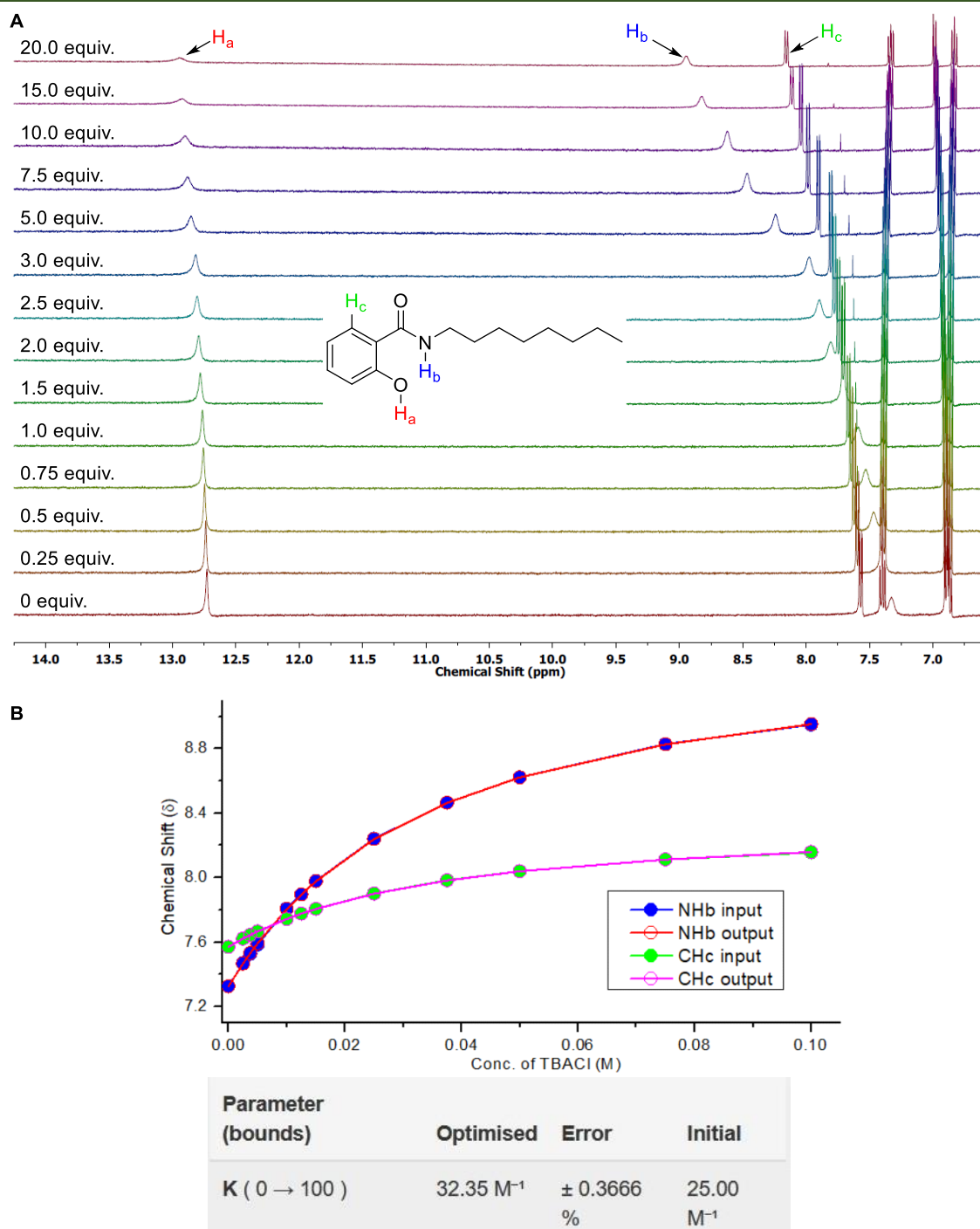


Figure 9: (A) ¹H NMR titration spectra of **1e** (5 mM) with stepwise addition of TBACl in CD₃CN. The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) Plot of chemical shifts (δ) of protons H_b and H_c vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

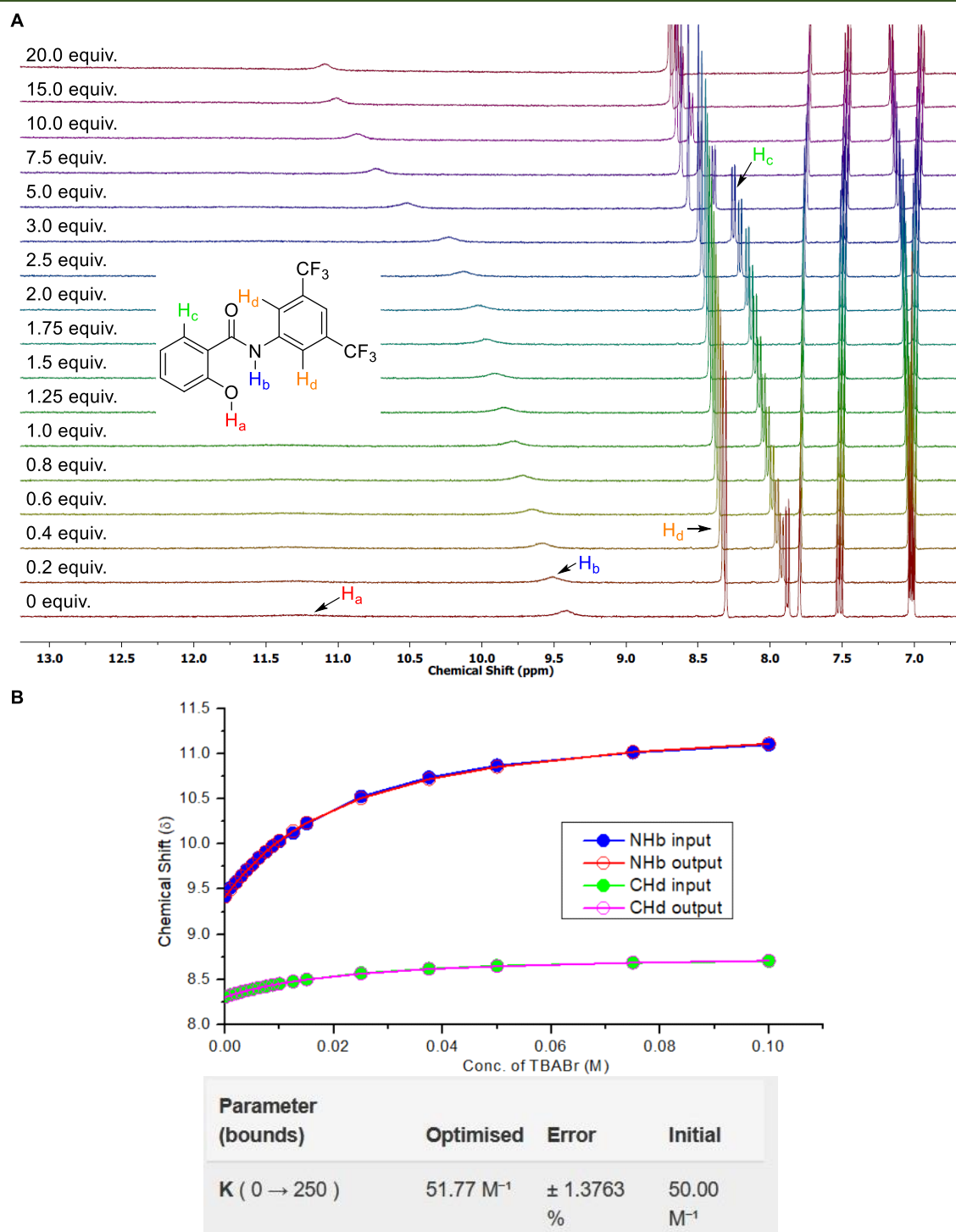


Figure 10: (A) ¹H NMR titration spectra of **1b** (5 mM) with stepwise addition of TBABr in CD₃CN. The number of TBABr equivalents added per reading is shown on the stacked spectra. (B) Plot of chemical shifts (δ) of protons H_b and H_d vs concentration of TBABr added, fitted to 1:1 binding model using BindFit v0.5.

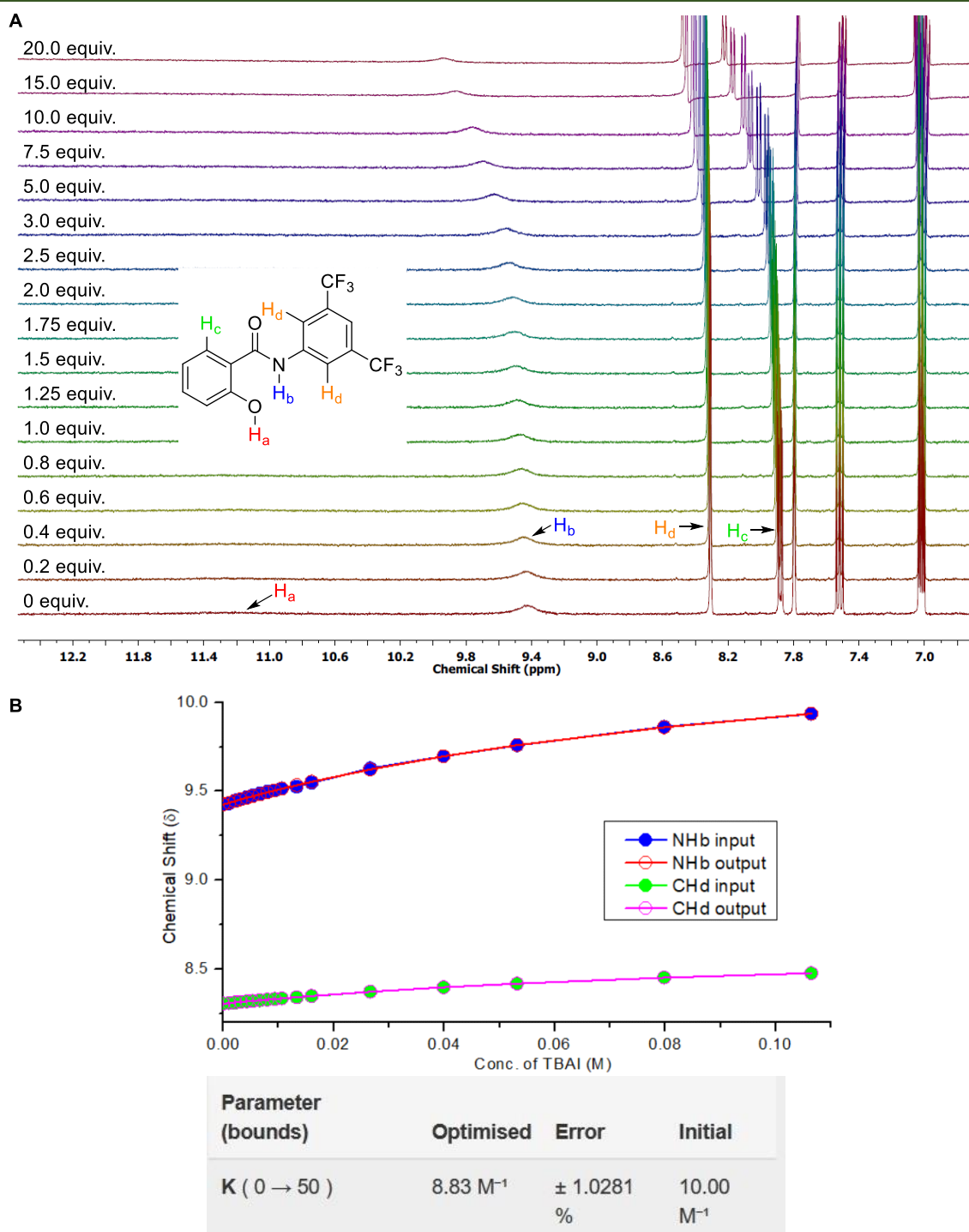
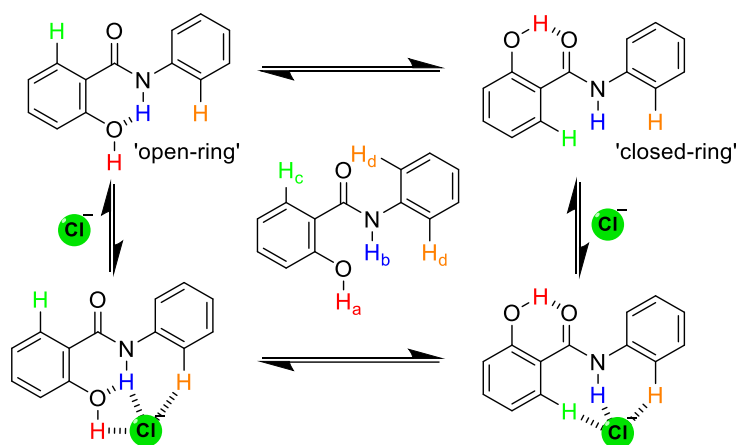


Figure 11: (A) ¹H NMR titration spectra of **1b** (5 mM) with stepwise addition of TBAI in CD₃CN. The number of TBAI equivalents added per reading is shown on the stacked spectra. (B) Plot of chemical shifts (δ) of protons H_b and H_d vs concentration of TBAI added, fitted to 1:1 binding model using BindFit v0.5.

Table 2: Summary of transporter-ion binding data from NMR titration experiments.

Compound	Titrated Ion	Binding	K_a (M ⁻¹)
		Stoichiometry	
1a	Cl ⁻	1:1	164.65 ± 1.63
1b	Cl ⁻	1:1	261.80 ± 9.01
1c	Cl ⁻	1:1	167.17 ± 1.07
1d	Cl ⁻	1:1	82.08 ± 0.41
1e	Cl ⁻	1:1	32.35 ± 0.12
1b	Br ⁻	1:1	51.77 ± 0.71
1b	I ⁻	1:1	8.83 ± 0.09

Salicylamides are known to exist in two hydrogen-bonded conformations, (i) an ‘open-ring’ form with intramolecular N–H···O–(C,H) interaction and (ii) a ‘closed-ring’ with O–H···O=C interaction³¹ (Figure 12). The participation of both conformations in anion binding was evident from the nature of peak shifts observed on increasing the concentration of chloride ion relative to the transporter in the NMR ion titration experiment. It was likely that there exists an equilibrium of ion-receptor complexes with the receptor in both ‘open’ and ‘closed’-ring conformations. This was verified through theoretical calculations.

**Figure 12:** Ion binding modes of salicylamides with Cl⁻ in ‘open-ring’ and ‘closed-ring’ conformations.

The compound **1b** was chosen for analysis, and the prediction of its probable geometries was conducted using CONFLEX 8 conformation search software package^{44, 45} using the MMFF94S (2010-12-04HG) force field and a search limit of 10 kcal/mol. A total of six conformation predictions were obtained with the major conformers, **Conf-1** and **Conf-2**, having Boltzmann populations 32.6812% and 32.3804%, respectively (Figure 13) and the subsequent two conformers, **Conf-3** and **Conf-4**, having Boltzmann populations 17.4307% and 17.4031% respectively (Figure 14). The remaining conformations (Boltzmann population < 0.1%) were rejected. Conformers **Conf-1** and **Conf-2** were almost identical and observed to be in the ‘open-ring’ conformation, while the highly similar **Conf-3** and **Conf-4** were of the ‘closed-ring’ conformation. This hints at the possibility of both ‘open’ and ‘closed’ states available for ion binding in solution, with the ‘closed-ring’ conformer being less abundant. The optimized structures, Hartree-Fock (HF) energies and zero-point energies (ZPE) of the ‘open-ring’ and ‘closed-ring’ conformations of **1b** were obtained through DFT geometry optimization and energy calculations of **Conf-1** (Figure 15A) and **Conf-3** (Figure 15B) respectively on Gaussian 09 program suite⁴⁶ using the B3LYP exchange-correlation functional^{47, 48} and the 6-31G(d,p) basis set.^{49, 50}

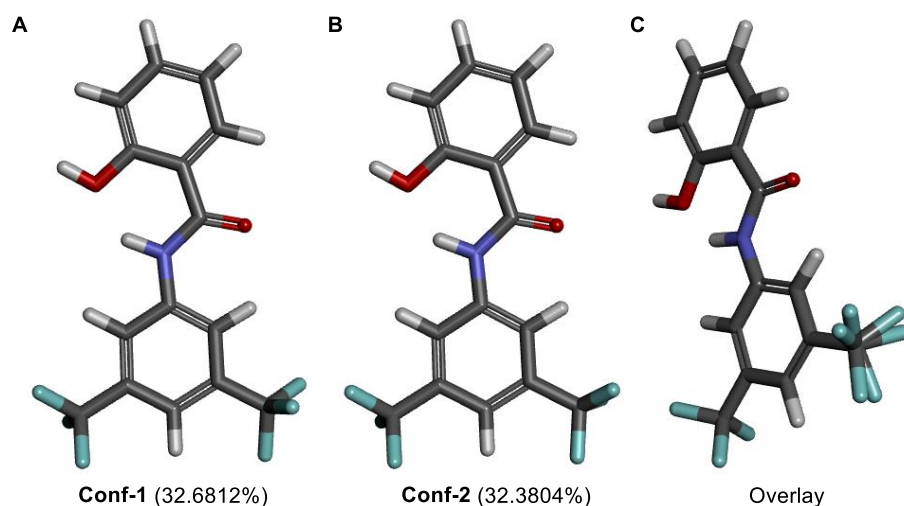


Figure 13: CONFLEX 8 predicted majority geometries, (A) **Conf-1** and (B) **Conf-2**, of **1b**, along with their Boltzmann populations (in brackets). Both conformations are in the ‘open-ring’ state, indicating its predominance. (C) Overlay of both conformations (**Conf-1** and **Conf-2**) exhibiting their near identical nature.

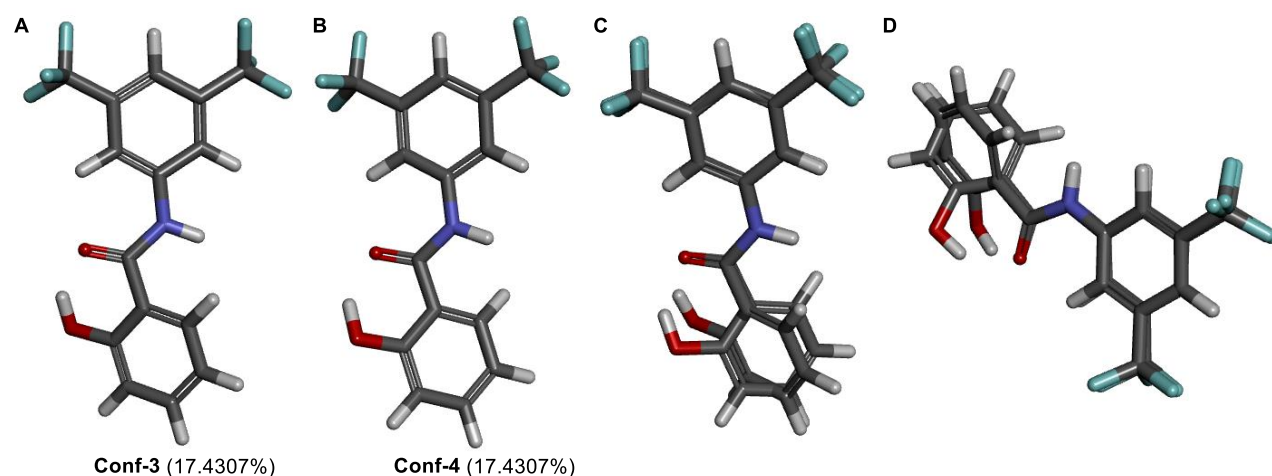


Figure 14: CONFLEX 8 predicted minority geometries, (A) **Conf-3** and (B) **Conf-4**, of **1b**, along with their Boltzmann populations (in brackets). Both conformations were observed to be in the ‘closed-ring’ state. (C) and (D) Two different perspectives of overlaid **Conf-3** and **Conf-4** exhibiting their similarity.

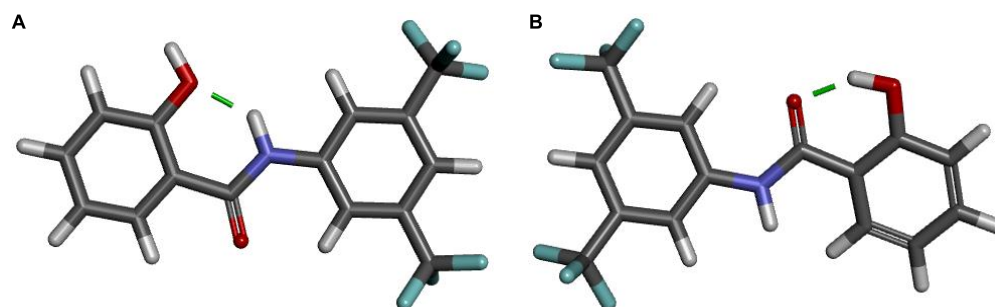


Figure 15: Gaussian 09 geometry optimized structure of (A) ‘open-ring’ **1b** (**Conf-1**) and (B) ‘closed-ring’ **1b** (**Conf-3**) using B3LYP/6-31G(d,p) basis set.

For **1b**-‘open-ring’ structure (from **Conf-1**):

- HF energy = -1381.3178131 Hartree
- Zero-point energy = 0.2227228 Hartree

For **1b**-‘closed-ring’ structure (from **Conf-3**):

- HF energy = -1381.3248189 Hartree
- Zero-point energy = 0.223063 Hartree

Free energy values of both geometry-optimized ‘open-ring’ and ‘closed-ring’ conformers of the salicylamide **1b** are observed to be -1381.3178131 Hartree and -1381.3248189 Hartree, respectively. This almost negligible difference in the free energy indicates that both the conformers can be freely interchangeable at room temperature (298 K), making both available for possible ion

binding interactions. The close correlation of the optimized ‘open-ring’ structure with the crystal structure of the molecule (Figure 20B) hints towards the prevalence of this conformer in solution. The parameters obtained from optimization and frequency calculations for ‘open’ and ‘closed-ring’ conformers of **1b** were utilized for the succeeding analyses of the complexation of transporter with chloride ion.

To better understand the 1:1 transporter-ion binding observed from NMR titration data, conformation prediction was attempted using CONFLEX 8 but yielded no logical results. As an alternative, a new model system was constructed by placing the chloride ion into the likely binding pocket of the geometry optimized structure of ‘open-ring’ **1b** (Figure 16A) using VMD molecular graphics viewer.⁵¹ This was carried forward as input for DFT geometry optimization (Gaussian 09,⁴⁶ B3LYP/6-31G(d,p) functional basis set).⁴⁷⁻⁵⁰ The geometry optimized output (‘open-ring’ [**1b** + Cl⁻]) exhibited interactions of chloride ion with phenolic O-H, amide N-H, and aromatic (N-aryl) C-H bonds (Figures 16B, C). Similarly, VMD molecular graphics viewer⁵¹ was used to construct a model system where the chloride ion was positioned into the likely binding pocket of the geometry optimized ‘closed-ring’ structure of **1b** (Figure 17A). This was carried forward as input for DFT geometry optimization (Gaussian 09, B3LYP/6-31G(d,p)). The geometry optimized output (‘closed-ring’ [**1b** + Cl⁻]) exhibited interactions of chloride ion with amide N-H and aromatic C-H bonds on both N-aryl and salicyl group rings (Figure 17B, C).

For ‘open-ring’ [**1b** + Cl⁻]:

- HF energy = -1841.6350307 Hartree
- Zero-point energy = 0.2219662 Hartree
- BSSE = 0.006123743967 Hartree
- Binding energy = -0.059617156 Hartree = -37.41036158 kcal/mol

For ‘closed-ring’ [**1b** + Cl⁻]:

- HF energy = -1841.6411709 Hartree
- Zero-point energy = 0.2229652 Hartree
- BSSE = 0.00559474179 Hartree
- Binding energy = -0.058621758 Hartree = -36.78573949 kcal/mol

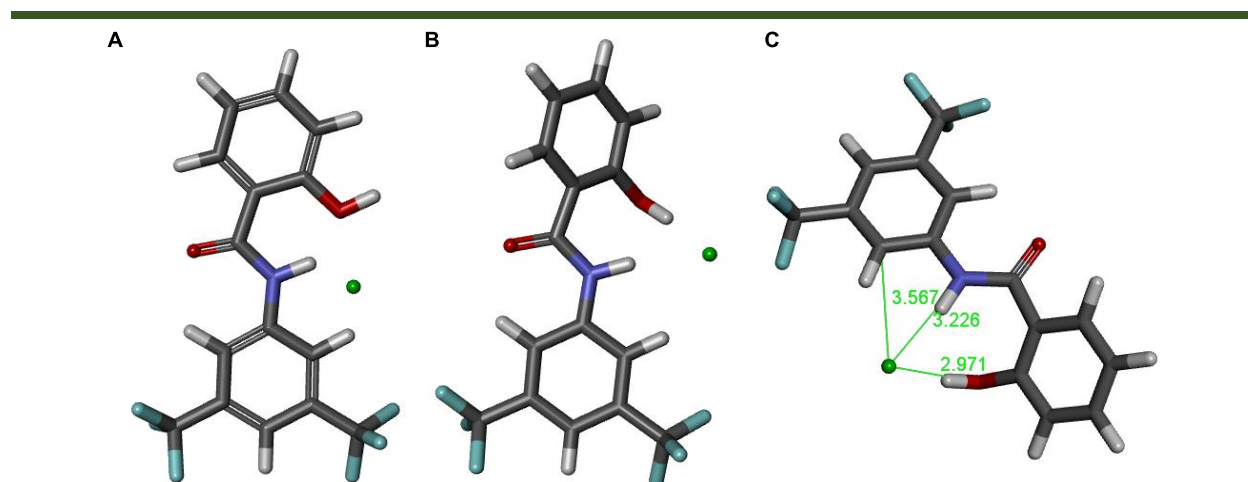


Figure 16: (A) Input coordinates for geometry optimization with Gaussian 09. (B) Geometry optimized output. (C) Distances of non-covalent interactions in optimized output.

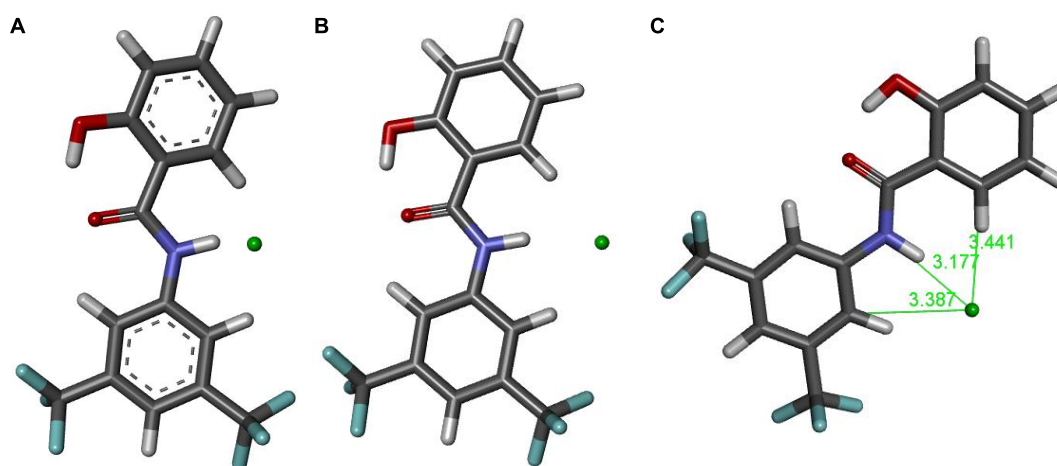


Figure 17: (A) Input coordinates for geometry optimization with Gaussian 09. (B) Geometry optimized output. (C) Distances of non-covalent interactions in optimized output.

The free energy values 1:1 complexes of the chloride with the ‘open-ring’ and ‘closed ring’ conformers of **1b** are −1841.6350307 Hartree and −1841.6411709 Hartree, and their binding energies are −37.41036158 kcal/mol and −36.78573949 kcal/mol respectively. These very close values confirm the existence of 1:1 chloride complexes of both conformers in solution in similar amounts. These results corroborate with data obtained from NMR ion titration studies, which exhibited downfield shifts in both salicylamide proton peaks uniquely available to only ‘open-ring’ (O-H) and ‘closed-ring’ (salicyl ArC-H) conformations.

The chloride ion binding properties of the protransporters **2** and **3** were also assessed through ¹H NMR titration experiments in CD₃CN. No significant peak shifts were observed for **2**

(Figure 18), even at high concentrations of Cl⁻, indicating loss of ion binding ability on pyrrolidinobenzoquinone caging of **1b**. Protransporter **3**, on titrating with Cl⁻ exhibited shifts in N-H_b, C-H_c, and C-H_d protons (Figure 19), indicating ion binding in a ‘closed-ring’ analogous conformation. Calculations with BindFit program⁴³ assuming 1:1 transporter-ion binding yielded $K_a(1:1) = 47.46 \pm 0.25 \text{ M}^{-1}$, which is only 18% of the binding constant of free transporter **1b**, indicating a significant loss of ion binding ability on caging with the trimethyl lock quinone system.

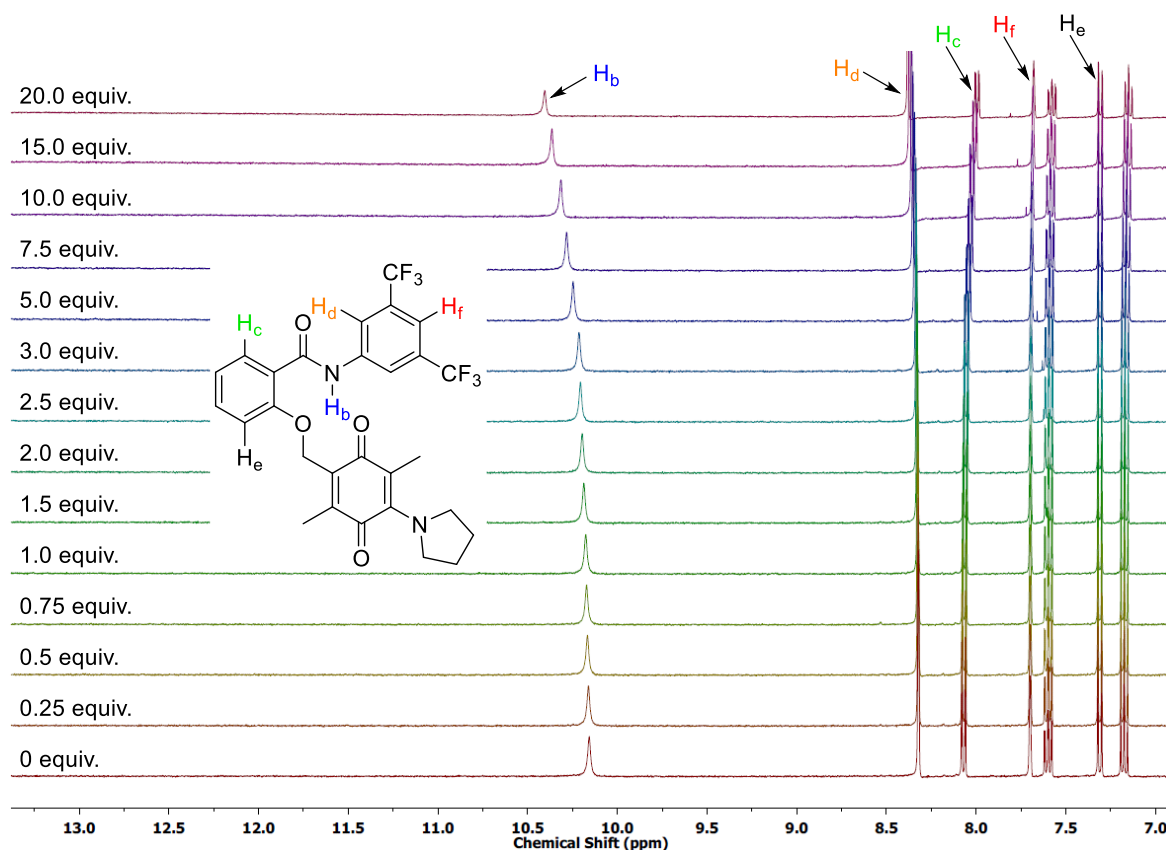


Figure 18: ¹H NMR titration spectra of **2** (5 mM) with stepwise addition of TBACl in CD₃CN. The number of TBACl equivalents added per reading is shown on the stacked spectra. No significant changes in chemical shifts (δ) of protons were observed. Fitting of data to different binding models was attempted using BindFit v0.5, but no proper fit was observed.

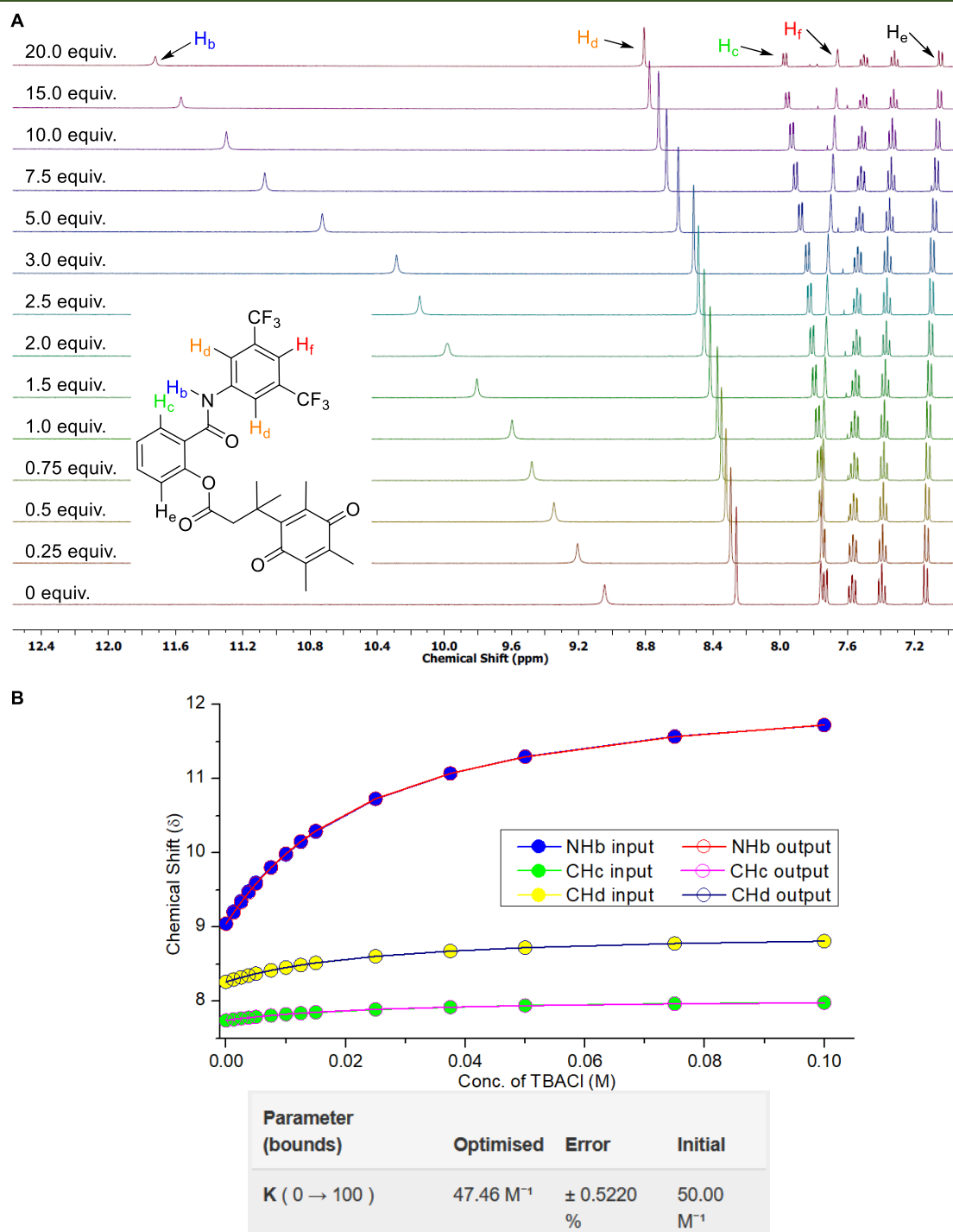


Figure 19: (A) ¹H NMR titration spectra of **3** (5 mM) with stepwise addition of TBACl in CD₃CN. The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) The plot of chemical shifts (δ) of protons H_b, H_c, and H_d vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

Table 3: Summary of protransporter-ion binding data from NMR titration experiments.

Compound	Titrated Ion	Binding	K_a (M ⁻¹)
		Stoichiometry	
2	Cl ⁻	1:1	N.D.
3	Cl ⁻	1:1	47.46 ± 0.25

2.2.4 Crystal Structure Data

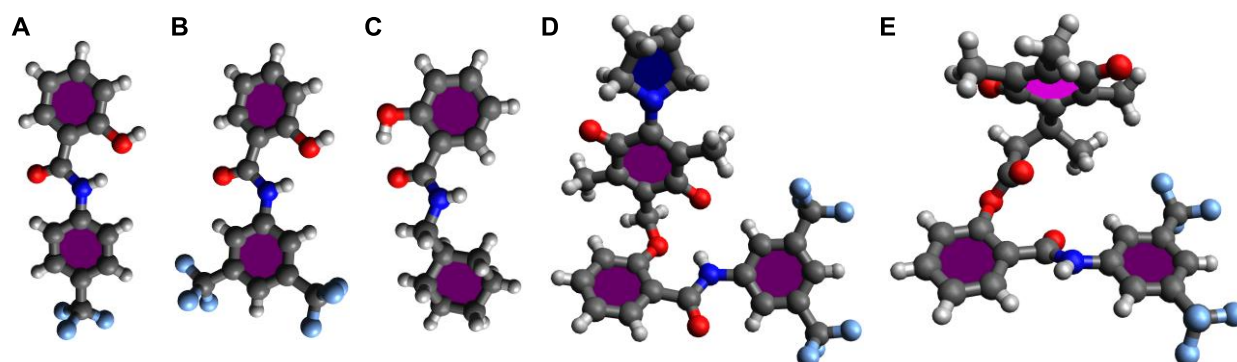


Figure 20: Molecular structures of **1a** (A), **1b** (B), and **1f** (C) obtained from SCXRD data. **1a** and **1b** are observed to be in the ‘open-ring’ conformation, while **1f** is in the ‘closed-ring’ conformation. Molecular structures of **2** (D) and **3** (E).

Compounds **1a-1b** were crystallized from CHCl₃, and their structures were solved through SCXRD (Figures 20A, B). Efforts to crystallize the transporters with electron-donating substituents (**1d**, **1e**) were unsuccessful; hence, the cyclohexylmethylamine derivative **1f** was synthesized and successfully crystallized from CHCl₃ (Figure 20C). Salicylamides with electron-withdrawing substituents (**1a-1b**) were observed to have ‘open-ring’ conformation with -N-H $\cdots$ O(OH) hydrogen bonding interaction, which was in agreement with the most probable conformation of **1b** obtained from theoretical predictions (Figure 15A). Meanwhile, the electron donating alkyl group substituted **1f** had O-H $\cdots$ O=C H-bonded ‘closed-ring’ structure. These results offered further proof of the existence of stable open and closed-ring conformations. A 7:3 ACN/water system was employed for the crystallization of protransporters **2** and **3**, and their structures were solved through SCXRD. Each molecule of **2** was observed to be in an ‘open-ring’ like conformation with an O $\cdots$ H(N) $\cdots$ O hydrogen-bonded, locked structure (Figure 20D), while each molecule of **3** was crystallized in a conformation similar to the ‘closed-ring’ salicylamide

system (Figure 20E). Multiple attempts were made for co-crystallization of the transporters (**1a-1c**) with chloride ion under a variety of conditions but yielded no success.

2.2.5 Ion Transport Studies

Ion transport activities of compounds **1a-1e** were examined across egg yolk phosphatidylcholine large unilamellar vesicles (EYPC-LUVs) with entrapped 8-hydroxypyrene-1,3,6-trisulfonate (HPTS) dye (1 mM) (i.e., EYPC-LUVs $\supset$ HPTS) suspended in HEPES (10 mM), NaCl (100 mM) buffer.⁵² A pH gradient ($\text{pH}_{\text{in}} = 7.0$, $\text{pH}_{\text{out}} = 7.8$) was applied by the addition of NaOH, and the collapse of this gradient on the addition of transporter was monitored at $\lambda_{\text{em}} = 510 \text{ nm}$ ($\lambda_{\text{ex}} = 450 \text{ nm}$) (Figures 21A, 77). Compounds **1a-1e** provided the activity sequence **1b** $\gg$ **1a** $>$ **1e** $>$ **1c** $>$ **1d** (Figure 21B). The dose-dependent studies of **1a-1e** (Figures 22-26) provided half maximal effective concentration (EC_{50}) = 2.10, 0.48, 5.45, 6.98, and 2.35 μM (i.e., 3.36, 0.77, 8.72, 11.17, and 3.76 mol%), respectively. The Hill coefficient values of **1a-1c** ($n = 1.70, 2.09$, and 1.90), having electron-withdrawing substituents, indicate the involvement of two molecules of a transporter in active transporter complex formation, while for **1d** and **1e** ($n = 2.97$ and 3.30), where electron-donating substituents are used, three molecules of transporter per ion are involved in active transporter formation (Table 4). This indicates that the electron withdrawing/donating nature of the amide substituent may play a role in the nature of the ion transporter-ion complex formed in the membrane during the transport process.

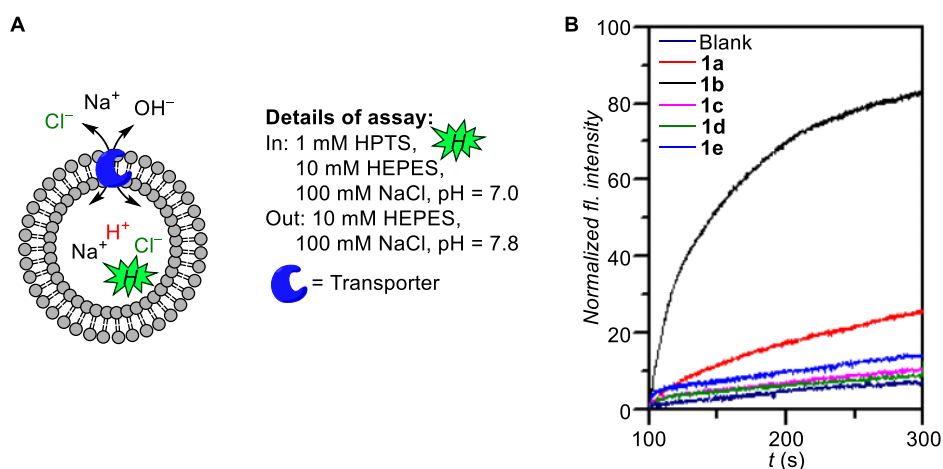


Figure 21: (A) Schematic of fluorescence-based assay of ion transport activity using EYPC-LUVs $\supset$ HPTS and (B) comparison of ion transport activities of salicylamides **1a-1e** (at 1 μM) across EYPC LUVs $\supset$ HPTS.

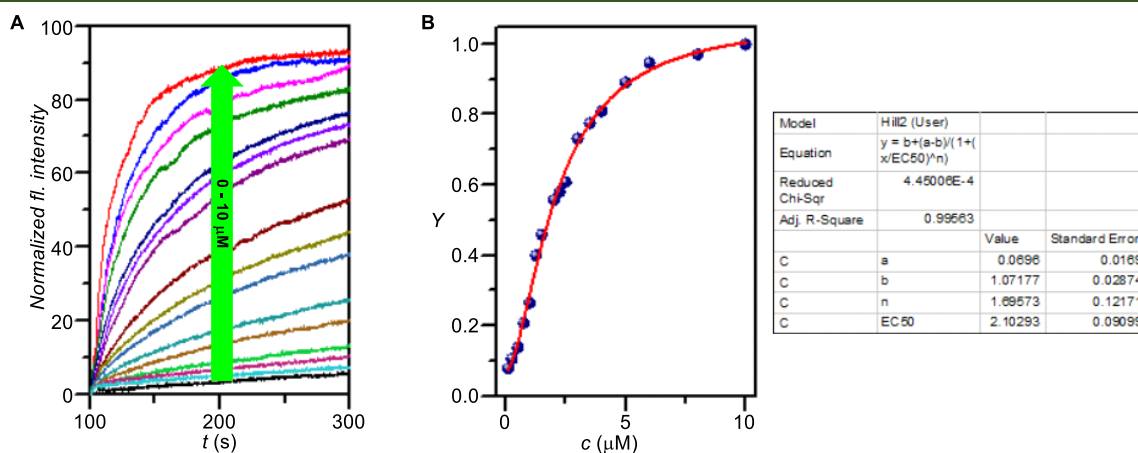


Figure 22: (A) Dose-dependent activity of compound **1a** across EYPC-LUVs⊃HPTS. (B) Hill plot analysis of **1a** at $t = 290$ s.

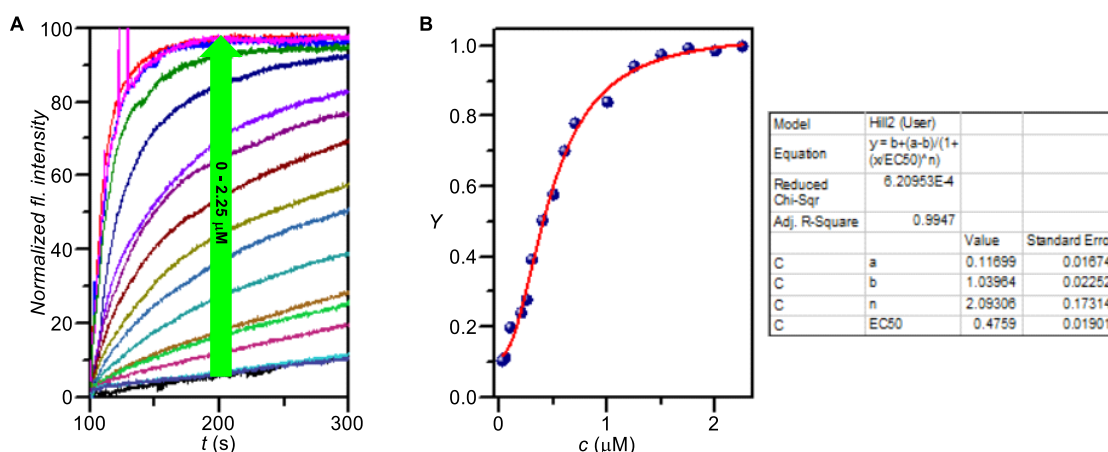


Figure 23: (A) Dose-dependent activity of compound **1b** across EYPC-LUVs⊃HPTS. (B) Hill plot analysis of **1b** at $t = 290$ s.

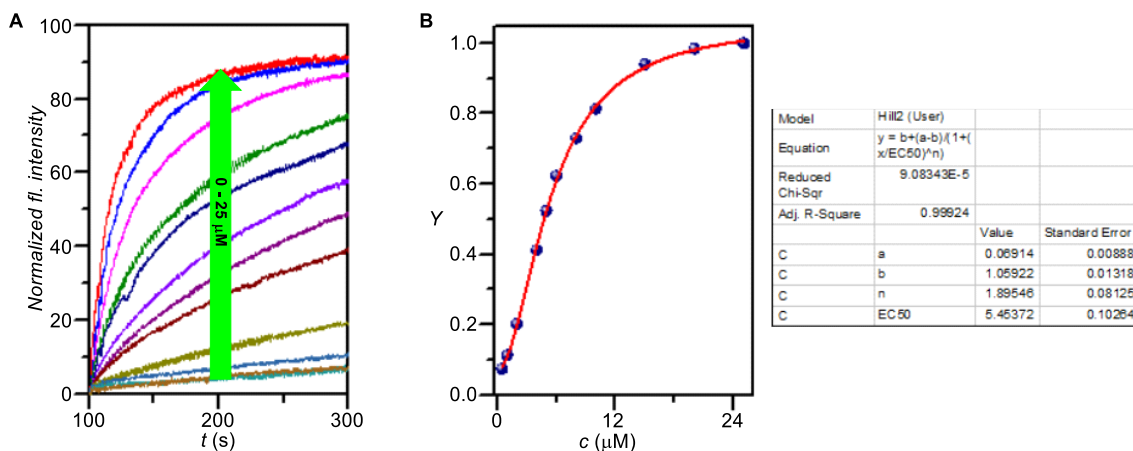


Figure 24: (A) Dose-dependent activity of compound **1c** across EYPC-LUVs⊃HPTS. (B) Hill plot analysis of **1c** at $t = 290$ s.

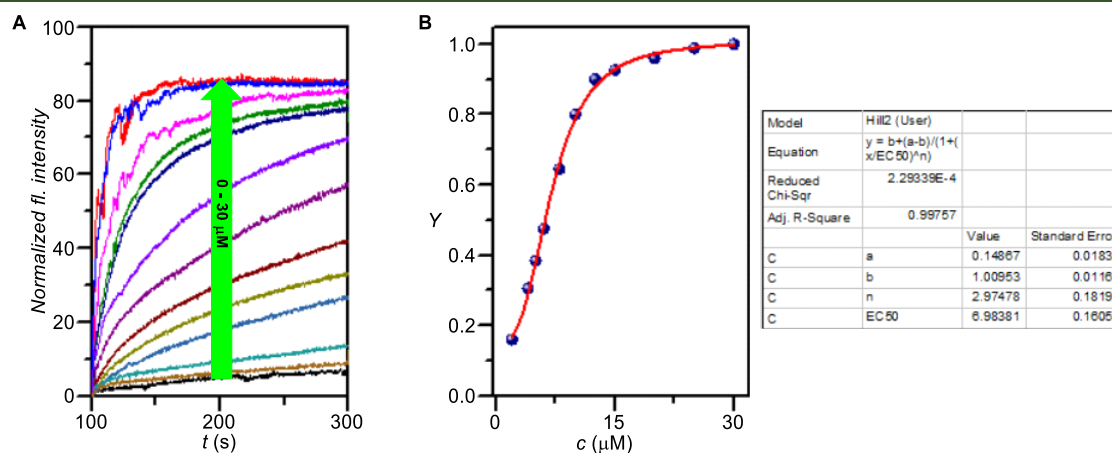


Figure 25: (A) Dose-dependent activity of compound **1d** across EYPC-LUVs⊃HPTS. (B) Hill plot analysis of **1d** at $t = 290$ s.

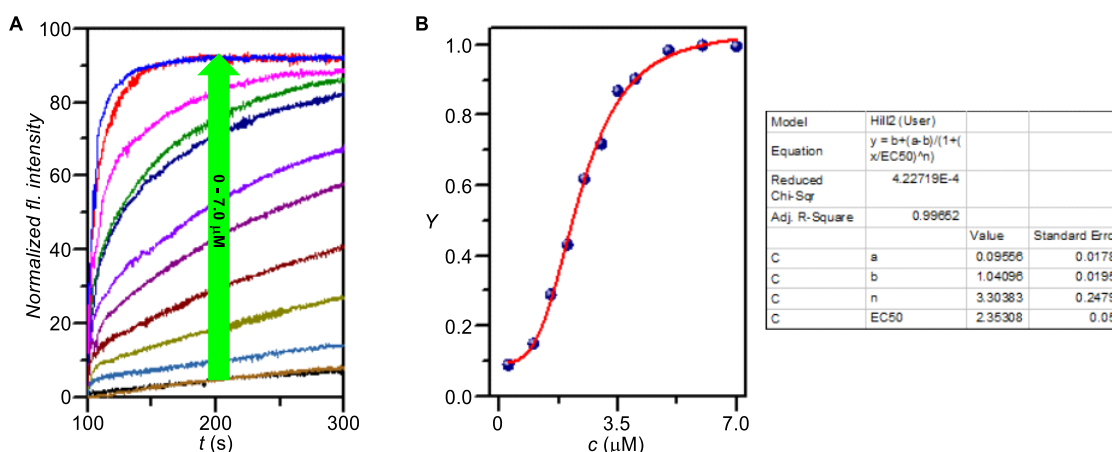


Figure 26: (A) Dose dependent activity of compound **1e** across EYPC-LUVs⊃HPTS. (B) Hill plot analysis of **1e** at $t = 290$ s.

Table 4: Summary of Hill analyses of dose dependent ion transporter studies across EYPC-LUVs⊃HPTS.

Compound	EC ₅₀ (μM)	Hill coeff. (n)
1a	2.10	1.70
1b	0.48	2.09
1c	5.45	1.90
1d	6.98	2.97
1e	2.35	3.30

To rule out the possibility of lipid membrane disruption by the transporter molecule, carboxyfluorescein (CF) leakage assay^{53,54} of **1b** was performed across EYPC-LUVs $\supset$ CF (50 mM CF) suspended in HEPES (10 mM), NaCl (100 mM) buffer (Figure 88). No increment in fluorescence of CF was observed even on the treatment of vesicles with **1b** (10 μ M, 16 mol%) (Figure 27) at greater than 20 times its EC_{50} value, indicating preservation of membrane integrity even at high concentrations of ion transporter, preventing CF leakage from vesicles.

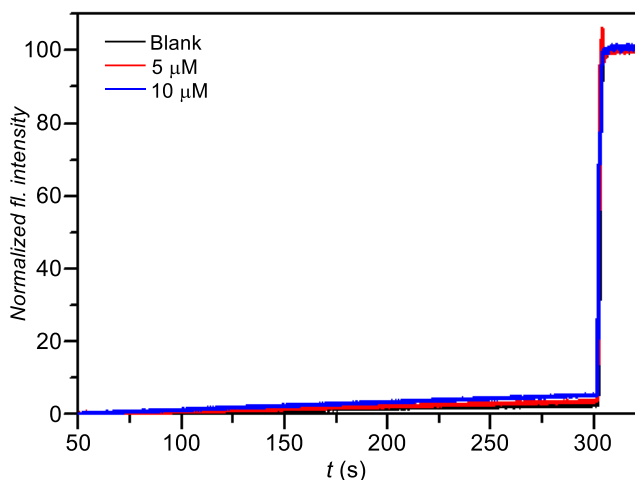


Figure 27: Activity of **1b** at 5 and 10 μ M across EYPC-LUVs $\supset$ CF.

The ion selectivity of the most active compound **1b** was studied across EYPC-LUVs $\supset$ HPTS. Variation of cations (i.e., M⁺ = Li⁺, Na⁺, K⁺, Rb⁺, and Cs⁺) in the extravesicular solution exhibited no considerable difference in ion transport rates (Figure 28A), indicating little involvement of cations in the transport process. However, the change of external anions (i.e., X⁻ = Cl⁻, Br⁻, I⁻, NO₃⁻, and ClO₄⁻) showed significant variation (Figure 28B), with Cl⁻ exhibiting the highest activity. These activity profiles indicate the involvement of extravesicular anions in the ion transport process. The observation of an initial quenching of HPTS fluorescence followed by its increment in the presence of extravesicular I⁻, NO₃⁻, and ClO₄⁻ suggests an initial competitive co-influx of H⁺, against the applied pH gradient, with the available extravesicular anion (I⁻, NO₃⁻, ClO₄⁻).⁵⁵⁻⁵⁷ This results from the hydrophobic nature of the anions (I⁻, NO₃⁻, and ClO₄⁻), which form transporter complexes with a higher membrane permeability relative to the corresponding chloride complex. The collapse of the pH gradient across the membrane during HPTS assay can be due to M⁺/H⁺ antiport, X⁻/OH⁻ antiport, H⁺/X⁻ symport, or M⁺/OH⁻ symport. The absence of

M^+/H^+ antiport or M^+/OH^- symport is indicated by the cation variation data, while the anion variation data is suggestive of X^-/OH^- antiport or H^+/X^- symport.

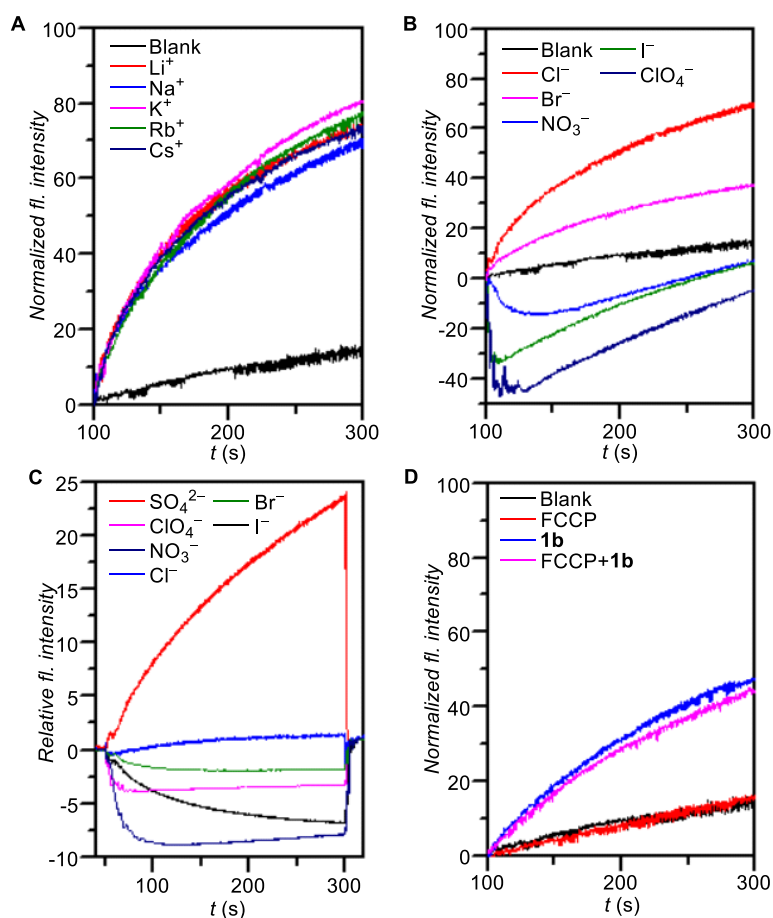


Figure 28: (A) Cation and (B) anion selectivity of **1b** (0.6 μ M) measured across EYPC-LUVs $\Rightarrow$ HPTS by varying extravesicular cations and anions, respectively. (C) Anion selectivity of **1b** (0.6 μ M), measured across EYPC LUVs $\Rightarrow$ HPTS by varying extravesicular anions without pH gradient. (D) Ion transport activity of **1b** (0.4 μ M) in the presence and absence of FCCP (0.2 μ M).

An assay was also carried out in EYPC-LUVs $\Rightarrow$ HPTS by varying the extravesicular anions without application of pH gradient ($pH_{in} = 7.0$, $pH_{out} = 7.0$) by suspension of NaCl entrapped vesicles in buffer containing NaCl, NaBr, NaI, $NaNO_3$, $NaClO_4$, and Na_2SO_4 salts (Figure 28C).^{55, 56} In the presence of extravesicular SO_4^{2-} , which is a poorly permeating ion, an immediate increase in HPTS fluorescence was observed, indicating H^+/Cl^- coefflux.⁵⁶ Use of extravesicular Cl^- exhibited no considerable change in HPTS fluorescence, while Br^- , I^- , NO_3^- , and ClO_4^- showed quenching of fluorescence, suggesting H^+/X^- influx owing to the lipophilicity of the anions. This was further

confirmed by ion transport studies across EYPC-LUVs $\supset$ HPTS in the presence and absence of the proton transporter carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP)⁵⁸ (Figure 81). Compound **1b** (0.4 μ M) was observed to exhibit similar transport activities in the presence and absence of FCCP (0.2 μ M) (Figure 28D). This indicated that the transmembrane pH gradient dissipation was via H⁺ efflux through H⁺/Cl⁻ symport rather than OH⁻ influx, excluding the possibility of X⁻/OH⁻ antiport.

The Cl⁻ transport activities of compounds were examined across EYPC-LUVs entrapping the chloride-sensitive lucigenin dye (1 mM) (i.e., EYPC-LUVs $\supset$ lucigenin) suspended in NaNO₃ solution (200 mM) (Figure 29A).⁵² An ion gradient was applied by providing an external pulse of NaCl and chloride influx monitored by a change in fluorescence intensity at λ_{em} = 535 nm (λ_{ex} = 455 nm) (Figure 82). At comparable concentration (5 μ M, 5.33 mol%), the salicylamides gave activity in the order **1b** >> **1a** > **1e** > **1c** > **1d** (Figure 29B), which was identical to that obtained from the HPTS assay. The dose-response studies of **1a** and **1b** (Figures 30, 31) provided EC_{50} = 4.26 μ M (4.54 mol%), 3.79 (4.04 mol%), and n = 2.52, 2.17, respectively, further confirming the involvement of two molecules each of the transporters in active ion transport complex.

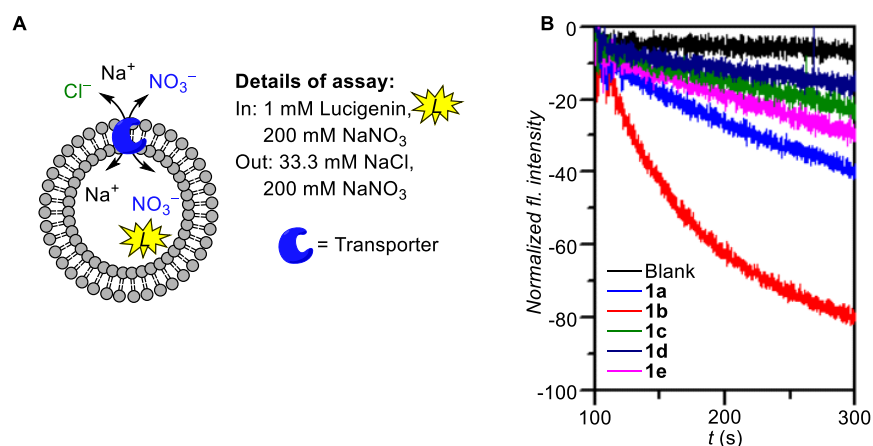


Figure 29: (A) Schematic of fluorescence-based assay of ion transport activity using EYPC-LUVs $\supset$ Lucigenin and (B) comparison of ion transport activities of salicylamides **1a-1e** (at 5 μ M) across EYPC-LUVs $\supset$ Lucigenin.

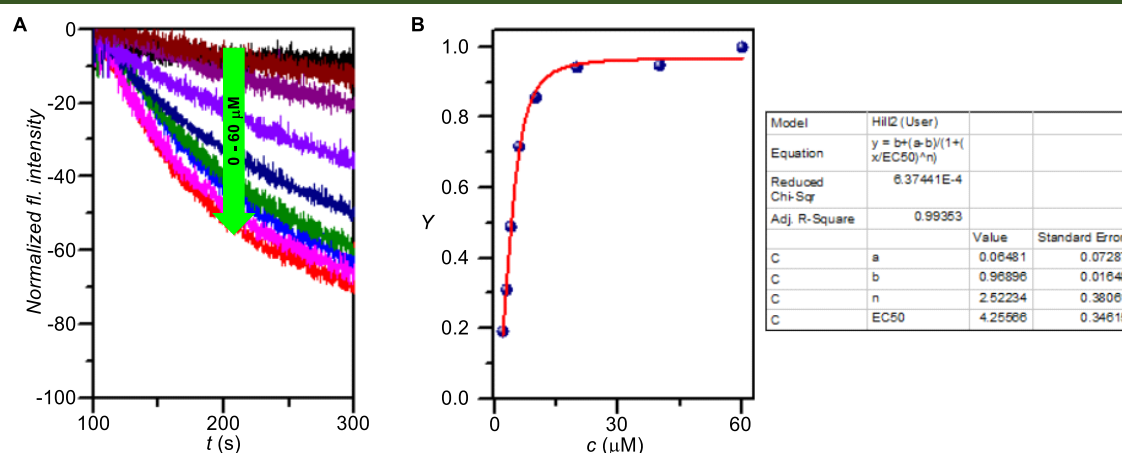


Figure 30: (A) Dose-dependent activity of compound **1a** across EYPC-LUVsΔLucigenin. (B) Hill plot analysis of **1a** at t = 290 s.

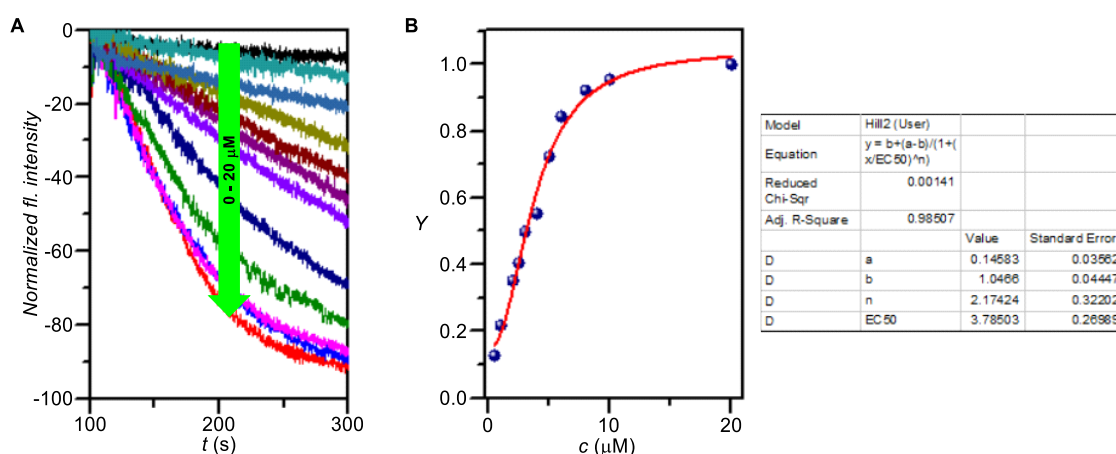


Figure 31: (A) Dose-dependent activity of compound **1b** across EYPC-LUVsΔLucigenin. (B) Hill plot analysis of **1b** at t = 290 s.

Table 5: Summary of Hill analyses of dose-dependent ion transporter studies across EYPC-LUVsΔLucigenin.

Compound	EC ₅₀ (μM)	Hill coeff. (n)
1a	4.26	2.52
1b	3.79	2.17

The Cl⁻ influx activity of **1b** (4 μM, 4.27 mol%) in the presence and absence of the K⁺ transporter valinomycin⁵⁹ (1 μM, 1.07 mol%) was comparable, indicating cation (or proton)/anion symport as the main transport mechanism (Figure 32B). Variation of cations in the extravesicular medium of

EYPC-LUVs $\supset$ lucigenin using M⁺Cl⁻ salts (i.e., M⁺ = Li⁺, Na⁺, K⁺, Rb⁺, and Cs⁺) displayed only slight differences in ion transport rates, further confirming H⁺/Cl⁻ symport mechanism (Figure 32A).

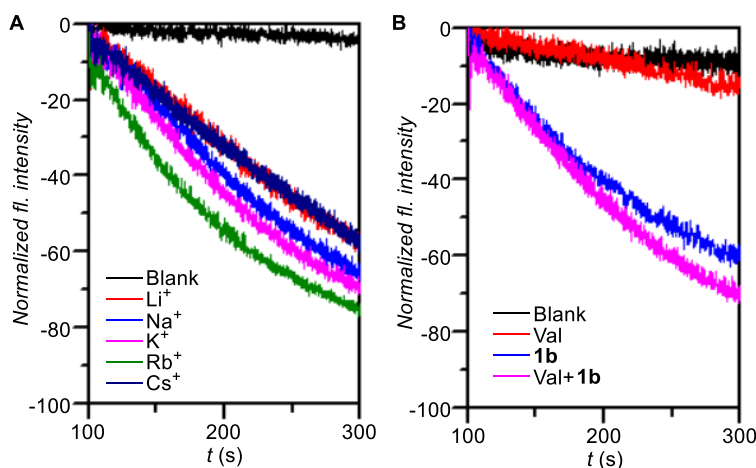


Figure 32: (A) Cation selectivity of **1b** (5 μM) measured by varying extravesicular cations, and (B) comparison of ion transport activity of **1b** (2.5 μM) in the presence and absence of valinomycin (Val, 1 μM) across EYPC-LUVs $\supset$ Lucigenin.

An ion-selective electrode (ISE) was used to monitor the chloride efflux activity of **1b** in the presence of monensin (Na⁺/H⁺ antiporter)⁶⁰ (Figure 86) and valinomycin (Figure 87) across EYPC-LUVs $\supset$ KCl (inside: 300 mM KCl, 10 mM potassium phosphate buffer, pH = 7.0) suspended in potassium gluconate (300 mM with 10 mM potassium phosphate buffer, pH = 7.0).⁶¹ An increment in the ion transport activity of **1b** was observed on coupling with monensin (Figure 33A). The pH gradient that builds up during the electroneutral H⁺/Cl⁻ symport by **1b** gets dissipated by monensin-facilitated K⁺/H⁺ antiport, which enhances the rate of ion transport by **1b**.⁶¹ Increased ion transport activity was also observed in the valinomycin coupled assay (Figure 33B), which indicates that the transporter **1b** is also capable of unidirectional transport of Cl⁻ when coupled with the K⁺ transport by valinomycin under the given experimental conditions. These studies confirm our initial hypothesis that H⁺/Cl⁻ symport is the dominant transmembrane ion transport mechanism in salicylamides.

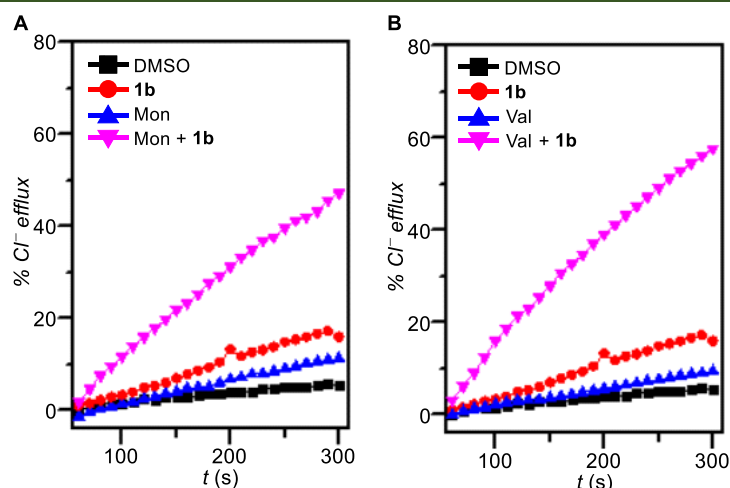


Figure 33: Ion transport activity of **1b** (20 μ M) in the presence and absence of (A) monensin (Mon, 1 μ M), and (B) valinomycin (Val, 1 μ M) studied across EYPC-LUVs $\Rightarrow$ KCl (300 mM) suspended in K gluconate (300 mM) solution (pH = 7.0).

To determine the structure of the [(**1b**)₂ + Cl⁻] active transporter complex (from experimental Hill coefficient (n) of ~ 2), prediction of the most probable geometry was first conducted with CONFLEX 8 software package^{44, 45} (MMFF94S force field, 10 kcal/mol search limit). An output of 764 possible geometries was obtained, of which the structure with the highest Boltzmann population (82.5015%) (**CF-1**) was taken (Figure 34A), and all conformations with Boltzmann population < 10% were rejected. The geometry optimization of **CF1** was then conducted (Gaussian 09, B3LYP/6-31G(d,p)) and the resulting geometry optimized structure of [(**1b**)₂ + Cl⁻] (Figure 34B-D) showed two molecules of ‘open-ring’ **1b** oriented in a tweezer-like arrangement which ‘clamps’ the ion on one side with each receptor binding to the chloride ion using its phenolic O-H group ($O_{\text{phenol}} \cdots \text{Cl}^- = 2.985 \text{ \AA}$). The $C_{\text{ortho}} \cdots \text{Cl}^-$ distance was measured to be $3.678 \text{ \AA}$ and the $C_{\text{ortho}}\text{-H} \cdots \text{Cl}^-$ angle of 131.8° , indicating $C_{\text{ortho}}\text{-H} \cdots \text{Cl}^-$ interaction.

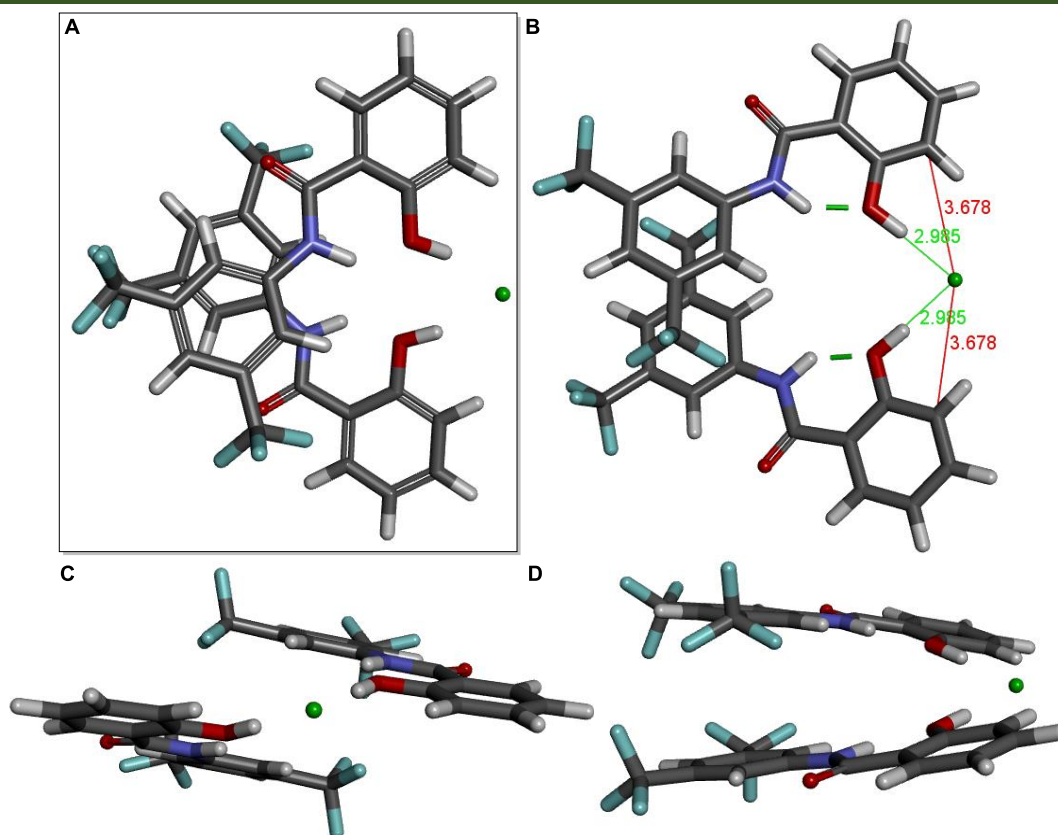


Figure 34: (A) CONFLEX 8 predicted majority geometry of $[(1b)_2 + Cl^-]$. (B) Geometry optimized $[(1b)_2 + Cl^-]$ complex (Gaussian 09, B3LYP/6-31G(d,p)) showing intramolecular H-bonds and distances of non-covalent interactions with ion. (C),(D) Different perspectives of geometry optimized $[(1b)_2 + Cl^-]$ complex.

For $[(1b)_2 + Cl^-]$:

- HF energy = -3223.0010315 Hartree
- Zero-point energy = 0.4462133 Hartree
- BSSE = 0.01638477191 Hartree
- Binding energy = -0.096019528 Hartree = -60.25321407 kcal/mol

We propose that two molecules of the salicylamide bind to a chloride ion like a pair of tweezers on one side of the lipid membrane, forming a $[(1b)_2 + Cl^-]$ complex. This complex travels through the membrane and releases the chloride ion along with a proton, obtained from deprotonation of the OH group of one of the transporters, effectively co-transporting H⁺/Cl⁻ across the membrane (Figure 35). The participating transporters, both the intact and the deprotonated molecules, move

back across the membrane to the side of lower pH where re-protonation of the second charged molecule takes place, and the transport process repeats. Planar bilayer conductance measurement for **1b** did not furnish any opening/closing event at varied potentials, ruling out ion channel formation (Figure 36).

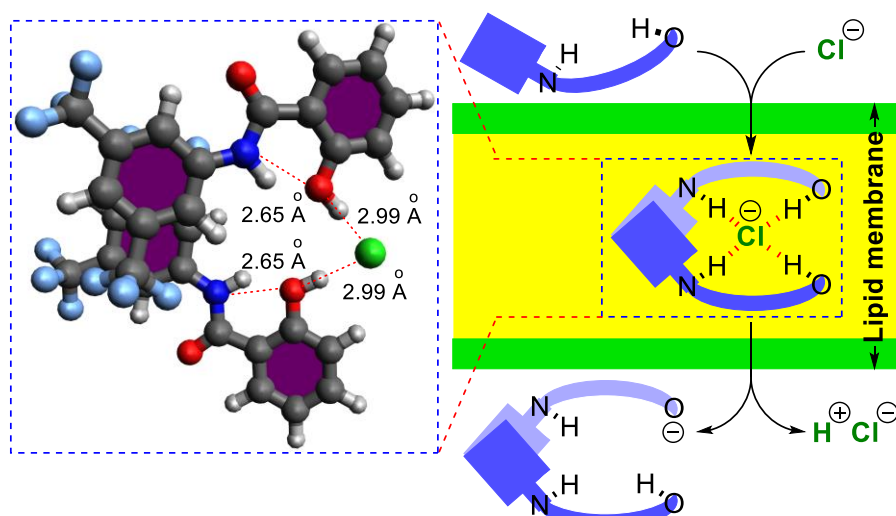


Figure 35: Geometry optimized conformation of $[(\mathbf{1b})_2 + \text{Cl}^-]$ showing binding interactions with chloride and its schematic showing proposed transmembrane H^+/Cl^- transport mechanism by salicylamide transporters.



Figure 36: Current traces at the applied voltage of -150 mV under symmetrical KCl (1.0 M) conditions across the diphytanoylphosphatidylcholine (diPhyPC) bilayer membrane before (A) and after (B) addition of compound **1b** (20 μM).

2.2.6 Ion Transport Activity Caging

Testing of the proton transporter **2** across EYPC-LUVs $\Rightarrow$ HPTS for verification of transport activity caging exhibited a quenching of dye fluorescence upon the addition of the compound (Figure 37A). This was likely due to the quenching of the dye fluorescence due to non-radiative energy transfer

when **2** enters the lipid membrane, resulting from the overlap of the fluorescence emission region of HPTS ($\lambda_{\text{ex}} = 450$ nm) with the absorption peak of **2** in the 450-750 nm region (Figure 37B).

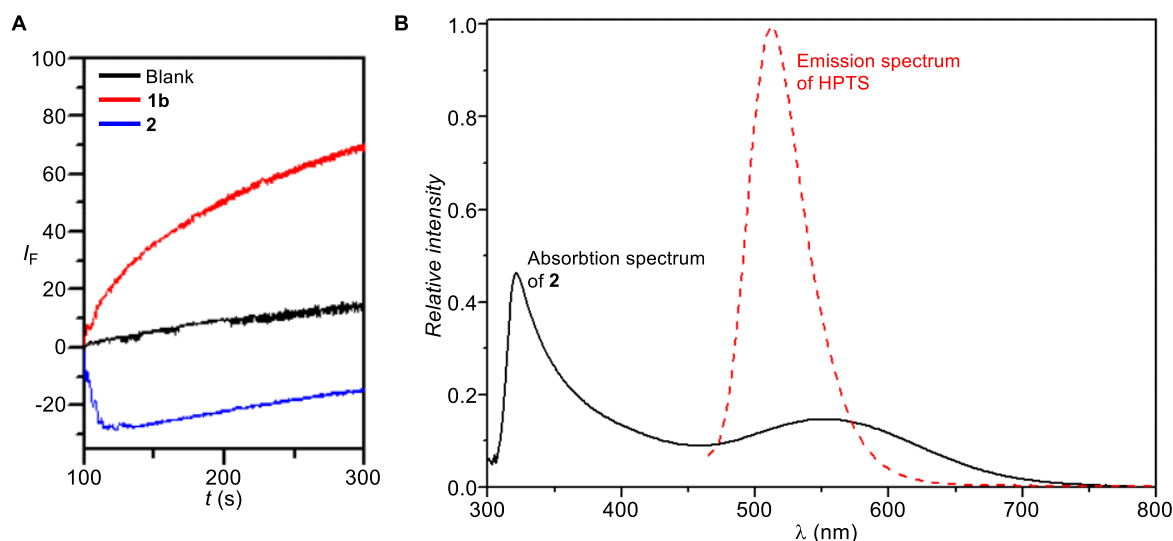


Figure 37: (A) Change in fluorescence intensity of HPTS dye in EYPC-LUVs on the addition of **1b** and **2** (0.6 μ M). (B) Absorption spectrum of **2** treated EYPC-LUVs and NaCl (500 mM) and emission spectrum of HPTS in EYPC-LUVs. Overlap of the fluorescence emission band ($\lambda_{\text{ex}} = 450$ nm) with the absorption band of **2** ($\lambda_{\text{max}} = 550$ nm) was observed.

To verify this hypothesis of quenching of fluorescence of HPTS dye via a non-radiative mechanism on the addition of **2**, the following experiment was devised. The emission spectrum of the HPTS vesicle solution was recorded ($\lambda_{\text{ex}} = 450$ nm) before and after treatment with varying concentrations of **2**. A decrease in fluorescence emission intensity ($\lambda_{\text{max}} = 512$ nm) of the HPTS dye trapped in EYPC-LUVs was observed, with increased fluorescence quenching on increment in dosage of **2**. DMSO treatment, used as the negative control, exhibited negligible fluorescence quenching of dye. Measurements of the fluorescence emission spectra following lysis of the treated vesicles with Triton X-100, and the release of the entrapped HPTS dye into the bulk solution showed recovery of fluorescence intensity of the solution irrespective of the amount of **2** used (Figures 38, 39). All plots were normalized with respect to fluorescence intensity at the emission maximum ($\lambda_{\text{max}} = 512$ nm) of vesicles before treatment with **2**.

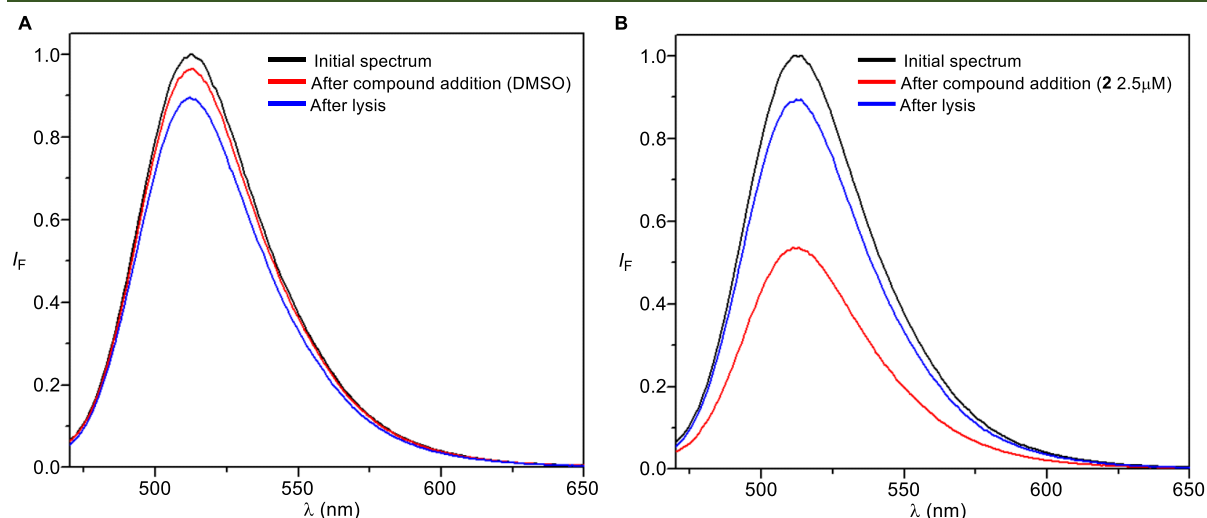


Figure 38: Emission spectra ($\lambda_{\text{ex}} = 450$ nm) of (A) DMSO, and (B) **2** (2.5 μ M) treated EYPC-LUVs $\supset$ HPTS, (i) initially, before the compound was added (black), (ii) after addition of the compound (red), and (iii) after lysis of the vesicle (blue). Quenching of fluorescence intensity was observed on the addition of **2**, followed by its recovery on lysis of the vesicles.

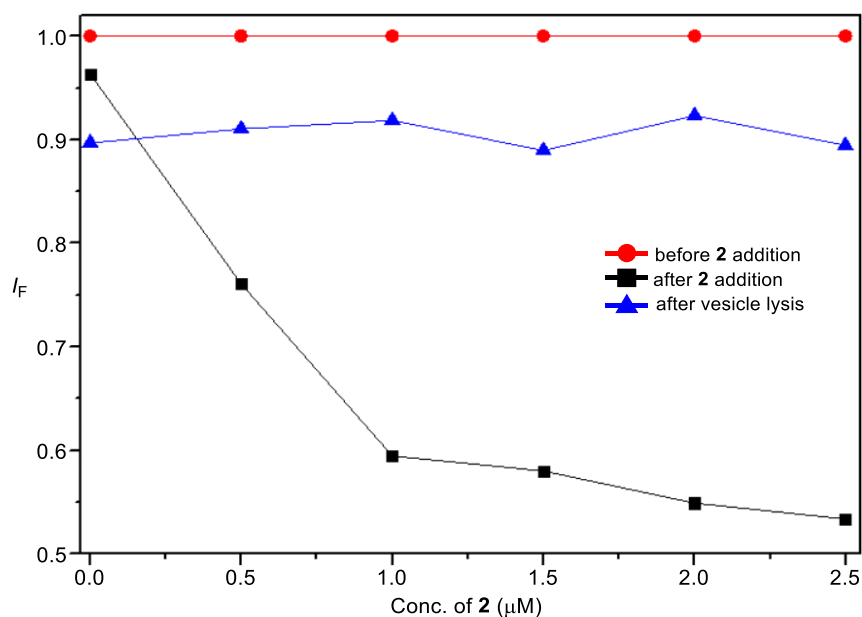


Figure 39: Relative fluorescence intensities at $\lambda_{\text{max(em)}} = 512$ nm (i) before (red) and (ii) after (black) the addition of the compound **2**, and (iii) after lysis of the vesicle (blue).

It is possible to conclude from these experiments that the proximity of the HPTS dye to molecules of **2** is essential for quenching the dye fluorescence. This is achieved in the EYPC-LUVs $\supset$ HPTS where **2** gets localized in the vesicular membrane, which can facilitate energy transfer following

excitation of HPTS and hence results in fluorescence quenching of the dye. On lysis of the vesicles with Triton X-100, the dye and the quencher are released into the bulk solution and can hence no longer maintain the distance necessary for non-radiative energy transfer, resulting in the recovery of dye fluorescence.

The ion transport activity of compound **2** was hence tested through measurement of Cl⁻ efflux from EYPC-LUVs⊃NaCl (500 mM) suspended in a phosphate buffered NaNO₃ (500 mM) solution using a Cl⁻-Ion-Selective Electrode (ISE) (Figures 84, 85). The protransporter **2** was found to be inactive at 30 μM (3.00 mol%) concentration, while the corresponding active transporter **1b** exhibited 70% activity at the same concentration (Figure 40A). The activity of **3** was checked across EYPC-LUVs⊃HPTS, and it was observed to be inactive at a concentration (0.5 μM, 0.8 mol%) where **1b** exhibited significant activity (65%), indicating complete caging of ion transport activity (Figure 40B).

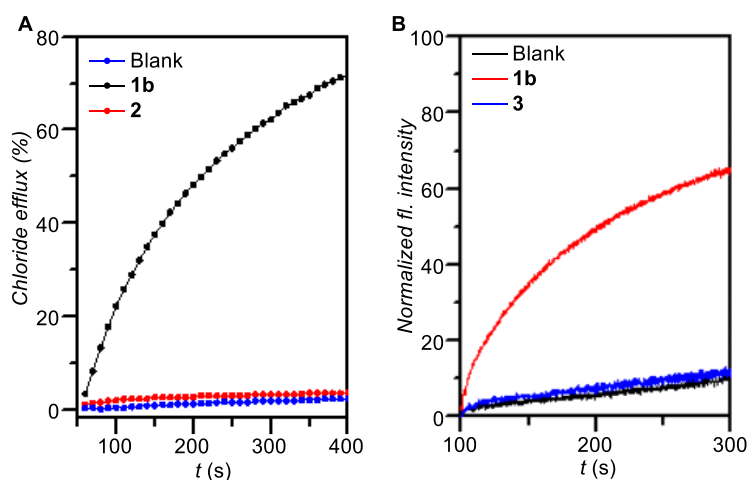
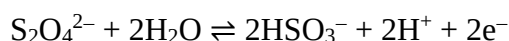


Figure 40: (A) Comparison of ion transport activities of **1b** and **2** (at 30 μM) through measurement of transporter-induced Cl⁻ efflux from EYPC-LUVs⊃NaCl using Cl⁻-ISE. (B) Comparison of ion transport activities of **1b** and **3** (at 0.5 μM) across EYPC-LUVs⊃HPTS. DMSO was used as the control in both cases.

2.2.7 Quinone Reduction Studies- Dithionite

The verification of quinone reduction triggered release of the free transporter from the respective protransporter was conducted by treatment of the protransporter with a freshly prepared aqueous solution of sodium dithionite (Na₂S₂O₄, DTi), a well-known reducing agent used in biochemical research which is known to reduce quinones to their corresponding hydroquinone.^{34, 62} In an

aqueous medium, it can undergo anaerobic oxidation to bisulphite as described by the following chemical equation:^{63, 64}



This allows sodium dithionite to reduce quinones to hydroquinones,^{34, 62} and can hence be used to study the reduction-induced cleavage of the quinone based protransporters to release active transporters.

Changes in the absorption spectrum of **2** (50 μM in 7:3 ACN/water) on the addition of increasing amounts of DTi was monitored, and the disappearance of quinone peaks at 256 nm and 550 nm to match the absorbance spectrum of **1b** at the same concentration, indicated quinone reduction and subsequent release of the transporter (Figure 41). This was further verified through NMR by recording the ¹H NMR spectra of **2** (in 7:3 CD₃CN/D₂O) before and after treatment with DTi (20 eq.) (Figure 42). The peaks in the aromatic region of the spectrum of **2** after DTi treatment were found to match with that of **1b** (¹H NMR recorded in 7:3 CD₃CN/D₂O), thus indicating its release. Further confirmation of transporter release on reduction of quinone was obtained from high resolution mass spectrum (HRMS) of a solution of **2** treated with DTi (Figure 43) that gave a base peak corresponding to the free transporter **1b** (*m/z* [**1b**+H]⁺ calc.: 350.0615; found: 350.0619) with the quinone methide **QM** peak (*m/z* [**QM**+H]⁺ calc: 220.1337, found: 220.1342).

Similar NMR and HRMS experiments were conducted to verify the release of **1b** from **3** upon the reduction of the quinone. The aromatic region of ¹H NMR spectrum (in 7:3 CD₃CN/D₂O) after the treatment of **3** with DTi (20 eq.) confirmed the formation of **1b** (Figure 44). HRMS analysis of the same reaction mixture gave a base peak of the free transporter **1b** (*m/z* [**1b**+H]⁺ calc.: 350.0615; found: 350.0615) along with lactone **L** (*m/z* [**L**+H]⁺ calc: 235.1334, found: 235.1338), confirming transporter release (Figure 45).

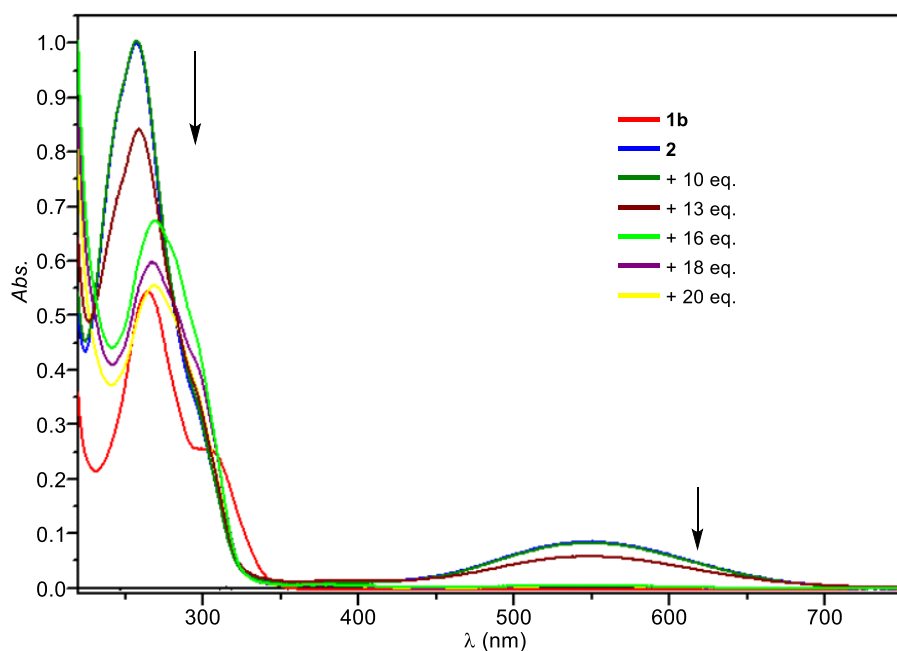


Figure 41: Absorption spectrum of **2** (50 μ M) treated with varying equivalents of sodium dithionite (DTi). Disappearance quinone peak at 550 nm was observed. Arrows indicate locations of the decrease in peak size. Solvent: ACN/water (7:3).

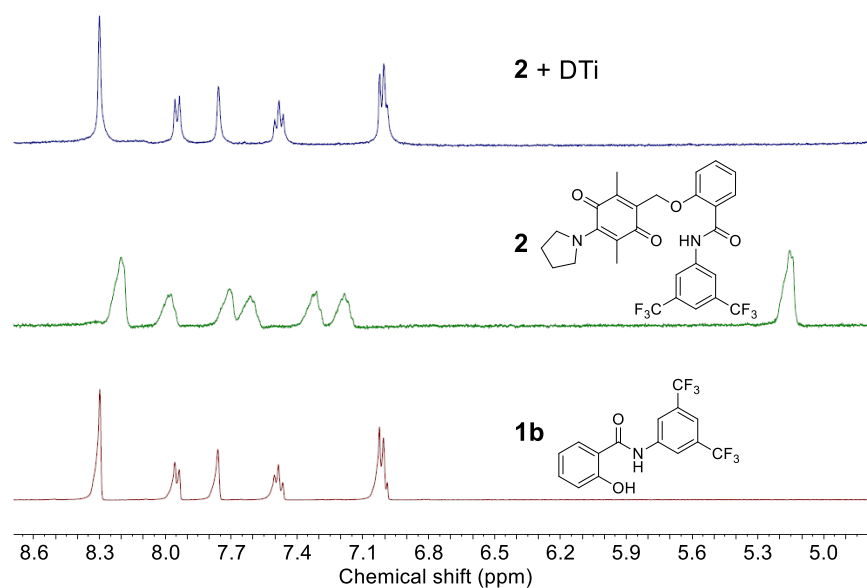


Figure 42: Comparison of ^1H NMR spectra of **2** and before and after DTi treatment (20 eq.) with spectra of **1b**. Peak structure after DTi treatment confirms quinone reduction induced release of the transporter. Solvent: 7:3 $\text{CD}_3\text{CN}/\text{D}_2\text{O}$.

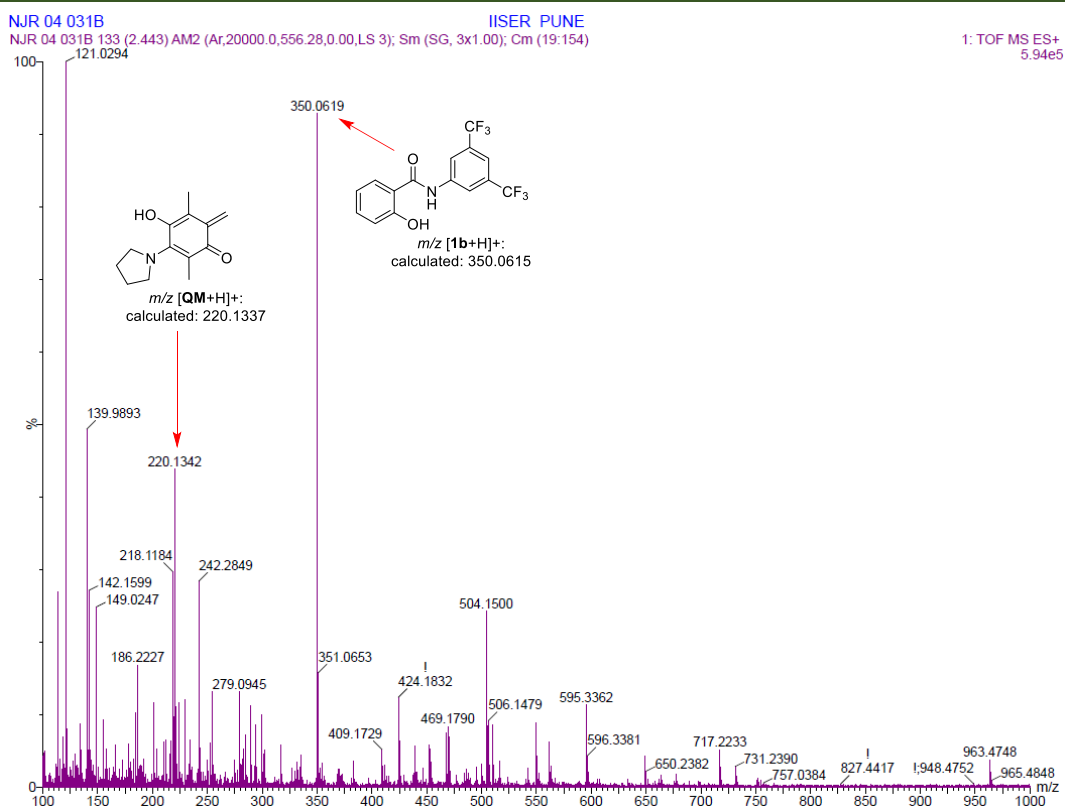


Figure 43: HRMS of **2** (in 7:3 ACN/water) after treatment with 20 eq. DTi.

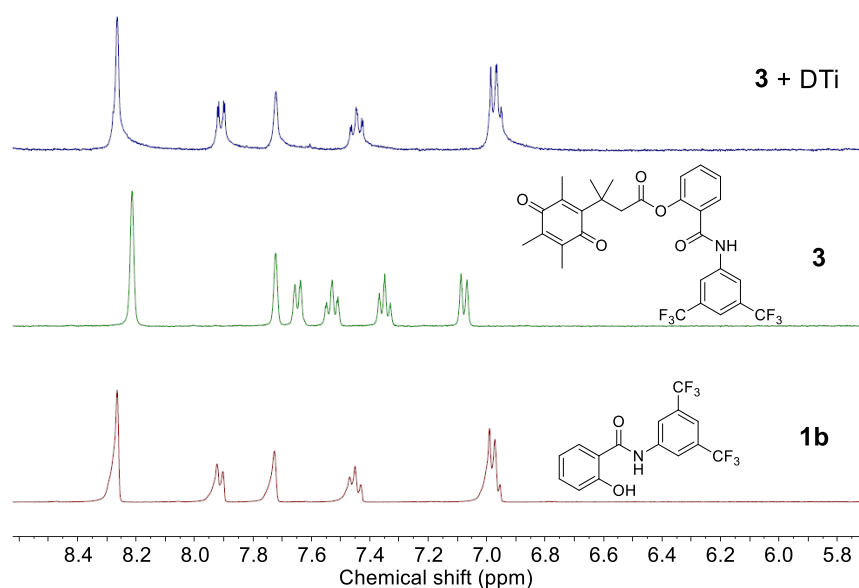


Figure 44: Comparison of ¹H NMR spectra of **3** and before and after DTi treatment (20 eq.) with spectra of **1b**. Peak structure after DTi treatment confirms quinone reduction induced release of the transporter. Solvent: 7:3 CD₃CN/D₂O.

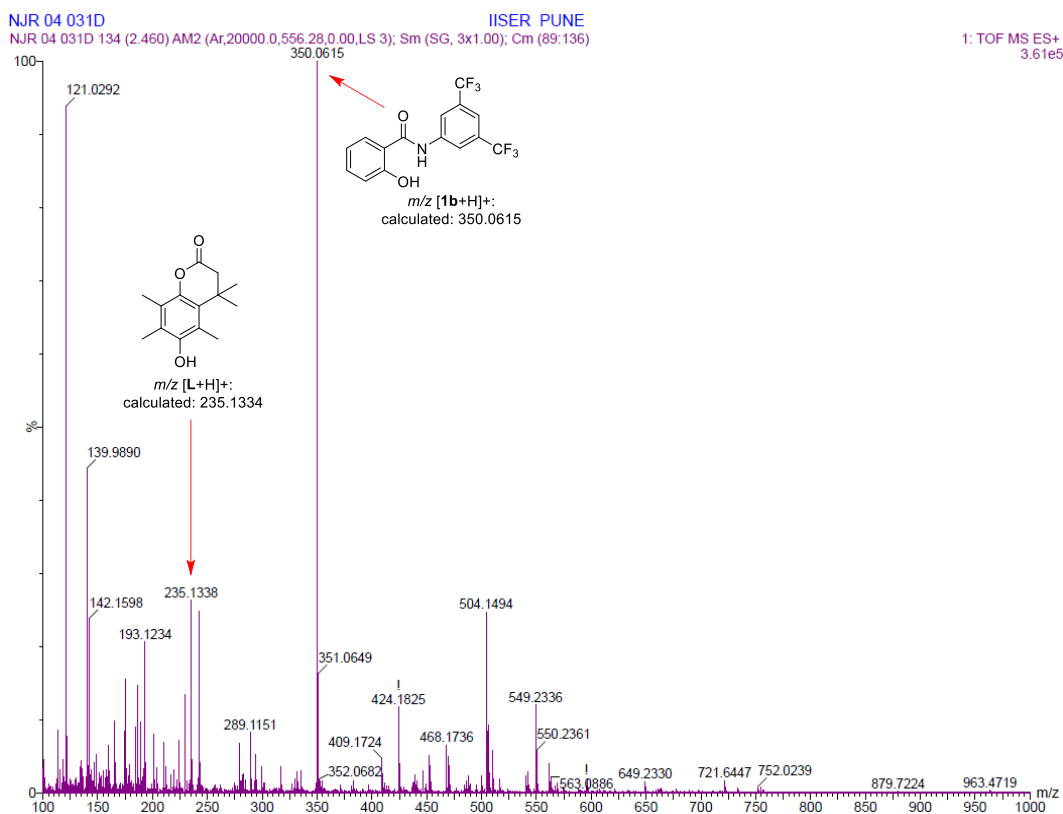


Figure 45: HRMS of **3** (in 7:3 ACN/water) after treatment with 20 eq. DTi.

2.2.8 Enzymatic (NQO1) Reduction Studies

The enzymatic reduction of **2** was verified using commercially available NQO1, by monitoring the absorbance of a reaction mixture of the protransporter (25 μ M), NQO1 (10 μ g/mL), and NADH (100 μ M) in phosphate buffer (10 mM, pH = 7.4) at 37 °C, during set time intervals using a UV-visible spectrophotometer fitted with a variable temperature cuvette holder. The partial disappearance of the quinone peak at 552 nm confirmed the enzymatic reduction of quinone, although not reaching completion even by 4 h, likely due to the quenching of the enzyme by the electrophilic quinone methide (**QM**) byproduct (Figure 46). This was resolved by setting up the same reaction in the presence of 250 μ M of *N*-acetylcysteine (NAC), a quinone methide trapping agent which also simulates the cellular environment, containing various nucleophilic species such as thiols. The complete disappearance of the quinone peak at 552 nm was observed, thus hinting towards the complete enzymatic conversion of the quinone **2** (Figure 47). The time-based decrease in absorbance at 552 nm given high substrate concentration (0-20 min) when fitted to one phase exponential decay model yielded a rate constant (k) of 0.06945 min⁻¹ and half-life of 9.981 min for the initial stages of the enzymatic reaction (Figure 48).

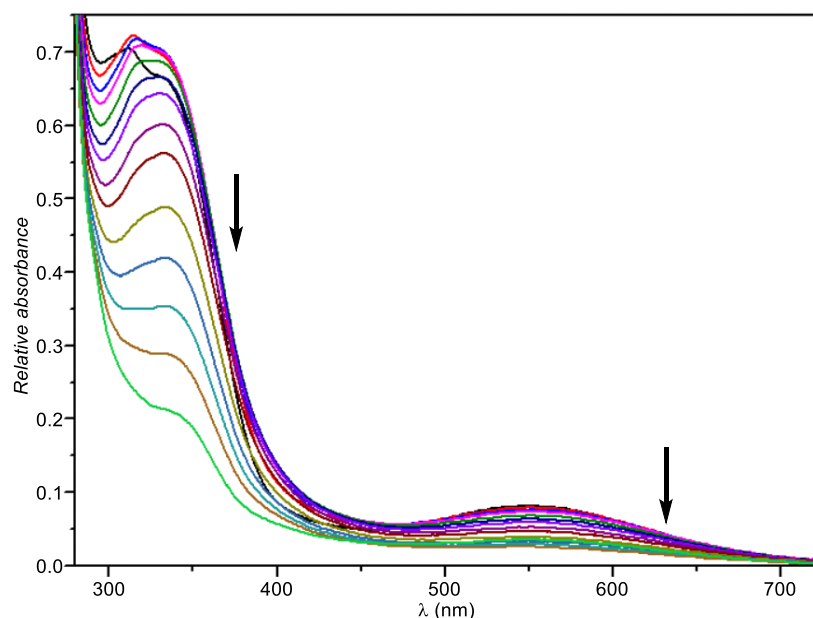


Figure 46: Change in absorbance spectrum of *reaction mixture 1*: of **2** (25 μM), NADH (100 μM), NQO1 (10 $\mu\text{g/mL}$) in phosphate buffer (10 mM), pH = 7.4, 37 $^{\circ}\text{C}$. Complete disappearance of the quinone peak at 550 nm is not observed, indicating incomplete conversion of the quinone in the given conditions.

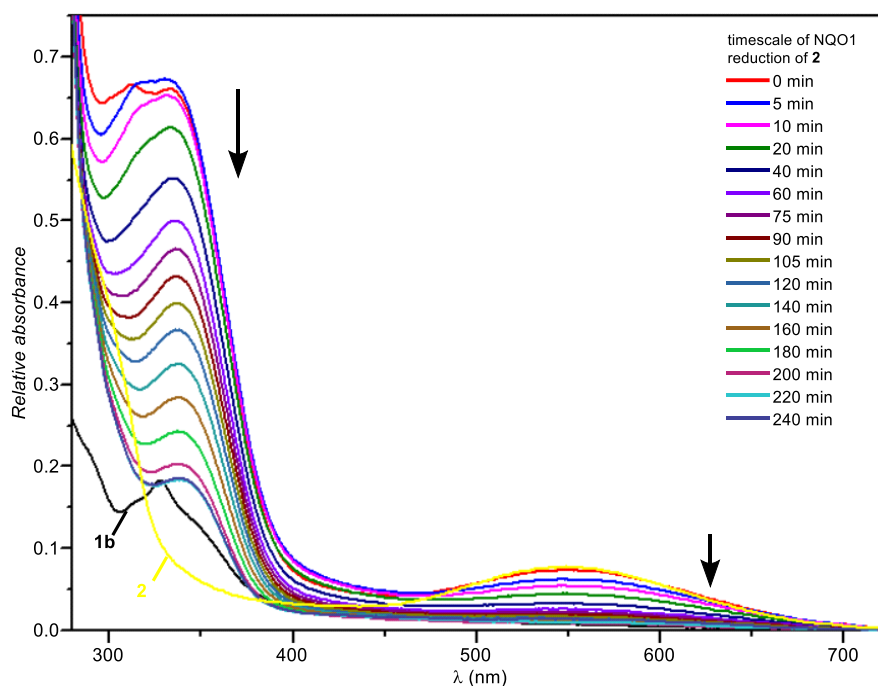


Figure 47: Change in absorbance spectrum of *reaction mixture 2*: of **2** (25 μM), NADH (100 μM), NQO1 (10 $\mu\text{g/mL}$), NAC (250 μM) in phosphate buffer (10 mM), pH = 7.4, 37 $^{\circ}\text{C}$. The complete disappearance of the quinone peak at 550 nm is observed, indicating the completion of the reaction.

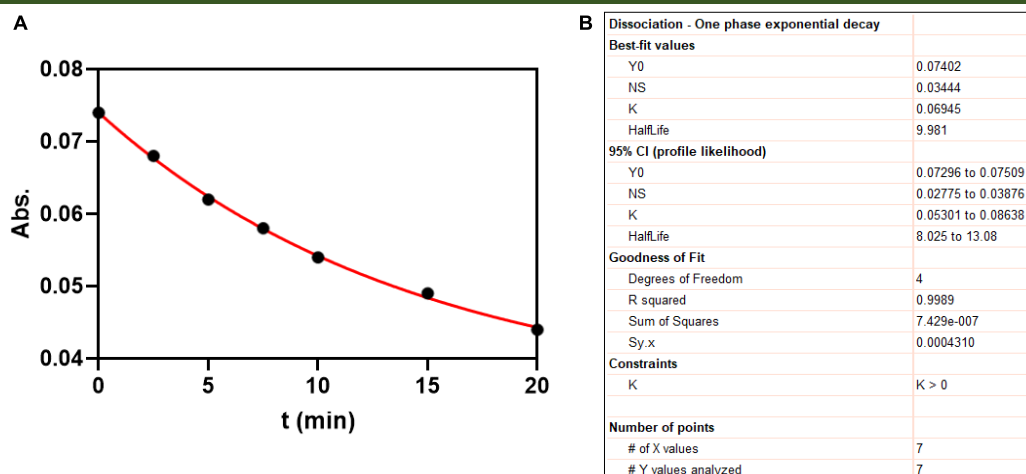


Figure 48: Fit of time-based decrease of absorbance in the initial stages of reaction in *reaction mixture 2*. Curve fitting was performed using GraphPad Prism 8.

HRMS analysis of a reaction mixture of **2** (25 μ M), NQO1 (10 μ g/mL), NADH (100 μ M) and NAC (250 μ M) in 10 mM phosphate buffer (pH = 7.4, 37 $^{\circ}$ C, 4 h) yielded mass peaks corresponding to the free transporter **1b** (m/z [**1b**+H]⁺ calc: 350.0615, found: 350.0612) (Figure 49), the quinone methide (m/z [**QM**+H]⁺ calc: 220.1337, found: 220.1340) (Figure 50), and the NAC-quinone methide adduct (m/z [**QM**-NAC+H]⁺ calc: 383.1640, found: 383.1644) (Figure 51), confirming the expected enzymatic reduction triggered rearrangement and release of free transporter.

Enzymatic reduction triggered rearrangement and release of **1b** from **3** was verified through HRMS analysis of a reaction mixture of **3** (25 μ M), NQO1 (10 μ g/mL), and NADH (100 μ M) in 10 mM phosphate buffer (pH = 7.4, 37 $^{\circ}$ C, 4 h). Mass peaks corresponding to the free transporter **1b** (m/z [**1b**+H]⁺ calc: 350.0615, found: 350.0616) (Figure 52), and the lactone byproduct (m/z [**L**+H]⁺ calc: 235.1334, found: 235.1347) (Figure 53) was observed, confirming transporter release via NQO1 reduction triggered lactonization of the trimethyl lock quinone cage.

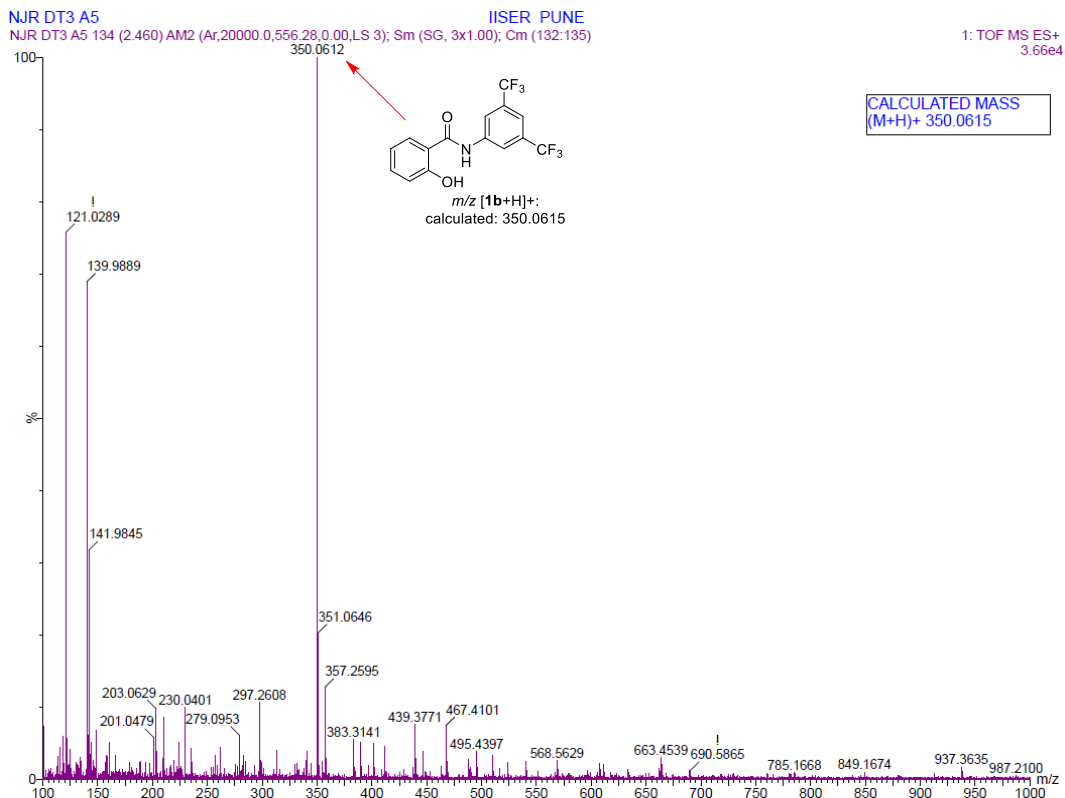


Figure 49: Mass spectrum of NQO1 reduction of 2 showing the mass peak of 1b.

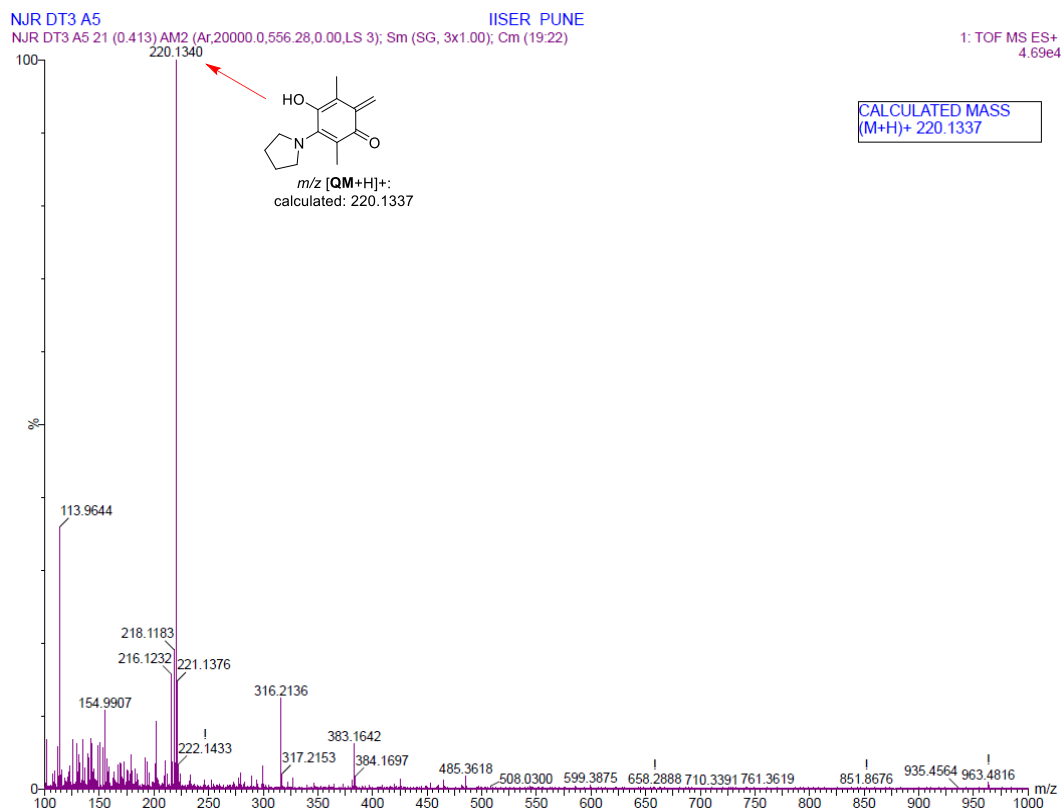


Figure 50: Mass spectrum of NQO1 reduction of 2 showing the mass peak of QM.

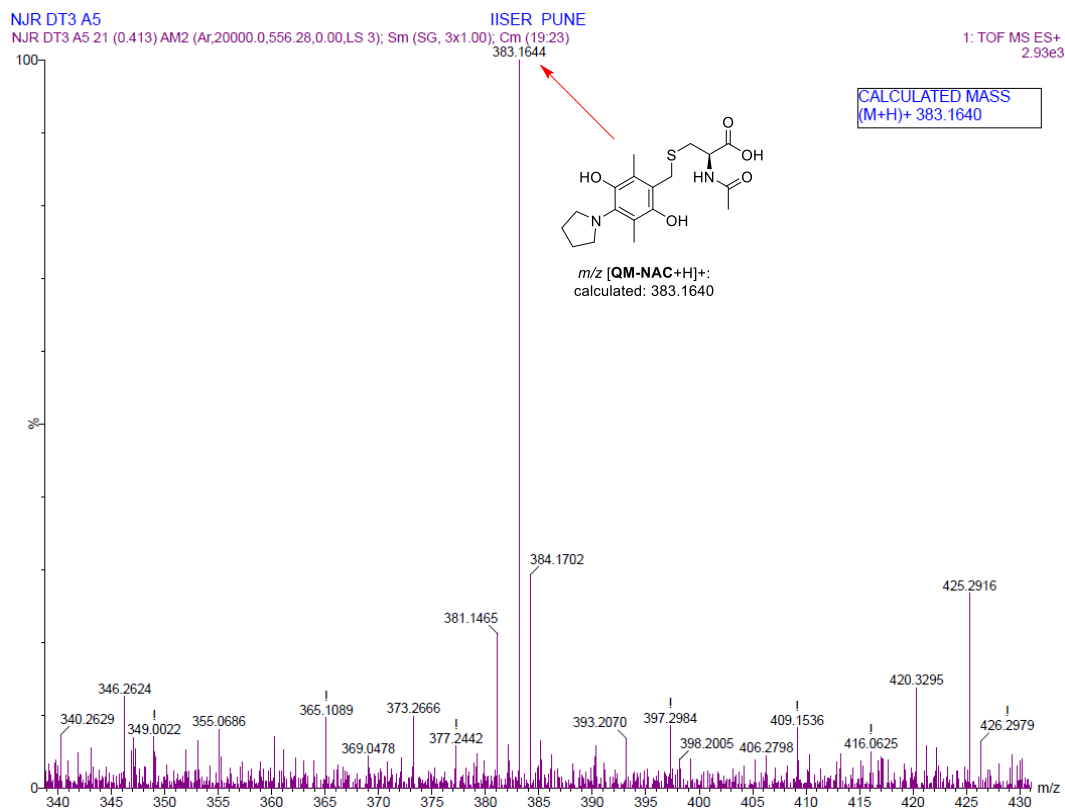


Figure 51: Mass spectrum of NQO1 reduction of **2** showing the mass peak of QM-NAC adduct.

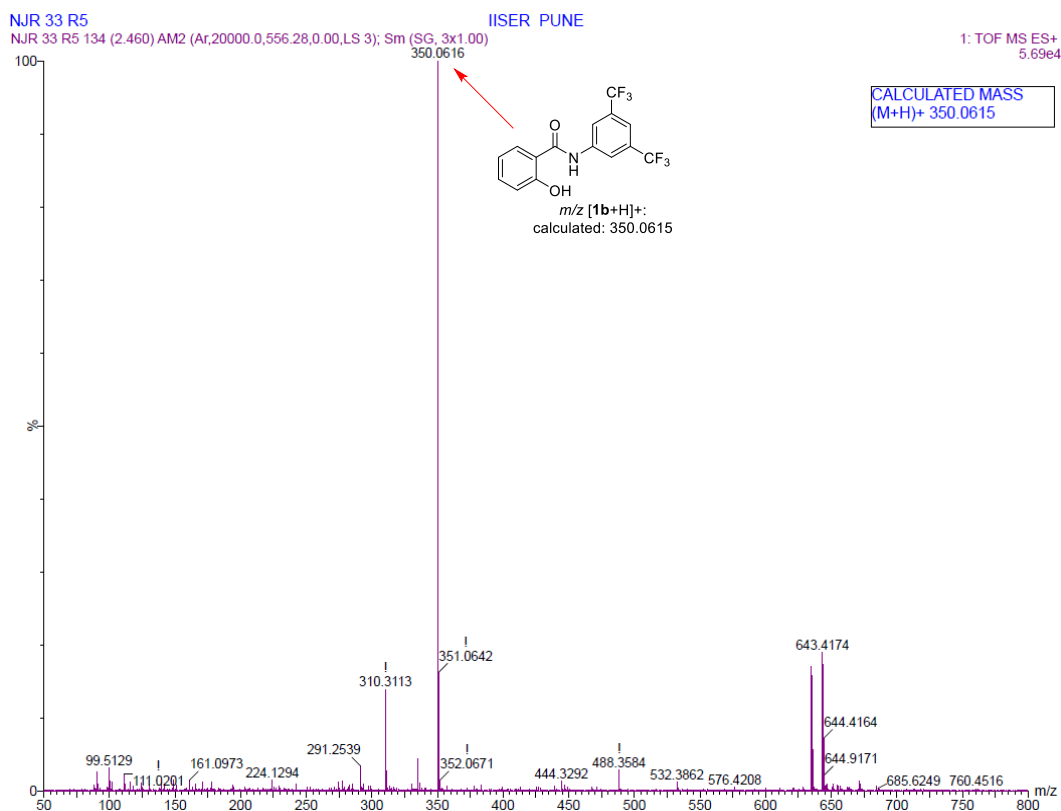


Figure 52: Mass spectrum of NQO1 reduction of **3** showing the mass peak of **1b**.

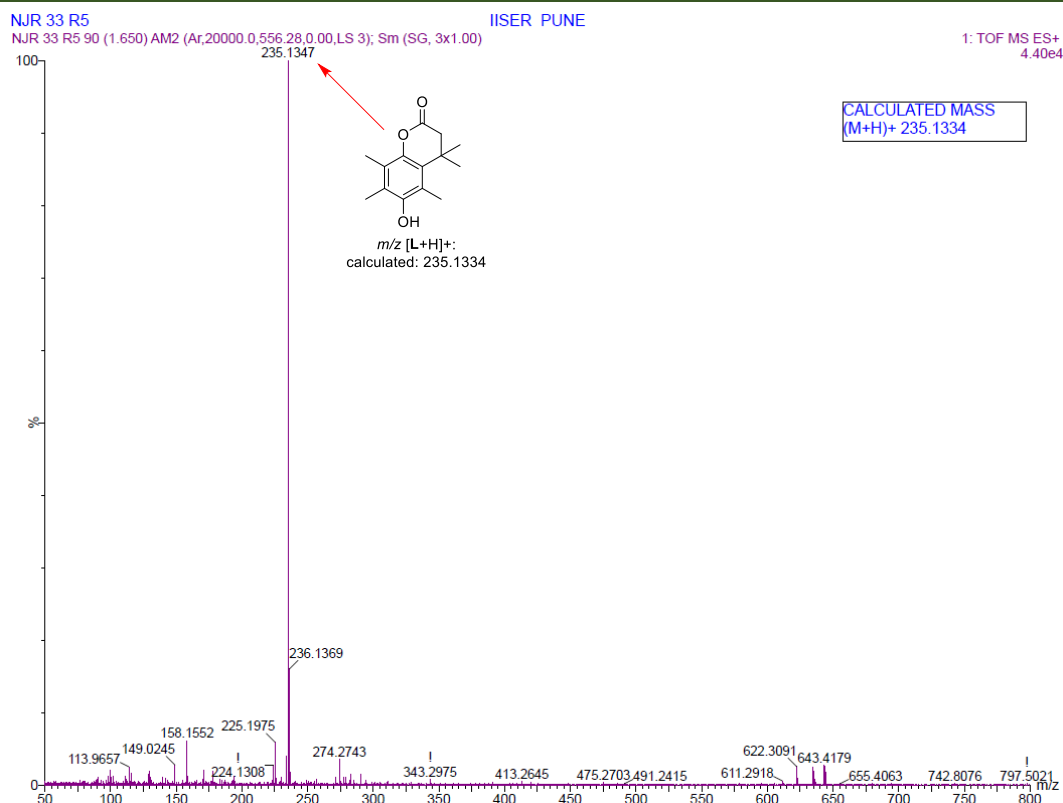


Figure 53: Mass spectrum of NQO1 reduction of **3** showing the mass peak of **L**.

NQO1 reduction triggered release of **1b** from the protransporters was further confirmed through analytical high performance liquid chromatography (HPLC) experiments. Aliquots were taken from the enzymatic reaction mixtures of **2** (RM2) and **3** (RM3) containing 50 μ M protransporter, 10 μ g/mL NQO1 and 100 μ M NADH in 10 mM phosphate buffer (pH = 7.4, 37 $^{\circ}$ C, 700 rpm shaking) at 0.5 h, 1 h, and 2 h time points, quenched by addition of an equal volume of ACN and analyzed through HPLC. An H₂O/ACN mobile phase was used for RM3, while the dragging of compound **2** in the column when injecting the reaction mixture necessitated the addition of 0.1% HCOOH to the water for analysis of RM2. The 0.5 h sample of RM2 yielded two peaks corresponding to the released transporter **1b** (retention time (RT) = 11.1 min) and the unreacted protransporter **2** (RT = 21.8 min), which were confirmed by spiking the reaction mixture aliquots with 25 μ M of pure **1b** (Figure 54B) and **2** (Figure 54C) respectively which resulted in an increase in the peak intensities. Similar spiking experiments were conducted with the 0.5 h aliquot of RM3 to confirm the presence of both the free transporter **1b** (RT = 13.2 min, Figure 55B) and protransporter **3** (RT = 20.8 min, Figure 55C). The presence of lactone **L** was also confirmed in RM3 using the pure compound as a reference (Figure 55A). Analysis of traces of reaction time

points (0.5 h, 1 h, and 2 h) showed a time dependent consumption of the protransporters (Figure 54A, 55A) in the reaction mixtures. This was better visualized by plotting the area under the curve (AUC) for **2** (Figure 54D) and **3** (Figure 55D) versus reaction time, from which the rate of consumption of **3** was observed to be faster.

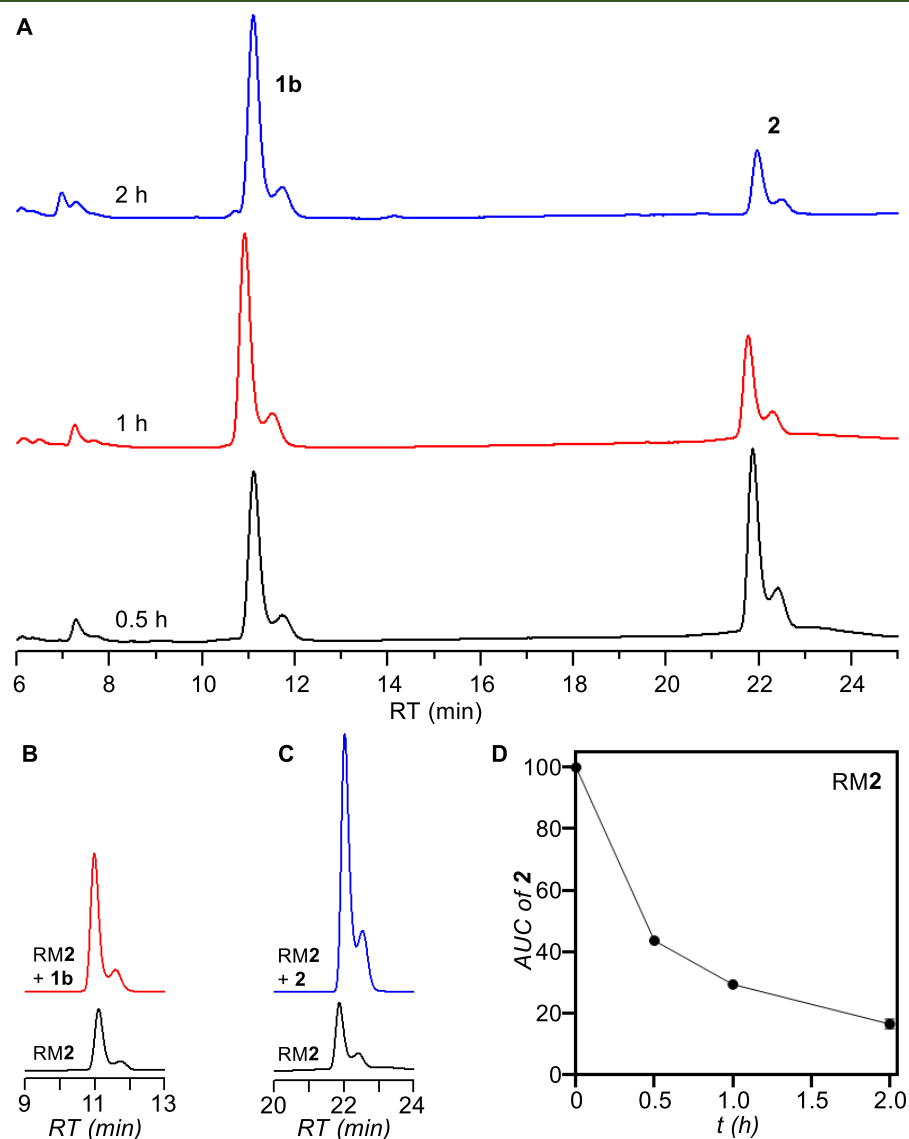


Figure 54: (A) HPLC analysis NQO1 reduction induced activation of protransporter 2. Reaction conditions (RM2)- 2 (50 μ M), NADH (100 μ M) and NQO1 (10 μ g/mL) in 10 mM PB (pH = 7.4). Reduction in protransporter (2) peak accompanied by increment of the free transporter (1b) peak was observed. Peak shoulders are due to the presence of HCOOH in the eluent. Spiking of the reaction mixture of 2 (RM2) at 30 min with 25 μ M of (B) 1b and (C) 2, respectively, showing the increase in the respective peak intensities, confirming positions of the transporter and protransporter peaks. (D) Plot of area under the curve (AUC) of 2 in RM2 with respect to time, indicating consumption of the protransporter on reduction by NQO1. Detector (DAD) set to 280 nm. Mobile phase: 0.1% HCOOH in H₂O/ACN.

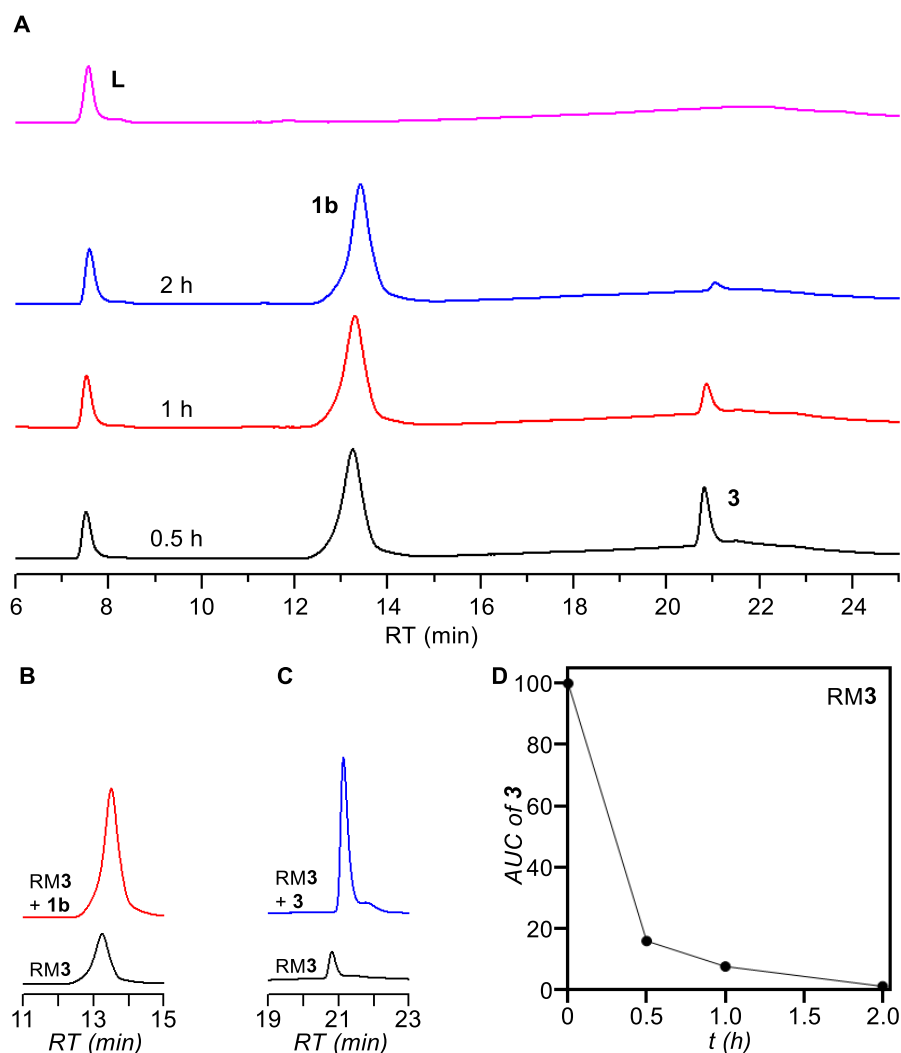


Figure 55: (A) HPLC analysis NQO1 reduction induced activation of protransporter 3. Reaction conditions (RM3)- 3 (50 μ M), NADH (100 μ M) and NQO1 (10 μ g/mL) in 10 mM PB (pH = 7.4). Reduction in protransporter (3) peak accompanied by increment of the free transporter (1b) peak was observed. Lactone L was used as a reference for the enzyme cleavage byproduct. Spiking of reaction mixture of 3 (RM3) at 30 min with 25 μ M of (B) 1b and (C) 3 respectively showing increase in the respective peak intensities, confirming positions of the transporter and protransporter peaks. (D) Plot of area under the curve (AUC) of 3 in RM3 with respect to time, indicating consumption of the protransporter on reduction by NQO1. Detector (DAD) set to 280 nm. Mobile phase: H₂O/ACN.

Stability analyses of the protransporters in phosphate buffer (37 °C, 2 h) ruled out decomposition in the absence of NQO1 (Figure 56). The presence of shoulder peaks in RM2 is attributed to the presence of HCOOH in the mobile phase. This was verified by purity analysis of **2** in H₂O/ACN mobile phase (without HCOOH), which showed only a single peak in HPLC trace (Figures 57, 58). Purity analysis of **3** in H₂O/ACN yielded a single peak of purity >95% (Figure 59).

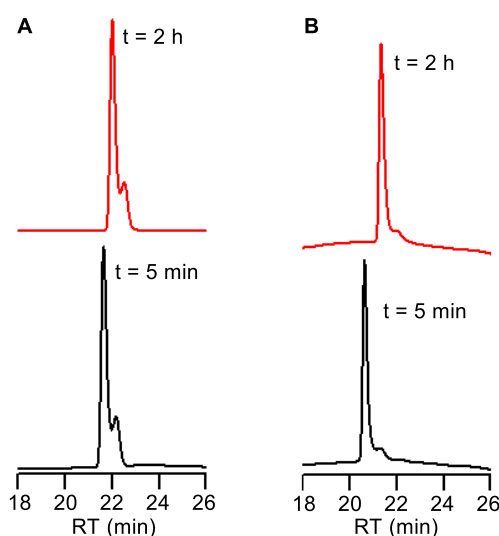


Figure 56: HPLC traces of stability assay of (A) **2** (mobile phase- 0.1% HCOOH in H₂O/ACN) and (B) **3** (mobile phase- H₂O/ACN) in 10 mM PB (pH = 7.4). Area under the peak (AUC) remains constant in both cases.

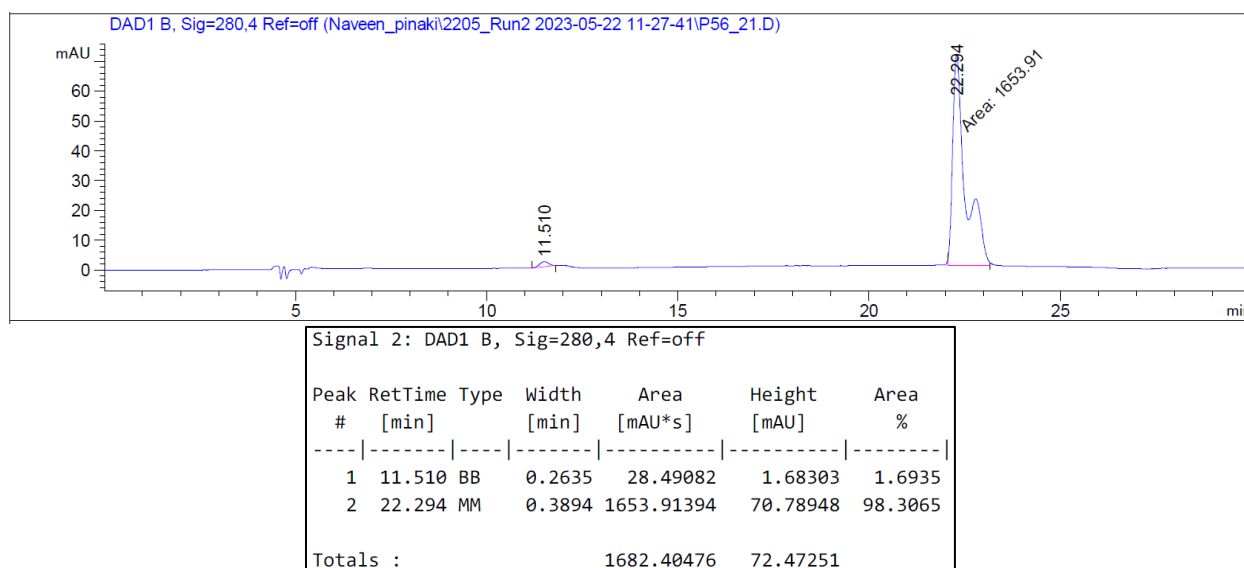


Figure 57: Purity analysis of **2** using 0.1% HCOOH in H₂O/ACN as mobile phase showing >95% purity for the compound. The shoulder observed in the compound peak is due to the effect of HCOOH present in the eluent.

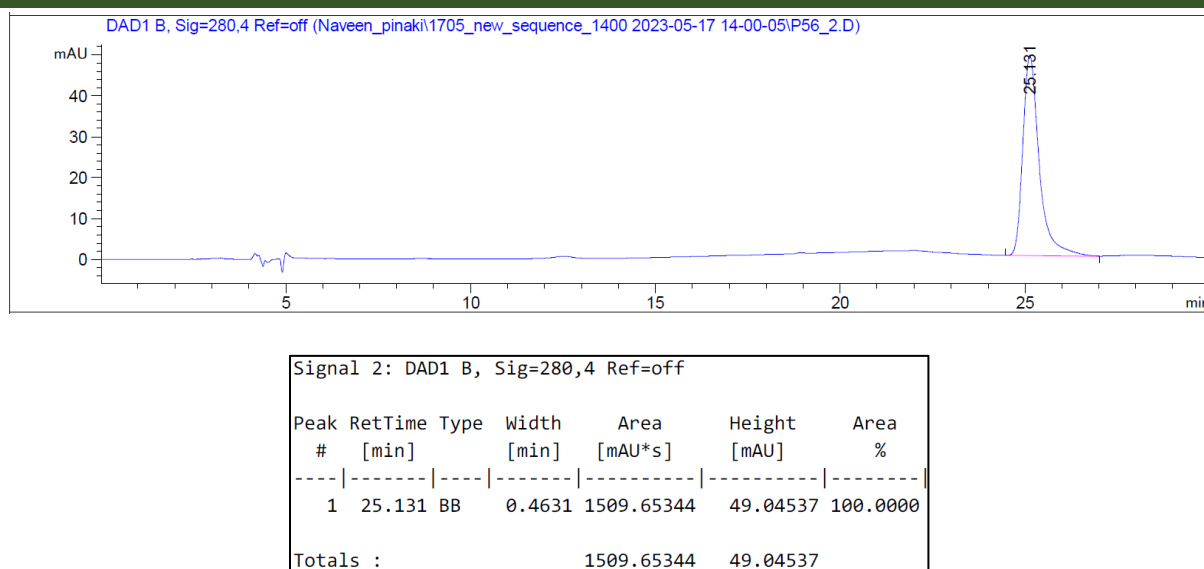


Figure 58: Purity analysis of **2** using H₂O/ACN as mobile phase showing >95% purity for the compound. Only a single peak was observed, confirming the presence of shoulder peak in the previous run (Figure 57) was due to the presence of HCOOH in the eluent.

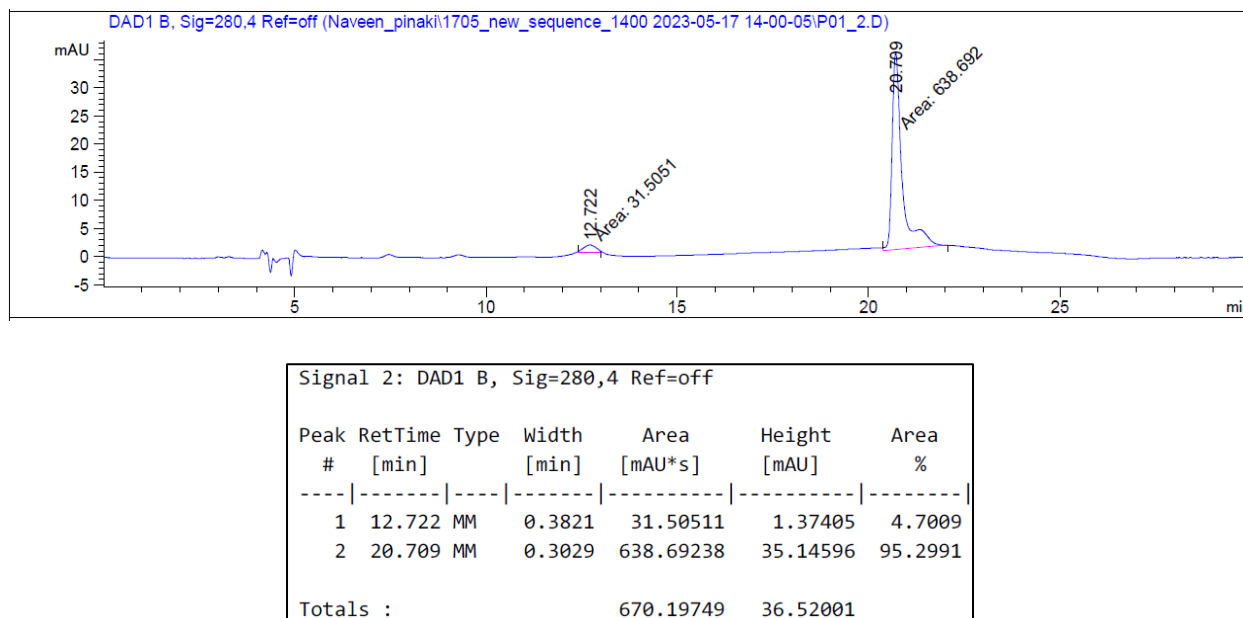


Figure 59: Purity analysis of **3** using H₂O/ACN as mobile phase showing >95% purity for the compound.

