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Initial Structural Studies of Charged Receptors That Bind to Inorganic Phosphate Anion and to an Anionic Phospholipid Found in Bacterial Membranes

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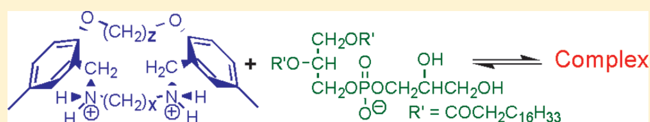
Supporting Information

ABSTRACT: ITC titration studies of a family of bis-ammonium receptors based upon a scaffold of two bis-linked phenol rings show that several of the receptors bind to both dihydrogenphosphate and phosphatidylglycerol anions in a similar binding motif. Thermodynamic properties determined from ITC show that anion binding is entropy driven. Job plots determined from ^{1}H NMR clearly demonstrate that anion−receptor binding stoichiometry is dependent on the receptor's length of its bis-amine linkage.



Due to the emergence of many strains of multidrug-resistant bacteria,[1−4] cationic peptides and their mimics have recently garnered interest as potential antibiotics.[5,6] Part of the innate immune system of such diverse organisms as plants, invertebrates, and vertebrates, cationic antimicrobial peptides exhibit a broad spectrum of activity against Gram-positive and Gram-negative bacteria.[7−9] They are believed to bind to the bacteria's inner membrane anionic lipid headgroups, insert into, and then disrupt the membrane, leading to cell death. However, even native cationic peptides can have substantial host effects, including toxicity.[5,10−12] Therefore, an essential requirement for any antimicrobial peptide mimic would be that it can selectively disrupt prokaryotic membranes and not eukaryotic membranes.

The goal of high membrane selectivity for a peptide mimic can be achieved by increasing the recognition ability of the synthetic antibiotic for unique bacterial membrane components. The outer leaflets of prokaryotic cell membranes contain an abundant supply of acidic (anionic) phospholipids, such as phosphatidylglycerol, whereas the outer leaflets of eukaryotic cell membranes, such as erythrocytes, are almost exclusively composed of zwitterionic phospholipids.[4] Antimicrobial cationic peptides utilize this difference in lipid head structure to recognize and bind to anionic phospholipids of the bacterial inner membrane. A receptor able to selectively bind to the accessible phosphatidylglycerol (PG) headgroup, via its phosphorus anion and glycerol dihidroxy functionality, is therefore an attractive target as a modular component of an antimicrobial therapeutic. The linkage of these receptors to membrane disruptors could result in synthetic antibiotics that exhibit bactericidal action similar to that of cationic peptides but with lessened host toxicity. Herein we present initial results toward the ultimate goal of preparing a selective phosphatidylglycerol receptor. Specifically, this study was undertaken to determine the necessary structural requirements for a receptor to bind the anionic phosphate group of PG using ammonium ions as receptor binding units.

Our modeling studies showed that a receptor with the preorganized (ortho-ring substituted) ammonium groups found in compound **1** (Scheme 1) would bind to the PG lipid's phosphate anion group via positively charged ammonium hydrogen bonds. Furthermore, appropriate elaboration of the receptor's phenol ring para-positions would orient functional groups that could then complex with the glycerol hydroxyl groups. Because both the ligand and receptor were charged and studies were to be conducted in polar solvents, it was expected that complex formation would be entropy driven.[13] It was hypothesized that such a receptor would still exhibit PG selectivity, however, because of the multiple binding domains found in the receptor. Each ammonium group would have two hydrogens for bonding, reducing conformational constraints in the binding pocket. Unknown was how the oxygen atoms in the phosphate anion portion of PG would interact with the two ammonium ions, i.e., would the most stable interaction be with two of the phosphate's oxygens or just one, or would there be an ensemble of binding modes. The difference in possible binding motifs for the complexation of PG could affect the presentation of the lipid's glycerol hydroxy groups to corresponding binding units on a receptor. To examine in detail the structural requirements for a receptor able to bind the phosphate anion portion of PG, we prepared a family of receptors whose linkages between the two phenolic oxygens and two benzyl amines were of different lengths. In this way receptors could be constructed that might support various binding motifs, and if so, potentially maximize the entropic contribution to complexation. Isothermal titration calorimetry (ITC) was used to probe the thermodynamic properties of the anion−receptor complexes, and ^{1}H NMR was used to determine the stoichiometry of binding.

