



# Synthetic receptors for phospholipid headgroups

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## Abstract

Small molecules that can selectively recognize phospholipids would likely be useful as tools for chemical biology and may have application as pharmaceuticals. Inspired by the phospholipid binding and transport systems found in nature, researchers have begun to develop synthetic receptors for phospholipid headgroups. These molecules range from ‘simple’ phosphate binders to more complex multi-topic receptors. This review provides an overview of the best characterized biological phospholipid receptors, as well as a comprehensive summary of the synthetic receptors reported thus far.

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**Keywords:** Phospholipid recognition; Biological receptors; Synthetic receptors; Phospholipid translocation; Fluorescent sensing

## 1. Introduction

The collection of review articles in this issue celebrating the 35th anniversary of the pioneering work of Park

and Simmons [1] are a testament to the intellectual interest, intriguing problems and growing importance of anion recognition to the field of supramolecular chemistry [2]. Anion recognition is a central topic in cell and

*Abbreviations:* BSP, bovine seminal plasma; BTABr, benzyltrimethylammonium bromide; CL, cardiolipin; DCPS, 1,2-dicaproyl-*sn*-phosphatidyl-L-serine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; GPE, glycerophosphoethanolamine; GPS, glycerophosphoserine; HDL, high density lipoproteins; LPS, lipopolysaccharide; lyso-PC, 1-acyl-phosphatidylcholine; lyso-PE, 1-acyl-phosphatidylethanolamine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEBP, phosphatidylethanolamine-binding protein(s); PG, phosphatidylglycerol; PI, phosphatidylinositol; PKC $\alpha$ , protein kinase C $\alpha$ ; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; PS, phosphatidylserine; SM, sphingomyelin; TREN, tris(2-aminoethyl)amine.

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molecular biology because many key biological molecules, such as DNA, proteins and carbohydrates, are anionic or at least have anionic substructures. Another extremely important class of biological molecules with anionic residues are the phospholipids. However, despite their prominence there have been remarkably few attempts by synthetic chemists to design and investigate artificial phospholipid receptors/hosts. Consequently, the purpose of this review is twofold. First we describe some of the inspiring biological phospholipid recognition systems that have been reported in the literature, focusing on those systems for which there is reasonable information on the atomic scale. Second, we summarize the handful of published efforts to prepare and evaluate synthetic phospholipid receptors.

## 2. Phospholipids as targets for molecular recognition

Phospholipids are critical constituents of most biological membranes (Fig. 1). Phospholipids are amphipathic in nature and their aggregation properties are the focus of intense study [3–5]. Phospholipids can be thought of as having three distinct structural regions: (1) a polar hydrophilic headgroup region which resides at the lipid–water interface when assembled into organized structures; (2) an interfacial region which is of intermediate polarity; and (3) a hydrophobic tail region. The common mammalian glycerol phospholipids (the main focus of this review) are known to adopt the general conformation shown in Fig. 2a [4]. The axis formed by the phospholipid headgroup is nearly perpendicular to the gamma chain, such that the headgroup essentially lies parallel to the surface of the membrane. Rotation of the headgroup about the C(1)–C(2) bond is known to be facile. Fig. 2b shows two possible ‘gauche’ conformations about the C(2)–C(3) bond [4]. Solution studies and X-ray crystallographic data indicates that both ‘gauche’ rotamers allow for parallel alignment of the alkyl chains, and that both rotamers represent energy minima [4]. Interconversion between the two

rotamers is believed to be facile and it is the parallel orientation (or chain stacking) of the hydrophobic chains that dictates the conformation about the C(2)–C(3) bond. While the non-polar tails determine the overall fluidity, the phase characteristics of the membrane (to some extent), and are generally essential for full biological function or activity, they are not normally considered to be the primary recognition targets [6]. As described below, it is the headgroup region that is usually involved in selective biological recognition events [6]. The structure of the headgroup also controls the rate of phospholipid diffusion through a bilayer membrane. It is well-established that lateral diffusion of a phospholipid through one leaflet of a bilayer membrane (in the absence of constraints) is very rapid, and that translocation of a phospholipid from one side of the bilayer to the other (a process also known as flip-flop) is quite slow [7]. In most animal cells, membrane-bound proteins called translocases control phospholipid translocation [8]. Some of these translocases are energy consuming, active transport systems, which means they produce transmembrane phospholipid distributions that are not symmetric. These asymmetric distributions control a range of important cellular functions such as signal transduction, membrane fusion, blood coagulation and cell apoptosis [9]. The phospholipids, and their metabolites are important secondary messengers in biology and phospholipid-binding proteins have been implicated in a wide array of biological processes [10]. Hence, synthetic receptors that selectively coordinate to phospholipid headgroups may act to disrupt these processes.

While phosphate recognition motifs will obviously be important elements in synthetic phospholipid receptors, the ability to distinguish between different headgroup architecture and electrostatic charge is a challenging task. For example, how does one design a receptor that can distinguish between phosphatidylcholine (PC) and sphingomyelin (SM), two phospholipids with the same headgroup but different interfacial/acyl chain components (Fig. 1)? How does one distinguish between the

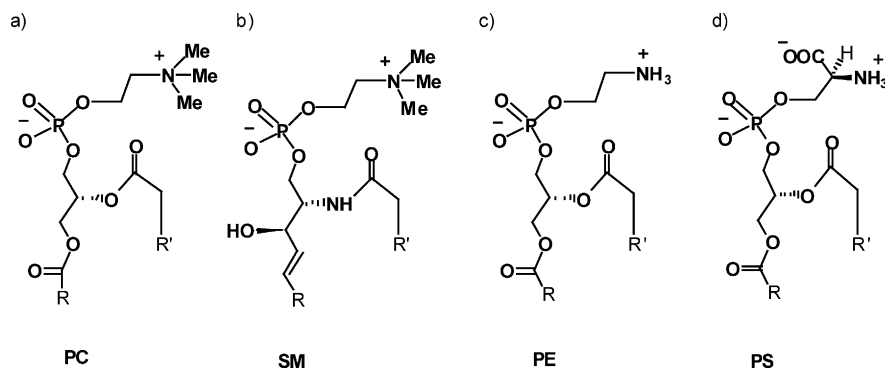


Fig. 1. Common mammalian glycerophospholipid headgroups (R = alkyl): (a) phosphatidylcholine, PC; (b) sphingomyelin, SM; (c) phosphatidylethanolamine, PE; and (d) phosphatidylserine, PS.

