Transmembrane ion transport by calixarenes

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Abstract

Regulation of transmembrane ion transport is a vital aspect in the maintenance of a healthy organism. To understand how this highly selective process occurs, how it can become impaired and how impairment may be treated, model compounds are useful tools. Several systems are presently being explored but one of the most widely applicable combines a rigid macrocycle, capable of size-based ion recognition, with membrane spanning substituents that allow the target ions to transverse a phospholipid bilayer. The calixarene class of macrocycles is ideally suited to this task. Previous work had shown that oxacalix[3]arenes could act as models for the filters in natural transmembrane ion channels. Nitrogen-containing analogues of these calixarenes, azacalix[3]arenes, were investigated with a view to constructing a chloride transporting system. Synthetic difficulties encountered when introducing lower rim substitutents precluded the use of azacalix[3]arenes and attention turned to 4-t-butylcalix[*n*]arenes. 4-t-Butylcalix[4]- and [6]arenes were derivatised with a commercial, membrane disrupting surfactant, Triton X-100®, forming compounds designed to form lipid bilayer-spanning, channel-like structures. The ion transporting ability of these, and other bilayer-spanning *O*-substituted calixarene derivatives, was determined by planar bilayer electrophysiological methods. Results showed that this modular approach to artificial ion channel construction was successful; calixarene derivatives formed transmembrane channels that allowed sodium ions to pass through but not the larger potassium ions.

Table of contents

List of Figures

List of Schemes and Tables

List of Abbreviations

THF	Tetrahydrofuran
NaOH	Sodium hydroxide
CH_2Cl_2	Dichloromethane
HCl (aq)	Hydrochloric acid
NaCl	Sodium chloride
NaH	Sodium hydride
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(2-aminoethyl)ether-*N*,*N*,*N*,*N*-tetra acetic acid
$CaCl_2$	Calcium chloride
$MgCl_2$	Magnesium chloride
NMR	Nuclear Magnetic Resonance
CPE	Buffer mix of citric acid, disodiumhydrogen phosphate and EDTA
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethansulfonic acid)

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Finally to my children Yasmin and Imran, you have been my rock! Thank you!

Thanks to God for everything

Dedication

I dedicate this thesis to my mum and my children Yasmin and Imran for all the unconditional love that I received from them.

Declaration

I declare that the research contained in this thesis, unless formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree, and does not incorporate any material already submitted for a degree.

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Chapter 1

1.1 Introduction

Several families of ion channel forming proteins allow transport of sodium (Na^+), potassium (K^+), calcium (Ca^{2+}), magnesium (Mg^{2+}), protons (H^+) and chloride (Cl^-) across cell membranes (Yu 2005; Swartz 2008; Payandeh 2011; Waldmann 1997; Jiang 2003; Bernardi 1999; Yan 2010). Similar structures almost certainly exist to selectively transport other ions such as the transition metals present in enzymes and metalloproteins (Althaus 1999). Although these ion channels are formed by immensely complex polypeptides some simple compounds, for example the bee sting protein melittin (Ruben 1996; Becucci 2007; Yang 1997) and antibiotic peptides (Wade 1990; Sengupta 2008) also induce transmembrane ion transport. These observations indicate that small molecules can mimic natural systems and inform artificial models of ion channel formation (Cragg 2002).

All cells contain cations and anions at specific concentrations that may vary greatly from the extracellular concentrations of those same chemical species thus the management of transmembrane ion flux is a crucial biological process (Ashcroft 1999). For example, the extracellular concentration of Na^+ is typically fifteen times greater than inside the cell. The reverse is true for K^+, which is thirty times more concentrated within cells than in the extracellular fluid. The difference between intra- and extracellular concentrations allows dynamic equilibria to power chemical reactions within cells (Cragg 2002).

As small variations in the intracellular concentrations can mean the difference between the cell living or dying, much work has been done to understand the mechanisms underlying

transmembrane ion transport and, in particular, the activation and inactivation of transmembrane ion channels (Prakriya 2002; Prawitt 2003; Thurm 2005; Sokolov 2008; Geiger 2009; Edgerton 2010; Okada 2011). The efficient function of ion channels is necessary if electrolytic homeostasis is to be maintained: when it is compromised through chemical attack or genetic misreading, the consequences for the health of the organism can be profound (Ashcroft 1999). Inactivation can be brought about by a wide range of toxins particularly those which block ion channels with positively charged groups (Elliott 1993; Huang 2004; Zhang 2007; Fletcher 2007; Sokolov 2008). The effective operation of these channels is also compromised when compounds keep them open or induce repetitive firing.

Na^+ channels are blocked by guanidinium-derived toxins, such as saxitoxin from shellfish (Wang 2003), and kept open by aconitine, from wolfsbane (Ceatele 2000), and the 'red tide' toxin, *Ptychodiscus brevis* (Purkerson 1999). K^+ channels are subject to a wider variety of inactivating compounds including the potassium channel blocking drugs tolbutamide and glibenclamide, both of which are sulphonylurea antihypoglycemic drugs used in the management of diabetes (Ashfield 1999; Edwards 1993).

Conditions resulting from defective Na^+ channels range from muscle related conditions, including temporary paralysis (Hurd 1996) and cardiac arrhythmia (Shinlapawittayatorn 2011), to neuronal diseases (Jentsch 2004), some of which may lead to epilepsy. Mutations in human skeletal muscle are associated with several forms of temporary, periodic and K^+-induced paralysis. Long QT syndrome, an arrhythmic cardiac disorder associated with fainting and sudden death, is associated with mutations in both Na^+ and K^+ channels. Compromised K^+ channel function has also been linked to many conditions including certain forms of ataxia, diabetes mellitus, epilepsy and muscular dystrophy (Jentsch 2004). A

particular theme running through many of these conditions is the effect of channel inactivation on neuronal function. As neurones react to electrochemical stimuli induced by channel activity any loss of that activity reduces their ability to function effectively. Neurodegeneration often becomes progressive as response to neurotransmitters decreases and the conditions worsen (Ashcroft 2000; Cragg 2002).

The impairment of Cl^- ion channel activity is well known to cause conditions such as cystic fibrosis (Collins 1992; Gabriel 1993), the most common lethal genetic defect in Caucasian populations, with an estimated 7500 sufferers in the UK alone. It arises from mutations in a protein that acts both as a Cl^--specific channel and as a regulator of other ion channels. These mutations reduce the ability of affected cells, predominantly epithelial cells lining the lung, to transport chloride effectively, which in turn affects fluid transport. Reduced fluid transport dehydrates mucosal secretions, leading to obstruction of organ passages, causing widespread and serious organ malfunction (Iqbal 2007a).

Model systems can help determine how transmembrane ion transport occurs, how it is sometimes impaired, and how impairment of malfunction can be rectified. The development of simple models to understand the mechanisms of ion channel function therefore has profound and wide reaching implications for health and a clear relevance to the design of therapeutics and strategies for management of disease. Artificial ion channels may be a way to treat such diseases. As the key interactions between ion channels and their targets are non-covalent, one approach to artificial ion design and synthesis is through the application of supramolecular chemistry (Cragg 2005).

1.2 Transmembrane ion transport

Ions traverse the cell membrane either actively, where chemical energy is required to facilitate transport (often against the cell's osmotic or electrochemical potential), or passively, where transmembrane diffusion requires no external energy and follows the concentration gradient. There are two general mechanisms by which inorganic ions are transported between extra- and intracellular environments: ionophore-mediated transport and the activation of transmembrane channels containing selectivity filters (Heinemann 1992; Zhou 2001, Dutzler 2002; Payandeh 2011) that allow the perfusion of particular species (Fig. 1.1). The former mechanism requires an ion-specific ligand to bind and transport species across the cell's phospholipid membrane at a rate of 10^4 ions per second. In the latter a physical channel is opened between the interior and exterior aqueous environments allowing transport at rates of 10^8 ions per second or higher (Iqbal 2007a). These differences in transport rate together with the average duration of each individual conduction event, as assessed by techniques such as patch clamping (see Chapter 3), give an indication of the mechanism. Relatively long 'open' events allowing high transport rates are indicative of channels whereas shorter, more sporadic activity is indicative of ionophore or gramicidin-type transport.

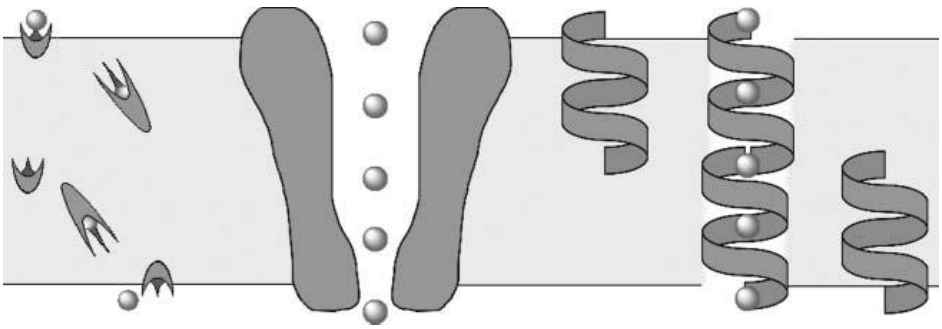


Figure 1.1 *Transmembrane ion transport mechanisms: ionophore (left), single molecule channel (centre), gramicidin-like aggregation (right) (from Iqbal 2007a)*

Ionophores and transmembrane ion channels must both discriminate in favour of one ion to the exclusion of all others if they are to control intracellular ionic concentrations (Licen 2009). Cyclic ionophores, like valinomycin (Duax 1996), achieve selectivity through a match between cavity size and the target ion's preferred coordination chemistry. The binding process must be reversible if the ion is ever to be released once across the lipid bilayer, however the complex must remain intact during its passage through the hydrophobic centre (Cragg 2002).

How do these process work? In the case of an ionophore-mediated mechanism a ligand binds to the ion and initiates water removal at the aqueous phase/bilayer interface. As well as binding the specific ion, the ligand shields it from the lipophilic region of the membrane by encapsulating the metal ion and striping off most or all of the water molecules that are bound to it. A change in the conformation of the ionophore follows, allowing the transport of the ion across the lipid bilayer. This also provides a lipophilic external surface for the complex that moves across the phospholipid membrane. Once this process has occurred it is reversed as the complex emerges from the opposite face of the bilayer and releases the ion, as long as it is not too tightly bound. The second mechanism for transmembrane ion transport is the activation of ion channels where a physical channel between the intra- and extracellular aqueous environments opens in response to a particular stimulus allowing the transport of ions (Cragg 2002). Ion channels incorporate a constricted region in the pore through which a selected ion may pass. While the design and synthesis of novel ionophores is an interesting challenge, models of ion channel activity are of more relevance to this project.

1.3 Ion channels

A cell membrane consists of phospholipids with water-soluble phosphate head groups attached to long lipid tails and are examples of amphiphiles. In an aqueous environment like the body's, the head groups are attracted to the surrounding medium (hydrogen bonding, dipolar interactions) while the organic tail is repelled. The result is a bilayer arrangement in which the organic components are all hidden away from the solvent while the hydrophilic portions face out (Cragg 2002).

The cell membrane (Fig. 1.2) is host to a variety of macromolecules having specific functions that can penetrate, or bind to, its surfaces. The bilayer may move to incorporate these macromolecules but remains unbroken unless seriously compromised. Changes in polarity through the lipid bilayer ensure that it forms an effective barrier between the extra and intracellular environment (Cragg 2002).

Figure 1.2 *The structure of a cell membrane (http://homepage.psyutexas.edu/ homepage:\winnt\profile).*

Transmembrane ion channels are a vital component of every cell membrane. They are able to regulate the transport of cations and anions across the phospholipid bilayers that form the

cellular boundary to optimise the intracellular concentrations of specific ions. Analysis of natural transmembrane ion channels show that they fall into two categories: those in which ions move through a pore formed at the confluence of several protein subunits, as in the voltage-gated potassium channel, KcsA (Doyle 1998) and those where they pass through channels formed within a single protein such as the ClC chloride channel (Dutzler 2002; Youxing 2003).

Ion channels exhibit three main properties which include their ability to catalyse high transport rates, the ability to discriminate between similar ions such as Na^+ and K^+ using a selectivity filter and the ability to be regulated by external stimuli such as ligand binding or a change in transmenbrane voltage (Woolley 2003; Fyles 1997; Mackinnon 2004). All channels must be able to open and close in response to external stimuli, particularly when preserving the optimal inter and extracellular concentration of ions. This 'gating' can occur through a variety of mechanisms. (Woolley 2003). Voltage gating of some K^+ channels occur when arginine residues react to transmembrane potential by moving through the phospholipid membrane to mechanically close the channel (Jiang 2003).

A similar reaction to potential may be responsible for glutamate blocking the ClC Cl^- channel. Small molecules may block a channel directly, by binding to the channel opening, or indirectly, by binding to a secondary site that signals a conformational change through an allosteric effect. Some channel-forming proteins respond mechanically to stimuli such as phospholipid tension, others to change in pH, where protonation disrupts intermolecular interactions that allow molecules to bridge the channel (Iqbal 2007a). Many ion channels that occur naturally in the body are high molecular weight membrane-spanning proteins and are believed to act via multiple α-helical strands that cross the membrane bilayer. The peptide

monomers such as alamethicin and gramicidin are examples of the most researched natural ion channels (Cragg 2002). Gramicidin is naturally synthesized by *Bacillus brevis* and forms a tubular helix channel in lipid membranes (Mobashery 1997) that is selective for monovalent cations. The mechanism of the gramicidin ion channel is unique. This pentadecapeptide is composed of alternating D- and L-amino acid residues. To form a channel, two molecules of gramicidin span the membrane via formation of an end-to-end dimer held together by hydrogen bonds as shown in Fig. 1.3.

Figure 1.3 *Gramicidin dimerization (Townsley 2001; PDB ID: 1JNO)*

The ion passage occurs through cation interactions with carbonyl oxygens on the inside the helix (Duax 2003; Burkhart 1998). Pohorille and colleagues studied molecular dynamic simulations of unassisted Na^+ and Cl^- ion transport across the bilayer membrane and observed that this generates a deep, local defect that allows the solvated water molecules and polar phospholipids head-groups to also enter the membrane (Wilson 1996). When the ion crosses the bilayer and is at the middle, the local defect switches to the outgoing side. Consequently, the ion remains solvated by water or by the phospholipid head-groups throughout the process.

Though it has limited similarity to a majority of natural channels, it is great interest in research of synthetic mimics. A better understanding of the mechanism of assisted ion transport came after the crystal structures of the two prokaryotic CLC Cl^- channels (Duzler 2002) and the KscA K^+ channel (Doyle 1998) were published.

Ion channel models, such as those in Fig. 1.4, are of particular interest as they allow different aspects of transmembrane transport to be analysed, for example the structural changes that can alter ion specificity or flux. To perform adequately the artificial channel must span a cellular phospholipid bilayer, implying a concerted length in excess of 4 nm, exhibit specificity for the target ion, and facilitate a flux of between 10^4 and 10^8 ions per second (Cragg 2002).

Figure 1.4 *Structures of synthetic channel-forming compounds: a) a photoactive gramicidin derivative, b) a membrane-spanning alamethicin dimer c) a synthetic relative of a naturally occurring polyester (Fyles 1997).*

1.4 Supramolecular chemistry and ion channels

1.4.1 Supramolecular chemistry

Jean-Marie Lehn, who shared the Nobel Prize for his work in the area in 1987, defined supramolecular chemistry as "the chemistry of molecular assemblies and the intermolecular bond". This may be also expressed as "the chemistry beyond the molecule" (Lehn 1998; Vogtle 1991). Other terms used are "inclusion phenomena", "host-guest chemistry" or "molecular

recognition". The former is broadly used and the latter two come from the origins of "lock and key" mechanism of biological catalysis proposed by Emil Fisher in 1894 (Fisher 1894). It has been expanding enormously for the last 15 years giving rise to a wide variety in chemical systems, some especially designed and others stumbled upon accidentally and claimed either in concept, origin or nature as being supramolecular. Supramolecular chemistry is based on the development of the chemistry of crown ethers and cryptands (Cragg 2005) and the study of the self-organization of molecules (for example, membranes and micelles) and of conductors and semiconductors (Steed 2000).

Supramolecular chemistry in its simplest sense involves some kind of non-covalent binding or complexation events. What is doing the binding? In this context, one molecule (a 'host') binds another molecule (a 'guest') to produce a 'host-guest' complex or supramolecule. Commonly the host is a large molecule or aggregate such as an enzyme or synthetic cyclic compound possessing a sizeable, central hole or cavity. The guest can be a monoatomic cation, a simple inorganic anion, or a more sophisticated molecule such as a hormone, pheromone or neurotransmitter. More formally, the host is defined as the molecular entity possessing *convergent* binding sites, e.g. Lewis basic donor atoms, hydrogen bond donors etc., and the guest possesses *divergent* binding sites, e.g. a spherical, Lewis acidic metal cation or hydrogen bond acceptor halide anion (Vogtle 1991).

There is a close relationship between supramolecular chemistry and both bioorganic and bioinorganic chemistry. Bioorganic chemistry is biomimetic, the chemistry of natural products and analogous compounds, which is done *in vitro.* 'Mimicking' life amounts to simplifying the process involved and is restricted to selected, well-defined properties of reactions in or on biological cells. These bioorganic properties, as compared to conventional

natural product chemistry, include the type of reactions, the reagent or the reaction conditions (Iqbal 2007b). When compared with organic chemical reactions, the reaction environment is very complex as it is composed of three components, water, polar surfaces and the less polar cavities of membranes and biopolymers. This system allows sequential reaction chains and also highly regioselective and stereoselective reaction.

The advances made in supramolecular chemistry were supported by the knowledge of the biological systems and the need for mimicking the structure and function of more complex biological processes. The artificial molecules and complexes that were synthesised in this way were referred to as models. Biological processes taking place in nature can be mimicked on a very small scale to be able to have a better understanding of such processes. The impairment of ion channel activity is one such example that can be interpreted through supramolecular chemical concepts. The regulation of ion transport is vital aspect of bioinorganic chemistry as it controls the balance of inorganic species between intracellular and extracellular environments. The concentrations of species present inside and outside the cells are very specific in all biological systems. For example the extracellular concentrations of Na^+ and K^+ are 140 mM and 5 mM respectively but the required intercellular concentrations are 5 mM and 140 mM in humans (Iqbal 2007a). This unequal distribution of alkali metal cations across the cell membrane is extremely important in maintaining the biological system giving rise to membrane potentials in these systems which, among other things, are used in information transfer in nerve cells. The importance of maintaining precise concentration gradients (Triggle 1999) is highlighted by the severe effects caused by a number of metabolic disorders, for example, high sodium intake leads to high blood pressure. Ion channels play critical roles in the generation of a response from cells to a wide variety of informational inputs, both chemical and physical which can be coupled directly or indirectly

to cellular responses through a number of biological effectors and transduction processes (Triggle 1999). To understand how different aspects of supramolecular chemistry contribute to ion channel selectivity and function we need to note some key "supramolecular" concepts.

1.4.2 Host-guest chemistry

In broad terms an ion channel can be considered to be a molecular "host" and the selected ion a "guest", the complex becoming an "inclusion compound". This relates directly to one of the definitions proposed by Powell, who coined the term 'clathrate' for a kind of inclusion compound "in which two or more components are associated without ordinary chemical union, but through complete enclosure of one set of molecules in a suitable structure formed by another" (Powell 1948).

A host-guest complex (Fig. 1.5) is composed of two or more molecules or ions that are held together by electrostatic forces other than those of full covalent bonds. The host could be a large molecule or aggregate such as an enzyme or a cyclic compound possessing a cavity or a central hole, and 'the guest' could be a monoatomic cation or a simple inorganic anion or even a hormone, pheromone or neurotransmitter (Steed 2000).

If the host-guest complex is held together primarily by electrostatic forces (ion-dipole, dipole-dipole, hydrogen bonding, etc.) the term 'complex' is used. On the other hand, if weaker forces (hydrophobic, van der Waals or crystal close packing effects) are involved then the terms 'cavitate' and 'clathrate' are more appropriate.

Figure 1.5 *Host-guest complexation (adapted from Steed 2000)*

1.4.3 Supramolecular host design

In order to design a host that will selectively bind a particular guest, the chelate and macrocyclic effects are used, as are the concepts of complementarity (matching of host and guest steric and electronic requirements) and, crucially, host preorganization. In the former effect, enhanced affinity is observed for ligands containing two or more metal ion binding sites compared to unidentate ligands providing the same number of binding sites; in the latter, macrocycles with three or more potential donor atoms exhibit strong and specific binding with metal ions that are of the correct size to fit the macrocyclic cavity

The first step in host design is a clear definition and careful consideration of the target. This leads immediately to conclusions about the properties of the new host system. If a metal cation is to be the guest, then its size (ionic radius), charge density and ‘hardness’ are important (Steed 2000). For anion complexation, these factors also affect spherical anions

such as chloride or bromide, but for non-spherical anionic guests, other factors such as shape, charge and hydrogen bond donor characteristics come into play (Davis 2010). Organic cations and anions may require hosts with both hydrophilic and hydrophobic regions, while neutral molecule guests may lack specific 'handles' such as polar groups that can strongly interact with the host (Steed 2000).

Several interactions contribute to a successful host-guest complex. The influence of each must be considered and a complementary site built into the host molecule if optimum binding is to occur.

1.4.3.1 Electrostatic interactions

Strong interactions between oppositely charged ions can lead to very stable host-guest complexes. Although most of the focus of supramolecular chemistry has been on binding cationic guests, the effect of charge complementarity is best illustrated in hosts designed to bind anions (Davis 2010). Schmidtchen produced two types of macrotricyclic quaternary ammonium hosts at a very early stage of his work and was able to find that they form complexes with a variety of anionic guests in water (Worm 1995). He also discovered that, because of their positive charge the counterions would compete for the anion-binding site. To solve this problem zwitterionic receptors were synthesized that were neutral (Schmidtchen 1977) and from NMR experiments in water were shown to bind strongly to chloride, bromide, and iodide ions better than the positively charged receptors. The coordination chemistry of group 1 and 2 metal and ammonium cations attracted most interest in the 1970s and consequently cation recognition is now a well developed area of supramolecular chemistry (Ma 2005).

1.4.3.2 Hydrogen bonding

Intramolecular hydrogen bonds (Rudkevich 2000) take an important part in the folding of proteins and are also responsible for enzymes catalytic pockets. Intramolecular and intermolecular hydrogen bonds are both very important as they can change the host conformation, shape and therefore its binding ability. Hydrogen bonding (Choi 2003) is a particularly appropriate interaction to build into hosts for amines, including peptides, or other guests with acidic hydrogen atoms. Crown ethers have been used for this purpose (Steed 2000) and a complementary hydrogen bond donor-acceptor motif is central to hosts designed to bind derivatives of barbituric acid (Chang 1988). Hydrogen bonds and electrostatic interactions can be used together to produce very effective receptors for anions. The first synthetic anion receptor was the polyammonium cryptand which bound the halide anions within a cavity through a combination of electrostatic interactions and hydrogen bonding (Simmons 1968). Further work was developed until Lehn reported a range of polyammonium macrocyles and cryptates with diverse binding selectivities (Graf 1976).

1.4.3.3 The hydrophobic effect

Hydrophobic effects relate to the exclusion from polar solvents, especially water, of large particles or those that are weakly solvated (through hydrogen bonds or dipolar interactions). When looking at the immiscibility of mineral oil and water, this effect is obvious. A combination of hydrophobic and electrostatic effects were demonstrated by Steed and Atwood (Atwood 1996) who used organometallic hosts based upon calix[4]arene and the related macrocycle, cyclotriveratrylene, to bind anionic guest species including BF_4^-, I^-, and $CF_3SO_3^-$.

Steed and co-workers coordinated transition metals to the outside of calixarenes in order to reduce the electron density present in the "cup", therefore making the cavity more adaptable to hydrophobic anionic guests (Steed 1994). Attachment of metals to the aromatic rings also rendered the calixarene cavity less electron rich and so tuned it to bind anions rather than cations.

1.4.3.4 Anion coordination

Anion recognition chemistry work began in the late 1960s around the time when Pedersen reported the synthesis and coordination chemistry of crown ethers and Lehn published the first accounts of cation coordination chemistry by cryptand (Lehn 1988). Only in the last 30 years has there been a development in the coordination chemistry of anions and the interest in the problems inherent in binding them (Beer 2001). Given that chloride, phosphate and other anions are essential constituents in biological systems, and that transmembrane transport routes for all exist, it is worth addressing the issues surrounding the design of anion hosts.

There are different reasons why anion receptors are quite challenging to design. Anions are larger than isoelectronic cations and therefore they have a lower charge to radius ratio. This means that electrostatic binding interactions are less effective that they would be for the smaller cation. Anions are even more sensitive to pH then cations or neutral molecules (becoming protonated at low pH and so losing their negative charge), thus receptors must function within the pH window of their target anion. Anionic species have a wide range of geometries and therefore a higher degree of design may be required to make receptors complementary to their anionic guest (Beer 2001).

When designing selective hosts for anions, it is necessary that the geometry and basicity of the anion and the nature of the solvent medium be taken into account. Complementarity between the receptor and anion is clearly crucial in determining selectivities. A valuable way of categorising anion receptors is to consider the types of non-covalent interaction used to complex the anionic guest. These take account of electrostatic interactions, hydrogen bonding, hydrophobicity, coordination to a metal ion, and combination of these interactions working together (Valiyaveettil 1993; Rudekevich 2000). Major research groups have synthesised receptors for anion coordination where hydrogen bonding and electrostatic interactions (Schmidtchen 1977), or both together, are used in binding the guest molecule (Beer 2001). Some functional groups, like amide, have been reported as convenient groups from which to synthesise receptors with the ability to coordinate anions (Pascal 1986; Reinhoudt 1993). Pyrrole containing macrocyles are also used as anion binding agents (Gale 1997). Urea and thiourea are especially good hydrogen bond donors, and are excellent receptors for anions such as carboxylate via the formation of two hydrogen bonds, and calixarenes have been used as scaffolds from which coordinating groups can be appended (Buhlmann 1997). Functionalised derivatives have been used as receptors for cations, anions and neutral guests (Beer 2001; Ma 2004; Le Gac 2007; Nikura 1999; Llinares. 2003) and consequently represent an ideal starting point from which to construct mimics for both cation and anion channels (Gokel 2001).

1.4.4 Calixarenes

The term “calixarene” was introduced by Gutsche as trivial name for a group of macrocycles prepared from para-substituted phenols (Gutsche 1989). They are great molecules for supramolecular chemistry having a three dimensional shape, concave surface and relatively

rigid structure, which make them convenient platforms for elaboration (Bohmer 1995). During the earliest stages of calixarene research it became clear that the unsubstituted hydroxyl groups at the lower rim are involved in strong intramolecular hydrogen bonding, both in solution (IR and NMR spectroscopy) and in solid state (X-ray analysis) (Rudkevich. 2000). The hydrogen bonding in the calixarenes, between lower rim phenolic groups, gives a cone shape resulting in deep cavities.

Calixarenes are derived from the condensation of phenols (e.g. 4-t-butylphenol) and formaldehyde (Fig. 1.6) under a variety of different conditions (Bohmer 1995). They contain bridged aromatic rings and are members of the cyclophane family. The compounds have been known for a long time (Bayer 1872; Zinke 1944; Hayes 1958), but only in the last 30 years have their structures been established definitely either in solution or in the solid state leading them to become popular in supramolecular chemistry (Gutsche 1985; Gutsche 1989).

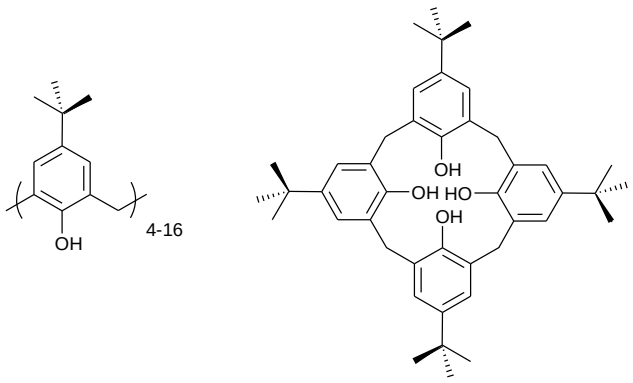


Figure 1.6 *Calixarenes: cyclic oligomers of 4-t-butylphenol and formaldehyde.*

In cyclophane nomenclature, they are named 'substituted [1.1.1.1]metacyclophanes'. C. David Gutsche coined the descriptive name calixarene, because the bowl-shaped conformation of the smaller calixarenes, resembled a Greek vase called a calix crater (Fig. 1.7) (Gutsche 1989; Steed 2000).

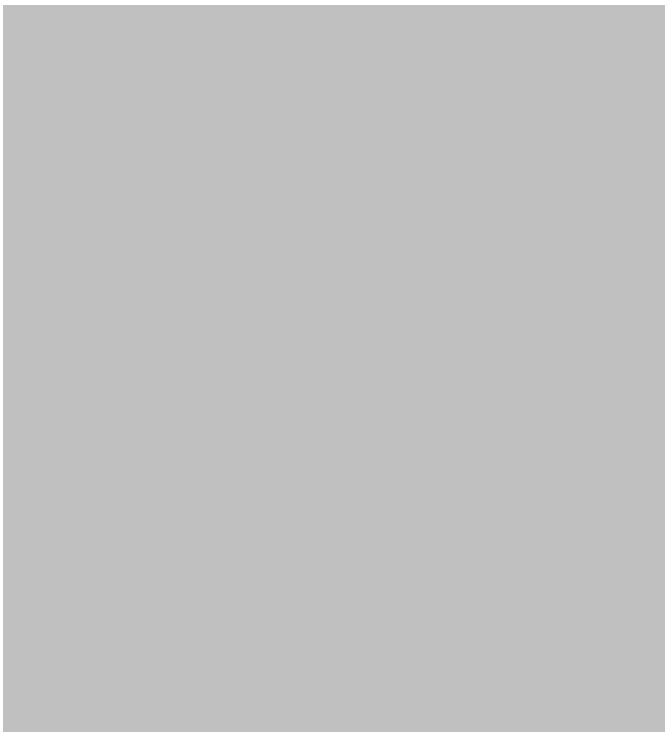


Figure 1.7 *The calix crater (http://uoch.vscht.cz/Skupiny/Lhotak/my_webs/ calix_1.gif)*

The calixarenes are extremely versatile host frameworks. They can act as hosts for cations, anions and neutral molecules, using the phenolic oxygens of the lower rim (Fig. 1.8). An additional subgroup of calixarenes compounds contains combinations of CH_2 groups and heteroatoms between the aromatic moieties, such as oxygen ("homooxacalixarenes") or nitrogen ("homoazacalixarenes"). Calixarenes and related macrocycles have been extensively studied due to their molecular recognition properties.

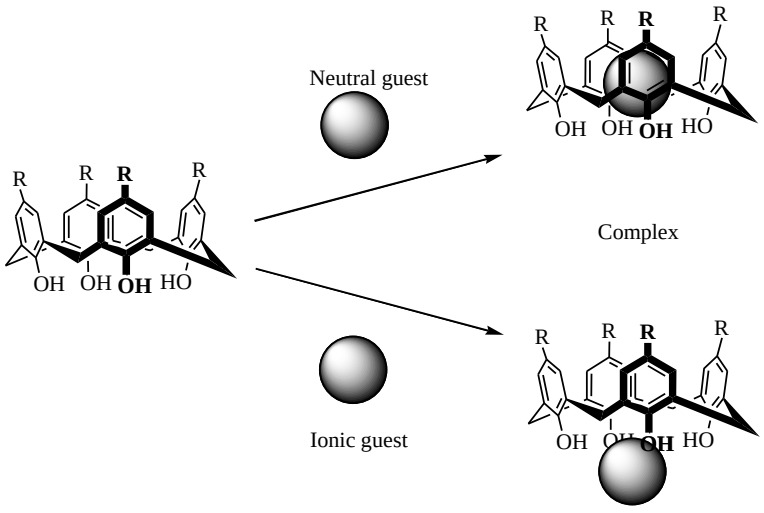


Figure 1.8 *Lower rim binding domains for neutral and cationic guests.*

1.4.4.1 Homooxacalixarenes

Homooxacalix[n]arenes (oxacalixarenes) are members of the calixarene group of macrocycles that have one or more ethereal bridges (CH_2OCH_2) in place of CH_2 groups, which link phenol moieties through the 2- and 6-positions (Fig. 1.9). The first reported synthesis of homoxacalixarene was by Hultzsch (Hultzsch 1962) who synthesised hexahomo-trioxacalix[3]arene through direct reaction of 4-t-butylphenol with paraformaldehyde. These compounds are also potential frameworks for artificial ion channels as they have been shown to desolvate sodium cations (Cragg 1999b).

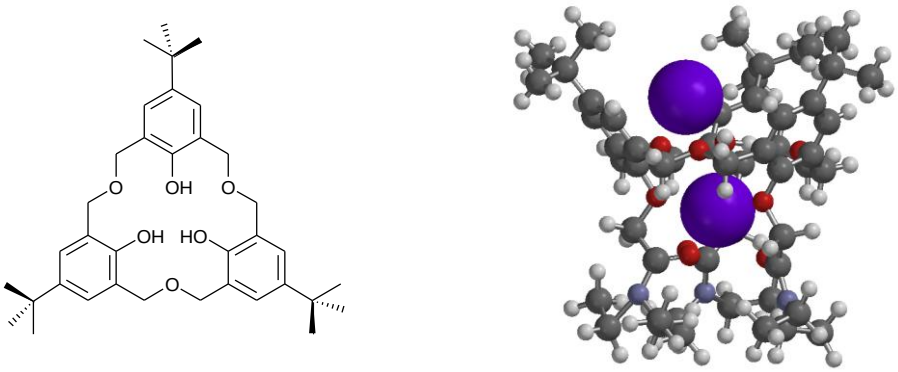


Figure 1.9 *Structure of an oxacalixarene (left) and the X-ray structure of the sodium complex formed by a derivative of oxacalix[3]arene (right).*

1.4.4.2 Homoazacalixarenes

The azacalixarenes have a similar structure to the oxacalixarenes but the linking oxygen atoms have been replaced by nitrogen atoms in the macrocycle ring which provide additional positions for substituents that can act as binding sites for guests (Fig. 1.10).

Azacalixarenes have been synthesized to be used as receptors for a large variety of guests (Hultzsch 1962). It is believed that azacalixarenes could be useful as receptors for a wide variety of guests particularly as they have a cationic and hydrophobic cavity, which provides binding sites for anionic guests and also the modification of the amines for the introduction of

additional functional groups. In addition the CH_2-NR-CH_2 bridges provide flexibility to the receptor. The pre-organized structures and the geometry of the donor sites are very important for the host's complexation ability and for the recognition of the guests. The increased affinity for spherical guest species is due to the highly symmetrical spatial arrangements of the binding sites and the converging of the dipoles (Beer 2001).

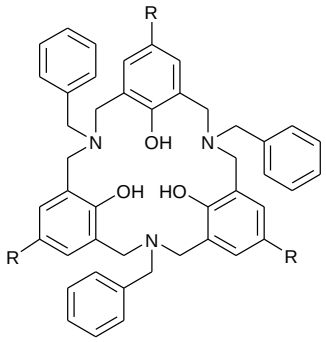


Figure 1.10 *Structure of an azacalix[3]arene*

The first reported synthesis of homoazacalixarene was by Hultzsch (Hultzsch 1962) who synthesised hexahomotriazacalix[3]arene through direct reaction of 4-t-butylphenol with hexamethylenetetramine. The first practical synthesis was done by Takemura (Takemura 1992) in which a new cyclization procedure was described leading to the formation of a hexahomotriazacalix[3]arene. The condensation reaction between 2,6-bis(hydroxymethyl)-4-methylphenol and benzylamine was believed to give a dihydrobenzoxazine derivative. However, a reinvestigation of the reaction determined that the product was the azacalixarene, triaza[3,3,3]cyclophane (Chirakul 2000).

A convergent synthesis was developed by Hampton (Chirakul 2000) based on the reaction of 2-(chloromethyl)-5-methylbenzaldehyde with benzylamine (forming a dimer), followed by the addition of bis(chloromethyl)-4-methylphenol, giving a product in 95% yield. Hampton's method and characterization of the compound indicated that Takemura generated a mixture of

oligomers in his procedure, predominantly the azacalix[3]arene and its azacalix[4]arene homologue.

A new synthetic route for the preparation of azacalix[3]arene macrocycles was reported by Hampton and co-workers which allows the formation of azacalix[3]arenes without the formation of higher homologous such as azacalix[4]arene. The two approaches used were the cyclization of an acyclic trimer or condensation of a dimer with a monomer to yield a cyclic trimer. The structure of the azacalix[3]arene macrocycle can be changed by varying the R group on each aromatic ring allowing the effects of variations in the geometric and electronic structure of the macrocycles on their affinity to be examined and selectivity in the metal ion binding to be assessed (Chirakul 1998).

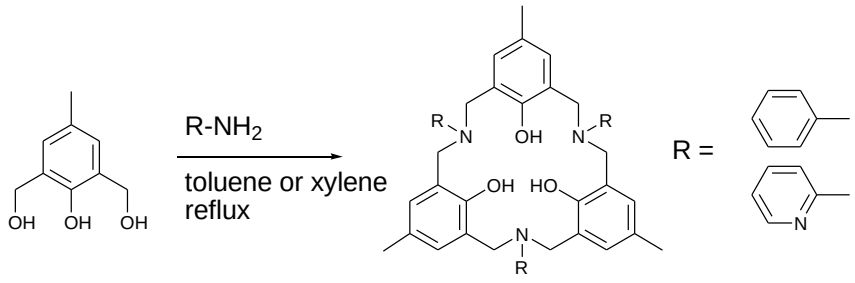
The *O*-alkylation of the phenolic oxygens in calixarenes and related macrocycles results in significantly higher affinities toward alkali and alkylammonium ions as reported in previous studies by Takemura and co-workers (Takemura 1994; Takemura 1996). Modification of the phenolic oxygens in the azacalix[3]arene eliminated the intramolecular hydrogen bonding, enhancing the coordination ability of the phenolic oxygens, and locked the macrocycle into either a *cone* or *partial-cone* conformation (Chirakul 1998)

1.4.4.3 Synthesis of azacalix[3]arenes (hexahomotriazacalix[3]arenes)

There are two approaches to the synthesis of azacalix[3]arene, either a one-pot method or the use of stepwise methods (Khan 1993; Takemura 1994; Takemura 1996; Takemura 2002; Kaewtong 2009). In the one-pot synthesis the generation of azacalix[3]arenes is achieved by the condensation of bis(hydroxymethyl)phenol derivatives with benzylamine following reflux in toluene or xylene for 3 days (Takemura 1994; Takemura 2002). A templating effect plays

an important role in the cyclization reaction where –OH··OH and -OH··N hydrogen bond interactions occur that are dependent on the type of non-polar solvents used (Takemura 1994). Advantageously these azacalix[3]arenes can be readily purified by column chromatography in moderate to high yields. The condensation reaction between the hydroxymethylphenols and amine groups is under relatively mild conditions compared to the condensation reaction between alcohols and amines. It has been suggested by Takemura that this is because the reaction is not a simple dehydration reaction but a Mannich-type reaction that proceeds via quinine intermediates (Takemura 2002).

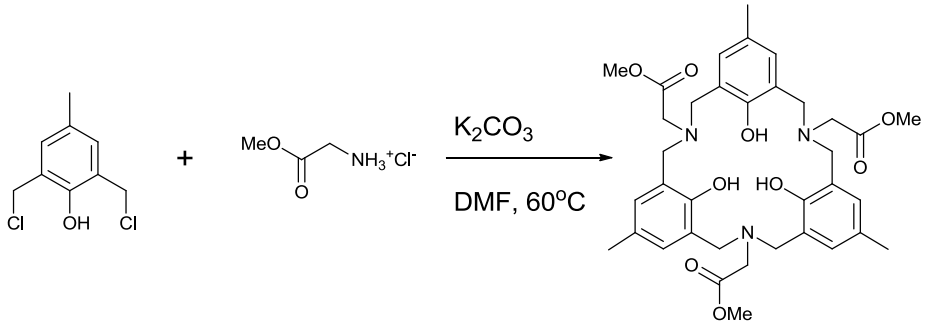
The first one-pot synthesis (Takemura 1992) of azacalix[3]arene afforded *p*-methyl-*N*-benzylhexahomotriazacalix[3]arene (Scheme 1.1). This was achieved by refluxing 2,6-bis(hydroxyl-methyl)phenol with benzylamine in toluene and generated azacalix[3]arene in reasonable yield (38%), following precipitation from methanol:acetone (4:1 v/v) and recrystallization from a benzene: methanol mixture (5:1 v/v). Importantly if the phenol contains a halogen the solvent of choice is xylene as these require higher temperatures to achieve cyclization (Takemura 2002).