The synthesis and structures of our initial receptor salts **4a−g** (PF_6 counterions) are shown in Scheme 1. ^{1}H NMR spectra

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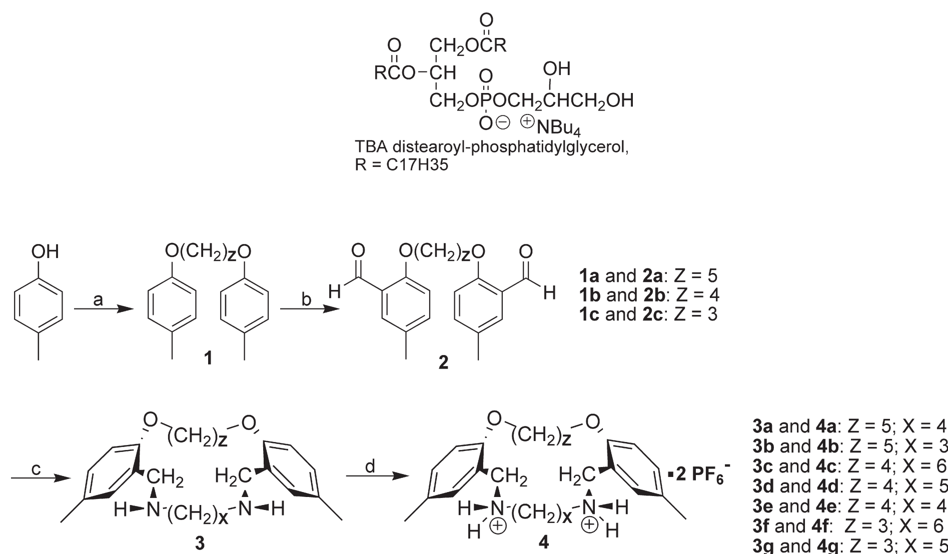
Scheme 1. Preparation of Receptors 4a−g and Lipid Salt Structure[a]



[a] Reagents and conditions: (a) $Br(CH_2)_zBr$, 18-crown-6, K_2CO_3, THF; (b) $(CH_2)_6N_4$, TFA, reflux; (c) (i) $H_2N(CH_2)_xNH_2$, EtOH, reflux, (ii) triethylsilane, Pd−C, MeOH; (d) HCl/MeOH then NH_4PF_6, CH_2Cl_2, H_2O.

Table 1. Results of Isothermal Titration Calorimetry

Receptor	*z*	*x*	S[a]	K_1 (M^{-1}) [error]	Δ*H*, cal/mol [error]	Δ*S*, cal/mol/°C	anion salt
4a	5	4	1:1	1.06×10^5 [$\pm 9.6 \times 10^3$]	5000 [±60]	39	TBAP
4b	5	3	1:1	1.01×10^5 [$\pm 5.6 \times 10^3$]	7300 [±50]	47	TBAP
4c	4	6	2:1	6.60×10^5 [$\pm 1.7 \times 10^5$]	4000 [±60]	40	TBAP
				1.84×10^4 [$\pm 5 \times 10^3$][b]	400 [±40]	21	
4d	4	5	mixture				TBAP
4e	4	4	1:1	4.67×10^4 [$\pm 4.6 \times 10^3$]	12000 [±300]	61	TBAP
4f	3	6	2:1	1.38×10^5 [$\pm 3.3 \times 10^4$]	5600 [±200]	42	TBAP
				5.77×10^3 [$\pm 1.2 \times 10^3$][b]	2000 [±500]	22	
4g	3	5	mixture				TBAP
4b	5	3	1:1	4.06×10^4 [$\pm 6.4 \times 10^3$]	4000 [±200]	35	TBA PG
4d	4	5	mixture				TBA PG
4e	4	4	1:1	2.26×10^4 [$\pm 6.1 \times 10^3$]	3000 [±300]	29	TBA PG