2.2.9 Biological Studies

Previous reports from our and other groups have shown that H⁺/Cl⁻ symport across the membrane is capable of inducing apoptosis in cells via disruption of ionic homeostasis. An initial screen of the salicylamide transporters (**1a-1e**) in MCF-7 cells (at 10 μ M concentration) showed that **1b**, the best working ion transporter in vesicles from our in vitro studies, was the most effective in inducing cytotoxicity in these cells (Figure 60). Thus, investigation of the effect of compound **1b** and its NQO1 activable protransporters (**2, 3**) on the viability of two cancerous (MCF-7 (human breast cancer), A549 (Adenocarcinoma human alveolar basal epithelial cells)) and non-cancerous cell lines (mouse embryonic fibroblasts (MEFs), human embryonic kidney (HEK293T) cells) was conducted by performing MTT assays (concentration range 0 – 20 μ M). Compounds **1b** (IC₅₀ – 7.2 μ M), **2** (IC₅₀ – 8.7 μ M) and **3** (IC₅₀ – 8.5 μ M) showed higher cytotoxicity in MCF-7 cells (Figure 61), while these were relatively non-toxic in the A549 cells (Figure 62), HEK293 cells (Figure 63) and MEFs (Figure 64). Since, the highest toxicity was observed for compound **1b** and its protansporters – **2, 3** in MCF-7 cells, all further experiments were performed in these cells.

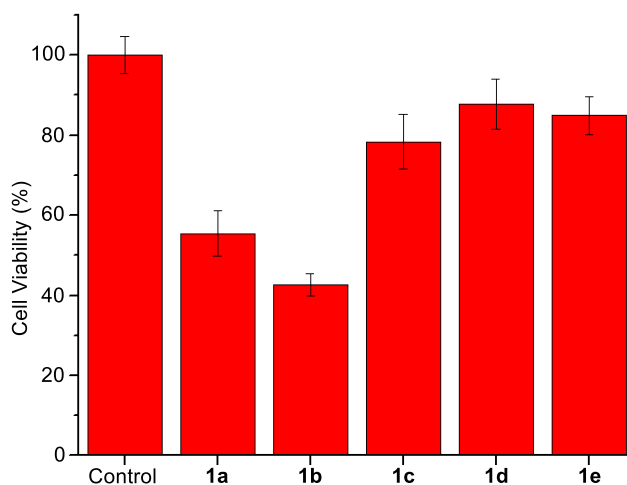


Figure 60: Comparison of cellular viability, as assessed by the MTT assay, in response to treatment of MCF-7 cells with free transporter library (**1a-1e**) at 10 μ M concentration.

Table 6: IC₅₀ Values for compound **1b**, its protransporters **2**, **3**, and controls **L**, **BA**, and **7**, as obtained by performing the MTT assay.

	1b (μM)	2 (μM)	3 (μM)	BA	L	7
MCF-7	7.2	8.7	8.5	Less toxic		
A549	Non-toxic					
MEFs	Non-toxic					Less toxic
HEK293T	Less toxic	Non-toxic				Less toxic

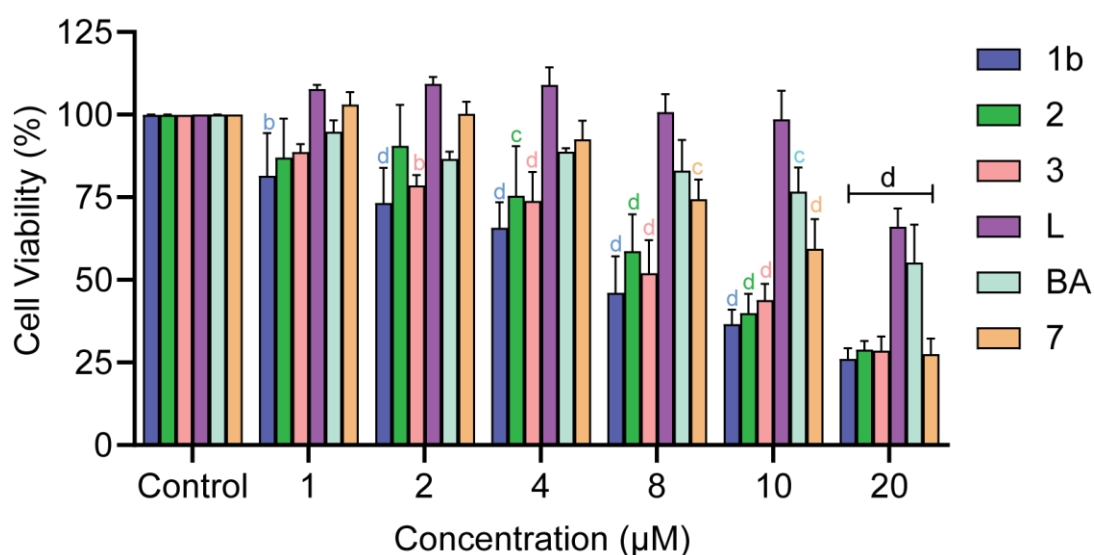


Figure 61: Cellular viability, as assessed by the MTT assay, in response to increasing concentrations for compound **1b**, its protransporters **2**, **3**, and controls **L**, **BA**, **7** in MCF-7 cell line. The untreated wells (Control) were set at 100% viability and the relative treatment for the different compounds has been depicted. a represents $p < 0.05$; b represents $p < 0.01$; c represents $p < 0.001$; and d for $p < 0.0001$ when compared to untreated control using Tukey HSD tests.

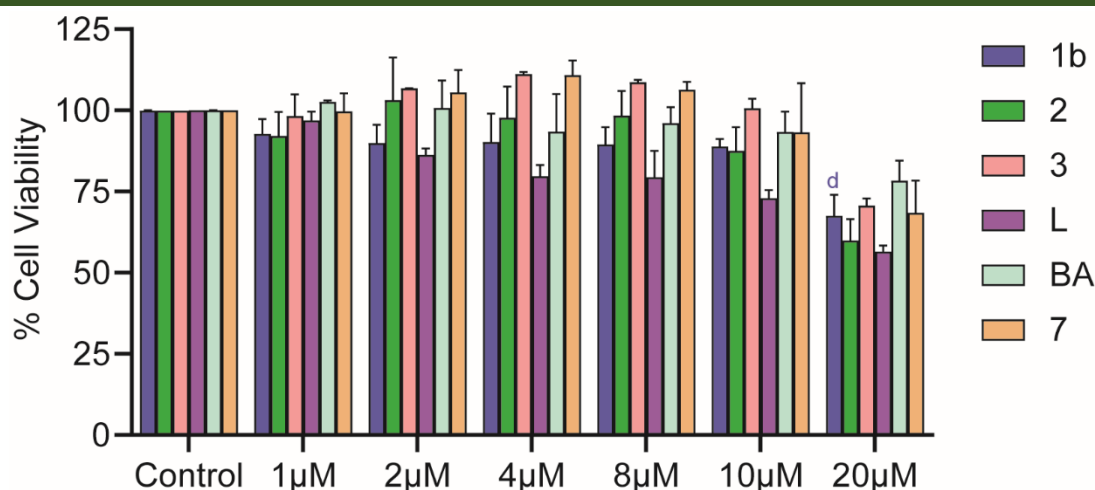


Figure 62: Cellular viability, as assessed by the MTT assay, in response to increasing concentrations for compound **1b**, its protransporters **2**, **3**, and controls **L**, **BA**, **7** in A549 cells. The untreated wells (Control) were set at 100% viability and the relative treatment for the different compounds has been depicted. a represents $p < 0.05$; b represents $p < 0.01$; c represents $p < 0.001$; and d for $p < 0.0001$ when compared to untreated control using Tukey HSD tests.

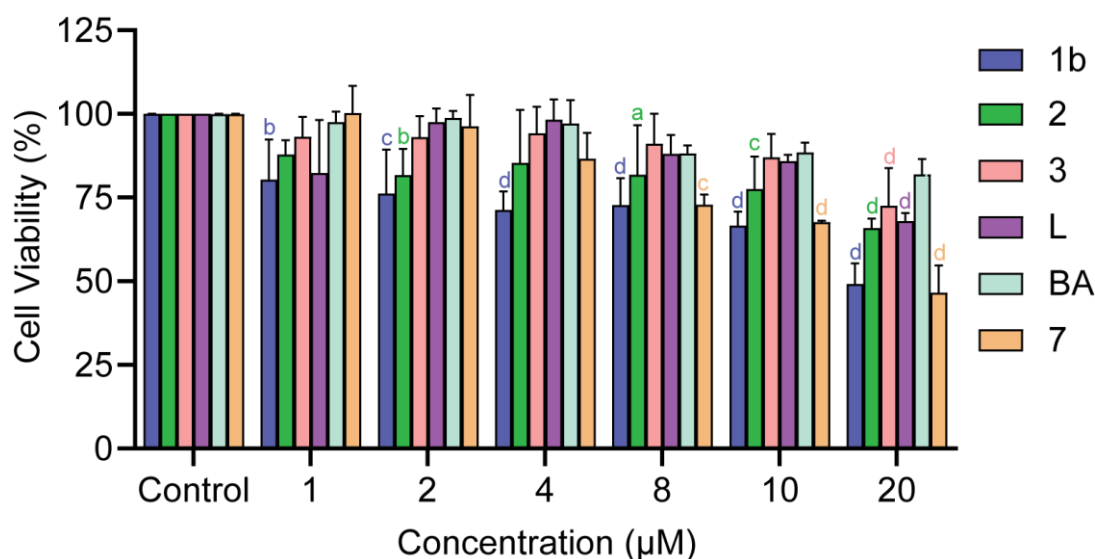


Figure 63: Cellular viability, as assessed by the MTT assay, in response to increasing concentrations for compound **1b**, its protransporters **2**, **3**, and controls **L**, **BA**, **7** in HEK293T cells. The untreated wells (Control) were set at 100% viability and the relative treatment for the different compounds has been depicted. a represents $p < 0.05$; b represents $p < 0.01$; c represents $p < 0.001$; and d for $p < 0.0001$ when compared to untreated control using Tukey HSD tests.

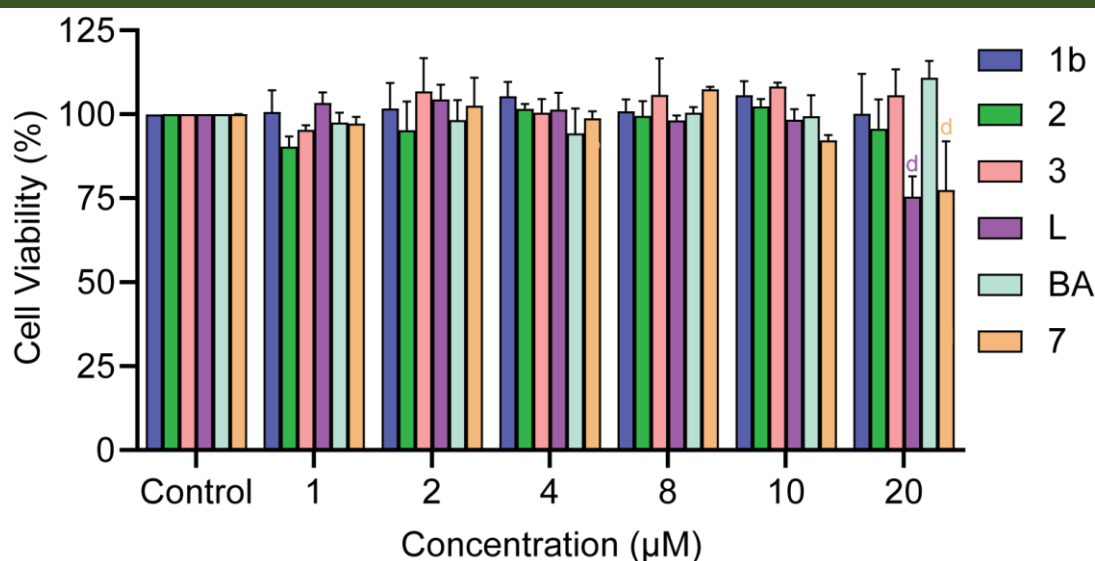


Figure 64: Cellular viability, as assessed by the MTT assay, in response to increasing concentrations for compound **1b**, its protransporters **2**, **3**, and controls **L**, **BA**, **7** in MEFs. The untreated wells (Control) were set at 100% viability and the relative treatment for the different compounds has been depicted. a represents $p < 0.05$; b represents $p < 0.01$; c represents $p < 0.001$; and d for $p < 0.0001$ when compared to untreated control using Tukey HSD tests.

The cytotoxicity of the byproducts following NQO1 activation of protransporters **2** and **3** were evaluated using controls **7** (generates byproduct **QM** *in situ* along with non-cytotoxic,⁶⁵ non-transporting **BA**) and **L** (the byproduct), respectively. Both controls were found to be relatively less cytotoxic in MCF-7 (Figure 61) and HEK293 cells (Figure 63) in comparison to **2** and **3**, indicating no significant contribution/synergy of byproducts in protransporter activity.

MCF-7 cells were found to undergo apoptosis (as observed by the increase in the cells in the G0 phase of the cell cycle) in the presence of compounds **1b**, **2**, and **3** in a dose-dependent manner (Figure 65). Indeed, higher permeation of the nucleic acid dye, propidium iodide, was observed in the MCF-7 cells treated with as low as 5 μM **1b** (Figure 66). The increase in cytotoxicity was associated with an increase in the generation of mitochondrial ROS species (Figure 67). Similarly, G0 arrest and increase in propidium iodide uptake were observed in MCF-7 cells upon treatment with the protransporters (**2**, **3**), along with significant ROS increase (Figures 65-67, respectively).

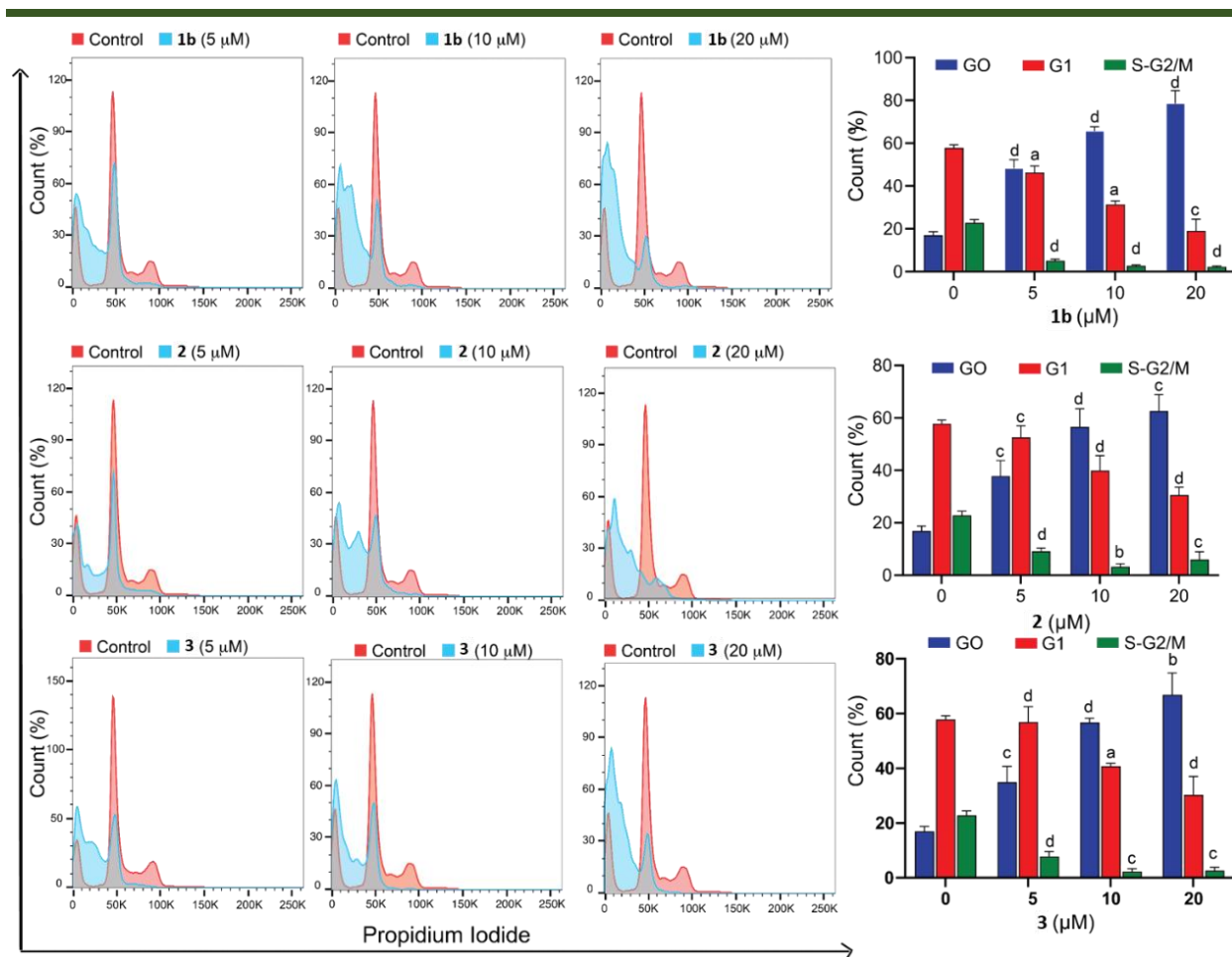


Figure 65: Representative cell cycle profiles for MCF-7 cells exposed to 5 μ M, 10 μ M, and 20 μ M of (A) compound **1b**, and its protransporters (C) **2**, (E) **3**. The quantitation of the cells in the different phases of the cell cycle has been depicted in (B), (D) and (F) for compound **1b**, and its protransporters **2** and **3**, respectively. a represents $p < 0.05$; b represents $p < 0.01$; c represents $p < 0.001$; and d for $p < 0.0001$ when compared to untreated control using Tukey HSD tests.

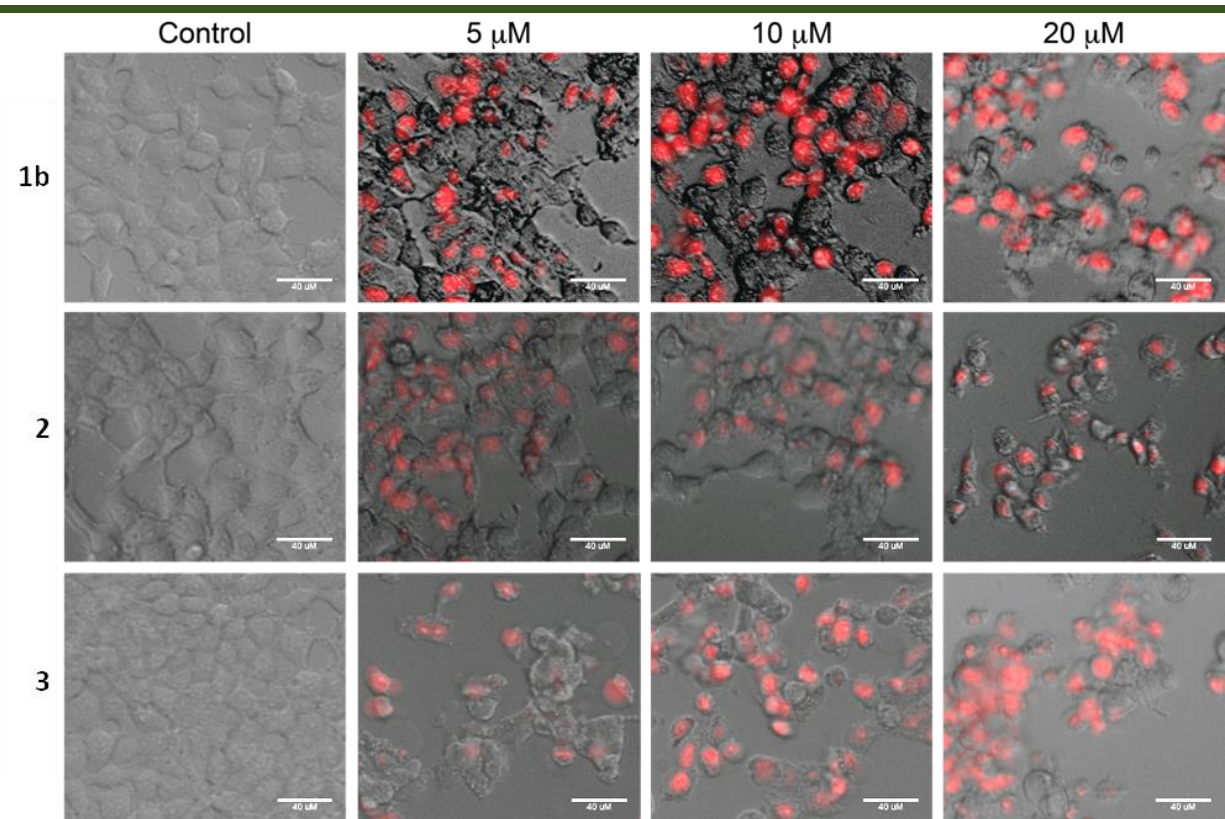


Figure 66: Live cell imaging of MCF-7 cells treated with and without increasing concentrations of compound **1b** and its protransporters **2**, **3** for 6 h, followed by staining with propidium iodide. The dead cells take up propidium iodide and appear red under the fluorescent microscope. Magnification: 20X; Scale Bar: 40 μ M.

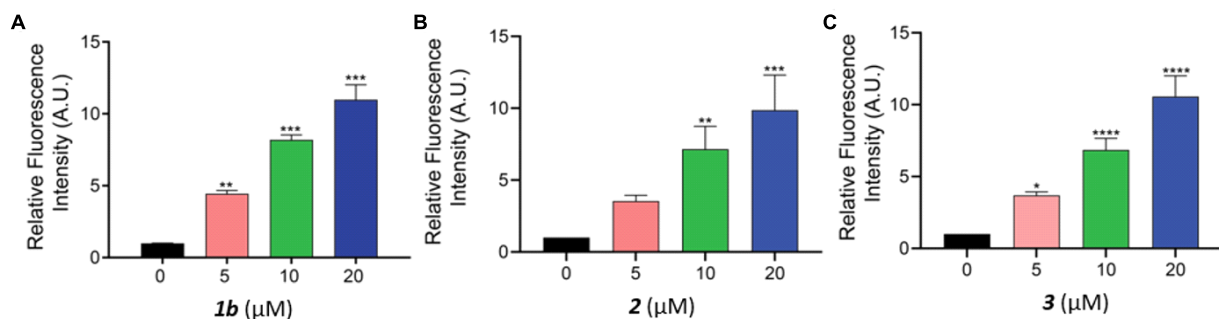


Figure 67: Measurement of Mitochondrial ROS production in MCF-7 cells incubated with increasing concentrations of a) **1b**, and its protransporters b) **2** and c) **3** using MitoSox Red. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; and **** for $p < 0.0001$ when compared to untreated control using Tukey HSD tests.

Increased ROS levels in the cell lead to a change in the mitochondrial membrane permeability, thereby resulting in the alteration of mitochondrial membrane potential. This is associated with the release of cytochrome *c* from the mitochondria into the cytosol, thereby triggering apoptosis in cells. MMP was assessed by using the MMP-sensitive JC-1, a cationic dye that exhibits MMP-dependent accumulation in mitochondria, where JC-1 forms J-aggregates which emit red fluorescence (~590 nm) in the healthy mitochondrial membrane, while J-monomers emit green fluorescence (~535 nm) in depolarized mitochondria. The addition of **1b** in MCF-7 cells, followed by incubation with JC-1 dye, showed a dose-dependent decrease in red fluorescence and an increase in green fluorescence (Figures 68, 69), thereby indicating a loss of MMP. Similar results were obtained for its protransporters (**2**, **3**) (Figure 68, 69).

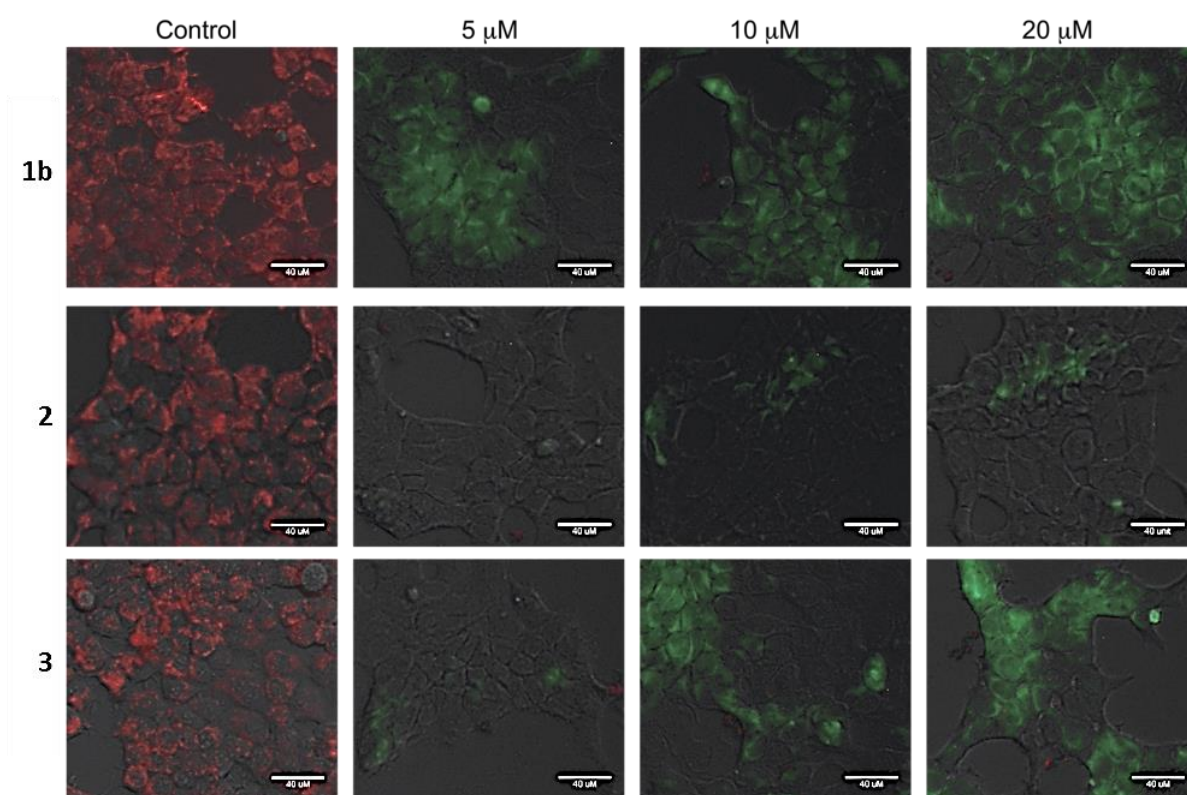


Figure 68: Representative merged images of JC-1 stained MCF 7 cells after treatment with different concentrations (0 (control), 5, 10, and 20 μ M) of compound **1b** and its protransporters **2**, **3** for 24 h. Magnification: 20X; Scale Bar: 40 μ M.

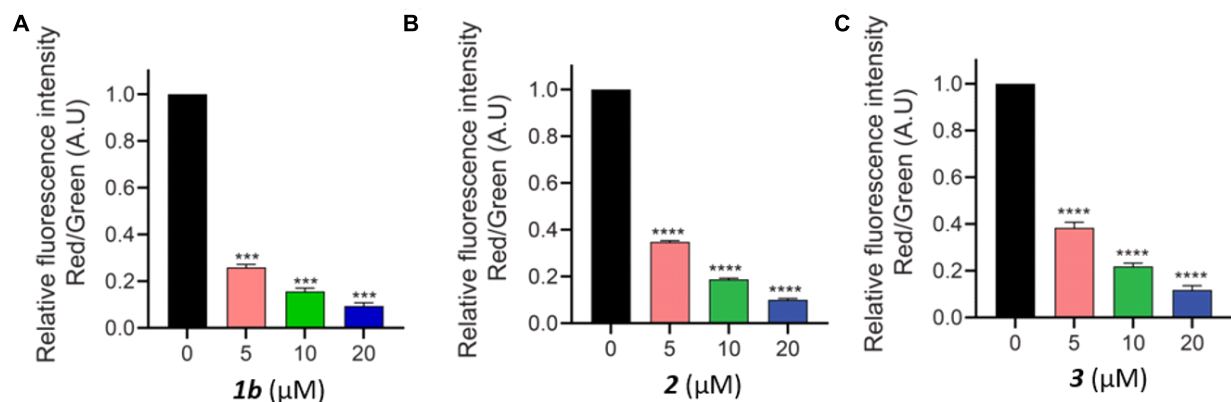


Figure 69: Quantification of change in the mitochondrial membrane potential (expressed as change in red/green fluorescence) with increasing concentrations of compound **1b**, and its protransporters **2**, **3** in MCF-7 cells. * represents $p < 0.05$; ** represents $p < 0.01$; *** represents $p < 0.001$; and **** for $p < 0.0001$ when compared to untreated control using Tukey HSD tests.

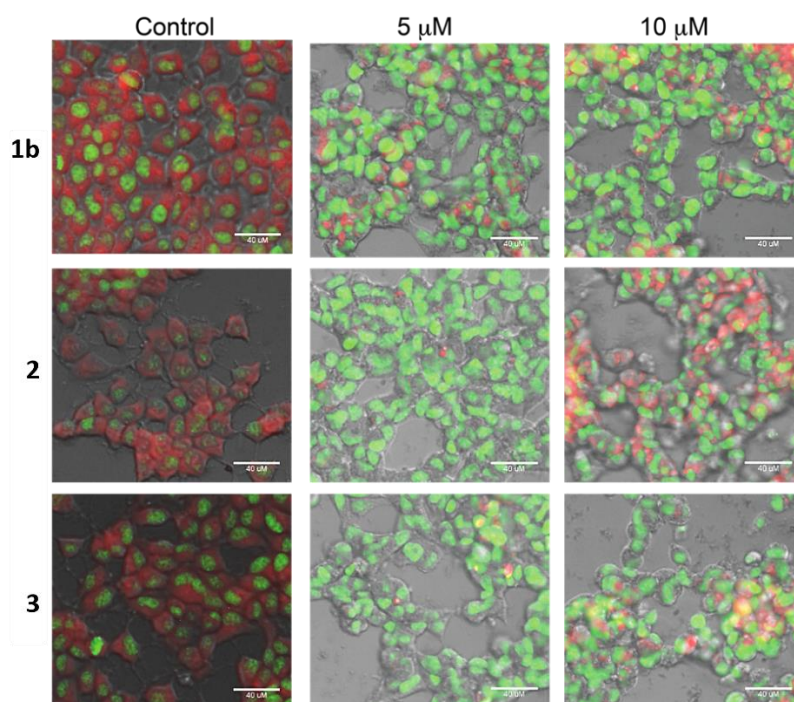


Figure 70: Live cell imaging of MCF-7 cells upon treatment with increasing concentrations of compound **1b**, and its protransporters **2**, **3** after staining with acridine orange dye. Magnification: 20X; Scale Bar: 40 μM.

The compound **1b** and its protransporters showed significant H^+/Cl^- transport through the membranes, which was associated with cytotoxicity. Thus, to understand the effect of **1b** (0, 5, and 10 μM) on the acidification of lysosomes, staining with AO was performed. A dose-dependent

decrease in red fluorescence and an increase in green fluorescence (a drop in acidity and an increase in pH) was observed (Figure 70). Similarly, in its protransporters (**2**, **3**) showed a dose-dependent increase in the lysosomal pH (Figure 70). These results indicate that the H⁺/Cl⁻ symport induced perturbations in the lysosomal pH and thus induced cytotoxicity in these cells.

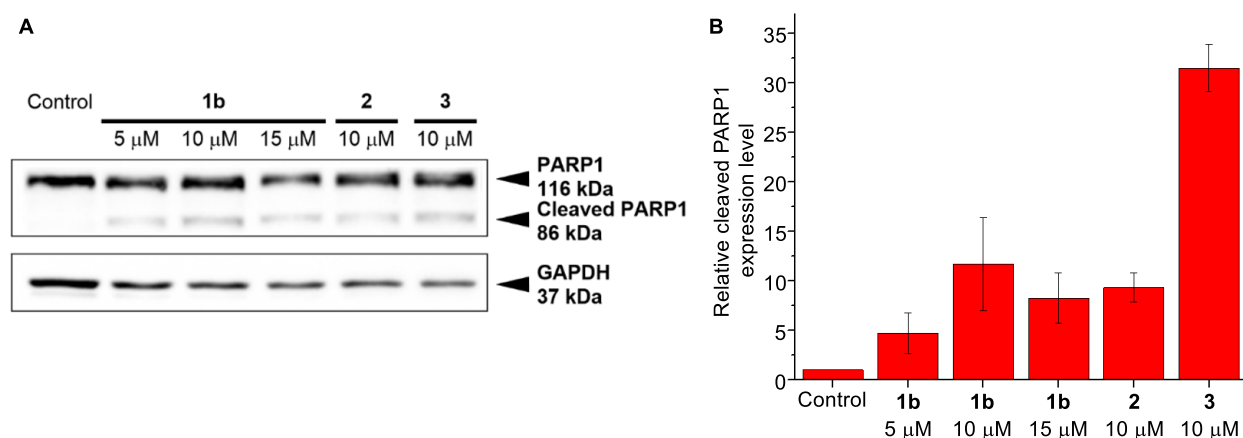


Figure 71: (A) Immunoblot assay of PARP1 cleavage in MCF-7 cells treated with transporter **1b** (5, 10 and 15 μM), and protransporters **2** (10 μM) and **3** (10 μM). (B) Relative levels of cleaved PARP1 expression with respect to GAPDH. DMSO treated cells were used as the negative control.

The cleavage of poly(ADP-ribose) polymerase-1 (PARP1) is a salient feature of apoptosis.⁶⁶ PARP1 plays a key role in DNA damage repair^{67, 68} and is cleaved by endogenous caspases during apoptosis to prevent futile DNA repair.^{66, 67, 69} MCF-7 cells treated with varying concentrations of the transporter **1b** were probed for PARP1 cleavage through immunoblot analysis. The expression of cleaved PARP1 was observed for treatment as low as 5 μM for **1b**, thus indicating towards the induction of apoptosis. PARP1 cleavage was also verified in cells treated with protransporters (10 μM each) (Figure 71A). The amounts of cleaved PARP1 expression for different compound treatments were quantified using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a loading control (Figure 71B).

2.3 Conclusions

In this work, an NQO1 enzyme activable ion transporter system has been presented that has possible application as a cancer tissue selective-ion transporting drug. Salicylamides were established to be efficient H⁺/Cl⁻ symporters capable of inducing cytotoxicity in cancer cells

through disruption of ion homeostasis. The ion transport activity of the salicylamide system was shown to be caged by blocking the OH group, and this strategy utilized to develop two quinone-tagged protransporters, activable by the cell protecting enzyme NQO1, which is overexpressed in cancer cells. The release of free transporter on enzymatic reduction of the protransporters was established through HRMS studies. The protransporters showed significantly higher toxicity in MCF-7 breast cancer cells when compared to non-cancerous HEK293T cells and MEFs. The anticancer activity of the protransporters **2** and **3** was established to be predominantly due to the release of active transporter **1b**. No synergistic effect was observed in the activity of protransporter **2** due to the quinone methide (**QM**) byproduct released after its bio-reduction. Both the transporter and its NQO1 activable protransporters were capable of inducing apoptotic cell death in MCF-7 cells by triggering mitochondrial membrane depolarization. This was associated with increased mitochondrial ROS generation, lysosomal deacidification and PARP1 cleavage. We are currently attempting attachment of these NQO1 activable quinones (pyrrolidinobenzoquinone and trimethyl lock quinone) groups to more complex ion transport systems for the creation of new, cancer tissue selective protransporters with better efficiency.

2.4 Experimental Section

2.4.1 General Methods

Reagents and solvents for synthesis were purchased from commercial sources (Sigma-Aldrich, TCI, Spectrochem, Rankem, Avra, SRL) and used without further purification. Purifications via column chromatography were performed on Spectrochem silica gel (100-200 mesh) (or 230-400 mesh silica gel where specified). Thin layer chromatography (TLCs) was performed on Merck silica gel 60-F254 plates. Egg yolk phosphatidylcholine (EYPC) as a chloroform solution (25 mg/mL), polycarbonate membranes of 100 nm and 200 nm pore size, and mini extruder set for vesicle preparation were purchased from Avanti Polar Lipids (Merck). HPTS, lucigenin, HEPES, Triton™ X-100, monensin sodium salt, valinomycin, NADH, NaOH and inorganic salts were of molecular biology grade purchased from Sigma-Aldrich. Dialysis was performed using Spectra/Por 6 dialysis membrane with 1 kD cutoff purchased from Spectrumlabs. All stock solutions were prepared in HPLC grade DMSO (Rankem) or acetonitrile (NICE). All buffer solutions were prepared in Milli-Q water.

2.4.2 Physical Measurements

All NMR spectra were recorded on Bruker Avance III HD 400 MHz and JEOL JNM-ECS 400 MHz nuclear magnetic resonance (NMR) spectrometers. ¹H NMR spectra were recorded at 400 MHz and ¹³C spectra at 101 MHz. All spectra were calibrated with respect to the residual solvent peak ($\delta_{\text{H}} = 7.26$ ppm and $\delta_{\text{C}} = 77.16$ ppm for CDCl₃, $\delta_{\text{H}} = 2.50$ ppm and $\delta_{\text{C}} = 39.52$ ppm for DMSO-*d*₆, $\delta_{\text{H}} = 1.94$ ppm for CD₃CN). The chemical shifts (δ) were reported in ppm and the coupling constants (*J*) in Hz. The following abbreviations were used to indicate peak splitting patterns: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), td (triplet of doublet), and dt (doublet of triplet). Infra-red (IR) spectra were obtained using a Bruker ALPHA-E FT-IR spectrometer and reported in ν (cm⁻¹). High resolution mass spectra (HRMS) were recorded on a Waters SYNAPT G2 mass spectrometer equipped with a Waters Z-SprayTM electrospray ionization (ESI) source. Melting points were measured in open glass capillaries on a Büchi Melting Point M-560 apparatus. Single crystal X-ray diffraction (SCXRD) measurements were conducted using Bruker D8 VENTURE or Bruker KAPPA APEX diffractometers.

Compounds for preparing stock solutions were weighed out using a Quintix® 125D Semi-Micro lab balance (0.01 mg least count) from Sartorius AG. UV-Vis absorption spectra were recorded on a Shimadzu UV-2600 UV-Vis spectrophotometer fitted with a Shimadzu S-1700 thermoelectric cell holder. Fluorescence spectra were recorded on a HORIBA Jobin Yvon Fluoromax®-4 spectrofluorometer equipped with injector port and magnetic stirrer. All buffer solutions were adjusted to the necessary pH using a Hanna Instruments pHep® (HI98107) pocket-sized pH tester. Chloride ion measurements were conducted using a Thermo Scientific OrionTM Chloride ionplusTM Sure-FlowTM Combination ISE, connected to a FisherbrandTM accumetTM AB250 Benchtop pH/ISE meter for recording ion concentrations (in ppm). Analytical HPLC (High Performance Liquid Chromatography) was performed on an Agilent 1260 Infinity II LC System fitted with a Phenomenex® Luna® C18 reverse phase LC column (250 x 4.6 mm, 5 μ m, 100 Å). Planar bilayer conductance studies were performed on a Planar Lipid Bilayer (BLM) Workstation from Warner Instruments (USA).

NMR spectra were processed using MestReNova (v6.0.2) software. All data from fluorescence studies were processed using OriginPro 8.5 software. ChemDraw 21.0.0 was used for drawing structures and processing figures. 3D structure images were generated using BIOVIA Discovery

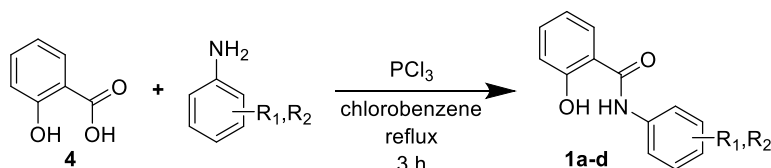
Studio Visualizer 2019. Theoretical predictions of pK_a and logP values were calculated using calculator plugins of MarvinSketch (v5.8) program. Molecular structure conformations were predicted using CONFLEX 8 software package and DFT calculations were performed on Gaussian 09 program suite.

Absorbance and fluorescence measurements on 96-well plate were recorded on a Hidex Sense multimode plate reader. Flow cytometry experiments were performed on a FACSCelesta™ cell analyzer from BD Biosciences (San Jose, CA) and analyzed using BD FACS flowJo software. Live cell imaging was performed on an EVOS® FL Auto automated multichannel fluorescence imaging system from Life Technologies (Thermo Fisher Scientific).

2.4.3 Synthetic Protocols

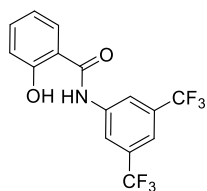
Synthesis of salicylamides:

General procedure A: Procedure as per a modified literature method.⁷⁰ PCl₃ (0.5 eq.) was added to a suspension of salicylic acid (1 eq.) and the substituted aniline derivative (1 eq.) in chlorobenzene (1.0 mL per 0.10 g salicylic acid) at rt and refluxed for 3 h. The mixture was quenched by pouring it into water and extracted with EtOAc (x 3). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and solvent removed *in vacuo*. The purification of crude product was performed using silica gel column chromatography.

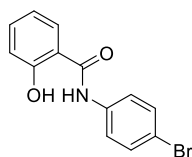


2-Hydroxy-N-(4-(trifluoromethyl)phenyl)benzamide (1a):

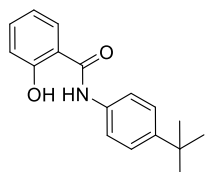
Eluent for column chromatography: 10% EtOAc in petroleum ether; **Physical appearance:** White solid; Yield = 59%; **M.P.:** 207.4-208.8 °C; **¹H NMR (400 MHz, DMSO-*d*₆):** δ 11.50 (s, 1H), 10.63 (s, 1H), 7.96 (d, *J* = 8.6 Hz, 2H), 7.91 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.73 (d, *J* = 8.7 Hz, 2H), 7.48 – 7.42 (m, 1H), 7.03 – 6.95 (m, 2H). **¹³C NMR (101 MHz, DMSO-*d*₆):** δ 166.6, 157.8, 142.0, 133.7, 129.4, 126.0 (q, *J* = 3.8 Hz), 124.3 (q, *J* = 272.3 Hz), 123.9 (q, *J* = 32.1 Hz), 120.5, 119.2, 118.2, 117.1, 39.5. **IR (ν̄ cm⁻¹):** 3843, 3737, 3616, 3028, 2916, 2849, 1605, 1544, 1455, 1320, 1227, 1167, 1102, 831, 743, 684, 598; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₄H₁₀F₃NO₂: 282.0722; Found: 282.0738.

***N*-(3,5-bis(trifluoromethyl)phenyl)-2-hydroxybenzamide (1b):**

Eluent for column chromatography: 8% EtOAc in petroleum ether; **Physical appearance:** White solid; Yield = 86%; **M.P.:** 182.4-184.0 °C; **¹H NMR (400 MHz, DMSO-*d*₆):** δ 11.31 (s, 1H), 10.83 (s, 1H), 8.47 (s, 2H), 7.87 (dd, *J* = 7.8, 1.5 Hz, 1H), 7.83 (s, 1H), 7.45 (m, 1H), 7.00 (m, 2H). **¹³C NMR (101 MHz, DMSO-*d*₆):** δ 167.0, 157.7, 140.4, 133.9, 130.7 (q, *J* = 32.8 Hz), 129.3, 123.2 (q, *J* = 271.6 Hz), 120.3, 119.2, 118.2, 117.1, 116.7. **IR (ν̄ cm⁻¹):** 3832, 3738, 3616, 3313, 3098, 1624, 1567, 1462, 1374, 1268, 1120, 881, 741, 666, 558; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₅H₉F₆NO₂: 350.0615; Found: 350.0614.

***N*-(4-bromophenyl)-2-hydroxybenzamide (1c):**

Eluent for column chromatography: 8% EtOAc in petroleum ether; **Physical appearance:** White solid; Yield = 69%; **M.P.:** 173.8-175.1 °C; **¹H NMR (400 MHz, CDCl₃):** δ 11.83 (s, 1H), 7.91 (s, 1H), 7.53 – 7.43 (m, 6H), 7.04 (dd, *J* = 8.4, 1.0 Hz, 1H), 6.95 – 6.90 (m, 1H). **¹³C NMR (101 MHz, CDCl₃):** δ 168.4, 162.0, 136.0, 135.1, 132.4, 125.5, 122.8, 119.2, 118.3, 114.5. **IR (ν̄ cm⁻¹):** 3846, 3738, 3611, 2923, 2682, 1602, 1537, 1459, 1377, 1318, 1225, 815, 741, 679; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₃H₁₀BrNO₂: 291.9973; Found: 291.9980.

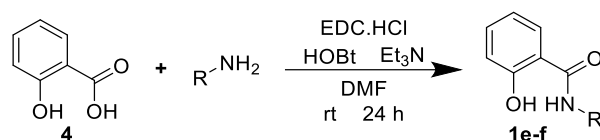
***N*-(4-(*tert*-butyl)phenyl)-2-hydroxybenzamide (1d):**

Eluent for column chromatography: 6% EtOAc in petroleum ether; **Physical appearance:** White solid; Yield = 81%; **M.P.:** 120.5-122.7 °C; **¹H NMR (400 MHz, CDCl₃):** δ 12.04 (s, 1H), 7.88 (s, 1H), 7.52 – 7.38 (m, 6H), 7.02 (dd, *J* = 8.3, 1.0 Hz, 1H), 6.91 (td, *J* = 7.7, 1.0 Hz, 1H), 1.32 (s, 9H). **¹³C NMR (101 MHz, CDCl₃):** δ 168.5, 162.0, 148.7, 134.8, 134.0, 126.2, 125.5, 121.2, 119.1, 119.1, 114.7, 34.7, 31.5. **IR (ν̄ cm⁻¹):** 3844, 3738, 3613, 3094, 2951, 1615, 1537, 1458, 1350, 1224, 826, 744, 671, 554; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₇H₁₉NO₂: 270.1494; Found: 270.1498.

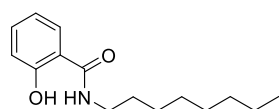
Formation of (*N*-aryl) salicylamides was confirmed from observation of phenolic O-H peak (s, 1H) in the 11 to 12 ppm region and the amide N-H peak (s, 1H) in the 7.8 to 11 ppm region in ¹H NMR.

General procedure B: To a solution of salicylic acid (1 eq.) in DMF (2 mL per 0.10 g salicylic acid) was added EDC·HCl (1.5 eq.), HOBT (1.5 eq.), octylamine (1 eq.) and triethylamine (2 eq.).

The mixture was stirred at rt for 24 h and then poured into water. The aqueous layer was extracted with EtOAc (x 3). The combined organic layers were washed with brine, dried over anhydrous Na₂SO₄ and solvent removed *in vacuo*. The purification of crude product was done using silica gel column chromatography.



2-Hydroxy-*N*-octylbenzamide (1e):

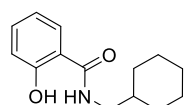


Eluent for column chromatography: 5% EtOAc in petroleum ether;

Physical appearance: White solid; Yield = 68%; **M.P.:** 47.0-48.6 °C;

¹H NMR (400 MHz, CDCl₃): δ 12.43 (s, 1H), 7.42 – 7.31 (m, 2H), 6.98 (dd, *J* = 8.3, 1.1 Hz, 1H), 6.87 – 6.79 (m, 1H), 6.36 (s, 1H), 3.43 (td, *J* = 7.2, 5.9 Hz, 2H), 1.62 (dt, *J* = 14.8, 7.3 Hz, 2H), 1.37 – 1.23 (m, 10H), 0.91 – 0.84 (m, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 170.0, 161.7, 134.2, 125.3, 118.7, 118.7, 114.5, 39.9, 31.9, 29.6, 29.4, 29.3, 27.1, 22.8, 14.2. **IR (ν̄ cm⁻¹):** 3831, 3738, 3609, 3400, 2912, 2849, 1634, 1579, 1524, 1486, 1359, 1301, 1231, 1137, 856, 809, 736, 686; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₅H₂₃NO₂: 250.1807; Found: 250.1812.

N-(cyclohexylmethyl)-2-hydroxybenzamide (1f):



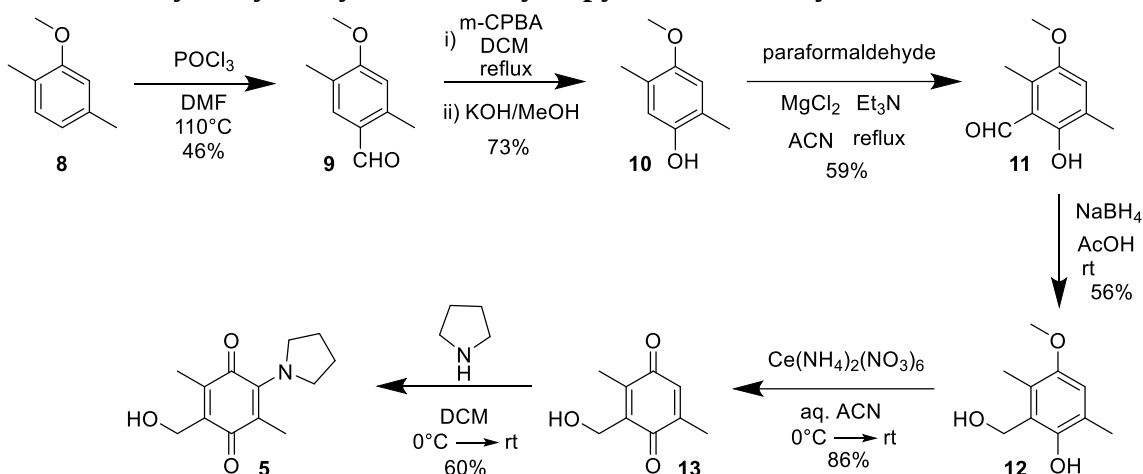
Eluent for column chromatography: 5% EtOAc in petroleum ether; **Physical**

appearance: White solid; Yield = 72%; **M.P.:** 72.3-75.5 °C; **¹H NMR (400 MHz,**

CDCl₃): δ 12.39 (s, 1H), 7.42 – 7.36 (m, 1H), 7.34 (d, *J* = 8.0 Hz, 1H), 6.98 (d, *J* = 8.1 Hz, 1H), 6.87 – 6.81 (m, 1H), 6.35 (s, 1H), 3.30 (t, *J* = 6.4 Hz, 2H), 1.82 – 1.73 (m, 4H), 1.71 – 1.65 (m, 1H), 1.63 – 1.55 (m, 1H), 1.32 – 1.14 (m, 3H), 1.06 – 0.95 (m, 2H). **¹³C NMR (101 MHz, CDCl₃):** δ 170.1, 161.7, 134.3, 125.3, 118.8, 118.7, 114.5, 45.9, 38.1, 31.0, 26.5, 25.9. **IR(ν̄ cm⁻¹):** 3845, 3736, 3611, 3294, 2920, 2846, 1593, 1554, 1487, 1441, 1327, 1212, 742, 688, 548; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₄H₁₉NO₂: 234.1494; Found: 234.1497.

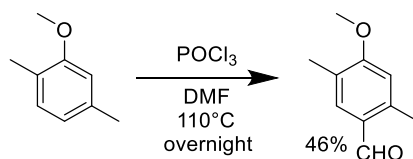
Formation of (*N*-alkyl) salicylamides was confirmed from observation of phenolic O-H peak (s, 1H) at approx. 12.4 ppm and the amide N-H peak (s, 1H) at approx. 6.36 ppm in ¹H NMR.

Synthesis of 2-Hydroxymethyl-3,6-dimethyl-5-pyrrolidino-2,5-cyclohexadiene-1,4-dione (5)

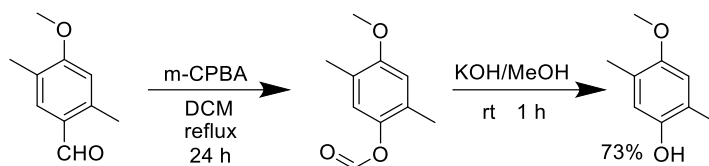


The photocleavable group, 2-hydroxymethyl-3,6-dimethyl-5-pyrrolidino-2,5-cyclohexadiene-1,4-dione was synthesized as per the procedure reported by Chen *et. al.*^{33, 71}

Synthesis of 4-methoxy-2,5-dimethylbenzaldehyde (9):

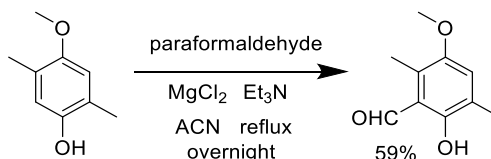


The reported procedure was followed.⁷² First, the Vilsmeier complex was prepared by the dropwise addition of freshly distilled POCl₃ (4.76 mL, 50.52 mmol) to dry *N,N'*-dimethylformamide (8.6 mL) over 15-20 min at 0°C and stirred 15 min. The complex was allowed to warm to room temperature and was then added during 1-1.5 h to a stirred solution of 2,5-dimethylanisole (4.30 g, 31.57 mmol) in dry DMF (8.6 mL) at 110°C. Heating and stirring were then continued overnight. The mixture was then poured into ice-water, made just basic by the addition of Na₂CO₃, and extracted with EtOAc (x3). The combined extracts were washed successively with 1 N HCl, water, and saturated brine, and finally dried over anhydrous Na₂SO₄. The solvent was removed *in vacuo* and the crude product was purified by silica gel column chromatography using 2% EtOAc in petroleum ether as eluent to obtain the product as clear oil. Yield = 2.79 g = 54%; ¹H NMR (400 MHz, CDCl₃): δ 10.12 (s, 1H), 7.58 (s, 1H), 6.64 (s, 1H), 3.89 (s, 3H), 2.65 (s, 3H), 2.21 (s, 3H). ¹³C NMR (101 MHz, CDCl₃): δ 191.4, 162.1, 141.4, 134.3, 127.2, 124.8, 112.7, 55.7, 19.7, 15.8. HRMS (ESI): *m/z* [M+H]⁺ Calc. for C₁₀H₁₂O₂: 165.0915; Found: 165.0922.

Synthesis of 4-methoxy-2,5-dimethylphenol (10):

The reported procedure was followed.⁷² The benzaldehyde **9** (2.46 g, 14.98 mmol) and *m*-chloroperbenzoic acid (4.18 g, 15.73 mmol) were refluxed in dry DCM (30 mL) for 24 h. The DCM was then removed *in vacuo* and the residue was dissolved in EtOAc. The organic layer was washed with aqueous NaHCO₃ solution, until effervescence ceased, and then with brine. The organic layer was dried over anhydrous Na₂SO₄, and the solvent was removed *in vacuo*. The residual crude 4-methoxy-2,5-dimethylphenyl formate was characterized through NMR. **¹H NMR (400 MHz, CDCl₃):** δ 8.28 (s, 1H), 6.81 (s, 1H), 6.67 (s, 1H), 3.81 (s, 3H), 2.18 (s, 6H). **¹³C NMR (101 MHz, CDCl₃):** δ 160.0, 155.9, 141.4, 127.6, 125.6, 123.5, 112.5, 55.8, 16.4, 15.9.

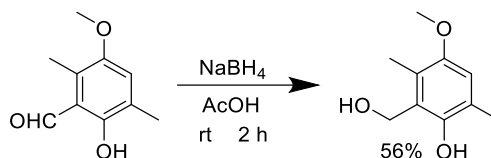
The crude formate was then dissolved in a little methanol and hydrolyzed under the nitrogen atmosphere (1 h) with a slight excess of aqueous 10% KOH at rt. The pH was made to 5 using dil. HCl (2 N) and the mixture were extracted exhaustively with DCM. The organic layers were combined, dried over anhydrous Na₂SO₄, and the solvent removed *in vacuo*. The crude product was purified by silica gel column chromatography using 5% EtOAc in petroleum ether as eluent to obtain the product as white solid. Combined yield = 1.66 g = 73%; **¹H NMR (400 MHz, CDCl₃):** δ 6.60 (s, 1H), 6.59 (s, 1H), 3.77 (s, 3H), 2.22 (s, 3H), 2.15 (s, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 151.8, 147.2, 125.2, 121.2, 117.7, 113.5, 56.2, 15.9, 15.9. **HRMS (ESI):** *m/z* [M]⁺ Calc. for C₉H₁₂O₂: 152.0837; Found: 152.0843.

Synthesis of 2-hydroxy-5-methoxy-3,6-dimethylbenzaldehyde (11):

Protocol was based on literature report.⁷³ To a solution of 4-methoxy-2,5-dimethylphenol (**10**, 3.43 g, 22.54 mmol) in dry acetonitrile (200 mL) was added Et₃N (8.9 mL, 64.23 mmol) and anhydrous MgCl₂ (6.74 g, 70.77 mmol) followed by dry paraformaldehyde (2.40 g) in portions. The mixture was refluxed overnight. Then ACN was removed *in vacuo* followed by the addition of 2 N HCl

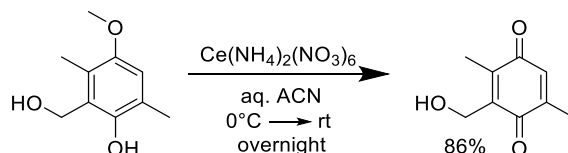
(100 mL), and the mixture was extracted with EtOAc (x3). The extracts were dried over anhydrous Na₂SO₄, the solvent was removed *in vacuo* and the crude product was purified by silica gel column chromatography using 2% EtOAc in petroleum ether as eluent to obtain the product as yellow solid. Yield = 2.40 g = 59%; **¹H NMR (400 MHz, CDCl₃):** δ 11.76 (s, 1H), 10.34 (s, 1H), 7.02 (s, 1H), 3.79 (s, 3H), 2.45 (s, 3H), 2.23 (s, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 196.1, 155.7, 149.5, 127.0, 124.6, 123.9, 118.2, 57.3, 15.5, 9.7. **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₀H₁₂O₃: 181.0864; Found: 181.0860.

Synthesis of 2-Hydroxy-5-methoxy-3,6-dimethylbenzenemethanol (12):



To a solution of 2-hydroxy-5-methoxy-3,6-dimethylbenzaldehyde (**11**, 1.03 g, 5.72 mmol) in glacial acetic acid (14 mL) was added NaBH₄ (0.87 g, 22.89 mmol) in portions. A little water was added to the mixture after 2 h and the mixture was neutralized with aq. NaHCO₃. The mixture was extracted exhaustively with EtOAc, the organic layers were combined, dried over anhydrous Na₂SO₄, and the solvent was removed *in vacuo*. The crude product was purified by silica gel column chromatography using 15% EtOAc+ PET ether→20% EtOAc+PET ether as eluent to obtain the product as white solid. Yield = 0.58 g = 56%; **¹H NMR (400 MHz, CDCl₃):** δ 7.28 (s, 1H), 6.63 (s, 1H), 4.91 (s, 2H), 3.76 (s, 3H), 2.23 (s, 3H), 2.11 (s, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 150.8, 148.6, 123.7, 122.6, 121.9, 113.7, 61.0, 56.7, 16.1, 11.1. **HRMS (ESI):** *m/z* [M-OH]⁺ Calc. for C₁₀H₁₃O₂: 165.0910; Found: 165.0918.

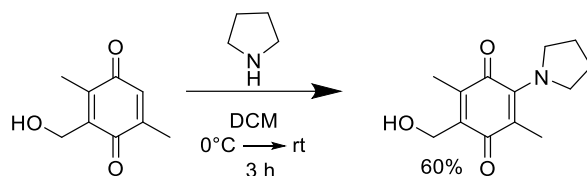
Synthesis of 2-Hydroxymethyl-3,6-dimethyl-2,5-cyclohexadiene-1,4-dione (13):



To a solution of 2-hydroxy-5-methoxy-3,6-dimethylbenzenemethanol (**12**, 0.58 g, 3.19 mmol) in ACN (3.2 mL) was added Ce(NH₄)₂(NO₃)₆ (5.23 g, 9.55 mmol) in water (29 mL) with cooling in ice. The mixture was stirred at rt overnight then extracted with CHCl₃ (x2). The organic layers were combined, washed with brine and dried over anhydrous Na₂SO₄. The solvent was removed

in vacuo and the crude product was purified by silica gel column chromatography using 15% EtOAc+ PET ether→30% EtOAc+PET ether as eluent to obtain the product as yellow solid. Yield = 0.46 g = 86%; **¹H NMR (400 MHz, CDCl₃):** δ 6.62 (dd, *J* = 3.1, 1.5 Hz, 1H), 4.57 (d, *J* = 6.1 Hz, 2H), 2.54 (t, *J* = 6.6 Hz, 1H), 2.09 (s, 3H), 2.05 (d, *J* = 1.6 Hz, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 189.2, 187.8, 145.6, 142.1, 140.8, 133.8, 57.7, 15.8, 11.6. **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₉H₁₀O₃: 167.0708; Found: 167.0708.

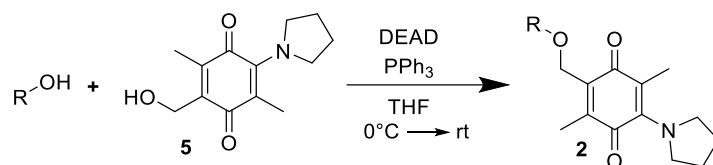
Synthesis of 2-Hydroxymethyl-3,6-dimethyl-5-pyrrolidino-2,5-cyclohexadiene-1,4-dione (5):



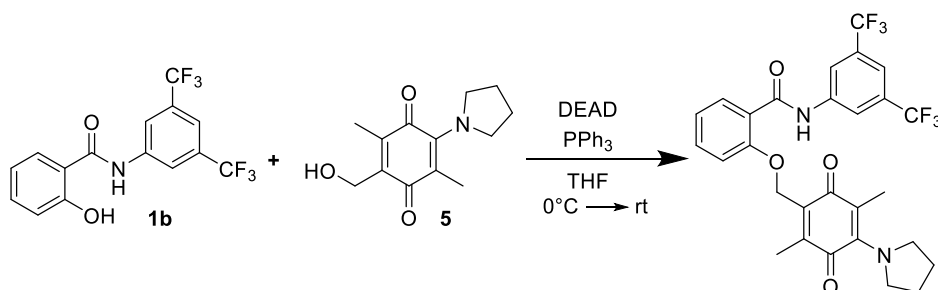
To a solution of 2-hydroxymethyl-3,6-dimethyl-2,5-cyclohexadiene-1,4-dione (**13**, 0.67 g, 4.04 mmol) in DCM (44 mL), was added dropwise a solution of pyrrolidine (1 mL, 12.13 mmol) in DCM (4 mL) with cooling in ice bath while keeping the reaction in dark. The mixture was then warmed to rt and stirred 3 h. The solvent was removed *in vacuo* and the crude product was purified by silica gel column chromatography using 30% EtOAc+ PET ether. to obtain product as a violet film; Yield = 0.57 g = 60%; **¹H NMR (400 MHz, CDCl₃):** δ 4.51 (d, *J* = 5.1 Hz, 2H), 3.72 – 3.62 (m, 4H), 2.02 (s, 3H), 1.98 (s, 3H), 1.91 – 1.85 (m, 4H). **¹³C NMR (101 MHz, CDCl₃):** δ 187.8, 187.0, 150.9, 141.0, 136.7, 111.4, 59.1, 53.4, 25.9, 12.8, 11.4. **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₃H₁₇NO₃: 236.1286; Found: 236.1286.

Attachment of photocleavable group via Mitsunobu reaction:

Mitsunobu reaction- General procedure C:⁷⁴ A solution of the alcohol (2-hydroxymethyl-3,6-dimethyl-5-pyrrolidino-2,5-cyclohexadiene-1,4-dione) (1 eq.) in THF (1 mL per 0.1 mmol salicylamide) kept in dark was cooled in ice and the salicylamide (1 eq.) followed by PPh₃ (1.7 eq.) was added. Then DEAD (1 eq.) was added slowly and the mixture was stirred at 0 °C for 1 h and then at rt for 21 h. The solvent was removed *in vacuo* and the crude product was purified by silica gel column chromatography. The column fractions were collected, the solvent was removed *in vacuo* and then washed with pentane to yield the pure product (unless specified otherwise).

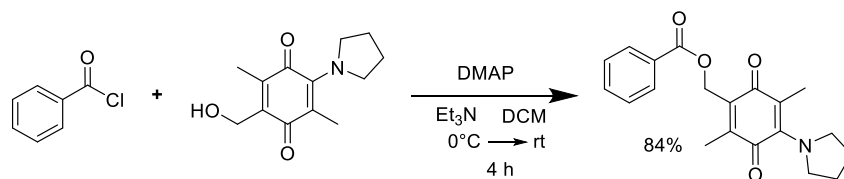


Synthesis of *N*-(3,5-bis(trifluoromethyl)phenyl)-2-((2,5-dimethyl-3,6-dioxo-4-(pyrrolidine-1-yl)cyclohexa-1,4-dien-1-yl)methoxy)benzamide (2):



The protransporter (**2**) was synthesized by Mitsunobu coupling of transporter **1b** to the quinone **5** using **General procedure C** to yield product as a violet solid; Yield = 77%; ¹H NMR (400 MHz, CDCl₃): δ 10.09 (s, 1H), 8.24 (s, 2H), 8.21 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.59 – 7.50 (m, 2H), 7.20 – 7.13 (m, 2H), 5.16 (s, 2H), 3.67 (t, *J* = 6.4 Hz, 4H), 2.10 (s, 3H), 2.01 (s, 3H), 1.92 – 1.85 (m, 4H). ¹³C NMR (101 MHz, CDCl₃): δ 186.9, 184.4, 164.1, 156.5, 150.5, 141.2, 140.3, 136.5, 133.9, 132.8, 132.1 (q, *J* = 33.2 Hz), 123.5 (d, *J* = 273.7 Hz), 122.4, 122.2, 120.4 (d, *J* = 3.6 Hz), 117.2 – 117.0 (m), 113.2, 111.9, 63.5, 53.4, 25.9, 12.9, 12.4. IR (ν̄ cm⁻¹): 3856, 3740, 3609, 3308, 2922, 2380, 2306, 1740, 1161, 1595, 1543, 1468, 1376, 1277, 1215, 1165, 1109, 993, 930, 885, 750, 671, 610; HRMS (ESI): *m/z* [M+H]⁺ Calc. for C₂₈H₂₄F₆N₂O₄: 567.1718; Found: 567.1730.

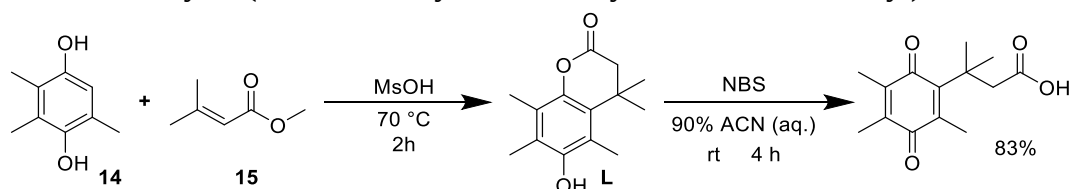
Synthesis of (2,5-dimethyl-3,6-dioxo-4-(pyrrolidin-1-yl)cyclohexa-1,4-dien-1-yl)methyl benzoate (7):



The reported procedure was followed.⁷⁵ To a solution of quinone **5** (49 mg, 0.21 mmol) in DCM (1.2 mL) was added DMAP (38 mg, 0.31 mmol) and triethylamine (43 μL, 0.31 mmol), and the mixture was cooled in ice. Benzoyl chloride (36 μL, 0.31 mmol) was then added dropwise and the mixture warmed to rt. After stirring for 4 h (ensuring complete consumption of starting material

via TLC), the solvent was removed *in vacuo* and the crude product was purified by silica gel column chromatography using 10% EtOAc+ PET ether as eluent. The column fractions were collected, solvent removed *in vacuo* and then washed with pentane to yield the pure product as violet powder. Yield = 59 mg = 84%; ¹H NMR (400 MHz, CDCl₃): δ 8.07 – 7.95 (m, 2H), 7.57 – 7.50 (m, 1H), 7.45 – 7.38 (m, 2H), 5.29 (s, 2H), 3.70 – 3.60 (m, 4H), 2.10 (s, 3H), 2.06 (s, 3H), 1.91 – 1.83 (m, 4H). ¹³C NMR (101 MHz, CDCl₃): δ 187.6, 184.2, 166.4, 150.4, 141.3, 137.3, 133.2, 130.0, 129.9, 128.5, 112.4, 58.4, 53.2, 26.0, 13.3, 12.3. IR (ν̄ cm⁻¹): 3844, 3737, 3612, 2920, 2859, 1707, 1652, 1543, 1452, 1382, 1327, 1256, 1099, 960, 704, 609, 555; HRMS (ESI): *m/z* [M+H]⁺ Calc. for C₂₀H₂₁NO₄: 340.1549; Found: 340.1550

Synthesis of 3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanoic acid (6):



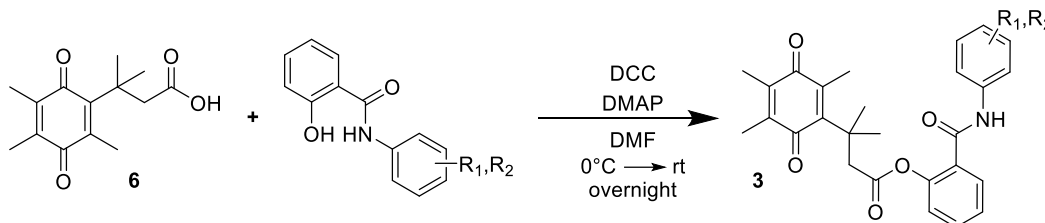
Procedure as per a modified literature method.⁷⁶ To a mixture of 2,3,5-trimethylhydroquinone (**14**, 0.83 g, 5.42 mmol) in methanesulfonic acid (5.5 mL) was added methyl 3,3-dimethylacrylate (**15**, 0.79 mL, 6.51 mmol) and stirred at 70 °C for 2 h. The mixture was then poured into water and extracted with DCM (x 3). The organic layers were combined, washed with water and brine, dried over anhydrous Na₂SO₄ and solvent removed *in vacuo* to yield crude 6-hydroxy-4,4,5,7,8-pentamethylchroman-2-one (**L**). The reaction was conducted on a smaller scale and the product **L** purified through column chromatography for use as negative control ¹H NMR (400 MHz, CDCl₃): δ 4.63 (brs, 1H), 2.54 (s, 2H), 2.36 (s, 3H), 2.22 (s, 3H), 2.18 (s, 3H), 1.45 (s, 6H). ¹³C NMR (101 MHz, CDCl₃): δ 169.0, 148.9, 143.6, 128.4, 123.6, 121.9, 119.0, 46.2, 35.6, 27.85, 14.6, 12.7, 12.4.

The crude chromanone was dissolved in 90% ACN (aq.) (10 mL) along with NBS (0.87 g, 4.88 mmol) and stirred 4 h at rt. The solvent was removed, and the residue was dissolved in DCM. The organic phase was washed with water and brine, dried over anhydrous Na₂SO₄, and the solvent was removed *in vacuo*. The crude product was purified by flash column chromatography on 230–400 mesh silica gel using a gradient of 20% → 50% EtOAc+ PET ether as eluent. The product was obtained as a yellow solid. Yield = 1.12 g = 83%; ¹H NMR (400 MHz, CDCl₃): δ 3.02 (s, 2H), 2.14 (s, 3H), 1.95 (d, *J* = 1.1 Hz, 3H), 1.92 (d, *J* = 1.1 Hz, 3H), 1.43 (s, 6H). ¹³C NMR (101

MHz, CDCl₃): δ 191.0, 187.6, 178.3, 152.2, 143.1, 139.2, 138.5, 47.3, 38.1, 28.9, 14.5, 12.7, 12.3.

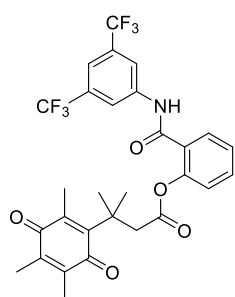
HRMS (ESI): m/z [M-OH]⁺ Calc. for C₁₄H₁₇O₃: 233.1178; Found: 233.1190. m/z [M-COOH]⁺ Calc. for C₁₃H₁₇O₂: 205.1229; Found: 205.1235.

Coupling of quinone 6 to transporter:



DCC-DMAP coupling- General procedure D: A solution of the carboxylic acid (1 eq.) and the phenol (1.1 eq.) were dissolved in DMF and cooled in ice. Then DMAP (0.2 eq.) followed by DCC (1.2 eq.) were added and stirred 30 min at 0 °C. The reaction mixture was then warmed to rt and stirred overnight. It was then diluted with EtOAc and washed with water. The aqueous layer was further extracted with EtOAc (x 2), the organic layers combined, dried over anhydrous Na₂SO₄, and the solvent removed *in vacuo*. The crude product was purified by flash column chromatography on 230-400 mesh silica gel.

2-((3,5-bis(trifluoromethyl)phenyl)carbamoyl)phenyl 3-methyl-3-(2,4,5-trimethyl-3,6-dioxocyclohexa-1,4-dien-1-yl)butanoate (3)



Eluent for column chromatography: 10%→15% EtOAc in petroleum ether;

Physical appearance: Yellow solid; Yield = 53%; **¹H NMR (400 MHz,**

CDCl₃): δ 8.61 (s, 1H), 8.28 (s, 2H), 7.96 (dd, J = 7.8, 1.6 Hz, 1H), 7.66 (s, 1H), 7.53 (td, J = 8.1, 1.7 Hz, 1H), 7.37 (td, J = 7.7, 1.0 Hz, 1H), 7.12 (dd, J = 8.2, 0.8 Hz, 1H), 3.36 (s, 2H), 2.15 (s, 3H), 1.89 (d, J = 1.1 Hz, 3H), 1.76 (d, J = 1.1 Hz, 3H), 1.49 (s, 6H). **¹³C NMR (101 MHz, CDCl₃)**: δ 191.2,

187.3, 170.9, 163.6, 150.6, 147.6, 142.4, 140.2, 139.4, 139.2, 132.9, 132.6, 132.3, 130.8, 126.9, 126.7, 123.3, 123.1 (q, J = 274.0 Hz), 119.9 (d, J = 3.4 Hz), 117.9 – 117.7 (m), 48.5, 38.1, 29.0, 14.6, 12.4, 12.2. **IR ($\tilde{\nu}$ cm⁻¹)**: 2930, 1744, 1641, 1539, 1378, 1279, 1176, 1127, 716, 681; **HRMS (ESI)**: m/z [M+Na]⁺ Calc. for C₂₉H₂₅F₆NO₅: 604.1534; Found: 604.1536.

2.4.4 ¹H NMR Titration Studies

¹H NMR titration studies were conducted at ambient temperature on a Bruker Avance III HD 400 MHz spectrometer. The titrations with Cl⁻ were conducted by the addition of aliquots of tetrabutylammonium chloride (TBACl) solution (0.5 M in CD₃CN) to a solution of the receptor (**1a-1e**) in CD₃CN (0.005 M). The spectra were calibrated using the residual signal from CD₃CN ($\delta_{\text{H}} = 1.94$) as an internal reference. Tetrabutylammonium bromide (TBABr) and tetrabutylammonium iodide (TBAI) solutions were used for Br⁻ and I⁻ titrations. The TBA salts were dried under vacuum in a desiccator prior to use.

2.4.5 Single Crystal X-ray Diffraction Studies

The compounds were crystallized from their solutions in HPLC grade solvents at ambient temperature. The crystals obtained were diffracted on Bruker diffractometers. For compounds **1b**, **1f**, **2** and **3** - Bruker D8 VENTURE single crystal X-ray diffractometer with Mo (Mo-K α = 0.71073 Å) or Cu (Cu-K α = 1.54178 Å) microfocus X-ray (I μ S) source and PHOTON 100 CMOS detector. For compound **1a** - Bruker KAPPA APEX single crystal X-ray diffractometer with Mo (Mo-K α = 0.71073 Å) ceramic sealed tube X-ray source and APEX II CCD detector. The sample temperature was maintained constant throughout the data collection using Cryostream coolers (Oxford Cryosystems).

Single crystal data for **1a** (CCDC 2223354)

Compound **1a** was crystallized from its saturated CHCl₃ solution in a glass vial to yield colorless plate like crystals. XRD data was collected with Mo (Mo-K α = 0.71073 Å) X-ray source. The data collection temperature was maintained at 100 K.

Table 7: Crystal data and structure refinement for **1a**

Empirical formula	C14 H10 F3 N O2
Formula weight	281.23
Temperature	100 K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P 1 21/c 1
Unit cell dimensions	a = 14.466(5) Å α = 90°. b = 11.682(4) Å β = 103.133(11)°. c = 7.361(3) Å γ = 90°.

Volume	1211.5(8) Å ³
Z	4
Density (calculated)	1.542 Mg/m ³
Absorption coefficient	0.134 mm ⁻¹
F(000)	576
Crystal size	0.430 x 0.300 x 0.064 mm ³
Theta range for data collection	1.445 to 32.031°.
Index ranges	-21 ≤ h ≤ 21, -17 ≤ k ≤ 17, -10 ≤ l ≤ 10
Reflections collected	24893
Independent reflections	4211 [R(int) = 0.0835]
Completeness to theta = 32.031°	100.0 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7465 and 0.6677
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	4211 / 93 / 214
Goodness-of-fit on F ²	1.027
Final R indices [I > 2σ(I)]	R1 = 0.0524, wR2 = 0.1214
R indices (all data)	R1 = 0.0924, wR2 = 0.1400
Extinction coefficient	n/a
Largest diff. peak and hole	0.385 and -0.389 e.Å ⁻³

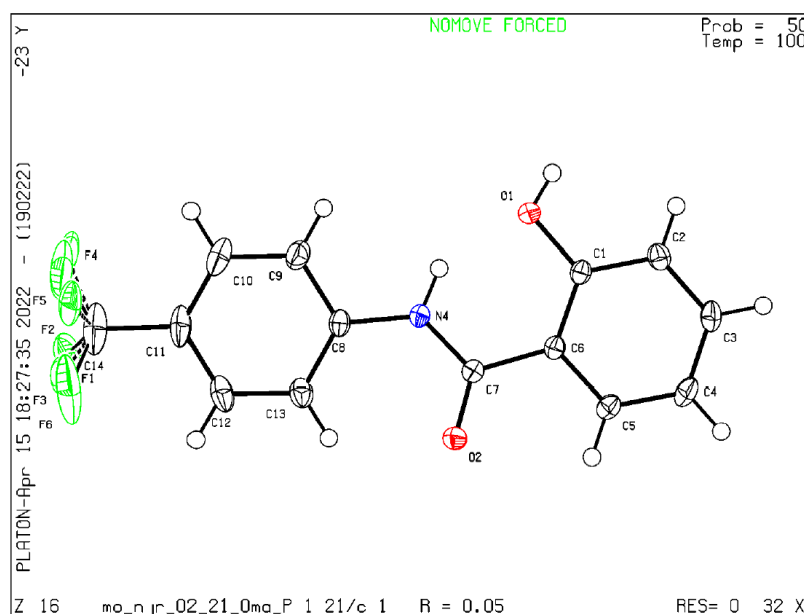


Figure 72: ORTEP diagram of 1a.

Single crystal data for 1b (CCDC 2223359)

Compound **1b** was crystallized from its saturated CHCl₃ solution in a glass vial to yield colorless rod-shaped crystals. XRD data was collected with Mo (Mo-K α = 0.71073 Å) X-ray source. The data collection temperature was maintained at 100 K.

Table 8: Crystal data and structure refinement for **1b**

Empirical formula	C15 H9 F6 N O2
Formula weight	349.23
Temperature	100(2) K
Wavelength	0.71073 Å
Crystal system	Triclinic
Space group	P -1
Unit cell dimensions	a = 8.6640(13) Å α = 86.339(4)°. b = 12.1337(17) Å β = 86.029(4)°. c = 13.8137(19) Å γ = 74.218(4)°.
Volume	1392.6(3) Å ³
Z	4
Density (calculated)	1.666 Mg/m ³
Absorption coefficient	0.164 mm ⁻¹
F(000)	704
Crystal size	0.550 x 0.164 x 0.151 mm ³
Theta range for data collection	1.479 to 26.370°.
Index ranges	-10 ≤ h ≤ 10, -15 ≤ k ≤ 15, -17 ≤ l ≤ 17
Reflections collected	32758
Independent reflections	5690 [R(int) = 0.065]
Completeness to theta = 26.370°	100.0 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7457 and 0.6917
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	5690 / 0 / 436
Goodness-of-fit on F ²	1.011
Final R indices [I > 2σ(I)]	R1 = 0.0461, wR2 = 0.1012
R indices (all data)	R1 = 0.0791, wR2 = 0.1179
Extinction coefficient	0.0025(6)
Largest diff. peak and hole	0.657 and -0.502 e.Å ⁻³

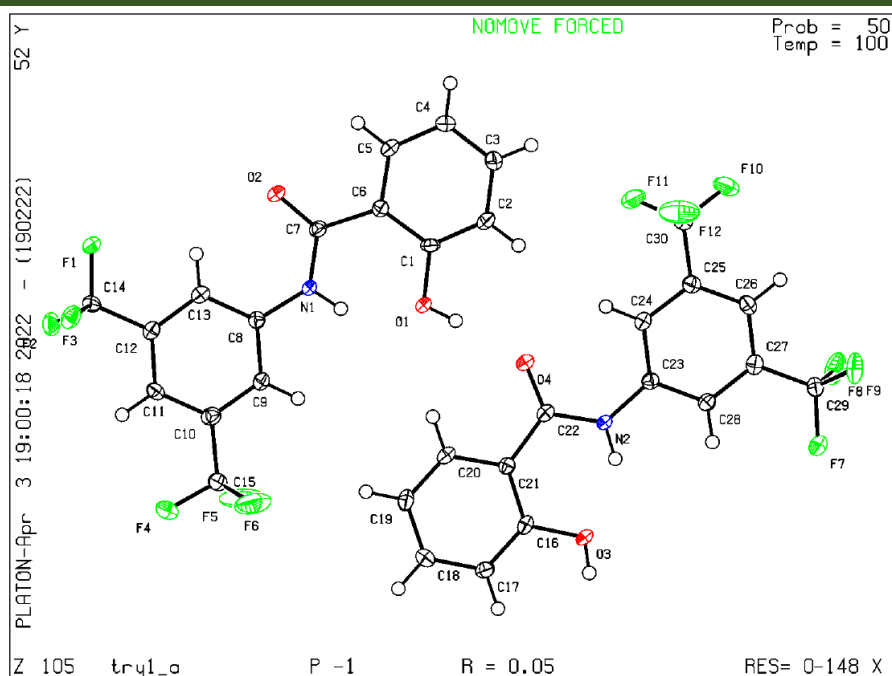


Figure 73: ORTEP diagram of **1b**.

Single crystal data for **1f** (CCDC 2223360)

Compound **1f** was crystallized from its saturated CHCl₃ solution in a glass vial to yield colorless plate like crystals. XRD data was collected with Cu (Cu-K α = 1.54178 Å) X-ray source. The data collection temperature was maintained at 296 K.

Table 9: Crystal data and structure refinement for **1f**

Empirical formula	C ₁₄ H ₁₉ N O ₂
Formula weight	233.30
Temperature	296(2) K
Wavelength	1.54178 Å
Crystal system	Orthorhombic
Space group	P bca
Unit cell dimensions	a = 12.2943(18) Å α =90°. b = 8.9674(12) Å β = 90°. c = 22.932(3) Å γ = 90°.
Volume	2528.2(6) Å ³
Z	8
Density (calculated)	1.226 Mg/m ³
Absorption coefficient	0.649 mm ⁻¹

F(000)	1008
Crystal size	0.543 x 0.354 x 0.174 mm ³
Theta range for data collection	3.855 to 68.231°.
Index ranges	-13 ≤ h ≤ 14, -10 ≤ k ≤ 10, -27 ≤ l ≤ 27
Reflections collected	24795
Independent reflections	2311 [R(int) = 0.1129]
Completeness to theta = 68.231°	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7542 and 0.6018
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	2311 / 0 / 158
Goodness-of-fit on F ²	0.727
Final R indices [I > 2σ(I)]	R1 = 0.0463, wR2 = 0.1483
R indices (all data)	R1 = 0.0547, wR2 = 0.1662
Extinction coefficient	n/a
Largest diff. peak and hole	0.273 and -0.277 e.Å ⁻³

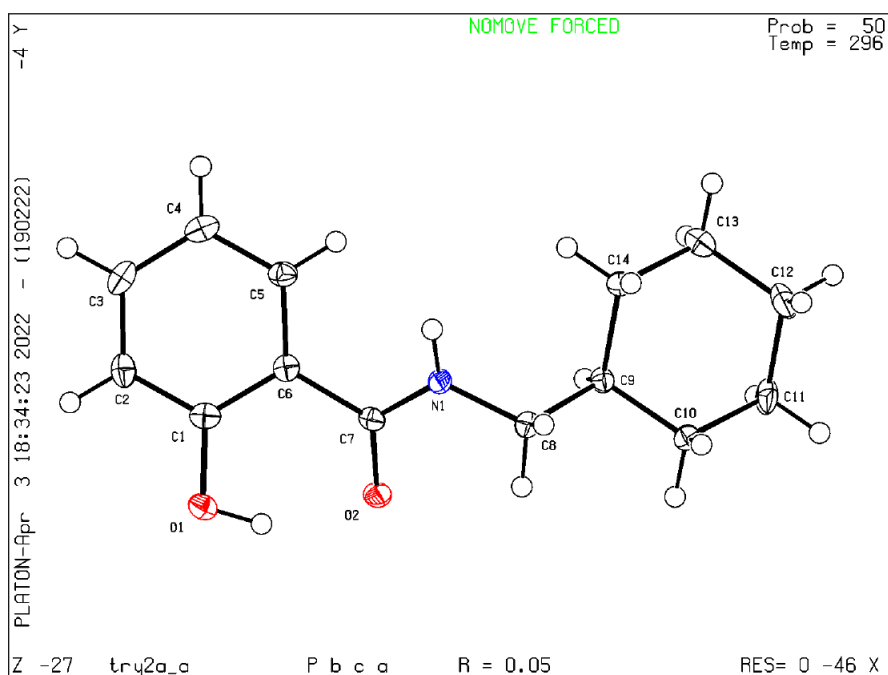


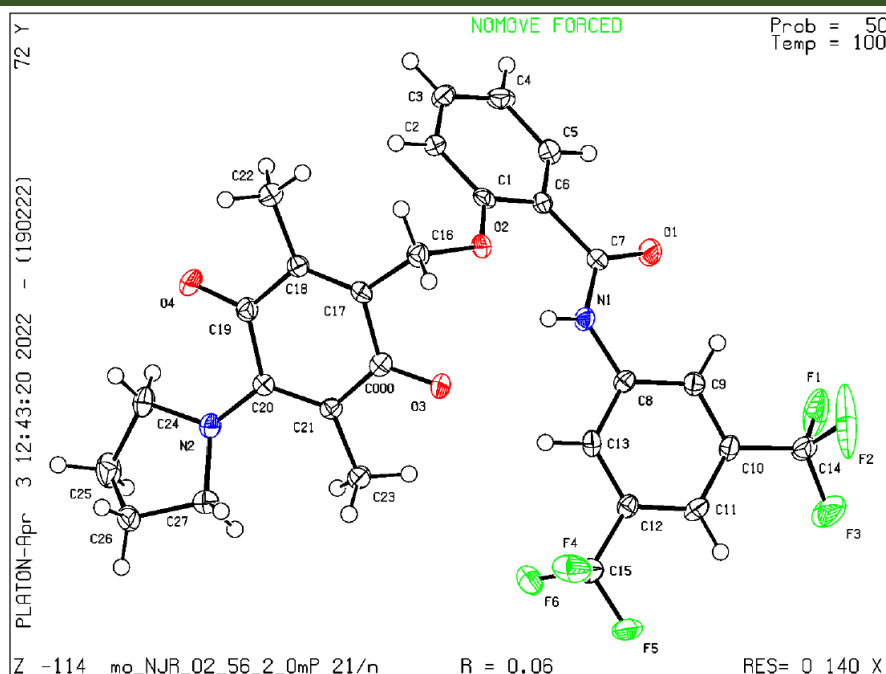
Figure 74: ORTEP diagram of **1f**.

Single crystal data for 2 (CCDC 2223361)

Compound **2** was crystallized by the slow evaporation of its solution in ACN/(MilliQ) water (7:3) mixture in a sealed crystallization tube to yield fine violet needle like crystals. XRD data was collected with Mo (Mo-K α = 0.71073 Å) X-ray source. The data collection temperature was maintained at 100 K.

Table 10: Crystal data and structure refinement for **2**

Empirical formula	C ₂₈ H ₂₄ F ₆ N ₂ O ₄
Formula weight	566.49
Temperature	100(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P2 ₁ /n
Unit cell dimensions	a = 4.5972(6) Å α = 90°. b = 20.927(2) Å β = 94.967(4)°. c = 25.803(3) Å γ = 90°.
Volume	2473.1(5) Å ³
Z	4
Density (calculated)	1.521 Mg/m ³
Absorption coefficient	0.132 mm ⁻¹
F(000)	1168
Crystal size	0.200 x 0.080 x 0.065 mm ³
Theta range for data collection	1.946 to 25.349°.
Index ranges	-5 ≤ h ≤ 5, -24 ≤ k ≤ 25, -30 ≤ l ≤ 30
Reflections collected	23044
Independent reflections	4524 [R(int) = 0.1041]
Completeness to theta = 25.349°	99.5 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7454 and 0.6925
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	4524 / 0 / 364
Goodness-of-fit on F ²	1.040
Final R indices [I > 2σ(I)]	R1 = 0.0597, wR2 = 0.1047
R indices (all data)	R1 = 0.1262, wR2 = 0.1263
Extinction coefficient	0.0046(6)
Largest diff. peak and hole	0.547 and -0.397 e.Å ⁻³

**Figure 75:** ORTEP diagram of **2**.**Single crystal data for 3 (CCDC 2223363)**

Compound **3** was crystallized by the slow evaporation of its solution in ACN/(MilliQ) water (7:3) mixture in a sealed crystallization tube to yield yellow needle like crystals. XRD data was collected with Mo (Mo-K α = 0.71073 Å) X-ray source. The data collection temperature was maintained at 100 K.

Table 11: Crystal data and structure refinement for **3**

Empirical formula	C ₂₉ H ₂₅ F ₆ N O ₅
Formula weight	581.50
Temperature	100(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P2 ₁ /c
Unit cell dimensions	a = 9.2284(9) Å α = 90°. b = 12.8799(14) Å β = 95.224(3)°. c = 45.515(5) Å γ = 90°.
Volume	5387.4(10) Å ³
Z	8
Density (calculated)	1.434 Mg/m ³

Absorption coefficient	0.125 mm ⁻¹
F(000)	2400
Crystal size	0.300 x 0.200 x 0.150 mm ³
Theta range for data collection	2.216 to 24.997°.
Index ranges	-9 ≤ h ≤ 10, -15 ≤ k ≤ 15, -54 ≤ l ≤ 54
Reflections collected	96861
Independent reflections	9690 [R(int) = 0.2235]
Completeness to theta = 25.349°	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.97 and 0.92
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	9690 / 307 / 815
Goodness-of-fit on F ²	1.107
Final R indices [I > 2σ(I)]	R1 = 0.0873, wR2 = 0.1418
R indices (all data)	R1 = 0.1717, wR2 = 0.1620
Extinction coefficient	0.00147(14)
Largest diff. peak and hole	0.869 and -0.925 e.Å ⁻³

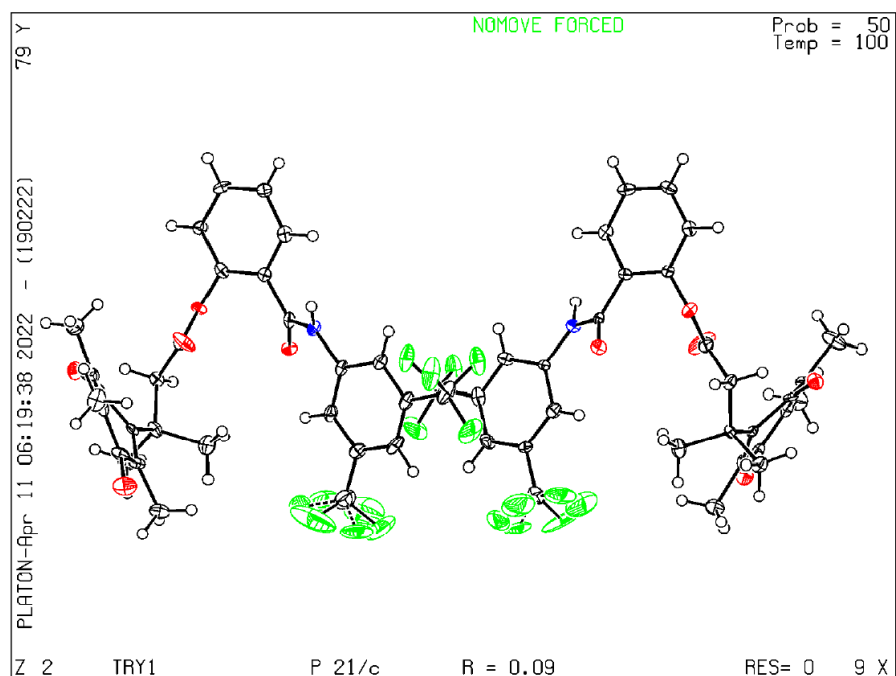


Figure 76: ORTEP diagram of **3**.

2.4.6 Ion Transport Experiments

Study of ion transport across EYPC-LUVs $\Rightarrow$ HPTS:

Preparation of HEPES buffer and stock solutions: The HEPES buffer (pH = 7.0) was prepared by dissolving solid HEPES (10 mM) and NaCl (100 mM) in autoclaved Milli-Q water, followed by adjustment of pH to 7.0 by addition of aliquots of 0.5 M NaOH solution. Stock solutions of all transporter molecules were prepared in HPLC grade DMSO.

Preparation of EYPC-LUVs $\Rightarrow$ HPTS in NaCl (Prep protocol P1): In a clean, dry 10 mL round bottom flask, 1 mL of egg yolk phosphatidylcholine (EYPC, 25 mg/mL in CHCl₃) was dried to a thin transparent film by purging nitrogen gas along with continuous rotation. The transparent film was kept under a high vacuum for 4-5 h to remove all traces of CHCl₃. The resulting film was then hydrated with 1 mL HEPES buffer (1 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0) by vortexing at 10 min intervals for a total of duration 1 h. This hydrated suspension was subjected to ≥ 11 cycles of freeze-thaw (liquid N₂, 55 °C). Extrusions were done using a Mini-extruder through a polycarbonate membrane (Avanti) of pore size 100 nm 25 times (must be an odd number). The untrapped HPTS dye was removed through size exclusion chromatography using Sephadex G-50 (Sigma-Aldrich) with HEPES buffer (10 mM HEPES, 100 mM NaCl, pH = 7.0). The collected vesicles were then diluted to 6 mL to get EYPC-LUVs $\Rightarrow$ HPTS. Final conditions: ~5 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 activity by HPTS assay (Assay protocol A1): In a clean, dry fluorescence cuvette, 1975 μ L of HEPES buffer (10 mM HEPES, 100 mM NaCl, pH = 7.0) was added, followed by 25 μ L of EYPC-LUVs $\Rightarrow$ HPTS. The cuvette was placed in the fluorescence instrument with a slow stirring condition by a magnetic stirrer equipped in the instrument ($t = 0$ s). The time dependent fluorescence emission intensity of HPTS was monitored at $\lambda_{em} = 510$ nm ($\lambda_{ex} = 450$ nm). A pH gradient (Δ pH = 0.8) was created across the intra- and extra-vesicular system by the addition of 20 μ L of 0.5 M NaOH at $t = 20$ s. The required concentrations of transporter molecules were added at $t = 100$ s, and finally, the vesicles were lysed by the addition of 25 μ L 10% Triton X-100 at $t = 300$ s to destroy the pH gradient. The collected data was then normalized to the percentage change in fluorescence intensity with respect to time using 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 (just before transporter addition), I_t is the intensity at time t , and I_∞ the final intensity after the addition of Triton X-100.

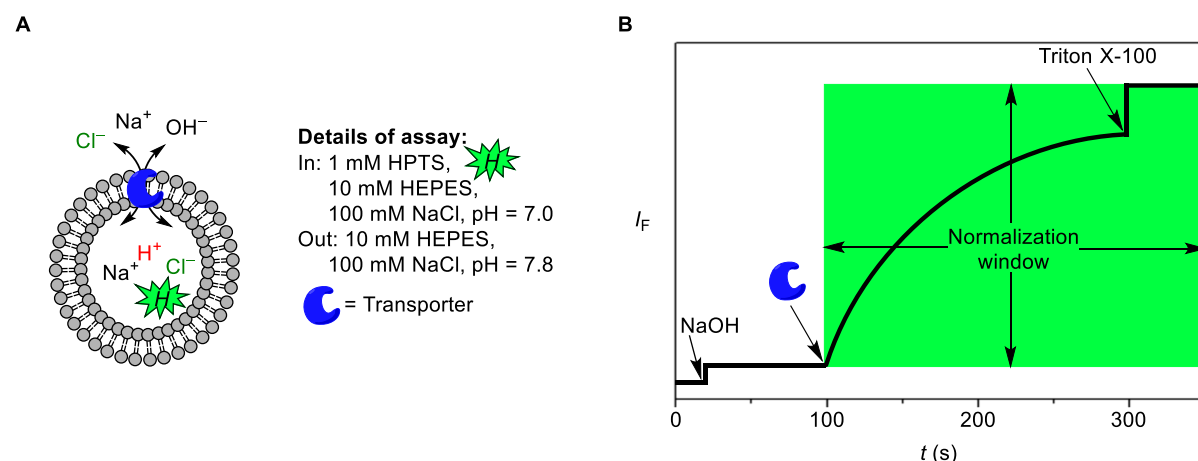


Figure 77: Schematic of fluorescence-based assay of ion transport activity using EYPC-LUVs⊃HPTS (A), and representative diagram of ion transport kinetics depicting the normalization window (B).

Dose response activity: The ion transport activities of all the transporters were measured at different concentrations. The I_F values were plotted against the respective concentration values for each concentration profile and were fitted using Hill equation (Equation 2) to obtain the corresponding EC_{50} (i.e. the concentration of transporter necessary to achieve 50% dissipation of pH gradient through ion transport):

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

where Y_0 = fluorescence intensity just before the transporter addition, Y_∞ = fluorescence intensity with excess transporter concentration, c = concentration of transporter compound, and n = Hill coefficient (i.e. indicative for the number of monomers needed to form an active supramolecular ion transport complex).

Ion selectivity studies across EYPC-LUVs⊃HPTS:

Preparation of HEPES buffers and stock solutions: The HEPES buffer (pH = 7.0) was prepared by dissolving solid HEPES (10 mM) and salts (100 mM) (NaCl, NaBr, NaI, NaNO₃, NaClO₄, Na₂SO₄ (33.3 mM), LiCl, KCl, RbCl, CsCl) in autoclaved Milli-Q water, followed by adjustment of pH to 7.0 using 0.5 M NaOH solution. The stock of the most active compound (**1b**) was prepared in HPLC grade DMSO.

Anion selectivity assay (varying only extravesicular anion) (Assay protocol A2): The EYPC-LUVs $\supset$ HPTS (Inside: 1 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0) were prepared as per the procedure given above. In a clean, dry fluorescence cuvette, 1975 μ L of HEPES buffer (10 mM HEPES, 100 mM NaX, pH = 7.0, X⁻ = Cl⁻, Br⁻, I⁻, NO₃⁻, and ClO₄⁻) was added, followed by 25 μ L of EYPC-LUVs $\supset$ HPTS. The cuvette was placed with a slow stirring condition in a magnetic stirrer-equipped fluorescence instrument ($t = 0$ s). The time dependent fluorescence emission intensity of HPTS was monitored at $\lambda_{em} = 510$ nm ($\lambda_{ex} = 450$ nm). A pH gradient (Δ pH = 0.8) was created across the intra- and extra-vesicular system by the addition of 20 μ L of 0.5 M NaOH at $t = 20$ s. The transporter **1b** (0.6 μ M) was added at $t = 100$ s, and finally, the vesicles were lysed by the addition of 25 μ L 10% Triton X-100 at $t = 300$ s to destroy the pH gradient. The collected data was then normalized to the percentage change in fluorescence intensity with respect to time using equation 1.

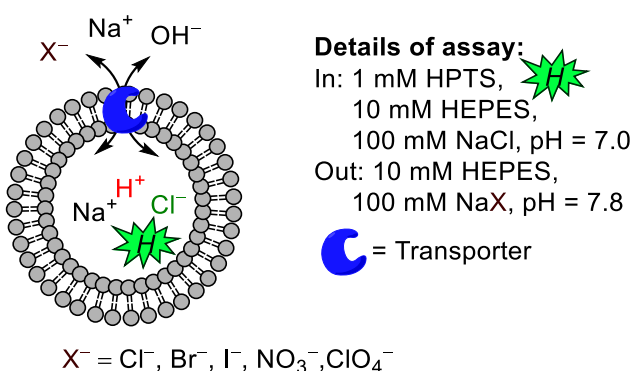


Figure 78: Schematic of fluorescence-based anion selectivity assay (by varying extravesicular anions) using EYPC-LUVs $\supset$ HPTS.

Cation selectivity assay (Assay protocol A3): Similarly, the cation selectivity assay of **1b** (0.6 μ M) was performed by varying the extravesicular cation in the HEPES buffer (10 mM HEPES, 100 mM MCl, pH = 7.0, M⁺ = Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺). The data was normalized to the percentage change in fluorescence intensity with respect to time using equation 1.

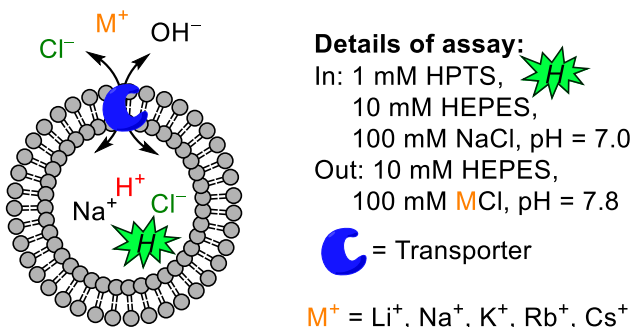


Figure 79: Schematic of fluorescence-based cation selectivity using EYPC-LUVs $\supset$ HPTS.

Anion selectivity assay without pH gradient (Assay protocol A4): The EYPC-LUVs $\supset$ HPTS (Inside: 1 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0) were prepared as per the procedure given above. In a clean, dry fluorescence cuvette, 1975 μL of HEPES buffer (10 mM HEPES, 100 mM NaX, pH = 7.0, $\text{X}^- = \text{Cl}^-, \text{Br}^-, \text{I}^-$, and NO_3^- ; 33.3 mM for SO_4^{2-}) was added, followed by 25 μL of EYPC-LUVs $\supset$ HPTS. The cuvette was placed with a slow stirring condition in a magnetic stirrer-equipped fluorescence instrument ($t = 0$ s). The time dependent fluorescence emission intensity of HPTS was monitored at $\lambda_{\text{em}} = 510$ nm ($\lambda_{\text{ex}} = 450$ nm). The transporter **1b** (0.6 μM) was added at $t = 50$ s, and finally, the vesicles were lysed by the addition of 25 μL 10% Triton X-100 at $t = 300$ s to destroy the pH gradient. The collected data was then normalized to the percentage change in fluorescence intensity with respect to time using equation 1.

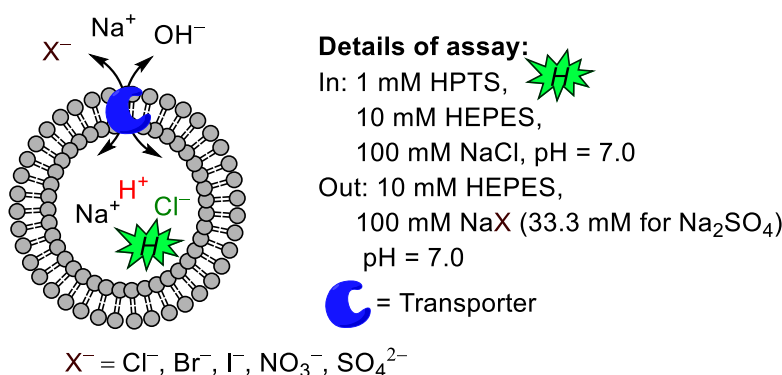


Figure 80: Schematic of fluorescence-based anion selectivity assay without pH gradient using EYPC-LUVs $\supset$ HPTS.

pH dissipation across EYPC-LUVs⊃HPTS in the presence of FCCP:

Confirmation between symport or antiport mechanism of ion transport of the molecule (**1b**) was carried out through FCCP coupled HPTS assay. The ion transport activity of **1b** was monitored across EYPC-LUVs⊃HPTS in the presence and absence of FCCP.

FCCP coupled HPTS assay (Assay protocol A5): The EYPC-LUVs⊃HPTS (Inside: 1 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0) were prepared as per the procedure given above. The fluorescence kinetics of the transporter (**1b**) was monitored in the presence and absence of FCCP. In a clean, dry fluorescence cuvette, 1975 μ L of HEPES buffer (10 mM HEPES, 100 mM NaCl, pH = 7.0) was added followed by 25 μ L of EYPC-LUVs⊃HPTS. The cuvette was placed with slow stirring condition in a magnetic stirrer equipped fluorescence instrument ($t = 0$ s). The time dependent fluorescence emission intensity of HPTS was monitored at $\lambda_{\text{em}} = 510$ nm ($\lambda_{\text{ex}} = 450$ nm). A pH gradient ($\Delta\text{pH} = 0.8$) was created across the intra- and extra- vesicular system by addition of 20 μ L of 0.5 M NaOH at $t = 20$ s. FCCP (in DMSO) was added at $t = 50$ s, followed by the transporter **1b** at $t = 100$ s, and finally the vesicles were lysed by addition of 25 μ L 10% Triton X-100 at $t = 300$ s to completely disrupt the pH gradient. The collected data was then normalized to the percentage change in fluorescence intensity with respect to time using the equation 1.

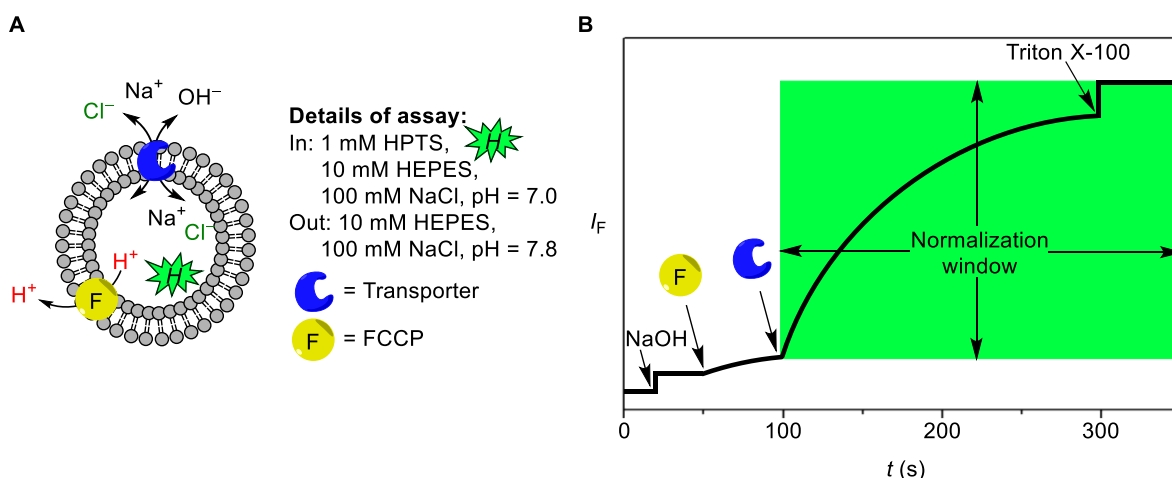


Figure 81: Schematic of FCCP coupled ion transport assay in EYPC-LUVs⊃HPTS (**A**), and representative diagram of ion transport kinetics depicting the normalization window (**B**).

Study of chloride transport across EYPC-LUVs⊃Lucigenin:

Preparation of stock solutions: Stock solutions of all transporter molecules were prepared in HPLC grade acetonitrile.

Preparation of EYPC-LUVs⊃Lucigenin in NaNO₃ (Prep protocol P2): In a clean, dry 10 mL round bottom flask, 1 mL of egg yolk phosphatidylcholine (EYPC, 25 mg/mL in CHCl₃) was dried to a thin transparent film by purging nitrogen gas along with continuous rotation. The transparent film was kept under a high vacuum for 4-5 h to remove all traces of CHCl₃. The resulting film was then hydrated with 1 mL NaNO₃ (200 mM, 1 mM Lucigenin) solution by vortexing at 10 min intervals for a total duration of 1 h. This hydrated suspension was subjected to ≥11 cycles of freeze-thaw (liquid N₂, 55 °C). Extrusions were done using a Mini-extruder through a polycarbonate membrane (Avanti) of pore size 200 nm 25 times (must be an odd number). The extravesicular lucigenin dye was removed through size exclusion chromatography using Sephadex G-50 with 200 mM NaNO₃ solution as eluent. The collected vesicles were then diluted to 4 mL to get EYPC-LUVs⊃Lucigenin. Final conditions: ~7.5 mM EYPC; Inside: 1 mM Lucigenin, 200 mM NaNO₃; Outside: 200 mM NaNO₃.

Ion transport activity by Lucigenin assay (Assay protocol A6): In a clean, dry fluorescence cuvette, 1975 μL of 200 mM NaNO₃ solution was added, followed by 25 μL of EYPC-LUVs⊃lucigenin. The cuvette was placed with a slow stirring condition in a magnetic stirrer-equipped fluorescence instrument ($t = 0$ s). The time dependent fluorescence emission intensity of lucigenin was monitored at $\lambda_{em} = 535$ nm ($\lambda_{ex} = 455$ nm). A chloride gradient was created across the intra- and extra-vesicular system by the addition of 33 μL of 2.0 M NaCl at $t = 20$ s. The required concentrations of transporter molecules were added at $t = 100$ s, and finally, the vesicles were lysed by the addition of 25 μL 10% Triton X-100 at $t = 300$ s to destroy the chloride gradient. The data was then normalized to the percentage change in fluorescence intensity with respect to time using 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 (just before transporter addition), I_t is the intensity at time t , and I_∞ the final intensity after the addition of Triton X-100.

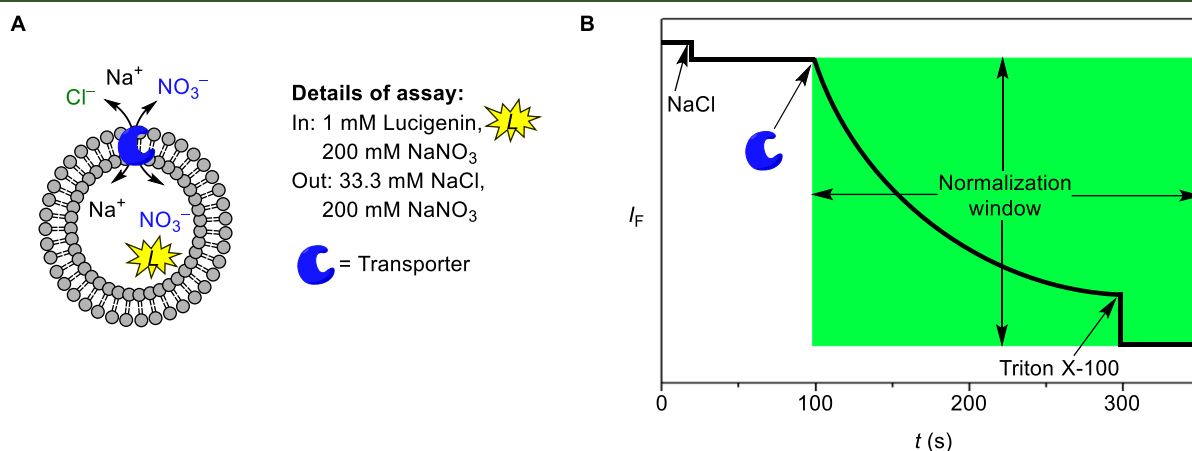


Figure 82: Schematic of fluorescence-based assay of ion transport activity using EYPC-LUVs⊃lucigenin (A), and representative diagram of ion transport kinetics depicting the normalization window (B).

Dose response activity: The ion transport activities of all the transporters were measured at different concentrations. The I_F values were plotted against the respective concentration values for each concentration profile and were fitted using Hill equation (Equation 2) to obtain the corresponding EC_{50} (i.e., the concentration of transporter necessary to achieve 50% chloride efflux from vesicles):

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

where Y_0 = fluorescence intensity just before the transporter addition, Y_{∞} = fluorescence intensity with excess transporter concentration, c = concentration of transporter compound, and n = Hill coefficient (i.e., indicative for the number of monomers needed to form an active supramolecular ion transport complex).

Cation selectivity assay across EYPC-LUVs⊃Lucigenin (Assay protocol A7): The vesicles were prepared as per the procedure given above. In a clean, dry fluorescence cuvette, 1975 μ L of 200 mM NaNO₃ solution was added, followed by 25 μ L of EYPC-LUVs⊃lucigenin. The cuvette was placed with a slow stirring condition in a magnetic stirrer-equipped fluorescence instrument ($t = 0$ s) and the time dependent fluorescence emission intensity of lucigenin was monitored at $\lambda_{em} = 535$ nm ($\lambda_{ex} = 455$ nm). A chloride gradient across the intra- and extra-vesicular system was created by the addition of 33 μ L of 2.0 M chloride salts (MCl) of different cations ($M^+ = Li^+, Na^+, K^+, Rb^+, Cs^+$) at $t = 20$ s. The transporter **1b** (5 μ M) was added at $t = 100$ s, and finally, the vesicles

were lysed by the addition of 25 μ L 10% Triton X-100 at $t = 300$ s to destroy the chloride gradient. The data was then normalized to the percentage change in fluorescence intensity with respect to time using equation 3.

Chloride transport across EYPC-LUVs $\supset$ Lucigenin in the presence of valinomycin:

Checking for antiport mechanism of ion transport of the molecule (**1b**) was carried out through valinomycin coupled lucigenin assay. The ion transport activity of **1b** was monitored in vesicles entrapping 1 mM lucigenin and 200 mM NaNO₃ suspended in 200 mM KNO₃ solution in the presence and absence of valinomycin.

Preparation of EYPC-LUVs $\supset$ Lucigenin: The vesicles (with 1 mM lucigenin and 200 mM NaNO₃ entrapped) were prepared as per *Prep protocol P2*.

Valinomycin coupled lucigenin assay (Assay protocol A8): In a clean, dry fluorescence cuvette, 1975 μ L of 200 mM KNO₃ solution was added, followed by 25 μ L of EYPC-LUVs $\supset$ lucigenin. The fluorescence kinetics of the transporter (**1b**) was monitored in the presence and absence of valinomycin. The cuvette was placed with a slow stirring condition in a magnetic stirrer-equipped fluorescence instrument ($t = 0$ s) and the time dependent fluorescence emission intensity of lucigenin was monitored at $\lambda_{em} = 535$ nm ($\lambda_{ex} = 455$ nm). A chloride gradient across the intra- and extra-vesicular system was created by the addition of 33 μ L of 2.0 M KCl at $t = 20$ s, followed by the addition of valinomycin (1 μ M in CH₃CN) at $t = 50$ s. The transporter **1b** (2.5 μ M) was added at $t = 100$ s, and finally, the vesicles were lysed by the addition of 25 μ L 10% Triton X-100 at $t = 300$ s to destroy the chloride gradient. The data was then normalized to the percentage change in fluorescence intensity with respect to time using equation 3.

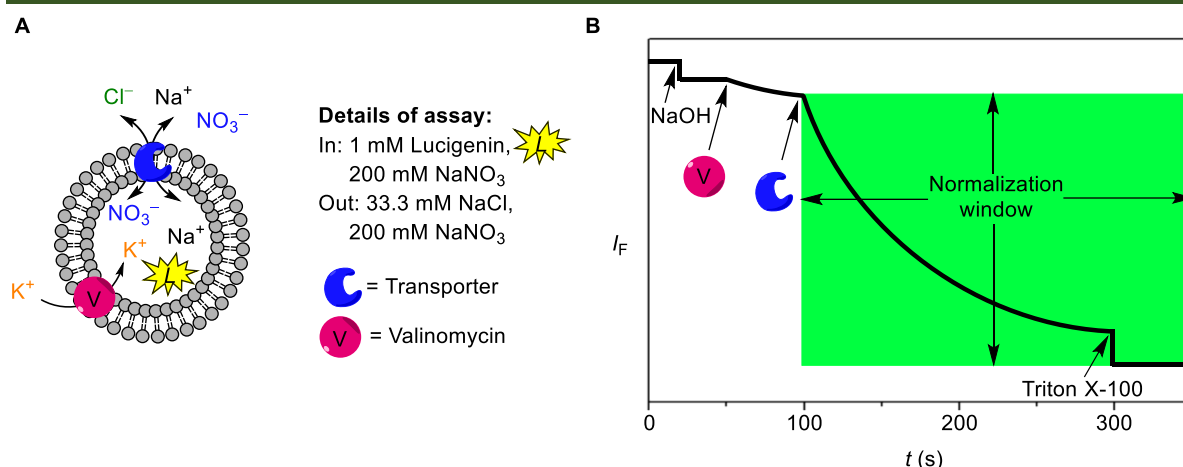


Figure 83: Schematic of valinomycin coupled ion transport assay in EYPC-LUVs $\supset$ lucigenin (**A**), and representative diagram of ion transport kinetics depicting the normalization window (**B**).

Cl⁻ -Ion selective electrode (ISE) based studies in EYPC-LUVs $\supset$ Cl⁻:

Chloride efflux, from vesicles entrapping Cl⁻, during transport experiments was measured using a Cl⁻ ion selective electrode (ISE) (Thermo Scientific Orion™). The electrode was calibrated against chloride solutions of known concentrations before each experiment.

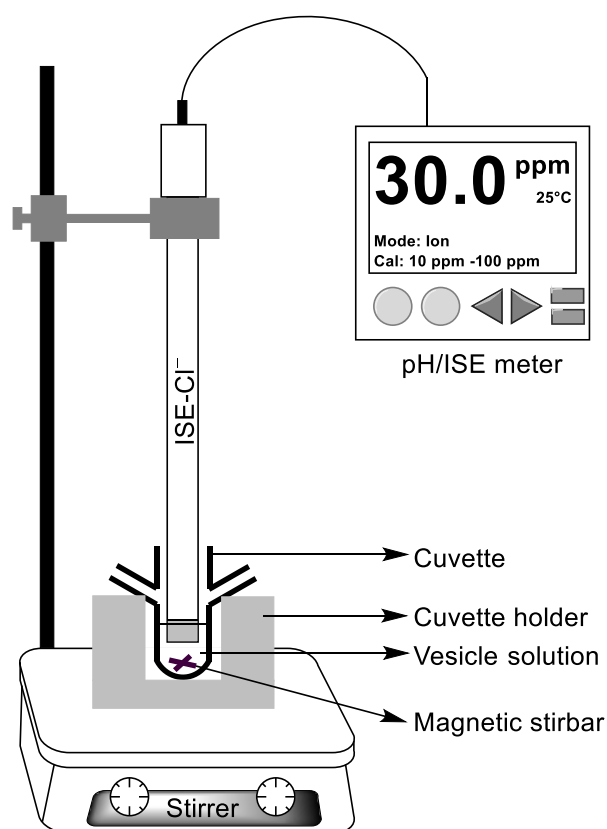


Figure 84: Schematic of setup for Cl⁻-ISE experiments in EYPC-LUVs.

Study of ion transport activities of 1b and 2 across EYPC-LUVs $\Rightarrow$ Cl⁻:

Chloride ion transport activities of transporter **1b** and protransporter **2** were compared across EYPC-LUVs $\Rightarrow$ NaCl (500 mM) suspended in NaNO₃ (500 mM) solution by monitoring the transporter-induced chloride efflux using Cl⁻-ISE.

Preparation of phosphate buffers and stock solutions: The NaCl-phosphate buffer was prepared by dissolving solid NaCl (500 mM) in 10 mM Na-phosphate buffer and pH adjusted to 7.0. The NaNO₃-phosphate buffer was prepared by dissolving solid NaNO₃ (500 mM) in 10 mM Na-phosphate buffer and pH adjusted to 7.0. Stock solutions of transporter molecules were prepared in HPLC grade DMSO.

Preparation of EYPC-LUVs for ISE experiments (Prep protocol P3): In a clean, dry 10 mL round bottom flask, 1 mL of egg yolk phosphatidylcholine (EYPC, 25 mg/mL in CHCl₃) was dried to a thin transparent film by purging nitrogen gas along with continuous rotation. The transparent film was kept under a high vacuum for 4-5 h to remove all traces of CHCl₃. The resulting film was then hydrated with 1 mL NaCl-phosphate buffer (500 mM NaCl, 10 mM Na-phosphates, pH = 7.0) by vortexing at 10 min intervals for a total duration of 1 h. This hydrated suspension was subjected to ≥ 11 cycles of freeze-thaw (liquid N₂, 55 °C). Extrusions were done using a Mini-extruder through a polycarbonate membrane (Avanti) of pore size 200 nm 25 times (must be an odd number). The resulting unilamellar vesicles were dialyzed (using MWCO 1 kD membrane) against NaNO₃-phosphate buffer (500 mM NaNO₃, 10 mM Na-phosphates, pH = 7.0) for 4 h (buffer changed at 2 h) to remove unencapsulated chloride ions. Final conditions: ~40 mM EYPC; Inside: 500 mM NaCl, 10 mM Na-phosphates, pH = 7.0; Outside: 500 mM NaNO₃, 10 mM Na-phosphates, pH = 7.0.

Transporter mediated Cl⁻ efflux across vesicles (Assay protocol A9): NaCl entrapped vesicles (50 μ L) were suspended in 1950 μ L of 500 mM NaNO₃-phosphate buffer (pH = 7.0) in a clean, dry cuvette. The chloride-ISE was dipped into the solution and a slow stirring condition was provided using a magnetic stirrer ($t = 0$ s). The time dependent chloride efflux was monitored using the ISE. The transporter was added at $t = 50$ s, and finally, the vesicles were lysed by the addition of 25 μ L 10% Triton X-100 at the end of the experiment ($t = 310$ s) to destroy the chloride gradient. The data was normalized to the percentage change in chloride concentration with respect to time,

setting the value at $t = 40$ s as 0% chloride efflux and the final chloride reading (stable reading after lysis of the vesicles) as 100% chloride efflux using equation 4.

$$\% \text{ chloride efflux} = \frac{([\text{Cl}^-]_t - [\text{Cl}^-]_0)}{([\text{Cl}^-]_\infty - [\text{Cl}^-]_0)} \times 100 \quad (\text{Equation 4})$$

Where $[\text{Cl}^-]_0$ is the initial chloride concentration (at $t = 40$ s), $[\text{Cl}^-]_t$ is the concentration at time t , and $[\text{Cl}^-]_\infty$ is the final stable value after lysis of the vesicles using Triton X-100.

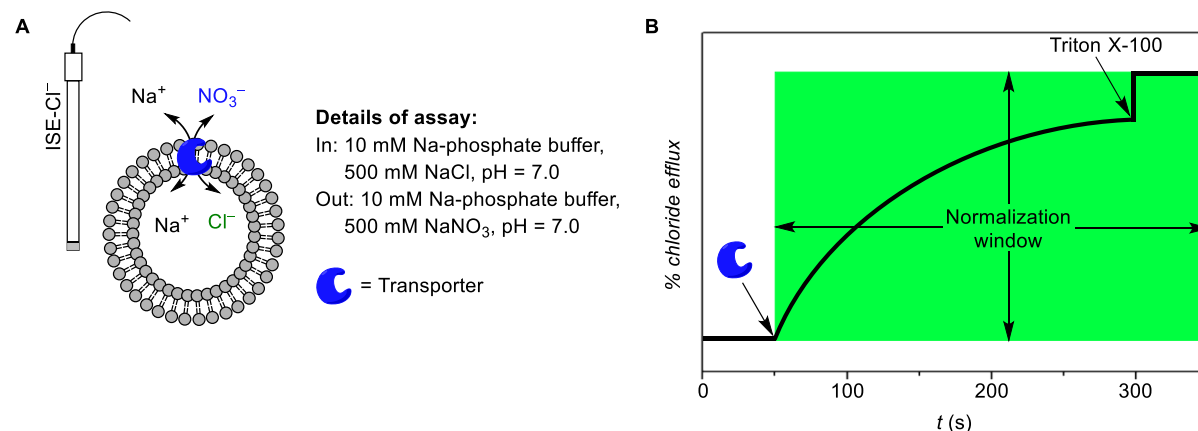


Figure 85: Schematic of ISE based chloride efflux assay using chloride entrapped EYPC-LUVs (A), and representative diagram of ion transport kinetics depicting the normalization window (B).

Ion transport studies in the presence of Monensin and Valinomycin using ISE vesicles:

The ion transport activities of compound **1b** in the presence of valinomycin and monensin were studied to provide further insights into the mechanism of ion transport.

Preparation of phosphate buffers and stock solutions: The KCl-phosphate buffer was prepared by dissolving solid KCl (300 mM) in 10 mM K-phosphate buffer and pH adjusted to 7.0. The K gluconate-phosphate buffer was prepared by dissolving solid potassium gluconate (300 mM) in 10 mM K-phosphate buffer and pH adjusted to 7.0. Stock solutions of the transporter molecule, monensin (in the form of monensin sodium salt), and valinomycin were prepared in HPLC grade DMSO.

Preparation of ISE vesicles for mechanistic studies (Perp protocol P4): In a clean, dry 10 mL round bottom flask, 1 mL of egg yolk phosphatidylcholine (EYPC, 25 mg/mL in CHCl₃) was dried to a thin transparent film by purging nitrogen gas along with continuous rotation. The transparent film was kept under a high vacuum for 4-5 h to remove all traces of CHCl₃. The resulting film was then hydrated with 1 mL KCl-phosphate buffer (300 mM NaCl, 10 mM K-phosphates, pH = 7.0)

by vortexing at 10 min intervals for a total duration of 1 h. This hydrated suspension was subjected to ≥ 11 cycles of freeze-thaw (liquid N₂, 55 °C). Extrusions were done using a Mini-extruder through a polycarbonate membrane (Avanti) of pore size 200 nm 25 times (must be an odd number). The resulting unilamellar vesicles were dialyzed (using MWCO 1 kD membrane) against K gluconate-phosphate buffer (300 mM potassium gluconate, 10 mM K-phosphates, pH = 7.0) for 4 h (buffer changed at 2 h) to remove unencapsulated chloride ions. Final conditions: ~40 mM EYPC; Inside: 300 mM KCl, 10 mM K-phosphates, pH = 7.0; Outside: 300 mM potassium gluconate, 10 mM K-phosphates, pH = 7.0.

Monensin coupled assay using ISE vesicles (Assay protocol A10): The KCl entrapped vesicles (50 μ L) were suspended in 1950 μ L of 300 mM K gluconate-phosphate buffer (pH = 7.0) in a clean, dry cuvette. The chloride-ISE was dipped into the solution and a slow stirring condition was provided using a magnetic stirrer ($t = 0$ s). The time dependent chloride efflux was monitored using the ISE. The monensin (1 μ M) was added at $t = 20$ s followed by transporter **1b** (20 μ M) addition at $t = 50$ s, and finally, the vesicles were lysed by the addition of 25 μ L 10% Triton X-100 at the end of the experiment ($t = 310$ s) to destroy the chloride gradient. The data was normalized to the percentage change in chloride concentration with respect to time, setting the value at $t = 40$ s as 0% chloride efflux and the final chloride reading (stable reading after lysis of the vesicles) as 100% chloride efflux using equation 4.

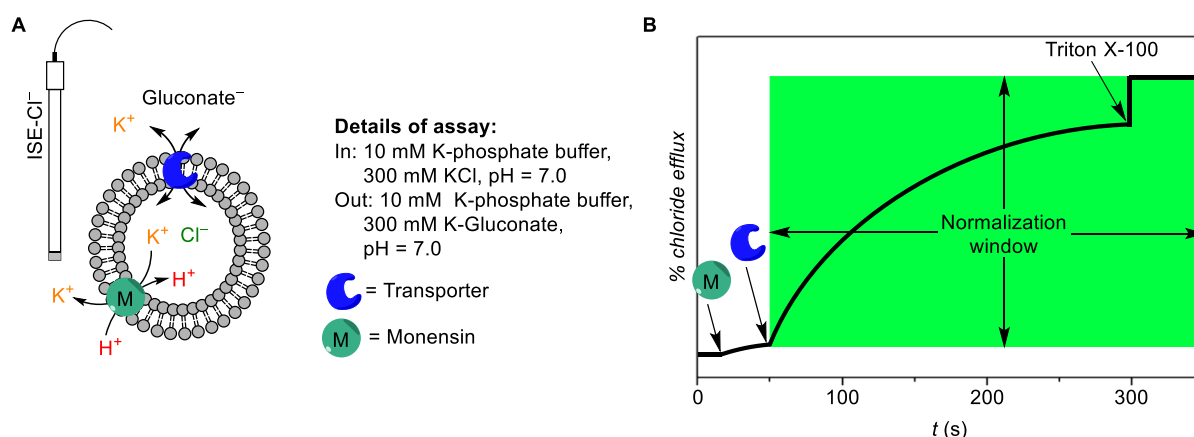


Figure 86: Schematic monensin coupled ISE assay (**A**), and representative diagram of ion transport kinetics depicting the normalization window (**B**).

Valinomycin coupled assay using ISE vesicles (Assay protocol A11): The assay for transporter **1b** (20 μ M) was performed in the same way as the monensin coupled assay, with valinomycin (1

μM) addition at $t = 20$ s. The data was normalized to the percentage change in chloride concentration with respect to time, setting the value at $t = 40$ s as 0% chloride efflux and the final chloride reading (stable reading after vesicle lysis) as 100% chloride efflux using equation 4.

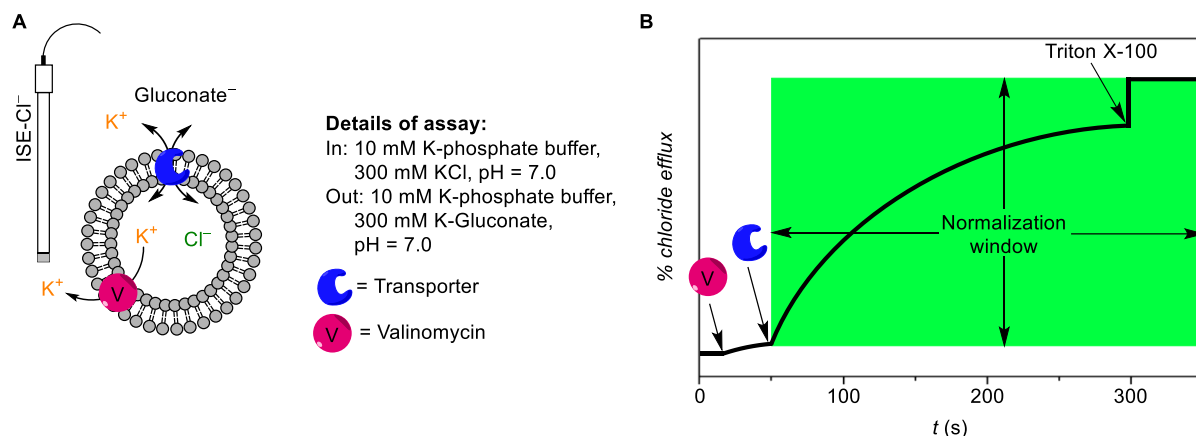


Figure 87: Schematic valinomycin coupled ISE assay (**A**), and representative diagram of ion transport kinetics depicting the normalization window (**B**).

Carboxyfluorescein (CF) leakage assay:

Preparation of buffer solutions: The CF-HEPES buffer was prepared by adding solid 5(6)-carboxyfluorescein (CF, 50 mM) in 10 mM NaCl, 10 mM HEPES solution, and dissolving it by addition of conc. NaOH aliquots, following which the pH was then adjusted to 7.0. The extravesicular buffer was prepared by dissolving NaCl (100 mM) and HEPES (10 mM) in MilliQ water and pH was adjusted to 7.0 using 0.5 M NaOH.

Preparation of CF entrapped vesicles (Prep protocol P5): In a clean, dry 10 mL round bottom flask, 0.5 mL of egg yolk phosphatidylcholine (EYPC, 25 mg/mL in CHCl₃) was dried to a thin transparent film by purging nitrogen gas along with continuous rotation. The transparent film was kept under a high vacuum for 4-5 h to remove all traces of CHCl₃. The resulting film was then hydrated with 0.5 mL CF-HEPES buffer (50 mM CF, 10 mM NaCl, 10 mM HEPES, pH 7.0) by vortexing at 10 min intervals for a total duration of 1 h. This hydrated suspension was subjected to ≥ 15 cycles of freeze-thaw (liquid N₂, 55 °C). Extrusions were done using a Mini-extruder through a polycarbonate membrane (Avanti) of pore size 200 nm 25 times (must be an odd number). The untrapped CF dye was removed through size exclusion chromatography using Sephadex G-50 (Sigma-Aldrich) with HEPES buffer (10 mM HEPES, 100 mM NaCl, pH = 7.0). The collected vesicles were then diluted to 3 mL to get EYPC-LUVs $\supset$ CF. Final conditions:

~ 5 mM EYPC; Inside: 50 mM CF, 10 mM HEPES, 10 mM NaCl, pH = 7.0; Outside: 10 mM HEPES, 100 mM NaCl, pH = 7.0.

CF leakage assay (Assay protocol A12): In a clean, dry fluorescence cuvette, 1975 μ L of HEPES buffer (10 mM HEPES, 100 mM NaCl, pH = 7.0) was added, followed by 25 μ L of EYPC-LUVs $\supset$ CF. The cuvette was placed in the fluorescence instrument with a slow stirring condition by a magnetic stirrer equipped in the instrument ($t = 0$ s). The time dependent fluorescence emission intensity of HPTS was monitored at $\lambda_{em} = 517$ nm ($\lambda_{ex} = 492$ nm). The required concentrations of transporter molecules were added at $t = 50$ s and the vesicles were lysed by the addition of 25 μ L 10% Triton X-100 at $t = 300$ s to destroy the pH gradient. The collected data was then normalized to the percentage change in fluorescence intensity with respect to time using the equation 1.

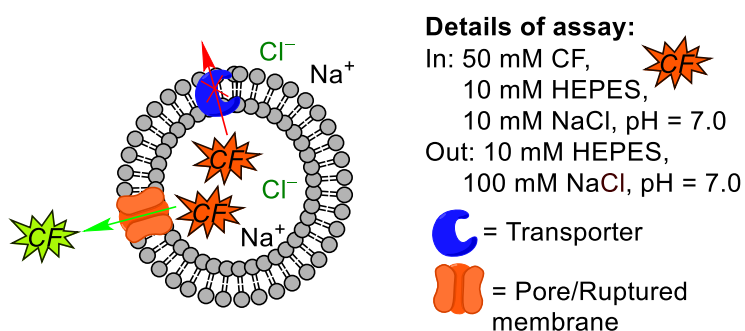


Figure 88: Schematic of CF leakage in EYPC-LUVs $\supset$ CF in the presence of ion transporter vs pore or membrane disrupting agent.

2.4.7 Vesicle Size Measurement- DLS

Vesicle size distribution was determined through dynamic light scattering (DLS) measurements with a Malvern Instruments Zetasizer Nano ZS90 Size Analyzer using a 633 nm red laser.

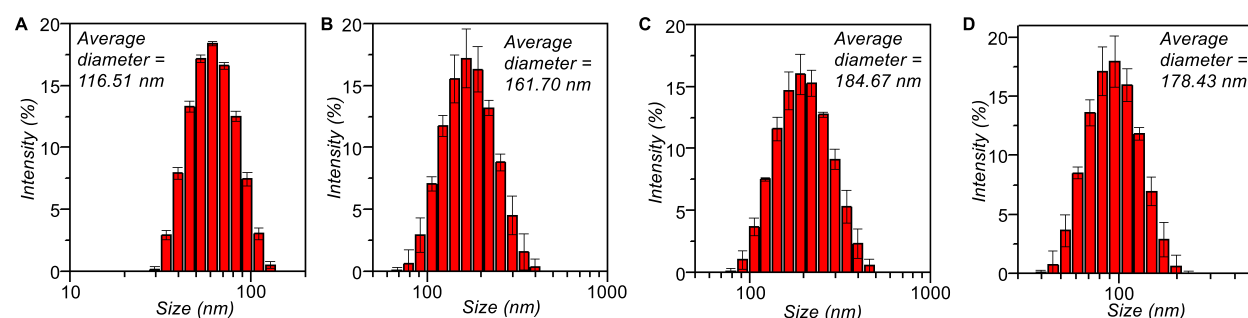


Figure 89: Size distributions of (A) EYPC-LUVs $\supset$ HPTS, (B) EYPC-LUVs $\supset$ Lucigenin, (C) EYPC-LUVs $\supset$ NaCl (Cl⁻ efflux assay), and (D) EYPC-LUVs $\supset$ KCl (Mon/Val coupled assay).

2.4.8 Fluorescence Emission Study of **2**

In a clean, dry fluorescence cuvette, 1975 μL of HEPES buffer (10 mM HEPES, 100 mM NaCl, pH = 7.0) was added, followed by 25 μL of EYPC-LUVs $\Rightarrow$ HPTS (Inside: 1 mM HPTS, 10 mM HEPES, 100 mM NaCl, pH = 7.0). The cuvette was placed in the fluorescence instrument with slow stirring conditions by a magnetic stirrer equipped in the instrument. The emission spectrum of the solution was recorded ($\lambda_{\text{ex}} = 450 \text{ nm}$). The vesicle solution was then treated with varying concentrations of **2** (stocks prepared in DMSO) in each experimental run and the emission spectrum was recorded ($\lambda_{\text{ex}} = 450 \text{ nm}$). Addition of an equivalent amount of DMSO was used for the control experiment. Finally, 25 μL of 10% Triton X-100 was added to the vesicle solution to completely lyse the lipid membranes and release the entrapped HPTS dye into the bulk solution and the emission spectrum recorded. All plots were normalised with respect to fluorescence intensity at the emission maximum ($\lambda_{\text{max}} = 512 \text{ nm}$) of vesicles before treatment with **2**.

2.4.9 Planar Bilayer Conductance Experiment

Bilayer membrane was formed across an aperture of 150 μm diameter in a polystyrene cup (Warner Instruments, USA) with the lipid diphytanoylphosphatidylcholine (Avanti Polar Lipids), dissolved in *n*-decane (20 mg/mL). Both chambers (cis and trans) were filled with symmetrical solution, containing 1.0 M KCl. The trans compartment was held at virtual ground and the cis chamber was connected to the BC 535 head-stage (Warner Instrument, USA) via matched Ag-AgCl electrodes. Compound **1b** (20 μM) was added to the trans chamber and the solution was stirred with magnetic stirrer for 10 min. No channel opening and closing events were observed after applying voltages. Currents were low pass filtered at 1 kHz using pClamp9 software (Molecular probes, USA) and analog-to digital converter (Digidata 1440, Molecular probes). The data were analyzed by the software pClamp 11.1.

2.4.10 Theoretical Calculations

Initial prediction of the most probable geometry of molecules/complexes were first conducted with CONFLEX 8 conformation search software package^{77, 78} using MMFF94S (2010-12-04HG) force field with a search limit of 10 kcal/mol. The output conformation with the highest predicted Boltzmann population was then used for geometry optimization. In the event of CONFLEX 8 yielding no logical results, the initial model system was constructed manually using VMD molecular graphics viewer.⁵¹ The DFT geometry optimization and energy calculation of the initial

model obtained/constructed was conducted on Gaussian 09 program suite⁴⁶ the B3LYP exchange-correlation functional^{47, 48} and the 6-31G(d,p) basis set.^{49, 50} The zero-point energy (ZPE) and basis set superposition error (BSSE) of each complex were calculated and used in the following equation to obtain the binding energy (BE)-

$$BE = [HF_{HG} + ZPE_{HG} + BSSE_{HG}] - n_H*[HF_H + ZPE_H] - n_G*[HF_G] \quad (\text{Equation 5})$$

where H = host (receptor), G = guest (Cl⁻), HF_{HG} = electronic energy of host-guest complex, ZPE_{HG} = ZPE of host-guest complex, BSSE_{HG} = BSSE of host-guest complex, HF_H = electronic energy of host, ZPE_H = ZPE of host, HF_G = electronic energy of guest, n_H = number of host (receptor) species, n_G = number of guest (Cl⁻) species.

Table 12: Atomic coordinates of ‘open-ring’ **1b** after geometry optimization by GAUSSIAN 09 using B3LYP/6-31G(d,p).

Charge = 0

Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	-6.40155	0.24276	0.00325
2	C	-5.4853	1.28994	-0.00848
3	C	-4.1125	1.02832	-0.00941
4	C	-3.63219	-0.29744	0.00186
5	C	-4.58311	-1.33078	0.0135
6	C	-5.94935	-1.07779	0.01418
7	C	-2.1914	-0.74836	0.00436
8	N	-1.23165	0.23986	-0.00897
9	O	-1.91739	-1.94465	0.01843
10	C	0.16205	0.09075	-0.00671
11	O	-3.2153	2.07075	-0.02113
12	C	0.92606	1.26875	-0.03803
13	C	2.31583	1.21141	-0.0338
14	C	2.97814	-0.01604	-0.00168
15	C	2.2156	-1.18222	0.0283

16	C	0.82067	-1.14865	0.02794
17	C	3.10951	2.49064	0.0045
18	F	2.44445	3.51306	-0.58098
19	F	4.29705	2.36388	-0.62413
20	F	3.37479	2.87285	1.27509
21	C	2.90982	-2.52084	-0.00209
22	F	4.13865	-2.45948	0.55791
23	F	3.07718	-2.96201	-1.27019
24	F	2.20907	-3.46688	0.65648
25	H	-7.46478	0.46258	0.00388
26	H	-5.83053	2.32175	-0.01691
27	H	-4.19909	-2.34448	0.02233
28	H	-6.6559	-1.90093	0.02333
29	H	-1.57499	1.1923	-0.0215
30	H	-3.68998	2.91189	-0.02787
31	H	0.43214	2.2346	-0.07179
32	H	4.05982	-0.0614	3.91E-4
33	H	0.24199	-2.05956	0.05876

Table 13: Atomic coordinates of ‘closed-ring’ **1b** after geometry optimization by GAUSSIAN 09 using B3LYP/6-31G(d,p).

