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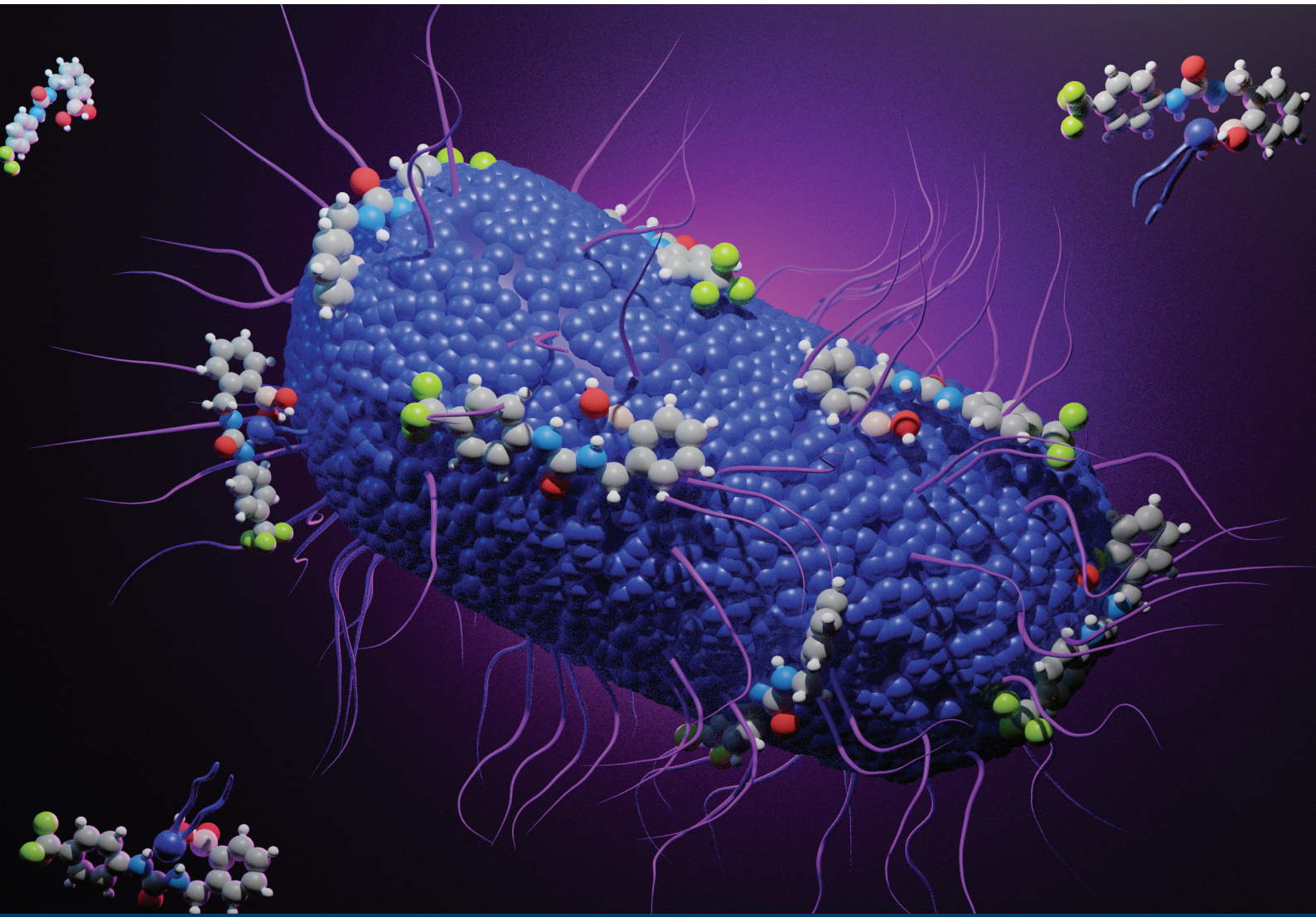

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Nathalie Busschaert *et al.*
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A supramolecular host for phosphatidylglycerol (PG) lipids with antibacterial activity†

Elliot S. Williams, Hassan Gneid, Sarah R. Marshall, Mario J. González, Jorgi A. Mandelbaum and Nathalie Busschaert *

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Lipids fulfill a variety of important physiological functions, such as energy storage, providing a hydrophobic barrier, and signal transduction. Despite this plethora of biological roles, lipids are rarely considered a potential target for medical applications. Here, we report a set of neutral small molecules that contain boronic acid and urea functionalities to selectively recognize the bacterial lipid phosphatidylglycerol (PG). The affinity and selectivity was determined using ^{1}H NMR titrations and a liposome-based Alizarin Red S assay. Minimum inhibitory concentrations (MIC) were determined to assess antibacterial activity. The most potent compounds display an association constant with PG in liposomes of at least 5×10^3 M^{-1}, function as antibacterial agents against Gram-positive bacteria (MIC = 12.5–25 μM), and show little hemolytic activity. Mode of action studies suggest that the boronic acids bind to the headgroup of the PG lipids, which leads to a change in membrane fluidity and ultimately causes membrane depolarization and cell death.