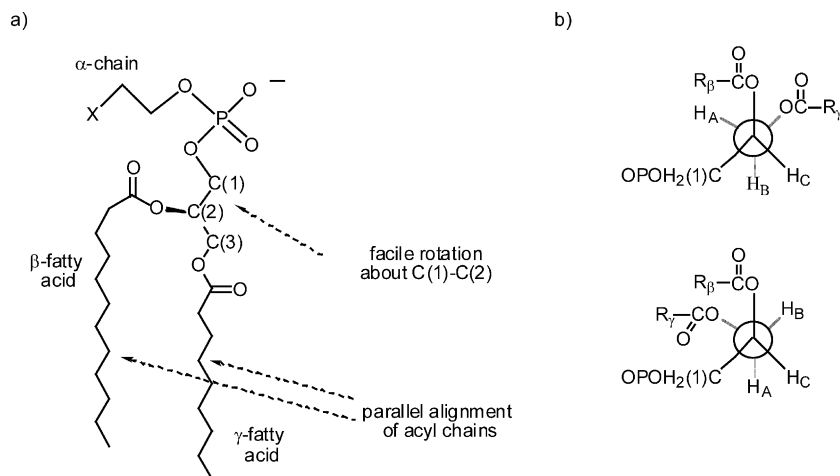


Fig. 2. (a) General conformation of phospholipids ( $R = \text{alkyl}$ ); (b) Newman projections along the  $C(2)-C(3)$  bond of the two favored 'gauche' rotamers [4].

zwitterionic aminophospholipid phosphatidylethanolamine (PE) and the triple ion (net-negative) phosphatidylserine (PS) headgroup? Additionally, a receptor may be required to bind a phospholipid and not a phosphorylated peptide for proper functioning *in vivo*. Water solubility, membrane partition constants and membrane diffusion rates are additional physicochemical factors that need to be considered.

## 2.1. Biological receptors for phospholipids

### 2.1.1. Biological receptors for PC/SM

A number of proteins have been reported to show affinity towards the PC headgroup. One such class is the major protein constituents of bovine seminal plasma (BSP) [11,12]. BSP proteins have molecular masses in the range of 15–16.5 kDa (BSP-A1, BSP-A2, BSP-A3) and one with a mass of 30 kDa (BSP-30 kDa). BSP-A1–A3 have identical amino acid composition but differ in their extent of glycosylation, while the 30 kDa protein shows only 66% amino acid homology to the first three [12]. Binding studies with immobilized lipid, and thin layer chromatography studies (utilizing radiolabeled phospholipids) have shown that BSP-A1, -A2 and -A3 exhibit high specificity towards the phosphocholine headgroup. For example, the proteins were found to bind strongly (in a calcium independent manner) to PC, 1-acyl-phosphatidylcholine (lyso-PC), SM and PC-plasmogen as well as the platelet activating factor; however, no binding to phosphatidic acid (PA), PE, phosphatidylinositol (PI), PS or PE-plasmogen was observed. Binding to lipids such as cholesterol and cardiolipin (CL) was also negligible [11]. The BSP-30 kDa protein was also found to bind the PC/SM headgroup; however, with less specificity. While the exact binding site for these proteins has not been elucidated, equilibrium dialysis experiments indicated that they complex two

equivalents of PC [12]. Additionally, the binding of these phosphocholine headgroups is known to exhibit negative cooperativity, i.e. that the binding of the first phosphocholine headgroup decreases the affinity for the second one [12]. It has been proposed that BSP are membrane docking proteins, in which the two 'PC docking' sites act to bring the sperm membrane and the high density lipoproteins (HDL) in close contact, thereby facilitating lipid exchange.

The phosphocholine binding site of the Fab region of the McPC603 antibody has recently been described, with binding assays [13–15] and crystallographic studies [16,17]. In the crystal structure of the McPC603-PC complex (3.1 Å resolution), phosphorylcholine was found to bind in an equimolar fashion within a large wedged shape cavity of dimensions 12 Å deep, 15 Å wide and 20 Å long [16]. The phosphorylcholine was bound asymmetrically to a small portion of this large cavity through hydrogen bonding, ionic and van der Waals interactions. The headgroup appeared to be in a '+ gauche' conformation while the O–C–N torsion appears to be trans. Furthermore, upon complexation of phosphorylcholine, the protein structure was found to undergo little re-organization, indicating a highly pre-organized site for PC complexation. Interactions were found between the phosphate portion of the PC headgroup and nearby Tyr33H and Arg52H residues (Fig. 3) [16]. More specifically, the Tyr33H hydroxyl and one of the amino groups of the Arg52H appear to be hydrogen bonded to one of the phosphate oxygens ( $\text{OH} \cdots \text{OP}$  and  $\text{NH} \cdots \text{OP}$  interactions). An electrostatic interaction between the Arg52H and the phosphate moiety has also been invoked (based on proximity,  $\sim 5$  Å) as a major contributor to the overall complex stability. The choline portion appears to be complexed via cation- $\pi$  interactions, with Tyr33H, Trp107L and Tyr100L, along with additional stability from an ion-ion pair interac-

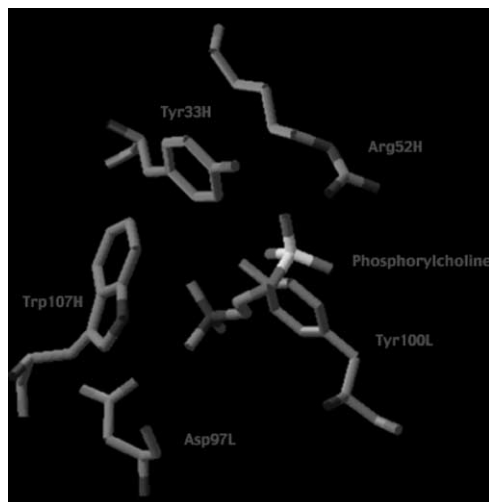


Fig. 3. Phosphocholine binding site of McPC603 [16].

tion with a nearby Asp97L. The walls of the cavity (main chain atoms of residues 102H and 103H) and Tyr33H also appear to be in van der Waals contact with the choline portion of the headgroup [16]. Binding studies have shown that the binding energy is largely driven by changes in entropy [13–15], implying that the specific contacts are more responsible for the PC selectivity than overall affinity [18].