Charge = 0

Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	6.21108	-1.17358	-0.09116
2	C	5.98007	0.15803	0.2095
3	C	4.67192	0.67459	0.20847
4	C	3.57713	-0.18999	-0.07238
5	C	3.85272	-1.53512	-0.39584
6	C	5.14588	-2.03075	-0.40758
7	C	2.20973	0.37744	-0.01087

8	N	1.16156	-0.51223	-0.03958
9	O	2.01558	1.60248	0.08198
10	C	-0.22118	-0.25632	-0.02182
11	O	4.5208	1.97672	0.48186
12	C	-1.07151	-1.37223	1.76E-4
13	C	-2.45256	-1.20638	0.0124
14	C	-3.01579	0.06942	-0.00233
15	C	-2.16599	1.17339	-0.0262
16	C	-0.77799	1.03106	-0.03408
17	C	-3.34502	-2.4168	0.1091
18	F	-2.76597	-3.51115	-0.43474
19	F	-4.522	-2.22061	-0.51993
20	F	-3.62831	-2.71949	1.39593
21	C	-2.75625	2.55989	-0.10681
22	F	-3.98419	2.61107	0.45382
23	F	-2.8899	2.9632	-1.39057
24	F	-1.98289	3.4717	0.51717
25	H	7.22889	-1.55247	-0.09306
26	H	6.79122	0.83957	0.4413
27	H	3.04688	-2.20616	-0.68141
28	H	5.33089	-3.06742	-0.6672
29	H	1.40894	-1.48894	0.00117
30	H	3.55693	2.17891	0.37732
31	H	-0.65677	-2.37556	0.00248
32	H	-4.09049	0.19943	0.00604
33	H	-0.13247	1.89619	-0.03882

Table 14: Atomic coordinates ‘open-ring’ of [**1b** + Cl⁻] after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p).

Charge = -1

Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	6.21314	0.48563	0.11032
2	C	5.42425	-0.3862	-0.63068
3	C	4.02415	-0.26479	-0.65781
4	C	3.41756	0.7789	0.08087
5	C	4.23519	1.66526	0.80195
6	C	5.61672	1.521	0.83693
7	O	3.32447	-1.10093	-1.46424
8	C	1.94923	1.11296	0.08257
9	N	1.06616	0.06037	0.06563
10	O	1.5934	2.29349	0.13873
11	C	-0.32933	0.13212	0.04487
12	C	-1.03359	-1.08958	0.05533
13	C	-2.42462	-1.09277	0.03134
14	C	-3.153	0.09886	-0.00643
15	C	-2.4504	1.30162	-0.0177
16	C	-1.05504	1.33661	0.00875
17	F	-3.46168	-2.72974	1.3982
18	C	-3.1678	-2.39752	0.11515
19	F	-2.47373	-3.42638	-0.40669
20	F	-4.3577	-2.34134	-0.54051
21	F	-2.5613	3.62911	0.44655
22	C	-3.21038	2.59304	-0.12934
23	F	-4.43402	2.51692	0.45356
24	F	-3.4297	2.94557	-1.42252
25	H	7.29349	0.36108	0.12002
26	H	5.86273	-1.18937	-1.21436

27	H	3.74888	2.48045	1.32756
28	H	6.22399	2.21179	1.41525
29	H	2.89603	-1.83982	-0.92848
30	H	1.44178	-0.89744	0.1376
31	H	-0.46951	-2.01935	0.0836
32	H	-4.23548	0.08633	-0.02637
33	H	-0.51864	2.27323	0.01268
34	Cl	1.94572	-3.03225	0.32371

Table 15: Atomic coordinates of ‘colsed-ring’ [**1b** + Cl⁻] after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p).

Charge = -1

Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	-6.19931	-0.42213	-0.01368
2	C	-5.82752	0.91426	0.02255
3	C	-4.47048	1.27567	0.03353
4	C	-3.46836	0.26002	0.00746
5	C	-3.87853	-1.08675	-0.02945
6	C	-5.22579	-1.42694	-0.03985
7	C	-2.04261	0.68903	0.01915
8	N	-1.05602	-0.25379	-0.00257
9	O	-1.76838	1.91573	0.04927
10	C	0.33107	-0.01464	0.00147
11	O	-4.18044	2.58843	0.06894
12	C	1.1679	-1.14958	0.05148
13	C	2.55056	-0.99672	0.05154
14	C	3.14351	0.26723	0.00348
15	C	2.3128	1.3822	-0.04801
16	C	0.9209	1.26079	-0.04998

17	C	3.43486	-2.21401	0.03708
18	F	2.87751	-3.27069	0.65429
19	F	4.62669	-1.97315	0.64843
20	F	3.73465	-2.60462	-1.2276
21	C	2.91848	2.75698	-0.03441
22	F	4.1795	2.76533	-0.53502
23	F	3.00425	3.26625	1.22158
24	F	2.1995	3.64534	-0.7581
25	H	-7.25519	-0.68355	-0.02185
26	H	-6.56187	1.7133	0.04312
27	H	-3.14349	-1.89169	-0.04986
28	H	-5.5096	-2.47415	-0.06831
29	H	-1.29818	-1.2695	-0.0173
30	H	-3.18042	2.62566	0.06933
31	H	0.70586	-2.13674	0.08502
32	H	4.22051	0.37626	0.00681
33	H	0.29462	2.13716	-0.09454
34	Cl	-1.34667	-3.41659	-0.05803

Table 16: Atomic coordinates of [(**1b**)₂ + Cl⁻] after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p).

Charge = -1

Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	2.91995	4.02198	5.2956
2	C	1.99456	3.0271	5.00903
3	C	1.48226	2.88784	3.70696
4	C	1.9197	3.7646	2.68167
5	C	2.85692	4.75912	3.0099
6	C	3.35701	4.89942	4.29643
7	O	0.57482	1.92765	3.4234

8	C	1.49197	3.72835	1.24287
9	N	0.52605	2.79531	0.91936
10	O	1.97786	4.49742	0.41312
11	C	0	2.50053	-0.33741
12	C	-1.01502	1.52807	-0.39583
13	C	-1.56875	1.15454	-1.61598
14	C	-1.13333	1.7368	-2.8069
15	C	-0.13208	2.70493	-2.74402
16	C	0.43521	3.09929	-1.53257
17	F	-3.89948	0.74595	-1.7053
18	C	-2.6841	0.14446	-1.6572
19	F	-2.68796	-0.65756	-0.57071
20	F	-2.60187	-0.6472	-2.7499
21	F	1.39274	2.50785	-4.54549
22	C	0.40747	3.27877	-4.02645
23	F	0.92355	4.51578	-3.86056
24	F	-0.55228	3.3697	-4.98045
25	H	3.30344	4.11237	6.30872
26	H	1.65164	2.33572	5.7721
27	H	3.17676	5.41296	2.20599
28	H	4.08025	5.67741	4.52157
29	H	0.38516	1.32187	4.20656
30	H	0.20952	2.22223	1.7
31	H	-1.35905	1.05357	0.51733
32	H	-1.55848	1.43692	-3.75651
33	H	1.20815	3.852	-1.49283
34	C	-2.91995	-4.02198	5.2956
35	C	-1.99456	-3.0271	5.00903
36	C	-1.48226	-2.88784	3.70696
37	C	-1.9197	-3.7646	2.68167
38	C	-2.85692	-4.75912	3.0099

39	C	-3.35701	-4.89942	4.29643
40	O	-0.57482	-1.92765	3.4234
41	C	-1.49197	-3.72835	1.24287
42	N	-0.52605	-2.79531	0.91936
43	O	-1.97786	-4.49742	0.41312
44	C	0	-2.50053	-0.33741
45	C	-0.43521	-3.09929	-1.53257
46	C	0.13208	-2.70493	-2.74402
47	C	1.13333	-1.7368	-2.8069
48	C	1.56875	-1.15454	-1.61598
49	C	1.01502	-1.52807	-0.39583
50	F	-0.92355	-4.51578	-3.86056
51	C	-0.40747	-3.27877	-4.02645
52	F	0.55228	-3.3697	-4.98045
53	F	-1.39274	-2.50785	-4.54549
54	F	3.89948	-0.74595	-1.7053
55	C	2.6841	-0.14446	-1.6572
56	F	2.68796	0.65756	-0.57071
57	F	2.60187	0.6472	-2.7499
58	H	-3.30344	-4.11237	6.30872
59	H	-1.65164	-2.33572	5.7721
60	H	-3.17676	-5.41296	2.20599
61	H	-4.08025	-5.67741	4.52157
62	H	-0.38516	-1.32187	4.20656
63	H	-0.20952	-2.22223	1.7
64	H	-1.20815	-3.852	-1.49283
65	H	1.55848	-1.43692	-3.75651
66	H	1.35905	-1.05357	0.51733
67	Cl	0	0	5.62875

2.4.11 Quinone Reduction Studies- Dithionite

Reduction-induced transporter release- NMR analysis:

Solutions of the protransporters **2** and **3**, and their corresponding transporter **1b** were prepared in 7:3 CD₃CN/D₂O (4 mg/mL) and their ¹H NMR spectra were recorded. The protransporter solutions were treated with 20 equivalents each of solid DTi, sonicated, and ¹H NMR spectra recorded. The peaks in the aromatic region of protransporter solutions after dithionite treatment were compared to that of **1b** to verify transporter release.

Reduction-induced transporter release- HRMS analysis:

Solutions of **2** and **3** were prepared as in the NMR experiment (solvent: 7:3 ACN/water) and treated with 20 equivalents of DTi and sonicated. Aliquots of the sample were submitted for HRMS analysis. Spectra were analyzed for mass peaks of **1b** (m/z [**1b**+H]⁺- calc.: 350.0615) and byproducts **QM** (m/z [**QM**+H]⁺- calc: 220.1337) and **L** (m/z [**L**+H]⁺- calc: 235.1334).

Reductive cleavage studies of 2- UV-Vis spectroscopy:

The absorbance spectrum of a solution of **2** (50 μM) in 7:3 ACN/water treated with varying equivalents of sodium dithionite (DTi) was recorded on a UV-Vis spectrophotometer (Shimadzu). Reference: 7:3 ACN/water.

2.4.12 Enzymatic (NQO1) Reduction Studies

Reductive cleavage studies of the protransporters using the quinone reductase enzyme NQO1 were conducted using commercially available enzyme purchased from Sigma-Aldrich. The reactions were conducted in 10 mM potassium phosphate buffer at pH = 7.4. NADH, also purchased from Sigma-Aldrich, was used as the electron donor substrate in the reactions.

NQO1 reduction of 2- UV-Vis spectroscopy:

The changes in the absorption spectrum of **2** on reduction with NQO1 were monitored in a 1 mL cuvette on a UV-Vis spectrophotometer (Shimadzu UV-2600) fitted with a thermoelectric cell holder (Shimadzu S-1700) to maintain constant temperature condition optimum for the reaction (37°C).

Reaction 1: A reaction mixture of **2** (25 μM), and NADH (100 μM) in phosphate buffer (10 mM, pH = 7.4) was prepared and NQO1 (10 μg/mL) was added. The absorbance of the spectrum of the sample was recorded at certain time intervals, maintaining 37 °C temperature in the cuvette holder.

Reaction 2: A reaction mixture of **2** (25 μ M), NADH (100 μ M), and N-acetylcysteine (NAC, 250 μ M) in phosphate buffer (10 mM, pH = 7.4) was prepared and NQO1 (10 μ g/mL) was added. The absorbance spectrum of the sample was recorded at certain time intervals, maintaining 37°C temperature in the cuvette holder. Fitting of the time-based decrease in absorbance at 552 nm, given high substrate concentration (0-20 min), to one phase exponential decay model was conducted using GraphPad Prism 8 software.

NQO1 reduction-induced transporter release- HRMS analysis:

The reaction mixture (500 μ L) was prepared in 1.5 mL microcentrifuge tubes and incubated for 4 h at 37°C on a heating block (Eppendorf Thermomixer) covered with Al-foil. The mixture was then transferred to a 10 mL round bottom flask and water was removed on a rotavapor (Heidolph), keeping the water bath temperature at 37 °C. HPLC grade acetonitrile (200 μ L) was added to the residue, sonicated and the suspension transferred to a clean 1.5 mL microcentrifuge tube (repeat twice). The supernatant was carefully transferred to a fresh microcentrifuge tube and the solvent was removed under a flow of nitrogen gas. The resultant residue was submitted for ESI-HRMS.

Reaction mixture for NQO1 reduction of 2: **2** (25 μ M), NADH (100 μ M), N-acetylcysteine (NAC, 250 μ M), and NQO1 (10 μ g/mL) in phosphate buffer (10 mM, pH = 7.4). NAC was used as a quinone methide trap.

Reaction mixture for NQO1 reduction of 3: **3** (25 μ M), NADH (100 μ M), and NQO1 (10 μ g/mL) in phosphate buffer (10 mM, pH = 7.4).

2.4.13 HPLC Analysis (NQO1 Reduction)

HPLC analysis was performed on an Agilent 1260 Infinity II LC system fitted with a C18 reverse phase column and a diode array detector (DAD). Detection wavelength was set to 280 nm. Samples were loaded in 2 mL glass HPLC vials with 250 μ L conical inserts (DAMCON Scientific) for injection.

Run parameters:

Mobile phase- water:ACN or 0.1% HCOOH in water:ACN; *Flow rate-* 0.5 mL/min; *Run time-* 30 min; *Run protocol-* (water:ACN) 20:80 $\rightarrow$ 0-5 min, 10:90 $\rightarrow$ 5-8 min, 5:95 $\rightarrow$ 8-10 min, 0:100 $\rightarrow$ 10-12 min, 5:95 $\rightarrow$ 12-14 min, 10:90 $\rightarrow$ 14-16 min, 20:80 $\rightarrow$ 16-18 min, 20:80 $\rightarrow$ 18-20 min, 20:80 $\rightarrow$ 20-25 min, 20:80 $\rightarrow$ 25-30 min.

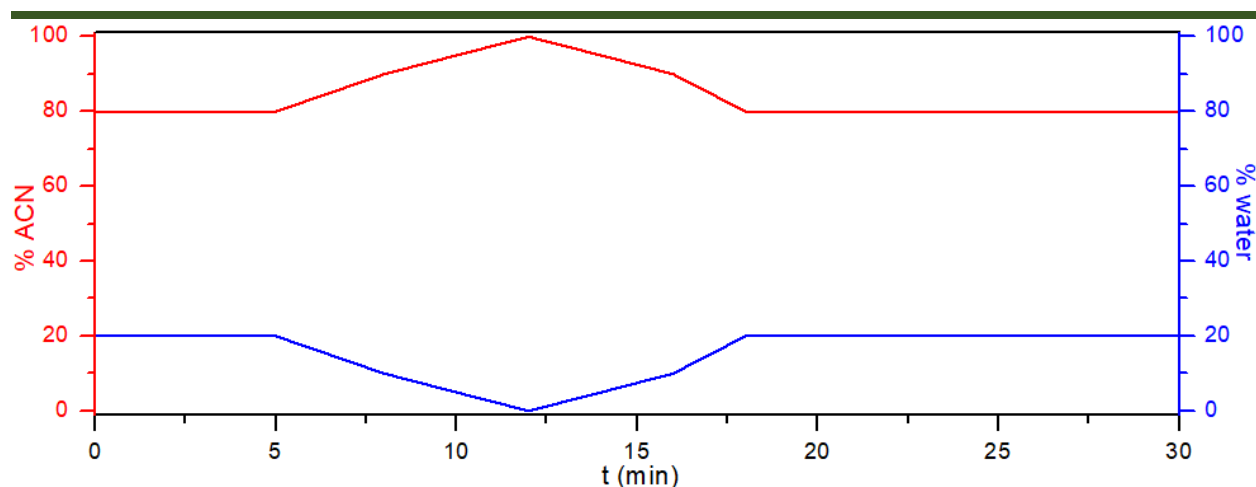


Figure 90: Time based solvent polarity chart of HPLC run with water (blue):ACN (red) eluent.

Purity analysis:

The stock solutions of the protransporters (**2**, **3**) (2 mM) were prepared in DMSO. A 25 μ M solution of the compound was prepared by addition of 12.5 μ L/2 mM stock into 987.5 μ L ACN and then injected (25 μ L) into the HPLC instrument.

Stability and NQO1 reduction studies of protransporters:

For assessment of protransporter stability in buffer, a solution of 50 μ M of the protransporter in 10 mM phosphate buffer (PB) (7.5 μ L/2 mM stock in 292.5 μ L PB, pH = 7.4) was incubated at 37°C on an Eppendorf ThermoMixer C at 700 revolutions per minute (rpm). Aliquots (150 μ L) were taken at 5 min and 2 h timepoints, diluted with an equal volume of ACN (150 μ L), and injected (25 μ L) into the HPLC instrument.

The reaction for NQO1 reduction induced release of the transporter was initiated by addition of 100 μ M NADH (25 μ L/2 mM) followed by 10 μ g/mL NQO1 enzyme (5 μ L/1 mg per mL) to 1.5 mL microcentrifuge tubes containing the protransporter **2/3** at 50 μ M concentration (12.5 μ L/2 mM) in 457.5 μ L PB (pH = 7.4). The reaction mixtures were incubated at 37°C on an Eppendorf ThermoMixer C with shaking at 700 rpm. Aliquots (150 μ L) were taken at 0.5 h, 1 h and 2 h timepoints, diluted with an equal volume of ACN (150 μ L), and centrifuged at 21130 relative centrifugal force (rcf) for 5 min. The supernatant was transferred to a fresh tube and again centrifuged at 21130 rcf for 5 min. The resulting supernatant was then injected (25 μ L) into the HPLC instrument.

For compound spiking experiments, the reaction mixtures were spiked with ~25 μM of the necessary compound by addition of 5 μL of 750 μM of compound stock (in ACN) to 145 μL of reaction mixture supernatant. This was then injected (25 μL) into the HPLC instrument.

2.4.14 Biological Studies

Cell lines and cell culture:

Non-cancerous cell lines from a non-human source namely, mouse embryonic fibroblasts (MEFs), and from human source namely, human embryonic kidney cells (HEK293T); and cancerous cell lines, namely, human breast cancer cells (MCF-7), and human adenocarcinoma alveolar basal epithelial cells (A549) were used for the study. All cell lines were cultured in the presence of high glucose Dulbecco's Modified Eagle media (DMEM; Lonza) containing 2 mM L-glutamine. The media was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; ThermoFisher Scientific), 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cells were maintained in tissue-culture treated T25 flasks at 37 °C in a humidified 5% CO₂ incubator (Thermo Scientific).

Assessment of cellular viability (MTT assay):

Cells were seeded in tissue-culture treated 96-well plates (Corning) at a density of 8×10^4 cells/well/100 μl in complete DMEM medium and were allowed to recover at 37 °C in a humidified 5% CO₂ incubator for 24 h. Post-incubation, the cells were incubated with increasing concentrations of the listed compounds for 24 h by maintaining the maximum amount of the solvent (DMSO) at 2 $\mu\text{l}/\text{well}$. Post incubation, the media was removed and the plates were incubated with 100 μL of MTT solution (0.5 mg/ml MTT in PBS) at 37 °C for a period of 4 h. The formazan crystals, thus formed, were dissolved using 100 μl solubilization buffer (10% SDS/0.1 N HCl in PBS) by incubating the plates overnight at rt. The absorbance was measured at 570 nm using a multi-mode plate reader (Hidex). All experiments were performed in triplicate (N = 3). The cellular viability was expressed as a percentage of viable cells for each treatment when compared with the untreated cells (control, set as 100%).

Cell cycle/propidium iodide (PI) staining:

The DNA content in the cells cultured in different experimental conditions was assessed using propidium iodide staining and flow cytometry using a previously standardized protocol.⁷⁹ Briefly, MCF7 cells were seeded in tissue-culture treated 35 mm plates at a density of 8×10^4 cells/ml in complete DMEM medium and incubated at 37 °C in a humidified 5% CO₂ incubator for 24 h. Cells were incubated with increasing concentrations of the compounds for 24 h. Post incubation,

cells were harvested and washed with ice-cold 1X PBS. After washing, the cells were fixed with 70% methanol overnight at -20°C. Following this, the cells were washed with 1X PBS containing 20 µg/ml RNAase A (20 mg/ml, HIMEDIA), to remove the RNA and the samples were further incubated for 15 min on ice. The samples were subsequently stained using 50 µg/ml Propidium iodide (1 mg/ml, Himedia) for 30 min in dark; acquired on FACSCelesta™ cell analyzer (BD Biosciences, San Jose, CA) and analyzed using BD FACS flowJo software (Ashland, OR). Ten thousand events were analyzed for each sample.

Fluorescence imaging of dead cells using propidium iodide (PI):

Dead cells take up propidium iodide and can be visualized by fluorescence imaging. Briefly, MCF-7 Cells were seeded at a density of 4×10^4 cells per well in tissue-culture treated 48-well plates in a complete DMEM medium and incubated at 37 °C in a humidified 5% CO₂ incubator for 24 h. Following this, the cells were incubated with increasing concentrations of compound for 6 h. Post incubation, cells were washed with ice-cold 1X PBS, and incubated with propidium iodide for ~20 minutes at 37°C in dark. Images were acquired after washing the cells with 1X PBS using EVOS FL Auto Imaging System (Life Technologies).

Measurement of reactive oxygen species (ROS):

MCF-7 cells were seeded in tissue-culture treated 96-well plates at a density of 8×10^4 cells/ml in complete DMEM medium and incubated at 37 °C in a humidified 5% CO₂ incubator for 24 h. After this, the cells were incubated with of compounds for 24 h. Post incubation, cells were washed with ice-cold 1X PBS, and incubated with MitoSox Red reagent (5 µM, Invitrogen) for ~20 minutes at 37 °C. After incubation, the cells were washed thrice with ice-cold 1X PBS and the fluorescence intensities were read using a multimode plate reader (Hidex) at (excitation 510 nm/emission 580 nm). All the experiments were performed in triplicate. The readings obtained were then normalized with MTT values.

Mitochondrial membrane potential (MMP) measurement:

To determine the changes in mitochondrial membrane permeability, MMP was assessed by using JC-1 dye (Invitrogen). In brief, MCF-7 cells were seeded in tissue-culture treated 96-well plates at a density of 8×10^4 cells/ml in a complete DMEM medium and incubated at 37°C in a humidified 5% CO₂ incubator for 24 h. Cells were incubated with increasing concentrations of compounds for 24 h. Post incubation, cells were washed with ice-cold 1X PBS, and incubated with JC-1 reagent (2 µg/ml) for 20 minutes at 37 °C in dark. Cells were washed twice with ice-cold 1X PBS and the

fluorescence intensities were read at 485/530 nm and 510/580 nm wavelengths. All experiments were performed in triplicate. The readings were then normalized with MTT values. For qualitative analysis, fluorescence images were acquired after washing with PBS in both red and green channels using EVOS FL Auto Imaging System (Life Technologies).

Immunoblot analysis:

MCF-7 cells were seeded at a density of 4×10^5 cells per well in 6-well tissue culture treated plates (Eppendorf) in complete DMEM medium and incubated at 37 °C in a humidified 5% CO₂ incubator for 16 h. The cells were then treated with the compounds. One well was kept as control and the cells were treated with an equal volume of DMSO. Following 24 h of treatment, the medium containing compounds was aspirated out and the cells were washed with 1 mL 1X Dulbecco's phosphate buffered saline (DPBS; Lonza). Then, 1X sample buffer (containing 60 mM Tris (pH 6.8), 6% glycerol, 2% sodium dodecyl sulfate (SDS), 0.1 M dithiothreitol (DTT) and 0.006% bromophenol blue) was added to each well, cells scraped out using a scraper and transferred to 1.5 mL centrifuge tubes. The cells were lysed by heating the samples at 95°C for 10 min and the lysates were stored at -55°C.

The cell lysates were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The lysates were run on 13.4 % acrylamide gel in a miniVE vertical electrophoresis system (GE Healthcare) at 130 V. The proteins were transferred to a polyvinylidenedifluoride (PVDF) membrane (Immobilon, Millipore) for 2 h at 4 °C using a TE 22 Mini Tank Transfer Unit (GE Healthcare), with a 245 mA constant current supply. Blocking was performed in 3% BSA prepared in 1X Tris buffered saline containing 0.1% Tween 20 (1X TBS-T) over 16 h at 4 °C. The blots were incubated in the primary antibody solution (1 h at RT for GAPDH and 16 h at 4 °C for PARP1). Following 3 x 10 min washes in 1X TBS-T, the blots were incubated with peroxidase-conjugated secondary antibody solution prepared in 3% BSA in 1X TBS-T for 1 h at RT. The blots were then developed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized using iBright™ CL1500 Imaging System (Invitrogen). The images were processed and quantified using ImageJ software.

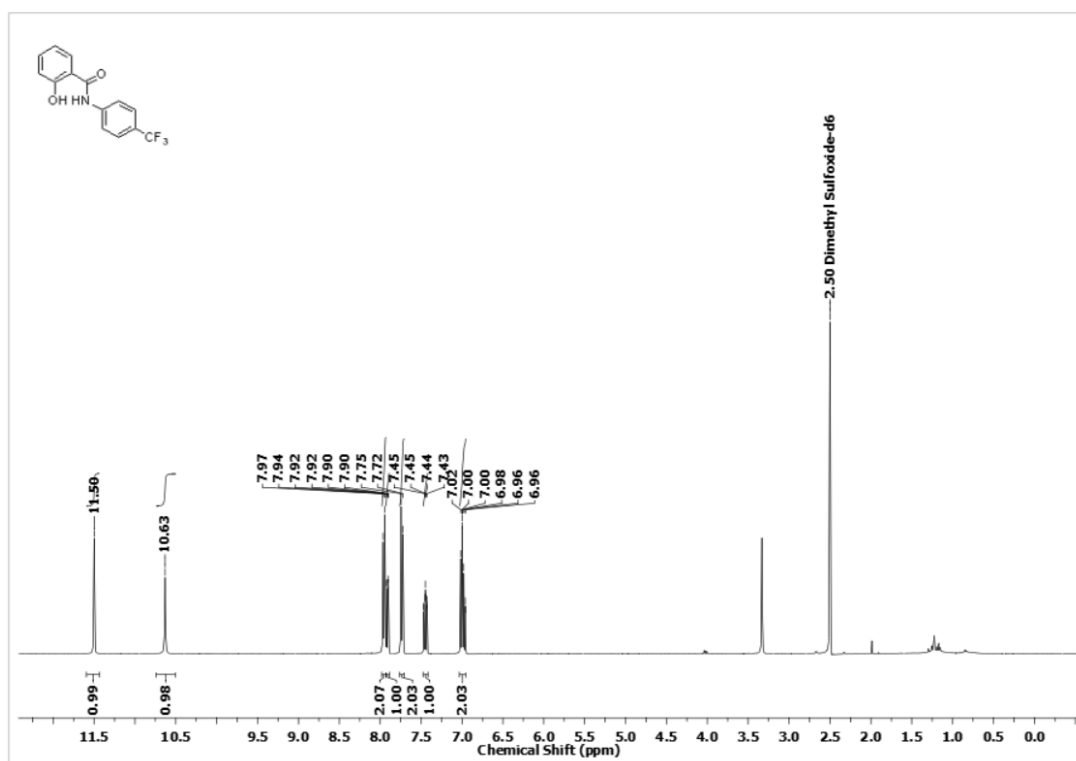
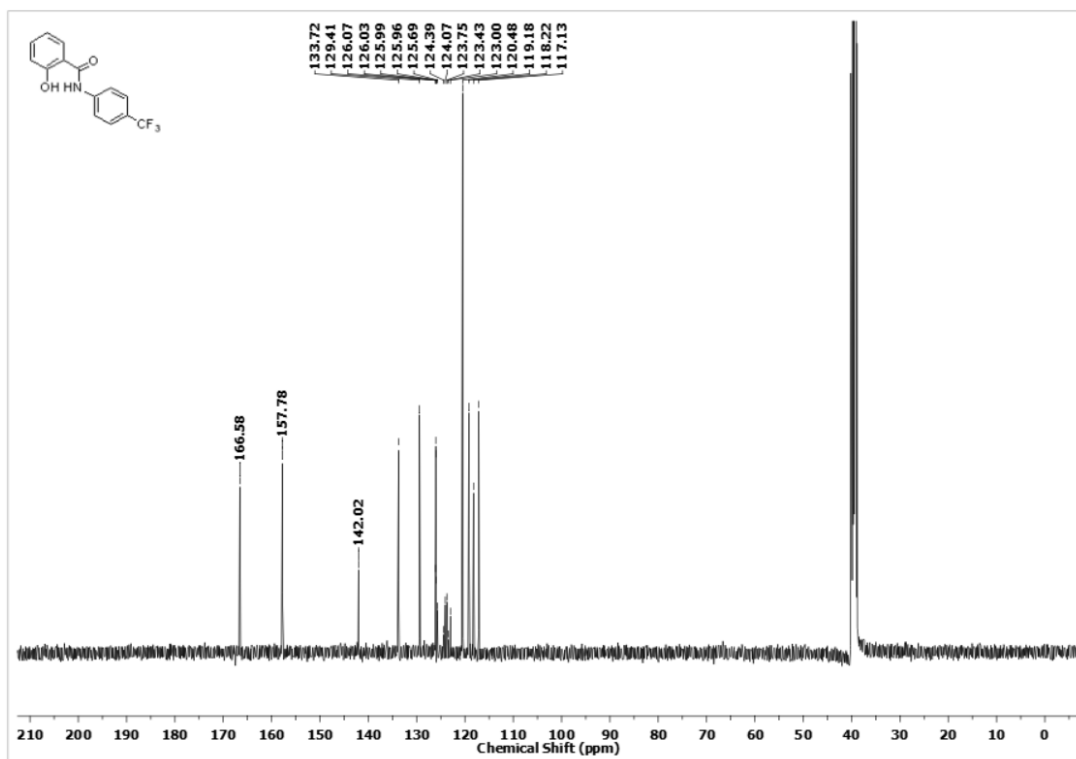
Acridine orange staining:

MCF7 cells were seeded in tissue-culture treated 24-well plates at a density of 8×10^4 cells/ml in a complete DMEM medium and incubated at 37 °C in a humidified 5 % CO₂ incubator for 24 h. After this, the cells were incubated with increasing concentrations of compounds for 24 h. Post incubation, cells were washed with ice-cold 1X PBS, and incubated with acridine orange reagent (5 µg/ml, Sigma) for ~20 minutes at 37 °C in dark. After incubation, the cells were washed thrice with ice-cold PBS, and fluorescence images were acquired in both red and green channels using EVOS FL Auto Imaging System (Life Technologies).

Statistical Analysis:

All experiments were performed in triplicate unless stated otherwise. The data has been represented as Mean $\pm$ SEM, where SEM represents the standard error mean and has been calculated by dividing the standard deviation of the samples by the square root of the number of replicates. Statistical analysis was performed by one-way ANOVA followed by Tukey HSD posthoc test. A p-value ≤ 0.05 was considered to be statistically significant between groups.

2.5 Characterization Spectra

Figure 91: 400 MHz ¹H spectrum of **1a** in DMSO-*d*₆Figure 92: 101 MHz ¹³C spectrum of **1a** in DMSO-*d*₆

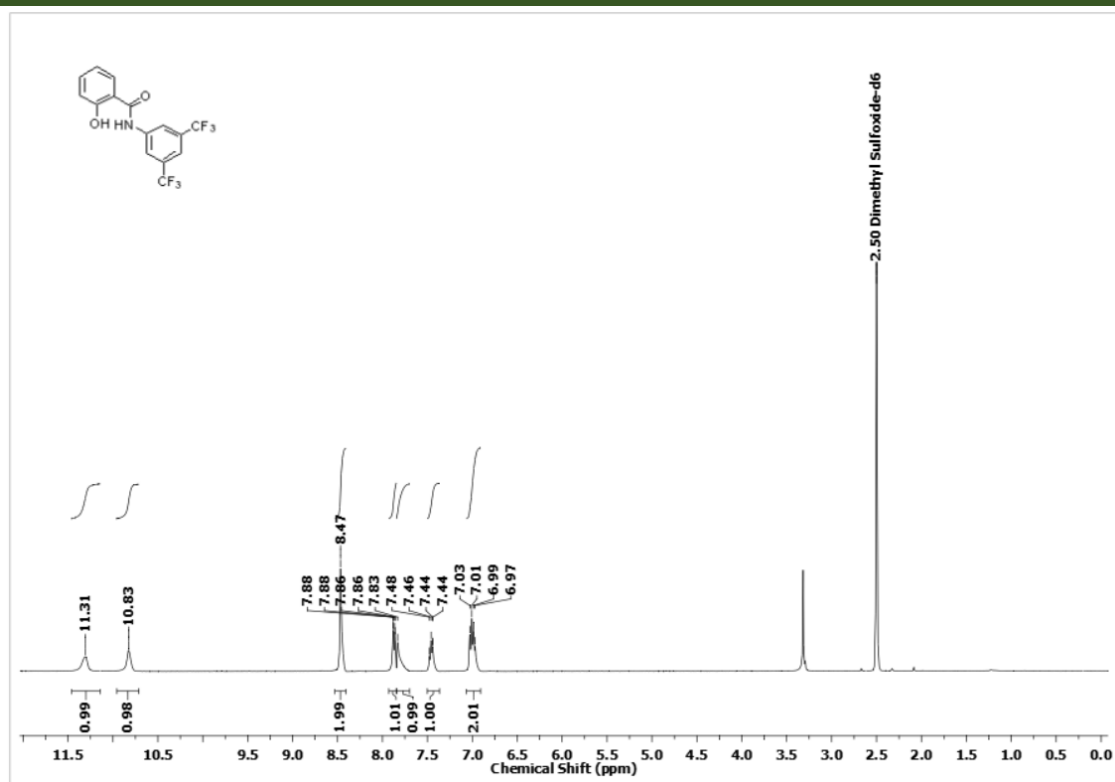


Figure 93: 400 MHz ¹H spectrum of **1b** in DMSO-*d*₆

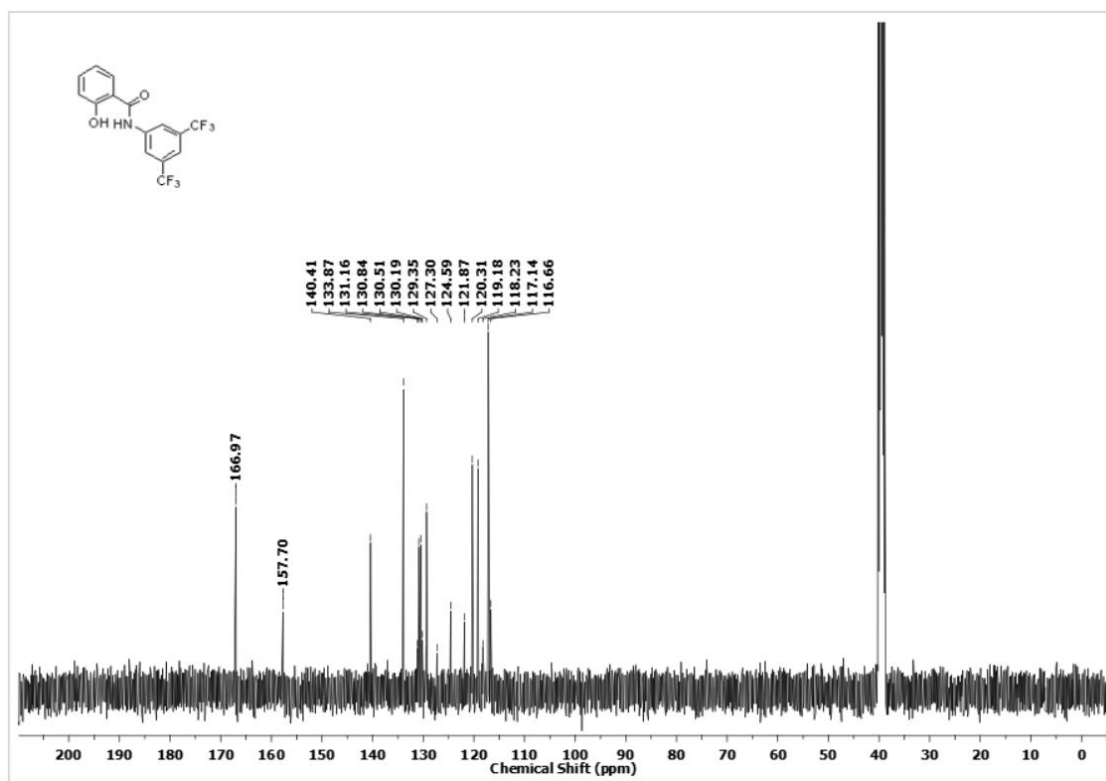


Figure 94: 101 MHz ¹³C spectrum of **1b** in DMSO-*d*₆

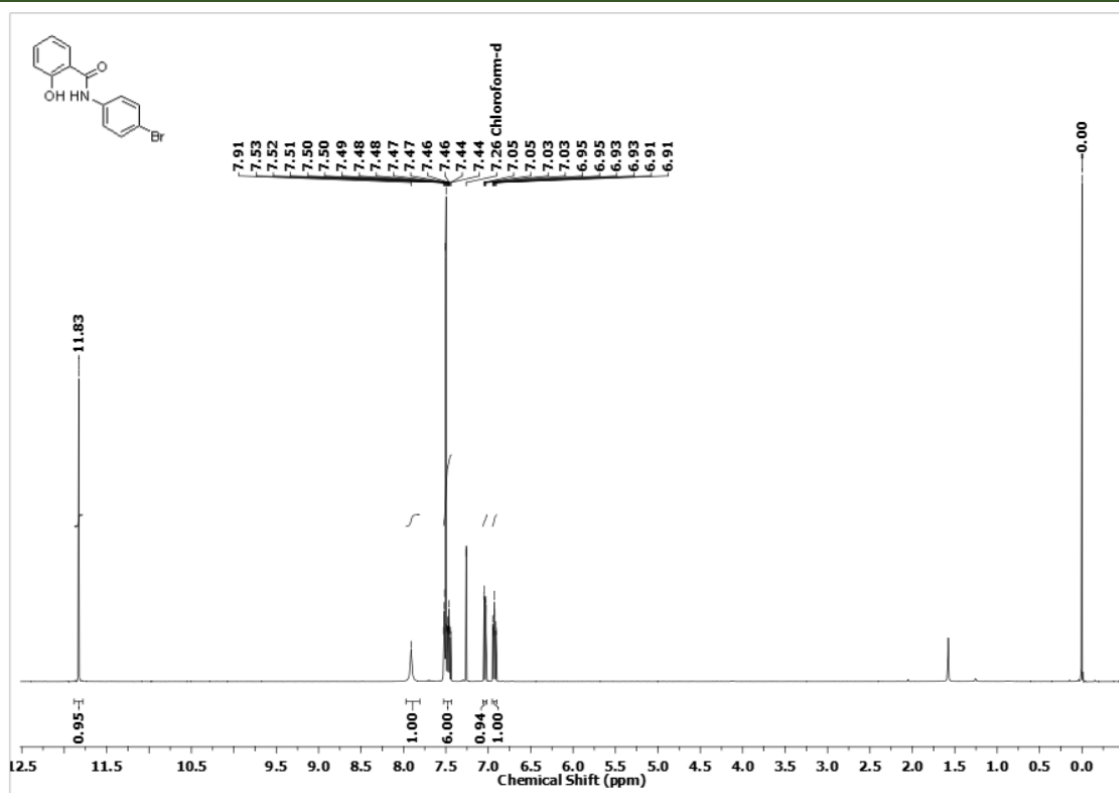


Figure 95: 400 MHz ¹H spectrum of **1c** in CDCl₃

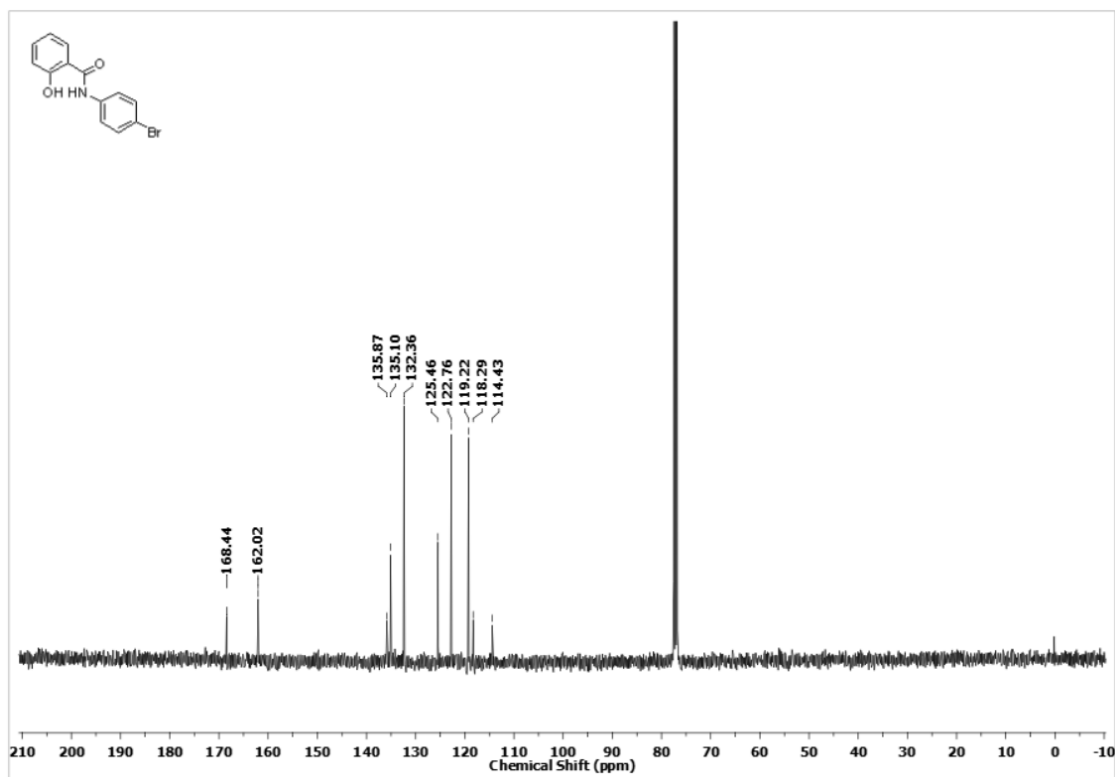


Figure 96: 101 MHz ¹³C spectrum of **1c** in CDCl₃

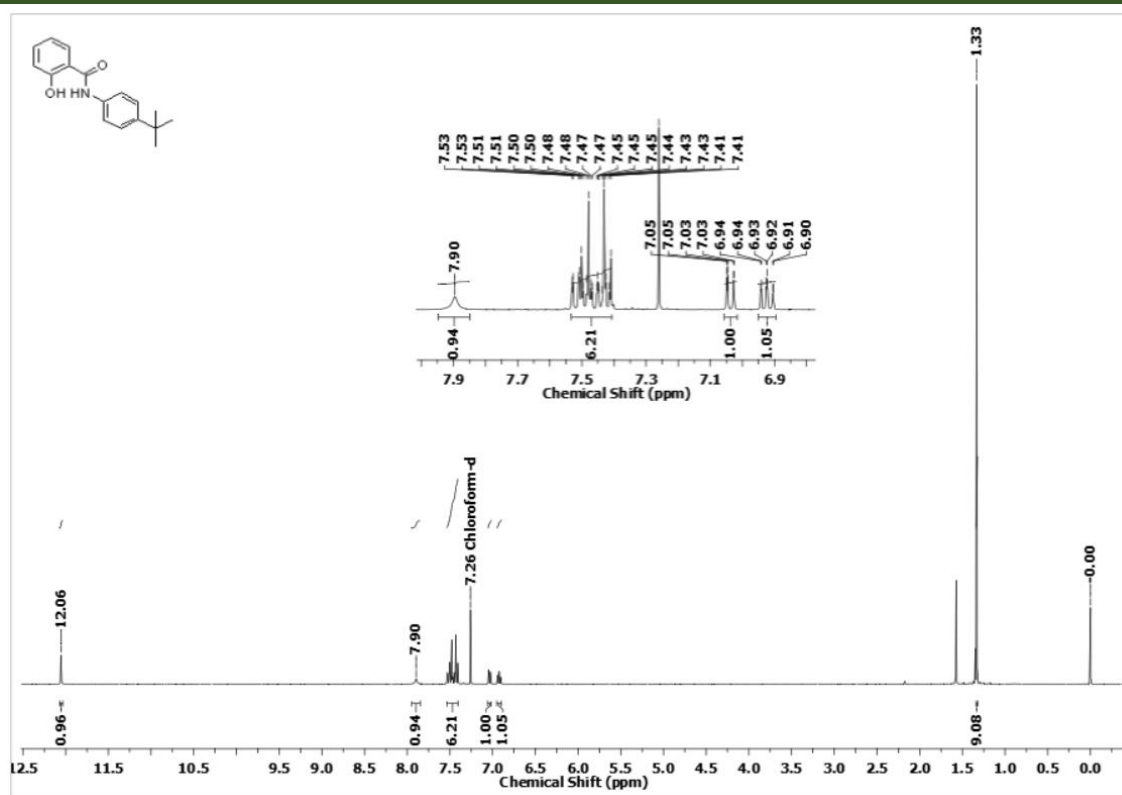


Figure 97: 400 MHz ¹H spectrum of **1d** in CDCl₃

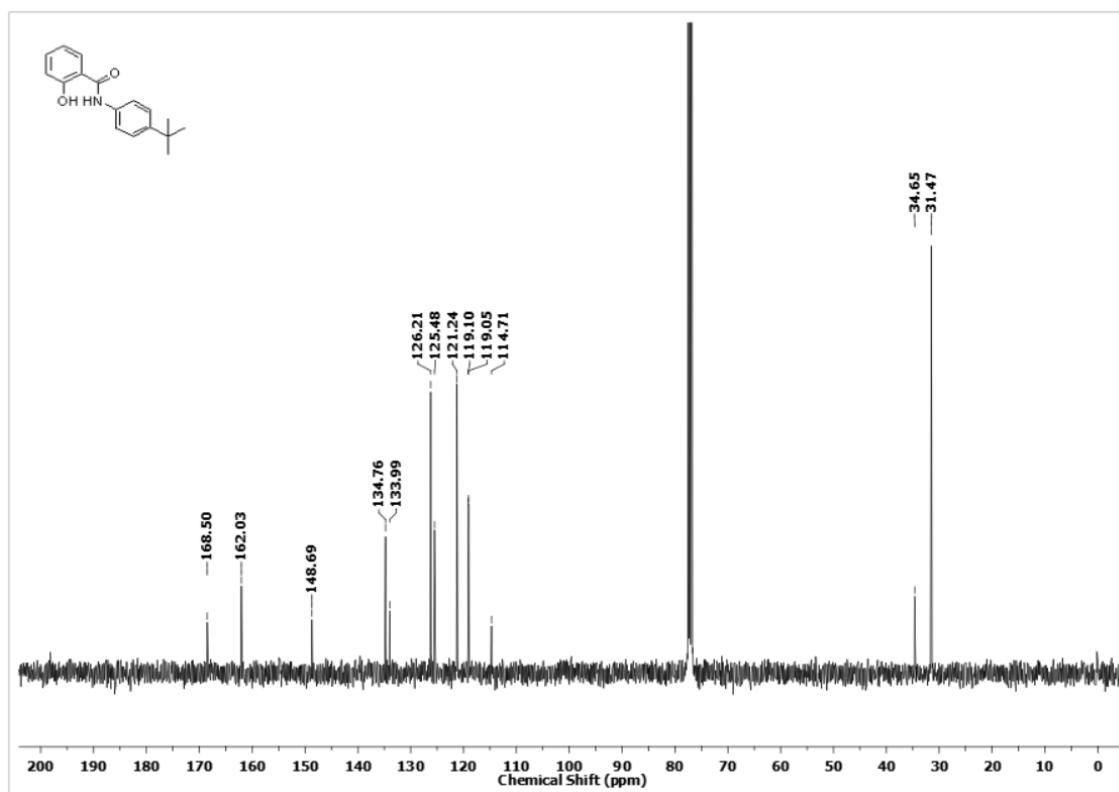


Figure 98: 101 MHz ¹³C spectrum of **1d** in CDCl₃

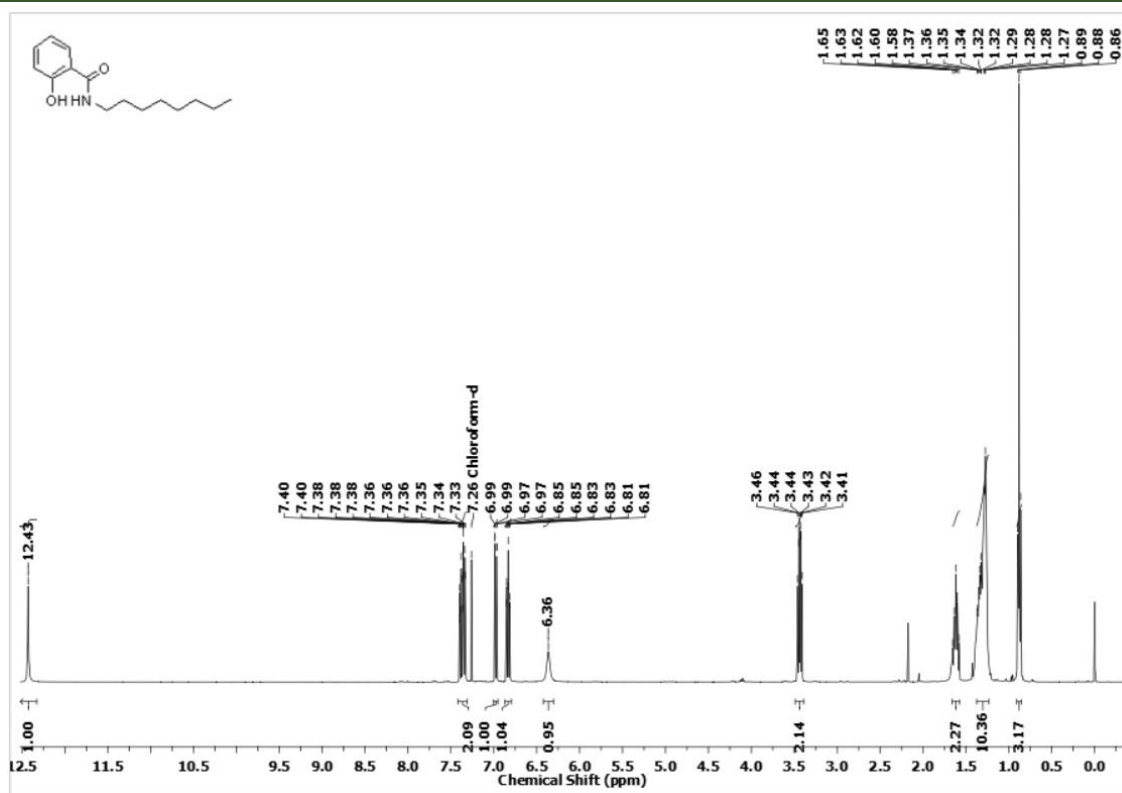


Figure 99: 400 MHz ¹H spectrum of **1e** in CDCl₃

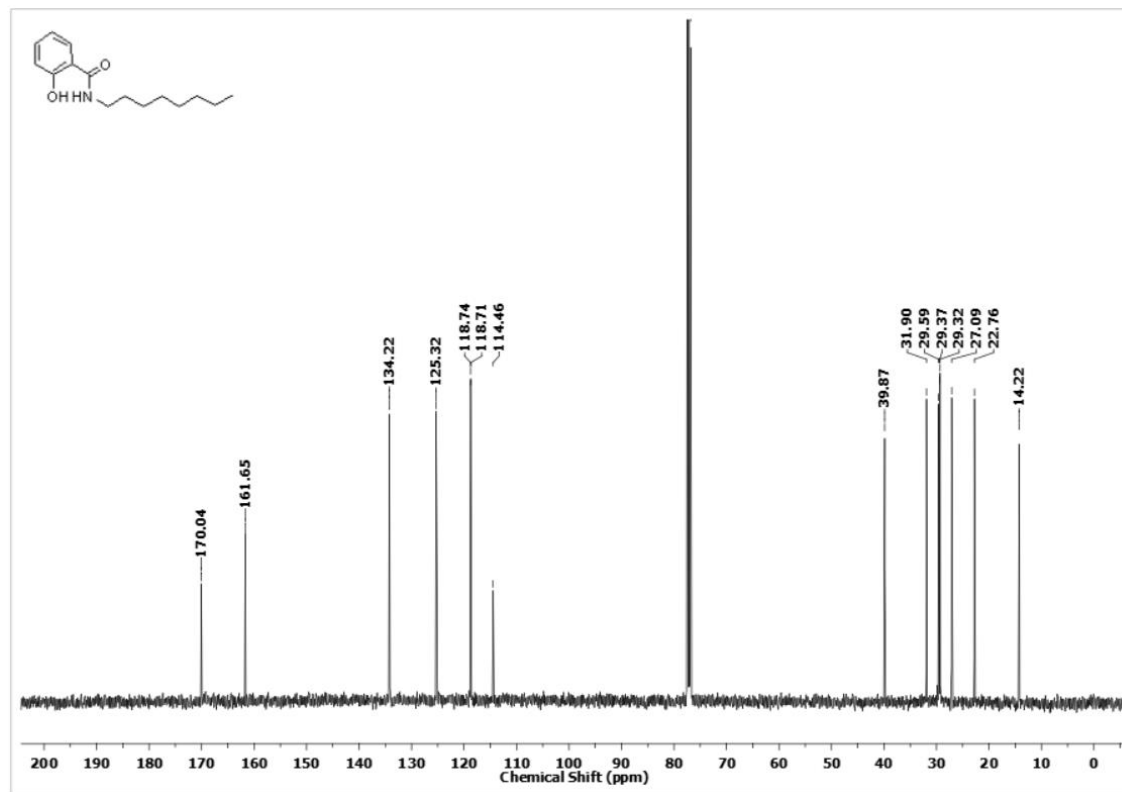


Figure 100: 101 MHz ¹³C spectrum of **1e** in CDCl₃

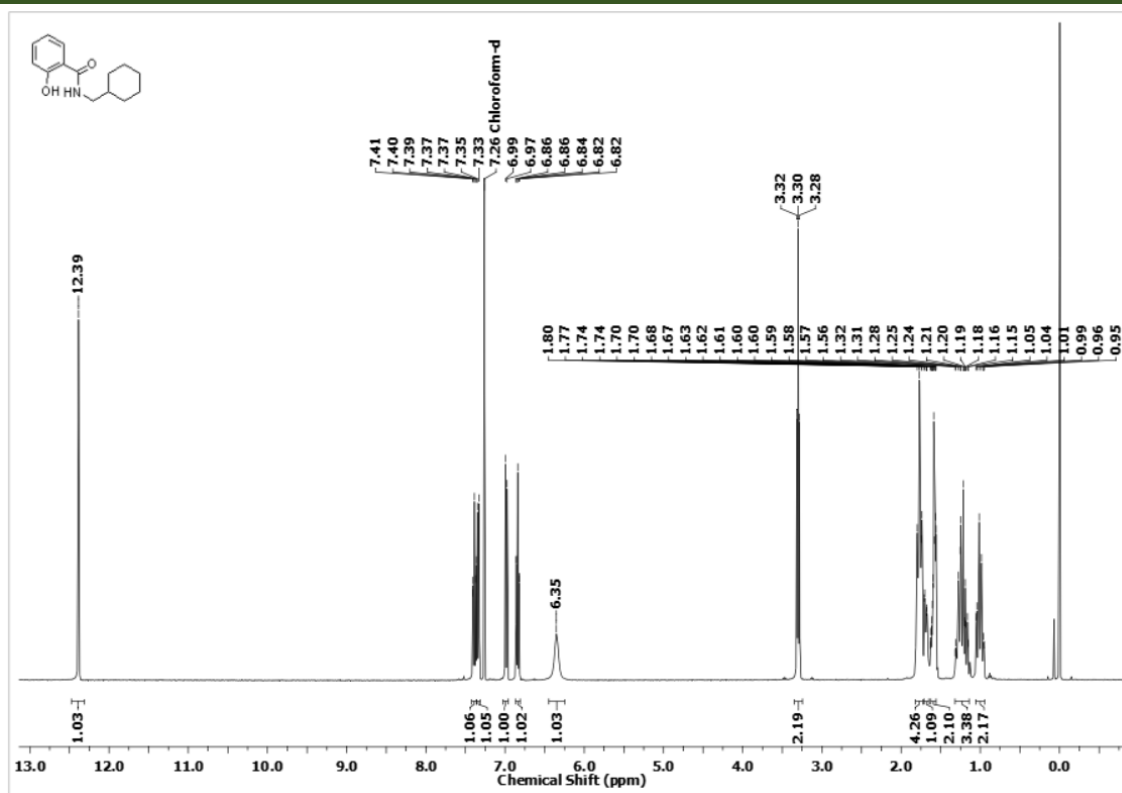


Figure 101: 400 MHz ¹H spectrum of **1f** in CDCl₃

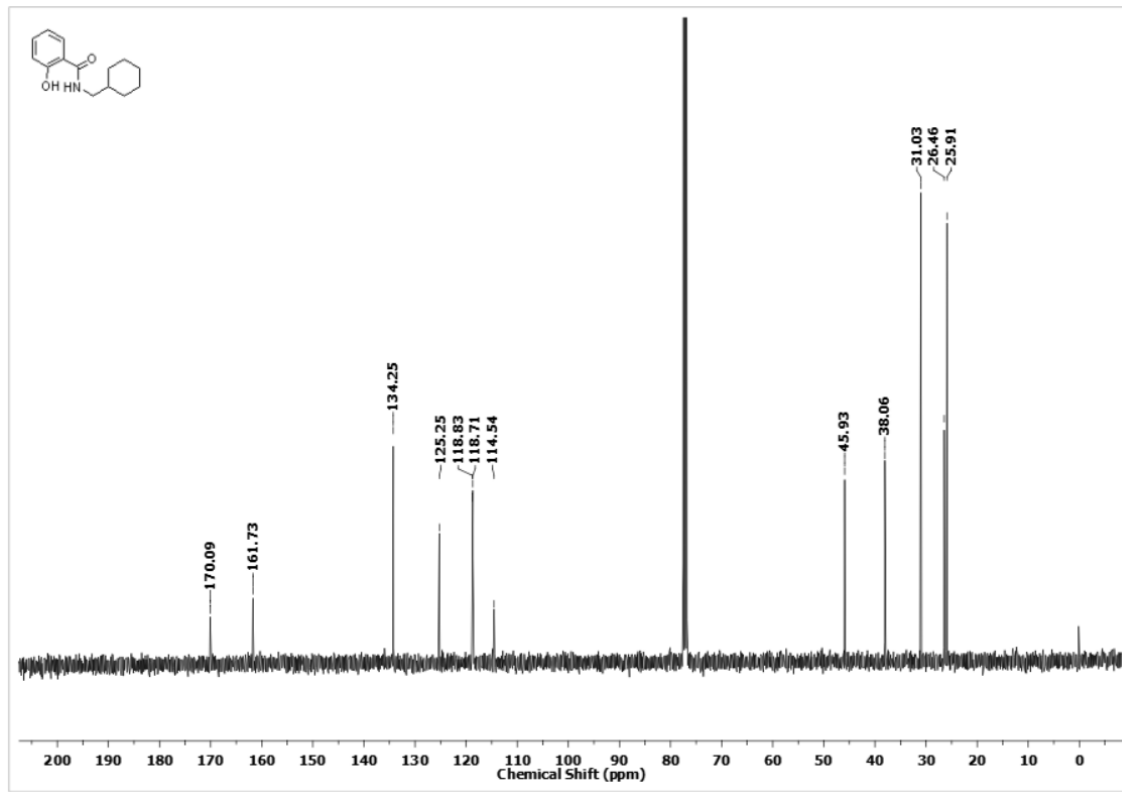


Figure 102: 101 MHz ¹³C spectrum of **1f** in CDCl₃

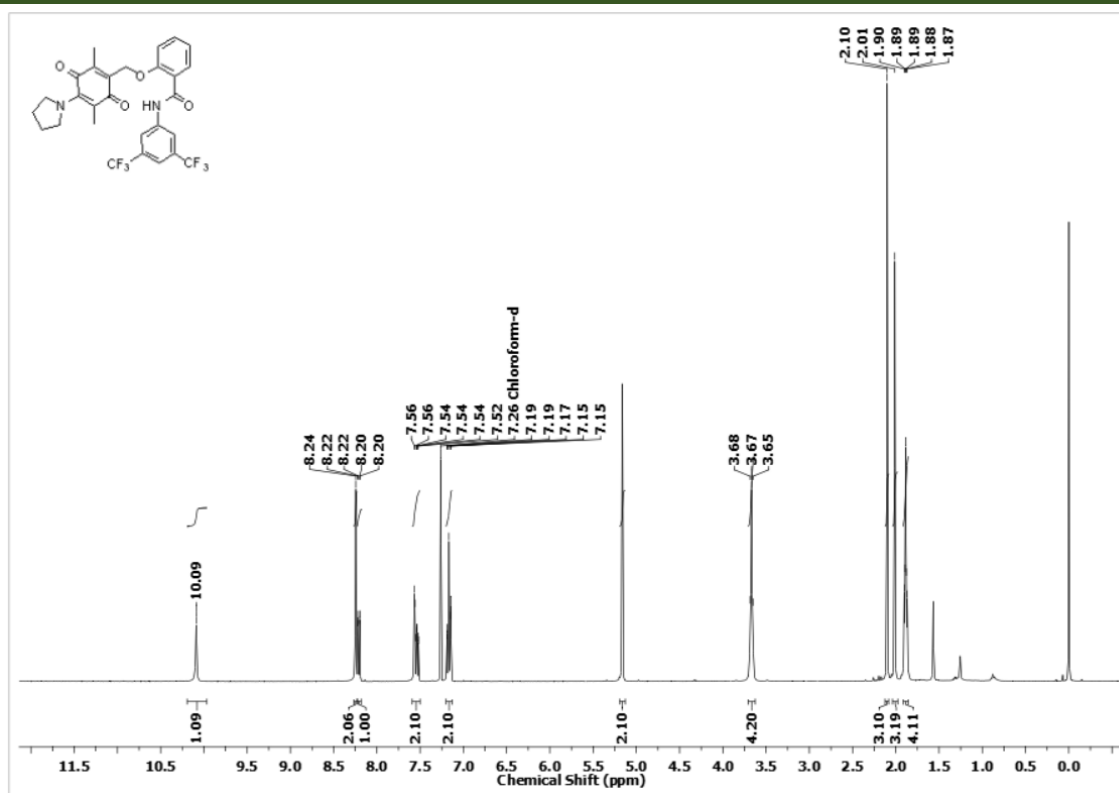


Figure 103: 400 MHz ¹H spectrum of 2 in CDCl₃

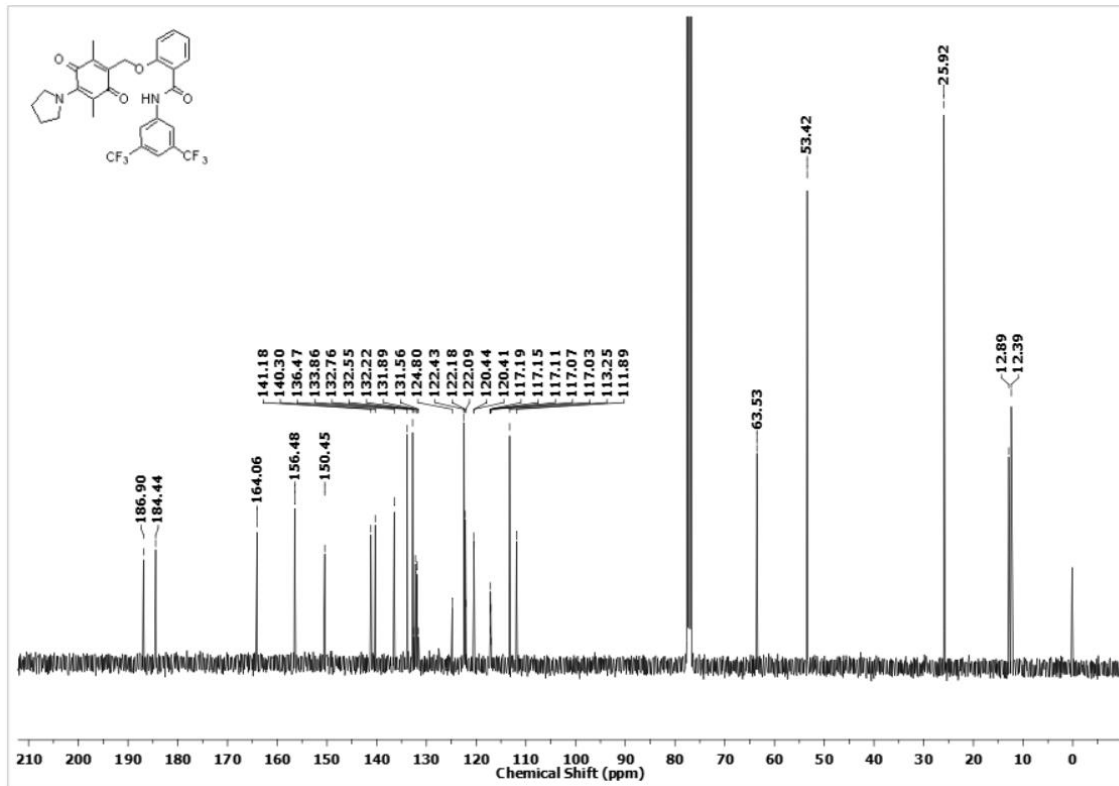
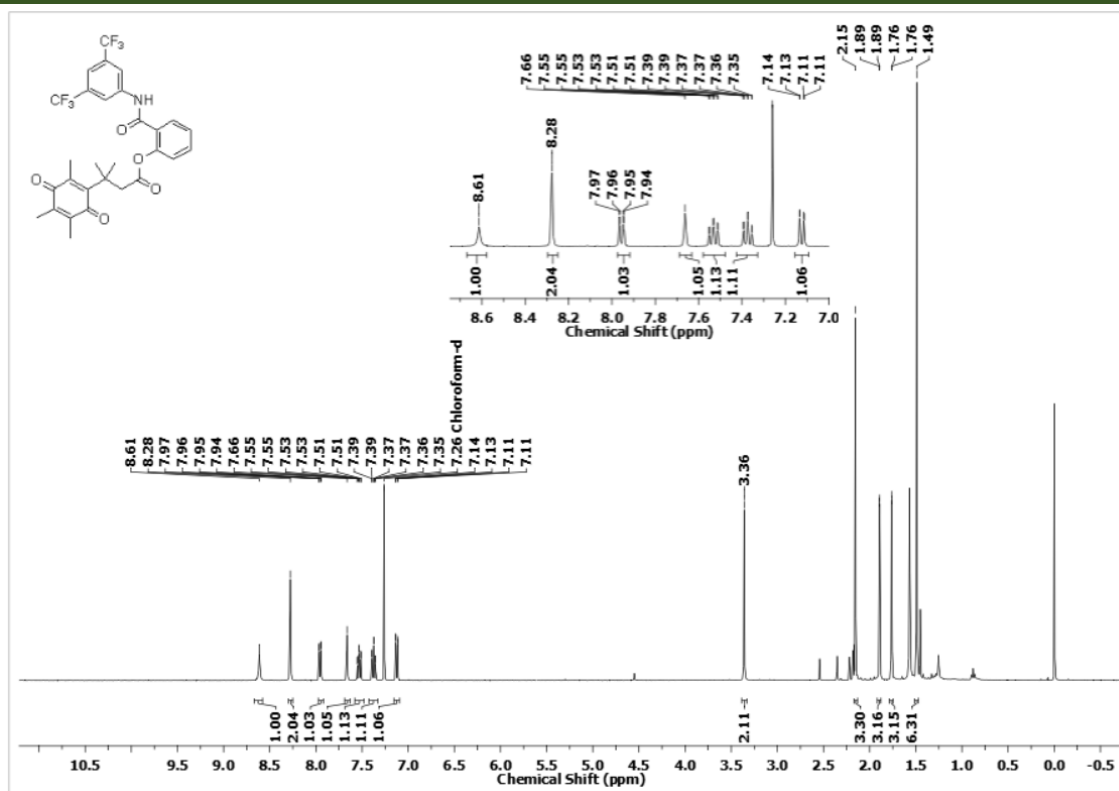
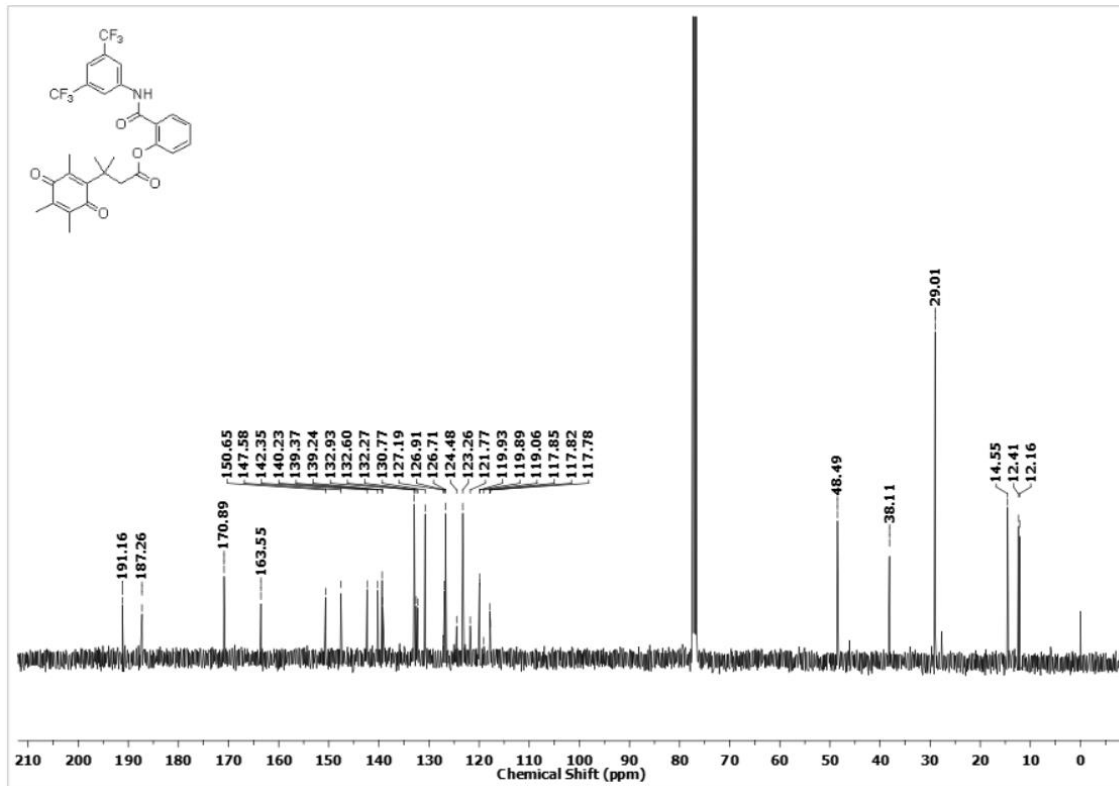


Figure 104: 101 MHz ¹³C spectrum of 2 in CDCl₃

**Figure 105:** 400 MHz ¹H spectrum of **3** in CDCl₃**Figure 106:** 101 MHz ¹³C spectrum of **3** in CDCl₃

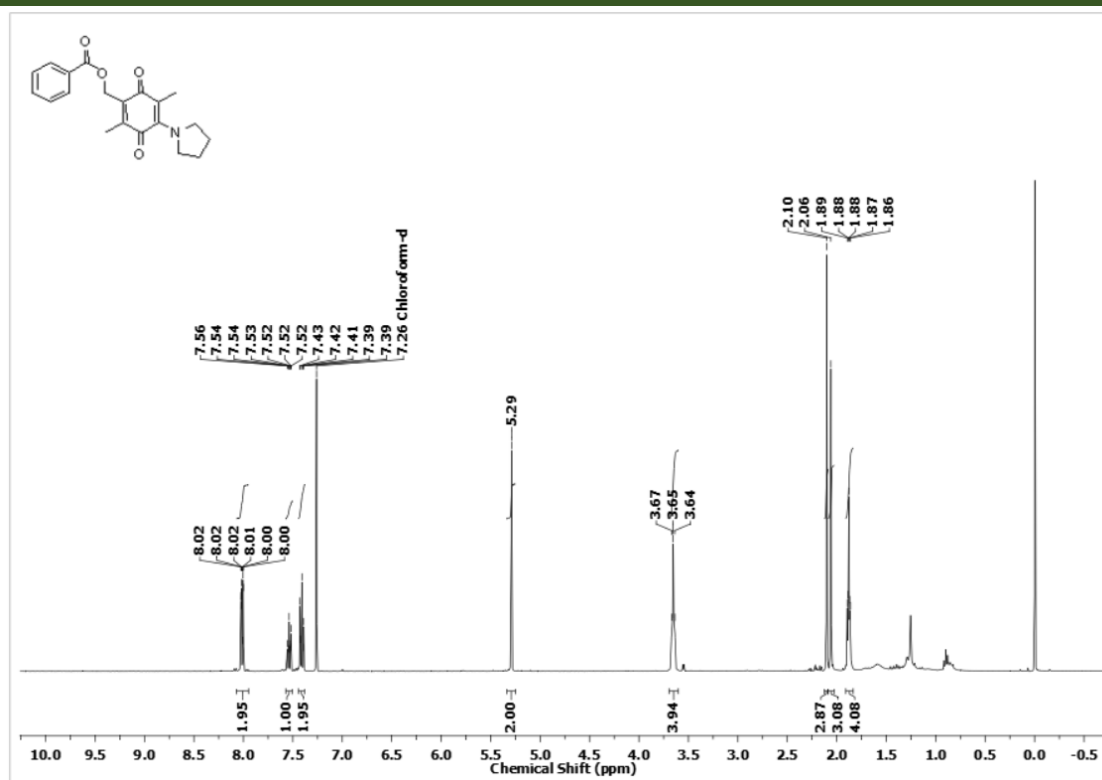


Figure 107: 400 MHz ¹H spectrum of 7 in CDCl₃

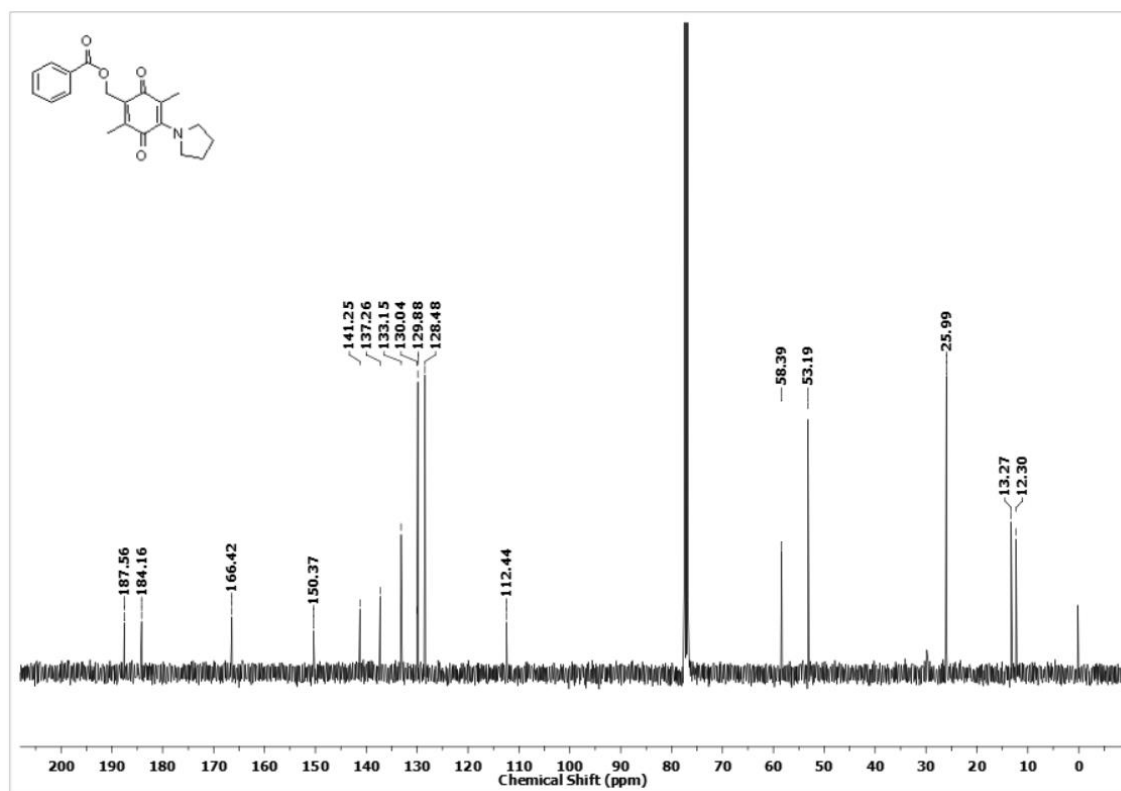


Figure 108: 101 MHz ¹³C spectrum of 7 in CDCl₃

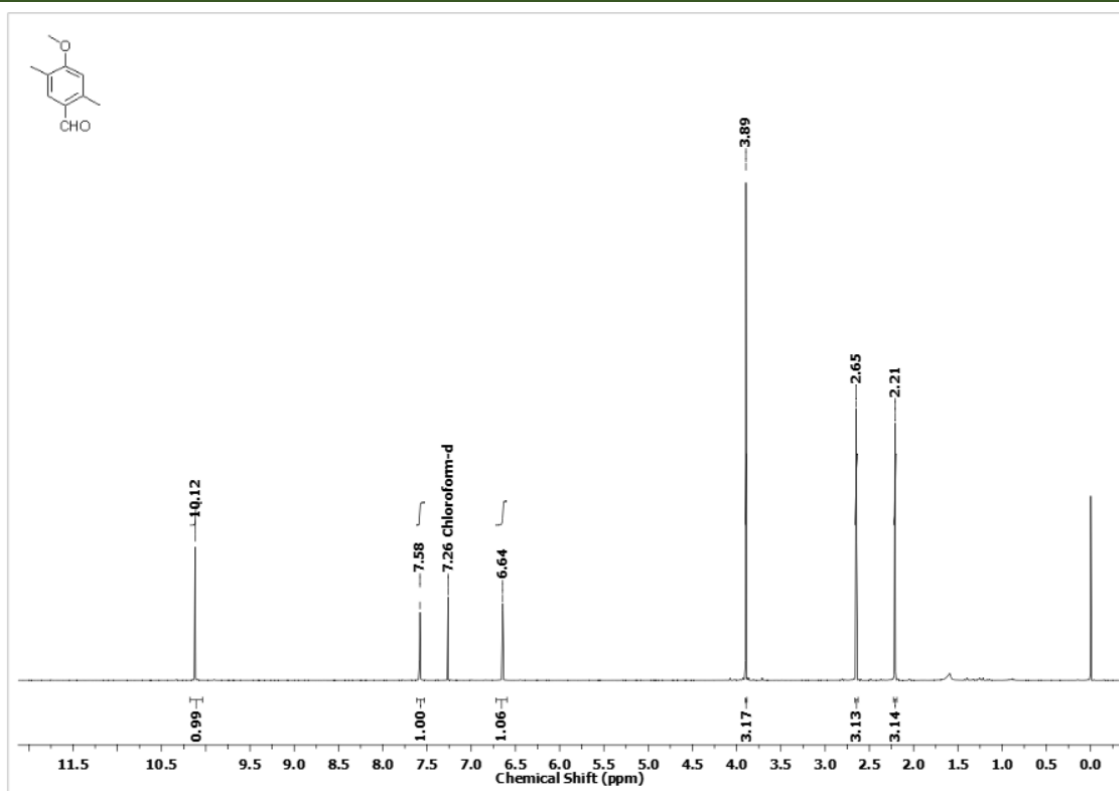


Figure 109: 400 MHz ¹H spectrum of **9** in CDCl₃

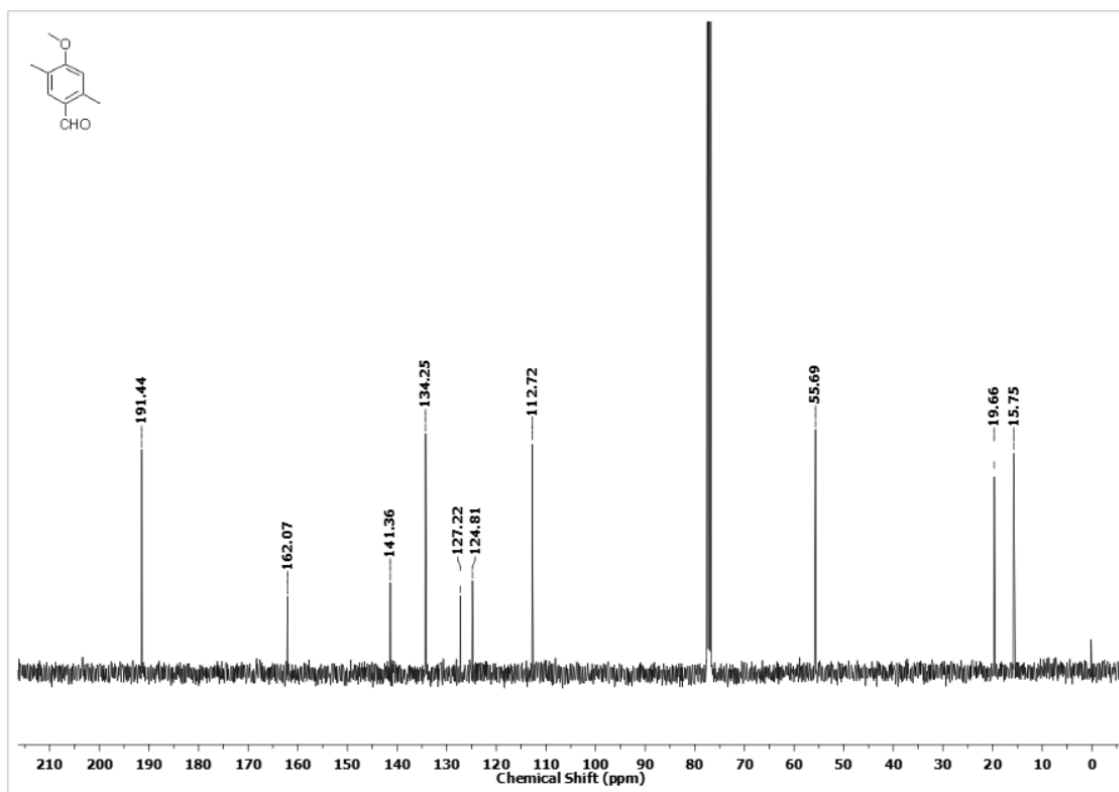


Figure 110: 101 MHz ¹³C spectrum of **9** in CDCl₃

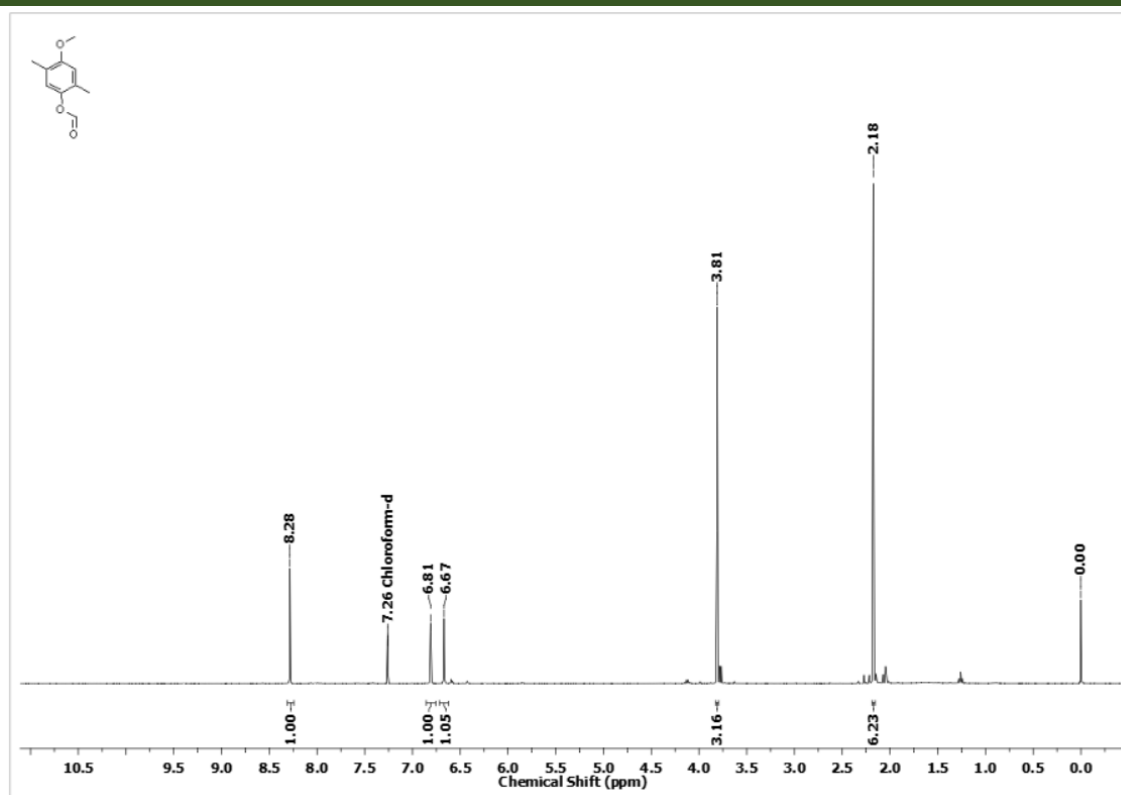


Figure 111: 400 MHz ¹H spectrum of 4-methoxy-2,5-dimethylphenyl formate in CDCl₃

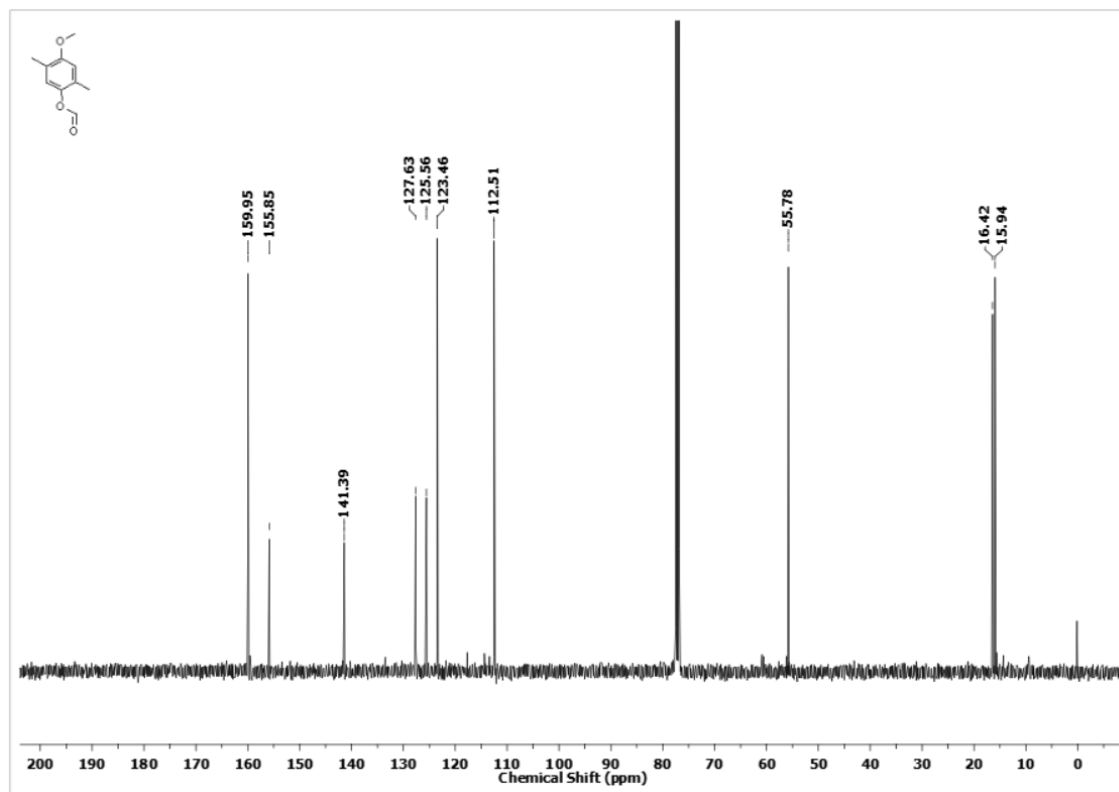


Figure 112: 101 MHz ¹³C spectrum of 4-methoxy-2,5-dimethylphenyl formate in CDCl₃

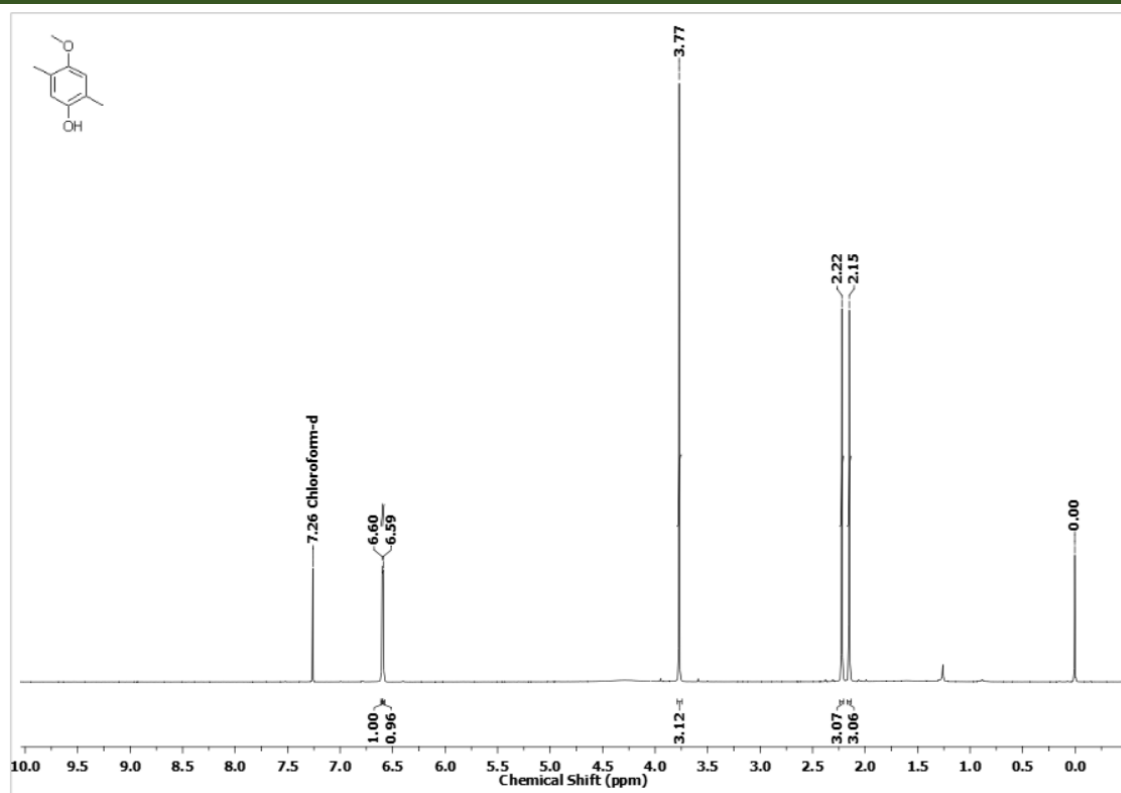


Figure 113: 400 MHz ¹H spectrum of **10** in CDCl₃

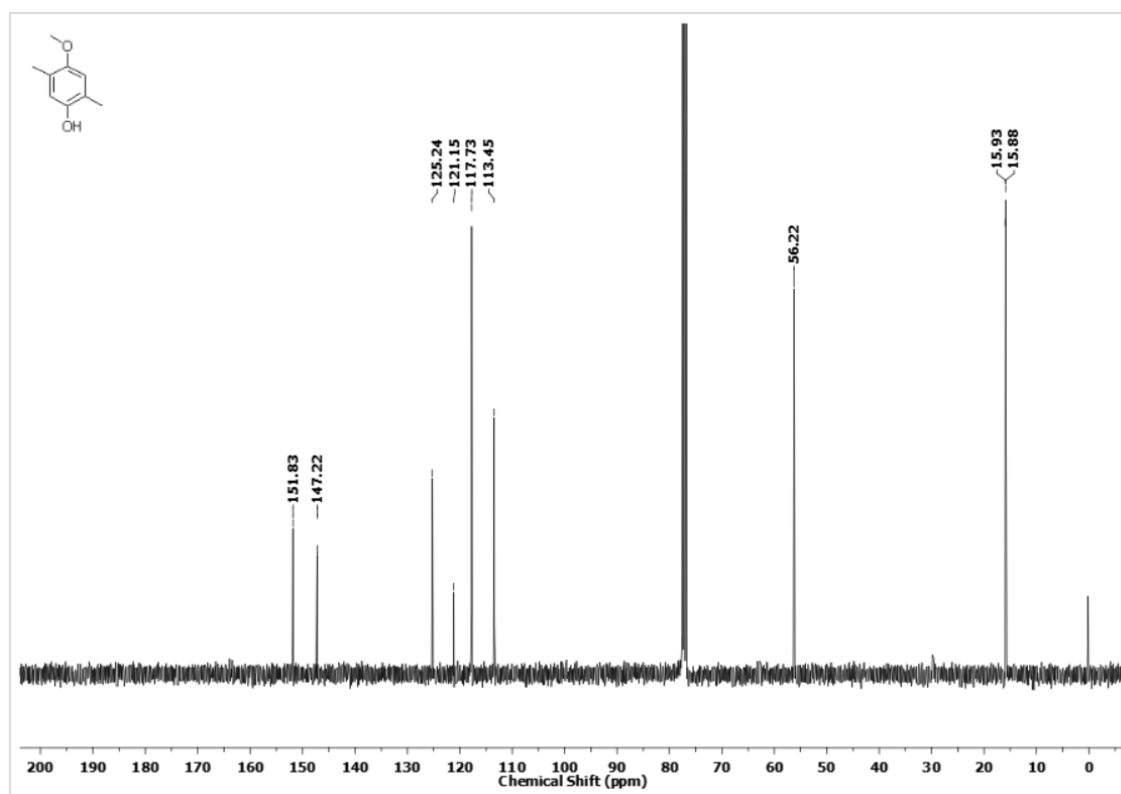


Figure 114: 101 MHz ¹³C spectrum of **10** in CDCl₃

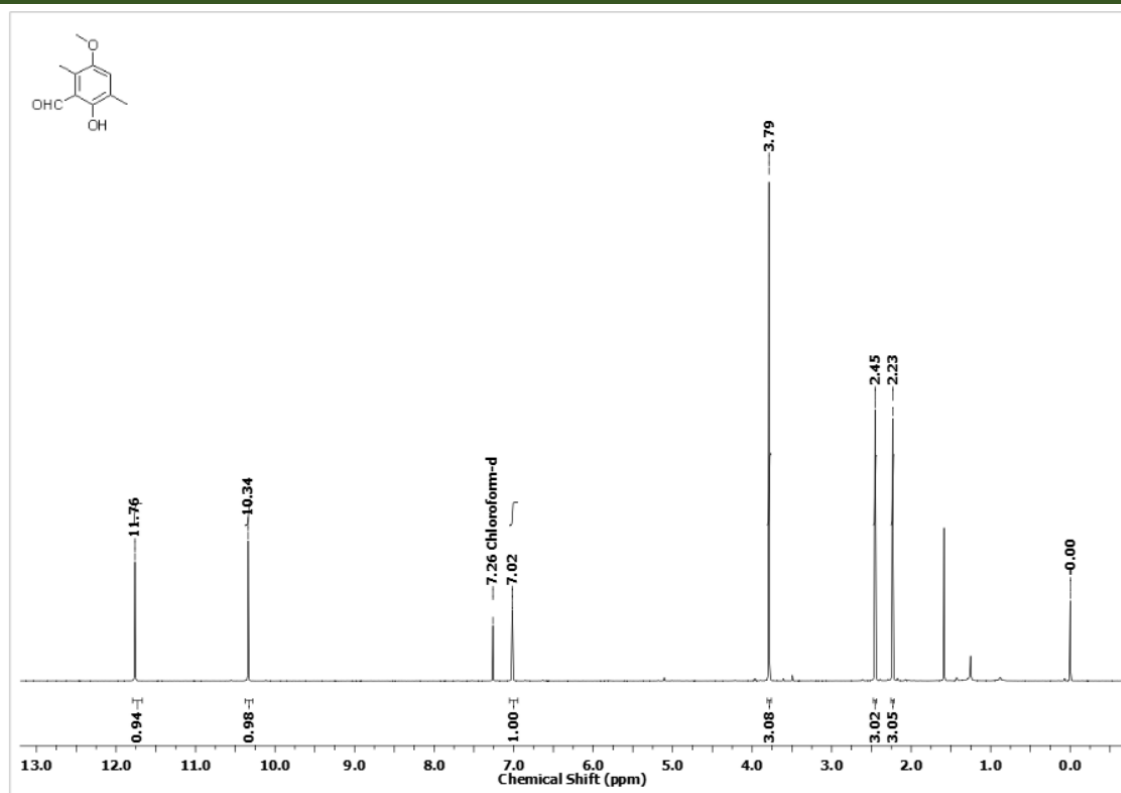


Figure 115: 400 MHz ¹H spectrum of 11 in CDCl₃

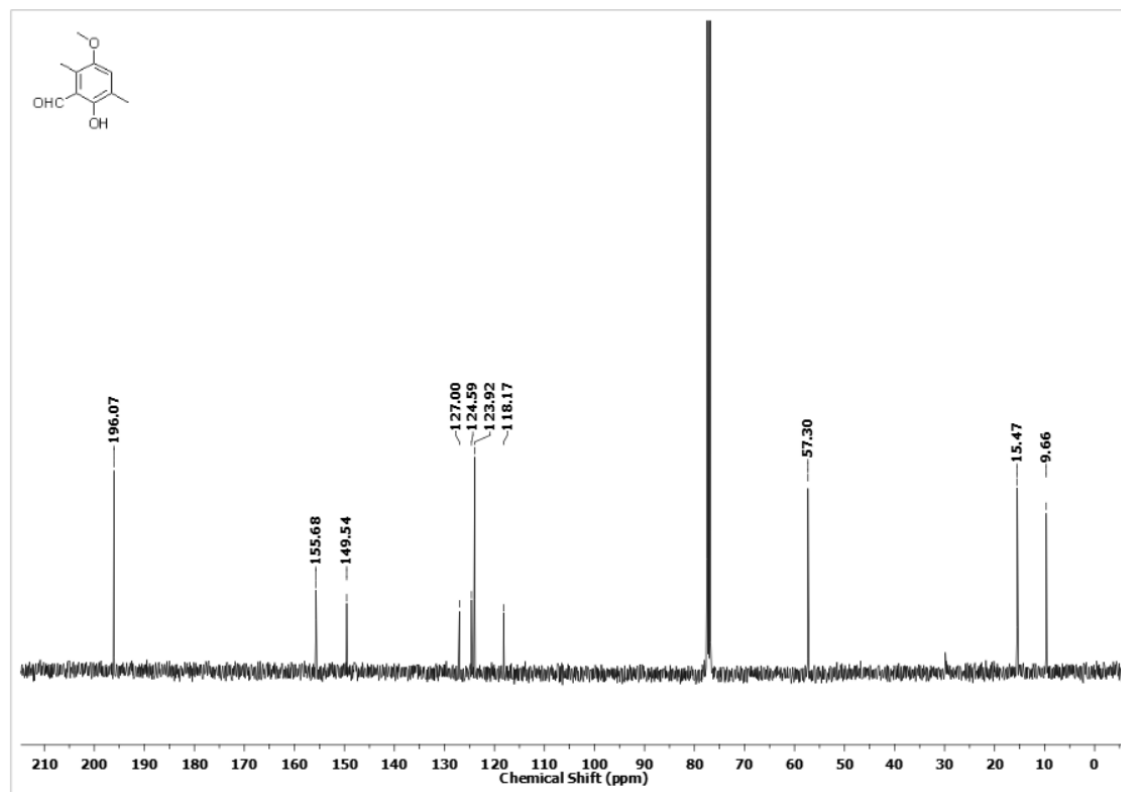


Figure 116: 101 MHz ¹³C spectrum of 11 in CDCl₃

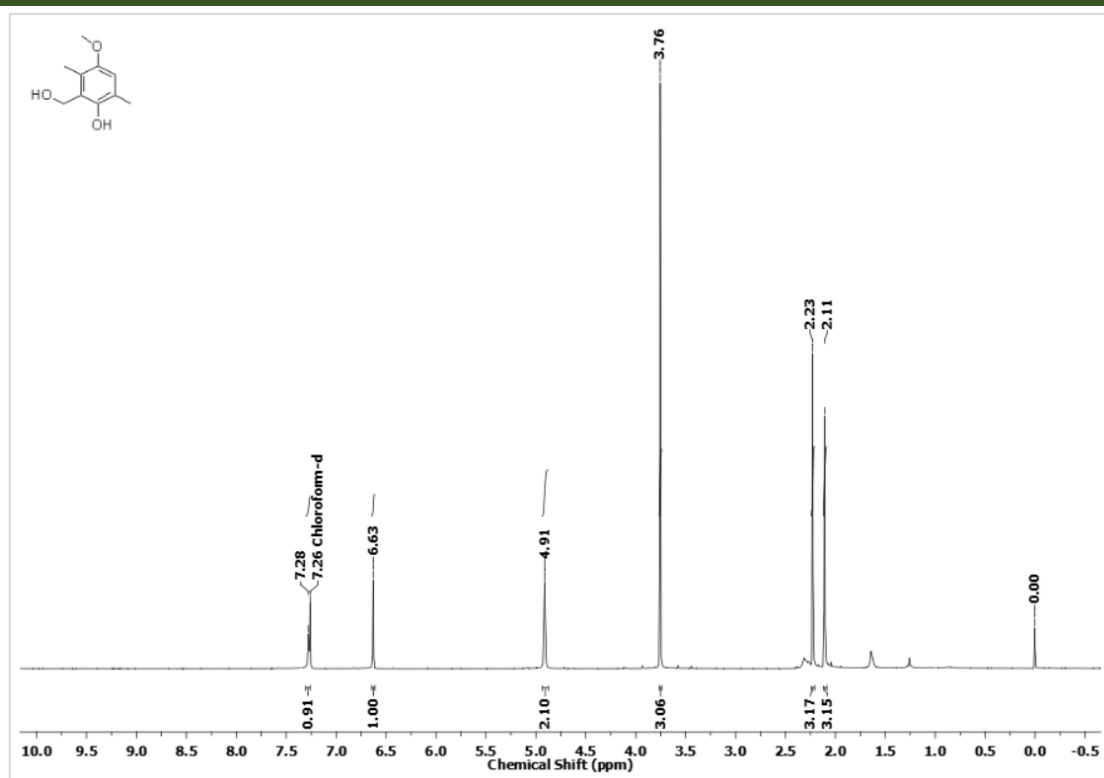


Figure 117: 400 MHz ¹H spectrum of **12** in CDCl₃

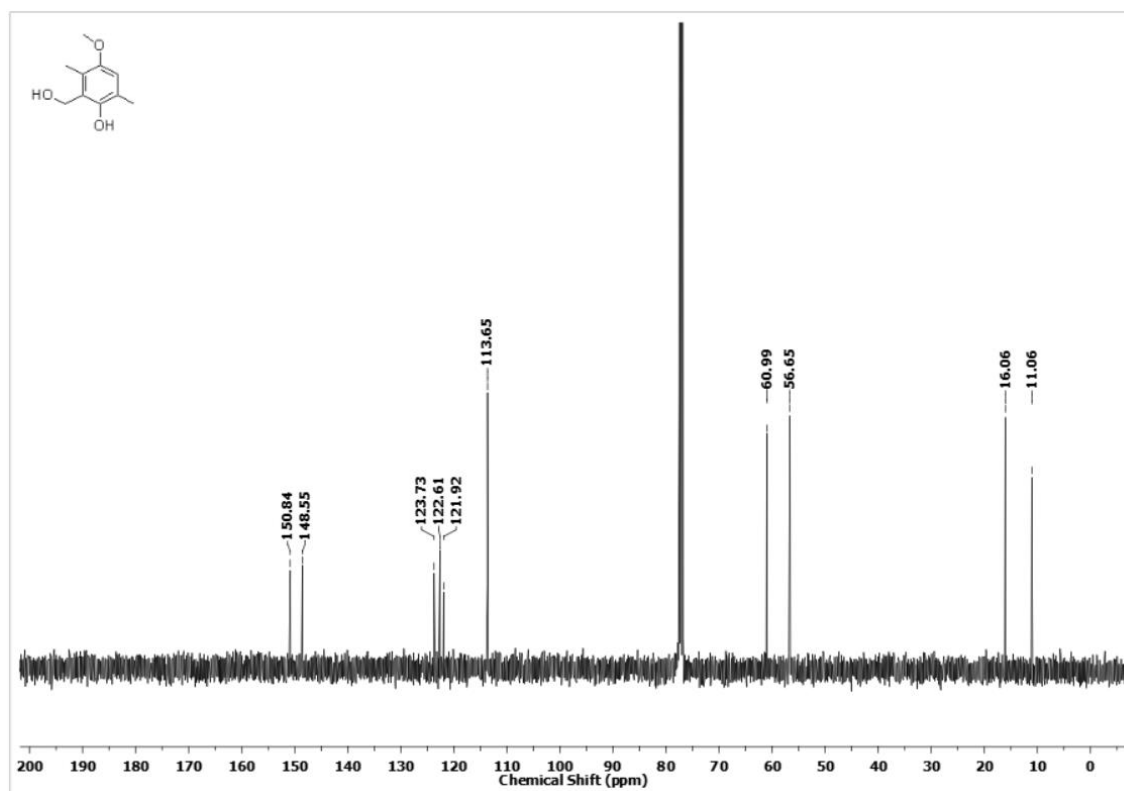


Figure 118: 101 MHz ¹³C spectrum of **12** in CDCl₃

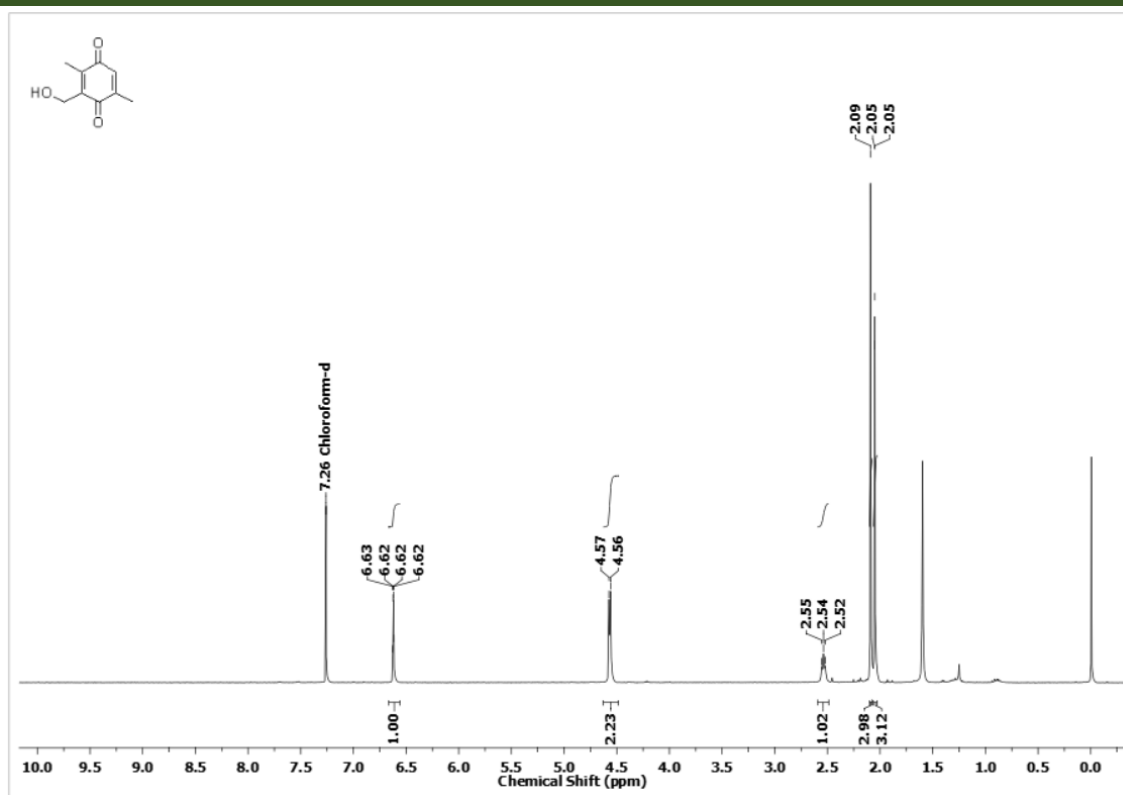


Figure 119: 400 MHz ¹H spectrum of **13** in CDCl₃

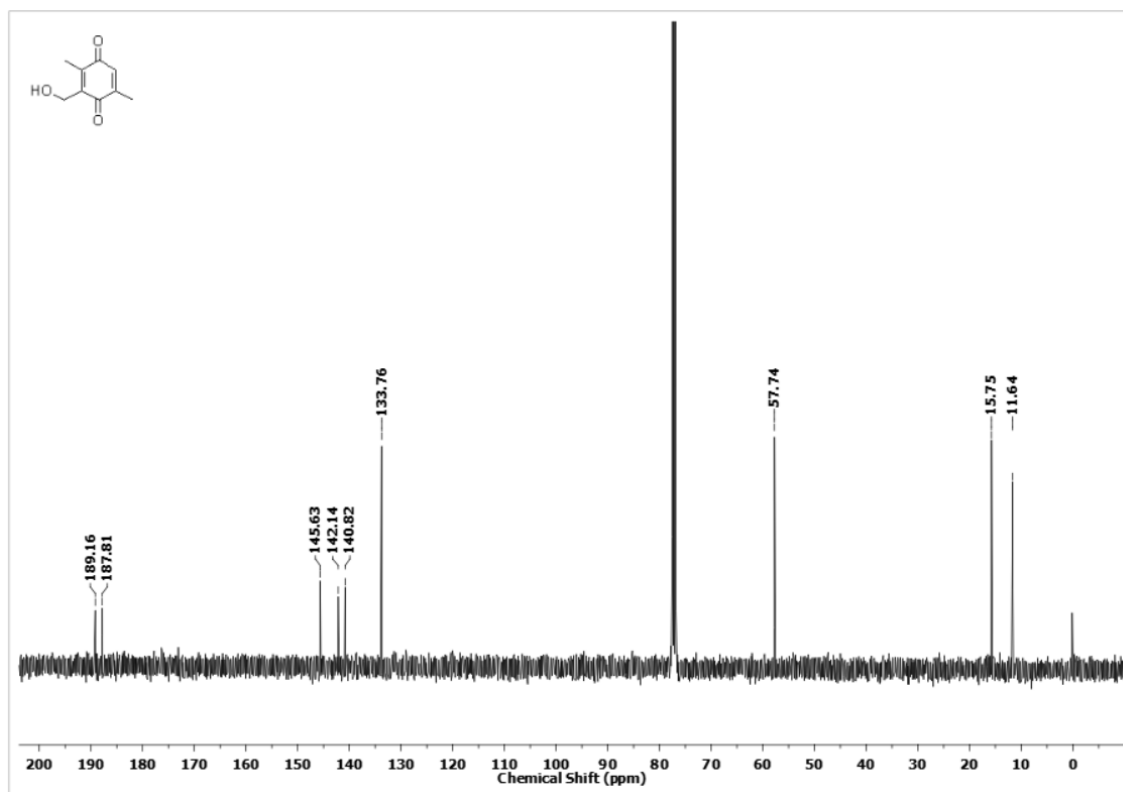
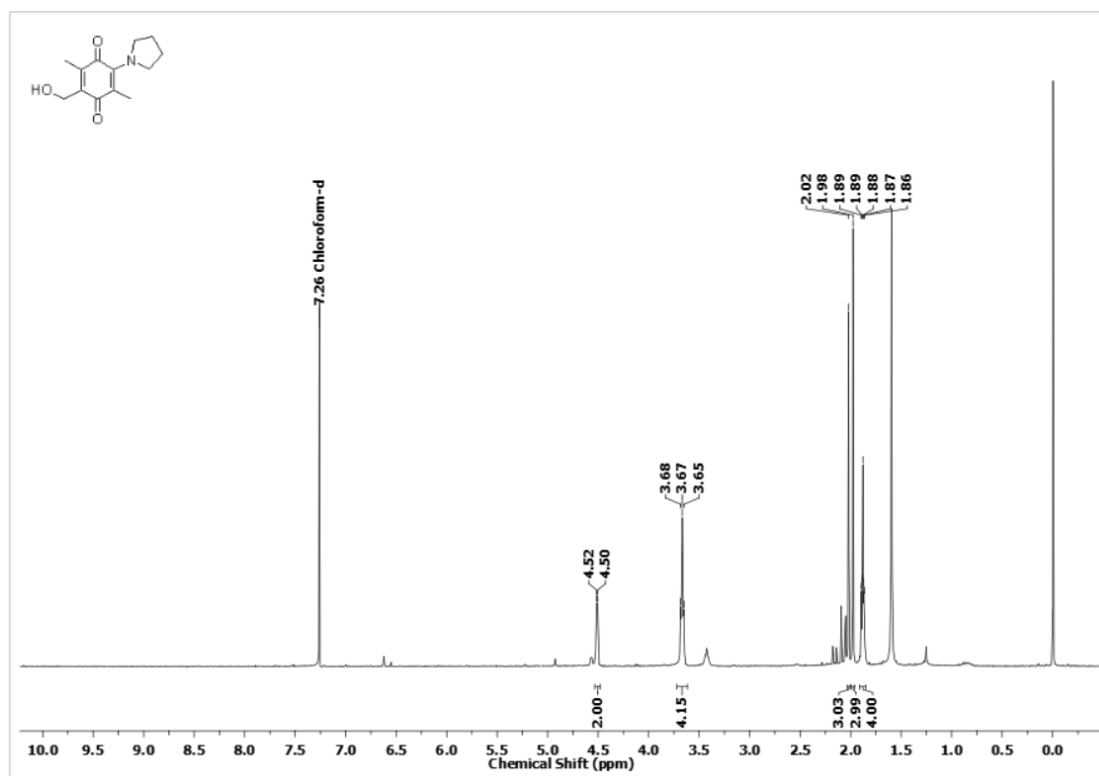
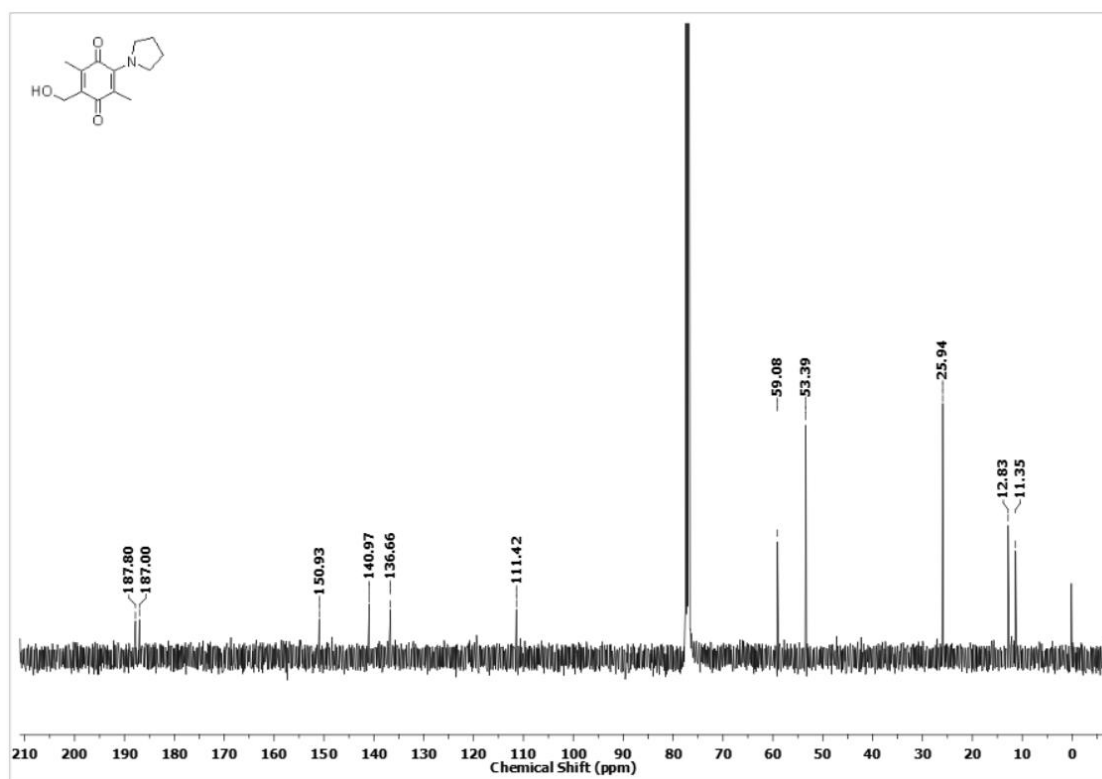


Figure 120: 101 MHz ¹³C spectrum of **13** in CDCl₃

**Figure 121:** 400 MHz ¹H spectrum of 5 in CDCl₃**Figure 122:** 101 MHz ¹³C spectrum of 5 in CDCl₃

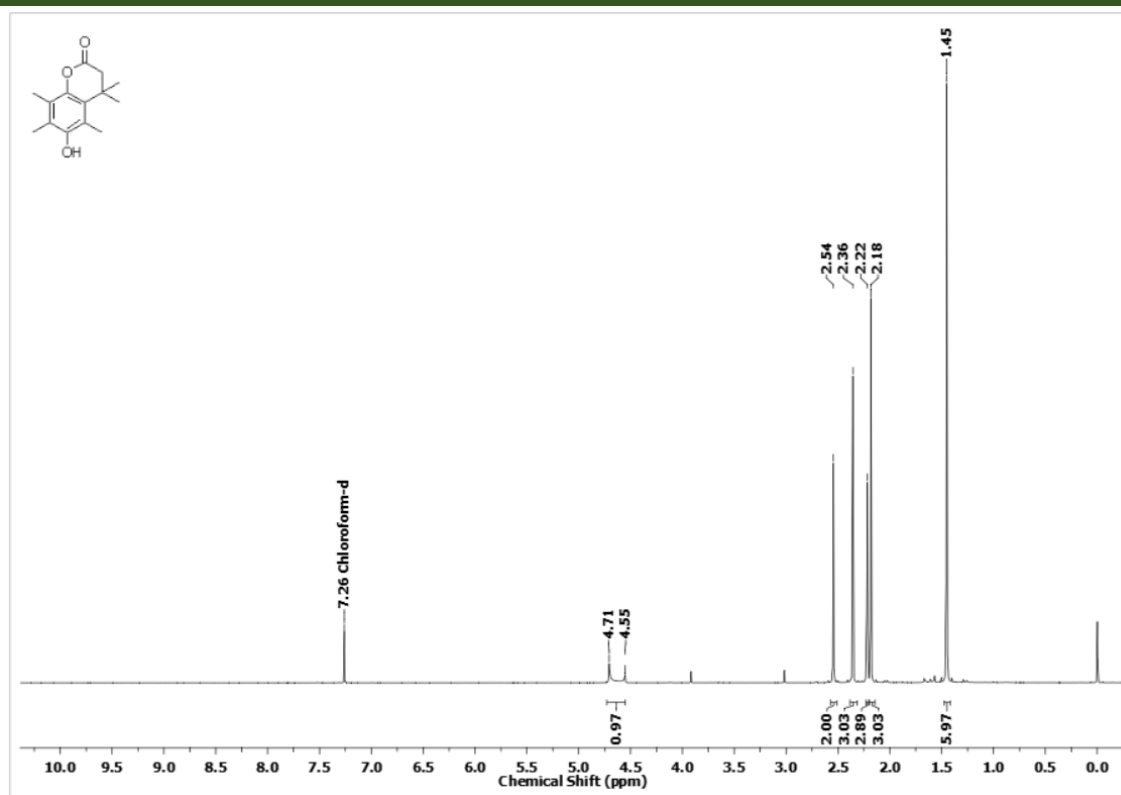


Figure 123: 400 MHz ¹H spectrum of **L** in CDCl₃

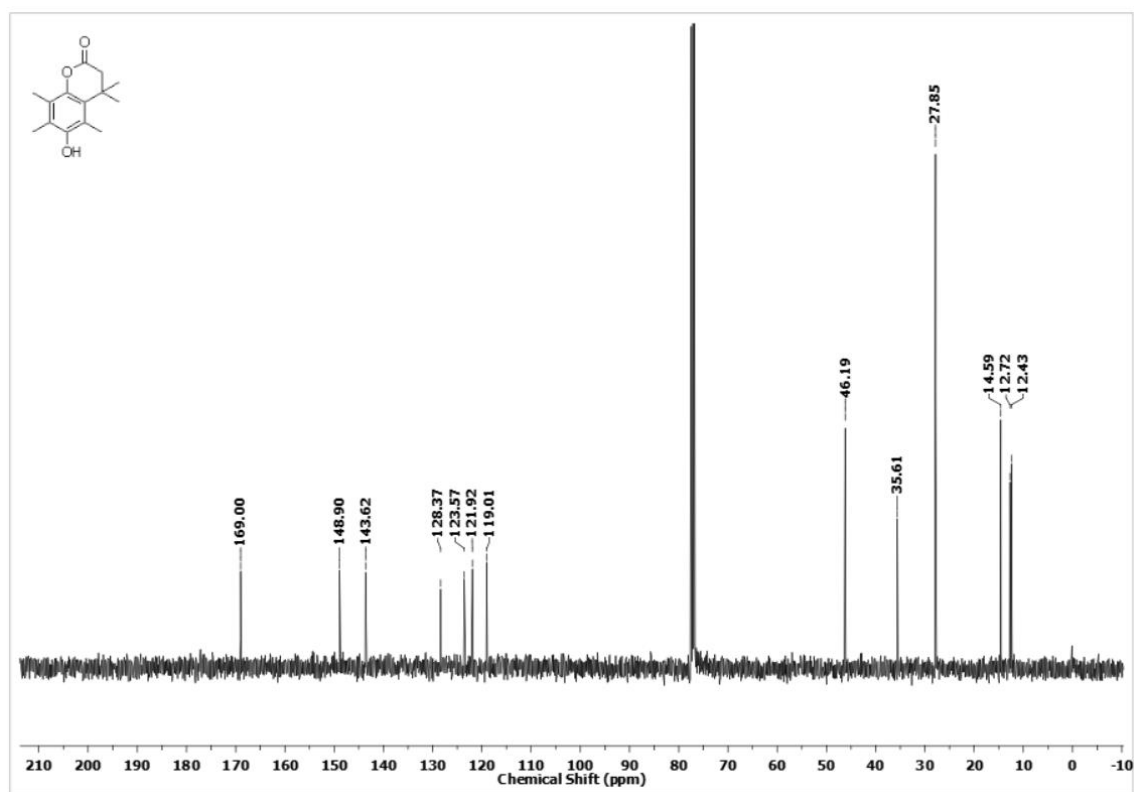


Figure 124: 101 MHz ¹³C spectrum of **L** in CDCl₃

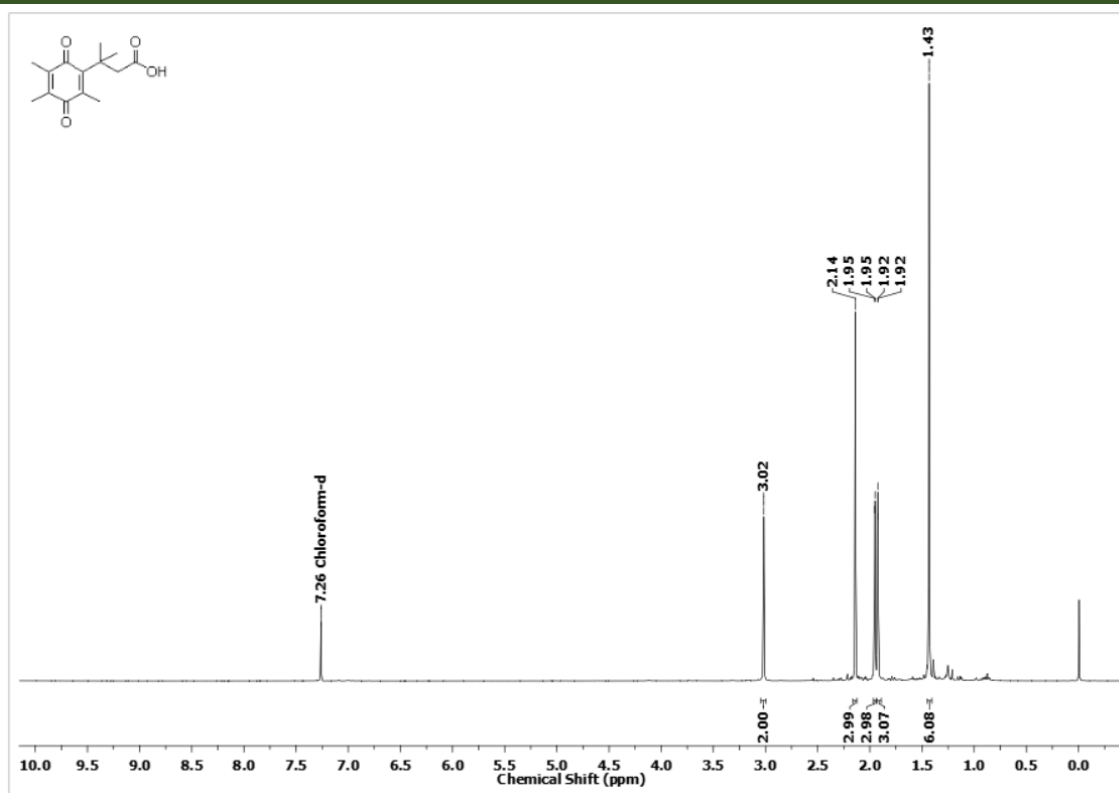


Figure 125: 400 MHz ¹H spectrum of **6** in CDCl₃

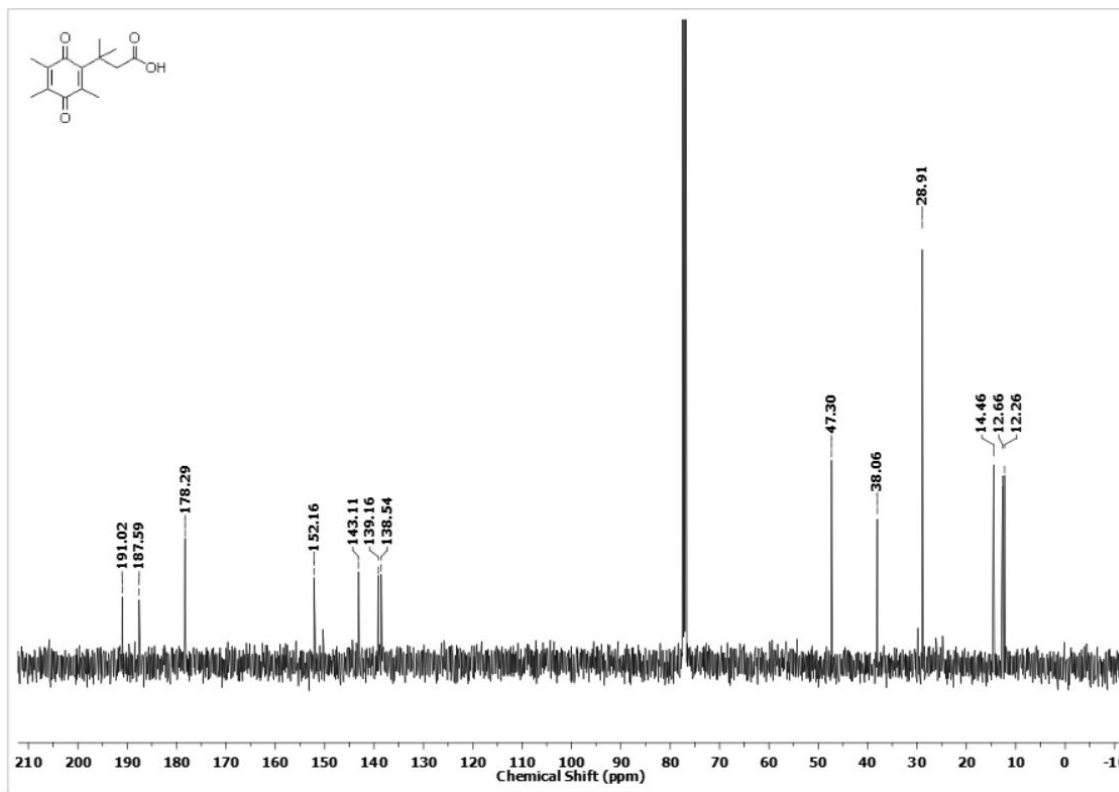


Figure 126: 101 MHz ¹³C spectrum of **6** in CDCl₃

2.6 References

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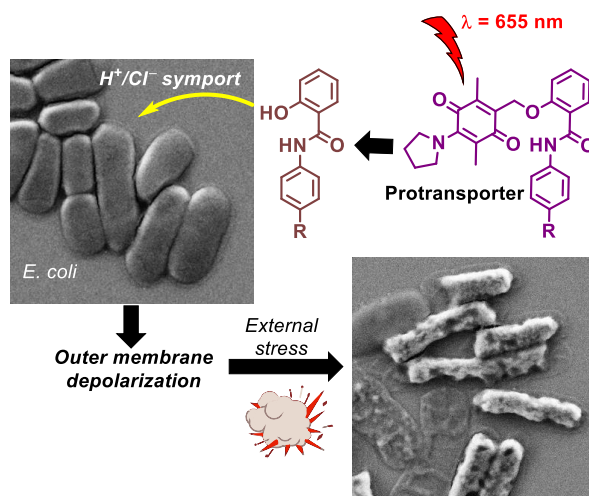
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Chapter 3

Salicylanilide H^+/Cl^- Transporters for the Inhibition of Gram-negative Bacterial Proliferation with Application in Photorelease Antimicrobial Therapy



3.1 Introduction

The emergence of antibiotic-resistant bacteria is a major threat to human health and is estimated to cause millions of infections and deaths worldwide.¹⁻⁴ Multi-drug resistance resulting from indiscriminate antibiotic use depletes the pool of antibiotics available for the treatment of bacterial infections, severely limiting therapeutic options, more so in countries lacking stringent regulation.^{2, 5, 6} The World Health Organization's recent global action plan⁷ and research agenda⁸ on antimicrobial resistance further emphasize the severity of this problem. The issue of the development of multi-drug resistance is more severe in Gram-negative bacteria, like the *Enterobacteriaceae* species (*Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*) and *Pseudomonas aeruginosa*, due to their complex outer membrane that acts as a multifaceted barrier to antibiotics restricting access to their intended targets in the cell.^{9, 10} In view of this crisis there is a severe need for the development of novel treatment methods while preserving the effectiveness of the existing antibiotic pool.^{1, 2}

Disruption of the bacterial outer membrane function is a potent strategy with a low likelihood of resistance development that can overcome many of the problems associated with traditional antibiotic treatment.^{11, 12} Transmembrane ion transport systems are suitably poised as ideal candidates in this regard. The ion homeostasis disrupting ability of transmembrane anion transporters has been applied to great effect in eukaryotic cells as potential anticancer agents,^{13, 14} but their utility in the antibiotic role remains much less explored. Work by Schmitzer,¹⁵⁻¹⁸ Gale-Sessler,¹⁹ Busschaert,²⁰⁻²² and Quesada²³ groups has demonstrated excellent antibiotic properties of various anion transporters in Gram-positive strains (including MRSA) but these systems exhibited very limited activity against Gram-negative strains. Report of the ability of prodigiosin,²⁴ a natural product capable of H⁺/Cl⁻ transport across lipid membranes,²⁵ to compromise the outer membrane in the Gram-negative bacterium *E. coli*,²⁶ inspired us to assess the antibacterial capability of the salicylanilide system, which is also an efficient H⁺/Cl⁻ co-transporter. We hypothesized salicylanilides to be capable of depolarizing the outer membrane of Gram-negative bacteria, inhibiting their growth, and rendering them susceptible to other antimicrobial agents.

One suitable application of these systems can be for topical application where there is a critical need for treatment methods with minimal possibility of resistance development. Bacterial incursion resulting from breaches in the skin is a very common cause of infection, making topical

antibiotics, necessary for its treatment/prevention, some of the most commonly prescribed drugs in use.^{27, 28} The development of antibiotic resistance resulting from their widespread use is a major cause of concern as it can also promote the spread of infections from opportunistic pathogens which gain drug resistance during the treatment regime.^{29, 30}

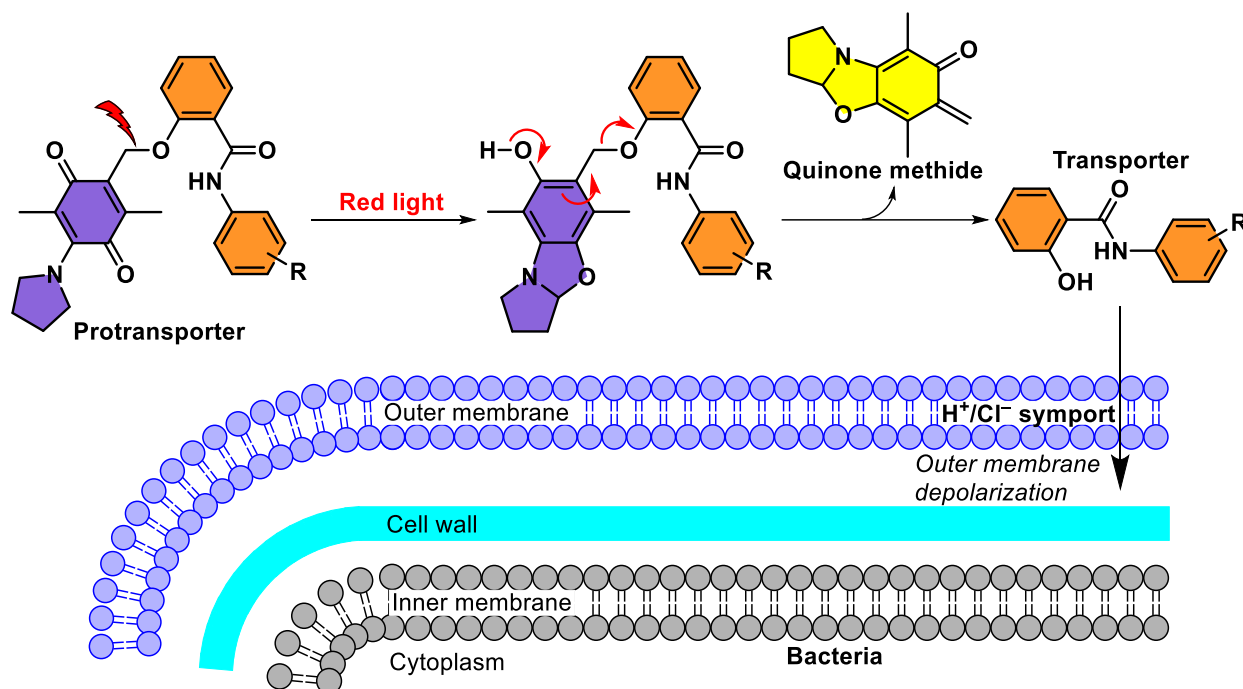


Figure 1: Red light activation of caged salicylanilide protransporter to induce transmembrane H⁺/Cl⁻ symport across the bacterial outer membrane.

The pyrrolidinobenzoquinone moiety used for the preparation of the salicylanilide protransporter in the previous chapter is also an efficient photocleavable protecting group (PPG) and enabling the extension of the salicylanilide system for photorelease antimicrobial therapy. Photoactivable antimicrobial therapies are gaining ground due to the high degree of spatiotemporal control achievable when using light and subsequent suitability for controlled-release applications.³¹⁻³³ The pyrrolidinobenzoquinone group is capable of photolysis under red light,^{34, 35} which makes it highly desirable for therapeutic use due to the biological compatibility of light in the red (and near infra-red) region (> 620-630 nm), which is biologically inert even at high fluences.^{36, 37} Most photoactivable antimicrobials reported in literature utilize light in the UV to blue region for their activation.^{31, 32, 38, 39} Studies in human keratinocytes and dermal fibroblasts indicated detrimental effects of visible light even up to 530 nm wavelength at high fluences,^{36, 37} precluding the use of

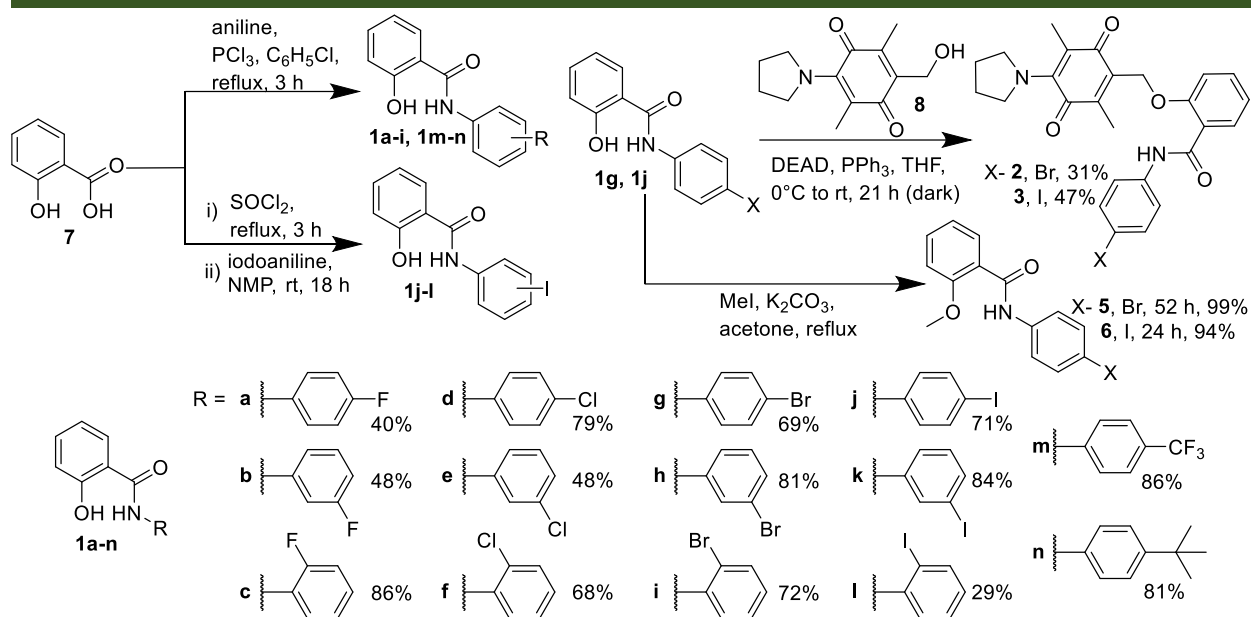
most PPGs for such applications.⁴⁰ Hence, the activation range of the pyrrolidinobenzoquinone group makes it highly suitable for this application, and it can effectively cage salicylanilide ion transport activity by attachment to the free OH via ether linkage (Figure 1). Even though the pyrrolidinobenzoquinone group is susceptible to quinone reductase activation, bacterial quinone reductases, like those that are part of the respiratory chain, are localized to the inner membrane and cytoplasm and hence should not initiate premature activation of the protransporters.^{41, 42}

In this work, a library of monosubstituted salicylanilide transporters was synthesized and screened in the Gram-negative model system *Caulobacter crescentus* to derive a structure activity relationship with respect to their ion transport properties. The best candidates were then tested in three (Gram-negative) *Enterobacteriaceae* species, including two common pathogens, namely *E. coli*, *S. enterica* and *K. pneumoniae* where they exhibited good results. The two most promising salicylanilides were then formulated in the photoactivable protransporter form by conjugation with the pyrrolidinobenzoquinone group. Photoactivated ion transport using red light was demonstrated in liposomes, followed by photoactivated antibiotic activity in two bacterial strains.

3.2 Results and Discussions

3.2.1 Synthesis

The monosubstituted salicylanilides (**1a-i**, **1m-n**) were synthesized by coupling the corresponding aniline to salicylic acid (**7**) in the presence of PCl_3 under reflux condition in chlorobenzene (Scheme 1) as reported in Chapter 2.⁴³ Preparation of the monoiodo-substituted salicylanilides (**1j-l**), for which the PCl_3 -reflux method was found to be unsuitable, was carried out via a two-step process, by conversion of salicylic acid to its acid chloride by refluxing in SOCl_2 followed by coupling to the iodoaniline in NMP under ambient conditions (Scheme 1).⁴⁴ The photoactivable protransporters (**2**, **3**) were prepared by Mitsunobu coupling⁴⁵ of the pyrrolidinobenzoquinone **8**^{34, 35} to salicylanilides **1g** and **1j** (Scheme 1). A control compound **4** was synthesized by coupling (non-transporting) benzoic acid (**BA**) to quinone **8** to assess the properties of the photocleavage byproduct (Experimental section). For the assessment of transporter activity on blocking of the OH group, salicylanilides **5** and **6** were synthesized by methylation of the phenolic OH using methyl iodide in the presence of base (K_2CO_3) under reflux conditions (Scheme 1).



Scheme 1: Synthesis of salicylanilide transporters and protransporters.

3.2.2 $\log P$ and pK_a Calculations

The $\log P$ of the compounds and the N-H and O-H pK_a values were predicted using calculator plugins of MarvinSketch (v23.11)⁴⁶ (Table 1). The O-H pK_a values were constant throughout the series (7.93), hence capable of protonation-deprotonation cycles at physiological pH, while the calculated amide N-H pK_a values were within the range of 15.23-16.16, depending on the type and position of the *N*-phenyl ring substitution. Calculation of $\log P$ values of the halogen-substituted salicylanilides (F- 2.90, Cl- 3.37, Br- 3.53, I- 3.69) indicated Cl, Br, and I substituted molecules to be highly suitable for transmembrane ion transport. $\log P$ values of protransporters **2** (5.19) and **3** (5.35), and controls **5** (3.68) and **6** (3.84) were also indicative of good membrane permeability of these molecules.