Introduction

Lipids have been largely ignored as a potential target by medicinal chemists and supramolecular chemists alike – especially compared to the other major biological entities such as proteins and DNA/RNA. Traditionally, lipids were merely seen as energy storage molecules and as the structural components that make up the cellular membranes, with little other biochemical function. While the majority of lipids do reside in the membrane, the recent advent of lipidomics and related biochemical studies have led to the realization that lipids play important roles in almost every area of cell biology and human health.[1,2] Thousands of unique lipids have been identified so far,[3] and it has become clear that some lipids function as primary or secondary messengers in signal transduction pathways (coined 'bio-active lipids').[1,2] Many of these bio-active lipids have been linked to diseases such as cancer and diabetes,[4–11] and there is therefore merit in the development of small molecules that can selectively bind to the headgroups of these bioactive lipids. Such molecules could be used to help investigate the biochemistry of these lipids, as sensors to detect the presence of these lipids (as biomarkers for certain diseases), or even as therapeutic molecules themselves.[12,13]

In addition to developing hosts that can bind bioactive lipids, there is also a need to develop molecules that can selectively bind to certain structural membrane lipids over other membrane lipids. The best-known example of this is the activity of antimicrobial peptides (AMPs).[14,15] AMPs are an extensive family of peptides that protect host cells from infection by pathogenic bacteria. Most AMPs are polycationic amphiphilic species, whereby the positive charges provide selectivity for the anionic bacterial membranes and the amphiphilic nature results in membrane destabilization.[16,17] Unfortunately, as potential therapeutics AMPs suffer from poor pharmacokinetics (including low *in vivo* stability), costly production, and resistance pathways intrinsic to their peptidic nature (*e.g.*, sensitivity to peptidases).[18,19] To overcome these problems, numerous AMP analogues, peptidomimetics and other polycationic molecules have been investigated as a potential solution.[20–31] However, the high molecular weight and the high positive charge of these molecules create a risk for off-target effects and similar pharmacokinetic problems as AMPs.[27,32,33] An alternative is therefore the use of rationally designed small molecules that selectively bind to bacterial lipids and thereby destabilize the membrane.[34–37]

In this manuscript, we utilize well-known supramolecular recognition motifs to develop a set of neutral small molecules that selectively bind to specific bacterial lipids. In opposition to AMPs that utilize non-selective coulombic interactions and as a result show limited lipid selectivity, the use of supramolecular chemistry allows the design of tailored hosts for specific lipid headgroups. The most common lipid in bacterial membranes is the anionic lipid phosphatidylglycerol (PG, Fig. 1a).

Department of Chemistry, Tulane University, New Orleans, Louisiana 70118, USA. E-mail: nbusschaert@tulane.edu

† Electronic supplementary information (ESI) available: Synthesis and characterization of the boronic acids, experimental details and additional graphs for the ^{1}H NMR titrations, ARS binding studies, liposome-based studies and bacterial investigations. See DOI: 10.1039/d1ob02298a

It is present in both Gram-positive and Gram-negative bacteria and often constitutes >50% of the phospholipids in Gram-positive bacteria.[38–41] In addition to acting as a major membrane constituent, PG performs essential roles in lipid biosynthesis,[42] protein transportation,[43] and protein folding.[44] In contrast, mammalian membranes predominantly exist of zwitterionic and neutral lipids, particularly phosphatidylcholine (PC, Fig. 1a) and cholesterol.[22–31] In a typical mammalian cell, PG concentrations are generally <1%.[45] Herein, we report a series of boronic acid containing hosts that can selectively bind the bacterial lipid PG over the mammalian lipid PC, and show that these molecules have potential as novel antibacterial agents.

Results and discussion

Design and synthesis

To bind PG effectively and selectively, we designed a set of small molecules containing functional groups intended to recognize both the phosphate and glycerol portions of the PG headgroup (Fig. 1b). A phenylboronic acid (BA) moiety was chosen to bind the 1,2-diol of the glycerol through boronic ester formation.[46] A urea moiety was used to synergistically recognize the phosphate of the headgroup through hydrogen bonding.[47] A schematic of the expected and modelled interaction between compound **1** and the PG headgroup is shown in Fig. 1c. The set varies in the relative position of the urea and boronic acid functionalities to identify the optimal scaffold for PG binding. The urea functionality is either directly attached to the phenylboronic acid (**2**, **4**, **7**, **8**) or *via* a methylene linker (**1**, **3**, **5**, **6**). In addition, the urea is either *ortho* (**1**, **4**, **5**, **6**) or *meta* (**2**, **3**, **7**, **8**) relative to the boronic acid. Compound **4** was designed to be the *ortho* analogue of compound **2** but underwent an irreversible intramolecular self-cyclization. The compounds also contain *para*-trifluoromethylphenyl (**1–5** and **7**) or 2-ethylhexyl (**6** and **8**) as lipophilic membrane anchors. All compounds were synthesized using a urea coupling reaction between the appropriate isocyanate and primary amine, and the final products were purified using the sorbitol extraction technique developed by Dennis Hall (see ESI†).[48] Compounds **2** and **4** have been previously reported,[49] and characterization was in agreement with these previous reports.