### 2.1.2. Biological receptors for PE

Phosphatidylethanolamine-binding proteins (PEBP) are a family of polypeptides (21 kDa) found in various plant and animals [19]. While their exact cellular and molecular roles are still to be clarified, they have been implicated in the modulation of membrane biogenesis and in signal transduction. In general the mammalian PEBP show a high degree of overall structural homology and their interaction with lipids and membranes is a dominant feature of this class. A number of PEBP have been shown to bind PE with a rather weak affinity [20]. Several X-ray crystal structures have been obtained (e.g. bovine PEBP [21], human PEBP [22], the plant protein *Antirrhinum centroradialis* [23], and *Escherichia coli* [24]) and each implicate a small cavity near the protein surface as the lipid binding site. The small volume of the cavity implies that it is only the polar headgroup of the phospholipid that is recognized. In general, the amino acids, which comprise this phosphate-binding cavity, are well conserved and the cavity is well defined by these residues. In the solid state structure of bovine PEBP with bound phosphorylethanolamine (resolution of 2.25 Å), hydrogen bonding interaction between residues His86 (NH $\cdots$ OP), Asp70 (OH $\cdots$ OP), Gly110 (NH $\cdots$ OP) and Tyr120 (OH $\cdots$ OP) and the phosphate of phosphorylethanolamine are implied [21]. Additionally, a hydrogen-bonding interaction between the carbonyl of Gly110 and the ammonium of the

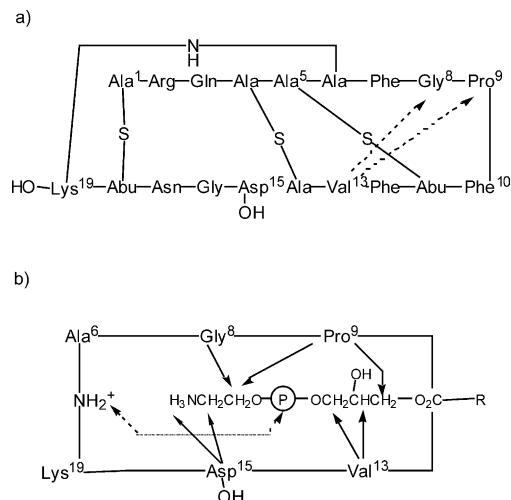


Fig. 4. (a) Primary structure of Ro09-0198 peptide [26]. Intramolecular interactions are indicated with dashed arrows [27]. (b) Inter-molecular interactions between Ro09-0198 and C12-lyso-phosphatidylethanolamine [27].

phosphorylethanolamine headgroup (C=O $\cdots$ HN $^+$ ) is also suggested. Hydrophobic interactions between the headgroup ethylchain and Trp43 were also found. Additionally, while PEBP binds selectively to solubilized PE in vitro, they do not bind to PE-enriched membranes, suggesting that their affinities toward membranes occurs through a different mechanism [25].

Another biological PE binding system is Ro09-0198, a tetracyclic nonadecapeptide (2041 Da) isolated from culture filtrates of *Streptovorticillium griseovorticillatum* [26–30] (Fig. 4). It is a rod-shaped, rigid amphiphilic structure comprised of a number of unusual amino acids (lanthionine,  $\beta$ -methylanthionine, lysinoalanine,  $\beta$ -hydroxyaspartic acid and D-phenylalanine), as well as a high degree of cross-linking with three thio and one imino bridge(s). During a study of its ability to induce hemolysis of erythrocytes, Ro09-0198 was found to absorb PE from membranes and form an equimolar complex [26]. For example, its hemolytic activity was decreased significantly when the erythrocytes were pre-incubated with PE; however, other phospholipids present in the membrane (e.g. PC, SM, CL, PA, PI and PS) had no inhibitory effect. In general, dialkylphosphatidylethanolamine (dialkyl-PE) and 1-acyl-phosphatidylethanolamine (lyso-PE) were found to have the same inhibitory effect as diacyl-PE while phosphatidyl-*N*-monomethylethanolamine and stearylamine were found to have no measurable effect. In summary, the interaction of Ro09-0198 with the PE-lipids (forming a 1:1 complex) is highly structure specific. The fact that Ro09-0198 recognizes both diacyl- and lyso-PE indicates that the peptide recognizes the monodispersed headgroup and not an assembled membrane surface [26].

The manner of complexation of Ro09-0198 with lyso-PE has been determined by NMR spectroscopy in



DMSO- $d_6$  [27]. The amino acids involved in the complexation of lyso-PE were identified by comparison of the chemical shifts of a number of proton resonances of the peptide in the absence and presence of lyso-PE, along with intermolecular NOESY experiments. In the absence of lyso-PE, intramolecular NOE studies indicated interactions between Gly8 and Val13, as well as between Pro9 and Val13 (Fig. 4a) [27]. Upon complexation, the intramolecular contacts between Gly8–Pro9 and Val13 are replaced with new interactions between the peptide and the phospholipid, indicating that the phospholipid headgroup has ‘inserted’ itself into this structure (Fig. 4b) [27]. More specifically, NOE studies indicated that Gly8, Pro9, Val13 and Asp15 were in close contact with the  $-\text{CH}_2\text{CH}_2-$  of the ethanolamine moiety and with the  $-\text{CH}_2\text{CHCH}_2-$  of the glycerol backbone. The  $\gamma$ -carboxylate of Asp15 was shown to be involved in a ion-pair interaction with the primary ammonium of the PE ( $\text{CO}^- \cdots \text{HN}^+$ ), while the secondary ammonium group of the Ala6–Lys19 bridge interacted with the negatively charged phosphate portion of the headgroup ( $\text{NH}^+ \cdots \text{OP}^-$ ) in a similar fashion. Interestingly, while a hydrophobic component of the lipid is required for complexation, no interactions between the acyl chain and Ro09-0198 were found. Hence, the complementary size and arrangement of charge displayed by Ro09-0198 leads to the *selective* interdigitation of the PE-headgroup, yielding a 1:1 complex [27,28]. The ability to selectively recognize the PE headgroup has allowed Ro09-0198 to be employed as a tool for biological membrane studies. For example, biotinylated Ro09-0198 (labeled through the alanine residue of the N-terminus) has been used successfully with fluorescently labeled streptavidin to monitor the transbilayer movement and redistribution of PE in eukaryotic cells [29,30].

### 2.1.3. Biological receptors for PS

A number of proteins have been identified with PS-selective recognition ability. In general these protein-PS interactions are classified into  $\text{Ca}^{2+}$  dependent PS-binding proteins (i.e.  $\text{Ca}^{2+}$  is required for the complexation of PS) [31–36] and non- $\text{Ca}^{2+}$  dependent PS-binding proteins [37–43]. The vast majority of these proteins belong to the former class of proteins and the following discussion is limited to this class of PS-binding proteins.

