STIMULI RESPONSIVE SMALL MOLECULE ANION TRANSPORTERS

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by

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Certificate

This is to certify that this dissertation entitled " Stimuli responsive small molecule anion transporters " towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents the study carried out by Anjana Sathyan at Indian Institute of Science Education and Research under the supervision of Dr. Pinaki Talukdar, Associate Professor, Department of Chemistry during the academic year 2017-2018

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Declaration

I hereby declare that the matter embodied in the report entitled " Stimuli Responsive Small Molecule Anion Transporters " are the results of the work carried out by me at the Department of Chemistry, Indian Institute of Science Education and Research, Pune, under the supervision of Dr. Pinaki Talukdar and the same has not been submitted elsewhere for any other degree.

Anjana Sathyan

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Dedicated to my *Amma*

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Abbreviations

DMF	Dimethyl formamide
TLC	Thin Layer Chromatography
EtOAc	Ethyl Acetate
DCM	Dichloromethane
MeOH	Methanol
THF	Tetrahydrofuran
$CHCl_3$	Chloroform
CH_3CN	Acetonotrile
DMSO	Dimethyl sulfoxide
LUV	Large Unilamellar Vesicle
EYPC	Egg yolk phosphitidyl choline
HPLC	High Pressure Liquid Chromatography
p-HP	*p*-hydroxy phenacyl
HPTS	8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt

Abstract

The transport of ions across the lipid bilayer is controlled by ion transport proteins. There are numerous synthetic ion transporters developed till date and some of these have shown promises to target certain life-threatening diseases. However, most of these molecules always remain active and fail to discriminate between diseased and healthy cells. Therefore, we have developed small-molecule procarriers which can be activated only by either external stimuli such as light or cell-specific chemical stimuli such as pH. These molecules were designed to facilitate chloride transport however, only after forming corresponding active transporters triggered by these stimuli. For these purpose, indole-based procarriers were designed for forming the active carrier by light. Bispidine-based molecules were designed for forming active carriers only under acidic pH and then facilitating chloride transport.

From NMR titration studies, indole-based active transporters were found to bind to chloride ions efficiently. Transport of chloride ions across lipid bilayer was established by lucigenin assay. The mechanism of ion transport determined by cooperative valinomycin (a K^+ carrier) assay showed the antiport mechanism. Procarriers were inactive because the anion binding site was blocked by a photocleavable group. For the bispidine-based system, the synthesis and characterization of molecules are done. In future, the photocleavage of the protecting group from indole-based procarriers and pH-dependent ion transport by bispidine-based molecules would be carried out.

Chapter 1

Introduction

1.1 Transmembrane transport in cells

The cells which are the building blocks of life, consists of cell organelles which are specialized for carrying out various functions. These organelles are found in the cytoplasm of the cell which is surrounded by the plasma membrane. This biological membrane separates and protects cellular matrix from its surrounding environment. It is made up of double layer of phospholipids, forming internal hydrophobic layer and external hydrophobic layer and thus it is amphiphilic in nature. This cell membrane is selectively permeable, allows small hydrophobic molecules like O_2, CO_2 and small uncharged polar molecules like H_2O to pass through it freely but prevents the movement of large uncharged polar molecules and ions.

The structure and chemical composition inside the cell is responsible for low electrical permittivity when compared to the surroundings. Hence the born energy required to move an ion from outside to hydrophobic interior of the membrane is quite high which makes the membrane impermeable to movement of ions. The transport of ions across the cell is essential for survival of cell and therefore, phospholipid bilayer contains proteins embedded in it acting as the channels, carriers or pumps that can transport ions into and out of the cell.[1]These proteins has hydrophobic part that interacts with the hydrophobic part of bilayer and the hydrophilic part of these proteins forms hydrated pores, channels (gated or non-gated) and ion-binding centers that act as transporters. There are mainly two types of transport in cells which are passive transport and active transport.

Passive transport of molecules depends on the concentration gradient, lipophilicity and size. Active transport happens against the concentration gradient. This can either be symport, antiport or uniport and needs specialized proteins to carry out the process. Symport and antiport are classified under co-transport. Anions and cations have specific intracellular and extracellular concentration and overall the

cell must be electrically neutral. The major cations inside the cell include Na^+, K^+, Mg^{2+}, Ca^{2+}, and H^+ while anions include Cl^-, HCO_3^-, PO_4^{3-} etc.

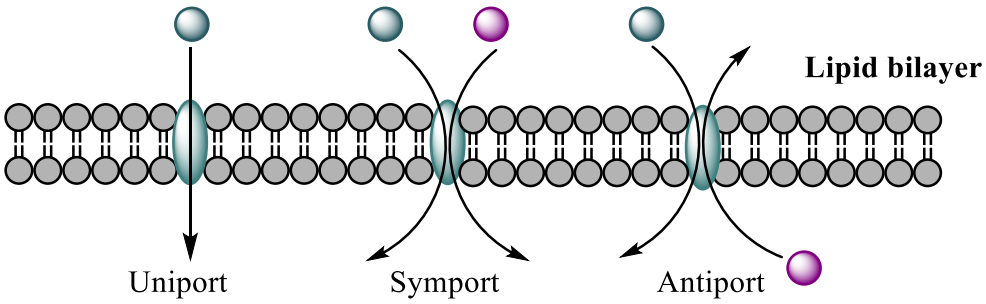


Figure 1.1. Mechanisms of transport of ions/molecules across lipid bilayer: Uniport, Symport and Antiport processes.

1.2 Importance of transmembrane anion transport

Transmembrane transport of anions is a very important biological process which ensures that the ion content of cell is different from that of outside the cell. It is essential for the maintenance of cell volume, cell homeostasis, regulation of intracellular pH, stabilization of resting membrane potential and signal transduction.[2] Malfunctioning of the proteins that form ion channels, carriers or pumps can result in diseases like cystic fibrosis, Dent's disease, myotonia, epilepsy, retinitis pigmentosa etc. These are classified under channelopathies.[3] The development of synthetic anion transporters which can replace the traditional therapies for these diseases became important in the field of supramolecular medicinal chemistry.[4] The concentration of chloride ions outside the cell is very high compared to the chloride ion concentration inside the cell. This concentration gradient is maintained by transmembrane anion transporters and channels. Synthetic anion transporters which are capable of transporting chloride ions into the cell are known to disrupt the pH gradient inside the tumor cells and hence trigger apoptosis. Therefore, these transporter molecules are important in terms of their anticancer activity.

1.3 Natural anion transporters and channels.

Natural anion transporters exhibit high selectivity and activity which is difficult to achieve in case of synthetic molecules. Prodigiosin is a natural anion transporter molecule which functions as HCl receptor and transporter. It is known to trigger

apoptosis of malignant cancer cells by disrupting the pH gradient inside the cell.[5] A synthetic derivative of prodigiosin named obatoclax is currently under clinical trials for its anticancer activity.

There are specific ion channels in the body like CLC for selective chloride transport, AE1-3 for Cl^-/HCO_3^- exchange and CFTR for regulated Cl^-/HCO_3^- transport.[6,7] Transport of chloride ions are controlled and activated by bio-orthogonal stimuli which includes pH, light, membrane potential and neurotransmitters. Hence, it is clear that most of the natural anionophores and channels are stimuli responsive. Chloride selective channel opens when GABA binds to the receptor in the channel. Light responsive halorhodopsins get activated by light and pump chloride ions against the concentration gradient. Na-K-2Cl (NKCCs) and K-Cl (KCCs) are natural ion channels whose activity is regulated by enzyme-catalyzed phosphorylation and dephosphorylation in the hydroxyl residues of aminoacids.[8]

1.4 Synthetic anion transporters

Synthetic anion transporters that can selectively transport anions are an active area of research in supramolecular chemistry. Many synthetic molecules are reported that shows selective chloride ion transport and have been studied for anticancer activity.

Synthetic anion transporters are designed in such a way that they can bind anions through non-covalent interactions like H-bonding, anion-pi interactions etc. which should be reversible. The designed molecules usually have acidic hydrogen atoms for forming H-bonding interactions with anions and also should have low molecular weight. Moreover, they should be highly lipophilic and hydrophobic so that it can efficiently partition into the lipid membranes as well as it should be soluble in water for efficient delivery.[8] It is important to achieve the optimum balance between these two. According to Lipinski rule, the logP value around 5 for the molecules allows it to pass through lipid bilayer. The ion transport activity of synthetic anion transporters is checked across liposomal vesicles by various methods which include HPTS assay, lucigenin assay etc.

It is very important to achieve selectivity in case of synthetic anionophores. In order to direct transport activity to a specific target of interest, stimuli responsive synthetic anion transporters has to be designed. Therefore, the developments of stimuli responsive anion transporters which can mimic the natural ion channels that work under bio-orthogonal stimuli have been focused.

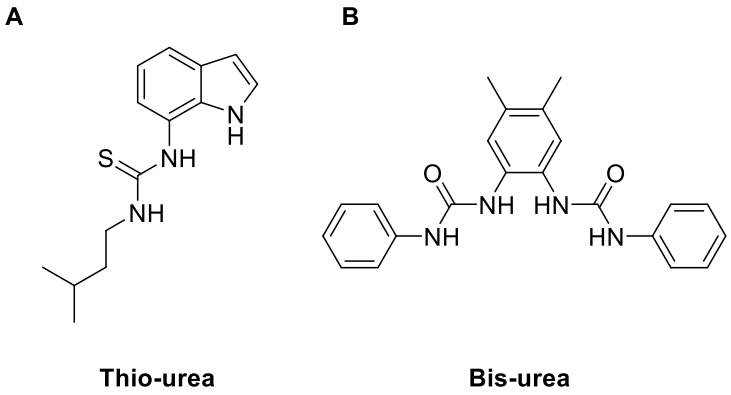


Figure 1.2. Synthetic anion transporters.

1.5 Stimuli responsive anion transporters and their importance

Spatiotemporal control of ion transport activity is a big challenge to achieve. Such control over ion transport helps in directing ion transport activity to specific areas of interest like tumor cells. To obtain this control, one strategy is to design stimuli responsive molecules that get activated only under specific external stimulus. Many research work going on in this area involves mainly the enzyme-responsive procarriers which on exposure to enzyme stimulus forms an active transporter. Therefore the procarriers which can be activated by an enzyme are very useful in biological systems because of biocompatibility, high selectivity and specificity.[8]

Another method is to design anion transporters that get activated by light as stimulus. To achieve this, a photo cleavable group can be blocking the anion binding site of the pro-carrier. However, on photolytic cleavage of blocker molecule forms the active anion transporter. In this way, we can control the site where anion transport has to be carried out. The photo cleavable group selected should have to be in such a way that the side products formed are not harmful to the cells (i.e. blocker molecules should be bioorthogonal).

In the first project, we are using a known photocleavable group, *p*-hydroxy phenacyl (*p*HP) as a blocker molecule to synthesize the pro-carrier that has an absorption maximum at 313 nm. The *p*-hydroxyphenacyl (*p*HP) protecting group is known to protect carboxylates, phosphates and thiophosphates in neurobiology, biochemistry and enzyme catalysis and therefore can be used as a linker for blocking ion binding site of transporters.[9]

Another way of applying external stimulus is chemical stimulus or change in pH. The inactive procarriers which lack sufficient anion binding sites and has protonation sites can be activated by the addition of protic acid or change in pH. Protic acid can protonate the procarrier, making it an active transporter that can form hydrogen bond interactions with anion. However light activated procarriers are superior as this radiation is chemical free source, can be used without involving directly in reaction medium (i.e., special access to medium).

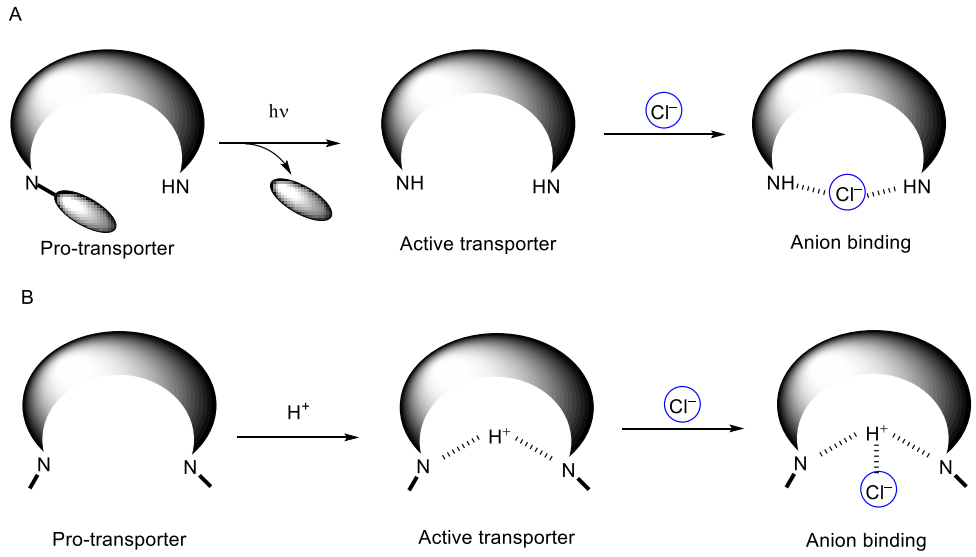


Figure 1.3. Cartoon diagram to represent formation of active transporter from the pro-transporters by external stimulus, by light (A), and pH (B).