[a] Binding stoichiometry determined from Job plots using ^{1}H NMR. [b] K_2 (M^{-1}).

show that upon acidification of receptor **3** the amine nitrogen protons move downfield to around 8.5 ppm (**3**'s NH protons are not observed) and integrate to four protons, consistent with the structure of receptor **4** (an X-ray crystal structure of bis-hexafluorophosphate bis-ammonium receptor **4c** is provided in the Supporting Information). The receptors were prepared without functional groups that bind to glycerol hydroxyl groups for two reasons: (1) these receptors would be needed anyway as control receptors for those PG receptors fully functionalized to bind both the lipid's phosphate and hydroxyl groups and (2) receptors that contain several binding units make the determination of binding stoichiometry for inorganic phosphate anion in the bis-ammonium pocket problematic (i.e., two or more anions could bind to the receptor via both binding domains). The latter would prove troublesome because our initial strategy was to probe the receptor−H_2PO_4 anion binding motif, including binding stoichiometry, in solution. Our working hypothesis was that the structure of the binding pocket found effective for binding dihydrogenphosphate anion would translate to a receptor pocket that would also bind the anionic phosphate group of the tetrabutylammonium distearoyl-phosphatidylglycerol salt (TBAPG).[14] Those receptors that bound to inorganic phosphate anion in a well-behaved manner with 1:1 stoichiometry would be selected from among the receptor family for binding studies with TBAPG. While ultimately a functional receptor for antibiotic use must bind to a membrane bound PG, it was believed that the use of solution NMR spectroscopy would most directly illustrate binding motifs, and thereby allows for the rational, iterative synthesis of a PG receptor's binding units.

The stoichiometry of phosphate anion binding for receptors **4a−g** was determined from Job plots in DMSO-d_6, using ^{1}H NMR[15,16] (Table 1). Downfield movement of the ammonium hydrogen ^{1}H NMR resonances upon addition of the anion showed that the phosphate anion was hydrogen bonding to the positively charged hydrogens (Supporting Information). The results showed that the binding stoichiometry for TBAP anion

was determined by the length of the bis-amine used to construct the receptor's link. Binding ratios of 1:1 were found only for the complexes made from receptors **4a**, **4b**, or **4e**, where $x = 3$ or 4, while binding ratios of 2:1 (anion: receptor) were found for receptors **4c** and **4f**, where $x = 6$ (an X-ray structure of the receptor−anion complex of **4f** bound to two dihydrogenphosphate anions is provided in the Supporting Information). Receptors **4d** and **4g**, where $x = 5$, showed no distinct stoichiometry of binding. Additionally, the ability to synthesize the shorter bis-amine link was dependent on the length of the bis-phenolic oxygen link, whereby $x = 3$ could only be prepared when $z = 5$. Notably, Job plots showed receptors **4b** and **4e** also bound PG in a 1:1 ratio as they did with dihydrogenphosphate anion (Table 1). Interestingly, receptor **4d**, which bound the dihydrogenphosphate anion in mixed ratios, also bound the TBAPG in the same not well-behaved manner. The TBAPG salt, and perhaps the receptor−PG complex, were not completely soluble in DMSO, and therefore the stoichiometric studies were conducted in 95% DMF-d_7/5% $CDCl_3$ with ^{1}H NMR.[16]

Isothermal titration calorimetry (MicroCal iTC200) was used to determine the thermodynamic properties of receptor−anion complexation. Receptors **4a**−**c**, **4e**, and **4f** (with 1:1 or 2:1 anion: receptor binding stoichiometries) were titrated with TBAP salt in DMSO. Not surprisingly, the ITC data show that the binding process for receptors **4a**−**c**, **4e**, and **4f** titrated with TBAP is entropy driven (Table 1). The ITC experiments were conducted first by adding the guest anion to receptor solution and then second by adding the receptor to a guest anion solution via an inverse titration. The thermodynamic properties of both additions were very similar, showing internal consistencies (i.e., state functions are not affected by pathway).[17]