Scheme 1.1 *Azacalix[3]arenes made by a one pot synthesis*

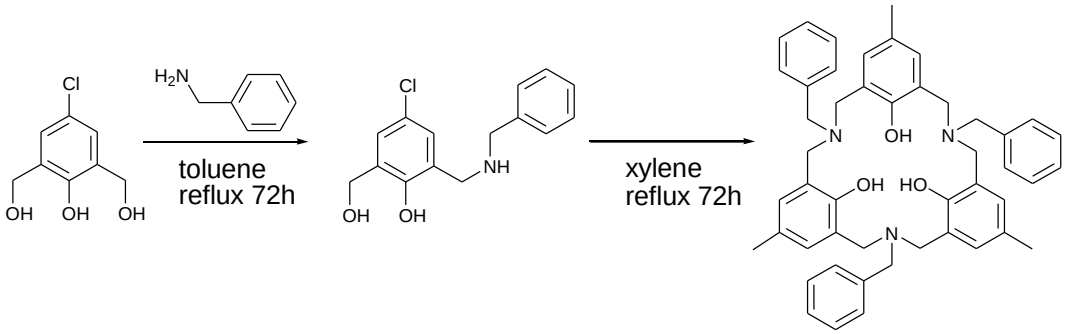
For reactions in which heat sensitive amines are present (Hampton 1996) has developed a methodology that involves a cyclooligomerization reaction between 2,6-bis-(chloromethyl)-

4-methylphenol and glycine methylester hydrochloride in low concentration in the solvent dimethylformamide in the presence of base (Scheme 1.2). It is important to note that the further reactions such as hydrogenation of azacalixarenes have been hampered due to the compounds' low solubility in common solvents (Asfari 1995).



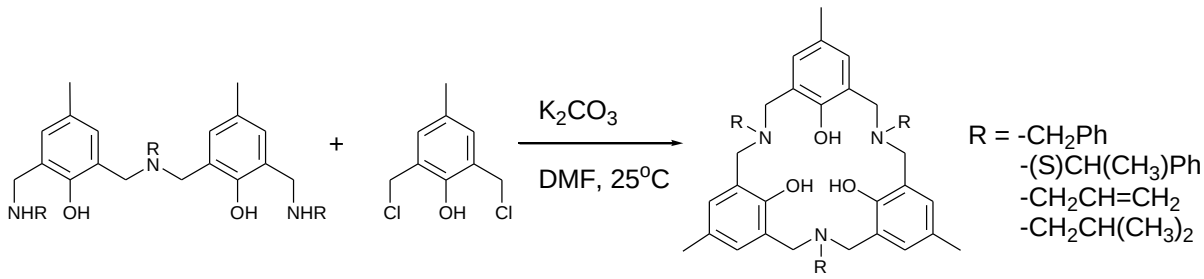
Scheme 1.2 *Synthesis of azacalix[3]arenes under basic conditions*

Single reagent methodologies have been devised (Takemura 1994; Lumetta 2000), the most efficient involving the cyclization of an aminomethylhydroxymethyl monomer attaining azacalix[3]arene (Scheme 1.3) in a yield of 34% (Takemura 1994).

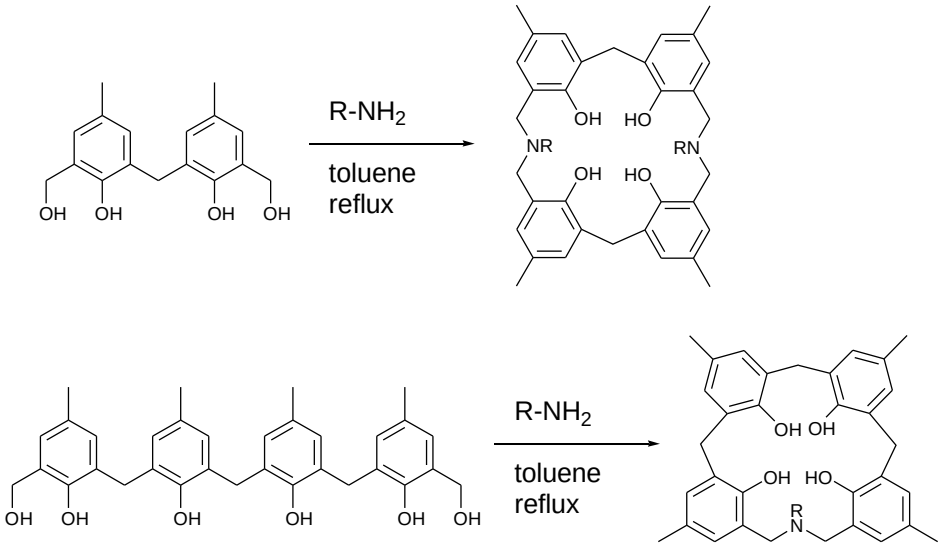


Scheme 1.3 *Generation of azacalix[3]arene via aminophenolic intermediates*

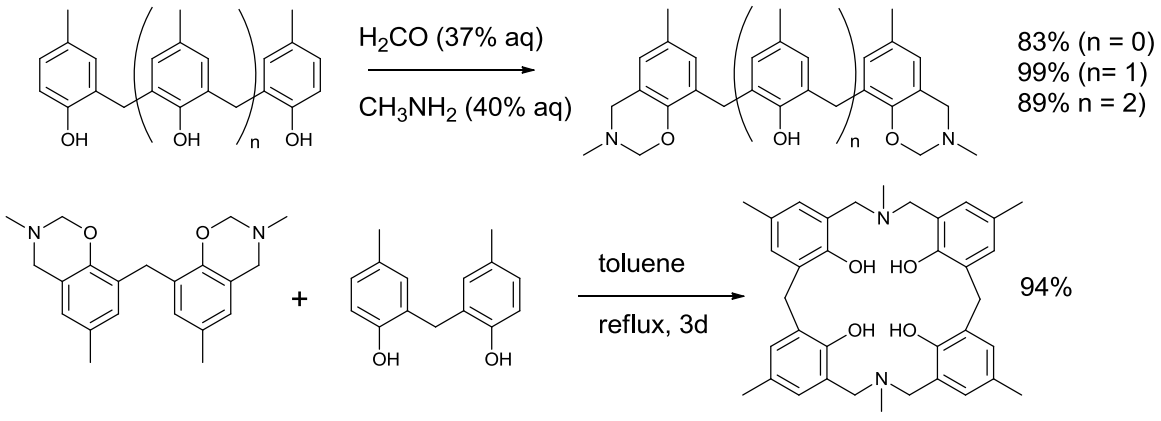
An important development in the synthesis of azacalix[3]arenes was achieved by Chirakul (Chirakul 2000). This stepwise synthesis involved a key condensation reaction involving the coupling of triamines with 2,6-bis-(chloromethyl)-4-methyl phenol (Scheme 1.4) which can attain remarkably high yield of up to 95% in the absence of side reactions.



Scheme 1.4. *Coupling synthesis of azacalix[3]arene*



Scheme 1.5. *Synthesis of azacalixarenes by reaction between linear bis(hydroxymethyl)phenols and amines.*



Scheme 1.6 *Preparation of azacalixarenes from dihydrobenzoxazine derivatives (Takemura 2011).*

Recently, a new synthesis has been described for azacalixarenes which involves the reaction of benzoxazine derivatives of phenols and the phenol oligomers. The reaction is carried out at

40–80 °C, because scrambling of the phenol units occurs at higher temperatures. The appropriate reaction conditions afforded the desired azacalixarene that are asymmetrical along the N···N_or N···CH_2 axis (Takemura 2011). This route is similar to other linear routes used to prepare azacalixarenes as shown in Schemes 1.5 and 1.6.

1.5 Artificial ion channels

Synthesis of artificial ion channels from simple organic molecules is an attractive approach for the future understanding of biological signal transduction systems. The transmembrane channel may contribute to its unique property in distinguishing specific metal ions from anions and other metal ions, allowing a large ionic flux and being regulated by external stimuli such as ligand binding or a change in transmenbrane voltage (Woolley 2003). These channels are also characterised as gated which is another challenging feature that has to be taken into account when trying to mimic these channels. Recent advances have contributed to the availability of artificial ion channels based on antibiotics, peptides, modified macrocycles such as crown ethers or cyclodextrins as well as oligoethers. The organic chemists interest in ion channels has been greatly influenced by three naturally occurring compounds: nystatin and amphotericin B, polyne antibiotics; gramicidin, a peptide that forms a cation-selective channel which is the best characterized of all channels and alamethicin, a peptide that forms voltage gated channels (Gokel 1996). Artificial ion channels synthesised from simple organic molecules are an attractive way to establish structure-function relationships and are expected to add significantly to the understanding of biological signal transduction systems (Kobuke 2001). The efficient functioning of ion transport in natural biological systems has increased the interest in understanding mechanisms by which these proteins mediate selective, regulated transmembrane ion transport. The ability of ion channels to effect electrical signalling under aqueous saline conditions has inspired the chemists to synthesise mimics to

use as biosensors, therapeutic agents and other materials. (Madhavan 2005). Gokel and co-workers have been working on an ion transporter that makes membranes permeable to chloride ions and alters chloride transport in epithelial cells (Pajewski 2007). Ferdani and co-workers have investigated the responses to the anchor chains and the fourth amino acid of heptapeptide ion channels and results have shown a variation of the chloride ion transport efficacy in liposomes (Ferdani 2005).

1.5.1 Requirements for a successful model of an ion channel

A cell membrane is comprised of a hydrophilic phosphate head, an amphiphilic mid region and a long water-repelling, fat-soluble tail. Due to the hydrogen bonding and dipolar interactions that exist in the body's aqueous environment the hydrophilic phosphate (head) group is attracted to the surrounding medium whereas the hydrophobic organic tail is repelled. This creates a bilayer arrangement with the organic (lipid) tail away from the solvent and the phosphate head groups facing out. The hydrated ions must be transported across the phospholipid bilayer. The transport of ions is highly specific allowing the passage of only few ions across the lipid bilayer through the thickness of about 40 Å in length (Iqbal 2007a).

Sodium and potassium ions for example are not lipid soluble. Therefore to cross the lipid bilayer they have to either have a lipophilic carrier or a hydrophilic channel in the membrane so they can diffuse through the cell wall. Polyethers, such as the commercial Triton non-ionic surfactants widely used to perturb liposome and cell membranes (Angelini 2011, Calvello 2011), exemplify the types of compounds that could be used to penetrate a phospholipid bilayer and so facilitate ion transport. Triton X-100®, in particular, is long enough (3 to 4 nm)

to penetrate lipid bilayers and appears to target areas of osmotic stress but is otherwise non-specific in its activity. The compounds can successfully insert because they contain both amphiphilic and lipophilic regions allowing them to interact favourably with the polar, solvated external and internal bilayer surfaces while passing through the hydrophobic core of the bilayer where lipid groups interdigitate (Ahyayauch 2011). The combination of rigid, calixarene-based ion filters and amphiphilic substituents would seem to be an ideal way to construct artificial ion channels and was the central idea behind the work described in this thesis.

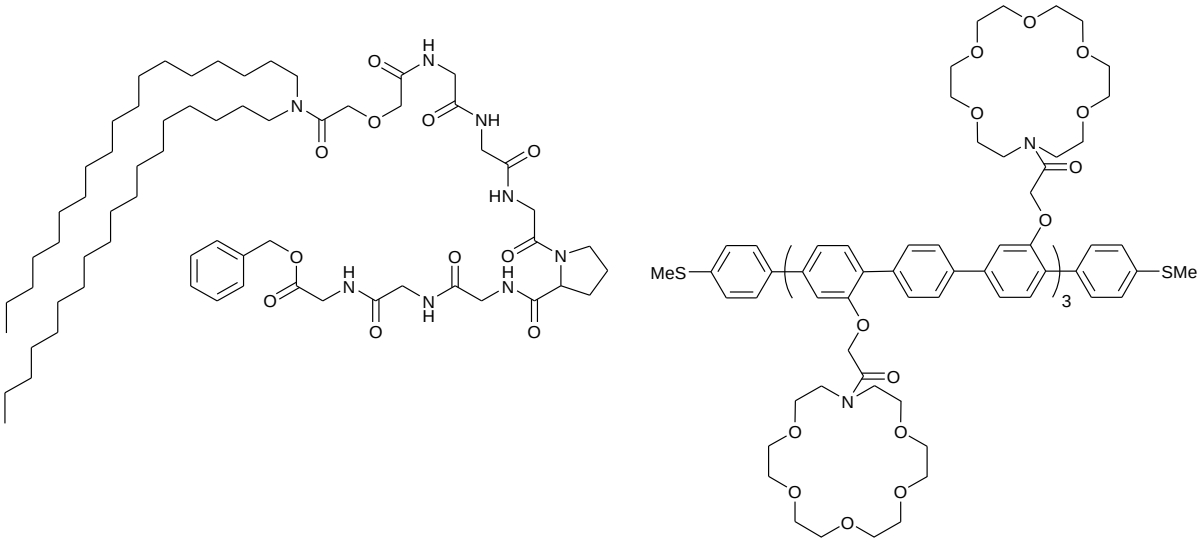


Figure 1.11 *Gokel's SCMTR (left) and Matile's rigid ion channel (right)*

Synthetic mimicry of ion channel activity has been achieved, to varying degrees of success, through a variety of approaches. A simplified analogue of a natural system may be found in Gokel's chloride transporting SCMTR compounds based on short protein fragments with alkyl and aryl termini (Gokel 2001).These compounds appear to aggregate and allow chloride to cross the cell membrane. Another tactic is to place crown ethers at regular intervals along a rigid (or polypeptide) backbone, as seen in the work of Matile and others (Matile 2004, Matile 2011). Finally there are numerous approaches based upon macrocycle bearing

membrane-spanning substituents (Iqbal 2007a) although these are the least similar to natural channels they have received substantial interest due to their modular construction.

In principle an ion specific channel could be designed by searching the literature for suitable "filter" molecules and attaching membrane–spanning substituents. When constructing these artificial channel forming compounds, two other factor must also be considered: ion flux and insertion of the channel in the bilayer. The first of these is often impossible to determine until experiments are actually attempted. The second requires that the model compound has properties that are complementary to those in the bilayer. Ideally the termini of the compound are polar to match the phospholipid's head groups but the bulk of the molecule is hydrophobic to match the lipid rich core of the bilayer. Incorporation of the amphiphilic substituents would therefore seem to be an essential feature in any model, however, many amphiphiles have been shown to disrupt phospholipid bilayers thereby compromising membrane integrity (Fuhrhop 1986).

Inherent requirements to be a successful model for a natural transmembrane ion channel the compounds used should be able to discriminate in favour of one ion, span the cell membrane, implying a concerted structure over 4 nm in length and achieve a transport rate in the region of 10^4 to 10^8 ions per second. A modular approach should therefore use an ion-specific macrocycle as a filter, have the length to incorporate amphiphilic polyethers or alkyl chains and the transport rate can be affected by many uncontrollable parameters.

One group of compounds known to increase transmembrane ion transport in an indiscriminate manner are those in the Triton class of surfactants. Triton X-100® (Fig. 1.12) is a non-ionic surfactant which has a hydrophilic polyethylene oxide group (on average it has

9.5 ethylene oxide units) and a hydrocarbon lipophilic or hydrophobic group. Triton X-100® is often quoted in literature as a simple amphiphile to exhibit channel-like behavior in phospholipid bilayers (Schlieper 1977; Wang 2009). The structure is 10 Å long when fully extended and it is not expected to form a pore in the cell membrane. In 1994, Rostovtseva and co-workers stated that "bilayers of DPhPC, DOPC or GMO, to which Triton X-100® was added only by injection into the aqueous layer had no channel activity" (Rostovtseva 1994), only exhibiting channel-type behaviour when added directly to an aqueous liposome suspension. Nothing is known in detail about the structure of channels formed by Triton X-100® and it is likely that they form through temporary aggregation of several molecules that allow ions and solvent to pass through a bilayer. Consequently Triton X-100® has the requirements that are necessary to produce a channel like behaviour in the cell membrane.

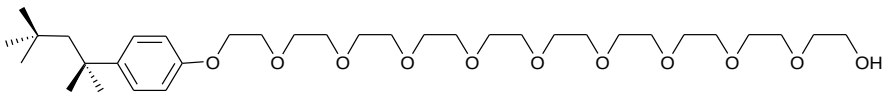


Figure 1.12 *Triton X-100®*

1.6 Recent synthetic mimics

A growing number of synthetic compounds have been demonstrated to ease ion and polar molecule transport across the bilayer membranes (Boon 2002). The different molecular scaffolds selective for cations that have been developed up to date are based on synthetic analogues that use cyclodextrins, crown ethers, peptides and calixarenes (Matile 2004). There are fewer examples of synthetic channels for anion selectivity. A limited number have been developed by Gokel and Matile (peptidic synthetic channels) and Regen (sterol mimics) which self-assembly within the phospholipid bilayer. One macrocyclic example should be

noted. Gin has developed a highly active, monomeric, cyclodextrin-based ion channel that shows not only selectivity for anions over cations, but also discriminates between halide anions, I^-, Br^- and Cl^- (Madhavan 2005). Gokel developed an hydraphile channel system based on the 4,13-diaza-18-crown-6 macrocycle (You 2006) with a crown ether believed to be fixed in the bilayer as the central relay group. The sodium transport rate of these channels is around 100-fold slower than gramidicin but much faster than a single crown ether acting as a carrier. Further examples of some of the many synthetic channels are shown below in Fig. 1.19. A similar compound, Lehn's tetracarboamido[18]crown-6, was synthesised as the potassium hydrate. The affinity of potassium ions for [18]crown-6 was greater than any other crown, the structure was thought to act as an ion channel (Dock 1983). To work as a true transmembrane channel the selectivity of [18]crown-6 had to be integrated within a much larger structure.

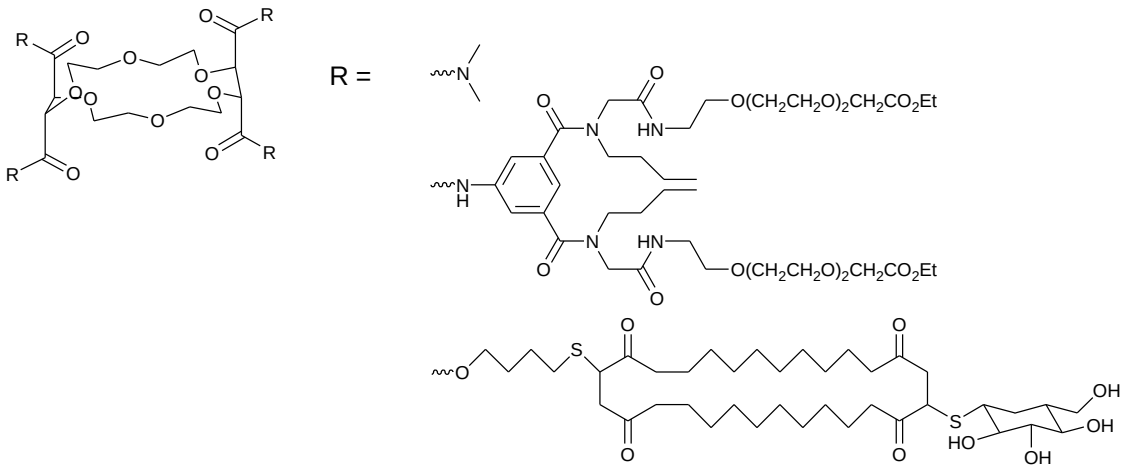


Figure 1.13 *Membrane-spanning compounds based on crown ethers.*

Calixarenes provide rigid, functionalisable, ion selective filters around which ionophore or channel frameworks can be constructed. They are available in a variety of sizes and their internal cavities can act as biomimetric filters. The aromatic rings of a regular cone conformer of calix[4]arene have a centroid-centroid separation of 6.8 Å giving the guest accessible cavity 3.2 Å in diameter; the cone conformation of calix[6]arene has a cavity of

6.1 Å and the oxacalix[3]arenes in the region of 4.0 Å (Iqbal 2007a). From the foregoing observation it can be determined that species with r ~ 1.6 Å can pass through calix[4]arene, r ~ 2.0 Å can pass through oxacalix[3]arene and r ~ 3.0 Å calix[6]arene-based filters (Cragg 2002). Most of the important species can only pass through calix[4]arene and oxacalix[3]arene filters if they are partially dehydrated in the process (Kiriukhin 2002). This is supported by crystallographic evidence: extensive dehydration occurs in natural K^+ channels (Doyle 1998) and desolvation is observed in a Na^+-oxacalix[3]arene complex (Cragg 1999b). A further reason for choosing calixarenes is the wealth of structural modifications that can be incorporated in the basic structure (Bohmer 1995) which has led to a range of binding motifs including those that encapsulate anions (Matthews 2005) and neutral molecules (Bott 1986). Weak, reversible binding attracts species of interest to the selectivity filter and then channels them through the macrocyclic host. This behaviour is responsible for initiation and maintenance of the transmembrane ion transport by artificial channels.

Calix[4]arenes in particular have another property which can be exploited to ion channels: they exist in different conformers as shown in Fig. 1.14 (Gutsche 1985). A symmetric channel can be prepared from *1,3-alt* conformer and an asymmetric channel from the cone conformer as shown in Fig. 1.15. Lower rim substituents in the region of 20 Å attached to the cone conformer can only span the phospholipid monolayer so dimerisation is necessary to span the entire membrane. If the same substituents are attached to the *1,3-alt* conformer the membrane can be spanned by a single molecule (Iqbal 2007a). In an interesting development Raston and co-workers reported the crystal structure of an *O*-alkyl substituted calix[4]arene with *n*-octadecyl substituents (Clark 2006). In the solid state the alkyl chains interdigitate to form crystalline bilayers approximately 33 Å thick. This ability to form bilayers indicates that

calixarenes, extended through upper or lower rim derivatisation, have the potential to insert themselves in biological membranes to form permanent channels.

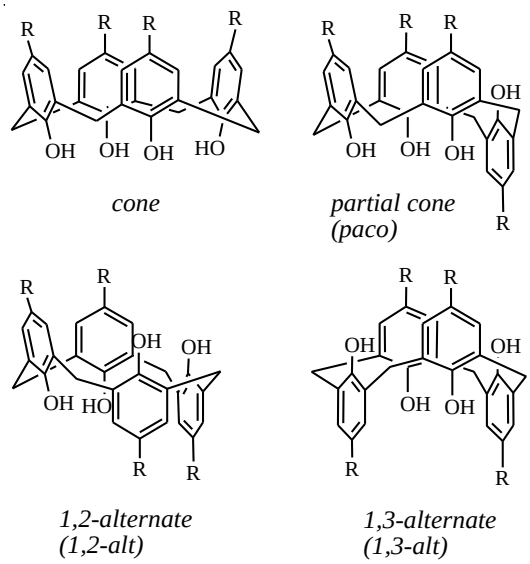


Figure 1.14 *Calix[4]arene conformers*

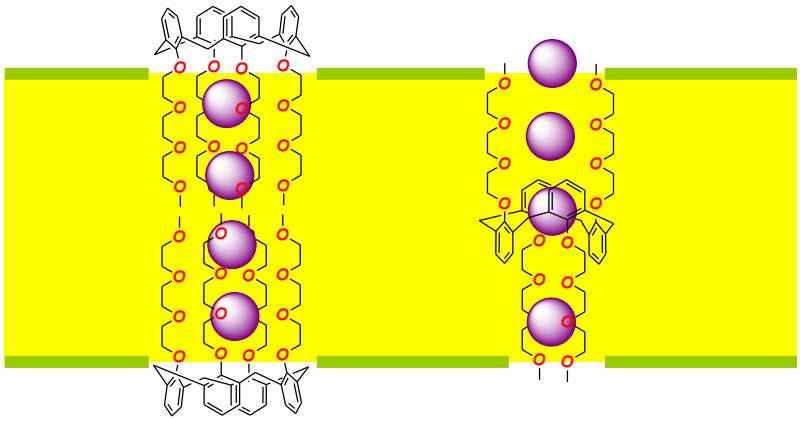


Figure 1.15 *Cone (left) and 1,3-alternate (right) calixarene-based ion channel models (Iqbal 2007a).*

1.7 Cation transport

The earliest indications that the calixarene framework could be used as a basis for artificial ion channels came when the research groups of Calestani, Ungaro and Ugozzoli reported the synthesis of *cone*-4-*tert*-butyl-calix[4]-arene-tetra-(diethylamide) (Fig. 1.16**, ii**) and X-ray structures of two K^+ complexes (Arduini 1988). These showed that a calix[4]arene could bind

alkali metals within an extended central cavity. Despite the apparently good fit between the macrocycle and K^+, the compound extracted Na^+ from aqueous solution with a selectivity factor 68 over K^+. Hydrophiles, composed of [18]crown-6 relays, display similar Na^+/ K^+ discrimination which has been attributed to the different rates of binding and release for the two cations: K^+ is bound better but Na^+ released more easily (Gokel 2000).This is highly relevant for ion channel dynamics for, while the channels must have specificity for one species over others, ions must not be bound too strongly if they are to pass through.

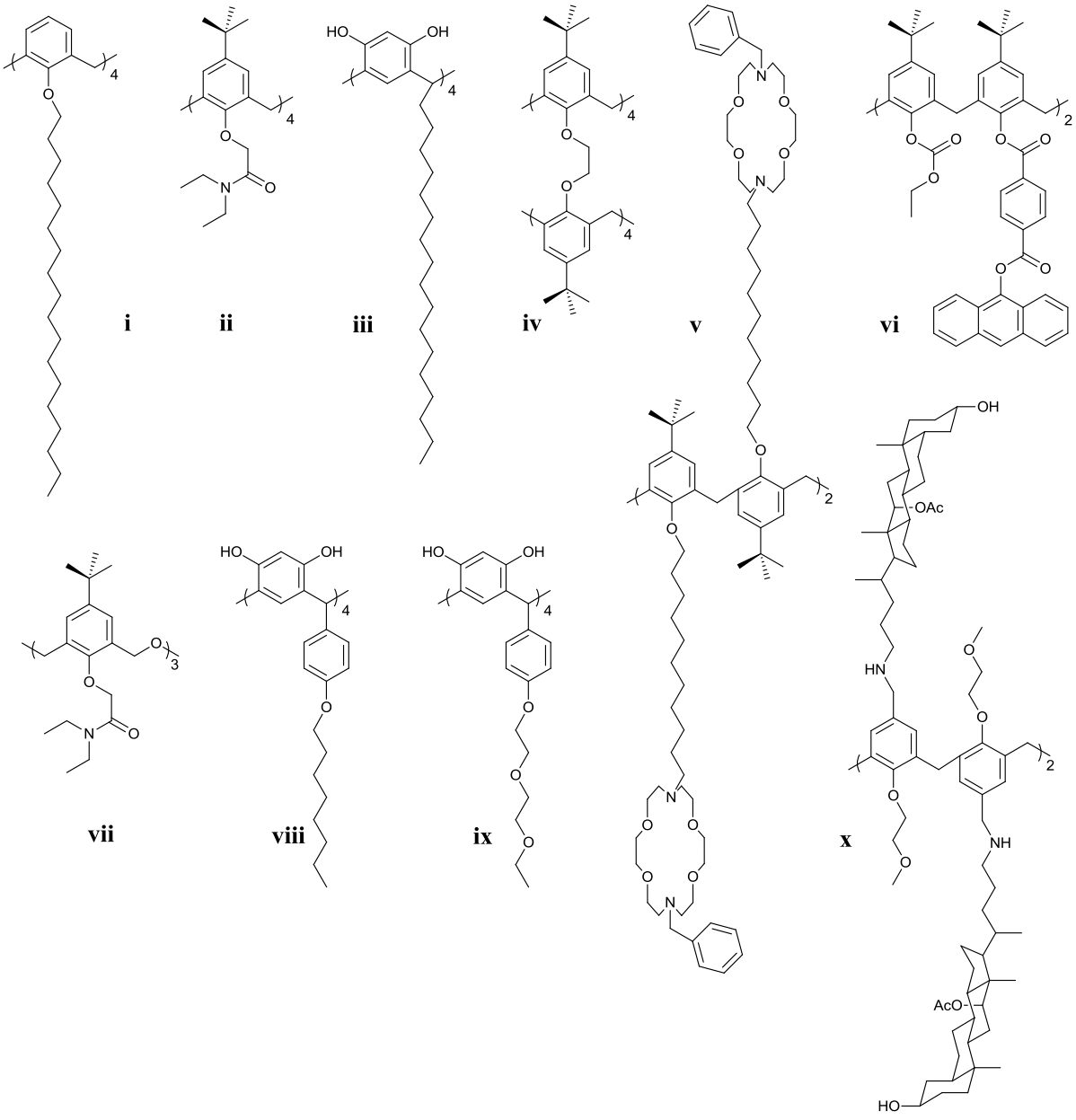


Figure 1.16 *Examples of calixarene derived ion channel mimics (adapted from Iqbal 2007a)*

Later work by Ogden and co-workers revealed that compound **ii** also forms crystalline complexes with several transition and main group metals (Beer 1995).

Noting that the resorcin[4]arenes are amphiphilic analogues of the calix[4]arenes, Kobuke and co-workers designed a derivative with heptadecyl substituents (Fig. 1.16, **iii**) to span a lipid monolayer. Experiments in planar bilayers demonstrated both Na^+ and K^+ conductance, as might be expected from earlier structural extraction data, but with selectivity for K^+ over Na^+ by factor of three (Tanaka 1995). Transport of the latter could be blocked by the addition of Rb^+. Conductance was not observed by analogous compounds prepared with shorter undecyl substituents leading the authors to speculate that the compound inserted in inner and outer phospholipids layers with cation transport occurring when two molecules met tail-to-tail in a manner reminiscent of the gramicidins.

Shinkai and co-workers (Otsuka 1995) showed that deposition of calix[4]arene and calix[6]arene ethyl esters on glassy carbon electrodes enhanced the response to Na^+ and Cs^+, respectively though whether selectivity was based on transport through intramolecular channels or intermolecular voids was not proven conclusively (Yagi 1996). These compounds could not function in a bilayer but the success of electrochemistry to characterise their channel-like behaviour directly suggests that artificial ion channels could have valuable applications in ion selective electrodes.

In 1997 Beer and co-workers reported several bis(calixarene)s (Fig. 1.16, **iv**) in which two calix[4]arenes in the cone conformer were linked by four ethyl bridges (Schmitt 1997). The synthesis is remarkable because it requires four concerted covalent links to be formed between the two macrocycles and achieved yields in excess of 50%. X-ray crystallography determined that one of the intercalixarene cavities was constricted and free from cations, in

the other K^+ had been captured within the cavity which in turn had been forced in to a more expanded geometry. Recent computer modelling indicated that the lowest energy pathway for the cation to enter the cavity is through the calixarene annulus rather than between the ethyl bridges (Felix 2002).

The importance of calixarene conformer was elegantly demonstrated by Gokel and co-workers who appended four dodecyl ethers, terminating in *N*-benzyldiaza[18]crown-6, from calix[4]arene in both cone and alternate conformations (De Mendoza 1998). The *1,3-alt* derivative **v** allowed Na^+ to " burst" through a planar lipid bilayer but the *cone* derivative exhibited no cation conduction as it could not span a bilayer fully. Other *1,3-alt* calix[4]arenes with appended guanosine moieties are belived to form self-assembled nanotubes in the presence of Na^+ though no ion transport has been measured (Siderov 2000).

In 2000 Jin published cation transport data for a bisanthroyl-calix[4]arene (Fig. 1.16, **vi**) which selectively transported Na^+ over K^+ across the lipid bilayer (Jin 2000). One interesting aspect of the calixarene was that anthracene substituents were close enough to be photodimerized. The effect of this was to close and open the selectivity filter in response to UV irradiation. Bilayer experiments show a rapid reduction in Na^+ currents during irradiation and slow rectification when the lamp was switch off. Earlier examples of calixarene photoactivity had been reported (Jin 2000) though this was the first example of its effect on transmembrane ion transport.

Interest was shown in the rich potential for metal coordination afforded by oxacalix[3]arenes for many years through their structural similarities with both calixarenes and [18]crown-6. The *cone*-tris(diethylamide) derivative, first prepared by Shinkai and co-workers (Matsumoto

1995) appears to be an ideal compound for alkali metal complexation (Fig. 1.16, **vii**). Although the central cavity in the metal-free ligand collapses in the absence of a guest the crystal structure of the Na^+ complex showed a water molecule bridging two Na^+ cations, thus supporting the current opinion of an alternating chain of cations and water through the filtering region of both Na^+ and K^+ selective channels. A patch clamp study of this compound indicated ion transport, possibly due to membrane disruption, at potentials consistent with Na^+ flux (Cragg 1999b).

A class of resorcin[4]arenes containing phenoxyalkyl ether, phenoxypolyether and phenoxycrown ether moieties, reported by Beer and co-workers, displayed K^+ selectivity over other metal cations (Fig. 1.16, **viii**) (Wright 2001). The acetylated derivatives were able to transport K^+ across a chloroform bulk liquid membrane with varying efficacies. Parent compounds with potential channel lengths in the region of 19 to 21 Å were active in the phospholipids bilayers, with four conductance levels, but derivatives containing shorter alkyl chains (ca 16 Å in length) were inactive. The polyether-containing derivatives (Fig. 1.16, **ix**) also conducted cations but only in bursts pointing to membrane perturbation rather than channel formation as the likely source of activity. As with Kobuke's resorcinarene system, gramicidin-like behaviour was proposed as the channel formation mechanism.

Calix[4]arene-cholic acid conjugates (Fig. 1.16, **x**) have been shown by Tecilla and co-workers to transport both Na^+ and H^+ across vesicle membranes. The rate constant for the *1,3-alt* conformer was similar to the membrane-spanning protein amphotericin B and an order of magnitude greater than the *cone* (Maulucci 2005). This is convincing evidence that membrane spanning compounds perform significantly better than those that must aggregate to transport ions.

1.8 Methods for determining transmembrane ion transport

Transmembrane ion transport can be studied directly through either a lipid bilayer or a cell membrane. In the former case a purified lipid is painted across a small hole in an impermeable wall between two compartments. The compounds to be studied are incorporated into the bilayer and the ion of interest introduced. Transmembrane currents are then measured across the bilayer. Transport through a living cell is often measured as a function of single channel activity using the technique of patch clamping, however, there is always the danger that the events recorded are due to endogenous channel activity rather than the model compound. Another useful technique involves the formation of liposomes (vesicles comprised of phospholipid bilayers) incorporating potential carrier or channel-forming compounds in the lipid matrix (Hardy 2001) This method is particularly attractive for studies on anion transport as Cl^--sensitive fluorescent dyes can be included within the liposomes. Influx of Cl^- can then be determined by measuring fluorescence quenching (Williams 1995).

1.9 Project rationale and summary of work undertaken

From the forgoing discussion it is clear that understanding mechanisms governing transmembrane ion transport, particularly those relating to selectivity, is of great importance. Evidence of natural protein channel mechanisms comes from a variety of techniques but none are able to identify the specific interactions that govern the movement of ions through the channels. X-ray structures are limited in number, relate to systems with different ion selectivities (e.g. KcsA for K^+, ClC for Cl^-) and give a structural snapshot rather than dynamic data. The fact that the structures are crystalline indicates that these molecules are not in their active biological forms and inferences made from crystallography may not reflect *in*

vivo mechanisms. Electrophysiological bilayer studies can determine ion selectivities and give an indication of the transport mechanism (e.g. channel or shuttle) but not the key interactions that determine selectivity. Site directed mutagenesis of natural proteins can determine which particular amino acid residues are essential for activity but not why. Computer simulations, though valuable as ways to visualise channels and test theories, are just models. They can be used to turn amino acid sequences into 3D structures, based on similarities to known examples, but cannot supplant *in vivo* or *in vitro* data. The hypothesis tested by this project is that simple *artificial* systems can give useful information on the essential requirements of natural ion selective transmembrane channel-forming molecules. Rigid macrocycles would be expected to provide ion selectivity, based on the size and coordination preferences of the ion, and, when coupled to membrane-penetrating substituents, it should be possible to observe the formation and activity of ion-selective transmembrane channels within phospholipid bilayers.

The objective of this project was therefore to use a modular approach that combined rigid macrocycles with membrane penetrating and spanning substituents to generate artificial ion-specific transmembrane channel forming compounds. To this end the following work was undertaken:

- synthesis and derivatization of oxacalix[3]arenes, azacalix[3]arenes, p-t-butylcalix[6]arene and calix[4]arene (Chapter 2)
- assessment of cation binding and transmembrane transport (Chapter 3)
- report of synthetic methods and full characterization of compounds (Chapter 4)
- summary of results (Chapter 5)

Chapter 2

2.1 Introduction

The aim of this project was to synthesise an artificial ion channel which can span across the phospholipid bilayer and discriminate against different ions. The idea was to use calixarenes as the source of cavity for specific ions to pass through. Attaching a long chain molecule in the lower rim of the calixarenes, long enough that it can span the cell membrane and with properties similar to the phospholipid bilayer was a primary driving force for the project. Calixarenes (Gutsche 1989; Vicens 1991) have different sizes of central cavities and can exist in different conformations depending on their size. Variations in size and conformation give the calixarenes potential to produce ion channels for different ions, such as cations (Cobben 1992; Arnaud-Neu 1993; Brzozka 1993), anions (Beer 1993; Morzherin 1993) and neutral molecules (Gutsche 1992; Loon 1992; Murakami 1993). With this in mind, it was decided to put some other calixarenes to the test. Expanded calix[3]arenes, specifically oxacalix[3]arenes and azacalix[3]arenes, are ideal frameworks for model ion channels as they can be constructed around a rigid, functionalisable macrocycle that has been shown to act as an ion selective filter (Cragg 1999a). Compounds described so far in the literature do not have the membrane spanning substituents necessary for them to act as ion channels but are able to function as ion transporters (Oluyomi 2007).

Oxacalix[3]arenes are a class of calixarenes which differ in the bridging between the aromatic rings which is $ArCH_2OCH_2Ar$ rather than the more usual $ArCH_2Ar$ motif. It is more flexible and for this reason the macrocycle can adopt different conformations (Araki 1993).

There are also differences between the central cavities in calix[n]arenes and oxacalix[3]arenes. This is discussed extensively in the literature by Cragg and co-workers (Cragg 1999a; Cragg 1999b; Cragg 2001; Miah 2002), where the preferable type of conformers adopted by the oxacalixarenes is explained in detail. Cragg and co-workers (Cragg 1999a) showed that the 4-t-butyloxacalix[3]arenetris(diethylamide) derivative (Scheme.2.1) works as a Na^+ filter.

Scheme 2.1 *Synthesis of an oxacalixarene 'Na$^+$ filter' (Cragg 1999a)*

Other calixarenes have also attracted interest as filters, notably calix[4]arenes, such as the synthetic Cl^- ion transporters from Davis (Oluyomi 2007), and the conformation studies from Shinkai and co-workers (Ikeda 1994), and the more flexible calix[6]arenes that has been reported by Gutsche (Gutsche 1985). In principle an ion-specific channel could be designed by searching the literature for suitable 'filter' molecules and attaching membrane-spanning substituents (Liang 2007). These substituents must have properties that are complementary to those of the bilayer. Ideally this requires that the termini of the compound are polar to match the phospholipid head groups but the bulk of the molecule is hydrophobic to match the lipid

rich core of the bilayer. Incorporation of amphiphilic substituents would therefore seem to be an essential feature in any model, however, many amphiphiles have been shown to disrupt phospholipid bilayers therefore compromising membrane integrity (Fuhrhop 1986; Fyles 1998; Eggers 2003; Kobuke 2001; Menger 1990; Jayasuriya 1990). Cornforth reported the effect of a polyether-containing molecule, Triton-A20, in 1951 (Cornforth 1951). In fact the earliest example of a calixarene-polyether conjugate is the antitubercular *p*-alkylcalix[8]arene macrocyclon prepared and tested by the Cornforth group (Cornforth 1955). This compound, illustrated in Fig. 2.1 (along with its calix[6]arene homologue), has been shown to have antimycobacterial properties that the unsubstituted parent did not (Colston 2004). Although ion channel formation was not cited as a source of this activity it may have been an important factor.