Table 1: Estimated $\log P$, and N-H, O-H pK_a values of salicylanilide derivatives. Values were estimated using calculator plugins of MarvinSketch.⁴⁶

Compound	$\log P$	N-H pK_a	O-H pK_a
1a	2.90	15.92	7.93
1b	2.90	15.72	7.93
1c	2.90	15.23	7.93
1d	3.37	15.96	7.93
1e	3.37	15.82	7.93
1f	3.37	15.58	7.93
1g	3.53	15.99	7.93
1h	3.53	15.89	7.93
1i	3.53	15.69	7.93
1j	3.69	16.00	7.93
1k	3.69	15.90	7.93
1l	3.69	15.71	7.93
1m	3.64	15.98	7.93
1n	4.31	16.16	7.93
2	5.19	15.13	-
3	5.35	15.14	-
5	3.68	15.15	-
6	3.84	15.16	-

3.2.3 Screening and Structure Activity Relationship

The ion transport activities of the salicylanilides (**1a-1n**) were compared across EYPC-LUVs $\Rightarrow$ HPTS. The compounds were tested at 10 μ M concentration. Salicylanilides with *para* (*p*) and *meta* (*m*) halogen (Cl, Br, I) substitution (relative to amide) were observed to exhibit high ion transport activities, while the *ortho* halogen (Cl, Br, I) substituted transporters showed negligible activity (Figure 2A). The fluorine substituted compounds exhibited very low transport activities (with **1b** being the most active) owing to their low $\log P$ values (Table 1). Compounds **1m** (*p*-CF₃) and **1n** (*p*-*t*Bu) also showed very good ion transport activities. The antimicrobial activities of the salicylanilides were then tested in the Gram-negative α -proteobacterium

Caulobacter crescentus. *C. crescentus* is representative of bacteria having an outer S-layer,⁴⁷ also found in pathogens including *Campylobacter* spp., *Aeromonas salmonicida*, etc. The S-layer is known to decrease the permeability of the bacterial membrane towards solutes and has been reported to confer resistance to certain antibiotic classes.⁴⁸ These factors make *C. crescentus* an ideal model organism for our initial screening of antimicrobial activity. We hypothesized that compounds effective against *C. crescentus* would also exhibit activity towards both Gram-negative pathogens with simpler envelope structure, and bacterial strains having protective outer layers such as mucoid capsules that block drug entry.^{49, 50}

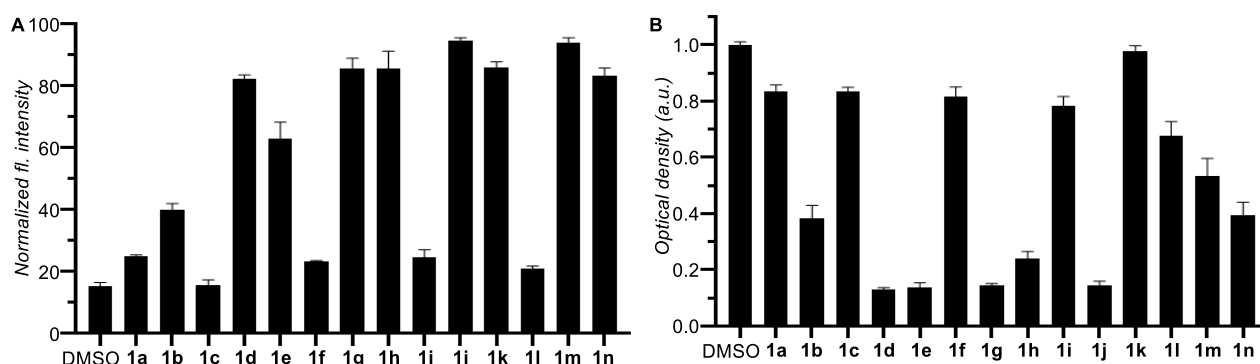


Figure 2: (A) Comparison of ion transport activities of transporters **1a-1n** (10 μ M) across EYPC-LUVs $\rightarrow$ HPTS (normalized fluorescence intensity at 290 s). DMSO was used as negative control. (B) Growth inhibition in *C. crescentus* on treatment with salicylanilides **1a-1n** (50 μ M) for 18 h. Data represented as culture OD relative to DMSO treated control culture.

C. crescentus cultures at OD (600 nm) 0.1 in PYE growth media were treated with the salicylanilides (50 μ M), and the cell density was measured at OD600 following 18 h incubation at 29 $^{\circ}$ C (Figure 2B). A strong correlation was observed between the ion transport and antibacterial activities of the compounds. Statistical analyses in the form of unpaired *t* tests (two tailed) were performed (Table 9) on the growth inhibition data and the data from cultures treated with transporters **1d**, **1e**, **1g**, **1h**, and **1j** gave a large effect size⁵¹ ($R^2 > 0.95$) relative to the DMSO treated control, indicating them to have the highest antibacterial activity. These *para* and *meta* halogen (Cl, Br, and I) substituted salicylanilides were also transporters that exhibited high ion transport activities. The only exception was **1k** (*m*-I), which in spite of being an efficient ion transporter exhibited negligible antibacterial activity due to precipitation in PYE media. Compounds with very low ion transport abilities, namely *ortho* Cl (**1f**), Br (**1i**), and I (**1l**), and the

fluoro derivatives (**1a**, **1c**) showed negligible antibacterial activity. The *m*-F derivative (**1b**) was an average transporter with a moderate antibacterial effect. The moderate antibacterial activities of **1m** (*p*-CF₃) and **1n** (*p*-*t*Bu), in contrast to their high ion transport abilities, were likely due to differences in membrane structure between the liposomal test system and the bacterial cell. Our analysis showed that salicylanilides with direct halogen substitution on the *N*-phenyl ring exhibit inhibitory activity against Gram-negative bacteria in relation to their ion transport ability observed across artificial liposomes.

The best working transporters **1d**, **1e**, **1g**, **1h**, and **1j** were selected for further studies. Data from dose-dependent studies of these transporters across EYPC-LUVs⇌HPTS (Figures 3-6) was fitted to the Hill equation to obtain the *EC*₅₀ values, which were found to be very close with the order of transport activity **1j** > **1h** > **1g** > **1d** > **1e** (Table 2). Hill coefficients (*n*) were determined to be ~2 for all five compounds (Table 2), indicating two molecules of the transporter assemble with the anion to form the transmembrane transport complex. This was in line with the findings in our prior report of salicylamide facilitated transmembrane H⁺/Cl⁻ symport (Chapter 2), where a 2:1 transporter-chloride complex formation was observed for salicylanilides with electron withdrawing group substitution. The chloride ion binding ability of the compounds **1d**, **1e**, **1g**, **1h**, and **1j** were also assessed through ion titration experiments using NMR (Figures 7-10). All compounds were observed to bind to Cl⁻ in a 1:1 stoichiometry and had similar binding constants (Table 3). Crystallization of the Br substituted derivatives (**1g-1i**) yielded structures in the ‘open-ring’ conformation of salicylanilide, with the N-H⋯O-(C,H) interaction (Figure 33A-C).⁵² This along with the nature of peak shifts in the NMR titration spectra confirms the salicylanilide conformation and the mode of anion binding to be consistent with our previous report (Chapter 2). The limited ion transport ability of the *ortho*-substituted derivatives can be attributed to the close proximity of the halogen to the ion binding site, as evident from the molecular structure of **1i** (Figure 33C) obtained from its crystal data, which could sterically hinder ion binding and disrupt transport complex formation.

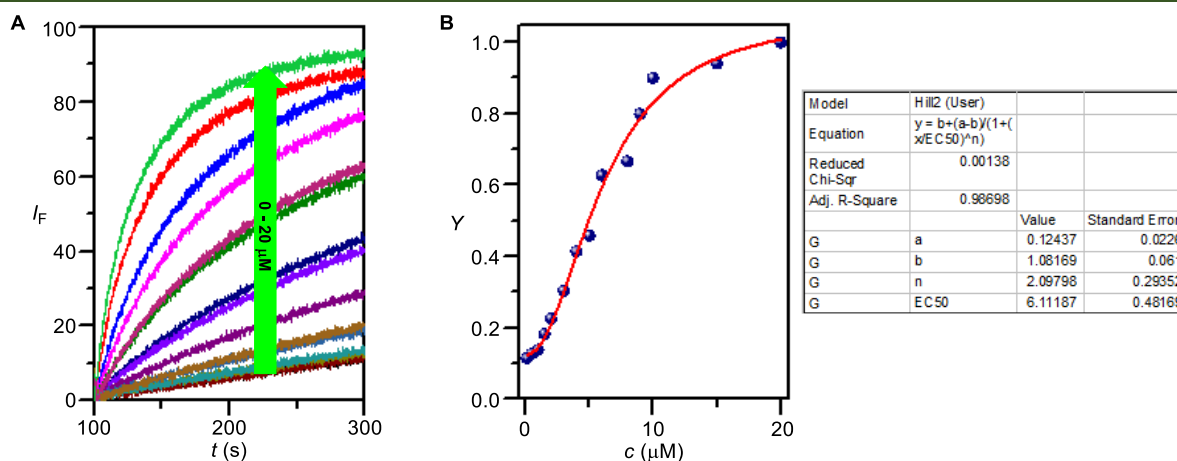


Figure 3: (A) Dose-dependent activity of compound **1d** across EYPC-LUVs to HPTS. (B) Hill plot analysis of **1d** at $t = 290$ s.

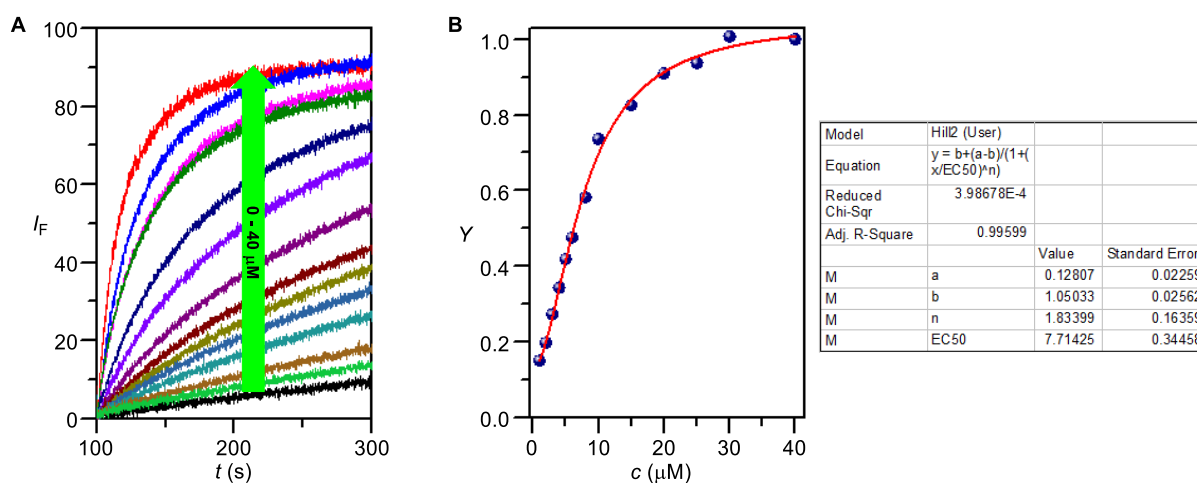


Figure 4: (A) Dose dependent activity of compound **1e** across EYPC-LUVs to HPTS. (B) Hill plot analysis of **1e** at $t = 290$ s.

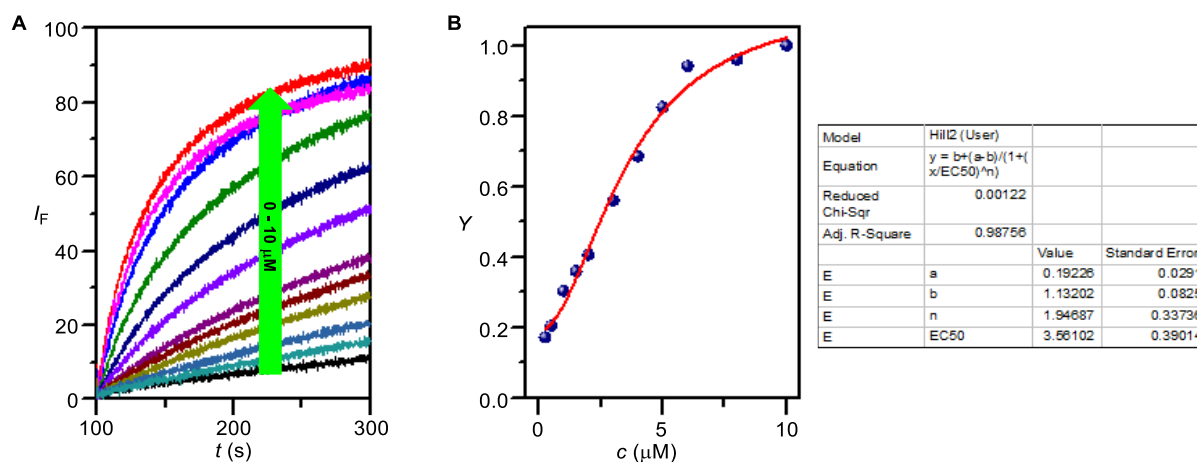


Figure 5: (A) Dose-dependent activity of compound **1h** across EYPC-LUVs to HPTS. (B) Hill plot analysis of **1h** at $t = 290$ s.

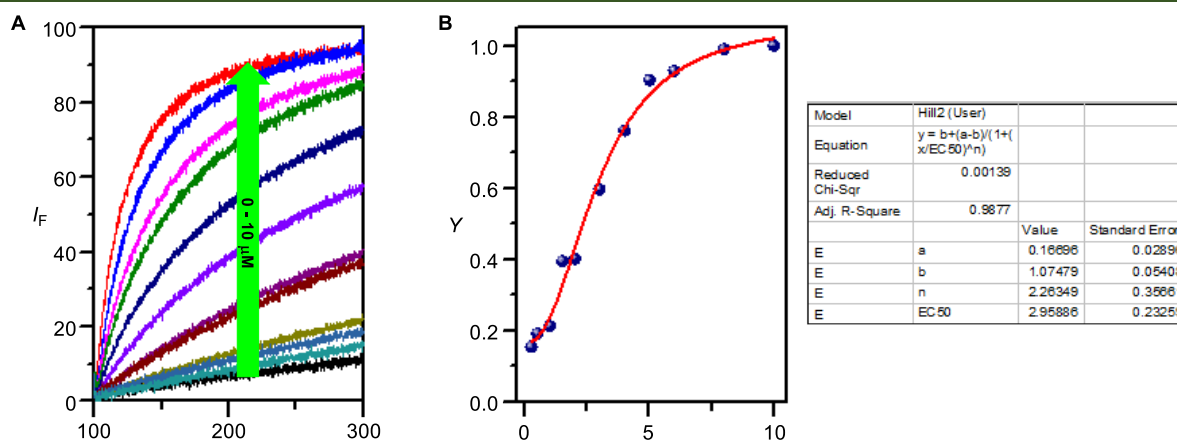


Figure 6: (A) Dose-dependent activity of compound **1j** across EYPC-LUVs to HPTS. (B) Hill plot analysis of **1j** at $t = 290$ s.

Table 2: Summary of Hill analyses of dose-dependent ion transporter studies across EYPC-LUVs to HPTS.

Compound	EC_{50} (μ M)	Hill coeff. (n)
1d	6.11	2.10
1e	7.71	1.83
1g	5.45	1.90
1h	3.56	1.95
1j	2.96	2.26

Table 3: Summary of transporter-ion binding data from NMR titration experiments.

Compound	Titrated Ion	Binding Stoichiometry	K_a (M^{-1})
1d	Cl^-	1:1	119.99 ± 2.05
1e	Cl^-	1:1	138.89 ± 2.39
1g	Cl^-	1:1	167.17 ± 1.07
1h	Cl^-	1:1	105.93 ± 0.75
1j	Cl^-	1:1	115.01 ± 1.64

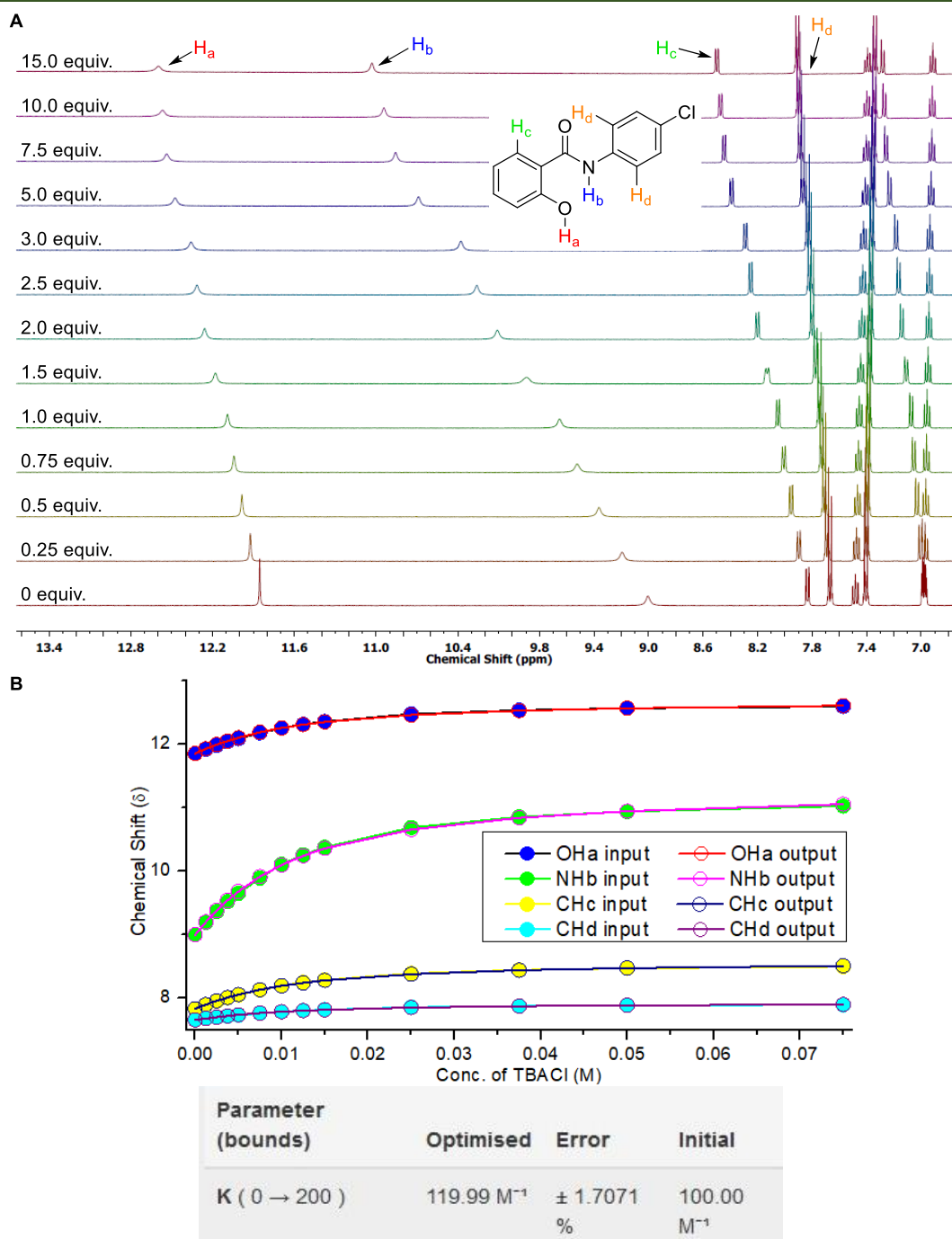


Figure 7: (A) ^1H NMR titration spectra of **1d** (5 mM) with stepwise addition of TBACl in CD_3CN . The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) The plot of chemical shifts (δ) of protons H_a , H_b , H_c , and H_d vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

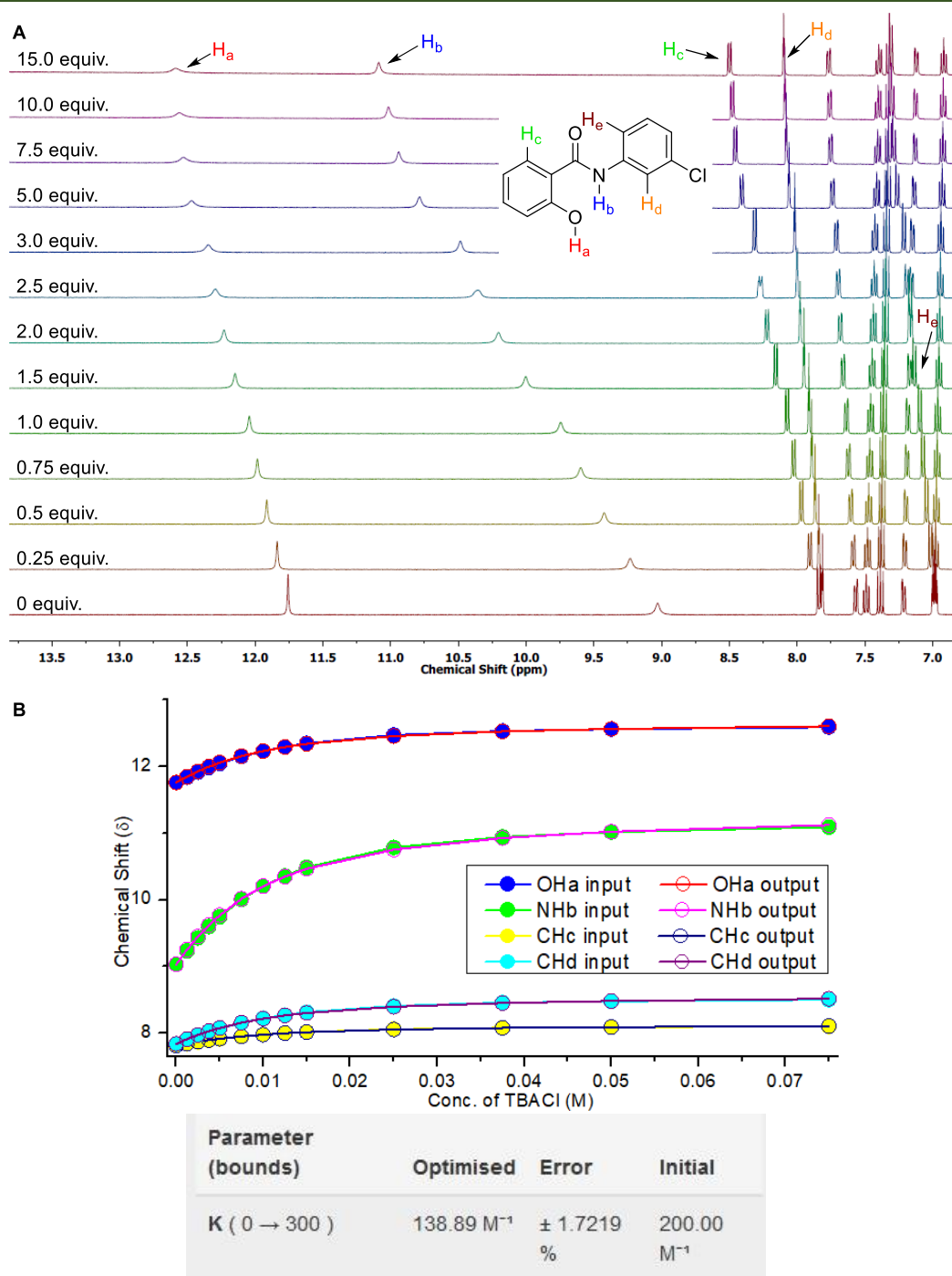


Figure 8: (A) ^1H NMR titration spectra of **1e** (5 mM) with stepwise addition of TBACl in CD_3CN . The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) The plot of chemical shifts (δ) of protons H_a , H_b , H_c , and H_d vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

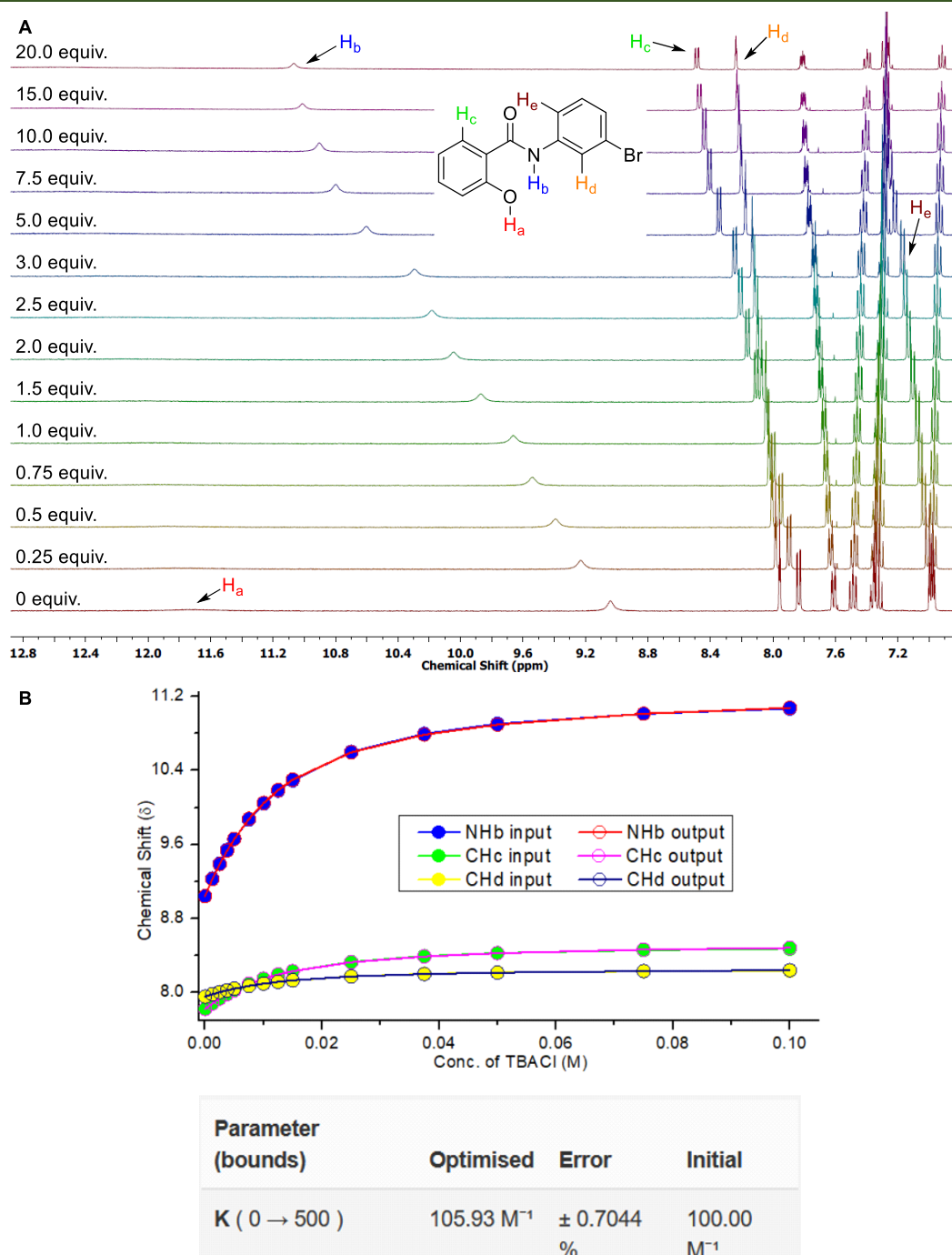


Figure 9: (A) ^1H NMR titration spectra of **1h** (5 mM) with stepwise addition of TBACl in CD_3CN . The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) The plot of chemical shifts (δ) of protons H_b , H_c , and H_d vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

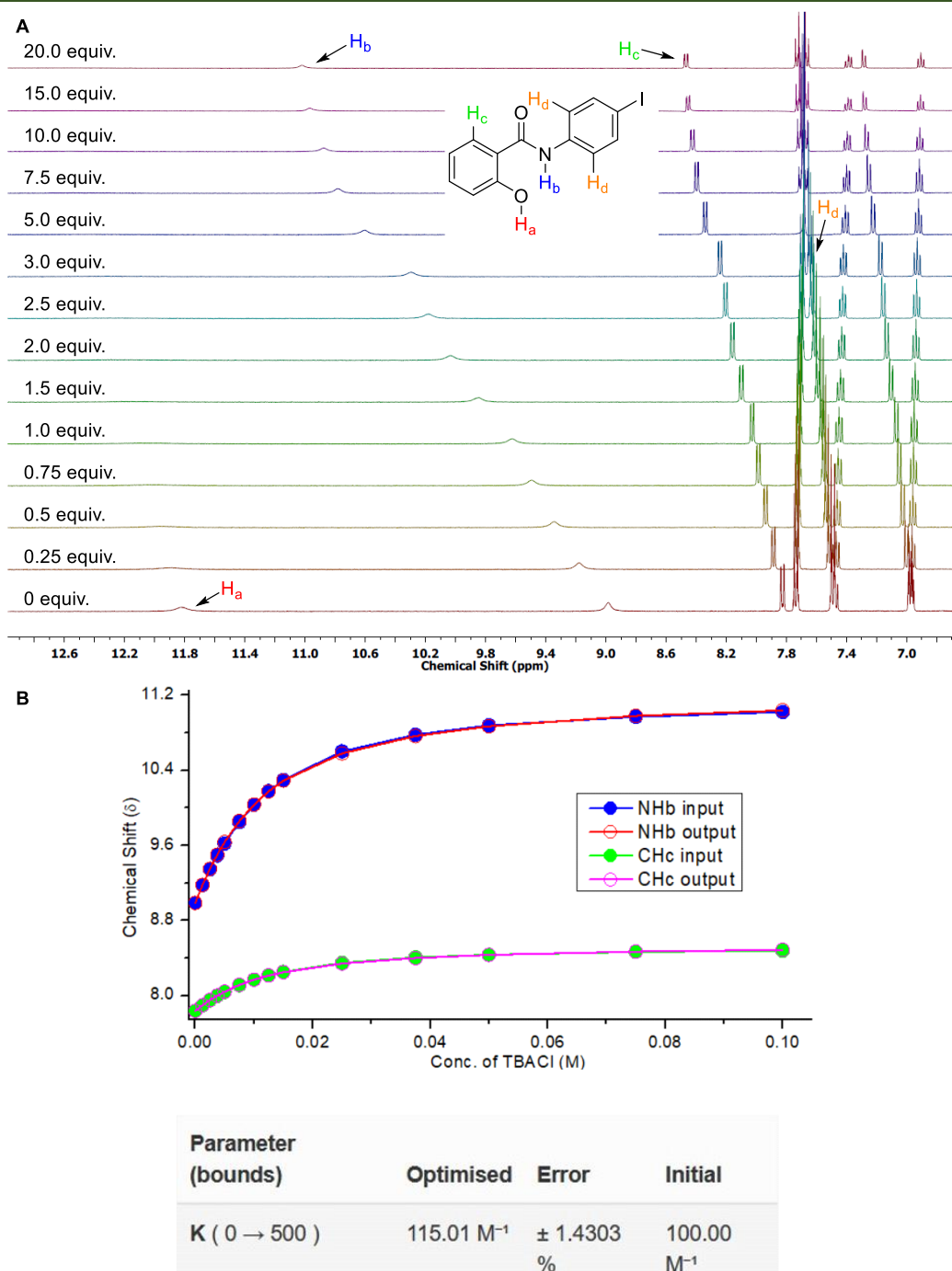


Figure 10: (A) ^1H NMR titration spectra of **1j** (5 mM) with stepwise addition of TBACl in CD_3CN . The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) The plot of chemical shifts (δ) of protons H_b , and H_c vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

Growth curves of *C. crescentus* in PYE broth following treatment with the compounds were recorded over 18 h (Figure 11). Inhibition of bacterial growth with increasing dosage of the compounds was observed and the order of activity was observed to be **1j** > **1g** > **1d** > **1e** $\approx$ **1h** based on their minimum inhibitory concentrations.

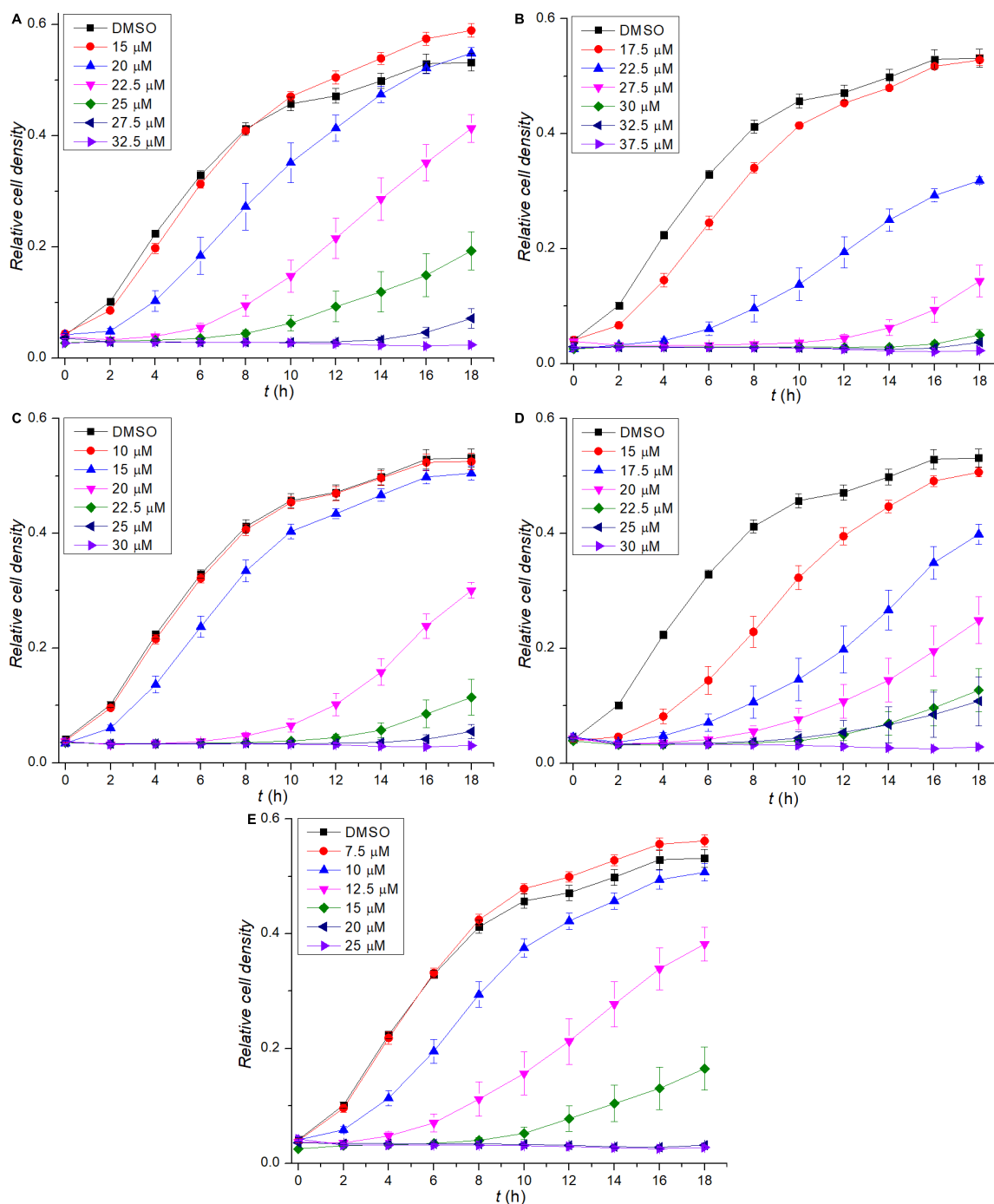


Figure 11: Growth curves of *C. crescentus* treated with varying doses of (A) **1d**, (B) **1e**, (C) **1g**, (D) **1h** and (E) **1j**.

To verify the role of H^+/Cl^- transport ability of salicylanilides in their antibacterial activity, compounds **5** and **6** were prepared by methylation of the OH groups in **1g** and **1j** (Scheme 1), effectively blocking the protonation-deprotonation center. The inhibition of the ion transport ability of the salicylanilides on methylation was verified by comparison of the compounds across EYPC-LUVs $\rightarrow$ HPTS at a high concentration of 15 μ M. The transport activities of the free transporters (**1g**, **1j**) were observed to quickly saturate at this concentration, while the methylated controls (**5**, **6**) exhibited only very low rates of ion transport (Figure 12A). The methylated salicylanilides (**5**, **6**) were observed to show negligible activity towards *C. crescentus* even at 50 μ M concentration (Figure 12B). Hence, we were able to conclude that the transmembrane H^+/Cl^- transport ability of the salicylanilide system was the dominant factor contributing to its antibacterial activity.

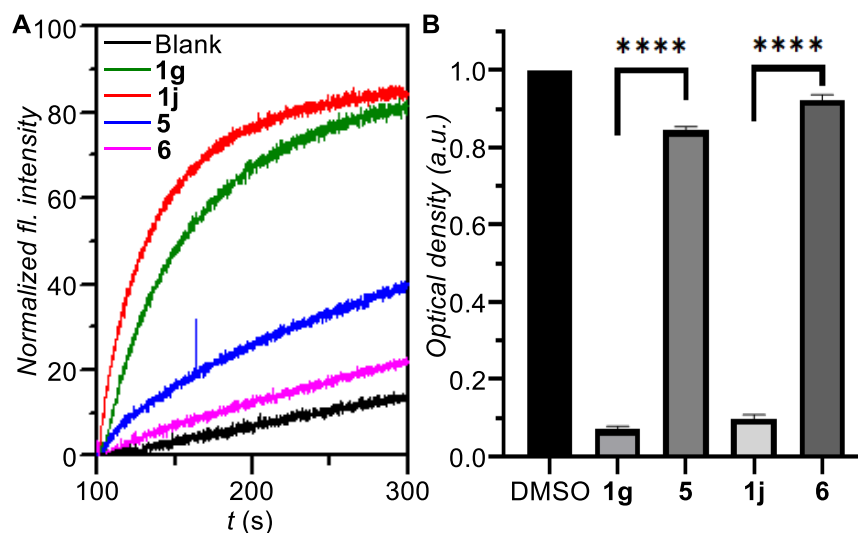


Figure 12: (A) Comparison of ion transport activities of transporters **1g** and **1j** with their OH methylated analogs **5** and **6**. (B) Comparison of growth in *C. crescentus* following treatment with **1g**, **1j**, **5** and **6** (50 μ M) for 18 h. Data represented as culture OD relative to DMSO treated control culture. **** represents $p < 0.0001$ when comparing free transporters (**1g**, **1j**) to their respective methylated controls (**5**, **6**) using unpaired t tests.

3.2.4 Antibacterial Effect in Enterobacteria

The antibacterial properties of selected compounds (**1d**, **1e**, **1g**, **1h** and **1j**) were then tested in *Escherichia coli* (MG1655 wild type strain), a Gram-negative model organism of the *Enterobacteriaceae* family. Growth curves following the treatment of *E. coli* cultures (in LB

media) with the compounds at 25, 50, and 100 μM concentrations were recorded over 12 h. All five selected compounds were observed to exhibit a dose-dependent inhibition in bacterial growth and showed similar activity profiles (Figure 13), confirming the successful screening of the salicylanilide library.

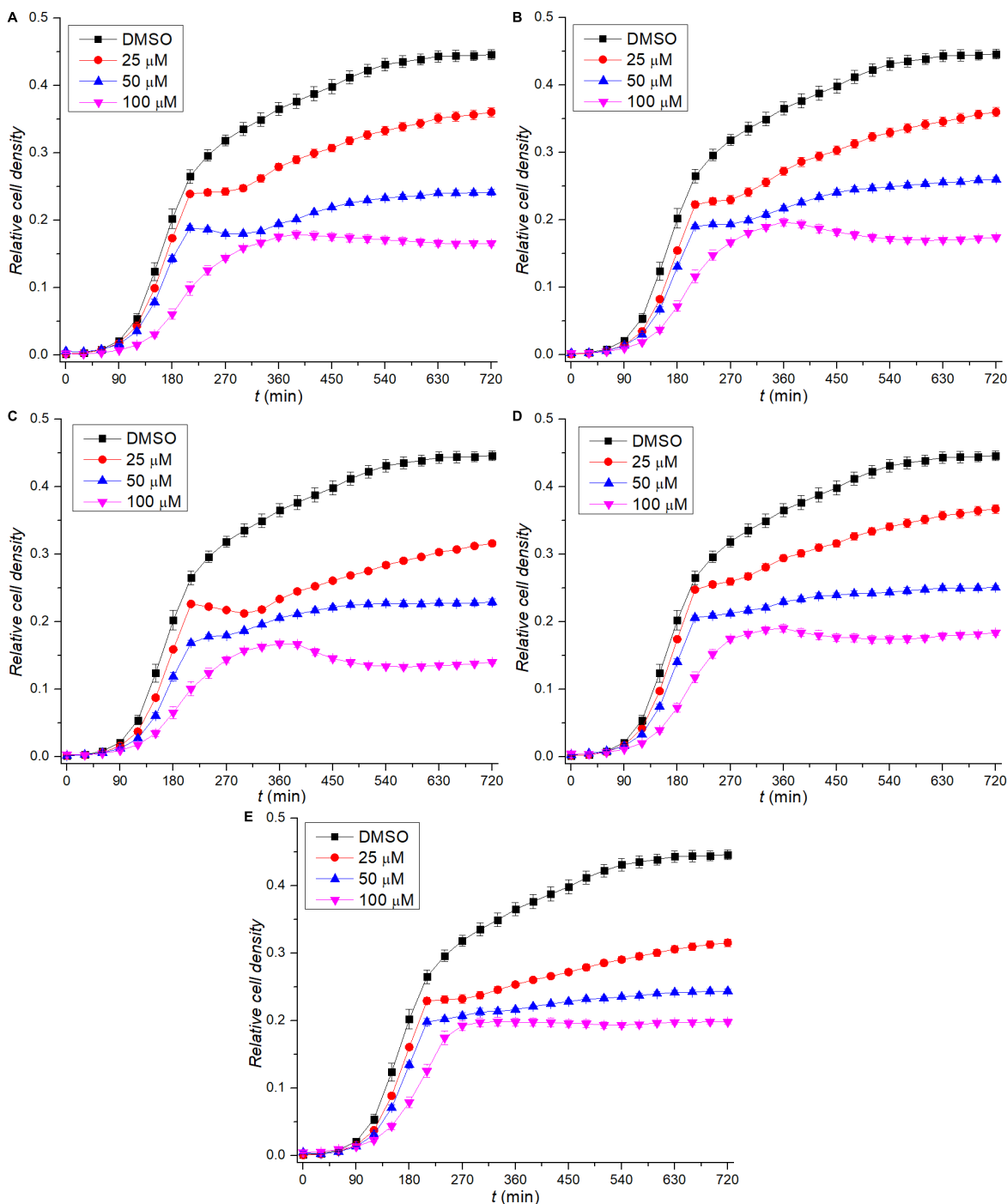


Figure 13: Growth curves of *E. coli* treated with varying doses of (A) **1d**, (B) **1e**, (C) **1g**, (D) **1h** and (E) **1j**.

Transporters **1g** and **1j** which showcased the best effects over both model organisms were chosen for further analysis. Testing of the antibacterial effects of the salicylanilide system was also conducted in two (Gram-negative) pathogenic strains of the *Enterobacteriaceae* family, namely *Salmonella enterica* (serovar Typhimurium) and *Klebsiella pneumoniae*. Both *S. enterica* (serovar Typhimurium) and *K. pneumoniae* along with *E. coli* are colonizing opportunistic pathogens commonly associated with nosocomial infections.⁵³⁻⁵⁵ Treatment with **1g** and **1j** (50 μ M) exhibited good inhibition of bacterial growth, similar to that in *E. coli*, in both pathogens (Figure 14), validating the effectiveness of the salicylanilide system as an antibacterial agent and attestation of *E. coli* as a suitable model organism for these studies. It was exciting to note that the transporters **1g** and **1j** exhibited significant (though lowered) antibacterial activity towards *K. pneumoniae* (Figure 14) despite the presence of a protective polysaccharide capsule that restricts accessibility to the bacterial cell.⁵⁰

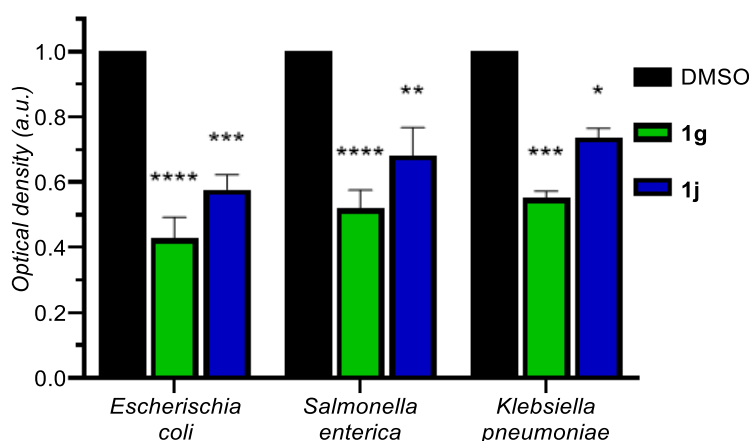


Figure 14: Growth inhibition in Enterobacteriaceae strains (*E. coli*, *S. enterica* and *K. pneumoniae*) on treatment with transporters **1g** and **1j** at 50 μ M for 6 h. Data represented as optical density relative to DMSO treated control culture. **** represents $p < 0.0001$, *** represents $p < 0.001$, ** represents $p < 0.01$ and * represents $p < 0.05$ when compared to untreated control.

3.2.5 Transporter Induced Membrane Depolarization

We had hypothesized the transporters to be capable of depolarizing the bacterial outer membrane, making the cells more susceptible to external changes. To test this, *E. coli* cultures treated with compounds **1g** and **1j** at 100 μ M over 6 h in LB media were pelletized and washed sequentially with decreasing concentrations of PBS (1X PBS $\rightarrow$ 0.1X PBS $\rightarrow$ water), subjecting the cells to osmotic shock, following which cell surface imaging was conducted in a scanning electron

microscope (SEM).⁵⁶ Neat DMSO was used as the negative control. A good percentage of cells from salicylanilide treated cultures were observed to have extensive damage, exhibiting membrane indentation, cytoplasmic shrinkage, and cell lysis (Figures 16-17). The cells in the DMSO treated control culture were mostly intact (Figure 15). The extent of damage was quantified by counting the percentage of damaged cells observed in the samples and around 50% percent of *E. coli* cells were found to be damaged in cultures treated with **1g** and **1j** while only <20% of cells exhibited damage due to osmotic stress in the control culture (Figure 18). The experiment was conducted in triplicate and the data is the average of the three independent experiments. This indicates outer membrane depolarization by the transporter molecules which in turn makes the bacterial cells highly susceptible to the changes in the osmolarity of the medium. To rule out the possibility of outer membrane disruption by the transporters, carboxyfluorescein (CF) leakage assay was performed across CF (50 mM) entrapped EYPC vesicles (EYPC-LUVs $\Rightarrow$ CF). Treatment with compounds **1g** and **1j** at 10 and 20 μ M concentrations yielded no considerable change in fluorescence intensity (Figure 19), indicating preservation of vesicle membrane integrity even at high levels of compound loading, confirming the mode of activity of the salicylanilides to be via ion transport induced membrane depolarization. Vesicle entrapped CF is in the fluorescence quenched state due to collisional quenching at high concentrations and damage to or disruption of the lipid membrane by membrane-disrupting agents would lead to leakage of the CF dye into the extravesicular buffer, thereby diluting it and leading to a significant increase in the dye fluorescence.

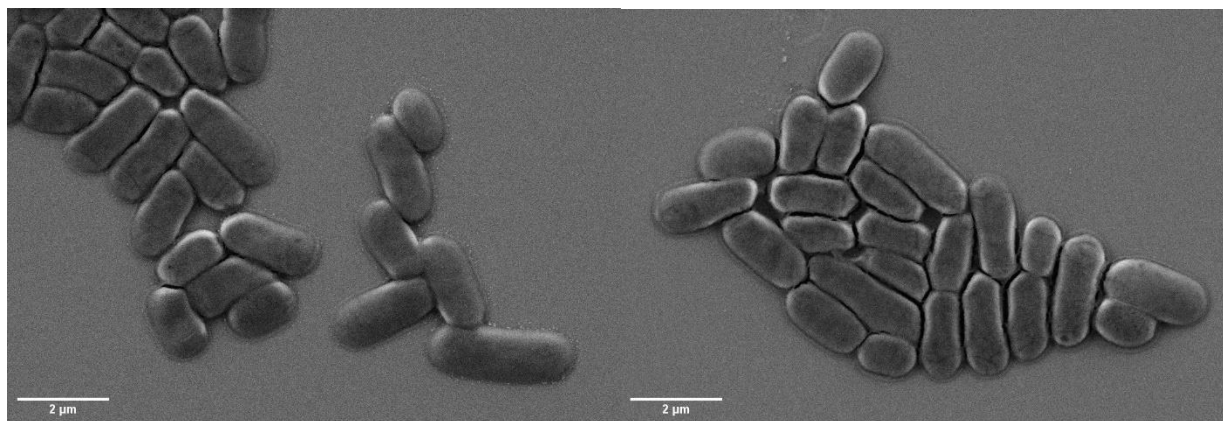


Figure 15: SEM surface imaging of *E. coli* cells treated with DMSO following osmotic shock. Negligible damage was observed. (Mag.- 16000X, Scale bar- 2 μ m, Imaging mode- SE2).

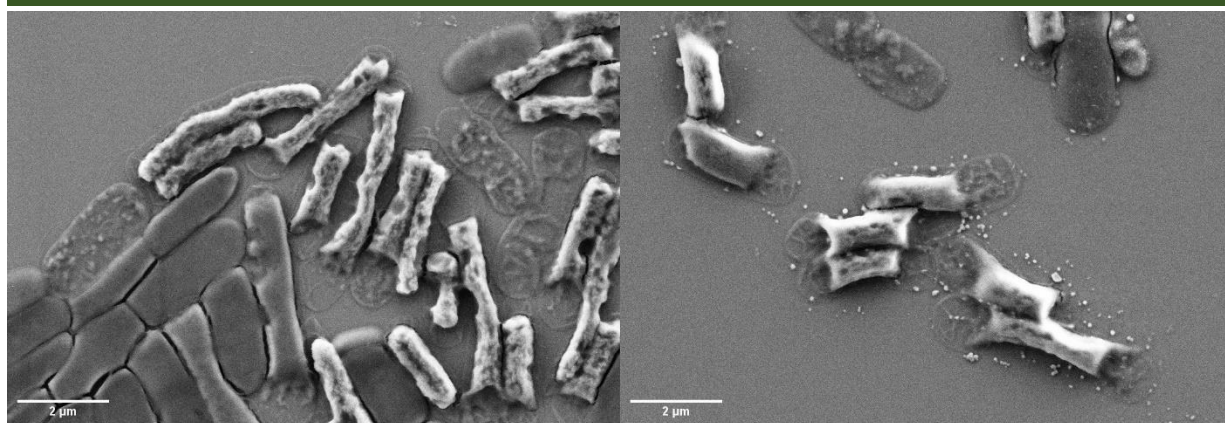


Figure 16: SEM surface imaging of *E. coli* cells treated with **1g** (100 μ M) following osmotic shock. Extensive damage was observed. (Mag.- 16000X, Scale bar- 2 μ m, Imaging mode- SE2).

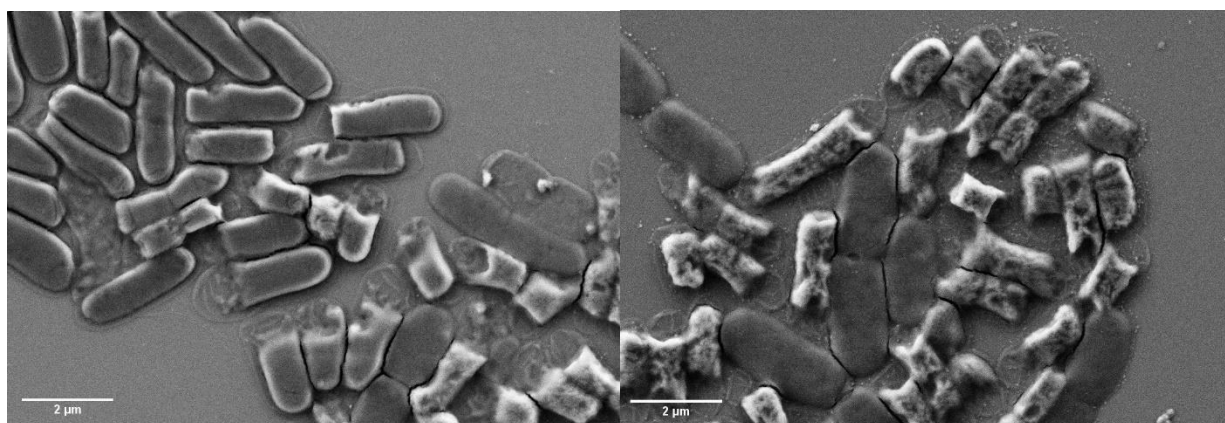


Figure 17: SEM surface imaging of *E. coli* cells treated with **1j** (100 μ M) following osmotic shock. Extensive damage was observed. (Mag.- 16000X, Scale bar- 2 μ m, Imaging mode- SE2).

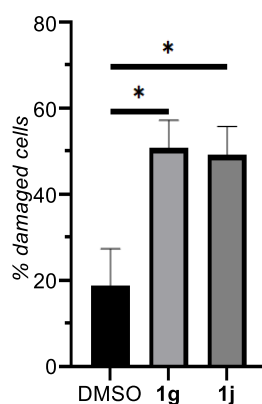


Figure 18: Quantification of damaged cells observed in SEM imaging as a percentage of the total cell count for each treatment. * represents $p < 0.05$ when compared to DMSO treated control using an unpaired t test.

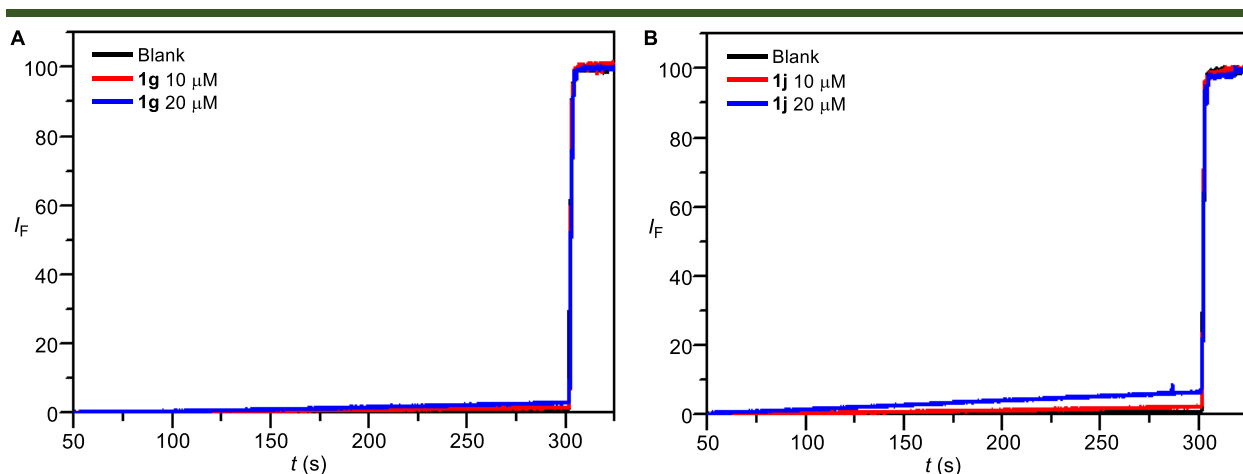


Figure 19: Activities of **1g** (A) and **1j** (B) across EYPC-LUVs $\supset$ CF. No increase in fluorescence signal was observed even for compound treatment at 20 μ M, corresponding to negligible CF leakage, indicating that the transporters do not compromise membrane integrity even at high loading.

3.2.6 Protransporter Photoactivation Studies

The ion transport activities of the selected compounds **1g** and **1j** were caged by blocking of the salicylamide OH group using photocleavable pyrrolidinobenzoquinone (PBQ) group^{34, 35} to yield protransporters **2** and **3** (Scheme 1). Crystallization of **2** (in ACN/water) and solution of its structure through SCXRD yielded an ‘open-ring’⁵² salicylanilide conformation with the ion binding site effectively blocked by C=O $\cdots$ H-N hydrogen-bonding with the quinone moiety (Figure 33D). Activation of the PBQ group under red light (655 nm) was first verified by monitoring the change in absorbance spectra of the protransporter (**2**, **3**) solution (50 μ M) in ACN/water (7:3) at different irradiation time points (Figures 20-21). Complete disappearance of the characteristic quinone peak at $\sim$ 550 nm over 10 min of photoirradiation was observed along with the appearance of a shoulder peak in the 294-380 nm region., which was consistent with literature reports indicating photolysis of the PBQ group.^{34, 35, 57} Similar experiments were conducted with **2** and **3** (50 μ M) in PYE and LB growth media (Figures 23, 24, 26A-B). Complete disappearance of the characteristic peaks of the protransporters (**2**, **3**) at $\sim$ 550 nm was observed over 5 min of irradiation under 655 nm light (radiant flux- 0.159 W/cm²), indicating their successful photolysis in both media.

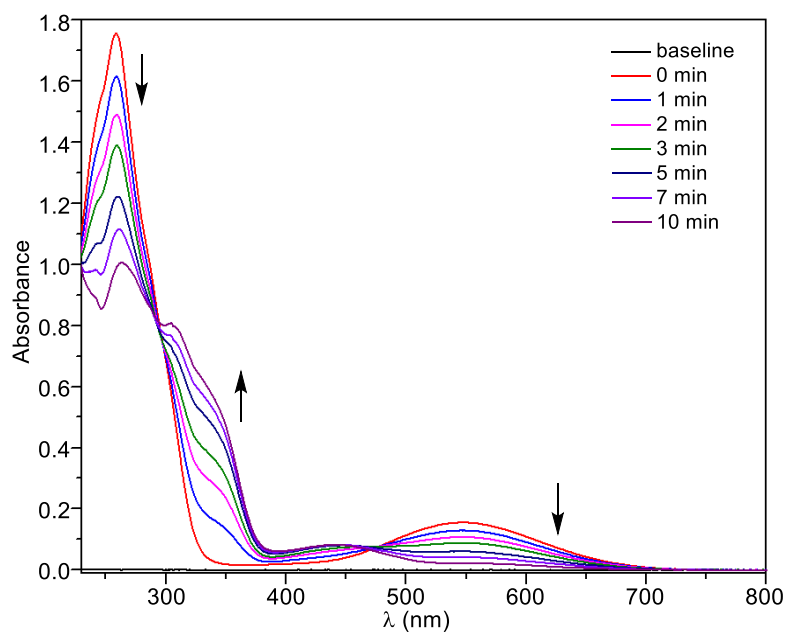


Figure 20: Change in the absorption spectrum of **2** on irradiation with 655 nm (red) light. Arrows indicate the direction of peak changes with increasing photoirradiation time. Solvent- 7:3 ACN/water.

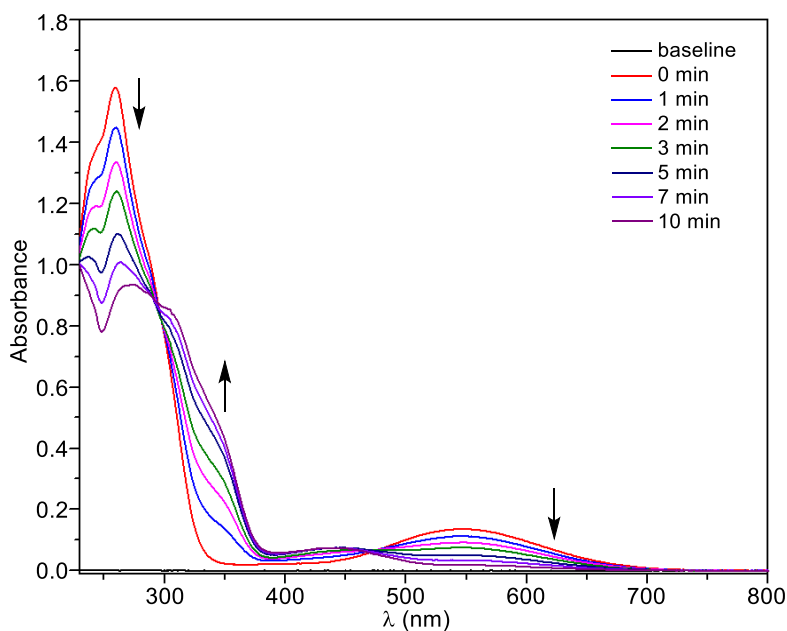


Figure 21: Change in the absorption spectrum of **3** on irradiation with 655 nm (red) light. Arrows indicate the direction of peak changes with increasing photoirradiation time. Solvent- 7:3 ACN/water.

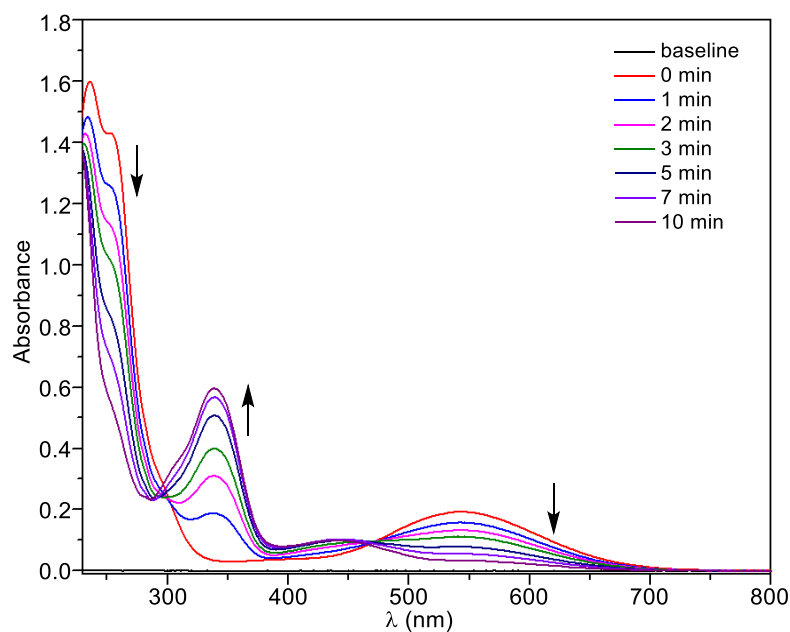


Figure 22: Change in the absorption spectrum of **4** on irradiation with 655 nm (red) light. Arrows indicate the direction of peak changes with increasing photoirradiation time. Solvent- 7:3 ACN/water.

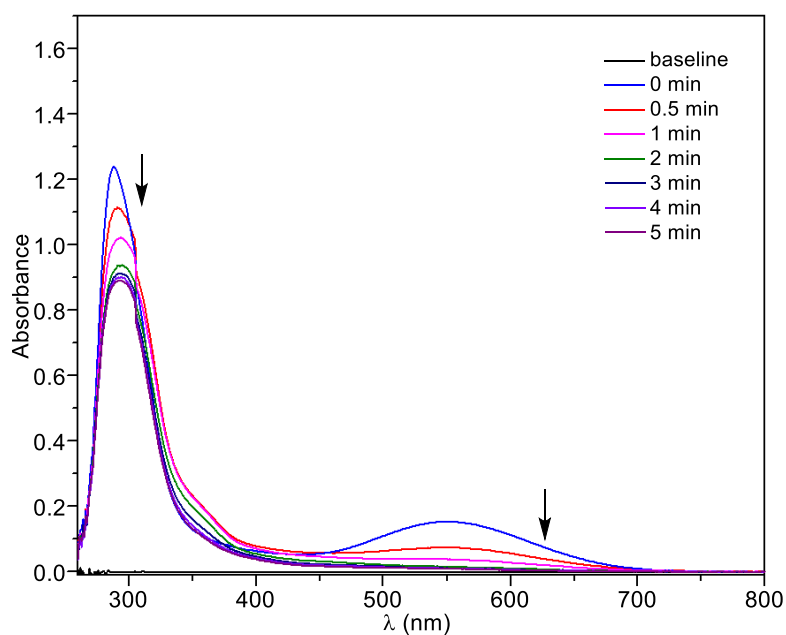


Figure 23: Change in the absorption spectrum of **2** in PYE on irradiation with 655 nm (red) light. Arrows indicate the direction of peak changes with increasing photoirradiation time.

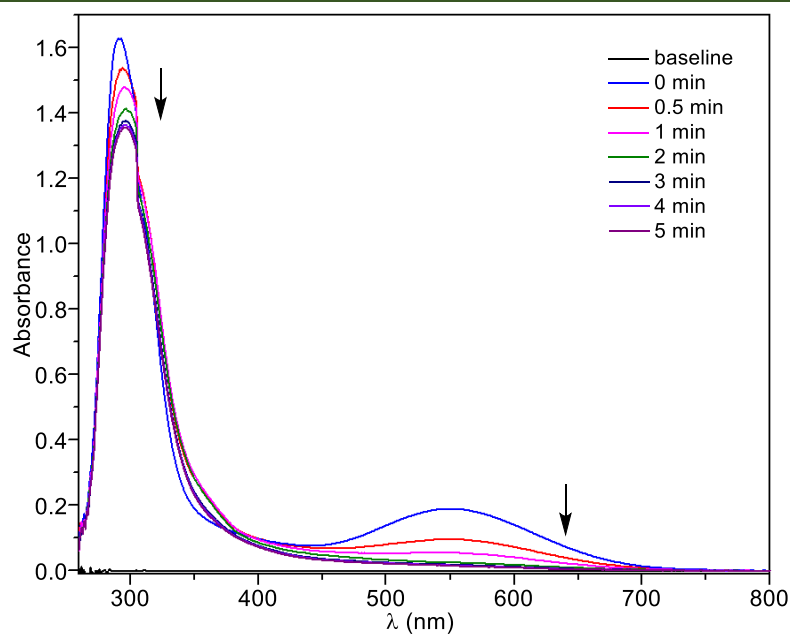


Figure 24: Change in the absorption spectrum of **3** in PYE on irradiation with 655 nm (red) light. Arrows indicate the direction of peak changes with increasing photoirradiation time.

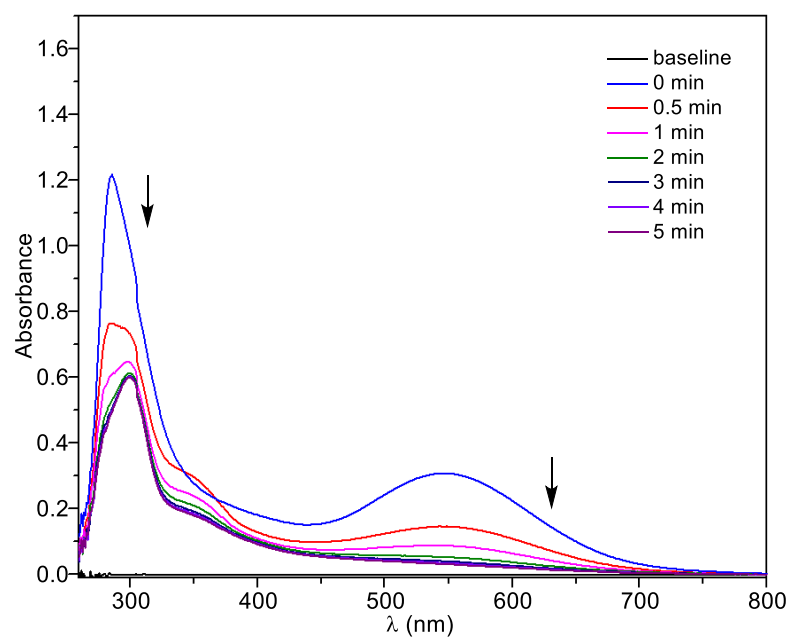


Figure 25: Change in the absorption spectrum of **4** in PYE on irradiation with 655 nm (red) light. Arrows indicate the direction of peak changes with increasing photoirradiation time.

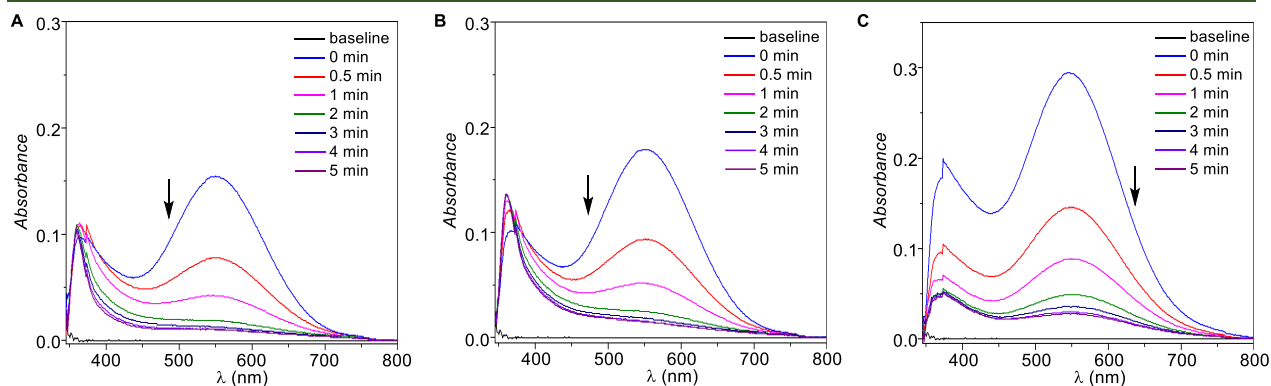


Figure 26: Change in the absorption spectrum of (A) **2**, (B) **3** and (C) **4** in LB on irradiation with 655 nm (red) light. Arrows indicate the direction of peak changes with increasing photoirradiation time.

Further verification of protransporter photolysis was obtained through NMR analysis of the solutions of **2** and **3** in CDCl_3 before and after photoactivation (Figures 28-29). The appearance of the salicylanilide OH peak (H_a) along with the disappearance of the PBQ CH_2 peak (H_c) following red light irradiation, confirmed the photoactivated release of the free transporter. A change in the color of the samples from violet to yellow on photoirradiation was also observed (Figure 27).⁵⁷ The same experiments were conducted for the control compound **4**, and the release of benzoic acid (**BA**) following photoirradiation was verified (Figures 22, 25, 26C, 30).

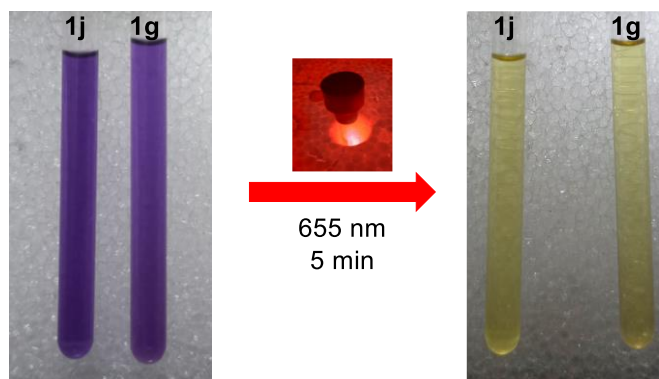


Figure 27: NMR samples of protransporters **1g** and **1j** before and after photoirradiation using 655 nm light (5 min). Color change observed from violet to yellow.

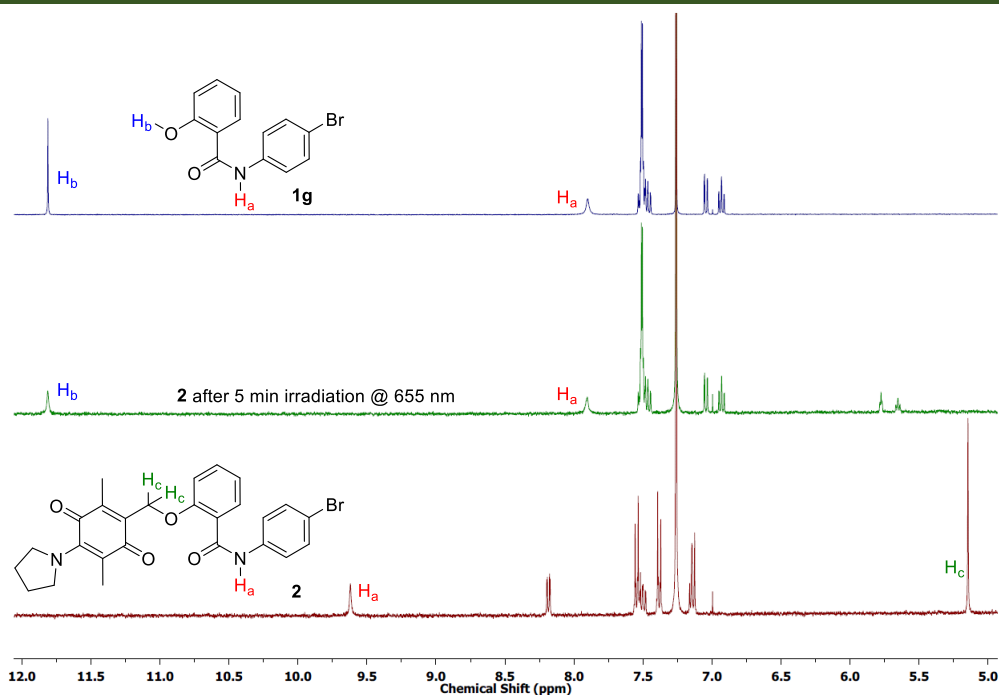


Figure 28: ^1H NMR spectrum of protransporter **2** before and after photoirradiation with 655 nm (red) light. Appearance of the salicylamide OH (H_b) peak and the disappearance of the CH_2 (H_c) peak of the salicylamide-quinone ether linkage are observed. Solvent- CDCl_3 .

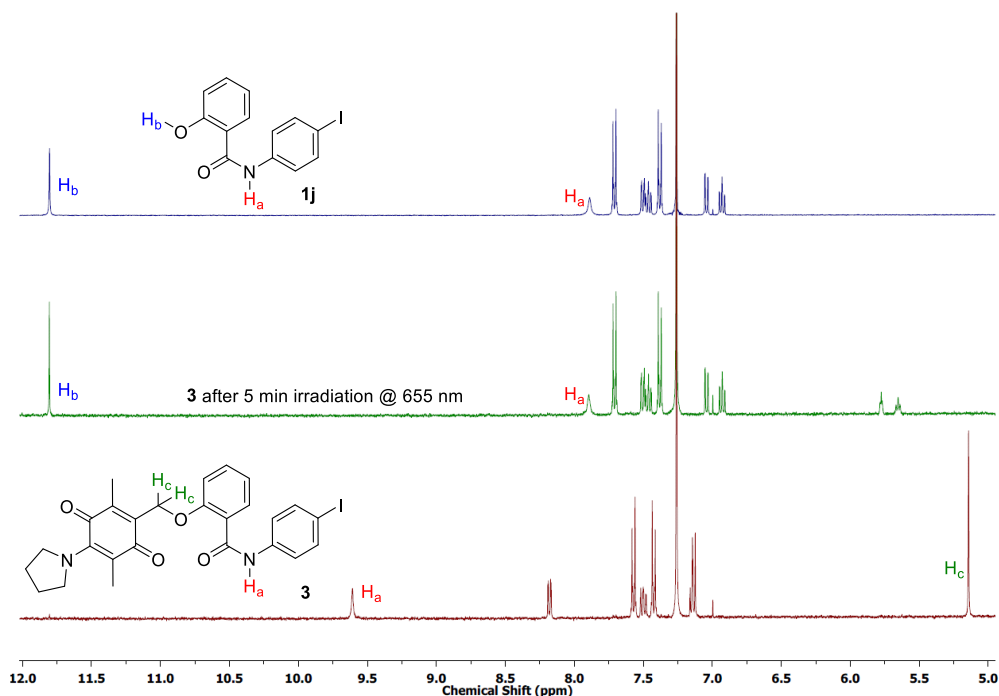


Figure 29: NMR spectrum of protransporter **3** before and after photoirradiation with 655 nm (red) light. The appearance of the salicylamide OH (H_b) peak and the disappearance of the CH_2 (H_c) peak of the salicylamide-quinone ether linkage are observed. Solvent- CDCl_3 .

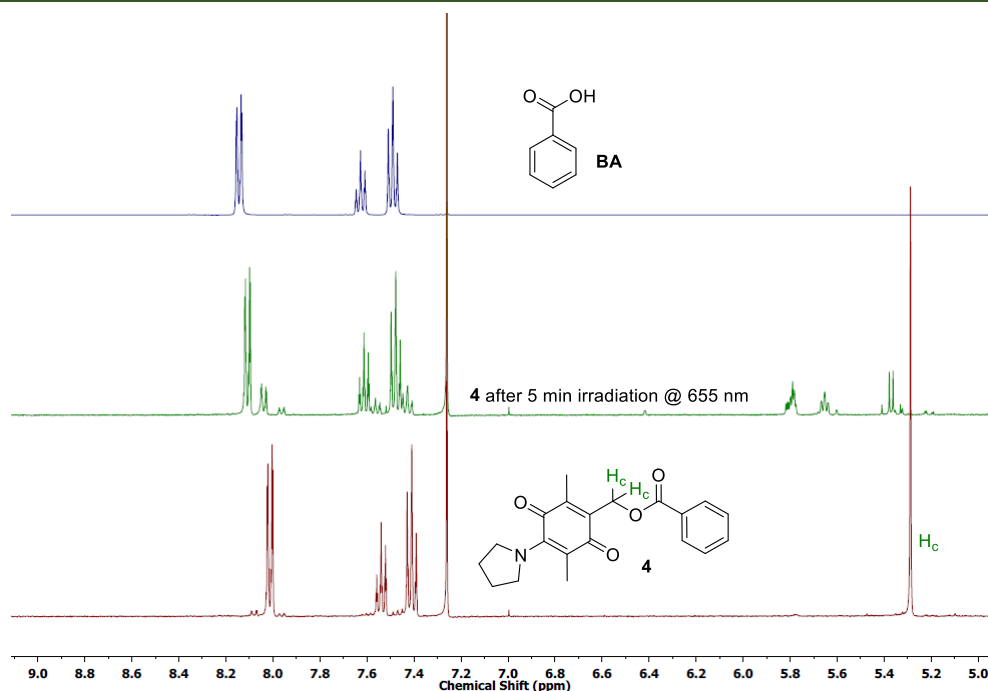


Figure 30: NMR spectrum of control compound **4** before and after photoirradiation with 655 nm (red) light. Disappearance of the CH₂ (H_c) peak of the salicylamide-quinone ether linkage is observed. Solvent- CDCl₃.

Ion transport activities of the transporters and protransporters were compared by monitoring transporter-mediated chloride leakage from EYPC-LUVs $\Rightarrow$ NaCl (inside: 500 mM NaCl, 10 mM Na-phosphate buffer, pH 7.0; outside: 500 mM NaNO₃, 10 mM Na-phosphate buffer, pH 7.0) using a chloride ion selective electrode (Cl⁻-ISE) (Figure 40). The active transporters (**1g**, **1j**) exhibited significant chloride efflux from the vesicle within 10 min of treatment (at 100 μ M) while protransporters (**2**, **3**) were observed to be inactive at the same concentration (Figure 31), indicating effective caging of transmembrane ion transport by the PBQ group. DMSO was used as the negative control for ion transport. The subdued activity of **1j** relative to **1g** was observed due to the partial precipitation of **1j** on addition to the vesicle solution. Recovery of ion transport activity following protransporter photolysis was verified by irradiating protransporter (**2**, **3**) (100 μ M) treated vesicles under 655 nm (red) light for 5 min and the extravascular chloride concentration was measured following a further 5 min in the dark using Cl⁻-ISE (Figures 40). Recovery of chloride efflux levels to that of the free transporter was observed following photolysis (Figure 31). To test the effect of the red light on the vesicles, measurement of extravascular chloride levels following 10 min irradiation of DMSO treated vesicles was conducted. Negligible change in

extravesicular chloride concentration was observed, indicating vesicle integrity was not affected during the photoirradiation process. Photoactivation of the control compound **4** was conducted across EYPC-LUVs $\supset$ NaCl to assess the effect on vesicles of the quinone methide byproduct formed during protransporter photolysis (Figure 31). Photolysis of **4** yields the quinone methide along with non-transporting **BA** and any possible contribution to membrane transport or membrane disruption by the quinone methide would be evident from increase the in extravesicular chloride ion detectable by the ISE. This control molecule was found to elicit no response in the vesicle both before and after photoirradiation, indicating that the photolysis byproduct (**QM**) neither damages the lipid membrane nor contributes to the ion transport process.

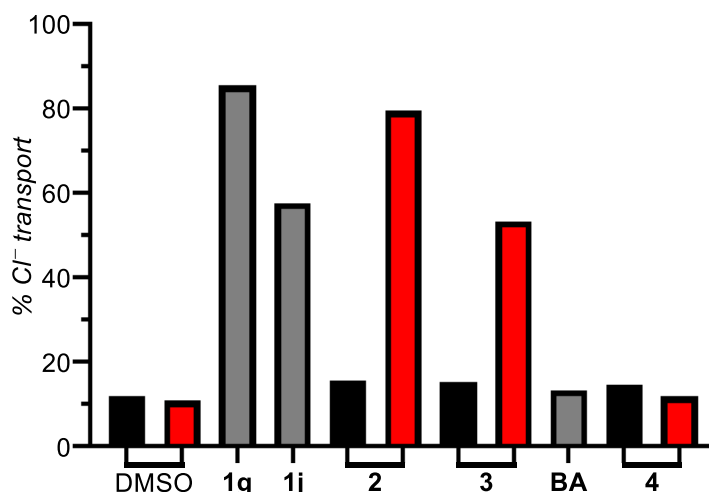


Figure 31: Photoactivated ion transport assay across EYPC-LUVs $\supset$ NaCl showing recovery in ion transport ability following photoactivation of protransporters **2** and **3**. **Red** bars represent compound (**DMSO**, **2**, **3**, **4**) treated vesicles irradiated under 655 nm light, while **black** bars represent the same treatments without photoactivation. **Grey** bars represent the free transporters (**1g**, **1j**)/control (**BA**) used for comparison.

3.2.7 Photoactivated Antibacterial Activity

Assessment of antibacterial properties of the protransporters on red light photoactivation was conducted in *E. coli*. Growth inhibition was observed in cultures treated with protransporters **2** and **3** following irradiation at 655 nm for 5 min which matched the activity levels observed during treatment with the active transporter **1g** and **1j** (Figure 32A). Protransporter treated cultures without photoactivation were found to exhibit similar growth as the DMSO treated control. The effect of the photolysis byproduct (**QM**) was assessed by treatment with **4** with and without

photoactivation. There was no inhibition in bacterial growth in either case or in cultures treated with **BA**, indicating the **QM** has no effect on bacterial growth (Figure 32A). Photoactivation studies for **2** and **3** were also conducted in *C. crescentus* and yielded similar results (Figure 32B), indicating the effectiveness of pyrrolidinobenzoquinone caged salicylanilide transporters as photoactivable antimicrobial agents.

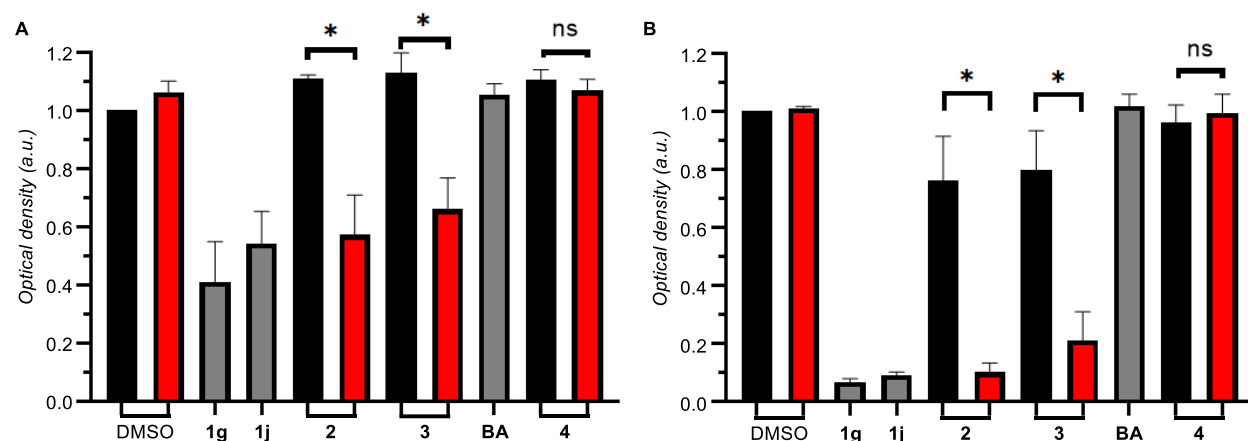


Figure 32: Effect of protransporter photoactivation in (A) *E. coli* and (B) *C. crescentus* using 50 μ M of compounds. **Red** bars represent compound (DMSO, **2**, **3**, **4**) treated cultures irradiated under 655 nm light for 5 min, while **black** bars represent the same treatments without photoactivation. **Grey** bars represent the free transporters (**1g**, **1j**)/control (**BA**) used for comparison. Data represented as percentage cell density relative to DMSO treated control culture. * represents $p < 0.05$ and ns represents ‘not significant’ when comparing effects of photoactivable compounds with and without photoirradiation.

3.3 Conclusions

Antimicrobial properties of salicylanilide transmembrane H^+/Cl^- transporters were verified in Gram-negative bacterial strains, including *Escherichia coli* and the opportunistic pathogens *Klebsiella pneumoniae* and *Salmonella enterica* (serovar Typhimurium). An initial screen against *C. crescentus* indicated *para* halogen substitution (with respect to amide NH) to be most effective for antibacterial use and compound activities correlated closely with ion transport ability. Blocking of salicylanilide ion transport via OH group methylation resulting in inhibition of antimicrobial activity demonstrated ion transport ability as a necessary criterion for bacterial growth inhibition. Extensive cell damage was observed in transporter-treated *E. coli* cells subject to osmotic shock, indicating H^+/Cl^- symport-induced outer membrane depolarization. This outer membrane

sensitization ability of salicylanilides can be applied in combination with existing antibiotics, achieving higher treatment efficiencies at lower drug concentrations. This work reports for the first time a photoactivable ion transporter with antimicrobial properties. Coupling of salicylanilide to the pyrrolidinobenzoquinone moiety yielded a protransporter useful as a red light activable antimicrobial agent. Successful caging of ion transport activity and its photoactivated recovery in 655 nm (red) light was demonstrated in liposomes. Red light-activated inhibition of bacterial growth in protransporter-treated *E. coli* and *C. crescentus* cultures was successfully demonstrated. Hence, this system can be potentially used for topical applications where it can be activated/released in a controlled manner. Further, due to their OM sensitizing ability, these molecules can potentially be used in conjunction with antimicrobial agents, which are by themselves ineffective against Gram negative bacteria due to their inability to permeate the OM and mucoid capsules. Such potential combination therapies can further expand the dwindling pool of useful antimicrobial drugs.

3.4 Experimental Section

3.4.1 General Methods

Reagents and solvents for synthesis were purchased from commercial sources (Sigma-Aldrich, TCI, Spectrochem, Rankem, Avra, SRL) and used without further purification. Purifications via column chromatography were performed on Spectrochem silica gel (100-200 mesh) (or 230-400 mesh silica gel where specified). Thin layer chromatography (TLCs) was performed on Merck silica gel 60-F254 plates. Egg yolk phosphatidylcholine (EYPC) as a chloroform solution (25 mg/mL), polycarbonate membranes of 100 nm and 200 nm pore size, and mini extruder set for vesicle preparation were purchased from Avanti Polar Lipids (Merck). HPTS, HEPES, Triton™ X-100, NaOH and inorganic salts were of molecular biology grade purchased from Sigma-Aldrich. Dialysis was performed using Spectra/Por 6 dialysis membrane with 1 kD cutoff purchased from Spectrumlabs. All stock solutions were prepared in HPLC grade DMSO. All buffer solutions were prepared in Milli-Q water. High power LEDs of 655 nm (Single color- Hyper red) were procured from Mouser Electronics India and their associated components were sourced locally.

3.4.2 Physical Measurements

All NMR spectra were recorded on Bruker and JEOL 400 MHz NMR spectrometers. ¹H NMR spectra were recorded at 400 MHz and ¹³C spectra at 101 MHz. All spectra were calibrated with

respect to the residual solvent peak ($\delta_{\text{H}} = 7.26$ ppm and $\delta_{\text{C}} = 77.16$ ppm for CDCl_3 , $\delta_{\text{H}} = 2.50$ ppm and $\delta_{\text{C}} = 39.52$ ppm for $\text{DMSO}-d_6$, $\delta_{\text{H}} = 1.94$ ppm for CD_3CN). ^{19}F NMR spectra were recorded at 377 MHz with a solution of TFA in CDCl_3 sealed in a capillary tube as internal reference, $\delta_{\text{F}} = -76.55$ ppm. The chemical shifts (δ) were reported in ppm and the coupling constants (J) in Hz. The following abbreviations were used to indicate peak splitting patterns: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), td (triplet of doublet), and dt (doublet of triplet). Infra-red (IR) spectra were obtained using a Bruker ALPHA-E FT-IR spectrometer and reported in ν (cm^{-1}). High resolution mass spectra (HRMS) were recorded on a Waters SYNAPT G2 mass spectrometer equipped with a Waters Z-SprayTM electrospray ionization (ESI) source. UV-vis absorption spectra were recorded on a Shimadzu UV-2600 UV-Vis. Single crystal X-ray diffraction (SCXRD) measurements were conducted using Bruker D8 VENTURE or Bruker KAPPA APEX diffractometers. Fluorescence studies were conducted on a HORIBA Jobin Yvon Fluoromax[®]+ spectrofluorometer with an injector port, and a Quantum Northwest, Inc. (QNW) Luma 40 Peltier-based temperature-controlled cuvette holder, with temperature control achieved using a QNW TC-1 temperature controller. All buffer solutions were adjusted to the necessary pH using a LAQUAtwin pH22 pocket pH meter from HORIBA. Chloride ion measurements were conducted using a Thermo Scientific OrionTM Chloride ionplusTM Sure-FlowTM Combination ISE, connected to a FisherbrandTM accumetTM AB250 Benchtop pH/ISE meter for recording ion concentrations (in ppm). Compounds for preparing stock solutions were weighed out using a Quintix[®] 125D Semi-Micro lab balance (0.01 mg least count) from Sartorius AG.

NMR spectra were processed using MestReNova software. All data from fluorescence studies were processed using OriginPro 8.5 software. ChemDraw 22.2.0 was used for drawing structures and processing figures. 3D structure images were generated using BIOVIA Discovery Studio 2021. Theoretical predictions of pK_{a} and $\log P$ values were calculated using calculator plugins of MarvinSketch program.

Absorbance and fluorescence measurements on 96-well microplate were recorded either on Thermo Scientific Varioskan[®] Flash or PerkinElmer EnSightTM multimode microplate readers. The data was processed and analyzed using code written on MATLAB R2020b (v9.9) by MathWorks, Inc. Optical density (OD) measurements of bacterial cultures in liquid media were

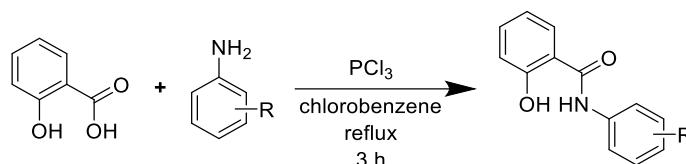
recorded at 600 nm on an Eppendorf BioPhotometer® in 1 mL plastic disposable cuvettes (BRAND®). Statistical analyses of data were performed using GraphPad Prism 8 software. Light intensity measurements were recorded using a Newport 842-PE model Optical Power/Energy Meter equipped with a photodetector.

Cell surface imaging was conducted using a ZEISS Ultra Plus Field Emission-Scanning Electron Microscope (FE-SEM). SEM samples were gold coated using a Q150T ES turbo-pumped sputter coater from Quorum Technologies.

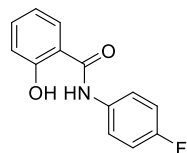
3.4.3 Synthetic Protocols

Synthesis of salicylamides:

Synthesis of **1a-1i**, **1m**, **1n** as per **General procedure A**.⁴³



N-(4-fluorophenyl)-2-hydroxybenzamide (**1a**):



Eluent for column chromatography: 12% EtOAc in petroleum ether; **Physical**

appearance: White solid; Yield = 40%; **¹H NMR (400 MHz, CDCl₃):** δ 11.90

(s, 1H), 7.90 (s, 1H), 7.57 – 7.49 (m, 3H), 7.46 (ddd, *J* = 8.6, 7.4, 1.5 Hz, 1H),

7.14 – 7.07 (m, 2H), 7.04 (dd, *J* = 8.4, 1.0 Hz, 1H), 6.95 – 6.90 (m, 1H). **¹³C NMR (101 MHz,**

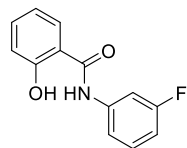
CDCl₃): δ 168.5, 162.0, 160.2 (d, *J* = 245.1 Hz), 135.0, 132.6 (d, *J* = 2.7 Hz), 125.5, 123.4 (d, *J* =

7.9 Hz), 119.2, 116.1 (d, *J* = 22.8 Hz), 114.5. **¹⁹F NMR (377 MHz, CDCl₃):** δ –117.17. **IR (ν̄ cm⁻¹)**:

3729, 2967, 1615, 1556, 1507, 1451, 1375, 1227, 1206, 830, 749, 681; **HRMS (ESI):** *m/z*

[*M*+*H*]⁺ Calc. for C₁₃H₁₀FNO₂: 232.0774; Found: 232.0769.

N-(3-fluorophenyl)-2-hydroxybenzamide (**1b**):



Eluent for column chromatography: 12% EtOAc in petroleum ether; **Physical**

appearance: White solid; Yield = 48%; **¹H NMR (400 MHz, CDCl₃):** δ 11.80

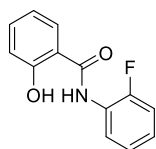
(s, 1H), 7.98 (s, 1H), 7.55 (dt, *J* = 10.7, 2.2 Hz, 1H), 7.51 (d, *J* = 8.0 Hz, 1H), 7.49

– 7.42 (m, 1H), 7.34 (td, *J* = 8.2, 6.5 Hz, 1H), 7.24 (d, *J* = 8.1 Hz, 1H), 7.04 (d, *J* = 8.2 Hz, 1H),

6.97 – 6.86 (m, 2H).. **¹³C NMR (101 MHz, CDCl₃):** δ 168.5, 163.1 (d, *J* = 245.5 Hz), 162.0, 138.3

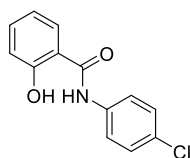
(d, $J = 10.9$ Hz), 135.1, 130.4 (d, $J = 9.2$ Hz), 125.54, 119.2, 119.2, 116.3 (d, $J = 2.3$ Hz), 114.5, 112.2 (d, $J = 21.4$ Hz), 108.7 (d, $J = 26.4$ Hz). **^{19}F NMR (377 MHz, CDCl_3):** δ -111.74. **IR ($\tilde{\nu} \text{ cm}^{-1}$):** 3704, 3058, 1610, 1551, 1456, 1230, 1148, 966, 864, 743, 682; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_{13}\text{H}_{10}\text{FNO}_2$: 232.0774; Found: 232.0771.

***N*-(2-fluorophenyl)-2-hydroxybenzamide (1c):**



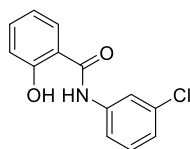
Eluent for column chromatography: 12% EtOAc in petroleum ether; **Physical appearance:** White solid; Yield = 86%; **^1H NMR (400 MHz, CDCl_3):** δ 11.83 (s, 1H), 8.36 – 8.29 (m, 1H), 8.19 (s, 1H), 7.55 (dd, $J = 8.3, 1.3$ Hz, 1H), 7.50 – 7.45 (m, 1H), 7.24 – 7.11 (m, 3H), 7.05 (dd, $J = 8.4, 1.2$ Hz, 1H), 6.98 – 6.92 (m, 1H). **^{13}C NMR (101 MHz, CDCl_3):** δ 168.4, 162.0, 153.2 (d, $J = 244.0$ Hz), 135.1, 125.7, 125.6, 125.5, 124.9 (d, $J = 3.6$ Hz), 122.6, 119.3, 119.5, 115.2 (d, $J = 19.2$ Hz), 114.6. **^{19}F NMR (377 MHz, CDCl_3):** δ -131.24. **IR ($\tilde{\nu} \text{ cm}^{-1}$):** 3748, 3063, 1636, 1603, 1454, 1333, 1234, 741, 681; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_{13}\text{H}_{10}\text{FNO}_2$: 232.0774; Found: 232.0769.

***N*-(4-chlorophenyl)-2-hydroxybenzamide (1d):**

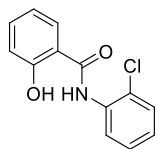


Eluent for column chromatography: 8% EtOAc in petroleum ether; **Physical appearance:** White solid; Yield = 79%; **^1H NMR (400 MHz, CDCl_3):** δ 11.83 (s, 1H), 7.92 (s, 1H), 7.57 – 7.53 (m, 2H), 7.51 (dd, $J = 8.0, 1.4$ Hz, 1H), 7.49 – 7.44 (m, 1H), 7.41 – 7.32 (m, 2H), 7.04 (dd, $J = 8.4, 1.0$ Hz, 1H), 6.97 – 6.89 (m, 1H). **^{13}C NMR (101 MHz, CDCl_3):** δ 168.5, 162.0, 135.4, 135.0, 130.6, 129.4, 125.5, 122.5, 119.2, 119.2, 114.5. **IR ($\tilde{\nu} \text{ cm}^{-1}$):** 3738, 3609, 2687, 2557, 2308, 1607, 1541, 1460, 1379, 1322, 1226, 1083, 816, 742, 679, 553; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_{13}\text{H}_{10}\text{ClNO}_2$: 248.0478; Found: 248.0480.

***N*-(3-chlorophenyl)-2-hydroxybenzamide (1e):**



Eluent for column chromatography: 8% EtOAc in petroleum ether; **Physical appearance:** White solid; Yield = 48%; **^1H NMR (400 MHz, CDCl_3):** δ 11.78 (s, 1H), 7.94 (s, 1H), 7.73 (t, $J = 2.0$ Hz, 1H), 7.54 – 7.40 (m, 3H), 7.32 (t, $J = 8.1$ Hz, 1H), 7.18 (ddd, $J = 8.0, 1.9, 1.0$ Hz, 1H), 7.05 (dd, $J = 8.4, 1.1$ Hz, 1H), 6.97 – 6.88 (m, 1H). **^{13}C NMR (101 MHz, CDCl_3):** δ 168.5, 162.0, 138.0, 135.1, 135.0, 130.3, 125.5, 121.3, 119.2, 119.1, 114.4. **IR ($\tilde{\nu} \text{ cm}^{-1}$):** 3840, 3733, 3614, 2638, 2545, 1600, 1547, 1461, 1326, 1224, 1090, 853, 685, 568; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_{13}\text{H}_{10}\text{ClNO}_2$: 248.0478; Found: 248.0475.

***N*-(2-chlorophenyl)-2-hydroxybenzamide (1f):**

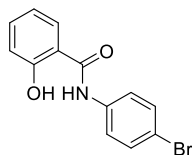
Eluent for column chromatography: 8%→10% EtOAc in petroleum ether;

Physical appearance: White solid; Yield = 68%; **¹H NMR (400 MHz, CDCl₃):** δ

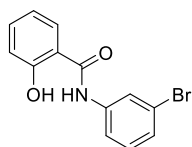
11.83 (s, 1H), 8.58 (s, 1H), 8.42 (dd, *J* = 8.3, 1.5 Hz, 1H), 7.58 (dd, *J* = 8.0, 1.5 Hz, 1H), 7.52 – 7.41 (m, 2H), 7.35 (td, *J* = 8.1, 1.4 Hz, 1H), 7.13 (ddd, *J* = 8.0, 7.5, 1.6 Hz, 1H), 7.05 (dd, *J* = 8.4, 1.1 Hz, 1H), 6.96 (ddd, *J* = 8.3, 7.3, 1.2 Hz, 1H). **¹³C NMR (101 MHz, CDCl₃):** δ

168.3, 162.1, 135.2, 133.8, 129.4, 128.0, 125.6, 123.9, 122.3, 119.4, 119.2, 114.7. **IR (ν̄ cm⁻¹):** 3839, 3736, 3609, 3040, 2706, 2560, 1616, 1533, 1445, 1374, 1313, 1228, 1135, 1039, 735, 659;

HRMS (ESI): *m/z* [M+H]⁺ Calc. for C₁₃H₁₀ClNO₂: 248.0478; Found: 248.0482.

***N*-(4-bromophenyl)-2-hydroxybenzamide (1g):**

Refer to Chapter 2.

***N*-(3-bromophenyl)-2-hydroxybenzamide (1h):**

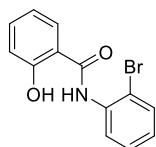
Eluent for column chromatography: 8% EtOAc in petroleum ether; **Physical**

appearance: White solid; Yield = 81%; **¹H NMR (400 MHz, DMSO-*d*₆):** δ

11.55 (s, 1H), 10.46 (s, 1H), 8.07 (s, 1H), 7.90 (d, *J* = 7.8 Hz, 1H), 7.70 – 7.63 (m, 1H), 7.47 – 7.40 (m, 1H), 7.36 – 7.29 (m, 2H), 7.02 – 6.93 (m, 2H). **¹³C NMR (101 MHz, DMSO-*d*₆):** δ

166.5, 158.0, 139.9, 133.7, 130.7, 129.2, 126.6, 123.0, 121.45, 119.5, 119.1, 117.9, 117.2. **IR (ν̄ cm⁻¹):** 3848, 3731, 3654, 2911, 2656, 2547, 1556, 1461, 1326, 1225, 1091, 851, 682;

HRMS (ESI): *m/z* [M+H]⁺ Calc. for C₁₃H₁₀BrNO₂: 291.9973; Found: 291.9973.

***N*-(2-bromophenyl)-2-hydroxybenzamide (1i):**

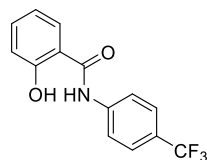
Eluent for column chromatography: 8% EtOAc in petroleum ether; **Physical**

appearance: White solid; Yield = 72%; **¹H NMR (400 MHz, CDCl₃):** δ

11.86 (s, 1H), 8.58 (s, 1H), 8.41 (dd, *J* = 8.3, 1.5 Hz, 1H), 7.60 (ddd, *J* = 8.0, 2.9, 1.5 Hz, 2H), 7.48 (ddd, *J* = 8.6, 7.3, 1.5 Hz, 1H), 7.42 – 7.36 (m, 1H), 7.10 – 7.03 (m, 2H), 6.96 (ddd, *J* = 8.3, 7.3, 1.2 Hz, 1H). **¹³C NMR (101 MHz, CDCl₃):** δ 168.3, 162.1, 135.1, 134.9, 132.6, 128.7, 126.1, 125.6, 122.5, 119.4, 119.2, 114.7, 114.6. **IR (ν̄ cm⁻¹):** 3844, 3726, 3075, 2704, 2558, 1624, 1590,

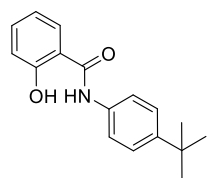
1532, 1442, 1368, 1313, 1229, 783, 652; **HRMS (ESI):** m/z $[M+H]^+$ Calc. for $C_{13}H_{10}BrNO_2$: 291.9973; Found: 291.9971.

2-Hydroxy-N-(4-(trifluoromethyl)phenyl)benzamide (1m):



Refer to Chapter 2.

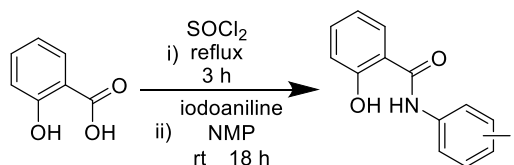
N-(4-(tert-butyl)phenyl)-2-hydroxybenzamide (1n):

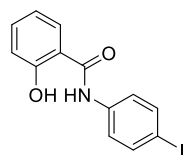


Refer to Chapter 2.

Formation of (*N*-aryl) salicylamides was confirmed from observation of phenolic O-H peak (s, 1H) in the 11 to 12 ppm region and the amide N-H peak (s, 1H) in the 7.8 to 11 ppm region in 1H NMR.

General procedure E: A modified literature method was used for synthesis of iodine substituted derivatives **1j-1l**.⁴⁴ A mixture of salicylic acid (1 eq.) and $SOCl_2$ (1 mL per 0.20 g salicylic acid) was refluxed for 3 h and then the excess $SOCl_2$ was removed under vacuum. The residue was then dissolved in dry 1-methyl-2-pyrrolidinone (NMP) (3 mL per 0.20 g salicylic acid) and a solution of the aniline (0.7 eq.) in NMP (2 mL per 0.20 g salicylic acid) was added dropwise. The mixture was then stirred at rt under a nitrogen atmosphere for 18 h. The reaction mixture was then poured into water and extracted with EtOAc (x2). The combined organic layers were washed with brine, dried over anhydrous Na_2SO_4 and solvent removed *in vacuo*. The purification of crude product was done using silica gel column chromatography.



2-Hydroxy-N-(4-iodophenyl)benzamide (1j):

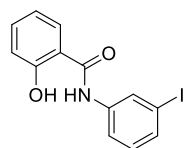
Eluent for column chromatography: 5%→7% EtOAc in petroleum ether;

Physical appearance: White solid; Yield = 71%; **¹H NMR (400 MHz, CDCl₃):**

δ 11.81 (s, 1H), 7.92 (s, 1H), 7.74 – 7.66 (m, 2H), 7.51 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.48 – 7.43 (m, 1H), 7.40 – 7.34 (m, 2H), 7.04 (dd, *J* = 8.4, 1.1 Hz, 1H), 6.96 – 6.87 (m, 1H).

¹³C NMR (101 MHz, CDCl₃): δ 168.5, 162.0, 138.3, 136.6, 135.1, 125.5, 123.0, 119.2, 114.5, 89.0. **IR (ν̄ cm⁻¹):** 3828, 3739, 3623, 2919, 2696, 2563, 1604, 1532, 1457, 1379, 1318, 1224, 807,

739, 663; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₃H₁₀INO₂: 339.9834; Found: 339.9836.

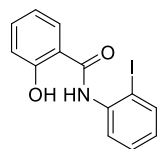
2-Hydroxy-N-(3-iodophenyl)benzamide (1k):

Eluent for column chromatography: 7% EtOAc in petroleum ether; **Physical**

appearance: White solid; Yield = 84%; **¹H NMR (400 MHz, CDCl₃):** δ 11.78

(s, 1H), 8.02 (t, *J* = 1.8 Hz, 1H), 7.89 (s, 1H), 7.59 – 7.44 (m, 4H), 7.12 (t, *J* = 8.0 Hz, 1H), 7.04 (d, *J* = 7.5 Hz, 1H), 6.96 – 6.89 (m, 1H). **¹³C NMR (101 MHz, DMSO-*d*₆):** δ 166.56, 158.16, 139.70, 133.72, 132.60, 130.76, 129.15, 128.87, 120.01, 119.10, 117.78, 117.19, 94.49.

IR (ν̄ cm⁻¹): 3832, 3735, 3605, 3019, 1610, 1556, 1456, 1322, 1224, 853, 765, 680; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₃H₁₀INO₂: 339.9834; Found: 339.9841.

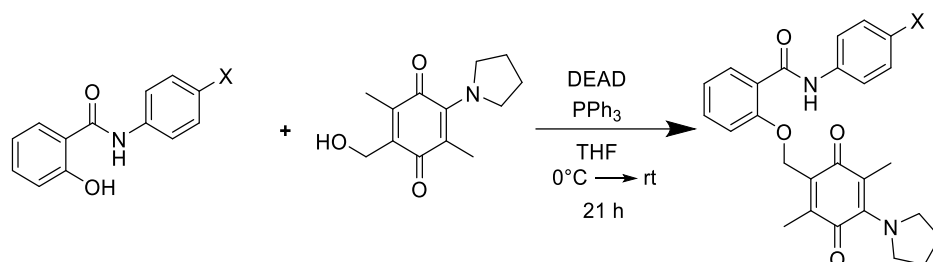
2-Hydroxy-N-(2-iodophenyl)benzamide (1l):

Eluent for column chromatography: 5%→7% EtOAc in petroleum ether;

Physical appearance: White solid; Yield = 29%; **¹H NMR (400 MHz, CDCl₃):** δ

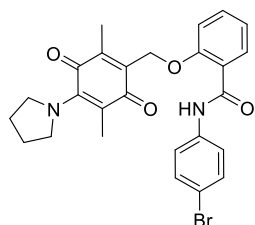
11.89 (s, 1H), 8.38 (s, 1H), 8.30 (dd, *J* = 8.2, 1.5 Hz, 1H), 7.85 (dd, *J* = 8.0, 1.4 Hz, 1H), 7.66 (dd, *J* = 8.0, 1.5 Hz, 1H), 7.48 (ddd, *J* = 8.6, 7.3, 1.5 Hz, 1H), 7.45 – 7.39 (m, 1H), 7.05 (dd, *J* = 8.4, 1.0 Hz, 1H), 7.00 – 6.88 (m, 2H). **¹³C NMR (101 MHz, CDCl₃):** δ 168.4, 162.2, 139.1, 137.4, 135.1, 129.5, 126.9, 125.8, 122.7, 119.4, 119.2, 114.6, 91.1. **IR (ν̄ cm⁻¹):** 3834, 3739, 3620, 3367, 2851, 1642, 1577, 1519, 1430, 1312, 1209, 1001, 745, 560; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₃H₁₀INO₂: 339.9834; Found: 339.9834.

Coupling of photocleavable group onto salicylamide:



Mitsunobu reaction as per **General procedure C**.⁴⁵

Synthesis of *N*-(4-bromophenyl)-2-((2,5-dimethyl-3,6-dioxo-4-(pyrrolidin-1-yl)cyclohexa-1,4-dien-1-yl)methoxy)benzamide (2):



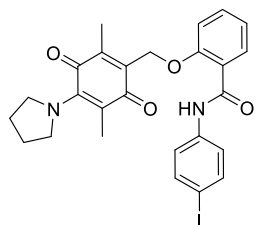
Eluent for column chromatography: 10% EtOAc in petroleum ether;

Product washed with hexane following purification. **Physical appearance:**

Violet solid; Yield = 31%; **¹H NMR (400 MHz, CDCl₃):** δ 9.62 (s, 1H), 8.18 (dd, J = 8.0, 1.6 Hz, 1H), 7.57 – 7.47 (m, 3H), 7.38 (d, J = 8.8 Hz, 2H), 7.18 – 7.09 (m, 2H), 5.14 (s, 2H), 3.66 (t, J = 6.2 Hz, 4H), 2.07 (s, 3H), 2.04

(s, 3H), 1.93 – 1.85 (m, 4H). **¹³C NMR (101 MHz, CDCl₃):** δ 187.1, 184.1, 163.5, 156.4, 150.8, 141.2, 137.9, 136.6, 133.4, 132.6, 131.7, 122.8, 122.2, 122.0, 116.4, 113.2, 111.6, 63.2, 53.4, 25.9, 13.2, 12.4. **IR ($\tilde{\nu}$ cm⁻¹):** 3847, 3738, 3612, 3371, 2924, 2378, 2306, 1738, 1663, 1591, 1530, 1449, 1380, 1322, 1277, 1230, 992, 826, 751, 691, 607; **HRMS (ESI):** m/z [M+H]⁺ Calc. for C₂₆H₂₅BrN₂O₄: 509.1076; Found: 509.1082.

Synthesis of 2-((2,5-dimethyl-3,6-dioxo-4-(pyrrolidin-1-yl)cyclohexa-1,4-dien-1-yl)methoxy)-*N*-(4-iodophenyl)benzamide (3)



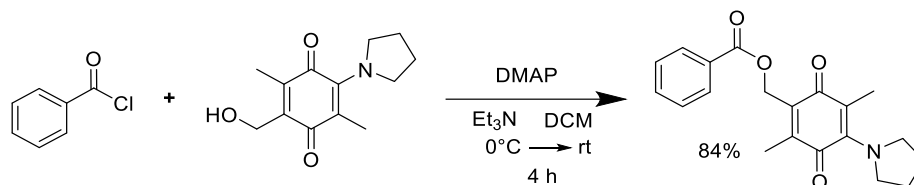
The crude product was purified through flash column chromatography on 230-400 mesh silica gel using 10%→20% EtOAc in petroleum ether as eluent; **Physical appearance:** Violet solid; Yield = 47%; **¹H NMR**

(400 MHz, CDCl₃): δ 9.61 (s, 1H), 8.17 (dd, J = 8.0, 1.8 Hz, 1H), 7.56 (d, J = 8.8 Hz, 2H), 7.52 – 7.45 (m, 1H), 7.42 (d, J = 8.8 Hz, 2H), 7.18 – 7.06

(m, 2H), 5.13 (s, 2H), 3.65 (t, J = 6.2 Hz, 4H), 2.06 (s, 3H), 2.04 (s, 3H), 1.93 – 1.85 (m, 4H). **¹³C NMR (101 MHz, CDCl₃):** δ 187.1, 184.1, 163.6, 156.4, 150.8, 141.2, 138.6, 137.7, 136.6, 133.4, 132.5, 122.7, 122.3, 122.2, 113.1, 111.5, 87.0, 63.2, 53.4, 25.9, 13.2, 12.4. **IR ($\tilde{\nu}$ cm⁻¹):**

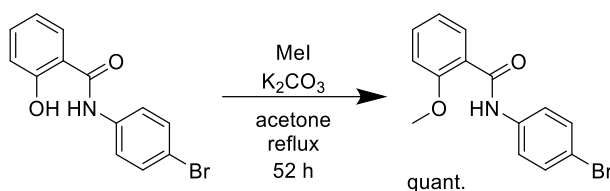
3860, 3740, 3336, 2966, 1661, 1589, 1525, 1484, 1450, 1389, 1319, 1278, 1244, 1218, 1163, 998, 823, 754; **HRMS (ESI):** m/z $[M+H]^+$ Calc. for $C_{26}H_{25}IN_2O_4$: 557.0937; Found: 557.0944.

Synthesis of (2,5-dimethyl-3,6-dioxo-4-(pyrrolidin-1-yl)cyclohexa-1,4-dien-1-yl)methyl benzoate (4):



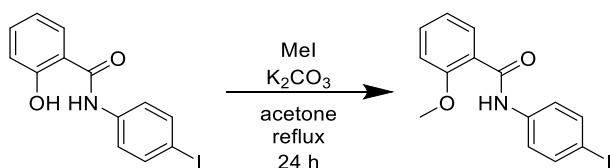
Refer to Chapter 2.

Synthesis of *N*-(4-bromophenyl)-2-methoxybenzamide (5)



To a stirred solution of the phenol (53 mg, 0.18 mmol) in acetone (5 mL) was added K_2CO_3 (28 mg, 0.20 mmol) followed by iodomethane (13 μ L, 0.20 mmol) and refluxed for 18 h. Then the mixture was cooled to rt and a further 0.20 mmol of K_2CO_3 and iodomethane were added and refluxed 36 h. The mixture was cooled to rt, quenched with sat. NH_4Cl solution and extracted with EtOAc (x2). removed under reduced pressure. The organic layers were combined, dried over anhydrous Na_2SO_4 and the solvent was removed to obtain the product as a viscous yellow oil in quantitative yield. **1H NMR (400 MHz, $CDCl_3$):** δ 9.83 (s, 1H), 8.28 (dd, J = 7.8, 1.8 Hz, 1H), 7.62 – 7.56 (m, 2H), 7.54 – 7.49 (m, 1H), 7.49 – 7.44 (m, 2H), 7.14 (t, J = 7.5 Hz, 1H), 7.04 (d, J = 8.3 Hz, 1H), 4.07 (s, 3H). **^{13}C NMR (101 MHz, $CDCl_3$):** δ 163.4, 157.3, 137.6, 133.6, 132.7, 132.1, 122.1, 121.9, 121.6, 116.7, 111.7, 56.4. **IR ($\tilde{\nu}$ cm^{-1}):** 3727, 3340, 2923, 1658, 1590, 1531, 1486, 1310, 1287, 1231, 1176, 1010, 831, 809, 651; **HRMS (ESI):** m/z $[M+H]^+$ Calc. for $C_{14}H_{12}BrNO_2$: 306.0129; Found: 306.0132.

Synthesis of *N*-(4-iodophenyl)-2-methoxybenzamide (6)



To a stirred solution of the phenol (150 mg, 0.44 mmol) in acetone (8 mL) was added K_2CO_3 (135 mg, 0.97 mmol) followed by iodomethane (61 μL , 0.97 mmol) and refluxed for 24 h. The mixture was cooled to rt, quenched with sat. NH_4Cl solution and extracted with EtOAc (x2). removed under reduced pressure. The organic layers were combined, dried over anhydrous Na_2SO_4 and the solvent was removed to obtain the product as a yellow-brown solid. Yield = 146 mg = 94%. **^1H NMR (400 MHz, CDCl_3):** δ 9.82 (s, 1H), 8.27 (dd, J = 7.8, 1.8 Hz, 1H), 7.69 – 7.62 (m, 2H), 7.54 – 7.44 (m, 3H), 7.17 – 7.11 (m, 1H), 7.04 (d, J = 8.3 Hz, 1H), 4.06 (s, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 163.4, 157.3, 138.3, 138.0, 133.6, 132.7, 122.4, 121.9, 121.6, 111.7, 87.3, 56.4. **IR ($\tilde{\nu}$ cm^{-1}):** 3726, 3550, 3332, 2316, 1660, 1588, 1531, 1482, 1316, 1230, 1012, 815, 782, 660; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_8\text{H}_8\text{O}_4$: 353.9991; Found: 353.9988.

3.4.4 Ion Transport Experiments

Study of ion transport across EYPC-LUVs $\supset$ HPTS:

Preparation of EYPC-LUVs $\supset$ HPTS in NaCl as per **Prep protocol P1**; HPTS assay as per **Assay protocol A1**.

Carboxyfluorescein (CF) leakage assay:

Preparation of EYPC-LUVs $\supset$ CF as per **Prep protocol P5**; Chloride transport assay as per **Assay protocol A12**.

3.4.5 ^1H NMR Titration Studies

Chloride binding affinities of the transporters were assessed through ^1H NMR titration of the transporter with chloride ion. ^1H NMR titration studies were conducted at ambient temperature on a Bruker Avance III HD 400 MHz spectrometer. The titrations with Cl^- were conducted by the addition of aliquots of tetrabutylammonium chloride (TBACl) solution (0.5 M in CD_3CN) to a solution of the receptor in CD_3CN (0.005 M). The spectra were calibrated using the residual signal from CD_3CN ($\delta_{\text{H}} = 1.94$) as internal reference. TBACl was dried under vacuum in a desiccator prior to use.

3.4.6 SCXRD

All compounds were crystallized from their solutions in HPLC grade solvents at ambient temperature. The crystals obtained were diffracted on Bruker diffractometers. For compounds **1h** and **2** - Bruker D8 VENTURE single crystal X-ray diffractometer with Mo ($\text{Mo-K}\alpha = 0.71073 \text{ \AA}$)

or Cu (Cu-K α = 1.54178 Å) microfocus X-ray (I μ S) source and PHOTON 100 CMOS detector. For compounds **1g** and **1i** - Bruker KAPPA APEX single crystal X-ray diffractometer with Mo (Mo-K α = 0.71073 Å) ceramic sealed tube X-ray source and APEX II CCD detector. The sample temperature was maintained constant throughout the data collection using Cryostream coolers (Oxford Cryosystems).

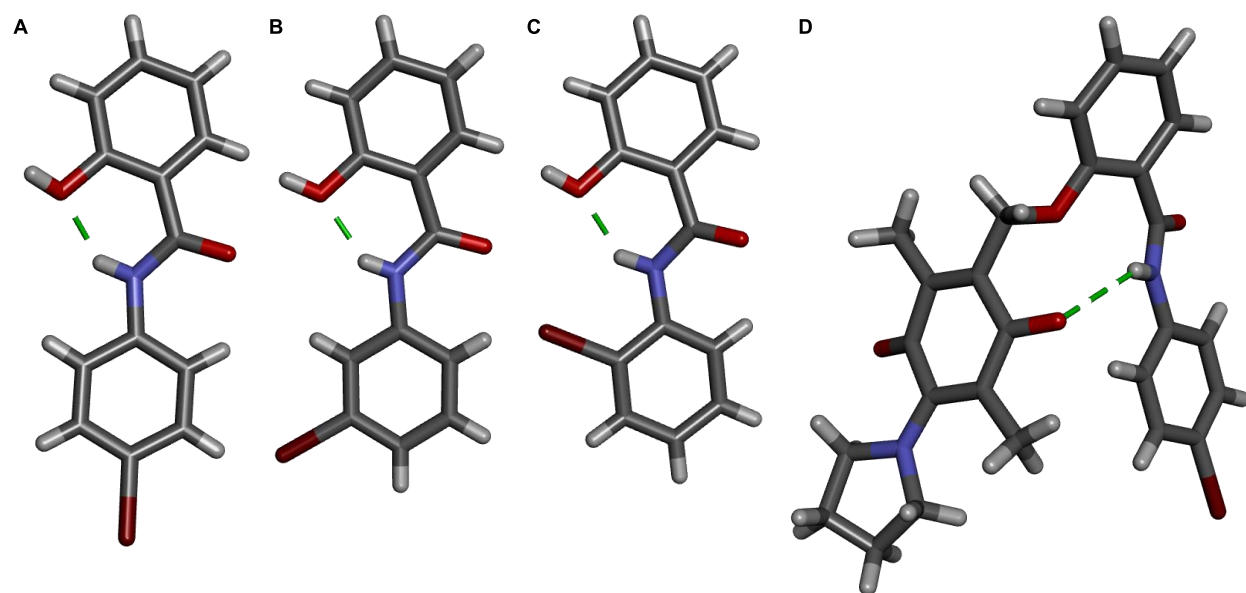


Figure 33: Molecular structures of (A) **1g**, (B) **1h**, (C) **1i**, and (D) **2** obtained from SCXRD data. H-bonding interactions are represented by dotted green lines.

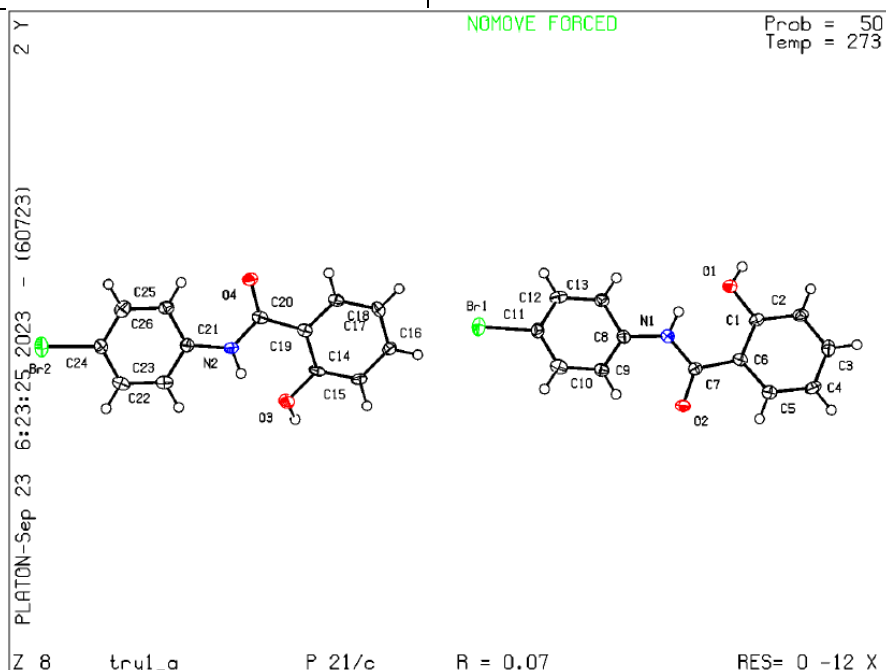
Single crystal data for **1g** (CCDC 2297002)

Compound **1g** was crystallized from the slow evaporation of its saturated CHCl₃ solution in a glass vial to yield crystals as colorless blocks. The data collection temperature was maintained at 273 K.

Table 4: Crystal data and structure refinement for **1g**

Empirical formula	C13 H10 Br N O2
Formula weight	292.13
Temperature	273(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P 21/c
Unit cell dimensions	a = 27.590(7) Å α = 90°. b = 11.709(3) Å β = 95.021(8)°.

	$c = 7.207(2) \text{ \AA}$ $\gamma = 90^\circ$.
Volume	$2319.3(11) \text{ \AA}^3$
Z	8
Density (calculated)	1.673 Mg/m^3
Absorption coefficient	3.532 mm^{-1}
F(000)	1168
Crystal size	$x \times x \text{ mm}^3$
Theta range for data collection	0.741 to 28.394° .
Index ranges	$-36 \leq h \leq 35$, $-15 \leq k \leq 14$, $-8 \leq l \leq 9$
Reflections collected	32132
Independent reflections	5782 [R(int) = 0.1409]
Completeness to $\theta = 28.394^\circ$	99.4 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.5462 and 0.7457
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	5782 / 0 / 310
Goodness-of-fit on F^2	1.033
Final R indices [$I > 2\sigma(I)$]	R1 = 0.0653, wR2 = 0.1378
R indices (all data)	R1 = 0.1415, wR2 = 0.1655
Extinction coefficient	0.0024(5)
Largest diff. peak and hole	1.002 and $-0.973 \text{ e.\AA}^{-3}$

Figure 34: ORTEP diagram of **1g**.

Single crystal data for 1h (CCDC 2297003)

Compound **1h** was crystallized from the slow evaporation of its saturated CHCl_3 solution in a glass vial to yield crystals as light brown blocks. The data collection temperature was maintained at 100 K.

Table 5: Crystal data and structure refinement for **1h**

Empirical formula	C13 H10 Br N O2
Formula weight	292.13
Temperature	100 K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P 21/c
Unit cell dimensions	$a = 13.644(3) \text{ Å}$ $\alpha = 90^\circ$, $b = 11.801(2) \text{ Å}$ $\beta = 99.496(7)^\circ$, $c = 7.0476(14) \text{ Å}$ $\gamma = 90^\circ$.
Volume	$1119.2(4) \text{ Å}^3$
Z	4
Density (calculated)	1.734 Mg/m^3
Absorption coefficient	3.660 mm^{-1}
F(000)	584
Crystal size	$0.319 \times 0.280 \times 0.268 \text{ mm}^3$
Theta range for data collection	2.295 to 28.383° .
Index ranges	$-18 \leq h \leq 18$, $-13 \leq k \leq 15$, $-9 \leq l \leq 9$
Reflections collected	32349
Independent reflections	2801 [$R(\text{int}) = 0.0663$]
Completeness to $\theta = 28.383^\circ$	99.8 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.5654 and 0.7457
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2801 / 0 / 162
Goodness-of-fit on F^2	1.093
Final R indices [$I > 2\sigma(I)$]	$R1 = 0.0459$, $wR2 = 0.1216$
R indices (all data)	$R1 = 0.0553$, $wR2 = 0.1276$
Extinction coefficient	n/a
Largest diff. peak and hole	1.766 and -0.825 e.Å^{-3}

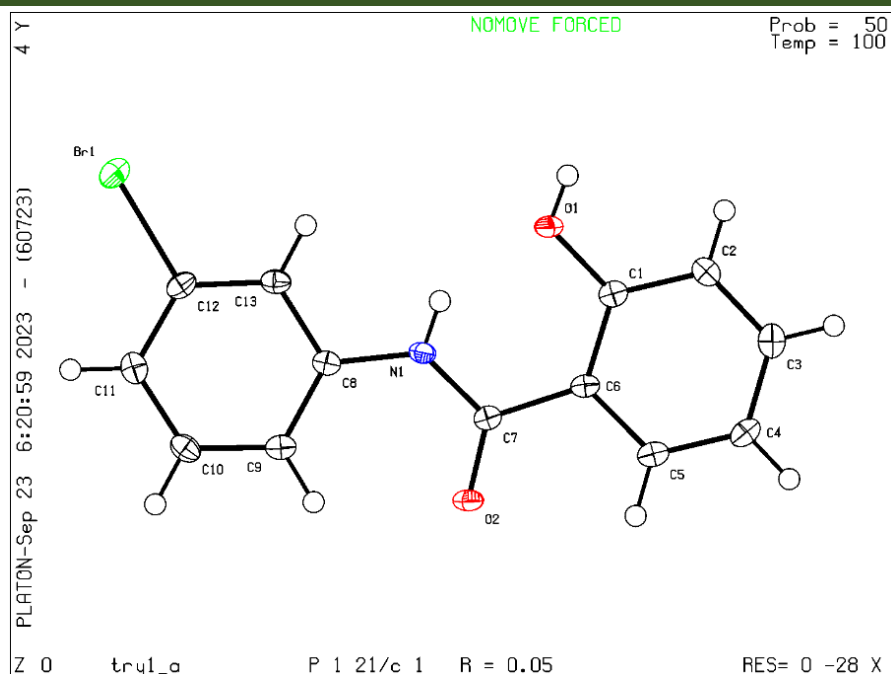


Figure 35: ORTEP diagram of **1h**.

Single crystal data for **1i** (CCDC 2297004)

Compound **1i** was crystallized from the slow evaporation of its saturated CHCl_3 solution in a glass vial to yield crystals as clear plates. The data collection temperature was maintained at 296 K.

Table 6: Crystal data and structure refinement for **1i**

Empirical formula	C ₅₂ H ₄₀ Br ₄ N ₄ O ₈
Formula weight	1168.52
Temperature	296(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	P c a 21
Unit cell dimensions	a = 24.01(2) Å α = 90°. b = 3.905(3) Å β = 90°. c = 12.155(11) Å γ = 90°.
Volume	1139.4(17) Å ³
Z	1
Density (calculated)	1.703 Mg/m ³
Absorption coefficient	3.595 mm ⁻¹
F(000)	584
Crystal size	0.159 x 0.156 x 0.086 mm ³

Theta range for data collection	1.696 to 33.270°.
Index ranges	-36≤h≤36, -5≤k≤6, -18≤l≤18
Reflections collected	29425
Independent reflections	4324 [R(int) = 0.1030]
Completeness to theta = 33.270°	99.6 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7465 and 0.5107
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	4324 / 1 / 156
Goodness-of-fit on F ²	0.981
Final R indices [I>2sigma(I)]	R1 = 0.0410, wR2 = 0.0735
R indices (all data)	R1 = 0.0959, wR2 = 0.0881
Extinction coefficient	0.0063(12)
Largest diff. peak and hole	0.333 and -0.379 e.Å ⁻³

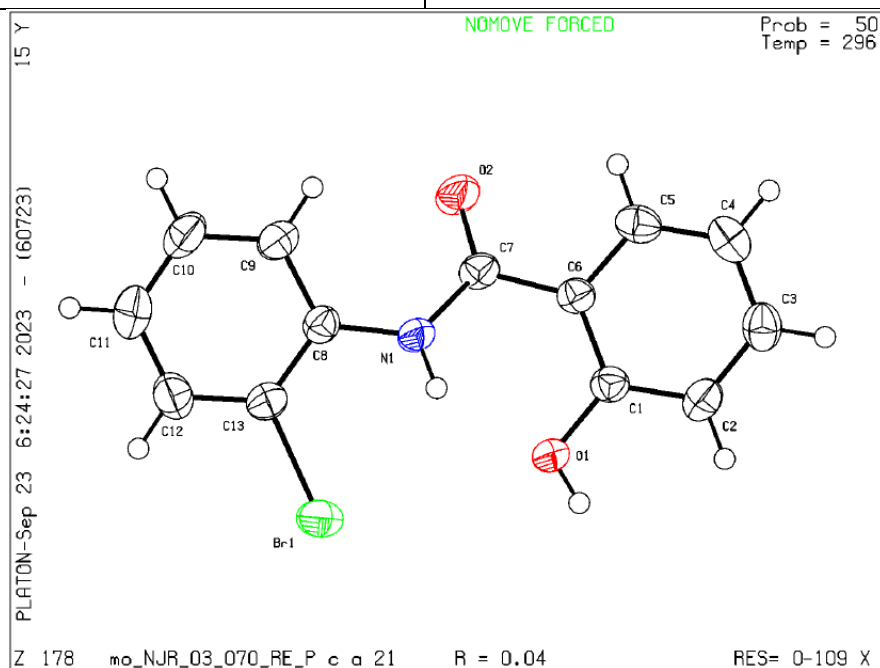


Figure 36: ORTEP diagram of **1i**.

Single crystal data for **2** (CCDC 2297005)

Compound **2** was crystallized by the slow evaporation of its solution in ACN/(MilliQ) water (7:3) mixture in a sealed crystallization tube to yield thick violet needle like crystals. The data collection temperature was maintained at 150 K.

Table 8: Crystal data and structure refinement for **2**

Empirical formula	C ₂₆ H ₂₅ Br N ₂ O ₄
Formula weight	509.39
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P2 ₁ /c
Unit cell dimensions	a = 12.069(3) Å α = 90°. b = 22.168(6) Å β = 112.49°. c = 9.234(3) Å γ = 90°.
Volume	2282.6(11) Å ³
Z	4
Density (calculated)	1.482 Mg/m ³
Absorption coefficient	1.837 mm ⁻¹
F(000)	1048
Crystal size	0.305 x 0.162 x 0.144 mm ³
Theta range for data collection	1.826 to 28.560°.
Index ranges	-15 ≤ h ≤ 15, -24 ≤ k ≤ 29, -12 ≤ l ≤ 12
Reflections collected	33484
Independent reflections	5738 [R(int) = 0.1270]
Completeness to theta = 25.349°	98.7 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.6453 and 0.7457
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	5738 / 0 / 301
Goodness-of-fit on F ²	1.032
Final R indices [I > 2σ(I)]	R ₁ = 0.0593, wR ₂ = 0.1241
R indices (all data)	R ₁ = 0.1446, wR ₂ = 0.1552
Extinction coefficient	0.0070(9)
Largest diff. peak and hole	0.963 and - 0.908 e.Å ⁻³

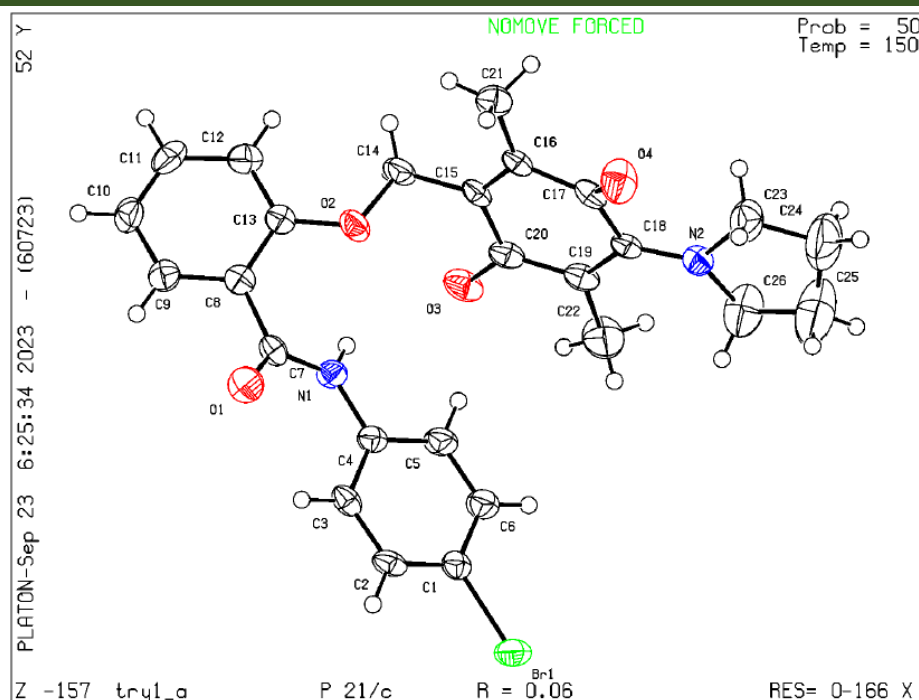


Figure 37: ORTEP diagram of **2**.

3.4.7 Photoactivation Setup

Photoactivation experiments of the protransporters in solution/media were conducted using red light of 655 nm wavelength supplied by three high power LEDs (Opulent Americas) directed to a point at their center where the sample was placed (in a cuvette/NMR tube/culture tube). A total radiant flux of 0.159 W/cm^2 was available to the sample (measured using an optical power meter). The setup was custom-built by N.J.R.

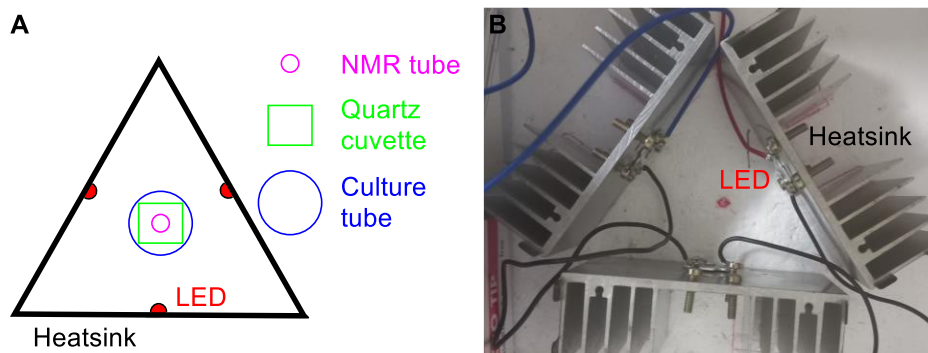


Figure 38: (A) Schematic of photoactivation (red, 655 nm) setup for protransporter photocleavage studies along with various sample (vessel) types. (B) Photoactivation setup showing the LEDs (integrated into PCB boards) and their heat sinks.

3.4.8 Photocleavage Studies

Photocleavage studies of the protransporters were conducted by placing a solution of the sample in a cuvette/NMR tube in the photoactivation setup and irradiating with red light (655 nm) for the specified duration of time. Total radiant flux- 0.159 W/cm^2

NMR analysis of photocleavage

Solutions of the protransporters **2** and **3**, and control **4** (0.5 mL, ~2 mg/mL) in CDCl_3 were prepared in borosilicate glass NMR tubes (Norell) and the ^1H NMR spectra (32 scans) recorded on a Bruker Avance III HD 400 MHz NMR spectrometer. The tubes were then irradiated with 655 nm light for 5 min in the photoactivation setup and the ^1H NMR spectra recorded again. ^1H NMR spectra of compounds **1g**, **1j**, and **BA** were recorded under similar conditions for comparison.

The color of the protransporter solutions was observed to change from violet to yellow following photoirradiation. ^1H NMR peaks of the protransporter samples were observed to match those of the corresponding free transporters after photoirradiation. The disappearance of the CH_2 peak of the ether linkage between the transporter and the quinone, along with the appearance of the OH peak of the free transporter confirmed photoactivated transporter release.

UV-Vis analysis of photocleavage

Solutions of the compounds (protransporters **2** and **3**, and control **4**) at $50 \mu\text{M}$ concentration were taken in 1 mL quartz cuvettes. The samples were irradiated with 655 nm light for specific time intervals and the absorption spectra recorded on a Shimadzu UV-2600 UV-Vis spectrophotometer.

Photoactivation in 7:3 ACN/water: The color of the protransporter solutions in ACN/water was observed to change from violet to yellow following photoirradiation. Disappearance of the characteristic quinone peak at ~550 nm with increasing photoirradiation was observed, indicating successful photoactivation, which is consistent with the reported literature.^{34, 35} Reference sample- 7:3 ACN/water.

Photoactivation in PYE media: For assessment of the ability of protransporters to undergo photoactivation in PYE media, $50 \mu\text{L}$ of 1 mM stock of compound in DMSO was added to 1 mL PYE in a quartz cuvette and mixed to yield a working concentration of $50 \mu\text{M}$, which was then irradiated using 655 nm light. Disappearance of the characteristic quinone peak at ~550 nm with

increasing photoirradiation was observed, indicating successful photoactivation. Reference sample- 50 μL DMSO in 1 mL PYE.

Photoactivation in LB media: For assessment of the ability of protransporters to undergo photoactivation in LB media, 50 μL of 1 mM stock of compound in DMSO was added to 1 mL LB in a quartz cuvette and mixed to yield a working concentration of 50 μM , which was then irradiated using 655 nm light. Disappearance of the characteristic quinone peak at ~ 550 nm with increasing photoirradiation was observed, indicating successful photoactivation. No spectrum was obtained below 350 nm due to the very strong absorbance of LB below that wavelength. Reference sample- 50 μL DMSO in 1 mL LB.

3.4.9 Photoactivated Ion Transport

Cl^- -Ion selective electrode (ISE) based studies in EYPC-LUVs $\Rightarrow \text{Cl}^-$:

Preparation of EYPC-LUVs $\Rightarrow$ HPTS in NaCl as per **Prep protocol P3**.

Phototriggered Cl^- efflux across vesicles: NaCl entrapped vesicles (50 μL) were suspended in 1950 μL of 500 mM NaNO_3 -phosphate buffer (pH = 7.0) in a clean, dry cuvette with slow stirring condition was provided using a magnetic stirrer. The setup was placed in the photoirradiation chamber. The initial chloride concentration ($[\text{Cl}^-]_0$) was measured using chloride-ISE. The stock solution of the transporter/protransporter in DMSO was added to the solution ($t = 0$ s) to obtain a final concentration of 100 μM . Then the solution was kept stirring under irradiation with 655 nm red light until $t = 5$ min (for photoactivation). (The setup was kept stirring in the dark for experimental runs not involving photoactivation.) The chloride-ISE was then dipped into the solution and the time dependent chloride efflux was monitored by taking readings every 1 min. Finally, the vesicles were lysed by the addition of 25 μL 10% Triton X-100 at the end of the experiment (after $t = 10$ min) to destroy the chloride gradient. The experiment was conducted in a dark room to prevent any interference from ambient light.

The data was normalized to the percentage change in chloride concentration with respect to time, setting the initial recorded value as 0% chloride efflux and the final chloride reading (stable reading after lysis of the vesicles) as 100% chloride efflux using equation 4.

$$\% \text{ chloride efflux} = \frac{([\text{Cl}^-]_t - [\text{Cl}^-]_0)}{([\text{Cl}^-]_\infty - [\text{Cl}^-]_0)} \times 100 \quad (\text{Equation 4})$$

Where $[Cl^-]_0$ is the initial chloride concentration, $[Cl^-]_t$ is the concentration at time t , and $[Cl^-]_\infty$ is the final stable value after lysis of the vesicles using Triton X-100.

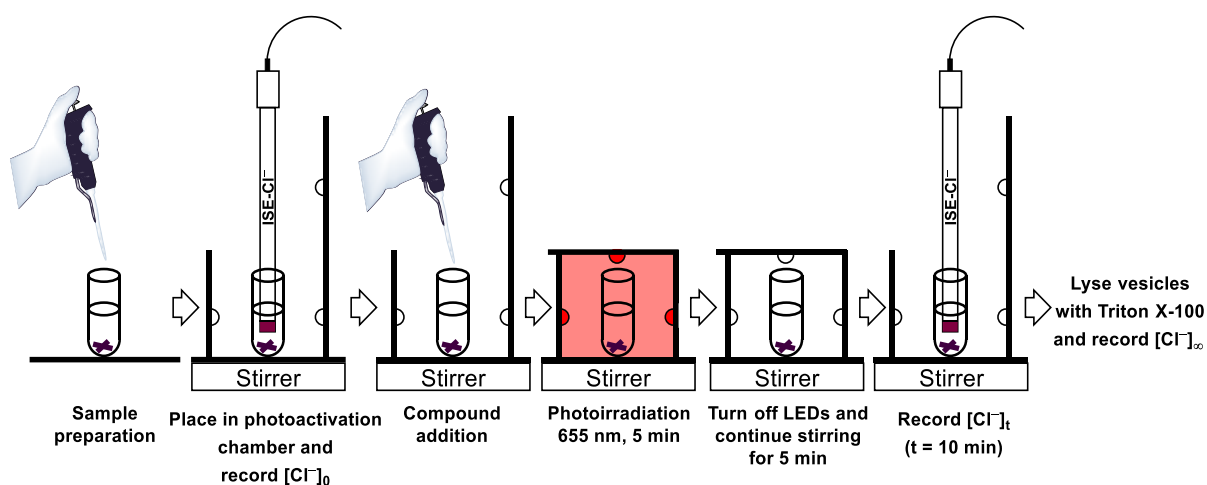


Figure 39: Schematic of protocol for verification of photoactivated ion transport across chloride-entrapped EYPC-LUVs.