The formation of receptor−H_2PO_4 complexes produced a large increase in the entropy of the system, from 39 to 61 eu, with positive enthalpies in the 4−12 kcal/mol range, resulting in binding constants ranging from 10^4 to 10^5. Receptors **4c** and **4f** exhibited slightly wider ranges (10^3 to 10^5) for both their K_1 and K_2 binding constants. While the binding of dihydrogenphosphate anion by receptors **4b** or **4e** in a 1:1 ratio affords the largest entropy gains in the receptor family, complex formation with either receptor requires the highest enthalpic costs as well. The high positive enthalpies suggest that desolvation of these receptors requires the disruption of a more stabilized solvent shell about the ammonium cations when $x = 3$ or 4. The high positive entropies additionally suggest that there may be an ensemble of low energy structures available to the binding partners, resulting in an increase in what has been termed configurational entropy.[13] Much less positive enthalpy or entropy is produced upon binding the second anion to receptors **4c** or **4f**. This is most likely due to the fact that the two bound phosphate anions are able to hydrogen bond with each other (see X-ray crystal structure in the Supporting Information), thus decreasing enthalpy (via H-bond stabilization) and entropy (via higher ordering in the complex).

ITC was also used to measure the thermodynamic properties of complex formation with TBAPG and receptors **4b** and **4e** (two receptors that bound TBAP with 1:1 binding stoichiometry). Although a different solvent system was required for binding studies with TBAP than for TBAPG, a comparison of the binding constants is still valid for the following two reasons: (1) the dielectric constants of DMSO and DMF are extremely close, but more importantly, (2) the ^{1}H NMR spectra of the receptor in either solvent system is nearly identical, suggesting that the chemical nature of the binding pocket is very similar in either solvent. As in the phosphate anion titration, downfield movement of the ammonium hydrogen ^{1}H NMR resonances occurred upon titration of the receptors with the PG anion. The receptor's binding constants determined for dihydrogenphosphate anion and the PG anion are very similar, only 2 times greater for the inorganic phosphate anion than for the PG anion. The ^{1}H NMR data along with the small difference in binding constants observed in the association of receptor and the two anions garnered from the ITC experiments suggest that, while structurally very different, the two phosphate anions are likely interacting with the receptor pocket in a similar binding motif, supporting our original hypothesis.

Drawing the same conclusion from a comparison of the binding enthalpies and entropies of the two anions when complexed to receptors **4b** or **4e** is problematic, however, since the two anions have such vastly different structures. Presumably the PG anion would have less configurational entropy because its anion portion is more sterically hindered, consequently less able to form a multitude of geometrical arrangements upon complex formation. This would lead to less positive entropy afforded upon formation of a receptor−PG complex, as is observed with both receptor **4b** and **4e**. The amount of binding enthalpy and entropy is larger for **4e** than **4b** upon complex formation with inorganic phosphate anion, but larger for **4b** than **4e** for complex formation with PG anion. Given that, it is interesting to note that the binding constants for both PG anion and inorganic phosphate anion are twice as large for receptor **4b** as for receptor **4e**. One thing is clear, that the strongest interaction between both receptor's ammonium cation and the anion's phosphorus−oxygen bond(s) is found when $x = 3$.

In summary, ITC titration studies of bis-ammonium receptors **4b** and **4e** show that they bind in a 1:1 stoichiometry to both dihdrogenphosphate anion and phosphatidygylcerol anion with similar binding constants, and that complexation is entropy driven in both systems. When titrated with either anion all the receptor's ^{1}H NMR spectra indicate the anion is binding to the ammonium hydrogens, forming charged hydrogen bonds. The stoichiometry of binding *for both anions* is determined by the length of the bis-amine spacer; 1:1 binding is seen with $x = 3$ or 4, not well-behaved binding is observed when $x = 5$, and 2:1 binding results when $x = 6$. All of these data taken together suggest that the binding motifs are similar for a complex formed between receptor **4b** or **4e** and either anion. The preparation of receptors containing functional groups that also bind to the glycerol headgroup of PG, based on the structures of receptors **4b** and **4e**, is in progress.