$\text{Ca}^{2+}$  is a secondary messenger for a number of biological signaling events. In all of the  $\text{Ca}^{2+}$  regulated signaling proteins there is a conserved targeting motif, the C2 domain, which is believed to bind  $\text{Ca}^{2+}$  ions and trigger docking to the intracellular membrane [44]. The specific affinities and the stoichiometry of  $\text{Ca}^{2+}$  uptake vary among these peptides and this variation is believed to modulate the mode (headgroup selectivity included) and extent of membrane docking [45]. The stabilizing

interactions that have been proposed for these  $\text{Ca}^{2+}$  mediated events are: (1) the direct inner sphere coordination of a protein-bound  $\text{Ca}^{2+}$  ion(s) by an anionic phospholipid; (2) a more general (outer-sphere) electrostatic headgroup recognition between anionic phospholipids and the protein- $(\text{Ca}^{2+})_n$  complex; (3) non-specific electrostatic interaction by a specific site on the protein; or (4) non-specific hydrophobic interactions between various side chains of the protein and the membrane [45]. One such example of inner-sphere coordination is the recent X-ray crystal structure (2.6 Å resolution) of a ternary complex between the C2 domain of a protein kinase C (PKC $\alpha$ -C2), a  $\text{Ca}^{2+}$  ion and 1,2-dicaproyl-*sn*-phosphatidyl-L-serine (DCPS) [46]. Both the ternary structure, as well as the ‘parent’ crystal structure of the protein- $(\text{Ca}^{2+})_n$  complex without phospholipid (2.4 Å resolution), show two bound  $\text{Ca}^{2+}$  ions, further referred to as Ca1 and Ca2. The Ca1 and Ca2 ions are coordinated by seven oxygens each (roughly in a pentagonal bi-pyramidal fashion), with six of the seven coming from the surrounding protein ligands: the side chains of five aspartate residues (Asp187, 193, 246, 248 and 254) the main chain residues Met186 and Trp247 [46]. Additionally, three of the aspartate ligands appear to be coordinated to both calciums (Asp246, 187 and 248). The seventh site on the Ca2 is occupied by a water, while the seventh site on Ca1 is occupied by either a water, in the binary complex, or the phosphate portion of the headgroup in the case of the ternary complex (Fig. 5) [46]. Two modes of DCPS headgroup binding were proposed from the diffraction data; however, they are actually quite similar [47]. In each case, at least one of the phosphate oxygens is coordinated to Ca1, while the phosphoserine carboxylate interacts specifically with the nitrogen atoms from a neighboring Asn189. The serine nitrogen atom is hydrogen bonded through a water molecule to either the glycerol *sn*-1 carbonyl (model 1) or to the *sn*-2 carbonyl

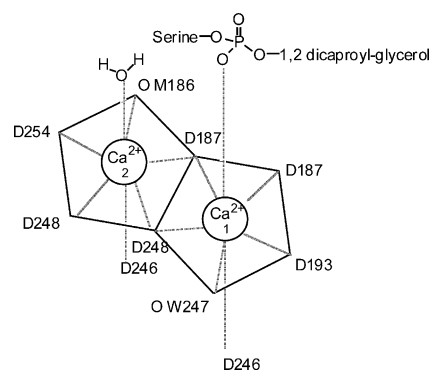


Fig. 5. (a) Schematic coordination scheme of the calcium ions found in the [(PKC $\alpha$ -C2)- $\text{Ca}^{2+}$ -DCPS] ternary complex [46]. Each  $\text{Ca}^{2+}$  ion is heptacoordinate in a pentagonal bipyramidal fashion; The seventh site on the Ca2 is occupied by a water, while the seventh site on Ca1 is occupied by the phosphate portion of the headgroup.

(model 2) which, in turn has its ester oxygen hydrogen bonding to a nearby Arg residue. Additionally, the fatty chains show hydrophobic interactions with the aliphatic carbons from Arg249. Hence, these interactions provide a very specific orientation of hydrogen bonding, electrostatics and van der Waals interactions which gives rise to the specific complexation of DCPS [46]. Additionally, mutation studies have indicated that the residues involved in the phospholipid binding are also important for the *in vivo* translocation of PKC $\alpha$  to the cell membrane [44].

Annexins are family of proteins that also bind PS in a calcium dependent fashion [48–56]. Annexins have been implicated in exocytosis, membrane fusion, the formation of ion-channels and as anti-coagulants of blood [57]. Binding studies have indicated that efficient binding requires the presence of a phosphate moiety and at least a short *sn*-2 acyl chain (only a few carbons required). A number of crystal structures for the annexin V protein (35 kDa) have been determined: (human [58], chicken [59] and rat [60]). These various annexin V homologues each consist of four canonical domains, with each domain containing multiple calcium ion binding sites near the protein surfaces. There are double calcium binding sites in the protein ‘AB loops’ (primarily responsible for protein–membrane binding), in which the calcium ions are separated by  $\sim 8.7$ – $8.8$  Å, as well as single calcium sites in the ‘DE loops’ (responsible for secondary protein–membrane interactions). The solid state structures again show calcium coordination to the protein via side chain carboxylate and main chain carbonyl oxygens [58–60]. Specific phospholipid binding interactions are revealed in the crystal structures of rat annexin V with a bound glycerophosphoserine (GPS) (1.9 Å resolution) or glycerophosphoethanolamine (GPE) (1.9 Å resolution) (Fig. 6) [61]. Annexins bind both PS and PE, but have a marked selectivity for PS, an affinity preference that is rationalized by these crystal structures. Both GPE and GPS directly coordinate their phosphate moiety to the protein-bound Ca $^{2+}$  ion (Ca1), in an apical ligand site, as seen in other calcium dependent phospholipid binding proteins [62]. In both phospholipid–annexin struc-

tures [61], Gly186 and Thr187 appear to play key roles in the phospholipid (GPS/GPE) binding. The carbonyl oxygen of Gly186 binds to Ca1 while its amide hydrogen interacts with the glycerol backbone of the phospholipid headgroup. The Thr187 carbonyl coordinates to the second calcium ion (Ca2), while its hydroxyl group stabilizes both phospholipid complexes: hydrogen bonded to the serine-NH $_3^+$  group in the case of GPS, and to a water molecule that is bound to the phosphate portion of the headgroup in the case of GPE. Most interestingly in the GPS–annexin structure, the phosphate is bound to Ca1 in a monodentate fashion while a serine carboxylate oxygen is bound to Ca2, also in a monodentate fashion. This ‘calcium-bridging structure’ is further stabilized by hydrogen bonds between the serine-NH $_3^+$  and the side chain hydroxyl of the Thr187 and with the main chain carbonyl of Glu226, both amino acids which are involved in the coordination of Ca1 and Ca2 (Fig. 6a) [61]. Furthermore, very little reorganization takes place upon GPS binding, indicating a receptor site that is highly pre-organized. Overall, the binding of GPE is less complementary, with the headgroup extending in the opposite direction of that found for GPS (Fig. 6b) [61]. The main binding interactions are between the Ca1 and phosphate, and a water bridged between the ammonium group and a carboxylate oxygen of Glu182. Additionally, there are no direct contacts between the protein and the ethylene chain of the GPE headgroup [61].