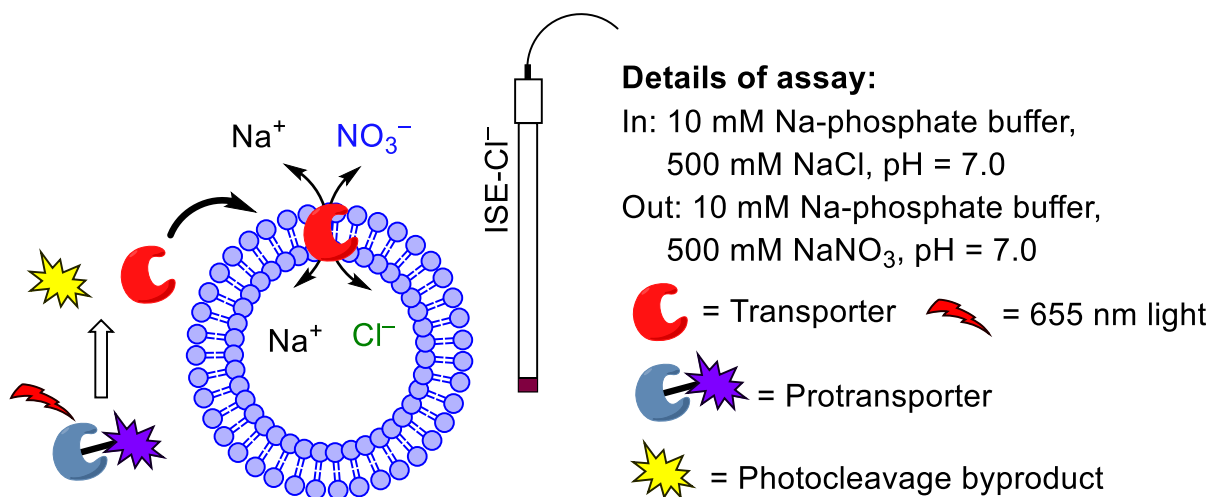


Figure 40: Schematic of photoactivated ion transport across chloride-entrapped EYPC-LUVs monitored using ISE.

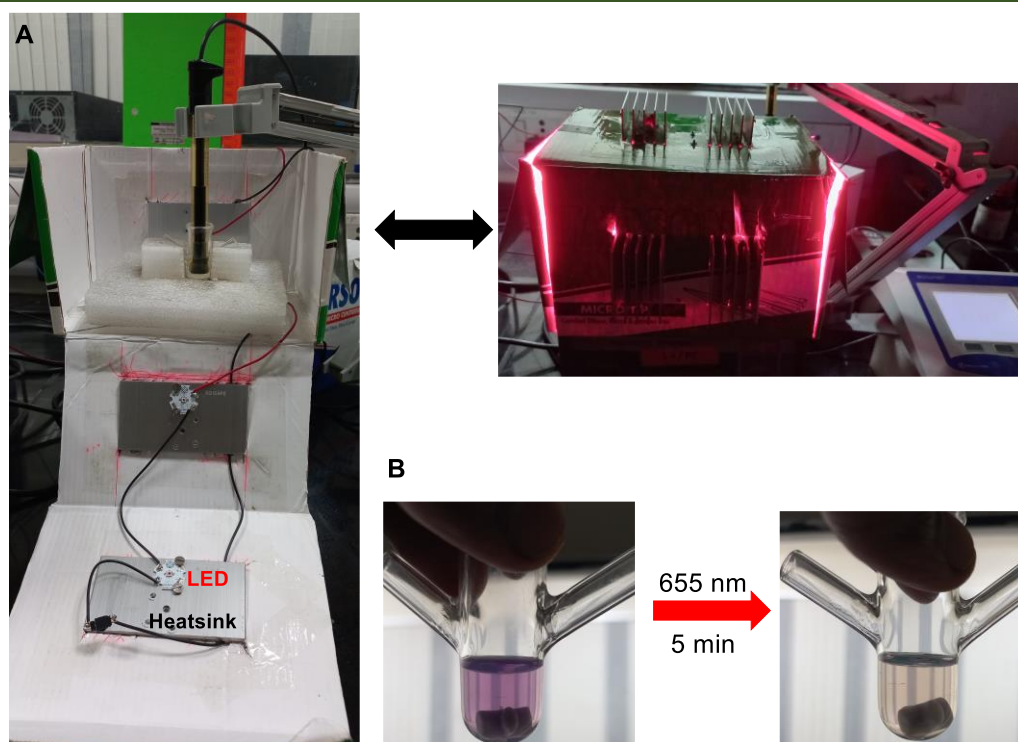


Figure 41: (A) Setup used for red light photoactivation of protransporters in EYPC-LUVs $\supset$ NaCl. (B) Protransporter (2) treated EYPC-LUVs $\supset$ NaCl before and after photoactivation showing color change from violet to yellow.

3.4.10 Microbial Studies

Bacterial strains and growth conditions

- *Caulobacter crescentus* (*C. crescentus*)- str. NA1000.⁵⁸

C. crescentus was grown in peptone yeast extract (PYE) media (0.2% peptone, 0.1% yeast extract, 1 mM MgSO₄, 0.5 mM CaCl₂) incubated at 29 °C.

- *Escherichia coli* (*E. coli*)- str. K-12 substr. MG1655; ATCC 700926.

E. coli was grown in lysogeny broth (LB) media incubated at 37 °C.

- *Salmonella enterica* (*S. enterica*) subsp. *enterica* (ex Kauffmann and Edwards) Le Minor and Popoff serovar Typhimurium - str. RC 907 [EMG 36]; ATCC 23564; MTCC 98.

S. enterica was grown in lysogeny broth (LB) media incubated at 37 °C.

- *Klebsiella pneumoniae* (*K. pneumoniae*) subsp. *pneumoniae* (Schroeter) Trevisan - str. NCTC 418 [Johns Hopkins Hosp. 240]; ATCC 15380; MTCC 109.

K. pneumoniae was grown in lysogeny broth (LB) media incubated at 37 °C.

Salicylanilide activity screening in *C. crescentus* (in 96 well plate)

C. crescentus grown in a culture tube (PYE media) was diluted to 0.1 OD (600 nm) in PYE and transferred to a 96 well plate (HiMedia), adding 145 μ L of the culture into each well. The compounds to be screened were added from stock solutions in DMSO, 5 μ L of 1.5 mM stock per well, to obtain a working concentration of 50 μ M per sample. DMSO (5 μ L/well) was used as a negative control. The media in each well was mixed using a micropipette to ensure uniform compound distribution and the plate was incubated at 29 °C for 18 h. The optical density of the cultures at 600 nm (OD600) in each well was recorded on a Thermo Scientific Varioskan® Flash multimode microplate reader. The data was normalized with respect to the negative control.

Table 9: Normalized mean, standard error (SE) of mean and calculated effect size (R squared) of salicylanilide treatment in *C. crescentus*.

Treatment	Mean	SE of mean	R squared
DMSO	100	1.09	-
1a	83.25	2.53	0.52
1b	38.21	4.70	0.87
1c	83.34	1.62	0.68
1d	12.84	0.87	0.99
1e	13.91	1.57	0.98
1f	81.65	3.34	0.44
1g	14.56	0.64	0.99
1h	23.87	2.63	0.96
1i	78.22	3.37	0.52
1j	14.53	1.47	0.99
1k	97.65	2.07	0.03
1l	67.47	5.29	0.52
1m	53.17	6.49	0.60
1n	39.34	4.66	0.82

Antibacterial activity testing in *C. crescentus* (in culture tubes)

C. crescentus culture was diluted to 0.1 OD₆₀₀ in PYE (3 mL) in culture tubes and treated with the compounds by the addition of corresponding stock solutions in DMSO. Neat DMSO was used for negative control. The tubes were then incubated at 29 °C with shaking (210 rpm) for 18 h. The OD₆₀₀ of the cultures were then recorded on an Eppendorf BioPhotometer® in 1 mL cuvettes. The data was normalized with respect to the negative control.

Bacterial growth curves

The chosen bacterial strain was diluted to the necessary initial OD₆₀₀ in its growth media and added to a 96 well plate (HiMedia) with 145 µL of the diluted culture per well. The compounds were added from their respective stocks in DMSO. Stock solutions were prepared via serial dilution such that 5 µL of the stock addition to each well ensures the required working concentration (final volume- 150 µL/well). The contents of each well were mixed using a micropipette to ensure uniform compound distribution. DMSO (5 µL/well) was used as a negative control. The plate was incubated in a PerkinElmer EnSight™ multimode microplate reader set to the required growth temperature with shaking (180 rpm). The optical density of the cultures in each well was recorded at 600 nm (OD₆₀₀) at specified time intervals.

Caulobacter crescentus: Media- PYE; Initial OD- 0.1; Temperature- 29 °C; Duration of run- 18 h; Measurement interval- 2 h.

Escherichia coli: Media- LB; Initial OD- 0.005; Temperature- 37 °C; Duration of run- 12 h; Measurement interval- 30 min.

Cell surface imaging (SEM)

E. coli culture (in LB) was diluted to 0.2 OD in culture tubes and treated with 50 µM each of compounds. The same volume of DMSO was added to the control culture. The tubes were incubated in a shaker for 7 h at 37 °C.

The cultures were then diluted to 0.2 OD (1 mL final volume) in LB media in 1.5 mL centrifuge tubes and the cells were pelletized (8000 rpm, 8 min, 4 °C). The supernatant was removed and the pellets were washed successively with 1 mL of ice cold 1X PBS and 0.1X PBS. Plastic 1 mL tips with cut ends were always used for resuspension of the pellets to prevent cell damage from

shearing during mixing. The cells were then pelletized (8000 rpm, 8 min, 4 °C), the supernatant removed and the pellets stored in ice.

Silicon wafers having a 300 nm SiO₂ surface layer were plasma cleaned (in Zepto Model 2 plasma cleaner from Diener Electronic) for 10 min (60 W power) to improve surface wettability. The pellets were resuspended in 1 mL ice cold MilliQ water (using a micropipette tip with a cut end) and two μ L each of the samples were immediately spotted on the silicon wafers. The samples were then dried under vacuum in a desiccator for a minimum of 12 h.

The samples were gold coated (8-10 μ M thickness) prior to imaging using a Q150T ES turbo-pumped sputter coater (Quorum Technologies). They were imaged using SE2 detector in a ZEISS Ultra Plus Field Emission-Scanning Electron Microscope (FE-SEM). Imaging was performed for three biological replicates. The number of damaged cells in the images was counted and normalized with respect to the total cell count (damaged and undamaged).

Photoactivation in bacterial cultures

Bacterial cultures were diluted to the required OD in their growth media and 2 mL each of the diluted cultures was transferred into culture tubes. The tubes were treated with 50 μ M of the respective compounds by the addition of 33.33 μ L of 3 mM stocks in DMSO. Cultures treated with 33.33 μ L of DMSO were used as controls. A total of 11 tubes with different compound treatments were used for each experiment. DMSO- 2, **1g**- 1, **1j**- 1, **2**- 2, **3**- 2, **BA**- 1, and **4**- 2. One each of the tubes containing the photoactivatable compounds **2**, **3** and **4** were irradiated with 655 nm red light for 5 min in the photoactivation setup. One tube of DMSO was also irradiated under 655 nm light to assess the effect of the electromagnetic radiation on the bacterial cultures. The tubes were then wrapped with Al-foil to shield them from ambient light and incubated with shaking (210 rpm). The OD₆₀₀ of the cultures was measured following the desired period of growth.

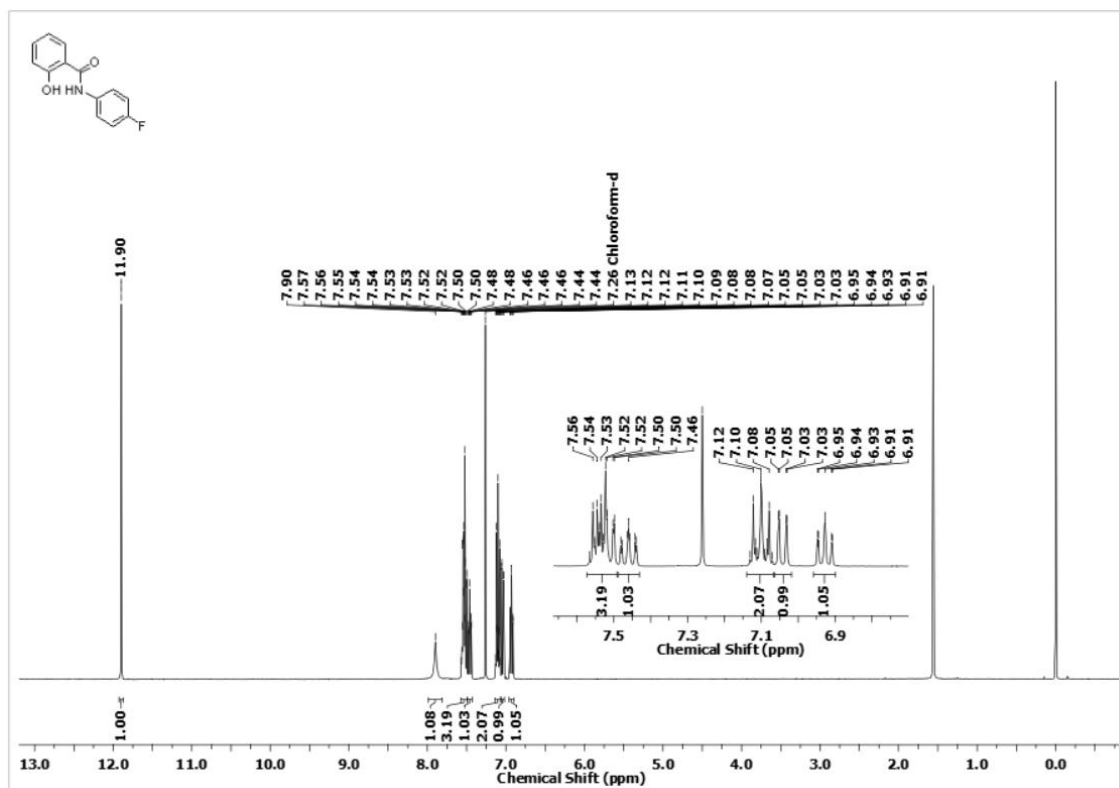
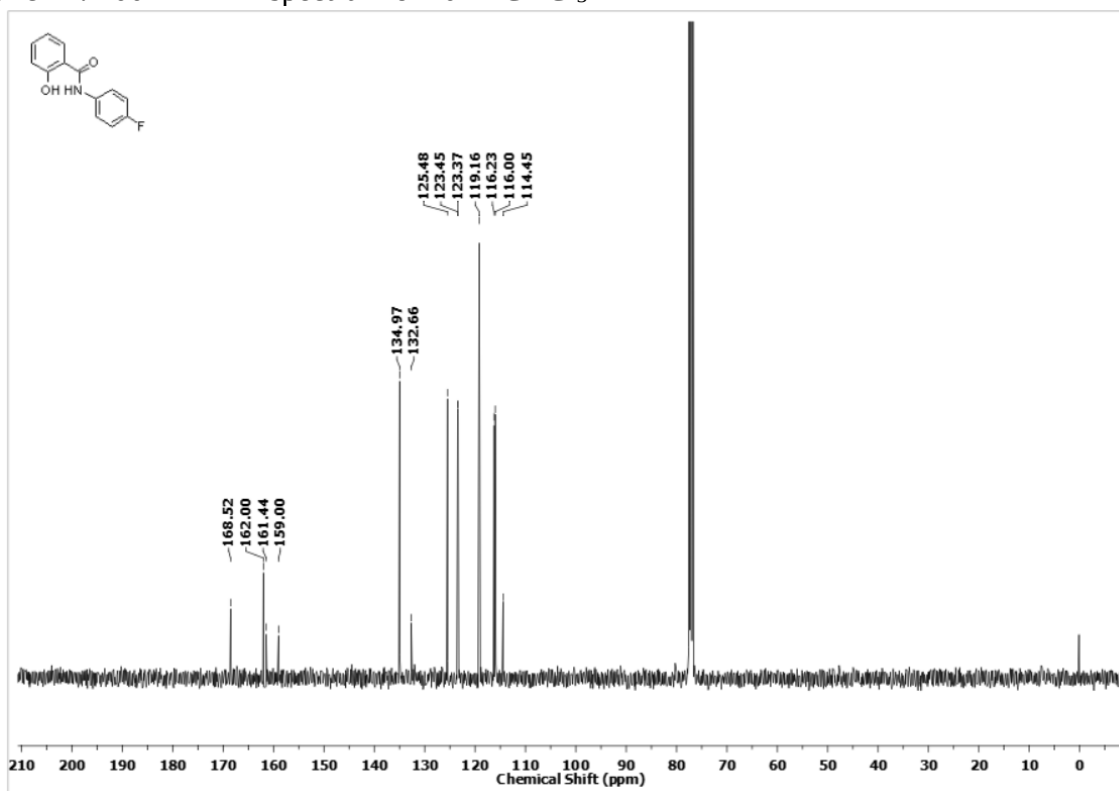
Caulobacter crescentus: Media- PYE; Initial OD- 0.1; Temperature- 29 °C; Incubation time- 18 h.

Escherichia coli: Media- LB; Initial OD- 0.005; Temperature- 37 °C; Incubation time - 6 h.

Statistical analysis

The data has been represented as Mean $\pm$ SE of mean (SE- standard error) and has been calculated by dividing the standard deviation of the samples by the square root of the number of replicates. Analyses of statistical significance between datasets were conducted in the form of unpaired *t*-tests (two-tailed). A *p*-value ≥ 0.05 was considered to be statistically significant between groups.

3.5 Characterization Spectra

Figure 42: 400 MHz ^1H spectrum of **1a** in CDCl_3 Figure 43: 101 MHz ^{13}C spectrum of **1a** in CDCl_3

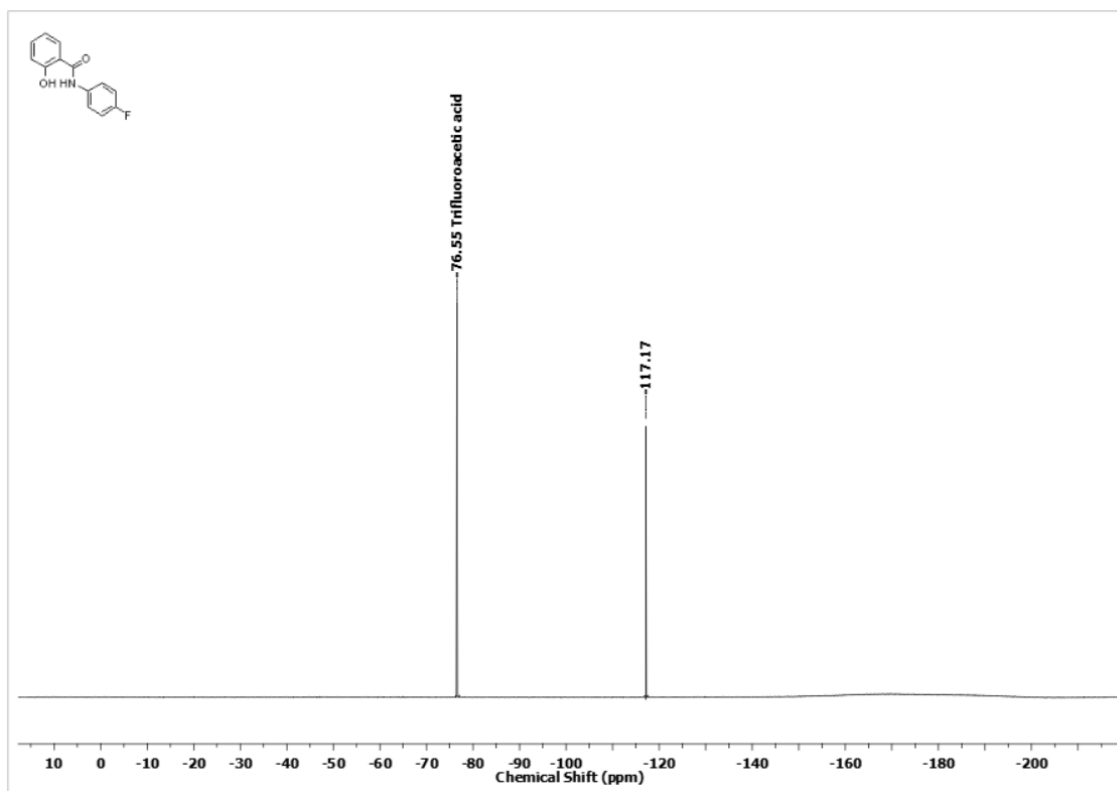


Figure 44: 377 MHz ^{19}F spectrum of **1a** in CDCl_3

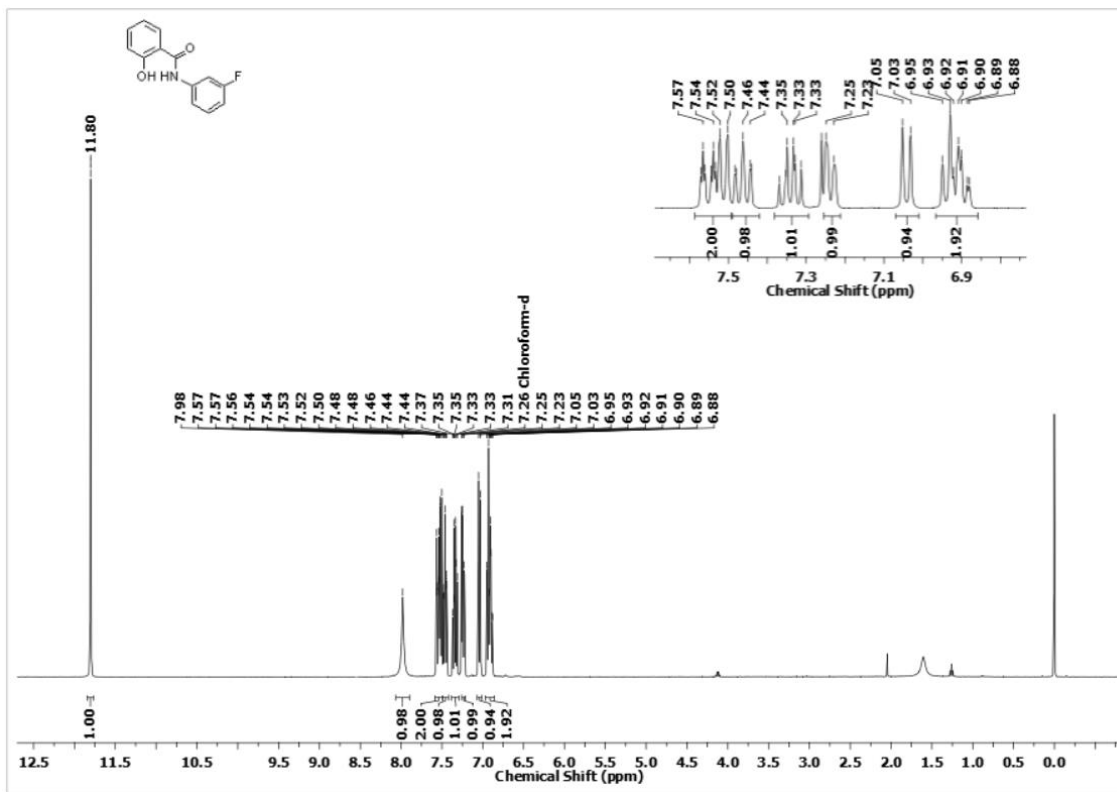


Figure 45: 400 MHz ^1H spectrum of **1b** in CDCl_3

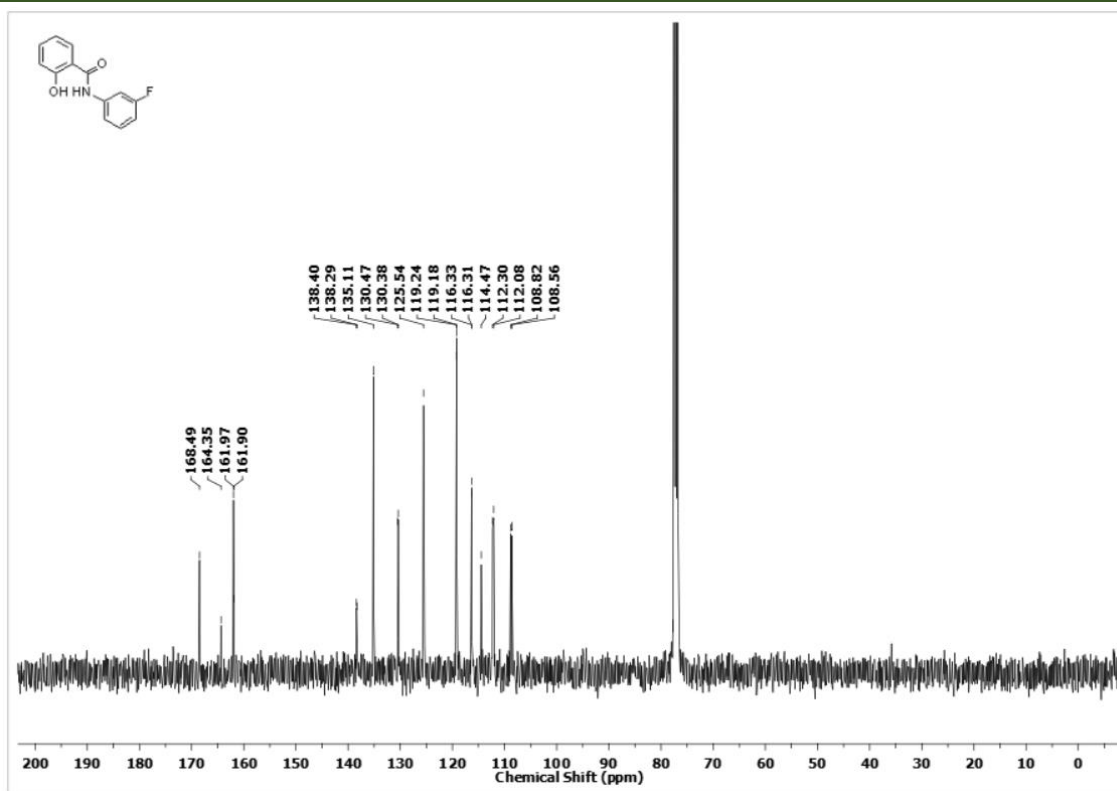


Figure 46: 101 MHz ^{13}C spectrum of **1b** in CDCl_3

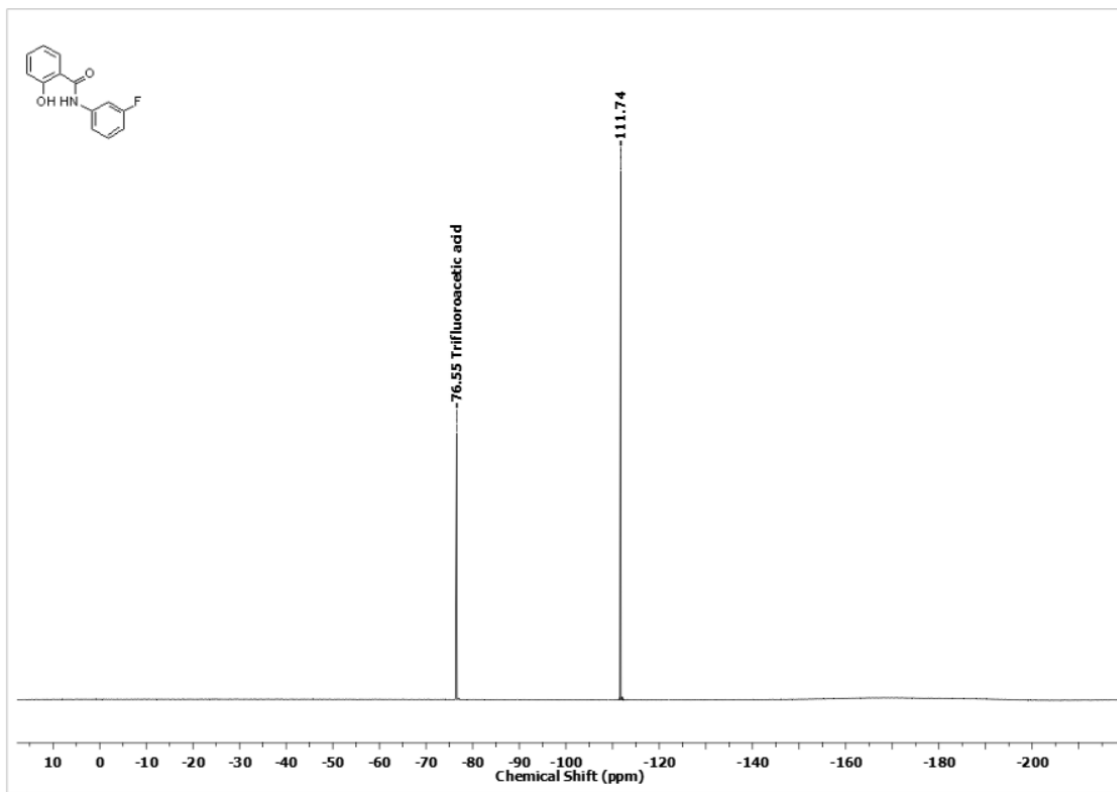


Figure 47: 377 MHz ^{19}F spectrum of **1b** in CDCl_3

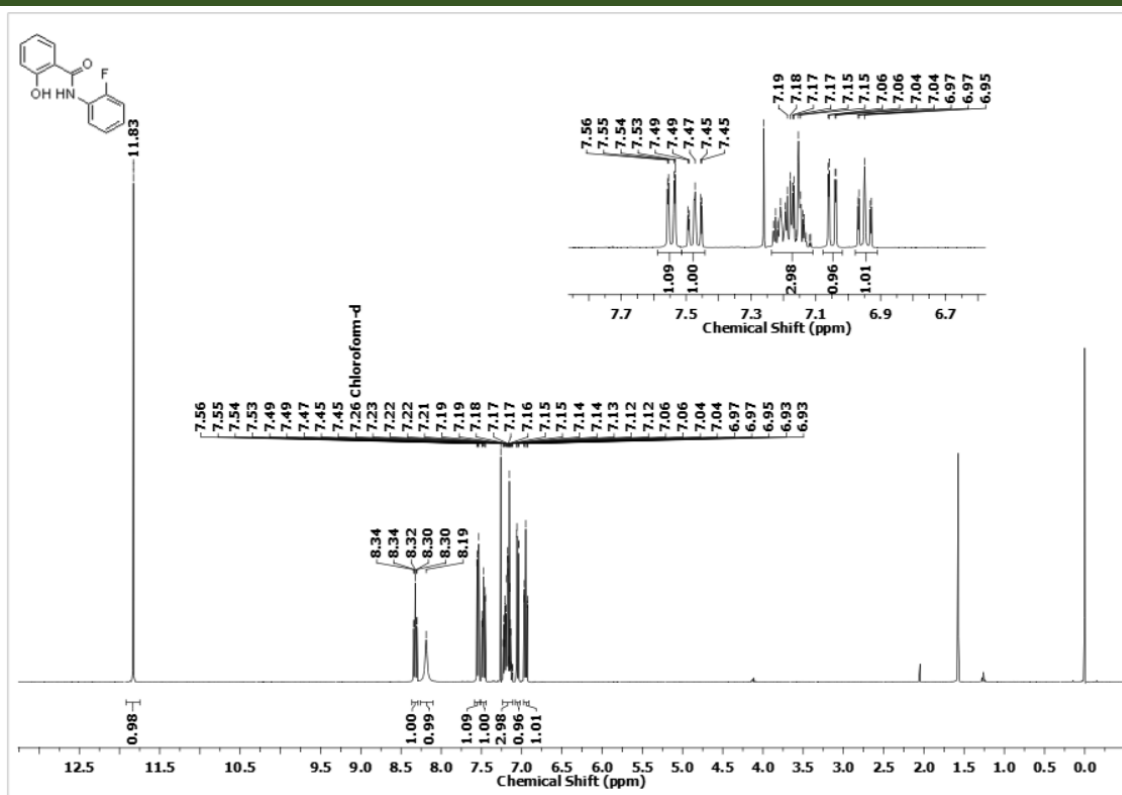


Figure 48: 400 MHz ^1H spectrum of **1c** in CDCl_3

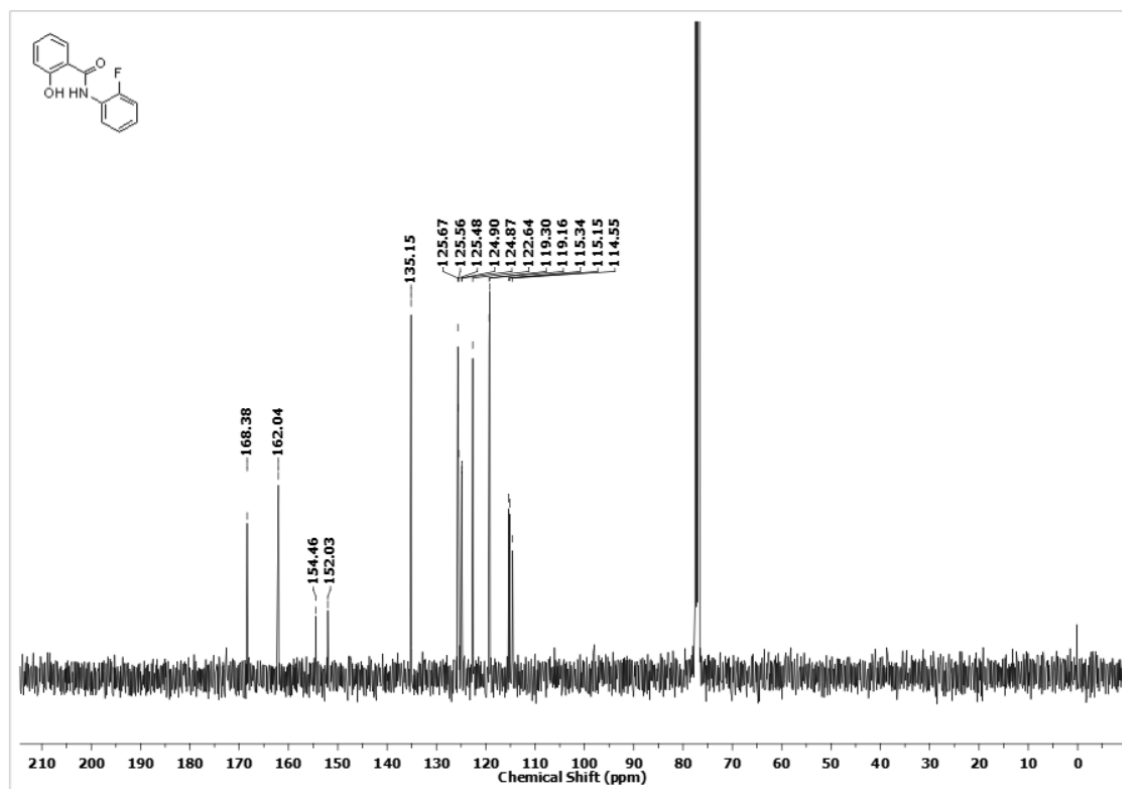


Figure 49: 101 MHz ^{13}C spectrum of **1c** in CDCl_3

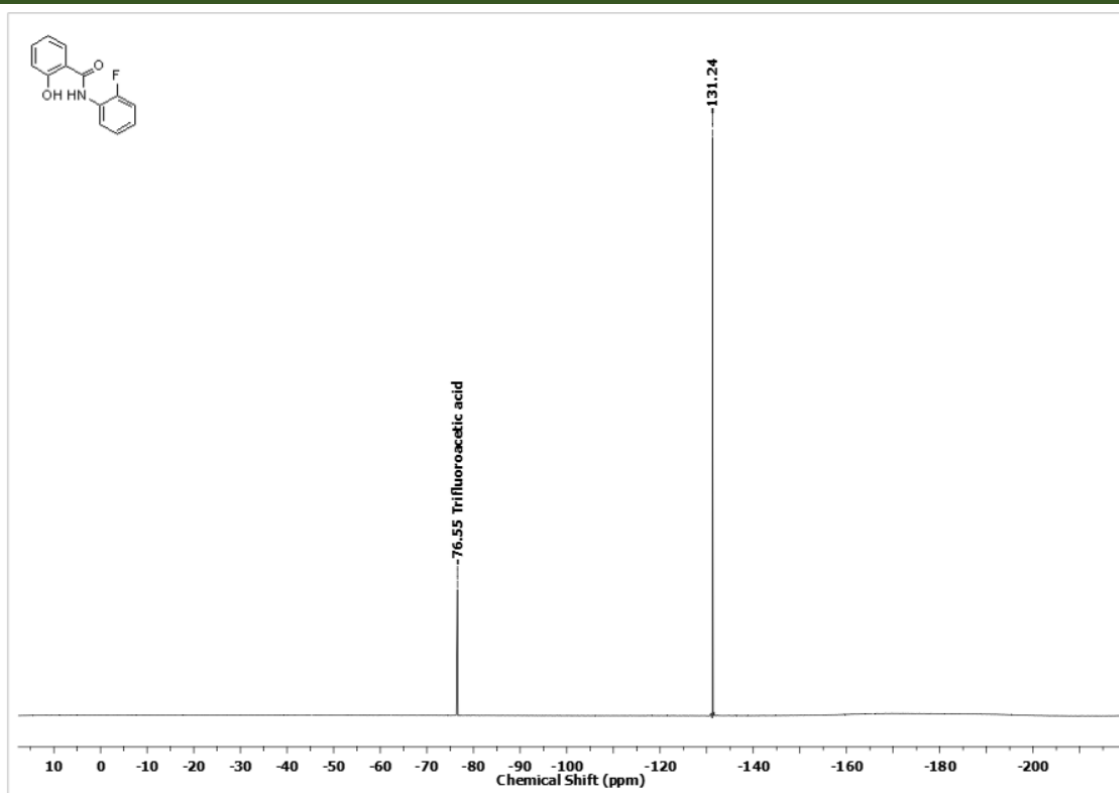


Figure 50: 377 MHz ^{19}F spectrum of **1c** in CDCl_3

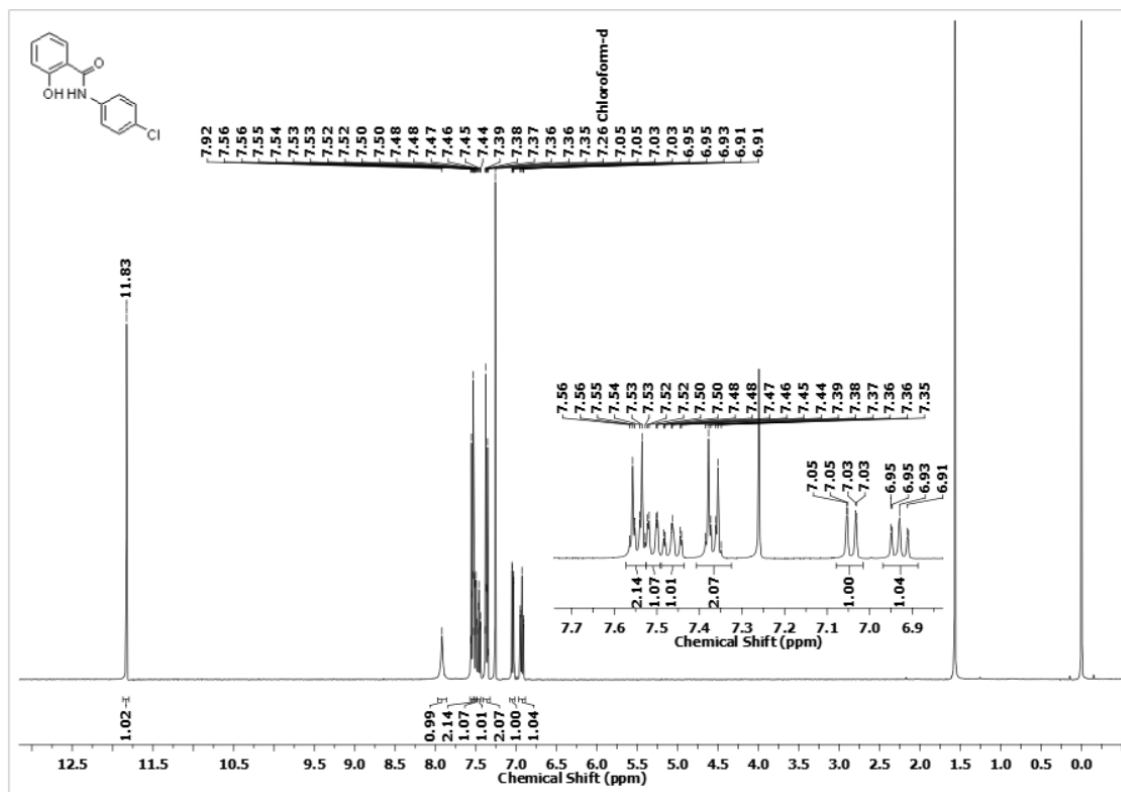


Figure 51: 400 MHz ^1H spectrum of **1d** in CDCl_3

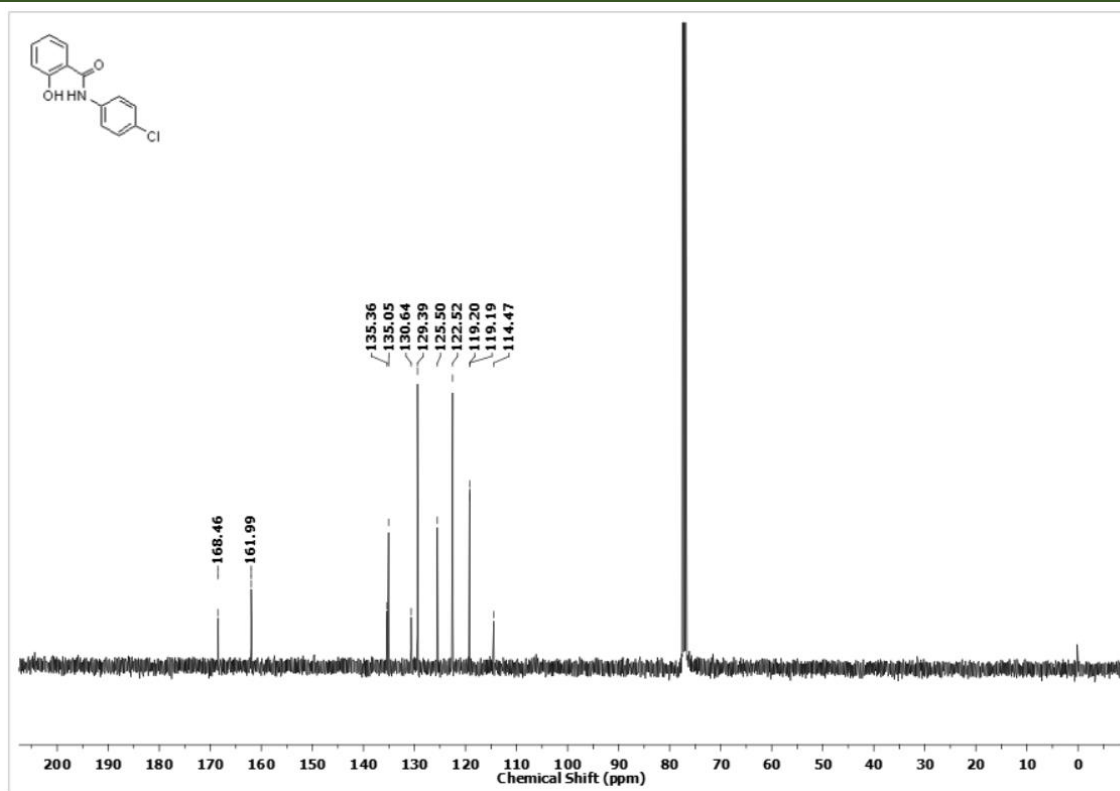


Figure 52: 101 MHz ^{13}C spectrum of **1d** in CDCl_3

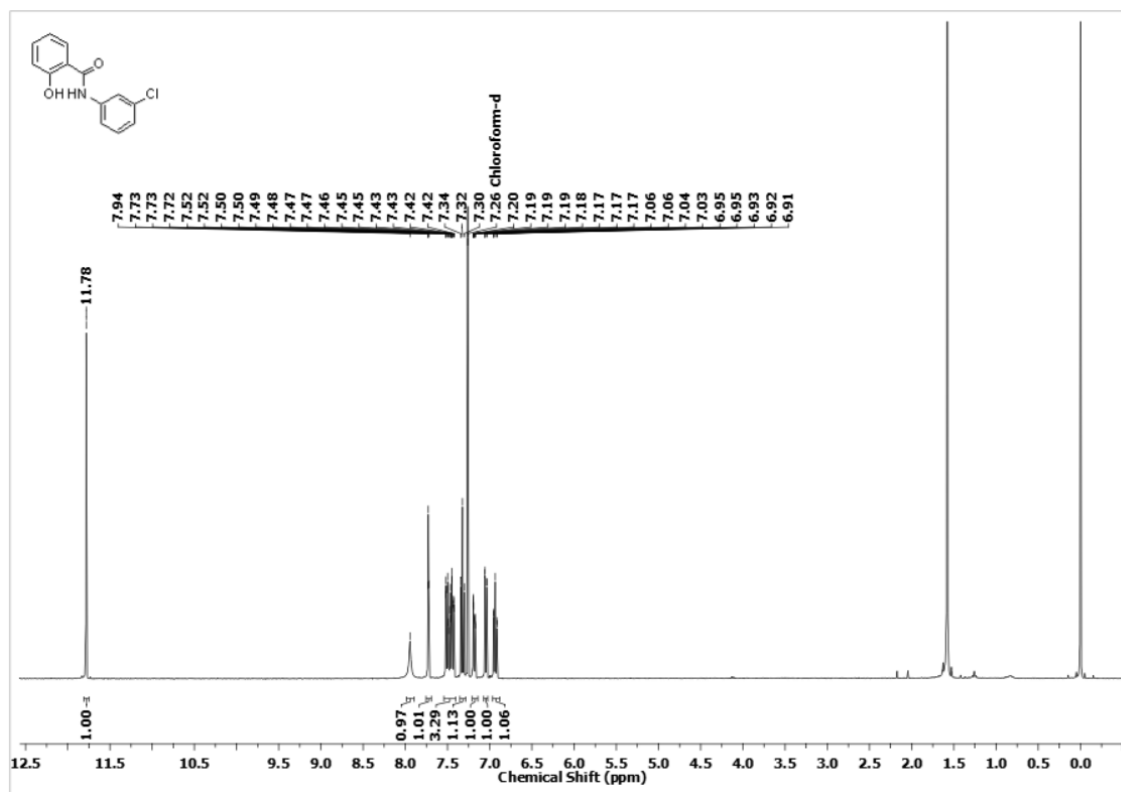


Figure 53: 400 MHz ^1H spectrum of **1e** in CDCl_3

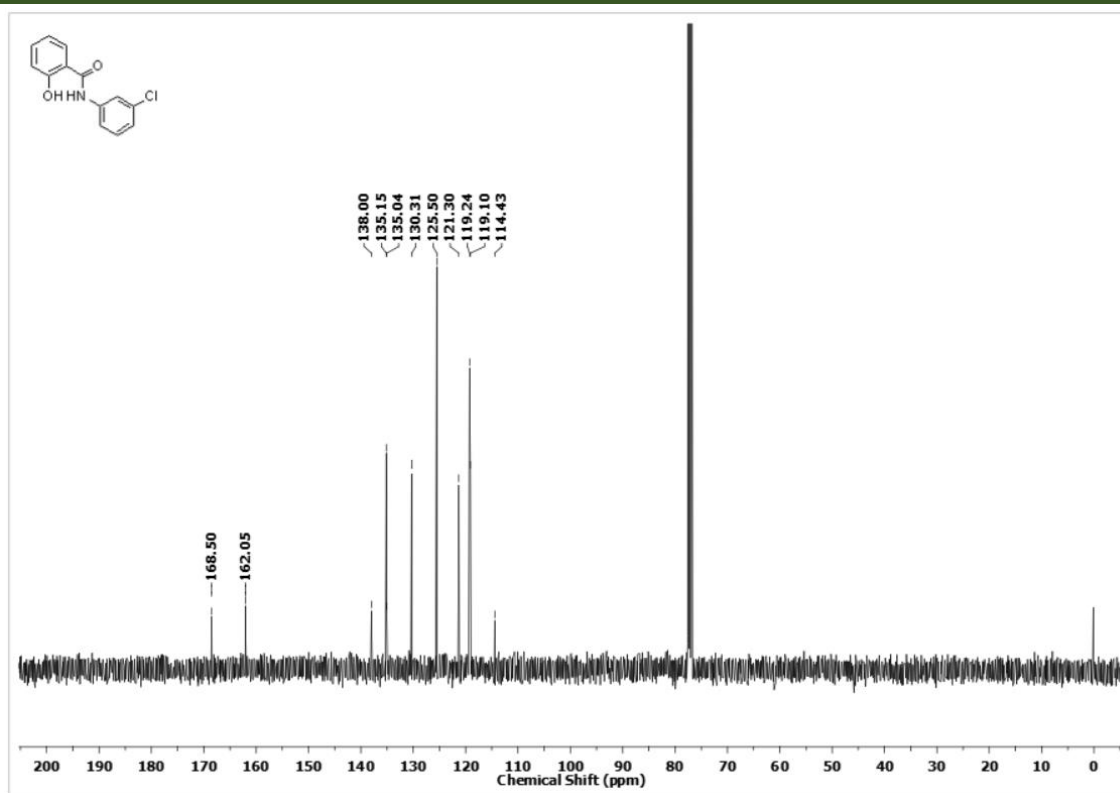


Figure 54: 101 MHz ¹³C spectrum of **1e** in CDCl₃

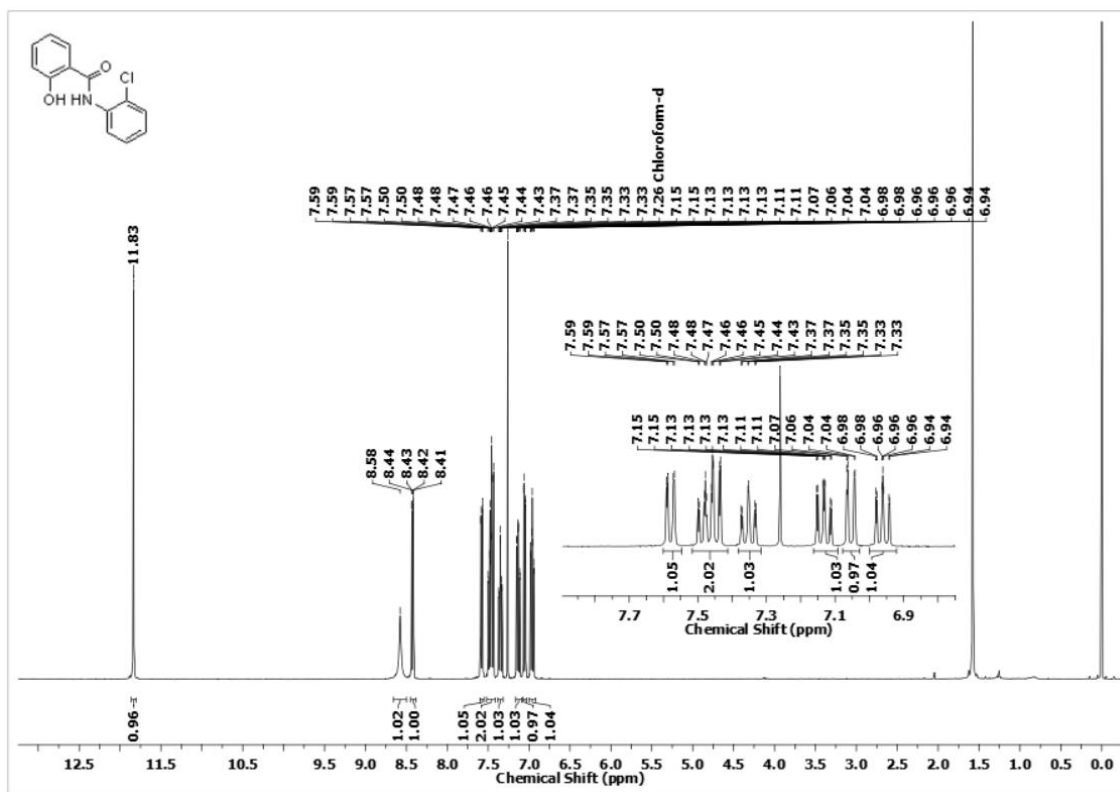


Figure 55: 400 MHz ¹H spectrum of **1f** in CDCl₃

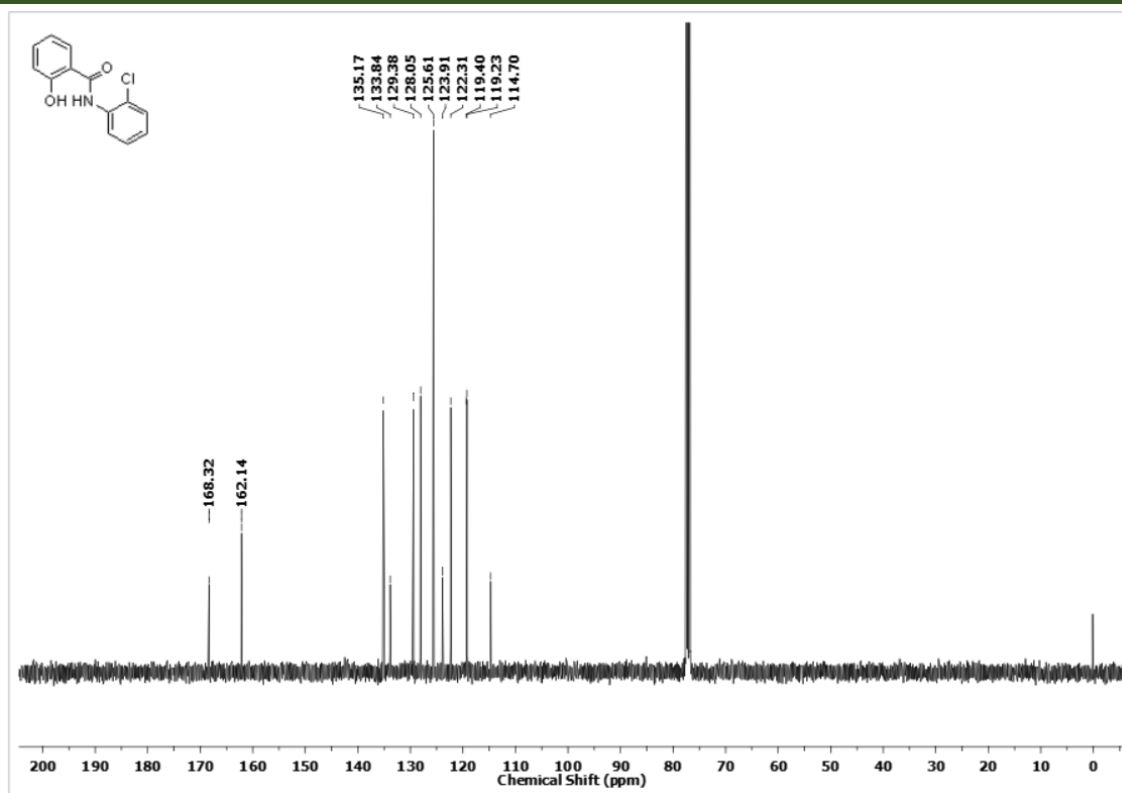


Figure 56: 101 MHz ¹³C spectrum of **1f** in CDCl₃

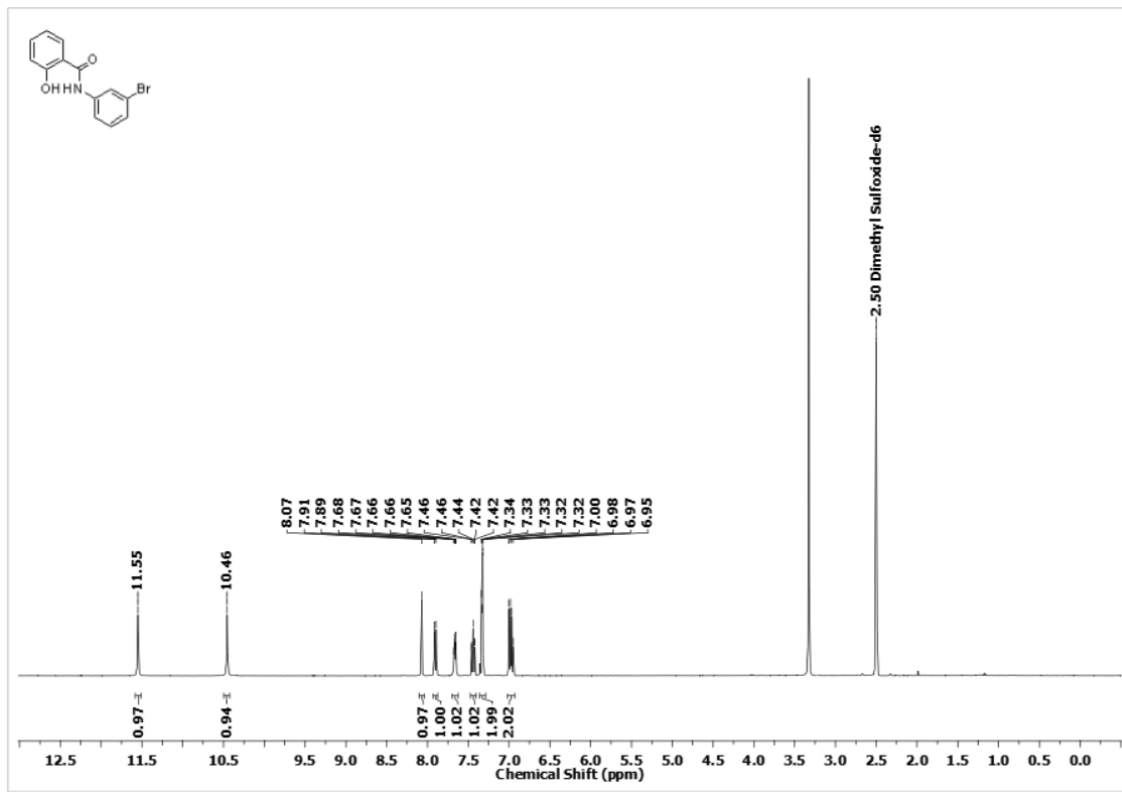


Figure 57: 400 MHz ¹H spectrum of **1h** in DMSO-*d*₆

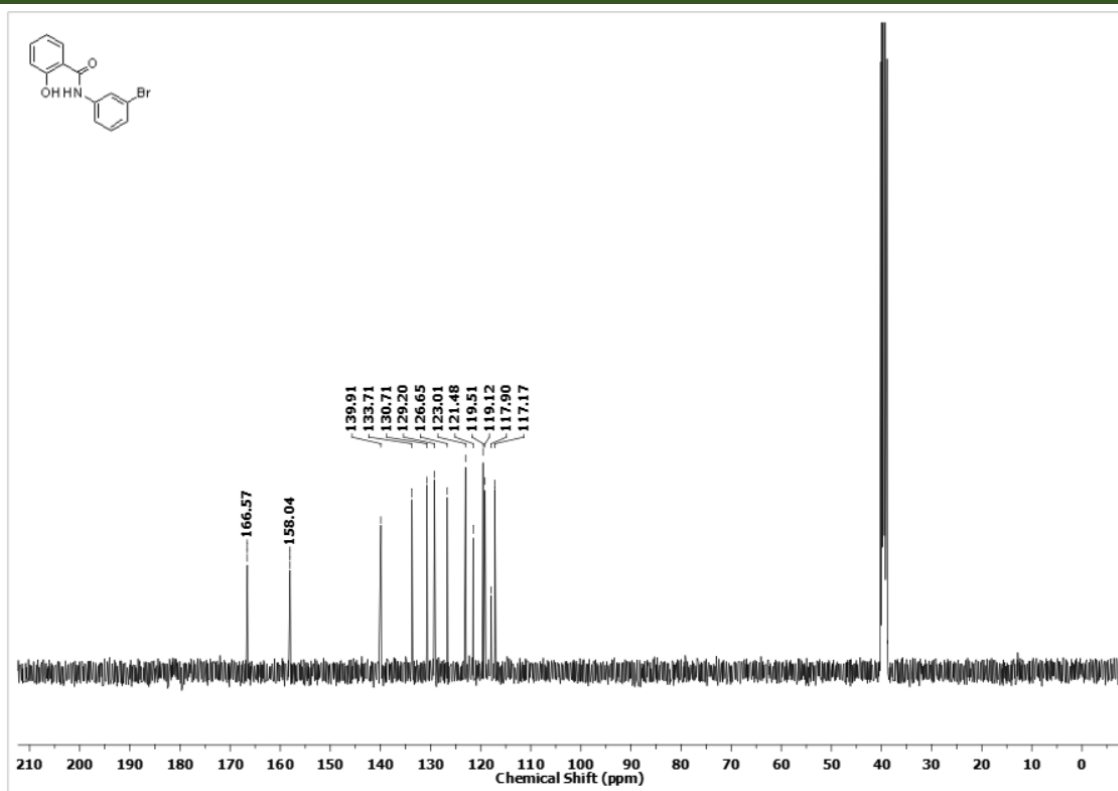


Figure 58: 101 MHz ¹³C spectrum of **1h** in DMSO-*d*₆

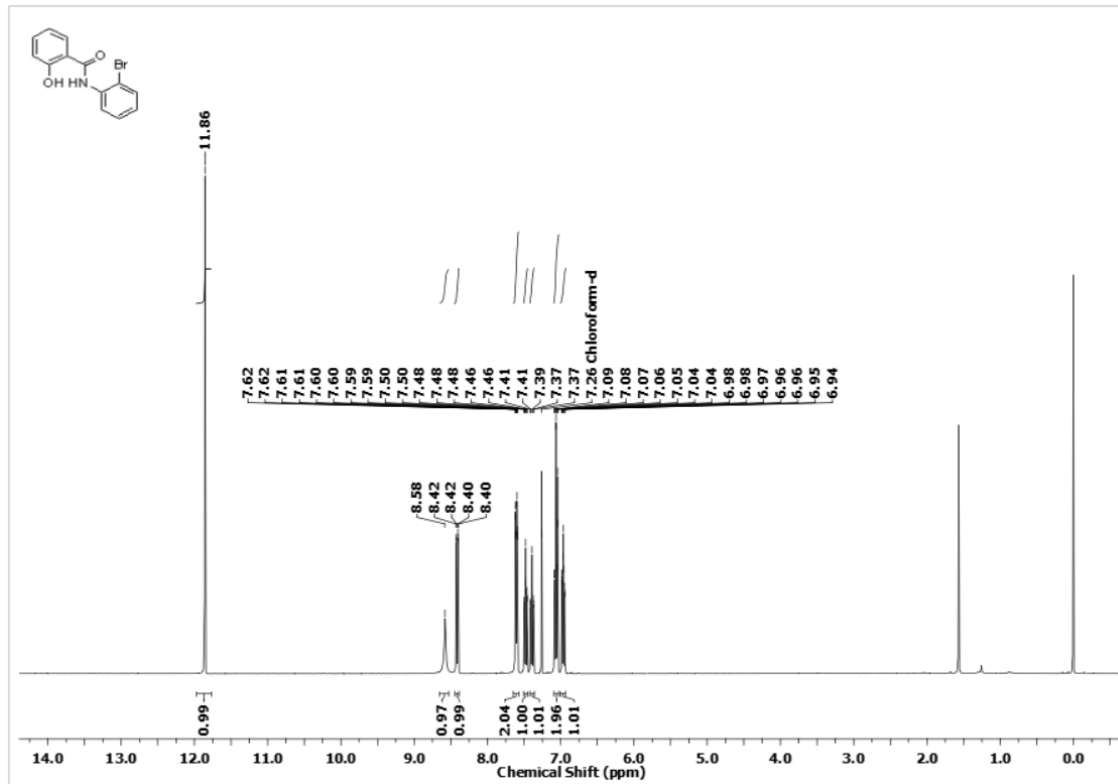


Figure 59: 400 MHz ¹H spectrum of **1i** in CDCl₃

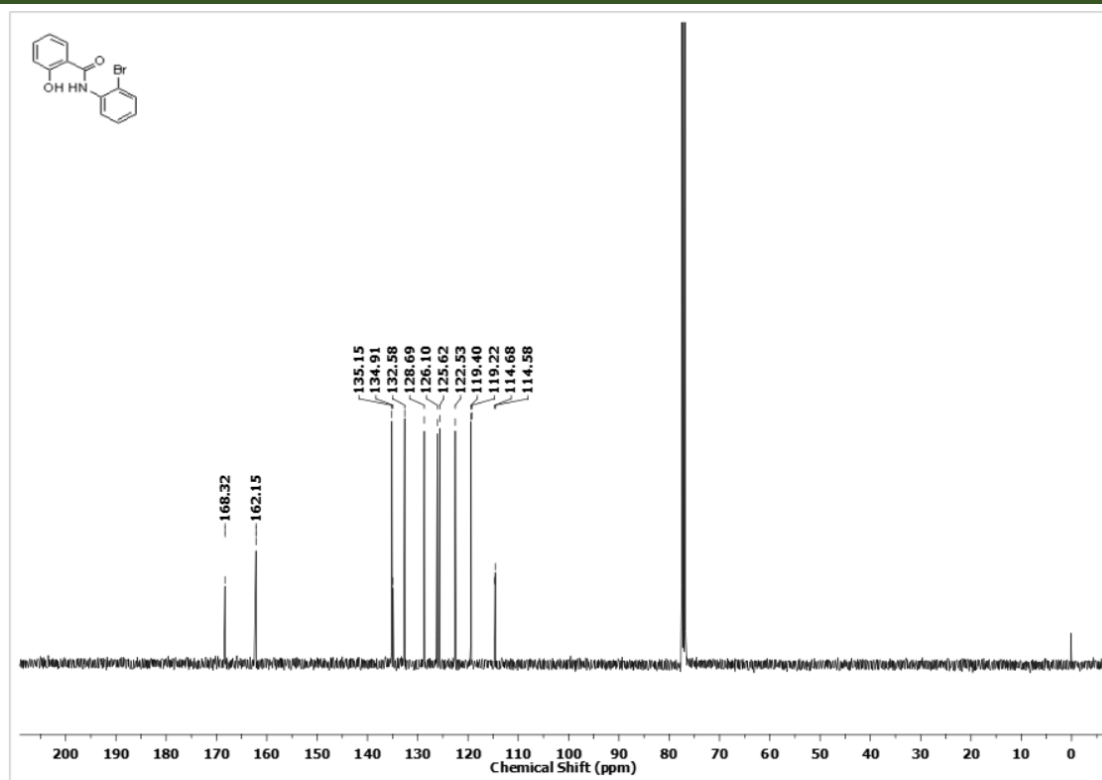


Figure 60: 101 MHz ¹³C spectrum of **1i** in CDCl₃

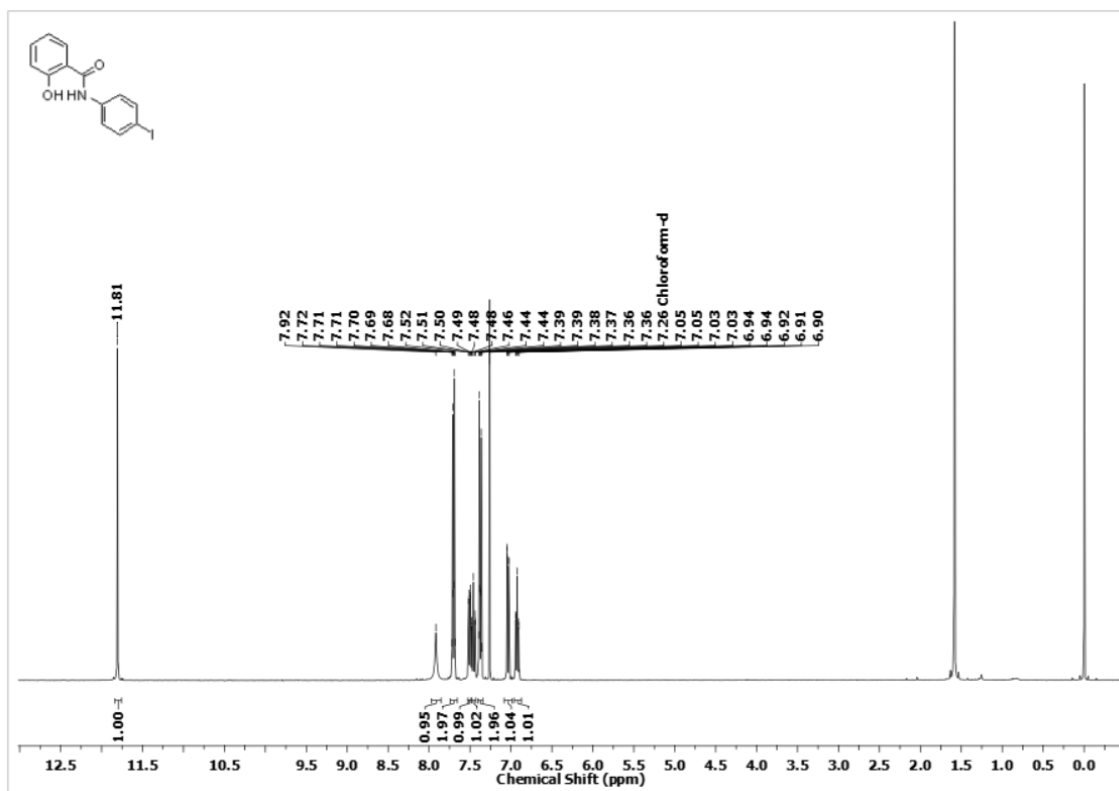


Figure 61: 400 MHz ¹H spectrum of **1j** in CDCl₃

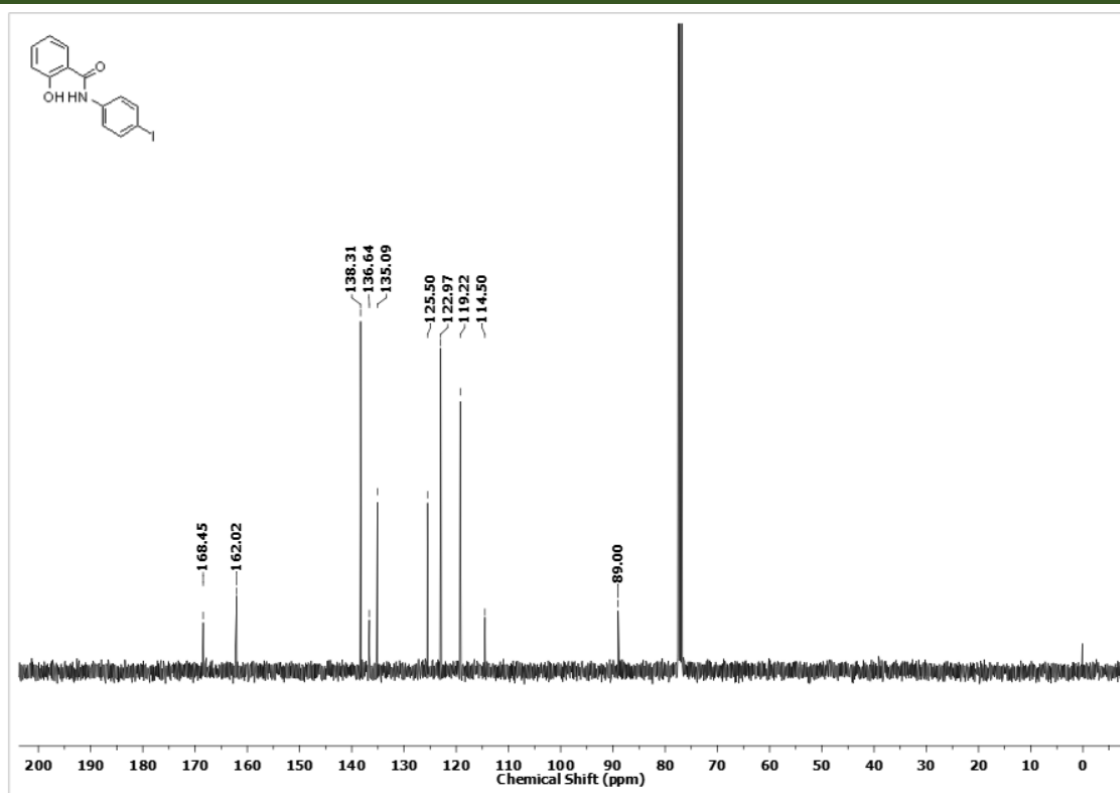


Figure 62: 101 MHz ¹³C spectrum of **1j** in CDCl₃

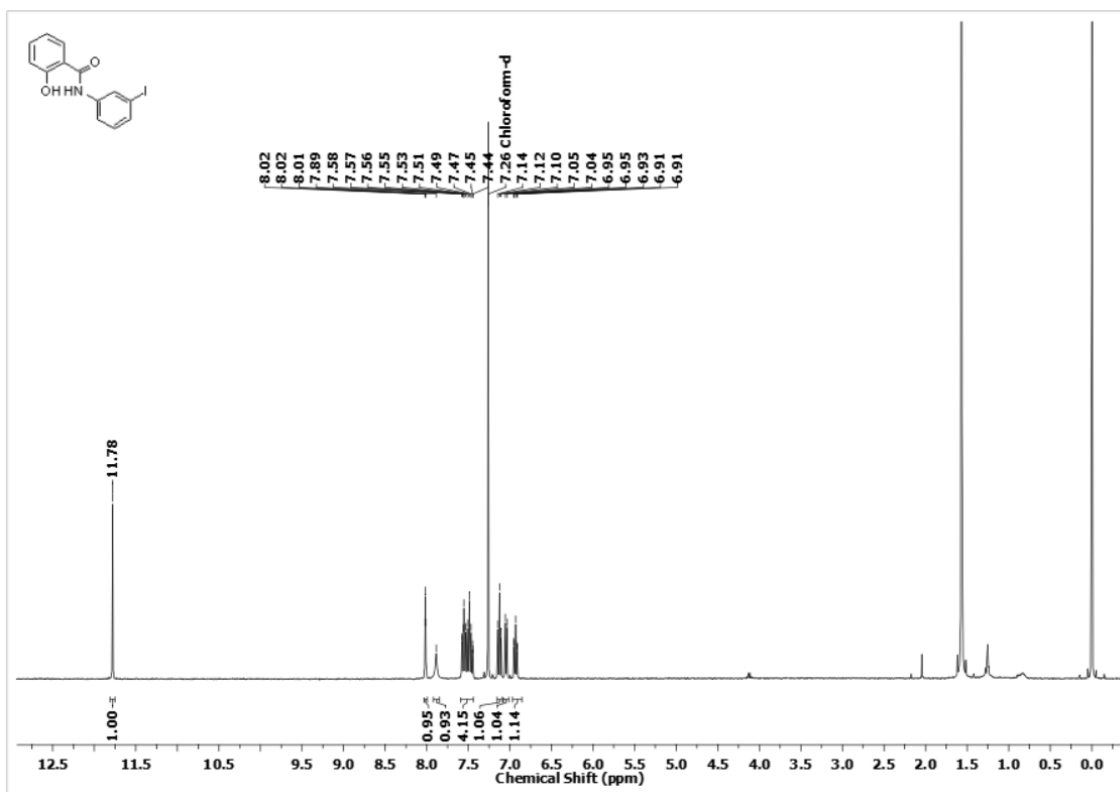


Figure 63: 400 MHz ¹H spectrum of **1k** in CDCl₃

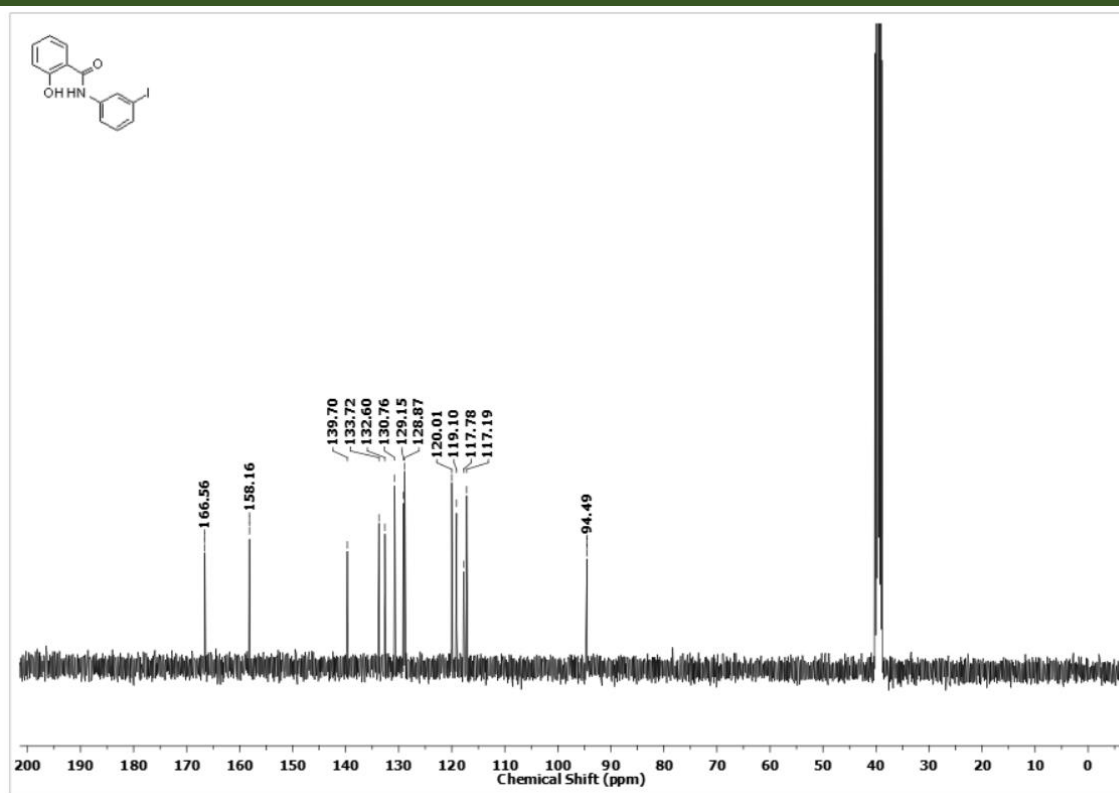


Figure 64: 101 MHz ¹³C spectrum of **1k** in DMSO-*d*₆

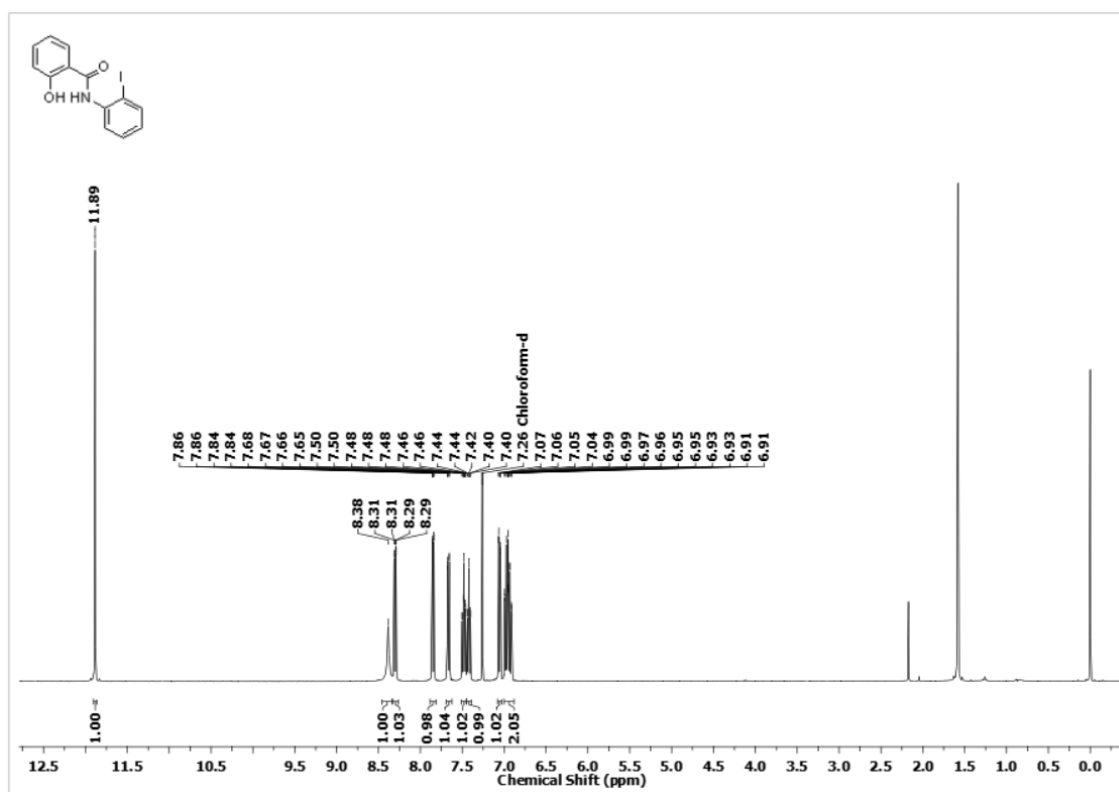


Figure 65: 400 MHz ¹H spectrum of **1l** in CDCl₃

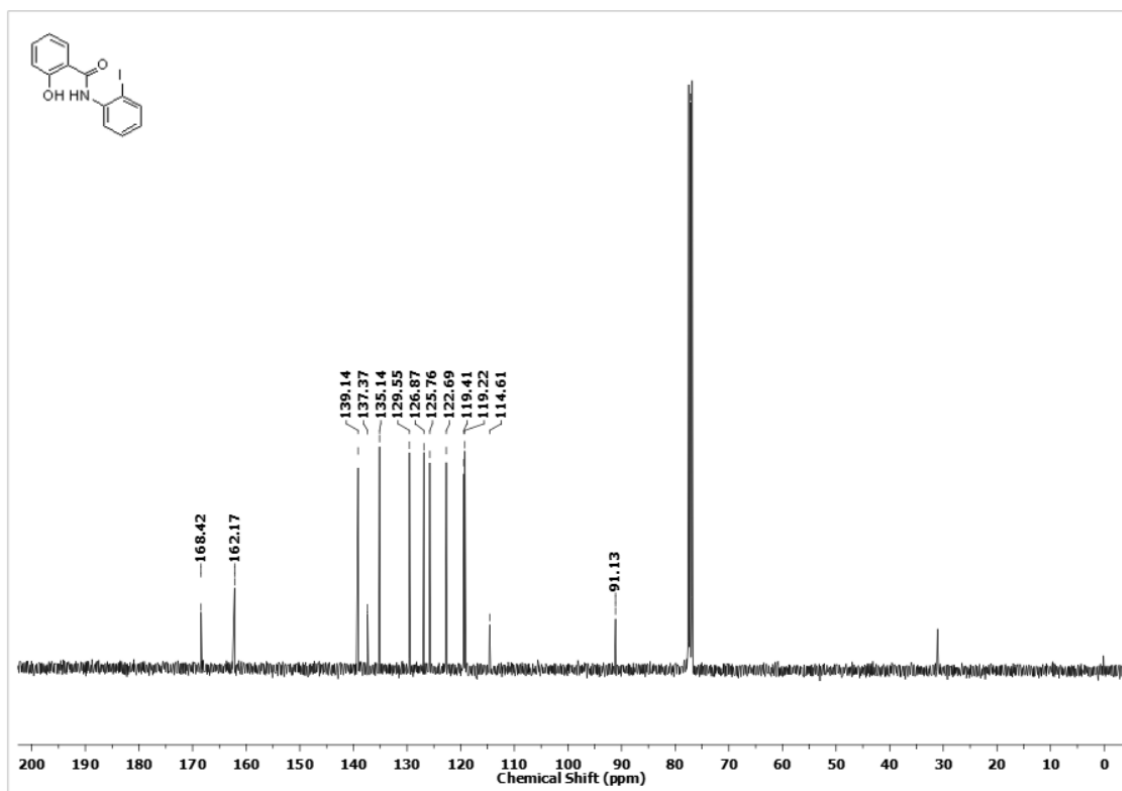


Figure 66: 101 MHz ¹³C spectrum of **1l** in CDCl₃

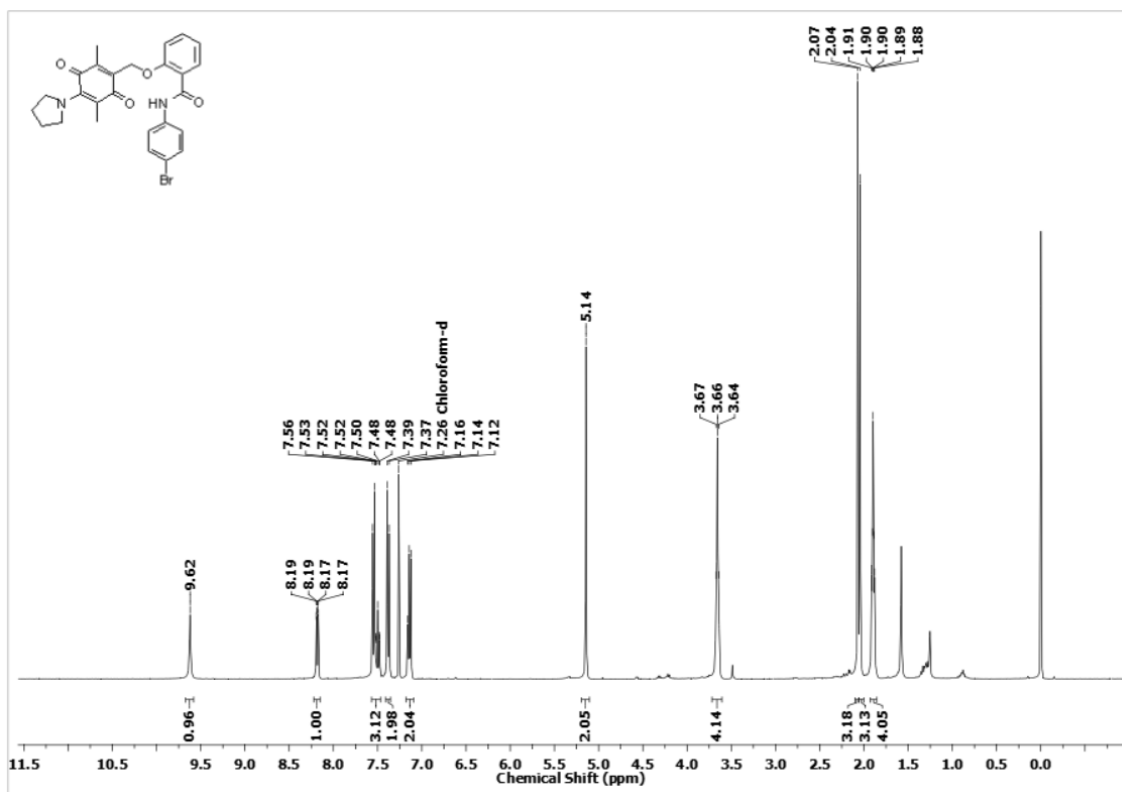


Figure 67: 400 MHz ¹H spectrum of **2** in CDCl₃

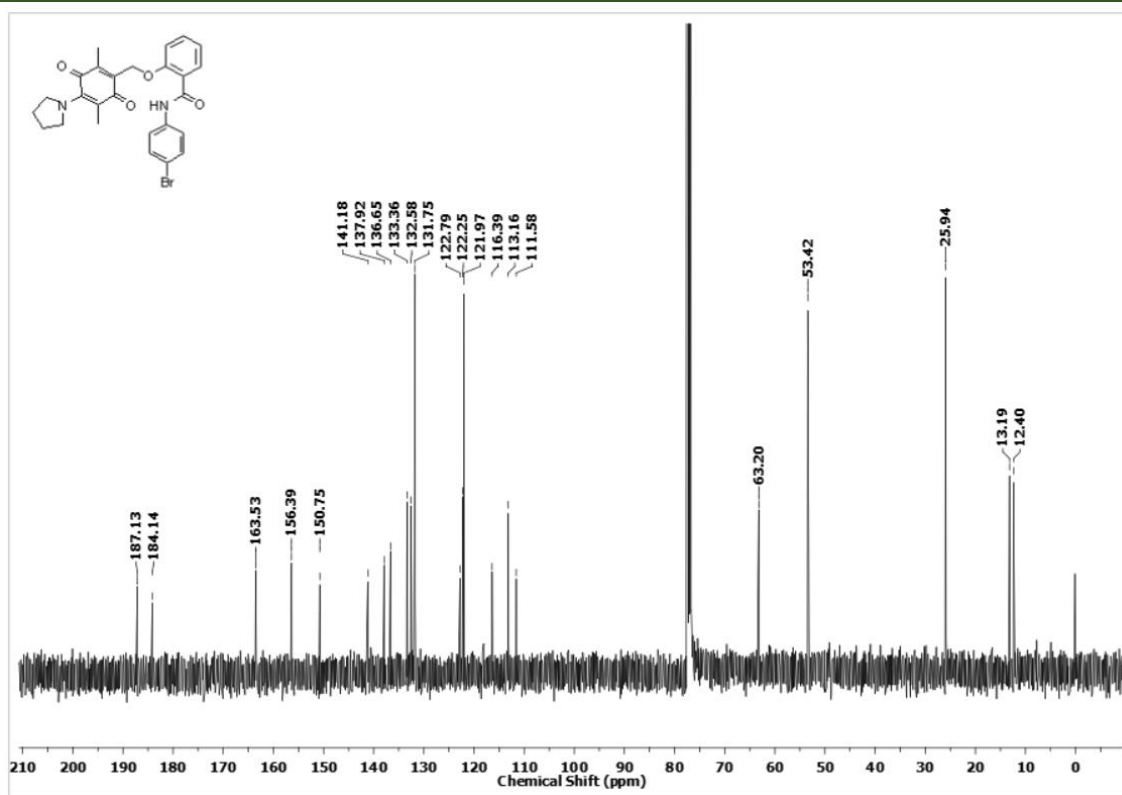


Figure 68: 101 MHz ^{13}C spectrum of 2 in CDCl_3

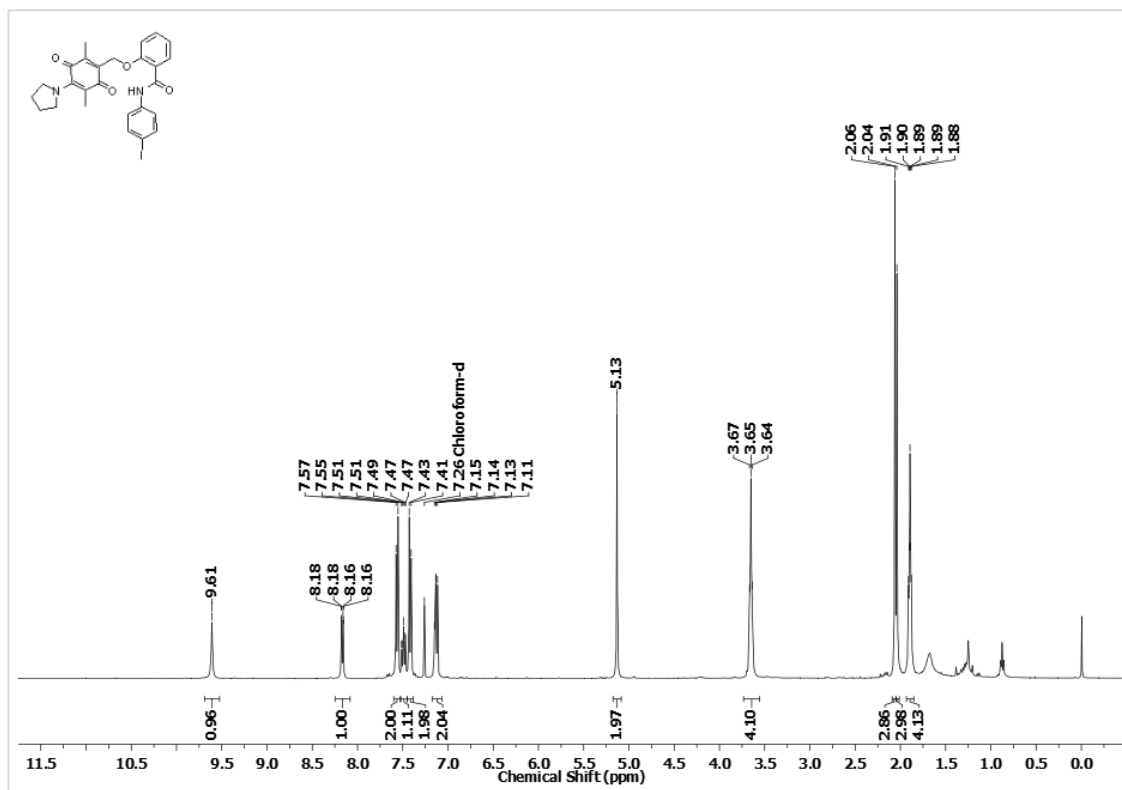


Figure 69: 400 MHz ^1H spectrum of 3 in CDCl_3

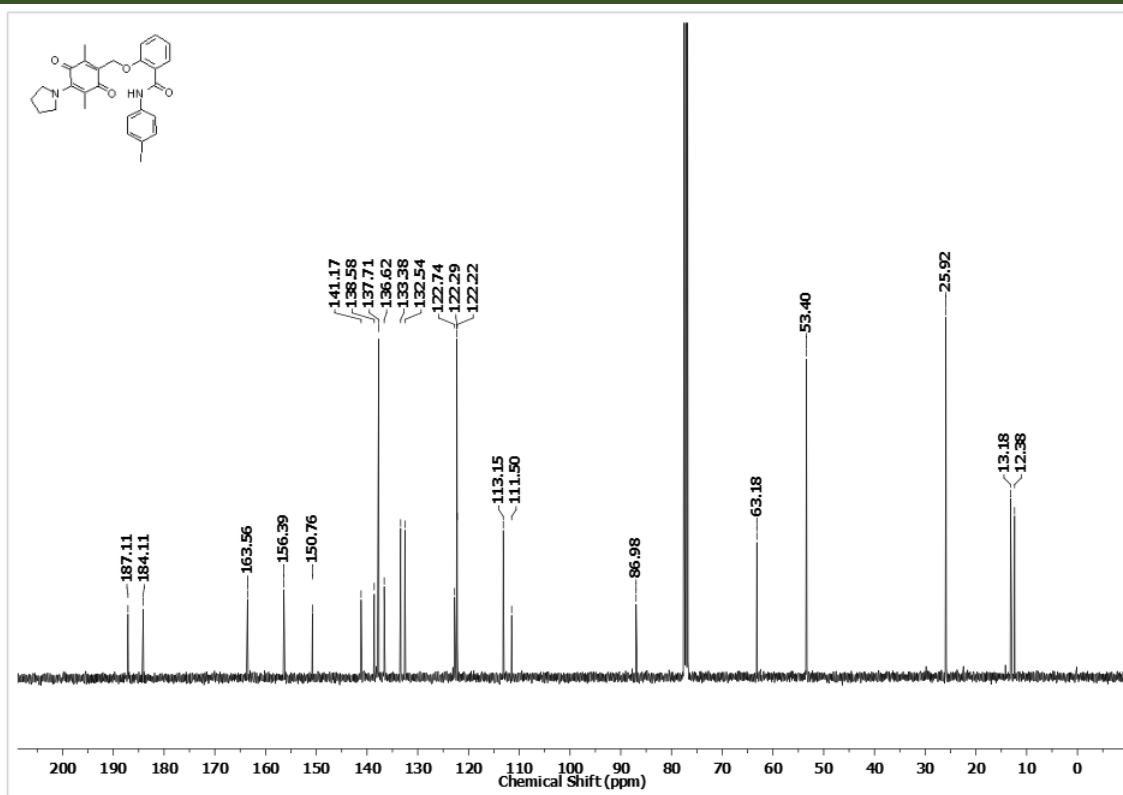


Figure 70: 101 MHz ^{13}C spectrum of **3** in CDCl_3

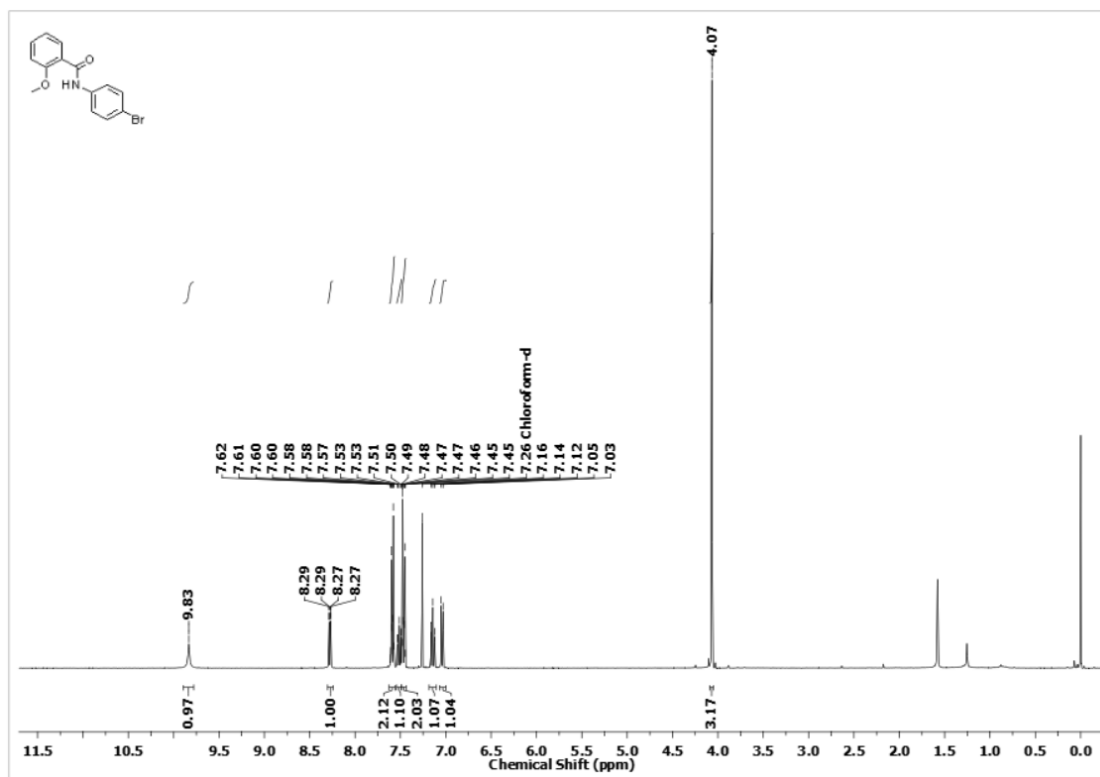


Figure 71: 400 MHz ^1H spectrum of **5** in CDCl_3

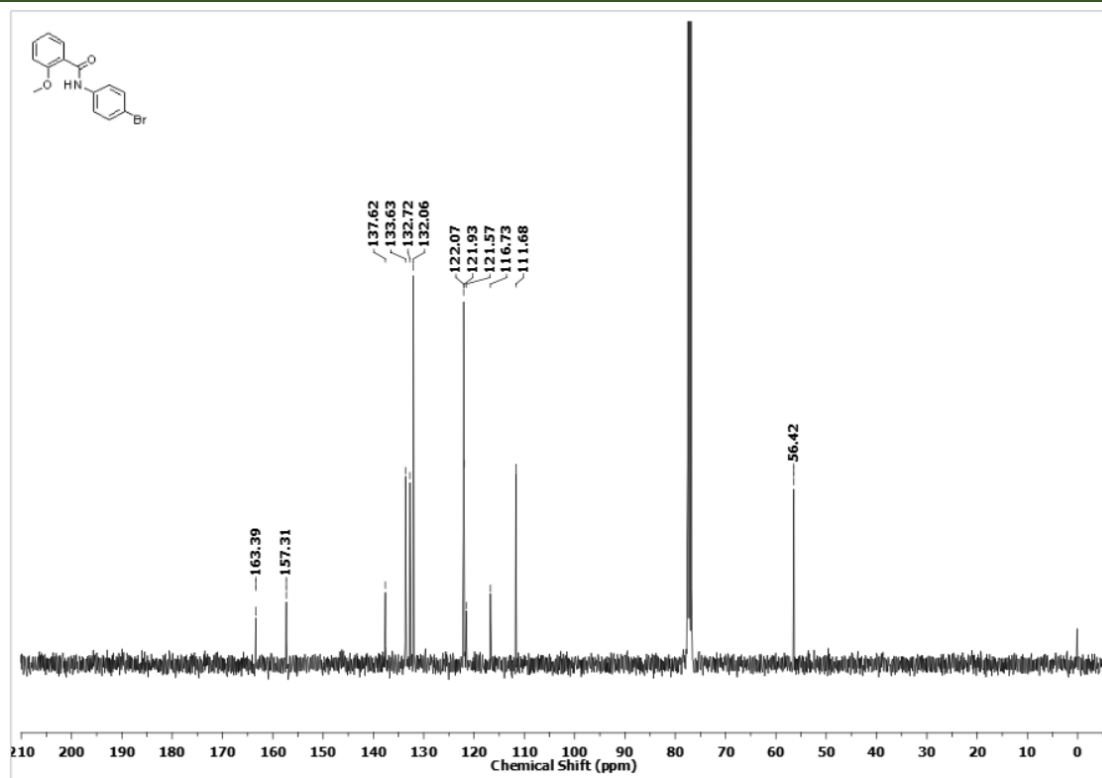


Figure 72: 101 MHz ^{13}C spectrum of 5 in CDCl_3

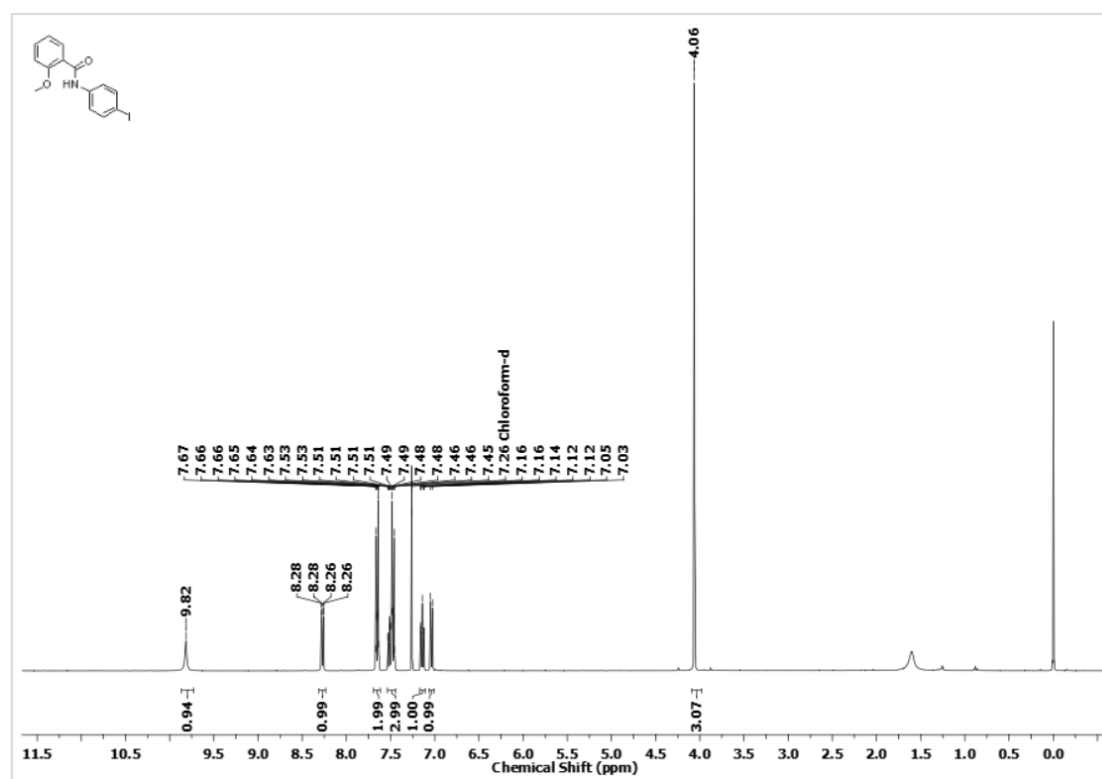


Figure 73: 400 MHz ^1H spectrum of 6 in CDCl_3

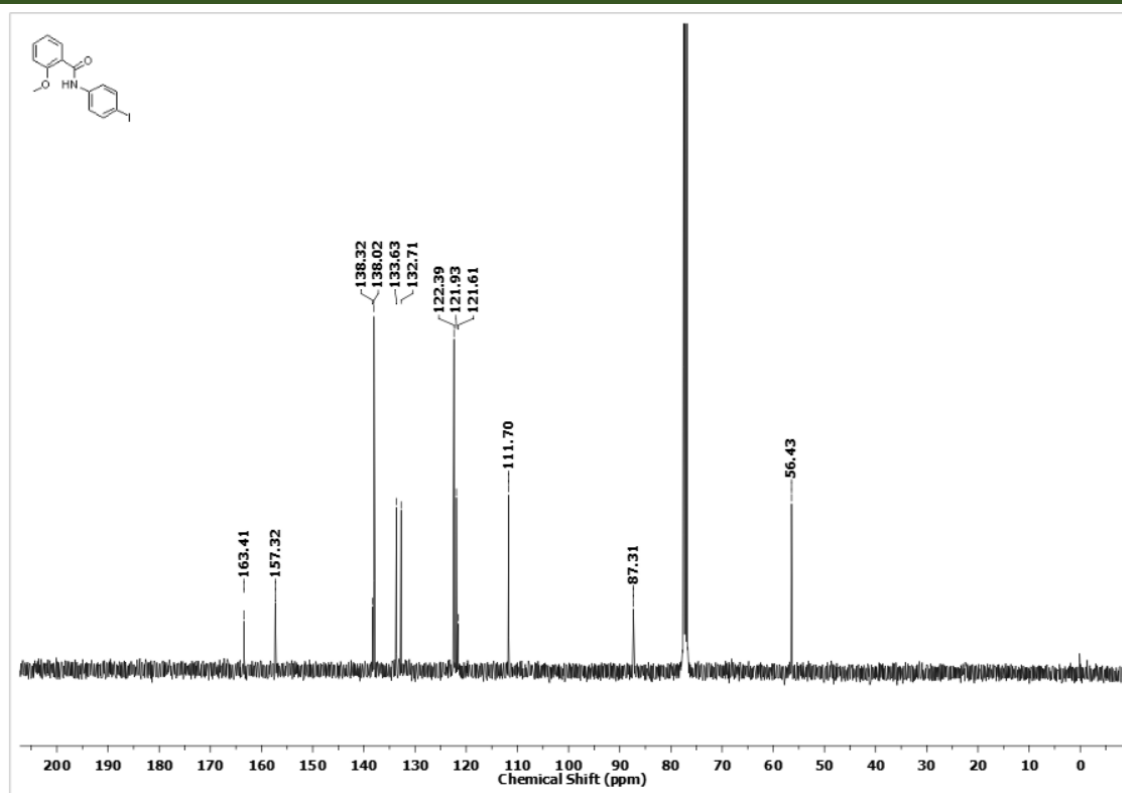


Figure 74: 101 MHz ^{13}C spectrum of **6** in CDCl_3

3.6 References

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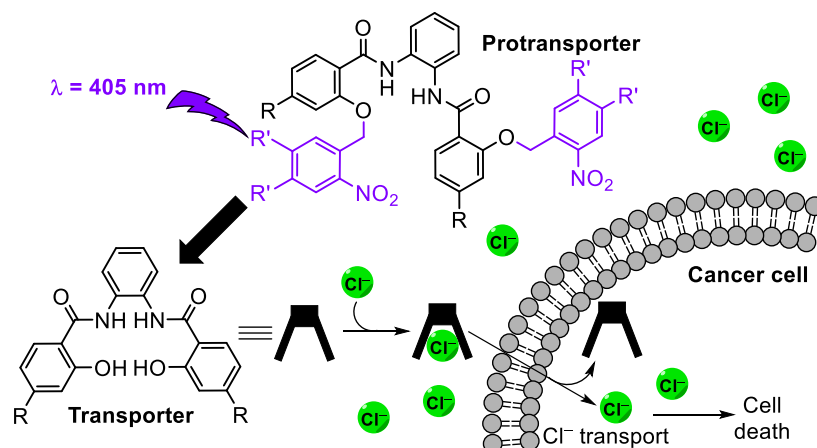
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Chapter 4

Photoactivable Bis(salicylamide) Transporters for Anticancer Applications



4.1 Introduction

The high level of spatiotemporal control offered by light and its benign nature make it an ideal external trigger for use in biological systems,¹⁻⁴ and hence suitable for protransporter activation in cancer tissue. Most existing reports of photoactivated protransporters utilized light in the UV region,⁵⁻⁸ which is harmful to the surrounding healthy tissue and has low penetration power.^{9, 10} There is a need for a shift to visible light for protransporter activation due to its non-toxic nature and better tissue penetrating properties.^{4, 11} This can be achieved by the use of photocleavable protecting groups (PPGs) removable in the visible region of the electromagnetic spectrum. The *o*-nitrobenzyl group is a highly versatile PPG whose activation wavelength can be tuned via variation of the phenyl ring substitution¹ and was chosen for preparation of the protransporter.

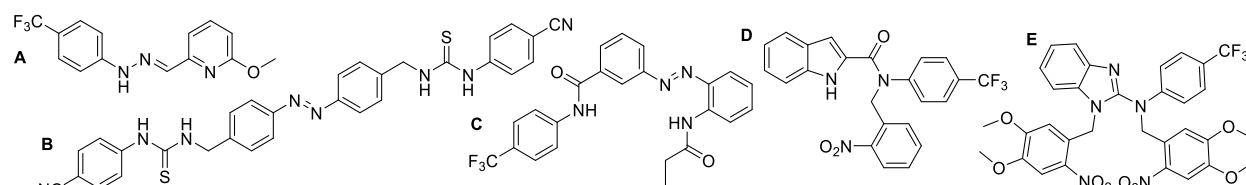


Figure 1: Photoactivable ion transporters.

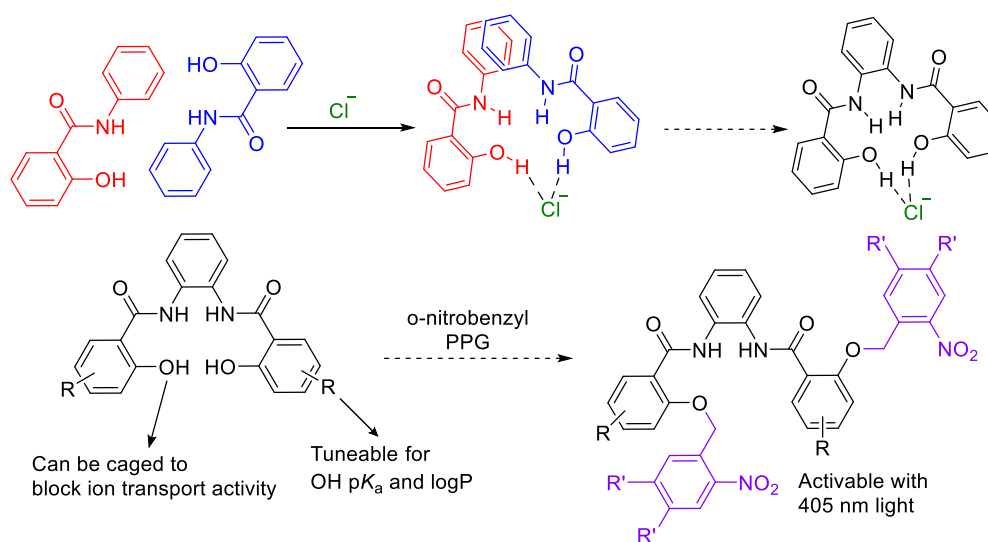


Figure 2: Design of bis(salicylamide) transporters and protransporters.

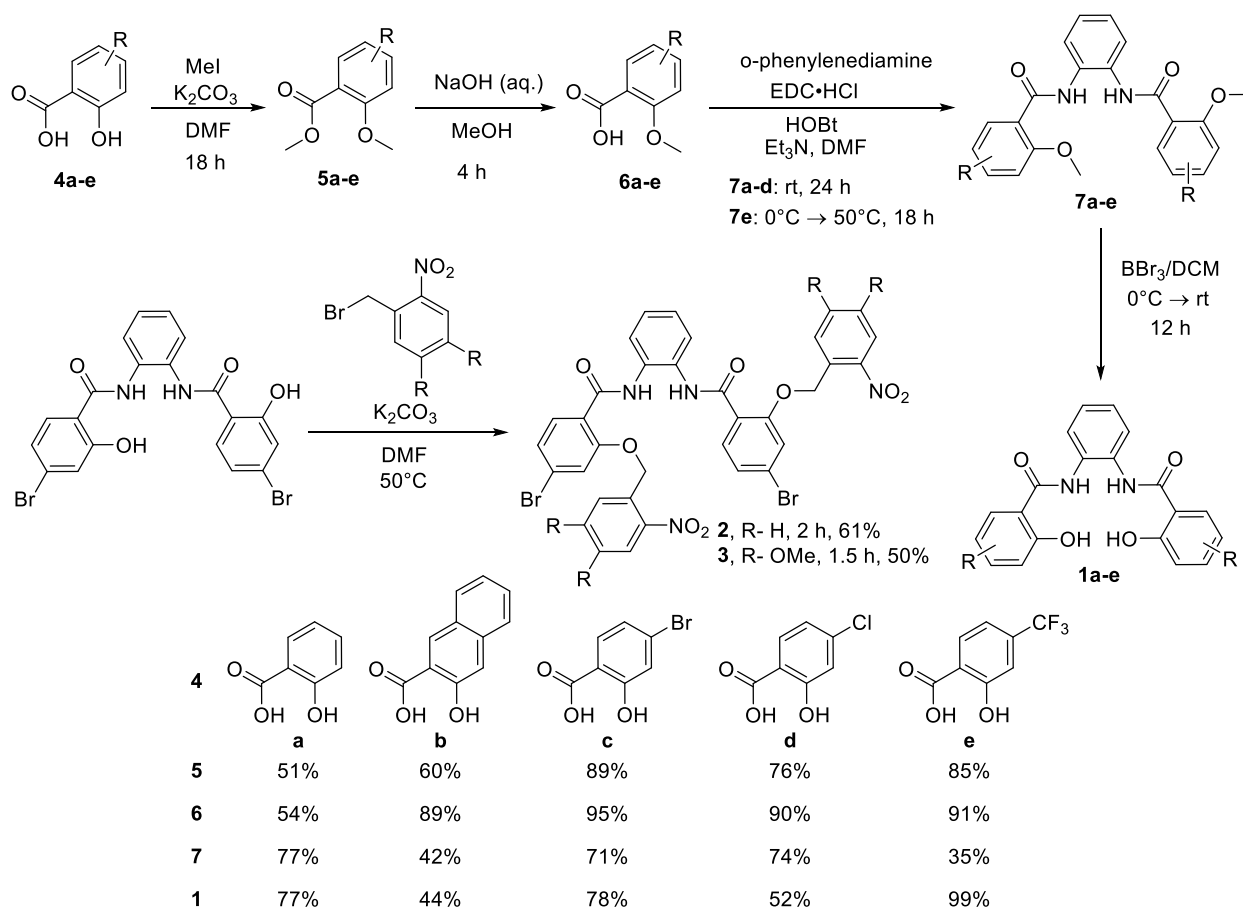
Following the successful demonstration of the salicylanilide system as an effective ion transporter, where the OH group played a key role in the transport process, it was extended to the larger bis(salicylamide) system. Here, tuning of both OH pK_a and $\log P$ would be possible through variation of substitution of the salicyl ring, and caging of ion transport activity can be achieved by attachment of *o*-nitrobenzyl moieties to the OH groups. Light in the violet region (405 nm) was

chosen for photoactivation as substituted *o*-nitrobenzyl groups are known to have absorbance in that spectral region. Although violet light has a low tissue penetrating ability, the advances in optical fibre technology allow for the internal application of light through minimally invasive surgical procedures,^{12, 13} making its use viable in a therapeutic setting.

4.2 Results and Discussions

4.2.1 Synthesis

The bis(salicylamide) transporters were synthesized starting from the respective substituted salicylic acid derivatives (**4a-4e**) (Scheme 1). Methylation of salicylic acid OH and COOH groups was conducted using iodomethane, which was followed by base hydrolysis of the resultant ester. The resultant methylated salicylic acid (**6a-6e**) was coupled to *o*-phenylenediamine using EDC·HCl/HOBt to form the amide, followed by demethylation of the OMe groups using BBr₃ to yield the described transporter derivatives (**1a-1e**).



Scheme 1: Synthesis of transporters **1a-1e** and protransporters **2-3**.

The protransporters **2** and **3** were synthesized by linking the corresponding *o*-nitrobenzyl moieties to transporter **1c** by reaction of their benzyl bromide derivatives in the presence of K₂CO₃ as the base. The photocleavage controls **8** and **9** were synthesized as per a previous report.¹⁴

4.2.2 log*P* and p*K*_a Calculations

The p*K*_a values of amide N-H protons of the *N*-salicylamides were estimated using calculator plugins of MarvinSketch v23.11.0¹⁵ (Table 1). The p*K*_a values of O-H protons varied from 6.97 to 8.23 depending on the salicyl group substitution, while the N-H groups gave values in the 15.13 to 16.08 range. The predicted log*P* values were between 3.55 and 5.53, in the optimal range for an ion transporter (Table 1). The log*P* values for the protransporters were predicted to be 8.71 and 8.08 for **2** and **3**, respectively, thereby indicating a good membrane permeability of these compounds (Table 1).

Table 1: Estimated log*P*, and N-H, O-H p*K*_a values of compounds **1a-1e**, **2**, and **3**. Values were estimated using calculator plugins of MarvinSketch.

Compound	log <i>P</i>	O-H _A /O-H _B p <i>K</i> _a	N-H _A /N-H _B p <i>K</i> _a
1a	3.55	8.23/7.63	16.08/15.40
1b	5.53	8.00/7.40	16.03/15.36
1c	5.09	7.06/7.69	15.96/15.28
1d	4.76	6.97/7.60	15.81/15.13
1e	5.31	7.30/7.96	15.82/15.14
2	8.71	-	14.09/14.77
3	8.08	-	14.09/14.77

4.2.3 Activity Screening

The ion transport abilities of the transporters **1a-1e** were tested across EYPC-LUVs⇌HPTS. Data from dose-dependent studies of **1a-1e** (Figures 3-7) were fitted to the Hill equation to yield the *EC*₅₀ values (Table 2), which showed the order of transport activity to be **1e** > **1c** > **1d** > **1b** >> **1a**. Hill coefficients (*n*) of transporters were found to be ~1, indicating the formation of an ion transport complex with one molecule of the transporter and one ion.

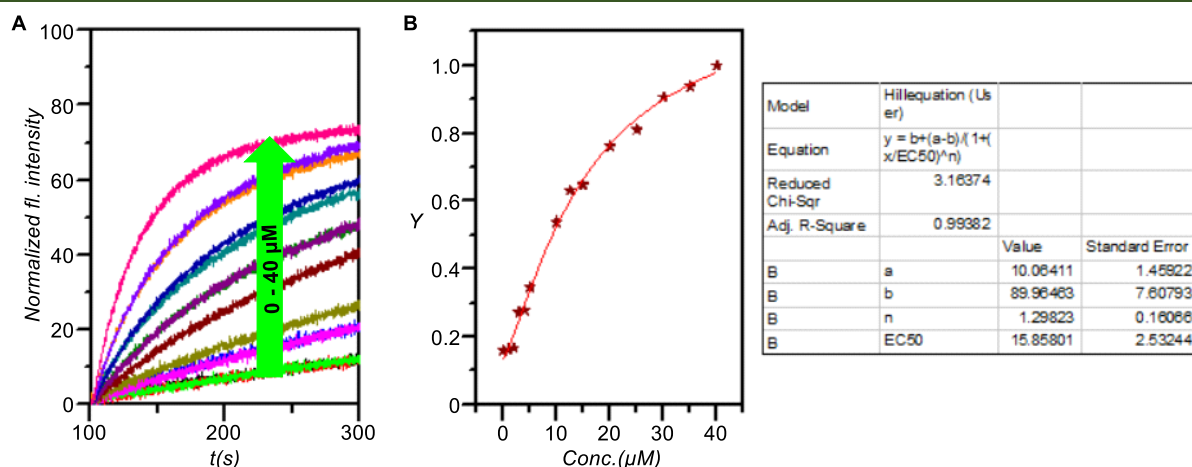


Figure 3: (A) Dose-dependent activity of compound **1a** across EYPC-LUVs>HPTS. (B) Hill plot analysis of **1a** at $t = 290$ s.

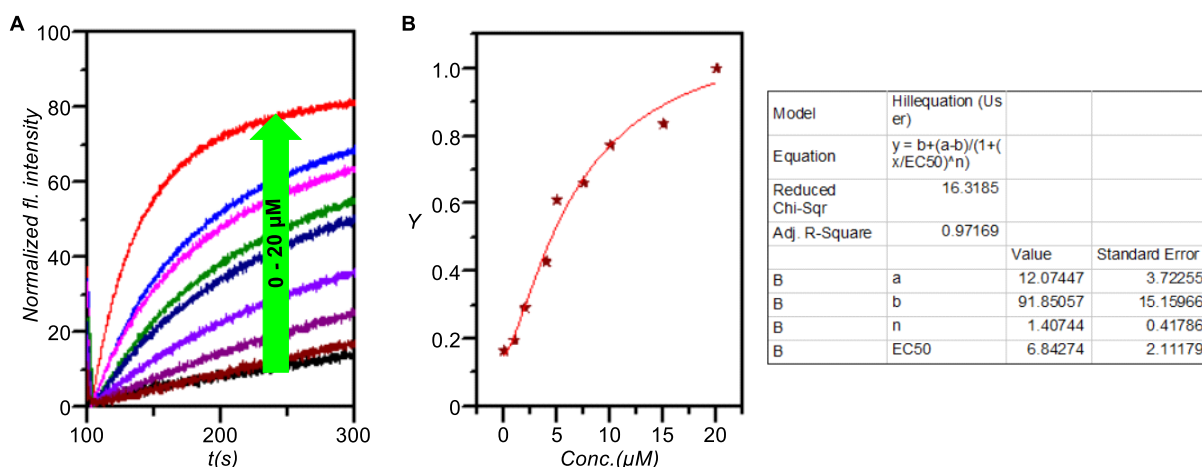


Figure 4: (A) Dose-dependent activity of compound **1b** across EYPC-LUVs>HPTS. (B) Hill plot analysis of **1b** at $t = 290$ s.

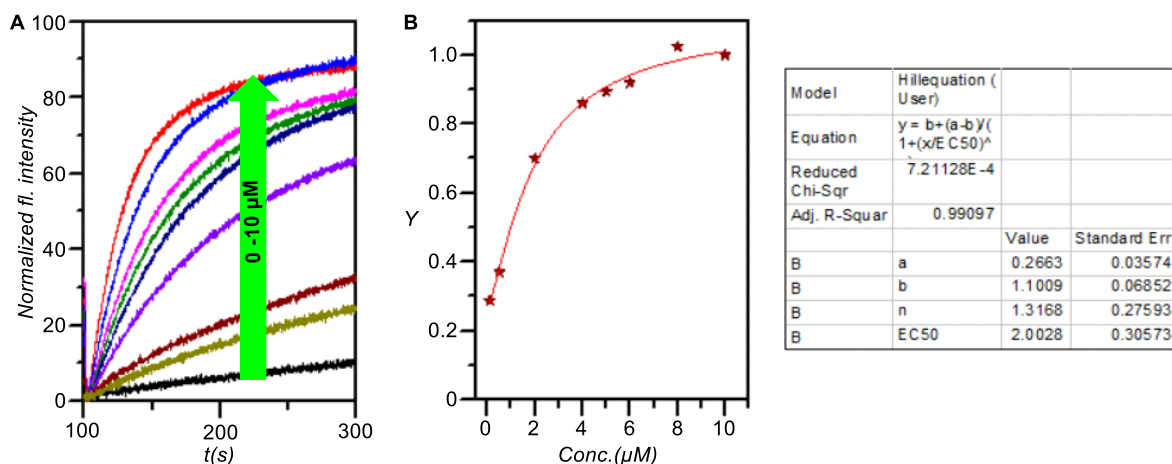


Figure 5: (A) Dose-dependent activity of compound **1c** across EYPC-LUVs>HPTS. (B) Hill plot analysis of **1c** at $t = 290$ s.

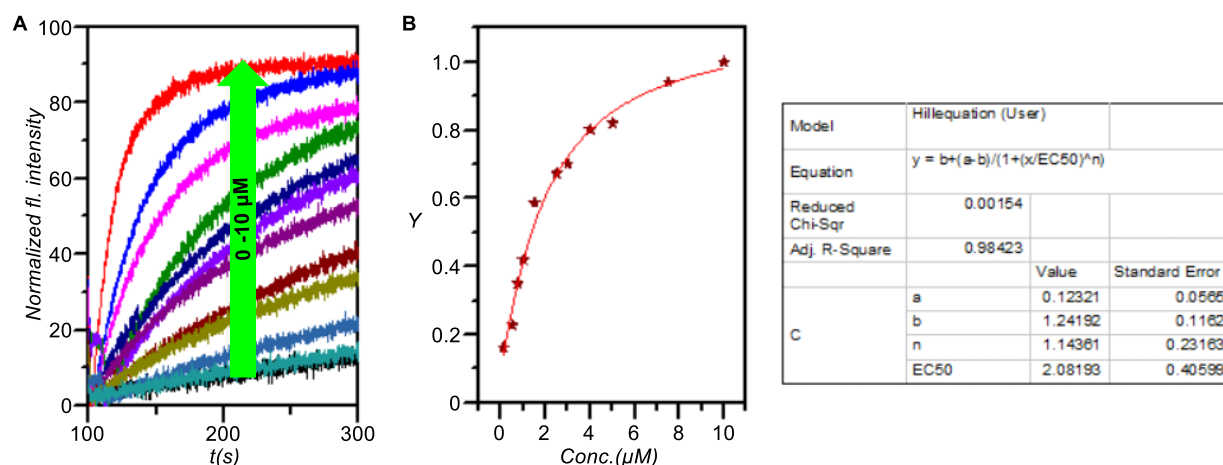


Figure 6: (A) Dose-dependent activity of compound **1d** across EYPC-LUVs⊃HPTS. (B) Hill plot analysis of **1d** at $t = 290$ s.

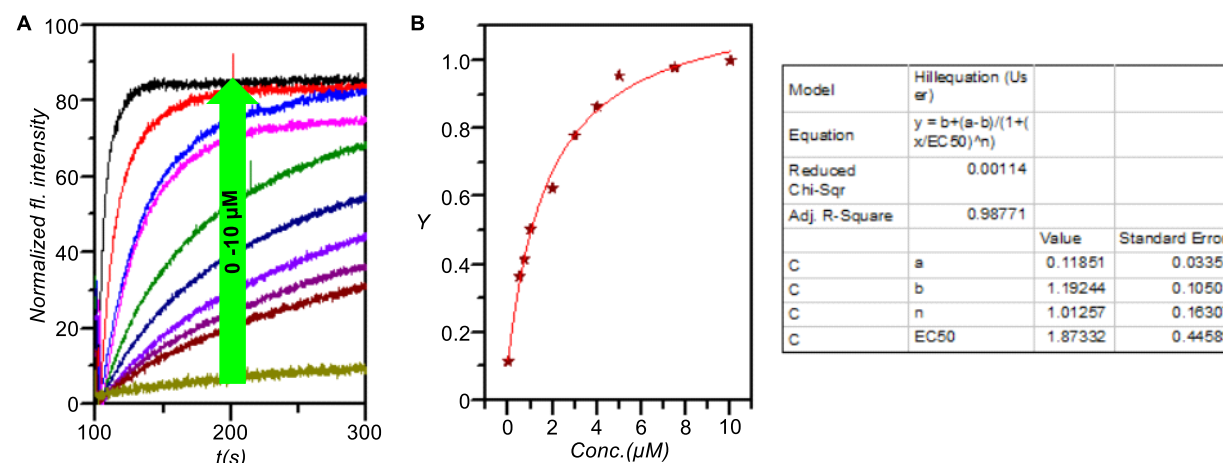


Figure 7: (A) Dose-dependent activity of compound **1e** across EYPC-LUVs⊃HPTS. (B) Hill plot analysis of **1e** at $t = 290$ s.

Table 2: Summary of Hill analyses of dose-dependent ion transporter studies across EYPC-LUVs⊃HPTS.

Compound	EC ₅₀ (μM)	Hill coeff. (n)
1a	15.86	1.30
1b	6.84	1.40
1c	2.00	1.31
1d	2.08	1.14
1e	1.87	1.01

The cytotoxicity of transporters **1a-1e** was assessed in the MCF-7 breast cancer cell line (Figure 8), where treatment at 50 μM concentration yielded activity of the order **1c** > **1d** > **1b** > **1e** > **1a**. The Br-substituted transporter was observed to exhibit the highest cytotoxicity despite being only the second most active ion transporter as assessed in EYPC liposomes. This was likely due to the differences in lipid membrane composition in the cancer cell compared to the liposome. Further studies were conducted using transporter **1c** since it was most suitable for anticancer applications. The IC_{50} of **1c** was determined to be 28.7 μM from dose-dependent studies (Figure 9).

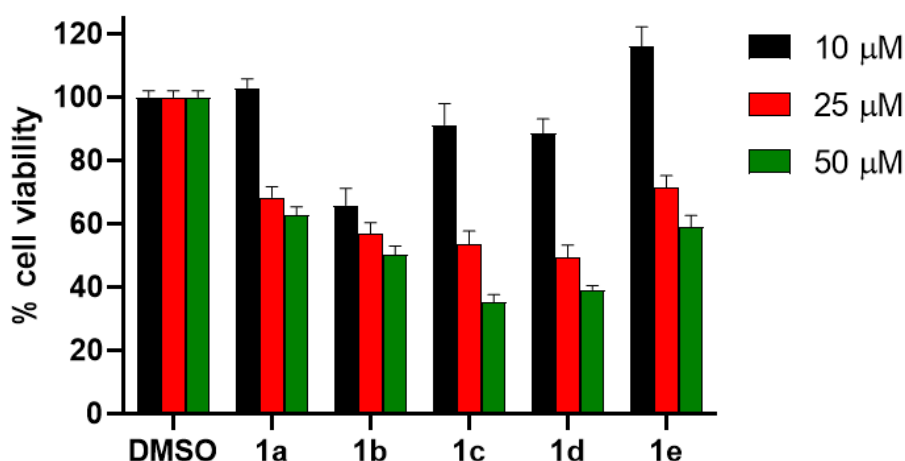


Figure 8: Cytotoxicity of bis(salicylamides) towards MCF-7 cells at 10, 25 and 50 μM concentrations.

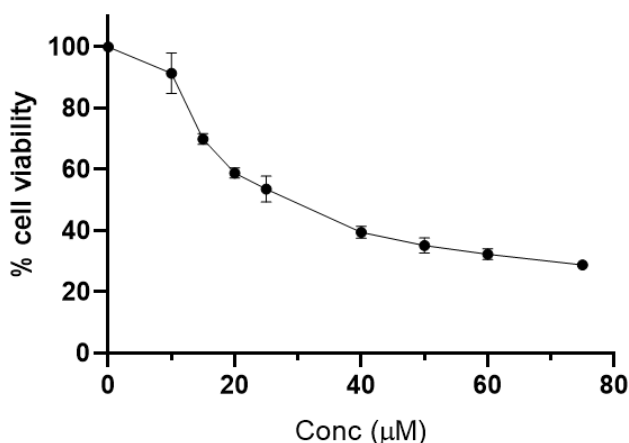


Figure 9: Dose-dependent activity of **1c** towards MCF-7 cells.

4.2.4 Mechanistic Studies and Theoretical Calculations

To elucidate the ion transport mechanism of the bis(salicylamide) system, the transport activity of **1c** (3 μM) was assessed across EYPC-LUVs $\Rightarrow$ HPTS while varying the anions (Cl^- , Br^- , I^- , NO_3^- ,

ClO_4^- , and AcO^-) in the extravesicular buffer. A significant difference in ion transport ability was observed, with external Cl^- exhibiting the highest activity (Figure 10A), indicating the transport process to be anion-dependent. Hence, the transporter was tested across EYPC-LUVs entrapping lucigenin (a Cl^- sensing dye), i.e., EYPC-LUVs $\supset$ lucigenin, where a Cl^- gradient was generated across the lipid membrane (Figure 10B). Dissipation of this gradient by **1a-1e** was observed with the order of activity **1e** > **1c** > **1d** > **1b** > **1a**, which was the same as that in the HPTS assay. Dose-dependent studies of **1c** yielded $n \approx 1$ ($n = 1.34$, $EC_{50} = 3.66$) (Figure 11), which was consistent with data from the HPTS assay. This was cross-verified using **1e**, which gave $n = 1.13$ and $EC_{50} = 2.15$ (Figure 12). Variation of extravesicular cations (Li^+ , Na^+ , K^+ , Rb^+ , and Cs^+) in EYPC-LUVs $\supset$ lucigenin showed no change in ion transport activity of **1c** (5 μM) confirming cation independent mechanism of ion transport (Figure 13A). The chloride transport ability of **1c** was observed to be significantly enhanced in the presence of valinomycin (K^+ transporter), which indicated anion antiport mechanism of ion transport (Figure 13B).

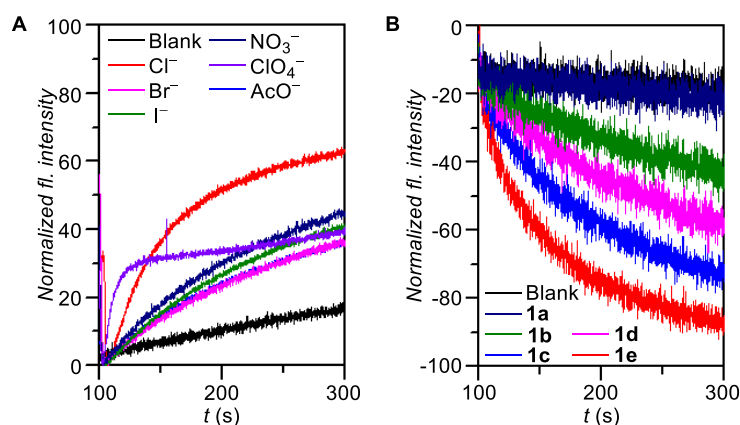


Figure 10: (A) Anion selectivity assay of **1c** (3 μM) across EYPC-LUVs $\supset$ HPTS. (B) Comparison of transport activities of **1a-1e** (5 μM) across EYPC-LUVs $\supset$ lucigenin.

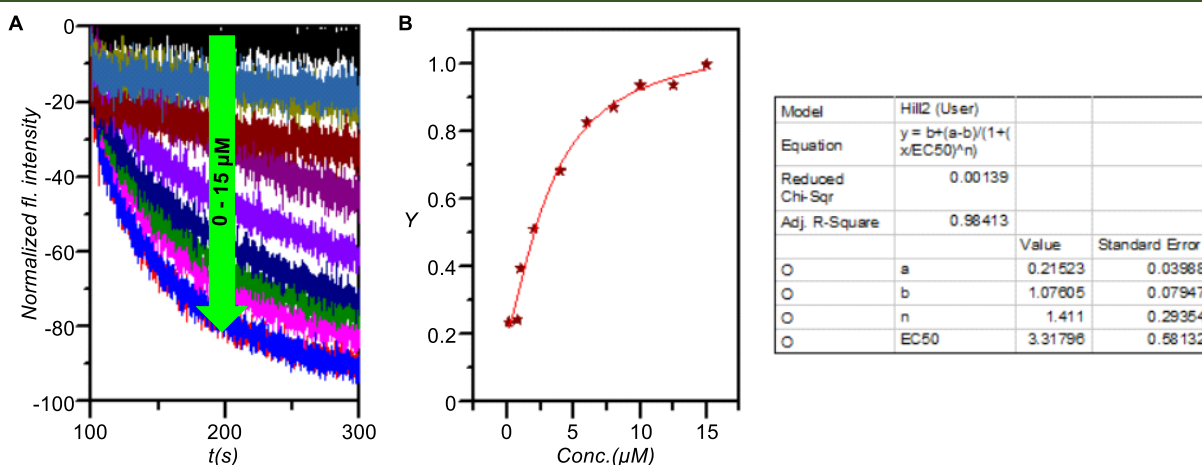


Figure 11: (A) Dose-dependent activity of compound **1c** across EYPC-LUVs⊃lucigenin. (B) Hill plot analysis of **1c** at $t = 290$ s.

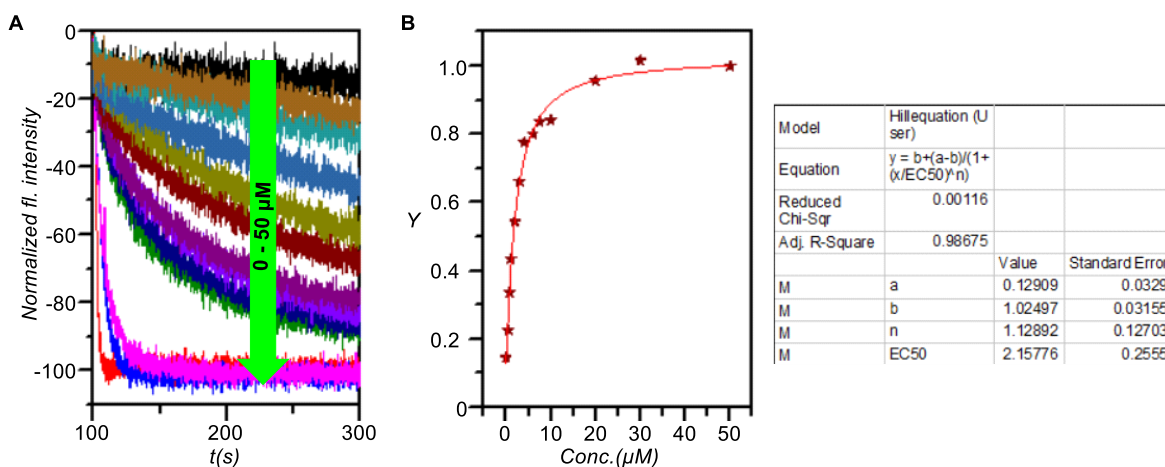


Figure 12: (A) Dose-dependent activity of compound **1e** across EYPC-LUVs⊃HPTS. (B) Hill plot analysis of **1e** at $t = 290$ s.

Table 3: Summary of Hill analyses of dose-dependent ion transporter studies across EYPC-LUVs⊃lucigenin.

Compound	EC ₅₀ (μM)	Hill coeff. (n)
1c	3.66	1.34
1e	2.15	1.13

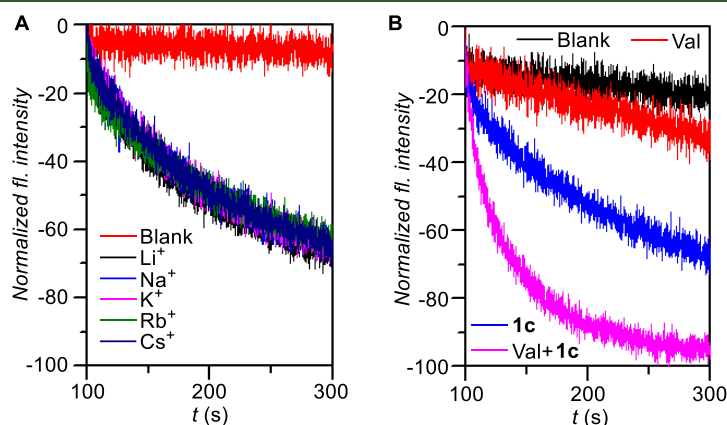


Figure 13: (A) Cation selectivity assay of **1c** (5 μ M) and (B) valinomycin (Val, 0.5 μ M) coupled assay of **1c** (3 μ M) across EYPC-LUVs containing lucigenin.

Carrier mechanism of transport by the bis(salicylamide) was verified through the classical U-tube experiment (Section 4.4.5).^{14, 16} Transport of chloride from the source arm (0.5 M NaCl) to the receiver arm (0.5 M NaNO₃) through bulk CHCl₃ in the presence of transporter **1c** (1 mM) was observed, which indicates carrier mode of anion transport (Figure 14). A dye leakage assay was performed across EYPC-LUVs containing carboxyfluorescein (CF) to probe for the possibility of lipid membrane disruption by the transporters. Negligible CF leakage was observed on treatment with **1c** and **1e** up to 20 μ M concentration (Figure 15), indicating liposomal membrane integrity is preserved during the transport process.

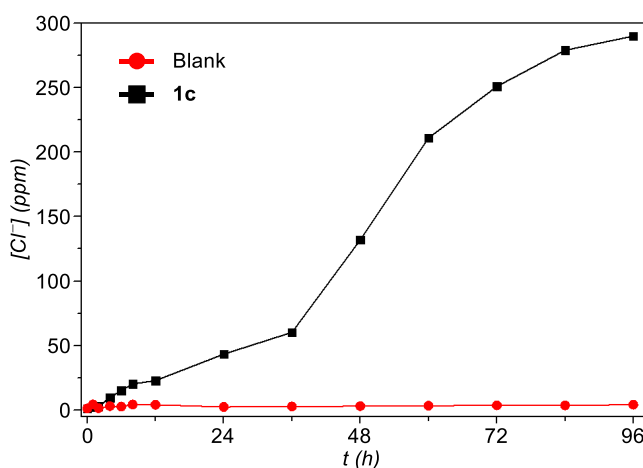


Figure 14: Chloride transport across CHCl₃ layer in U-tube experiment in the presence (2 mM) and absence of transporter **1c**.