1.6 Mechanism of photo cleavage

The *p*-hydroxyphenacyl group when cleaved undergo photo-Favorskii rearrangement and finally in the presence of water forms 4-hydroxyphenyl acetic acid which is a not a harmful side product. The photo cleavage of *p*-hydroxyphenacyl

group is reported to be effective at pH < 7.5, when cleaved from protected aliphatic primary and secondary amines in aqueous solutions.[10]

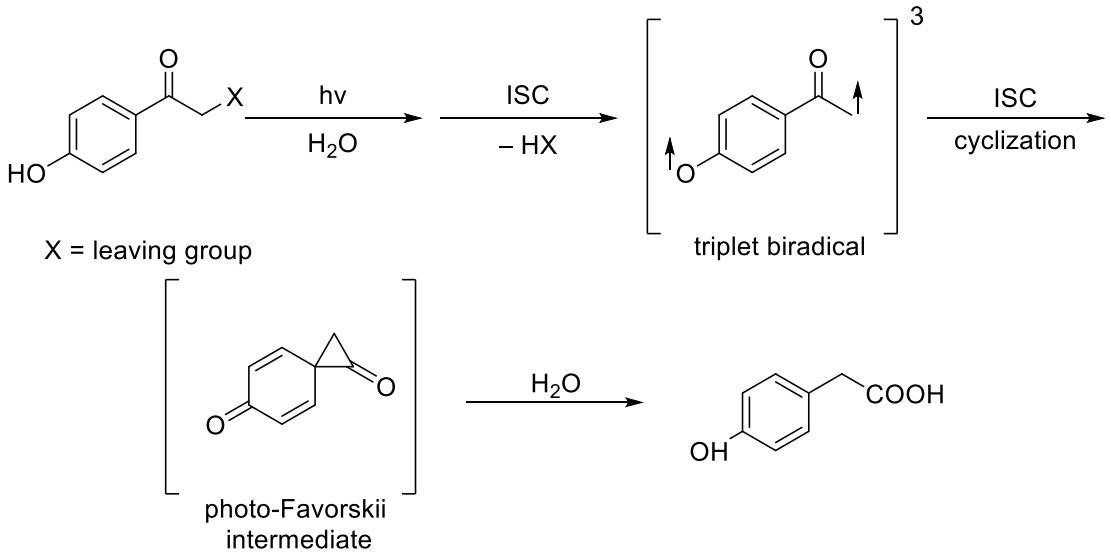


Figure 1.4. General mechanism of photo-Favorskii rearrangement.

Chapter 2

Light responsive anion transporters

2.1 Objective

Anion transporters are designed based on an indole backbone (**1a-c**) which contains two acidic N–H hydrogens (one amide and one indole hydrogen) capable of forming hydrogen bonding interactions with anion. The lipophilicity of the transporter is controlled by varying the length of aliphatic chain. As per the calculations using Marvin sketch, highest logP (partition coefficient) value is found for designed transporter **1a,** the octyl derivative (4.17), followed by **1b,** hexyl derivative (3.28) and the least is for **1c,** butyl derivative (2.39).The acidity of both hydrogens are found to be same for all compounds. pK_a for indole hydrogen is found to be 12.37 and for amide hydrogen is 14.67 in all compounds. Thus, designed anion transporter is expected to transport anions across lipid bilayer through reversible hydrogen bonding interactions.

The aim of this project is to synthesize the designed anion transporters along with the pro-transporter with a photo cleavable group attached to it. Here the photo labile protecting group blocks one of the anion binding site of the free transporter making it reluctant to transport the anions. On irradiation with light of suitable wavelength the photo cleavable group gets cleaved and the pro-transporter becomes active (Figure 1.4). Here, *p*-hydroxyphenacyl group which has absorption at 300-350 nm is used as protecting group. This group has an absorption maximum at 313 nm and reaction occurs through photo-Favorskii rearrangement forming 4-hydroxyphenylacetic acid. The side products formed in this photoreaction is non-toxic and hence this photo removable protecting group can be used for biological studies.[9] Another advantage of using this *p*-hydroxyphenacyl group is that the presence of hydroxyl group increases the hydrophilicity of the molecule which increases the solubility in water. In situ cleavage of these pro-transporters generates active hydrophobic carriers that can be easily incorporated into lipid bilayer. The

photo cleavable group acts as a hydrophilic appendage which increases the deliverability of hydrophobic synthetic anion transporters.

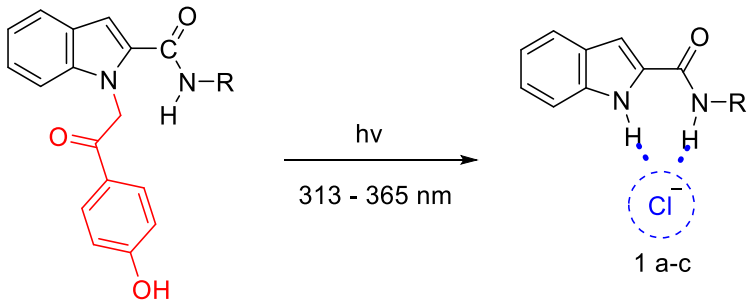
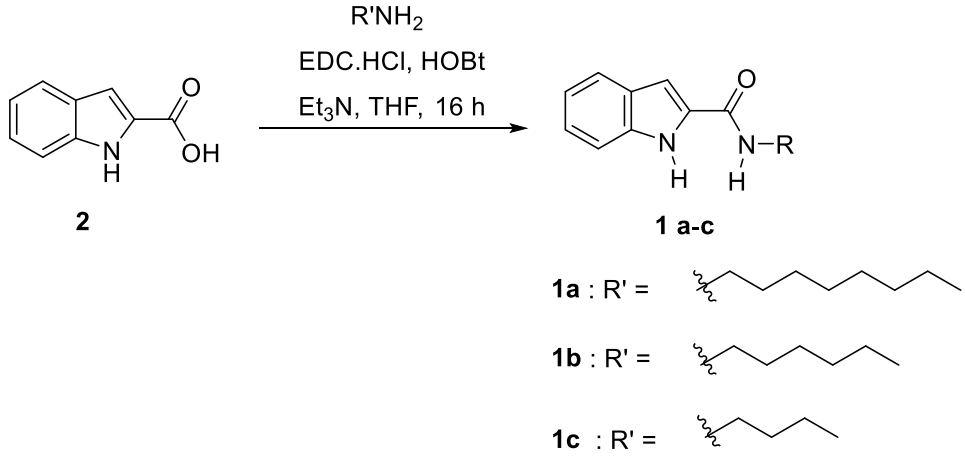


Figure 2.1. Formation of active transporter from the photo cleavage of pro-transporter.

2.2 Results and Discussion

2.2.1 Synthesis of indole core based anion transporter

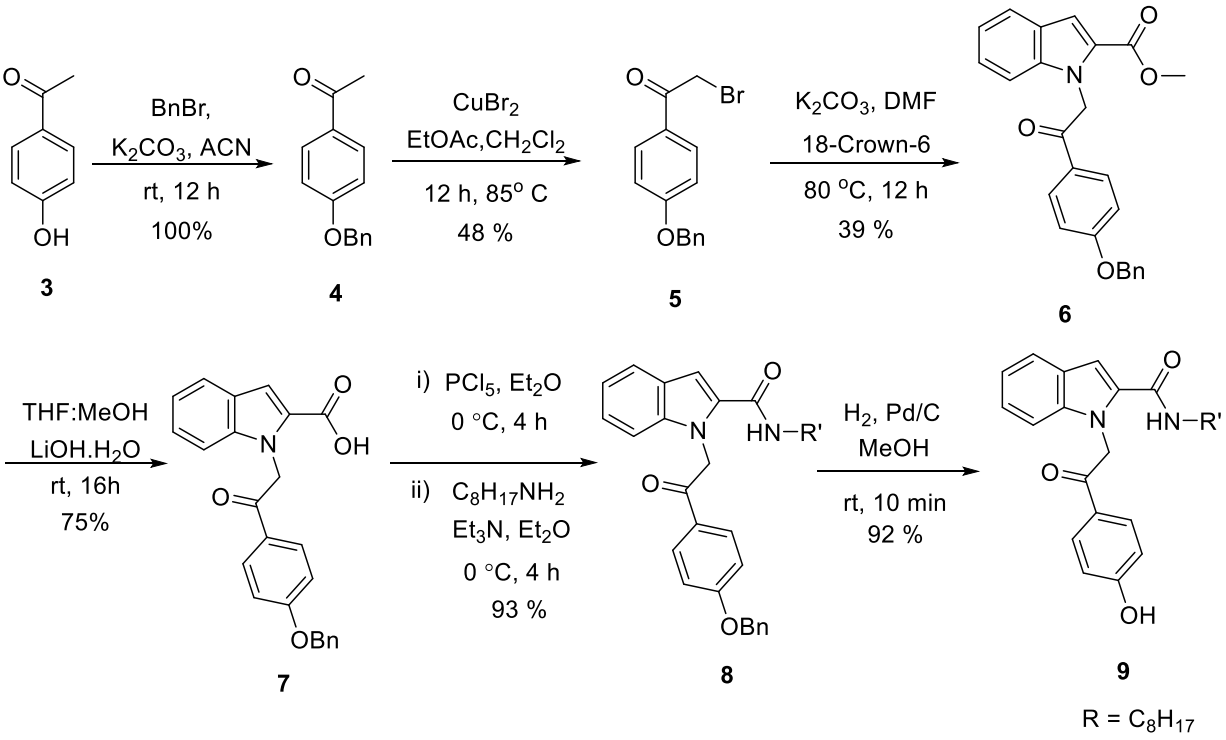
According to our design we have synthesized the indole core based anion transporter which will employ the indole N-H as well as amide N-H to bind with the anions. For the synthesis of amide derivatives, indole-2-carboxylic acid was reacted with different amines in the presence of EDC·HCl and Et_3N as a base which lead to the formation of amides in good yield.



Scheme 1. Synthesis scheme for indole core based anion transporters **1a-c**.

2.2.2 Synthesis of indole based *p*-HP photo cleavable group attached pro-transporter

For the synthesis of the photo cleavable group protected anion transporter of **1a,** we have started our synthesis from commercially available 4-hydroxyacetophenone **3**. At first the hydroxyl group was protected by benzyl group by the reaction with benzyl bromide to get compound **4**. The benzyl protected compound **4** was brominated by $CuBr_2$ in CH_2Cl_2: EtOAc (1:1) to get the compound **5** in 48% yield. Benzyl protected bromo compound **5** was reacted with methyl indole-2-carboxylate in the presence of K_2CO_3 and 18-Crown-6 to get compound **6** in 39% yield. Compound **6** was hydrolyzed using $LiOH.H_2O$ in 3:1:1 THF/MeOH/H_2O to get acid compound **7** in 75% yield. Compound **7** was reacted with PCl_5 in diethyl ether to get corresponding acid chloride which was reacted with octyl amine in the presence of Et_3N to get compound **8** in 93% yield. The debenzylation of compound **8** in the presence of H_2 and Pd/C resulted in protected compound **9** in quantitative yield.



Scheme 2. Synthesis of pro-transporter of compound **9**.

2.2.3 Anion Binding Studies by ^{1}H NMR Titration

The ability of compound **1a** (5 mM) to bind anions in solution was determined by ^{1}H NMR titration in acetonitrile-d_6 in the presence of tetrabutylammonium chloride

(TBACl). The addition of TBACl resulted in the downshield shift of two acidic N–H hydrogens at δ = 9.81 ppm and δ = 7.05 ppm in compound **1a,** which increased with increase in concentration of TBACl. This clearly indicated that these acidic protons are binding with chloride ion as expected by N–H···Cl^- hydrogen bonding interactions. Change in NMR signal of one proton at δ = 9.81 ppm was considered for jobs plot analysis with mole fraction of compound. [**1a**] / ([**1a**] + [Cl^-]) was plotted against Δδ ∗ [**1a**] / ([**1a**] + [Cl^-]) to obtain jobs plot. From jobs plot analysis Δδ was found to be maximum at 0.33 indicating 1:2 binding of transporter with anions. It was confirmed from this that one transporter molecule binds to two chloride ions.