■ EXPERIMENTAL SECTION

The following experimental procedure is provided as representative of the preparation for all corresponding compounds **1**−**4**:

1,5-Bis(4-methylphenoxy)pentane (1a). *p*-Cresol (5.17 g, 0.048 mol) was dissolved in 475 mL of dry THF under N_2 and to this mixture was added K_2CO_3 (6.54 g, 0.047 mol) and 18-crown-6 (2.59 g, 0.007 mol). 1,5-Dibromopentane (3.32 mL, 0.024 mol) was added and the reaction mixture was refluxed for 20 h. The reaction was allowed to cool to room temperature and quenched with 10% HCl. The layers were separated and the aqueous layer was extracted with CH_2Cl_2 (3 × 40 mL). The combined organic layer was washed with a saturated sodium bicarbonate solution (2 × 40 mL) and brine (40 mL) and dried with anhydrous Na_2SO_4. Removal of the solvent under reduced pressure yielded the crude product as a white solid. Column chromatography

(silica gel) eluting with 35% ethyl acetate and 65% hexanes furnished the pure product as a white crystalline solid (6.73 g, 0.023 mol, 99% yield): mp 58−60 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.61−1.66 (m, 2H), 1.80−1.87 (m, 4H), 2.27 (s, 6H), 3.94 (t, 4H, J = 6.7 Hz), 6.78 (d, 4H, J = 7.9 Hz), 7.06 (d, 4H, J = 8.5 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 21, 23, 29, 68, 115, 129.9, 130.1, 157; MS (ESI) m/z 307.1 $(M + Na)^+$; HRMS (ESI) calcd for $C_{19}H_{24}O_2$ $(M + Na)^+$ 307.1675, found 307.1740.

1,5-Bis(2-formyl-4-methylphenoxy)pentane (2a). Compound **1a** (1.58 g, 0.0055 mol) and Duff reagent (1.82 g, 0.0129 mol) were dissolved in 20 mL of TFA and the reaction mixture was allowed to reflux under N_2 for 24 h. The mixture was concentrated, water was added, and the aqueous layer was extracted with 50 mL of CH_2Cl_2. The organic layer was separated, and the aqueous layer was acidified with concentrated HCl and extracted with CH_2Cl_2 (2 × 50 mL). The combined organic layer was washed sequentially with 4 N HCl (2 × 50 mL), 50 mL of a saturated Na_2CO_3 solution, and 50 mL of brine. Removal of the solvent under vacuum furnished the crude product as a brown gluey mass. Column chromatography (silica gel) eluting with hexanes furnished the pure product as a white crystalline solid (0.75 g, 0.0022 mol, 40%): mp 76−77 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.66−1.72 (m, 2H), 1.88−1.95 (m, 4H), 2.29 (s, 6H), 4.07 (t, 4H, J = 6.2 Hz), 6.96 (d, 2H, J = 8.7 Hz), 7.32 (d, 2H, J = 8.6 Hz), 7.61 (s, 2H), 10.46 (s, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20, 23, 29, 68, 113, 125, 130, 137, 160, 190; MS (ESI) m/z 409.0 $(M + Na)^+$; HRMS (ESI) calcd for $C_{21}H_{24}O_4$ $(M + Na)^+$ 363.1572, found 363.1584.