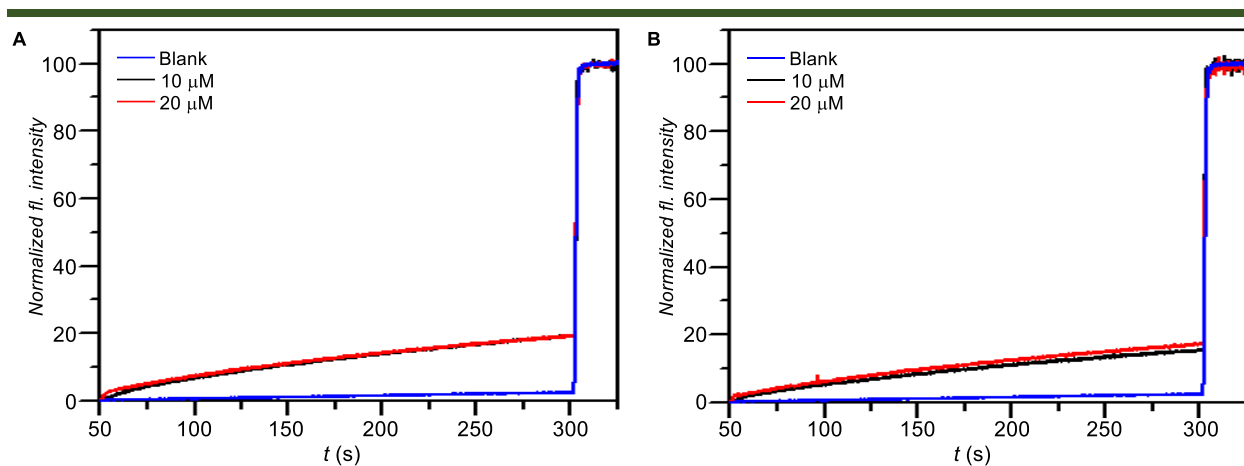


Figure 15: CF leakage assays of (A) **1c** and (B) **1e** showing little change in dye fluorescence, indicating preservation of vesicle integrity in the presence of the transporters.

The transporter **1c** was titrated with TBACl (Cl^- source) and monitored through ^1H NMR to assess its anion binding ability. Both N-H (H_a) and O-H (H_b) protons were observed to show downfield shifts along with aromatic C-H protons H_c and H_d (Figure 16). The binding constant was calculated from the titration data using BindFit program,¹⁷ where it fit to the 1:1 transporter-ion binding model with $K_a(1:1) = 205.7 \pm 5.3 \text{ M}^{-1}$. The binding stoichiometry was the same as that observed for the ion transporter complex where $n \approx 1$. The locations of protons involved in the anion binding indicate the presence of more than one ion binding mode, as it is not possible for all four protons to interact with the anion in a single conformation. This was likely due to the salicylamide arms adopting ‘open-ring’ and ‘closed-ring’ conformations,¹⁸ both of which contribute to chloride binding in different binding modes. The evidence of these conformers was obtained from the crystals of **1a** and **1b**, where the salicylamide arms adopted ‘closed-ring’ and ‘open-ring’ structures, respectively (Figure 17). Attempts at co-crystallization of transporters with Cl^- yielded no results, and hence, theoretical calculations were conducted to shed light on the structure of the $[\mathbf{1c}+\text{Cl}^-]$ complex based on evidence from ion transport and ion titration (NMR) experiments.

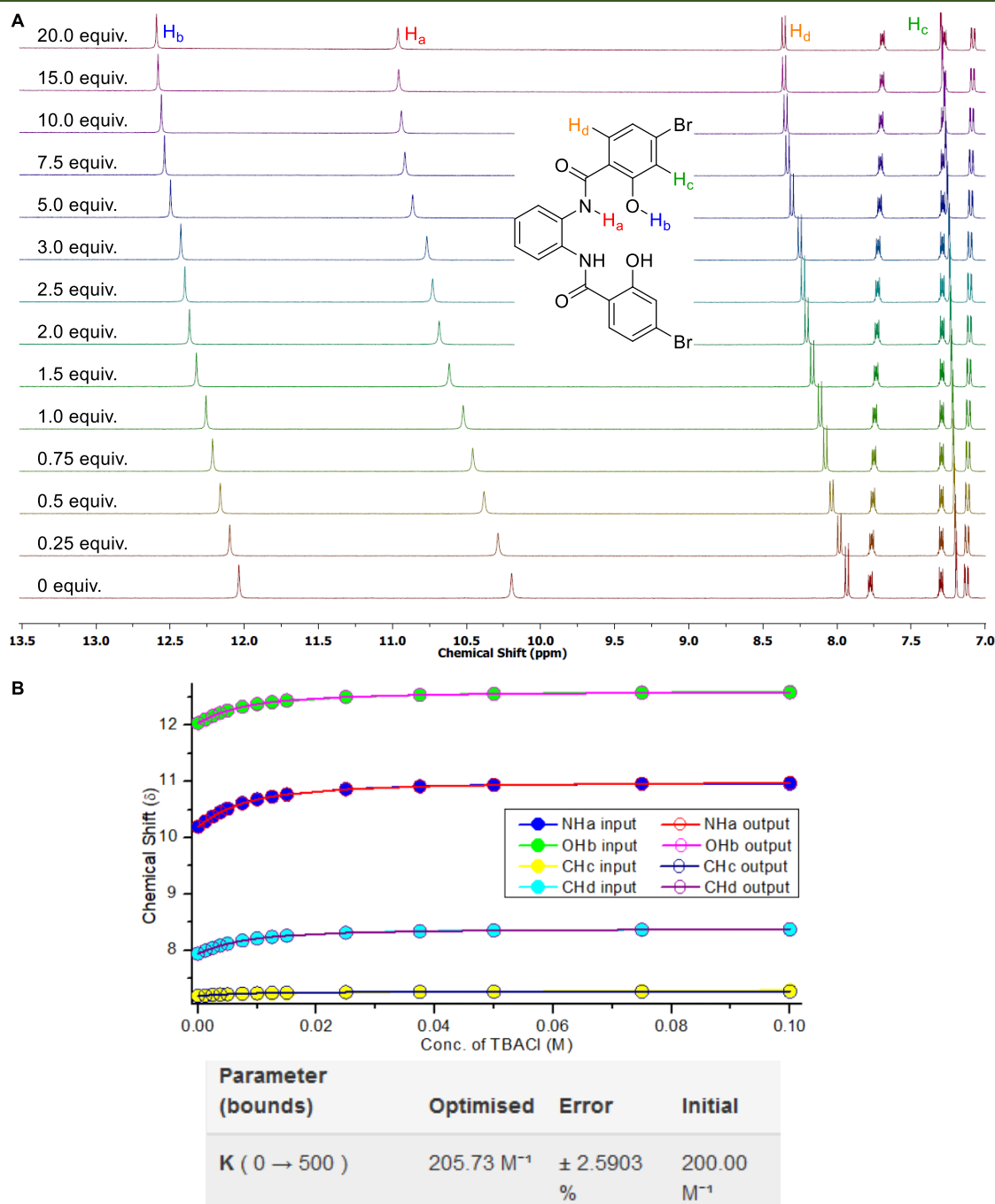


Figure 16: (A) ^1H NMR titration spectra of **1c** (5 mM) with stepwise addition of TBACl in 13.5% DMSO- d_6 in CD_3CN . The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) The plot of chemical shifts (δ) of protons H_a , H_b , H_c , and H_d vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

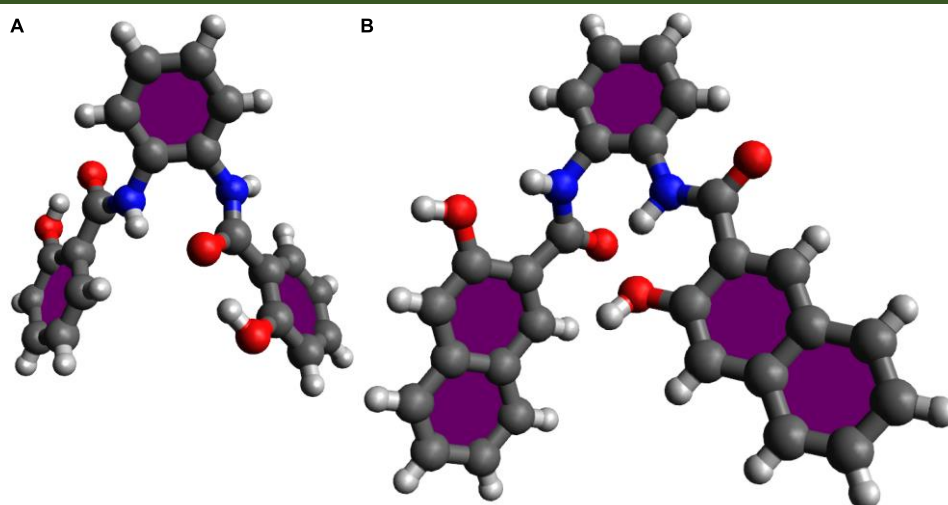


Figure 17: Molecular structures of (A) **1a** and (B) **1b** showing salicylamide arms in ‘closed-ring’ and ‘open-ring’ conformations respectively. Structures obtained from SCXRD data.

Predictions for the most probable geometry of $[2c+Cl^-]$ were carried out with CONFLEX 8 conformation search software package^{19, 20} using MMFF94S forcefield. The output gave one major conformation with Boltzmann populations 99.99% (**CBr1**), which had salicylamide arms in the ‘open-ring’ conformation (Figure 18A). To obtain the conformer with ‘closed-ring’ salicylamide arms, the structure was built using Chem3D (v22.2.0), and the chloride ion was positioned in the likely binding site (**CBr2**) (Figure 18B). The geometry optimization of these conformers was then conducted on Gaussian 09 program suite²¹ using B3LYP exchange-correlation functional^{22, 23} and 6-31G(d,p) basis set.^{24, 25} The geometry optimized outputs of the two possible interchangeable conformers of $[1c+Cl^-]$ were obtained, wherein the ‘open-ring’ **CBr1** conformation the O-H_b and C-H_c protons are involved in anion binding (Figure 18C), while in the ‘closed-ring’ **CBr2** conformation the N-H_a and C-H_d protons bind to the anion (Figure 18D). Both structures have similar HF and binding energies, indicating both are equally likely as ion transport complexes and might exist in an equilibrium with each other.

For $[1c+Cl^-]$ (**CBr1**):

- HF energy = -6784.7876644 Hartree
- Zero-point energy = 0.3058071 Hartree
- BSSE = 0.007549698086 Hartree
- Binding energy = -0.075309702 Hartree = -47.25759105 kcal/mol

For $[1c+Cl^-]$ (CBr2):

- HF energy = -6784.7891867 Hartree
- Zero-point energy = 0.3056581 Hartree
- BSSE = 0.007304092 Hartree
- Binding energy = -0.077226608 Hartree = -48.46046876 kcal/mol

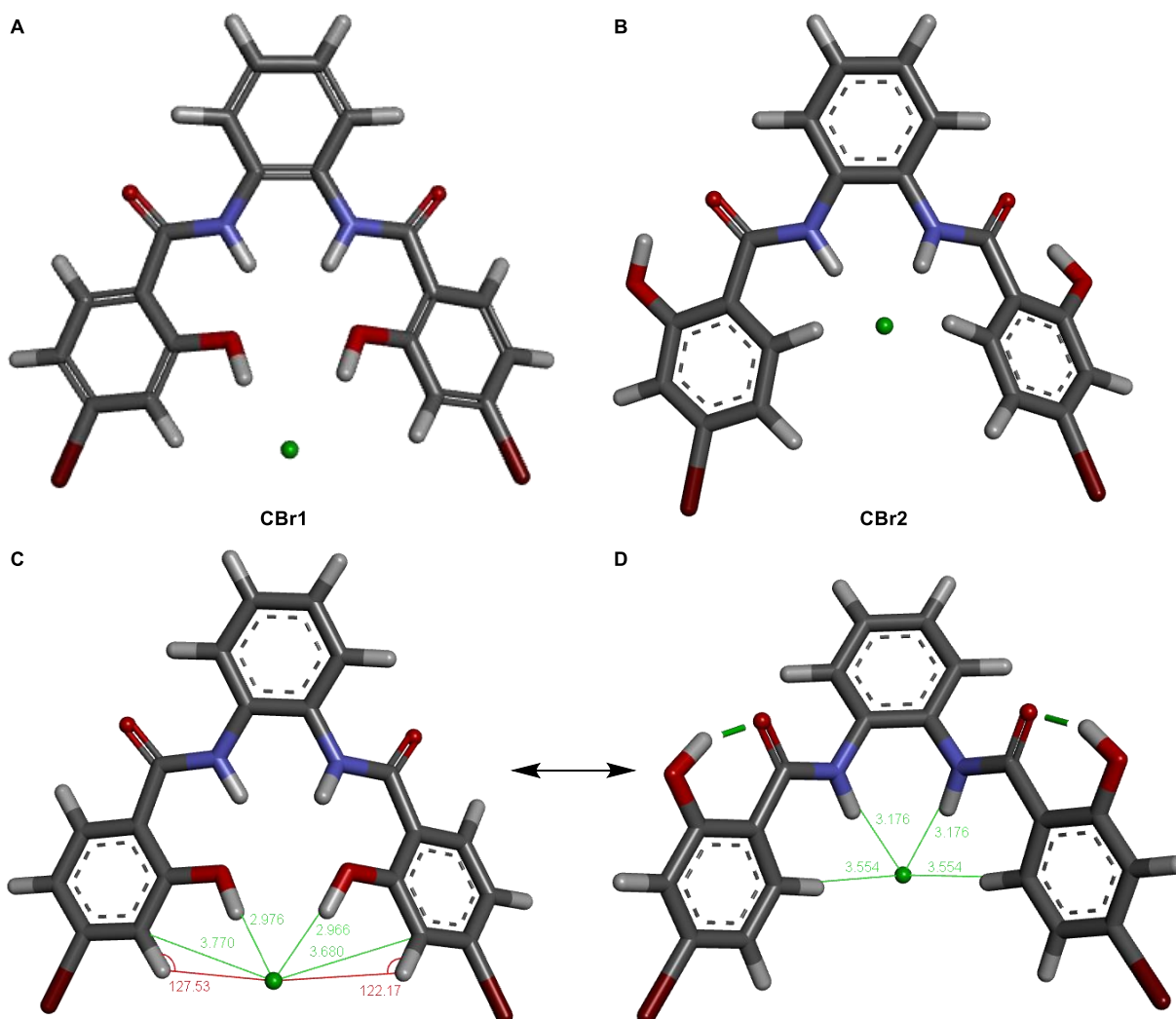


Figure 18: (A)-(B) Predicted majority geometries of $[1c+Cl^-]$. (C)-(D) Geometry optimized structures of ion transporter complexes.

4.2.5 Photoactivation Studies

The ion transport activities of protransporters **2** and **3** ($5\ \mu\text{M}$) were tested across EYPC-LUVs>HPTS. Both compounds exhibited negligible ion transport activity compared to free

transporter **1c** (Figure 19A), indicating successful caging of activity by the *o*-nitrobenzyl groups. Absorbance spectra of **2** and **3** were recorded in DMSO, where protransporter **3** was found to have a significant absorbance at 405 nm while **2** showed only a very mild absorption at that wavelength (Figure 19B). Hence, **3** was expected to undergo faster photoactivation under violet light in comparison to **2**.

The photoactivated release of the active transporter **1c** from **2** and **3** on photoirradiation was monitored using NMR (Figures 20-23). The appearance of a new set of peaks, which then disappeared with an increase in photoirradiation time was observed that corresponded to the formation of an intermediate with one free OH. This indicated the photorelease to be a two-step process, where one of the *o*-nitrobenzyl groups gets released forming an intermediate that then undergoes a second photoactivation step. Overall, with increasing time of photoirradiation, the disappearance of benzyl CH₂ (H_d) peak was observed along with the appearance of the free OH (H_b) peak. Formation of the CHO (H_c) peak from the *o*-nitrosobenzaldehyde byproduct was transiently observed owing to the unstable nature of the molecule.

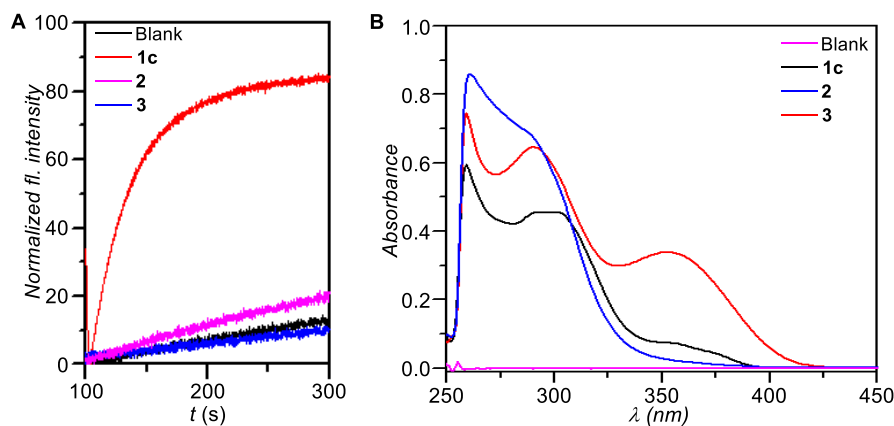


Figure 19: (A) Comparison of ion transport activities of transporter **1c** and protransporters **2** and **3** (5 μ M) across EYPC-LUVs⊃HPTS. (B) Absorbance spectra of **1c**, **2**, and **3** (25 μ M) in DMSO.

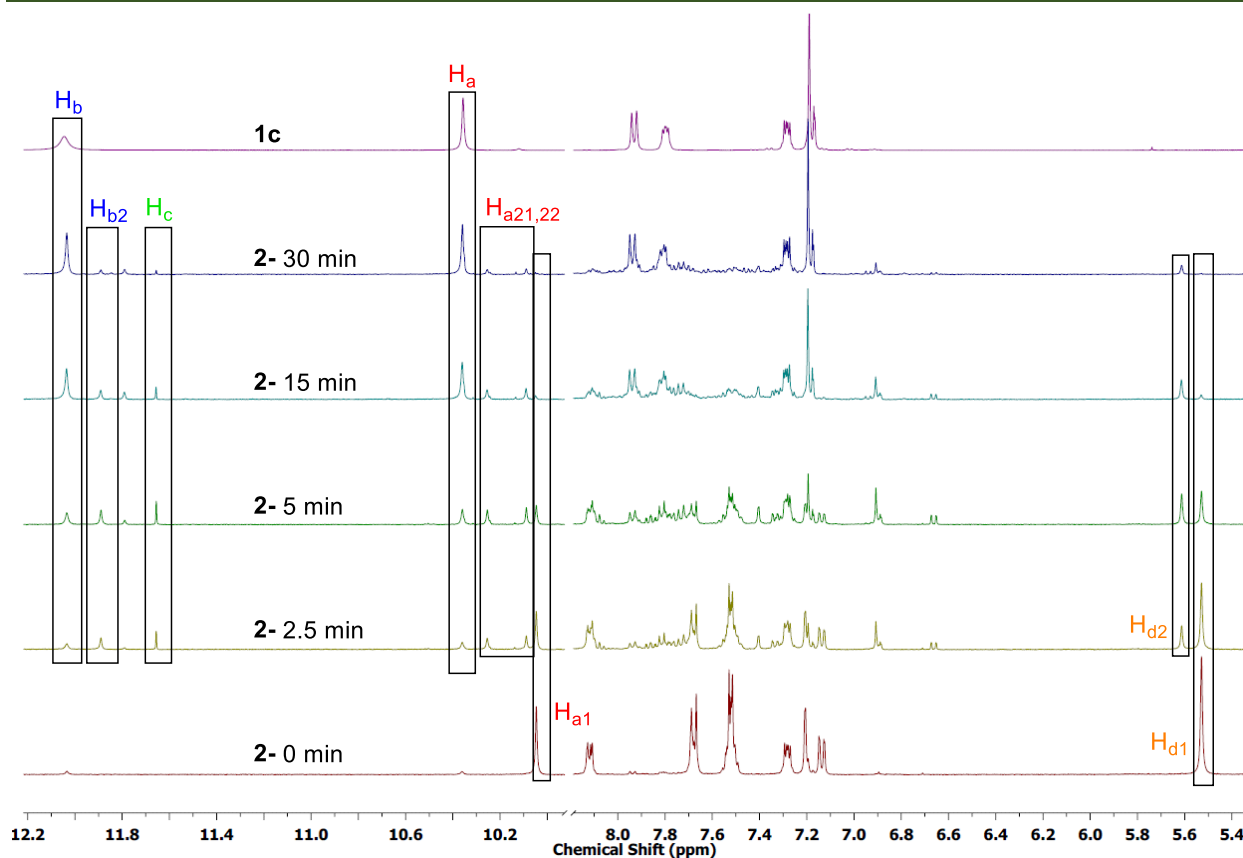


Figure 20: Change in ^1H NMR spectrum of **2** on irradiation with 405 nm light. Solvent- $\text{DMSO-}d_6$.

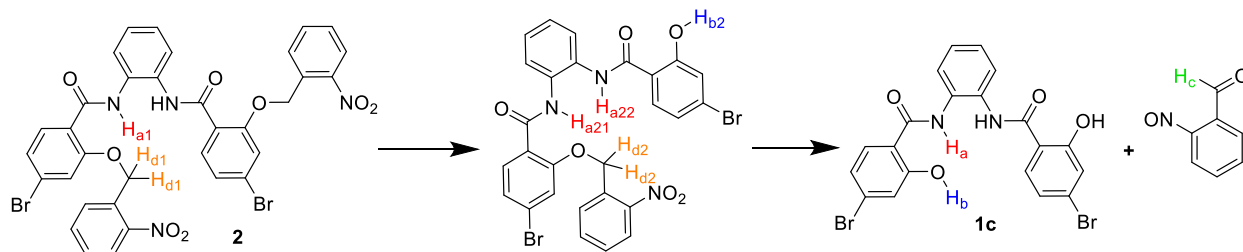


Figure 21: Mechanism of two step photoactivation of protransporter **2** on irradiation with 405 nm light.

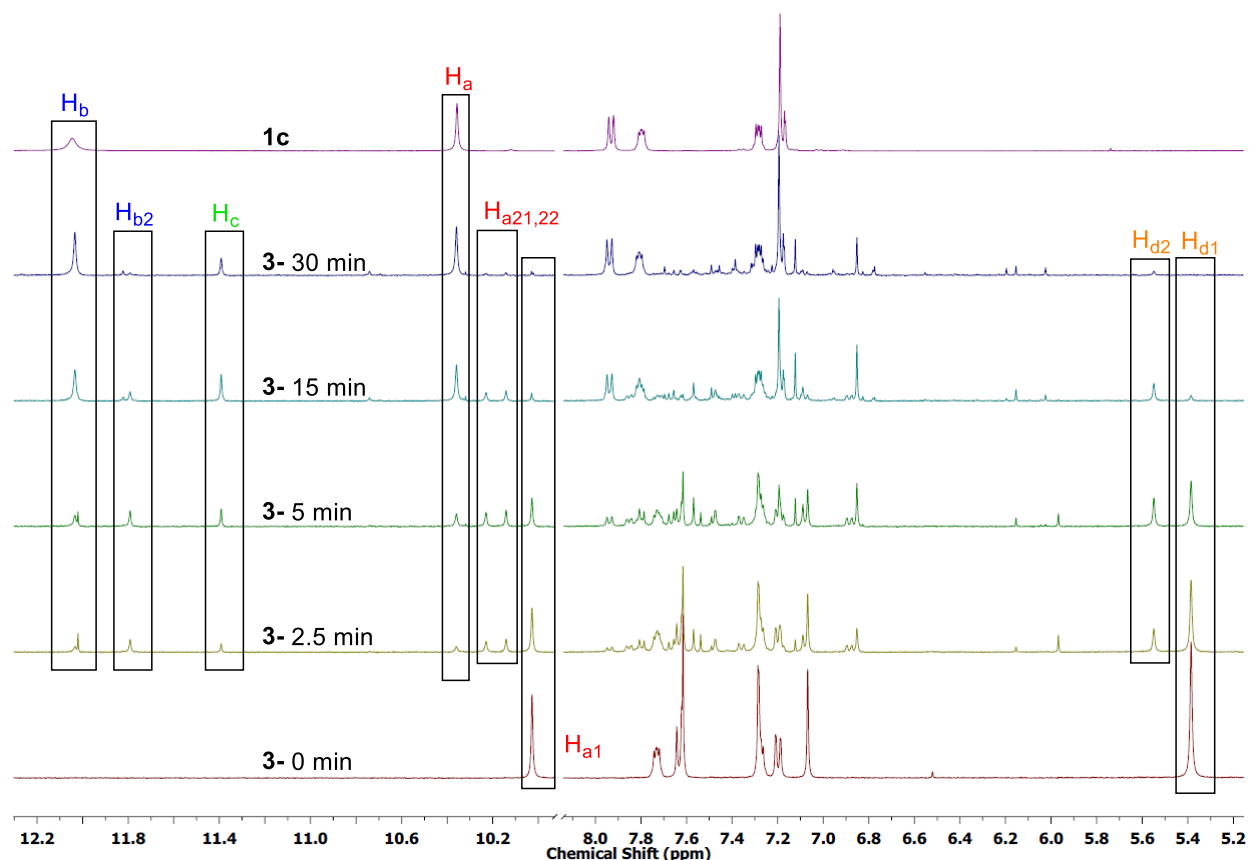


Figure 22: Change in ^1H NMR spectrum of **3** on irradiation with 405 nm light. Solvent- $\text{DMSO-}d_6$.

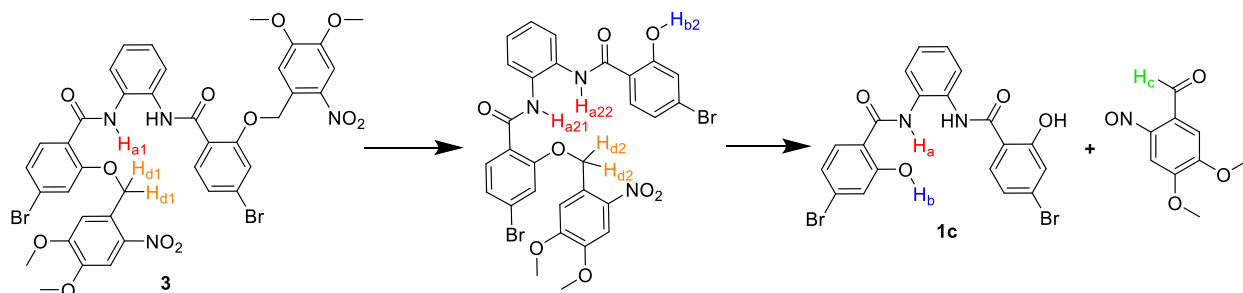


Figure 23: Mechanism of two-step photoactivation of protransporter **3** on irradiation with 405 nm light.

Following verification of photoactivated transporter (**1c**) release, activation of ion transport activity on irradiation under violet light was verified. Stock solutions of protransporters **2** and **3** (0.5 mM) in DMSO were irradiated under 405 nm and then tested for ion transport activity across EYPC-LUVs $\Rightarrow$ HPTS at a final working concentration of 5 μM . Recovery of ion transport activity was observed with increasing photoirradiation time (Figure 24). Transport activity in protransporter **3**, which had a stronger absorbance at 405 nm, was observed to recover in 3 min,

while **2** having unsubstituted *o*-nitrobenzyl group took longer at 10 min. The protransporters were then tested in MCF-7 cells, where they were found to be inactive under normal conditions but on irradiation of treated cells under 405 nm light, both compounds exhibited concentration dependent cytotoxicity (Figure 25). Compound **3** was observed to show excellent cytotoxicity within 10 min of photoirradiation due to its faster activation time. Compound **2** only exhibited moderate toxicity following 10 min irradiation but showed much higher activity on increasing the irradiation time to 20 min. To assess the effect of photocleavage byproduct on cells, the same photoactivation experiments were performed using control compounds **8** and **9** that release photocleavage byproducts corresponding to those of **2** and **3**, respectively, along with non-toxic methoxyphenol (**MP**). Compounds **8**, **9**, and **MP** exhibited negligible cytotoxicity even at 50 μ M concentration following 20 min of irradiation under 405 nm light (Figure 26).

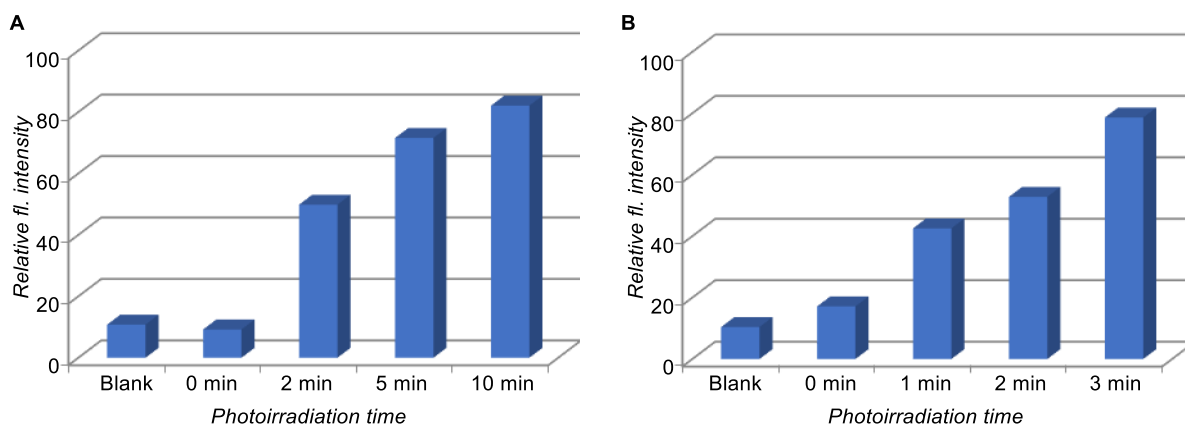


Figure 24: Recovery of ion transport activity of (A) **2** and (B) **3** on activation with 405 nm light verified across EYPC-LUVs $\Rightarrow$ HPTS.

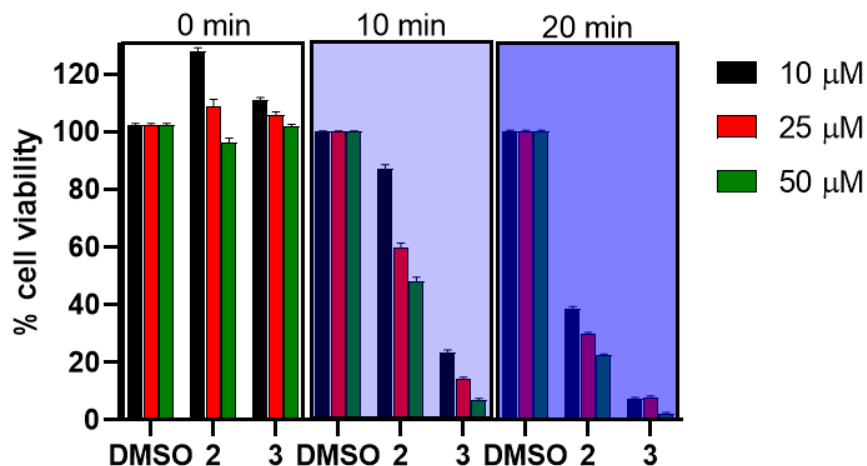


Figure 25: Cell viability assay of protransporter treated MCF-7 cells following irradiation under 405 nm light for 0, 10 and 20 min.

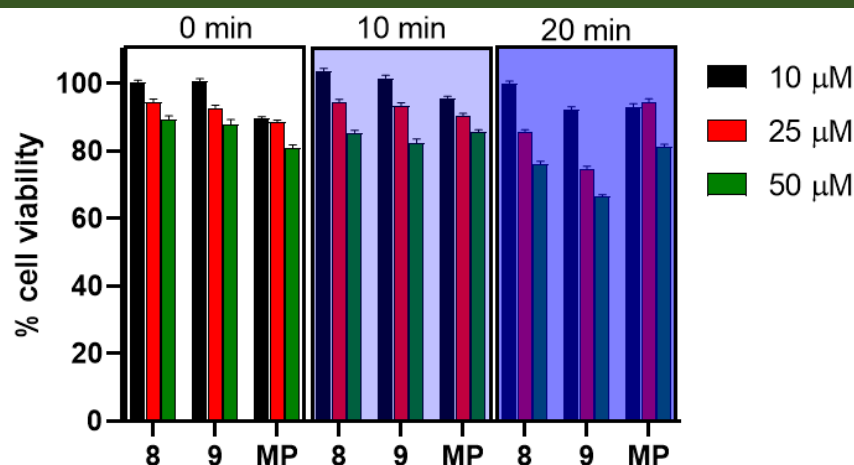


Figure 26: Cell viability assay of MCF-7 cells treated with controls **8**, **9**, and **MP** following irradiation under 405 nm light for 0, 10 and 20 min.

4.3 Conclusions

Bis(salicylamides) were demonstrated to be efficient transmembrane anion antiporters capable of inducing death in MCF-7 breast cancer cells. Protransporters were prepared by caging the transporter OH groups with *o*-nitrobenzyl photocleavable groups. Photoactivation of protransporters was conducted using 405 nm light, following which the release of active transporter and recovery of ion transport activity was verified. Irradiation of protransporter treated MCF-7 cells with 405 nm light resulted in extensive cell death, indicating their successful activation in cells.

4.4 Experimental Section

4.4.1 General Methods

Reagents and solvents for synthesis were purchased from commercial sources (Sigma-Aldrich, TCI, Spectrochem, Rankem, Avra, SRL) and used without further purification. Purifications via flash column chromatography were performed on Spectrochem 230-400 mesh silica gel. Thin layer chromatography (TLCs) was performed on Merck silica gel 60-F254 plates. Egg yolk phosphatidylcholine (EYPC) as a chloroform solution (25 mg/mL), polycarbonate membranes of 100 nm and 200 nm pore size, and mini extruder set for vesicle preparation were purchased from Avanti Polar Lipids (Merck). HPTS, HEPES, Triton™ X-100, lucigenin, CF, NaOH, and inorganic salts were of molecular biology grade purchased from Sigma-Aldrich. All stock solutions were prepared in HPLC grade DMSO, ACN, and MeOH. All buffer solutions were prepared in Milli-Q

water. High power LED of 405 nm (Single color- Violet) was procured from Mouser Electronics India, and associated components were sourced locally.

4.4.2 Physical Measurements

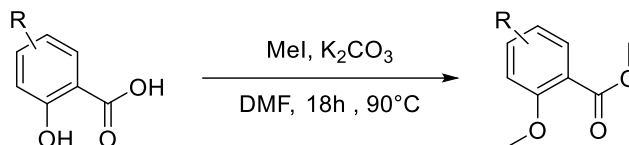
All NMR spectra were recorded on a Bruker Avance III HD 400 MHz NMR spectrometer. ^1H NMR spectra were recorded at 400 MHz and ^{13}C spectra at 101 MHz. All spectra were calibrated with respect to the residual solvent peak ($\delta_{\text{H}} = 7.26$ ppm and $\delta_{\text{C}} = 77.16$ ppm for CDCl_3 , $\delta_{\text{H}} = 2.50$ ppm, and $\delta_{\text{C}} = 39.52$ ppm for $\text{DMSO}-d_6$, $\delta_{\text{H}} = 1.94$ ppm for CD_3CN). ^{19}F NMR spectra were recorded at 377 MHz with a solution of TFA in CDCl_3 sealed in a capillary tube as an internal reference, $\delta_{\text{F}} = -76.55$ ppm. The chemical shifts (δ) were reported in ppm and the coupling constants (J) in Hz. The following abbreviations were used to indicate peak splitting patterns: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), td (triplet of doublet), and dt (doublet of triplet). Infra-red (IR) spectra were obtained using a Bruker ALPHA-E FT-IR spectrometer and reported in ν (cm^{-1}). High resolution mass spectra (HRMS) were recorded on a Waters SYNAPT G2 mass spectrometer equipped with a Waters Z-SprayTM electrospray ionization (ESI) source. UV-Vis absorption spectra were recorded on a Shimadzu UV-2600 UV-Vis. Single crystal X-ray diffraction (SCXRD) measurements were conducted using Bruker D8 VENTURE or Bruker KAPPA APEX diffractometers. Fluorescence studies were conducted on a HORIBA Jobin Yvon Fluoromax[®]+ spectrofluorometer with an injector port and a Quantum Northwest, Inc. (QNW) Luma 40 Peltier-based temperature-controlled cuvette holder, with temperature control achieved using a QNW TC-1 temperature controller. All buffer solutions were adjusted to the necessary pH using a LAQUAtwin pH22 pocket pH meter from HORIBA. Chloride ion measurements were conducted using a Thermo Scientific OrionTM Chloride ionplusTM Sure-FlowTM Combination ISE, connected to a FisherbrandTM accumetTM AB250 Benchtop pH/ISE meter for recording ion concentrations (in ppm).

ChemDraw 22.2.0 was used for drawing structures and processing figures. 3D structure images were generated using BIOVIA Discovery Studio Visualizer 2021 and Avogadro 1.2.0. Theoretical predictions of pK_a and $\log P$ values were calculated using calculator plugins of MarvinSketch 23.11. Absorbance and fluorescence measurements on 96-well microplate were recorded on a Thermo Scientific Varioskan[®] Flash multimode microplate reader. Light intensity measurements were recorded using a Newport 842-PE model Optical Power/Energy Meter equipped with a

photodetector. Molecular structure conformations were predicted using CONFLEX 8 software package. DFT calculations were performed on Gaussian 09 program suite using the PARAM Brahma supercomputing cluster.

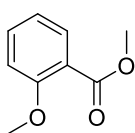
4.4.3 Synthetic Protocols

Synthesis of substituted methyl 2-methoxybenzoates:



General Procedure E: To a stirred suspension of the substituted salicylic acid (1 eq.) and K_2CO_3 (2.5 eq.) in DMF (1 ml per 100 mg substituted salicylic acid) was added MeI (2.42 eq.) and stirred for 18 h at 90°C. It was then diluted with EtOAc and washed with water. The aqueous layer was further extracted with EtOAc (3x), the organic layers combined, washed with brine, dried over anhydrous Na_2SO_4 , and the solvent removed *in vacuo*.

Methyl 2-methoxybenzoate (5a)

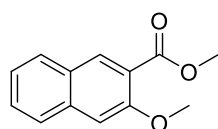


Physical appearance: Light yellow oil; Yield = 51%; 1H NMR (400 MHz, $CDCl_3$):

δ 7.79 (dd, J = 7.9, 1.8 Hz, 1H), 7.52 – 7.43 (m, 1H), 7.03 – 6.94 (m, 2H), 3.91 (s, 3H), 3.89 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.9, 159.2, 133.7, 131.8,

120.2, 120.1, 112.1, 77.2, 56.1, 52.2.; IR ($\tilde{\nu}$ cm^{-1}): 2951, 2840, 1723, 1599, 1491, 1461, 1435, 1301, 1244, 1131, 1081, 1021, 707; HRMS (ESI): m/z $[M-OCH_3]^+$ Calc. for $C_9H_{10}O_3$: 135.0446; Found: 135.0434.

Methyl 3-methoxy-2-naphthoate (5b)



The residue was purified by silica gel column chromatography. **Eluent for**

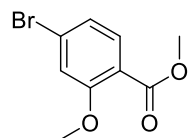
column chromatography: 8% EtOAc in petroleum ether; **Physical**

appearance: Yellow oil; Yield = 60%; 1H NMR (400 MHz, $CDCl_3$): δ 8.30

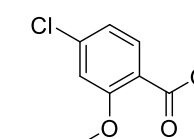
(s, 1H), 7.82 (d, J = 8.2 Hz, 1H), 7.74 (d, J = 8.2 Hz, 1H), 7.52 (ddd, J = 8.2, 6.9, 1.2 Hz, 1H), 7.38 (ddd, J = 8.1, 6.9, 1.1 Hz, 1H), 7.21 (s, 1H), 4.00 (s, 3H), 3.96 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$): δ 166.8, 155.8, 136.2, 133.0, 128.8, 128.6, 127.6, 126.6, 124.5, 121.8, 106.9, 56.1, 52.4.

IR ($\tilde{\nu}$ cm^{-1}): 2950, 1725, 1630, 1503, 1467, 1359, 1335, 1207, 1131, 1014, 954, 783, 746, 714, HRMS (ESI): m/z $[M-OCH_3]^+$ Calc. for $C_{13}H_{12}O_3$: 185.0603; Found: 185.0601.

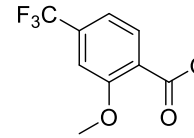
Methyl 4-bromo-2-methoxybenzoate (5c)

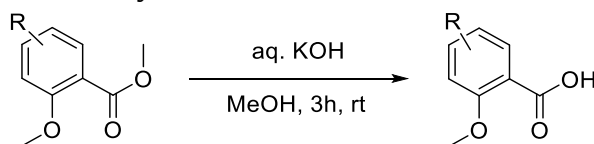
**Physical appearance:** Orange oil (liquid); Yield= 89%; **¹H NMR (400 MHz, CDCl₃):** δ 7.69 – 7.66 (m, 1H), 7.12 (dt, *J* = 4.0, 1.8 Hz, 2H), 3.90 (s, 3H), 3.87 (s, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 166.0, 159.8, 133.0, 127.9, 123.5, 119.0, 115.8, 56.4, 52.3. **IR (ν̄ cm⁻¹):** 2949, 1728, 1588, 1485, 1461, 1434, 1395, 1287, 1241, 1089, 1023, 858, 722; **HRMS (ESI):** *m/z* [M–OCH₃]⁺ Calc. for C₉H₉BrO₃: 212.9551; Found: 212.9546.

Methyl 4-chloro-2-methoxybenzoate (5d)

**Physical appearance:** Light Yellow oil; Yield= 76%; **¹H NMR (400 MHz, CDCl₃):** δ 7.77 – 7.74 (m, 1H), 6.96 (td, *J* = 4.4, 1.9 Hz, 2H), 3.90 (s, 3H), 3.88 (s, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 165.9, 160.0, 139.6, 133.0, 120.5, 118.5, 112.9, 56.4, 52.2. **IR (ν̄ cm⁻¹):** 2950, 1729, 1595, 1571, 1488, 1435, 1398, 1287, 1241, 1083, 1025, 963, 878, 773; **HRMS (ESI):** *m/z* [M–OCH₃]⁺ Calc. for C₉H₉ClO₃: 169.0056; Found: 169.0047.

Methyl 2-methoxy-4-(trifluoromethyl)benzoate (5e)

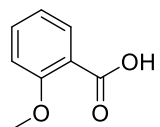
**Physical appearance:** Yellow oil; Yield = 85%; **¹H NMR (400 MHz, CDCl₃):** δ 7.86 (d, *J* = 7.9 Hz, 1H), 7.24 (d, *J* = 8.0 Hz, 1H), 7.18 (s, 1H), 3.95 (s, 3H), 3.91 (s, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 165.9, 159.1, 135.1 (q, *J* = 65.3, 32.6 Hz), 132.2, 123.6, 123.6 (q, *J* = 272.8 Hz), 117.0 (q, *J* = 3.8 Hz), 109.0 (q, *J* = 3.7 Hz), 56.4, 52.5. **IR (ν̄ cm⁻¹):** 2954, 1734, 1412, 1328, 1240, 1171, 1125, 1093, 964, 892.20, 862, 742; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₀H₉F₃O₃: 235.0582; Found: 235.0575

Synthesis of substituted 2-methoxybenzoic acids

General Procedure F: To a solution of the ester in MeOH (2.7 ml per 100 mg ester) was added 6N aqueous KOH (26.5 eq.) and stirred for 3 h at rt. MeOH was then removed *in vacuo*. The mixture was quenched by the addition of water (10 ml) followed by conc. HCl until pH ~ 2. It was then diluted with EtOAc and then washed with water. The aqueous layer was extracted

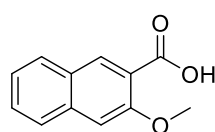
exhaustively with EtOAc, the organic layers combined, washed with brine, dried over anhydrous Na_2SO_4 , and the solvent removed *in vacuo*.

2-Methoxybenzoic acid (6a)



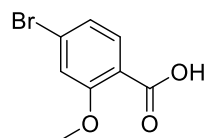
Physical appearance: white powder; Yield= 54%; **^1H NMR (400 MHz, CDCl_3):** δ 10.69 (s, 1H), 8.19 (t, J = 7.1 Hz, 1H), 7.58 (t, J = 7.8 Hz, 1H), 7.14 (dd, J = 12.8, 7.3 Hz, 1H), 7.06 (d, J = 8.4 Hz, 1H), 4.08 (s, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 165.5, 158.2, 135.2, 134.0, 122.4, 117.7, 111.8, 56.8. **IR ($\tilde{\nu}$ cm^{-1}):** 1696, 1670, 1493, 1258, 829; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_8\text{H}_8\text{O}_3$: 153.0552; Found: 153.0549.

3-Methoxy-2-naphthoic acid (6b)



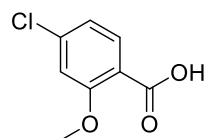
Physical appearance: white powder; Yield= 89%; **^1H NMR (400 MHz, CDCl_3):** δ 10.94 (s, 1H), 8.79 (s, 1H), 7.91 (d, J = 8.1 Hz, 1H), 7.78 (d, J = 8.2 Hz, 1H), 7.59 (t, J = 7.5 Hz, 1H), 7.46 (t, J = 7.5 Hz, 1H), 7.31 (s, 1H), 4.18 (s, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 165.6, 154.4, 136.7, 136.4, 129.6, 129.6, 128.5, 126.7, 125.6, 118.0, 107.3, 56.8. **IR ($\tilde{\nu}$ cm^{-1}):** 2965, 2660, 1694, 1675, 1627, 1459, 1284, 1254, 1219, 1193, 1070, 865, 749; **HRMS (ESI):** m/z $[\text{M}-\text{OH}]^+$ Calc. for $\text{C}_{12}\text{H}_{10}\text{O}_3$: 185.0603; Found: 185.0595.

4-Bromo-2-methoxybenzoic acid (6c)



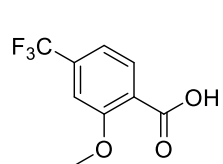
Physical appearance: yellowish white powder; Yield= 95%; **^1H NMR (400 MHz, CDCl_3):** δ 8.02 (d, J = 8.4 Hz, 1H), 7.28 (dd, J = 8.4, 1.7 Hz, 1H), 7.22 (d, J = 1.7 Hz, 1H), 4.07 (s, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 165.3, 158.6, 134.9, 129.5, 125.6, 116.8, 115.6, 57.12; **IR ($\tilde{\nu}$ cm^{-1}):** 2946, 2813, 1682, 1588, 1562, 1295, 1238, 1016, 858, 829, 772; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_8\text{H}_7\text{BrO}_3$: 212.9551; Found: 212.9546.

4-Chloro-2-methoxybenzoic acid (6d)



Physical appearance: white powder; Yield= 90%; **^1H NMR (400 MHz, CDCl_3):** δ 8.11 (d, J = 8.4 Hz, 1H), 7.13 (dd, J = 8.4, 1.8 Hz, 1H), 7.06 (d, J = 1.8 Hz, 1H), 4.08 (s, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 164.8, 158.6, 141.2, 135.0, 122.7, 116.4, 112.6, 57.2. **IR ($\tilde{\nu}$ cm^{-1}):** 3119, 3017, 1683, 1593, 1565, 1298, 1241, 1019, 883, 833, 774; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_8\text{H}_7\text{ClO}_3$: 187.0162; Found: 187.0151.

2-Methoxy-4-(trifluoromethyl)benzoic acid (6e)



Physical appearance: white powder; Yield= 91%%; **¹H NMR (400 MHz,**

CDCl₃): δ 8.28 (d, *J* = 8.1 Hz, 1H), 7.40 (dd, *J* = 8.1, 0.7 Hz, 1H), 7.28 (s, 1H),

4.14 (s, 3H). **¹³C NMR (101 MHz, CDCl₃):** 165.4, 158.5, 136.5 (q, *J* =

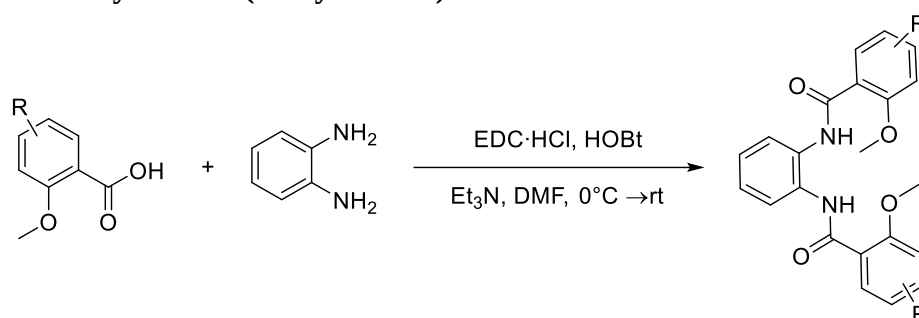
33.1 Hz), 134.5, 123.2 (q, *J* = 2.7 Hz), 121.1 (d, *J* = 0.9 Hz), 118.6 (q, *J* = 0.0 Hz), 109.0 (q, *J* =

7.6, 3.8 Hz), 57.1. **¹⁹F NMR (377 MHz, CDCl₃):** δ −63.32. **IR (ν̃ cm^{−1}):** 2981, 1690, 1327, 1303,

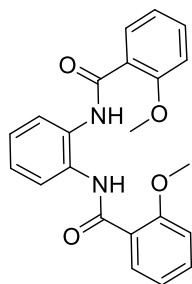
1148, 1100, 1077, 1022, 898, 789; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₉H₇F₃O₃: 221.0426;

Found: 221.0413.

Synthesis of *o*-methylated bis(salicylamides)



General Procedure G: To a solution of the substituted 2-methoxybenzoic acid (2.2 eq.) in DMF (6 ml per 100 mg *o*-phenylenediamine) was added *o*-phenylenediamine (1 eq.) followed by Et₃N (3 eq.) and cooled to 0 °C in an ice bath. Then HOBT (2.6 eq.) was added, and the mixture was stirred at 0 °C for 10 min. Then EDC·HCl (2.6 eq.) was added, and the mixture was stirred at 0 °C for 10 min. The mixture was then allowed to reach rt and stirred overnight for 24 h. It was then diluted with EtOAc and washed with water. The aqueous layer was further extracted with EtOAc (3x), the organic layers combined, washed with brine, dried over anhydrous Na₂SO₄, and the solvent removed *in vacuo*. The residue was purified by silica gel flash column chromatography.

N,N'-(1,2-phenylene)bis(2-methoxybenzamide) (7a)

Eluent for column chromatography: 0.5% MeOH in CHCl₃; **Physical**

appearance: White powder; Yield = 77%; **¹H NMR (400 MHz, CDCl₃):** δ 9.84

(s, 2H), 8.31 (dd, *J* = 7.8, 1.8 Hz, 2H), 7.78 (dd, *J* = 5.9, 3.6 Hz, 2H), 7.52 – 7.46

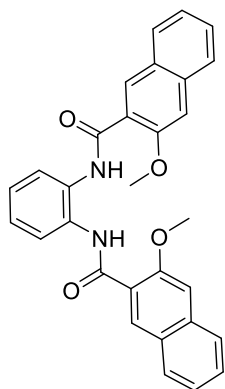
(m, *J* = 8.4, 7.4, 1.8 Hz, 2H), 7.28 (dd, *J* = 6.7, 3.2 Hz, 2H), 7.16 – 7.05 (m, 2H),

6.98 (d, *J* = 8.3 Hz, 2H), 3.83 (s, 6H).; **¹³C NMR (101 MHz, CDCl₃):** δ 164.2,

157.8, 133.6, 132.8, 131.4, 126.3, 126.0, 121.5, 121.4, 111.5, 56.0. **IR (ν̄ cm⁻¹):**

3330, 3078, 1659, 1597, 1511, 1475, 1296, 1237, 1179, 1130, 1018, 754; **HRMS (ESI):** *m/z*

[*M*+*H*]⁺ Calc. for C₂₂H₂₀N₂O₄: 377.1501; Found: 377.1505

N,N'-(1,2-phenylene)bis(3-methoxy-2-naphthamide) (7b)

Eluent for column chromatography: 30% EtOAc in petroleum ether;

Physical appearance: Light yellow powder; Yield = 42%; **¹H NMR**

(400 MHz, CDCl₃): δ 9.99 (s, 2H), 8.88 (s, 2H), 7.92 (d, *J* = 8.1 Hz, 2H), 7.88

(dt, *J* = 7.2, 3.6 Hz, 2H), 7.74 (d, *J* = 8.1 Hz, 2H), 7.57 – 7.51 (m, 2H), 7.45 –

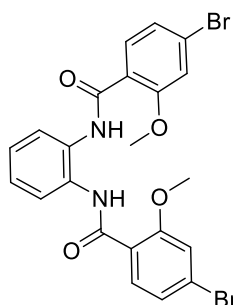
7.38 (m, 2H), 7.35 – 7.29 (m, 2H), 7.21 (s, 2H), 3.89 (s, 6H). **¹³C NMR**

(101 MHz, CDCl₃): δ 164.1, 154.8, 136.1, 134.5, 131.3, 129.3, 128.7, 128.35,

126.4, 126.3, 125.8, 124.9, 122.2, 106.7, 56.0. **IR (ν̄ cm⁻¹):** 3330, 3294, 3046,

2935, 1661, 1626, 1593, 1531, 1482, 1455, 1454, 1170, 1073, 1006, 827, 746,

704, 654; **HRMS (ESI):** *m/z* [*M*+*H*]⁺ Calc. for C₃₀H₂₄N₂O₄ 477.1814; Found: 477.1808.

N,N'-(1,2-phenylene)bis(4-bromo-2-methoxybenzamide) (7c)

Eluent for column chromatography: 0.5% MeOH in CHCl₃; **Physical**

appearance: White powder; Yield = 71%; **¹H NMR (400 MHz, CDCl₃):** δ

9.67 (s, 2H), 8.16 (d, *J* = 8.4 Hz, 2H), 7.73 – 7.66 (m, 2H), 7.31 – 7.24 (m,

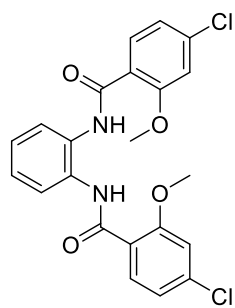
4H), 7.14 (d, *J* = 1.6 Hz, 2H), 3.88 (s, 6H). **¹³C NMR (101 MHz, CDCl₃):** δ

163.4, 158.0, 134.0, 131.3, 127.7, 126.6, 126.2, 124.9, 120.4, 115.2, 56.4.

IR (ν̄ cm⁻¹): 3344, 3303, 1662, 1586, 1533, 1510, 1466, 1394, 1236, 1180,

1017, 896, 846, 760; **HRMS (ESI):** *m/z* [*M*+*H*]⁺ Calc. for C₂₂H₁₈Br₂N₂O₄: 532.9712; Found:

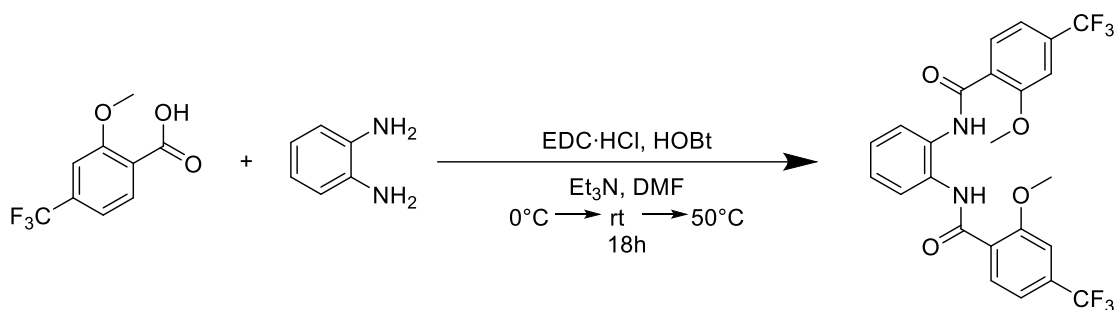
532.9717.

N,N'-(1,2-phenylene)bis(4-chloro-2-methoxybenzamide) (7d)

Eluent for column chromatography: 15% EtOAc in petroleum ether

Physical appearance: White powder; Yield = 82%; **¹H NMR (400 MHz, CDCl₃):** δ 9.68 (s, 2H), 8.24 (d, *J* = 8.5 Hz, 2H), 7.70 (dd, *J* = 5.9, 3.6 Hz, 2H), 7.28 (dd, *J* = 6.0, 3.5 Hz, 2H), 7.10 (dd, *J* = 8.5, 1.9 Hz, 2H), 6.98 (d, *J* = 1.8 Hz, 2H), 3.88 (s, 6H); **¹³C NMR (101 MHz, CDCl₃):** δ 163.3, 158.1, 139.4, 133.9, 131.3, 126.6, 126.2, 121.9, 119.9, 112.3, 56.4. **IR(ν̄ cm⁻¹):** 3343,

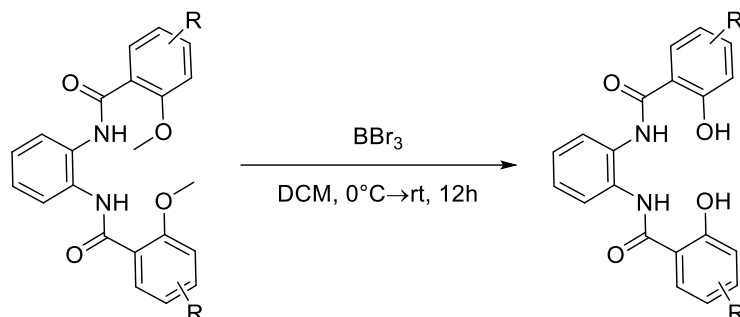
1658, 1591, 1531, 1511, 1459, 1400, 1295, 1238, 1130, 1020, 902, 876, 840, 762; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₂₂H₁₈Cl₂N₂O₄: 445.0722; Found: 445.0715

Synthesis of N,N'-(1,2-phenylene)bis(2-methoxy-4-(trifluoromethyl)benzamide) (7e)

Procedure: To a solution of the substituted 2-methoxybenzoic acid (300 mg, 1.36 mmol, 3.5 eq) in DMF (9.5 ml) was added *o*-phenylenediamine (42 mg, 0.39 mmol, 1 eq.) followed by Et₃N (215 μL, 1.6 mmol, 4 eq.), cooled to 0 °C in an ice bath and stirred for 5 min. Then HOBT (189 mg, 1.4 mmol, 3.6 eq.) was added and the mixture was stirred at 0 °C for 15 min. Then EDC·HCl (340 mg, 1.7 mmol, 4.5 eq.) was added and the mixture was stirred at 0 °C for 15 min. The mixture was then allowed to reach rt and stirred at 50 °C for 18 h. It was then diluted with EtOAc and washed with water. The aqueous layer was further extracted with EtOAc (3x), the organic layers combined, washed with brine, dried over anhydrous Na₂SO₄, and the solvent removed in vacuo. The residue was purified by silica gel column chromatography using 1% MeOH in chloroform as eluent to obtain the product as a white powder. Yield = 215 mg = 42%; **¹H NMR (400 MHz, CDCl₃):** δ 9.78 (s, 2H), 8.41 (d, *J* = 7.9 Hz, 2H), 7.73 – 7.65 (m, 2H), 7.38 (d, *J* = 7.5 Hz, 2H), 7.36 – 7.28 (m, 2H), 7.21 (s, 2H), 3.96 (s, 6H); **¹³C NMR (101 MHz, CDCl₃):** δ 163.0, 157.7, 135.1 (q, *J* = 32.7 Hz), 133.5, 131.3, 126.8, 126.4, 124.4, 123.5 (q, *J* = 272.9 Hz), 118.2 (d, *J* = 3.7 Hz), 108.7 (q, *J* = 3.6 Hz), 56.4. **¹⁹F NMR (377 MHz, CDCl₃):** δ –63.07. **IR(ν̄ cm⁻¹):** 3346,

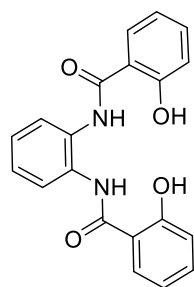
1673, 1598, 1538, 1459, 1413, 1328, 1233, 1171, 1125, 1021, 747, **HRMS (ESI):** m/z $[M+H]^+$ Calc. for $C_{24}H_{18}F_6N_2O_4$: 513.1249; Found: 513.1253.

Synthesis of bis(salicylamides)



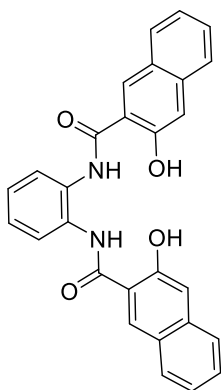
General Procedure H: To a 0°C cooled solution of substituted methylated bis(salicylamide) (100 mg, 1 eq) in DCM (8 ml) was added dropwise BBr_3 (1 M in DCM, 5.8 eq). After the addition of BBr_3 , the mixture was stirred at rt for 12 h. The progress of the reaction was monitored using TLC. Once the reaction was done, DCM in the reaction mixture was removed, and the crude reaction was cooled in an ice bath, followed by the addition of methanol to quench the excess BBr_3 . The formation of precipitate was observed with the addition of the methanol. The solvent was removed *in vacuo* and distilled water was added to the crude reaction mixture. The precipitate was collected using a Buchner funnel and dried under vacuum inside a desiccator for 18h.

N,N'-(1,2-phenylene)bis(2-hydroxybenzamide) (1a)

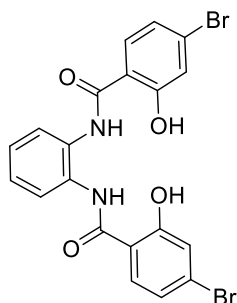


Physical appearance: White powder; yield = 77%; **1H NMR (400 MHz, DMSO- d_6):** δ 11.75 (s, 2H), 10.43 (s, 2H), 8.01 (dd, J = 8.2, 1.4 Hz, 2H), 7.80 (dd, J = 5.9, 3.6 Hz, 2H), 7.43 (td, J = 8.1, 1.6 Hz, 2H), 7.32 – 7.25 (m, 2H), 7.05 – 6.90 (m, 4H). **^{13}C NMR (101 MHz, DMSO- D_6):** δ 166.4, 158.3, 134.0, 131.2, 129.7, 125.8, 125.5, 119.4, 117.3, 117.1. **IR ($\tilde{\nu}$ cm^{-1}):** 3257, 3022, 1637, 1588, 1522, 1495, 1338, 1314, 1223, **HRMS (ESI):** m/z $[M+H]^+$ Calc. for $C_{20}H_{16}N_2O_4$:

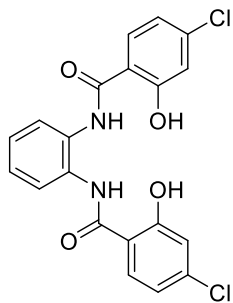
349.1188; Found: 349.1190.

N,N'-(1,2-phenylene)bis(3-hydroxy-2-naphthamide) (1b)

Physical appearance: r; yield = 44%; **¹H NMR (400 MHz, DMSO-*d*₆):** δ 10.69 (s, 2H), 8.66 (s, 2H), 8.00 – 7.79 (m, 4H), 7.73 (d, *J* = 8.3 Hz, 2H), 7.51 (t, *J* = 7.4 Hz, 2H), 7.38 – 7.25 (m, 6H). **¹³C NMR (101 MHz, DMSO-*D*₆):** δ 165.8, 154.0, 136.2, 131.7, 131.3, 129.1, 128.6, 127.1, 126.01, 125.9, 125.5, 124.0, 120.3, 111.0. **IR (ν̄ cm⁻¹):** 3050, 1623, 1598, 1523, 1450, 1358, 1210.24, 874, 771, 746, **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₂₈H₂₀N₂O₄: 449.1501; Found: 449.1499.

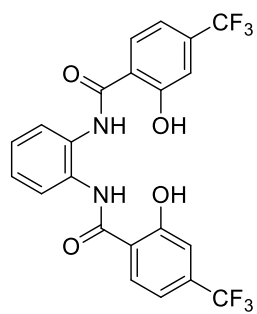
N,N'-(1,2-phenylene)bis(4-bromo-2-methoxybenzamide)(1c)

Physical appearance: White powder; yield = 78%; **¹H NMR (400 MHz, DMSO-*d*₆):** δ 12.05 (s, 2H), 10.36 (s, 2H), 7.93 (d, *J* = 8.3 Hz, 2H), 7.80 (dd, *J* = 5.7, 3.6 Hz, 2H), 7.28 (dd, *J* = 6.0, 3.6 Hz, 2H), 7.21 – 7.15 (m, 4H). **¹³C NMR (101 MHz, DMSO-*d*₆):** δ 165.3, 158.7, 131.7, 131.0, 126.6, 125.9, 125.4, 122.6, 119.8, 116.9. **IR (ν̄ cm⁻¹):** 3344, 3303, 1662, 1586, 1533, 1510, 1466, 1394, 1236, 1180, 1017, 896, 846, 759, **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₂₀H₁₄Br₂N₂O₄: 504.9399; Found: 504.9393.

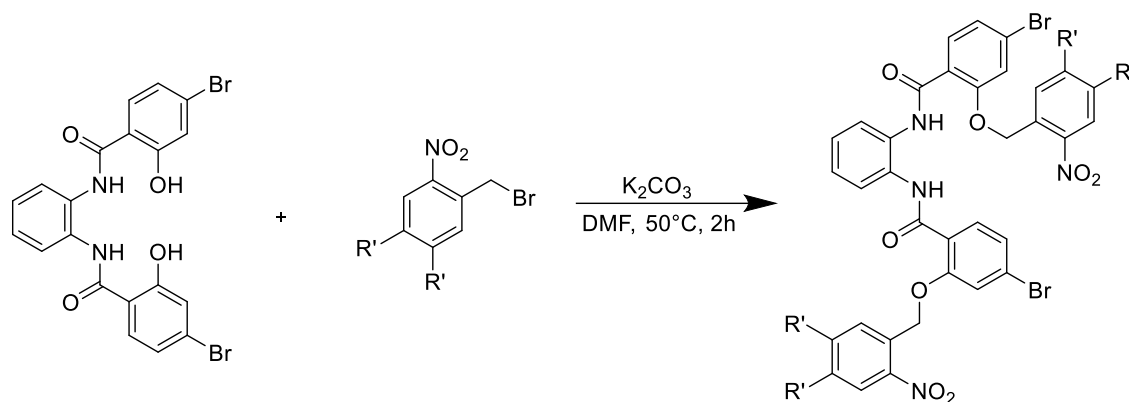
N,N'-(1,2-phenylene)bis(4-chloro-2-hydroxybenzamide) (1d)

Physical appearance: Gray powder; yield=52%; **¹H NMR (400 MHz, DMSO- *d*₆):** δ 12.08 (s, 2H), 10.36 (s, 2H), 8.01 (d, *J* = 9.1 Hz, 2H), 7.80 (dd, *J* = 5.7, 3.6 Hz, 2H), 7.29 (dd, *J* = 6.0, 3.6 Hz, 2H), 7.05 (dd, *J* = 6.7, 2.0 Hz, 4H) **¹³C NMR (101 MHz, DMSO- *d*₆):** δ 165.3, 158.9, 137.8, 131.7, 131.0, 126.0, 125.5, 119.7, 117.0, 116.6. **IR (ν̄ cm⁻¹):** 3353, 3301, 1637, 1586, 1554, 1490, 1363, 1328, 1295, 1224, 1207, 1066, 920, 752, **HRMS (ESI):** *m/z*

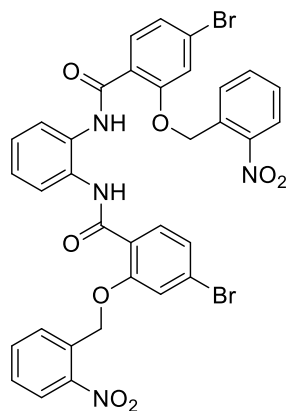
[M+H]⁺ Calc. for C₂₀H₁₄Cl₂N₂O₄: 417.0409; Found: 417.0400.

N,N'-(1,2-phenylene)bis(2-hydroxy-4-(trifluoromethyl)benzamide) (1e)

Physical appearance: white powder; yield = 99%; ^1H NMR (400 MHz, DMSO- d_6): δ 12.04 (s, 2H), 10.47 (s, 2H), 8.18 (d, J = 8.1 Hz, 2H), 7.82 (dd, J = 5.7, 3.6 Hz, 2H), 7.31 (dd, J = 9.2, 6.0 Hz, 6H). ^{13}C NMR (101 MHz, DMSO- d_6): δ 164.6, 157.7, 133.1 (q, J = 31.6 Hz), 131.4, 130.9, 126.0, 125.4, 123.5 (q, J = 272.8 Hz), 121.6, 115.6 (d, J = 3.4 Hz), 113.8 (d, J = 3.6 Hz). ^{19}F NMR (377 MHz, CDCl_3): δ -62.36. IR ($\tilde{\nu}$ cm^{-1}): 3293, 1649, 1603, 1552, 1515, 1334, 1120, 752, **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_{22}\text{H}_{14}\text{F}_6\text{N}_2\text{O}_4$: 485.0936; Found: 485.0939.

Synthesis of Bissalicylamide protransporters

General Procedure I: To a solution of N,N'-(1,2-phenylene)bis(4-bromo-2-methoxybenzamide) in DMF kept in a dark environment was added K_2CO_3 (2.6 eq.) and the substituted benzyl bromide (2.5 eq.) and stirred at 50°C for 2 h. The progress of the reaction was monitored using TLC. Once the reaction was complete, it was diluted with EtOAc and washed with water. The aqueous layer was further extracted with EtOAc (x3), the organic layers combined, washed with brine, dried over anhydrous Na_2SO_4 , and the solvent removed *in vacuo*. The residue was purified by silica gel flash column chromatography.

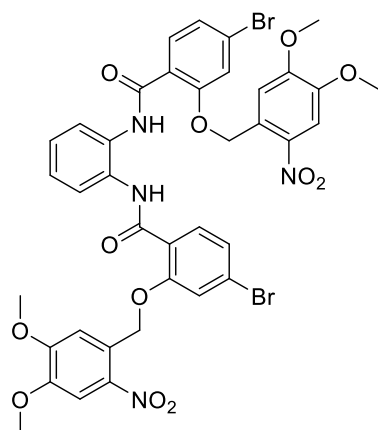
N,N'-(1,2-phenylene)bis(4-bromo-2-((2-nitrobenzyl)oxy)benzamide) (2)

Eluent for column chromatography: 30% EtOAc in petroleum ether

Physical appearance: white powder; yield = 61%; **¹H NMR (400 MHz, CDCl₃):** δ 9.56 (s, 2H), 8.16 (d, *J* = 7.3 Hz, 2H), 7.96 (d, *J* = 8.4 Hz, 2H), 7.64 – 7.59 (m, 2H), 7.57 – 7.47 (m, 6H), 7.29 – 7.23 (m, 2H), 7.12 (dd, *J* = 8.4, 1.6 Hz, 2H), 6.89 (d, *J* = 1.6 Hz, 2H), 5.57 (s, 4H). **¹³C NMR (101 MHz, CDCl₃):** δ 163.3, 156.4, 146.9, 134.6, 133.9, 131.59, 131.1, 129.5, 128.5, 127.6, 126.7, 125.9, 125.7, 125.7, 120.9, 116.4, 68.9.

IR (ν̄ cm⁻¹): 3628, 3257, 3022, 2379, 2314, 1637, 1588, 1522, 1494,

1443, 1338, 1314, 1223, 1163, **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₃₄H₂₄Br₂N₄O₈:775.0039; Found: 775.0054.

N,N'-(1,2-phenylene)bis(4-bromo-2-((4,5-dimethoxy-2-nitrobenzyl)oxy)benzamide) (3)

Eluent for column chromatography: 40% EtOAc in petroleum

ether **Physical appearance:** white powder; yield = 50%; **¹H NMR (400 MHz, CDCl₃):** δ 9.60 (s, 2H), 7.90 (d, *J* = 8.4 Hz, 2H), 7.68 (s, 2H), 7.65 – 7.59 (m, 2H), 7.26 – 7.22 (m, 2H), 7.15 (dd, *J* = 8.4, 1.6 Hz, 2H), 7.01 (d, *J* = 1.6 Hz, 2H), 6.99 (s, 2H), 5.50 (s, 4H), 3.98 (s, 6H), 3.68 (s, 6H). **¹³C NMR (101 MHz, DMSO- *d*₆):** δ 163.6, 155.7, 153.3, 147.4, 138.5, 131.8, 130.7, 127.1, 125.7, 125.0, 124.3, 122.8, 116.3, 109.2, 107.9, 79.2, 67.4, 56.0, 55.5.

IR (ν̄ cm⁻¹): 3729, 3628, 2378, 2314, 1729, 1657, 1584, 1521, 1479, 1328.25, 1277, 1219, 1066, 751, **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₃₈H₃₂Br₂N₄O₁₂:895.0462; Found:895.0466

Synthesis of controls 8 and 9

4-Methoxyphenol releasing photoactivation controls **8** and **9** were synthesized as per reported protocol.¹⁴

4.4.4 Ion Transport Experiments**Study of ion transport across EYPC-LUVs⊃HPTS:**

Preparation of EYPC-LUVs⊃HPTS in NaCl as per **Prep protocol P1**; HPTS assay as per **Assay protocol A1**.

Anion selectivity assay across EYPC-LUVs⊃HPTS: As per **Assay protocol A2**.

Study of chloride transport across EYPC-LUVs $\rightarrow$ Lucigenin:

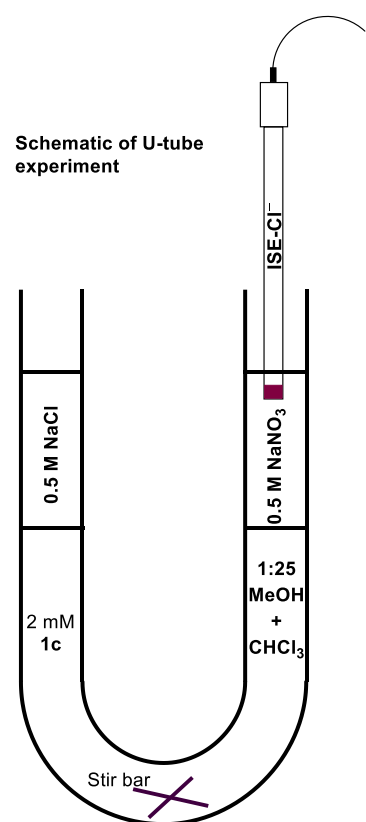
Preparation of EYPC-LUVs $\rightarrow$ lucigenin as per **Prep protocol P2**; Chloride transport assay as per **Assay protocol A6**.

Cation selectivity assay across EYPC-LUVs $\rightarrow$ Lucigenin: As per **Assay protocol A7**.

Valinomycin coupled lucigenin assay: As per **Assay protocol A8**.

Carboxyfluorescein (CF) leakage assay:

Preparation of EYPC-LUVs $\rightarrow$ CF as per **Prep protocol P5**; Chloride transport assay as per **Assay protocol A12**.

4.4.5 U-tube Experiment

The U-tube experiment simulates a bilayer membrane with an applied ion gradient where the thin lipid bilayer is replaced with a hydrophobic bulk solvent that separates the aqueous phases. Due to movement provided by stirring, ion carriers can bind to ions and carry them to the opposite arm through the hydrophobic layer, which can be detected by ion selective electrode. Channel forming molecules will not be able to assemble into ion channels spanning the length of this bulk solvent layer and are hence unable to transfer ions from the aqueous layer in one arm to the other. Hence, this experiment can be used as proof of the carrier mechanism of transport.

For this experiment, a 2 mM solution of transporter **1c** in 1:25 MeOH/CHCl₃ was placed into the upright U-tube along with a stir bar. The whole setup was clamped on top of a magnetic stirrer. Solutions of NaCl (0.5 M) and NaNO₃ (0.5 M) in Milli-Q water were

filled carefully into the left and right arms of the U-tube, respectively. The Cl⁻ concentration in the right arm was measured using a Cl⁻-ISE to get the zero-point reading. Then, the stirring was turned on and the Cl⁻ concentration in the right arm was measured at determined time points for up to 96 h. The data was plotted as Cl⁻ concentration (U-tube right arm) vs time. A control experiment was set up without a transporter in the organic layer and monitored in parallel.

4.4.6 ^1H NMR Titration Study

The ^1H NMR titration study was conducted at ambient temperature on a Bruker Avance III HD 400 MHz spectrometer. The titration with Cl^- ion was conducted by the addition of aliquots of tetrabutylammonium chloride (TBACl) solution (0.5 M, Solvent: 13.5% $\text{DMSO}-d_6$ in CD_3CN) to a solution of the receptor **1c** (0.005 M, Solvent: 13.5% $\text{DMSO}-d_6$ in CD_3CN). The spectra were calibrated using the residual signal from CD_3CN ($\delta_{\text{H}} = 1.94$ ppm) as an internal reference. TBACl was dried under vacuum in a desiccator prior to use.

4.4.7 SCXRD

The compounds were crystallized from their organic solvent solutions at ambient temperature. The crystals obtained were diffracted on Bruker diffractometers using Mo ($\text{Mo-K}\alpha = 0.71073 \text{ \AA}$) as the X-ray source. Sample temperatures were maintained constant throughout the data collection using Cryostream coolers (Oxford Cryosystems).

Single crystal data for **1a**

The transporter **1a** was crystallized from its saturated solution in CHCl_3 in a glass vial to yield crystals as colorless rods. Single crystal X-ray diffraction data was collected on a Bruker D8 VENTURE single crystal X-ray diffractometer with Mo ($\text{Mo-K}\alpha = 0.71073 \text{ \AA}$) microfocus X-ray ($\text{I}\mu\text{S}$) source and PHOTON 100 CMOS detector. The data collection temperature was maintained at 150 K.

Table 4: Crystal data of **1a**

Empirical formula	$\text{C}_{20}\text{H}_{16}\text{N}_2\text{O}_4$
Formula weight	348.35
Temperature	150 K
Wavelength	$0.71073 \text{ \AA}$
Crystal system	Monoclinic
Space group	P 21/n
Unit cell dimensions	$a = 8.8379(5) \text{ \AA}$ $\alpha = 90^\circ$. $b = 14.3279(9) \text{ \AA}$ $\beta = 101.642(2)^\circ$. $c = 13.3357(9) \text{ \AA}$ $\gamma = 90^\circ$.
Volume	$1653.94(18) \text{ \AA}^3$
Z	4

Density (calculated)	1.399 Mg/m ³
Absorption coefficient	0.099 mm ⁻¹
F(000)	728
Crystal size	0.626 x 0.180 x 0.096 mm ³
Theta range for data collection	2.110 to 28.348°.
Index ranges	-11≤h≤10, -19≤k≤18, -17≤l≤17
Reflections collected	42609
Independent reflections	4124 [R(int) = 0.0659]
Completeness to theta = 28.348°	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7457 and 0.7102
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	4124 / 0 / 239
Goodness-of-fit on F ²	0.638
Final R indices [I>2sigma(I)]	R1 = 0.0486, wR2 = 0.1377
R indices (all data)	R1 = 0.0813, wR2 = 0.1806
Extinction coefficient	n/a
Largest diff. peak and hole	0.299 and -0.284 e.Å ⁻³

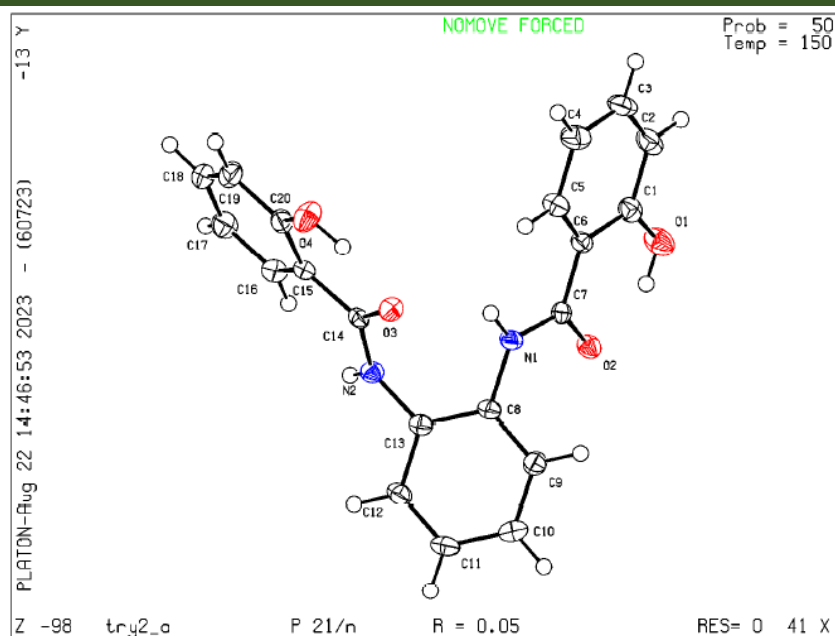


Figure 27: ORTEP diagram of **1a**.

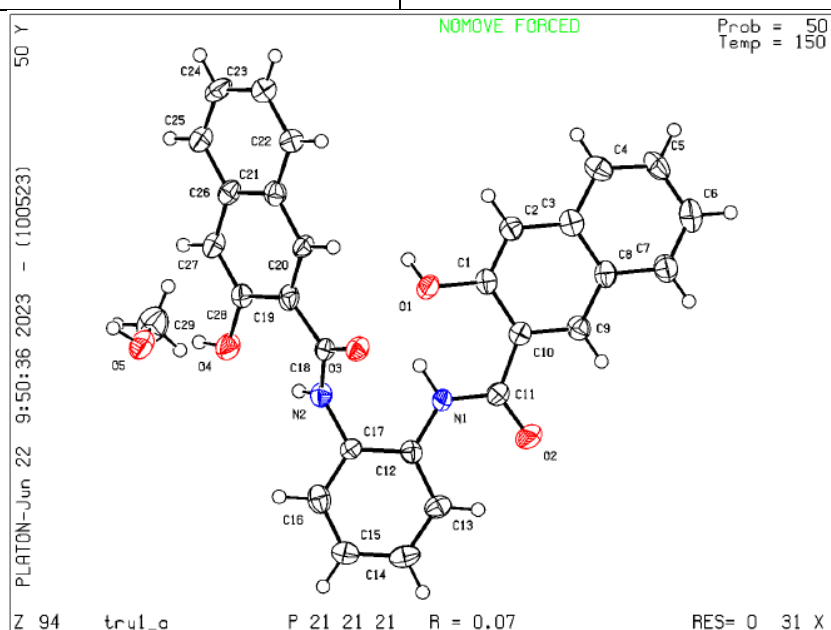
Single crystal data for **1b**

The transporter **1b** was crystallized from its saturated solution in MeOH in a glass vial to yield crystals as yellow needles. Single crystal X-ray diffraction data was collected on a Bruker KAPPA APEX single crystal X-ray diffractometer with Mo (Mo-K α = 0.71073 Å) ceramic sealed tube source and Apex II CCD detector. The data collection temperature was maintained at 150 K.

Table 5: Crystal data of **1b**

Empirical formula	C ₂₉ H ₂₄ N ₂ O ₅
Formula weight	480.50
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	Orthorhombic
Space group	P 21 21 21
Unit cell dimensions	a = 4.9007(18) Å α = 90°. b = 20.554(7) Å β = 90°. c = 23.071(9) Å γ = 90°.
Volume	2323.9(15) Å ³
Z	4
Density (calculated)	1.373 Mg/m ³

Absorption coefficient	0.095 mm ⁻¹
F(000)	1008
Crystal size	0.562 x 0.283 x 0.164 mm ³
Theta range for data collection	1.327 to 28.207 °.
Index ranges	-6 ≤ h ≤ 6, -27 ≤ k ≤ 27, -28 ≤ l ≤ 30
Reflections collected	34202
Independent reflections	5740 [R(int) = 0.1962]
Completeness to theta = 28.207 °	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7457 and 0.6597
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	5740 / 0 / 338
Goodness-of-fit on F ²	0.829
Final R indices [I > 2σ(I)]	R1 = 0.0687, wR2 = 0.1678
R indices (all data)	R1 = 0.1952, wR2 = 0.2580
Extinction coefficient	0.020(5)
Largest diff. peak and hole	0.257 and -0.276 e.Å ⁻³

Figure 28: ORTEP diagram of **1b**.

4.4.8 Theoretical Calculations

Generation of the DFT geometry optimization inputs was done either through prediction of the most probable geometries of the molecule/complex using CONFLEX 8 conformation search software package^{26, 27} or were constructed using Chem3D v22.2.0 (PerkinElmer ChemOffice). CONFLEX 8 conformation search was performed using MMFF94S (2010-12-04HG) force field with a search limit of 10 kcal/mol and the output conformation with the highest predicted Boltzmann population was used for geometry optimization. The DFT geometry optimization and energy calculation of the initial model obtained/constructed was conducted on Gaussian 09 program suite²¹ the B3LYP exchange-correlation functional^{22, 23} and the 6-31G(d,p) basis set.^{24, 25} The zero-point energy (ZPE) and basis set superposition error (BSSE) of each complex were calculated and used in Equation 5 to obtain the binding energy (BE).

For **1c** (free transporter)

Prediction of possible geometries of transporter **1c** in CONFLEX 8 yielded a total of 47 conformers, of which the major conformer **BSA-1** gave a Boltzmann population of 90.74%. The remaining conformations (Boltzmann population < 5.0%) were rejected. The optimized structure, Hartree-Fock (HF) energy and zero-point energy (ZPE) of **1c** were obtained through DFT geometry optimization and energy calculations of **BSA-1** (Figure 29).

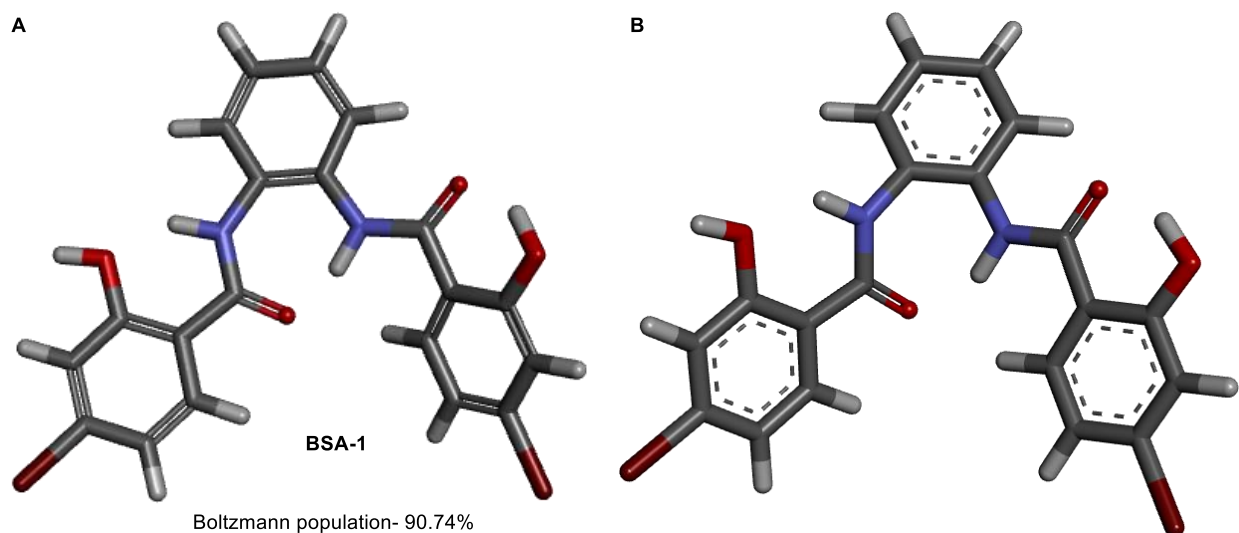


Figure 29: (A) Input coordinates of **1c** for geometry optimization with Gaussian 09. (B) Geometry optimized output.

For **1c** (BSA-1):

- HF energy = -6324.4529827 Hartree
- Zero-point energy = 0.3062181 Hartree

Table 6: Atomic coordinates of **1c** after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p) functional basis set.

Charge = 0

Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	1.0972	5.85221	-0.38138
2	C	-0.29026	5.98245	-0.30855
3	C	-1.0767	4.83984	-0.22403
4	C	-0.50203	3.56178	-0.19704
5	C	0.90287	3.43005	-0.26544
6	C	1.68423	4.59198	-0.36955
7	N	1.48076	2.14458	-0.25296
8	N	-1.40573	2.47543	0.00356
9	C	-1.46932	1.2881	-0.65773
10	C	2.68644	1.80119	0.284
11	C	3.1226	0.38614	0.15432
12	O	3.40149	2.63777	0.87595
13	C	-2.63214	0.3813	-0.35787
14	O	-0.61044	0.92928	-1.47871
15	C	-2.64454	-0.83978	-1.05013
16	C	-3.6489	-1.78446	-0.88489
17	C	-4.68309	-1.5025	0.00561
18	C	-4.71814	-0.30561	0.71199
19	C	-3.69937	0.63313	0.53021
20	O	-3.73316	1.81714	1.21965
21	C	4.37456	0.02116	0.72652
22	C	4.8312	-1.3057	0.62614

23	C	4.05653	-2.24461	-0.0291
24	C	2.82123	-1.91873	-0.59999
25	C	2.37272	-0.61034	-0.49946
26	O	5.15777	0.89221	1.37036
27	Br	-6.07946	-2.77056	0.26713
28	Br	4.68872	-4.04361	-0.16147
29	H	1.7276	6.73299	-0.45266
30	H	-0.75727	6.96195	-0.32382
31	H	-2.15927	4.92263	-0.17956
32	H	2.75907	4.48636	-0.41294
33	H	0.92533	1.45164	-0.75664
34	H	-2.19296	2.6865	0.60459
35	H	-1.82527	-1.02337	-1.73539
36	H	-3.63182	-2.71908	-1.43166
37	H	-5.53273	-0.10129	1.4
38	H	-4.50222	1.84231	1.80397
39	H	5.7865	-1.5594	1.06817
40	H	2.23347	-2.67259	-1.10854
41	H	1.41175	-0.3746	-0.9449
42	H	4.67237	1.76515	1.33735

For [1c+Cl⁻] (CBr1)**Table 7:** Atomic coordinates of [1c+Cl⁻] (CBr1) after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p) functional basis set.

Charge = -1 Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	-0.78423	6.10236	0.25906
2	C	0.60526	6.10098	0.36624
3	C	1.28936	4.88942	0.36139

4	C	0.60715	3.67384	0.2389
5	C	-0.80738	3.67493	0.11783
6	C	-1.48636	4.90668	0.1394
7	N	-1.47709	2.44275	-0.00534
8	N	1.32513	2.44841	0.24387
9	C	2.52626	2.25663	-0.40434
10	C	-2.82034	2.22722	-0.21778
11	C	-3.28151	0.79319	-0.25541
12	O	-3.63941	3.13495	-0.37756
13	C	3.17009	0.90132	-0.26629
14	O	3.05629	3.12629	-1.09524
15	C	4.30693	0.69179	-1.06092
16	C	4.98442	-0.51965	-1.08661
17	C	4.49771	-1.55469	-0.28739
18	C	3.38826	-1.40134	0.52754
19	C	2.7213	-0.16192	0.55228
20	O	1.66967	0.02725	1.37444
21	C	-2.52396	-0.34343	0.11887
22	C	-3.11202	-1.62285	0.10045
23	C	-4.43064	-1.75314	-0.30215
24	C	-5.20346	-0.65776	-0.68987
25	C	-4.61316	0.59807	-0.65176
26	O	-1.24112	-0.18929	0.48769
27	Br	5.40661	-3.24688	-0.31085
28	Br	-5.22555	-3.50133	-0.33168
29	H	-1.33533	7.03904	0.26513
30	H	1.15705	7.03216	0.45796
31	H	2.36851	4.86488	0.43193
32	H	-2.56124	4.89849	0.04378
33	H	-0.91708	1.59924	0.06213
34	H	1.02218	1.68143	0.84057

35	H	4.63463	1.52469	-1.67317
36	H	5.8577	-0.6649	-1.71099
37	H	3.01014	-2.21552	1.1345
38	H	1.24455	-0.84586	1.64835
39	H	-2.5212	-2.47802	0.40844
40	H	-6.23233	-0.78825	-1.00242
41	H	-5.17855	1.4832	-0.92133
42	H	-0.84582	-1.02317	0.89484
43	Cl	0.12105	-2.45658	1.85104

For [1c+Cl⁻] (CBr2)**Table 8:** Atomic coordinates of [1c+Cl⁻] (CBr2) after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p) functional basis set.

Charge = -1 Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	0.69756	5.73905	-0.41401
2	C	-0.69799	5.73911	-0.41401
3	C	-1.39066	4.5334	-0.42348
4	C	-0.7063	3.31067	-0.41448
5	C	0.70562	3.3106	-0.41442
6	C	1.39009	4.53326	-0.42347
7	N	1.41907	2.08099	-0.4296
8	N	-1.41977	2.08108	-0.42986
9	C	-2.50112	1.82894	0.35159
10	C	2.5001	1.82887	0.3523
11	C	3.18592	0.51006	0.2365
12	C	-3.18642	0.50983	0.23604
13	C	-2.79342	-0.50104	-0.65815
14	C	-3.46762	-1.71492	-0.73071

15	C	-4.55959	-1.91773	0.11373
16	C	-4.98893	-0.95717	1.01206
17	C	-4.30689	0.27041	1.08349
18	C	4.30595	0.2706	1.08451
19	C	4.98856	-0.95665	1.01279
20	C	4.5602	-1.91688	0.11363
21	C	3.46868	-1.714	-0.73139
22	C	2.79393	-0.50044	-0.65858
23	H	4.13395	1.96247	1.85218
24	O	2.9265	2.68134	1.16939
25	O	-2.9282	2.68159	1.16815
26	O	-4.75422	1.17507	1.96391
27	O	4.75235	1.17494	1.96573
28	Br	-5.50581	-3.58937	0.02871
29	Br	5.50715	-3.58806	0.02827
30	H	1.24879	6.67518	-0.41328
31	H	-1.24913	6.67529	-0.41319
32	H	-2.4726	4.51999	-0.41904
33	H	2.47204	4.51978	-0.41901
34	H	1.08753	1.38413	-1.11539
35	H	-1.08802	1.3842	-1.11547
36	H	-1.94483	-0.35529	-1.32441
37	H	-3.15002	-2.48116	-1.42682
38	H	-5.83725	-1.11961	1.66502
39	H	-4.13596	1.96269	1.85049
40	H	5.83654	-1.11906	1.66619
41	H	3.15184	-2.47996	-1.42815
42	H	1.94569	-0.35471	-1.3253
43	Cl	1.03E-4	0.35071	-2.68429

4.4.9 Photoactivation studies

Photocleavage studies of the protransporters were conducted by placing a solution of the sample in a cuvette/NMR tube approx. 1 cm from the 405 nm LED (~3.8 W radiant flux output) and irradiating for the specified duration of time.

NMR analysis of photocleavage

Solutions of the protransporters **2** and **3** (0.5 mL, ~2 mg/mL) in DMSO-*d*₆ were prepared in borosilicate glass NMR tubes (Norell) and the ¹H NMR spectra (32 scans) recorded on a Bruker Avance III HD 400 MHz NMR spectrometer. The tubes were then irradiated with 405 nm light for specified time periods and the ¹H NMR spectra were recorded. ¹H NMR spectrum of compound **1c** was recorded under similar conditions for comparison.

Photoactivated ion transport

Stock solutions of the protransporters **2** and **3** (0.5 mM) in DMSO were taken in 1 mL quartz cuvettes and irradiated with 405 nm light for specified time periods. The transport activities of the samples were then assessed across EYPC-LUVs→HPTS (**Assay protocol A1**) by the addition of 20 µL of the irradiated stocks at the 100 s time point of the assay. Transporter **1c** (5 µM working concentration) was used as the positive control and DMSO as the blank.

4.4.10 Biological Studies

Cell lines and cell culture:

The human breast cancer cell line (MCF-7) was used for studies. All cell lines were cultured in the presence of high glucose Dulbecco's Modified Eagle media (DMEM; Lonza) containing 2 mM L-glutamine. The media was supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; ThermoFisher Scientific), 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were maintained in tissue-culture treated 60 mm dishes at 37 °C in a humidified 5% CO₂ incubator (Thermo Scientific).

Assessment of cellular viability (MTT assay):

The cells were dispersed in a 96-well flat bottom tissue culture treated plates (Eppendorf) at the density of 8000 cells/well (per 100 µL) and incubated at 37 °C in a humidified 5% CO₂ incubator for 24 h. Compounds were added to each well at the required concentrations, maintaining the maximum amount of DMSO at 1 µL. Following 24 h incubation at 37 °C the media was aspirated out and replaced with 110 µL of MTT solution (0.5 mg/mL) in DMEM and incubated for 4 h in

identical conditions. Then, the media was removed and 100 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was recorded in a Varioskan Flash (Thermo Scientific) multimode plate reader at 570 nm wavelength. All experiments were performed in triplicate, and the relative cell viability (%) was expressed as a percentage of vehicle (DMSO- 1 μL) treated control.

Photoactivation of compounds in cells:

MCF 7 were dispersed in a 96-well flat bottom tissue culture treated plates (Eppendorf) in complete L-15 medium at a density of 8000 cells/well (per 100 μL) and incubated at 37 °C in a 5% CO_2 incubator for 24 h. The cells were then treated with compounds (stocks in DMSO) at different concentrations, maintaining a maximum amount of DMSO at 1 μL per well, and the plate was irradiated for the specified time at 405 nm light of 0.0412 W/cm^2 radiant flux using a high-power LED. The plate was then incubated for 24 h at 37 °C (5% CO_2). Post incubation, the media was aspirated out and replaced with 110 μL (per well) of MTT solution (0.5 mg/mL) in DMEM. Following incubation at 37 °C (5% CO_2) for 4 h, the media was removed, and the formazan crystals formed were dissolved using 100 μL DMSO. The absorbance for each well was measured at 570 nm using a Varioskan Flash (Thermo Scientific) multimode plate reader. All experiments were performed in triplicate, and the relative cell viability (%) was expressed as a percentage of vehicle (DMSO- 1 μL) treated control.

4.5 Characterization Spectra

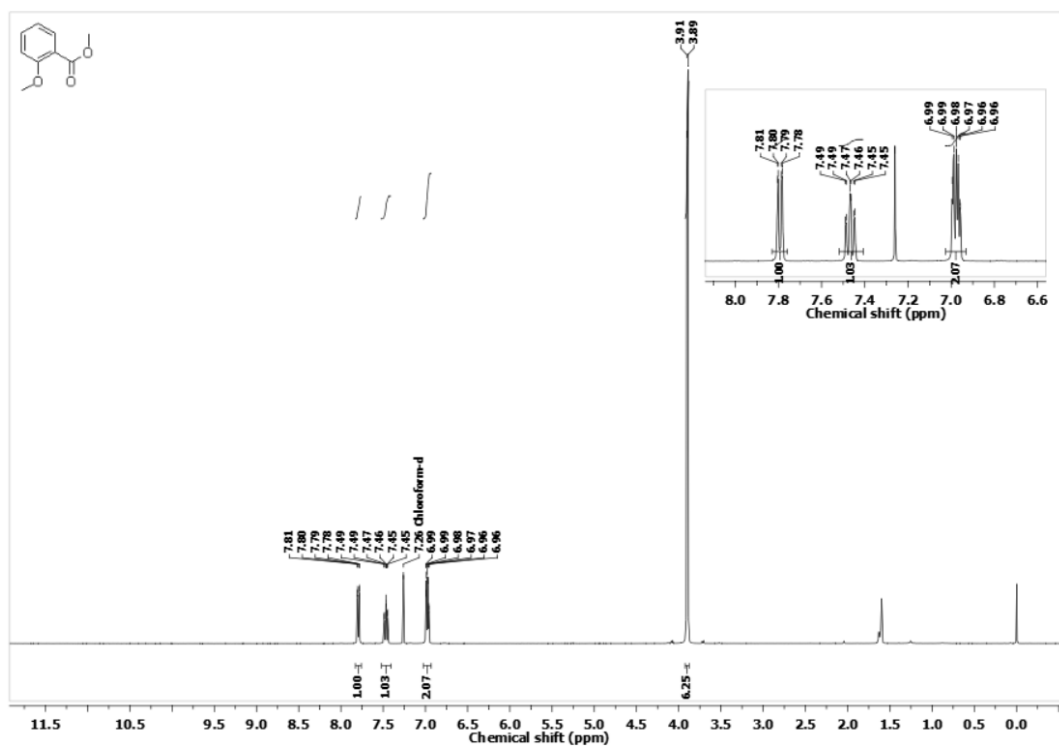


Figure 30: 400 MHz ^1H NMR spectrum of 5a in CDCl_3 .

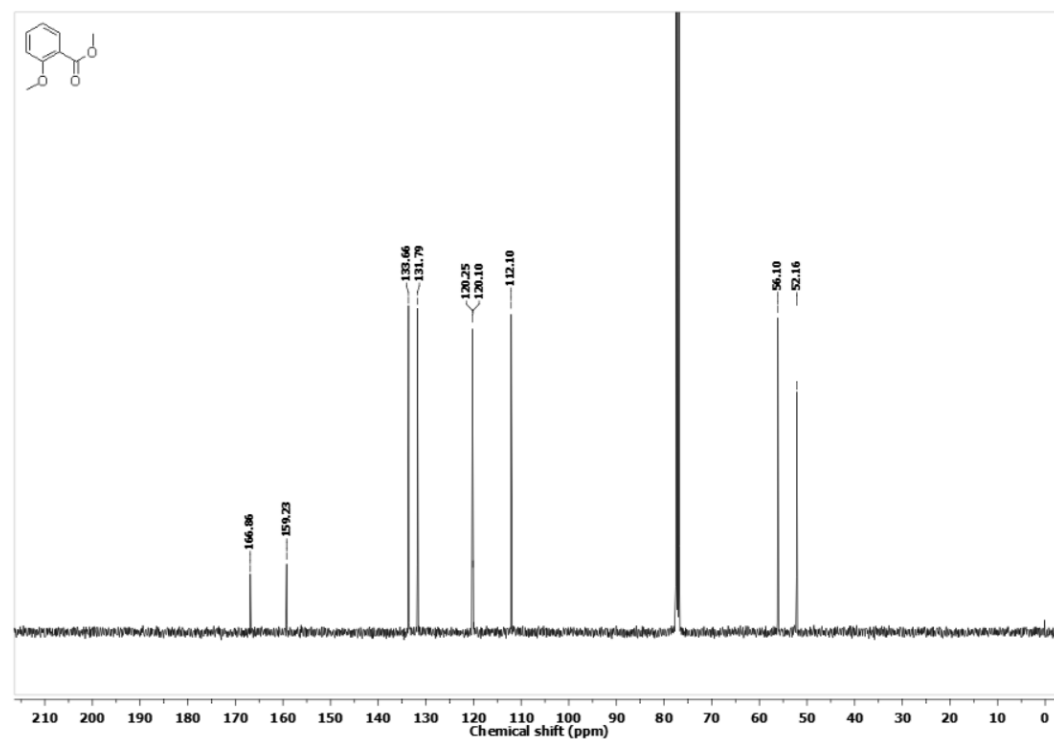


Figure 31: 101 MHz ^{13}C NMR spectrum of 5a in CDCl_3 .

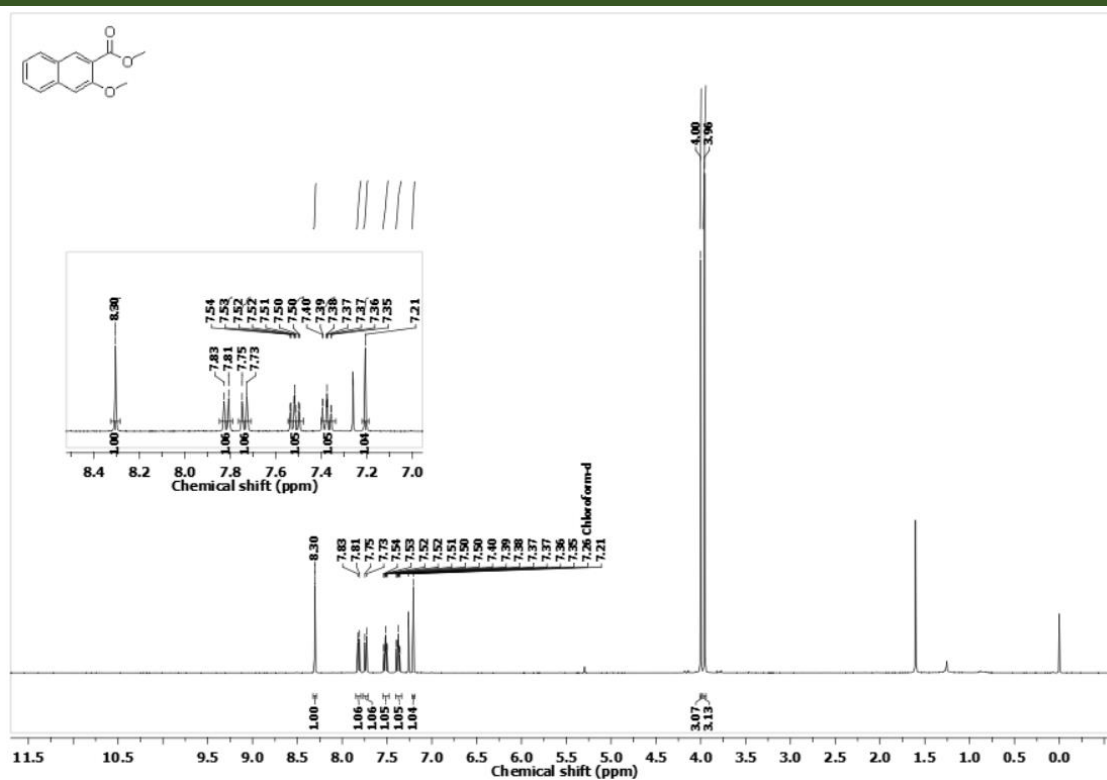


Figure 32: 400 MHz ^1H NMR spectrum of **5b** in CDCl_3 .

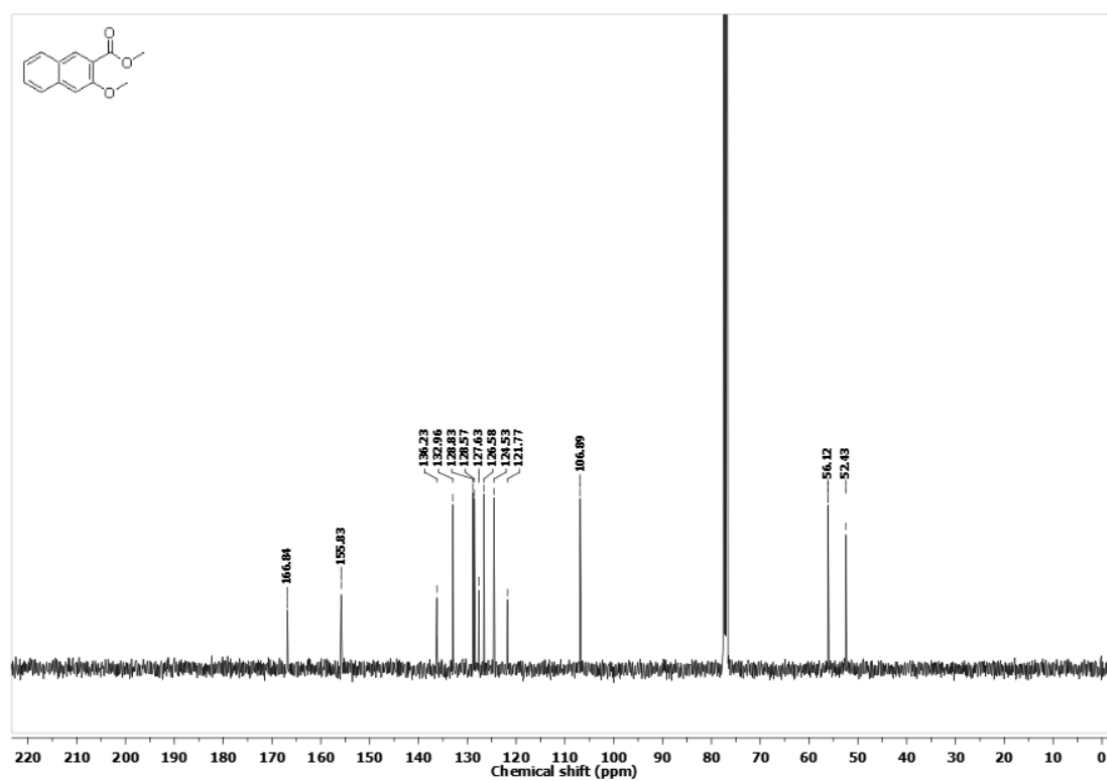


Figure 33: 101 MHz ^{13}C NMR spectrum of **5b** in CDCl_3 .

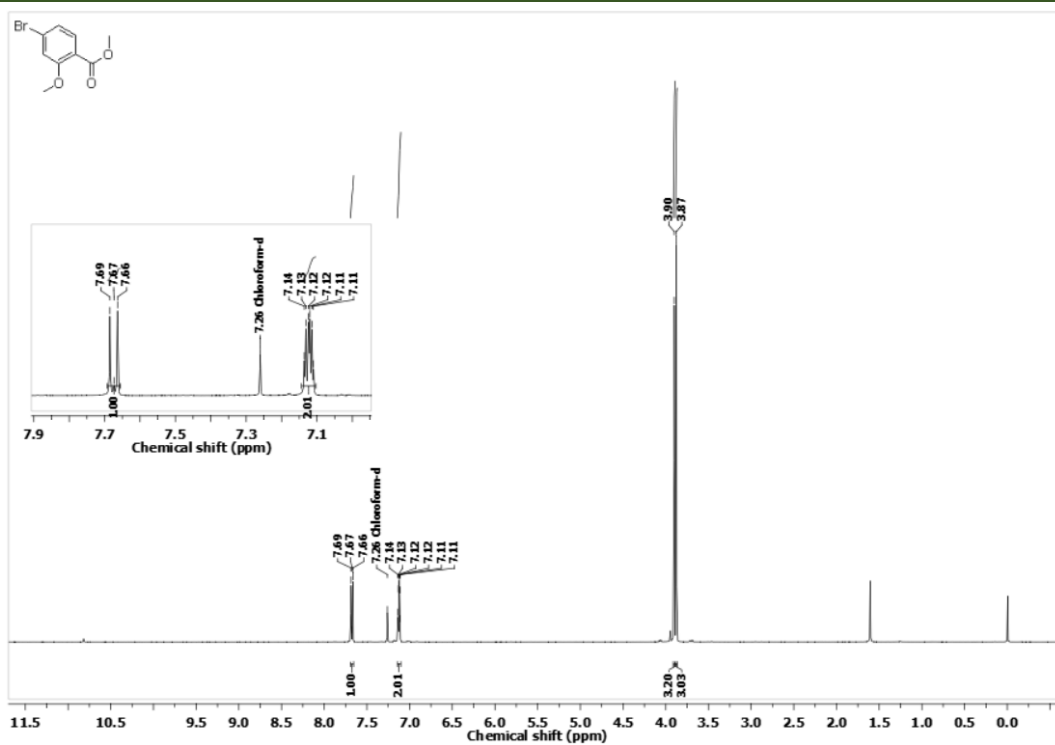


Figure 34: 400 MHz ^1H NMR spectrum of 5c in CDCl_3 .

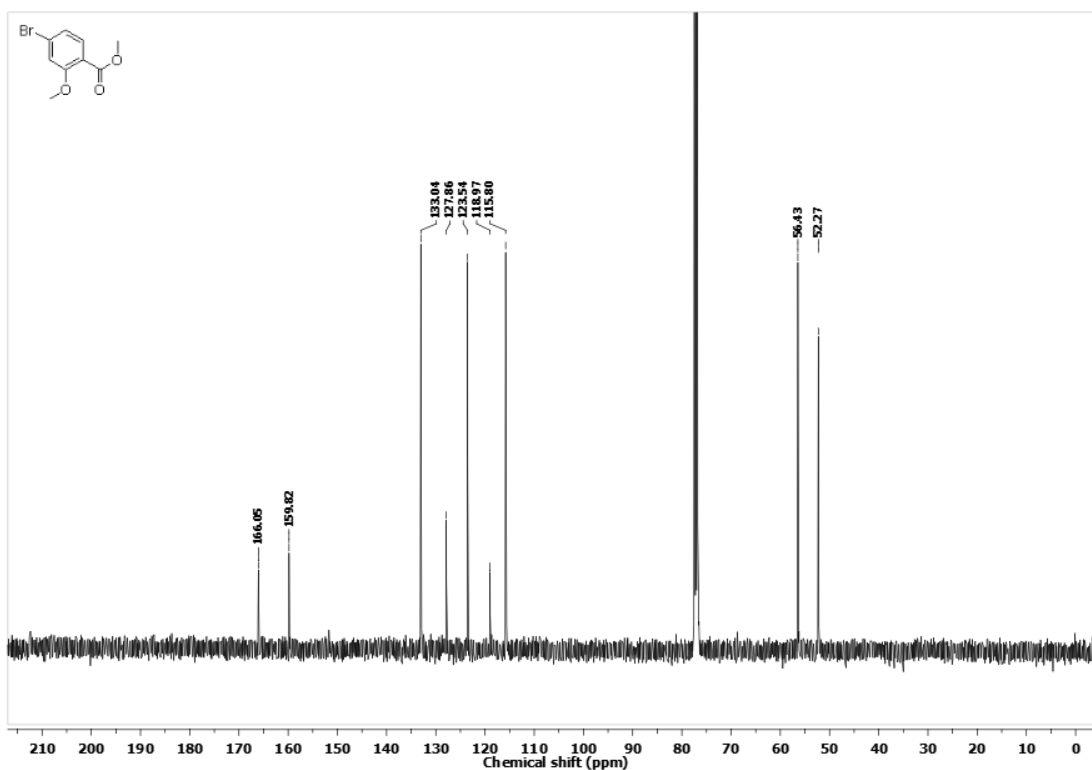


Figure 35: 101 MHz ^{13}C NMR spectrum of 5c in CDCl_3 .

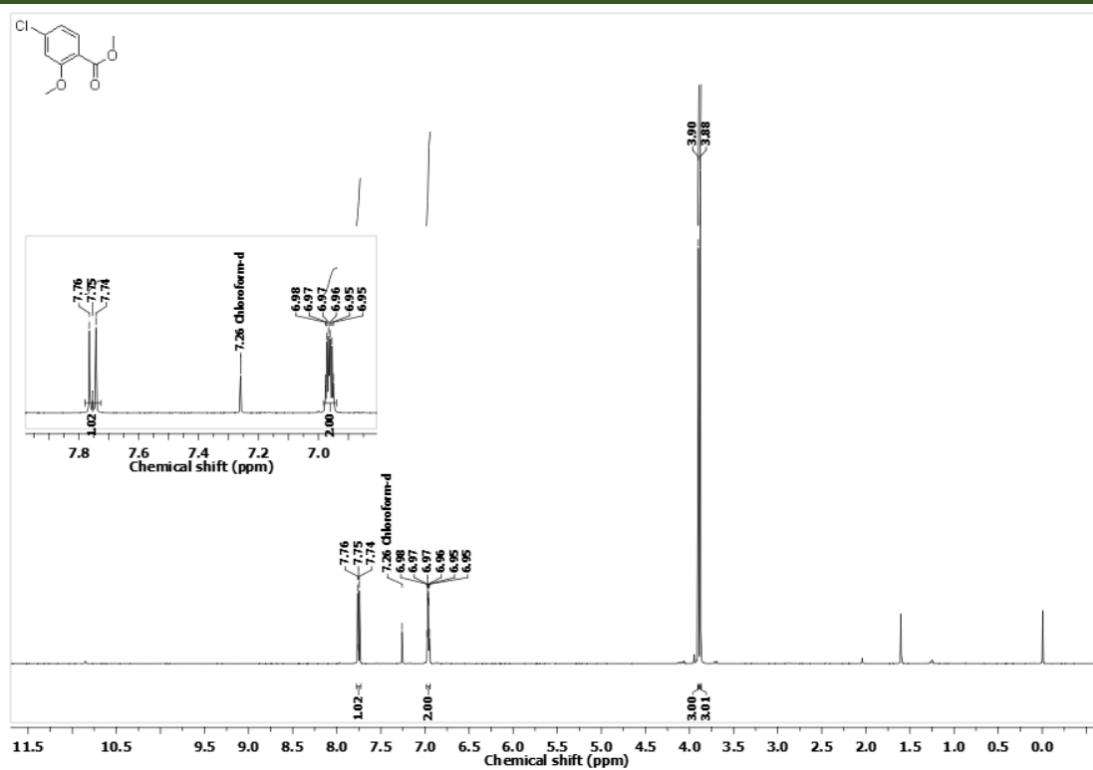


Figure 36: 400 MHz ¹H NMR spectrum of **5d** in CDCl₃.

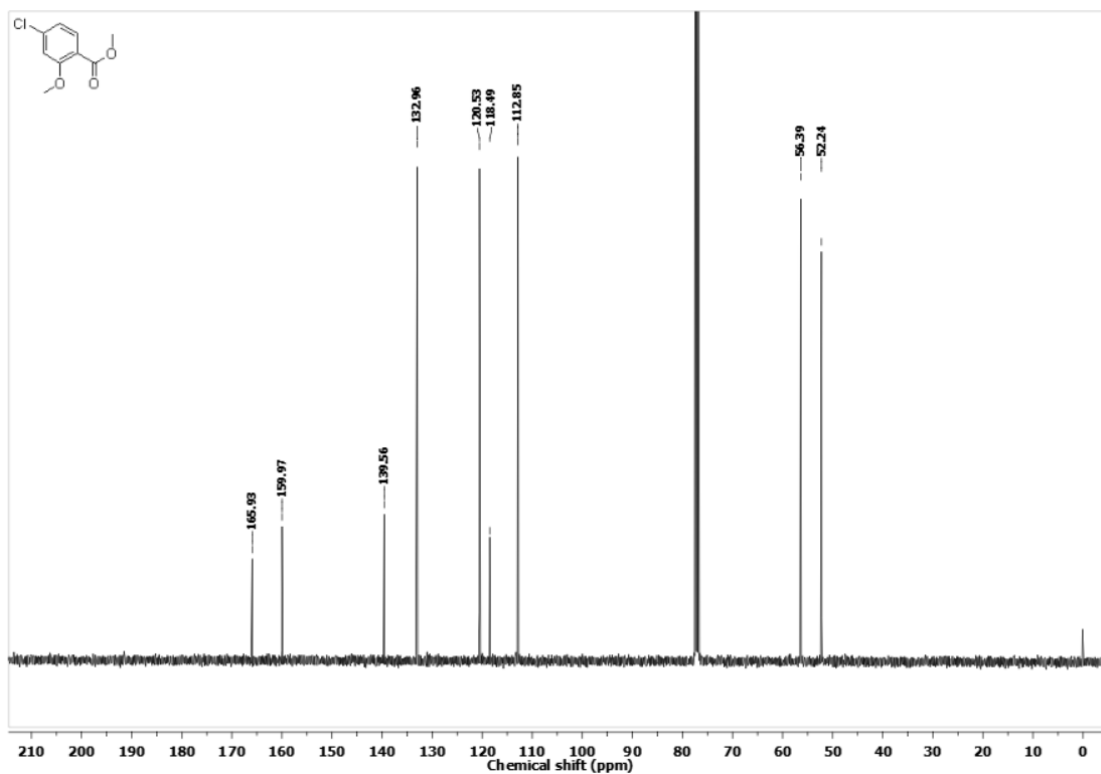


Figure 37: 101 MHz ¹³C NMR spectrum of **5d** in CDCl₃.

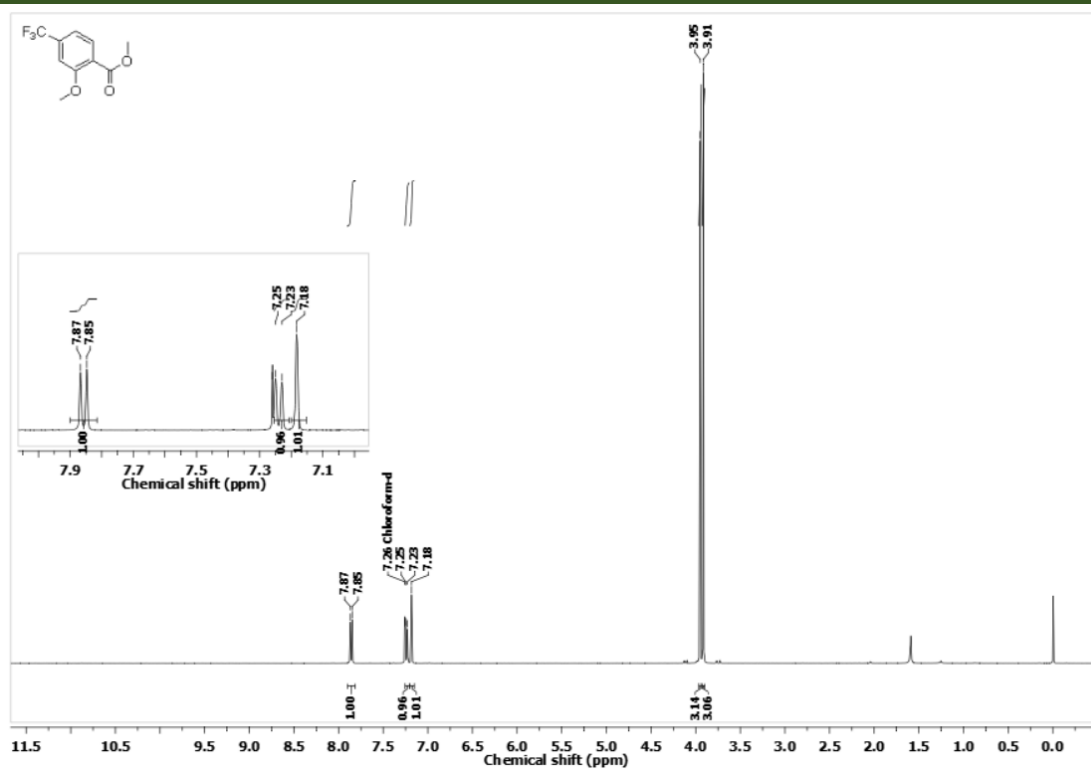


Figure 38: 400 MHz ^1H NMR spectrum of **5e** in CDCl_3 .

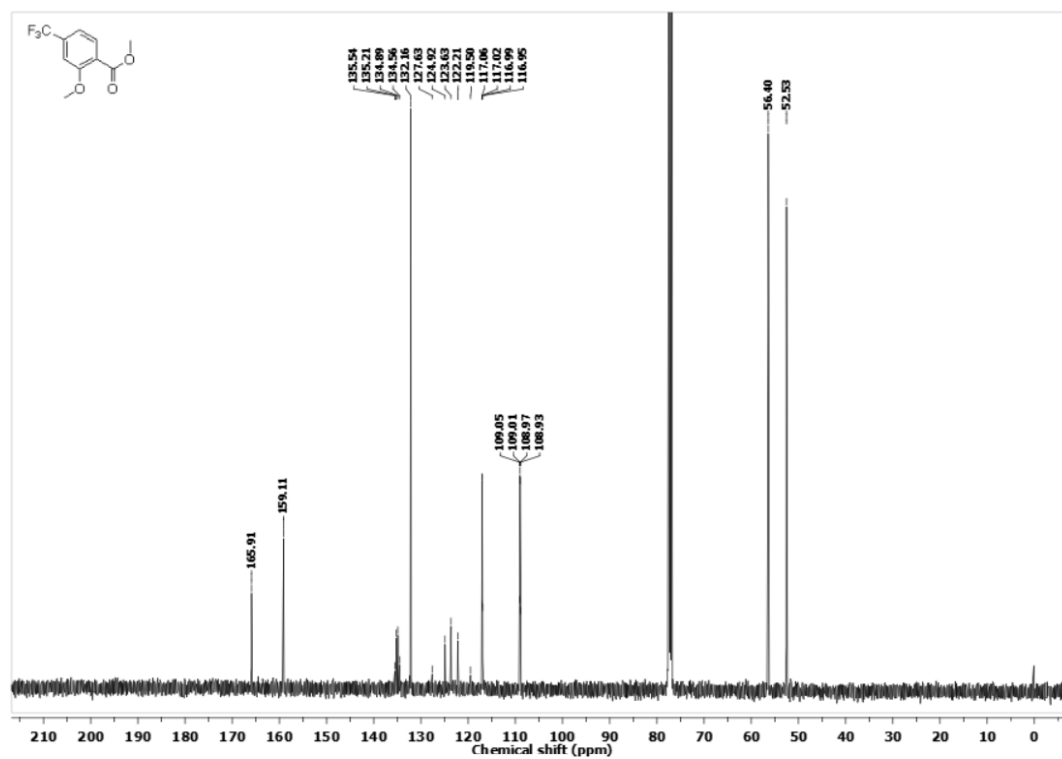


Figure 39: 101 MHz ^{13}C NMR spectrum of **5e** in CDCl_3 .

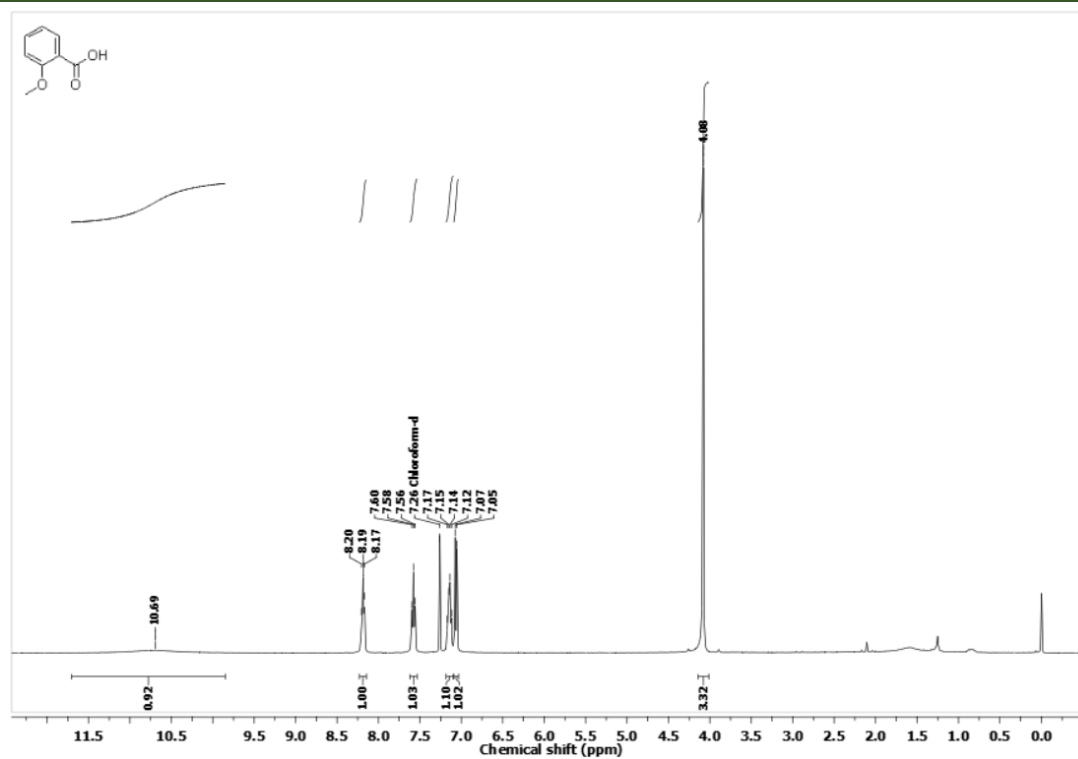


Figure 40: 400 MHz ^1H NMR spectrum of **6a** in CDCl_3 .

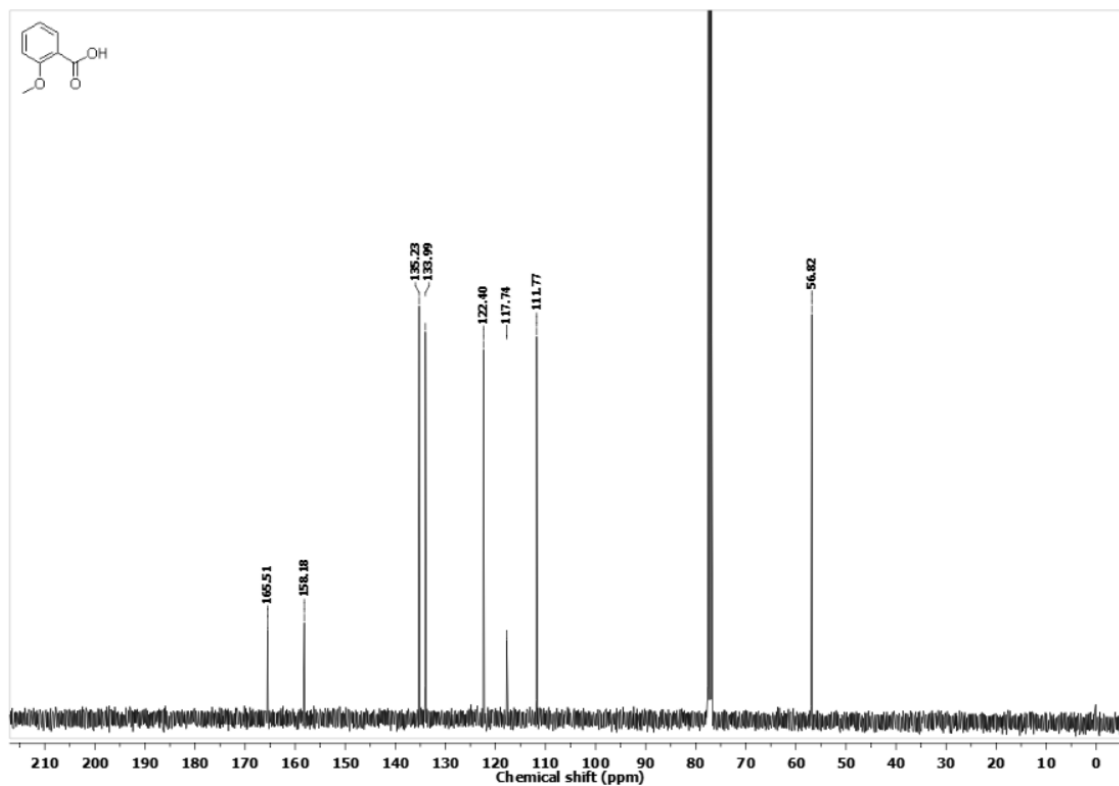


Figure 41: 101 MHz ^{13}C NMR spectrum of **6a** in CDCl_3 .

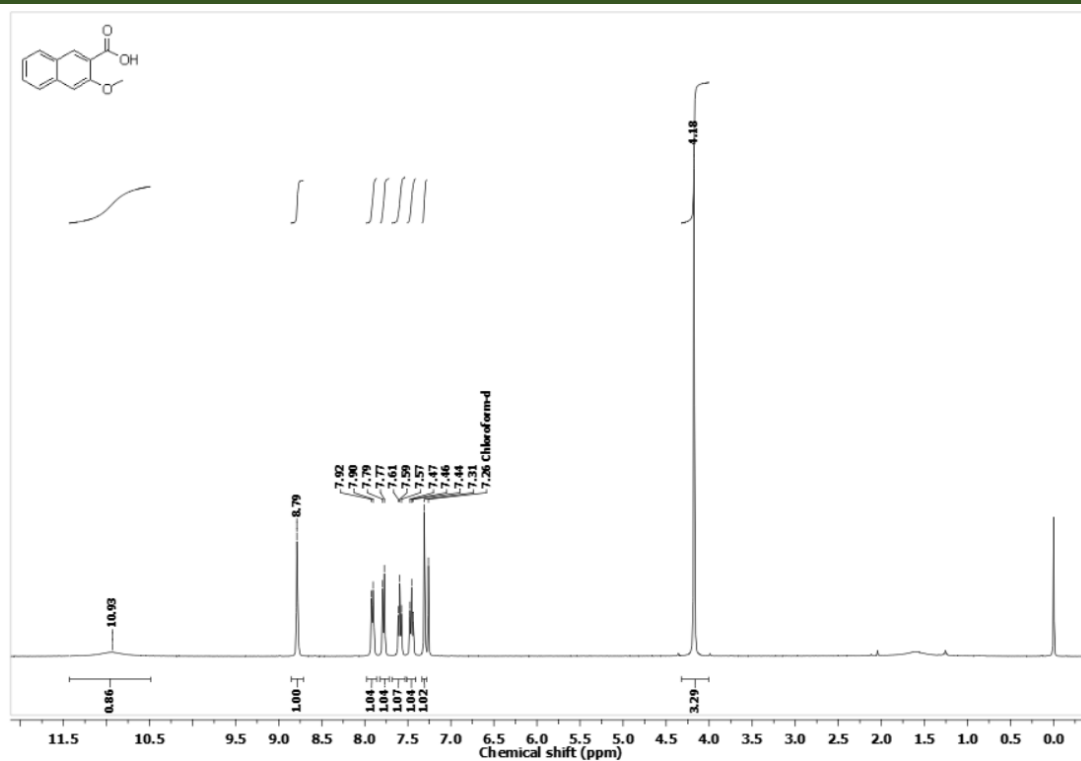


Figure 42: 400 MHz ^1H NMR spectrum of **6b** in CDCl_3 .

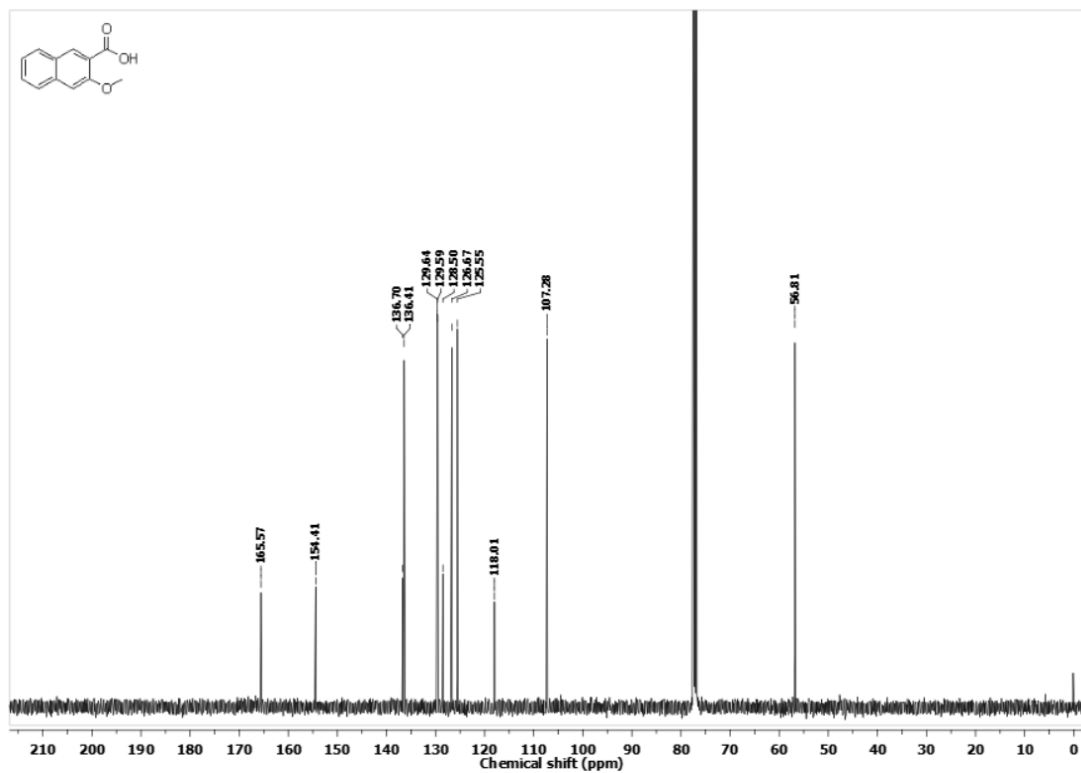


Figure 43: 101 MHz ^{13}C NMR spectrum of **6b** in CDCl_3 .

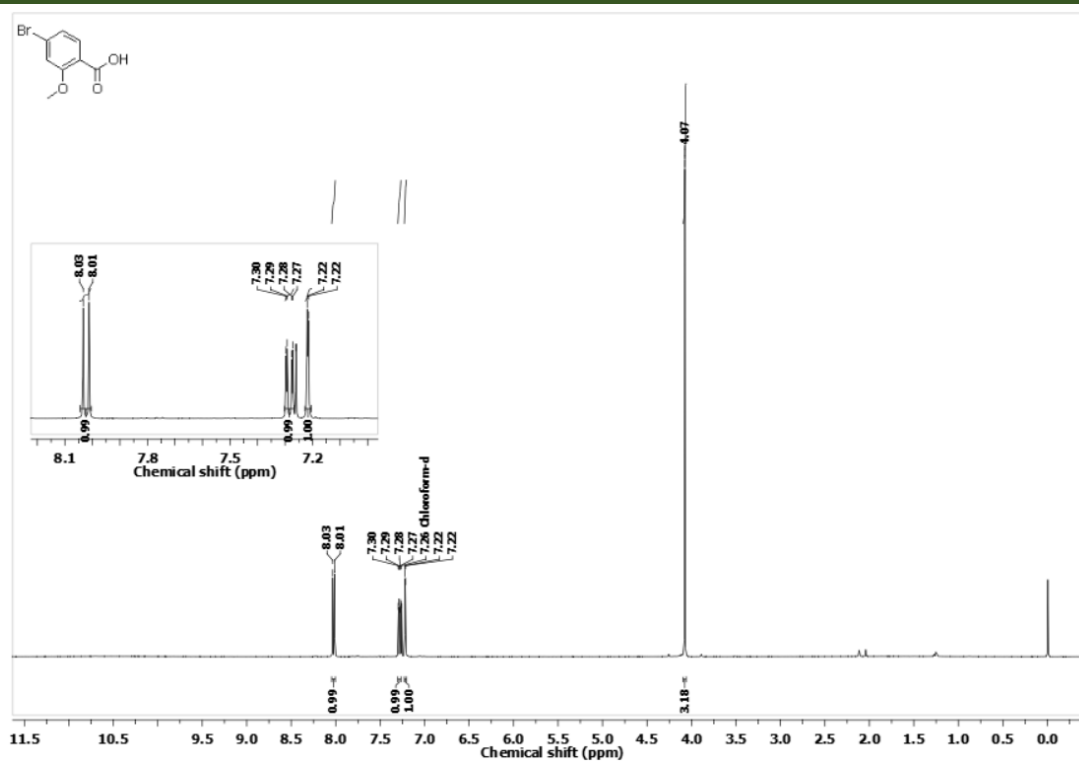


Figure 44: 400 MHz ^1H NMR spectrum of **6c** in CDCl_3 .

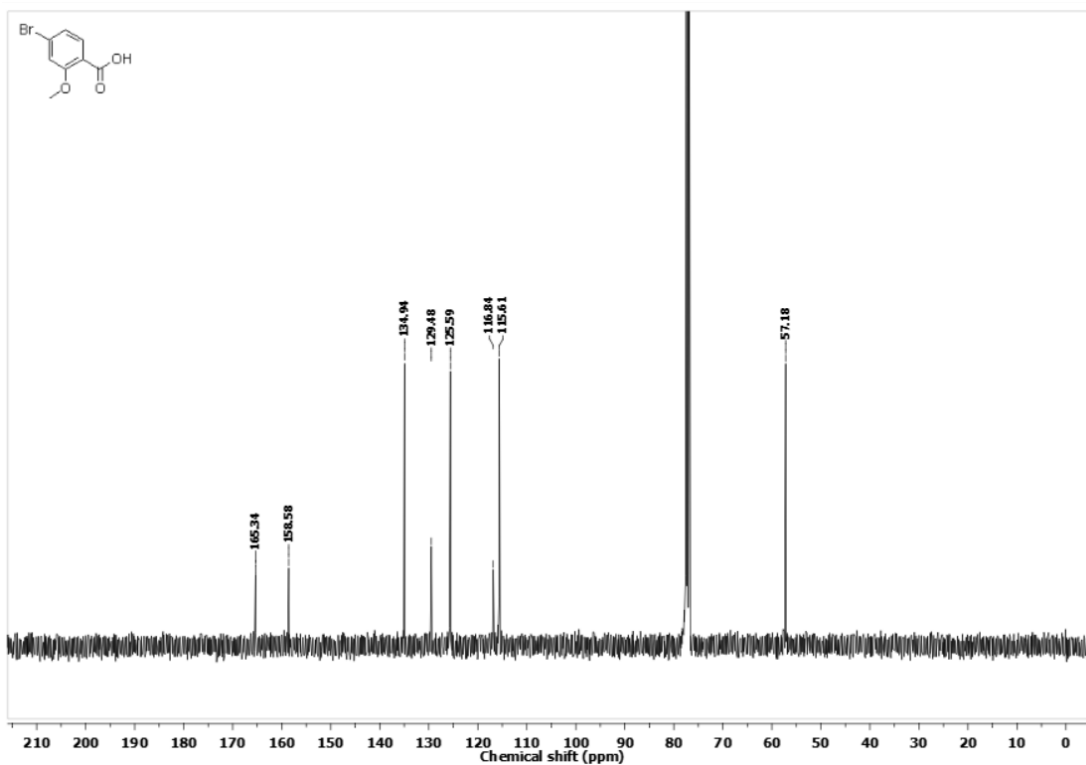


Figure 45: 101 MHz ^{13}C NMR spectrum of **6c** in CDCl_3 .

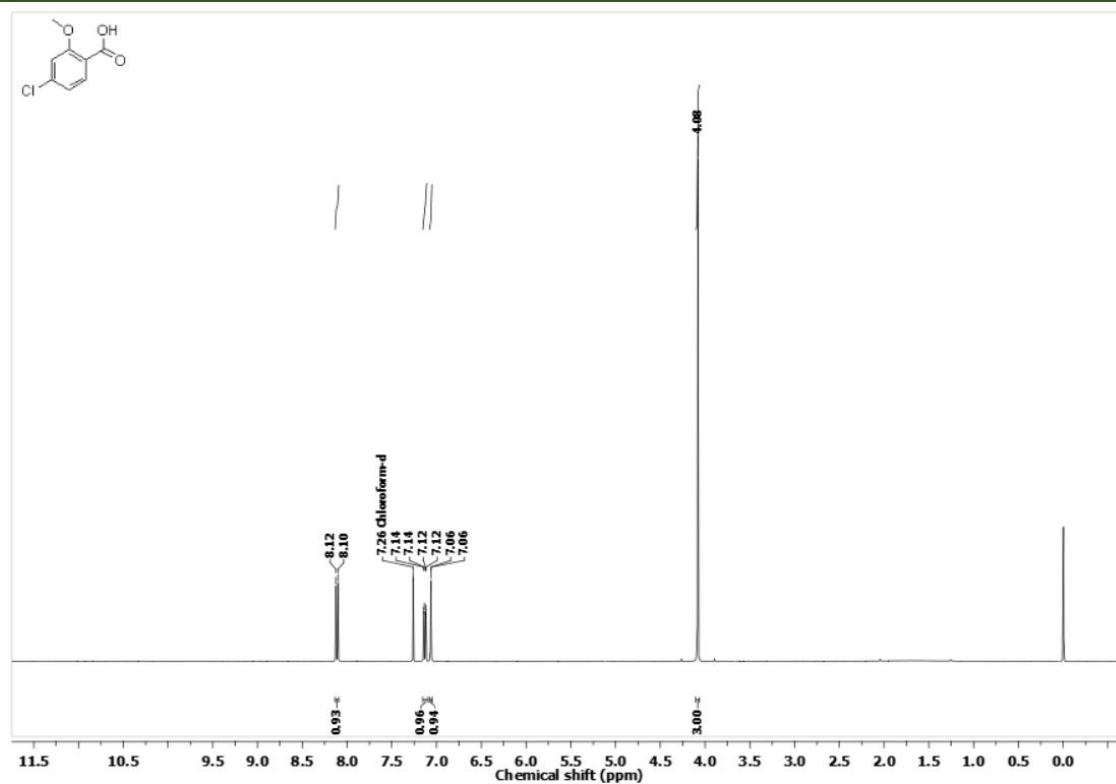


Figure 46: 400 MHz ^1H NMR spectrum of **6d** in CDCl_3 .