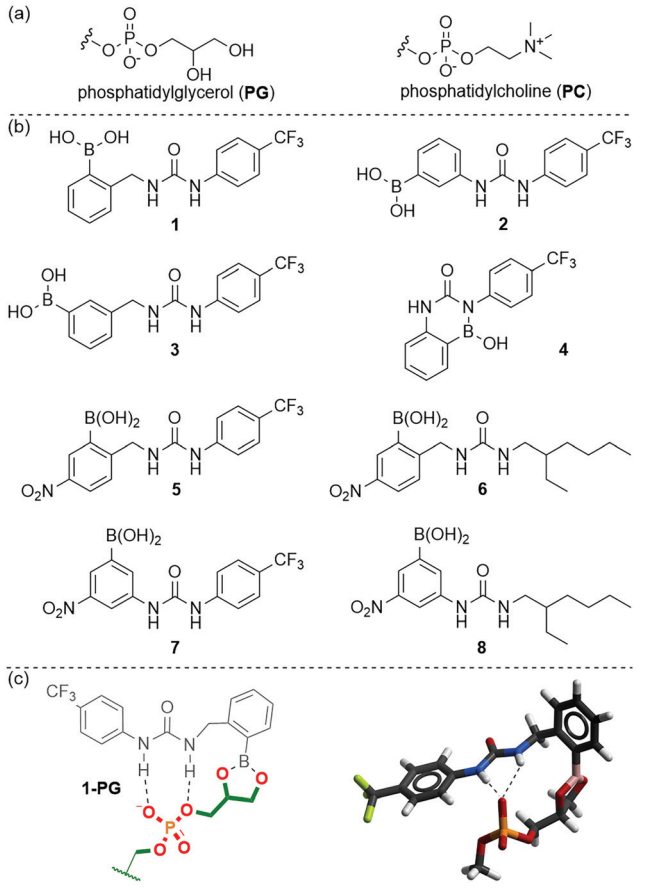

Fig. 1 (a) Structure of the PG and PC headgroups. (b) Structures of the phenylboronic acids (BA) **1–8**. (c) Structure of the complex between **1** and PG, as well as the lowest energy conformer of the complex computed using Molecular Operating Environment 2018, MMFF94x force field with generalized Born solvation model for water. Structure for the neutral boronic ester is shown.

Boronic acid pK_a

Boronic acids initially gained popularity as sugar sensors due to their ability to overcome the competitive high hydration of saccharides.[50] In the presence of a *cis*-1,2 or *cis*-1,3 diol at the appropriate pH, BAs readily exchange into cyclic phenylboronic esters. For this reason, BA-based receptors have been developed to bind a variety of diol containing molecules and anions, including the lipid phosphatidylinositol-(4,5)-bisphosphate (PIP_2).[50–57] However, the use of boronic acids to design small-molecule receptors for the bacterial lipid PG has not yet been reported. Boronic ester formation depends on the structure and pK_a of the boronic acid and the diol, the pH of the solution, and the buffer composition. A number of groups have shown that the optimal pH for boronic ester formation is the average of the pK_a of the diol and BA.[58] Since the pK_a of the diol is typically above the BA, it is believed that a lower BA pK_a increases diol affinity at physiological pH. However, Wang and co-workers have revealed that the lowest BA pK_a is not always the strongest binder with a diol.[58]

We determined the pK_a of the boronic acids using UV-Vis pH titrations, which utilize the difference in absorbance of the neutral boronic acid and the anionic boronate (Fig. 2). Due to partial aqueous solubility of **1–8**, 25% acetonitrile was incorporated into a 75 mM phosphate buffer during the titration, and a correction factor was necessary to estimate the pK_a in pure water (see ESI†). The obtained adjusted pK_a values of **1–8** are given in Table 1. All compounds show a lower pK_a than unsubstituted **PBA**, indicating modest activation of the boronic acid. The effect is most pronounced for the compounds containing the electron-withdrawing nitro substituent (**5–8**), which have a pK_a below physiological pH of 7.4, whereas the unsubstituted compounds **1–3** have pK_a values >8. The lowest pK_a was obtained for cyclized compound **4**, which has the most different electronic environment around the boron atom due to the presence of a boron–nitrogen bond.

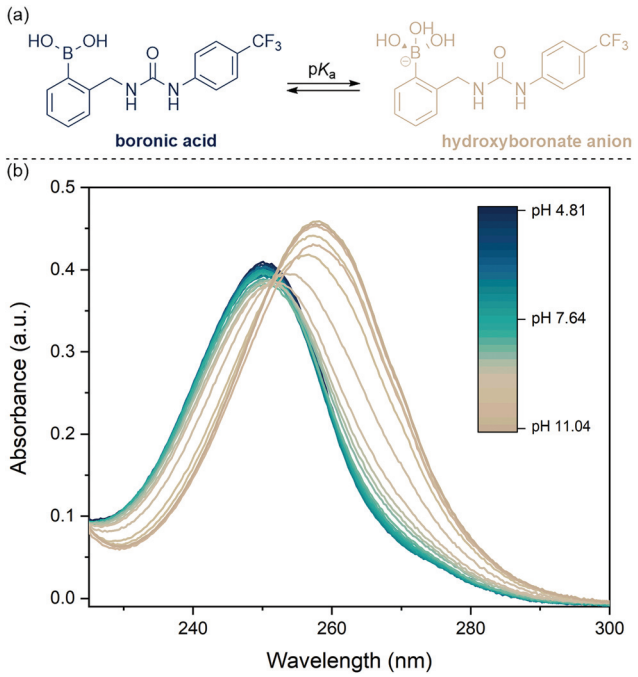

Fig. 2 (a) Structure of the neutral boronic acid and anionic hydroxyboronate form of compound **1**. (b) UV-Vis spectra of compound **1** (100 μM) at various pH conditions in 25% acetonitrile, 75% buffer (75 mM phosphate buffer). The change in absorbance was used to determine the pK_a of **1**.