## 2.2. Synthetic receptors for phospholipids

### 2.2.1. Synthetic receptors for PC/SM

A calix[6]arene with a bicyclic guanidinium appended to the upper rim **1** has been prepared as a simplified non-peptidic mimic of the McPC603 antibody (see Section 2.1.1) [63]. A combination of calixarene choline encapsulation [64–70] (cation- $\pi$  interactions) and phosphate-guanidinium [71,72] hydrogen bonding dictates the complexation. The presence of these interactions was inferred from NMR spectroscopic studies of the 1:1 complex of **1** and PC. For example, addition of one equivalent of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) to host **1** in CDCl $_3$  resulted in upfield shifts of the methyl ( $\Delta\delta = 0.51$  ppm) and methylene protons ( $\Delta\delta = 0.35$  ppm) bound to the quaternary nitrogen of the choline, which is consistent with the inclusion of the choline portion of the lipid inside the calixarene cavity. This mode of complexation was further supported by a intermolecular NOE cross peak between aromatic protons of the calixarene (specifically, the aryl ring with the guanidinium attached) and the methyl groups of the choline. NMR-ROE spectroscopic studies indicated that the host–guest complex was rigidified to some extent (primarily in the linker region) because cone inversion of the calixarene was slowed upon complexation. Down-

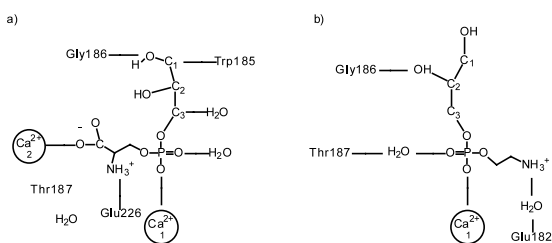


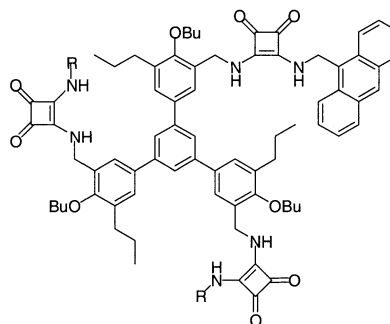
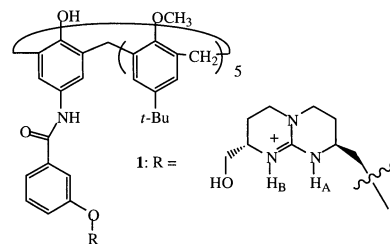
Fig. 6. (a) Schematic coordination scheme of: (a) the glycerolphosphatidylserine headgroup; and (b) glycerolphosphatidylethanolamine headgroup binding to the annexin V protein [61]. Favorable interactions are indicated by dashed lines.

field shifts for the amide hydrogen of **1** ( $\Delta\delta = 1.76$  ppm), as well as the guanidinium hydrogen nearest the calixarene ( $H_A$ ,  $\Delta\delta = 0.81$  ppm) indicated strong hydrogen bonding with the phosphate portion of the headgroup. Interestingly, the other guanidinium ( $H_B$ ) exhibited an upfield shift ( $\Delta\delta = 0.51$  ppm) in the complex, implying that only one of the guanidinium hydrogens ( $H_A$ ) is binding to the phosphate oxygen [63].

The association constant for the 1:1 complex of **1**–DOPC in  $CHCl_3$  was found to be  $K_a$  (298 K) =  $(7.3 \pm 0.5) \times 10^4 M^{-1}$  ( $\Delta G = -6.7$  kcal mol $^{-1}$ ), a value that is very similar to that obtained for the McPC603-PC complex in water ( $\Delta G = -6.6$  kcal mol $^{-1}$ ). Control studies indicated that both the calixarene cavity and the guanidinium sub-units were required for effective complexation of the PC headgroup. Additionally, van't Hoff plots indicated that the complexation of the PC headgroup in organic solvents is enthalpically driven [63], unlike the natural receptor (McPC603) which is entropically driven in water [13–15].

Taking a different approach, compounds **2** and **3** were designed 'de novo' to coordinate with the phosphocholine headgroup [73]. Similar tripodal squaramides were previously reported to complex tetralkylammonium salts via  $C=O \cdots NR_4^+$  interactions and hence **2** and **3** were expected to be good hosts for the complexation of choline-type headgroups [74]. Results from  $^1H$ -NMR spectroscopy studies indicated that the manner of complexation for of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and SM was similar to that of simple tetra-alkylammonium salts such as benzyltrimethylammonium bromide (BTABr), where the choline portion of the headgroup is bound in the 'pocket' of the tripod. Additionally, the conformational changes of compounds **2** and **3** upon complexation of DPPC or SM induce a change in the fluorescence intensity (quenching). Hence, these compounds act as fluorescence sensors for the phosphorylcholine headgroup. Based on fluorescent quenching data in  $CHCl_3$  (295 K), compounds **2** and **3** were found to have a higher affinity for DPPC (81,000 and 158,000  $M^{-1}$ , respectively) and SM (53,000 and 111,000  $M^{-1}$ , respectively) than the simple BTABr (10,000 and 35,000  $M^{-1}$ , respectively) [73]. The higher affinity for DPPC over SM may be attributed to secondary interactions between the squaramide NH's and the phosphate oxygens of DPPC. More useful, however, was the lack of complexation by other cell constituent phospholipids such as PE, PS and PI. Hence, these compounds may be able to measure micromolar concentrations of PC/SM in cellular extracts [73].

Tris(2-aminoethyl)amine (TREN) sulfonamide and amide derivatives are well known for their ability to complex phosphates via hydrogen-bonding in organic solvents [75–77]. Our research group has examined these compounds as synthetic receptors that can translocate



2: R = *n*-octyl, 3: R = 4-(*N,N*-dimethylamino)phenyl

phospholipids across biological membranes [78,79]. It was envisioned that such 'simple' phosphate binders would hydrogen bond to the phospholipid headgroup, decreasing the headgroup polarity and promoting translocation across the lipophilic membrane interior (a process also known as flip-flop, see Fig. 7). Indeed, TREN sulfonamide and amide derivatives do transport fluorescent phospholipid analogs across vesicle and erythrocyte membranes in the order of phosphatidylglycerol (PG) > PC > PE > PS. The ability of compounds **4**–**10** to complex with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) in  $CDCl_3$  at 298 K was evaluated using  $^1H$ -NMR spectroscopy [80]. The order of POPC association constants was found to be **9** ( $9.1 \times 10^3 M^{-1}$ ) > **8** ( $2.5 \times 10^3 M^{-1}$ ) ~ **7** ( $2.2 \times 10^3 M^{-1}$ ) ~ **4** ( $2.1 \times 10^3 M^{-1}$ ) > **10** ( $6.4 \times 10^2 M^{-1}$ ) > **5** ( $2.0 \times 10^1 M^{-1}$ ) >> **6** ( $< 1 M^{-1}$ ). In general, the observed association constants are consistent with a hydrogen bonded complex, and correlate with the acidity of the receptor NH groups. For example, all of the aryl sulfonamides were found to have a greater affinity than the propyl sulfonamide **10**, which in turn was better than amide **5**, while ester **6** had no discernible binding ability. The higher affinity displayed by sulfonamide **9** relative to the other aryl sulfonamides was attributed to its lower  $pK_a$  [81]. The binding constants for **4**, **7** and **8** were the same within experimental error of  $\pm 15\%$ . On the basis of acidity, a lower binding constant was predicted for **8**