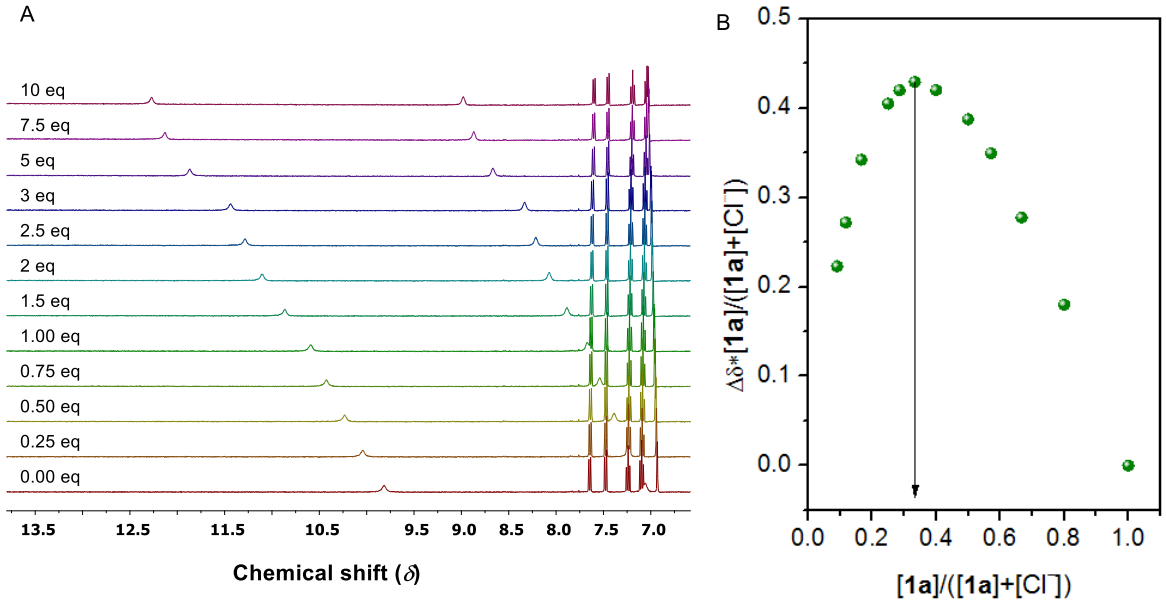


Figure 2.2. ^{1}H NMR titration of **1a** with TBACl (A), and Jobs Plot of N-H proton shift (B).

2.2.4 Ion transport activity by Lucigenin assay for the active transporters

The ion transport activities of the free transporters across large unilamellar vesicles (EYPC vesicles) were checked by fluorescence kinetics experiments. For that the EYPC vesicles was prepared (entrapped with fluorescent dye Lucigenin, $NaNO_3$) and suspended in a solution of $NaNO_3$. The salt gradient was brought by the addition of 2N NaCl. The transport of chloride ion influx was measured by the decrease in fluorescence intensity of lucigenin. After the achievement of gradient across the bilayer, compounds were added and fluorescence intensity was measured. The designed free transporters compounds **1a-c** were checked for their

ion transport activity. Compound **1a** was found to be the most active followed by compound **1b** and **1c** (15 μM).

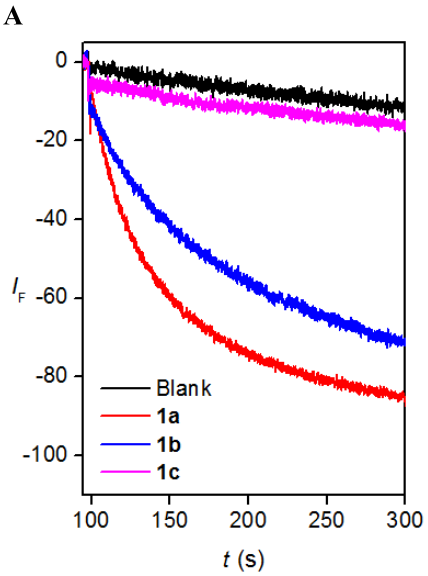


Figure 2.3. Comparison of chloride ion transport by compounds **1a-c** in lucigenin assay.

The concentration dependent ion transport activity of compound **1a** was studied which clearly showed an increase in chloride ion influx with increase in concentration.

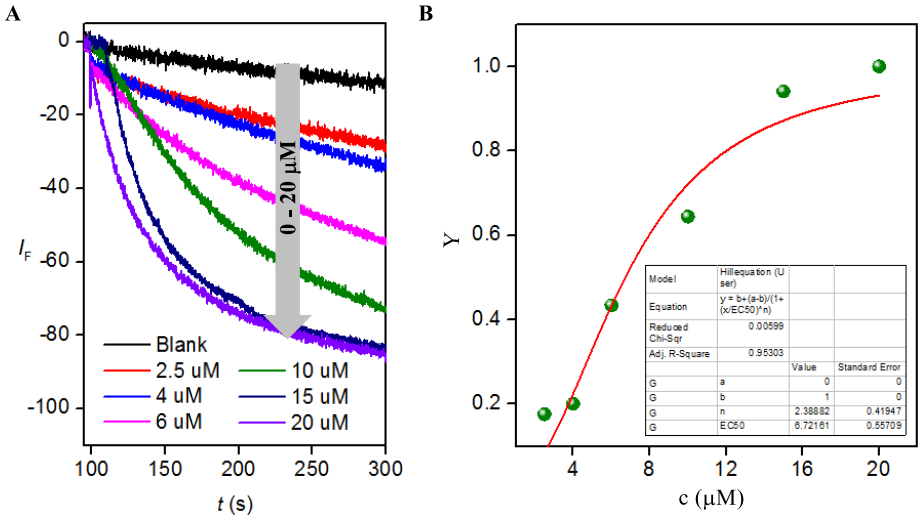


Figure 2.4. Concentration dependent chloride ion transport of compound **1a** by lucigenin assay (A), and Hill plot analysis to calculate Hill coefficient and EC_{50} (B).

2.2.5 Symport and Antiport assay using lucigenin

The influx of chloride ions can be through either Na^+/Cl^- symport transport or through chloride /nitrate antiport process. To check if the influx of chloride ions is due to M^+/Cl^- symport process, lucigenin assay was carried out by varying the cations in the extra vesicular medium. Different salt solutions of 2N concentration ($M^+ = Li^+, K^+, Rb^+, Cs^+$) were used to bring the salt gradient across the vesicles and chloride ion influx for compound **1a** was measured at 10 µM concentration. No significant change in the ion transport activity was observed with the change in cations in extra vesicular medium which rules out the possibility of M^+/Cl^- symport mechanism.

To confirm the chloride/nitrate antiport transport, lucigenin assay was done in the presence of valinomycin and 2N KCl in the extra vesicular medium. Valinomycin is selective K^+ ion transporter. At first, chloride ion influx rate after addition of 0.5 µM of valinomycin was measured. Later, chloride ion influx rate of compound **1a** at 4.5 µM concentration was measured with and without valinomycin (0.5 µM). Increase in ion transport activity in the presence of valinomycin was observed which is due to the co-operative effect of two transporters. This confirms the chloride/nitrate antiport mechanism.

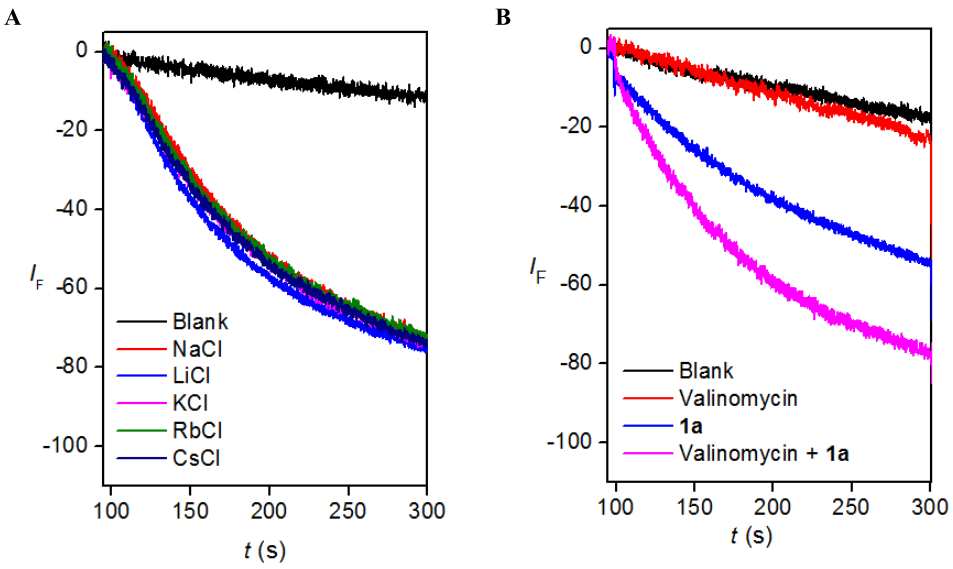


Figure 2.5. Lucigenin assay using different extra vesicular alkaline cations (A), and Lucigenin assay with and without valinomycin for checking antiport mechanism (B).

2.2.6 Comparison of ion transport activity of free transporter and pro-transporter

The octyl derivative **1a** was found to be the most active among the anion transporters. Therefore, compound **9** was synthesized with a photo cleavable group (*p*-hydroxyphenacyl) attached to indole nitrogen of compound **1a** according to synthetic procedure mentioned above. This is a pro-transporter of compound **1a** where one of the anion binding sites is blocked by the photo cleavable group. On irradiation with light of suitable wavelength the group will get cleaved releasing the free anion transporter. The ion transport activity of pro-transporter compound **9** and most active free transporter compound **1a** was checked using lucigenin assay at 15 µM concentration. The pro-transporter did not show any significant increase in chloride ion influx when compared with free transporter at 15 µM concentration as expected.

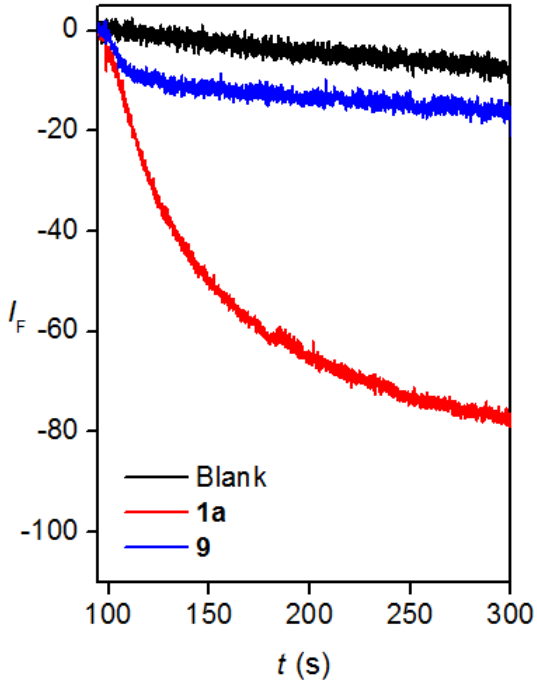


Figure 2.6. Ion transport activity of compound **1a**, free transporter and compound **9**, photo cleavable group attached pro-transporter.

2.2.7 UV absorption spectra for pro-transporter

Photo cleavable group (*p*HP) has an absorption range from 300-350 nm with absorption maxima at 313 nm. The UV absorption spectrum of protected compound **9** was measured at 40 and 80 µM concentration with MeOH as solvent. Absorption maxima were found at 218.4 and 280.4 nm. This protected pro-transporter has to be activated by irradiating with light of appropriate wavelength to get back the active transporter.

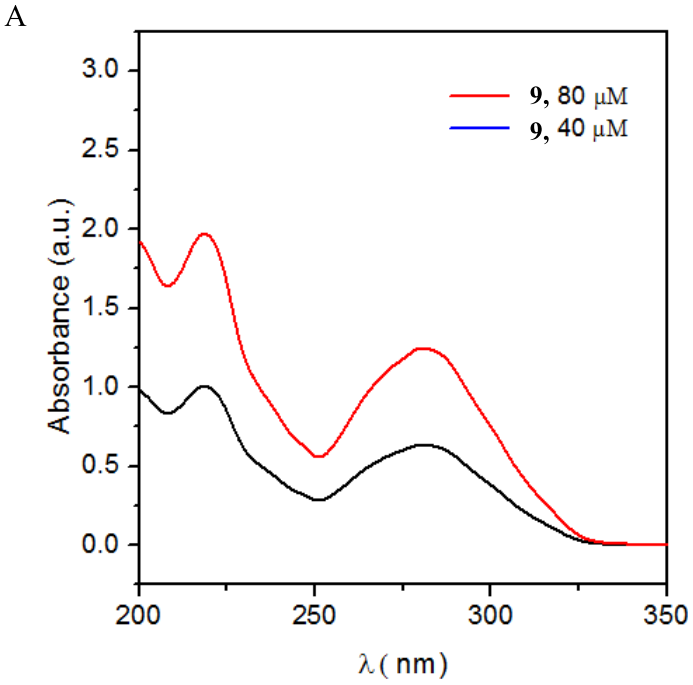


Figure 2.7. UV Absorption spectrum of compound **9**.

2.3 Conclusion

Free anion transporters based on indole backbone were synthesized and checked for their transport activity across liposomal vesicles. Compound **1a** (octyl derivative) was found to be the most active and compound **1c** (butyl derivative) being the least active. This result is as expected from their calculated logP value. The ion transport activity was studied using lucigenin assay including symport and antiport studies. The pro-transporter of compound **1a** was also synthesized successfully and found to be a non-transporter of anion when checked for ion transport activity.

2.4 Future plans

The pro-transporter has to be activated by irradiating it at 313 nm and then it has to be checked for transport activity. Controlled ion transport activity of protransporter has to be studied in a time dependent irradiation.

2.5 Experimental Section

2.5.1 Methods

General Methods

Reagents and compounds used for the synthesis were purchased from Sigma-Aldrich, Avra chemicals, Spectrochem, Alfa Aesar and used without further purification. For dry reaction MeCN, CH_2Cl_2 and DMF were purchased from commercial suppliers and used without further purification.Egg yolk phosphatidylcholine (EYPC) was purchased from Avanti Polar Lipids as a solution of 25 mg/mL in $CHCl_3$.