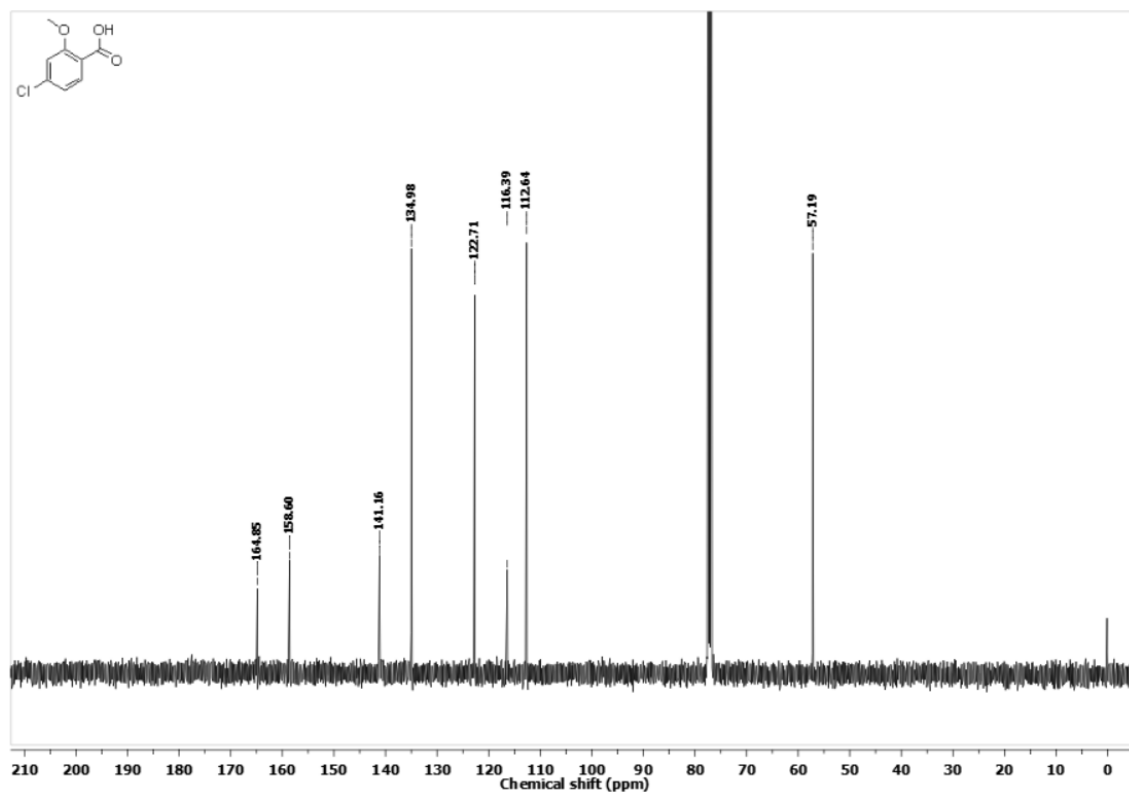


Figure 47: 101 MHz ^{13}C NMR spectrum of **6d** in CDCl_3 .

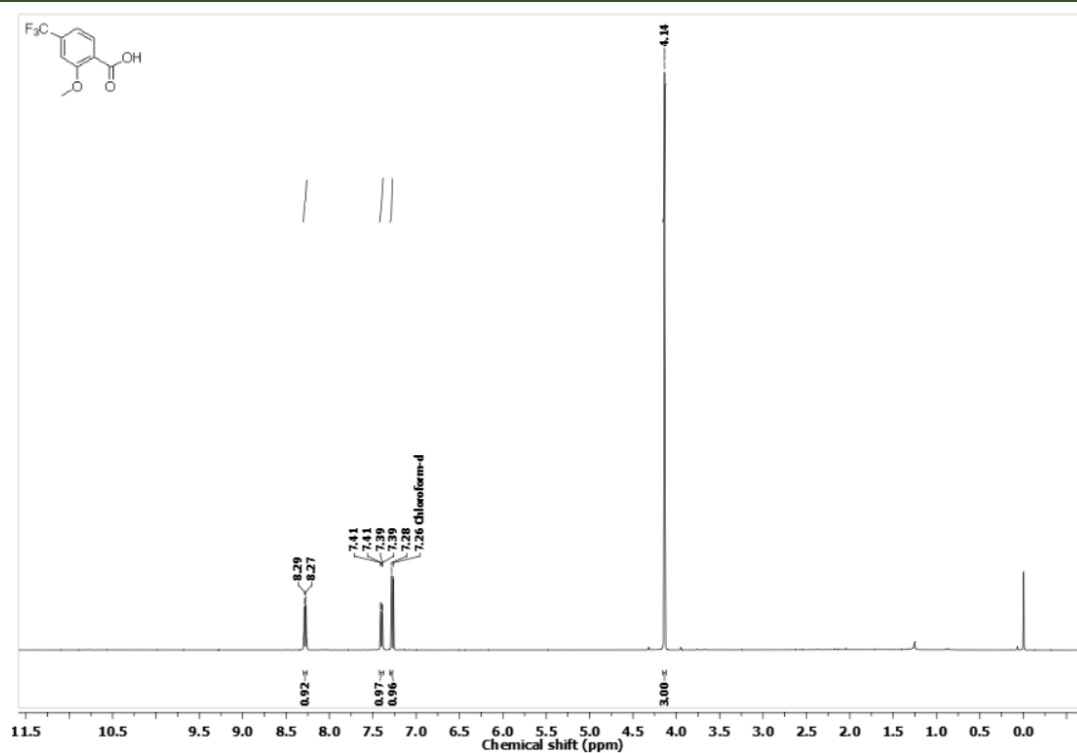


Figure 48: 400 MHz ^1H NMR spectrum of **6e** in CDCl_3 .

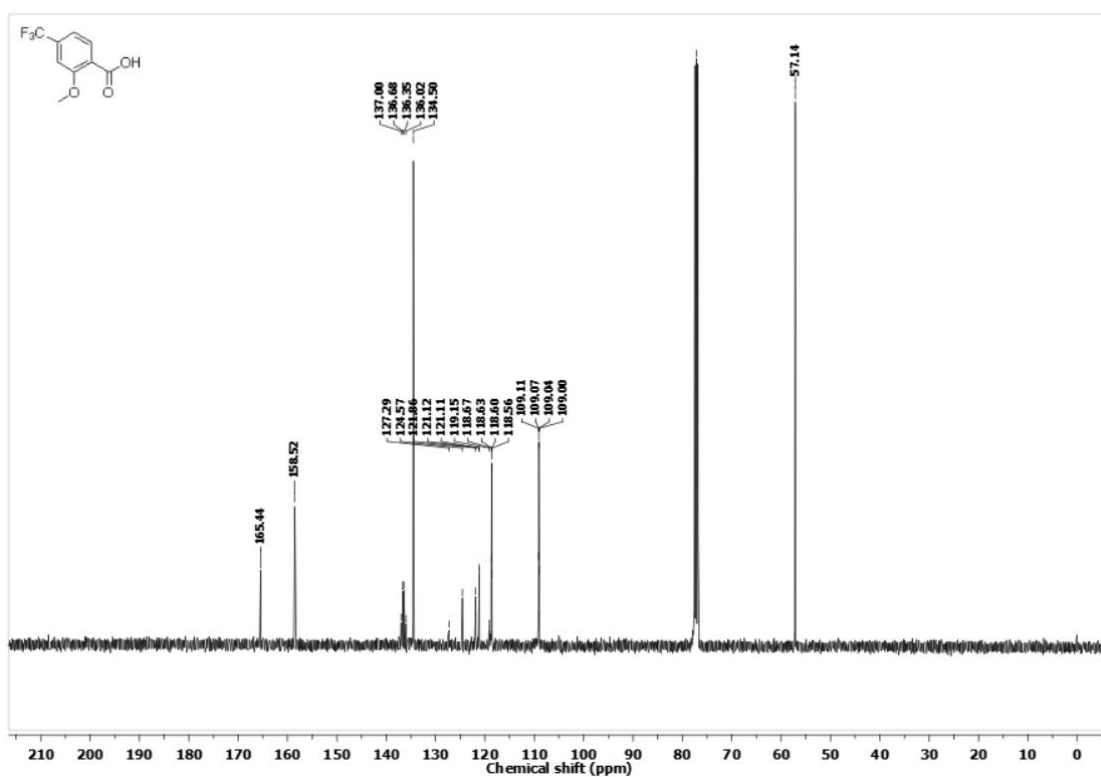


Figure 49: 101 MHz ^{13}C NMR spectrum of **6e** in CDCl_3 .

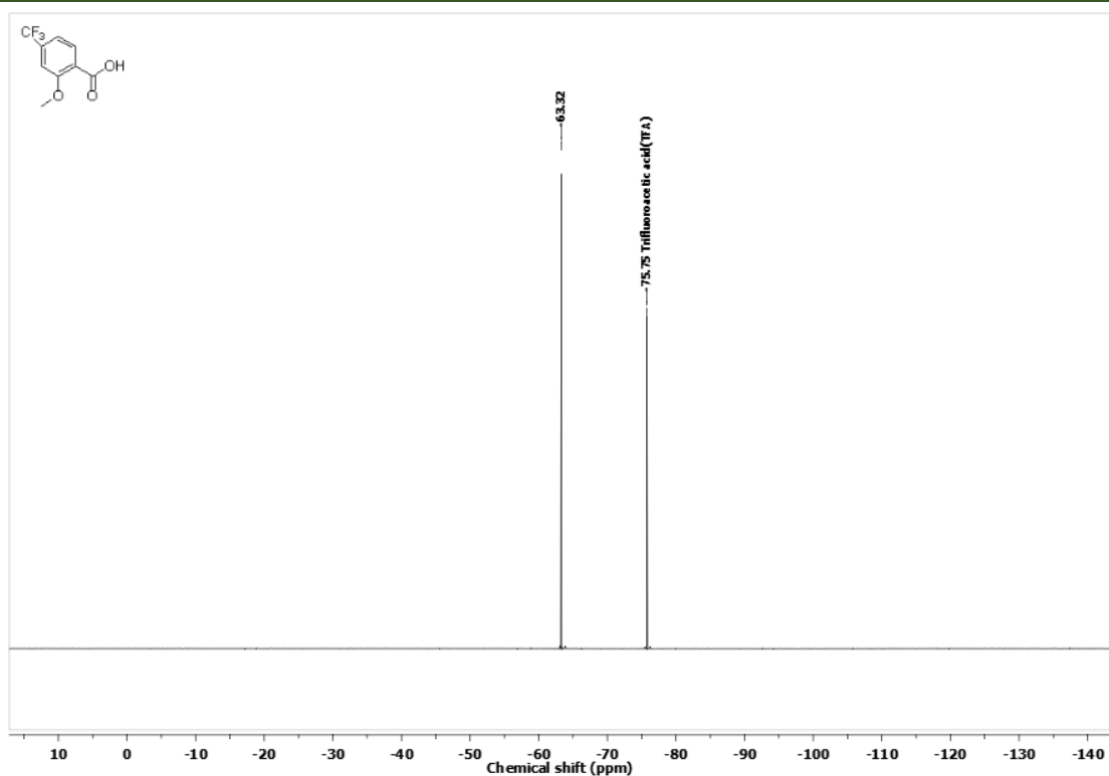


Figure 50: 377 MHz ^{19}F NMR spectrum of **6e** in CDCl_3 .

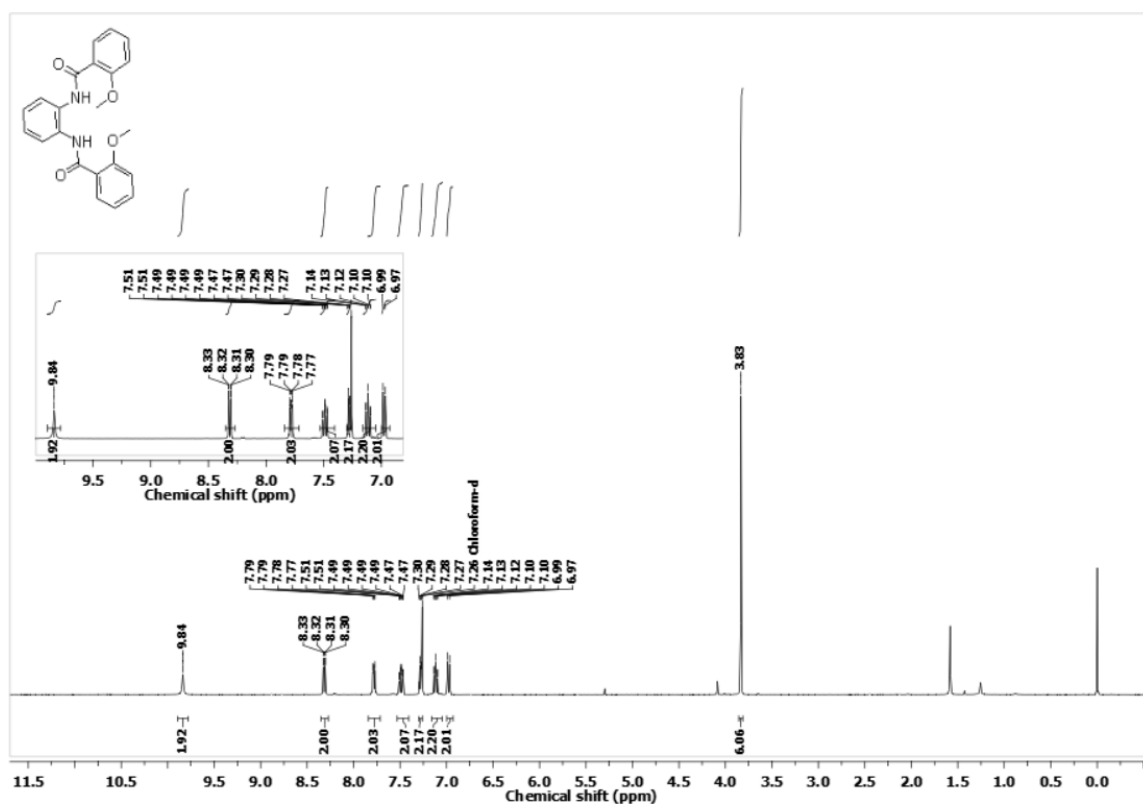


Figure 51: 400 MHz ^1H NMR spectrum of **7a** in CDCl_3 .

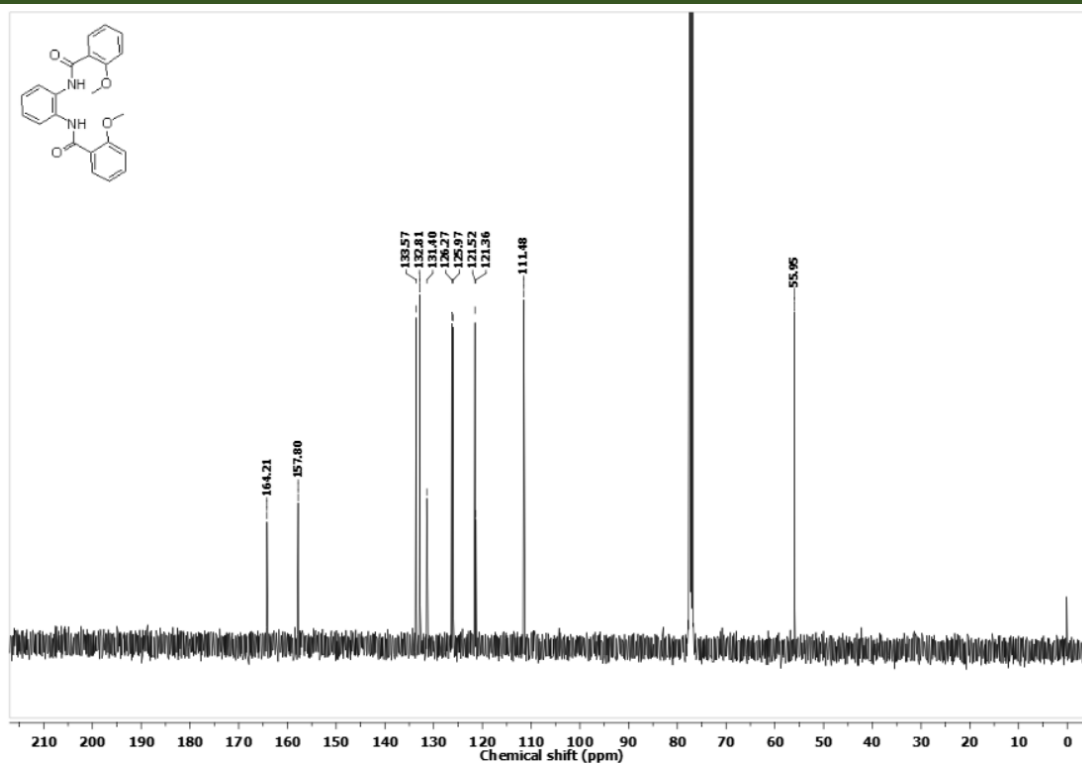


Figure 52: 101 MHz ¹³C NMR spectrum of **7a** in CDCl₃.

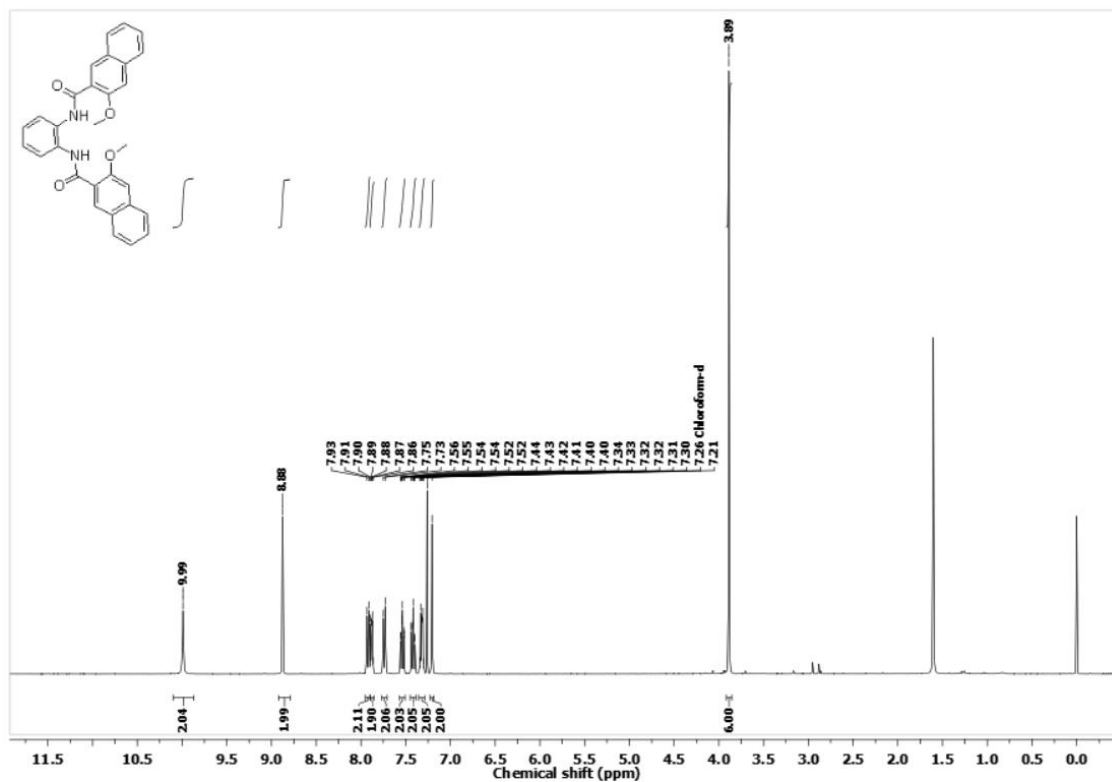


Figure 53: 400 MHz ¹H NMR spectrum of **7b** in CDCl₃.

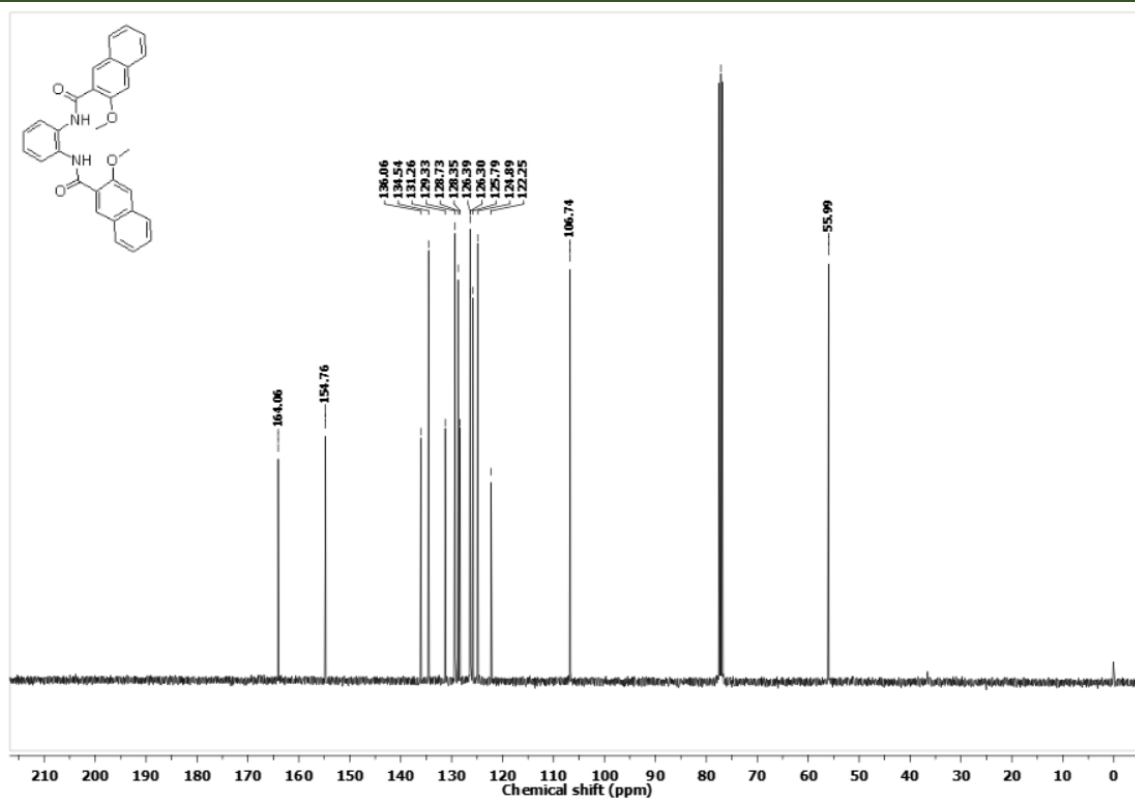


Figure 54: 101 MHz ^{13}C NMR spectrum of **7b** in CDCl_3 .

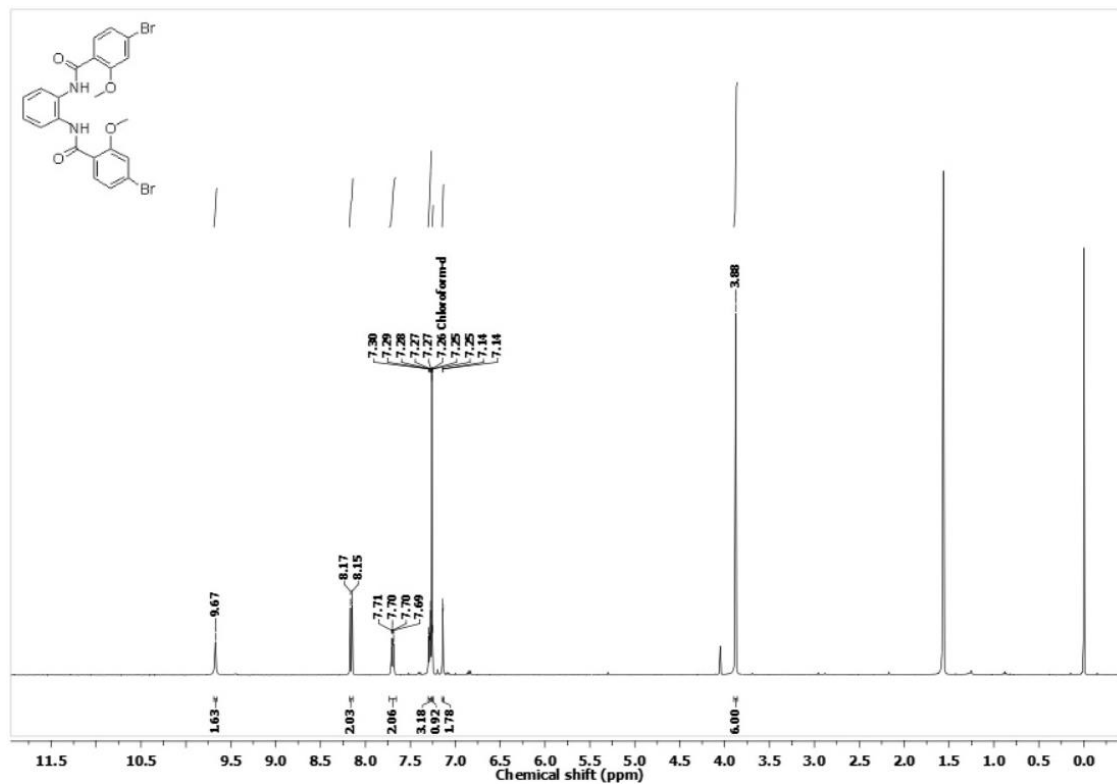


Figure 55: 400 MHz ^1H NMR spectrum of **7c** in CDCl_3 .

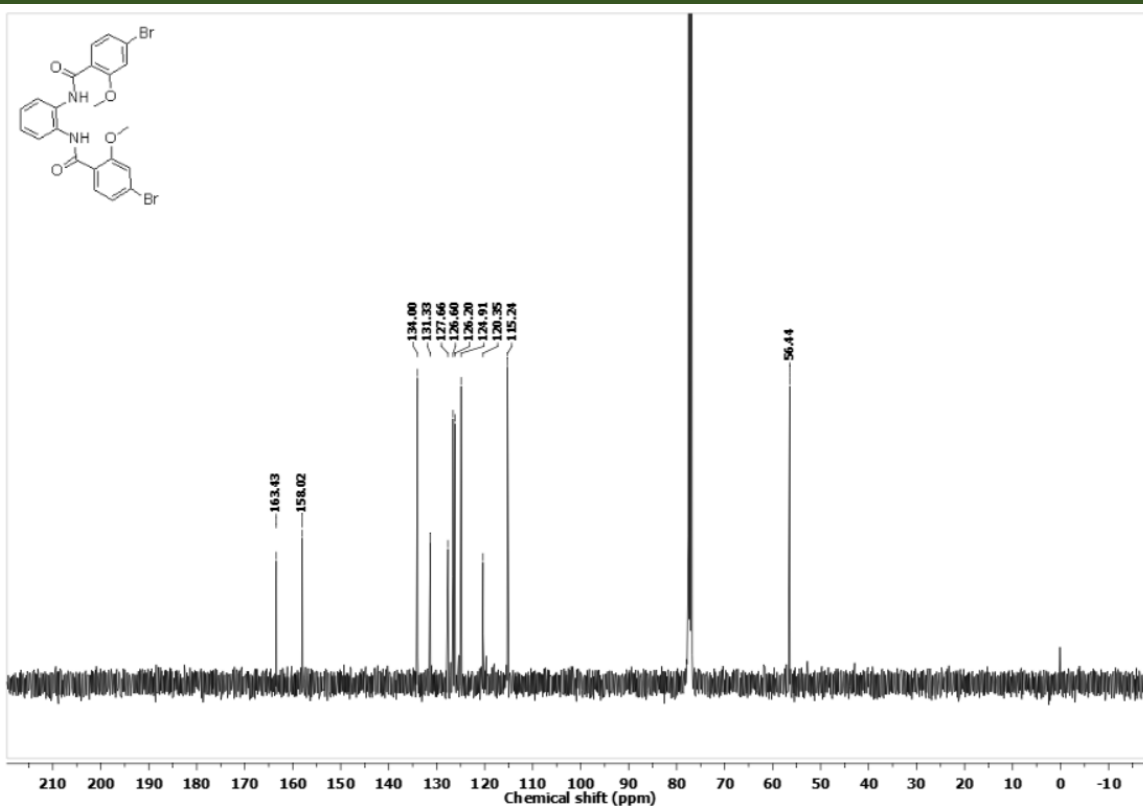


Figure 56: 101 MHz ^{13}C NMR spectrum of 7c in CDCl_3 .

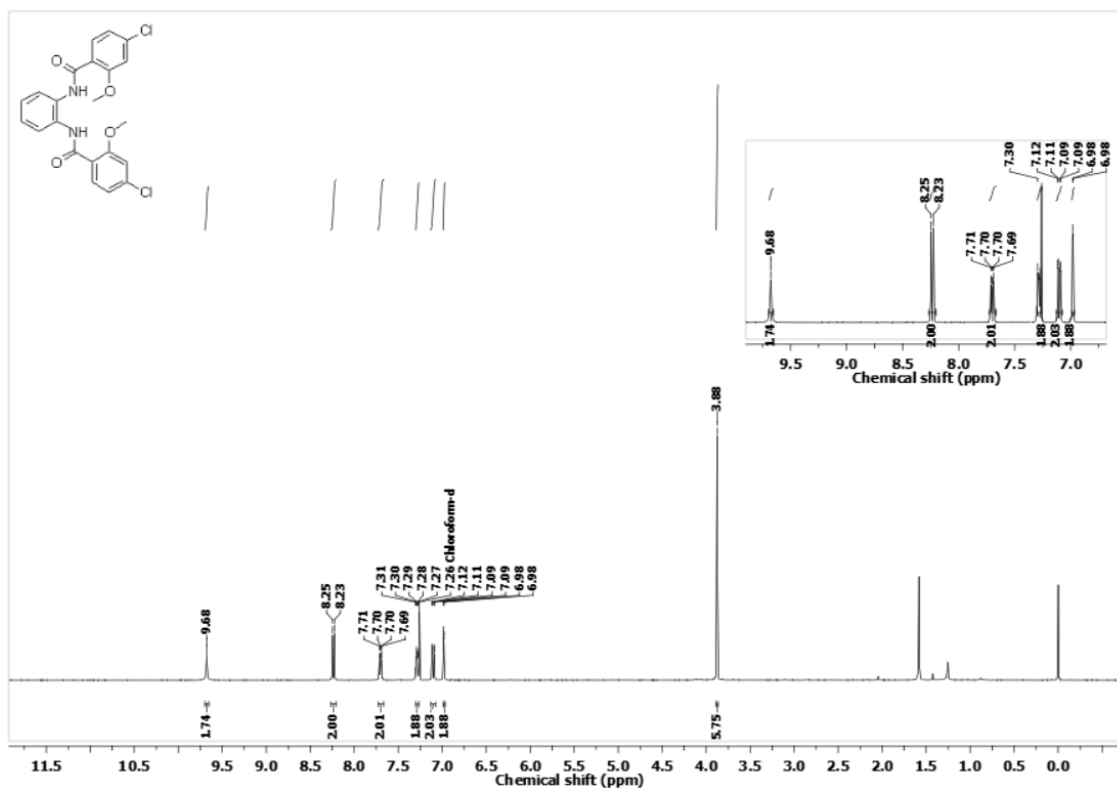


Figure 57: 400 MHz ^1H NMR spectrum of 7d in CDCl_3 .

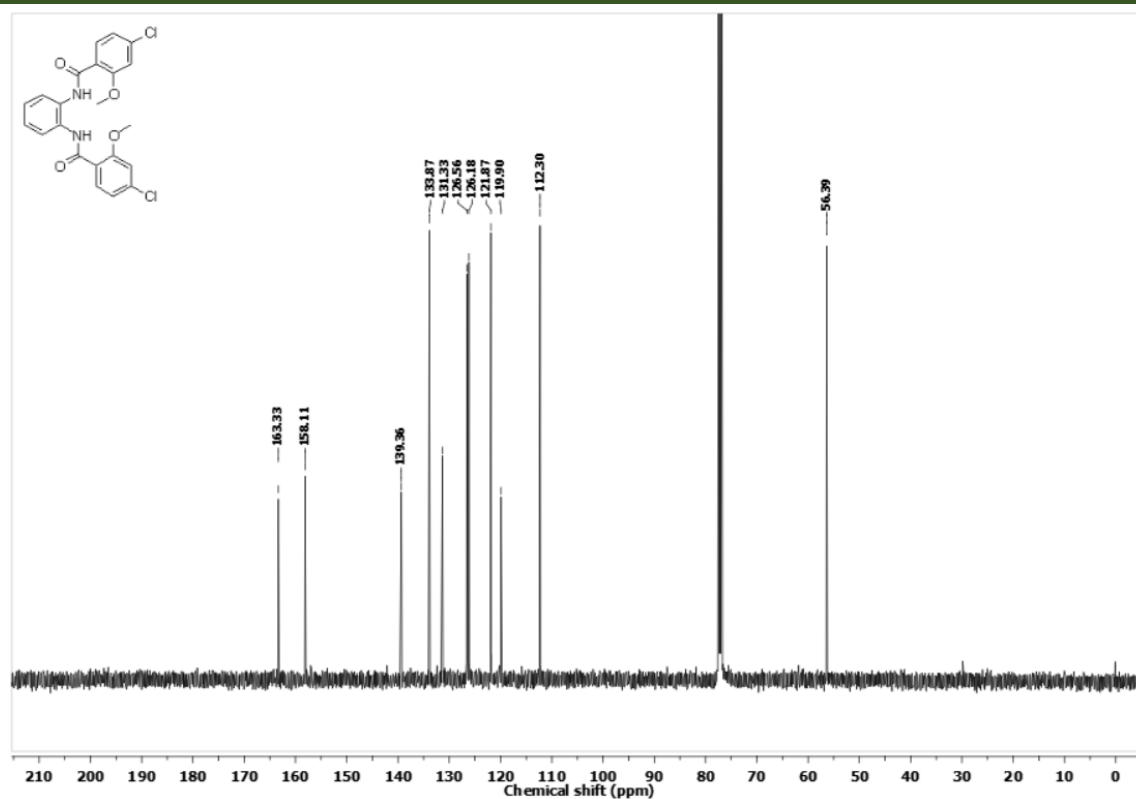


Figure 58: 101 MHz ^{13}C NMR spectrum of 7d in CDCl_3 .

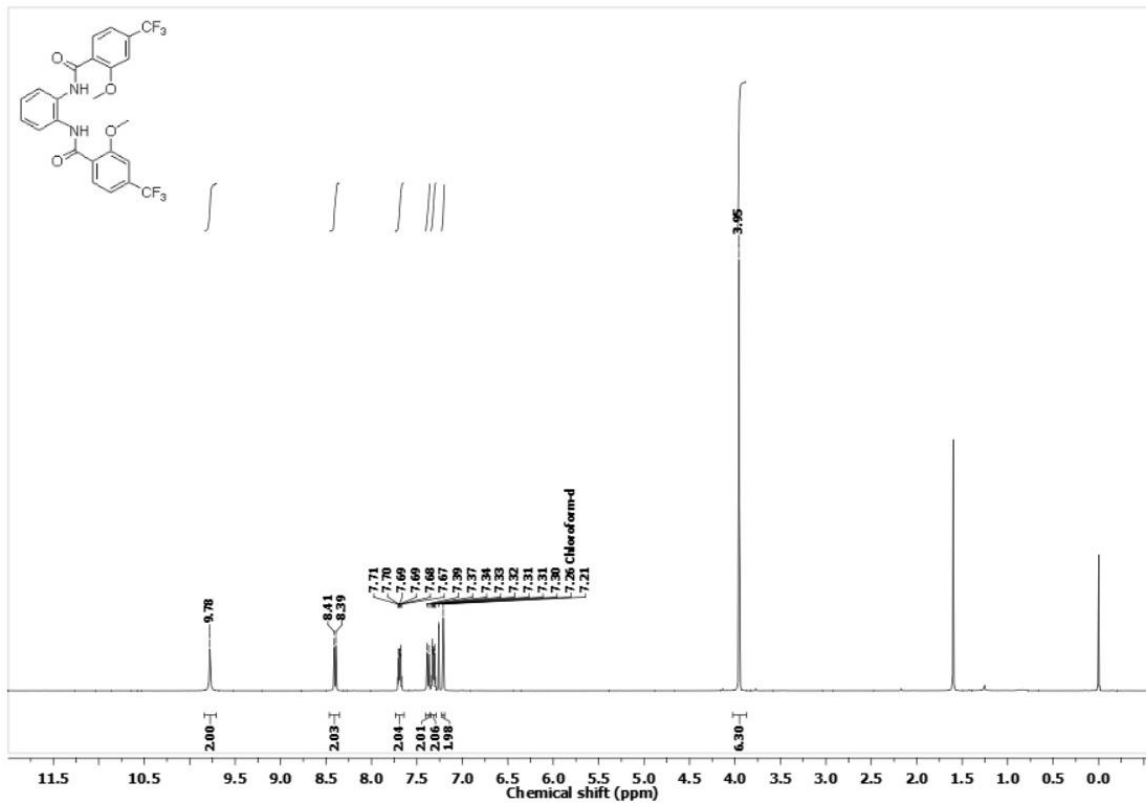


Figure 59: 400 MHz ^1H NMR spectrum of 7e in CDCl_3 .

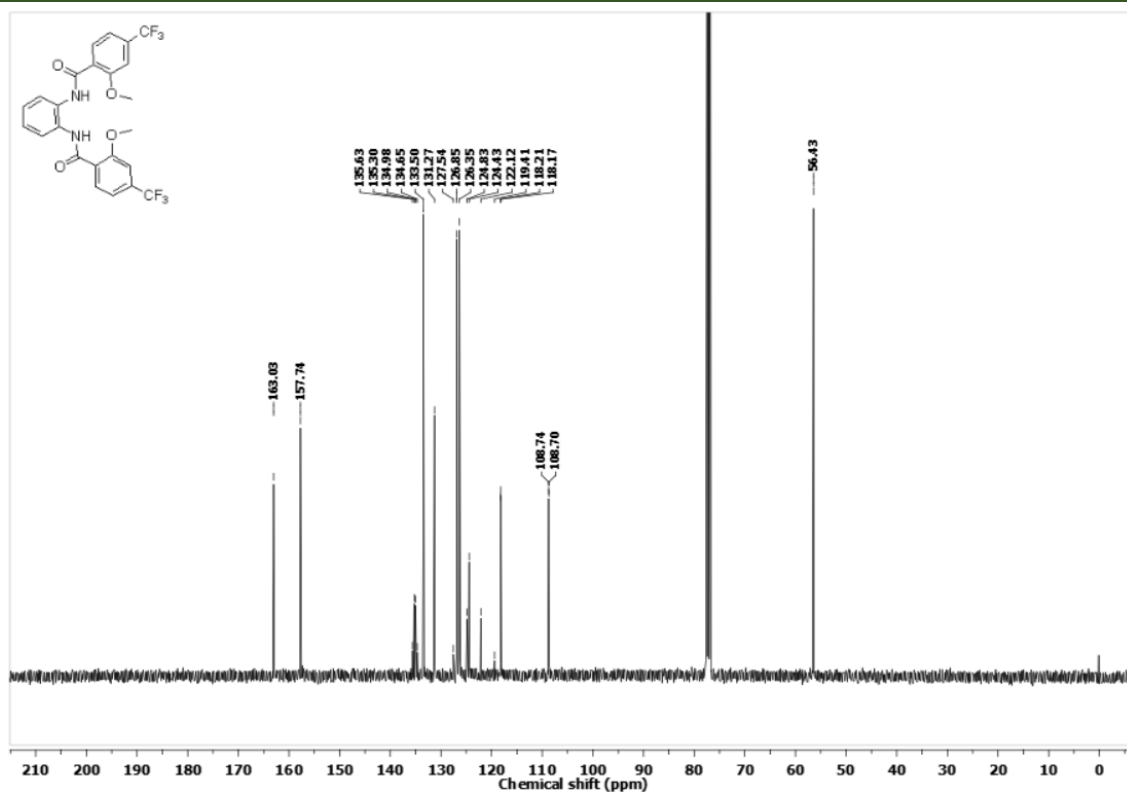


Figure 60: 101 MHz ^{13}C NMR spectrum of **7e** in CDCl_3 .

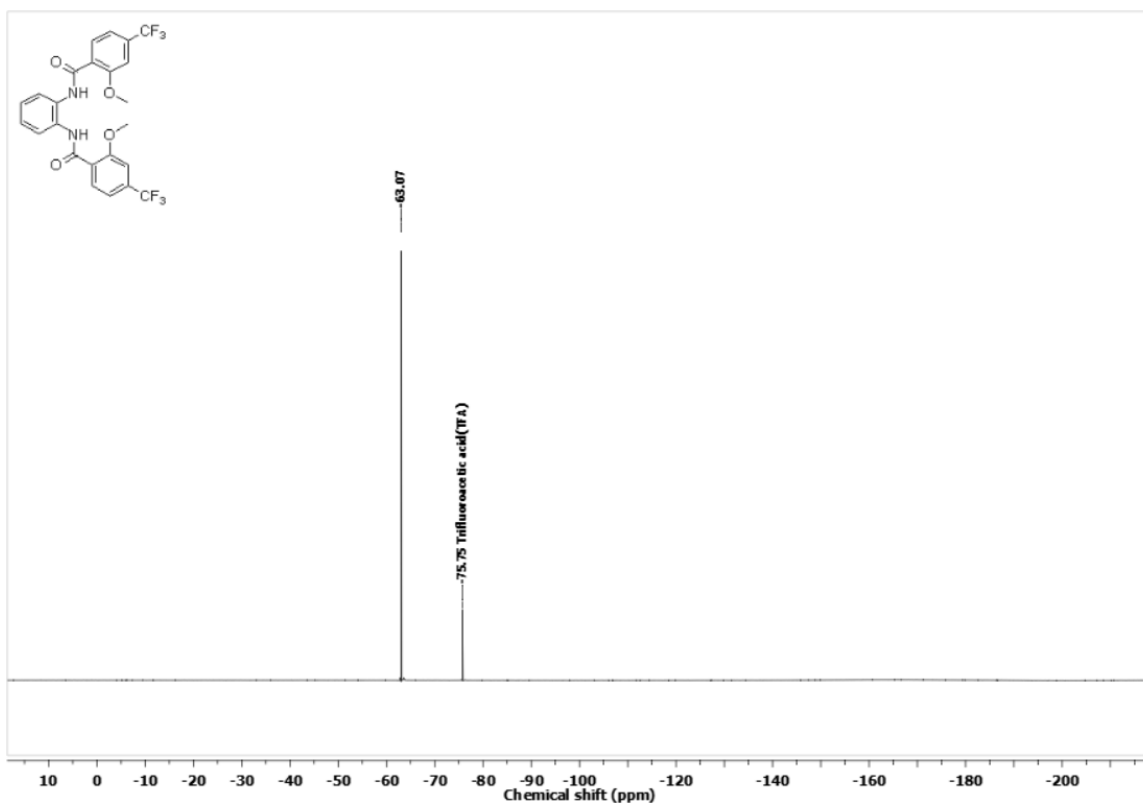


Figure 61: 377 MHz ^{19}F NMR spectrum of **1e** in CDCl_3 .

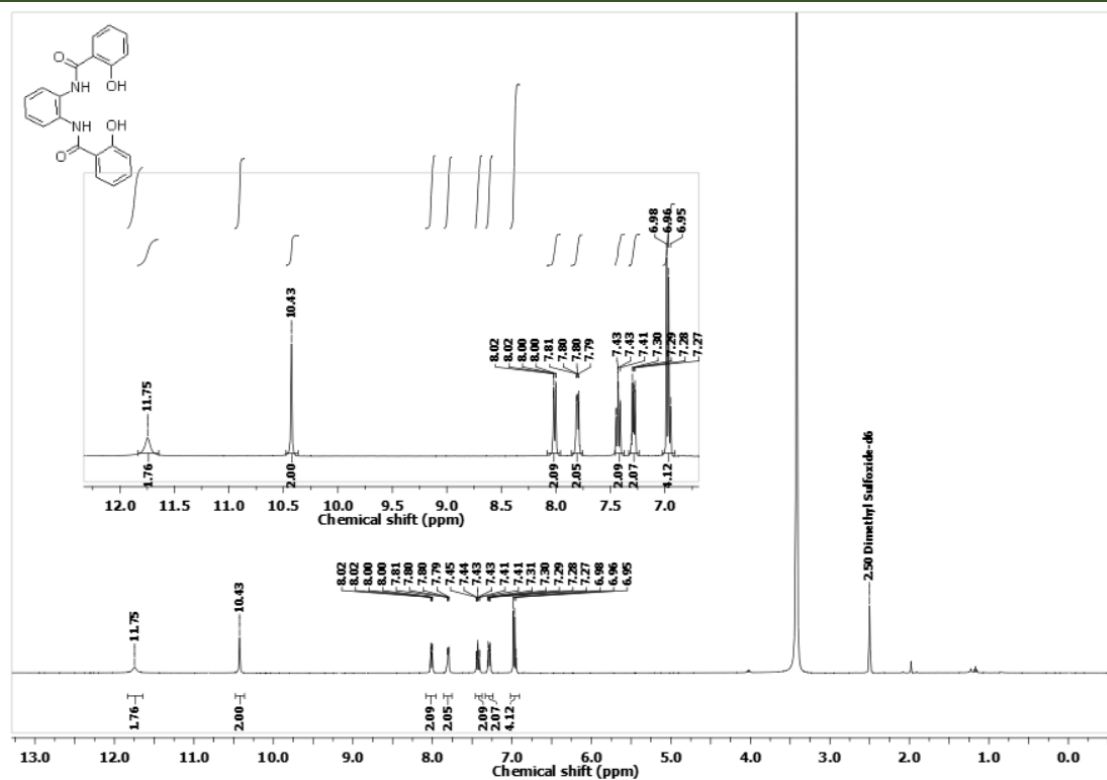


Figure 62: 400 MHz ^1H NMR spectrum of **1a** in $\text{DMSO}-d_6$.

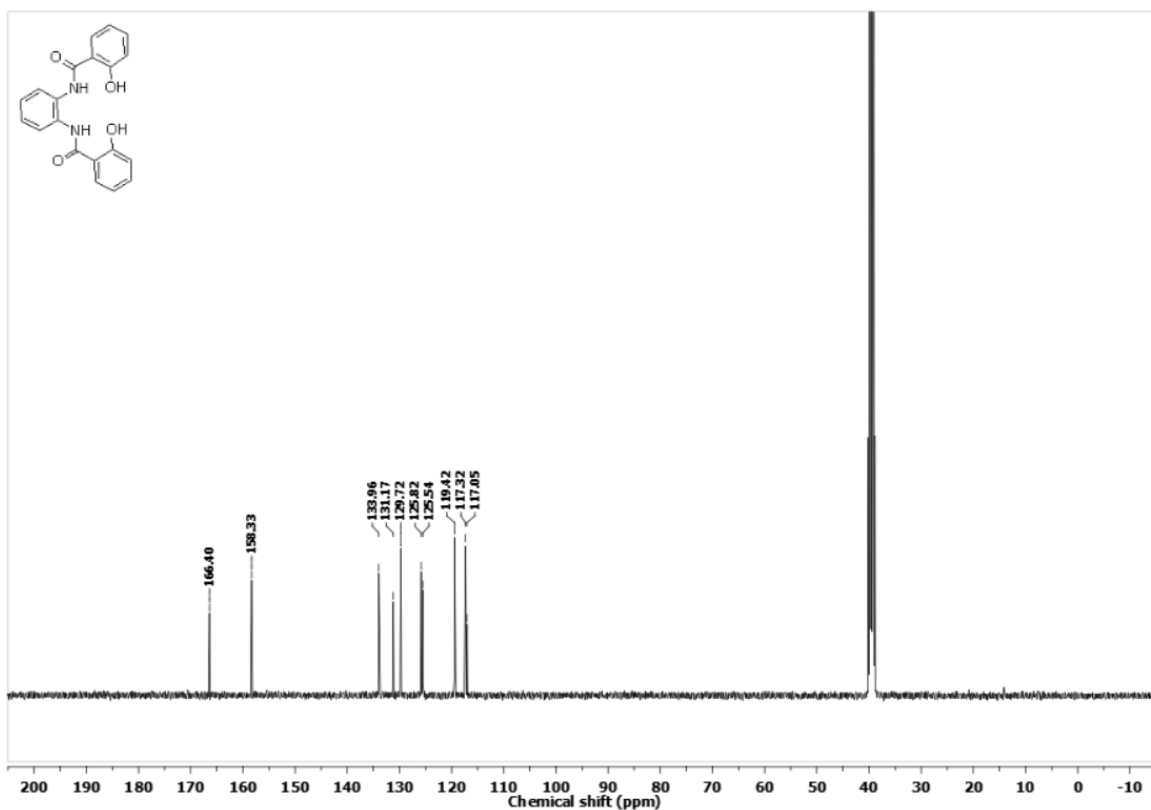


Figure 63: 101 MHz ^{13}C NMR of **1a** in $\text{DMSO}-d_6$.

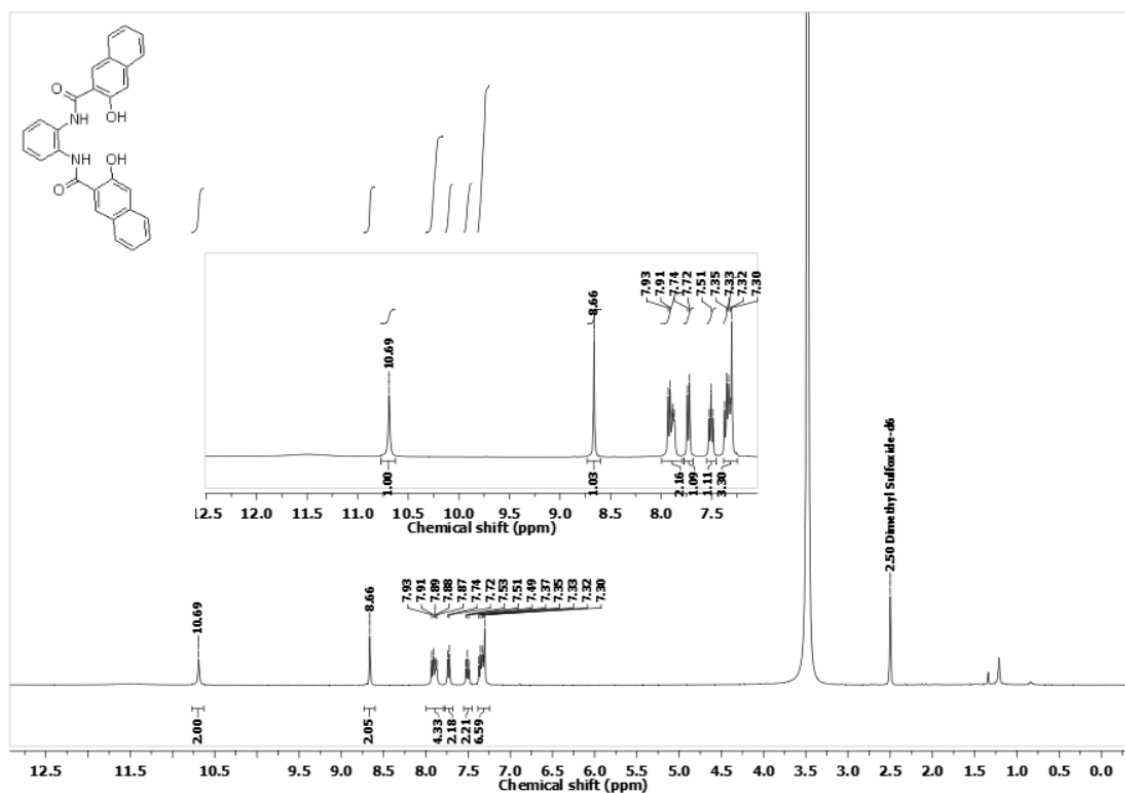


Figure 64: 400 MHz ¹H NMR spectrum of **1b** in DMSO-*d*₆.

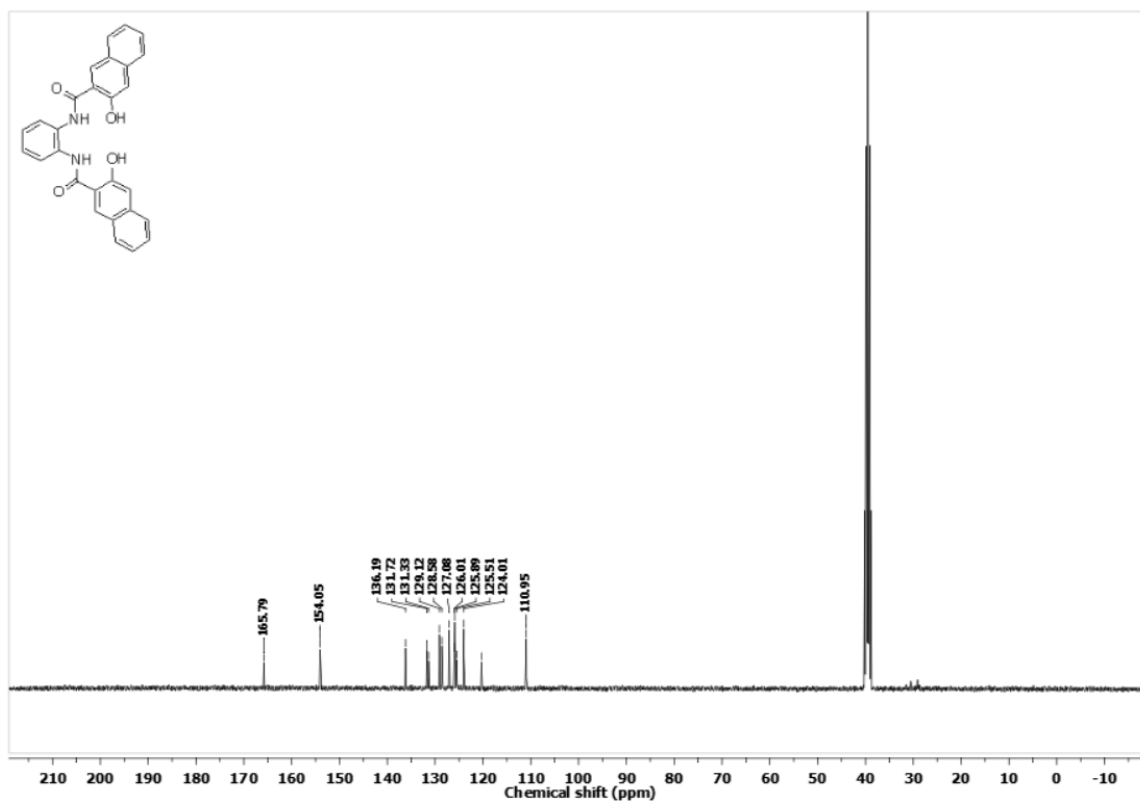


Figure 65: 101 MHz ¹³C NMR of **1b** in DMSO-*d*₆.

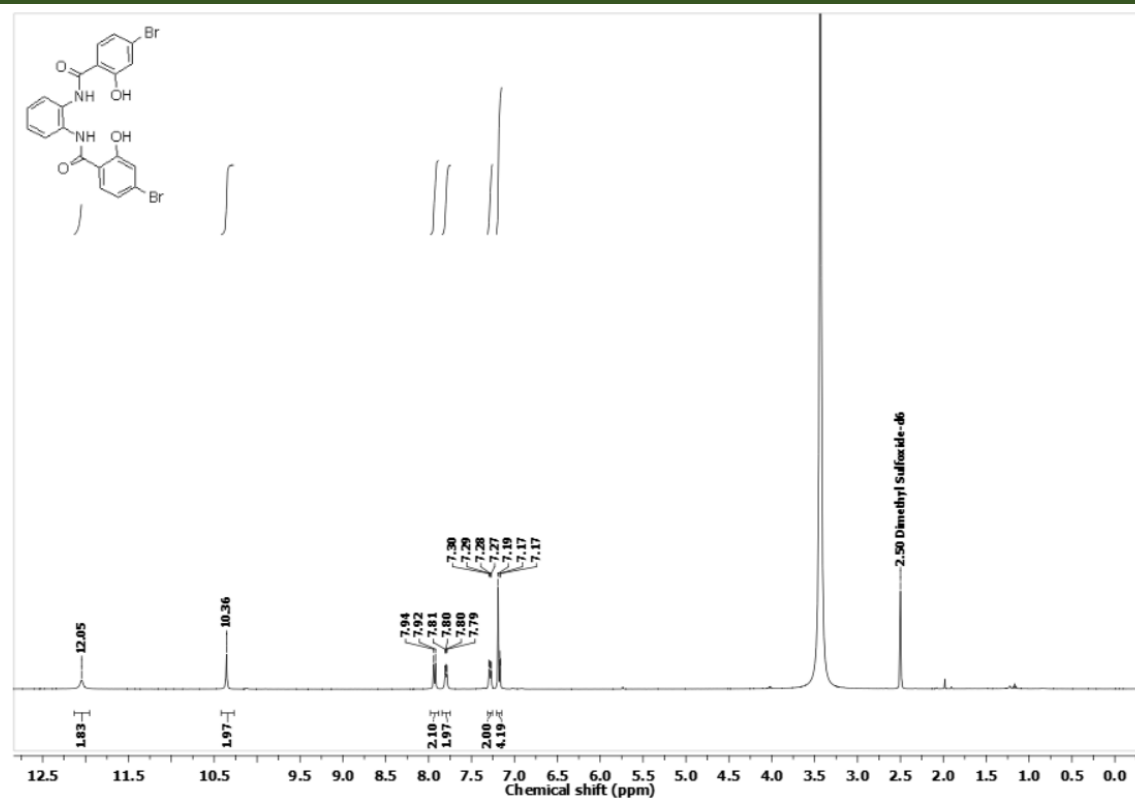


Figure 66: 400 MHz ^1H NMR spectrum of **1c** in $\text{DMSO}-d_6$.

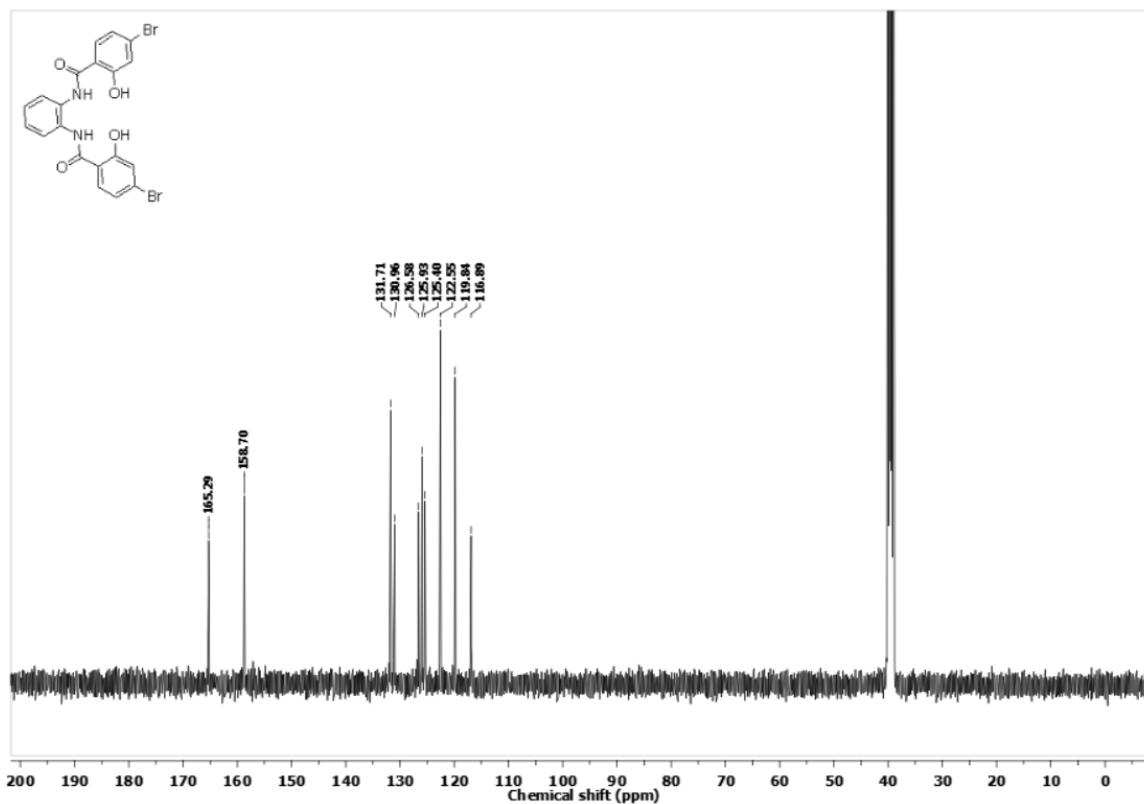


Figure 67: 101 MHz ^{13}C NMR of **1c** in $\text{DMSO}-d_6$.

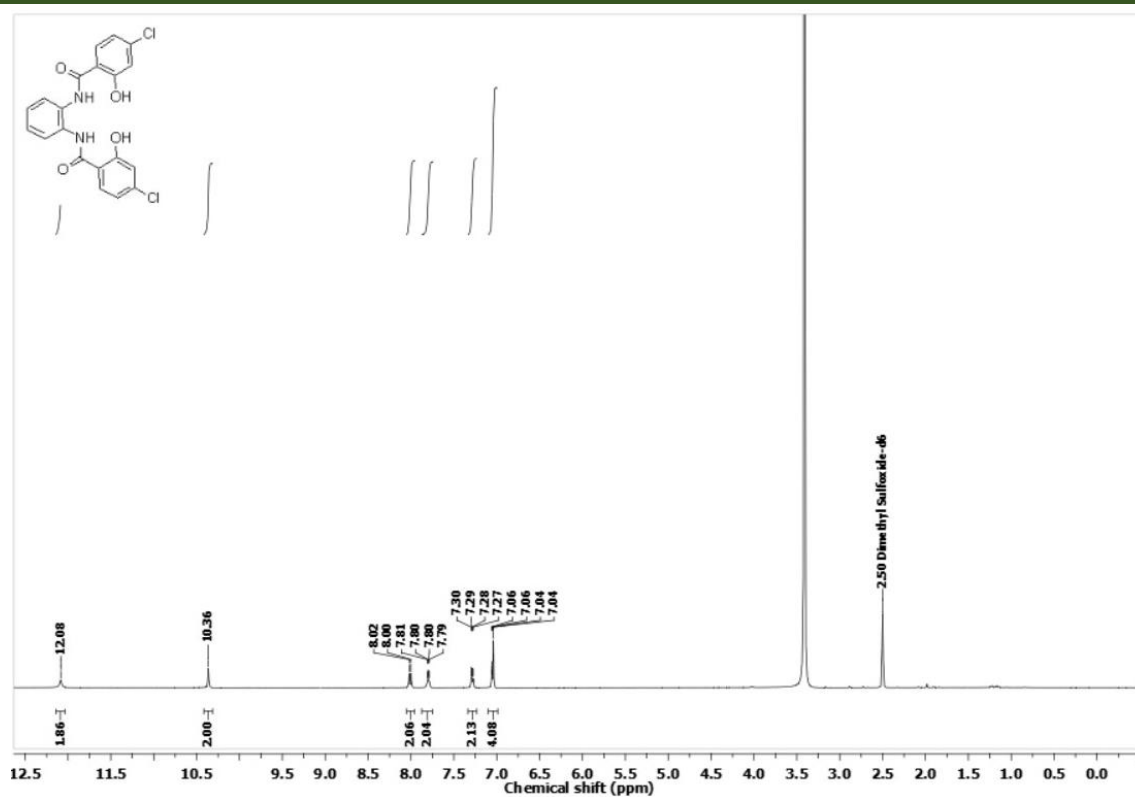


Figure 68: 400 MHz ^1H NMR spectrum of **1d** in $\text{DMSO}-d_6$.

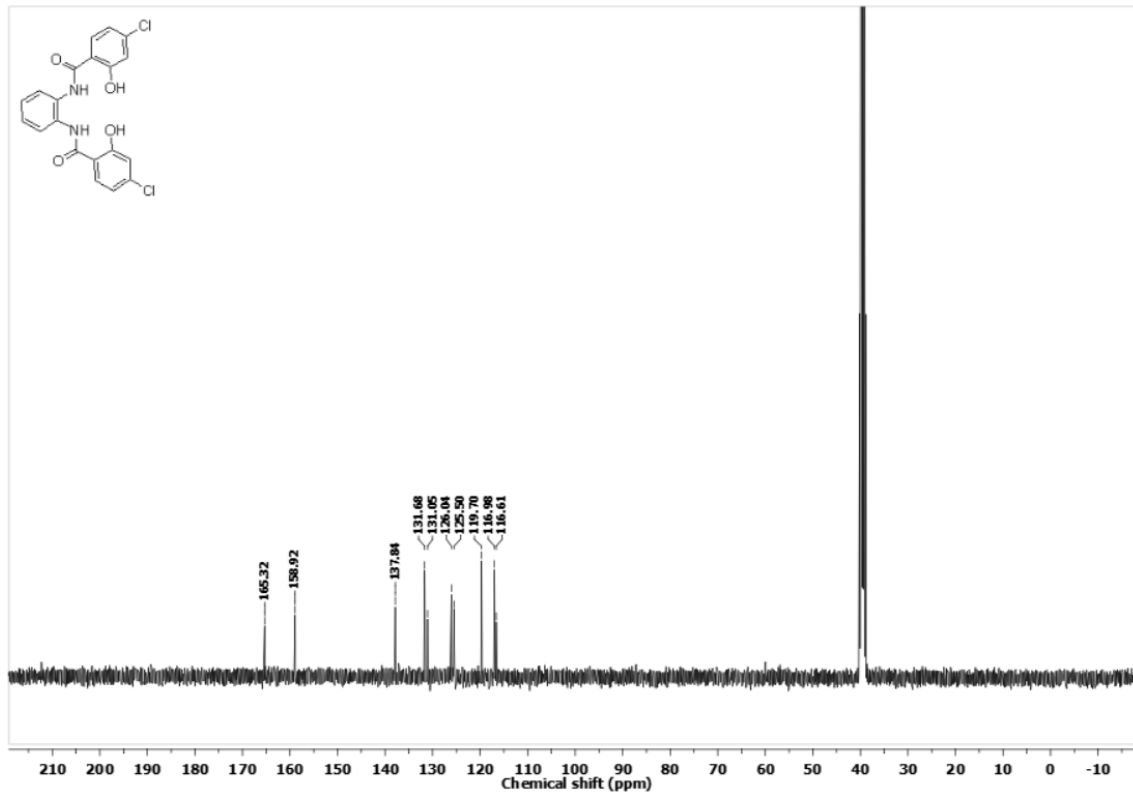


Figure 69: 101 MHz ^{13}C NMR of **1d** in $\text{DMSO}-d_6$.

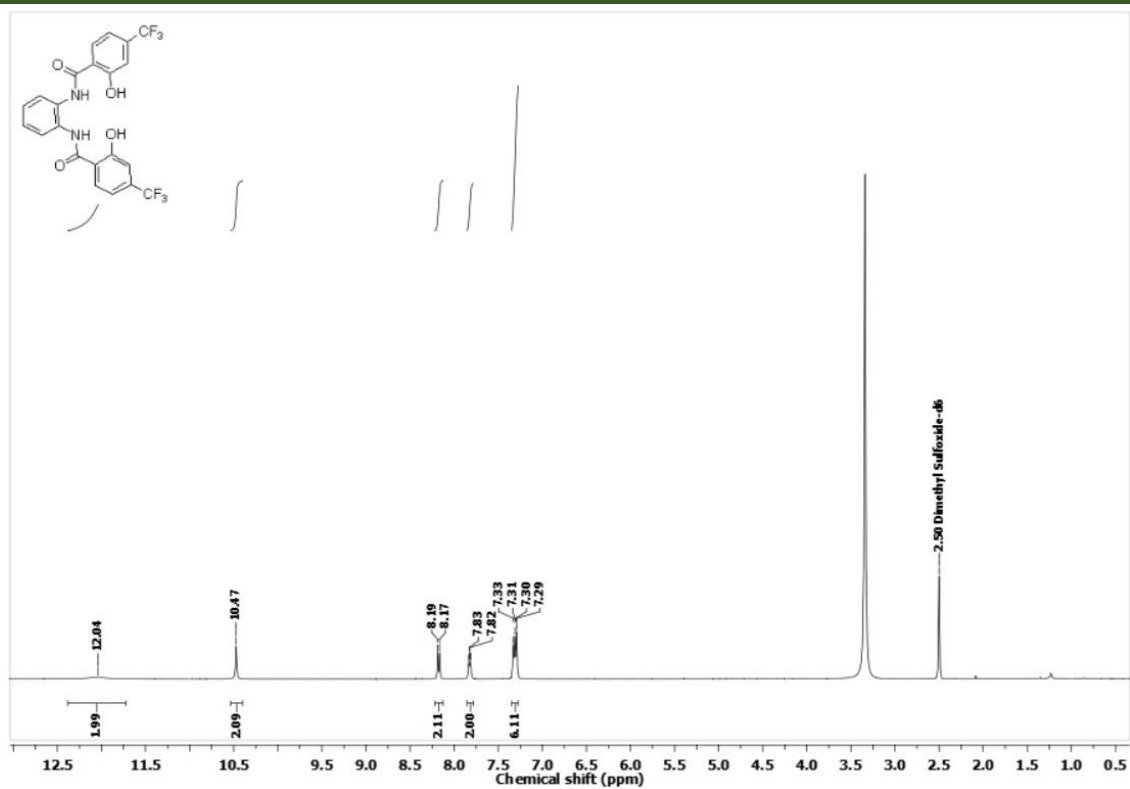


Figure 70: 400 MHz ^1H NMR spectrum of **1e** in $\text{DMSO}-d_6$.

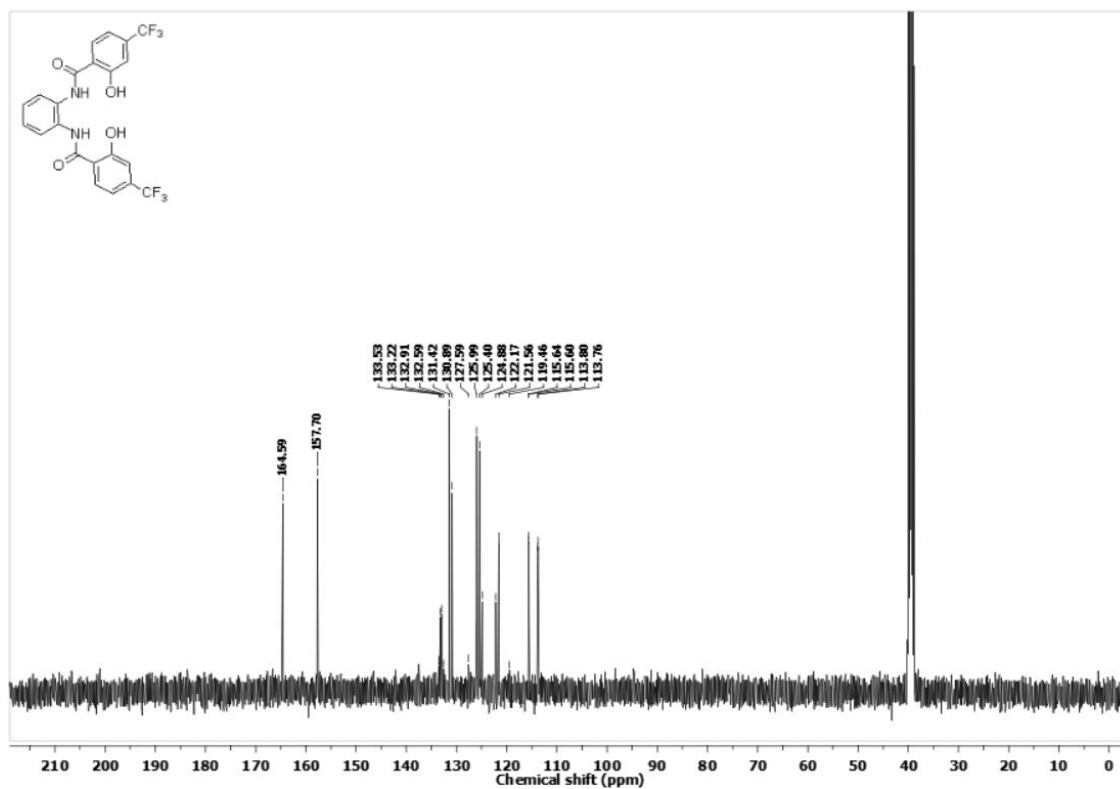


Figure 71: 101 MHz ^{13}C NMR of **1e** in $\text{DMSO}-d_6$.

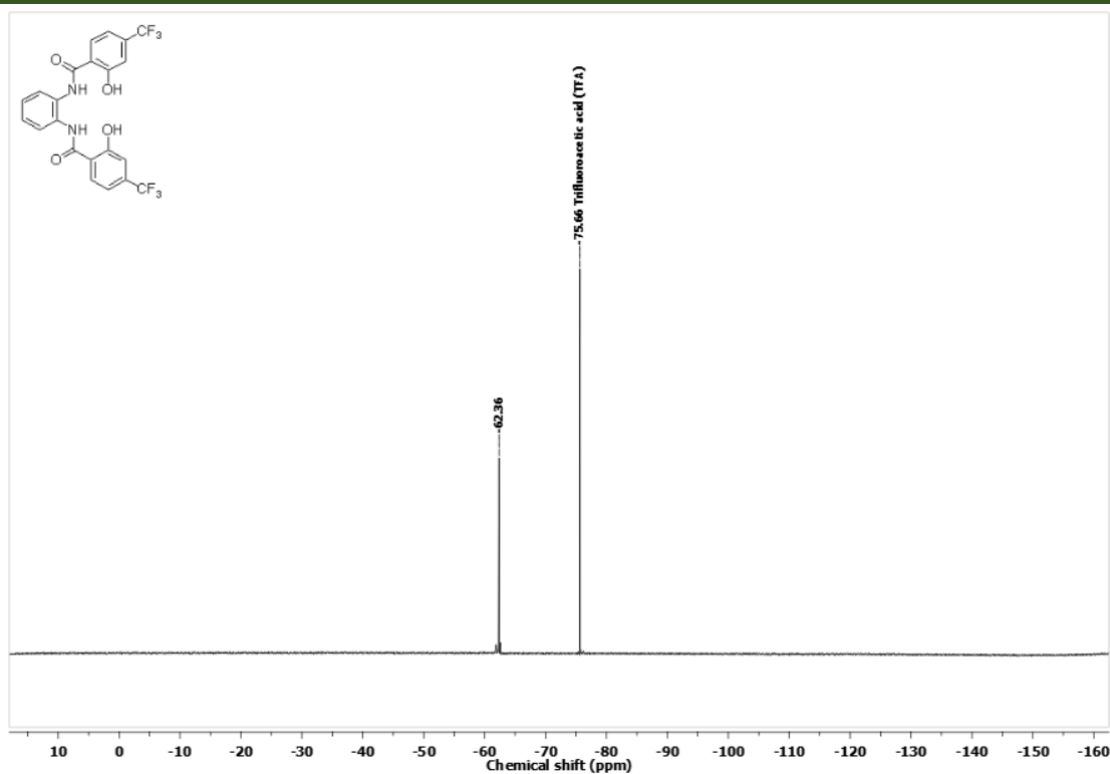


Figure 72: 377 MHz ^{19}F NMR of **1e** in $\text{DMSO}-d_6$.

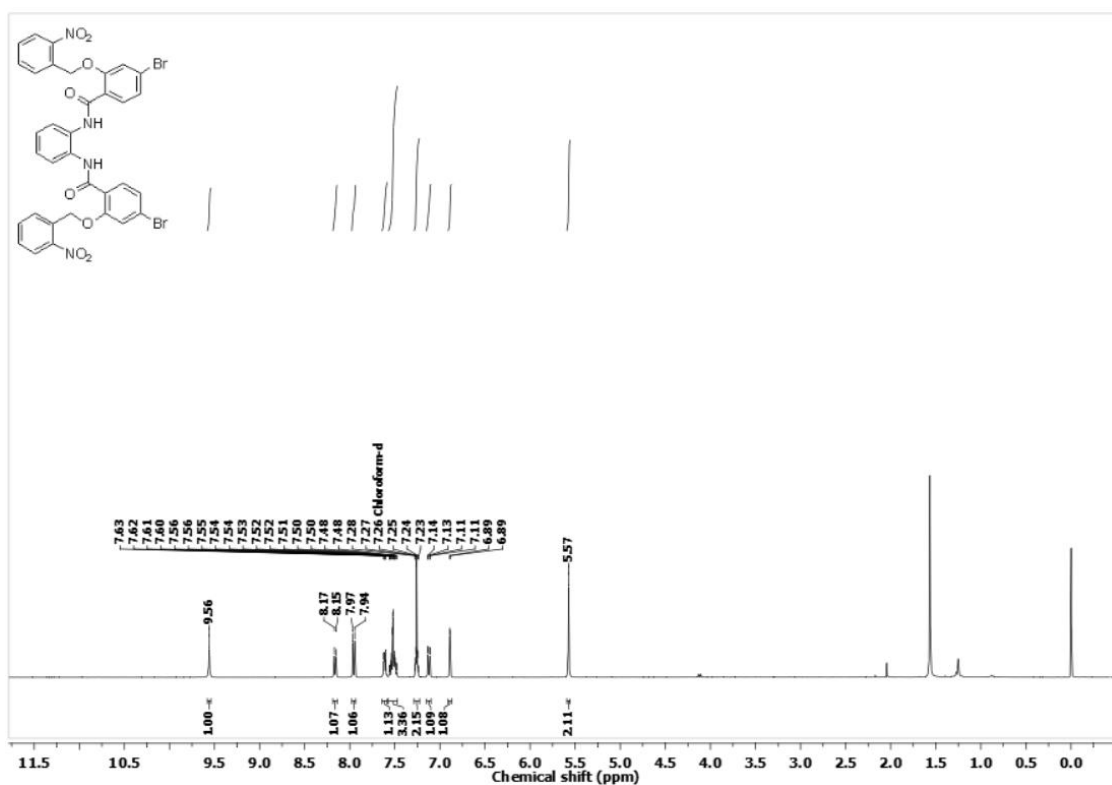


Figure 73: 400 MHz ^1H NMR spectrum of **2** in CDCl_3 .

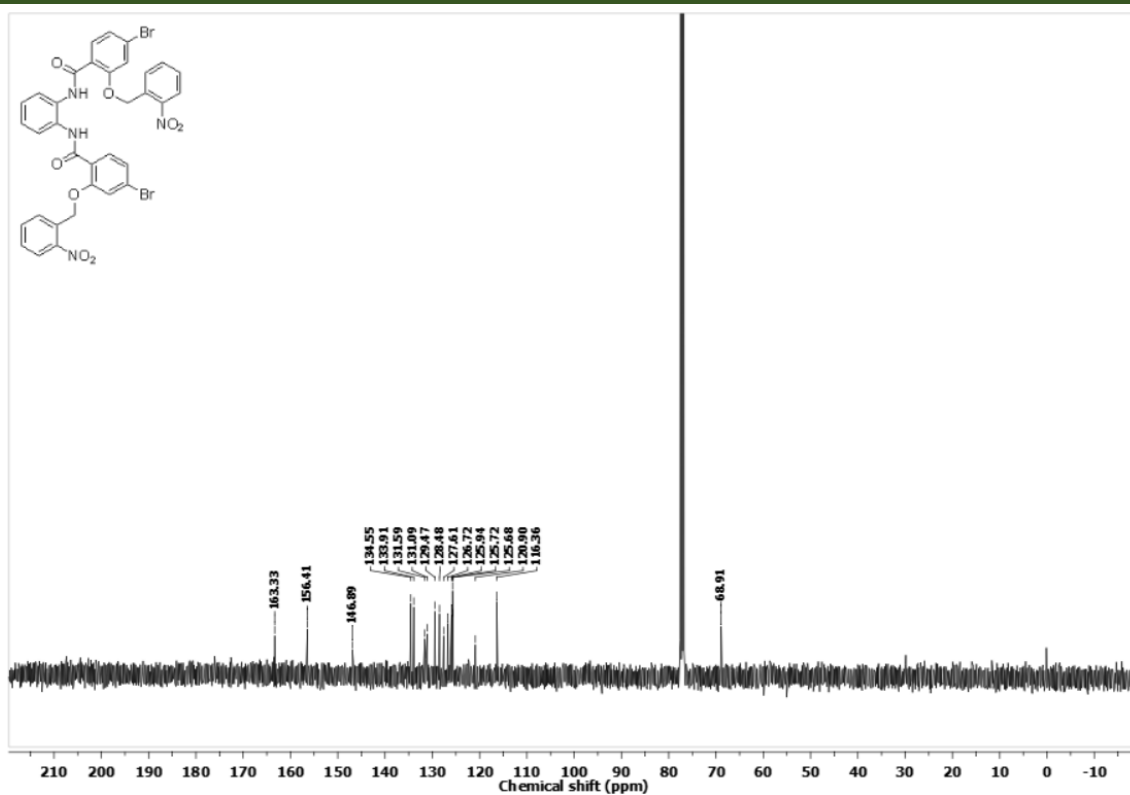


Figure 74: 101 MHz ¹³C NMR spectrum of 2 in CDCl₃.

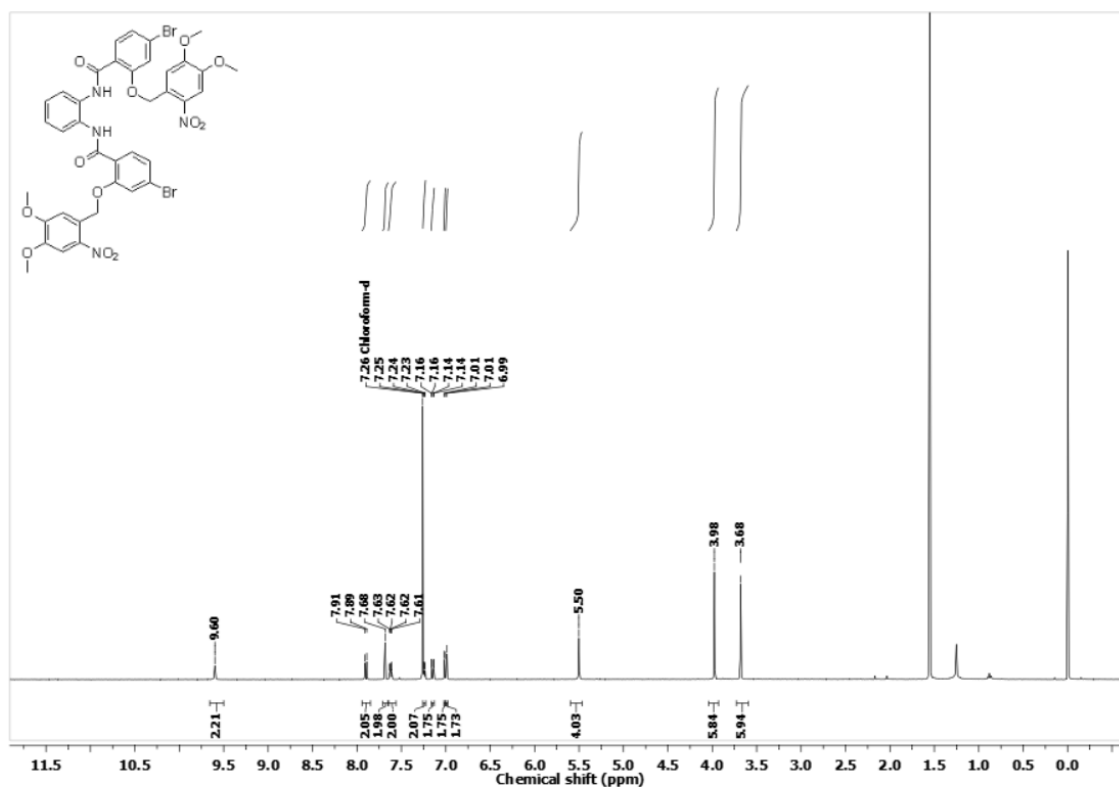


Figure 75: 400 MHz ¹H NMR spectrum of 3 in CDCl₃.

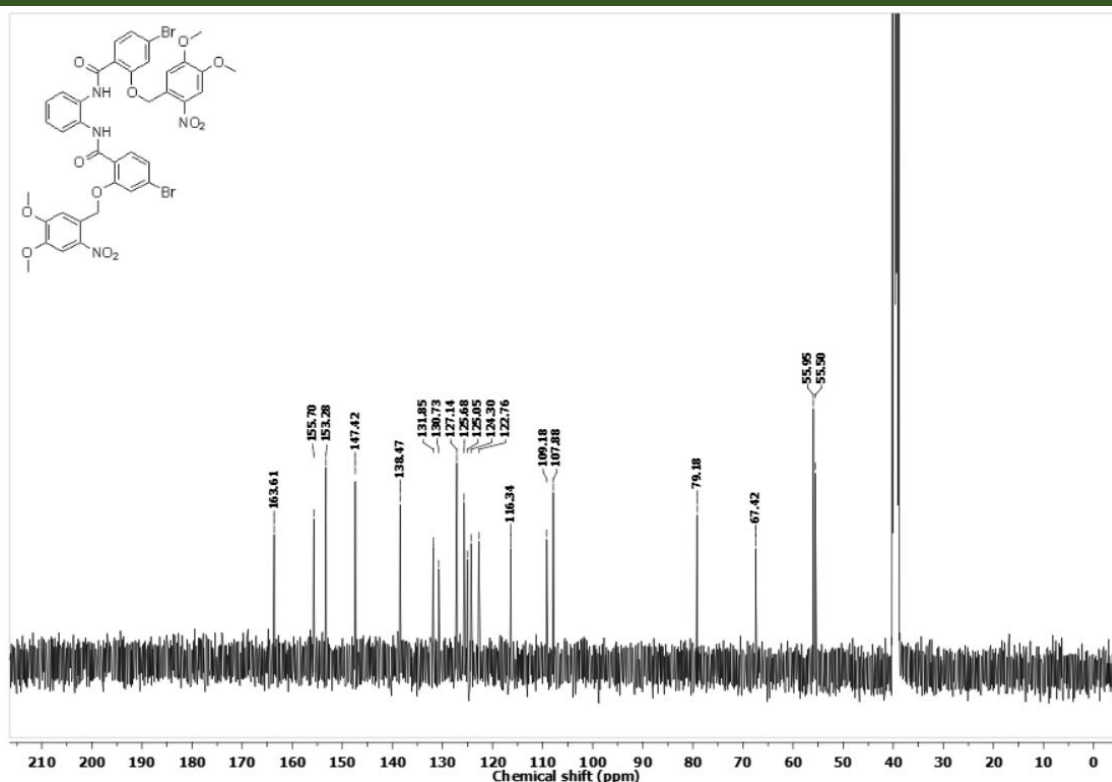


Figure 76: 101 MHz ^{13}C NMR spectrum of **3** in $\text{DMSO}-d_6$.

4.6 References

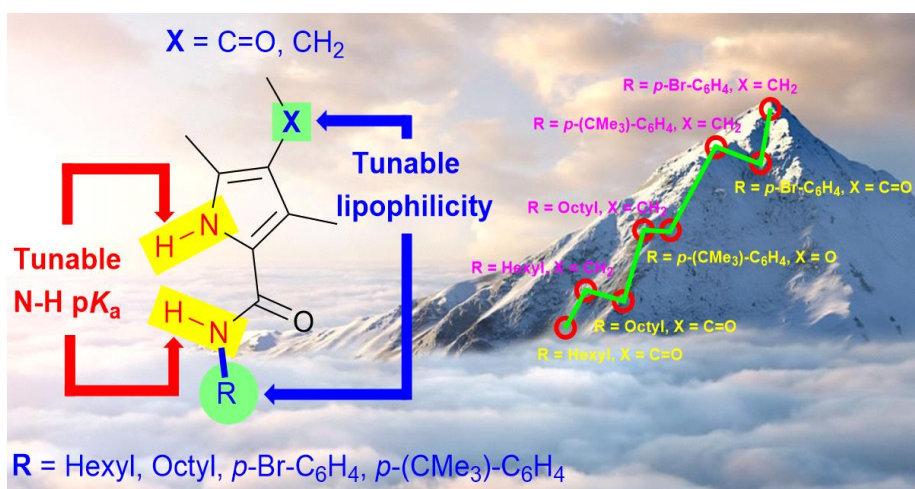
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Chapter 5

Pyrrole-2-carboxamide System as a Step Toward Tunable, Targeted Ion Transport



5.1 Introduction

The salicylamide and bisalicylamide systems discussed in the preceding chapters, though having excellent ion transport properties, have limited flexibility with respect to structural tunability. A more versatile system allows for a much broader range of ion transport properties achievable through small structural changes that are synthetically easily accessible. Hence, attention was turned to the pyrrole moiety, which is well known as a key component in various biologically active scaffolds and natural products, including bile pigments (bilirubin), porphyrins (haem, chlorophyll), anticancer drugs, and antibiotics.¹⁻⁴ The chemical and biological importance of pyrrole has led to decades of research into the synthesis of pyrrole heterocycles, resulting in various methodologies for the synthesis of pyrrole derivatives with desired substitutions,^{1,3} starting from classical methods such as Knorr,⁵ Paal-Knorr⁶ and Hantzsch⁷ pyrrole synthesis. The ability of the pyrrole motif to reversibly hydrogen bond with anions and the high N-H pK_a that prevents its anion-induced deprotonation⁸ has led to its extensive use in anion receptors and sensors.⁹⁻¹⁴ Pyrrole-incorporated ion transport systems include prodigiosenes (including obatoclax),¹⁵⁻¹⁹ tambjamines,²⁰⁻²² perenosins,^{23, 24} and (strapped) calixpyrroles.²⁵⁻²⁹ These structurally diverse systems are capable of various transmembrane ion transport processes, including anion antiport, H⁺/Cl⁻ symport, and M⁺/Cl⁻ symport. Some of these systems have demonstrated potent anticancer effects, being capable of disrupting cellular ion homeostasis and inducing apoptosis.^{16-18, 23, 24, 28}

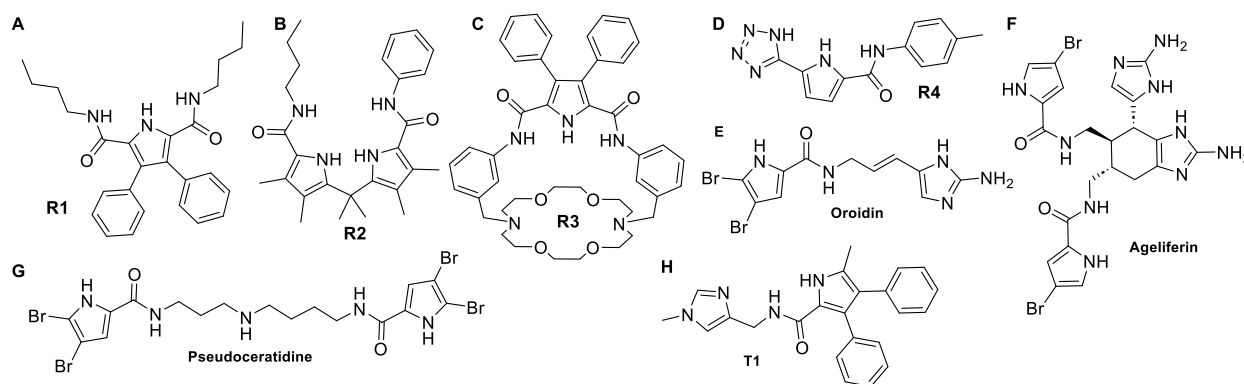


Figure 1: Pyrrole-2-carboxamide systems as (A)-(D) ion receptors, (E)-(G) bioactive natural products, and (H) ion transporter.

When considering these systems, the pyrrole-2-carboxamide motif is of special interest, as the addition of the hydrogen bond capable amide functionality in proximity to the pyrrole N-H offers scope for modulation of anion binding affinity, along with the possibility of intramolecular H-

bonding interactions that can result in favorable molecular conformations. Additionally, modifications to the substitution on the pyrrole ring itself are known to greatly vary the ion binding properties of pyrrole-2-carboxamide systems,^{11, 30, 31} making them ideal candidates for highly tuneable anion transport systems. Extension of this system by the addition of H-bond donor groups to the pyrrole-2-carboxamide core, appending to other moieties such as crown ethers or calixarenes, and incorporation into macrocyclic systems has yielded receptors capable of binding to ion pairs and more complex anions (Figure 1A-D),^{11, 13, 31-34} offering a myriad of possibilities for transmembrane transporters with interesting properties. This was first demonstrated through an aminomethylimidazole appended pyrrole-2-carboxamide system that was capable of H^+/Cl^- co-transport (Figure 1H), which, as of the time of publication of this report remained the only such application of the moiety.³⁵ Pyrrole-2-carboxamides are also found in many natural products, including various bioactive marine alkaloids extracted from sponges (Figure 1E-G).^{4, 36-38} Given the diversity of properties offered by these systems, we envisioned a transporter system with pyrrole-2-carboxamide as the core/foundation, keeping the simplicity of structure and ease of synthesis as the guiding principles of its design.

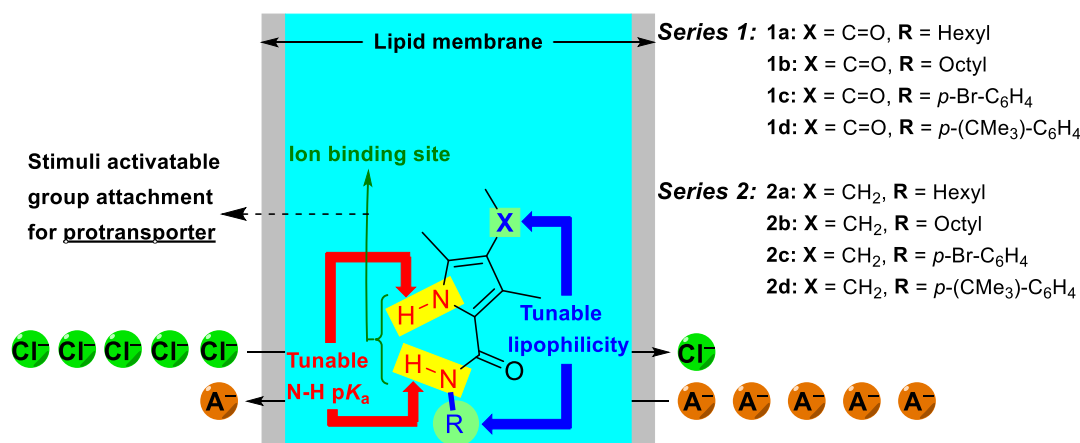


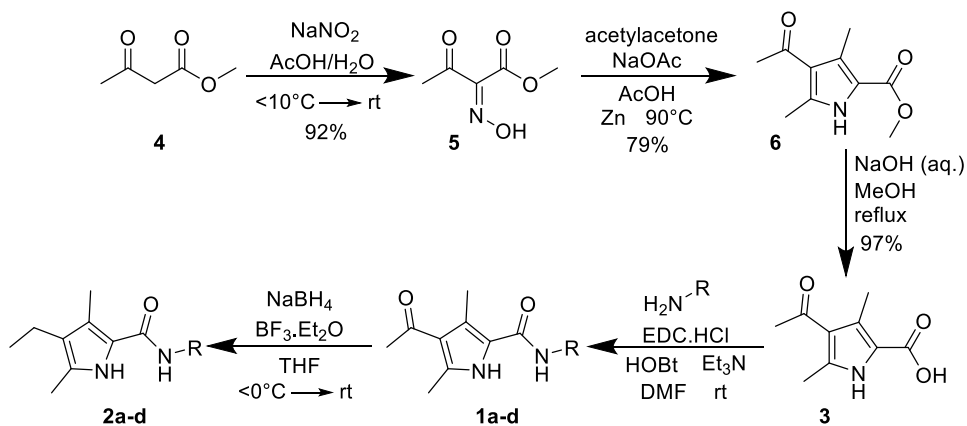
Figure 2: Pyrrole-2-carboxamide transporter design and the range of substitutions implemented for the tuning of ion transport property.

In this chapter, an attempt was made to study the ion transport properties of substituted pyrrole-2-carboxamides, along with the effect of variation of substituents toward their activity. Bimodal structural changes on the pyrrole ring and the amide moiety by variation of their substitution can be used to modulate the respective N-H pK_a values (controls ion binding affinity) and the $\log P$ (enhance lipophilicity) of the transporter molecule (Figure 2). Blocking of the N-H moieties with

labile groups will cage the ion transport activity, yielding stimuli-responsive protransporters. This study was conducted with a set of eight compounds with two variations on the pyrrole substitution and four variations of the amide substituent.

5.2 Results and Discussions

5.2.1 Synthesis



Scheme 1: Synthetic route for pyrroleamides **1a-d** and **2a-d**.

The pyrrole-2-carboxylic acid **3** was synthesized via the Zanetti and Levi modification³⁹ of the Knorr pyrrole synthesis (Scheme 1). Methyl acetoacetate was converted to 2-oximinoacetoacetate, which was then condensed with acetylacetone to form the substituted pyrrole **6**, whose ester group was hydrolyzed under basic conditions to yield **3**. The *Series 1* pyrroleamide (pyrrole-2-carboxamide) ion transporters (**1a-1d**) were synthesized by EDC·HCl/HOBt coupling of pyrrole-2-carboxylic acid **3** to the respective commercially available alkyl/arylamines (hexyl, octyl, 4-bromophenyl and 4-(*tert*-butyl)phenyl). The 4-acetyl groups on *Series 1* systems were reduced to ethyl groups using NaBH₄ with BF₃·Et₂O to obtain the *Series 2* transporter systems (**2a-2d**).

5.2.2 log*P* and p*K*_a Calculations

Calculations of log*P* and p*K*_a values of the transporters were performed using calculator plugins of MarvinSketch⁴⁰ (Table 1). Both series gave a good range of log*P* values (2.5-5.3), which are in the window ideal for transmembrane ion transporters. Reduction of the 4-acetyl to ethyl groups from *Series 1* to *Series 2* was accompanied by a significant increase in the log*P* value (by 1.4). The p*K*_a values of pyrrole and amide N-H groups were observed to vary with the amide substituent across each series. Conversion from *Series 1* to *Series 2* was accompanied by an increase in the pyrrole N-H p*K*_a, while a decrease in the value was observed for amide N-H (Table 1).

Table 1: Estimated log*P* and N-H p*K*_a values of compounds **1a-1d** and **2a-2d**. Values were estimated using calculator plugins of MarvinSketch.⁴⁰

Compound	log <i>P</i>	Pyrrole N-H p <i>K</i> _a	Amide N-H p <i>K</i> _a
1a	2.53	12.25	15.37
1b	3.42	12.25	15.37
1c	3.10	11.75	14.38
1d	3.88	11.77	14.77
2a	3.93	15.84	15.86
2b	4.82	15.84	15.86
2c	4.50	18.02	12.96
2d	5.28	18.02	13.38

5.2.3 Ion Transport Studies

The ion transport activities of the compounds **1a-1d** and **2a-2d** were studied across EYPC-LUVs∅HPTS, suspended in HEPES (10 mM), NaCl (100 mM) buffer⁴¹ with a pH gradient of 0.8 (pH_{in} = 7.0 and pH_{out} = 7.8) generated across the membrane. The compounds exhibited activity in the order **2c** > **2d** > **1c** > **1d** ≈ **2b** > **2a** > **1b** > **1a** (Figure 3). Dose-dependent studies of both series compounds were conducted, followed by Hill analysis to obtain half maximal effective concentrations (*EC*₅₀) and Hill coefficients (*n*) (Figures 4-11). The transporters in *Series 1* (**1a-1d**) gave *EC*₅₀ = 34.76, 3.23, 0.69, and 1.38 μM with *n* = 2.58, 2.94, 2.14, and 2.25, respectively, while *Series 2* (**2a-2d**) transporters gave *EC*₅₀ = 4.17, 1.30, 0.31, and 0.68 μM with *n* = 2.09, 3.93, 1.83, and 2.24 respectively (Table 2). The relative ion transport activities of compounds in relation to the amide substituents were found to be consistent across both series, with 4-bromophenyl > 4-(*tert*-butyl)phenyl > octyl > hexyl (**1c** > **1d** > **1b** > **1a** for *Series 1* and **2c** > **2d** > **2b** > **2a** for *Series 2*), with aromatic *N*-amides exhibiting higher ion transport activity their aliphatic counterparts. A considerable increase in the ion transport activity was observed from *Series 1* to 2 on the reduction of the pyrrole 4-acetyl group, indicating the subsequent increase in log*P* greatly favors ion transport activity. On analysis of the Hill coefficient (*n*) values, the *N*-aryl pyrroleamides were observed to consistently give a value of ~2 (2.14, 2.25, 1.83, and 2.24, respectively for **1c**, **1d**, **2c**, and **2d**), indicating the involvement of two molecules of the transporter when forming the transmembrane ion transport complex. In the case of *N*-alkyl pyrroleamides, the *N*-hexyl

derivatives provided a Hill coefficient value of ~ 2 (2.58 and 2.09, respectively for **1a** and **2a**), while the *N*-octyl derivatives surprisingly gave higher values of n , with $n = 2.94$ (~ 3) for the Series 1 transporter **1b** and $n = 3.93$ (~ 4) for Series 2 transporter **2b**. This shift corresponds to minor changes in the transporter substitution, namely the length of the *N*-alkyl chain, and the substitution on the pyrrole-4 position (acetyl vs ethyl). A few ion transporter systems have been reported in literature where changes in the substitution around the core moiety correspond to significant variations of Hill coefficient values.⁴²⁻⁴⁵ These n values could be interpreted as the involvement of three (for **1b**) and four (for **2b**) transporter molecules for forming the active transport complex, indicating wider ranges of property change on varying the alkyl groups. This observed variability, though very interesting, is also surprising at the same time and should perhaps be treated with caution, pending further investigation. Overall, **2c** was identified to be the most active compound ($EC_{50} = 0.31 \mu\text{M}$, $n = 1.83$) and was chosen for further mechanistic studies.

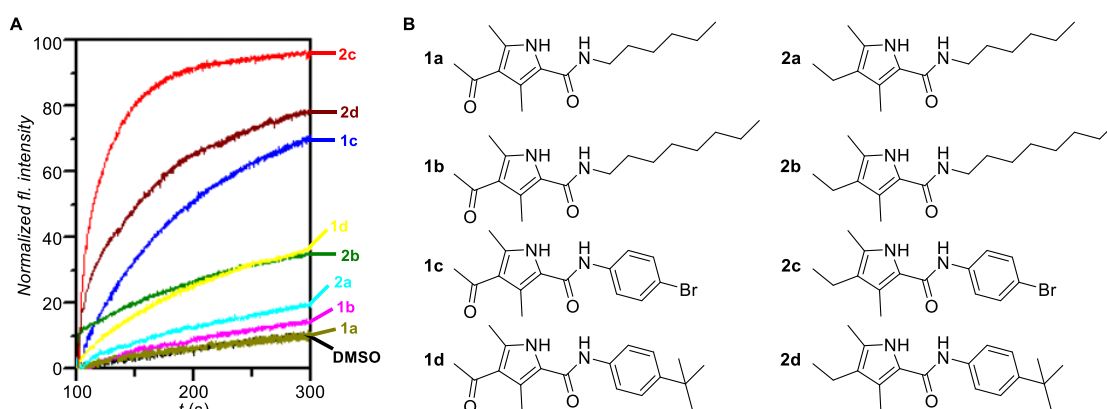


Figure 3: (A) Comparison of ion transport activities of pyrroleamides ($1 \mu\text{M}$) across EYPC-LUVs $\supset$ HPTS. (B) Structures of the tested pyrroleamide systems.

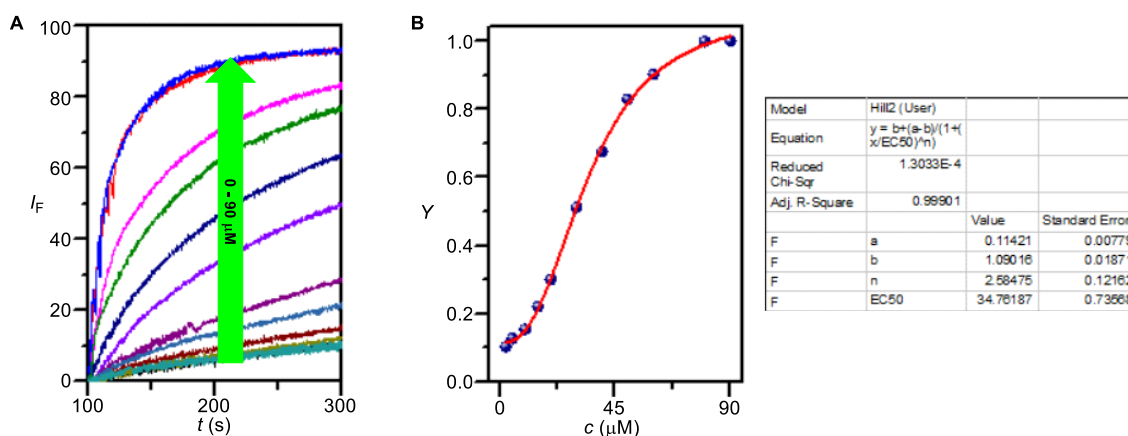


Figure 4: Pyrrole (A) Dose-dependent activity of compound **1a** across EYPC-LUVs $\supset$ HPTS. (B) Hill plot analysis of **1a** at $t = 290 \text{ s}$.

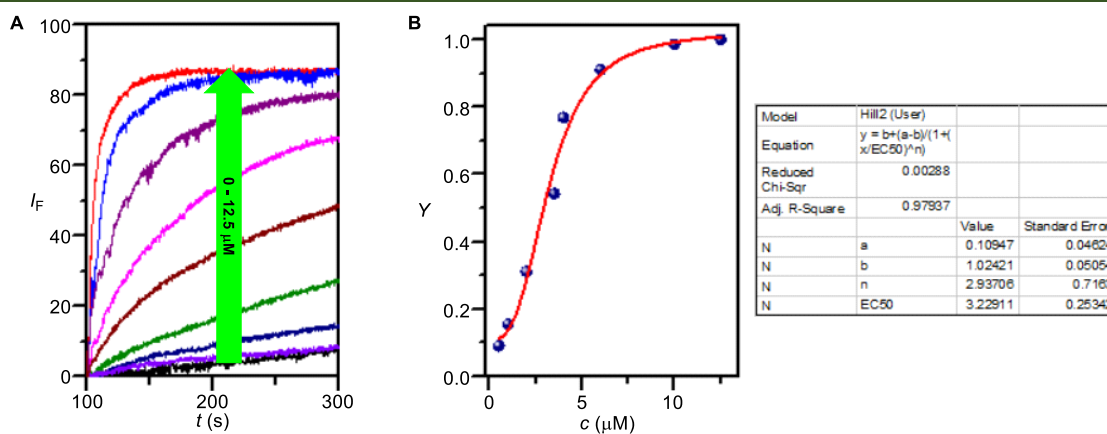


Figure 5: (A) Dose-dependent activity of compound **1b** across EYPC-LUVs to HPTS. (B) Hill plot analysis of **1b** at $t = 290$ s.

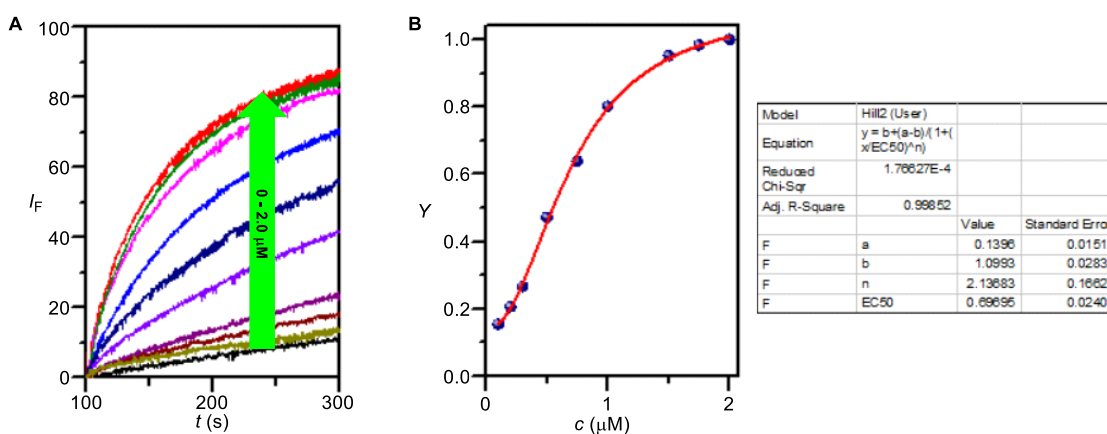


Figure 6: Dose-dependent activity of compound **1c** across EYPC-LUVs to HPTS. (B) Hill plot analysis of **1c** at $t = 290$ s.

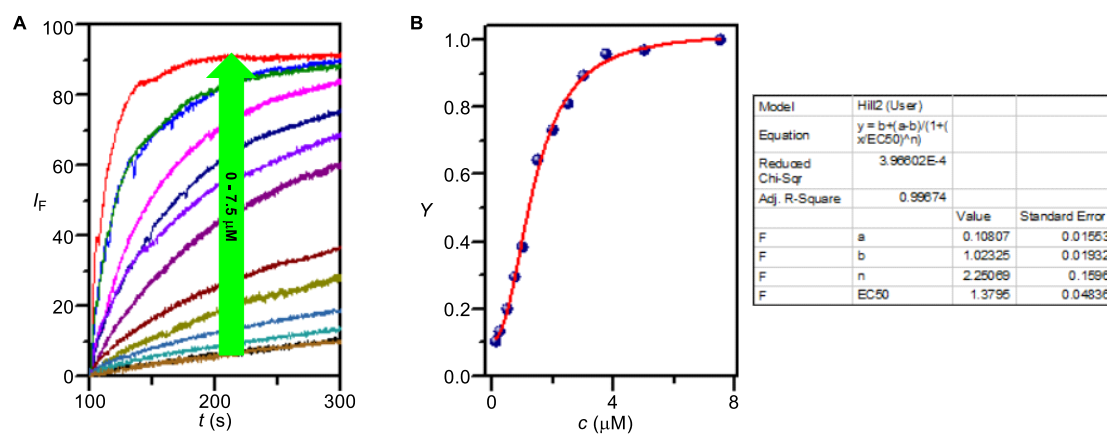


Figure 7: (A) Dose-dependent activity of compound **1d** across EYPC-LUVs to HPTS. (B) Hill plot analysis of **1d** at $t = 290$ s.

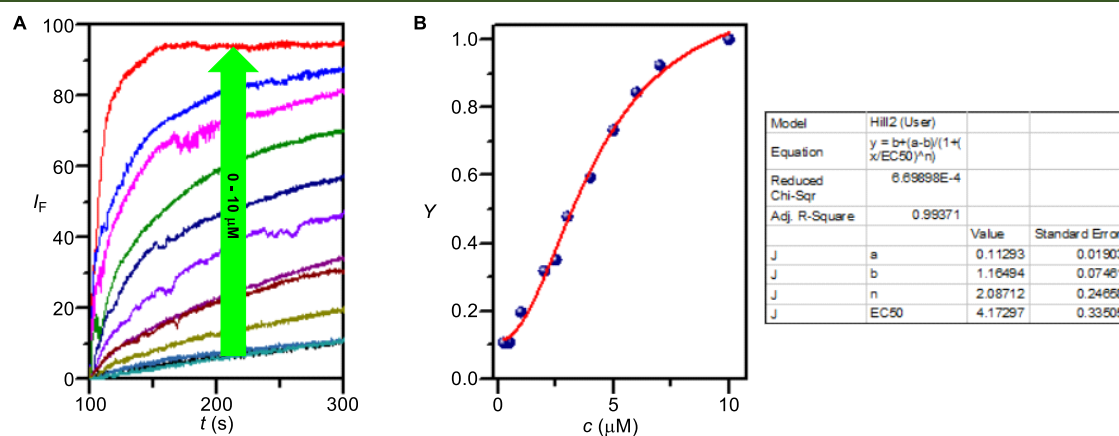


Figure 8: (A) Dose-dependent activity of compound **2a** across EYPC-LUVs Δ HPTS. (B) Hill plot analysis of **2a** at $t = 290$ s.

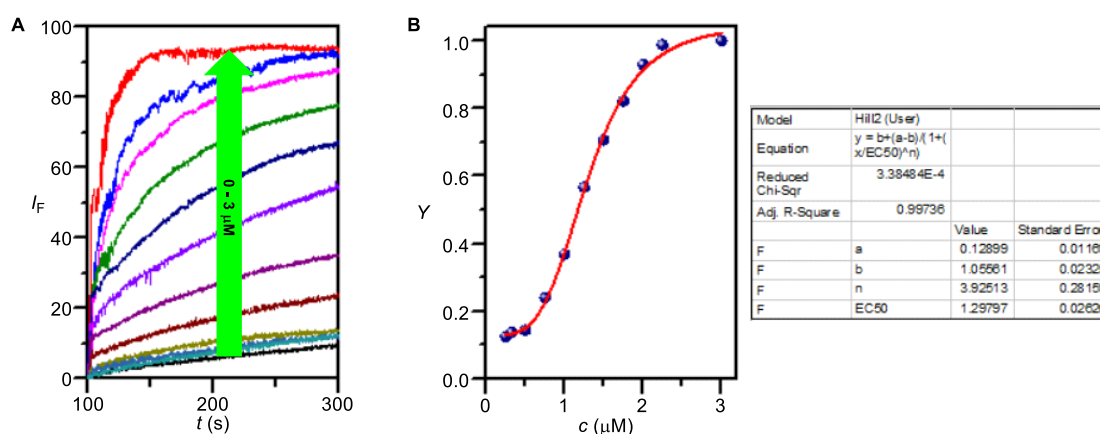


Figure 9: (A) Dose-dependent activity of compound **2b** across EYPC-LUVs Δ HPTS. (B) Hill plot analysis of **2b** at $t = 290$ s.

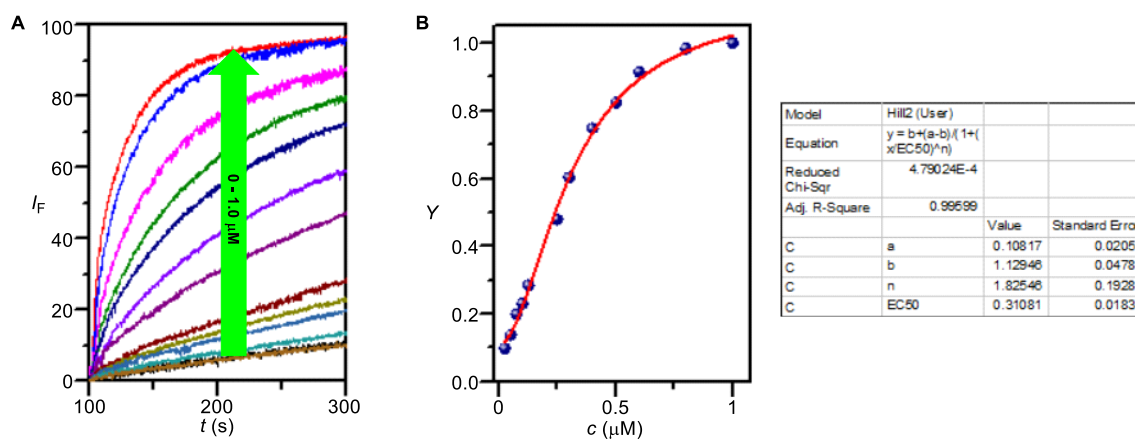


Figure 10: (A) Dose-dependent activity of compound **2c** across EYPC-LUVs Δ HPTS. (B) Hill plot analysis of **2c** at $t = 290$ s.

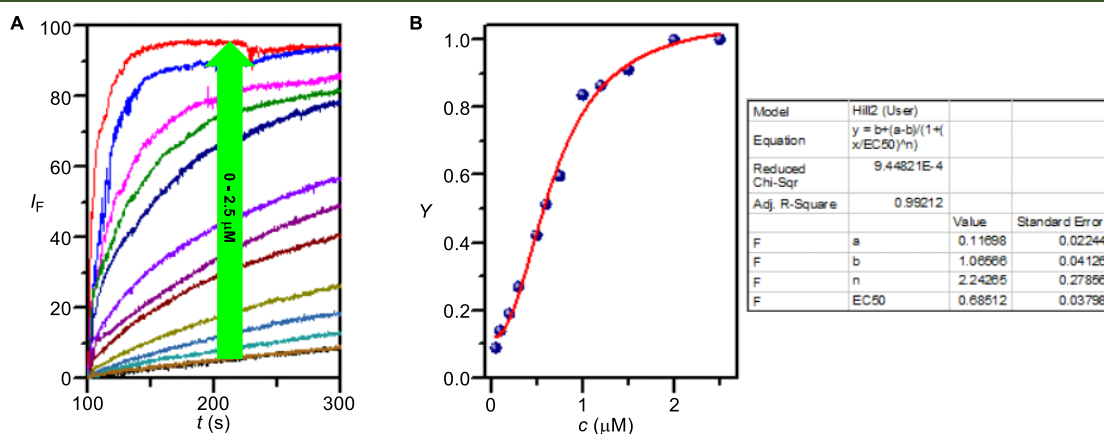


Figure 11: (A) Dose-dependent activity of compound **2d** across EYPC-LUVs⊃HPTS. (B) Hill plot analysis of **2d** at $t = 290$ s.

Table 2: Summary of Hill analyses of dose dependent ion transporter studies across EYPC-LUVs⊃HPTS.

Compound	EC_{50} (μM)	Hill coeff. (n)
1a	34.76	2.58
1b	3.23	2.94
1c	0.69	2.14
1d	1.38	2.25
2a	4.17	2.09
2b	1.30	3.93
2c	0.31	1.83
2d	0.68	2.24

Anion selectivity of **2c** (0.2 μM) was studied across EYPC-LUVs⊃HPTS by varying the vesicular anions (Cl^- , Br^- , NO_3^- , ClO_4^- , and SO_4^{2-}), keeping them the same both inside and outside the vesicles (Figure 28). Significant variation was observed in the ion transport activity of the order $\text{NO}_3^- > \text{Br}^- > \text{ClO}_4^- > \text{Cl}^-$ (Figure 12A), indicating the dependence of the transport process on the nature of the anion. No ion transport activity was observed in the case of SO_4^{2-} due to the inability of the transporter to dehydrate and transport the hydrophilic anion. Similarly, the cation selectivity of **2c** (0.2 μM) was studied across EYPC-LUVs⊃HPTS by simultaneous variation of the intra and extra-vesicular cations (Li^+ , Na^+ , K^+ , Rb^+ , Cs^+) (Figure 29). No significant change in ion transport

activity was observed (Figure 12B), indicating the process to be cation independent. Of the four ion transport modes possible (X^-/OH^- antiport, M^+/H^+ antiport, H^+/X^- symport, and M^+/OH^- symport) that lead to pH gradient dissipation in HPTS assay, M^+/H^+ antiport, and M^+/OH^- symport can be ruled out due to independence of the ion transport process on the nature of cation.

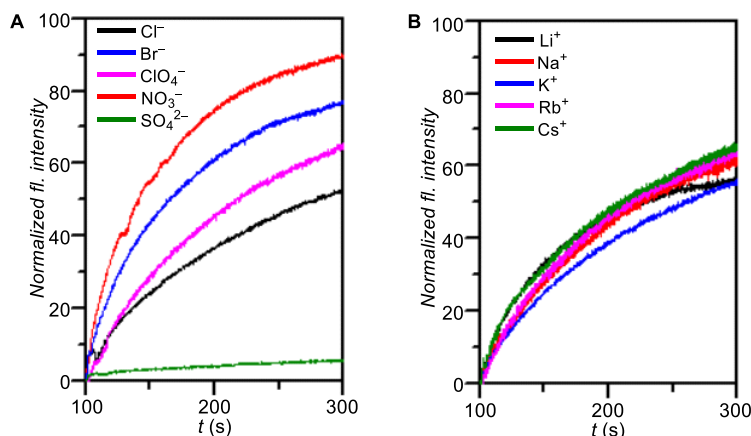


Figure 12: (A) Anion and (B) Cation selectivity assays of **2c** (0.2 μ M) across EYPC-LUVs Δ HPTS where both intra and extravesicular ions were simultaneously varied.

The Cl^- transport ability of both series, **1a-1d** and **2a-2d**, were assessed across EYPC-LUVs Δ lucigenin,⁴¹ through monitoring transporter-induced dissipation of the chloride gradient. Comparison of chloride transport activities of the molecules at 2.5 μ M yielded activities in the order **2c** > **2d** > **1c** > **1d** $\approx$ **2b** > **2a** > **1b** > **1a** (Figure 13A), which is identical to the result from the HPTS assay. Dose-dependent studies of the most active derivatives from *Series 1* (**1c**) and *Series 2* (**2c**) were conducted across EYPC-LUVs Δ lucigenin to yield EC_{50} = 4.04, 0.93 μ M, and n = 2.03, 1.79 for **1c** and **2c** respectively (Figures 14-15). The Hill coefficient values were found to match closely with those obtained in the HPTS assay. Activities of **2c** (1 μ M) across EYPC-LUVs Δ lucigenin on variation of the extravesicular cations by changing the M^+Cl^- (M^+ = Li^+ , Na^+ , K^+ , Rb^+ , Cs^+) salt used for ion pulse generation showed no significant changes (Figure 13B), further corroborating the cation independent mechanism of ion transport. The Cl^- influx activity of **2c** (0.6 μ M) was observed to drastically increase in the presence of the K^+ transporter valinomycin⁴⁶ (0.5 μ M) (Figure 13C), confirming anion (X^-/OH^-) antiport as the mechanism of ion transport by the pyrroleamides. To probe for possible detergent effect by the transporters on the lipid membrane, CF leakage assay⁴⁷ was performed by treating EYPC-LUVs Δ CF with transporter **2c**. No CF leakage was observed even at 3 μ M of **2c** (dosage $\sim$ 10 times of EC_{50} obtained

in HPTS assay) (Figure 16), indicating membrane integrity of vesicles is preserved during the transport anion process.

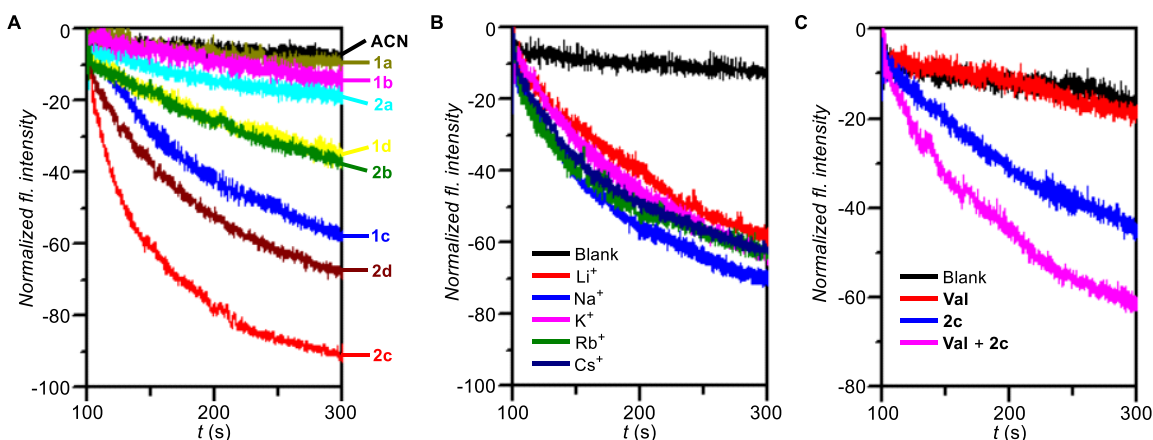


Figure 13: (A) Comparison of chloride transport activities of pyrroleamides (2.5 μM) across EYPC-LUVs $\supset$ lucigenin. (B) Cation selectivity of 2c (1 μM) measured across EYPC-LUVs $\supset$ Lucigenin by varying extravesicular cations. (C) Valinomycin (Val, 0.5 μM) coupled chloride transport assay of 2c (0.6 μM) across EYPC-LUVs $\supset$ Lucigenin. Enhancement of transport activity in the presence of valinomycin confirms anion antiport as the dominant transport mechanism.

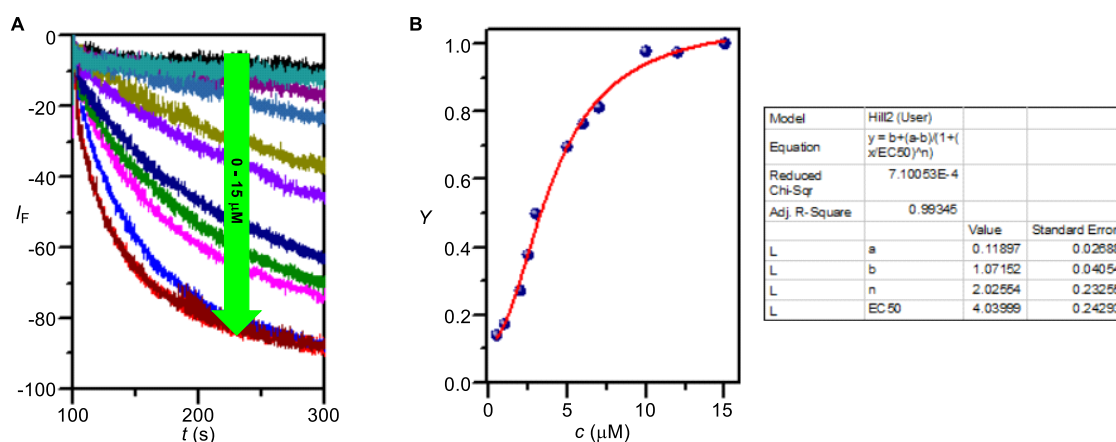


Figure 14: (A) Dose-dependent activity of compound 1c across EYPC-LUVs $\supset$ Lucigenin. (B) Hill plot analysis of 1c at $t = 290$ s.

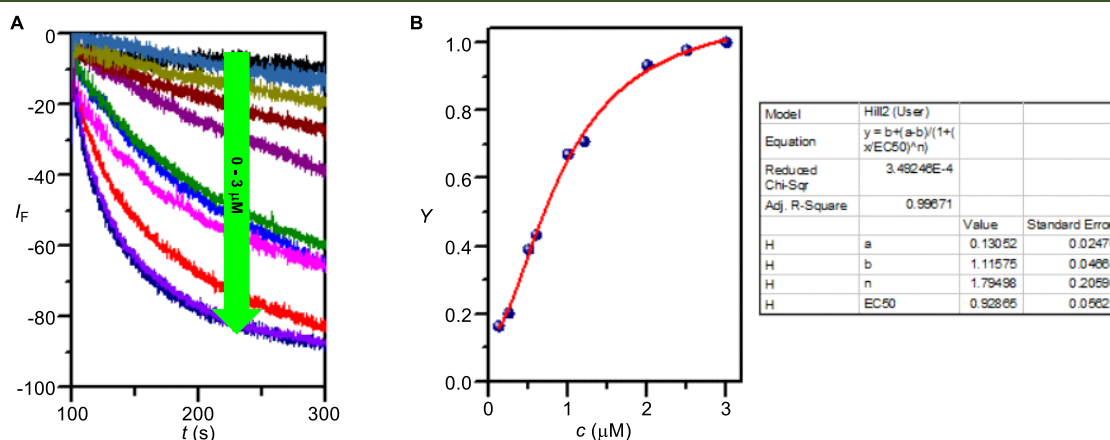


Figure 15: (A) Dose-dependent activity of compound **2c** across EYPC-LUVs $\Rightarrow$ Lucigenin. (B) Hill plot analysis of **2c** at $t = 290$ s.

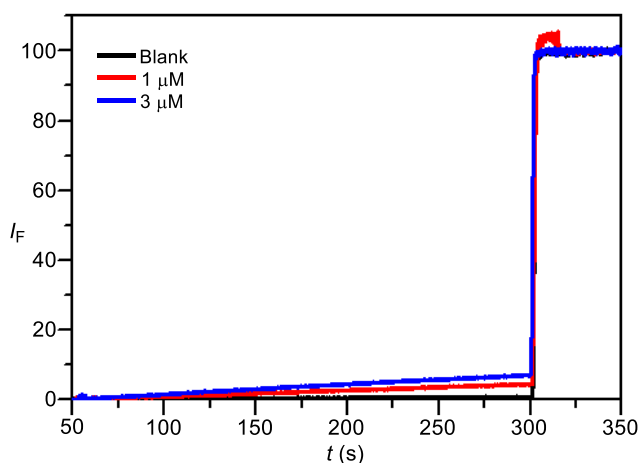


Figure 16: Activity of **2c** across EYPC-LUVs $\Rightarrow$ CF. Little increase in fluorescence signal was observed even for compound treatment at $3 \mu\text{M}$, corresponding to negligible CF leakage, indicating pyrroleamides do not compromise membrane integrity even at high dosages.

5.2.4 Anion Binding Studies

To obtain insight into the ion binding ability of pyrroleamides, co-crystallization of the derivatives with TBACl was attempted, of which only **1c** was successful. In the single crystal structure of free **1c** (crystallized from EtOAc), the pyrroleamide was observed to exist in a conformation where the N-H_a (pyrrole) and N-H_b (amide) bonds were *anti* to each other (Figure 17). Co-crystallization of **1c** with TBACl (in ACN) gave a 1:1 complex of transporter with Cl⁻ that showed N-H_a (pyrrole) and N-H_b (amide) in a *syn* orientation with N-H_a, N-H_b and C-H_c (aryl C-H *ortho* to amide N) participating in the anion binding (Figure 17). The distances of these atoms to Cl⁻ were measured to be N(H_a)- $3.067 \text{ \AA}$, N(H_b)- $3.296 \text{ \AA}$, and C(H_c)- $3.691 \text{ \AA}$, respectively.

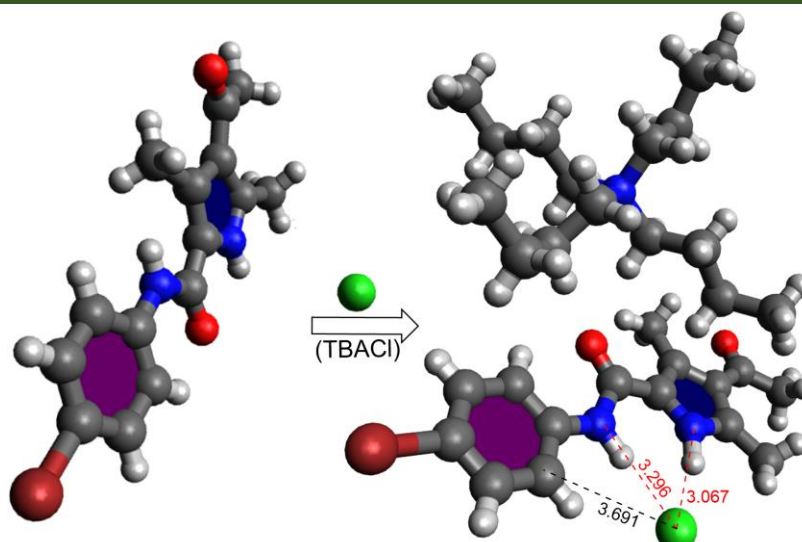


Figure 17: Molecular structures of transporter **1c** and co-crystal of **1c** with TBACl (showing noncovalent interactions with chloride; distance measurements in Å units) obtained from SCXRD data. The amide N-H, which was initially *anti* to the pyrrole N-H, flips into the *syn* conformation to bind with the chloride ion.

The role of these three protons in the ion binding was further verified through ^1H NMR titration experiments of **2c** in CDCl_3 . Upon addition of incremental quantities of the anions Cl^- , Br^- and NO_3^- in the form of their tetrabutylammonium (TBA) salts to **2c** (2 mM), downfield shifts were observed in N-H_a , N-H_b and C-H_c protons (Figures 18-20), indicating their involvement in anion binding. Binding constants were calculated from the ^1H NMR titration data using the BindFit program,⁴⁸ assuming 1:1 binding for **2c** with anion. Obtained $K_a(1:1) = 1394.8 \pm 91.8 \text{ M}^{-1}$, $123.9 \pm 5.7 \text{ M}^{-1}$ and $121.2 \pm 4.0 \text{ M}^{-1}$ for Cl^- , Br^- and NO_3^- respectively.

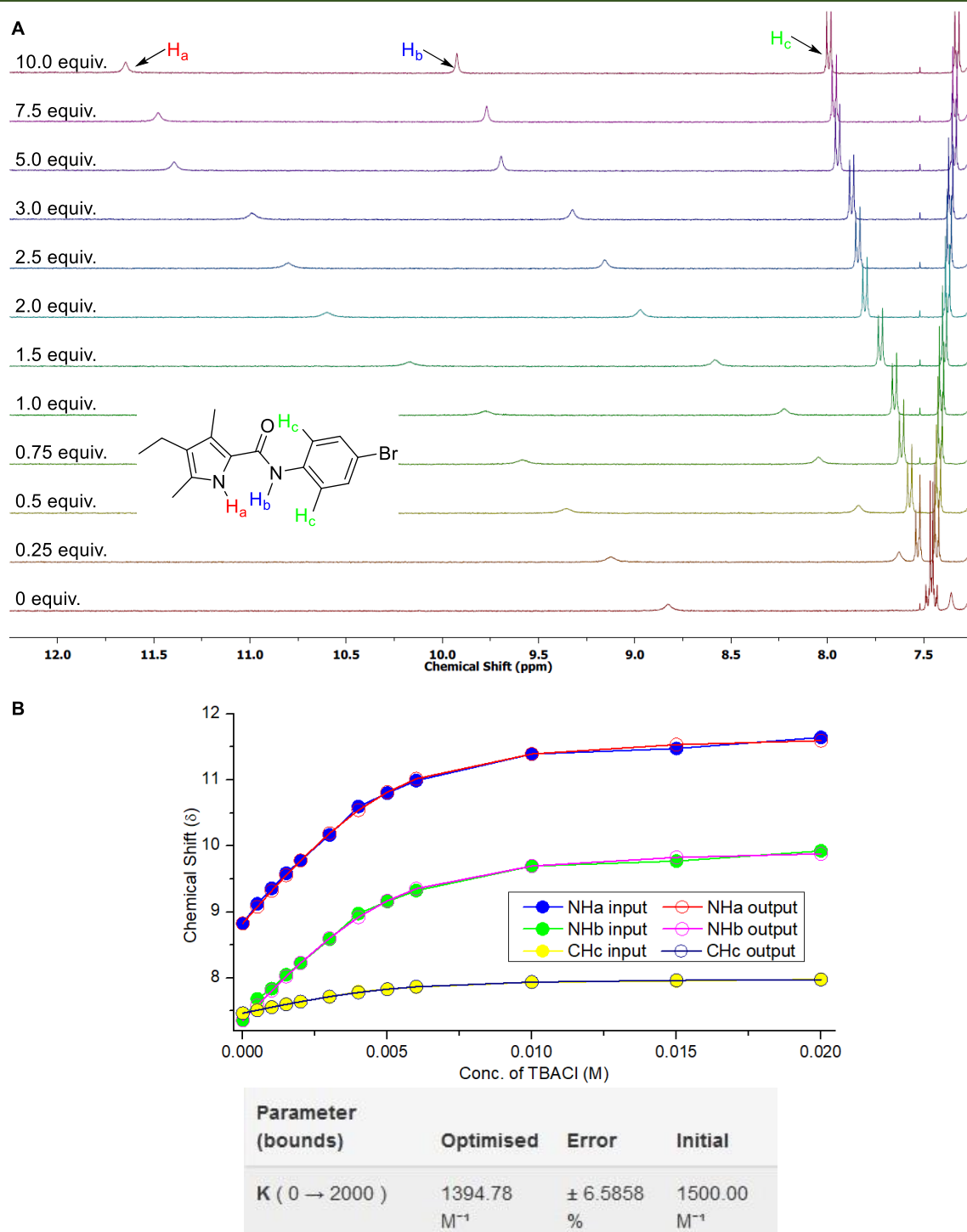


Figure 18: (A) ^1H NMR titration spectra of **2c** (2 mM) with stepwise addition of TBACl in CDCl_3 . The number of TBACl equivalents added per reading is shown on the stacked spectra. (B) Plot of chemical shifts (δ) of protons H_a , H_b , and H_c vs concentration of TBACl added, fitted to 1:1 binding model using BindFit v0.5.

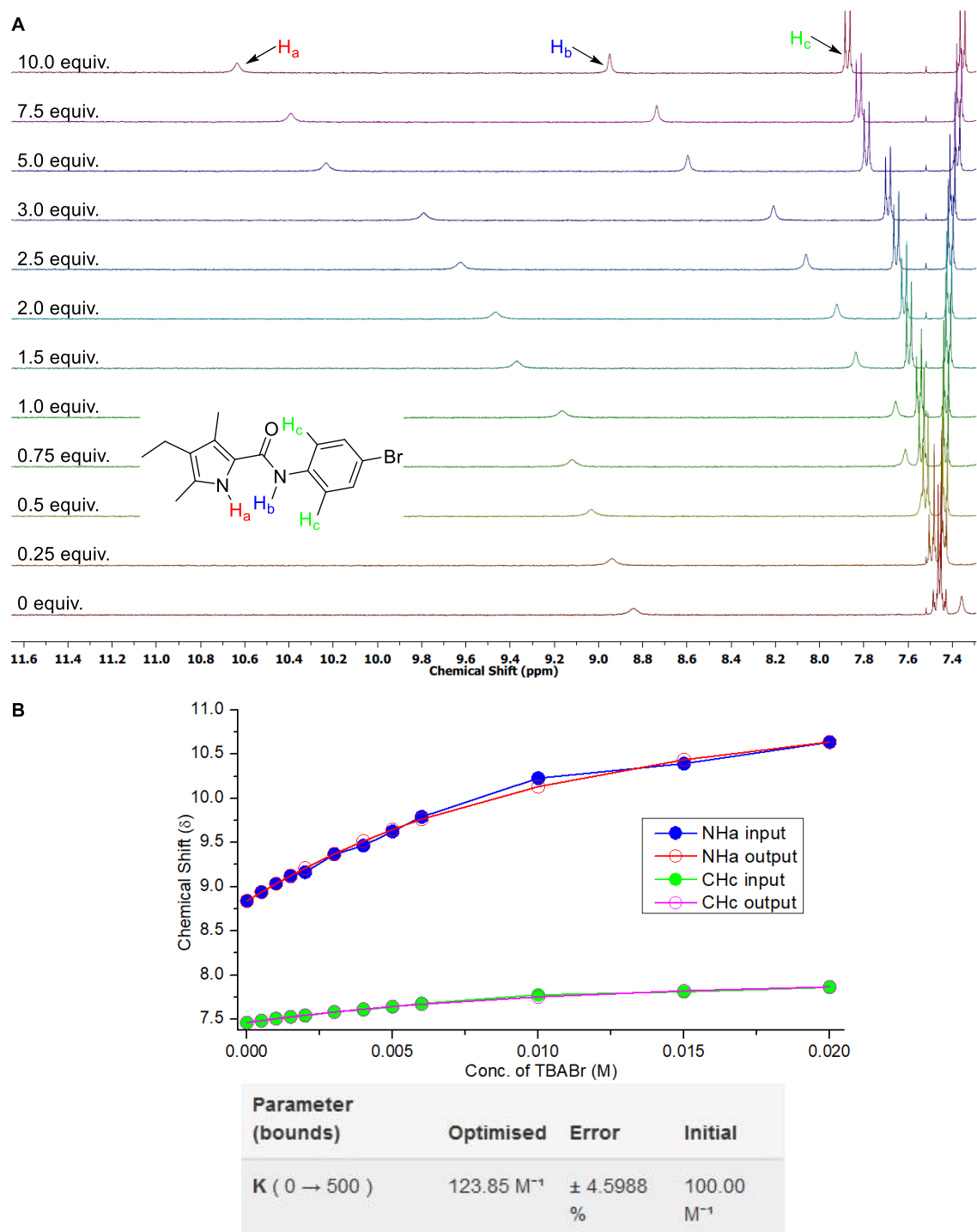


Figure 19: (A) ^1H NMR titration spectra of **2c** (2 mM) with stepwise addition of TBABr in CDCl_3 . The number of TBABr equivalents added per reading is shown on the stacked spectra. (B) Plot of chemical shifts (δ) of protons H_a and H_c vs concentration of TBABr added, fitted to 1:1 binding model using BindFit v0.5.

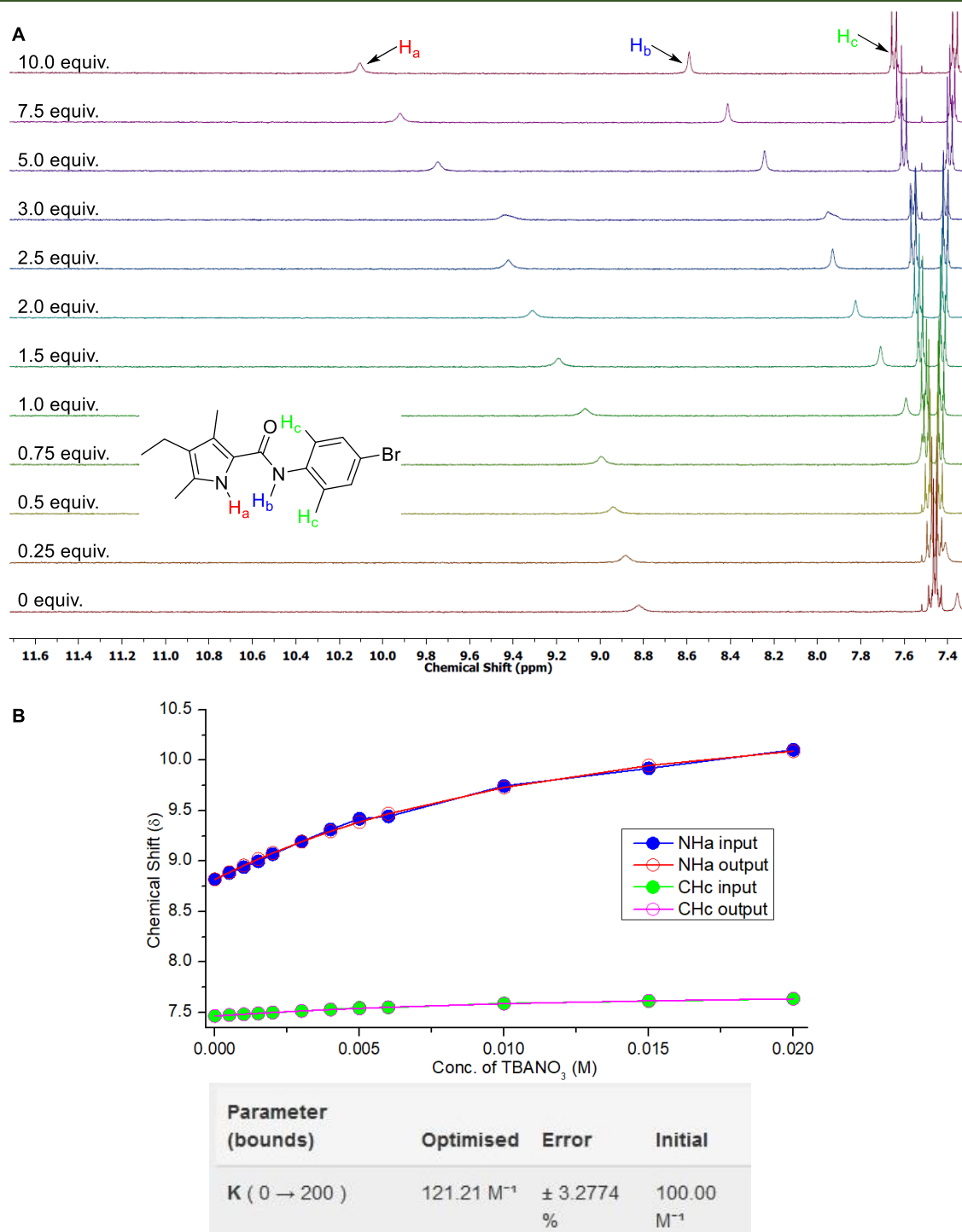


Figure 20: (A) ^1H NMR titration spectra of **2c** (2 mM) with stepwise addition of TBANO₃ in CDCl₃. The number of TBANO₃ equivalents added per reading is shown on the stacked spectra. (B) Plot of chemical shifts (δ) of protons H_a and H_c vs concentration of TBANO₃ added, fitted to 1:1 binding model using BindFit v0.5.