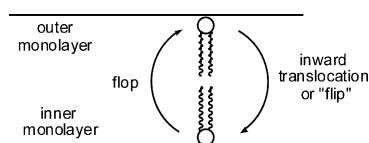
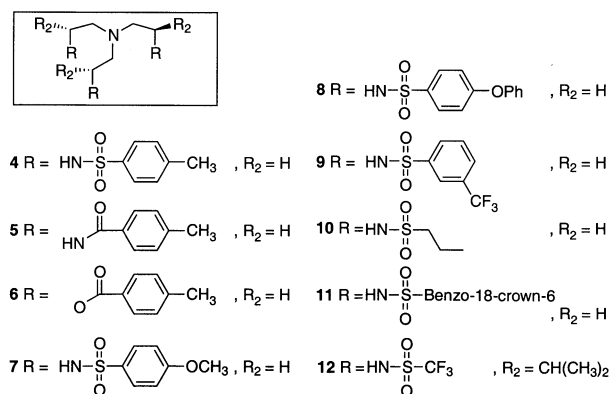


Fig. 7. Phospholipid translocation or flip-flop.

because the order of electron donating ability is phenoxy > methoxy > methyl [81]. It is possible that there are additional weak interactions between the receptor **8** and the PC headgroup. For example,  $\pi$ -stacking of the phenoxy rings in **8** with the cationic choline portion may compensate for the weakened hydrogen bonding to the phosphate group. The  $pK_a$ s of the protonated apical nitrogen atom of the TREN receptors **4**, **5** and **6** were obtained via  $^1\text{H-NMR}$  titrations in 9:1  $\text{CD}_3\text{OD-D}_2\text{O}$  and shown to be: **4** ( $4.00 \pm 0.01$ ), **5** ( $5.52 \pm 0.02$ ), **6** ( $3.75 \pm 0.01$ ). These values suggest that compounds **4–6** exist primarily in their free base form at neutral pH.



A comparison of the X-ray crystal structures of sulfonamide **4** and amide **5** provides structural reasons why **4** is a much better anion binder. The crystal structure of tris-sulfonamide **4**· $\text{H}_2\text{O}$  shows that the three secondary arylsulfonamides adopt their characteristic conformation, in which the nitrogen lone pair and the aromatic  $p$ -orbital lie in a plane that bisects the O–S–O internuclear angle (Fig. 8) [82–84]. The molecular geometry of **4** is roughly a  $\text{C}_3$ -symmetric tripod arrangement with all three NHs hydrogen-bonded to a water molecule ( $\text{NH}\cdots\text{O}$  distances are 2.43, 2.48 and 2.18 Å) which is also hydrogen-bonded to a sulfonyl oxygen in the same molecule ( $\text{SO}\cdots\text{H}$  distance of 1.89 Å). Overall, the solid state structure of **4** provides a clear visual picture of its ‘anion binding’ pocket.

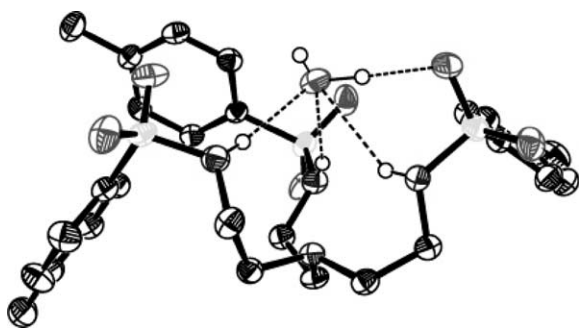


Fig. 8. Crystal structure of sulfonamide **4**· $\text{H}_2\text{O}$  with 50% ellipsoid probability [80]. Only sulfonamide and water hydrogen atoms are shown for clarity. Dashed lines indicate hydrogen bonds.

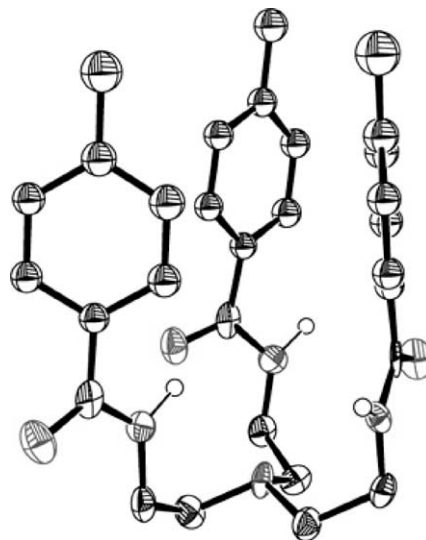


Fig. 9. Crystal structure of amide **5** with 50% ellipsoid probability [80]. Only amide hydrogen atoms are shown for clarity.

The crystal structure of tris-amide **5**, shows that the secondary amides adopt a  $Z$  conformation [85] about the amide C–N bond (Fig. 9) [86]. Because of this conformational constraint, the putative tripodal ‘anion binding pocket’ in receptor **5** is effectively closed due to the steric bulk of the three aromatic rings, which are oriented edge-to-face with each other. While the amide  $E/Z$  conformational equilibrium can be altered by non-covalent interactions [87], it is an energetically costly process [85]. Thus, it appears that aromatic TREN tris-amides such as **5** cannot bind anions in the same tridentate fashion as the analogous tris-sulfonamide **4**. In summary, the TREN-derived tris-sulfonamide receptors (such as **4**) are superior to the tris-amide versions (such as **5**) for two reasons: (1) the increased acidity of the TREN sulfonamide NHs; and (2) a molecular geometry that is able to achieve the tridentate coordination of one of the oxygens.

A crystal structure of the protonated form of sulfonamide **4** complexed with dibenzyl phosphate is shown in Fig. 10. In this case, only one sulfonamide NH and the protonated tertiary amine were found to be hydrogen-bonded to one of the phosphate oxygens ( $\text{NH}\cdots\text{O}$  distances of 1.94 and 1.77 Å, respectively). While this ionic structure is not so relevant to the binding/translocation of zwitterionic PC (which we believe involves a neutral translocase/phospholipid complex, Fig. 11a), it is relevant to the facilitated translocation of anionic phospholipids such as PG, which most likely requires a protonated translocase/phospholipid complex (Fig. 11b).