Physical Measurements

All ^{1}H NMR (400 MHz) and ^{13}C (100 MHz) spectra were recorded on Bruker 400 MHz and JEOL 400 MHz spectrometers. The chemical shifts (δ) in parts per million were referenced to the residual proton signal of deuterated solvents (^{1}H NMR $CDCl_3$: δ 7.26 ppm; ^{13}C NMR $CDCl_3$: δ 77.2 ppm; ^{1}H NMR DMSO-d_6: δ 2.50 ppm; ^{13}C NMR DMSO-d_6: δ 39.52 ppm). The multiplicities of the peaks are s (singlet), d (doublet), q (quartet), t (triplet), dd (doublet of doublet), m (multiplet). Column chromatography was performed using ethyl acetate in hexane as solvent on silica (100-200 mesh, 230-400 mesh) and also methanol in chloroform as solvent on silica (100-200 mesh).

2.5.2 Ion transport activity using Lucigenin assay

Preparation of salt and stock solution for Lucigenin assay

Salt solution was prepared by dissolving $NaNO_3$ (200 mM) in autoclaved water. Lucigenin dye was added later into this salt solution to make 1 mM solution.

All the compounds were dissolved in HPLC grade 1:1 of ACN: MeOH solvent to make stock solutions.[11]

Preparation of EYPC-LUVs ⊂ Lucigenin for concentration dependent assay and symport assay.

1 mL off egg yolk phosphatidylcholine (EYPC, 25 mg/mL in $CHCl_3$) was taken in a round bottom flask. It was dried by purging nitrogen along with continuous rotation to form thin film of EYPC. Then round bottom flask was kept in high vacuum for 6 h to remove trace amount of $CHCl_3$. Later 1 mL solution(1 mM lucigenin, 200 mM $NaNO_3$) was added into the thin film to hydrate and was vortexed occasionally (4-5 times) for 1 h. Then it was subjected to freeze-thaw cycle (> 15 times). Extrusions were done using mini extruder with polycarbonate membrane (pore diameter of 200nm). All extra vesicular dyes were removed by gel filtration with Sephadex G-50 using same buffer without Lucigenin dye. The vesicle obtained was diluted to 6 ml using the same buffer to get EYPC-LUVs ⊂ Lucigenin ~ 5.0 mM EYPC; inside: 1 mM Lucigenin, 200 mM $NaNO_3$.Outside: 200 mM $NaNO_3$.[11]

Concentration dependent and comparative Ion transport activity:

In a clean and dry fluorescence cuvette, 1950 µL 100 mM $NaNO_3$ solution was taken and into it 25 µL of EYPC-LUVs ⊂ lucigenin was added and this cuvette was placed on fluorescence instrument equipped with magnetic stirrer (t = 0 s). Fluorescence emission intensity of Lucigenin dye, F_t was monitored at λ_{em} = 535 nm (λ_{ex} = 455 nm). Then a chloride ion concentration gradient was created between intra and extra vesicular system by adding 33 µL of 0.5 NaCl to the same cuvette at t = 50 s. Compounds were added at different concentration at t = 100 s each time and finally at t = 300 s, 25 µL of 10% Triton-X-100 was added to lyse all the vesicles which resulted in the destruction of chloride ion concentration gradient and saturation of fluorescence intensity.

Fluorescence time courses (F_t) were normalized to fractional emission intensity I_F using Equation given below:

$$\%\ F_I\ Intensity\ (I_F) = [(F_t - F_0) / (F_\infty - F_0)] \times (-100)$$

Where F_0 = Fluorescence intensity just before the compound addition (at 0 s). F_∞ = Fluorescence intensity at saturation after complete leakage. F_t = Fluorescence intensity at time *t*.

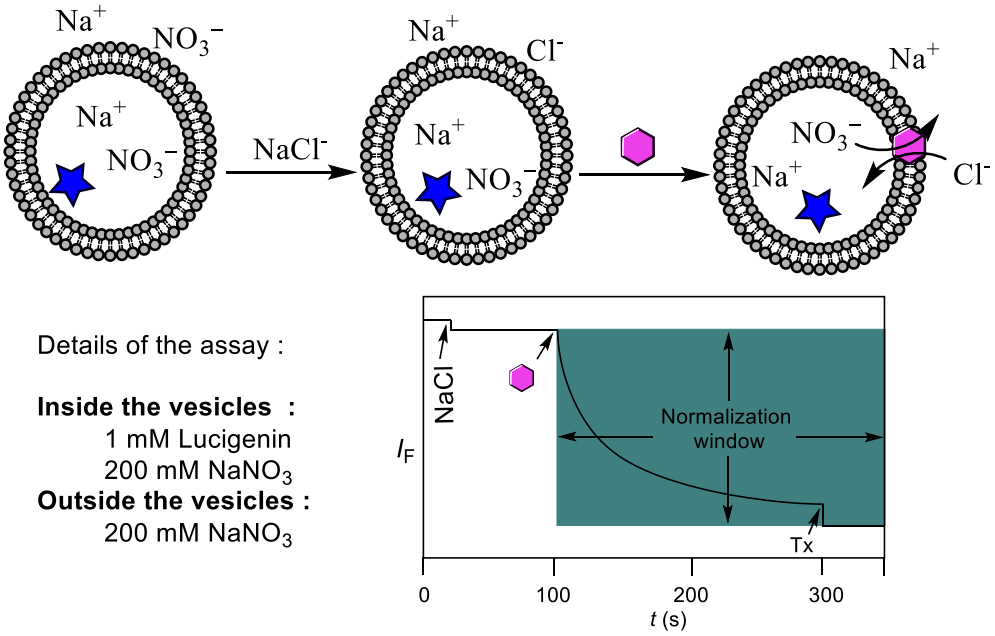


Figure 2.8.Schematic representation of determination of ion transport activity using lucigenin assay.

Ion transport activity by symport assay:

In a clean and dry fluorescence cuvette, 1950 µL of salt solution (100 mM $NaNO_3$) was taken and into it 25 µL of EYPC-LUVs ⊂ lucigenin was added and this cuvette was placed on fluorescence instrument equipped with magnetic stirrer (*t* = 0 s). Fluorescence emission intensity of Lucigenin dye, F_t was monitored at λ_{em} = 535 nm (λ_{ex} = 455 nm). Then a chloride ion concentration gradient was created between intra and extra vesicular system by adding 33 µL of 2N MCl solution (where M^+ = Li^+, Na^+, K^+, Rb^+, and Cs^+) to the same cuvette at *t* = 50 s. Compound was added at *t* = 100 s and finally at *t* = 300 s, 25 µL of 10% Triton-X-100 was added to lyse all the vesicles which resulted in the destruction of chloride ion concentration gradient and saturation of fluorescence intensity.

Fluorescence time courses (F_t) were normalized to fractional emission intensity I_F using Equation given below:

$$\%\ Fl\ Intensity\ (I_F) = [(F_t - F_0) / (F_\infty - F_0)] \times (-100)$$

Where F_0 = Fluorescence intensity just before the compound addition (at 0 s). F_∞ = Fluorescence intensity at saturation after complete leakage. F_t = Fluorescence intensity at time t.[11]

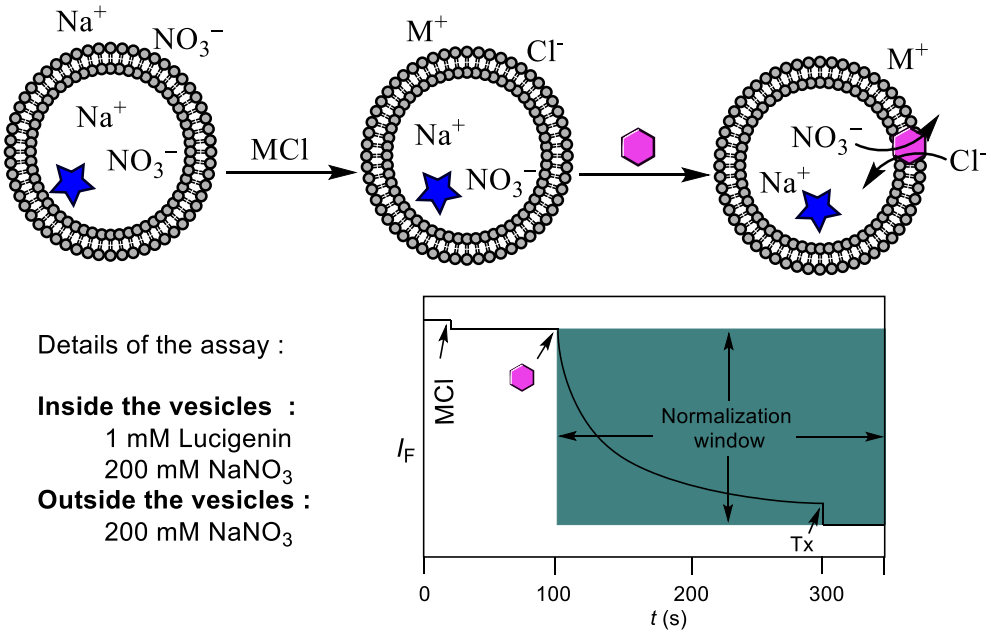


Figure 2.9. Schematic representation of chloride ion transport by symport assay.

Ion transport activity by antiport assay using valinomycin:

In a clean and dry fluorescence cuvette, 1950 µL 100 mM $NaNO_3$ solution was taken and into it 25 µL of EYPC-LUVs ⊂ HPTS was added and this cuvette was placed on fluorescence instrument equipped with magnetic stirrer (t = 0 s). Fluorescence emission intensity of Lucigenin dye, F_t was monitored at λ_{em} = 535 nm (λ_{ex} = 455 nm). Then a chloride ion concentration gradient was created between intra and extra vesicular system by adding 33 µL of 2N KCl to the same cuvette at t = 20 s. Later, 0.5 µM valinomycin was added at t = 50 s. 18 µL of 500 µM compound solution was added at t = 100 s and finally at t = 300 s, 25 µL of 10% Triton-X-100 was added to lyse all the vesicles which resulted in the destruction of chloride ion concentration gradient and saturation of fluorescence intensity.

Fluorescence time courses (F_t) were normalized to fractional emission intensity I_F using Equation given below:

$$\%\ \textit{Fl Intensity}\ (I_F) = [(F_t - F_0) / (F_\infty - F_0)] \times (-100)$$

Where F_0 = Fluorescence intensity just before the compound addition (at 0 s). F_∞ = Fluorescence intensity at saturation after complete leakage. F_t = Fluorescence intensity at time *t*.[11]

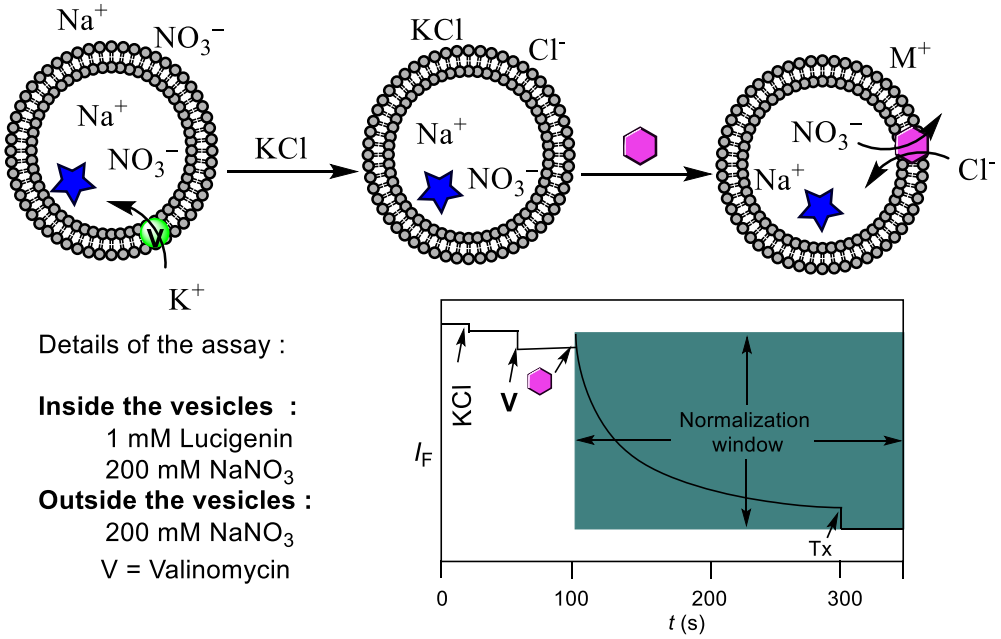
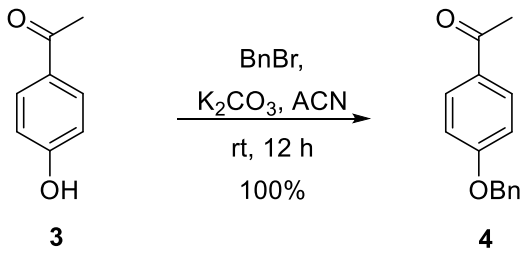


Figure 2.10. Schematic representation of chloride ion transport by antiport assay.