Figure 2.1. *Schematic structure of macrocyclon (n = 8, R_1 = t-octyl, x = 9-10) and analogues (Coulston 2004)*

2.2 Expanded calix[3]arene ionophores

Hultzsch, Gutsche and Hampton all worked with expanded calix[3]arenes (Hultzsch 1962; Gutsche 1989; Hampton 1996; Chirakul 1998). An oxacalixarene derivative that appeared to act as a sodium specific ionophore was synthesised by Cragg and co- workers (Cragg 1999a). Azacalix[3]arenes, analogues of the oxacalix[3]arenes but with nitrogens in the macrocycle ring, have been synthesized as receptors for a large variety of guests (Hultzsch 1962). Azacalix[3]arenes have the potential to act as receptors for anions as they can be protonated to give a hydrophobic cationic cavity to provide binding sites for these guests (Takemura

1999). In addition the amine groups can be modified to introduce additional functional groups. The CH_2-NR-CH_2 bridges also provide flexibility. Azacalixarenes are ideal for the use as the basis of an artificial ion channel, as they can be prepared in a *cone* conformation which is the intended shape for an ion channel. A number of substituents can be attached to the nitrogen bridgehead which is not possible in the oxacalixarene analogue. Certain substituents, such as phenyl rings, should be able to anchor the azacalixarene in a phosphlipid bilayer making the channel more stable for the ion passage. Consequently it was proposed that azacalix[3]arenes should be investigated as potential ion channel filters.

2.3 From ionophores to transmembrane channels

The ion specific binding properties of the macrocycles described above can be used to filter specific ions through a phospholipid bilayer but their orientation needs to be elongated so that they can span the membrane. One way to do this is to incorporate amphiphilic membrane spanning substituents through either the upper or lower rim. In practice it is easier to modify the lower rim through the phenolic oxygen atoms so then a choice must then be made regarding which substituents to choose. The commercial Triton® non-ionic surfactants (Iqbal 2007; Gao 2009) widely used to destroy liposome and cell membranes would therefore appear to make ideal substituents for membrane spanning artificial ion channels (Delamaza 1994; Velluto 2011). They are long enough, at 3 to 4 nm, to penetrate lipid bilayers and appear to target areas of osmotic stress but are otherwise non-specific in their activity (Robson 1977). These compounds can successfully insert because they contain both amphiphilic and lipophilic regions allowing them to interact favourably with the polar, solvated external and internal bilayer surfaces while passing through the hydrophobic core of the bilayer where lipid groups interact. It was also decided to prepare another group of ‘tails’

based on triethylene glycol monomethyl ether, a shorter amphiphile than Triton-X100®. The iodo derivative is analogous to the Triton iodide and makes it possible to attach different types and sizes of tails to form channel-like structures that could be tested in bilayer membranes. The investigation can relate different lengths of tails to transmembrane activity. The idea came from the short, natural gramicidins where two molecules span the membrane through the formation of end-to-end dimer held together by hydrogen bonds (Burkhart 1998). As gramicidin is shorter than Triton-X100® it consequently requires compound aggregation to span a bilayer.

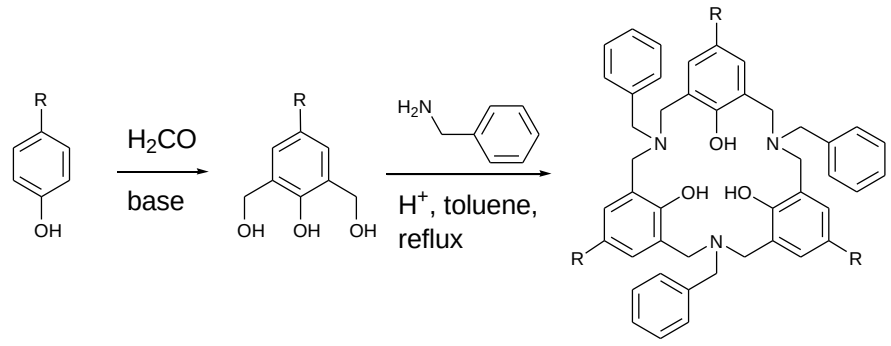
2.4 Attempts to synthesise an ion channel filter with azacalix[3]arenes

To determine if oxa- or azacalix[3]arenes could form the filter region of an artificial ion channel several derivatives were prepared with the intention that membrane-spanning polyether groups could be introduced as lower rim esters. To do this the expanded calix[3]arenes would need to be prepared as the tris(acid) derivatives and in the *cone* conformer. Acid derivatives can be prepared through cleavage of either the tris(ethyl ester) or tris(amide). Formation of the ester from the parent calix[3]arene results in a mixture of *cone* and *alternate* conformers and leads to a mixture of acid conformers however cleavage of the easily isolated *cone*-tris(amide) would yield the *cone*-tris(acid) as the only product (Cragg 1999a).

The most frequently reported functionalization of azacalix[3]arene is through *O*-substitution which generate *cone* and *partial cone* conformers (Takemura 1994, Takemura, 2002, Kaewtong 2008, Kaewtong 2006). Following these substitutions a ratio of 1:1 *cone* : *partial cone* is usually obtained even when a strong base such as NaH (in contrast to K_2CO_3 or

$NaBH_4$) is utilized (Kaewtong 2009). Surprisingly, the presence of a substituent chain with more than three atoms, such as methoxyacetyl, does not inhibit *cone* to *partial cone* conversion following further modification (Kaewtong 2008). This has been explained by the inherent increased flexibility afforded by the azacalix[3]arene structure in comparison to calix[4]arene. To date the only cone selective functionalization of azacalix[3]arene has been achieved with 1-(trimethylsilyl)imidazol and 1,1,1,3,3,3-hexamethyidisilazane. A selective synthesis of N-benzylhexahomotriaza-p-chlorocalix[3]trinaphthylamide in partial cone conformation has been achieved by using 1-aminomethylnapthalene as the molecule to control steric effects (Kaewtong 2008).

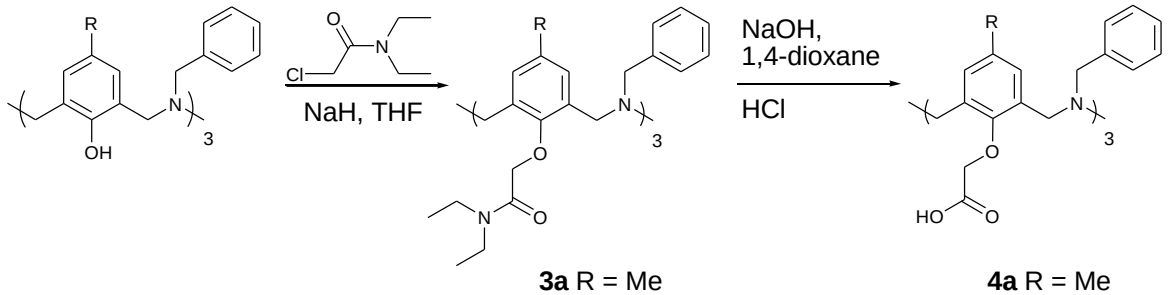
Previous work in the group had focused on oxacalix[3]arenes so it was decided to target the azacalix[3]arenes in this project (Schemes 2.2 and 2.3). The azacalix[3]arenes have the additional potential to form charged quaternary ammonium derivatives that may be attractive to anions.The methyl derivative is less sterically hindered than the t-butyl so should lead to greater flexibility in the macrocycle. The phenyl derivative extends the depth of the macrocyclic cavity and should make it more attractive to cations due to the increased density of electrons in the π-system.



1a R = Me
1b R = ^{t}Bu
1c R = C_6H_5

2a R = Me
2b R = ^{t}Bu
2c R = C_6H_5

Scheme 2.2 *Synthesis of azacalix[3]arenes discussed in this chapter.*

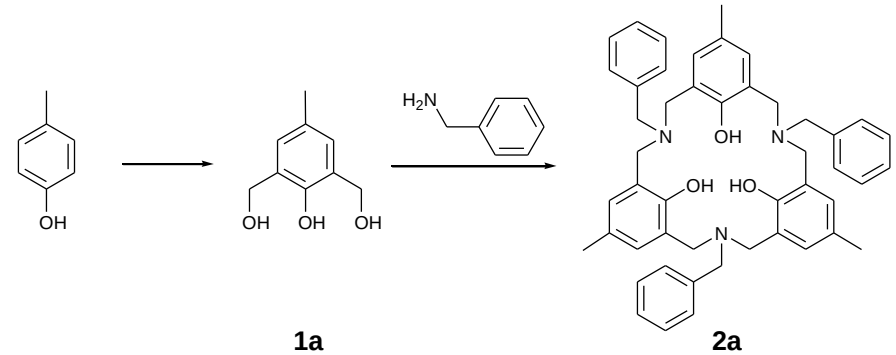


3a R = Me
3b R = ^{t}Bu
3c R = C_6H_5

4a R = Me
4b R = ^{t}Bu
4c R = C_6H_5

Scheme 2.3 *Azacalix[3]arene derivatives discussed in this chapter.*

2.4.1 4-Methyl(*N*-benzyl)hexahomotriazacalix[3]arene (**2a**)



1a **2a**

Scheme 2.4 *Synthesis of **2a***

2.4.1.1 Preparation of 2,6-bis(hydroxymethyl)-4-methylphenol (**1a**)

Sodium hydroxide was added to *p*-cresol in THF and following subsequent addition of formaldehyde the reaction was left at room temperature for seven days. Addition of propan-2-ol resulted in precipitation of the sodium 2,6-bis(hydroxymethyl)-4-methyl phenolate salt which was then isolated by filtration. The product was then recrystallized with toluene to give 2,6-bis(hydroxymethyl)-4-methylphenol (**1a**) (Hampton 1994). The yield obtained was a reasonable 55%. ^{1}H NMR, recorded in $CDCl_3$, shows a singlet at 6.85 ppm for the aromatic protons (2H). The singlet at 4.71 ppm is consistent with the methylene groups present on the 1,4-disubstituted aromatic ring, with the integration for four protons Also present is the singlet at 2.25 ppm for the methyl group attached to the aromatic ring (3H). The mass

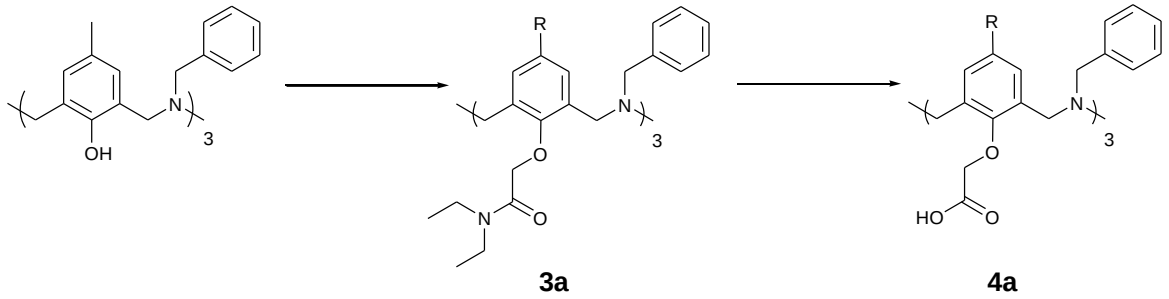
spectrum of 191.07 was obtained which correlated with the expected value for $[M + Na]^+$ of 191.07, confirming the identity of this compound.

2.4.1.2 Preparation of 4-methyl(*N*-benzyl)hexahomotriazacalix[3]arene (**2a**)

4-Methyl(*N*-benzyl)hexahomotriazacalix[3]arene was prepared by the addition of 2,6-bis(hydroxymethyl)-4-methylphenol to a solution of benzylamine in toluene and heating at reflux for 24 hours with *p*-toluenesulfonic acid. The solution was evaporated under pressure and the crude product was collected as a yellow oil. The product was purified by the dropwise addition of methanol to a solution of the product dissolved in dichloromethane and left overnight. The expected compound was isolated as a yellow microcrystalline solid precipitate.

The product was obtained in 30% yield which, according to the literature (Takemura 1992) was quite low. ^{1}H NMR, recorded in $CDCl_3$, shows a singlet for the proton at 9.2 ppm of the phenol in the aromatic ring. At 7.87 ppm a singlet representing the six protons which are in a similar environment in the aromatic ring and between 6.75 and 7.40 ppm a multiplet is observed which represents the 15 protons from the benzyl groups attached to the nitrogens in the azacalix[3]arene ring. The singlet at 4.33 ppm represents the methylenes in the ring. Finally, a singlet signal in the NMR spectra at 1.39 ppm which integrated as nine protons of the methyl groups in the azacalix[3]arene ring. The high resolution mass spectra found a peak at 718.3953 which agreed with the expected value of 718.3930 for $[M]^+$. Thus compound **2a** was synthesised and confirmed by the techniques used.

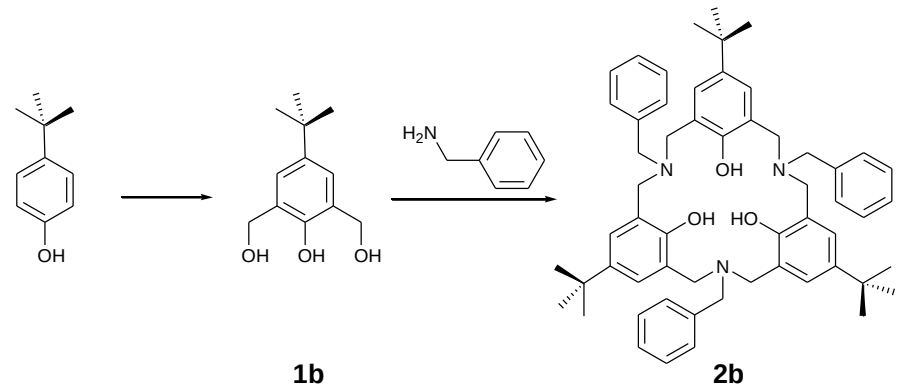
The synthesis of 4-methyl(*N*-benzyl)hexahomotriazacalix[3]arenetris(*N*,*N*-diethylacetamide) (**3a**), and subsequent cleavage to the acid, was attempted. No products were isolated.



Scheme 2.5 *Attempted syntheses of* ***3a*** *and* ***4a****.*

The methods used to attempt the preparation of **3a** and **4a** were adapted from those used by Shinkai (Matsumoto 1995) and Cragg (Cragg 2005) to synthesise the oxacalix[3]arene analogues. The same methods applied to the azacalix[3]arene **2a** gave inconclusive results and the molecular ion was not found in the mass spectroscopy.

2.4.2 4-t-Butyl(*N*-benzyl)hexahomotriazacalix[3]arene (**2b**)



Scheme 2.6 *Synthesis of* ***2b***

2.4.2.1 Preparation of 2,6-bis(hydroxymethyl)-4-t-butylphenol (**1b**)

Following the method of Hampton, sodium hydroxide was added to 4-t-butylphenol in THF (Hampton 1994). Once the reaction mixture cooled, formaldehyde was added and the reaction

was left at room temperature for seven days. When the reaction had gone to completion, propan-2-ol was added to the paste producing the precipitate of sodium 2,6-bis(hydroxymethyl)-4-t-butyl phenolate. This was then isolated by filtration and recrystallized with toluene to give 2,6-bis(hydroxymethyl)-4-t-butylphenol in 36% yield (Matsumoto 1995). ^{1}H NMR, recorded in $CDCl_3$, shows a singlet at 7.05 ppm for the two aromatic protons. The singlet at 4.79 ppm is consistent with the methylene groups present in the 1,4-disubstituted aromatic ring. Also present is the singlet for the t-butyl group attached to the aromatic ring which integrated to nine protons. The mass spectrum of 232.1130 was obtained which correlated with the expected value for $[M + Na]^+$ of 232.11, confirming the identity of this compound.

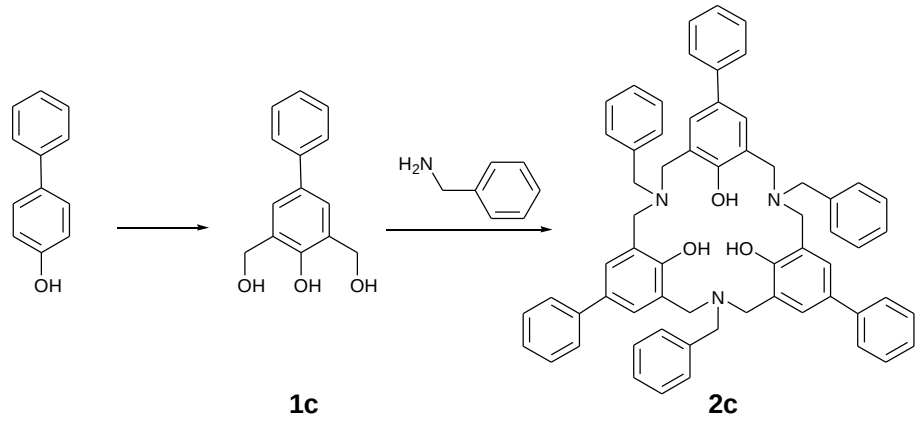
2.4.2.2 Preparation of 4-butyl(*N*-benzyl)hexahomotriazacalix[3]arene (**2b**)

4-t-Butyl(*N*-benzyl)hexahomotriazacalix[3]arene (Hampton 1996) was prepared by the addition of 2,6-bis(hydroxymethyl)-4-butylphenol to a solution of benzylamine in toluene and heating at reflux for 24 hours with *p*-toluenesulfonic acid. The solution was evaporated under pressure and a yellow oil was collected as the crude product. The product was purified by the addition of diethyl ether and recovered as a yellow microcrystalline powder in 30% yield.

^{1}H NMR, recorded in $CDCl_3$, shows a singlet for the proton at 9.2 ppm of the phenol in the aromatic ring. At 7.87 ppm a singlet represents the two protons which are in similar chemical environments in the aromatic ring; between 6.75 and 7.40 ppm a multiplet is observed which represents the five protons from the benzyl groups attached to the nitrogens in the azacalix[3]arene ring. The singlet at 3.62 ppm represents the methylene groups. Finally, a singlet is shown in the NMR spectrum at 1.30 ppm which confirms the nine protons of the t-

butyl groups in the azacalix[3]arene ring. The high resolution mass spectra gave a peak at 844.5457 which agreed with the expected value of 844.5417 for $[M + H]^+$. Thus compound **2b** was synthesised and confirmed by the techniques used.

2.4.3 4-Phenyl(*N*-benzyl)hexahomotriazacalix[3]arene (**2c**)



1c **2c**

Scheme 2.7 *Synthesis of* ***2c***

2.4.3.1 Preparation of 2,6-bis(hydroxymethyl)-4-phenylphenol (**1c**)

Following the method of Gutsche, 2,6-bis(hydroxymethyl)-4-phenylphenol was prepared by dissolving 4-phenylphenol in THF, then adding sodium hydroxide and formaldehyde(Gutsche 1982). After seven days at room temperature propan-2-ol was added to the mixture and the precipitated phenolate was suspended in acetone and acidified with glacial acetic acid. The sodium acetate recipitate was removed by filtration. Finally the off white solid product was collected after the solvent was removed under vacuum. The product was dissolved in hot toluene and left to crystallize to give the title compound.

The yield of the product was 36%. ^{1}H NMR, recorded in $CDCl_3$, shows two triplets and one doublet with an integration for the seven protons in both aromatic rings. The singlet at 4.80

ppm represents the four protons from the methylene on the hydroxyl ring. From the ^{1}H NMR spectrum it can be deduced that the 4-phenylphenol derivative was synthesised. The mass spectrum showed a peak at 253.0848 which was calculated as 253.08 $[M + Na]^{+}$. This compound was synthesised and confirmed by the techniques used.

2.4.3.2 Preparation of 4-phenyl(*N*-benzyl)hexahomotriazacalix[3]arene (**2c**)

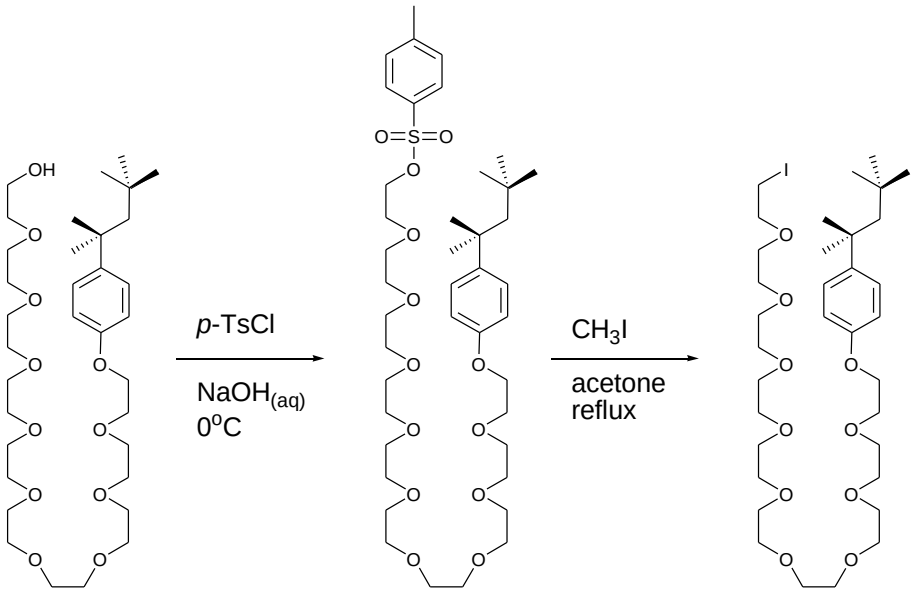
2,6-Bis(hydroxymethyl)-4-phenylphenol was added to a solution of benzylamine in toluene. *p*-Toluenesulfonic acid was added with additional toluene and this mixture was left to heat at reflux overnight. After the solvent was evaporated the resulting oily yellow precipitate was collected. With the addition and evaporation of diethyl ether the product was collected as a yellow fluffy precipitate and this was left overnight to dry and isolated as a yellow powder. The results show a very small yield was obtained. ^{1}H NMR, recorded in $CDCl_3$, shows multiplets between 7.00 and 7.65 ppm for the aromatic protons. At 3.75 ppm a doublet is seen for the methylenes between the aromatic rings representing the 12 protons which are in a similar environment. The high resolution mass spectrum gave a peak at 904.4291 m/z which agreed with the expected value of 904.4478 for $[M + H]^{+}$ fully supporting the proposed structure for compound **2c.**

2.4.4 Attempts to prepare azacalix[3]arenes with membrane penetrating substituents

As discussed above incorporation of commercially available Triton® surfactants as the membrane spanning part of the molecules was an attractive prospect. These amphiphiles are polyether derivatives with a terminal hydroxyl group so can be derivatised to react with the phenolic groups of the azacalix[3]arenes. Triton® surfactants are composed of a polyether section with different numbers of polyether repeat units and an octylphenol terminus. In

Triton-X100® there are ten polyether groups whereas Triton-X45® is a mixture of four and five polyethers. Many commercially available polyethers are sold by average molecular mass, which would make characterisation of compounds difficult, but fortunately Triton-X100® was shown by ^{1}H NMR to be at least 90% pure based on ten polyether repeats.

2.4.5 Preparation of Triton-X100® tosylate (**5**) and Triton-X100® iodide (**6**).



Scheme 2.8 *Synthesis of Triton® derivatives.*

The easiest way to couple Triton-X100® to the phenolic groups of the azacalix[3]arene would be through the tosylate which should form readily. Other workers (Loiseau 2007) had reported that the bulky tosylates proved difficult to react with calix[n]arenes, where presumably the leaving groups became bound in the macrocyclic cavity, but that the iodo derivatives were more successful. Consequently it was decided to prepare Triton-X100® tosylate and then generate the iodide.

2.4.5.1 Preparation of Triton® tosylate (**5**)

Compound **5** was prepared by dissolving Triton-X100® in THF and distilled water. An aqueous solution of sodium hydroxide as added to the mixture which was stirred in an ice bath until the solution temperature fell below 5°C. *p*-Toluenesulfonyl chloride was then added and the temperature was maintained below 5°C. After stirring for 30 minutes the THF was removed under vacuum and the resulting aqueous solution extracted into toluene. The solvent was removed and the product readily identified as Triton® tosylate (Cragg 2005). ^{1}H NMR data, recorded in $CDCl_3$, shows doublets at 7.79 and 7.32 ppm for a 1,4-disubstututed aromatic ring (4 H, *J* = 8.*6 Hz*) which is consistent with the presence of the tosyl group (2 and 3 in Fig. 2.2). At 7.25 and 6.81 ppm doublets (4H, *J* = 8.6 Hz) are also present representing the 1,4-disubstututed aromatic ring at the alkyl terminus of the molecule (7 and 8 in Fig. 2.2). Signals showing triplets at 4.14 ppm (2 H) and at 4.10 ppm (2 H) arise from the methylene protons next to the two aromatic rings (4 and 6 in Fig. 2.2). At 3.83 ppm there is a triplet which represents the methylene protons on the second carbon from the tosyl group (5 in Fig. 2.2). The remaining 34 protons in the polyether ($-OCH_2CH_2$) region are all in a similar environment and appear as a multiplet from 3.71 to 3.56 ppm. The singlet at 2.43 ppm is from the three equivalent protons in the tosyl $-CH_3$ group (1 in Fig. 2.2), the singlet at 1.69 ppm is from two methylene protons in the alkyl region of the molecule (10 in Fig. 2.2) and a singlet at 1.33 ppm is from six methyl protons in the groups closest to the aromatic ring (9 in Fig. 2.2). Finally a singlet is present at 0.7 ppm (9 H) from the remaining three methyl groups in the alkyl region (11 in Fig. 2.2).

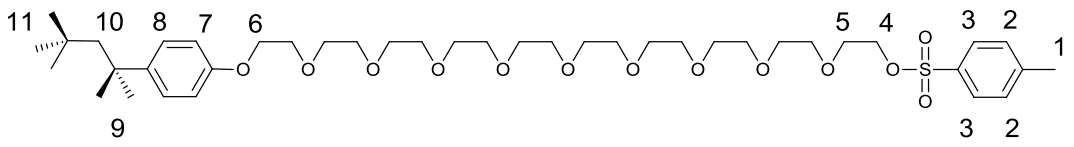


Figure 2.2 *Triton-X100® tosylate NMR signal assignment.*

The high resolution electrospray ionoization mass spectrum also showed the molecular ion of the Triton® tosylate, as the sodium salt, which was found to be 823.4467 in agreement with the expected value of 823.4279.

2.4.5.2 Preparation of Triton® iodide (**6**)

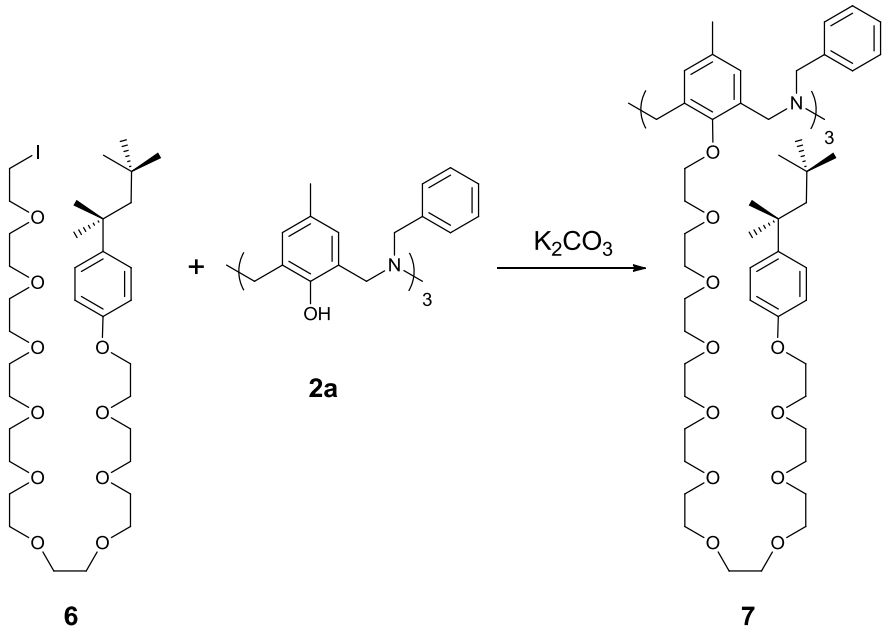
Compound **6** was prepared when potassium iodide was added in a ratio of 5:1 to a solution of Triton tosylate in acetone. The solution was heated at reflux for 24 hours then the inorganic salts were removed by filtration and the filtrate evaporated. The residue was dissolved in ethyl acetate and water in a ratio of 1:1. After washing with water and saturated sodium thiosulfate, the product was dried using magnesium sulfate, filtered and the solution evaporated by the use of the rotary evaporator resulting in the expected product as the Triton® iodide (Loiseau 2004).

^{1}H NMR data shows doublets at 7.25 and 6.81 ppm (4 H, *J*= 8.6 Hz) which correspond to the aromatic protons. A triplet at 4.10 ppm, downfield from the majority of the signals for the polyether, is from the methylene group next to the aromatic ring ($ArOCH_2$-) and the triplet further upfield at 3.83 ppm is from the methylene group attached to the iodine. Between 3.71-3.56 ppm is a multiplet from the remaining 34 polyether protons (-OCH_2CH_2-). The remaining signals below 2 ppm are, as before, from the terminal alkyl chain.

When analysing the NMR results for triton tosylate and triton iodide it shows that the signals observed are consistent with the transformations that occurred during the synthesis of this compound. The doublets observed at 7.79 and 7.32 ppm for the aromatic tosylate disappeared when the tosyl group was replaced by iodine and a triplet at 3.25 ppm became distinct from the remaining polyether protons as a result of that substitution. The mass spectrum was also

consistent with the structure of Triton® iodide as a mass of 779.3299 compared favourably with the calculated value of 779.3207 for the sodium salt.

2.4.5.3 Attempted synthesis of 4-methyl(*N*-benzyl)hexahomotriazacalix[3]arene-(Triton)$_3$ (**7**)



Scheme 2.9 *Attempted synthesis of 4-methyl(N-benzyl)hexahomotriazacalix[3]arene(Triton)$_3$ (**7**)*

4-Methyl(*N*-benzyl)hexahomotriazacalix[3]arene, **2a**, and potassium carbonate were suspended in dry acetonitrile. Triton iodide was added to this stirred suspension and the resulting mixture was heated under reflux and under nitrogen atmosphere for 4 days. The suspension was allowed to cool and the solvent removed under pressure to give a mass of white solid. Analysis gave inconclusive results due to solubility problems in the work up.

As discussed above, it was shown to be difficult to obtain the tris(amide) derivatives due to solubility problems in the work up so further development in the method would be necessary to apply the method used to derivatise oxacalix[3]arenes to the azacalix[3]arenes. Because it was successful with the oxacalix[3]arenes (Cragg 1999a), it was thought that it would also be successful with the azacalix[3]arenes too, but difficulties in the synthesis of the trisamide

arose and the last step was left incomplete. It was decided to explore the possibility of using oxacalix[3]arenes in place of the azacalix[3]arenes to overcome the difficulties encountered in extending the lower rim. Further experiments would need to be performed to substantiate the true cause of the problems surrounding the lack of formation of the trisamide and trisacid for the azacalix[3]arene compounds.

2.5 Attempts to synthesise an ion channel filter with oxacalix[3]arenes

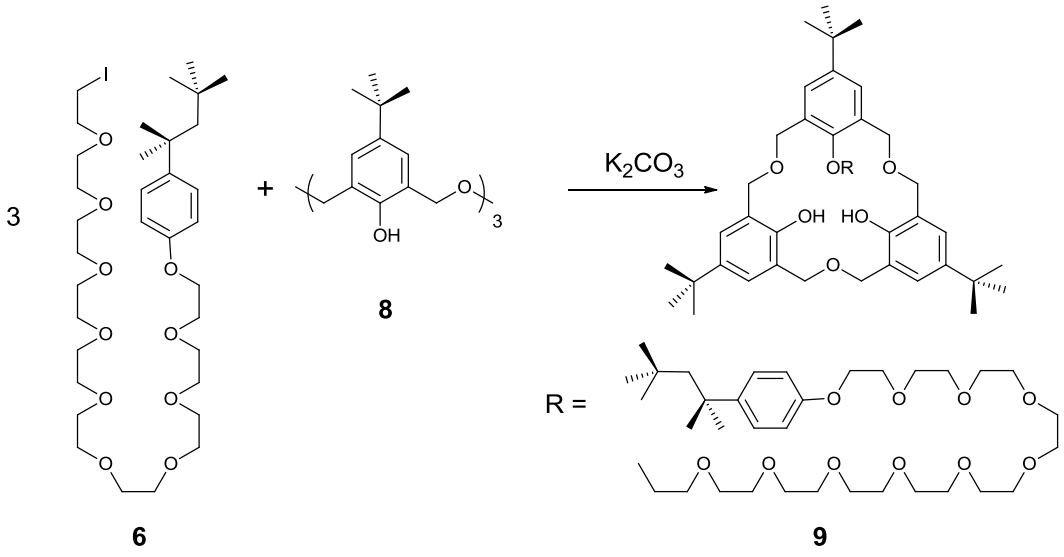
2.5.1 Synthesis of an oxacalix[3]arene-Triton-X100® conjugate

Difficulties encountered in the attempted syntheses of azacalix[3]arene derivatives led to a rethink in the approach. First it was important to determine if the method was sound. Assuming that the lack of success with the azacalix[3]arenes was due to the presence of the nitrogen bridgeheads, which may become protonated or interfere with further reactions through some other mechanism, the oxacalix[3]arenes should be easier to work with and react cleanly with the Triton® derivatives. The group has considerable experience with derivatives of this class of macrocycle (Cragg 1999a; Cragg 1999b; Cragg 2002; Cragg 2005) and has shown that they mimic the filter region of a sodium selective ion channel. To test this a sample of the group's 4-t-butyloxacalix[3]arene (**8**) was reacted with Triton® iodide.

A suspension of 4-t-butyloxacalix[3]arene, anhydrous potassium carbonate and three equivalents of Triton iodide, **6**, was refluxed in anhydrous acetonitrile under nitrogen for seven days. When cooled the solvent was removed on a rotary evaporator and the residue was suspended in dichloromethane. This was washed with hydrochloric acid and brine then the

organic phase was separated. The solvent was removed under reduced pressure to yield an yellow oil.

Analysis of ^{1}H NMR, recorded in $CDCl_3$, indicated that rather than three substituents attaching to the macrocycle only one was present. This was confirmed by high resolution mass spectrometry which gave a peak at 1245.7703 corresponding to the unusual $[M + K + H]^+$ species with an expected *m/z* of 1245.7386. Potassium was presumably picked up during synthesis and remained bound in the final product. The reaction used a greater than three equivalents of Triton iodide with the expectation that three Triton groups would be attached but it was concluded from the mass spectrum that only one Triton substituent had been attached. As the oxacalix[3]arenes are conformationally flexible this result would suggest that the steric bulk of Triton iodide made it difficult for it to react with all three phenolic groups. In addition the oxygen atoms present in the bridge can hydrogen bond to rotating phenolic protons which could affect the reaction. The ^{1}H NMR confirmed the mass spectral evidence as two aromatic signals, at 7.15 and 7.01 ppm, integrated in a 1:2 ratio implying that one of the three phenolic groups had been substituted.



Scheme 2.10 *Reaction of 4-t-butyloxacalix[3]arene (**8**) with Triton-X100® iodide*

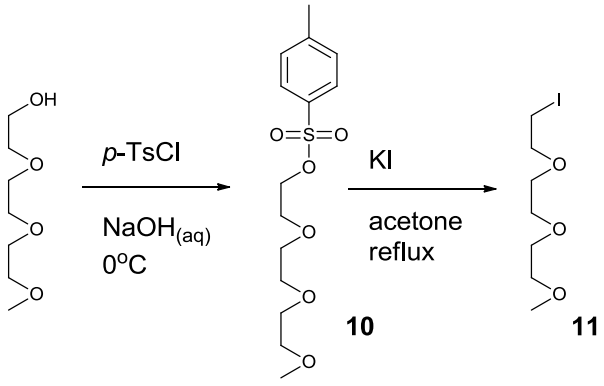
2.5.2 Use of less bulky substituents

As well as difficulties with the nitrogen bridgehead it appeared that the steric bulk of the substituents were causing problems. To assess this it was decided to introduce less bulky substituents. Triethylene glycol monomethyl ether was readily available and could be transformed into the iodo derivative (Scheme 2.8) in the same manner as the Triton-X100® compounds. This was undertaken so that the monomethyl ether of triethylene glycol iodide could be conjugated with 4-t-butyloxacalix[3]arene.

Successful conjugation of three substituents would support the hypothesis that steric bulk was also a factor in the lack of success encountered when coupling azacalix[3]arenes with Triton-X100® derivatives.

2.5.3 Preparation of triethylene glycol tosylate, monomethyl ether (**10**) and triethylene glycol iodide, monomethyl ether (**11**)

2.5.3.1 Synthesis of triethylene glycol tosylate, monomethyl ether (**10**)



Scheme 2.11 *Synthesis of triethylene glycol iodide, monomethyl ether (**11**)*

The tosylation of triethylene glycol monomethyl ether was done following the same method as the Triton-X100® tosylate (Loiseau 2004). Triethylene glycol monomethyl ether was dissolved in a mixture of THF and aqueous sodium hydroxide and stirred in an ice bath until the solution temperature fell below 5 ºC. A solution of *p*-toluenesufonyl chloride in THF was added to the stirred solution at a rate that kept the temperature below 5 ºC. Once the addition was complete, the solution was left to stir for a further 1 h below 5 ºC, poured onto iced water and stirred until all the ice melted. Most of the THF was removed by rotary evaporation the product extracted into toluene. The organic extract was dried over calcium chloride, filtered and the solvent removed by rotary evaporation. Triethylene glycol tosylate, monomethyl ether, **10**, was obtained as a colourless oil in almost 90% yield and was readily identified as triethylene glycol monomethyl ether tosylate (Iqbal 2007; Cragg 2005).

Analytical data confirmed the proposed identity of the product. As is common for polyethers, mass spectrometry indicated the formation of a sodium salt with the experimentally determined mass of 341.1056 agreeing with the mass for the $[M + Na]^+$ species of 341.1035. ^{1}H NMR data shows doublets at 7.80 and 7.35 ppm (2.5 Hz) for the aromatic protons which is consistent with the presence of the tosyl group. A triplet at 4.16 ppm for the methylene group next to the tosyl region integrated to two protons. A multiplet is present at 3.50-3.75 ppm for the six remaining methylene polyether protons ($-OCH_2CH_2-$) of the tosylated triethylene glycol monomethyl ether. The singlet at 3.39 ppm is for the three methoxy protons and the singlet at 2.45 ppm is from the tosyl methyl group. The presence and the integration of the signals especially of the aromatic tosylate show that the reaction occurred.

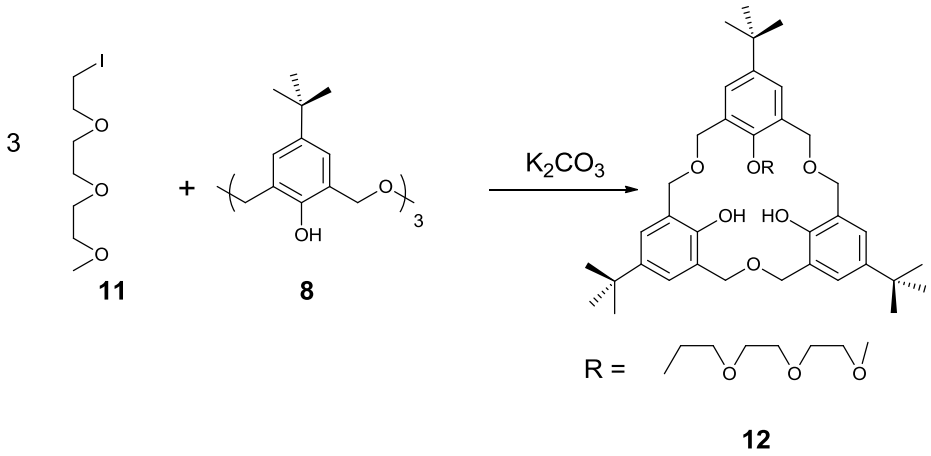
2.5.3.2 Synthesis of triethylene glycol iodide, monomethyl ether (**11**)

Using the method of Loiseau (Loiseau 2007), triethylene glycol tosylate, monomethyl ether, **10**, was dissolved in acetone. Potassium iodide (5 equivalents) was added and the mixture heated at reflux overnight. The solution was cooled to room temperature and filtered, the solvent was removed under reduced pressure and the residue dissolved in a mixture of ethyl acetate and distilled water. The mixture was separated and the ethyl acetate phase washed with water then saturated sodium thiosulfate. The organic phase was dried over magnesium sulfate, filtered and solvent removed to give triethylene glycol iodide, monomethyl ether as a colourless oil in 61% yield. Again, analytical data indicated that the expected product had been synthesised.