PG binding in solution

To determine the association constant (K_a) between the boronic acids and PG lipids, we performed ^{1}H NMR titrations according to our previously reported method.[59] In this assay, the lipids are freely dissolved in an organic solvent, and do not form an organized bilayer. While this is not an ideal mimic of a biological membrane, it allows the accurate determination of binding strength and selectivity, and provides structural details of the binding event. 1,2-Didecanoyl-*sn-glycero*-3-phospho-(1′-*rac*-glycerol) (sodium salt) (10:0 PG or DDPG) served as the experimental bacterial lipid and 1,2-dihexanoyl-*sn-glycero*-3-phosphocholine (6:0 PC or DHPC) was the mammalian control lipid. The slight difference in chain length ensured complete solubility of all species in the same solvent system (99.5% DMSO-d_6 : 0.5% H_2O). The native sodium counterion of DDPG was exchanged for tetramethylammonium (TMA) because it forms a weaker ion pair with phosphate (see ESI†).[60] The temperature of each titration was set to 37 °C to improve peak resolution, decrease the equilibration time between each measurement, and mimic biological conditions. The obtained association constants are summarized in Table 1.

When the boronic acids were titrated with DHPC, the host–guest interaction exhibited fast-exchange on the NMR timescale and revealed weak binding with PC (Table 1). In contrast, when titrated with DDPG-TMA, five compounds (**1** and **5–8**) exhibited slow-exchange compared to the NMR timescale due to the slow rate of boronic ester formation. The formation of the boronic ester is evident from the decrease of the ^{1}H NMR signal corresponding to the boronic acid OH upon the addition of PG (Fig. 3). In addition, the intensity of the urea NH signals of the host compound decreases, and a new set of NH signals is seen further downfield (Fig. 3). These two observations provide evidence that compounds **1** and **5–8** bind PG through both the formation of a boronic ester and hydrogen bonding involving the urea moiety. Consequently, these compounds show strong binding to PG and significant PG/PC selectivity (Table 1). The integrations of the NH signals of the

Table 1 Overview of the pK_a values, lipid binding ability (K_a with PG and PC lipids), antibacterial activity (MIC values against *B. subtilis*, *S. aureus*, and *E. faecalis*) and hemolytic activity (HC_{50}) of hosts **1–8** and phenylboronic acid (**PBA**) and indolicidin. Results are the average of minimum two independent repeats and the reported errors indicated the standard deviation

Host	pK_a (adjusted)[a]	K_a (M^{-1}), NMR[b]		K_a (M^{-1}), ARS assay[c]	MIC[d] (μM)			HC_{50}[e] (μM)
		PG	PC	PG[d]	*B. subtilis*	*S. aureus*	*E. faecalis*	
1	8.34 ± 0.14	(7.6 ± 1.1) × 10^2	36 ± 26	(5.81 ± 0.45) × 10^3	25	25	50	297 ± 39
2	8.69 ± 0.18	42 ± 10	33.5 ± 3.4	n.d.[f]	>100	>100	>100	358 ± 54
3	8.54 ± 0.13	35 ± 14	23 ± 14	(5.94 ± 0.51) × 10^3	25	12.5	25	164 ± 12
4	6.37 ± 0.13	Weak[g]	Weak[g]	n.d.[f]	>100	>100	>100	332 ± 39
5	6.85 ± 0.02	(1.44 ± 0.25) × 10^3	37.8 ± 1.6	n.d.[f]	50	50	50	233 ± 11
6	7.25 ± 0.08	(6.20 ± 0.83) × 10^2	Weak[g]	(1.12 ± 0.07) × 10^4	100	>100	100	169 ± 15
7	6.59 ± 0.20	(1.32 ± 0.51) × 10^2	75 ± 13	(6.67 ± 0.53) × 10^3	50	50	75	209 ± 17
8	7.05 ± 0.14	(1.12 ± 0.25) × 10^2	17.7 ± 3.3	(2.63 ± 0.43) × 10^3	75	75	100	162 ± 14
PBA	8.87 ± 0.11	n.d.[f]	n.d.[f]	n.d.[f]	>100	>100	>100	356 ± 21
Indolicidin	n.d.[f]	n.d.[f]	n.d.[f]	n.d.[f]	6.25	>25	>25	107 ± 7

[a] pK_a values were determined by UV-Vis titrations in 25% MeCN, 75% aqueous buffer (75 mM phosphate), and were corrected by subtracting a value of 0.77 to obtain the estimated pK_a in pure aqueous solution (see ESI† for details). [b] Association constant (K_a, M^{-1}) obtained *via* ^{1}H NMR titrations with TMA-DPPG (PG) or DHPC (PC) in 99.5% DMSO-d_6, 0.5% H_2O, at 37 °C. [c] Association constant between the hosts and 50 nm POPG liposomes obtained using a fluorescence indicator displacement assay with ARS in 10 mM HEPES, 150 mM NaCl, pH 7.4, and 1% DMSO. [d] Minimum inhibitory concentration (MIC) for the hosts against the Gram-positive bacteria *B. subtilis*, *S. aureus*, and *E. faecalis* obtained using standard broth microdilution methods. [e] Hemolytic activity of the compounds was determined by the concentration of host needed to achieve 50% hemolysis in washed single-donor human red blood cells (HC_{50}). [f] n.d. = not determined. [g] Change in chemical shift was too small to quantify (<0.03 ppm).