2.5.3 Synthetic procedures

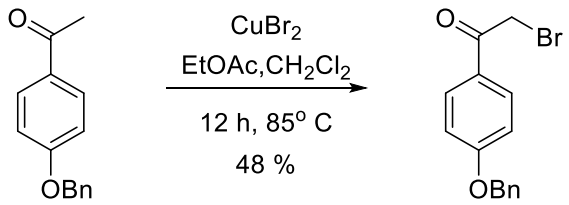
Synthesis of 1-(4-(benzyloxy) phenyl) ethan-1-one 4:



Compound **4** was synthesized according to reported procedure. In a 50 ml round bottom flask *p*-hydroxyacetophenone (2g, 14.68 mmol) was dissolved in CH_3CN followed by the addition of Benzyl bromide (2.63g, 15.42 mmol) and K_2CO_3 (8 g, 58.72 mmol). The reaction mixture was allowed to stir at room temperature overnight. Reaction was monitored by TLC. After completion of reaction salt formed

was filtered out and solvent was evaporated to get crude product. This crude product was purified using column chromatography (8% E.A/Hexane) to yield white solid compound **4.** Yield: 100 %. NMR data is matching with reported data.[12]

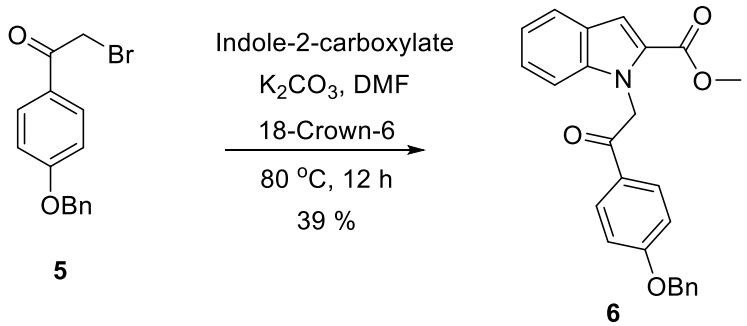
Synthesis of 1-(4-(benzyloxy) phenyl)-2-bromoethan-1-one 5:



4 **5**

Compound **5** was synthesized according to reported procedure. In a 50 ml round bottom flask compound **4** (1g, 4.41 mmol) was dissolved in $CHCl_3$ (6 mL) which was added to a suspension copper (II) Bromide in EtOAc (6 mL).The reaction mixture was stirred under reflux condition overnight. After completion of reaction solvent was filtered out passing through celite bed and was evaporated to get crude product. This crude product was purified using column chromatography (4% E.A/Hexane) to yield white crystalline compound **5.** Yield: 55 %. NMR data is matching with reported data.[9]

Synthesis of methyl 1-(2-(4-(benzyloxy) phenyl)-2-oxoethyl)-1H-indole-2-carboxylate 6:

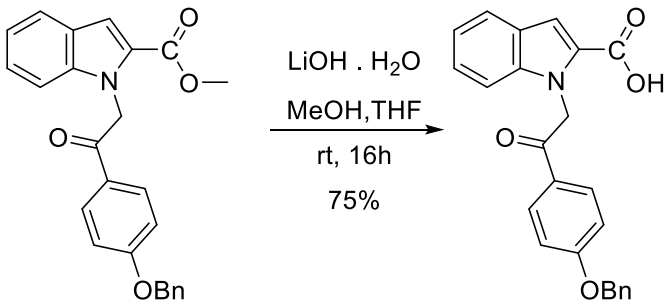


5 **6**

In a 50 ml round bottom flask, indole ester (693 mg, 3.9 mmol) was dissolved in DMF (5 mL) followed by the addition of compound **5** (1.19 g, 7.79 mmol) and K_2CO_3 (2.1 g, 15.6 mmol) under nitrogen atmosphere.18-Crown-6 was added to the reaction mixture as a catalyst. The reaction mixture was allowed to stir at 100˚C

overnight. Reaction was monitored by TLC. After completion of reaction DMF was evaporated by vacuum and crude product was washed with water and extracted using ethyl acetate. Then organic layer was washed with brine solution.[13] The product was purified using column chromatography (4% E.A/Hexane in 230-400 silica) to yield white solid compound **6. Yield:** 39 %. **^{1}H NMR (400 MHz, $CDCl_3$):** δ 8.08 – 8.02 (m, 2H), 7.73 (d, *J* = 8.0 Hz, 1H), 7.48 – 7.29 (m, 7H), 7.23 – 7.14 (m, 2H), 7.10 – 7.04 (m, 2H), 5.99 (s, 2H), 5.17 (s, 2H), 3.83 (s, 3H). **^{13}C NMR (101 MHz, $CDCl_3$):** δ 191.97, 163.25, 162.86, 139.95, 136.19, 130.46, 128.87, 128.43, 128.33, 127.60, 126.33, 125.54, 123.07, 121.07, 115.08, 111.32, 109.87, 70.33, 51.81, 50.69, 31.06.

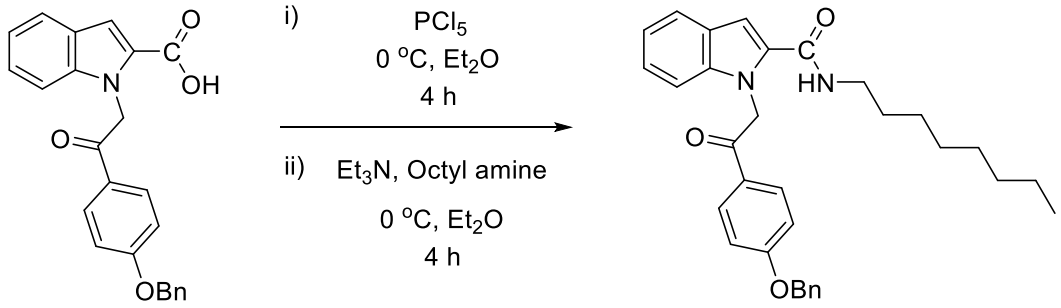
Synthesis of 1-(2-(4-(benzyloxy) phenyl)-2-oxoethyl)-1H-indole-2-carboxylic acid 7:



6 **7**

In a 50 ml round bottom flask, compound **6** (300 mg, 0.751 mmol) was dissolved in THF: MeOH: H_2O (3:1:1) mL followed by the addition $LiOH.H_2O$ (157.35 mg, 3.75 mmol) .The reaction mixture was allowed to stir at room temperature overnight. Reaction was monitored by TLC. After completion of reaction, 2N HCl was added till pH became 3.0. Compound was extracted using EtOAc after washing with H_2O and brine solution.[14] The product was purified using column chromatography (4% methanol/chloroform) to yield white solid compound **7. Yield:** 75 %. **^{1}H NMR (400 MHz, DMSO):** δ 8.11 – 8.05 (m, 2H), 7.71 (d, *J* = 8.0 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 7.49 (d, *J* = 7.9 Hz, 2H), 7.42 (dd, *J* = 8.0, 6.8 Hz, 2H), 7.39 – 7.33 (m, 1H), 7.29 (d, *J* = 7.4 Hz, 2H), 7.20 (d, *J* = 7.5 Hz, 2H), 7.13 (s, 1H), 6.11 (s, 2H), 5.26 (s, 2H). **^{13}C NMR (101 MHz, DMSO):** δ 192.75, 163.00, 162.66, 139.66, 136.52, 131.44, 130.45, 128.63, 127.99, 127.87, 125.63, 124.72, 122.29, 120.55, 115.00, 110.86, 109.92, 69.63, 50.94.

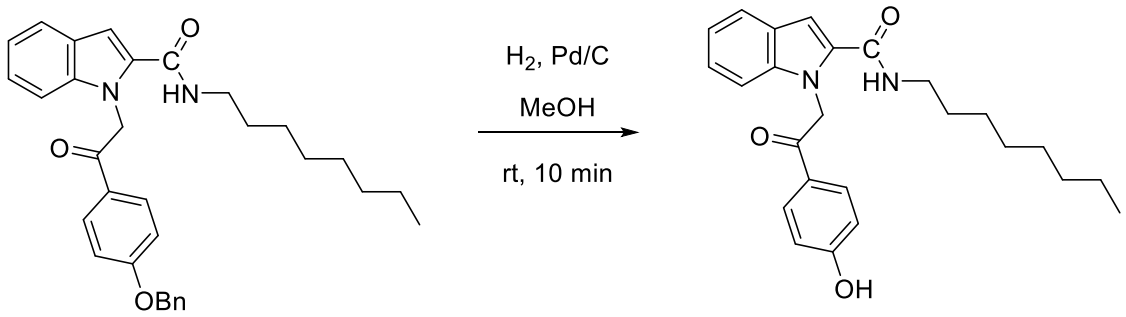
Synthesis of 1-(2-(4-(benzyloxy) phenyl)-2-oxoethyl)-N-octyl-1H-indole-2-carboxamide 8:



7 **8**

In a 50 ml round bottom flask, compound **7** (55 mg, .142 mmol) was dissolved in Et_2O and cooled under ice bath. PCl_5 (148 mg, 0.713 mmol) was added to this solution. The reaction mixture was allowed to stir at 0 °C for 3 hours. Reaction was monitored by TLC. When starting material was completely consumed, reaction mixture was directly taken for further reaction. The reaction mixture kept under ice bath, Et_3N was added followed by the addition of octyl amine. The mixture was allowed to stir for 10 minutes and monitored by TLC. On completion of reaction, Et_2O was removed under vacuum. Crude product was washed with water and extracted with EtOAc. Later, organic layer was washed with brine solution and dried over sodium sulfate. The product was purified using column chromatography (15% E.A/ Hexane) to obtain white solid. **Yield :** 93%.**^{1}H NMR (400 MHz, $CDCl_3$)** : δ 8.08 – 8.01 (m, 2H), 7.68 – 7.62 (m, 1H), 7.47 – 7.33 (m, 5H), 7.26 (s, 1H), 7.20 (d, *J* = 8.1 Hz, 1H), 7.17 – 7.11 (m, 1H), 7.08 – 7.02 (m, 2H), 6.93 (s, 1H), 6.29 (s, 1H), 6.08 (s, 2H), 5.16 (s, 2H), 3.30 (d, *J* = 6.2 Hz, 2H), 1.53 (d, *J* = 6.7 Hz, 2H), 1.37 – 1.21 (m, 10H), 0.91 – 0.84 (m, 3H). **^{13}C NMR (101 MHz, $CDCl_3$) :** δ 195.73, 166.45, 165.95, 142.40, 139.46, 135.29, 133.77, 132.11, 131.66, 130.85, 129.72, 127.65, 125.45, 124.13, 118.25, 113.01, 107.76, 73.55, 53.87, 42.91, 35.17, 32.97, 32.65 , 30.32, 26.02, 17.49.

Synthesis of 1-(2-(4-hydroxyphenyl)-2-oxoethyl)-N-octyl-1H-indole-2-carboxamide 9:



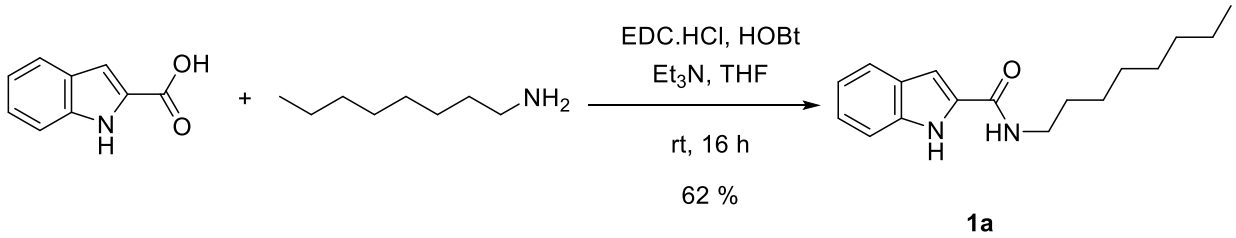
8 **9**

Compound **8** was taken in a round bottom flask dissolved in MeOH and was purged with nitrogen. Catalytic amount of Pd/C was added into the reaction mixture and hydrogen gas was purged through it for 15 minutes. Reaction was monitored using TLC. After completion of reaction the reaction mixture was passed through celite bed and solvent was evaporated to get crude product. Pure product was isolated using column chromatography (30% E.A/Hexane).**Yield:** 98%.**^{1}H NMR (400 MHz, DMSO) :** δ 10.44 (s, 1H), 8.47 (t, *J* = 5.7 Hz, 1H), 7.95 (d, *J* = 8.7 Hz, 2H), 7.65 (d, *J* = 7.9 Hz, 1H), 7.47 (d, *J* = 8.4 Hz, 1H), 7.24 – 7.16 (m, 2H), 7.09 (t, *J* = 7.4 Hz, 1H), 6.91 (d, *J* = 8.7 Hz, 2H), 6.09 (s, 2H), 3.14 (dd, *J* = 13.0, 6.7 Hz, 2H), 1.51 – 1.40 (m, 2H), 1.23 (s, 10H), 0.84 (t, *J* = 6.7 Hz, 3H). **^{13}C NMR (101 MHz, DMSO-D6) :** δ 192.31 , 162.45 , 161.70 , 138.78 , 131.84 , 130.54, 126.52, 125.76, 123.41, 121.49, 120.15, 115.35, 110.49, 104.44, 50.56, 38.58, 31.27, 29.15, 28.77, 28.70, 26.43 , 22.11 , 13.98.

Synthesis of free amide compounds 1a-c:

General procedure: Indole-2-carboxylic acid (1 eq) was taken in a 25 mL round bottom flask and was dissolved in dry THF followed by the addition of Et_3N. To this solution was added HOBt (1.2 eq) and EDC.HCl (1.2 eq) and was allowed to stir for 1 hour. Later corresponding amine was added to the mixture and the reaction mixture was allowed to stir overnight. On completion of reaction, THF was evaporated under vaccum and the crude product was washed with water and extracted using EtOAc, followed by washing with brine solution, dried over sodium sulfate. The pure product was isolated by column chromatography (12% E.A/ Hexane) to yield white solid compound in 80-90 % yield.