^{1}H NMR data shows that the aromatic signals disappeared as the tosyl group was removed. A new signal was present for the methylene group next to the iodine at 3.26 ppm and the remaining methylene protons observed between 3.76 and 3.54 ppm. The terminal methyl group appeared as a singlet at 3.39 ppm. Mass spectrometry indicated the presence of the sodium salt with the detected mass of 296.9998 agreeing with 296.9964 calculated for the $[M + Na]^{+}$ species.

With this polyether derivative in hand the final step was to couple it to an oxacalix[3]arene. As in the previous oxacalix[3]arene experiment the 4-t-butyl derivative was used. Given the conformational mobility of the oxacalix[3]arenes it was expected that all three phenolic sites could be substituted although whether the compound formed in the *cone* conformation, *partial cone* or mixture of the two remained to be seen.

2.5.4 Synthesis of an oxacalix[3]arenetriethylene glycol iodide, monomethyl ether conjugate (**12**)



Scheme 2.12 *Synthesis of 4-t-butyloxacalix[3]arene(triethyleneglycol monomethyl ether)$_3$* ***(12)***

A suspension of 4-t-butylhexahomotrioxacalix[3]arene, anhydrous potassium carbonate and three equivalents of triethylene glycol iodide, monomethyl ether was refluxed in anhydrous acetonitrile under nitrogen for seven days. When cooled the solvent was removed on a rotary evaporator and the residue was suspended in dichloromethane. This was washed with hydrochloric acid and brine and the organic phase separated. Solvent was removed under reduced pressure and the product was collected as a yellow oil in 42% yield. This was characterized with different techniques which supported the proposed structure but not the expected trisubstituted derivative. ^{1}H NMR, recorded in $CDCl_3$, showed signals characteristic of the oxacalix[3]arene ring only reacted at one of the phenols, leaving the other two phenols available for further reaction. The signal at 7.21 ppm integrates to four protons for the aromatics that did not react with the glycol molecule attached and the signal at 7.13 ppm integrates to the two protons at the aromatic part attached to the glycol molecule. The characteristic signal for the methylene groups were observed as a singlet at 4.72 with an integration of twelve protons. The resonances for the glycol molecule were observed at 3.74, and 3.58 ppm as triplets the first with the integration of four protons and the latter with the

integration of eight protons. The characteristic signal for the methyl group at the end of the glycol molecule is seen at 3.26 ppm. The important part of this analysis is seen in the presence of the two different signals for the t-butyl groups, one with the double integration of the other as expected if only one of the phenols had reacted and the other two phenolic groups remained the same. Mass spectrometry gave a peak of 745.4296 corresponding to the mass of 745.4286 calculated for the $[M + Na]^+$ species.

2.6 Assessment of the results for expanded calix[3]arenes

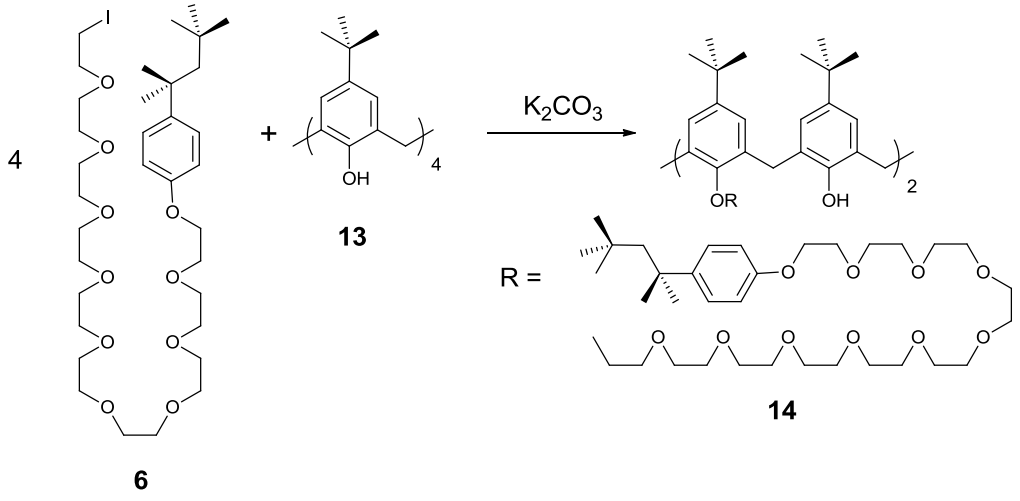
It was clear from the experiments that two problems were being encountered. Firstly, *O*-substitution reactions undertaken as part of the project on azacalix[3]arenes had so far proved unsuccessful suggesting that the nitrogen bridgeheads were interfering with the successful coupling of lower rim substituents. This was confirmed when an oxacalix[3]arene-Triton-X100® conjugate was successfully prepared. However, only one of the three possible sites had reacted suggesting that the second problem, one of steric bulk, remained an issue. Reaction of an oxacalix[3]arene with three equivalents of a small polyether also gave the monsubstituted product which would seem so confirm that steric effects were partially responsible.

At this stage it was clear that a different macrocyclic platform was required. Calixarenes were still potential filters but would have to have no oxygens or nitrogens in the ring. Consequently attention was turned to the most well known members of the calixarene class, the calix[4]arenes and calix[6]arenes. The calix[6]arenes retain the threefold symmetry present in the expanded calix[3]arenes but would require careful derivatisation. They are also considerably more flexible than the expanded calix[3]arenes which may make the synthesis

of the correct conformer a challenge. The calix[4]arenes may be frozen out in the *cone* or *1,3-alternate* conformers but lack the threefold symmetry that may be essential for cation selectivity.

2.7 Attempts to synthesise an artificial ion channel with 4-t-butylcalix[4]arene

Despite the influence of steric bulk on the reaction between Triton-X100® iodide it was decided to try reacting it with commercially obtained 4-t-butylcalix[4]arene under the same conditions as had been used earlier.



Scheme 2.13 *Synthesis of 4-t-butylcalix[4]arene(OH)$_2$(Triton)$_2$ (**14**)*

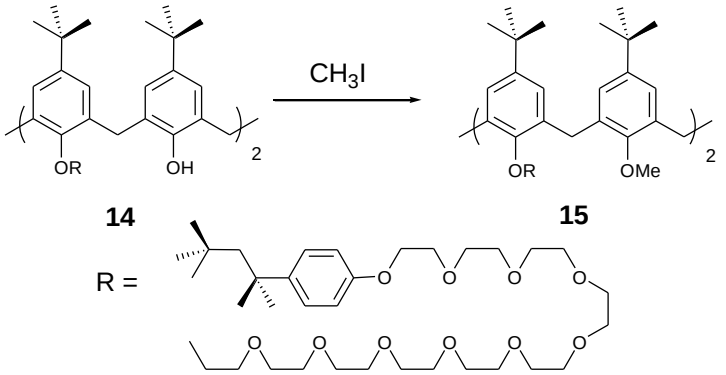
2.7.1 Synthesis of 4-t-butylcalix[4]arene(OH)$_2$(Triton)$_2$ (**14**)

Compound **14** was synthesised by dissolving 4-t-butylcalix[4]arene in anhydrous acetonitrile. Four equivalents of Triton® tosylate and potassium carbonate were added to the solution and the mixture was heated at reflux under nitrogen for 72 hours. The cooled mixture was filtered

to remove solid byproducts and the solvent was removed under vacuum to give 4-t-butylcalix[4]arene$(OH)_2(Triton)_2$ as colorless, viscous oil. A good yield of 50% was obtained for the resulting compound. ^1H NMR, recorded in $CDCl_3$, gave the characteristic doublets at 4.38 and 3.30 ppm for the calix[4]arene methylene signals indicating that the resulting compound remained in the *cone* conformation. A singlet at 7.74 ppm, integrating to two protons and assigned to ArOH, indicated that only the disubstituted compound was formed. The Triton aromatic protons were observed as doublets at 7.25 and 6.82 and singlets at 7.03 and 6.95 ppm corresponded to the calixarene aromatic protons. This demonstrated that there were two different types of aromatic rings present as two of the four phenols in the ring were attached to the Triton X-100® forming a two tailed derivative. Conformation that the compound was in the *cone* conformation came from the characteristic pair of doublets observed for the methylene protons at 4.38 ppm (J = 12.96) and 3.30 pm (J = 12.60). Mass spectrometry indicated the formation of the sodium complex as the observed mass of 1929.2877 agreed with the calculated mass of 1929.2486 for the $[M + Na]^+$ species.

A preliminary electrophysiological experiment showed no change in conductivity across a phospholipid bilayer in a buffer containing 150 mM Na^+ when **14** was added. Ten experiments were attempted and in six of these the compound clearly inserted in the bilayer (as determined by changes in bilayer capacitance after addition) but no channels were observed to form. It was proposed that compound **14** bound Na^+, as indicated by mass spectrometry, and consequently would not allow the cations to cross the bilayer. To test this, a dimethoxy derivative, **15**, was required. In principle it would not be possible for this derivative to form a salt with Na^+. Although it could still interact with cations through the methoxy groups the binding would be much weaker which would allow the ions to pass through rather than bind.

2.7.2 Attempted synthesis of 4-t-butylcalix[4]arene$(OMe)_2(Triton)_2$ (**15**)



Scheme 2.14 *Proposed synthesis of 4-t-butylcalix[4]arene$(OMe)_2(Triton)_2$* ***(15)***

4-t-Butylcalix[4]arene$(OH)_2(Triton)_2$ was dissolved in dry acetone followed by addition of potassium carbonate and methyl iodide. This was left to reflux for 18 hours. The solvent was then evaporated and the organic phase collected as yellow oil. The mass spectrum, calculated as 1934.3826 for the sodium salt, gave a value of 1934.2726 for the proposed target, however, the ^{1}H NMR did not support the presence of the proposed structure.

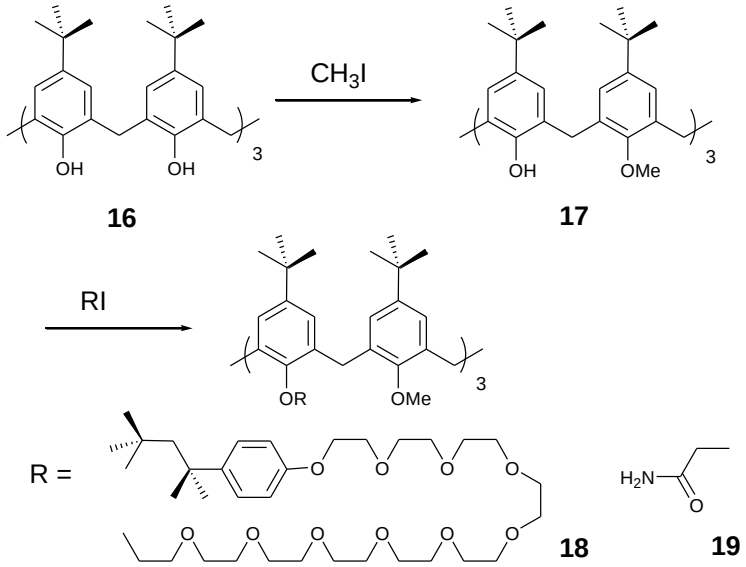
2.8 Attempts to synthesise an artificial ion channel with 4-t-butylcalix[6]arene

Following on from the successful double substitution of 4-t-butylcalix[4]arene it was decided to move to a larger calixarene, 4-t-butylcalix[6]arene, so that the effect of size on ion channel selectivity could be investigated. A derivative of 4-t-butylcalix[6]arene had been prepared in which alternate phenolic groups had been blocked by methyl substituents in order to introduce functionality into the other three positions (Groenen 1991). Achieving the correct

regioselectivity requires very careful monitoring of the experimental conditions but is possible (Otsuka 1994).

One other synthesis was attempted, that of the tris(amide) derivative. If this were to be successful it may give a route to ion channel mimetics through Schiff base condensation with polyether aldehyde derivatives.

2.8.1 Preparation of 4-t-butylcalix[6]arene$(OMe)_3(OH)_3$ (**17**)



Scheme 2.15*.Preparation of 4-t-butylcalix[6]arene$(OMe)_3(R)_3$ derivatives*

Using the method of Otsuka (Otsuka 1994) 4-t-butylcalix[6]arene, potassium carbonate and methyl iodide were added to dry acetone. The mixture was stirred and refluxed for 24 hours at room temperature under nitrogen. Acetone was removed under vacuum and the residue treated with hydrochloric acid followed by the addition of dihloromethane. The organic layer was isolated and washed with distilled water then dried with sodium sulphate, filtered and evaporated to give crude 4-t-butylcalix[6]arene$(OMe)_3(OH)_3$, **17**, as a white powder. Flash chromatography was performed using hexane : THF (9:1) afforded the purified compound as

a white powder in 53% yield. Mass spectrometric and NMR analysis, in agreement with literature data (Groenen 1991), showed that the required regioisomer had been isolated.

2.8.2 Preparation of 4-t-butylcalix[6]arene$(OMe)_3(Triton)_3$ (**18**)

The synthesis of the 4-t-butylcalix[6]arene$(OMe)_3(Triton)_3$ was performed by dissolving 1,3,5-trimethoxy(4-butyl)calix[6]arene in anhydrous acetonitrile. Triton-X100® tosylate and potassium carbonate were added and the mixture refluxed for 4 days under nitrogen. The cooled mixture was filtered and the solvent evaporated, resulting in colourless viscous oil, which was the product.

The product was isolated in a reasonable 42% yield. ^{1}H NMR shows singlets for the calixarene and Triton aromatic protons, similar to those in compound **15**. This is characteristic for the compound to which three Triton tails had been attached. There are two different sets of environments for the aromatic rings created by the attaching of the Triton tails to the 1,3,5-alternate hydroxyl groups of the lower rim. The signal at 3.70 ppm is characteristic for the methyl group attached to the hydroxyl in the lower ring and no signal for the phenol groups are present in the spectra, demonstrating that they all been substituted. This was supported by the mass spectra for M^{3+} 966.7254, which compared well with the calculated value of 966.6432.

2.8.3 Attempted synthesis of 4-t-butylcalix[6]arene$(OMe)_3(acetamide)_3$ (**19**)

A solution of 1,3,5-trimethoxyhexa(4-t-butyl)calix[6]arene in anhydrous THF was added to a suspension of sodium hydride also in anhydrous THF. The reaction was left to stir for 20

minutes at room temperature. Bubbling was observed and after 20 minutes the solution was clear yellow. A solution of bromoacetamide in anhydrous THF was introduced to the mixture and left to reflux for 24 hours. The solvent was then removed under pressure to leave a white powder. The residue was dissolved in dichloromethane and washed with distilled water. The organic layer was isolated and the solvent evaporated under pressure resulting in a white solid precipitate. The crude product was subjected to column chromatography but analysis was inconclusive. Given the success of the other 4-t-butylcalix[4]arene and 4-t-butylcalix[6]arene reactions this approach was not taken any further. Subsequently Reinaud and Jabin published the synthesis of a trisacetamide calix[6]arene derivative from 4-t-butylcalix[6]arene-$(OMe)_3(OH)_3$ (Darbost 2004) but, by that point, the project was focusing on bilayer studies.

2.8.4 Preparation of tetrakis(triethylene glycol monomethyl ether) calix[4]arene, 1,3-alternate conformer (**21**)

***Scheme 2.16**. Preparation of 1,3-alt calix[4]arene(triethylene glycol monomethyl ether)$_4$ (**21**) by Lawal (Lawal 2009)*

A suspension of calix[4]arene (**20**), anhydrous caesium carbonate and iodotriethylene glycol monomethyl ether (1:2:4) was refluxed in anhydrous acetonitrile under nitrogen for 7 days.

Caesium carbonate was used in place of the more usual potassium carbonate to promote the formation of the *1,3-alternate* product. After cooling at room temperature the solvent was removed under reduced pressure and the solids taken up in dichloromethane. The suspension was washed up with 1 M hydrochloric acid and brine, the organic phase separated and solvent removed under pressure. The crude oil was subjected to flash chromatography on silica and eluted with ethanol. Fractions containing the product, as assessed by TLC (silica plates, ethanol eluent, R_f 0.84) were combined. Removal of solvent left a viscous residue that slowly solidified. The residue was dissolved in hot diethyl ether and left in the freezer overnight. Despite having the same R_f value, the residue contained a number of products. An off white powder, isolated by filtration, was shown to be tetrakis(triethylene glycol monomethyl ether)calix[4]arene in the 1,3-*alternate* conformation. Solvent was removed from the filtrate to yield a mobile oil containing an intractable mixture of mono-, tri- and tetrasubstituted calix[4]arenes. Based on pairs of doublets present in the methylene region of the ^{1}H NMR it was presumed that these existed in the *cone* conformer. The yield of *1,3-alt*-**21** was 12% (139 mg). ^{1}H NMR shows a peak at 3.73-3.67 a multiplet for 16 protons and at 3.60-3.54 ppm a signal integrating to eight protons for the methylene bridges. This was supported by the mass spectral peaks for M^+ 527.2638, 1031.4727, which compared well with the calculated value of 527.2621 $[M + 2\ Na]^{2+}$, 1031.5344 $[M + Na]^+$.

2.8.5 NMR binding studies

Further investigation by NMR was undertaken to see if any cation-calixarene interactions could be detected. Compound *1,3-alt* **21** was dissolved in $CDCl_3$ and the ^{1}H and ^{13}C spectra recorded at 25°C on a Bruker Avance 360 spectrometer at 360 MHz and 90 MHz for ^{1}H and ^{13}C respectively. An excess of solid $NaPF_6$ was added and the spectra recorded again after 2

min, 27 min, 87 min and 18.5 h. Samples were kept at ambient temperature (ca. 22°C) between data acquisitions. Upon addition of $NaPF_6$, a new peak appeared as a broad feature between 7.9 and 7.5 ppm. It is probable that this was from trace water present in the Na^+ salt. The peak became progressively deshielded until, after 18 h, it had sharpened and was centred at 10.5 ppm. Integration indicated that it corresponded to four protons for each calixarene, consistent with two water molecules to every Na^+·*1,3-alt* **21** complex. Hydration was evident within two minutes of the salt being added but took almost 20 h to reach completion. Analogous experiments were undertaken by researchers Siddiqui and Walizadeh using NH_4^+, K^+ and Cs^+ salts (Siddiqui 2009). Based on changes in ^1H signals for the protons recorded over 18 h at constant ambient temperature in the macrocyclic ring and lower rim substituents it was clear that only Na^+ interacted with the annulus of the macrocycle as no significant shifts in the compound's ^1H NMR were observed with the other salts tested. No evidence of bound water was observed and no changes in signal integration were seen for the other cations indicating that the calixarene was indeed selective for Na^+.

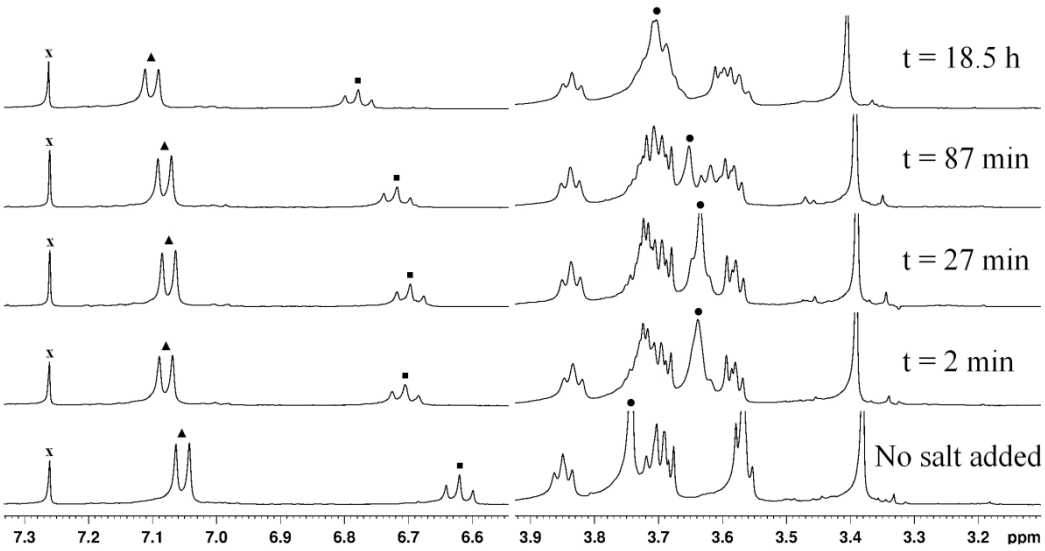
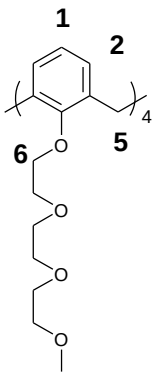


Figure 2.3 *Time dependent changes in ^1H signals for 1,3-alt **21** with Na^+ (salt in approximately ten-fold excess, $CDCl_3$, ambient temperature and humidity).*



H	0 min	2 min	27 min	84 min	18.5 h
1	6.62	6.70	6.70	6.72	6.78
2	7.05	7.08	7.07	7.08	7.10
5	3.75	3.64	3.63	3.65	3.69
6	3.85	3.83	3.84	3.84	3.84
H_2O	-	7.77	8.60	9.25	10.50

Table 2.1 *^1H signals (ppm) over time for 1,3-alt **21** with added Na^+ (H_2O not shown on spectrum)*

Taken together these changes suggested that the cation was not associated with the polyether region of the molecule but resided inside the annulus of the calixarene initially interacting most strongly with the aryl carbons and protons on the methylene bridge.

Why did this particular conformer of the calixarene act as a Na^+ channel when the crude *cone* mixture showed no activity and other compounds related to calix[4]arenes are K^+ selective? Only the *1,3-alt* conformer, at approximately 3 nm in length, has the potential to span a lipid bilayer. Furthermore, Shinkai has proposed that the *1,3-alternate* conformer of calix[4]arene provides an extended electron rich environment that is attractive to monovalent cation (Ikeda 1994). Finally, molecular modelling studies (Lawal 2009) indicated that the sodium cation interacts with two phenolic oxygen atoms, two phenolic rings and two water molecules. This gives the preferred six coordinate environment for Na^+ as seen in the acid-sensing ion channel (Jasti 2007) and contrasts with the eight coordinate environment favoured by K^+ as seen in the crystal structure of KcsA potassium channels (Doyle 1998).

2.9 Conclusions

Results showed that while expanded calix[3]arenes would appear to be the ideal platforms from which to construct model ion channels there are practical difficulties in extending the compounds through the phenolic oxygens. The tris(amide) derivatives of 4-methyl(*N*-benzyl)azacalix[3]arene, (**3a**), 4-t-butyl(*N*-benzyl)azacalix[3]arene, (**3b**), and 4-phenyl(*N*-benzyl)azacalix[3]arene, (**3c**), analogues of the Na^+ selective 4-t-butyloxacalix[3]arene(*N*,*N*-diethylacetamide)$_3$, were hard to synthesise and could not be isolated. Reactions of the parent 4-t-butyloxacalix[3]arene (**8**) with iodinated polyethers was more successful. Following the preparation of Triton iodide (**6**) and triethylene glycol iodide, monomethyl ether (**11**) via the tosylate derivatives, both were appended to 4-t-butyloxacalix[3]arene, though not to 4-t-butyl(*N*-benzyl)azacalix[3]arene, but only as monosubstituted compounds.

Attention was turned to 4-t-butylcalix[4]arene (**13**) and 4-t-butylcalix[6]arene (**16**). Triton tosylate was prepared and then was attached to the lower rim of 4-t-butylcalix[4]arene successfully according to literature procedures for 1,3-dialkylation of calixarenes (Lankshear 2007) ^{1}H NMR showed characteristic doublets at 4.38 ppm and 3.30 ppm that indicated that the disubstituted calixarene, compound **14**, remained in the *cone* conformer when synthesised. The synthesis of the dimethylated compound **15** was partially successful but following reaction of **14** with methyl iodide a mixture of the two compounds was present. The synthesis of 4-t-butylcalix[4]arene(OMe)$_2$(Triton)$_2$ was successful but would need more work to increase the yield of the methylated form.

The same method was applied in the synthesis of 4-t-butylcalix[6]arene(OMe)$_3$(Triton)$_3$ (**18**) with a good yield of 42%. The difference was that 4-t-butylcalix[6]arene(OMe)$_3$(Triton)$_3$ was

first methylated in three alternated phenolic positions on the lower rim. After methylation the Triton chain was attached to the remaining phenolic positions. The absence of signals for free OH groups on the lower rim confirmed that all the hydroxyl groups had been substituted. The *cone* conformation was confirmed by the ^{1}H NMR signals at 3.87 and 3.50 ppm integrating to 12 protons in total. The compound was later tested for activity in a lipid bilayer.

Chapter 3

3.1 Introduction

As noted in the introduction to Chapter 1, transmembrane ion channels are a vital component of every cell. They are able to regulate the transport of cations and anions across the phospholipid bilayers that form the cellular boundary to optimise the intracellular concentrations of specific ions. Analysis of natural transmembrane ion channels shows that they fall into two broad categories: those in which ions move through a pore formed at the confluence of several protein subunits, as in a voltage gated potassium channel, KcsA (Doyle 1998) and those where they pass through channels formed within a single protein such as the ClC chloride channel (Dutzler 2002).

3.2 How channels work

One of the ways we can study the properties of the channels and their behaviour is by using single channel current recordings. At resting potential the current shows no deflections of the trace and is constant, but at certain points along the recording deflections in the current are seen which represent the opening of the channels. After short intervals the channels tend to close for random lengths of time. To find the probability of the opening of the channels, mean open time can be obtained by dividing the sum of all the open times by the duration of the recording. 0.5 of open probability indicates that the channel spends 50% of its time in an open state (Ashcroft 1999).

The equilibrium potential controls the movement of ions across the cell membrane. The equilibrium potential is the electric force on the ion which balances exactly the opposing force of the concentration gradient. This equilibrium potential is given by the Nernst equation.

The Nernst equation

For an ion X,

$$E_x = RT/zF \log_e [X]_O/[X]_I \qquad \text{Equation 1}$$

where E_x is the equilibrium potential (in volts), R is the gas constant (8.314 $Jk^{-1}mol^{-1}$), T is the absolute temperature (in Kelvin), z is the ion valence (positive for cations and negative for anions), F is the Faraday constant (96,500 C mol^{-1}), $[X]_O$ and $[X]_I$ are the external and internal concentrations of X.

The equation can be written in a simplified form where E_x is given in mV and the temperature is assumed to be 293 K (or 20^oC).

$$E_x = 58/z \log_{10} [X]_O/[X]_I \qquad \text{Equation 2}$$

In mammalian biology the cellular potassium concentrations are internally 140 mM and externally 5 mM (Tucker 1998). The calculated K^+ equilibrium potential is -84 mV. The resting membrane potential in most cells is between -60 mV to -100 mV and this indicates that the resting permeability is determined mainly by the potassium selective channels. Looking back at Equation 2 it can be seen that the external potassium concentration is plotted

in a logarithmic scale against the equilibrium potential, (E_x). This relationship gives a straight line with the slope of 58 mV for a tenfold change in $[K^+]_o$. When the concentrations of the K^+ ions are equal inside and outside of the cell membrane the equilibrium potential is 0 mV. If there is a divalent cation such as Ca^{2+}, the slope would be 29 mV per tenfold change in calcium ions due to the doubling of the charge.

3.2.1 The current-voltage relationship

A typical single channel current-voltage can be obtained by plotting the current amplitude against the applied membrane potential. If the ion concentrations on either side of the membrane are not very different, the current flow through the open channel is determined by Ohm's law. Three forms of Ohm's law are commonly encountered in lipid bilayer electrochemistry:

$$I = V/R \qquad \text{Equation 3a}$$

$$I = VG \qquad \text{Equation 3b}$$

$$I = V\gamma \qquad \text{Equation 3c}$$

where I is the single channel current (in amps), V is the voltage gradient across the membrane (in volts), R is the resistance to current flow through the open channel (in ohms), G is conductance (in siemens), γ is the single channel conductance (in siemens).

Ohms law predicts the linear relationship between membrane potential and the single channel current amplitude. The single channel conductance (γ) is given by the slope of this relationship, as conductance is the reciprocal of resistance. If the ion concentrations inside

and outside of the cell membrane shows a large difference than Ohm's law does not hold. This is the case for Ca^{2+} ions which have a large difference in concentrations: inside it is less than 500 nM compared to the concentration of the plasma which is 2-5 mM. Therefore it is less easy for the Ca^{2+} ions to move out of the cell, giving a non-linear single channel current-voltage relationship. Sometimes there is a presence of blocking ions which will also give a non-linear current-voltage relationship. For example Mg^{2+} ions block the inwardly rectifying K^+ channels, which can enter the pore of the channel from the cytoplasmic side but can pass through the extracellular solutions (Matsuda 1987; Sackman 1984b; Vanderburg 1987).

The movement of the ions through an open channel is not only dependent of its electrochemical gradient. It is also affected by the relative permeability of the channel to the ion. Ion channels do not act just as pores, through which all ions can diffuse, but are essentially molecular sieves and discriminate between ions (Hille 1992). For example, Na^+ channels are highly permeable to Na^+ but not K^+ ions, whereas K^+ channels are ~100 times more permeable to K^+ than to Na^+ ions. Because Na^+ has a smaller ionic radius than K^+ this high selectivity for potassium ions cannot be due to the size of the ion. It is thought that the ion selectivity is better when the pore is at the narrowest, at the place called selectivity filter where the side chains from a specific amino-acid sequence determines which ions can permeate. Mullins put forward the idea that the pore is a cage where ions lose their hydration shell, at least partially, to fit snugly and allow differentiation between Na^+ and K^+ (Mullins 1959), a concept that is not too far away from what we know today. The charges on the amino acids determine which ion is going to attract and which is going to be repelled. If the amino acid is charged negatively it will attract a cation and vice versa.

3.2.2 Gating

Gating is the process that occurs when a pore opens or closes. Some channels, such as the K^+ channels which determine the resting potential of the cell (Bernstein 1902), open and close randomly at all membrane potentials (Ashcroft 1999). Their gating is said to be voltage independent but other channels can be voltage or ligand gated. Voltage gated channels (Payandeh 2011) open when there is a change in membrane potential and ligand gated channels are affected by intracellular or extracellular ligand binding (Lepore 2011).

Channels that are open at the membrane resting potential can be closed, as shown in Fig. 3.1. On the left hand side is the ligand binding channel and on the right hand side is the voltage gated channel.

Figure 3.1 *Voltage-dependent gated* ***(a)*** *and ligand activated* ***(b)*** *channels (from Ashcroft, 1999).*

3.2.3 Ligand-gated channels

Ligand gated channels are named after the ligand that binds to them (Chen 2011). They can be extracellular ligands such as neurotransmitters such as acetylcoline and glycine and intracellular ligands such as cyclic AMP, Ca^{2+} and ATP. When a ligand binds to the specific

sites of the channel protein, this produces a conformational change that allosterically opens the ion pore. The ligand remains bound to the channel and the channel may open and close several times. The channel permanently closes when the ligand dissociates from the channel site. Some channels results in a channel closure when the legend is present. One example of this type of channel is the inhibition of the ATP-sensitive K^+ channel by intracellular ATP.

3.2.4 Voltage-gated channels

At the resting potential of the cell, most voltage-gated channels are closed. When there is a change in membrane potential the channel undergoes a series of conformational changes resulting in the opening of the channel pore (Shaya 2011). This is an activation state and is then followed by an inactivation state where the channel does not conduct ions. It is also unresponsive to any further changes in voltage and subsequent depolarization. Another conformational change will take place then it will return to the closed state. The voltage dependent channels undergo conformational change when they sense a change in voltage field across the membrane. Charged amino-acids tend to be the voltage sensors in the membrane-spanning domains. They will trigger a further conformational change that will open the pore (Linnertz 2011). Not enough is known about the location of the voltage sensors and how this results in the channel opening and closing.

Almost all ion channels are modulated by a series of substances present in the body such as H^+ (Qu 2000), Ca^{2+}, metabolites (e.g. ATP and MgADP), fatty acids, phosphorylation, GTP-binding proteins and gases, such as oxygen (Kaczmarek 1987). Many hormones and neurotransmitters mediate their effects in the ion channels, not by binding directly to the

channel like a ligand but by activation of a secondary messenger system that modulates ion channel activity (Dilly 2011).

3.2.5 Kinetic analysis of single-channel currents (Aidley 1996; Hille 1992)

If we consider a simple mechanism where the channel only exists as open (O) or closed (C) state then the kinetic behaviour of the channel is given by the equilibrium:

$$C \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} O$$

where k_1 and k_{-1} are the rate constants (in sec^{-1}) for entering and leaving the open state, respectively. The mean time open is $1/K_{-1}$ and the mean closed time is $1/K_1$. The rate constants can then be obtained by measuring the mean open and closed times. The open probability of the channel is the mean open divided by total time. This can be represented in the form of a histogram. The time the channel is open is variable and the mean opening can be obtained from the open-time histogram (Fig.3.2).

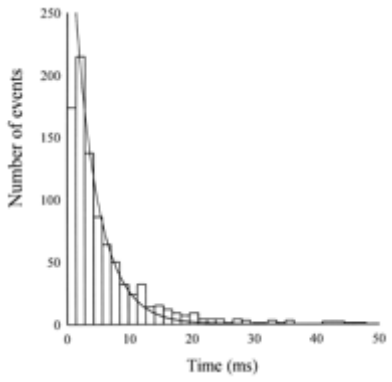


Figure 3.2 *An open-time histogram*

3.2.6 The membrane current

Cell membranes contain several types of channels and this produces a current across the membrane. The total current that flows across the cell membrane is due to all the ion fluxes. To measure the current of a single channel it is necessary to block all current flowing from other channels. It can be achieved by the use of selective channel blockers such as tetrodotoxin, for example, which blocks only voltage gated Na^+ channels or by the removal of permeating ions (Andrikopoulos 2011)

3.3 Investigating ion channel function

Channel function can be investigated directly by recording the current, which flows through an open channel or by measuring the changes in the membrane potential that this produces.

3.3.1 Voltage clamp

Electrophysiologists use the voltage clamp technique to measure ion currents across the membrane of excitable cells such as neurons, while holding the membrane voltage at a set level. Marmot and Cole (Cole 1941) were the first to invent the voltage clamp technique which was more fully developed by Hodgkin and Huxley (Hodgkin 1952). A current is set up which is equal in amplitude but opposite in sign to that which flows across the cell membrane. The membrane potential thus remains constant as there is no net current flow across the membrane. The current required to clamp the potential is measured as well as the current flowing across the membrane. Cations flowing towards the amplifier are by

convention downwards (Ashcroft 1999). The whole-cell current is the sum of the current flow of all ion channels existing in the cell membrane.

Figure 3.3 *Showing a voltage clamp technique used in studying membrane currents of a squid axon (from http://life.nthu.edu.tw/~g864264/Neuroscience/min/Voltage.html).*

The difficulties are that the speed of the voltage response is limited by the rate at which the current can pass through the microelectrode. This also depends on the electrode resistance; the higher the electrode resistance the harder it is for the current to pass. Microelectrodes must be inserted into the cell membrane, which may damage the bilayer and compromise its high resistance, so it is sometimes better to use the patch clamp technique (Sakmann 1984). The main problem with this method is that it measures total change in membrane resistance due to the movement of affected ions, or whole cell current, and cannot discriminate between different ion channels or ions. To record data originating from single channels the pach clamp metod is generally adopted.

3.3.2 Patch clamp

This technique was invented by Neher and Sackmann in 1976 (Neher 1976) and refined by Hamill (Hamill 1981). It can be used to record the activity of single ion channels and to

measure whole cell currents in small cells. The revolutionary technique made it possible to measure the single channel conductance directly in the cell. The technique works by forming a seal between the wall of the micropipette and cell membrane. It creates a high resistance seal so that the current flows through the ions channels in the patch of the membrane that is sealed and can be recorded at very low level of background current noise (Fishman 1973). Sometimes the current produced by the channel is so small that it can be confused with the recording of background current noise (Stevens 1972). This can be resolved by using a technique called noise analysis which can be used to obtain an estimate of the single-channel current amplitude and the mean channel open time. This method is reliant on the fact that the channel fluctuates randomly between open and closed states. Patch clamping noise analysis is a useful technique to use when a single channel current cannot be resolved (Anderson 1973) but the method relies on assumptions which suggest the channel population behaves homogeneously with a single conductance state.

3.4 Methods for studying intracellular ion channels

Ion channels in intracellular membranes are very difficult to study and are not accessible to the patch clamp technique. This problem can be solved by isolating intracellular membranes such as the sarcoplasmic reticulum, by cell fractionation. These intracellular membranes, when isolated spontaneously form vesicles of approximately 300 nm diameter that can be studied following fusion with an artificial lipid bilayer (Aoki 2010). Fig. 3.4, below, illustrates the bilayer recording method. The bilayer can be voltage clamped, that is, the transmembrane potential is held at a constant known value, to record a current. Ions are not permeable in pure lipid bilayers and the background current noise level is low and therefore if a few vesicles diffuse through it is possible to record a single-channel current. Another

approach is to reconstitute the purified channel protein into the bilayer. The first recordings obtained were made by Hladky and Haydon, (Hladky 1970; Ashcroft 1999) where single channel currents were obtained by adding the pore-forming antibiotic gramicidin to an artificial bilayer.

Figure 3.4 *Schematic of a voltage clamp experiment. A cuvette (a), with a small hole cut into one face, is immersed in an electrolyte (b) and the bilayer is formed by painting lipid across the hole. Electrical contact with Ag/AgCl reference electrodes (e) in a reference electrolyte (d) is via Agar salt-bridges (c). A potential is applied and the current measured as a function of time (Fyles 2007).*

The advantages of using the lipid bilayer method are that different lipid environments of the channels can be studied, as there are charged and uncharged types of lipid. The disadvantages of this method are that the channel is in a different environment than what is in the body and that has to be taken in consideration when studying the channel in question. Sodium and potassium channels are the main focus of this study. This is due to the diameter of the macrocycles synthesised, these would not accommodate or let through the larger ions.

3.4.1 Voltage-gated Na^+ channels

Voltage-gated Na^+ channels (Payandeh 2011) are responsible for the action potential in nerve and muscle fibres (Catterall 2000). Mutations of these channels genes results in diseases of the nerve and muscle, including cardiac muscle called channelopathies (Andavan 2011). Other mutations of the channels in the skeletal muscle gives rise to a group of diseases known as the periodic paralysis (George, Jr. 2005; Cannon 2002) and mutations in the cardiac muscle sodium channel gene result in QT syndrome (Kramer 2011). Mutations in the β subunit of the human neuronal Na^+ channel give rise to febril convulsions and epilepsy (Catterall 2010).