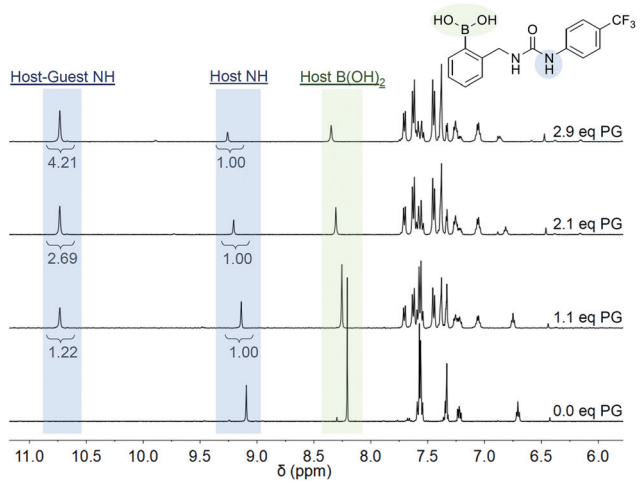

Fig. 3 ^{1}H NMR titration (500 MHz NMR) of 3 mM **1** with DDPG-TMA in 99.5% DMSO-d_6 : 0.5% H_2O at 37 °C. Slow exchange is observed, whereby the original host signals disappear and new host–guest signals appear (*e.g.*, urea NH, blue). The boronic acid $B(OH)_2$ peak also decreases in intensity upon the addition of PG (green).

host and host–guest complex were used to calculate the K_a values (see ESI†).[61] Compounds **2** and **3** exhibited fast exchange with DDPG-TMA, and showed little PG/PC selectivity. This indicates that **2** and **3** do not form a boronic ester under the conditions of the ^{1}H NMR titrations and bind to PG and PC only through the formation of hydrogen bonds with the phosphate unit. This is not surprising because **2** and **3** are the compounds with the highest BA pK_a (Table 1) and therefore the least activated for boronic ester formation. Self-cyclized compound **4** exhibited no change in chemical shift for either lipid because it is unable to form boronic esters or hydrogen bonds with the lipid headgroups.

In summary, compounds **5** > **1** > **6** demonstrated the strongest binding to PG and the greatest preference for PG over PC ($K_{a,(PG)}/K_{a,(PC)}$ > 20). These three compounds all have the boronic acid *ortho* to the urea functionality and utilize a methylene linker to separate both binding moieties. It is also clear that the trifluoromethylphenyl group is superior to the 2-ethylhexyl group due to its positive effect on the acidity and hydrogen bond donating ability of the urea NHs, and that the nitro substituent of compound **5** increases binding to PG due to its effect on the pK_a of the boronic acid.

PG binding in liposomes

The ability of compounds **1–8** to bind PG in lipid bilayers was investigated using an indicator displacement assay with Alizarin Red S (ARS). This displacement assay was first reported by Wang and co-workers to quantify the interaction between boronic acids and carbohydrates in water.[62] ARS is a catechol-based dye that results in a red solution with low fluorescence intensity in aqueous media. In the presence of a boronic acid, a boronic ester is formed from the catechol and the BA, causing an increase in fluorescence intensity with a concomitant change in the color of the solution from red to yellow. When a competing diol is titrated to this solution, it can displace the ARS dye from the BA host, leading to a decrease in fluorescence intensity and a shift in color from yellow back to red (Fig. 4a).

Aliquots of 50 nm small unilamellar vesicles (SUVs) composed of 16:0–18:1 PG (POPG) were titrated into a solution containing ARS and BA. The small liposome size was chosen to minimize interference from scattering (see ESI†). Most compounds showed a decrease in fluorescence intensity upon the addition of POPG liposomes, consistent with the displacement of the ARS dye and the formation of a BA-PG complex (Fig. 4b). The change in fluorescence was processed using the formulas derived by Wang and co-workers[62] and the resulting association constants are reported in Table 1. The association constant with PG could not be determined for compounds **2**, **4**, **5** and **PBA** due to solubility issues, interference, or weak binding (see ESI†). As a result, it is not possible to provide a detailed quantitative assessment of the ARS displacement assay, but qualitative analysis is still possible. In general, all compounds that showed PG binding in the ^{1}H NMR titrations, also indi-