We have also recently examined a number of cholate derivatives **13–18** for their ability to bind phospholipids and induce phospholipid flip-flop. The lipophilic cholate bis-urea esters **13** and **14** were found to be more effective



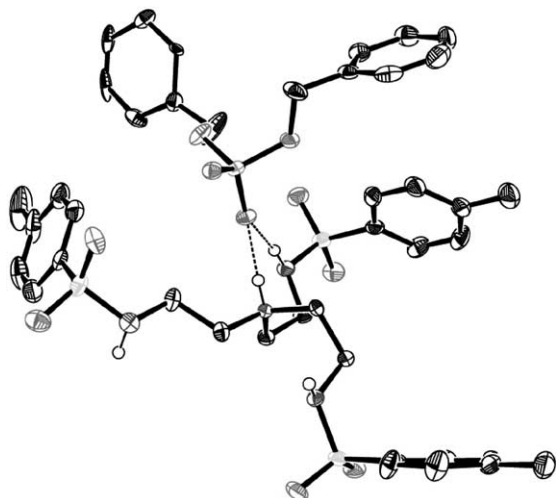


Fig. 10. Crystal structure of  $(4+H)^+-(\text{dibenzylphosphate})^-$  ionic complex with 50% ellipsoid probability [80]. Only relevant hydrogen atoms are shown. Included  $\text{CHCl}_3$  has been omitted for clarity. Dashed lines indicate hydrogen bonds.

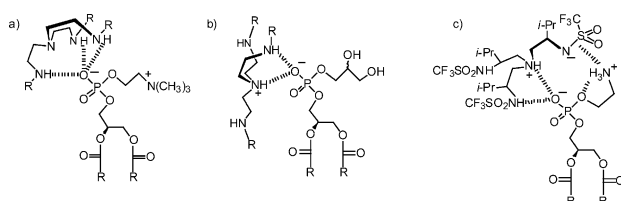


Fig. 11. Proposed supramolecular complexes with TREN-derived receptors ( $R = \text{alkyl}$ ): (a) PC headgroup [80]; (b) PG headgroup [80]; and (c) sulfonamide **12** and PE headgroup [91].

at PC binding and transport than the TREN-derived sulfonamides, such as **4** [88–90]. The binding of POPC to **13** or **14** in  $\text{CDCl}_3$  was too strong ( $> 10^5 \text{ M}^{-1}$ ) to be determined quantitatively by NMR spectroscopy; however, the complexed-induced changes in chemical shift were consistent with the supramolecular complex illustrated in Fig. 12. This conclusion was confirmed by a series of UV titration studies in 99:1  $\text{CHCl}_3\text{--CH}_3\text{OH}$ . For example, addition of POPC to translocase **13** produced an increase in absorbance and a slight bathochromic shift in absorbance wavelength. An association constant of for the proposed 1:1 complex was determined to be  $(1.2 \pm 0.1) \times 10^5 \text{ M}^{-1}$ . In contrast, addition of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) or 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS) to **13** produced such

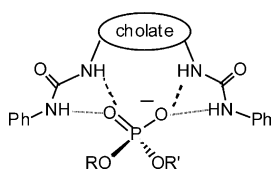
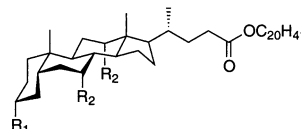


Fig. 12. Proposed interaction of bis-(phenylurea) **13** or **14** with a phosphocholine headgroup [88].

slight changes in absorbance that association constants could not be determined reliably. This very weak binding of POPE and POPS has tentatively been attributed to internal hydrogen bonding between the cationic ammonium and anionic phosphate (and carboxylate) residues of the phospholipids. A more hydrophilic methyl ester derivative of **13** was prepared and shown to be more effective than sulfonamide **4** at translocation of a fluorescent PC analogue across human erythrocyte membranes.



<b>13</b> $R_1 = \text{O}(\text{CO})\text{CH}_3$	<b>16</b> $R_1 = \text{O}(\text{CO})\text{CH}_3$
$R_2 = \text{NH}(\text{CO})\text{NHC}_6\text{H}_5$	$R_2 = \text{NH}(\text{CO})\text{OC}(\text{CH}_3)_3$
<b>14</b> $R_1 = \text{H}$	<b>17</b> $R_1 = \text{O}(\text{CO})\text{CH}_3$
$R_2 = \text{NH}(\text{CO})\text{NHC}_6\text{H}_5$	$R_2 = \text{NH}_2$
<b>15</b> $R_1 = \text{O}(\text{CO})\text{CH}_3$	<b>18</b> $R_1 = \text{OH}$
$R_2 = \text{NH}(\text{CO})\text{CH}_3$	$R_2 = \text{OH}$

### 2.2.2. Synthetic receptors for PE

Our first attempt to design a selective receptor for PE was the sulfonamide crown ether **11** [91]. The benzo-18-crown-6 ring is well known to strongly associate with ammonium cations in organic solvents and weakly in aqueous solution [92]. Thus, it was reasoned that the sulfonamide NH residues in **11** would form hydrogen bonds with the PE phosphate moiety while one of the crown ether moieties in **11** would form hydrogen bonds with the PE ammonium group [93]. Compound **11** was able to selectively discriminate between the PE and PC headgroups as determined by the translocation of fluorescently labeled phospholipid analogs across surface differentiated vesicles, although the facilitated transport was rather slow. The PE headgroup selectivity is most likely due to the recognition properties of the benzo-18-crown-6 ring, while the longer half-life observed with **11** compared to **4** can be attributed to its larger molecular size and increased amphiphilicity, which means **11** takes longer to diffuse through the hydrophobic interior of the vesicle membrane. TREN sulfonamide structures with only one crown ether are likely to be more effective translocators due to an increase in diffusion rate.

Unexpectedly, the ‘simple’ TREN-based phosphate receptor **12** was found to be a highly effective and selective PE translocase [91]. This is likely due to the following structural features: (1) the increased acidity of the sulfonamide NHs (due to the electron withdrawing effect of the  $-\text{CF}_3$  group); (2) the isopropyl chains located on the backbone make the compound more lipophilic and likely pre-organize the ‘binding pocket’. It was determined from NMR titration studies that the neutral form of **12** binds to POPE in  $\text{CDCl}_3$  at 298 K

with a rather weak binding constant of  $23 \text{ M}^{-1}$ , while POPC was bound with an affinity of  $2.5 \times 10^3 \text{ M}^{-1}$ . Therefore, binding by the neutral form of **12** does not appear to induce the headgroup selectivity observed in the translocation assay. A possible explanation is that **12** exists as a zwitterionic species in aqueous solution (due to a low  $\text{p}K_{\text{a}}$  value of the trifluoromethyl-sulfonamide NH), leading to the supramolecular complex illustrated in Fig. 11c. While, molecular models suggest that this flexible zwitterionic version of **12** can adopt conformations that nicely complement the structure of the PE headgroup, further studies are needed to verify this proposed mode of complexation.