Predictions for the most probable geometry of free **2c** and $[\mathbf{2c}+\text{Cl}^-]$ were carried out with CONFLEX 8 conformation search software package^{49, 50} (MMFF94S forcefield, search limit-10 kcal/mol). Out of 4 predictions obtained for free **2c**, the Boltzmann populations of the major conformers **Conf-1** and **Conf-2** were 55.3960% and 44.5702%, respectively (Figure 21 A, B), which were highly similar to pyrrole and amide N-H groups in *anti* orientation. The remaining two conformations (Boltzmann population <0.02%) were rejected. The DFT geometry optimization and energy calculation of **Conf-1** was conducted, and the conformation of the obtained structure was observed to be highly similar to that of free **1c** obtained from the crystal data (Figure 21C).

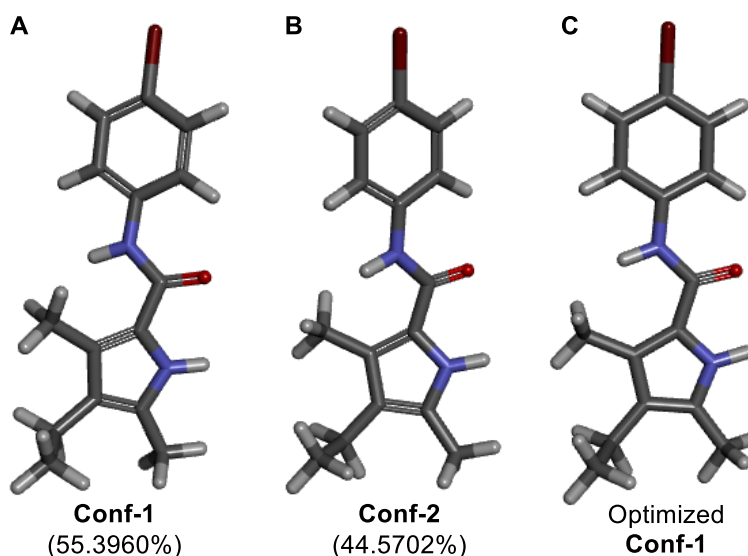


Figure 21: CONFLEX 8 predicted majority geometries, (A) **Conf-1** and (B) **Conf-2**, of **2c** along with their Boltzmann populations (in brackets). Both conformations are in the N-H *anti* state, indicating its predominance. (C) DFT geometry optimized structure of **2c** (**Conf-1**).

For **2c** (from **Conf-1**):

- HF energy = -3338.3341679 Hartree
- Zero-point energy = 0.2933699 Hartree

Only a single prediction (**Conf-3**) (Figure 22A) was obtained from the conformation search of $[\mathbf{2c}+\text{Cl}^-]$ complex, on which DFT geometry optimization and energy calculation were conducted. The conformation of the obtained structure (Figure 22B) was observed to be highly similar to that of $[\mathbf{1c}+\text{Cl}^-]$ obtained from crystal data (Figure 17), showing N-H_a (pyrrole) and N-H_b (amide) in a *syn* orientation interacting with the anion along with C-H_c (aryl C-H *ortho* to amide N). The

distances of these atoms to Cl^- were measured to be $\text{N}(\text{H}_a)$ - 3.113 Å, $\text{N}(\text{H}_b)$ - 3.283 Å, and $\text{C}(\text{H}_c)$ - 3.683 Å, respectively (Figure 22C). Binding energy was calculated using equation 5.

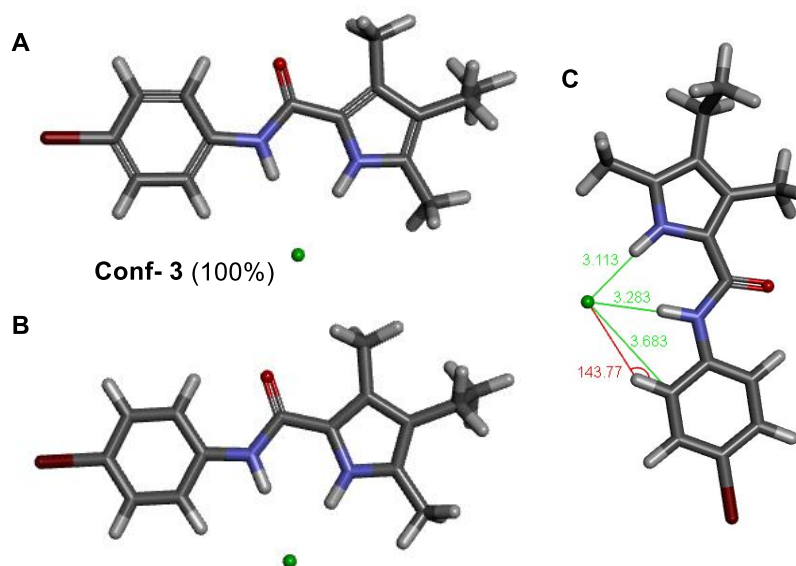


Figure 22: (A) CONFLEX 8 predicted geometry **Conf-3** of $[\mathbf{2c}+\text{Cl}^-]$ along with their Boltzmann populations (in brackets). (B) DFT geometry optimized structure of $[\mathbf{2c}+\text{Cl}^-]$ (**Conf-3**). (C) Distances of non-covalent interactions in optimized output.

For $[\mathbf{2c}+\text{Cl}^-]$ (from **Conf-3**):

- HF energy = -3798.651537 Hartree
- Zero-point energy = 0.2930112 Hartree
- BSSE = 0.00644884078 Hartree
- Binding energy = -0.059045659 Hartree = -37.05174162 kcal/mol

5.2.5 Prediction of Ion Transport Complex Structure

Experimental data from ion transport experiments evidenced the formation of a $[(\mathbf{2c})_2+\text{Cl}^-]$ active transporter complex (from Hill coefficient ≈ 2) in the lipid membrane. The initial prediction of the most probable geometry of $[(\mathbf{2c})_2+\text{Cl}^-]$ with CONFLEX 8 yielded a total of 8 possible geometries as output. The structure with the highest Boltzmann population (42.784%) (**CF-A**) (Figure 23A) was selected, and all conformations with a Boltzmann population < 30% were rejected. The DFT geometry optimization of **CF-A** (Gaussian 09, B3LYP/6-31G(d,p)). The resulting structure showed two molecules of **2c** binding to Cl^- in an almost orthogonal fashion with N-H_a and N-H_b

H-bonding with the anion (Figure 23B-D). The *ortho* C-H on the phenyl ring (C-H_c) was also observed to contribute to the binding. Binding energy was calculated using equation 5.

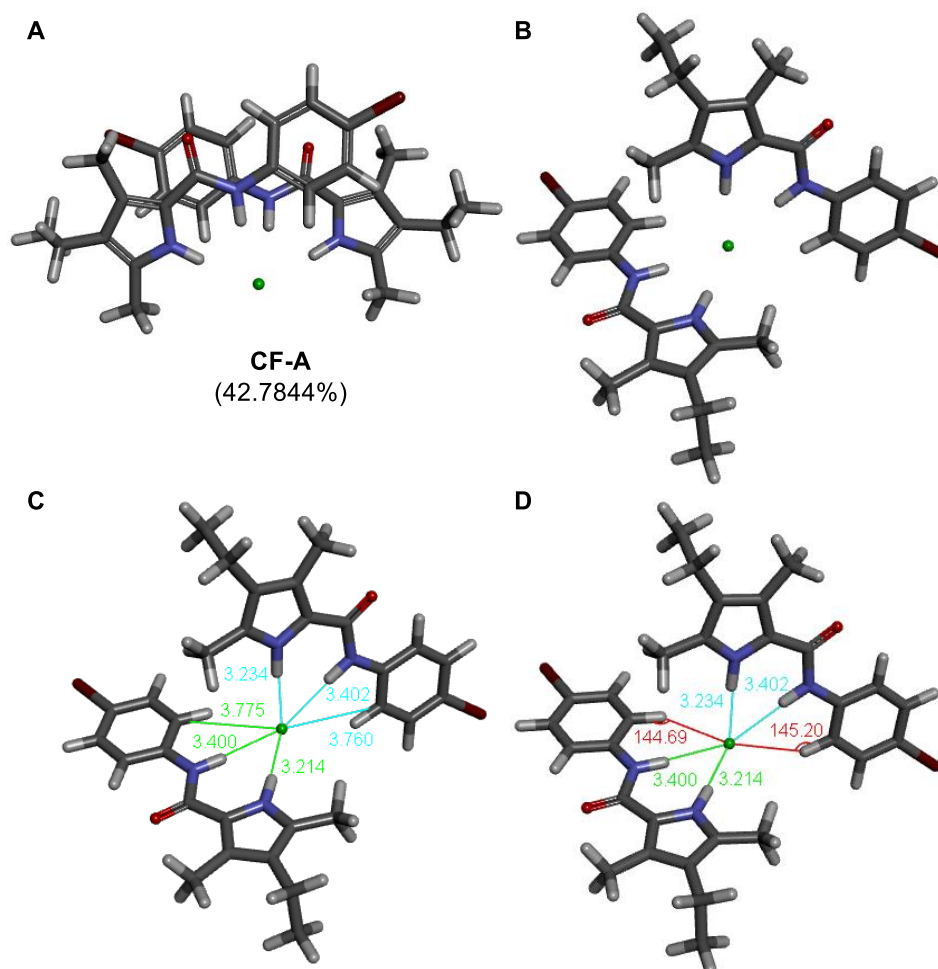


Figure 23: (A) CONFLEX 8 predicted geometry **CF-A** of $[(2c)_2+Cl^-]$ along with their Boltzmann population (in brackets). (B) DFT geometry optimized structure of $[(2c)_2+Cl^-]$ (**CF-A**). (C) and (D) Distances and angles of non-covalent interactions in optimized output.

For $[(2c)_2+Cl^-]$ (from **CF-A**):

- HF energy = -7137.0204539 Hartree
- Zero-point energy = 0.5871497 Hartree
- BSSE = 0.01338758919 Hartree
- Binding energy = -0.086087311 Hartree = -54.02064841 kcal/mol

Hill analysis of dose-dependent studies of transporter **2b** hinted at the possibility of formation of a $[(2b)_4+Cl^-]$ active transporter complex (Hill coefficient ~ 4). Out of curiosity, its structure

prediction was attempted. First, prediction of possible geometries of the transporter **2b** was conducted. A total of 10241 predictions were obtained, with the Boltzmann population of the most probable conformer **Conf-4** being 2.01307% (Figure 24A). Analysis of the first 100 conformers (cumulative Boltzmann population- 42.84737) showed a highly similar pyrroleamide core, the pyrrole and amide N-H groups were in *anti* orientation, with fluctuations only in the conformation of the alkyl (octyl) chain. Hence, DFT geometry optimization and energy calculation of **Conf-4** was conducted, and the conformation of the free pyrroleamide core (Figure 24B) was observed to be highly similar to that of free **1c** and **2c** obtained from the crystal data (Figure 17) and theoretical calculations (Figure 21C) respectively.

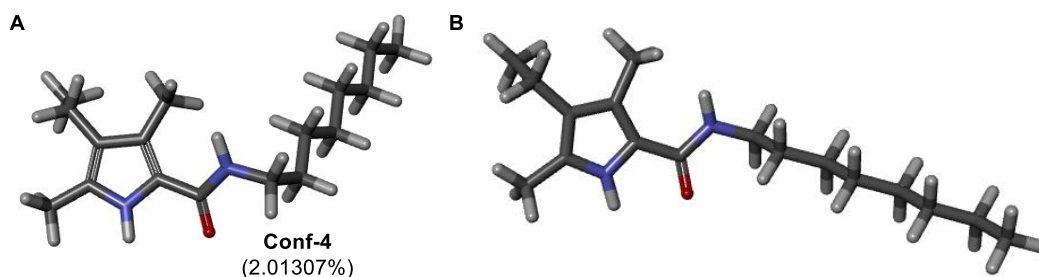


Figure 24: (A) CONFLEX 8 predicted majority geometry **Conf-4** of **2b** along with Boltzmann population (in brackets). (B) DFT geometry optimized structure of **2b** (**Conf-4**).

For **2b** (from **Conf-4**):

- HF energy = -850.7030017 Hartree
- Zero-point energy = 0.4500029 Hartree

The initial geometry prediction of $[(\mathbf{2b})_4 + \text{Cl}^-]$ with CONFLEX 8 yielded 13620 conformers, with the highest Boltzmann population being 0.232% (Figure 25A). Analysis of the first 100 conformers (cumulative Boltzmann population- 9.762%) showed the conformers to be highly similar, with only minor fluctuations in the alkyl chain conformation. Hence, geometry optimization was performed on the most probable conformer (Boltzmann population- 0.232%) (**CF-B**) and the resulting structure showed a chloride ion H-bonded by four pyrroleamide cores (total 8 $\text{N-H}\cdots\text{Cl}^-$ bonds) in a pseudo-octahedral fashion with the alkyl groups wrapping around this polar core to form a lipophilic capsule for transmembrane transport of Cl^- (Figures 25B, 26). HF energy of **2b** (**Conf-4**) was considered for binding energy calculations (Equation 5).

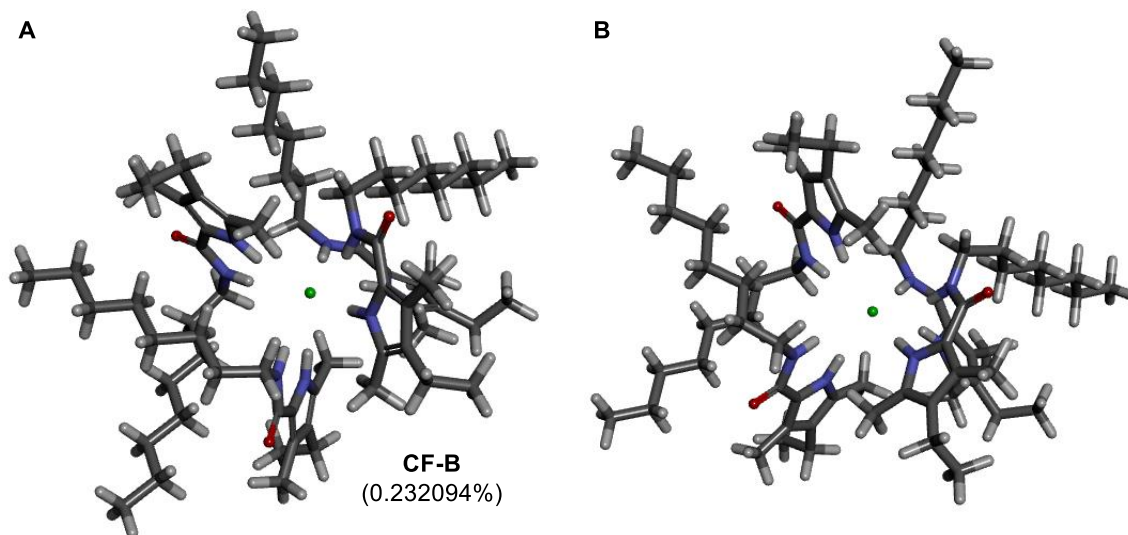


Figure 25: (A) CONFLEX 8 predicted geometry CF-B of $[(2b)_4+Cl^-]$ along with their Boltzmann population (in brackets). (B) DFT geometry optimized structure of $[(2b)_4+Cl^-]$ (CF-B).

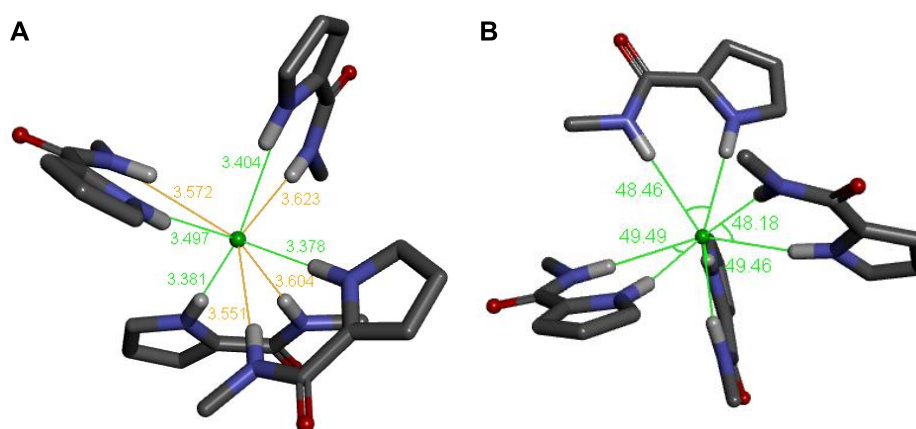


Figure 26: Core of the geometry optimized structure of $[(2b)_4+Cl^-]$ (CF-B) showing (A) distances (pyrrole N-H...Cl in green and amide N-H...Cl in orange), and (B) and angles of non-covalent interactions in optimized output.

For $[(2b)_4+Cl^-]$ (from CF-B):

- HF energy = -3863.180908 Hartree
- Zero-point energy = 1.801867 Hartree
- BSSE = 0.038198597559 Hartree
- Binding energy = -0.076613902 Hartree = -48.07598992 kcal/mol

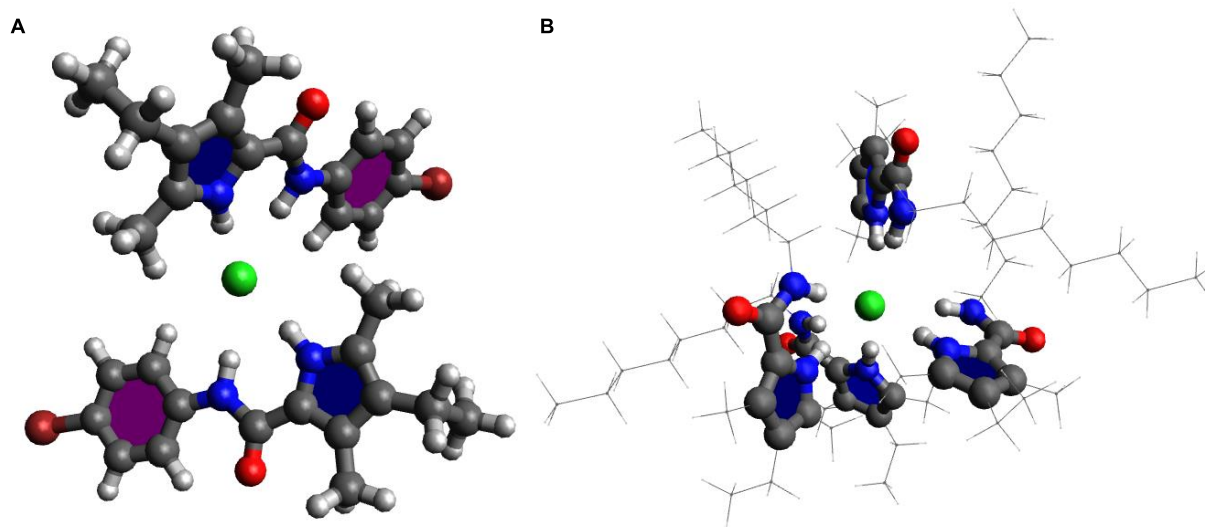


Figure 27: Comparison of geometry-optimized structures of predicted ion transporter complexes of (A) **2c** ($[(2c)_2^+ Cl^-]$) and (B) **2b** ($[(2b)_4^+ Cl^-]$) with chloride ion. Alkyl groups of $[(2b)_4^+ Cl^-]$ were drawn as a wireframe for ease of visualization.

5.3 Conclusions

In this work, pyrrole-2-carboxamides have been demonstrated to be highly versatile transmembrane ion transport systems that are easily tunable for a wide range of properties achievable by small variations in structure. The *Series 1* of four pyrrole-2-carboxamides (**1a-1d**) were synthesized with two alkyl (hexyl, octyl) and two aryl (4-bromophenyl and 4-(tert-butyl)phenyl) substituents. The (pyrrole) 4-acetyl groups on these compounds were reduced to ethyl to obtain a second series (*Series 2*) (**2a-2d**) of transporters. The synthesized molecules were observed to have a wide range of values for $\log P$ and N-H pK_a . Ion transport assays across EYPC-LUVs $\Rightarrow$ HPTS showed the derivatives to be efficient anion transporters, with *Series 2* having higher activity than the corresponding members of *Series 1* and compound **2c** exhibiting the highest ion transport activity. Hill analyses of dose-dependent activities of the compounds indicated the participation of two to four molecules of the transporter (Hill coefficient $n = 2-4$) in the formation of the active ion transport complex depending on the substituents, indicating the high degree of transporter tunability with small variations in the structure. Mechanistic studies with **2c** across HPTS and lucigenin-entrapped vesicles established anion antiport to be the preferred mechanism of ion transport. The mode of anion binding was verified through crystallization, NMR titration experiments and theoretical calculations. Structures of the active transporter complexes $[(2c)_2^+ Cl^-]$ (aryl substituted) and the probable $[(2b)_4^+ Cl^-]$ (alkyl

substituted) were predicted using CONFLEX 8 software followed by DFT geometry optimization in Gaussian 09. This highly versatile system has the potential to be modified as a stimuli responsive anion transporter by blocking the pyrrole and/or amide NH groups with labile groups activatable by external stimuli.

5.4 Experimental Section

5.4.1 General Methods

Reagents and solvents for synthesis were purchased from commercial sources (Sigma-Aldrich, TCI, Spectrochem, Finar, Avra, SRL) and used without further purification. Purifications via column chromatography were performed on Spectrochem silica gel (100-200 mesh) (or 230-400 mesh silica gel where specified). Thin layer chromatography (TLCs) was performed on Merck silica gel 60-F254 plates. Egg yolk phosphatidylcholine (EYPC) as a chloroform solution (25 mg/mL), polycarbonate membranes of 100 nm and 200 nm pore size, and mini extruder set for vesicle preparation were purchased from Avanti Polar Lipids. HPTS, lucigenin, Triton™ X-100, valinomycin, NaOH, and inorganic salts were of molecular biology grade purchased from Sigma-Aldrich (Merck). HEPES was purchased from SRL. All stock solutions were prepared in HPLC grade DMSO (Rankem) or acetonitrile (NICE). All buffer solutions were prepared in Milli-Q water.

5.4.2 Physical Measurements

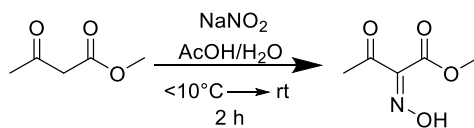
All ^1H NMR spectra were recorded at 400 MHz and ^{13}C spectra at 101 MHz on Bruker Avance III HD 400 MHz and JEOL JNM-ECS 400 MHz NMR spectrometers. All spectra were calibrated with respect to the residual solvent peak ($\delta_{\text{H}} = 7.26$ ppm and $\delta_{\text{C}} = 77.16$ ppm for CDCl_3 , $\delta_{\text{H}} = 2.50$ ppm and $\delta_{\text{C}} = 39.52$ ppm for $\text{DMSO}-d_6$). The chemical shifts (δ) were reported in ppm, and the coupling constants (J) in Hz. The following abbreviations were used to indicate peak splitting patterns: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), dd (doublet of doublet), td (triplet of doublet), and dt (doublet of triplet). Infra-red (IR) spectra were obtained using a Bruker ALPHA II FT-IR spectrometer and reported in ν (cm^{-1}). High resolution mass spectra (HRMS) were recorded on a Waters SYNAPT G2 mass spectrometer equipped with a Waters Z-Spray™ electrospray ionization (ESI) source. Melting points were measured in open glass capillaries on a Büchi Melting Point M-560 apparatus. Single crystal X-ray diffraction (SCXRD) measurements were done on Bruker D8 VENTURE or Bruker KAPPA APEX diffractometers. Fluorescence

spectra were recorded on a HORIBA Jobin Yvon Fluoromax®-4 spectrofluorometer equipped with an injector port and magnetic stirrer. All buffer solutions were adjusted to the necessary pH using a Hanna Instruments (HI98108 pHep+) pocket size pH meter.

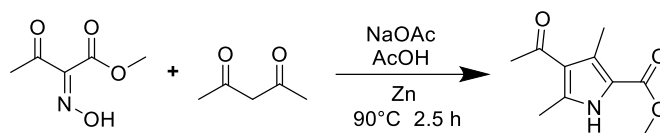
Compounds for preparing stock solutions were weighed out using a Quintix® 125D Semi-Micro lab balance (0.01 mg least count) from Sartorius AG. NMR spectra were processed using MestReNova software. All data from fluorescence studies were processed using OriginPro 8.5 software. ChemDraw 21.0.0 was used for drawing structures and processing figures. 3D structure images were generated using BIOVIA Discovery Studio Visualizer 2019 and Avogadro 1.2.0. Theoretical predictions of pK_a and $\log P$ values were calculated using calculator plugins of MarvinSketch program (v5.8.0). Molecular structure conformations were predicted using CONFLEX 8 software package. DFT calculations were performed on Gaussian 09 program suite.

5.4.3 Synthetic Protocols

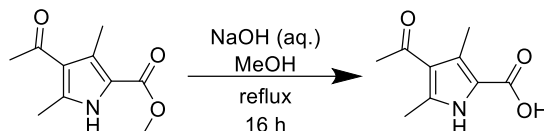
Synthesis of methyl (Z)-2-(hydroxyimino)-3-oxobutanoate (5)



Compound 5 was synthesized using a modified literature protocol.⁵¹ To a solution of methylacetoacetate (10 mL, 92.7 mmol) in acetic acid (18 mL), maintained below 10°C in an ice bath, was added a chilled solution of NaNO_2 (8.3 g, 120.5 mmol) in distilled water (18 mL) dropwise over 15 min. The mixture was then allowed to reach rt and stirred for 1 h, following which 66 mL of distilled water was added and stirred for a further 2 h. The mixture was then extracted with diethyl ether (100 mL x3), the organic layers combined, and washed with sat. NaHCO_3 solution (100 mL) and brine (100 mL). The organic layer was then dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo* to yield the product as a yellow oil, which was used as such for the next reaction. The formation of the desired product was confirmed through ^1H NMR. Yield = 12.4 g = 92%; ^1H NMR (400 MHz, CDCl_3): δ 3.91 (s, 5H), 2.41 (s, 5H), 2.11 (s, 2H).

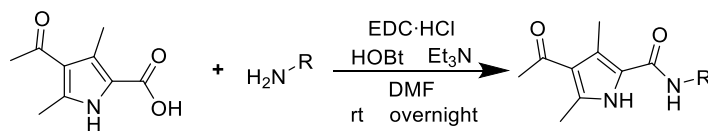
Synthesis of methyl 4-acetyl-3,5-dimethyl-1H-pyrrole-2-carboxylate (6)

Compound **6** was synthesized using a modified literature protocol.⁵² A solution of the oxime (12.4 g, 84.5 mmol), acetylacetone (10.4 mL, 101.4 mmol), and sodium acetate (17.3 g, 211.3 mmol) in acetic acid (90 mL) was heated to 60 °C and zinc powder (13.8 g, 211.3 mmol) was slowly added in parts. The mixture was then heated to 90 °C and stirred for 2.5 h. The reaction mixture was then poured into 800 mL of cold ice water, and the resulting precipitate was filtered out. The precipitate was dissolved in DCM and filtered to remove zinc. Then, the solvent was removed and the residue dried under vacuum to yield the product as an off-white solid. Yield = 13 g = 79%; **¹H NMR (400 MHz, CDCl₃)**: δ 8.96 (s, 1H), 3.86 (s, 3H), 2.59 (s, 3H), 2.52 (s, 3H), 2.45 (s, 3H). **¹³C NMR (101 MHz, CDCl₃)**: δ 195.6, 162.2, 138.5, 129.7, 123.7, 117.9, 51.6, 31.5, 15.4, 12.8. **MP**: 157.2-160.6 °C; **IR (ν̄ cm⁻¹)**: 3295, 2955, 1643, 1558, 1505, 1440, 1401, 1272, 1201, 1100, 1067, 776, 756; **HRMS (ESI)**: m/z [M+H]⁺ Calc. for C₁₀H₁₃NO₃: 196.0973; Found: 196.0972.

Synthesis of 4-acetyl-3,5-dimethyl-1H-pyrrole-2-carboxylic acid (3)

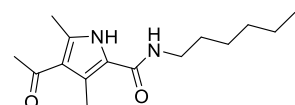
To a solution of the ester **5** (1.52 g, 7.80 mmol) in MeOH (25 mL) was added 2 M aq. NaOH (20 mL) and the mixture stirred at reflux for 16 h. The MeOH was then removed *in vacuo* and the residue was diluted with water. It was then made to pH 3-4 using conc. HCl. The resulting precipitate was filtered, washed with water, and dried under vacuum in a desiccator to yield the product as a white solid. Yield = 1.37 g = 97%; **¹H NMR (400 MHz, CDCl₃)**: δ 12.45 (s, 1H), 11.74 (s, 1H), 2.48 (s, 3H), 2.43 (s, 3H), 2.34 (s, 3H). **¹³C NMR (101 MHz, CDCl₃)**: δ 194.4, 162.4, 138.4, 128.4, 122.4, 118.0, 31.1, 14.4, 12.3. **MP**: melting and decomposition above 213 °C; **IR (ν̄ cm⁻¹)**: 3271, 2927, 1641, 1570, 1514, 1438, 1280, 1070, 953, 783, 736; **HRMS (ESI)**: m/z [M+H]⁺ Calc. for C₉H₁₁NO₃: 182.0817; Found: 182.0820.

Synthesis of pyrrole amides (Series 1) through acid-amine coupling



EDC·HCl-HOBT coupling- General procedure A: To a solution of the pyrrole carboxylic acid (1 eq.) in DMF (1 ml per 100 mg pyrrole) was added EDC·HCl (1.2 eq.) followed by HOBT (1.2 eq.) and stirred 30 min at rt. Then the amine (1 eq.) was added followed by Et₃N (1.7 eq.) and the mixture was stirred overnight at rt. It was then diluted with EtOAc and washed with water. The aqueous layer was further extracted with EtOAc (x2), the organic layers combined, washed with brine, dried over anhydrous Na₂SO₄, and the solvent removed *in vacuo*. The residue was purified by silica gel column chromatography.

4-Acetyl-*N*-hexyl-3,5-dimethyl-1*H*-pyrrole-2-carboxamide (1a)

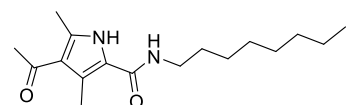


Eluent for column chromatography: 50% EtOAc in petroleum ether;

Physical appearance: White solid; Yield = 79%; **MP:** 138.3-142.6 °C;

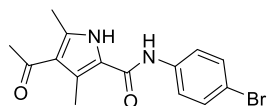
¹H NMR (400 MHz, CDCl₃): δ 9.99 (s, 1H), 5.80 (s, 1H), 3.44 (dd, *J* = 13.0, 7.1 Hz, 2H), 2.54 (s, 3H), 2.53 (s, 3H), 2.43 (s, 3H), 1.64 – 1.55 (m, 2H), 1.40 – 1.27 (m, 6H), 0.89 (t, *J* = 6.9 Hz, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 195.7, 162.1, 136.5, 123.1, 122.3, 121.7, 39.9, 31.6, 31.4, 29.9, 26.8, 22.7, 15.4, 14.1, 12.8. **IR (ν̄ cm⁻¹):** 3226, 2957, 2853, 1613, 1521, 1408, 1283, 1249, 1070, 944, 802; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₉H₂₄N₂O₂: 265.1916; Found: 265.1911.

4-Acetyl-3,5-dimethyl-*N*-octyl-1*H*-pyrrole-2-carboxamide (1b)



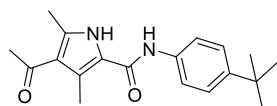
Eluent for column chromatography: 30% EtOAc in petroleum ether; **Physical appearance:** White solid; Yield = 61%; **MP:** 111.6-113.9 °C;

¹H NMR (400 MHz, CDCl₃): δ 10.08 (s, 1H), 5.81 (s, 1H), 3.44 (dd, *J* = 13.0, 7.0 Hz, 2H), 2.54 (s, 3H), 2.53 (s, 3H), 2.43 (s, 3H), 1.60 (p, *J* = 7.3 Hz, 2H), 1.39 – 1.23 (m, 10H), 0.87 (t, *J* = 6.8 Hz, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 195.7, 162.1, 136.5, 123.1, 122.3, 121.7, 39.9, 31.9, 31.4, 29.9, 29.4, 29.3, 27.1, 22.8, 15.3, 14.2, 12.8. **IR (ν̄ cm⁻¹):** 3210, 2925, 2852, 1613, 1524, 1411, 1280, 1205, 1072, 815; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₇H₂₈N₂O₂: 293.2229; Found: 293.2230.

4-Acetyl-N-(4-bromophenyl)-3,5-dimethyl-1H-pyrrole-2-carboxamide (1c)

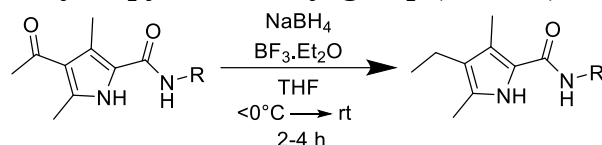
Eluent for column chromatography: 30% EtOAc in petroleum ether;

Physical appearance: White solid; Yield = 50%; **MP:** 225.1-226.6 °C; **¹H NMR (400 MHz, DMSO-*d*₆):** δ 11.66 (s, 1H), 9.69 (s, 1H), 7.67 – 7.60 (m, 2H), 7.54 – 7.46 (m, 2H), 2.48 (s, 3H), 2.47 (s, 3H), 2.37 (s, 3H). **¹³C NMR (101 MHz, DMSO-*d*₆):** δ 194.4, 159.8, 138.6, 137.0, 131.5, 125.4, 122.1, 121.7, 121.6, 114.7, 31.2, 14.6, 12.5. **IR (ν̄ cm⁻¹):** 3259, 1630, 1525, 1481, 1389, 1306, 1233, 1066, 821, 753; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₉H₂₄N₂O₂: 335.0395; Found: 335.0394.

4-Acetyl-N-(4-(*tert*-butyl)phenyl)-3,5-dimethyl-1H-pyrrole-2-carboxamide (1d)

Eluent for column chromatography: 30% EtOAc in petroleum ether;

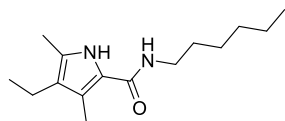
Physical appearance: White solid; Yield = 57%; **MP:** 210.1-211.7 °C; **¹H NMR (400 MHz, CDCl₃):** δ 9.98 (s, 1H), 7.53 – 7.46 (m, 3H), 7.40 – 7.35 (m, 2H), 2.66 (s, 3H), 2.50 (s, 3H), 2.45 (s, 3H), 1.32 (s, 9H). **¹³C NMR (101 MHz, CDCl₃):** δ 195.6, 160.1, 147.9, 137.3, 134.9, 126.1, 123.4, 122.5, 122.4, 120.5, 34.6, 31.5, 15.4, 12.9. **IR (ν̄ cm⁻¹):** 3236, 2959, 1619, 1510, 1361, 1267, 823, 769; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₉H₂₄N₂O₂: 313.1916; Found: 313.1923.

Reduction of pyrrole-4-acetyl to pyrrole-4-ethyl group (Series 2):

BF₃.Et₂O reduction- General procedure B: A modified literature protocol was followed.⁵³ To a solution of the 4-acetylpyrrole (0.3 mmol, 1 eq.) in THF (2 mL) was added NaBH₄ (2.5 eq.) and the solution cooled to below 0 °C in a freezing mixture (ice + NaCl) bath. Then, boron trifluoride etherate (3.5 eq.) was added dropwise such that the temperature in the reaction vessel does not exceed 0 °C. The reaction mixture was then warmed to rt and stirred 2-4 h, allowing the reaction to reach completion (monitored through TLC). The mixture was then quenched with slow addition of water (0.2 mL) followed by conc. HCl (0.07 mL). The mixture was then neutralized using sat. NaHCO₃ solution and the THF was removed *in vacuo*. The residual water layer was then extracted with DCM (x2), organic layers combined, and washed with water (x2) and brine. The organic layer

was dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The residue was purified by silica gel column chromatography.

4-Ethyl-*N*-hexyl-3,5-dimethyl-1*H*-pyrrole-2-carboxamide (2a)



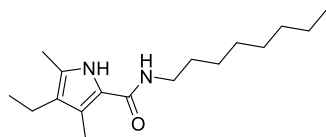
Eluent for column chromatography: 20% EtOAc in petroleum ether;

Physical appearance: White solid; Yield = 92%; **MP:** 67.3-69.1 °C; **^1H**

NMR (400 MHz, CDCl_3): δ 8.97 (s, 1H), 5.63 (s, 1H), 3.42 (dd, J = 13.0,

6.8 Hz, 2H), 2.38 (q, J = 7.5 Hz, 2H), 2.23 (s, 3H), 2.19 (s, 3H), 1.63 – 1.52 (m, 2H), 1.44 – 1.21 (m, 8H), 1.04 (t, J = 7.5 Hz, 3H), 0.89 (t, J = 6.6 Hz, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 162.3, 127.1, 123.1, 120.6, 118.2, 39.5, 31.6, 30.1, 26.8, 22.7, 17.4, 15.8, 14.2, 11.3, 11.1. **IR ($\tilde{\nu}$ cm^{-1}):** 3334, 3182, 2955, 2920, 2853, 1606, 1576, 1320, 1129, 720; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_{19}\text{H}_{24}\text{N}_2\text{O}_2$: 251.2123; Found: 251.2122.

4-Ethyl-3,5-dimethyl-*N*-octyl-1*H*-pyrrole-2-carboxamide (2b)



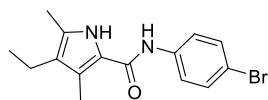
Eluent for column chromatography: 5%→8% EtOAc in petroleum

ether; **Physical appearance:** White solid; Yield = 74%; **MP:**

98.9-101.3 °C; **^1H NMR (400 MHz, CDCl_3):** δ 9.06 (s, 1H), 5.64 (s,

1H), 3.42 (dd, J = 12.9, 7.1 Hz, 2H), 2.38 (q, J = 7.5 Hz, 2H), 2.23 (s, 3H), 2.19 (s, 3H), 1.63 – 1.51 (m, 2H), 1.37 – 1.23 (m, 10H), 1.04 (t, J = 7.5 Hz, 3H), 0.88 (t, J = 6.9 Hz, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 162.3, 127.0, 123.1, 120.6, 118.2, 39.6, 31.9, 30.1, 29.4, 29.4, 27.2, 22.8, 17.4, 15.8, 14.2, 11.3, 11.1. **IR ($\tilde{\nu}$ cm^{-1}):** 3235, 2958, 2922, 2850, 1580, 1540, 1320, 1291, 1131, 747; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_{17}\text{H}_{30}\text{N}_2\text{O}$: 279.2436; Found: 279.2438.

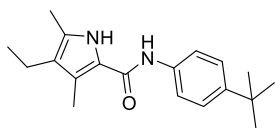
N-(4-Bromophenyl)-4-ethyl-3,5-dimethyl-1*H*-pyrrole-2-carboxamide (2c)



Eluent for column chromatography: 10% EtOAc in petroleum ether;

Physical appearance: White solid; Yield = 66%; **MP:** 175.2-176.6 °C; **^1H**

NMR (400 MHz, CDCl_3): δ 8.97 (s, 1H), 7.51 – 7.41 (m, 4H), 7.37 (s, 1H), 2.41 (q, J = 7.6 Hz, 2H), 2.37 (s, 3H), 2.21 (s, 3H), 1.06 (t, J = 7.6 Hz, 3H). **^{13}C NMR (101 MHz, CDCl_3):** δ 160.0, 137.5, 132.1, 129.1, 124.0, 121.6, 120.3, 119.6, 116.4, 17.4, 15.8, 11.4, 11.3. **IR ($\tilde{\nu}$ cm^{-1}):** 3298, 3274, 2963, 1640, 1507, 1483, 1428, 1388, 1301, 1234, 1067, 826, 754, 733; **HRMS (ESI):** m/z $[\text{M}+\text{H}]^+$ Calc. for $\text{C}_{15}\text{H}_{17}\text{BrN}_2\text{O}$: 321.0602; Found: 321.0604.

***N*-(4-(*tert*-Butyl)phenyl)-4-ethyl-3,5-dimethyl-1*H*-pyrrole-2-carboxamide (2d)**

Eluent for column chromatography: 15% EtOAc in petroleum ether;

Physical appearance: White solid; Yield = 84%; **MP:** decomposes above 169 °C without melting; **¹H NMR (400 MHz, CDCl₃):** δ 8.96 (s, 1H), 7.52

– 7.45 (m, 2H), 7.40 – 7.33 (m, 3H), 2.45 – 2.38 (m, 2H), 2.36 (s, 3H), 2.21 (s, 3H), 1.32 (s, 9H), 1.06 (t, *J* = 7.6 Hz, 3H). **¹³C NMR (101 MHz, CDCl₃):** δ 160.1, 147.0, 135.6, 128.3, 126.0, 123.7, 120.6, 120.0, 119.1, 34.5, 31.5, 17.4, 15.8, 11.4, 11.2. **IR (ν̄ cm⁻¹):** 3273, 2957, 2925, 2863, 1628, 1518, 1428, 1241, 834, 759; **HRMS (ESI):** *m/z* [M+H]⁺ Calc. for C₁₉H₂₆N₂O: 299.2123; Found: 299.2127.

5.4.4 Ion Transport Experiments

Study of ion transport across EYPC-LUVs⊃HPTS:

Preparation of EYPC-LUVs⊃HPTS in NaCl as per **Prep protocol P1**; HPTS assay as per **Assay protocol A1**.

Ion selectivity studies across EYPC-LUVs⊃HPTS (varying both intra and extravesicular ions):

EYPC-LUVs⊃HPTS entrapping various MX salts were prepared as per **Prep protocol P1**.

Anion selectivity assay (Assay protocol A13): The EYPC-LUVs⊃HPTS (Inside: 1 mM HPTS, 10 mM HEPES, 100 mM NaX or 33.3 mM Na₂SO₄, pH = 7.0) were prepared as per the procedure given above. In a clean, dry fluorescence cuvette, 1975 μL of HEPES buffer (10 mM HEPES, 100 mM NaX or 33.3 mM Na₂SO₄, pH = 7.0, X⁻ = Cl⁻, Br⁻, NO₃⁻, and ClO₄⁻) was added, followed by 25 μL of EYPC-LUVs⊃HPTS. The cuvette was placed with a slow stirring condition in a magnetic stirrer-equipped fluorescence instrument (*t* = 0 s). The time-dependent fluorescence emission intensity of HPTS was monitored at λ_{em} = 510 nm (λ_{ex} = 450 nm). A pH gradient (ΔpH = 0.8) was created across the intra- and extra-vesicular system by the addition of 20 μL of 0.5 M NaOH at *t* = 20 s. The transporter **2c** (0.2 μM) was added at *t* = 100 s, and finally, the vesicles were lysed by the addition of 25 μL 10% Triton X-100 at *t* = 300 s to destroy the pH gradient. The collected data was then normalized to the percentage change in fluorescence intensity with respect to time using Equation 1.

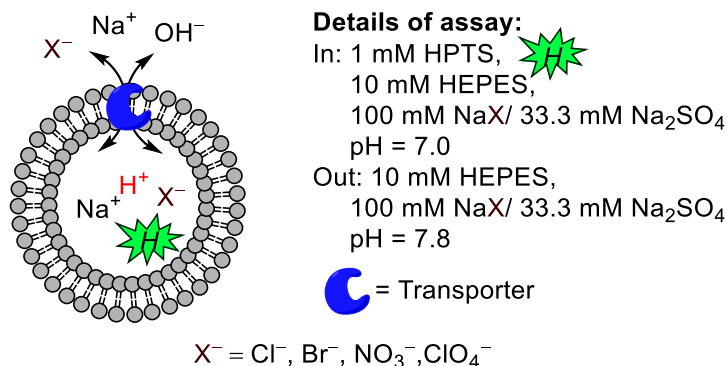


Figure 28: Schematic of fluorescence-based anion selectivity assay (by varying extravesicular anions) using EYPC-LUVs $\supset$ HPTS.

Cation selectivity assay (Assay protocol A14): Similarly, the cation selectivity assay of **2c** (0.2 μ M) was performed by varying the extravesicular cation in the HEPES buffer (10 mM HEPES, 100 mM MCl, pH = 7.0, M⁺ = Li⁺, Na⁺, K⁺, Rb⁺, Cs⁺). The data was normalized to the percentage change in fluorescence intensity with respect to time using equation S1.

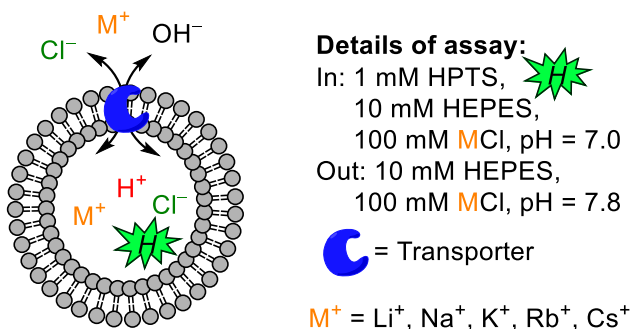


Figure 29: Schematic of fluorescence-based cation selectivity using EYPC-LUVs $\supset$ HPTS.

Study of chloride transport across EYPC-LUVs $\supset$ Lucigenin:

Preparation of EYPC-LUVs $\supset$ lucigenin as per **Prep protocol P2**; Chloride transport assay as per **Assay protocol A6**.

Cation selectivity assay across EYPC-LUVs $\supset$ Lucigenin: As per **Assay protocol A7**.

Valinomycin coupled lucigenin assay: As per **Assay protocol A8**.

Carboxyfluorescein (CF) leakage assay:

Preparation of EYPC-LUVs $\supset$ CF as per **Prep protocol P5**; Chloride transport assay as per **Assay protocol A12**.

5.4.5 Vesicle Size Measurement- DLS

The size distribution of vesicles prepared for anion selectivity assay was determined through DLS as per protocol in Chapter 2 (Section 2.4.7).

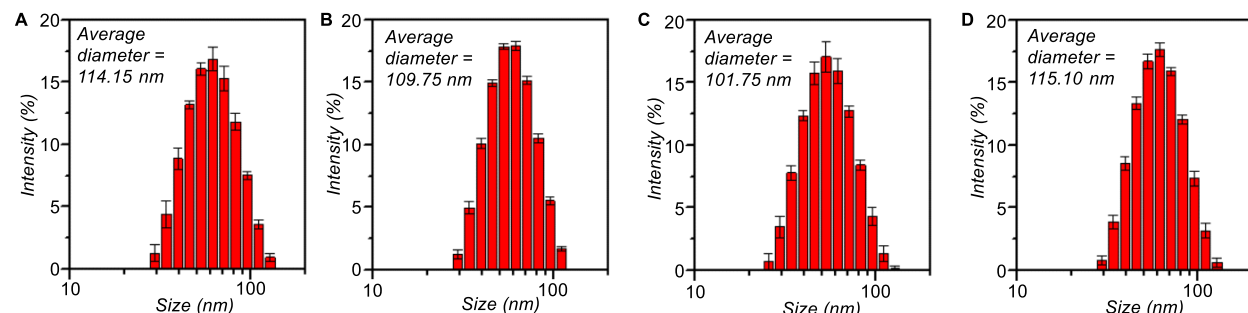


Figure 30: Size distributions of EYPC-LUVs with HPTS entrapping (A) NaBr (100 mM), (B) NaNO₃ (100 mM), (C) NaClO₄ (100 mM), and (D) Na₂SO₄ (33.3 mM).

5.4.6 ¹H NMR Titration Studies

¹H NMR titration studies were conducted at ambient temperature on a Bruker Avance III HD 400 MHz spectrometer. The titrations with anion were conducted by the addition of aliquots of the respective tetrabutylammonium (TBA) solution (0.2 M in CDCl₃) to a solution of the receptor (**2c**) in CDCl₃ (0.002 M). The spectra were calibrated using the residual signal from CDCl₃ (δ_H = 7.26 ppm) as internal reference. Tetrabutylammonium chloride (TBACl), tetrabutylammonium bromide (TBABr) and tetrabutylammonium nitrate (TBANO₃) solutions were used for Cl⁻, Br⁻ and NO₃⁻ titrations. The TBA salts were dried under vacuum in a desiccator prior to use.

5.4.7 SCXRD

The compounds were crystallized from their organic solvent solutions at ambient temperature. The crystals obtained were diffracted on Bruker diffractometers using Mo (Mo-K α = 0.71073 Å) as the X-ray source. Sample temperatures were maintained constant throughout the data collection using Cryostream coolers (Oxford Cryosystems).

Single crystal data for **1c** (CCDC 2241948)

The transporter **1c** was crystallized from its saturated solution in EtOAc in a conical flask to yield plate-like crystals. Single crystal X-ray diffraction data was collected on a Bruker KAPPA APEX single crystal X-ray diffractometer with Mo (Mo-K α = 0.71073 Å) ceramic sealed tube source and Apex II CCD detector. The data collection temperature was maintained at 150 K.

Table 3: Crystal data of **1c**

Empirical formula	C15 H15 Br N2 O2
Formula weight	335.20
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	Triclinic
Space group	P -1
Unit cell dimensions	a = 7.588(4) Å α = 67.863(17)°. b = 13.617(8) Å β = 80.098(17)°. c = 14.984(9) Å γ = 77.161(16)°.
Volume	1391.6(14) Å ³
Z	4
Density (calculated)	1.600 Mg/m ³
Absorption coefficient	2.957 mm ⁻¹
F(000)	680
Crystal size	0.559 x 0.254 x 0.101 mm ³
Theta range for data collection	1.474 to 29.574°.
Index ranges	-9 ≤ h ≤ 10, -18 ≤ k ≤ 18, -20 ≤ l ≤ 20
Reflections collected	22357
Independent reflections	7792 [R(int) = 0.0882]
Completeness to theta = 29.574°	99.8 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7462 and 0.5360
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	7792 / 0 / 368
Goodness-of-fit on F ²	0.963
Final R indices [I > 2σ(I)]	R1 = 0.0509, wR2 = 0.1003
R indices (all data)	R1 = 0.1174, wR2 = 0.1196
Extinction coefficient	0.0007(3)
Largest diff. peak and hole	0.620 and -0.804 e.Å ⁻³

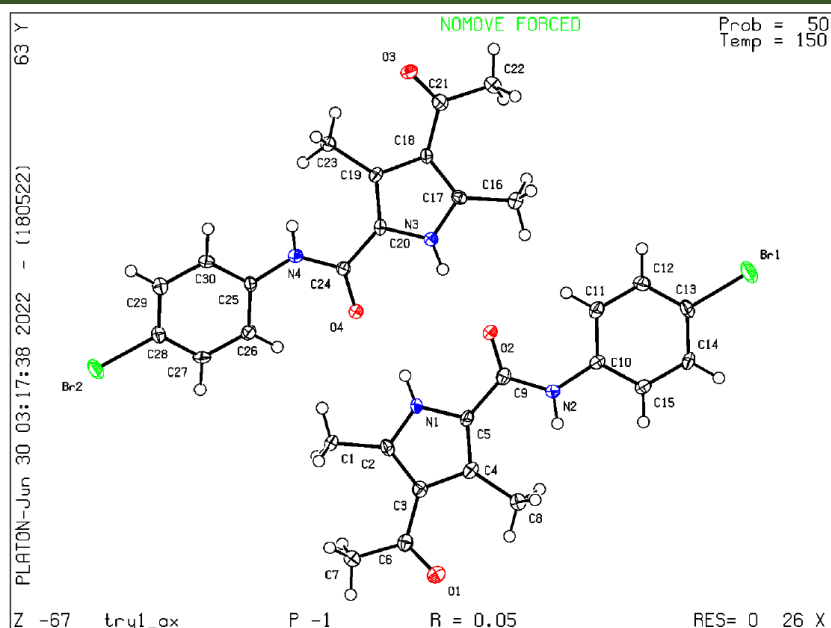


Figure 31: ORTEP diagram of **1c**.

Single crystal data for **1c** + TBACl cocrystal (CCDC 2241950)

1c and TBACl were cocrystallized from their acetonitrile solution in a crystallization tube. Single crystal X-ray diffraction data of the co-crystal of **1c** with TBACl was collected on a Bruker D8 VENTURE single crystal X-ray diffractometer with Mo (Mo-K α = 0.71073 Å) microfocus X-ray (μ S) source and PHOTON 100 CMOS detector. The data collection temperature was maintained at 150 K using a Cryostream cooler (Oxford Cryosystems).

Table 4: Crystal data of **1c** + TBACl cocrystal

Empirical formula	C ₃₁ H ₅₁ Br Cl N ₃ O ₂
Formula weight	613.10
Temperature	150(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P2 ₁ /c
Unit cell dimensions	a = 7.5114(14) Å α = 90°. b = 23.535(4) Å β = 90.010(10)°. c = 18.064(3) Å γ = 90°.
Volume	3193.4(10) Å ³
Z	4

Density (calculated)	1.275 Mg/m ³
Absorption coefficient	1.402 mm ⁻¹
F(000)	1304
Crystal size	0.470 x 0.290 x 0.110 mm ³
Theta range for data collection	2.065 to 24.999°.
Index ranges	-8 ≤ h ≤ 8, -27 ≤ k ≤ 27, -20 ≤ l ≤ 21
Reflections collected	63359
Independent reflections	5609 [R(int) = 0.2346]
Completeness to theta = 24.999°	100.0 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.88 and 0.65
Refinement method	Full-matrix least-squares on F ²
Data / restraints / parameters	5609 / 636 / 359
Goodness-of-fit on F ²	1.082
Final R indices [I > 2σ(I)]	R1 = 0.0842, wR2 = 0.1778
R indices (all data)	R1 = 0.1339, wR2 = 0.2097
Extinction coefficient	n/a
Largest diff. peak and hole	1.162 and -1.495 e.Å ⁻³

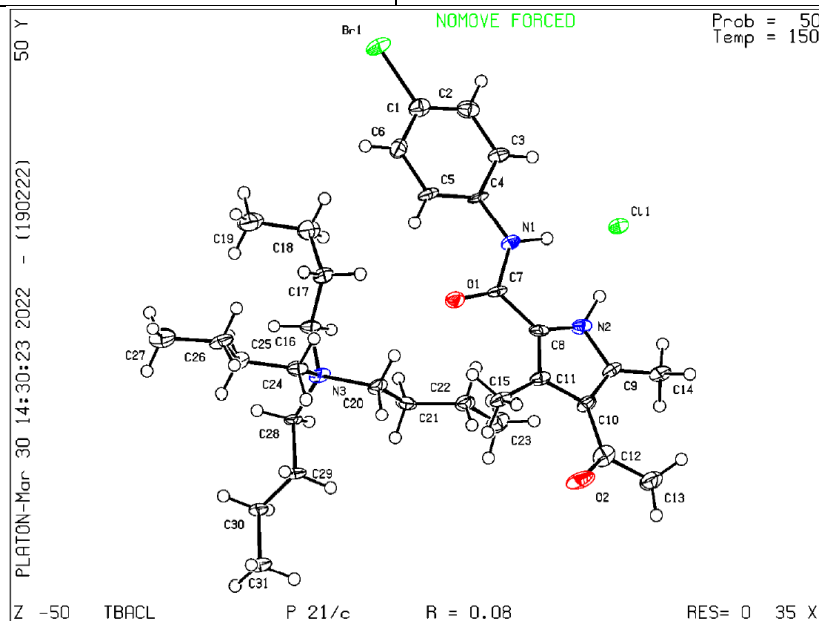


Figure 32: ORTEP diagram of **1c** + **TBACl** cocrystal.

5.4.8 Theoretical calculations

Initial prediction of the most probable geometry of molecules/complexes were first conducted with CONFLEX 8 conformation search software package^{54, 55} using MMFF94S (2010-12-04HG) force field with a search limit of 10 kcal/mol. The output conformation with the highest predicted Boltzmann population was then used for geometry optimization. The DFT geometry optimization and energy calculation of the initial model obtained/constructed was conducted on Gaussian 09 program suite⁵⁶ the B3LYP exchange-correlation functional^{57, 58} and the 6-31G(d,p) basis set.^{59, 60} The zero-point energy (ZPE) and basis set superposition error (BSSE) of each complex were calculated and used in equation 5 to obtain the binding energy (BE).

For 2c (free transporter)

Table 5: Atomic coordinates of **2c** after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p) functional basis set.

Charge = 0

Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	5.86006	1.99184	-4.32E-4
2	C	4.68653	1.06891	-0.09096
3	N	3.40936	1.52935	-0.00549
4	C	2.50759	0.49372	-0.09587
5	C	3.24427	-0.68579	-0.24537
6	C	4.62258	-0.31815	-0.24279
7	C	5.80192	-1.248	-0.35084
8	C	6.23545	-1.86648	0.99069
9	C	2.71371	-2.08493	-0.3975
10	C	1.09394	0.88111	-0.0281
11	O	0.77953	2.0709	0.07778
12	N	0.17453	-0.14779	-0.08516
13	C	-1.22861	-0.0752	-0.04418
14	C	-1.94366	1.13066	0.04517
15	C	-3.33697	1.10979	0.0773

16	C	-4.02299	-0.1003	0.02175
17	C	-3.32874	-1.30463	-0.06658
18	C	-1.93855	-1.28655	-0.09932
19	Br	-5.93545	-0.11379	0.06773
20	H	6.08906	2.2676	1.03673
21	H	6.75397	1.52308	-0.41842
22	H	5.68178	2.91964	-0.55509
23	H	3.09225	2.48373	0.09453
24	H	5.57474	-2.05358	-1.06031
25	H	6.6525	-0.70963	-0.78571
26	H	7.09533	-2.53215	0.85905
27	H	6.51497	-1.08843	1.70821
28	H	5.42352	-2.4482	1.439
29	H	3.52638	-2.79431	-0.56939
30	H	2.18355	-2.43144	0.49992
31	H	2.0304	-2.18088	-1.25145
32	H	0.55219	-1.08063	-0.15122
33	H	-1.40318	2.06479	0.08995
34	H	-3.88619	2.04214	0.14624
35	H	-3.86522	-2.24559	-0.10941
36	H	-1.39794	-2.22761	-0.1686

For [2c+Cl⁻] (1:1) complex**Table 6:** Atomic coordinates of [2c+Cl⁻] after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p) functional basis set.

Charge = -1 Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	4.87319	2.2372	-0.08284
2	C	4.16224	0.91881	-0.12849

3	N	2.80799	0.86155	-0.07572
4	C	2.3873	-0.45291	-0.12837
5	C	3.5311	-1.25861	-0.21804
6	C	4.65452	-0.38694	-0.21821
7	C	6.1073	-0.77414	-0.27539
8	C	6.70199	-1.17222	1.08846
9	C	3.56819	-2.75704	-0.30522
10	C	0.98832	-0.89472	-0.10936
11	O	0.70387	-2.10123	-0.12703
12	N	0.02656	0.10142	-0.07232
13	C	-1.362	-0.05749	-0.03775
14	C	-2.02212	-1.30188	-0.04294
15	C	-3.41559	-1.35573	-0.00546
16	C	-4.1561	-0.18011	0.0361
17	C	-3.52462	1.06187	0.04141
18	C	-2.13631	1.12156	0.00492
19	Br	-6.08184	-0.26893	0.08766
20	H	5.38761	2.39635	0.8739
21	H	5.63074	2.31242	-0.87213
22	H	4.15888	3.05473	-0.21472
23	H	2.20246	1.7052	-0.02893
24	H	6.24641	-1.60881	-0.97596
25	H	6.69205	0.05959	-0.68584
26	H	7.76152	-1.44487	1.00235
27	H	6.61984	-0.34758	1.80438
28	H	6.16522	-2.02712	1.51246
29	H	4.5521	-3.10613	-0.63863
30	H	3.36047	-3.22634	0.66464
31	H	2.8038	-3.13208	-0.98886
32	H	0.32638	1.0861	-0.06478
33	H	-1.43348	-2.20681	-0.07622

34	H	-3.91833	-2.31715	-0.0092
35	H	-4.10815	1.97549	0.0742
36	H	-1.62619	2.08222	0.01002
37	Cl	0.84858	3.27886	-0.00677

For [(2c)₂+Cl⁻] (2:1) complex**Table 7:** Atomic coordinates of [(2c)₂+Cl⁻] after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p) functional basis set.

Charge = -1

Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	1.39457	-4.11621	1.25832
2	C	-0.09869	-4.20273	1.18511
3	N	-0.82798	-3.21283	0.60407
4	C	-2.17505	-3.52865	0.64433
5	C	-2.29361	-4.77178	1.27826
6	C	-0.97934	-5.19562	1.6195
7	C	-0.58818	-6.48148	2.29621
8	C	-0.48242	-7.68306	1.33898
9	C	-3.56786	-5.51581	1.55402
10	C	-3.26654	-2.70143	0.12356
11	O	-4.44111	-3.08236	0.19743
12	N	-2.91196	-1.48598	-0.44771
13	C	-3.75967	-0.5354	-1.03317
14	C	-5.16061	-0.65644	-1.09387
15	C	-5.92317	0.34422	-1.69558
16	C	-5.30274	1.46523	-2.23602
17	C	-3.91776	1.60493	-2.18669
18	C	-3.15265	0.60878	-1.58941
19	Br	-6.36738	2.83765	-3.06239

20	H	1.76716	-4.50678	2.21089
21	H	1.72751	-3.07883	1.16334
22	H	1.8812	-4.69033	0.45925
23	H	-0.40658	-2.35876	0.22417
24	H	-1.31133	-6.72164	3.08662
25	H	0.37488	-6.34845	2.80539
26	H	-0.19539	-8.59683	1.87335
27	H	0.26367	-7.49655	0.55946
28	H	-1.43832	-7.86878	0.83862
29	H	-4.30615	-4.87492	2.04256
30	H	-3.38154	-6.38903	2.18777
31	H	-4.04025	-5.86288	0.6282
32	H	-1.92518	-1.23257	-0.48481
33	H	-5.63038	-1.53193	-0.67096
34	H	-7.0024	0.24485	-1.73996
35	H	-3.43713	2.47925	-2.61136
36	H	-2.07104	0.70622	-1.55223
37	Cl	0.32463	-0.4524	-0.57202
38	C	-2.07316	1.73175	2.19056
39	C	-0.70839	2.30968	2.40181
40	N	0.35426	1.87999	1.66784
41	C	1.49577	2.57401	2.03213
42	C	1.13219	3.47724	3.03738
43	C	-0.26165	3.30785	3.2704
44	C	-1.11191	4.07654	4.24585
45	C	-1.61257	5.42908	3.70633
46	C	2.04325	4.43782	3.74496
47	C	2.83552	2.38083	1.47134
48	O	3.79046	3.06293	1.86308
49	N	2.96841	1.40589	0.49185
50	C	4.12978	1.04057	-0.20139

51	C	5.3959	1.61488	0.01941
52	C	6.4973	1.18486	-0.72007
53	C	6.34869	0.18714	-1.6771
54	C	5.10502	-0.39439	-1.91141
55	C	4.00355	0.03141	-1.1773
56	Br	7.8768	-0.397	-2.68998
57	H	-2.02507	0.65155	2.01967
58	H	-2.57915	2.17001	1.32113
59	H	-2.70851	1.90672	3.06292
60	H	0.28466	1.1426	0.95991
61	H	-0.54905	4.25121	5.17199
62	H	-1.97825	3.46946	4.53725
63	H	-2.22326	5.95505	4.4502
64	H	-2.21964	5.28869	2.80606
65	H	-0.77264	6.07774	3.43694
66	H	1.51164	4.96605	4.54298
67	H	2.45464	5.18341	3.05628
68	H	2.90705	3.92524	4.17742
69	H	2.14276	0.87685	0.21495
70	H	5.49818	2.38908	0.7652
71	H	7.47015	1.6316	-0.54548
72	H	4.99337	-1.17116	-2.65963
73	H	3.02887	-0.41476	-1.35574

For 2b (free transporter)**Table 8:** Atomic coordinates of **2b** after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p) functional basis set.

Charge = 0

Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	5.49349	2.53199	0.3571

2	C	4.56586	1.36251	0.25959
3	N	3.33375	1.47466	-0.30962
4	C	2.67885	0.26549	-0.30107
5	C	3.52947	-0.6646	0.29863
6	C	4.72353	0.03319	0.65517
7	C	5.94902	-0.55652	1.30194
8	C	6.93496	-1.19269	0.30486
9	C	3.25767	-2.12426	0.53715
10	C	1.33006	0.26638	-0.88668
11	O	0.87437	1.29636	-1.40061
12	N	0.6225	-0.90596	-0.85805
13	C	-0.75448	-0.96587	-1.33034
14	C	-1.78892	-0.53577	-0.28059
15	C	-3.2274	-0.60079	-0.80658
16	C	-4.27243	-0.15577	0.22401
17	C	-5.71254	-0.20799	-0.30057
18	C	-6.75646	0.24476	0.72734
19	C	-8.19687	0.19727	0.20294
20	C	-9.23251	0.65412	1.23464
21	H	6.27423	2.34582	1.09851
22	H	5.99238	2.74565	-0.59689
23	H	4.96095	3.44107	0.6587
24	H	2.89588	2.29281	-0.71007
25	H	6.47246	0.2204	1.87233
26	H	5.65146	-1.31026	2.04166
27	H	7.80567	-1.612	0.82099
28	H	6.45745	-1.9981	-0.26263
29	H	7.29217	-0.45191	-0.41768
30	H	4.1447	-2.63224	0.92296
31	H	2.45958	-2.28575	1.27526
32	H	2.96836	-2.64426	-0.38427

33	H	0.96596	-1.66187	-0.28681
34	H	-0.8213	-0.30992	-2.202
35	H	-0.95327	-1.99057	-1.666
36	H	-1.6903	-1.17185	0.61031
37	H	-1.5509	0.48755	0.03213
38	H	-3.3129	0.0289	-1.70324
39	H	-3.45381	-1.62635	-1.1332
40	H	-4.18938	-0.78604	1.12097
41	H	-4.0423	0.86779	0.55141
42	H	-5.79338	0.41965	-1.19949
43	H	-5.94395	-1.23248	-0.62589
44	H	-6.67785	-0.38311	1.6266
45	H	-6.52426	1.26874	1.05358
46	H	-8.27456	0.82406	-0.69588
47	H	-8.43009	-0.82609	-0.12142
48	H	-10.249	0.60804	0.83039
49	H	-9.20286	0.02493	2.13158
50	H	-9.04752	1.6869	1.55141

For [(2b)₄+Cl⁻] (4:1) complex

Table 9: Atomic coordinates of [(2b)₄+Cl]⁻ after geometry optimization by Gaussian 09 using B3LYP/6-31G(d,p).

Charge = -1 Multiplicity = 1

Atom #	Atom type	Coordinates (Angstroms)		
		X	Y	Z
1	C	1.05458	-2.49265	4.11604
2	C	1.85157	-2.61217	5.24959
3	C	2.75075	-1.50516	5.26077
4	C	2.48117	-0.73846	4.12723
5	N	1.44761	-1.36763	3.44455

6	C	-0.04295	-3.36832	3.59892
7	C	1.79622	-3.72251	6.26339
8	C	2.74131	-4.89809	5.95222
9	C	3.79066	-1.23636	6.31001
10	C	3.13396	0.50291	3.67469
11	O	3.90417	1.14697	4.39882
12	N	2.85106	0.93217	2.40128
13	C	3.40266	2.18425	1.89696
14	C	4.86057	2.10395	1.41999
15	C	5.1022	1.16575	0.23203
16	C	6.54478	1.23015	-0.28576
17	C	6.83502	0.28724	-1.46015
18	C	8.26584	0.42255	-1.9965
19	C	8.58332	-0.52564	-3.1589
20	C	10.00553	-0.36286	-3.70451
21	H	0.98242	-1.02345	2.61056
22	H	-0.562	-3.8685	4.42173
23	H	-0.78613	-2.79376	3.03863
24	H	0.33217	-4.15286	2.92896
25	H	2.04299	-3.32243	7.25508
26	H	0.77037	-4.10517	6.34043
27	H	2.6769	-5.67615	6.72207
28	H	2.49285	-5.35378	4.98814
29	H	3.78171	-4.56159	5.89737
30	H	4.51353	-2.05949	6.37878
31	H	4.32427	-0.31376	6.08502
32	H	3.33197	-1.13642	7.30261
33	H	2.26628	0.39032	1.77936
34	H	2.75554	2.51336	1.07702
35	H	3.33397	2.92549	2.69922
36	H	5.17179	3.12308	1.14727

37	H	5.48155	1.80387	2.27139
38	H	4.86127	0.13506	0.52203
39	H	4.41485	1.41775	-0.58822
40	H	6.76844	2.26393	-0.58913
41	H	7.23545	1.00506	0.54023
42	H	6.65787	-0.75023	-1.14578
43	H	6.12111	0.476	-2.27322
44	H	8.43369	1.45963	-2.32316
45	H	8.98165	0.24561	-1.17978
46	H	8.43705	-1.56303	-2.82786
47	H	7.85815	-0.36167	-3.96649
48	H	10.20186	-1.05513	-4.53045
49	H	10.17217	0.65455	-4.07755
50	H	10.75389	-0.55317	-2.92594
51	C	-1.09828	-4.02816	-1.43029
52	C	-1.97154	-5.11066	-1.38098
53	C	-2.91133	-4.85603	-0.33939
54	C	-2.58745	-3.61729	0.21937
55	N	-1.47788	-3.1391	-0.46487
56	C	0.09795	-3.77916	-2.29642
57	C	-1.941	-6.31971	-2.27685
58	C	-2.9114	-6.23512	-3.46973
59	C	-4.02764	-5.77309	0.06958
60	C	-3.27109	-2.8945	1.30487
61	O	-4.24524	-3.37554	1.902
62	N	-2.79568	-1.65109	1.651
63	C	-3.37112	-0.93338	2.78572
64	C	-4.69201	-0.2092	2.48177
65	C	-4.56314	1.01517	1.56927
66	C	-5.92057	1.62969	1.20278
67	C	-5.81954	2.90733	0.36038

68	C	-7.18369	3.46954	-0.05823
69	C	-7.08506	4.75424	-0.88999
70	C	-8.44583	5.28693	-1.34963
71	H	-0.96791	-2.28278	-0.26574
72	H	0.00636	-4.31471	-3.24485
73	H	0.21212	-2.71553	-2.52753
74	H	1.03385	-4.10368	-1.82467
75	H	-0.92358	-6.47577	-2.65543
76	H	-2.18116	-7.21586	-1.68966
77	H	-2.86629	-7.14073	-4.08626
78	H	-3.9438	-6.11008	-3.12791
79	H	-2.67155	-5.37768	-4.10705
80	H	-3.67503	-6.8097	0.14188
81	H	-4.44096	-5.46417	1.0285
82	H	-4.84509	-5.76704	-0.66361
83	H	-2.00296	-1.23606	1.17871
84	H	-3.54311	-1.66028	3.58526
85	H	-2.61701	-0.2187	3.13451
86	H	-5.38181	-0.94161	2.04832
87	H	-5.13207	0.10049	3.44079
88	H	-3.93856	1.77495	2.0604
89	H	-4.03386	0.74069	0.64805
90	H	-6.51519	0.88059	0.65972
91	H	-6.48201	1.84642	2.12417
92	H	-5.26676	3.67044	0.92605
93	H	-5.22079	2.71496	-0.53883
94	H	-7.72591	2.70733	-0.63708
95	H	-7.79605	3.6603	0.83599
96	H	-6.57223	5.52656	-0.30011
97	H	-6.44602	4.56887	-1.76279
98	H	-8.34112	6.20563	-1.93704

99	H	-8.96768	4.55203	-1.97376
100	H	-9.09512	5.51092	-0.49472
101	C	-0.98638	3.604	1.51164
102	C	-1.53967	4.82504	1.13232
103	C	-2.15739	4.63684	-0.13932
104	C	-1.96437	3.30097	-0.50089
105	N	-1.24849	2.69905	0.52395
106	C	-0.25623	3.20801	2.75713
107	C	-1.51676	6.09491	1.93894
108	C	-2.79429	6.33137	2.76546
109	C	-2.87624	5.69123	-0.92981
110	C	-2.43316	2.6158	-1.71593
111	O	-3.13248	3.19687	-2.55853
112	N	-2.08723	1.29691	-1.89226
113	C	-2.44461	0.60767	-3.13123
114	C	-3.90675	0.1408	-3.20159
115	C	-4.25555	-1.0317	-2.27916
116	C	-5.75174	-1.37267	-2.29248
117	C	-6.1152	-2.61397	-1.46893
118	C	-7.62136	-2.89933	-1.42683
119	C	-7.98458	-4.14746	-0.61309
120	C	-9.49226	-4.40897	-0.54316
121	H	-0.88012	1.75195	0.52905
122	H	0.42697	2.37337	2.57508
123	H	0.33537	4.04201	3.14584
124	H	-0.94315	2.89951	3.55615
125	H	-0.6524	6.08713	2.61463
126	H	-1.36601	6.95328	1.27093
127	H	-2.73559	7.26856	3.33151
128	H	-3.6755	6.38204	2.11784
129	H	-2.95587	5.51465	3.47675

130	H	-2.43741	6.67975	-0.75232
131	H	-2.84652	5.46416	-1.99512
132	H	-3.93587	5.75273	-0.64943
133	H	-1.45356	0.83196	-1.2551
134	H	-2.25321	1.29067	-3.9654
135	H	-1.76789	-0.24809	-3.23333
136	H	-4.54271	1.00599	-2.9858
137	H	-4.11603	-0.145	-4.24253
138	H	-3.67532	-1.91695	-2.5759
139	H	-3.94853	-0.80113	-1.25116
140	H	-6.31792	-0.50683	-1.91903
141	H	-6.08206	-1.51977	-3.33198
142	H	-5.58965	-3.48558	-1.88315
143	H	-5.74123	-2.50443	-0.44304
144	H	-8.14306	-2.02894	-1.00207
145	H	-8.00594	-3.00911	-2.45218
146	H	-7.48254	-5.02163	-1.04984
147	H	-7.57917	-4.04575	0.40162
148	H	-9.71877	-5.30794	0.0405
149	H	-10.01849	-3.56866	-0.07513
150	H	-9.91924	-4.54606	-1.54402
151	C	3.48422	-3.01966	-0.46331
152	C	4.3693	-3.5607	-1.39135
153	C	4.35519	-2.71345	-2.54111
154	C	3.45856	-1.677	-2.28117
155	N	2.94241	-1.88989	-1.01168
156	C	3.11234	-3.46729	0.91519
157	C	5.21043	-4.79486	-1.20863
158	C	6.64412	-4.50672	-0.72656
159	C	5.14522	-2.932	-3.79855
160	C	3.06349	-0.56704	-3.16653

161	O	3.65909	-0.33883	-4.22825
162	N	1.99558	0.20675	-2.78208
163	C	1.4858	1.25317	-3.66358
164	C	2.17836	2.61626	-3.51268
165	C	1.9126	3.31234	-2.17423
166	C	2.56484	4.69719	-2.06968
167	C	2.28991	5.40429	-0.737
168	C	2.90672	6.80563	-0.64546
169	C	2.64217	7.50456	0.69367
170	C	3.2587	8.90418	0.7804
171	H	2.23396	-1.32903	-0.54902
172	H	2.02522	-3.49911	1.04739
173	H	3.50163	-4.46945	1.10961
174	H	3.51073	-2.80152	1.69005
175	H	4.7261	-5.47325	-0.49538
176	H	5.26062	-5.34639	-2.15656
177	H	7.22219	-5.43221	-0.61871
178	H	7.17298	-3.85876	-1.43307
179	H	6.63418	-3.99708	0.24243
180	H	6.18774	-2.60881	-3.68148
181	H	5.16842	-3.99517	-4.06714
182	H	4.72238	-2.35724	-4.62186
183	H	1.4802	0.00963	-1.93405
184	H	1.61003	0.9015	-4.69143
185	H	0.41453	1.35916	-3.46268
186	H	3.25467	2.47475	-3.66701
187	H	1.82616	3.26036	-4.3312
188	H	0.82893	3.41384	-2.02368
189	H	2.27444	2.67501	-1.35661
190	H	3.65063	4.60226	-2.21632
191	H	2.20278	5.32817	-2.89468

192	H	1.2052	5.47064	-0.57747
193	H	2.67692	4.78766	0.08635
194	H	3.99233	6.73909	-0.80981
195	H	2.5157	7.4298	-1.46241
196	H	1.55832	7.57043	0.85853
197	H	3.03387	6.88155	1.50913
198	H	3.05377	9.37587	1.74755
199	H	4.34696	8.86595	0.65348
200	H	2.86018	9.56178	-0.00113
201	Cl	0.22871	-0.33598	0.33379

5.5 Characterization Spectra

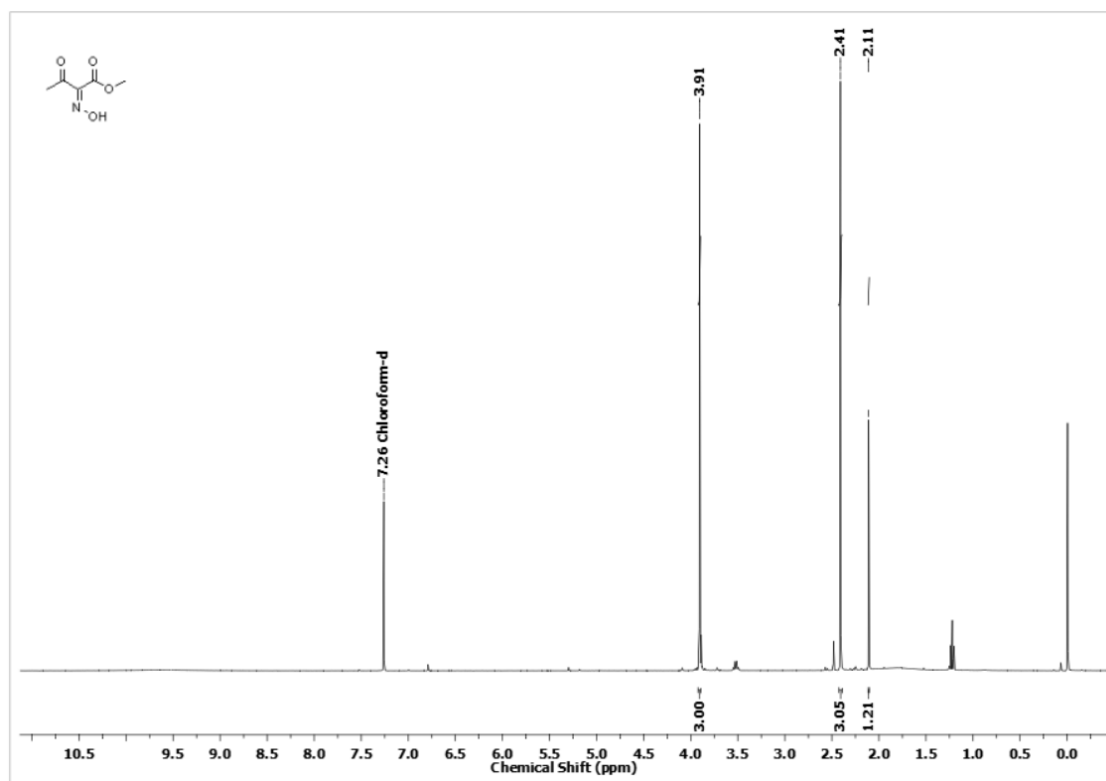


Figure 33: 400 MHz ^1H NMR spectrum of 5 in CDCl_3

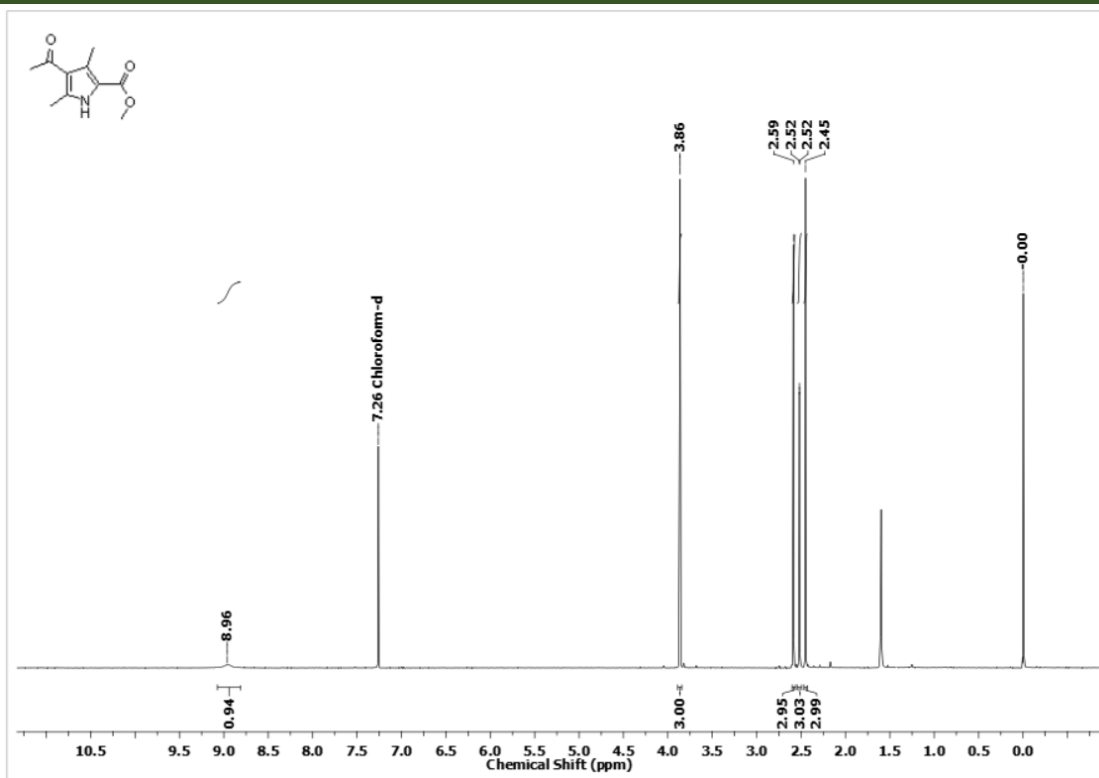


Figure 34: 400 MHz ^1H NMR spectrum of **6** in CDCl_3

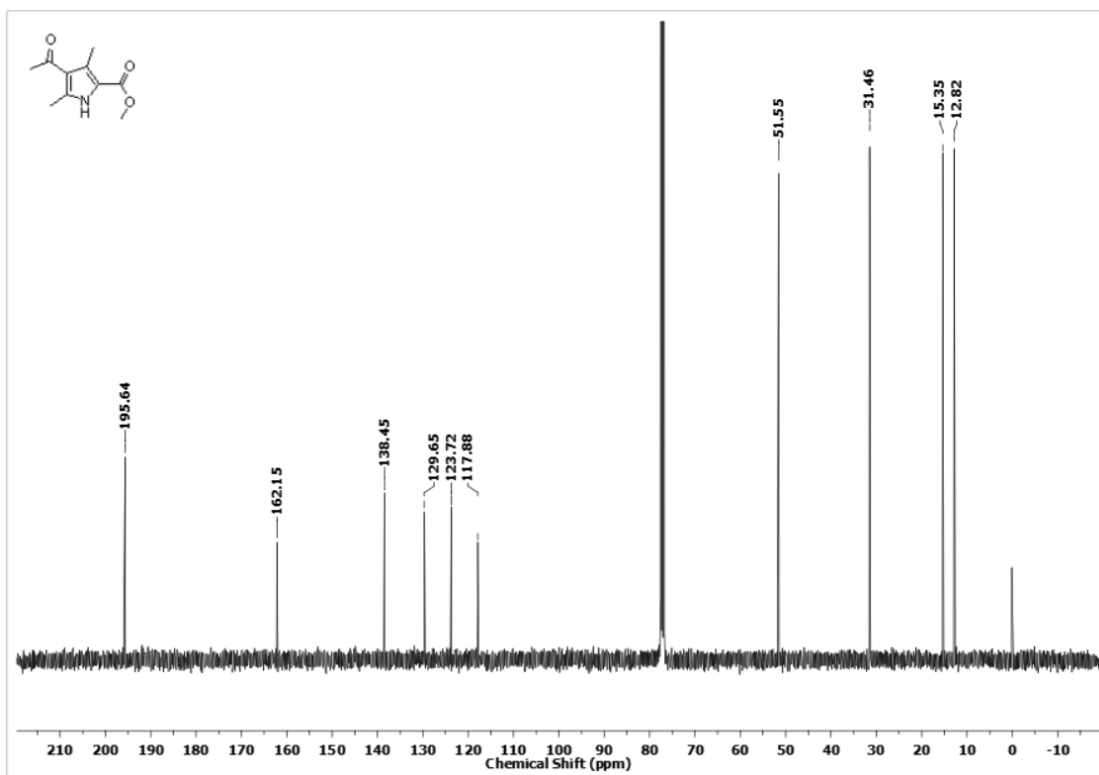


Figure 35: 101 MHz ^{13}C NMR spectrum of **6** in CDCl_3

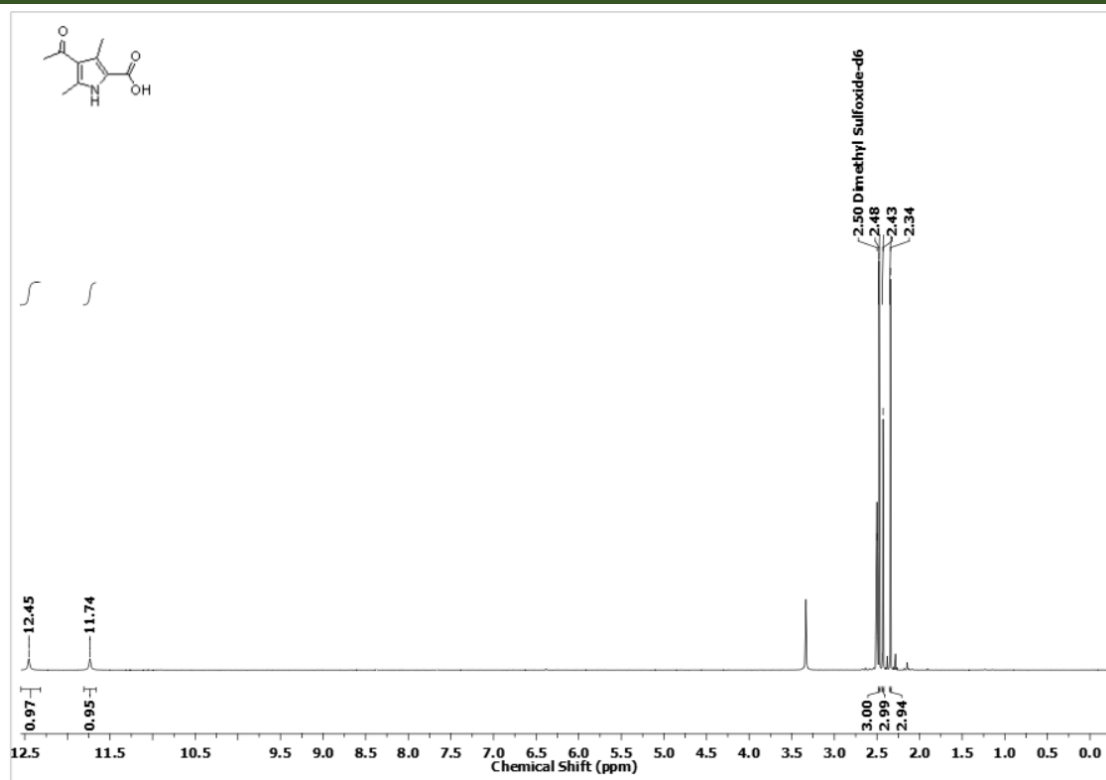


Figure 36: 400 MHz ^1H NMR spectrum of **3** in CDCl_3

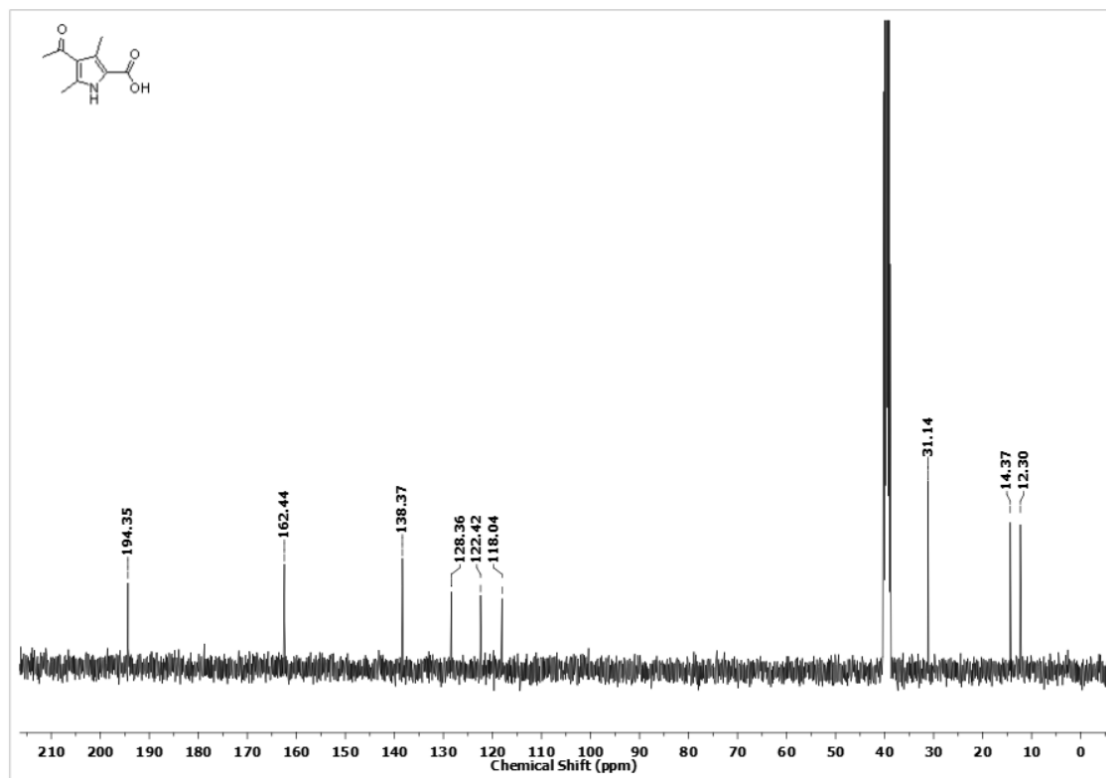


Figure 37: 101 MHz ^{13}C NMR spectrum of **3** in CDCl_3

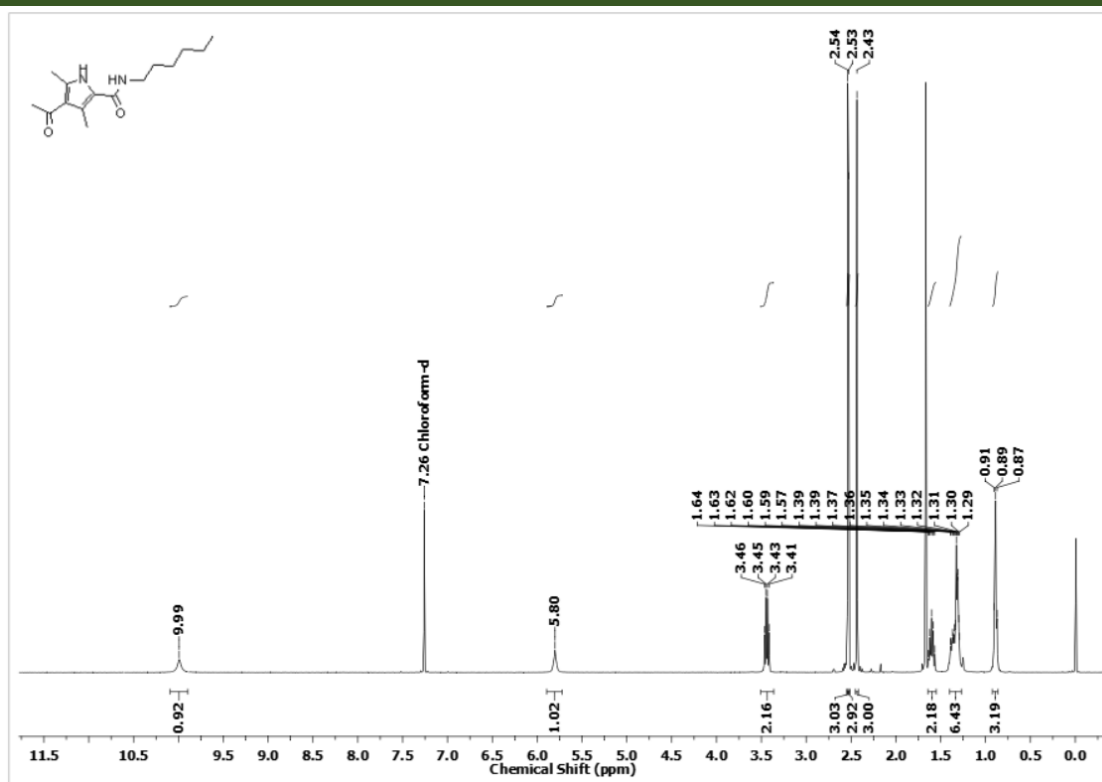


Figure 38: 400 MHz ^1H NMR spectrum of **1a** in CDCl_3

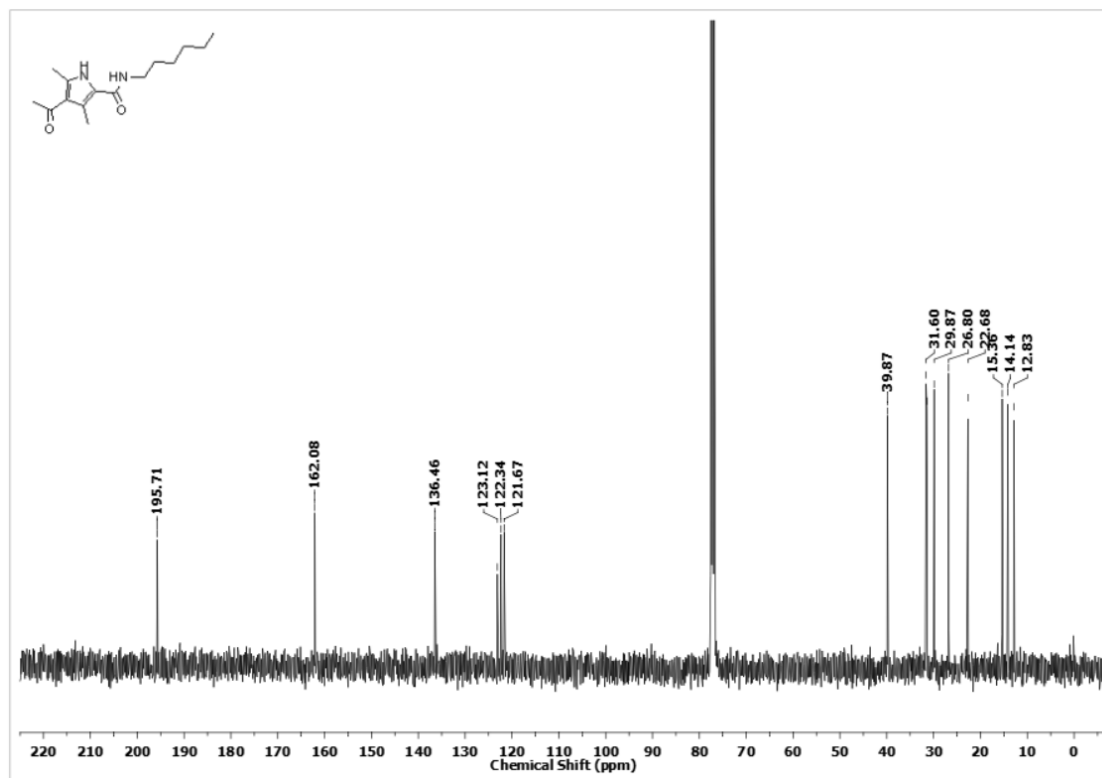


Figure 39: 101 MHz ^{13}C NMR spectrum of **1a** in CDCl_3

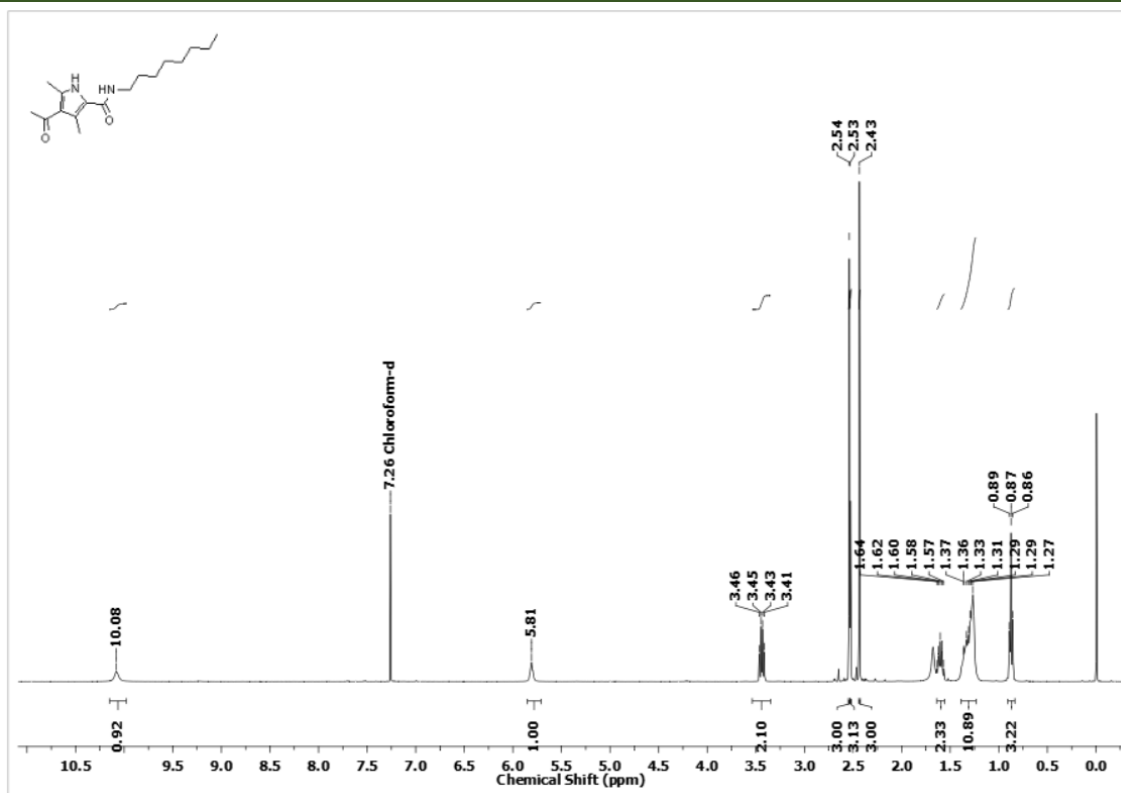


Figure 40: 400 MHz ^1H NMR spectrum of **1b** in CDCl_3

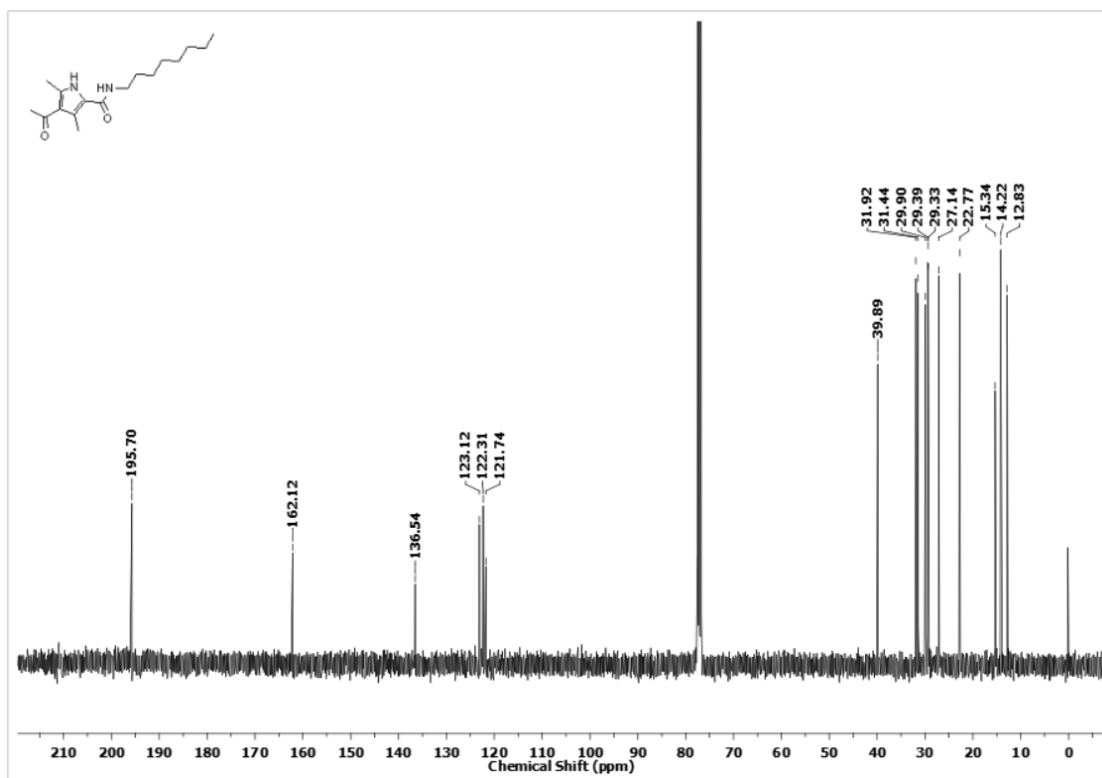


Figure 41: 101 MHz ^{13}C NMR spectrum of **1b** in CDCl_3

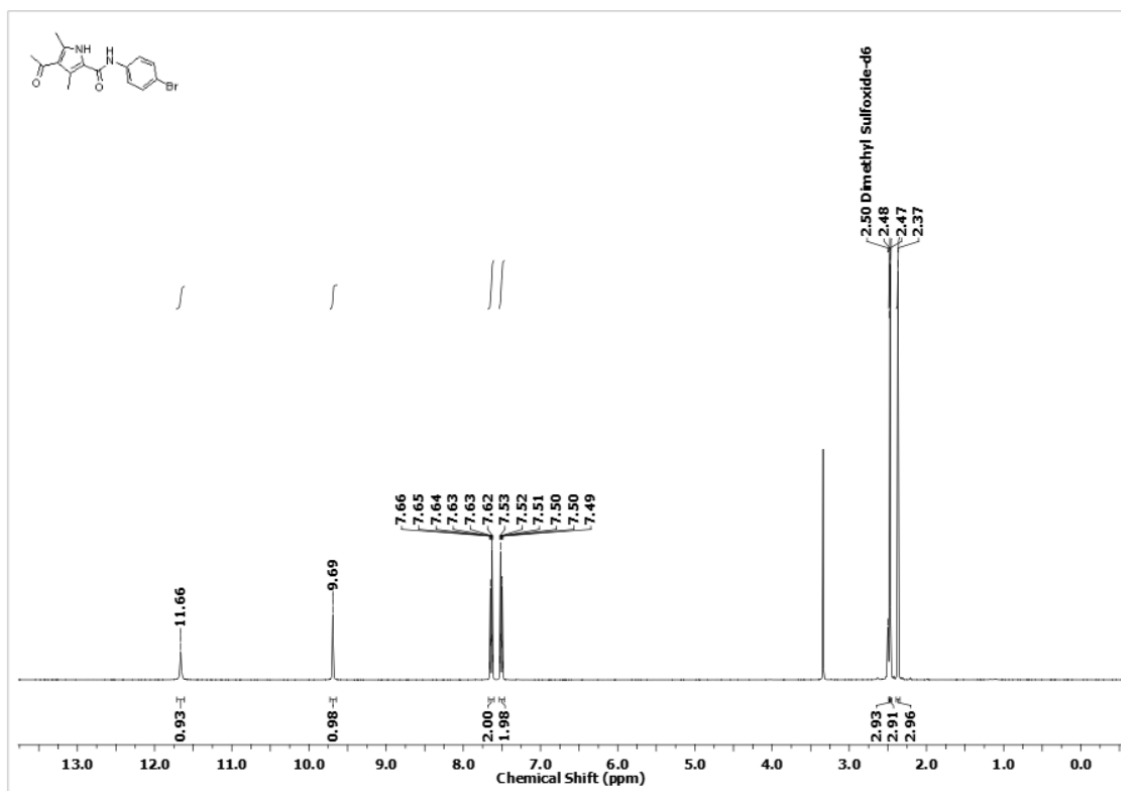


Figure 42: 400 MHz ^1H NMR spectrum of **1c** in $\text{DMSO}-d_6$

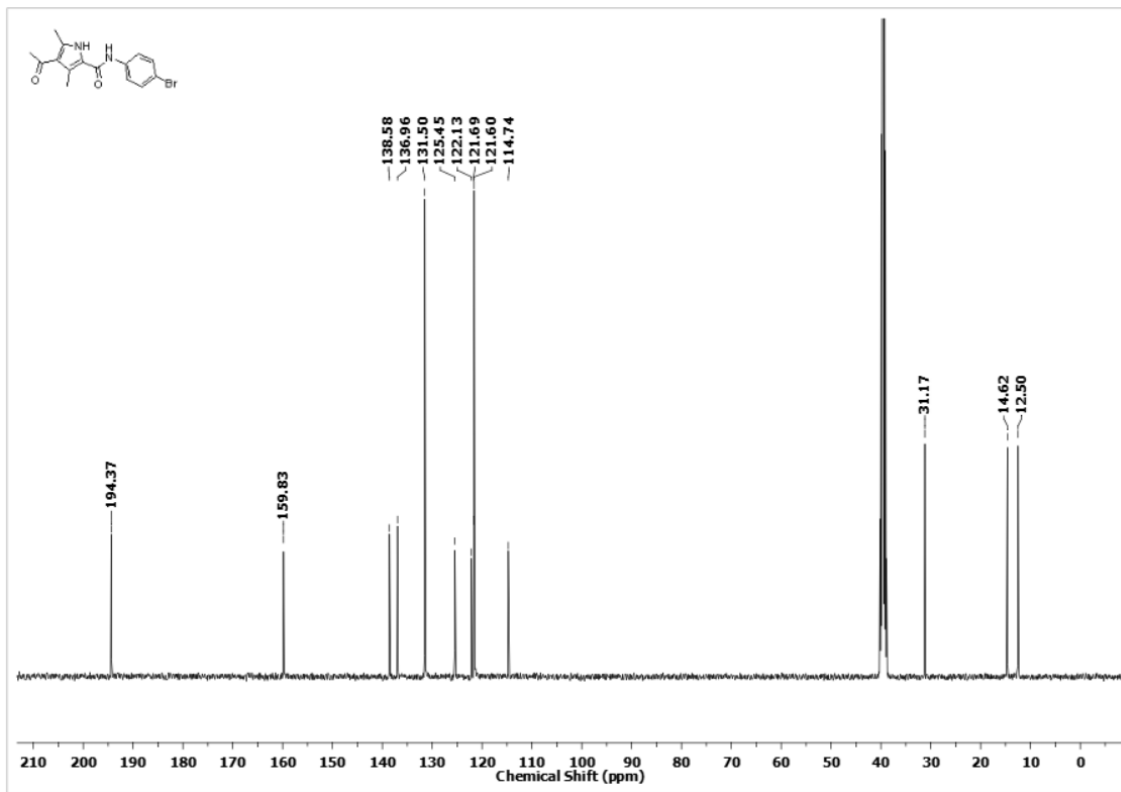


Figure 43: 101 MHz ^{13}C NMR spectrum of **1c** in $\text{DMSO}-d_6$

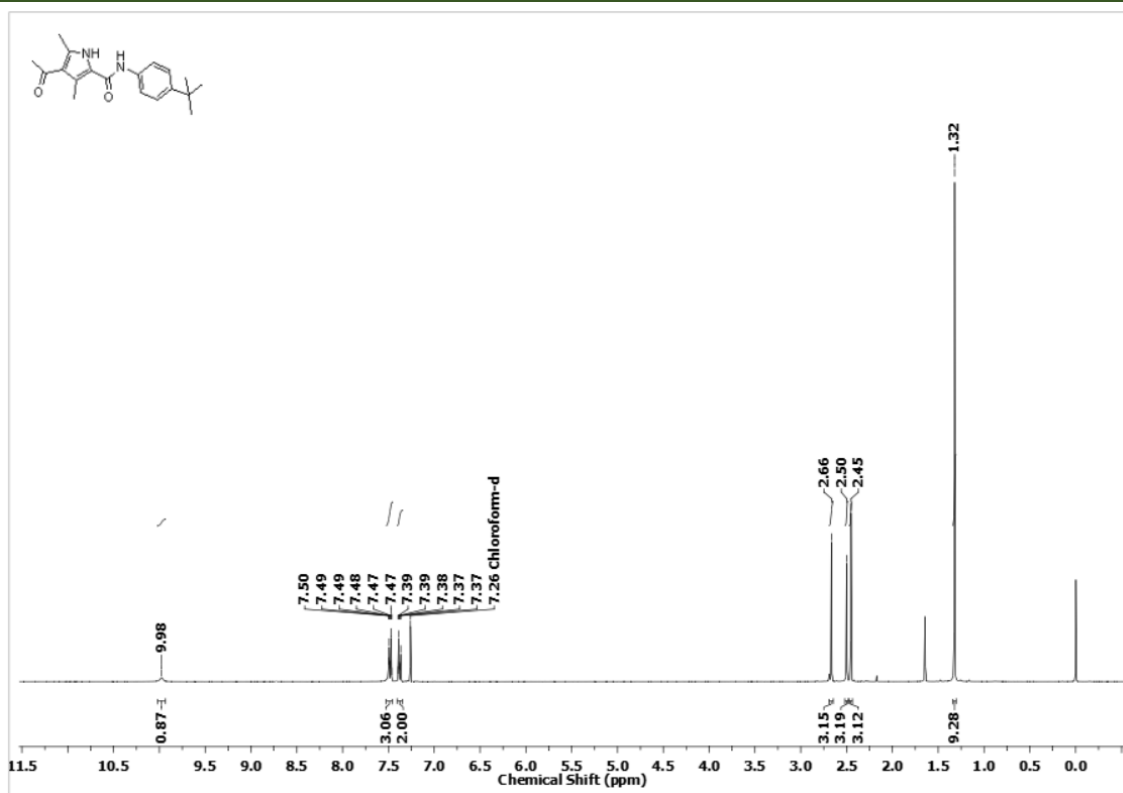


Figure 44: 400 MHz ¹H NMR spectrum of **1d** in CDCl₃

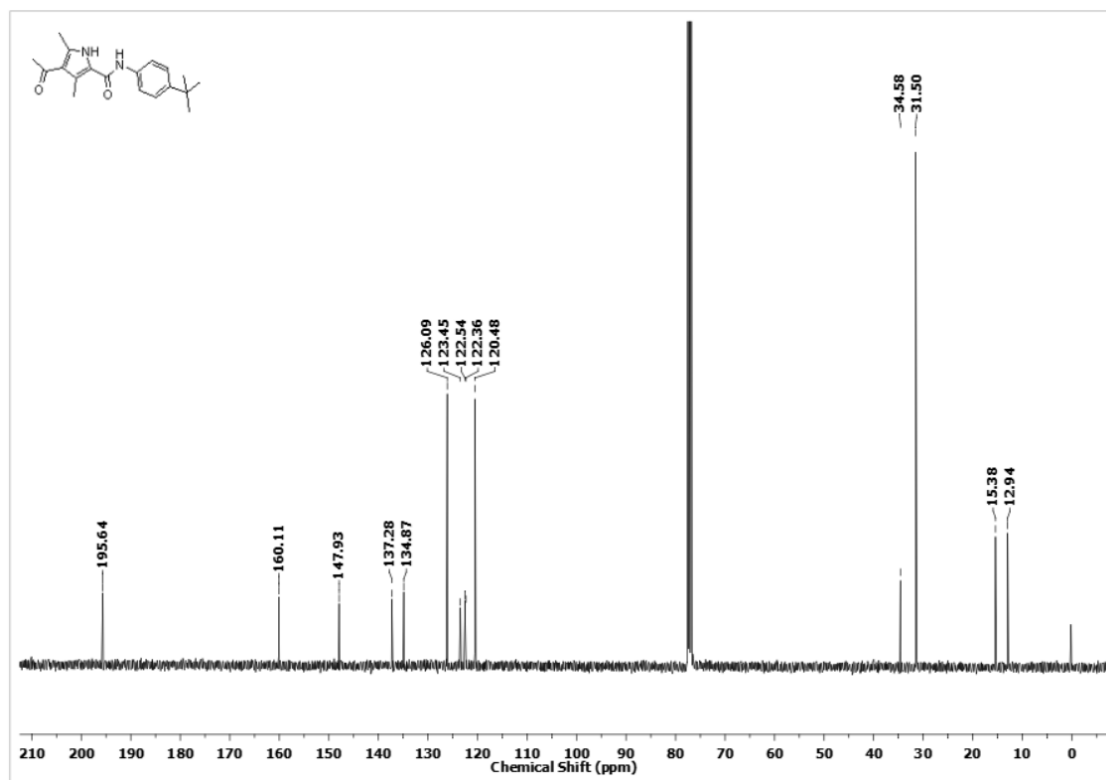


Figure 45: 101 MHz ¹³C NMR spectrum of **1d** in CDCl₃

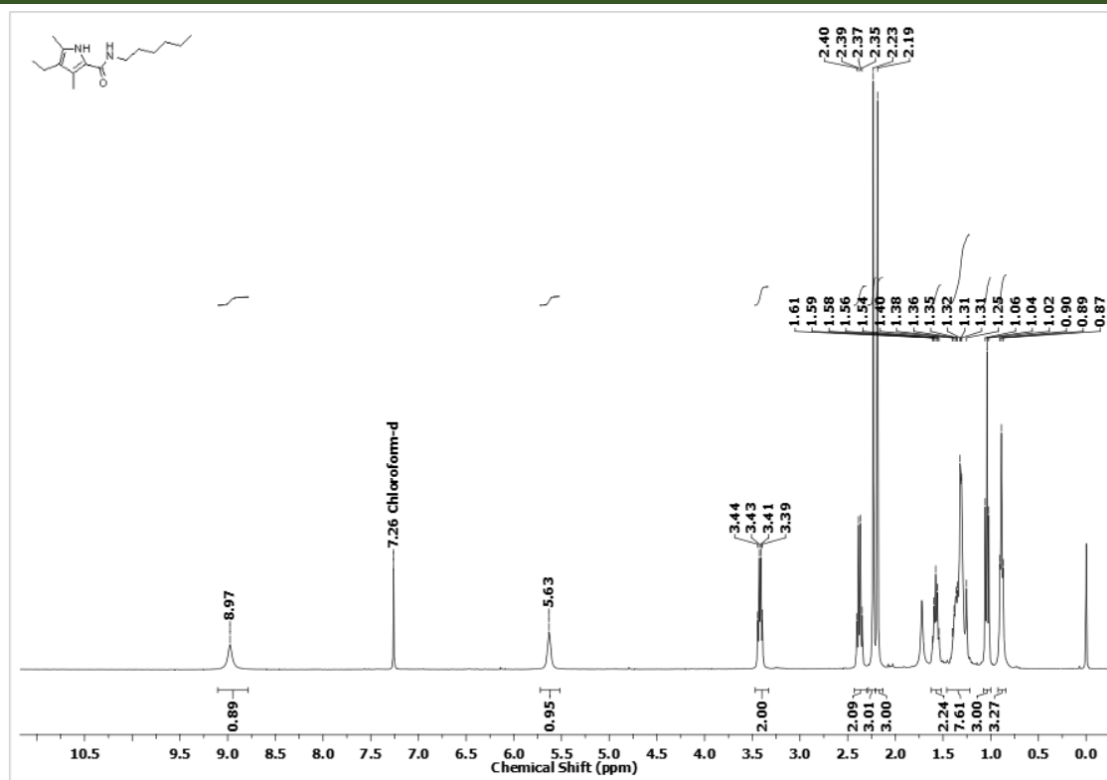


Figure 46: 400 MHz ^1H NMR spectrum of **2a** in CDCl_3

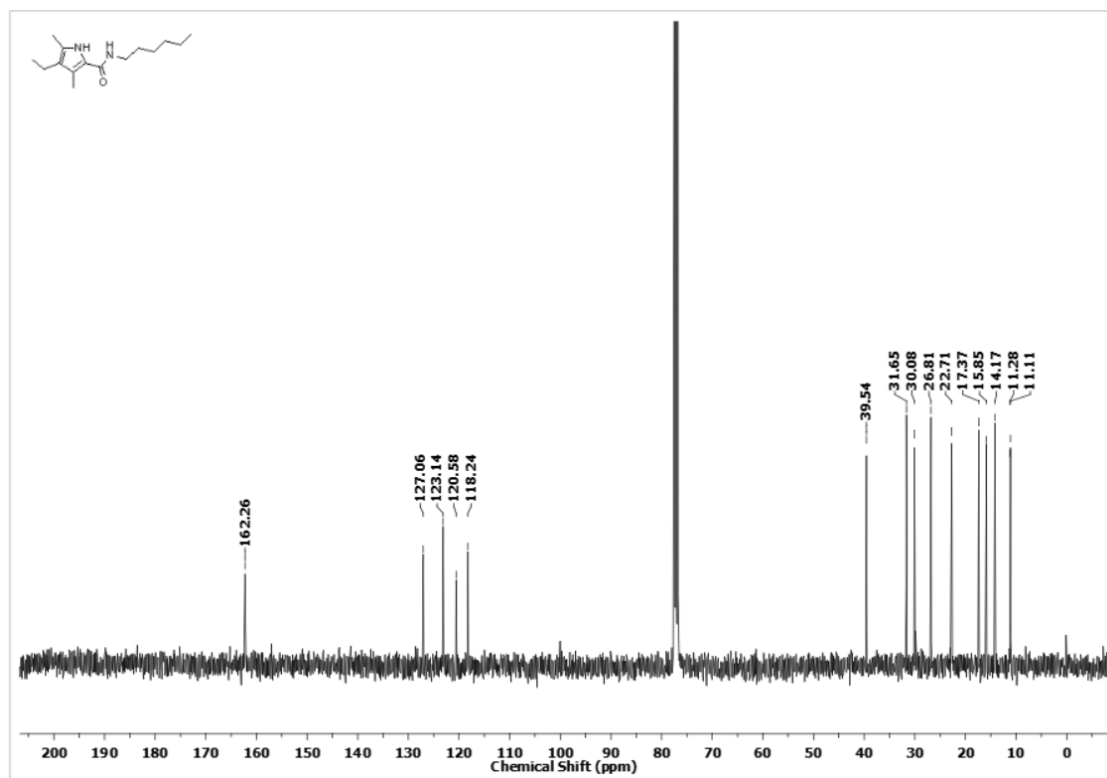


Figure 47: 101 MHz ^{13}C NMR spectrum of **2a** in CDCl_3

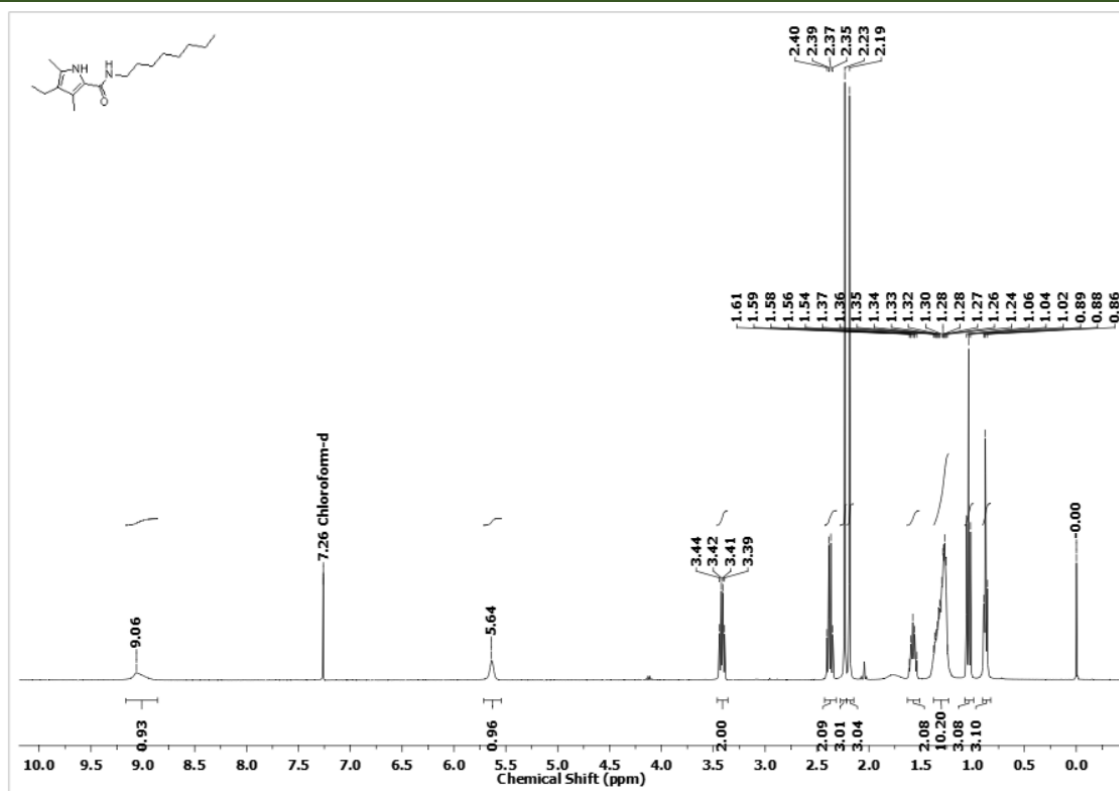


Figure 48: 400 MHz ^1H NMR spectrum of **2b** in CDCl_3

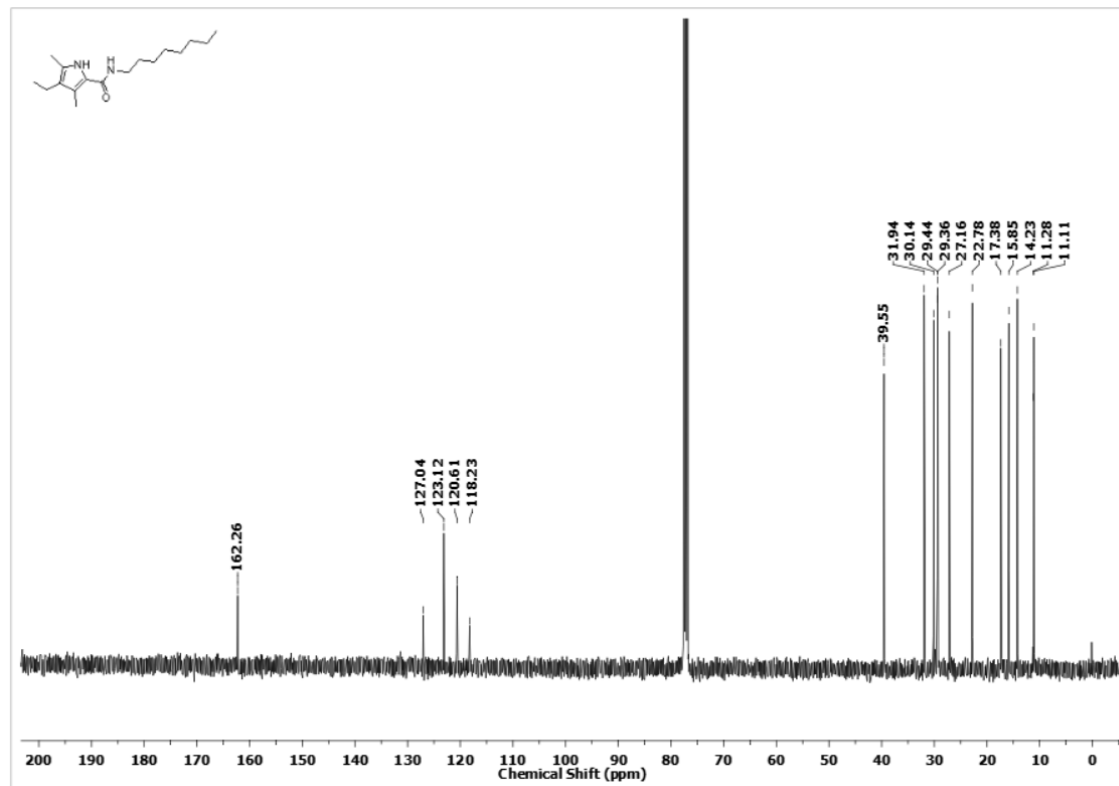


Figure 49: 101 MHz ^{13}C NMR spectrum of **2b** in CDCl_3

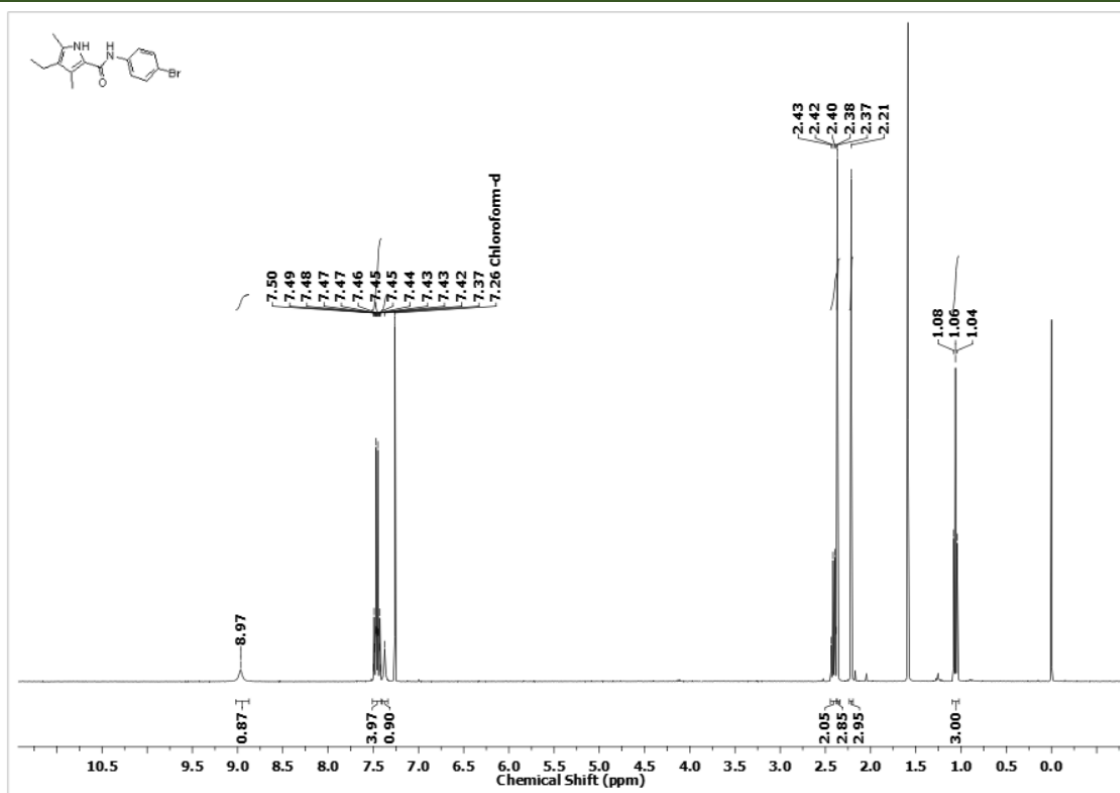


Figure 50: 400 MHz ^1H NMR spectrum of 2c in CDCl_3

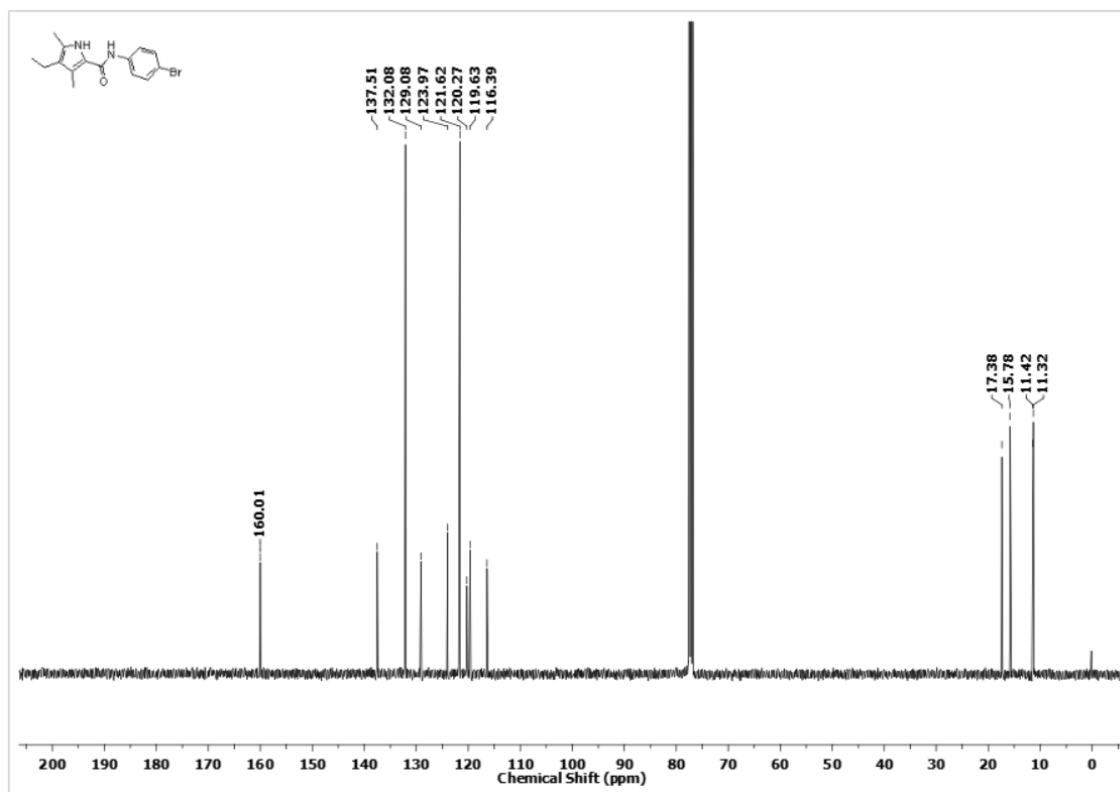


Figure 51: 101 MHz ^{13}C NMR spectrum of 2c in CDCl_3

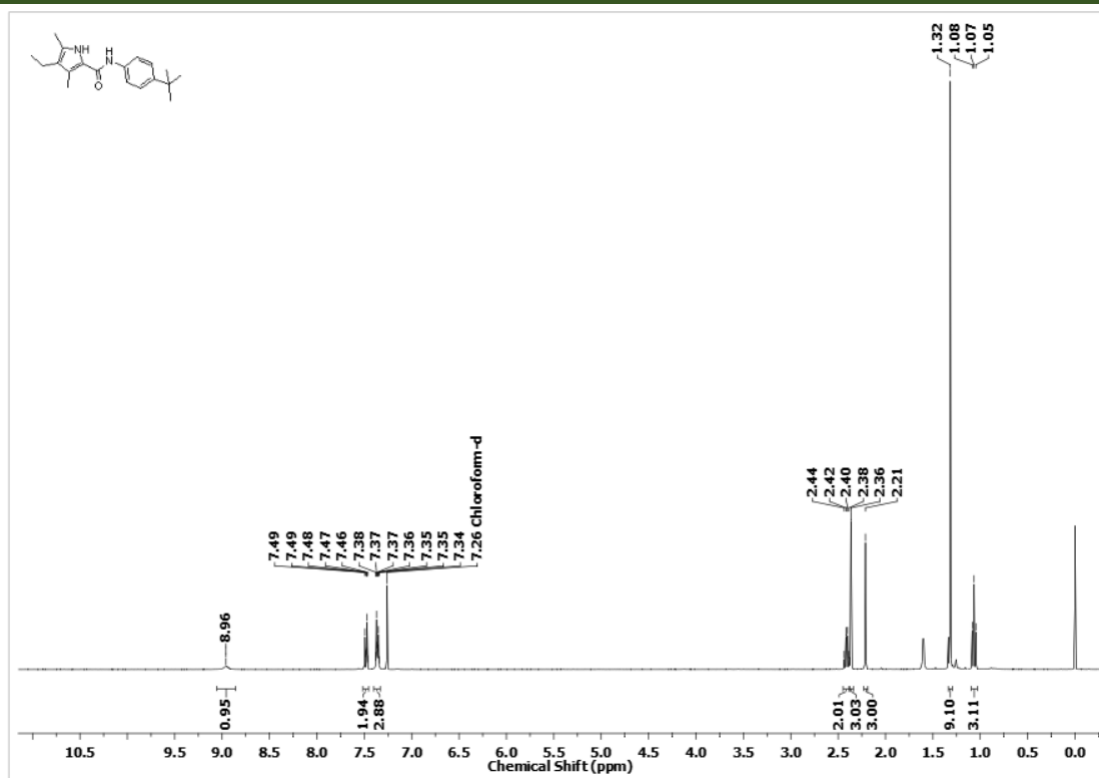


Figure 52: 400 MHz ^1H NMR spectrum of **2d** in CDCl_3

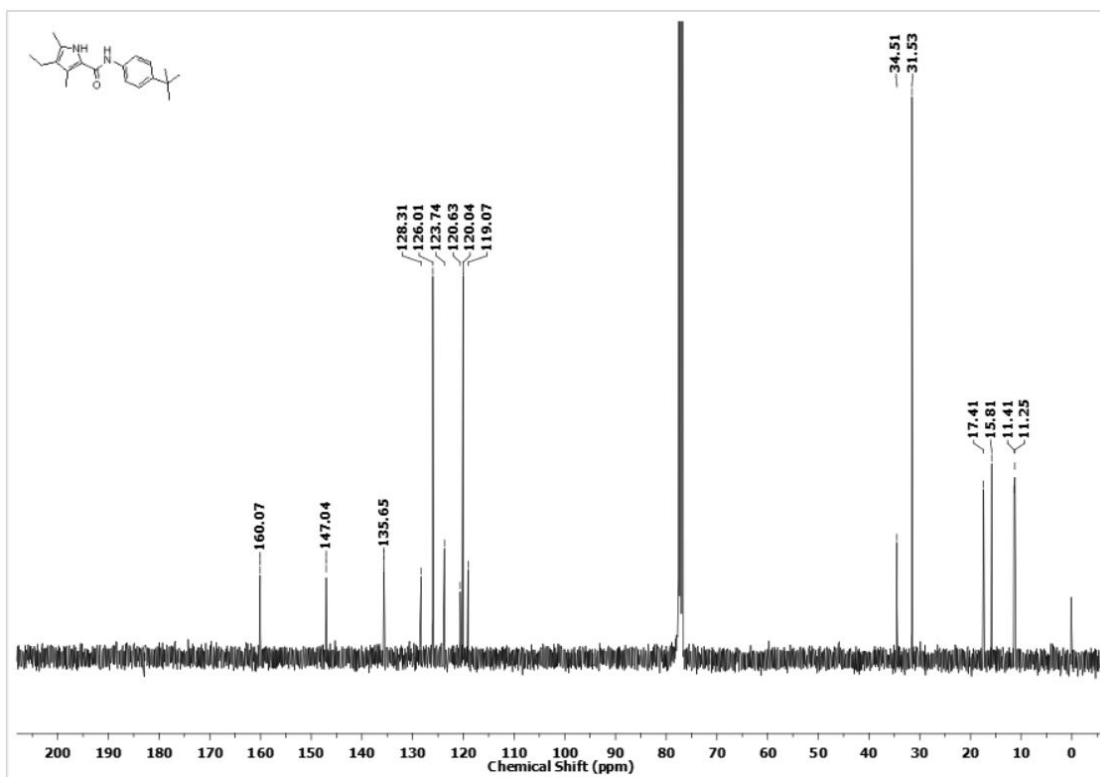


Figure 53: 101 MHz ^{13}C NMR spectrum of **2d** in CDCl_3

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Summary, Conclusions, and Future Perspectives

Summary

In this work, three classes of transmembrane ion transporters, namely salicylamides, bis(salicylamides), and pyrrole-2-carboxamides, with modifications for application in targeted ion transport, were synthesized and studied. Initially, five derivatives of the salicylamide system were prepared and demonstrated to be an efficient transmembrane H^+/Cl^- symporter. The transport activity of this system was successfully caged by the attachment of labile quinone moieties that could undergo reductive release in the presence of the NQO1 enzyme, which is overexpressed in cancer cells. The selective activation of these protransporters on enzymatic reduction was demonstrated and selective toxicity toward cancer cells was verified. Both the salicylamide transporter and its protransporters were found to induce mitochondrial membrane depolarization, increase oxidative stress, and trigger lysosomal deacidification in MCF-7 breast cancer cells, resulting in apoptotic cell death. Next, 14 derivatives of the salicylanilide system were prepared and tested against Gram-negative bacteria. An initial screen in *C. crescentus* showed salicylanilides with *para*- and meta-Cl, Br, and I substitutions to have excellent antibacterial activity. The screened compounds also showed excellent activity against the Gram-negative *Enterobacteriaceae*, namely *E. coli*, *S. enterica* (serovar Typhirium) and *K. pneumoniae*. Transmembrane H^+/Cl^- transport by salicylanilides was found to depolarize the bacterial outer membrane in *E. coli* and render it susceptible to external stress factors. The system was modified into a red light activable protransporter by attachment of the pyrrolidinobenzoquinone group, which exhibited an antibacterial effect on irradiation with red light.

The bis(salicylamide) system was prepared as an extension of the salicylamide system. Five derivatives were prepared and shown to have good ion transport and anticancer (in MCF-7 cells) activities. Mechanistic studies showed the system to be an anion antiporter. Conjugation of the bis(salicylamide) with substituted and unsubstituted *o*-nitrobenzyl groups yielded protransporters activable under violet light. Photoactivated ion transport and cancer cell toxicity were demonstrated for the protransporters on irradiation under 405 nm light. As the last project of this thesis, a set of eight pyrrole-2-carboxamide transporters was prepared and assessed for ion transport activity. They were shown to be capable of anion antiport and a wide range of transport

activities was observed with small changes in substitution. This system is pending modification into a stimuli-activated protransporter.

Conclusions

This thesis demonstrates the effectiveness of various structurally simple transmembrane ion transporter systems as targeted anticancer and antimicrobial agents. Both the salicylamide and bis(salicylamide) systems were easy to synthesize and functionalize with labile groups to form protransporters activable under various stimuli. Both were demonstrated to be efficient transporters capable of anticancer activity activable under light and enzymes as stimuli. The simplicity of these systems allows for easy functionalization with various other stimuli-responsive groups suitable for different biomedical applications. The halogen-substituted salicylanilide system was demonstrated to be an outer membrane depolarizing agent effective against Gram-negative bacteria. This was further modified to a red light activable protransporter. These properties make this system ideal for topical applications that require controlled release. The OM depolarizing effect of this system allows for its use in combination with other antimicrobial agents to yield synergistic activity. The substituted pyrrole-2-carboxamide system developed as an easy-to-functionalize core where small structural changes can result in a high degree of variation in ion transport properties. Such a highly tuneable core can be applied as the base for a wide range of transporter and protransporter systems for targeted ion transport.

Future Perspectives

The work carried out in this thesis can represent a significant step forward toward the development of viable ion transporter-based anticancer and antimicrobial drugs. The systems and methodologies utilized in this study can be further optimized and built upon to realize, maybe in the not-so-distant future, the practical application of this attractive class of molecules as a treatment method superior to current therapeutic strategies.

List of Publications

Publications that are part of the thesis work

- “NAD(P)H:Quinone Acceptor Oxidoreductase 1 (NQO1) Activatable Salicylamide H⁺/Cl⁻ Transporters”, **Naveen J. Roy**, Shreyada N. Save, Virender Kumar Sharma, Benchamin Abraham, Abhijith Kuttanankuzhi, Shilpy Sharma, Mayurika Lahiri, Pinaki Talukdar, *Chemistry - A European Journal* **2023**, 29, e202301412.
- “Bimodal structural tuning of pyrrole-2-carboxamide-based transmembrane ion transport systems”, **Naveen J. Roy**, Parappa L. Pujari, Pinaki Talukdar, *Organic & Biomolecular Chemistry* **2023**, 21, 3323-3329.
- “A Class of Photoactivable H⁺/Cl⁻ Transporters Having Antimicrobial Potential Against Gram-negative Bacteria”, **Naveen J. Roy**, Unnimaya Anilkumar, Parappa L. Pujari, Sunish K. Radhakrishnan, Pinaki Talukdar, *Manuscript submitted*.
- “Bissalicylamide Protransporters for Photoactivable Anticancer Applications”, **Naveen J. Roy**, Ronedy Naorem, Benchamin Abraham, Arjun Jagdish, Parappa L. Pujari, Manzoor Ahmad, Mayurika Lahiri, Pinaki Talukdar, *Manuscript under preparation*.

Publications apart from thesis work

- “Reversible Stimuli-Responsive Transmembrane Ion Transport Using Phenylhydrazone-Based Photoswitches”, Manzoor Ahmad, Debashis Mondal, **Naveen J. Roy**, Thangavel Vijayakanth, Pinaki Talukdar, *ChemPhotoChem* **2022**, 6, e202200002.
- “Photocontrolled activation of doubly o-nitrobenzyl-protected small molecule benzimidazoles leads to cancer cell death”, Manzoor Ahmad, **Naveen J. Roy**, Anurag Singh, Debashis Mondal, Abhishek Mondal, Thangavel Vijayakanth, Mayurika Lahiri, Pinaki Talukdar, *Chemical Science* **2023**, 14, 8897-8904.
- *Three more are under preparation.*