10,21-Dimethyldibenzo-[*h*,*r*]-1,7-dioxa-13,18-diaza-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclononadecene (3a). Compound **2a** (0.06 g, 0.176 mmol) was suspended in 22 mL of dry ethanol and purged with dry N_2 for 30 min, then the reaction was brought to reflux. Butane-1,4-diamine (0.0224 mL, 0.222 mol) in 9 mL of dry ethanol was added dropwise to the reaction mixture. The reaction was allowed to reflux for 20 h, and the solvent was then removed under reduced pressure yielding the bis-imine as a yellow solid, which was subjected to reduction without further purification. The crude bis-imine was redissolved in 2 mL of anhydrous methanol under N_2. Pd−C (10%, 0.1 g) was added and the reaction mixture was allowed to stir. Triethylsilane (1.0 mL, 0.006 mol) was added dropwise into the methanolic bis-imine solution with an addition funnel, the top of which was attached to a rubber balloon. After the addition was complete, the mixture was stirred for an additional 6 h at room temperature. The reaction mixture was filtered through Celite, and removal of the solvent under vacuum gave the crude product as a yellow oil. Column chromatography (silica gel) eluting with 10% $CHCl_3$, 4% isopropylamine, and 86% diethyl ether furnished compound **3a** as an off-white solid (0.060 g, 0.151 mmol, 86% after both steps): mp 95−96 °C; ^{1}H NMR (400 MHz, DMSO) δ 1.39−1.42 (m, 4H), 1.62−1.67 (m, 2H), 1.77−1.84 (m, 4H, J = 6.87 Hz), 2.21 (s, 6H), 2.47 (t, 4H, J = 5.6 Hz), 3.61 (s, 4H), 3.96 (t, 4H, J = 5.9 Hz), 6.85 (d, 2H, J = 8.2 Hz), 6.99 (d, 2H, J = 8.8 Hz), 7.04 (s, 2H); ^{13}C NMR (100 MHz, DMSO) δ 21, 25, 27, 30, 48.3, 48.5, 69, 113, 128.7, 128.9, 129.4, 131, 156; MS (ESI) m/z 397.1 $(M + H)^+$; HRMS (ESI) calcd for $C_{25}H_{36}O_2N_2$ $(M + H)^+$ 397.2855, found 397.2852.

10,21-Dimethyldibenzo-[*h*,*r*]-1,7-dioxa-13,18-diaza-2,3,4,5,6,7,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclononadecenediium bis(hexafluorophosphate (4a). Bis-amine **3a** (15 mg, 0.038 mmol) was dissolved in 3 mL of methanol, 6 N HCl (2 mL) was added, and the reaction mixture was allowed to stir for 3 h at room temperature. The solvent was removed under vacuum and the crude reaction mixture was redissolved in 3 mL of H_2O. Ammonium hexafluorophosphate (>30 equiv) dissolved in CH_2Cl_2 was added and the reaction mixture was stirred overnight during which time **4a** precipitated from the solvent mixture as an off-white solid (24 mg, 0.034 mmol, 92%): mp >165 °C dec; ^{1}H NMR (400 MHz, DMSO) δ 1.71 (s, broad, 6H), 1.82−1.87 (m, 4H, J = 5.9 Hz), 2.26 (s, 6H), 2.92 (s, broad, 4H), 4.04 (t, 4H, J = 5.4 Hz), 4.08 (s, 4H), 7.01 (d, 2H, J = 8.7 Hz), 7.21−7.23 (m, 4H), 8.49 (s, 4H); ^{13}C NMR (100 MHz, DMSO) δ 21, 23, 25, 30, 45, 46, 69, 113, 120, 130, 132, 133, 156; MS (ESI) m/z 397.1 $(M - H)^+$, 199.2 $(M^{2+}/2)^+$, 543.1$(M^{2+}\cdot PF_6^-)^+$; HRMS (ESI) calcd for $C_{25}H_{37}O_2N_2$ $(M - H)^+$ 397.2855, found 397.2864.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Full characterizations for **1b**, **1c**, **2b**, **2c**, **3b**, **3c**, **3e**, **3f**, **4b**, **4c**, **4e**, and **4f**, HRMS, ^{1}H NMR, and ^{13}C NMR spectra of **1a**−**c**, **2a**−**c**, **3a**, **3b**, **3c**, **3e**, **3f**, and **4a**−**g**, ^{1}H NMR spectra of TBAPG, Job plots of receptors **4a**, **4b**, **4c**, and **4f** with TBA $H_2PO_4^-$ and of **4e** with TBA $H_2PO_4^-$ or TBAPG, stacked plots of ^{1}H NMR titrations of **4e** + TBA H_2PO_4 or TBAPG, and ITC binding isotherms of **4e** + TBA H_2PO_4 or TBAPG, and X-ray crystal structures for anion−receptor complexes of **4f** + $2H_2PO_4^-$ and for **4c** + $2PF_6^-$, and estimated complex structure **4b** + $H_2PO_4^-$. This material is available free of charge via the Internet at http://pubs.acs.org.

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