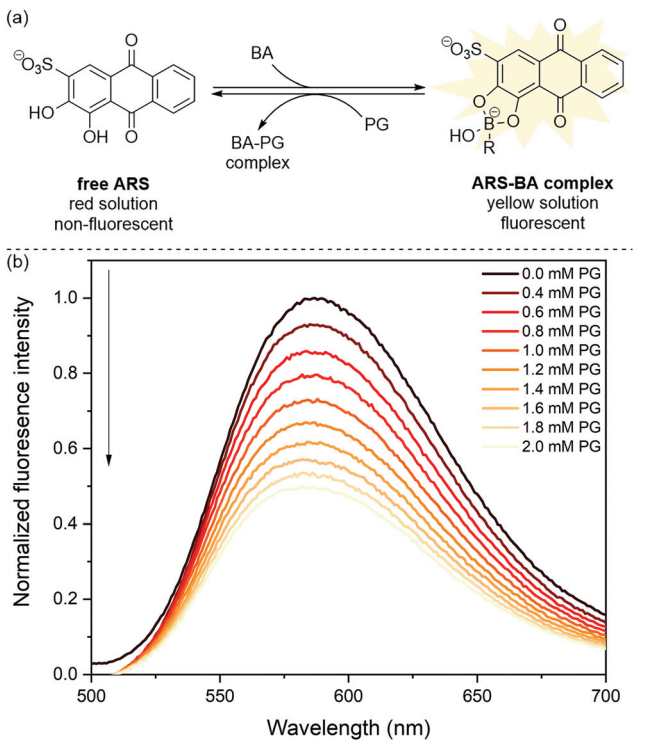

Fig. 4 (a) Concept of the ARS displacement assay. Free ARS is non-fluorescent and gives a reddish color in aqueous solutions, whereas the ARS-boronic acid complex is fluorescent and yellow in color. Upon the addition of PG lipids to the ARS-BA complex, the ARS is displaced and a decrease in fluorescence is observed. (b) Fluorescence titration involving the addition of 50 nm POPG liposomes to a solution of 100 μM **1**, 10 μM ARS, 10 mM HEPES, 150 mM NaCl, pH 7.4, and 1% DMSO. Results are normalized to the maximum fluorescence at 582 nm before the addition of POPG liposomes, and corrected for liposome scattering.

cated PG binding in the liposome-based ARS assay, with K_a values of 5000–10 000 M^{-1}. Furthermore, the fact that the ARS dye can be displaced by PG liposomes, indicates that binding of **1–8** to PG in lipid bilayers involves the formation of a boronic ester. Interestingly, compound **3** clearly shows evidence of PG binding in the ARS displacement assay, but not during the ^{1}H NMR titrations (Table 1). Compound **3** has its boronic acid placed *meta* from the urea group, which is unfavorable for 1 : 1 binding to PG headgroups, but might favor alternative binding modes with lipids that are closely packed inside a membrane (*e.g.*, 2 : 1 lipid : host). It is also worth noting that the K_a values obtained using the liposome-based assay are an order of magnitude higher than those obtained using ^{1}H NMR titrations. We hypothesize that there is a significant hydrophobic effect that enhances the binding of the lipophilic BAs to the membrane.

As a control, we also performed the ARS displacement assay with 50 nm SUVs composed entirely of 16:0–18:1 PC (POPC) lipids. In this case, a pronounced increase in fluorescence intensity was observed, which cannot be due to binding of the compounds to the PC headgroup (see ESI†). We assume that the ARS-BA complex partitions into the membrane without displacement of the ARS fluorophore. The hydrophobic environment of the membrane provides less solvent interaction with the ARS-BA complex, leading to an increase in fluorescence.[63] These results thus confirm that boronic acids **1–8** can bind to PG lipids in lipid bilayers, but not to PC lipids.

Antibacterial and hemolytic activity

After the promising results with model lipid systems, we wanted to test the biological activity of **1–8**. As the compounds display selective binding of the bacterial lipid PG over the mammalian lipid PC, we expect them to function as antibacterial agents with limited human toxicity. There is an urgent need for new antibacterial agents due to the increasing threat of bacterial resistance against existing antibiotics. Both the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) have declared the antimicrobial resistance crisis to be one of the greatest contemporary challenges to public health.[64,65] The bacterial membrane is considered an excellent target for antibiotic development because it is less prone to bacterial resistance (due to the limited possibilities of alterations in the lipid headgroups and the fast-acting bactericidal effect of membrane disruption).[66,67]

To assess the antibacterial activity of the boronic acids, the minimum inhibitory concentration (MIC) was determined for a variety of bacteria (*B. subtilis*, *S. aureus*, *E. faecalis*, and *E. coli*) using standard broth microdilution methods.[68] None of the compounds showed any activity against the Gram-negative bacterium *E. coli* (see ESI†). Gram-negative bacteria contain two membranes and PG lipids are primarily located in the inner membrane at low concentrations (<20%),[69,70] rendering access to this target challenging in Gram-negative bacteria. In contrast, many of the boronic acids did possess potent antibacterial activity against the Gram-positive bacteria *B. subtilis*, *S. aureus*, and *E. faecalis* (Table 1). In general, the MIC values against *E. faecalis* are higher than those against *B. subtilis* and *S. aureus*, which agrees with the fact that *E. faecalis* has a lower PG content (<30%)[71,72] than *B. subtilis* and *S. aureus* (>50%),[73–75] and suggests that the boronic acids exert their antibacterial activity through their interaction with PG lipids. Unsubstituted **PBA** did not show any antibacterial activity, confirming that the urea functionality is necessary for sufficient PG binding and antimicrobial activity. The lowest MICs were found for compounds **1**, **3**, and **5** (MIC ≤ 50 μM). All three compounds showed potent PG binding ability in the ^{1}H NMR titrations and/or ARS assays discussed above. Another agreement with the PG binding studies is the observation that the 2-ethylhexyl substituent is less effective than the *p*-trifluoromethylphenyl substituent (**6** < **5**, and **8** < **7**). More surprising was the modest antibacterial activity of the boronic acids containing a nitro group (**5–8**), which could be due to degradation of these nitroaromatic compounds by the bacteria.[76–80]

One of the most common side effects of membrane-active agents is lysis of red blood cells (hemolysis).[81] We therefore determined the concentration of **1–8** that induces 50% hemolysis in single-donor human red blood cells (HC_{50}), using protocols commonly used for antimicrobial peptides.[82] The results are given in Table 1. The lowest HC_{50} values were noted for **6** and **8**, which both contain the 2-ethylhexyl substituent, suggesting that this substituent enforces a general detergent-like effect rather than selective lipid headgroup binding. On the other hand, the most potent antibacterial compounds **1**, **3** and **5** show HC_{50} values that are at least 10x higher than their MIC value, which is comparable to the biological activity of the PG-targeting antimicrobial peptide indolicidin (Table 1).[83] The results confirm our initial hypothesis that the boronic acid containing ureas can selectively bind to PG lipids and thereby function as selective antimicrobial agents.