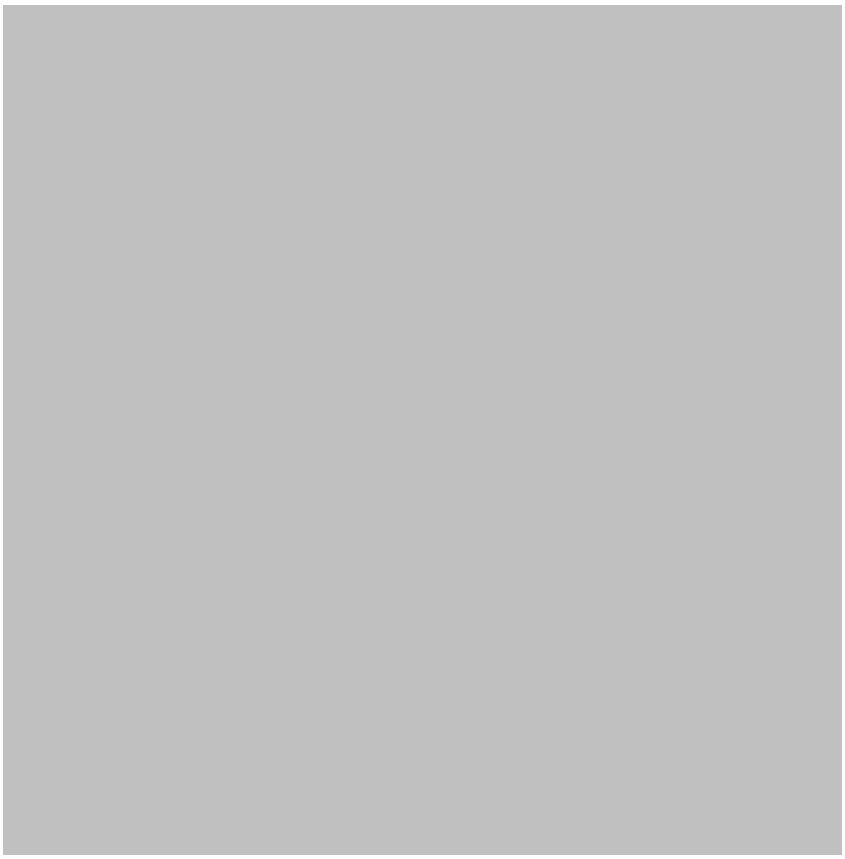


Figure 3.5 *Functional properties of NaVChs. (**A**) Schematic representation of an NaVCh undergoing the major gating transitions. (**B**) Voltage-clamprecording of NaVCh activity in response to membrane depolarization. Downward deflection of the current trace (red) corresponds to inward movement of Na^+ (George Jr. 2005).*

An understanding of how Na^+ channel mutations gives rise to all these different diseases started when the first channel was cloned in 1984 (Noda 1984). The most important properties of sodium ion channels are their voltage-dependent activation in response to depolarization, their inactivation during a maintained depolarization and the high selectivity of the channel pore to Na^+, illustrated in Fig. 3.6 (George Jr. 2005).

The probability of the sodium channel at the resting potential (Venosa 2011) being open is very low. Depolarization of the cell membrane increases the probability of the channel opening and can require as little as a 9 mV depolarization. The open probability of the channel also depends on the time. Sodium channels are open briefly for less than 1 ms and then they follow an inactivated state which maintains until the membrane hyperpolarizes. Voltage-gated Na^+ channels are highly selective to sodium ions and have a single channel condunctance of 10-30 pS in 100 mM extracellular Na^+ (Hille 1992).

3.4.2 Voltage-gated K^+ channels

Potassium channels exist in most cells. There are two types of potassium channels: those which possess six transmembrane domains and those which are formed from only two transmembrane domains. The six transmembrane K^+ channels are further divided into six conserved gene families. These are the voltage-gated K^+ channels (K_v channels), the KCNQ channels (Mathieu 2003) the eag-like K^+ channels and the three types of Ca^+ activated K^+ channels (BK, IK and SK) (Gallin 1984; Zhou 2011; Gu 2011). Mutations in the genes encoding members of these K^+ channels subfamilies give rise to diseases such as episodic ataxia, long QT syndrome and epilepsy (Pischalnikova 2009).

Data can be gathered by using a variety of related techniques such as patch and voltage clamping. The compounds described in this thesis were tested using a planar lipid bilayer method.

3.5 Bilayer recording methods

The dynamics of ion transport can be studied either through a cell membrane or lipid bilayer. Ion transport through a living cell, or through part of a cell membrane is often measured as a function of single channel activity due either to opening of endogenous channels or to the effects of ion channel mimics. The technique of patch clamping (Hamill 1981; Wiegand 2000) when used on whole cells, requires that a micropipette containing an electrode makes a good seal with the cell membrane to generate a 10 GΩ resistance between the cell and the pipette wall. The resulting transmembrane currents, measured by microelectrodes inside and outside the cell, generate extremely low noise to allow single channel events to be monitored. This method allows for variation in the holding potential to investigate voltage-gated effects. Ion current that flows through an ion channel is only due to electric driving force that is defined by Ohm's law as:

$$I = \frac{V - V_R}{R} \qquad \text{Equation 4}$$

where I is the current, V the actual membrane potential, V_R the reversal potential and R is the cell resistance.

VR depends on the intracellular and extracellular concentration of each permeable ion. The reversal potential for a mixture of monovalent and divalent ions may be calculated according to Goldmann-Hodgkin-Katz equation or evaluated experimentally as the membrane potential at which the amplitude of the respective ion current is zero (Karmazinova 2010). There are three functional states of voltage operated ion channels: closed, open or inactivated. The whole cell patch clamp has two recording modes and depends on the configuration of a patch clamp amplifier. In the voltage clamp technique the signal recorded is the transmembrane current and the controlled input is the "clamped" membrane voltage delivered to the cell examined. In the "current-clamp" technique the input is the current that passes into the cell and changes are recorded in the membrane potential, for example, generation of an action potential. The voltage operated ion channels exist in one of three functional states: closed, open or inactivated (Fig. 3.6).

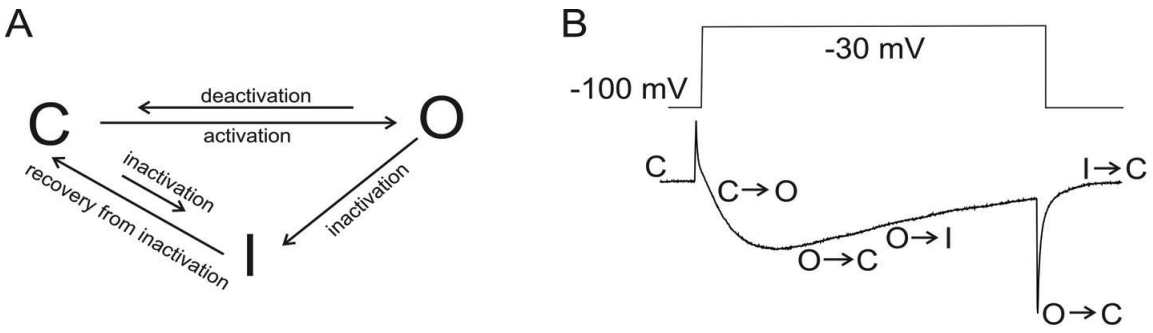


Figure 3.6 *Gating transitions of a voltage-dependention channel (**A**) and an example of inward current recorded from T-type calcium channel during depolarizing pulse (**B**). Letters C, O and I represent a closed or resting channel state, an open or activated channel state and an inactivated channel state, respectively. The size of the arrows illustrates relative probability of individual transitions.*

Closed or resting state is when the channel has the conformation in which the channel is ready for activation. This means that the channel is capable of opening upon membrane depolarization. The most common depolarizing impulse used in patch clamp experiments is a rectangular voltage pulse. When the channel turns into its open or activated state the ion current passes through the channel pore and inward current increases. Prolonged

depolarization of the cell membrane shifts the channel into its inactivated state. This process is reflected by slow current decay. The closed and inactivated channels are not conductive. The closed channel can open at any time and the inactivated channel has to recover from the inactivated state before reopening. This is a very slow process and can take from seconds to minutes. The voltage dependence of the distribution of channels among their functional states obeys Boltzmann distribution. Current I(V) flowing through an ion channel at specific membrane potential V is proportional to both driving force and opening probability. Fig. 3.7 shows two theoretical situations: Fig 3.7A shows a current carried by an ion with positive reversal potential, Fig.3.7 B shows current carried by an ion with negative reversal potential. While the former example describes quite closely voltage dependent sodium and calcium channels, the latter corresponds to a hypothetical potassium channel lacking rectification phenomenon (Karmazinova, 2010).

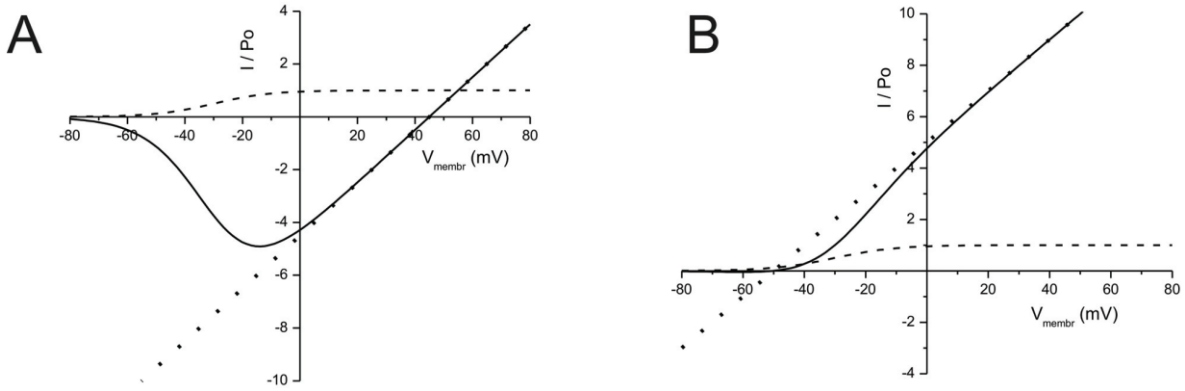


Figure 3.7. *Voltage dependence of channel distribution. Dashed lines illustrate Boltzmann-type voltage dependence of an open probability P_O of a channel. Dotted lines demonstrate linear Ohmic driving force for a situation in which reversal potential has a positive value (**A**) and a negative value (**B**). Solid lines represent product of both relations mimicking current-voltage.*

The lipid bilayer technique, which is useful for artificial channel mimics (Fyles, 2007), requires purified lipids to remove any influence from natural proteins. Lipid bilayers, formed from a dispersion of 15 mg/ml palmitoyloleoylphosphatidylethanolamine and 15 mg/ml palmitoyloleoylphosphatidylserine in n-decane, are drawn across a 0.4 mm diameter hole in a

polystyrene cup separating two solution-filled chambers designated cis and trans and are allowed to thin to a bilayer (Hardy 2001). The compounds to be studied are incorporated into the bilayer and the ion of interest is introduced into one compartment. Transmembrane currents are measured across the bilayer, which itself may be voltage clamped where the transmembrane potential is held at a constant value, e.g. -50 mV. The clamp may be applied at different potentials to determine the point at which ions permeate the lipid and will record the total current which is produced by the insertion of the channel. For the experiment to be performed with accuracy a Faraday cage (Handel 2009) should be used to eliminate external electromagnetic interference. Any electrical fields from external sources will produce force fields which will act on the electrons in the metal of the cage within the conductor which will generate a current that rearranges the charges and neutralizes them. Once the charges have been rearranged and the electrical field has been cancelled inside the cage, the current stops. Any small interference in the bilayer recording will create noise and will destroy any recording of the channel in the bilayer. When a channel has inserted in the bilayer membrane it is necessary for no noise recording to be registered, as it would be impossible to prove that it was an insertion and not some sort of noise interference, as the insertion of the channels can be small enough to be confused with a noise recording (Ghosh 2009).

Fig. 3.8 illustrates typical results expected for single-channel currents (Hardy, 2001). Recording A is from a planar lipid bilayer after adding 10 μg CPE to the *cis* (ground) chamber. The bathing solution was symmetrical, 100 mM NaCl, 5 mM HEPES/NaOH (pH 7.2), and the applied voltages are given. The open and closed levels are indicated by the dashed and dotted lines respectively. Note the reversal in the direction of current between negative and positive voltages. The current–voltage (I–V) relationship of CPE-induced channels bathed in 100 mM NaCl is shown in B. Values represent the mean ± S.E.M. of nine

bilayers. The solid line is plotted by regression analysis and, from the slope, the single-channel conductance is calculated as 270 ± 2 pS (mean ± S.E.M.). Note that the error bars are present, but hidden within the symbols. Voltage-dependent closure of CPE-induced channels is shown in C. The probability of single-channel opening (P_o) is plotted versus applied voltage demonstrates the tendency of the channels to close at voltages over +10 mV. Channel lifetime analysis is illustrated in D. The mean open and closed lifetimes of typical CPE-induced single-channel recordings, plotted versus applied voltage. As the applied voltage becomes more positive the mean open time (τ_o; ϒ) decreases and the mean duration of the longer closed times increases. The duration of the brief closed times (mean lifetime <10 ms) does not change.

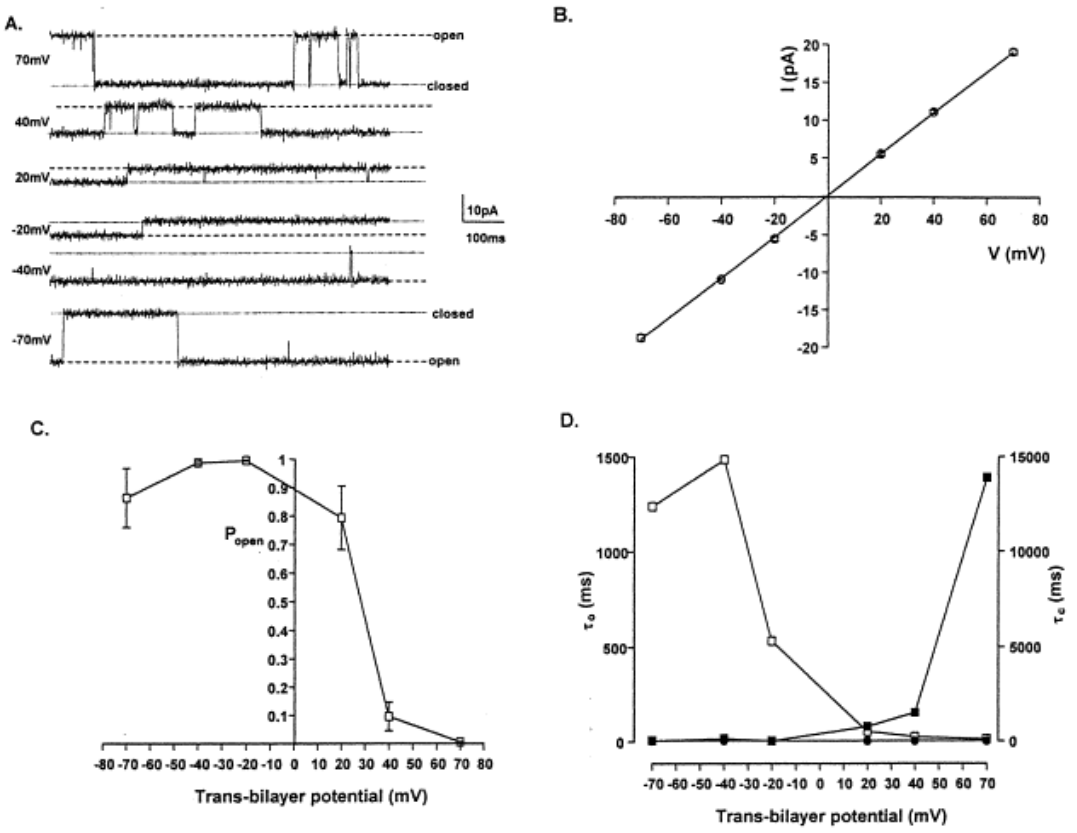


Figure 3.8 *Examples of different types of analysis of recordings.*

In bilayer conductance experiments performed in this project, calixarene compounds were added to one side of the bilayer, designated the *cis* side, with the opposite side designated *trans*. When the channel-like compounds fused with and then were incorporated into the bilayer, a pore opened. The pore size relates to the size of the central calixarene cavity in the compound tested. It is also possible, indeed likely, that several calixarenes insert as a coherent group and that channels may exist between molecules. When channels form, ions present in the buffer solution in the *cis* compartment cross the bilayer membrane to the *trans* side, producing a current. The current produced can be calculated from changes in the resistance of the membrane. The current can be used to estimate the number of channels that the bilayer incorporated. The Nernst equation calculates the equilibrium potential for an ion based on the charge of that ion, concentrations across the membrane and temperature. This potential will only occur if a concentration gradient exists across the membrane and if selective ion channels exist that allow the movement of ions across the membrane. When there is more than one ion present the Nernst equation predicts the reverse potential of the current-voltage relationship when the selective filter favours one ion over the other, as it was in our case. This should give a plot of current-voltage which does not go through zero as would be the case of a single channel IV plot (Fig.3.8 B).

In this project conductance measurements were performed on a planar bilayer composed of a mixture of palmitoyl triglycerides(15 mg/ml palmitoyloleoylphosphatidylethanolamine and 15 mg/ml palmitoyloleoylphosphatidylserine in n-decane) with an electrolytic solution containing Na^+ at physiological levels (150 mM) and buffered at pH 7.2. An electrolytic solution of K^+ at 150 mM, also buffered at pH 7.2, was used to test for K^+ conductance of some of the compounds. The following calixarene derivatives were investigated by this method: 4-t-butylhexahomotrioxacalix[3]arene$(OH)_2$(Triton) (**9**), 4-t-butylcalix[6]arene-

$(OMe)_3(Triton)_3$ (**18**), tetrakis(triethyleneglycol monomethyl ether) calix[4]arene, *cone* conformer (*cone*-**21**), tetrakis(triethyleneglycol monomethyl ether) calix[4]arene, *1,3-alternate* conformer (*1,3-alt*-**21**).

Experiments were undertaken in buffered solutions containing either 150 mM NaCl or 150 mM KCl, 10mM HEPES, 1 mM EGTA, 1.05 mM $CaCl_2$, 1mM $MgCl_2$ and 50 μM free calcium. Specificity was determined through asymmetric experiments where Na^+ and K^+ were present in the *cis* and *trans* chambers during the experiment. The specificity of the ion channel can be calculated through the Nernst equation.

3.6 Results and discussion

The experiments performed in the lipid bilayer were mainly to identify if the novel compounds produced a channel like behaviour. This is seen as typical recording of insertions (Fyles 2007) in the lipid bilayer where the ions tested are crossing over to the other side of the bilayer.

3.6.1 Symmetrical experiments (NaCl buffer in *cis* and *trans* compartments)

t-Butylhexahomotrioxacalix[3]arene$(OH)_2$Triton (**9**) and 4-t-butylcalix[6]arene$(OMe)_3$-$(Triton)_3$ (**18**) were tested at 64 mM concentration with 150 mM NaCl buffer used in both compartments of the lipid bilayer**.**

3.6.1.1 4-*t*-Butylhexahomotrioxacalix[3]arene(OH)$_2$Triton (**9**)

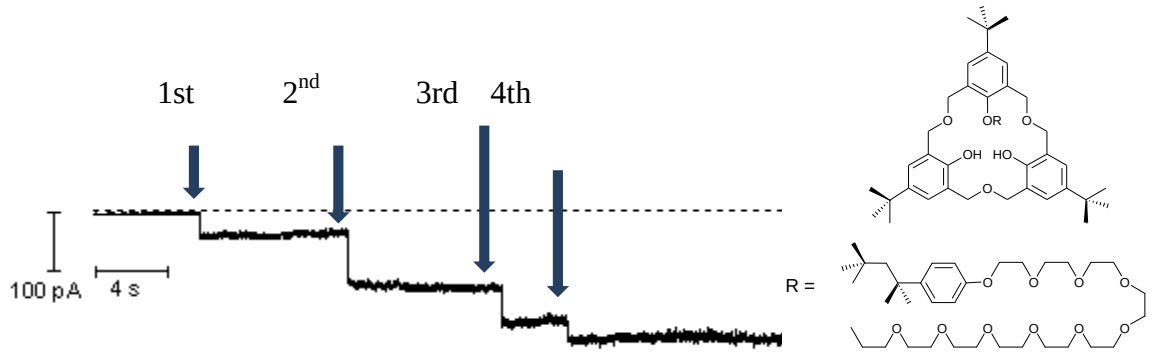


Figure 3.9 *Bilayer data for 4-t-butylhexahomotrioxacalix[3]arene(OH)$_2$Triton (with 150 mM NaCl)*

4-t-Butylhexahomotrioxacalix[3]arene(OH)$_2$Triton (**9**) was added to give a total of 64 mmol in 150 mM NaCl solution. Four insertions can be seen in the Fig. 3.9. The first feature, a single insertion, happens at 399.9 s with a current of -3.418 pA and voltage of -50.00 mV. It is followed by a second insertion at 408.2 s and a third channel at 4.16.3 s. The recording continued for longer where another insertion were observed. In total, four different channels were detected in the 500 s recording.

Although the tris(amide) derivative of 4-t-butylhexahomo-trioxacalix[3]arene has been shown to bind Na^+ and initiate a change in transmembrane conductance in cells (Cragg 1999b) this was the first evidence that a 4-t-butylhexahomotrioxacalix[3]arene derivative functions by a channel-like mechanism.

3.6.1.2 4-t-Butylcalix[6]arene(OMe)$_3$(Triton)$_3$ (**18**)

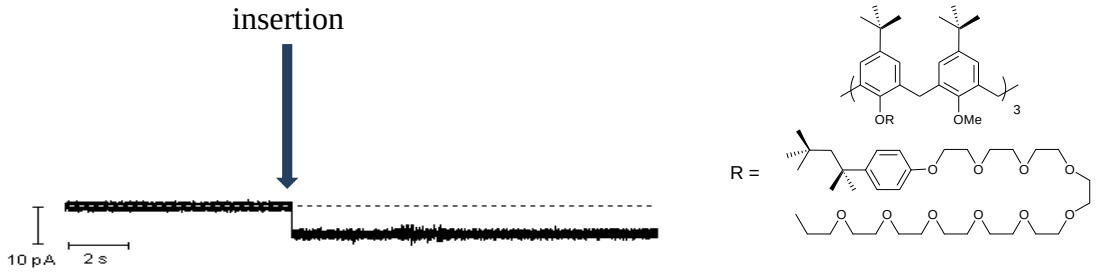


Figure 3.10 *Bilayer data for 4-t-butylcalix[6]arene(OMe)$_3$(Triton)$_2$ (with 150 mM NaCl)*

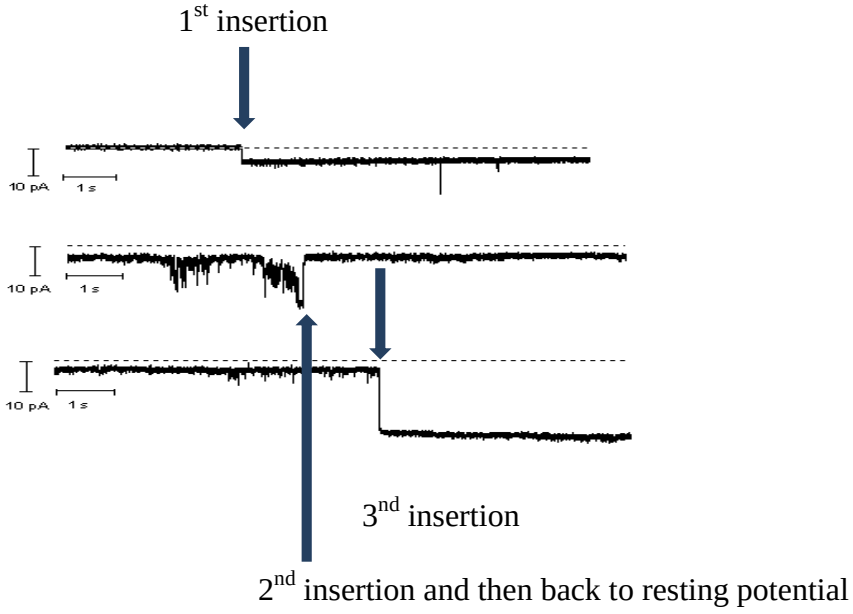


Figure 3.11 *Data for 4-t-butylcalix[6]arene(OMe)$_3$(Triton)$_3$(**18**) on a second bilayer.*

4-t-Butylcalix[6]arene(OMe)$_3$(Triton)$_3$ was added in 16 mmol aliquots to a pH 7.2 buffered solution containing 150 mM NaCl. Activity was observed after 64 mmol of the compound had been added as shown in Fig. 3.10. Data were collected from 0 to 522 s. One insertion occurred after 502.2 seconds giving a step with of -7.610 pA and the channel remained open for the remainder of the experiment.

In a second experiment, under the same conditions as in Fig. 3.10, data were collected for 500 sec (Fig. 3.11). Three stages can be identified. Stage 1 shows a current step (1st insertion) at 48.47 s with a current of -7.524 pA. The channel remained in place until at 84.08 s there was a disturbance in the bilayer. It is believed that another insertion occurred in here as it can be seen a current of -15.45 pA. The compound is released from the lipid bilayer, and the channels close, leaving the bilayer in a stable condition. At 109.8 seconds another insertion occurs with a current of -4.837 pA in the same bilayer. These experimental results show that different types of activity are occurring but the lipid bilayer remained stable throughout the whole recording.

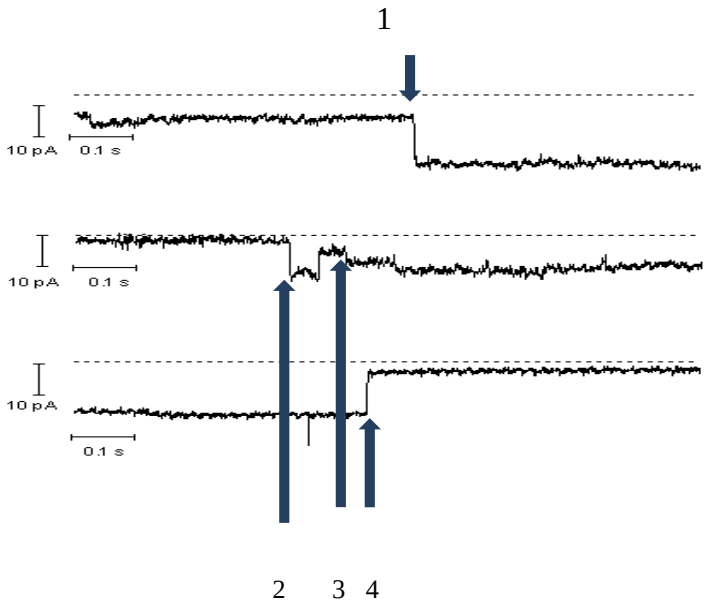


Figure 3.12 *Data for 4-t-butylcalix[6]arene(OMe)$_3$(Triton)$_3$* ***(18)*** *on a third bilayer.*

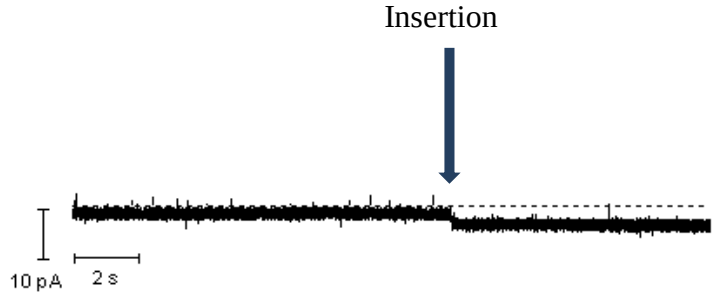


Figure 3.13 *Data for 4-t-butylcalix[6]arene(OMe)$_3$(Triton)$_3$* ***(18)*** *on a fourth bilayer.*

Two further experiments indicate that channels form with this compound. The conditions were the same as in the previous experiment and the data recorded in new bilayer for up to 1000s. Fig. 3.12 shows the effect of double the amount (128 mmol) of 4-t-butylcalix[6]arene$(OMe)_3(Triton)_3$ added to the bilayer. Here three steps were observed. At 503.15 seconds a first insertion with a -10.56 pA current was detected. The bilayer remained stable and the channel later closed. At 505.5 seconds a second insertion is observed with a current of –14.41 pA. With such a large insertion, it can be presumed that a group of channels had inserted at the same time to create such a big current, allowing more ions to pass through. The channel partially closed but smaller channels remained in the bilayer (3). Before the end of the recording the channels closed (4) and the membrane returned to the resting potential of -50mV. The bilayer remained stable throughout the experiment lasting 1000 seconds. In Fig. 3.13, an insertion occurred at 501s with a step size of -2.274 pA, under the same conditions as previous experiments. The channel remained in the bilayer until the end of the recording. The bilayer remained stable all through the recording. The recording was 515 seconds long and, as the channel occurred almost at the end of the recording, no other activity was observed.

These four sets of data demonstrate that the novel compound 4-t-butylcalix[6]arene$(OMe)_3$-$(Triton)_3$ (**18**) behaves as a channel in the lipid bilayer but in different ways depending on the concentration of compound. At low concentrations of 4-t-butylcalix[6]arene$(OMe)_3(Triton)_3$ small stepwise changes were observed in the current, corresponding to a flux of 7×10^6 ions s^{-1}, with channel conductance typically lasting from 0.5 to 20 seconds. At high concentrations, stepwise insertion of several molecules was detected and much larger step changes in current were observed. When Triton X-100® was added at the same concentration it caused the bilayer to burst whereas it had remained intact in the presence of the calixarene.

The large change in conductance suggested that several calixarenes inserted into the bilayer simultaneously as sequential insertions would give a characteristic step pattern. Many synthetic and natural transmembrane channels are formed by the confluence of several intertwining molecules (Schlesinger 2002) but observations suggest an additive effect through the concerted action of three or four calixarenes as the currents detected were exact multiples of the single channel events. Calix[6]arenes incorporating surfactant groups can therefore operate as Na^+ channel mimics at low concentrations but aggregate to form multiple channels at higher concentrations shown in Fig.3.11; the small step shows that a small number of ions pass through the membrane. The second, much larger, step demonstrates a greater number of sodium ions passing through the membrane, possibly due to a micelle type structure or a multiple number of channels opening at the same time. The next step of this project was to identify if the channel-like structure showed specificity to certain ions so the compound was tested for potassium and sodium selectivity, as described below.

3.6.2 Asymmetric experiments (150 mM Na^+, *cis*, and 50mM Na^+/100mM K^+, *trans*).

Several experiments were performed but only one showed promising results (Fig. 3.13). An experiment was undertaken in a mixed buffer (50 mM NaCl, 100 mM KCl, 150 mM total cation concentration) in the *cis* chamber and 150mM NaCl in the *trans*. The voltage was set at -50 mV and +50mV was applied. 4-t-Butylcalix[6]arene$(OMe)_3(Triton)_3$ (**18**) was added at 64 mmol. The asymmetric experiment was designed to show specificity for a particular cation, in this case either specificity for sodium ions or potassium ions were tested. Fig. 3.13 **A** shows two channels forming at 69 s and with a current of 16.01 pA and 28.92 pA, respectively. The lipid bilayer remained stable. The voltage across the bilayer was altered from +50mV to -50mV in four sequential 25mV steps and the current changed to – 8 pA. In

B the current is plotted against voltage to obtain the reversal potential. The calculated reversal potential for sodium using the Nernst equation is -27 mV. The reverse potential of this experiment was -27.6 mV consistent with the channel being sodium selective.

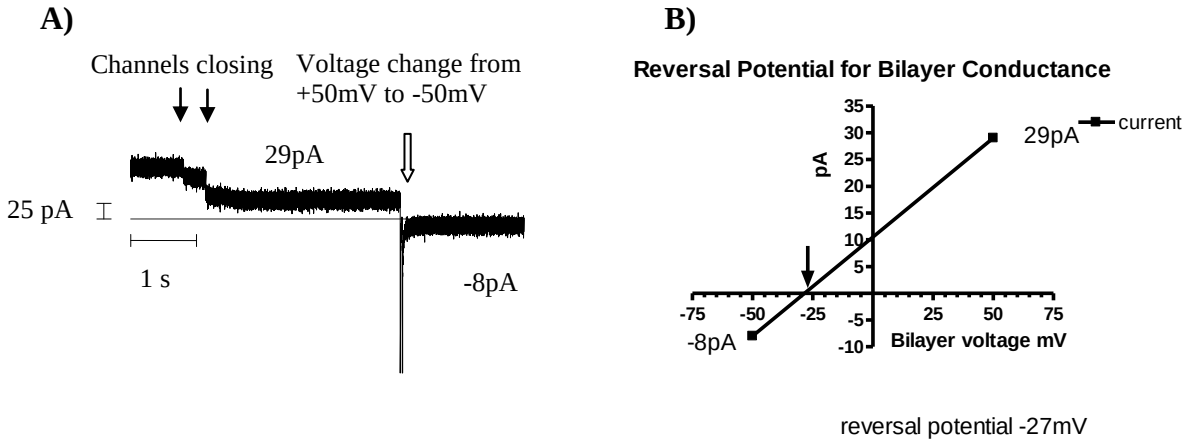


Figure 3. 14 *A bilayer recording of 4-t-butylcalix[6]arene(OMe)$_3$(Triton)$_3$ (64mmol) in an asymmetric buffer* (150 mM Na^+, *cis*, and 50mM Na^+/100mM K^+, *trans)*

3.6.3 Results obtained from tetrakis(triethyleneglycol ether)calix[4]arene (**21**)

3.6.3.1 Experiments with crude *cone*-**21**

Tetrakis(triethyleneglycol monomethyl ether)calix[4]arene, prepared as part of a final year undergraduate project by Lawal, was investigated for potential channel forming activity in the *cone* and *1,3-alternate* conformers. When *1,3-alt*-**21** was extracted from the reaction mixture, the remaining residue was identified, primarily by mass spectrometry and NMR spectroscopy, as partially substituted *cone*-calix[4]arene with either one, three or four substituents attached (Lawal 2009). It was decided to test this as a mixture because separation on a thin layer chromatography plate proved difficult to achieve. If significant bilayer activity was observed then the mixture would be separated by column chromatography and the active component isolated.

A crude mixture of *cone* calix[4]arene **21** was added to a standard 150 mM KCl buffer at pH 7.2 in aliquots to a total of 32 mmol. Compound insertion was detected at 232.8 s, with a current of 109.7 pA, and the channel remained active for about 220 seconds as shown in Fig. 3.15.

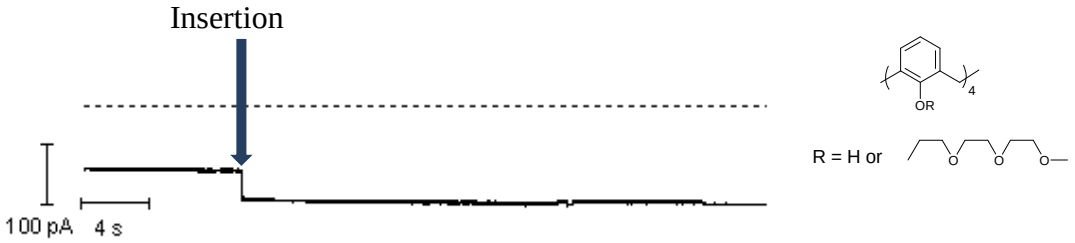


Figure 3.15 *Bilayer data for a crude mixture of cone-calix[4]arene derivatives (cone-**21**) in KCl (150 mM K^+).*

In a second experiment, a crude mixture of *cone* calix[4]arene **21** derivatives was applied to a bilayer (Fig. 3.16). The experiment was run at -50 mV in 150 mM KCl buffer, pH 7.2. Addition of 32 mmol of the tetrakis(triethyleneglycol monomethyl ether)calix[4]arene mixture resulted in very uneven activity, as shown. Insertion clearly occurred but the bilayer was unstable.

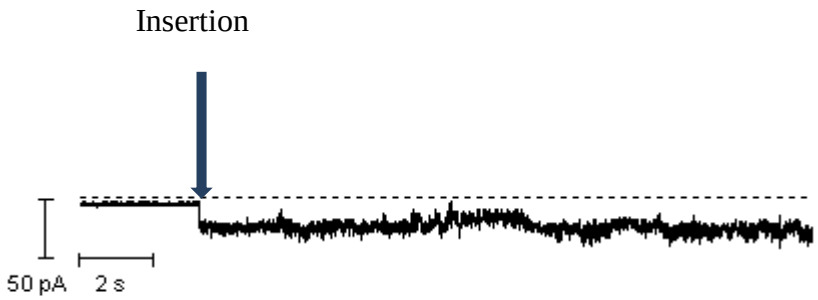


Figure 3.16 *Bilayer data for a crude mixture of tetrakis(triethyleneglycol monomethyl ether)calix[4]arene cone conformers with addition of KCl.*

3.6.3.2 Experiments with *1,3-alt*-**21**

When 32 mmol of *1,3-alternate* tetrakis(triethyleneglycol monomethyl ether)calix[4]arene (*1,3-alt*-**21**) was added to a pH 7.2 buffered solution containing 150 mM KCl, insertion was

indicated by a perturbation of the membrane shortly after addition (Fig. 3.17). The membrane remained intact but no features corresponding to channels were observed.

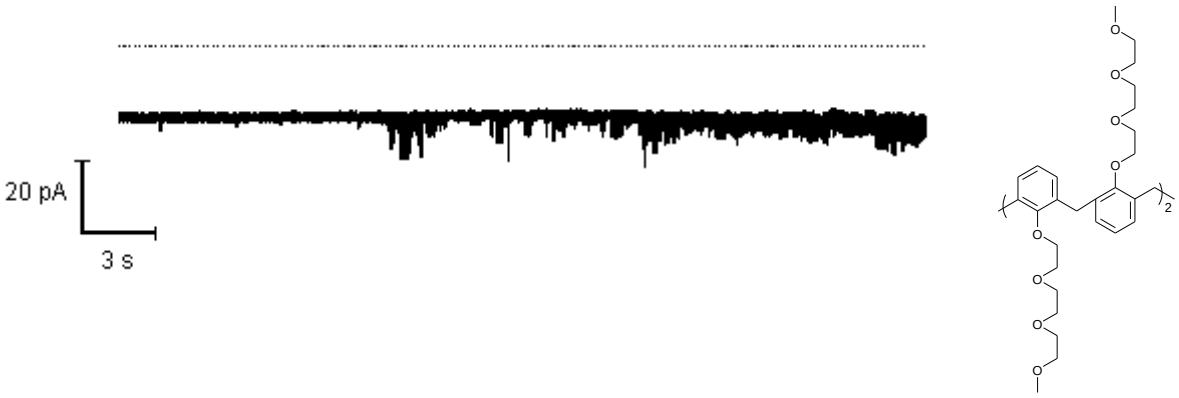


Figure 3.17 *Bilayer data for 1,3-alternate-tetrakis(triethyleneglycol monomethyl ether)calix[4]arene (1,3-alt-**21** **(**32 mmol) in KCl (150 mM).*

In these experiments it can be seen that in the cone conformation the compound lets potassium ions through. A step can be seen in Fig. 3.15 and Fig. 3.16 but not in Fig. 3.17 indicating that in the alternate form there is no channel forming in the bilayer to allow passage of potassium ions.

The next step was to test tetrakis(triethyleneglycol monomethyl ether)calix[4]arene in the *cone* and *1,3-alternate* conformers using a sodium buffer to test for sodium selectivity. Both were tested in the lipid bilayer but only the *1,3-alternate* results are shown (Fig. 3.18). The *1,3-alternate* conformer of tetrakis(triethyleneglycol monomethyl ether)calix[4]arene was introduced to the bilayer as in the previous experiment but with NaCl, buffered solution containing 150 mM NaCl to a total of 32 mmol and pH 7.2. The voltage was held at -50mV. The first insertion of the channel like structure was observed at 10 seconds after compound addition and lasted for up to 60 s. A second insertion occurred shortly after and this was followed by a third. The bilayer maintained stable and was followed by closure of the channel before a fourth insertion was recorded. The molecule had channel activity in the presence of

sodium ions. The steps were on the scale of 100 pS implying insertion of several calixarenes similar to what was assumed to occur with the calix[6]arene derivative. The integrity of the bilayer held throughout the experiments.