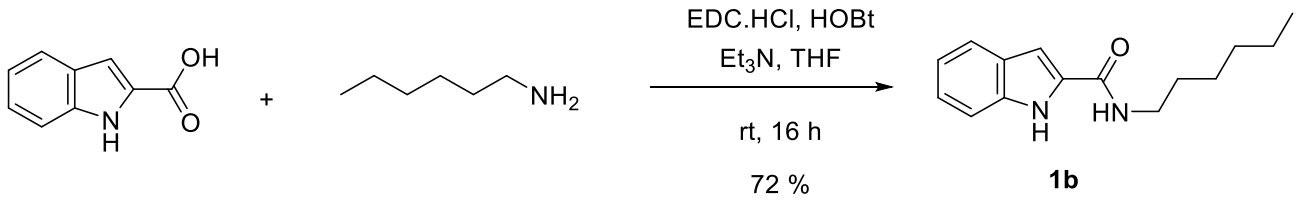
N-octyl-1H-indole-2-carboxamide:



1a

Yield : 62%.**^{1}H NMR (400 MHz, $CDCl_3$):** δ 9.76 (s, 1H), 7.64 (d, *J* = 8.0 Hz, 1H), 7.45 (d, *J* = 8.3 Hz, 1H), 7.26 (s, 1H), 7.14 (ddd, *J* = 8.0, 7.1, 0.9 Hz, 1H), 6.83 (s, 1H), 6.23 (s, 1H), 3.51 (dd, *J* = 13.5, 6.7 Hz, 2H), 1.65 (dt, *J* = 14.8, 7.3 Hz, 2H), 1.45 – 1.22 (m, 10H), 0.88 (t, *J* = 6.9 Hz, 3H). **^{13}C NMR (101 MHz, $CDCl_3$) :** δ 161.85, 136.48 , 131.04, 127.77, 124.46, 121.94, 120.70, 112.19, 101.69 , 76.84, 39.95, 31.93, 29.93, 29.40, 27.14, 22.78, 14.23.

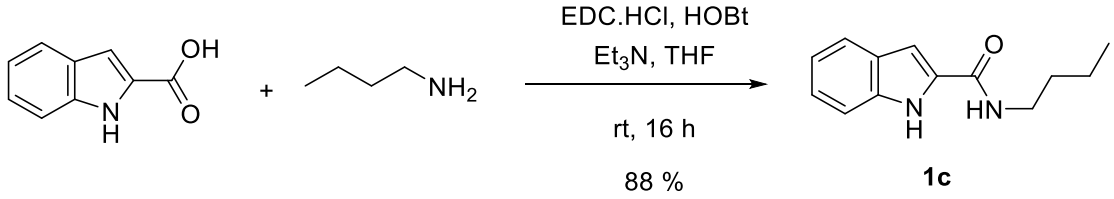
N-hexyl-1H-indole-2-carboxamide:



1b

Yield : 72 % **^{1}H NMR (400 MHz, $CDCl_3$) :** δ 9.44 (s, 2H), 7.67 (d, *J* = 8.0 Hz, 2H), 7.47 (d, *J* = 8.3 Hz, 2H), 7.35 – 7.28 (m, 3H), 7.16 (t, *J* = 7.5 Hz, 2H), 6.84 (s, 2H), 6.19 (s, 2H), 3.51 (q, *J* = 6.6 Hz, 4H), 1.67 (dt, *J* = 14.6, 7.2 Hz, 9H), 1.36 (td, *J* = 7.2, 3.8 Hz, 8H), 0.96 – 0.87 (m, 6H). **^{13}C NMR (101 MHz, $CDCl_3$)**: δ 161.89, 136.52, 131.03, 127.74, 124.44, 121.93, 120.68, 112.22, 101.70, 39.94, 31.64, 29.87, 26.80, 22.71, 14.17.

N-butyl-1H-indole-2-carboxamide:



1c

Yield : 88%.**^{1}H NMR (400 MHz, $CDCl_3$) :** δ 9.66 (s, 1H), 7.64 (dd, *J* = 8.0, 0.8 Hz, 1H), 7.45 (dd, *J* = 8.3, 0.8 Hz, 1H), 7.32 – 7.26 (m, 1H), 7.14 (ddd, *J* = 8.0, 7.0, 1.0 Hz, 1H), 6.82 (dd, *J* = 2.1, 0.8 Hz, 1H), 6.21 (s, 1H), 3.52 (td, *J* = 7.1, 6.1 Hz, 2H), 1.64 (t, *J* = 7.2 Hz, 2H), 1.50 – 1.40 (m, 2H), 0.98 (t, *J* = 7.3 Hz, 3H). **^{13}C NMR (101 MHz, $CDCl_3$)**: δ 161.85, 136.45, 131.01, 127.76, 124.48, 121.95, 120.72, 112.18, 101.70, 39.62, 31.96, 20.28, 13.93.

Chapter 3

Pro-carriers responsive to chemical stimuli

3.1 Objective

The aim of this project was to synthesize anion transporters based on a bicyclic moiety (bispidine) which has conformational preorganization and thus can form stable host-guest complexes with anions (Figure 3.1). The binding of anions can be through acidic amide hydrogen in the molecule. Preorganization in bispidine backbone helps in bringing two arms together for efficient binding with anion. The bicyclic system is heterocyclic with two highly basic nitrogen centers that can get protonated at physiological pH. Thus it forms a "proton sponge'' like system [N···H^+···N] and increases binding efficiency and co-transport anions.

$HClO_4$ can be used to protonate the molecule where ClO_4^- being a non-binding anion will stay at the vicinity of the protonated species (Figure 3.1 A). The ClO_4^- leaves the N-H binding site open for binding with chloride ions and remains as a counter anion. During the ion transport ClO_4^-can be replaced easily by chloride ions and will not affect the ion transport. Protonation is necessary for the molecule to transport anions. Hence, the chemical stimuli applied on the molecule make it an active anion transporter.

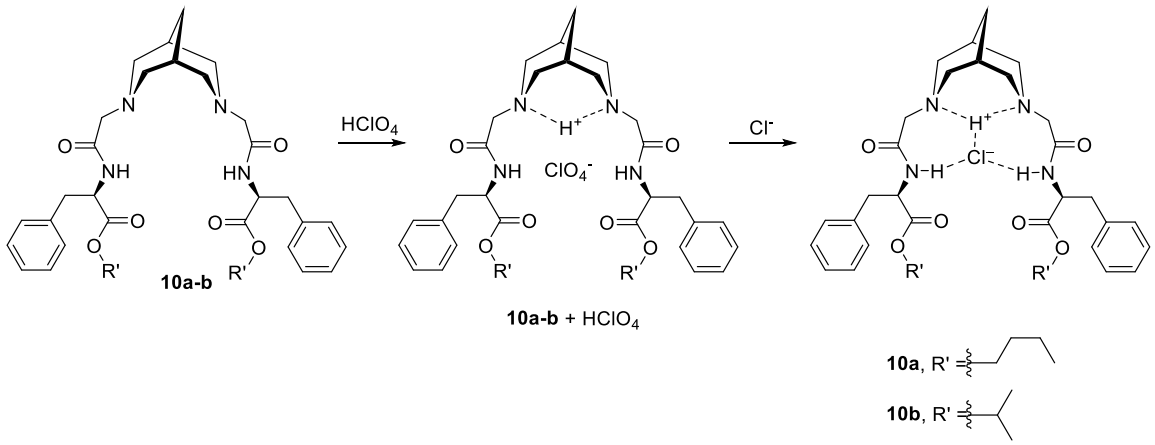
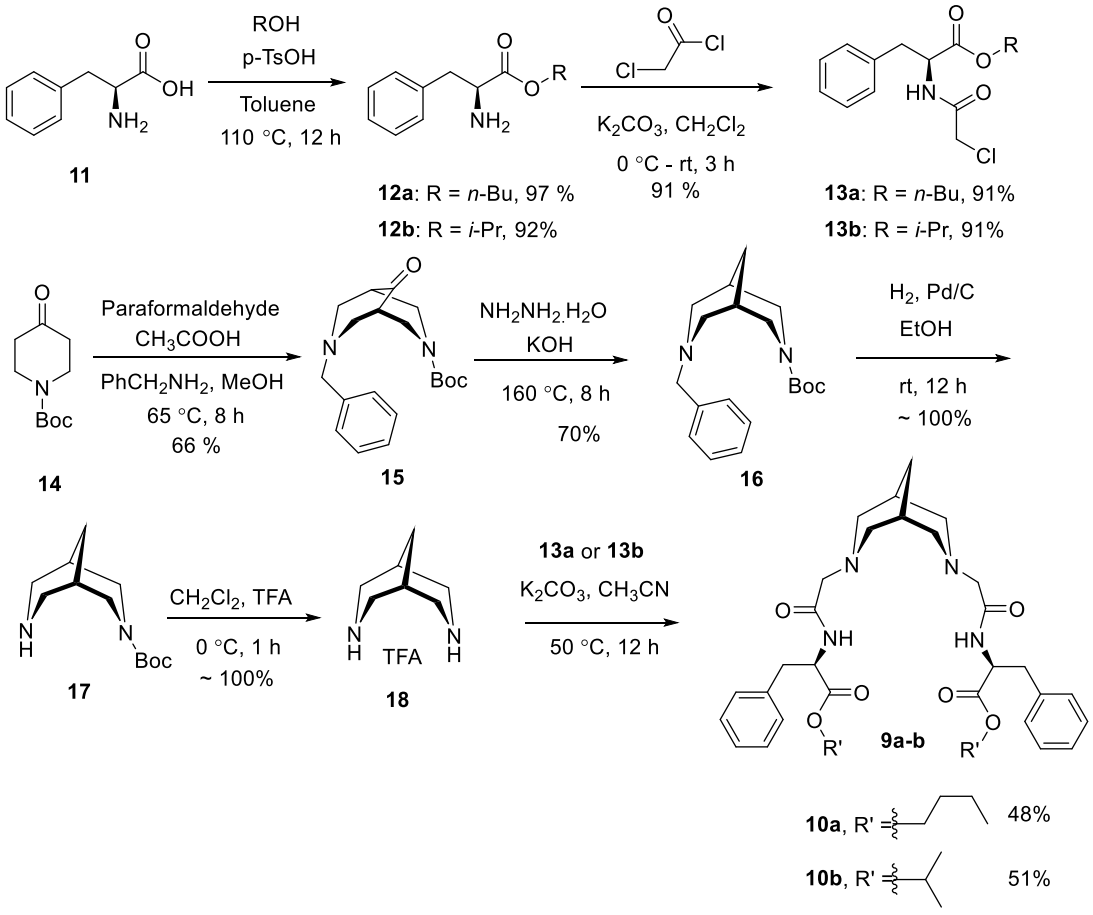


Figure 3.1. Formation of active anion transporters **10a-b**·$HClO_4$ and **10a-b**·HCl from the bispidine core based anion transporters **10a-b**.

3.2 Results and Discussion

3.2.1 Synthesis of bispidine-based derivatives:

Synthesis was started from commercially available L-Phenylalanine and boc-piperidone (Scheme 3). At first, the L-Phenylalanine **11** was converted to its esters **13a** or **13b** by reacting with isopropyl alcohol or n-butyl alcohol in the presence of p-TsOH and toluene in 92-97% yield. Then the reaction of esters with chloroacetyl chloride was done in the presence of K_2CO_3 which lead to the formation of **14a-b** in 77% and 91% yield respectively. The boc-piperidone **15** was converted to compound **19** in 4 steps according to reported procedure.[15] The final compounds were synthesized from compound **19** by reacting with **14a** or **14b** in presence of K_2CO_3 as a base in acetonitrile solvent in moderate yield.



Scheme 3. Synthesis scheme for bispidine core based anion transporters **10a**-**b**.

3.2.2 Ion transport activity by HPTS assay for the bispidine core based compounds

The ion transport activities of the bispidine based compounds across large unilamellar vesicles (EYPC vesicles) were checked by fluorescence kinetics experiments. For that the EYPC vesicles was prepared (entrapped with fluorescent dye HPTS, NaCl, HEPES buffer and inside pH 7.0) and suspended in a solution of pH 7.8. The destruction of pH gradient (either H^+ efflux or OH^- influx) was monitored with time after the addition of transporter molecules (**10a** and **10b**). Here the bispidine based compounds have displayed significant increase in fluorescence intensity (λ_{ex} = 450 nm and λ_{em} = 510 nm). Both bispidine core based compounds (**10a-b**) showed ion transport activity through phospholipid bilayer but with different result than expected. The increase in intensity was rapid at initial stage and then intensity remained constant which was quite unexpected as there was no constant increase in intensity. Compound **10a** showed oversaturation at 20 uM concentration.

The unexpected behavior by the compound in ion transport assay may be due to the deprotonation of HPTS by transporter molecules resulting in sharp increase in intensity which further becomes constant. To get some evidence in support of deprotonation of HPTS by transporter molecule we have done the fluorescence experiment by adding compound **10a** directly to 1 mM HPTS solution which has shown the increase in fluorescence intensity by gradual addition transporter molecule. From the result obtained it was confirmed that compound is deprotonating HPTS.