Mechanism of antibacterial activity

The mechanism of action was studied in more detail in *B. subtilis* for the most promising antibacterial compounds **1** and **3** (MIC ≤ 25 μM). We first investigated the effect of **1** and **3** on the membrane potential in *B. subtilis* using the $Disc_3(5)$ (3,3′-dipropylthiadicarbocyanine iodide) method reported by te Winkel *et al.*[84] $Disc_3(5)$ is a membrane-permeable fluorescent dye that accumulates in polarized cells, where it self-quenches.[85] Thus, a kinetic assay can be performed whereby the fluorescence intensity upon the addition of membrane-active agents is monitored over time. The results for compounds **1** and **3** are shown in Fig. 5a. Both boronic acids clearly depict depolarization of the membrane, giving rise to fluorescence intensities comparable to the known membrane depolarizer gramicidin.[86] The $Disc_3(5)$ assay can also be visualized using fluorescence microscopy. Healthy, polarized cells accumulate $Disc_3(5)$ and can therefore be seen as red fluorescent cells (despite the self-quenching inside the cells). When the cells are depolarized, the dye leaks out of the cells and no fluorescence can be seen under the microscope. A selection of the obtained images is shown in Fig. 5b (additional images can be found in the ESI†). The overlay of

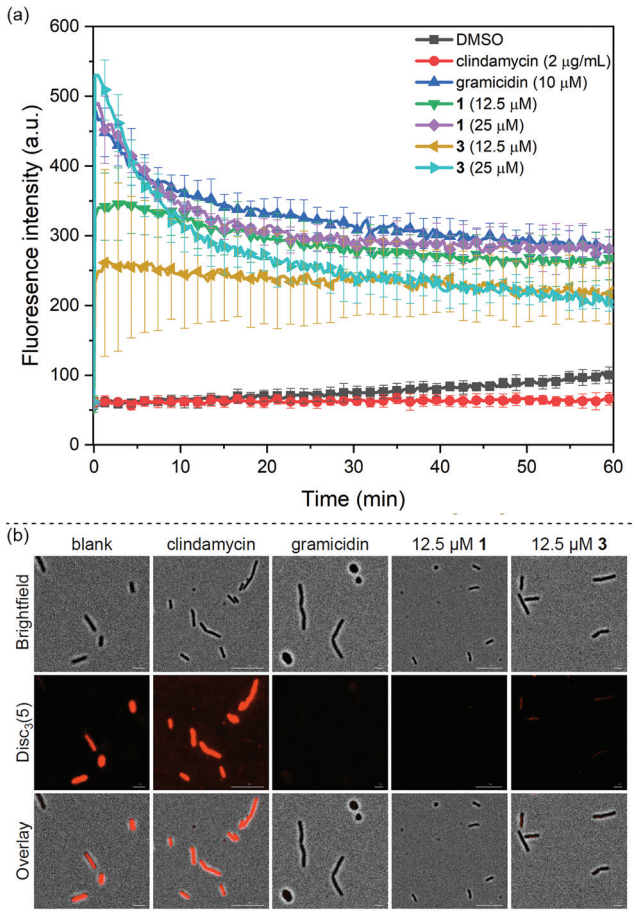

Fig. 5 Membrane depolarization of *B. subtilis* induced by BA **1** and **3**. (a) Bulk fluorescence intensity of $Disc_3(5)$ in *B. subtilis* upon the addition of DMSO (1%, blank), clindamycin (2 μg mL^{-1}, negative control), gramicidin (10 μM, positive control), 12.5 μM **1**, 25 μM **1**, 12.5 μM **3**, or 25 μM **3**. Results are the average of at least 2 technical × 2 biological repeats and error bars represent standard deviations. (b) Images of membrane depolarization of *B. subtilis* incubated for 10–15 minutes with 1% DMSO (blank), clindamycin (2 μg mL^{-1}, negative control), gramicidin (10 μM, positive control), 12.5 μM **1** or 12.5 μM **3**. Absence of fluorescence indicates that the cells are depolarized.

the brightfield and fluorescence images clearly shows loss of the electrochemical gradient in the presence of **1** and **3**.

The membrane depolarization induced in *B. subtilis* by BA **1** and **3** can be the result of various membrane-related processes. We therefore conducted additional experiments to elucidate how PG headgroup binding can lead to antibacterial activity. First, we used Sytox Green to determine if compounds **1** and **3** can form large pores or lead to membrane lysis. Sytox Green is a membrane-impermeable dye whose fluorescence intensity increases upon interaction with DNA.[87] Thus, an increase in fluorescence intensity indicates large membrane disturbances that allow the fluorophore and/or DNA to leak through the membrane, but neither compound **1** nor **3** led to an increase in fluorescence (see ESI†). We then investigated the effect of compounds **1** and **3** on the membrane fluidity of *B. subtilis* using Laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) and DPH (1,6-diphenyl-1,3,5-hexatriene). Laurdan is a hydrophobic dye whose generalized polarization (GP), calculated from measuring the fluorescence intensity at two different wavelengths, can be used to monitor changes in membrane fluidity over time.[88] Similarly, DPH is a hydrophobic rod-shaped dye whose fluorescence polarization (anisotropy) is highly sensitive to membrane fluidity.[89] As shown in Fig. 6, compounds **1** and **3** exhibit an increase in membrane fluidity in *B. subtilis* comparable to the known membrane fluidizer benzyl alcohol.[88] Compound **1** has a smaller effect on membrane fluidity than compound **3** in both the Laurdan and DPH assay, consistent with its higher MIC value. Based on these observations, we have further reasons to believe that the antimicrobial mode of action of boronic acids **1–8** involves