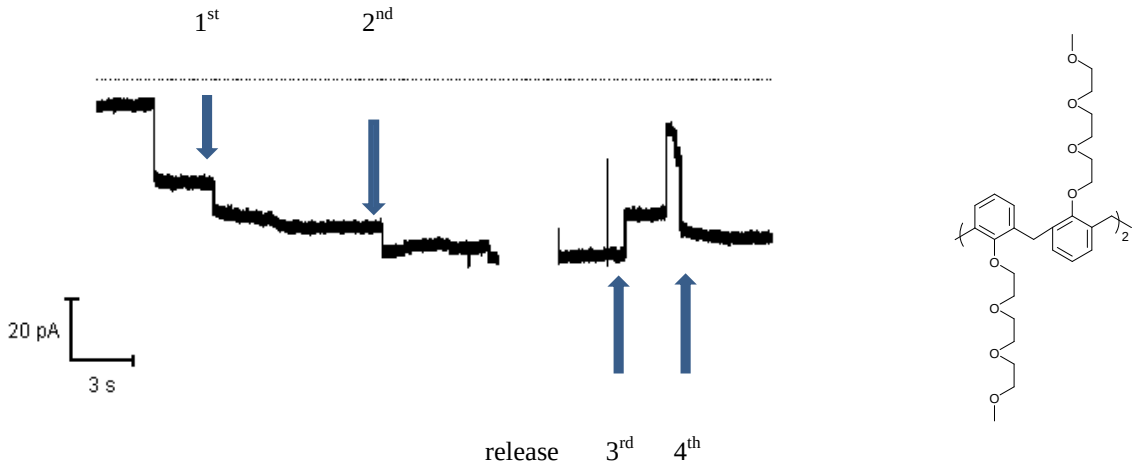


Figure 3.18 *Bilayer data for 1,3-alternate-tetrakis(triethyleneglycol monomethyl ether)calix[4]arene (1,3-alt-**21**) (32 mmol) in NaCl (150 mM).*

The results are reminiscent of those reported by Gokel for a tetrasubstituted calix[4]arene in the *cone* and *1,3-alt* conformers, however, no ion selectivity (e.g. Na^+ over K^+) was reported in the paper (De Mendoza 1998). In the case of calix[4]arenes with lower rim cholic acid substituents (Maulucci 2005), ion transport was determined by release of Na^+ or H^+ from large unilamellar vesicles and was measured by the response of a pH-sensitive pyranine fluorophore or $^{23}Na^+$ NMR signals, respectively. Increasing the concentration of calixarenes increased the rate of transport of both cations with a linear dependence. For both ions the *1,3-alt* conformer was observed to be significantly more active. In neither case was the mechanism of transport determined, the authors assumed the compounds acted as ionophores rather than channel-forming systems, nor was any size selectivity investigated. The data reported here are therefore far more significant as they demonstrate both conformer-dependent selectivity and ion-dependent selectivity.

In summary, bilayer recordings have demonstrated that 4-*t*-butylhexahomotrioxa-calix[3]arene$(OH)_2$Triton (**14**) and 4-*t*-butylcalix[6]arene$(OMe)_3(Triton)_3$ (**18**) had shown that these have channel like activity. The 4-*t*-butyhexahomooxacalix[3]arene (**9**) derivative was tested, with promising results, and 4-*t*-butylcalix[6]arene$(OMe)_3(Triton)_3$ (**18**) demonstrated channel activity and selectivity toward Na^+ over K^+ as shown by the Nernst equation and the reverse potential calculation.

Tetrakis(triethyleneglycol monomethyl ether)calix[4]arene was prepared from calix[4]arene by Lawal by the general method of Verboom (Verboom 1992). The *1,3-alt* conformer could be isolated cleanly from the reaction mixture. Lipid bilayer experiments confirmed that a crude mixture of *cone*-**21** derivatives were able to insert into a lipid bilayer but that it had no channel forming activity. The single component *1,3-alt*-**21** gave the response expected for an artificial transmembrane ion channel in the presence of Na^+ but not K^+. Why did this particular conformer of the calixarene act as a Na^+ channel when the crude *cone* mixture showed no activity and other compounds related to calix[4]arenes are K^+ selective? Firstly, only the *1,3-alt* conformer, at approximately 3 nm in length, has the potential to span a lipid bilayer. Secondly, Shinkai has proposed that the *1,3-alt* conformer of calix[4]arene provides an extended electron rich environment that is attractive to monovalent cations (Ikeda 1994). Finally, molecular modelling studies (Lawal 2009) indicated that the sodium cation interacts with two phenolic oxygen atoms, two phenolic rings and two water molecules. This gives the preferred six coordinate environment for Na^+, observed in many systems from NaCl to more complex protein-based ion channels, and contrasts with the eight coordinate environment favoured by K^+ in potassium channels and observed in the crystal structure of KcsA (Doyle 1998).

Table 3.1 *Summary of electrophysiological studies on lipid bilayers*

Compound	Number of experiments undertaken	Insertion into bilayer observed	Channel formation with K^+	Channel formation with Na^+
Triton X-100® control	8	8	NR	MD
4-*t*-Butylhexahomotrioxacalix[3]-arene$(OH)_2$ Triton (**9**)	3	1	NR	1
Oxacalix[3]arene-tryethylene glycol, monomethyl ether conjugate (**12**)	1	0	NR	0
4-*t*-Butylcalix[4]arene$(OH)_2$$(Triton)_2$(**14**)	10	6	0	0
4-*t*-Butylcalix[6]arene$(OMe)_3$$(Triton)_3$ (**18**)	54	29	0	13
1,3-alt- Tetrakis(triethylene glycol monomethyl ether)calix[4]arene (*1,3-alt*-**21**)	8	6	0	3
cone-Tetrakis(triethylene glycol monomethyl ether)calix[4]arene (*cone*-**21**)	7	1	NR	0

MD – membrane disruption NR – no recording made

3.6.4 Proposed mode of multichannel conductance

In the case of 4-t-butylcalix[6]arene$(OMe)_3$$(Triton)_3$ it was often observed that steps were of equal size indicating that exact multiples of ions were moving across the bilayer. This is rationalised in Fig. 3.19 which shows single molecule inserting to give a current of I (left), four molecules inserting, interacting primarily through hydrophobic effects, giving a current of 4 I (centre), and a group of three molecules with an interstitial channel (right). The latter arrangement would result in a current oscillating between 3 I and 4 I, depending on the permittivity of the interstitial channel, denoted by the blue triangle.

Had interstitial channels formed between the calixarenes then non-quantized changes in current would have been observed indicative of transmembrane leakage. A similar

interpretation has been made by Fyles to explain the large step changes in conductivity observed for channel-forming oligoesters (Fyles 2006).

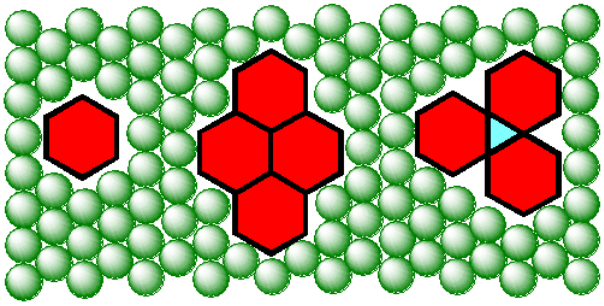


Figure 3.19 *Proposed mode of multichannel conductance.*

3.6.5 Proposed mode of selectivity for 4-t-butylcalix[6]arene$(OMe)_3(Triton)_3$

Experiments with K^+ in place of Na^+ showed no conductance even though the calix[6]arene cavity should be able to accommodate a hydrated K^+ ion. The reason for this lies in the preferred geometry of 4-t-butylcalix[6]arene trimethylether derivatives. Crystallographic data for *cone* conformers, extensively discussed in the supplementary information to Iqbal 2007b, show three of the t-butylphenol groups meeting over the central cavity while alternating, methoxy substituted rings adopt open, or winged, positions as shown in Fig. 3.20. In brief, a 2007 search of the Cambridge Structural Database revealed 41 *cone*-1,3,5-trimethoxycalix[6]arene derivatives with bulky substituents in the 2,4,6-positions. Of these, 17 had no bound metal atoms and methyl groups blocking the cavity. The remaining metal ion complexes exhibited geometries in which allowed access to the macrocyclic cavity. From these structures it is proposed that, even when bulky substituents are present, metal ions can pass through the calix[6]arene's central cavity. For this to occur, the t-butyl groups must also rotate to allow metal ions to pass through the upper rim. Computer models indicate that while Na^+ can traverse the cavity, K^+ is too large to pass through (Iqbal 2007b).

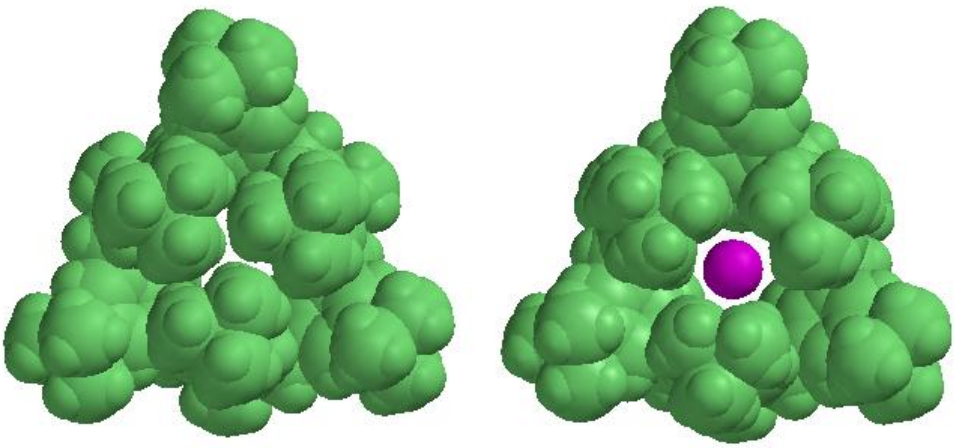


Figure 3.20 *Proposed conductance mechanism for 4-t-butylcalix[6]arene(OMe)$_3$(Triton)$_3$: closed (left) and open (right) conformer.*

The asymmetric experiments performed using Na^+ and K^+ indicated that this compound is specific for Na^+ over K^+ but more experiments would need to be done in order to determine the transmembrane ion flux.

3.6.6 Proposed mode of 4-t-butylcalix[6]arene(OMe)$_3$(Triton)$_3$ insertion in the planar bilayer

The calixarene derivatives has a lipophilic head group and amphiphilic 'tails' so molecules are expected to form micelles at concentrations above the critical micelle concentration, interacting with each other predominantly through the hydrophobic effect. At 64 μM the solutions used were often visibly opalescent, indicative of micelle formation. As indicated in Fig. 3.21, it is proposed that a micelle, composed of the calixarene with amphiphilic polyethers on its exterior, approaches the phospholipid bilayer (left). A portion of the micelle inserts in the bilayer (centre) and is left when the micelle moves away from the bilayer (right). Small steps may be due to single molecules inserting with the larger steps due to the insertion of more molecules simultaneously. This is consistent with the well-known fusion method of introducing channel-forming proteins to bilayers (Burkhart 1998).

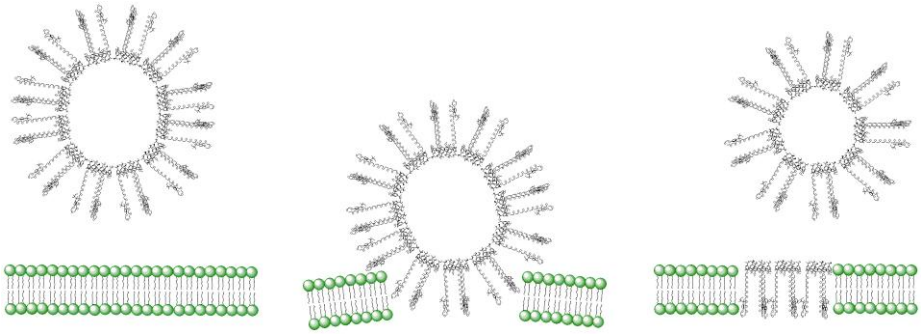


Figure 3.21 *Calixarene insertion hypothesis.*

3.7 Conclusions

The results obtained are evidence of channel-like behaviour for the compounds tested by electrophysiological methods. One result obtained for 4-t-butylhexahomotrioxacalix[3]arene-$(OH)_2$Triton (**9**) showed that the compound formed channel-like steps in sequence with channels inserting one after another. The evidence described demonstrated that even when channels started to insert one after another, the bilayer remained very stable throughout the 500 seconds of the recording. The steps observed were for sodium ions and the channels remained open throughout the whole recording.

The activity for 4-t-butylcalix[6]arene$(OMe)_3$$(Triton)_3$ (**18**) was the most interesting revelation in this project. Channel-like behaviour was observed in several experiments where small steps (for one channel) and bigger steps for micelle-like insertion in the bilayer were observed. The stability of the bilayer was demonstrated throughout the experiments where recordings were made for 500s or longer. The channels inserted and were released from the lipid bilayer constantly with no signs of instability in the lipid bilayer. The patterns observed were the insertion of the channels in the bilayer in exact multiples of the single channel events. This compound demonstrated that they were single channel insertions at lower

concentration and multiple insertions at a higher concentration. These channels behaved differently throughout the recording. In the presence of sodium ions a conductance of –7.610 pA was detected which could presumably be only one opening of these channels or several openings at the same time. The micelle behaviour of this compound was explained as a portion of the micelle inserts in the bilayer and is left embedded. When the micelle moves away from the bilayer the compound inserted in the bilayer as a multiple unit. These can be one, two, four or more multiple insertions of the channel-causing agent. At low concentrations of 32 mmol solution the bilayer shows small steps that match a single channel insertion and at larger concentrations the larger steps appear more often that match a multiple insertion. In further experiments, another form of insertion was observed. The compound inserted as multiple channels in the bilayer then a few small and apparently reversible insertions occurred. Finally the multiples of this compound dislodged leaving behind a very stable bilayer. Calix[6]arenes incorporating surfactant moieties can therefore operate as single channel Na^+ channel mimics at low concentrations but aggregate to form multiple channels at higher concentrations The recordings taken for any experiment has its downside too. It can be seen in one of the 4-t-butylcalix[6]arene$(OMe)_3(Triton)_3$ experiments that the bilayer recordings were very sensitive to noise. If any noise was around the recording could be damaged as some of the noise data can be confused with channel insertion, especially for very small steps.

The asymmetric experiments for the 4-t-butylcalix[6]arene$(OMe)_3(Triton)_3$ proved that this channel is specific for sodium ions. Channels appeared then disappeared but were all the same size, implying that discrete channels were forming. The potassium ions are not passing through as no activity was observed. The results indicate that this compound is selective for sodium ions. The selectivity can be explained by molecular models that show the possibility

of three of the t-butylphenol groups meeting over the central cavity while alternating, methoxy substituted rings adopt open, or winged, positions. This can be backed up by crystallographic data for the *cone* conformers of 4-t-butylcalix[6]arene$(OMe)_3(R)_3$ complexes in the Cambridge Crystallographic Database that all show the methoxy groups pointing away from the central cavity (Iqbal 2007b). It would appear that the t-butyl groups must rotate to allow metal ions to pass through the upper rim. Although Na^+ can traverse the cavity, K^+ is too large to pass through.

Previously, 4-t-butylcalix[4]arene$(OH)_2$$(Triton)_2$ was tested in the lipid bilayer but no results were obtained and it was believed at that time that sodium ions were blocking the channel as they were binding to the two OH groups in the aromatic lower rim. This is consistent with the mass spectrometric data that support the existence of a sodium complex. Further development of artificial ion channels with calix[4]arenes occurred in parallel with this project. Tetrakis(triethyleneglycol monomethyl ether)calix[4]arene was synthesised successfully by a project student in the group and was tested in the lipid bilayer. Results obtained demonstrated that the *cone* conformer of this compound did not work well as a channel for sodium ions and the *1,3-alternate* form of these compound shown positive results for sodium ions and not potassium ions (Lawal 2009).

Finally the results demonstrated that the molecules with long Triton substituents and short triethylene glycol monomethyl ether substituents both resulted in channel-like behaviour. 4-t-Butylcalix[4]arene$(OMe)_2Triton_2$ has a potential to form channels. *1,3-alt*-Tetrakis(triethyleneglycol monomethyl ether)calix[4]arene and a mixture of calix[4]arene *cone* deivatives were also investigated. Results obtained show clearly that the *1,3-alt* conformer works as a channel mimic for sodium ions. It is believed that the formation of

these channels is due to the polyether tails stretching to the upper layer of the lipid bilayer and the other half spreading in the opposite side. This would go across the whole width of the lipid bilayer and open a pore where the sodium ions could go through. The pure *1,3-alternate* form produced exciting results of insertions in the bilayer. As discussed above, the mixture of *cone* derivatives had some activity but did not form distinct channels. The results also demonstrated that the *1,3-alternate* conformer does not let potassium ions through, making it specific to sodium ions only.

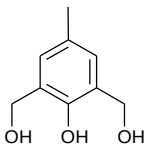
In this molecule molecular modelling suggests that Na^+ interacts with two phenolic oxygen atoms, two phenolic rings and two water molecules when the sodium ions pass through the channel (Lawal 2009). This gives the preferred sixfold octahedral environment for Na^+, observed in many systems and proposed for protein-based ion channels (Jasti 2007), rather than the eightfold cubic environment favoured by K^+ in the crystal structure of KcsA in which each cation interacts with eight carbonyl oxygen atoms as it traverses the central cavity in the transmembrane protein (Doyle 1998). ^1H NMR experiments for the other cations showed no evidence of shifts in the aromatic and bridging protons as were observed for sodium. This is strong evidence for the calixarene to be indeed selective for Na^+.

Chapter 4

4.1 Experimental work

Unless otherwise stated, all materials and solvents are from Sigma-Aldrich (Gillingham, Dorset, UK). NMR spectra were recorded on a Bruker AM-360 spectrometer at 360 MHz and 90 MHz for ^{1}H and ^{13}C respectively. All spectra were recorded in deuterochloroform unless otherwise stated and chemical shifts were referenced to tetramethylsilane (TMS). High resolution mass measurements (HRMS) were determined in electrospray ionization (ESI) mode using a Bruker Daltonics MicroToF spectrometer operating in the positive mode. Infrared absorption spectra were recorded directly on a Nicolet avatar 320 FT-IR fitted with a Smart Golden Gate®. Melting points were determined on an Electrothermal melting point apparatus and are uncorrected.

4.1.1 2,6-Bis(hydroxymethyl)-4-methylphenol (**1a**)

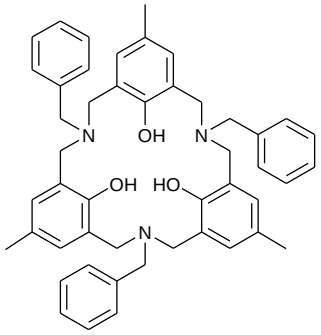


Compound **1a** was prepared according to the literature method of Hampton (Hampton 1994). p-Cresol (27.55 g, 0.25 mol) was dissolved in tetrahydrofuran (55 ml). Sodium hydroxide (10 g) in 25 ml of water was added to the reaction mixture. Formaldehyde (60 ml, 37% aqueous solution) was added to the reaction mixture which was left at room temperature for 7 days. Propan-2-ol (300 ml) was added to the paste and the sodium 2,6-bis(hydroxymethyl)-4-

methyl phenolate precipitated. The precipitate was suspended in acetone (125 ml) and acidified with glacial acetic acid (10 ml in 100 ml of acetone), to precipitate the sodium as sodium acetate which was removed by filtration. The remaining solvent was removed under vacuum to give an off-white solid. This was then recrystallized to give 2,6-bis(hydroxymethyl)-4-methylphenol.

Yield: 23.18 g (55 %); m p.: 108-110 °C; ^{1}H NMR ($CDCl_3$) δ: 6.85 (s, 2 H, Ar-CH), 4.71 (s, 4 H, -CH_2-), 2.25 (s, 3 H, -CH_3); ESI HRMS found: m/z 168.0800; calculated: 168.0786 $[M]^+$. Data agree with those in the literature (Hampton 1994).

4.1.2 4-Methyl(*N*-benzyl)hexahomotriazacalix[3]arene (**2a**)

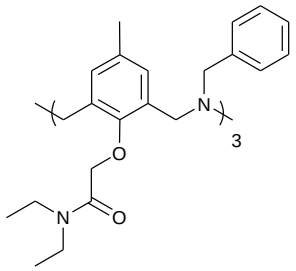


Compound **2a** was prepared according to the literature method of Takemura (Takemura 1992). 2,6-Bis(hydroxymethyl)-4-methyl phenol, **1a**, (4.0 g, 24 mmol) was added to a solution of benzylamine (2.57 ml, 24 mmol) in toluene (125 ml) in a flask equipped with a Dean-Stark trap and condenser. p-Toluenesulfonic acid (1.52 g, 8 mmol) was added, a further 25 ml of toluene poured into the Dean-Stark trap, and the mixture heated at reflux for 24 hours. The resulting solution was decanted from the remaining precipitate and the solvent removed under vacuum to yield a yellow oil. Purification was achieved through the dropwise addition of methanol to the product dissolved in dichloromethane which was left overnight.

The precipitate was separated from the solution by decantating. The solvent was evaporated and the product, 4-methyl(*N*-benzyl)hexahomo-triazacalix[3]arene, collected as a yellow microcrystalline solid.

Yield: 7.2 g (30 %); m.p.: 75-78 ºC; ^{1}H NMR ($CDCl_3$) δ: 9.2 (s, 3 H, Ar-OH), 7.87 (s, 6 H, Ar-H), 6.67-7.40 (m, 15 H, benzyl-H), 4.77 (s, 12 H, -N-CH_2-), 4.33 (s, 6 H, -CH_2-), 1.39 (s, 9 H, -CH_3); ^{13}C NMR ($CDCl_3$) δ: 165.99, 160.01, 131.46, 128.84, 123.79, 70.78, 60.64, 29.51; IR ν: 3397.4, 3317.1, 2975.6, 2915.4, 2855.1, 1717.0, 1908.4, 1315.4, 1197.3, 1076.8, 1031.6, 765.5 cm^{-1}; ESI HRMS *m/z* found: 718.3953 calculated: 718.3930 $[M]^+$. Data agree with those in the literature (Takemura 1992).

4.1.3 Attempted synthesis of 4-methyl(*N*-benzyl)hexahomotriazacalix[3]arenetris(*N*,*N*-diethylacetamide) (**3a**)

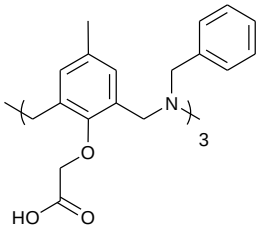


4-Methyl(*N*-benzyl)hexahomotriazacalix[3]arene, (**2a**), (0.7 g, 0.98 mmol) was added to THF (25 ml), dried using a small amount of sodium hydride (60 % in mineral oil). The mixture was stirred until all the solids had dissolved. Sodium hydride (0.28 g, 6 mmol, 60% dispersed in mineral oil) was added carefully, a little at the time. Once the solid had been added and the solution stopped effervescing, any solids remaining were rinsed into the flask by pouring additional dry THF (25 ml). The mixture was left to stir for 30 minutes. During this time the colour changed to a bright orange. After the 30 minutes stirring under reflux 2-chloro-*N*,*N*-diethylacetamide (0.90 g, 0.82 ml, 6 mmol) was added carefully to the reaction mixture. After

the addition was complete the neck was resealed with a glass stopper and the mixture refluxed for two and half hours. After this the reaction was left to cool. Once cool, distilled water (30 ml) was added, the residue was acidified to pH 1 with 4 M HCl and extracted with CH_2Cl_2 (3 x 25 ml). The organic phase was washed with water (30 ml), the organic phase separated and the solvent was removed under vacuum to give a yellow oily residue.

The compound was dissolved in a minimum amount of CH_2Cl_2 and triturated in stirred diethyl ether (150 ml). The product was left for 24 hours to settle at the bottom of the flask. The product was isolated as a white/yellow powder (0.48 g) and taken to the next stage. No data were collected.

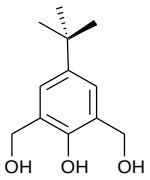
4.1.4 Attempted synthesis of 4-methyl(*N*-benzyl)hexahomotriazacalix[3]arene-tris(acetic acid) (**4a**)



4-Methyl(*N*-benzyl)hexahomotriazacalix[3]arenetris(*N*,*N*-diethylacetamide) (**3a**), (0.07 g, 0.66 mmol) was dissolved in 1,4-dioxane (30 ml). Aqueous 1 M NaOH (30 ml) was added and the mixture was refluxed for 72 hours. The reaction was topped up with 1,4-dioxane occasionally to avoid evaporation. The solution was then concentrated. A white residue present at the bottom of the flask was acidified to pH 1 with 2 M HCl. Ethyl acetate was added but an emulsion formed. The emulsion was extracted with more ethyl acetate (2 x 20 ml) then washed with water (2 x 20 ml) and saturated aqueous NaCl. The solvent was

evaporated resulting in a white powder. ^{1}H NMR analysis indicated that the reaction had not achieved synthesis of the target compound with recovery of the starting materials.

4.1.5 2,6-Bis(hydroxymethyl)-4-t-butylphenol (**1b**)



Compound **1b** was prepared according to the literature method of Hampton (Hampton 1994). 4-t-Butylphenol (37.55 g, 0.25 mol) was dissolved in THF (55 ml). Sodium hydroxide (10 g) was dissolved in 25 ml of water, stirred and left to cool before it was added to the reaction mixture. Formaldehyde (60 ml, 37% aqueous solution) was added to the reaction mixture which was left at room temperature for 7 days. After the reaction had gone to completion, propan-2-ol (300 ml) was added to the paste and the sodium 2,6-bis(hydroxymethyl)-4-t-butyl phenolate precipitated. The precipitate was suspended in acetone (125 ml) and acidified with glacial acetic acid (10 ml in 100 ml of acetone), to precipitate the sodium as sodium acetate which was removed by filtration. The remaining solvent was removed under vacuum to give an off-white solid which was recrystallized to give 2,6-bis(hydroxymethyl)-4-t-butylphenol.

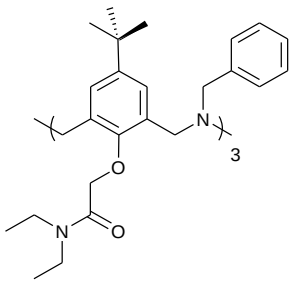
Yield: 19.11 g (36 %); m.p.: 41-43 °C; ^{1}H NMR ($CDCl_3$) δ: 7.05 (s, 2 H, Ar-CH), 4.79 (s, 4 H, -CH_2-), 2.35 (s, 2 H, -OH), 1.25 (s, 9 H, ^{t}Bu); ESI HRMS *m/z* found 232.11298: calculated: 232.11 $[M + Na]^{+}$. Data are in agreement with those in the literature (Hampton 1994).

4.1.6 4-t-Butyl(*N*-benzyl)hexahomotriazacalix[3]arene (**2b**)

Compound **2a** was prepared according to the literature method of Hampton (Hampton 1996). A mixture of 2,6-bis(hydroxymethyl)-4-t-butylphenol, **2a**, (5.05 g, 24 mmol) was added to a solution of benzylamine (2.57 ml, 24 mmol) in toluene (125 ml) in a round bottomed flask equipped with a magnetic stirrer bar and fitted with a Dean-Stark trap and condenser. p-Toluenesulfonic acid (1.52 g, 8 mmol) was added, a further 25 ml of toluene poured into the Dean-Stark trap, and the mixture refluxed for 24 hours. The solvent was evaporated and a yellow oily precipitate was collected. Diethyl ether was added and removed under vacuum to azeotrope any remaining solvent. This was repeated a further three times to leave a yellow fluffy compound which was left to dry overnight and 4-t-butyl(*N*-benzyl)-hexahomotriazacalix[3]arene was isolated as a pale yellow microcrystalline powder.

Yield: 6.17 g (30.5 %); m.p.: 86-88 °C; ^{1}H NMR ($CDCl_3$) δ: 9.2 (s, 3 H, Ar-OH), 7.35 (s, 6 H, Ar-H), 6.75 and 7.40 (m, 15 H, benzyl-H), 4.77(s, 12 H, -N-CH_2-), 3.62 (s, 6 H, -CH_2-), 1.30 (s, 27 H, ^{t}Bu); ^{13}C NMR ($CDCl_3$) δ: 139.91, 137.58, 127.31, 127. 07, 126.61, 126.44, 126.02, 125.57, 125.10, 124.33, 123.70, 123.36, 31.59, 31.53, 29.32, 29.30, 29.27, 29.19; IR *v*: 3024.66, 2950.94, 1302.55 cm^{-1}; ESI HRMS *m/z* found: 844.5457 calculated: 844.5457 $[M]^{+}$. Data are in agreement with those in the literature.

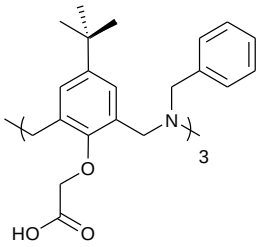
4.1.7 Attempted synthesis of 4-t-butyl(*N*-benzyl)hexahomotriazacalix[3]arenetris(*N*,*N*-diethylacetamide) (**3b**)



4-t-Butyl(*N*-benzyl)hexahomotriazacalix[3]arene, **2b**, (0.846 g, 1.0 mmol) was added to THF (25 ml), dried using a small amount of sodium hydride (60 % in mineral oil). The mixture was stirred until all the solids had dissolved. Sodium hydride (0.28 g, 6 mmol, 60% dispersed in mineral oil) was added carefully. Once the solid had been added and the solution stopped effervescing, any solids remaining were rinsed into the flask by additional dry THF (25 ml). The mixture was left to stir for 30 minutes. Unlike the methyl analogue, no colour change observed. After 30 minutes stirring under reflux 2-chloro-*N*,*N*-diethylacetamide (0.50 ml, 0.45 g, 3 mmol) was added carefully to the reaction mixture. After the addition was complete the mixture refluxed for two and half hours. After this the reaction was left to cool. Once cool, distilled water (30 ml) was added, the residue was acidified to pH 1 with 4 M HCl and extracted with CH_2Cl_2 (3 x 25 ml). The organic phase was washed with water (30 ml), the organic phase separated and the solvent was removed under vacuum to give a yellow oily residue.

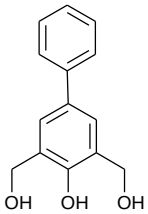
The compound was dissolved in a minimum amount of CH_2Cl_2 and triturated in stirred diethyl ether (150 ml) in a 250 ml conical flask. The product was left for 24 hours to settle at the bottom of the flask. 4-t-Butyl(*N*-benzyl)hexahomotriaza-calix[3]arenetris(*N*,*N*-diethylacetamide was isolated as a white/yellow powder (0.195 g) and taken to the next stage.

4.1.8 Attempted synthesis of 4-t-butyl(*N*-benzyl)hexahomotriazacalix[3]arene-tris(acetic acid) (**4b**)



4-t-Butyl(*N*-benzyl)hexahomotriazacalix[3]arenetris(*N*,*N*-diethylacetamide, **3b**, (0.07 g, 0.6 mmol) was dissolved in 1,4-dioxane (30 ml). Aqueous 1 M NaOH (30 ml) was added and the mixture was refluxed for 72 hours. The reaction was topped up with 1,4-dioxane occasionally to avoid evaporation. Following the reflux the solution was concentrated by evaporating on the rotary evaporator. A white residue present at the bottom of the flask was acidified to pH 1 with 2 M HCl. Ethyl acetate was added but an emulsion formed. The emulsion was extracted with more ethyl acetate (2 x 20 ml) then washed with water (2 x 20 ml) and saturated aqueous NaCl. The solvent was evaporated resulting in a white powder. ^{1}H NMR analysis indicated that the reaction had been unsuccessful.

4.1.9 2,6-Bis(hydroxymethyl)-4-phenylphenol (**1c**)

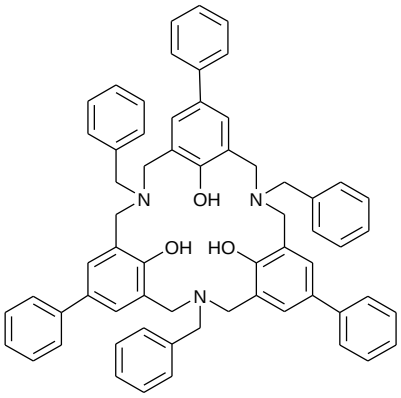


Compound **1c** was prepared according to the literature method of Gutsche (Gutsche 1982). 4-Phenylphenol (37.55 g, 0.25 mol) was dissolved in THF (55 ml). A solution of sodium hydroxide (10 g in 25 ml of water) was added to the reaction mixture. Formaldehyde (60 ml,

37% aqueous solution) was added to the reaction mixture which was left at room temperature for 7 days. After the reaction had gone to completion, propan-2-ol (300 ml) was added to the paste and the sodium 2,6-bis(hydroxymethyl)-4-phenyl phenolate precipitated. The precipitate was suspended in acetone (125 ml) and acidified with glacial acetic acid (10 ml in 100 ml of acetone), to precipitate the sodium as sodium acetate which was removed by filtration by a Büchner funnel. The remaining solvent was removed under vacuum to give an off-white solid. This was then recrystallized to give 2,6-bis(hydroxymethyl)-4-phenylphenol.

Yield: 21.22 g (36 %); m.p.: 94-96 °C; ^{1}H NMR ($CDCl_3$) δ: 7.20-7.60 (t, 7 H, Ar-CH), 4.80 (s, 4 H, -CH_2-); ESI HRMS *m/z* found: 253.08484, calculated: 253.08 $[M + Na]^+$. Data are in agreement with those in the literature (Gutsche 1982).

4.1.10 4-Phenyl(*N*-benzyl)hexahomotriazacalix[3]arene (**2c**)

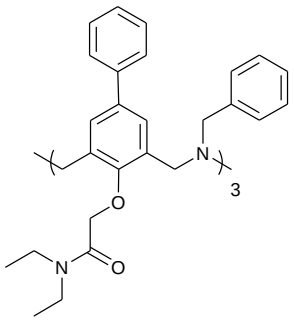


A mixture of 2,6-bis(hydroxymethyl)-4-phenylphenol, **1c**, (5.55 g, 24 mmol) was added to a solution of benzylamine (2.57 ml, 24 mmol) in toluene (125 ml) in a 250 ml 2-necked round bottomed flask equipped with a magnetic stirrer bar and fitted with a Dean-Stark trap and condenser. p-Toluenesulfonic acid (1.52 g, 8 mmol) was added, a further 25 ml of toluene

poured into the Dean-Stark trap, and the mixture refluxed for 24 hours. The solvent was evaporated and a yellow oily precipitate was collected. Diethyl ether was added and removed on the under vacuum to azeotrope any remaining solvent. This was repeated a further three times to leave a yellow fluffy compound which was left to dry overnight and 4-t-butyl(*N*-benzyl)hexahomotriazacalix[3]arene was isolated as a pale yellow microcrystalline solid.

Yield: 0.77 g (3.55 %); m.p.: 145-149 ºC; ^{1}H NMR ($CDCl_3$) δ: 7.00-7.65 (m, 36 H, Ar-H), 3.75 (t, 6H, -CH_2-); 4.80 (s, 12H, N-CH_2-); ^{13}C NMR ($CDCl_3$) δ: 156.71, 142.03, 139.96, 129.80, 128.77, 128.65, 128.22, 127.19, 126.68, 126.59, 126.48, 125.93, 63.39, 57.77; IR ν: 3052.78, 1599, 1454 cm^{-1}; ESI HRMS *m/z* found: 844.5457 calculated: 844.5457 $[M]^{+}$.

4.1.11 Attempted synthesis of 4-phenyl(*N*-benzyl)hexahomotriazacalix[3]arenetris (*N*,*N*-diethyl-acetamide) (**3c**)

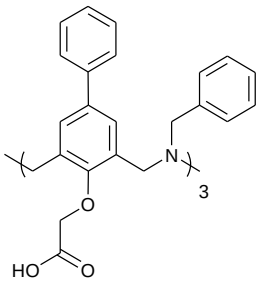


4-Phenyl(*N*-benzyl)hexahomotriazacalix[3]arene, (**2c**), (0.45 g, 0.53 mmol) was added to THF (25 ml), dried using a small amount of sodium hydride (60 % in mineral oil). The mixture was stirred until all the solids had dissolved. Sodium hydride (0.12 g, 3 mmol, 60% dispersed in mineral oil) was added carefully, a little at the time, through a glass funnel placed in the second neck of the flask. Once the solid had been added and the solution stopped effervescing, any solids remaining were rinsed into the flask by pouring additional

dry THF (25 ml) through the funnel. The funnel was removed, the neck sealed with a stopper and the mixture was left to stir for 30 minutes. During this time the colour changed to a bright orange. After the 30 minutes stirring under reflux 2-chloro-*N*,*N*-diethylacetamide (0.50 ml, 0.45 g, 3 mmol) was added carefully to the reaction mixture. After the addition was complete the neck was resealed with a glass stopper and the mixture refluxed for two and half hours. After this the reaction was left to cool. Once cool, distilled water (30 ml) was added, the residue was acidified to pH 1 with 4 M HCl and extracted with CH_2Cl_2 (3 x 25 ml). The organic phase was washed with water (30 ml), the organic phase separated and the solvent was removed under vacuum to give a yellow oily residue and taken to the next stage.

The compound was dissolved in a minimum amount of CH_2Cl_2 and triturated in stirred diethyl ether (150 ml) in a 250 ml conical flask. The product was left for 24 hours to settle at the bottom of the flask. The product (0.195 g) was isolated as a white/yellow powder. Analysis was inconclusive.

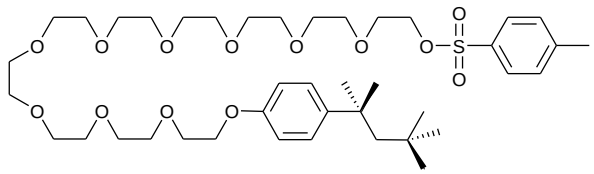
4.1.12 Attempted synthesis of 4-t-butyl(*N*-benzyl)hexahomotriazacalix[3]arene-tris(acetic acid) (**4c**)



4-Phenyl(*N*-benzyl)hexahomotriazacalix[3]arenetris(*N*,*N*-diethylacetamide), **3c**, (0.07 g, 0.56 mmol) was dissolved in 1,4-dioxane (30 ml). Aqueous 1 M NaOH (30 ml) was added and the mixture was refluxed for 72 hours. The reaction was topped up with 1,4-dioxane occasionally to avoid evaporation. Following the reflux the solution was concentrated by evaporating on

the rotary evaporator. A white residue present at the bottom of the flask was acidified to pH 1 with 2 M HCl. Ethyl acetate was added but an emulsion formed. The emulsion was extracted with more ethyl acetate (2 x 20 ml) then washed with water (2 x 20 ml) and saturated aqueous NaCl. The solvent was evaporated resulting in a white powder. Analysis indicated that the reaction had been unsuccessful.

4.1.13 Triton® tosylate (**5**)

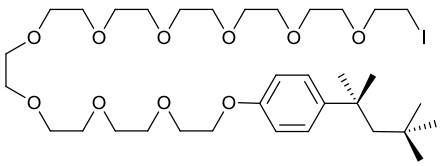


Triton-X100® (15.18 ml, 16.2 g, 0.0025 mol) was dissolved in THF (15 ml) and distilled water (5 ml) to which a solution of NaOH (1.42 g in 5.5 ml water) was added. The mixture was stirred in an ice bath until the solution temperature fell below 5 ºC. *p*-Toluenesufonyl chloride (5.33 g, 0.027 mol) in THF (10 ml) was added to the solution at a rate that kept the temperature below 5 ºC. Once the addition was complete the solution was left to stir for a further 30 minutes whereupon it was poured onto ice (20 g) and water (10 ml), then added to toluene (30 ml). The product was extracted into toluene, dried over anhydrous calcium chloride and filtered. Solvent was removed under vacuum to give Triton® tosylate as a colourless oil.

Yield: 10.2 g (51 %); ^{1}H NMR ($CDCl_3$) δ: 7.79 (d, 4H, *J* = 7.9 Hz, tosylAr*H*), 7.32 (d, 4H, *J* = 7.9 Hz, tosylAr*H*), 7.25 (d, 4H, *J* = 8.6 Hz, Ar*H*), 6.81 (d, 4H, *J* = 8.6 Hz, Ar*H*), 4.14 (t, 2H, *J* = 4.9 Hz, tosylArCH_2), 4.10 (t, 2H, *J* = 4.9 Hz, ArCH_2), 3.83 (t, 2H, *J* = 4.9 Hz,

tosylArCH_2CH_2), 3.71-3.56 (m, 34H, OCH_2CH_2), 2.43 (s, 3H, tosylCH_3), 1.69 (s, 2H, CH_2CH_3), 1.33 (s, 6H, ArC(CH_3)$_2$), 0.70 (s, 9H, C(CH_3)$_3$); ^{13}C NMR ($CDCl_3$) δ: 156.4, 144.7, 142.3, 137.8, 133.1, 129.8, 129.0, 128.0 ,127.0, 125.3, 113.8, 72.5, 71.0, 70.6, 70.3, 69.8, 69.2, 68.7, 67.3, 57.0, 37.9, 32.3, 31.8, 21.6; IR ν: 1609, 1243, 1176 cm^{-1} ESI HRMS *m/z* found: 823.4467 [M + Na]$^+$, calculated: 823.4279.