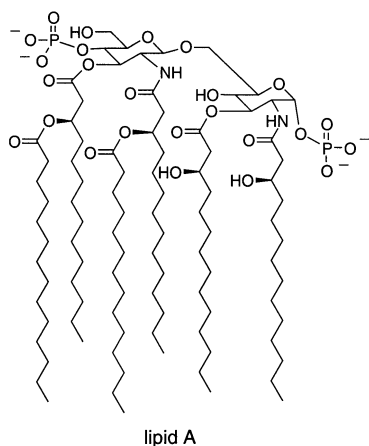
### 2.2.3. Synthetic receptors for PS

To date, no synthetic receptors have been reported to selectively recognize the PS headgroup. Receptors for this headgroup will need to be polytopic, with an ability to complex both the phosphate and the zwitterionic serine moiety of the headgroup in an aqueous environment. It is likely that polypeptide systems may be one approach to this problem. For example, combinatorial assemblies of peptides on an appropriate spacer/scaffold could be screened for their ability to selectively complex the PS headgroup. Alternatively, biomimetic receptors that can bind PS as a [Receptor– $\text{Ca}^{2+}$ –PS] ternary complex may be feasible [94].

### 2.2.4. Synthetic receptors for other phospholipids

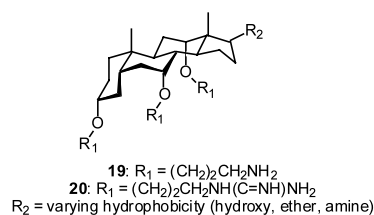
Although not a mammalian phospholipid, the anionic and amphiphilic lipid A is an interesting recognition target. For the sake of completeness, a brief overview of this lipid's biological function will be provided, as well as some of the synthetic receptors that have been designed for complexation/sequestration purposes [95].

Lipid A is the main component of the outer membrane of Gram negative bacteria. It rigidifies the outer membrane via salt-bridges with  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$ . Lipid A is also known to be the toxic component of the lipopolysaccharide (LPS) endotoxins that play a key



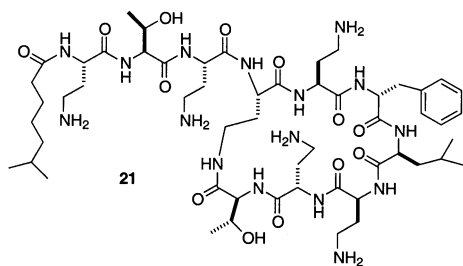
role in pathogenesis of septic shock, a leading cause of death in intensive care units. Lipid A is comprised of a bis-phosphorylated  $\beta$ -1,6-linked D-glucosamine disaccharide backbone with up to seven acyl chains [96]. It is highly conserved in Gram negative bacteria and hence is an attractive target for drug development via sequestration. Molecules that successfully recognize and bind to lipid A/LPS may prevent septic shock or 'blood poisoning'. A review of the strategies used to date to bind LPS/lipid A has recently been published [95]. Polyclonal/monoclonal antibodies, non-antibiotic LPS-binding proteins, peptides and non-peptidic molecules have all been examined for binding and/or neutralization. The data indicates that a specific arrangement of hydrophobic and cationic regions (to match the  $13.9 \text{ \AA}$  separation of the anionic phosphates) is critical for LPS binding and neutralization [95]. Two recent examples of synthetic lipid A receptors will now be described.

Cholic acid based structures of the type **19** and **20** have been studied as mimics of the polymyxin antibiotics, which are known to be bactericidal and to act via complexation with lipid A. The cholic acid serves as a hydrophobic scaffold to arrange the amine or guanidinium functionalities in a similar conformation to that as predicted for polymyxin B (**21**) [97]. It was expected that the appropriate arrangement of these cationic residues would permeabilize the bacterial outer-membrane and a number of these compounds were shown to be effective sensitizers for antibiotics such as erythromycin. In general, the guanidinium compounds were found to be more active than their amine counterparts, presumably because they could coordinate with the phosphate anions of lipid A (rather than just an ionic balancing). Additionally, by lengthening the alkyl chain on the C-24 position (increasing the hydrophobicity of  $\text{R}_2$  in compound **19** or **20**), the molecules were found to have antibiotic properties themselves. This behavior was attributed to their self-promoted transport across the membrane of Gram negative bacteria [97,98].

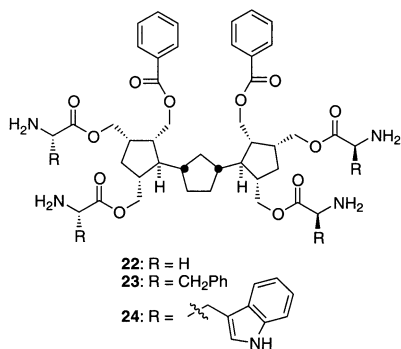


Substituted *ter*-cyclopentanes **22–24** have also been utilized as a receptors for lipid A [99]. Molecular modeling indicates that these structures are conformationally rigid and allow for the presentation of various functionalities capable of binding to lipid A. In particular, it was expected that the amine and hydrophobic

functionalities would be suitably arranged for disruption of the  $(\text{Ca}^{2+}/\text{Mg}^{2+})$ –lipid A network. UV methods were used to measure dissociation constants of 587 and 592 nM, for compounds **23** and **24**, respectively, in pH 7.4 phosphate-buffered saline. These affinities are similar to those obtained for polymyxin–lipid A complexation [100]. Compound **22** was found to only interact weakly with lipid A, which is consistent with the notion that the hydrophobic side chains are important in the complexation. Job's plots indicated that a 1:1 complex between **24** and lipid A was formed at lower lipid concentration and a 1:2 complex was observed at higher lipid A concentrations [99]. In summary, these rigid *ter*-cyclopentane structures bind strongly to lipid A in water and may be useful as endotoxin neutralizing agents.



### 3. Summary



We believe that the design of synthetic phospholipid receptors is a tractable and potentially significant research problem that warrants increased attention by supramolecular chemists. To date, three types of supramolecular functions have been explored: polar lipid sequestration, fluorescent sensing, and membrane translocation. Most of the synthetic receptors reported so far have been designed to bind zwitterionic PC which has limited biological activity. Future studies are likely to focus on structurally more complex polar lipids that are known to regulate important recognition and signal transduction processes, especially the aminoglycerophospholipids (e.g. PS, PE) [8,9], PIs (e.g. PIP<sub>2</sub>) [101], sphingoglycolipids (e.g. gal-ceramide) and the glycerophospholipids (e.g. lipid A) [95,96].

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