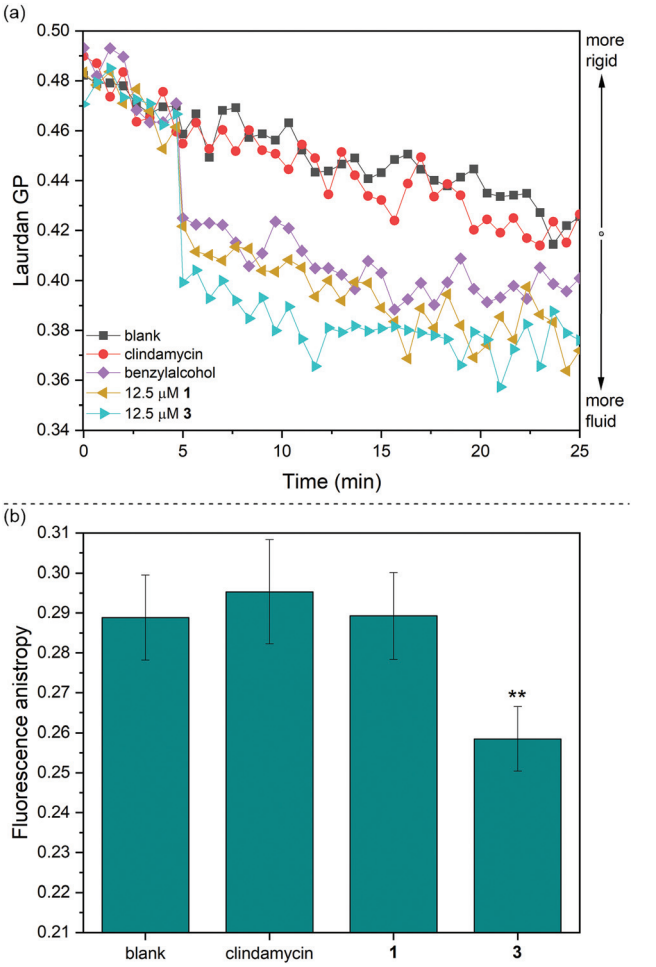

Fig. 6 Increase in membrane fluidity in *B. subtilis* induced by BA **1** and **3**. (a) Laurdan generalized polarization (GP) in *B. subtilis* (OD_{600} = 0.2) stained with 10 μM Laurdan. Compounds were added at time t = 5 min: DMSO (1%, blank), clindamycin (2 μg mL^{-1}, negative control), benzyl alcohol (50 mM, positive control), 12.5 μM **1**, or 12.5 μM **3**. Additional repeats are given in the ESI.† (b) Fluorescence anisotropy of 10 μM DPH in *B. subtilis* after 1.25 h incubation with blank (1% DMF), clindamycin (2 μg mL^{-1}, negative control), 25 μM **1**, or 25 μM **3**. Results are the average of at least 3 independent repeats and error bars represent standard deviations. The anisotropy value for **3** is significantly different from the blank (p-value = 0.0036).

binding to the headgroup of PG lipids in the bacterial membrane. We hypothesized that binding to the PG headgroup by the BA compounds functions as a wedge that pushes neighbouring lipids away from each other, leading to an increase in the fluidity of the lipid alkyl chains (as observed using Laurdan and DPH). The altered fluidity can change the permeability of the membrane,[90] as well as impact mechanosensitive ion channels in the membrane,[91] which ultimately leads to loss of concentration gradients and membrane depolarization (as observed using $Disc_3(5)$). Membrane depolarization is often lethal to cells,[92] and thus accounts for the antibacterial activity observed for the boronic acid-containing ureas.

Conclusions

In this manuscript, we report a series of small molecules that can selectively bind one type of lipid (PG) over other types of lipid (PC). ^{1}H NMR titrations in organic solvents, as well as liposome-based studies using the ARS dye, revealed that the compounds can bind to the bacterial lipid PG *via* the formation of a boronic ester with the PG glycerol unit and hydrogen bonding interactions involving the urea moiety. The most potent compounds selectively bind the bacterial lipid PG over the mammalian lipid PC with a selectivity ratio >20, and association constants with PG in aqueous solutions of 5000–10 000 M^{-1}. In addition, the best PG binders display antibacterial activity against a variety of Gram-positive bacteria (MIC $\approx$ 12.5–25 μM) with little toxicity to red blood cells. Mechanistic studies suggest that the antibacterial activity of the boronic acids is due to their ability to bind the PG headgroup, which leads to an increase in membrane fluidity and membrane depolarization.

Author contributions

ESW and NB were involved in the conceptualization, data analysis and writing of the manuscript. ESW designed and synthesized the compounds, performed the ^{1}H NMR titrations and hemolysis assay, and optimized and coordinated the pK_a determinations and ARS displacement assay. HG performed the antibacterial studies (MIC determinations) and mechanism of action studies in *B. subtilis*. SRM performed the ARS displacement assay. MJG and JAM performed the pK_a determination measurements. NB supervised all activities and was responsible for funding acquisition.

Conflicts of interest

There are no conflicts to declare.

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