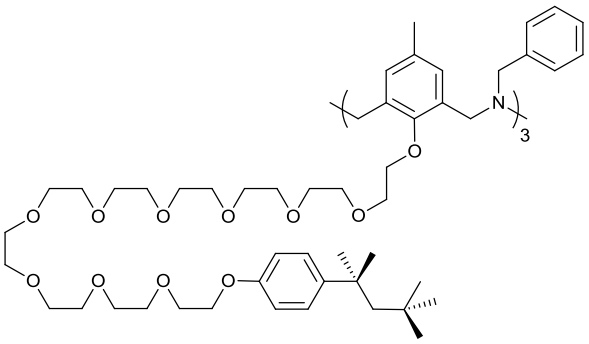
4.1.14 Triton® iodide (**6**)



In a method adapted from Loiseau (Loiseau 2007), potassium iodide (0.83 g, 5 mmol) was added to a solution of Triton tosylate, **5**, (0.81g, 15 mmol) in acetone (70 ml) and the solution was heated at reflux for 24 hours. After this time, the inorganic salts were removed by filtration and the filtrate was reduced in volume. The residue was dissolved in a mixture of ethyl acetate and water (1:1 v/v) and separated. The combined organic extracts were washed with water and saturated aqueous sodium thiosulfate, dried over magnesium sulfate, filtered and evaporated under vacuum.

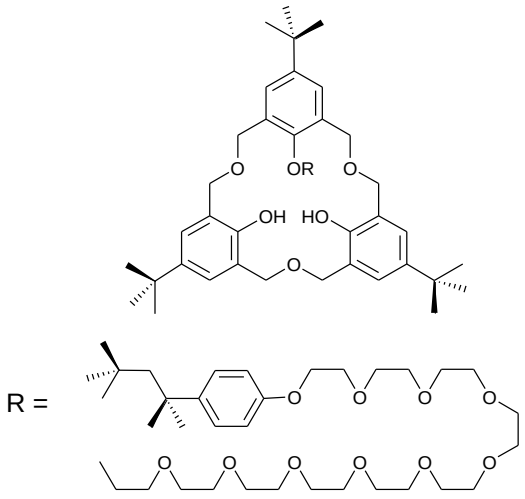
Yield: 1.23 g, (65.8 %); ^{1}H NMR ($CDCl_3$) δ: 7.25 (d, 4 H, *J* = 8.6 Hz, Ar*H*), 6.81 (d, 4 H, *J* = 8.6 Hz, Ar*H*), 4.10 (t, 2 H, *J* = 4.8 Hz, ArOCH_2), 3.83 (t, 2 H, *J* = 4.8 Hz, -CH_2I), 3.71-3.56 (m, 34 H, OCH_2CH_2), 1.69 (s, 2 H, CH_2CH_3), 1.33 (s, 6 H, ArC(CH_3)$_2$), 0.70 (s, 9 H, C(CH_3)$_3$); ^{13}C NMR ($CDCl_3$) δ: 126.99, 142.33, 126.99, 113.77, 72.66, 71.96, 70.78, 70.65, 70.56, 70.21, 69.81, 67.29, 61.65, 56.98, 48.35, 41.00, 37.92, 32.30, 31.76, 31.67; IR ν: 1609 1186 cm^{-1} ; ESI HRMS *m/z* found: 779.3299 [M + Na]$^+$ calculated: 779.3280.

4.1.15 Attempted synthesis of 4-methyl(*N*-benzyl)hexahomotriazacalix[3]arene(Triton)$_3$ (**7**)



4-Methyl(*N*-benzyl)hexahomotriazacalix[3]arene, **2a**, (1.4 g, 1.96 mmol) and potassium carbonate (0.54 g, 12 mmol) were suspended in dry acetonitrile (40 ml). To this stirred suspension Triton® iodide, **6**, (2.9 g, 9.8 mmol) was added and the resulting mixture was heated under reflux and under nitrogen atmosphere for 4 days. The suspension was allowed to cool and the solvent removed under pressure to give a mass of white solid. ^{1}H NMR analysis gave inconclusive results.

4.1.16 4-t-Butylhexahomotrioxacalix[3]arene(OH)$_2$(Triton) (**9**)

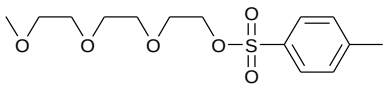


A suspension of 4-t-butylhexahomotrioxacalix[3]arene, **8**, (0.174 g, 0.302 mmol), anhydrous potassium carbonate (0.084 g, 0.60 mmol) and Triton iodide, **6**, (0.70 g, 0.91 mmol) was

refluxed in anhydrous acetonitrile (60 ml) under nitrogen for 7 days. When cooled the solvent was removed on a rotary evaporator and the residue was dissolved/suspended in CH_2Cl_2 (30 ml). This was washed with 1 M HCl (2 x10 ml) and brine (10 ml) and the organic phase separated. Solvent was removed under reduced pressure. The product was collected as a yellow oil.

Yield: 0.51g (42 %); ^{1}H NMR ($CDCl_3$) δ: 7.15 (d, 2 H, *J* = 8.6 Hz tritonAr*H*), 7.01 (s, 4 H, Ar*H*), 6.80 (s, 4 H, Ar*H*), 4.10 (t, 6 H, *J* = 4.8 Hz, ArOCH_2), 4.63 (m, 12 H, ArCH_2 OCH_2Ar), 3.85 (t, 2 H, *J* = 4.8 Hz ArOCH_2), 3.55-3.70 (m, 108 H, OCH_2CH_2), 1.69 (s, 6 H, CH_2CH$_3$), 1.33 (s, 18 H, ArC(CH_3)$_2$), 1.32 (s, 9 H, ArC(CH_3)$_3$, 0.71 (s, 9H, C(CH_3)$_3$), ^{13}C NMR ($CDCl_3$) δ 156.3, 142.44, 126.97, 113.74, 70.11, 67.25, 61.6, 56.93, 37.88, 34.24, 32.27, 31.75; IR ν: 3362, 1612, 1197 cm^{-1} ESI HRMS *m/z* found: 1245.7703 $[M + K + H]^+$, calculated: 1245.7386.

4.1.17 Triethylene glycol tosylate, monomethyl ether (**10**)

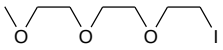


Triethylene glycol monomethyl ether (16.1 ml, 16.4 g, 0.1 mol) was dissolved in a mixture of tetrahydrofuran (50 ml) and aqueous sodium hydroxide (5.7 g, 0.14 mol in 30 ml H_2O) and stirred in an ice bath until the solution temperature fell below 5ºC. A solution of *p*-toluenesufonyl chloride (20.7 g, 0.11 mol) in tetrahydrofuran (30 ml) was added to the stirred solution at a rate that kept the temperature below 5 ºC. Once the addition was complete, the solution was left to stir for a further 1 h below 5 ºC, poured onto iced water (75 ml water, 75 g ice) and stirred until all the ice melted. Most of the tetrahydrofuran was removed by rotary

evaporation the product was extracted into toluene (3 x 30 ml). The organic extract was dried over calcium chloride, filtered and the solvent removed by rotary evaporation. Triethylene glycol tosylate, monomethyl ether, **2**, was obtained as a colourless oil.

Yield: 27 g (87%); ^{1}H NMR ($CDCl_3$) δ: 7.80 (d, *J* = 8.3 Hz, 2H Ar*H*), 7.35 (d, *J* = 8.3 Hz, 2H Ar*H*), 4.16 (t, *J* = 4.9 Hz, 2H, -C*H*$_2$OTs), 3.50-3.75 (m, 6H, -C*H*$_2$C*H*$_2$O-), 3.39 (s, 3H, -OC*H*$_3$), 2.45 (s, 3H, TsC*H*$_3$); ^{13}C NMR ($CDCl_3$) δ: 144.8, 132.9, 129.5, 127.8, 72.4, 71.8, 71.0, 70.6, 70.1, 69.7, 58.8, 21.5; IR ν: 2874, 1598, 1452, 1174 cm^{-1}; ESI HRMS m/z found: 341.1056, calculated: 341.1035 $[M + Na]^+$. (Lawal 2009).

4.1.18 Triethylene glycol iodide, monomethyl ether (**11**)

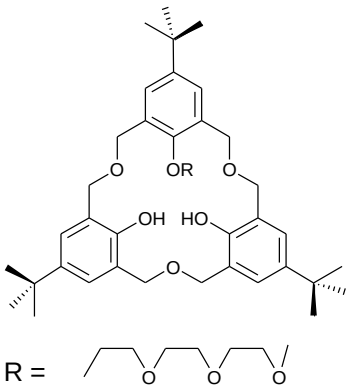


Using a method adapted from Loiseau (Loiseau 2007), triethylene glycol tosylate, monomethyl ether, **10**, (10 g, 0.03 mol) was dissolved in acetone (125 ml). Potassium iodide (25 g , 0.15 mol) was added and refluxed overnight. The solution was cooled to room temperature and filtered to remove inorganic salts. The solvent was removed under reduced pressure and the residue dissolved in a mixture of ethyl acetate (75 ml) and distilled water (75 ml). The mixture was separated and the ethyl acetate phase washed with water (30 ml) then saturated sodium thiosulfate (50 ml). The organic phase was dried over magnesium sulfate, filtered and solvent removed to give triethylene glycol iodide, monomethyl ether, **11**, as a colourless oil.

Yield: 5.2 g (61%); ^{1}H NMR ($CDCl_3$) δ: 3.76 (t, *J* = 8.1 Hz, 2H, -C*H*$_2$OCH$_3$), 3.65-3.70 (m, 6H, -C*H*$_2$C*H*$_2$O-), 3.54-3.60 (m, 2H, -OC*H*$_2$CH$_2$I), 3.39 (s, 3H, -OC*H*$_3$), 3.26 (t, *J* = 8.1 Hz,

2H, -OCH_2CH_2I); ^{13}C NMR ($CDCl_3$) δ: 72.5, 71.6, 70.5 (x 2), 70.1, 59.0, 3.3; IR ν: 2872, 1454, 1097 cm^{-1}; ESI HRMS m/z found: 296.9998, calculated: 296.9964 $[M + Na]^+$. (Lawal 2009)

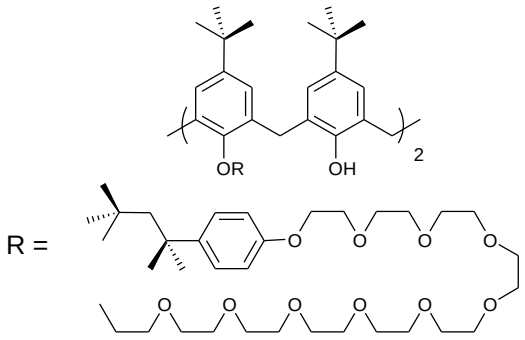
4.1.19 4-t-Butylhexahomotrioxacalix[3]arene$(OH)_2$(triethyleneglycol monomethyl ether) (**12**)



A suspension of 4-t-butylhexahomotrioxacalix[3]arene, **8**, (0.174 g, 0.302 mmol), anhydrous potassium carbonate (0.0835g, 0.60 mmol) and iodotriethylene glycol monomethyl ether, **11**, (0.262 g, 0.906 mmol) was refluxed in anhydrous acetonitrile (30 ml) under nitrogen for 7 days. When cooled the solvent was removed on a rotary evaporator and the residue was dissolved/suspended in CH_2Cl_2 (30 ml). This was washed with 1 M HCl (2 x10 ml) and brine (10 ml) and the organic phase separated. Solvent was removed under reduced pressure. The product was a yellow oil.

Yield: 2.42 g (42%); ^1H NMR ($CDCl_3$) δ: 7.21 (s, 4 H, ArH), 7.13 (s, 2 H, Ar*H*), 4.72 (s, 12 H, $ArCH_2OCH_2$ Ar), 3.74 (t, *J* = 8.1 Hz, 4 H, $ArOCH_2CH_2$), 3.58 (t, *J* = 8.1 Hz, 8 H, OCH_2CH_2), 3.26 (t, *J* = 8.1 Hz, 3 H, OCH_3), 1.24 (s, 18 H, ^{t}Bu), 1.23 (s, 9 H, ^{t}Bu), ^{13}C NMR ($CDCl_3$) δ 153.46, 126.84, 123.66, 71.99,71.94, 33.94, 31.48, 31.34, 29.70; ESI HRMS *m/z* found: 745.4296 $[M + Na]^+$, calculated: 745.4286.

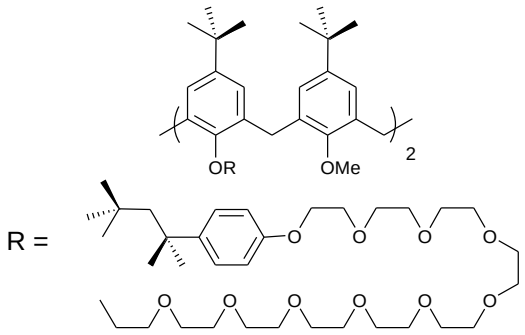
4.1.20 4-t-Butylcalix[4]arene$(OH)_2$$(Triton)_2$ (**14**)



4-t-Butylcalix[4]arene, **13**, (1.28 g, 1.96 mmol) was dissolved in anhydrous acetonitrile (40 ml). Triton tosylate, **6**, (3.93 g, 4.9 mmol) and potassium carbonate (0.54 g, 4 mmol) were added and the mixture refluxed under nitrogen for 72 h. The cooled mixture was filtered to remove solid byproducts and solvent was removed under vacuum to give the product as a colourless, viscous oil.

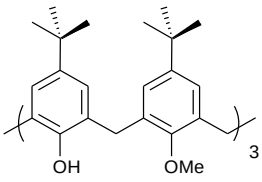
Yield: 1.86 g (50 %); ^{1}H NMR ($CDCl_3$) δ: 7.74 (s, 2 H, ArO*H*), 7.25 (d, 4 H, *J* = 8.64 Hz, TritonAr*H*), 7.03 (s, 4 H, Ar*H*), 6.95 (s, 4 H, Ar*H*), 6.82 (d, 4 H, *J* = 8.28 Hz, TritonAr*H*), 4.38 (d, 4 H, *J* = 12.96, ArCH_2Ar), 4.11 (t, *J* = 4.32 Hz, 4 H, ArOCH_2), 3.82 (m, 2 H, ArOCH_2CH_2), 3.71-3.57 (m, 68 H, OCH_2CH_2), 3.30 (d, 4 H, *J* = 12.60 Hz, ArCH_2Ar), 1.69 (s, 4 H, Ar$(CH_3)_2$C$H_2$$(CH_3)_3$), 1.33 (s, 12 H, Ar$(CH_3)_2CH_2$$(CH_3)_3$), 1.25 (s, 18 H, Ar$(CH_3)_2CH_2$$(CH_3)_3$), 1.08 (s, 18 H, ArC$(CH_3)$), 0.70 (s, 18 H, C$(CH_3)_2CH_2CH_3$); ^{13}C NMR ($CDCl_3$) δ: 156.4, 150.6, 149.9, 146.7, 142.3, 141.2, 132.6, 129.8, 127.9, 127.7, 127.0, 125.5, 125.0, 113.8, 71.0, 70.7, 70.3, 69.8, 69.2, 67.3, 57.0, 37.9, 33.9, 32.3, 31.8, 31.7; IR ν: 3317, 1609, 1186 cm^{-1}; ESI HRMS m/z found: 1929.2877 $[M + Na]^+$ calculated: 1929.2486.

4.1.21 4-t-Butylcalix[4]arene$(OMe)_2(Triton)_2$ (**15**)



t-Butylcalix[4]arene$(OH)_2(Triton)_2$, **14**, (0.2080 g, 1.1 mmol) was dissolved in dry acetone (150 ml). Potassium carbonate (0.0507 g, 0.37 mmol) and methyl iodide (0.139 g, 0.061 ml, 0.98 mmol) was added and the solution and left to reflux for 18 hours. The resulting solvent of the mixture was removed by vacuum and quenched with 2 M HCl. The organic phase was separated and evaporated to give the product as yellow oil which was identified as starting material.

4.1.22 1,3,5-Trimethoxyhexa(4-t-butyl)calix[6]arene (**17**) (Casnati 1991, Janssen 1993)

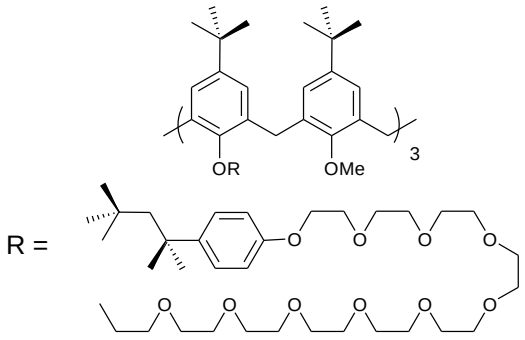


4-t-Butylcalix[6]arene, **16**, (6.0 g, 6.2 mmol) was placed in a 2-necked 500 ml round bottomed flask to which was added dry acetone (300 ml), potassium carbonate (2.56 g, 18.6 mmol) and methyl iodide (3.4 ml, 24.8 mmol). The mixture was stirred and refluxed for 24 hours at room temperature under nitrogen. The acetone was removed under vacuum and the residue treated with 2 M HCl (140 ml) and followed by the addition of CH_2Cl_2 (140 ml). The two phases were transferred into a separating funnel and the organic layer isolated and washed twice with distilled water (2 x 100 ml). The organic phase was dried with sodium

sulphate, filtered and evaporated to give crude 1,3,5-trimethoxyhexa(4-t-butyl)calix[6]arene as a white powder. Flash chromatography was performed using hexane: THF (9:1) to afford the purified compound as a white powder.

Yield: 3.37 g (53.5%), m.p.64^{o}C; ^{1}H NMR ($CDCl_3$) δ: 1.15 (27H, s,$C(CH_3)_3$), 1.16 (27H, s, $C(CH_3)_3$, 3.37 (9H, s, OCH_3),3.88 (12H, s, $ArCH_2Ar$), 6.67 (3H, s, OH), 6.89 (6H, s, ArH, 6.99 (6H, s,ArH), ^{13}CNMR($CDCl_3$) δ: 153.2, 148.82, 142.67, 132.62, 126.39, 124.8, 77.01, 31.83; IR ν: 2957.49, 1295.06, 1000.86 cm^{-1}). Data are in agreement with those in the literature (Casnati, 1991).

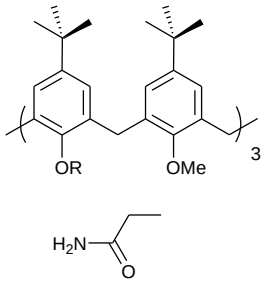
4.1.23 4-t-Butylcalix[6]arene$(OMe)_3(Triton)_3$ (**18**)



1,3,5-Trimethoxyhexa(4-t-butyl)calix[6]arene, **17**, (1.99 g, 1.96 mmol) was dissolved in anhydrous acetonitrile (40 ml). Triton tosylate, **5**, (5.9 g, 7.35 mmol) and potassium carbonate (0.54 g, 4 mmol) were added and the mixture refluxed under nitrogen for 72 h. The cooled mixture was filtered to remove solid byproducts and the solvent was removed under vacuum to give 4-t-butylcalix[4]arene$(OMe)_3(Triton)_3$ as a colourless, viscous oil.

Yield: 2.42g (42 %); ^{1}H NMR ($CDCl_3$) δ: 7.24 (d, 6 H, *J* = 8.6 Hz, TritonAr*H*), 7.01 (s, 6 H, Ar*H*), 6.90 (s, 6 H, Ar*H*), 6.82 (d, 6 H, *J* = 8.6 Hz, TritonAr*H*), 4.10 (t, 6 H, *J* = 4.8 Hz, ArOCH_2), 3.87 (m, 6 H, ArCH_2Ar), 3.85 (t, 6 H, *J* = 4.8 Hz, ArOCH$_2$CH_2), 3.70 (s, 9 H, OCH_3), 3.55-3.70 (m, 108 H, OCH_2CH_2), 3.50 (m, 6 H, ArCH_2Ar), 1.69 (s, 6 H, CH_2CH$_3$), 1.33 (s, 18 H, ArC(CH_3)$_2$), 1.22 (s, 27 H, ArC(CH_3)), 1.08 (s, 27 H, ArC(CH_3)), 0.71 (s, 27 H, C(CH_3)$_3$); ^{13}C NMR ($CDCl_3$) δ: 156.5, 153.3, 152.4, 149.8, 149.4, 146.8, 146.6, 142.4, 132.3, 126.7, 126.0, 125.7, 124.9, 113.9, 70.6, 69.9, 67.4, 61.5, 57.1, 38.0, 33.9, 32.3, 31.8, 31.7; IR ν: 3317, 1602, 1116 cm^{-1}; ESI HRMS *m/z* found: 2898.0798, calculated 2898.9174 (M-2H); M^{3+} 966.7254, calculated: 966.9777.

4.1.24 Attempted synthesis of 1,3,5-trimethoxyhexa(t-butyl)calix[6]arene-tris(acetamide) (**19**)



A solution of 1,3,5-trimethoxyhexa(4-t-butyl)calix[6]arene, **17**, (1.23 g, 1.2 mmol) in anhydrous THF (10 ml) was added to a suspension of NaH (0.1675 g, 4.135 mmol, 60% dispersed in mineral oil) in anhydrous THF (40 ml). The reaction was left to stir for 20 minutes at room temperature. Bubbling was observed and after 20 minutes the solution was clear yellow. A solution of bromoacetamide (1.009 g, 7.275 mmol) in anhydrous THF (5 ml) was introduced to the mixture and left to reflux for 24 hours. The solvent was then removed under pressure to leave a white powder. The residue was dissolved in CH_2Cl_2 (50 ml) and washed with distilled water (50 ml). The organic layer was isolated and the solvent

evaporated under pressure resulting in a white solid precipitate. The crude product was subjected to column chromatography but analysis was inconclusive.

4.2 Planar bilayer experiments

Planar lipid bilayer recordings were carried out as described in Hardy (Hardy, 2001). Pure synthetic lipids (Avanti Polar Lipids, Birmingham, AL USA) were dispersed in chloroform and stored at -70^{o}C under nitrogen. Lipid bilayers were formed from a dispersion of 15 mg/ml 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE) and 15 mg/ml 1-palmitoyl-2-oleoyl phosphatidylserine (POPS) in *n*-decane, which was drawn across a 0.25 mm diameter hole in a polystyrene cup separating two solution filled chambers, designated *cis* and *trans*. The *cis* chamber (to which compounds were added) was held at ground, and the *trans* chamber was clamped at -50mV using a Warner PC501A patch clamp amplifier equipped with a 10GB (10GΩ) bilayer headstage (Warner Instruments). The sign of the membrane potential refers to the *trans* chamber, and currents are defined as positive when cations flow from *trans* to *cis*. Transmembrane currents were low pass filtered at 500Hz (4 pole Bessel) digitised at 10 KHz and recorded directly to disk via a CED Micro 1401 Mark II AD interface. Membrane capacitance was measured by differentiating a triangular wave input of 0.2 kHz. Only bilayers that had a resting conductance of less than 10 pS and an initial capacitance of at least 150 pF were used. Unless otherwise stated bilayers were bathed in symmetrical solutions containing 150 mM NaCl; 10mM HEPES; 1 mM EGTA; 1.05 mM $CaCl_2$; 1mM $MgCl_2$; 50 μM free Calcium. All recordings were made at room temperature. Recordings were analysed off line using win EDR v2.3.9 software (Strathclyde electrophysiological software). Maximum current amplitudes were determined from the peaks of Gaussian functions fitted to amplitude histograms.

Chapter 5

5.1 Conclusions

Currently, our understanding of transmembrane ion transport relies on the few high resolution crystal structures such as cation selective proteins (Zhorov 2004), KcsA channels (Doyle 1998), MthK (Jiang 2002), the Mg^{2+} transporter MgtE (Hattori 2007), Na^{+}/K^{+} channels (Shi 2006; Gyrup, 2007) and an acid-sensing ion channel, ASICl (Jasti 2007). These structures in turn can act as models for other, less well-characterised, channel proteins such as the K^{+}-selective region of the protein product of the hERG gene (Farid 2006). Despite a vast amount of research there is still a lack of knowledge of ion transport mechanism and gating processes.

Synthetic ion channel models allow individual aspects of transmembrane transport to be probed, in particular the structural changes that can alter specificity or transport rate (Hall 1999). Rigid macrocycles have also been employed in cation channel mimetic compounds with calixarenes, in particular calix[4]arene, being seen as potential ion selective filters around which channel frameworks can be constructed (Iqbal 2007; de Mendoza 1998). Furthermore, it has been suggested that *1,3-alt* calix[4]arenes exhibit enhanced ionophoricity (Baklouti 2006).

Previous work in this group indicated that an oxacalix[3]arene derivative has sodium selectivity (Cragg 1999b) and has similarities in structure with the ion selective filters in natural channels. Initially this project aimed to prepare anion selective channels based on azacalix[3]arenes due to their potential to form charged quarternary ammonium derivatives that may attract anions. Attempts to synthesise tris(N,N-diethylacetamide) derivatives of *p*-

methyl-, *p*-t-butyl- and *p*-phenyl(*N*-benzyl)azacalix[3]arene using the same method as has been successfully applied to the oxacalix[3]arenes (Takemura 1992; Matsumoto 1995; Cragg 2005) gave inconclusive results. Subsequent cleavage to prepare the tris(acid) derivatives, from which membrane-spanning substituents would be appended, was also unsuccessful.

Known compounds *p*-methyl- and *p*-t-butyl(*N*-benzyl)azacalix[3]arenes were successfully synthesised and their identification confirmed by NMR and mass spectrometry. The novel *p*-phenyl(*N*-benzyl)azacalix[3]arenes was also prepared by this route and fully characterised. A different strategy was then adopted in the synthesis of ion channel model compounds.

Triton-X100® is an interesting molecule with all the properties necessary to form a pore as it is long enough to span a cell membrane. Instead of going through the amide and carboxylic acid derivatives, and then attaching a polyether chain, Triton could be attached straight onto the calixarenes through the phenolic group. To test the idea a sample of oxacalix[3]arene was treated with Triton-X100® iodide, prepared from the tosylate. The results indicated that the monosubstituted oxacalix[3]arene could be prepared using Loiseau's method (Loiseau 2004).

The oxacalix[3]arenes are conformally flexible which made it difficult to attach Triton-X100® to all three phenolic groups. The bridging oxygen atoms in the oxacalix[3]arene can form hydrogen bonds with the rotating phenolic protons which could affect the efficiency of the reaction. ^{1}H NMR gave two aromatic signals, at 7.15 and 7.01 ppm, integrating to a 1:2 ratio implying that one of the three phenolic groups had been substituted. The expected mass for this derivative (*m/z* found: 1245.7703) was detected by mass spectrometry. The same methodology was used to synthesise an oxacalix[3]arene with a triethelene glycol tosylate monomethyl ether substituent. The product was characterised by ^{1}HNMR and mass

spectrometry (*m/z* found: 745.4296 $[M + Na]^+$). No results were obtained with the azacalix[3]arenes using this method. The successful synthesis of 4-t-butyloxacalix[3]arene$(OH)_2$Triton-X100® suggested that the nitrogen bridgeheads were interfering with the successful coupling of lower rim substituents. Only one Triton-X100® had attached to the ring suggesting that there could also have been a problem of steric bulk. Monosubstitution also occurred when triethylene glycol monomethyl ether was attached to the oxacalix[3]arene, possibly for the same reason.

Calixarenes have potential as filters and should exhibit less steric interference as they have no bridging oxygens or nitrogens. It was decided to concentrate on the most well known members of the calixarene class, the calix[4]arenes (Iqbal 2007a) and calix[6]arenes (Böhmer 1995). The calix[6]arenes retain the threefold symmetry present in the aza- and oxacalix[3]arenes but require careful derivatization. They are more flexible than the expanded calix[3]arenes which made the synthesis of the correct conformer a challenge. The calix[4]arenes may be frozen out in the *cone* or *1,3-alternate* conformers but lack the threefold symmetry that may be essential for cation selectivity.

Triton-X100® iodide was reacted with calix[4]arene under the same conditions as the oxacalix[3]arene experiment and the results (^{1}H NMR and *m/z*: 1929.2877 $[M + Na]^+$) showed that the compound incorporating two substituents in the 1 and 3-positions had been synthesized. Bilayer experiments were not successful. It was proposed that the compound bound Na^+, as indicated by mass spectrometry, and consequently would not allow the cations to cross the bilayer. The dimethoxy derivative was prepared successfully but in very low yield. The compound was tested in a phospholipid bilayer but no positive results were obtained.

The same method was applied in the synthesis of 4-t-butylcalix[6]arene$(OMe)_3$-$(Triton)_3$ with a reasonable yield of 42% with appropriate ^1H NMR and mass spectrometric data for the proposed compound. In this case 4-t-butylcalix[6]arene was methylated first on the three alternate hydroxyl groups in the lower rim. After methylation the Triton-X100® chains were attached, as the iodide derivatives, to the rest of the lower rim hydroxyl groups.

Having prepared these novel calixarene derivatives the next step was to determine if they had any activity in artificial phospholipid bilayers using electrophysiological testing methods. 4-t-Butylhomotrioxacalix[3]arene$(OH)_2$Triton inserted and the bilayer remained stable. Insertion in the lipid bilayer created a pore and allowed sodium ions to pass through.

In experiments with 4-t-butylcalix[6]arene$(OMe)_3(Triton)_3$ it was observed that several types of insertions, single and multiple, occurred in the bilayer. It was hypothesised that the multiple insertions were due to micelle forming and insertion of portions of these micelles. When small concentrations of this compound were added single channels tend to be formed and at higher concentrations a bigger step is observed and this is explained by the micelle behaviour of the compound. Experiments with K^+ in place of Na^+ showed no conductance even though the calix[6]arene cavity should be able to accommodate a hydrated K^+ ion. The reason for this lies in the sixfold octahedral coordination geometry for Na^+, observed in many systems and proposed for protein-based ion channels (Jasti 2007), rather than the eightfold cubic environment favoured by K^+ in the crystal structure of KcsA (Doyle 1998). Similar observations were made by MacKinnon (Doyle 1998) to explain why the potassium channel-forming protein, KcsA, selects potassium over the smaller sodium cations.

The synthesis of the tetrakis(triethyleneglycol monomethyl ether)calix[4]arenes has been described and the *1,3-alt* conformer compound shown by NMR to bind Na^+. Lipid bilayer experiments confirmed that the compound functions as an artificial transmembrane ion channel in the presence of Na^+ but not K^+. In this case evidence from NMR experiments and molecular simulations suggests that Na^+ is in its preferred six-coordinate environment interacting with two water molecules, two phenolic oxygens and two aromatic rings. This is consistent with the current thinking on cation channel mechanisms that indicate that conductance occurs as water linked chains of partially dehydrated cations pass through channel selectivity filters (Lawal 2006; Doyle 2004; Noskov 2004). This calix[4]arene derivative is therefore ideally suited as a synthetic ion channel for Na^+ (Lawal 2006).

This work focused on planar lipid bilayer techniques to investigate transmembrane ion transport. To determine if the compounds are active in cells it would be necessary to extend the project to the use of the patch clamp technique however time did not allow for this.

In conclusion, a number of novel calixarene derivatives were synthesised, characterised and tested for ion transport and selectivity on phospholipid bilayers. The project demonstrated that it is possible to design artificial sodium selective ion channel mimics and revealed a link between cations' preferred coordination environments and channel selectivity.

References

Aidley, D.J., Stanfield, P.R., *Ion channels*, Cambridge University Press, 1996

Andavan, G. S. B., Lemmens-Gruber, R., Voltage-gated sodium channels: mutations, channelopathies and targets, *Curr. Med. Chem.*, **2011**, *18*, 377

Anderson, C. R., Stevens, C.F., Voltage clamp analysis of acethylcholine-produced end plate current fluctuation at the frog neuromuscular junction, *J. Physiol*., **1973**, *235*, 655

Andrikopoulos,P., Fraser, S. P., Patterson, L., Ahmad, Z., Burcu, H., Ottaviani, D., Diss, J. K. J., Box, C., Eccles, S. A., Djamgoz, M. B. A., Angiogenic functions of voltage-gated channels in human endothelial cells: modulation of vascular endothelial growth factor (VEGF) signalling, *J. Biol. Chem.*, **2011**, *286*, 16846

Althaus, E. W., Outten, C. E., Olson, K. E., Cao, H., O'Halloran, T. V., The ferric uptake regulation (Fur) repressor is a zinc metalloprotein. *Biochem.* **1999**, *38*, 6559

Aoki, T., Hirano, M., Takeuchi, Y., Kobayashi, T., Yanagida, T., Ide, T., Single channel properties of lysenin in artificial lipid bilayer and their applications to biomolecule detection, *P. Jpn. Acad. B-Phys.*, **2010**, *88*, 920

Arduini, A., Ghidini, E. Pochini, R., Ungaro, R,,. Andreetti, D.G., Calestani, G., Ugozzoli, F., Molecular inclusion in functionalized macromolecules 15. Para-tert-butyl[4]arene tetra-acetamide- A new strong receptor for alkali cations, *J. Inclusion Phenom.* **1988**, *6*, 119

Arnaud-Neu, F., Barret, G., Harris, S.J., Owens, M. A., Schwing-Weill, M.-J., Schwinte, P., Cation complexation by chemically modified calixarenes. 5. Protonation constants for calixarene carboxylates and stability constants of their alkali and alkaline-earth complexes, *Inorg. Chem*., **1993**, 2644

Asfari, Z., Wenger, S., Vicens, J. Calixcrowns and related molecules. *Pure & Appl. Chem.* **1995**, *67*, 1037.

Ashcroft, F.M. *Ion Channels and Disease*, Academic Press, San Diego **1999**.

Ashfield, R., Gribble, F. M., Ashcroft, S. J. H., Ashcroft, F. M., Identification of the high-affinity tolbutamide site on the SUR1 subunit of the K-ATP channel. *Diabetes* **1999**, *48*, 1341

Atwood, J.L., Holman, K.T., Steed. J. W. Laying traps for elusive prey: Recent advances in the non-covalent binding of anions. *Chem. Commun.* **1996**, 1401

Baklouti, L., Harrofield, J., Pulpoka, B., Vicens, J., 1,3-Alternate, the smart conformation of calix[4]arenes, *Mini-Rev. Org. Chem.*, **2006**, *3*, 355

Bayer, A. Uber die Verbindungen der Aldehyde mit den Phenolen. *Ber. Dtsch. Chem. Ges.*, **1872**, *5*, 25

Becucci, L., Guidelli, R. Kinetics of channel formation in bilayer lipid membranes (BLMs) and tethered BLMs: Monazomycin and melittin. *Langmuir*, **2007**, *23*, 5601.

Beer, P. D., Chen, Z., Goulden, A. J., Graydon, A., Stokes, S. E., Wear, T. J., Selective electrochemical recognition of the dihydrogen phosphate anion in the presence of hydrogen sulfate and chloride ions by new neutral ferrocene anion receptors*, J. Chem. Soc. Chem, Comm*., **1993**, 1834

Beer, P. D., Drew, M. G. B., Leeson, P. B., Ogden, M.I., *J. Chem. Soc, Dalton Trans.* **1995**, 1273

Beer, P.D. and Gale, P.A., Anion recognition and sensing: The state of the art and future perspectives, *Angew. Chem. Int. Ed.* **2001**, *40*, 486

Bernardi, P., Mitochondrial transport of cations: Channels exchangers, and permeability transition, *Pharm. Rev.* **1999**, *79,* 1127

Bernstein, J., Untersuchungen zur Thermodynamik der bioelecktrischen Strome. *Erster Thiel Arch ges Physiol*, **1902**, *92*, 521

Bohmer, V., Calixarenes, macrocycles with (almost) unlimited possibilities, *Angew. Chem. Int. Ed. Engl.***1995**, *34*, 713

Boon, J. M. and Smith, B. D., Synthetic membrane transporters, *Cur. Opin. Chem. Biol.* **2002**, *6*, 749

Bott, S. G., Coleman, A. W., Atwood, J.L. Inclusion of both cation and neutral molecule by a calixarene-structure of the [para-tert-butylmethoxycalix[4]arene-sodium-toluene]$^{+}$ cation. *J. Am. Chem. Soc,* **1986**, *108*, 1709

Brzozka, Z., Lammerink, B., Reinhout, D.N., Ghidini, E., Ungaro, R., Transduction of selective recognition by preorganized ionophores; K+ selectivity of the different 1,3-diethoxycalix[4]arene crown ether conformers, *J.Chem. Soc., Perkin Trans, 2*, **1993**, 1037

Buhlmann, P., Nishizawa, S., Xiao, K. P., Umezawa, Y., Strong hydrogen bond-mediated complexation of $H_2PO_4^-$ by neutral bis-thiourea hosts, *Tetrahedron*, **1997**, *53*, 1647

Burkhart, B. M., Gassman, R. M., Langs, D. A., Pangborn, W.A. and Duax, W. L., Heterodimer formation and crystal nucleation of gramicidin D*, Bioyphys. J.* **1998**, *75*, 2135

Cannon, S. C., George, A. L., Pathophysiology of myotonia and periodic paralysis, in *Diseases of the nervous system: clinical neuroscience and therapeutic principles*. Eds., Asbury, A. K., McKhann, G. M., McDonald, W. I., Goadsby, P. J., McArthur, J. C., Cambridge University Press, Cambridge, **2002**

Casnati, A., Minari, P., Pochini, A., Ungaro, R., Conformational freezing of p-tert-butylcalix[6]arene in the cone structure by selective functionalization at the lower rim: synthesis of new preorganized ligands, *J.Chem. Soc., Chem. Commun*., **1991**, 1413

Catterall W.A., From ion currents to molecular mechanisms: the structure and function of voltage-gated sodium, *Neuron,* **2000**, *26*, 13

Catterall, W. A., Na^+ channel mutations and epilepsy, *Epilepsia*, **2010**, *51*, 59

Cestele, S. Catterall, W. A., Molecular mechanisms of neurotoxin action on voltage-gated sodium channels, *Biochimie* **2000**, *82*, 883

Chang, S.-K., Hamilton, A. D., Molecular recognition of biologically interesting substrates: Synthesis of an artificial receptor for barbiturates employing six hydrogen bonds, *J. Am. Chem. Soc.* **1988**, *110*, 1318

Chen, X., Whissell, P., Orser, B. A., MacDonald, J. F., Functional modifications of acid-sensing ion channels by ligand-gated chloride channels, *PLoS ONE ,* **2011**, 7, 21960

Chirakul, P., Hampton, P. D., Duesler, E. N., Synthesis and crystal structure of an *O*-silylated hexahomotriazacalix[3]arene. *Tetrahedron Lett.* **1998**, *39*, 5473.

Chirakul, P., Hampton, P. D., Bencze, Z., A convergent synthesis of hexahomotriazacalix[3]arene macrocycles, *J. Org. Chem.* **2000**, *65*, 8297

Choi, K., Hamilton, A. D., Macrocyclic anion receptors based on directed hydrogen bonding interactions, *Coord. Chem. Rev*. **2003**, *240*, 101

Clark, T. E., Makha, M., Raston, C. L., Sobolev, A. N., Supersized bilayers based on an *O*-alkyl substituted calix[4]arene. *CrystEngComm.* **2006**, *8*, 707

Cobben, P. L. H. M., Egberink, R. J. M., Bomer, J. G., Bergveld, P., Verboom, W., Reinhoudt, D. N., Transduction of selective recognition of heavy metal ions by chemically modified field effect transistors (CHEMFETs), *J. Am. Chem. Soc*., **1992**, 114, 10573

Cole, K.S., Rectification and induction in the squid giant axon, *J. Gen. Physiol.*, **1941**, *25*, 29

Collins, F. S., Cystic-fibrosis - molecular biology and therapeutic implications. *Nature* **1993**, *256,* 774

Cragg, P. J., Drew, M. G. B., Steed, J. W. Conformational preferences of O-substituted oxacalix[3]arenes. *Supramol. Chem.,* **1999a**, *11*, 5

Cragg, P. J., Allen, M. C., Steed, J. W. A 'toothpaste tube' model for ion transport through trans-membrane channels, *Chem. Commun.*, **1999b**, 553

Cragg, P. J., Artificial transmembrane channels for sodium and potassium, *Sci. Prog.,* **2002**, *85*, 219

Cragg, P. J., *A Practical Guide to Supramolecular Chemistry*, John Wiley & Sons, Ltd., Chichester, **2005**

Colston, M. J., Hailes, H. C., Stavropoulos, E., Hervé, A. C., Goodworth, K. J., Hill, A. M. , Jenner, P., Hart P. D., Tascon, R. E., Antimycobacterial calixarenes enhance innate defense mechanisms in murine macrophages and induce control of *Mycobacterium tuberculosis* infection in mice, *Infect. Immun*., **2004**, 72, 6318.