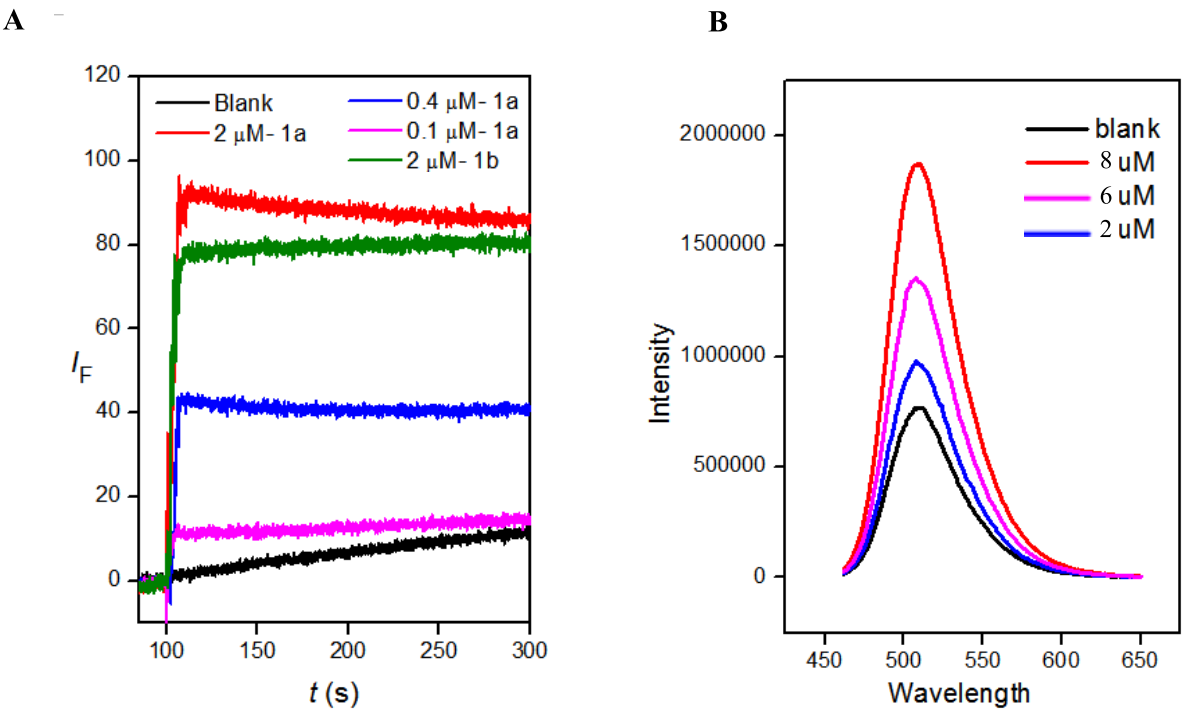


Figure 3.2. Ion transport activity of **10a** and **10b** across EYPC vesicles in different concentration, HPTS assay (A), and increase in intensity of HPTS dye on addition of compound **10a** in different concentration (B).

3.2.3 Ion transport activity by lucigenin assay for the bispidine based compounds

The ion transport activities of the bispidine based compounds across large unilamellar vesicles (EYPC vesicles) were checked by fluorescence kinetics experiments. For that the EYPC vesicles was prepared (entrapped with fluorescent dye Lucigenin, $NaNO_3$, HEPES buffer and inside pH 7.0) and suspended in a solution of pH 7.0. Salt gradient was brought by the addition of 25 µL of 2N NaCl. Compounds **10a-b** was added to check for transport activity and there was no significant decrease in the fluorescent intensity which indicates that the compounds are not transporting anions. Therefore it is confirmed that the compounds are non-transporter of chloride ions initially and can act as a pro-transporter which becomes active on protonation using $HClO_4$/HPF_6.Compound 10a was protonated using $HClO_4$ and ion transport activity was checked for the same. Increase in chloride ion influx was observed in lucigenin assay compared to the pro-carrier at similar concentration.

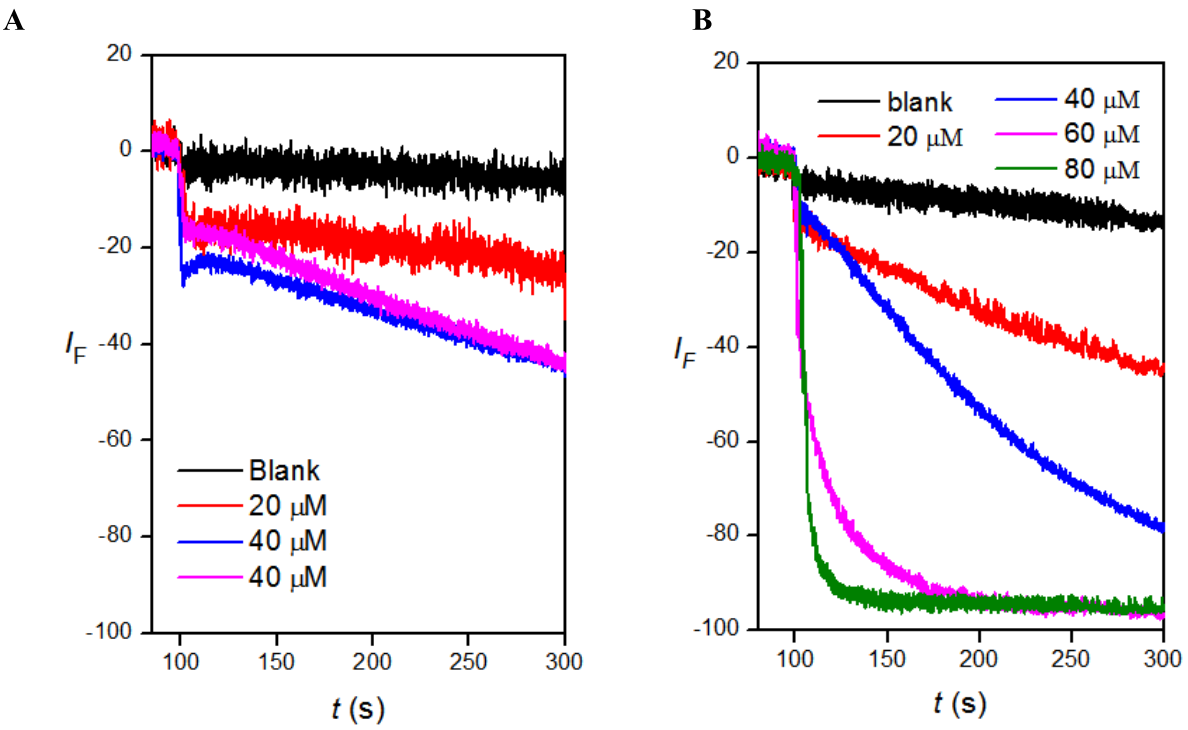


Figure 3.3. Ion transport activity of compound **10a** at 20 and 40 μM concentration using lucigenin assay(A), compound **10a**.**HClO$_4$** (B)

3.3 Conclusion

The pro-transporter of bispidine derivatives proved that they abstract protons from HPTS dye resulting in the increase in intensity in HPTS assay. From the lucigenin assay, it is confirmed that compound **10a-b** initially is a non-transporter before protonation. Protonation of these molecules with $HClO_4$/HPF_6 is found to make the transporter active.

3.4 Future Plans

The future plans include confirming the protonation of the molecule with $HClO_4$/HPF_6 using NMR spectroscopy and checking the ion transport activity of the same using lucigenin and HPTS assay.

3.5 Experimental Section:

3.5.1 Ion transport activity using HPTS assay:

Preparation of buffer and stock solution for HPTS assay:

HEPES buffer was prepared by dissolving HEPES (10 mM) and NaCl (100Mm) in autoclaved water. The pH of the solution was adjusted to 7.0 by adding NaOH solution and was monitored using the pH meter. HPTS dye was added later into this buffer to make 1 mM solution of HPTS in the buffer. All the compounds were dissolved in HPLC grade DMSO solvent to make stock solutions.

Preparation of EYPC- LUVs ⊂ HPTS

1 mL off egg yolk phosphatidylcholine (EYPC, 25 mg/mL in $CHCl_3$) was taken in a round bottom flask. It was dried by purging nitrogen along with continuous rotation to form thin film of EYPC. Then round bottom flask was kept in high vacuum for 6 h to remove trace amount of $CHCl_3$. Later 1 mL buffer (1 mM HPTS, 10 mM HEPES buffer, 10 mM NaCl, pH = 7.0) was added into the thin film to hydrate and was vortexed occasionally (4-5 times) for 1 h. Then it was subjected to free-thaw cycle (> 15 times). Extrusions were done using mini extruder with polycarbonate membrane (pore diameter of 100nm). All extra vesicular dyes were removed by gel filtration with Sephadex G-50 using same buffer without HPTS dye. The vesicle obtained was diluted to 6 mL using the same buffer to get EYPC-LUVs ⊂ HPTS ~ 5.0 mM EYPC; inside: 1 mM HPTS, 10 mM HEPES, 10 mMNaCl, pH = 7.0. Outside: 10 mM HEPES, 100 mM NaCl, pH = 7.0.

Determination of Ion transport activity by HPTS assay:

In a clean and dry fluorescence cuvette, 1975 µL of HEPES buffer (10 mM HEPES, 100 mM NaCl, pH =7.0) was taken and into it 25 µL of EYPC-LUVs ⊂ HPTS was added and this cuvette was placed on fluorescence instrument equipped with magnetic stirrer (t = 0 s). Fluorescence emission intensity of pH sensitive dye HPTS, F_t was monitored at λ_{em} = 510 nm (λ_{ex} = 450 nm). Then a pH gradient was created

between intra and extra vesicular system by adding 20 µL of 0.5 N NaOH to the same cuvette at t = 20 s. Compounds were added at t = 100 s each time and finally at t=300 s, 25 µL of 10% Triton-X-100 was added to lyse all the vesicles which resulted in the destruction of pH gradient and saturation of fluorescence intensity.

Fluorescence time courses (*F*t) were normalized to fractional emission intensity *I*F using Equation given below:

$$\%\ F_I\ Intensity\ (I_F) = [(F_t - F_0) / (F_\infty - F_0)] \times 100$$

Where F_0 = Fluorescence intensity just before the tripodal molecule addition (at 0 s). F_∞ = Fluorescence intensity at saturation after complete leakage (at 275 s). F_t = Fluorescence intensity at time *t*.

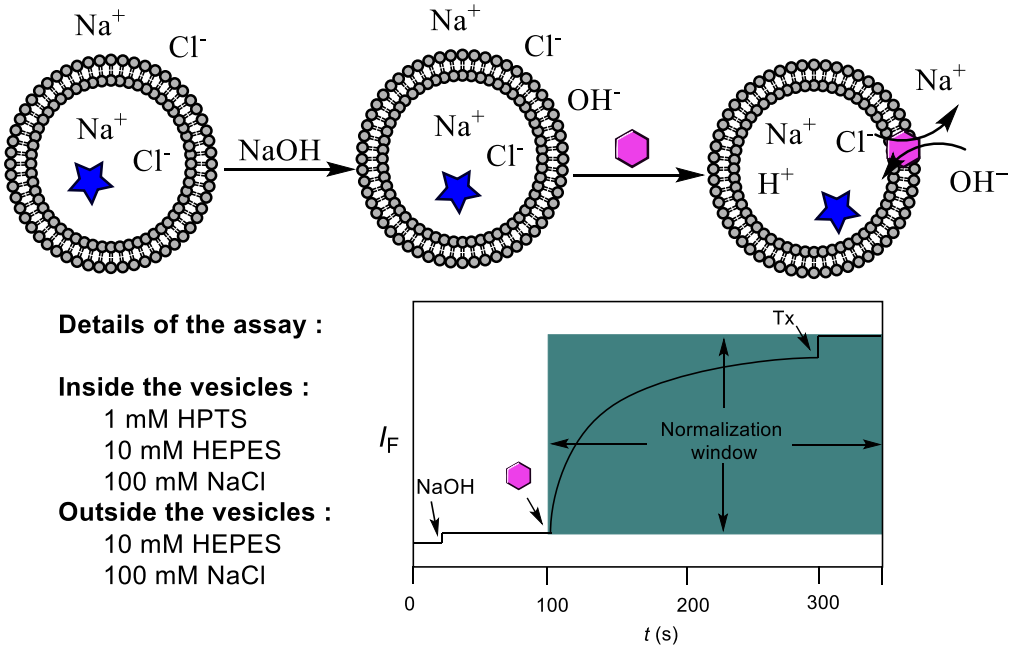
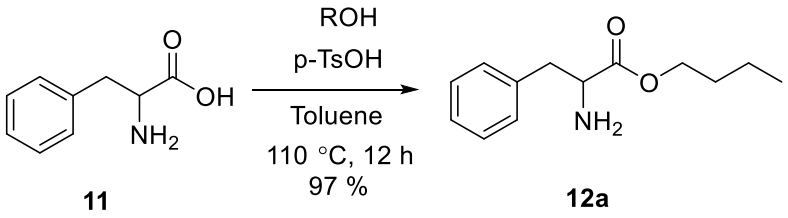


Figure 3.4: Schematic representation of ion transport activity determination across liposomal vesicles using HPTS assay