Cornforth, J. W., Hart, P. D. , Rees R. J. W., Stock, J.A., Antituberculous effect of certain surface-active polyoxyethylene ethers in mice, *Nature* **1951**, *168*, 150

Cornforth, J. W., Hart, P. D., Nicholls, G. A., Rees, R. J. W., Stock, J. A., Antituberculous effects of certain surface-active polyoxyethylene ethers, *Brit. J. Pharmacol.* **1955**, 10, 73

Darbost, U., Giorgi, M., Reinaud, O., Jabin, I., A novel C_{3v}-symmetrical calix[6](aza)-cryptand with a remarkably high and selective affinity for small ammoniums, *J. Org. Chem.*, **2004**, *69*, 4879

Davis, J. T., Recent advances in the transmembrane transport of anions, *Chem. Soc. Rev.*, **2010,** *39*, 3843

Delamaza, A., Parra, J. L., Vesicle-micelle structural transition of phosphatidylcholine bilayers and Triton x-100, *Biochem. J.*, **1994**, *303*, 907

de Mendoza, J., Cuevas, F., Prados, P., Meadows, E. S., Gokel, G. W., A synthetic cation-transporting calix[4]arene derivative active in phospholipid bilayers, *Angew. Chem. Int. Ed. Engl.* **1998**, *37*, 1534

Dilly, S., Lamy C., Marrion, N. V., Liegeois, J. F., Seutin, V., Ion channel modulators: more diversity than previously thought, *Eur. J. Chem. Biol.*, **2011**, 12, 1808

Dock, A. C., Moras, D., Behr, J. P., and Lehn, J. M. Structure of the dimeric complex formed by a tetrafunctional macrocyclic polyether with the potassium ion, $2C_{24}H_{44}N_4O_{10}{\cdot}3KBr.7H_2O$ - a model of the transmembrane channel, *Acta Cryst. C,* **1983**, *39*, 1001

Doyle, D. A., Cabral, J. M., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., MacKinnon, R., The structure of the potassium channel: Molecular basis of K^+ conduction and selectivity, *Science*, **1998**, *280*, 69.

Doyle, D. A. Molecular insights into ion channel function, *Mol. Membr. Biol.* **2004**, *21*, 221

Duax, W. L., Griffin, J. F., Langs, D. A., Smith, G. D., Grochulski, P., Pletnev, V., Ivanov, V., Molecular structure and mechanisms of action of cyclic and linear ion transport antibiotics. *Biopolymers* **1996**, *40*, 141

Duax, W.L., Pletnev, V., Burkhart, B.M., Mechanism of ion transport and gating in gramicidin nanotubes, *J. Mol. Structure,* **2003**, *647*, 97

Duzler, R., Campbell , E. B., Cadene, M.,Chait, B.T., MacKinnon, R., X-Ray structure of a ClC chloride channel at 3.0 Å reveals the molecular basis of anion selectivity, *Nature*, **2002,** *415*, 287

Dutzler R., Campbell , E. B. MacKinnon, R. Gating the selectivity filter in CIC chloride channels. *Science* **2003**, *300*, 108

Edgerton, G. B., Blumenthal, K. M., Hanck, D. A. Inhibition of the activation pathway of the T-type calcium channel Ca(V)3.1 by ProTxll. *Toxicon* **2010**, *56*, 624

Edwards, G. Weston, A. H. The pharmacology of ATP-sensitive potassium channels. *Ann. Rev. Pharmacol. Toxicol.* **1993**, *33*, 597

Eggers, P. K., Fyles, T. M., Mitchell, K. D. D., Sutherland, T. J., Ion channels from linear and branched bola-amphiphiles, J. Org. Chem. 2003, 68,150

Elliott, A. A., Elliott, J. R. Characterization of TTX-sensitive and TTX-resistant sodium currents in small cells from adult rat dorsal root ganglis. *J. Physiol. (Lond.)* **1993**, *463*, 39

Farid, R., Day. T., Friesner, R. A. Pearlstein, R. A., New insights about HERG blockade obtained from protein modelling, potential energy mapping, and docking studies. *Bioorg. Med. Chem.* **2006**, *14*, 3160

Felix, V., Matthews, S. E., Beer, P. D., Drew, M. G. B., Selectivity of calix[4]tubes towards metal ions: A molecular dynamics study, *Phys. Chem. Chem. Phys.* **2002**, *4*, 3849

Ferdani, R., Pajewski, R., Djedovic, N., Pajewska, J.,Schlensinger, P.H. and Gokel, G.W., Anion transport in liposomes responds to variations in the anchor chains and the fourth amino acid of heptapeptide ion channels, *New J. Chem.* **2005**, *29*, 673

Fisher, E., Einfluß der Configuration auf die Wirkung der Enzyme, *Ber. Deutsch, Chem. Ges,* **1894**, 27, 2985

Fishman, H.M., Relaxation spectra of potassium channel noise from squid axon membrane, *Proc. Nat., Acad. Sci*., **1973**, 70, 876

Fletcher, D. I., Ganellin, C. R., Piergentili, A., Dunn, P. M., Jenkinson, D. H., Synthesis and pharmacological testing of polyaminoquinolones as blockers of the apamin-sensitive Ca^{2+}-activated K^+ channel (SKCa), *Bioorg. Med. Chem.* **2007**, *15*, 5457

Fuhrhop, J.-J., David, H.-H., Mathieu, J. Liman, U., Winter, H-J., Boekema, E., Bolaamphiphiles and monolayer lipid membranes made from 1,6,19,24-tetraoxa-3,21-cycloheax tricontadiene-2,5,20,23-tetrone., *J. Am. Chem. Soc.* **1986**, *108*, 1785.

Fuhrhop, H. J., David, H. H., Mathieu, J., Liman, L., Winter, H. J., In calixarenes for separations, Eds. Lumetta, G. J., Rogers, R. D., Gopalan, A. S., ACS Symposium Series Washington DC, **2000**, p 195.

Fyles, T. M., Bilayer menbranes and transporter models, *Curr. Opin. Chem. Biol.* **1997**, *1*, 497.

Fyles, T.M., Loock, D., Zhou,X., Ion channels based on bis-macrocyclic bolaamphiphiles: effects of hydrophobic substitutions, Can. J. Chem. **1998**, *76*, , 1015

Fyles, T. M., Hu, C.-W., Luong, H., Solid-phase synthesis of oligoester ion channels, *J. Org. Chem.*, **2006,** *71*, 8545

Gabriel, S. E., Clarke, L. L., Boucher, R. C., Stutts, M. J., CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship *Nature*, **1993**, *363*, 263

Gale, P. A., Sessler, J. L., Allen, W. E., Tvermoes, N. A., Calix[4]pyrroles: C-rim substitution and tenability of anion binding strength, *Chem. Commun.* **1997**, *7*, 665

Gallin, E. K., calcium and voltage activated potassium channels in human macrophages, *Biophys. J.*, **1984**,46, 821

Gao, W., Starkov, V. G., He, Z., Wang, Q-H., Tsetlin, V. I., Utkin, U. N., Lin, Z-J., Bi, R.-C., Functions, structures and Triton X-100 effect for the catalytic subunits of heterodimeric phospholipases A2 from *Vipera nikolski*i venom, *Toxicon*, **2009,** 54, 709

Geiger, D., Scherzer, S., Mumm, P., Stange, A., Marten, I., Bauer, H., Ache, P., Matschi, S. , Liese, A., Al-Rasheid, K. A. S., Romeis, T., Hedrich, R., Activity of guard cell anion channel SLAC1 is controlled by drought-stress signalling kinase-phosphatase pair. *Proc. Nat. Acad. Sci. USA,* **2009**, *106*, 21425

George, A. L., Inherited disorders of voltage-gated sodium channels, *J. Clin. Invest.*, **2005**, *115*, 1990

Ghosh, S., Bhattacharjee, A., Banerjee, J., Manna, S., Bhatraju, N. K., Verma, M. K., Das, M. K., Electrical Noise in Cells, Membranes and Neurons, Complex Dynamics in Physiology Systems: from Heart to Brain, Springer Science, 2009, 255

Graf E., Lehn J.-M., Anion cryptates – highly stable and selective macrotricyclic anion inclusion complexes *J. Am. Chem. Soc.*, **1976**, *98*, 6403.

Groenen, L.C., Ruel, B. H. M., Casnati, A., Timmerman, P., Verboom, M., Harkema, S., Pochini, A., Ungaro, R., Reinhoudt, D. N., Syn-1,2-dialkylated calix[4]arenes - general intermediates in the NAH/DMF tetraalkylation of calix[4]arenes *Tetrahedron Lett*. **1991**, *32*, 2675

Gokel, G.W., Murillo, O., Synthetic organic chemical models for transmenbrane channels, *Acc. Chem. Res*.,**1996,** *29*, 425

Gokel, G. W., Hydraphiles: design, synthesis and analysis of a family of synthetic, cation-conducting channels., *Chem. Commun.* **2000**, 1

Gokel, G. W., Ferdani, R., Liu J., Pajewski, R., Shabany,H., Uetrecht, P., Hydraphile channels: Models for transmembrane, cation-conducting transporters, *Chem. Eur. J.*, **2001**, *7*, 33

Gokel. G. W., Carasel, I. A., Biologically active synthetic ion transporters, *Chem. Soc. Rev*., **2007**, *36*, 378

Gyrup, C., Vuust, J. M., Morth, J. P., Nissen, P., Olesen, C., Oxvig, C., The structural basis of calcium transport by the calcium pump, *Nature,* **2007**, *450*, 1043

Gu, C., Barry, J., Function and mechanism of axonal targeting of voltage-sensitive potassium channels, *Prog. Neurobio.*, **2011**, *94*, 115

Gutsche, C. D., Kwang, H. N., Calixarenes 7. *p*-Phenylcalix[4]arene, *J. Org. Chem.*, **1982**, *47*, 2708

Gutsche, C. D., Bauer, L. J., Calixarenes.13. The conformational properties of calix[4]arenes, calix[8]arenes and oxacalixarenes, *J. Am. Chem. Soc*., **1985**, *107*, 6052

Gutsche, C. D., *Calixarenes*, Monographs in supramolecular chemistry; Ed., Stoddart, J. F., The Royal Society of Chemistry, Cambridge, **1989**, p 27

Gutsche, C. D., See, K. A., Calixarenes. 27. Synthesis, characterization, and complexation studies of double-cavity calix[4]arenes, *J. Org. Chem.,* **1992**, *57*, 4527

Hall, C. D, Kirkovits, G. J., Hall, A. C., Towards a redox-active artificial ion channel, *Chem. Commun.*, **1999**, 1897

Hamill, O. P., Marty, A., Neher, E., Sackmann, B., Sigworth, F. J., Improved patch – clamping techniques for high-resolution current recording from cells and cell-free membrane patches, *J. Physiol.*,**1981,** *391*, 85

Hampton, P. D., Bencze, Z., Tong, W. D., Daitch, C. E., A new synthesis of oxacalix[3]arene macrocycles and alkali-metal-binding studies. *J. Org. Chem.* **1994**, *59*, 4838

Hampton, P. D., Tong, W., Wu, S., Duesler, E. N., Synthesis, X-ray structure and alkali-metal binding properties of a new hexahomotriazacalix[3]arene, *J. Chem. Soc., Perkin Trans. 2* **1996**, 1127

Handel, P. H., George, T. F., 1/f Noise Inside a Faraday Cage, *AIP Conf. Proc.*, **2009**, 1129, 491

Hao, S., Bao, Y-M., Zhao, R. G., Wang, H.-S., Bi, J., An, L-J., Jiang, B., Effects of resibufogenin on voltage-gated sodium channels in cultured rat hippocampal neurons, *Neurosci. Lett*., **2011**, *501*, 112

Hardy, S. P., Ritchie, C., Allen, M. C., Ashley, R. H., Granum, P. E., *Clostridium perfringens* type A enterotoxin forms mepacrine-sensitive pores in pure phospholipid bilayers in the absence of putative receptor proteins, *Biochim. Biophys. Acta* , **2001**, *1515*, 38

Hayes, B. T., Hunter, R. F., Phenol formaldehyde and allied resins VI: Rational synthesis of a 'cyclic' tetranuclear *p*-cresol novolak. *J. Appl. Chem.*, **1958**, *8*, 743

Heinemann, S. H., Teriau, H., Stuhmer, W., Imoto, K., Numa, S., Calcium-channel characteristics conferred on the sodium channel by single mutations, *Nature* **1992**, *356*, 441

Hille, B., *Ionic channels of excitable membranes*, 2nd Ed. Sinauer Associates, Sunderland, MA, **1992**

Hladky, S. B., Haydon, D.A., Discreteness of conductance change in bimolecular lipid mambranes in presence of certain antibiotics, *Nature*, **1970**, *225*, 451

Hodgkin, A. L., Huxley, A. F., A quantitative description of membrane current and its application to conduction and excitation in nerve, *J. Physiol*., **1974**, 117, 500

Huang, X. Q., Lih, H., Cui, M., Fu, W., Yu, K. Q., Chen, K. X., Luo, X. M., Shen, J. H., Jiang, H. L., Simulating the interactions of toxins with K^+ channels. *Curr. Pharmaceut. Des.* **2004**, *10*, 1057

Hultzsch, K., Ring- Kondensate in Alkylphenolharzen, *Kunststoffe,* **1962**, *52*, 10

Hurd, D. D., Saxton, W. M., Kinesin mutations cause motor neuron phenotypes by disrupting fast axonal transport in *Drosophila Genetics* **1996**, *144*, 1075

Ikeda, A., Shinkai, S., On the origin of high ionophoricity of 1,3-alternate calix[4]arenes: a donor participation in complexation of cations and evidence for metal-tunneling through the calix[4]arene cavity, *J. Am. Chem. Soc.,* **1994**, 116, 3102

Iqbal, K. S. J., Cragg, P. J., Transmembrane ion transport by calixarenes and their derivatives, *Dalton Trans.*, **2007a**, 1, 26.

Iqbal, K. S. J., Allen, M. C., Fucassi F., Cragg, P. J., Artificial transmembrane ion channels from commercial surfactants, *Chem. Commun.*, **2007b**, 3951

Itzhak, N., Biali, S. E., selective functionalization of a single methylene bridge of a calix[6]arene, *J. Org. Chem.*, **2010**, *75*, 3437

Jasti, J., Furukawa, H., Gonzalez, E. B., Gouaux, E., Structure of acid-sensing ion channel 1 at 1.9A resolution and low pH, *Nature*, **2007**, *449*, 316

Jayasuriya, N., Bosak, S., Regen, S. L., Design, synthesis, and activity of membrane-disrupting bolaphiles, *J. Am. Chem. Soc.*, **1990**, *112*, 5845

Jentsch, T. J., Hubner, C. A., Fuhrmann, J. C., Ion channels: Function unravelled by dysfunction, *Nat. Cell Biol.,* **2004**, *6*, 1039

Jiang, Y., Lee., A., Chen, J., Cadene, M., Chait, B.T., MacKinnon, R., Crystal structure and mechanism of a calcium-gated potassium channel, *Nature*, **2002**, *417*, 515

Jiang, Y. , Lee, A., Chen, J. Y., Ruta, V., Cadene, M., Chait, B. T., MacKinnon, R., X-ray structure of a voltage-dependent K^+ channel, *Nature*, **2003**, *423*, 33

Jin, T., Photocontrol of Na^+ transport across a phospholipid bilayer containing a bisanthroylcalix[4]arene carrier, *Chem. Comm.*, **2000**, 1379

Jog, P. V., Gin, M. S., A light-gated synthetic ion channel, *Org. Lett.*, **2008**, *10*, 3693

Kaewtong, C., Pulpoka, B., Azacalix[3]arenes: chemistry and recent developments in functionalization for specific anion and cation recognition. *J. Incl. Phenom. Macro. Chem.* **2009**, *65*, 129

Karmazinova, M., Lacinova, L., Measurement of cellular excitability by whole cell patch clamp technique, *Physiol. Res.,* **2010**, *59*, S1

Kazmarek, L. K., Levitan, I. B., *Neuromodulation: The biochemical control of neuronal excitability* OUP, Oxford, **1987**

Kiriukhin, M. Y., Collins, K. D., Dynamic hydration numbers for biologically important ions, *Biophys. Chem.* **2002**, *99*, 155

Kobuke, Y., Nagatani, T., Transmembrane ion channels constructed of cholic acid derivatives *J, Org. Chem.* **2001**, *66*, 5094

Kramer, D. B., Zimelbaum, P. J., Long-QT syndrome, *Cardiol. Rev.*, **2011**, *19*, 217

Lankshear M. D. Cowley, A. R. Beer, P. D., Cooperative AND receptor for ion-pairs., *Chem. Commun.* **2006**, 612

Lawal, O., Iqbal, K. S. J., Mohamadi, A., Razavi, P., Dodd, H. T., Allen, M. C., Siddiqui, S., Fucassi F., Cragg, P. J., An artificial ion channel from calix[4]arene in the 1,3-alternate conformation, *Supramol. Chem*., **2009**, *21*, 55

Le Gac, S., Luhmer, M., Reinaud, O., Jabin, I. Self assembly via ionic interactions of calix[6]arene-based receptors displaying remarkable host-guest properties toward neutral guests, *Tetrahedron*, **2007**, *63*, 10721

Liang, X., Campopiano, D. J., Sadler, P. J., Metals in membranes, *Chem. Soc. Rev.*, **2007**, *36*, 968

Lehn, J.M., Supramolecular chemistry - scope and perspectives. Molecules, supermolecules and molecular devices (Nobel lecture), *Angew. Chem. Int. Ed.,* **1988**, *27*, 90

Lepore, B. W., Indic, M., Pham, H., Hearn, E. M., Patel, D. R., Van den Berg, B., Ligand-gated diffusion across the bacterial outer membrane, *Proc. Nat. Acad. Sci.*, **2011**, *25*, 10121

Licen, S., C. Coppola, C., D'Onofrio, J., Montesarchio, D., Tecilla, P., CyPLOS: a new family of synthetic ionophores, *Org. Biomol. Chem.*, **2009**, *7*, 1060

Linnertz, R., Wurm, A., Pannicke, T., Krugel, K., Hollbeorn, M., Hartig, W., Iandiev, I., Wiedemann, P., Reichenbach, A., Brinmann, A., Activation of voltage-gated Na^+ and Ca^{2+} channels is required for glutamate release from retinal glial cells implicated in cell volume regulation, *Neurosci.*, **2011**, *188*, 23

Llinares, M. J., Powel, D., Bowman-James, K., Ammonium based anion receptors, *Coord. Chem. Rev.*, **2003**, *240*, 57

Loiseau, F. A. Hill, A. M. and Hii, K. K., Multgram synthesis of well defined extended polyethylene glycol (PEG) chains, *J. Org. Chem.*, **2004**, 69, 639

Loiseau, F. A., Hill, A. M., Hii, K. K., Preparation of macrocyclon analogues: Calix[8]arenes with extended polyethylene glycol chains, *Tetrahedron*, **2007**, *63*, 9947

Calixarenes for separations, Eds. Lumetta, G.J., Rogers, R.D., Gopalan, A.C. S., **2000**, 195

Ma, S.-L., Qi, C.-M., Zhu, W.-X., Guo, Q.-L., Liu, Y.-C., Zhang, J., The synthesis of phenol-containing azamacrocycles and liquid membrane transports of alkali cations, *J. Mol. Structure,* **2005**, *733*, 77

MacKinnon, R. Potassium channels and the atomic basis of selective ion conduction (Nobel Lecture), *Angew. Chem. Int. Ed.* **2004,** *43*, 4265

Madhavan, N., Robert, E. C., Gin, M. S., A highly active anion-selective aminocyclodextrin ion channel. *Angew. Chem. Int. Ed,* **2005**, *44*, 7584

Mathie, A., Clarke, C. E., Ranatunga, K. M., Veale, E. L. What are the roles of the many different types of potassium channel expressed in cerebellar granule cells? *Cerebellum,* **2003**, *2*, 11.

Matile, S., Som A., Sorde,, N., Recent synthetic ion channels and pores, *Tetrahedron* **2004**, *60*, 6405

Matile, S., Jentzsch, A. V., Montenegro, J., Fin, A., Recent synthetic transport systems, *Chem. Soc. Rev.*, **2011**, *40*, 2453

Matsuda, H., Saigusa, A., Irisawa, H., Ohmic conductance through the inwardly rectifying K^+ channel and blocking by internal Mg^{2+}, *Nature*, **1987**, *325*, 156

Matsumoto, H., Nishio, S., Takeshita, M., Shinkai, S., Synthesis and ion selectivities of tri-amide derivatives of hexahomotrioxacalix[3]arene – remarkably large metal template effect on the ratio of cone vs partial –cone conformers. *Tetrahedron* **1995**, *51*, 4647

Matthews, S. E., Beer, P. D. Calixarene-based anion receptors *Supramol. Chem.* **2005**, *17*, 411

Maulucci, N., De Riccardis, F. Botta, C.B., Casapullo, A., Cressina,, E., Fregonese, M., Tecilla, P., Izzo, I., Calix[4]arene-cholic acid conjugates: a new class of efficient synthetic ionophores, *Chem. Comm.,* **2005**, 1354

Menger, D. S., Davis, D. S., Persichetti R. A., Lee, J. J., Synthetic flux-promoting compounds. Exceeding the ion transporting ability of gramicidin, *J. Am. Chem. Soc.* **1990,** *112,* 2451

Miah, M., Romanov, N. N., Cragg, P. J., Acid catalysed formation of hexahomooxacalix[3]arenes, *J. Org. Chem.*, **2002**, *67*, 3124

Mobashery, N., Nielsen, C., Andersen, O. S., The conformational preference of gramicidin channels is a function of lipid bilayer thickness, *Febs Lett*. **1997**, *412*, 15

Morzherin, Y., Rudkevich, D. M., Verboom, W., Reinhoudt, D. N., Chlorosulfonylated calix[4]arenes: precursors for neutral anion receptors with a selectivity for hydrogen sulphate *J. Org. Chem.,* **1993**, *58*, 7602

Mullins, L. J., The penetration of some cations into muscle, *J. Gen. Physiol.,* **1959**, *42*, 1013

Murakami, H., Shinkai, S., Metal-induced conversion of a closed receptor to an open receptor on a p-tert-butylcalix[4]arene diamide derivative - fluorescence detection of a molecular recognition process, *J. Chem. Soc., Chem. Commun.,* **1993**, 1533

Neher, E., Sakmann, B., Single channel currents recorded from membrane of denervated frog muscle-fibres. *Nature* **1976,** *260*, 799

Nikura, K., Anslyn, E. V., Azacalixarene:synthesis, conformational analysis, and recognition behaviour toward anions, *J. Chem. Soc., Perkin Trans*. **1999**, 2, 2769

No, K. H., Gutsche, C. D., Calixarenes. 8. Short, stepwise synthesis of *p*-phenylcalix[4]arene, *p*-phenyl-*p*-tert-butylcalix[4]arene, and derived products. *J. Org. Chem.* **1982**, *47*, 2713

Noda, M., Shimizu, S., Tanabe, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Miyata, T., Numa, S., Primary structure of *electrophorus electricus* sodium-channel deduced from CDNA sequence, *Nature,* **1984**, *312*, 121

Noskov, S. Y. Bernche, S. Roux, B., Control of ion selectivity in potassium channels by electrostatic and dynamic properties of carbonyl ligands, *Nature*, **2004**, *431*, 830

Okada, Y., Imendra. K. G., Miyazaki, T., Hotokezaka, H., Fujiyama, R., Toda, K., High extracellular Ca^{2+} stimulates Ca^{2+}-activated Cl^- currents in frog parathyroid cells through the mediation of arachidonic acid cascade, *PLOS 1* **2011**, *6*, e19158

Oluyomi, A.O., Seganish, J. L., Zavalij, P. Y., Davis, J. T., Membrane-active calixarenes: toward 'gating' transmembrane anion transport, *Tetrahedron* **2007**, *63*, 10743

Otsuka, H., Araki, K., Shinkai, S., Syntheses of all possible *O*-methylation products derivable from 5,11,17,23,29,35-hexa-tert-butylcalix[6]arene-37,38,39,40,41,42-hexol, *J. Org. Chem.*, **1994**, *59*, 1542

Otsuka, H., Araki, K., Shinkai, S., Syntheses of all possible calix[6]arene derivatives with MeO- and $ROCOCH_2O$- substituents and their metal binding, *Tetrahedron*,**1995**, *51*, 8757

Payandeh, J., Scheuer, T., Zheng, N., Catterall, W.A., The crystal structure of a voltage-gated sodium channel, *Nature,* **2011,** *475*, 353

Pajewski, R., Garcia-Medina, R., Brody, S. L., Leevy, W. M., Schlesinger P. H., Gokel, G. W., A synthetic chloride-selective channel that alters chloride transport in ephithelial cells, *Chem. Commun.,* **2006**, 329

Pascal, R., Synthesis and x-ray crystallographic characterization of a (1,3,5) cyclophane with three amide N-H groups surrounding a central cavity. A neutral host for anion complexation, *Tetrahedron. Lett.* **1986**, *27*, 4099

Pischalnikova, A. V., Sokolova O. S., The domain and conformational organization in potassium gated ion channels, *J. Neuroimmune Pharmacol.,* **2009**, 4, 71

Pohorille, A., Wilson, M.A., Unassisted and assisted ion transport across membranes: Insights from computer simulations. *Cell Mol. Biol. Lett.* **2001**, *6*, 369

Powell, H. M., Compound formation by molecular imprisonment, *Research*, **1948**, *1*, 353

Prakriya, M., Lewis, R. S., Separation and characterization of currents through store-operated CRAC channels and Mg^{2+} inhibited cation (MIC) channels, *J. Gen. Physiol.* **2002**, *119*, 487

Prawitt, D., Monteilh-Zoller, M. K., Brixel, L., Spangenberg, C., Zabel, B., Fleig, A., Penner, R., TRPM5 is a transient Ca^{2+} activated cation channel responding to rapid changes in $[Ca^{2+}]_i$ *Proc. Nat. Acad. Sci. USA*, **2003**, *100*, 15166

Purkerson, S. L., Baden, D. G., Fieber, L. A., Brevetoxin modulates neuronal sodium channels in two cell lines derived from rat brain, *Neurotoxicol.* **1999**, *20,* 909

Qu, Z., Yang, Z. Cui, N., Zhu, G., Liu, C., Xu, H., Chanchevalap, S., Shen,W., Li, Y., Jiang, C., Gating of inward rectifier K1 channels by proton-mediated interactions of N- and C-terminal domains, *J. Biol. Chem.*, **2000**, *41*, 31573

Reinhout, D.N., van Veggel, F.C.J.M., Complexation of neutral molecules by synthetic hosts, *Pure Appl. Chem*.**1993**, 65, 965

Robson, R. J., Dennis, E. A., Size, shape of non-ionic surfactant micelles-Triton X-100. *J. Phys. Chem.* **1977**, 81, 1075

Rostovtseva, T. K. Bashford, C. L. Lev, A. A. Pasternak, C. A., Triton channels are sensitive to divalent-cations and protons. *J. Membr. Biol.***1994**, *141,* 83

Ruben, L., Akins, C. D., Haghighat, N. G., Xue, L., Calcium influx *Trypanosoma brucei* can be induced by amphiphillic peptides and amines, *Mol. Biochem. Parasitol.,* **1996**, *81*, 191

Rudkevich, D. M., Intramolecular hydrogen bonding in calixarenes, *Chem. Eur. J*., **2000**, *6*, 2679.

Siddiqui, S., Walizadeh, M., Cragg, P. J., unpublished results, **2009**.

Sakmann, B., Naher, E., Patch clamp techniques for studying ion channels in excitable-membrane*, Ann. Rev. Physiol.*, **1984a** 45, 455

Sakmann, B., Trube, G., Conductance properties of single inwardly rectifying potassium channels in ventricular cells from guinea-pig heart, *J. Physiol.*, **1984b**, *347*, 641

Schlesinger, P. H., Ferdani, R., Liu, J., Pajewska, J., Pajewski, R., _Saito, M., Shabany, H., Gokel, G. W., SCMTR: A chloride-selective, membrane-anchored peptide channel that exhibits voltage gating, *J. Am. Chem.Soc.*, **2002**, *124*, 1848

Schlieper, P., De Robertis, E., Triton X-100 as a channel-forming substance in artificial lipid bilayer membranes, *Arch. Biochem. Biophys.,* **1977**, *184*, 204

Schmidtchen, F. P., Inclusion of anions in macrotricyclic quaternary ammonium salts, *Angew Chem*. *Int. Ed. Engl.,* **1977**, *16*, 720

Schmitt, P., Beer, P. D., Drew, M. G. B., Sheen, P. D., Calix[4]tube: A tubular receptor with remarkable potassium ion selectivity., *Angew. Chem. Int. Ed. Engl.* **1997**, *36*, 1840

Sengupta, D., Leontiadou, H., Mark, A. E., Marrink, S. J., Toroidal pores formed by antimicrobial peptides show significant disorder, *BBA Biomembranes* **2008**, *1778,* 2308

Shaya, D., Kreir, M., Robbins, R. A., Wong, S., Hammon, J., Brüggemann, A., Minor Jr., D. L., Voltage-gated sodium channel (Na_V) protein dissection creates a set of functional pore-only proteins, *Proc. Nat. Acad. Sci. U S A*, **2011**, *108*, 12313

Shi, N. Ye, S., Alam, A., Chen, L. and Jiang, Y., Atomic structure of a Na+- and K+-conducting channel, *Nature*, **2006**, *440*, 570

Shinlapawittayatorn, K., Du, X. X., Liu, H. Y., Ficker, E., Kaufman, E. S., Deschenes, I., A common SCN5A polymorphism modulates the biophysical defects on SCN5A mutations, *Heart Ryth.,* **2011**, *8*, 455

Siddiqui, S., Cragg, P. J., Design and synthesis of transition metal and inner transition metal binding calixarenes., *Mini-Rev. Org. Chem.* **2009**, *6*, 283

Siderov, V., Kotch, F. W., El-Kouedi, M., Davis, J.T., Toward artificial ion channels: self assembled nanotubes from calix[4]arene-guanosine conjugates, *Chem. Comm.,* **2000**, 2369

Simmons, H. E., Park, C. H., Macrobicyclic amines I. Out-in isomerism of (l,k+2)diazabicyclo[k.l.m]alkanes, *J. Am. Chem. Soc.*, **1968**, *90*, 2428

Sokolov, S., Kraus, R. L., Scheuer, T., Catterall, W. A., Inhibition of sodium channel gating by trapping the domain II voltage sensor with protoxin II, *Mol. Phamacol.* **2008**, *73*, 1020

Sokolov, S., Scheuer, T., Catterall, W. A., Depolarization-activated gating pore current conducted by mutant sodium channels in potassium-sensitive normokalemic periodic paralysis. *Proc. Nat. Acad. Sci. USA*, **2008**, *105*, 19980

Steed, J. W., Juneja, R. K., Burkhalter, R. S., Atwood, J. L., Synthesis of cationic organometallic calixarene hosts by direct metalation of the outer face, *J. Chem. Soc., Chem. Commun.*, **1994**, 2205

Steed, J. W., Atwood, J. L., *Supramolecular Chemistry* John Wiley & Sons, Ltd., Chichester, **2000**, p. 2

Stevens, C. F., Inferences about membrane properties from electrical noise measurements, *Biophys. J.*, **1972**, *12*, 1028

Swartz, K. J., Sensing voltage across lipid membranes, *Nature*, **2008,** *456,* 891

Takemura, H., Yoshimura, K., Inazu, T., Shinmyozu, T., Inazu, T., The first synthesis and properties of hexahomotriaza[3]arene, *Tetrahedron Lett*., **1992,** *33*, 5775

Takemura, H., Shinmyozu, T., Miyura, H., Khan, I. U., Inazu, T., Synthesis and properties of *N*-substituted azacalix[n]arenes, *J. Incl. Phenom. Macro. Chem*., **1994**, *19*, 193

Takemura, H., Shinmyozu, T., Inazu, T., Nitrogen-bridged macrocycles: synthesis, structures and inclusion phenomena, *Coord. Chem. Rev.* **1996**, *156,* 183

Takemura, H., Kozima, Y., Inazu, T., Synthesis of azacalix[4]arene betaine, *Tetrahedron Lett.*, **1999**, *40*, 6431

Takemura, H., Azacalixarenes: Synthesis, complexation and structures. *J. Incl. Phenom. Macro. Chem*., **2002**, *42*, 169

Takemura, H., Takahashi, A., Suga, H., Fukuda, M., Iwanaga, T., Synthesis of azacalixarenes through benzoxazine derivatives of phenols, *Eur. J. Org. Chem.,* **2011**, 3171

Tanaka, Y., Kobuke, Y. Sokabe, M., A nonpeptidic ion-channel with K^+ selectivity, *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 693.

Thurm, H., Fakler, B., Oliver, D., Ca^{2+} independent activation of BKCa channels at negative potentials in mammalian inner hair cells, *J. Physiol. London,* **2005**, *569*, 137

Tucker, S. J., Ashcroft, F. M., A touching case of channel regulation: the ATP-sensitive K^+ channel, *Cur. Opin. Neurobiol.*, **1998**, *8*, 316

Townsley, L. E., Tucker, W. A., Sham, S., Hinton, J. F., Structures of gramicidins A, B, and C incorporated into sodium dodecyl sulfate micelles, *Biochem.*, **2001**, *40***,** 11676

Triggle, D. J., The Pharmacology of ion channels: with particular reference to voltage-gated Ca^+ channels, *Eur. J. Pharmacol*., **1999**, 375, 311

Valiyaveettil, S., Enghersen, J. F. J., Verboom, W., Reinhoudt, D. N., Synthesis and complexation studies of neutral anion receptors, *Angew Chem. Int. Ed. Engl.,* **1993**, *32*, 900

Vanderburg, C. A., Inward rectification of a potassium channel in cardiac ventricular cells depends on internal magnesium ions, *Proc. Nat. Acad. Sci. USA,* **1987**, *84*, 2560

Velluto, D., Gasbarri, C., Angelini, G. Tontana, A., Use of simple kinetic and reaction-order measyrements for the evaluation of the mechanism of surfactant-liposome interactions, *J. Phys. Chem.* **2011**, *115*, 8130

Venosa, R. A., Resting and action potentials under hypotonic conditions, unlike Na^+ pump activity, depend only on the alteration of intracellular [Na^+] and [K^+] in frog skeletal muscle, *J. Exp. Biol.,* **2011**, *214*, 858

Verboom, W., Datta, S., Asfari, Z., Harkema, S., Reinhoudt, D. N., Tetra-*O*-alkylated calix[4]arenes in the *1,3-alternate* conformation, *J. Org. Chem*., **1992**, *5,* 5395

Vicens, J., Bohmer, V., *Calixarenes: A versatile class of macrocyclic compounds*, Kluwer Academic Press, Dordrecht, **1991**, p. 3

Vogtle, F., *Supramolecular chemistry*, Wiley, New York, **1991**, p. 1

Wade, D., Boman, A., Wahlin, B., Drain, C. M., Andreu, D., Bohman, H. G., Merrifield, R. B., All-D amino acid-containing channel-forming antibiotic peptides, *Proc. Nat. Acad. Sci. USA,* **1990**, *87*, 4761

Waldmann, R., Champigny, G., Bassilana, F., Heurteaux, C., Lazdunski, M., A proton-gated cation channel involved in acid sensing, *Nature,* **1997**, *386,* 173

Wang, S. Y., Wang, G. K., Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins, *Cellular Signal.* **2003**, *15*, 151

Wang, W., Li, R., Gokel, G. W., Membrane-length amphiphiles exhibiting structural simplicity and ion channel activity, *Chem. Eur. J.*, **2009**, *15*, 10543

Wiegand, G., Neumaier, K.R., Sackmann, E., Fast impedance spectroscopy: General aspects and performance study for single ion channel measurements, *Rev. Sci. Instr.*, **2000,** *71*, 2309

Williams, A. J., in *Ion Channels, a Practical Approach*, Ed., R. H. Ashley, IRL/OUP, Oxford, **1995**, p. 43

Wilson, M. A., Pohorille A., Mechanism of unassisted ion transport across membrane bilayers, *J. Am. Chem. Soc.,* **1996**, *118*, 6580

Woolley, G. A., Lougheed T., Modeling ion channel regulation, *Cur. Opin. Chem. Biol.,* **2003**, *7*, 710

Worm, K., Schmidtchen, F. P., Molecular recognition of anions by zwitterionic host molecules in water, *Angew, Chem, Int. Ed. Engl.,***1995**, *34*, 65

Wright, A. J. Matthews, S. E., Fisher, W. B., Beer, P. D., Novel resorcin[4]arenes as potassium-selective ion-channels, *Chem. Eur. J.,* **2001**, *7*, 3474

Yagi, K. Khoo, S. B. Sugawara, M., Sakaki, T., Shinkai, S., Odashima, K., Umezawa, Y., Channel mimetic sensing membranes for alkali metal cations based on oriented monolayers of calixarene esters, *J. Electroanal. Chem.*, **1996**, *401*, 65.

Yan, J. S., Aldrich, R. W., LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium, *Nature*, **2010**, *466*, 513

Yang, S., Carrasquer, G., Effect of melittin on ion transport across cell membranes, *Acta Pharm. Sinica*, **1997**, *18*, 3

You, L., Ferdani, R. Gokel, G. W., Chloride ion efflux from liposomes is controlled by sidechains in a channel-forming heptapeptide. *Chem. Commun.* **2006**, 603

Youxing, J., Lee, A., Chen, J., Ruta, V., Cadene, M., Chait, B. T., MacKinnon, R., X-Ray structure of a voltage-dependent K^+ channel, *Nature*, **2003**, *6935*, 33

Yu, F. H., Yarov-Yarovoy, V., Gutman, G. A., Catterall, W. A., Overview of molecular relationships in the voltage-gated ion channel super family, *Pharmacol. Rev.,* **2005**, *57*, 387

Zhang, M., Liu, X.S., Diochot, S., Lazdunski, M., Tseng, G. N., APETx1 from sea anemone *Anthopleura elegantissima* is a gating modifier peptide toxin of the human ether-a-go-go-related potassium channel, *Mol. Pharmacol.* **2007**, *72*, 259

Zhang, D., Thimmapaya, R., Zhang, X.-F., Anderson, D. J., Baranowski, J. L., Scanio, M., Perez-Medrano, A., Peddi, S., Wang, Z., Patel, J. R., DeGoey, D. A., Gopalakrishnan,M., Honore, P., Yao, B. B., Surowy, C. S., KCNQ2/3 openers show differential selectivity and site of action across multiple KCNQ channels, *J. Neurosci. Meth.*, **2011**, *200*, 54

Zhorov, B. S., Tikhonow, D. B., Potassium, sodium, calcium and glutamate-gated channels:pore architecture and ligand action, *J. Neurochem.*, **2004**, *88*, 782

Zhou, Y. F., Morais-Cabral, J. H., Kaufman, A., MacKinnon, R., Chemistry of ion coordination and hydration revealed by a K^+ channel-Fab complex at 2.0 Angstrom resolution, *Nature,* **2001**, *414*, 43

Zhou, Y., Xia, X.-M., Lingle, C. J., Cysteine scanning and modification reveal major differences between BK channels and Kv channels in the inner pore region*, Proc. Nat. Acad. Sci. USA*, **2011**, *108*, 12161

Zinke, A., Ziegler, E., Zur Kenntnis des Härtungsprozesses von Phenol-Formaldehyd-Harzen, X. Mitteilung. *Chem. Ber.*, **1944**, *77B*, 264