3.5.2 Synthetic procedures

Compound **18** was synthesized according to reported procedure.[15]

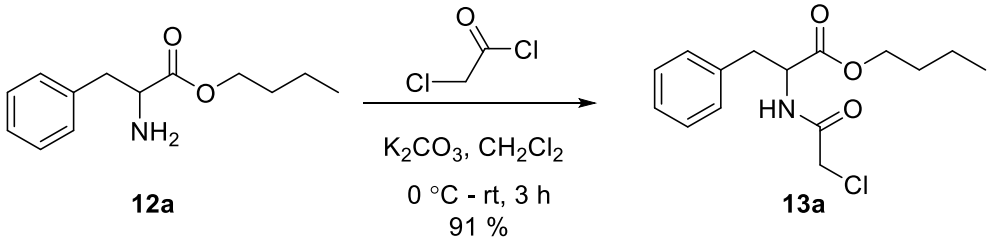
Synthesis of Butylphenylalaninate 12 a:



11 **12a**

In a 25 ml round bottom flask, phenylalanine **11** was taken (400 mg, 2.42 mmol) and was dissolved in ~30 mL of Toluene. n-butanol (801 µL, 2.90 mmol) was added into it followed by the addition of *p*-toluenesulfonic acid (499 mg, 2.90 mmol). The reaction mixture was allowed to stir at 110 °C overnight. Reaction was monitored by thin layer chromatography. After completion of reaction solvent was evaporated using rotary evaporator and crude product was washed with 1M NaOH solution, compound was extracted using 2% MeOH/$CHCl_3$ and was washed with brine solution.[16] The crude product was purified by column chromatography to afford pure product in 97% yield.**^{1}H NMR (400 MHz, $CDCl_3$)** δ 7.29 (ddd, *J* = 18.6, 11.3, 5.9 Hz, 5H), 4.15 (t, *J* = 6.7 Hz, 2H), 3.76 (dd, *J* = 7.8, 5.4 Hz, 1H), 3.12 (dd, *J* = 13.5, 5.4 Hz, 1H), 2.91 (dd, *J* = 13.5, 7.8 Hz, 1H), 1.83 – 1.51 (m, 2H), 1.39 (dt, *J* = 15.1, 7.4 Hz, 2H), 0.96 (t, *J* = 7.4 Hz, 3H).NMR matching with reported compound.

Synthesis of butyl (2-chloroacetyl) phenylalanine 13a:

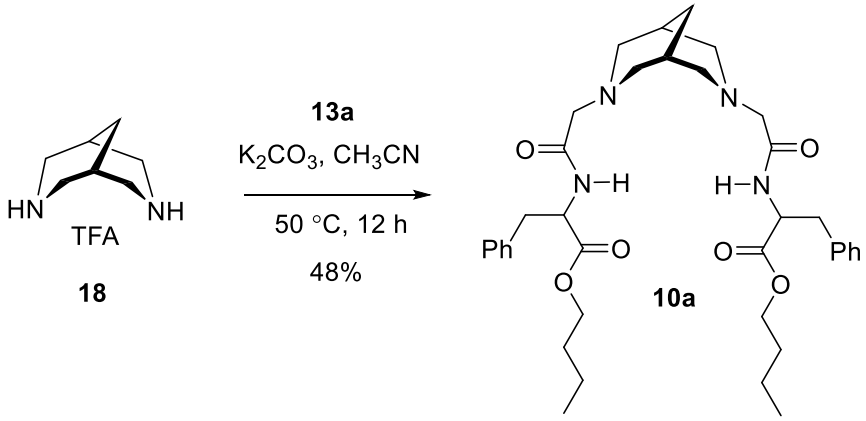


12a **13a**

In a 25 ml round bottom flask, compound **12a** was taken (200 mg, 0.903mmol) and was dissolved in 7 mL of dry CH_2Cl_2 followed by the addition of K_2CO_3 and cooled under ice bath. Chloroacetyl chloride (90 µL, 1.083mmol,) was added into it under nitrogen atmosphere. Reaction progress was monitored by TLC. On completion of reaction, the reaction mixture was washed with water followed by extraction of compound using CH_2Cl_2 which was washed with brine solution.[17] The crude product obtained was purified using column chromatography to yield pure product in 91% yield.**^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.29 (d, *J* = 7.4 Hz, 3H), 7.13 (d, *J* = 6.8 Hz, 2H),

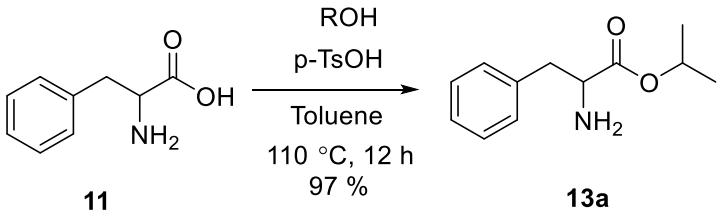
6.99 (d, *J* = 7.5 Hz, 1H), 4.86 (dd, *J* = 13.8, 6.0 Hz, 1H), 4.28 – 4.08 (m, 2H), 4.03 (s, 2H), 3.15 (dd, *J* = 5.9, 2.6 Hz, 2H), 1.58 (dd, *J* = 14.8, 6.7 Hz, 2H), 1.33 (dd, *J* = 15.0, 7.5 Hz, 2H), 0.92 (t, *J* = 7.4 Hz, 3H). NMR is matching with reported compound.

Synthesis of dibutyl 2,2'-((2,2'-(3,7-diazabicyclo[3.3.1]nonane-3,7-diyl)bis(acetyl))bis(azanediyl))bis(3-phenylpropanoate) 10a:



In a 25 ml round bottom flask, compound **18** was taken (41 mg, 0.324mmol) and was dissolved in ~2 mL of dry DMF. Et_3N (452 µL, 3.24 mmol) was added into it under nitrogen atmosphere. To this compound **13a** (241 mg, .81 mmol) was added and reaction mixture was kept for stirring at 50 °C for 12 hours. Reaction progress was monitored using TLC. On completion of reaction DMF was evaporated using high vaccum and reaction mixture was washed with water, compound extracted using DCM and washed with brine solution.[18] The crude product was purified by column chromatography to yield pure product in 48% yield.**^{1}H NMR (400 MHz, $CDCl_3$)**: δ 7.54 (t, *J* = 33.4 Hz, 2H), 7.36 – 6.91 (m, 10H), 4.79 (q, *J* = 6.7 Hz, 2H), 4.09 – 3.94 (m, 4H), 3.15 (qd, *J* = 14.0, 6.5 Hz, 4H), 3.00 (d, *J* = 16.2 Hz, 2H), 2.75 (d, *J* = 16.2 Hz, 2H), 2.64 (d, *J* = 10.7 Hz, 2H), 2.47 (d, *J* = 10.5 Hz, 2H), 2.27 (dd, *J* = 22.7, 9.7 Hz, 4H), 1.70 (s, 2H), 1.60 – 1.47 (m, 4H), 1.42 (s, 2H), 1.36 – 1.21 (m, 5H), 0.98 – 0.79 (m, 6H). **13C NMR (101 MHz, $CDCl_3$):** δ 171.87, 170.97, 136.53, 129.37, 128.51, 126.98, 77.48, 77.16, 76.84, 65.30, 61.78, 57.78, 57.54, 53.05, 37.81, 30.82, 30.60, 29.66, 19.16, 13.79.

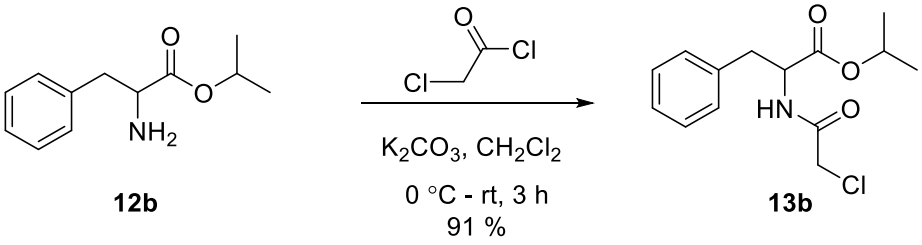
Synthesis of Isopropylphenylalaninate 12b:



11 **13a**

In a 25 ml round bottom flask, phenylalanine was taken (200 mg, 1.21) and was dissolved in ~30 mL of toluene. Isopropyl alcohol (111 µL, 1.45 mmol) was added into it followed by the addition of *p*-toluenesulfonic acid (249mg, 1.45 mmol). The reaction mixture was allowed to stir at 110 °C overnight. Reaction was monitored by thin layer chromatography. After completion of reaction solvent was evaporated using rotary evaporator and crude product was washed with 1M NaOH solution, compound was extracted using 2% MeOH/$CHCl_3$ and was washed with brine solution.[16] The crude product was purified by column chromatography to afford pure product in 62% yield.**^{1}H NMR (400 MHz, $CDCl_3$)**:δ 7.44 – 6.81 (m, 5H), 5.02 (hept, *J* = 6.3 Hz, 1H), 3.67 (dd, *J* = 7.8, 5.6 Hz, 1H), 3.06 (dd, *J* = 13.5, 5.5 Hz, 1H), 2.86 (dd, *J* = 13.5, 7.8 Hz, 1H), 1.21 (dd, *J* = 18.0, 6.3 Hz, 6H).NMR is matching with reported compound.

Synthesis of Isopropyl (2-chloroacetyl) phenylalaninate 13b:

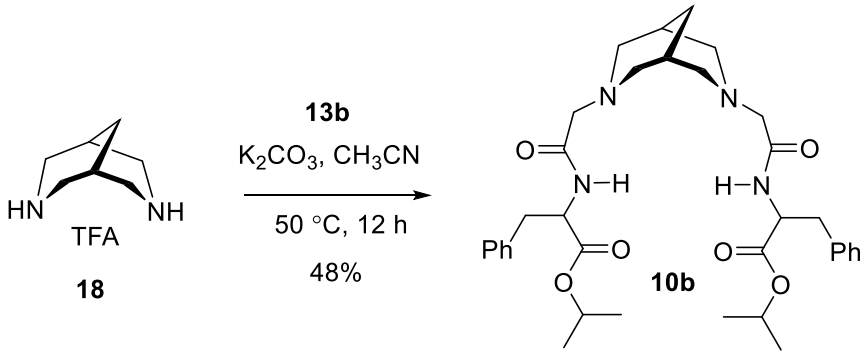


12b **13b**

In a 25 ml round bottom flask, compound **12b** was taken (200 mg, .903 mmol) and was dissolved in ~7 mL of dry CH_2Cl_2 followed by the addition of K_2CO_3 and cooled under ice bath. Chloroacetyl chloride (90 µL, 1.083 mmol) was added into it under nitrogen atmosphere. Reaction progress was monitored by TLC. On completion of reaction, reaction mixture was washed with water followed by extraction of compound using CH_2Cl_2 which was washed with brine solution.[17] The crude product obtained was purified using column chromatography to yield pure product in 91% yield.**^{1}H NMR (400 MHz, $CDCl_3$):**δ 7.28 (ddd, *J* = 16.5, 7.8, 2.9 Hz, 3H), 7.18 – 7.11

(m, 2H), 6.99 (d, *J* = 7.2 Hz, 1H), 5.02 (dq, *J* = 12.5, 6.3 Hz, 1H), 4.81 (dt, *J* = 7.9, 6.0 Hz, 1H), 4.07 – 3.98 (m, 2H), 3.19 – 3.09 (m, 2H), 1.22 (t, *J* = 6.6 Hz, 6H).NMR is matching with reported compound.

Synthesis of diisopropyl 2,2'-((2,2'-(3,7-diazabicyclo[3.3.1]nonane-3,7-diyl)bis(acetyl))bis(azanediyl))bis(3-phenylpropanoate) 10b:



In a 25 ml round bottom flask, compound **18** was taken (83 mg, 0.663 mmol) and was dissolved in ~ 2 mL of dry DMF. K_2CO_3 (916 mg, 6.63 mmol) was added into it under nitrogen atmosphere. To this, compound **13b** (470 mg, 1.657 mmol) was added and reaction mixture was kept for stirring at 50 °C for 12 hours. Reaction progress was monitored using TLC. On completion of reaction DMF was evaporated using high vacuum and reaction mixture was washed with water ,compound extracted using DCM and washed with brine solution.[18] The crude product was purified by column chromatography to yield pure product in 51% yield**.^{1}H NMR (400 MHz, $CDCl_3$):** δ 7.64 (t, *J* = 16.1 Hz, 2H), 7.44 – 6.98 (m, 10H), 5.09 – 4.87 (m, 2H), 4.73 (q, *J* = 6.5 Hz, 2H), 3.24 – 3.05 (m, 4H), 3.00 (t, *J* = 11.3 Hz, 2H), 2.84 (s, 2H), 2.75 (d, *J* = 16.2 Hz, 2H), 2.65 (d, *J* = 9.6 Hz, 2H), 2.48 (d, *J* = 10.4 Hz, 2H), 2.35 – 2.20 (m, 2H), 1.86 (s, 2H), 1.69 (s, 2H), 1.42 (s, 6H), 1.29 – 1.06 (m, 6H). **^{13}C NMR (101 MHz, $CDCl_3$):** δ 171.26, 170.95, 136.52, 129.49, 128.45, 126.95, 69.18, 61.71, 57.77, 57.48, 53.13, 37.78, 30.83, 29.63, 21.84.

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